Electron Spin Resonance Spectroscopy for Determination of the Oxidative Stability of Food Lipids

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ABSTRACT: Evaluation of the oxidative stability of food lipids based on the tendency of formation of radicals is shown to be possible using electron spin resonance (ESR) spectroscopy and the spin-trapping technique. Induction time can be determined for mildly accelerated conditions (50°C for lipid fraction from mayonnaise enriched with fish oil), and the length of the induction time decreases during storage and γ-tocopherol depletion. The protection by ethylenediaminetetraacetic acid against initiation of lipid oxidation is also detected in the new assay. For more oxidatively stable lipids (butter, rapeseed oil, dairy spread) the mildly accelerated conditions can be used in the assay, provided that difference in signal height for fixed times replaces determination of induction time. ESR spin trapping provides a sensitive method for evaluating the oxidative stability of food lipids. Detection of radicals in the lipid as an early event in oxidation allows mild conditions to be used, and future experiments should also include sensory evaluation in relation to determination of practical shelf life.

Paper no. J9359 in *JAOCS 77*, 725–730 (July 2000).

KEY WORDS: Electron spin resonance, ESR, food lipids, lipid oxidation, peroxide value, radicals, spin trapping.

Especially in highly processed foods, lipid oxidation is one of the major deteriorative reactions, resulting in undesirable flavors and formation of toxic oxidation products and further affecting the nutritional value negatively (1). Accordingly it is important to predict the oxidative stability of a given food by rapid and reliable methods in order to determine shelf life and to evaluate the effect of protective antioxidants. Stability testing at ambient storage conditions is often too slow for practical use in quality control, and consequently the oxidative stability is tested at forced conditions using high temperature or addition of prooxidants. Several accelerated methods are routinely used to characterize the stability of food lipids such as the Schaal oven test, the Rancimat method, and the active oxygen (AO) method. However, these methods are often criticized for giving wrong predictions (1). The Rancimat method involves heating of the sample to a high temperature (100–140°C) and measuring the levels of volatile acids produced by oxidation. The mechanism of oxidation changes significantly at elevated temperatures due to large differences in activation energies for various reactions, making comparison to oxidation at normal storage conditions unreliable (1). Furthermore, levels of volatile acids are only significant at elevated temperatures and may not be relevant to normal storage conditions. The Schaal oven test involves a sensory panel, which periodically examines the sample, heated to 50–60°C, until a definite rancid flavor develops. It requires minimal laboratory equipment, but large samples and a trained sensory panel, and furthermore it takes a relatively long time (4–8 d) to complete each evaluation (2).

Electron spin resonance (ESR) spectroscopy is a new technique in food science but has already proven to be a valuable tool in the study of the very early stages of oxidation in different kinds of products. In beer a rapid method for prediction of the flavor stability using ESR spectroscopy has been developed based upon the spin-trapping technique (3,4), and for whole milk powder (5) and cheese (6) direct ESR measurement seems promising for prediction of the oxidative stability during subsequent storage. Thermal decomposition of grape seed oil has also been studied using the spin-trapping method (7). For food emulsions like mayonnaise, it became possible to quantify the rate of production of radical precursors for hydroperoxides and secondary lipid oxidation products (8). In contrast to most other methods used in food science, ESR spectroscopy provides the possibility of studying the very early stages of lipid oxidation. At this stage the sensory attributes of foods are still unchanged and by evaluating the oxidative status of a given food at this early stage, action can be taken to change process parameters before the consumers experience a deteriorated product.

The objective of this study was to evaluate whether it is possible to develop a rapid test in which the resistance to oxidation can be expressed as an induction time for formation of radicals using only mildly accelerated conditions with a significantly lower temperature compared to the existing alternatives for determination of the oxidative stability of pure food lipids or foods containing high amounts of fat. Rapeseed oil, oil separated from mayonnaise containing fish oil, and the lipid fraction separated from butter, and dairy spread as four

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examples of foods with a different resistance to oxidation were used to establish this accelerated test based on ESR.

