Antioxidative Effects of Polyamines

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The antioxidative effects of spermine, spermidine and putrescine were determined by measurement of primary and secondary oxidation products of polyunsaturated fatty acids, using gas and liquid chromatography as well as spectrophotometric recordings. It was demonstrated that polyamines inhibit the oxidation of polyunsaturated fatty acids, α-tocopherol and carotenoid pigments. Both linear and nonlinear dose/response relationships have been observed. The efficiency of a given polyamine was correlated with the number of amine groups in the molecule. Spermine was, thus, more efficient than spermidine, which in turn had a higher efficiency than putrescine. The relative antioxidative effect was as follows: spermine (100.0), spermidine (61.0), putrescine (23.0), ethoxyquin (27.6), ascorbyl palmitate (18.3), octyl gallate (7.9), tert butylhydroquinone (6.3), butylated hydroxyanisole (3.6) and α-tocopherol (3.4).

KEY WORDS: Antioxidant, α-tocopherol, carotenoid pigments, lipid peroxidation, polyamines, spermine.

There has been a steady increase in the use of antioxidants for medical, nutritional and technical purposes. There is also an increasing interest in defining new antioxidants of high potency, low toxicity and good solubility properties in aqueous as well as organic phases.

Of the chemical components of organic matter, lipids are the most prone to autoxidation (1). Possible deleterious effects of this oxidation may be due to the formation of toxic oxidation products as well as reactions of the latter with proteins, amino acids, carbohydrates, vitamins, pigments and nucleic acids (2). Autoxidation proceeds initially very slowly. At a specific time (designated "the induction time") there is an abrupt increase in the reaction rate, indicating positive feedback from a reaction product.

It has been suggested that peroxides, the primary oxidation product, may act as initiation centers for further propagation of the reaction (3). It is, however, conceivable that secondary oxidation products like acids, aldehydes, alcohols, ketones, epoxides etc. may have a similar deleterious effect. It is, for example, known that in the presence of acid the decomposition of peroxides is first order in substrate and first order in acid (4).

It has been known for some time that certain aliphatic amines have antioxidative properties. A well known example is diethylenetriaminepentaacetate (DTPA), which by virtue of its ability to occupy at least three positions in the sphere of a heavy metal coordination complex, is referred to as a polydentate coordinating compound. DTPA-Fe^{+++} will not catalyze the production of hydroxyl radicals ($OH^{\bullet -}$) from superoxide radicals ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), thus preventing the Fenton reaction from occurring (5). Other aliphatic amines are used as sulphite antioxidants (6), corrosion inhibitors (7) or stabilizers of edible fats and oils (8,9). Examples of such aliphatic amines are polyethyleneamines, 9-aminomethylstearylamine and 2,2′-diaminodiethylamine.

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The polyamines described in the present paper are, in contrast to the above-mentioned compounds, natural constituents of living organisms and may, as such, have special significance for food, feed and medical applications.

At physiological pH, polyamines are fully protonated and polycationic. The compounds are easily soluble in organic as well as aqueous media, thus making introduction into a wide variety of products/samples feasible. In lipophilic environments the polyamines probably exist as neutral compounds.

The aim of the present study was to stabilize fish oils and concentrates of polyunsaturated fatty acids against oxidation. We searched for a lipid-soluble antioxidant of higher potency than α-tocopherol which was of natural origin.

MATERIALS AND METHODS

Materials. The α-tocopherol was a gift from Hoffmann-LaRoche (Nutley, NJ). The butylated hydroxyanisole, 2,(3)-tert-butyl-4-hydroxyanisole (BHT), tert butylhydroquinone (TBHQ), octylgallate, and ascorbyl palmitate were purchased from Aldrich-Chemie, Steinheim, Germany. Ethoxyquin (6-ethoxy-1,2,-dihydro-2,2,4-trimethylquinoline), spermine, spermidine and putrescine were purchased from Sigma Chemical Company, St. Louis, MO. Stock-solutions of polyamines were made up in butanol. All organic solvents were of Pro Analysis-grade from Merck, Darmstadt, Germany.

