Determination of Neutral Lipid Hydroperoxides by Size Exclusion HPLC with Fluorometric Detection. Application to Fish Oil Enriched Mayonnaises during Storage

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A fast (12 min) high-performance size exclusion chromatography (HPSEC) method for the separation of neutral lipid class hydroperoxides in the oil phases from fish oil enriched mayonnaises was developed. Detection and quantification were performed using the postcolumn fluorometric (FL) diphenyl-1-pyrenylphosphine oxidation principle. The reproducibilities judged by intra- and interassay variations were 0.64 and 7.2%, respectively. The HPSEC-FL method was applied to assess the effect of supplementations with emulsifier, gallic acid, and EDTA on the oxidative processes in the mayonnaises during storage. Substantial amounts of hydroperoxy triacylglycerols (TAGOOH) and cholesterol esters (CEOOH), together with traces of TAGOOH-dimers, were detected. All supplementations significantly decreased the levels of TAGOOH and to a lesser degree CEOOH. Supplementations with EDTA and gallic acid resulted in constant and slightly increasing levels of TAGOOH, respectively, thus affecting the oxidation mechanisms seen in reference mayonnaise. The emulsifier Panodan TR DATEM reduced the levels of TAGOOH as compared to the appropriate controls.

Keywords: *Diphenyl-1-pyrenylphosphine; emulsions; lipid oxidation; postcolumn high-performance liquid chromatography; triacylglycerol hydroperoxides*

INTRODUCTION

Lipid oxidation has attracted much attention in biological research due to the potential influence on aging and on a variety of disorders (Vendemiale et al., 1999), for example, atherosclerosis (Witztum and Berliner, 1998). In addition, lipid oxidation has been shown to have great impact on food quality through flavor deterioration (Coupland and McClements, 1996; St. Angelo, 1996). Therefore, accurate and reproducible measurements of lipid hydroperoxides, the primary product of lipid oxidation, are important for elucidation and thereby control of the oxidation processes in foods as well as in the intact organism.

Traditionally, lipid oxidation is monitored by the peroxide value (PV) obtained from simple nonchromatographic methods with low-cost equipment, as, for example, described by Asakawa and Matsushita (1980). Today, the need for more specific methods has rendered the use of chromatography necessary. Several investigations using high-performance liquid chromatography (HPLC) in combination with UV detection at 234 nm have been reported. However, the UV detection is not selective for the conjugated diene structure of lipid hydroperoxides, and the results are therefore difficult to interpret when complex matrices, namely, mayonnaises, are analyzed. More selective and sensitive methods include electrochemical detection (Terao et al., 1988) and a series of postcolumn reaction principles, for example, colorimetric detection with iron thiocyanate (Müllertz et al., 1990), chemiluminescence detection with isoluminol/microperoxidase (Yamamoto et al., 1987) or luminol/cytochrome *c* (Miyazawa et al., 1987), and fluorometric (FL) detection with diphenyl-1-pyrenylphosphine (DPPP; Akasaka et al., 1988). These detection principles have been applied in combination with various normal- and reverse-phase HPLC separation systems, but in complex food systems containing a wide range of fatty acids, none of these methods are capable of determining the neutral lipid hydroperoxides within a lipid class as a single group. High-performance size exclusion chromatography (HPSEC) has been shown by several authors, for example, Hopia et al. (1993), to perform neutral lipid class separation of oils including autoxidized oils. However, direct combination of HPSEC with one of the selective detection principles has not yet been reported.

To comprehend the underlying mechanisms of lipid oxidation and food deterioration in complex food matrices, namely, fish oil enriched mayonnaises, the need for chemical measurements of the different stages of oxidation, that is, free radicals, lipid hydroperoxides, and secondary lipid oxidation products, volatiles as well as nonvolatiles, is mandatory (Coupland and McClements, 1996). Recently, methods for measurement of free radicals (Thomsen et al., 1999) and characterization of the volatile and the olfactometric profiles (Hartvigsen et al., 2000) have been reported.

In the present study an HPLC method for the separation, detection, and quantification of neutral lipid hydroperoxides from dietary lipids and lipid systems, namely, mayonnaises, is described. Separation was performed by size exclusion chromatography and combined with a specific lipid hydroperoxide detection system composed of postcolumn oxidation of DPPP

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Table 1. Supplemental Additives to the Mayonnaises Included in This Study

	supplementation (ppm)		
mayonnaise[a]	emulsifier (Panodan TR DATEM)	antioxidant (gallic acid)	metal chelator (EDTA)
Ref			
Em-Ref	200		
GA		200	
Em-GA	200	200	
EDTA			200
Em-EDTA	200		200

[a] Ref, reference mayonnaise; Em, emulsifier; GA, gallic acid; EDTA, ethylenediaminetetraacetate.

followed by measurement of the FL intensity. The HPSEC-FL method was compared with a traditional method for PV determination and applied to six different fish oil enriched mayonnaises to monitor the formation of lipid hydroperoxides during storage. The mayonnaises were supplemented with emulsifier, polar antioxidant, and metal chelator to assess their effect in the oxidative progress.

