Chemiluminescence as a Method for Oxidative Rancidity Assessment in Autoxidized Marine Oils

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Chemiluminescence based on light emission from excited oxygen species, *e.g.* singlet oxygen and triplet carbonyls, has been used to measure the oxidative rancidity of fish oils. The luminescence was recorded after sodium hypochlorite addition to the oils dissolved in *tert*-butanol. In addition to high sensitivity and ability to detect small changes in rancidity, the method is fast and may be used as a supplement to the standard chemical methods for quality assessment and antioxidant evaluation. However, care has to be taken in interpretation of the chemiluminescence data of different fish oils, as the light emission depends on both the composition and the rancidity level of the oil. The autoxidized oils were also characterized by peroxide value, thiobarbituric acid value, anisidine value, Kreis rancidity index, iodine value, ultraviolet measurements, capillary gas chromatography, size-exclusion chromatography and sensoric evaluation.

KEY WORDS: Anisidine value, antioxidant evaluation, autoxidation, chemiluminescence, fish oil, peroxide value, rancidity assessment, sensoric evaluation, thiobarbituric acid value, tocopherol.

Lipids become rancid as a result of autoxidative and hydrolytic reactions. In marine oils, which contain mainly long-chain fatty acids, hydrolysis is not important and deterioration is normally caused by autoxidation. Due to their high degree of unsaturation, marine lipids are especially vulnerable, with damage of product quality and formation of harmful substances as a result. Much work has therefore been carried out to determine the chemical, nutritional and metabolic aspects of rancid fats and oils (1–3).

Identification of the oxidation products and assessment of the rancidity level are thus important, and a variety of methods are available. Sensoric evaluation is most commonly used for quality evaluation of oils and is often regarded as the ultimate measure of rancidity as no analytical test is capable of assessing the composite sensory attributes of food. However, the method is expensive, and very often no correlation is found between the sensoric scores and the chemical methods used (4,5). The chemical methods available are also hampered by limitations (6–8). Determination of peroxide value (PV), although the most widely used method, is of limited value as a general method due to the transitory nature of the peroxides. Thiobarbituric acid value (TBAV), anisidine value (AV) and the Kreis test are used to determine secondary oxidation products formed from peroxides, but all methods are rather insensitive and nonspecific. As with the peroxides, the carbonyl compounds responsible for the TBAV, AV and Kreis test, may react further to form other secondary products, with a decrease in these rancidity indices as a result.

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During autoxidation, conjugated dienes are formed from polyunsaturated fatty acids. Due to the absorption around 230 nm by these compounds, ultraviolet (UV) spectroscopy has been widely used to determine the degree of rancidity. Unfortunately, the UV method is only useful in the early stage of oxidation because the conjugated diene structure is partly lost by formation of secondary products (9). More sophisticated spectroscopic and chromatographic methods are often employed for detailed structural analysis of the oxidation products, but in general these methods are too expensive and time-consuming to be used as a simple rancidity test.

None of the methods mentioned above alone fulfill the criteria for a simple test for rancidity assessment, and the search for an alternative analysis is thus important. Measurement of the ultra-weak chemiluminescence (CL), which accompanies autoxidation, has been reported to be a potentially useful method (10–14), but the sensitivity is low. However, by adding sodium hypochlorite to an oxidized oil, a strong light emission from the excited oxygen species is observed (15–17). This hypochlorite-activated CL has been reported to be a highly sensitive method for detection of low levels of lipid hydroperoxides, and the method can therefore be applied to estimate the extent of autoxidation. In the present study, the correlation between hypochlorite-activated CL and the methods normally used for oxidative rancidity assessment is described.

