Screening of pesticides and polycyclic aromatic hydrocarbons in feeds and fish tissues by gas chromatography coupled to high-resolution mass spectrometry using an atmospheric pressure chemical ionization (GC-(APCI)QTOF MS).

Jaime Nácher-Mestre[a], Roque Serrano[a], Tania Portolés[a], Marc H. G. Berntssen[b], Jaume Pérez-Sánchez[c], Félix Hernández[a]*

[a]*Research Institute for Pesticides and Water (IUPA). Avda. Sos Baynat, s/n. University Jaume I, 12071 Castellón, Spain*

[b]*National Institute of Nutrition and Seafood Research, PO Box 2029 Nordines, N-5817 Bergen, Norway*

[c]*Institute of Aquaculture of Torre la Sal (IATS), C.S.I.C., 12595 Ribera de Cabanes, Castellón, Spain*

Abstract

This paper describes the development, validation, and application of a wide screening for pesticides and polycyclic aromatic hydrocarbons (PAHs) in feeds and fish tissues. The study involves different matrices such as vegetable and marine ingredients, vegetable and fish oils, feed compositions and fish tissues derived from aquaculture. QuEChERS methodology was applied for sample treatment and the method was qualitatively validated to test the detection capabilities for pesticides and PAHs according to European guidelines. Freezing was incorporated to the QuEChERS sample treatment as an additional clean-up in order to remove protein, lipids and other interferences of the sample extract. Analysis were carried out by GC-HRMS system, consisting of gas chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry with atmospheric pressure chemical ionization (GC-(APCI)QTOF MS). The qualitative validation was carried out for over 133 representative pesticides and 24 PAHs at 0.01 and 0.05 mg/kg. Subsequent application of the screening methodology to aquaculture samples made it possible to detect several compounds from the target list, such as the chlorpyrifos-methyl, pirimiphos-methyl, ethoxyquin, among others. Light PAHs (≤ 4 rings) were found in both animal and vegetable samples. The reliable identification of the compounds detected was supported by accurate mass measurements and the presence of at least two representative *m/z* ions in the spectrum together with the retention time of the peak, in agreement with the reference standard. Additionally, the searching was widening with other pesticides for which standard were not available thanks to the expected presence of the protonated molecule and/or molecular ion in the APCI spectrum. This could allow the detection and tentative identification of other pesticides different from those included in the validated target list.

Keywords: gas chromatography, high resolution mass spectrometry, QuEChERS, screening, organic contaminants, QTOF MS, feed, fish, qualitative validation

** Corresponding author. Tel. +34-964-387366; e-mail address: felix.hernandez@qfa.uji.es*

1. Introduction

One of the goals of the aquaculture is the reduction of fish origin ingredients in feeds by using new plant-based alternative feed ingredients in order that feed producers become less dependent on fish meal and fish oil. There is a notable interest to know the impact of these substitutions on the quality of farmed fish species and also on food safety of the final product. The use of vegetable origin raw materials reduces the total load of potentially hazardous persistent organic pollutants (POPs) among others but may load new undesirables, different from POPs. [1-3] Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants that are widely deposited in vegetable samples so their inclusion in priority lists becomes relevant.[4] In addition, pesticides are among the most relevant contaminants when dealing with samples from vegetable origin.

The analysis of organic undesirable compounds in fatty samples from aquaculture activities is commonly conducted by gas chromatography coupled to mass spectrometry (GC-MS). Generally, a time-consuming sample treatment is required to achieve low detection limits, including one or more clean-up steps to eliminate matrix components that negatively affect analysis (pigments, proteins, lipids…). [5-9] A widely used sample preparation approach is QuEChERS, initially developed for determination of pesticides in fruits and vegetables.[10,11] Modifications of this approach have been developed for different compounds and matrices making this stage highly flexible depending on the sample matrix. [12-18] One of the most distinguishing features of QuEChERS over previous sample preparation techniques is the use of dispersive solid-phase extraction (d-SPE) for clean-up. Following quick and easy steps it is possible to obtain clean extracts well-suited for both GC-MS and LC-MS analysis.

Large-scope screening approaches are becoming attractive in the last years, as conventional target analysis offers a limited overview of a (normally) reduced number of organic compound candidates. The combined use of GC-HRMS and LC-HRMS is currently one of the most efficient strategies for wide-scope screening of organic pollutants.[19] The qualitative validation of

the screening methodology previous application to real samples is required to support that the methodology fits properly at least for selected "model compounds". In a wide screening of organic contaminants, the number of targets investigated is, in principle, unlimited. Among the full spectrum acquisition analyzers, the time-of-flight analyzer (TOF) is especially suited for this purpose due to the high sensitivity and accurate mass data generated. [19-22] Additionally, for the GC coupling, if combined with the recently revived atmospheric pressure chemical ionization source (APCI), the investigation of target compounds is easier and more successful due to its softer ionization character in comparison with the highly fragmentation pattern observed with the widely accepted electronic ionization (EI). Thus, working with this soft fragmentation source, the molecular ion ($M^{+\cdot}$) or the protonated molecule ($[M+H]^+$) is commonly presented in the APCI spectrum (in most cases as a base peak) which improves both selectivity and sensitivity of the screening detection. [23] Also, the availability of a QTOF instrument allows performing MS/MS and/or MS^E experiments to go further in the confirmation of the identity of the compounds detected due to the structure information given by the fragmentation pathways.

The aim of the present work is to complement a previous developed screening based on LC-HRMS, pursuing one of the main challenges in food safety and toxicology: the combination of GC-HRMS and LC-HRMS, which seems to be the closest approach to the ideal "universal" screening where all type of analytes, independently of their polarity or volatility could be detected in the analysis. A QuEChERS-based sample treatment has been applied, with some modification. A critical stage was to validate the GC-QTOF MS methodology for both pesticides and PAHs establishing the screening detection limit (SDL) in complex aquaculture samples. The validated methodology was applied for GC-undesirables in commercially and experimentally available real samples.

