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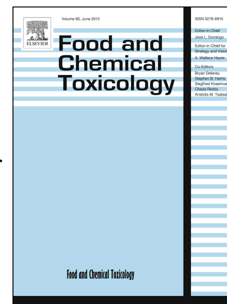

Fast methodology for the determination of a broad set of antibiotics and some of their metabolites in seafood

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Fast methodology for the determination of a broad set of antibiotics and some of their metabolites in seafood

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2 **List of abbreviations:**

3

4 ACN: acetonitrile; CAFOs: confined animal feeding operations; dSPE: dispersive solid
5 phase extraction; dw: dry weight; EU: european union; IS: internal standards; MDLs:
6 method detection limits; MQLs: method quantification limits; MRLs: maximum residue
7 limits; QuEChERS: quick, easy, cheap, effective, rugged, and safe; SPE: solid phase
8 extraction; UHPLC-MS/MS: ultra high pressure liquid chromatography- tandem mass
9 spectrometry; US: ultrasonic extraction; ww: wet weight.

10

11 **Keywords:** Antibiotics; Multi-residue; Seafood; UHPLC-MS/MS; Microbial growth
12 inhibition test

13

14 **1. Introduction**

15 Antibiotics usage in human and veterinary medicine has become a common
16 therapeutic practice (Manzetti and Ghisi, 2014). This high antibiotic consumption,
17 resulted in a gradual accumulation of antibiotics in the water bodies, being wastewater
18 discharges, agricultural runoff and aquaculture the most important sources of this type
19 of contamination into the environment (Loos et al., 2013; Nödler et al., 2014). It is well
20 known that antibiotics pose a significant risk to environment, even at low
21 concentrations (Kümmerer, 2009). For example antibiotics like bacitracin, flumequine,
22 lincomycin and aminosidine showed to be harmful to aquatic organisms such as
23 *Artemia* (Migliore et al., 1997), or metronidazole which showed a toxic effect to
24 *Chlorella spp* and *Selenastrum capricornutum* (Lanzky and Halting-Sørensen, 1997). In
25 addition, the occurrence of antibiotics in the natural aquatic systems may pose a risk
26 for the wild organisms due to their bioaccumulative potential as for instance
27 roxithormycin that showed a bioaccumulation factor higher than 600 L/Kg in different
28 aquatic organisms (Xie et al., 2015). Furthermore, the bioaccumulation factor of some

1 antibiotics in fish has been reported to be higher than 3000 L/Kg (Gao et al., 2012) in
2 agreement with this, Chen et al., (Chen et al., 2014) reported a bioaccumulation factor
3 of 6488 L/Kg for trimethoprim in fish *(Lutjanus russelli*). Residues of these drugs can
4 remain in fish tissues with the consequent potential risk of exposure for fish
5 consumers (Cabello, 2006); especially when antibiotics are accumulated in seafood
6 species highly consumed by the population. The use of antibiotics in food producing
7 animals may provoke undesirable effects on consumer's health. If antibiotics are
8 present at high enough concentrations in food producing animals then they may cause
9 allergies or development of antibiotic resistant bacteria (Alderman and Hastings, 1998;
10 Cañada-Cañada et al., 2009) causing treatment resistant illness, which can be a human
11 health problem when treating infections (Heuer et al., 2009).
12 In order to protect human health and avoid the potential risks above mentioned,
13 regulatory authorities like the European Union (EU) establishes Maximum Residue
14 Limits (MRLs) for some pharmaceutical compounds, including antibiotics, in different
15 foodstuffs from animal origin like fish and others seafood species (EU No 37/2010).
16 Seafood for human consumption produced in aquaculture are likely to contain
17 antibiotic residues since many antibiotics are commonly used in confined animal
18 feeding operations (CAFOs) and aquaculture activities in order to treat or prevent
19 bacterial infections (Stolker and Brinkman, 2005). Therefore, information regarding the
20 presence of antibiotics in seafood is crucial for evaluating the fate, environmental
21 effects, and human health risks of these substances. Most of the analytical methods
22 developed so far have focused on one (Samanidou et al., 2008) or few (Evaggelopoulou
23 and Samanidou, 2013) antibiotic families. Moreover, most of them were specific for
24 one organism class like fish (Cháfer-Pericás et al., 2010a) or shrimps (Villar-Pulido et

1 al., 2011). Analytical methods able to detect a broad spectrum of antibiotics are still
2 scarce (Dasenaki and Thomaidis, 2010; Fedorova et al., 2013; Li et al., 2012). The
3 limited number of analytical methods covering the detection of antibiotics belonging
4 to several chemical families may be explained by the difficulty of the simultaneous
5 extraction of antibiotics with different physicochemical properties. The extraction
6 procedure technique and the solvents used are key issues for the simultaneous
7 extraction of different antibiotics. Usually a compromise should be made between the
8 extraction conditions and good performance of the method in terms of recovery,
9 sensitivity, reproducibility, etc. Furthermore, most of the methods developed focus on
10 pharmaceutical compounds administered to humans or animals, but few of them
11 include antibiotics metabolites (Fernandez-Torres et al., 2011). The inclusion of
12 antibiotics metabolites in multi-residue analytical methods is of great interest since
13 they can be accumulated even at higher degree than the antibiotics themselves (Gros
14 et al., 2013), and can be as bioactive or even more than the corresponding parent
15 compound. As example, García-Galan et al., (García-Galán et al., 2012) found that
16 acetylated metabolites of some sulfonamides can be more toxic than the parent
17 compound. According to this paper a risk classification ranked N_4-acetylsulfapyridine
18 metabolite as toxic, whereas its parent compound, sulfapyridine, was classified as
19 harmful (European Commission, 2002). However, other studies suggested that
20 metabolites of antibiotics like sulfonamides may reduce their toxicity in microalgae
21 (Eguchi et al., 2004)
22 Most of the methods mentioned above for the analysis of antibiotics in seafood are
23 based on detection with LC-MS/MS (i. e. Dasenaki and Thomaidis, 2010; Fedorova et
24 al., 2013). However, alternative detection methodologies like immunoassay techniques

or microbial growth inhibition tests have been tested for the analysis of antibiotics in seafood, but its applicability is still scarce. Immunoassays were applied for the detection of oxytetracycline (Cháfer-Pericás et al., 2010c) and sulfonamides (Cháfer-Pericás et al., 2010b) in fish samples. Some of them are commercially available, such as ELISA test kits for the specific detection of antibiotics like tetracyclines, β-lactams or chloramphenicol in seafood and meat ("Randoxfood," 2016). A microbial growth inhibition test was applied for the analysis of three antibiotic families including quinolones, sulfonamides and tetracyclines in shrimps (Dang et al., 2010); whereas Barker et al. (Barker, 1994) applied this methodology for the specific analysis of quinolones in fish. Some kits based on microbial growth inhibition are also commercially available i.e. PremiTest Antibiotic Test ("Nelsonjameson," 2016), which provides a qualitative detection of a broad spectrum of antibiotics . Microbial growth inhibition tests are not as sensitive as LC-MS/MS methods and do not allow to distinguish between individual compounds. This type of test is rather intended as a screening methodology for the preliminary detection of some antibiotic residues and its metabolites with a similar mode of action in different types of food from animal origin. Furthermore the application of this screening technique does not require the use of complex instrumentation. This would reduce the cost of the analysis and facilitate the implementation of this technique as routine method for the analysis of seafood in laboratories or aquaculture facilities.