MATERIALS AND METHODS

Chemicals. The cod liver oil (CLO) was obtained from Peter Möller a/s (Oslo, Norway), the capelin oil (CO) was supplied by J. C. Martens a/s (Bergen, Norway), and the chromatographically purified fish oil 30 (CPL) was obtained from Karlshamns ab (Karlshamn, Sweden). The hydrogenated fish oil M30/32 [iodine value (IV) = 70] was delivered by a/s Denofa og Lilleborg Fabriker (Fredrikstad, Norway). The oils contained no added antioxidants. Ronoxan was supplied by F. Hoffmann-La Roche (Basle, Switzerland). All chemicals used were of *p.a.* quality supplied by Sigma (St. Louis, MO) Aldrich (Steinheim, Germany) or Merck (Darmstadt, Germany), except the solvents, which were of high-performance liquid chromatography (HPLC) grade and obtained from Rathburn Chemicals (Walkerburn, Scotland).

General methods. Chemiluminescence (CL) was either determined on a 3M™ Lumac Biocounter M2010 (Schaesberg, The Netherlands) after injection of 3 × 100 μL 1 M NaOCl in 0.1 M NaOH, with recording of the luminescence for a period of 30 s at 37°C after each injection, or on a Bio-Orbit 1251 Luminometer (Turku, Finland) equipped with Dispense SVD and Dispenser Controller DC after injection of 200 μL hypochlorite solution, with recording of the luminescence for a period of 60 s at 30°C. With both instruments, samples containing 100 μL oil dissolved in 500 μL *tert*-butanol were analyzed.

Ultraviolet measurements were performed on a Shimadzu UV-160 spectrophotometer (Kyoto, Japan) at 233 nm of samples containing 0.400 mg oil/mL heptane. PVs were measured by iodometric titration (18); TBAV were determined as described by Ke and Woyewoda (19); and the Kreis test was determined according to BS 684 (20). Anisidine and iodine values were measured according to IUPAC 2.504 (21) and the Wijs method (22), respectively. The amount of free fatty acids was determined by titration (23).

Gas-chromatographic analyses were performed on a Carlo Erba 5300 Mega series chromatograph (Milano, Italy) equipped with flame-ionization detector (FID) and a Chrompack tailor-made fatty acid methyl esters (FAME) (CP-Sil 88) fused-silica capillary column (50 m, i.d. 0.25 mm, film thickness 0.2 μm) (Middelburg, The Netherlands). Hydrogen was used as carrier gas and peak integration was carried out with an LDC/Milton Roy CI-10 integrator (Rochester, NY). The methyl esters of fatty acids were prepared by methanolysis with potassium hydroxide followed by neutralization (24). The method for polymer analysis is previously described (25).

The oxidation experiments were performed at 35°C with irradiation from an artificial daylight fluorescent tube (420–750 nm, 1.1×10^{19} $Qm^{-2}s^{-1}$) (26). The oils (40 g) were oxidized in open beakers (4.2 cm i.d.) with stirring.

RESULTS AND DISCUSSION

Oxidative rancidity assessment. To determine the optimum conditions for chemiluminescence measurements, a number of exploratory experiments were carried out with cod liver oil of different oxidation levels as test material. From these experiments, CL was found to be highly dependent on the experimental conditions employed, *i.e.* sample amount, solvent, amount of hypochlorite added, measuring time, temperature and mixing of the sample. Especially the composition of the emulsions (27) formed after hypochlorite addition to the oils dissolved in *tert*-butanol was found to be critical for reproducible results. The optimum conditions are dscribed in Materials and Methods.

Compared with the classical methods for rancidity assessment, CL is much more sensitive and it enables detection of small changes in oxidative rancidity. Storage of a capelin oil for seven months at 5°C showed a chemiluminescence increase of 89%, while PV decreased, and TBAV and fatty acid composition were essentially unchanged (Table 1). An unaltered amount of free fatty acids after storage indicates that the observed change in CL was not caused by hydrolysis. The reproducibility of the CL measurements was high, with standard deviation for sample-to-sample and day-to-day analyses less than 3%. These figures are low compared to the values generally observed for the chemical methods used for rancidity assessment (28).

