New Analytical Methods

# Determination of the synthetic antioxidant ethoxyquin and its metabolites in fish and fishery products using liquid chromatography-fluorescence detection and stable isotope dilution analysis liquid chromatography-tandem mass spectrometry

Andreas Kranawetvogl, and Paul Wilhelm Elsinghorst

*J. Agric. Food Chem.*, **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.9b01508 • Publication Date (Web): 16 May 2019

# Determination of the synthetic antioxidant ethoxyquin and its metabolites in fish and fishery products using liquid chromatography-fluorescence detection and stable isotope dilution analysis liquid chromatography-tandem mass spectrometry

Andreas Kranawetvogl,[†] Paul W. Elsinghorst[*,†,‡]

[†] Central Institute of the Bundeswehr Medical Service Munich, Ingolstädter Landstrasse 102, 85748 Garching, Germany

[‡] Pharmaceutical Institute, Pharmaceutical Chemistry I, University of Bonn, An der Immenburg 4, 53121 Bonn, Germany

[*] **Corresponding author:**

Priv.-Doz. Dr. Paul W. Elsinghorst, Central Institute of the Bundeswehr Medical Service Munich, Ingolstädter Landstrasse 102, 85748 Garching, Germany; Phone: +49-89-3755849-5310, Fax: +49-89-3755849-5209, e-mail: paulelsinghorst@bundeswehr.org

**Abstract**

The use of the synthetic antioxidant ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline, EQ) as a flame retardant in fish meal transported by sea is required by international authorities to prevent self-ignition. Due to extensive carry-over within the food chain, selective and sensitive analytical methods are required for investigations on human exposure and safety of EQ and its metabolites. Therefore, a simple, fast and rugged liquid chromatography method was developed for detection of EQ and its metabolites in fish and fishery products after liquid-liquid extraction using QuEChERS. For screening purposes a fluorescence detector was used (LC-FLD) with the EQ-analogue methoxyquin serving as internal standard. For stable isotope dilution analysis by liquid chromatography-tandem mass spectrometry (SIDA-LC-MS/MS) deuterated analogues of EQ and its metabolites were synthesized for the first time and allowed for sensitive quantification and thus confirmation of screening results. Both methods were validated and successfully applied to commercially available fish samples.

**Keywords**

ethoxyquin, Skraup-Doebner-von Miller quinoline synthesis, fishery products, metabolite, fluorescence detection, stable isotope dilution analysis

## Introduction

The antioxidant ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline, EQ) was first described by the German chemist Emil Knoevenagel in 1921, when working on the condensation of aromatic amines with aliphatic ketones.[1] Although initially used as a rubber additive to impede cracking from isoprene oxidation, EQ has also a long history of application as an antioxidant in feed, especially for poultry and fish.[2] Fish meal, because of its high content in polyunsaturated fatty acids, is prone to oxidation and requires stabilization to preserve its high nutritional value. Moreover, international regulations by the International Maritime Organization require addition of EQ to fish meal and fish scrap when transported by sea to prevent spontaneous combustion.[3] Currently, minimum levels of EQ at the time of consignment are set at 100 mg/kg (100 ppm).

Previous studies revealed that EQ and metabolites thereof are detectable in various tissues of fish fed with EQ-containing feed, indicating that human exposure may occur as a result of carry-over.[4–7] Notably, the EQ dimer (6,6'-diethoxy-2,2,2',2',4,4'-hexamethyl-1',2'-dihydro-2*H*-1,8'-biquinoline, EQDM) has been reported as the main metabolite showing considerably higher concentrations than EQ itself.[6] Other metabolites formed by demethylation/rearomatization (2,4-dimethyl-6-ethoxyquinoline, DMEQ) or the de-ethylation product hydroxyquin (1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline, DEQ) and its corresponding quinone imine (2,6-dihydro-2,2,4-trimethyl-6-quinolone, QI) have also been detected, but often at negligible intensity. Recently, even more oxidation products were reported to be present in salmon feed, of which several were shown to carry-over into fish including EQDM as the most abundant but also QI.[8,9]

Safety of EQ was assessed by the European Food Safety Authority (EFSA), which found EQ to be neither genotoxic nor carcinogenic and not to cause developmental toxicity in offspring.[10] However, despite its high antioxidant capacity EQ may also act as a prooxidant facilitating formation of reactive oxygen species that are known for their adverse health effects,

in particular at higher concentrations.[10,11] Typical symptoms observed in different animals after oral administration of EQ were liver and kidney damages as well as changes in the gastrointestinal tract. With respect to the toxicity of EQ metabolites data are incomplete except for EQDM, whose toxicological profile is considered to reflect that of EQ. Toxicity of QI has not been investigated yet, however, it is assumed to be capable of forming covalent DNA adducts due to its quinone imine structure and might thus be mutagenic and carcinogenic.[10]

Due to this considerable lack of data the European Commission decided to suspend the authorization of EQ as a feed additive in June 2017, pointing out that the suspension should be reviewed upon submission and evaluation of additional data with focus on consumer exposure.[12] Consequently, appropriate analytical methods are required in order to investigate levels of EQ and its metabolites in fish and fishery products for human consumption. Several methods for this purpose have been described in the literature including gas chromatography with flame ionization detection (GC-FID) and mostly liquid chromatography using fluorescence (LC-FLD) or more recently electrochemical (LC-EC) detection.[4,7,13–18] Also coupling of the respective chromatographic systems with mass spectrometry (GC-MS, LC-MS) are common.[13,16,19] Quantification of analytes in these methods is either performed by external calibration or with the aid of internal standards like quinoline or methoxyquin (1,2-dihydro-6-methoxy-2,2,4-trimethylquinoline, MQ), the latter allowing for correction of analyte extraction losses during sample preparation.[15,17] However, until now no method has been reported applying stable isotope dilution analysis (SIDA) using stable-labeled isotopomers of the respective analytes as internal standards, although SIDA can be considered state of the art in modern residue analysis.[20,21] A reason for this might be the lack of commercially available stable-labeled isotopomers, which in SIDA will allow to compensate extraction losses or matrix effects during mass spectrometry because of their almost identical physicochemical properties.

Therefore, deuterium-labeled isotopomers of EQ and its metabolites were synthesized and a stable isotope dilution analysis liquid chromatography-tandem mass spectrometry (SIDA-

LC-MS/MS) method was developed for highly selective and sensitive determination of the analytes in fish and fishery products. Additionally, a simple, fast and rugged LC-FLD method was established for screening of samples prior to confirmation of residue contents using SIDA-LC-MS/MS. Both methods were validated and successfully applied to a set of commercially available salmon samples.

## Materials and Methods

**Chemicals and Reagents.** 4-Ethoxyaniline (≥ 98%, for synthesis) was purchased from Sigma-Aldrich (Taufkirchen, Germany). 4-Methoxyaniline (≥ 98%, for synthesis), ethyl acetate (Emsure®, p.a.), ethanol (absolute, p.a.), ammonium acetate (Emsure®, ACS reagent), hydrochloric acid (Titripur®, 2 M), solid sodium hydroxide (≥ 97%), sodium hydroxide (Titrisol®, 1 M) and anhydrous sodium sulfate (Emsure®, p.a.) were purchased from Merck (Darmstadt, Germany). Petroleum ether (boiling range 40-60 °C, Chemsolute®, p.a.) was obtained from Th. Geyer (Renningen, Germany). Crystalline iodine (≥ 99.5%) and *tert*-butyl hydroperoxide (70% aqueous solution) were from Thermo Fisher (Karlsruhe, Germany). Toluene (Chromasolv®, p.a.) was purchased from Honeywell Riedel-de-Haën (Seelze, Germany) and $d_6$-acetone from Deutero (Kastellaun, Germany). Acetonitrile (MeCN, LC grade) was purchased from VWR (Darmstadt, Germany) and ascorbic acid (ACS reagent, p.a.) and ammonium ferrous sulfate hexahydrate (≥ 99%, ACS reagent) were from Fluka (Buchs, Switzerland). Water (LC grade) was prepared using a Milli-Q® Gradient water purification system from Merck. ALUGRAM® Xtra SIL G/$UV_{254}$ (silica gel 60, layer thickness 0.2 mm) plates for thin-layer chromatography, silica gel 60 (0.063–0.2 mm) for column chromatography, the Chromabond® QuEChERS citrate extraction mix (Mix I) as well as the Chromabond® QuEChERS clean-up mix (Mix VI) were obtained from Macherey-Nagel (Düren, Germany).

