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Human Exposure Biomarkers of Some Commonly Used Pesticides

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2020

Document Version:
Publisher's PDF, also known as Version of record

Link to publication

Citation for published version (APA):
Faniband, M. (2020). *Human Exposure Biomarkers of Some Commonly Used Pesticides*. [Doctoral Thesis (compilation), Department of Laboratory Medicine]. Lund University, Faculty of Medicine.

Total number of authors:
1

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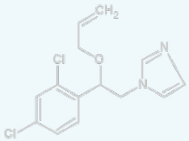
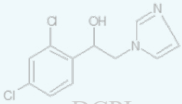
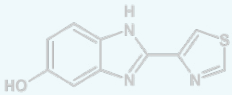
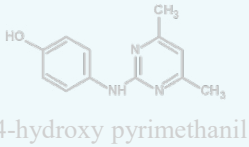
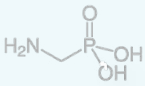




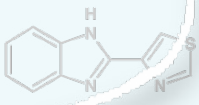


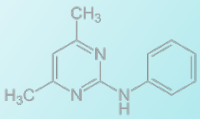


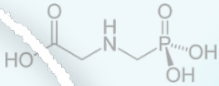


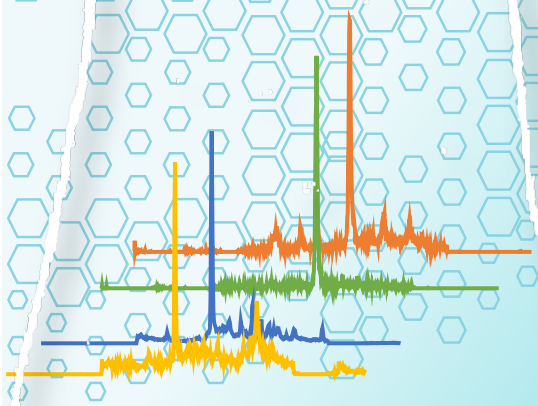
Human Exposure Biomarkers of Some Commonly Used Pesticides

MOOSA HASSAN FANIBAND
DEPARTMENT OF LABORATORY MEDICINE | LUND UNIVERSITY









Human Exposure Biomarkers of Some Commonly Used Pesticides

Human Exposure Biomarkers of Some Commonly Used Pesticides

Moosa Hassan Faniband

LUND UNIVERSITY

DOCTORAL DISSERTATION

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To be defended at Hörsalen, Medicon Village on 24th of January 2020 at 09:15 am.

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Organization
LUND UNIVERSITY
Division of Occupational and Environmental Medicine

Document name
DOCTORAL DISSERTATION

Date of issue
January 24th, 2020

Sponsoring organization

Author Moosa Hassan Faniband

Title Human Exposure Biomarkers of Some Commonly Used Pesticides

Abstract

The use of pesticides has caused pollution of surface and groundwater, soil and air across the world. Many pesticides cause health effects in humans. General populations are exposed to some degree and diet is reported to be a main source of exposure. It is important to study exposure and exposure-effect relationships in occupational groups and in general populations. For such studies, good methods to monitor exposure, such as human biomonitoring are needed. However, for many pesticides, there is a lack of knowledge on exposure biomarkers and analytical methods to measure exposure. Thus, there is a need for validated biomarkers of exposure and analytical methods.

In this thesis, new methods were developed and validated to analyze the fungicides thiabendazole (TBZ), imazalil (IMZ) and pyrimethanil (PYM) and their exposure biomarkers in urine using LC-MS/MS. Further, an LC-MS/MS method for analysis of glyphosate (GLY) and one of its metabolite in urine was established. The analytical methods were sensitive enough to measure a wide range of concentrations of exposure biomarkers in populations and showed good precisions.

Human experimental exposures (oral and/or dermal) with TBZ, IMZ, PYM and GLY were performed with 2-3 volunteers to investigate basic toxicokinetics. The excretion of biomarkers in urine was rapid with short half-lives for all the four pesticides. The biomarkers of IMZ, TBZ and PYM were found conjugated with glucuronides and sulfates. The concentrations of biomarkers varied quantitatively with the degree of exposure.

The analytical methods were applied to biomonitor the exposure in occupationally exposed groups and in general populations. The measured biomarkers in population groups in Sweden reflect concentrations which seem to be far below the excreted concentrations after an intake of a dose equivalent to a dose half or equal to the ADIs. The diet of the general population could be a possible source of exposure. The measured exposure biomarkers of IMZ in greenhouse workers and of PYM in orchardists were higher than the general population and reflected concentrations that were sometimes close to those following an exposure at a dose half or equal to the ADIs.

In conclusion, the analytical methods performed well and can be applied in biomonitoring studies. The identified biomarkers of all four pesticides were related to the exposures and the human experiments facilitated validation of the biomarkers. The short urinary excretion half-lives require a well-planned sampling strategy.

Key words exposure biomarkers, biomonitoring, pesticides, human experimental exposures, mass spectrometry

Classification system and/or index terms (if any)

Supplementary bibliographical information

Language English

ISSN and key title
1652-8220 Lund University, Faculty of Medicine
Doctoral Dissertation Series 2020:6

ISBN
978-91-7619-866-7

Recipient's notes

Number of pages 65

Price

Security classification

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Human Exposure Biomarkers of Some Commonly Used Pesticides

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Division of Occupational and Environmental Medicine
Department of Laboratory Medicine
Faculty of Medicine
Lund University

ISBN 978-91-7619-866-7
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2020

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MADE IN SWEDEN

To my parents, to them I owe everything.

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Populärvetenskaplig sammanfattning

Användningen av kemiska bekämpningsmedel i jordbruket kan förorena yt- och grundvatten, mark och luft i hela världen. Yrkesarbetande inom den gröna sektorn kan exponeras för bekämpningsmedlen liksom i viss mån allmänbefolkningen främst via livsmedel. Bekämpningsmedel kan orsaka hälsoeffekter och det är angeläget att undersöka exponering och effekter av dem. För att studera detta behöver man kunna mäta exponeringen på ett tillförlitligt sätt. Detta kan man göra genom att mäta biologiska nedbrytningsprodukter av bekämpningsmedlen dvs. exponeringsbiomarkörer i kroppsvätskor, t ex i urin. Sådana metoder för att mäta biomarkörer finns för ett antal bekämpningsmedel men för merparten saknas de. Därför finns ett stort behov av analysmetoder för att mäta bekämpningsmedel och deras metaboliter. I denna avhandling har nya analysmetoder för att bestämma biomarkörer av vissa bekämpningsmedel i urin hos människor tagits fram för att sedan tillämpa dessa metoder för att mäta exponering för bekämpningsmedlen i olika befolkningsgrupper. Arbetet är en del av den forskning som utförs inom detta område på Avdelningen för Arbets och miljömedicin vid Lunds universitet.

För att mäta koncentrationen av biomarkörerna i urinen användes metodiken vätskekromatografi med masspektrometrisk detektion (LC-MS/MS). Metoder har utvecklats för antimögelmedlen tiabendazol (TBZ), imazalil (IMZ) och pyrimetanil (PYM) och deras exponeringsbiomarkörer har utvecklats och validerats. En LC-MS/MS-metod för analys av glyfosat (GLY) i urin, som finns i Roundup, och en metabolit har etablerats. Analysmetoderna validerades dvs. kontrollerades med tester som visade att analysresultaten var pålitliga och robusta. De framtagna metoderna var utvecklade för att kunna mäta många prov och såväl höga som mycket låga koncentrationer av dessa biomarkörer kunde mätas med god precision i olika befolkningsgrupper, såväl bland yrkesexponerade som i allmänbefolkning i Sverige.

För att undersöka om biomarkörerna verkligen härstammade från de fyra olika bekämpningsmedlen och hur snabbt de utsöndras från kroppen utfördes experiment där två eller tre försökspersoner exponerades på vårt laboratorium. För att studera hur mycket som tas upp i magtarmkanalen fick försökspersonerna dricka små doser av ämnena och för att studera hur mycket som tas upp i huden satte man små doser direkt på armen. Doserna som försökspersonerna exponerades för bestämdes utifrån det fastställda gränsvärdet ADI (accetabelt dagligt intag) som motsvarar ”den mängd en person kan få i sig varje dag under en hel livstid utan att hälsan påverkas”. Resultaten visade att ämnena togs upp både genom magtarmkanalen och huden och kunde sedan mätas i urinen. Urinutsöndringen var snabb, några timmar, för alla fyra bekämpningsmedlen.

Metoderna för att mäta biomarkörerna användes också för att undersöka halter i både yrkesgrupper och allmänbefolkning i Sverige. Biomarkörer för TBZ och PYR

kunde påvisas i hälften av de urinprov vi analyserade från den sydsvenska allmänbefolkningen, medan man i färre prov kunde mäta IMZ och GLY. Halterna av biomarkörerna som uppmättes i grupper av allmänbefolkningen som relaterades till frågeformulär antydde att kosten kunde vara en möjlig exponeringskälla. Dock var halterna långt under de vi observerade vid ett intag nära ADI i våra experimentella exponeringar. I Sverige verkar exponeringen av allmänbefolkningen för dessa bekämpningsmedel vara mycket låg. De uppmätta halterna bland IMZ-exponerade arbetare i växthus och PYM-exponerade äppelodlare var högre än i allmänbefolkningen. I några fall kunde biomarkörhalterna nå nivåer i närheten av de vi observerade vid ett intag nära ADI i våra experimentella exponeringar.

Sammanfattningsvis var de utvecklade analysmetoderna pålitliga och robusta och de kan användas i studier av exponering i stora befolkningsgrupper. Exponeringsförsöken visade att biomarkörerna var tydligt kopplade till exponering för bekämpningsmedlen. De undersökta bekämpningsmedlens snabba utsöndring kräver en välplanerad provtagningsstrategi.

List of papers

I. Ekman E, **Faniband MH**, Littorin M, Maxe M, Jönsson BA, Lindh CH. Determination of 5-hydroxythiabendazole in human urine as a biomarker of exposure to thiabendazole using LC-MS/MS. *Journal of Chromatography B, 2014, 973, 61-67*.

