# Biomarkers of exposure to pesticides in humans

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# Biomarkers of Exposure to Pesticides in Humans

# Biomarkers of Exposure to Pesticides in Humans

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LUNDS UNIVERSITET

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**Abstract**

Almost every human is exposed to pesticides, in work environments, by domestic use and via diet, drinking water and personal products. Current research expresses concern that low dose exposure over time can lead to adverse health effects. It is therefore important to biomonitor exposure to pesticides in different groups, especially vulnerable groups such as pregnant women. However, there is a general lack of validated bioanalytical methods in order to generate reliable biomonitoring data for the quantification of exposure biomarkers to pesticides. The present thesis describes efforts directed at addressing this shortage.

Three new high throughput LC-MS/MS (liquid chromatography coupled with tandem mass spectrometry) methods were developed and validated for the quantification of the exposure biomarkers for ethylenebisdithiocarbamates (EBDCs): ethylenethiourea (ETU); thiabendazole (TBZ): 5-hydroxythiabendazole (5-OH-TBZ) and pyrimethanil (PYR): 4-hydroxypyrimethanil (OH-PYR). Human experimental studies, where two volunteers were orally and dermally exposed, were conducted to confirm that these three biomarkers are metabolites of their parent compounds. For ETU, however, only dermal exposure was studied. In these studies, also basic pharmacokinetics were determined. In an epidemiological cohort study of 445 pregnant women living close to banana plantations in Costa Rica, the LC-MS/MS method for ETU and a modified version of the combined methods for 5-OH-TBZ and OH-PYR were applied to assess exposure to EBDCs, TBZ, PYR and chlorpyrifos, pesticides used on the plantations. Commonly used pyrethroids and 2,4-dichlorophenoxyacetic acid were also assessed. Exposure to TBZ and PYR had not been studied earlier in pregnant women or other human populations.

The LC-MS/MS methods were selective and had excellent sensitivity; the limit of detection was ≤0.2 ng/mL. The precision and accuracy were also excellent; the coefficient of variation was ≤15%. In all the experimental studies, the exposure biomarkers ETU, 5-OH-TBZ and OH-PYR were excreted in urine as conjugates. In the dermal exposure experiments, the urinary elimination half-life ($t_{½}$) was a few days for ETU and hours for 5-OH-TBZ and OH-PYR. In the oral exposure experiments, the $t_{½}$ was a few hours both for 5-OH-TBZ and OH-PYR.The exposure biomarkers of TBZ and PYR were determined in hundreds of samples from a general Swedish population; half of them had levels of 5-OH-TBZ and of OH-PYR above the limit of detection.

In 909 urine samples repeatedly collected from the 445 pregnant women, ETU was detected in 100%, 5-OH-TBZ in 65% and OH-PYR in 87% of the samples. The concentrations of ETU seem comparable to levels in Italian agricultural workers. Further, the other pesticide exposure biomarkers were detected in almost all the samples.

The pregnant women working at the banana plantations were significantly more exposed to TBZ and EBDCs than the nonworking pregnant women in the cohort and, in addition, the exposure to chlorpyrifos was slightly higher among the working women.

In conclusion, the developed LC-MS/MS **methods** can be used in biomonitoring of EBDCs, TBZ and PYR in large exposure studies of general populations. The metabolites ETU, 5-OH-TBZ and OH-PYR were confirmed to be reliable urinary exposure biomarkers after dermal and oral exposure. Hydrolysis to release the analyte from the conjugate in urine is essential in the LC-MS/MS methods. Some new pharmacokinetic information on ETU, TBZ and PYR were obtained from exposure experiments in two volunteers. Because of the short $t_{½}$ of the **biomarkers**, repeated sampling is recommended in exposure assessments. The exposure to pesticides in pregnant women in Costa Rica is of great concern.

Key words: biomarker, biomonitoring, exposure, mass spectrometry, pesticides, pharmacokinetics, pregnant women, urine

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# Populärvetenskaplig sammanfattning

Idag används årligen över 2 miljoner ton bekämpningsmedel (BM) i världen. Av dessa står Sverige för drygt 9000 ton, eller knappt en halv procent. Den här avhandlingen handlar om att ta fram metoder för att bestämma rester av vissa bekämpningsmedel i kroppsvätskor (urin) hos människor och sedan använda metoderna för att mäta exponering för bekämpningsmedlen i en befolkningsgrupp. Arbetet är en del av forskningen inom detta område på Avdelningen för Arbets- och miljömedicin vid Lunds universitet.

I avhandlingen behandlas svampmedlena mankozeb, tiabendazol och pyrimetanil, insektsmedlen klorpyrifos och gruppen pyretroider samt ogräsmedlet 2,4-diklorfenoxi-ättiksyra. Alla används som växtskyddsmedel, pyretroiderna också som biocider för att skydda människor och byggnader mot t.ex. mygg eller löss. Även om BM är framtagna för att verka mer eller mindre specifikt mot skadegörare är de oftast också giftiga (toxiska) för människor. Exponering för BM kan leda till akuta besvär och olika kroniska sjukdomar. Hanteringen av BM är därför reglerad med bland annat olika typer av gränsvärden. Ett exempel på gränsvärde är ADI (acceptable daily intake) ”den mängd en person kan få i sig varje dag under en hel livstid utan att hälsan påverkas”.

För att kunna koppla en exponering för BM till en hälsoeffekt måste exponeringsdata vara tillförlitliga. I avhandlingen har metoder för bestämning av exponeringsbiomarkörer tagits fram. Dessa exponeringsbiomarkörer är metaboliter dvs. biologiska nedbrytningsprodukter av bekämpningsmedlen. För att mäta koncentrationen av en specifik biomarkör i urinen användes analystekniken vätskekromatografi med masspektrometrisk detektion (LC-MS/MS). Eftersom bra analysmetoder saknades har tre nya LC-MS/MS metoder utvecklats eller förfinats för att mäta exponering för mankozeb, tiabendazol och pyrimetanil. Metoderna validerades – dvs. kontrollerades med tester så att analysresultaten är pålitliga och genom att testa respektive exponeringsbiomarkörs pålitlighet. Genom att två försökspersoner fick en låg dos (25-100% av ADI-värdet) av bekämpningsmedelet på huden eller oralt kunde det fastställas att biomarkören i urinprovet verkligen härstammade från bekämpningsmedlet.

Resultaten visade att BM togs upp både genom huden och magtarmkanalen för att sedan utsöndras bl a i urinen. Resultaten visade också att utsöndringen till urinen tog olika lång tid för de tre nämnda ämnena (timmar till dag/ar/). Det är intressant eftersom kort utsöndringstid kräver upprepade mätningar för att en god uppfattning om exponeringen ska erhållas. 5-OH-TBZ och OH-PYR kunde också påvisas i hälften av hundratals urinprov från den sydsvenska allmänbefolkningen.

Sättet att använda BM på påverkar hur det sprids i miljön och därmed hur vi människor exponeras. Vissa, exempelvis jordbruksarbetare, kan exponeras i arbetsmiljön. Allmänheten exponeras i sin hemmiljö via maten men också genom egen användning av ogräs- och insektsmedel. Människor som lever i områden där BM används, kan exponeras indirekt, t ex genom drift av BM med luften. Exponeringsförhållandena motiverar undersökning av halter av BM-rester i olika befolkningsgrupper, särskilt i känsliga grupper som gravida kvinnor och deras foster. Avhandlingen syftar till att bidra till ökad kunskap inom området.

Inom avhandlingen mättes också totalt 8 exponeringsbiomarkörer av BM i 900 urinprov från 445 gravida kvinnor boende nära bananplantager i Costa Rica. I proverna detekterades ETU i 100%, 5-OH-TBZ i 64% och OH-PYR i 87%. Övriga exponeringsbiomarkörer hittades i stort sett i alla prov och värdena var i nivå med vad som hittats hos gravida kvinnor i varierande miljöer världen över. ETU-halterna var nära halterna hos italienska jordbruksarbetare. 5-OH-TBZ och OH-PYR har inte tidigare uppmätts i en befolkningsgrupp. Några gravida kvinnor i studien arbetade på bananplantagerna med att packa bananer; de hade signifikant högre halter av biomarkören för tiabendazol jämfört med de som inte arbetade. De hade också högre halter av exponeringsbiomarkörerna för mankozeb och klorpyrifos. BM-exponeringen för de gravida kvinnorna är oroande och understryker vikten av att utföra tillförlitliga exponeringsmätningar – som också ska leda till lämpliga åtgärder att minska exponeringen.

# List of Papers

The Thesis is based on the Papers listed, find them attach in the end of the book.

I. High-throughput method for the analysis of ethylenethiourea with direct injection of hydrolysed urine using online on-column extraction liquid chromatography and triple quadrupole mass spectrometry.
Eva Ekman, Margaretha Maxe, Margareta Littorin, Bo A.G. Jönsson, Christian H. Lindh, *Journal of Chromatography B*, 934 (2013) 53– 59.

II. Determination of 5-hydroxythiabendazole in human urine as a biomarker of exposure to thiabendazole using LC/MS/MS
Eva Ekman, Moosa H. Faniband, Margareta Littorin, Margareta Maxe, Bo A.G. Jönsson, Christian H. Lindh, *Journal of Chromatography B*, 973, (2014) 61–67

III. Determination of hydroxypyrimethanil in human urine as a biomarker of exposure to pyrimethanil using LC/MS/MS
Moosa H. Faniband, Eva Ekman, Margareta Littorin, Margareta Maxe, Bo A.G. Jönsson, Christian H. Lindh. *Manuscript* 2016

IV. Pesticide exposure in pregnant women from the Infants' Environmental Health Study (ISA), Costa Rica
Berna van Wendel de Joode, Eva Ekman, Ana Maria Mora, Leonel Córdoba, Karin Broberg, Christian H. Lindh. *Manuscript* 2016

# Abbreviations

| | |
|---|---|
| 2D-LC: | two dimensional on column separation |
| 2,4-D: | 2,4-dichlorophenoxyacetic acid |
| 3PBA: | 3-phenoxybenzoic acid |
| 4F3PBA: | 4-flour-3-phenoxybenzoic acid |
| ADI: | accepted daily intake |
| APCI: | atmospheric chemical ionization |
| CPF: | chlorpyrifos |
| CV: | coefficient of variation |
| CYP450: | cytochrome P450 enzymes, (monooxygenase) |
| DCCA: | cis- and trans-2,2-(dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acids |
| EDC: | endocrine disrupting chemicals |
| ESI: | electrospray ionization |
| ETU: | ethylenethiourea |
| IS: | stable isotopic internal standard |
| ISA: | Infants' Environmental Health Study |
| LC-MS/MS: | liquid chromatography tandem mass spectrometry |
| LOD: | limit of detection |
| OP: | organophosphate pesticide |
| PYR: | pyrimethanile |
| QC: | quality control |
| SRM: | selected reaction monitoring |
| $t_{½}$: | half-life |
| TCP: | 3,5,6-trichloro-2-pyridinol |
| TBZ: | thiabendazole |

# Introduction

## General background

There are thousands of pesticides in use today. According to US EPA over 2 million tonnes are in use world-wide. Almost everyone is exposed via work environments or domestic use and also through e.g. diet, drinking water and personal products. There is also an overall concern that low dose exposure can lead to adverse health effects. Therefore, it is important to have reliable methods to monitor and survey exposure to pesticides in the different groups of both general populations and occupational workers.

## Pesticides studied in this Thesis

In this thesis the pesticides: mancozeb, thiabendazole, pyrimethanil, chlorpyrifos, pyretroids and 2,4-dichlorophenoxyacetic acid have been studied. Pesticides are divided into different groups such as fungicides, insecticides and herbicides.

The fungicide mancozeb is used in large scale in the growth of, for example potatoes, grapes and bananas to protect against different fungi e.g. moulds. In the banana industry, the crop is often sprayed by light aircraft over large areas. Mancozeb belongs to the ethylenebisdithiocarbamates (EBDCs) and is transformed to ethylenethiourea (ETU) in water and by plants and animals (1,2). In animal studies, ETU has caused a large spectrum of adverse effects, mainly concerning mutagenic, teratogenic, carcinogenic and hepatogenic effects (1,3-5). ETU is of major toxicological concern since it has been classified to be "reasonably anticipated to be a human carcinogen" based on sufficient evidence from animal experiments (6). On the other hand, is ETU placed in Group 3 "Not classifiable as to its carcinogenicity to humans" by IARC (7). ETU is considered to be an endocrine disruptor in mammals and is used to induce decreased ano-genital distances in animal studies (8-11). Also, in humans ETU is suspected to be an endocrine disruptor, since some data suggest effects on the thyroid gland among heavily exposed workers (12,13).

The fungicide thiabendazole (TBZ) is currently widely used for pre-planting and post-harvest treatment of vegetables and fruits. TBZ was introduced in the 1960's, first as an anthelmintic in humans and animals and later also as a fungicide. In animal studies, adverse kidney and liver effects as well as teratogenic and reproductive toxicity, have been reported at high doses (14-18). In general, TBZ is very toxic to aquatic organisms. Humans treated with TBZ as an anthelmintic have experienced adverse effects, like abdominal pain and nausea, dizziness and other cognitive complaints (19-21). Serious effects such as liver diseases have also been reported (22).

Pyrimethanil (PYR) is a fungicide, introduced in the 1990's (23). It is used both pre- and post-harvest to protect growing plants and crops like grapes, bananas and strawberries from pathogens that cause mould. Adverse effects have been reported in mammals. These effects are primarily seen in the main target organs of PYR, the liver and the thyroid (24). Even though PYR has not been shown to have any acute effects in humans, it still is of particular toxicological concern as it has in vitro been found to be an endocrine disruptor and is suspected to affect the thyroid-pituitary homeostasis in humans (25).

The insecticide chlorpyrifos (CPF) is a chlorinated organophosphate, frequently used in agriculture to protect the growing crops. The organophosphates (OPs) are cholinesterase enzyme inhibitors and exposure is associated with neurologic effects (26). CPF has high acute toxicity, but the toxicity is lower compared to other OPs due to fast detoxification. The main target of OPs and CPF is acetylcholinesterase (AChE), which hydrolyses acetylcholine, a major neurotransmitter in the central and peripheral nervous systems. Thus, inhibition of AChE results in overstimulation of the cholinergic receptors located in the whole body. This leads to the "cholinergic syndrome" characterised by symptoms such as increased sweating, and salivation, profound bronchial secretion, bronchoconstriction, muscular twitching and various central nervous system effects. Deadly outcomes are believed to be respiratory failure due to inhibition of respiratory centres in the brain stem, bronchial secretion, bronchoconstriction and paralysis of the respiratory muscles (27). CPF is also reported to be an antagonist to androgen activity (28) and thus a suspected endocrine disruptor.

Synthetic pyrethroids are a group of insecticides including permethrin, cypermethrin and cyfluthrin. Pyrethroids are frequently used in agriculture and domestically to protect humans and pets from insects. In mammals, pyrethroids can exhibit toxic effects on the central and peripheral nervous systems. They are grouped into two subclasses (Types I and II) based on chemical structure and on the toxic symptoms they cause in rats (29). Pyrethroids are suspected to be endocrine disruptors (30); cypermethrin, for instance, has shown estrogenic effects (28). Moreover, pyrethroids have effects on the immune system (31).

The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is one of the most used pesticides world-wide. It is applied to corn, grain and lawns. It is a chemical analogue to plant growth hormone and is ground sprayed to suppress the growth of broadleaf weeds but does not affect grass. 2,4-D has androgenic effects when combined with testosterone (28, 32). It has low to moderate-acute toxicity in mammals. The effects are primarily seen on the main target organs of 2,4-D, the kidney, liver and the central nervous system (33). Cases of acute poisoning due to ingestion of 2,4-D have been reported to have caused clinical symptoms such as vomiting, abdominal pain, hypotension and symptoms of the central nervous system including coma (27).

Exposure to pesticides is often determined through analyses of environmental sampling of air, food and water. In biological monitoring (biomonitoring) all sources of exposure important to human uptake may be covered in one sample. In environmental monitoring, several analyses from many different sites and sources are needed for a complete assessment (34). Unfortunately, there are not many methods for analyses of biomarkers of human exposure to pesticides described in the literature, at least for pesticides mainly used today. For valid biomonitoring, it is therefore important to develop such bioanalytical methods and to find appropriate biomarkers of exposure.

# Biomarkers of Exposure

Biomarkers of exposure are the chemical substances, parent compound itself, or its metabolites that can be detected and quantified in a biologic matrix and used as a measure of an internal exposure. In addition, it could be the product of the reaction between the chemical substance or its metabolite and an endogenous substance. There are several advantages with biomarkers of exposure compared to environmental monitoring. All routes and sources of exposure are taken into account and thus fewer samples need to be collected and analysed. Furthermore, the level of internal exposure is measured and differences in exposure levels between individuals can be detected. Thus, it can be applied in large scale epidemiological studies on an individual basis. However, the biomarker should be selective for the pesticide or group of pesticides measured, be stable in the biological matrix (35) and without any artefactual formation of the measured compound. For the interpretation of exposure data from humans, basic knowledge of the metabolic fate is important and reliable analytical methods with sufficient sensitivity and precision are needed (34-39).

# Metabolism

Metabolism includes absorption, distribution, biotransformation (metabolism) and elimination (ADME) of a substance. Factors influencing the metabolism can be internal (e.g. species, genetic, age, sex, hormones and disease) or external (e.g. diet, smoking and environment).

## Absorption

Main routes for pesticides to enter the human body are via inhalation, the gastro intestinal tract (oral) or via the skin (dermal). The route of absorption is a factor that influences the amount of absorption and the distribution of the substance.

Inhalation of pesticides may be considered as the most important route of occupational exposure to pesticides. Oral exposure may be an important route of exposure via contaminated hands of workers or through the diet of the general population. The uptake via inhalation and oral route is affected by the physio-chemical properties of the substance.

The dermal route of exposure can be significant when handling pesticides. Direct skin contact of pesticide mixtures, treated plants, fruit or vegetables and contaminated personal protection equipment may result in dermal absorption. Also, exposure via contaminated water and deposition of airborne particles or vapours may be considered as sources of exposure. Once the pesticide is on the skin it has to be transferred through the skin into the body. The factors influencing dermal uptake include the physio-chemical properties of the substance, the condition of the skin, time of exposure, the area and anatomic site that is exposed. Also the vehicle in which the substance is dissolved or suspended are important as it may alter the conditions for the skins absorption of the substance (40).

Knowledge of the metabolism of the pesticides depending on different routes of absorption is important for the establishment of adequate biomarkers of exposure. However, only a few pesticides have been characterized in these respect, e.g., by human experimental exposure studies.

Human experimental studies, looking at oral exposure to estimate basic pharmacokinetics, have been performed for e.g. mancozeb (ETU) (41), TBZ (42), CPF (43,44), pyrethroids e.g permethrin (45), cypermethrine (46) and 2,4-D (47,48).

Human experimental studies after dermal exposure have been performed to estimate basic pharmacokinetics for e.g. CPF (43,44,49), pyrethroids e.g permethrin (50), cypermethrine (51) and 2,4-D (52).

## Biotransformation

Biotransformation of pesticides may occur through several different pathways and can be divided into two phases, phase I and II reactions. Phase I reactions create, expose or change functional groups and include oxidation, reduction, hydrolysis, and hydration. For phase I oxidation reactions the mixed function oxygenase also called cytochrome P (CYP) 450 is the most important. This multi substrate enzyme is found inside the cells and is plentiful in liver, kidney, lung and intestine. The final chemically reactive functional groups -OH, -NH2 and -COOH are the most common in the phase I reactions. A substance can have repeated phase I reactions. The phase II reactions include formation of for example, glucuronic- and sulfate conjugates. These reactions make the products more water-soluble than the parent compound and more easily excreted (27).

The chemical structure of the pesticides and the metabolites analysed in this thesis are shown in Table 1.

Kinetics for the EBDCs, for example mancozeb, zineb, maneb have been examined in animals (e.g. rats, monkeys, mice) and ETU is found to be the main metabolite, which is mainly excreted in urine. While ETU is rapidly absorbed from the intestinal tract, mancozeb is not. Thus, it seems like mancozeb has to be metabolised in the intestine to be absorbed (3). In animals ETU is, to some extent, further metabolised to carbon disulphide, ethylene urea and methylated to S-methyl ETU (53). The toxic mechanism of ETU is thought to include bioactivation via CYP450 enzymes and flavin containing monooxygenases (FMOs) to produce reactive metabolites. These have been suggested to form protein adducts (11,54,55). In humans exposed to mancozeb ETU is the main urinary metabolite found but the biotransformation in humans still needs to be further studied (41,56).

The metabolism of TBZ has been studied in several different animal species (e.g. sheep, rats and dogs) and also in humans and been reported to possess nephrotoxic, hepatotoxic and teratogenic capacity. The major biotransformation pathway of TBZ, in animals as well as in humans is the hydroxylation catalyzed by CYP450 enzymes to form 5-hydroxy(OH)-TBZ and thereafter conjugation to glucuronic acid and sulphate (42,57). It is also suggested that 5-OH-TBZ, due to its structure is bioactivated to a quinone imine by enzymes such as CYP450 or peroxidases before the phase II reactions (58). Fujutani et al (59) has also found the isomer 4-OH-TBZ. Two other metabolic pathways are suggested and studied in vitro and in vivo. The first suggested biotransformation pathway via CYP450 is the oxidative cleavage of the thiazole moiety to form thioformamide via epoxidation (60). The other pathway is that TBZ, to a small extent, via methyltransferase can be transformed to N-methyl-TBZ (57).

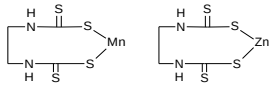
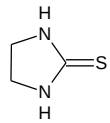
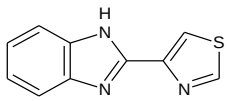
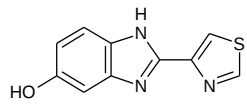
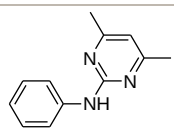
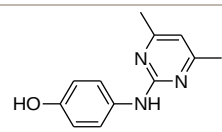
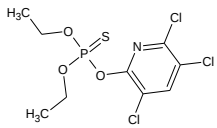
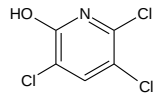
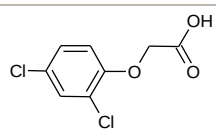
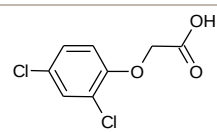
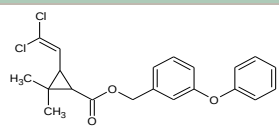
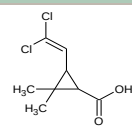
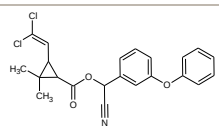
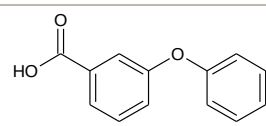
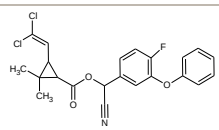
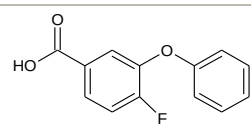
The metabolism of PYR has been studied in animals, but not yet in humans. In animals PYR is initially biotransformed to the major metabolite 4-hydroxypyrimethanil (OH-PYR) followed by conjugation with glucuronic acid and sulphate (24).

The metabolism of CPF has been studied and found similar in several different animal species as well as in humans. CPF is biotransformed by phase I oxidation of the thiophosphate moiety to diethyl thiophosphate and via hydrolysis of the organic moiety to, the metabolite, 3,5,6-trichloro-2-pyridinol (TCP) specific for CPF. The reactions are mainly catalysed by the CYP450 enzymes. In one of the CYP 450 pathways, CPF is desulphonated and bioactivated to an oxon that is an efficient inhibitor of the AChE. When the CPF oxon binds to AChE, TCP is released (61). In human urine the majority of TCP is found as a glucoronoide conjugate (43). Another metabolite is diethyl phosphate (44).

The biotransformation of pyrethroids (e.g. permethrin, cypermethrin, cyfluthrin) has been studied in rodents and metabolites found in human urine are similar to those in animals. The pyrethroides consist of two groups, one alcohol moiety and one carboxyl acid moiety, joined by an ester bond (62). Concirning the pyrethroids in this thesis, the ester linkage breaks, either by oxidation by CYP 450, to form e.g. 3-phenoxybenzoic acid (3PBA), or 4-flour-3-phenoxybenzoic acid (4F3PBA) or through hydrolysis by a carboxylesterase enzymes, to form the cis- and trans-2,2-(dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acids (cis- and trans-DCCA). Then conjugation follows including glucuronic acid or sulphate (29).

The metabolism of 2,4-D has been studied in several different species and in humans. In rats 2,4-D is not biotransformed but in dogs several urinary metabolite conjugates were found including glucuronic acid and sulphate (63). In humans the results are not consistent. Lindh et al. (2008) (47) could not see any biotransformation but Sauerhoff et al. (1977) found small amounts of conjugated 2,4-D but also found that the amount varied between individuals (48). It is worth mentioning that there is a difference in dose given see Table 2.

**Tabel 1.**
Stuctures of pesticides and their metabolites used as exposure biomarker

| Pesticide | Structure | Biomarker | Strucrure |
|---|---|---|---|
| Mancozeb |  | ETU |  |
| Thiabedazole |  | 5-OH-TBZ |  |
| Pyrimethanil |  | OH-PYR |  |
| Chlopyrifos |  | TCP |  |
| 2,4-D |  | 2,4-D |  |
| **Pyrethroids** | | | |
| e.g permethrine |  | DCCA |  |
| e.g. cypermethrin |  | 3PBA |  |
| cyfluthrin |  | 4F3PBA |  |

## Elimination

Pesticides are eliminated via biotransformation and/or excretion, i.e. via exhalation, the bile to faeces, via the urine and other routes. Excretion via the kidneys into the urine is through glomerular filtration. Passive diffusion or active transport may occur in both directions across the tubules. For each and every individual compound there is a specific relation between the levels in blood and urine.

However, since the urine has a difference in dilution, it ought to be adjusted for when comparing urinary concentrations between samples, both within individuals and between individuals (64,65). To adjust for urinary dilution three standard procedures are applied urinary elimination rate, biomarker creatinine ratio and normalisation with urinary density. Within the field of occupational and environmental medicine the adjustment by creatinine, the endogenous final product of muscle breakdown, has been commonly applied. However it has been reported from many studies of diverse populations that creatinine differs between different age groups, ethnical groups, sex and diet (64). Thus, in studies of diverse groups adjustment for urinary density can be a better choice. On the other hand, for substances that are rapidly reabsorbed in the tubules, non-adjusted concentrations may be preferred.

When studies using biomonitoring are planned, one parameter that is very important is the rate of elimination, i.e., the half-life ($t_{½}$), the time it takes for the compound to decrease to 50% of its current concentration. This can be obtained from concentration measurements in biological matrices over time. A few older pesticides have been rather well characterised some of which today are prohibited or restricted in use in most developed countries. However, more recently introduced pesticides have not been studied to the same extent. Some pesticides that are used in medical treatments like TBZ and some pyrethroids have been studied in humans although, there are only few scientific publications. However, there are reports such as registration reports to FDA (http://www.fda.gov/Drugs/default.htm) or European medical agency (http://www.ema.europa.eu/ema).

The human experimental studies of pesticides in this thesis with estimated urinary $t_{½}$ after oral or dermal exposure are summarised in Table 2.

**Tabel 2.**

Human experimental studies after oral or dermal exposure to pesticides in this thesis. Estimated urinary $t_{½}$ and the recoveries of the biomarkers are presented for comparison.

| Pesticide | biomarker | route | ADI ($_{mg/kg}$) | dose ($_{mg/kg}$) | recovery (%) | $t_{½}$ (h) slope 1 (slope 2) | Auther/ Paper |
|---|---|---|---|---|---|---|---|
| Mancozeb | ETU | oral | 0.05 | 0.4 mg | 69-82 | 17-23 | Lindh et al. 2008 |
| ETU | ETU | oral | 0.004 | 0.03 mg | 76 | 20 | Lindh et al. 2008 |
| ETU | ETU | dermal | 0.004 | 0.3 mg | 10 | 53 | Paper I |
| TBZ | 5-OH-TBZ | oral | 0.10 | 1 g ($^{14}$C) | 48 | | Tocco et al. 1966 |
| TBZ | 5-OH-TBZ | oral | 0.10 | 6 mg | 23 | 2 (15) | Paper II |
| TBZ | 5-OH-TBZ | dermal | 0.10 | 2 mg | 6 | 14 | Paper II |
| PYR | OH-PYR | oral | 0.17 | 5 mg | 79 | 4 (15) | Paper III |
| PYR | OH-PYR | dermal | 0.17 | 5 mg | 21 | 7 (24) | Paper III |
| CPF | TCP | oral | 0.01 | 0.5 | 70 | 27 | Nolan et al. 1984 |
| CPF | TCP | dermal | 0.01 | 0.5 & 5 | 3 | 27 | Nolan et al. 1984 |
| CPF | TCP | dermal | 0.01 | 5 & 15 | $4_{5}$& $11_{15}$ | 41 | Meuling et al. 2005 |
| 2,4-D | 2,4-D | oral | 0.05 | 0.2 mg | 96 | 9-12 | Lindh et al. 2008 |
| 2,4-D | 2,4-D | oral | 0.05 | 5 | 95 | | Sauerhoff et al. 1977 |
| 2,4-D | 2,4-D | dermal | 0.05 | 10 mg | 4.5 | 40 | Ross et al. 2005 |
| **Pyrethroids** | | | | | | | |
| permethrine | DCCA | oral | 0.25 | 0.1 | 45 | 5 | Ratell et al. 2015 |
| | 3PBA | oral | 0.25 | 0.1 | 45 | 6 | |
| permethrine | DCCA | dermal | 0.25 | 215 mg | 0.3 | 33 | TomalikScharte 2005 |
| cypermethrin | DCCA | oral | 0.05 | 0.1 | 45 | 6 | Ratell et al. 2015 |
| | 3PBA | oral l | 0.05 | 0.1 | 45 | 6 | |
| cypermethrin | DCCA | dermal | 0.05 | 31 mg | 1.2 | | Woollen et al. 1992 |

# Bioanalytical Methods

There are many different methods for the monitoring of pesticides or its metabolites in biological matrices, e.g. immunological and chromatographic methods. Many of these have a rather low specificity. Nowadays, mainly mass spectrometric (MS) methods are used. Reviews of bioanalytical methods for the determinations of pesticides and or their metabolites have been published (39, 66-69).

