UNIVERSITAT DE BARCELONA

MÀSTER UNIVERSITARI EN QUÍMICA ANALÍTICA

Analytical optimization for the determination of pesticide metabolites in urine samples. Exposure assessment in vulnerable population groups.

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Barcelona, Juliol 2024

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FA CONSTAR

Que el present Màster Universitari en Química Analítica titulat:

Analytical optimization for the determination of pesticide metabolites in urine samples. Exposure assessment in vulnerable population groups.

ha estat realitzat sota la seva direcció per ***Paula Ruiz Francisco*** en els laboratoris de l'**Institut de Diagnòstic Ambiental i Estudis de l'Aigua (IDAEA-CSIC).**

Barcelona, ***Juliol 2024***

Mercè Garí de Barbarà

Aquest treball de final de màster es presenta en format article científic.

Aquest treball de màster ha estat tutoritzat per la Doctora Elisabet Fuguet Jorda del Departament d'Enginyeria Química i Química Analítica de la Universitat de Barcelona.

M’agradaria agrair a la Dra. Mercè Garí tota la seva ajuda, paciència i entrega. Gràcies per donar-me suport i guiar-me en tot moment. El teu compromís i dedicació han sigut fonamentals en aquest projecte. M’has obert les portes a un món que, fins ara, mirava de lluny, la investigació. Donar les gràcies també a la Natàlia, la Carolina, el Martí, l’Antonia i el David, per fer de l’espai de feina un ambient tan agradable i ser sempre de gran ajuda. Sense oblidar a la Clara, la Isabel i la Marianda, sense elles els dinars i els cafès no serien aquell moment tan esperat.

Per acabar, agrair a la meva família i amics per aguantar els meus maldecaps i acompanyar-me en tot moment.

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Summary

Pesticides are biocides designed to control harmful organisms, used both in agriculture and in domestic applications in daily life. This extensive use represents a danger to both human health and the environment.

A fast and robust analytical methodology has been optimized with the aim of determining up to 23 pesticides and specific metabolites of organophosphates, pyrethroids, triazoles, carbamates/thiocarbamates and neonicotinoids. The method is based on solid-phase extraction with methanol and acetone followed by ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). The 5-window method with a duration of 22 minutes was optimized with acceptable results in terms of accuracy (recovery > 75 %), precision (coefficients of variation <26 %) and linearity (R^2> 0.9915). Detection limits ranged from 0.012 ng/ml to 0.058 ng/ml.

After the optimization, an exposure assessment encompassing the analysis of 153 urine samples from two European birth cohort studies has been performed. On the one hand, 94 samples were collected from 14-year-old adolescents from the Polish REPRO_PL cohort. On the other hand, the remaining 59 samples were from pregnant women during the first trimester of gestation from the La Garrotxa Pediatric Environmental Health Unit (PEHSU Olot).

The results of the analyses showed that the most abundant compounds in both cohorts were the metabolites of acetamiprid (DM-ACE) and tebuconazole (TEB-OH). An international comparison showed that Polish adolescents had the lowest concentrations of DEAMPY (metabolite of pirimiphos), IMPY (metabolite of diazinon), PNP (metabolite of parathion), TCPY (metabolite of chlorpyrifos), 3PBA (metabolite of several pyrethroids) and 4F3PBA (metabolite of cyfluthrin) compared to other studies conducted in Spain, Italy, Slovenia, USA, Thailand and Australia. Conversely, pregnant women from La Garrotxa had the highest concentrations of DEAMPY in relation to the respective comparison studies.

This work has allowed to improve a methodological approach for detecting and quantifying a large broad of pesticides and their metabolites in urine samples. The method is suitable for human biomonitoring studies, as shown in the current exposure assessment performed in two birth cohorts. Further studies are required to evaluate the health risks and impacts posed by the exposure to pesticides in vulnerable population groups such as pregnant women, children and adolescents.

Resum

Els pesticides són biocides dissenyats per controlar organismes nocius, utilitzats tant en l'agricultura com en aplicacions domèstiques en la vida quotidiana. Aquest ús extensiu representa un perill tant per a la salut humana com per al medi ambient.

S'ha optimitzat una metodologia analítica ràpida i robusta amb l'objectiu de determinar fins a 23 pesticides i metabòlits específics d'organofosfats, piretroides, triazoles, carbamats/tiocarbamats i neonicotinoids. El mètode es basa en l'extracció en fase sòlida amb metanol i acetona seguida de cromatografia líquida d'ultra-rendiment acoblada a espectrometria de masses en tàndem (UPLC-MS/MS). El mètode de 5 finestres amb una durada de 22 minuts es va optimitzar amb resultats acceptables en termes d'exactitud (recuperació > 75 %), precisió (coeficients de variació <26 %) i linealitat (R2> 0,9915). Els límits de detecció oscil·len entre 0.012 ng/ml i 0.058 ng/ml.

Després de l'optimització, s'ha realitzat una avaluació de l'exposició que inclou l'anàlisi de 153 mostres d'orina de dos estudis europeus de cohorts de naixement. D'una banda, es van recollir 94 mostres d'adolescents de 14 anys de la cohort polonesa REPRO_PL. D'altra banda, les 59 mostres restants eren de dones embarassades durant el primer trimestre de gestació de la Unitat de Salut Ambiental Pediàtrica de la Garrotxa (PEHSU Olot).

Els resultats de les anàlisis van mostrar que els compostos més abundants en ambdues cohorts eren els metabòlits de l'acetamiprid (DM-ACE) i el tebuconazol (TEB-OH). Una comparació internacional va mostrar que els adolescents polonesos tenien les concentracions més baixes de DEAMPY (metabòlit de pirimifos), IMPY (metabòlit de diazinon), PNP (metabòlit de paratió), TCPY (metabòlit de clorpirifos), 3PBA (metabòlit de diversos piretroides) i 4F3PBA (metabòlit de la ciflutrina) en comparació amb altres estudis realitzats a Espanya, Itàlia, Eslovènia, EUA, Tailàndia i Austràlia. En canvi, les embarassades de la Garrotxa van presentar les concentracions més altes de DEAMPY en relació amb els respectius estudis comparatius.

Aquest treball ha permès millorar l'enfocament metodològic per detectar i quantificar una gran varietat de pesticides i els seus metabòlits en mostres d'orina. El mètode és adequat per a estudis de biomonitorització humana, tal com es mostra en l'avaluació d'exposició actual realitzada en dues cohorts de naixement. Es requereixen estudis addicionals per avaluar els riscos i impactes per a la salut que suposa l'exposició a pesticides en grups de població vulnerables com ara dones embarassades, nens i adolescents.

Sustainable Development Goals

In 2015, in order to achieve a more just, peaceful, prosperous and sustainable world, the United Nations established a set of 17 interconnected goals called Sustainable Development Goals (SDGs). These goals encompass a wide range of topics, from poverty eradication to gender equality, quality education, climate action and peace and justice. The SDGs are a call to action for all stakeholders, including governments, the private sector, civil society and citizens, to work together to achieve a sustainable future for all. This project will mainly affect two main areas: people and the planet.

Within these two broad areas are the following four main goals; firstly, there is goal number 3 (Good health and well-being). This goal seeks to improve people's health in recent years. Pesticides are used in agriculture to eradicate pests; however, their abusive use can have serious consequences for the environment and the human health. Children and pregnant women are particularly vulnerable as the foetus can be affected causing developmental problems, among others. This study highlights the need for preventive and mitigation policies to protect health of these exposed populations. Some of the targets involved on this goal are 3.2 (end preventable deaths of newborns and children), 3.4 (reduce premature mortality from non-communicable diseases) and 3.9 (reduced the number of deaths and illnesses from hazardous chemicals).

Pesticides not only affect humans, but also have a significant impact on terrestrial and aquatic ecosystems. Through agriculture, pesticides can affect drinking water quality, soil health and biodiversity, reaching diverse species and ecosystems. This study provides valuable information on the presence of pesticides in human biological systems, reflecting environmental contamination and the need to better manage and regulate pesticides use to protect terrestrial and aquatic sources, ensuring clean and safe water for all. This information is essential for implementing conservation and ecosystem restoration measures, promoting a more sustainable use of land and natural sources. For these reasons, this study can be related to goals 6 (Clean water and sanitation) and 15 (Life on land), particularly to targets 6.3 (improve water quality by reducing pollution) and 15.1 (sustainable use of terrestrial and inland freshwater ecosystems).

Finally, it is important to highlight the importance of a proper pesticide management and to promote the use of safer alternatives and sustainable agricultural practices. For these reasons goal 12 (Responsible consumption and production) with its target 12.4 (environmentally sound management of chemicals and all wastes) should be mentioned. By providing evidence on exposure to pesticides, my research can encourage policies that promote more responsible production and consumption, reducing environmental impact and promoting public health.

To conclude, the analysis of pesticides in urine of children and pregnant women does not only address the concern on the effect of pesticide exposure impacts on human health and the environment, but also aligns with the UN Sustainable Development Goals by promoting sustainability, health and well-being globally.

Analytical optimization for the determination of pesticide metabolites in urine samples. Exposure assessment in vulnerable population groups

1. INTRODUCTION

Pesticides are chemical compounds used to control harmful organisms, such as insects, fungi, rodents or unwanted plants. They are mainly used in agriculture and livestock to eradicate pests that affect crops or herds, and in daily life applications, such as insect repellents, gardening or ornamental plants and in products for pets (European Comission, n.d.-b; World Health Organization, 2020). They are also used for health applications, including creams for dermatological disorders (e.g. scabiosis, mooring).

Although pesticides play a very important role in food production, protecting and increasing crop yields (Pesticide Residues in Food, n.d.), an extensive use causes pollution in various environmental compartments, including water, air and soil, thus entering in the food chain (Bravo et al., 2019). Pesticide residues have been found in freshwater fish for human consumption (Bille et al., 2017). These events have triggered an increase in public concern, not only because of the great impact on the environment, but also because of human exposure and its impact on population's health. Pesticides such as carbamate/thiocarbamate (CBM), neonicotinoids (NEO), organophosphates (OP) and pyrethroids (PYR) are neurotoxic chemicals because of their potential to interfere with neurotransmission, either blocking specific receptors, or to alter the normal function of neural membrane channels, respectively, producing erroneous transduction of nerve signals (Mouskeftara et al., 2021; Rani et al., 2021) (Figure S1). Some studies highlight the association between exposure to pesticides and various adverse health effects, such as cancer, diabetes or reproductive and endocrine complications (Engel et al., 2017) (Kamijima et al., 2004). Human exposure can occur via ingestion, direct skin contact or inhalation (e.g. due to proximity to fumigation areas or domestic application of pesticides in sprays), although the diet has been identified as the main source (Becker et al., 2006). Once pesticides are found in the human body, they are metabolized or hydrolysed, and the resulting products (metabolites) are excreted in urine, both in its free form and attached to glucuronide or sulphatase. (Barr, 2008).

Children are especially susceptible to environmental toxins, especially their brain and nervous system, due to rapid growth during this developmental period. Their still immature metabolic pathways do not allow them to fully detoxify and excrete toxic pollutants, causing an increased risk on neurotoxicity compared to adults (Landrigan et al., 2019). The detection of pesticides in amniotic fluid and meconium (Berton et al., 2014) suggests that fetuses are exposed to these chemical compounds. Prenatal exposure to pesticides has been related to neurodevelopmental problems, (Biosca-Brull et al., 2021; González-Alzaga et al., 2014), obesity and diabetes (Debost-Legrand et al., 2016; Pinos et al., 2021), respiratory difficulties (Raanan et al., 2016) and shorter gestation time (Eskenazi et al., 2004). On the other hand, early postnatal exposures, occuring during the first months of the baby's life, either through breastfeeding, or by repeated and unintentional ingestion due to hand-to-mouth behaviour (Landrigan et al., 2019; Zartarian et al., 2000), can also impact their neurological development (González-Alzaga et al., 2014). In this regard, prenatal and postnatal exposures to pesticides have been linked to an increased risk of autism spectrum disorders (Biosca-Brull et al., 2021) and respiratory

problems (Raanan et al., 2016), among others. For these reasons, pregnant women and children are vulnerable population groups.

