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SIMULTANEOUS DETERMINATION OF SELECTED PESTICIDES AND/OR THEIR METABOLITES IN URINE BY OFF-LINE SOLID PHASE EXTRACTION AND ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY / HYBRID QUADRUPOLE TIME-OF-FLIGHT MASS SPECTROMETRY

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ABSTRACT

An innovative method for the determination of pesticides of different uses and from different chemical families and/or their known metabolites in human urine was developed. The protocol includes an enzymatic hydrolysis followed by an off-line solid phase extraction (SPE) step and then a targeted quantitative analysis by ultra-high performance liquid chromatography coupled with hybrid quadrupole time-of-flight mass spectrometry (UHPLC/QTOFMS). Still rarely used for this purpose, this technique nevertheless offers excellent quantitative performance with high selectivity, but requires two separate injections for the same sample, one in the negative electrospray ionization mode (ESI-), and the other in the positive mode (ESI+). Two analytical methods were thus developed to cover 40 substances corresponding to 23 different pesticides plus nicotine and cotinine. The first method allows the determination of 15 biomarkers (SPE on a weak anion exchange polymeric support; ESI- mode) and the second 27 (SPE on a reverse phase polymeric support; ESI+ mode) with detection limits for a 2-mL urine sample varying from 0.02 to 25 $\mu\text{g}\cdot\text{L}^{-1}$. Replicate analysis of three pools of urine non-spiked and spiked at an intermediate level on about 20 different days showed most often excellent performance in terms of inter-day precision (< 30%) and accuracy (between 80 and 125%), confirming that the UHPLC/QTOFMS coupling is perfectly adapted to a targeted quantitative analysis. The proposed methods were then applied to fifteen real urine samples, demonstrating the presence of pesticides, predominantly in metabolized form, in the urine of French pregnant women, and the capabilities of the proposed methods to detect them. They were finally applied to a study of the impact of thawing/freezing cycles on the conservation of the target substances, showing for the first time to our knowledge that the thawing/freezing cycles and the composition of urine samples could have an influence on the stability of some of the target substances.

KEYWORDS

High resolution mass spectrometry; enzymatic hydrolysis; quantitative analysis; biomonitoring; exposure biomarker; stability study

1. Introduction

Pesticides are widely used for crop protection, as medicines and biocides to control pest in agriculture and at home, and to prevent molds. Their application can lead to environmental contaminations via soil, water, air or settled dust, resulting in human exposures, as was already shown for some chemical families [1,2]. Most of the pesticides are considered hazardous for human health, particularly in occupational situations, in which increased risks of cancer, developmental defects, or neurologic problems have been observed. In cases of lower dose environmental exposures, malformations, fetal growth impairment and behavioral disorders later in life may be associated to prenatal pesticide exposure [3]. Biomonitoring is interesting for assessing aggregate and cumulative exposure: biomarkers may be indicators of the body burden of chemicals and reflect all routes of exposure, and the measurement of common metabolites may be the reflect of an exposure to an entire chemical family such as organophosphorus pesticides or pyrethroids. Any of the body storage or excretion compartments can serve potentially as a biomonitoring matrix (blood, exhaled air, milk, urine, meconium...). Moreover, urine has the advantage to be easy to collect and in relatively high quantity and is recommended for non-persistent compounds [4]. Non persistent pesticides have a short-life in the human body, so they are found in low concentrations in blood, and consequently urine may be the matrix of choice [5].

The determination of pesticides and/or their metabolites in environmental and biological samples is increasingly carried out using multi-residue approaches [6]. Such approaches are useful for cost reasons, but generally suffer from poorer performance compared to more specific approaches [7,8], especially in terms of sensitivity. Given the physicochemical properties of non-persistent pesticides and their metabolites, liquid chromatography is generally preferred for these analyses, ultra-high performance liquid chromatography (UHPLC) being already widely used for many years in the

field of food or environmental matrices [6] and increasingly popular in the field of biomonitoring [5,9–12]. Combined with liquid chromatography, triple quadrupole tandem mass spectrometry is the technique of choice for quantitative analysis, but high resolution mass spectrometry (HRMS) is progressively becoming a reliable and robust alternative, offering today excellent quantitative performance with high sensitivity and selectivity. To our knowledge, only a few works reported to date the use of UHPLC/HRMS coupling for the simultaneous determination of a wide range of pesticides in urine using a multi-residue approach [13,14].

Furthermore, chemical degradation due to oxidation or hydrolysis influences the composition of a biological sample. This degradation can be physical (temperature, light), biological (microorganisms) but also related to the individual characteristics of the urine (metabolic, nutritional or pathological factors). When biological samples are collected, they are generally aseptically treated with addition of acid and quickly frozen to avoid degradation of targeted substances. They can sometimes be used for different projects and thus undergo several thawing/freezing cycles. But little is known about the potential degradation of targeted substances during these thawing/freezing cycles. The literature mainly mentions studies on the urinary analysis of endogenous metabolites rather than those of xenobiotics. It concludes either that thawing/freezing cycles have no impact on the measurement of endogenous urinary metabolites (glucose, citrate, lactate, PAG, urea, creatinine and albumin after 5 cycles) [15,16], or that inter-individual variability is more important than the variability related to sample preservation [17–20]. In contrast, the pH of urine increases with storage time, which can lead to changes in the concentrations of pH-sensitive substances [21,22]. It is therefore needed to investigate the impact of thawing/freezing cycles on the conservation of pesticides and their metabolites in urine.

In this context, two methods for the targeted quantitative determination in human urine of a wide range of pesticides and/or their metabolites (n=40) of different use (insecticides, herbicides,

fungicides...) and from different chemical families (organophosphorus, pyrethroids, carbamates...) plus nicotine and cotinine were developed to assess human exposure. Methods were based on ultra-high performance liquid chromatography coupled with hybrid quadrupole time-of-flight mass spectrometry (UHPLC/QTOFMS), technique that offers excellent quantitative performance with high selectivity, but requires two separate injections for the same sample, one in the negative electrospray ionization mode (ESI-), and the other in the positive mode (ESI+) because the hybrid quadrupole time-of-flight mass spectrometers are generally not fast enough to work in both modes simultaneously (unlike the triple quadrupole mass spectrometers) and because not all target substances can be optimally detected with the same ionization mode. Target pesticides were selected at a regional scale according to available data on agricultural practices in order to evaluate the exposure of the general population in Brittany. The proposed method was then applied to a study of the impact of thawing/freezing cycles on the conservation of the target substances and finally to fifteen urine samples of French pregnant women from Brittany.

2. Experimental

2.1 Selection of pesticides

The targeted pesticides were selected according to three criteria: i) the potential for exposure, ii) the toxicity, and iii) the analytical feasibility. Given the origin of the urine samples used in this study, a first list was constituted from different surveys on agricultural practices in Brittany (France): a survey on cultivation practices on cereals in 2001 (<https://agreste.agriculture.gouv.fr/agreste-web/>), a survey on pesticide uses on wheat in 2005 (<https://draaf.bretagne.agriculture.gouv.fr/Contacter-le-secteur-Sante-des>), a survey on pesticide uses on wheat and corn crops in 2003-2004, and a cadaster of pesticide emissions into the air carried

out in 2003 from watershed studies. This was completed with the recommendations of the 2005 phytosanitary index and the chambers of agriculture in Brittany. For each pesticide, toxicity data were retrieved from CLP classification and acceptable daily intake if available, and human metabolites were searched in the WHO international program on chemicals safety database (www.inchem.org) and in the literature. The list was then completed with the pesticides that were already found in a previous study conducted on the same samples [23] and the pesticides that contribute the most to French dietary exposures according to total diet studies [24]. Then the final list was established taking into account the compatibility of each substance with the chosen analytical approach. Forty substances corresponding to twenty three pesticides belonging to different chemical families were thus included (parent compounds and/or known metabolites). Nicotine and cotinine were also included to measure smoking status. The final list of forty two targeted substances is presented in Table 1.