Oils used in this work included a concentrate of fish oil containing 30% eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) delivered from Martens, Bergen, Norway. The fatty acid composition was typically: 14:0 (7.9%), 16:0 (16.7%), 16:1 (9.4%), 18:0 (13.1%), 18:2 (2.1%), 20:0 (4.7%), 20:1 (3.3%), 20:5 (18.7%), 22:1 (4.9%), 22:5 (2.2%) and 22:6 (10.1%). Crude capelin oil was delivered from local factories. Oil from capelin (*Mallotus villosus*) contains about 20% of polyunsaturated fatty acids, mainly 20:5 and 22:6, and is prone to oxidation. The typical composition of this oil was: 14:0 (7.5%), 16:0 (16.7%), 16:1 (1.4%), 18:0 (2.8%), 18:2 (3.3%), 18:3 (1.5%), 18:4 (2.6%), 20:1 (11.9%), 20:4 (0.5%), 22:5 (2.2%) and 22:6 (9.8%).

Antioxidants were added to fish oils or fish oil concentrates, and the efficiency of the antioxidant was measured in a Rancimat (10), by peroxide detection, by the thiobarbituric acid (TBA) reaction (11–13) or by gas chromatography (GC) detection of polyunsaturated fatty acids.

Oxidation of volatile oxidation products (Rancimat). A Rancimat measures the development of volatile oxidation products in samples exposed to high temperatures and a stream of air. Oxidation products are transported by the air stream into a collection chamber containing distilled water and give rise to an increase in conductivity which is measured by a pair of platinum electrodes. Typically, there is a slow linear rise in conductivity, followed by an abrupt rise. The onset of the rise, termed the induction time, is characteristic of the type and amount of antioxidant added.

Experiments in the Rancimat were performed on crude fish oils at temperatures ranging from 70° to 100° C. Fish oil (2.5 mL) was added to the oxidation chamber and exposed to an air stream of 18 mL/hr at the indicated temperature. The instrument was calibrated with acetic acid, giving a linear relationship between the amount of acetic acid added and the conductivity (0.5 μS/μmole acetic acid). The rate of the propagation reaction was, thus, transformed from μS/hr to "acetic acid equivalents"/hr. Conductivity is expressed as Siemens (S), which is the inverse value of the resistance (Ohm). Experiments were performed at different temperatures to keep the induction time within reasonable limits. Crude oil from capelin (20% unsaturated fatty acids) was thus oxidized at a higher temperature than EPA/DHA oil (30% unsaturated fatty acids).

Gas chromatography of fatty acids. The fish oils were hydrolyzed and derivatized to methyl esters by acid-catalyzed transmethylation of triglycerides as follows: 10 μL of oil was added to 1 mL 3 M HCl in methanol. The mixture was heated to 100° C for 30 min, then cooled, and 2 mL water and 1 mL of hexane was then added. After mixing and phase separation, the hexane phase was diluted 1:15 with hexane. A 1-μL sample was injected to a Dani 8500 chromatograph (Dani, Milan, Italy) equipped with a programmable temperature injector and a 0.25 mm × 30 m fused silica glass capillary column coated with OV101 (Supelco, Bellefonte, PA). Injection was by a "solvent split" technique. The temperature program was as follows: 60°C for 4.2 min, then a steep temperature rise of 27.5°C/min to 180°C followed by a slow temperature rise of 4° C/min to 240°C. A flame ionization detector kept at 300°C was used. Signals were integrated by a Nelson Analytical Model 2600 system (Nelson Analytical, Cupertino, CA).

Peroxide detection. The effect of antioxidants was also investigated at ambient temperature. Samples containing 30% EPA/DHA (eicosapentaenoic acid/docosahexaenoic acid) were exposed to open air at a temperature of 27°C, and the development of peroxides was measured by a spectrophotometric method essentially as described by Løvaas and Palmieri (14).

