LC-MS/MS method for the determination of organophosphorus pesticides and their metabolites in salmon and zebrafish fed with plant-based feed ingredients

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Abstract

The composition of Atlantic salmon feed has changed considerably over the last two decades from being marine-based (fishmeal and fish oil) to mainly containing plant ingredients. Consequently concern related to traditional persistent contaminants typically associated with fish-based feed has been replaced by other potential contaminants not previously associated with salmon farming. This is the case for many pesticides, which are used worldwide to increase food production, and may be present in plant ingredients. Earlier studies have identified two organophosphorus pesticides, chlorpyrifos-methyl and pirimiphos-methyl, in plant ingredients used for aquafeed production. In the present study, we developed a reliable and sensitive analytical method, based on liquid chromatography coupled to tandem mass spectrometry, for the determination of these pesticides and their main metabolites in warm-water (zebrafish) and cold water (Atlantic salmon) species, where possible differences in metabolites could be expected. The method was tested in whole zebrafish and in different salmon tissues, such as muscle, bile, kidney, fat and liver. The final objective of this work was to assess kinetics of chlorpyrifos-methyl and pirimiphos-methyl and their main metabolites in fish tissue, in order to fill the knowledge gaps on these metabolites in fish tissues when fed over prolonged time.

Keywords: Chlorpyrifos-methyl; Pirimiphos-methyl; Metabolites; Atlantic salmon, Zebrafish; LC-MS/MS

INTRODUCTION

Plant ingredients are the main substitutes for fish oil and fishmeal and currently typically constitute about 70% of the ingredients in commercial salmon feed in Norway [1, 2]. The use of plant ingredients, together with commercial decontamination techniques, decreases the content of persistent organic pollutants (POPs) traditionally associated with fish oil and other marine ingredients [3, 4]. However, plant ingredients may introduce novel contaminants not previously associated with salmon farming [4]. Among them, pesticides are the group of major concern [5]. Earlier studies in the EU projects "AQUAMAX" and "ARRAINA" identified novel feed contaminants, such as polyaromatic hydrocarbons (PAHs), mycotoxins and none organochlorine pesticides (OCP) in plant ingredients and fish feed with low or non-detectable transfer of the parent compounds to the edible part of the fish [4, 5].

Until recently, research has focused on the analysis of organochlorine compounds in fish, and less information has been available concerning other groups of pesticides. In the last five years the number of scientific articles related to pesticides in fish matrices has notably increased reflecting growing concern regarding these contaminants [6–8]. Most recent literature dealing with pesticide residue analysis is based on the use of liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) with triple quadrupole (QqQ) [6–10]. This technique is applicable for currently used pesticides, mostly polar in nature, and is especially suitable for metabolites and transformation products (TPs), which are usually more polar than the parent compound. LC-MS/MS is a powerful technique in this field due to its excellent sensitivity and selectivity, as well as robustness and less sample treatment required (e.g. in comparison with GC-MS methods).

Our previous work indicated that from all new compounds screened, pesticides were the major contaminants present in novel fish feed [11]. Among more than 400 pesticides investigated,

chlorpyrifos-methyl and pirimiphos-methyl were found in several vegetable feed ingredients as well as in salmon feed. Further surveillance of commercially produced Norwegian salmon feed and feed ingredients showed that 55% of the analyzed rapeseed oils contained pirimiphos-methyl. For most food products, maximum residue levels (MRLs) for none OCP pesticides have been established in the EU; however, no specific MRLs have been defined yet for fish or seafood and default precautionary MRLs are currently applied. Knowledge on the effect of dietary plant-derived pesticides and their metabolites in Atlantic salmon (*Salmo salar*) is needed to set appropriate limits for pesticides to ensure good fish health and food safety.

In a benefit-risk assessment of fish and fish products, it was highlighted that knowledge on the feed-to-fillet transfer of plant-derived pesticides from feed to fish is lacking [12]. With regards to chlorpyrifos-methyl and pirimiphos-methyl, one might expect that bioavailability and accumulation are high due to their lipophilic nature and relatively small molecular size. Bioaccumulation of chlorpyrifos-ethyl has been reported in body, head and viscera of tilapia (*Oreochromis mossambicus*) [13]. However, biotransformation may be crucial in the process of accumulation of the parent compound. Particularly, for non-persistent pesticides metabolism plays an important role in the bioavailability and potential transfer to edible parts of fish. It is known that 3,5,6-trichloropyridinol (TCP free and conjugated) is the major metabolite of both chlorpyrifos-ethyl and chlorpyrifos-methyl in products of animal and plant origin [14, 15], while pirimiphos-methyl is mainly metabolized into 2-(diethylamino)-6-methyl-4-pyrimidinol (2-DAMP), O-[2-(ethylamino)-6-methylpyrimidin-4-yl]O,O-dimethylphosphorothioate (N-Des-PM) and 2-amino-6-methyl-4-pyrimidinol (2-AMP). The first two metabolites are considered of toxicological significance by the European Food Safety Authority (EFSA) [16].

In the project "Aquasafe" the main objective was to investigate the bioaccumulation, biotransformation and elimination kinetics of dietary chlorpyrifos-methyl and pirimiphos-methyl in whole zebrafish (*Danio rerio*) and Atlantic salmon tissues. For this purpose, the present study is aimed to develop a modern, fast and sensitive analytical method, based on LC-MS/MS with QqQ, for the quantification of these two pesticides and their main metabolites in zebrafish, and also in salmon muscle, bile, kidney, fat and liver.

MATERIALS AND METHODS

Chemicals

Pirimiphos-methyl (PM), chlorpyrifos-methyl (CLP-M), 3,5,6-trichloro-2-pyridinol (TCP), chlorpyrifos-methyl-oxon (CLP-M-oxon), N-desethyl-pirimiphos-methyl (N-Deset-PM) and 2-diethylamino-6-methyl-4-pyrimidinol (2-DAMP) were purchased from Sigma-Aldrich (Pestanal ® analytical standard, St Louis, MO, USA). Stock standard solutions (around 500 $mg \cdot L^{-1}$) were prepared in acetone. Working standard solutions containing all compounds were prepared by dilution of mixtures with acetonitrile. Both stock standard solutions and working solutions were stored in a freezer at -27 ºC.

Stable Isotopic Labelled Internal Standards (SIL-IS) CLP-M D_6, PM D_6 and TCP $^{13}C_3$ were purchased from Dr. Ehrenstorfer (Augsburg, Germany).

HPLC-grade water was obtained from water passed through a Milli-Q water purification system (Millipore LTD, Bedford, MA, USA). LC-MS grade acetonitrile (ACN) and methanol (MeOH), residue analysis grade acetone, extra pure anhydrous magnesium sulphate ($MgSO_4$), sodium hydroxyde and LC-MS grade formic acid (FA) were obtained from Scharlau (Barcelona, Spain).

