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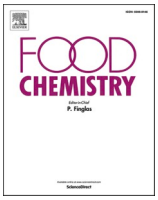

Extraction method for the multiresidue analysis of legacy and emerging pollutants in marine mussels from the Adriatic Sea

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ABSTRACT

The release of hazardous chemicals into aquatic environments has long been a known problem, but its full impact has only recently been realized. This study presents a validated liquid chromatography–mass spectrometry (HPLC–MS/MS) method for detecting pharmaceutical and pesticide residues in mussels (*Mytilus galloprovincialis*). An innovative MS-compatible extraction method was developed and validated, demonstrating successful recovery rates for analytes at three different concentration levels (25–95%). The method detected the target analytes at ng/g concentrations with high accuracy (−7% to 11%) and low relative standard deviation (<10%) for both intra-day and inter-day analyses. After validation, the method was applied to mussel samples collected from a commercial farm near Senigallia, Adriatic Sea, detecting different contaminants in the range of 2–40 ng/g (dry weight). The study provides a valuable tool for investigating the potential threats posed by diverse contaminant classes with high annual tonnage, including analytes with known persistence and/or illegal status.

1. Introduction

The improper management of hazardous chemicals and their related waste can acutely and chronically affect the biome and ecosystem health for present and future generations. The fate and effects of this pollution have been studied in many terrestrial organisms. However, less is known about this pollution in coastal marine ecosystems (Carrasco De La Cruz, 2021).

Worldwide agriculture and farming are the leading cause of water pollution, which is due to the extensive use of bioactive compounds for crop protection and animal husbandry (Mateo-Sagasta et al., 2017). Antibiotics are commonly used in animal husbandry worldwide to promote growth and prevent disease. In 2006, the European Union banned the use of antibiotics as growth promoters. New regulations that began in 2022 include a ban on antibiotic-medicated feeds for disease prevention and a ban on imported meat raised using antibiotics as growth promoters (Regulation (EU), 2019).

From a human and veterinary health perspective, the presence of antibiotics in aquatic environments is a serious problem because it accelerates the proliferation and spread of antibiotic-resistant bacteria, thus reducing the therapeutic effect of antibiotics themselves.

Along with animal husbandry, agriculture is another major cause of pollution because of the large-scale use of pesticides and herbicides to increase the quantity and quality of crops. The released contaminants can reach many different and geographically distant environments. Pesticides can leach through the soil into groundwater and thus reach the marine ecosystem. The diffusion of individual pesticides in the environment depends on their water solubility and chemical stability.

Given the potential effects, persistence, and widespread use of many pesticides and herbicides, these compounds pose a major risk to humans

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and to the environment. The European Green Deal (Communication from the Commission to the European Parliament, the European Council, 2019) therefore aims to reduce the use of chemical pesticides by 50% by 2030. The zero-pollution action plan, farm-to-fork strategy, and biodiversity strategy are designed to achieve this aim.

In light of the above, it is clearly important to use sensitive and reliable techniques to monitor contaminants in the various ecosystems. However, assessment of aquatic pollution levels cannot be based solely on the quantification of hazardous compounds in environmental samples (e.g. water, sediments, soil). Rather, one must also consider the bioaccumulation of xenobiotics in organisms inhabiting the specific environment. This enables a system-level vision of the chemical treatment of the entire ecosystem, including the biome (i.e. microbiome), in accordance with the One Health approach (Mackenzie & Jeggo, 2019). In this context, bivalves are a useful sentinel organism for monitoring how anthropogenic substances affect the health of the aquatic environment (Burns & Smith, 1981).

To monitor the pollution of aquatic ecosystems, it is therefore important to determine residues in sentinel marine organisms like mussels (Blanco-Rayón et al., 2020), which serve as proxies for those ecosystems (Galimany et al., 2023).

Furthermore, consumption of mussels as food is very high all over the world (in the EU, the yearly average mussel consumption per capita is 1.28 kg (Commission et al., 2021)). The presence of contaminant residues in mussels or other edible marine species is therefore a critical public health concern that must be monitored via specific analyses. One important aspect of this issue is to develop analytical methods to detect pollution residues in various edible aquatic species, combining environmental monitoring with risk assessment of dietary intake.

In recent years, a few multiresidue methods have been developed to quantify different classes of organic pollutants (Chiesa et al., 2018; Kalogeropoulou et al., 2021; Lerebours et al., 2021; Martínez-Morcillo et al., 2020; Mezzelani et al., 2020; Miranda et al., 2022).

But despite optimization efforts, the comprehensive extraction, chromatographic separation, and quantification of organic pollutants is an unsolved challenge, especially in various animal tissues (Han et al., 2016). Indeed, the soft tissues of marine animals are very complex matrices. They contain various compounds (e.g. lipids, proteins) that may interfere with detection and quantification, affecting selectivity and sensitivity (Costa et al., 2022; Wang et al., 2019).

Given the differences in the physicochemical properties of the molecules involved in multiresidue analyses, it is necessary to use extraction methods based on different strategies. Several sample clean-up techniques are usually combined to increase the extraction capacity (a greater number of analytes) and the recovery rate. The most common preprocessing techniques usually involve first liquid–solid extraction using solvents with different polarity, often enhanced with microwave-assisted extraction (MAE) (Wille et al., 2011) or pressurized liquid extraction (PLE) (Li et al., 2012). If needed, the obtained fractions can be further purified e.g. by solid phase extraction (SPE) to concentrate the analytes. Quick, easy, cheap, effective, rugged, and safe (QuEChERS) methods comprising both the extraction and clean-up steps have been widely adopted for their simplicity and reliability. They are now the standard approach for matrices that are not excessively fatty.

After the sample pretreatment, liquid chromatography coupled to mass spectrometry (LC-MS) is the analytical technique of choice for multiresidue methods. The chromatographic system usually comprises a column with nonpolar stationary phase, C18, with different end-capping and bonding technologies. For example, the Alvarez-Muñoz group used two different columns for positive and negative ionization modes, an Acquity HSS T3 and an Acquity BEH C18 respectively, both by Waters (Alvarez-Muñoz et al., 2015). This functionalization enhances polar compound retainment. The Mijangos group used a method with Kinetex F5 column, obtaining good results on an ample set of analytes (Mijangos et al., 2019). Almost all developed methods used a gradient for analyte elution. The most frequently used mobile phases were water with different concentrations of formic acid or ammonium formate, methanol, or acetonitrile as organic phase (Alvarez-Muñoz et al., 2015; Mijangos et al., 2019).

Detection occurs mainly with electrospray ionization (ESI) and triple quadrupole or high-resolution MS (QTOF or orbitrap) as analyzers. The highest sensitivity is achieved with the multiple reaction monitoring (MRM) acquisition mode, which increases the signal-to-noise ratio.

To substantially contribute to this issue, we developed and fully validated an analytical method based on LC-MS to quantify up to eleven anthropogenic xenobiotics of different families of contaminants in the soft tissues of Mediterranean mussels (*Mytilus galloprovincialis*). These families of contaminants are pharmaceuticals (including antibiotics), personal care products, and pesticides.

Here, to improve and validate a reliable new sample pretreatment, we tested and compared the most popular methods of extraction and clean-up of mussel samples. Different protocols were investigated to establish the best strategy for each specific class of chemicals. Finally, we optimized an original sample cold extraction and preconcentration, followed by a clean-up with surfactant CHAPS, recognized as a valid protocol for analyzing all the investigated analytes.

