Observations concerning…

☑ ***a compound*** ☑ ***a matrix*** ☑ ***a method*** ☐ ***other***

Improvement of Ethoxyquin Recoveries by QuEChERS Through the Addition of Ascorbic Acid

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Version 2 (last update: 31.03.2015)

Brief description:

Being an antioxidant ethoxyquin is prone to oxidative losses. Applying the QuEChERS method, notoriously low ethoxyquin recoveries are observed from commodities having poor antioxidative potential such as pears, apples, cucumbers and cereals. In contrast, recovery rates from commodities exhibiting strong antioxidative potential, such as citrus fruit and berries are typically nearly quantitative. Decomposition may occur both in crops and in the laboratory during most stages of analytical procedures, such as sample homogenization and extraction. Losses are also observed during the storage of frozen homogenates and particularly pronounced during the storage of extracts with ethoxyquin decomposition being faster in extracts of commodities with poor antioxidative potential. This is to be considered in analysis, e.g. when preparing calibration standards based on blank extracts. Degradation rates in extracts significantly decline following dSPE with PSA but increase again when extracts are re-acidified with formic acid. To prevent oxidative losses and to increase ethoxyquin recoveries the addition of antioxidants such as ascorbic acid (AA) are shown to be very helpful. If the commodity to be analyzed does not exhibit antioxidative protection, the addition of AA should occur as early in the procedure as possible to minimize degradation. Adding AA to the analytical sample prior to spiking with ethoxyquin typically leads to virtually quantitative recoveries. AA addition after spiking results in oxidative losses, with their extend depending on a number of factors including type of commodity, temperature and the time passed between spiking and AA-addition. Although not part of the current residue definition, ethoxyquin transformation products can be used as indicators for the presence of ethoxyquin especially in those cases, where routine multiresidue procedures lead to a complete or almost complete decomposition of the parent compound.

Analysis of 26 pear samples from the market resulted in 3 samples from Italy with positive ethoxyquin results all of them exceeding the MRL. All these cases indicate a misuse of ethoxyquin, as ethoxyquin authorizations expired in September 2012.

Compound profile:

Ethoxyquin is a quinoline-based **antioxidant** widely applied to inhibit superficial scald (formation of brown spots) in **pears** and **apples**. The Codex Alimentarius Commission classifies ethoxyquin as a scald control agent. As scald is often accompanied by fungus infections, ethoxyquin is also often listed as a fungicide. For treatment, the fruits are typically dipped into a solution containing ethoxyquin but ethoxyquin-impregnated fruit wraps are also often employed. Pre-harvest applications have been reported for apples.

In 2011[1] the commission decided not to include ethoxyquin in Annex I of Reg. 91/414/EEC[2] with a **grace period for use ending in September 2012**. EU-MRLs are set at the agreed LOQ of 0.05 mg kg^{-1} for all commodities with exception of pears where the MRL is 3 mg kg^{-1} (=Codex MRL). Diphenylamine (DPA), the main alternative to ethoxyquin, was also not included in Annex I (grace till May 2011), other alternatives including 1-MCP (1-methylcyclopropene) are currently being tested.
An additional food-related use of ethoxyquin concerns spices such as **chili**, **paprika** and **curcuma powder.** Here ethoxyquin is applied as an additive to prevent oxidative discoloration of carotenoid pigments during storage (US tolerance 100 mg kg^{-1}).

In addition to the applications directly on food, there are numerous feed related applications with indirect food relevance, including the following:

- In the EU register on feed additives[3] ethoxyquin is listed as **E324** and is allowed to be applied alone or in combination with butyl hydroxyanisole (BHA, E320) and/or butylated hydroxytoluene (BHT, E321) up to a total concentration of 150 mg kg^{-1} [4].

- Ethoxyquin is commonly used to treat **dried forage crops** such as corn, wheat and oats to preserve them from oxidative loss of vitamin E and carotenes. The US tolerance is set at 150 mg kg^{-1}.