$MgSO_4$ was dried overnight at 300ºC before its use. Leucine enkephaline was provided by Sigma-Aldrich.

Instrumentation

UHPLC-MS/MS.

A UPLC™ system (Acquity, Waters, Milford, MA, USA) was interfaced to a triple quadrupole mass spectrometer (Xevo TQ-S, Waters Corporation, Manchester, UK). LC separation was performed with a 50 x 2.1mm, 1.7 μm particle size Acquity UPLC BEH C_{18} analytical column (Waters). The mobile phases employed consisted on water (A) and acetonitrile (B) both with 0.0025% HCOOH, at a flow rate of 0.3 $mL \cdot min^{-1}$. The gradient program started with 50% B, increased linearly to 90% of B for 1.5 min and maintained during 1.5 min. Finally the gradient was held to initial conditions in order to re-equilibrate the column. Temperature column was set to 25ºC. 2 μL were selected as injection volume.

In the Selected Reaction Monitoring (SRM) method applied, dwell time values ranging from 5 to 90 ms were used in order to obtain 12 points per peak. Source temperature was set to 150 ºC. Drying and nebulising gas was nitrogen (Praxair, Valencia, Spain). Desolvation gas flow was set to 1200 $L \cdot h^{-1}$ and the cone gas to 250 $L \cdot h^{-1}$. For operating in MS/MS mode, argon (99.995%; Praxair, Valencia, Spain) was used as collision gas at 0.25 mL·min-1). Capillary voltage and desolvation gas temperature were set at 3.2 kV (1.9 kV in ESI^- mode) and 650ºC respectively. TargetLynx (MassLynx v. 4.1, Waters, Manchester, UK) software was used to process the quantitative data.

UHPLC-(Q)TOF MS.

UHPLC-(Q)TOF MS analysis was performed following the conditions used by Portolés et al. [17]. A UPLC^{TM} system (Acquity, Waters) was coupled to a hybrid QTOF mass spectrometer (XEVO G2, Waters Micromass, Manchester, UK) with an orthogonal Z-spray electrospray ionization interface. The chromatographic separation was performed using a Cortecs C18 (Waters) (100 × 2.1 mm, 2.7 μm) analytical column at a flow rate of 0.3 mL/min. The column temperature was set to 40°C. The mobile phases used were H_2O with 0.01% HCOOH (A) and MeOH with 0.01% HCOOH (B) performing a phase gradient as follows: 10% of B at 0 min, 90% of B at 14 min linearly increased, 90% of B at 16 min, and finally 10% B at 18 min to return to initial conditions. The injection volume was 20 μL.

For MS^E experiments, two acquisition functions with different collision energies were created and applied sequentialy in each sample injection: the low energy function (LE), selecting a collision energy of 4 eV, and the second one, the high energy function (HE), with a collision energy ramp ranging from 15 to 40 eV. The TOF resolution was 20.000 at FWHM at m/z 556,2771.

Samples

Muscle, liver, kidney, bile and fat tissue samples were obtained from seawater adapted Atlantic salmon, that was fed with pirimiphos-methyl spiked diets to a level of 15.2 mg·kg-1 for 81 days. The pirimiphos-methyl was vacuum top coated to commercially produced (Skretting ARC, Stavanger, Norway) salmon feed pellets with 2% fish oil at an ambient temperature of 15ºC. No pirimiphos-methyl was detected in the unspiked feed pellets. Post-smolt Atlantic salmon (Salmo salar L.) of both genders (SalmoBreed strain) were distributed among fifteen flow-through fiberglass tanks (100L; 0.80m x 0.95m x 0.5m). Initial weight and length (fork-tail) were

150 respectively 132 ± 25 g and 18 ± 2 cm (mean ± standard deviation; n = 375). The experiment
151 complied with the guidelines of the Norwegian Regulation on Animal Experimentation and EC
152 Directive 86/609/EEC. The experiment was ethically approved by the Norwegian Animal
153 Research Authority (now the Norwegian Food Safety Authority; approval number 12091) and
154 performed according to national and international ethical standards.
155
156 **Sample treatment**
157 LC-QTOF MS screening of salmon liver, kidney and muscle
158 For each matrix, a control sample (not exposed to contaminants) and the most exposed one to
159 contaminated diets were subjected to a screening analysis, in order to identify potential metabolites
160 of the pesticides under study To this aim, 1 g of muscle (0.5 g for liver and kidney) was accurately
161 weighed into a 15 mL Falcon tube and 2 mL of ACN:acetone (80:20) with 1% HCOOH (1 mL in
162 the case of liver and kidney) was added, and the tube was vigorously shaken by Vortex for 1 min.
163 After that, 0.5 g of $MgSO_4$ per gram of sample were added and the tube was immediately shaken
164 for 1 min. Subsequently, the tube was centrifuged at 6,000 rcf for 5 min, and 200 μL of the
165 supernatant were evaporated to dryness at 30ºC under a gentle stream of nitrogen. The residue was
166 dissolved in 200 μL of water and filtered through 0.45 μm nylon filters (Phenomenex, Torrance,
167 CA, USA). Finally, 20 μL of the extract was injected into the LC-QTOF MS system.
168
169 LC-MS/MS QqQ analysis (see Figure 1A)
170 For LC-MS/MS analysis, 1 g of zebrafish or salmon muscle (0.5 g for liver and kidney, and 0.1 g
171 for fat) was accurately weighed into a 15 mL Falcon tube (2 mL Eppendorf tube for fat). Then, 2
172 mL per gram of ACN:acetone (80:20) with 1% HCOOH were added for zebrafish, muscle, liver

and kidney (5 mL per gram for fat), and the tube was vigorously shaken by Vortex for 1 min. After that, 0.5 g of $MgSO_4$ per gram of sample was added and the tube was immediately shaken for 1 min. Subsequently, the tube was centrifuged at 6000 rcf for 5 min and 100 μL of the supernatant was diluted with 800 μL of water and 100 μL of 25 $ng \cdot mL^{-1}$ SIL-IS solution. Finally, the diluted extracts were filtered through 0.45 μm nylon filters and 2 μL were injected into the LC-MS/MS system.

For the analysis of bile samples, 400 μL of ACN:acetone (80:20) with 1% HCOOH were added to 100 μL of bile in a 2 mL Eppendorf tube. The tube was shaken by Vortex for 1 min and centrifruged at 12600 rcf for 5 min. Then, 250 μL of the extract were 4-fold diluted with 650 μL of water and 100 μL of 25 $ng \cdot mL^{-1}$ SIL-IS solution. Finally, the diluted extract was filtered through 0.45 μm nylon filters and 2 μL were injected into the LC-MS/MS system.

