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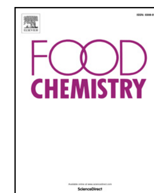
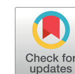


Research Article

Identification of ethoxyquin and its transformation products in salmon after controlled dietary exposure via fish feed



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ABSTRACT

Ethoxyquin (EQ) is an additive present in fish feed and its fate in fish should be carefully characterized due to food safety concerns regarding this compound. Therefore, the objective of this work was to identify the transformation products (TPs) of EQ in Atlantic salmon. Salmon in independent tanks were given feed containing ethoxyquin concentrations of 0.5 mg/kg, 119 mg/kg or 1173 mg/kg for 90 days. After the feeding trial, salmon fillets were extracted in acetonitrile and analyzed by liquid chromatography with traveling-wave ion mobility spectrometry coupled to high resolution mass spectrometry (UHPLC-TWIMS-QTOFMS). EQ was transferred from the feed to salmon fillets and 23 TPs were characterized, resulting from dimerization, oxygenation, cleavage, cleavage combined with oxygenation, cleavage combined with conjugation, and other uncategorized alterations. Moreover, EQ and some TPs were also detected in commercial salmon randomly sampled from different Norwegian fish farms. This study confirmed that the dimer 1,8′-EQDM was the main TP of EQ and, together with previous research, brought the overall number of characterized TPs to a total of 47.

1. Introduction

Ethoxyquin (EQ) is a synthetic antioxidant frequently used as an additive in animal feeds (Blaszczyk, Augustyniak, & Skolimowski, 2013) to prevent the oxidation of lipids and stabilize fat-soluble vitamins. In the European Union (EU), the directive 70/524/EEC and the regulation EC 1831/2003 previously authorized the inclusion of EQ as a feed additive for all farmed species with a maximum content of 150 mg/kg. However, the regulation EC 2017/962 suspended this authorization. Nonetheless, specific transitional measures allow feed produced from certain materials containing EQ to be placed on the market until 31 March 2020.

The presence of EQ in animal feed may lead to the presence of residues in several food products of animal origin. For instance, previous studies have reported the occurrence of EQ in poultry, egg and farmed fish (Aoki et al., 2010; Hobson-Frohock, 1982). In farmed Atlantic salmon, EQ has been detected in fillet samples (Bohne, Lundebye, & Hamre, 2008) at concentrations up to 55 µg/kg (Lundebye, Hove, Måge, Bohne, & Hamre, 2010). However, occurrence studies should not only consider EQ but also its transformation products (TPs). Due to its antioxidant properties, EQ is readily transformed into several TPs which can be formed due to the presence of oxygen during the storage of EQ-containing fish feed before being subsequently transferred to farmed fish. In addition, the residual of EQ in fish feed would also trigger the formation of other TPs after ingestion by animals such as farmed salmon (Bohne, Hamre, & Arukwe, 2006, 2007).

Among the previously characterized TPs, the EQ dimer 1,8′-EQDM is usually predominant (Ørnsrud, Arukwe, Bohne, Pavlikova, & Lundebye, 2011). It has been detected following chemical oxidation of EQ in the laboratory as well as in fish feed stored for considerable time (Negreira, Regueiro, Valdersnes, Berntssen, & Ørnsrud, 2017) and concentration of 1,8′-EQDM up to 730 µg/kg have been reported in fish fillets (Lundebye et al., 2010). Other commonly found TPs include dehydrodemethylethoxyquin (DHMEQ) detected in fish feed (Negreira et al., 2017), dihydroethoxyquin (DEQ) detected in Atlantic salmon after long term dietary exposure to EQ (Bohne et al., 2008), and EQ quinone-imine (EQI) detected in fish feed (Negreira et al., 2017) as well as in fish fillets (Bohne et al., 2008) but not in quantifiable amount. Moreover, a recent study on bench-scale chemical oxidation of EQ and the analysis of multiple fish feed samples described thirty-one other TPs (Negreira et al., 2017) using liquid chromatography coupled to traveling wave ion mobility spectrometry and high resolution mass

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spectrometry (UHPLC-TWIMS-QTOFMS).

The occurrence of EQ and a large number of its TPs in fish feed raises concern regarding the potential human exposure through the consumption of farmed fish. The hypothesis is that EQ contained in fish feed is transferred to farmed fish where a fraction is accumulated while the remaining fraction forms TPs following detoxification metabolism. Therefore the aim of this study was to apply UHPLC-TWIMS-QTOFMS in order to assess the fate of EQ in Atlantic salmon after controlled dietary exposure. Indeed, while the application of UHPLC coupled to QTOFMS is commonly used to screen and identify trace organic compounds, the second dimensional separation provided by traveling wave ion mobility spectrometry (TWIMS) further enhances the characterization of target compounds and the identification of unknown TPs (Lanucara, Holman, Gray, & Eyers, 2014; May & McLean, 2015; Regueiro, Negreira, & Berntssen, 2016; Regueiro, Negreira, Hannisdal, & Berntssen, 2017), particularly in complex samples such as fish extracts.

2. Materials and methods

2.1. Materials and reagents

HPLC-grade acetonitrile used for sample extraction was purchased from Sigma-Aldrich (Steinheim, Germany). Liquid chromatography was performed with LCMS-grade methanol and formic acid (98–100%) procured from Merck (Darmstadt, Germany), ultrapure water (18.2 MΩ·cm) freshly produced from a Milli-Q Gradient system from Merck (Darmstadt, Germany), and LCMS-grade ammonium acetate from VWR International (Oslo, Norway). The CCS Major Mix used to calibrate the high resolution mass spectrometer as well as the leucine-enkephalin standard used as lock mass were purchased from Waters (Manchester, U.K.).

The antioxidant EQ used for the production of experimental fish feed (Capsoquin batch no. S-5162, 99% purity) was provided by Industrial Técnica Pecuaria, S.A., (Barcelona, Spain) while the analytical standard was purchased from Sigma-Aldrich (Steinheim, Germany). The standards of TPs 1,8′-EQDM (99.2% pure), DEQ (99.8% pure), DHEQ (97.5% pure) and EQI (98.6% pure) were acquired from HPC Standards (Cunnersdorf, Germany) while 6-ethoxy-2,2,4-trimethyl-quinolin-8-one (TP-232B) was provided by Dr. F. Thrun from BASF (Ludwigshafen, Germany).

2.2. Production of experimental fish feed

A control, EQ-free, fish feed (5-mm extruded pellets) was produced specifically for this study by EWOS Innovation (Dirdal, Norway), according to an established feed formulation (Harmony Debio, organic fish feed). However, the control feed was not totally free of EQ and contained a background concentration of 0.5 mg/kg. Some of the feed was then mixed with EQ. Briefly, EQ (Capsoquin batch no. S-5162, 99% purity; courtesy of Industrial Técnica Pecuaria, S.A., Barcelona, Spain) was added at ambient temperature (18 °C) to the oil which was subsequently vacuum coated at 0.1 bar for 10 min onto the control pellets. Two treated feeds were produced that contained 119 mg EQ/kg and 1173 mg EQ/kg. In order to prevent potential degradation of EQ, the produced feeds were immediately stored in darkness at −20 °C and kept in these conditions throughout the experimental period, until fed to the fish.

2.3. Feeding trial

Ethical statement

The experiment was approved by the Norwegian National Animal Research Authority (Mattilsynet; FOTS ID: 9004) and performed in compliance with national and international ethical standards.

