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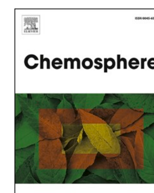

Analytical research of pesticide biomarkers in wastewater with application to study spatial differences in human exposure

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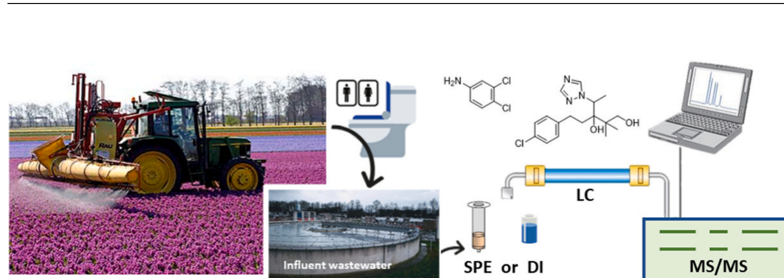
HIGHLIGHTS

- Specific urinary biomarkers of pesticide exposure were determined in urban wastewater.
- SPE and direct injection were assayed followed by LC-MS/MS measurement.
- Analytical challenges were identified and discussed in detail.
- The applicability for assessing human exposure to pesticides was illustrated.
- The developed method can be applied for future wastewater-based epidemiology studies.

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewater-based epidemiology (WBE) relies on the assessment and interpretation of levels of biomarkers in wastewater originating from a well-defined community. It has provided unique information on spatial and temporal trends of licit and illicit drug consumption, and has also the potential to give complementary information on human exposure to chemicals. Here, we focus on the accurate quantification of pesticide biomarkers (*i.e.,* predominantly urinary metabolites) in influent wastewater at the ng L^{-1} level to be used for WBE. In the present study, an advanced analytical methodology has been developed based on ultra-high-pressure liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS), for the simultaneous determination of 11 specific human biomarkers of triazines, urea herbicides, pyrethroids and organophosphates in urban wastewater. The sample treatment consisted of solid-phase extraction using Oasis HLB cartridges. Direct injection of the samples was also tested for all compounds, as a simple and rapid way to determine these compounds without sample manipulation (*i.e.,* minimizing potential analytical errors). However, if extraction recoveries are satisfactory, SPE is the preferred approach that allow reaching lower concertation levels. Six isotopically labelled internal standards were evaluated and used to correct for matrix effects. Due to the difficulties associated with this type of analysis, special emphasis has been placed on the analytical challenges encountered. The satisfactory validated methodology was applied to urban wastewater samples collected from different locations across Europe revealing the presence of 2,6-EA, 3,4-DCA, 3-PBA and 4-HSA *i.e,* metabolites of metolachlor-s, urea herbicides, pyrethroids and chlorpropham, respectively. Preliminary data reported in this paper illustrate the applicability of this analytical approach for assessing human exposure to pesticides through WBE.

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1. Introduction

Quantitative analysis of specific human lifestyle biomarkers in influent wastewater has been largely employed and provided unique information on spatial and temporal trends of licit and illicit drug consumption (Castiglioni et al., 2015; González-Mariño et al., 2020; Ryu et al., 2016). This cutting-edge and transdisciplinary approach *i.e.,* wastewater-based epidemiology (WBE) relies on the assessment and interpretation of levels of biomarkers in wastewater originating from a well-defined community. The approach has been endorsed by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) and the United Nations Office on Drugs and Crime (UNODC) for increased knowledge to support strategic and policy decision-making on illicit drugs (European Monitoring Centre for Drugs and Drug Addiction (2021)). However, WBE has occasionally also been used and can conceptually be applied as a complementary source of information about other health-related aspects such as exposure to chemicals and disease (Daughton, 2018; González-Mariño et al., 2021; Gracia-Lor et al., 2017; Rousis et al., 2017a; Sims and Kasprzyk-Hordern, 2020).

When developing new WBE applications, two key aspects need to be considered. First, biomarker selection is pivotal and ideally ubiquitous biomarker that have no other source than human excretion and are stable in wastewater are selected (Castiglioni and Gracia-Lor, 2016). Second, highly sensitive and selective analytical methodologies are required that allow for the detection and quantification of the biomarkers at trace level (ng L^{-1} - μg L^{-1}) concentrations. Ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS), with triple quadrupole mass analyzers is the most suitable analytical technique to comply with the requirements needed for accurate WBE measurements. UHPLC-MS/MS has demonstrated excellent performance in terms of robustness, dynamic range, sensitivity, and selectivity, specifically when measuring targeted polar compounds in aqueous samples (Hernandez et al., 2018). However, a pre-concentration step is often required in order to reach the necessary limits of quantification. This step, performed prior to analysis, is normally carried out by employing solid phase extraction (SPE). Yet, due to the high sensitivity provided, modern analytical instruments even allow the measurement of the more sensitive compounds by direct injection (DI) of filtered or centrifuged wastewater (Boix et al., 2015; Reymond et al., 2022; Rousis et al., 2016). Although pre-concentration frequently leads to lower quantification limits, less matrix is introduced in the system when employing direct injection (Bijlsma et al., 2014). This is an important facet since matrix effects (ME) may complicate accurate WBE measurements. Therefore, a compensation for this phenomenon is generally recommended and the use of isotopically labelled internal standards (ILIS) is the strategy of choice. However, ILIS are not always available or are very expensive especially when measuring specific human biomarkers *e.g.*, metabolites.

The prospects of WBE to estimate human exposure to pesticides is of particular interest. Exposure to pesticides has been linked to pose risks for human health and associated to for example cancer and Parkinson's disease (Allen and Levy, 2013; Weichenthal et al., 2012; Zhang et al., 2019). WBE can provide information about the exposure of the general population to pesticides via combined exposure routes including diet, household use and environmental exposure, and evade in this way time consuming and costly human biomonitoring sampling campaigns. Moreover, WBE can provide information on spatial differences in exposure and in temporal trends. The determination of pesticide biomarkers in influent wastewater is, however, an analytical challenge and as compared to applications of WBE to illicit drugs (Huizer et al., 2021) very few studies are available (Devault et al., 2018; Rousis et al., 2017a, 2017b, 2021), applying the same methodology (Rousis et al., 2016).

The objective of the present study is to develop and validate a rapid and sensitive methodology for the simultaneous determination of 11 pesticide biomarkers in urban wastewater. Specific human biomarkers (*i.e.,* all metabolites, except asulam) of triazines, pyrethroids and organophosphates were identified and subjected to study. Validation was performed using real wastewater samples collected from different locations, in order to support robustness considering the large diversity in water sample composition among wastewater treatment plants (WWTPs). Both SPE and DI were assayed followed by UHPLC-MS/MS measurement. Special emphasis has been placed on a detailed discussion of the analytical challenges encountered, the implications for the applicability of WBE to study pesticides exposures, and to the reliability of the identification in positive samples. The methodology developed has been applied to the analysis of 24-h composite influent urban wastewater samples collected from different locations across Europe to obtain preliminary results and illustrate its applicability for future WBE studies.