The procedure for feed samples was as follows: 1 g of feed was accurately weighed into a 15 mL Falcon tube. Then, 10 mL of ACN:acetone (80:20) with 1% HCOOH were added and the tube was vigorously shaken by Vortex for 1 min. After that, 0.5 g of $MgSO_4$ was added and the tube was immediately shaken for 1 min. Subsequently, the tube was centrifuged at 6000 rcf for 5 min. 20 μL of the supernatant were diluted with 880 μL of water and 100 μL of 25 $ng \cdot mL^{-1}$ SIL-IS solution. Finally, the diluted extracts were filtered through 0.45 μm nylon filters and 2 μL were injected into the LC-MS/MS system.

<u>Thermal stability experiment</u> (see Figure 1B)

1 g of feed was accurately weighed into a 15 mL Falcon tube (in quadruplicate). Then, 150 μL of a 20 $ng \cdot \mu L^{-1}$ standard solution containing CLP-M and PM were added in each tube and kept aging for 30 min (spiking level, 3 $mg \cdot kg^{-1}$). Then, 2 mL of ACN:acetone (80:20) with 1% HCOOH were added to one tube (QC tube) which was vigorously shaken by Vortex for 1 min. The other three tubes were subjected to the simulated conditions of the feed production process. To this aim, samples were heated in an oven at 50ºC for one hour. After that, they were extracted identically to the QC tube. The samples were centrifuged at 6000 rcf for 5 min and 100 μL of the extract were diluted to 100 mL with water. Finally, 100 μL of 25 $ng \cdot mL^{-1}$ SIL-IS solution were added to 1 mL of the diluted extract, which was filtered through 0.45 μm nylon filters, and 2 μL were injected into the LC-MS/MS QqQ system.

Validation study

Quantitative validation of the method was performed by evaluating the following parameters:

-Linearity: The calibration curves were obtained by injecting ten reference standards in solvent (except for bile, where matrix-matched calibration was applied) in the range 0.025-25 $ng \cdot mL^{-1}$ at the beginning and the end of the validation batch. Linearity was assumed when the regression coefficient was higher than 0.99 with residuals lower than 20% and the difference between initial and final calibration curves did not exceed 30% (RSD ≤ 30% of the SIL-IS signal for those compounds whose quantification was carried out using relative areas).

-Trueness and precision: Trueness was evaluated by means of recovery experiments, analyzing zebrafish, muscle, liver and bile matrices in sextuplicates at three concentrations: 1, 10 and 100 $\mu g \cdot kg^{-1}$ ($ng \cdot mL^{-1}$ for bile). Blank matrices were not available for kidney and fat tissue hence validation was performed by the analysis of the lowest contaminated samples spiked at 10 and 100

218 µg·kg^{-1}, and 500 and 5000 µg·kg^{-1}, respectively. No replicates could be performed for fat tissue,
219 due to the small amount of sample available. Feed matrix was validated at 500 and 5000 µg·kg^{-1}
220 due to the characteristics of the samples. Precision, expressed as the repeatability of the method,
221 was evaluated in terms of relative standard deviation (RSD) from recovery experiments at each
222 fortification level (n=6). Quantification was performed by means of calibration curves in solvent
223 using relative responses to the selected SIL-IS (see Table 1), except for bile which was quantified
224 using matrix-matched calibration curves. Recoveries (between 70-120%) and RSDs (below 20%)
225 were considered as satisfactory, according to SANTE/11813/2017 guideline [18].
226 -Limit of quantification (LOQ) was defined as the lowest concentration satisfactorily validated,
227 following the SANTE/11813/2017 guideline criteria (recoveries 70-120 and RSDs < 20%) [18].
228 -Limit of detection (LOD) was estimated, from the quantification transition, as the analyte
229 concentration that produced a peak signal with a signal-to-noise ratio of 3 from the chromatogram
230 at the lowest fortification level.
231 -Specificity was evaluated by verification of the absence of interfering peaks at the retention times
232 of each compound in blank samples. To this aim, the response of a potential peak in the blank
233 sample should be lower than 30% of the lowest level validated.
234
235 **RESULTS AND DISCUSSION**
236 **Screening of salmon liver, kidney and muscle using LC-(Q)TOF MS**
237 As stated in the "Reasoned opinion on the review of the existing maximum residue levels (MRLs)
238 for pirimiphos-methyl according to Article 12 of Regulation (EC) Nº 396/2005" [16], PM is mainly
239 metabolized into the metabolites shown in Table S1 in the case of lactating goat milk, muscle,
240 liver, kidney and fat. However, to our knowledge, information regarding dietary pirimiphos-

methyl metabolism in teleost fish is currently lacking. Therefore, LC-(Q)TOF MS screening was applied in order to investigate the potential PM metabolites present in our samples. The Extracted Ion Chromatograms (XICs) at LE (0.005 Da mass window) were obtained for the theoretical masses of the (de)protonated molecules of the expected metabolites. As shown in Figure 2, PM seemed to be metabolized mainly into 2-DAMP (R46382) and N-Des-PM (R36341) in muscle, liver and kidney. Both metabolites have been reported to be toxicologically significant by the EFSA in order to generate appropriate MRLs [16]. The other hydroxypyrimidine metabolites reported in warm-blooded animals were not detected in the fish samples. The identity of the compounds was determined by comparing the LE and HE spectra with those of the standards in solvent. Mass errors for the protonated molecules were in all cases below ±1.5 ppm, and the main fragment ions did not exceeded ±4 ppm mass errors.

From the results obtained after screening of metabolites, a LC-MS/MS QqQ quantitative method was developed for the determination of PM, 2-DAMP and N-Des-PM in different fish tissues.

Optimization of LC-MS/MS QqQ conditions

The MS parameters were optimized by direct infusion of 0.1 $ng \cdot \mu L^{-1}$ individual standard solutions in methanol:water (1:1) 0.01% FA at a flow rate of 10 $\mu L \cdot min^{-1}$ (25 $\mu L \cdot min^{-1}$ for CLP and TCP). The optimal cone voltage and collision energies finally selected are shown in Table 1**.**

Regarding LC conditions, different mobile phases (H_2O, MeOH and ACN) and additives (HCOOH and NH_4OAc) were tested. For most of the compounds except TCP, sensitivity improved using a mobile phase containing 0.01% HCOOH. Decreasing the HCOOH concentration to 0.0025%,

improved the peak shape for TCP and sensitivity was not substantially affected. Finally, H_2O:ACN with 0.0025% HCOOH was used for the analysis of samples.

The q_i/Q ratio (q_i identification transition; Q quantification transition), of the chromatographic peaks in samples were compared with those of the reference standard (average value for standard solutions at 1, 5, 10 and 25 $ng \cdot mL^{-1}$; see Table 1) for identification of the compounds, with a tolerance in deviations ±30%.

Table 1. Experimental conditions of the optimized UHPLC-ESI-MS/MS method. Quantification (Q) and identification (q_i) ions, collision energy, cone voltage, q_i/Q ratio and linear range.

