A reliable analytical approach based on gas chromatography coupled to triple quadrupole and time of flight analyzers for the determination and confirmation of polycyclic aromatic hydrocarbons in complex matrices from aquaculture activities

Jaime Nácher-Mestre[a], Roque Serrano[a*], Tania Portolés[a], Felix Hernández[a]

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

Laura Benedito-Palos[b] , Jaume Pérez-Sánchez[b]

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

Abstract

The potential of gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) with triple quadrupole analyzer (QqQ) has been investigated for the quantification and reliable identification of 16 EPA priority list of polycyclic aromatic hydrocarbons (PAHs) in animal and vegetable samples from aquaculture activities, which fat content ranged from 5 to 100%. Matrices analyzed included fish fillet, fish feed, fish oil and linseed oil. Combining optimized saponification and solid-phase extraction led to high efficiency in the elimination of interfering compounds, mainly fat, from the extracts. The procedure developed minimized the presence of these compounds in the extracts and provided satisfactory recoveries of PAHs. The excellent sensitivity and selectivity of GC-(QqQ)MS/MS in selected reaction monitoring (SRM) allowed to reach limits of detection at pg/g levels. Two SRM transitions were acquired for each analyte to ensure a safe identification of compounds detected in samples. Confirmation of positive findings was performed by GC coupled to high resolution time-of-flight mass spectrometry (GC-TOF MS). The accurate mass information in full acquisition mode together with its high mass resolution makes GC-TOF MS a powerful analytical tool for the unequivocal confirmation of PAHs in the matrices tested.

The method developed was applied to the analysis of real-world samples of each matrix studied with the result of detecting and confirming the majority of analytes at the μg/kg level by both QqQ and TOF MS analyzers.

* *Corresponding author. Tel. +34-964-387358; e-mail address: serrano@qfa.uji.es.*

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental pollutants from both natural and anthropogenic origins, such as the partial combustion of organic compounds, or pollution from petrochemical activities [1]. PAHs are lipophillic contaminants and tend to accumulate in the biotic compartment of the environment [1-6]. Concern has arisen as consequence of their potential adverse effects on organisms, including human beings, which has led to the inclusion of sixteen PAHs in the list of priority contaminants by the United States Environmental Protection Agency [7].

Marine aquaculture has suffered strong development in the last few decades as a consequence of increased fish consumption by the world population and decreasing wild stocks. Fish culture operates in parallel to traditional fisheries and nowadays both cultured and wild fish are important components of the Mediterranean diet [8,9]. Aquaculture products are subject to increasingly strict control and regulation. As an example, the European Commission Regulation (EC) 1881/2006 [10], have fixed a maximum level of 2 μg/kg (wet weight) for benzo[a]pyrene in fish.

PAHs enter into the marine environment through the atmosphere depositions and surface runoff, and due to of their lipophillic character they are accumulated by marine organisms [2-4,6,11-13]. As consequence of the artificial food chain in aquaculture activities, fish used as raw material for the manufacture of fish feed ingredients (fish oils and meals) are a potential source of PAHs in fish feed, which might be bioaccumulated by cultured fish [14]. Likewise, vegetable oils used in fish feed manufacture are another possible source of these family of contaminants [5,15].

PAHs determination in aquaculture matrices is difficult due to their complexity and the presence of interfering substances, mainly fats that are co-extracted with the analytes when

using techniques such as Soxhlet, microwave assisted extraction, pressurized liquid extraction, etc. [6,16-22], making necessary an efficiency clean-up before analysis. Traditionally, the clean up step is performed by SPE or preparative column chromatography using Florisil, silica, alumina or other absorbents available [6,18,21,23]. Nowadays, other modern techniques are applied, like accelerated solvent extraction followed by gel permeation chromatography [19], solid phase microextraction [22] or microwave-assisted extraction [21].

High-performance liquid chromatography (HPLC) with fluorescence detection has been widely used for PAHs determination [16]. However, in contemporary analysis of complex matrices, gas chromatography (GC) rather than LC is often preferred for separation, identification and quantification because GC generally affords greater selectivity, resolution and sensitivity for PAHs determination. GC coupled to mass spectrometry (MS) using single quadrupole has been widely used for the determination of organic compounds in environmental samples [13,15,19]. In recent years, tandem mass spectrometry (MS/MS) is increasingly being used, as a more valuable approach because of its higher sensitivity and selectivity, minimizing or even removing many interferences. GC-MS/MS using ion trap (ITD) or triple quadrupole (QqQ) analyzers, has been successfully applied to the analysis of PAHs in a variety of matrices [21,24]. The use of two stages of mass analysis in MS/MS systems based on QqQ offers the possibility of applying selected reaction monitoring (SRM), one of the most selective and sensitive approaches for quantification and confirmation, especially at trace levels. Thus, GC-MS/MS (QqQ) applications in the environmental and food analysis fields are notably increasing in the last few years.

In recent years, high resolution time-of-flight mass analyzer (TOF MS) has become increasingly prevalent in environmental analysis. This technique can provide conclusive information for the reliable confirmation of target analytes and also for the elucidation of non-

target compounds due to its unrivalled full spectra sensitivity together with its elevated mass resolution and mass accuracy [25,26].

The aim of this paper is to develop a reliable and sensitive methodology based on the use of advanced GC-MS techniques for the quantitative determination and safe identification of the 16 PAHs EPA priority contaminants in complex matrices from the aquaculture activities. In order to reach this objective we have studied the extraction and clean-up steps, searching for extracts with a minimal content of fats. The developed methodology has been applied to real-world samples of fish feed, raw materials and fish speciments from feeding trials performed with gilthead sea bream (*Sparus aurata L.*) carried out as a part of the European Union project "Sustainable Aquafeeds to Maximise the Health Benefits of Farmed Fish for consumers" (AQUAMAX). Contract number: 016249-2. The acquisition of two selective SRM transitions for each target analyte by GC-(QqQ)MS/MS has allowed the quantification and identification of PAHs at the low µg/kg level. Besides, positive GC-(QqQ)MS/MS findings have been confirmed by GC-TOF MS, taking advantage of the high mass resolution and mass accuracy provided by this technique.

EXPERIMENTAL

Materials and reagents.

PAHs analytical standard 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, benzo(*g,h,i*)perylene was purchased from Dr Ehrenstorfer (Promochem, Wesel, Germany) with a purity 97-99.8% at 10 µg/mL in cyclohexane. Stock solution (1 µg/mL) were prepared by dissolving reference standard in n-hexane and stored in

120 a freezer at –20 ºC. Working solutions were prepared by diluting stock solution in n-hexane
121 for sample fortification and calibration curves.

122 In addition, benzo(*a*)anthracene-D_{12} from Dr Ehrenstorfer (Promochem, Wesel, Germany)
123 was used as surrogate internal standard in the validation study and real samples analysis.
124 Working solutions of labeled standards were prepared by dilution of commercial solutions
125 with n-hexane and stored at 4ºC.

126 Methanol, dichloromethane, n-hexane and ethyl acetate (ultratrace quality) were purchased
127 from Scharlab (Barcelona, Spain). Anhydrous sodium sulfate of pesticide residue quality
128 (Scharlab) was dried for 18 h at 300°C before use. Potassium and sodium hydroxide were
129 purchased from Scharlab. The 0.2µm filters were from Serviquimia (Castellón, Spain). Silica
130 cartridges (Strata 0.5g and 1g; Phenomenex, USA) and Supelclean LC-florisil SPE tube (0.5g
131 and 1g; Sigma-Aldrich, Madrid, Spain) were used in SPE experiments.

132

133 **Sample material.**

134 Gilthead sea bream (*Sparus aurata L.*) specimens of Atlantic origin (Ferme Marine de
135 Douhet, Ile d'Oléron, France) were cultured at the Instituto de Acuicultura de Torre la Sal,
136 Spain (IATS, CSIC) and collected when fish accomplished commercial size (≈500g). The
137 left-side fillets (denuded from skin and bone) were excised and stored at -20 ºC until analysis.
138 Fish feed supplied to sea bream during feeding trials from AQUAMAX project experiments
139 were stored at -20ºC until analysis. Fish oil and linseed oil used in fish feed manufacture, used
140 usually as raw materials of fish feed, were also stored at -20ºC until analysis. Ingredients and
141 chemical composition of fish feed are shown in **Table 1**.

142

143

GC instrumentation

Two GC systems (Agilent 6890N; Agilent Palo Alto, USA) equipped with an autosampler (Agilent 7683) were coupled to 1) triple quadrupole mass spectrometer (Quattro Micro GC; Micromass, Boston, USA) and 2) time-of-flight mass spectrometer (GCT, Waters Corporation, Manchester, U.K.), both operating in electronic ionisation (EI). In both cases, the GC separation was performed using a fused silica HP-5MS capillary column with a length of 30 m, an internal diameter of 0.25 mm and a film thickness of 0.25 μm (J&W Scientific, Folson, CA, USA). The injector temperature was set to 250°C. Splitless injections of 1 μL of the sample were carried out. Helium (99.999%; Carburos Metálicos, Valencia, Spain) was used as carrier gas at a flow rate of 1 mL/min. The interface and source temperature were set to 250°C in both systems and a solvent delay of 3 min was selected.