**Instrumentation.** A syringe pump KDS 100 Legacy from KD Scientific Inc. (Holliston, MA, USA) was used for continuous addition of reagents for synthesis of $d_{10}$-EQ and MQ as described below. Melting points were determined using a capillary tube apparatus B-540 from Büchi (Essen, Germany). GC-MS analysis for compound characterization was performed on an 7890B GC system (injector: splitless, 250 °C; carrier gas: He; column: HP-5 MS, 30 m × 0.25 mm × 0.25 µm; temperature gradient, min/°C: 0/60, 2/60, 21/250, 22.5/280, 35/280) coupled to a 5977A mass spectrometer (EI, scan range *m/z* 50-500) from Agilent Technologies (Waldbronn, Germany). NMR spectra were recorded on an Avance III ($^1$H NMR: 600 MHz, $^{13}$C NMR: 150 MHz) and DRX 500 ($^1$H NMR: 500 MHz, $^{13}$C NMR: 125 MHz) spectrometer from Bruker (Rheinstetten, Germany) and chemical shifts δ are given in parts per million referring to the signal center using the respective solvent peaks for reference ($CDCl_3$, 7.26/77.0 ppm; $d_6$-DMSO, 2.50/39.5 ppm). To characterize the spin multiplicity, the following abbreviations are used: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet.

***d*$_{10}$-Ethoxyquin (*d*$_{10}$-EQ).** In a round-bottom flask 4-ethoxyaniline (2 g, 14.6 mmol) and iodine (50 mg, 0.20 mmol) were dissolved in toluene (6.5 mL) under vigorous stirring. After purging with nitrogen, $d_6$-acetone (4.68 g, 73 mmol) was added to the refluxing reaction mixture using a syringe pump (0.26 g/h, 18 h). Subsequently the reaction mixture was filtered through silica using petroleum ether/ethyl acetate 80:20 v/v (500 mL) and the filtrate was evaporated *in vacuo.* Purification of the residue was achieved by column chromatography using petroleum ether/ethyl acetate 90:10 v/v (detection: i) blue fluorescence upon excitation at λ = 365 nm, ii) fast degradation to a brownish product under the influence of daylight) to provide $d_{10}$-EQ (1.69 g, 51%) as a yellowish oil. GC-MS: purity > 94%. $^1$H NMR ($d_6$-DMSO) δ: 1.13 (m, $^2J_{H,D}$ = 9.5 Hz, 2-$CH_3$, 24% H), 1.26 (t, 3H, $^3J_{H,H}$ = 6.9 Hz, $CH_2C\underline{H}_3$), 1.84 (m, 4-$CH_3$, 11% H), 3.88 (q, 2H, $^3J_{H,H}$ = 6.9 Hz, $C\underline{H}_2CH_3$), 5.29 (m, 3-H, 13% H), 5.31 (s, 1H, NH), 6.39 (m, 8-H, 11% H), 6.53 (m, 2H, 5/7-H); $^{13}$C NMR ($d_6$-DMSO) δ: 14.90 ($CH_2\underline{C}H_3$), 17.56 (m, 4-$CH_3$),

29.52 (m, 2-$CH_3$), 50.58 (m, C-2), 63.49 ($\underline{C}H_2CH_3$), 110.14 (C-5), 112.65 (m, C-8), 114.52 (C-7), 121.37 (C-4a), 127.32 (C-4), 129.11 (m, C-3), 138.38 (C-8a), 149.61 (C-6).

***d*$_7$-Dimethylethoxyquinoline (*d*$_7$-DMEQ).** $d_{10}$-EQ (0.5 g, 2.20 mmol) was heated to 180 °C for 30 min under vigorous stirring. The crude reaction product was purified by column chromatography using petroleum ether/ethyl acetate 50:50 v/v (detection: blue fluorescence upon excitation at λ = 254 nm) and recrystallization from petroleum ether to obtain $d_7$-DMEQ (10 mg, 2%) as light brown crystals, m.p. 83-84 °C, lit.[22] 86-87 °C (non-deuterated). GC-MS: purity > 95%. $^1$H NMR ($CDCl_3$) δ: 1.49 (t, 3H, $^3J_{H,H}$ = 7.0 Hz, $CH_2C\underline{H}_3$), 2.58 (m, 2-$CH_3$, 14% H), 2.65 (m, 4-$CH_3$, 26% H), 4.15 (q, 2H, $^3J_{H,H}$ = 7.0 Hz, $C\underline{H}_2CH_3$), 7.10 (s, 3-H, 11% H), 7.15 (d, 1H, $^4J_{H,H}$ = 2.7 Hz, 5-H), 7.33 (d, 1H, $^4J_{H,H}$ = 2.7 Hz, 7-H), 7.96 (app. d, $^3J_{H,H}$ = 9.2 Hz, 8-H, 13% H); $^{13}$C NMR ($CDCl_3$) δ: 14.80 ($CH_2\underline{C}H_3$), 18.17 (4-$CH_3$), 24.16 (2-$CH_3$), 63.76 ($\underline{C}H_2CH_3$), 102.97 (C-5), 121.30 (C-7), 122.65 (C-3), 127.39 (C-4a), 129.90 (C-8), 142.91 (C-4), 143.12 (C-8a), 155.62 (C-6), 156.50 (C-2).

***d*$_{18}$-Ethoxyquin dimer (*d*$_{18}$-EQDM).** A solution of $d_{10}$-EQ (0.51 g, 2.25 mmol) in ethanol (50 mL) was cooled to 0 °C and an equimolar amount of *tert*-butyl hydroperoxide (70%, 0.31 mL) and aqueous ammonium ferrous sulfate hexahydrate (0.88 g in 20 mL) were added under vigorous stirring. The mixture was allowed to react at 0 °C for 18 h before it was acidified (1 M HCl, 50 mL) and extracted with petroleum ether (3 × 50 mL). The combined extracts were washed (1°M HCl, 2 × 25 mL), dried using $Na_2SO_4$ and evaporated *in vacuo*. The remaining residue was purified by column chromatography using petroleum ether/ethyl acetate 90:10 v/v (detection: i) blue fluorescence upon excitation at λ = 365 nm, ii) fast degradation to a reddish product under the influence of daylight) to obtain $d_{18}$-EQDM (0.35 g, 69%) as a yellowish solid, m.p. 109-110 °C, lit.[22] 107-109 °C (non-deuterated). GC-MS: purity 100%. $^1$H NMR ($CDCl_3$) δ: 0.93 (m, $^2J_{H,D}$ = 11.7 Hz, 2'-H, 27% H), 1.14 (m, $^2J_{H,D}$ = 11.2 Hz, 2'-H, 24% H), 1.18 (m, $^2J_{H,D}$ = 11.2 Hz, 2-H, 31% H), 1.27 (m, $^2J_{H,D}$ = 11.7 Hz, 2-H, 33% H), 1.37 (t, 3H, $^3J_{H,H}$ = 7.0 Hz, 8-$OCH_2C\underline{H}_3$), 1.39 (t, 3H, $^3J_{H,H}$ = 7.0 Hz, 8'-$OCH_2C\underline{H}_3$), 2.01 (m,