II. **Faniband MH**, Littorin M, Ekman E, Jönsson BA, Lindh CH. LC-MS/MS analysis of urinary biomarkers of imazalil following experimental exposures. *Journal of Analytical Toxicology, 2015;39, 691–697*

III. **Faniband MH**, Ekman E, Littorin M, Maxe M, Larsson E, Lindh CH. Biomarkers of exposure to pyrimethanil after controlled human experiments *Journal of Analytical Toxicology, 2019, 43, 277-283*.

IV. **Faniband MH**, Norén E, Littorin M, Larsson E, Axelsson J, Lindh CH. Human experimental exposure to glyphosate and determination of urinary exposure biomarkers in young Swedish adults (*manuscript*).

V. **Faniband MH**, Lindh CH, Taj T, Littorin M. Biomonitoring of some commonly used fungicides in adults residing in southern Sweden (*manuscript*).

VI. **Faniband MH**, Lindh CH, Löfkvist K, Littorin M. Exposure to imazalil and pyrimethanil in greenhouse gardeners and orchardists in Sweden: A pilot study (*manuscript*)

Abbreviations

ADI	Acceptable daily intake
AMPA	Aminomethylphosphonic acid
AOEL	Acceptable operator exposure level
CYP	Cytochrome P450
DCPI	1-(2,4-dichlorophenyl)-2-(1H-imidazole-1-yl)-1-ethanol
GC-MS/MS	Gas chromatography tandem mass spectrometry
GLY	Glyphosate
IMZ	Imazalil
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
OH-PYM	4-hydroxy pyrimethanil
OH-TBZ	5- hydroxy thiabendazole
PIS	Product ion scan
PPE	Personal protection equipment
PPP	Plant protection pesticides
PYM	Pyrimethanil
QC	Quality control
SPE	Solid phase extraction
TBZ	Thiabendazole
UER	Urinary excretion rate

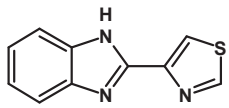
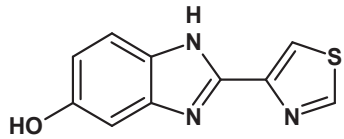
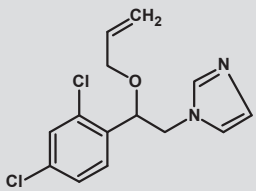
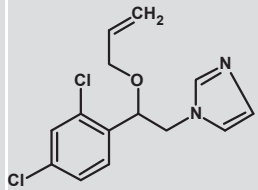
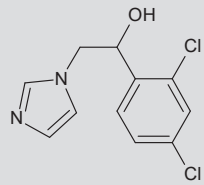
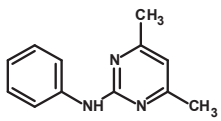
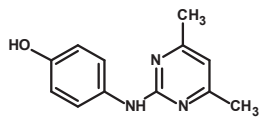
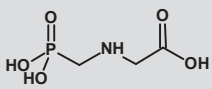
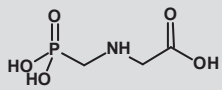
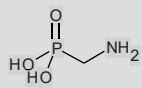
Introduction

General introduction

Plant protection pesticides (PPP) are excessively used in conventional agro and horticulture to chemically control pests, weeds, plant growth and fungal attacks on the cultivation. As a result, pesticides have caused pollution and are ubiquitously detected in air, water and soil across the world. In Sweden, the total estimate of active substances sold to agriculture sector in 2017 was 1447 tons, of which 1153 tons was herbicides and 237 tons was fungicides (Statistics Sweden, 2017). Higher usage of pesticides is seen in southern Sweden due to its intensively cultivated agricultural land, compared to other regions. However, the concentrations of pesticide residues in Swedish grown crops were in general low, compared to those found in commodities imported from other countries in or outside of the EU (Jansson et al. 2016). Fresh fruit and vegetables dominate the import of agricultural commodities in Sweden (Strandberg et al. 2016).

Fungicides are one of the PPP that are used in all the growing stages of crops. Fungicides such as imazalil (IMZ), thiabendazole (TBZ) and pyrimethanil (PYM) are commonly used on a wide variety of vegetable and fruit crops. Residues of IMZ and TBZ were frequently found in fruit samples tested by the National Food Agency of Sweden (Jansson et al. 2016). In Sweden, IMZ is permitted for seed treatment of potatoes and barley and in the cultivation of cucumbers in green-houses (Swedish Chemicals Agency). PYM is permitted for use, for e.g. in apple and strawberry cultivation. Currently, TBZ is not licensed for plant protection in Sweden. Herbicides account for a large portion of pesticide use worldwide and glyphosate (GLY) is the most used pesticide. GLY present in e.g. Round-Up products is a broad-spectrum herbicide also used for crop desiccation. In Sweden, as well, GLY is extensively used but with some restrictions. Some products are also available for household purposes. The sale of glyphosate in Sweden was 485 tons in 2017 and was the most commonly used PPP (Swedish Chemicals Agency). In the current thesis work, TBZ, IMZ, PYM and GLY are the studied compounds (Table 1).

Table 1.
Structures of the parent pesticides and their urinary exposure biomarkers selected in the human experimental exposures. The exposure biomarkers shown below were used in the method development and biomonitoring of exposure in populations. The biomarkers were found as conjugates of glucuronides (as in case of IMZ and DCPI) or glucuronides and sulfates (as in case of OH-TBZ and OH-PYM). The biomarkers of GLY were measured as unconjugated compounds.

Parent pesticide	Chemical structure	Urinary exposure biomarker	
Thiabendazole (TBZ)		 OH-TBZ	
Imazalil (IMZ)		 IMZ	 DCPI
Pyrimethanil (PYM)		 OH-PYM	
Glyphosate (GLY)		 GLY	 AMPA

Scientific literature on pesticide exposure is increasingly being linked to a wide range of adverse health outcomes (Eddleston et al. 2002, Sisman et al. 2010, Orton et al. 2011, Mnif et al. 2011, Ntzani et al. 2013, Kim et al. 2017). The imidazole fungicides such as imazalil may influence the cytochrome P450 isoforms (Muto et al. 1997) and are known to cause anti-androgenic effects as shown in animal studies (Vinggaard et al. 2006). The fungicide TBZ is linked to liver and kidney diseases (Bion et al. 1995, Tada et al. 2001). In rodent studies, PYM is suspected to adversely affect the endocrine and reproductive system and may also cause other adverse effects (Hurley et al. 1998, Medjakovic et al. 2014, Orton et al. 2011). GLY is classified by the International Agency for Research on Cancer as ‘’probably carcinogenic to humans’’ (IARC, 2015). However regulatory bodies such as EFSA, ECHA and US EPA are not in agreement with the decision (EFSA Journal 2015,

ECHA 2017, US EPA 2016). Although very limited knowledge is available; these compounds are commonly used. The Swedish National Food Agency (Livsmedelsverket) has reported residues of TBZ, IMZ, PYM and GLY in food products (Jansson et al. 2016). Occupational and general populations are exposed to residues of pesticides and may be at health risk. Thus, monitoring of human exposure to pesticides is important.

Biomonitoring of pesticide exposure

Human biomonitoring of exposure is a measurement of concentrations of chemicals and/or their metabolites in human biological samples. Human biomonitoring of exposure is increasingly being applied in public health, in detection of risk of exposure, and in environment and health policy making (Joas et al. 2012). There are programs such as e.g. National Health and Nutrition Examination Surveys (NHANES) in United States, the German Environmental Surveys (GerES, Federal Environmental Agency) in Germany and Health-related Environmental Monitoring (HÄMI) in Sweden, which biomonitor exposure of populations to pesticides. In biomonitoring studies, various biological samples or matrices are used, however blood and urine are the most common matrices (Barr et al. 2005). Whole blood, serum and plasma have been used to measure lipophilic and persistent organic pollutants (POPs) such as organochlorine pesticides which have long half-lives. For non-persistent pesticides such as current use pesticides, urine is preferred due to abundance and ease of sampling (Yusa et al. 2015).

Biomarkers of exposure

A biomarker of exposure in humans can be described as a chemical, or its metabolite or a product of an interaction between a chemical and a target molecule that is measured in different matrices (Manno et al. 2010). Exposure biomonitoring estimates the internal exposure of a chemical by taking into account all routes of exposure, however it may not differentiate between the different routes (Manno et al. 2010, Barr et al. 2006). Measurement of specific biomarkers is advantageous to identify the parent pesticide which may help in exposure assessment. It may be challenging to assess exposure when a parent compound is likely to degrade environmentally and the exposure may occur to a metabolite of the parent pesticide (Yusa et al. 2015). It is important to validate exposure biomarkers by testing whether they are specific to the exposure, and that there is a dose-concentration relationship. Human experiments provide suitable samples for such investigations.

A good biomarker of pesticide exposure (cf. Hoet et al. 1997):

- does not require sampling method which causes much discomfort
- is specific to the chemical of interest
- varies quantitatively with the degree of exposure
- can be measured sensitively to detect low level exposure
- is stable in the matrix under storage conditions
- can be measured by a validated analytical method
- can be measured with a method that is time-efficient and cost-effective.

A good biomarker may address pesticide exposure in general population, and can be useful in occupational settings, when PPE may fail and can be used as a pedagogic tool to visualize exposure for the individuals.

Some aspects of pesticide metabolism

The role of human metabolism studies of pesticides is important e.g. from the point of view of biomonitoring (Hoet et al. 1997). Most of the available literature on kinetics and metabolism of pesticides is based on animal studies. This may serve as a good starting point for method development. However, the data from animal studies may be difficult to extrapolate to humans due to interspecies differences.

An exposure may lead to uptake of a pesticide. Pesticides may enter the blood circulation via different ways based on the routes of exposure. The physicochemical characteristics of the pesticides may play a major role in their uptake. Water soluble compounds are less readily absorbed in the alimentary canal and through skin, compared to the lipophilic compounds (Casarett and Doull, sixth edition 2001). However, the absorption of extremely lipophilic compounds is low. In the oral exposure, the absorption of some compounds may already start in the oral cavity and continue throughout the alimentary canal. In the dermal exposure, the compounds pass through the epidermis before entering the blood capillaries in the dermis. In inhalation exposure, gases, aerosol or small particles of pesticides, may be absorbed in the respiratory tract and pass into blood.