Among the chromatographic methods, gas chromatography (GC) is still preferred if the analyte is volatile. Otherwise liquid chromatography (LC) most often is the better choice. To directly ionize non-volatile compounds eluting from the LC was first possible by the introduction of the electrospray ionization (ESI) and atmospheric chemical ionization (APCI) interphases and have revolutionised the overall use of the LC-MS. The robustness of the MS detectors has also increased enabling direct injection of crude urine samples. The LC column-materials have improved further by for example wider diversity and better stability providing fast and reproducible chromatography and, in addition, also the ability to separate both very polar and basic compounds. These new LC-columns enable very efficient separation of the analyte from the matrix. Moreover, when an LC is connected to a tandem mass spectrometer (MS/MS), the detector will provide the final separation. The mass separation takes place in the two quadrupoles. This result in shorter chromatography requieres less peak separation, and the total time for the final analytical run may be less than 7 min.

In biomonitoring, often large population groups, exposed to several pesticides, are studied. Thus it is desirable to have bioanalytical methods providing high throughput of samples, without compromising the reliability of either the identity or quantity of the exposure. The use of LC-MS/MS provides this possibility if analytical standards and internal standards are used. Additionally, often several compounds can be determined by the same method. Analytical standard is used both for the quantification and the identification of the analyte. For the quantification of an analyte the ratio between analyte and internal standard signals are used. This approach is used to remove reproducibility issues that may arise during sample preparation and analysis and also between analytical runs. The stable isotope labelled-internal standard (IS) is the same compound as the analyte but where one or more atoms have been substituted with stable isotopes. Stable isotopes commonly used for LC-MS IS are $^{2}H$ (D), $^{13}C$, $^{15}N$ or $^{17}O$. Thus, the IS and analyte will behave in a similar fashion during sample preparation, in the LC-column and during ionization and be exposed to the same matrix effects in the interphase during ionization. However the D IS may deviate a little in retention

time. It is first in the MS that the IS and analyte will be separated due to the heavier mass weight of the IS (70).

The MS/MS detector is very sensitive and maintenance with a following mass calibration is needed on a regular basis. Furthermore, a lot of parameters that may or may not be controllable may well affect the final quantification result. Therefore, if possible, collected samples should be run during a short period of time and quality control (QC) samples should be used to keep track on time trends.

In biological monitoring, unwanted contamination of samples during sample handling, preparation and analysis can be a large problem for compounds that have an environmental background noise. Therefore, to be able to adjust for possible contamination during sample preparation and analysis blank samples are used.

# Exposure Assessment

A validated bioanalytical method is needed to be able to conduct valid biomonitoring studies. Moreover, data on the metabolism is needed to develop studies for biomonitoring of the compound, including data on time of sampling and if repeated sampling is recommended. With such a bioanalytical method and with an optimized sample collection it will be possible to perform exposure assessments in workers as well as in the general population.

Exposure to mancozeb and its metabolite ETU has been shown to occur via inhalation and the oral and dermal route. For workers the mixing and filling spraying equipment can result in high air levels and exposure and also bring about a risk of skin contact (13,71). Hand wash samples of pesticide formulators have been observed to be highly correlated ($r^2 > 0.9$) with urinary ETU concentrations (72). Aerial spraying has been shown to be a source of exposure to the general population as the airborne pesticides can drift into villages nearby (73,74). Further, in agricultural workers´ wives, washing of contaminated working clothes are correlated with increased exposure levels of ETU (73). Associations have been observed between ETU when used as exposure biomarker to EBDCs and smoking, wine drinking and consumption of fruit and vegetables (74,75).

Workers may be occupationally exposed to TBZ but studies on exposure are missing. In the general population there is a potential source of exposure by residues of the fungicide in the diet. Also, for some, medical treatment is an obvious source of exposure.

There are no studies describing exposure to the fungicide PYR but workers may be occupationally exposed. There are no reports about exposure to PYR in the general

population but there is a potential source of exposure by residues of PYR in the diet.

Occupational exposure to the insecticide CPF has been shown to occur in workers (76,77). TCP has been found in urine samples from family members and general populations living near areas where CPF is used (78,79). The general population can be exposed via diet or due to domestic use. However in many countries CPF is not used in domestic products any longer. The exposure to CPF may also be monitored through the analysis of alkyl phosphates, e.g. diethyl phosphate and diethyl thiophosphate but these biomarkers are not specific to a certain OP (80).

Exposure to pyrethroid insecticides occur via inhalation and the oral and dermal routes (81). Occupational exposure is one way to get exposed to pyrethroids. The general population is also exposed via diet, through domestic or public health vector control. An obvious source of exposure is also medical treatment (lice and scabies) with some pyrethroids e.g. permethrin (82). The most commonly measured metabolite is 3-PBA which is as an exposure biomarker for many of the pyrethoids.

Occupational exposure to the herbicide 2,4-D is also common as it is widely used in agriculture and forestry. The general population may also be exposed from contaminated food or to some extent from the spraying on public grass areas or household gardening (83).

Exposure assessments to the pesticides above, TBZ and PYR excluded have been performed in different population groups, including agricultural workers and pregnant women. Pregnant women is a group studied both due to the susceptibility of the pregnant woman but especially for the vulnerability of the foetuses. Exposure assessment in the general population is often part of a surveillance program in a province, country or large region - e.g. the European Union.

# Aim

*General aim*

To determine if reliable biomonitoring data can be generated by the use of LC-MS/MS methods developed for the analysis of urine samples collected from humans exposed to low levels of pesticides.

*Specific aims*

I. To develop reliable LC-MS/MS methods for the quantification of biomarkers of exposure to the three fungicides mancozeb, TBZ and PYR in human urine.

II. To determine if reliable biomarkers can be found in urine collected in human experimental pilot studies, after dermal exposure to ETU, TBZ and PYR

III. To determine if reliable biomarkers can be found in urine collected in human experimental pilot studies, after oral exposure to TBZ and PYR.

IV. To study the concentrations of urinary biomarkers of pesticide exposure in an epidemiological cohort study of pregnant women.

# Methods

## Urine Samples

The urine samples analysed in this project have different origins. For the development of the analytical methods, urine was collected from co-workers in the laboratory. Also, urine used in the preparation of blank urine samples, calibration curves and QC-samples were obtained from co-workers in our laboratory. Moreover, urine samples have been collected from participating volunteers in the experimental exposure studies (Papers I, II, II). In Papers II and III urine from general population in the south of Sweden has been analysed. In the final study (Paper IV), repeated urine samples were collected from 451 women from the prospective cohort study (ISA) in Costa Rica. All urine samples handled in this PhD-project were transferred to 15 mL tubes and stored at –20°C (during shipment (4°C)) at the Division of Occupational and Environmental Medicine at Lund University, Sweden.

## Bioanalytical Methods

Papers I, II and III describe LC-MS/MS methods for the quantification exposure biomarkers ETU, 5-OH-TBZ and OH-PYR in human urine. A smaller validation of each method, for the use in experimental and environmental exposure assessments, is also performed. In Paper IV, the methods in Papers II and III are merged and modified to analyse the five biomarkers, TCP, DCCA, 3PBA, 4F3PBA and 2,4-D. Figure 1 show how the different biomarkers are analysed and in what Paper.

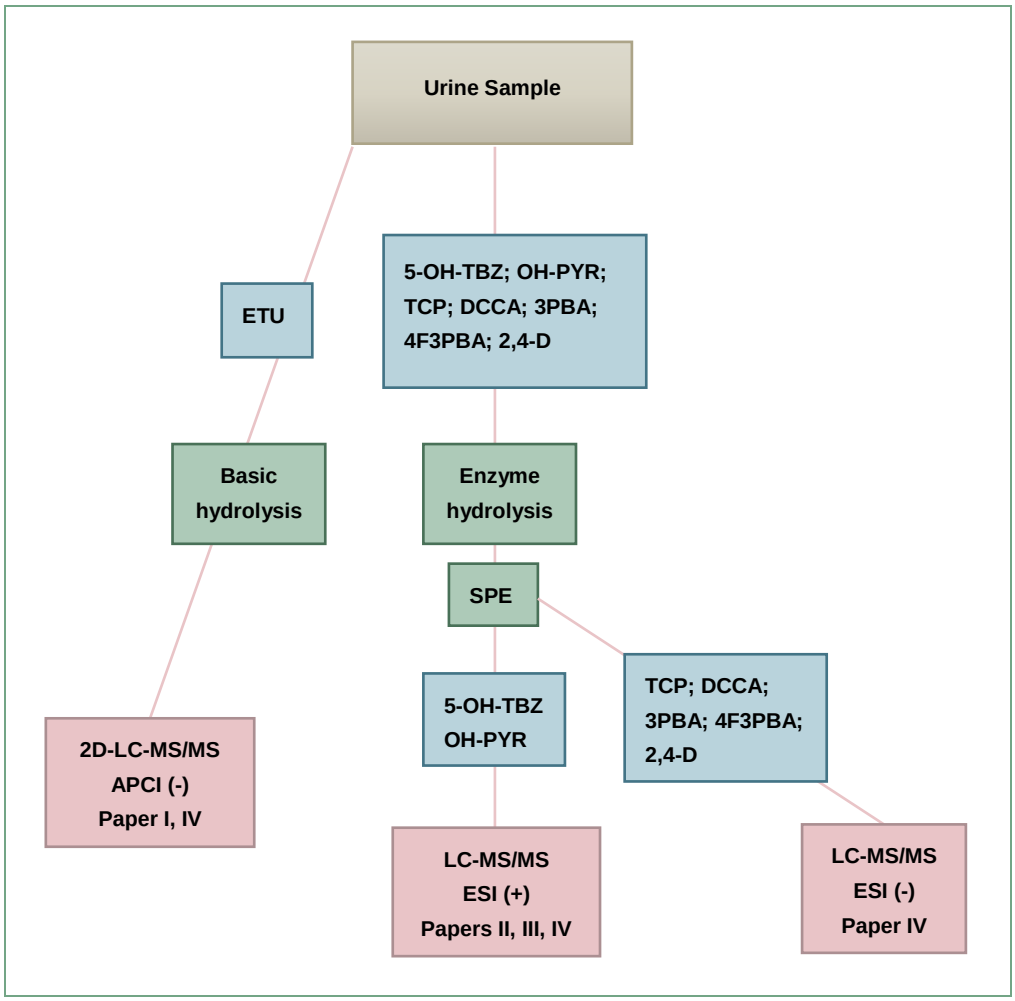



**Figure 1**
Figure 1 show how different biomarkers are analysed and in what Paper. Papers I, II and III describe how LC-MS/MS methods for the exposure biomarkers ETU, 5-OH-TBZ and OH-PYR in human urine were developed for the use in experimental and environmental exposure assessments. In Paper IV, a modified version of the method in Papers II and III is described and used to analyse seven biomarkers.

## Sample Preparation

*Preparation of calibration standards, quality control and samples*

Stock solutions were prepared in duplicates by dissolving accurately weighed amounts of analytical standard and IS in methanol. The analytical standard and IS

stock solutions were diluted further in methanol and stored at -20°C. In Paper IV, all analytes were mixed into a single stock solution. This procedure was used for both the analytes standard stock solution and IS. Urinary samples with no or very low levels of the measured chemicals were used for the preparation of calibration standards. If it was not possible to obtain completely blank urine for preparation of standards, the calibration curve was corrected for this amount.

The chemical blanks were all prepared from Millie-Q water. Blanks were treated in exactly the same way as the samples all the way through the bioanalytical method. The mean signal from the chemical blanks is subtracted from the analyte signal in the sample before quantification.

The QC-samples used, in Papers I-III, were prepared from authentic urine samples already containing appropriate amounts of the analyte or additionally spiked with the analyte to a low, medium and high concentration. If the QC-samples needed to be spiked with an additional amount of analyte the analytes standard stock solution was used. In Paper IV authentic blank urine samples were spiked, to low and high QC-levels, with one stock solution containing all analytes. All QC-samples were divided into several aliquots and stored at -20°C.

All samples were prepared in duplicates in 96-well plates containing, besides the samples, at least six levels of calibration standards, one urine blank, three chemical blanks, and duplicate QC-samples on each plate. The standard stock solutions, IS, urine samples and QC-samples were always vortex-mixed before samples were taken out. Aliquots of 0.5 mL urine were analysed. The IS solution was added and the samples then hydrolysed according to one of the methods described below.

## Hydrolysis

Deconjugation of ETU was performed by hydrolysis with 0.09 M sodium hydroxide in 100°C for one hour (Paper I). In Papers II, III and IV the hydrolysis of the conjugations of 5-OH-TBZ, OH-PYR, TCP, DCCA, 3PBA, 4F3PBA and 2,4-D were instead performed by an enzymatic method. The urine samples were buffered to a pH of 6.5 prior to the addition of β-glucuronidase/arylsulfatase and then hydrolysed overnight at 37°C.

## Analyte Extraction

Extraction of ETU, in Paper I, was performed online during separation in the chromatographic system using two identical analytical columns (Genesis® Lightn AQ (C18, 4.6 × 100 mm, 4 µm, Grace Vydac) and a diverter valve, i.e. by 2 dimensional (2D) LC. The first column separates most of the urinary matrix to

waste and after 2.5 min a small portion of the sample was diverted for 1 min into the second analytical column for further separation. After about 2 min separation on the second column the now even smaller portion of the sample containing the ETU analytes was diverted for 2.5 min into the MS/MS for detection.

Solid phase extraction (SPE) was used to separate the biomarkers 5-OH-TBZ, OH-PYR, TCP, DCCA, 3PBA, 4F3PBA and 2,4-D from the urine matrix. SPE columns containing a hydroxylated polystyrene divinylbenzene co-polymer fixed in 96 well-plates were used. Thus, after the enzyme hydrolysis the urine samples were mixed and transferred to a conditioned 96-SPE plate using a 96-multichannel pipette. Then they were washed once with purified water, once with an acidified organic solvent and once with acetonitrile. In Paper IV, TCP, DCCA, 3PBA, 4F3PBA and 2,4-D were eluted into a separate 96-well plate in the third step when the acetonitrile was added.

While, in Papers II and III acetonitrile containing 5% ammonia was used to elute 5-OH-TBZ and TBZ into a 96-well plate.

In Paper I special glass vials placed in aluminium 96-well plates used and sealed with a capmat tightened by an aluminium lid for leak proof closure to prevent evaporation during the heated hydrolysis step. In Papers II, III and IV the hydrolysis was performed in polypropylene 2 mL 96-well plates and sealed with an airtight sealing mat, or, for analysis, sealed with hard plastic pierceable sealing capmat for leak proof closure. After the hydrolysis the analytes were extracted from the urine samples in one of the clean-up procedures described above. The samples were mixed thoroughly and centrifuged for about 10 min before analysis.

# LC-MS/MS Analysis

## Instrumentation

In this thesis a triple quadrupole linear ion trap mass spectrometer was used (QTRAP 5500; AB Sciex, Foster City, CA, USA) equipped with a TurboIonSpray source and coupled to a liquid chromatography system (UFLC$^{RX}$, Shimadzu Corporation, Kyoto, Japan). The liquid chromatography system was equipped with rack changer, autosampler, two sets of ultra-high pressure pumps i.e. four pumps in total and a column heater oven with an integrated valve with ten (we used only six) connections and room for two columns or more. This set up make it possible to maintain two columns both with gradient elution and as described in Paper I.

The MS/MS analyses were carried out using selected reaction monitoring (SRM) in both positive and negative ionisation mode. Pure nitrogen was used as curtain gas and collision gas. To find the best SRM conditions, standard solutions were infused into the MS/MS for optimisation. Collision-induced dissociation of each $[M+H]^+$ was performed and the product ions giving the best signal to noise ratio were selected for the SRM analysis in the final acquisition method. The quantifier ions (I-IV) and the qualifiers ions (I-III ) are tabulated in the Papers together with their collision energy (CE) and declustering potential (DP). All data acquisition and processing was performed using the Analyst 1.6.1 application software and in Paper IV also Multiquant 2.1 application software (Applied Biosystems).

In Paper I atmospheric pressure chemical ionisation (APCI) ion source was used and air was used as nebuliser spraying gas and the APCI temperature was set at 450◦C. Moreover the instrument was tuned to a peak resolution of 0.5 ± 0.1 Da at half the peak height to get a better signal for ETU. In Papers II-IV electrospray ionisation mode (ESI) was used with air as nebuliser and auxiliary gas. The settings of the temperature of the auxiliary gas and the ion spray voltage can be found in the separate Papers.

## Analysis

The sample aliquot injected was 20 µL (Papers I and IV) and 3 µL or 6 µL (Papers II, III and IV).

In all the four bioanalytical methods two types of mobile phases were used, mobile phase A consisting of 0.1% formic acid in Millie-Q water and mobile phase B consisting of 0.1% formic acid in methanol.

Two different types of reversed phase chromatography were used. In Papers I and IV the analytical columns Genesis® Lightn AQ (C18, 4.6 × 100 mm, 4 µm, Grace Vydac) were applied. In Papers II, III and IV Poroshell 120EC-C18 column (4.6 × 233 mm, 2.7 µm, Agilent Technologies) analytical columns were used. In all the analytical methods the columns were maintained at 40°C.

The total analytical run time per sample, including equilibration time, was 7 min. The flow rate was 0.7 mL/min. The columns were reconditioned with 95% of the methanol based eluent and then equilibrated to the initial conditions, between each analytical run. Post-column, the effluent was diverted into the MS during an appropriate time window during which the peaks were eluted.

In all Papers except in Paper I, the separation was carried out using gradient elution using a linear gradient of mobile phase B. In Paper I, 2D-LC with two analytical columns was used. Here the two sets of LC pumps were essential to perform the separation. During the separation of ETU on one column with one set

of LC-pumps, the other column was reconditioned and equilibrated to initial conditions with the other set of LC-pumps. Isocratic elution was used over both columns.

In Paper IV the exposure biomarkers of pesticides used on banana plantations were determined. The bioanalytical method developed in Paper I was used to measure ETU and the methods developed in Papers II and III were combined and used to determine 5-OH-TBZ and OH-PYR. The other biomarkers, TCP; DCCA; 3PBA; 4F3PBA; and 2,4-D, were analysed according to a modified version of the methods developed in Papers II and III.

### Quantification

The urinary sample concentrations were determined from peak area ratios between the analyte and the IS corrected for the average concentration in the chemical blanks.

Each batch, was analysed with single injections, and then evaluated by its own calibration curve. In the calibration curve the urine blank sample was included and only the slope of the regression line is used to calculate sample concentrations from the calibration curve. The concentration calculations were performed in MS Excel 2010.

## Exposure Studies

### Ethics

In the exposure studies described in Papers I-III, the two healthy volunteers had given their written informed consent to participate in the experimental oral and dermal studies. The studies were ethically approved by The Regional Ethical Review Board in Lund, Lund University, Sweden.

In Paper IV, written informed consent was obtained from all the 451participating women. If the woman was <18 years of age, additional written informed consent was obtained from her legal representative. All study activities were approved by the Scientific Ethics Committee of the Universidad Nacional in Costa Rica and by The Regional Ethical Review Board in Lund, Lund University, Sweden.

## Study Design Experimental Exposure

The human experimental pilot studies were all conducted at the Division of Occupational and Environmental Medicine in Lund, Sweden.

In the experimental oral and dermal studies described in the Papers I-III, two healthy volunteers participated, one female in her late sixties and one male in his early forties, both non-smokers.

In the conducted exposure studies, described in Papers I-III, the volunteers received one single low-dose with exposure levels between 25-100% of the accepted daily intake (ADI) for ETU, TBZ and PYR. Moreover, to lower the environmental intake of the compounds the volunteers minimised the intake of conventionally grown food a few days before as well as during the study.

The ADI for ETU is 0.004 mg/kg/day (84); for TBZ 0.1 mg/kg/day and for PYR 0.17 mg/kg/day (85).

*Oral Exposure*

Papers II and III describe oral exposure to TBZ or PYR. TBZ (2.5 mg/mL, about 3 mg) was dissolved in acetone while PYR (2.05 mg/mL, about 5 mg) was dissolved in ethanol. The volunteers received the oral dose corresponding to 50% of ADI in 250 mL organic fruit juice (orange (TBZ) and cranberry (PYR)).

*Dermal Exposure*

Papers I-III describe dermal exposure to ETU, TBZ and PYR for the duration of eight hours. The volunteers received the dermal dose on the inner forearm, within a fixed area of either 50 or 75 $cm^2$. The compounds were dissolved in organic solvents commonly used in dermal exposure studies 50 and 75% ethanol (ETU, PYR) or acetone (TBZ) (40).

The doses corresponded to 100%; 25% and 50% of ADI for ETU, TBZ and PYR respectively. The dose (about 0.3 mg) of ETU was administrated in 0.1 mL and TBZ (about 2 mg) and PYR (about 5 mg) in about 0.6 mL. The dose was evenly distributed over the whole surface and the vehicle was allowed to evaporate before the skin was occluded with aluminium foil. The exposure was stopped after eight hours and the dose remaining on the skin was wiped off with a cotton swab moistened with the vehicle and the exposed area washed with soap and water, see Figure 2. In Paper III, the exposed area was also tape-stripped before the exposed area was washed with soap and water. The aluminium foil, swabs and tapes were stored separately at -20◦C until analysis. The aluminium foil, the cotton swabs and tapes were all extracted in suitable solvents. In paper II only the mean recovery from the two volunteers was calculated.

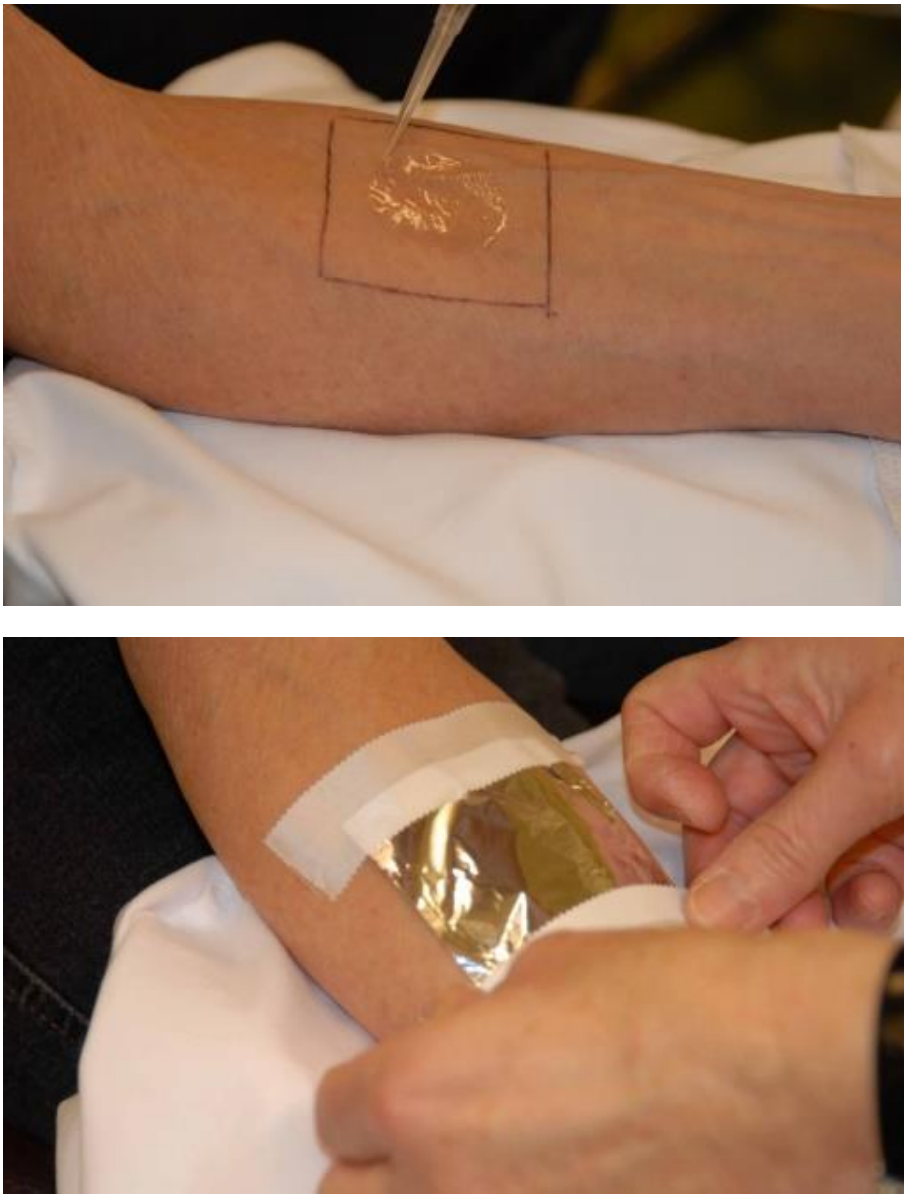


**Figure 2.**
Administration of the pesticide on the inner forarm (picture above) . The exposed area was coverd with aluminium foil during the 8 h long exposure (picture below)

## Sample Collection and Adjustments for Urinary Dilution

In the Papers I-III, a first pre-exposure urine sample was collected immediately prior to the exposure. After exposure urine was voided on pre-defined time points up to 12 hours, and then ad libitum for 4-7 days. All urine voided was collected. The volume of the samples was measured and used in the recovery calculations. In addition, creatinine and density were determined for each sample and used to adjust for the urinary dilution. Creatinine was analysed with an enzymatic method and density with a hand refractometer. In the calculation of the density adjusted concentration, the specific density in the urine sample is first normalized against the mean reference urine density ($\rho_{mean}$). The mean reference values for the density in Papers I-III were calculated for each volunteer, the individual $\rho_{mean}$ was calculated as the mean density in the collected urine samples. In Paper IV, however, the $\rho_{mean}$ was calculated as the mean density in the collected urine samples for the whole cohort. However, the calculations were performed using the same equation, in all the Papers (I-IV). The concentration adjusted for urinary density, $C_d$, was calculated according to $C_d = C\ (observed) \times (\rho_{mean}-1)/(\rho-1)$, where C (observed) is the obtained concentration in the urine sample, $\rho$ is the measured specific density and $\rho_{mean}$ was used as the mean reference urine density (65).

## Calculation and Pharmacokinetic data

In Papers I-III, the time zero was set as the time the dose was administrated. The elimination $t_{½}$ in urine was estimated from the slope of the elimination curve in the natural logarithm-linear concentration versus time plot. The elimination $t_{½}$ was calculated for unadjusted concentrations and for concentrations adjusted for creatinine or density. The correlation coefficient r of the slope was also estimated. Additional parameters estimated were $t_{max}$ the time of the maximum concentration ($C_{max}$).The time was given as the mid time point between two sample collection time points. Each data point shows the mean excreted biomarker concentration (27).

To calculate the dose recovery correctly when the urinary biomarker was a metabolite, the unit mole was used. For ETU and PYR the total dermal recovery was calculated by dividing the urinary biomarker concentration with the available dose. The available dermal dose was the administrated dose minus the amount of the parent compound found in the aluminium foil, swabs and tapes, also accounted for as loss of dose found in the aluminium foil, swabs and tapes.

# The Exposure Assessment Study

To study exposure to the pesticides TBZ and PYR the analytical methods presented in Papers II and III were applied to analyse urine samples collected from groups of a general population in southern Sweden. The population consisted of men and women, living and working in cities or the countryside. First morning void urine samples (n = 285) were analysed by the analytical method presented in Paper II, to determine the urinary biomarker 5-OH-TBZ. Additionally, first morning void urine samples (n=413) were analysed by the analytical method presented in Paper III, to determine the urinary biomarker OH-PYR.

In Paper IV the exposure to pesticides in a cohort of pregnant women was described by the determination of eight different exposure biomarkers found in collected urine samples. A total of 451 pregnant women were participants in the ISA study, a prospective community-based birth cohort study in Matina County, Limón, Costa Rica. This cohort was designed to examine possible effects of prenatal and early-life pesticide and manganese exposure on children's growth and neurodevelopment (73). The study population included pregnant women of 15 years or older with less than 33 weeks of gestation who expected a singleton birth and lived within 5 km of a banana plantation. The study was conducted according to the following procedures. Women were interviewed in their homes and a urine sample was obtained one to three times during pregnancy. Out of the 451 women enrolled in the study, six were excluded from data analysis because gestational age was unknown or no urine samples were provided. In total 909 spot urine samples collected from 445 women were analysed for eight pesticide biomarkers. Further details are described in Paper IV.

## Evaluation of Exposure Biomarkers

Urinary density was determined using a hand refractometer. To correct pesticide metabolites for urinary dilution, we adjusted measured pesticide metabolite concentrations for density using the mean density of 1.017 $g/cm^3$ of all samples (n=909) as a reference. Biomarker concentrations below LOD were substituted with the value obtained/calculated for the lowest corrected concentration that was detected divided by the square root of 2. Descriptive statistics to examine the associations between the exposure biomarkers was used. Continuous variables were tested for normal distribution and non-normally distributed variables were $log_{10}$-transformed. Correlations between continuous variables were performed using Pearson's r correlation coefficient. The intra class correlation (ICC) of pesticide metabolite concentrations from repeated samples collected from the same woman was estimate for each participant, using variance components models with

random intercepts, restricted maximum likelihood (REML). Additionally, when applicable in the statistical analysis, linear mixed-effects regression models with random intercepts (REML) were used, to take into account correlation among repeated samples collected from the same woman. Mixed-effects models were also run to see if biomarker concentrations were significantly higher for women working in agriculture during their pregnancy.