The aim of this paper was to develop a fast methodology based on ultra high pressure liquid chromatography-triple quadrupole mass spectrometry (UHPLC-MS/MS) for the detection of antibiotics (from different chemical families), and some of their major metabolites, in several seafood matrices, especially in highly consumed species.

Different extraction and clean-up procedures were tested in order to obtain a simple and fast method covering the maximum number of antibiotics possible. The method allowed the detection and identification of 23 individual compounds (including four of their major metabolites). After that, the method was applied for the analysis of real seafood samples of highly consumed species collected from aquaculture and natural environments. In addition, a method based on the inhibition of susceptible bacterium in the presence of antimicrobial residues was tested as an alternative technique for the detection of antibiotic families such as tetracyclines, quinolones, macrolides/β-lactams, amino-glycosides and sulfonamides.

2. Material and Methods

2.1. Chemical and reagents

A list with the antibiotics included in the analysis based on UHPLC-MS/MS detection is presented on the supplementary material (Table S1). Antibiotic standards were of high purity grade (> 90%). All antibiotic standards were purchased from Sigma-Aldrich except N-acetylsulfadiazine, N-acetylsulfamerazine and N-acetylsulfamethazine that were obtained from Toronto Research Chemicals (TRC), clarithromycin was purchased from Fluka and clindamycin from European Pharmacopeia (EP). Isotopically labelled compounds used as internal standards, azithromycin-d3, ampicilin-d5, erythromyicin-d13, ibuprofen-d3, lincomycin-d3 and sulfamethoxazole-d4 were obtained from TRC whereas ronidazole-d3, ofloxacin-d3 and ciprofloxacin-d8 were purchased from Sigma-Aldrich.

The cartridges used for solid phase extraction OASIS HLB (200 mg, 6mL), the QuEChERS extract tubes (AOAC method), and the QuEChERS for dispersive solid phase extraction

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(dSPE) (15 ml, fatty acids tubes) were obtained from Water Corporation (Milford, MA, U.S.A.). PVDF filters (0.45µm pore) were purchased from Merck Millipore Corporation (Darmstadt, Germany). HPLC grade methanol, water and acetonitrile were purchased from Merck (Darmstadt, Germany), whereas formic acid (98% purity), EDTA 0.01 mol/L, hydrochloric acid 0.1 mol/L and sodium hydroxide 1 mol/L were obtained from Sharlab (Barcelona, Spain).

Stock standards and isotopically labelled internal standards were prepared in methanol at a concentration of 1000 mg/L and stored at -20°C. Working standard solutions containing all antibiotics and isotopically labelled internal standards (1mg/L) were prepared in methanol/water (50/50, v/v) before each analytical run.

2.2. Sample collection and pre-treatment

Clams (*Chamelea gallina*) were the organisms selected to perform the different extraction procedures in order to find out which one was the most suitable one for antibiotics. This organism has low fat content minimizing the co-extraction of undesirable compounds (mainly fats) that possibly will interfere in the detection of the analytes (Huerta et al., 2013). In addition, C. *gallina* are abundant and easy to capture. They were collected from the Ebro Delta, Tarragona, Spain, between November and December 2013.

The sample pre-treatment consisted in removing clam's shell and a pool with 50 individual organisms was prepared with the edible content. After homogenization, samples were freeze-dried, grounded in a mortar and kept at -20°C until its analysis. Freeze-drying of the samples was aimed at the preservation of antibiotics in the samples, as the water content in non-dried samples may degrade the compounds.

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Furthermore, as antibiotics are not volatile compounds, the freeze-drying process should not affect the final amount of antibiotics present in the samples. A previous experiment regarding stability of pharmaceuticals after freeze drying was carried out and showed no loss of compounds after freeze-drying process (data not shown). Once the extraction procedure was optimized, the method based on detection and quantification of analytes using UHPLC-MS/MS was validated for the analysis of antibiotics in clams, mussels (*Mytilus galloprovincialis*), and fish (*Platichthys flesus*). Mussels were collected from the Ebro Delta, Tarragona, Spain, whereas fish was taken from the Scheldt estuary, Netherlands. Mussels were pre-treated in the same way than clams, whereas for fish samples the skin was removed and only muscle tissue was further freeze-dried, grounded in a mortar and kept at -20°C for the analysis.

Once the method was optimized and validated, it was applied for the analysis of real samples. Eight samples were taken from aquaculture facilities (five mussels and three fish). The *Mytilus galloprovincialis* from Spain, *Mytilus galloprovincialis* from Italy, *Mytilus spp* from Netherlands, *Pangasius spp* from Vietnam, *Salmo salar* from Scotland, and *Salmo salar* from Norway were bought from local supermarkets. Whereas the two *Mytilus spp* from Greece were directly sampled in the aquaculture facility, pooled, homogenized and snap frozen before the transport. After this all the samples were freeze-dried and kept at 20°C until their analysis. All aquaculture samples were commercialized in European countries *(Pangasius spp* was imported). Four samples (three mussels and one fish) were collected from natural environments: *Mytilus Galloprovincialis* from the bay of Saint-brieuc, France, *Mytilus galloprovincialis* from Po Delta, Italy, *Mytilus spp* from Tagus estuary, Portugal, and *Platichthys flesus* from The Scheldt estuary, Netherlands.

For the microbial growth inhibition test evaluation, the mussel sample *Mytilus Galloprovincialis* collected from the bay of Saint-brieuc, France, was selected.

2.3. Extraction and clean-up procedure optimization

Four different extraction and clean-up procedures were tested and a recovery study was performed in order to evaluate the efficiency of each extraction procedure. Approximately 0.5 g of freeze-dried clam tissue were weighted and placed in a glass tube. Samples were then spiked with a mixture of antibiotics and some metabolites at a final concentration of 50 ng/g (dw); half of the MRLs established by the authorities for those compounds included in the method and regulated by the authorities (sulphonamides, tetracycline, tilmicosin, tylosin and lincomycin) (European Commission, 2010). All compounds added to the spiking mix and their corresponding internal standards are listed in the supplementary information (table S2). Besides, control samples were also analysed in order to determine the background levels of the target compounds. Both spiked and control samples were analysed in triplicate. The detection and quantification of the target compounds were done with UHPLC-MS/MS. Recoveries were then calculated by comparing the concentrations measured in the sample after the analytical procedure with the initial spiked concentration. The concentrations measured in the sample were determined by using internal sample calibration. The internal standard curve was made in clam extract in a range of 0.01 to 50 ng/g (dw).