$^{2}J_{H,D}$ = 9.7 Hz, 4/4'-H, 18% H), 3.95 (q, 2H, $^{3}J_{H,H}$ = 7.0 Hz, 8-OC$\underline{H}_2$CH$_3$), 3.96 (q, 2H, $^{3}J_{H,H}$ = 7.0 Hz, 8'-OC$\underline{H}_2$CH$_3$), 5.37 (s, 3'-H, 29% H), 5.44 (s, 3-H, 54% H), 6.06 (d, $^{3}J_{H,H}$ = 8.8 Hz, 8-H, 13% H), 6.49 (d, 1H, $^{4}J_{H,H}$ = 2.9 Hz, 7-H), 6.68 (d, 1H, $^{4}J_{H,H}$ = 2.5 Hz, 7'-H), 6.72 (d, 1H, $^{4}J_{H,H}$ = 2.5 Hz, 5'-H), 6.76 (d, 1H, $^{4}J_{H,H}$ = 2.9 Hz, 5-H); $^{13}$C NMR ($CDCl_3$) δ: 15.03 (CH$_2\underline{C}$H$_3$), 15.07 (CH$_2\underline{C}$H$_3$), 56.56 (C-2), 63.95 ($\underline{C}$H$_2$CH$_3$), 64.27 ($\underline{C}$H$_2$CH$_3$), 109.99 (C-7), 110.76 (C-5'), 113.71 (C-5), 114.70 (m, C-8), 117.40 (C-7'), 124.32 (C-4a), 124.62 (C-8a'), 127.83 (C-4a'), 127.95 (C-4/4'), 128.48 (C-4/4'), 130.61 (C-3/3'), 130.98 (C-3/3'), 137.89 (C-8a), 150.35 (C-6'), 150.86 (C-6).

**$d_{10}$-Quinone imine ($d_{10}$-QI).** The combined acidic layers obtained following the procedure for the synthesis of $d_{18}$-EQDM were basified (4 M NaOH, 50 mL) and extracted with petroleum ether (5 × 50 mL). After washing (1 M NaOH, 2 × 25 mL) and drying with $Na_2SO_4$, the solvent was evaporated *in vacuo* and the crude residue purified by column chromatography using petroleum ether/ethyl acetate 50:50 v/v (detection: i) fluorescence extinction upon excitation at λ = 254 nm, ii) yellow spots) to obtain $d_{10}$-QI as an auburn solid (73 mg, 17%), m.p. 47-49 °C, lit.[22] 52-53 °C (non-deuterated). GC-MS: purity > 90%. $^{1}$H NMR ($CDCl_3$) δ: 1.38 (m, $^{2}J_{H,D}$ = 9.7 Hz, 2-CH$_3$, 29% H), 1.95 (m, 4-CH$_3$, 14% H), 6.33 (s, 1H, 5-H), 6.44 (s, 3-H, 10% H), 6.65 (m, 1H, 7-H), 7.19 (d, $^{3}J_{H,H}$ = 9.9 Hz, 8-H, 12% H); $^{13}$C NMR ($CDCl_3$) δ: 16.97 (4-CH$_3$), 28.01 (2-CH$_3$), 61.60 (C-2), 120.64 (C-5), 126.64 (C-8a), 131.51 (C-4), 133.25 (C-7), 140.34 (C-3), 145.44 (C-8), 154.76 (C-4a), 188.46 (C-6).

**Methoxyquin (MQ).** In a round-bottom flask 4-methoxyaniline (1.8 g, 14.6 mmol) and iodine (50 mg, 0.20 mmol) were dissolved in toluene (6.5 mL) under vigorous stirring. After purging with nitrogen acetone (4.24 g, 73 mmol) was added to the refluxing reaction mixture using a syringe pump (0.24 g/h, 18 h). Subsequently the reaction mixture was filtered through silica using petroleum ether/ethyl acetate 80:20 v/v (500 mL) and the filtrate was evaporated *in vacuo*. Purification of the residue was achieved by column chromatography using petroleum ether/ethyl acetate 90:10 v/v (detection: i) blue fluorescence upon excitation at λ = 365 nm, ii)

fast degradation to a brownish product under the influence of daylight) to provide MQ (1.65 g, 55%) as a yellowish oil. GC-MS: purity > 96%. $^{1}$H NMR ($d_6$-DMSO) δ: 1.16 (s, 6H, 2-$CH_3$), 1.88 (d, 3H, $^{4}J_{H,H}$ = 1.4 Hz, 4-$CH_3$), 3.64 (s, 3H, $OCH_3$), 5.30 (m, 1H, 3-H), 5.33 (s, 1H, NH), 6.41 (m, 1H, $^{3}J_{H,H}$ = 8.8 Hz, 8-H), 6.53 (dd, 1H, $^{3}J_{H,H}$ = 8.7 Hz, $^{4}J_{H,H}$ = 2.8 Hz, 7-H), 6.55 (app. s, 1H, 5-H); $^{13}$C NMR ($d_6$-DMSO) δ: 18.19 (4-$CH_3$), 30.19 (2-$CH_3$), 50.96 (C-2), 55.43 ($OCH_3$), 109.27 (C-5), 113.00 (C-8), 113.87 (C-7), 121.36 (C-4a), 127.52 (C-4), 129.56 (C-3), 138.42 (C-8a), 150.49 (C-6).

**Sample preparation.** Samples for LC-FLD and SIDA-LC-MS/MS analysis were obtained from local retailers and originated either from Norwegian aquaculture or from the North-East Pacific. Fillets were cut into pieces and homogenized using a knife mill (Grindomix GM 200; Retsch, Haan, Germany). Samples (5 g) were exactly weighed into amber glass centrifuge tubes (50 mL) and spiked either with MQ (500 µL, 1 µg/mL, MeCN) for LC-FLD analysis or a mixture of $d_{10}$-EQ, $d_7$-DMEQ, $d_{18}$-EQDM and $d_{10}$-QI (500 µL, 1 µg/mL for $d_{10}$-EQ, $d_7$-DMEQ and $d_{10}$-QI, 100 ng/mL for $d_{18}$-EQDM, MeCN) in the case of SIDA-LC-MS/MS. Subsequently, water was added (6,5 mL) and samples were shaken vigorously by hand (1 min) prior to addition of MeCN (9,5 mL) and further mixing (15 min, 200 rpm) using a laboratory shaker (KS 501 digital; IKA® Labortechnik, Staufen, Germany). Subsequently, QuEChERS citrate extraction mix (Mix I) was added and the centrifuge tubes were shaken vigorously by hand (1 min) before samples were centrifuged (3345 × *g*, room temperature (rt), 5 min) using a laboratory centrifuge (Megafuge 1.0R; Heraeus, Hanau, Germany). Finally, an aliquot of the supernatant (6 mL) was transferred into centrifuge tubes (15 mL) containing QuEChERS clean-up mix (Mix VI) followed once more by vigorous shaking (1 min) by hand and another centrifugation step (3345 × *g*, rt, 5 min). An aliquot (1 mL) of the supernatant was transferred into amber glass vials and subjected directly to LC-FLD or SIDA-LC-MS/MS analysis.

**LC-FLD analysis.** Sample analysis was performed using an Infinity 1200 series LC-FLD system (Agilent Technologies) consisting of an autosampler, a binary pump, and a column

thermostat coupled to a fluorescence detector. Post-column derivatization was achieved using an Infinity 1260 isocratic pump (Agilent Technologies) and an LC-MS effluent optimizer (LEO) module (Gerstel, Mülheim, Germany). Samples (10 µL) were separated at 40 °C on a Kinetex® EVO C18 column (100 mm × 4.6 mm, 2.6 µm; Phenomenex, Aschaffenburg, Germany) protected by a precolumn (SecurityGuard Ultra cartridge for C18 ultra-high performance LC; Phenomenex) using gradient elution with solvent A (10 mM ammonium acetate) and solvent B (MeCN) at a flow of 500 µL/min. The following gradient was applied, min/% B: 0/40, 10/80, 10.2/100, 14.8/100, 15/40, 20/40. Within each run the six-way valve installed in the column oven was switched from configuration 1 to configuration 2 after 9 min and backwards after 20 min (see Supporting Information, **Figure S1**) allowing for regular backflushing and thus cleaning of the precolumn. Aqueous ascorbic acid (0.1% w/v) was continuously added post-column at a flow of 50 µL/min using the LEO module to increase detection sensitivity by reduction of QI to DEQ. The following parameters were used for detection: excitation wavelength 235 nm; emission wavelength, min/nm: 0/440, 6/365, 8/440; photomultiplier gain 16; time constant 4 s. Data acquisition, processing, and analysis were performed using ChemStation (Rev. B.04.03, Agilent Technologies).

