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Protection of fish feed, made directly from marine raw materials, with natural antioxidants

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ABSTRACT

The present experiments were designed to study the effects of different natural antioxidants in an experimental fish feed, made directly from marine raw materials. A rosemary extract (Herbalox®) and crystalline ascorbic acid were the most effective antioxidants in this feed and the effect of ascorbic acid was enhanced by adding a tocopherol mix. Ascorbyl palmitate, citric acid, a phosphate mix designed to enhance the effect of ascorbic acid, and spermine had minor antioxidant effects, no effect or pro-oxidant effects. It was necessary to add higher concentrations of the rosemary extract than the vitamin C/E combination to obtain an optimal antioxidant effect. A minor effect of adding ethoxyquin to a diet with tocopherol mix and ascorbic acid was detected in one of the experiments, but this effect was not reproduced in the other experiments. It is therefore concluded that the diet used in the present study can be protected against oxidation using natural antioxidants. Since antioxidants must be tested in the oxidising system in which they are going to be used, the present results have to be confirmed before applying them to commercial fish feeds and feed ingredients.

1. Introduction

Fish feeds are partly or fully based on marine ingredients, with high levels of polyunsaturated $n-3$ fatty acids, and are therefore susceptible to lipid oxidation. Presently, fish meal and fish oil used in feeds are protected with synthetic antioxidants, mainly ethoxyquin (EQ) in fish meal and butylated hydroxytoluene (BHT) in fish oil (Lundebye, Hove, Bohne, & Hamre, unpublished). There is a substantial carry-over of these antioxidants to the fish fillet and the mandatory 2 weeks starvation period before slaughter of farmed fish is not sufficient for clearance of these antioxidants from the fillet (Bohne, Lundebye, & Hamre, 2008; Hamre & Bohne, unpublished). Food safety concerns connected to synthetic antioxidants have urged authorities to reduce maximal residual levels of antioxidants allowed in food for human consumption (Bohne et al., 2008) to levels which are just slightly above the levels found in farmed fish (Lundebye et al., unpublished). Therefore, a switch to natural antioxidants in fish feed ingredients would be an advantage both for the aquaculture industry and with regard to consumer health and well-being. The present study was connected to the development of a new method of fish feed production, where whole fish or fish offal were blended with micronutrients and heated using electromagnetic energy (microwaves; Hemre, Sandnes, Lie, Torrisen, & Waagbø, 1995). This method would reduce energy costs, compared to conventional methods, where fish oil and fish meal are first separated and then blended again to make the final feed.

Lipid oxidation is initiated when a free radical ($X^{\cdot}$, $OH^{\cdot}$ or others) abstracts a hydrogen atom from a polyunsaturated fatty acid (PUFA). The PUFA radical formed reacts with oxygen to form a peroxyl radical. The chain reaction propagates when the lipid peroxyl radical abstracts a hydrogen atom from a new PUFA, which enters a new turn in the reaction cycle (Hølmer, 1993). The primary products of lipid oxidation are the conjugated dienes and lipid hydroperoxides, which may undergo cleavage to form different secondary products of low molecular weight, i.e., aldehydes, alkanes, alkenes, alcohols and acids (Horton & Fairhurst, 1987; Hølmer, 1993). Malondialdehyde is often analysed as a representative of the secondary products, for example, by the thiobarbituric acid-reactive substances (TBARS) analyses. Transition metals and reactive oxygen species are known initiators of lipid oxidation.

The antioxidants studied in the present experiments were crystalline ascorbic acid; a mix of phosphates designed to enhance the activity of ascorbic acid; a mix of natural tocopherols with approximately 27%, 1%, 40% and 30% of α, β, γ and δ-tocopherol, respectively; a commercially available rosemary extract, Herbalox®; the lipid-soluble form of vitamin C, ascorbyl palmitate; spermine; and citric acid. Tocopherols act as primary antioxidants by donating a hydrogen to the lipid peroxyl radical, preventing it from starting a new cycle in the auto-oxidation reaction chain. A tocopheoxyl radical is formed in this reaction, which can be reduced to tocopherol by ascorbic acid. Tocopherol also scavenges reactive oxygen

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species in the lipid phase (Frankel, 1998). In addition to the regeneration of tocopherol and perhaps other antioxidants which act as hydrogen donors, ascorbic acid scavenges radicals in the water phase and inactivates metal ions (Frankel, 1998). Antioxidants from rosemary leaves are often extracted by organic solvents (Frankel, 1998), and the main active components are phenolic compounds, the most effective being carnosic acid, carnosol and rosmarinic acid (Erkan, Ayranci, & Ayranci, 2008). Phenolic compounds may act as chain-breaking antioxidants, radical scavengers and metal chelators (Frankel, 1998; Rice-Evans, 1999). Citric acid acts mainly as a metal chelator (Frankel, 1998). Spermine is a polyamine present in all animal tissues and was proposed as an important antioxidant, protecting skin against UV radiation (Løvaas, 1995). The compound was shown to be 30 times more efficient than α-tocopherol in protecting fish oil against lipid oxidation (Løvaas, 1991). Antioxidants such as ascorbic acid and tocopherols may act as pro-oxidants under certain conditions, especially at very high concentrations or when the antioxidants are involved in recycling reactions with transition metals. The antioxidants also show different efficiencies in different oxidising systems, for example in bulk oil, emulsions and complex food/feed systems (Frankel, 1998). It is therefore difficult to predict the efficiencies of different antioxidants and it is necessary to study them in the specific system in which they are going to be used.

The purpose of the present study was to evaluate the protective effects of seven different natural antioxidant preparations on a fish feed produced directly from marine raw materials. Two multivariate reduced factorial designs were used to compare the main effects of different antioxidants and study their interactions. The most active preparations were then tested individually and together for optimal concentrations. Feeds protected with tocopherol mix and ascorbic acid were finally compared to feeds containing added ethoxyquin and phosphate in addition to the natural antioxidants.

2. Materials and methods

2.1. General

2.1.1. Origin of antioxidants

Rosemary: Oleoresin Rosemary Herbalox®, Brand HT-W, Kalsec, Kalamazoo, MI.

Tocopherol mix: MTS-70 (70% tocopherols), Archer Daniels Midland Company, Illinois.

Ascorbic acid, ascorbyl palmitate, citric acid, spemine, ethoxyquin: Sigma Aldrich Inc. St. Luis, MO.

Phosphates: Merck, Darmstadt, Germany.

2.2. Diets

An outline of the production and storage plan for the diets is given in Fig. 1 and diet compositions in Table 1. Whole herring or herring offal was finely minced in a food processor and divided into diet batches according to the number of diets that were going to be produced. The batches of mince were then blended thoroughly with Suprex® wheat (Codrico BV, Rotterdam, the Netherlands), vitamin (Hoffmann-La Roche, Basel, Switzerland) and mineral (Merck, Darmstadt, Germany) mixes according to National Research Council, NRC (1993), and the different combinations of antioxidants. The diet blends were divided into finger-thick strings and cooked for 2 min in a microwave oven to obtain 35–45% dry matter. The strings were then cut into pellets.

