Sample preparation method to analyse 15 pesticide metabolites in human urine using HPLC-MS/MS

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Introduction

Pesticides are heavily used and applied in agriculture, but also in residential places, which leads to their spreading to the whole environment. Their wide use makes them inevitable. Most of the acute effects of exposure are well known, but chronic low-dose exposure can cause serious problems. To monitor levels in human matrices, it is usually better to focus on metabolites than on parent compounds. Metabolites can be found in urine, usually in much higher concentrations and for a longer time than for example in blood. Pesticide metabolites include various compounds with different physical-chemical properties, structure, environmental fate and toxic effects on humans and living organisms. Therefore, they also behave differently during the sample preparation or instrumental analysis. There are many different approaches to determine pesticide metabolites, but most of them have some disadvantages. Because of that, the aim of this study is to develop a new suitable, robust and reproducible method to simultaneously determine 15 specific and non-specific pesticide metabolites in urine samples using a single optimized sample preparation method.

Methods

Sample preparation method

- 500 µL of urine + isotopically labelled standards + β-glucuronidase solution (*Helix pomatia*)
- vortex, incubation at 37 °C for 90 minutes
- solid-phase extraction (SPE)

Specific metabolites:

- elution from SPE with 3 mL of methanol
- sample evaporation, transfer into conical vial
- reduction to minimal volume using nitrogen
- + 500 µL of 50% methanol, vortex

Non-specific metabolites:

permeate from the SPE column into a new Eppendorf tube QuEChERS method - four non-specific metabolites (DEP, DMP, DETP and DMTP, further referred as DAPs):

- permeate + 6 M hydrochloric acid + and 500 µL of acetonitrile
- tube vortexed for 10 seconds, shaken vigorously for one minute
- + salts (i.e. 0.2 g of $MgSO_4$ and 0.1 g of NaCl), shaken vigorously for one minute
- centrifugation at 11,000 rpm for five minutes → formation of two layers, only the top layer collected
- into a new Eppendorf tube, + 10 mg of C18 sorbent (Bondesil LMS) and 60 mg of $MgSO_4$
- tube shaken vigorously for one minute, centrifugation
- 200 µL of supernatant transferred into a conical vial

LC-MS/MS analysis

Agilent 1290 HPLC system for separation

- three different methods

AB Sciex Qtrap 5500 for analyte detection

- both positive and negative mode

DAPs	DEET, IMPY	Other specific pesticide metabolites
Analytical column Tosoh TSK gel amide (150 x 2 mm, 3 µm)	Analytical column Acquity UPLC BEH C18 (100 x 2.1 mm, 1.7 µm)	Analytical column Acquity UPLC BEH C18 (100 x 2.1 mm, 1.7 µm)
Mobile phases A – 10 mM ammonium acetate in Milli-Q water B – 1mM ammonium acetate in acetonitrile	Mobile phases A – Milli-Q water B – methanol	Mobile phases A – 1 mM ammonium fluoride in Milli-Q water B – 1 mM ammonium fluoride in methanol

