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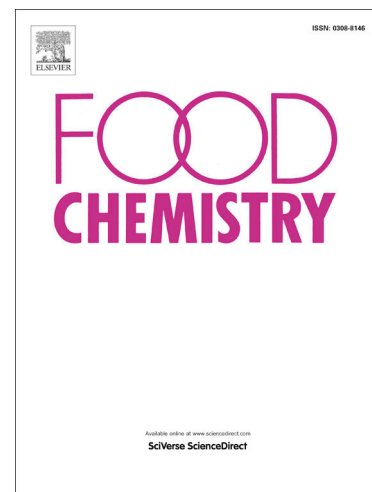
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Development and validation according to the SANTE guidelines of a QuEChERS-UHPLC-QTOF-MS method for the screening of 204 pesticides in bivalves

Thierno Diallo ^{a, b}, Yassine Makni ^a, Adélaïde Lerebours ^b, Hélène Thomas ^b, Thierry Guérin ^c, Julien Parinet ^{a, *}

^a ANSES, Laboratory for Food Safety, F-94701 Maisons-Alfort, France

^b Littoral Environnement et Sociétés (LIENSs), UMR 7266, CNRS-Université de La Rochelle, 2 rue Olympe de Gouges, F-17042 La Rochelle Cedex 01, France

^c ANSES, Strategy and Programmes Department, F-94701 Maisons-Alfort, France

* Corresponding Author: ANSES, Laboratory for Food Safety, F-94701 Maisons-Alfort, France
E-mail address: julien.parinet@anses.fr (J. Parinet)

Abstract

A qualitative screening high resolution mass spectrometry method was developed and validated according to the EU SANTE/12682/2019 guidelines for the analysis of 204 pesticides in seven commercial bivalve species spiked at three concentrations (0.01, 0.05 and 0.1 mg.kg⁻¹). Samples were extracted using QuEChERS and analysed using ultra-high-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry. The QuEChERS method was optimised by the Taguchi Orthogonal Array approach. The best conditions were obtained with pure ACN, MgSO₄/NaCl as extraction salts, MgSO₄/PSA/C18 as clean-up, and the non-dilution of extracts. The impact of different HRMS acquisition modes on detection and identification rates were also evaluated. The screening detection limits were determined to be 0.01 mg.kg⁻¹ and 0.1 for 66% and 87% of pesticides, respectively. These screening procedure was finally applied to different bivalve samples using target and suspect analysis. This allowed the identification of diuron and its metabolite 1-(3,4-dichlorophenyl)-3-methylurea in the investigated samples.

Keywords

Qualitative screening; LC-HRMS acquisition modes; Pesticides; Bivalves; QuEChERS; Taguchi Orthogonal Array

1. Introduction

During the last few decades, the use of pesticides to protect crops has facilitated mass food production. However, the use of pesticides can lead to chemical pollution in water located near crop fields due to transport into surface and groundwater (Tudi et al., 2021). Once in water, pesticides and their metabolites become bioavailable to aquatic organisms and can bioaccumulate in their tissues via the skin, gills and/or diet, depending on their solubility (van der Oost et al., 2003). Biomagnification of chemicals through the food chain can lead to toxic effects to predators and humans (van der Oost et al., 2003). Therefore, the use of pesticides near surface waters poses a high risk to the aquatic ecosystem and potentially to consumers.

Bivalves are indicator species of environmental quality. They filter large volumes of water and concentrate xenobiotics, allowing for the assessment of pollutant concentrations in their ecosystems (Breitwieser et al., 2018). Moreover, bivalves are a reliable source of information on human exposure to contaminated seafood (Rodríguez-González et al., 2015). Bivalves are also crucial in aquatic environment since they provide habitats for other organisms (algae, macroinvertebrates) and contribute to nutrient cycling and storage (Vaughn and Hoellein, 2018). In addition, bivalves accounted for 17.7 million tons of coastal and marine animal aquaculture in 2018, with an estimated market value of USD 34,6 billion (FAO, 2020). For all these reasons, high mortality of bivalves such as oysters (Vohmann et al., 2010) and mussels (McFarland et al., 2016) worldwide is not only a major ecological issue but also an economic concern.

Analytically, whole-body bivalves are complex matrices. The presence of proteins and lipids in high concentrations (between 2 to 10% and between 10 to 18% of lipids and proteins, respectively) makes it difficult to analyse compounds such as pesticides with lower concentrations in the sample (US Department of Agriculture, 2016). This matrix effect needs to be reduced to avoid bias in the overall analysis. Sample preparation of bivalves normally requires time-consuming steps to remove these lipids and proteins in order to obtain acceptable recoveries for the target compounds. The most commonly used extraction techniques for bivalve samples are accelerated solvent extraction (Galvao et al., 2012), pressurized liquid extraction (Rodrigues et al., 2016), and microwave extraction (Uluturhan et al., 2019). Extraction methods based on these techniques are usually followed by solid phase analysis (SPE). The Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) extraction method is a preferable alternative to these different methods due to its fast protocol, reliable results, and

affordable cost. This method has already been successfully applied for the analysis of pesticides in bivalves (Cruzeiro et al., 2016; Álvarez-Muñoz et al., 2019).

Despite the advantages of QuEChERS, to achieve the best extraction efficiency, many parameters need to be optimised, including sample amount, extraction solvent, solvent pH, partition salt, dispersive-SPE (dSPE) sorbent, concentration to increase sensibility of the compounds, and dilution of extracts to reduce matrix effects. Because of the high number of parameters that can influence the analysis, the possible interactions between them, and the time needed to process data, it is not feasible to analyse all of them independently. As a result, it is recommended to use so-called split factorial design methods to save time. One of these methods, called Taguchi Orthogonal Array (TOA), is characterized by its simplicity and efficiency in designing parameter combinations and in evaluating each parameter. Taguchi proposed a design matrix that allows the study of all parameters with selectively reduced experimental size (Narendran et al., 2019). This matrix can be used to determine the influence of each designed parameter and to identify the best combination of parameters to obtain the best result (Narendran et al., 2019). The TOA method was recently used to optimise the QuEChERS method for wide-scope multi-residue analysis of pesticides in beef (Pang et al., 2021).

Following the sample preparation optimisation step comes the analytical step, to highlight the presence or absence of target compounds and to quantify them in the reference matrices. To determine pesticides in bivalves, gas chromatography (GC) and liquid chromatography (LC) coupled to triple quadrupole mass spectrometry operating in multiple reaction monitoring (MRM) mode are the most common techniques, due to their selectivity and sensitivity (Cruzeiro et al., 2016). However, these low-resolution (LR) techniques are limited to the target compounds, so compounds not included in the method are not analysed. In addition, the number of compounds that can be screened simultaneously is limited (López et al., 2014). More recently, there has been increasing interest in the benefits of high-resolution mass spectrometry (HRMS) (Rajski et al., 2019). This is partly due to its ability to perform simultaneous target, suspect, and non-target analyses to increase the number of analytes (the scope) that can be included in multi-residue methods (Rajski et al., 2019). HRMS increases selectivity needed to overcome interferences in more complex matrices (Rajski et al., 2021). It also allows for retrospective analysis by reprocessing the acquired data for potential new compounds discovered without reinjection of the sample (Arroyo-Manzanares et al., 2021), which limits sample stability problems. Although HRMS, such as time-of-flight (TOF) instruments, has

emerged as a conventional means for pesticide detection and identification (López et al., 2014), it is still a major challenge to analyse low-level and trace pesticides in matrices as complex as bivalves. Therefore, the quality of MS/MS spectra is critical to obtain high confidence in the identification of small molecules (Schymanski et al., 2014). Currently, the main methods for acquiring a simultaneous MS-MS/MS mass spectrum are data-dependent acquisition (DDA) and data-independent acquisition (DIA) methods. DDA and DIA are also referred to as IDA (information-dependent acquisition) and SWATH® (sequential window acquisition of all theoretical fragment ion spectra) in Sciex instruments, respectively. The MS/MS data for the SWATH® produce more fragments due to the ability of SWATH® to fragment all ions, including isotopes. This results in spectra that are richer in information but also more complex. In IDA mode, only the most intense ions are fragmented. The number of most intense ions (*top-n*) to be fragmented is set by the user. The MS/MS spectra in IDA mode are less rich than those obtained in SWATH, but clearer and easier to interpret (Álvarez-Ruiz and Picó, 2019).

