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Accumulation and depuration of the synthetic antioxidant ethoxyquin in the muscle of Atlantic salmon (*Salmo salar* L.)

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Abstract

The biological fate of the fish feed additive, ethoxyquin (EQ) was examined in the muscle of Atlantic salmon during 12 weeks of feeding followed by a 2 weeks depuration period. Parent EQ (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline), quinone imine (2,6-dihydro-2,2,4-trimethyl-6-quinolone), de-ethylated EQ (6-hydroxy-2,2,4-trimethyl-1,2-dihydroquinoline) and EQDM (EQ dimer or 1,8′-di(1,2-dihydro-6-ethoxy-2,2,4-trimethyl-quinoline) were found to be the ubiquitous metabolites of dietary EQ, with EQDM as a main metabolite. A rapid decrease in the level of EQ (2.4 days of half-life) was balanced by an increase in EQDM, giving an unchanged net sum following 2 weeks of depuration. The mandatory 14 days depuration period prior to slaughtering of farmed salmon in Norway was not sufficient for complete elimination of EQ-derived residuals. Post depuration, EQDM accounted for 99% of sum of the two compounds in all treatment groups; possible toxicological effects of EQDM are not known. The individual concentrations of EQ and EQDM and their sum are dependent on EQ level in the feed, consequently, their residual concentrations may be controlled. The theoretical amount of EQ and EQDM consumed in one meal of farmed salmon would be under the recommended ADI, provided that the fish were raised on feed with no more than 150 mg EQ/kg feed, which is the EU maximum limit for EQ in fish feed.

Keywords: Antioxidant; Amine; Feed additive; Ethoxyquin; Salmon; Metabolites

1. Introduction

Ethoxyquin (EQ; 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline) is an aromatic amine with the ability to scavenge lipid peroxide radicals and thus terminate the spontaneous oxidation of unsaturated lipids in fish feeds and feed ingredients. According to the national surveillance program EQ is the most widely used preservative in fish feed in Norway (Maage et al., 2006). The upper limit for EQ in fish feed established in the EU is 150 mg/kg, alone or in combination with butylated hydroxianisole (BHA) and/or butylated hydroxytoluene (BHT) (Council Directive, 2003). In contrast to BHT and BHA, EQ is not an approved food additive in the EU, nevertheless it may be carried over from feed to food products originating from farmed terrestrial or aquatic animals. A National surveillance programme monitors the level of contaminants and additives (including EQ) in fish feed and feed ingredients to document that levels comply with regulations (http://www.mattilsynet.no). Over the three decades that EQ has been used in salmon feed, only one study has been published on the depuration of EQ from salmon muscle (Skaare and Roald, 1977). The biological fate of parent EQ alone and unspecified bulk of EQ-derived residue was traced and elimination of EQ-derived residue was shown to be rapid. The difference in the levels of EQ-derived metabolites in fish fed 150 and 900 mg EQ/kg feed indicated a possible correlation with the EQ level in the feed, however, no elimination data or residue concentrations of EQ alone were presented (Skaare and Roald, 1977).

Despite the fact that EQ may protect against cancer caused by chemical agents (Hayes and McMahon, 2001;

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Hayes et al., 1998, 2000; Bammler et al., 2000; Manson et al., 1997; Prochaska and Talalay, 1988; Kahl and Kahl, 1983), the Acceptable Daily Intake was re-evaluated by the JMPR in 1998 and reduced from 0.06 to 0.005 mg/kg body weight (JMPR, 1998). Moreover, EQ was recently shown to cause several unfavourable side-effects in animals fed diets containing this compound, but also adverse effects have been observed in people exposed to EQ (Blaszczyk and Skolimowski, 2005).

With the current focus on food safety, particularly in relation to animal feeds the current study was designed to evaluate the potential transfer of EQ from feed to the edible parts of fish. A new analytical method for the simultaneous detection of EQ and it's metabolites in salmon and fish feed was used to produce the results reported in this paper. The commercial production of salmon in Norway includes a mandatory depuration period of 2 weeks for the elimination of feed additives out of the fish flesh. Therefore, muscle levels of EQ and EQDM was measured before and after depuration.

2. Materials and methods

2.1. Diet composition

The diet formulation (Table 1), including ecological EQ-free fish meal (NorsEco-LT, Sildoljefabrikk A/S) was used to produce five experimental diets, with nominated concentrations of EQ (0, 15, 150, 1500 and 15000 mg/kg dry feed) at Fiskeriforskning (Bergen, Norway). The feeds were produced in one batch, as 4 mm extruded pellets. The levels of EQ were adjusted by mixing commercially available EQ (Rexoquin 100, Grace Rexolin, >97%) with the EQ-free oil coating (NorsEcoOil, Sildoljefabrikk A/S). The EQ-free pellets were produced from EQ-free fish meal and coated with EQ-free oil, both stabilised with commercial mixture of natural antioxidants (Vitalox, Helm). The diets were stored at −20 °C through the whole experimental period. The feed requirement was calculated with a start weight of 0.2 kg per fish, predicted daily growth rate of 1% and a feed conversion factor of 1 (dry weight feed/fresh weight fish).