Two extraction techniques were used; QuEChERS and ultrasonic bath, and four different extraction procedures were tested. Two of them based on QuEChERS (i and ii)

1 whereas the other two were based on ultrasonic bath (iii and iv). The methods were
2 performed as follows:
3 (i) QuEChERS extraction only: spiked samples were placed in a 50 mL
4 polypropylene tube, 2 mL of HPLC water and 10 mL of ACN:MeOH (75:25, v/v) were
5 added and shaken in a rotator shaker for 15 min. Then, the extraction salts
6 (magnesium sulphate 6g and sodium acetate 1.5g) were added and the mix was
7 shaken again for 15 min in a rotator shaker. The samples were centrifuged 5 min at
8 10.000 rpm. Four mL of the extract were taken out, evaporated to dryness, and
9 reconstituted in 1 ml of MeOH. Then, the samples were filtered through PVDF filters of
10 0.45µm and kept at -20°C until its analysis.
11 (ii) QuEChERS extraction followed by dispersive solid phase extraction (dSPE):
12 spiked samples were placed in a 50 mL polypropylene tube. 5 mL of HPLC water were
13 added and vortexed for 30 seconds followed by the addition of 10 mL of acetonitrile
14 (ACN) with the subsequent vortex for 1 minute. Then, the QuEChERS extraction salts
15 composed by magnesium sulphate 6g and sodium acetate 1.5g were added and the
16 mix was hand shacked for 1 min. Samples were centrifuged 5 min at 10.000 rpm. The
17 ACN layer was transferred to a tube containing the dispersive sorbents (primary
18 secondary amine (PSA) 149.9 mg; octadecyl (C18) 149.9 mg and magnesium sulphate
19 900.2 mg) in order to carry out a dSPE. The sample was vortex for 1 min and
20 centrifuged 10 min at 5000 rpm. Finally, 6 mL of the extract were evaporated to
21 dryness, reconstituted in 1 ml of methanol (MeOH) and kept at -20°C until its analysis.
22 (iii) Ultrasonic extraction (US) with ACN:water followed by solid phase
23 extraction (SPE): spiked samples were placed in a 50 mL polypropylene tube, 5 mL of
24 ACN:H_2O (3:1) were added; the mixture was vortexed 1 min and sonicated for 15 min.

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After that, the samples were centrifuged for 10 min at 3500 rpm and the supernatant was collected. This process was repeated another time. Later on, SPE was performed as follows: 240 µL of EDTA was added to each sample, and the pH was adjusted to 2.5 using hydrochloric acid. The cartridges (Oasis HLB 200mg, 6ml) were conditioned with 5 mL of MeOH followed by 5 mL of HPLC water at pH 2.5. After sample loading the cartridges were rinsed with 5 mL of HPLC water and dried under a gentle stream of nitrogen for 5 min. Finally, samples were eluted with 6 mL of methanol, dried down under nitrogen, reconstituted in 1 ml of MeOH and kept at -20°C until its analysis.

iv) Ultrasonic extraction (US) with NaOH y NaCl followed by solid phase extraction (SPE): spiked samples were placed in a 50 mL polypropylene tube, 5 mL of 0.1M sodium hydroxide (NaOH) and 0.1 g of sodium chloride (NaCl) were added to each sample. The mixture was vortexed 1 min and sonicated for 15 min. After that, the samples were centrifuged for 10 min at 3500 rpm and the supernatant was collected. This process was repeated two times. Then, solid phase extraction was performed as follows: Oasis HLB (200 mg 6 ml) cartridges were conditioned with 6 mL of methanol followed by 6 mL of HPLC water. After sample loading, cartridges were rinsed with 6 mL of HPLC water. Finally, samples were eluted with 6 mL of methanol, dried down under nitrogen, reconstituted in 1 ml of MeOH and kept at -20°C until its analysis.

All purified samples were evaporated, re-dissolved in 1 mL of methanol-water (50:50) and 10 µL of internal standard (IS) mixture 1mg/L (table S2) was added to each extract before UHPLC-MS/MS analysis.

2.4. Instrumental analysis

The sample extracts were analysed using an ultra high pressure liquid chromatography coupled to a quadrupole linear ion trap tandem mass spectrometry (UHPLC-QqLIT) following the method of (Gros et al., 2013). The chromatographic separations were performed using a Water Acquity Ultra-Performance™ liquid chromatography system, equipped with two binary pumps (Milford, MA, USA), using an Acquity HSS T_3 column (50 mm x 2.1 mm i.d., 1.8 µm particle size) with a precolumn Acquity UPLC HSS T3 1.8 µm particle size. The chromatographic separation conditions were: solvent (A) Acetonitrile, solvent (B) HPLC grade water acidified with 0.1% of formic acid. The flow rate was 0.5 mL/min and the gradient elution was: initial conditions 5% A; 0-3 min 5-70% A; 3.0-5.0 min, 100% A; 5.0-5.1 return to initial conditions and from 5.1-6.0 equilibrium of the column. The sample volume injected was 5 µL. The UHPLC instrument was coupled to a 5500 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) with a turbo Ion Spray source. All the compounds were analysed under positive electrospray ionization except for chloramphenicol that was analysed under negative ionization. Chloramphenicol was analysed with the same instrument describe above following the method developed by Gros et al. (Gros et al., 2012) and using an Acquity BEH C18 column (50 mm x 2.1 mm i.d., 1.7 um particle size). The chromatographic separation conditions were: solvent (A) Acetonitrile, solvent (B) 5mM ammonium acetate/ammonia (pH 8). The flow rate was 0.6 mL/min and the gradient elution was: 0-1.5min, 0-60% A; 1.5-2.0min, 100% A; 2.0-3.0, 100% A; 3.20 return to the initial conditions; 3.20-3.70 equilibration of the column. The sample volume injected was 5 µL. Blank samples (MeOH and MeOH:H_2O 50:50) were run every 3 samples on the sample queue both between standards, spiked and non-spiked in order to detect any possible carryover effect. Two selected reaction

monitoring (SRM) transitions were monitored for each antibiotic. The first transition was used for antibiotics quantification and for the calculation of the validation parameters, whereas the second transition was used for confirmation of the identity. The relative abundance of the two transitions was compared with those in the standards and the difference was within ± 20% in all cases.

2.5. Statistical analysis

For the determination of significant differences between the different extraction procedures tested, one way ANOVAs were performed using R software (i386 3.1.0) comparing the different recoveries obtained for each compound in each extraction procedure. The normality and homogeneity of the data was tested before ANOVAs by using Shapiro-Wilk test and Levene's test respectively. For those compounds that the data showed no normality or homogeneity, a kruskal-Wallis test was performed using the same software. Results are presented in supporting information, tables S4 and S5.

