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# Title: Suspect screening and targeted analyses: two complementary approaches to characterize human exposure to pesticides

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## ABSTRACT

Characterizing human exposure to pesticides is challenging because their metabolites have a broad diversity of chemical structures. We aimed to study the interest of associating suspect screening and targeted analysis to identify multiple pesticide exposure of pregnant women. Approximately 300 urinary samples collected during early pregnancy (France) were analyzed by UHPLC/HRMS using both a suspect screening approach and targeted multiresidue analysis. Sixty-eight pesticides were selected according to available data on agricultural practices and analytical feasibility and 435 known or putative metabolites were added based on the literature. Compounds detected using the two approaches were compared and the place of residence was studied as a determinant of exposure for illustrative purposes. Suspect screening resulted in the characterization of 28 pesticide metabolites, corresponding to three fungicides (azoxystrobin, fenpropimorph, and procymidone), three herbicides (an arylphenoxypropionic acid derivative, chlorpropham, and phenmedipham), and one insecticide (carbofuran). The targeted approach led to the identification of pyrethroids, organophosphorus, fluazifop-P-butyl, and chlorpyrifos in > 60% of samples and prochloraz, bromoxynil, diazinon, and procymidone in 10 to 50%. Both urban and rural areas were identified as being determinants of exposure, depending on the active substance. This combined strategy better characterizes pesticide mixtures. Suspect screening allows the detection of metabolites of pesticides that are rarely studied and not measured in biomonitoring studies, mainly because it allows the detection of conjugated phase II metabolites (azoxystrobin and fenpropimorph). The targeted approach complements it with the detection of highly polar and low molecular-weight metabolites, the confirmation of the parent compound, and the quantification of compounds for which analytical standards are commercially available or may be synthesized (organophosphorous, pyrethroids, and fluazifop-P-butyl). In addition, we detected several metabolites that have never been



described in humans (fenpropimorph and azoxystrobin), which deserve to be candidates in the selection of markers of exposure.

**KEYWORDS:** plant protection products; biocides; biomarkers; mass spectrometry; mixtures

## 1. INTRODUCTION

Pesticides include plant-protection products (PPP) used to protect crops and plant products and biocides used for general hygiene and public health purposes. A single substance can be authorized for agricultural, medicinal, and biocidal uses. According to the Food and Agriculture Organization of the United Nations (FAO), the agricultural use of pesticides in the European Union (EU, 27 countries) varied from 354,320 tons in 2008 to 348,493 tons in 2018 (<http://www.fao.org/faostat/en/#data/RP/visualize>). During the same period, France used from 78,577 to 85,072 tons, respectively, making it one of the main pesticide users in Europe. In France, and especially in Brittany, more than 60% of land use is devoted to agricultural activities, with a large part (50%) made up of wheat and maize crops, which require the use of substantial amounts of PPPs each year. In 2018, total PPP sales in this region reached more than 3,300 tons for a cultivated area of 1,700,000 ha (<https://www.data.gouv.fr/fr/datasets/ventes-de-pesticides-par-departement>).

As their application can lead to environmental contamination *via* air, water, dust, particles, and residues in food, there are valid reasons to be concerned about the possible adverse health effects in the general population resulting from pesticide exposure. Assessment of the potential human health risk related to pesticide use requires knowledge that is as precise as possible about the exposure of the general population to these substances. Traditional pesticide risk-assessment approaches have considered single sources of exposure and individual active substances, probably because pesticides are authorized individually and because PPPs and biocides are separately regulated. However, recent advances in risk-assessment methodology suggest taking into consideration aggregate and cumulative exposure (Fox et al. 2017; Kennedy et al. 2015a, 2015b, 2015c). The study of such exposure is challenging due to the large number of substances to be considered (i.e. 400 active substances

currently authorized as PPPs within the EU, according to Rebouillat et al. (Rebouillat et al. 2020)), as well as the various sources and potential routes of exposure.

Human biomonitoring (HBM) is a useful approach for assessing human exposure to chemicals such as pesticides. It consists of measuring the parent chemical and/or the corresponding biotransformation products in biological samples and can reflect co-exposure to multiple compounds from different sources or routes. Although various biological matrices, such as blood, plasma, and hair, have been used for HBM, urine samples are generally considered to be the best choice due to the relative ease with which they can be obtained (Bevan et al. 2017). Most studies that have used HBM for estimating pesticide exposure have focused on one or two specific compounds, or one category of compounds, such as organophosphates. However, over the past five years, several teams have developed multiresidue analytical methods for pesticides in human urine (Appenzeller et al. 2017; Baudry et al. 2019; Hyland et al. 2019; Llop et al. 2017; Pirard et al. 2020; Roca et al. 2014; Shin et al. 2019; Sierra-Diaz et al. 2019; Yusa et al. 2015). Although such methods can sometimes detect and quantify several dozen PPPs, they do not account for compounds that are not on the predefined list of active substances to be measured or for those for which no analytical standards corresponding to urinary metabolites are commercially available.

Recently, so-called non-targeted approaches have been published (Andra et al. 2017; Baduel et al. 2015; Cortéjade et al. 2016; Pourchet et al. 2020; Rejczak and Tuzimski 2015). Non-targeted analysis involves the detection and identification of a very large panel of chemicals and metabolites by high-resolution mass spectrometry (HRMS) analyses including those for which reference chemical standards are currently unavailable. In the field of pesticides, they mainly concern PPP residues in various food categories (see (Guo et al. 2020) for review), showing the complementarity of this approach with the more traditional targeted analysis (Picó et al. 2018). In addition, a study that attempted untargeted profiling of pesticides in a

limited number of non-occupationally exposed individuals was published (Jamin et al. 2014), showing promising results and potential applications in HBM. Pourchet et al. (2020) distinguished between three methodological approaches for HBM: i) targeted analysis for compounds of known chemical name and structure, for which quantitative targeted methods are available; ii) suspect screening for compounds of known chemical name and structure that are suspected to be present in a sample but for which analytical standards are not readily available and, therefore, relevant analytical methods are not validated and compound identities not definitive; suspect screening aims to generate semi-quantitative data and contribute to better prioritization for further targeted development; and iii) non-targeted screening, which aims to detect unknown compounds without any *a priori* criteria.

This study aims to combine a suspect screening approach and targeted quantitative analysis using urine samples from pregnant women from the PELAGIE cohort recruited in an agricultural region of Brittany, France (Chevrier et al. 2011) to demonstrate the strong complementarity of the two approaches and provide a broader spectrum of information than that usually given by multiresidue analyses in human biomonitoring. This is the first time that these two approaches have been used simultaneously on the same set of human biological samples to characterize aggregate pesticide exposure. This combination of approaches was then used to study the place of residence as a determinant of pesticide exposure. Despite the growing evidence that proximity to crops contributes to pesticide exposure (Teyssere et al. 2021), it is not sufficiently considered in risk assessment, contrary to the assessment of occupational or dietary exposure.