For all statistical tests, the significance level was set to 0.05. JMP 8 (SAS Institute Inc., Cary, NC, USA) was used for statistical analysis.

# Results with Comments

## Bioanalytical Methods

Papers I, II and III describe how LC-MS/MS methods for the exposure biomarkers ETU, 5-OH-TBZ and OH-PYR in human urine were developed, Moreover, validations of each method for the use in experimental and environmental exposure assessments are described. In Paper IV, a modified version of the method in Papers II and III is also described and the validation parameters, sensitivity and precision, were evaluated for each of the eight biomarkers, to verify the reliability of the exposure data, see Table 3.

### Mass spectrometry and Chromatography

The use of 96-well plates and LC-MS/MS detection enabled efficient analysis of a large number of samples. The results of exposure in general population studies in Papers I, II, III and IV shows that the analytical methods are applicable to use in large scale biomonitoring studies.

The chosen analytical columns provided consistent and reproducible chromatography and retained the analytes with stable retention times and good separation. Thousands of injections were performed on the analytical columns before signs of degradation were observed as e.g. bad peak shapes, fluctuating retention times and bad separation.

The exposure biomarkers and there IS analogues showed an excellent sensitivity in APCI and positive ion mode for ETU, ESI and positive ion mode for 5-OH-TBZ and OH-PYR, ESI and negative ion mode for TCP, DCCA, 3PBA, 4F3PBA and 2,4-D.

Papers I, II and III, describe a second SRM chosen as a qualifier ion to strengthen the identity of the analytes. The transition giving the best signal to noise ratio was chosen as the quantifier ion for the analyte. Paper IV describes the quantifier ion for TCP, DCCA, 3PBA, 4F3PBA, and 2,4-D. The transitions with optimum collision energies are tabulated in each Paper.

## Validation

The following validation parameters were evaluated for the new analytical methods: selectivity, LOD, LOQ, calibration model (linearity), accuracy (bias), precision (repeatability) and stability (86). Additionally, in paper I a cross method validation is described and in papers II and III a recovery experiment.

*Selectivity and Sensitivity*

The selectivity for the biomarkers, ETU, 5-OH-TBZ and OH-PYR were identified by comparison with known standards and no interfering signals were detected in chemical blanks or in authentic urinary samples, except in the case of ETU in urine. Since there seems to be a ubiquitous exposure to ETU 11 authentic urinary blank samples were used in the determination the sensitivity. This may have had an influence on the value of the LOD. However, in quantitative methods small interferences can be accepted as long as the precision is acceptable, and for ETU the precision at LOD was less than 20 % (86). Furthermore was the LOD concentration in the same range as reported in previous methods (41,87-89). The LOD levels were sufficient for the measurement of environmental human exposure to mancozeb, ETU, TBZ and PYR. The LOD and limit of quantification (LOQ) were determined in authentic urine as the average concentration (peak area ratio) plus three and ten times the standard deviation, respectively (86,90). Additionally in Paper I, LOD and LOQ for ETU were also assessed in chemical blank samples. Paper IV reports LOD assessed from chemical blank samples. The LOD and LOQ are shown in Table 3.

*Matrix Effect*

The matrix effect was described in Paper II, and reported to be minor as the geometric mean of the ratio between the IS in the chemical blanks and the IS in authentic urine samples were close to one. The low matrix effect probably is explained by the use of SPE, the use of IS and the analytical column, giving a good separation of the analyte from the matrix. Furthermore, the sensitive LOD, good linearity, precision and bias of the method further showed that the matrix effects does not pose a problem in the method. This approach to measure the matrix effect was doable since the IS-5-OH-TBZ [$^{13}C_2$][$^{15}N$] is a stable isotopic analogue of 5-OH-TBZ and co-elutes with the analyte and thus the matrix effect was considered to affect the signals in the same manner. Matrix effects have a smaller impact when IS and an APCI interface are used. Therefore, matrix effects were not described in Paper I.

*Linearity*

The calibration curves obtained were described for each method in Papers I-III, and the linearity of the curves had correlation coefficients between 0.997 and 0.999. The linear range of the calibration curves was somewhere between 0.1 and 200 ng/mL in the different methods and determined from about ten urinary concentration levels.

*Repeatability and Accuracy (bias)*

The repeatabilities of the LC-MS/MS methods are described in Papers I-III as within-run, between-run and between-batch precision and reported to be between 4 and 13% coefficient of variation (CV) (Table 3). In bioanalytical methods, a repeatability below or equal to 15% CV is considered excellent (86).

Papers I-III describe the within-run precision at three concentration levels, representing the lower, mid and higher part of the calibration curve. Each level was spiked in ten consecutive urine samples prepared in one sample batch during one day. The bias (accuracy) was good, as the reported calculated concentrations were close to the nominal values and the precisions were below 15% in the spiked within-run precision samples.

Further, papers I-III, describe the between-run precision determined by including duplicates of the three to four QC-samples in at least seven analytical sample batches. The sample batches were prepared and analysed on separate days during a period of at least four months. A CV % was calculated for each concentration level of the QC-samples. Paper IV reports the between-run precision for QC-samples at two levels. The precision varied between 4 and 15% CV for the eight biomarkers, see Table 3.

The between-batch precision CV % was calculated from the differences between the duplicate samples prepared and analysed in separate analytical batches as previously described by Lindh et al. 2008 (41). The between-batch precision in the methods was determined from the duplicates of around 100 urine samples collected in the experimental exposure studies described in Papers I-III, see Table 3. Paper IV reports a between-batch precision below 15% CV determined from the duplicates of the study samples in the range of LOQ to 99$^{th}$ percentile for each of the analysed biomarkers, except for 4F3PBA. The latter had a CV of 19%. This higher CV can probably be explained by the low concentration of 4F3PBA at the 90$^{th}$ percentile, only 0.1 ng/mL. The analyses of 3PBA and TCP are part of a biannual round robin inter-laboratory program (University of Erlangen-Nuremberg, Germany) with results within the tolerance limits.

*Stability*

Stability data found in literature show that ETU is stable in authentic urine samples for at least 12 months when stored at -20°C (74). In papers I and II, the standard stock solutions of ETU and 5-OH-TBZ dissolved in methanol were reported to be stable for at least 7 and 12 months and 5-OH-TBZ in authentic urine samples for at least 5 months when stored at -20◦C.

*Recovery*

Papers II and III report a recovery of 91-100% after SPE extraction of the analytes from spiked urine samples. The use of an IS standard through the sample preparation makes the recovery less critical. However a high recovery can enable lower LOD.

*Cross-method Comparison*

A cross-method comparison between the ETU LC-MS/MS method by Lindh et.al (41) and the new LC-MS/MS method was described in Paper I. The results from the cross-method comparison showed that if the samples were hydrolysed before analysis, as described in Paper I, an excellent correlation was obtained between the two methods.

**Tabel 3.**

Validation data for the developed bioanalytical methods in Papers I-III and when applying the methods in an exposure assessment study in Paper IV.

| Biomarker | LOD (n) urine (ng/mL) | LOD (n) chemical blank (ng/mL) | LOQ Urine (ng/mL) | LOQ chemical blank (ng/mL) | Precision[a] (n) Between-run (CV%) | Precision (n) Between-batch (CV%) | Paper |
|---|---|---|---|---|---|---|---|
| ETU | 0.2 (11) | 0.08 (14) | 0.5 | 0.16 | 4 (11) | 6 (88) | Paper I |
| 5-OH-TBZ | 0.05 (7) | 0.03 (85)[b] | 0.13 | 0.08[b] | 8 (7) | 9 (110) | Paper II |
| OH-PYR | 0.11 (8) | 0.06 (82)[b] | 0.26 | 0.16[b] | 7 (12) | 12(162) | Paper III |
| ETU | | 0.08 (32) | | 0.24 | 15 (57) | 11 (827) | Paper IV |
| 5-OH-TBZ | | 0.03 (85) | | 0.08 | 4 (96) | 8 (172) | Paper IV |
| OH-PYR | | 0.06 (82) | | 0.16 | 9 (95) | 8 (301) | Paper IV |
| TCP | | 0.05 (92) | | 0.11 | 4 (84) | 10 (387) | Paper IV |
| DCCA | | 0.04 (92) | | 0.10 | 7 (88) | 15 (323) | Paper IV |
| 3PBA | | 0.03 (92) | | 0.06 | 7 (80) | 14 (394) | Paper IV |
| 4F3PBA | | 0.01 (92) | | 0.03 | 13 (46) | 19 (126) | Paper IV |
| 2,4-D | | 0.02 (92) | | 0.04 | 8 (68) | 13 (323) | Paper IV |

a) Medium QC-sample level
b) LOD from chemical blank determined in Paper IV

# Biomarker Validation

Papers I, II and III describe how the identity of the exposure biomarkers ETU, 5-OH-TBZ and OH-PYR was studied in human experimental exposures. Some basic pharmacokinetics were studied, as well.

## Effect of Hydrolysis - deconjugation

Paper I reports that the maximum concentration during hydrolysis of ETU was reached after 1 hour. In the following evaluation of the effect of hydrolysis, the levels of ETU increased between 2 and 20 times when ten authentic urine samples were hydrolysed prior to analysis. A linear trend was also reported when the ETU concentrations from the hydrolysed and non-hydrolysed samples were compared using linear regression. This suggests that the higher the ETU concentration in the sample was from the beginning, the higher impact the hydrolysis had on the concentration levels. In summary, this suggests that ETU is conjugated in urine. Thus, for a correct exposure assessment, hydrolysis is an essential part of the bioanalytical method. However, previously published methods for the analysis of urinary ETU have not addressed conjugation of ETU (41,87-89). Most earlier published analytical methods perform acidic or basic hydrolysis to some extent, but since the effect of hydrolysis was not evaluated they may have underestimated the exposure to EBDCs and ETU (41,87,88). The conjugate of ETU is still unknown. However, it has been suggested that ETU can form protein adducts in blood, which can be released with mild acid hydrolysis (55).

In Paper II, it was verified that 5-OH-TBZ is conjugated to both glucuronic acid and sulphate. The highest concentration levels were found after 18 hours of enzyme hydrolysis with β-glucuronidase/arylsulfatase. The enzymes β-glucuronidase/arylsulfatase and β-glucuronidase were studied regarding hydrolysis of the 5-OH-TBZ conjugates in authentic urine samples. Interestingly, the increase in concentration differed 1:4 and 1:2 in yields between the two individuals. Thus, it seems that the level of conjugation to sulphate varied between the two individuals; these results agree with earlier findings (42). Furthermore, for one individual the conjugate ratio seemed to differ between the oral and dermal route. This may contribute to differences in metabolism due to absorption route and biotransformation enzymes, i.e., individual polymorphism. This will probably not have an impact on the recovery of the analyte if the urine samples are hydrolysed. This emphasizes that hydrolysis is an essential part of the bioanalysis if the biomarker is conjugated and the importance of having information about the metabolism to perform a reliable exposure assessment. Moreover, evaluation was performed on samples from the oral exposure experiments which were screened

for metabolites using a compound-specific, predicted SRM/ IDA method. In the metabolite evaluation, 5-OH-TBZ and its conjugates were the best metabolites to use as biomarker of exposure to TBZ. It has been suggested, that the benzene ring in TBZ can undergo ring cleavage and, sulfoxidation (42). In the screening, small peaks of other mono-OH-TBZ and di-OH-TBZ (transition 234.0-188) and their conjugates were found.

In Paper III, it was suggested that OH-PYR is conjugated to both glucuronic acid and sulphate. Unless enzyme hydrolysis overnight using β-glucuronidase/aryl-sulfatase was conducted, OH-PYR was not detected in the urine samples. In the metabolite screening, a compound-specific, predicted SRM/ IDA method was used on samples from the oral exposure experiments. OH-PYR and its conjugates were probably the best metabolites to use as biomarker of exposure to PYR.

## Human Exposure

In Papers I, and II, the pre-exposure samples i.e. urine sample collected prior to the experimental exposure, were reported to contain biomarker levels of 1 ng/mL for ETU and 5-OH-TBZ levels below LOD in the TBZ exposure experiment. In Paper III, the pre-exposure samples were reported to contain biomarker levels of OH-PYR close to the LOD in the oral and 0.2 and 1.6 ng/mL in the dermal exposure studies.

In Papers I, II and III, the reported mean urinary $t_{½}$ for ETU, 5-OH-TBZ and OH-PYR in the dermal exposure studies were between 14 and 53 hours, for adjusted values. In Papers II and III, the reported mean urinary $t_{½}$ for 5-OH-TBZ and OH-PYR in the oral exposure studies, were between 1 and 15 hours for adjusted values. The separate values of $t_{½}$ are summarized in Table 4. For the five exposure biomarkers TCP, DCCA, 3PBA, 4F3PBA, and 2,4-D, measured in Paper IV, the half-lives were found in literature to be between 5 and 41 hours, see summary in see Table 2.

The half-lives of ETU, correlation coefficient (r) for the regression line and urinary elimination curves of ETU are shown in Paper I. After the dermal exposure of ETU, the urinary levels increased slowly for 24 hours and then a plateau was reached. The maximum concentration ($C_{max}$) of ETU 4 ng/mL was reached after about 40 hours. The urinary elimination can be described by a one-compartment model and to follow first-order kinetics. Attempts made earlier, estimating the elimination half-lives in urine after occupational dermal and inhalation exposure to EBDC, varies considerably in their estimates of the half-life of ETU between 32 and 100 hours (56,71). The half-life after an experimental oral exposure to EBDC contaminated with ETU was estimated to around 20 hours for

two volunteers and described by a one-compartment model and followed first-order kinetics (41).

The half-lives of 5-OH-TBZ with correlation coefficient (r) for the regression line and urinary elimination curves are shown in Paper II. After oral exposure to TBZ, the urinary levels increased rapidly and $C_{max}$ was reached after 1 hour. The excretion of 5-OH-TBZ in urine seemed to be described by a two-compartment model and both the first rapid and the second slower elimination phase followed first-order kinetics, with estimated elimination half-lives of 2 hours and between 9 and 12 hours. After the dermal exposure to TBZ, the urinary levels increased slowly and the urinary $C_{max}$ was reached after 13 hours. The excretion seemed to be described by a one compartment model and it followed first order kinetics, with estimated elimination half-life between 9 and 18 hours. If there was a first rapid elimination phase, it was not observed. This may be caused by a continuous absorption phase.

The half-lives of OH-PYR with correlation coefficient (r) for the regression line and urinary elimination curves are shown in Paper III. After oral exposure to PYR, the urinary levels increased rapidly and $C_{max}$ was reached after 2 hours. After the dermal exposure to PYR, the urinary levels increased slowly and the urinary $C_{max}$ was reached after 8 hours. After both the administration routes the urinary elimination seemed to be described by a two-compartment model and they followed first-order kinetics.

The compounds are probably distributed into different compartments e.g. organs, tissues or different biotransformation pathways before excretion.

The dose recoveries were estimated, in Papers I, II and III, after the dermal and in Paper II, III also after oral exposure, see Table 4. The means of the available doses are summarized in Table 2 and the mean dermal recoveries of the administrated doses were 7, 1 and 14% for ETU, TBZ and PYR, respectively. The loss of dose was about 30% for ETU, 85% for TBZ and 33% for PYR. The large uncertainty in the available dose can depend on several factors, such as uncertainty in the determination of the loss of parent compound from aluminium foil, swabs and tapes, stability of parent compound in extraction media, and stability of parent compound on the skin.

The oral recovery for TBZ was between 21 and 24% of the administrated oral dose and 96% was recovered within 24 hours. In an earlier study of oral exposure to 1 g of $^{14}$C-TBZ in humans, 87% of the radioactivity was found in urine, of which 38% was recovered as 5-OH-TBZ conjugates, (42); this is comparably higher than in our study. As reported by Tocco et al, (1966) several phase I metabolites of TBZ are formed and excreted in urine. The absorption may be dose-dependent, since the dose (3 mg) in Paper II represents only 0.3% of the dose administrated by

Tocco et al.(1966) (42). Also, TBZ has a very low solubility in water and thus was dissolved in acetone. The solubility of TBZ may be a dependent factor and precipitation may have occurred in the vehicle.

The mean oral recovery of PYR was 79% of the administrated oral dose, of which 99% was found within the first 24 hours.

In oral experiments, the loss of dose within the gastrointestinal tract may also occur in the described exposure experiments, since the oral doses were not analysed for concentration in the juice and faeces was not collected.

**Tabel 4.**

Human experimental studies after oral or dermal exposure to pesticides in this thesis. Estimated urinary $t_{½}$ and the dose recoveries of the biomarkers for the two volounteers, one female (f) and one male (m).

| biomarker | route | t½ (h) creatinine | | t½ (h) density | | t½ (h) unadjusted | | recovery (%) | sex | Paper |
|---|---|---|---|---|---|---|---|---|---|---|
| | | slope1 | slope 2 | slope1 | slope 2 | slope1 | slope 2 | | | |
| ETU | dermal | 75 | | 72 | | 103 | | 10 | f | I |
| ETU | dermal | 29 | | 34 | | 64 | | 10 | m | I |
| 5-OH-TBZ | oral | 2 | 12 | 2 | 12 | 2 | 12 | 21 | f | II |
| 5-OH-TBZ | oral | 2 | 9 | 1 | 9 | 1 | 9 | 24 | m | II |
| 5-OH-TBZ | dermal | 18 | | 18 | | 20 | | 6[a] | f | II |
| 5-OH-TBZ | dermal | 9 | | 9 | | 10 | | 6[a] | m | II |
| OH-PYR | oral | 4 | 14 | 5 | 14 | 5 | 15 | 77 | f | III |
| OH-PYR | oral | 3 | 15 | 4 | 15 | 3 | 32 | 80 | m | III |
| OH-PYR | dermal | 6 | 30 | 10 | 31 | 11 | 29 | 15 | f | III |
| OH-PYR | dermal | 8 | 18 | 9 | 20 | 8 | 20 | 26 | m | III |

a) Samples from Aluminium foil and swab from the two volunteers were pooled before analysis and only the mean recovery was calculated.

## Adjustments for urinary dilution

ETU, 5-OH-TBZ and OH-PYR, all showed that creatinine and density adjustment gave better correlation for the elimination curves compared to unadjusted urinary concentrations. For ETU, density and for 5-OH-TBZ, creatinine gave the best fit of the adjusted values. For OH-PYR, both creatinine and density adjustment seemed to be usable.

In our study, it should be emphasized that only two subjects participated in the experimental exposure studies.

Generally, adjustment for urinary dilution is often recommended. Traditionally, creatinine adjustment has been preferred. However, density adjustment may be an option. It should be considered that creatinine levels are affected by several factors, such as gender, age, muscle mass, and meat consumption. Sometimes,

density adjustment may be a better choice, e.g., when comparing individuals, groups or populations of both sexes and various ages and large differences in muscle mass and meat intake (64,65). However, adjustment for urinary density may be affected by similar factors. Studies, for example, in a study on urinary cadmium levels adjusted for creatinine, was more affected by gender, age, body size and meat intake than adjustment using urinary density (91).

## Exposure-Assessment using Biomarkers

Paper I reports the urinary concentrations for the exposure biomarker ETU in samples obtained from the cohort described in paper IV including urine samples from the women after giving birth. All urine samples (about 1300) had detectable concentrations between 0.3 and 210 ng ETU/mL. The CVs for the QC-samples were between 9 and 19%. In Papers II and III, the urinary concentrations for the exposure biomarkers 5-OH-TBZ and OH-PYR were reported for samples obtained from a general population (around 300 samples) in southern Sweden. Urinary levels of 5-OH-TBZ were detected in 52% of the population with a median of 0.05 ng/mL and 1.6 ng/mL $90^{th}$ percentile. The CVs for the QC-samples were between 3 and 5%. Urinary levels of OH-PYR were detected in 48% of the population with a median of 0.07 ng/mL and the $95^{th}$ percentile was 3.7 ng/mL.

Thus, the high throughput methods described in the Papers I, II and III are applicable in large studies to biomonitor exposure in general population in Sweden as well as in Costa Rica.

Paper IV reports the urinary concentrations of eight biomarkers ETU, 5-OH-TBZ, OH-PYR, TCP, DCCA, 3PBA, 4F3PBA and 2,4-D in pregnant women participating in the ISA-study. The exposure biomarker levels in the cohort were compared to literature data on exposure levels in other groups of pregnant women. Additionally, the difference in the biomarker levels from women occupationally exposed was compared to biomarker levels in women not working in agriculture in the cohort.

In Paper IV the pregnant women in the ISA-study were reported to be young; more than half were less than 25 years of age, economically impoverished, and half of the women had less than 6 years of education. Almost half of the women lived less than 200 meters from a banana plantation and 63% of their partners worked in agriculture. Only about 8% of the women worked in banana plantations during pregnancy. However, living in agricultural areas or with agricultural workers has been reported earlier as a source of exposure to OPs and EBDCs (73,78,92-94).

The concentrations for the eight pesticide biomarkers determined in the 909 urine samples were reported in Paper IV. These samples were collected as spot urine samples, 1-3 times for each of the 445 women during pregnancy. For the analysis of the urine samples the analytical method described in Paper I (ETU), and a merged and slightly modified version of the two methods described in Papers II and III were used to analyse 5-OH-TBZ, OH-PYR, TCP, DCCA, 3PBA, 4F3PBA and 2,4-D. Biomarker concentrations below LOD were substituted with the lowest corrected concentration that was detected, divided by the square root of 2. The reason for this was that an analytical sample above LOD adjusted for urinary dilution could become smaller than the substituted value. Similarly, a value below LOD could become larger than LOD, when adjusted for the urinary dilution. Hence the smallest adjusted value above LOD in the data set was chosen instead of LOD. The reliability of the exposure data was confirmed by good precision and low LODs for the eight exposure biomarkers, see Table 3.

For descriptive statistics and comparison between the women, density adjusted values were used. Density was decided to be a better choice than the creatinine, because the excretion of creatinine changes during pregnancy (95,96). This change may affect the density as well but according to literature to a smaller extent (64,91,97).

The distributions of the concentrations were skewed for all eight biomarkers and therefore $\log_{10}$-transformed for normal distribution before statistical calculations. Because of the repeated sampling and that the women contributed with different number of samples (n=1, 2 or 3) multivariate mixed effects model were used when comparing the exposure in women working in agriculture versus women not working in agriculture in the cohort.

The highest pesticide concentrations, in paper IV, were reported for the metabolite of mancozeb, ETU, followed by the metabolite of CPF, TCP, with median concentrations of 3.1 and 1.6 ng/mL respectively.

Maximum concentrations (33 to 934 ng/mL) were reported for all biomarkers except for 4F3PBA (1.2 ng/mL). For some biomarkers, e.g. OH-PYR and 5-OH-TBZ, more than thousand-fold higher concentrations were observed than their median concentrations. Median concentrations of 5-OH-TBZ and OH-PYR were 0.06 and 0.4ng/mL respectively, with detection frequencies of 65% and 87%. For 4F3PBA the median was 0.03ng/mL with a detection frequency of 79%; however, the levels were all close to LOD. With respect to metabolites of synthetic pyrethroids, DCCA showed a median concentration of 1.2 ng/mL.

A few studies have investigated exposure to pesticides in pregnant women (98-105). To be able to compare the exposure found in Paper IV with that in other studies, creatinine adjusted values and unadjusted values were calculated. ETU

concentrations reported in Paper IV were more than five times higher than those reported for other general populations(74,88,106) and comparable with urinary ETU concentrations of Italian agricultural workers (107,108). In one study however, the median ETU concentration was below LOD (102) but since the analytical method used in that study does not hydrolyse the urine samples, the results are questionable.

As to the results reported in Paper IV of the CPF metabolite, TCP, the concentrations are in the same range as in many studies in the literature. An exception is the study by Berkowitz et al. which reports a median TCP concentration a tenfold higher (98). However, in that study an unspecific analytical method was used (LC-UV).

The concentrations of the pyrethroid metabolites DCCA and 3PBA in Paper IV were also in the same range as in many studies in the literature. However, Berkowitz et al. reports a tenfold higher median concentration for 3PBA (98). For 4F3PBA all studies show very low concentrations (102-104).

Several studies have measured the biomarker of 2,4-D, but only one study detected levels in more than 50% of the samples; the geometric mean was in the same range as in our study (102,104).

The exposure to TBZ (5-OH-TBZ) or PYR (OH-PYR) was described for the first time in pregnant women in paper IV.

Except for the metabolites of synthetic pyrethroids (DCCA, 3PBA and 4F3PBA), the pesticide biomarker levels were poorly correlated, with Pearson´s r ranging from -0.05 to 0.23. DCCA was highly correlated with 3PBA (Pearson's r=0.81) and moderately with 4F3PBA (Pearsons' r=0.41). That the metabolites of synthetic pyrethroids (DCCA, 3PBA and 4F3PBA correlate suggests an exposure to permethrin or cypermethrin since these pyrethroids both metabolize to 3-PBA and DCCA. Several pyrethroids metabolize to 3-PBA but not DCCA, but only cyfluthrine metabolizes to DCCA and 4F3PBA. However, exposure to other pyrethroids e.g. deltamethrin, allethrin, resmethrin and fenvalerate is also possible as they are biotransformed to 3PBA (109).

In Paper IV, it is reported that pregnant women (n=36) working in agriculture had higher urinary 5-OH-TBZ concentrations compared to the not working women (n=409). An explanation could be that women working in banana plantations usually work in the packing plant, where thiabendazole is applied on bananas. During packing process several manual tasks that may result in high dermal exposures, including take the fruit to the spraying cabin, pull the bananas out of the cabin, putting on stickers and packing the bananas in boxes, see Figure 3.

**Figure 3.**
Women working in a banana plantation in the packing plant, where thiabendazole is applied on bananas. The packing process includ several manual tasks that may result in high dermal exposures: take the fruit to the spraying cabin, pull the bananas out of the cabin, put stickers on and packing the bananas in boxes.

Women working, also had higher urinary ETU (4.21 ng/mL) and TCP (2.07 ng/mL) concentrations compared to women who did not work. These differences are perhaps explained by frequent contact, at work, with bananas contaminated with aerially-sprayed fungicides (73) and plastic bags that contain CPF. For example, the CPF-impregnated bags are removed from the banana bunches in the packing plant and if they still contain residues of CPF this may result in dermal exposures. Metabolites of synthetic pyrethroids (i.e., DCCA, 3PBA, 4F3PBA) and

2,4-D were also present in the women's urine samples, but did not differ between women who worked in banana plantations and those who did not. This finding is possibly due to the fact that these pesticides were not used on banana plantations, but at home and for some of the pyrethroids in vector control programs.

Values of ICC indicated a relatively low temporal reliability for ETU, reflecting considerable fluctuations in metabolite concentrations measured within the same woman on repeated occasions during pregnancy. The best temporal reliability were found for TCP and 5-OH-TBZ with ICC = 0.36 and 0.43, respectively. This suggests that the women are exposed irregularly for EBDCs which fits with the weekly air spraying of mancozeb. The airborne contribution of CPF may come from the bags covering the bananas in the fields. For TBZ the data suggest that occupationally exposed women are continuously highly exposed. It is interesting to see, that in this population, 5-OH-TBZ has the best temporal reliability even if it has the shortest $t_{½}$ among the biomarkers.

# Ethics

An often mentioned drawback when biomonitoring data shall be interpreted is the lack of reliability of the biomarker. To be reliable, the chosen exposure biomarker should be verified as metabolite of the pesticide or group of pesticides of the exposure and in the species that are studied. One way is to perform human experimental exposure studies. In recent years, few studies have been performed, mainly hindered by ethical issues, because of the toxicity of pesticides. However, advanced LC-MS/MS techniques, enables low-dose exposure studies in humans with doses below the recommended ADIs. These exposure levels are in same range or even less than what the general population can be exposed to via diet. Thus, the ethical problem could be considered to be minor (41). However, many countries do not allow human experimental exposure studies of pesticides.

# Strengh and Weaknesses

## Some Strengths

- Access to modern state-of-the-art LC-MS/MS equipment
- Well validated analytical methods have been developed
- The methods were high throughput enabling repeated measurements in large populations
- Participation in inter-laboratory biannual round robin program (TCP and 3-PBA)
- Experimental exposure studies were conducted in humans
- The biomarkers were excreted as conjugates; hydrolysis methods were optimized
- The collected cohort data is being used in an interdisciplinary study

## Some Weaknesses

- Some biomarkers are specific to a group of compounds and not to specific ones, e.g. EBDCs (ETU) and pyrethroids (3PBA)
- Only two volunteers participated in the human experimental studies
- Incomplete knowledge of the absorbed dose and its further fate after dermal and oral exposure
- The biomarker 5-OH-TBZ has a half-life less than 2 hours, obstructing exposure assessment
- It is difficult with urine sampling in reality - it was difficult to get one sample per trimester from all of the women

# Conclusions

From the results obtained in this thesis the following conclusion were made.

I. Three specific high throughput LC-MS/MS methods for the quantification of the urinary exposure biomarkers ETU, 5-OH-TBZ and OH-PYR were developed and validated. Hydrolysis to release the analyte from the conjugates was essential. The methods were specific with excellent sensitivity and repeatability and can be used in exposure assessment of EBDCs, TBZ and PYR in large exposure studies of general populations exposed to low levels of these pesticides. The exposure biomarkers of TBZ and PYR were determined in samples from a general Swedish population.

II. Experimental exposure studies studies, examining the elimination of ETU, TBZ and PYR in humans were performed. The metabolites ETU, 5-OH-TBZ and OH-PYR are reliable exposure biomarkers of exposure in urine. In the studies the exposure biomarkers were excreted in urine as conjugates. The biomarkers had rather short half-lives in urine after oral and dermal exposure, thus repeated sampling can be recommended.