2.6. Microbial growth inhibition test

Once the extraction procedure was optimized a microbial growth inhibition test was performed using Water-Scan plates supplied by RIKILT (Wageningen University, Netherlands) as alternative detection technique. The test system contains five plates, one for each antibiotic family considered: tetracyclines, quinolones, macrolides/β-lactams, amino-glycosides and sulfonamides. The preparation of the Water-Scan plates, including the test organisms, the agar mediums and the supplements was done following the method of (Pikkemaat et al., 2008). The test requires samples to be in liquid phase and, therefore, a prior extraction procedure of seafood samples was

mandatory. In this sense, the extraction method showing the best performance among the four previously tested was employed (QuEChERS extraction only, full details in section 3.1). However, this extraction procedure was not suitable for a further analysis with the microbial growth inhibition test, probably due to interferences with the extraction salts used (data not shown). Therefore, an alternative extraction procedure based on ultrasonic extraction (US) and solid phase purification (SPE) was applied. Full details of the extraction procedure and plates preparation are given in supporting information. Three samples were analyzed with the microbial growth inhibition test: a procedure blank (sample treated with the same extracting procedure but without biological matrix), a control sample (mussel sample previously analyzed with QuEChERS extraction and UHPLC-MS/MS that did not show the presence of any antibiotics), and the same control sample extract spiked with 100 µg/L of oxytetracycline, 200µg/L of flumequine, 100 µg/L of erythromycin, and 100 µg/L of sulfamethoxazole, the spiking values have been chosen as they are in the range of the MRLs established by the regulatory authorities (European Commission, 2010). In addition, a solvent blank (1:1) methanol:demineralised water, and demineralised water only, were used as negative controls. A positive control was also used in each plate for tetracyclines 100 µg/L of oxytetracycline; for quinolones 200 µg/L of flumequine; for macrolides/ß-lactam 100 µg/L of amoxicillin; for sulfonamides 100 µg/L of sulfamethoxazole and for aminoglycosides 100 µg/L of neomycine.

3. Results and Discussion

3.1 Extraction procedure optimization

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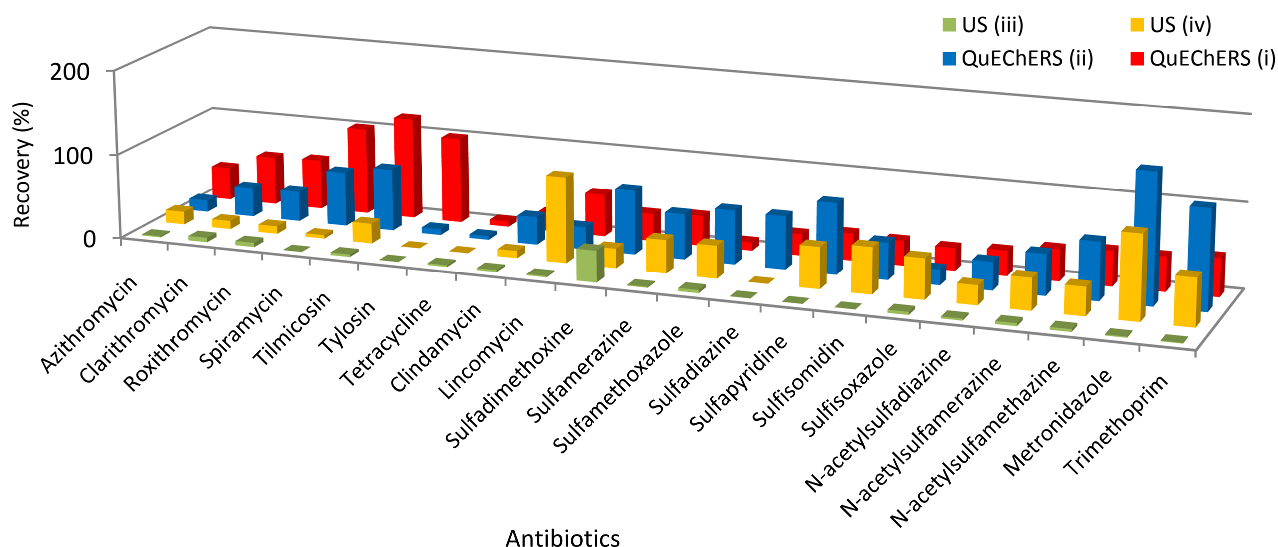
1 Initially the following antibiotics families were targeted for their inclusion in the multi-
2 residue method: macrolides, tetracyclines, fluoro(quinolones), lincosamides,
3 sulfonamides, nitroimidazoles, dihydrofolate reductase inhibitors and amphenicols
4 (table S2) but due to the recoveries obtained with the extraction methods tested some
5 of them had to be removed. This is the case of (fluoro)quinolones, which presented
6 very poor recoveries for the methods i and ii (table S3). The method based on
7 ultrasonic bath using NaOH as extraction solvent and NaCl (method iv) achieved the
8 highest recoveries for this group of antibiotics. However, this method was discarded
9 due to the bad recoveries obtained for macrolides antibiotics group (Figure 2). All the
10 antibiotics included in the method (except for metronidazole-OH and chloramphenicol
11 that were added in the spiking mixture in a further stage of the extraction method
12 development) and the recoveries obtained for each procedure tested are shown in
13 figure 2. Table S4 provides the standard deviation and statistical differences between
14 the different treatments. Within the different extraction and clean-up procedures
15 tested, the method based on ultrasonic bath using ACN:H_2O (3:1) as extraction solvent
16 (method iii) was discarded due to the low recoveries for most of the compounds
17 analysed (Figure 2). The two methods based on QuEChERS showed similar recoveries
18 for the majority of the compounds except for macrolides where QuEChERS extraction
19 using ACN:MeOH (75:25 v/v) presented higher percentages of recoveries. Besides, this
20 method was able to extract a higher number of antibiotic families and also presented
21 good reproducibility with smaller standard deviation (Figure 2, table S4).



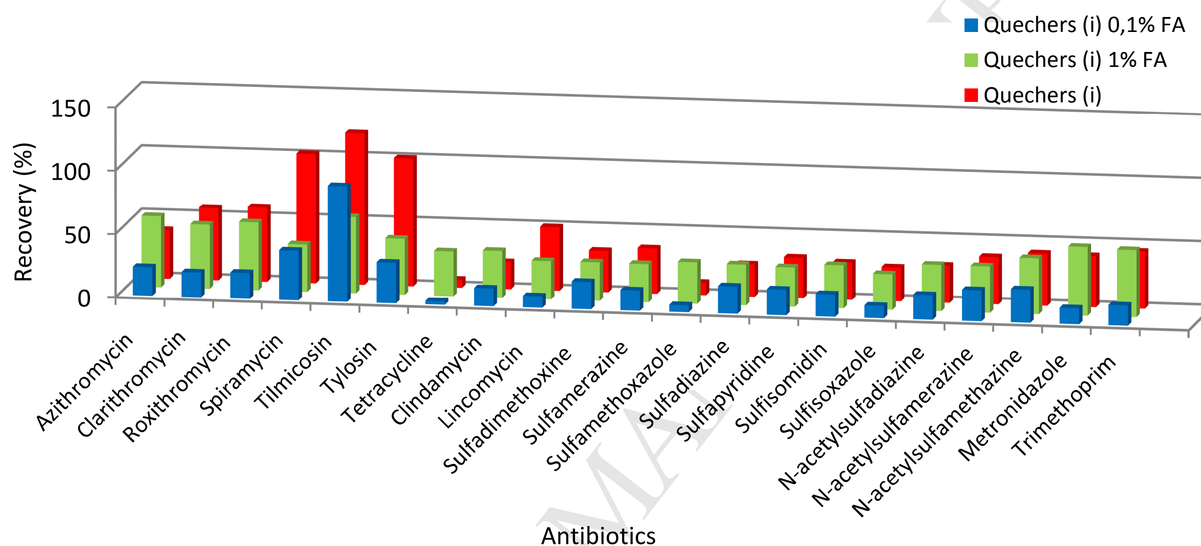
1

2 **Fig. 2**. Comparison of extraction efficiencies (%) obatined for each extraction procedure: QuEChERS (i),
3 QuEChERS (ii) US (iii) and US (iv). Mean of 3 replicates (n=3). Metronidazole-OH and Chloramphenicol
4 are not represented because these compounds were included in a later stage of the method
5 development.