- Within the EU ethoxyquin is furthermore allowed to be used at levels up to 2000 mg kg^{-1} in **salmon and trout feed** based on *Phaffia Rhodozyma,* a yeast variety rich in Astaxanthin, a carotenoid responsible for the reddish color of these fish.

- In the US ethoxyquin 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 ethoxyquin in these cases is set at 3000 mg kg^{-1}.

- Another interesting application concerns **dried fishmeal[5,6],** which is very prone to auto-oxidation due to the high unsaturation of fish oil. Lipid auto-oxidation is highly exothermous and if uncontrolled can even lead to spontaneous combustion of the material during long storage and shipping

[1] 2011/143/EU: Commission Decision of 3 March 2011 concerning the non-inclusion of ethoxyquin in Annex I to Council Directive 91/414/EEC
[2] now Reg. (EC) No 1107/2009
[3] 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)
[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**

periods. The International Maritime Organization (IMO) as well as the US legislation prescribes the treatment of fish meal with ethoxyquin at concentrations not less than 400 mg kg^{-1} (1000 mg kg^{-1} if fat content exceeds 12 %).

The use of ethoxyquin as an animal feed additive often leads to residues in food of animal origin such as poultry meat and eggs[7] as well as in farmed fish[8] and shrimp[9].

Table 1: Ethoxyquin facts at a glance

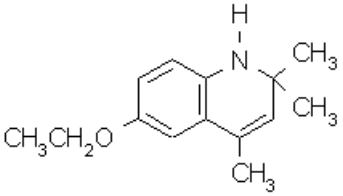
Parameter	Value	Notes	Ethoxyquin
pKa	4.56	at 22 °C (weak base)	
LogP	3.39	at pH7 (intermediately polar compound)	
Water solubility	60 mg L^{-1}	at 20 °C	
Hydrolytic behavior DT50	pH 5: 3.7 days pH 7: 6.7 days pH 9: 9.3 days	at 25 °C	
Residue definition EU	Ethoxyquin (F)		
Approved in…	Not approved within the EU		

Table 2: Instrumentation details (exemplary)

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		

Materials:

Ethoxyquin (purity 98.5%), purchased from Dr. Ehrenstorfer (CA13310000)
L-Ascorbic acid, reagent grade (Sigma Aldrich, CAS 50-81-7)

[7] http://onlinelibrary.wiley.com/doi/10.1002/jsfa.2740331213/pdf
[8] http://lib3.dss.go.th/fulltext/Journal/Journal%20of%20food%20science/2000%20v.65/no.8/jfsv65n8p1312-1314ms20000212%5B1%5D.pdf
[9] http://www.iffo.net/default.asp?contentID=813

Initial observations:

Initial recovery experiments with QuEChERS for ethoxyquin spiked at 0.1 mg kg^{-1} on pear homogenates resulted in very low and strongly variable recovery rates. Recovery experiments on apples (results not shown here) showed a similar trend. From cucumber and wheat flour ethoxyquin could even not be recovered at all (0 %). In contrast to these results, very good average recoveries were obtained from red currants and rehydrated raisins (103 % and 96 % respectively).
We attributed this to the stronger antioxidative potential of these two commodities. Red currants contain ca. 40 mg ascorbic acid (AA) per 100 g. The AA levels in grapes are comparable to apples and pears and rather low (4-10 mg/100 g), but grapes contain higher levels of catechins and polyphenols that also exhibit antioxidative activity.

Experiment 1: Addition of AA before spiking

To prove that the low antioxidative potential of some matrices is the cause for low ethoxyquin recoveries, we decided to use AA as an antioxidant prior to extraction.
The addition of 0.5 g solid AA per analytical portion of cucumber, pear, raisin, red currant and wheat before spiking with ethoxyquin resulted in satisfactory recoveries (between 95 and 105 %) and RSDs (<5 %) in all cases using QuEChERS method. Recoveries with and without the addition of AA prior to extraction are shown in Figure 1.

