Residues of Ethoxyquin and Ethoxyquin Dimer in Ocean-Farmed Salmonids Determined by High-Pressure Liquid Chromatography

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ABSTRACT: The occurrence of residues of ethoxyquin (EQ) and ethoxyquin dimer (DM) from fish feeds in the liver and muscle of farmed salmon and steelhead was studied. For quantitative analysis of DM, the tissue was partially hydrolyzed with 50% NaOH, and then the lipid and antioxidant residues were extracted with hexane. After solvent removal and recovery into acetonitrile, DM was determined by reversed-phase high-pressure liquid chromatography (HPLC) with a fluorescence detector, with the excitation wavelength set at 360 nm and the emission wavelength at 440 nm. The mobile phase was acetonitrile:0.01M ammonium acetate (80:20, v:v). The EQ levels were very low, but the DM levels were higher. The recoveries from the spiked samples were 88±4% for DM. Sample site determinations indicated that the DM residue level could be associated with depot fat except in the liver, where the tissue content of DM was 60% to 70% less. Similar results appear satisfactory for ethoxyquin extracted from fish oils.

Key Words: ethoxyquin, ethoxyquin dimer, antioxidants, fish muscle, salmon

Introduction

ETHOXYQUIN (EQ; 1,2-DIHYDRO-6-ETHOXY-2,2,4-TRIMETHylquinoline) is usually added as an antioxidant to fish meals when produced and also to fish feeds when they are being compounded for aquaculture because the omega-3 fatty acids involved are highly susceptible to autoxidation. During storage of fish meal, EQ is oxidized to a quinolone compound (QI; 2,6-dihydro-2,2,4-trimethyl-6-quinolone) and a dimer (DM; 1,8′-di(1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline)) as described by Thorisson and others (1992). All of the 3 compounds can usually be found in commercial fish feeds (He and Ackman 2000a). Thus, it is logical to suspect that they could also accumulate in farmed fish bodies and might be absorbed by consumers. Whether these compounds, especially the oxidation products, have beneficial or toxic effects on human health is not fully understood, but we are unaware of any information on the residue levels of these compounds in farmed fish.

This study was carried out to confirm the occurrence of DM in ocean-farmed salmonids using gas chromatography-mass spectrometry (GC-MS) and to develop a high-pressure liquid chromatography (HPLC) method to quantitatively determine the residues in fish muscle and liver tissues.

Materials and Methods

Materials and apparatus

Fresh steelhead, *Oncorhynchus mykiss*, ranging from 1 to 1.5 kg, were received from a marine aquaculture operation in Nova Scotia. These fish had been starved for a short period before slaughter. Whole fish were protected by crushed ice during the 1 d of transportation to Halifax where they were **be**headed and gutted. The samples were then double-bagged in polyethylene bags, tied off, and stored at −30 °C for about 1 wk before analysis. Fresh steaks of farmed Atlantic salmon, *Salmo salar*, were obtained from local supermarkets on the same d as their analysis. The muscle fat content of such commercial fish is nominally 10% of a whole fillet. Wild Pacific salmon (species unknown), gutted and **be**headed, were also obtained from a local supermarket and stored briefly at −20 °C before use.

HPLC consisted of a CSC-Select ODS-2 column (15 × 0.46 cm, 5 μm, Chromatographic Sciences Company, Montreal, Quebec) with a guard column (3 × 0.30 cm) from the same manufacturer, a Waters 6000A solvent delivery system, a Waters Model U6K HPLC injector, and a Waters 474 scanning fluorescence detector (Waters, Milford, Mass.). The mobile phase was acetonitrile and 0.01 N CH_3COONH_4 (80:20, v:v) at a flow rate of 1 ml min^{-1}. The same fluorescence detector was also used to obtain the emission spectra of the standard and the objective peaks. For recovery studies, a DM standard was prepared freshly in our laboratory as described elsewhere (He and Ackman 2000b). The concentrations of standard solutions then made up were 0.10, 0.20, 0.40, 0.60, 0.80, 1.00 μg ml^{-1}.

GC-MS was carried out with a PE 990 Gas Chromatograph (Perkin-Elmer, Norwalk, Conn.) fitted with a DB-1 fused silica capillary column (59 m × 0.25 mm i.d., Supelco, Oakville, Ontario) passed directly into a Finnigan 700 Ion Trap Detector (Finnigan Corp., San Jose, Calif.), as the mass spectrometer. The column temperature was 260 °C. Data acquisition was executed by a computer program provided by Finnigan Corp.

Sample analysis and recovery study

One ml 50% NaOH in water and 2 ml ethanol were added to a 1.0-g sample of well-homogenized fish muscle or macerated liver in a 15-ml test tube. The tube was fully flushed with nitrogen and capped. The sample was partially alkali-hydrolyzed with magnetic stirring at 100 °C on a water bath until the tissue just disappeared (normally less than 1.5 min). The tube was immediately cooled under running water, and 2 ml distilled water was added. The aqueous solution was extracted with hexane (4 × 2.5 ml), and the hexane solutions were combined. A small amount of NaCl could effectively destroy any emulsion if it formed during the extraction operation. The hexane solutions were washed with 2 ml distilled water and dried over Na_2SO_4. The hexane was removed by a stream of nitrogen, and the residues were dissolved in exactly 1 ml of acetonitrile before HPLC analysis. The eluate of the suspected DM peak was collected, recovered into hexane, and then analyzed by GC-MS.