MATERIALS AND METHODS

Materials. DPPP was purchased at Dojindo Laboratories (Kumamoto, Japan). Cholesterol (Ch), cholesteryl linoleate, dilinoleoylglycerol, 2,6-di-*tert*-butyl-*p*-cresol (BHT), ethylenediaminetetraacetate (EDTA), gallic acid (GA), linoleic acid, 1-palmitoyl-2-linoleoyl-*sn*-glycerophosphocholine (GroPCho), tocopherols, and trilinoleoylglycerol were purchased from Sigma-Aldrich (St. Louis, MO). The emulsifier Panodan TR DATEM (Em; diacetyl tartaric acid esters of monoacyl- and diacylglycerols) and the stabilizer Grindsted FF DC (guar gum and sodium alginate) were kindly supplied by Danisco Ingredients (Brabrand, Denmark). All solvents used were of analytical or HPLC grade.

Preparation of Mayonnaises. Mayonnaises (pH 4.0) were prepared as previously described (Jacobsen et al., 1999a) and contained, by weight, 64.0% rapeseed oil (refined and deodorized), 16.0% fish oil (refined and deodorized), 10.4% water, 4.0% vinegar, 0.3% NaCl, 1.0% sucrose, 0.1% potassium sorbate, 4.0% egg yolk, and 0.15% Grindsted FF DC. Six mayonnaises, with different contents of Panodan TR DATEM, gallic acid, and EDTA, were prepared (Table 1). Aliquots (300 g) of the mayonnaises, stored in nontransparent jars at 20 °C in darkness, were sampled after 0, 1, 2, 3, and 4 weeks and kept at −80 °C. For processing the samples were thawed and centrifuged at 2500*g* for 10 min at 4 °C. A clear oil phase was obtained, dissolved in cold, degassed dichloromethane containing 0.002% BHT, and kept in nitrogen atmosphere at −80 °C.

Determination of PV. The PVs of the oil phase solutions (∼250 mg/mL) were quantified in triplicates (intra-assay variations) by the colorimetric microassay described by Asakawa and Matsushita (1980). The levels of PV (milliequivalents per kilogram of oil) were given as the means obtained by triplicates from three different aliquots of the oil phases (inter-assay variations).

External Reference Preparations. Trilinoleoylglycerol (500 mg) was photooxidized using 0.1 mM methylene blue as a photosensitizer in dichloromethane/methanol (2:1, v/v) at 4 °C according to the method of Miyazawa et al. (1995). After 8 h of oxidation, the solvent was gently removed by rotary evaporation at 30 °C, and methanol was added and evaporated, followed by extraction of lipids with hexane. The monohydroperoxide of trilinoleoylglycerol (TAG18:2-OOH; R_f 0.49) was separated from the corresponding bis- and trishydroperoxides (R_f 0.36 and 0.22, respectively) and the nonoxidized trilinoleoylglycerol (R_f 0.61) by preparative thin-layer chromatography (TLC) with hexane/diethyl ether/acetic acid/BHT (50:50:1:0.002, v/v/v/w) as developing solvent. The TAG18:2-OOH was scraped off and extracted with dichloromethane/methanol (2:1, v/v) containing 0.002% BHT, the solvent was evaporated under nitrogen, and the residue was dissolved in cold, degassed dichloromethane containing 0.002% BHT and kept in a nitrogen atmosphere at −80 °C in nontransparent vials. The reference TAG18:2-OOH was detected as a single chromatographic peak, when tested by the HPSEC-FL method and by a method combining reverse-phase HPLC, performing partial separation of nonoxidized triacylglycerol (TAG) and mono-, bis-, and tris-TAGOOH (Yamamoto et al., 1987), and the DPPP detection principle. The PV of the TAG18:2-OOH standard was determined by the colorimetric microassay (Asakawa and Matsushita, 1980), and samples were diluted appropriately in the range of 2.5−2000 pmol of TAG18:2-OOH/30 μL of dichloromethane containing 0.002% BHT.

Reference free fatty acid hydroperoxide (FFAOOH), that is, 13*S*-hydroperoxy-*cis*,*trans*-9,11-octadecadienoic acid, was produced enzymatically from linoleic acid by soybean lipoxygenase (Iacazio et al., 1990). Reference lipid hydroperoxide preparations of cholesterol (ChOOH), cholesterol ester (CEOOH), diacylglycerol (DAGOOH), and diacyl-GroPCho were produced by mild autoxidation, that is, 3 h at 25 °C, of cholesterol, cholesteryl linoleate, dilinoleoylglycerol, and 1-palmitoyl-2-linoleoyl-*sn*-GroPCho, respectively.

HPLC. The HPLC system consisted of a multi-HPLC pump (model 600E; Waters, Milford, MA), an autosampler (model 717, Waters; injection volume = 30 μL), a multiwavelength UV detector (model 490, Waters; 234 and 292 nm), a postcolumn HPLC pump (Bioclean model 350, Waters), a stainless steel T-connector, stainless steel reaction (20 m, 0.5 mm i.d., 80 °C) and cooling (1 m, 0.5 mm i.d., 30 °C) coils, and a fluorescence detector (model F1000; Hitachi, Tokyo, Japan; excitation = 352 nm, emission = 380 nm). The system was controlled by Millennium 2010 software (Waters).