The oven temperature program in GC-QqQ was as follows: 90°C (1 min); 10°C/min to 250°C; 5°C/min to 300°C (3min). The oven program in GC-TOF analysis was programmed as follows: 90 °C (1min); 5 °C/ min to 300 °C (2 min). In both cases helium was used as carrier gas at 1 mL/min. The QqQ system operated in MS/MS mode using 99.995% Argón (Carburos Metálicos) as collision gas at a pressure of 0.28 Pa in the collision cell, and a dwell time per channel between 0.1 and 0.3 s. The time-of-flight mass spectrometer was operated at 1 spectrum/s, acquisition rate over the mass range m/z 50-300, using a multichannel plate voltage of 2650 V. TOF-MS resolution was approximately 7000 (FWHM). Heptacosa standard, used for the daily mass calibration and as lock mass, was injected via syringe in the reference reservoir at 30 °C for this purpose; the m/z ion monitored was 218.9856. The application manager TargetLynx and QuanLynx were used to process the qualitative and quantitative data obtained from calibration standards and from sample analysis.

Analytical procedure

Before analysis, samples were thawed at room temperature and, in the case of fish fillet and fish feed, were carefully ground using mill Super JS (Moulinex, Ecully Cedex, France). Approximately 2 g of sample were homogenized with 6 g of anhydrous sodium sulfate and the blend was spiked with 100µL of surrogate solution (25 ng/mL). Ten milliliters of methanolic solution KOH 1 M were added to the mixture and were submitted to a saponification for 3 h at 80°C. Then, analytes were extracted twice with 8 mL of n-hexane and the solution was filtered through 0.2 µm filter and concentrated under gentle nitrogen stream at 40°C to 1 mL (in the case of oils, extracts were concentrated to 5 mL). The 1mL extract was passed through a Florisil SPE cartridge, previously conditioned with 6 mL of n-hexane, and eluted with 8 mL dichloromethane:hexane (DCM:Hx) (20:80). The eluate was evaporated under a gentle nitrogen stream at 40ºC and the final residue was redisolved in 0.25 mL of n-hexane. The final extracts obtained after clean-up were analyzed by GC-QqQ under the experimental conditions shown in **Table 2**. Quantification of samples was carried out by means of calibration curves with standard in solvent using the internal standard method. Positive real samples were reanalyzed by GC-TOF MS for an additional confirmation of the compounds detected by QqQ.

Validation

GC-(QqQ) MS/MS statistical validation of the method was performed by evaluating the following parameters:

– Linearity. The calibration curves were obtained by injecting reference standard solutions in triplicate. The concentration range tested was 0.5–200 ng/mL (eight points). Linearity was

191 assumed when the regression coefficient was greater than 0.99 with residuals randomly
192 distributed and lower than 30%.

193 – Accuracy. It was evaluated by means of recovery experiments, analyzing "blank" samples
194 of each matrix spiked at three concentration levels in fish fillet and fish feed (0.125, 1.25 and
195 2.5 µg/kg, in sixtuplicate) and two levels (1.25 and 2.5 µg/kg) in fish oil and linseed oil (in
196 triplicate). Previously, "blank" samples were analyzed to determine the concentration of the
197 analytes present in the matrices (fish fillet and fish feed in sixtuplicate, and oils in triplicate)
198 (see **Tables 3 and 4**).

199 – Precision. The precision, expressed as repeatability of the method, was determined in terms
200 of relative standard deviation in percentage (RSD, %) from recovery experiments at each
201 fortification level.

202 – Limit of quantification (LOQ) objective. The LOQ was established as the lowest
203 concentration that was validated following the overall analytical procedure with satisfactory
204 recovery (between 70-110%) and precision (RSD<20%).

205 – Limit of detection (LOD). It was statistically estimated, from the quantification transition,
206 as the analyte concentration giving a peak signal of three times the background noise from the
207 chromatograms at the lowest fortification level tested. When the analytes were present in the
208 "blank", LODs were calculated from the chromatogram of the analyzed "blank" sample. The
209 LOD was calculated using the software option for estimating the S/N ratio and referring this
210 value to a S/N value of three [27].

211 – Confirmation criteria: The Q/q ratio, defined as the ratio between the intensity of the
212 quantification (Q) and the confirmation (q) transitions, was used to confirm the identity of the
213 compounds detected in samples. A safe confirmation was assumed taken into account the
214 European Commission Decision (2002/657/CE) [28]. Briefly, to confirm a finding as an actual

positive, a maximum ratio tolerance ±20% was accepted when the relative intensity of the confirmative transition was >50% as regards the quantitative one (Q/q ratio 1-2). For higher Q/q ratios, the tolerances increased. Thus, deviations ±25% (relative intensity 20-50%, Q/q ratio 2-5), ±30% (relative intensity 10-20%, Q/q ratio 5-10) and 50 % (relative intensity ≤ 10%, Q/q ratio >10) were accepted. This criterion was originally defined on measures to monitor certain substances and residues thereof in live animals and animal products, and it is being increasingly used in other fields like environmental and biological samples analysis [29,30]. Obviously, the agreement in the retention time for sample and reference standard was also required to confirm a positive finding. The Q/q ratio for each compound was empirically determined as the average value calculated from eight standard solutions injected in triplicate.

RESULTS AND DISCUSSION

Cleanup optimization.

Clean up is an important step in environmental and food analysis due to the complexity of the matrices and the selectivity required. Traditionally, analytical methods for PAHs in fatty samples include an alkaline saponification. This treatment offers a satisfactory lipid removal of the extracts [5,6,31]. In our work, as consequence of the complexity of the matrices analyzed (especially fish and vegetable oils), a modification of the commonly applied saponification procedures was necessary in order to reach the complete reaction of lipids [6,32]. Different alkaline solutions were proved, including NaOH/MeOH 1M, NaOH/EtOH 1M, NaOH/MeOH saturated, NaOH/EtOH saturated and KOH/MeOH 1M, KOH /EtOH 1M, KOH/MeOH saturated, KOH/EtOH saturated. The effect of adding hexane or water was also tested but poor results were observed, so their addition was discarded. In all cases, more than 2 hours at temperature higher than 60ºC were required to remove lipid interferences and to hydrolyze

lipids and esters produced in the process. As can be seen in **Figure 1**, fluorene, fluoranthene and pyrene were masked when the saponification time was below three hours. In those conditions, esters and free fatty acids were not hydrolyzed, producing matrix effects. On the contrary, when the sample was submitted to a saponification of three or more hours at 80ºC, the matrix effect decreased improving selectivity and sensitivity. Thus, a poor saponification led to unsatisfactory data. These results are in the line of previous data reported on low organic compounds recoveries in marine matrices, suggesting poor saponification as the cause [6,31]. We finally obtained the most satisfactory results when using KOH/MeOH 1M during 3 hours at 80ºC (**Figure 1**). Under these conditions, saponification process removed the most of fats after extraction with n-hexane and filtration (see analytical procedure).

An additional step using SPE clean-up was still necessary to remove several interfering compounds. Silica and Florisil cartridges with 0.5 g and 1 g stationary phase, and different elution procedures were compared as regards efficiency in lipid removal, recovery of analytes, elution volume and elution time.

Florisil cartridges (1g) were finally selected, and elution was performed with DCM:Hx (20:80), which led to cleaner extracts and better recoveries than other mixtures assayed, such as n-hexane, DCM or ethyl acetate. Using DCM:Hx (20:80), interfering compounds seemed to be more retained in the cartridge, while PAHs eluted in cleaner eluates.

Other SPE purification procedures reported, using Silica, Florisil [18] or C_{18} cartridges [33] did not allow the determination of light PAHs as naphthalene and acenaphthylene, this being a notable difference with the methodology developed in the present work.

262 **GC-MS/MS optimization**

263 Chromatographic conditions for PAHs determination are particularly complex due to their
264 similar structure and properties. These commonly lead to similar retention times for them with
265 poor peak separation at the base line, especially when using liquid chromatography [16].

266 High resolution GC coupled to MS using single quadrupole or ITD working in single ion
267 monitoring has been widely used [16,19,34]. However, there is little information about the use of
268 tandem mass spectrometry with QqQ for the determination of PAHs in complex aquaculture
269 samples. Optimization of GC-MS/MS method was performed in this work by injecting
270 hexane standard solutions into the GC-(QqQ)MS/MS system operating in EI mode. Full-scan
271 spectra for all PAHs congeners showed basically the molecular ion, with poor fragmentation.
272 So, the molecular ion was selected as the precursor ion for fragmentation purposes in the
273 collision cell. Different values of collision energy (between 20 and 60 eV) were tested to
274 perform the subsequent fragmentation of the selected precursor ion. Two MS/MS transitions
275 were selected for each compound, normally the most sensitive ones, in order to have a reliable
276 confirmation of the identity of the analyte. The dwell time parameter was also optimized
277 between 0.1 and 0.3 s in order to obtain peaks with at least ten points maintaining satisfactory
278 sensitivity for each compound. **Table 2** shows the precursor and product ions corresponding
279 to the quantitative and confirmation transitions monitored. Linearity of the relative response
280 of analytes was established by analyzing hexane standard solutions in the ranges 0.5–200
281 ng/mL. Regression coefficients above 0.995 were obtained for all the compounds with
282 residuals lower than 30% and without any clear trend in their distribution.