Many of the absorbed pesticides are metabolized by a combination of several chemical reactions. The metabolism is majorly initiated by the CYP enzymes during the phase I biotransformation, and liver is the major site for these enzymes. The phase I induced reactions may add or expose a functional group of a compound, which makes the compounds hydrophilic. The most commonly induced reactions by the phase I enzymes are oxidation, hydrolysis, dealkylation, epoxidation, desulfuration, etc. In the phase II biotransformation, the sites exposed by the phase I reactions are conjugated with glucuronic acid, sulfates, glutathione, etc. which increase the hydrophilicity of the compounds and facilitate excretion. In some cases, the phase II enzymes may induce the metabolic reactions of the compounds, without

the involvement of the phase I enzymes. Thus, the metabolism of pesticides is important to study. In this thesis we have included studies on some conjugation reactions. The biomarkers of exposure were studied in urine because there were reports of excretion of metabolites in this matrix to some extent. Further, urine is easy to sample and causes little discomfort to an individual.

LC-MS/MS analysis of pesticides

Various analytical methods are described in the literature for pesticide analysis in biological samples of populations (e.g. Barr et al. 2008, Lewis et al. 2014, Yusa et al. 2015, Connolly et al. 2018b, Conrad et al. 2017, Chang et al. 2016, Knudsen et al. 2017, Mandic-Rajcevic et al. 2019, McKelvey et al. 2013, Tao et al. 2019). The majority of pesticide analyses in biological samples is performed by gas chromatography mass spectrometry (GC-MS) or LC-MS/MS. However, the method of choice is increasingly LC-MS/MS. Polar pesticides and their metabolites which are usually more polar than the parent compound, can be conveniently analysed, when comparing to gas chromatography (GC) methods. LC-MS/MS is a highly sensitive technique, and performs well in the analysis of thermolabile and less-volatile compounds. Such compounds are usually problematic in GC analysis and often require derivatization steps and rigorous sample preparation. Analysing pesticides by LC-MS/MS works well even with simple or minimum sample preparation.

LC-MS/MS analysis combined with solid phase extraction (SPE) can achieve trace level analysis with improved LODs. SPE is a widely applied sample extraction and concentration method in targeted analysis since most common biological samples are in liquid phase. The availability of 96-wells plates for SPE and sample preparation, the possibility of multi-residue analysis methods due to improved chromatographic columns and high performance of current generation MS, makes LC-MS/MS a high-throughput and time-efficient analytical technique.

The validation of the methods described in this thesis was performed following the guidelines of Food and Drug Administration (FDA, 2001), Eurachem and Oxford journal's (Journal of Analytical Toxicology) validation requirements, with some modifications. Availability of pure chemical standards and appropriate internal standards are necessary for method development and a validation process. The validation may demonstrate the performance of the method by estimation of essential parameters such as specificity, linearity, limit of detection, precision of the method and stability of the analyte during the sample preparation process and storage. Although there is scientific literature on analysis of pesticides in environmental samples, literature on exposure biomarkers in humans is limited, particularly concerning currently used non-persistent pesticides (Yusa et al. 2015). Thus, analytical methods for monitoring of human pesticide exposure are important to develop.

Human experimental exposure studies

The interpretation of biomonitoring data for public health is mostly based on animal studies. However, the value of animal data for humans is limited due to interspecies differences. Biotransformation products of pesticides in animal studies may differ from those in humans, which may lead to differences in interpretation of exposure biomarker (Wilkes et al. 1993). Human experimental exposure studies have been lagging behind and one of the reasons is ethics (London et al. 2010). Human exposure studies may aid in the interpretation of biomonitoring data (Bahadori et al. 2007). There is scarcity of literature on toxicokinetic data based on human volunteer studies for currently used pesticides. Some studies are available such as Griffin et al. 1999 on chlorpyrifos; Garfitt et al. 2002 on propetamphos and diazinon; Lindh et al. 2007, 2008 and 2011 on 3,5-dichloroaniline, vinclozolin, iprodione, 4-chloro-2-methylphenoxyacetic acid (MCPA), 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), ethylenethiourea (ETU) and chlormequat (CCC); Sams et al. 2011, 2012 and 2016 on deltamethrin, pirimicarb, chlorpyrifos-methyl and penconazole; Berthet et al. 2010 on captan and folpet; Ekman et al. 2013 on ETU; Garner et al. 2014 on methamidophos; Khemiri et al. 2017 and 2018 on lambda-cyhalothrin; Harada et al. 2016 on acetamiprid, clothianidin, dinotefuran and imidacloprid; Oerlemans et al. 2019 on tebuconazole. Hence, controlled human experimental exposure studies with low oral and dermal dose, may be performed to investigate human metabolism, excretion and dermal uptake of pesticides. Due to advancements in the sample preparation and analytical techniques, it is possible to perform low dose studies and still detect trace concentrations of compounds in samples. Thus controlled human experiments are important to perform.

Some issues in biomonitoring

In population studies, temporal variability in the biomarker concentrations may be an issue (Barr et al. 2006) and sampling of urine at an appropriate time is necessary to assess the exposure to pesticides. Morning void or spot urine sampling is generally used in general populations as a sampling strategy in biomonitoring studies (Barr et al. 2005). A single sample may not sufficiently reflect an individuals' exposure which may be especially so in case of currently used pesticides which have short half-lives. Knowledge of toxicokinetics of pesticides from e.g. human experimental exposures may help in planning of sampling strategy. For adequate exposure assessment in individuals, it has been suggested to collect multiple samples. Thus, it is important to develop high-throughput multi-analyte methods which are time-efficient and cost effective, which can be applied to analyse many samples.

The concentrations of exposure biomarkers measured in urine may have large variations due to e.g. hydration level of individuals (Hoet et al. 1997). Hence, it is recommended to correct biomarker concentrations for dilution of urine. Correction is generally performed by adjustment with creatinine content or density measurement of the urine sample. Creatinine and density levels vary with some important factors such as gender, age and body mass (Suwazono et al. 2005). Creatinine correction has been suggested for compounds that have a renal excretion similar to the excretion of urinary creatinine and density correction has been suggested when the population is comprised of mixed genders and diverse age groups (Sauvé et al. 2015). Other methods such as osmolality and urinary excretion rate have also been investigated (Weaver et al. 2014, Bulka et al. 2017, Lassen et al. 2013, Middleton et al. 2016). Research suggests that all correction methods have drawbacks and the best method of correcting for urinary dilution is debatable. Further work would be required to investigate other possible methods to adjust for urine dilution.

The determination of LOD affects the classification of biomonitoring results as below the LOD or above. Such below LOD data is substituted by various methods such as ½ LOD, LOD/$\sqrt{2}$ or data imputation, which is further used for statistical analysis of biomonitoring data. Recently it has been suggested that the use of raw data instead of substitution methods can be applied (Gyllenhammar et al. 2017). The variation in the LOD values may present difficulties in comparing results between different studies, e.g. the descriptive statistics of the data may be affected based on the chosen LOD. This may be important in describing biomonitoring data of general populations where the exposure levels are low. It is therefore important to address this issue.

Biomonitoring of exposure to pesticides in populations

Exposure of general population

In general populations, exposure to pesticides may occur mainly via oral but also via dermal and respiratory route. Studies have suggested diet as the main source of pesticide residues found in urine of general populations (Angerer et al. 2007, Ye et al. 2015; Gavelle et al. 2016; Fortes et al. 2013; Berman et al. 2013; McKelvey et al. 2013). Other sources of exposure may be household use of pesticides, exposure at public places treated with pesticides or living in close proximities to farms treated with pesticides, etc.

Recent investigations suggest that the choice of food may affect the exposure of general population to pesticides. Higher consumption of vegetables and fruits can be linked with higher urinary pesticide biomarkers (Ye et al. 2015, Fortes et al. 2013,

Berman et al. 2013). The sections of populations consuming high amounts of vegetables and fruits, such as vegetarians and women (Wallström et al. 2000) might be more likely to be exposed to pesticides via diet. Thus to address sources of pesticide exposure in general populations, questionnaires and diet diaries can be used and then correlated to exposure biomarker data.

Occupational exposure

Pesticides are extensively used in conventional agriculture and workers especially in the developing countries may be substantially exposed. Farmers and others working in agro- or horticulture are potentially in more contact with pesticides than the general population. The work tasks such as mixing and loading of spraying equipment, it's maintenance and re-entry into the treated fields increase the risk of pesticide exposure. Literature suggests that dermal exposure may be a prominent route of exposure in workers (Barr et al. 2006, Baldi et al. 2014, Connolly et al. 2019a, Mercadante et al. 2019), but inhalation and unintentional ingestion may also contribute to the total exposure. The use of personal protection equipment (PPE) is recommended during handling and spraying of pesticides. The workers performing other tasks may express casualness due to lack of information, inconvenience caused by PPE or language barrier in case of migrant workers (Lopez-Galvez et al. 2018). Biomarkers of exposure can be useful in occupational settings, when PPE may fail and can be used as a pedagogic tool to visualize exposure to the individuals. Assessment of the time of exposure in workers can be difficult and some recent studies have closely investigated on-field exposures of workers (e.g. Connolly et al. 2019a, Mandic-Rajcevic et al. 2019). It is important to have a good sampling strategy related to the half-life of pesticides. Measurement of exposure biomarkers may help to identify individuals with different degrees of exposure. Thus, exposure biomarkers need to be evaluated in occupational settings.

Ethics

The human volunteer studies are regulated by ethical bodies which provide protection to the study participants. Strict rules on data use for regulatory risk assessment from human volunteer studies are in place in US and Europe. The United States Environmental Protection Agency (US EPA) has prohibited participation of pregnant or nursing women, children and individuals who cannot consent for themselves in human experimental exposure to environmental chemicals (London et al. 2010). Volunteer studies with non-pregnant adults are permitted based on ethics in accordance with the Declaration of Helsinki (2004), if the data use is not intended for regulatory risk assessment. The European Commission (EC) has stated that for ethical reasons, data collected from volunteer studies shall not be considered

to determine a no observed effect level (NOEL) and shall not be used to lower the safety margins that are in place, except in cases when the data suggests that humans are more sensitive. However, EFSA is supportive towards using human volunteer studies data to derive reference values when the data is obtained ethically and is scientifically acceptable (EFSA 2006). In our human volunteer studies, pregnant or nursing women, children and individuals who could not consent for themselves were not qualified for recruitment. We further implied that individuals in fertile ages were not qualified to participate. Further, our criteria of inclusion were that the participating volunteers were generally healthy and not under medication. The volunteers were under the observation of a physician, a specialist in Occupational and Environmental Medicine. In our human experiments we have administered low doses to the individuals and our developed analytical methods were capable of detecting concentrations above LOD after several days after exposure.

