EURL-SRM - Analytical Observations Report

concerning the following…

- **Compound(s)**: Ethoxyquin, Ethoxyquin dimer, EQI, DHEQ
- **Commodities**: Fish and seafood
- **Extraction Method(s)**: QuEChERS, addition of ascorbic acid
- **Instrumental analysis**: LC-MS/MS, ESI (pos.)

Analysis of Ethoxyquin and its Metabolites in Fish Using the QuEChERS Method

Version 1 (last update: 10.05.2016)

Background information / Initial Observations:

Ethoxyquin (EQ) is a quinoline-based **antioxidant** with various food and feed related applications. It was originally used as a rubber additive to prevent isoprene oxidation and elasticity loss[1].

Feed related applications with indirect food relevance include the following:

- In the EU register on feed additives[2] EQ is listed as **E324.** It is currently **authorized as an antioxidant in feed** for all animal species with the maximum content of EQ alone or in combination with BHA (E 320) and/or BHT (E 321) being 150 mg/kg (in dog feed EQ must additionally not exceed 100 mg/kg)[3]. Similar provisions apply in the US[4]. Here EQ is among others used to treat **dried forage crops** such as corn, wheat and oats to preserve them from oxidative loss of vitamin E and carotenes. In a recent proposal the applicant proposed a maximum EQ content for feed in general of 50 mg/kg complete feed and for the combination of EQ with BHA and/or BHT of 150 mg/kg.
- The UN International Maritime Organization (IMO) prescribes the treatment of **fish meal**[5,6] with EQ at concentrations not less than 400 mg/kg (1000 mg/kg if the fat content exceeds 12 %)[7].

[1] A. J. de Koning, "The antioxidant ethoxyquin and its analogues: a review," International Journal of Food Properties, vol. 5, no. 2, pp. 451–461, 2002

[2] Annex I of Regulation (EC) No1831/2003: **http://ec.europa.eu/food/food/animalnutrition/feedadditives/comm_register_feed_additives_1831-03.pdf** (European Union Register of Feed Additives)

[3] List of the authorised additives in feedingstuffs published in application of Article 9t (b) of Council Directive 70/524/EEC concerning additives in feedingstuffs (2004/C 50/01)

[4] http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=573.380

[5] **http://www.iffo.net/default.asp?contentID=717**

[6] **http://www.iffo.net/downloads/Research%20reports/IFOMA%201978-2001/1993/Uses%20of%20ethoxyquin%20and%20alternatives%201993-7.pdf**

[7] 'Stabilization of fishmeal shall be achieved to prevent spontaneous combustion by effective application: of between 400 and 1000 mg/kg (ppm) ethoxyquin, or liquid BHT (butylated hydroxy toluene); or between 1000 and 4000 mg/kg (ppm) BHT in powder form at the time of production '(IMO, 2014) and that: 'fish scrap of fish meal shall contain at least 100ppm of antioxidant (ethoxyquin) at the time of consignment' in UN (United Nations), 2014. Recommendations on the Transport of Dangerous Goods, Model regulations, Volume I, 18th revised edition. United Nations Publications

- This application applies worldwide. Due to the high degree of unsaturation of fish oil, dried fishmeal is very prone to autocatalytic oxidation by radical chain reaction mechanisms. This lipid auto-oxidation is highly exothermic and if uncontrolled can even lead to spontaneous combustion of the material during long storage and shipping periods.
- Within the EU EQ is allowed to be used at levels up to 2000 mg/kg in **salmon and trout feed** based on *Phaffia Rhodozyma,* a yeast variety rich in astaxanthin (the carotenoid naturally occurring in crustaceans and responsible for the reddish color of these fishes).
- In the US EQ is allowed to be added to **Aztec marigold** and **dried algae meals.** Both are rich in carotenoid pigments and are thus **added to feed for hens** with the aim to intensify the color of the egg yolks produced. The US tolerance for EQ in these cases is set at 3000 mg/kg.

The use of EQ as animal feed additive often leads to residues in food of animal origin such as poultry meat and eggs[8] as well as in farmed fish[9] and shrimp[10]. A comprehensive review article concerning the use of EQ in feed was recently published by Alina Błaszczy et. al.[11]

In the **food area** EQ is not any more authorized in the EU but is still in use elsewhere. It is used for the treatment of **pears** and, to a lesser extent, apples to reduce superficial scald (formation of brown spots). Fruits are typically dipped into a solution containing EQ and/or wrapped with EQ-impregnated fruit wraps. Pre-harvest applications have been reported for apples. An additional food-related use of EQ concerns spices such as **chili**, **paprika** and **curcuma powder.** Here EQ is applied as an additive to prevent oxidative discoloration of carotenoid pigments during storage (US tolerance 100 mg/kg)[12]. According to the US regulations EQ is also permitted as preservative of **uncooked fat of meat from animals** with a tolerance of 5 mg/kg (3 mg/kg for poultry fat and liver).
In the EU EQ is not allowed to be used as a preservative in food products. The authorisation of EQ as "pesticide" was initially withdrawn in 2009[13]. A new application was submitted followed by a reevaluation, during which a number of concerns have been identified including the inability to set a residue definition for EQ and its metabolites due to insufficient data submission by the applicant. In 2011[14] the commission finally decided not to include EQ in Annex I of Reg. 91/414/EEC[15]. The **grace period for EQ use ended in September 2012**. In January 2015 the MRL for EQ in pears dropped from 3 mg/kg to 0.05 mg/kg[16].