283

284

285

GC-TOF MS optimization

In our study, GC-TOF MS was used for additional confirmation of the PAHs detected in samples by QqQ. For these purpose a TargetLynx processing method was using reference standards solutions in solvent. The MS spectrum for each compound was obtained and four ions were selected, for which elemental compositions were proposed (see **Table 5**). Narrow mass windows of 0.02 Da were chosen as a compromise between sensitivity, peak shape and accurate mass measurements. Q/q intensity ratios were used as confirmation parameter. Theoretical Q/q ratios were calculated from solvent standard solutions as the ratio between the most sensitive ion (Q, quantitative) and each of the other measured ions (q, confirmative). The selection of four ions provided up to three Q/q intensity ratios that could be used for the reliable confirmation of compounds in samples. Tolerances accepted were in accordance with the European Commission Decision (2002/657/CE) [28]. The agreement in the retention time of the analyte in sample and the reference standard was also required to confirm a positive result (relative error ± 0.5 %).

Analytical parameters

"Blank" samples used for validation purposes contained appreciable concentrations of several PAHs. Therefore, it was necessary to accurately calculate this concentration in order to correct the quantitative results in recovery experiments [27]. One labelled standard (benzo(*a*)anthracene-D_{12}) was added at the initial stage of the procedure as quality control (surrogate) in order to correct possible losses during the overall procedure and instrumental deviations for all compounds except for naphthalene and acenaphthylene which were processed without surrogate.

309 Fortified samples of the four matrices studied in this work were analyzed applying the
310 developed methodology with satisfactory results (see **Tables 3 and 4**).

311

312 **Fish fillet and fish feed**

313 Pools of fish fillet and fish feed, blending 325 g of left-hand fillets and 50 g of fish feed,
314 respectively, were used for the statistical validation experiments. First, six replicates of each
315 matrix were analyzed by applying the analytical procedure proposed to determine the content
316 of selected analytes in these samples. Results showed the presence of trace levels of most of
317 them, as could be expected because of the usual presence of PAHs in marine samples [2,3,5],
318 especially in fish feed due to the concentration of contaminants during the manufacture
319 process with fish derivatives [35]. Fluorene, phenanthrene, fluoranthene and pyrene presented
320 the highest concentrations in these "blank" samples. As can be seen in **Table 3**, samples were
321 fortified at 0.125, 1.25 and 2.5 μg/kg (n=6) and submitted to the developed procedure. In
322 general, recoveries were satisfactory with average values between 80 and 110% and RSDs
323 lower than 30 %, except for naphthalene which presented poor precision. LODs were found to
324 be at sub-μg/kg level in both sample matrices (all ≤ 0.1μg/kg). Such low LODs were achieved
325 thanks to the efficient clean-up applied together with the selectivity and sensitivity provided
326 by QqQ in SRM mode.

327 The average Q/q intensity ratios calculated from reference standards in solvent (see **Table 2**)
328 were compared to those experimentally obtained from spiked sample extracts to test the
329 robustness of these values and potential matrix interferences that might affect Q/q ratios and,
330 consequently, the confirmation process. Average deviations obtained were in all cases in
331 accordance with the criteria indicated in the validation section.

As an example, **Figure 2** shows representative chromatograms of fish fillet (naphthalene, dibenzo(*a,h*)anthracene) and fish feed (acenaphthene and benzo(*g,h,i*)perylene) fortified at 0.125 µg/kg (except for naphthalene, 1.25 µg/kg).

Q/q ratios for all analytes were between 1 and 5, except for pyrene which showed by far the highest Q/q ratio with a value of 28.17 (**Table 2**). This fact allows the determination of pyrene at LOQ level, but makes difficult its confirmation at low concentrations below the LOQ. No more transitions with the precursor ion selected (m/z=202) nor other precursors were found leading to better sensitivity.

Fish and Linseed oil

Frequently oils added in fish feed, as fish oil and linseed oil, are a source of contamination [36]. These ingredients are main part of the fish feed compositions, necessary to reach healthy composition of the diets (**Table 1**). For these reasons, data obtained in the analysis of the "blank" sample used for validation reveal appreciable concentrations of PAHs. As reported by several authors, fats and oils represent one of the major sources of contamination in the diet because of their lipophilic nature [37]. **Table 4** shows the analytic parameters for the oil matrices studied, with validation data expressed as µg/kg lipid weight. Fluorene, phenanthrene, fluoranthene and pyrene were, similarly to fish fillet and fish feed, the most abundant PAHs found in the oils. "Blanks" were fortified at 1.25 and 2.5µg/kg and submitted to the developed procedure with satisfactory recoveries in all cases, and RSD lower than 30 %. Limits of detection were at the low µg/kg levels in fish oil and linseed oil. LODs, LOQs and Q/q ratios were calculated in the same way than fish fillet and fish feed obtaining in all cases satisfactory results in accordance with the criteria established.

356 **Application to real samples**

357 The optimized sample procedure followed by GC-(QqQ)MS/MS and GC-TOF MS was
358 applied (by triplicate) to the analysis of raw materials, fish feeds and fillets samples from
359 "AQUAMAX" long term feeding trials with gilthead sea bream (*Sparus aurata L.*) in the
360 frame work of an European project. Fish were exposed through the entire productive cycle to
361 experimental diets with graded levels of fish oil replacement, studing the health and welfare
362 of the farmed fish, and maximising the health-promoting properties, safety, quality and
363 acceptability of the final product to the consumer [35]. PAHs were determined in 19 fish fillet
364 samples, 8 fish feed, 1 fish oil, 1 linseed oil, 1 rapeseed oil and 1 palm oil from fish exposed
365 through the productive cycle (14 months) to experimental diets with different percentages of
366 fish oil replacement with vegetable oils.

367 Regarding GC-QqQ analysis, **Table 6** shows the PAH concentrations detected in aquaculture
368 samples. All PAHs studied were found in fish feed at concentrations range of 0.2-12.7 μg/kg.
369 The only exception was naphthalene in six fish feed samples, where it was found at
370 concentrations around 200 μg/kg fresh weight. All fish fillet samples analyzed were positive
371 for phenanthrene, fluoranthene and pyrene (range of 0.2-11.4 μg/kg). Benzo(*a*)pyrene was
372 detected only in one sample of fish fillet, but a concentration (3.9 μg/kg) above the maximum
373 established by the European Commission Regulation (EC) No 1881/2006. Rapeseed and palm
374 oils presented lower total load of PAHs (12 μg/kg) in comparison with fish and linseed oils,
375 which had a total load of 65.7 μg/kg and 47 μg/kg respectively, principally due to the major
376 presence of fluorene, phenanthrene, fluoranthene and pyrene (see values in **Table 4**).

377 Illustrative chromatograms for real samples analyzed are shown in **Figure 3,** where the
378 quantification and confirmation transitions monitored for several PAHs can be seen in the
379 different matrices analyzed. As shown in the chromatograms, both quantification and

380 confirmation was feasible at sub-µg/kg levels with satisfactory peak shape. Benzo(*a*)pyrene
381 was not detected in fish fillet, whereas it was quantified and confirmed in the diets.

382 GC-TOF MS was used for the additional confirmation of PAHs previously detected by QqQ.
383 The GC-TOF detection and identification of target PAHs in the samples was carried out by
384 obtaining up to 4 narrow window eXtracted Ion Chromatograms (nw-XIC) (0.02 Da) at
385 selected m/z ions for every compound (**Table 5**). The software application TargetLynx
386 automatically processed data and reported qualitative data. Analyte confirmation was carried
387 out by comparison of the Q/q intensity ratios obtained in samples with obtained from
388 reference standards in solvent. In all cases, the presence of chromatographic peaks at the
389 expected retention time and the attainment of all Q/q ratios when comparing with the
390 reference standard allowed the confirmation of these findings in samples. Additionally, the EI
391 accurate mass spectra generated by TOF MS were obtained and the mass errors for
392 representative ions were calculated, giving high confidence to the confirmation process.

393 As an example, **Figure 4** shows illustrative GC-TOF chromatograms (nw-XICs) for pyrene
394 and naphthalene detected in fish fillet and fish feed, respectively. A reliable confirmation was
395 feasible as all Q/q ratios were in agreement with the European Commission Decision
396 (2002/657/CE) [28]. In addition, EI accurate mass spectra provided mass errors for the four ions
397 monitored below 2.9 mDa.