2. Material and methods

2.1. Reagents and chemicals.

Individual pesticide reference standards were purchased from Dr. Ehrenstorfer (Scharlab, Spain) with purity between 93-99%. Stock standard solutions (around 500 mg/L) were prepared in acetone and were stored in a freezer at -20 ºC. Nineteen mixtures of pesticide standards (individual concentration of each pesticide around 50 mg/L) were prepared by dilution of stock individual solutions in acetone. A working standard solution containing all pesticides at 1 mg/L was prepared by dilution of mixtures with acetone. In our target list, ethoxyquin is included in the pesticide list as a preservative. It is mainly considered as a synthetic preservative but it is also used as pesticide (under commercial name as "Stop-Scald") in order to prevent oxidation in vegetable and fruit samples.

Benzo[*j*]fluoranthene, 5-methylchrysene, benzo[*c*]fluorene, dibenzo[*a,e*]pyrene, dibenzo[*a,h*]pyrene, dibenzo[*a,i*]pyrene, dibenzo[*a,l*]pyrene and cyclopenta[*cd*]pyrene individual standard solutions and mixture PAH MIX 9 containing naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, indeno[*1,2,3-cd*]pyrene, dibenzo[*a,h*]anthracene and benzo[*g,h,i*]perylene at10 mg/L were purchased from Dr. Ehrenstorfer. A working standard solution containing all compounds at 1 mg/L except for cyclopenta[*cd*]pyrene which was at 0.5 mg/L, were prepared by combining the standard mixtures and diluting in n-hexane.

Acetone (pesticide residue analysis quality), n-hexane (ultra-trace quality), acetonitrile (reagent grade), toluene (for GC residue analysis) and glacial acetic acid were purchased from Scharlab (Barcelona, Spain). Anhydrous magnesium sulphate (extra pure) and anhydrous sodium acetate (reagent grade) were purchased from Scharlab. The QuEChERS commercial products composed by 2 mL microcentrifuge tubes for d-SPE containing 50 mg primary secondary amine (PSA), 150 mg anhydrous $MgSO_4$ and 50 mg C18, were purchased from Teknokroma (Barcelona, Spain). This was the kit selected in our recommended procedure. Moreover, another QuEChERS kit with the same composition together graphitized carbon black

(GCB, 50 mg) was also purchased from Teknokroma. It was also studied in the optimization of the methodology (Scharlab, Barcelona, Spain).

2.2. Samples

Feed ingredients that are either used or tested and aquafeed were directly purchased or provided from manufacturers. Protein feed ingredients were pea protein (2 samples), pea (1), wheat (3), wheat gluten (4), corn gluten (3), soya protein (4), sunflower meal (1), rapeseed cake (1), fish meal (2), krill meal (1) and fish protein (1). Oil ingredients such as rapeseed oil (5), palm oil (2), linseed oil (1) and fish oil (2) were also studied. As regards feed, five different aquafeeds were analysed that had different composition of marine ingredients and plant ingredients. With regard to fish, three fish species (atlantic salmon (salmon salar), sea bass (Dicentrarchus labrax) and sea bream (Sparus aurata) were directly purchased from supermarkets. Sea bream fillets (3) and one fish liver from other growing experiments were also collected from IATS facilities.

2.3. GC-QTOF MS instrumentation.

GC system (Agilent 7890A, Palo Alto, CA, USA) was equipped with an autosampler (Agilent 7693) and coupled to a hybrid quadrupole-orthogonal acceleration-TOF mass spectrometer (XEVO G2 QTOF, Waters Micromass, Manchester, UK), using an APCI (APGC® by Waters Corporation). A fused silica DB-5MS capillary column (length 30 m × I.D. 0.25 mm × df 0.25 µm) (J&W Scientific, Folson, CA, USA) was used for GC separation. Injector was operated in splitless mode, injecting 1 µL at 280 ºC. The oven temperature was programmed as follows: 90 ºC (1 min), 5 ºC/min to 315 ºC (5 min). Helium was used as carrier gas at 2 mL/min. The interface temperature was set to 280 ºC using N_2 (from liquid N_2) as auxiliary gas at 250 L/h and as cone gas at 170 L/h, and N_2 (from gas cylinder quality 99.9990%) as make-up gas at 320 mL/min. The APCI corona pin was operated at 1.8 µA. The ionization process occurred within a closed ion volume, which enabled control over the

protonation/charge transfer processes. The water, used as modifier when working under proton-transfer conditions, was placed in an uncapped vial, which was located within a specially designed holder placed in the source door. In these conditions, the most critical separation was between benzo[*b*]fluoranthene, benzo[*j*]fluoranthene and benzo[*k*]fluoranthene, by one side, and between dibenzo[*a,i*]pyrene and dibenzo[*a,h*]pyrene, by other side, whose results should be treated as primary data.

For MS^E experiments, two acquisition functions with different collision energies were generated. The low energy function (LE), selecting a collision energy of 4 eV, and the high energy (HE) function, with a collision energy ramp ranging from 15 to 40 eV in order to obtain a greater range of fragment ions. It should be noted that all the exact masses shown in this work have a deviation of 0.55 mDa from the 'true' value, as the calculation performed by the MassLynx software uses the mass of hydrogen instead of a proton when calculating $[M + H]^+$ exact mass. However, because this deviation is also applied during mass axis calibration, there is no negative impact on the mass errors presented in this article. MS data were acquired in centroid mode and were processed by the ChromaLynx XS application manager (within MassLynx v 4.1; Waters Corporation).

2.4. Recommended analytical procedure.

Before analysis, ingredients and feed samples were thawed at room temperature and ground using a Super JS mill from Moulinex (Bagnolet Cedex, France). Fish tissues were also thawed at room temperature and processed in a crushing machine (Thermomix, Vorwerk España M.S.L., S.C., Madrid). As a result, homogenized samples were obtained in both cases. 5 g of sample was accurately weighed (precision 0.1 mg) into centrifuge tubes (50 mL), and mixed in a Vortex with 10 mL of acetonitrile (Figure 1). Then, 4 g of $MgSO_4$ was added and it was again shaken in a Vortex during 30 s. Following, extract is centrifuged at 4500 rpm for 5 min (Consul centrifuge, Orto-Alresa, Madrid, Spain) and the upper layer of the extract was transferred to a new centrifuge tube (15 mL) and stored overnight in a freezer to precipitate proteins and fix

lipids to the tube walls (freezing clean-up). Expired this time, 1 mL of the extract was carefully transferred to the clean-up QuEChERS vial (50 mg PSA + 150 mg $MgSO_4$ + 50 mg C18) and it was shaken 30 s and centrifuged at 12000 rpm for 5 min. After this clean-up, 0.5 mL were transferred to a new eppendorf vial adding 0.1 mL of hexane. The extract was concentrated to dryness at 30 ºC (to remove acetonitrile) under a gentle stream of nitrogen, reconstituted with 0.2 mL of n-hexane and finally transferred to a vial for GC injection. The samples were run twice, using water as modifier to favour in-source protonation and without adding water for those compounds for which no protonation was observed.