Subsamples of each diet, according to the number of sampling points during the frozen storage period and the number of analyses planned, were put into Nunc boxes (Nunc GmbH and Co. Ltd., Langenselbold, Germany) and frozen at −20 °C where they were stored for different periods of time. If not analysed immediately at the sampling day, the boxes were cooled to −80 °C and stored there until analyses. Dry matter and lipid levels in the diets, sampled after the heat treatment, are also given in Table 1.

2.3. Antioxidant concentrations and experimental designs

Four experiments were conducted and the antioxidant combinations added to the diets are given in Table 2, while Table 3 gives an overview of the experimental designs. In all experiments, a diet without added antioxidants (blank) and a diet with 0.7 g/kg tocopherol mix, 1.0 g/kg ascorbic acid, 0.85 g/kg phosphate mix and 0.12 g/kg ethoxyquin (dry matter basis, control) were included. The choice of control diet was based on previous experience at NIFES.

Experiments 1 and 2 were carried out as a $2^{(7-3)}$ and $2^{(6-2)}$ reduced factorial designs, respectively (Table 3a and b). In the case of Experiment 1, this means that the seven different antioxidants were added at two levels (1, −1) and that the number of antioxidant combinations (cases) was systematically reduced from 128 to 16, according to Box, Hunter, and Hunter (1978). With a full factorial design it would be possible to calculate effects of the single antioxidants (main effects) and all the interactions, but with seven variables one would need 128 treatments. Reducing the design to $2^{(7-3)}$ and 16 treatments gives loss of resolution, since the main effects and interactions overlap with one another. In this particular design, it is possible to calculate the main effects separated from each other and from the effects of two-factor interactions. There are three possible interpretations (overlap) of each two-factor interaction (Tables 4 and 5) and the three-factor interactions

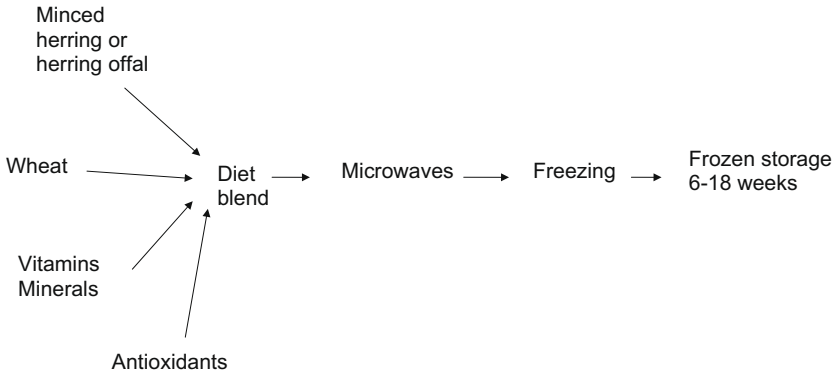

Fig. 1. Overview of the feed production and storage.

Table 1
Diet compositions.

Experiment	1	2	3	4
	g/kg wet diet			
Wheat	20.2	18.4	20.2	20.2
Herring offals		975		973
Whole herring	973		972	
Vitamins	3.3	3.3	3.7	3.3
Minerals	3.3	3.3	3.7	3.3
Dry matter	292	332	366	335
Lipid	93	132	160	131

Table 2
Concentrations of antioxidants (g/kg dry weight) added to the diets.

Experiment	1	2	3	4
Ascorbic acid	1.0	1.0	0.5; 1.0; 2.0	1.0
Ascorbyl palmitate	1.17[a]	2.34[b]		
Rosemary	0.12	3.0	1.5; 3.0; 6.0	
Tocopherol mix (70% TOH)	0.71	0.71	0.35; 0.7; 1.4	0.7
Citric acid	1.0	1.0		
Spermine	0.5			
Phosphate mix[c]	0.85	0.85		0.85
Ethoxyquin (75%)	0.12	0.12	0.12	0.12

[a] 0.5 g Ascorbate equivalents.
[b] 1 g Ascorbate equivalents.
[c] Sodium pyrophosphate and sodium hexametaphosphate 1:1.

overlap with the main effects. Three-factor interactions are usually small, compared to main effects and two-factor interactions, which were considered in the present study. The advantage of this design is the possibility to screen a greater number of variables than in traditional designs. The use of one replicate per dietary treatment may be considered a disadvantage, but the fact that every nutrient level is replicated eight times, increases the confidence of the study. Four replicate diets were included with intermediate concentrations (0) of all the antioxidants, to get an indication of the variation in the study. Factorial experiments are described by Langsrud, Ellakjær, and Næs (1994), and Sundberg (1994). The main differences between experiments 1 and 2 are that the concentrations of ascorbyl palmitate and rosemary were increased in Experiment 2 and that spermine was added in Experiment 1 but omitted in Experiment 2.

Experiment 3 tested three levels of the most active natural antioxidants, as judged form the results of experiments 1 and 2 (Table 3c), after 6 weeks of storage, while in Experiment 4, possible effects of adding phosphate mix and ethoxyquin to a diet protected with ascorbic acid and tocopherol mix were studied during a storage time of 18 weeks (Table 3d).

2.4. Chemical analyses

All analyses were performed in duplicate according to routine laboratory methods and procedures, using control charts and reference materials for quality assurance. Dry weight was determined gravimetrically after drying the samples at 105 °C overnight and total lipid, gravimetrically, according to Lie, Waagbø, and Sandnes (1988). Thiobarbituric acid-reactive substances (TBARS) were determined according to Hamre, Næss, Espe, Holm, and Lie (2001) and total ascorbic acid was analysed by HPLC, according to Mæland and Waagbø (1998).