398 Most of positives detected and confirmed by triple quadrupole could be confirmed by TOF.
399 When findings could not be confirmed by TOF, the reason was that no chromatographic peak
400 was present in the nw-XIC. All these cases occurred when analyte concentration was quite
401 low (generally $\leq$ 1µg/kg), as a consequence of the lower sensitivity of TOF compared with
402 triple quadrupole working in SRM mode.

However, in the "full-analysis" field, TOF's capability of interrogating full-spectrum data after acquisition seems really interesting, as it allows screening for unexpected compounds and metabolites at the time of injection without re-analyzing the sample [38,39]. The main advantage is the huge number of compounds that might be investigated, with the obvious restrictions deriving from the requirements of GC and MS analysis. High-occurrence metabolites, or new compounds after being properly identified with TOF-MS, might then be included in analytical methods using QqQ if the commercial reference standards are available. This attractive approach is under study at present and research in our laboratories for the samples analyzed in this work.

CONCLUSIONS

A rapid, sensitive and selective analytical methodology for the determination PAHs in high lipid content aquaculture samples has been developed, rearching low quantification levels by means of an efficient clean-up step combining saponification and SPE procedures previously to the injection of the extracts in GC coupled to both QqQ and TOF analyzers. Especial attention was paid to naphthalene, the earliest eluting chromatographic peak , which was of major difficulty in part also due to the high levels found in the "blank" fish fillet and fish feed samples used for validation experiments. The combined use of triple quadrupole and time-of-flight analyzers gives an extraordinary reliability to the confirmation process of the compounds detected in samples.

ACKNOWLEDGEMENTS

The authors are thankful to the financial support of the European Union Project: Sustainable Aquafeeds to Maximise the Health Benefits of Farmed Fish for Consumers (AQUAMAX). Contract number: 016249-2. The authors acknowledge the Serveis Centrals d´Instrumentació Científica (SCIC) of University Jaume I for using the GC-TOF MS.

446 **FIGURE CAPTIONS**

447 **Figure 1.** GC-TOF MS extracted ion chromatograms (mass window 0.02 Da) for Fluorene
448 (m/z=166.0782) and Fluoranthene and Pyrene (m/z= 202.0782) in fortified fish fillet extracts
449 (50µg/kg) submitted to a saponification at 80ºC for an hour (A) and for three hours (B).
450 Numbers below the m/z show the intensity of the ion acquired.

451 **Figure 2. "**Blank" sample, spiked sample (0.125 µg/kg) and standard (1 ng/mL, equivalent to
452 0.125 µg/kg in sample)) SRM chromatograms from fish fillet and fish feed. Naphthalene
453 (128>102 transition acquired) and dibenzo(*a,h*)anthracene (278>276 transition acquired) in
454 fish fillet; acenaphthene (152>126 transition acquired) and benzo(*g,h,i*)perylene (276>274
455 transition acquired) in fish feed. *Spiking level for naphthalene was 1.25 µg/kg, and reference
456 standard was 10ng/mL, equivalent to 1.25 µg/kg in sample. Numbers below the transition
457 show the intensity of the transition acquired.

458 **Figure 3.** SRM Chromatograms for selected PAHs in real aquaculture samples. Two
459 transitions were necessary to the correct confirmation of the identity of the compounds, "Q"
460 quantification transition and "q" confirmation transition. ✓: Q/q ratio within tolerance limits.
461 Transitions monitored: acenaphthene: 154>152, 153>126; pyrene: 202>200, 202>152;
462 benzo(*a*)pyrene: 252>250, 250>248; fluoranthene: 202>200, 202>150; benzo(*a*)anthracene:
463 228>226, 228>224. Numbers below the transition show the intensity of the transition
464 acquired.

465 **Figure 4.** GC-TOF MS extracted ion chromatograms at four m/z (mass window 0.02 Da) for
466 Pyrene detected in fish fillet and Naphthalene in fish feed. Accurate mass spectra (bottom). ✓:
467 Q/q ratio within tolerance limits. St: reference standard; S: sample. Ions acquired are shown in
468 **Table 5**. Numbers below the transition shows the intensity of the transition acquired.

469

REFERENCES

1. Menzie CA, Potocki BB, Santodonato J. *Environ. Sci. Technol.* 1992; **26**: 1278.

2. Baumard P, Budzinski H, Garrigues P, Sorbe JC, Burgeot T, Bellocq J. *Mar. Pollut. Bulletin* 1998; **36**: 951.

3. Filipkowska A, Lubecki L, Kowalewska G. *Anal. Chim. Acta* 2005; **547**: 243.

4. Hellou J, Leonard J, Collier TK, Ariese F. *Mar. Pollut. Bull.* 2006; **52**: 433.

5. Moret S, Conte LS. *J. Chromatogr. A* 2000; **882**: 245.

6. Perugini M, Visciano P, Giammarino A, Manera M, Di Nardo W, Amorena M. *Chemosphere* 2007; **66**: 1904.

7. USEPA, Toxicological profile for polycyclic aromatic hydrocarbons (PAHs), /http://www.usepa.gov/S.

8. Martí-Cid R, Bocio A, Llobet JM, Domingo JL. *Food Chem. Toxicol.* 2007; **45**: 1968.

9. Martí-Cid R, Llobet JM, Castell V, Domingo JL. *Food Chem Toxicol.* 2008; **46**: 3163.

10. Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. (eurlex.europa.eu/LexUriServ/site/en/oj/2006/l_364/l_36420061220en00050024.pdf)

11. Baumard P, Budzinski H, Garrigues P, Narbonne JF, Burgeot T, Michel X, Bellocq J. *Mar. Environ. Res.* 1999; **47**: 415.

12. Hellou J, Haya K, Steller S, Burridge L. *Aquat. Conserv.* 2005; **15**: 349.

13. Malik A, Ojha P, Singh KP. *B. Environ. Contam. Tox.* 2008; **80**: 134.

14. Hellou J, Haya K, Steller S, Burridge L. *Aquat. Conserv.* 2005; **15**: 349.

15. Patel K, Fussell RJ, Hetmanski M, Goodall DM, Keely BJ. *J. Chromatogr. A* 2005; **1068**: 289.

493 16. Poster DL, Schantz MM, Sander LC, Wise SA. *Anal. Bioanal Chem.* 2006; **386**: 859.

494 17. Pena T, Pensado L, Casais C, Mejuto C, Phan-Tan-Luu R, Cela R. *J. Chromatogr. A*
495 2006; **1121**: 163.

496 18. Navarro P, Cortazar E, Bartolomé L, Deusto M, Raposo JC, Zuloaga O, Arana G,
497 Etxebarria N. *J. Chromatogr. A* 2006; **1128**: 10.

498 19. Liguori L, Heggstad K, Hove HT, Julshamn K. *Anal. Chim. Acta* 2006; **573-574**: 181.

499 20. Fernández-González V, Concha-Graña E, Muniategui-Lorenzo S, López-Mahía P, Prada-
500 Rodríguez D. *J. Chromatogr. A* 2007; **1176**: 48.

501 21. Fernández-González V, Muniategui-Lorenzo S, López-Mahía P, Prada-Rodríguez D. *J.*
502 *Chromatogr. A* 2008; **1207**: 136.

503 22. Campíns-Falcó P, Verdú-Andrés J, Sevillano-Cabeza A, Molins-Legua C, Herráez-
504 Hernández R. *J. Chromatogr. A* 2008; **1211**: 13.

505 23. Loutfy N, Fuerhacker M, Tundo P, Raccanelli S, Ahmed MT. *Chemosphere* 2007; **66**:
506 1962.

507 24. Pitarch E, Medina C, Portolés T, López FJ, Hernández F. *Anal. Chim. Acta* 2007; **583**:
508 246.

509 25. Portolés T, Pitarch E, López FJ, Sancho JV, Hernández F. *J. Mass Spectrom.* 2007; **42**:
510 1175.

511 26. Čajka T, Hajšlová J. *J. Chromatogr. A* 2004; **1058**: 251.

512 27. Hernández F, Portolés T, Pitarch E, López FJ, Beltrán J, Vázquez C. *Anal. Chem.* 2005;
513 **77**: 7662.

514 28. Commission Decision 2002/657/EC implementing Council Directive 96/23/EC
515 concerning the performance of analytical methods and the interpretation of results.
516 (eur-lex.europa.eu/pri/en/oj/dat/2002/l_221/l_22120020817en00080036.pdf)

517 29. Hernández F, Sancho JV, Pozo OJ. *Anal. Bioanal. Chem.* 2005; **382**: 934.

518 30. I. Ferrer, E.M. Thurman (Editors), Liquid Chromatography Mass Spectrometry/Mass
519 Spectrometry, MS/MS and Time-of-Flight MS: Analysis of Emerging Contaminants,
520 American Chemical Society Symposium Series 850, Oxford University Press, New York,
521 USA, 2003.