2.2 Sample collection

To test both methods, fifteen samples were selected from the PELAGIE mother-child cohort, which includes 3421 pregnant women in Brittany (France) enrolled from general population in early pregnancy between 2002 and 2006. The objective of the PELAGIE study is to assess the consequences of environmental and occupational exposures (solvents, persistent organic pollutants, pesticides...) on the pregnancy, birth outcomes and psychomotor development in infant. A detailed description of this cohort is provided elsewhere [25]. At her inclusion during the first trimester of the pregnancy (4th to 15th week), each woman had to return a first morning void urine sample that she collected and transferred into two vials containing nitric acid to avoid bacterial degradation. Samples were mailed to the study laboratory in a pre-stamped package at ambient

temperature, with routine delivery taking from 1 to 3 days. Upon receipt, samples were frozen and stored at -20 °C.

In the PELAGIE cohort, gynecologists informed women about the nature of the study and asked them to participate, after providing written consent. This consent was accompanied by a letter of information describing the goal of the study, the consortium, data collection procedures, follow-up after birth through mailed questionnaires or health examinations. Reference (Nu902076; 31 may 2002) to the approval of the National Commission in charge of Data Protection (CNIL) was also indicated. The right to refuse participation and the fact that this refusal would not have any consequence on the relation with her physician was explicitly mentioned.

2.3 Reagents and chemicals

Acetonitrile and methanol (ULC/MS grade) were purchased from Biosolve Chimie (Dieuze, France). Acetone (PESTIPUR-For pesticide analysis) and glacial acetic acid (for LC/MS) was purchased from CARLO ERBA Reagents S.A.S (Val de Reuil, France). Ammonium hydroxide (ACS reagent grade), 28-30% solution in water, was purchased from Fisher Scientific S.A.S. (Illkirch, France). Ammonium acetate for analysis EMSURE[®] ACS (Reagent European Pharmacopoeia), potassium hydroxide solution 1 N Tritipur[®] (Reagent European Pharmacopoeia) and potassium dihydrogen phosphate for analysis EMSURE[®] ACS (Reagent European Pharmacopoeia) were purchased from Merck KGaA (Darmstadt, Germany). Nitric acid 67-69% NORMATOM[®] were purchased from VWR International (Radnor, PA, USA). Anhydrous sodium acetate salt, β -glucuronidase from limpets (*Patella vulgata*) and sulfatase from *Helix pomatia* were purchased from Sigma Aldrich (Saint Louis, MO, USA). Strata-X and Strata-X-AW cartridges (200 mg, 6 mL) were purchased from Phenomenex France (Le Pecq, France).

Certified standards of 1-(3,4-dichlorophenyl)-3-methylurea (DCPMU), 1-(3,4-dichlorophenyl)urea (DCPU), 1-(4-isopropylphenyl)-3-methylurea (IPPMU), 2-methyl-6-ethylaniline, 2-phenylphenol, 2,4,6-trichlorophenoxyacetic acid (2,4,6-T), 3-chloroaniline, 3-phenoxybenzoic acid (3PBA), 3,5-dichloroaniline, 3,5,6-trichloro-2-pyridinol (TCPy), 6-chloronicotinic acid, acetochlor, azoxystrobin, bromoxynil, carbendazim, carbofuran, carbofuran phenol, chlorpropham, chlorpyrifos-ethyl, chlorpyrifos-methyl, dichlorvos, dimethoate, fenpropimorph, iprodione, isoproturon, malathion, nicotine, phorate and quizalofop (free acid) were purchased from LGC Labor GmbH (Augsburg, Germany). Standards of 2-(4-hydroxyphenoxy)-propionic acid and diethylthiophosphate (DETP) were purchased from Sigma Aldrich (Saint Louis, MO, USA). Standards of 2-isopropyl-6-methyl-4-pyrimidinol (IMPy) and diethylphosphate (DEP) were purchased from Chem Service Inc. (West Chester, PA, USA). Standards of 2-amino-5,6-dimethyl-4-hydroxypyrimidine (ADHP), dimethyldithiophosphate (DMDTP) and cotinine were purchased from Alfa Aesar (Heysham, UK). Standard of dimethylphosphate (DMP) was purchased from Fisher Scientific S.A.S. (Illkirch, France). Standard of methyl-2-(2-hydroxyphenyl)-3-methylacrylate was obtained by donation. The purity of standards was above 95%. Individual standard stock solutions (1 g.L^{-1}) were prepared in acetone, methanol or acetonitrile by accurately weighing 5, 10 or 25 mg ($\pm 0.1 \text{ mg}$) of standards using a Sartorius Cubis MSE 225P semi-micro balance (Sartorius AG, Göttingen, Germany) into 5-, 10- or 25-mL volumetric flasks, depending on the compound, and stored at $-18 \text{ }^{\circ}\text{C}$. Acetonitrile solution (100 mg.L^{-1}) of fluazifop (free acid) was purchased from LGC Labor GmbH (Augsburg, Germany). Acetonitrile solutions of cis/trans 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (DCCA) (100 mg.L^{-1}), 4-fluoro-3-phenoxybenzoic acid (4F3PBA) (100 mg.L^{-1}) and dimethylthiophosphate (DMTP) (1 g.L^{-1}) were purchased from Cambridge Isotope

Laboratories, Inc. (Andover, MA, USA). Calibration solutions were prepared in methanol by appropriate dilution of individual standard stock solutions and commercial solutions.

Nonane solutions of bromoxynil $^{13}\text{C}_6$ (50 mg.L $^{-1}$) and 3PBA $^{13}\text{C}_6$ (100 mg.L $^{-1}$), MTBE solution (100 mg.L $^{-1}$) of 6-chloronicotinic acid $^{13}\text{C}_6$ and acetonitrile solutions (100 mg.L $^{-1}$) of 4F3PBA $^{13}\text{C}_6$, trans-DCCA $^{13}\text{C}_2$ D $_1$, TCPy $^{13}\text{C}_3$, 2,4-dichlorophenoxyacetic acid $^{13}\text{C}_6$ and phorate $^{13}\text{C}_4$ were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Acetone solutions (100 mg.L $^{-1}$) of quizalofop (free acid) D $_3$, dimethoate D $_6$, acetochlor D $_{11}$, diazinon D $_{10}$, chlorpyrifos-methyl D $_6$, chlorpyrifos-ethyl D $_{10}$ and isoproturon D $_6$, acetonitrile solutions (100 mg.L $^{-1}$) of mecoprop D $_6$ and iprodione D $_7$, and cyclohexane solution (100 mg.L $^{-1}$) of dichlorvos D $_6$ were purchased from LGC Labor GmbH (Augsburg, Germany). Methanol solutions (100 mg.L $^{-1}$) of DEP $^{13}\text{C}_2$, DETP $^{13}\text{C}_2$, DMP $^{13}\text{C}_2$, DMTP $^{13}\text{C}_2$ and DMDTP $^{13}\text{C}_2$ were purchased from Chiron AS (Trondheim, Norway). Acetonitrile solution (100 mg.L $^{-1}$) of nicotine D $_4$ was purchased from Cerilliant Corporation (Round Rock, TX, USA). Methanol solution (100 mg.L $^{-1}$) of cotinine D $_3$ was purchased from LGC GmbH (Luckenwalde, Germany). Acetone solution (100 mg.L $^{-1}$) of carbendazim D $_4$ and acetonitrile solution (100 mg.L $^{-1}$) of 3-chloroaniline D $_3$ were purchased from A2S - Analytical Standard Solutions (Saint Jean d'Ilac, France). Standard of 2-methyl-6-ethylaniline D $_{13}$ was purchased from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada). Standard of azoxystrobin D $_4$ was purchased from Toronto Research Chemicals (Toronto, ON, Canada).

Pooled urine samples for optimization experiments, method validation and quality controls were obtained by anonymous donation. SurineTM Negative Urine Control was purchased from Sigma Aldrich (Saint Louis, MO, USA).

2.4 Sample preparation (enzymatic hydrolysis)

After adding the labelled internal standards (ISTDs) and adjusting the pH of the urine sample to pH 5 in order to ensure maximum activity for each enzyme, 2 mL of a 0.2 M sodium acetate buffer at pH 4.8 were added to 2 mL of urine. Then, 200 μL of a solution of β -glucuronidase from limpets (*Patella vulgata*) ($25 \text{ g}\cdot\text{L}^{-1} / 25 \text{ units}\cdot\mu\text{L}^{-1}$) and sulfatase from *Helix pomatia* ($2.5 \text{ g}\cdot\text{L}^{-1} / 0.025 \text{ units}\cdot\mu\text{L}^{-1}$) prepared in a 0.2 M sodium acetate buffer at pH 4.8 were added and the sample was subsequently incubated for 2 hours at 50 °C. After return to room temperature, 16 mL of a 100 mM phosphate buffer at pH 6 were added to reach a final volume of 20 mL.