The method was subsequently applied to assess the occurrence of target compounds in real mussel samples (*Mytilus galloprovincialis*) collected in a farm in the Northwestern Adriatic Sea.

2. Materials and methods

2.1. Chemicals

Water of HPLC-MS grade (Millipore) was produced using the depurative system Milli-Q Synthesis A 10 (Molsheim, France). Methanol (MeOH), hexane, cyclohexane, dichloromethane (DCM), acetone, and acetonitrile (ACN), all of HPLC-grade, were purchased from Merck (Darmstadt, Germany). Acetic acid (98% pure), magnesium sulfate monohydrate (MgSO4·H2O) (97% pure), ethylenediaminetetraacetic acid (EDTA) (>98.5% pure), and sodium hydroxide (98% pure) were purchased from Fluka (Buchs, Switzerland).

Metolachlor (≥95.0% pure), alachlor (≥98.0% pure), atrazine (≥98.0% pure), sulfamethoxazole (≥98.0% pure), erythromycin A dehydrate (≥90.0% pure), tetracycline (≥95.0% pure), doxycycline hyclate (≥95.0% pure), and amoxicillin trihydrate (≥95.0% pure) standards were purchased from Merck Life Science BV (Overijse, Belgium). Carbamazepine (≥99.0% pure), atrazine-desethyl-desisopropyl (≥95.0% pure), N,*N*-diethyl-meta-toluamide (≥95.0% pure) standards, and 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS) (>98% pure) were purchased from Merck KGaA (Darmstadt, Germany). Isotopically labeled internal standards (IL-ISs) metolachlor-(2-ethyl-6-methylphenyl-d11) (≥97.0% pure), alachlor-d_{13} (≥98.0% pure), atrazine-d_5 (≥99.0% pure), carbamazepine-$^{13}C_6$ (99.9% pure), sulfamethoxazole-(phenyl-$^{13}C_6$) (≥99.0% pure) were from Merck Life Science BV (Overijse, Belgium).

Supel QuE Z-Sep + Tube, Supel QuE PSA/C18 Tube, Supel QuE Citrate (EN) extraction tubes, Supel QuE Acetate (AC) extraction tubes, and LiChrolut EN 200 mg 6 mL SPE materials were acquired from Merck KGaA (Darmstadt, Germany). C18 (500 mg, 6 mL) SPE columns were purchased from SiliCycle (Quebec, Canada).

2.2. Standard solutions

Single stock solutions (1 mg/mL) of metolachlor, alachlor, atrazine, sulfamethoxazole, erythromycin A dehydrate, carbamazepine, tetracycline, doxycycline hyclate, amoxicillin trihydrate standards and respective IL-ISs (metolachlor-(2-ethyl-6-methylphenyl-d11), alachlor-d_{13}, atrazine-d_5, and sulfamethoxazole-(phenyl-$^{13}C_6$) were prepared in methanol and stored until use at −80° C.

Carbamazepine- $^{13}C_6$ was already available at a concentration of 100 μg/mL in methanol. Stock solution of atrazine-desethyl-

desisopropyl was prepared in methanol at a concentration of 100 μg/mL and stored until use at −80° C.

Standard solutions used for method validation were obtained by diluting stock solutions in mobile phase.

Spiked sample solutions (QC) used for optimizing the extraction procedure and for matrix matched calibration curve were obtained by adding diluted stock solution in the range 0.002–500 ng/mL to the sample. For preparation, see Section 2.5.

2.3. Instrumentation conditions

Liquid chromatography was performed using a 2690 Alliance system (Waters, Milford, MA, USA) coupled to a triple quadruple mass spectrometer (Quattro-LC, Micromass), equipped with an ESI source, operating in the multiple reaction monitoring (MRM) acquisition mode. The optimal analytical separation was achieved by using an Atlantis T3 Column (5 μm, 2.1 mm X 150 mm, Waters) already used for pharmaceutical analysis in a previous publication by Alvarez-Muñoz (Alvarez-Muñoz et al., 2015). The gradient elution mode was performed with a mobile phase comprising 0.01% acetic acid in water (A) and 0.01% acetic acid in a solution of methanol and acetonitrile 65:35 (v/v) (B). The initial conditions, 10% of solvent B, were held for 5 min, then solvent B was increased to 60% over 7 min, followed by a further rise to 80% over 3 min, and then a further rise to 90% over 2 min. These conditions were held for 25 min. Finally, mobile phase B was returned to its initial conditions over 10 min. The separation was completed within 37 min. The flow rate was 0.14 mL/min, the column temperature was maintained at 20 °C with an injection volume of 5 μL.

The MS/MS experimental conditions were tuned by direct infusion of the single analytes. The detection was performed in positive mode (2500 V) and the spectra were acquired in multiple reaction monitoring (MRM) mode. Argon gas was selected as collision gas and nitrogen as nebulizer and heater gas. Nitrogen was used as nebulizer gas at 117 L/h flow rate and as desolvation gas at 622 L/h. Ion source block and desolvation temperatures were set at 120 °C and 180 °C, respectively. Capillary and cone voltages were 2.90 kV and 60 V, respectively. For optimization of MS parameters, individual standard solutions were prepared in methanol (10 mM) and introduced into ESI source by direct infusion at a flow rate of 20 mL/min. Table S1 summarizes the optimal MS/MS parameters for each standard and internal standard including precursor ions, product ions, collision energies, and cone voltages.

2.4. Sample collection, pooling, and storage

Mussel handling and sampling procedures were performed in line with recommendations and technical protocols from the OSPAR Commission (2013) (https://www.ospar.org/).

Specimens of *M. galloprovincialis* of commercial size (4–6 cm in length) were collected in October 2019 by fisherman of the "Cooperativa Copr.al.mo" (Cesenatico, Italy) within a farm location approved for direct commercialization according to European legislation 91-492-CEE. The sampling site is located in the Northwestern Adriatic Sea, an area characterized by shallow waters and high riverine inputs from the Italian border, which results in widespread chemical contamination. The chemicals analyzed in this study have been documented in coastal waters (Nödler et al., 2014) and in mussels (Chiesa et al., 2018; Mezzelani et al., 2020).

Upon collection, mussels were transferred to the laboratory in seawater tanks. 30 mussels were immediately processed for whole soft tissue dissection, homogenization, and lyophilization, as outlined below. Additional animals were acclimated in aquaria containing 35-psu filtered seawater at 16 °C with continuous aeration (>90% oxygen saturation). During acclimatization, mussels were fed once a day with a commercial algal slurry (Koral, Xaqua). These mussels were used to provide samples for spiking experiments and for the further steps of protocol validation. In any case, the mussels were immediately analyzed to assess their good initial health status according to the lysosomal membrane stability (neutral red retention assay) (Buratti et al., 2013) (data not shown).

Whole mussel soft mass was dissected from each mussel and pooled to obtain samples made up from 3 animals/group. 10 pooled samples were then homogenized using a UltraTurrax system (IKA), frozen at −20 °C, and finally lyophilized.