The aim of this study was to develop a simple, rapid and wide-scope QuEChERS sample extraction method, followed by a UHPLC-QTOF-MS comprehensive target screening analysis for 204 pesticides in bivalves. The list of target compounds included pesticides from different families with very distinct physicochemical properties, which therefore serve as a template to prepare the analysis in suspect screening. To our knowledge, this study is the first to perform HRMS analysis for such a comprehensive list of pesticides in bivalves. So far, only one study determined pesticides (12 in total) in bivalves by HRMS (Álvarez-Muñoz et al., 2019), while others have used LR mass spectrometers to analyse up to 60 pesticides in bivalves (Cruzeiro et al., 2016). The TOA method was used to evaluate optimum parameters for the sample extraction step. To the best of our knowledge, this is the first time that the TOA method has been used to optimise a QuEChERS extraction for the analysis of pesticides in bivalves. Moreover, different IDA and SWATH methods were tested to optimise detection and identification rates. The selected method was also validated according to the European Union SANTE/12682/2019 guideline (European Commission, 2019). For each pesticide, the screening detection limits (SDLs) was established in bivalve samples. Although it is not required for the qualitative screening method validation according to the SANTE guidelines, the limit of identification (LOI) was also established. The number of false positives was assessed using blank bivalve samples out of the 204 pesticides. Once validated, the screening method was applied to the analysis of different bivalve samples, including mussels and oysters by target and suspect analysis.

2. Materials and methods

2.1. Chemical substances and analytical standards

Acetonitrile (ACN), obtained from Fisher Scientific (Illkirch, France), and ultrapure water (18.2 M Ω .cm), obtained by purifying distilled water with a Milli-Q system from Merck Millipore (Saint-Quentin-en-Yvelines, France), were used for the mobile phases and sample preparation. Formic acid (FA) and acetic acid (AA) were supplied by Fisher Scientific (Illkirch, France), to prepare LC-MS mobile phases and the extraction solvent. QuEChERS extraction pouches based on the original method (4.0 g of magnesium sulphate (MgSO₄), 1.0 g of sodium chloride (NaCl)), AOAC Method 2007.1 (6.0 g anhydrous MgSO₄, 1.5 g sodium acetate) and EN Method 15662 (4.0 g of MgSO₄, 1.0 g of NaCl, 1.0 g of sodium citrate, and 0.5 g of sodium citrate sesquihydrate) were provided by Agilent Technologies (Santa Clara, CA, USA). A dispersive solid phase extraction (d-SPE) kit containing 900 mg of MgSO₄, 150 mg of Primary and Secondary Amines (PSA) and 150 mg of octadecylsilane (C18), and Enhanced Matrix Removal-Lipid (EMR-lipid) d-SPE was obtained from Agilent Technologies (Santa Clara, CA, USA). Zirconium-based Z-Sep/C18 (120 mg Z-Sep, 300 mg Discovery DSC-18) was supplied by Supelco (Bellefonte, PA, USA).

The standard consists of 204 pesticides (Table S1) divided into ten mixtures (100 mg.L⁻¹ of each compound in ACN). The ten pesticide standard stock solutions were obtained from Restek Corporation (Les Ulis, France), transferred and stored in screw-capped amber glass tubes at -20 °C. A mixture working solution (2 mg.L⁻¹) was prepared through suitable dilution of the ten intermediate solutions with ACN, and stored in screw-capped amber glass tubes at -20 °C.

2.2. Equipment and apparatus

A Genie 2 vortex mixer (Scientific Industries, Bohemia, NY, USA) was used for the extraction procedure. An IKA Ultra-turrax T25 digital homogenizer and a GM 200 Grindomix (Retsch Technology GmbH, Haan, Germany) were used for the extraction procedure. A Mettler Toledo (Columbus, USA) XS 204 analytical balance was used to weigh the samples.

An UHPLC Thermo Fisher Scientific U3000 system composed of a binary pump and autosampler (Waltham, MA, USA) coupled with a 5600 Q-TOF AB Sciex mass spectrometer (Darmstadt, Germany) with an electrospray ionization source (ESI) was used for analyses.

2.3. *Orthogonal array design for optimisation of QuEChERS extraction and clean-up*

Four factors (extraction solvent acidification, extraction salts, dSPE and dilution ratio) were considered for the optimisation of QuEChERS extraction and clean-up. Three levels were used for each factor (Table 1).

To optimise these four factors simultaneously, a Design of Experiment (DoE) was performed using L9 TOA. As shown in Table S2, nine experiments with three replicates for each were performed to evaluate responses such as pesticide detection rates (%) and signal-to-noise (S/N) of the 204 pesticides.

2.4. *Bivalve samples*

The bivalve samples (oysters, mussels, cockles, clams, sea almonds and two types of scallops) used for method optimisation and validation were purchased from local markets (Maisons-Alfort, France). To test the screening method applicability, a total of fourteen bivalves (seven mussels and seven oysters) were collected at three sites located in the Pertuis Charentais littoral area (southwest France) in May 2019, September 2019 and January 2020 and frozen at -20 °C. Before shucking, the oysters were stored at 4 °C overnight and then brought to room temperature. The samples were homogenized with a GM 200 Grindomix (Retsch Technology GmbH, Haan, Germany) and kept at -20 °C until analysis.

2.5. *Sample preparation*

Extraction solvent acidification, extraction salts, dSPE and dilution ratio were simultaneously optimised using L9 TOA. For the enrichment experiment, 7.5 µL of the mix working solution were added to 50-mL polypropylene centrifuge tubes, which contained 1.50 ± 0.05 g of blank oyster sample, to provide a supplementation at 0.01 mg.kg^{-1} of pesticides. A contact time of 24 h at 4 °C was allowed for the pesticides to bind to the matrix. Then, 8.5 mL of Milli-Q water were added to each tube, and the sample was then ground using an IKA Ultra-Turrax mixer for 3 min at 5,000 rpm. Then, 10 mL of extraction solvent were added to each centrifuge tube to perform pesticide extraction, and tubes were vortexed again for 1 min. After that, the salts were added to the fortified samples, and the resulting mixture was centrifuged for 5 min at 4,000 rpm.

When EMR lipid was used as the dSPE reagent, 5 mL water was added to a 15-mL centrifuge tube containing the EMR-Lipid and vortexed for 3 s to activate the sorbent. Then, 5 mL of the

supernatant (50-mL polypropylene centrifuge tube) were transferred to activated 15-mL centrifuge tube. The mixture was vortexed immediately for 1 min. The tube was centrifuged at 4,000 rpm for 5 min, and 5 mL of the supernatant was transferred to the polishing tube containing MgSO_4 to remove excess water. When using the other dSPE reagents, 6 mL of the supernatant were transferred to a 15-mL centrifuge tube containing the dSPE reagent. The tube was vortexed for 1 min, and then centrifuged for 5 min at 4,000 rpm. The supernatant was then combined with ACN according to the dilution ratio. The mixture was then vortexed, filtered, and deposited into a 2-mL auto-sampler vial for injection in the UHPLC-QTOF system.

To determine background contamination, matrix-free ACN was subjected to the same extraction and purification protocols as previously described.