## 2. MATERIALS AND METHODS

### 2.1. Study population and sample collection

The population selected for this study was based on the PELAGIE cohort, which included approximately 3500 pregnant women in Brittany (France) between 2002 and 2006 for a longitudinal follow-up of children's health. A detailed description of this cohort has been provided elsewhere (Chevrier et al. 2011). For this study, we selected samples stored under the same conditions and for the same length of time, as described in (Bonvallot et al. 2013), corresponding to 338 women for whom urinary samples were collected in 2004. Most lived in a town with < 20,000 inhabitants (>80%), were 25-34 years old (76%), and did not drink alcohol (84%) or smoke (71%) during their pregnancy. The median maternal age at inclusion was 30.2 years. Most (59.8%) had a high educational level (post-secondary). A detailed description of the population and the collection of urinary samples is provided in Supplemental Material S1.

## 2.2. Analyses of pesticides and their metabolites in urine

*Suspect screening* was performed in 2014 by ultra-high-performance liquid chromatography combined with high-resolution mass spectrometry (UHPLC–HRMS) using an RSLC3000 chromatography system (Thermo Scientific, Les Ulis, France) and an LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, Les Ulis, France), as previously described (Jamin et al. 2014). Briefly, samples were diluted with an equal volume of mobile phase A and centrifuged at 9000 x g for 5 min. Samples were applied onto a hypersil Gold C18 column (100x2.1 mm, 1.9µm) from Thermo Scientific (Les Ulis, France) using a gradient program of H<sub>2</sub>O/CH<sub>3</sub>OH/CH<sub>3</sub>CO<sub>2</sub>H 95/5/0.1 (v:v:v) and CH<sub>3</sub>OH/CH<sub>3</sub>CO<sub>2</sub>H 100/0.1 (v:v), at a flow rate of 0.3mL/min. Ionization by electrospray in the negative mode was used according to our previous data because positive mode did not provide any specific additional information (Jamin et al. 2014). High resolution mass spectra were acquired between mass to charge ratios ( $m/z$ ) of 80 and 800 at a resolution of 30000 ( $m/z$  400). Samples were randomly analyzed in

eight batches composed of 40 or 41 samples and six injections of the same quality control (QC) sample (corresponding to a pool of all samples). Ethephon was used as an internal standard to verify the quality of data acquisition. The intra-batch relative standard deviation (RSD) of ethephon intensity measured in QC samples was below 14.3% and the RSD of measured retention times (RT) of ethephon was below 1.0%. The batch effect was corrected using the Workflow4metabolomics batch correction tool (Giacomoni et al. 2015). Variation among sample data was normalized using probabilistic quotient normalization (Dieterle et al. 2006). Sixty-eight pesticides were selected according to available data on the agricultural practices of 2004, use recommendations, and analytical feasibility. The year 2004 was chosen to be consistent with the urine samples collection. The selected pesticides correspond to insecticides, herbicides, and fungicides belonging to various chemical families (see Supplemental Material S2). From them, a list of 435 potential metabolites was assembled from known metabolites described in the literature, in particular from reports of pesticide residues in food of the FAO, as well as all putative phase II metabolites, if not already reported (Supplemental Material Table S3). Thus, the intensities of a list of 503  $m/z$  ratios of  $[M-H]^-$  of candidate compounds (i.e., pesticides and their metabolites) (Supplemental Material S3) were measured using an Xcalibur system (Thermo Scientific, Les Ulis, France) (i.e. peak picking, retention time alignment, signal area measurement) according to a signal to noise ratio above 3, a  $m/z$  precision of  $\pm 5$  ppm, and non-detection in blank samples. Structures of putatively detected metabolites were characterized similarly to as in Jamin et al. (2014). Isotopic patterns were studied to confirm, or not, the chemical formula. Tandem mass spectrometry (MS/MS) experiments were targeted for suspected metabolites in the QC sample, using the linear ion trap of an LTQ-Orbitrap hybrid mass spectrometer. Phase II conjugated metabolites were characterized by observation of characteristic MS/MS fragmentation patterns. The structures of phase II metabolites were also characterized by

interpretation of MS<sup>3</sup> spectra. Similarly, the structures of phase I metabolites were characterized by interpretation of MS/MS spectra. At this stage, detected metabolites were only tentatively identified (level 3) according to the nomenclature of (Schymanski et al. 2014). In the absence of commercially available compounds, an animal experiment was conducted to produce *in vivo* reference metabolites by administration of the parent compounds (Jamin et al. 2014). Observation of the same RT, MS/MS, and MS<sup>3</sup> spectra achieved on the same instrument allowed confirmation of the structures (level 1 of Schymanski et al. 2014) of several metabolites in human urine samples.

*Targeted analyses* were carried out in 2017-2018. Two multi-residue methods were used to cover the targeted substances. Methods n°1 and n°2 allowed the determination of 15 (acid metabolites, dialkyl phosphate metabolites (DAPs), TCPy and bromoxynil) and 27 (other targeted substances) molecules, respectively, with detection limits for a 2-mL urine sample ranging from 0.02 µg/L for the most sensitive molecules to 25 µg/L for 2-phenylphenol (Supplemental Material S4). Both methods included i) enzymatic hydrolysis (β-glucuronidase and sulfatase from *Helix pomatia*), ii) off-line solid phase extraction (SPE) using a Gilson GX-274 ASPEC automatic extraction system (Gilson, Middleton, WI, USA) on Phenomenex Strata-X-AW cartridges for method n°1 and Phenomenex Strata-X cartridges for method n°2, and iii) quantitative analysis using a SCIEX ExionLC<sup>TM</sup> AD / SCIEX X500R QTOF UHPLC/HRMS system (AB Sciex, Framingham, MA, USA) operated in the negative ESI mode for method n°1 and in the positive ESI mode for method n°2. Chromatographic separation was performed at a flow rate of 0.3 mL min<sup>-1</sup> on a Waters Acquity UPLC HSS T3 column (150 mm length x 2.1 mm I.D., 1.8 µm 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 µL. 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). A detailed description of both methods including reagents and chemicals and quality assurance and quality control (QA/QC) is provided in Supplemental Material S5.