6

7 However, tetracyclines were not extracted with this procedure and due to their
8 frequent use in aquaculture (De la Cruz et al., 2013; Rico et al., 2013) a decrease of pH
9 in the extraction solvent was tested in order to improve their extraction. This has been
10 previously reported to increase the recoveries in certain compounds (Lopes et al.,
11 2012). Different amounts of formic acid (FA) were added to the extraction solvent:
12 ACN:MeOH (0.1% FA) and ACN:MeOH (1% FA), and the results obtained are shown in
13 Figure 3.Table S5 provides the standard deviation for each compound and the
14 statistical differences between the treatments. No significant increase in the extraction
15 recoveries were found when adding 0.1% of FA to the extraction solvent. However,
16 when 1% of formic acid was added tetracycline antibiotic was extracted with an
17 acceptable recovery (35.4%). Besides, lincosamides, sulfonamides, nitroimidazoles,

1 dihydrofolate reductase inhibitors and amphenicols were still satisfactory extracted
2 with the addition of 1% formic acid. Although macrolides recoveries decreased due to
3 the addition of formic acid (ranging from 38.6% to 119.6% without acid and from
4 37.4% to 60.15% with the addition of 1 % formic acid), their recoveries were still
5 satisfactory (Figure 3, table S5).



6
7
8 **Fig. 3.** Extraction efficiency (%) obtained with the method developed by using QuEChERS (method ii,
9 without FA) and with the addtionof formic acid in the extraction solvent at 0.1% and 1%. Mean of 3
10 preplicates (n=3).
11
12 After all the test performed the method showing the best performance was QuEChERS
13 only (i) with the addition of 1% of formic acid in the extraction solvent. No further
14 clean-up procedure was needed, but some evaporation steps were performed under a
15 gentle stream of nitrogen at room temperature. These concentration steps didn't
16 affect the stability of the compounds and neither the recoveries. An additional
17 filtration was carried out before running the samples on the mass spectrometry.

1 Consequently, the final method developed is simple, effective and fast, only one
2 extraction with QuEChERS followed by evaporation and filtration of the sample was
3 undertaken. In addition the cost of sample analysis was also considerably reduced.
4 The total time of analysis was less than 3 hours allowing simultaneously analysis of 30
5 samples per day.
6
7 **3.2 Method performance evaluation**
8 The performance of the final method was evaluated for clams (*Chamalea gallina*),
9 mussels (*Mytilus galloprovincialis*), and fish (*Platichtys flesus*). The recoveries obtained
10 for the three seafood species are presented in table 1. Twenty-three different
11 compounds belonging to seven chemical families were analysed using this
12 methodology. Recoveries for most of the compounds ranged between near 30% and
13 70%. Concretely, for clams it varies between 28% for sulfisoxazole and 60 % for
14 tilmicosin, for mussels between 29% for sulfisoxazole and 59% for tilmicosin and for
15 fish between 28% for chloramphenicol to 70% for tilmicosin. In other methods referred
16 in the literature for the analysis of antibiotics in seafood the recoveries were higher
17 than the ones reported in the present work ranging from 50% to 104% (Dasenaki and
18 Thomaidis, 2010; Evaggelopoulou and Samanidou, 2013). However, as mentioned
19 above, most of them focused on one or two families of antibiotics with similar physic-
20 chemical properties which facilitate the development of a more specific methodology
21 than in multi-residue methods. Next to this, when applying multi-residues methods in
22 biota samples, recoveries are usually considered acceptable when they are over 30%
23 due to the analytical challenge of developing a method for diverse compounds with
24 different lipophilicity and pKa (Huerta et al., 2013). The method developed covers

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1 antibiotics commonly used in aquaculture as macrolides, sulfonamides and
2 tetracyclines (Cañada-Cañada et al., 2009) and four of their major metabolites (N-
3 acetylsulfadiazine, N-acetylsulfamerazine, N-acetylsulfamethazine and metronidazole-
4 OH). Besides, the banned substance chloramphenicol was also included. Despite the
5 fact that chloramphenicol is not authorised for its use in food-producing animals in the
6 European Union (EFSA, 2014) some residues are still detected in seafood (EFSA, 2014)
7 due to illegal practices.
8

9 **Table 1.** Mean percentage recoveries (%) and standard deviation (n=3) of the target compounds in
10 *Chamalea gallina*, *Mytilus galloprovincialis* and *Platichthys flesus* spiked at 50ng/g dry weight.

Therapeutic family	**Antibiotic**	**Recovery (%) ± SD**		
		C. gallina	*M. galloprovincialis*	*P. flesus*
Macrolides	Azithromycin	56 ± 3	55 ± 0	52 ± 4
	Clarithromycin	51 ± 4	46 ± 3	43 ± 2
	Roxithromycin	54 ± 1	50 ± 2	47 ± 2
	Spiramycin	37 ± 5	38 ± 3	47 ± 11
	Tilmicosin	60 ± 2	60 ± 2	71 ± 5
	Tylosin	44 ± 6	51 ± 2	59 ± 7
Tetracyclines	Tetracycline	35 ± 9	33 ± 4	48 ± 6
Lincosamides	Clindamycin	37 ± 1	37 ± 5	41 ± 4
	Lincomycin	30 ± 2	31 ± 3	32 ± 6
Sulfonamides	Sulfadimethoxine	30 ± 7	34 ± 3	53 ± 1
	Sulfamerazine	30 ± 3	29 ± 3	40 ± 1
	Sulfamethoxazole	33 ± 8	30 ± 2	31 ± 2
	Sulfadiazine	32 ± 8	40 ± 4	45 ± 4
	Sulfapyridine	31 ± 13	34 ± 7	32 ± 7
	Sulfisomidin	34 ± 9	30 ± 4	33 ± 2
	Sulfisoxazole	28 ± 4	29 ± 3	33 ± 1
(Metabolite)	N-acetylsulfadiazine	37 ± 4	38 ± 8	38 ± 4
(Metabolite)	N-acetylsulfamerazine	37 ± 3	39 ± 4	43 ± 3
(Metabolite)	N-acetylsulfamethazine	44 ± 3	40 ± 3	42 ± 2
Nitroimidazoles	Metronidazole	54 ± 11	45 ± 4	48 ± 2
(Metabolite)	Metronidazole-OH	40 ± 4	39 ± 6	32 ± 3
Dihydrofolate reductase inhibitors	Trimethoprim	53 ± 12	50 ± 5	41 ± 2