2.5. Sample extraction procedure

The optimized extraction procedure reported in Fig. 1 offered the best compromise between recoveries, limit of detection, and matrix effect. Aliquots of 250 mg dry weight (dw) of whole mussel powder were transferred into a centrifuge tube and stored at −20 °C until sample clean-up. The extraction procedure was as follows: 1) Aliquot was thawed, 10 μL of IS 10 μM were added, and the freeze-dried sample was extracted with 1 mL of cold ACN:MeOH (50:50 v/v) mixture (extraction mixture A), 10 μL of EDTA 25 mM and 0.25 g of $MgSO_4$ were added. The sample was vortexed for 2 min, cooled for 10 min at −20 °C, and centrifuged at a controlled temperature of 4 °C at 3600 rpm for 2 min for protein precipitation. The supernatants, obtained from two identical replicated extractions, were collected. 2) The sample powder pellet underwent a second double extraction with 1 mL of a previously refrigerated solution of hexane:acetone (50:50 v/v), extraction mixture B, and the addition of 10 μL of EDTA 25 mM. The sample was vortexed for 2 min, cooled for 10 min at −20 °C and centrifuged at a controlled temperature of 4 °C at 3600 rpm for 2 min. 3) The supernatants were collected and mixed with those obtained with ACN:MeOH (50:50 v/v) mixture. The extracted solution was vacuum-dried with a UNIVAPO Vacuum Concentrator (UniEquip, Monaco). 4) The oily residue was redissolved in 200 μL of CHAPS 0.6% (m/v) aqueous solution. The sample was vortexed for 1 min and centrifuged at 3600 rpm for 10 min. The supernatant was collected and stored separately. 5) 100 μL of MeOH were added to the remaining residues, the sample was vortexed for 1 min, and centrifuged at 3600 rpm for 7 min. 6) The supernatant was collected and stored with the previously collected supernatant. 7) This solution was centrifuged at a controlled temperature of 4 °C at 13400 rpm for 10 min. 100 μL of the filtered (0.45 μm syringe filter) supernatant were injected into the LC-MS system.

2.6. Optimization of extraction procedure

To determine the optimum extraction procedure, different organic solvents were tested: hexane, cyclohexane, dichloromethane, acetonitrile, methanol, and acetone. The optimization of the solvents was conducted to maximize recovery of the analytes and, when possible, to decrease matrix effects.

As described in Section 2.5, we obtained the best compromise by using two separated extraction mixtures: the first with ACN:MeOH (50:50 v/v) solution and the second with a hexane:acetone (50:50 v/v) solution.

To improve recovery of analytes, microvolumes of a solution of EDTA (25 mM) and 0.25 g of $MgSO_4$ were added to the mussel pellet during extraction.

The extraction temperature was also optimized. Different thermal sample treatments were evaluated. In particular, the sample preparation as described in Section 2.5 was conducted in parallel at room temperature, or using refrigerated solvents and a rapid cooling cycle (-20 °C for 20 min). Percentage agreement of target analytes from cold-treated versus room temperature was calculated.

Sample clean-up with solid phase extraction (SPE) was evaluated. After solvent extraction with ACN/MeOH and hexane/acetone as reported in Section 2.5, the sample was dried and resuspended with 1 mL of the mobile phase, and SPE was tested. Two different SPEs were tested: LiChrolut EN (200 mg, 6 mL) and Silicycle C18 (500 mg, 6 mL). SPE columns were activated with 5 mL of MeOH and 5 mL of H_2O. The

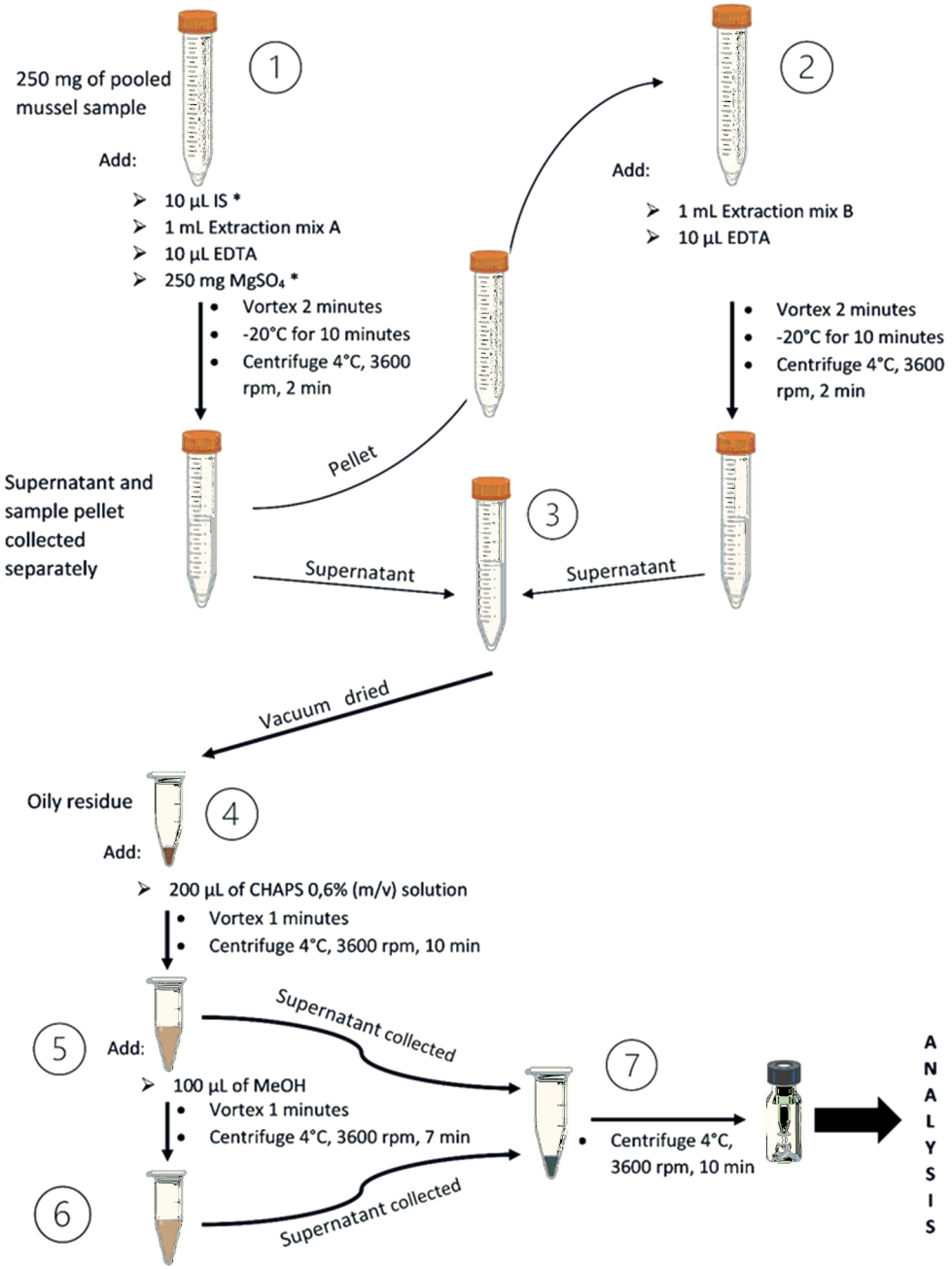

Fig. 1. Optimized extraction procedure for the investigated compounds in mussels using CHAPS detergent.

resuspended sample was added to the SPE cartridge. The cartridge was washed with 1 mL of water, and the analytes were eluted with MeOH. Aliquots of 1 mL were collected, dried, and quantified as per the optimized procedure described in Section 2.3. The recoveries and matrix effect were calculated.