Separation of Lipid Hydroperoxides. The method for the separation of lipid class hydroperoxides was a modification of that described by Hopia et al. (1992). Separation was performed at ambient temperature by size exclusion chromatography with dichloromethane as the mobile phase (0.6 mL/min) and a series of size exclusion columns (PLgel, 5 μm; Polymer Laboratories, Amherst, MA; kindly provided by Dr. Anu Hopia, Department of Applied Chemistry and Microbiology, University of Helsinki, Finland): one 100 Å precolumn (7.5 × 50 mm), two 100 Å, and one 50 Å analytical columns (7.5 × 300 mm). Lipid classes were characterized by cochromatography and spiking of samples with appropriate reference compounds.

Detection of Lipid Hydroperoxides. The method for the detection of lipid hydroperoxides was a modification of that described by Akasaka et al. (1992). The DPPP reagent solution (3.8 mg of DPPP and 250 mg of BHT were dissolved in 2 mL of dichloromethane prior to the addition of 500 mL of cold, degassed methanol) was mixed with the column effluent after the UV detector in the T-connector at a rate of 0.3 mL/min. The mixture was allowed to react and cool in the coils, before the FL intensity of the DPPP oxides was measured. The DPPP reagent solution (kept on ice in the dark) and the dichloromethane were continuously degassed with 30 mL/min helium (99.997%, Hydrogas, Fredericia, Denmark).

Lipid hydroperoxides were quantified using TAG18:2-OOH as an external standard (picomoles of lipid hydroperoxide per milligram of oil). The oil phase solutions (∼25 mg/mL) were analyzed directly by triple injections (intra-assay variations). The levels of lipid hydroperoxides were reported as the means of PV given as milliequivalents per kilogram of oil (2 × mmol of lipid hydroperoxide/kg of oil) obtained by triple injections from three different aliquots of the oil phases (inter-assay variations).

Statistics. The results are given as means ± SD. Student's *t* test for unpaired variates and Kruskal−Wallis followed by Dunn's method for pairwise multiple comparisons were used when appropriate. Probability values <0.05 were considered to be statistically significant.

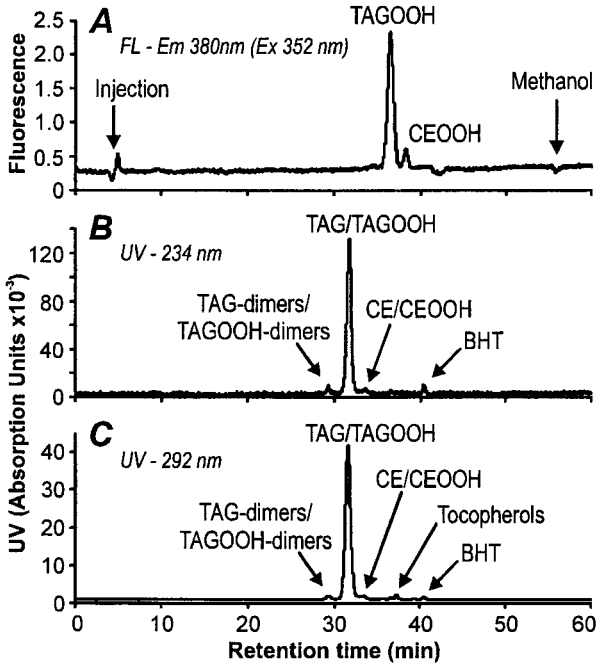

Figure 1. HPSEC-UV/FL analysis of the oil phase of Em-EDTA mayonnaise stored for 4 weeks (Table 1): (A) FL chromatogram of the detected lipid class hydroperoxides; (B and C) UV absorption chromatograms for detection of lipids and conjugated dienes at 234 nm and of tocopherols at 292 nm, respectively. Retention times are as indicated in Table 2.

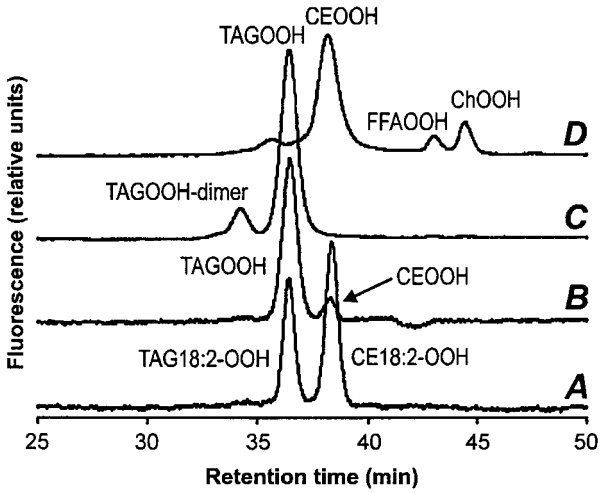

Figure 2. HPSEC-FL chromatograms for comparison of retention times and for the characterization of the TAG and CE lipid class hydroperoxides: (A) resolution of reference TAG18:2-OOH and CE18:2-OOH; (B) detected lipid class hydroperoxides in the Em-EDTA mayonnaise stored for 4 weeks; (C and D) TLC separated TAG and CE, respectively, from the Em-EDTA mayonnaise (B) prior to mild autoxidation. Retention times are as indicated in Table 2.