522 31. Numata M, Yarita T, Aoyagi Y, Yamazaki M, Takatsu A. *Chemosphere* 2005; **58**: 865.

523 32. Baumard P, Budzinski H, Garrigues P. *Polycycl. Aromat. Comp.* 1996; **9**: 241.

524 33. Barranco A, Alonso-Salces RM, Bakkali A, Berrueta LA, Gallo B, Vicente F, Sarobe M.
525 *J. Chromatogr. A* 2003; **988**: 33.

526 34. Santos FJ, Galceran MT. *TrAC - Trends Anal. Chem.* 2002; **21**: 672.

527 35. Nácher-Mestre J, Serrano R, Benedito-Palos L, Navarro J C, Lopez F J, Pérez-Sanchez J.
528 *Chemosphere.* Accepted for publication.

529 36. Dennis MJ, Massey RC, Cripps G, Venn I, Howarth N, Lee G. *Food Addit. Contam.*
530 1991; **8**: 517.

531 37. Van der Lee MK, van der Weg G, Traag WA, Mol HGJ. *J. Chromatogr. A* 2008; **1186**:
532 325.

533 38. Hernández F, Portolés T, Pitarch E, López FJ. *Anal. Chem*. 2007; **79**:9494

534 39. Hernández F, Portolés T, Pitarch E, López FJ. *J. Mass Spectrom*. 2009; **44**:1

535

536

537

538

539 **Table 1.** Ingredients and chemical composition of fish feed analyzed.

Ingredient (%)	*FO*	*33VO*	*66VO*
Fish meal (CP 70%) [1]	15	15	15
CPSP 90 [2]	5	5	5
Corn gluten	40	40	40
Soybean meal	14.3	14.3	14.3
Extruded wheat	4	4	4
Fish oil [3]	15.15	10.15	5.15
Rapeseed oil	0	0.85	1.7
Linseed oil	0	2.9	5.8
Palm oil	0	1.25	2.5
Soya lecithin	1	1	1
Binder	1	1	1
Mineral premix [4]	1	1	1
Vitamin premix [5]	1	1	1
$CaHPO_4.2H_2O$ (18%P)	2	2	2
L-Lys	0.55	0.55	0.55
Approximate composition			
Dry matter (DM, %)	93.13	92.9	92.77
Protein (% DM)	53.2	52.81	52.62
Fat (% DM)	21.09	21	20.99
Ash (% DM)	6.52	6.69	6.57

540 [1]Fish meal (Scandinavian LT)

541 [2]Fish soluble protein concentrate (Sopropêche, France)

542 [3]Fish oil (Sopropêche, France)

543 [4]Supplied the following (mg · kg $diet^{-1}$, except as noted): calcium carbonate (40% Ca)
544 2.15 g, magnesium hydroxide (60% Mg) 1.24 g, potassium chloride 0.9 g, ferric
545 citrate 0.2 g, potassium iodine 4 mg, sodium chloride 0.4 g, calcium hydrogen
546 phosphate 50 g, copper sulphate 0.3, zinc sulphate 40, cobalt sulphate 2, manganese
547 sulphate 30, sodium selenite 0.3.

548 [5]Supplied the following (mg · kg $diet^{-1}$): retinyl acetate 2.58, DL-cholecalciferol
549 0.037, DL-α tocopheryl acetate 30, menadione sodium bisulphite 2.5, thiamin 7.5,
550 riboflavin 15, pyridoxine 7.5, nicotinic acid 87.5, folic acid 2.5, calcium pantothenate
551 2.5, vitamin B_{12} 0.025, ascorbic acid 250, inositol 500, biotin 1.25 and choline
552 chloride 500.

553 FO: Reference fish feed; 33VO: fish feed with 33% fish oil replacement; 66VO: fish
554 feed with 66% fish oil replacement

555

556

557

558 **Table 2**. Experimental conditions of the optimized GC-EI(SRM) method.

t_R (min)	Window (min)	Compounds	Precursor Ion (m/z)	Product Ion (m/z)	Q/q	Dwell time (sec)	Collision Energy (eV)	Q/q[c] Ratio
5.84	3-7	Naphthalene	128	102	Q	0.1	30	1.20 (5)
			128	77	q		30	
8.80	7-9.1	Acenaphthylene	152	126	Q	0.2	20	1.09 (6)
			152	150	q		30	
9.20	8.9-10.5	Acenaphthene	154	152	Q	0.2	35	3.58 (5)
			153	126	q		30	
10.44	9.7-11.5	Fluorene	165	115	Q	0.15	30	1.03 (10)
			166	164	q		35	
12.67	11.5-14	Phenanthrene[b]	178	152	Q	0.1	20	3.12 (4)
12.80		Anthracene[b]	178	176	q	0.1	35	2.62 (9)
15.51	14-15.9	Fluoranthene	202	200	Q	0.1	35	3.41 (5)
			202	150	q		45	
16.02	15.8-17	Pyrene	202	200	Q	0.1	30	28.17 (3)
			202	152	q		20	
18.90	17-21	B(*a*)Anthracene-D_{12}[a]	240	236		0.1	30	
19.10		B(*a*)Anthracene[b]	228	226	Q	0.1	20	4.00 (11)
19.20		Chrysene[b]	228	224	q	0.1	55	3.48 (12)
22.33	21-23	B(*b*)Fluoranthene[b]	252	250	Q	0.2	35	4.38 (7)
22.42		B(*k*)Fluoranthene[b]	250	248	q	0.2	35	4.02 (10)
23.31	23-25	B(*a*)Pyrene	252	250	Q	0.2	35	4.24 (4)
			250	248	q		30	
26.75	25-27,6	Indeno(*1,2,3-cd*)Pyrene	276	274	Q	0.1	40	2.89 (10)
			276	272	q		60	
26.90		Dibenzo(*a,h*)Anthracene	278	276	Q	0.1	30	3.27 (9)
			278	274	q		30	
27.50	27,2-28,5	B(*g,h,i*)Perylene	276	274	Q	0.3	30	3.54 (7)
			274	272	q		30	

559 *[a]Internal Standard used as surrogate. [b]The same transitions for both compounds. [c]Average*
560 *value calculated from standard solutions at eight concentration levels each injected three*
561 *times and RSD, in brackets. Q: Quantification transition, q: confirmation transition.*

562

Table 3. Validation parameters obtained for the analysis of PAHs in fish fillets and fish feeds (n=6, at each fortification level). Analysis performed by GC-(QqQ)MS/MS.

Compound	"Blank" (µg/kg) (n=6)		Recoveries (%) (n=6)						LOD (µg/kg, fresh weight)	
			Fortification levels (µg/kg)							
			0.125		1.25		2.5			
	Fish Fillet	Fish Feed	Fish Fillet	Fish Feed	Fish Fillet	Fish Feed	Fish Fillet	Fish Feed	Fish Fillet	Fish Feed
Naphthalene	2.4 (6)	2.9 (8)	-*	-*	105(36)	76(36)	107(37)	83(37)	0.06	0.09
Acenaphthylene	0.2(16)	0.2(2)	112(10)	80(20)	85(16)	85(17)	80(12)	61(17)	0.05	0.09
Acenaphthene	0.2 (17)	0.3 (15)	106(20)	102(27)	73(13)	108(5)	64(9)	87(13)	0.02	0.1
Fluorene	0.8 (6)	1.7 (6)	108(22)	82(16)	91(12)	104(13)	89(10)	108(12)	0.1	0.1
Phenanthrene	2.6 (20)	3.9 (3)	-*	-*	71(9)	92(4)	71(9)	102(8)	0.1	0.1
Anthracene	0.6 (14)	0.8 (1)	90(6)	100(22)	88(7)	101(7)	91(4)	96(12)	0.03	0.1
Fluoranthene	1.7 (1)	4(4)	-*	-*	76(14)	124(5)	86(7)	105(10)	0.1	0.1
Pyrene	4.4 (1)	7(1)	-*	-*	84(18)	105(2)	89(4)	99(6)	0.1	0.1
Benzo(*a*)anthracene	0.06 (19)	0.7 (4)	88(17)	83(15)	103(2)	99(2)	104(4)	108(4)	0.02	0.1
Chrysene	0.1 (19)	1.5 (3)	83(7)	98(25)	101(4)	101(2)	100(2)	108(9)	0.04	0.1
Benzo(*b*)fluoranthene	-	0.8 (5)	93(9)	93(19)	100(4)	100(5)	100(3)	108(8)	0.06	0.1
Benzo(*k*)fluoranthene	-	0.3 (17)	93(13)	103(6)	99(4)	102(2)	101(3)	102(3)	0.09	0.1
Benzo(*a*)pyrene	-	0.45 (6)	98(8)	95(9)	101(3)	100(6)	101(3)	103(5)	0.09	0.09
Indeno (*1,2,3-cd*)pyrene	-	0.3 (6)	97(13)	97(13)	96(6)	98(6)	97(7)	98(9)	0.09	0.07
Dibenzo(*a,h*)anthracene	-	0.2 (16)	98(12)	92(14)	94(11)	81(3)	93(8)	74(9)	0.09	0.06
Benzo(*g,h,i*)perylene	-	0.4 (8)	105(10)	109(9)	99(4)	101(4)	100(3)	104(10)	0.07	0.1

*- not validated due to the high analyte content in the "blank" sample.