Rt (min)	Compound	Internal standard		Precursor ion	Product ion	Collision energy (eV)	Cone voltage (V)	q_i/Q	Linear range ($ng \cdot ml^{-1}$)
0,53	2-DAMP	PM D_6	Q	182.1	84.1	20	30		
			q_1		109.1	25		0.41	
			q_2		137.1	20		0.38	0.025 - 25
			q_3		126.1	20		0.21	
			q_4		99.0	20		0.14	
1,13	TCP $^{13}C_3$		Q	203	203	5	20		
1,14	TCP	TCP $^{13}C_3$	Q	196	196	5	10		
			q_1	198	197.9	5		0.86	0.25 - 25
			q_2	200	199.8	5		0.26	
1,24	N-Des-PM	PM D_6	Q	278,1	125.1	25	40		
			q_1		67.1	35		1.2	
			q_2		108.1	25		1.0	0.025 - 25
			q_3		246	15		0.62	
			q_4		100.1	20		0.46	
2,79	CLP-M D_6		Q	330	131	20	20		
2,82	CLP-M	CLP-M D_6	Q	322	290	15	30		
			q_1		125.1	20		2.6	
			q_2		79.1	30		0.62	0.25 - 25
			q_3		109.1	20		0.38	
			q_4		212	30		0.21	
2,86	PM D_6		Q	312,2	284.2	20	10		
2,87	PM	PM D_6	Q	306,1	108.1	30	10		
			q_1		67.1	40		0.90	
			q_2		164.1	20		0.84	0.025 - 25
			q_3		95.1	25		0.45	
			q_4		136.2	25		0.26	

Sample treatment optimization and matrix effect study

Sample treatment for solid matrices was optimized in order to get the maximum recovery with the simplest method possible. Different extraction systems, followed by several clean-up sorbents were tested. Recovery experiments were carried out at 50 $\mu g \cdot kg^{-1}$ in triplicate using salmon fillet as the reference matrix (spiked samples were aged for 45 min). Quantification was performed by matrix-matched calibration in each experiment.

The following solvents were firstly tested: ACN, ACN:acetone (80:20) and ACN:acetone (80:20) containing 1% FA using mechanical agitator for 1 hour. It was found that CLP-M-oxon was rapidly converted to TCP after spiking the sample, causing the overestimation of TCP. This instability indicated that CLP-M-oxon should not be present in the samples, and therefore it was removed from the analytical method. Using ACN the less polar compounds (PM and CLP-M) showed low recoveries (68 and 56 %, respectively), which improved using ACN:acetone (80:20). The addition of 1% FA to the later solvent mixture improved extraction efficiency (83-103% recoveries) with a maximum RSD of 11% (see Figure 3.A). Thus, ACN:acetone (80:20) 1% FA was chosen as the extractant solution in further studies.

Once the extractant was selected, different extraction times and techniques were evaluated. For this purpose, mechanical agitator (1 hour), vortex (1 min + 1 min after adding $MgSO_4$) and ultrasonic assisted extraction (US, 15 min) were tested, selecting finally 2 min Vortex, as the most suitable and simplest system (see Figure 3.B**).** In order to ensure its extraction efficiency, an extra

294 experiment consisting of the analysis of three spiked samples aged for 2 days at 7ºC was
295 performed, obtaining recoveries between 83 and 93%, with RSD < 5%.
296 Several clean-up treatments were also evaluated: Z-Sep, Z-Sep^{+}, freezing and 10-fold dilution. As
297 can be seen in Figure 3.C, 10-fold dilution showed excellent recoveries (72-108%), with RSD
298 <6%, and was selected for the analysis of samples.
299
300 Prior to the analysis of samples, we performed an evaluation of matrix effects in the samples under
301 study. To this aim, matrix-matched calibrations were prepared according to the sample treatment
302 showed in Figure 1, in which 100 µL of the corresponding standard solution in ACN (between 1
303 and 250 ng·mL^{-1}), instead of 100 µL of the SIL-IS solution, were added to the final extract,
304 resulting in final analyte concentrations between 0.1 and 25 ng·mL^{-1}. Matrix effect was evaluated
305 by calculating the relative error between the slopes of the calibration graphs obtained with
306 standards in solvent and in matrix [10].
307
308 Bile showed strong matrix effects for 2-DAMP and N-Des-PM (77 and 41% signal suppression,
309 respectively), whereas the rest of the compounds were not substantially affected (suppression of
310 7-21%). Despite the notable ionization suppression observed, the required concentrations were
311 still reached due to the high sensitivity of the method. In order to compensate matrix effects, the
312 accurate quantification in bile samples was ensured by using matrix-matched calibration (with
313 relative responses to SIL-IS only for PM, CLP and TCP). Regarding salmon fillet and liver, matrix
314 effect ranged 4-28% signal suppression for 2-DAMP, N-Des-PM, PM and TCP. CLP-M signal
315 was 46 and 50% suppressed in salmon and liver, respectively. Quantification using calibration in

316 solvent with relative responses to the selected SIL-IS (see Table 1) provided satisfactory results in
317 salmon fillet, liver, fat, kidney and feed.
318
319 **Method validation**
320 Validation of the method was carried out with zebrafish, salmon tissues (fillet, liver, kidney, bile
321 and fat), and salmon feed.
322 The study of linearity in solvent revealed that correlation coefficients (R^2) were higher than 0.99
323 with residuals lower than 20% for 2-DAMP, N-Des-PM and PM in the range 0.025-25 ng·mL^{-1}
324 and 0.25-25 ng·mL^{-1} for CLP-M and TCP. Matrix matched calibration for bile analysis also
325 showed correlation coefficients (R^2) higher than 0.99 with residuals lower than 20% for PM and
326 its TPs in the range 0.025-25 ng·mL^{-1}, and 0.25-25 ng·mL^{-1} for CLP-M and TCP.
327 Blank samples were pre-analyzed (except salmon kidney and salmon fat which were not available)
328 in order to ensure the absence of interfering peaks at the retention time of the analytes of study.
329 The method was found to be highly specific as no relevant signals were observed.
330 Trueness and precision data are shown in Table 2. For zebrafish, salmon muscle, liver and bile,
331 recoveries ranged from 72 to 106%, with RSD ≤ 16%, for PM and its metabolites; and from 71 to
332 112%, with RSD ≤ 12%, for CLP-M and TCP. CLP-M and TCP could only be validated at 10 and
333 100 μg·kg^{-1} (ng·mL^{-1}) spiking levels. Although no EU regulations exist for marine products, the
334 concentrations tested were lower than the precautional maximum residue limits (MRLs). Thus,
335 LOQs were established at 1 μg·kg^{-1} (ng·mL^{-1} in bile) for PM and its metabolites, and 10 μg·kg^{-1}
336 for CLP-M and TCP. For these matrices, LODs were in the range 0.1 – 0.6 μg·kg^{-1} (ng·mL^{-1} in
337 bile) and 2.5 – 8,0 μg·kg^{-1} (ng·mL^{-1} in bile), respectively.