The high sensitivity and ability to detect early changes in rancidity were also demonstrated by comparing CL with sensoric evaluation. Subjecting a hydrogenated fish oil (IV = 70) to autoxidative conditions for two weeks at 25°C gave a CL increase of 24%, while the sensoric scores of the oils were only slightly altered (1%) (Table 2). However, for samples from different productions of the same hydrogenated fish oil not subjected to autoxidative conditions, no correlation was found between chemiluminescence and sensoric scores. Although some correlation with off-flavor has been reported (16), the observation is in agreement with the lack of correlation between sensoric scores and chemical evaluation methods that are found so often (4,5). Despite the disagreement with sensoric evaluation, CL gives valuable and additional information regarding the quality of the oil. Because CL is measuring active oxygen species *(vide infra)*, an oil with high CL, even though the smell and taste is acceptable, may contain components that can cause adverse effects on consumption and should therefore be analyzed further.

Reasonably good correlations (r = 0.920–0.999) were found between CL and PV, TBAV, anisidine value, Kreis rancidity index, UV measurements at 233 nm and sensoric score for CLO and the chromatographically purified fish oil subjected to autoxidative conditions at 35°C for four days (Table 3). The correlation coefficients depended on the oil tested, and the best correlation was found between CL and UV measurement of conjugated dienes at 233 nm. For more oxidized samples, a decrease in UV absorbance was found (25), with subsequent loss

TABLE 1

Chemiluminescence (CL), Change in Chemiluminescence (ΔCL), Amount of Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA), Peroxide Value (PV) and Thiobarbituric Acid Value (TBAV) of Capelin Oil Stored in Closed Vials for Seven Months at 5°C

	CL (counts)	ΔCL (%)	EPA + DHA[a] (%)	PV (meq/kg)	TBAV (μmol/g)
Start	3,350	0	14.2	3	0.06
Seven months	6,330	89	14.0	<1	0.08

[a]Analyzed by gas chromatography.

TABLE 2

Chemiluminescence (CL) and Sensoric Score (SS) of Hydrogenated Fish Oils[a] Stored for Two Weeks at 25°C

	CL (counts)	ΔCL[b] (%)	SS[c]	ΔSS[b] (%)
Start	830		7.8	
Two weeks	1,030	24	7.7	1

[a]Mean value of seven different samples.

[b]Change in percent.

[c]On a 0–10 scale with highest score 10.

TABLE 3

Correlation Coefficient (r) for Chemiluminescence (CL) *vs.* Peroxide Value (PV), Thiobarbituric Acid Value (TBAV), Anisidine Value (AV), Kreis Rancidity Index (Kreis), Ultraviolet Measurement at 233 nm (UV) and Sensoric Score (SS) of Fish Oils[a] Oxidized at 35°C (0–4 d)[b]

	PV	TBAV	AV	Kreis	UV	SS
CLO	0.998	0.998	0.989	0.920	0.998	0.963
CPL	0.993	0.994	0.977	0.999	0.997	0.981

[a]CLO, cod liver oil; CPL, chromatographically purified fish oil.

[b]Samples analyzed at start and after one, two, three and four days autoxidation.

of correlation with CL. This effect was not observed for the other oxidation detection methods used (Table 4).

We concluded that chemiluminescence can be used as a fast supplement (approximately 5 min/sample) to the standard chemical methods used for rancidity assessment for oils subjected to autoxidative conditions (Fig. 1). The method can also be used to analyze severely oxidized samples with polymer content around 30% (Table 4). Compared to the established oxidation detection method, CL has several advantages, but much work has to be conducted before it eventually can replace any of the traditional methods.

A direct comparison of the chemiluminescence for different fish oils is of limited value as the oils give CL values within certain limits depending on the rancidity level (Table 4). A somewhat unexpected result was the high CL value for the starting sample of CO, although low PV and TBAV were measured. Concerning the stability, the relative CL increase of CO is less than that of CLO and CPL.