III. The developed bioanalytical methods were applied in an epidemiological cohort study of pregnant women living close to banana plantations in Costa Rica. All pesticides measured could be detected. ETU and TCP were detected in all samples, 5-OH-TBZ in 65% and OH-PYR in 87 %. The concentrations of ETU in these pregnant women seemed comparable to levels in Italian agricultural workers. The pregnant women working at the banana plantations were significantly more exposed to TBZ and EBDCs than the nonworking pregnant women in the cohort and, in addition, the exposure to chlorpyrifos was slightly higher among the working women.The exposure to pesticides in pregnant women in Costa Rica is of concern and need to be further studied for adverse health effects also in the children.

# Future Perspectives

- Simplified on-line clean-up methods enabling direct injection of crude urine
- ETU conjugation to be studied
- Experimental exposure studies with at least 6 subjects
- Optimise urinary sampling strategy – e.g. 24 h, morning, spot, multiple sampling
- Handle short half-lives of biomarkers in exposure assessment
- Long term stability of samples should be investigated further
- Improved methods for adjustment of urinary dilution
- International agreement on urinary adjustments
- Use the collected data in further exposure – response studies
- Complementary environmental monitoring to identify sources
- New pesticides are continuously developed – new biomarker methods needed.

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# Paper I

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# High-throughput method for the analysis of ethylenethiourea with direct injection of hydrolysed urine using online on-column extraction liquid chromatography and triple quadrupole mass spectrometry

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ABSTRACT

Ethylenethiourea (ETU) is of major toxicological concern, since in experimental animal studies, ETU has shown a large spectrum of adverse effects. High occupational exposure can be found among agricultural workers or during manufacturing of ethylenbisdithiocarbamates (EBDC). For the general public, sources of environmental exposure may be residues of ETU in commercial products, food and beverages. For the determination of ETU in human urine we present a high-throughput online on-column extraction liquid chromatography triple quadrupole mass spectrometry method using direct injection of hydrolysed urine samples. This method is simple, user- and environmentally friendly and all sample preparation is performed in 96-well plates. A labelled ETU internal standard was used for quantification. The method showed a good sensitivity with a limit of quantification (LOQ) of 0.5 ng ETU/mL urine and the calibration curve was linear in the range 0.25–200 ng ETU/mL urine. The within-run, between-run and between-batch precision was between 6% and 13%. Alkaline hydrolysis considerably increased the levels of ETU indicating a potential conjugate. The method was applied in an experimental dermal exposure study in humans, with sample concentrations ranging from 0.4 to 5.0 ng ETU/mL urine. The excretion in urine was 10% of the applied dose. The elimination profile seemed to differ between the two individuals. The results show an estimated half-life of ETU between 34 and 72 h. Although the experiment is limited to two individuals, the data provide valuable and new information regarding the toxicokinetics of ETU after dermal exposure.



## 1. Introduction

Ethylenbisdithiocarbamates (EBDCs) is a group of fungicides of which ethylenethiourea (ETU) is an environmental degradation product as well as a metabolite and impurity. Potential sources of ETU exposure to humans may be occupational or environmental. Occupational exposure may be high among agricultural workers and among workers manufacturing EBDCs [1–9]. Further, ETU is used as a vulcanization agent in the production of polychloroprene (neoprene) and polyacrylate rubbers and in several other products such as dyes. In the general population, residues of ETU in products can be one of many sources of environmental exposure to ETU. Associations have been observed between ETU, and smoking, wine drinking and consumption of fruit and vegetables [4,10]. ETU is of major toxicological concern. In animal studies, ETU has caused a large spectrum of adverse effects, mainly concerning mutagenic, teratogenic, carcinogenic and hepatogenic effects [11–13]. However, the evidence for such effects in humans is less well founded. ETU has been classified to be "reasonably anticipated to be a human carcinogen" based on sufficient evidence of carcinogenicity from animal experiments [14]. Some data suggest that ETU affects the lymphocyte genome and the thyroid gland among heavily exposed workers [1,2,15]. Both for EBDCs and ETU, there is a need for large scale epidemiological studies of exposure-effect relationships. In such studies, an accurate exposure assessment is required. Biomarkers have many advantages in comparison with other methods of exposure assessment, but reliable analytical methods are needed. Many analytical methods for measurement of ETU in biological samples have been presented [16]. Several mass spectrometry (MS) based analytical methods for quantification of the low levels of ETU present in human urine after occupational or environmental exposures, using gas chromatography/mass

*Abbreviations:* ADI, acceptable daily intake; b.w., body weight; CID, collision induced dissociation; EBDC, ethylenbisdithiocarbamates; ETU, ethylenethiourea; IS, internal standard; LC/MS/MS, liquid chromatography triple quadrupole mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; QC, quality control.

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spectrometry (GC/MS) [17], and liquid chromatography/tandem mass spectrometry (LC/MS/MS) have been developed [18–21]. However, these methods are all laborious, time-consuming and not suitable for epidemiological studies.

Dermal exposure is thought to play an important role in the risk assessment of pesticides [22]. However, the knowledge of dermal exposure to pesticides is limited. Moreover, there is only little knowledge of exposure assessment techniques for quantification of dermal exposure. For data of biomarker levels to be useful, supporting toxicokinetic data are needed. Knowledge of the parent compound and the major metabolites excreted in urine, including the excretion half-life, is important [23]. In guinea pigs, dermally exposed to ETU, 14% of the applied dose was absorbed after 24 h [24]. There are several occupational studies addressing dermal EBDC and ETU exposure. Dermal exposure to ETU is related to the presence of ETU as contaminant in EBDC-based formulation, or in re-entry workers that come in contact with treated crops where EBDC degradation products may be present. In studies of agricultural workers exposed to EBDC, dermal exposure to ETU was measured using filter pads [8,25]. A significant correlation was found between end-shift ETU levels in urine and the measured levels on pads [25]. This result is in agreement with a study of workers at a production plant where ETU levels in urine correlated with EBDC contamination on the hands [5]. Earlier, no studies of experimental dermal exposure to ETU have been performed in humans. On the other hand very few pesticide dermal exposure studies have been performed in humans [22,26–29].

The aim of this study was to develop a high-throughput online on-column extraction LC/MS/MS method for analysis of ETU in human urine. The method was applied in a human experimental dermal exposure study of ETU.

## 2. Materials and methods

### 2.1. Chemicals and materials

The internal standard (IS) [$^2H_4$]-ETU was purchased from Dr. Ehrenstorfer (Augsburg, Germany). Hexane was from Lab-Scan (Dublin, Ireland). Sodium hydroxide (NaOH), hydrochloric acid (HCl) and methanol (hyper grade for LC–MS) were from Merck (Darmstadt, Germany). ETU was a PESTANAL® analytical standard, formic acid (FA), pentafluorobencyl bromide (PFBr) and tetrabutylammonium hydrogen sulphate (TBA) were from Sigma–Aldrich Inc. (St. Louis, MO, USA). Purified water from a Millie-Q Integral 5 system (Millipore, Billerica, MA, USA) was used.

Plastic 96-well-plates SQW block with clear glass insert vials 1.5 mL, SQW 45 × 7.7 mm, sealed with a welled sealmat block cover, was from La-Pha-Pack®GmbH (Langerwehe, Germany) and 96-well-aluminium plates with1.5 mL aluminium block cover, was from J.G. Finneran Associates, Ltd (Surrey, United Kingdom).

### 2.2. Instrumentation

Quantitative analysis was conducted using a triple quadrupole linear ion trap mass spectrometer, equipped with TurboIonSpray source (QTRAP 5500; AB Sciex, Foster City, CA, USA) coupled to a liquid chromatography system with four pumps (UFLC$^{RX}$, Shimadzu Corporation, Kyoto, Japan). The MS/MS analyses were carried out using selected reaction monitoring (SRM) in positive atmospheric pressure chemical ionization (APCI) mode. Air was used as nebulizer spraying gas. Pure nitrogen was used as curtain gas and collision gas. The APCI temperature was set at 450 °C. The instrument was tuned to a peak resolution of 0.5 ± 0.1 Da at half the peak height in high resolution mode. To establish the appropriate SRM conditions, standard solutions were infused into the MS/MS for optimization. Collision-induced dissociation (CID) of each $[M+H]^+$ was performed and the product ions giving the best signal to noise ratio were selected for the SRM analysis. All data acquisition and processing was performed using the Analyst 1.5.1 application software (Applied Biosystems, Foster City, CA, USA).

### 2.3. Preparation of calibration standards, quality control and samples

Stock solutions were prepared in duplicates by dissolving accurately weighed amounts of [$^2H_4$] ETU (IS) and ETU in methanol. The IS and ETU standard stock solutions were diluted further in methanol and stored at −20 °C. Urine samples for the calibration standards and for quality control samples were obtained from healthy volunteers at our laboratory.

For the calibration curve, 475 μL blank urine was spiked with 25 μL of the standard solutions and 25 μL of the IS solution, giving a urinary concentration between 0.25 and 200 ng ETU/mL and 5 ng [$^2H_4$] ETU/mL urine. The calibration curve was corrected with the amount found in the urine. As quality control (QC) urine samples naturally containing 2 and 7 ng ETU/mL and the 7 ng/mL QC spiked to 32 ng/mL were used. The low, medium and high QC-samples were divided into several aliquots and stored at −20 °C. The chemical blank was prepared from Millie-Q water and thereafter treated like the other samples. The urine samples and QC-samples were vortex-mixed after thawing and aliquots of 500 μL were transferred into 1.5 mL glass vials and placed in an aluminium 96-well plate, and 25 μL of IS solution was added.

For the hydrolysis, 20 μL of 2.5 M NaOH was added to the samples, standards, QC-samples and chemical blanks, giving a final concentration of 0.09 M NaOH. To prevent evaporation during hydrolysis the glass-vials were sealed with a sealmat and a cover was screwed on. After sealing, the samples were mixed thoroughly for 1 min and then transferred to a heating oven. Hydrolysis was performed for 1 h at 100 °C. The aluminium 96-well-plates was used in the hydrolysis step, because of the possibility to secure the cover of the vials with four screws and the capacity of fast temperature transfer throughout the plate. After hydrolysis, the samples were cooled to room temperature and the glass vials were moved to plastic 96-well-plates compatible with the autosampler. Aliquots of 15 μL of 5 M HCl were added to acidify the samples. The samples were mixed thoroughly and centrifuged for 10 min at 2600 × *g* before analysis.

### 2.4. Analysis

The two mobile phases used consisted of 0.1% (v/v) FA in water (mobile phase A) and 0.1% (v/v) FA in methanol (mobile phase B). The two dimensional separation was carried out, using two identical analytical columns Genesis® Lightn AQ (C18, 4.6 × 100 mm, 4 μm, Grace Vydac, Hesperia, CA, USA) and two sets of LC pumps, each set containing two pumps. The columns and LC pumps were connected through a diverter valve. An aliquot of 20 μL of the sample was injected on the first column and the separation was carried out by isocratic elution, using 100% mobile phase A and a flow rate of 0.7 mL/min. After 2.5 min, the diverter valve switched over and the effluent was diverted into the second column during 1 min and thereafter the diverter valve switched over again. The second set of pumps continued the isocratic elution of the analytes on the second column, using 100% mobile phase A. A diverter valve on the MS diverted the column effluent to the MS between 4.6–6.9 min. The first column was cleaned with 95% mobile phase B at a flow rate of 1.2 mL/min for 1 min, followed by equilibration with 100% mobile phase A for 2.5 min, during the time ETU was eluting on the second column. The second column was reconditioned with 95% mobile phase B for 0.5 min in the end of the analytical run and then

**Table 1**
Summary of the SRM transitions for ETU and [$^2H_4$] ETU used in the LC/MS/MS analysis.

| Compound | Transitions (Da) | Collision energy (V) |
|---|---|---|
| | Quantifier ions | |
| ETU | 103.1 → 60.0 | 42 |
| [$^2H_4$]ETU | 107.1 → 48.0 | 25 |
| | Qualifier ions | |
| ETU | 103.1 → 44.0 | 20 |
| [$^2H_4$]ETU | 107.1 → 64.0 | 42 |

equilibrated with 100% mobile phase A for 2.5 min in the beginning of the next analytical run. The columns were maintained at 40 °C. The LC/MS/MS analysis was performed using SRM transitions and collision energies for ETU and IS quantifier ion and qualifier ions as tabulated in Table 1. All samples were prepared in duplicates and analyzed by single injections. Concentrations were determined by peak area ratios between analyte and the IS. All values were corrected for the chemical blank.

### 2.5. Sample preparation for cross method comparisons

Sample preparation and analysis were performed according to a previously described method using an extractive derivatisation [18]. In this method, there was an incomplete hydrolysis, thus we included hydrolysis at 0.09 M and 100 °C as a minor modification. The samples were prepared in 13 mL glass tubes. Samples and calibration standards were prepared as in Section 2.3. The sample was hydrolysed for 1 h at 100 °C after an addition of 20 μL of 2.5 M NaOH. After cooling, the derivatisation reagents 0.4 M TBA, 10 M NaOH, dichloromethane and PFBr were added. The samples were derivatised for 90 min in an ultrasonic bath and analyzed by LC/MS/MS.

### 2.6. The impact of hydrolysis

Determination of the optimal time for hydrolysis was performed using an authentic urine sample, previously quantified to contain 7 ng ETU/mL urine. Samples were prepared in triplicates for nine time points between 0 and 240 min. The test was performed at a constant amount of 0.09 M NaOH and a temperature of 100 °C, except for the non-hydrolysed sample.

To test the impact of hydrolysis on real samples, ten urine samples were selected from an experimental oral exposure to an EBDC (mancozeb) [18]. Five samples were from a male volunteer (A) and five samples from a female volunteer (B). All samples were prepared in duplicates and prepared with and without hydrolysis according to the presented method. In the samples without hydrolysis, NaOH and HCl were mixed just before addition to the samples to avoid hydrolysis.

### 2.7. Validation of the analytical method

Limit of detection (LOD) and limit of quantification (LOQ) were determined as the mean level of the peak, within 0.1 min of the expected retention time of ETU, plus three and ten times the standard deviation, respectively [30]. LOD and LOQ were determined in chemical blank samples ($n = 14$). To assess the LOD and LOQ in urine matrix, 11 samples with known low ETU levels were selected and then analyzed.

The linear range of the calibration curve was determined from ten concentration levels between 0.25 and 200 ng ETU/mL urine. The equation of the curve was calculated by linear regression and the correlation coefficient ($r$) was used as a measure of the fit of the curve.

The precision of the method was determined using three different approaches, within-run, between-run and between-batch precision. The precision was calculated as the coefficient of variation (CV) of repeated measurements. The within-run precision was obtained from spiked urine at three concentration levels, 1, 10 and 100 ng ETU/mL urine. The samples were divided into ten aliquots and the sample preparation was performed in one sample batch, during one day.

The between-run precision was determined by including the three QC urine samples, containing 2, 7 and 32 ng/mL of ETU, in eleven analytical sample batches. The QC-samples were prepared in duplicates in each different analytical sample batch. The batches were prepared and analyzed on separate days, during a period of six months. Thus, the standard deviation was calculated from 11 individual mean results.

A between-batch precision of the method was determined by analysis of 88 urine samples collected in the dermal exposure study (see below). Each urine sample was divided into two aliquots which were then subjected to the entire analytical procedure. The samples were prepared and analyzed in separate analytical batches and on different days. The CV was calculated as previously described [18].

The mean obtained concentration was measured at three concentration levels for the spiked samples used to determine the within-run precision method.

The stability of the ETU standard solutions in methanol was investigated by reanalysis after storage in −20 °C for 7 months.

A cross-method comparison was conducted. The ten urine samples described in Section 2.6 were prepared using the method in Section 2.5 and compared with the here presented method. The results were compared using linear regression.

### 2.8. Human experimental study of dermal exposure

Two healthy volunteers, one male (age 42; weight 75 kg) and one female (age 65; weight 56 kg) participated in the study. They had given their written informed consent and the study was ethically approved by The Regional Ethical Review Board in Lund, Lund University, Sweden (721-1395-05 mM and Dnr2013/6). The participants minimized the intake of conventionally grown food a few days before as well as during the study. The two subjects received one single dose, administrated topically on the inner forearm on an area of 75 $cm^2$. The dose to the male volunteer consisted of an accurately weighed amount of ETU, dissolved to a concentration of 3 mg/mL of ethanol/water 50% (v/v), giving 0.30 mg in 100 μL. The female received an aliquot, diluted with ethanol/water 50% (v/v) to a final concentration of 0.22 mg in 100 μL. The doses corresponded to the accepted daily intake (ADI) for ETU, 4 μg/kg b.w./day [31] assuming 100% absorbtion. After administration, the vehicle was allowed to evaporate to dryness before the skin was covered with aluminium foil. After 8 h of exposure, the cover was removed. The remainder of the dose was wiped off the exposed area four times using ethanol/water 50% (v/v) and cotton swabs. Thereafter, the subjects cleaned their forearms with soap and water. The aluminium foil and cotton swabs were stored at −20 °C until analysis. Before analysis, ETU on the aluminium foil and cotton swabs was recovered by extraction into 1 L of Millie-Q water.

The first urine sample was collected immediately prior to the exposure and then in intervals ad libitum during 144 h for the male subject and 170 h for the female. All urine voided was collected. All samples were stored at −20 °C until analysis. Sample volume, creatinine and density were determined in all the collected samples to adjust for the urinary dilution. Creatinine was analyzed with an enzymatic method [32] and density with a hand refractometer. The concentration adjusted for urinary density, $C_d$, was calculated according to the equation $C_d = C(\text{observed}) \times (1.016 - 1)/(\rho - 1)$, where $C$(observed) is the obtained concentration in the urine sample, $\rho$ is the measured specific density and 1.016 was used as the average urine density [33]. The half-life ($t_{½}$) of the elimination in

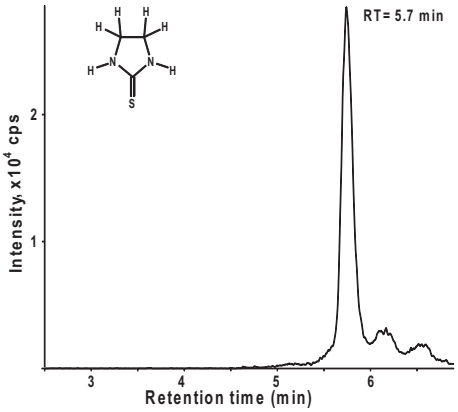


**Fig. 1.** LC/MS/MS SRM chromatogram showing a urine sample naturally containing ETU and quantified to a concentration of 7 ng/mL, for the quantifier transition 103.1–60.0 Da.

urine was estimated from the slope of the curve in the natural logarithm-linear (ln-linear) concentration versus time plot, where time is given as the mid time points between two sample collection time points.

## 3. Results and discussion

### 3.1. Mass spectrometry and chromatography

Previously, published methods for the analysis of ETU in biological samples are too time-consuming for large scale epidemiological studies. We here present a high-throughput method for the analysis of ETU with direct injection of hydrolysed urine using 2 dimension-LC set up as an online on-column extraction. This method is simple and all sample preparation is performed in 96-well plates. Thus, the method is environmental friendly with a significant reduction in solvent consumption. Also, we omitted dichloromethane commonly used for sample preparation [9,17–21,34].

The chosen column provided consistent and reproducible chromatography and retained ETU with stable retention times even in 100% aqueous eluent. The direct injection of urine was possible since an online on-column extraction LC separation and two separate switching valves were used. Thus, most of the urine matrix was discarded, which reduced the chemical background and kept the ion source clean. The cycle time for an analytical run, including equilibration time, was 7.0 min. Thousands of injections were performed on the analytical columns before signs of degradation were observed.

ETU showed an excellent sensitivity in APCI and positive ion mode. The transition 103.1/60 was chosen as the quantifier ion, since it gave the best signal to noise ratio. A second SRM was chosen as a qualifier ion and used to strengthen the identity of the analyzed ETU in urine (Supplementary data Figs. S1a and S1b). The transitions and optimum collision energies are shown in Table 1. When the analysis was performed with the instrument in high resolution mode, a better signal to noise and a reduction of interfering peaks was obtained. A typical chromatogram of a urine sample is shown in Fig. 1 (Supplementary data Fig. S1c).

**Table 2**
Increase in ETU levels after hydrolysis in ten urine samples, five samples collected from two individuals [27].

| Sample | Subject | Non hydrolysed ETU concentration (ng/mL) | Hydrolysed ETU concentration (ng/mL) |
|---|---|---|---|
| 1 | A | 2.4 | 9.7 |
| 2 | A | 3.8 | 18 |
| 3 | A | 4.0 | 21 |
| 4 | A | 0.4 | 7.7 |
| 5 | A | 0.2 | 1.2 |
| 6 | B | 0.2 | 2.7 |
| 7 | B | 2.3 | 16 |
| 8 | B | 1.9 | 13 |
| 9 | B | 2.9 | 12 |
| 10 | B | 1.9 | 3.5 |

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2013.06.035.

### 3.2. Hydrolysis

To evaluate the effect of hydrolysis, a urine sample was treated with 0.09 M NaOH at 9 different time point during 240 min. The level in the non-hydrolysed urine was 0.1 ng ETU/mL. The levels increased rapidly, and the highest ETU levels were found after 45 min. However, we chose 60 min hydrolysis time in the method (Supplementary data Fig. S2).

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2013.06.035.

To further evaluate the effect of hydrolysis, ten urine samples from an earlier oral experimental exposure of two subjects was analyzed with and without hydrolysis [18]. It was found that the levels of ETU increased considerably in all samples after hydrolysis. The levels are shown in Table 2. The levels of hydrolysis and non-hydrolysed urine samples were also compared using linear regression. The curve shows a linearity with $r = 0.88$ and a slope of $y = 4.7x$. Previously published methods for urine analysis have not addressed hydrolysis or conjugation of ETU. Interestingly, it has been shown that ETU can form protein adducts that can be released with mild acid hydrolysis [35,36].

### 3.3. Validation of the analytical method

The LOD and LOQ for ETU determined from analysis of 14 chemical blanks were 0.08 ng/mL and 0.16 ng/mL respectively. Since there seems to be a ubiquitous exposure to ETU, it is difficult to obtain urine samples with low ETU levels and this has an influence on the value of the LOD. After selecting 11 urine samples with low levels of ETU, the LOD was 0.2 ng/mL and the LOQ 0.5 ng/mL (Supplementary data Figs. S3a–S3c). The LOD was in the same range as reported in previous methods [9,17–21,34] and sufficient for the measurement of environmental human exposure. The CV was 19% in urine samples spiked with 0.25 ng/mL ($n = 6$) and 10% at LOQ, 0.5 ng/mL urine.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2013.06.035.

For the calibration curves, urine with a low amount of ETU were selected and then corrected with the amount found in the urine. The obtained calibration curves in the range 0.25–200 ng/mL urine showed excellent linearity with $r = 0.999$ and a slope of $0.0248 \pm 0.0016$ ($n = 6$).

The within-run, between-run and between-batch precisions are presented in Table 3. The between-run precision was determined

**Table 3**
Precision of the method at different concentration levels. The within-run precision was calculated from spiked urine samples and the between-run and batch precision from authentic urine samples.

| Precision | ETU concentration (ng/mL) | *n* | Mean obtained ETU concentration (ng/mL) | CV (%) |
|---|---|---|---|---|
| Within-run | 1 | 10 | 1.2 | 9.9 |
| | 10 | 10 | 11 | 13 |
| | 100 | 10 | 98 | 12 |
| Between-run | 2 | 11 | 1.9 | 6.2 |
| | 7 | 11 | 6.5 | 4.0 |
| | 32 | 11 | 28 | 6.1 |
| Between batch | Range 0.3–5.0 | 88 | 1.7 | 6.2 |

over a period of six months. In Table 3, the obtained concentrations at three concentration levels for spiked samples are presented.

The stability of the standard solutions of ETU dissolved in methanol was examined and found to be stable for 7 months at −20 °C. Several studies have found that ETU is stable in urine samples: in room temperature and in darkness for 24 h, at −20 °C for 6 months [17], at 8 °C for 2 weeks, at −20 °C for 3 months [18], and at −18 °C for 12 months [4].

A cross-method comparison was conducted using ten urine samples from an oral experimental exposure of two subjects and analyzed after hydrolysis using the here presented method and a method using an extractive derivatisation and analysis using LC/MS/MS [18]. The obtained results showed an excellent correlation when the levels were compared using linear regression giving a linearity with $r = 0.975$ and the slope 1.00 (Fig. 2).

### 3.4. Human experimental study of dermal exposure to ETU

In urine sampled before the experimental exposure, the ETU levels were for the female subject 0.8 ng/mL and for the male subject 0.7 ng/mL, density adjusted. After the dermal exposure of ETU, corresponding to a dose of 4 μg/kg of b.w., the urinary levels increased slowly. After about 24 h, a plateau was reached which lasted for about 50 h for the female and 30 h for the male subject Fig. 3a and for the female subject, Fig. 3b. Similar plateaus have also been observed in human dermal exposure studies of other pesticides [27–29,37]. The maximum ETU level was reached after 39 h and was

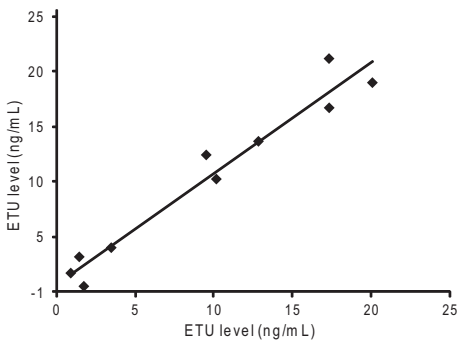


**Fig. 2.** Ten hydrolysed urine samples analyzed with two different analytical methods in the cross-method comparison. Urinary levels of ETU obtained by the modified method described by Lindh et al. [18] were plotted versus levels of ETU obtained by the method reported in this paper. The equation of the straight line was obtained by linear regression and found to be $y = 1.00x + 0.20$ ($r = 0.975$).

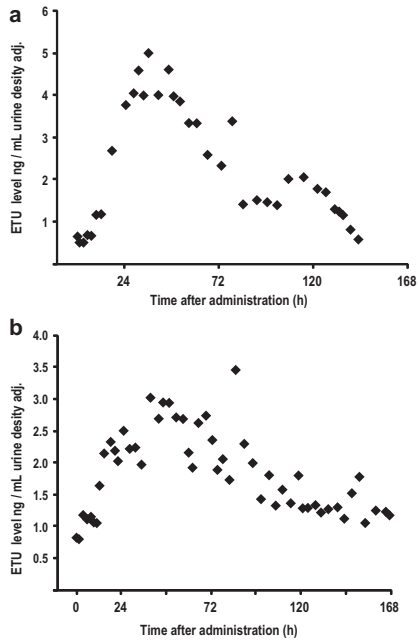


**Fig. 3.** (a) Urinary absorption and elimination for the male volunteer with the midpoint time in the interval 0–143 h was plotted against the density adjusted urinary levels (ng/mL). The volunteer was dermally exposed to a dose of 4 μg/kg b.w. on the inner forearm for 8 h. The first two urine samples were collected just before and 1 h after administration of the dose. (b) Urinary absorption and elimination for the female volunteer with the midpoint time in the interval 0–169 h was plotted against the density adjusted urinary levels (ng/mL). The volunteer was dermally exposed to a dose of 4 μg/kg b.w. on the inner forearm for 8 h. The first two urine sample was collected just before and 1 h after administration of the dose.

3.0 ng/mL (density adjusted) in the female subject and 5.0 ng/mL (density adjusted) after 36 h in the male. The decline in elimination of ETU was estimated to begin at 39 h in the female and at 36 h in the male. For the calculation of the half-life of elimination, values after 143 h for the female and 101 h for the male were discarded due to low levels and a suspected interfering environmental exposure. The half-lives of ETU with correlation coefficients are shown in Table 4. The urinary elimination of ETU for the male volunteer is shown in Fig. 4a and for the female volunteer in Fig. 4b. Earlier, attempts have been made to estimate elimination half-lives in urine after occupational dermal and inhalation exposure to EBDC. The half-lives vary considerably between the studies and are in the

**Table 4**
Estimated half-life of ETU found in the two volunteers, dermally exposed to a dose of 4 μg/kg b.w. of ETU for 8 h on an area of 75 cm².

| ETU urinary levels adjusted for | Female $t_{½}$[a] (h) | $r$[b] | Male $t_{½}$[a] (h) | $r$[b] |
|---|---|---|---|---|
| Density | 72 | 0.89 | 34 | 0.94 |
| Creatinine | 75 | 0.75 | 29 | 0.89 |
| Unadjusted | 103 | 0.42 | 64 | 0.71 |

[a] The half-life of elimination ($t_{½}$) in urine estimated from the slope of the curve in the natural logarithm-linear (ln-linear) concentration versus mid time plot.
[b] Correlation coefficient ($r$) for the regression line.