2.5. Method Validation.

Validation of the screening method was performed for qualitative purposes on the basis of European analytical guidelines.[24-25] 20 different samples (section 2.2) were spiked with over 133 pesticides and 24 PAHs at two levels, 0.01 and 0.05 mg/kg (0.005 and 0.025 mg/Kg for cyclopenta[*c,d*]pyrene), and analysed together with their non-spiked samples ("blanks"). Additionally, two method blanks were analysed to ensure that no laboratory contamination was introduced along the procedure. The SDL was set-up as the main validation parameter to estimate the threshold concentration at which detection becomes reliable. SDL was established as the lowest concentration tested at which a compound was detected in at least 95% of 20 spiked samples under study (i.e. detected in at least 19 samples at each concentration level) independently of its recovery and precision. The detection was made by using the most abundant ion measured at its accurate mass (typically the protonated molecule). This means that, at least, one peak (SDL) had to be observed in the respective narrow-window eXtracted Ion Chromatogram (nw-XIC), at the same retention time (tolerance of ±0.5% with respect to standard) and measured at accurate mass (mass error < 5 ppm). Table 1 and 2 show the results obtained at each spiked level.

3. Results and Discussion

Feed ingredients, feed compositions and fish tissues are complex matrices that contain a large number of interferences that may hamper detection and identification of undesirable compounds. In order to investigate the presence of any GC-amenable organic contaminant in this kind of samples, clean-up steps are normally applied to improve sensitivity and selectivity. [4,7,12-15,18] However, when the screening is focused on different chemical families of compounds, the situation is more problematic since analytes have rather different chemical and physical properties, and the analytical strategy should be suitable for all of them. [22,26] In this work, the screening was focused on many different pesticides and PAHs in a single analysis. As the objective was the detection and subsequent identification of the compounds detected in samples, no recoveries and precisions were calculated in this work.

3.1. Sample treatment optimization

One of the goals of a wide screening methodology is to minimize the possible analyte losses along sample treatment; so any restrictive step should be carefully studied. Acetonitrile solvent was selected since it is not highly amenable with lipid content and offers good recoveries for many GC-amenable compounds. In addition, in order to reduce the amount of extracted interferences, different conditions were tested, like (1) the addition of water, (2) addition of toluene and/or hexane together with acetonitrile for the extraction, and (3) the addition of sodium acetate.

1- The addition of water did not offer better results than the only use of acetonitrile. Thus, many compounds could not be detected at the lowest spiked level. Although some authors reported that water incorporation to fatty samples improves the determination of many pesticides, [27] in the samples under study the addition of water did not represent a relevant improvement for pesticides.

2- The use of organic solvents such as hexane or toluene reduced the co-extractives in the acetonitrile layer but also the presence of several non-polar compounds, like DDTs,

241 heptachlors, HCH-isomers in the sample extract, as they have more affinity to the
242 hexane or toluene layer. [27,28]

243 3- The addition of sodium acetate seemed not much favorable since it generated a turbid
244 extract. Although this situation does not represent a great disadvantage as reported by
245 other authors, [29] in our case better results were obtained without using sodium acetate.

246 One of the easiest ways to reduce the amount of matrix interferences is to place the organic
247 extract stored in a freezer. Thus, the solution obtained is rather clean as most proteins and lipids
248 are fixed on the bottom and walls of the tube, respectively. As previously reported, the
249 application of low temperature before d-SPE cleanup substantially reduces the amount of co-
250 extractives.[13,14] Moreover, an improvement of chromatographic peak shapes, reduction of signal
251 suppression and minimization of retention time shifts were observed for some compounds, as
252 supported by the bibliography. [13,14] After the freezing, an aliquot can be easily taken and
253 centrifuged to improve the solid-liquid separation.

254 The d-SPE clean-up step was also studied by using two commercially available QuEChERS kits
255 (d-SPE with 150 mg Mg_2SO_4, 50 mg PSA and 50 mg C18; and d-SPE with 150 mg Mg_2SO_4, 50
256 mg PSA, 50 mg C18 and 50 mg GCB). The kit containing GCB was tested trying to improve
257 the removal of matrix that hampers the detection.[12,27] After using these clean-up cartridges,
258 several pesticides, as HCB and DDTs, were not detected at the spiked levels. GCB seems to
259 properly remove additional matrix components from vegetable extracts, but it also tends to
260 retain certain pesticides, such as terbufos, thiabendazole, HCB, and other planar-ring analytes.[12]
261 Finally, the QuEChERS kit without GCB was selected for sample clean-up.

262

263 3.2. QTOF MS data processing

264 The acquired data files from the GC-QTOF MS were processed by using ChromaLynx
265 software. A *txt* file with the list of the molecular formula for representative ions was collected
266 together with the retention time per compound. This information was available when the

267 reference standard was injected, and was used to search in the recorded masses for each file.
268 The software searches for $[M+H]^+$, $M^{+\cdot}$ and/or fragment ions at a pre-fixed retention time
269 (target approach).

270 When the reference standard was not available, the only information was that either the
271 molecular ion and/or the protonated molecule would be expected upon GC-(APCI)QTOF MS
272 analysis. In this case, both ions were included in the processing screening method, as the
273 behavior in the APCI source could not be previously evaluated for these compounds. Any
274 detection being made by this way would indicate potential presence of the compound and more
275 information would be required for further identification (e.g. MS/MS experiments,…).
276 Obviously, as no reference standard was injected, no experimental data on the behavior of the
277 compound along sample preparation and GC-MS sensitivity was available. The acquisition of
278 reference standard and injection in the GC-QTOF MS system would be needed for unequivocal
279 confirmation of the tentative identification.