In biomonitoring studies, the protection of the individuals' health over research is important. Non-invasive methods of sampling are recommended. Well defined methodology of the study, benefits of risk assessment and selection of the population shall be justified during the ethical application (Manno et al. 2010). A written informed consent must be asked for from each study individual and adequate information about the study, the right to withdraw, the risks and benefits involved and interpretation of results must be provided (ICOH 2014). A balanced reporting of results should be achieved without worrying the individual and the public. Sharing of individual biomonitoring data must strictly be followed with the consent of the individual and in case of workers' data, only collective data must be shared with the employer (Manno et al. 2014). In reporting of results, individuals should not be identifiable and privacy should be maintained. Thus, in biomonitoring studies it is important to consider ethical aspects.

Aims

General aim

To develop and validate LC-MS/MS methods to measure exposure biomarkers of some pesticides and to apply them in populations. To validate the exposure biomarkers by performing human experimental exposures.

Specific aims

- To develop and validate LC-MS/MS methods to measure IMZ, TBZ and PYM and their metabolites DCPI, OH-TBZ, OH-PYM, respectively, in human urine.
- To conduct human experimental exposures (oral and/or dermal) with TBZ, IMZ, PYM and GLY to investigate basic toxicokinetics and to validate the biomarkers.
- To examine the possible exposure of general population groups in Sweden to the compounds studied.
- To investigate exposure biomarkers of IMZ in green house workers and of PYM in orchardists in Sweden.

Methods

Sample preparation

Preparation of calibration standards and quality controls

For details of the sample preparation, please refer to Papers I-IV. The standards of all the compounds were accurately weighed in 10 mL flasks in duplicates, and dissolved in an organic solvent, e.g. methanol or acetonitrile or in milliQ water acidified with formic acid. The resulting stock solutions were diluted in the range of concentrations preferred for calibration standards. Using these solutions, a blank urine obtained from a healthy volunteer was spiked to the preferred concentration to prepare the calibration standards. Generally, a blank urine of 475 µL was spiked with 25 µL of stock solution containing the compounds to prepare calibration standards with 6-9 concentration levels.

Quality control (QC) samples were prepared by spiking a blank urine with the analytes to have 2-3 concentration levels between 1 and 25 ng/mL. The QC samples were prepared in bulk (50 mL of each) for the preferred concentrations and stored at -20 ºC as 2 mL aliquots to facilitate for a single use.

Preparation of urine samples

The urine samples (500 µL) were pipetted into 2mL 96 well-plate and added with 1 M ammonium acetate buffer (6.5 pH), β-glucuronidase or β-glucuronidase/aryl sulfatase and internal standard. The plates were covered with silicon mats, vortex-mixed and incubated for the optimized time period at 37°C with agitation at 400 rpm. The samples were applied on SPE columns and extracted according to the developed protocols. The eluted samples were mixed and centrifuged at 3000 x g for 10 min before analysis.

For GLY analysis, the urine samples (500 µL) were pipetted into 2mL 96 well-plate and added with 25 µL of 2% formic acid and 25µL (5ng/mL) internal standard. The samples were vortex-mixed and centrifuged. The plates were covered with silicon sealing mats, vortex-mixed and centrifuged at 3000 x g for 10 min before analysis.

Enzyme hydrolysis of urine samples

For details of the enzyme hydrolysis of samples, please refer to Papers I-III. In the Papers I-III, the samples were hydrolysed with β-glucuronidase or β-glucuronidase/aryl sulfatase. Based on the mass spectrometric analysis of untreated urine samples from the experimental exposures and literature data on animal studies, the need for enzyme hydrolysis was verified. The enzyme was chosen by further tests and the hydrolysis time was optimized. For GLY, the literature on animal studies suggested unchanged GLY excretion and conjugation with glucuronides or sulfates was not reported.

Solid-phase extraction (SPE)

The extraction of analytes was performed by SPE in paper I-III. The SPE helped in reducing matrix effects and better chromatography of the analytes on LC columns. The SPE column was selected and the extraction methods were developed by optimizing each step of the process. The plates were pre-conditioned with methanol and water, and wash steps were included before elution of the analytes. The extraction process was performed using 96-channel equipment CEREX 96 under a positive pressure of nitrogen gas for a uniform extraction.

Analysis of samples

The studied pesticides and their metabolites are polar and hence, polar mobile phases were used in the chromatographic separation of the analytes. Formic acid was used as an additive in the mobile phases to decrease the pH and facilitate ionization of the compounds in ESI (electrospray ionization). The chromatographic columns used in the analysis provided good separation of the analytes and stable retention times.

In paper I and III, the chromatographic column Poroshell 120EC-C18 (4.6 x 100 mm, 2.7 µm) from Agilent technologies was used for separation of the analytes. The mobile phases 0.1 % formic acid in methanol and 0.1% formic acid in milliQ water were used for elution of the analytes. In paper II, IMZ and DCPI were separated on a Grace Genesis Lightn (USA) C18 (4 mm, ID 2.1 mm, length 50 mm) column using gradient elution with mobile phases 0.1 % formic acid in methanol and 0.1 % formic acid in milliQ water. The total analytical run time per sample, including equilibration, was around 7.0 min in the three methods. In paper IV, the chromatographic separation of GLY and AMPA was performed on a BIO-RAD Micro-guard Cation H+ (30 x 4.6 mm) column (CA, USA). The mobile phase consisted of 0.1 % formic acid in MQ-water, acetonitrile and 0.2 % phosphoric acid. Gradient elution with progressive flow-rate of the mobile phases was performed and the total analytical run time per sample, including column cleaning and equilibration, was 11 min.

Validation of the analytical methods

Specificity

Blank urine obtained from 10 healthy volunteers at our laboratory were used to test the specificity of the analytical method. Blank urine was subjected to SPE extraction procedure similar to the other samples. The eluates were analysed without IS to test for possible interferences caused due to other compounds present in the matrix. An analyte-free blank urine was spiked with a known amount of IS and was analysed to test for interference caused by the IS. Further, a blank urine was spiked only with a known amount of analyte and was analysed to test for interference caused by the analyte.

Linearity

The linearity of the instrument signal was determined by replicate analyses of the calibration standards over an extended period. The calibration standards were prepared using blank urine samples spiked with 6-9 concentrations covering the expected range of analytes. Every batch of samples contained a set of calibration standards and several batches were prepared on different occasions over an extended period. The linearity was presented as a mean value of the slope of regression lines with 95% confidence intervals and the coefficient of correlation (r2) for the calibration standards range.

Limit of detection and quantification

Blank urines from 10 healthy volunteers were collected which were used to determine LOD and LOQ of the methods. The LOD was calculated as three times the standard deviation of concentration of the peak at the analyte retention time. The LOQ was calculated as 10 times the standard deviation of concentration of the peak at the analyte retention time. In paper I, the LOD was calculated as mean plus three times the standard deviation of concentration of the peak at the analyte retention time, and LOQ was mean plus 10 times the standard deviation of concentration of the peak at the analyte retention time.

Precision

For the measurement of precision, the coefficient of variation (CV) was determined during the within-run, between-run and between-batch analysis. The within-run precision was determined by analysis of 10 blank urines spiked each with three concentration levels. The samples were analysed in the same batch. The between-

run precision was determined by analyses of QC samples which were spiked with three concentrations levels. The QC samples were analysed with every analytical batch in duplicates, for an extended period. The between-batch precision was determined by comparing the duplicate analyses of samples from the exposure studies. The samples were prepared and analysed in separate analytical batches. After analysis, the concentrations were grouped into similar concentration ranges to determine the CV.

Matrix effects

In paper I, the matrix effect was studied based on the signal of the IS. The ratio between the mean peak area of the IS in six chemical blanks (from the same sample batch) and the mean peak area of IS in a sample batch (containing 80 samples), was calculated to investigate the possible matrix effects.

In paper II and III, the matrix effects were studied by applying the post-extraction addition approach. Blank urine samples were obtained from 10 healthy individuals at the department. The samples were divided into 2 set of aliquots and extracted by SPE to clean up the matrix. Then, one set containing 10 aliquots was spiked with a low concentration of analytes and another set of 10 aliquots with a higher concentration of analytes. IS was added to the samples and the precision of the measurements in the 10 different matrices was estimated as CV.

Recovery

For measuring the recovery of the method, the blank urines from 10 healthy volunteers were spiked with the analytes at two concentration levels. One set containing 10 aliquots was spiked with a low amount of analyte and another set of 10 aliquots with higher amount of analyte. The aliquots were SPE extracted and IS was added to the two sets. Further, two sets containing 10 aliquots were extracted using SPE and then one set of aliquots was spiked with the same low amount of the analytes and the second set of aliquots was spiked with the same higher amount of the analytes, as in the first two sets. The aliquots were SPE extracted and IS was added. The sets of aliquots spiked after the SPE extraction were considered as equivalent to 100% recovery. After analysis, the quantified concentrations were compared at both concentrations and recovery was presented in percentages.

Stability

The stability of the compounds was investigated by analysis of the standard solutions and quality control samples, under storage conditions of -20°C. Additionally, in paper II and III, the degradation of analytes was investigated during enzyme hydrolysis, by continuous incubation of samples at 37°C for up to six days.

Human experimental exposures

Healthy non-smoking volunteers (2-3 persons) participated in the experimental exposures and the details of the experiments are described in the papers I – IV. The volunteers gave their written informed consent prior to the studies. Generally, the volunteers had minimized or avoided the intake of conventionally grown foods throughout the study period, especially the foods known to contain the pesticides of interest. The oral and dermal exposures were usually performed with a time gap of at least 6 weeks. For the GLY study in paper IV, the volunteers observed a fasting period of two hours before and two hours after the oral dose. A pre-exposure urine sample was collected from the volunteers before all the experiments and henceforth all voided urine was collected for up to 100 h -120 h after the dose. If convenient, an hourly urine void was collected for the first few hours after the dose was administered. The time of voiding and the total volume of each sample were registered. All the samples were aliquoted and stored at -20ºC until analysis.