2.5 Solid phase extraction (SPE)

Two SPE methods were used to fully cover the list of target substances, differing by the nature of the phase and the washing and elution conditions of the cartridge. They are described below and in Table 2. Extractions were performed using a Gilson GX-274 ASPEC automatic extraction system (Gilson, Middleton, WI, USA).

2.5.1 Method n°1

The Strata-X-AW cartridge was conditioned with successively 10 mL of MeOH, 10 mL of a 95% ACN / 5% MeOH mixture and 10 mL of ultra-pure water. Then, the pre-treated urine sample (20 mL) was loaded. The cartridge was washed with successively 5 mL of a 25 mM ammonium acetate buffer (pH 6-7) and 5 mL of a 95% ACN / 5% MeOH mixture and subsequently completely dried. Analytes were eluted with 4 x 2.5 mL of a 95% ACN / 5% MeOH + 5% NH_4OH mixture. Organic extracts were evaporated almost to dryness under a nitrogen stream using a N-EVAP 111 Organomation Nitrogen Evaporators and reconstituted in 500 μL of the mobile phase under the

initial conditions of the gradient (90% ultra-pure water / 10% methanol mixture acidified with 0.01% formic acid) prior to be transferred into a 2 mL clear glass vial.

2.5.2 Method n°2

The Strata-X cartridge was conditioned with successively 10 mL of MeOH, 10 mL of a 95% ACN / 5% MeOH mixture and 10 mL of ultra-pure water. Then, the pre-treated urine sample (20 mL) was loaded. The cartridge was washed with 2 x 5 mL of ultra-pure water and subsequently completely dried. Analytes were eluted with 4 x 2.5 mL of a 95% ACN / 5% MeOH mixture. Organic extracts were evaporated almost to dryness under a nitrogen stream using a N-EVAP 111 Organomation Nitrogen Evaporators and reconstituted in 500 μ L of the mobile phase under the initial conditions of the gradient (90% ultra-pure water / 10% methanol mixture acidified with 0.01% formic acid) prior to be transferred into a 2 mL clear glass vial.

2.6 UHPLC/HRMS analysis

Analyses were performed using a SCIEX ExionLCTM AD / SCIEX X500R QTOF UHPLC/HRMS system (AB Sciex, Framingham, MA, USA) equipped with the TurboIonSpray ion source and operated in the negative ESI mode with the method n°1 and in the positive ESI mode with the method n°2. Chromatographic separation was performed at a flow rate of 0.3 mL min⁻¹ on a Waters Acquity UPLC HSS T3 column (150 mm length x 2.1 mm I.D., 1.8 μ m particle size) maintained at a constant temperature of 45 °C. The binary mobile phase was composed of ultra-pure water (solvent A) and methanol (solvent B), both acidified with 0.01% formic acid. The sample injection volume was 10 μ L. The gradient elution program was as follows: 0-1.5 min, 90% A; 1.5-15 min, 90-0% A; 15-17 min, 0% A; 17-17.1 min, 0-90% A (return to initial conditions); 17.1-20 min, 90% A (equilibration). The ion source parameters were as follows: ionspray voltage, 4.5 kV for the

negative ESI mode and 5.5 kV for the positive ESI mode; curtain gas (nitrogen, Air Liquide) pressure, 35 psi; ion source gas 1 and 2 (nitrogen, Air Liquide) pressure, 50 and 70 psi, respectively; desolvation temperature, 550 °C. The mass spectrometer was operated in the high resolution multiple reaction monitoring (MRM^{HR}) mode. The two most sensitive and specific transitions were monitored for each compound using positive or negative ESI mode depending on the method. Accumulation time was 30 msec. Analytical characteristics of measured compounds are summarized in Table 3 (method n°1) and in Table 4 (method n°2). SCIEX OS software (1.2) was used for instrument control, data acquisition and quantification.

2.7 Validation

The limits of detection (LODs) are defined as the lowest concentration of a substance that can be distinguished from the absence of that substance. LODs were estimated from the replicate analysis of a blank sample. Limits of quantification (LOQs) were defined as the lowest concentration of a substance for which the relative standard deviation (RSD) of the raw signal (n = 5) was lower than or equal to 20%, the signal-to-noise ratio (S/N) was greater than or equal to 10, and the raw signal was greater than or equal to 5 times the signal of the blank sample.

Matrix interference can affect the detection of pesticides in urine samples due to the presence of non-target compounds that may react with the target analytes or that may be similar to and co-elute with the target analytes. To control internal accuracy and precision and identify possible interference due to the matrix, spiking experiments were conducted on the three pools of urine available at the lab (pools #1, #2 and #3 for the method n°1, and pools #2 and #3 for the method n°2). Inter-day method accuracy and precision were assessed via replicate analysis of these pools non-spiked and spiked at an intermediate level on 20 different days for the method n°1 and 17

different days for the method n°2. Measured concentrations (arithmetic mean) were compared to theoretical concentrations, and method precision was defined as the RSD of the replicates.

The proposed method was then applied to fifteen urine samples of pregnant women.

2.8 Study of the impact of thawing/freezing cycles on the conservation of the target substances

The real urine samples used for this work were previously aliquoted for other research projects and were thus not all thawed the same number of times (one to three times). This study of the impact of thawing/freezing cycles (-25°C, three cycles) on the conservation of the target substances was carried out on two urine pools (pools #1 and #3) using plastic tubes such as those in which the urine samples were stored. These 2 urine pools were stored in 10 mL brown glass tubes (5 mL aliquots) and were acidified before freezing (addition of nitric acid). The study was conducted according to the following schedule:

Step 1: i) Both urine pools were thawed, ii) 9 mL of each of the two urine pools were dispatched in three 10 mL plastic tubes for each of the two methods (i.e. 12 tubes), iii) each tube was spiked at an intermediate concentration level using spiking solutions prepared in methanol and then homogenized, iv) 2 mL of urine from each tube were sampled and the first extractions (T_0) were performed with both methods, and v) the 12 tubes were immediately refrozen after sampling;

Steps 2, 3 and 4: i) The 12 tubes were thawed (3 hours at room temperature on the bench), ii) 2 mL of urine from each tube were sampled and extractions (1st, 2nd and 3rd cycles) were performed with both methods, and iii) the 12 tubes were immediately refrozen after sampling (except for step 4);

Step 5: All the organic extracts were evaporated, reconstituted and injected at the same time.

A total of 78 analyses were performed for this study in 8 days (48 urine samples, 16 procedural calibration samples, 8 procedural blank samples and 6 procedural matrix QC samples).

2.9 Quality Assurance and Quality Control (QA/QC)

Several labeled substances were selected to best cover the physical and chemical properties of the targeted analytes and were added prior to the extraction step and used as internal standards (ISTDs). All the compounds were quantified using the appropriate ISTD to compensate for the variability associated with the SPE-UHPLC/HRMS analysis, from calibration curves generated for each compound by analyzing at least five different calibration samples. A quadratic fit (origin ignored, no weighting) was used to compensate for the nonlinearity of the instrument response over a wide working range [26].

Each batch included: i) eight procedural calibration samples and one procedural calibration blank sample prepared from a real urinary matrix (pooled urine sample) and analyzed as regular samples to generate quadratic calibration curves intended for quantification and to assess whether contamination may have occurred during analysis, respectively, ii) several procedural calibration samples analyzed at the beginning, throughout and at the end of the batch to check for the stability of the detector response, and iii) two procedural matrix QC samples (2-mL real urine sample non-spiked and spiked at an intermediate level) analyzed as regular samples to check for method accuracy.

Positive values for each substance were confirmed by comparing retention times and MRM^{HR} transitions ratios between calibration samples and urine samples. The data validation protocol included several conditions: i) the determination coefficient of the calibration curve had to be greater than 0.995, ii) the response of a substance (ISTD response ratio) in the procedural calibration blank sample had to be lower than 60% of that in the calibration sample at the LOQ level, iii) the concentration of a substance measured in the procedural calibration samples analyzed at the beginning, throughout and at the end of the batch had to be within $\pm 25\%$ of its theoretical concentration value, iv) the concentration of a substance measured in the procedural calibration

samples had to be within $\pm 50\%$ of its theoretical concentration value at the LOQ level and $\pm 25\%$ at all other levels, and v) the concentration of a substance measured in the procedural matrix QC sample spiked at an intermediate level had to be within $\pm 30\%$ of its theoretical concentration value. If all these conditions were not met, results were not validated and samples were reanalyzed if possible.