2.6. UHPLC-QTOF-MS conditions

Reversed-phase liquid chromatography separation was performed using A Phenomenex Aqua[®] C18 column (150 x 2 mm, 3 μm particle size, 125 Å pore diameter; Phenomenex Inc, Torrance, CA, USA). A gradient using Milli-Q water (Solvent A) and acetonitrile (Solvent B) with both containing 0.01% of FA was used at a constant flow rate of 0.500 $\text{mL}\cdot\text{min}^{-1}$. The gradient profile started with 10% B (0 min); increased linearly to 100% B for 9 minutes (0-9 min); fixed for 3 minutes (9–12 min) and returned to the initial conditions in 0.1 min (12.1 min), followed by a re-equilibration step for 3.9 minutes. The total run time was 16 min. The auto sampler was thermo-regulated at 15 °C to avoid sample degradation during analysis-runs. The injection volume was 10 μL and the column temperature was set at 50 °C. The detection was carried out in positive (ESI+) ionization mode. The instrument was tuned and calibrated before each batch, in high-resolution mode. The automated calibration device system (CDS) was scheduled to perform automatic calibration every five injections using APCI Positive Calibration Solution for the 5600 system (AB Sciex technology). ESI+ parameters were as follows: the ion spray voltage floating (ISVF) was set at 5.5 kV, the source temperature (TEM) was 500 °C, the curtain gas (CUR) was set at 30 psi, the nebulizing gas (GS1) at 40 psi, and the heater gas (GS2) at 45 psi.

2.7. Evaluation and comparison of IDA and SWATH[®] workflows for pesticide detection and identification

In this study, four acquisition methods were implemented using the UHPLC-QTOF-MS system for pesticide detection and identification in spiked sample at 0.01 $\text{mg}\cdot\text{L}^{-1}$ of the target 204

pesticides. The source condition was the same for all methods as well as the mass range of 50–1,000 Da for TOF MS and TOF MS/MS.

Method A consisted in implementing the IDA method. The accumulation time for the TOF MS full scan was set to 490 ms, while in TOF MS/MS it was set to 100 ms. For the product ion scan, the parameters were set as follows: collision energy (CE) was fixed at 40 eV with ± 20 eV and declustering potential (DP) was 80 eV (+). Isotope exclusion was switched off. The 4 most intense ions (*Top4*) following precursor ion intensity greater than 50 cps with ion tolerance ≤ 10 ppm were monitored per cycle, and dynamic background subtraction was turned on. The total cycle run time was 940 ms.

Method B used IDA-PIL (Precursor Ions List). The parameters were the same as the IDA but with an inclusion list of the 204 target pesticides.

Method C and method D consisted in using the fixed (F-SWATH) and variable (V-SWATH) methods, respectively, with the specific settings listed in Table S3. Different numbers of SWATH® windows (20, 30, 40, and 50) were evaluated with F-SWATH (F-SWATH20, F-SWATH30, F-SWATH40 and F-SWATH50) or V-SWATH (V-SWATH20, V-SWATH30, V-SWATH40 and V-SWATH50) window width for Q1 isolation (overlap 1 Da). F-SWATH selects and fragments all detected precursor ions with an m/z window size Q1 that is uniform throughout the LC-MS run. For V-SWATH, the m/z windows are variable depending on the density of precursor ions by adjusting the isolation window to the number of precursor ions in the window (Álvarez-Ruiz and Picó, 2019).

Different acquisition methods were evaluated and compared using the pesticide detection rates and identification rates. For the latter, two different MS/MS search algorithms (*purity score* and *fit score*) at two levels ($>70\%$ and $> 50\%$) were evaluated according to the literature (Gómez-Romero et al., 2011).

2.8. Method validation

The QuEChERS-LC-HRMS qualitative screening method was validated according to the European Union SANTE/12682/2019 guidelines (European Commission, 2019). For this purpose, seven species of commercial bivalves were used: oysters, mussels, cockles, clams, sea almonds and two types of scallops (*Pecten maximus* and *Mimachlamys varia*). For each species, four groups were randomly formed. Individuals from each group were pooled, then 1.5 g of each was weighed and transferred into 50-ml polypropylene centrifuge tubes. Sub-samples were

spiked before extraction with a mixture containing 204 pesticides at 0.01, 0.05, and 0.1 mg.kg⁻¹, (to a total of 28 samples for each concentration level). The SDL was established as the lowest concentration level at which a compound was detected (not necessarily meeting the MS-identification criteria) in at least 95% of the samples (i.e., 27 out of 28 samples analysed) (European Commission, 2019). SDL was estimated as the concentration level at which the thresholds of (i) T_R (variation ± 0.1 min) and (ii) mass accuracy (± 5 ppm) of the precursor ion were satisfied (European Commission, 2019). The LOI (not required according to SANTE guidelines) was established as the lowest concentration tested for which a compound was satisfactorily identified in at least 95% of the samples. LOI used the same thresholds as for SDL, as well as an MS/MS library score. Non-spiked (blank) samples were also extracted and analysed to assess the number of false positives out of the 204 targeted pesticides.

2.9. Software and statistical analysis

Analyst[®] 1.7 software (AB Sciex, Les Ulis, France) was used for equipment control and data acquisition. Data were processed and evaluated using PeakView[®] software version 2.2 comprising the AB Sciex MasterView[™] module (Les Ulis, France), with intensity > 50 cps and $S/N > 3$. Pesticide detection was performed using a previously developed home-made database containing information on molecular formulae, retention time (T_R), accurate m/z , and isotopic pattern for 204 target pesticides (see Fig. S1). Pesticide identification was performed by MS/MS spectral matching and not by mass accuracy of the product ions as recommended by SANTE guidelines (European Commission, 2019). Indeed, MasterView[™] software allows to obtain automatically the mass error only on the precursor ions and not on the product ions. The MS/MS spectral library of 204 pesticides was built using AB Sciex LibraryView[™] software, version 1.0.3 (Les Ulis, France). The library was acquired in IDA mode by injecting separately each of the ten standard intermediate solutions diluted to 0.2 mg.L⁻¹ in ACN. It should be noted that LibraryView[™] software does not enable creation of a SWATH[®] spectral library. Different MS/MS search algorithms were used for pesticide identification, including *purity score* (Purity) and *fit score* (Fit). Purity is guaranteed in the range of 0–100 and is a measure of the similarity between the library spectrum and the acquired spectrum (Gómez-Romero et al., 2011). Fit reflects the degree of agreement between the masses and intensities of the library spectrum and those found in the acquired spectrum, ignoring other masses contained in the acquired spectrum (Gómez-Romero et al., 2011). A high Fit and low Purity indicate that the acquired spectrum is likely impure but contains the library compound.

The SWATHTM Variable Window Calculator tool (version 1.1) was used to calculate V-SWATH using an IDA total ion chromatogram (TIC).

Statistical analyses were performed using RStudio (Boston, MA, USA). The normality of the data was verified by the Shapiro–Wilk test. According to the distribution of the data, analysis of variance (ANOVA) with Tukey's multiple comparison post-hoc test or Kruskal–Wallis's test with Dunn's post-test were used (confidence limit of 95%, $p < 0.05$).

3. Results and discussion

3.1. *Optimisation of sample preparation using the TOA method*

Several parameters can have an impact on the efficiency of a QuEChERS extraction. This is the case for the sample amount, the extraction solvent, the acidification of the solvent, the partition salt, the clean-up, the concentration and the dilution of the extracts. To simplify the optimisation of the QuEChERS method, the Taguchi approach was chosen to simultaneously optimise the acidification of the extraction solvent, extraction salts, dSPE and dilution ratio. Responses evaluated were detection rates (%) and S/N of the 204 pesticides. The sample amount was not tested in this study as the aim of this work is to apply the optimised method to analyse a large number of bivalve samples (in particular oysters and mussels) pooled and individually (to test inter-individual variability). As the flesh of a mussel can weigh between 1 and 2 g, the choice of a sample weight of 1.5 g was considered appropriate.