8 **http://onlinelibrary.wiley.com/doi/10.1002/jsfa.2740331213/pdf**

9 **http://lib3.dss.go.th/fulltext/Journal/Journal%20of%20food%20science/2000%20v.65/no.8/jfsv65n8p1312-1314ms20000212%5B1%5D.pdf**

10 **http://www.iffo.net/default.asp?contentID=813**

11 Ethoxyquin: An Antioxidant Used in Animal Feed ; Alina Błaszczyk, Aleksandra Augustyniak and Janusz Skolimowski; International Journal of Food Science- Volume 2013 (2013), Article ID 58593 (**http://www.hindawi.com/journals/ijfs/2013/585931/**)

12 **https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=172.140**

13 Commission Decision 2008/941/EC of 8 December 2008 concerning the non-inclusion of certain active substances in Annex I to Council Directive 91/414/EEC and the withdrawal of authorisations for plant protection products containing these substances. OJ L 335, 13.12.2008, p. 91

14 2011/143/EU: Commission Decision of 3 March 2011 concerning the non-inclusion of ethoxyquin in Annex I to Council Directive 91/414/EEC

15 now Reg. (EC) No 1107/2009

16 Reg. (EU) No 703/2014 (applicable from 16/01/2015)

EQ converts into a multitude of transformation products, some of which also exhibit antioxidant properties themselves. The following table shows a compilation of some important EQ-related compounds:

Metabolite	Information
Ethoxyquin dimer (EQDM) = 1,8´-dimer	Information on antioxidant activity of EQDM is contradictive[17,18]. Considerably longer half-life than EQ. Formed in EQ-treated fishmeal. Found in e.g. Atlantic salmon, halibut, trout, cod, caviar[18,19,20]. Also found in pears[11]. Among the 4 main EQ-related compounds found in feed materials and in animals[18]
N-N´- Ethoxyquin Dimer	Found in pears[11]
Ethoxyquin quinone imine (EQI or QI) = 2,2,4-Trimethylquinolinon = 2,6-Dihydro-2,2,4-trimethyl-6-quinolone	Antioxidant properties[17]. Formed in fishmeal treated with EQ. Found in Atlantic salmon. Among the 4 main EQ-related compounds found in feed materials and in animals [18]
EQI N-oxide	Among the 4 main EQ-related compounds found in feed materials and in animals [18]
Deethylated EQ (DEQ) = 6-hydroxy-2,2,4-trimethyl-1,2-dihydroquinoline = 2,2,4-trimethyl-1H-quinolin-6-ol	Antioxidant properties[17]. Reported in Atlantic salmon at much lower concentrations compared to EQDM and EQ[18,20]
Dihydroethoxyquin (DHEQ) = 6-Ethoxy-2,2,4-trimethyl-1,2,3,4-tetrahydroquinoline	Found in pears[11]
Dehydrodemethylethoxyquin (DHMEQ) 2,4-dimethyl-6-ethoxyquinoline	Among the 4 main EQ-related compounds found in feed materials and in animals [18]. Reported in pears[11]
Methylethoxyquin (MEQ) = 6-Ethoxy-1,2,2,4-tetramethyl-1,2-dihydroquinoline	Found in pears[11]

According to a recent EFSA reasoned opinion **EQI** shows structural alerts for mutagenicity, carcinogenicity and DNA binding. However, no conclusion on the absence of genotoxicity of EQI was possible. For **EQDM** it is stated that its genotoxic profile reflects that of EQ. By specification EQ polymers should not exceed 8% of the additive.
The formulation byproduct **p-phenetidine**, which by specification should not exceed 3 % of the additive, is a recognized possible mutagen and thus also considered toxicologically critical.

The main **EQ degradation products and byproducts can be useful as indicators of EQ treatments**, especially in those cases where routine multiresidue procedures lead to an excessive decomposition of the parent compound at levels below the LOQ (e.g. analysis of EQ in pears via

17 **http://www.iffo.net/system/files/Ethoxyquin%201987-2.pdf**

18 **http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/4272.pdf**

19 Ortelli et al.: **http://ge.ch/dares/SilverpeasWebFileServer/ORTELLI_poster_Ethoxyquin_Rafa2011_vf.pdf?ComponentId=kmelia704&SourceFile=1325690106231.pdf&MimeType=application/pdf&Directory=Attachment/Images/** (poster presentation)

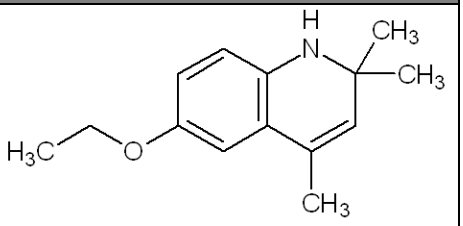
20 Lundebye et al. in Levels of synthetic antioxidants (ethoxyquin, butylated hydroxytoluene and butylated hydroxyanisole) in fish feed and in commercially farmed fish. Food Additives and Contaminants, 27, 1652–1657 (2010).