3. Results and discussion

3.1 Optimization experiments

Optimization experiments focused mainly on the extraction step. The first extraction experiments were carried out on a partial list of 33 substances (see Table S1 in the supplementary material) on Evian water spiked at $10 \mu\text{g}\cdot\text{L}^{-1}$ at pH 2 and 7 with Phenomenex Strata-X and Waters Oasis HLB cartridges ($n = 3$ for each condition). Cartridges were conditioned with successively 10 mL of a 60% DCM / 40% ACN mixture, 10 mL of MeOH and 10 mL of ultra-pure water at pH 2 or 7, and analytes eluted with 10 mL of a 60% DCM / 40% ACN mixture. The results are presented in Table S1 in the supplementary material. Overall, the recovery rates and RSDs were better with the Strata-X cartridges under these conditions. Therefore, Strata-X cartridges were selected for the continuation of these experiments. Regarding the pH, the results were globally better at pH 2 except for some substances like nicotine, cotinine and some anilines and organophosphorus compounds. In addition, some substances were not extracted at all regardless of the pH or the type of cartridge. This was the case of DAPs in particular. On the basis of these first results, it seemed impossible to extract all the targeted substances with a single extraction method. At this stage, at least two extraction methods seemed necessary, the first with Strata-X cartridges at neutral or acidic pH and

the second with cartridges capable of retaining DAPs such as those made of a weak anion-exchange functionalized polymeric sorbent that allows for retention of acidic compounds.

The following experiments aimed at testing such cartridges to extract DAPs, acidic compounds (2-(4-hydroxyphenoxy)propanoic acid, 6-chloronicotinic acid, 2,4,6-T, DCCA, 3PBA, 4F3PBA, fluazifop (free acid) and quizalofop (free acid)) and other substances likely to be retained (bromoxynil and TCPy). Waters Oasis WAX and Phenomenex Strata-X-AW cartridges were tested on Evian water. The Oasis WAX cartridge was conditioned with successively 5 mL of MeOH and 5 mL of acidified ultra-pure water (2% formic acid). Then, the Evian water acidified with H₃PO₄ (2%) and spiked at 1 µg.L⁻¹ was loaded. The cartridge was washed with successively 5 mL of acidified ultra-pure water (2% formic acid) and 5 mL of MeOH and subsequently completely dried. Analytes were eluted with 2 x 5 mL of a 95% MeOH / 5% NH₄OH mixture. The Strata-X-AW cartridge was conditioned with successively 5 mL of MeOH and 5 mL of ultra-pure water at pH 6-7. Then, the Evian water adjusted at pH 6-7 and spiked at 1 µg.L⁻¹ was loaded. The cartridge was washed with successively 5 mL of a 25 mM ammonium acetate buffer (pH 6-7) and 5 mL of MeOH and subsequently completely dried. Analytes were eluted with 2 x 5 mL of a 95% MeOH / 5% NH₄OH mixture. These experiments were also conducted on both types of cartridges without the MeOH washing step (n = 3 for each condition). The results are presented in Table S2 in the supplementary material. Recovery rates and RSDs were globally better under these conditions with the Strata-X-AW cartridges that were therefore selected for the continuation of this study. Recovery rates were excellent for the acidic compounds, bromoxynil and TCPy (above 75%) but globally low or very low for DAPs (2-3% for DMP, 10% for DEP, 30% for DMTP and 70% for DETP). In addition, the results were comparable with and without the MeOH washing step which was therefore integrated to the method.

The following experiments aimed at optimizing the extraction conditions to improve recovery rates for DAPs without degrading them for all other substances. These tests made it possible to define the most suitable solvent mixture for the elution step of both methods, i.e. 95% ACN / 5% MeOH (+ 5% NH₄OH with the Strata-X-AW cartridge). However, despite extensive experiments on the conditioning, washing and eluting steps (pH of the sample and nature and volume of solvents or solvent mixtures), recovery rates remained low for DAPs, especially for DMP and DEP. Labeled analogues were thus used as ISTD for each DAP to compensate for these low recovery rates and LODs/LOQs in matrix were set accordingly.

In the end, two extraction methods were developed to cover the list of compounds of interest, one on Strata-X-AW cartridges for acidic compounds, and the other on Strata-X cartridges for the other compounds. Thereafter, as the large majority of the compounds extracted on the Strata-X-AW cartridges could be ionized in the ESI⁻ mode and the large majority of the compounds extracted with the Strata-X cartridges could be ionized in the ESI⁺ mode, it was possible to group all the compounds compatible with an extraction on Strata-X-AW cartridges and an ionization in the ESI⁻ mode in a first method (method n°1) and all the compounds compatible with an extraction on the Strata-X cartridges and an ionization in the ESI⁺ mode in a second method (method n°2), thus avoiding injecting each organic extract twice, once in the ESI⁻ mode and once in the ESI⁺ mode (the hybrid quadrupole time-of-flight mass spectrometer used for this work was not fast enough to work in both modes simultaneously). Finally, methods n°1 and n°2 allowed the determination of 15 and 27 compounds respectively (see Table 3 and Table 4). Furthermore, nine substances could not be integrated during the development phase into either of the two methods developed for various reasons: i) fluctuation of the retention time and deformation of the chromatographic peak depending on the matrix and the concentration level for DEDTP, ii) incompatibility with the two extraction protocols developed on the Phenomenex Strata-X and Strata-X-AW cartridges for 2-

aminobenzimidazole (2-AB), 2,5-dihydroxybiphenyl, 4-hydroxypyrimidine (4-HP), ethylene thiourea (ETU), malathion dicarboxylic acid (MDCA), 3-acetamidophenol and 2-methyl-2-phenylpropanoic acid, and iii) interference between the parent compound and its metabolite for diazinon. These nine substances are therefore not mentioned in the rest of the article.

Finally, the last important step consisted in defining the most appropriate mode of quantification, taking into account i) significant matrix effects (ion suppression) not compensated by the use of a labelled analogue as ISTD for some substances, and ii) differences in retention time and peak shape in presence of matrix for some DAPs. Several solutions were tested, all based on a quantification from calibration curves generated for each compound by analyzing at least five different calibration samples prepared: i) from ultra-pure water, ii) from a synthetic urinary matrix (SurineTM Negative Urine Control), or iii) from a real urinary matrix (pooled urine sample). The best results were obtained with the real urinary matrix, the synthetic urinary matrix giving results very close to those of ultra-pure water. Calibration samples were thus prepared from 2 mL of the urine pool #1 for the method n°2 and from only 100 µL of the urine pool #2 plus 1.9 mL of ultra-pure water for the method n°1 because it was impossible to obtain a real urine sample not contaminated with DAPs.

3.2 Method validation and application

3.2.1 Assessment of the accuracy and precision of both methods from spiking experiments

Inter-day method accuracy and precision were assessed via replicate analysis of the three pools of urine available at the lab (pools #1, #2 and #3 for the method n°1, and pools #2 and #3 for the method n°2) non-spiked and spiked at an intermediate level on 20 different days for the method n°1 and 17 different days for the method n°2. The results are presented in Table S3 (method n°1) and in Table S4 (method n°2) in the supplementary material and illustrated in Figure 1.

Overall, measured concentrations were in very good agreement with theoretical concentrations with inter-day accuracy ranging from 84 (chlorpropham) to 124% (methyl-2-(2-hydroxyphenyl)-3-methylacrylate), except for carbofuran (168%), carbofuran phenol (522%) and fenpropimorph (154%). Moreover, the method showed good inter-day precision, with RSD most often much lower than 30%, except for ADHP (49%), methyl-2-(2-hydroxyphenyl)-3-methylacrylate (34%), carbofuran (36%), carbofuran phenol (37%), fenpropimorph (33%) and 2-phenylphenol (32%). 2-Phenylphenol was quantified without ISTD, while the other five compounds (ADHP, methyl-2-(2-hydroxyphenyl)-3-methylacrylate, carbofuran, carbofuran phenol and fenpropimorph) were quantified using an ISTD to compensate for the variability associated with the SPE-UHPLC/HRMS analysis but not a labeled analogue because it was not commercially available. The ISTD chosen for these compounds from the labeled compounds included in the method (nicotine D₄ for ADHP, isoproturon D₆ for methyl-2-(2-hydroxyphenyl)-3-methylacrylate and carbofuran phenol, cotinine D₃ for carbofuran, and diazinon D₁₀ for fenpropimorph) probably did not perfectly cover their physical and chemical properties, which may explain the slightly poorer results for these compounds.