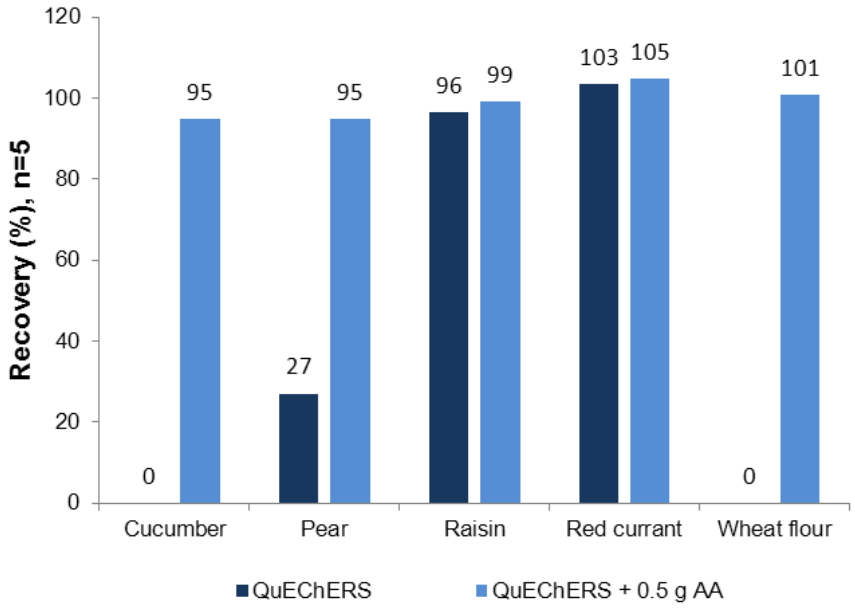


Figure 1: Impact of AA-addition on the recoveries of ethoxyquin from different commodities

Remarks on this experiment:

- *For cucumber, pear, raisin, red currents 0.5 g of solid AA was added to the analytical portions followed by vortexing for one minute (to evenly distribute the AA) and by spiking with ethoxyquin (onto the semi-thawed material).*
- *In the case of raisins 13.5 g of the rehydrated homogenate was used (containing 5 g raisins)*
- *In the case of wheat flour 0.5 g of solid AA was added to the dry material. Following spiking with ethoxyquin, cold water was added to the sample and the spiked sample was left for 10 minutes to soak before the extraction was started.*
- *In all cases the first QuEChERS extraction step was conducted by mechanical shaking for 15 minutes*
- *No dSPE cleanup with PSA-sorbent was conducted*
- *In all cases suitable matrix-matched calibration solutions were used.*

Experiment 2: Variation of AA amount used

In experiment 2 we varied the amount of solid AA added to the pear matrix before spiking with ethoxyquin (0.1, 0.25, 0.5 and 1 g). As shown in Figure 2 the protection effect was sufficient in all cases with recoveries ranging between 87 and 97 %. We also compared the recoveries achieved with and without dSPE cleanup with PSA sorbent and the recovery rates remained good in all cases. Appropriate matrix-matched calibration solutions were used in all cases.