2. Materials and methods

2.1. Chemicals and reagents

The pesticide biomarkers studied are shown in Table 1, together with their abbreviation, parent pesticide, CAS number, molecular structure, formula and weight. Prioritized were those biomarkers used for human biomonitoring and the selection was based on the scientific literature, the Dutch OBO report and Board for the Authorization of Plant Protection Products and Biocides (Ctgb) (Gooijer et al., 2019; https://www.ctgb.nl/; Rousis et al., 2016; Yusa et al., 2015). These compounds were obtained from Merck – Sigma Aldrich (Zwijndrecht, the Netherlands), Toronto Research Chemicals (North York, Ontario, Canada) and Cambridge Isotope Laboratories (Tewksbury, MA, USA) as solids or in the case of DBCA as solution in acetonitrile. Standard stock solutions of each compound were prepared at 1000 mg L^{-1} in methanol. Mixed working solutions containing all analytes were prepared from intermediate solutions at different concentrations by appropriate dilution with methanol and were used for the preparation of the calibration line and for spiking samples in the validation study.

Isotopically labelled compounds of 3-PBA-$^{13}C_6$, 3,4-DCA-d_2, acetamiprid-d_3, atrazine-d_6, imidaclorprid-d_4 and thiacloprid-d_4 were purchased from Merck – Sigma Aldrich and Cambridge Isotope Laboratories and used as surrogate internal standards. 3-PBA-$^{13}C_6$, 3,4-DCA-d_2 were dissolved in methanol, acetamiprid-d_3, atrazine-d_6 in acetonitrile and imidaclorprid-d_4 and thiacloprid-d_4 in acetone. All solutions were stored in amber glass bottles at −20 °C.

LC-MS grade methanol (MeOH), LC-MS grade acetonitrile (ACN), LC-MS grade acetone, ammonium acetate (NH_4Ac, >98%) and formic acid (HCOOH, LC-MS grade) were acquired from Scharlau (Barcelona, Spain). HPLC-grade water was obtained by purifying demineralized water in a Milli-Q plus system from Millipore (Bedford, MA, USA).

2.2. Samples

Influent wastewater (IWW) commonly shows temporal and specifically spatial differences, due to for example distinct agricultural activities or divergences in dietary habits between sites. Therefore, 24-h time-proportional (every <15 min) composite IWW samples of different geographical origin 1. Castellón (Spain), 2. Amsterdam (the Netherlands), 3. Lisbon (Portugal), 4. Dortmund (Germany) and 5. Saarbrücken (Germany) were selected and collected for the validation of the analytical methodology. Moreover, these samples were analyzed for the pesticide biomarkers selected to illustrate the applicability of the approach.

After collection, samples were transferred frozen to Castellon and stored at −20 °C until sample treatment.

2.3. Sample treatment

2.3.1. solid phase extraction

2,6-EA, 3,4-DCA, DES, 3-PBA, HCBA and 4-HSA were concentrated

by Solid Phase Extraction (SPE) using Oasis HLB 3 cc/60 mg cartridges, previously conditioned with 2 × 5 mL MeOH and 2 × 5 mL Milli-Q water. Influent wastewater sample (50 mL) was centrifuged at 5000 rpm for 5 min, spiked with ILIS (1 ng of ILIS, 20 ng L^{-1} in sample and 1 μg L^{-1} in vial after SPE), and percolated through the cartridges by gravity. After drying under vacuum during 20 min, the analytes were eluted with 5 mL MeOH. Subsequently, 900 μL of Milli-Q water was added, and the extracts were evaporated to 900 μL at 40 °C under a gentle stream of nitrogen. Then, 100 μL of MeOH was added to obtain a final volume of 1 mL (MeOH:water, 10:90, v/v). The sample extract was injected (10 μL) in the UHPLC–MS/MS system.

2.3.2. Direct injection

N-DMA, TEB-OH, ASU, 6-CN and Boscalid-OH were analyzed in influent wastewater samples through DI. 1 mL of sample was centrifuged at 5000 rpm during 5 min and spiked with ILIS at 1 μg L^{-1} in vial. Analyses were performed by injecting 100 μL of the sample directly into the UHPLC–MS/MS system.

The recommended procedures for both SPE and direct injection are illustrated in Fig. 1.

Table 1
The studied pesticide biomarkers, together with their abbreviation, parent pesticide, CAS number, molecular structure, formula and weight M.W. (g/mol).

Biomarker (target compound)	Abbreviation	Parent pesticide	Molecular structure	Molecular Formula	M.W. (g/mol)	CAS number
2-Methyl-6-ethylaniline	**2,6-EA**	Metolachlor-S		$C_9H_{13}N$	135.2	24549-06-2
6-Chloronicotinic acid	**6-CN**	Imidacloprid, Thiacloprid, Acetamiprid		$C_6H_4ClNO_2$	157.5	5326-23-8
3,4-Dichloroaniline	**3,4-DCA**	Linuron, Diuron, Neburon, Propanil		$C_6H_5Cl_2N$	162.0	95-76-1
Terbuthylazine desethyl	**DES**	Terbuthylazine		$C_7H_{12}ClN_5$	201.6	30125-63-4
Cis-3-(2,2-dibromovinyl)-2,2dimethylcyclopropane carboxylic acid	**DBCA**	Deltamethrin		$C_8H_{10}Br_2O_2$	298.0	63597-73-9
N-desmethylacetamiprid	**N-DMA**	Acetamiprid		$C_9H_9ClN_4$	208.6	190604-92-3
Asulam	**ASU**	Asulam		$C_8H_{10}N_2O_4S$	230.2	3337-71-1
Tebuconazole-1-hydroxy	**TEB-OH**	Tebuconazole		$C_{16}H_{22}ClN_3O_2$	323.8	212267-64-6
Boscalid-5-hydroxy	**Boscalid-OH**	Boscalid		$C_{18}H_{12}Cl_2N_2O_2$	359.2	661463-87-2
2,4,6-Trichlorophenoxy acetic acid	**2,4,6-TCP**	Prochloraz		$C_8H_5Cl_3O_3$	255.5	575-89-3
3,5,6-Trichloro-2-pyridinol	**TCPy**	Chlorpyriphos, Chlorpyriphos-methyl		$C_5H_2Cl_3NO$	198.4	6515-38-4
3-Phenoxybenzoic acid	**3-PBA**	Pyrethroids (>14)		$C_{13}H_{10}O_3$	214.2	3739-38-6
3-(2-Chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylic acid	**HCBA**	Cyhalothrin-Lambda (pyrethroid)		$C_9H_{10}ClF_3O_2$	242.6	72748-35-7
4-Hydroxychlor propham-O-sulphonic acid	**4-HSA**	Chlorpropham		$C_{10}H_{12}ClNO_6S$	309.7	28705-88-6

2.4. Instrumentation

An Acquity H-class UPLC system (Waters Corp., Milford, MA, USA) was interfaced to a triple quadrupole mass spectrometer (Xevo TQ-S, Waters Corp., Manchester, UK) equipped with an electrospray ionization interface (Z-Spray, ESI) operated in positive and negative ionization mode. The chromatographic separation was performed using a Waters Acquity UPLC BEH C_{18} column, (50 × 2.1 mm i.d., particle size 1.7 μm) and the mobile phases consisted of Milli-Q water with 0.1% HCOOH (solvent A) and of MeOH (solvent B). The percentage of solvent B changed linearly as follows: 0 min, 5%; 2.5 min, 60%; 8 min, 99%; 9 min, 99%; 9.1 min 5%; 11 min, 5%. The flow rate was 0.3 mL min^{-1}. Dry nitrogen was used as cone gas as well as desolvation gas (Praxair, Valencia, Spain), with flows set to 250 and 1200 L h^{-1}, respectively. For operation in MS/MS mode, collision gas was argon 99.995% (Praxair, Madrid, Spain). Further parameters optimized included: capillary voltage, 1.0 kV in positive mode, −1.5 kV in negative mode; source temperature, 150 °C and desolvation temperature, 650 °C. Dwell times were automatically selected, being at least 0.01 s/transition.