### 2.3. The place of residence as a determinant of exposure

The place of residence was tested as a determinant of exposure to pesticides identified by both the suspect screening and the targeted analysis. Geographical indicators used are those available in the PELAGIE cohort:

- District of residence (Côtes-d'Armor, Finistère, Ille-et-Vilaine)
- Urban or rural areas (> or < 20,000 inhabitants)
- Percentage of land use dedicated to various types of crops (corn, wheat, other cereals) in the municipality of residence retrieved from the general agricultural census
- Presence of other types of crops (vegetables, peas, and potatoes) in the municipality of residence, is also retrieved from the general agricultural census
- Presence of crops within a 500-meters radius of the residence. This last indicator was estimated with a geographical information system using georeferencing, satellite pictures, and the 2006 agricultural parcel registry database, for corn, wheat, and colza crops (Chevrier et al. 2014).

Three groups were constituted for corn, wheat, and other cereals according to terciles. For vegetables, peas, and potatoes, two groups according to the absence or presence of crops were used (Bonvallot et al. 2013; Chevrier et al. 2014).

As the data generated varied according to the approach used, i.e., MS peak integrations for suspect screening or absolute concentrations for targeted analysis, different statistical analyses

were performed to associate the detection or quantification of pesticide metabolites in urine and the place of residence.

*For the suspect screening approach*, if several metabolites were detected for one parent compound, the most frequently detected and specific metabolite with a signal that did not show interference for further analyses was selected as the response variable. The intensities of the response variables were transformed ( $\log_{10}$ ) depending on the distribution of each signal and normalized against an internal standard (ethephon) to correct for inter-injection variation within the same batch. Missing data were replaced by a random log normal value between 0 and the minimum value (non-quantifiable detection limit). The effect of each previously mentioned geographical indicator was addressed by analysis of variance (ANOVA) using the `lm` function (R software). Each ANOVA model also included the following factors: BMI, age, number of children, smoking status, and season of urine collection. The normality of the residuals was tested to check the assumption of linear models. Student-Newman-Keuls tests were used to compare the levels of the various factors.

*For targeted analyses*, pesticide metabolites were grouped according to their respective parent compound, chemical family, or their similar use. For this purpose, molar concentrations were summed. Left-censored values ( $<LOD$ ) were imputed using a random draw which completes a left-censored normal distribution using the QRILC method (quantile regression approach for the imputation of left-censored missing data) (Wei et al. 2018). In addition to linear regression models, a Bayesian statistical approach was used to introduce uncertainty into the parameters (Bayes and Price 1763), allowing the use of more flexible models to manage complex data (Shoari and Dubé 2018). Both Bayesian logistic and linear regression models that included the various place-of-residence indicators as the main explanatory variables were generated. Other known determinants of exposure were additionally considered as potential confounding factors in the models (sociodemographic factors, physiological characteristics, smoking

status, season of urine collection, dietary and domestic uses, creatinine concentrations, and freezing/thawing events). Parameters were estimated using MCMC (Monte Carlo Markov Chain) (R software). Convergence of the models was verified using linear regression residues, auto-correlation, density and variable magnitude for each iteration (Hamra et al. 2013). Bayesian model averaging was used specifically for each targeted compound for covariable selection considering all available variables (R software, BMA package) (Hoeting et al. 1999). Models were compared using BIC (Bayesian information criteria), according to the Laplace approximation method (Raftery et al. 2005).

These analyses concerned 273 to 327 and 244 samples for suspect screening and targeted analysis, respectively, depending on the availability of data for each woman. A flowchart explaining the selection steps of the populations studied for each type of statistical analysis and the reasons for excluding certain samples are presented in Supplemental Material S6.

### 3. RESULTS

#### Detected pesticides

Approximatively 70 potential metabolites or pesticides were detected by suspect screening according to their accurate  $m/z$  ratio. The structures of detected metabolites were tentatively identified (level 3 according to Schymanski et al. 2014) according to the interpretation of  $MS^n$  fragmentation patterns. The metabolites listed in Supplemental Material S3 displayed MS/MS fragmentations patterns in concordance with their suspected structures, for example with a diagnostic loss of 176 u ( $C_6H_8O_6$ ) or 80 u ( $SO_3$ ) for glucuronide or sulfate conjugates, respectively. Tandem mass spectrometry experiments by  $MS^3$  carried out based on the loss of  $C_6H_8O_6$  or  $SO_3$  yielded fragmentation patterns compatible with the suspected structures. The structure of several metabolites was confirmed (level 1 according to Schymanski et al. 2014) by the observation of the same results (i.e., RT and MS/MS spectra) as those obtained during

the previously reported animal experiment in which urinary metabolites were generated from 7 pesticides (see Jamin et al. 2014 for details). Since this previous publication, additional pesticide metabolites were produced allowing five metabolites of phenmedipham to be successfully identified in rat urine samples, enabling their confirmation in human urine samples (i.e., same RT and MS/MS on the same instrument). Thus 28 metabolites were at least tentatively identified, corresponding to six pesticides: azoxystrobin, fenpropimorph, procymidone, fluazifop-P-butyl or quizalofop-P-ethyl, phenmedipham, and chlorpropham (Supplemental Material S7). In total, 27 compounds (parents or metabolites) were detected using the targeted approach, corresponding to two families and 17 pesticides: organophosphates and pyrethroids, chlorpyrifos, fluazifop-P-butyl, prochloraz, bromoxynil, cyfluthrin, diazinon, procymidone, carbendazim, quizalofop-P-ethyl, azoxystrobin, dichlorvos, 2-phenylphenol, imidacloprid, chlorpropham, carbofuran, malathion and phorate (Supplemental Material S8). The detection frequencies of most detected metabolites by suspect screening and targeted analysis and the corresponding semi-quantitative and quantitative data distributions are presented in Tables 1 and 2, respectively.

Combining the two approaches, the most frequently detected pesticides (as corresponding metabolites) were: fenpropimorph (11 metabolites), procymidone (1 metabolite), azoxystrobin (3 metabolites), phenmedipham (5 metabolites), fluazifop-P-butyl (3 metabolites specific to the arylphenoxypropionic acid derivatives, and the specific free acid), organophosphorus compounds (dialkylphosphates and TCPy, a chlorpyrifos metabolite), and pyrethroids (3-phenoxybenzoic acid and to a lesser extent, 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid, a metabolite of permethrin, cypermethrin and cyfluthrin). These metabolites were detected at frequencies > 80%. Prochloraz, bromoxynil, cyfluthrin, and diazinon were

less frequently detected, with frequencies of approximately 20 to 30%. Except for bromoxynil, all detected pesticides were metabolites.