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Amphenicols	Chloramphenicol	28 ± 2	32 ± 2	28 ± 1

1

2 Method detection limits (MDLs) and method quantification limits (MQLs) were
3 calculated for *C. gallina* (clam), *M. galloprovincialis* (mussel) and *P. flesus* (fish). Results
4 are shown in table 2. MDLs and MQLs both determined in spiked samples were
5 calculated using the first SRM considering the minimum amount of analyte with a
6 signal-to-noise ratio of 3 and 10 respectively. MDLs ranged between 0.02-0.31 ng/g
7 (dw), 0.01-0.29ng/g (dw) and 0.01-0.20 ng/g (dw), whereas MQLs ranged between
8 0.06-1.03ng/g (dw) 0.05-0.97 ng/g (dw) and 0.02-0.66 ng/g (dw) for clam, mussel and
9 fish respectively (table 2). The method detection and quantification limits obtained in
10 the present work were lower than those previously reported for the analysis of
11 antibiotics in seafood by other authors (Dasenaki and Thomaidis, 2010; Dickson, 2014),
12 and in the same range that those calculated by Ferderova et al., (Fedorova et al.,
13 2013).

14 **Table 2.** Method detection limits (MDLs) and method quantification limits (MQLs) of the target
15 compounds in clam (*C. gallina*), mussel (*M. galloprovincialis*) and fish (*P. flesus*)

Antibiotic	**MDLs (ng/g dw)**			**MQLs (ng/g dw)**		
	Chamalea gallina	***Mytilus galloprovincialis***	***Platichthys flesus***	***Chamalea gallina***	***Mytilus galloprovincialis***	***Platichthys flesus***
Azithromycin	0.06	0.03	0.05	0.18	0.10	0.17
Clarithromycin	0.05	0.04	0.07	0.16	0.15	0.23
Roxithromycin	0.20	0.17	0.13	0.67	0.56	0.43
Spiramycin	0.18	0.03	0.01	0.59	0.09	0.03
Tilmicosin	0.02	0.02	0.06	0.07	0.05	0.20
Tylosin	0.05	0.05	0.03	0.17	0.17	0.10
Tetracycline	0.10	0.05	0.13	0.33	0.15	0.45
Clindamycin	0.05	0.07	0.03	0.16	0.23	0.08
Lincomycin	0.13	0.03	0.09	0.42	0.09	0.29
Sulfadimethoxine	0.18	0.12	0.01	0.61	0.40	0.02
Sulfamerazine	0.08	0.14	0.06	0.26	0.46	0.21
Sulfamethoxazole	0.21	0.25	0.04	0.69	0.84	0.12
Sulfadiazine	0.10	0.18	0.08	0.34	0.60	0.26
Sulfapyridine	0.09	0.25	0.14	0.30	0.83	0.47
Sulfisomidin	0.31	0.29	0.06	1.03	0.97	0.19
Sulfisoxazole	0.07	0.08	0.03	0.24	0.25	0.09
N-acetylsulfadiazine	0.02	0.10	0.03	0.06	0.34	0.11

N-acetylsulfamerazine	0.05	0.02	0.13	0.17	0.05	0.44
N-acetylsulfamethazine	0.07	0.03	0.20	0.25	0.10	0.66
Metronidazole	0.07	0.01	0.06	0.24	0.05	0.19
Metronidazole-OH	0.07	0.10	0.06	0.22	0.32	0.20
Trimethoprim	0.15	0.07	0.02	0.51	0.24	0.08
Chloramphenicol	0.09	0.18	0.04	0.31	0.61	0.13

Calibration curves were generated using linear regression analysis ($r^2 \geq 0.990$ see table S6), they were prepared in the corresponding seafood extract (clam, mussel and fish) and used for the quantification of their corresponding matrix samples. The preparation of the standard curves in seafood matrix is of great interest as matrix effects may strongly influence the compounds analysis using UHPLC-MS/MS especially when dealing with complex matrices like biota (Alvarez-Muñoz et al., 2015; Gros et al., 2009). Therefore, the matrix effect on the MS signal was evaluated for each compound in each matrix comparing the peak areas of the calibration curve prepared in the seafood extract and those prepared in solvent (MeOH:H_2O 50:50) both spiked at 5, 10, 25 and 50 ng/ml. The percentages of reduction or enhancement are presented in figure S2. The majority of the compounds presented ion suppression. Only 5 compounds out of the 23 included in the method presented ion enhancement, three macrolides (azithromycin, spiramycin and tilmicosin), one tetracycline (tetracycline), and chloramphenicol (only in fish matrix). Ion enhancement in some antibiotics (e. g. azithromycin) has been previously reported in seafood matrices (Álvarez-Muñoz et al., 2015). The "internal sample calibration approach", calibration curve made up in the matrix with addition of isotopically labeled internal standards, was used to minimize matrix interferences and to avoid any under or over estimation during quantification. This approach has been previously demonstrated to be effective when analyzing target compounds in complex samples such as biota (Huerta et al., 2013; Stüber and Reemtsma, 2004).

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Accuracy of the whole method for each seafood matrix was calculated intra-day from five repeated injections of a sample spiked at 50 ng/g and extracted, and inter-day from three injections of this sample on three different days (table 3). Accuracy was calculated according to Bogialli et al., 2003 as the deviation of the measured mean concentration from the spiked concentration, expressed in percentage, and for most of the cases the values were lower than 20%. The instrumental precision was calculated intra-day (repeatability) and inter-day (reproducibility) as the relative standard deviation of the measured concentration (table 3). Both values were lower than 20% for the majority of the compounds, indicating good repeatability and reproducibility, demonstrating the effectiveness of the method for quantification purposes.

Table 3. Accuracy and precision of the target compounds in clam (C. *gallina*), mussel (M. *galloprovincialis*) and fish (P. *flesus*).