For better understanding its fragmentation, TCPY, 2,4,5-TCP and DCBA were further studied using a Waters Acquity UPLC system (Waters Corp., Milford, MA, USA) interfaced to a hybrid quadrupole-orthogonal acceleration-ToF mass spectrometer (Xevo G2 QTof, Waters Corp., Manchester, UK).

Data were acquired and processed using MassLynx v4.1 software (Waters Corp., Manchester, UK).

2.5. Method validation

Acquisition was performed in Selected Reaction Monitoring (SRM) mode, with the (de)protonated molecule of each biomarker selected as precursor ion. Typically, the most abundant product ion of each target analyte was used for quantification (*Q*) and two additional product ions (*q*) were used for confirmation. Chromatographic retention times (RTs) and *q/Q* ratios were also compared with those of the reference standards (*i.e.,* within ±0.1 min and ±30%, respectively) for confirmation of the identity of the compounds detected in samples (SANTE/12682/2019, 2019). Each compound was quantified using its corresponding isotope labelled internal standard (ILIS). When the own analyte ILIS was not available, an analogue ILIS, if appropriate was used as surrogate. Some other compounds were determined using absolute responses, as no suitable ILIS was found.

Method validation was done with five different IWW and included the most relevant parameters affecting the performance of the method, following the spirit of the SANTE guideline (SANTE/12682/2019, 2019): linearity, limit of quantification (LOQ), limit of detection (LOD), accuracy and precision (both parameters estimated by recovery experiments at two concentration levels *i.e.,* LOQ and 10xLOQ in the case of the SPE, and four concentration levels for the DI method. The linearity of the method was studied in the range 5–10,000 ng L^{-1}, using as criterion an R^2 value higher than 0.99. The LODs and LOQs were estimated using the lowest spiked concentration level which yielded a recovery within the established acceptance criteria and considering signal to noise (S/N) ratios of 3 for the *q* transition and 10 for the *Q* transition, respectively. Owing to the difficulty and variability associated with these analytes and complex matrices (*i.e.,* the composition of IWW can differ in time or in space, due to for example meteorological conditions or the impact of distinct industries), the LOQs estimated should be taken as an indicative value. It would be possible to quantify analytes in a given sample at concentrations slightly lower than the estimated LOQs if the above criteria mentioned were met. This means, that both identification and quantification of the analyte in such sample would be ensured. Obviously, the amount quantified should be always above the lowest calibration level. It is the task of the analysts to critically evaluate their results before reporting data.

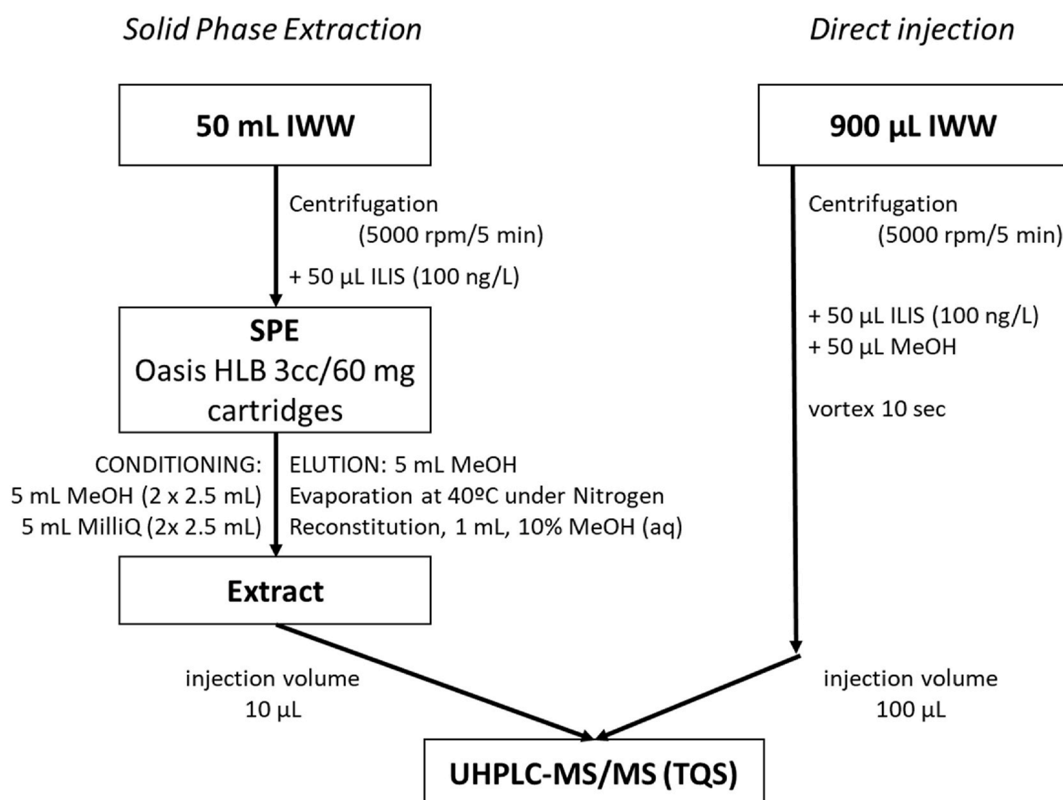

Fig. 1. Graphical workflow of the recommended sample pretreatment procedures.

3. Results and discussion

3.1. UHPLC and MS/MS optimization

The list of pesticide biomarkers includes compounds showing very different physicochemical properties and polarity. An adequate optimization of the UHPLC conditions was important to distribute the elution of the analytes and obtain satisfactory chromatographic performance. Parameters such as S/N, analyte response and peak width were evaluated. The mobile phase consisted of (A) Milli-Q water and (B) MeOH, but different elution gradients and mobile phase modifiers *i.e.,* variations in concentration of buffer ammonium acetate (0.5–5 mM) and pH (addition of formic acid at 0.1 and 0.01%, v/v), were tested. Milli-Q water with 0.1% of formic acid as mobile phase (A) showed the best peak shape, resolution and efficiency. Retention times were stable in solvent and IWW, and the total analysis time was fixed at 11 min. Eight analytes out of the 14 compounds investigated, showed higher response in ESI+ and 6 were monitored in ESI- using $[M+H]^+$ and $[M-H]^-$ as precursor ion, respectively. In general, the most abundant product ion of each analyte was used for quantification (*Q*) and one or two additional product ions were used for confirmation (*q1* and *q2*), taking also into account the specificity of the transitions.

The MS/MS parameters (cone voltage (CV) and collision energy (CE)) were optimized for maximum sensitivity for each analyte. All the MS/MS parameters, as well as SRM transitions, ion ratios and chromatographic retention times (RTs), are listed in Table 2. In spite of performing two different sample treatments, both extracts were analyzed using the same instrumental parameters, as no limitations were observed when working in polatiry switching mode at low dwell times.

3.2. Analytical challenges

The investigation of pesticides biomarker of exposure in wastewater presents several analytical challenges derived from the high complexity of the matrix and chemical properties of the compounds (mostly pesticide metabolites) that require the use of different sample treatments and chromatographic and MS measurement conditions. Hence, prior to method validation and application, several major issues were taken into account, from the mass spectrometry point of view, and were carefully addressed, as discussed below.