**SIDA-LC-MS/MS.** An Infinity 1260 series LC-MS/MS-system (Agilent Technologies) was used consisting of an autosampler, a binary pump, and a column thermostat coupled to a 6460 triple quadrupole mass spectrometer equipped with an Agilent Jet Stream ion source. Chromatographic parameters including sample volume, column type, column temperature, solvent composition, flow, gradient and time-dependent valve switching were identical to those described above for LC-FLD. The following parameters were used for detection by mass spectrometry: gas temperature 300 °C, gas flow 9 L/min, nebulizer 15 psi ($1.03 \times 10^5$ Pa), sheath gas temperature 350 °C, sheath gas flow 12 L/min, nozzle voltage +500 V, capillary voltage +3500 V. MS/MS transitions were recorded in dynamic multiple reaction monitoring mode with the following parameters including expected retention time ($t_R$) ± corresponding

time window, fragmentor voltage (FV), collision energy (CE), and cell acceleration voltage (CAV): QI *m/z* 188.1 → 145.0 ($t_R$ 4.3 ± 2 min, FV 100 V, CE 24 V, CAV 4 V), $d_{10}$-QI *m/z* 198.2 → 152.1 ($t_R$ 4.2 ± 2 min, FV 85 V, CE 32 V, CAV 4 V), DMEQ *m/z* 202.1 → 174.0 ($t_R$ 6.6 ± 2 min, FV 110 V, CE 24 V, CAV 4 V), $d_7$-DMEQ *m/z* 209.2 → 181.1 ($t_R$ 6.5 ± 2 min, FV 125 V, CE 24 V, CAV 4 V), EQ *m/z* 218.2 → 203.0 ($t_R$ 10.2 ± 2 min, FV 80 V, CE 8 V, CAV 4 V), $d_{10}$-EQ *m/z* 228.2 → 210.1 ($t_R$ 10.0 ± 2 min, FV 80 V, CE 8 V, CAV 4 V), EQDM *m/z* 433.3 → 216.1 ($t_R$ 15.3 ± 2 min, FV 135 V, CE 24 V, CAV 4 V), $d_{18}$-EQDM *m/z* 451.4 → 225.0 ($t_R$ 15.2 ± 2 min, FV 130 V, CE 28 V, CAV 4 V). Data acquisition, processing, and analysis were performed with MassHunter Workstation (version B.08.00, Agilent Technologies).

**Method validation.** Linearity was assessed by spiking blank samples (wild salmon) with EQ, DMEQ, EQDM and QI at six different levels resembling the natural occurrence in samples from aquaculture: 0, 40, 80, 120, 160, and 200 µg/kg. For LC-FLD analysis the internal standard MQ was added to each sample at a concentration of 100 µg/kg, as it was the case with the deuterated isotopomers $d_{10}$-EQ, $d_7$-DMEQ and $d_{10}$-QI for analysis by SIDA-LC-MS/MS. However, due to extensive ion suppression at higher concentrations EQDM was added at 1/10 of the aforementioned levels (0, 4, 8, 12, 16, and 20 µg/kg), including the internal standard $d_{18}$-EQDM, which was spiked at a concentration of 10 µg/kg. All spiked samples were subsequently extracted as described above and analyzed by LC-FLD or SIDA-LC-MS/MS. Linear calibration curves were obtained by plotting the peak area ratios (analyte/internal standard) versus the concentration ratios (analyte/internal standard). Limits of detection (LOD) and quantification (LOQ) were derived from the obtained calibration lines by calculation according to ISO 11843-2.[23]

Matrix effects on ionization were evaluated in a separate experiment by dividing the slope of a corresponding calibration curve obtained from spiked blind matrix by the slope from solvent-based calibration, where a ratio < 1 indicates ion suppression and a ratio > 1 ion

enhancement.[24] Recovery rates were subsequently derived for each linearity level from either solvent-based (LC-FLD, MeCN) or matrix-matched calibration (SIDA-LC-MS/MS) data. For estimation of intraday precision, six replicates were prepared by spiking blank samples to the respective LOQ (**Table 1**) and analyzed within one day using MQ (LC-FLD) or the respective deuterated isotopomers (SIDA-LC-MS/MS) as internal standards for recovery correction. The calculated coefficients of variation served as a measure for precision.

## Results and Discussion

### Synthesis

Although the product of a rather simple condensation reaction, EQs exact chemical structure has puzzled researchers for many years. Initially referred to as "aceton-*p*-phenetidil",[1] *i.e.*, the condensation product of *p*-phenetidine and acetone obtained in the presence of iodine, it was not until 1961 that Elliott & Yates were finally able to unambiguously identify its structure as 1,2-dihydro-2,2,4-trimethylquinoline, thus refuting the idea of an "acetone anil" formation.[25] Despite this problem significant effort has been put into optimizing synthetic strategies for EQ production to meet industrial demand. Today, the underlying reaction mechanism is known as the Skraup-Doebner-von Miller quinoline synthesis, named after three chemists that had already worked on this topic in the early 1880s.[26,27] It has been applied not only for the efficient synthesis of EQ and closely related analogues like MQ, but also for the corresponding metabolites recently discovered in fish and fishery products.[22,28]

Notably, the yield of EQ may be increased by slow and continuous addition of acetone into the flask by means of a syringe pump, which is most presumably due to the reduced evaporation of unreacted acetone despite the required high temperature of 110 °C (refluxing toluene).[29] Accordingly, application of $d_6$-acetone provided deuterium-labeled EQ and subsequently deuterium-labeled metabolites (**Figure 1**). Comparison of the NMR spectra of unlabeled EQ and the product obtained from the reaction with $d_6$-acetone clearly demonstrated

the success of isotopic labeling. However, in contrast to initial expectations deuteration did not only occur at carbon 3, 11, 12, and 13 but also at C-8, which was also confirmed by the NMR spectra of deuterated DMEQ, EQDM and QI (**Figure 2a**). Residual proton NMR signals at these deuteration sites allowed for estimation of deuterium incorporation ranging from 46% at certain positions of deuterated EQDM to 89% at certain sites of deuterated EQ and DMEQ. Additionally, GC-MS of the deuterated products revealed a quite broad isotopic distribution (**Figure 2b**), which is in accordance with the aforementioned varying deuteration efficiency and may be explained by H/D exchange processes during synthesis.

Indeed, using 4-ethoxyaniline and $d_6$-acetone as starting materials the initial step is most likely formation of the corresponding Schiff base (see Supporting Information, **Figure S2**).[30] Water released by this condensation reaction may then participate in H/D exchange reactions either with unreacted $d_6$-acetone or any of the deuterated intermediates. Moreover, the resulting Schiff base is considered to subsequently react with its enamine in a kind of a self-condensation followed by cyclization and elimination of deuterated 4-ethoxyaniline.[30,31] The latter most likely serves as a source of deuterium leading to the observed labeling of deuterated EQ at position C-8. In fact, EQ was shown to exist partly as a free radical produced by dehydrogenation of the quinoline nitrogen through free radicals or simply UV light.[32] Because of its aromatic ring system, EQ is capable of stabilizing this radical through delocalization with one of the corresponding resonance structures bearing the radical at position C-8 (see Supporting Information, **Figure S3**). Quenching of this radical by deuterium donors like deuterated 4-ethoxyaniline, $d_6$-acetone or $D_2O$ and rearomatization by imine-enamine tautomerism may produce EQ deuterated at C-8 as observed by NMR spectroscopy. The well-established dimerization of EQ through a covalent bond between C-8 and the quinoline nitrogen (EQDM) serves not only as an explanation for its antioxidant properties but also corroborates this hypothesis.[22,32,33]

**Sample preparation**

Various procedures for the extraction of EQ and some of its oxidation products have been described in the literature mostly comprising simple liquid extraction of homogenized samples using organic solvents[13,34] or the QuEChERS protocol.[35,36] Other methods make use of solid phase extraction[17] or include a further saponification step with sodium hydroxide for enhanced matrix disruption prior to liquid extraction.[4,14] However, due to the high fat content of certain fish species like salmon, the biggest challenge remains the efficient separation of the most lipophilic analyte EQDM from disturbing matrix components.