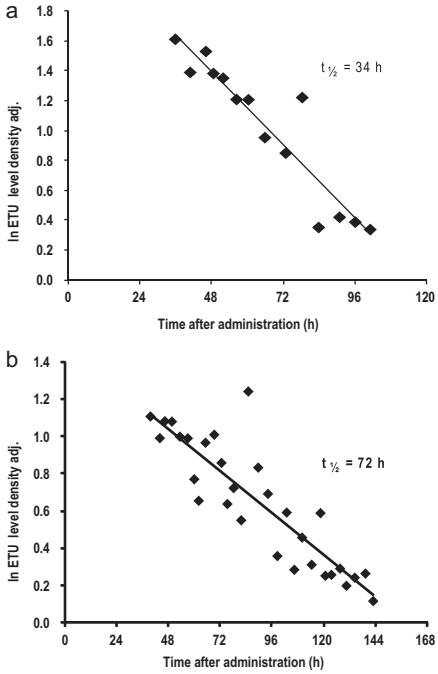


**Fig. 4.** (a) Urinary elimination of ETU for the male volunteer, after dermal exposure to a dose of 4 μg/kg b.w. of ETU for 8 h on an area of 75 $cm^2$ on the inner forearm. The half-life of elimination ($t_{½}$) in urine is estimated from the slope of the curve in the log-linear concentration versus time plot. Urinary elimination with the midpoint time in the interval 36–101 h was plotted against logarithm of the density adjusted urinary levels (ng/mL). (b) Urinary elimination of ETU for the female volunteer, after dermal exposure to a dose of 4 μg/kg b.w. of ETU for 8 h on an area of 75 $cm^2$ on the inner forearm. The half-life of elimination ($t_{½}$) in urine is estimated from the slope of the curve in the log-linear concentration versus time plot. Urinary elimination with the midpoint time in the interval 39–143 h was plotted against logarithm of the density adjusted urinary levels (ng/mL).

range 32–100 h [7,8]. The half-life after an experimental oral exposure to EBDC contaminated with ETU was estimated to 19–23 h for one female and one male [18]. Our study also contains only two individuals of each sex, thus one should be careful to draw to many conclusions. The half-life reported here differed more between the male and female volunteer compared to that of the oral exposure. This may indicate that the differences between sex and age have larger impact on dermal absorption than on oral absorption. The longer plateau for the female subject may possibly be explained by the fact that skin properties change with age and differ between individuals and that may have an impact on the dermal absorption. This is not surprising, since it is well known that, xenobiotic metabolism and excretion kinetics varies greatly depending on age, sex and activity level [22].

Of the administrated dermal dose, 35% was recovered from the skin washings from the female and 24% from the male at the end of the 8 h exposure. After deduction of the amounts in the washings, the total recovery of ETU in the female urine was 10% of the dose after 143 h. Correspondingly, in the male, 10% of the dose was recovered after 101 h. The results indicate a high uptake of ETU after dermal exposure. After an oral exposure of EBDC contaminated with ETU, the recovery was 69–82% of the dose. [18].

The toxicokinetic data in this study indicate that an adjustment for the urinary dilution is recommended and both creatinine levels and urinary density may be applied. Density gave the best fit of the adjusted values. Most often, though, creatinine levels have been used to adjust for urinary dilution. However, creatinine levels may be affected by several factors such as gender, age, muscularity, and consumption of meat. Also urinary density may be affected by similar factors, but a recent study has shown that urinary cadmium levels adjusted for creatinine was more affected by gender, age, body size and meat intake than adjustment using urinary density [38]. When comparing individuals or populations with large differences in muscle mass, meat intake, or when both males and females of various ages are included, density adjustment may be the more applicable.

In our study, 0.1 mL of ethanol/water 50% (v/v) solution was chosen to get an even dispersal of ETU over the application area. The solution evaporated within a minute before the skin was occluded to protect the surface. We therefore assume that the use of an ethanol solution had minimal effect on the permeation of ETU. The use of ethanol as vehicle and washing solution has been used in other dermal absorption studies [27,28]. Occlusion of the exposed skin may also affect the dermal absorption by enhancing the hydration and temperature of the skin. Further, washing of the exposed skin may enhance the dermal absorption, especially if using soap. Surfactants have shown to alter the properties of the skin barrier [22].

We chose to expose the forearm of the subjects, the most commonly used anatomic site in dermal experimental exposure studies [22,26–29]. However, it has been shown that the forearm is less permeable compared to the forehead and the neck. This difference is due to skin thickness and amount of appendage like hair follicles, sweat- and sebaceous glands. Thus, the absorption may be higher, if other more permeable areas of the body are exposed [5,8].

### *3.5. Application of high-through put method in an epidemiological study*

Spot urine samples ($n$ = 1282) were collected from individuals environmentally exposed to EBDCs and ETU. Data from this study will be published elsewhere. All samples were divided into two aliquots and analyzed in duplicate sample batches. A total of 32 96-well plates were prepared with six QC samples per plate. Sample concentrations ranged from 0.3 to 210 ng ETU/mL. The between-run precision determined from QC samples were CV = 19% at 2.3 ng/mL ($n$ = 63), CV = 11% at 7.1 ng/mL ($n$ = 62) and CV = 9.3% at 30 ng/mL ($n$ = 63). The samples were prepared and analyzed during about 2 months. Thus, the method is applicable in large epidemiology studies.

## 4. Conclusions

We present a high-throughput method for the analysis of ETU with direct injection of hydrolysed urine using online on-column extraction LC/MS/MS. This method is simple and user-friendly and all sample preparation is performed in 96-well plates. The method is environmentally friendly with a significant reduction in solvent consumption. The urinary levels of ETU increased considerably after alkaline hydrolysis at 0.09 M NaOH. The method has excellent within-run, between-run and between-batch precisions. The method has a sufficiently low LOD to enable detection of ETU in environmentally exposed populations.

The method was applied in a human experimental dermal exposure study where about 10% of the applied dose of ETU

was recovered in urine. The elimination profile seemed to differ between the two subjects. Although this study is limited to two individuals, the data provide valuable and new information regarding the toxicokinetics of ETU after dermal exposure. The method was also applied in an epidemiological study, showing that 1282 samples could be analyzed with a good precision during a short time.

## Acknowledgements

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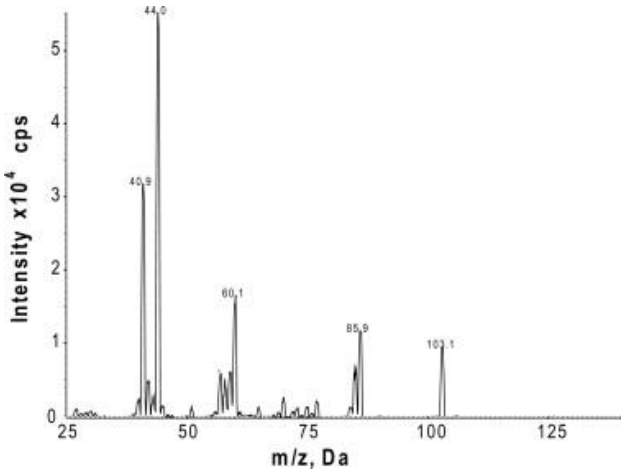


Fig. S1a. A product ion spectrum of m/z 103 Da obtained by infusion of ETU.

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**High-throughput method for the analysis of ethylenethiourea with direct injection of hydrolysed urine using online on-column extraction liquid chromatography and triple quadrupole mass spectrometry ☆**

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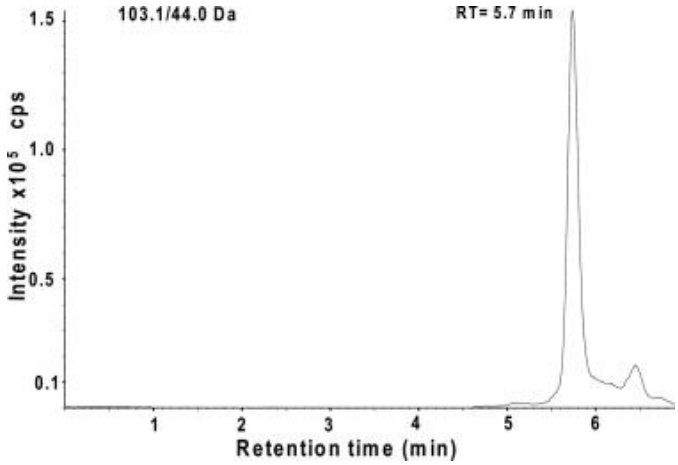


Fig. S1b. LC/MS/MS SRM chromatogram showing the same urine sample as Fig. 1, for the quantifier transition 103.1–44.0 Da.

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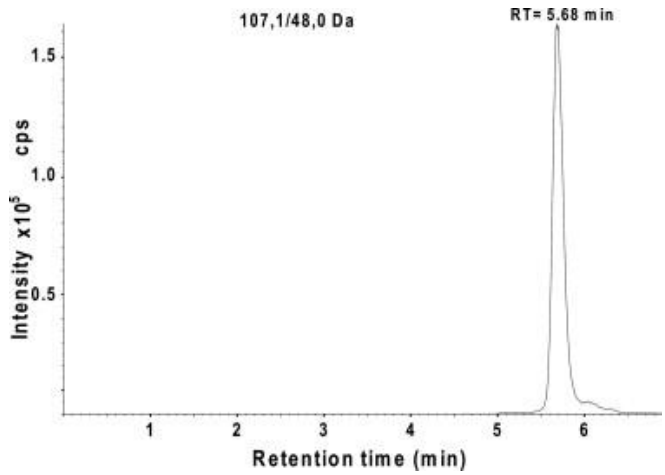


Fig. S1c. LC/MS/MS SRM chromatogram showing the same urine sample as Fig. 1, for the internal standard, transition 107.1–48.0 Da.

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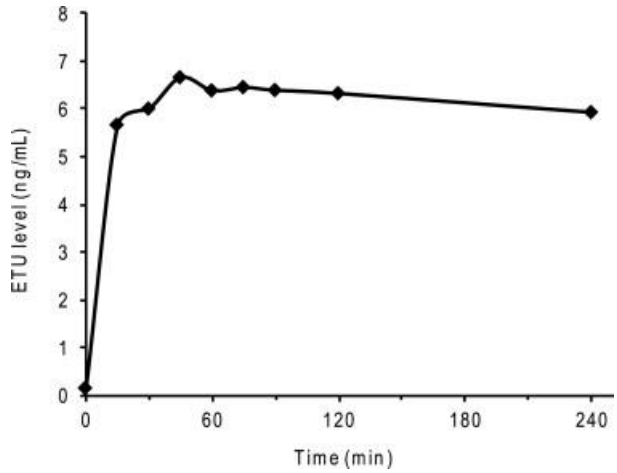


Fig. S2. Change in concentration versus time of hydrolysis at 100℃, for an authentic urine sample containing ETU.

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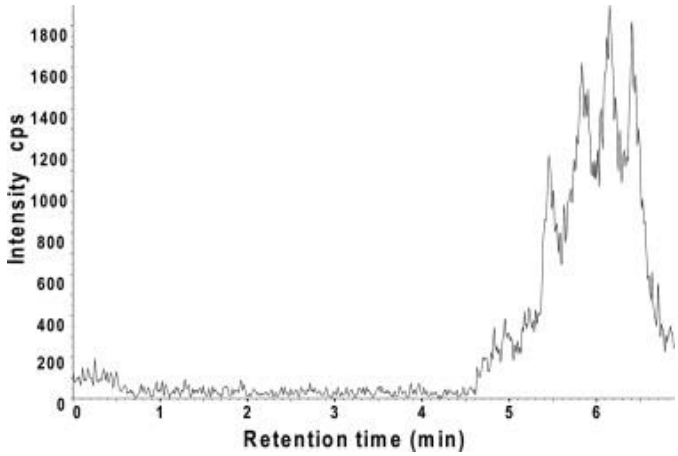


Fig. S3a. Chromatogram of ETU in a blank urine sample.

Eva Ekman, Margaretha Maxe, Margareta Littorin, Bo A.G. Jönsson, Christian H. Lindh

**High-throughput method for the analysis of ethylenethiourea with direct injection of hydrolysed urine using online on-column extraction liquid chromatography and triple quadrupole mass spectrometry ☆**

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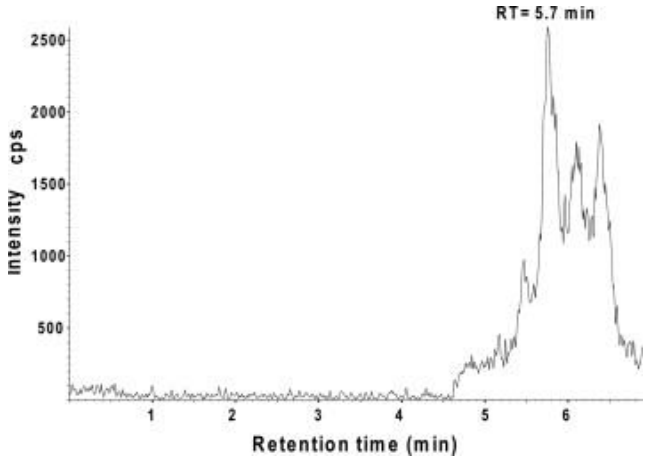


Fig. S3b. Chromatogram of ETU close to LOD, in a spiked urine sample.

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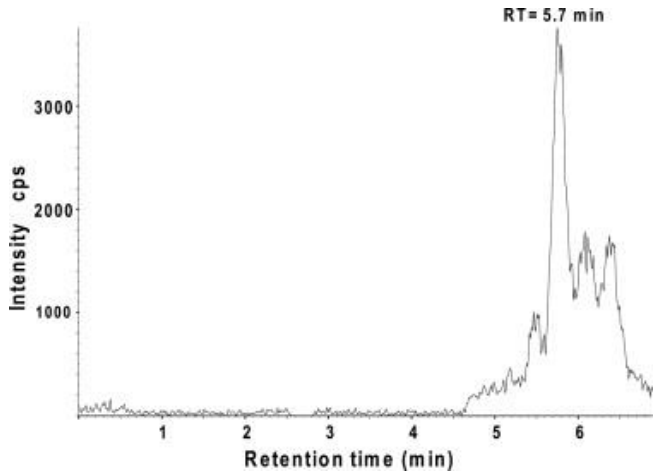


Fig. S3c. Chromatogram of ETU at LOQ in a spiked urine sample.

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# Paper II

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# Determination of 5-hydroxythiabendazole in human urine as a biomarker of exposure to thiabendazole using LC/MS/MS

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ABSTRACT

Thiabendazole (TBZ) is widely used as a pre-planting and post-harvest agricultural fungicide and as an anthelminthic in humans and animals. TBZ is of toxicological concern, since adverse effects including nephrogenic, hepatogenic, teratogenic and neurological effects have been reported in mammals. Occupational exposure can occur among agricultural workers and the general public may be environmentally exposed to TBZ through the diet. The metabolite 5-hydroxythiabendazole (5-OH-TBZ) was chosen as biomarker of exposure to TBZ and a LC/MS/MS method for the quantification of 5-OH-TBZ in human urine was developed. The method includes enzyme hydrolysis, as 5-OH-TBZ is conjugated to glucuronide and sulphate in urine. Sample through put was optimised using 96-well plates for sample handling as well as for solid phase extraction (SPE). The method has excellent, within-run, between-run and between-batch precision between 4 and 9%. The limit of detection (LOD) of 0.05 and a limit of quantification (LOQ) of 0.13 ng 5-OH-TBZ/mL urine enable detection in environmentally exposed populations. When applying the method in a general Swedish population, 52% had levels above LOD. The method was also applied in one oral and one dermal human experimental exposure study in two individuals. After oral exposure, the excretion of 5-OH-TBZ in urine was described by a two-compartment model and both the first rapid and the second slower elimination phase followed first-order kinetics, with estimated elimination half-life of 2 h and 9–12 h. The recoveries in urine were between 21 and 24% of the dose. Dermal exposure was described by a one compartment model and followed first order kinetics, with estimated elimination half-life of 9–18 h. The recovery in urine was 1% of the administrated dose of TBZ. Although these studies are limited to two individuals, the data provide new basic information regarding the toxicokinetics of TBZ after oral and dermal exposure.



## 1. Introduction

Thiabendazole (TBZ) was introduced in the 1960s first as an anthelmintic in humans and animals and later also as an agricultural fungicide. It is currently widely used as a fungicide, registered mainly for pre-planting and post-harvest treatment of vegetables (potatoes) and fruits.

Agricultural workers may be occupationally exposed to TBZ, but studies on exposure are missing. In the general public, there is a potential source of exposure by residues of TBZ in food [1]. Also, for some, medical treatment is an obvious source of exposure.

In general, mammalian toxicity is low [2]. However, humans treated with TBZ as an anthelmintic, have experienced adverse effects, like abdominal pain and nausea, dizziness and other cognitive complaints [3–5]. Serious effects such as liver diseases have also been reported [6] and in animal studies adverse kidney and liver effects, as well as teratogenic and reproductive toxicity at high doses [7–10]. TBZ is very toxic to aquatic organisms, and release of TBZ-containing waste water into the environment is prohibited within the EU [2].

In the risk assessment of pesticides, dermal as well as, oral and inhalation routes of exposure should be considered [11]. However, studies on dermal uptake of TBZ are missing, a shortcoming in view of safety control among agricultural workers. Further, there is a need for epidemiological studies of exposure–response relationships; in such studies an accurate exposure assessment is

*Abbreviations:* ADI, acceptable daily intake; b.w, body weight; CID, collision induced dissociation; IS, internal standard; LC/MS/MS, liquid chromatography triple quadrupole mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; QC, quality control; SPE, solid phase extraction; SRM, selected reaction monitoring; TBZ, thiabendazole; 5-OH-TBZ, 5-hydroxy thiabendazole.

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required. Biomarkers have many advantages in comparison with other methods of exposure assessment, i.e., all routes of exposure are taken into account. However, for the interpretation of exposure data, basic knowledge of the metabolic fate is important. After human oral exposure to 1 g $^{14}C$ radiolabeled TBZ, 87% of the radioactive dose was recovered in urine [12]. Of the recovered dose, 38% of the metabolites was identified as conjugates of 5-hydroxythiabendazole (5-OH-TBZ) and less than 1% was found as TBZ or unconjugated 5-OH-TBZ. Thus, to measure the total 5-OH-TBZ in urine samples enzymatic hydrolysis prior to measurement is suggested.

A biomarker of exposure should be selective and validated [13]. Thus, reliable analytical methods are needed. Methods for the analysis of TBZ and 5-OH-TBZ in serum, using liquid chromatography (LC) with fluorescence detection [14], and in urine, using LC-UV have been reported [15]. However, the limit of detection, and selectivity in these methods are not sufficent for the use in studies of environmental exposure. No mass spectrometry based methods have been presented previously.

The aim of this study was to develop an analytical method for the quantification of total 5-OH-TBZ as a biomarker of exposure to TBZ in human urine using LC/MS/MS. The method was applied in one oral and one dermal human experimental exposure pilot study in two individuals to validate 5-OH-TBZ as biomarker of exposure and to estimate some basic toxicokinetic data for TBZ. The method was also applied in samples from a Swedish general population.

## 2. Materials and method

### 2.1. Chemicals and materials

The standards 5-OH-TBZ (10 ng/μL in methanol) and TBZ were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Acetone (analytical grade ACS Reag Ph Eur) and acetic acid (glacial) were from Fisher Scientific (Loughborough, UK). Methanol and acetonitrile (hyper grade for LC-MS), ammoniumacetate (EMSURE ACS, Reag. Ph Eur), and ammonia (25%) ($NH_3$) were from Merck (Darmstadt, Germany) and the enzyme β-glucuronidase/arylsulfatase from *Helix pomatia* and β-glucuronidase from *E. coli* from Roche Diagnostics Scandinavia AB (Bromma, Sweden).

Formic acid (FA) was from Sigma–Aldrich Inc. (St. Louis, MO, USA). The IS [$^{13}C_2$, $^{15}N$] 5-OH-TBZ was purchased from Toronto Research Chemicals (North York, ON, Canada). Purified water from a Millie-Q Integral 5 system (Millipore, Billerica, MA, USA) was used.

Polypropylene (PP) Riplate® Squarwell (SW) 2 mL 96-well-plates from Ritter (Schwabmünchen, Germany) were used and sealed during long term storage in −20 °C with an airtight sealing mat, 96 square well from Kinesis (Cambridgeshire, UK) or, for HPLC analysis, sealed with hard plastic ISOLUTE® pierceable sealing capmat for leak proof closure. The different cap mats are needed to prevent $NH_3$ to evaporate. Solid phase extraction (SPE) column plate, ISOLUTE®-96 ENV+ 50 mg fixed well plate, was from Biotage (Uppsala, Sweden).

### 2.2. Instrumentation

Quantitative analysis was conducted using a triple quadrupole linear ion trap mass spectrometer, equipped with TurboIonSpray source (QTRAP 5500; AB Sciex, Foster City, CA, USA) coupled to a liquid chromatography system with two pumps (UFLC$^{RX}$, Shimadzu Corporation, Kyoto, Japan). Pure nitrogen was used as curtain gas and collision gas. Air was used as nebuliser and auxiliary gas. The temperature of the auxiliary gas was set at 650 °C and the ion spray voltage at 5500 V. The MS/MS analyses were carried out using selected reaction monitoring (SRM) in positive ionisation mode. To establish the appropriate SRM conditions, standard solutions were infused into the MS/MS for optimisation. Collision-induced dissociation (CID) of each $[M+H]^+$ was performed and the product ions giving the best signal to noise ratio were selected for the SRM analysis. All data acquisition and processing was performed using the Analyst 1.6.1 application software (Applied Biosystems, Foster City, CA, USA).

The solutions used for SPE extraction were added with a 96-multichannel pipette (Liquidator 96, Rainin pipetting 360°) from Rainin Instruments LLC, Mettler-Toledo International Inc., Greifensee, Switzerland. To accelerate the flow of the liquid through the SPE columns, positive pressure processing of the SPE-plates using nitrogen together with a multichannel pressure processor (CEREX 96 II multi-channel SPE) SPEware Corporation, Baldwin Park, CA, USA was used.

### 2.3. Preparation of calibration standards and quality control samples

The 5-OH-TBZ standard was purchased dissolved. Accurately weighed amount of IS [$^{13}C_2$, $^{15}N$] 5-OH-TBZ was dissolved in methanol. The IS and standard stock solutions were diluted further in methanol and stored at −20 °C. Standard solutions were prepared in duplicates. For the calibration curve, 475 μL blank urine was spiked with 25 μL of the standard solutions and 25 μL of the IS solution, giving a urinary concentration between 0.05 and 100 ng 5-OH-TBZ/mL and 5 ng IS-5-OH-TBZ/mL urine. The calibration curve was corrected with the amount found in the chemical blank prepared from Millie-Q water and thereafter treated like the other samples. Urine blank samples and quality control (QC) samples were obtained from healthy volunteers at our laboratory. Blank urine samples were used for preparation of calibration curves and zero samples. As QC-samples, two authentic urine samples were pooled and then quantified to urinary concentrations 0.1, 1.0, 8.0 and 15 ng 5-OH-TBZ/mL urine. The QC-samples were divided into aliquots before stored at −20 °C.

### 2.4. Sample preparation

The urine samples and QC-samples were vortex-mixed after thawing and aliquots of 500 μL were transferred into a 96-well-plate and then 25 μL of IS solution, 150 μL 1 M ammonium acetate buffer pH 6.5 and 10 μL β-glucuronidase/arylsulphatase obtained from *H. pomatia* was added. The plate was sealed and mixed thoroughly for about 1 min before incubation. The enzyme incubation was performed at 37 °C with agitation at 400 rpm for about 18 h, i.e. overnight. After incubation, the samples were carefully mixed and transferred to a conditioned 96-SPE-plate using the Liquidator. The SPE-plate was conditioned in two steps with 1 mL of methanol and 1 mL of water. After the samples were applied, they were washed in three steps with 1 mL of water, 1 mL 40% methanol with 1% acetic acid and 1 mL acetonitrile. To elute 5-OH-TBZ and TBZ, 1 mL acetonitrile containing 5% $NH_3$ was manually added. The samples were eluted into a freeze-cooled 96-well-plate. Thus, the evaporation was minimised. The samples were gently mixed for 30 s and centrifuged for 10 min at 3000 × *g* immediately before analysis. The plate was stored in −20 °C if not analysed directly. If a sample concentration was above the linear range, a volume of 0.5 mL urine was diluted with Millie-Q water until an appropriate level was reached.

### 2.5. Analysis

The separation of the analytes was carried out, using a Poroshell 120EC-C18 column (4.6 × 233 mm, 2.7 μm, Agilent Technologies, Santa Clara, CA, USA). The two mobile phases used consisted of 0.1% (v/v) formic acid in water (mobile phase A) and 0.1% (v/v)

**Table 1**
Summary of the SRM transitions for 5-OH-TBZ and internal standard used in the LC/MS/MS analysis.

| Compound | Transitions (Da) | Collision energy (V) |
|---|---|---|
| *Quantifier ions* | | |
| 5-OH-TBZ | 218.0 → 191.2 | 36 |
| [$^{13}C_2$][$^{15}$N] 5-OH-TBZ | 221.0 → 194.0 | 35 |
| *Qualifier ions* | | |
| 5-OH-TBZ | 218.0 → 147.0 | 45 |
| [$^{13}C_2$][$^{15}$N] 5-OH-TBZ | 221.0 → 150.0 | 45 |

formic acid in methanol (mobile phase B). An aliquot of 3.0 μL of the sample was injected on the column. The separation started with isocratic elution with 30% of mobile phase B for 1.0 min followed by a linear gradient of mobile phase B to 84% in 2.5 min, during which the analytes eluted. The column was washed with 95% mobile phase B during 1.0 min and then equilibrated with 30% mobile phase B during 1.5 min. Post-column, the effluent was diverted into the MS between 1.0 and 4.0 min. The total analytical run time per sample, including equilibration time, was 6.5 min. The flow rate was 0.7 mL/min and the column was maintained at 40 °C. The LC/MS/MS analysis was performed using SRM transitions. The quantifier- and qualifier-ions for 5-OH-TBZ and the IS are tabulated in Table 1. All samples were prepared in duplicates and analysed by single injections. Concentrations were determined by peak area ratios between analyte and IS. All values were corrected for the chemical blank.

### 2.6. Test of deconjugation by enzyme hydrolysis

The enzymes β-glucuronidase/arylsulfatase and β-glucuronidase were tested regarding hydrolysis of the 5-OH-TBZ conjugates. The described analytical method was used to analyse twenty authentic samples, ten from each volunteer participating in the oral and dermal exposure studies described below, either with 10 μL β-glucuronidase/arylsulfatase or with 20 μL of the β-glucuronidase enzyme. The concentration of 5-OH-TBZ was determined for each sample. The ratios were then calculated.

To determine an appropriate time for enzyme incubation, a pooled authentic urine sample was used: from each volunteer and study one sample was pooled. Aliquots from this sample were prepared in duplicates for nine time points between 0 and 48 h. The incubation starting point was scheduled for each aliquot so that enzyme incubation was completed at the same time, for all aliquots, and SPE sample preparation was performed for the aliquots together. The zero-sample aliquot was added with enzyme just before it was applied to the SPE-column. Then, sample preparation and analysis were performed according to the method. The standards and QC-samples were incubated for 18 h.

### 2.7. Validation of the analytical method

To assess limit of detection (LOD) and limit of quantification (LOQ) in urine matrix, seven blank authentic urine samples were used. LOD and LOQ were determined as the mean level of the peak area concentration at the same retention time as 5-OH-TBZ, plus three and ten times the standard deviation, respectively [16].

The linear range of the calibration curve was determined from ten concentration levels 0.1, 0.3, 0.5, 1.3, 2.5, 5.0, 10, 20, 50 and 100 ng 5-OH-TBZ/mL urine. The equation of the curve was calculated by linear regression and the correlation coefficient ($r$) was used as a measure of the fit of the curve.

The precision of the method was determined using three different approaches, within-run, between-run and between-batch precision. The precision was calculated as the coefficient of variation (CV) of repeated measurements. The within-run precision was obtained from spiked urine at three concentration levels 0.1, 5.0 and 50 ng 5-OH-TBZ/mL urine. Each level was spiked in ten consecutive samples and prepared in one sample batch during one day. The mean concentrations at the three concentration levels were calculated.

The between-run precision was determined by including duplicates of the four QC-samples, containing 0.1, 1.0, 8.0 and 15 ng 5-OH-TBZ/mL urine, in seven analytical sample batches. The QC-samples are authentic urine samples. The batches were prepared and analysed on separate days during a period of four months. Thus, the standard deviation was calculated from fourteen individual results.

A between-batch precision of the method was determined by analysis of 110 urine samples collected in the dermal and oral exposure studies (see below). Each urine sample was divided into two aliquots which were then subjected to the entire analytical procedure. The samples were prepared and analysed in separate analytical batches and on different days. The CV was calculated from the differences between the duplicate samples as previously described [17].

The signal of the IS was used to determine the matrix effect. The ratio between the mean peak area of the IS in the chemical blanks ($n$ = 6) and the mean peak area of the IS in authentic urine samples ($n$ = 80) in an analytical batch was calculated. The geometric mean of the matrix ratio was calculated for two analytical batches.

The recovery of 5-OH-TBZ after SPE extraction was determined by comparing obtained concentrations in two sets of blank urine samples, spiked to a final concentration of 20 ng 5-OH-TBZ/mL. One set of samples was spiked before applied to the SPE columns. The other set of samples were also applied to the SPE columns but spiked after they had eluted. Addition of IS was made to all samples after they were eluted from the SPE columns. The urine samples spiked after eluting from the SPE columns were set as 100%. The recovery was calculated for three spiked blank urine samples.

The stability of the 5-OH-TBZ standard solutions in methanol was investigated by reanalysis after storage at −20 °C for 12 months and the authentic samples after storage at −20 °C for 5 months.

### 2.8. Ethical approval

These studies and the investigation of environmental exposure to TBZ in the general population in Sweden were ethically approved by The Regional Ethical Review Board in Lund, Lund University, Sweden (Dnr463/2005; Dnr2010/41; Dnr2010/465 and Dnr2013/6).

### 2.9. Human exposure studies

Two healthy volunteers, one female (age 67; weight 57 kg) and one male (age 42; weight 75 kg) had given their written informed consent to participate in the experimental studies. They minimised the intake of conventionally grown food a few days before as well as during the study.