Table 2
UHPLC-MS/MS parameters established for the SRM acquisition mode (quantification (*Q*) and confirmation (*q*1 and *q*2) transitions).

Biomarker	ESI	R_t (min)	Precursor ion (*m/z*)	CV[a] (V)	CE[b] (eV)	Product ion (*m/z*)	*q/Q* ratio, solvent[c] (RSD%)	*q/Q* ratio, matrix SPE[d] (RSD%)	*q/Q* ratio, matrix DI[e] (RSD%)
2,6-EA	+	**4.2**	136.0	30	15	117.0			
					20	91.0	3.5 (10)	5.4 (7)	5.1 (11)
					15	98.8	1.3 (2)	1.1 (2)	0.98 (1)
6-CN	+	**3.8**	158.1	30	15	122.0			
					20	77.9	0.38 (6)	–	0.36 (6)
					20	112.0	0.20 (7)	–	0.27 (11)
3,4-DCA	+	**4.9**	162.0	30	15	127.0			
					25	109.0	0.13 (15)	0.14 (8)	0.10 (10)
					25	145.0	0.12 (8)	0.10 (7)	0.10 (15)
DES	+	**4.8**	202.6	30	15	146.0			
					25	110.1	0.25 (22)	0.13 (54)	–
					25	103.9	0.12 (26)	0.12 (63)	–
N-DMA	+	**3.8**	209.6	10	15	127.0			
					35	99.0	0.22 (18)	–	0.19 (47)
					30	90.8	0.32 (6)	–	0.28 (55)
ASU	+	**2.6**	231.1	10	10	155.9			
					20	92.0	0.56 (3)	–	0.42 (11)
					20	108.0	0.36 (8)	–	0.28 (11)
TEB-OH	+	**5.9**	324.8	10	15	69.9			
					35	125.0	0.26 (14)	0.20 (8)	0.25 (9)
					30	151.9	0.05 (27)	0.04 (11)	0.05 (39)
Boscalid-OH	+	**4.8**	360.2	5	25	140.0			
					15	323.9	0.87 (7)	0.79 (5)	1.1 (19)
					40	112.0	0.56 (13)	0.50 (17)	0.59 (21)
3-PBA	-	**6.1**	213.0	40	35	92.8			
					15	168.9	1.7 (10)	3.7 (12)	3.4 (18)
					50	65.0	0.01 (28)	0.004 (56)	0.07 (54)
HCBA	-	**6.4**	240.8	30	10	204.8			
					20	120.8	0.23 (18)	0.19 (15)	0.21 (37)
4-HSA	-	**4.2**	308.0	20	15	227.9			
					30	140.9	0.47 (13)	0.50 (3)	0.46 (7)
					30	167.8	0.13 (12)	0.14 (3)	0.14 (17)
3,4-DCA-*d*$_2$	+	**4.9**	165.9	30	20	131.0			
3-PBA-^{13}C$_6$	-	**6.1**	219	40	35	98.9			
Atrazine-*d*$_5$	+	**4.7**	221.0	10	20	178.9			
Acetamiprid-*d*$_3$	+	**3.8**	226.1	10	35	125.9			
Thiacloprid-*d*$_4$	+	**4.0**	257.0	10	40	98.8			
Imidacloprid-*d*$_4$	+	**3.6**	260.0	15	15	213.0			

Note: 3,4-DCA-d$_2$ was used to correct 3,4-DCA; 3-PBA ^{13}C$_6$ for 3-PBA and 4-HSA; acetamiprid-d$_3$ for N-DMA; atrazine-d$_5$ for DES and TEB-OH; imidacloprid-d$_4$ for 6-CN; thiacloprid-d$_4$ for 2,6-EA and Boscalid-OH.

[a] Cone voltage.
[b] Collision energy.
[c] *q/Q* ratio in solvent.
[d] *q/Q* ratio in matrix after performed SPE.
[e] *q/Q* ratio in matrix when applying DI.

3.2.1. Use of ILIS for correcting sample treatment and ionization efficiency

Matrix effects in UHPLC-ESI-MS/MS analyses leads to important signal variations (both suppression and enhancement) produced by co-eluting compounds that modify the ionization process (Gosetti et al., 2010). In the case of wastewater samples, signal suppression has been widely reported (Bijlsma et al., 2013). This behavior was also observed for pesticides biomarkers included in this study (see Fig. 2 using SPE as sample treatment, and Table S1 of the supplementary information for DI analysis). The benefits of using ILIS for correcting ME have been clearly demonstrated in UHPLC-ESI-MS/MS determinations (Mirmont et al., 2022; Van Eeckhaut et al., 2009). The basis of ME correction using ILIS is that isotopically-labelled compounds in theory present an ionization behavior in ESI similar to unlabeled compounds. For this reason, when using ILIS in the SPE method ME was substantially corrected (Fig. 2) for those compounds for which their corresponding ILIS were available (*i.e.,* 3,4,-DCA and 3-PBA). The ME correction for other compounds using analogue ILIS was not always satisfactory, despite the similarities between structures, for example when using atrazine-d_5 to correct for DES, or having similar retention times like thiacloprid-d_4 for 2,6-EA. This fact illustrates the need of using the corresponding ILIS for each analyte for an appropriate ME correction in this type of analysis, as well as the limitations of selecting analogue ILIS to this aim. However, this is not always feasible given the unavailability of ILIS for some molecules and the high cost of labelled compounds.

3.2.2. SRM transitions for 3,4-DCA and 3,4-DCA-d_2

The use of appropriate ILIS for correcting ME, as well as sample treatment recoveries, when added in the first step of the analysis (*i.e.,* as surrogates) has evident advantages. Nevertheless, it should be kept in mind that isotopically-labelled compounds may produce "cross-talk" if the mass difference between natural and labelled compound is less than 3 Da, as the isotope peaks of the unlabeled analyte may interfere with the signal of the ILIS (Stokvis et al., 2005). This was the case for one of the pesticide metabolites included in this work, for which the requirement of > 3Da was not met, causing challenges. The 3,4-DCA-d_2 is, to the best of our knowledge, the only isotopically-labelled standard available of 3,4-DCA. This compound has two chlorine atoms in its structure, producing the well-known isotope pattern shown in Fig. S1. Thus, the 3,4-DCA spectrum overlaps the 3,4-DCA-d_2 spectrum, *i.e.,* leading to the fact that protonated molecule of 3,4-DCA-d_2 is overlapping with the chlorine isotopic pattern of 3,4-DCA. This made the selection of the SRM transitions for the ILIS crucial to avoid cross-talk from the natural compound to the ILIS.

As the SRM transition of 3,4-DCA-d_2 should be selected keeping in mind the isotope distribution, product ion scan spectra of natural and labelled compound were acquired for the three main ions of the chlorine isotope cluster (Fig. 3). It can be seen that the SRM transition 166 > 131 observed for the labelled compound is not present in the natural one, making it suitable for the ME correction of 3,4-DCA. In order to assure that there was no cross-talking, the three SRM transitions selected for the natural compound and the 166 > 131 transition selected for the labelled compound were acquired in the analysis of individual standards of 3,4-DCA and 3,4-DCA-d_2. No cross-talking was observed (Fig. 3), confirming the suitability of the SRM transitions selected.