Recovery studies were carried out at different levels of DM

added to steelhead muscle. All standards, in solution, were spiked into the sample just before flushing with nitrogen so that oxidation was minimized. At least 2 analyses were carried out at each level of addition.

Results and Discussion

STEELHEAD AND ATLANTIC SALMON ARE 2 COMMON SPECIES OF salmonids reared in the Atlantic area. Originally, residues of EQ, QI, and DM in liver and muscle tissues of these 2 species of fish were studied by HPLC with a UV detector. It was unexpected to find that both EQ and QI would be very low in these tissues (He 1998). However, a relatively large amount of an unidentified compound was observed and suspected to be the DM whose structure is shown in Figure 1.

Extracts of the muscle and liver of farmed steelhead, the muscle of farmed Atlantic salmon, as well as the muscle tissues of wild Pacific salmon were examined by HPLC (Figure 2). No peak for DM was found when wild salmon muscle samples were analyzed, but a peak having the same elution time as the DM standard on HPLC was observed in all farmed fish samples.

To identify this unknown peak, the eluate between the putative DM peak beginning and end was collected. The materials were recovered into hexane and analyzed by GC-MS. A group of compounds was found on the GC chromatogram, among which 1 peak with the retention time of DM was confirmed as DM according to the match of the MS spectrum with that of the standard and the spectrum published by Thorisson and others (1992). The emission spectrum of the major peaks seen in the steelhead sample of Figure 2 at λ_{ex} = 360 nm was scanned on the fluorescence detector, and it was quite similar to that of the standard. Thus, the DM residues in tissues of farmed fish produce the appropriate fluorescence detector response.

The reliability of sample preparation was evaluated by determining the recoveries of the analytes from the spiked samples. The spiking range was 0.02 to 0.10 mg kg $^{-1}$ for EQ and 0.2-1.0 mg kg^{-1} for DM, since these were practical levels found in the salmon muscle in the preliminary experiments. Recoveries of DM (Table 1) varied from 83% to 93% with an average of 88% and a variation of 3.7%. The saponification procedure assured the complete release of the antioxidant residues no matter in which matrix of biological tissue they were found. In addition, DM was quite stable under the saponification conditions, and no loss was found after 10 min of heating. The partial loss of DM was possibly due to bioactive tissue components released during alkaline hydrolysis.

The contents of DM in farmed fish tissues were determined in duplicate. The results were corrected for the recoveries and are given in Table 2. The average DM residues were 0.9 mg kg^{-1}, 0.25 mg kg^{-1}, and 0.7 mg kg^{-1} in steelhead muscle, steelhead liver, and salmon muscle, respectively. The variations for results among different fish for both steelhead muscle and salmon muscle were large. Sample-to-sample differences were as great as 4-fold in the steelhead. On the other hand, if the tissue comparisons were carried out on the same fish, it is noteworthy that the liver contained a lower level of DM than the corresponding muscle in all of the 3 steelhead samples. The reason is not clear, but differential partitioning ability of DM in various body components must be one of the factors that affects the residue levels in different tissues. Another factor could be due to the enzymatic systems in the biological models. For example, the distribution and retention in organs of some common organic toxicants, such as polycyclic aromatic hydrocarbon (PAH) and polychlorinated biphenyl (PCB), is mainly dependent on their susceptibility to biotransformation by various xenobiotic-metabolizing enzymes after absorption (Van Veld 1990).

A high content of DM (about 5 times higher than that in muscle) on HPLC chromatograms was observed when the mesenteric adipose tissue of the local steelhead samples were examined. Mesenteric tissue includes much fat in adipocyte deposits. The result agreed closely with the findings of Hobson-Frohock (1982) in which the highest level of EQ in poultry tissues occurred in the depot fat. This is an indication that both EQ and DM levels may be closely related to fat content in the tissue or to other fat-relat-