Antibiotic	*Chamalea gallina*				*Mytilus galloprovincialis*				*Platichthys flesus*			
	Intra-day		Inter-day		Intra-day		Inter-day		Intra-day		Inter-day	
	RSD	Accuracy	RSD	Accuracy	RSD	Accuracy	RSD	Accuracy	RSD	Accuracy	RSD	Accuracy
Azithromycin	3.7	-1.4	6.3	3.0	3.2	-1.3	1.9	-2.6	1.7	-1.3	2.9	2.5
Clarithromycin	5.0	-1.7	8.1	-0.3	3.5	2.8	11.1	19.1	3.5	-0.3	14.9	-6.8
Roxithromycin	4.9	7.1	9.5	10.6	2.0	-0.6	12.7	13.7	2.8	0.0	12.5	-6.8
Spiramycin	4.8	2.1	1.9	11.0	2.7	3.6	14.6	-9.5	5.1	-15.3	20.4	5.8
Tilmicosin	2.4	-0.7	5.1	3.0	3.1	-3.3	3.6	-4.5	2.5	2.7	2.6	3.7
Tylosin	5.5	6.9	9.7	13.5	3.2	-2.9	6.5	2.9	3.1	-0.5	0.6	-1.7
Tetracycline	3.6	-6.9	12.3	-14.7	6.6	-5.5	7.4	-10.2	7.4	3.6	12.5	-1.8
Clindamycin	2.5	3.2	7.6	1.2	9.7	1.2	19.3	8.1	3.6	1.2	0.8	5.1
Lincomycin	3.7	-3.8	4.7	0.5	6.8	0.1	11.2	2.2	9.5	9.8	5.2	14.0
Sulfadimethoxine	8.4	17.4	6.9	7.9	4.2	3.6	10.0	-6.5	10.0	3.9	17.6	-3.3
Sulfamerazine	3.9	10.1	20.3	-3.0	4.0	-2.1	5.3	-6.8	8.6	19.0	8.0	13.9
Sulfamethoxazole	5.3	15.7	14.6	-0.1	5.3	-4.0	2.3	-10.5	5.8	14.8	11.0	13.8
Sulfadiazine	2.5	10.1	17.0	-1.4	5.4	-2.1	7.1	-9.6	8.1	10.2	16.9	5.3
Sulfapyridine	4.8	20.2	1.9	10.2	9.7	-0.2	11.0	-13.9	9.5	12.3	9.8	13.9
Sulfisomidin	3.4	12.6	7.6	-1.4	7.5	-5.5	5.5	-12.0	8.8	-1.7	16.0	-10.7
Sulfisoxazole	3.0	17.0	167	8.0	2.3	-1.2	5.1	-9.9	8.7	8.8	13.7	2.8
N-acetylsulfadiazine	1.,2	1.5	7.7	-18.0	13.6	3.7	15.8	-5.9	8.3	2.5	19.9	-0.8
N-acetylsulfamerazine	9.4	1.2	11.2	-9.9	7.3	-4.1	7.0	-14.7	8.2	-3.2	14.7	-4.8
N-acetylsulfamethazine	5.5	-1.2	4.1	-8.6	7.4	0.3	7.0	-13.3	5.7	7.5	18.8	14.1
Metronidazole	6.4	0.0	16.1	-15.9	5.9	6.5	6.8	5.8	4.1	-3.4	18.9	-6.3
Metronidazole-OH	4.1	-1.5	16.1	-11.3	9.5	-0.3	8.6	-1.9	8.0	1.9	11.3	-2.0
Trimethoprim	2.0	2.3	11.6	-8.8	7.1	11.6	11.3	-7.6	8.7	-0.3	10.6	-2.0
Chloramphenicol	17	-8.4	12.9	8.7	4.7	0.1	5.7	-1.4	12.1	0.0	3.4	-0.01

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1 **3.3 Method application to farmed and wild seafood samples**

2 The method developed was applied to seafood samples (fish and mussel) taken from
3 different aquaculture and natural environments. Antibiotics concentrations found in
4 the different organisms analysed are represented in table 4. Six out of the twelve
5 samples analysed showed the presence of at least one antibiotic with concentrations
6 above MDLs, including three samples from aquaculture facilities and another three
7 from natural environments. Nine different antibiotics out of the 23 included in the
8 method were detected with levels above MDLs. These compounds belong to three
9 different antibiotic families: macrolides, tetracyclines and sulfonamides (table 4).
10 Among these nine compounds, seven were detected in aquaculture samples, three of
11 them (Clarithromycin, sulfadimethoxine and sulfamethoxazole) at levels below MQLs,
12 and the other four (roxithromycin, tilmicosin, tylosin and tetracycline) above MQLs in
13 at least three out of the eight species analysed. Their quantifiable levels ranged from
14 0.19ng/g (dw) of tylosin in salmon from Scotland, up to 4.96 ng/g (dw) of tetracycline
15 in the same sample. In the seafood samples collected from natural environments, only
16 4 antibiotics were found at levels above MDLs, and among them only 2 were above
17 MQLs. Concretely, azithromycin and tetracycline with levels ranging from 0.77 ng/g
18 (dw) in *Mytilus spp* from Tagus estuary to 5.63 ng/g (dw) in *Platichtys flesus* from
19 Scheldt estuary. These results showed that samples coming from aquacultures have a
20 higher amount of antibiotics than those coming from natural environments. These
21 results are in line with previous studies which reported that seafood from aquacultures
22 have higher presence of man-made chemicals such as antibiotics than the wild
23 organisms (Cole et al., 2009). Unfortunately, water sample from the same location
24 where seafood samples were taken was not available for analysis so the concentration

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1 of the contaminants in the surrounding media was not measured, and therefore, their
2 bioaccumulation factor could not be calculated.
3 Regarding the occurrence of antibiotics in the samples analysed, macrolides was the
4 most frequently detected group with at least one antibiotic from this family detected
5 in six out of the twelve samples analysed. Macrolides are potent antimicrobials used in
6 veterinary practices against a wide bacteria range, furthermore, they are some of the
7 most effective medicine against diseases produced by *Mycoplasmas*, and therefore,
8 they are commonly used in food-producing animals in order to treat or prevent
9 bacterial infections (Cañada-Cañada et al., 2009; Horie et al., 2003). In the particular
10 case of azithromycin, it was only detected in environmental samples. This antibiotic is
11 commonly indicated for human treatment but is rarely used in aquaculture, which may
12 explain that this compound was not found in any aquaculture sample. Similar
13 azithromycin concentrations in mussels from natural environments (Ebro delta, Spain)
14 have been previously reported in the same concentration range (Álvarez-Muñoz et al.,
15 2015). In the case of sulfonamides sulfadimethoxine, sulfamethoxazole and
16 sulfisoxazole were detected but none of them showed levels above MQLs.
17 Sulfonamides are synthetic antimicrobials widely used in fish cultures (Huet et al.,
18 2010). However, its occurrence in edible tissues of seafood has been rarely reported
19 (Baran et al., 2011). Indeed, only in few commercial seafood samples the presence of
20 sulfonamides have been reported with levels between non-detected to 20 ng/g (dw)
21 (Done and Halden, 2014; Fedorova et al., 2013). Despite the fact that some
22 sulfonamides metabolites were included in the analytical method (N-
23 acetylsulfadiazine, N-acetylsulfamerazine and N-acetylsulfamethazine), none of them
24 were detected above MDLs in the samples, probably due to the low concentrations of

the parent compounds detected, being all of them below MQLs. Tetracycline was the most ubiquitous compound being present in four out of the twelve samples analysed. It was also the antibiotic which presented the highest concentrations in natural environments, 5.63 ng/g (dw) in *Platichthys flesus* from Netherlands, and also in aquaculture samples 4.96 ng/g (dw) in *Salmo salar* from Scotland (table 4). Tetracycline antibiotic is commonly used in aquacultures as it is a broad-spectrum antibiotic, and it is also used for promoting growth in the farming industry (Cañada-Cañada et al., 2009). Similar values of tetracycline antibiotic (from non-detected to 13.1 ng/g (dw) were detected in fish (*Sparus aurata*) collected from marine farms from Cartagena, Spain (Cháfer-Pericás et al., 2011); whereas Na et al., 2013 analysed different marine species in coastal waters from China and found tetracycline antibiotic at concentrations around 1.73 ng/g wet weight (ww).