Tocopherols were analysed by HPLC by the method of Lie, Sandvin, and Waagbø (1994), modified to detect and quantify the non-α-tocopherols. Homogenised sample (0.1–0.5 g) was weighed accurately into a 10-ml screw-capped glass tube, followed by 4 ml ethanol, 0.01 g pyrogallol, 0.01 g ascorbic acid, 0.5 ml saturated EDTA and 0.5 ml 20% (w/w) KOH. The mixture was shaken vigorously, heated at 100 °C for 20 min and cooled in water at 25 °C. Distilled water (1 ml) and 2 ml hexane were added, the samples were again shaken and centrifuged at 3000 rpm in a bench centrifuge for a few minutes to separate the solvent and water layers. The upper hexane layer was then transferred to another tube. The extraction with hexane was repeated three times. The solvent was then evaporated under nitrogen and the samples were diluted with a fixed amount of hexane before injection into the HPLC. The HPLC system consisted of a pump (TSP, SpectraSYSTEM, P1000, Thermo Fisher Scientific, 0.9 ml min^{-1}), an auto-injector (TSP, SpectraSYSTEM, AS3000, injection volume 20–50 μl), a fluorescence detector (TSP, SpectraSYSTEM, FL3000, emission 330 nm, excitation 295 nm), an analytical column (LiChroCART, 4.6 × 125 mm, Purospher STAR Si, 3 μm; Merck, Darmstadt, Germany) and Totalchrom software from Perkin Elmer (Waltham, MA). The mobile phase consisted of 5% tetrahydrofuran in *n*-hexane. The run time was 10 min. The peaks were identified and quantified by external standards. Validation of the method gave detection and quantification limits at 0.75 and 2.5 ng injected component, respectively, for all the isomers. The accuracy when analysing different commercial reference materials, varied between 88% and 108% for α, β and γ-tocopherol and between 49 and 98% for δ-tocopherol. The relative standard deviation, when analysing six replicates of the same salmon fillet, was 2–4% for α, β and γ-tocopherol and 12% for δ-tocopherol, and recovery ranged from 90–94% for α, β and γ-tocopherol and amounted to 58% for δ-tocopherol.

2.5. Statistical analyses

The software Statistica (Version 8.0, StatSoft Inc., Tulsa, OK) was used for the statistical analyses. The multivariate designs were analysed by multiple regression to find significant effects of the antioxidants and their interactions on TBARS and concentrations of vitamins C and E. Two-way analysis of variance (ANOVA) was used to determine effects of processing/storage time (repeated measurements ANOVA) and antioxidant supplementation on TBARS and vitamin C and E concentrations. The data were tested for homogeneous variances by Levene's test and were log-transformed in the cases of significant results. Differences and effects were considered significant at $p < 0.05$.

3. Results

3.1. Experiment 1

In Experiment 1, average TBARS was not affected by production and freezing of the diet, but increased on average from 77 to 212 nmoles g^{-1} during storage at −20 °C for 16 weeks (Table 4). Ascorbic acid had a profound effect on TBARS at all sampling points (p varied between 0.005 and 0.0006), except in the diet blend ($p > 0.05$), keeping the oxidation rate near to zero when added. Ascorbyl palmitate significantly reduced oxidation in the diet blend ($p = 0.04$), after heat treatment ($p = 0.005$) and after 3 weeks of frozen storage ($p = 0.015$). The positive interaction effect at 3 weeks of storage ($p = 0.018$) is probably that between ascorbic acid and ascorbyl palmitate, since the additive effect of them both would give a TBARS value of –44 nmoles g^{-1}. Adding tocopherol mix reduced TBARS significantly, only after heat treatment ($p = 0.029$), while adding phosphate mix increased TBARS after heat treatment and after freezing ($p = 0.01$), but had no effect at the other sampling points. There were no significant effects of adding rosemary, spermine or citric acid to the diet on TBARS in this experiment.

Table 3
Experimental designs. In the multivariate designs in Experiments 1 and 2, 1 and −1 indicate that the diets are supplemented or not with the antioxidant in question. 0 (centre points) indicates that the antioxidant is supplemented at 0.5 times the amounts added in diets denoted 1. The concentrations of antioxidants are given in Table 2.

Diet no.	Phosphate mix	Tocopherol mix	Ascorbic acid	Rosemary	Spermine	Ascorbylpalmitate	Citric acid	Ethoxyquin
(a) Experiment 1 (reduced factorial design 2^{7-3})								
1 (blank)	−1	−1	−1	−1	−1	−1	−1	−1
2	1	−1	−1	−1	1	−1	1	−1
3	−1	1	−1	−1	1	1	−1	−1
4	1	1	−1	−1	−1	1	1	−1
5	−1	−1	1	−1	1	1	1	−1
6	1	−1	1	−1	−1	1	−1	−1
7	−1	1	1	−1	−1	−1	1	−1
8	1	1	1	−1	1	−1	−1	−1
9	−1	−1	−1	1	−1	1	1	−1
10	1	−1	−1	1	1	1	−1	−1
11	−1	1	−1	1	1	−1	1	−1
12	1	1	−1	1	−1	−1	−1	−1
13	−1	−1	1	1	1	−1	−1	−1
14	1	−1	1	1	−1	−1	1	−1
15	−1	1	1	1	−1	1	−1	−1
16	1	1	1	1	1	1	1	−1
17	0	0	0	0	0	0	0	−1
18	0	0	0	0	0	0	0	−1
19	0	0	0	0	0	0	0	−1
20	0	0	0	0	0	0	0	−1
Control	1	1	1	−1	−1	−1	−1	1

Diet no.	Phosphate mix	Tocopherol mix	Ascorbic acid	Rosemary	Ascorbyl palmitate	Citric acid	Ethoxyquin
(b) Experiment 2 (reduced factorial design 2^{6-2})							
1 (blank)	−1	−1	−1	−1	−1	−1	−1
2	1	−1	−1	−1	1	−1	−1
3	−1	1	−1	−1	1	1	−1
4	1	1	−1	−1	−1	1	−1
5	−1	−1	1	−1	1	1	−1
6	1	−1	1	−1	−1	1	−1
7	−1	1	1	−1	−1	−1	−1
8	1	1	1	−1	1	−1	−1
9	−1	−1	−1	1	−1	1	−1
10	1	−1	−1	1	1	1	−1
11	−1	1	−1	1	1	−1	−1
12	1	1	−1	1	−1	−1	−1
13	−1	−1	1	1	1	−1	−1
14	1	−1	1	1	−1	−1	−1
15	−1	1	1	1	−1	1	−1
16	1	1	1	1	1	1	−1
17	0	0	0	0	0	0	−1
18	0	0	0	0	0	0	−1
19	0	0	0	0	0	0	−1
20	0	0	0	0	0	0	−1
Control	1	1	1	0	0	0	1

(c) Experiment 3
Diets: ascorbic acid low, medium, high; tocopherols low, medium, high; rosemary, low, medium, high. All low, all medium, all high, blank, control. No replicates.

Diet	Tocopherol mix	Ascorbic acid	Phosphate mix	Ethoxyquin
(d) Experiment 4				
1	1	1	–	–
2	1	1	1	–
3	1	1	1	1
Blank	–	–	–	–

1: Added according to the concentrations given in Table 2.
–: Not added.