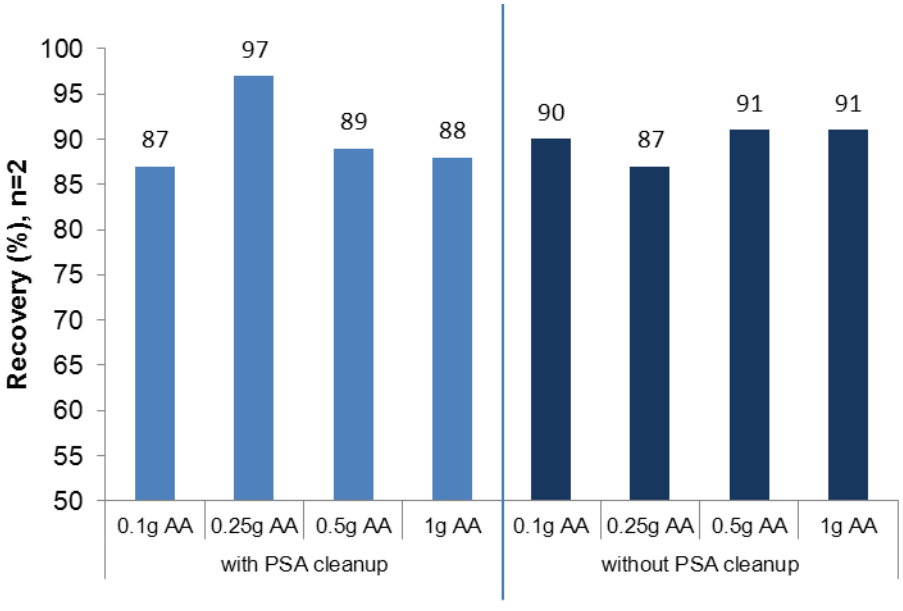


Figure 2: Ethoxyquin recoveries in pears depending on different amounts of AA added to the matrix prior to spiking

Experiment 3: Addition of aqueous AA solution

With practicability in mind we further tested whether AA can be more conveniently added as aqueous solution. For this experiment we prepared a nearly saturated AA solution in water (containing ca. 0.3 g AA /mL) and added 1 mL (this corresponds to 300 mg AA) to 10 g analytical portions of **pear** before spiking with ethoxyquin. As shown in Fig. 3, protection following addition of "liquid AA" was also sufficient.

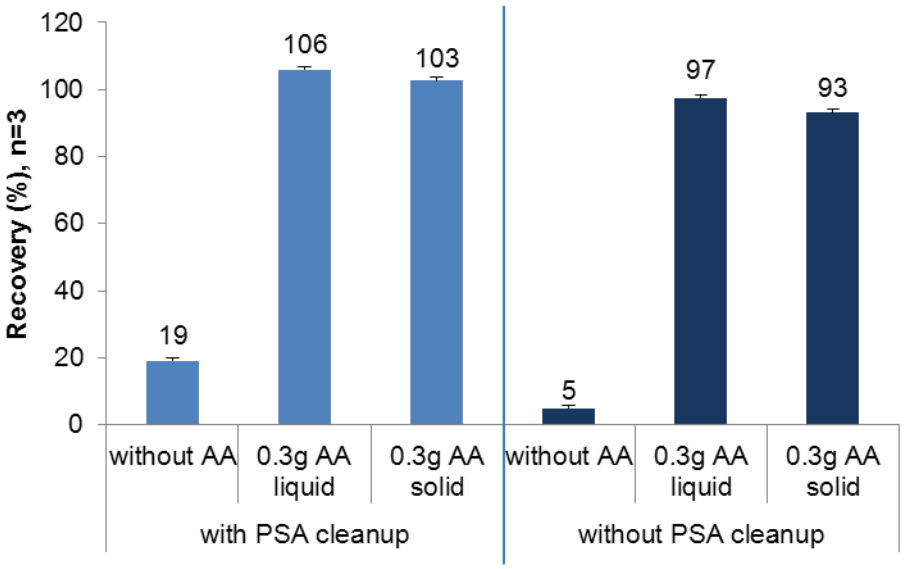


Figure 3: Ethoxyquin recoveries with AA-addition to the pear sample in solid and liquid form as well as without AA-addition

Experiment 4: Prevention of ethoxyquin degradation during milling

In a next experiment we studied the degradation of ethoxyquin during milling and checked whether it can be reduced by adding AA prior to milling. For this we spiked a large number of whole fresh pears superficially with a solution containing 0.5 mg ethoxyquin /mL and left them standing in the dark for 5 days**.** The spiked fruits were divided into two main groups. The first one was intended for cryogenic milling (CRYO-group) and the second one for milling in fresh condition (RT-group). Before milling the fruits of both groups were cut into 8 segments each and the segments were randomly divided into two subgroups. The material of the CRYO-group was first put in the freezer overnight and then milled in frozen condition with the assistance of dry ice. The material of the RT-group was milled directly. One of the two sub-groups of the CRYO- and the RT-Group was milled following addition of 1 g AA (solid) /100 g pears and the other one without. From each of the homogenates of the four sub-groups six analytical portions were weighed and put in the freezer until extraction. In each case 3 of the portions were extracted normally using QuEChERS and the other 3 following addition of 1 mL AA-solution (containing 0.3 g AA per mL). In total 24 samples were extracted. Measurements were conducted

both directly from the raw extracts as well as from the extracts following d-SPE cleanup with PSA. This resulted in a total of 48 determinations. An appropriate matrix-matched calibration was prepared in each case. The following table gives an overview of the experimental setup.