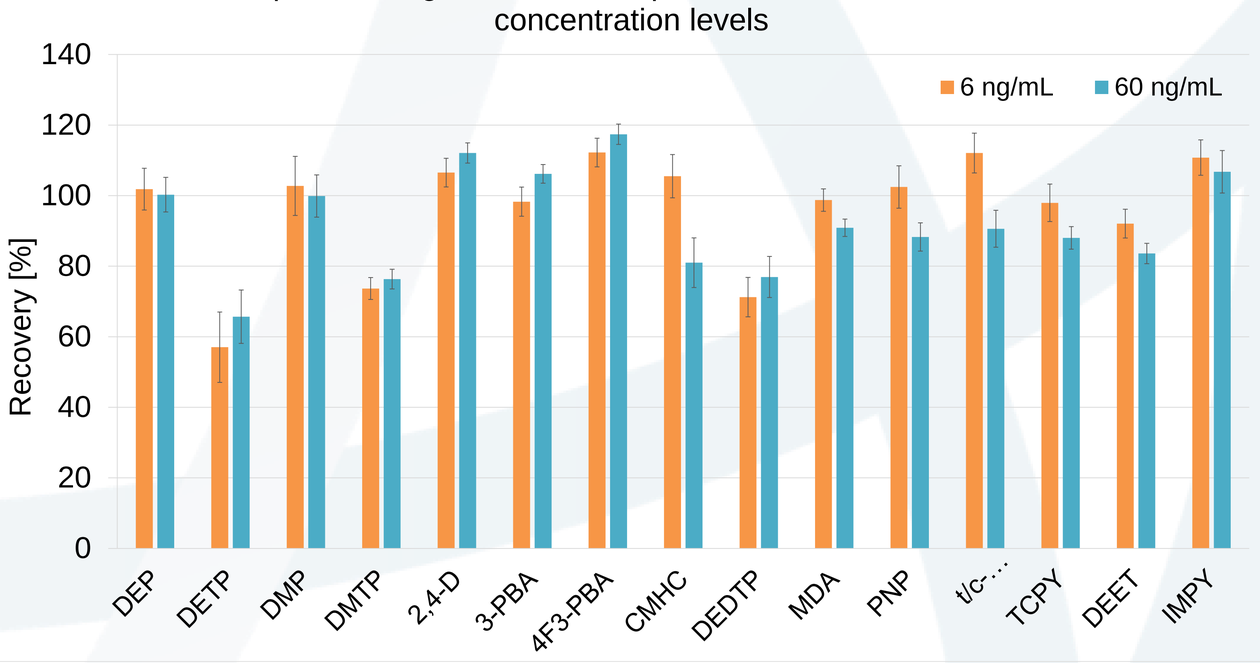
Results and discussion

- Recovery tests on two concentration levels, quality control samples on three concentration levels. Average recoveries of individual compounds were 80.0-120.0 %. Lower recoveries for DETP (57.0-65.7 %) and DMTP (73.3-76.3 %). Relative standard deviations under 10.0 % (Graph 1). Quantitation of pesticide metabolites - isotopic dilution method.
- Method validation - 20 urine samples from South Africa (children in age from 9 to 15)
- Detection frequency: 3-PBA, 4F3-PBA, MDA and IMPY detected in all 20 samples. DAPs generally found in most of the samples (DETP, DMP and DMTP in at least 19/20 samples). On the contrary, 2,4-D, CMHC, DEDTP and PNP detected in less than 5/20 samples, DEET never detected.
- Pesticide metabolite concentrations: generally similar levels among the children, median range of < LOD-5.50 µg/g creatinine for all compounds. Exception - DMP, 4F3-PBA and TCPY with the median range of 0.09-21.97 µg/g creatinine. Concentrations are shown in Graph 2, two outliers for DMP (50.65 µg/g) and TCPY (91.43 µg/g) excluded for clarity.
- Sample preparation optimisation - recovery troubles with DAPs metabolites. They had non-acceptable recoveries using deconjugation and SPE. Next, lyophilisation was tested, suitable only for DETP and DMTP. Finally, modified QuEChERS method - acceptable recoveries
- Combination with the original method, therefore presented method requires only 500 µL of urine sample.