EXPERIMENTAL PROCEDURES

Materials. *N-t*-Butyl-α-phenylnitrone (PBN) was obtained from Molecular Probes Inc. (Eugene, OR) and was used as received. Di-*tert*-butylnitroxide was from Sigma (St. Louis, MO). Delios® V MCT (medium-chain triglycerides) oil was from Brøste A/S (Lyngby, Denmark). Barium chloride dihydrate, iron(II) sulfate heptahydrate, ammonium thiocyanate, hydrochloric acid (32%), and hydrogen peroxide (30%) were obtained from Merck (Darmstadt, Germany), and chloroform and methanol from Labscan Limited (Dublin, Ireland). All were of analytical grade and were used as received. Refined, unhydrogenated rapeseed oil was obtained from Aarhus Olie A/S (Aarhus, Denmark). Raw fish oil was obtained from Esbjerg Fiskeindustri (Esbjerg, Denmark). The fish oil was refined and deodorized at the pilot plant of Department of Biotechnology, Technical University of Denmark. Liquid frozen egg yolk with 3% salt (NaCl) was from Danæg (Copenhagen, Denmark). Tarragon vinegar (7%) was purchased from A/S Dansk Eddikecentral (Copenhagen, Denmark). Lemon juice was from ItalLemon® (Milano, Italy). Potassium sorbate was purchased from Merck and ethylenediaminetetraacetic acid (EDTA) was from Sigma (Steinheim, Germany). The food-grade stabilizer Grindsted FF DC was donated by Danisco Ingredients (Brabrand, Denmark).

Gas chromatrography (GC) analysis. The fatty acid composition of the lipids was determined from their methyl esters by GC using a 5890 A-II chromatograph (Hewlett-Packard Co., San Fernando, CA) with HP-FFAP column (Hewlett-Packard Co.) with the dimensions 25 m × 0.20 mm × 0.33 μm. The following conditions were employed for all samples: injector temperature at 250°C; flame-ionization detector temperature at 300°C; flow 0.95 mL/min; and helium as carrier gas with a split ratio of 1:10. For rapeseed oil and lipid from dairy spread and butter, the following oven program was employed: 50°C for 1 min; from 50 to 180°C at 15˚C/min; from 180 to 220°C at 5°C/min; 220°C for 10 min. For mayonnaise the oven program used was: 50°C for 1 min; from 50 to 180°C at 10°C/min; from 180 to 220°C at 2.5°C/min; 220°C for 15 min.

Peroxide value (PV). PV, defined as meq O_2/kg fat, was determined according to Reference 9. The sample was dissolved in a 70:30 mixture of chloroform/methanol, and iron(II) chloride and ammonium thiocyanate were added and the absorbance of the red Fe(III)-complex measured at 500 nm on a Cintra 40 spectrophotometer (GBC Scientific Equipment Pty. Ltd., Victoria, Australia).

Analysis of tocopherols. The amount of α- and γ-tocopherols in the lipid phases was analyzed by liquid chromatography (HPLC) with fluorescence detection using an external standard. To samples of lipid (50 mg) was added 2 mL pyrogallol (5 g/L absolute ethanol), and the samples were saponified with 0.5 mL KOH (500 g/L) at 70°C for 30 min in a water bath. The samples were cooled on ice and then extracted twice with hexane containing butylated hydroxytoluene (0.01 g/L). The pooled hexane extracts were evaporated under nitrogen and resuspended in absolute ethanol using 5 mL for lipid from mayonnaise and rapeseed oil and 0.5 mL for lipid samples originating from butter and dairy spread. Quantification of tocopherols in samples was performed using a 250 mm × 4.6 mm, 5μ C18 stainless column (Chrompack, Middleburg, The Netherlands) using methanol/H_2O (97:3) (1.7 mL/min) as the mobile phase. Excitation was at 292 nm and emission at 330 nm.

Food samples and storage experiments. Two mayonnaises containing 0 and 75 ppm EDTA were produced as previously described (10): rapeseed oil (64% w/w), fish oil (16% w/w), egg yolk (4.0% w/w), NaCl (0.3% w/w), sugar (1.0% w/w), vinegar (4.0% w/w), lemon juice (1.2% w/w), potassium sorbate (0.1% w/w), Grindsted FF DC as stabilizer (0.2% w/w), and distilled water (9.2% w/w). Mayonnaises were stored at 20°C for 0, 2, and 4 wk in the dark and subsequently frozen for a minimum of 24 h in order to separate the phases. After thawing, the oil phase was centrifuged for 15 min at 2,000 rpm and 10°C to remove traces of water. The pure oil was frozen and kept at –50°C until further analysis.