Table 3: Overview of the experimental design concerning analysis of ethoxyquin in laboratory-spiked intact pears with and without addition of AA

Code	Milling conditions	AA-Addition prior to milling	AA-Addition prior to extraction	dSPE with PSA
CRYO-YYY	Frozen samples were milled, dry ice was added and milling was continued (Cryo-milled)	YES	YES	YES
CRYO-YYN		YES	YES	NO
CRYO-YNY		YES	NO	YES
CRYO-YNN		YES	NO	NO
CRYO-NYY		NO	YES	YES
CRYO-NYN		NO	YES	NO
CRYO-NNY		NO	NO	YES
CRYO-NNN		NO	NO	NO
RT-YYY	at Room Temperature (RT-milled)	YES	YES	YES
RT-YYN		YES	YES	NO
RT-YNY		YES	NO	YES
RT -YNN		YES	NO	NO
RT -NYY		NO	YES	YES
RT -NYN		NO	YES	NO
RT -NNY		NO	NO	YES
RT -NNN		NO	NO	NO

A summary of the results of this experiment is shown in Figure 4. The addition of AA during milling gave the highest degree protection to ethoxyquin. Where milling was done without AA-addition, ethoxyquin losses were massive during both RT- and CRYO-milling. Addition of supplementary AA to the analytical portions just prior to extraction increased ethoxyquin levels in these samples. Milling in absence of AA contributed roughly 40% of the overall ethoxyquin losses whereas extraction in absence of AA corresponded to ca. 60% of the overall losses.

Where AA was added during milling losses of ethoxyquin were minimized. In the case of RT-milling, however, considerable losses occurred during the subsequent extraction step. These losses could be prevented by the addition of supplementary AA to the analytical portion prior extraction. This fact is supported by the observation that the RT-milled samples showed a clearly darker color compared to the CRYO-milled ones. This browning is most probably a result of enzymatic (polyphenol oxidase – catalyzed) browning that goes along with a higher consumption of AA. Overall, the supplementary addition of AA to the test portions before the extraction was not necessary when comminuting cryogenically with the addition of AA. In all other cases including when the sample was comminuted at room temperature with the addition AA the supplementary addition of AA during extraction was proven essential to minimize further losses.

The use dSPE cleanup with PSA had in most cases no clear effect but it had a clearly positive impact on ethoxyquin yields in those cases where no AA was added during extraction (results not shown).