Table 4. Validation parameters obtained for the analysis of PAHs in fish oil and linseed oil (n=3, at each fortification level). Analysis performed by GC-(QqQ)MS/MS.

Compound	"Blank" (µg/kg) (n=3)		Recoveries (%) (n=3)				LOD (µg/kg, lipid weight)	
			Fortification levels (µg/kg)					
			1.25		2.5			
	Fish Oil	Linseed Oil	Fish Oil	Linseed Oil	Fish Oil	Linseed Oil	Fish Oil	Linseed Oil
Naphthalene	-	-	78(28)	75(24)	80(21)	71(22)	0.4	0.1
Acenaphthylene	-	-	116(1)	74(21)	89(29)	82(24)	1	0.9
Acenaphthene	0.3 (8)	-	97(8)	88(15)	105(28)	90(13)	0.2	1.25
Fluorene	4.3 (5)	2.6(4)	112(3)	87(13)	100(25)	115(4)	0.8	1.25
Phenanthrene	38.2 (2)	15.2(1)	-*	-*	113(9)	93(3)	1.25	1.25
Anthracene	-	-	94(11)	71(4)	104(8)	93(7)	1.25	1.25
Fluoranthene	13.3(7)	16.7(4)	-*	-*	98(3)	105(1)	1.25	1.25
Pyrene	9(1)	11.3(1)	-*	-*	92(1)	111(2)	1.25	1.25
Benzo(*a*)anthracene	-	-	113(7)	104(15)	112(4)	100(1)	0.6	1.25
Chrysene	-	-	119(3)	108(9)	104(5)	111(4)	0.6	1.25
Benzo(*b*)fluoranthene	0.3(22)	0.5(4)	111(6)	112(6)	111(4)	120(4)	0.3	0.4
Benzo(*k*)fluoranthene	0.3(2)	0.4(5)	108(6)	115(8)	107(4)	103(9)	0.3	0.4
Benzo(*a*)pyrene	-	0.3(8)	80(5)	87(11)	110(4)	95(4)	0.7	0.3
Indeno (*1,2,3-cd*)pyrene	-	-	84(10)	107(14)	97(3)	103(7)	0.5	0.4
Dibenzo(*a,h*)anthracene	-	-	89(7)	99(19)	98(7)	117(2)	0.15	0.2
Benzo(*g,h,i*)perylene	-	-	110(4)	105(8)	115(8)	101(8)	0.4	0.2

*- not validated due to the high analyte content in the "blank" sample.

Table 5. Experimental ions selected for the confirmation of PAHs by GC-TOF MS.

Compound	Ion $_1$	m/z $_1$	Ion $_2$	m/z $_2$	Ion $_3$	m/z $_3$	Ion $_4$	m/z $_4$
Naphthalene	$C_{10}H_8$	128.0626	$C_{10}H_7$	127.0548	$C_{10}H_6$	126.0452	C_8H_6	102.047
Acenaphthylene	$C_{12}H_8$	152.0626	$C_{12}H_7$	151.0548	$C_{12}H_6$	150.047	$C_{10}H_6$	126.047
Acenaphthene	$C_{12}H_9$	153.0707	$C_{12}H_{10}$	154.0782	$C_{12}H_8$	152.0626	$C_{10}H_6$	126.047
Fluorene	$C_{13}H_9$	165.0704	$C_{13}H_{10}$	166.0782	$C_{13}H_8$	164.0621	$C_{11}H_7$	139.0544
Phenanthrene	$C_{14}H_{10}$	178.0782	$C_{14}H_8$	176.0626	$C_{12}H_8$	152.0626	$C_{12}H_6$	150.047
Anthracene	$C_{14}H_{10}$	178.0774	$C_{14}H_8$	176.0626	$C_{12}H_8$	152.0626	$C_{12}H_6$	150.047
Fluoranthene	$C_{16}H_{10}$	202.0782	$C_{16}H_8$	200.0626	$C_{14}H_6$	174.047	$C_{12}H_6$	150.047
Pyrene	$C_{16}H_{10}$	202.0782	$C_{16}H_9$	201.0621	$C_{16}H_8$	200.0621	$C_{14}H_6$	174.047
Benzo(*a*)anthracene	$C_{18}H_{12}$	228.0939	$C_{18}H_{10}$	226.0783	$C_{16}H_8$	202.0626	C_9H_6	114.047
Chrysene	$C_{18}H_{12}$	228.0939	$C_{18}H_{10}$	226.0783	$C_{16}H_8$	202.0626	C_9H_6	114.047
Benzo(*b*)fluoranthene	$C_{20}H_{12}$	252.0939	$C_{20}H_{10}$	250.0783	$C_{10}H_6$	126.047	$C_{10}H_6$	126.047
Benzo(*k*)fluoranthene	$C_{20}H_{12}$	252.0939	$C_{20}H_{10}$	250.0783	$C_{10}H_6$	126.047	$C_{10}H_6$	126.047
Benzo(*a*)pyrene	$C_{20}H_{12}$	252.0939	$C_{20}H_{10}$	250.0783	$C_{10}H_6$	126.047	$C_{10}H_6$	126.047
Indeno (*1,2,3-cd*)pyrene	$C_{22}H_{12}$	276.0939	$C_{22}H_{10}$	274.0783	$C_{11}H_6$	138.047	$C_{11}H_6$	138.047
Dibenzo(*a,h*)anthracene	$C_{22}H_{12}$	276.0939	$C_{22}H_{10}$	274.0783	$C_{11}H_7$	139.0548	$C_{11}H_7$	139.0548
Benzo(*g,h,i*)perylene	$C_{22}H_{12}$	276.0939	$C_{22}H_{10}$	274.0783	$C_{11}H_6$	138.047	$C_{11}H_6$	138.047

Table 6. PAH concentrations in aquaculture samples analyzed by the application of the developed method.

Compound	Fish Fillet[(a)]		Fish Feed[(b)]		Rapeseed Oil[(c)]		Palm Oil[(d)]	
	Positives	conc. range (µg/kg)	Positives	conc. range (µg/kg)	Positives	conc. range (µg/kg)	Positives	conc. range (µg/kg)
Naphthalene	2	2.4-3.9	7	2.9-242	1	1.9	1	0.8
Acenaphthylene	1	0.2	7	0.2-1.6	1	0.2	1	0.2
Acenaphthene	1	0.2	7	0.2-1.7	1	0.6	1	0.5
Fluorene	18	0.2-0.8	7	1.7-6.4	1	0.9	1	0.4
Phenanthrene	19	0.5-3.7	8	0.4-12.7	1	1.2	1	1.3
Anthracene	2	0.6-1	8	0.4-2.1	1	1.6	-	-
Fluoranthene	19	0.2-3.9	7	2-4	-	-	-	-
Pyrene	19	0.2-11.4	8	0.8-7	-	-	-	-
Benzo(*a*)anthracene	6	0.06-4.9	8	0.4-0.9	1	0.9	1	1.2
Chrysene	12	0.1-6.9	8	0.6-1.5	1	0.8	-	-
Benzo(*b*)fluoranthene	1	5.9	8	0.6-2.7	1	0.4	1	1.3
Benzo(*k*)fluoranthene	1	5.9	8	0.3-2.3	1	0.4	1	1.3
Benzo(*a*)pyrene	1	3.9	2	0.45-0.5	1	0.7	1	1.4
Indeno (*1,2,3-cd*)pyrene	-	-	1	0.3	1	0.7	1	1.1
Dibenzo(*a,h*)anthracene	-	-	1	0.2	1	0.9	1	1.3
Benzo(*g,h,i*)perylene	1	3.4	1	0.4	1	0.8	1	1.2

Concentrations detected from fish oil and linseed oil are presented in **Table 4**. Total number of samples analyzed: (a) 19, (b) 8, (c) 1 and (d) 1.