### 3.1. The place of residence as a determinant of pesticide exposure

Five highly detected (> 90%) and specific metabolites were used for statistical analyses for the suspect screening (see the Table 1). For the targeted approach, 10 compounds or groups of compounds were used for statistical analyses because they were detected > 10% of the samples (see the Table 2). In this case, frequently detected compounds (> 60%) were analyzed by linear regression, leading to an estimate of the  $\beta$  parameter, whereas less well detected compounds (< 50%) were analyzed using logistic regression with the binary variable detected/non-detected, leading to an estimated odds ratio (OR). The associations between urinary metabolites and their potential determinants are presented in Tables 3 and 4 for the suspect screening and targeted analysis, respectively.

These analyses of the data obtained by both suspect screening and targeted approaches show that urbanization and proximity to crops play a role in pesticide exposure. The determinants were different depending on the pesticide studied: Women living in towns with > 20,000 inhabitants showed higher concentrations of phenmedipham and procymidone metabolites than women living in smaller towns (Figure 1). We observed a similar trend fluazifop-p-butyl. This trend was confirmed by the *p*-values observed for phenmedipham and the density of cereal crops, as well as for procymidone and the presence of vegetable crops near the residence (Table 3).

Conversely, women living in urban areas had a mean urinary concentration of bromoxynil that was four times lower than those living in rural areas (Table 4). In addition, women who

lived within a 500-m radius of vegetable crops had a 30% higher urinary concentration of chlorpyrifos than those who did not. There was also a positive association between urinary concentrations chlorpyrifos and the presence of cereal crops in the town of residence. Surprisingly, we found an inverse association between the presence of potato and pea crops in the town of residence and urinary metabolites concentrations of pyrethroids.

#### 4. DISCUSSION

It has become apparent in recent years that one of the main weakness of risk assessment methods for pesticide exposure is related to the lack of consideration of exposure to mixtures of these substances. Potential interactions between pesticide residues may result in unexpected adverse effects related to exposure to such mixtures. Thus, methods for the rapid and sensitive determination of these chemicals in biological matrices is required. A combination of suspect screening and targeted analysis was proposed as a means to increase the number of monitored analytes (Andra et al. 2017; Pourchet et al. 2020). Here, we tested this analytical strategy to determine pesticide exposure in Brittany (France) and its determinants on the basis of urine samples collected in 2004 from 338 women living in this area. Although the samples used were old, this approach is nonetheless relevant as a proof of concept for the study of exposure to mixtures, and the more complete characterization of its determinants. A number of geographical indicators of exposure to pesticides were determined based on individual responses to questionnaires, geocoding of the place of residence, an agricultural parcel registry database, and satellite pictures. It is possible that these geographical indicators of exposure were suboptimal, due to the exclusion of women who had not been geolocalized and considering the hypothesis that women living in highly rural areas, who are less frequently geolocalized, are potentially more likely to be exposed to pesticides.

Fenpropimorph, procymidone, azoxystrobin, phenmedipham, fluazifop-P-butyl, and organophosphates and pyrethroid compounds were the most frequently detected pesticides. We also found that exposure to phenmedipham, procymidone, and, potentially, fluazifop-p-butyl was higher in urban than rural areas. Conversely, exposure to bromoxynil was higher in rural areas and chlorpyrifos exposure was associated with the presence of and proximity to cereal crops. These observations indicate that both rural and urban areas may be considered in pesticide exposure studies, but that the active substances involved are generally different. Until now, it is rather the proximity to rural areas or agricultural activities that have been considered in studies focusing on the determinants of pesticide exposure (Dereumeaux et al. 2020). For example, proximity to spraying or living near treated orchards or agricultural fields were shown to be associated with higher urinary concentrations of organophosphorus compounds (Bradman et al. 2011; Loewenherz et al. 1997; Lu et al. 2000) and proximity to corn crops with higher urinary concentrations of herbicides (acetochlor, metolachlor, atrazine) (Chevrier et al. 2014).

Fenpropimorph and azoxystrobin are fungicides that were used in combination in France and in Brittany to treat cereal crops until 2010. Approximately 200 tons of azoxystrobin was used at the national level and 10 tons at the regional level whereas the corresponding quantities for fenpropimorph were 250 and 20 tons (according to the National database for pesticide sales (<https://www.data.gouv.fr/fr/datasets/donnees-de-vente-de-pesticides-par-departement/>)). For comparison, up to 112 tons of acetochlor, an herbicide widely used on corn crops and largely detected in the urine of women in Brittany (Chevrier et al. 2014), was used in Brittany over the same period, i.e., 5 to 10 times more than fenpropimorph and azoxystrobin. Both fenpropimorph and azoxystrobin were also used as biocidal products. Indeed, azoxystrobin has also often been detected in fruits and vegetables in France (ANSES 2016). Procymidone is a fungicide and was also used as a PPP on fruit and vegetable crops, and as a biocide over

the same period (it was then banned in Europe in 2007 due to its toxicity). Our observations may be due to differences in the supply chain between urban and rural areas, with higher consumption of products by individual producers (Ravache 2003), or to biocidal use in materials and buildings, which are more prevalent in large municipalities. The free acid form of fluazifop was present in almost 70% of samples analyzed by the targeted approach; the non-specific metabolite (2,4-hydroxyphenoxypropanoic acid) found by suspect screening probably came from fluazifop-P-butyl rather than quizalofop-P-ethyl. We observed a higher frequency of detection (*ca.* 95%) for 2,4-hydroxyphenoxypropanoic acid, as a glucuronide conjugate, using the suspect screening approach than the free form using the targeted approach (approximately 20%, data not shown). This discrepancy may be due to a higher sensitivity of detection of this metabolite in its conjugated than free form after enzymatic hydrolysis, resulting from a better ionization yield of the conjugate with electrospray. Another explanation could be resistance of the conjugated 2,4-hydroxyphenoxypropanoic acid to hydrolysis by beta-glucuronidase or limited stability of the aglycone in the presence of the enzyme, leading to an underestimation of this metabolite when the targeted approach is applied. Incomplete deconjugation of glucuronides has been reported by several groups (Choi et al. 2010; Dickinson et al. 1984; Dwivedi et al. 2018). Fluazifop-P-butyl was used in 2004 in agriculture to treat cereals, corn, colza, peas, beetroots, vegetables, and sunflowers. However, sales in Brittany and France were very low (390 kg in Brittany and 13 tons in France in 2008). In addition, phenmedipham and fluazifop-p-butyl are two herbicides that may have been used for communal weed control. It has also been shown that fungicides and herbicides are the pesticide class profiles that predominate in urban air samples (Airparif 2016), which could explain the higher concentrations observed in urban areas. Differences in the supply chain cannot be excluded in explaining our results as phenmedipham and fluazifop-p-butyl were also used on fruits and vegetables, and for the former, on medicinal

plants, condiments, aromatics, and spices. Prochloraz (fungicide) and bromoxynil (herbicide) were highly used in the 2000's (more than 40 tons per year in both cases). This could explain the detection frequencies above 20%. As confirmed by the high detection levels (> 90%), organophosphates and pyrethroids are ubiquitous substances: they have been largely used in agriculture and at home for plant protection, as well as for repellent and household products with biocidal activities and for antiparasitic uses. The results obtained for chlorpyrifos are in accordance with those already obtained by Gunier et al., who found a positive correlation between chlorpyrifos concentrations in carpet dust and the proximity of agricultural activity in California (Gunier et al. 2011). The surprising results obtained for pyrethroids may thus be explained by their multitude of existing uses other than for agriculture.