A saturated solution of KI was made by dissolving excess KI in high quality distilled water (conductance less than 10^{-9} mho). Excess solids must remain. The solution was stored in the dark, and tested when appropriate by adding 0.5 mL to 30 mL (HOAc:MeOH) (3:2) and 2 drops of 1% starch solution. If the solution turned blue, requiring more than 1 drop of 0.1 N $Na_2S_2O_3$ to discharge color, then fresh solution was prepared. Further details appear in American Oil Chemists' Society Method no. 28.025 (*Official Methods of Analysis*, edited by Sidney Williams, Academic Press, Arlington, VA, 1984, p. 507).

For spectrophotometric measurements, 1 2:1 mixture of methanol:butanol, containing 3% of saturated KI-solution, was used. The solution is stable when stored in the dark, and sufficiently polar to dissolve both KI, iron salts and lipids. Precipitates may form if iron or lipid concentrations are higher than reported in this work.

A 12.5 mM stock solution of ferrous salt was prepared in high quality water. The pure ferrous salt is stable. The stock ferrous salt must not be prepared in buffered media at neutral pH, where it can rapidly autoxidize, and it must not be prepared in the presence of chelating agents (*e.g.* ethylenediaminetetracetic acid (EDTA or DTPA) because the chelate may be unstable.

A solution of 25 mM HCl was made by diluting 3M methanolic HCl in methanol.

The standard assay was performed as follows: We added 2.2 mL solvent (MeOH:BuOH:KI) to a 3.0 mL cuvette, then added 100 μL of 25 mM HCl in methanol, 100 μL of 12.5 mM Fe^{2+} solution, and 100 μL of lipid sample (diluted 1:100 in butanol). The absorbance was read at 15 min on a recording spectrophotometer at 360 nm.

Peroxide value (PV, in mEq/kg lipid) was calculated by equation 1:

$$PV = A_{15}/18.3 \times D \times V_t/V_s \qquad [1]$$

A_{15} is the absorbance value at 360 nm after 15 min, D is the dilution factor for the lipid sample in butanol, V_t is the total volume added to the cuvette and V_s is the sample volume added. The calculations were based on a mM extinction coefficient for the I_3^- ion of 18.3 (360 nm).

TBA-reaction. Thiobarbituric acid (TBA) reacts with saturated and unsaturated aldehydes and gives a red complex with a high extinction coefficient (15,16). The method was originally assumed to be specific for malondialdehyde, for which the molar extinction coefficient is 1.74×10^5 (15), but was later demonstrated to detect other aldehydes (16–18).

The TBA-positive compounds were detected essentially as described by Vyncke (19). Oil (0.1 mL) was transferred to 5 mL of 10 mM TBA in butanol and boiled for 40 min. Absorbance was read at 532 nm against a blank similarly treated, but without addition of oil.

Diene conjugation. A frequently used method for detecting lipid peroxidation is measuring conjugated diene absorbance at 232 nm (molar extinction coefficient = 27.000) (20). In simple model systems, as the one used in this work, the measurements are relatively straightforward. Oil (200 μL) was diluted in 2.8 mL of butanol, and the absorbance read at 232 nm.

Viscosity. The viscosity of oil oxidized in the Rancimat was measured by the rate of a sinking spherical tin-ball of diameter 1.3 mm and weight 9.4 mg (specific gravity = 8.17 kg/L). The viscosity, expressed as poise (n), was calculated in equation 2 according to:

$$n = 2 \times (D1\text{–}D2) \times g \times r/(9 \times v), \qquad [2]$$

where D1 is the density of the tin-ball, D2 is the density of the oil, g is the gravitational constant (981 cm/sec^2), r is the radius of the tin-ball in cm, and v is the speed of the sinking in cm/sec.