#### 2.9.1. Oral exposure study

The two healthy volunteers received one single oral dose corresponding to 50% of the accepted daily intake (ADI) of TBZ. The ADI for TBZ suggested by the European Union is 0.1 mg/kg/day [18]. The TBZ was dissolved in acetone to a concentration of 2.5 mg/mL and was then added to 250 mL organic orange juice. The final amounts of TBZ in the orange juice were 2.85 mg and 3.75 mg for the female and male, respectively. The first urine sample was collected immediately prior to the exposure. After exposure urine was voided in 1 h intervals for the first 8 h. Then, all urine voided, ad libitum for 4 days was collected.

*2.9.2. Dermal exposure study*

The dermal study was performed twelve weeks before the oral study. TBZ was administrated topically on an area of 75 $cm^2$ of the inner forearm of the two volunteers. The dose of TBZ corresponded to 30% and 25% of the ADI. An accurately weighed amount was dissolved in 6 mL of acetone and a volume of 600 μL containing 1.75 mg was administrated to the female and 640 μL containing 1.88 mg to the male volunteer. After administration, the vehicle evaporated to dryness and then the skin was occluded with aluminium foil. After 8 h of exposure, the remainder of the dose was wiped off with acetone, and the exposed area washed with soap and water. The first urine sample was collected immediately prior to the exposure. After exposure urine was voided in 2 h intervals for the first 12 h. Then, all urine voided, ad libitum for 6–7 days was collected.

*2.10. Sample collection and adjustments for urinary dilution*

The samples collected in the two studies were stored at −20 °C until analysis. Sample volume was determined and creatinine and density were determined in all samples to adjust for the urinary dilution. Creatinine was analysed with an enzymatic method [19] and density with a hand refractometer. The concentration adjusted for urinary density, Cd, was calculated according to $Cd = C(\text{observed}) \times (1.016 - 1)/(\rho\text{-}1)$, where $C$ (observed) is the obtained concentration in the urine sample, $\rho$ is the measured specific density and 1.016 was used as the average reference urine density [20].

*2.11. Calculation of half-lives*

The half-life ($t_{1/2}$) of the elimination in urine was estimated from the slope of the curve in the natural logarithm linear (ln-linear) concentration versus time plot, where time is given as the mid time points between two sample collection time points.

*2.12. Application of the analytical method in an environmental exposure study*

Spot urine samples ($n = 285$) were collected from a general population in Sweden and the analytical method presented above was applied to determine 5-OH-TBZ. The population consisted mainly of men and women, living and working in cities or the countryside in the south of Sweden.

## 3. Results and discussion

*3.1. Mass spectrometry and chromatography*

A method for the analysis of 5-OH-TBZ in human urine using SPE and LC/MS/MS was developed. A large number of samples can efficiently be analysed with the analytical method, due to the use of 96-well plates throughout the sample preparation and LC/MS/MS analysis. The chosen analytical column provided consistent and reproducible chromatography and retained 5-OH-TBZ with stable retention times and good separation (supplementary data Fig. S1). Both 5-OH-TBZ and the IS showed an excellent sensitivity in ESI and positive ion mode. The transition giving the best to noise ratio signal was chosen as the quantifier ion for 5-OH-TBZ. A second SRM was chosen as a qualifier ion to strengthen the identity of the analyte. The transitions and optimum collision energies are tabulated in Table 1.

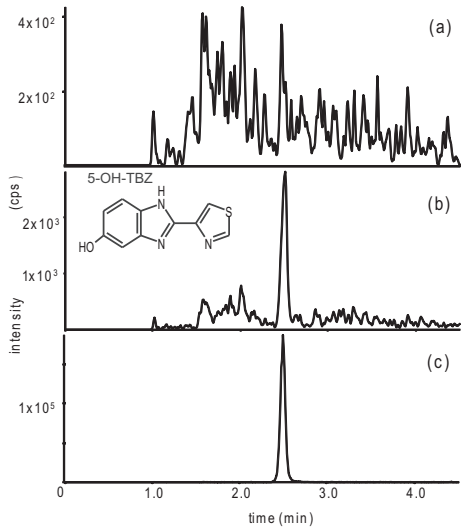


**Fig. 1.** LC/MS/MS SRM chromatogram showing (a) urinary blank, transition 218.0–191.2 Da (b) LOD in a urine sample spiked to a of 0.05 ng 5-OH-TBZ/mL urine, transition 218.0–191.2 Da. (c) Internal standard spiked to 5 ng/mL urine, transition 221.0–194.0 Da. Retention time of 5-OH-TBZ and IS is 5.2 min.

*3.2. Deconjugation by enzyme hydrolysis*

When the enzymes β-glucuronidase/arylsulfatase and β-glucuronidase were tested regarding hydrolysis of the 5-OH-TBZ conjugates, higher concentrations of 5-OH-TBZ were obtained when β-glucuronidase/arylsulfatase was used to hydrolyse the authentic urine samples. The concentrations increased about four times and almost two times in urine samples from the female and male volunteer, respectively (supplementary data Table S1). There seems to be differences between the two subjects regarding the level of conjugation; these results agree with earlier findings [12].

The optimal time for enzyme incubation was found to be 18 h (supplementary data Fig. S2).

*3.3. Validation of the analytical method*

The LOD was found to be 0.05 ng/mL and the LOQ 0.13 ng/mL. Chromatograms of a urine blank (a), a urine sample at LOD (b), and of the IS (c) are shown in Fig. 1. The LOD in urine is sufficiently low for the measurement of environmental human exposure.

The obtained calibration curves in the range 0.13–100 ng 5-OH-TBZ/mL urine showed excellent linearity with $r = 0.997$, a slope of $0.278 \pm 0.015$ (CV of 6.9%) and an intercept of $0.0395 \pm 0.046$ (CV of 14.7%) at 95% confidence level ($n = 6$).

The within-run, between-run and between-batch precisions are presented in Table 2.

The obtained concentrations at three concentration levels for spiked samples are shown in Table 2.

The assessment of the matrix effect was performed using [$^{13}C$, $^{15}N_2$] 5-OH-TBZ which co-elutes with 5-OH-TBZ [21]. The geometric mean of the ratio between the IS in the chemical blanks and the IS in authentic urine samples was 0.99. The minor matrix effect probably is explained by the use of SPE, the use of a [$^{13}C$, $^{15}N_2$] IS

**Table 2**
Precision of the method at different concentration levels. The within-run precision was calculated from spiked urine samples and the between-run and batch precision from authentic urine samples.

| | 5-OH-TBZ concentration (ng/mL) | *n* | Mean obtained 5-OH-TBZ concentration (ng/mL) | CV (%) |
|---|---|---|---|---|
| Within-run precision | 0.13 | 10 | 0.14 | 7.0 |
| | 5.0 | 10 | 5.0 | 5.0 |
| | 50 | 10 | 47 | 3.6 |
| Between-run precision | 0.1 | 14 | 0.2 | 7.8 |
| | 1.0 | 14 | 1.2 | 7.7 |
| | 8.0 | 14 | 7.9 | 7.5 |
| | 15 | 14 | 16 | 6.8 |
| Between batch precision | Range 0.1–64 | 110 | 8.2 | 8.6 |

**Table 3**
Estimated half-life of 5-OH-TBZ and conjugates in urine of the two volunteers orally exposed to TBZ. The dose was 0.05 mg TBZ/kg of b.w (50% of ADI) for both volunteers. For the calculation values after 101 h for the female and 90 h for the male were excluded due to levels below LOD.

| Oral route | [a,b] $t_{½}$ (h) creatinine | | [a,b] $t_{½}$ (h) density | | [a,b] $t_{½}$ (h) unadjusted | | [c] $C_{max}$ (nmol/mmol creatinine) | $T_{Cmax}$ (h) |
|---|---|---|---|---|---|---|---|---|
| | Phase 1 | Phase 2 | Phase 1 | Phase 2 | Phase 1 | Phase 2 | | |
| Female | 2 | 12 | 2 | 12 | 2 | 12 | 2385 | 1 |
| Male | 2 | 9 | 1 | 9 | 1 | 9 | 1729 | 1 |

[a] The half-life of elimination ($t_{½}$) in urine estimated from the slope of the curve in the natural logarithm-linear concentration versus mid time plot.
[b] Correlation coefficient ($r$) for the regression line of adjusted $t_{½}$ were 0.94–0.98 and 0.90–0.94 for unadjusted.
[c] $C_{max}$ urinary concentration creatinine adjusted values.

and the analytical column, giving a good separation of the analyte from the matrix.

The recovery in spiked urine determined after SPE elution was found to be 100.3% ($n = 3$).

The standard solutions of 5-OH-TBZ dissolved in methanol were found to be stable for 12 months and the authentic urine samples for at least 5 months when stored at −20 °C.

### 3.4. Oral exposure to TBZ

In urine collected before the exposure, the 5-OH-TBZ levels were below LOD in both volunteers. After the oral exposure to TBZ, the urinary levels of 5-OH-TBZ increased rapidly and the urinary $C_{max}$ was reached after 1 h, see Table 3 and Fig. 2a. The urinary elimination can be described by a two-compartment model and both the first rapid and the second slower elimination phase followed first-order kinetics; see the excretion curve in Fig. 3a. The half-lives of 5-OH-TBZ with correlation coefficients are shown in Table 3. The first rapid phase was found to be only 2 h. This short half-life of 5-OH-TBZ (Table 3) limits its use as a biomarker and when collecting samples for biomonitoring, the toxicokinetics should be considered [22].

In chromatograms from the oral study, two small peaks with the same transitions as 5-OH-TBZ eluting close to the 5-OH-TBZ peak was observed (supplementary data Fig. S1). Thus, samples were screened for other metabolites using a compound-specific, predicted SRM/Information-Dependent Acquisition method (supplementary data Figs. S3 and S4).

The main metabolite was 5-OH-TBZ with glucuronide and sulphate conjugation. Other minor metabolites were OH-TBZ isomers, di-hydroxy-TBZ and TBZ. The metabolic specificity was confirmed since the concentrations increased after exposure to TBZ and decreased as the metabolites were rapidly excreted in the urine (supplementary data Fig. S3).

The amount of TBZ recovered in the study samples, was quantified and found to be less than 0.05% with regard to the administrated dose in both volunteers. Hence, TBZ was not included in the validation of the method.

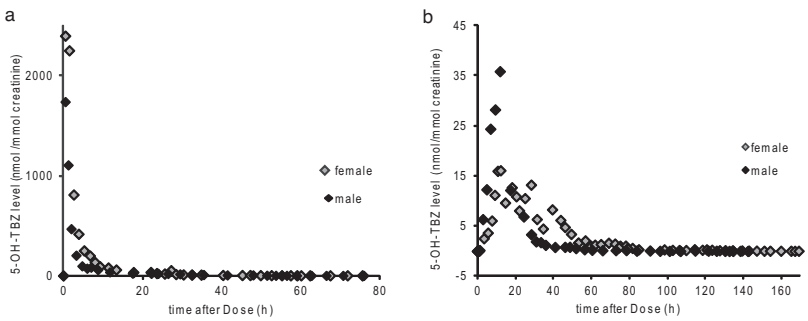


**Fig. 2.** The concentration versus time urinary excretion curves of 5-OH-TBZ for the female and male volunteer after oral (a) and dermal (b) exposure to TBZ. The creatinine adjusted urinary levels (nmol/mmol creatinine) were plotted versus the mid time points. (a) The oral dose corresponded to 50% of the accepted daily intake (ADI) mixed in 250 mL of organic orange juice. In both studies the first two urine samples were collected just before and 1 h after administration of the dose. (b) The volunteers were dermally exposed on the inner forearm for 8 h with a dose corresponding to 25% of the ADI (0.1 mg TBZ/kg b.w.).

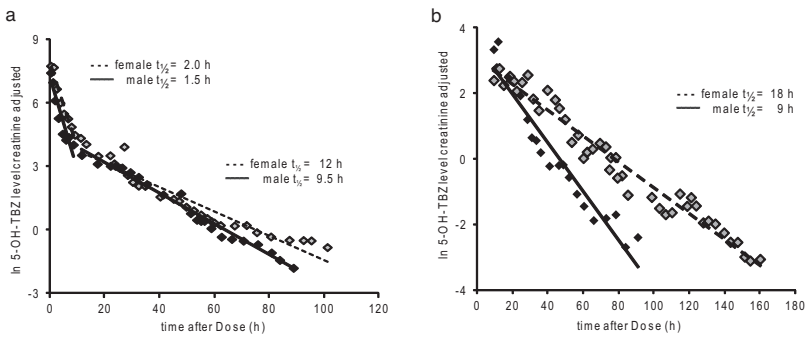


**Fig. 3.** Semi logarithmic urinary elimination curves of 5-OH-TBZ for the female and male volunteer after oral (a) and dermal (b) exposure to TBZ. The oral dose corresponded to 50% of the accepted daily intake (ADI) (0.1 mg TBZ/kg b.w.) and the dermal dose corresponded to 25% of the ADI. The half-life of elimination ($t_{½}$) in urine is estimated from the slope of the log-lin concentration versus time plot. The natural logarithm of the creatinine adjusted urinary levels (nmol/mmol creatinine) were plotted versus the mid time points.

In urine, the total recovery, measured as 5-OH-TBZ, was found to be 21–24% of the dose of TBZ. Of the recovered dose, 96% was found within 24 h. In an earlier study of oral exposure to $^{14}$C-TBZ in human volunteers, 87% of the radioactivity was found in urine, of which 38% was recovered as 5-OH-TBZ conjugates, after 5 days [12]. The difference in uptake of TBZ may be due to the difference in doses administrated (3 mg versus 1 g) or that the dosage form (solution versus suspension) to administrate the substance, differed. An oral study in mice has shown that the vehicle has great influence on the uptake of TBZ. When the mice were given $^{14}$C ring radiolabeled TBZ in olive oil, instead of aqueous solution, TBZ was absorbed 12 times faster and the plasma levels became 5 times higher [23]. The solubility of TBZ may be the dependent factor. Moreover, metabolites not found by the analytical method are likely to be formed; since the benzene ring in TBZ can undergo ring cleavage and, sulfoxidation has also been suggested [12].

### 3.5. Dermal exposure to TBZ

Before the experimental exposure, the 5-OH-TBZ levels in urine were below LOD in both volunteers. The limited solubility of TBZ in acetone and the limited volume possible to disperse evenly on the application site resulted in a lower dose than in the oral study above. After the dermal exposure of TBZ, the urinary levels increased slowly. The urinary $C_{max}$ was reached after 13 h in both volunteers compared to 1 h by the oral route (Table 4). After 9 h, the level of 5-OH-TBZ in the female urine reached a balance between absorption and elimination, followed by a plateau (Fig. 2b). No plateau was seen in the male volunteer. The urinary elimination of 5-OH-TBZ seemed to follow first-order kinetics and a one-compartment model (Fig. 3b). Also, in other studies of dermal exposure to pesticides in humans, a plateau in the urinary excretion curve has been observed [24–29]. After 13 h, the levels started to decrease in both volunteers, probably reflecting the end of exposure. If there is a first rapid elimination phase it was not observed, probably obscured by the absorption phase. The dermal half-life is within the same range as that of the second slower phase in the oral study (Tables 3 and 4).

Of the administrated dermal dose, the total recovery of 5-OH-TBZ in urine was 1% for both volunteers. The results indicate a low uptake of TBZ. Acetone was chosen as vehicle, because of its ability to dissolve TBZ and because it is commonly used in dermal experimental exposure studies. The choice of vehicle and use of occlusion may affect the absorption. The permeability of the skin can be increased by occlusion through increased hydration and temperature of the skin. Also the washing after exposure has been shown to enhance the dermal absorption [11]. The inner forearm was chosen as site of exposure as it is the most commonly used anatomic site in dermal experimental exposure studies [28,30]. However, it has been shown that the forehead and the neck are more permeable than the forearm. Thus, the absorption is probably higher, if other more permeable areas of the body are exposed [31]. This should be considered in studies of exposure and effect.

The toxicokinetic profiles were different between the oral and dermal administration routes. This may be because the uptake is both slower and lower in the dermal experiment. After dermal uptake, the substance enters the blood directly, i.e., the whole system, without passing the liver first, as it does when entering via the gastro intestinal tract. Moreover, the toxicokinetic profiles seemed to differ between the two individuals in the dermal study. This is not surprising, since it is well known that xenobiotic metabolism and excretion kinetics vary greatly depending on, i.e., age, sex, and activity level [11].

**Table 4**
Estimated half-life of 5-OH-TBZ and conjugates in urine for the two volunteers dermally exposed to TBZ for 8 h on an area of 75 cm$^2$. The dose was 0.030 and 0.025 mg TBZ/kg of b.w (ca 25% of ADI 0.1 mg/kg of b.w) for the female and male respectively. For the calculation values after 160 h for the female and 91 h for the male were excluded due to levels below LOD.

| Dermal route | [a,b] $t_{½}$ (h) creatinine | [a,b] $t_{½}$ (h) density | [a,b] $t_{½}$ (h) unadjusted | [c] $C_{max}$ (nmol/mmol creatinine) | $T_{Cmax}$ (h) |
|---|---|---|---|---|---|
| Female | 18 | 19 | 20 | 16 | 13 |
| Male | 9 | 9 | 10 | 17 | 13 |

[a] The half-life of elimination ($t_{½}$) in urine estimated from the slope of the curve in the natural logarithm–linear concentration versus mid time plot.
[b] Correlation coefficient ($r$) for the regression line of adjusted $t_{½}$ were 0.94–0.98 and 0.90–0.94 for unadjusted.
[c] $C_{max}$ urinary concentration creatinine adjusted values.

### 3.6. Adjustments for urinary dilution

The toxicokinetic data in this study indicate that an adjustment for the urinary dilution should be recommended and that adjustment for creatinine rather than for density should be applied. However, creatinine levels are affected by several factors (such as gender, age, muscle mass, and meat consumption) and when comparing individuals, groups or populations of both sexes and various ages, with large differences in muscle mass and meat intake, density adjustment may be the best choice.

### 3.7. Application of the analytical method in an environmental exposure study

Sample concentrations in the population samples ranged from <LOD to 33 ng 5-OH-TBZ/mL urine and 52% were above the LOD. The median was 0.05 ng/mL and the 90th percentile was 1.6 ng/mL.

The between-run precisions determined from QC-samples were CV = 4.9% at 1 ng/mL ($n = 21$) CV = 3.9% at 5 ng/mL ($n = 21$) and CV = 2.7% at 10 ng/mL ($n = 21$). The samples were prepared and analysed during 4 months. Thus, the method is applicable for running large sample batches. More detailed data from this study will be published elsewhere.

## 4. Conclusions

The metabolite 5-OH-TBZ was used as a biomarker of exposure to TBZ and a LC/MS/MS method for the quantification of 5-OH-TBZ in human urine was developed. The sample through put is high as all sample preparation is performed in 96-well plates.

The biomarker is conjugated to glucuronide and sulphates in urine. Thus enzyme hydrolysis using glucuronidase/arylsulfatase is suggested.

The method has excellent precision and a low LOD which enables detection of 5-OH-TBZ in environmentally exposed populations. When applying the method in a general Swedish population, 52% had concentration levels above LOD.

The method was applied in one oral and one dermal human experimental exposure study in two individuals. After oral exposure to TBZ, 21–24% was recovered as 5-OH-TBZ in urine, which was excreted in two phases with a rapid elimination half-life of 2 h and a slower of 9–12 h. Moreover, 96%, of the recovered dose was found within 24 h. For a correct exposure assessment of TBZ, the fast elimination requires consideration of the time of sampling.

After dermal exposure to TBZ, 1% was recovered as 5-OH-TBZ in urine with an elimination half-life of 9–18 h. Although these studies are limited to two individuals, the data provide new information regarding the toxicokinetics of TBZ after oral and dermal exposure and will make the interpretation of the biomarker levels and, thus, risk assessment for TBZ easier.

## Acknowledgements

This work was supported by the Swedish Environmental Protection, Agency; Swedish Research Council for Environment, Agricultural Sciences, and Spatial Planning; The Swedish Agency for International Development Cooperation; The Department for Research Cooperation; Swedish council for working life and social research; Skåne Regional Council; and the Medical Faculty at Lund University, Sweden.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2014.10.003.

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**Paper II Supplementary data**

# Determination of 5-hydroxythiabendazole in human urine as a biomarker of exposure to thiabendazole using LC/MS/MS

## Tables

**Table S1**

Increase in 5-OH-TBZ levels after incubation with β–glucuronidase/arylsulfatase from *Helix pomatia* and β–glucuronidase from *E.Coli* in twenty urine samples. Five samples collected in the dermal study and five samples collected in the oral study from the two subjects. The increase of using β–glucuronidase/arylsulfatase compared to β–glucuronidase is calculated.

| Sample | Study | volunteer | Incubation with enzyme β concentration (ng/mL) | Incubation with enzyme β–A concentration (ng/mL) | ratio between β–glucuronidase/arylsulfatase and β–glucuronidase |
|---|---|---|---|---|---|
| 1 | Oral | male | 31.4 | 45.0 | 1.4 |
| 2 | | | 39.9 | 54.6 | 1.4 |
| 3 | | | 8.59 | 12.1 | 1.4 |
| 4 | | | 1.35 | 1.90 | 1.4 |
| 5 | | | 0.55 | 0.79 | 1.4 |
| 6 | Dermal | male | 5.76 | 8.03 | 1.4 |
| 7 | | | 9.15 | 13.7 | 1.5 |
| 8 | | | 2.47 | 3.45 | 1.4 |
| 9 | | | 0.49 | 0.63 | 1.3 |
| 10 | | | 0.19 | 0.26 | 1.4 |
| 11 | Oral | female | 36.7 | 129 | 3.5 |
| 12 | | | 18.9 | 58.9 | 3.1 |
| 13 | | | 9.78 | 28.0 | 2.9 |
| 14 | | | 7.39 | 18.9 | 2.6 |
| 15 | | | 1.72 | 5.52 | 3.2 |
| 16 | Dermal | female | 0.34 | 1.74 | 5.1 |
| 17 | | | 1.83 | 8.77 | 4.8 |
| 18 | | | 1.03 | 5.18 | 5.0 |
| 19 | | | 1.97 | 8.44 | 4.3 |
| 20 | | | 0.72 | 3.40 | 4.7 |

**Table S2**

Summary of the SRM transitions for TBZ and internal standard used in the LC/MS/MS analysis.

| Compound | Transitions (Da) | Collision Energy (V) |
|---|---|---|
| | *Quantifier ions* | |
| TBZ | 202.0 ⟶175.0 | 35 |
| [$^2H_6$] TBZ | 208.0 ⟶180.1 | 37 |
| | *Qualifier ions* | |
| TBZ | 202.0 ⟶131.0 | 43 |
| [$^2H_6$] TBZ | 208.0 ⟶136.0 | 45 |

**Paper II Supplementary data**

**Determination of 5-hydroxythiabendazole in human urine as a biomarker of exposure to thiabendazole using LC/MS/MS**

## Figure Captions

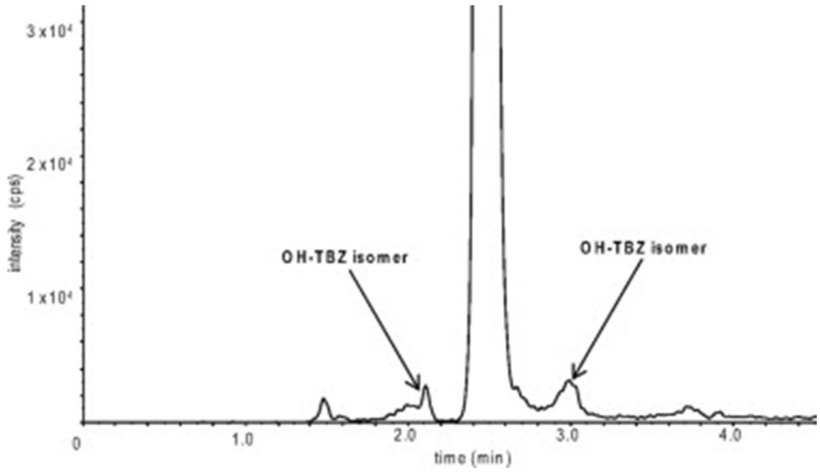


**Fig. S1** LC/MS/MS SRM chromatogram; of a urine sample taken 5.5 h after oral exposure, for the 5-OH-TBZ quantifier transition 218.0-191.2 Da. Three consecutive separate peaks were observed and the largest peak in the middle was identified as 5-OH-TBZ, with a reference standard. Of the remaining two peaks, one could be 4-OH-TBZ, which has been identified as a metabolite in rodents using 1H nuclear magnetic resonance (NMR) spectrum (T. Fujitani, M. Yoneyama, A. Ogata, T. Ueta, K. Mori, H. Ichikawa, Food and Chemical Toxicology, 29 (1991) 265-274.).

**Paper II Supplementary data**

**Determination of 5-hydroxythiabendazole in human urine as a biomarker of exposure to thiabendazole using LC/MS/MS**

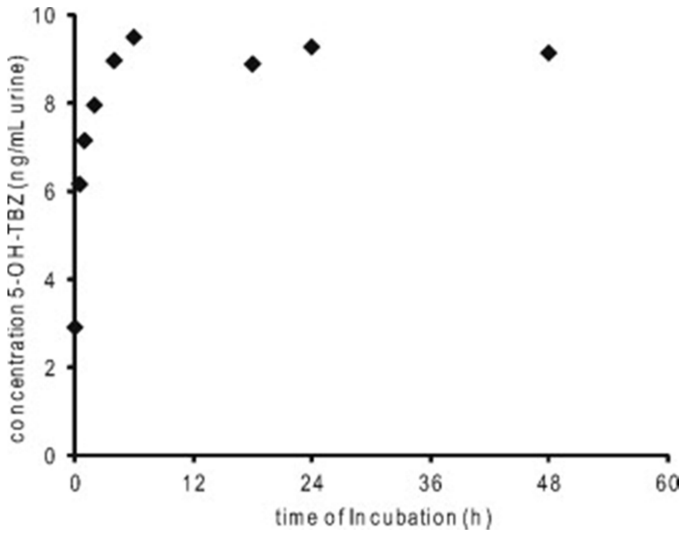


**Fig. S2** Change in concentration versus time of enzyme incubation at 37°C, for cleavage of glucuronide and sulphate conjugates, in a pooled authentic urine sample containing 5-OH-TBZ. The sample contains a total of four samples, one sample from each of the two volunteers from both the dermal and the oral study.

**Paper II Supplementary data**

**Determination of 5-hydroxythiabendazole in human urine as a biomarker of exposure to thiabendazole using LC/MS/MS**

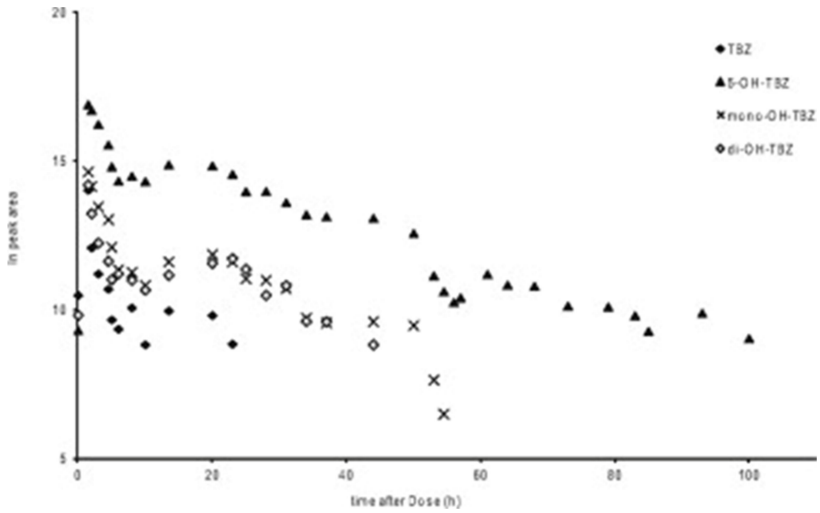


**Fig. S3** Semi logarithmic urinary elimination curves of 5-OH-TBZ, TBZ, mono-OH-TBZ and di-OH-TBZ for the male volunteer after oral exposure to TBZ. The oral dose corresponded to 50 % of accepted daily intake (0.1 mg TBZ /kg b.w.). The natural logarithm of the peak area versus mid time was plotted. For both volunteers the peak area of the selected biomarker of exposure 5-OH-TBZ, increased after exposure to TBZ and decreased as the metabolite was rapidly excreted. The other metabolites peak areas were minor compared to that of 5-OH-TBZ.

Method:

In the SRM LC/MS/MS method, used to analyse the crude urinary samples from the oral study, the SRM transitions and the collision energies, for selected metabolites, were obtained by screening one of the urine samples from the oral study. The sample was screened for metabolites using the QTRAP® 5500 System and the metabolite biotransformation data acquisition and processing was performed using the LightSight® Software 2.2.1 application software (AB Sciex, Foster City, CA, USA). This software creates compound-specific, predicted SRM information-dependent acquisition methods. The main metabolites suggested by the LightSight ®software, were oxidation of TBZ with glucuronide and sulphate conjugation.

The separation was carried out, using a Poroshell 120EC-C18 column (4.6 x 233 mm, 2.7 µm, Agilent Technologies, Santa Clara, CA, USA).The two mobile phases used consisted of 0.1 % (v/v) formic acid in water (mobile phase A) and 0.1% (v/v) formic acid in methanol (mobile phase B). An aliquot of 3.0 µL of the sample was injected on the column. The separation started with isocratic elution with 20 % of mobile phase B for 1.0

min followed by a linear gradient of mobile phase B to 95 % in 7.0 min, during which the analytes eluted. The column was washed with 95 % mobile phase B during 1.0 min and then equilibrated with 20 % mobile phase B during 2.0 min. Post-column, the effluent was diverted into the MS between 2.0 and 9.0 min.