280

281 3.3. Qualitative validation of the screening methodology

282 Firstly, different samples of each matrix were injected in order to find the lowest contaminated
283 matrix for spiking. In this previous analysis, we found some matrices positives for several target
284 compounds. It is noteworthy that ethoxyquin and light PAHs were present in the wide majority
285 of samples analyzed. The lowest contaminated samples for each type of matrix were then spiked
286 with a mixture of pesticides and PAHs at a concentration of 0.01 and 0.05 mg/Kg for each
287 analyte (0.005 and 0.025 mg/Kg for cyclopenta[*c,d*]pyrene). 133 pesticides and 24 PAHs were
288 selected in order to qualitatively validate the screening by GC-QTOF MS. Twenty different
289 samples of interest for marine seafood were used for validation experiments. Table 1 and 2
290 show the number of positive/negative findings for pesticides and PAHs, respectively, at each
291 spiked level in the samples studied. At 0.01 mg/Kg, 76% of pesticides and 83% of PAHs were
292 detected. At the highest level validated (0.05 mg/Kg), these values improved up to 91 % of
293 detected pesticides and up to 100 % of PAHs. Figure 2 shows the percentage of detections for

294 the different matrices studied. As it can be seen, oils were the most problematic matrices
295 followed by feeds and tissues while feed ingredients represented lower difficulty for detections.
296 Regarding fish tissues, liver was trickier than fish fillets.

297 Figure 3 shows different examples of the qualitative validation at the 0.01 mg/Kg level. Four
298 groups are illustrated, attending at the samples studied: (A) ingredients, (B) oils, (C) feeds and
299 (D) fish tissues. The bottom of each figure shows the nw-XIC for the non-spiked sample and,
300 top shows the nw-XIC for the spiked sample at 0.01 mg/Kg with the most abundant ion used for
301 detection, measured at accurate mass (mass error in ppm is also given). In the case of
302 ingredients (A), HCH isomers were properly detected at 0.01 mg/Kg in fish meal. These
303 compounds were satisfactorily validated in all samples at 0.01 mg/Kg except for oils so, a SDL
304 at 0.05 mg/Kg was finally proposed (Table 1). The group of dibenzo[*al*]pyrene,
305 dibenzo[*ae*]pyrene, dibenzo[*ai*]pyrene and dibenzo[*ah*]pyrene were validated at 0.05 mg/Kg
306 since they could not be detected in at least 95 % of samples at the lowest level, despite that in
307 corn gluten these isomers were detected at 0.01 mg/Kg; The figure for oils (B) illustrates the
308 validation of benzo[*a*]pyrene, as a toxicity referent, at 0.01 mg/Kg. The validation of
309 chlorpyrifos methyl was of relevance since it is widely used as an insecticide. In both cases, the
310 detection at 0.01 mg/Kg was feasible in vegetable oils within low mass errors below 5 ppm; In
311 the case of feeds (C), the widely known DDTs, included in the target list due to their common
312 presence in marine resources as part of the larger group of fat-soluble POPs that readily
313 accumulate along the marine food chain, were satisfactorily validated at 0.01 mg/Kg for
314 ingredients, feeds and tissues but not for oils so, a SDL at 0.05 mg/Kg was finally proposed.
315 The herbicide carfentrazone-ethyl is used in wheat productions and sub-products widely
316 incorporated in feed compositions. Thus, it was included in the target list and a SDL at 0.01
317 mg/Kg was achieved in all samples studied. Finally, in fish tissues (D), the nw-XICs illustrate
318 the presence of phenanthrene and anthracene in the non-spiked samples, a fact that was also
319 observed in other types of samples, supporting the ubiquitous of light PAHs in many
320 environmental and food samples. The same occurred for ethoxyquin, synthetic preservative

widely used in fatty compositions to prevent lipids oxidation. The presence of at least two representative ions for each compound at the expected retention time with accepted mass errors (< 5ppm) allowed the reliable identification in positive samples.

3.4. Screening of real samples.

The validated screening was applied to different types of samples, searching for the target list of validated compounds. After the detection of any compound in the samples, the reliable identification was required in order to avoid reporting false positives. Although the presence of a *m/z* ion (commonly $[M+H]^+$), measured at accurate mass with low mass error, and the agreement in retention time, gives confidence to the analysis, we followed strict criteria for confirmation, which was based on the presence of, at least, another representative *m/z* ion (commonly fragment ion) with low mass error. This is feasible working in the QTOF MS instrument that allows the use of MS^E mode (details in section 2.3).

Figure 4 shows different positive findings in oils, proteins and feeds commonly used in animal farming. As shown, at least two representative *m/z* ions were necessary to unequivocally identify the presence of the compound in the sample, at the expected retention time (deviation ≤ ± 0.5 %, in comparison to standards) and measured at accurate mass within acceptable mass deviation (≤ 5ppm). The main pesticides found were chlorpyrifos methyl and pirimiphos methyl which were detected in several vegetable samples. Ethoxyquin, which use is currently authorized in feed ingredients, was also found in feeds but, additionally, it was identified in the edible part of several commercial fish samples. It seems that this synthetic preservative (and possibly its transformation products) can arrive to consumers. Earlier studies also reported the overall presence of synthetic antioxidants, such as ethoxyquin, in several commercially important species of farmed fish, namely Atlantic salmon, halibut, cod and rainbow trout and their aqua feeds, [30] as well as the carry-over from feed to fillet. [31] Therefore, quantitative methods directed towards this compound and its derivatives will surely be necessary in the near future.

As regards PAHs, "light" PAHs (e.g. phenanthrene, pyrene…) were in nearly all samples analyzed. Although they are not the carcinogenic PAHs according to EFSA, they are contaminants that can give (non-carcinogenic) toxic reactions in fish.[32] PAHs present poor MS-fragmentation, a fact that makes their identification troublesome. In this work, after evaluating the presence of the protonated molecule in the LE function, collision induced dissociation (CID) fragments, or characteristic isotopic ions, were also evaluated for positive samples to achieve a proper identification. As illustrative examples, at the bottom of Figure 4, positive findings for light PAHs are presented for samples of rapeseed, linseed and wheat.