Blank samples were not available for kidney and fat tissue. Consequently, analyzed samples with the lowest contamination levels were subsequently spiked for validation experiments, at a level at least three times the concentration present. Recoveries were then calculated by subtracting "blank" concentration. In kidney, the spiking levels were 10 and 100 $\mu g \cdot kg^{-1}$ as the concentrations of PM and 2-DAMP in the "blank" sample were 2.4 and 2.9 $\mu g \cdot kg^{-1}$, respectively. Trueness and precision were estimated in sextuplicates, obtaining recoveries between 70 – 82% (RSD < 9%) for PM and its metabolites, and 72 - 87% (RSD < 15%) for CLP-M and TCP. LODs were calculated from the "blank" samples used. Fat could be validated by a single QC spiked at 500 and 5000 $\mu g \cdot kg^{-1}$ due to the low amount of sample available. The spiking levels were selected based on the concentrations found in the "blank" samples (666, 56.5 and 102 $\mu g \cdot kg^{-1}$ for PM, 2-DAMP and N-Des-PM, respectively). Recoveries ranged 71 to 105%.

Salmon feed was validated at 500 and 5000 $\mu g \cdot kg^{-1}$ as the experimental design of the study established 3000 $\mu g \cdot kg^{-1}$ as the approximated concentration of PM and CLP for feeding trials. Recoveries ranged 74 – 84% with RSD<6% for parent compounds. N-Des-PM and TCP were not evaluated as they were not of interest in the analysis.

Table 2. Validation of the analytical method. Mean recoveries (%) and RSD (%, in brackets) of the overall procedure (n=6). Estimated limits of detection (LOD).

	Zebrafish ($\mu g \cdot kg^{-1}$)				Salmon muscle ($\mu g \cdot kg^{-1}$)			
	1	10	100	LOD	1	10	100	LOD
PM	73 (6)	89 (7)	91 (4)	0.1	84 (7)	82(7)	83 (11)	0.1
2-DAMP	96 (8)	83 (9)	83 (6)	0.2	97 (10)	94 (10)	101 (14)	0.2
N-Des-PM	91 (13)	87 (9)	82 (8)	0.3	82 (6)	86 (9)	87 (11)	0.6
CLP-M	-[a]	80 (6)	81 (6)	2.5	-[a]	90 (12)	86 (8)	2.0
TCP	-[a]	71 (2)	105 (10)	3.3	-[a]	112 (9)	100 (12)	8.0
	Salmon liver ($\mu g \cdot kg^{-1}$)				Salmon bile ($ng \cdot mL^{-1}$)			
	1	10	100	LOD	1	10	100	LOD
PM	77 (1)	80 (4)	82 (9)	0.1	90 (6)	94 (9)	106 (5)	0.1
2-DAMP	91 (10)	83 (4)	72 (10)	0.3	89 (16)	89 (10)	101 (15)	0.3
N-Des-PM	107 (10)	95 (2)	92 (6)	0.4	75 (5)	72 (11)	77 (5)	0.3

CLP-M	-[a]	111 (4)	104 (8)	2.0	-[a]	100 (9)	107 (4)	1.8
TCP	-[a]	96 (3)	105 (7)	7.6	-[a]	92 (11)	107 (4)	4.5

	Salmon kidney (μg·kg^{-1})			Salmon fat (μg·kg^{-1})			Feed (μg·kg^{-1})		
	10	100	LOD	500	5000	LOD	500	5000	LOD
PM	80 (9)	82 (6)	0.1	-[b]	87	[d]	74 (2)	75 (2)	[d]
2-DAMP	73 (3)	70 (5)	0.3	96	105	[d]	85 (3)	92 (4)	[d]
N-Des-PM	80 (8)	78 (5)	0.4	94	89	[d]	-[c]	-[c]	[d]
CLP-M	72 (15)	74 (7)	2.0	81	78	[d]	81 (2)	84 (6)	[d]
TCP	80 (6)	87 (15)	7.3	71	95	[d]	-[c]	-[c]	[d]

[a] Limit of detection > lowest spiking level (1 μg·kg^{-1} (ng·mL^{-1})).
[b] Blank concentration > spiking level.
[c] Compounds out of interest from the purpose of the analysis.
[d] Very high concentrations to calculate LODs

Thermal stability study of parent compounds

In order to assess the thermal stability of CLP and PM in feed, a trial simulating the conditions employed in feed production process (1 h, 50ºC) was carried out. The experiment was performed in triplicate, and results were compared with a QC that was not subjected to elevated temperature. The percentage of pesticide degradation was calculated by using **Equation 1**:

$$\%\ \text{degradation} = 100 - \frac{\%\ \text{recovery Trial}}{\%\ \text{recovery QC}} \times 100$$

As shown in **Table S2**, CLP and PM did not show relevant degradation at the production temperature conditions, with partial degradation of 15 and 17%, respectively. It was found that PM was degraded to 2-DAMP, generating a considerable background in the final diets (see **Table 3**).

Quantitative analysis of samples in dietary exposed fish

The developed method was applied for the analysis of zebrafish samples, salmon fillet, salmon liver, salmon kidney, salmon bile and diets. A reagent blank, a reagent blank spiked with SIL-IS (to evaluate SIL-IS stability), a blank (non-spiked) sample and 9 spiked samples (3 at each validation level) were included in each batch. Each matrix was analyzed in different batches. The

results summarized in Table 3 corresponding to the analysis of solid fish tissues are expressed in a wet weight basis, whereas those which correspond to bile analysis, are expressed in $ng \cdot mL^{-1}$. The q_i/Q ratios obtained for all positive samples were in agreement with those of the reference standards with deviations lower than the maximum tolerance accepted (30%). This data confirmed the identity of the compounds in samples according to the SANTE/11813/2017 guideline [18].

In whole zebrafish fed with CLP-M, TCP was the main metabolite and was present in higher concentrations (approximately two fold higher) than the parent compound. For PM, both 2-DAMP and N-Des-PM metabolites were identified in whole zebrafish, but at lower levels than the parent compound (see Figure 4). As for zebrafish, also for Atlantic salmon the main PM metabolites were 2-DAMP and N-Des-PM. The distribution of the PM metabolites showed highest concentrations for 2-DAMP, higher than the parent compound, in the liver which is likely the main organ of metabolisation. This is confirmed by the higher concentrations of 2-DAMP in the bile. The second metabolite, N-Des-PM, was found in all tissues (muscle, liver, kidney) at concentrations in the same range (3-6 $\mu g \cdot kg^{-1}$). The parent compound, PM, had highest levels in the fat tissue. Similarly, EFSA concludes that PM in commodities of animal origin is fat soluble and, in goat, parent pirimiphos-methyl was the main compound, accounting for 55 % of the total radioactive residue [16].