The flow rate was 0.5 mL/min and the column was maintained at 40°C. The LC/MS/MS analysis was performed using the following SRM transitions; TBZ, transition 202.0-175.0 Da; 5-OH-TBZ and mono-OH-TBZ (its isomers), transition 218.0-191.2 Da; di-OH-TBZ, transition 234.0-188.0 Da for.

Both TBZ and its internal standard have excellent sensitivity in ESI and positive ion mode. The transitions giving the best signal to noise ratio was chosen as the quantifier ion for TBZ. A second SRM was chosen as a qualifier ion to strengthen the identity of the analyte. The transitions and optimum collision energies are tabulated in Table S2. TBZ and the internal standard (IS) [$^2H_6$]-TBZ (100 ng/µL in acetone) was purchased from Dr. Ehrenstorfer (Augsburg, Germany).

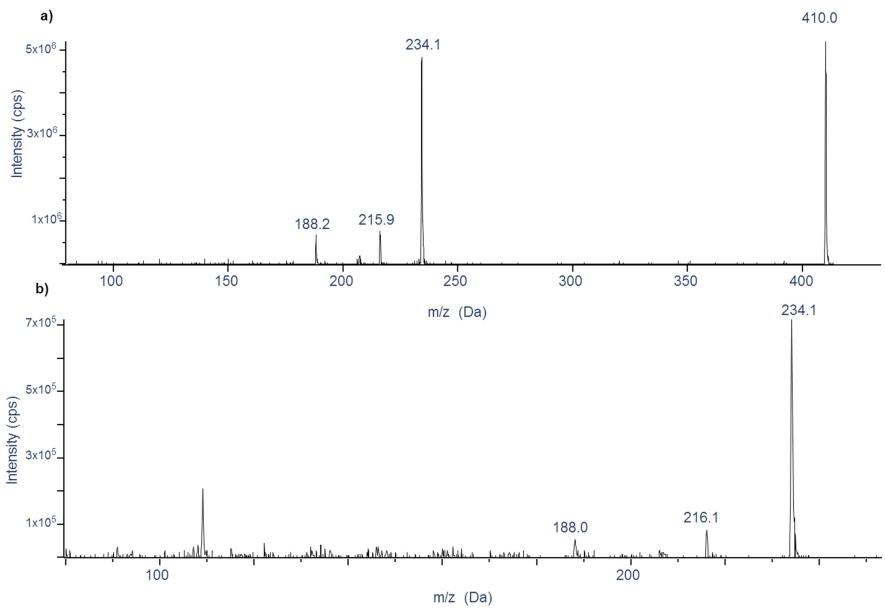


**Fig. S4 a**) product ion MS/MS spectra in positive mode of the di-OH-TBZ- glucuronide conjugate 410 m/z Da and **b**) product ion MS/MS spectra in positive mode of the di-OH-TBZ 234 m/z Da. The peaks were obtained from the same crude urine sample from the oral study.

# Paper III

# Determination of hydroxypyrimethanil in urine, using LC/MS/MS, after oral and dermal exposure to pyrimethanil

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## ABSTRACT

Pyrimethanil (PYR) is a fungicide used pre- and post-harvest on many crops. It has a low acute toxicity but is of toxicological concern because of its endocrine-disrupting properties. Exposure occurs to workers in agri- and horticulture and also to the general population, primarily via diet.

To study the exposure to PYR in workers or general populations, a reliable analytical method as well as an exposure biomarker are required, both of which are currently lacking. The metabolite hydroxypyrimethanil (OH-PYR) was chosen as biomarker of exposure to PYR and an LC/MS/MS method for the quantification in human urine was developed. The LC/MS/MS method showed excellent precision with a coefficient of variation between 6 and 12 % and a low limit of detection (LOD) of 0.1 ng/mL urine, which enables detection in both occupationally and environmentally exposed populations.

The method was also applied in one oral and one dermal human experimental exposure study in two individuals. After the oral exposure, 80% of the dose was recovered as OH-PYR in urine excreted in two elimination phases with half-lives of 4 and 15 h. After the dermal exposure, 21 % of the dose was recovered as OH-PYR and excreted in two elimination phases, with half-lives of 7 and 24 h. The method was also applied in a general south Swedish population (n=413). In 48 % of the samples, the levels of OH-PYR was above LOD and the $95^{th}$ percentile was 3.7 ng OH-PYR/mL.

**Keywords:** Biomarker, Dermal, LC/MS/MS, Oral, Pesticide, Pyrimethanil

**Abbreviations:** ADI, acceptable daily intake; CID, collision induced dissociation; ESI, electro spray ionisation; IS, internal standard; LC/MS/MS, liquid chromatography triple quadrupole mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; OH-PYR, hydroxypyrimethanil; PYR, pyrimethanil; QC, quality control; SPE, solid phase extraction; SRM, selected reaction monitoring.

# 1 Introduction

Pyrimethanil (PYR), 4,6-Dimethyl-N-phenyl-2-pyrimidinamine, is a fungicide used in the cultivation as well as post-harvest on fruits and vegetables, to control pathogens, that cause blue, grey and green mould. PYR was first introduced in the 1990's, and has a mode of action that is different from many other fungicides [1]. It controls the sporulation in the pathogens by inhibiting the methionine biosynthesis and the secretion of cell wall degrading enzymes.

PYR is considered to have a low acute toxicity in humans but adverse effects have been reported in animal studies. These effects are primarily seen on the main target organs of PYR, the liver and the thyroid [2, 3]. PYR is an agonist to the aryl hydrocarbon receptor [4]. It is of particular toxicological concern as it has been found (in vitro) to be an endocrine disruptor and is suspected to disrupt the thyroid-pituitary homeostasis [5]. The metabolism of PYR has been studied in animals, but not yet in human. In animals, the major metabolite has been identified as 4-hydroxypyrimethanil (OH-PYR). In animals PYR is metabolised through aromatic oxidation with a further conjugation to glucuronic acid and sulphate [3].

In the risk assessment of pesticides, all routes of exposure namely - oral, dermal and inhalatory – should be considered important [6]. But, despite this, studies on uptake in humans of PYR through these different routes are lacking, a shortcoming in the view of safety control among agricultural workers. Further, there is a need for epidemiological studies of exposure-response relationship. Compared to other methods of exposure assessment, biomarkers of exposure are advantageous, as they give an estimate of the internal exposure, where all routes of exposure are taken into account. However, the biomarker needs to be validated for its intended use [7]. There is no established exposure biomarker for PYR in humans. Work in agriculture involves a risk of exposure to pesticides like PYR. The general population may also be exposed, mainly via diet.

The aim of this study was to develop a method for exposure assessment of PYR by quantification of total OH-PYR in human urine using LC/MS/MS. To validate the biomarker, experimental exposures of two human volunteers by oral and dermal administration were performed. The method was also applied in samples from a Swedish population.

# 2 Methods and Materials

## *2.1 Chemicals and materials*

The standard PYR was purchased from Dr. Ehrenstorfer (Augsburg, Germany). Acetone (analytical grade ACS Reag Ph Eur) and acetic acid (glacial) were from Fisher Scientific (Loughborough, UK). Methanol and acetonitrile (hyper grade for LC-MS), ammonium acetate (EMSURE ACS, Reag. Ph Eur) and ammonia (25%) ($NH_3$) were from Merck (Darmstadt, Germany) and the enzyme β–glucuronidase/arylsulfatase from *Helix pomatia* was purchased from Roche Diagnostics Scandinavia AB (Bromma, Sweden).
Formic acid (FA) was from Sigma-Aldrich Inc. (St. Louis, MO, USA). The standard OH-PYR (2-(4-hydroxyanilino)-4,6-dimethylpyrimidine) and the internal standard (IS) [2H4] OH-PYR were both purchased from Toronto Research Chemicals (North York, ON, Canada). Purified water from a Millie-Q Integral 5 system (Millipore, Billerica, MA, USA) was used.

Polypropylene (PP) Riplate® Squarwell(SW) 2 mL 96-well-plates from Ritter (Schwab-münchen, Germany) were used and sealed during long term storage in -20°C with an airtight

sealing mat, 96 square well from Kinesis (Cambridgeshire, UK) or, for HPLC analysis, sealed with hard plastic ISOLUTE® pierceable sealing capmat. The solid phase extraction (SPE) column plate was an ISOLUTE®-96 ENV+ (50 mg) fixed well plate from Biotage (Uppsala, Sweden). The solutions used for the SPE extraction were added with a 96-multichannel pipette (Liquidator 96, Rainin pipetting 360° from Rainin Instruments LLC, Mettler-Toledo International Inc., Greifensee, Switzerland). To accelerate the flow of the liquid through the SPE columns, positive pressure processing of the SPE-plates using nitrogen together with a multichannel pressure processor (CEREX 96 II multi-channel SPE, SPEware Corporation, Baldwin Park, CA, USA) was applied. For the tape-stripping, a medical tape (Fixomull® self-adhesive gauze, BSN medical GmbH, Hamburg, Germany) was used.

*2.2 Instrumentation*

The quantitative analysis was conducted using a triple quadrupole linear ion trap mass spectrometer, equipped with TurboIonSpray source (QTRAP 5500; AB Sciex, Foster City, CA, USA) coupled to a liquid chromatography system with two pumps (UFLC$^{RX}$, Shimadzu Corporation, Kyoto, Japan). Pure nitrogen was used as curtain gas and collision gas. Air was used as nebulizer and auxiliary gas. The temperature of the auxiliary gas was set at 650°C and the ion spray voltage at 5500 V. The MS/MS analyses were carried out using selected reaction monitoring (SRM) in a positive ionisation mode. To establish the appropriate SRM conditions, the standard solutions were infused into the MS/MS for optimisation. The collision-induced dissociation (CID) of each $[M+H]^+$ was performed and the product ions giving the best signal to noise ratio, were selected for the SRM analysis. All the data acquisition and the processing were performed using the Analyst 1.6.1 software (Applied Biosystems, Foster City, CA, USA).

*2.3 Preparation of calibration standards and quality control samples*

The OH-PYR and IS [$^2H_4$] OH-PYR standards were accurately weighed and dissolved in methanol. The standard and IS stock solutions were diluted further in methanol and stored at -20°C. The standard solutions were prepared in duplicates. For the calibration curve, 475 µL authentic blank urine was spiked with 25 µL of the standard solutions and 25 µL of the IS solution, giving a urinary concentration between 0.1 and 25 ng OH-PYR/mL and 5 ng IS [$^2H_4$] OH-PYR /mL urine. The authentic blank urine samples and the quality control (QC) samples were obtained from healthy volunteers at our laboratory. The QC-samples were also prepared from authentic blank urine spiked to concentrations of 1.0, 5.0 and 10 ng OH-PYR/mL urine. The QC- samples were divided into aliquots before storage at -20°C.

*2.4 Sample preparation*

The urine samples and the QC-samples were vortex-mixed after thawing and aliquots of 500 µL were transferred into a 96-well-plate. Then 25 µL of IS solution, 150 µL 1 M ammonium acetate buffer with pH 6.5 and 10 µL of the enzyme β–glucuronidase/arylsulphatase were added. The plate was sealed and mixed thoroughly for about 1 minute before incubation. The enzyme incubation was performed at 37ºC with agitation at 400 rpm for about 18 h, i.e. overnight. A 96 well SPE-plate was conditioned in two steps with 1 mL of methanol and 1 mL of water. Thereafter, the samples were mixed and transferred to the plate, using the multichannel pipette. The plates were then washed in three steps with 1 mL of water, 1 mL 40% methanol acidified with 1% acetic acid and 1 mL acetonitrile. To elute OH-PYR and

PYR, 1 mL acetonitrile, containing 5% $NH_3$, was manually added. To minimize evaporation, the samples were eluted into a freeze-cooled 96-well-plate. Before analysis, the samples were gently mixed for 30 s and centrifuged for 10 min at 3000 rpm (1500g). The plate was stored at -20°C, if not analysed directly. If a sample concentration was above the linear range of the method, the sample was diluted with Millie-Q water until the concentration fell within the calibration curve.

*2.5 LC/MS/MS analysis*

The separation of the analytes was carried out, using a Poroshell 120EC-C18 column (4.6 x 100 mm, 2.7 µm, Agilent Technologies, Santa Clara, CA, USA). The two mobile phases used consisted of 0.1% (v/v) formic acid in water (mobile phase A) and 0.1% (v/v) formic acid in methanol (mobile phase B). An aliquot of 3.0 µL of the sample was injected on the column. The separation started with isocratic elution with 30 % of mobile phase B for 1.0 min followed by a linear gradient of mobile phase B to 84% in 2.5 min. Thereafter, the column was washed with 95% mobile phase B during 1.0 min and then equilibrated with 30% mobile phase B during 2 min. The effluent was diverted into the MS between 2.0 and 6.0 min. The total analytical run time per sample was 7.0 min. The flow rate was 0.7 mL/min and the column was maintained at 40°C. The LC/MS/MS analysis was performed using SRM transitions. The quantifier and qualifier ions for OH-PYR, PYR and the IS are tabulated in Table 1. One set of the calibration standards and two sets of the QC-samples were added and analysed with each plate. All the samples were prepared in duplicates and analysed by single injections. Concentrations were determined by peak area ratios between the analyte and the IS. All values were corrected for the average concentrations in the blanks, to control for environmental contaminations. The concentrations were determined in the urine used for preparation of standards in each batch and the calibration standard samples were corrected for these concentrations.

*2.6 Validation of the analytical method*

To assess the limit of detection (LOD) and the limit of quantification (LOQ) in the urine matrix, eight authentic blank urine samples were analysed. The LOD and LOQ were determined as the mean level of the concentration at the same retention time as OH-PYR, plus three and ten times the standard deviation, respectively [8].

The linear range of the calibration curve was determined from eight concentration levels: 0.25, 0.5, 2.5, 5.0, 10, 25, 50 and 100 ng OH-PYR/mL urine. The equation of the curve was calculated by linear regression and the correlation coefficient (r) was used as a measure of the fit of the curve.

The precision of the method was determined using three different approaches, within-run, between-run and between-batch precision. The precision was calculated as the coefficient of variation (CV) of the repeated measurements. The within-run precision was obtained from spiked urine at the three concentration levels; 1.0, 5.0 and 10 ng OH-PYR/mL urine. Each level was spiked in ten urine samples and prepared in one sample batch during one day. The mean concentrations at the three concentration levels were calculated.

The between-run precision was determined from the three QC-samples, in 12 analytical sample batches, prepared and analysed during a period of 18 months. The standard deviation was finally calculated using 23 quantified QC-samples at each concentration level.

The between-batch precision of the method was determined from the duplicate quantification of 162 urine samples collected from the two exposure studies (see section 2.8). The samples were prepared and analysed in separate analytical batches. The CV was calculated from the differences between the duplicate samples as previously described by Lindh et.al [9].

The recovery of OH-PYR after SPE extraction was determined by comparing the concentrations obtained in two sets of eight spiked urine samples, each with a final concentration of 25 ng OH-PYR/mL urine. One set of samples was spiked before application to the SPE columns. The other set of samples was spiked when the samples had been applied to the SPE columns and eluted. This set of samples was equivalent to full recovery. After elusion from the SPE columns, the IS was added, to both sets of samples.

### *2.7 Ethical approval*

The human experimental studies and the investigation of exposure to PYR in a population from southern Sweden were ethically approved by The Regional Ethical Review Board in Lund, Lund University, Sweden (Dnr463/2005; Dnr2010/41; Dnr2010/465 and Dnr2013/6).

### *2.8 Human exposure studies*

Two healthy volunteers, one female (age 69; weight 57 kg) and one male (age 45; weight 75 kg), both non-smokers, had given their written informed consent to participate in the experimental oral and dermal studies. They minimized the intake of conventionally grown food a few days before as well as during the study. The oral and dermal studies were performed twelve weeks apart.

#### *2.8.1 Oral exposure study*

The two volunteers received one single oral dose corresponding to 50% of the accepted daily intake (ADI) of PYR. The ADI for PYR suggested by the European Union is 0.17 mg/kg body weight /day [3, 10]. PYR was dissolved in 10 mL of ethanol to a concentration of 2.05 mg/mL and was then added to 250 mL organic cranberry juice. The final doses of PYR in the cranberry juice were 4.85 mg and 6.37 mg for the female and male, respectively. The first urine sample was collected immediately prior to the exposure. After exposure, urine was voided in 1 h intervals for the first 8 hours. Then, all urine voided was collected *ad libitum* for 130h and 85h, for the female and male, respectively.

#### *2.8.2 Dermal exposure study*

The administered dermal dose of PYR was the same as in the oral experiment, 4.85 mg for the female volunteer and 6.38 mg for the male volunteer. An accurately weighed amount of PYR was dissolved in 5 mL of the vehicle containing ethanol and water (75:25 v/v). The dose was administrated topically in 658 µL and 866 µL on an area of 50 $cm^2$ of the inner forearm. After administration, the vehicle evaporated to dryness and then the skin was occluded with aluminium foil. After 8 h of exposure, the remainder of the dose was wiped off with cotton swabs moistened with the vehicle. Thereafter, the exposed area was tape-stripped with fifteen tapes, cut in pieces of 10 x 5 cm, the size of the exposed area. The tapes were carefully applied, one after the other, on the skin and stripped off using clean forceps. The aluminium foil, swabs and tapes were stored separately. The aluminium foil was extracted in 50 mL and

the swabs and tapes in 20 mL of methanol. To quantify the amount of PYR extracted from the aluminium foil, swabs and tapes, we used a PYR standard calibration curve and OH-PYR IS as an internal standard.

A pre-exposure urine sample was collected immediately prior to the exposure. After exposure, urine was voided in 1 h intervals for the first 8 h. Then, all urine voided was collected *ad libitum* for 120h. The available dermal dose was calculated as total administrated dose minus the amount of recovered PYR in the extracts from the aluminium foil, swabs and tapes, which were regarded as a loss of the dose. The total recovery of urinary OH-PYR was then calculated by dividing the urinary recovery with the available dose on a molar basis.

### *2.9 Sample collection and adjustments for urinary dilution*

The samples collected in the two studies were stored at -20°C until analysis. To adjust for the urinary dilution, the sample volumes as well as creatinine and density were determined in all the samples. Creatinine was analysed with an enzymatic method [11] and density with a hand refractometer. The concentration adjusted for urinary density, $C_d$, was calculated according to $C_d$=C(observed) x (1.016-1)/(ρ-1), where C(observed) is the obtained concentration in the urine sample, ρ the measured specific density and 1.016 the average reference urine density [12].

### *2.10 Calculation of half-lives*

The half-life (t½) of the elimination in urine was estimated from the slope of the curve in the natural logarithm linear (ln-linear) concentration versus time plot, where time is given as the mid time points between two sample collection time points [9].

### *2.11 Application of the analytical method in a general population*

The first morning void urine samples (n=413) were collected from groups of a general population in southern Sweden. The analytical method was applied to determine OH-PYR in the urine samples. The population consisted of men and women, living and working in cities or the countryside. The samples were prepared and analysed during 4 months.

## 3 Results and Discussion

### *3.1 Mass spectrometry and chromatography*

A method for the analysis of OH-PYR in human urine using SPE and LC/MS/MS was developed. The chosen analytical column provided consistent and reproducible chromatography and retained OH-PYR and PYR with stable retention times and good separation. Both PYR and OH-PYR and the internal standard for OH-PYR showed an excellent sensitivity for electro spray ionisation (ESI) in positive ion mode. The chromatograms are presented in Fig. 1. The transition giving the best signal to noise ratio was used as the quantifier ion for OH-PYR. A second SRM transition was chosen as a qualifier ion to strengthen the identity of the analyte. The transitions and optimum collision energies are tabulated in Table 1. The analytical method can efficiently analyse a large number of samples thanks to the use of 96-well plates and LC/MS/MS detection.

**Table 1**
Summary of the quantifier and qualifier SRM transitions for hydroxypyrimethanil (OH-PYR), the internal standard [$^2H_4$] OH-PYR and pyrimethanil (PYR) used in the LC/MS/MS analysis.

| Compound | Transitions (Da) | Collision Energy (V) |
|---|---|---|
| | *Quantifier ions* | |
| OH-PYR | 216.1 ⟶ 107.0 | +32 |
| [$^2H_4$] OH-PYR | 220.1 ⟶ 111.0 | +32 |
| PYR | 200.2 ⟶ 107.1 | +32 |
| | *Qualifier ions* | |
| OH-PYR | 216.1 ⟶ 159.2 | +36 |
| [$^2H_4$] OH-PYR | 220.1 ⟶ 163.0 | +36 |

### *3.2 Validation of the analytical method*

The sensitivity of the analytical method was good. The LOD and LOQ, was found to be 0.11 ng/mL and 0.36 ng/mL, respectively. The LOD is sufficiently low to allow for the measurement of environmental human exposure.

In the range 0.25-25 ng OH-PYR/mL urine, the calibration curves showed excellent linearity with r = 0.998 and the precision of the slope (1.38 ± 0.06) of 14 other curves had a CV of 7.1% (95% confidence level). Actually, the calibration curves were linear also up to 100 ng/mL.

The within-run, between-run and between-batch precisions were all excellent (Table 2).

The concentrations obtained in the samples spiked at the three concentration levels were all close to the spiked values.

The recovery in eight spiked urine samples determined after SPE elution was found to be 91%.

**Table 2**
Precision of the method at different concentration levels. The within-run precision, the between-run and batch precision were calculated from spiked urine samples.

| | OH-PYR concentration (ng/mL) | n | Mean measured OH-PYR concentration (ng/mL) | CV (%) |
|---|---|---|---|---|
| Within-run precision | 1.0 | 10 | 1.1 | 8.0 |
| | 5.0 | 10 | 5.9 | 7.4 |
| | 10 | 10 | 12 | 7.1 |
| Between-run precision | 1.0 | 23 | 1.0 | 8.0 |
| | 5.0 | 23 | 5.4 | 6.6 |
| | 10 | 23 | 11 | 5.9 |
| Between batch precision | range 0.1-10 | 69 | 3.3 | 12 |
| | range 10-100 | 50 | 39 | 10 |
| | range 100-9300 | 43 | 1300 | 7.4 |

### *3.4 Oral exposure to PYR*

In the samples from the oral exposure, the major metabolite OH-PYR was identified by the use of the synthesized standard (2-(4-hydroxyanilino)-4,6-dimethylpyrimidine). In the urine collected before the exposure to PYR, the OH-PYR levels were close to the LOD in both volunteers (0.15 and 0.12 ng/mL in the female and male, respectively). After the oral exposure, the urinary levels of OH-PYR increased rapidly and the $C_{max}$ was reached after 2 h (Table 3; Fig. 2a). No PYR was recovered in the urine from the two volunteers. The urinary elimination can be described by a two-compartment model and both the first rapid and the second slower elimination phase followed first-order kinetics (Fig. 3a). The half-life in the male, obtained from the rapid elimination phase was 3 h and in the female 4h. In the second slower elimination phase it was 15 h in the male and 14 h in the female (Table 3).The first rapid phase was found to last for 50 and 30 h for the female and male, respectively. The total recovery in urine, measured as OH-PYR, was found to be 77-80% of the dose, of which 99% was found within the first 24 h. The results agree with those found in animal studies [3].

**Table 3**
Estimated half-life of OH-PYR and conjugates in urine of the two volunteers orally exposed to PYR. The dose was 0.085 mg PYR /kg of b.w. equal to 50% of ADI

| Oral route | [a]$t_{½}$ (h) creatinine | | [a]$t_{½}$ (h) density | | [a]$t_{½}$ (h) unadjusted | | [b]$C_{max}$ (µmol/mmol crea) | $T_{Cmax}$ (h) |
|---|---|---|---|---|---|---|---|---|
| | phase 1 | phase 2 | phase 1 | phase 2 | phase 1 | phase 2 | | |
| Female | 4 | 14 | 5 | 14 | 5 | 15 | 13 | 2.5 |
| [c] r | 0.97 | 0.64 | 0.97 | 0.82 | 0.98 | 0.81 | | |
| male | 3 | 15 | 3 | 15 | 3 | 32 | 11 | 2 |
| [c] r | 0.99 | 0.83 | 1.0 | 0.78 | 0.99 | 0.38 | | |

[a] The half-life of elimination ($t_{½}$) in urine estimated from the slope of the curve in the natural logarithm-linear concentration versus mid time plot.
[b] $C_{max}$ Maximum urinary concentration of creatinine adjusted values.
[c] Correlation coefficients (r) for the regression line of t½

*3.6 Dermal exposure to PYR*

Before the dermal experimental exposure to PYR, the OH-PYR levels in urine were 0.2 and 1.6 ng/mL in the female and male, respectively. After the exposure, the urinary levels increased rapidly but $C_{max}$ was not reached until 8 h after exposure (Table 4; Fig. 2b). In both volunteers, the urinary elimination of OH-PYR seemed to follow a two-compartment model, and first-order kinetics (Fig. 3b). After 8 h, the levels started to decrease, probably reflecting the end of exposure. The half-life in the female, obtained from the rapid elimination phase, was 6h and in the male 8h. In the second slower elimination phase the half-life was 31 h in the female and 20h in the male (Table 4).

The total recovery of OH-PYR in urine, calculated from the available dose described in section 2.8.2, was 15% and 26% for the female and male volunteer, respectively. The loss of the dose recovered as PYR from the aluminum foil, swabs and tapes were 39% and 26% of the total administrated dose of PYR and the recovery of OH-PYR in urine from the total administrated dose of PYR was 9.4 % and 19 %, in the female and male volunteer, respectively.

**Table 4**
Estimated half-life of OH-PYR and conjugates in urine for the two volunteers dermally exposed to PYR on an area of 50cm$^2$ for 8 h. The administrated dose was 0.085 mg PYR/kg bw, equal to 50% of ADI.

| Dermal route | [a]$t_{½}$ (h) creatinine | | [a]$t_{½}$ (h) density | | [a]$t_{½}$ (h) unadjusted | | [b]$C_{max}$ (nmol/mmol crea) | $T_{Cmax}$ (h) |
|---|---|---|---|---|---|---|---|---|
| | phase 1 | phase 2 | phase 1 | phase 2 | phase 1 | phase 2 | | |
| Female | 6 | 30 | 10 | 31 | 11 | 29 | 489 | 8 |
| [c] r | 0.99 | 0.96 | 0.96 | 0.87 | 0.87 | 0.74 | | |
| Male | 8 | 18 | 9 | 20 | 8 | 20 | 682 | 8 |
| [c] r | 0.98 | 0.98 | 0.96 | 0.88 | 0.85 | 0.64 | | |

[a] The half-life of elimination ($t_{½}$) in urine estimated from the slope of the curve in the natural logarithm-linear concentration versus mid time plot.
[b] $C_{max}$ Maximum urinary concentration of creatinine adjusted values.
[c] Correlation coefficients (r) for the regression line of t½

Parameters that may affect the absorption include physiochemical properties of the compound, the choice of vehicle, the choice of application area, the use of occlusion, and the washing and tape stripping after exposure. Due to the number of cell layers and chemistry in the stratum corneum, the extent of absorption varies over the surface of the body. The forearm skin is less permeable than the forehead and the neck, at least for some substances [6, 13]. Still, the inner forearm was chosen as site of exposure, because it was convenient and it is commonly used in dermal experimental exposure studies [14]. For the same reason, ethanol/water was the choice of vehicle. Occlusion, used for protection of the area during the exposure, may have increased the permeability of the skin and the absorption, through an increase in the hydration and temperature. Additionally, washing of the skin after exposure may also have enhanced the dermal absorption [6]. Finally, enhanced blood flow due to the tape stripping, can have contributied to an elevated turnover of the substance from the skin into the blood stream. At ocular inspection, the skin was flushed which can indicate an increased blood flow.

In summary, the toxicokinetic profiles of the two volunteers followed a two compartment model and first order kinetics, both in the oral and dermal studies. Even though the profiles

were similar, differences were found in the toxicokinetics parameters, both between the two routes of exposure and between the two volunteers. Differences in toxicokinetic profiles between oral and dermal route agree with results from other experimental exposure studies [15-18]. It is well known that the toxicokinetics, i.e., the xenobiotic absorption, biotransformation and excretion kinetics vary greatly depending on, e.g., route of exposure, age, sex, and activity level [6, 19]. The difference in elimination rate for the two routes of administration might be explained by the fact that at dermal exposure, the compound enters the blood directly, without passing the liver while via the oral route, the substance passes via the liver before entering the blood circulation [3].

### *3.7 Adjustments for urinary dilution*

Both creatinine and density adjustment gave better correlation for the elimination curves compared to unadjusted urinary concentrations, see Table 3 and 4. This indicates, that adjustment for urinary dilution is recommended and both creatinine as well as density adjustment can be used. However, creatinine levels are affected by several factors, such as gender, age, and muscle mass, and meat consumption. Density adjustment may thus, be a better choice when comparing individuals, groups or populations [12, 19].

### *3.8 Application of the analytical method in a general population*

OH-PYR was above the LOD in 48% of samples obtained from a general population in southern Sweden the median was 0.07 and the 95$^{th}$ percentile was 3.7 ng OH-PYR/mL. However, concentrations of up to 750 ng OH-PYR/mL urine were observed. More detailed data from this study will be published elsewhere. The method was found to be suitable for analysis of large sample batches.

## 4 Conclusion

The metabolite OH-PYR was used as a biomarker of exposure to PYR and an LC/MS/MS method for the quantification of OH-PYR in human urine was developed. The method has a high through put of samples, due to the use of 96-well plates for sample preparation and analysis. This method shows a good precision and a sufficient LOD which enables detection of OH-PYR even in environmentally exposed populations.

We applied the method in two experimental exposure studies in two volunteers.

After the oral exposure to PYR, about 80% of the dose was recovered as OH-PYR, within 24 h, and excreted in two phases with elimination half-lives of 3-4 h and 14-15 h, respectively.

After the dermal exposure, 15-26% was recovered as OH-PYR, and excreted in two phases with elimination half-lives of 6-8 h and 18-30 h, respectively.

