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ETHOXYQUIN AND ITS OXIDATION PRODUCTS IN FISH MEALS, FISH FEEDS AND FARMED FISH

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EXECUTIVE SUMMARY

Research undertaken by Ping He and R G Ackman and financed by the Natural Sciences and Engineering Research Council of Canada has been kindly forwarded to IFOMA for publication as a Research Report.

The major oxidation products of ethoxyquin in fish meals, fish feeds and farmed fish were studied. This work confirmed IFOMA sponsored research work (Research Report 1987-2) that the two principal oxidation products of ethoxyquin in fish meal are a quinone imine (QI) (called quinolone in IFOMA Research Reports) and the dimer (DM).

The authors developed two new HPLC methods for quantitative determination of these compounds. These methods were contrasted with the IFOMA sponsored method (Research Reports 1993-2; 1993-9; 1996-4). The new HPLC methods were able to detect all three products unlike the earlier GC method which required two separate runs for EQ and its oxidation products. The analysis time required to finish one HPLC run is short at about twenty minutes. Recovery of the products using the new methods was also greater than the older GC method.

Five commercial fish feeds were examined by the method developed. Four of them contained EQ, QI and DM, while the fifth, from another manufacturer, contained no QI and no DM but only traces of EQ. The health Canada regulations specify 150 PPM as the maximum level of EQ that can be added to animal feeds, and all the samples were far below this legal limit. (See table 4.2) Legal maximum levels of QI and DM in fish meal or feeds have not been specified.

Wild salmon muscle served as a control, while the muscle of farmed salmon as well as the muscle and the liver of farmed steelhead were examined by HPLC method. Low concentrations of EQ could occur in farmed fish muscle and livers. Higher concentrations of DM were found in these same tissues and confirmed by GC-MS. The contents of EQ and DM in farmed fish tissues were determined and corrected for recoveries. The EQ residues in most samples were less than but close to 0.01 PPM. The DM residues were on average 0.9 PPM, 0.25 PPM and 0.7 PPM in steelhead muscle and liver and salmon muscle respectively.

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Introduction

Ethoxyquin (EQ, trade name Santoquin or Santoflex, 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline), is an antioxidant used in fish meals and animal feeds to prevent autoxidation of unsaturated fatty acids and fat-soluble vitamins. The oxidation of EQ includes a series of complex reactions. Only some of the oxidation products have been identified (Lin and Olcott, 1975; Bharucha et al, 1987; Taimr and Prusíková, 1991; Thorisson et al, 1992). Among these oxidation products, a quinone imine (QI) and a dimer (DM) were identified as the major oxidation products if EQ was added to the oxidized anchovy fish meal or to oxidized anchovy fish oil followed by extraction with a chloroform-based solvent system (Thorrisson et al, 1992).

Studies on the biological disposition of EQ in the rat (Skaare, 1979; Skaare and Nafstad, 1979) confirmed that EQ was rapidly absorbed and distributed throughout most tissues. Accumulation took place in liver, kidney and adipose tissue. Several kinds of metabolic products were examed by gas chrtomatography-mass spectroscopy (GC-MS) and the proposed structures were similar to those chemically synthesized and identified later by Thorisson et al (1992). Compared with other common antioxidants, less work has been done on the physiological benefit or toxicity of EQ. However, there was some evidence indicating that EQ was effective in preventing potentially excessive peroxidation in poultry tissues (Bailey et al, 1996). High dose intakes of EQ (1000mg/Kg in diet) through feeding may cause several adverse effects on fur animals (Rouvinen and Laine,

1991). The ability of EQ to enhance or decrease the acute toxicity of other chemicals has also been reported (Kahl, 1984).