α-Tocopherol. The α-tocopherol was measured by high-performance liquid chromatography (HPLC) on a Resolve C-18 column (Waters, Sundbyberg, Sweden). Isocratic elution with acetonitrile, isopropanol and acetic acid (90:8:2) was used. Detection was by a fluorescence detector with excitation at 290 nm and emission at 320 nm. Sample preparation for α-tocopherol analysis was as follows: 5 mL of oil was mixed with 25 mL of ethanol and centrifuged. A portion (0.5 mL) of the clear supernatant was then mixed with 1 mL of water and 2 mL of ethyl acetate. Some of the ethyl acetate fraction (20μL) was injected on the chromatograph.

Pigments. The pigment-stability of naturally pigmented oil, which predominantly contains astaxanthine, was

recorded as a function of oxidation-time at 80°C in the Rancimat. Pigments were measured spectrophotometrically at 474 nm after dilution of the oil in butanol. Typically, 1 mL of naturally pigmented oil was diluted in 9 mL of butanol. Astaxanthine (1ppm) gave an absorbance of 0.212. Butanol was used as a reference. The recordings were performed in a Hewlett-Packard (Palo Alto, CA) diode array spectrophotometer (HP 8451A). The concentration of astaxanthine (in ppm) was calculated by dividing the absorbance by 0.212.

RESULTS

Effect of acid on lipid oxidation. The stability of capelin oil in the presence of 0–1.0 mM HCl was tested on the Rancimat as described in the Materials and Methods section. We found that HCl acted as a pro-oxidant at concentrations of less than 0.1 mM. The induction time (as defined under Materials and Methods) was reduced by approximately 50% for each μmole HCl added to 2.0 mL capelin oil (Fig. 1).

Effects of polyamines on lipid oxidation at high temperatures—polyunsaturated fatty acids. The addition of 0.1 to 2 mM of spermine to a 30% EPA/DHA concentrate oxidized in the Rancimat at 70°C increased the induction time from 6.0 hr (control) to 10.0–52.6 hr (Fig. 2), depending on the spermine concentration. Other polyamines exhibited similar effects, although to a lesser extent, so that spermine was a better antioxidant than spermidine, which again was better than putrescine. There was a linear relationship between the amount of polyamine added and the increase in stability. The dose-effect curve displayed in Figure 3 indicates a relative efficiency of 1.00:0.61:0.23 for spermine, spermidine and putrescine, respectively. The dose-effect curve was, however, not always linear. For crude capelin oil oxidized at 100°C in the Rancimat, we have observed an escalating effect at increasing antioxidant concentrations (Hill coefficient for spermine = 1.44). The rate of oxidation in the propagation phase, measured as acetic acid equivalents, was, in the control sample, 260 μmole/hr, suggesting that 5–10% of the fatty acids were oxidized to secondary degradation products each hr. The addition of antioxidants not only prolonged the induction time, but also reduced the rate of oxidation in the propagation phase.

The effect of polyamines was further compared to those of other antioxidants. Spermine had, under all conditions tested, a much better action than the other compounds tested (Table 1). For example, in crude oil oxidized at 100°C, α-tocopherol had an antioxidative effect which was only 3.4% of the spermine effect.

It was observed that the viscosity of the control oil increased during oxidation in the Rancimat, while no such changes took place for the oil stabilized with spermine. This effect was further studied on a crude capelin oil oxidized at 80°C (Fig. 4), giving an induction time of 15 hr for the control and more than 35 hr for oil stabilized with 1 mM (200 ppm) spermine.

Experimental data from the Rancimat were confirmed by measurements of peroxides as well as secondary degradation products (TBA reaction), by measurements of diene conjugation and by gas-chromatography of fatty acids (Table 2).