In addition to the characterization of exposure to pesticides and its determinants, our study clearly shows the complementarity of suspect screening and targeted analysis, as illustrated in Figure 2.

Most studies focusing on pesticide monitoring in human samples have been based on substances that can be routinely measured by targeted approaches, such as organochlorine, organophosphates, and pyrethroids and/or molecules that were extensively used, such as triazine or chloroacetamide herbicides in Brittany, France (Chevrier et al. 2011, 2014). However, multi-residue targeted methods are often limited by the number of monitored signals because of the need of (i) numerous analytical standards and (ii) efficient sample preparation and detection methods, which are all the more difficult to develop, as the number of targeted substances and chemical families is large. This leads to prioritization of the targeted pesticides, which generally corresponds to those that are the most widely studied. Alternatively, suspect screening is not limited by the number of monitored signals. Thus,

rarely analyzed pesticides can be included in the list of screened suspect compounds, without a decrease in analytical performance. In the present study, pesticides, such as the fungicides fenpropimorph, procymidone, azoxystrobin, and the herbicides fluazifop-P-butyl and phenmedipham, were frequently detected by the suspect screening method, although they have only very rarely been analyzed up to now. Some of these pesticides are of toxicological concern and could interact with each other. For example, fenpropimorph and bromoxynil are possibly reprotoxic under European CLP regulations (<http://echa.europa.eu/>) (European Food Safety Authority (EFSA) 2008) and procymidone is considered to be an endocrine disruptor by antagonizing the androgen receptor (European Commission 2002). Nevertheless, suspect screening is only semi-quantitative and solely enables the comparison between samples (i.e., populations) analyzed together. The comparison of several sets of samples (i.e., populations) analyzed separately or from different HBM studies requires absolute quantification obtained from validated methods, which is routinely achieved with targeted approaches. As illustrated by the detection of azoxystrobin in this study, suspect screening can be applied as a first step in a biomonitoring strategy to identify particular pesticides that are rarely or not measured by targeted approaches. Following such untargeted detection, efforts could be made (purchase or synthesis of standards and method development) to implement a targeted approach for the absolute quantification of the major metabolites of these pesticides.

Another challenge of this complementary approach concerns access to analytes with diverse physicochemical properties. Highly polar low molecular-weight metabolites require particular analytical conditions (i.e., for chromatographic separation, limitation of matrix effects, ionization), which can be achieved with good sensitivity using targeted approaches, as shown for organophosphorus compounds; highly polar dialkylphosphates were detected with good sensitivity relative to the non-targeted screening. Similarly, the targeted approach allows differentiation between quizalofop and fluazifop exposure by quantifying specific free acids.

Indeed, suspect screening can use simple sample preparation (i.e., direct injection after dilution) and generic chromatographic conditions and ionization parameters to ensure the detection of as many compounds as possible. However, this limits the identification of highly polar low molecular-weight metabolites for which both the sample preparation step and the chromatographic system are not optimized as mentioned by several authors (Kiefer et al. 2019; Newton et al. 2018).

Moreover, suspect screening allows for the detection of conjugated phase II metabolites (i.e., glucuronides, sulfates) with high sensitivity, without the need of chemical or enzymatic hydrolysis (deconjugation), because these forms are highly ionizable. Although most targeted approaches apply a deconjugation step during sample preparation to transform phase II metabolites into phase I metabolites, for which standard compounds may be more readily available, the efficiency of this deconjugation step is questionable, as (i) conditions may vary depending on the compound, and (ii) the deconjugation yield may not be measurable due to the lack of commercially available analytical standards for conjugated forms. This was illustrated in this study by the detection of a very prevalent azoxystrobin metabolite by the suspect screening (sulfate conjugate of methyl (2E)-2-(2-Hydroxyphenyl)-3-methoxyacrylate), whereas the targeted approach was not sufficiently sensitive to detect the corresponding deconjugated compound. This also confirms that analyses of parent compounds are generally not the most relevant in the urinary matrix.

In most cases, targeted approaches are more sensitive than non-targeted approaches due to the specific sample preparation and the specific acquisition of data. Conversely, suspect screening is nonspecific, by definition, to allow the broadest detection of compounds. The LOD observed for most of the targeted molecules was below 1 µg/L of collected urine (Supplemental Material S4), whereas the LOD of the suspect screening could only be estimated, as no standards were available. In this study, the LOD was estimated to be

approximately 200 µg/L based on other analyses achieved on the instrument used. However, the LOD could be much lower depending on the physico-chemical properties of each metabolite in the gas phase, and would be improved with the use of new instruments or new methods, such as nanoscale chromatography (Chetwynd and David 2018).

Identification is still challenging with untargeted methods. However, the expansion of MS/MS databases of xenobiotics and their metabolites, such as HMDB 4.0 (the human Metabolome DataBase) with the implementation of metabolite prediction (Wishart et al. 2018) or the improvement of *in silico* MS/MS solutions (Blaženović et al. 2018), would enable the putative identification of an increasing number of metabolites of xenobiotics. In the particular case of phase II metabolites, MS/MS spectra generally display a loss of the conjugate moiety, with weak information about the structure of the initially conjugated compound, requiring MS<sup>n</sup> experiments. Nevertheless, unambiguous identification is provided by analysis of the standard in all cases (Schymanski et al. 2014). As it will be almost impossible to chemically synthesize all metabolites, the biological production of metabolite standards, *in vivo* or *in vitro*, appears to be a promising alternative (Jamin et al. 2014).