Determination methods for EQ in fruit, spices, animal feeds and foodstuffs have been studied in many publications (Vega et al., 1990; Winell, 1976; Perfetti, et al., 1983). Techniques include spectrometry, thin layer chromatography (TLC), high performance thin layer chromatography (HPTLC), gas chromatography (GC) and high performance liquid chromatography (HPLC). GC and HPLC are the most important means of determining EQ residues. GC with a flame ionization detector (FID) has the advantage of very high sensitivity but the disadvantage of possible decomposition of EQ at high temperature in the column (Skaare and Dahle, 1975). In contrast with GC, HPLC methods avoid EQ decomposition but the sensitivity is less.

The work on quantitative determination of EQ oxidation products was carried out by de Koning and van der Merwe (1992), who developed a GC method using methoxyquin (MQ) and methoxyquin dimer (MQDM) as the internal standards to determine EQ, QI and DM in fish meal. The recoveries from spiked samples were 90~100% for EQ and DM, but only 55% for QI. In addition, the method requires separate sample preparation and GC runs for the three analytes.

Because QI, EQ and DM can occur in fish feeds, it is logical to suspect that they can accumulate in farmed fish bodies. Whether these compounds, especially the oxidation products, have beneficial or toxic functions in human health is not fully understood. As

yet we do not know how much of these compounds are taken into human body through fish as food, but it could increase as farmed fish replace traditional fisheries.

Steelhead and Atlantic salmon are two common species of salmonids, and are a large proportion of fresh fish consumed in Atlantic area. The muscle of the fish is the tissue that human consume. The residues of EQ and its oxidation products in this tissue will directly decide the daily uptake dose of the consumer. On the other hand, the liver is the organ where most catabolism or metabolic changes take place. These two tissues are the most important to examine if either human health or fish health is of concern. Using the HPLC method, this project was carried out to determine EQ and its major oxidation products in fish meal, fish feed and the muscle and liver tissues of the farmed steelhead and salmon.

Experimental

Materials

EQ was obtained from the Canada Packers Chemicals (Mississauga, ON). It was purified in the laboratory by vacuum distillation followed by column chromatography. QI and DM were synthesized and purified in the laboratory basicly following Thorisson's method (1992), except that DM was purified through recrystallization in MeOH. All inorganic reagents used were analytical grade. All solvents were either HPLC grade or were redistilled before use in our laboratory.

Freshly produced, EQ untreated, herring fish meal was provided by Connors Bros., Ltd. (Blacks Harbour, NB). EQ was spiked to a portion of this fish meal in our laboratory immediately on receipt. Four commercial salmon feeds were obtained from Moore-Clark (St. Andrews, NB), and the fifth was from Corey Feed Mill Ltd. (Fredericton, NB).

Steelhead samples, 1~1.5 kg per fish, were obtained from an aquaculture operation in Nova Scotia. Whole fish was protected by ice for one day during transportation to Halifax where they were headed and gutted. The samples were then stored at -30 ^{0}C before analysis. Fresh Atlantic salmon steaks were obtained from local supermarkets on the same day as analysis. Frozen wild Pacific salmon (gutted and headed) were also obtained from local supermarkets and stored under -20 ^{0}C before analysis.

Equipment

HPLC was carried on a Waters 6000A solvent delivery system (Waters, Milford, MA) with a Waters Model U6K HPLC injector (Waters, Milford, MA). Chromatograms

and peak integration were processed with a PE LCI-100 laboratory computing integrator (Perkin-Elmer, Norwalk, CT). 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}. For fish meal and feed, a CSC-S ODS2 column (30 × 0.39 cm, 10 μm, CSC Inc., Montreal, PQ) with a guard column (3 cm, Waters, Milford, MA) and a Waters 450 variable wavelength UV detector (Waters, Milford, MA) was used; For the farmed fish, a CSC-Select ODS2 column (15 × 0.46 cm, 5 μm, CSC Inc., Montreal, PQ) with a guard column (3 × 0.30 cm) from same manufacturer and a Waters 474 Scanning Fluorescence detector (Waters, Milford, MA) was used. The same fluorescence detector was also used to scan the emission spectrums.