Despite the fact that some antibiotics residues were found in seafood samples their levels were far away from the Maximum Residue Limits established by the authorities being between 100 and 600 ng/g (ww) for the compounds detected in the analysed samples (EU No 37/2010). Furthermore, the banned substance chloramphenicol, which can provoke serious toxic effects in humans, was not detected in any sample. Therefore it is very unlikely that antibiotics present in seafood could cause an adverse effect in consumers due to the single intake of seafood. However, other dietary and non-dietary sources needs to be taken into consideration in order to assess their potential risk and identify if the levels ingested are below the acceptable daily intake advice by authorities (Australian Government Department of Health - Office of Chemical Safety, 2016). Besides, the risk for individual allergic people should be taken into consideration. Furthermore, the additive toxic effect of antibiotics together with

1 other contaminants also present in seafood like mercury, polychlorinated biphenols
2 (PCBs) and dioxins is not known yet, as well as the effect of chronic exposure to low
3 concentrations of this cocktail of pollutants (Cole et al., 2009; Jones et al., 2004).
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Table 4. List of antibiotics which were found in at least one sample with values above MDLs in different fish and mussel species collected from aquacultures and natural environments around Europe. Concentrations are represented in ng/g (dw) mean of 3 replicates and standard deviation (n=3 ± sd).

Therapeutic family	**Antibiotic**	**Aquaculture samples (ng/g dw) ± SD**								**Environmental samples (ng/g dw) ± SD**			
		Pangasisus spp (Vietnam)	Salmo salar (Scotland)	Salmo salar (Norway)	Mytilus galloprovincialis (Spain)	Mytilus galloprovincialis (Italy)	Mytilus galloprovincialis (Netherlands)	Mytilus spp (Greece)	Mytilus spp (Greece)	Mytilus galloprovincialis (Po delta)	Mytilus spp. (Tagus estuary)	Platichthys flesus (Scheldt)	Mytilus Gallorpovincialis (Bay Saint-Brieuc)
Macrolides	**Azithromycin**	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	2.13 ± 0.09	0.77 ± 0.04	< MDL	< MDL
	Clarithromycin	< MDL	**< MQL**	**< MQL**	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL
	Roxithromycin	1.12 ± 0.14	< MDL	0.48 ± 0.05	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	**< MQL**	< MDL
	Tilmicosin	< MDL	0.23 ± 0.10	0.42 ± 0.08	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL
	Tylosin	< MDL	0.19 ± 0.05	0.24 ± 0.02	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL
Tetracyclines	**Tetracycline**	2.38 ± 1.56	4.96 ± 0.50	3.36 ± 0.28	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	5.63 ± 0.41	< MDL
Sulfonamides	**Sulfadimethoxine**	< MDL	**< MQL**	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL
	Sulfamethoxazole	**< MQL**	**< MQL**	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL
	Sulfisoxazole	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	**< MQL**	< MDL

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1 **3.4 Microbial growth Inhibition test**

2 A detection technique based on the microbial growth inhibition was preliminarily
3 evaluated for the screening of antibiotics in seafood samples. The response in the
4 microbial growth inhibition test was checked for a procedure blank sample, a positive
5 and negative control sample, a clean sample (with no presence of any antibiotics) and
6 a spiked sample (fortified with 100 µg/L of oxytetracycline, 200µg/L of flumequine, 100
7 µg/L of erythromycin, and 100 µg/L of sulfamethoxazole). Positive controls showed
8 inhibition in all corresponding plates, and negative controls presented no inhibition
9 zone in any plate analysed (figure 4) and therefore the performance of the test was
10 considered correct. Regarding the samples analyzed, the procedure blank sample did
11 not show inhibition in the plate for any antibiotic family. However, the clean sample
12 showed inhibition for almost all antibiotic families and some interference due to the
13 biological matrix was postulated. Furthermore amino-glycosides plate showed
14 inhibition although no amino-glycoside compound was added to the spiking mix, which
15 may indicate some cross-reactive interferences. Only for macrolides/β-lactams plate a
16 clear differentiation between the clean sample and the spiked one was observed.
17 Therefore, the application of the microbial inhibition test was only feasible for a
18 qualitative identification of macrolides/β-lactams. Application of the microbial
19 inhibition test to other antibiotic families will need further investigation in order to
20 improve the extraction procedure and to assure the removal of matrix interferences.

21

Antibiotic group → Sample↓	Tetracyclines	Macrolides / ß-Lactam	Quinilones	Sulfonamides	Amino-glycosides
Procedure blank					
Clean sample					
Spiked sample					
Positive control		a b			
Negative control (methanol)					

Fig. 4. Schematic representation of the results for the three samples analysed extracted using ultrasonication followed by solid phase purification, the results for the positive and negative controls are also presented. The black circle represents the well to which samples were added, the red circle represents the inhibition zone (no growth of bacteria). a) It corresponds to macrolides positive control, and b) to β-lactams positive control.

4. Conclusions

A methodology for the analysis of antibiotics in seafood based on QuEChERS extraction followed by detection using UHPLC-MS/MS was developed. The method allowed the simultaneous analysis of twenty-three antibiotics belonging to seven different therapeutic families, and including four major metabolites. The performance of the method was good for the analysis of antibiotics in seafood (fish, mussels and clams) in terms of recoveries, accuracy, precision, MDL and MQL, proving the effectiveness of this methodology for a fast routine analysis of these compounds. The method was

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1 applied for the analysis of antibiotics in seafood species from aquacultures and natural
2 environments and a total of nine antibiotics were detected with levels above MDLs in
3 six out of the twelve samples analyzed. Aquaculture samples presented higher amount
4 of antibiotics than those samples coming from natural environments, however no toxic
5 effect for consumers is expected as all concentrations detected were lower than the
6 MRLs established.
7 An alternative detection technique based on microbial growth inhibition for the
8 detection of antibiotics in seafood was also tested. The method allowed a rapid and
9 simple detection of macrolides and β-lactams antibiotics in seafood. However, some
10 drawbacks of this methodology were observed (matrix interferences and cross-
11 reactivity) when analyzing other antibiotics families in seafood. Based on these
12 limitations, further experiments will be needed in order to improve the response of
13 the test for seafood samples.
14
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Highlights:

- A methodology was developed for the detection of 23 antibiotics in seafood
- QuEChERS extraction followed by UHPLC-MS/MS showed an optimal performance
- Nine antibiotics were detected in real seafood, all of them with levels below MRL
- A microbial inhibition test allowed a preliminary detection of macrolides/β-lactams

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