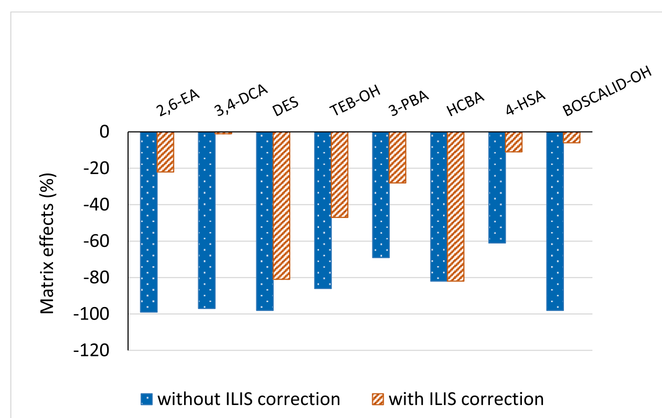

Fig. 2. Matrix effect (%) in the SPE-based method with and without ILIS correction.

3.2.3. The case of TCPy and 2,4,6-TCP

The initial selection of analytes included 3,5,6-trichloro-2-pyridinol (TCPy) and 2,4,6-trichlorophenoxy acetic acid (2,4,6-TCP), as metabolites of chlorpyriphos/chlorpyriphos-methyl and prochloraz, respectively (Gooijer et al., 2019; Yusa et al., 2015). However, both compounds were discarded during method optimization due to several limitations.

UHPLC-MS/MS determination of TCPy was successfully optimized for the standard solutions, but the low analytical response in ESI-MS/MS for this compound, and the high signal suppression observed in SPE extracts and in raw IWW when applying DI, made it impossible to reach an acceptable LOQ, useful in WBE applications. In the case of 2,4,6-TCP, we could not identify this compound in the stock analytical standard during MS/MS optimization. Instead, we observed 2,4,6-trichlorophenol, but were not able to verify if its presence was due to the degradation of the 2,4,6-TCP during storage, or to formation during ionization as a consequence of a complete in-source fragmentation. This last hyphotesis seems unlikely considering that other groups have successfully developed ESI-MS methods for this compound (Lindh et al., 2008). The stock solution was also analyzed by UHPLC-high resolution mass spectrometry (UHPLC-HRMS), obtaining the same results as with the triple quadrupole. Therefore, as the identity of the compound was not assured, 2,4,6-TCP was not included in the analytical method.

3.2.4. Ionization behavior of DBCA

DBCA (*cis*-2,2-dibromovinyl-2,2-dimethylcyclopropane-1-carboxylic acid) was initially selected in the UHPLC-ESI-MS/MS method development, as a metabolite of pyrethroid insecticides (Jurewicz et al., 2020; Yusa et al., 2015). We observed an unusual ionization and fragmentation behavior of this compound in the preliminary UHPLC-MS/MS experiments, and therefore an in-depth analysis by UHPLC-HRMS was performed for better understanding. Fig. 4 shows the full-range accurate-mass spectra of a DBCA standard obtained under the instrumental conditions and data processing workflow for fragmentation evaluation reported by Fabregat-Safont et al. (2019).

As DBCA presents a carboxylic acid moiety, it was expected to be observed as deprotonated molecule ($[M-H]^-$). Nevertheless, the compound was found as a double deprotonated dimeric sodium adduct ($[2M\text{-}2H+Na]^-$), obtaining an ion with a single negative charge. The observed fragmentation presented an unexpected behavior: the first fragment was produced by the loss of a single monomer of the dimer, but maintaining a bromide in the remaining intact sodiated molecule (fragment 1). More unusual is fragment 2, produced by fragment-fragment and/or fragment-adduct reactions between two bromide ions (fragment 3) and the sodium ion. The structure of dimer and the three observed fragment ions fitted with the accurate mass of the ions (mass error below 5 ppm) and the isotopic pattern, as can be seen in Fig. 4.

In spite of the possibility to form the dimer and fragment ions (as observed by HRMS), the UHPLC-MS/MS analytical methods reported for DBCA used the 295(297) > 79(81) SRM transition (Jeong et al., 2019), where the deprotonated molecule was selected as precursor ion and bromide as product ion for both isotopes. The unusual ionization and fragmentation behavior observed is likely due to dimerization during the ionization process as a consequence of intermolecular forces (hydrogen bonding) (Clifford et al., 2007). For that reason, also DBCA was not included in the analytical methodology.

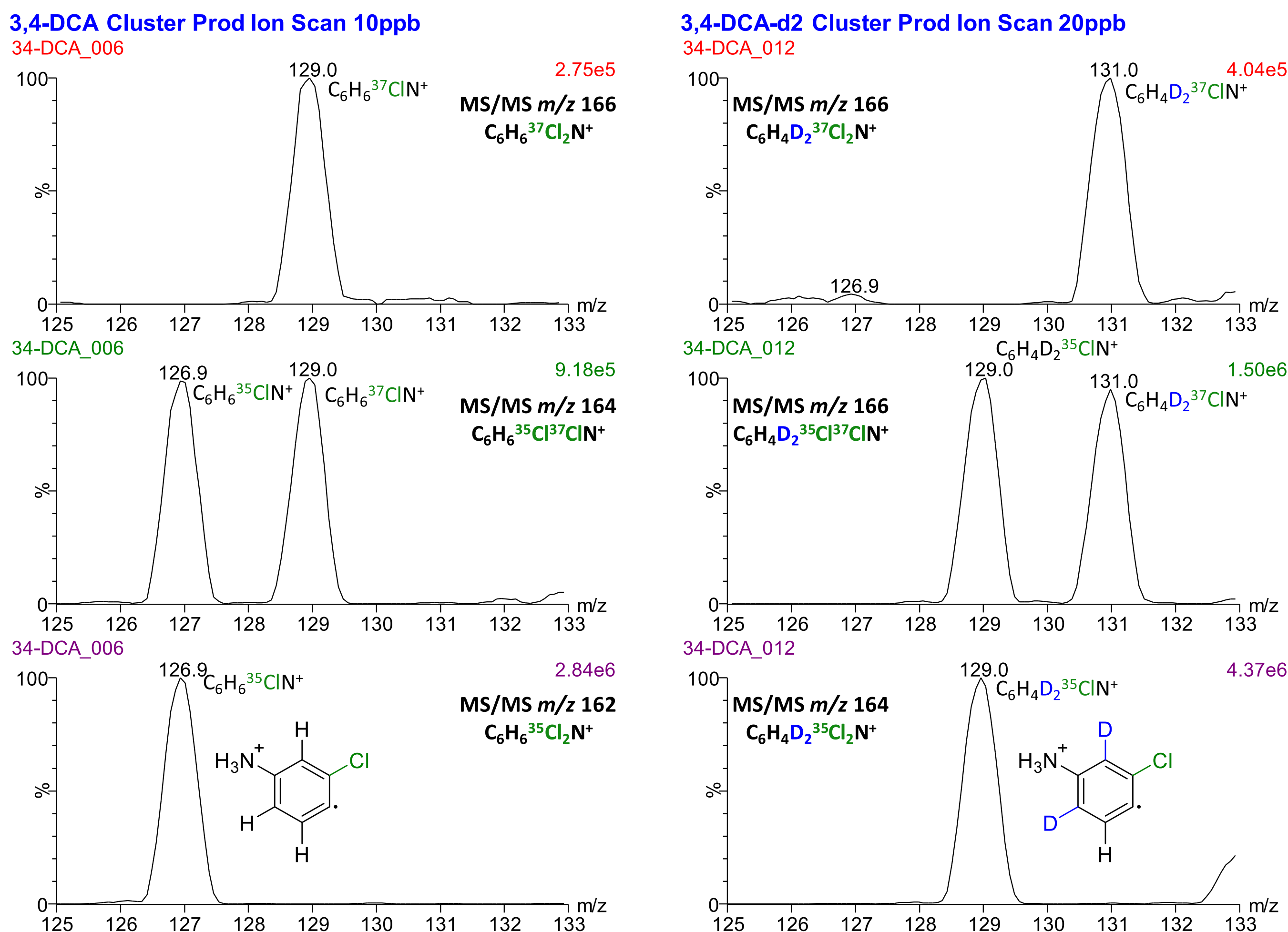

Fig. 3. Experimental product ion scan spectra of the chlorine isotope pattern ions of 3,4-DCA (left) and 3,4-DCA-d_2 (right). Elemental composition indicating isotopes are included in the observed product ions as well as the selected precursor ions.