3.2.2 Study of the impact of the enzymatic hydrolysis on the quantitative analysis of the target substances

Experiments were carried out with the method n°1 only on two urine pools (pools #1 and #2). Both non-spiked urine pools were analyzed with and without enzymatic hydrolysis to assess the impact of this step on the quantitative analysis of the target substances. The results are detailed in Table S5 in the supplementary material. Overall, the results obtained with and without enzymatic hydrolysis were very close for all substances except for 3PBA and TCPy that were quantified in both urine pools with enzymatic hydrolysis, but not detected without. These results suggest that

these two substances were predominantly present in both urine pools in their conjugated forms (glucuronide and/or sulfate conjugates), and that the enzymatic hydrolysis step was therefore essential for the determination of their total concentration (concentration of free and conjugated forms). Further experiments should be conducted on a wider list of substances to confirm these results and especially to assess the efficiency of the enzymatic deconjugation step.

3.2.3 Study of the impact of thawing/freezing cycles on the conservation of the target substances

The results of this study are summarized in Table S6 in the supplementary material. These results indicates a potentially low impact of thawing/freezing cycles for most substances of interest. Indeed, the variation in urinary concentrations for these substances remains systematically lower than the tolerance accepted during this analysis campaign ($\pm 30\%$) on the accuracy at the concentration level considered, even if it is sometimes possible to identify downward or upward trends for certain substances.

In contrast, for chlorpyrifos (methyl and ethyl) and phorate, there is a clear decrease in urinary concentrations when the sample is thawed and then refrozen several times, and this from the first thawing/freezing cycle for chlorpyrifos-ethyl, as shown in Figure 2. Thus, thawing/freezing cycles could have an influence on the preservation of these molecules and therefore on the exploitation of results. It should be noted that these first results are sample-dependent, as chlorpyrifos did not behave in the same way in the two urine samples, probably due to a difference in composition between the two samples. The composition of an urine sample could therefore have an influence on the stability of certain substances against thawing/freezing cycles, and probably also on the stability of these same molecules over time.

3.2.4 Application to fifteen human urine samples

Both methods were applied to fifteen urine samples (noted as sample #1 to sample #15) collected from French pregnant women between 2002 and 2006. The results are detailed in Table S7 (method n°1) and in Table S8 (method n°2) in the supplementary material and summarized in Table 5. DEP, DETP and TCPy were detected in all samples, 8 other compounds (3,5-dichloroaniline, DMP, DMTP, DMDTP, 2-(4-hydroxyphenoxy)propanoic acid, 2,4,6-T, fluazifop (free acid) and 3PBA) in more than 30% of the samples, and 25 of the 42 target compounds in at least one sample. The following 17 compounds were never detected: nicotine, ADHP, dimethoate, methyl-2-(2-hydroxyphenyl)-3-methylacrylate, dichlorvos, 2-methyl-6-ethylaniline, carbofuran phenol, DCPU, fenpropimorph, IPPMU, DCPMU, isoproturon, chlorpropham, malathion, iprodione, acetochlor and 6-chloronicotinic acid. Concentrations were widely varied, ranging from a few tens of ng.L^{-1} to more than $100 \mu\text{g.L}^{-1}$ (DMP and 2-phenylphenol). The 17 compounds detected in more than 20% of the samples were metabolites with the exception of bromoxynil and carbendazim (carbendazim is an active substance but also a metabolite of benomyl and thiophanate-methyl). This confirms that parent compounds are generally not the most relevant compounds to measure in urine. These results demonstrate the presence of pesticides, predominantly in metabolized form, in the urine of French pregnant woman and confirm the capabilities of the proposed methods to detect them.

4. Conclusions

The results obtained with the proposed methods confirm that the UHPLC/QTOFMS coupling is perfectly adapted to a targeted quantitative analysis in human urine of a large panel of pesticides. The gap in sensitivity of the QTOFMS systems compared to the most sensitive triple quadrupole

systems is reduced with each new generation and this gap is partially compensated by a gain in specificity depending on the matrix and the compounds considered. However, these systems remain more expensive and always require two injections of the same sample (ESI- and +). The application of the proposed methods to real human urine samples moreover demonstrated the presence of pesticides in the urine of French pregnant woman, predominantly in their metabolized form. The results of the present study also demonstrated that the enzymatic hydrolysis step was essential for the determination of the total concentration (concentration of free and conjugated forms) of the substances that are predominantly present in urine in their conjugated forms (glucuronide and/or sulfate conjugates). Further experiments should therefore be conducted to control the efficiency of this deconjugation step but the means available to laboratories are still limited today, whatever the method used, in particular because the conjugated forms are not commercially available for a large majority of substances of interest. Finally, this study showed that the thawing/freezing cycles and the composition of urine samples could have an influence on the stability of a few substances, which prompts further studies to be carried out.

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Table 1

Final list of target compounds.

Target compounds	Parent compounds
Chlorpyrifos-ethyl	<i>Chlorpyrifos-ethyl</i>
Chlorpyrifos-methyl	<i>Chlorpyrifos-methyl</i>
3,5,6-Trichloro-2-pyridinol (TCPy)	<i>Chlorpyrifos-methyl and ethyl</i>
Dichlorvos	<i>Dichlorvos</i>
2-Isopropyl-6-methyl-4-pyrimidinol (IMPy)	<i>Diazinon</i>
Malathion	<i>Malathion</i>
Dimethoate	<i>Dimethoate</i>
Phorate	<i>Phorate</i>
Dimethylphosphate (DMP)	<i>Organophosphorus pesticides</i>
Dimethylthiophosphate (DMTP)	<i>Organophosphorus pesticides</i>
Dimethyldithiophosphate (DMDTP)	<i>Organophosphorus pesticides</i>
Diethylphosphate (DEP)	<i>Organophosphorus pesticides</i>
Diethylthiophosphate (DETP)	<i>Organophosphorus pesticides</i>
Carbendazim	<i>Carbendazim and benomyl</i>
Carbofuran	<i>Carbofuran, carbosulfan and benfuracarb</i>
Carbofuran phenol	<i>Carbofuran, carbosulfan and benfuracarb</i>
2-Amino-5,6-dimethyl-4-hydroxypyrimidine (ADHP)	<i>Pirimicarb</i>
Cis/trans 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (DCCA)	<i>Permethrin, cypermethrin and cyfluthrin</i>
4-Fluoro-3-phenoxybenzoic acid (4F3PBA)	<i>Cyfluthrin</i>
3-Phenoxybenzoic acid (3PBA)	<i>Pyrethroids</i>
Bromoxynil	<i>Bromoxynil and esters</i>
Acetochlor	<i>Acetochlor</i>
2-Methyl-6-ethylaniline	<i>Metolachlor</i>
Quizalofop (free acid)	<i>Quizalofop-P-ethyl</i>
Fluazifop (free acid)	<i>Fluazifop-P-butyl</i>
2-(4-Hydroxyphenoxy)-propionic acid	<i>Quizalofop-P-ethyl and fluazifop-P-butyl</i>
Chlorpropham	<i>Chlorpropham</i>
3-Chloroaniline	<i>Chlorpropham</i>
Fenpropimorph	<i>Fenpropimorph</i>
Methyl-2-(2-hydroxyphenyl)-3-methylacrylate	<i>Azoxystrobin</i>
Azoxystrobin	<i>Azoxystrobin</i>
3,5-Dichloroaniline	<i>Procymidone</i>
Iprodione	<i>Iprodione</i>
6-Chloronicotinic acid	<i>Imidacloprid</i>
1-(3,4-Dichlorophenyl)urea (DCPU)	<i>Linuron</i>
1-(3,4-Dichlorophenyl)-3-methylurea (DCPMU)	<i>Linuron</i>
1-(4-Isopropylphenyl)-3-methylurea (IPPMU)	<i>Isoproturon</i>
Isoproturon	<i>Isoproturon</i>
2,4,6-trichlorophenoxyacetic acid (2,4,6-T)	<i>Prochloraz</i>
2-Phenylphenol	<i>2-Phenylphenol</i>
Cotinine	<i>Nicotine</i>
Nicotine	<i>Nicotine</i>

Table 2

Description of methods n°1 & n°2.