QuEChERS). The most prominent among the EQ-metabolites would potentially qualify for a future residue definition.

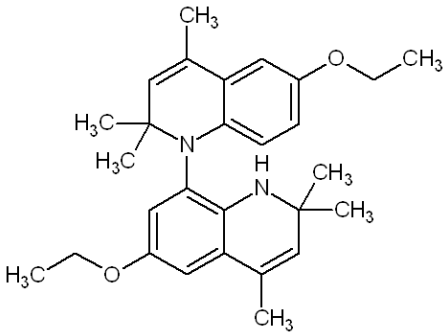
Analytical observations concerning EQ analysis in pears were presented in a separate EURL-SRM Analytical Observations document[21]. It was observed that applying the QuEChERS method on fruits and vegetables, recovery rates from commodities having poor antioxidative potential, such as pears, apples, cucumbers and cereals were low. In contrast, nearly quantitative recoveries were typically obtained from commodities exhibiting strong antioxidative potential, such as citrus fruit and berries. Losses were also observed during the storage of frozen homogenates and in QuEChERS extracts. The addition of antioxidants such as ascorbic acid (AA) prior to extraction was shown to minimize oxidative losses leading to nearly quantitative EQ recoveries. To tackle the problem of EQ losses during sample comminution, ascorbic acid was added prior to sample milling. By doing this, yields of incurred EQ residues could be increased dramatically. Despite the non-authorization of EQ containing PPPs within the EU (grace period ended in September 2012), still, analyses of 26 pear samples from the market in 2014 revealed that 3 pear samples from Italy contained EQ residues indicating an illegal use of EQ.

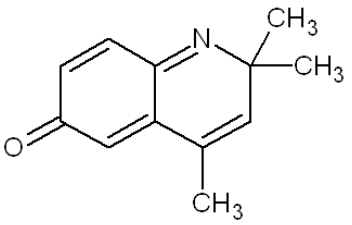
Compound profile:

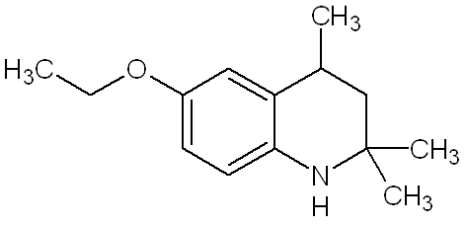
Some information of the EQ metabolites that have been studied within the frame of this work are compiled below:

Ethoxyquin (EQ) 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline			
Parameter	**Value**	**Notes**	
PKa	4.56	at 22 °C (weak base)	
LogP	3.39	at pH7 (intermediate polarity)	
Water solubility	60 mg L^{-1}	at 20 °C	
Hydrolytic behavior	pH 5: 3.7 days pH 7: 6.7 days pH 9: 9.3 days	at 25 °C sensitive to strong acids	
Chemical behavior, other	Tends to oxidize and to polymerize; protect from light, air and heat		
Residue definition EU	Food of plant and animal origin (except fish): Ethoxyquin (F) ; Fish: not regulated Feed: Ethoxyquin (max levels of EQ as additive refer to the combination of EQ/BHA/BHT)		
Approved in…	As pesticide: Not approved within the EU but approved elsewhere As feed additive: Approved within the EU Listed as E324 in the EU. Allowed at levels up to 2000 mg/kg in salmon and trout feed based on an astaxanthin-containing yeast variety (for color protection). Prescribed by IMO for use on fishmeal to prevent oxidation and potential auto-inflammation of fishmeal during long-term transport (see main text)		

[21] EURL-SRM, "Improvement of Ethoxyquin Recoveries by QuEChERS Through the Addition of Ascorbic Acid", Version 2 (last update: 31.03.2015); http://www.crl-pesticides.eu/userfiles/file/EurlSRM/EurlSrm_Observations_Ethoxyquin_V2.pdf

Ethoxyquin dimer (EQDM) (CAS: 74681-77-9) = 1,8'-Di(1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline)			
Parameter	**Value**	**Notes**	
PKa*	4.6	Weakly basic On the secondary amine	
LogP*	6.22 (at pH>6.5)	LogD is pH dependent At pH4: ~5.5 At pH3: ~4.8	
Water solubility	No data		
Chemical behavior	No data		
Residue definition EU	Not included		

Ethoxyquin quinone imine (EQI or QI) (CAS: 4071-18-5) = 2,6-Dihydro-2,2,4-trimethyl-6-quinolone = 2,2,4-Trimethylquinolinon			
Parameter	**Value**	**Notes**	
Pka*	5.3	Weakly basic On imine	
LogP*	2.36 (at pH>7)	LogD is pH dependent At pH4: ~1 At pH3: ~0	
Water solubility	No data		
Chemical behavior	Reported being an antioxidant too[22]		
Residue definition EU	Not included		