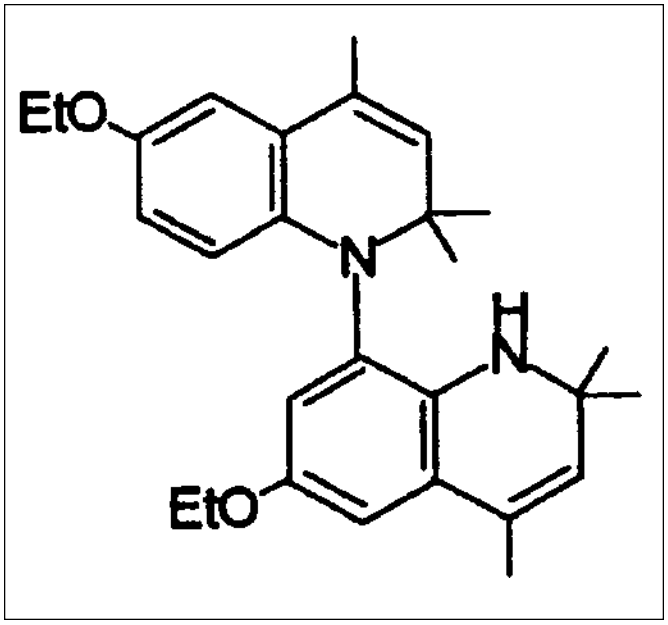

Figure 1—Ethoxyquin dimer (DM) structure

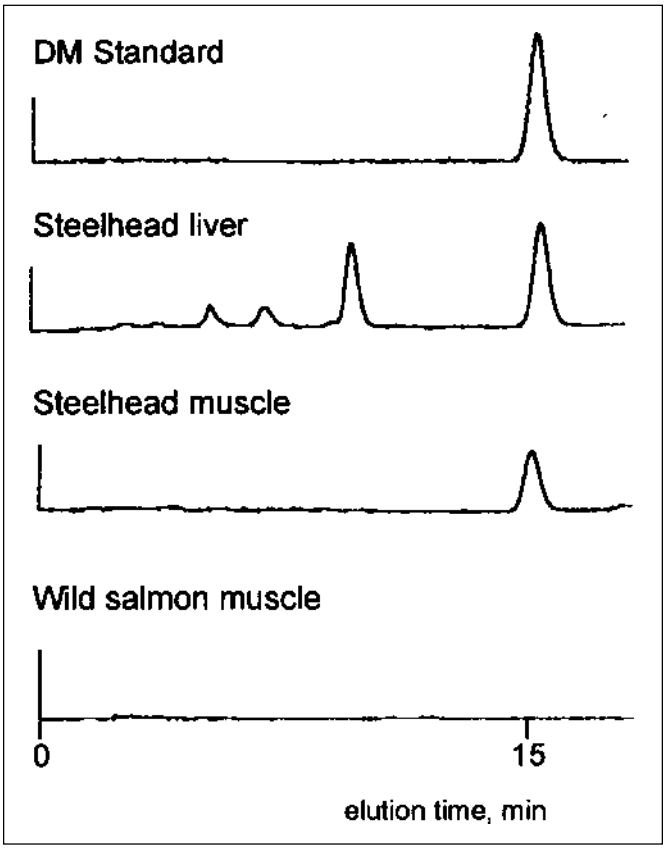

Figure 2—Reversed-phase HPLC analyses with fluorescence detection for DM in fish tissues: DM standard, steelhead liver (containing DM), steelhead muscle (containing DM), wild salmon muscle (no DM)

Table 1—Replication of recoveries of DM from spiked fish muscle samples

Added (mg)	Recovered (mg)	Recovery (%)
0.20	0.168	84
0.20	0.166	83
0.20	0.175	87
0.20	0.185	93
0.50	0.443	89
0.50	0.452	90
1.00	0.865	87
1.00	0.906	91
1.00	0.827	83
1.00	0.848	85
1.00	0.912	91
1.00	0.925	93

Table 2—DM contents (mg kg^{-1}) in different farmed fish tissues (duplicate analyses)

	Steelhead muscle	Steelhead liver	Salmon muscle
Fish 1	1.17	0.31	0.91
	1.14	0.29	0.93
Fish 2	1.27	0.41	0.43
	1.27	0.42	0.42
Fish 3	0.25	0.05	0.79
	0.25	0.04	0.80

ed factors. The distribution of fat in the muscle of salmon is notoriously uneven (Aursand and others 1994; Zhou and others 1995, 1996) and could account for some of the scatter of replicates from any 1 fish or even differences among fish from 1 lot even when tissue sampling was anatomically standardized. Unfortunately, the HPLC method developed in this research could not be applied to pure fat samples without modification because the final volume of 1 ml acetonitrile was not able to dissolve the lipid extracts when even as little as 0.5 g adipose tissue was used for alkaline hydrolysis.

Addendum

A MODIFICATION TESTED WHEN REQUIRED FOR FISH OILS AND esters involved 4 successive extractions of 2 ml with equal volumes of CH_3CN. The CH_3CN extracts were pooled, and the bulk of the CH_3CN was removed under a steady stream of nitrogen. An accurate volume of 2 ml could be injected onto the HPLC column, in this case a Whatman (Cat. No. 4692-1017) EQC 10μ C18 from Mandel Scientific Co. Ltd. (Guelph, Ontario), with a guard column (3 × 0.3 cm) from the same supplier. The detector, mobile phase, and flow rate were as described above. Recovery from spiked refined fish oil (EQ content below detectable limits) was approximately 85% for the EQ peak eluting at 5.2 min. Ethyl esters of fish-oil fatty acids did not need to be extracted as suitable amounts dissolved freely in CH_3CN. This method has not yet been tested with high-fat fish or for QI and DM.

Conclusions

ETHOXYQUIN AND ITS DIMERIC FORM ORIGINATING IN FISH meals or feeds were detected in muscle and liver of ocean-farmed salmonids. Recovery was through alkali digestion of tissue, recovery into hexane, and measurement by HPLC with fluorescence detection.

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