Fig. 2a shows the development of TBARS with time, when diets containing equal combinations of ascorbic acid and ascorbyl palmitate were combined. TBARS in the diet without added antioxidants (blank) increased to a maximum of 745 nmoles g^{-1} after 3 weeks of frozen storage and was thereafter reduced to between 440 and 520 nmoles g^{-1}. The TBARS of the control diet, with added ascorbic acid, tocopherol mix, phosphate mix and ethoxyquin increased slightly from 56 to 79 nmoles g^{-1} from after heating until 16 weeks of storage. There was no significant effect of adding ascorbyl palmitate to the diets containing ascorbic acid ($p = 0.07$) and the average TBARS in all the diets with 1 g/kg ascorbate was reduced from 76 to 59 nmoles g^{-1} during heating and increased thereafter to 76 nmoles g^{-1} after 16 weeks of storage. The diets with added ascorbyl palmitate but no ascorbic acid increased their average TBARS level from 71 to 327 nmoles g^{-1} from after heating until 16 weeks of storage, while the diets with no ascorbyl palmitate and no ascorbic acid increased their average TBARS at 16 weeks to 488 nmoles g^{-1}. The reduction in TBARS as a result of adding ascorbyl palmitate to the diets not containing ascorbic acid was significant ($p = 0.03$). Average TBARS in the diets with added intermediate concentrations of all antioxidants (centre points) increased from 66 to 85 nmoles g^{-1} from after heating until 16 weeks of storage and the standard deviation varied between 8% and 13% of the average. Similar variations were seen in the diet combinations containing

Table 4
TBARS (nmoles g^{-1} dry weight) in fish feed during production and frozen storage in Experiment 1. Average of all feeds and quantitative effects and two-factor interactions of antioxidants after multiple regression analyses. Only significant effects are given ($p < 0.05$).

	Average	1. Phosphate mix	2. Tocopherol mix	3. Ascorbic acid	4. Rosemary	5. Spermine	6. Ascorbyl palmitate	7. Citric acid	Overlapping interactions						
									12	13	14	15	16	17	24
									67	47	56	46	27	26	57
									35	25	37	23	45	34	36
Diet blend	79						−7.6								
After heat treatment	67	4.2	−3.8	−7.9			−5.3								
After freezing	77	8.2		−15.7											
Frozen storage															
1 week	115			−50											
3 weeks	181			−134			−91								87
9 weeks	166			−113											
16 weeks	212			−168											

Table 5
TBARS (nmoles g^{-1} dry weight) in fish feed during production and frozen storage (−20 °C) in Experiment 2. Average of all feeds and quantitative effects and interactions of antioxidants after multiple regression analyses. Only significant effects are given ($p < 0.05$).

	Average	1. Phosphate mix	2. Tocopherol mix	3. Ascorbic acid	4. Rosemary	5. Ascorbyl palmitate	6. Citric acid	Overlapping interactions						
								15						
								12	13	14	23	16	24	26
								35	25	56	46	45	36	34
Diet blend	46			−15	−18	−14								14
After heating	81		−63	−70	−70		−66				65		64	69
After freezing	117			−109	−94									94
1 week	137		−79	−113	−110		−81				70		87	122
6 weeks	227		−102	−136	−120									
2 days at 4 °C	227		−70	−240	−105	−147		149						

added 1 g/kg ascorbic acid. Development of TBARS in the centre point diets was thus similar to that of the diets with added 1 g/kg ascorbic acid. The variation in results increased sharply as the oxidation process became active in the diets with no added ascorbic acid (Fig. 2a).

Data on development of vitamin C and E concentrations with time in Experiment 1 are given in Fig. 2b and c. The diets with no added ascorbic acid had an average vitamin C level of 69 mg/kg, those with 500 mg/kg added had an average level of 420 mg/kg and those with 1000 mg/kg added had an average level of 608 mg/kg. The ascorbic acid levels in the three diet groups were all significantly different from each other ($p < 2 \times 10^{-4}$). There was no change in ascorbic acid concentration over time and no effect of the other antioxidants on the ascorbic acid concentration in the diets.

Adding 250 mg/kg tocopherols gave approximately 350 mg/kg total tocopherols in the diets, which is an approximate sum of tocopherols in the ingredients and added tocopherol. However, adding 500 mg/kg tocopherols gave only about 430 mg/kg tocopherols in the diets, showing that there was a great loss of tocopherols at this level of inclusion. The sum of tocopherols was significantly higher in diets where they were added at 500 mg/kg than in diets with no added tocopherols ($p < 0.02$), while the levels in diets with 250 mg/kg tocopherols added were intermediate and not different from the other two. The apparent reduction in tocopherol concentration with time seen in Fig. 2c in the diets with 500 mg/kg tocopherols added, was not significant ($p = 0.2$). However, there was a significant positive effect of adding ascorbic acid on the total tocopherol concentration after 16 weeks of storage in these diets ($p = 0.04$), restoring total tocopherols to above the average initial level in the diet blend. There was no change in tocopherol concentration with time in the diets with 0 or 250 mg/kg tocopherols added. In the diets with no added tocopherols, no effect of the other antioxidants on tocopherol concentration was detected.

3.2. Experiment 2

In Experiment 2, the average TBARS concentration increased both during heating and freezing from 46 to 81 and then to 117 nmoles g^{-1}. After 6 weeks of storage at −20 °C, average TBARS had increased to 227 nmoles g^{-1} (Table 5). In this experiment, addition of ascorbic acid and rosemary had the most pronounced effects on TBARS in the diets ($p = 0.04$–0.007), with consistency through the whole experiment. Tocopherol mix, citric acid and ascorbyl palmitate addition also reduced TBARS significantly ($p = 0.04$–0.03), but only at some of the sampling points. Phosphate mix had no effect on TBARS in this experiment. Storage of the diet blend for 2 days at 4 °C gave an increase in average TBARS from 46 to 227 nmoles g^{-1} with large inhibiting effects of ascorbic acid ($p = 0.004$) and ascorbyl palmitate ($p = 0.03$). The interaction effects given in Table 5 show that the effect of adding more than one of the active antioxidants is smaller than the sum of the effects of individual antioxidants.

Development of TBARS over time in diets with 1 g/kg ascorbic acid, grouped according to the combinations of rosemary and tocopherol mix is given in Fig. 3a. TBARS in the diet not supplemented with antioxidants (blank) increased to a maximum of 1068 nmoles g^{-1} after 1 week of frozen storage, while the control diet, added tocopherol mix, ascorbic acid, phosphate mix and ethoxyquin, had only minor variation in TBARS and developed from 29 nmoles g^{-1} in the diet blend to 21 nmoles g^{-1} after 6 weeks of storage. The diets with added ascorbic acid and tocopherol mix had a very similar development in TBARS to the control diet. The diets with the combination of ascorbic acid and rosemary and those with