Rapeseed oil was stored at 20°C for 0, 2, and 4 wk in the dark and after storage was frozen and kept at –50°C until further analysis.

Cultured butter (800 g fat/kg, 10 g salt/kg) and dairy spread (800 g fat/kg, 10 g salt/kg) based on milk fat and rapeseed oil (3:1) were bought locally and kept at –50°C until further analysis. Thawing lasted for 24 h in a refrigerator at 5°C after which the products were melted at 65°C for 1 h. After melting, the fat was separated by centrifugation for 15 min at 2,000 rpm at room temperature. The lipid fractions were kept at –50°C until further analysis.

Rapeseed oil and oil separated from mayonnaise containing 0 ppm EDTA (0 wk samples) and lipid fractions from butter and dairy spread were used for optimizing the temperature conditions in the ESR measurements (see below).

ESR spectroscopy measurements. Differences in the fatty acid composition of the four food products made it necessary to determine the optimal temperature conditions for each lipid sample prior to actual measurements of induction times for formation of radicals. Samples of all four types of food were tested at the following temperatures: 50, 60, 70, and 80°C and the lipid fraction of mayonnaise also at 55°C, with 10 mg PBN dissolved in 10 g lipid sample in each case. For each lipid sample, aliquots were filled into 10 ESR-tubes (700-PQ-7, heavy wall; Wilmad Glass, Buena, NJ) and placed in a water bath at the given temperature. Every 30 min, one tube was transferred from the water bath to an ECS 106 ESR spectrometer (Bruker, Rheinstetten, Germany), and the ESR spectrum was recorded. The following parameters were used in all ESR measurements: microwave power, 20 mW; modulation amplitude, 1.0 G; modulation frequency, 100 kHz; conversion time, 40.96 ms; time constant, 81.92 ms. For each spectrum, the peak-to-peak amplitude (A_{pp}) of the first line was determined by the use of the Winepr software program (Bruker, Billerica, MA) (see Fig. 1),

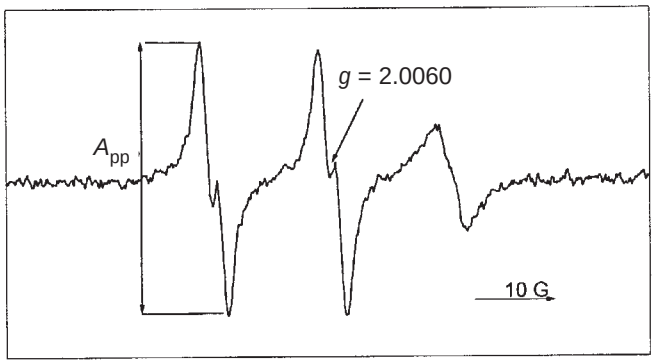

FIG. 1. Electron spin resonance spectrum of the spin-trap N-*t*-butyl-α-phenylnitrone in rapeseed oil after 278 min at 70°C. The *g*-value was determined to be 2.0060. The peak-to-peak amplitude A_{pp} is determined as shown.

and these relative values were plotted against time to show the development of the ESR signal at each temperature. The limit of detection was defined as the mean blank signal plus three times the standard deviations. For the actual instrument parameters, the blank value was 500 in average on a relative scale with a standard deviation of 50, resulting in a limit of detection of 650. To determine the induction time for production of radicals in the mayonnaise and rapeseed oil stored at different times, the same procedure for measurements was followed. All measurements were made in duplicate.

RESULTS AND DISCUSSION

Characterization of the food lipids. Four different types of food lipids were investigated using the ESR method. Mayonnaise is an oil-in-water emulsion in contrast to butter and dairy spread, which both are water-in-oil emulsions, the latter product with vegetable oil added to the milk fat. Rapeseed oil was included in the investigation as an example of a cooking oil. The main composition of the lipids studied is summarized in Table 1. The four lipids have different fatty acid profiles as expected (11,12). The mayonnaise lipid contains a significant amount of long polyunsaturated fatty acids (PUFA) due to the inclusion of 20% fish oil. Butter contains a very low amount of PUFA, and rapeseed oil is characterized by a high content of the monounsaturated oleic acid. The fatty acid profile of the dairy spread reflects the partial substitution of milk fat with rapeseed oil. The ratios of PUFA to saturated fatty acids (SAT) are 2.43, 4.73, 0.26, and 0.08 for lipid from mayonnaise, rapeseed oil, dairy spread, and butter, respectively. However, the lipid fraction from mayonnaise contains a significantly higher amount of eicosapentanoic acid (EPA) + docosahexanoic acid (DHA) compared to the other lipids (Table 1), and based on an overall consideration the anticipated order of oxidation stability is accordingly butter > dairy spread > rapeseed oil > mayonnaise for equal storage conditions.