A feeding trial was carried out on 6 months old Atlantic salmon smolt (*Salmo salar*, L.) at NOFIMA (Sunndalsøra, Norway) between July and October 2016 (Bernhard et al., 2019). Nine indoor flow-through tanks were each stocked with 70 fish with an initial weight of 150–200 g kept in 840 L of water which temperature was maintained at 8.0 ± 0.3 °C throughout the entire experimental period. Fish were acclimatized in their tanks for a 14 days period with 24 h light exposure. During this period, all fish received the control feed without added EQ (0.5 mg/kg). When the 90 days feeding trial started, fish in three tanks received the control feed, three tanks received feed containing 119 mg EQ/kg, and three tanks received feed containing 1173 mg EQ/kg. Automatic feeders provided feed every four hours and unconsumed feed pellets were collected and weighed daily. In addition, a high water flow (~15 L min^{-1}) was maintained through the tanks in order to avoid potential water contamination by EQ from the diet and faeces. At sampling, the fish were anaesthetised with tricaine methanesulfonate (MS-222; ~60 mg L^{-1}) before being sacrificed by a blow on the head. For each tank, Norwegian quality cut (NQC) muscle samples were taken from five fish, pooled together, homogenized, flash frozen and subsequently stored at −80 °C until sample preparation and analysis by UHPLC-TWIMS-QTOFMS.

2.4. Salmon samples from fish farms

The Norwegian food safety authority provided samples of commercial Atlantic salmon intended for human consumption and randomly sampled from Norwegian fish farms. NQC muscle samples were taken from five fish from the same net pen, pooled together, homogenized and stored at −20 °C until sample preparation and analysis by UHPLC-TWIMS-QTOFMS. For twelve of these pooled samples from commercial salmons, a retrospective data analysis was carried out in order to screen for EQ and its TPs.

2.5. Sample preparation

A 10 g muscle sample aliquot was placed into a 50 mL polypropylene centrifuge tube and 10 mL of acetonitrile were added along with a ceramic homogenizer (Agilent, Ref. 5982-9313). The tube was placed on a shaking table with 3 mm orbit at 2500 RPM (calculated equivalent to 4 g) for one hour and was subsequently centrifuged at 3000*g* for ten minutes. The supernatant was collected and stored at −30 °C for at least 1 h, which allowed the precipitation of lipids and other macromolecules. After slowly thawing the tube at room temperature, 6 mL of the upper phase were placed at 45 °C and evaporated to 0.3 mL under a gentle stream of nitrogen. The concentrated liquid was transferred to an Eppendorf tube containing 30 mg of Z-sep sorbent (Sigma Aldrich). After adding 0.3 mL of water with 0.1% formic acid and vortex shaking for 20 s, the tube was centrifuged at 18,000*g* for 10 min. Finally, the supernatant was passed through a 0.2 µm regenerated cellulose syringe filter and collected into a 2 mL vial for UHPLC-TWIMS-QTOFMS analysis.

2.6. Ultra-high performance liquid chromatography (UHPLC)

Liquid chromatography was performed using the ACQUITY UPLC I-Class device from Waters (Manchester, U.K.), including a binary pump, a solvent degasser, an auto-sampler and a column compartment equipped with a thermostat. A volume of 5 µL of sample was injected onto a reverse phase C18 ACQUITY UPLC BEH column with 100 mm length, 2.1 mm internal diameter and 1.7 µm particle size from Waters (Manchester, U.K.). While the column temperature was kept at 45 °C, the analytes were eluted with a 0.45 mL/min gradient of water and methanol (both containing 10 mM ammonium acetate) previously used for the analysis of ethoxyquin and pesticides in fish feed (Negreira et al., 2017; Regueiro et al., 2016). Briefly, the organic fraction was kept at 2% during 0.25 min, linearly increased to 99% by 12.25 min and held until 18 min. Before injecting another sample, the initial

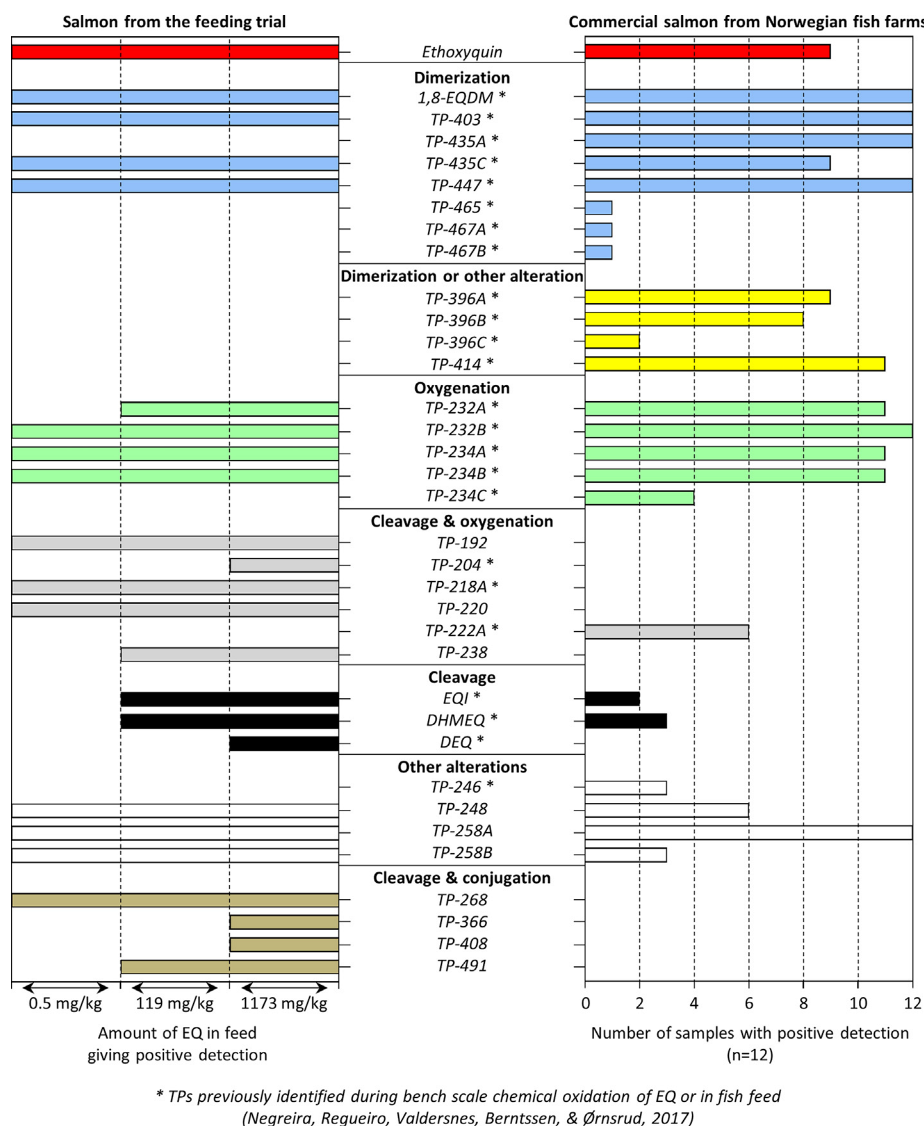

Fig. 1. Detection of ethoxyquin and its transformation products in Atlantic salmon (*Salmo salar* L.). Salmon from feeding trial refer to Atlantic salmon exposed to three graded levels of EQ through their diet for 90 days. Salmon intended for Human consumption refer to twelve Atlantic salmon sampled from Norwegian fish farms.

conditions of the gradient were restored and the column was allowed to equilibrate for 4 min.