After the first screening for which reference standards were available, we focused our screening to find any other pesticides for which reference standard was not available in our lab. Although tentative detection for some of these pesticides occurred, based on the presence of $[M+H]^+$ and/or $M^{+\cdot}$ in the LE function, further investigation of fragment ions, from the LE and/or HE function, did not allow us to confirm its identity, so they could not be reported as positive identifications.

With this work we pursued the achievement of an essential tool in food safety and toxicology: the use of wide-scope screening for detection of large number of compounds. The combination of GC-HRMS and LC-HRMS is nowadays the closest approach to the ideal "universal" screening. The GC-QTOF MS screening developed has been qualitatively validated for a notable number of pesticides and PAHs in complex samples from aquaculture activities, allowing the detection of these compounds in a rapid and efficient way at the low SDL established. The combination of this methodology in combination with the LC-QTOF MS screening previously developed allows searching of many undesirables of different polarity and volatility in distinct sample matrices. [22,33,34]

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498 Table 1. Validation results for pesticides. Screening detection limit (SDL).
499

Compound	positive/negative results (n=20) 0.01 mg/kg +/-	0.05 mg/kg +/-	SDL (mg/Kg)	Compound	positive/negative results (n=20) 0.01 mg/kg +/-	0.05 mg/kg +/-	SDL (mg/Kg)
2-Phenylphenol [a]	20/0	20/0	0.01	Fipronil	20/0	20/0	0.01
4-4'-Dichlorobenzophenone	20/0	20/0	0.01	Flucythrinate	11/9	20/0	0.05
Alachlor	19/1	20/0	0.01	Fludioxonil	17/3	19/1	0.05
Aldrin	4/16	14/6	-	Folpet	19/1	20/0	0.01
alpha-endosulphan	14/6	20/0	0.05	**gamma-HCH**	13/7	19/1	0.05
alpha-HCH [b]	14/6	19/1	0.05	**HCB**	13/7	19/1	0.05
Atrazine	20/0	20/0	0.01	Heptachlor	18/2	20/0	0.05
Atrazine desethyl	20/0	20/0	0.01	Heptachlor epoxide A	5/15	20/0	0.05
Atrazine desisopropyl	20/0	20/0	0.01	Heptachlor epoxide B	5/15	20/0	0.05
Azinphos methyl	20/0	20/0	0.01	**Hexachlorobutadiene**	10/10	16/4	-
Azoxystrobin	20/0	20/0	0.01	Imazalil	5/15	10/10	-
beta-endosulfan	12/8	20/0	0.05	Iprodione	20/0	20/0	0.01
beta-HCH	13/7	19/1	0.05	Isodrin	4/16	14/6	-
Bifenthrin	20/0	20/0	0.01	lamba-Cyhalothrin	7/13	20/0	0.05
Bromophos	20/0	20/0	0.01	Leptophos	19/1	20/0	0.01
Bromophos ethyl	19/1	20/0	0.01	Malathion	20/0	20/0	0.01
Bromopropilate	19/1	20/0	0.01	Metalaxyl	20/0	20/0	0.01
Buprofezin	19/1	20/0	0.01	Methamidophos	15/5	19/1	0.05
Cadusafos	18/2	20/0	0.05	Methidathion	12/8	15/5	-
Captafol	1/19	3/17	-	Methiocarb	20/0	20/0	0.01
Captan	4/16	4/16	-	Methoxychlor	20/0	20/0	0.01
Carbaryl	7/13	20/0	0.05	Metolachlor	20/0	20/0	0.01
Carbofuran	8/12	20/0	0.05	Metribuzin	20/0	20/0	0.01
Carbophenothion	1/19	12/8	-	**Mirex**	10/10	18/2	-
Carfentrazone ethyl	19/1	20/0	0.01	Molinate	20/0	20/0	0.01
Chinomethionat	20/0	20/0	0.01	Oxadixyl	13/7	19/1	0.05
Chlorfenapyr	20/0	20/0	0.01	Oxyfluorfen	20/0	20/0	0.01
Chlorfenson	13/7	15/5	-	**p,p'-DDD**	16/4	20/0	0.05
Chlorfenvinphos	20/0	20/0	0.01	**p,p'-DDE**	14/6	20/0	0.05
Chlorothalonil	3/17	19/1	0.05	**p,p'-DDT**	14/6	19/1	0.05
Chlorpropham	20/0	20/0	0.01	Parathion ethyl	20/0	20/0	0.01
Chlorpyrifos ethyl	20/0	20/0	0.01	Parathion methyl	20/0	20/0	0.01
Chlorpyrifos methyl	20/0	20/0	0.01	Pendimethalin	9/11	14/6	-
Coumaphos	20/0	20/0	0.01	**Pentachlorobenzene**	14/6	20/0	0.05
Cyanazine	18/2	20/0	0.05	Permethrin	2/18	20/0	0.05
Cyanophos	20/0	20/0	0.01	Phorate	12/8	19/1	0.05
Cyfluthrin	1/19	3/17	-	Phosmet	12/8	20/0	0.05
Cypermethrin	0/20	3/17	-	Pirimicarb	20/0	20/0	0.01
Cyprodinil	20/0	20/0	0.01	Pirimiphos methyl	20/0	20/0	0.01
delta-HCH	13/7	19/1	0.05	Procymidone	20/0	20/0	0.01
Deltamethrin	0/20	4/16	-	Propetamphos	1/19	9/11	-
Diazinon	20/0	20/0	0.01	Propham	20/0	20/0	0.01
Dichlofenthion	20/0	20/0	0.01	Propiconazole	20/0	20/0	0.01
Dichloran	20/0	20/0	0.01	Propoxur	10/10	20/0	0.05
Dichlorvos	20/0	20/0	0.01	Propyzamide	20/0	20/0	0.01
Dieldrin	14/6	20/0	0.05	Pyriproxyfen	20/0	20/0	0.01
Diflufenican	20/0	20/0	0.01	Quinalphos	20/0	20/0	0.01
Dimethoate	15/5	19/1	0.05	Resmethrin	20/0	20/0	0.01
Dioxathion	16/4	20/0	0.05	Simazine	20/0	20/0	0.01
Diphenylamine	20/0	20/0	0.01	tau-Fluvalinate	1/19	12/8	-
Endosulfan ether	20/0	20/0	0.01	Tefluthrin	14/6	20/0	0.05
Endosulfan sulfate	4/16	12/8	-	Terbacil	20/0	20/0	0.01
Endrin	12/8	20/0	0.05	Terbumeton	20/0	20/0	0.01
EPN	20/0	20/0	0.01	Terbumeton desethyl	19/1	20/0	0.01
Ethalfluralin	20/0	20/0	0.01	Terbuthylazine	18/2	20/0	0.05
Ethion	12/8	20/0	0.05	Terbuthylazine desethyl	20/0	20/0	0.01
Ethoxyquin	20/0	20/0	0.01	Terbutryn	20/0	20/0	0.01
Etofenprox	19/1	20/0	0.01	Tetradifon	19/1	19/1	0.01
Famphur	20/0	20/0	0.01	Thiabendazole	4/16	20/0	0.05
Fenamiphos	17/3	20/0	0.05	Tolclofos methyl	19/1	20/0	0.01
Fenarimol	20/0	20/0	0.01	Tolyfluanid	10/10	12/8	-
Fenhexamid	20/0	20/0	0.01	trans-Chlordane	0/20	4/16	-
Fenitrothion	20/0	20/0	0.01	Triadimefon	20/0	20/0	0.01
Fenoxycarb	19/1	20/0	0.01	Triflumizole	18/2	20/0	0.05
Fenthion	20/0	20/0	0.01	Trifluralin	20/0	20/0	0.01
Fenvalerate	0/20	5/15	-	Vinclozolin	19/1	20/0	0.01