	Method n°1	Method n°2
Sample preparation	Adding labeled internal standards (27) to 2-mL urine sample Adjusting the pH of the sample to pH 5 Adding 2 mL of 0.2 M sodium acetate buffer at pH 4.8 Adding 200 µL of a solution of β-glucuronidase (25 g/L / 25 units/µL) and sulfatase (2.5 g/L / 0.025 units/µL) from Helix pomatia prepared in 0.2 M sodium acetate buffer at pH 4.8 Incubating for 2 hours at 50 °C then return to room temperature Adding 16 mL of a 100 mM phosphate buffer at pH 6	
	<i>Strata-X-AW Cartridge</i>	<i>Strata-X Cartridge</i>
Solid phase extraction	Conditioning of the cartridge with successively 10 mL of MeOH, 10 mL of a 95% ACN / 5% MeOH mixture and then 10 mL of ultra pure water Sample loading (20 mL)	
	Washing the cartridge with successively 5 mL of 25 mM ammonium acetate buffer (pH 6-7) and then 5 mL of a 95% ACN / 5% MeOH mixture Drying the cartridge Eluting with 4 x 2.5 mL of a 95% ACN / 5% MeOH + NH ₄ OH mixture	Washing the cartridge with 2 x 5 mL of ultra pure water Drying the cartridge Eluting with 4 x 2.5 mL of a 95% ACN / 5% MeOH mixture
Evaporation	Evaporation to the drop, reconstitution in the mobile phase and then transfert in vials	
Analysis	UHPLC/HRMS (ESI-)	UHPLC/HRMS (ESI+)

Table 3

Analytical characteristics of measured compounds, method n°1 (ESI-).

Compound ^a	CAS number	ISTD	t _R (min)	Quantifier MRM ^{HR} transition Precursor > Product (DP (V) / CE (V))	Qualifier MRM ^{HR} transition Precursor > Product (DP (V) / CE (V))	LOD ^b (µg L ⁻¹)	LOQ ^b (µg L ⁻¹)	
Target compounds								
1	DMP	813-78-5	DMP ¹³ C ₂	1.5	125 > 78.9592 (80 / 32)	125 > 62.9642 (80 / 21)	0.63	1.3
2	DMTP	1112-38-5	DMTP ¹³ C ₂	1.9	141 > 62.9642 (80 / 48)	141 > 125.9546 (80 / 15)	0.63	1.3
3	DEP	598-02-7	DEP ¹³ C ₂	2.5	153 > 78.9591 (65 / 35)	153 > 125.0008 (80 / 12)	0.63	1.3
4	DMDTP	756-80-9	DMDTP ¹³ C ₂	2.8	157 > 78.9414 (80 / 44)	157 > 141.9317 (80 / 16)	0.25	0.50
5	DETP	5871-17-0	DETP ¹³ C ₂	4.0	169 > 94.9362 (65 / 21)	169 > 140.9781 (65 / 14)	0.13	0.25
6	2-(4-Hydroxyphenoxy)propanoic acid	67648-61-7	6-Chloronicotinic acid ¹³ C ₆	7.7	181 > 109.0297 (65 / 16)	-	0.25	0.50
7	6-Chloronicotinic acid	5326-23-8	6-Chloronicotinic acid ¹³ C ₆	8.3	156 > 111.9958 (65 / 12)	-	0.13	0.25
8	Bromoxynil	1689-84-5	Bromoxynil ¹³ C ₆	11.9	274 > 78.9190 (80 / 35)	274 > 273.8509 (65 / 14)	0.031	0.063
9	TCPy	6515-38-4	TCPy ¹³ C ₃	12.9	196 > 195.9129 (65 / 12)	-	0.25	0.50
10	2,4,6-T	575-89-3	6-Chloronicotinic acid ¹³ C ₆	13.2	253 > 194.9178 (65 / 22)	-	0.063	0.13
11	DCCA	55701-05-8	Trans-DCCA ¹³ C ₂ D ₁	13.2	207 > 206.9985 (65 / 12)	-	0.63	1.3
12	Fluazifop (free acid)	69335-91-7	Quizalofop (free acid) D ₃	13.3	326 > 254.0430 (80 / 23)	326 > 226.0483 (80 / 32)	0.031	0.063
13	3PBA	3739-38-6	3PBA ¹³ C ₆	13.4	213 > 93.0348 (65 / 31)	213 > 169.0659 (80 / 14)	0.13	0.25
14	4F3PBA	77279-89-1	4F3PBA ¹³ C ₆	13.5	231 > 93.0347 (65 / 35)	231 > 187.0564 (65 / 16)	0.063	0.125
15	Quizalofop (free acid)	76578-12-6	Quizalofop (free acid) D ₃	14.4	343 > 271.0279 (80 / 22)	343 > 243.0331 (80 / 33)	0.031	0.063
Labeled ISTDs								
a	DMP ¹³ C ₂	157487-95-1		1.6	127 > 78.9587 (80 / 37)			
b	DMTP ¹³ C ₂	n/a		1.9	143 > 126.9575 (80 / 18)			
c	DEP ¹³ C ₂	n/a		2.5	155 > 126.0043 (80 / 14)			
d	DMDTP ¹³ C ₂	1329745-95-0		2.8	159 > 142.9351 (80 / 16)			
e	DETP ¹³ C ₂	n/a		4.0	171 > 141.9816 (80 / 16)			
f	6-Chloronicotinic acid ¹³ C ₆	n/a		8.3	162 > 117.0121 (60 / 12)			
g	Bromoxynil ¹³ C ₆	n/a		11.9	284 > 80.9158 (80 / 43)			
h	TCPy ¹³ C ₃	n/a		12.9	201 > 200.9193 (80 / 10)			
i	Trans-DCCA ¹³ C ₂ D ₁	n/a		13.2	210 > 209.9996 (65 / 12)			
j	3PBA ¹³ C ₆	n/a		13.4	219 > 99.0542 (80 / 33)			
k	4F3PBA ¹³ C ₆	n/a		13.5	237 > 99.0540 (80 / 39)			
l	Quizalofop (free acid) D ₃	n/a		14.3	346 > 271.0269 (80 / 20)			

^a Compounds listed in order of retention times; ^b for a 2-mL sample of urine

Table 4

Analytical characteristics of measured compounds, method n°2 (ESI+).