The detection, for the first time, of numerous metabolites of pesticides in human urine, as well as their relative proportion in the same sample, provides valuable information for the selection of relevant biomarkers for future biomonitoring studies. Although data on the urinary excretion of pesticides are generally available in rodents, they have not or have only rarely been described in humans. For example, several authors have highlighted important differences in the metabolic pathways for procymidone between species, including humans using *in vitro* models and/or chimeric mice with humanized hepatocytes (Abe et al. 2018; Tomigahara et al. 2018). Carboxylated-procymidone was found to be a major urinary end-product in rats. On the contrary, hydroxylated-procymidone-glucuronide was a major component in rabbit and monkey urine, but only a minor component in rat urine (Tomigahara

et al. 2015). In our study, we mainly detected 1,2-dimethylcyclopropane-1,2-dicarboxylic acid (99%), whereas 3,5-dichloroaniline was rarely detected (only 10%), suggesting that urinary 1,2-dimethylcyclopropane-1,2-dicarboxylic acid, although undetected or only a minor metabolite in rodents (APVMA (Australian Pesticides and veterinary medicines authority) 2017; Shiba et al. 1991; Tomigahara et al. 2015) could be an appropriate candidate for monitoring procymidone exposure in humans.

Concerning fenpropimorph or azoxystrobin we detected several metabolites that have never been described in humans. These biotransformation compounds deserve to be candidates in the selection of markers of exposure to fenpropimorph or azoxystrobin as summarized in Table 5.

An additional advantage of non-targeted approaches is the possibility of extending contaminant searches to molecules other than pesticides, without the need for additional analysis. Indeed, one of the challenges of exposure measurements in the general population is to be able to link chemical exposure of these populations to possible health effects. It is therefore likely that certain health effects cannot be linked to a single substance or family of substances (e.g., pesticides) but rather to a complex mixture of several xenobiotics that may interact with each other. Because the detrimental effect of one contaminant may be modified by the presence of other compounds, it is useful to be able to identify as many contaminants in human biological fluids as possible. This is consistent with the ongoing expansion of the exposome concept (Niedzwiecki et al. 2019; Wild 2005) and the subsequent development of suspect screening methods, such as that developed here (Pouchet et al., 2020).

## 5. CONCLUSION

An accurate estimate of population exposure to pesticides is necessary to investigate the link between exposure to these chemicals and health. The biomonitoring methods currently used to measure pesticide exposure, all routes taken together, only offer a fragmentary view. The complementarity of the suspect screening and targeted approaches that we have developed substantially increases the number of pesticides that can be considered and advocates a strategy for sequential analysis of pesticides in biological fluids. A first step based on non-targeted analysis would allow extensive detection of exposure biomarkers, while a second step, consisting of targeted analysis, would aim to quantify the previously detected analytes. This promising approach nevertheless requires further analytical development and validation procedures. Because the focus is on urine samples, it is crucial to gain a better understanding of the urinary metabolites of pesticides in humans, as few active substances are eliminated as such *via* this route. A detailed knowledge of the toxicokinetics of each active substance would also help to identify which of the metabolites detected are the most relevant to consider, as well as establish the link between urinary concentrations, levels in target tissues, and the external dose of exposure. Moreover, an effort should be undertaken to make analytical standards that correspond to these metabolites available for quantification by targeted analyses of the biomarkers previously identified by non-targeted approaches.

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## Tables

Table 1. Pesticide metabolites (and corresponding parent compound) primarily detected using the suspect screening approach. The distribution of the signal integration values (peak areas), i.e., 50th and 90th percentiles are expressed in arbitrary units, as suspect screening measures relative abundances. These values do not allow comparison of the concentrations of metabolites with each other.

<b>Most frequently detected urinary metabolite (corresponding pesticide)</b>	<b>%&gt;LOD</b>	<b>P50</b>	<b>P90</b>
2-Methyl-2-[4-(2-carboxypropyl)phenyl]propanoic acid glucuronide (Fenpropimorph)	100	1.6 <sup>E6</sup>	5.1 <sup>E6</sup>
1,2-dimethylcyclopropane-1,2-dicarboxylic acid (Procymidone)	99.1	32.1 <sup>E6</sup>	60.3 <sup>E6</sup>
Methyl-2-(2-hydroxyphenyl)-3-methoxyacrylate sulfate (Azoxytrobins)	98.2	40.2 <sup>E6</sup>	198.6 <sup>E6</sup>
3-methylphenyl-carbamic acid glucuronide (Phenmedipham)	97.9	0.9 <sup>E6</sup>	2.5 <sup>E6</sup>
2-(4-hydroxyphenoxy)propanoic acid glucuronide (Fluazifop- p-butyl and/or quizalofop-p-ethyl)	94.8	2.1 <sup>E6</sup>	5.6 <sup>E6</sup>

LOD: limit of detection, P50: 50th percentile, P90: 90th percentile.

Table 2. Pesticide metabolites (and corresponding parent compound) detected in > 10% of samples using the targeted approach and their respective urinary concentrations in nmol/L. The urinary concentrations are expressed as 50th and 90th percentiles.

<b>Pesticides (analyte measured)</b>	<b>%&gt;LOD</b>	<b>P50</b>	<b>P90</b>
DEP, DETP (Ethyl-organophosphates)	97.5	49.9	212.0
3,5,6-trichloro-2-pyridinol TCPy (Chlorpyrifos)	94.7	5.0	18.7
DMDTP, DMP, DMTP (Methyl-organophosphates)	93.0	51.1	312.0
DCCA, 3PBA, 4F3PBA (Pyrethroids)	89.3	2.0	13.1
Free acids of fluazifop and quizalofop, and 2-(4-hydroxyphenoxy)-propanoic acid (Fluazifop-p-butyl and quizalofop-p-ethyl)	73.8	0.2	4.0
Free acid of fluazifop (Fluazifop-p-butyl)	67.2	0.2	0.7
2,4,6-trichlorophenoxyacetic acid (Prochloraz)	29.5	<LOD	0.8
Bromoxynil (parent compound, bromoxynil)	21.7	<LOD	0.1
2-isopropyl-4-methyl-6-hydroxypyrimidine IMPH (Diazinon)	16.0	<LOD	1.1
3,5-dichloroaniline (Procymidone)	10.2	<LOD	3.3

LOD: limit of detection, P50: 50th percentile, P90: 90th percentile, DCCA: cis/trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid, 3PBA: 3-phenoxybenzoic acid, 4F3PBA: 4-fluoro-3-phenoxybenzoic acid, DMDTP: dimethyldithiophosphate, DMP: dimethylphosphate, DMTP: dimethylthiophosphate, DEP: diethylphosphate, DETP: diethylthiophosphate.

Table 3. *P*-values (and F test for *p*-values < 0.1) for the analysis of variance (lm function, R software) for potential geographical determinants of pesticide exposure based on suspect screening.