2.7. Ion mobility and high resolution mass spectrometry (TWIMS-QTOFMS)

After chromatographic separation, the analytes were detected using the Vion IMS QTOF mass spectrometer from Waters (Manchester, U.K.) as described in a previous publication on EQ degradation (Negreira et al., 2017). The electrospray ionization source (ESI) was operated in positive and negative mode with 0.45 kV capillary voltage, 10 V cone voltage, 80 V source offset, 110 °C source temperature, 450 °C desolvation gas temperature, 900 L/h and 40 L/h nitrogen (> 99.5%) respectively as desolvation gas flow and cone gas flow. In order to maintain mass accuracy, a solution of leucine-enkephalin was infused through a dedicated reference sprayer with a 3 kV capillary voltage and the corresponding ion was used as lock mass.

After ionization, the analytes were further separated by traveling-wave ion mobility spectrometry (TWIMS) as described in a previous study on ethoxyquin transformation products (Negreira et al., 2017). Briefly, TWIMS was applied with the following operating conditions: trap bias, 40 V; stopper height, 40 V; gate height, 40 V; trap wave velocity, 100 m/s; trap pulse height A, 20 V; trap pulse height B, 5 V, IMS wave velocity, 250 m/s; IMS wave height, 45 V; gate release, 2 ms, and nitrogen (> 99.5%) as trap and IMS buffer gas at 1.6 L/min and 25 mL/min, respectively. CCS calibration was performed regularly using a mixture of calibrants prepared in acetonitrile/water/formic acid (50:49.9:0.1, v/v/v).

Following ion mobility separation, ions were detected through high-definition MS^E (Negreira et al., 2017). Briefly, without any precursor ion selection in the quadrupole, data acquisition was performed in the range m/z 50–1000 with 0.2 s/scan and ultra-high purity (≥ 99.999%) argon for collision-induced dissociation (CID) at different collision energies (CE) alternatively. Within the same analysis, the acquisition of a low-energy scan (CE 4 eV) allowed monitoring the molecular ions with different charge carriers while the acquisition of a high-energy scan (CE ramp 8–45 eV) allowed monitoring their fragment ions. A high mass accuracy was maintained through a dual point correction based on the acquisition every 30 s of ions $[C_{21}H_{25}N_4O_4]^+$ at m/z 397.1870 and $[C_8H_{10}N]^+$ at m/z 120.0808 resulting from the 25 eV CID of leucine-enkephalin (100 ng/mL) continuously infused at 5 µL/min through the reference probe. In addition, an average resolving power (m/Δm) of

approximately 40,000 full width at half maximum (FWHM) was obtained in the range of *m/z* acquired as the TOF was operated in sensitivity mode. Finally, both data acquisition and data processing were performed using the version 1.8 of the UNIFI software from Waters, with the minimum intensity threshold set to 20 counts and the background noise filter set to "low" in order to improve the observation of compounds with low abundances.

The application of this analytical method for screening purposes was validated with performance described in two previous publications (Regueiro et al., 2016, 2017). In this study, a compound was identified with respect to the library when the following conditions were gathered: the retention time error was less than 0.1 min, the mass difference was lower than 10 ppm and the error on the CCS value was lower than 2%.

3. Results and discussion

The fate of EQ in salmon was initially assessed through the feeding trial, screening fillet samples for the occurrence of parent EQ and known TPs. Subsequently, the acquired data was further evaluated in order to identify new TPs and compare the relative abundances of all detected compounds. Finally, fillets of commercial salmon from Norwegian fish farms were analyzed with the same analytical method in order to screen for EQ and all the TPs previously characterized.

3.1. Fate of ethoxyquin in salmon during feeding trial

3.1.1. Residual of ethoxyquin in salmon

EQ was detected in salmon fillets regardless of the amount contained in the feed (Fig. 1). In the extracts, EQ was identified with the highest confidence level (Schymanski et al., 2014) using an analytical standard. According to the analytical details provided in Table 1, EQ showed deviation lower than 0.02 min for retention time, 0.8 ppm for accurate mass and 0.4% for collision cross section (CCS). Moreover, characteristic fragment ions were also observed (Fig. S1).

3.1.2. Screening of known transformation products

TPs of EQ (chemical structures provided in Table S1) identified in previous studies (Negreira et al., 2017) represent 37 compounds grouped into 6 classes according to their formation mechanism: dimerization (12 compounds), dimerization or other alteration (4 compounds), oxygenation (6 compounds), cleavage (3 compounds), cleavage and oxygenation (9 compounds), and other alterations (3 compounds). In order to screen for the occurrence of these compounds in salmon fillets, their respective analytical details provided in Table 1 were included in a library and used to process UHPLC-TWIMS-QTOFMS data.

Overall, 13 of the 37 TPs were identified in salmon from the feeding trial. Among them, 4 (1,8′-EQDM, TP-403, TP-435C and TP-447) resulted from a dimerization, 4 others (TP-232A, TP-232B, TP-234A and TP-234B) resulted from an oxygenation, 2 others (TP-204 and TP-218A) resulted from a cleavage and an oxygenation, while the last 3 (DEQ, DHMEQ and EQI) resulted from a simple cleavage (Fig. 2).

The detection of these TPs in salmon fillets from the feeding trial was also considered with respect to the amount of EQ in the feed (Fig. 1). For instance, the compounds 1,8′-EQDM, TP-403, TP-435C, TP-447, TP-232B, TP-234A, TP-234B, and TP-218A were detected in salmon fillets regardless of the amount of EQ in the feed. However, the compounds TP-232A, DHMEQ and EQI were detected in salmon given feed containing a concentration of EQ of 119 mg/kg or higher. The other compounds, TP-204 and DEQ, were only detected in salmon fed with feed containing EQ at 1173 mg/kg, a concentration which greatly exceeds the maximum allowed in the EU.

Finally, screening data evaluation also included looking for the occurrence of phenetidine ($C_8H_{11}NO$), an impurity resulting from the production of EQ. However, no significant signal attributed to the ion $[C_8H_{12}NO]^+$ was observed in any of the salmon extracts regardless of the amount of EQ in the feed. This is consistent with the high purity of the EQ solution added in to the feed for the feeding trial.

3.1.3. Identification of new transformation products

The analysis of salmon extracts by UHPLC-TWIMS-QTOFMS revealed an additional ten TPs (chemical structures provided in Table S1) which have not previously been observed. They were added to the library for subsequent screening in commercial salmon fillets and their respective analytical characteristics are provided in Table 1.

Three of these new compounds (TP-192, TP-220 and TP-238) were formed by "cleavage and oxygenation" (Fig. 2). The first compound, TP-192, was detected at a retention time of 5.74 min with an observed CCS value of 145.7 Å and was attributed to the ion $[C_{11}H_{14}NO_2]^+$ with a mass error of 0.6 ppm. Tentatively, TP-192 would be formed by the O-dealkylation of EQ followed by the cleavage of one methyl group located near the nitrogen and a hydroxylation eliminating the double bond of the heterocycle (Fig. 2). All three characteristic fragments (Table 1) would be consistent with this hypothesis. The second compound, TP-220, detected at 6.71 min with an observed CCS value of 156.8 Å and was attributed to the ion $[C_{12}H_{14}NO_3]^+$ with 1 ppm mass error. TP-220 would be formed by the O-dealkylation of EQ followed by a double oxygenation (Fig. 2). The first oxygenation would form a ketone on the aromatic ring while the second oxygenation would remove the double bond of the heterocycle to form another ketone moiety. Both characteristic ions at *m/z* 174.0552 and *m/z* 146.0598 would be consistent with this theory. The third compound, TP-238, was detected at a retention time of 7.01 min with an observed CCS value of 162.1 Å, and was assigned to the ion $[C_{13}H_{20}NO_3]^+$ with 1.1 ppm mass error. It was hypothesized that TP-238 resulted from the cleavage of a methyl group located close to the nitrogen followed by a dual hydroxylation (Fig. 2). The first hydroxylation would occur on the aromatic ring while a second hydroxylation would occur on the heterocycle and eliminate the double bond. However, the single characteristic ion *m/z* 192.1020 did not provide enough information to confirm this hypothesis.