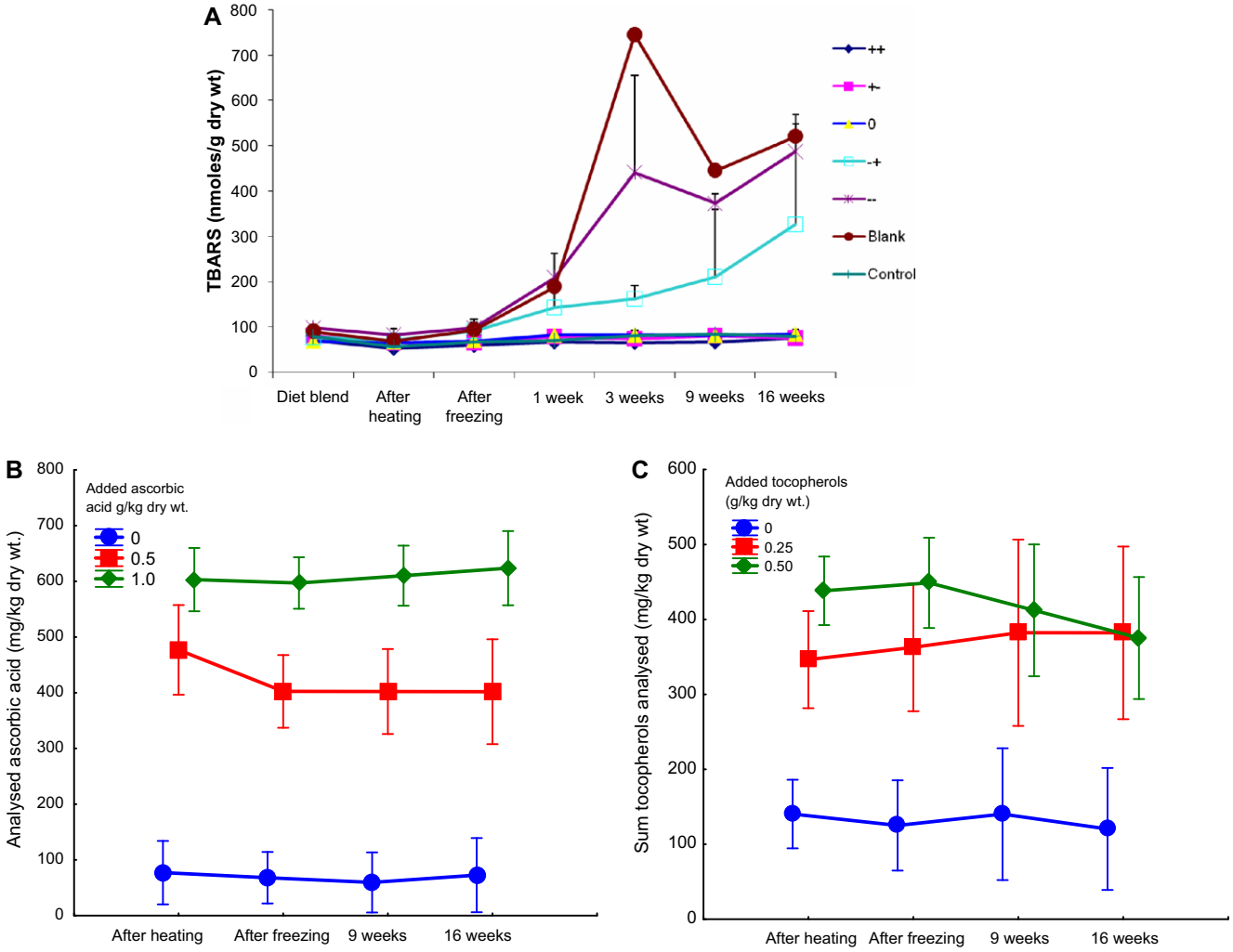

Fig. 2. Experiment 1. (A) The development of lipid oxidation measured as thiobarbituric acid-reactive substances (TBARS, nmoles g^{-1} dry weight) in the diets during production and storage. Diets grouped according to added levels of ascorbic acid (AA) and ascorbyl palmitate (AP): ++, both added; +- only AA added; -+, only AP added; – none of them added; blank, no antioxidants added; control, added AA, tocopherol mix, ethoxyquin and phosphate mix. (B) Concentrations of ascorbic acid and (C) total tocopherols (mg/kg dry weight) in diets with no addition or medium or high addition of these antioxidants.

all three antioxidants added appeared slightly higher in TBARS, but were not significantly different from the diets with the ascorbic acid and tocopherol mix. Diets containing added ascorbic acid only had significantly elevated TBARS after 6 weeks of storage, compared to the other diets with added ascorbic acid.

Development of vitamin C and E in Experiment 2 is given in Fig. 3b and c. Ascorbic acid concentrations in the diet blend, according to the added amounts and the three levels of supplementation, gave significant differences in ascorbic acid concentration between the three diet groups ($p = 0.04– <10^{-6}$, Fig. 3b). There was a reduction in ascorbic acid concentration during heating of 66% when all diets were combined ($p < 10^{-6}$), while freezing and storage of the diets gave no further decrease in ascorbic acid. When ascorbate was not added to the diets, ascorbyl palmitate (diet blend, $p = 0.004$), tocopherol mix and citric acid (after heating, $p = 0.015$ for both) increased the concentration of ascorbic acid in the diets. From after freezing and during frozen storage, none of the other antioxidants affected the ascorbate concentration in these diets. In the diets with added ascorbic acid, supplementation with rosemary ($p = 0.02$), ascorbyl palmitate ($p = 0.001$) and citric acid ($p = 0.02$) gave a reduction in ascorbic acid after heating. From after freezing until 6 weeks of storage, supplementation of ascorbyl palmitate ($p = 0.02–0.007$), but not the other antioxidants, gave a reduction in concentration of ascorbic acid in the diets. Also, during storage of the diet blends for 2 days at 4 °C, ascorbyl palmitate had a negative effect on the ascorbic acid concentration in the diets with added ascorbate ($p = 0.009$), but no such effect was seen in the diets without addition of ascorbate.

Average total vitamin E in the diet blends was 190 mg/kg in diets with no added tocopherol mix, 355 mg/kg in diets with 250 mg/kg tocopherols added and 497 mg/kg in diets with 500 mg/kg tocopherols added (Fig. 3c). The concentration of tocopherols in the unsupplemented diets was lower than in the supplemented diets ($p = 0.0015$ and 10^{-6} for medium and high, respectively), which were not significantly different from each other. There was a significant decrease in total tocopherols from before heat treatment until 6 weeks of storage ($p < 10^{-6}$), where the most pronounced loss occurred during heating. However, there was also a significant reduction in total tocopherols during frozen storage ($p = 0.0014$), which was most pronounced in the diets with 500 mg/kg tocopherols added. There were no effects of the other antioxidants on total tocopherol concentration in the diets.