Human biomonitoring studies are currently performed to evaluate the degree of exposure to environmental pollutants, including pesticides, from general populations worldwide. Non-persistent pesticides are mainly excreted in urine after 48-72h of exposure, depending on the compound (F. Fernández et al., 2020). Therefore, urine is the simplest and least intrusive analysis matrix that allows to evaluate exposure to pesticides at a population level. The methods previously published for the analysis of urine metabolites are based on both gas chromatography and liquid chromatography and mainly use mass spectrometry techniques for their detection (Cequier et al., 2016; Koureas et al., 2012; Roca, Leon, et al., 2014). Target and suspect screening methods provide extensive and accurate quantitative analysis of pollutants (Bonvallot et al., 2021). The method developed by Garí et al. (2018) allowed the characterization of several types of pesticides, mainly organophosphates and pyrethroids, in a single methodology, and since then (2018) it has been used in various human biomonitoring studies including birth cohorts from Spain, Italy, Slovenia, Finland and Poland (Bravo et al., 2019; Bravo, Grimalt, et al., 2020; Bravo, Peralta, et al., 2020; Garí et al., 2018). It has also been applied to occupational studies (mainly farmworkers) from Spain and Argentina (Bravo et al., 2022; Filippi et al., 2021).

This study aims to optimize the existing methodology on the determination of organophosphate (OP) and pyrethroid (PYR) pesticide metabolites, to include further pesticide gropus, including neonicotinoids (NEO), carbamates/thiocarbamates (CBM) and triazoles (TRI). The new methodology will allow the identification and characterization of up to 23 pesticides and their metabolites from five different families in urine, to be used in human biomonitoring studies. Urine samples from, two European cohorts in Catalonia (PEHSU La Garrotxa, Olot) and in Poland (REPRO_PL) will be analysed and an exposure assessment will be performed.

2. MATERIALS AND METHODS

2.1 Standards, solvents and reagents

A total of 23 pesticides and their metabolites are included in this assessment, including 5 OP metabolites (2-isopropyl-6-methyl-4-pyrimidiol, IMPY; 2-diethylamino-6-methyl pyrimidin-4-ol, DEAMPY; 3,5,6-trichloro-2-pyridinol, TCPY; 4-nitrophenol, PNP; 3-chloro-4-methyl-7-hydroxicoumarin, CMHC), 2 PYR metabolites (3-phenoxybenzoic acid, 3PBA; and 4-fluoro-3-phenoxybenzoic acid, 4F3PBA), 3 CBM metabolites (Carbofuran, CARBO; 3-hydroxycarbofuran, 3OH-CARBO; and cis-1,2,3,6-tetrahydrophthalimide, THPI), 2 TR metabolites (tebuconazole, TEB; and 4-hydroxy-4-(1H-1,2,4-triazole-1-ylmethyl)-5,5-dimethyl-hexanoic acid, TEB-OH) and 11 NEO and their metabolites (imidacloprid, IMI; 5-hydroxy-imidacloprid, 5OH-IMI; imidacloprid-olefin , IMI-OLE; 6-chloronicotinic acid, 6ClNA; clothianidin, CLO; clothianidin-desmethyl, DM-CLO; dinotefuran, DIN; dinotefuran-demethyl, DM-DIN; thiamethoxam-demethyl, DM-THX; acetamiprid, ACE; and N-(6-Chloro-3-pyridylmethyl)-N-cyano-acetamidine, DM-ACE).

Standards of IMPY and TCPY were purchased from Sigma-Aldrich (Madrid, Spain), PNP from Supelco (Madrid, Spain), CMHC from Acros Organics (Geel, Belgium). ACE, DM-CLO and 5OH-IMI were purchased from Camdrigde Isotope Laboratories (Andover, MA, USA). Whereas, DEAMPY, MDA, 3PBA, 4F3PBA, CLO, DIN, DM-DIN, IMI, 6ClNA, IMI-OLE, DM-THX, DM-ACE, CARBO, 3OH-CARBO, TEB, TEB-OH and THPI from Dr. Ehrenstorfer (Augsburg, Germany).

The isotopically-labeled standards of 3PBA, IMPY, PNP, IMI, IMI-OLE, CLO, DM-CLO, DIN, ACE and DM-ACE were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Whereas, THPI isotopically-labeled was purchased from TRC (Toronto, Canada) and CARBO and TEB from Dr. Ehrenstrofer (Augsburg, Germany).

All solvents used were of analytical grade. Acetonitrile was from Panreac (Barcelona, Spain), methanol, acetone and water for HPLC analysis were from Merck (Darmstadt, Germany), glacial acetic acid was from Scharlab (Barcelona, Catalonia, Spain), and sodium acetate anhydrous and β-glucuronidase type H-1 from Helix pomatia were from Sigma-Aldrich (Madrid, Spain) and synthetic urine (Surine, Preserve Free).

2.2 Sample preparation and extraction procedure

Sample preparation followed the method described in (Garí et al., 2018). In a 10 ml centrifugal tube, 1ml of urine was introduced, previously centrifugated, and 10 µl of a mixture of internal standards isotopically labelled at a concentration of 200 ppb was added. To hydrolyse possible conjugate metabolites of glucuronide or sulphatase, H-1 type glucoronidase of Helix pomatia was used with a specific activity (~1000 units/mg). The samples were incubated overnight at 37 °C and subsequently extracted using solid phase extraction (SPE, Oasis HLB 3 cm^3, Waters, Milford, MA, USA). SPE cartridges were pre-conditioned with 1 ml of methanol/acetone (25:75 v/v) and then with 1 ml of 1% (v/v) of acetic acid in H_2O HPLC. Incubated samples were added and passed through cartridges, which were subsequently washed with 500 µl of 1% (v/v) of acetic acid in H_2O HPLC. After drying the cartridges for 30 minutes in vacuum, the target chemicals were released with 1.5 ml of a methanol:acetone solution (25:75 v/v). The collected extracts were evaporated to almost dry under a soft current

of pure nitrogen at 40 °C. Then, they were transferred quantitatively to chromatographic vials by adding 120 µl of methanol:water solution (25:75 v/v).

2.3 Instrumental analysis

Identification and quantification of compounds was carried out using an Ultra Performance Liquid Chromatography (UPLC Acquity H-Class, Waters, Milford, MA, USA) coupled to a Triple Quadrupole Mass Spectrometer (XEVO-TQ-S, Waters, Milford, MA, USA) equipped with an electrospray ionization (ESI) interface. The chromatographic separation of the compounds was carried out following the method proposed by Garí et al., 2018, optimizing it to detect a greater number of compounds. It was carried out in a Betasil C18 column (100 mm × 2.1 mm, 3 µm particle size, Thermo Scientific, West Palm Beach, FL, USA). To extend the life of the column, a guard holder and guard column of the same sorbent material (2.1 µm and 3.0 mm id, Universal Uniguard Holder, Thermo Scientific, West Palm Beach, FL, USA) were installed inline before the column. The injection volume was 10 µl, at a flow rate of 0.3 ml/min. The column temperature was kept at 30 °C during the analysis.

A solution gradient consisting of a moving phase of acetonitrile and a mixture of H_2O HPLC with 1% CH_3COOH and 5% CH_3OH was used. The gradient began with ACN/Mix 2:98, increased to 20:80 in 4 min, then to 40:60 in 3 min, to 50:50 in the 14th minute, and ended with ACN 100% in the 16.5 minute. During the next 3 min, the column was cleaned with 100% ACN, adjusting to the initial conditions in minute 19.5 to minute 22.

During operating time, MS acquisition parameters changed following 5 different timed segments. In segments 1 to 3, data were acquired in positive ionization mode: the 1st segment began at minute 2.5, 2nd at minute 6 and 3rd at minute 11. On the other hand, in segments 4 and 5, data were acquired in negative ionization mode: 4th segment began at minute 4.5 and 5th at minute 11. The total time was 22 minutes.

2.4 Quality assurance procedure

Blanks and quality control material (QCs) were prepared using synthetic urine. Blanks were used to measure laboratory contamination, both by staff manipulation and by the use of material and solvents. One blank was analysed for every 15 urine samples. Two synthetic urine samples were enriched with different concentrations of analytes, one with a low concentration (QCL, 1 µg/l) and one with a high concentration (QCH, 10 µg/l). Calibration lines were prepared by adding 25 µl of the different pattern solutions with concentrations of a range of 2.5 ppb to 560 ppb in 1 ml of synthetic urine, reaching final concentrations of 0.06, 0.12, 0.24, 0.48, 0.95, 1.9, 2.4, 4.8, 9.5 and 13.3 ng/ml in urine. Quantification was performed using internally marked isotopic patterns (Garí et al., 2018). A batch includes 10 calibration points, 1 blank, 1 QCL, 1 QCH and up to 10 urine samples.

Recoveries (in percentage, %) were calculated for each target analyte, based on matrix concentration relative to standard concentration, using QCH/QCL quantified with calibration curves. Intra-day precision (coefficient of variation (CV), %) was evaluated by ten QCL and QCHs duplicate analyses. Duplicate QCL and QCHs analyses were performed in seven different days to obtain the inter-day precision (CV, %). The acceptance criteria for recovery and precision are 75% and 25%, respectively.

Matrix effects were calculated using real urine samples (pooled) and synthetic urine samples, both spiked after the extraction, according to Panuwet et al., 2016. Most of the compounds showed up to 30% of signal suppression (Figure 1). Only IMI-OLE showed a signal suppression of 50%, and THPI, DIN and DM-ACE showed a minor signal enhancement (<110%).

Limits of detection (LDs) were calculated by the DIN 32645 methodology (equivalent to ISO 11843) using calibration straight lines. The calculation was carried out following the previous descriptions (Massart, 1997) and was implemented through the chemCal Package (Ranke, 2015) of the statistical software R (R Core Team, 2023).

The methodology has been externally validated by participating in rounds of the German External Quality Assessment Scheme (G-EQUAS) since 2016, which includes two OP metabolites (PNP and TCPY), two PYR metabolites (3PBA and 4F3PBA and one NEO metabolite (6ClNA).

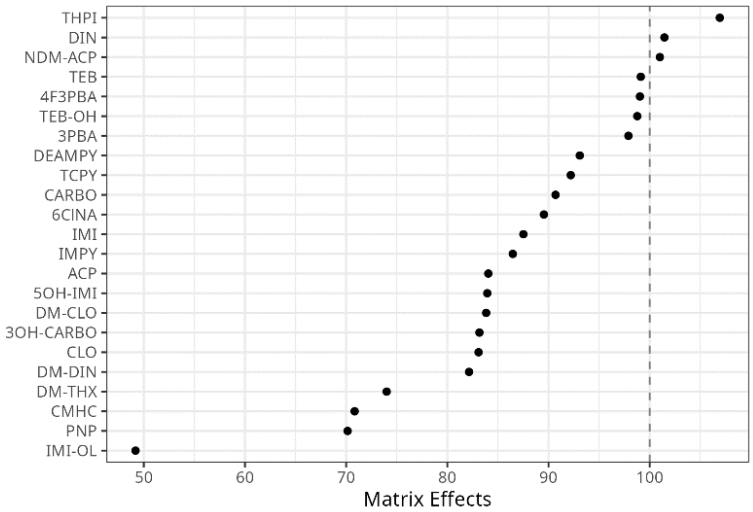

Figure 1. Matrix effects percentages (%) for each compound.

2.5 Data analysis

Data acquisition, data management, chromatograms and instrumental control were performed with version 4.1 of Masslynxs software (Waters Inc., 2008). Data analysis for method development (calculation of detection limits, linearity of calibration curves, recoveries, intra-day and inter-day precision), descriptive statistics for exposure assessment (geometric means (GM) and confidence intervals (CI) at 95%, medians, percentiles, range) and graphics were performed using the statistical software R (R Development Core Team, 2023).