The values of the mean detection rates oscillated between 56% and 86% (Table 2) and the S/N between 97 and 190 (Table 3) for all experiments. Overall, the best performances were found with experiment 9 and 1 for detection rates and S/N, respectively. The performance of each experiment depends on the pesticide considered as shown in the chromatograms of fenpropimorph, methoprotetryne, hydramethylnon and azoxystrobin in Fig. S2. According to the R values for the mean detection rates and S/N, the most impactful factor is dilution ($R=28.55$ and 56.76), followed by extraction solvent acidification ($R=3.98$ and 9.69) or dSPE ($R=2.39$ and 43.19), and finally the extraction salts ($R=1.40$ and 8.92). The effects of the four studied factors on the pesticide detection rates and S/N are also shown in Fig. S3. Detection rates and S/N graphs can be read in two different ways depending on whether the levels are represented by column chart or connected by curves. When the levels are connected by curves (as in the case of dilution), the flatter the slope, the less significant the factor on the response studied. Conversely, the steeper the slope, the more significant the factor. When the detection rate and

S/N ratio are represented by column chart (as in the case of extraction solvent acidification, extraction salts and dSPE), the greater the difference between the best and worst performing level, the more significant the factor. Only dilution was a significant factor ($p < 0.05$) for the detection rates, while all factors were significant for the S/N (Table 2 and 3).

3.1.1. Extraction solvent acidification

For the extraction solvent, only ACN was tested as extraction solvent because the use of ACN leads to much less interference from compounds such as lipophilic compounds, lipids and pigments than other extraction solvents such as acetone, ethyl acetate (Anastassiades et al., 2003). In addition, ACN is the most widely used organic solvent for QuEChERS extraction due to its ability to extract a wide polarity range of pesticides from several animal and plant foodstuffs. Due to its miscibility with water, it is able to penetrate the aqueous fraction of the sample matrices, allowing for easy separation of the two phases upon salt addition. In order to increase the extraction efficiency of certain pesticides (e.g., acidic compounds), ACN can be acidified with acetic acid (AA) or formic acid (FA), for instance (Pang et al., 2021). For this study, we therefore chose to evaluate the impact of ACN with 1% (v/v) FA or 1% (v/v) AA on the detection rates of target pesticides and their S/N. The best detection rates and S/N were obtained with pure ACN as extraction solvent (Fig. S3).

3.1.2. Extraction salts

In the QuEChERS method, MgSO_4 and NaCl are the original salts used for liquid-liquid phase separation by binding to water (Anastassiades et al., 2003). MgSO_4 has good water absorption and allows the release of acetonitrile, while NaCl affects the polarity of the extraction solvents and, therefore, increases the selectivity of the extraction (Kim et al., 2019). To avoid ionization or degradation of pesticides due to pH changes during extraction, two modifications of the original QuEChERS method were developed: (1) AOAC Method (acetate buffer) (Lehotay, 2007) and (2) EN Method 15662 (citrate buffer) (Anastassiades et al., 2007). Both methods stabilize the pH at 5–5.5, which facilitates the extraction of pesticides sensitive to low pH or those with stability problems. However, the ability of the primary secondary amine PSA (used as a sorbent) may decrease to retain the co-extractives at this pH level, especially for samples with high fat content (Kim et al., 2019). As shown in Fig. S3, a mixture of MgSO_4 and NaCl without buffer yielded better results in terms of detection rates and S/N than the methods using buffers.

3.1.3. *dSPE*

As illustrated in Fig. S3, the best detection rates and S/N were obtained with a mixture of MgSO_4 /PSA/C18 as dSPE reagent. As mentioned in the previous paragraph, MgSO_4 is commonly used as a drying salt for ACN extraction because it effectively removes residual water. PSA has been shown to remove many polar organic acids from the extract, as well as polar pigments, sugars and fatty acids, whereas C18 can retain fats, vitamins, and minerals (Kim et al., 2019). Z-Sep/C18 is composed of zirconium oxide and C18. This mixture reduces fat and pigment levels in matrices and it is recommended for matrices with >18% of lipid (Álvarez-Ruiz et al., 2021), which is not the case for bivalve samples, whose lipid content is usually below 10%. EMR-Lipid, whose structure is a proprietary secret, removes lipids from sample matrices (Álvarez-Ruiz et al., 2021; Pang et al., 2021) but its ability to remove other interferents such as proteins has not yet been described in literature.

3.1.4. *Dilution*

As observed in Fig. S3, non-dilution was necessary to obtain the best detection rates and S/N. Dilution combined with extraction and purification steps showed decreased matrix effects in apple, tea and broccoli during analysis (Guo et al., 2019). However, sample dilution reduces the overall sensitivity of the method, thus a compromise is needed to reduce matrix effects while maintaining high method sensitivity. In addition to the dilution of extracts, the concentration of the sample can be important on the sensitivity of the target compounds and on matrix effects. To have a simple, reliable and not time-consuming method to apply it to a large number of bivalve samples, the sample concentration was not considered in this work. However, this aspect should be tested in a future research to obtain a good compromise between increasing sensitivity and minimising matrix effects.

The conclusion based on the R values associated with the K1, K2, and K3 values and the main effects plot for both responses is that the optimal QuEChERS extraction conditions were obtained with pure ACN, Original QuEChERS method, MgSO_4 /PSA/C18 as dSPE, and the non-dilution of extracts.

3.2. *Evaluation and comparison of IDA and SWATH[®] workflows for pesticide detection and identification*

As shown in Fig. 1a, detection rates ranged from an average of 75% to 96% for all methods, with the IDA and IDA-PIL methods being the most effective with detection rates of 96% on average, for both modes. The detection rates of F-SWATH mode ranged from an average of

75% for F-SWATH50 to 94% for F-SWATH20 and the detection rates of V-SWATH ranged from an average of 77% for V-SWATH50 to 94% for V-SWATH20. The detection similarity between IDA and IDA-PIL is expected because they have the same TOF-MS conditions. PIL only affects TOF-MS/MS (identification). The same observation was made with F-SWATH and V-SWATH which have the same detection rates when the number of windows is the same. For the SWATH® methods, the detection rates decreased with the increase of the number of windows. This is because, for the same accumulation time, if the number of windows increases, the number of cycles and points per peak decreases, which reduces the sensitivity of the precursor ions. In terms of detection, the performance of the IDA modes is equivalent to that of the best SWATH modes.

The TOF-MS used gave information on the accurate m/z of the targeted pesticides. However, pesticides were only considered identified in cases where the characteristic MS/MS of the compound was obtained. As can be seen in Fig. 1b-e, the IDA-PIL mode achieved a better identification rate than the IDA mode. This is due to the fact that in the IDA-PIL mode, the targeted precursor ions have the highest priority for MS/MS data acquisition, even though these precursor ions were not the most intense. For the IDA mode, only the *top n* most intense precursor ions are fragmented, which can be problematic in case of complex matrix or low concentration. The IDA modes were slightly impacted by the decrease of the MS/MS score threshold (from > 70% to > 50%) with an average increase of 5% of pesticides identified for both IDA and IDA-PIL. The same observation is made for the impact of the MS/MS search algorithm. From Purity to Fit, an average increase in pesticide identification rates of 2 and 6% was observed for the IDA-PIL and IDA modes, respectively.

For SWATH modes, V-SWATH achieved overall higher identification rates than F-SWATH because it adjusts the isolation window to the number of precursor ions in the window. For F-SWATH, the m/z Q1 window size is uniform throughout the LC-MS run. Compared to IDA modes, SWATH modes are more affected by the decrease of the MS/MS score threshold (from >70% to >50%), especially for the Purity algorithm with an average increase of 15% of identified pesticides (Fig. 1b-c). For MS/MS search algorithms, the impact was even more pronounced. From Purity to Fit, an average increase of 20% in pesticide identification rates was observed. The Fit algorithm was therefore found to be the best for identifying compounds in SWATH modes.