α-Tocopherol and carotenoid pigments. The effect of spermine on the stability of α-tocopherol in capelin oil kept

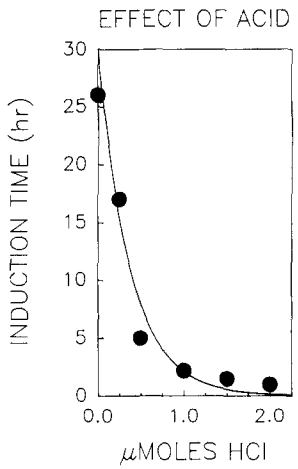

FIG. 1. Induction time of capelin oil in the presence of 0–100 μL of dry hydrochloric acid in methanol added to 2.5 mL of oil. The oxidations were performed in the Rancimat at 100°C.

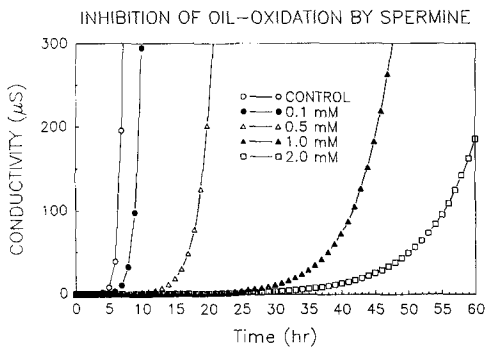

FIG. 2. Development of volatile degradation products measured as the increase in conductivity (μS) from the oxidation of polyunsaturated fatty acids (30% EPA/DHA concentrate) at 70°C in the Rancimat. Spermine was added to the indicated concentrations.

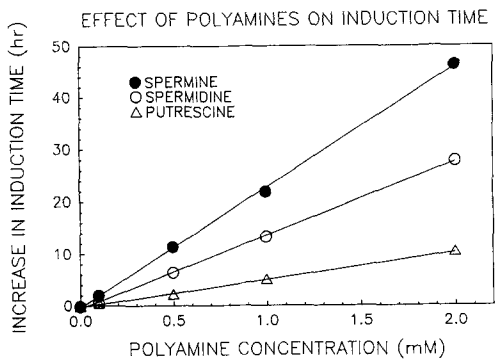

FIG. 3. Concentration-dependent increase in the stabilizing effect of various polyamines on polyunsaturated fatty acids (30% EPA/DHA concentrate) at 70°C in the Rancimat.

TABLE 1

Relative Effects of Antioxidants on Induction Time in Crude Fish Oil and EPA/DHA Concentrate

Antioxidant	Crude oil[a] 100°C[c] 2 mM[d]		EPA/DHA[b] 70°C[c] 2 mM[d]	
α-tocopherol	3.4[e]	(0.8)[f]	—	—
BHA	3.6	(1.1)	—	—
TBHQ	—	—	—	—
Octylgallate	—	—	—	—
Ascorbylpalmitate	18.3	(2.1)	—	—
Ethoxyquin	27.6	(2.0)	—	—
Putrescine	—	—	23.0	(3.0)
Spermidine	74.5	(6.1)	61.0	(4.8)
Spermine	100.0	(8.1)	100.0	(7.6)
Control induction time	2.1[g]	(0.4)	6.0	(0.8)
Spermine induction time	85.2	(6.9)	52.6	(4.0)

[a]Contained 20% unsaturated fatty acids.

[b]Methyl esters of eicosapentaenoic acid/docosahexaenoic acid.

[c]Investigation performed in the Rancimat at the indicated temperature.

[d]Concentration of antioxidant.

[e]The antioxidative effects, expressed as induction times relative to spermine. The antioxidative effect of spermine is set at 100.

[f]Standard deviation (n = 3) is shown in parentheses.

[g]Actual induction times.

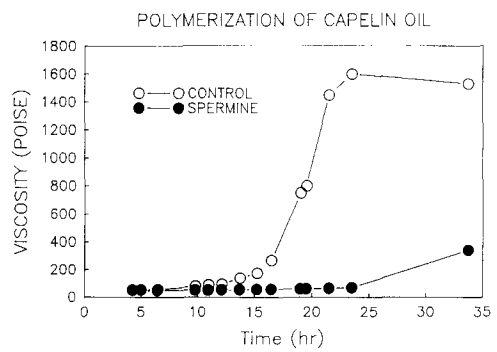

FIG. 4. Polymerization of capelin oil at 80°C in the Rancimat. Spermine was added to a final concentration of 1 mM (200 ppm).