Dihydroethoxyquin (DHEQ) 6-Ethoxy-2,2,4-trimethyl-1,2,3,4-tetrahydroquinoline			
Parameter	**Value**	**Notes**	
PKa*	5.8	Weakly basic On the secondary amine	
LogP*	3.11 (at pH>8)	LogD is pH dependent At pH4: ~1.5 At pH3: ~1.2	
Water solubility	No data		
Hydrolytic behavior	No data		
Residue definition EU	Not included		

*pKa and logP/logD values computed by chemicalize.org

[22] http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/4272.pdf

Table 1: Materials used in the test (excluding typical QuEChERS chemicals)[23]

Substance	Purity	CAS	Source
Ethoxyquin (EQ)	98.5%	CAS 91-53-2	Dr. Ehrenstorfer
2,2,4-Trimethylquinolinon (EQI)	98.6%	CAS 4071-18-5	HPC Standards GmbH
Ethoxyquindimer (EQDM)	99.2%	CAS 74681 77-9	
Dihydroethoxyquin (DHEQ)	unknown	CAS 16489-90-0	Sigma Aldrich
Chlorpyrifos D10	97.0%	CAS 285138-81-0	Dr. Ehrenstorfer
L-Ascorbic acid	reagent gr.	CAS 50-81-7	Sigma Aldrich
Sodium ascorbate	≥98 %	CAS 134-03-2	

Table 2 Instrumentation details

LC	WATERS Acquity UPLC		
MS/MS	ABSCIEX API 4000 Q-Trap, run in ESI positive mode		
MRMs	218.2/148.0; 218.2/160.1; 218.2/174.1		
Column	Acquity BEH C18, 2.1x100 mm, 1.7 µm		
Pre-column	Acquity BEH C18, 2.1x5 mm, 1.7 µm		
Mobile Phase	A: 5 mmol NH_4formate in purified water + 5% methanol B: 5 mmol NH_4formate in methanol		
Gradient	**Time** (min)	**Mobile Phase A** (%)	**Mobile Phase B** (%)
	0	95	5
	0.5	60	40
	2	10	90
	5	10	90
	5.1	95	5
	10	95	5
Flow	0.35 mL min^{-1}		
Injection volume	2 µL, partial loop with needle overfill		
Column temperature	40°C		

23 **Disclaimer**: Names of companies are given for the convenience of the reader and do not indicate any preference by the EURL-SRM towards these companies and their products.

Table 3 MS/MS parameters (exemplary for ABI 4000 Qtrap)

Compound	Intensity ranking	Q 1	Q 3	DP	CE	CXP
EQ	1	**218**	**148**	86	31	8
	2	**218**	**160**	86	47	8
	2	**218**	**174**	86	43	10
EQDM	1	**433**	**216**	101	37	12
	2	**433**	**375**	101	43	4
	2	**433**	**188**	101	59	10
EQI	1	**188**	**145**	66	37	8
	2	**188**	**173**	66	23	10
	3	**188**	**143**	66	51	8
DHEQ	1	**220**	**91**	61	55	4
	2	**220**	**65**	61	83	2
	3	**220**	**136**	61	39	6
Chlorpyrifos D10	-	**360**	**199**	66	23	12

Analysis of EQ in Atlantic salmon

Preliminary considerations and experiments:

The varying lipid content of salmon was of concern as QuEChERS recoveries of highly lipophilic compounds (such as EQDM with a $\log K_{ow}$ of 6.6[24]), are strongly influenced by the lipid content of the samples. Lipids are not well soluble in acetonitrile, thus forming a separate phase into which lipophilic analytes tend to partition. According to the packaging label, the wild salmon samples used for the recovery studies contained 6% lipids. According to Hamilton et al.[25] farmed salmon shows much higher total lipid levels than wild salmon (16.6% versus 6.4% on average). Apart of the seasonal fluctuations of the fat content fat, is not always uniformly distributed throughout the flesh. Analysis of the entire fish or only fillets also makes a difference, as much of the fat is localized below the skin.

Partitioning losses can be reduced by reducing the analytical portion size. At the same time, however, sub-sampling variability increases. As a compromise, it was decided to use 5 g rather than 10 g analytical portions of salmon.

Wild and farmed salmon samples were homogenized cryogenically and tested for the absence of EQ, EQDM, EQI or DHEQ. Unfortunately all farmed salmon samples tested contained EQ and EQDM and were thus considered unsuitable for recovery experiments. To avoid cross-effects between the components, it was decided to spike all components individually.