TABLE 4

Chemiluminescence (CL), Peroxide Value (PV), Thiobarbituric Acid Value (TBAV), Anisidine Value (AV), Iodine Value (IV) and Polymer Content of Fish Oils[a] Oxidized at 35°C (0–8 d)

	CL (counts)	PV (meq/kg)	TBAV (μmol/g)	AV	IV	Polymers[b] (w/w %)
CLO						
0	670	4	0.7	12	169	1
4	1,370	82	5	23	169	4
8	18,600	940	39	360	139	30
CPL						
0	810	3	0.4	2	222	1
4	3,600	130	7	20	220	3
8	20,600	740	33	180	206	20
CO						
0	3,500	4	0.3	10	139	1
4	4,190	45	3	16	136	2
8	14,500	740	29	210	122	22

[a]CLO, cod liver oil; CPL, chromatographically purified fish oil; CO, capelin oil.

[b]Polymeric triacylglycerols determined by size-exclusion high-performance liquid chromatography.

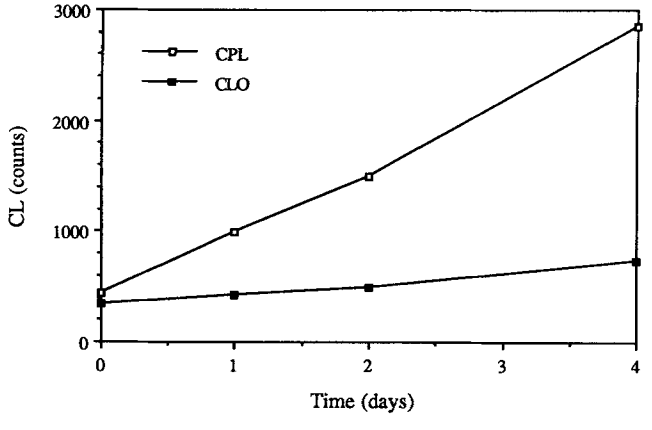


FIG. 1. Chemiluminescence (CL) of autoxidized fish oils (0–4 d at 35°C); CPL, chromatographically purified fish oil; CLO, cod liver oil.

However, the conditions employed in this study were deliberately chosen to induce autoxidation and are not representative of those employed normally for the storage of bulk oils. For this reason, and because the past history of the oils was not known, no conclusions can be drawn regarding the stability of the different oils to autoxidation under commercial conditions.

It is well known that hypochlorite-activated decomposition of hydroperoxides gives singlet oxygen (29–31). The intensity of the luminescence has been reported to depend on both the concentration and the structure of the hydroperoxide (15,17), but the exact mechanism for light emission is not understood. However, not only peroxides are causing the change in hypochlorite-activated chemiluminescence. Even a decrease in PV was followed by a CL increase (Table 1), and comparison of CLO, CPL, and CO, which exhibit identical PVs (and TBAVs), gave CL values varying from 670–3500 counts (Table 4). These great differences are most probably not caused by any of the products formed during autoxidation.

Antioxidant evaluation. Hypochlorite-activated chemiluminescence is useful for antioxidant evaluation (Fig. 2). The analysis is simply carried out by measuring the CL change during storage of oils, to which various levels of antioxidants have been added, relative to the CL change for the pure oil with no antioxidants. Due to the high sensitivity and ability to detect small changes in rancidity, the antioxidants can be tested and evaluated at ambient temperatures within 1–2 d. The results from these experiments are in agreement with long-time stability tests conducted at 5–10°C in the dark, which are more typical storing conditions for oils and oil-containing products. The CL results have been confirmed by PV, AV and TBAV measurements. Compared with the evaluation method based on ultraweak CL (12,17,32,33), our method of using hypochlorite-activated CL also gives information on the influence of the oil and storing conditions on the antioxidant activity.