3.3. Method optimization and validation

After excluding TCPy, 2,4,6-TCP and DCBA, for the above mentioned reasons, method validations were performed for both the SPE and DI procedures using IWW samples from different locations. IWW samples used for validation were previously analyzed and positive findings signals were subtracted from the spiked samples. Although SPE is more labour intensive, lower LOQ levels can generally be reached which is an important advantage considering the low concentration levels normally required for WBE. However, not all pesticide biomarkers were retained by SPE and therefore DI was applied as a complementary approach. Despite that LOQs are generally higher, DI allowed including five important pesticide biomarkers in the overall approach providing valuable information for future WBE studies.

3.3.1. Solid phase extraction

Different cartridges were tested for SPE optimization, from hydrophilic-lipophilic balance polymers (Oasis HLB and Strata-X) to mixed-mode polymeric sorbents, such as cationic and anion exchange based cartridges (MCX and MAX, respectively). Moreover, several amounts of sorbents (3 cc/60 mg and 6 cc/200 mg) were tested. Due to the broad range in the type of compounds, Oasis HLB was the sorbent which provided the best compromise in terms of retention, and the cheaper 3 cc/60 mg sorbent was selected for method validation in IWW as it provided results similar to 6 cc/200 mg cartridges. Table S2 shows satisfactory recoveries for six pesticide biomarkers (2,6-EA; 3,4-DCA; DES; 3-PBA; HCBA and 4-HSA) during the SPE-based method optimization in Milli-Q spiked water. The other compounds (N-DMA; TEB-OH; ASU; 6-CN and Boscalid-OH) were not retained well by the SPE cartridges tested. These biomarkers were analyzed by means of DI, as indicated in section 3.3.2 below.

After these preliminary results, method accuracy was studied for the six compounds in terms of trueness (recovery) and precision (intra-day, expressed as RSD) at two concentrations levels (20 and 200 ng L^{-1}) in five different IWW. The SPE recoveries were evaluated by comparing the signals obtained in a sample spiked before and after the SPE process. Table 3 shows the SPE recoveries (calculated by spiking an IWW sample at 200 ng L^{-1} before and after SPE extraction) and overall method recoveries (including SPE recovery and ME). It is noteworthy that during the evaporation step (after SPE elution), 900 μL of Milli-Q water was added and the sample extracts were evaporated not to complete dryness in order to avoid losses of analyte. When there was only water left in the evaporation tube, 100 μL of MeOH was added for injection into the UHPLC-MS/MS for improving extract solubility and compatibility with the chromatographic separation.

SPE recoveries were satisfactory (ranging from 71 to 121%) for the studied compounds. However, the overall method recoveries were only adequate for three compounds (3,4-DCA and 4-HSA at the two spiked levels; 3-PBA only at the higher level). The fact that validation was performed under the worst-case scenario (*i.e.,* five different real IWW samples instead of five replicates of the same sample) led to high RSDs for most of the compounds. For the three remaining compounds (2,6-EA; DES and HCBA), the average overall recoveries were below 50%. These low recoveries were probably due to an inefficient ME correction *i.e.,* the

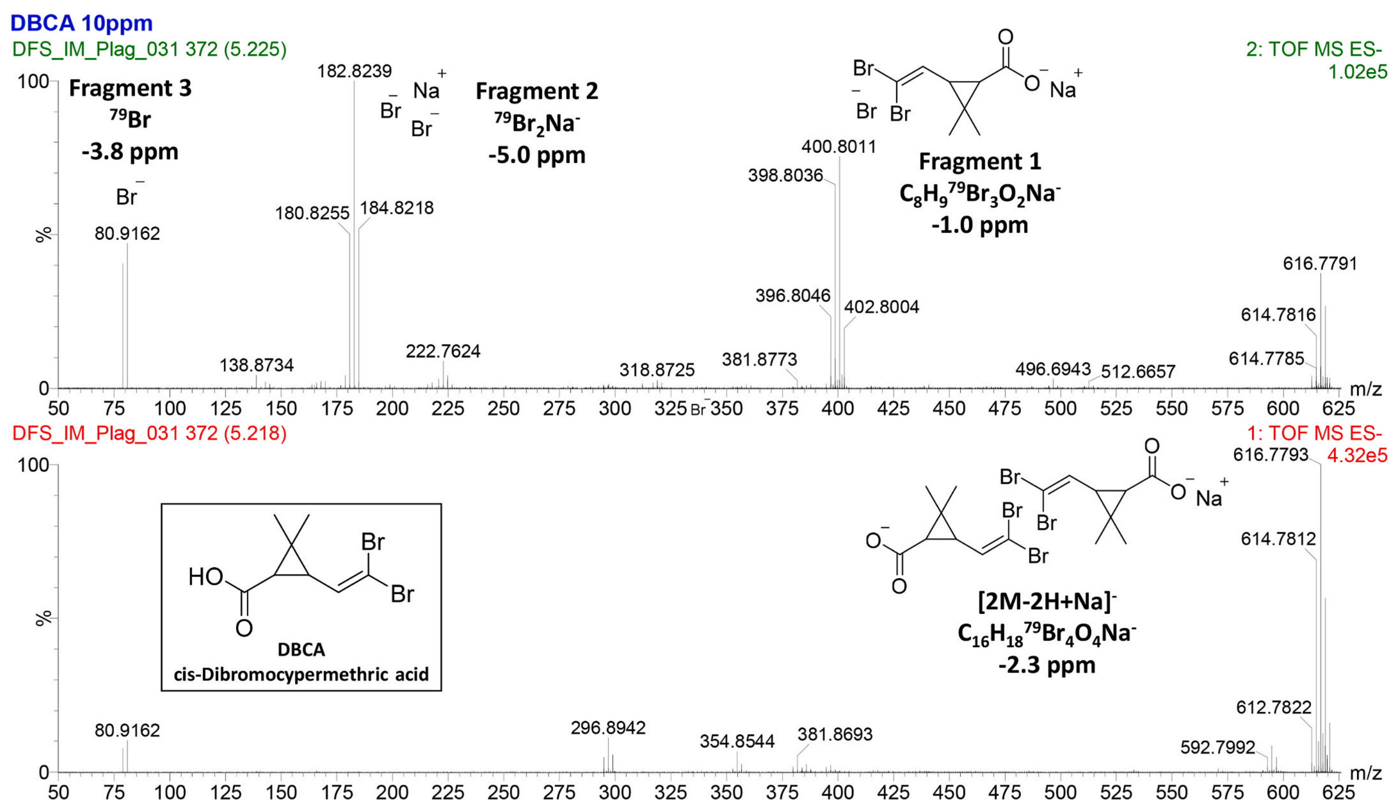

Fig. 4. Full-range accurate-mass spectra of DBCA analytical standard analyzed in ESI negative mode. Information of the intact molecule (bottom spectrum) and its fragments (top spectrum) is included in terms of elemental composition, mass accuracy and structure proposal. Mass errors (in ppm) are calculated using the lightest ion of the isotope pattern, as indicated in the determined elemental composition.