Oral exposure to pesticides

The volunteers received a single dose of the pesticides in the form of spiked organic juice. In the GLY exposure in paper IV, the dose was administered in milliQ water. The pesticides were dissolved in ethanol, acetone or milliQ water, based on the solubility, to prepare stock solutions. A full dose of IMZ, which is equivalent to the established ADI of 0.025 mg/kg body weight per day was administered, as described in paper I. In the experiments for TBZ, PYM and GLY, a dose equivalent to 50% of the ADI was administered, as described in the papers II, III and IV. The established ADI for TBZ is 0.1 mg/kg body weight per day, for PYM is 0.17 mg/kg body weight per day and for GLY is 0.5 mg/kg body weight per day.

Dermal exposure to pesticides

The volunteers received a single dermal dose of the fungicides TBZ, IMZ and PYM on the inner side of the forearm. The pesticides were dissolved in acetone, ethanol, or a mixture of ethanol and water, based on the solubility. The solutions were applied on a pre-marked area of the forearm, which was 50 or 75 cm^2. After the

application, the vehicle was evaporated to dryness and the skin was covered with aluminium foil. After 8 h of exposure, the remainder of the dose was wiped with cotton swabs which were moistened with the vehicle. Further, in paper II and III, the exposed area was tape-stripped using a self-adhesive gauze with the size of the exposed area. The tapes were applied on the forearm one after the other, and stripped off using clean forceps. The aluminium foil, swabs and tapes were stored separately in 250 mL glass bottles and added with methanol. The bottles were sonicated and shaken to homogenize the solution and further analysis was performed using a simplified LC-MS/MS method (without the enzyme hydrolysis and SPE extraction) to quantify the amount of fungicides in the aluminium foil, swabs and tapes. For IMZ experiment, a dose equivalent to twice the amount of the established ADI was administered, as described in paper II. In the experiments for TBZ and PYM, the administered dose was equivalent to 25% and 50% of the ADI respectively, as described in the papers I and III.

Investigation of urinary metabolites

For the investigation of biotransformation products of TBZ, IMZ and PYM in urine, the samples obtained from the exposed volunteers were used. The urine samples collected within short time after the experimental exposures were expected to contain high concentrations of biomarkers and were selected. The untreated samples were injected into the LC-MS/MS with similar experimental conditions as described in the respective methods and scanned for metabolites using the LightSight® software. The program was run in parallel with the LC-MS/MS to scan for predicted metabolite mass ranges. The prediction of possible metabolites was based on the phase I and phase II biotransformation of analytes. The software generated optimized SRMs for the predicted metabolites using information dependent acquisition method and a list of possible metabolites was created. The possible metabolites were evaluated by the peak intensities and their detection in urine (by acquisition of product ion scans (PIS) and comparison with pure standards) obtained from both volunteers at different time-points. In some cases, further evaluation was performed by estimation of half-lives of the metabolites. In paper III, a QTOF coupled to an LC system was used to acquire PIS of the selected possible metabolites and compared with the PIS of the pure standards.

Biomonitoring of exposure to pesticides in populations

Environmentally exposed population

TBZ, IMZ and PYM were measured in urine samples of a population in a cross-sectional study. For details, please refer to paper V. The recruited study population consisted of five subgroups that represented different sections of the general population in southern Sweden. The participants were recruited between the years 2005 and 2011. The five subgroups formed a total population of 413 participants. Written and oral information about the project was conveyed and a voluntary participation with a written informed consent was required. The participants supplied a morning urine void and a filled in questionnaire. The questionnaire included information on some socio-demographic and health-lifestyle related questions. In addition, the participants filled in a diet diary for three days preceding the urinary sampling.

Occupationally exposed population

For details please refer to paper VI. The developed analytical methods were applied to measure occupational exposure of greenhouse workers to IMZ and orchardists to PYM in southern Sweden. The green house workers (n=28) were recruited from eight companies producing cucumbers. The workers were examined in June, August and September in 2012, 2013 and 2014. The orchardists (n=20) were recruited who reported the use of PYM in their cultivation. The orchardists were examined during the months of April, May and June in 2013 and 2014.

Measurement of creatinine and density of urine samples

Creatinine concentrations and density were measured in all the urine samples for dilution correction. Creatinine was measured by an enzymatic method (Mazzachi et al, 2000) at the Department of Clinical Chemistry, University Hospital, Lund. The department has accreditation for creatinine analysis. Density of the urine was measured with a hand-held refractometer. The adjustment for urinary density, C_d, was calculated according to the formula, $C_d = C_{(observed)} \times (1 - \rho_{mean}) / (1 - \rho_{sample\ density})$, where $C_{(observed)}$ is the concentration of the analyte in the urine sample, ρ_{mean} is the mean specific density of the samples from the study participants and $\rho_{sample\ density}$ is the density of the sample.

Calculation of elimination half-lives

The elimination half-life was estimated by using the quantified concentrations of the compounds in the urine obtained from the oral and dermal experimental exposures. The excretion half-lives were calculated by the slope of the curve obtained from the plot of the natural log-linear of concentration of the compounds versus mid-time points between the two sample collection times. A pre-exposure sample was collected as a time-zero sample.

Statistical analysis

For details please refer to paper V and VI. The statistical analyses were performed using SPSS version 23 (IBM, Chicago, IL, USA). The descriptive statistics of the urinary exposure biomarker concentrations of the studied fungicides were calculated for the density corrected (μg/L) values. In paper V, the concentrations below the LOD were substituted with the lowest density corrected value divided by the square root of two. Non-parametric tests (Mann-Whitney) were performed to evaluate the differences in the concentrations of the urinary exposure biomarkers of the population, based on the consumption of selected food items/groups. For this analysis, the five population groups were merged into one population of 413 participants. When the differences were statistically significant, they were further explored using binary logistics. A multivariate analysis was performed for each dietary item/item group and was adjusted for age (≤ 39, 40-59 and ≥60), gender and smoking and/or moist tobacco use (No/Yes), as possible confounders to test their effect on the model. The correlation between the urinary exposure biomarkers was examined using Spearman's rho.

Ethics

The human experimental studies and the investigation of exposure to IMZ, TBZ and PYM in the populations of southern Sweden were ethically approved by The Regional Ethical Review Board in Lund, Lund University, Sweden (Dnr2005/463; Dnr2010/41; Dnr2010/465 and Dnr2013/6). The investigation of occupational exposure to IMZ in the green house workers and to PYM in the orchardists was ethically approved by the board (Dnr2010/465, Dnr2013/6). The investigation of GLY exposure in young adults in Sweden was ethically approved by the board (Dnr2018/139). All participants had given their written informed consent.

Results with comments

Validation of analytical methods

Prior to validation of the methods, sample preparation steps were optimized by experimentation. For details, please refer to papers I-III. The biomarkers of TBZ, IMZ and PYM were found conjugated with glucuronides and sulfates. The authentic urine samples obtained from experimental exposures were used to test the efficiency of β-glucuronidase and β-glucuronidase/arylsulfatase enzyme. Further tests were performed to optimize the sample incubation time using the selected enzyme. In the IMZ study, long incubation time (48 h) with β-glucuronidase was found more efficient compared to the β-glucuronidase/arylsulfatase. In the TBZ and PYM study, the results indicated β-glucuronidase/arylsulfatase enzyme to be more effective in deconjugation of OH-TBZ and OH-PYM, respectively. The optimum incubation time for deconjugation of OH-TBZ was 18 h and for OH-PYM was 6 h.

The developed analytical methods are high throughput and all the steps of sample preparation were optimized for 96-well plates format. Thus, the methods can be applied in large biomonitoring studies. The validation of the methods was performed based on the parameters described below. For details, please refer to papers I-III. Validation ensures that the method of measurement is specific, which accounts for interferences, and can detect low concentrations, and is able to quantitatively differentiate biomarker concentrations in different samples, and supplies information on stability of the biomarkers in the matrix. These factors are important in reliable measurement of biomarkers of exposures.

Specificity

The tests for specificity (or selectivity) of the analytical method is recommended to evaluate the interferences which may be caused by chemicals, endogenous compounds and degradation products of analyte or internal standard in the matrix that may co-elute. Hence, chemical blanks, urine blanks, IS-free blanks and analyte-free blanks are used to ensure the specificity of the method. Blank urines obtained from healthy volunteers were used to perform specificity experiments. We found no co-eluting compounds that caused interference with the signal of the analytes. Further, there was no interfering signal with the IS, caused by the presence of analytes in spiked urine samples. Analyte-free urine samples spiked with IS caused

no interference with the signal of analytes. No degradation of IS was found during the sample preparation. Besides, the specificity of the methods was tested in our work through human experiments.

Linearity

Linearity of calibration range is recommended to be evaluated with at least 6-8 non-zero concentrations with an additional zero matrix sample (FDA guidelines). The calibration range should possibly include concentrations close to LOQ. In our calibration model, we used 6-9 non-zero concentrations and a zero concentration sample prepared in blank urine to evaluate the calibration range. The linear range of the calibration curve for OH-TBZ (0.1-100 ng/mL), IMZ and DCPI (0.5-200 ng/mL), OH-PYM (0.25-100 ng/mL), and GLY and AMPA (0.25-200 ng/mL) was determined. Good linearity was observed and the correlation coefficient (r2) was above 0.995. In all our analyses, the batches of samples contained a set of calibration standards which was analysed at the beginning and at the end of each batch, and mean of the slope of both readings was used for quantification of samples. Several batches were analysed on different occasions over an extended period and the linearity of the calibration range was described.

Limit of detection and quantification

In the evaluation of the limit of detection and quantification, we used blank urine from different volunteers. It is suggested to use chemical blanks when a matrix blank is not possible to obtain. In our studies, several urines were analysed to obtain 7-10 different blanks. The LOD was calculated as, three times the standard deviation of concentration of the peak at the analyte retention time in 10 different blank urines. The LOQ was calculated as 10 times the standard deviation of concentration of the peak at the analyte retention time in 10 different blank urines. The determined LODs and LOQs were 0.05 µg/L and 0.1 µg/L respectively for OH-TBZ; 0.2 µg/L and 0.8 µg/L respectively for IMZ and DCPI; and 0.1 µg/L and 0.4 µg/L respectively for OH-PYM. The obtained LODs were low enough to detect concentrations in general population. The sample extraction by SPE contributed to achieve low LODs.