TABLE 2

Measurement of Olive Oil Deterioration by Different Methods After Oxidation in the Rancimat at 100°C for Up to 9 Hr

Hours	μS/cm[a]	GC[b]	PV[c]	TBA[d]	UV[e]
0	0	5,04	9	0	30
2	6	4,58	14	4,1	40
4	36	4,33	40	9.8	50
5	84	4,14	95	20,5	110
7	216	3,16	234	50,3	330
9	354	1,00	454	79,5	450

[a]Conductivity expressed in μS/cm.

[b]Linolenic acid (%) remaining in the oil measured by GC.

[c]Peroxide value (mEq) in the oil.

[d]TBA-reactive products distilled into the aqueous phase.

[e]μM dienes, measured by ultraviolet (UV)-spectroscopy, in the oil.

at 80°C in the Rancimat is shown in Figure 5. In the control experiment α-tocopherol was consumed at a rate of 250 ppm/hr. The addition of 1 mM spermine reduced the decay rate to 40 ppm/hr.

It was also observed that the natural pigmentation of the oil, mainly due to astaxanthine, was much better conserved in the presence of spermine. This effect was further examined, and the influence of 200 ppm of butylated hydroxyanisole (BHA), ascorbyl palmitate and ethoxyquin was compared with that of spermine (Fig. 6). The pigment (starting level = 16.0 ppm) rapidly disappeared in the absence of added antioxidants. The initial decay rates (ppm/hr) were as follows: control (3.75), ethoxyquin (2.04), BHA (1.46), ascorbyl palmitate (0.87) and spermine (0.18). The sample stabilized with spermine was stable for 11.5 hr, whereafter a slow deterioration of the pigment took place. The other samples had no pigment left after 8 hr or less.

Effect of polyamines on lipid oxidation at room temperature. During 60 days of storage at ambient temperature (27°C), there was no peroxide development in an EPA/DHA oil stabilized with spermine, whereas there was a steady increase in the peroxide value for the control and the samples stabilized with tert butylhydroquinone (TBHQ) and octyl gallate (Fig. 7). Oil stabilized with spermine darkened upon prolonged storage (as observed visually), while samples without spermine maintained their original color.

DISCUSSION

The present work demonstrates that the stability of polyunsaturated fatty acids is greatly diminished in the presence of small amounts of acid. Since acids are included among the secondary degradation products of fatty

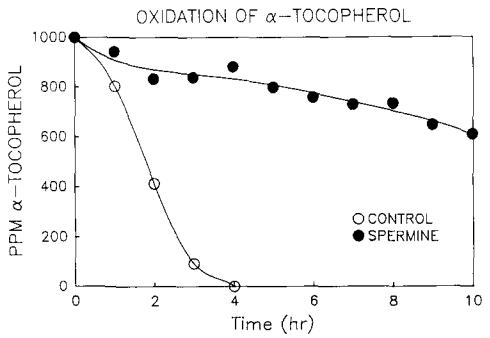

FIG. 5. Effect of 1 mM (200 ppm) spermine on the oxidation of 2.3 mM (1000 ppm) α-tocopherol. The experiment was performed at 80°C in the Rancimat. α-Tocopherol was measured by HPLC.

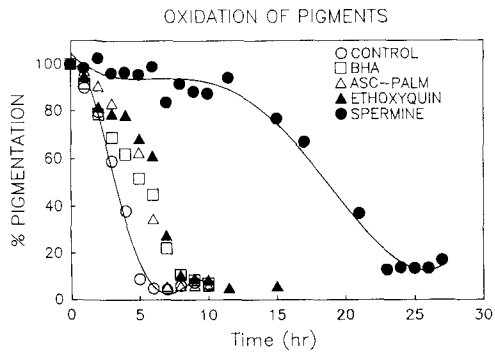

FIG. 6. Decay of naturally pigmented capelin oil containing 16.0 ppm astaxanthine, when oxidized in the Rancimat at 80°C. The antioxidative effect of 200 ppm butylated hydroxyanisole (GHA), ascorbyl palmitate and ethoxyquin was compared with that of spermine. Astaxanthine was measured by ultraviolet (UV)-spectroscopy at 474 nm.