Three other new compounds (TP-248, TP-258A and TP-258B) were formed by "other alteration" (Fig. 2). TP-248 was observed at a retention time of 8.99 min with a CCS value of 163.5 Å and assigned to the ion $[C_{15}H_{22}NO_2]^+$ with a mass error of 0.9 ppm. According to the fragment ions *m/z* 202.1225, *m/z* 188.1068 and *m/z* 173.0834, TP-248 could result from the loss of one methyl group on the heterocycle of EQ followed by a hydroxylation of the aromatic ring and the binding of an ethyl group on the nitrogen (Fig. 2). TP-258A and TP-258B were respectively detected at 9.42 min and 10.66 min on the chromatogram with a CCS value of 167 Å. Both compounds were assigned to the ion $[C_{17}H_{24}NO]^+$ with a mass error of 1.3 ppm and were considered to be isomers. According to their characteristic fragments, these TPs would result from the formation of a tertiary amine with a C_3H_5 moiety on the nitrogen of EQ (Fig. 2).

The remaining compounds (TP-268, TP-366, TP-408 and TP-491) represent a new class of TPs resulting from "cleavage and conjugation" (Fig. 2). In particular, three of these compounds (TP-268, TP-408 and TP-491) were only detected when extracts were analyzed using negative electrospray ionization. TP-268 was detected at 5.21 min on the chromatogram with a CCS value of 168.8 Å and was assigned to the ion $[C_{12}H_{14}NO_4S]^-$ with a mass error of 1.1 ppm. This chemical formula corresponds to the loss of two carbon atoms and six hydrogen atoms by EQ along with a gain of sulfur and three oxygen atoms. Consequently, TP-268 would be formed by O-dealkylation of EQ in order to form a sulfate conjugate. All three characteristic fragments sustain this hypothesis and sulfate conjugates are common TPs of xenobiotics formed by the sulfotransferase enzyme. TP-366 and TP-408 were respectively observed at 4.33 min and 6.94 min. While TP-366 was assigned to the ion $[C_{18}H_{24}NO_7]^+$ with 0.6 ppm mass error, TP-408 was assigned to the ion $[C_{20}H_{26}NO_8]^-$ with less than 0.1 ppm mass error. Both compounds are consistent with glucuronide conjugates formed respectively after O-

Table 1
Analysis of ethoxyquin (EQ) with its transformation products (TPs) by UHPLC-TWIMS-QTOFMS.