Table 2. Molecular Weights of Reference Compounds and Their Retention Times by HPSEC-UV/FL

		retention time[a] (min)	
compound	MW	UV[b]	FL[c]
TAG18:2	879	31.5	36.5
DAG18:2	617	32.5	37.5
CE18:2	649	33.6	38.4
tocopherols	403−431	36.9	nd
FFA18:2	280	37.9	43.0
cholesterol	387	39.4	44.5
GroPCho16:0/18:2	758	39.6	44.7
BHT	220	40.4	nd
methanol	46	50.7	55.8

[a] The difference in retention time between the UV and FL response is due to the delay in the reaction and cooling coils. [b] Detection of oxidized and nonoxidized lipids. [c] Detection of lipid hydroperoxides. nd, not determined.

RESULTS

Neutral Lipid Hydroperoxides in Mayonnaises. Figure 1 shows typical HPSEC-UV/FL chromatograms of neutral lipid hydroperoxides in fish oil enriched mayonnaise supplemented with emulsifier and EDTA following 4 weeks of storage (Em-EDTA, Table 1). Figure 1A shows the FL chromatogram with retention times (RT) of 36.5 and 38.4 min for TAGOOH and CEOOH, respectively. The baseline disturbance at 4.8 min is due to the injection. The total time of chromatography is ∼60 min, as verified by spiking of the sample with methanol (RT = 55.8 min). Because the only compounds detected, the lipid hydroperoxides, appear within 7 min, samples may be injected consecutively at 12 min intervals. Parts B and C of Figure 1 show the corresponding UV absorption chromatograms at 234 and 292 nm, respectively. Peaks at RT = 29.3, 31.6, 33.5, 36.9, and 40.4 min represent TAG-dimers/TAGOOH-dimers, TAG/TAGOOH, CE/CEOOH, tocopherols, and BHT, respectively. The tocopherols were detected only at 292 nm (Figure 1C). As judged from the UV chromatogram at 234 nm (Figure 1B) the TAG constitutes ∼94% and the TAG-dimers and CE constitute ∼3% each. The 5 min difference between the UV and FL responses is due to the delay in the reaction and cooling coils.

Lipid Class Separation. Figure 1 further illustrates the cochromatography within the separated lipid classes, namely, TAG/TAGOOH and CE/CEOOH, despite a wide spectrum of fatty acids, ranging from 14:0 to 22:6*n*3. The simultaneous measurement of conjugated dienes (Figure 1B) and lipid hydroperoxides (Figure 1A) revealed no separation between oxidized and nonoxidized lipids, which indicates no interaction/affinity between the hydroperoxy group and the column matrix.

Figure 2 illustrates the separation and characterization of TAGOOH and CEOOH by HPSEC-FL. Figure 2A shows the separation of the references TAG18:2-OOH and CE18:2-OOH at RT = 36.5 and 38.4 min, respectively. Figure 2B corresponds to the 25−50 min part of Figure 1A. Parts C and D of Figure 2 represent HPSEC-FL analysis of TAG and CE, respectively, purified by TLC from the 4-week-stored Em-EDTA mayonnaise (Figure 2B) prior to mild autoxidation. The TAGOOH was characterized by cochromatography with reference TAG18:2-OOH, mayonnaise TAGOOH, and TLC separated and autoxidized mayonnaise TAG (parts A, B, and C of Figure 2, respectively), as also performed for CEOOH (Figure 2A,B,D). The additional peaks in parts C and D of Figure 2 represent compounds originating in autoxidation and concentration of the lipids. The compound at RT = 34.2 min in Figure 2C corresponds well with the TAGOOH-dimers, whereas the compounds at RT = 43.0 and 44.5 min in Figure 2D coelute with external reference compounds of FFAOOH and ChOOH, respectively. The identity of the TAGOOH and CEOOH classes was further verified in experiments in which samples were spiked with reference TAG18:2-OOH and CE18:2-OOH. Table 2 summarizes the retention times for reference 18:2-lipid compounds, for example, the hydroperoxides of TAG, DAG, CE, diacyl-GroPCho, and FFA and free cholesterol.

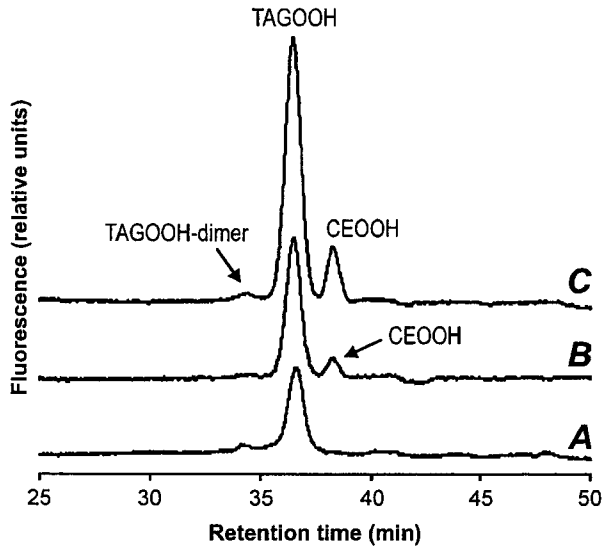

Figure 3. HPSEC-FL chromatograms for comparison of the oils before and after preparation of the mayonnaises: (A) rapeseed oil; (B) Em-EDTA mayonnaise stored for 4 weeks; (C) fish oil. The FL responses in (A) and (C) are enhanced 10-fold in comparison to their respective amounts in the mayonnaise. Retention times are as indicated in Table 2.