Table 1
Formulation and proximate composition of the basal fish diet (g/kg diet)

Ingredients	Dry weight (g/kg)
EQ free fish meal	66
EQ free NorSea oil	27
Mais Suprex	15
Vitamin mix[a]	1.1
Mineral mix[b]	0.44
Astaxantin (CAPROPHYLL® PINK, 8%)	0.09
Chemical analyses (% in feed wet weight)	
Ash	8.7
Water	6.3
Protein	43.3
Lipid	30.1
Carbohydrate	11.1
Energy (kJ/g)	24.2

[a] Vitamin mixture (g/kg dw) was composed as follows: 1 g retinyl acetate, 0.4 g cholecalciferol, 20 g α-tocopheroyl acetate, 0.5 g menadione, 28.57 g ascorbic acid, 162 mg choline, 4.55 g thiamine mononitrate, 0.63 g riboflavin, 0.5 g pyridoxine, 5 g niacin, 0.5 g folic acid, 5 g biotin, 0.1 g cobalamin, 2 g Ca-pantothenate and 769.25 g casein (filler).

[b] Mineral mixture (g/kg dw) was composed as follows: 506 g Mg (as $MgSO_4 \cdot 7H_2O$), 44 g Zn (as $O_4SZn \cdot 7H_2O$), 24.9 g $FeSO_4 \cdot 7H_2O$), 4.6 g Mn (as $MnSO_4 \cdot 7H_2O$), 2 g Cu (as $CuSO_4 \cdot 7H_2O$) and 418.5 g casein (filler).

2.2. Fish, husbandry and feeding experiment

A total of 900 individuals of Atlantic salmon (*Salmo salar* L.) produced from a March 2001 egg batch from Aqua Gen AS (Kyrkaeterøra, Norway) were used in the feeding experiment. The experimental fish were ½ years smolt with an initial mean weight of 0.2 kg. The fish were kept in $5.0 \times 5.0 \times 1.5\ m^3$ indoor tanks with seawater, for at least 2 months for acclimatization to the environment. During the last month the fish were fed the experimental control diet. Fish were subsequently stocked in 15 $1.5 \times 1.5 \times 0.5\ m^3$ glass fibre tanks with 60 fish per tank. The five experimental diets were randomly assigned to the 15 tanks (in triplicate). The salinity and temperature during the experiment were monitored and maintained between $27 \pm 2‰$ and 9.5 ± 0.8 °C, respectively. The daily light and dark cycle was artificially adjusted to follow the natural photoperiod for Matre (near Bergen, Norway, 60°N) and feeding was performed automatically every 5 min from 8 a.m. to 4 p.m. in the winter months, and from 8 a.m. to 8 p.m. from April to July.

Five fish from each tank were randomly caught and anesthetised in a bath of benzocaine (ethyl aminobenzoate), prepared from a 5 ml of stock solution of 0.1% benzocaine in 96% ethanol in 10 l seawater. Fish were sacrificed by a blow to the head. The skinless muscle from the right side of the body was carefully removed, homogenised and stored in plastic tubes with a screw cap at −80 °C in light tight containers until analysing within following 2–3 months. The average fish weight per tank was measured three times during the experiment as biomass to number of fish ratio, for determining growth rate, also the whole fish and muscle weights were recorded during sampling. Samples of muscle were taken on day 0, 3, 7, 14, 28, 84 during the feeding period and day 0 (the same as day 84 in the feeding period), 3, 7 and 14 in the depuration period, for all diets, except the control group. The control group received an EQ-free diet and thus should not contain EQ or any metabolites of dietary EQ. Therefore, samples were taken on day 0, 84 and at the end of depuration period.

2.3. Quantitative determination of EQ metabolites and total lipid content in muscle and feeds samples

Frozen samples of homogenised muscle were protected from the light when thawed. The 0.500 g of sample was submerged into a reaction blend consisting of ethanol, NaOH, saturated EDTA, ascorbic acid and pyrogallol and extracted as described elsewhere (Bohne et al., 2007a). Samples were extracted with hexane, which prior to the HPLC was exchanged by 0.1% (w/v) solid acetic acid in acetonitrile. EQ and metabolites were separated by elution with acetonitrile/ascorbic acid/acetic acid/diethyl amine mobile phase and detected at 358 and 433 nm for excitation and emission, respectively. The detection limit of matrix-spiked EQ compounds was 0.02 μg/l for EQ, 0.06 μg/l for EQDM. The repeatability and reproducibility of the method were less than 11% and 21%, respectively. Triplicate feed samples were extracted in a one step procedure, as described in the AOAC official method (Schreier and Greene, 1997) and analysed as described above. Analyses of total lipid content of the muscle and feed samples were performed according to Lie et al. (1988).

2.4. Data analyses and statistical analyses

Growth rates during the feeding trial and weight loss under the starvation period were determined by fitting all fish weight data to an exponential model (ln weight $= a + b \times$ time (days), where a is a constant and b is the growth rate). Biological feed conversion ratio (FCR) was corrected for mortalities and sampled fish:

$$\mathrm{FCR} = \frac{\text{Feed consumed (kg)}}{\text{Weight gain (kg)}} \quad (1)$$