The PV of the products are typical for these types of products (Table 1), and the relatively high PV for mayonnaise oil

TABLE 1
Fatty Acid Composition, Content of α- and γ-Tocopherols, and Peroxide Values of the Food Lipids Studied[a]

	Mayonnaise	Rapeseed oil	Butter	Dairy spread
C < 14	n.d.	n.d.	9.65	6.66
14:0	1.48 (0.01)	0.06 (0.01)	11.73 (0.04)	8.35 (0.03)
15:0	n.d.	n.d.	1.17 (0.02)	0.82 (0.01)
16:0	8.43 (0.01)	4.78 (0.01)	33.61 (0.02)	25.03 (0.31)
18:0	2.45 (0.02)	1.62 (0.01)	11.57 (0.01)	8.54 (0.18)
20:0	n.d.	n.d.	0.21 (0.01)	0.32 (0.01)
SAT	12.36	6.46	67.93	49.72
14:1	n.d.	n.d.	0.28 (0.01)	0.23 (0.02)
16:1	2.98 (0.02)	0.25 (0.01)	1.79 (0.01)	1.38 (0.02)
18:1	53.30 (0.01)	61.14 (0.03)	24.51 (0.06)	35.18 (0.80)
22:1	0.73 (0.01)	0.60 (0.03)	n.d.	0.28 (0.01)
MUFA	57.00	61.99	26.58	37.17
18:2n-6	18.12 (0.01)	20.03 (0.01)	2.88 (0.01)	8.26 (0.19)
18:3n-3	8.19 (0.01)	9.96 (0.06)	0.90 (0.07)	3.62 (0.08)
18:4n-3	0.93 (0.01)	n.d.	0.57 (0.01)	0.41 (0.01)
20:4n-6	n.d.	n.d.	0.17 (0.01)	n.d.
20:5n-3	1.41 (0.01)	n.d.	n.d.	n.d.
22:6n-3	1.37 (0.01)	0.54 (0.04)	0.62 (0.01)	0.63 (0.03)
PUFA	30.02	30.53	5.14	12.92
EPA + DHA	2.78	0.54	0.62	0.63
PUFA/SAT	2.43	4.73	0.08	0.26
PV[b]	1.28 (0.01)	1.14 (0.05)	0.45 (0.03)	0.54 (0.03)
α-Tocopherol[c]	189.02 (6.46)	241.39 (7.55)	15.64 (0.94)	86.93 (3.54)
γ-Tocopherol[c]	253.86 (10.28)	277.34 (7.66)	n.d.	92.36 (2.51)

[a]Expressed as mass percentage. Values are expressed as mean ± standard deviation where means are results of duplicate determinations. SAT, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated acid; EPA, eicosapentanoic acid (20:5n-3); DHA, docosahexanoic acid (22:6n-3); n.d., not detected.
[b]Peroxide value (PV) expressed as milliequivalents O_2 per kg anhydrous lipid. Means are results of duplicate determinations with standard deviation in parentheses. The peroxide values are based on the International Dairy Federation method (9), and the numbers should be divided by a factor of two to be comparable with the AOCS method (18).
[c]The amount is expressed as μg /g lipid. Means are result of duplicate determination.

reflects the high content of PUFA and the treatment of the oil before PV analysis. The content of α- and γ-tocopherol in the food lipids (Table 1) is within normal range for this type of product, with only α-tocopherol in an appreciable amount in butter (13); the γ-tocopherol found in the dairy spread originates from the rapeseed oil.

Optimization of assay temperature. The temperature screening ESR experiments demonstrated that PBN spin adducts could be detected in all lipid samples at elevated temperatures. All observed signals were triplets of doublets, as shown for rapeseed oil in Figure 1, with a hyperfine constant of 14.78 G for a_N. Only rudiments of the hyperfine splitting into doublets ($a_H \sim 2.1$ G) were found and not in all spectra recorded, which can be ascribed to the decreased mobility of the radicals due to the viscous nature of the oils. To determine the g-value (the proportionality factor for resonance for the radical) of the spin adducts, the radical di-*tert*-butyl nitroxide was used as a reference, since the g-value for this species is well known in a wide variety of solvents (14). The g-value of the PBN spin adducts was determined to be 2.0060, which is typical for nitroxyl radicals (15).