[24] computed by the ACD Labs Software

[25] Hamilton, M. C., R. A. Hites, S. J. Schwager, J. A. Foran, B. A. Knuth, und D. O. Carpenter. „Lipid Composition and Contaminants in Farmed and Wild Salmon." Environmental Science and Technology, 2005: pp. 8,622-8,629

Recovery experiments on wild salmon

Wild Atlantic salmon (fillets) were cryogenically milled in two ways:

(a) After addition of 1g ascorbic acid (AA) per 100 g sample
(b) Directly (without AA-addition)

The homogenates containing AA were spiked and extracted normally (a).
The homogenates not containing AA were either extracted following addition of AA to the sample portions (1 mL AA-solution[26]) just prior to extraction (b1) or normally (b2).
To protect the final extracts of (b2) from potential oxidation 50 µL of AA-mix[27]/mL extract was added).

5 g of frozen homogenates were weighed into 50 mL extraction tubes. Spiking was done at 0.2 mg/kg level with either EQ or EQDM or EQI or DHEQ (addition of 100 µL of standard solutions of c=10 µg/mL acetonitrile in each case). Following spiking and AA-addition (only for (b1)) water was added to adjust the volume to ca. 10 mL (6.5 mL for (a) and (b2) and 5.5 mL for (b1). Extraction was conducted following addition of 10 mL acetonitrile and mechanical shaking for 15 min. Phase separation was induced by adding the QuEChERS salts mixture (as described in EN-15662) followed by a 1 min shaking and centrifugation. The final extract was cleaned-up via dSPE using C18 sorbent (25 mg/mL extract). The flow-chard of the procedure can be found in the Annex.

From previous experience it was known that chlorpyrifos-D10 ($logK_{ow}$ 4.7) experiences partitioning losses into the lipid phase. These losses increase as the amount of lipids present in the analytical portion increases. Any recovery loss of the internal standard translates in a positive shift of the calculated recoveries of all analytes. Thus, additionally a second internal standard -diuron-D6- which is more polar ($logK_{ow}$ 2.46), was tested. All experiments were conducted in quintuplicate. EQ, EQI and DHEQ were validated at two levels each (0.025 µg/mL and 0.1 µg/mL) and EQDM at the 0.1 mg/kg level. The results of this recovery study are summarized in Table 4 and Figure 1.

[26] AA-solution: aqueous solution containing 0.3 g/mL AA

[27] AA-mix: aqueous solution containing 0.075 g/mL AA and 0.075 g/mL sodium-ascorbate

Table 4: Validation data of EQ, EQDM, DHEQ and EQI from wild Atlantic salmon using chlorpyrifos-D10 as internal standard

Spiking Level [mg/kg]	Extraction	A	B	C	D	E	Mean	RSD
EQ								
0.025	**No AA***	88	83	81	83	91	**85**	**4.7**
	AA prior to cryo-milling of blank	99	107	98	96	100	**100**	**3.9**
	AA-Mix to anal. portion prior to QuEChERS	111	109	112	107	102	**108**	**3.6**
0.1	**No AA***	79	72	70	63	70	**71**	**8.2**
	AA prior to cryo-milling of blank	103	108	103	103	97	**103**	**3.7**
	AA-Mix to anal. portion prior to QuEChERS	103	99	107	104	102	**103**	**3.0**
EQDM								
0.1	**No AA***	113	80	41	110	91	**98**	**16.2**
	AA prior to cryo-milling of blank	77	74	80	82	84	**79**	**5.1**
	AA-Mix to anal. portion prior to QuEChERS	82	89	92	87	81	**86**	**5.3**
DHEQ								
0.025	**No AA***	70	70	75	77	71	**73**	**4.5**
	AA prior to cryo-milling of blank	102	97	101	99	99	**100**	**1.9**
	AA-Mix to anal. portion prior to QuEChERS	99	105	101	98	91	**99**	**4.9**
0.1	**No AA***	84	69	72	74	80	**76**	**8.0**
	AA prior to cryo-milling of blank	106	103	98	104	103	**103**	**2.8**
	AA-Mix to anal. portion prior to QuEChERS	97	92	99	100	107	**99**	**5.3**
EQI								
0.025	**No AA***	19	20	18	19	17	**19**	**5.8**
	AA prior to cryo-milling of blank	98	95	94	97	100	**97**	**2.4**
	AA-Mix to anal. portion prior to QuEChERS	101	94	100	103	101	**100**	**3.7**
0.1	**No AA***	11	13	10	9	14	**11**	**18.6**
	AA prior to cryo-milling of blank	96	86	98	100	98	**96**	**5.9**
	AA-Mix to anal. portion prior to QuEChERS	111	102	99	97	99	**102**	**5.4**

*AA added to the final extract (see text)