Compound ^a	CAS number	ISTD	t _R (min)	Quantifier MRM ^{HR} transition Precursor > Product (DP (V) / CE (V))	Qualifier MRM ^{HR} transition Precursor > Product (DP (V) / CE (V))	LOD ^b (µg L ⁻¹)	LOQ ^b (µg L ⁻¹)	
Target compounds								
1	Nicotine	54-11-5	Nicotine D ₄	1.8	163 > 132.0810 (80 / 17)	163 > 117.0575 (65 / 31)	4.0	8.0
2	ADHP	3977-23-9	Nicotine D ₄	2.2	140 > 98.0601 (65 / 21)	-	0.50	1.0
3	Cotinine	486-56-6	Cotinine D ₃	3.5	177 > 80.0496 (65 / 27)	-	2.0	4.0
4	Carbendazim	10605-21-7	Carbendazim D ₄	7.1	192 > 160.0503 (50 / 19)	192 > 105.0449 (61 / 47)	0.050	0.10
5	IMPy	2814-20-2	Dimethoate D ₆	7.4	153 > 84.0444 (80 / 21)	-	0.10	0.20
6	Dimethoate	60-51-5	Dimethoate D ₆	8.9	230 > 124.9820 (65 / 23)	230 > 198.9646 (46 / 11)	0.050	0.10
7	3-Chloroaniline	108-42-9	3-Chloroaniline D ₃	9.6	128 > 93.0575 (50 / 20)	128 > 128.0263 (80 / 10)	0.25	0.50
8	Methyl-2-(2-hydroxyphenyl)-3-methylacrylate	125808-20-0	Isoproturon D ₆	9.8	209 > 78.0465 (65 / 53)	-	8.0	16
9	Dichlorvos	62-73-7	Dichlorvos D ₆	11.0	221 > 109.0047 (80 / 20)	221 > 220.9532 (80 / 10)	0.50	1.0
10	2-Methyl-6-ethylaniline	24549-06-2	2-Methyl-6-ethylaniline D ₁₃	11.2	136 > 136.1121 (80 / 11)	136 > 77.0386 (80 / 41)	0.25	0.50
11	Carbofuran	1563-66-2	Cotinine D ₃	11.2	222 > 123.0431 (60 / 22)	222 > 165.0899 (60 / 14)	0.050	0.10
12	Carbofuran phenol	1563-38-8	Isoproturon D ₆	11.5	165 > 123.0441 (80 / 15)	-	4.0	8.0
13	DCPU	2327-02-8	Isoproturon D ₆	11.7	205 > 127.0173 (60 / 31)	205 > 204.9919 (60 / 10)	0.25	0.50
14	Fenpropimorph	67564-91-4	Diazinon D ₁₀	12.0	304 > 147.1160 (50 / 37)	304 > 98.0962 (50 / 37)	0.10	0.20
15	IPPMU	34123-57-4	Isoproturon D ₆	12.1	193 > 94.0641 (60 / 22)	193 > 136.1107 (60 / 20)	0.10	0.20
16	DCPMU	3567-62-2	Isoproturon D ₆	12.2	219 > 127.0172 (60 / 29)	221 > 163.9828 (60 / 18)	0.10	0.20
17	Isoproturon	34123-59-6	Isoproturon D ₆	12.4	207 > 165.1008 (60 / 18)	207 > 72.0434 (60 / 20)	0.10	0.20
18	3,5-Dichloroaniline	626-43-7	3-Chloroaniline D ₃	12.6	162 > 127.0186 (50 / 22)	162 > 161.9875 (50 / 11)	0.50	1.0
19	2-Phenylphenol	90-43-7	None	13.0	171 > 153.0709 (80 / 16)	171 > 171.0820 (80 / 11)	25	50
20	Azoxystrobin	131860-33-8	Azoxystrobin D ₄	13.0	404 > 372.0971 (46 / 21)	404 > 329.0787 (65 / 38)	0.020	0.040
21	Chlorpropham	101-21-3	Acetochlor D ₁₁	13.2	172 > 154.0052 (50 / 14)	214 > 172.0156 (50 / 10)	0.10	0.20
22	Malathion	121-75-5	Acetochlor D ₁₁	13.5	331 > 99.0074 (56 / 28)	331 > 127.0386 (56 / 14)	0.020	0.040
23	Iprodione	36734-19-7	Iprodione D ₇	14.2	330 > 244.9862 (60 / 18)	-	0.10	0.20
24	Acetochlor	34256-82-1	Acetochlor D ₁₁	14.2	224 > 148.1117 (80 / 19)	224 > 133.0885 (80 / 28)	0.050	0.10
25	Phorate	298-02-2	Phorate ¹³ C ₄	14.9	261 > 75.0262 (50 / 11)	-	0.10	0.20
26	Chlorpyrifos-methyl	5598-13-0	Chlorpyrifos-methyl D ₆	15.1	322 > 124.9820 (66 / 23)	-	0.10	0.20
27	Chlorpyrifos-ethyl	2921-88-2	Chlorpyrifos-ethyl D ₁₀	15.9	350 > 96.9506 (65 / 36)	352 > 199.9243 (61 / 30)	0.10	0.20
Labeled ISTDs								
a	Nicotine D ₄	350818-69-8		1.7	167 > 136.1056 (80 / 18)			
b	Cotinine D ₃	110952-70-0		3.4	180 > 80.0494 (80 / 27)			
c	Carbendazim D ₄	291765-95-2		7.0	196 > 164.0739 (80 / 20)			
d	Dimethoate D ₆	1219794-81-6		8.7	236 > 205.0024 (80 / 10)			
e	3-Chloroaniline D ₃	347840-11-3		9.4	131 > 96.0794 (80 / 22)			
f	Dichlorvos D ₆	203645-53-8		10.9	227 > 115.0427 (80 / 20)			
g	2-Methyl-6-ethylaniline D ₁₃	1219794-93-0		10.9	147 > 98.0977 (80 / 27)			
h	Isoproturon D ₆	1007461-76-8		12.4	213 > 78.0812 (60 / 22)			
i	Azoxystrobin D ₄	1346606-39-0		13.0	408 > 333.1020 (80 / 41)			
j	Acetochlor D ₁₁	1189897-44-6		14.1	235 > 159.1796 (80 / 20)			
k	Iprodione D ₇	n/a		14.2	337 > 244.9861 (60 / 20)			
l	Diazinon D ₁₀	100155-47-3		14.7	315 > 98.9626 (80 / 37)			

Compound ^a	CAS number	ISTD	t _R (min)	Quantifier MRM ^{HR} transition Precursor > Product (DP (V) / CE (V))	Qualifier MRM ^{HR} transition Precursor > Product (DP (V) / CE (V))	LOD ^b (µg L ⁻¹)	LOQ ^b (µg L ⁻¹)
m Phorate ¹³ C ₄	n/a		14.9	265 > 75.0264 (80 / 10)			
n Chlorpyrifos-methyl D ₆	2083629-84-7		15.0	328 > 131.0195 (80 / 29)			
o Chlorpyrifos-ethyl D ₁₀	285138-81-0		15.8	360 > 98.9631 (80 / 37)			

^a Compounds listed in order of retention times; ^b for a 2-mL sample of urine

Table 5

Pesticide concentrations in fifteen human urine samples.

Measured concentrations in 15 human urine samples ($\mu\text{g}\cdot\text{L}^{-1}$)					
Method n°1 (ESI-)			Method n°2 (ESI+)		
Compound ^a	n > LOD	Range	Compound ^a	n > LOD	Range
DMP	13	[<0.63;100]	Nicotine	0	[<4.0;<8.0]
DMTP	12	[<0.63;65]	ADHP	0	[<1.0;<2.0]
DEP	15	[3.1;68]	Cotinine	4	[<2.0;9.6]
DMDTP	14	[<0.25;20]	Carbendazim	4	[<0.050;1.6]
DETP	15	[0.48;6.6]	IMPy	4	[<0.10;1.3]
2-(4-Hydroxyphenoxy)propanoic acid	5	[<0.25;4.2]	Dimethoate	0	[<0.050;<0.10]
6-Chloronicotinic acid	0	[<0.13;<0.25]	3-Chloroaniline	1	[<0.25;0.87]
Bromoxynil	3	[<0.031;0.057]	Methyl-2-(2-hydroxyphenyl)-3-methylacrylate	0	[<8.0;<16]
TCPy	15	[0.27;14]	Dichlorvos	0	[<0.50;<5.0]
2,4,6-T	6	[<0.063;0.21]	2-Methyl-6-ethylaniline	0	[<0.25;<0.50]
DCCA	4	[<0.63;13]	Carbofuran	1	[<0.050;0.68]
Fluazifop (free acid)	8	[<0.031;0.20]	Carbofuran phenol	0	[<4.0;<32]
3PBA	14	[<0.13;5.4]	DCPU	0	[<0.25;<0.50]
4F3PBA	1	[<0.063;0.083]	Fenpropimorph	0	[<0.10;<0.20]
Quizalofop (free acid)	3	[<0.031;0.18]	IPPMU	0	[<0.10;<0.20]
			DCPMU	0	[<0.10;<0.20]
			Isoproturon	0	[<0.10;<0.20]
			3,5-Dichloroaniline	11	[<0.50;3.8]
			2-Phenylphenol	1	[<25;140]
			Azoxystrobin	1	[<0.020;0.24]
			Chlorpropham	0	[<0.20;<0.40]
			Malathion	0	[<0.020;<0.040]
			Iprodione	0	[<0.10;<0.20]
			Acetochlor	0	[<0.050;<0.10]
			Phorate	1	[<0.10;0.13]
			Chlorpyrifos-methyl	1	[<0.10;0.48]
			Chlorpyrifos-ethyl	2	[<0.10;0.78]