	<b>Residence in a town with &gt; or &lt; 20,000 inhabitants (n = 327)</b>	<b>% cereal crops within a 500-meter radius of the residence<sup>2</sup> (n = 273)</b>	<b>% corn crops within a 500-meter radius of the residence<sup>2</sup> (n = 273)</b>	<b>% vegetable crops within a 500-meter radius of the residence<sup>3</sup> (n = 273)</b>
<b>Azoxystrobin</b>	0.7713	0.5369	0.9818	0.1719
<b>Fenpropimorph</b>	0.5681	0.4151	0.6704	0.5769
<b>Phenmedipham</b>	<b>0.0044 (F=8.2)</b> (U>R) <sup>1</sup>	<i>0.0561 (F=2.9)</i> (1>2>3)	0.6803	0.6647
<b>Procymidone</b>	<b>0.0244 (F=5.1)</b> (U>R) <sup>1</sup>	0.1419	0.6740	<i>0.0880 (F=2.9)</i> (0>1)
<b>Fluazifop-p-butyl</b>	<i>0.0755 (F=3.2)</i> (U>R) <sup>1</sup>	0.5986	0.9508	0.3885

1 U: urban: > 20,000 inhabitants, R: rural: < 20,000 inhabitants, 2 three groups according to the tercile of percentages, 3 two groups: presence (1) or absence (0), bold p-values < 0.05, bold p-values in italics < 0.1.

Table 4. Estimation of parameters for Bayesian linear ( $\beta$ ) or logistic (odds ratio) regressions studying the determinants of pesticide exposures based on targeted analyses (n = 244)

Pesticides	Determinants of pesticides	$\beta$	OR	HDI 95%
<b>Pyrethroids</b> (nmol/l, log-scale)	Presence of pea crops in the town of residence	-0.5 <sup>a,b</sup>		[-0.8; -0.2]
	Presence of potato crops in the town of residence	-0.3 <sup>a,b</sup>		[-0.6; 0]
<b>Chlorpyrifos</b> (nmol/l, log-scale)	Presence of vegetable crops within a 500-meter radius of the residence	0.3 <sup>a</sup>		[0; 0.6]
	Percentage of cereal crops in the town of residence:			
	-2 <sup>nd</sup> tercile vs 1 <sup>st</sup> tercile	0.3 <sup>a</sup>		[0; 0.5]
	-3 <sup>rd</sup> tercile vs 1 <sup>st</sup> tercile	0.3 <sup>a</sup>		[0.1; 0.6]
<b>Fluazifop-p-butyl</b> (nmol/l, log-scale)	District of residence (Côtes-d'Armor or Finistère vs Ille-et-Vilaine)	-0.4		[-0.8; -0.1]
	Presence of wheat crops within a 500-meter radius of the residence	0.2		[-0.2; 0.7]
<b>Bromoxynil</b> (detected vs not detected)	Urbanization within a 500-meter radius of the residence		0.2 <sup>a</sup>	[0.1; 0.8]

$\beta$ : Bayesian linear regression coefficient, OR: odds ratio for bromoxynil and diazinon, HDI: highest density interval, HDI is the Bayesian confidence interval. Adjusted for: a urinary creatinine concentrations, b freezing/thawing events, using the Bayesian Model Averaging technique

Table 5. Pesticides for which specific and robust biomarkers were identified using the combination of suspect screening and targeted analysis.

<b>Azoxystrobin</b>	The deconjugated metabolite (2E)-2-methyl (2-Hydroxyphenyl)-3-methoxyacrylate is commercially available, but poor analytical performances was observed with the multi-residue method developed here in urine. However, three conjugated forms of this compound, which is specific for azoxystrobin, were detected by suspect screening in 68 to 98% of samples (n = 333). Their structures were confirmed with an animal experiment (Jamin et al. 2014)
<b>Fenpropimorph</b>	Two metabolites, phenylpropanoic acid derivatives, are commercially available, and were detected in 94 and 82% of samples (n = 333) by suspect screening. Ten other metabolites of the same pesticide were detected in 59 to 100% of these samples by suspect screening. No quantitative measurements were possible with the multi-residue method developed here (incompatibility with the two extraction protocols used for this study), but their structures were confirmed with an animal experiment (Jamin et al. 2014).
<b>Phenmedipham</b>	Five metabolites were detected in >80% of samples by suspect screening. Quantitative measurement was not possible with the multi-residue analysis chosen for this study, but one metabolite is commercially available (methyl-(3-hydroxyphenyl)carbamate).
<b>Fluazifop-P-butyl</b>	The free acid of fluazifop was detected in 68% of samples with the targeted approach. The conjugated form of the 2,4-hydroxyphenoxypropanoic acid was also well detected by suspect screening (94.8% of samples) and its free form was well detected with

	the targeted approach (73.8%).
<b>Chlorpyrifos</b>	3,5,6-trichloro-2-pyridinol (TCPy) was detected in 94% of samples with the targeted approach. DAPs (diethylphosphate and diethylthiophosphate), two other major metabolites of chlorpyrifos, were also well detected in the same samples (97 and 84%, respectively) with the same approach.
<b>Pyrethroid compounds</b>	The 3-phenoxybenzoic acid was detected in 89% of samples with the targeted approach. This metabolite is already widely used in biomonitoring and epidemiological studies.

## FIGURES LEGENDS

Figure 1. Mean levels of phenmedipham and procymidone metabolites according to the number of inhabitants in the town of residence, expressed as the logarithm of MS intensities.

U: urban areas. Number of inhabitants in the town of residence > 20,000. R: rural areas.

Number of inhabitants in the town of residence < 20,000.