Unlike what was observed for detection rates (Fig. 1a), which decrease with increasing the number of SWATH® windows (decreasing precursor ion sensitivity), this is not always the case

for identification rates (Fig. 1b-e). Increasing the number of SWATH® windows with narrower widths, allows fewer precursor ions to be selected for MS/MS fragmentation, resulting in higher specificity and selectivity (Demianova et al., 2017). A compromise is therefore necessary to increase sensitivity while maintaining high precursor ion specificity and selectivity for confident pesticide identification.

In terms of identification, the best performance was obtained with IDA-PIL, regardless of the algorithm or MS/MS threshold chosen, which demonstrates the importance of adding PIL to increase the probability of fragmenting the desired precursor ions. A previous study by Álvarez-Ruiz and Picó (2019) also compared the IDA mode (without PIL) with the F-SWATH and V-SWATH modes. In this mentioned study, the SWATH modes performed better than the IDA mode.

For the identification of pesticides in complex matrices such as bivalves, the quality of the MS/MS spectrum is also crucial. It was therefore taken into account in this work. As an example of the difference between the IDA modes and SWATH® methods, Fig. 2 shows the results obtained with IDA-PIL and V-SWATH methods for zoxamide in spiked sample at 0.01 mg.L⁻¹. The MS spectra showed the two characteristic signals of zoxamide (one with ³⁵Cl and one with ³⁷Cl) (Fig. 2a). The MS/MS data of the SWATH® acquisition showed zoxamide product ions with ³⁵Cl and ³⁷Cl (Fig. 2b). In the IDA acquisition, only the monoisotopic ion (zoxamide with ³⁵Cl) was selected and fragmented (Fig. 2c), so the MS/MS data lose isotopic abundance but with a cleaner spectrum than that obtained in SWATH®. These results are consistent with the study conducted by Álvarez-Ruiz and Picó (2019) who found that the quality of MS/MS spectra is lower in SWATH than in IDA and more complex to interpret. Indeed, not all MS/MS data acquired with SWATH is useful as it may come from several precursor ions that are not all of interest. Thus, some fragments of other precursors may interfere with the signal of the analyte of interest, leading to additional verification by the user. Despite these described drawbacks, SWATH modes have shown good performance for the analysis of small molecules such as pharmaceuticals and personal care products in complex matrices, although these modes are more commonly used for protein analysis (Álvarez-Ruiz and Picó, 2019; Baduel et al., 2015).

For the search algorithm, the library scores (Fig. 2b-c) showed that the Purity algorithm does not adapt the SWATH® data, especially when compared to a home-made library in IDA. Of note, LibraryView™ does not allow for the implementation of a home-made spectral library in SWATH® mode, which is an issue. It is therefore necessary to find ways in the future with

improved software to take better advantage of SWATH modes. Indeed, SWATH modes have interesting unique features, namely the generation of chemical fingerprints (MS and MS/MS spectra) of the sample that can be exploited retrospectively as new knowledge is gained and the possibility of using samples in multiple studies.

According to the results obtained in terms of detection, identification rates and quality of MS/MS spectra, the IDA-PIL mode was selected for the validation method. The identification rates obtained with the evaluated algorithms being similar in the IDA-PIL mode, the Purity algorithm was chosen because it allows to evaluate the quality of the obtained spectra in terms of interfering peaks. As the score used does not have a great influence on the identification rates in the IDA-PIL mode, the score threshold $> 70\%$ was set to increase identification confidence. Furthermore, for IDA-(PIL) modes, the Purity $> 70\%$ is most often used in the literature by authors working with the 5600 Q-TOF AB Sciex mass spectrometer (Baduel et al., 2015; Romera et al., 2018).

3.3. *Selectivity of the screening method*

In order to determine the selectivity of the chosen screening method, the possible presence of false positives was also checked. In the SANTE guideline no criteria have been set with respect to the occurrence of false positives. However, it is important that the selectivity of the method is such that it reduces them to a sufficiently low number to avoid manual verification or confirmatory analysis that may reduce the effectiveness of the screening methods. The number of false positives was checked by analysing blank samples of each of the seven bivalve species prepared in triplicate. According to the results, a total of 5 false positives (2.5% of the 204 target pesticides) were obtained across all blank samples, when applying tolerances of ± 5 ppm and ± 0.1 min for mass accuracy and T_R , respectively. False positive pesticides for fenpropimorph ($C_{20}H_{33}NO$) and formetanate-HCl ($C_{11}H_{16}ClN_3O_2$) were each detected once across the different blank samples, whereas false positives for spiroxamine ($C_{18}H_{35}NO_2$), aminocarb ($C_{11}H_{16}N_2O_2$) and propham ($C_{10}H_{13}NO_2$) were observed in two, three and five samples, respectively. When additional criteria were set, such as the MS/MS library score (Purity $> 70\%$) the number of false positives dropped to zero. These results demonstrate the good selectivity of the method both in terms of detection and identification.

3.4. *Method validation*

The objective of this work was to develop and validate an analytical methodology according to the SANTE guidelines for the simultaneous detection and identification of 204 pesticides (used

as model compounds) in bivalve matrices. Therefore, the validation of the qualitative screening methodology included the determination of SDL. Although it is not required for the SANTE guidelines, the LOI was also established. Seven species of bivalves were tested for method validation: oysters, mussels, cockles, clams, sea almonds and two species of scallops (*Pecten maximus* and *Mimachlamys varia*). Four groups of each species were spiked at three concentration levels (0.01, 0.05, and 0.1 mg.kg⁻¹). For each individual pesticide, the number of positive detections and identifications at each concentration level was recorded and the SDL and LOI were established. Table S4 shows the number of detections and identifications for each compound in the 28 samples and the corresponding SDL and LOI. A summary of this table is also shown in Fig. 3. As it can be seen, about 93% of the pesticides could be detected at 0.1 mg kg⁻¹ in bivalves, while the percentage of identification decreased down to 77% at this same concentration level. The SDL was determined to be equal to or less than 0.1 mg.kg⁻¹ for 87% of the pesticides, whereas the percentage of pesticides with an LOI equal to or less than 0.1 mg.kg⁻¹ decreased to 63%. The criteria of SDL and LOI were therefore not reached for 13 and 37% of pesticides, respectively. However, the proposed screening method was able to detect and identify them in most of the samples at the highest concentration level. In this regard, it is worth noting the complexity of bivalve matrices, which generally present a significant amount of co-extractives, even after sample clean-up, which may increase SDLs and LOI compared to other simpler matrices. For 50 pesticides (representing 25% of the selected pesticides), it was possible to establish the SDL at least at one of the three concentration levels, but not the LOI. For these compounds, validation was limited to detection (only SDL could be set-up). Regarding maximum residue limits (MRLs), no values have yet been established in bivalves for any of the pesticides studied, so the SDLs obtained, in most cases 0.01 mg.kg⁻¹, can be considered satisfactory and the proposed methodology suitable. In addition, no qualitative pesticide screening was performed on bivalves by QuEChERS-LC-HRMS to compare our results. Previous work has been done on other matrices such as fish fillet (Munaretto et al., 2016), salmon feed (Regueiro et al., 2017), and fruits and vegetables (López et al., 2014) for qualitative screening of 182, 156, and 199 pesticides, respectively. In these three studies, the percentage of pesticides with an SDL of 0.05 mg.kg⁻¹ ranged from 79% to 84%. In our study, the SDL was determined to be equal to or less than 0.05 mg.kg⁻¹ for 81% of the pesticides which is quite similar to the literature and done on more complex matrix like bivalves.