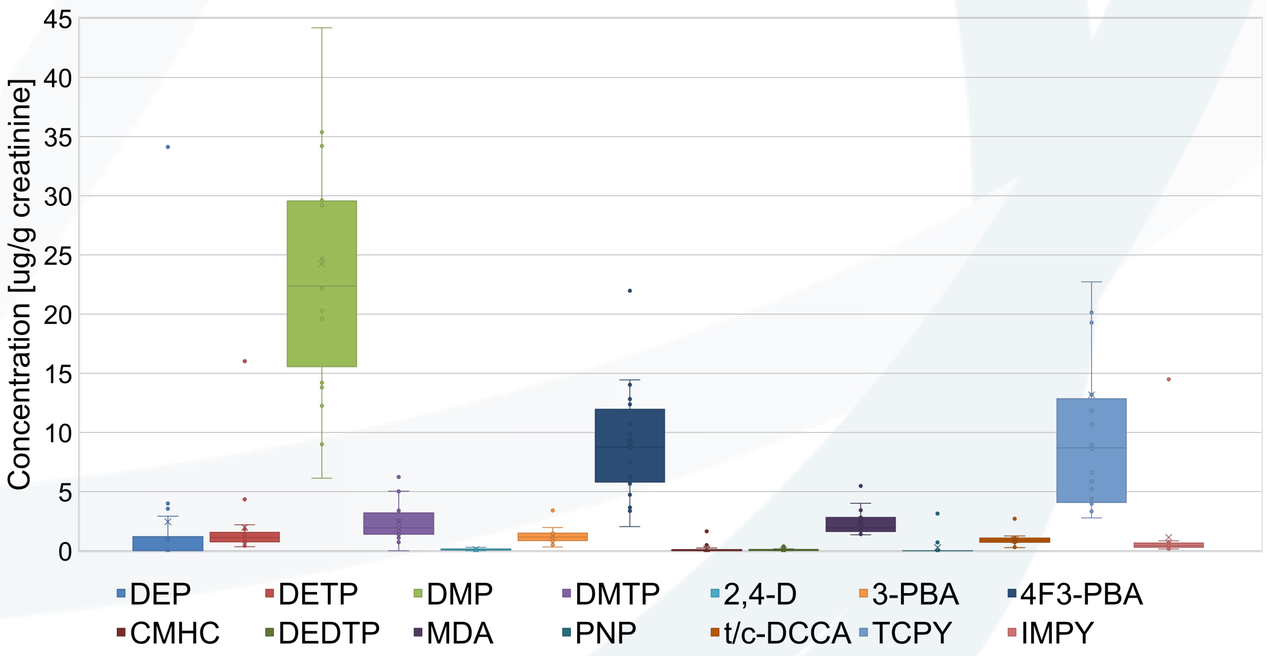
Table 1 Recoveries (with standard deviations) in % for DAPs using different types of extraction

Compound	Deconjugation + SPE		Lyophilisation		QuEChERS	
Concentration level	Low	High	Low	High	Low	High
DEP	2 (26)	1 (25)	9 (18)	10 (2)	101 (6)	99 (5)
DETP	2 (10)	1 (145)	116 (5)	0 (0)	57 (10)	67 (8)
DMP	5 (73)	6 (24)	10 (10)	7 (10)	102 (8)	105 (6)
DMTP	0 (24)	0 (15)	135 (7)	0 (0)	74 (3)	77 (3)

Graph 1 Average recoveries of pesticide metabolites on two concentration levels



Graph 2 Boxplot graph for pesticide metabolites in µg/g creatinine



Conclusions

This study presents a novel sensitive, selective, robust and reproducible method for determination of 15 pesticide metabolites, both specific and non-specific. The method was validated using different sets of recovery standards and real urine samples. Such method could be easily implemented as routine in various cohort studies assessing the influence of pesticide mixtures on human health, even at the trace level concentrations.

Abbreviations

Dimethylphosphate (DMP), diethylphosphate (DEP), dimethylthiophosphate (DMTP) potassium salt, diethylthiophosphate (DETP) potassium salt, diethyldithiophosphate (DEDTP), 3,5,6-trichloro-2-pyridinol (TCPY), malathion dicarboxylic acid (MDA), p-nitrophenol (PNP), 2-isopropyl-4-methyl-6-hydroxypyrimidine (IMPY), 3-phenoxybenzoic acid (3-PBA), coumaphos (CMHC), 4-fluoro-3-phenoxybenzoic acid (4F3-PBA), *trans/cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (*t/c*-DCCA), N,N-diethyl-3-methylbenzamid (DEET); 2,4-dichlorophenoxyacetic acid (2,4-D)

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