Descriptive statistics were focused on the metabolites found above limit of detection in more than 50% of the samples. Values under the LDs were replaced by one-half of the LD.

2.6 Human biomonitoring

An exposure assessment in two European birth cohorts has been performed. On the one hand, the study is based on the mother-child cohort (REPRO_PL), a prospective birth cohort established in 2007 in Poland, with the recruitment of pregnant women. Details concerning the methodology of the cohort have been published previously. The study comprised four phases covering the prenatal phase (phase I: 2007-2011 (Polańska et al., 2009), the examination of the

child at 1 to 2 years of age (phase II: 2008-2013) (Polańska et al., 2011) the examination of the child at the age of 7 to 9 (phase III: 2014-2019) (Polańska et al., 2016) and the examination of children during the age of 14 (phase IV: 2020-2024) (Janc et al., 2022). REPRO_PL aims to assess the impact of a variety of environmental and lifestyle-related factors on pregnancy outcomes and children's health. To do this, a sample of the Polish population was selected, sampling pregnant women (and their children) from various regions, including large cities with more than 500 thousand inhabitants, as well as small towns and cities. A preliminary urine sample of 94 children who were followed-up until the age of 14 years old (phase IV) has been recruited in 2023-2024. The four phases of REPRO_PL were approved by the Bioethics Committee of the Nofer Institute of Occupational Medicine, Lodz, Poland (Decision No. 7/2007, 3/2008, 22/2014, 3/2021).

On the other hand, the cohort of pregnant women in La Garrotxa, Olot, is part of the first Pediatric Environmental Health Unit of Catalonia (PEHSU), from where activities such as environmental screening of pregnancy are carried out. A total of 59 urine samples were collected from pregnant women during the first trimester of pregnancy in the period September 2023 to March 2024. The study was approved by the IDIAPJGol Clinical Research Ethics Committee (CREC), Catalonia, Spain (Ref. Code 20/222-P).

3. RESULTS AND DISCUSSION

3.1 Method optimization

Preliminary MS/MS optimization experiments were carried out using single-compound standard solutions (800 ng /ml) prepared in ACN, to obtain the precursor ion and products. The preliminary collision energy value for each MS transition was obtained by checking the best signal from 10 to 60 eV with an increment of 10 eV. When the collision energy value was selected, the optimal cone voltage value was obtained from the best production transition signal achieved.

Two selected reaction monitoring (SRM) were chosen for each analyte; the first was selected for quantification (Q -SRM) based on abundance, and the second for confirmation (C-SRM).

SRM transitions and their corresponding ion ratios (IRs) were used to identify the analytes in the samples and to distinguish between possible coelutions.

The retention time (RT), IRs, optimum value of collision and cone energies for the quantitative transition, the MRM windows (MRMw) and the ion mode selected for each analyte (positive or negative) are summarized in Table 1.

Table 1. List of pesticides and their metabolites and instrumental analytical data of the analytes considered in the present study.

Parent Pesticide	*Analyte*	*Acronym*	*Q-SRM*	*C-SRM*	*IR*	*CE[a]*	*CV[a]*	*RT*	*MRMw*
Neonicotinoids (NEOs)									
Imidacloprid	Imidacloprid	IMI	256-175	256-209	1.1	20	20	4.53	2 (ESI+)
	5-hydroxy-imidacloprid	5OH-IMI	272-225	272-191	1.1	20	20	4.06	2 (ESI+)
	Imidacloprid-olefin	IMI-OLE	254-207	254-81	9.9	30	10	3.86	4 (ESI-)
	6-chloronicotinic acid	6ClNA	158-122	158-78	1.8	20	30	4.33	2 (ESI+)
Clothianidin	Clothianidin	CLO	250-169	250-132	0.69	20	10	4.36	2 (ESI+)
	Clothianidin-desmethyl	DM-CLO	236-132	236-155	1.6	10	20	4.08	2 (ESI+)
Dinotefuran	Dinotefuran	DIN	203-129	203-113	1.6	20	10	2.87	1 (ESI+)
	Dinotefuran-desmethyl	DM-DIN	189-99	189-100	1.9	20	10	2.41	1 (ESI+)
Thiamethoxam	Thiamethoxam-desmethyl	DM-THX	278-197	278-167	1.9	30	10	4.79	2 (ESI+)
Acetamiprid	Acetamiprid	ACE	223-126	223-56	4.7	20	30	8.37	2 (ESI+)
	N-(6-Chloro-3-pyridylmethyl)-N-cyano-acetamidine	DM-ACE	209-126	209-90	5.3	20	25	7.57	2 (ESI+)
Carbamates/Thiocarbamates (CBMs/TCBMs)									
Carbofuran	Carbofuran	CARBO	222-165	222-123	1.2	10	30	10.31	2 (ESI+)
	3-hydroxycarbofuran	3OH-CARBO	238-181	238-163	3.4	10	25	7.48	2 (ESI+)
Captan	Cis-1,2,3,6-tetrahydrophthalimide	THPI	152-81	152-79	5.8	20	10	6.02	1 (ESI+)
Triazoles (TRIs)									
Tebuconazole	Tebuconazole	TEB	308-70	308-125	15.3	20	20	16.17	3 (ESI+)
	4-hydroxy-4-(1H-1,2,4-triazole-1-ylmethyl)-5,5-dimethyl-hexanoic acid	TEB-OH	324-70	324-125	4.2	30	20	11.69	3 (ESI+)
Organophosphates (OPs)									
Pirimiphos	2-diethylamino-6-methyl pyrimidin-4-ol	DEAMPY	182-154	182-84	1.3	20	40	4.65	1 (ESI+)
Diazinon	2-isopropyl-6-methyl-4-pyrimidiol	IMPY	153-84	153-70	1.9	20	40	5.05	1 (ESI+)
Parathion	4-nitrophenol	PNP	138-108	138-92	8.2	20	45	8.66	4 (ESI-)
Chlorpyrifos	3,5, 6 -trichloro-2-pyridinol	TCPY	196-196	198-198	1.0	7	10	11.28	4 (ESI-)
Coumaphos	3-chloro-4-methyl-7-hydroxicoumarin	CMHC	209-145	209-117	3.2	25	20	9.70	4 (ESI-)
Pyrethroids (PYRs)									
Several PYR	3-phenoxybenzoic acid	3PBA	213-93	213-169	1.6	20	30	12.87	5 (ESI-)
Cyfluthrin	4-fluoro-3-phenoxybenzoic acid	4F3PBA	231-187	231-93	1.3	15	25	13.04	5 (ESI-)

Q-SRM: Quantification Selected Reaction Monitoring; C-SRM: Confirmation Selected Reaction Monitoring; IR: Ion ratio; CE: Collision energy; CV: Cone voltage; RT: Retention time; MRMw: MRM window.

[a] CEs and CVs refer always to the Q-SRM

[b] 6ClNA is a common metabolite of imidacloprid and acetamiprid

3.2 Method validation

Linearity was evaluated using calibration straight lines. Good linearity (R2>0.9915) was obtained by these calibration straight lines for all the target analytes (Table 2).

Sensitivity of the method was assessed by the limit of detection, which were in the range of 0.013 ng/ml and 0.058 ng/ml (Table 2). Limits of detection were below 0.058 ng/ml for OP metabolites, between 0.04 ng/ml and 0.052 ng/ml for PYR metabolites could be achieved (Table 2). As for CBM and TRI, limits of detection were between 0.013 ng/ml and 0.048 ng/ml, whereas for NEOs, LDs were below 0.041 ng/ml. These values demonstrate an adequate sensitivity for trace analysis of pesticides in human urine. Accuracy and precision of the method were assessed from two concentration levels, 1 ng/ml and 10 ng/ml, QCL and QCH, respectively, using synthetic urine. They are expressed as coefficients of variance (CVs), and are shown in Table 2.

Intra-day and inter-day CVs were below 25% in all the cases, except for TEB-OH, for which a percentage of 26% was calculated (in the case of QCH inter-day CV). Together with TEB-OH, THPI and 3OH-CARBO were the analytes with the highest CVs. Recoveries ranged from 78% to 99%, depending on the compound. In general, linearity, recoveries and CVs were within the acceptable variability limits, indicating that the accuracy and precision of the method were satisfactory for a routine human biomonitoring purpose.

Table 2. Limits of detection, calibration curve linearity, recoveries, intra-day and inter-day precision for the studied analytes.

Acronym	*LD (ng/ml)*	*Linearity (R^2)*	*Recovery (%)*	*Intra-day precision (CV, %)*		*Inter-day precision (CV, %)*	
				QCL	*QCH*	*QCL*	*QCH*
IMI	0.029	0.9983	98 (±15)	14	14	15	13
5OH-IMI	0.035	0.9987	86 (±11)	15	12	13	13
IMI-OLE	0.041	0.9926	82 (±13)	8	14	18	10
6ClNA	0.022	0.9957	91 (±28)	7.7	12	16	9.7
CLO	0.018	0.9982	99 (±14)	12	13	18	15
DM-CLO	0.012	0.9999	95 (±8.9)	6	7.7	6.3	7.3
DIN	0.017	0.9969	87 (±16)	9.1	10	17	14
DM-DIN	0.023	0.9970	83 (±26)	13	8.5	18	21
DM-THX	0.026	0.9995	95 (±12)	11	10	11	15
ACE	0.017	0.9988	83 (±14)	7.4	9.6	10	15
DM-ACE	0.037	0.9994	78 (±14)	8.8	8.6	7.7	14
CARBO	0.013	0.9967	97 (±10)	8.4	7.8	10	8.8
3OH-CARBO	0.017	0.9953	90 (±7.5)	16	18	23	16
THPI	0.048	0.9966	97 (±24)	16	20	18	25
TEB	0.019	0.9969	96 (±16)	5.3	9.7	11	19
TEB-OH	0.032	0.9988	93 (±25)	9.1	25	18	26
DEAMPY	0.045	0.9984	86 (±9.1)	6.6	2.2	6.5	5.3
IMPY	0.030	0.9961	92 (±7.1)	6.2	1.0	6.9	6.3
PNP	0.058	0.9944	92 (±23)	7.8	4.8	11	6.3
TCPY	0.054	0.9976	81 (±26)	14	21	22	12
CMHC	0.039	0.9915	98 (±21)	11	18	24	7.7
3PBA	0.041	0.9983	90 (±26)	14	4.1	11	9.7
4F3PBA	0.052	0.9941	99 (±12)	13	3.1	13	9.9

Analysis of proficiency testing materials obtained from G-EQUAS rounds provided results in accordance with the reference result and their acceptable ranges (Table 3).

Table 3. Results obtained in the analysis of proficiency testing materials from G-EQUAS program (RV-59 sample 14-15A for PNP and TCPY, and RV-65 sample 9A for 3PBA, 4F3PBA and 6ClNA). Reference values and tolerance ranges provided by G-EQUAS are also shown. Concentration units are expressed in ng/ml.

	PNP	*TCPY*	*3PBA*	*4F3PBA*	*6ClNA*
	(RV-59)	*(RV-59)*	*(RV-65)*	*(RV-65)*	*(RV-65)*
Result A	35.11	12.35	3.52	1.67	1.46
Ref. value A	34.4 [26.9 - 41.9]	12.72 [10.71 -14.73]	3.21 [2.45 - 3.97]	1.83 [1.38 - 2.28]	1.26 [0.84 - 1.68]

The total ion chromatograms (TIC) of synthetic and real urine extracts previously fortified with the analytes of interest are compared in Figure 2. As shown in this figure, real urine extracts present many peaks which may interfere in the real signals of calibration straight lines, whereas fortified synthetic urine samples only show the peaks of the selected analytes.