Figure 3 shows HPSEC-FL chromatograms of the lipid hydroperoxides in the bulk rapeseed oil and fish oil (parts A and C of Figure 3, respectively) together with the oil phase of the Em-EDTA mayonnaise after 4 weeks of storage (Figures 3B and 2B). The elution patterns of TAGOOH and CEOOH from rapeseed oil and fish oil compared to the oil phase of the mayonnaise further verify the class separation. As expected, the fish oil initially had a higher level of TAGOOH than the rapeseed oil and revealed substantial amounts of CEOOH.

Detection of Lipid Hydroperoxides. The DPPP detection was reproducible in the range 40−2000 pmol for the reference TAG18:2-OOH, as indicated by relative standard deviations (RSD) <1% for triple injections and a correlation coefficient >0.999 obtained with nine different amounts. The detection limit was 5.0 pmol of TAG18:2-OOH (signal-to-noise ratio ∼3). The FL response of 250 pmol of TAG18:2-OOH was stable for at least 20 h of analysis, as indicated by an RSD <2% determined by double injections every 2.5 h. Furthermore, samples were also stable in this time period, as indicated by an RSD of 2.5% for triple injections of 20 identical samples.

Comparison of HPSEC-UV/FL and Colorimetric Microassay. A fish oil enriched mayonnaise, stored for 4 weeks at 20 °C, was analyzed by the HPSEC-FL method and the colorimetric microassay. Table 3 shows PV obtained by the two methods along with the corresponding SD and RSD. The intra- and inter-assay variations for the HPSEC-FL system were 0.64 and 7.2%, respectively, as judged by triple injections on three oil solutions from the same mayonnaise. The corresponding average RSD for the intra- and inter-assay variations of the colorimetric microassay were 11.5 and 16.9%, respectively, indicating significantly less reproducible results ($p < 0.001$ and 0.01, respectively). The two methods gave similar PV profiles, but the levels of PV obtained by the colorimetric microassay were consistently lower (1.0−1.3 mequiv/kg of oil).

Effect of the Supplementations. The HPSEC-FL chromatograms showed that all of the mayonnaises contained TAGOOH and CEOOH (Figure 1A). Some samples also contained traces of TAGOOH-dimers; that is, the responses were below the quantification limit (signal-to-noise ratio ∼10).

Table 3. Comparisons between Determination of TAGOOH by HPSEC-FL and PV by Colorimetric Microassay

	TAGOOH by HPSEC-FL		PV by colorimetric microassay	
storage time	PV ± SD (mequiv/kg)	RSD (%)	PV ± SD (mequiv/kg)	RSD (%)
	Intra-assay[a]			
week 0	1.85 ± 0.01	0.32	0.52 ± 0.09	17.0
	1.57 ± 0.01	0.84	0.53 ± 0.05	9.4
	1.39 ± 0.01	0.68	0.42 ± 0.04	10.3
week 1	3.48 ± 0.00	0.14	1.86 ± 0.29	15.6
	3.16 ± 0.04	1.21	1.97 ± 0.07	3.7
	3.00 ± 0.01	0.36	2.11 ± 0.16	7.6
week 2	3.07 ± 0.00	0.16	1.86 ± 0.08	4.1
	2.60 ± 0.03	0.97	1.60 ± 0.44	27.4
	2.70 ± 0.04	1.46	1.99 ± 0.16	8.1
week 3	5.13 ± 0.00	0.08	4.84 ± 0.14	2.8
	5.16 ± 0.00	0.10	3.21 ± 0.21	6.5
	4.97 ± 0.06	1.19	3.08 ± 0.30	9.8
week 4	2.84 ± 0.01	0.45	1.27 ± 0.32	25.1
	2.42 ± 0.02	1.00	1.33 ± 0.20	14.9
	2.75 ± 0.02	0.59	1.67 ± 0.17	10.1
	Inter-assay[b]			
week 0	1.60 ± 0.20	12.6	0.49 ± 0.08	15.3
week 1	3.22 ± 0.21	6.6	1.98 ± 0.20	10.2
week 2	2.79 ± 0.22	7.8	1.82 ± 0.29	16.2
week 3	5.09 ± 0.09	1.8	3.71 ± 0.87	23.4
week 4	2.67 ± 0.19	7.1	1.43 ± 0.28	19.5

[a] Intra-assay variations, analyses of reproducibility of the triplicate determination of PV in the same oil phase solution. $p < 0.001$ between RSD of values obtained by the two methods. [b] Inter-assay variations, analyses of reproducibility of the triplicate determinations of PV in different preparations of the same oil phase. $p < 0.01$ between RSD of values obtained by the two methods.