There are different opinions on the estimation of LOD for the analytical methods and several methods are followed. A LOD is generally described as a concentration at which an instrument signal for the analyte is significantly different from the signal of a blank urine matrix. Ideally, the LOD may be estimated based on the analysis of samples which were analysed together with other test samples and quantified using a same calibration curve (Eurachem 2014). Some commonly used methods are: (1) the instrumental signal for an analyte at a concentration which is equal to a signal for a blank sample plus three times the standard deviation of the blanks (Miller and Miller, 2005), (2) three times the standard deviation of concentration of the peak at

the analyte retention time in 6-15 different blank samples or chemical blanks when it is not possible to obtain a blank sample (Eurachem 2014, IUPAC 2002), (3) measurement of 10 replicates each, of the five spiked samples or five 1:2 dilutions of each spiked blank sample (concentrations close to the expected LOD of the method) and determining the reliability of the lowest measured concentration (Theodorsson et al. 2012), (4) five times the standard deviation of the concentration measured in 10 blank samples (Theodorsson et al. 2012), (5) 3.3 times the standard deviation of the concentration measured in < 20 blank samples (Stöckl et al. 2009). Due to differences in the preferred methods, it may be difficult to reach at a consensus to recommend a single method. The variation in descriptive statistics of a population data based on LOD may cause problems in data comparison between studies. Hence, an appropriate determination method of LOD based on the purpose of the study may be applied. It is important that the method is specified in the scientific publications.

Precision

In our studies in paper I-III, the precision of the methods was determined as within-run, between-run and between-batch precisions. All the precisions were under 15 % CV. The CV for the within-run precision or repeatability typically suggested higher variation at lower concentrations and vice-versa. The CV for between-run precision was determined at three concentrations (low, medium and high) using QC samples, which were analysed over an extended period by different analysts. Further, a between-batch precision was determined as previously described (Lindh et al. 2008), by comparing duplicate analysis of samples, which were analysed in different batches. The between-batch precision further explained the variation during an extended period. The reproducibility of the measurements may be tested by inter-laboratory programs. This is important for comparison of results between laboratories. For pesticides in this thesis, only GLY has recently been included in an inter-laboratory program.

Matrix effects

In paper I, the matrix effects were determined by variation of the signal for IS in chemical blanks and urine samples. The mean peak area of IS in the chemical blanks was slightly lower than that for urine samples. The ratio between the means for IS in chemical blanks and urine samples was 0.99 which suggested low matrix effects. In paper II and III, the post-extraction addition approach was used to assess the signal variation due to matrix effects. Post-column infusion and post-extraction addition are the most common methods used to examine matrix effects in LC-MS/MS analysis. The post-extraction addition method provides a quantitative estimate of matrix effects (Eeckhaut et al. 2009). In our experiments, we used 10

blank matrix samples from different volunteers which allowed to quantify variation in the signal for the analytes. Our results suggested low matrix effects and the variation of measurements were within 15 % CV. In our analytical methods in paper I-III, labelled IS were used and sample clean-up was performed using SPE. It is suggested that the use of IS may compensate for the ion suppression or enhancement (Eeckhaut et al. 2009) and sample clean-up by SPE may lower the matrix effects to some extent (Saar et al. 2009).

Recovery

In our experiments, the recovery of an analyte was tested in spiked urine samples. The measurement of recovery may be performed usually when sample preparation involves extraction of samples such as SPE. The average recovery reported in our experiments was close to or above 90 %. The use of an IS may aid to correct for the loss of analyte and to improve precision of the measurements.

Stability

Stability of analytes in the matrix and the standard solutions may be evaluated during storage conditions, when such data is unavailable. Stability tests can be important when sample preparation includes processes such as hydrolysis and incubation of samples to detect any degradation of analytes. In paper I-III, stability of the analytes was evaluated under storage conditions in the spiked urine samples (QC samples) and in the standard solutions. The analytes were found to be stable for at least 12 months. The degradation of biomarkers of IMZ and PYM in urine samples were tested under extended incubation period and were found to be stable for at least 72 h at 37 ºC.

Human experimental exposure

Human experiments provided suitable samples to study biomarkers of exposure to TBZ, IMZ, PYM and GLY. The experiments have helped to investigate basic kinetics and to validate the biomarkers of exposure to these pesticides. Further, the experiments have facilitated in development and validation of analytical methods which were sensitive enough to monitor a wide-range of concentrations of exposure biomarkers in populations.

Oral exposure to pesticides

Animal studies have shown that TBZ is rapidly absorbed and excreted (61 % in urine and 24 % in faeces) mostly as OH-TBZ (free or conjugated) in 48 h after dosage (Tocco et al, 1964). A human study (Tocco et al, 1966) reported maximum concentrations in plasma within 1-2 h after an oral dose and 80 % of the dose excreted within first 24 h. Nearly 50 % of the dose was excreted in urine as conjugates of glucuronides and sulfates and less than 1 % was excreted as parent TBZ (Tocco et al, 1966). In our study in paper I, the data indicated similar results to some extent. The urinary concentrations of OH-TBZ increased rapidly after the oral dose and half-life in the rapid phase was 1-2 h and in the slower phase was 9-12 h. The OH-TBZ was excreted as glucuronide and sulfate conjugates and the dose recovery (21-24%) was lower than the recovery reported in Tocco et al, 1966. The administered doses in Tocco et al. (1964, 1966) were higher than our experiments.

In paper II, the excretion of the biomarkers IMZ and DCPI seemed to follow first-order kinetics and a two-phase excretion. The half-life estimations indicated a rapid excretion phase between 2 and 3 h and a slower excretion phase between 7 and 11 h (density adjusted) and almost 10 % of the dose was excreted within the first 24 h. Similar results were reported in a human case study where half-life of IMZ was found to be 2 h in serum (Stiller et al, 1986); and a half-life of 1 h in plasma of rats administered with a radioactively labelled IMZ (EMEA, 1998). Animal studies have suggested rapid absorption, distribution, metabolism and excretion of IMZ after oral dosing, with almost a complete excretion within 96 h and slightly more amount was excreted in urine than in faeces (Marrs, 2000). Around 10 % of the dose was reported to be excreted as parent IMZ in urine and 3 % in faeces. In our experiments no unconjugated IMZ was detected in urine. We only measured the conjugated IMZ and DCPI as exposure biomarkers in urine after enzyme hydrolysis and reported a recovery of 10 % of the orally administered dose.

In paper III, the elimination of OH-PYM seemed to follow first-order kinetics and a two-phase excretion. The estimated half-life of OH-PYM in urine in the rapid phase was between 3 and 5 h and in the slower phase was between 14 and 15 h. The data suggested rapid uptake of PYM which was extensively metabolized to OH-PYM, and found mostly as conjugates of sulfates. A dose recovery of around 80 % was reported and no unconjugated or conjugated parent PYM was detected. Our results were similar to previously reported animal studies in the document of JMPR-2007 meeting on pesticide residues. In rats, about 80 % of the oral dose of PYM was rapidly excreted in urine as conjugates of glucuronides or sulfates of OH-PYM. The excretion half-life of about 5 h was reported and most of the dose was excreted in the first 24 h (JMPR-part I, 2007).

In paper IV, the excretion of GLY and AMPA seemed to follow first-order kinetics and a two-phase excretion. The urinary concentrations increased rapidly after the oral administration and the half-life in the rapid phase was between 6 and 9 h and in

the slower phase was between 18 and 33 h. The dose recovery was between 1 and 6 % in urine which was excreted as parent GLY. Our half-life estimations are similar to the estimations described by Connolly et al. (2019b), which were between 5 and 10 h. We also reported half-life of AMPA, which was between 4 h and 14 h in the short phase and between 29 h and 56 h in the longer phase. The excretion of GLY seemed similar to the published animal studies (Anadón et al. 2009). The elimination half-life of GLY and AMPA in rats after an oral dose of parent GLY was 14 h and 15 h (in blood), respectively. However, excretion of the dose in urine (10-30%) was higher in animal studies (Williams et al. 2000, Anadón et al. 2009) compared to our experiments.

Differences in the excretion of the pesticides were observed in the volunteers, which may be due to differences in metabolic rate, age, gender and body mass, among other factors. The experiments were limited to 2-3 volunteers, and results may vary in a larger group.

Dermal exposure to pesticides

The dermal experimental exposures to TBZ, IMZ and PYM are described in papers I-III. The data in paper I indicated that the urinary elimination of OH-TBZ seemed to follow first-order kinetics and a single phase excretion. Only around 1 % of the administered dose was found in urine as OH-TBZ, which was excreted as conjugates of glucuronides and sulfates. The half-life of OH-TBZ was around 18 h and 9 h in the female and male volunteer respectively, and the time of maximum excretion was 13 h.

In paper II, the urinary elimination of the biomarkers of IMZ seemed to follow first-order kinetics and a single phase excretion. Of the administered dose, 2-5% was excreted in the urine of the volunteers as IMZ and DCPI. The biomarkers were excreted mainly as conjugates of glucuronides. The excretion half-life was between 7 h and 10 h in both female and male volunteers, and the majority of the recovered dose was excreted within the first 24 h.

In paper III, the elimination of OH-PYM followed first-order kinetics and the excretion seemed to proceed in two phases. The biomarker OH-PYM was found as conjugates of mainly sulfates, but also minor amounts of glucuronides. The maximum concentrations were excreted between 10 and 12 h after the dermal exposure. The half-lives for the female and male volunteer, in the rapid phase were around 8 h, and in the slower phase were between 20 and 30 h respectively. Of the applied dose of PYM, 9 and 19 % was recovered as urinary OH-PYM in the female and male volunteer respectively.

In all three dermal exposure studies, no unconjugated parent compounds were detected in the urine samples. The data from tape-stripping experiments performed in paper II and III suggested that both IMZ and PYM penetrated through the skin of

the volunteers. The excretion of the compounds seemed slow compared to the oral exposure studies. This may be expected due to delay caused by the time required for chemicals to penetrate the skin, before being slowly released into the blood circulation (Fennell et al, 2006). The dermal doses in our experiments were administered in organic solvents (ethanol or acetone). We chose the inner side of fore-arm as the site of exposure due to convenience and it is often a commonly used site in dermal exposure studies. The diffusion of chemicals through skin may depend upon various factors such as e.g. chemical properties of the compound, site of exposure, choice of vehicle used, volume of the vehicle and occlusion of the exposed skin (Ngo et al, 2010, Berthet et al 2010). Skin properties such as density of skin appendages, thickness of stratum corneum, lipid content and aging of skin may affect dermal absorption of chemicals (Ngo et al, 2010). Dermal absorption of chemicals is much higher in liquids than in the dried residue form (Clarke et al, 2018). Hence, higher volume of solvent may increase the time of evaporation of liquids and further influence absorption of chemicals. Occlusion of the exposed skin may increase perspiration, which may further promote uptake (Ngo et al, 2010). All these factors may have differential effects on the dermal absorption of chemicals.