As described above, this study is the first to perform a qualitative multi-residue analysis of pesticides in bivalves. The other studies performed so far were quantitative analyses. This is the case for the studies carried out by Álvarez-Muñoz et al. (2019) and Cruzeiro et al. (2016) for the quantitative analysis of 13 and 55 pesticides, respectively. The authors of these two works obtained LOD and LOQ values below 0.01 mg.kg^{-1} for all the pesticides studied. Of all the pesticides investigated in the two studies mentioned above, nine (acetamiprid, azoxystrobin, difenoconazol, dimethoate, imidacloprid, metribuzin, simetryn, terbutryn and thiabendazole) are included in the list of 204 pesticides analysed in the present study. For all of these nine pesticides, the SDL was established at least at one of the three concentration levels tested in the present study and only one of these pesticides (simetryn) had an LOI greater than 0.01 mg.kg^{-1} .

3.5. *Application to real samples*

3.5.1. *Target screening analysis*

To evaluate the applicability of the proposed method, 14 bivalve samples (seven mussels and seven oysters) from three sites (Ile de Ré, Sèvre Niortaise and Charente) located in the Pertuis Charentais littoral area were analysed. For target screening, the home-made database of the 204 target pesticides (with information on T_R , MS, isotopic pattern, and MS/MS spectra) was used. Pesticides were detected and identified using the criterion applied in this work. Table S5 summarizes pesticides detected and identified in mussel and oyster samples. Diuron was the most frequently detected and identified compounds (in 8 of the 14 bivalve samples). Although it has been banned in France since 2008, diuron is still detected at high concentrations in coastal and fresh waters (Caquet et al., 2013). Other pesticide such as fungicide (fenpropimorph) was also detected and identified in one mussel sample in Sèvre Niortaise estuary in January 2020. To best our knowledge, this study is the first to show the presence of fenpropimorph in bivalves.

3.5.2. *Suspect screening analysis*

In target screening, most of the peaks resulting from sample analysis are ignored during data processing. The feasibility of the developed method has also been demonstrated on “known unknown” compounds in bivalve real samples. Post-acquisition data processing was carried out using a suspect screening of accurate masses. The database of compounds (with information of exact mass and molecular formula) has been expanded to include approximately 545 organic contaminants, including many pesticides and their metabolites as well as some flame retardants and plasticizers. A total of five additional compounds were tentatively

identified. The identification obtained from the suspect screening was considered tentative (level 3) until confirmed by a standard compound to reach identification confidence level 1 (Schymanski et al., 2014). As in the case of diuron in the target analysis, its metabolite 1-(3,4-dichlorophenyl)-3-methylurea was the most frequently tentatively identified. Other pesticide such as fungicide (diphenylamine) and personal insect repellent (N,N-diethyl-*m*-toluamide (DEET)) were also tentatively identified. Diphenylamine has been frequently detected in environmental waters because, in addition to its wide use as a fungicide, it is an important intermediate used in the chemical and pharmaceutical industries (Chen et al., 2014). DEET has previously been reported in mussels collected in San Francisco Bay, California with a concentration (mean 0.014 mg.kg⁻¹) positively correlated with their lipid content (Klosterhaus et al., 2013). The organophosphate flame retardant and plasticizer (2-ethylhexyl diphenyl phosphate (EHDPP) and tris(2-chloroethyl) phosphate (TCEP)) were tentatively identified. EHDPP and TCEP have previously been detected in bivalves collected on several points along the northern coast of Spain and along the Korean coast, with a concentration of around 0.002 mg.kg⁻¹ for both (Castro et al., 2020; Choi et al., 2020). As shown in Table S6, all tentatively identified compounds were subsequently confirmed with their respective reference standard with MS, MS/MS and T_R matching.

4. Conclusion

The development and validation of a QuEChERS-LC-HRMS method using IDA-PIL mode enabling screening of 204 pesticides in bivalves was carried out according to the European Union SANTE/12682/2019 guidelines. The list of target compounds included pesticides from different families with very distinct physicochemical properties. The use of L9 TOA for QuEChERS parameter optimisation greatly simplified the method development process and made it scientifically sound. We evaluated the impact of the different IDA and SWATH[®] modes on a QTOF 5600 on detection and identification rates and demonstrated the effectiveness of the IDA-PIL approach, especially since LibraryView[™] software does not allow for building of a SWATH library, only an IDA library. Most of the target list compounds were detected at all three concentration levels (0.01, 0.05 and 0.1 mg.kg⁻¹) with false positives averaging 2.5% on blank matrices. Unequivocal identification was problematic at the lowest concentration (0.01 mg.kg⁻¹) due to the lower abundance of product ions but was possible for most analytes at the highest concentration level (0.1 mg.kg⁻¹). The SDL and LOI were equal to or less than 0.1 mg.kg⁻¹ for 87% and 67% of pesticides, respectively.

The applicability of this approach for pesticide screening was finally demonstrated by analysing several samples of mussels and oysters collected in southwestern France. Diuron and fenpropimorph were the only identified analytes in the investigated samples using target analysis. The method also demonstrated its versatility to identify other organic contaminants (pesticides or other compounds) than those included in the method validation by suspect screening. This allowed the identification of diuron metabolite 1-(3,4-dichlorophenyl)-3-methylurea and other contaminants such as flame retardants and plasticizers.

Although the proposed methodology is very suitable, it is still necessary to develop workflows on different open-source software to follow SANTE guidelines regarding identification (product ion mass accuracy ± 5 ppm). Importantly, MasterViewTM software can be used to automatically obtain the mass error only on the precursor ions and not on the product ions. Another important point will be to find ways to realize an MS/MS library in SWATH methods in order to better exploit the advantages of these acquisition methods, especially on the richness of the generated MS/MS spectra and the retrospective analysis of the data.

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Declaration of competing interest

The authors declare no conflicts of interest.

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Table and Figure captions

Table 1. Control factor and levels in the QuEChERS extraction and clean-up.

Table 2. Pesticide detection rates of L9 TOA (3⁴) for the optimisation of QuEChERS extraction and clean-up.

Table 3. Pesticide S/N of L9 TOA (3⁴) for the optimisation of QuEChERS extraction and clean-up.

Fig. 1. Evaluation and comparison of IDA, IDA-PIL, F-SWATH and V-SWATH methods with 20, 30, 40 and 50 windows for pesticide detection (a) identification rates using Purity > 70% as search algorithm (b), identification rates using Purity > 50% as search algorithm (c), identification rates using Fit > 70% as search algorithm (d), and identification rates using Fit > 50% as search algorithm (e) at 0.01 µg.mL⁻¹ in ACN of the target 204 pesticides.

Fig. 2. Zoxamide MS spectrum with isotopic profile pattern (a), MS/MS spectrum acquired in SWATH® (b) and IDA-PIL mode (c) in spiked sample at 0.01 µg.mL⁻¹.

Fig. 3. Percentage of pesticides presenting SDL (a) and LOI (b) at 0.01, 0.05, 0.1 and > 0.1 mg.kg⁻¹ under optimised conditions. Detection (c) and identification rates (d) across different bivalve samples spiked at 0.01, 0.05 and 0.1 mg.kg⁻¹.

Electronic Supplementary Material

Table S1: List of the target pesticides with their corresponding formula, adduct or in-source fragment, theoretical *m/z* and retention time.

Table S2. L9 TOA for factor optimisation.

Table S3. SWATH acquisition MS settings for fixed windows and variable windows size.

Table S4. Validation results for 204 pesticides across 28 samples mainly spiked at 0.01, 0.05 and 0.1 mg.kg⁻¹ with corresponding SDL and LOI.

Table S5. Pesticide detected and identified by target screening analysis in mussel and oyster samples collected in the Pertuis Charentais littoral area.

Table S6. Pesticide detected and identified by suspect screening analysis in mussel and oyster samples collected in the Pertuis Charentais littoral area.