500 [a]Non-black marked compounds are better detected as $[M+H]^+$ after adding water in the APCI source.
501 [b]Compounds in black are better detected as $M^{+\cdot}$ without water in the APCI source.

Table 2. Validation results for PAHs. Screening detection limit (SDL).

	positive/negative results		
	0.01 mg/kg	0.05 mg/kg	SDL
Compound[a]	+/-	+/-	
Naphthalene	20/0	20/0	-[b]
Acenaphthylene	20/0	20/0	-[b]
Acenaphthene	20/0	20/0	-[b]
Fluorene	20/0	20/0	-[b]
Phenanthrene	20/0	20/0	-[b]
Anthracene	20/0	20/0	-[b]
Fluoranthene	20/0	20/0	-[b]
Pyrene	20/0	20/0	-[b]
Benzo[*c*]fluorene	20/0	20/0	0.01
Cyclopenta[*c,d*]pyrene[c]	20/0	20/0	0.005
Benzo[*a*]anthracene	20/0	20/0	0.01
Chrysene	20/0	20/0	0.01
5-Methylchrysene	19/1	20/0	0.01
Benzo[*j*]fluoranthene	19/1	20/0	0.01
Benzo[*b*]fluoranthene	19/1	20/0	0.01
Benzo[*k*]fluoranthene	19/1	20/0	0.01
Benzo[*a*]pyrene	19/1	20/0	0.01
Indeno[*1,2,3,cd*]pyrene	19/1	20/0	0.01
Dibenzo[*a,h*]anthracene	19/1	20/0	0.01
Benzo[*g,h,i*]perylene	19/1	20/0	0.01
Dibenzo[*a,l*]pyrene	9/11	20/0	0.05
Dibenzo[*a,e*]pyrene	9/11	20/0	0.05
Dibenzo[*a,i*]pyrene	9/11	20/0	0.05
Dibenzo[*a,h*]pyrene	9/11	20/0	0.05

[a] PAHs are better detected as $[M+H]^+$ after adding water in the APCI source.
[b] The evaluation of the SDL was not feasible due to the presence of the analyte in all samples used for validation.
[c] Cyclopenta[*c,d*]pyrene was spiked at 0.005 and 0.025, respectively.

Figure captions.

Figure 1. Sample methodology based on QuEChERS clean-up method.

Figure 2. Validation results. Number of pesticides detected at 0.01 and 0.05 mg/Kg in different type of samples.

Figure 3. Validation. nw-XICs with the representative $[M+H]^+$ and/or $M^{+\cdot}$ molecule for the sample spiked at 0.01 mg/Kg (top) and non-spiked sample (bottom): (A) α, β, γ, δ-HCH isomers and dibenzo[*a,l*]pyrene, dibenzo[*a,e*]pyrene, dibenzo[*a,i*]pyrene, dibenzo[*a,h*]pyrene in feed ingredients (B) benzo[*a*]pyrene and chlorpyrifos methyl in oils (C) p,p'-DDD, p,p'-DDT and carfentrazone-ethyl in feeds (D) phenanthrene, anthracene and ethoxyiquin in fish tissues. ✓: accurate mass deviations within tolerance limits.

Figure 4. Real samples. nw-XICs for identified compounds in oils, proteins and feeds. For each matrix, the LE function (bottom) and HE (top) are shown to illustrate the presence of the protonated molecule (LE) and fragment ions (HE). ✓: accurate mass deviations within tolerance limits.