Table 3. Concentration of PM, 2-DAMP, N-Des-PM, CLP-M and TCP in zebrafish, salmon muscle, liver, kidney, fat and bile.

	($\mu g \cdot kg^{-1}$)	PM	2-DAMP	N-Des-PM	CLP-M	TCP
Zebrafish	**Trial 1**	n.d	0.7	n.d	5.5	16.0
	Trial 2	5.6	2.1	0.6	n.d	n.d
	Trial 4	n.d	0.6	n.d	n.d	n.d
Feed	**Trial 1**	n.d	200	n.d	1600	n.d

	Trial 2	2300	500	n.d	n.d	n.d
	Trial 4	n.d	200	n.d	n.d	n.d
muscle	**T81**	28.2	18.0	3.4	n.d	n.d
liver	**T81**	6.1	28.8	5.9	n.d	n.d
kidney	**T81**	22.4	16.8	3.1	n.d	n.d
Fat	**T81**	2757	75.5	346	n.d	n.d
	($ng \cdot mL^{-1}$)	**PM**	**2-DAMP**	**N-Des-PM**	**CLP-M**	**TCP**
Bile	**T81**	48.0	205	89.1	n.d	n.d

n.d: not detected. Concentration < LOD

CONCLUSIONS

A fast, simple and sensitive method for the determination of PM, CLP-M and their main metabolites in different fish tissues has been developed. Previous LC-(Q)TOF screening demonstrated that cold-blooded fish show a different metabolism of PM than in warm-blooded animals, with 2-DAMP and N-Des-PM being the most abundant metabolites in salmon. This was supported by analysis performed in the present work. The application of this method to zebrafish fed with CLP-M also allowed the identification of TCP as the most abundant metabolite. This work has generated analytical information essential for developing a kinetic model of accumulation and elimination of PM in salmon, and will contribute to establish relevant MRLs for fish.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest: The authors state that there is no conflict of financial and non-financial interest.

Research involving animals: The experiment complied with the guidelines of the Norwegian Regulation on Animal Experimentation and EC Directive 86/609/EEC. The National Animal Research Authority approved the protocol (ID 12091).

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FIGURE CAPTIONS

Fig. 1 Scheme of the analytical procedure for quantification of pesticides and TPs in solid and bile samples (A) and thermal stability experiment for CLP-M and PM in feed

Fig. 2 Screening of salmon liver, kidney and muscle

Fig. 3 Sample treatment optimization (A) extraction solvent, (B) extraction technique and (C) clean-up treatment. Percentage recoveries are calculated as means of triplicate experiments at 50 $\mu g \cdot kg^{-1}$

Fig. 4 UHPLC-(ESI)-MS/MS chromatograms obtained for the quantification and identification of A) PM (5.6 $\mu g \cdot kg^{-1}$), B) 2-DAMP (2.1 $\mu g \cdot kg^{-1}$), C) N-Des-PM (0.6 $\mu g \cdot kg^{-1}$), D) CLP-M (5.5 $\mu g \cdot kg^{-1}$) and E) TCP (16.0 $\mu g \cdot kg^{-1}$); in zebrafish samples. Q: quantification transition; q_i: identification transitions. ✓ q/Q within accepted tolerances

FIGURES

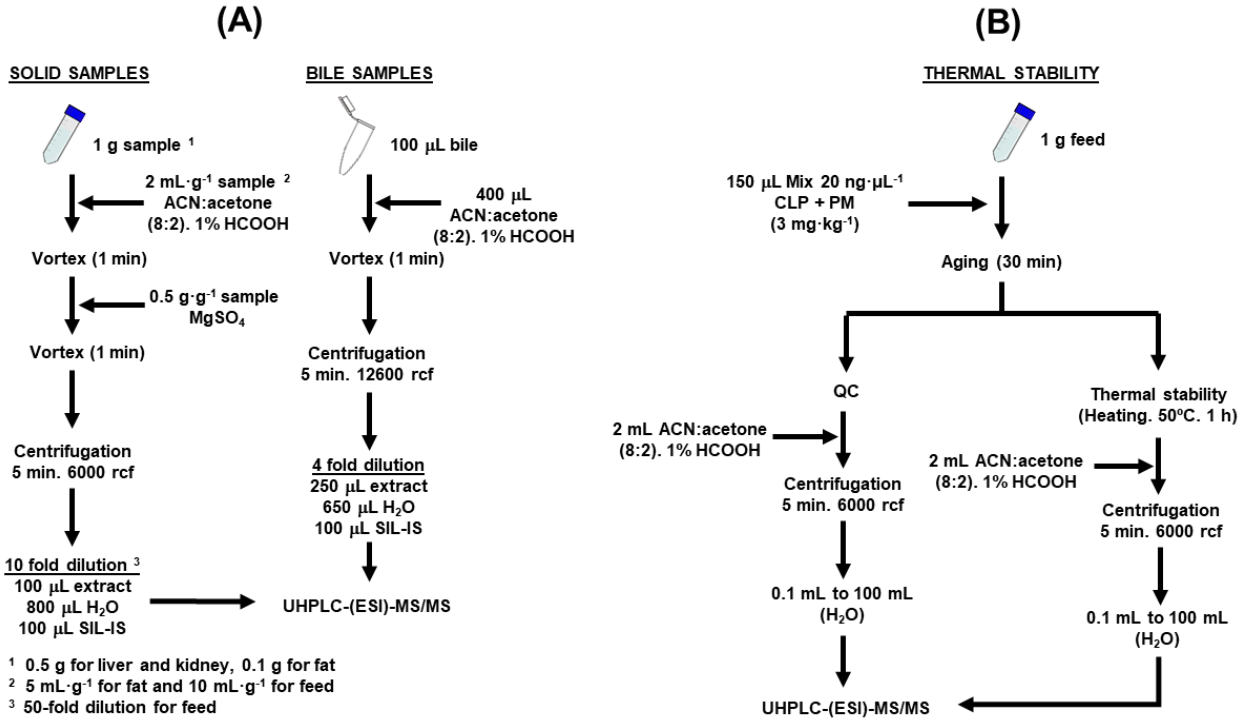
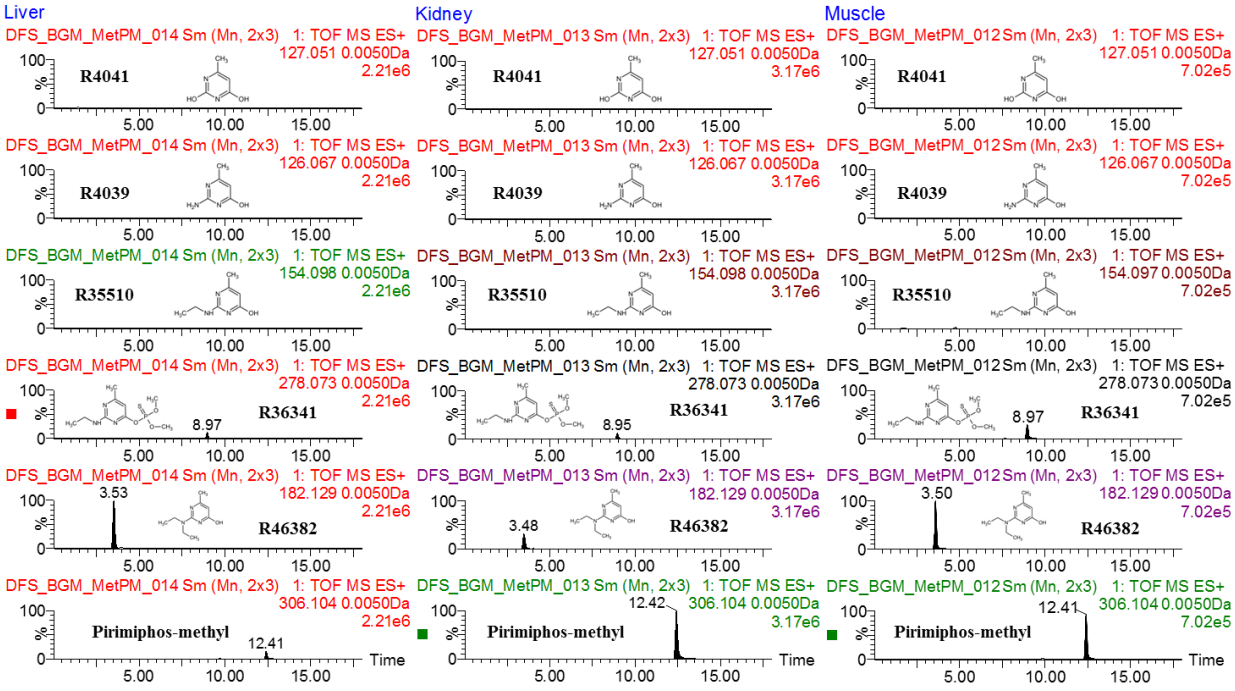