Fig. S1. Extracted Ion Chromatogram (top), MS spectra (bottom left) and MS/MS (bottom right) observed in the MasterView™ software interface.

Fig. S2. Chromatograms of fenpropimorph (a), methoprotetryne (b), hydramethylnon (c) and azoxystrobin (d) in oyster samples spiked at 0.01 mg.kg⁻¹.

Fig. S3. Effects of extraction solvent acidification (i), extraction salts (ii), dSPE (iii) and dilution (iv) on pesticide detection rates (a) and pesticide S/N (b).

CRedit authorship contribution statement

Thierno Diallo: Methodology, Data Curation, Formal analysis, Validation, Visualization, Investigation, Writing - Original Draft, Writing - Review & Editing.

Yassine Makni: Methodology, Data Curation, Investigation.

Adélaïde Lerebours: Validation, Writing - review & editing

Hélène Thomas: Validation, Resources, Writing - review & editing

Thierry Guérin: Validation, Writing - review & editing

Julien Parinet: Conceptualization, Methodology, Validation, Writing - Review & Editing, Supervision, Funding acquisition, Project administration.

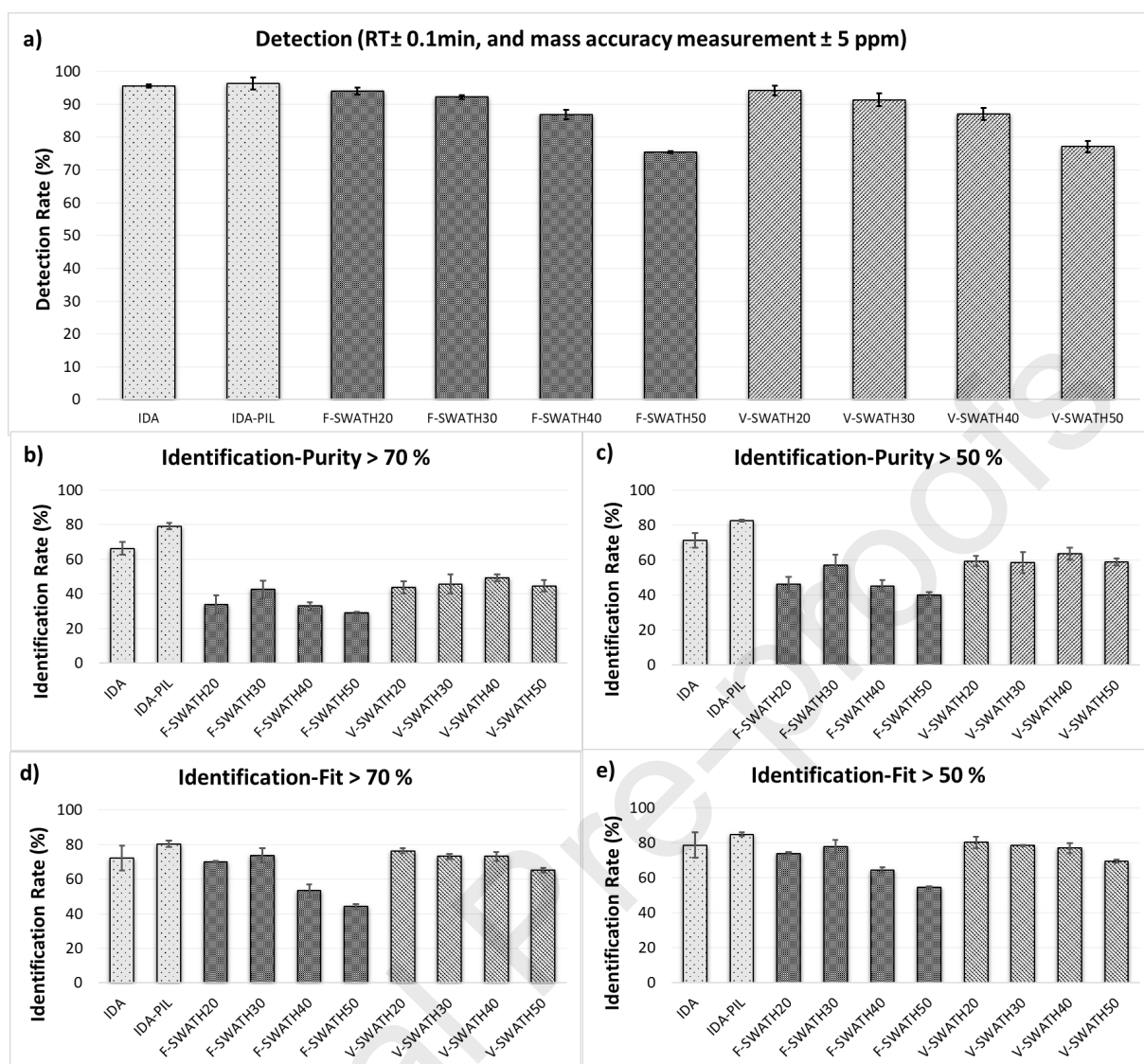


Fig. 1.

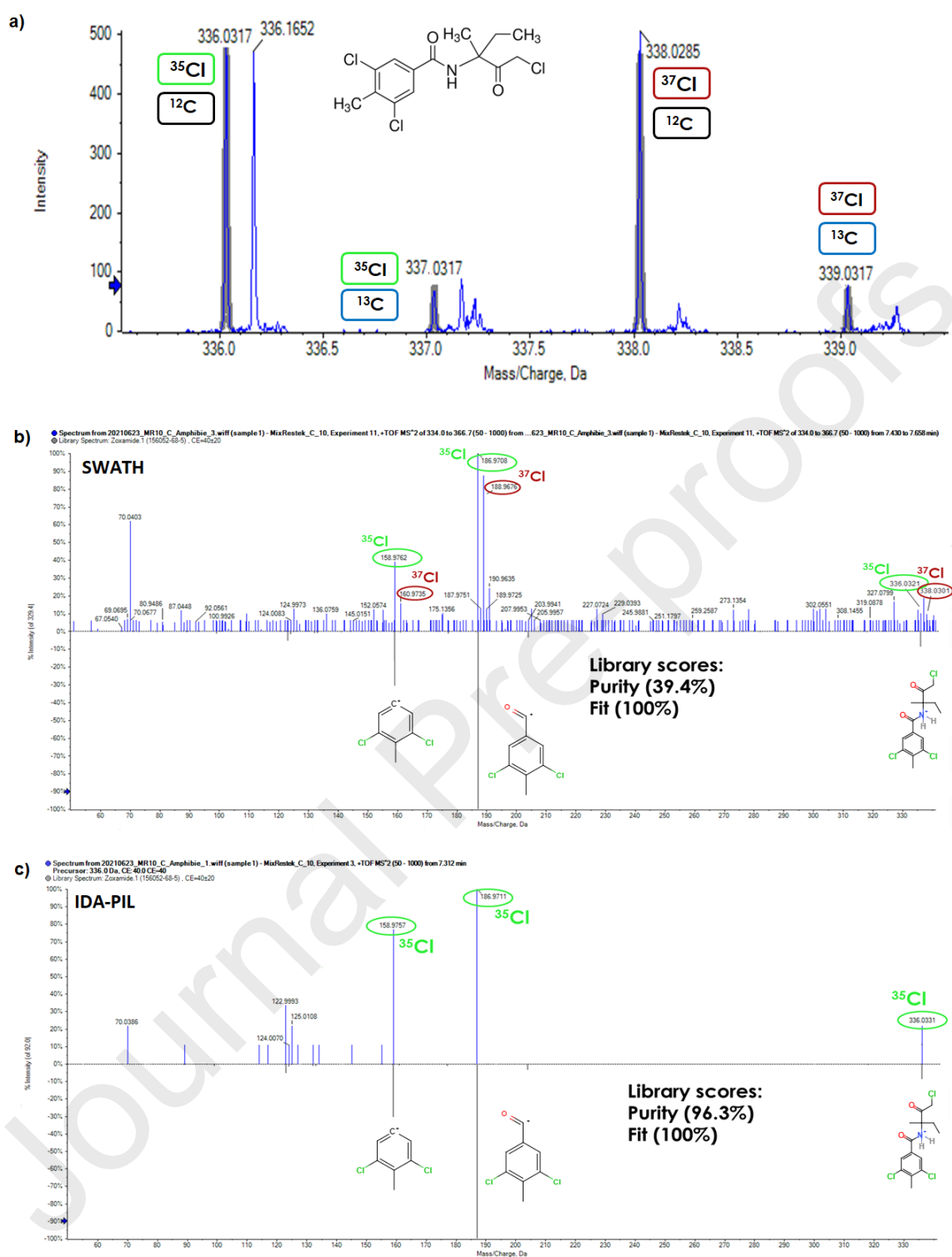


Fig. 2.