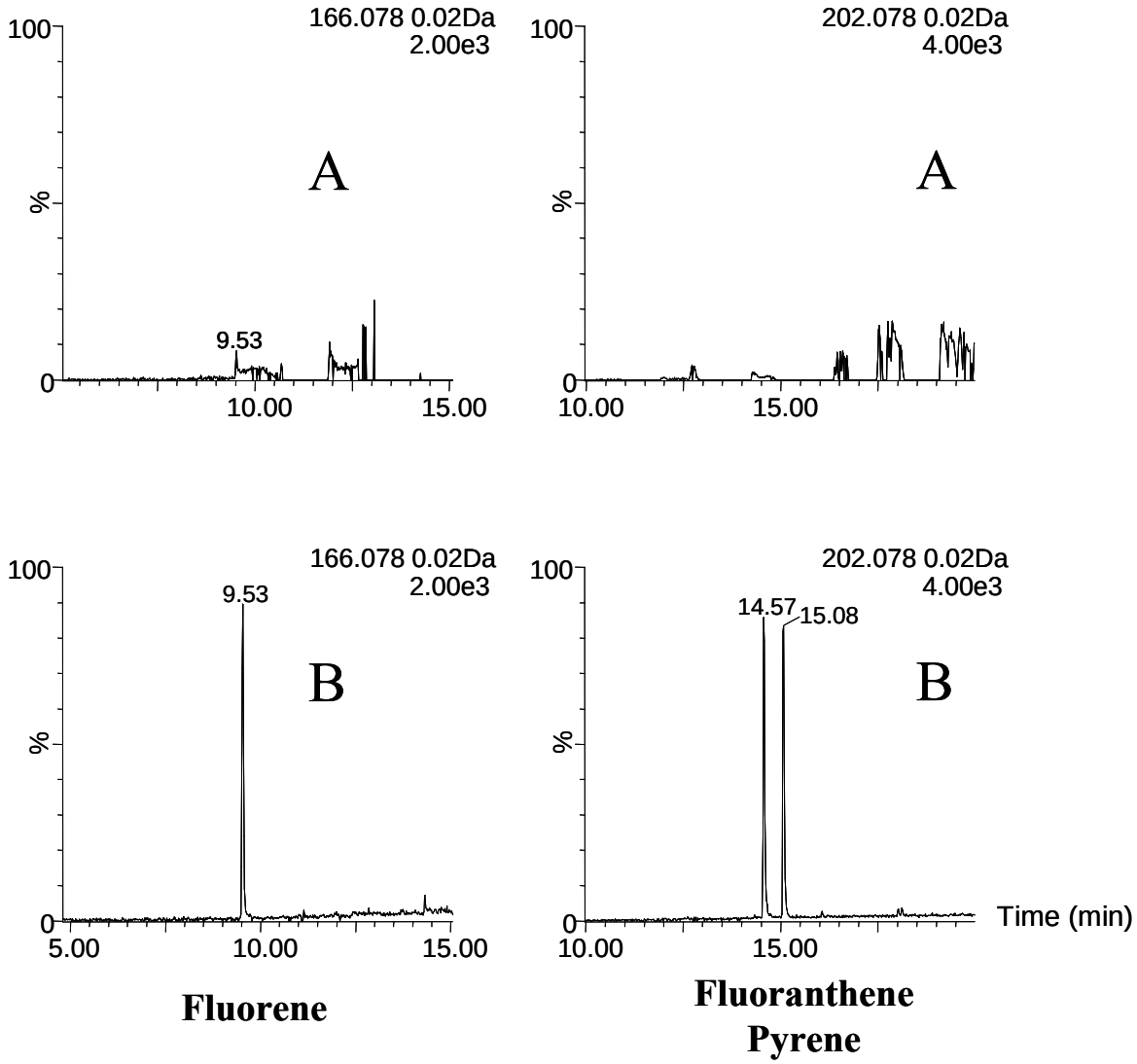
166.078 0.02Da
2.00e3
100
%
0
A
9.53
10.00
15.00
202.078 0.02Da
4.00e3
100
%
0
A
10.00
15.00
166.078 0.02Da
2.00e3
100
%
0
B
9.53
5.00
10.00
15.00
Fluorene
202.078 0.02Da
4.00e3
100
%
0
B
14.57
15.08
10.00
15.00
Time (min)
Fluoranthene
Pyrene

Figure 1.

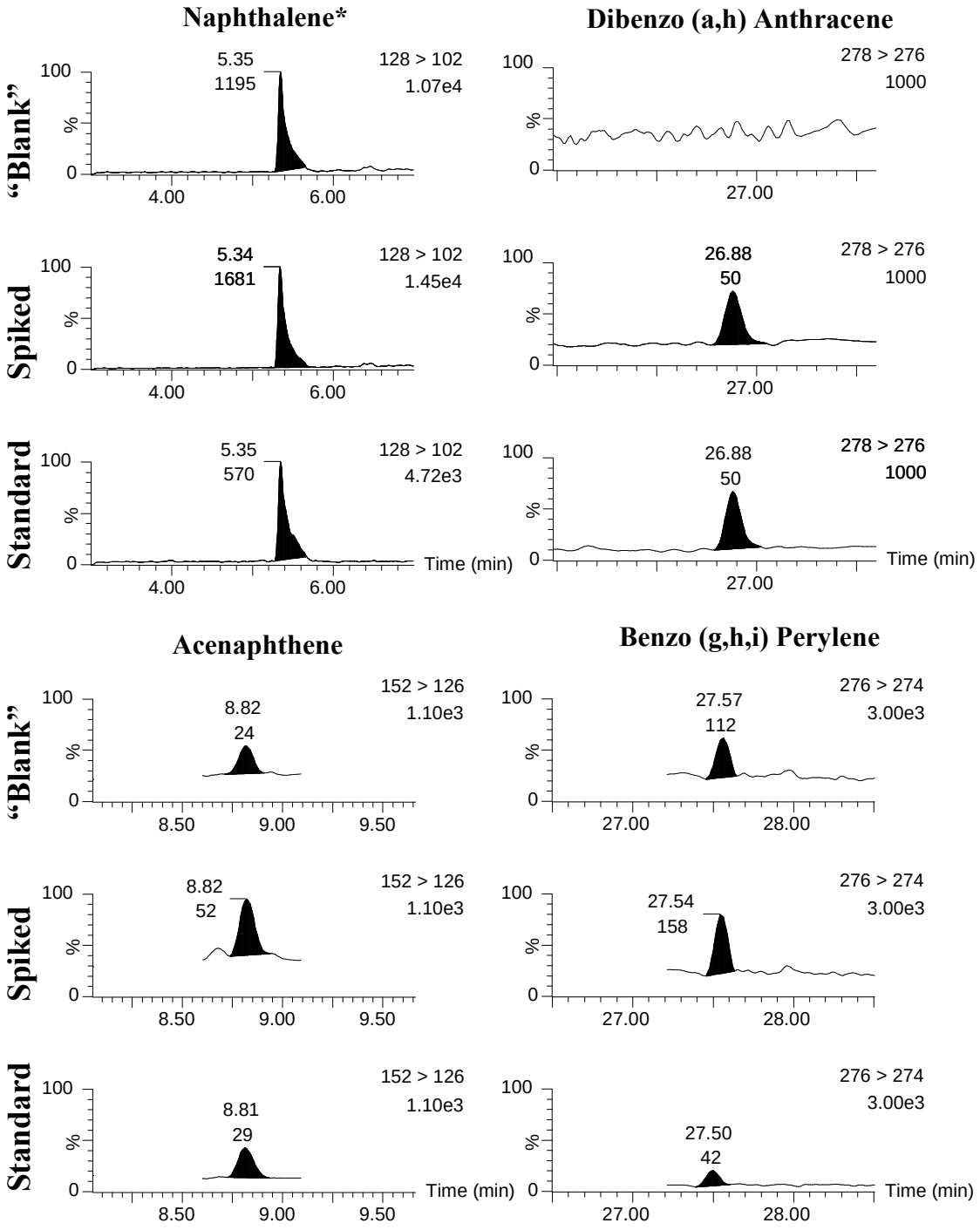
Naphthalene*
Dibenzo (a,h) Anthracene
"Blank"
Spiked
Standard
5.35
1195
128 > 102
1.07e4
278 > 276
1000
5.34
1681
128 > 102
1.45e4
26.88
50
278 > 276
1000
5.35
570
128 > 102
4.72e3
26.88
50
278 > 276
1000
Time (min)
Acenaphthene
Benzo (g,h,i) Perylene
8.82
24
152 > 126
1.10e3
27.57
112
276 > 274
3.00e3
8.82
52
152 > 126
1.10e3
27.54
158
276 > 274
3.00e3
8.81
29
152 > 126
1.10e3
27.50
42
276 > 274
3.00e3
Time (min)

Figure 2.