An induction time, defined as the period of time where radicals are formed very slowly before a sudden sharp linear increase in signal intensity (A_{pp}), was experienced and is shown for rapeseed oil at three different temperatures in Figure 2. This pattern of signal intensity resembled what is commonly experienced in other accelerated oxidative tests (2) and is also experienced for accelerated oxidation of beer (3). The parameter Δ(peak-height), defined as [A_{pp}(end) – A_{pp}(start)] turned out to be a suitable measure of formation of radicals as well, especially in cases where almost no radicals were formed during the 250 min selected as assay time. Under such conditions, no induction time could be determined directly because of very weak signals, but Δ(peak-height) could be used as an alternative parameter. It was found that the induction time and the intensity of the ESR signal were strongly product- and temperature-dependent. Hence, the temperature dependence of the individual lipids was investigated in order to determine a suitable temperature for each individual food product. In Table 2 the induction times and Δ(peak-height) for the four types of lipids at 50 and 80°C are summarized.

TABLE 2
Evaluation of Oxidative Stability Based on Induction Time and Signal Difference in ESR Spin-Trapping Assay for Rapeseed Oil and Lipid Fraction Isolated from Food Samples. Optimization of Temperature Conditions

	Induction time (min)		Δ(peak-height) (arbitrary units)[b]	
Sample type	50°C	80°C	50°C	80°C
Mayonnaise	95	~ 0	2250	82750
Rapeseed oil	>300	15	(550)	4550
Dairy spread	n.d.[a]	70	n.d.[a]	5200
Butter	n.d.[a]	70	n.d.[a]	2550

[a]ESR, electron spin resonance; n.d., not detected.
[b]Detection limit for Δ(peak-height) is 650.

Signals could be detected in rapeseed oil and in lipid samples from mayonnaise for 50°C and at higher temperatures with the highest signal intensity in lipid from mayonnaise. In contrast, no signal could be detected in butter and dairy spread at 50 and 60˚C for up to 250 min. For dairy spread, ESR signals could only be detected for lipid samples evaluated at 80°C; however Δ(peak-height) at 80°C for dairy spread was approximately twice the size of Δ(peak-height) for butter at the same temperature. Hence, based on these experiments, the oxidative stability of the examined lipids was butter > dairy spread > rapeseed oil > mayonnaise, being in good agreement with the conclusions drawn from the fatty acid composition and the tocopherol content, showing that the method can rank different food lipids according to their oxidative stability from the very early events of oxidation.

Storage experiments with mayonnaise and rapeseed oil. The assay temperature at which the induction time for formation of radicals was found to be approximately 100 min, as determined by a sharp increase in signal intensity, was selected for the evaluation of oxidation stability during storage of mayonnaise and rapeseed oil, i.e., 60°C for rapeseed oil and 50°C for mayonnaise. In Figure 3, the result of measurements on the lipid sample isolated from mayonnaise containing 0 ppm EDTA (week 0) is shown, and the induction time is marked together with

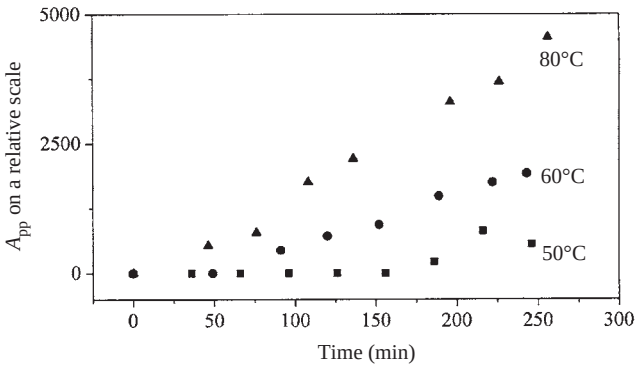

FIG. 2. Peak-to-peak amplitude (A_{pp}), as determined in Figure 1, plotted against assay time for rapeseed oil at 50, 60, and 80°C.