GC-MS was carried out on PE 990 Gas Chromatograph (Perkin-Elmer, Norwalk, CT) with a DB-1 fused silica capillary column (59 m × 0.25 mm i.d.) passed into a Finnigan 700 Ion Trap Detector (Finnigan Corp., San Jose, CA) as the mass spectrometer. The column temperature was 150 ^{0}C for EQ and QI determination, and 260 ^{0}C for DM determination. Data acquisition was done by a computer program provided by Finnigan Corp. (San Jose, CA).

Extraction Procedures

For fish meal: To a 15 ml test tube, 1.0 g sample and 10 ml hexane was added. After fully flushing with N_2, the tube was tightly capped, vortexed for 1 min and sonicated in a 40 ^{0}C water bath for 10 min. Then it was vortexed for another 30 sec. After 5 min of centrifuging at 2000 r min^{-1}, the clear hexane layer was transferred to another tube. The extraction was repeated twice without the sonicating procedure with 5 ml hexane each

time. The hexane solutions were combined and the hexane was removed with a stream of N_2. The fish meal lipid extract was then extracted with acetonitrile three times using 1 ml each time. The combined acetonitrile solutions were concentrated to an anexact 1 ml before HPLC analysis.

For fish feed: After grinding in a glass mortar, a 1.0 g subsample was extracted by the procedure for fish meal.

For fish tissue: To a 1.0 g well-homogenized sample of muscle or of macerated liver weighed into a 15 ml test tube was added 1 ml 50% NaOH and 2 ml ethanol. The tube was fully flushed with N_2 and capped. The sample was partially alkaline hydrolyzed with stirring at 100 ^{0}C on a water bath until the tissue had just disappeared (normally less than 1.5 min). Immediately the tube was cooled under running water and 2 ml distilled water was added. The aqueous solution was extracted by hexane (2.5 ml × 4) and the hexane solutions were combined. A small amount of NaCl could effectively destroy any elmusion if it formed during the extraction operation. The EQ residue was separated into aqueous phase by extraction with 1M HCl (2 ml × 3); the DM remained in the hexane. After adjusting the aqueous solution to pH>10 with NaOH while cooling under running water, EQ was again recovered into hexane (2 ml × 3). Both the hexane solutions were washed with 2 ml distilled water and dried over Na_2SO_4. The hexane was removed by a stream of N_2 and the residues were dissolved into exactly 1 ml of acetonitrile before HPLC analysis.

Results and Discussion

The HPLC system was set up similarly to that of the AOAC method (Schreier and Greene, 1997). In order to determine the major oxidation products of EQ (i.e. QI and DM) with the antioxidant itself in one run, a small modification of the mobile phase was made. The proportion of acetonitrile to the buffer (i.e. 0.01 N ammonium acetate solution) was changed from 70:30 (v:v) to 80:20 (v:v). QI, EQ and DM elute sequentially. The solvent prepared in this proportion can elute DM in a reasonable time and still keep a good separation between QI and EQ. This HPLC system also proved to be able to successfully separate other commonly used antioxidants from QI, EQ and DM. Ascorbic acid, tertiary-butylhydroquinone (TBHQ) and butylated hydroxyanisole (BHA) eluted before QI. Butylated hydroxytoluene (BHT) eluted neatly between EQ and DM. Tocopherol and tocopherol acetate did not elute in 30 min in this system. Thus even if a sample contains the above antioxidants, they will not disturb the determination of EQ and the two oxidation products.

Although QI and DM were reported as autoxidation products of EQ in oxidized fish meal and fish oil (Thorisson et al, 1992), one could argue about two points. First, they extracted the analytes with a chloroform-based solvent, which could probably introduce EQ oxidation products during sample preparation. Secondly, the anchovy fish meal they used was already oxidized, but in practice, EQ is added to newly produced fish meal before oxidation has happened.