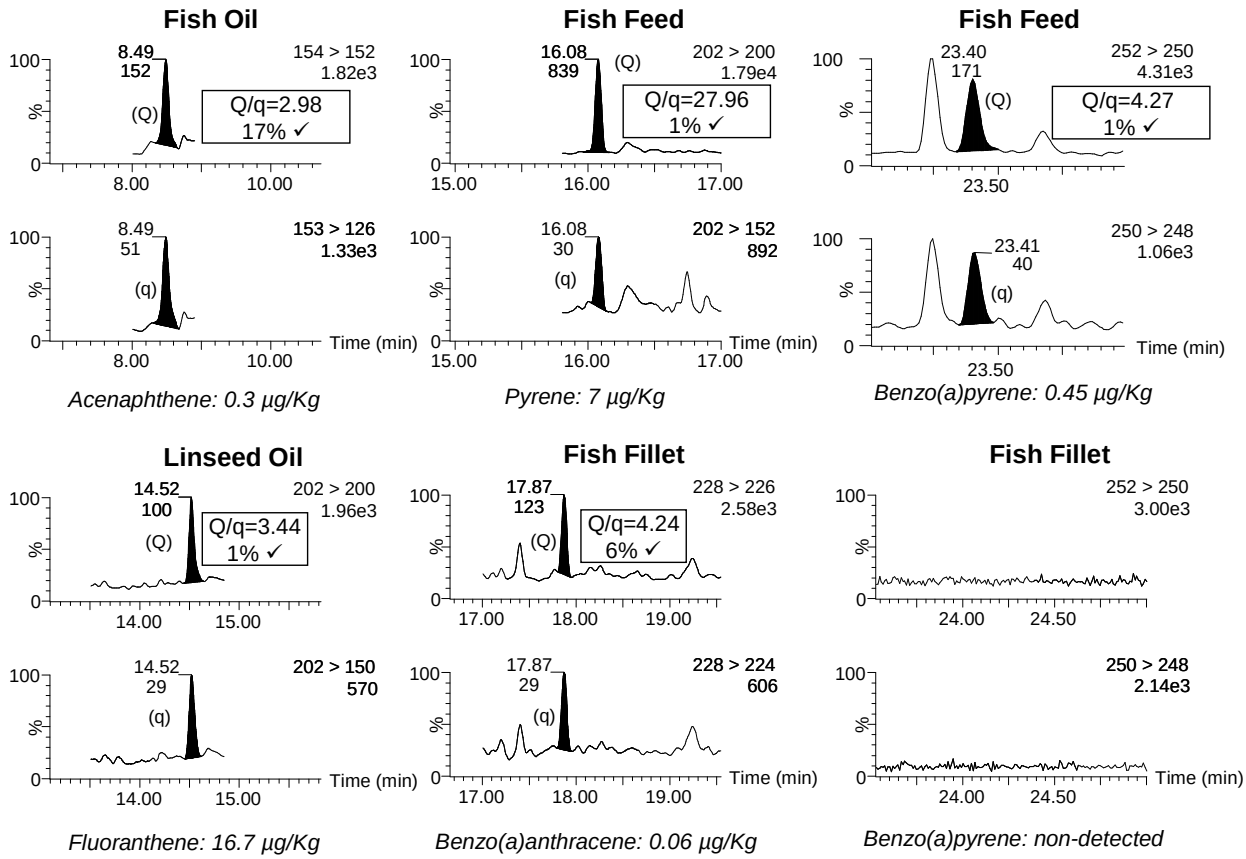
Fish Oil
8.49
152
154 > 152
1.82e3
(Q)
Q/q=2.98
17% ✓
8.49
51
153 > 126
1.33e3
(q)
Time (min)
Acenaphthene: 0.3 µg/Kg
Fish Feed
16.08
839
(Q)
202 > 200
1.79e4
Q/q=27.96
1% ✓
16.08
30
202 > 152
892
(q)
Time (min)
Pyrene: 7 µg/Kg
Fish Feed
23.40
171
(Q)
252 > 250
4.31e3
Q/q=4.27
1% ✓
23.41
40
250 > 248
1.06e3
(q)
Time (min)
Benzo(a)pyrene: 0.45 µg/Kg
Linseed Oil
14.52
100
(Q)
202 > 200
1.96e3
Q/q=3.44
1% ✓
14.52
29
202 > 150
570
(q)
Time (min)
Fluoranthene: 16.7 µg/Kg
Fish Fillet
17.87
123
(Q)
228 > 226
2.58e3
Q/q=4.24
6% ✓
17.87
29
228 > 224
606
(q)
Time (min)
Benzo(a)anthracene: 0.06 µg/Kg
Fish Fillet
252 > 250
3.00e3
250 > 248
2.14e3
Time (min)
Benzo(a)pyrene: non-detected

Figure 3.

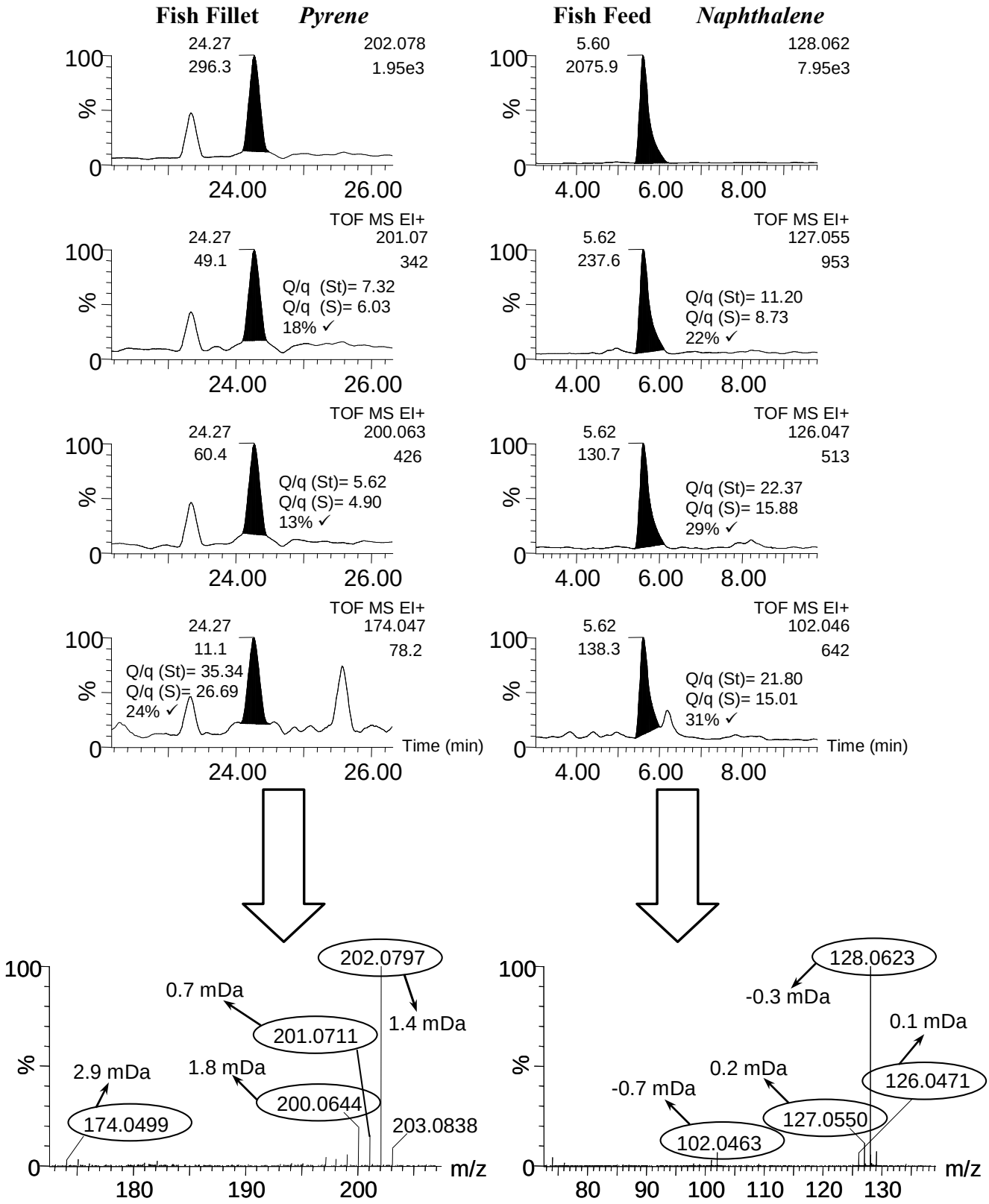
Fish Fillet
Pyrene
Fish Feed
Naphthalene
24.27
296.3
202.078
1.95e3
5.60
2075.9
128.062
7.95e3
TOF MS EI+
24.27
49.1
201.07
342
Q/q (St)= 7.32
Q/q (S)= 6.03
18% ✓
5.62
237.6
127.055
953
Q/q (St)= 11.20
Q/q (S)= 8.73
22% ✓
24.27
60.4
200.063
426
Q/q (St)= 5.62
Q/q (S)= 4.90
13% ✓
5.62
130.7
126.047
513
Q/q (St)= 22.37
Q/q (S)= 15.88
29% ✓
24.27
11.1
174.047
78.2
Q/q (St)= 35.34
Q/q (S)= 26.69
24% ✓
5.62
138.3
102.046
642
Q/q (St)= 21.80
Q/q (S)= 15.01
31% ✓
Time (min)
202.0797
0.7 mDa
1.4 mDa
201.0711
2.9 mDa
1.8 mDa
200.0644
174.0499
203.0838
m/z
128.0623
-0.3 mDa
0.1 mDa
0.2 mDa
126.0471
-0.7 mDa
127.0550
102.0463

Figure 4.