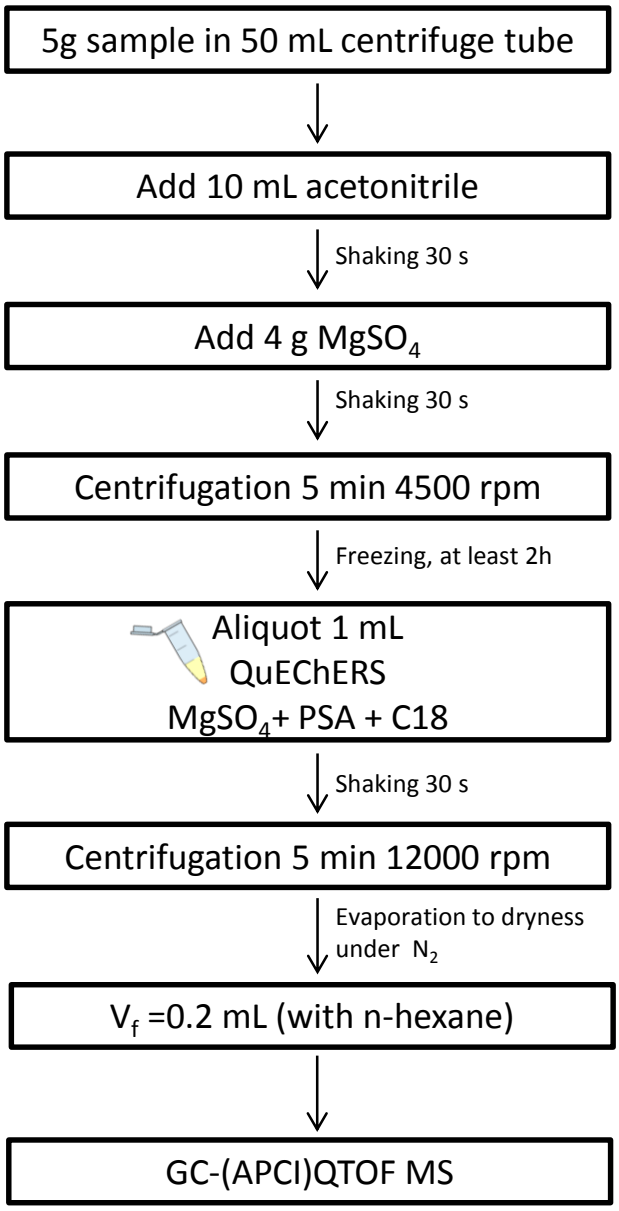
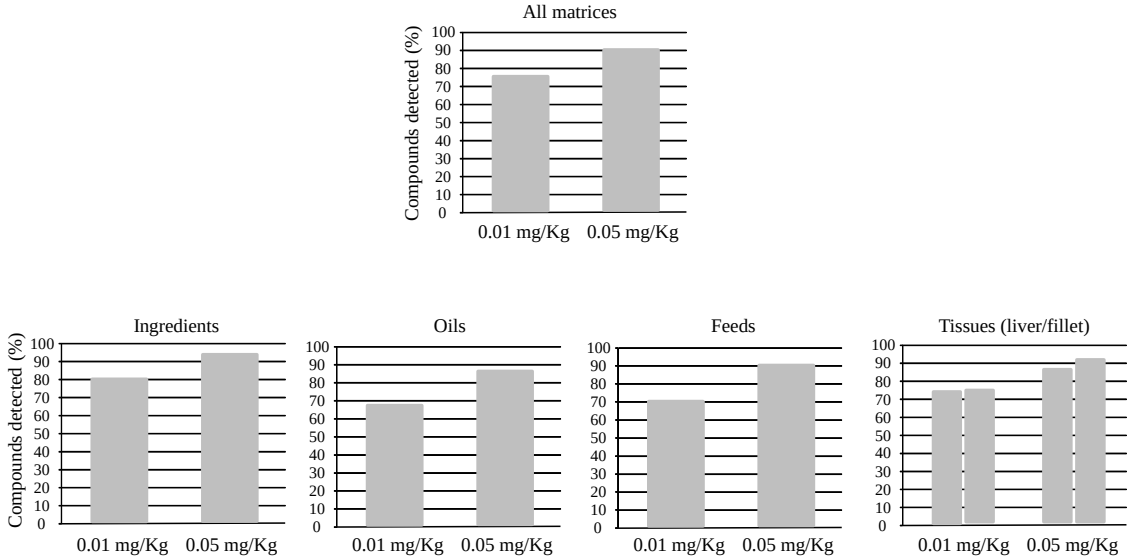

5g sample in 50 mL centrifuge tube
Add 10 mL acetonitrile
Shaking 30 s
Add 4 g MgSO4
Shaking 30 s
Centrifugation 5 min 4500 rpm
Freezing, at least 2h
Aliquot 1 mL
QuEChERS
MgSO4+ PSA + C18
Shaking 30 s
Centrifugation 5 min 12000 rpm
Evaporation to dryness
under N2
Vf =0.2 mL (with n-hexane)
GC-(APCI)QTOF MS

Figure 1.