To reconfirm the major autoxidation products of EQ in fish meal, EQ was added to a freshly produced herring fish meal at a level of about 200 ppm. Solvents used to extract the analytes were hexane and acetonitrile, both of which have earlier proven to be reliable solvents for EQ study. The whole procedure was conducted under nitrogen. No distinct formation of QI and DM was found during the first two months storage at room temperature. However, at the ninetieth day, a clear formation of QI and DM in fish meal was observed by HPLC analysis. The same sample was also examed by GC-MS, and two small peaks were identified as QI and DM, their MS spectra matching those of the standards.

The recovery tests from the spiked samples were carried out at three levels for each compound to evaluate the efficiency of extraction. The spiked samples contained 3 ppm QI / 40 ppm EQ / 20 ppm DM as low level, 15 ppm QI / 200 ppm EQ / 100 ppm DM as middle level, and 30 ppm QI / 400 ppm EQ / 200 ppm DM as high level. The recoveries for all three levels were 73~89%, 89~104% and 91~106% for QI, EQ and DM, respectively. The average recoveries were 83% for QI, 95% for EQ and 98% for DM, which were higher than or the same as the recovery results by the GC method (de Koning and van der Merwe, 1992). The method detection limits were down to 0.5 ppm for QI, 5 ppm for EQ and 5 ppm for DM, which was also similar to those of the GC method.

The HPLC method with the simple extraction procedure can be used as an alternative to the GC method (de Koning and van der Merwe, 1992). Although the

detection limits restrict its use to avoid samples containing very low levels of EQ and the oxidation products, the method provides enough sensitivity for fish meal and fish feed samples. Unlike the GC method which requires two separate runs for EQ and its oxidation products, the present HPLC method can analyze the three compounds in one run. The analysis time required to finish one run is short, only about 20 min. In addition, HPLC does not produce any possible decompostion products during detection.

Five commercial fish feed products were examined by the developed method. Four of them contained QI, EQ and DM, while the fifth, from another manufacturer, contained no QI and no DM but only traces of EQ. The Health Canada regulations specify 150 ppm as the maxium level of EQ that can be added to animal feeds, and all the samples were far below the legal limit. The maxium levels of QI and DM in fish meal or feed have not been specified.

Preliminary studies of farmed fish tissues were carried out with the same HPLC system as was used for fish meal analysis. The useful results ~~included: the occurrence of~~ DM was observed in farmed salmon muscle and liver, but QI was not found. The occurrence of EQ was neither confirmed nor denied due to low concentrations of interfering components. In general, the results of the prelimilary experiments strongly suggested that a fluorescence detector should be used to improve sensitivity and to diminish interference, although it means that QI determination has to be given up since QI does not give any response to a fluorescence detector.

With the high sensitivity of the fluorescence detector, the required tissue weight for an effective determination of EQ and DM could be less than one gram. Such a small amount of tissue permitted a much more easier operation for sample preparation. Instead of solvent extraction, EQ and DM could be released from the tissue through alkaline hydrolysis in a capped test tube under the protection of nitrogen. This procedure assured the complete release of the antioxidant residues no matter in which matrix of biological tissue they were found. However, the sample should not be saponified for a long period since a high temperature would result in loss of EQ even under N_2.

The muscle of the wild salmon, the muscle of the farmed salmon, as well as the muscle and the liver of the farmed steelhead were examined by HPLC after the hydrolysis procedure. A very small peak having a elution time close to that of EQ standard indicated that low concentrations of EQ could occur in farmed fish muscle and livers. Unfortunately, it was impossible to identify the structure and the fluorescence spectrum of the component since the concentration was too low to collect effectively. In some samples, even this small peak was not observed. On the other hand, a peak having the same elution time as the DM standard on HPLC was observed in all farmed fish samples, indicating that DM was a component of the fish tissue. To confirm the occurrence of DM, the elute between the peak beginning and end was collected. The materials were recovered into hexane and analyzed by GC-MS. A group of compounds were found on GC chromatogram, among which one peak was confirmed as DM according to the MS spectrum matching that of the standard. The emission spectrum of the peak at $\lambda_{ex} = 360$

nm was scanned on the fluorescence detector, and it was quite similar to that of standard in respect to the major peaks.