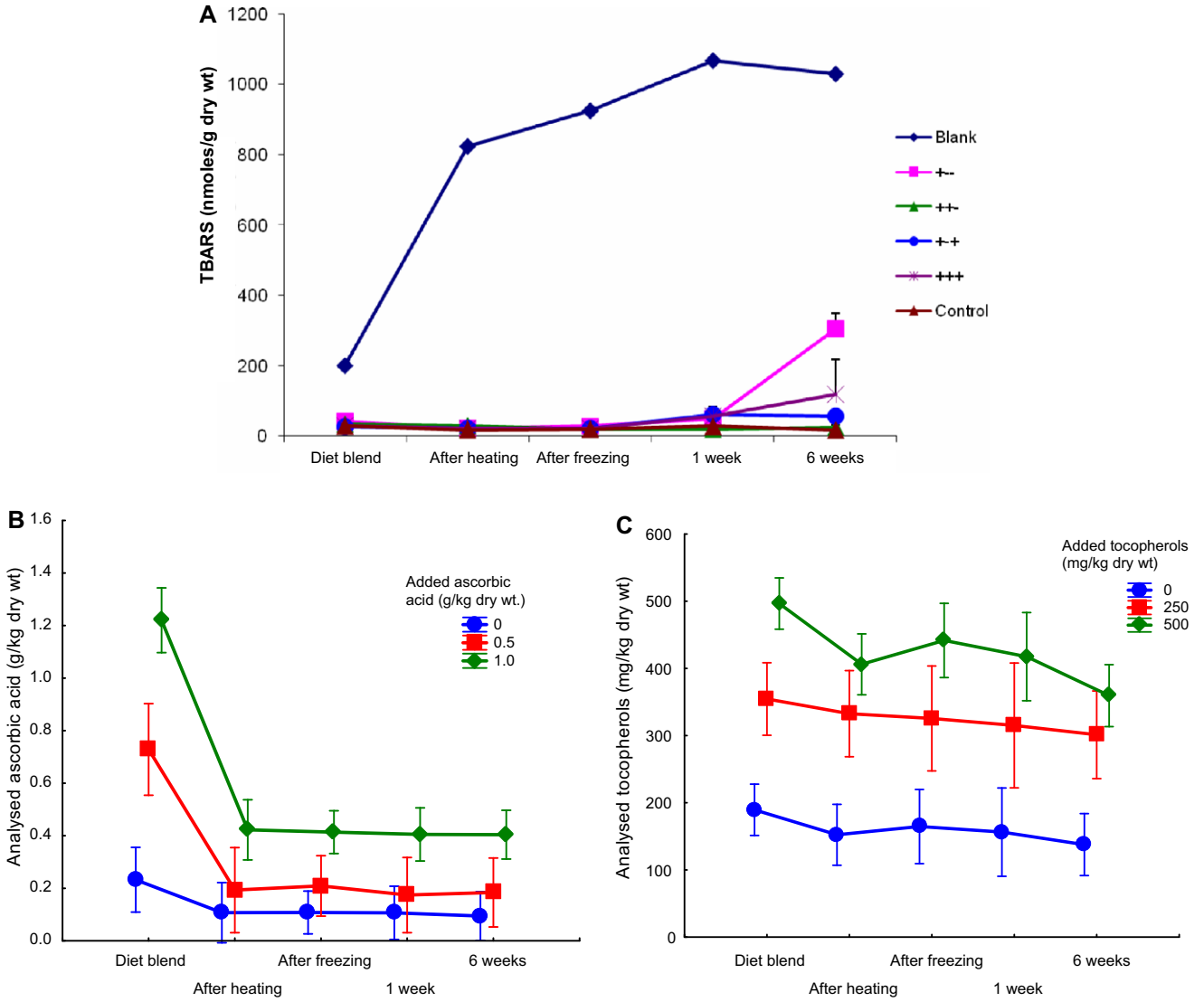

Fig. 3. Experiment 2, the development of lipid oxidation measured as thiobarbituric acid-reactive substances (TBARS, nmoles g^{-1} dry weight) in the diets during production and storage. Diets contain added AA and grouped according to addition of tocopherol mix (TOH) and rosemary (herb): +–, no TOH or herb added; ++-, TOH added; +-+, herb added; +++, both added. Blank and control as in Fig. 2A. (B) Concentrations of ascorbic acid and (C) total tocopherols (mg/kg dry weight) in diets with no addition or medium or high addition of these antioxidants.

3.3. Experiment 3

In Experiment 3, where the results from 6 weeks of storage are given in Fig. 4, ascorbate alone gave the lowest levels of TBARS when added at the low concentration, compared with the other two antioxidants. Increase in ascorbic acid concentration from low to medium (0.5 to 1.0 g/kg) gave only a slight decrease in TBARS. A slight increase in TBARS was seen with increase in ascorbate from 1.0 to 2.0 g/kg. Tocopherols had limited antioxidant effect at additions of 0.35 and 0.7 g/kg, but an effect could be seen at 1.4 g/kg. The antioxidant effect of rosemary increased from additions of 1.5 to 3.0 and further to 6.0 g/kg, the high inclusion level giving a lower level of TBARS than in the control diet. When all three antioxidants were added at the low level, TBARS was similar to TBARS in the control diet, while increasing concentrations reduced TBARS to a similar level as in the high rosemary diet.

3.4. Experiment 4

The effects of adding phosphate mix and ethoxyquin to a diet protected by tocopherol mix and ascorbic acid were studied in Experiment 4. Running a repeated measurements ANOVA on this design, where the diets were stored for 18 weeks, showed that the diet with added phosphate mix and ethoxyquin had a stable TBARS level during the whole experiment (Fig. 5). There was a significant increase in TBARS in the other two diets from before heat treatment until 3 weeks of storage ($p = 0.04$). After 8 weeks of storage, TBARS was reduced to initial levels in these diets, but increased again at 18 weeks ($p = 0.04$). When treating the whole data set, TBARS was significantly lower in the diet with added ethoxyquin than in the other diets ($p = 0.03$ and 0.02 for the diets with and without added phosphate mix, respectively). The diets with added tocopherol mix and ascorbic acid, with or without phosphate mix, did not have different TBARS levels.