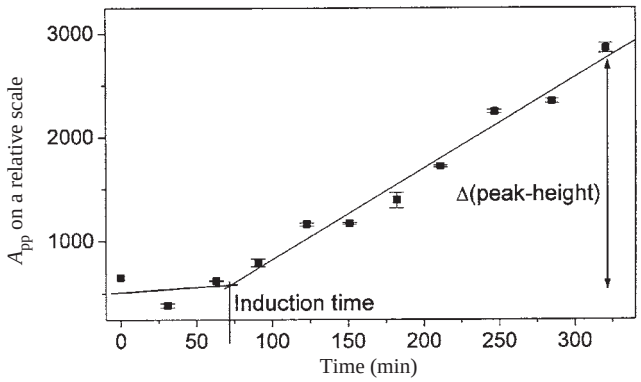

FIG. 3. A_{pp} plotted against assay time for lipid fraction isolated from mayonnaise containing 0 ppm ethylenediamine tetraacetic acid, stored for 2 wk at 20°C in the dark. Assay temperature was 50°C. The induction time is shown together with the Δ(peak-height), defined as described in the text. See Figure 2 for abbreviations.

TABLE 3
Evaluation of Oxidative Stability of Rapeseed Oil at 60°C and Lipid Fraction Isolated from Mayonnaise at 50°C During Storage at 20°C for 4 wk for Both Products, Based on Induction Time and Signal Difference in ESR Spin-Trapping Assay with 300 min Run Time

	Induction time (min)			Δ(peak-height) (arbitrary units)[b]			PV values (meq O_2/kg lipid)[c]		
Sample type	0 wk	2 wk	4 wk	0 wk	2 wk	4 wk	0 wk	2 wk	4 wk
ML[a], 0 ppm EDTA	94	70	0	1450	2,200	5,300	1.28	2.54	4.10
ML[a], 75 ppm EDTA	>300	>300	109	(350)	1,350	2,500	1.25	1.90	2.19
Rapeseed oil	>300	>300	>300	750	1,200	1,100	1.14	1.50	1.80

[a]ML = lipid separated from mayonnaise. PV, peroxide value.
[b]Detection limit for Δ(peak-height) is 650.
[c]For experiments with nonseparated mayonnaise, see Reference 11.

Δ(peak-height). In Table 3 the resulting induction times and Δ(peak-height) from the mayonnaise samples tested (0 or 75 ppm EDTA) in the storage experiment are shown. Mayonnaise containing no EDTA had significantly lower induction time and higher signal intensities compared to the lipid samples from mayonnaise containing EDTA, confirming previous findings (11). As can be seen from these results, a significant antioxidative effect of EDTA for lipid samples from mayonnaise was noted. The results further show that the new method can be used to detect the (small) changes in oxidative stability occurring during relative short-term storage. The content of α-tocopherol did not change during this storage, while γ-tocopherol showed a significant lowering in mayonnaise without EDTA added after 4 k (0 wk: 254 ppm, 2 wk: 255 ppm, and 4 wk: 207 ppm), showing that γ-tocopherol is oxidized before α-tocopherol, as has previously been reported for rapeseed oil (16). In the samples containing EDTA, the content of γ-tocopherol did not change during storage showing that EDTA is active in protecting against initiation of oxidation.

No significant change could be detected for rapeseed oil during the storage period applying the ESR method. Only small signal intensities were noted [Δ(peak-height) in Table 3], confirming that rapeseed oil oxidizes only slightly under the given conditions at 20°C. The PV (with standard deviation) increased likewise only moderately during storage: 1.14 (0.05), 1.50 (0.07) and 1.80 (0.07), meq O_2/kg lipid at week 0, 2, and 4, respectively, and the content of α- and γ-tocopherol did not change during the period, as reported for nonstripped rapeseed oil (17).

ACKNOWLEDGMENTS

The authors wish to thank Kirsten Metz and Lars Månsson for the excellent technical assistance. The work of MKT was financed by the Danish Association of Fish Meal and Fish Oil Manufacturers, Danisco Ingredients, Denmark and the Danish Food Research Program, FØTEK, through LMC-Center for Advanced Food Studies. The work of DK was part of a cooperation between MD Food Research and Development Center and the Danish Ministry of Food, Agriculture, and Fisheries.

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[Received August 23, 1999; accepted May 13, 2000]