The reliability of saponification processing and solvent extraction was evaluated by determining the recoveries of the analytes from the spiked samples. The spiking range was 0.02~0.10 ppm for EQ and 0.2~1.0 ppm for DM, since these were practical levels found in the salmon muscle in the prelimilary experiments. Recoveries for EQ varied from 48% to 71%, and recoveries of DM varied from 83% to 93%. The average recovery of EQ (59%) was lower than that of DM (88%), and the variation of EQ recovery (6.4%) was larger than that of DM (3.7%). The reason was not clear although it was probably due to partial oxidation of EQ by the oxidative components released during tissue alkaline hydrolysis.

The contents of EQ and DM in farmed fish tissues were determined and were corrected for the recoveries. The EQ residues in most samples were less than but close to 0.01 ppm, while only one liver sample contained 0.02 ppm. That is to say, EQ levels in most samples were lower than the detection limit, since the lower limit of the method after recovery correction was 0.02 ppm. The results indicate that only very low levels of EQ accumulated in the farmed fish muscle. It seems likely that EQ residues in animal muscle are normally very low. In an earlier study (Hobson-Frohock, 1982), EQ was also found to be less than 0.005 ppm in both broiler muscle and layer muscle.

The DM residues were much higher than EQ levels, with averages of 0.9 ppm, 0.25 ppm and 0.7 ppm in steelhead muscle and liver, and salmon muscle, respectively.

The DM level variations in both steelhead muscle and salmon muscle were so large that no significant difference (P>0.05) was found between the two species statistically. It is believed that the intestinal absorption of lipophilic toxicants is associated with and enhanced by concomitant dietary fat digestion and absorption (Van Veld, 1990). When the triglycerids are hydrolyzed with lipases and colipases into monoglycerides and fatty acids, bile salts solubilize them into micelles. As DM is non-polar, it can easily be dissolved in fat and accompany the monoglycerides to form micelles entering fish body. The larger molecular weight makes it difficult for DM to be hydrolyzed as easily as EQ, thus DM is excreted more slowly out the body and is accumulated at higher levels. The source for DM accumulation in fish tissue is the feed which contains DM as we observed in the fish feed study.

On the other hand, if the comparison was carried out in the same fish, the liver always contained a lower DM level than the muscle did in any one of the three steelhead samples. The reason is not clear, but differential partitioning ability of DM in various components must be one of the factors that affect the residue levels in different tissues. Another factor could be the enzymatic systems in the biological models. For instance, the distribution and retention in organs of some common organic toxicants, such as polycyclic aromatic hydrocarbons(PAH) and polychlorinated biphenyls(PCB), is mainly dependent on their susceptibility to biotransformation by various drug-metabolizing enzymes after absorption (Van Veld, 1990).

A further experiment on fish mesenteroic adipose tissue demonstrated a high content of DM (about five times higher than that in muscle) and an enlarged EQ peak on the HPLC chromatogram. This is a indication that both EQ and DM levels may be closely related to fat content in the tissue and to other fat-related factors. This result also supported our earlier hypothesis that biological dispositions of EQ and DM were similar to other lipophilic toxicants such as PAH. Unfortunately, the newly developed HPLC method could not be applied for pure fat samples without modification because the final volumn of 1 ml acetonitrile was not able to dissolve the lipid extracts after only 0.5 g adipose tissue was used for alkaline hydrolysis.