Investigation of urinary metabolites

The literature on metabolism of TBZ both in animal and human experiments suggested OH-TBZ as the main metabolite, which was formed by hydroxylation reaction (Tocco 1966). In humans, OH-TBZ was reported to be excreted as conjugates of glucuronide and sulfate, and unconjugated parent TBZ in trace concentrations. In our studies in paper I, OH-TBZ was confirmed as a biomarker of exposure to TBZ, which was found in urine as conjugates of glucuronide and sulfates. Unchanged parent TBZ detected in urine was 0.05 % of the dose. The PIS of OH-TBZ obtained by LC-MS/MS after enzyme hydrolysis was compared with the pure standard for identification. Other minor metabolites detected were isomers of OH-TBZ and dihydroxy-TBZ, but were not quantified. The dose-concentration relationship of these metabolites confirmed their formation and were related to TBZ exposure.

Animal studies have suggested extensive metabolism of IMZ and 25 metabolites were reported, with DCPI being the major metabolite formed by oxidative O-dealkylation reaction (Marrs, 2000). Other major metabolites reported were di-hydroxy IMZ formed by epoxide hydration and imidazole oxidation of di-hydroxy IMZ. In our study in paper II, IMZ and DCPI were confirmed as urinary biomarkers of IMZ exposure, which were found as conjugates of glucuronides. The concentrations of parent IMZ-glucuronide conjugate were higher than in the animal studies. The metabolites IMZ and DCPI were quantified after enzyme hydrolysis using pure synthesized chemical standards of IMZ and DCPI. Other metabolites

detected in our study were single, di-, and tri-oxidation products of IMZ (as conjugates). The PIS of these metabolites were compared with that of the parent IMZ which confirmed as biotransformation products of IMZ. Additionally, the excretion curves of these metabolites suggested that the resulting metabolites were related to IMZ exposure.

The major urinary metabolite of PYM reported in animal studies was OH-PYM (around 80%) formed by aromatic oxidation and excreted as conjugates of glucuronide and sulfate (JMPR 2007). Other minor metabolites reported were products of different oxidation reactions. Our data in paper III also suggested OH-PYM as a main urinary metabolite after experimental exposure to PYM. Around 80% of the oral dose was detected in urine as OH-PYM, which was excreted majorly as conjugates of sulfate and in minor quantities as glucuronide conjugates. No unconjugated PYM or OH-PYM were detected. The mass spectra of enzyme hydrolysed OH-PYM conjugates acquired by a QTOF were matched with that of pure OH-PYM standard to confirm its identification.

Our results are based on two individuals and interindividual differences due to various factors exist. Investigation of basic metabolism of the compounds has helped to study the metabolites which may serve as specific biomarkers of exposure. Our studies in paper V and VI suggest that these biomarkers can be applied to biomonitor exposure of populations.

Correction for urinary dilution

The data from human experiments (paper I-IV) indicated better correlation coefficients for the excretion curves when the data was corrected with either creatinine, density or urinary excretion rate (UER), compared to the uncorrected data and we recommend to perform dilution correction of the biomarker concentrations. Dilution correction methods have limitations and the limitations of each method should be considered before its application. Creatinine concentrations in urine are affected by several factors such as gender, age, BMI, muscularity and diet. Higher creatinine excretion is often observed in males compared to females (Barr et al. 2005, Wang et al. 2018). The gender and age related differences may be due to the body composition and muscle mass. Urinary density is suggested to be less affected by variability causing parameters such as age, gender and body mass, when compared to creatinine excretion (Suwazono et al. 2005, Hoet et al. 2015, Lermen et al. 2019). Some studies have further addressed these issues by investigating 24 h urine sampling to study variability in biomarker values due to urinary dilution (e.g. Akerstrom et al. 2012, Lassen et al. 2013, Lermen et al 2019). A comparative study of spot urine and 24 h sampling to investigate urinary cadmium excretion has suggested that creatinine adjustment tended to overestimate cadmium

excretion in females (Akerstrom et al. 2012). In biomonitoring studies, correction with urinary density is suggested to be an appropriate approach if the studies include mixed genders and diverse age groups (Sauvé et al. 2015). Furthermore, use of different mean values of urinary density for males and females is proposed to reduce the variability caused by gender differences. Density correction may seem an interesting option, when studying mixed populations.

There are other alternative methods such as e.g. correction with osmolality of urine and UER (Weaver et al. 2014, Bulka et al. 2017, Lassen et al. 2013, Middleton et al. 2016). Osmolality measurement is not available at our laboratory and is expensive. Urinary excretion rate may seem a viable option, however it is usually less feasible in biomonitoring studies to collect full urine voids (Barr et al. 2005, Lassen et al. 2013) and to obtain an accurate information about the time of urine voids. Several density values have been used for dilution correction. This could be a problem for comparability of data, since it may cause over/underestimation of results. For example, in paper VI, the mean value for density in the green house workers and orchardists was 1.022. In this case, using the reference density value e.g. 1.016 may result in underestimation of the biomarker values by 30 %. Further research is required to investigate better methods for correction of urinary dilution.

Biomonitoring of exposure to pesticides in populations

In paper IV, 20 % of the young Swedish adults' population had detectable concentrations of GLY. The median concentrations were below the LOD of 0.1 µg/L and the 95th percentile was 0.2 µg/L (density adjusted). The measured biomarkers in the urine samples suggest that the concentrations were far below the maximum concentrations found in our human exposure study (paper IV), after an intake of a dose equivalent to half of ADI (0.5 mg/kg body weight per day). Similar low concentrations of GLY have been reported in general populations from other parts of Europe (Hoppe et al. 2013, Conrad et al. 2017, Knudsen et al. 2017, Connolly et al. 2018b). In our human experiments, the urinary GLY concentrations in 3-4 days after the oral exposure in three volunteers were between 3 and 10 µg/L (density adjusted), which is much above the median or 95th percentile values of the young Swedish adults' population. Therefore, it seems that the exposure of general population to GLY is low.

In paper V, low concentrations of IMZ, DCPI, OH-TBZ and OH-PYM were detected in 18, 13, 44 and 48 % respectively, of the study population of 413 participants. The concentrations below the LOD were substituted with the lowest density corrected value divided by the square root of two. The median concentrations for all 413 urine samples were below or close to LOD, but occasional higher concentrations were reached in a few cases. The data suggested that the

concentrations of OH-TBZ, IMZ and DCPI were weakly correlated in bivariate analysis (data not shown). In Mann-Whitney U test, vegetables and fruits suspected to be contaminated with TBZ, IMZ or PYM were considered for evaluation of differences in biomarker concentrations between the consumers group (Yes) and the non-consumers group (No). The consumption frequency of food items was based on questionnaire data for the day before the urine sampling. There were statistically significant differences (p-value ≤ 0.005 after Bonferroni correction) in the urinary OH-TBZ and IMZ concentrations between the consumers (Yes) and the non-consumers (No) of citrus fruits. Similarly, a difference in urinary OH-TBZ concentrations was found between groups Yes/No of bananas, and a difference in urinary OH-PYM concentrations was found between the groups Yes/No of wine. These differences were further evaluated using binary logistic regression analysis. The results indicated that the consumers of fruits and wine were more likely to have higher concentrations of urinary biomarkers than others (for details please refer to paper V). Overall, low concentrations of the exposure biomarkers of fungicides IMZ, TBZ and PYM were found in the general populations, except in one sample which contained 448 µg/L (density adjusted) OH-PYM. The measured biomarkers of the fungicides in general population suggest that the concentrations were far below the maximum urinary concentrations measured in our human exposure studies (paper I-III), after an intake of a dose half or equivalent to the ADIs. In our human experiments, in 3.5-5 days after the oral exposure, the urinary concentrations of OH-TBZ (0.3-0.8 µg/L, density adjusted), IMZ (0.2-0.5 µg/L, density adjusted) and OH-PYM (0.2-12 µg/L, density adjusted) in the two volunteers were above the median concentrations of the general population in Sweden. Thus, it seems that the exposure of general population to the monitored fungicides is low.

In paper VI, the recruited greenhouse workers were 6 females and 22 males (n=28) and aged between 18 and 67 years (median 33 years). The workers provided 198 urine samples in total, on multiple occasions. The IMZ concentrations in the urine samples varied between <LOD and 53 µg/L (density adjusted) and the metabolite DCPI varied between <LOD and 9.4 µg/L (density adjusted), and three participants had higher concentrations than the others. The data suggested that the urinary biomarker concentrations of workers who provided multiple samples during the sampling period were above their baseline concentrations. The urinary biomarker concentrations measured in the workers were high compared to the general population (paper V). The maximum measured concentrations of IMZ and DCPI in the urine of occupationally exposed greenhouse workers were below the maximum urinary concentrations measured in the human exposure study (paper II), where the oral dose was equivalent to an ADI (0.025 mg/kg bodyweight per day). But, the concentrations of IMZ and DCPI in the urine of greenhouse workers were close to the maximum urinary concentrations measured in the dermal exposure study (paper II), where the dose was equivalent to the acceptable operator exposure limit (AOEL, 0.5 mg/kg bodyweight per day).

The recruited orchardists were 20 males, aged between 28 and 74 years (median 53 years). In total 109 urinary samples were collected from the orchardists. The concentrations of OH-PYM varied between <LOD and 1352 µg/L (density adjusted) during the study period and only 4 % of samples were below LOD. In comparison to the general population (paper V), the urinary concentrations of OH-PYM in the orchardists were high. The measured urinary biomarkers reflected concentrations that could be close to the concentrations following an exposure of a dose equal to half of ADI (0.17 mg/kg bodyweight per day).