Sample clean-up was also considered by using two alternative extraction procedures based on dispersive SPE (d-SPE) such as QuEChERS method, including Supel QuE Citrate (EN) + Supel QuE Z-Sep + extraction tubes and Supel QuE Acetate (AC) + Supel QuE PSA/C18 extraction tube. These last specific powder mixes were used because they are especially indicated for improving recovery of pesticides from fat matrixes. Prior to extraction with ACN:MeOH, salts were added and the standard QuEChERS and d-SPE procedure was applied. The obtained solution was then evaporated, resuspended in 200 µL of mobile phase, centrifugated, and the supernatant was stored separately. The remaining residues were treated as described in points 5, 6, and 7 in Section 2.5 (without using CHAPS solution).

The use of a surfactant was assayed. CHAPS is a zwitterionic surfactant typically used to solubilize biological macromolecules. We used it in our procedure to improve our recoveries by dissolving the oily residues obtained after drying our extraction solvent. We conducted a side-by-side comparison of the quantification of CHAPS-treated vs. untreated samples.

2.7. Method validation

For each analyte, the method performance was evaluated by determining the retention time (RT), transition ion ratios, recovery, accuracy (trueness), precision (expressed as the intra- and inter-day repeatability), linearity, method detection limits (MDLs), and method quantification limits (MQLs).

Selectivity was evaluated by comparing the chromatograms obtained

from standards, samples, and spiked sample solutions.

The instrumental linearity was also assessed with six-point calibration curves in matrix-matched curve, containing each IS (fix concentration 10 MQL). Spiked mussel matrices were obtained by adding stock solution in the range 0.002–500 ng/mL.

MDL and MQL were determined in the samples spiked before the extraction (n = 3) and considered as the minimum detectable amount of analyte with signal-to noise ratio (S/N) of 3 and 10, respectively.

The method's overall accuracy and precision were calculated intra (n = 3) and inter-day (n = 9) from three repeated injections of spiked sample solution (QC) at three different concentrations (low, medium, high) and extracted. Low concentration was the MQL, medium concentration was 10 MQL and high concentration was 100 MQL for each analyte.

Accuracy was calculated following this expression: bias (%) = (STD_m-STD_s)/ STD_s were STD_m and STD_s indicate mean calculated concentration and spiked concentration, respectively.

To evaluate potential matrix effects, we adopted the following approach: a pooled mussel sample (mussel matrix without analytes) was extracted as per the protocol (Section 2.5); the final supernatant was then spiked with analyte standard solutions at three concentration levels (low, medium, high) and analyzed. Quantification on this sample was compared to results obtained on a mobile phase standard solution at the same analyte concentration levels. The percentage matrix effect (matrix ion suppression/enhancement) was calculated. If ME ≈ 0%, then there is no observed matrix effect. If ME > 0%, then an ion enhancement occurred. If ME less than 0%, then an ion suppression occurred.

Recovery experiments were performed in triplicate at three concentration levels: low (MQL), medium (10 MQL), high (100 MQL). This was done by comparing the area ratio of the analyte to the IS of sample fortified before and after extraction. In these conditions, both samples are subjected to the same matrix effects, making eventual differences dependent only on the efficiency of the extraction. The different samples were analyzed, and percentage absolute recoveries were calculated.

2.8. Short-term storage stability

To investigate the effects of different storage methods during daily operations, stability of mixed standard solutions was assessed. Mixed standard solutions placed in 2 mL amber glass LC vials, were stored under three different conditions, room temperature, at 4 °C in the autosampler, at −20 °C, for 8 h to assess the possible loss of analytes during sample processing and analysis time. The t = 0 and t = 8 h standard solutions at the same concentrations were analyzed and percentage relative recovery was calculated.

3. Results and discussion

3.1. Analytical separation method development

To select the optimal separation conditions, single solutions and mixture of standards underwent a series of iterated analyses, using a conventional experimental design approach. Two different reversed phase separation columns were trialed for the separation of the target bioactive molecules. Of the two columns tested, the XBridge C18 showed co-elution of various analytes and unacceptable separative performances, thus the Atlantis T3 column was selected because it provided good chromatographic separation and peak symmetry. In a second step, different mobile phase compositions were evaluated at different solvent gradients with common solvents and buffer used for fatty matrix analysis. As organic phases, we tested pure methanol, pure acetonitrile, and mixture of both. As water phases, we tested pure water, water with different amounts of formic acid and acetic acid from 0.01 to 0.1% (v/v), and ammonium acetate buffer at three different pH levels (6-7-8). Mobile phases with low pH levels were not tested because of potential epimerization processes of the investigated antibiotic compounds (Libinson & Ushakova, 1976; Mohammed-Ali, 2012).

In terms of chromatographic resolution, peak shapes, and analysis times, the best compromise was achieved with a mobile phase comprising 0.01% acetic acid in water and a solution of methanol and acetonitrile 65:35 (v/v) with 0.01% acetic acid.

Finally, we investigated the influence of flow rate in the range 0.10–0.50 mL/min and column temperature in the range 20–60 °C. The flow rate was set at 0.14 mL/min, because higher flow rates led to poorer peak shape and loss of resolution, and the temperature was set at 20 °C.

Fig. S1 shows the chromatograms relative to the different analytes included in the method at medium concentration (10 MQL).

3.2. Optimization of the extraction procedure

3.2.1. Solid-liquid extraction solvents

It is challenging to analyze multiresidues in fatty matrices such as mussels. This is because of the relatively low concentration of the chemically different analytes and the intrinsic complexity of the matrix. Mussels contain large amounts of lipids and proteins that could interfere with chromatographic analysis (Martinez et al., 2004). The lipid content in *Mytilus galloprovincialis* has been established at 2–4% w/w and proteins at around 15% (Dernekbaşı, 2015). This poses a great challenge when developing efficient extraction procedures.

For this reason, we compared several sample preparation strategies reported in the literature, knowing that no single strategy would be suitable for all the analytes in our study. It is challenging to purify the analyte of interest, avoiding the co-extraction of fatty material, which may hamper proper detection by affecting recovery and matrix effect. Furthermore, some of the pesticides we wished to quantify are fat-soluble nonpolar compounds (e.g. organochlorine), which tend to concentrate and accumulate in fat. Given the different chemical physical properties of our analytes and based on several articles reporting similar approaches, we essayed the extractions with different pure solvents first and then with mixtures. We tested the nonpolar solvents hexane, cyclohexane, and dichloromethane. Cyclohexane and hexane showed similar recoveries for carbamazepine, sulfamethoxazole, and erythromycin, but cyclohexane showed much lower recoveries for all the other compounds. Hexane and DCM showed comparable recoveries for pesticides, but DCM showed better recoveries for pharmaceuticals. However, DCM showed an increase in matrix effects for most analytes, prompting us to choose hexane. Given that recoveries were too low for some specific analytes (i.e. sulfamethoxazole, erythromycin) and given their specific solubility in acetone (Guo et al., 2020; Wang et al., 2006), we also decided to test acetone in the extraction mixture. Pure acetone and a mixture of hexane and acetone 50:50 (v/v) were tested. The latter was the best solution, with a significant increase in sulfamethoxazole recovery and an increase in nearly every other compound. With this extraction, we obtained acceptable recoveries and matrix effects for some of the compounds, especially pesticides. Recovery and matrix effects of each single solvent and binary mixture are reported in Figure 2 and 3, respectively. Recovery was calculated as a percentage of our final method described in Section 2.5 (dash line), which was considered to be 100%.