Figure 1


Liver
Kidney
Muscle
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DFS_BGM_MetPM_013 Sm (Mn, 2x3) 1: TOF MS ES+ 127.051 0.0050Da 3.17e6
DFS_BGM_MetPM_012 Sm (Mn, 2x3) 1: TOF MS ES+ 127.051 0.0050Da 7.02e5
R4041
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DFS_BGM_MetPM_013 Sm (Mn, 2x3) 1: TOF MS ES+ 126.067 0.0050Da 3.17e6
DFS_BGM_MetPM_012 Sm (Mn, 2x3) 1: TOF MS ES+ 126.067 0.0050Da 7.02e5
R4039
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R35510
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DFS_BGM_MetPM_013 Sm (Mn, 2x3) 1: TOF MS ES+ 278.073 0.0050Da 3.17e6
DFS_BGM_MetPM_012 Sm (Mn, 2x3) 1: TOF MS ES+ 278.073 0.0050Da 7.02e5
8.97
8.95
8.97
R36341
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DFS_BGM_MetPM_013 Sm (Mn, 2x3) 1: TOF MS ES+ 182.129 0.0050Da 3.17e6
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3.48
3.50
R46382
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DFS_BGM_MetPM_013 Sm (Mn, 2x3) 1: TOF MS ES+ 306.104 0.0050Da 3.17e6
DFS_BGM_MetPM_012 Sm (Mn, 2x3) 1: TOF MS ES+ 306.104 0.0050Da 7.02e5
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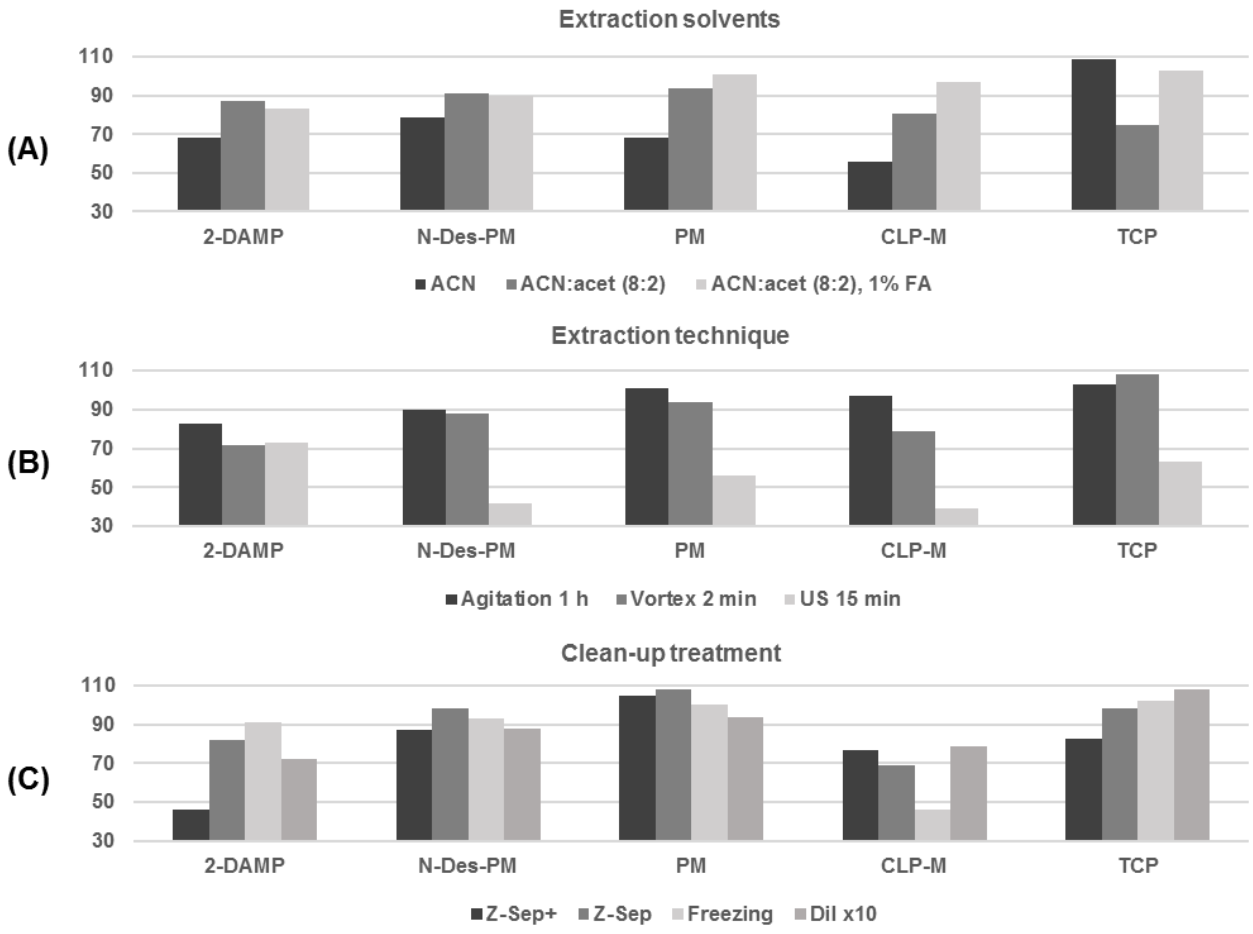
12.41
12.41
Pirimiphos-methyl
Time
100
%
0
5.00
10.00
15.00