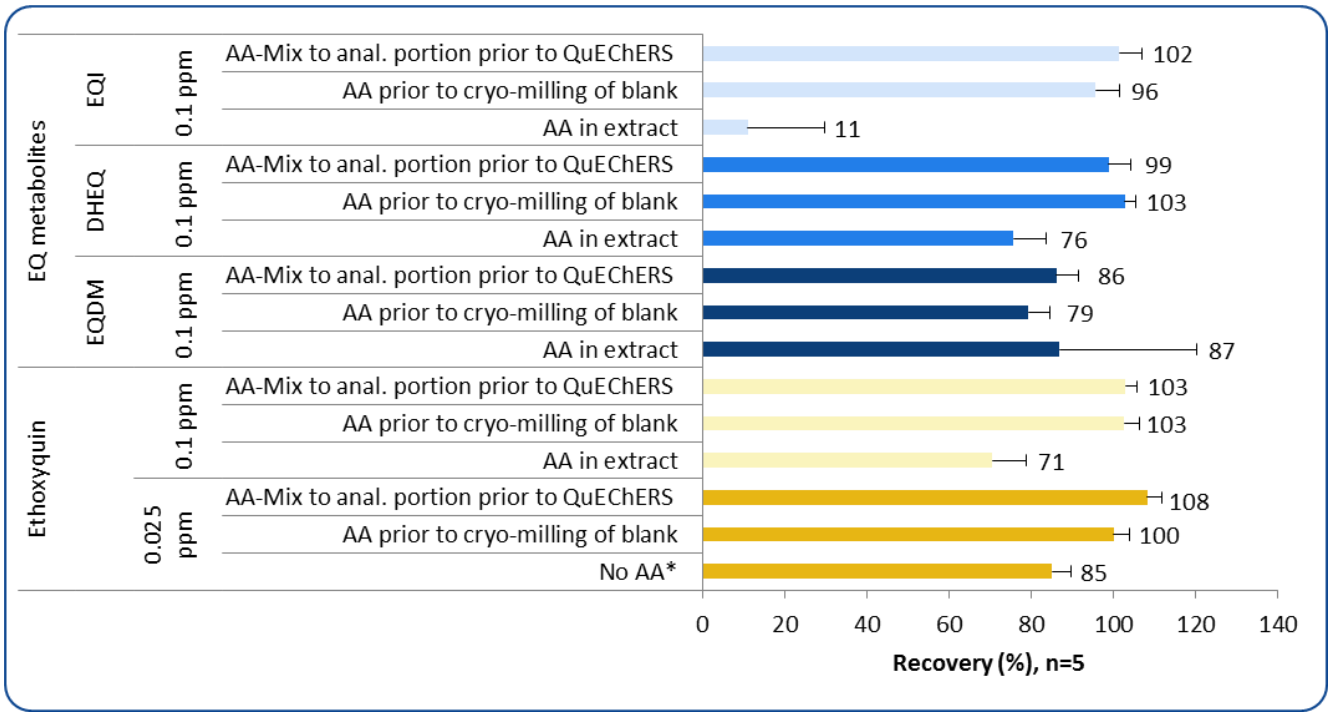


Figure 1 Recoveries of EQ and its metabolites in salmon (level 0.1 mg/kg, n=5)

In contrast to previous experiments using pears, the impact of AA-addition on EQ recovery rates was more moderate with the average recoveries rising by 32% (from 71 to 103%) at the 0.1 mg/kg level, and by 21% (from 85 to 104%) at the 0.025 mg/kg level. DHEQ recoveries also improved moderately upon AA-addition (by 25% from 76 to 101%). In contrast, EQDM recoveries appeared to be unaffected by AA addition (87 versus 86% with some uncertainty associated with the higher RSD).

In the case of EQI the impact of AA was dramatic. Without the addition of AA prior to extraction the average recovery rate was 11%. When AA was added prior to extraction the recovery rate was nearly quantitative (93%). This indicates that EQI is a much more potent antioxidant than EQ.

Recoveries using chlorpyrifos-D10 as IS were in general ca. 8-10% higher than those using diuron-D6, which essentially reflects the recovery difference between the two compounds. RSDs were similar using both internal standards. Looking at the cases where AA was already present or added during extraction the EQDM recoveries were ca. 15% lower than those of the other components. This is attributed to the higher lipophilicity of this compound and the associated partitioning losses which cannot be matched by the respective partitioning losses of chlorpyrifos-D10. As losses of EQDM are expected to be higher in farmed salmon, the use of chlorpyrifos-D10 as IS seems to be a better compromise than diuron-D6.

Influence of ascorbic acid (AA) addition on EQ and EQDM yields from farmed salmon

To check the impact of AA-addition on the residue findings in real samples, five conventionally farmed frozen salmon products were analyzed for the presence of EQ and its metabolites using the procedure described above. A first screening revealed residues of EQ and EQDM in all five samples, but no detectable residues of the metabolites EQI and DHEQ.

AA was added either during cryogenic milling or during QuEChERS extraction or to the final extracts. The samples were analyzed in 4 variations as follows:

- AA **added during cryo-milling** (1 g/100 g sample) ***and*** **to sample portions (1 mL AA-solution[28])** before QuEChERS extraction;
- AA **added during cryo-milling** (1 g/100 g sample);
- AA only **added to sample portions (1 mL AA-solution**) before QuEChERS extraction;
- AA only added **to final extract** (50 µL of AA-mix[29]/mL extract)

The samples were extracted in triplicate. Calibration was accomplished using matrix-matched standards prepared from wild salmon extracts. Chlorpyrifos D10 was used as IS. The results of this experiment are shown in Figure 2.