To improve our results on antibiotics, we tested two more solvents with higher polarity: acetonitrile and methanol. Acetonitrile was efficient for the extraction of pesticides, with an acceptable matrix effect for most compounds, but its pharmaceuticals recovery was not satisfactory. Methanol reduced recoveries for pesticides, but it drastically increased recoveries for pharmaceuticals, especially doxycycline and tetracycline, increasing the matrix effects for most compounds by extracting more interferences. We found an acceptable compromise between recoveries and matrix effects using a mixture (50:50 v/v) of the two solvents (Figures 2 and 3). Finally, we conducted two separate extractions with two different solvent mixtures (ACN:MeOH) (50:50 v/v) and hexane: acetone (50:50 v/v)) on the same freeze-dried sample.

The recovery and matrix effects of the different solvent extractions

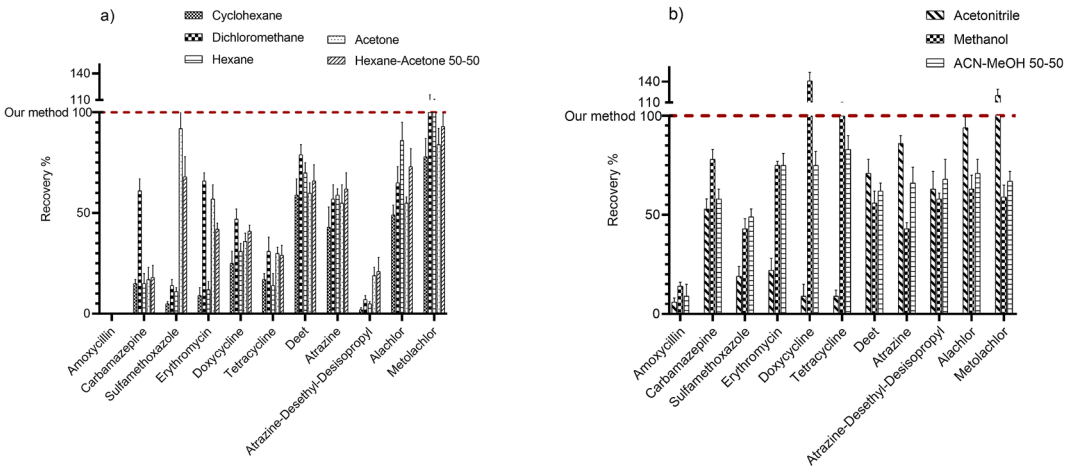

Fig. 2. Recovery and relative standard deviations (RSD) of different extraction solvents: a) nonpolar solvents; b) polar solvents. Dash line represents the recovery obtained, with our final method (Section 2.5) considered to be 100% recovery.

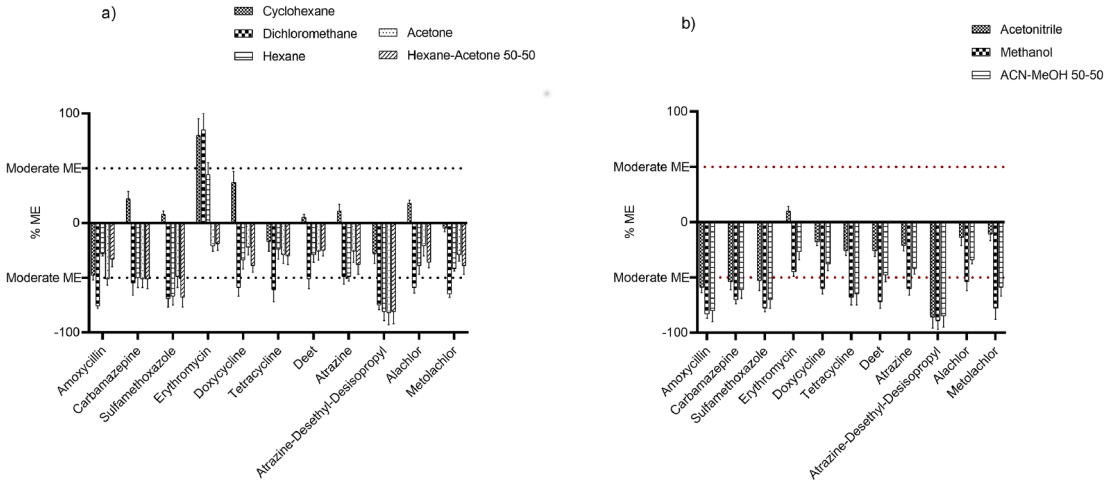

Fig. 3. Matrix effects and relative standard deviations (RSD) of different extraction solvents: a) nonpolar solvents; b) polar solvents.

and the complete extraction method are shown in Tables S2–S10.

3.2.2. *Short-term storage stability*

Antibiotic stock solutions in a suitable solvent (i.e. methanol) stored at appropriate temperature (i.e. −80 °C or −20 °C) are generally stable over time, but this stability might be compromised in suboptimal storage conditions (such as acidic phases) (Emami et al., 2022). We conducted stability studies on QC samples stored for 8 h at three different temperatures (room temperature, 4 °C, −20 °C). In agreement with the literature, our studies showed a decrease in the content of some compounds, especially antibiotics (Table S11).

Based on the information gathered during our short-term stability study, the whole extraction procedure followed a cold process, which minimized the solubilization of contaminants from the matrix and increased the stability of some analytes under the extraction conditions.

The cold processing was obtained by using previously refrigerated solvents, centrifuged at 4 °C, with a decanting interval of 10 min at −20 °C after each centrifuging step. Overall, compared to room temperature extraction, the cold processing showed higher recovery for amoxicillin, tetracycline, DEET, and atrazine-desethyl-desisopropyl, and comparable values for other compounds. Matrix effects were comparable or slightly better. This improvement could be ascribed to lowering the degradation of the analytes and the concentration of interferents (results in Table S12). The latter effect was obtained by extracting fewer impurities and by favoring their precipitation.

3.2.3. *SPE and d-SPE*

Other attempts were made to optimize the sample clean-up and the recovery of the oily residue formed after extraction with optimized solvent mixture in the dried joined supernatants (see Section 2.5). In order to recover this residue, we made several attempts to solubilize and extract the compounds of interest by testing SPE and QuEChERS procedures. Indeed, for further sample purification, a clean-up with SPE is part of the most common approach for residue extraction in fatty matrices. Hence, we tested two different SPE cartridges, LiChrolut EN and Silicycle C18, as described in Section 2.6. The eluate in MeOH was dried and the oily residue was still present. Of the two SPE cartridges, the LiChrolut EN column provided a low level of matrix effect and improved recoveries for most pharmaceuticals (i.e. erythromycin, tetracycline and doxycycline). Unfortunately, it also drastically decreased recoveries for some pesticides compared to Silicycle C18 (i.e. alachlor, metolachlor, DEET) and our final method. The two columns did not improve the matrix effect enough and did not improve recovery at all for most analytes (i.e. metolachlor, carbamazepine, sulfamethoxazole, alachlor, DEET, etc.), relative to the time cost. The resulting recoveries and matrix effects are reported in Figures 4a and 5and in Tables S13-S14 in the Supplementary Information.