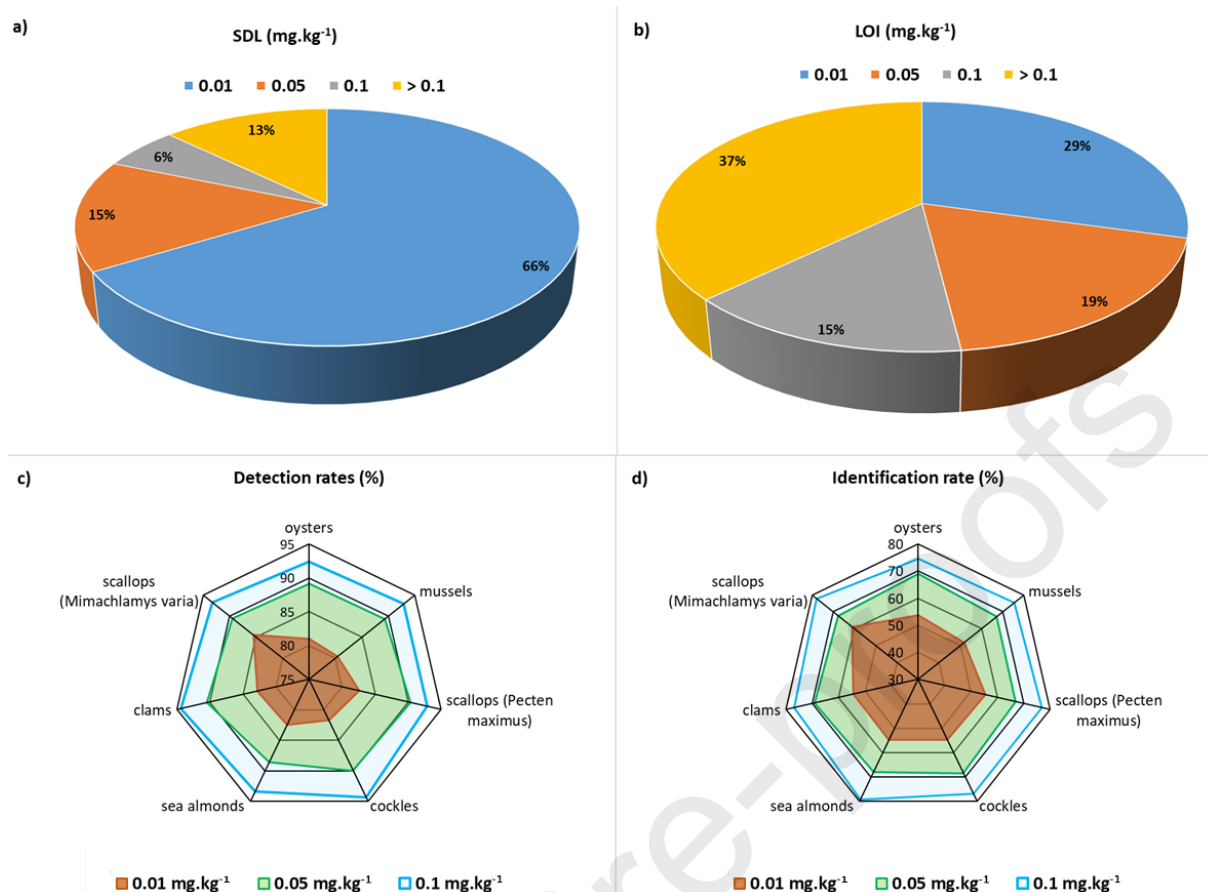


Fig. 3.

Table 1.

Level	Factor			
	Solvent acidification	Salt	dSPE	Dilution
1	ACN (1% AA)	AOAC Method*	MgSO ₄ /PSA/ C18	Non-dilution
2	ACN (1% FA)	EN Method**	Z-sep/C18	1:2
3	ACN	Original Method***	EMR lipid	1:4

* 6.0 g anhydrous MgSO₄, 1.5 g NaOAC.** 4.0 g of MgSO₄, 1.0 g of NaCl, 1.0 g of sodium citrate, and 0.5 g of sodium citrate sesquihydrate.*** 4.0 g of MgSO₄, 1.0 g of NaCl.

Table 2.

Experiment	Extraction solvent acidification	Extraction salts	dSPE	Dilution	Mean of Detection Rates (%)	Relative SD (%)
1	1	1	1	1	84.98	2.36
2	1	2	2	2	75.29	3.88
3	1	3	3	3	55.97	3.50
4	2	2	1	3	55.65	8.09
5	2	3	2	1	82.10	4.93
6	2	1	3	2	72.82	3.34
7	3	3	1	2	80.06	9.80
8	3	1	2	3	56.13	12.40
9	3	2	3	1	86.33	4.39
K1*	72.08	71.31	73.56	84.47		
K2	70.19	72.42	71.17	76.06		
K3	74.17	72.71	71.71	55.92		
R**	3.98	1.40	2.39	28.55		
F-value	1.75	0.24	0.69	95.09		
p-value***	0.20	0.79	0.51	< 0.001		

* The average detection rate for each level (Ki) in different factors. It is used to select the best level for each factor.

** The range of detection rates for different factors. It is used to rank the factors by their impacts on the detection rate. The higher this value, the greater the impact of the given parameter on the detection rate.

*** One-way ANOVA test.

Table 3.

Experiment	Extraction solvent acidification	Extraction salts	dSPE	Dilution	Mean of S/N	Relative SD (%)
1	1	1	1	1	189.73	6.57
2	1	2	2	2	117.11	1.42
3	1	3	3	3	96.87	4.20
4	2	2	1	3	127.81	14.36
5	2	3	2	1	150.95	1.63
6	2	1	3	2	114.99	2.48
7	3	3	1	2	174.95	6.09
8	3	1	2	3	96.79	1.48
9	3	2	3	1	151.07	0.21
K1*	134.57	133.83	164.16	163.92		
K2	131.25	132.00	121.62	135.68		
K3	140.94	140.93	120.98	107.16		
R**	9,69	8,92	43,19	56,76		
F-value	4.80	4.40	121.44	159.65		
p-value***	0.0213	0.0277	< 0.001	< 0.001		

* The average detection rate for each level (Ki) in different factors. It is used to select the best level for each factor.

** The range of detection rates for different factors. It is used to rank the factors by their impacts on S/N. The higher this value, the greater the impact of the given parameter on the S/N.

*** One-way ANOVA test.

Highlights

- First qualitative screening of a mix of 204 pesticides in bivalves by UHPLC-QTOF-MS
- Taguchi orthogonal array used to simplify the optimisation of the QuEChERS method
- IDA-PIL provides better quality and easier to interpret MS/MS spectra than SWATH
- Method validation was performed according to SANTE/12682/2019
- Screening detection limits were ≤ 0.05 mg.kg for 81% of pesticides

Journal Pre-proofs