In our hands, neither liquid extraction in combination with a freeze-out step nor solid-phase extraction did sufficiently remove co-extracted lipids. Alternative saponification followed by extraction provided sufficiently lipid-free extracts, but chromatography was impaired by co-eluting matrix components in case of EQ and EQDM. We also considered to apply gel permeation chromatography, a common sample preparation technique for lipid removal, but did not succeed in sufficient separation of EQDM. Finally we opted for the QuEChERS method obtaining high recovery rates for all analytes, albeit being aware that the extracts still contained considerable amounts of lipophilic matrix components. Therefore, in order to reduce the matrix load and thus extend the lifetime of the analytical column a precolumn was used serving as a kind of "matrix trap". Continuous cleaning of this precolumn was achieved by operating the chromatographic system in a backflush mode (for details see Materials and Methods as well as Supporting Information, **Figure S1**). In this way, lipophilic matrix components are prevented from reaching the analytical column as they are first trapped on the precolumn (configuration 1) and then flushed away by the inverted eluent flow containing more than 76% MeCN (configuration 2). Altogether, the combination of quickly preparing samples using the QuEChERS protocol and continuously preventing the protective precolumn from matrix overcharging allowed for high-throughput sample analysis without affecting the lifetime of the analytical column.

**Sample analysis using LC-FLD**

Several chromatographic methods have been reported in the literature for the analysis of EQ and some of its metabolites. Notably, GC-FID or GC-MS used to be the methods of choice some decades ago,[15–17,19] despite the drawback of a low temperature stability of EQ, quickly degrading to DMEQ at temperatures applied in the injector or the column oven during GC analysis.[17] Today, LC-based methods can be considered state of the art.[4,7,13,14,18] Moreover, the dihydroquinoline moiety of EQ and EQDM as well as the quinoline in DMEQ make these compounds excellent fluorophores allowing for selective and sensitive detection using a fluorescence detector after LC separation (LC-FLD). The reported drawback that QI can hardly be detected by LC-FLD as its quinone imine structure shows only weak fluorescence,[4] however, can easily be overcome by post-column reduction of QI to DEQ (**Figure 1**). We succeeded in enhancing method sensitivity for QI detection by infusing 0.1% (w/v) aqueous ascorbic acid at 1/10 of the total flow prior to fluorescence detection.

The resulting LOD and LOQ of QI (detected as DEQ) were therefore of the same order of magnitude as for the lead compound EQ (**Table 1**). Moreover, comparison of the obtained LOD and LOQ values for EQ and EQDM demonstrated the suitability of the developed method for the analysis of commercial fish samples. Lundebye *et al.* reported on levels of EQ in fillets of different fish species ranging from 9.5 to 55 µg/kg and of EQDM either in the range > 700 µg/kg or not detectable.[7] The latter values of EQDM are in line with the findings of He & Ackman, who found EQDM levels in fish meals and feeds at least four times higher than in fillets.[34] In contrast, no values have been reported in the literature for QI, mainly because levels found were always lower than the respective LOQs.[4]

Analysis of a small set of fish fillets including wild salmon caught in the North-East Pacific and salmon obtained from Norwegian aquaculture allowed distinction between these two types of commercial fish using the developed LC-FLD method. Whereas wild salmon did not show any additional peaks (**Figure 3a**), there were distinct peaks in salmon from

aquaculture indicating the presence of EQ, EQDM and a further compound of unknown identity (**Figure 3b**). Notably, a highly abundant but also unknown compound termed UMEQ (unknown metabolite of EQ) has already been described in previous studies by Bohne *et al.*[4–6] but it remains unclear, whether this compound is identical to the one observed in the present study. Analysis of wild salmon spiked with EQ, MQ, EQDM, QI and DMEQ demonstrated the suitability of the developed gradient for sufficient separation of all analytes and the internal standard MQ (**Figure 3c**).

Unfortunately, recovery rates of QI remained low despite correction using the internal standard MQ. Significant differences in polarity between the more lipophilic MQ, EQ, DMEQ or EQDM and the much more polar QI containing a basic nitrogen in its quinone imine moiety may explain this behavior. Actually, this difference in polarity is the key feature allowing for efficient separation from EQDM by liquid-liquid extraction during synthesis (see above). Application of stable-labeled isotopomers of EQ and its metabolites, especially QI, in course of SIDA-LC-MS/MS was expected to solve this problem.

**Sample analysis using SIDA-LC-MS/MS**

Validation data obtained by SIDA-LC-MS/MS of EQ and its metabolites clearly showed the advantages of this methodology compared to LC-FLD (**Table 1**). Both, LODs and LOQs of QI, DMEQ and EQDM were significantly lower, especially for EQDM, where LOD and LOQ accounted for only 1/10 of the levels obtained for LC-FLD. Results for EQ were slightly higher, but still in comparable range and sufficiently low for analysis of commercial fish samples. Most probably this observation results from EQ being thermally unstable leading to in-source degradation to DMEQ during electrospray ionization.[17] Evaluation of matrix effects on ionization revealed a significant matrix influence, especially for the more lipophilic analytes (QI: -9.9%, DMEQ: 6.1%, EQ: -13.9%, EQDM: -67.8%), which was successfully compensated by combining a matrix-matched calibration with SIDA. Even more important, SIDA

significantly improved the recovery rate of QI, as the almost identical polar properties of QI and its internal standard $d_{10}$-QI provided an efficient correction for additional extraction losses resulting in recoveries of > 78% compared to < 35% obtained by LC-FLD (**Table 1**). Moreover, recovery rates reached almost 100% as the corresponding isotopomers of EQ, EQDM and DMEQ were applied for SIDA (**Table 1**).

Concluding, the developed SIDA-LC-MS/MS method is simple, fast, rugged and highly sensitive and exceeds existing methods for the quantification of EQ and its metabolites in fish and fishery products. First-time synthesis of the deuterated isotopomers of EQ and its metabolites did not only allow for SIDA but provided additional insight into the complex mechanism of the Skraup-Doebner-von Miller quinoline synthesis. The accompanying LC-FLD procedure proofed sufficiently selective and sensitive to serve as a reliable screening method followed by SIDA-LC-MS/MS for confirmation if required. Analysis of an extensive set of commercial samples will allow for monitoring current levels of EQ and its metabolites in fish and fishery products. These data will support assessment of human exposure due to carry-over and facilitate surveillance of the current ban of EQ use as an antioxidant in fish meal and feed.

406 **Supporting Information**

407 LC valve wiring diagram for backflush operation; proposed mechanisms for the formation of

408 deuterated EQ (PDF)

409 **Acknowledgements**

410 We gratefully thank J. Lang (Central Institute of the Bundeswehr Medical Service Munich) for

411 his excellent technical support.

## References

(1) Knoevenagel, E. Zur Kenntnis der Keton-anile, I.: Darstellung aliphatischer Keton-anile und alkalische Spaltung von Keton-anil-Jodalkylaten. *Ber. Dtsch. Chem. Ges.* **1921**, *54* (8), 1722–1730. https://doi.org/10.1002/cber.19210540807.

(2) de Koning, A. J. The antioxidant ethoxyquin and its analogues: a review. *Int. J. Food Prop.* **2002**, *5* (2), 451–461. https://doi.org/10.1081/JFP-120005797.