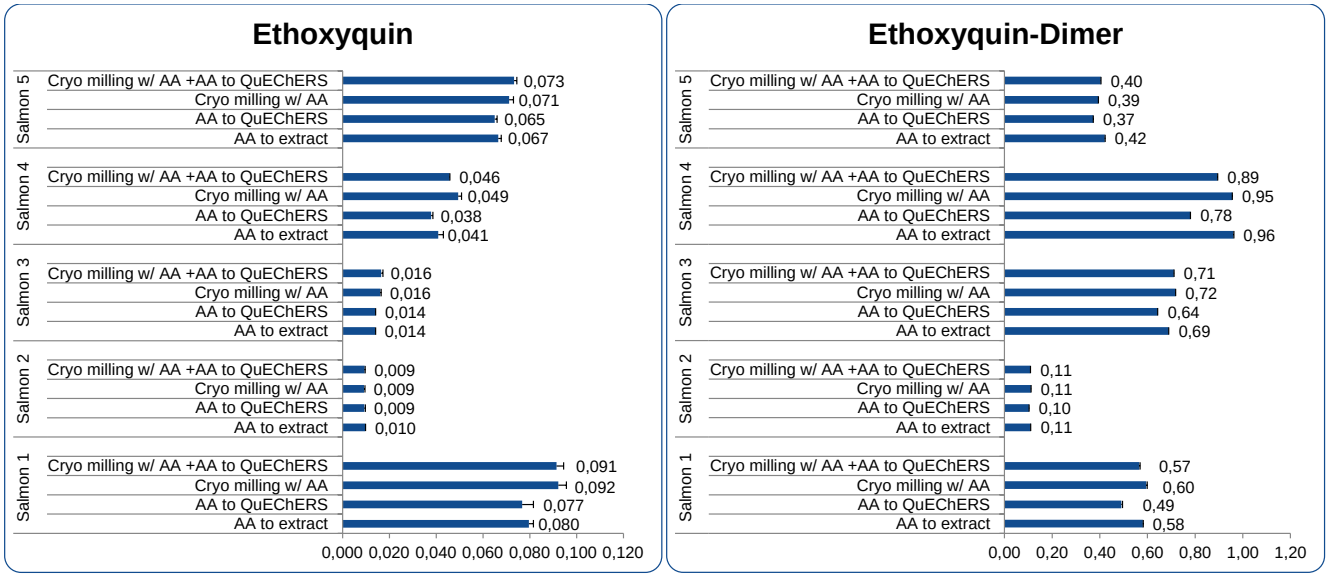


Figure 2 Analysis of EQ and EQDM residues in salmon samples (n=3)

EQDM levels (0.21 to 0.89 mg/kg) were clearly higher than the EQ levels (0.01 to 0.15 mg/kg).
As with the recovery experiment, AA-addition had no visible impact on the results in the case of EQDM. Where AA was only added to the final extract the determined EQDM levels are most likely

[28] AA-solution: aqueous solution containing 0.3 g/mL AA

[29] AA-mix: aqueous solution containing 0.15 g/mL AA and 0.15 g/mL sodium-ascorbate

overestimated. In a later experiment it was observed that EQDM signals are enhanced when injecting solutions to which AA was added.
Adding AA during milling resulted in a rather minor but still visible increase of EQ-yields. The yield-increase within the individual samples ranged between 8% and 14% and the average increase was 12%.
Overall farmed salmon appears to be more protective towards EQ compared to wild salmon. This is surely related to the fact that farmed salmon is fed with feed containing antioxidants such as EQ, BHT, BHA and propyl gallate. The feed is also enriched with carotenoids such as astaxanthin and capsaxanthin to achieve a better flesh color. This surely reduces the presence of oxidizing molecules (hydroxyl and peroxyl radicals) in the samples. According to Hamilton et al.[25], wild salmon contains much higher rates of polyunsaturated fatty acids (which are highly sensitive to oxidation) compared to farmed salmon, e.g. cis-9,12-octadecadienoic acid (6.5% versus 0.7%), cis-5,8,11,14,17-eicosapentaenoic acid (11.8% versus 4.1%) and cis-4,7,10,13,16,19-docosahexenoic acid (15.7% versus 6.3%).

Analysis of further fish and seafood samples from the market

A number of additional samples (10 Atlantic salmon fillets, 4 prawns, 1 gilthead, 1 panga catfish fillets) were taken from local supermarkets and analyzed as described above for both EQ and EQDM. Matrix-matched calibration solutions based on blank wild salmon extracts were used and chlorpyrifos-D10 was employed as internal standard.
Whereas all farmed Atlantic salmon samples contained both EQ and EQDM none of the other samples contained measurable levels of these two compounds. In accordance with the experiments described above and other studies[18-20], EQDM levels in farmed salmon were significantly higher than those of EQ.

The results of the 10 farmed Atlantic salmon samples are compiled in Figure 3.