We tested two different dispersive solid phase extraction (d-SPE)

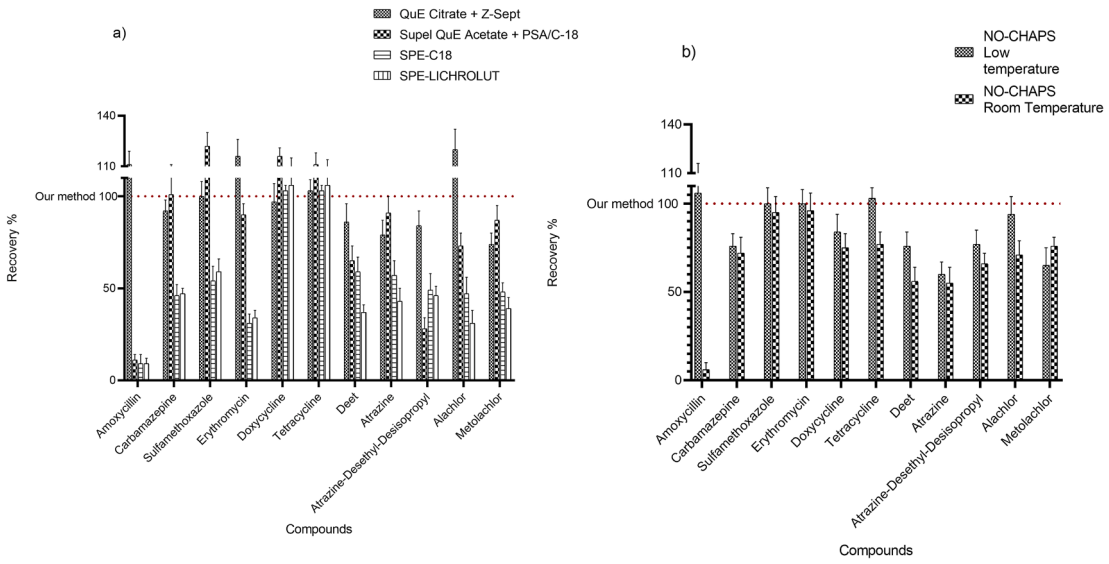

Fig. 4. Recovery and relative standard deviations (RSD) of different clean-up methods: a) SPE (LiChrolut EN column and Silicycle C18 column) and d-SPE (QuE Citrate (EN) + Supel QuE Z-Sep + Extraction tubes and Supel QuE Acetate (AC) + Supel QuE PSA/C18 extraction Tube); b) without using CHAPS at different temperatures. Dash line represents the recovery obtained, with our final method (Section 2.5) considered to be 100% recovery.

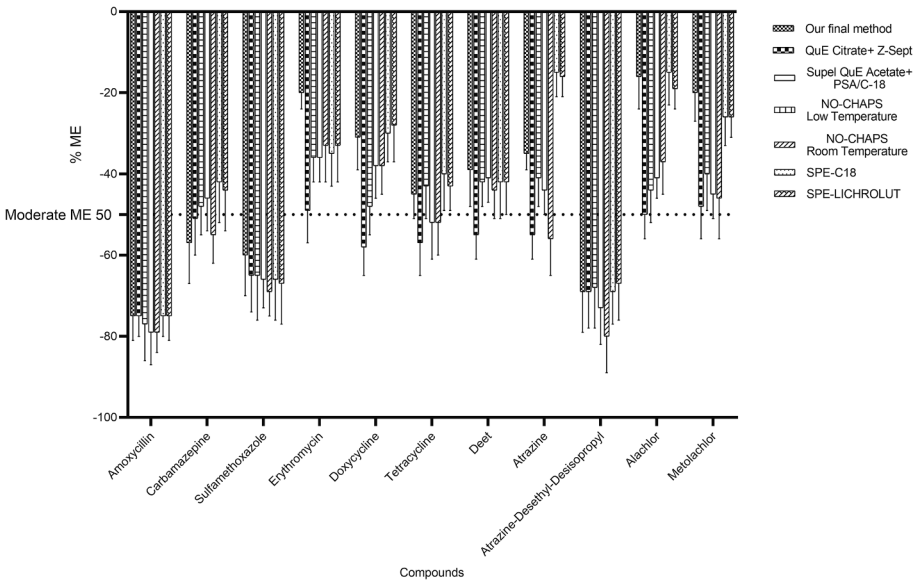

Fig. 5. Matrix effect and relative standard deviations (RSD) of different methods.

mixtures (QuE Citrate (EN) + Supel QuE Z-Sep + extraction tubes and Supel QuE Acetate (AC) + Supel QuE PSA/C18 extraction tube). The first powder mix was a QuECHERS and d-SPE method, which is especially used to determine pesticide residues in complex matrices containing >15% fat. The second powder mix is described in an AOAC Official Method to determine pesticides in several food matrices (Lehotay et al., 2007).

For the two d-SPE using PSA/C18, we observed a decrease in matrix effects and an improvement in recovery for doxycycline, tetracycline (two of the analytes for which we consistently had low recoveries), atrazine, and sulfamethoxazole. However, compared to Z-SEPTs, we observed around a 90% loss in recovery for amoxicillin, a comparable loss for atrazine-desethyl-desisopropyl, and lower recoveries for alachlor, DEET, and erythromycin. However, while drying the samples, we still observed the formation of an oily residue.

For both the d-SPE systems, the overall effects on recovery and matrix effect were not satisfactory. Except for a few analytes (i.e. alachlor, doxycycline, sulfamethoxazole), we did obtained a poor compromise of method recovery, and we obtained an increased matrix effect for several compounds (i.e. metolachlor, erythromycin, atrazine).

Resulting recoveries and matrix effect are reported in Figures 4 and 5 and in Table S15–S16 in the Supplementary Information.

3.2.4. *Chaps*

Since none of the pretreatments described above (SPE and QuEChERS) were satisfactory in avoiding oil formation in the dried sample, an aqueous solution with CHAPS 0.6 % (m/v) instead of FM was used to redissolve the oily residue. CHAPS is a mixture of zwitterionic detergents that are particularly suitable for mass spectrometry. A substance with detergent properties is used to help the redissolution of analytes trapped in the fatty oily residue. The use of CHAPS improved recoveries, especially at low temperature and for pesticides (i.e. DEET, atrazine, atrazine-desethyl-desisopropyl, metolachlor), and delivered comparable results for pharmaceuticals such as tetracycline and

doxycycline. Figure 4b compares the recoveries for our final method with CHAPS at low temperature (dash line) and without CHAPS. Recovery was calculated as the percentage of our final method's recovery (Section 2.5, dash line), which was considered to be 100%. The matrix effects were significantly lower in almost all cases, making the method with CHAPS the best of those tested for the extraction and purification of these compounds in mussel samples (Fig. 5). Final method recoveries and matrix effects at medium concentration are reported in Section 3.2.2 and in Table S17 (low to high concentration) in the Supplementary Information.

3.3. Method validation

Table S18 compares the main characteristics of our proposed method with other recently reported methods, which use different extraction and clean-up procedures and analytical techniques to assess pharmaceuticals in mussels. Notably, some of the recently reported methods use time-consuming analytical procedures such as SPE- FUSCLE (ultrasonic solid–liquid extraction) and HLB-SPE and PLE.

Given the methods published so far, it is challenging to develop a comprehensive analytical method for the main chemicals potentially present in mussels. This is due to the different analytes' physical–chemical properties and the complex matrix. The chemicals potentially present in mussels include tetracycline and penicillin antibiotics, pesticides and several other pharmaceuticals with different polarities. This makes it impossible to tailor the extraction and clean-up procedure to a specific class of compounds, i.e. by the exclusive use of a single solvent extraction step. This makes it necessary to find the best compromise between recovery method performance and matrix effect, obtaining the highest sensitivity as described in our study.

3.3.1. Linearity, accuracy, and precision

Calibration curve parameters for all the considered compounds, in the specific concentration range for each analyte, were obtained by plotting the peak area ratio between analyte and IS of the spiked sample solution against their theoretical concentration through a linear least squares regression analysis.