Compound	RT (min)	Molecular ion	*m/z*	CCS (Å)	Characteristic fragment ions (*m/z*)	Formation	Reference
EQ	8.99	$[C_{14}H_{20}NO]^+$	218,15394	155.8	202.1225, 190.1228, 188.1070, 174.0912, 160.0757, 148.0757	Native Compound	Negreira et al. (2017)
1,8′-EQDM	12.23	$[C_{28}H_{37}N_2O_2]^+$	433,28495	216.6	375.2065, 347.1743, 333.1596, 216.1379, 188.1069	Dimerization	Negreira et al. (2017)
TP-403	11.07	$[C_{26}H_{31}N_2O_2]^+$	403,23800	204.3	373.1911, 345.1596, 329.1280, 315.1492, 188.1074	Dimerization	Negreira et al. (2017)
TP-419A	8.49	$[C_{26}H_{31}N_2O_3]^+$	419,23292	203.0	333.1593, 305.1279, 276.1251	Dimerization	Negreira et al. (2017)
TP-419B	8.69	$[C_{26}H_{31}N_2O_3]^+$	419,23292	198.8	N.A.	Dimerization	Negreira et al. (2017)
TP-433	11.47	$[C_{28}H_{37}N_2O_2]^+$	433,28495	203.6	N.A.	Dimerization	Negreira et al. (2017)
TP-435A	10.79	$[C_{28}H_{39}N_2O_2]^+$	435,30060	154.6	218.1532	Dimerization	Negreira et al. (2017)
TP-435B	11.22	$[C_{28}H_{39}N_2O_2]^+$	435,30060	216.5	218.1548, 178.1218, 150.0931	Dimerization	Negreira et al. (2017)
TP-435C	11.59	$[C_{28}H_{39}N_2O_2]^+$	435,30060	154.8	420.2762, 218.1531, 188.1064, 148.0779	Dimerization	Negreira et al. (2017)
TP-447	8.85	$[C_{28}H_{35}N_2O_3]^+$	447,26422	222.9	389.2214, 373.1901, 345.1591, 317.1277, 289.1330, 273.1020	Dimerization	Negreira et al. (2017)
TP-465	9.6	$[C_{28}H_{37}N_2O_4]^+$	465,27478	214.0	N.A.	Dimerization	Negreira et al. (2017)
TP-467A	10.94	$[C_{28}H_{39}N_2O_4]^+$	467,29043	219.3	N.A.	Dimerization	Negreira et al. (2017)
TP-467B	11.09	$[C_{28}H_{39}N_2O_4]^+$	467,29043	220.3	373.1909, 359.1742, 345.1594, 329.1283	Dimerization	Negreira et al. (2017)
TP-396A	7.91	$[C_{24}H_{30}NO_4]^+$	396,21693	194.8	353.1619, 352.1549, 228.1383	Dimerization or other alteration	Negreira et al. (2017)
TP-396B	8.14	$[C_{24}H_{30}NO_4]^+$	396,21693	205.8	216.1381, 202.1225, 174.0913	Dimerization or other alteration	Negreira et al. (2017)
TP-396C	8.22	$[C_{24}H_{30}NO_4]^+$	396,21693	197.9	340.1550, 228.1383, 202.1228, 200.1063, 167.0707	Dimerization or other alteration	Negreira et al. (2017)
TP-414	5.43	$[C_{24}H_{32}NO_5]^+$	414,22750	201.0	396.2174, 356.1854, 340.1539, 228.1382, 188.1069, 160.0756	Dimerization or other alteration	Negreira et al. (2017)
TP-232A	6.49	$[C_{14}H_{18}NO_2]^+$	232,13321	156.4	204.1379, 176.1071, 160.0734	Oxygenation	Negreira et al. (2017)
TP-232B	8.17	$[C_{14}H_{18}NO_2]^+$	232,13321	154.0	204.1019, 189.0783, 176.1069, 161.0827, 134.0963, 132.0806	Oxygenation	Negreira et al. (2017)
TP-234A	5.6	$[C_{14}H_{20}NO_2]^+$	234,14886	157.2	216.1383, 188.1072, 173.0832, 148.0754	Oxygenation	Negreira et al. (2017)
TP-234B	5.86	$[C_{14}H_{20}NO_2]^+$	234,14886	157.3	216.1380, 188.1069, 173.0829, 148.0753	Oxygenation	Negreira et al. (2017)
TP-234C	8.28	$[C_{14}H_{20}NO_2]^+$	234,14886	156.9	190.0862, 188.1076, 176.0704	Oxygenation	Negreira et al. (2017)
TP-250	6.86	$[C_{14}H_{20}NO_3]^+$	250,14377	162.2	162.0914	Oxygenation	Negreira et al. (2017)
TP-204	6.06	$[C_{12}H_{14}NO_2]^+$	204,10191	144.0	186.0921, 172.0762, 160.0762	Cleavage and oxygenation	Negreira et al. (2017)
TP-206	6.12	$[C_{12}H_{16}NO_2]^+$	206,11756	144.3	N.A.	Cleavage and oxygenation	Negreira et al. (2017)
TP-218A	6.55	$[C_{13}H_{16}NO_2]^+$	218,11756	151.1	203.0942, 160.0761	Cleavage and oxygenation	Negreira et al. (2017)
TP-218B	6.83	$[C_{13}H_{16}NO_2]^+$	218,11756	150.0	202.0863	Cleavage and oxygenation	Negreira et al. (2017)
TP-218C	7.26	$[C_{13}H_{16}NO_2]^+$	218,11756	147.7	175.0985, 146.0959	Cleavage and oxygenation	Negreira et al. (2017)
TP-222A	3.05	$[C_{12}H_{16}NO_3]^+$	222,11247	149.7	204.1022, 190.0867, 150.0917	Cleavage and oxygenation	Negreira et al. (2017)
TP-222B	3.82	$[C_{12}H_{16}NO_3]^+$	222,11247	148.7	204.1018, 190.0868, 150.0911	Cleavage and oxygenation	Negreira et al. (2017)
TP-222C	3.96	$[C_{12}H_{16}NO_3]^+$	222,11247	148.3	150.0550	Cleavage and oxygenation	Negreira et al. (2017)
TP-236	6.68	$[C_{13}H_{18}NO_3]^+$	236,12812	154.4	N.A.	Cleavage and oxygenation	Negreira et al. (2017)
DEQ	6.74	$[C_{12}H_{16}NO]^+$	190,12264	144.7	188.1071, 174.0900, 145.0880	Cleavage	Negreira et al. (2017)
DHMEQ	8.08	$[C_{13}H_{16}NO]^+$	202,12264	146.3	174.0915, 159.0681, 145.0885, 130.0653	Cleavage	Negreira et al. (2017)
EQI	6.73	$[C_{12}H_{14}NO]^+$	188,10699	140.6	173.0832, 145.0880, 130.0652	Cleavage	Negreira et al. (2017)
TP-246	8.72	$[C_{15}H_{20}NO_2]^+$	246,14886	160.1	228.1382, 218.1542, 202.1221, 188.1073, 174.0909, 162.0537	Other Alteration	Negreira et al. (2017)
TP-276	8.25	$[C_{17}H_{26}NO_2]^+$	276,19581	168.5	218.1542, 202.1220, 190.1256, 178.1228, 162.0924	Other Alteration	Negreira et al. (2017)
DHEQ	9.03	$[C_{14}H_{22}NO]^+$	220,16959	156.2	204.1383, 202.1225, 190.1228, 188.1070, 160.0757, 148.0757	Other Alteration	Negreira et al. (2017)
TP-192	5.74	$[C_{11}H_{14}NO_2]^+$	192,10191	145.7	166.0863, 138.0549, 94.0650	Cleavage and oxygenation	N.A.
TP-220	6.71	$[C_{12}H_{14}NO_3]^+$	220,09682	156.8	174.0552, 146.0598	Cleavage and oxygenation	N.A.
TP-238	7.01	$[C_{13}H_{20}NO_3]^+$	238,14377	162.1	192.1020	Cleavage and oxygenation	N.A.
TP-248	8.99	$[C_{15}H_{22}NO_2]^+$	248,16451	163.5	202.1225, 188.1068, 173.0834	Other Alteration	N.A.
TP-258A	9.42	$[C_{17}H_{24}NO]^+$	258,18524	167.8	242.1539, 228.1382, 215.1296, 187.0990, 158.0963	Other Alteration	N.A.
TP-258B	10.66	$[C_{17}H_{24}NO]^+$	258,18524	167.1	243.1577, 228.1380, 215.1280, 187.0990, 158.0963	Other Alteration	N.A.
TP-268	5.21	$[C_{12}H_{14}NO_4S]^-$	268,06490	168.8	252.0337, 188.1083, 172.0764	Cleavage and Conjugation	N.A.
TP-366	4.33	$[C_{18}H_{24}NO_7]^+$	366,15473	192.9	N.A.	Cleavage and Conjugation	N.A.
TP-408	6.94	$[C_{20}H_{26}NO_8]^-$	408,16639	202.2	N.A.	Cleavage and Conjugation	N.A.
TP-491	5.55	$[C_{22}H_{27}N_4O_7S]^-$	491,16059	208.5	N.A.	Cleavage and Conjugation	N.A.