Figure 4 illustrates the progress in the oxidation of the mayonnaises during storage, judged from the concentration of TAGOOH (PV in milliequivalents per kilogram) in the corresponding oil phases. The inter-assay variation was 4.5%. Reference mayonnaise with and without emulsifier revealed the expected autoxidation pattern (Em-Ref and Ref), that is, an exponential increase in the level of lipid hydroperoxides (except for Ref in week 2) followed by a decreasing level due to an increased ratio between the rates of decomposition and formation. Supplementation with the polar antioxidant, gallic acid, with and without emulsifier (Em-GA and GA), significantly decreased the levels of PV ($p < 0.05$); however, both mayonnaises revealed significant increments in PV during storage ($p < 0.05$). Supplementation with the metal chelator, EDTA, with and without emulsifier (Em-EDTA and EDTA), significantly suppressed the levels of PV ($p < 0.05$), which were almost constant throughout the storage period. Supplementation with the emulsifier, Panodan TR DATEM, revealed slightly but significantly decreased levels of PV ($p < 0.05$) when compared with all of the appropriate controls.

Figure 5 illustrates the progress in oxidation of the mayonnaises during storage, judged from the concentration of CEOOH (PV in milliequivalents per kilogram) in the corresponding oil phases. The amounts of CEOOH were very low, that is, 0.11−0.34 mequiv/kg, inter-assay variation of 8.0%. Mayonnaises with and without emulsifier revealed the expected autoxidation pattern as also

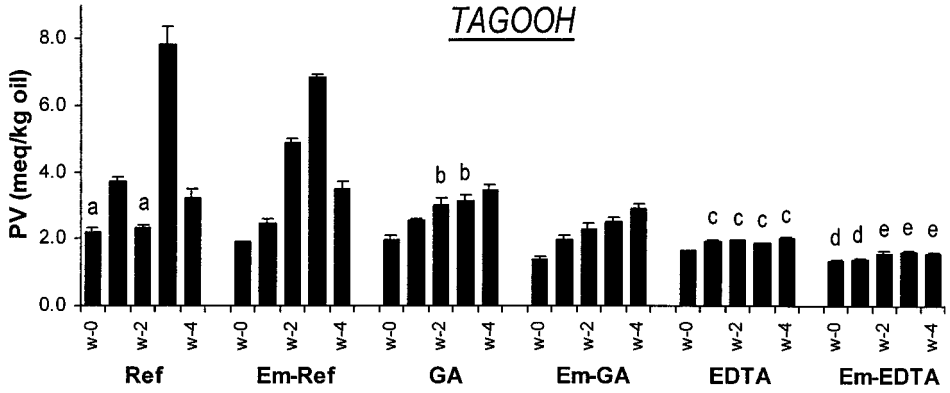

Figure 4. Histogram showing the weekly variations in TAGOOH levels from the 20 °C stored mayonnaises with supplementations of emulsifier, gallic acid, and EDTA. Columns with the same letters are not significantly different ($p > 0.05$). Comparisons are performed only within groups or with and without emulsifier at corresponding times, and only insignificant differences are indicated. Abbreviations are as given in Table 1.

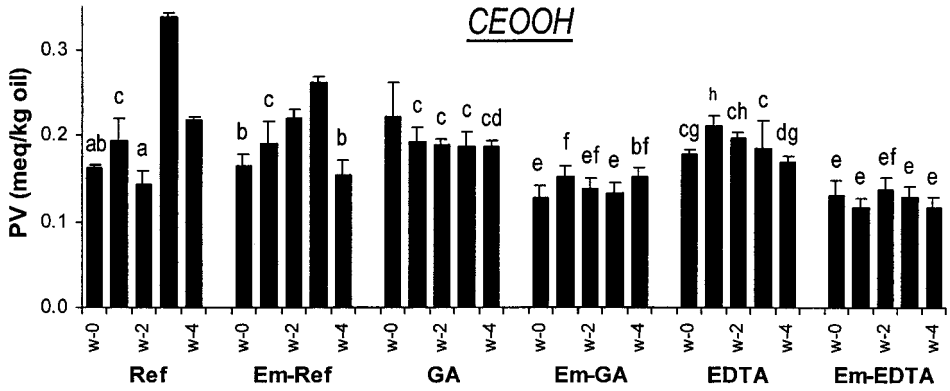

Figure 5. Histogram showing the weekly variations in CEOOH levels from the 20 °C stored mayonnaises with supplementations of emulsifier, gallic acid, and EDTA. Columns with the same letters are not significantly different ($p > 0.05$). Comparisons are performed only within groups or with and without emulsifier at corresponding times, and only insignificant differences are indicated. Abbreviations are as given in Table 1.

observed for TAGOOH (Em-Ref and Ref), with an especially pronounced effect of the emulsifier at the end of the storage period. The mayonnaises supplemented with gallic acid and EDTA, with and without emulsifier (Em-GA, GA, Em-EDTA, and EDTA), showed no significant differences in the levels of CEOOH during storage; however, supplementation with emulsifier significantly decreased the levels of CEOOH as compared with the appropriate controls ($p < 0.05$).