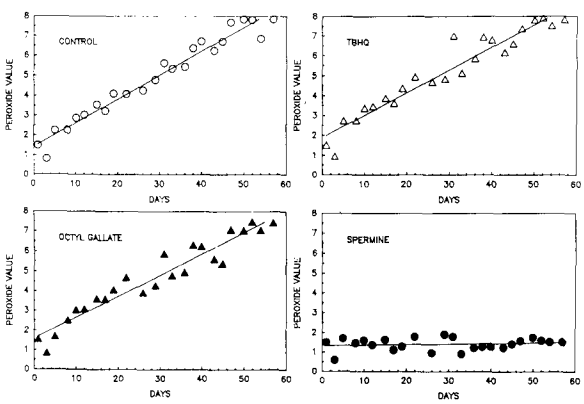

FIG. 7. Development of peroxides in a sample of polyunsaturated fatty acids (30% EPA/DHA concentrate) at 27°C. The oil was stabilized with 200 ppm of tert butylhydroquinone (TBHQ), octyl gallate and spermine, and stored in the dark for 60 days.

acid oxidation, they may constitute a positive feedback link which can lead to rapid oxidation once the "buffer capacity" of the oil is exceeded. If acid catalysis is an important aspect of lipid oxidation, it is possible that organic bases, like polyamines, could act as antioxidants.

This hypothesis was confirmed by investigating the stability of fish oil and polyunsaturated fatty acids in the presence of polyamines. Experiments performed at 100°C demonstrated that spermine was 30 times more efficient than α-tocopherol and 3.6 times more efficient than ethoxyquin. It was also demonstrated that the antioxidative effect correlated with the number of amine groups in the polyamine—spermine being more efficient than spermidine, and spermidine more efficient than putrescine.

It was also demonstrated that spermine has a stabilizing effect on α-tocopherol and carotenoid pigments. This was expected on the basis of the antioxidative effect on polyunsaturated fatty acids. However, the magnitude of the effect was surprisingly high compared to the other antioxidants tested. The ability of spermine to inhibit polymerization of oils is a consequence of its properties as an antioxidant.

The reaction kinetics of fatty acid oxidation are complicated, and it is difficult to extrapolate the results from high temperature conditions to low temperatures. Most of the experiments in the present work have been performed at elevated temperatures (70°-100°C). While such studies are of importance for technical applications, they give little information of antioxidative effects *in vivo*, or at ambient temperatures. It was therefore of interest to check whether the antioxidative effect of spermine also was present at ambient temperature. When experiments were conducted at 27°C, no fatty acid oxidation of 30% EPA/DHA concentrate was induced during a 60-day period, although there was a steady increase in the peroxide value in the control sample and the tert butylhydroquinone (TBHQ)- and octyl gallate-samples. The sample stabilized by spermine remained stable. The darkening of this sample upon prolonged storage may be due to a reaction product between lipid hydroperoxides and spermine (21).

In past years the role of polyamines as cellular antioxidants and membrane stabilizers has been suggested (22-25). Recently it has also been shown that polyamines (spermine, spermidine, putrescine and cadaverine) have the ability to scavenge superoxide radicals and hydroxyl radicals (26-29). The efficacy of the polyamine-scavenging appears to be correlated to the extent of amination, suggesting the involvement of amino groups (30).

Polyamines are natural constituents of all aerobic organisms investigated (31), and may find use in medical, cosmetic and technical applications, as well as in food and feed technology. Their use as antioxidants is under patent protection (32).

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