Care has to be taken in interpretation of the CL data because light emission depends on the antioxidant added (Table 5), as well as on oil composition and rancidity

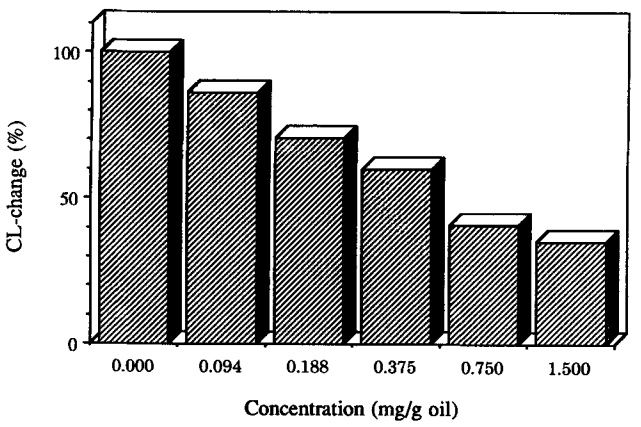


FIG. 2. The antioxidant activity of different concentrations of Ronoxan (dl-α-tocopherol [5%], ascorbyl palmitate [25%] and lecithin [70%]) on cod liver oil stored for 24 h at 35°C analyzed by chemiluminescence (CL). The results are presented as CL change in percent relative to the oil without antioxidant.

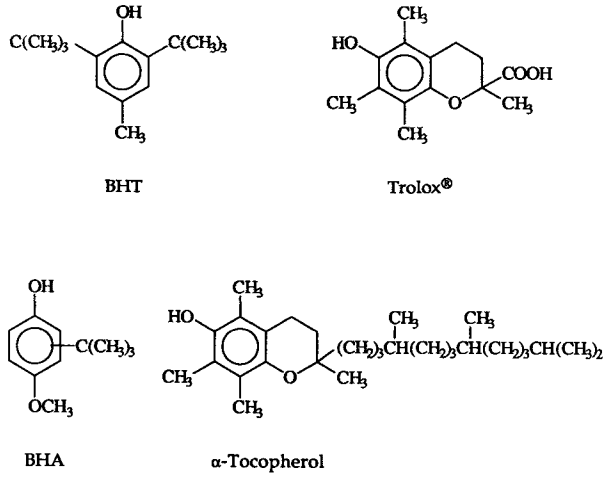
TABLE 5

Change in Chemiluminescence (in percent) by Addition of Different Antioxidants[a] to Cod Liver Oil (not stored)

Concentration (mg/g oil)	BHT	BHA	α-Tocopherol	α-Tocopheryl acetate	Trolox®
0.094	0	−4	−2	−1	0
0.188	+1	−7	−2	−3	0
0.375	+1	−16	−4	−3	−3
0.750	+2	−20	−4	−3	−3
1.500	+2	−22	−6	−4	−5

[a]BHA, butylated hydroxyanisole (2[3]-*tert*-butyl-4-hydroxyanisole); BHT, butylated hydroxytoluene (2,6-di-*tert*-butyl-*p*-cresol); Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

level (Table 4). A direct comparison of the chemiluminescence value of unknown samples may therefore give misleading results. All phenolic antioxidants (Scheme 1), the addition of butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), α-tocopherol, α-tocopheryl acetate and Trolox to CLO influenced the CL measurements to different extents, depending on the concentration. Most dramatical was the difference observed when BHT and BHA were used. While BHT (0.75–1.50 mg BHT/g CLO) gave a 2% increase in CL, BHA at the same concentrations gave a decrease of 20–22%. The main structural difference between BHT and BHA is the methoxy group in the latter one. The ether oxygen in the heterocyclic ring of α-tocopherol did not, however, influence the luminescence to the same extent as the methoxy group did and gave values comparable with those observed by addition of phenol. The change in CL of α-tocopheryl acetate was almost identical to the values observed for α-tocopherol itself, and the carboxylic group in Trolox did not significantly change the CL compared to the hydrocarbon side chain in α-tocopherol. The CL of oils with these phenolic antioxidants added is clearly influenced by substituents on the aromatic part of the molecule, but the basis for the changes is not understood. More extensive studies on the use of CL for antioxidant evaluation, including comparison with the Rancimat test, are currently underway.



SCHEME 1

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