DISCUSSION

Neutral Lipid Hydroperoxides in Mayonnaises. The mayonnaise oil phases were shown to have detectable amounts of TAG/TAGOOH, CE/CEOOH, and TAG-dimers/TAGOOH-dimers and tocopherols (Figure 1). The lipids in commercial mayonnaises originate from vegetable oils and egg yolk. Enrichment with fish oil for nutritional benefits makes the products more susceptible to lipid oxidation due to the contents of 20:5*n*3 and 22:6*n*3 fatty acids. The fish oil and rapeseed oil both consist of approximately 95% TAG, 3% CE/sterol esters, 2% Ch/sterols, and traces of FFA, as judged by TLC and HPSEC-UV. The oil phase of the mayonnaises contained 94% TAG and 3% CE, whereas no detectable amounts of free Ch/sterols were found, as judged by TLC and HPSEC-UV (Figure 1B), probably due to their association with the oil−water interface of mayonnaise. The egg yolk neutral lipids have a low degree of unsaturation (Kuksis, 1992) corresponding to <1 wt %, which is why hydroperoxides from egg yolk neutral lipids may be neglected.

Lipid Class Separation. Several reverse- and normal-phase HPLC systems, using isocratic elution (Yamamoto et al., 1987; Akasaka et al., 1992; Miyazawa et al., 1995) and gradient elution (Akasaka et al., 1993a, 1999), combined with selective and sensitive detection principles for lipid hydroperoxides have been reported for separation of reference compounds such as TAGOOH and CEOOH. However, application of these systems to the oil phase of mayonnaise results in separation of the individual lipid hydroperoxide species within the classes, due to the differences in polarity caused by the wide spectrum of fatty acids, that is, 14:0 to 18:3*n*3 from rapeseed oil, 14:0 to 22:6*n*3 from fish oil, and 14:0 to 20:4*n*6 from egg yolk. Therefore, partition and adsorption chromatography were not found to be suitable.

The present HPSEC-UV/FL system performed separation of the neutral lipid classes including the hydroperoxides, that is, TAG-dimers, TAG, DAG, CE, Ch, and FFA, of vegetable, marine, and mayonnaise-derived oil, without separation of the individual species (Figures 1−3). Furthermore, compounds such as tocopherols, BHT, diacyl-GroPCho, and sample solvents, usually encountered, were also resolved (Figures 1−3 and Table 2).

Separation by HPSEC is dependent on the hydrodynamic volume of the molecular species, of which the

molecular weight may serve as an approximate index (Barth, 1998). Separation was not strictly governed by size exclusion, because interactions between polar compounds and the column matrix occurred; for example, 16:0/18:2-GroPCho showed a relatively long retention time compared to its molecular weight (Table 2). Addition of 5% methanol in dichloromethane indicated such interactions, as the phospholipid eluted 8 min earlier and thereby coeluted with TAG18:2. Thus, the chromatographic separation is sensitive to polar additives in the mobile phase. The obtained separation and resolution using dichloromethane are in accordance with the results by Hopia et al. (1992) using tetrahydrofuran.

Detection of Lipid Hydroperoxides. Dichloromethane was chosen as eluting solvent because the solvents normally used for HPSEC, for example, tetrahydrofuran and toluene, were incompatible with the DPPP detection principle, probably due to unspecific oxidation of DPPP by solvent impurities, unremovable by glass distillation. Dichloromethane exerts a strong UV absorption, with a cutoff at 226 nm, which renders UV detection of isolated double bonds at 205 nm impossible and of conjugated dienes at 234 nm difficult. Due to the class separation the lipids detected at 234 nm in this study are mainly nonoxidized. In flow injection analysis, dichloromethane decreased the sensitivity by a factor 10 in comparison with 4% 1-butanol in glass-distilled hexane (Jacobsen et al., 2000a). However, substituting parts of the dichloromethane either interfered with the chromatographic resolution (e.g., methanol and 1-butanol) or had no effect on the DPPP detection (e.g., glass-distilled hexane). The high amounts of applicable lipid (>1 mg) to the size exclusion columns compensates for the 5-fold higher detection limit for TAG18:2-OOH as compared with the method described by Akasaka et al. (1999). Therefore, further optimization of the DPPP detection principle was not pursued. Miyazawa et al. (1995) have shown that only monohydroperoxy triacylglycerols were detected in soybean oil at PV < 10 mequiv/kg of oil, which was therefore also anticipated for the oil phase of fish oil enriched mayonnaise. The monohydroperoxide reference, TAG18:2-OOH, was used for quantification of both TAGOOH and CEOOH, as TAG18:2-OOH and CE18:2-OOH yield similar molar responses within RSDs of 4% (Akasaka et al., 1993b); furthermore, the response factor may vary considerably within the same lipid class due to different contents of fatty acid species (Akasaka et al., 1992, 1993b).

Previously, Christensen and Hølmer (1996) determined the level of lipid hydroperoxides in butter and dairy spreads using reverse-phase HPLC and luminol−cytochrome *c* chemiluminescence detection as described by Yamamoto et al. (1987) and Miyazawa et al. (1987), respectively. However, application of this system to the oil phases of mayonnaise revealed difficulties due to resolution within lipid classes and the cochromatography with the tocopherols, causing chemiluminescence quenching. Although the chemiluminescence detection was very sensitive, with a detection limit of 0.1 pmol of TAG18:2-OOH, the responses were time-dependent with resulting inappropriate inter-assay variations in contrast to the DPPP detection.