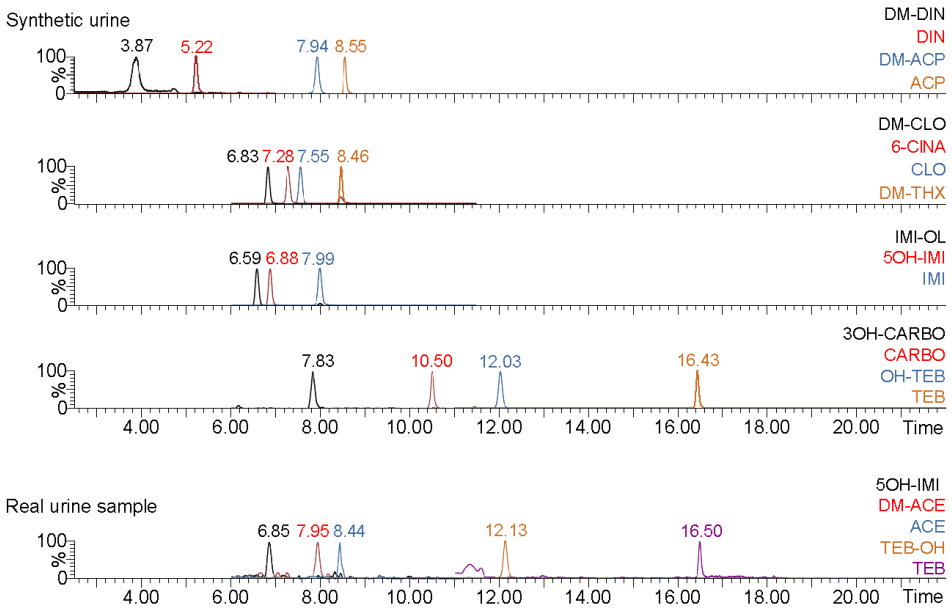

Figure 2. TIC chromatograms of a spiked synthetic urine extract (QCH) and a real urine extract. (Bustamante et al., 2024)

3.3 Analysis of urine samples from birth cohorts

Descriptive statistics for the measured urinary concentrations of the analysed metabolites are reported in Table 4.

The application of this procedure to human urine samples from pregnant women living in La Garrotxa, Olot, shows that almost all mothers presented detectable levels of OPs, PYR, TRI and NEOs. Detection frequencies of each compound ranged from non-detected to 92%, being DM-ACE (92%) and TEB-OH (86%) the most frequently detected compounds, followed by DEAMPY (83%) and 3PBA (81%). These four most abundant metabolites have geometric mean (GM) concentrations of 0.82 ng/ml (DM-ACE), 0.32 ng/ml (TEB-OH), 1.6 ng/ml (DEAMPY) and 0.42 ng/ml (3PBA) (Table 4). TEB, CMHC, ACE and THPI had detectable concentrations lower than 5% (Table 4). Regarding pyrethroids, 3PBA metabolite was the one found at highest concentration (GM of 0.41 ng/ml) followed by 4F3PBA (GM of 0.020 ng/ml). A higher concentration of 3PBA is expected since it reflects contributions from several pyrethroids whereas 4F3BA is the only specific metabolite of cyfluthrin.

Concerning the Polish cohort, detection frequencies in the studied children ranged from non-detected to 92% of detection, being TEB-OH (82%), DM-ACE (86%) and PNP (84%) the most frequently detected compounds, followed by TCPY (83%), and 3-PBA (61%). GM concentrations of these five most abundant metabolites were 0.31 ng/ml, 0.42 ng/ml, 0.57 ng/ml, 0.30 ng/ml and 0.15 ng/ml, respectively. As regards pyrethroids, 3PBA was the one found at highest concentration (GM of 0.15 ng/ml).

Considering the two cohorts, notable differences can be observed between them. In the case of OP metabolites PNP and TCPY, detection levels of children from Poland were a bit higher than in pregnant women from La Garrotxa (84% vs. 60% for PNP, respectively, and 83% and 76% for TCPY, respectively). These metabolites are originated from parathion and chlorpyrifos, and despite there are currently prohibited, they are still found in human urines of European

populations, probably due to consumption of contaminated food of non-European origin (Supporting material, section 2). The concentrations of these metabolites were slightly higher in adolescents from Poland than in pregnant women from La Garrotxa, with GM of 0.57 ng/ml and 0.20 ng/ml for PNP, respectively, and 0.30 ng/ml and 0.22 ng/ml for TCPY, respectively. In contrast, detection levels of DEAMPY and 3PBA in children from Poland were significantly lower than for pregnant women from La Garrotxa, with percentages below 61% in the former and >80% in the latter. Besides, concentrations in Poland (0.26 ng/ml for DEAMPY and 0.15 ng/ml for 3PBA) were significantly lower than concentrations in La Garrotxa (1.6 ng/ml for DEAMPY and 0.42 ng/ml for 3PBA). On the other hand, detection levels of DM-ACE and TEB-OH were similar in both populations, although GM concentration of DM-ACE were higher in La Garrotxa (0.82 ng/ml) than in Poland (0.42 ng/ml). These comparisons are shown in Figure 3.

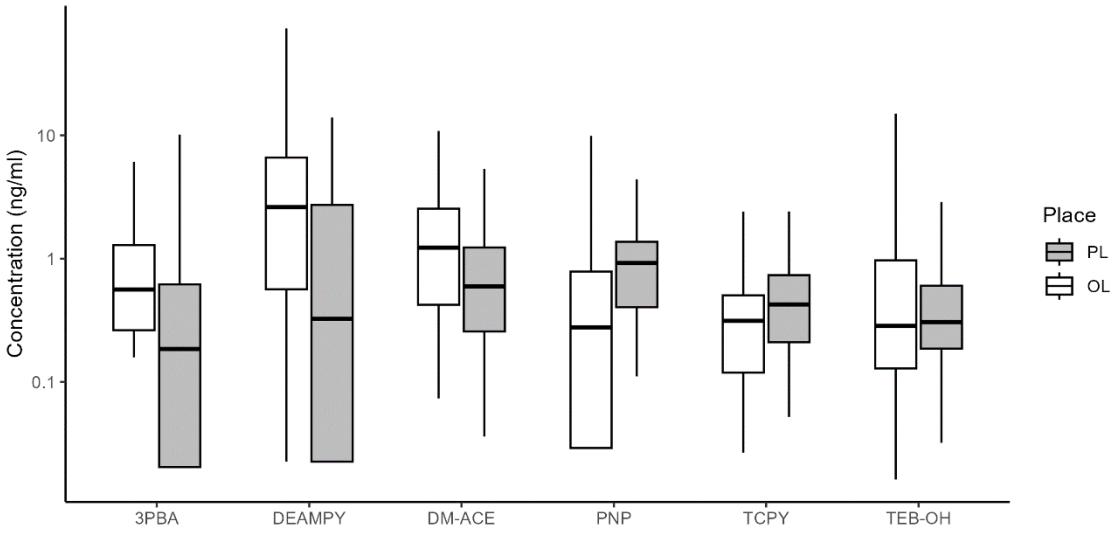

Figure 3. Boxplot comparing concentrations from Poland and La Garrotxa.

Table 4. Detection frequencies (DF, %), geometric means (GM) and 95% confidence intervals (CI), medians and concentration ranges of pesticide metabolites in urine of the two studied populations. Concentrations are expressed in ng/ml.

		Pregnant women from La Garrotxa				*Children from Poland*			
		DF (%)	*GM (95% CI)*	*Median*	*Range*	*DF (%)*	*GM (95% CI)*	*Median*	*Range*
NEOs	IMI	29	0.038 (0.025-0.058)	<LD	nd-2.3	0	<LD	<LD	<LD
	5OH-IMI	55	0.10 (0.060-0.17)	0.081	nd-5.8	14	<LD	<LD	nd-0.56
	IMI-OLE	7	<LD	<LD	nd-1.1	<LD	<LD	<LD	<LD
	6ClNA	11	<LD	<LD	nd-0.12	5	<LD	<LD	nd-0.11
	CLO	31	0.022 (0.016-0.032)	<LD	nd-0.57	11	<LD	<LD	nd-2.0
	DM-CLO	11	<LD	<LD	nd-0.81	34	0.017 (0.011-0.026)	<LD	nd-0.41
	DIN	7	<LD	<LD	nd-0.84	8	<LD	<LD	nd-0.10
	DM-DIN	7	<LD	<LD	nd-0.066	0	<LD	<LD	<LD
	DM-THX	22	<LD	<LD	nd-3.0	0	<LD	<LD	<LD
	ACE	2	<LD	<LD	nd-0.081	<LD	<LD	<LD	<LD
	DM-ACE	92	0.82 (0.54-1.3)	1.23	nd-11	86	0.42 (0.31-0.57)	0.60	nd-5.4
CBMs/ TCBMs	CARBO	20	0.013 (0.0090-0.017)	0.007	nd-0.37	0	<LD	<LD	<LD
	3OH-CARBO	<LD	<LD	<LD	<LD	2	<LD	<LD	nd-1.2
	THPI	3	<LD	<LD	nd-18	1	<LD	<LD	nd-23
TRIs	TEB	5	<LD	<LD	nd-0.24	0	<LD	<LD	<LD
	TEB-OH	86	0.32 (0.21-0.49)	0.29	nd-38	92	0.31 (0.25-0.39)	0.31	0.016-9.4
OPs	DEAMPY	83	1.63 (0.90-3.0)	2.62	nd-73.75	54	0.26 (0.016-0.43)	0.34	0.023-13.92
	IMPY	10	<LD	<LD	nd-0.38	1	<LD	<LD	nd-0.077
	PNP	60	0.20 (0.013-0.31)	0.28	nd-9.9	84	0.57 (0.42-0.77)	0.92	0.029-9.2
	TCPY	76	0.22 (0.16-0.31)	0.31	nd-2.4	83	0.30 (0.24-0.39)	0.43	0.027-2.4
	CMHC	5	<LD	<LD	nd-0.35	0	<LD	<LD	<LD
PYRs	3PBA	81	0.42 (0.28-0.64)	0.56	nd-6.1	61	0.15 (0.11-0.22)	0.19	nd-10
	4F3PBA	7	<LD	<LD	nd-0.41	10	<LD	<LD	nd-1.6

3.4 International comparison

Comparison of the OP and PYR metabolite concentrations in pregnant women from La Garrotxa with other studies worldwide (performed in general adult populations or pregnant women) is shown in Table 5. The present cohort shows the lowest concentrations for PNP (0.20 ng/ml) when compared with measurements in Sucs (Catalonia), València (Spain), Ljubljana (Slovenia), Puerto Rico, Queensland (Australia) and China (median concentrations ranging 0.50-5.0 ng/ml). Only in the case of Ljubljana and Puerto Rico, 3PBA concentrations (0.13 and 0.2 ng/ml, respectively) were lower than in the present study (0.42 ng/ml). As well as 3PBA, TCPY concentration was also lower in Ljubljana (0.16 ng/ml) than in La Garrotxa (0.22 ng/ml). Finally, DEAMPY, metabolite of pirimiphos, an OP pesticide, is the compound at higher concentration (1.63 ng/ml), when compared with other populations, such as Sucs (0.60 ng/ml) and Ljubljana (<LD) (Table 5).

Table 5. Comparison of geometric means of organophospahate and pyrethroid pesticide metabolites in urine of pregnant women with other similar studies. Concentrations are expressed in ng/ml.