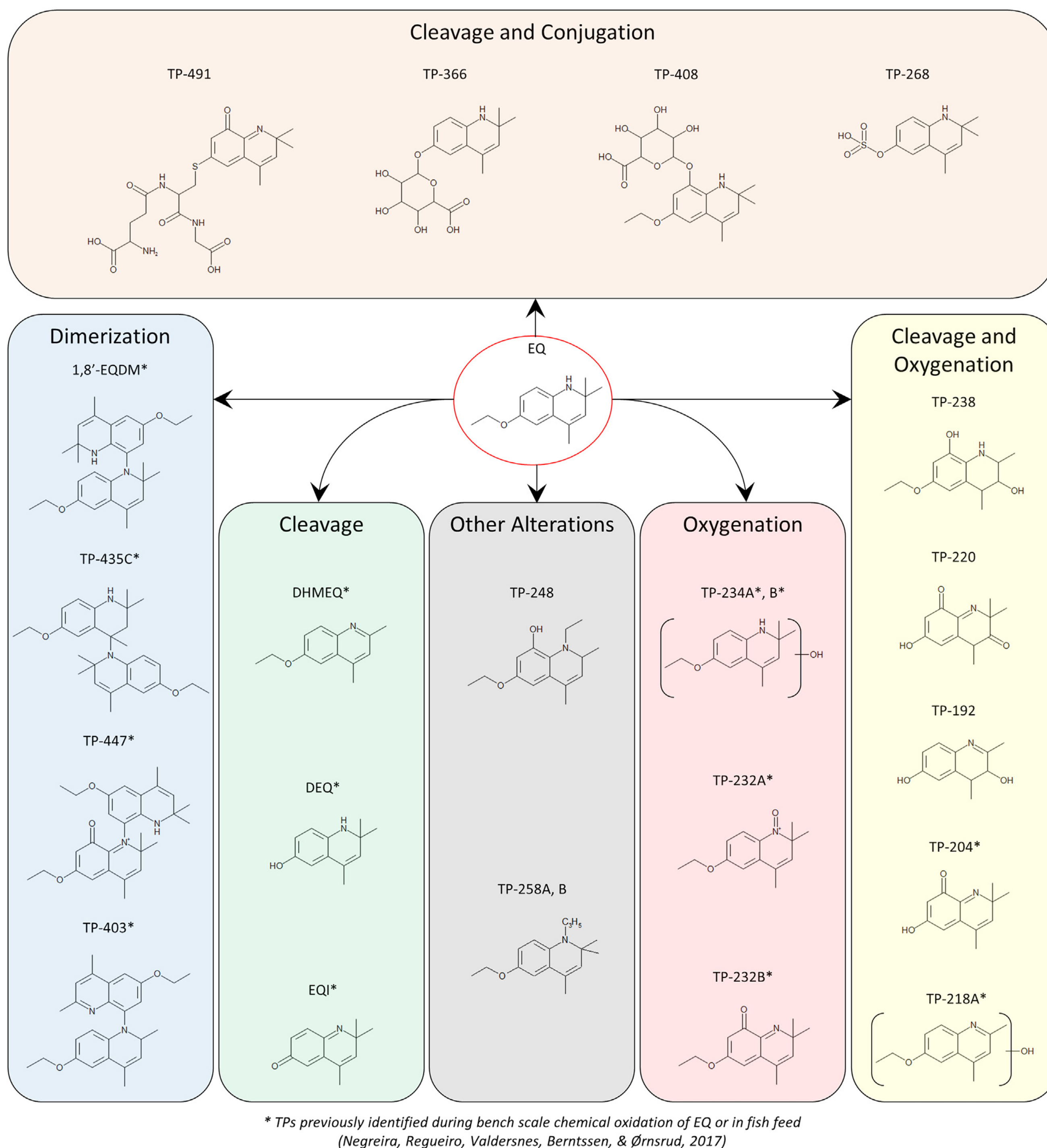

Fig. 2. Tentative transformation pathway of ethoxyquin (EQ) in Atlantic salmon (*Salmo salar* L.) exposed through their diet for 90 days.

dealkylation of EQ and after hydroxylation of the aromatic ring. However, no characteristic ion could be observed to sustain the location of the glucuronide moiety. The last compound, TP-491 was observed at 5.55 min retention time with a CCS value of 208.5 Å and was assigned to the ion $[C_{22}H_{27}N_4O_7S]^-$ with a mass error lower than 0.2 ppm. This compound would result from the oxygenation of the aromatic ring of EQ in order to form a ketone, followed by the cleavage of the ether moiety in order to form a glutathione conjugate. However, no characteristic fragment could confirm this hypothesis.

Finally, the detection of these new TPs should also be considered along with the concentration of EQ in the feed (Fig. 1). Most of them (TP-192, TP-220, TP-248, TP-258A, TP-258B, TP-268 and TP-491) were detected in salmon exposed to any of the doses of EQ present in the feed. However, TP-238 was detected in salmon given feed containing EQ at 119 mg/kg or more while TP-366 and TP-408 were only detected in salmon which received the feed with the highest concentration of EQ (1173 mg/kg).

3.1.4. Abundances of EQ and its TPs in salmon from the trial

The lack of analytical standards and the application of a screening method designed specifically for compound identification did not allow an accurate quantification but allowed the absolute and relative

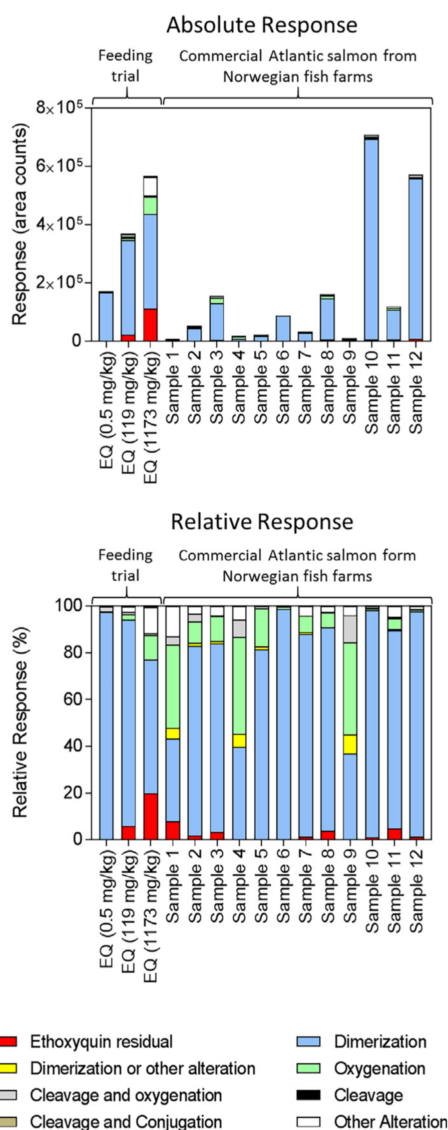

Fig. 3. Absolute and relative responses of ethoxyquin (EQ) and its transformation products in Atlantic salmon (*Salmo salar* L.) exposed to graded levels of EQ through their diet for 90 days (feeding trial) and in Atlantic salmon (n = 12) intended for Human consumption sampled from Norwegian fish farms.

responses of each class of TPs in salmon fillets to be compared across all levels of EQ spiked in the feed (Fig. 3).

Overall, the absolute abundances of EQ and its TPs in fish muscle clearly increased with the amount of EQ in fish feed (Fig. 3, Fig. S2). For instance, the absolute response of EQ in salmon fillet was low (peak area, 552 counts) when feed contained EQ at 0.5 mg/kg, but increased when feed contained EQ at 119 mg/kg (peak area, 22,130 counts) and 1173 mg/kg (peak area, 112,645 counts). In addition, the same trend was observed when considering the relative response of EQ (Fig. 3).

The first and most predominant class of TPs gathered compounds resulting from "dimerization" which represented 57% to 97% of the relative responses (Fig. 3). More specifically, the dimer 1,8′-EQDM itself was the most significant TP within this class with an abundance 100-fold higher than that of TP-435C, 1000-fold higher than that of TP-403, and 2000-fold higher than that of TP-447 (Fig. S3). The second class of TPs included those formed by oxygenation (TP-232A, TP-232B, TP-234A and TP-234B in Fig. S4) which relative abundance in muscle extracts increased with the amount of EQ in the feed and reached 10% with the highest dose (1173.1 mg/kg). The third class of TPs comprised those formed by other alterations (TP-248, TP-258A and TP-258B in Fig. S5) which overall relative abundance reached up to 11% at the highest dose of EQ in feed. The other classes of TPs gathered respectively those formed by cleavage (DHMEQ, DEQ and EQI in Fig. S6), by cleavage and conjugation (TP-268, TP-366, TP-408 and TP-491 in Fig. S7), and by cleavage and oxygenation (TP-192, TP-204, TP-218A, TP-220 and TP-238 in Fig. S8). None of these classes of TPs showed a relative abundance exceeding 2% regardless of the concentration of EQ in the feed.

3.2. EQ and TPs in commercial salmon

The relevance of the TPs identified during the feeding trial as well as in previous EQ degradation tests was assessed by analyzing commercial salmon (n = 12) collected randomly from different Norwegian fish farms. Muscle extracts were analyzed by UHPLC-TWIMS-QTOFMS, screening for the occurrence of EQ and its forty-seven TPs known to date (Table 1).

The antioxidant EQ was detected in nine out of twelve fillets from commercial fish, (Fig. 1). Even though EQ was not accurately quantified by UHPLC-TWIMS-QTOFMS, its absolute response was limited (Fig. 3). In fact, the maximum response for EQ in commercial salmon remained below the response of EQ in salmon from the feeding trial given the feed containing EQ at 119 mg/kg.