Comparison of HPSEC-UV/FL and Colorimetric Microassay. The major difference between the results obtained by the two methods lies in the intra-assay variations (Table 3). Sample handling in the analysis of lipid hydroperoxides is critical, even when sample processing is fast and simple, as indicated by the large difference between intra- and inter-assay variations obtained with the HPSEC-FL method. Regardless of the higher reproducibility of the HPSEC-FL method, the colorimetric microassay qualitatively gave similar PV profiles (Table 3); however, the levels of PV were consistently lower (1.0−1.3 mequiv/kg). In addition to the longer sample handling, the significantly larger intra- and inter-assay variations may be due to interference with the sample matrix or the low pH (4.0) during measurements by the colorimetric microassay.

Effect of Supplementations. The oxidation processes in the six mayonnaises were monitored by HPSEC-UV/FL to assess the effects of supplementation with emulsifier, gallic acid, and EDTA (Table 1). The results indicated that the natural content of tocopherols in the rapeseed oil and fish oil was unable to protect the mayonnaise from oxidative deterioration (Ref and Em-Ref; Figure 4). This was also concluded in our previous study, showing an exponential increase of volatile secondary oxidation products, as assayed by a dynamic headspace gas chromatography−mass spectrometry method (Hartvigsen et al., 2000) or several other methods including sensory assessment (Jacobsen et al., 2000b). The low level of PV in week 2 for Ref was unexpected, but reproducible, as a corresponding Ref showed the same pattern, following analysis both by HPSEC-UV/FL and colorimetric microassay (inter-assay variations; Table 3). The corresponding mayonnaise supplemented with emulsifier (Em-Ref) showed the expected exponential oxidation progress, followed by a decrease in PV (Em-Ref; Figure 4). This irregular variation in PV may be ascribed to a "sample effect" during storage.

All of the supplementations resulted in lower levels and impaired progressions of PV than in Ref mayonnaise during storage. However, beyond progress in oxidation, a low PV may be the result of a prooxidant, which primarily enhances the decomposition of the lipid hydroperoxides and thereby the formation of secondary oxidation products. One example is ascorbic acid and its ability to form complexes with Fe(II), which is more active than Fe(III) in the homolytic decomposition of lipid hydroperoxides (Kanner et al., 1977; Jacobsen et al., 1999b). Therefore, further investigations on, for example, the volatile profiles of the secondary oxidation products have been conducted to assess the antioxidative effect of the supplementations (Jacobsen et al., 2000c).

The primary antioxidant, gallic acid, decreased the ratio between the rates of formation and decomposition of the lipid hydroperoxides (Ref versus GA; Figure 4); however, the pro- or antioxidative effects of GA cannot be evaluated from the present results only. The secondary antioxidant, EDTA, is known to chelate metal ions, especially Fe(II) and Fe(III) (Decker, 1998), and may thereby inhibit this redox system and its participation in the initiation of lipid oxidation through the Fenton-type reactions, which are believed to be the predominant initiation reaction in emulsions (Frankel, 1998). The results indicate that EDTA is a potent antioxidant in mayonnaise (Ref versus EDTA; Figure 4), probably acting through the aforementioned chelation of iron. The emulsifier, Panodan TR DATEM, seems to delay the oxidation through presently not clarified mechanisms (Figure 4), which is why it is concluded that Panodan

TR DATEM may contribute to the oxidative stability of mayonnaise.

The oil phase of the mayonnaises consists of ~94% TAG and only ~3% CE/sterol esters; nevertheless, substantial amounts of CEOOH were detected and quantified, that is, on average 6.8% of the total amounts of lipid hydroperoxides. This indicates that CE was more susceptible to oxidation than TAG, as also seen for fish oil (Figure 3C). The oxidation progress for the CEOOH levels was not as evident as for the TAGOOH levels. As mayonnaise CE and TAG are believed to be located predominantly within the core of the lipid droplet of the oil-in-water emulsion system, they are consequently assumed to follow the same oxidation progress. Considering the low levels of CEOOH, the actual variations may be too small to be detected by the present method without further optimization. The sensitivity could be improved by using a mobile phase of at least 5% methanol in dichloromethane; however, this affects separation of other lipid classes and may thereby cause additional problems.

In conclusion, the present HPSEC-UV/FL method is reliable and rapid and may become a powerful tool in monitoring the oxidation processes by determination of the concentrations of neutral lipid class hydroperoxides in a variety of lipids and lipid systems from different food products or biological materials of vegetable, animal, and marine origin. In our laboratory, this method has also been successfully applied to several types of dairy products.

ABBREVIATIONS USED

BHT, 2,6-di-*tert*-butyl-*p*-cresol; CE, cholesterol ester; Ch, cholesterol; DAG, diacylglycerol; DPPP, diphenyl-1-pyrenylphosphine; EDTA, ethylenediaminetetraacetate; Em, emulsifier; FFA, free fatty acid; FL, fluorometric; GA, gallic acid; GroPCho, glycerophosphocholine; HPLC, high-performance liquid chromatography; HPSEC, high-performance size exclusion chromatography; PV, peroxide value; Ref, reference mayonnaise; RSD, relative standard deviation; RT, retention time; TAG, triacylglycerol; TAG18:2-OOH, reference trilinoleoylglycerol monohydroperoxide; TLC, thin-layer chromatography.

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