	Population/Year	*DEAMPY*	*IMPY*	*PNP*	*TCPY*	*3PBA*	*Study*
La Garrotxa (Catalonia, Spain)	Women 2023	1.63	<LD	0.20	0.22	0.42	Present study
Sucs (Catalonia, Spain)	General 2016	0.60	<LD	1.5	1.9	0.87	(Bravo et al., 2022)
Valencia (Spain)	Women 2015			0.80	1.5	1.4	(Fernández et al., 2020)
Ljubljana (Slovenia)	Women 2015	0.05	<LD	0.45	0.16	0.13	(Bravo et al., 2020)
Puerto Rico	Women		<LD	0.50	0.40	0.20	(Lewis et al., 2014, 2015)
Queensland (Australia)	Women 2013		0.36	1.4	21.3	1.1	(Heffernan et al., 2016)
Wuhan (China)	Pregnant Women 2017			2.30	1.86	0.20	(A. Wang et al., 2023)
China	General 2014		0.20	5.0	3.7	0.70	(Li & Kannan, 2018)

Although the study of neonicotinoids and triazoles is limited, it has been possible to compare the concentrations obtained from the present cohort with other populations in the world. Table 6 shows that the concentrations of TEB-OH and CLO in the Catalan (0.29 and 0.022 ng/ml, respectively) were lower than those of other populations (Czech Republic, China, Taiwan and Ghana). In contrast, the concentration of DM-ACE (1.23 ng/ml) in La Garrotxa was the highest, except in Ghana (0.41 ng/ml).

Table 6. Comparison of geometric means of triazoles and neonicotinoids pesticide metabolites in urine of pregnant women with other similar studies. Concentrations are expressed in ng/ml.

	Year	*TEB-OH*	*CLO*	*DM-ACE*	*6-ClNA*	*Study*
La Garrotxa (Catalonia, Spain)	2023	0.32 [0.29]	0.02 [<LD]	0.82 [1.23]	<LD	Present study
Czech Republic	Adults 2019	[0.44]				(Šulc et al., 2022)
Laizhou (China)	Pregnant women 2013			[0.18]	[5.03]	(Pan et al., 2022)
Wuhan (China)	Pregnant women 2017		0.16 [0.15]	1.17 [1.13]		(A. Wang et al., 2023)
Taiwan	Pregnant women 2016		[12.4]			(P.-W. Wang et al., 2024)
Ghana	General 2021		0.05	0.79 [0.41]		(Nimako et al., 2021)

[a] medians between square brackets.

Comparison with the OP and PYR metabolite concentrations of the Polish children participating in the study has been performed with another series of reference cohorts (Table 7). As shown in Table 7, the concentrations of OP and PYR metabolites in the Polish children were generally among the lowest described in previous studies from Valencia, Trieste, Ljubljana, North Carolina, Thailand and Queensland. Only in a few places, such as DEAMPY (0.19 ng/ml) and TCPY (0.069 ng/ml) in Ljubljana and DEAMPY (0.14 ng/ml) and 3PBA (0.07 ng/ml) in North Carolina, the concentrations were lower than those from the present study (Table 7).

Table 7. Comparison of geometric means of organophosphate and pyrethroid pesticide metabolites in urine of children with other similar studies. Concentrations are expressed in ng/ml.

	Year	*DEAMPY*	*IMPY*	*PNP*	*TCPY*	*3PBA*	*4F3PBA*	*Study*
Poland	2023	0.26 [0.34]	<LD	0.57	0.30	[0.19]	<LD	Present study
Valencia (Spain)	2010	0.47 [<LD]	3.31	0.96	3.36			(Roca et al., 2014)
Trieste (Italy)	2015	2.70 [3.0]	<LD	1.10	0.23	[0.56]	0.022	(Bravo et al., 2019)
Ljubljana (Slovenia)	2015	0.19 [0.30]	0.015	0.70	0.069	[0.4]	0.021	(Bravo et al., 2020)
North Carolina (US)	2004	[0.14]	0.56	1	1.92	[0.07]		(Arcury et al., 2007)
Thailand	2007	0.16 [0.14]	<LD	2.68	2.35	[0.07]	0.15	(Panuwet et al., 2009)
Queensland	2013		0.33	1.76	24.16	[1.63]	<LD	(Heffernan et al., 2016)

[a] medians between square brackets.

Table 8 shows the comparison between concentrations of triazole and neonicotinoids pesticide metabolites from different populations. The concentration of TEB-OH has been compared with the Czech Republic and Sweden, where the concentration in Poland (0.31 ng/ml) was higher than in Sweden (0.09 ng/ml), but lower than in the Czech Republic (0.46 ng/ml). In contrast, the concentration of DM-ACE has been compared with populations in Japan and China, where the concentration in the Polish cohort (0.60 ng/ml) was higher than in Japan (0.34 ng/ml) but lower than in China (1.06 ng/ml).

Table 8. Comparison of geometric means of triazoles and neonicotinoids pesticide metabolites in urine of children with other similar studies. Concentrations are expressed in ng/ml.

	Year	*TEB-OH*	*CLO*	*DM-ACE*	*Study*
Poland	2023	[0.31]	<LD	[0.60]	Present study
Czech Republic	2019	[0.46]			(Šulc et al., 2022)
Sweden	2013	[0.09]			(Norén et al., 2020)
Japan	2016		[0.14]	[0.34]	(Ikenaka et al., 2019)
China	2019		[0.43]	[1.06]	(Yang et al., 2024)

[a] medians between square brackets.

4. CONCLUSIONS

The isotope dilution solid phase extraction UPLC-MS/MS method optimized in the present study is a fast and sensitive method for the determination of 23 pesticides and their metabolites in urine samples. The method has been validated regarding several parameters, including linearity, limits of detection, accuracy, precision, recoveries and matrix effects. The method is able to determine up to five families of pesticides, including neonicotinoids, carbamates/thiocarbamates, triazoles, organophosphates and pyrethroids, and shows detection limits in the range of 0.012 ng/ml and 0.058 ng/ml, appropriate for human biomonitoring studies. An exposure assessment using this methodology has been performed in children and pregnant women from two European birth cohorts, detecting three metabolites of organophosphate pesticides (DEAMPY, PNP and TCPY), a common metabolite of several pyrethroids (3PBA), one metabolite of triazole (TEB-OH) and one of neonicotinoid (DM-ACE) in <50% of the samples analysed. Tebuconazole and acetamiprid, followed by pirimiphos in pregnant women from La Garrotxa and chlorpyriphos in children from Poland, are the pesticides showing a more general degree of exposure in the studied cohorts. Further studies are required in order to determine the risk of exposure associated to these pesticides, most of them still available in the market.

Supporting material

SECTION 1 (S-1): **Pesticide effects on neural system**

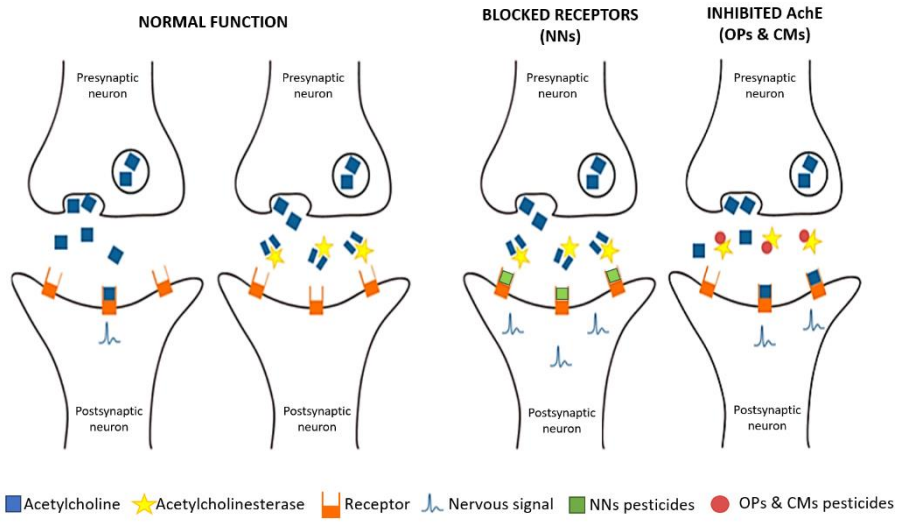

Figure S1. Interaction of organophosphates, carbamates and neonicotinoids in the nervous system.

OPs are synthetic and non-systemic pesticides that act on the nervous system and cause death through neuronal dysfunction by inhibiting acetylcholinesterase (AChE), the enzyme that hydrolyses acetylcholine (ACh), a neurotransmitter involved in nerve signal transduction (Figure S1) (Gupta et al., 2018). Inhibition of AChE makes ACh persist in the synaptic cleft and cause continuous neuronal impulse, leading to death from respiratory failure caused by the inhibition of the respiratory centers in the brain stem and flaccid paralysis of the respiratory muscles (Rezende-Teixeira et al., 2022). The carbamate compounds toxicity varies according the molecular structure, but in general they have shorter duration than that of organophosphates and the latter inhibits acetyl cholinesterase (Hassaan & El Nemr, 2020).

Pyrethroids works on the central nervous system, which causes fluctuations in the dynamics of sodium cation channels in the membrane of the nerve cell, which leads to an increase in the time of opening of the sodium channels. The sodium cation stream extends across the membrane leading to a neuronal hyperexcitation. (Hassaan & El Nemr, 2020).

NEOs were the first insecticide with acetylcholine (ACh)-mimicking properties, and their interaction with nicotinic receptors produces an initial stimulation followed by prolonged depolarisation, leading to paralysis of the receptor. The insecticidal activity of NEOs is attributed to activation of nicotinic receptors, where it remains bound to postsynaptic ACh receptors. Uninterrupted activation of the receptors leads to hyperexcitation of the nervous system causing paralysis and death of the insect (Rezende-Teixeira et al., 2022).

SECTION 2 (S-2): Assessment status of pesticides

Table S1 shows the list of pesticides classified as "approved" or "not approved" according to Regulation (EC) No 1107/2009 (European Comission, n.d.), the regulation in force during the years 2023-2024, when the samples were taken. This regulation sets out the criteria for the authorization and safe use of plant protection products in the European Union. Pesticides included in the "approved" category meet the safety requirements for human health and the environment, while those in the "not approved" category have been banned due to unacceptable risks identified during their evaluation. This classification is essential to ensure the responsible and safe use of pesticides in agriculture and other applications.

Table S1. Assessment status of the studied pesticides in Europe

Parent Pesticide	*Analyte*	*Acronym*	*Assessment status*
Imidacloprid	Imidacloprid	IMI	Not approved
	5-hydroxy-imidacloprid	5OH-IMI	
	Imidacloprid-olefin	IMI-OLE	
	6-chloronicotinic acid	6ClNA	
Clothianidin	Clothianidin	CLO	Not approved
	Clothianidin-desmethyl	DM-CLO	
Dinotefuran	Dinotefuran	DIN	Not approved
	Dinotefuran-desmethyl	DM-DIN	
Thiamethoxam	Thiamethoxam-desmethyl	DM-THX	Not approved
Acetamiprid	Acetamiprid	ACE	Approved
	N-(6-Chloro-3-pyridylmethyl)-N-cyano-acetamidine	DM-ACE	
Carbofuran	Carbofuran	CARBO	Not approved
	3-hydroxycarbofuran	3OH-CARBO	
Captan	Cis-1,2,3,6-tetrahydrophthalimide	THPI	Approved
Tebuconazole	Tebuconazole	TEB	Approved
	4-hydroxy-4-(1H-1,2,4-triazole-1-ylmethyl)-5,5-dimethyl-hexanoic acid	TEB-OH	
Pirimiphos-methyl	2-diethylamino-6-methyl pyrimidin-4-ol	DEAMPY	Approved
Diazinon	2-isopropyl-6-methyl-4-pyrimidiol	IMPY	Not approved
Parathion	4-nitrophenol	PNP	Not approved
Chlorpyrifos	3,5, 6 -trichloro-2-pyridinol	TCPY	Not approved
Coumaphos	3-chloro-4-methyl-7-hydroxicoumarin	CMHC	Not approved
Several PYR (*)	3-phenoxybenzoic acid	3PBA	Depending on the pyrethroid
Cyfluthrin	4-fluoro-3-phenoxybenzoic acid	4F3PBA	Not approved

***Several PYR. Not approved**: Permethrin, Cyfluthrin, Fenvalerate, Tetramethrin, Resmethrin, Bioallethrin. **Approved:** Cypermethrin, Deltamethrin, Esfenvalerate

SECTION 3 (S-3): **Chromatograms**

The following figures show the chromatograms of the analysed compounds, ordered according to their corresponding window.