The analysis of commercial salmon extracts also revealed the occurrence of multiple TPs (Fig. 4) with a large difference of detection frequency (Fig. 1) and abundances (Fig. 3). Out of forty-seven TPs considered (Table 1), twenty-four compounds belonging to six classes of TPs were observed at least once. As for the feeding trial, the major class of TPs gathered those compounds formed by dimerization (1,8′-EQDM, TP-403, TP-435A, TP-435C, TP-447, TP-465, TP-467A and TP-467B) which represented 35% to 97% of the relative response (Fig. 3). In particular, 1,8′-EQDM was detected in all the samples with a response at least 10-fold higher than the response of the other dimers. The second major class of TPs in commercial salmon comprised compounds resulting from oxygenation (TP-232A, TP-232B, TP-234A, TP-234B and TP-234C) which relative response varied between 1% and 41%. The third class of TPs gathered compounds resulting from "other alteration" (TP-246, TP-248, TP-258A and TP-258B), with a relative abundance ranging from 1% to 13%. The fourth class of TPs was represented by TP-225A formed by cleavage and oxygenation which relative abundance did not exceed 11%. The fifth class of TPs was formed by compounds resulting from dimerization or other alteration (TP-396A, TP-396B, TP-396C and TP-414) with a relative response not exceeding 8%. The last class of TPs gathered compounds resulting from the cleavage of EQ (DHMEQ and EQI) which relative response never exceeded 1%.

Finally, TPs resulting from cleavage and conjugation were not detected in commercial salmon. However, it should be noticed that this could be explained since (except from TP-366) these compounds were previously characterized using negative electrospray ionization while only data from positive electrospray ionization were available for commercial salmon. Six TPs (TP-192, TP-204, TP-218A, TP-220, and TP-238) were detected with a very low abundance in salmon from the fish trial and were not detected in commercial salmon. On the contrary, 11 TPs (TP-435A, TP-465, TP-467A, TP-467B, TP-396A, TP-396B, TP-396C, TP-414, TP-234C, TP-222A and TP-246) were detected in commercial salmon but not in salmon from the fish trial. A potential explanation for this observation is that these TPs occurred in the feed used in the fish farms and were transferred to commercial salmon but they did not occur in the freshly produced feed used during the feeding trial.

3.3. Overall fate of EQ and comparison with previous studies

The observations from the feeding trial and commercial salmon along with results from previous studies provide an understanding of the overall fate of EQ (Fig. 5). While previous studies on the fate of EQ in fish are scarce, some have established that the antioxidant may be

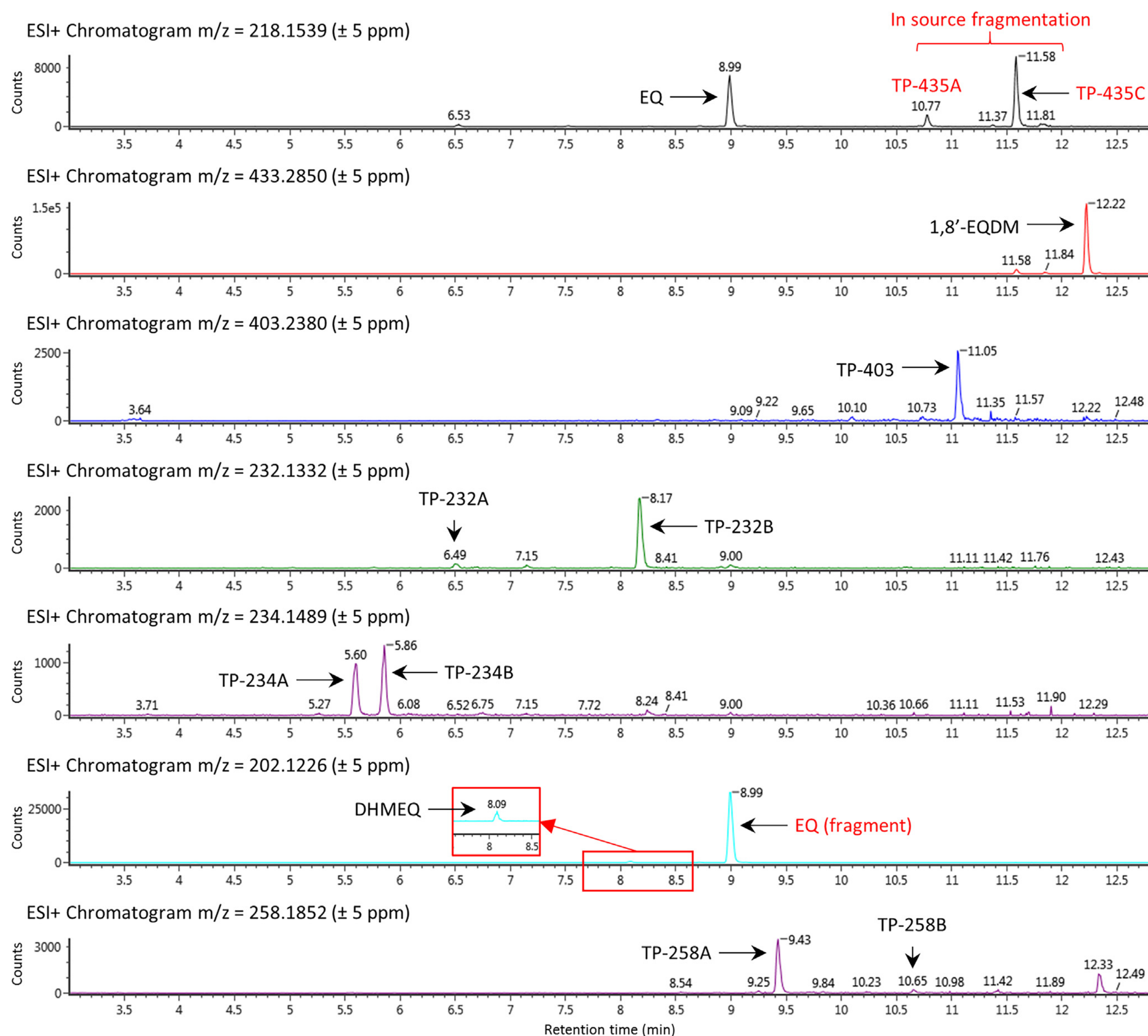

Fig. 4. Chromatogram of EQ and some of its TPs in commercial Atlantic salmon sampled from Norwegian fish farms.

transferred from feed to fish fillet (Bohne et al., 2008; He & Ackman, 2000; Lundebye et al., 2010). Therefore, results from the feeding trial and the analysis of commercial salmon are in agreement with the published literature. The feeding trial also suggested a link between the abundance of EQ in salmon fillet and the amount contained in the feed but this observation should be considered with caution since the current data set was limited to three doses and did not include replicates.

The analysis of salmon from the feeding trial and from Norwegian fish farms revealed that 1,8′-EQDM was the main TP of EQ, which is consistent with findings from previous studies (Bohne et al., 2007; Lundebye et al., 2010; Ørnsrud et al., 2011). Moreover, the detection of the TPs EQI and DEQ during the feeding trial is also in agreement with previous studies which also detected them after dietary exposure to EQ but without providing any quantification due to their low abundance (Bohne et al., 2006, 2007, 2008). Finally, another study on the metabolism of EQ based on a feeding trial and relying on sample analysis by liquid chromatography with fluorescence detection also reported the occurrence of more than 10 potential other TPs in salmon muscle (Bohne, Hove, & Hamre, 2007). Therefore, the detection of multiple TPs in salmon resulting from the feeding trial (Fig. 1, Fig. 2) was consistent with the existing literature and the application of UHPLC-TWIMS-QTOFMS improved the existing knowledge by allowing their identification.