Table 3
Method validation in different IWW samples (n = 5): SPE and overall method recoveries and RSD.

ILIS	Compound	SPE Recoveries (200 ng L^{-1}) (RSD, %)	Method Recoveries (RSD, %)	
			20 ng L^{-1}	200 ng L^{-1}
Thiacloprid-d_4	**2,6-EA**	71 (7)	50 (32)	47 (25)
3,4-DCA-d_2	**3,4-DCA**[a]	80 (14)	77 (41)	71 (37)
Atrazine-d_5	**DES**	116 (25)	–	20 (12)
3-PBA-$^{13}C_6$	**3-PBA**	98 (6)	30 (26)	71 (9)
–	**HCBA**	121 (23)	35 (22)	17 (9)
3-PBA-$^{13}C_6$	**4-HSA**[a]	93 (9)	85 (56)	75 (51)

[a] High variation observed between the IWW matrices used in SPE validation.

unavailability of the analyte ILIS. This hypothesis is supported by the satisfactory data obtained in Milli-Q water and unsatisfactory recoveries in real IWW.

ME were evaluated, in terms of ion suppression or enhancement, by comparing the signal of a sample spiked after the SPE (*i.e.,* spiking the SPE eluate) with the signal of a standard in Milli-Q. All compounds were affected by strong signal suppression (60–99%), which hinders their determination in IWW if an appropriate ILIS is not available to correct the heavy ME (Table 4). As stated above, this strong ME also influences the recoveries of the method when there is no ILIS available, as in the case of 2,6-EA, DES and HCBA.

3.3.2. Direct injection

The DI method was optimized and validated as a complementary method to the SPE as explained above. All pesticide biomarkers were studied, but special attention was paid to those compounds that showed low recoveries when performing SPE. Several filters with different pore sizes were tested (polytetrafluoroethylene (PTFE) 0.22 μm, Nylon 0.22 and Nylon 0.45 μm) as a prior step to injection. In comparison with the centrifugation of the sample, better results were obtained if the samples were only centrifuged before UHPLC-MS/MS injection, as previously described in the literature for some organic compounds analyzed by DI-based methods (Fabregat-Safont et al., 2021). Thus, when performing DI, sample handling was only limited to a centrifugation step and the addition of 100 μL of MeOH containing ILIS.

Table 4
Limits of quantification and detection, linear range and matrix effect of the SPE method.

Compound	Estimated LOQ (ng L^{-1})	Estimated LOD (ng L^{-1})	Linear range (ng L^{-1})	ME (%) (200 ng L^{-1}) (n = 5)
2,6-EA	5	1.5	5–10000	−99 (0.3)
3,4-DCA	0.8	0.2	5–1000	−97 (0.2)
DES	160	50	100–10,000	−98 (0.3)
3-PBA	3	1	5–10,000	−60 (2)
HCBA	40	13	50–10,000	−82 (6)
4-HSA	3	1	5–10,000	−61 (16)

Trueness was calculated in terms of recovery by comparison of the average analyte concentration obtained with the real concentration of the IWW samples ((n = 5 different IWW samples) and spiked at four concentrations levels (5, 10, 50 and 100 ng L^{-1}). Table 5 shows the results obtained in the validation of the DI method. The experimental LOQs were calculated in spiked IWW and ranged from 5 to 50 ng L^{-1}. Only N-DMA and 6-CN recoveries were corrected by an ILIS, despite that their own analyte ILIS were not available; anyway, the correction with analogue ILIS was quite satisfactory with good recoveries. Recoveries for TEB-OH were between 70 and 80% at all concentrations, and for

Table 5
DI method validation in IWW samples: LOQs, LODs, and linear range.

ILIS	Compound	Experimental LOQ (ng L^{-1})	Estimated LOD (ng L^{-1})	Linear range (ng L^{-1})	Method Recoveries (RSD) (%) (n = 5)			
					5 ng L^{-1}	10 ng L^{-1}	50 ng L^{-1}	100 ng L^{-1}
Acetamiprid-d_3	**N-DMA**	50	25	50–10,000	–	–	91 (19)	151 (2)
–	**TEB-OH**	10	3	10–10,000	–	81 (12)	78 (13)	69 (13)
–	**ASU**	5	2	5–10,000	15 (8)	12 (7)	11 (5)	11 (7)
Imidacloprid-d_4	**6-CN**	10	3	10–10,000	–	99 (12)	97 (6)	90 (6)
–	**Boscalid-OH**	50	23	50–10,000	–	–	59 (19)	55 (24)

Boscalid-OH were around 50–60%, without ME correction as the analogue ILIS tested did not worked well. The recoveries for asulam were below 20%, but the RSD was less than 10% in all cases, even though the validation was performed for five different wastewater samples. The good reproducibility allows one to apply a correction factor if this compound was detected in IWW samples.

ME were evaluated by comparing the signal of a spiked sample with the signal of a standard in Milli-Q at 100 ng L^{-1} without ILIS correction. Table S1 shows the ME obtained during the validation in each IWW and the mean ME for each compound. All compounds showed signal suppression (31–89%) and RSDs less than 15% in all cases. The latter is interesting considering that the IWW were of different origin.

3.4. Application to influent wastewater analysis

In order to demonstrate the applicability of the method developed, several IWW samples from WWTP of different countries were analyzed for 11 pesticide biomarkers using the validated procedures, *i.e.,* by DI (5 biomarkers) and SPE (6 biomarkers). Internal quality controls (QC) were included in each sequence of analyses consisting of spiked IWW at three different concentration levels (20, 100 and 200 ng L^{-1}). The results for the QC were in line with the previous data with recoveries between 60 and 140%, with one exception for 2,6-EA with a QC recovery of 50%. No compounds were detected by DI, while up to four pesticide metabolites could be identified in the IWW samples via the SPE-based method, at the concentrations shown in Table 6. The metabolites 4-HSA and 3,4-DCA, were found in 4 out of the 5 samples, and 3-PBA was detected in all samples.

The compounds 4-HSA and 3,4-DCA were found at the highest concentrations. 3,4-DCA is a biodegradation product of the herbicide propanil, phenylurea (diuron, linuron, neburon) and phenylcarbamate pesticides (Yusa et al., 2015). Due to the harmful eco-toxicologial properties of both propanil and 3,4-DCA, this herbicide has been excluded from the list of authorized phytosanitary products in Europe (EFSA, 2013). However, 3,4-DCA has been used as an intermediate in the production of several widely-used herbicides (*e.g.,* diuron, linuron and propanil) and industrial chemicals, such as azo dyes, paints, cosmetics (Brüschweiler et al., 2014; Crossland, 1990). Hence 3,4-DCA is not an exclusive metabolite and humans can be also directly exposed to this compound. Fig. 5 shows a positive of 3,4-DCA in one of the samples analyzed, complying with strict identification and confirmation criteria: retention time agreement, three transitions and the *q/Q* ratio with a deviation below 30% with respect to the reference standard. Moreover, 3-PBA was quantified in all samples in the range 21.5–87.2 ng L^{-1}. 3-PBA is the most frequently measured and detected metabolite of

Table 6
Results obtained in the analysis of five IWW samples by SPE UHPLC-MS/MS.