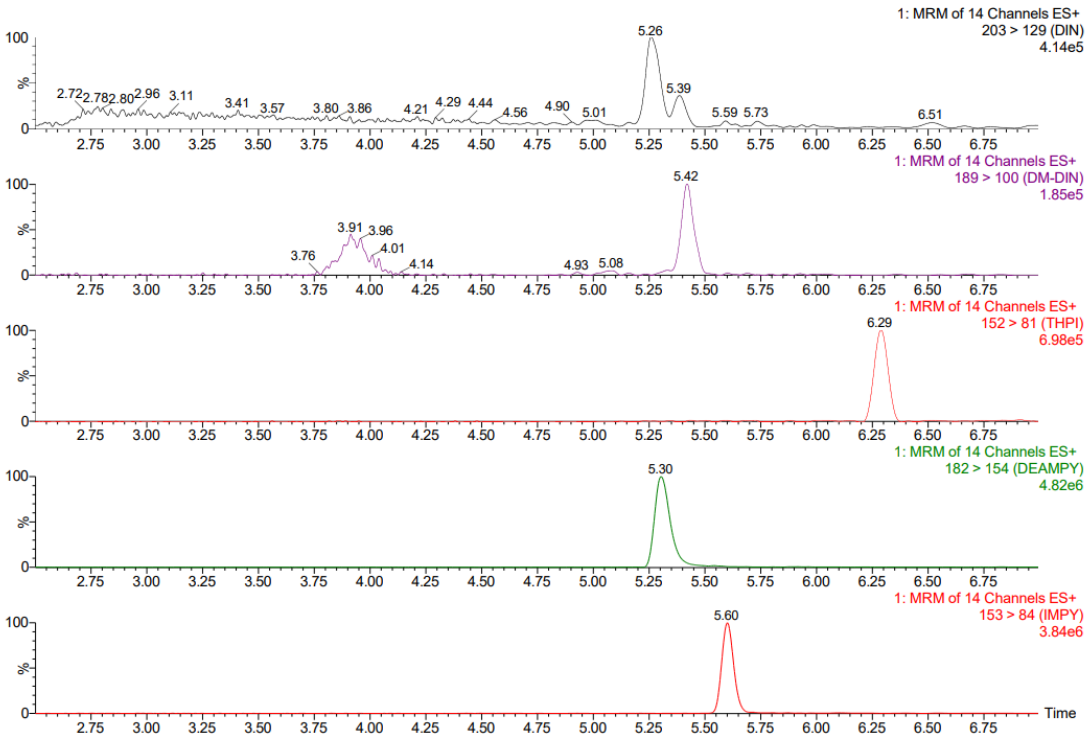

Figure S2. Chromatogram of the compounds analysed in window 1.

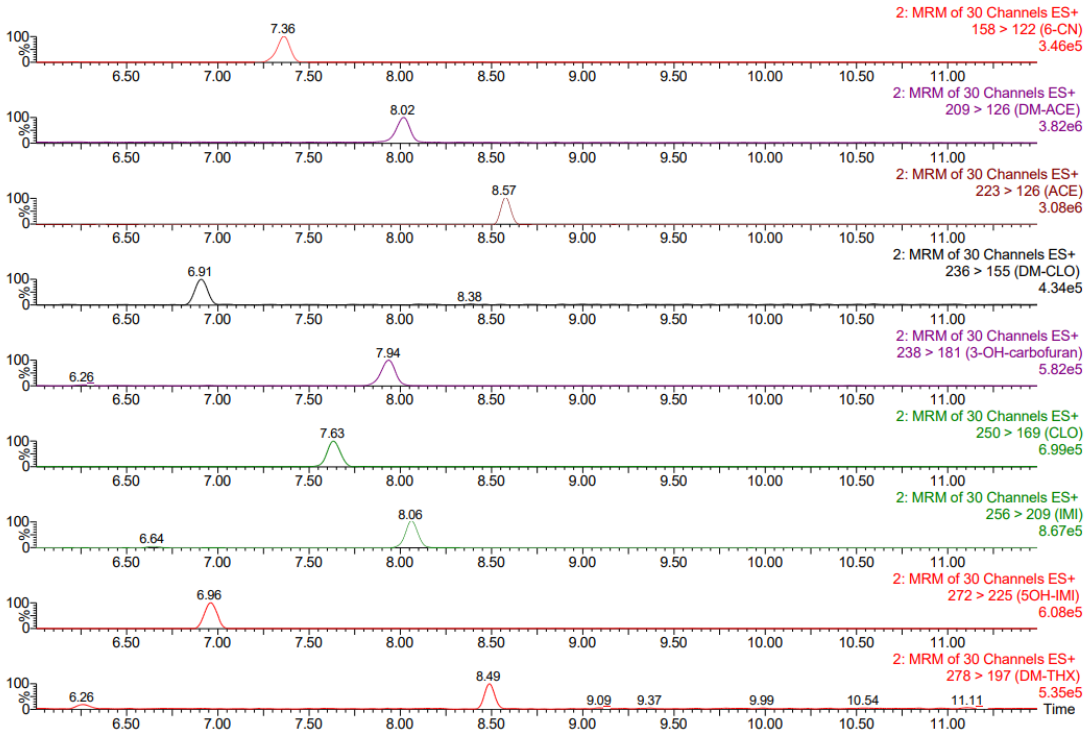

Figure S3. Chromatogram of the compounds analysed in window 2.

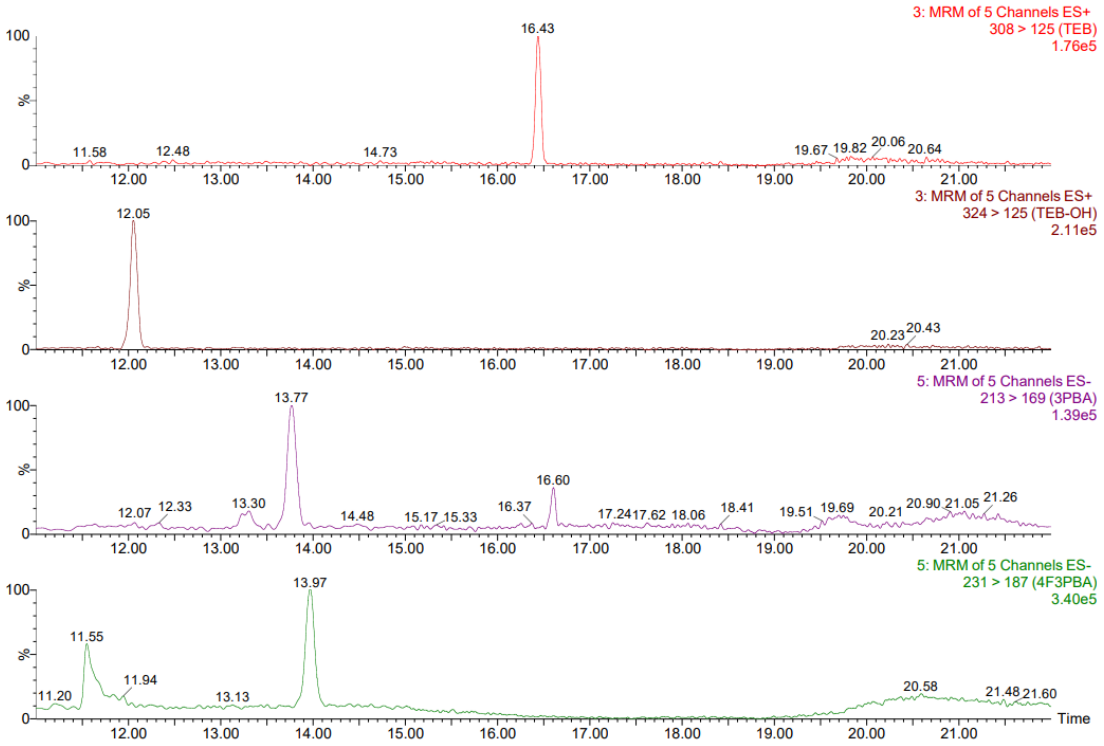

Figure S4. Chromatogram of the compounds analysed in windows 3 and 5.

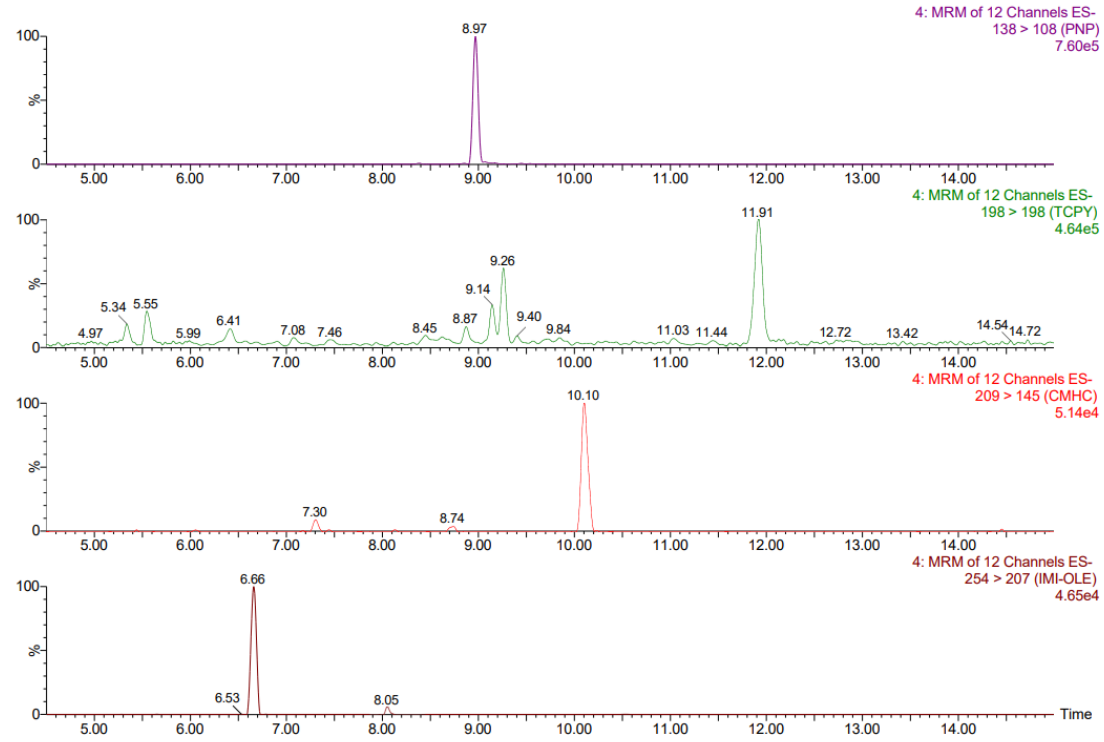

Figure S5. Chromatogram of the compounds analysed in window 4.

SECTION 4 (S-4): **International comparison**

The following two comparative graphs (Figure S6 and S7) illustrate the pesticide concentrations in the two populations studied (pregnant women from La Garrotxa and children from Poland) against the concentrations observed in populations around the world. These graphs provide a global perspective on pesticide levels, allowing us to assess how our samples rank in the international context.