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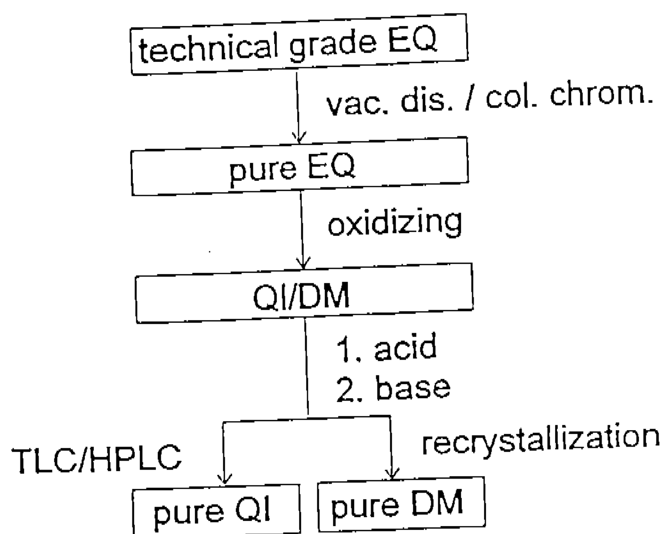
Preparation of Pure Standards



HPLC System

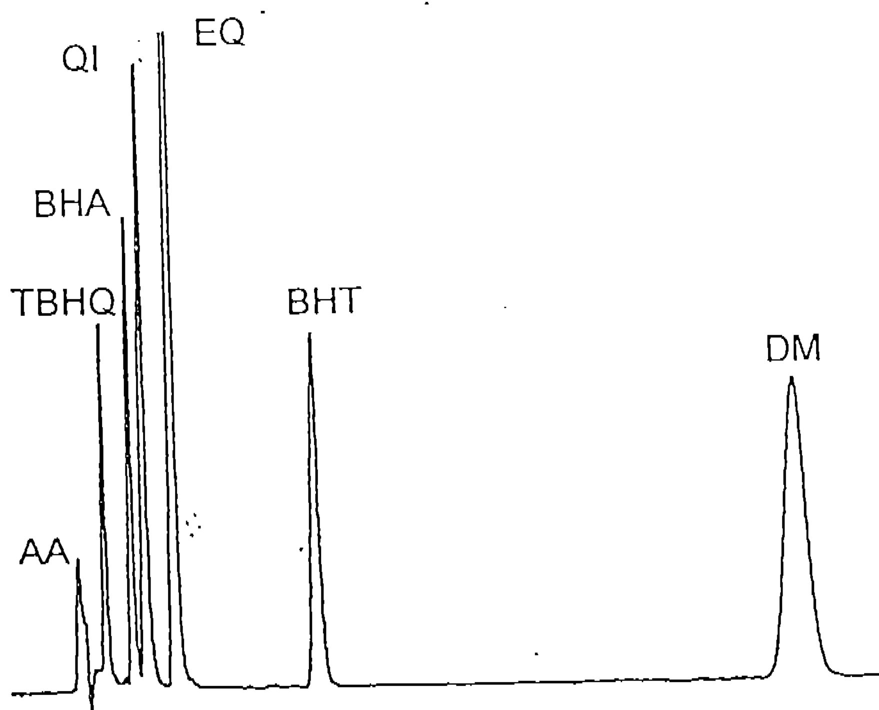


Table 4.2 EQ and its oxidation products in commercial fish feeds [a]

Sample ID	EQ (mg kg^{-1})	QI (mg kg^{-1})	DM (mg kg^{-1})
Moore-Clark 1#	58±2.7	14.2±0.5	44±1.5
Moore-Clark 2#	52±1.4	8.0±0.5	30±1.2
Moore-Clark 3#	28±0.8	3.4±0.1	29±0.3
Moore-Clark 4#	17±0.4	2.8±0.1	36±0.3
Corey 1#	6±0.3	BDL [b]	BDL [b]

a. mean ± s.d. (n=3).

b. BDL = below the detection limit (0.5 mg kg^{-1} for QI, 5 mg kg^{-1} for EQ, and 5 mg kg^{-1} for DM)