Compound	Concentration (ng L^{-1})				
	IWW 1	IWW 2	IWW 3	IWW 4	IWW 5
2,6 - EA			<5	<5	
3,4 - DCA	5.0	9.2	3.6	1.4	
3-PBA	87.2	53.5	27.8	47.3	21.5
4-HSA	10.2		6.2	32.2	54.2

pyrethroids and it can be used as a biomarker to assess low-level environmental exposure to pyrethroids (Ueyama et al., 2010). However, 3-PBA might not be the result of human pyrethroids metabolism only, as pyrethroids are also used as household products and in gardening, and may therefore be transformed and end up in the sewer after cleaning or run-off (Chen et al., 2012; Richards et al., 2017).

Future research will focus on the application of the methodology described in this paper to wastewater samples collected in the surrounding of flower bulb fields in the Netherlands to evaluate the human exposure to pesticides, including an overall risk assessment of the data obtained.

4. Conclusions

Advanced analytical methodology based on UHPLC–MS/MS has been developed for the simultaneous quantification and confirmation of specific human biomarkers of pesticides in urban wastewater at trace (ng L^{-1}) level. The overall analytical procedure, based on an off-line SPE step using Oasis HLB cartridges (to measure 2,6-EA; 3,4-DCA; DES; 3-PBA; HCBA and 4-HSA) and DI (to measure N-DMA; TEB-OH; ASU; 6-CN and Boscalid-OH), has been validated with five different wastewater samples at realistic concentration levels. The problems observed for biomarkers of chlorpyriphos/chlorpyriphos-methyl, prochloraz and deltamethrin (TCPy, 2,4,6-TCP and DBCA, respectively), that prevented the inclusion of these compounds in the analytical method, were identified and discussed. Due to the complexity of this type of analysis, special emphasis was placed in the reliable identification of the compounds in samples, which was guaranteed by acquiring 3 SRM transitions per compound and the accomplishment of the retention time and ion ratio deviations. All compounds were affected by matrix effects leading to severe ion suppression. In absence of complex sample treatments, the use of appropriate ILIS was essential for efficient ME correction and accurate quantification. Thus, the use of own-analyte ILIS (if available) is highly recommended, especially when applying DI. In absence of efficient ILIS correction, analyte concentrations in samples might be corrected based on internal quality control samples included in each sequence of analysis, if robust and reproducible recoveries are obtained. The methodology developed was successfully applied to IWW samples from different locations. The metabolites 2,6-EA, 4-HSA, 3,4-DCA and 3-PBA were identified in several samples at quantification levels, illustrating the applicability of the approach for assessing human exposure to pesticides through WBE.

Credit author statement

Marina Campos-Mañas: Writing – original draft; Methodology, Formal analysis; **David Fabregat-Safont:** Writing – original draft; Investigation, Formal analysis; **Félix Hernández**: Conceptualization, Resources, Writing – review & editing; **Eva de Rijke**: Investigation, Writing – review & editing; **Pim de Voogt**: Conceptualization, Writing – review & editing; **Annemarie van Wezel**: Project administration, Funding acquisition, Resources, Writing – review & editing; **Lubertus Bijlsma:** Conceptualization, Supervision, Data curation, Visualization, Writing – original draft

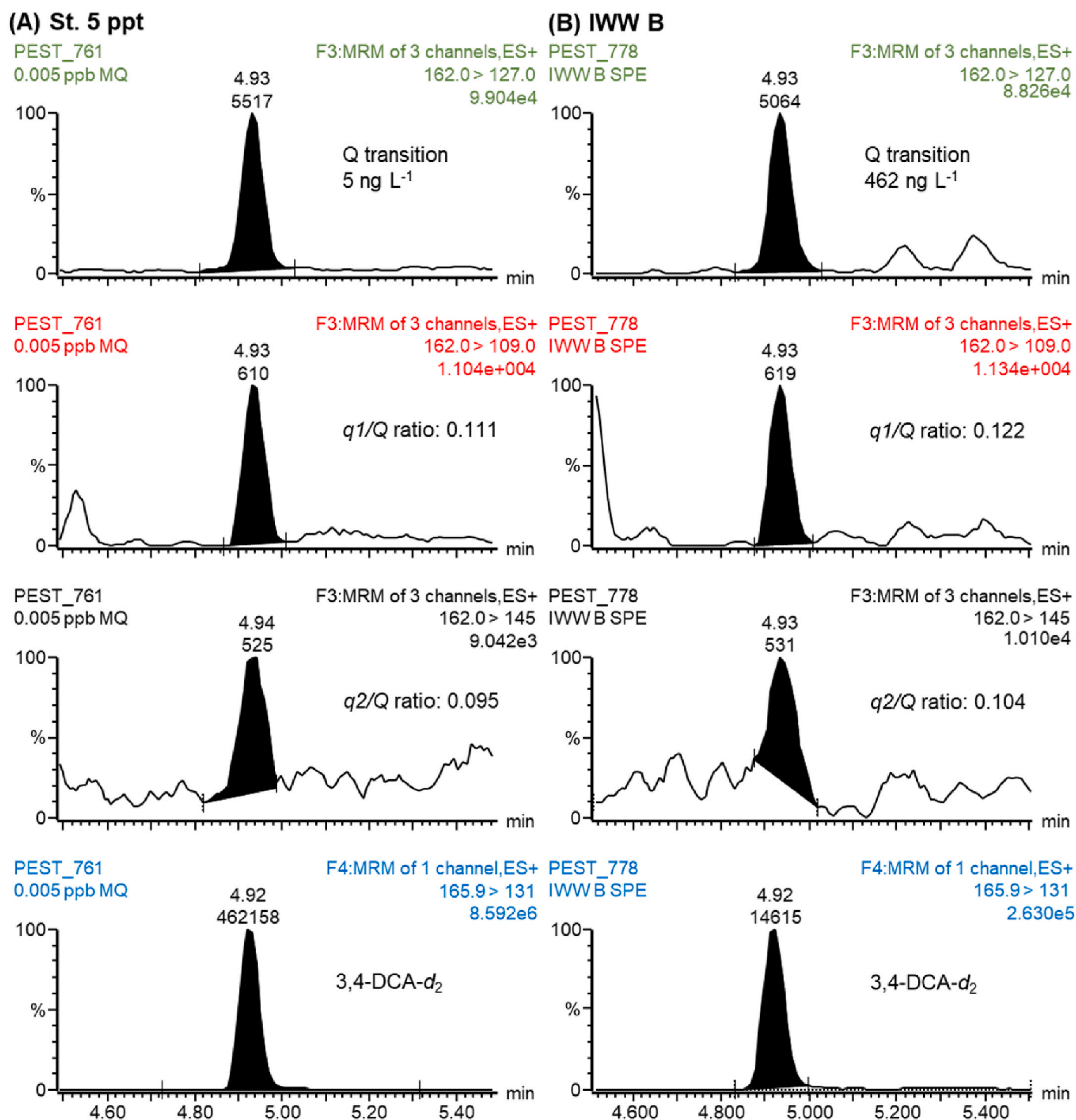

Fig. 5. Identification and quantification of 3,4-DCA in IWW. (A) MS/MS chromatograms for a 5 ng L^{-1} standard. (B) MS/MS chromatograms for the IWW sample (concentration 9.3 ng L^{-1}). Bottom chromatograms for A and B correspond to the ILIS used for this metabolite 3,4-DCA-d_2.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.135684.

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