The detection of TPs in salmon fillet is also consistent with their occurrence previously reported in fish feed (Negreira et al., 2017). For instance, while EQI was shown to occur in fish feed, the corresponding signal was much lower in samples of Atlantic salmon even when given the feed containing EQ at 1173 mg/kg (Fig. S9). However, it should also be noticed that the detection of EQ and its TPs in fish given the control feed might be either the consequence of the background level of EQ (0.5 mg/kg) in the feed or the result of the regular commercial feed used prior to the feeding trial and the two-week acclimation period.

3.4. Implications for food analysis and consumers

The application of UHPLC-TWIMS-QTOFMS proved successful in order to screen for specific components and TPs in food samples. In addition, the high mass accuracy and the ion mobility allowed a higher confidence in the identification than detection with fluorescence or usual tandem mass spectrometry. The results presented in this study

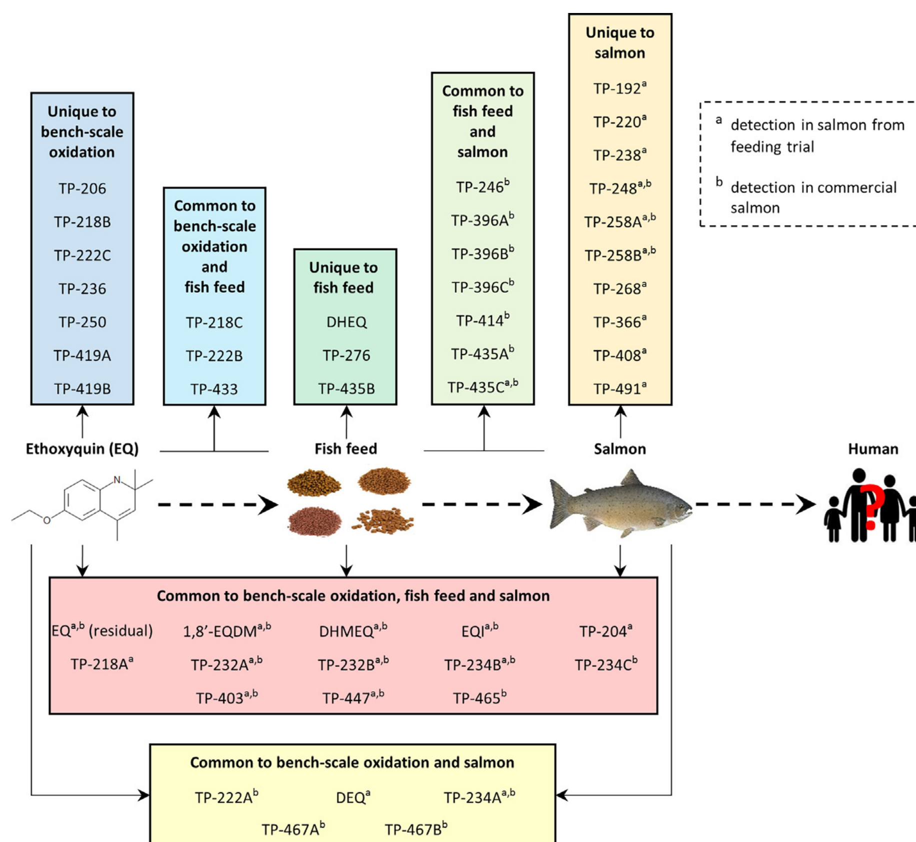

Fig. 5. Overview of the fate of ethoxyquin (EQ) during oxidation experiment (Negreira et al., 2017), in fish feed (Negreira et al., 2017) and in Atlantic salmon (this study).

provide a first global assessment of the potential occurrence of EQ and its TPs in salmon due to the EQ contained in the feed. However, these results must be considered cautiously since the samples from the feeding trial did not include replicates and commercial salmon only included 12 samples. Moreover, since this work only aimed at identifying and screening TPs, no accurate quantification was performed and extraction efficiency as well as matrix effect were not investigated. Accurate quantification of TPs should be performed in future work including the analysis of a large number of commercial salmon.

The exposure of the consumer to EQ and its TPs and the potential health effect should be determined by future risk assessment studies. While the current research provides initial information regarding the tentative chemical structure of the TPs, additional studies are required to assess their potential biological activity. For instance, several studies are available regarding the toxicity of EQ (Bernhard, et al., 2019; Blaszczyk, 2006; Blaszczyk, Osiecka, & Skolimowski, 2003; Blaszczyk & Skolimowski, 2007; Manson, Green, & Driver, 1987; Manson, Green, Wright, & Carthew, 1992) but little is known with respect to the TPs. To date, detailed information is only available for 1,8′-EQDM (Bernhard et al., 2018; Ørnsrud et al., 2011) while EQI showed structural alerts for mutagenicity and carcinogenicity (EFSA, 2015) according to quantitative structure–activity relationship (QSAR) analysis. Therefore a similar QSAR approach should be carried out on the TPs reported in salmon fillets. Finally, food products available from supermarkets should also be tested since it is not known how EQ and its TPs will behave during the industrial processing of farmed salmon.

4. Conclusion

The antioxidant EQ used as an additive in fish feed can be transferred to fish muscle. Following the dietary exposure to three different doses of EQ over 90 consecutive days, this study confirmed the occurrence of the antioxidant in fillets of Atlantic salmon (*Salmo salar*, L.) and assessed the occurrence of related TPs. After the feeding trial, the application of UHPLC-TWIMS-QTOFMS revealed the occurrence of EQ along with twenty-three TPs in fish muscle. These transformation products were formed through six mechanisms such as 1) dimerization, 2) cleavage, 3) oxygenation, 4) cleavage and oxygenation, 5) cleavage and conjugation, and 6) other alterations. This study also considered the relative abundances of each TP and showed that the dimer 1,8′-EQDM was the most abundant. Combining these results with those of previous studies performed on fish feed leads to a total of forty-seven TPs of EQ characterized so far.

Through the application of the same UHPLC-TWIMS-QTOFMS method, this study also revealed the occurrence of EQ and twenty-four TPs (out of forty-seven characterized to date) in fillets of commercial Atlantic salmon randomly sampled from different Norwegian fish farms. In these commercial salmon, the TP with the largest abundance was also the 1,8′-EQDM. However, EQI, another TP showing structural alerts for mutagenicity and carcinogenicity, was also detected at trace level in two out of twelve samples. Therefore, future studies should aim at quantifying EQ and its TPs in salmon food products to assess human exposure. In order to evaluate the potential health effects of EQ and its TPs, additional studies should also determine the toxicological properties of each compound, for instance through quantitative structure-–activity relationship (QSAR) models.

The novel application of ion mobility with liquid chromatography and high resolution mass spectrometry is promising for both screening and identification of transformation products in complex matrices such as extracts of salmon fillets. CCS values obtained from the application of ion mobility represent an additional criterion for compound identification which improves the confidence level, particularly for analytes for which the abundance is too low to allow the detection of characteristic fragments.

Conflict of interest

The authors declare that there is no personal conflict of interest. However, the authors disclose that the study was funded by the Norwegian Seafood Research Fund (FHF), the Marine Ingredients Organisation (IFFO), Marine Harvest ASA, EWOS AS/Cargill Aqua Nutrition, Biomar AS, Skretting AS and Europharma (FHF project no. 901327). The feed trial was performed in facilities made available by Nofima AS, using feed produced by EWOS AS. The Norwegian Seafood Federation (SjømatNorge) mediated contact with Industrial Técnica Pecuaria S.A. (Spain), which provided the ethoxyquin used to fortify the feed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.03.054.

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