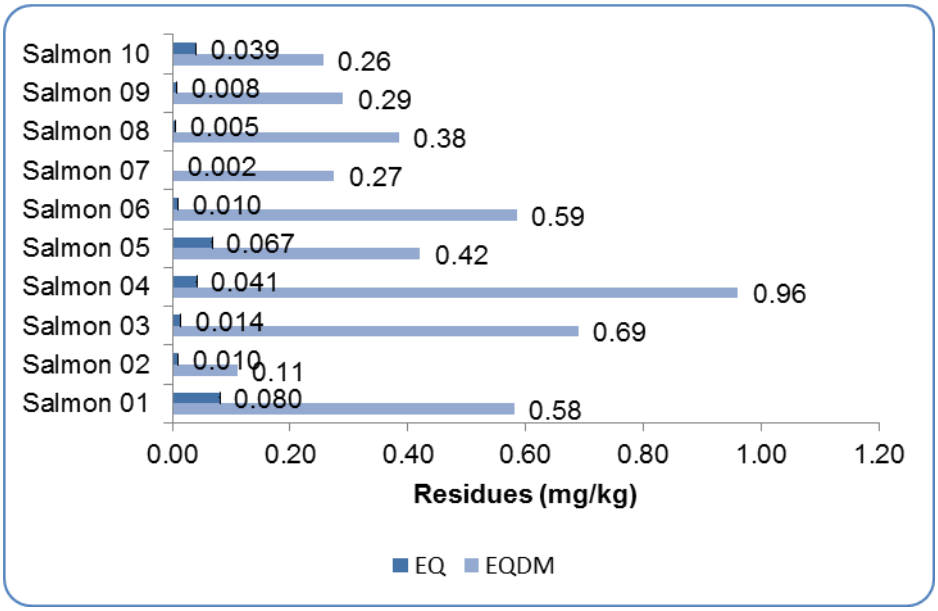


Figure 3 EQ and EQDM levels in Atlantic salmon samples from local markets

Unknown peak in farmed Atlantic salmon

Figure 4 shows a chromatogram of a farmed Atlantic salmon extract, where an additional peak within the mass transition trace (m/z 218 to 148) of EQ can be seen. Measurements on a high-resolution LC-TOF instrument showed that the mass of the unknown substance corresponded with the mass of EQ ($[M+H]^+$ = 218.1539 g/mol). This could be explained either by an isomer of EQ or a reaction product, which is fragmented into EQ in the ESI source. A similar observation is reported in an FAO report[30].

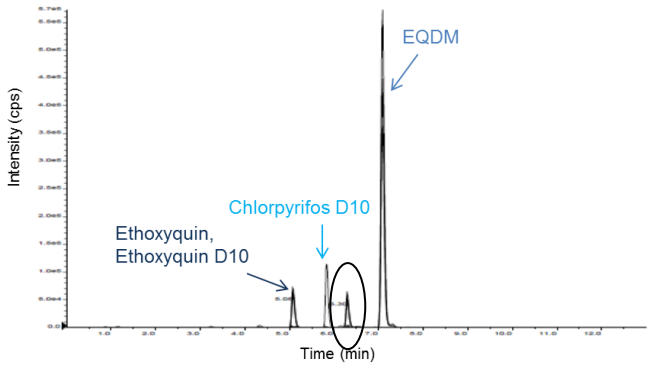


Figure 4 Chromatogram of an Atlantic salmon extract

[30] Pesticide Residues in Food 1999 Evaluations: Residues, Part 1 by Food and Agriculture Organization of the United Nations (page 223)

Conclusion:

While the addition of AA during the cryogenic milling process is essential to prevent EQ degradation in apples and pears (see Analytical Observation on Ethoxyquin, Version 2, 31.03.2015[31]), its impact in the case of farmed salmon was more moderate. EQDM appeared to be stable irrespective if AA was added or not.

When conducting recovery studies on wild Atlantic salmon the impact of adding AA during QuEChERS extraction on the protection of EQ was clearly stronger than in farmed fish (recovery increases in the range of ca. 25%). EQI was by far the most sensitive among the components and addition of AA before the QuEChERS extraction led to a dramatic increase of the recovery rate from just 9% to 93%. In the case of DHEQ AA-addition also led to a moderate recovery increase in the range of ca. 25%.

Chlorpyrifos-D10 ($logK_{ow}$ 4.7) was deemed as a suitable IS as it, at least partly, matched the partitioning losses experienced by the highly non-polar EQDM ($logK_{ow}$ 6.6) whereas in the case of EQ ($logK_{ow}$ 3.9) it caused only a very minor overstep of the 100% recovery mark.

All of analyzed Atlantic salmon samples from the market contained both EQ and EQDM. The other two tested metabolites QI and DHEQ were not encountered. The concentrations of EQDM were significantly higher than those of EQ. Validation experiments on EQ, EQDM, DHEQ and EQI showed satisfying recoveries and variation coefficients in salmon. The use of matrix-matched or procedural calibrations is recommended.

Document History

Date	Action	Changes
February – September 2015	Experiments	
May 2016	Publication of V1	

[31] http://www.crl-pesticides.eu/userfiles/file/EurlSRM/EurlSrm_Observations_Ethoxyquin_V2.pdf

Ethoxyquin extraction in salmon with ascorbic acid at a glance