4. Discussion

The present study points to ascorbate and rosemary as the most effective of the studied antioxidants. However, Herbalox® must be added at higher concentrations than ascorbate to be effective. This may be a logical consequence of the fact that while ascorbate is a crystalline concentrated powder, Herbalox® is a rosemary extract

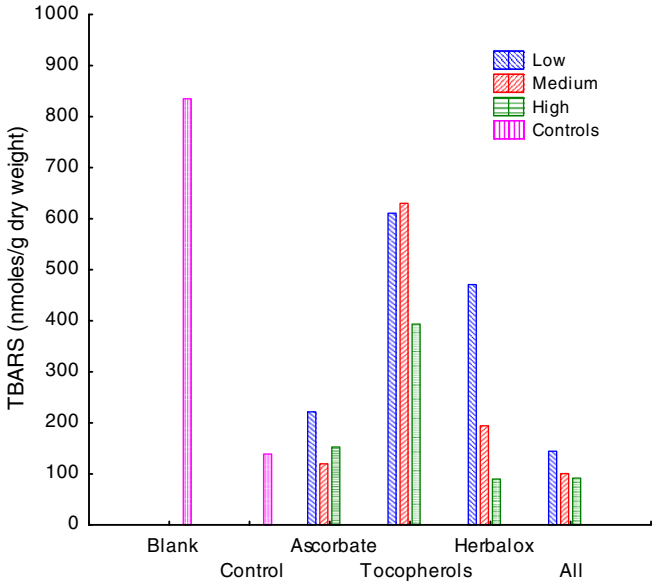

Fig. 4. Experiment 3. Thiobarbituric acid-reactive substances (TBARS, nmoles g^{-1} dry weight) in diets supplemented with either ascorbic acid, tocopherol mix or rosemary at three different concentrations and stored at −20 °C for 6 weeks. All, all three antioxidants added at either low, medium or high levels; blank, no antioxidants added; control, added ascorbic acid, tocopherol mix, ethoxyquin and phosphate mix.

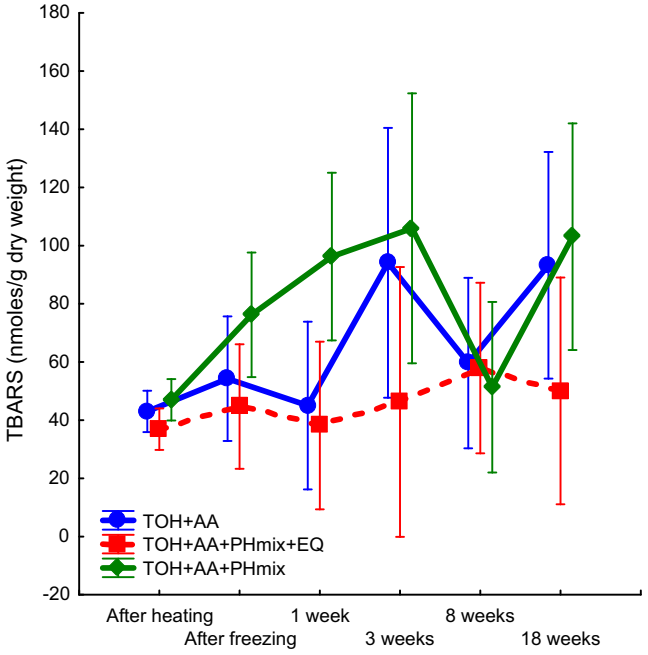

Fig. 5. Experiment 4. Thiobarbituric acid-reactive substances (TBARS, nmoles g^{-1} dry weight) in diets with added ascorbic acid (AA), tocopherol mix (TOH), ethoxyquin (EQ) and phosphate mix (PH mix) and stored for 18 weeks at −20 °C.

and will contain substances that are not antioxidants. Furthermore, rosemary is dissolved in vegetable oil, also contributing to lower concentrations of active antioxidants in the product.

Tocopherol mix prevented accumulation of TBARS only after heat treatment in Experiment 1. In Experiment 2, tocopherol mix also reduced TBARS during storage, both at −20 °C and at 4 °C. Experiment 3 shows that tocopherol mix had low efficiency when added to the feed as the only antoxidant. However, the proposed interaction between vitamin E and vitamin C, where vitamin C reduces the tocopheroxyl radical formed when vitamin E reacts with oxidising lipid (Packer & Kagan, 1993; Packer, Slater, & Willson 1979; Tappel, 1962), may explain the substantial antioxidative effect of tocopherol mix seen in Experiment 2. Another mode of interaction of these antioxidants is that they have different mechanisms of preventing oxidation. Vitamin C, in addition to the regeneration of vitamin E, also scavenges oxygen radicals in the water phase and chelates metals. Vitamin E scavenges reactive oxygen species in the lipid phase (Frankel, 1998). This prevents the radicals and transition metals becoming involved in further oxidation reactions. At 6 weeks of frozen storage and 2 days of cold storage, there was no interaction between ascorbate and tocopherol mix, showing that the effect of adding both antioxidants was similar to the sum of their individual effects. This indicates that adding a combination of vitamin C and E gives good protection, compared to many of the other combinations where the sums of individual effects were higher than the effect of the added combination. Experiment 2 shows that ascorbic acid alone, without addition of tocopherol mix may give increased oxidation. Furthermore, in Experiment 1 the levels of tocopherols in the diets were increased by addition of ascorbic acid at 16 weeks of storage, indicating an interaction between the two antioxidants.

Experiment 2 also shows that the concentration of ascorbic acid in the diets decreased sharply during heating, but after the heat treatment there was very little change in ascorbic acid levels. A similar result was seen in Experiment 1, but unfortunately the diets were not sampled before heat treatment in this experiment. For tocopherols, the reduction in concentration was already apparent in the diet blend in Experiment 2, and a minor reduction was seen both during heat treatment and during storage. In Experiment 1 the concentration of tocopherol was not significantly changed from after heating until 16 weeks of storage. It appears that ascorbic acid is most unstable during heating, while the tocopherols are most unstable during the blending process. Further freezing and storage of the feed only had a small effect on the concentrations. Furthermore, addition of 500 mg/kg tocopherols, compared to 250 mg/kg, had a limited effect on the concentrations in the feeds, showing that added tocopherols become increasingly unstable at higher concentrations.

Rosemary had little effect on oxidation in Experiment 1, but when the concentration was raised in Experiment 2, the effect of rosemary was of a similar size to the effect of ascorbic acid. Experiment 3 also shows that increasing the concentration from 1.5 to 3.0 and then to 6.0 g/kg gave a gradual increase in protection and that the highest concentration of rosemary appeared to give a better protection than the most effective concentration of ascorbic acid. Rosemary contains several compounds of which some have antioxidant activity. It is a lipid-soluble product, dissolved in vegetable oil and will mainly be in the lipid phase of the feed. It is possible that the different compounds in rosemary have different antioxidant functions and that they interact with each other, for example, through recycling reactions, in a similar manner to vitamin C and E. This may enhance the antioxidant effect of rosemary. In the results from Experiment 2, there is a positive interaction effect that may be either ascorbate with rosemary or tocopherol mix with citric acid. Since large main effects more often show interactions with each other than small main effects, this interaction is probably that between ascorbate and rosemary, i.e., the reduction in TBARS due to adding ascorbate and rosemary in combination is lower than the sum of the individual effects of these antioxidants. The lack of synergy may be caused by an enhanced efficiency of rosemary, due to interactions between compounds already present, in such a way that addition of ascorbate has no further effect.