^a Compounds listed in order of retention times

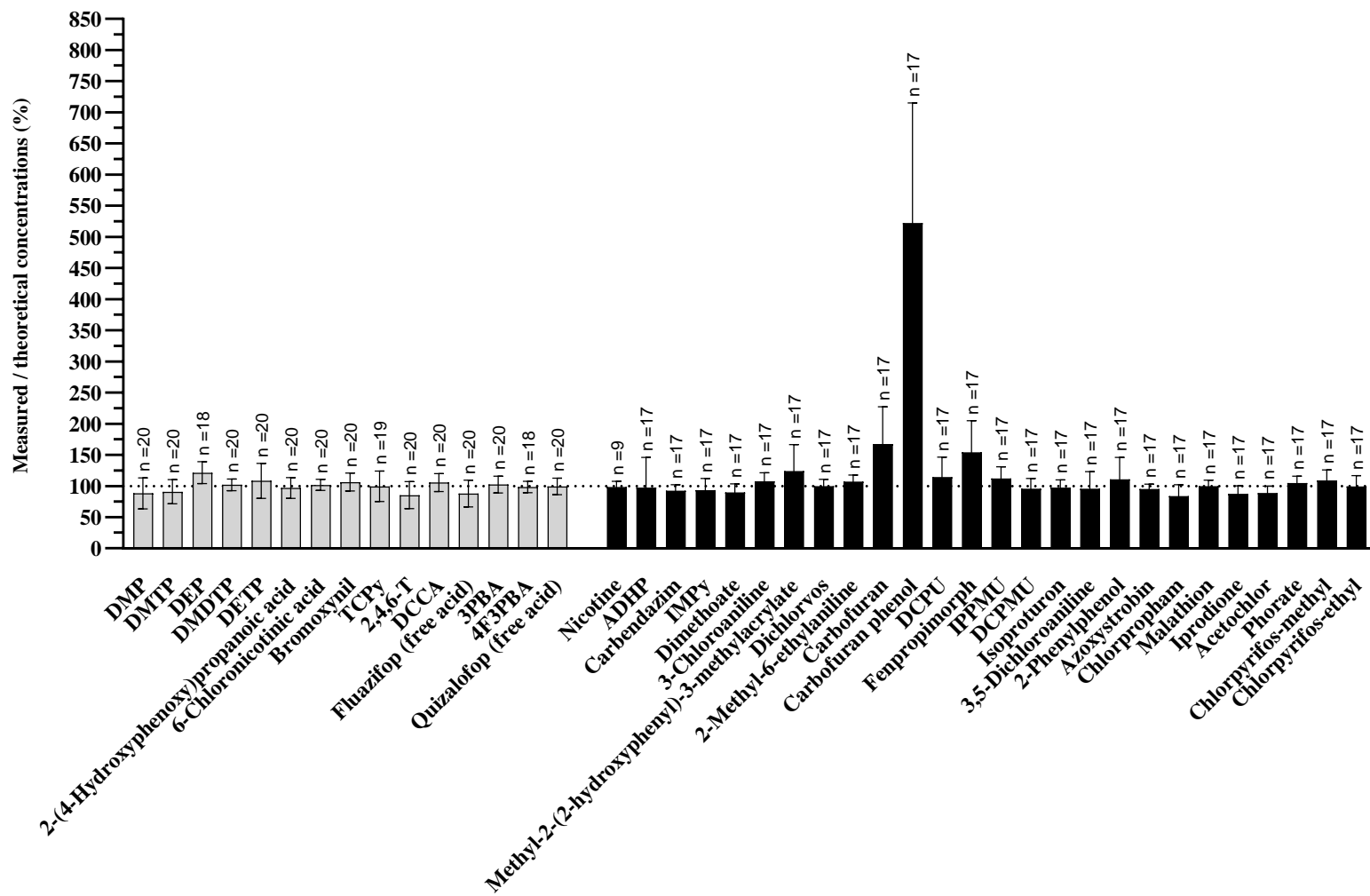


Figure 1

Spiking experiments on two (method n°2, black bars) or three (method n°1, grey bars) different urine pools non-spiked and spiked at an

intermediate level: bar chart showing arithmetic mean and standard deviation of measured to theoretical concentrations ratios (%).

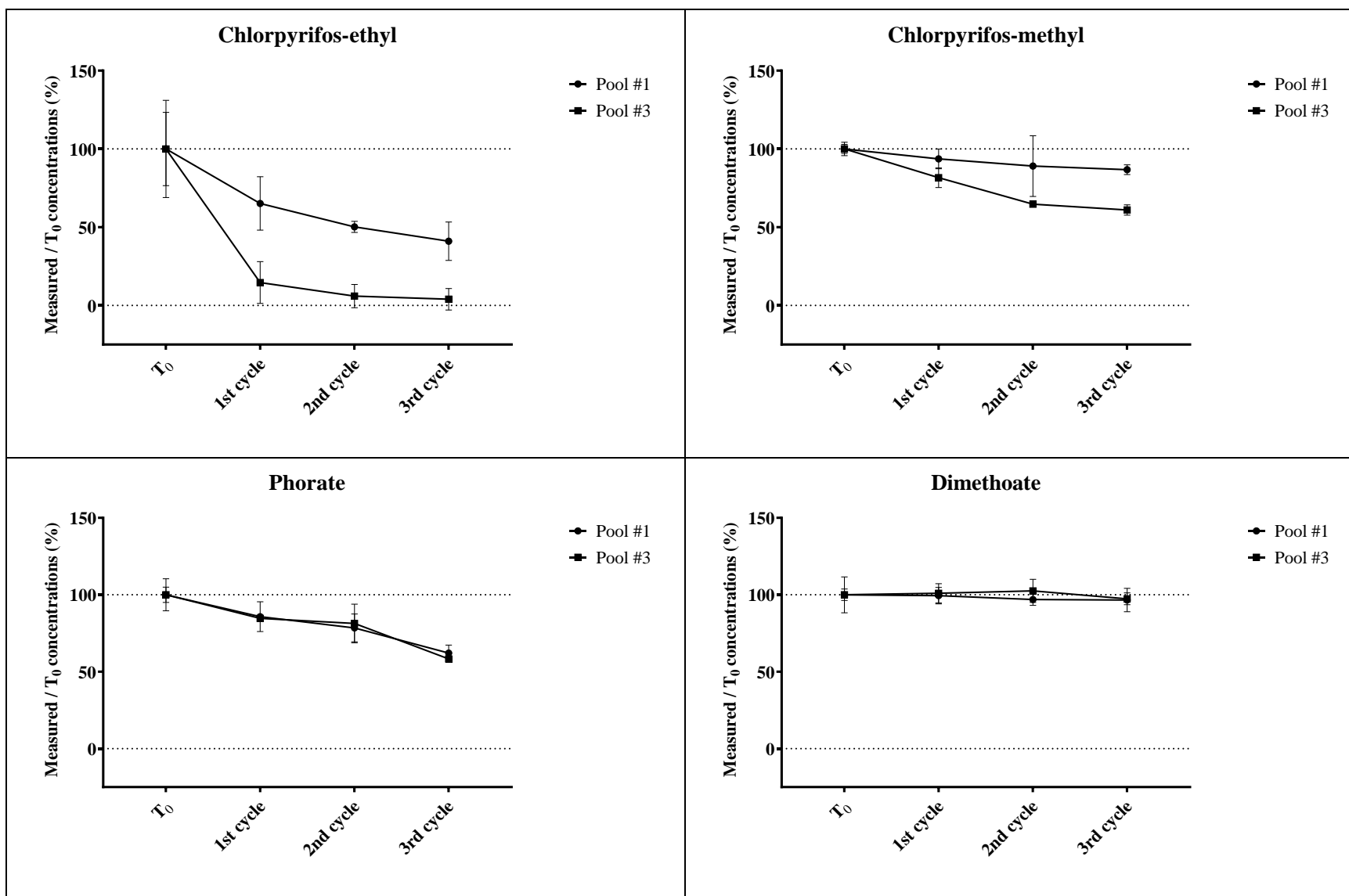


Figure 2

Study of the impact of thawing/freezing cycles on the conservation of chlorpyrifos-ethyl, chlorpyrifos-methyl, phorate and dimethoate: experiments on two urine pools (pools #1 and #3) spiked at an intermediate level.