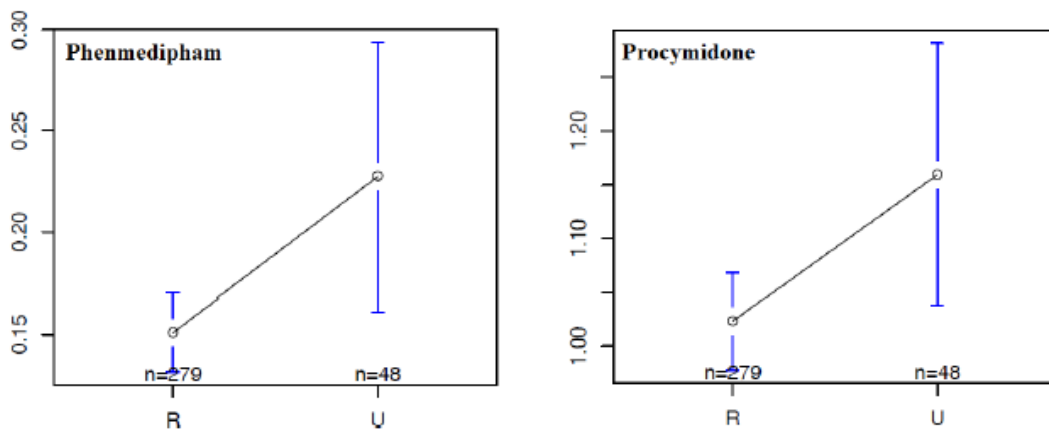
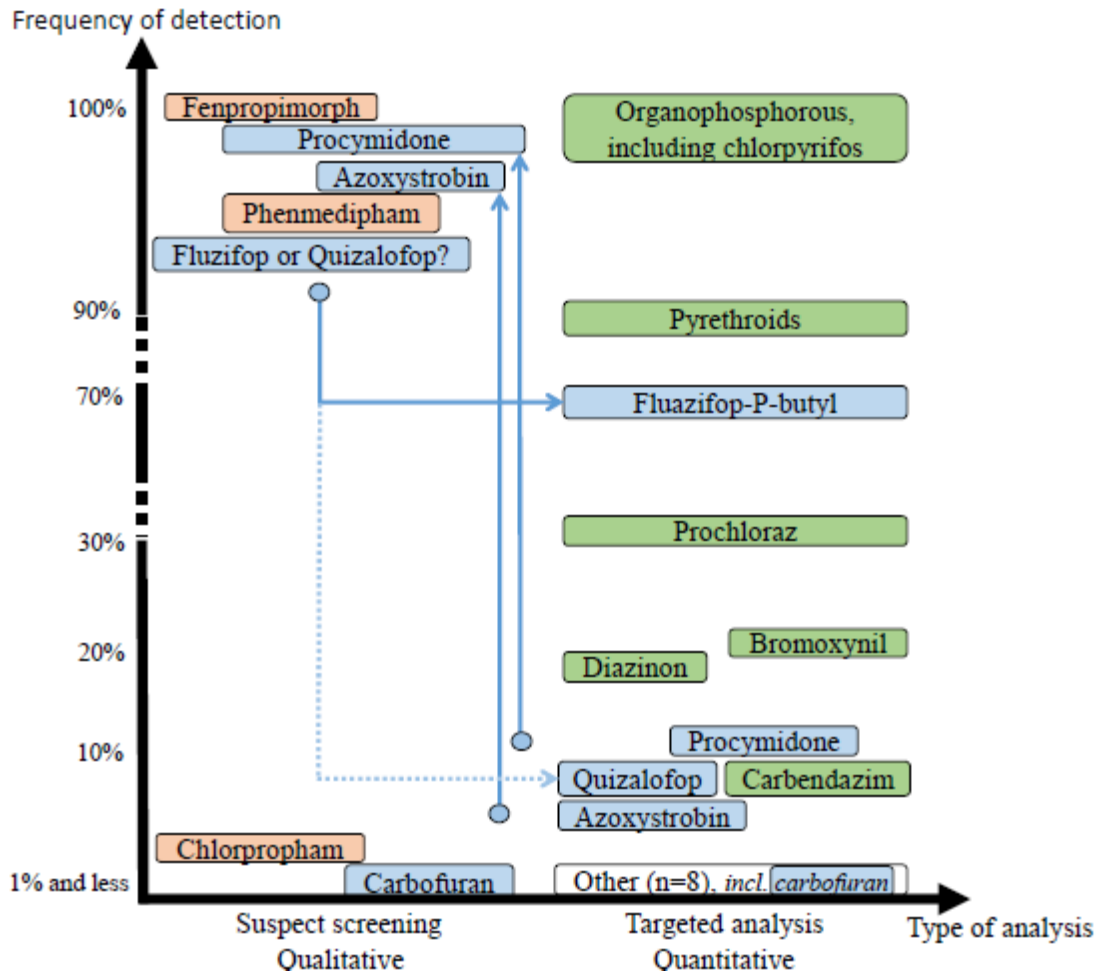


Figure 2: Illustration of the complementarity of suspect screening and targeted analysis to increase the detection of analytes: the case of pesticide exposure. In orange: the compounds only identified using the suspect screening approach, in green: the compounds only identified using targeted analysis, in blue: the compounds identified with the two approaches.

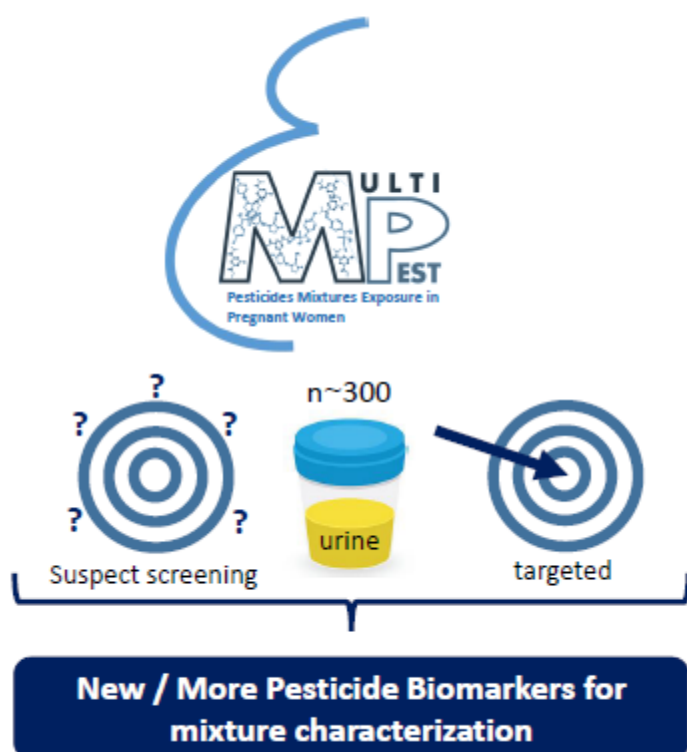


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**Declaration of competing interest**

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.



Graphical Abstract. Illustration of two complementary approaches to characterize human exposure to pesticides. Suspect screening for the detection of pesticide metabolites that are not routinely measured in biomonitoring studies. Targeted quantitative analysis for the detection of highly polar and low molecular-weight metabolites, the confirmation of parent compounds, and the quantification of compounds.

## **Highlights**

- Suspect screening and target analyses were combined for mixture identification.
- Both approaches were used on the same urine samples from pregnant women.
- Exposure to various pesticide families occurred both in rural and urban areas.
- Suspect screening identified highly detected metabolites that are rarely studied.
- Target analysis is a complementary approach for the quantification of specific compounds.