(3) International Maritime Organization. Annex to Resolution MSC.406(96): Amendments to the International Maritime Dangerous Goods Code (38-16). **2016**.

(4) Bohne, V. J. B.; Hove, H.; Hamre, K. Simultaneous quantitative determination of the synthetic antioxidant ethoxyquin and its major metabolite in Atlantic salmon (Salmo salar, L), ethoxyquin dimer, by reversed-phase high-performance liquid chromatography with fluorescence detection. *J. AOAC Int.* **2007**, *90* (2), 587–597.

(5) Bohne, V. J. B.; Hamre, K.; Arukwe, A. Hepatic biotransformation and metabolite profile during a 2-week depuration period in Atlantic salmon fed graded levels of the synthetic antioxidant, ethoxyquin. *Toxicol. Sci.* **2006**, *93* (1), 11–21. https://doi.org/10.1093/toxsci/kfl044.

(6) Bohne, V. J. B.; Lundebye, A.-K.; Hamre, K. Accumulation and depuration of the synthetic antioxidant ethoxyquin in the muscle of atlantic salmon (Salmo salar l.). *Food Chem. Toxicol.* **2008**, *46* (5), 1834–1843. https://doi.org/10.1016/j.fct.2008.01.028.

(7) Lundebye, A.-K.; Hove, H.; Måge, A.; Bohne, V. J. B.; Hamre, K. Levels of synthetic antioxidants (ethoxyquin, butylated hydroxytoluene and butylated hydroxyanisole) in fish feed and commercially farmed fish. *Food Addit. Contam. A* **2010**, *27* (12), 1652–1657. https://doi.org/10.1080/19440049.2010.508195.

(8) Negreira, N.; Regueiro, J.; Valdersnes, S.; Berntssen, M. H. G.; Ørnsrud, R. Comprehensive characterization of ethoxyquin transformation products in fish feed by traveling-wave ion mobility spectrometry coupled to quadrupole time-of-flight mass

spectrometry. *Anal. Chim. Acta* **2017**, *965*, 72–82. https://doi.org/10.1016/j.aca.2017.02.021.

(9) Merel, S.; Regueiro, J.; Berntssen, M. H. G.; Hannisdal, R.; Ørnsrud, R.; Negreira, N. Identification of ethoxyquin and its transformation products in salmon after controlled dietary exposure via fish feed. *Food Chem.* **2019**, *289*, 259–268. https://doi.org/10.1016/j.foodchem.2019.03.054.

(10) EFSA Panel on Additives and Products or Substances used in Animal Feed. Scientific opinion on the safety and efficacy of ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) for all animal species. *EFSA J.* **2015**, *13* (11), 4272–4329. https://doi.org/10.2903/j.efsa.2015.4272.

(11) Błaszczyk, A.; Augustyniak, A.; Skolimowski, J. Ethoxyquin: an antioxidant used in animal feed. *Int. J. Food Sci.* **2013**, *2013*, 1–12. https://doi.org/10.1155/2013/585931.

(12) Commission Implementing Regulation (EU) 2017/962 of 7 June 2017 suspending the authorisation of ethoxyquin as a feed additive for all animal species and categories. *OJ* **2017**, *L 145*, 13–17.

(13) Aoki, Y.; Kotani, A.; Miyazawa, N.; Uchida, K.; Igarashi, Y.; Hirayama, N.; Hakamata, H.; Kusu, F. Determination of ethoxyquin by high-performance liquid chromatography with fluorescence detection and its application to the survey of residues in food products of animal origin. *J. AOAC Int.* **2010**, *93* (1), 277–283.

(14) He, P.; Ackman, R. G. Residues of ethoxyquin and ethoxyquin dimer in ocean-farmed salmonids determined by high-pressure liquid chromatography. *J. Food Sci.* **2000**, *65* (8), 1312–1314. https://doi.org/10.1111/j.1365-2621.2000.tb10603.x.

(15) Dahle, H. K.; Skaare, J. U. Gas chromatographic determination of ethoxyquin in feed and food products. *J. Agric. Food Chem.* **1975**, *23* (6), 1093–1095.

(16) Skaare, J. U.; Dahle, H. K. Gas chromatographic-mass spectrometric studies of ethoxyquin in some organic solvents. *J. Agric. Food Chem.* **1975**, *23* (6), 1091–1093.

(17) de Koning, A. J.; van der Merwe, G. Determination of ethoxyquin and two of its oxidation products in fish meal by gas chromatography. *Analyst* **1992**, *117* (10), 1571–1576. https://doi.org/10.1039/AN9921701571.

(18) Rodríguez-Gómez, R.; Vandeput, M.; Zafra-Gómez, A.; Kauffmann, J.-M. Liquid chromatography-electrochemical detection for the determination of ethoxyquin and its dimer in pear skin and salmon samples. *Talanta* **2018**, *177*, 157–162. https://doi.org/10.1016/j.talanta.2017.08.078.

(19) Guo, L.; Xie, M.-Y.; Yan, A.-P.; Wan, Y.-Q.; Wu, Y.-M. Simultaneous determination of five synthetic antioxidants in edible vegetable oil by GC-MS. *Anal. Bioanal. Chem.* **2006**, *386* (6), 1881–1887. https://doi.org/10.1007/s00216-006-0738-1.

(20) Lehmann, W. D. A timeline of stable isotopes and mass spectrometry in the life sciences. *Mass Spectrom. Rev.* **2017**, *36* (1), 58–85. https://doi.org/10.1002/mas.21497.

(21) Ciccimaro, E.; Blair, I. A. Stable-isotope dilution LC–MS for quantitative biomarker analysis. *Bioanalysis* **2010**, *2* (2), 311–341. https://doi.org/10.4155/bio.09.185.

(22) Thorisson, S.; Gunstone, F. D.; Hardy, R. Some oxidation products of ethoxyquin including those found in autoxidising systems. *Chem. Phys. Lipids* **1992**, *60* (3), 263–271.

(23) ISO 11843-2:2000. Capability of Detection - Part 2: Methodology in the Linear Calibration Case.

(24) Zhou, W.; Yang, S.; Wang, P. G. Matrix effects and application of matrix effect factor. *Bioanalysis* **2017**, *9* (23), 1839–1844. https://doi.org/10.4155/bio-2017-0214.

(25) Elliott, Jr., I. W.; Yates, P. The structure of "acetone anil", 2,2,4-trimethyl-1,2-dihydroquinoline. *J. Org. Chem.* **1961**, *26* (4), 1287–1289. https://doi.org/10.1021/jo01063a602.

(26) Skraup, Z. H. Eine Synthese des Chinolins. *Monatsh. Chem. Verw. Tl.* **1880**, *1* (1), 316–318. https://doi.org/10.1007/BF01517073.

(27) Doebner, O.; von Miller, W. Über eine dem Chinolin homologe Base. *Ber. Dtsch. Chem. Ges.* **1881**, *14* (2), 2812–2817. https://doi.org/10.1002/cber.188101402258.

(28) de Koning, A. J.; Milkovitch, S. The synthesis of a number of ethoxyquin analogues and their evaluation as antioxidants in fish oil. *Fat Sci. Technol.* **1991**, *93* (10), 378–382. https://doi.org/10.1002/lipi.19910931004.

(29) Tu, S.; Cui, Y.; Chen, X.; Ye, L.; Yin, Y. Method for producing ethoxyquin. People's Republic of China patent CN 105085392A, November 25, 2015.

(30) Forrest, T. P.; Dauphinee, G. A.; Miles, W. F. On the mechanism of the Doebner-Miller reaction. *Can. J. Chem.* **1969**, *47* (11), 2121–2122. https://doi.org/10.1139/v69-345.

(31) Denmark, S. E.; Venkatraman, S. On the mechanism of the Skraup-Doebner-von Miller quinoline synthesis. *J. Org. Chem.* **2006**, *71* (4), 1668–1676. https://doi.org/10.1021/jo052410h.