Effects of ascorbyl palmitate on oxidation were mainly seen in the diet blend, after heat treatment and in cold-stored feed. Only

at one point (Experiment 1, 3 weeks of storage), was there an effect of this antioxidant after freezing and storage of the feed. There was no effect of adding ascorbyl palmitate to diets already containing ascorbate, and adding ascorbyl palmitate alone gave a considerable increase in TBARS, compared to the control diet. However, TBARS was still lower than in the diets containing no ascorbate and no ascorbyl palmitate. The reason that the analysed ascorbic acid concentration in the diets did not show a reproducible increase in response to adding ascorbyl palmitate may be that our analytical method did not contain a step for hydrolysis to free ascorbate and palmitate. Therefore, ascorbyl palmitate was probably not detected in the samples. On the other hand, ascorbyl palmitate led to a general reduction in measured ascorbic acid in diets with added ascorbate in Experiment 2, indicating that ascorbyl palmitate in this situation may have had a pro-oxidant effect.

Citric acid acts mainly as a metal chelator (Frankel, 1998) and fish feed contains appreciable amounts of transition metals, both from the raw ingredients and from the mineral mixes. However, in the present feed production system, citric acid had no effect on oxidation in Experiment 1 and only a minor effect in Experiment 2. The phosphate mix had no effect in Experiment 1 and a minor pro-oxidant effect during feed production and freezing, but not during storage, in Experiment 2. The results from Experiment 4 further confirm that the phosphate mix has no enhancing effect on the antioxidative activity of vitamin C in the present feed production and storage system. Although spermine had a 30 times higher activity than α-tocopherol in protecting fish oil from oxidation (Løvaas, 1991), there was no effect of spermine on either TBARS or dietary vitamin C and E concentrations in the present study, even though the concentration of spermine used (500 vs 200 mg/kg) was higher than that used by Løvaas (1991). Spermine was therefore omitted after Experiment 1.

The main question of this study was whether the synthetic antioxidant ethoxyquin, can be replaced by natural antioxidants. In Experiments 1 and 2, the diets with addition of the combination ascorbate and tocopherol mix gave similar TBARS results to the control diet containing added ascorbate, tocopherol mix, ethoxyquin and phosphate mix. In Experiment 4, the diets with added ethoxyquin had slightly better performance than those with added ascorbate and tocopherol mix, with or without phosphate mix. Furthermore, rosemary protected well against oxidation both in Experiments 2 and 3. The positive effect of ethoxyquin in Experiment 4 was small and was not reproduced in the other experiments, while the positive consequences with regard to food safety and consumer wellbeing, of protecting fish feed with natural antioxidants is important in a society where awareness of food additives is increasing. However, the present results cannot be used directly for any feed type, since the effect of antioxidants are dependent on the oxidising system in which they are used (Frankel 1998). We therefore recommend the examination of the effects of natural antioxidants in fish feeds produced using commercial methods and in feed ingredients of marine origin, to promote a change from synthetic to natural antioxidants in fish feeds.

References

Bohne, V. J. B., Lundebye, A.-K., & Hamre, K. (2008). Accumulation and depuration of the synthetic antioxidant ethoxyquin in the muscle of Atlantic salmon (*Salmo salar* L.). *Food and Chemical Toxicology, 46*, 1834–1843.

Box, G. E. P., Hunter, W. G., & Hunter, J. S. (1978). *Statistics for experimenters. An introduction to design, data analyses and model building*. New York: Wiley.

Erkan, N., Ayranci, G., & Ayranci, E. (2008). Antioxidant activities of rosemary (*Rosmarinus Officinalis* L.) extract, blackseed (*Nigella sativa* L.) essential oil, carnosic acid, rosmarinic acid and sesamol. *Food Chemistry, 110*(7), 6–82.

Frankel, E. N. (1998). *Lipid oxidation*. Dundee, Scotland: The Oily Press Ltd.

Hamre, K., Næss, T., Espe, M., Holm, J. C., & Lie, Ø. (2001). A formulated diet for Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae. *Aquaculture Nutrition, 7*, 123–132.

Hemre, G.-I., Sandnes, K., Lie, Ø., Torrisen, O., & Waagbø, R. (1995). Carbohydrate nutrition in Atlantic salmon I. Growth and feed utilization. *Aquaculture Research, 26*, 149–154.

Hølmer, G. (1993). Mechanisms of oxidation – autooxidation and enzymic oxidation. In A. T. Diplock, J. M. C. Gutteridge, & V. K. S. Shukla (Eds.), *Antioxidants, free radicals and polyunsaturated fatty acids in biology and medicine* (pp. 15–30). Lystrup, Denmark: International Food Science Centre A/S.

Horton, A. A., & Fairhurst, S. (1987). Lipid peroxidation and mechanisms of toxisity. *CRC Critical Reviews in Toxicology, 18*, 27–79.

Langsrud, Ø., Ellakjær, M. R., & Næs, T. (1994). Identifying significant effects in fractional factorial experiments. *Journal of Chemometrics, 8*, 205–219.

Lie, Ø., Sandvin, A., & Waagbø, R. (1994). Transport of α-tocopherol in Atlantic salmon (Salmo salar) during vitellogenesis. *Fish Physiology and Biochemistry, 13*, 241–247.

Lie, Ø., Waagbø, R., & Sandnes, K. (1988). Growth and chemical composition of adult Atlantic salmon (*Salmo salar*) fed dry and silage based diets. *Aquaculture, 69*, 343–353.

Løvaas, E. (1991). Antioxidative effect of polyamines. *JOACS, 68*, 353–358.

Løvaas, E. (1995). Hypothesis: Spermine may be an important epidermal antioxidant. *Medical Hypotheses, 45*, 59–67.

Mæland, A., & Waagbø, R. (1998). Examination of the qualitative ability of some cold water marine teleosts to synthesise ascorbic acid. *Comparative Biochemistry and Physiology, 121*, 249–255.

National Research Council, NRC (1993). Nutrient requirements of fish. *Nutrient requirements of domestic animals* (pp. 62–63). Washington DC: National Academy Press.

Packer, L., & Kagan, V. E. (1993). Vitamin E: The antioxidant harvesting center of membranes and lipoproteins. In L. Packer & J. Fuchs (Eds.), *Vitamin E in health and disease* (pp. 179–192). New York, Basel, Hong Kong: Marcel Dekker Inc.

Packer, J. E., Slater, T. F., & Willson, R. L. (1979). Direct observation of a free radical interaction between vitamin E and vitamin C. *Nature, 278*, 737–738.

Rice-Evans, C. (1999). Screening of phenolica and flavonoids for antioxidant activity. In L. Packer, M. Hiramatsu, & T. Yoshikawa (Eds.), *Antioxidant food supplements in human health* (pp. 239–255). San Diego, London: Academic Press.

Sundberg, R. (1994). Interpretation of unreplicated two-level factorial experiments, by examples. *Chemometrics and Intelligent Laboratory Systems, 24*, 1–17.

Tappel, A. L. (1962). Vitamin E as the biological lipid antioxidant. *Vitamins and Hormones, 20*, 493–510.