Linearity was assessed via six-point calibration curves in matrix-matched curve due to the presence of medium or high matrix effects for most analytes. The resulting calibration curve equations were in the form of $Y = a\ (\pm\delta a)X + b\ (\pm\delta b)$. Calibration curve determination coefficients (r^2) were $\geq$0.995 for all molecules in the linearity ranges (0.002–500 ng/g). Table S19 reports the regression coefficients and the linearity range for each analyte.

Accuracy was defined as the deviation of the measured mean concentration from the spiked concentration, expressed in percentage, as described by Muñoz et al. (Alvarez-Muñoz et al., 2015). Precision was expressed as the relative standard deviation of the measured concentration on replicated analysis.

Accuracy values ranged between −7% and 11% at three different concentration levels. RSD values were between 1% and 6% for the intra-day analysis (repeatability) and between 2% and 6% for the inter-day analysis (reproducibility). This demonstrates the method's repeatability and reproducibility with an error of less than 20%, making it effective for quantification. Table S20 reports the results.

3.3.2. Method detection limits (MDL) and method quantification limits (MQL)

MDLs and MQLs for all the analytes ranged from 0.9 pg/g to 10 ng/g and from 3 pg/g to 30 ng/g, respectively. Table S21 reports the results. As mentioned above, Table S18 reports the limits of the analytical methods in the mussels analysis. Most of the cited papers with the best sensitivity do not include both pharmaceuticals and pesticides. The simultaneous presence of analytes covering a wide polarity spectrum makes it necessary to use solvent mixtures. Consequently, there is an increase in matrix effect caused by co-extraction of other compounds and a decrease in method sensitivity. The MDL/MQL values are sometimes presented in ng/g dry weight concentration (as in this study) and sometimes in ng/g wet weight (ww) units, making it difficult to directly compare these data.

For the pharmaceuticals, we obtained lower MDL/MQL for amoxycillin (Chiesa et al., 2018), carbamazepine (James et al., 2020; Mezzelani et al., 2020; Mijangos et al., 2019), sulfamethoxazole (James et al., 2020; Mijangos et al., 2019) erythromycin (Chiesa et al., 2018; James et al., 2020; Martínez-Morcillo et al., 2020), and tetracycline (Chiesa et al., 2018; James et al., 2020). Some researchers have reported lower MDL/MQL values, such as for carbamazepine (Martínez-Morcillo et al., 2020) and doxycicline (Chiesa et al., 2018; James et al., 2020).

For the same compounds, our MDLs were similar to those obtained with the method developed by the Álvarez-Muñoz group (Alvarez-Muñoz et al., 2015) (sulfamethoxazole, erythromycin, carbamazepine), who used UHPLC–MS/MS in the same matrix (*Mytilus galloprovincialis*, Mediterranean mussel), and by the Li group (sulfamethoxazole, erythromycin), who used HPLC-MS/MS (Li et al., 2012). However, the latter two reports both used more time-consuming methods (PLE and SPE). A more recent paper from the Álvarez-Munoz group did not consider tetracycline or macrolide antibiotics (Álvarez-Muñoz et al., 2019). They reported comparable MDLs for sulfamethoxazole and carbamazepine. However, their procedure used ACN. In our investigations, we found poor efficiency for penicillin and tetracycline antibiotics in our method using ACN. Another reported method for determining antibiotics and their metabolites in seafoods obtained better results for tetracycline, and comparable results for sulfamethoxazole (Serra-Compte et al., 2017).

For pesticides, we obtained better MDL/MQL for deet (James et al., 2020) and metolachlor (Lerebours et al., 2021). For atrazine we obtained better and comparable values (Mijangos et al., 2019; Petrarca et al., 2022). Our values for alachlor were higher than those reported with previous methods (Miranda et al., 2022; Petrarca et al., 2022).

Comparable MDL values have been reported for atrazine (Álvarez-Muñoz et al., 2019). However, that method uses H_2O and ACN as extraction solvents, and the extraction and purification take longer. Our method improved the sensitivity for metolachlor.

Interestingly, one group has reported a method for extracting two anticonvulsants and their transformation products in marine mussels, comparing PLE and QuEChERS extraction (Martínez Bueno et al., 2013). This paper used QuEChERS extraction, with the MDL for carbamazepine being higher than our method.

A method with MQLs higher than ours has been reported for metolachlor and atrazine (Chang et al., 2016), with comparable results for alachlor using the QuEChERS method in hard clam and oyster.

3.3.3. Matrix effects

Matrix effects can greatly influence the sensitivity, linearity, accuracy, and precision of quantitative LC MS/MS determinations, particularly with complex matrices. All compounds included in this method were subjected to ion suppression. Three analytes (erythromycin, alachlor, metolachlor) showed no matrix effect ($\leq$20%, because this variation is close to the repeatability values), four analytes (doxycycline, tetracycline, DEET, atrazine) showed a medium effect (20–50%), and four analytes showed a high effect (>50%) (amoxycillin, carbamazepine, sulfamethoxazole, atrazine-desethyl-desisopropyl). Coextracted matrix components had the greatest effect on the analytical response of early-eluting analytes (i.e. amoxycillin and atrazine-desethyl-desisopropyl).

With respect to the published method focused on pesticides, endocrine disruptors, and pharmaceuticals (Álvarez-Muñoz et al., 2019), we obtained higher matrix effects for atrazine, metolachlor, carbamazepine, and sulfamethoxazole. As mentioned, we sought the best compromise for sample pretreatment to obtain good results for physically and chemically diverse compounds. Given the presence of penicillin and tetracycline antibiotics, we therefore could not use ACN only as the best extraction solvent for the above-mentioned pesticides.

Instead, we used an ACN/MeOH mixture that slightly increased the matrix effect.

No significant matrix effects (lower than 15%) were obtained for pharmaceuticals (amoxicillin, doxycycline, tetracycline, erythromycin) in the method developed by Chiesa et al (Chiesa et al., 2018). No information about matrix effects were reported for the other published methods.

In light of our results, it is not feasible to accurately quantify analytes in mussel extracts using calibration standards prepared in mobile phase. It was necessary to use matrix-matched calibration curves, with addition of isotopically labelled internal standards when available.

3.3.4. Recovery

Recoveries varied between 32 and 95% with relative standard deviations (RDS) below 10%, which indicates high reproducibility of the extraction. Only four analytes were extracted with less than 40% efficiency, but acceptable RSD were observed also in these cases (Table 1).

We obtained higher recovery than the published method focused on pharmaceuticals (Alvarez-Muñoz et al., 2015) for sulfamethoxazole, carbamazepine, and erythromycin. We obtained lower recovery than in another recent paper by the same research group (Álvarez-Muñoz et al., 2019) for atrazine, metolachlor, carbamazepine, and sulfamethoxazole, although that group did not consider tetracycline antibiotics.

Our results for tetracycline and sulfamethoxazole were comparable to the method developed by the Compte group (Serra-Compte et al., 2017). They used d-SPE with QuEChERS during the extraction procedure, but d-SPE did not significantly improve results, supporting our decision not to use these chemicals. As reported above, some recently published methods obtained better recovery than our proposed method, but focused on a specific class of molecules.