Figure 2



502

503 **Figure 3**

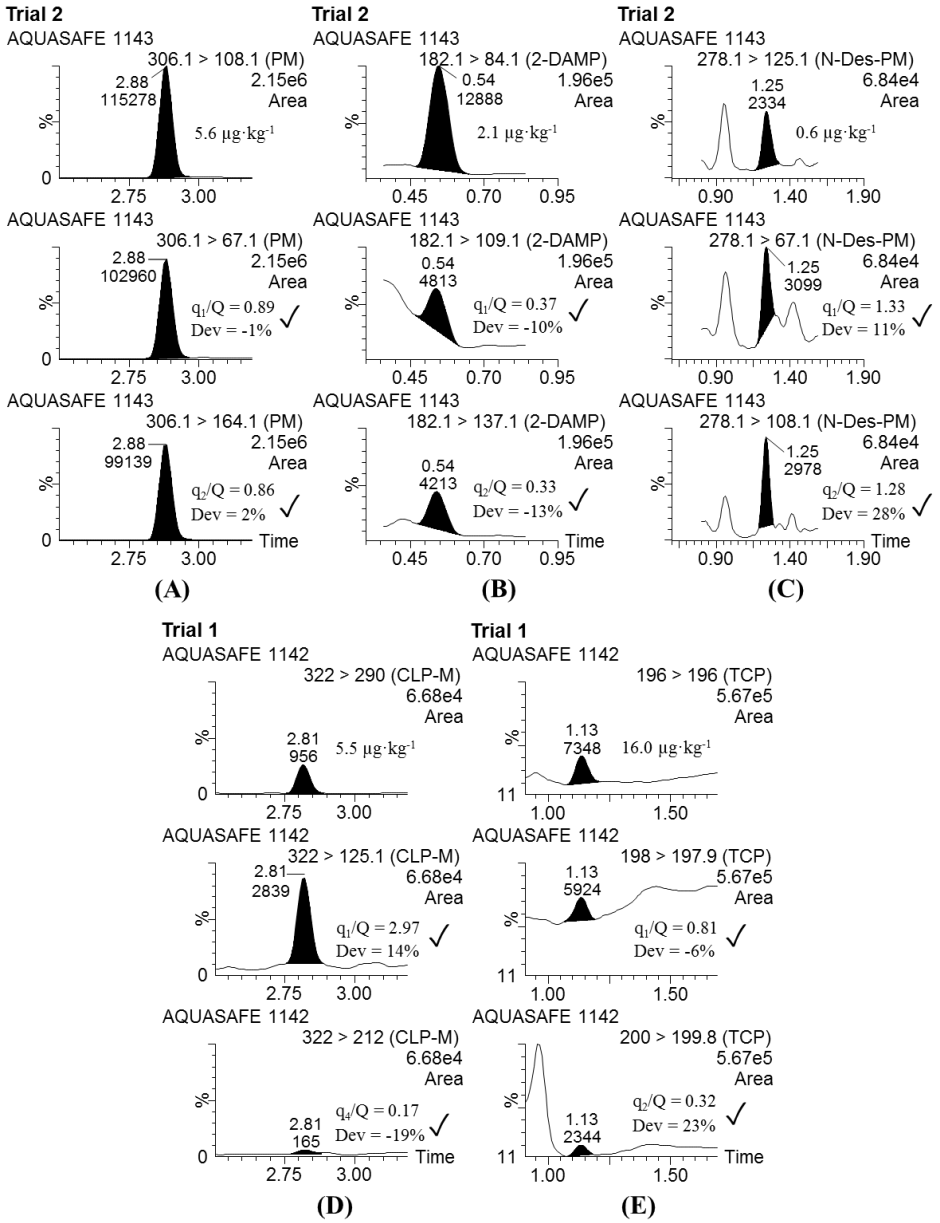

Trial 2
AQUASAFE 1143
306.1 > 108.1 (PM)
2.88
115278
2.15e6
Area
5.6 µg·kg-1
306.1 > 67.1 (PM)
2.88
102960
2.15e6
Area
q1/Q = 0.89
Dev = -1%
306.1 > 164.1 (PM)
2.88
99139
2.15e6
Area
q2/Q = 0.86
Dev = 2%
Time
(A)
Trial 2
AQUASAFE 1143
182.1 > 84.1 (2-DAMP)
0.54
12888
1.96e5
Area
2.1 µg·kg-1
182.1 > 109.1 (2-DAMP)
0.54
4813
1.96e5
Area
q1/Q = 0.37
Dev = -10%
182.1 > 137.1 (2-DAMP)
0.54
4213
1.96e5
Area
q2/Q = 0.33
Dev = -13%
Time
(B)
Trial 2
AQUASAFE 1143
278.1 > 125.1 (N-Des-PM)
1.25
2334
6.84e4
Area
0.6 µg·kg-1
278.1 > 67.1 (N-Des-PM)
1.25
3099
6.84e4
Area
q1/Q = 1.33
Dev = 11%
278.1 > 108.1 (N-Des-PM)
1.25
2978
6.84e4
Area
q2/Q = 1.28
Dev = 28%
Time
(C)
Trial 1
AQUASAFE 1142
322 > 290 (CLP-M)
2.81
956
6.68e4
Area
5.5 µg·kg-1
322 > 125.1 (CLP-M)
2.81
2839
6.68e4
Area
q1/Q = 2.97
Dev = 14%
322 > 212 (CLP-M)
2.81
165
6.68e4
Area
q4/Q = 0.17
Dev = -19%
Time
(D)
Trial 1
AQUASAFE 1142
196 > 196 (TCP)
1.13
7348
5.67e5
Area
16.0 µg·kg-1
198 > 197.9 (TCP)
1.13
5924
5.67e5
Area
q1/Q = 0.81
Dev = -6%
200 > 199.8 (TCP)
1.13
2344
5.67e5
Area
q2/Q = 0.32
Dev = 23%
Time
(E)

Figure 4