569

570 Figure 2.

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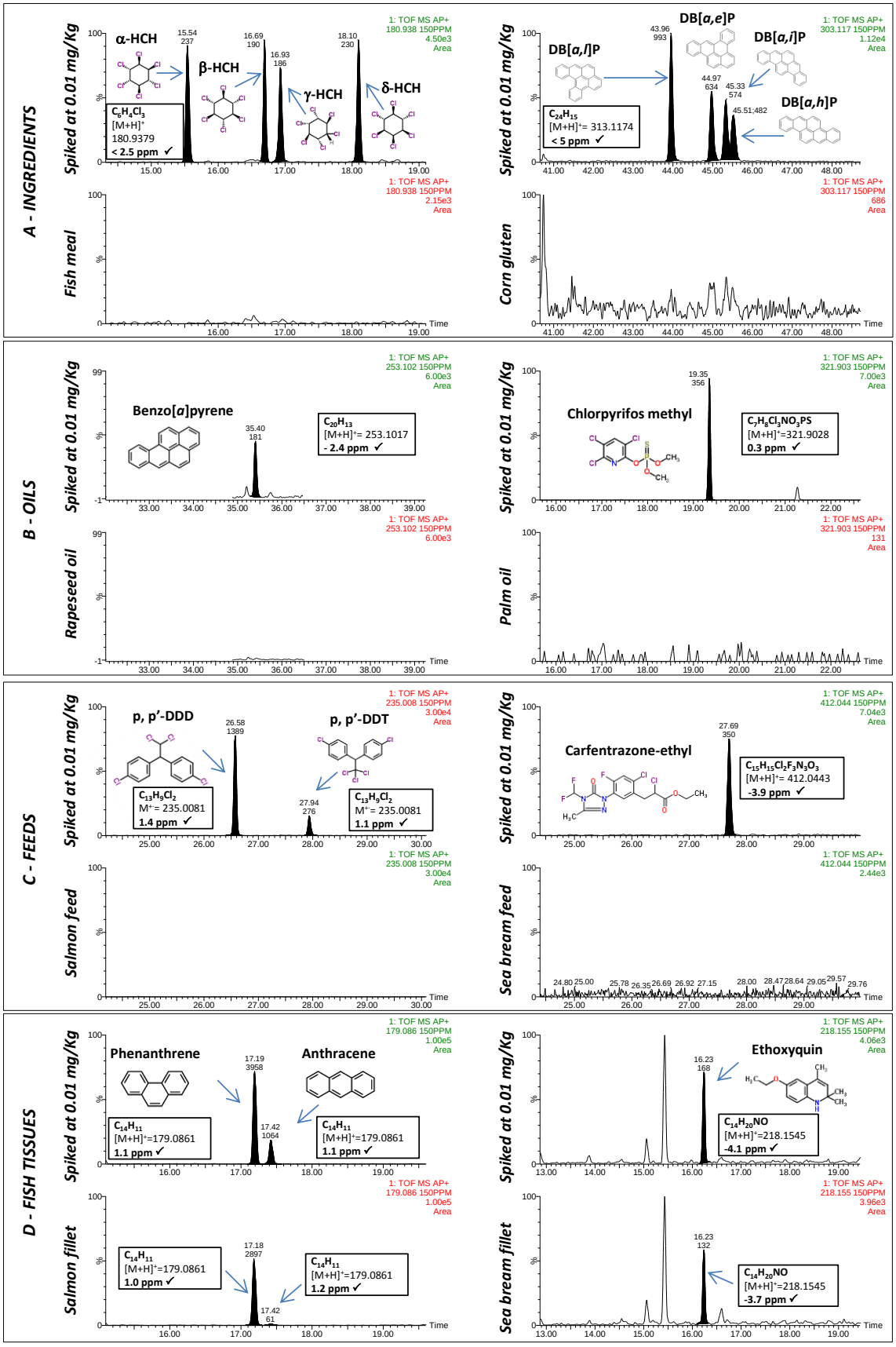
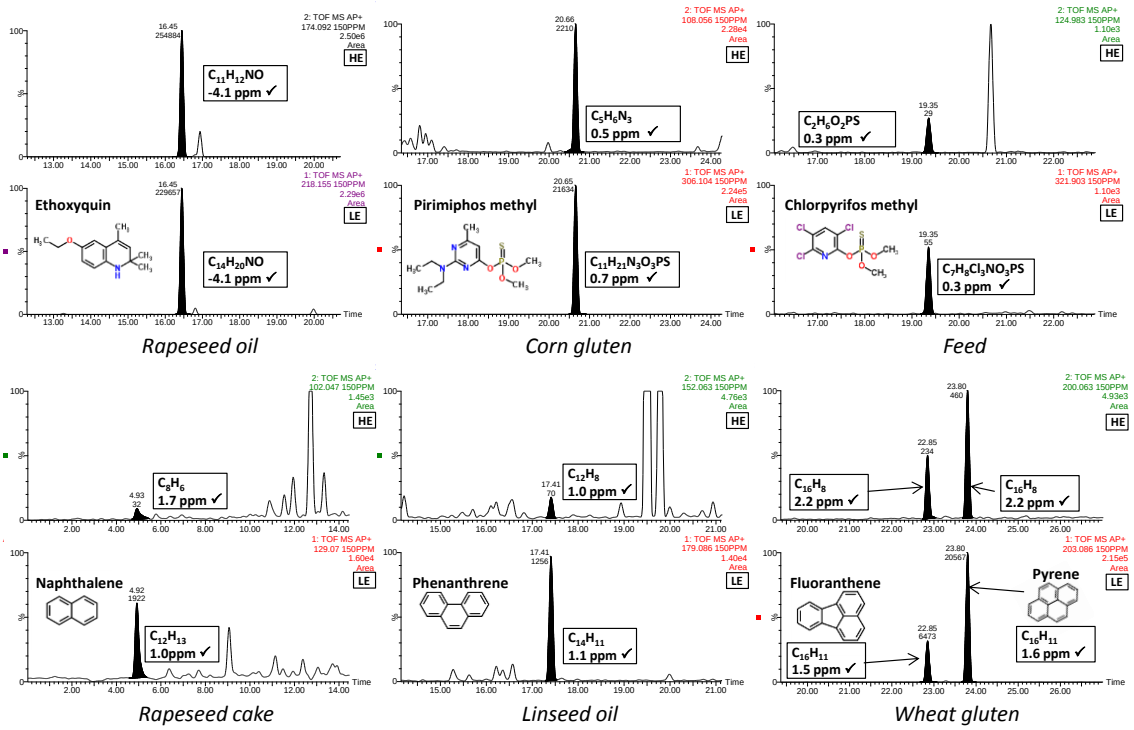

A - INGREDIENTS
Spiked at 0.01 mg/Kg
α-HCH
β-HCH
γ-HCH
δ-HCH
C6H4Cl3 [M+H]+ 180.9379 < 2.5 ppm ✓
DB[a,l]P
DB[a,e]P
DB[a,i]P
DB[a,h]P
C24H15 [M+H]+= 313.1174 < 5 ppm ✓
Fish meal
Corn gluten
B - OILS
Benzo[a]pyrene
C20H13 [M+H]+= 253.1017 - 2.4 ppm ✓
Chlorpyrifos methyl
C7H8Cl3NO3PS [M+H]+=321.9028 0.3 ppm ✓
Rapeseed oil
Palm oil
C - FEEDS
p, p'-DDD
p, p'-DDT
C13H9Cl2 M+= 235.0081 1.4 ppm ✓
C13H9Cl2 M+= 235.0081 1.1 ppm ✓
Carfentrazone-ethyl
C15H15Cl2F3N3O3 [M+H]+= 412.0443 -3.9 ppm ✓
Salmon feed
Sea bream feed
D - FISH TISSUES
Phenanthrene
Anthracene
C14H11 [M+H]+=179.0861 1.1 ppm ✓
C14H11 [M+H]+=179.0861 1.1 ppm ✓
Ethoxyquin
C14H20NO [M+H]+=218.1545 -4.1 ppm ✓
Salmon fillet
C14H11 [M+H]+=179.0861 1.0 ppm ✓
C14H11 [M+H]+=179.0861 1.2 ppm ✓
Sea bream fillet
C14H20NO [M+H]+=218.1545 -3.7 ppm ✓
Time

Figure 3.

578



579

580 Figure 4.