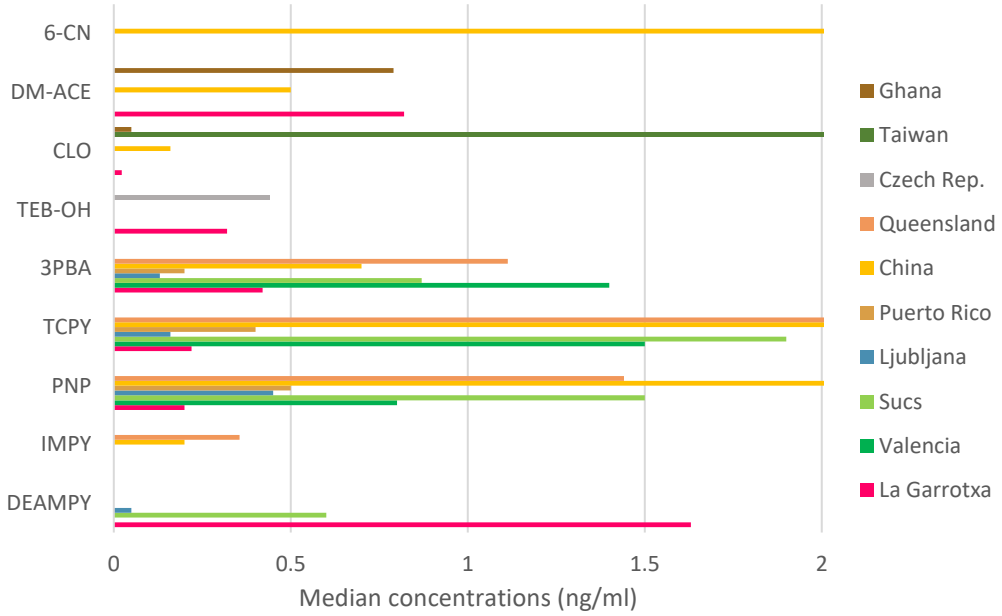

Figure S6. Comparison of geometric means in pregnant women's urine samples with other similar studies. Concentrations are expressed in ng/ml.

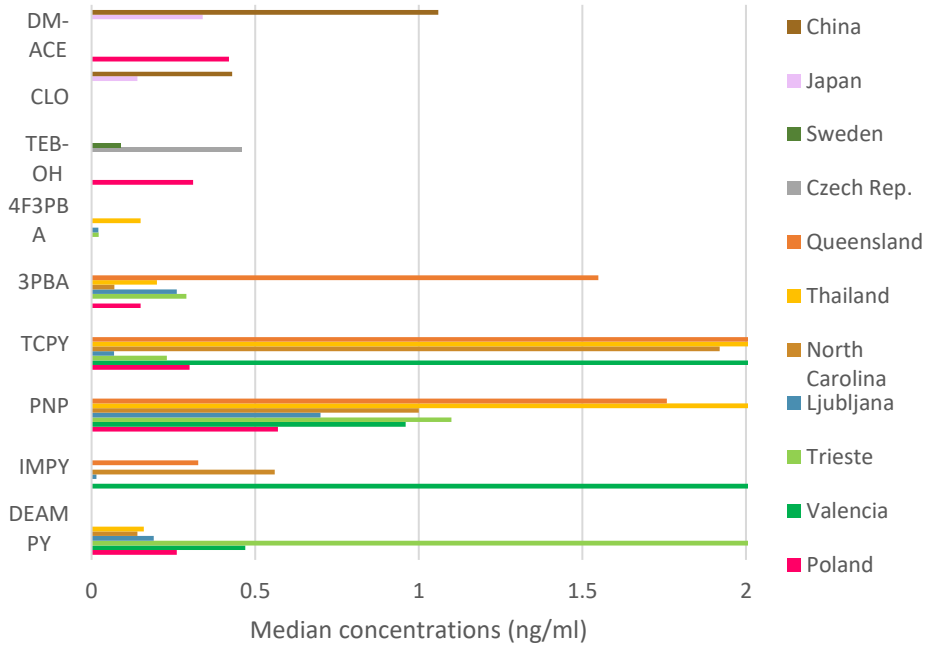

Figure S7. Comparison of geometric means in children's urine samples with other similar studies Concentrations are expressed in ng/ml.

References

Arcury, T. A., Grzywacz, J. G., Barr, D. B., Tapia, J., Chen, H., & Quandt, S. A. (2007). Pesticide urinary metabolite levels of children in Eastern North Carolina farmworkers households. *Environmental Health Perspectives*, *115*(8), 1254–1260. https://doi.org/10.1289/ehp.9975

Barr, D. B. (2008). Biomonitoring of exposure to pesticides. *Journal of Chemical Health and Safety*, *15*(6), 20–29. https://doi.org/10.1016/j.jchas.2008.07.001

Becker, K., Seiwert, M., Angerer, J., Kolossa-Gehring, M., Hoppe, H.-W., Ball, M., Schulz, C., Thumulla, J., & Seifert, B. (2006). GerES IV Pilot Study: Assessment of the exposure of German children to organophosphorus and pyrethroid pesticides. *International Journal of Hygiene and Environmental Health*, *209*(3), 221–233. https://doi.org/https://doi.org/10.1016/j.ijheh.2005.12.002

Berton, T., Mayhoub, F., Chardon, K., Duca, R.-C., Lestremau, F., Bach, V., & Tack, K. (2014). Development of an analytical strategy based on LC–MS/MS for the measurement of different classes of pesticides and theirs metabolites in meconium: Application and characterisation of foetal exposure in France. *Environmental Research*, *132*, 311–320. https://doi.org/https://doi.org/10.1016/j.envres.2014.03.034

Bille, L., Binato, G., Gabrieli, C., Manfrin, A., Pascoli, F., Pretto, T., Toffan, A., Dalla Pozza, M., Angeletti, R., & Arcangeli, G. (2017). First report of a fish kill episode caused by pyrethroids in Italian freshwater. *Forensic Science International*, *281*, 176–182. https://doi.org/https://doi.org/10.1016/j.forsciint.2017.10.040

Biosca-Brull, J., Pérez-Fernández, C., Mora, S., Carrillo, B., Pinos, H., Conejo, N. M., Collado, P., Arias, J. L., Martín-Sánchez, F., Sánchez-Santed, F., & Colomina, M. T. (2021). Relationship between autism spectrum disorder and pesticides: A systematic review of human and preclinical models. In *International Journal of Environmental Research and Public Health* (Vol. 18, Issue 10). MDPI. https://doi.org/10.3390/ijerph18105190

Bonvallot, N., Jamin, E. L., Regnaut, L., Chevrier, C., Martin, J. F., Mercier, F., Cordier, S., Cravedi, J. P., Debrauwer, L., & Le Bot, B. (2021). Suspect screening and targeted analyses: Two complementary approaches to characterize human exposure to pesticides. *Science of the Total Environment*, *786*. https://doi.org/10.1016/j.scitotenv.2021.147499

Bravo, N., Garí, M., & Grimalt, J. O. (2022). Occupational and residential exposures to organophosphate and pyrethroid pesticides in a rural setting. *Environmental Research*, *214*. https://doi.org/10.1016/j.envres.2022.114186

Bravo, N., Grimalt, J. O., Bocca, B., Pino, A., Bin, M., Brumatti, L. V., Rosolen, V., Barbone, F., Ronfani, L., Alimonti, A., & Calamandrei, G. (2019). Urinary metabolites of organophosphate and pyrethroid pesticides in children from an Italian cohort (PHIME, Trieste). *Environmental Research*, *176*. https://doi.org/10.1016/j.envres.2019.05.039

Bravo, N., Grimalt, J. O., Mazej, D., Tratnik, J. S., Sarigiannis, D. A., & Horvat, M. (2020). Mother/child organophosphate and pyrethroid distributions. *Environment International*, *134*. https://doi.org/10.1016/j.envint.2019.105264

Bravo, N., Peralta, S., Grimalt, J. O., Martínez, M. Á., Rovira, J., & Schuhmacher, M. (2020). Organophosphate metabolite concentrations in maternal urine during pregnancy. *Environmental Research*, *182*. https://doi.org/10.1016/j.envres.2019.109003

Bustamante, C. M., Bravo, N., Ruiz, P., Grimalt, J. O., & Garí, M. (2024). Method optimization for a simultaneous determination of neonicotinoid, carbamate/thiocarbamate, triazole, organophosphate and pyrethroid pesticides and their metabolites in urine using UPLC-MS/MS. *Journal of Chromatography A*, *1730*, 465054. https://doi.org/10.1016/j.chroma.2024.465054

Cequier, E., Sakhi, A. K., Haug, L. S., & Thomsen, C. (2016). Development of an ion-pair liquid chromatography–high resolution mass spectrometry method for determination of organophosphate pesticide metabolites in large-scale biomonitoring studies. *Journal of Chromatography A*, *1454*, 32–41. https://doi.org/10.1016/j.chroma.2016.05.067

Debost-Legrand, A., Warembourg, C., Massart, C., Chevrier, C., Bonvallot, N., Monfort, C., Rouget, F., Bonnet, F., & Cordier, S. (2016). Prenatal exposure to persistent organic pollutants and organophosphate pesticides, and markers of glucose metabolism at birth. *Environmental Research*, *146*, 207–217. https://doi.org/https://doi.org/10.1016/j.envres.2016.01.005

Eskenazi, B., Harley, K., Bradman, A., Weltzien, E., Jewell, N. P., Barr, D. B., Furlong, C. E., & Holland, N. T. (2004). Association of in utero organophosphate pesticide exposure and fetal growth and length of gestation in an agricultural population. *Environmental Health Perspectives*, *112*(10), 1116–1124. https://doi.org/10.1289/ehp.6789

European Comission. (n.d.-a). *EU Pesticides Database (v3.2)*. Retrieved July 1, 2024, from https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/start/screen/active-substances

European Comission. (n.d.-b). *Pesticides*. Retrieved July 1, 2024, from https://food.ec.europa.eu/plants/pesticides_en

F. Fernández, S., Pardo, O., Corpas-Burgos, F., & Yusà, V. (2020). Exposure and cumulative risk assessment to non-persistent pesticides in Spanish children using biomonitoring. *Science of the Total Environment*, *746*. https://doi.org/10.1016/j.scitotenv.2020.140983

Fernández, S. F., Pardo, O., Adam-Cervera, I., Montesinos, L., Corpas-Burgos, F., Roca, M., Pastor, A., Vento, M., Cernada, M., & Yusà, V. (2020). Biomonitoring of non-persistent pesticides in urine from lactating mothers: Exposure and risk assessment. *Science of the Total Environment*, *699*. https://doi.org/10.1016/j.scitotenv.2019.134385

Filippi, I., Bravo, N., Grimalt, J. O., Butinof, M., Lerda, D., Fernández, R. A., Muñoz, S. E., & Amé, M. V. (2021). Pilot study of exposure of the male population to organophosphate and pyrethroid pesticides in a region of high agricultural activity (Córdoba, Argentina). *Environmental Science and Pollution Research*, *28*(38), 53908–53916. https://doi.org/10.1007/s11356-021-14397-1

Garí, M., González-Quinteiro, Y., Bravo, N., & Grimalt, J. O. (2018). Analysis of metabolites of organophosphate and pyrethroid pesticides in human urine from urban and agricultural populations (Catalonia and Galicia). *Science of the Total Environment*, *622–623*, 526–533. https://doi.org/10.1016/j.scitotenv.2017.11.355

González-Alzaga, B., Lacasaña, M., Aguilar-Garduño, C., Rodríguez-Barranco, M., Ballester, F., Rebagliato, M., & Hernández, A. F. (2014). A systematic review of neurodevelopmental effects of prenatal and postnatal organophosphate pesticide exposure. *Toxicology Letters*, *230*(2), 104–121. https://doi.org/10.1016/j.toxlet.2013.11.019

Gupta, R. C., Sachana, M., Mukherjee, I. M., Doss, R. B., Malik, J. K., & Milatovic, D. (2018). Chapter 37 - Organophosphates and Carbamates. In R. C. Gupta (Ed.), *Veterinary Toxicology (Third Edition)* (pp. 495–508). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-811410-0.00037-4