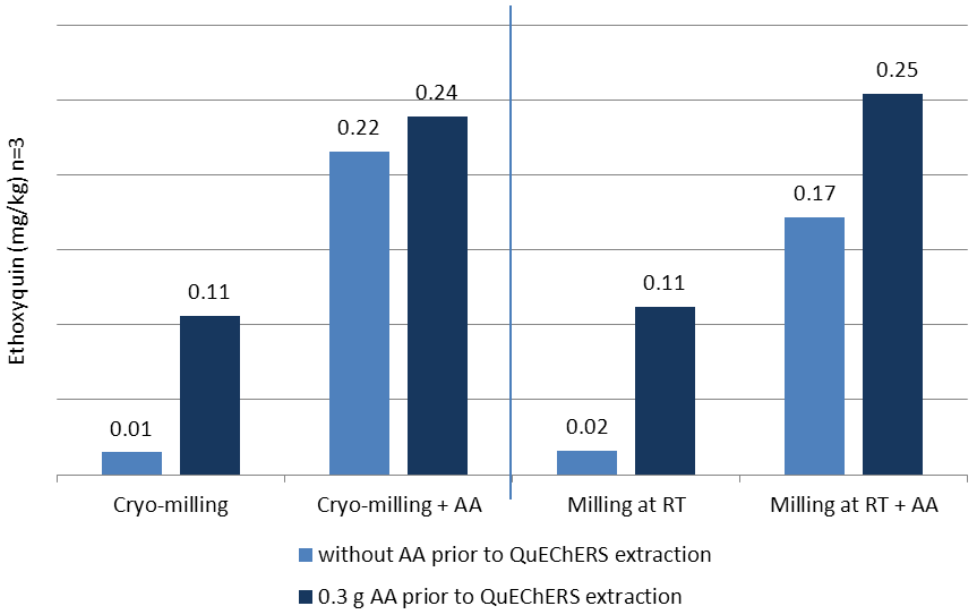


Figure 4: Ethoxyquin levels in pears spiked with ethoxyquin prior to comminution with and without the addition of AA before milling

Experiment 5: Analysis of pears with incurred residues of ethoxyquin

We repeated Experiment 4 using pear samples obtained from the US market containing incurred ethoxyquin residues[10] but only focusing on cryogenic milling. As expected, the addition of AA resulted in a dramatic increase in the determined ethoxyquin levels (see Figure 5). The ethoxyquin losses when no AA was used during milling ranged between ~15 and ~80% depending on the sample.

[10] Special acknowledgement to our colleague Dr. Ingrid Kaufmann-Horlacher for spending some of her holiday time in the US in search for pears and especially for bringing so many kilograms to Germany in her hand luggage

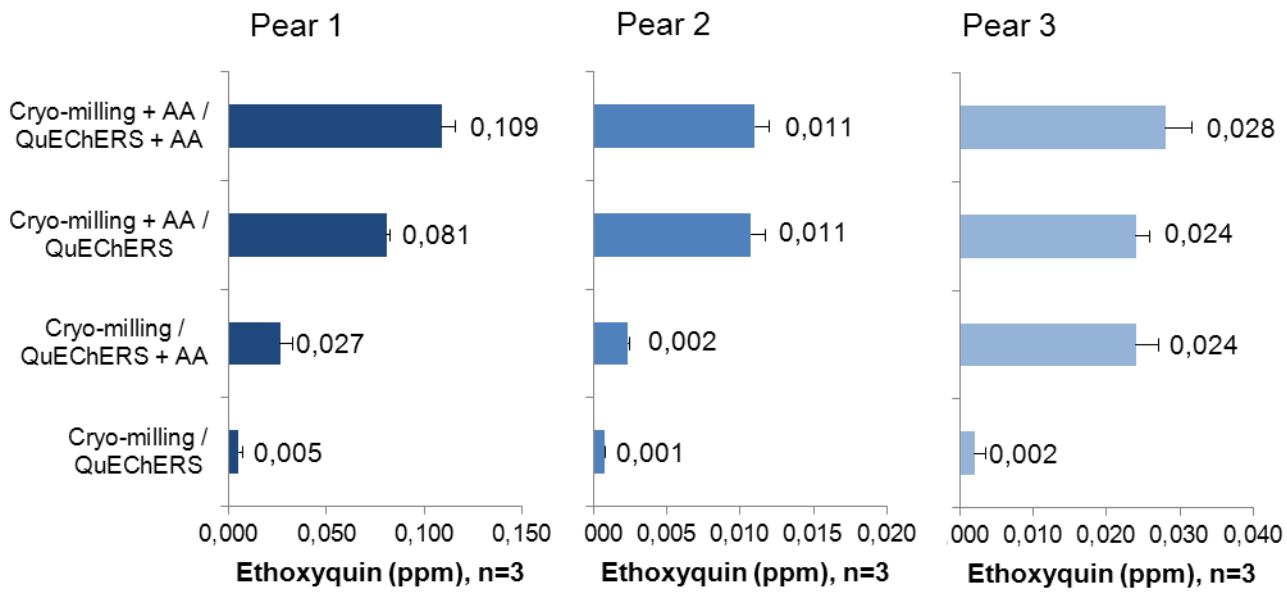


Figure 5: Impact of AA on ethoxyquin levels determined in pears from the market of US origin containing incurred residues