(32) Skaare, J. U.; Henriksen, T. Free radical formation in the antioxidant ethoxyquin. *J. Sci. Food Agr.* **1975**, *26* (11), 1647–1654. https://doi.org/10.1002/jsfa.2740261105.

(33) He, P.; Ackman, R. G. Purification of ethoxyquin and its two oxidation products. *J. Agric. Food Chem.* **2000**, *48* (8), 3069–3071. https://doi.org/10.1021/jf991193g.

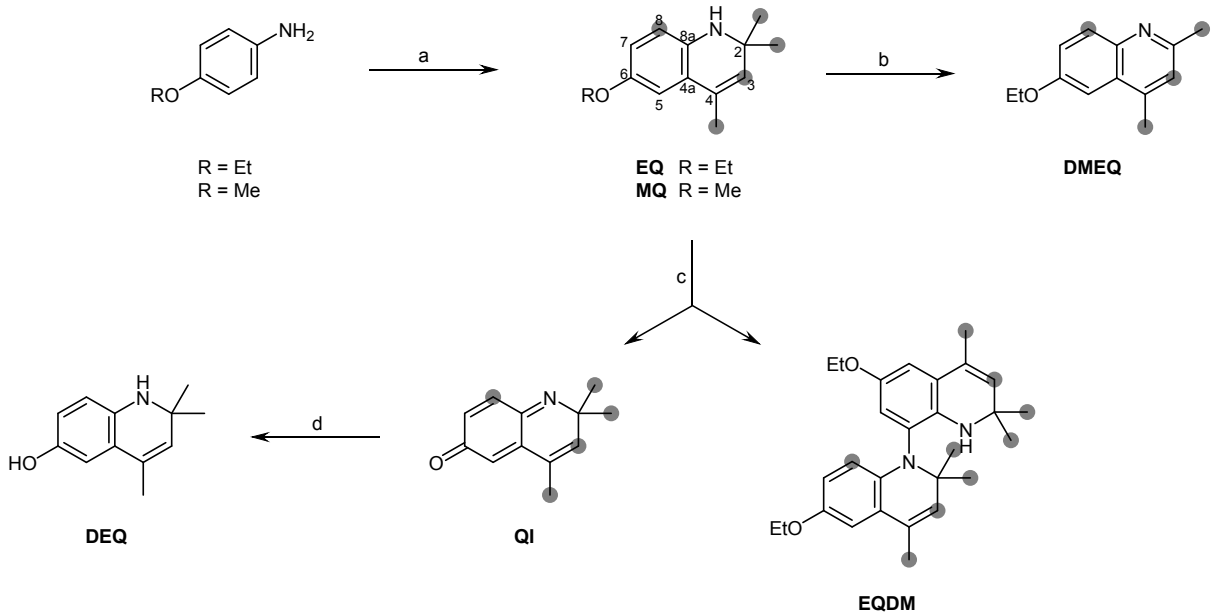
(34) He, P.; Ackman, R. G. HPLC determination of ethoxyquin and its major oxidation products in fresh and stored fish meals and fish feeds. *J. Sci. Food Agr.* **2000**, *80* (1), 10–16. https://doi.org/10.1002/(SICI)1097-0010(20000101)80:1<10::AID-JSFA478>3.0.CO;2-T.

(35) Anastassiades, M.; Lehotay, S. J.; Stajnbaher, D.; Schenck, F. J. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. *J. AOAC Int.* **2003**, *86* (2), 412–431.

514 (36) EU Reference Laboratory for Single Residue Methods (EURL-SRM), CVUA Stuttgart.
515 Analysis of ethoxyquin and its metabolites in fish using the QuEChERS method.
516 October 5, 2016.