Hassaan, M. A., & El Nemr, A. (2020). Pesticides pollution: Classifications, human health impact, extraction and treatment techniques. In *Egyptian Journal of Aquatic Research* (Vol. 46, Issue 3, pp. 207–220). National Institute of Oceanography and Fisheries. https://doi.org/10.1016/j.ejar.2020.08.007

Heffernan, A., English, K., Toms, L., Calafat, A., Valentin-Blasini, L., Hobson, P., Broomhall, S., Ware, R., Jagals, P., Sly, P., & Mueller, J. (2016). Cross-sectional biomonitoring study of pesticide exposures in Queensland, Australia, using pooled urine samples. *Environmental Science and Pollution Research*, *23*(23), 23436–23448. https://doi.org/10.1007/s11356-016-7571-7

Ikenaka, Y., Miyabara, Y., Ichise, T., Nakayama, S., Nimako, C., Ishizuka, M., & Tohyama, C. (2019). Exposures of children to neonicotinoids in pine wilt disease control areas. *Environmental Toxicology and Chemistry*, *38*(1), 71–79. https://doi.org/10.1002/etc.4316

Janc, M., Jankowska, A., Weteska, M., Brzozowska, A., Hanke, W., Jurewicz, J., Garí, M., Polańska, K., & Jerzyńska, J. (2022). REPRO_PL-Polish Mother and Child Cohort—Exposure, Health Status, and Neurobehavioral Assessments in Adolescents—Design and Cohort Update. *International Journal of Environmental Research and Public Health*, *19*(21). https://doi.org/10.3390/ijerph192114167

Kamijima, M., Hibi, H., Gotoh, M., Taki, K., Saito, I., Wang, H., Itohara, S., Yamada, T., Ichihara, G., Shibata, E., Nakajima, T., & Takeuchi, Y. (2004). A Survey of Semen Indices in Insecticide Sprayers. *Journal of Occupational Health*, *46*(2), 109–118. https://doi.org/10.1539/joh.46.109

Koureas, M., Tsakalof, A., Tsatsakis, A., & Hadjichristodoulou, C. (2012). Systematic review of biomonitoring studies to determine the association between exposure to organophosphorus and pyrethroid insecticides and human health outcomes. In *Toxicology Letters* (Vol. 210, Issue 2, pp. 155–168). https://doi.org/10.1016/j.toxlet.2011.10.007

Landrigan, P. J., Fuller, R., Fisher, S., Suk, W. A., Sly, P., Chiles, T. C., & Bose-O'Reilly, S. (2019). Pollution and children's health. *Science of the Total Environment*, *650*, 2389–2394. https://doi.org/10.1016/j.scitotenv.2018.09.375

Lewis, R. C., Cantonwine, D. E., Anzalota Del Toro, L. V., Calafat, A. M., Valentin-Blasini, L., Davis, M. D., Montesano, M. A., Alshawabkeh, A. N., Cordero, J. F., & Meeker, J. D. (2015). Distribution and determinants of urinary biomarkers of exposure to organophosphate insecticides in Puerto Rican pregnant women. *Science of the Total Environment*, *512–513*, 337–344. https://doi.org/10.1016/j.scitotenv.2015.01.059

Lewis, R. C., Cantonwine, D. E., Del Toro, L. V. A., Calafat, A. M., Valentin-Blasini, L., Davis, M. D., Baker, S. E., Alshawabkeh, A. N., Cordero, J. F., & Meeker, J. D. (2014). Urinary biomarkers of exposure to insecticides, herbicides, and one insect repellent among pregnant women in Puerto Rico. *Environmental Health: A Global Access Science Source*, *13*(1). https://doi.org/10.1186/1476-069X-13-97

Li, A. J., & Kannan, K. (2018). Urinary concentrations and profiles of organophosphate and pyrethroid pesticide metabolites and phenoxyacid herbicides in populations in eight countries. *Environment International*, 1148–1154. https://doi.org/10.1016/j.envint.2018.10.033

Massart, D. L. , V. B. G. , B. L. M. , L. P. J. , S.-V. J. , & J. S. D. (1997). *Handbook of chemometrics and qualimetrics: Vol. Part a*. Elsevier Science Inc.

Mouskeftara, T., Virgiliou, C., Iakovakis, A., Raikos, N., & Gika, H. G. (2021). Liquid chromatography tandem mass spectrometry for the determination of nine insecticides and fungicides in human postmortem blood and urine. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *1179*. https://doi.org/10.1016/j.jchromb.2021.122824

Nimako, C., Ikenaka, Y., Akoto, O., Bortey-Sam, N., Ichise, T., Nakayama, S. M. M., Asante, K. A., Fujioka, K., Taira, K., & Ishizuka, M. (2021). Human Exposures to Neonicotinoids in Kumasi, Ghana. *Environmental Toxicology and Chemistry*, *40*(8), 2306–2318. https://doi.org/10.1002/etc.5065

Norén, E., Lindh, C., Rylander, L., Glynn, A., Axelsson, J., Littorin, M., Faniband, M., Larsson, E., & Nielsen, C. (2020). Concentrations and temporal trends in pesticide biomarkers in urine of Swedish adolescents, 2000–2017. *Journal of Exposure Science and Environmental Epidemiology*, *30*(4), 756–767. https://doi.org/10.1038/s41370-020-0212-8

Pan, C., Yu, J., Yao, Q., Lin, N., Lu, Z., Zhang, Y., Zhao, S., Wang, Z., Lei, X., Tian, Y., & Gao, Y. (2022). Prenatal neonicotinoid insecticides Exposure, oxidative Stress, and birth outcomes. *Environment International*, *163*. https://doi.org/10.1016/j.envint.2022.107180

Panuwet, P., Hunter, R. E., D'Souza, P. E., Chen, X., Radford, S. A., Cohen, J. R., Marder, M. E., Kartavenka, K., Ryan, P. B., & Barr, D. B. (2016). Biological Matrix Effects in Quantitative Tandem Mass Spectrometry-Based Analytical Methods: Advancing Biomonitoring. In *Critical Reviews in Analytical Chemistry* (Vol. 46, Issue 2, pp. 93–105). Taylor and Francis Ltd. https://doi.org/10.1080/10408347.2014.980775

Panuwet, P., Prapamontol, T., Chantara, S., & Barr, D. B. (2009). Urinary pesticide metabolites in school students from northern Thailand. *International Journal of Hygiene and Environmental Health*, *212*(3), 288–297. https://doi.org/10.1016/j.ijheh.2008.07.002

Pesticide residues in food . (n.d.).

Pinos, H., Carrillo, B., Merchán, A., Biosca-Brull, J., Pérez-Fernández, C., Colomina, M. T., Sánchez-Santed, F., Martín-Sánchez, F., Collado, P., Arias, J. L., & Conejo, N. M. (2021). Relationship between prenatal or postnatal exposure to pesticides and obesity: A systematic review. In *International Journal of Environmental Research and Public Health* (Vol. 18, Issue 13). MDPI AG. https://doi.org/10.3390/ijerph18137170

Polańska, K., Hanke, W., Gromadzińska, J., Ligocka, D., Gulczyńska, E., Sobala, W., & Wąsowicz, W. (2009). Polish mother and child cohort study — defining the problem, the aim of the study and methodological assumptions. *International Journal of Occupational Medicine and Environmental Health*, *22*(4). https://doi.org/10.2478/v10001-009-0037-0

Polańska, K., Hanke, W., Jurewicz, J., Sobala, W., Madsen, C., Nafstad, P., & Magnus, P. (2011). Polish mother and child cohort study (REPRO_PL) — Methodology of follow-up of the children. *International Journal of Occupational Medicine and Environmental Health*, *24*(4). https://doi.org/10.2478/s13382-011-0026-y

Polańska, K., Hanke, W., Król, A., Potocka, A., Waszkowska, M., Jacukowicz, A., Gromadzińska, J., Wąsowicz, W., Jerzyńska, J., Stelmach, W., & Stelmach, I. (2016). Polish Mother and Child Cohort Study (REPRO_PL) – Methodology of the follow-up of the children at the age of 7. *International Journal of Occupational Medicine and Environmental Health*, *29*(6), 883–893. https://doi.org/10.13075/ijomeh.1896.00811

Raanan, R., Balmes, J. R., Harley, K. G., Gunier, R. B., Magzamen, S., Bradman, A., & Eskenazi, B. (2016). Decreased lung function in 7-year-old children with early-life organophosphate exposure. *Thorax*, *71*(2), 148–153. https://doi.org/10.1136/thoraxjnl-2014-206622

Rani, L., Thapa, K., Kanojia, N., Sharma, N., Singh, S., Grewal, A. S., Srivastav, A. L., & Kaushal, J. (2021). An extensive review on the consequences of chemical pesticides on human health and environment. In *Journal of Cleaner Production* (Vol. 283). Elsevier Ltd. https://doi.org/10.1016/j.jclepro.2020.124657

Ranke, J. (2015). *chemCal: calibration functions for analytical chemistry. R package version 0.1-37*. http://www.r-project.org, http://chem.uft.uni-bremen.de/ranke/?page= chemCal, http://cgit.jrwb.de/chemCal.

Rezende-Teixeira, P., Dusi, R. G., Jimenez, P. C., Espindola, L. S., & Costa-Lotufo, L. V. (2022). What can we learn from commercial insecticides? Efficacy, toxicity, environmental impacts, and future developments. In *Environmental Pollution* (Vol. 300). Elsevier Ltd. https://doi.org/10.1016/j.envpol.2022.118983

Roca, M., Leon, N., Pastor, A., & Yusà, V. (2014). Comprehensive analytical strategy for biomonitoring of pesticides in urine by liquid chromatography-orbitrap high resolution mass spectrometry. *Journal of Chromatography A*, *1374*, 66–76. https://doi.org/10.1016/j.chroma.2014.11.010

Roca, M., Miralles-Marco, A., Ferré, J., Pérez, R., & Yusà, V. (2014). Biomonitoring exposure assessment to contemporary pesticides in a school children population of Spain. *Environmental Research*, *131*, 77–85. https://doi.org/10.1016/j.envres.2014.02.009

Šulc, L., Janoš, T., Figueiredo, D., Ottenbros, I., Šenk, P., Mikeš, O., Huss, A., & Čupr, P. (2022). Pesticide exposure among Czech adults and children from the CELSPAC-SPECIMEn cohort: Urinary biomarker levels and associated health risks. *Environmental Research*, *214*. https://doi.org/10.1016/j.envres.2022.114002

Wang, A., Wan, Y., Mahai, G., Qian, X., Li, Y., Xu, S., & Xia, W. (2023). Association of Prenatal Exposure to Organophosphate, Pyrethroid, and Neonicotinoid Insecticides with Child Neurodevelopment at 2 Years of Age: A Prospective Cohort Study. *Environmental Health Perspectives*, *131*(10). https://doi.org/10.1289/EHP12097

Wang, P.-W., Huang, Y.-F., Fang, L.-J., & Chen, M.-L. (2024). Prenatal and childhood neonicotinoid exposure and neurodevelopment: A study in a young Taiwanese cohort. *Science of The Total Environment*, 174232. https://doi.org/10.1016/j.scitotenv.2024.174232

World Health Organization. (2020, October 26). *Chemical safety: Pesticides*. https://www.who.int/news-room/questions-and-answers/item/chemical-safety-pesticides

Yang, Z., Wang, Y., Tang, C., Han, M., Wang, Y., Zhao, K., Liu, J., Tian, J., Wang, H., Chen, Y., & Jiang, Q. (2024). Urinary neonicotinoids and metabolites are associated with obesity risk in Chinese school children. *Environment International*, *183*. https://doi.org/10.1016/j.envint.2023.108366

Zartarian, V. G., Ozkaynak, H., Burke, J. M., Zufall, M. J., Rigas, M. L., & Furtaw, E. J. (2000). A modeling framework for estimating children's residential exposure and dose to chlorpyrifos via dermal residue contact and nondietary ingestion. *Environmental Health Perspectives*, *108*(6), 505–514. https://doi.org/10.1289/ehp.00108505