Analysis of samples from the market

Starting in July 2014 a total of 26 pear samples from the local market were analyzed for ethoxyquin residues with ascorbic acid (1g AA per 100g pear) being added during the comminution step (cryo-milling). An overview of the results is shown in Figure 6. Three of the 26 samples (all from Italy) were found to contain ethoxyquin residues at levels between 0.25 and 0.58 mg kg^{-1}. In all three cases the ethoxyquin levels exceeded the MRL of 0.05 mg kg for pears. At the same time these findings indicate a **misuse** of ethoxyquin as according to Commission decision 2011/143/EU authorizations of ethoxyquin had to be withdrawn in all EU member states with the grace period ending in September 2012.

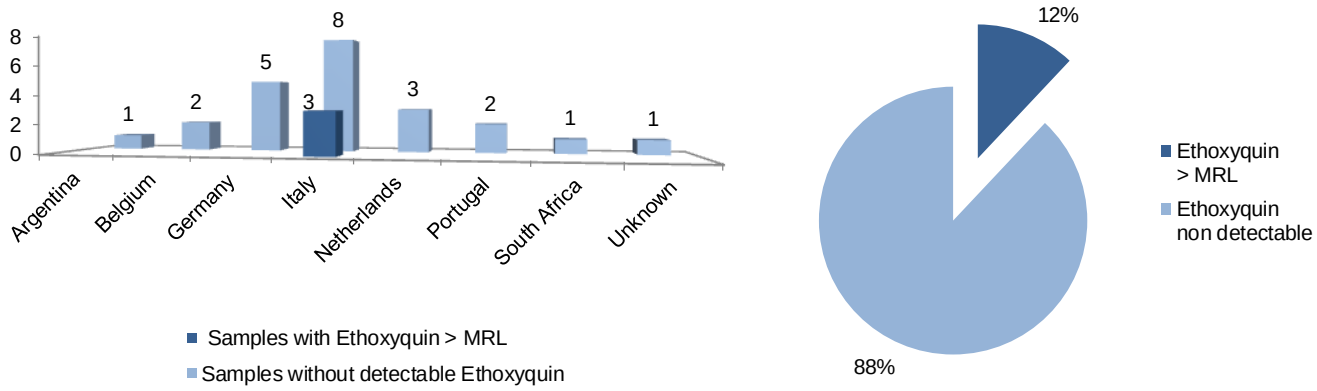


Figure 6: Number of samples with and without ethoxyquin findings in pears and country of origin of the analyzed pears

All 26 samples were also comminuted without the addition of AA and analyzed using QuEChERS method. The results achieved were compared. There are no hints so far that other pesticide residues are negatively influenced by the addition of AA.

Discussion and Conclusions:

Ethoxyquin recoveries from pears and apples (and some other crops) using the QuEChERS method are low due to oxidative losses. Addition of ascorbic acid (AA) to analytical portions prior to extraction increased recoveries. AA addition to sample portions was accomplished by adding 1 mL of an aqueous solution containing 300 mg mL^{-1} AA. For achieving quantitative recoveries, however, the AA addition had to be done prior to spiking, thus indicating a very fast degradation of ethoxyquin in homogenates of apples and pears.
To minimize ethoxyquin degradation throughout the analytical procedure AA-addition had to be done during the homogenization step. Using pear samples with incurred ethoxyquin residues as well as pears that were superficially-spiked in the lab, it could be shown that degradation occurs both during cryogenic and ambient temperature milling. When conducting cryogenic milling the addition of AA prior to homogenization was enough to protect ethoxyquin throughout the procedure. When milling pears at an ambient temperature, however, a supplementary addition of AA was necessary prior to QuEChERS extraction to minimize degradation. When dealing with pears containing incurred residues, we recommend adding AA to coarsely cut pieces prior to freezing and cryogenic milling.

Document History

Date	Action	Changes
07.04.2014	Publication of V1	
31.03.2015	Publication of V2	Inclusion of results from the analyses auf pears from the local market, minor adaptions in the text and figures for better under-standing