Although the measured occupational exposure reflected the concentrations which may be within the limits, the use of IMZ and PYM should be minimized due to the frequent close contact of workers with the fungicides. In biomonitoring studies, it should be noted that the excretion half-lives of most of the currently used pesticides are short and therefore, the window of peak excretion may be missed. This may lead to misclassification of the exposure (Connolly et al. 2018a). For example, according to Paper II, a suitable time of urine sampling in general population to measure IMZ exposure would be somewhere around 2 – 2.5 h after the exposure. Assuming that the route of exposure is most likely via diet (or oral route), there may be better time points to measure exposure of short half-life pesticides than a morning void. Hence a proper strategy of sampling must be adopted. The aim of a study/project may play an important role to decide the time of sampling. For occupationally and environmentally exposed populations, the sampling strategy may be different, such as the times of multiple sampling.

Strengths and limitations

Strengths

- The developed LC-MS/MS methods perform well to measure specific biomarkers of the studied compounds.
- In IMZ, TBZ and PYM studies, both oral and dermal exposures were performed, which are major routes of pesticide exposure in populations.
- The generated data in the human exposure studies contributes to fill the lack of knowledge in the field. The data may be valuable in the exposure assessment of populations in biomonitoring studies.
- The selective recruitment of different sub-sections of the general population enabled to investigate groups which may be more likely to be exposed to pesticides.
- In the investigation of occupational exposure to IMZ and PYM, repeated samples from the workers made it possible to study the range of exposure.

Limitations

- The human experimental exposures were performed with only a few individuals and results of the study may vary in larger group of volunteers.
- No controlled airway exposure to pesticides was performed, which is an important route of exposure.
- Low recovery of some compounds in urine in the human experiments was not investigated further.
- In the general population study, the extrapolation of the results to the whole population in Sweden may not be possible due to selective recruitment of groups.
- The exposure biomarkers of the studied compounds have short excretion half-lives. Hence, biomonitoring based on a single urine void may lead to

misinterpretation of the exposure. A better sampling strategy may be required to monitor exposure of these compounds in general population as well as in occupationally exposed population.

- Information obtained by measuring exposure biomarkers in biological fluids may not be sufficient to distinguish between different sources of exposure. Other methods such as ambient monitoring can complement to reflect exposure.

Conclusions

The key conclusions of the thesis are as follows,

- LC-MS/MS methods were developed for the quantification of exposure biomarkers of IMZ, TBZ and PYM in human urine. The methods showed a good precision and a low limit of detection was achieved. The methods performed well and enabled detection of low concentrations of biomarkers in environmentally exposed populations. The methods were high throughput and thus aided in performing sample analyses in large studies.
- In the oral and dermal experimental exposures of IMZ, TBZ and PYM, the excretion of biomarkers in urine was rapid with short half-lives. The biomarkers were excreted as conjugates and enzyme hydrolysis with glucuronidase and sulfatases increased the biomarker concentrations. The measured biomarkers were specific to the exposures, and are suggested as suitable biomarkers in biomonitoring studies.
- After an oral dose, the excretion of unchanged GLY and its metabolite AMPA in urine suggested short half-lives. The recovery of GLY was low and varied between the individuals. Low concentrations of GLY exposure biomarkers were found in young adults of a general population from southern Sweden.
- Low concentrations of biomarkers of IMZ, TBZ and PYM were detected in the general population groups in southern Sweden, with occasional high values. However, the measured biomarkers in 413 urine samples from population groups in Sweden reflect concentrations which seem to be far below the excreted concentrations following an exposure at a dose half or equal to ADI values for the three fungicides. The diet of the population seemed to be a possible source of exposure.
- The measured exposure biomarkers of the greenhouse workers and the orchardists to IMZ and PYM respectively, in Sweden, could be high. However, the biomarker concentrations in the workers reflected levels that could reach concentrations close to those following an exposure at a dose half or equal to the ADIs.

Future research perspectives

- The data generated in the current thesis contributes to the field of biomonitoring of pesticides. The developed methods and data may contribute in future studies.
- It seems relevant to investigate the low turnover of TBZ, IMZ and GLY in human exposure studies. Other urinary metabolites may be explored and quantified, e.g. the di-hydroxy IMZ, because its formation indicates a potentially reactive epoxide.
- The chelating property of GLY is known and has been described in literature. Can this affect the uptake of GLY? GLY may form complexes in the human body, which may be important to investigate.
- Development of better methods to adjust for urinary dilution.
- Recent literature is indicating towards the possible effects of pesticides on human gut microbiota. Altering of the gut microbiome is being associated with digestive tract disorders. It may be interesting to investigate pesticide exposure in diet cohorts and explore the possible links to outcomes related to digestive tract diseases.
- There is a vast number of pesticides used globally which may be relevant for further studies. More methods need to be developed for analysis.

Acknowledgements

This work was supported by the Swedish Environmental Protection Agency; SIDA- The Swedish Agency for International Development Cooperation; National Institutes of Health (NIH)/National Institute of Environmental Health Sciences (NIEHS); FORMAS- Swedish Research Council for Sustainable Development; FORTE- Swedish Council for Working Life and Social Research; Stiftelsen Lantbruksforskning- Swedish Farmers' Foundation for Agricultural Research; Skåne Regional Council; and the Medical Faculty at Lund University, Sweden.

There are so many, to whom I would like to thank for their help and support throughout my PhD studies. Here I will make an attempt to express my sincerest gratitude.

Firstly, I thank my supervisor Christian Lindh, for his guidance, engagement, support and patience throughout these years. I am grateful to you for teaching me LC-MS/MS when I was new in lab and up until today. I am sincerely thankful for giving me opportunities and involving me in various projects, which has helped me to gain knowledge and learn new skills especially in the lab work. Thank you for the invaluable discussions and sharing your vast experience and knowledge, that has helped my understanding about the research area.

I thank my co-supervisor Margareta Littorin, for your enthusiasm and active involvement all through the years. Thank you for all the discussions, guidance and conversations (scientific and others) and your tireless reading and reviewing of manuscripts and thesis. Thank you for sharing your vast knowledge and thoughtful insights.

I thank late Bo Jönsson (R.I.P), for his scientific guidance and support during my early days at the department, and for his great sense of humour and casual conversations.

Thank you Margareta Maxe, for teaching me everything in the lab. Thank you for your endless support, kindness, important advices and always being there no matter what. You always brought smiles and humour to the lab.

Thank you Agneta Kristensen, Åsa Amilon and Birgitta Björk for your kindness, cheerful nature and all the help throughout the years.

Thank you Karin Broberg Palmgren for your insightful advices and support during the years and the opportunity to work with you in the filaggrin project.

Shegufta A, it was great to share office and speak Hindi with you all the time and discuss bollywood sometimes. Thank you Bakhtiar H for all the humour and kindness. Thank you to both, for giving me a family feeling and most importantly for all the shahi Bengali cuisine.

Huiqi L and Yiyi X, thank you for your friendship, social meetings and home-made authentic Chinese cuisine. It was perfect to be your neighbour at work and also at home. I felt safe knowing that I could ask you about statistics at any point.

Ayman, thanks bro for all the training, sports sessions and social activities and above all being a great friend.

Thank you Tahir for all your statistical help and advices. It was amazing to have a friend and colleague to speak Hindi and share similar culture, and your Pakistani style biryani is excellent. Thank you that I could always count on you.

Lina, thank you and Ibaa for your close friendship, for all the social events and delicious Iraqi cuisine. It was wonderful to have you as a colleague in my early days at the department and to be able to speak with you about different things. Above all, thank you for always being around no matter what.

Thank you Zeeshan and Rabia for your unlimited friendship. Those barbeques we had are memorable. You guys reminded me of home. Thank you for the best achar gosht ever.

Thank you Annette K for reviewing my work during half-time and final assessment and being a wonderful office-mate, friend, colleague and a fantastic innebandy team-mate. Thanks for all the social events and helping me out with transporting my bike in your VW Golf.

Emelie R-L and Erika N, it is wonderful to be able to talk to you about anything and everything. At work, you two have been my go-to people. Thank you for your support, intelligent advices, encouragement and for always having a listening ear for me. Erika, after the Lana's incidence I say you together with Anna R are the best recovery agents.

Mats G, thanks for being a great friend and colleague. Thank you for all the casual discussions and fun innebandy and badminton sessions.

Yona (Julie) E, it is a pleasure to share office with you. Thank you for being easy to talk to and for being a great training pal.

Thank you Eva E for your help and being the colleague to discuss about pesticides among other things. Thank you Harriet M, Gabriella Å, Neserin A, Julia B and Hanna L for being great colleagues.

Thank you to Marie B, Hamideh R, Anna R, Bo S, Monika K, Karin W, Daniella P, Helene Å, Karen C, Carina N, Maria W, Estelle L, Kristoffer M, Jonathan L and Ralf R for all your help, great working atmosphere, fun conversations and for your

kind and friendly attitude. Marie B, thank you for your company during the trip to the workshop in Wageningen; Hamideh R, Anna R and Karen C, thank you for your help during filaggrin exposure study; Bo S, thank you for the sports activities, advices on presentations and casual talks; Thanks Estelle L for reviewing my work and your input during my half-time assessment; Thomas L for reviewing my work and your input for my final assessment; Ralf R for your timely help related with computers.

Thank you Eva Assarsson and Eleni for all your help during the exposure study. We were a good strong team. Eleni thanks for your always friendly attitude.

Thank you to my co-authors Eva E, Erika N, Estelle L, Jonatan A, Tahir T and Klara L for your help and excellent comments on the manuscripts.

Thank you Ewa K and Pawel K, for your unlimited friendship. You have always been there for us on so many occasions. Your kindness and generosity was very much appreciated.

Thank you to my friends in India for keeping me updated with anything and everything on what's up back home.

Thank you to my Indian and Polish family for their love, infinite support and best wishes. Thank you for inviting and hosting us on our short and long stays. Thank you to my daughter Sara who has been my sun, constantly showering me with her energy and innocence. She has helped me see the other side of life which I was unaware of. Finally, thank you to my lovely wife Ewa for her encouragement and standing behind me through all.

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Printed by Media-Tryck, Lund 2020 NORDIC SWAN ECOLABEL 3041 0903

Division of Occupational and Environmental Medicine
Department of Laboratory Medicine

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2020:6
ISBN 978-91-7619-866-7
ISSN 1652-8220

LUND UNIVERSITY

FACULTY OF MEDICINE