517 **Figures**

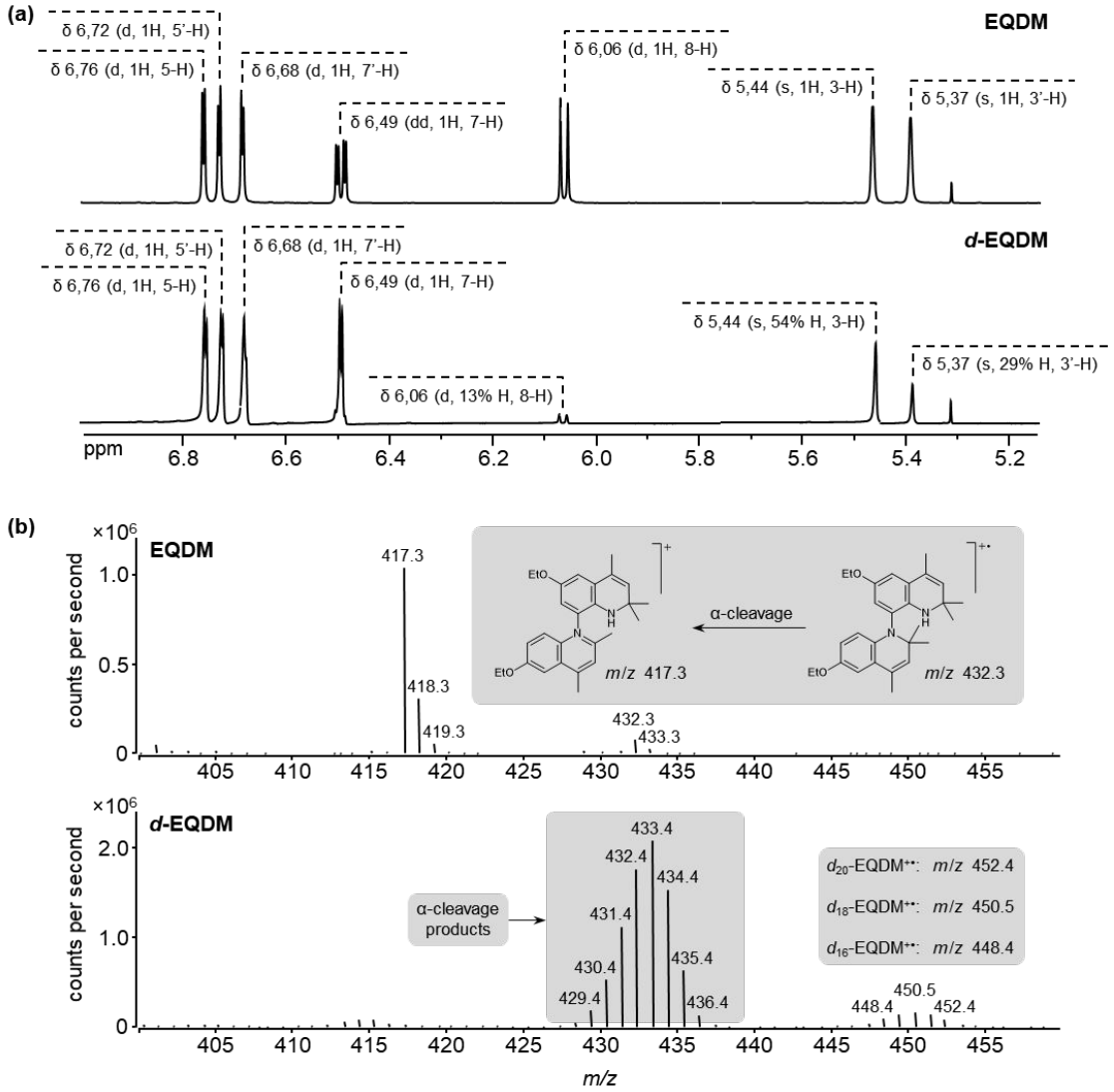
518




519

520 **Figure 1**

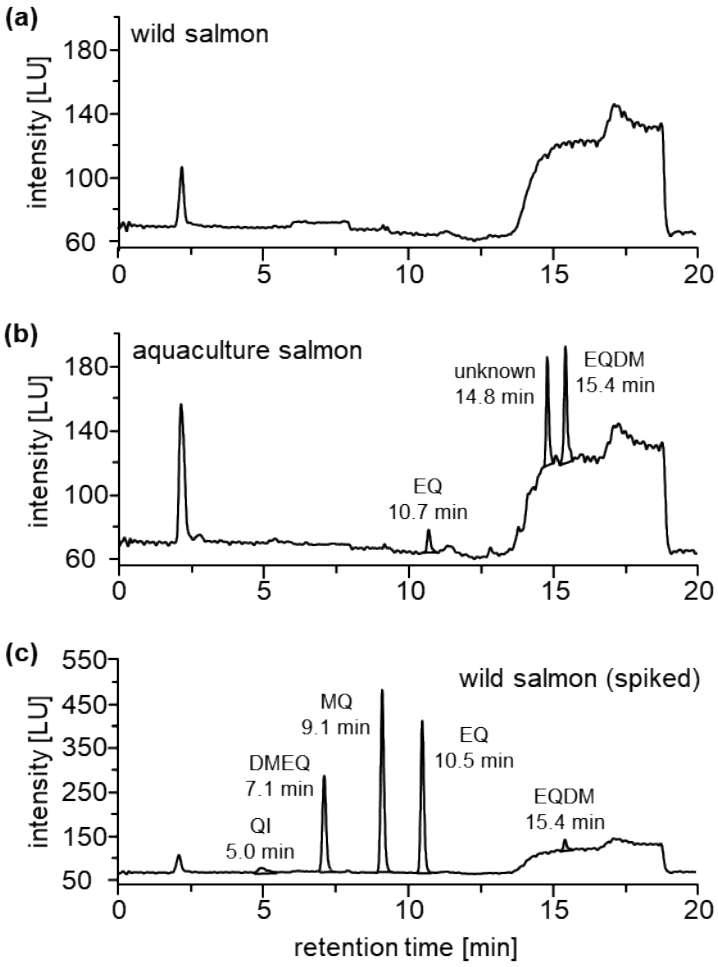
521 Synthesis of (deuterated) ethoxyquin and its (deuterated) metabolites. Reagents and conditions:
522 a) iodine, ($d_6$-)acetone, toluene, 110 °C, 18 h; b) 180 °C, 1.5 h; c) $(NH_4)_2Fe(SO_4)_2$ (aq.), *tert*-
523 butyl hydroperoxide (70%, aq.), EtOH, 0 °C, 18 h; d) ascorbic acid (0.1%, aq.), rt. Carbons
524 marked with an ● are potential deuteration sites.




525

526 **Figure 2**

527 Characterization of the reaction product obtained from the synthesis of deuterated EQDM (*d*-
528 EQDM). (a) Comparison of $^{1}$H NMR spectra of EQDM and *d*-EQDM reveals that deuteration
529 also occurs at position C-8. Apart from reduced intensity of the corresponding 8-H signal at δ
530 6.06 ppm the multiplicity of the signal at δ 6.49 ppm belonging to the adjacent 7-H is reduced
531 from a doublet of doublets to a simple doublet. (b) GC-MS analysis of both compounds
532 demonstrates that the reaction product obtained from the synthesis of *d*-EQDM does not only
533 consist of one isotopomer but represents a complex mixture of deuterated analogues including
534 $d_{16}$- to even $d_{20}$-EQDM. However, $d_{18}$-EQDM is the main component as the corresponding
535 signal at *m/z* 450.5 in the mass spectrum shows the highest intensity.




536

537 **Figure 3**

538 Analysis of commercial fish fillets using LC-FLD. Wild salmon (a) is clearly distinguishable
539 from breeding salmon (b) due to the absence of peaks at $t_R$ 10.7 min (EQ), $t_R$ 15.4 min (EQDM)
540 and a further peak at $t_R$ 14.8 min (unknown compound). The chromatogram in (c) was obtained
541 from wild salmon spiked with 80 µg/kg QI, DMEQ, EQ, EQDM and 100 µg/kg MQ.

542 **Tables**

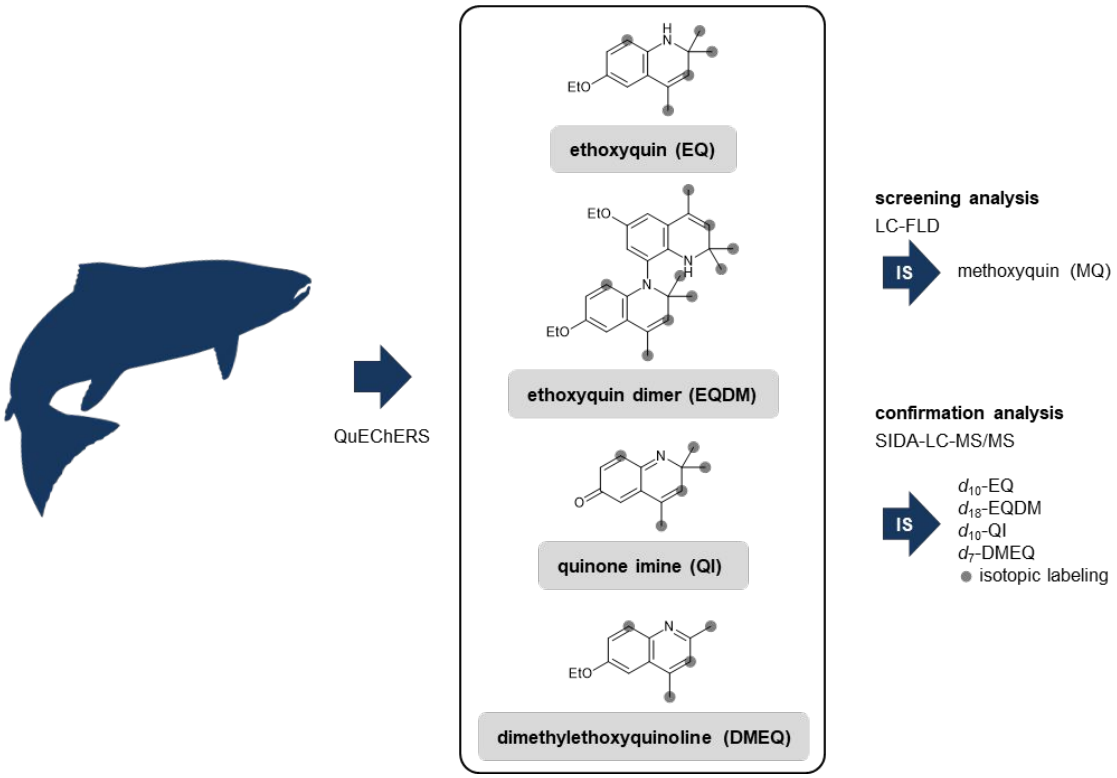
543 **Table 1**

544 Validation parameters obtained for both LC-FLD and SIDA-LC-MS/MS of EQ and its metabolites in wild salmon samples.

| | **LC-FLD[a]** | | | | | **SIDA-LC-MS/MS[a]** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $R^2$ | LOD (µg/kg) | LOQ (µg/kg) | RR (%) | P (%) | $R^2$ | LOD (µg/kg) | LOQ (µg/kg) | RR (%) | P (%) |
| QI | ≥ 0.99 | 17.2 | 61.0 | 26.5 - 34.8 | 13.7 | ≥ 0.99 | 12.7 | 45.9 | 78.3 - 85.3 | 11.5 |
| DMEQ | | 2.7 | 10.2 | 91.3 - 92.9 | 9.7 | | 2.2 | 8.4 | 98.5 - 101.0 | 1.1 |
| EQ | | 6.8 | 25.6 | 100.0 - 103.5 | 5.3 | | 8.6 | 31.9 | 91.9 - 100.0 | 8.1 |
| EQDM | | 12.2 | 44.1 | 82.2 - 94.5 | 14.4 | | 1.1 | 4.0 | 95.6 - 103.1 | 4.6 |

545 [a] $R^2$: coefficient of determination, LOD: limit of detection, LOQ: limit of quantification, RR: recovery range, P: intraday precision.

546 **Table of Contents Graphics**




547