3.4. Analysis in real samples from the Adriatic Sea

As a successful application, we analyzed real mussel samples (*Mytilus galloprovincialis*) collected from a farm in the Northwestern Adriatic Sea. Widespread contamination by different classes of chemicals has been extensively documented in this area, including metals, polyaromatic hydrocarbon (PAHs), pesticides, and, more recently, microplastics and pharmaceuticals. (Mezzelani et al., 2020; Strafella et al., 2019). Furthermore, the combination of shallow waters and high riverine inputs makes this one of the most eutrophic and productive areas in the Mediterranean, promoting intense mussel farming activity (Brigolin et al., 2017). Table S21 presents the results of the analysis of these samples. Of the 11 contaminants targeted by our method, seven were determined at concentrations exceeding their respective MDLs. Two pesticides, atrazine desethyl-desisopropyl and alachlor, and two pharmaceuticals, amoxicillin and doxycycline hyclate, were below their MDLs.

For the pharmaceuticals, our results are in agreement with other studies in mussels. Carbamazepine concentrations have been reported in *Cassostrea gigas* in the Ebro delta (2.1 ± 0.04 ng/g (dw) (Alvarez-Muñoz et al., 2015). In *Mytilus galloprovincialis*, carbamazepine was been reported in concentrations from 0.5 to 3.5 ng/g (dw) in samples collected from Mediterranean Sea cultures in southeastern France (Martínez Bueno et al., 2013) and in concentrations from 21.9 to 299.7 ng/g dw (depending on season and year) in samples collected from 2014 to 2017 in Italy (Senigallia, Torrette, Portonovo) (Mezzelani et al., 2020). Tetracycline was also found in low concentrations (0.55 ng/ g w/w) in a pool of farmed mussels from Atlantic Spain, depurated in a plant in North Italy (Chiesa et al., 2018).

Carbamazepine is one of the ten pharmaceuticals that are most frequently detected in aquatic systems, with known effects on the organisms, including bivalves, inhabiting those systems (Almeida et al., 2020). Carbamazepine has refractory properties, such as resistance to conventional water treatments (coagulation, flocculation, sand filtration, chlorination), biotreatments, and photodegradation (Almeida et al., 2020).

The detected tetracycline is one of the most common antibiotic drugs in the world. Its overuse is associated with significant variations in environmental microflora that are detrimental to environmental health. Several sources (e.g. hospitals, pharmaceutical industries, livestock) contribute to tetracycline accumulation in wastewater systems (Ahmad et al., 2021).

The detected erythromycin and sulfamethoxazole are members of the macrolide and sulfonamide families, respectively, with activities against most gram-positive and certain gram-negative bacteria. These antibiotics are reported to have negative effects on the environment and on organisms.

Erythromycin is reported to inhibit the growth and development of photosynthetic aquatic entities, even at low concentrations (Ayankojo et al., 2020), and to produce biochemical, physiological, and behavioral disturbances in different fish species (Minski et al., 2021). Sulfamethoxazole is among the top 30 most frequently detected wastewater contaminants with a half-life of 85–100 days and more (Prasannamedha & Kumar, 2020).

However, the levels of pharmaceutical residues that we detected in mussels are far below the Maximum Residue Limits (Regulation and (EU) No 37/2010 of 22 December 2009), established by the authorities as being between 100 and 600 ng/g (ww).

Of the pesticides and herbicides, we detected metolachlor and DEET at concentrations near to the MQL, while the concentration of atrazine was 7 times higher than its MQL.

In previous studies, metolachlor has been found in concentrations between 0.5 and 1.67 ng/g in blue mussels (Lerebours et al., 2021). DEET has been found in concentrations between 0.483 and 0.532 ng/g dw in bay mussels (*Mytilus trossulus*) (James et al., 2020).

Pesticides cause serious health problems because they accumulate in fat-rich foods and affect the food chain.

Metolachlor is the most frequently used amide herbicide, mostly in corn crops. It is regulated by the authorities because of its high persistency in soil, with a half-life of about 15–70 days (Liu et al., 2021). Moreover, metolachlor is listed as a possible cancer-causing substance by the World Health Organization, so pollution with metolachlor and its metabolites must be recorded (Silver et al., 2015).

DEET is primarily used an insect repellent. It is available in diverse forms from aerosols to lotions. It is complex to ascertain how DEET reaches an aquatic environment due to the different possible routes of introduction. Domestic use of DEET is considered the primary route of introduction to the aquatic environment (Marques Dos Santos et al., 2019). The DEET manufacturing process is another potential route of introduction, with contaminated process effluents being discharged into surface water streams. However, due to its anti-biodegradation, water self-purification and conventional wastewater treatment plant technologies are inefficient for removing DEET (Marques Dos Santos et al., 2019).

2-chloro-4-ethylamino-6-isopropylamino-s-triazine (i.e. atrazine) is

Table 1
Recovery, matrix effect, and their respective relative standard deviations (RSD) (n = 3) for each analyte at medium concentration (10 MQL).

COMPOUND	RECOVERY	MATRIX EFFECT
Amoxycillin	35% ± 6%	−75% ± 6%
Carbamazepine	72% ± 5%	−57% ± 10%
Sulfamethoxazole	37% ± 8%	−60% ± 10%
Erythromycin	67% ± 3%	−20% ± 4%
Doxycycline	32% ± 5%	−31% ± 8%
Tetracycline	35% ± 7%	−45% ± 6%
DEET	86% ± 9%	−40% ± 9%
Atrazine	58% ± 10%	−35% ± 4%
Atrazine-Desethyl-Desisopropyl	95% ± 10%	−69% ± 10%
Alachlor	51% ± 9%	−16% ± 8%
Metolachlor	46% ± 5%	−20% ± 7%

a pre- and post-emergent herbicide used to control broad-leafed weeds and grasses for agricultural and nonagricultural purposes (Mohammadi et al., 2019). The half-life of ATR varies from several weeks to about two years in different environmental matrices (Triassi et al., 2022) giving it the characteristic of a product with a long permanence in the environment.

3.5. Conclusions

The high consumption of pharmaceuticals and the extensive use of pesticides and herbicides is an important global issue because of its negative impacts on the environment and on human health.

Here, we developed a new method to simultaneously identify and quantify pharmaceuticals and pesticides. We tested different solvents and solid-phase extraction approaches in order to comprehensively evaluate and compare the methods reported in the literature. We then developed an original, improved, and economical method for sample treatment. In particular, we avoided time-consuming and costly methodologies, such as SPE and d-SPE (QuEChERS). This strategy improved the method's overall performance. Indeed, the best compromise for all the investigated compounds was achieved by using different organic solvents and nonionic detergent.

The developed method is a powerful tool for the analytical detection of major pollutants in aquatic fauna, specifically for high-fat matrixes like mussels.

To the best of our knowledge, the reported results are similar to or better than those reported in recent papers. Moreover, it is the first time that so many different classes of molecules have been studied in a similar way in a high-fat matrix like mussels. For each compound, we obtained data on the different solid–liquid extractions, clean-up procedures, use of detergents, chemical stability, effect of chromatographic condition, and other parameters. These data should form the basis of further work on pollutant analysis in complex matrices.

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CRediT authorship contribution statement

Nicolò Interino: Investigation, Visualization, Writing - original draft. **Rossana Comito:** Formal analysis, Writing – original draft, Visualization, Investigation. **Patrizia Simoni:** Writing – review & editing. **Silvia Franzellitti:** Writing – review & editing. **Simone Rampelli:** Writing – review & editing. **Anastasiia Mosendz:** Writing – review & editing. **Roberto Gotti:** Writing – review & editing. **Aldo Roda:** Writing – review & editing. **Marco Candela:** Writing – review & editing, Funding acquisition. **Emanuele Porru:** Conceptualization, Methodology, Writing – review & editing, Investigation. **Jessica Fiori:** Conceptualization, Supervision, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.136453.

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