Although these studies are limited to two individuals, the data provide new information regarding the toxicokinetics of PYR after oral and dermal exposure and will make the interpretation of the biomarker levels and, thus, risk assessment for PYR more reliable. However, the presented data should be interpreted cautiously since only two individuals were observed.

## Acknowledgements

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## Figures

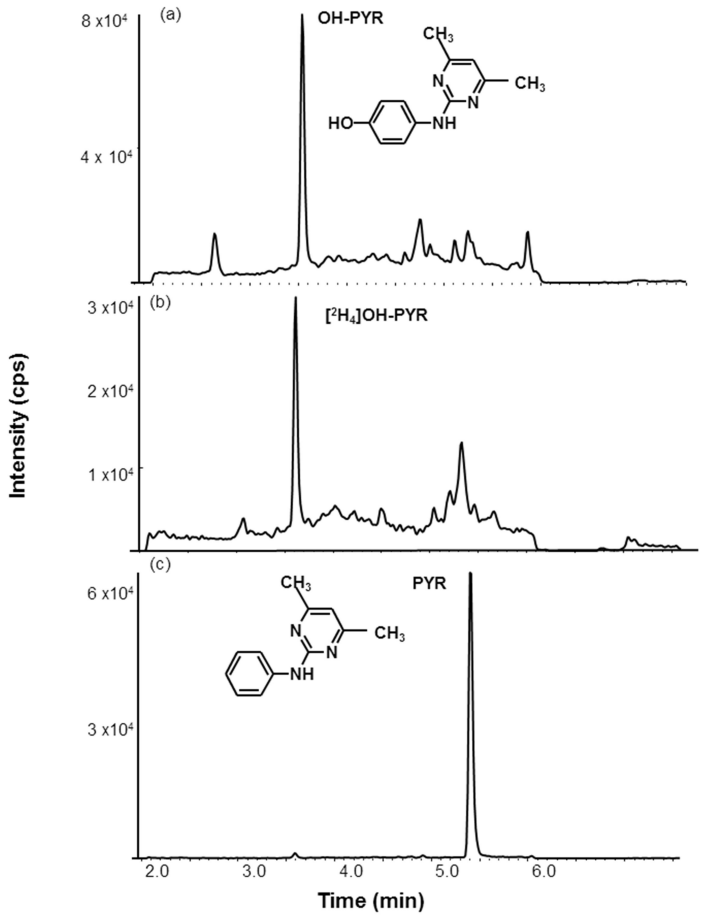


**Fig. 1** LC/MS/MS SRM chromatogram for (a) a urine sample spiked to 2.5 ng OH-PYR/mL urine, retention time 3.54 min, transition 216.1-107.0 Da (b) Internal standard spiked to 5 ng [$^2H_4$] OH-PYR /mL urine, retention time 3.53 min transition 220.1-111.0 Da (c) a urine sample spiked to 2.5 ng PYR/mL urine, retention time 5.35 min, transition 200.2-107.1 Da.

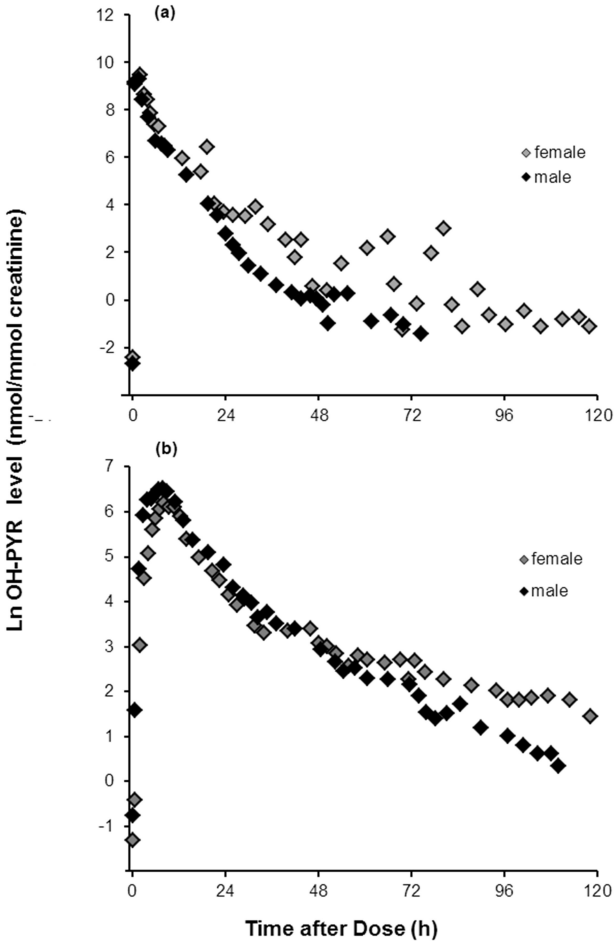


**Fig. 2** The urinary elimination curves of OH-PYR for the female and male volunteer after oral (a) and dermal (b) exposure to PYR. In the plot, the creatinine adjusted urinary levels (nmol/mmol creatinine) were log-normalized and plotted versus the mid time points. In both studies the first two urine samples were collected just before and 1 h after administration of the dose. (a) The oral dose, corresponding to 50% of the accepted daily intake (ADI) 0.17 mg PYR/kg body weight, was mixed in 250 mL of cranberry juice. (b) The volunteers were dermally exposed on the inner forearm for 8 h to a dose corresponding to 50% of the ADI.

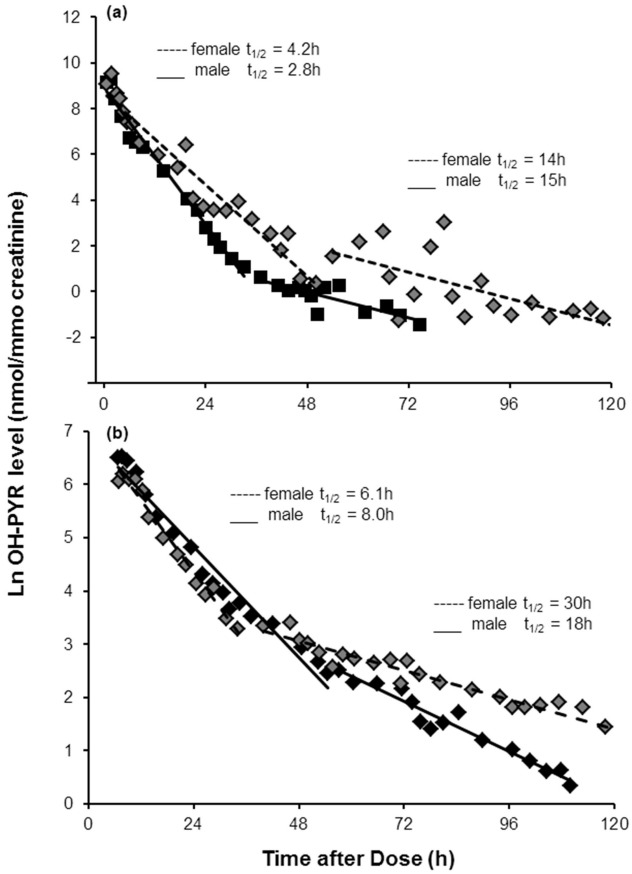


**Fig. 3** Semi logarithmic urinary elimination curves of OH-PYR for the female and male volunteer after oral (a) and dermal (b) exposure to PYR. Both the oral and dermal dose corresponded to 50% of the accepted daily intake (ADI) 0.17 mg PYR/kg body weight. The half-life of elimination ($t_{½}$) in urine is estimated from the slope of the log-normal urinary concentration versus time plot. The natural logarithm transformed creatinine adjusted OH-PYR urinary concentration levels (nmol/mmol creatinine) were plotted versus the mid time points.

# Paper IV

# Pesticide exposure in pregnant women from the Infants' Environmental Health Study (ISA), Costa Rica

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## Abstract

The extensive use of pesticides on banana plantations may result in substantial human exposure. Pesticide exposure was assessed in subjects participating in the ISA study, a prospective community-based birth cohort study in Matina County, Limón, Costa Rica, that examines possible effects of prenatal and early-life pesticide exposure on children's growth and neurodevelopment in pregnant women.

Repeated urine samples (n = 909) were collected from 445 women. Exposure biomarker concentrations were analysed using liquid chromatography tandem mass spectrometry for ethylenethiourea (ETU); 5-hydroxythiabendazole (5-OH-TBZ); hydroxypyrimethanil (OH-PYR); 3,5,6-trichloro-2-pyridinol (TCP); cis/trans 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid (DCCA); 3-phenoxybenzoic acid (3PBA); 4-fluoro-3-phenoxybenzoic acid (4F3PBA) and 2,4-diphenoxyacetic acid (2,4-D).

ETU was detected in 100% of the samples, the mean concentration was 4.2 µg/L and the median concentration 2.9 µg/L. Concentrations of 5-OH-TBZ and OH-PYR were detected in 65% and 87% of the samples respectively. The biomarkers TCP, DCCA, 3PBA and 2,4-D were detected in over 99% and 4F3PBA in 79% of the samples.

ETU concentrations in the present study were over five times higher than reported for other general populations and comparable with levels observed in some occupationally exposed populations. The biomarkers 5-OH-TBZ and OH-PYR were measured for the first time in a human population. The biomarkers TCP, DCCA, 3PBA, 4F3PBA and 2,4-D were in the same range as earlier reported for pregnant women.

Conclusion: The women had higher concentration levels of ETU compared with other general populations. Women who worked in banana plantations had higher concentrations of pesticide biomarkers, especially of 5-OH-TBZ, than women not occupationally exposed.

## Abbreviations

2,4-D, 2,4 dichlorophenoxy acetic acid; 3PBA, 3-phenoxybenzoic acid; 4F3PBA, 4-fluoro-3-phenoxybenzoic acid; 5-OH-TBZ, 5- hydroxy-thiabendazole;ADI, accepted daily intake; DCCA 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid; ETU, ethylenethiourea; OH-PYR, hydroxypyrimethanil; ICC, intra class correlation; IS, internal standard; ISA, Infants' Environmental Health Study [Infantes y Salud Ambiental]; LOD, limit of detection; QC, quality control; TCP, 3,5,6-trichloro-2-pyridinol.

# 1 Introduction

Pesticides are extensively used in agriculture and other settings, which may result in substantial human exposure causing acute [1] as well as chronic illnesses [2, 3]. The nervous system, liver, and thyroid are particularly targeted [4-6]. Neurologic diseases, cancer, and childhood leukaemia have been associated with a broad range of pesticides; however, only few studies have been able to link these diseases with exposure to specific pesticides [2, 3, 7].

Early life is the most vulnerable period of development, and therefore pesticide exposure in pregnant women and young children is of main concern [8, 9]. Results from birth cohort studies indicate delayed mental development and attention deficit hyperactivity disorders in relation to organophosphate (OP) exposures that include the insecticide chlorpyrifos [9, 10], and possibly synthetic pyrethroids [11, 12]. A cross-sectional study showed that exposure to chlorpyrifos, mancozeb and synthetic pyrethroids seemed to affect neurodevelopment [13].

In the Limon province of Costa Rica, pesticides are intensively and extensively used, approximately 50 kg active ingredient per hectare per year, on bananas grown for export purposes [14, 15]. About 27 different pesticides are reported to be applied on bananas and include weekly aerial applications of fungicides, ground applications of nematicides and herbicides, permanent use of insecticide-treated bags to protect the fruits, and post-harvest application of fungicides [14, 15]. Epidemiological studies have observed elevated urinary concentrations of ethylenethiourea (ETU), a specific metabolite of mancozeb, and 3,5,6-trichloro-2-pyridinol (TCP), a specific metabolite of chlorpyrifos, in pregnant women and school-aged children living near banana plantations [13, 16, 17]. In addition, pesticides are used in vector control programs of the Ministry of Health and inside the homes; for example, synthetic pyrethroids, such as cypermethrin, are used against mosquitoes that can carry the Dengue and Zika virus [16-19].

The aim of this study was to determine the levels of urinary biomarkers of pesticides in a prospective cohort of pregnant women, the Infants' Environmental Health Study (ISA).

# 2 Material and methods

## 2.1 Study

*Study population*

Subjects were participants in the ISA study, a prospective community-based birth cohort study in Matina County, Limón, Costa Rica, that examines possible effects of prenatal and

early-life pesticide and manganese exposure on children's growth and neurodevelopment. A detailed description of the study population has been published previously [17, 20]. Briefly, pregnant women were eligible if they were <15 years old, had less than 33 weeks of gestation, expected a singleton birth, and lived in one of 40 villages in Matina County located within 5 km of a banana plantation. A total of 451 out of 480 eligible pregnant women were enrolled. Written informed consent was obtained from each woman. For women <18 years of age, additional written informed consent was obtained from their legal representative. The women did not receive any incentives for their participation. All study activities were approved by the Scientific Ethics Committee of the Universidad Nacional in Costa Rica (CECUNA-11-2009) and Lund University (Dnr 2009/208) [17].

*Study procedures*

Women were interviewed in their homes, one to three times during pregnancy, depending on their gestational age at enrollment. A urine sample was obtained at each visit. Out of the 451 women enrolled in the study, three lacked information on gestational age and three did not provide any urine sample, so these women were therefore excluded from data analysis. For the remaining 445 women, 909 spot urine samples collected throughout pregnancy were analyzed for pesticide biomarkers.

During each visit, interviewers used structured questionnaires to obtain information on women's sociodemographic characteristics, medical and occupational history, partners' occupation, pesticide use at work and at home, lifestyle habits, quality and type of housing, basic dietary information (including frequency of fruit and vegetable intake), and source of drinking water [17]. The participants' home coordinates were determined using a global positioning system (GPS) receiver. Euclidean distances were then measured from the home to the nearest border of the closest banana plantation [17].

### 2.2 Biomarkers of pesticide exposure

Table 1 shows the pesticides used at banana plantations and their specific urinary pesticide biomarkers: ETU for mancozeb; hydroxypyrimethanil (OH-PYR) for pyrimethanil; 5-hydroxythiabendazole (5-OH-TBZ) for thiabendazole; and TCP for chlorpyrifos [21]. In addition, metabolites of common synthetic pyrethroids, reported to be used in vector control programs and inside the homes, were measured: the sum of cis/trans 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid (DCCA) for the pyrethroids permethrin, cypermethrin, and cyfluthrin; 3-phenoxybenzoic acid (3PBA) for permethrin, cypermethri¬ deltamethrin, allethrin, resmethrin, and fenvalerate; and 4-fluoro-3-phenoxybenzoic acid

(4F3PBA) for cyfluthrin [22]. The herbicide 2,4-D, used to control broadleaf in pasture and rice, was also measured.

### 2.3 Analytical methods

*Urinary sampling*

Urine samples were collected in 100 mL beakers (Vacuette®, sterile), transferred to 15 mL tubes (PerformR™ Centrifuge tubes, Labcon®, sterile), and stored at –20°C until shipment (4°C) to the Division of Occupational and Environmental Medicine at Lund University, Sweden for analysis.

*Instrumentation*

Quantitative analysis was conducted using liquid chromatography (UFLCXR with four pumps, Shimadzu Corporation, Kyoto, Japan), coupled to a triple quadrupole linear ion trap mass spectrometer (LC-MS/MS; QTRAP 5500, AB Sciex, Foster City, CA, USA) equipped with either an electrospray ion source (ESI) or atmospheric pressure chemical ionization source (APCI). Air was used as nebulizer and auxiliary gas, pure nitrogen as curtain and collision gas. LC-MS/MS analyses were carried out by selected reaction monitoring (SRM) in both positive and negative ionization mode (Table 2). The data acquisition and processing was performed using Analyst 1.6.1 and Multiquant 2.1 software (AB Sciex).

*Urinary analysis*

ETU was analysed according to Ekman et al. (2013) [23]. Briefly, 500 µL urine were added with [$^{2}H_4$]-ETU as an IS and hydrolysed in 0.09 M NaOH for 1 hr at 100°C. Sample aliquots of 20 µL were injected and analysed using two dimensional LC/LC-MS/MS with two C18 columns (Genesis Lightn AQ C18 2.1 x 100 mm, 4.0 µm, Grace Vydac, Hesperia, CA, USA). The separation was carried out using an isocratic elution on the first column followed by an isocratic elution on the second using water and methanol with 0.1% formic acid (Table 2).

Analysis of 5-OH-TBZ, OH-PYR, TCP, 2,4-D, DCCA, 3PBA, and 4F3PBA was performed according to a modified method by Ekman et al. (2014) [24]. Briefly, 500 µL urine were added with [$^{13}C_2$][$^{15}N$]-5-OH-TBZ, [$^{2}H_4$]-OH-PYR, [$^{13}C_3$]-TCP, [$^{2}H_3$]-2,4-D, [$^{2}H_6$]-DCCA, [$^{13}C_6$]-3PBA and [$^{13}C_6$]-4F3PBA as ISs and hydrolysed using aryl/sulphates enzyme at 37°C for 18 hrs. Biomarkers were extracted from the urinary matrix using 96 well format solid phase extraction plates (ISOLUTE®-96 ENV+, 50 mg, Biotage, Uppsala, Sweden) and were eluted in two steps. The first eluate (1) was eluted with 1 mL of acetonitrile and the secon

eluate (2) using 1 mL of acetonitrile with 5% ammonia. The eluates were collected in two separate 96 well plates. Sample aliquots of 6 µL of eluate 1 were injected and separated on a C18 column (Genesis Lightn AQ C18 2.1 x 100 mm, 4.0 µm, Grace Vydac, Hesperia, CA, USA) using a gradient elution using water and methanol with 0.1% formic acid. The samples were analysed for TCP, 2,4-D, DCCA, 3PBA and 4F3PBA. Sample aliquots of 3 µL of eluate 2 were injected and separated on a C18 column (Poroshell 120EC-C18 column, 4.6 x 100 mm, 2.7 µm, Agilent Technologies, Santa Clara, CA, USA) using a gradient elution using water and methanol with 0.1% formic acid. The samples were analysed for 5-OH-TBZ and OH-PYR (Table 2).

*Method validation*

All samples were prepared in duplicates in 96 well plates, containing, besides of the samples, at least six levels of calibration standards, chemical blanks, and quality control (QC) samples. Concentrations were determined by peak area ratios of the analyte versus the IS. All values were corrected for the average concentrations in the blanks and the average concentration of the duplicate samples was used in further calculations.

The limit of detection (LOD) for each biomarker was defined as the average concentration of the chemical blanks plus three times the standard deviation [25]. The between-run precision of the methods was determined from the QC samples for each biomarker and reported as the coefficient of variation (CV; Table 3). The between-batch precision of the methods was determined from the duplicate quantified samples for each biomarker and reported as the CV [26] (Table 3).

The analyses of 3-PBA and TCP are part of a biannual round robin inter-laboratory program (University of Erlangen-Nuremberg, Germany) with results within the tolerance limits.

*Adjustments for urinary dilution*

The density of the urine samples was determined using a hand refractometer. Urinary concentrations were adjusted for density according to $C_d = C(\text{observed}) \times (1.017 - 1)/(\rho - 1)$, where C(observed) was the determined concentration in a urine sample,ρthe measured density in each sample, and 1.017 kg/L is the average density of all urine samples in this study [27]. Creatinine concentrations were analysed according to an enzymatic method described by Mazzachi et al. [28].

**2.4 Statistical analysis**

Concentrations <LOD were set at the lowest density-corrected concentration that was detected divided by the square root of 2. Descriptive statistics to examine the associations between the exposure biomarkers was used. Continuous variables were tested for normal distribution using the Shapiro-Wilk test. Non-normally distributed variables were $\log_{10}$-transformed. Correlations between continuous variables were performed using Pearson's r correlation coefficient.

To estimate temporal reliability of biomarkers we used variance components models with random intercepts [restricted maximum likelihood (REML)] for each participant to estimate intraclass correlation coefficients (ICC) of pesticide metabolite concentrations from repeated samples collected from the same woman. Subsequently, linear mixed-effects regression models with random intercepts (REML), taking into account correlation among repeated samples collected from the same woman, was performed. Mixed-effects models were run for each biomarker using the $\log_{10}$-transformed density-corrected concentrations (µg/L) and the variable work in agriculture during pregnancy (yes/no).
For all statistical tests, the significance level was set at 0.05. JMP 8 (SAS Institute Inc., Cary, NC, USA) was used for statistical analysis.

## 3 Results and discussion

Women in the study were young (64% <25 years of age), economically impoverished (59% below Costa Rica poverty line), and had low educational attainment (52% ≤6 years of education). Almost half of the women lived less than 200 meters from a banana plantation. Relatively few women worked in agriculture during pregnancy (8%, of whom most worked in banana plantations), whereas the large majority of their partners did (63%, of whom about half worked in banana plantations).

The highest pesticide concentrations were observed for the metabolite of Mancozeb, ETU, with a median concentration (p10; p90) of 3.1 (1.5; 7.1) µg/L followed by the metabolite of chlorpyrifos, TCP, with a median concentration (p10; p90) of 1.6 (0.7; 4.3) µg/L (Table 4a). For most biomarkers high maximum concentrations were measured. For some biomarkers, e.g. OH-PYR and 5-OH-TBZ, more than thousand-fold higher concentrations were observed than their median concentrations. Concentrations of 5-OH-TBZ and 4F3PBA were low, with median concentrations of 0.06 and 0.03 µg/L, respectively, and detection frequencies of 65 and 79% respectively. With respect to metabolites of synthetic pyrethroids, DCCA showed highest concentrations (median=1.2, p10=0.3; p90=3.8) µg/L.

Several studies have investigated exposure to pesticides in pregnant women, a comparison of these studies with the pesticide metabolite concentrations in the present ISA study is shown in Tables 4a-c and Tables 5a-d [29-37]. Comparisons between studies of exposure biomarker concentrations should be performed with caution, especially when different analytical methods are used.

Few other studies have investigated exposures to bisdithiocarbamate fungicides (ETU) in pregnant women. In Castorina et al. the median ETU concentrations was below LOD[30]. However, ETU concentrations in the ISA study were more than five times higher than those reported for other general populations[38-40], and comparable with post-shift urinary ETU concentrations of Italian agricultural workers[41, 42].

The chlorpyrifos metabolite, TCP, has been measured in several studies and the TCP concentrations are in the same range as in our study. However, a study by Berkowitz et al., showed a median TCP concentration that was a tenfold higher compared to the others studies [29]. In this study an unspecific analytical method was used (LC-UV).

For the pyrethroid metabolites, DCCA and 3PBA concentrations were also in the same range as in our study. However in Berkowitz et al. a tenfold higher concentrations for 3PBA was observed. For 4F3PBA all studies show very low concentrations [30-32].

Several studies have measured the exposure to 2,4-D, only one has more than 50% of the samples above LOD, but the geometric mean(GM) is in the same range as in our study[30, 32].

No other studies have described exposure to TBZ (5-OH-TBZ) or PYR (OH-PYR).

Values of ICC (Table 4a) indicated a relatively low temporal reliability for ETU, reflecting considerable fluctuations in metabolite concentrations measured for the same women on repeated occasions during pregnancy. Highest ICCs were found for TCP and 5-OH-TBZ with ICC = 0.36 and 0.43, respectively.

Description of density corrected pesticide biomarker concentrations of 36 women working in agriculture during pregnancy (n=77 urine samples) compared with 409women who did not work in agriculture during pregnancy (n= 832 urine samples) estimated with mixed-effects multiple regression models and shown in Table 6. Pregnant women who worked in agriculture during pregnancy had in particular higher urinary 5-OH-TBZ concentrations. This can be explained by the fact that women working in banana plantations usually work in the packing plant, where thiabendazole is applied on bananas as a post-harvest fungicide before shipping them abroad. During this process, women take the fruit to the spraying cabin, push a button to apply the pesticide, and then pull the bananas out of the cabin. The packing proc[illegible] contains several manual tasks that may result in high dermal exposures.

Women who worked in agriculture during pregnancy had higher urinary ETU (GM=4.21 μg/L; 95% CI: 3.58, 4.96) and TCP (2.07 μg/L; 95% CI: 1.69, 2.53) concentrations compared to women who did not. These differences can be explained by the frequent contact that women may have at work with bananas contaminated with aerially-sprayed fungicides [17] and plastic bags that contain chlorpyrifos. For example, the chlorpyrifos-impregnated bags are removed from the banana bunches in the packing plant and these bags could still contain residues of chlorpyrifos that may result in dermal exposures. It is important to mention that, even though metabolites of synthetic pyrethroids (i.e., DCCA, 3PBA, 4F3PBA) were also present in the women's urine samples, concentrations did not differ between women who worked in banana plantations and those who did not (Table 6). This finding is possibly due to the fact that these pesticides were not used on banana plantations, but at home and in vector control programs.

Results presented in Table 7 show, except for the metabolites of synthetic pyrethroids (DCCA, 3PBA and 4F3PBA), that the pesticide biomarkers were poorly correlated, with Pearson´s r) ranging from -0.05 to 0.23 between logtransformed biomarkers. DCCA highly correlated with 3PBA (Pearson's r=0.81, 95%CI= 0.87 – 0.83) and moderately with 4F3PBA (Pearsons' r=0.41, 95%CI=0.36 – 0.47). This correlation can be expected since several pyrethroids metabolize both to 3-PBA and DCCA and this suggests an exposure to permethrin or cypermethrin. This has been observed in other studies on pyrethroid exposures [22].

This study has several strengths. We performed one to three measures of pesticide biomarkers during pregnancy which is important since these exposure biomarkers have relatively short half-lives. The latter is illustrated by the poor to moderate ICCs, which indicate a relatively low temporal reliability for the pesticide biomarkers. Thus, this indicates that the concentrations may fluctuate considerably during the pregnancy within each woman. Repeated measurements improve the reliability of the exposure assessments of the pesticides. Another strength was that we measured several different pesticides or pesticide biomarkers from different groups of pesticides, e.g. fungicides, insecticides and herbicides. This provides a better understanding of the pesticide exposure profile of pregnant women living near banana plantation. Also, the analytical methods that we used are well validated, and the biomarkers that we analysed are also validated in human pharmacokinetic studies [23, 24, 26]. Finally, the LC-MS/MS technique, analytical reference standards and stable isotope labelled internal standards that we used, provide a very selective, sensitive and reliable quantitative analytical methods with high detection frequency [23, 24, 26].

## 4 Conclusions

The women had higher concentration levels of ETU compared with other general populations. Women who worked in agriculture had higher biomarker concentrations levels especially of 5-OH-TBZ.

## Acknowledgement

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## Tables

**Table 1**: Description of pesticides and biomarkers evaluated in this study.

| Group | Use | Chemical family | Pesticide | Urinary biomarker (metabolite) |
|---|---|---|---|---|
| **Fungicides** | Banana, Aerial application | Dithiocarbamates | Mancozeb | Ethylenethiourea (ETU) |
| | | Anilinopyrimidines | Pyrimethanil | Hydroxypyrimethanil (OH-PYR) |
| | Banana, Post-harvest | Benzimidazoles | Thiabendazole | 5-Hydroxythiabendazole (5-OH-TBZ) |
| **Insecticides** | Banana, Treated bags | Organophosphate insecticides | Chlorpyrifos | 3,5,6-Trichloro-2-pyridinol (TCP) |
| | Vector control / residential use | Synthetic pyrethroids | Permethrin, Cypermethrin, Cyfluthrin | 3-(2,2-dichlorovinyl)-2,2-Dimethylcyclopropanecarboxylic acid (DCCA) |
| | | | Permethrin, Cypermethrin, Deltamethrin, Allethrin, Resmethrin, Fenvalerate | 3-Phenoxybenzoic acid (3PBA) |
| | | | Cyfluthrin | 4-Fluoro-3-phenoxybenzoic acid (4F3PBA) |
| **Herbicides** | Pasture, rice, to combat broadleaf | Dichlorophenoxyacetic acid | 2,4-D | 2,4-D |

**Table 2** Summary of the SRM quantifier transitions used in the LC-MS/MS analysis, for the pesticide exposure biomarkers, and their corresponding isotopic labelled internal standards.

| Compound | Transitions (Da) | CE[1] (V) | DP[2] (V) | Ionization mode | Ion source |
|---|---|---|---|---|---|
| ETU | 103.1 → 60.1 | 42 | 110 | + | APCI[3] |
| 5-OH-TBZ | 218.0 → 191.2 | 36 | 136 | + | ESI[4] |
| OH-PYR | 216.1 → 107.0 | 32 | 80 | + | ESI |
| TCP | 197.7 → 35.0 | -40 | -60 | - | ESI |
| 2,4-D | 219.1 → 161.0 | -20 | -60 | - | ESI |
| DCCA | 207.0 → 35.0 | -40 | -60 | - | ESI |
| 3PBA | 213.0 → 93.0 | -50 | -64 | - | ESI |
| 4F3PBA | 231.1 → 93.0 | -36 | -70 | - | ESI |
| [$^{2}H_4$] ETU | 107.1 → 48.0 | 25 | 110 | + | APCI |
| [$^{13}C_2$][$^{15}N$] 5-OH-TBZ | 221.0 → 194.0 | 35 | 136 | + | ESI |
| [$^{2}H_4$] OH-PYR | 220.1→ 111.0 | 32 | 80 | + | ESI |
| [$^{13}C_3$] TCP | 200.7 → 35.0 | -40 | -60 | - | ESI |
| [$^{2}H_3$] 2,4-D | 224.1 → 166.0 | -20 | -60 | - | ESI |
| [$^{2}H_6$] DCCA | 213.0 → 35.0 | -40 | -60 | - | ESI |
| [$^{13}C_6$] 3PBA | 219.1 → 99.0 | -40 | -60 | - | ESI |
| [$^{13}C_6$] 4F3PBA | 237.1 → 98.9 | -40 | -70 | - | ESI |

1) Collision Energy; 2) Declustering Potential; 3) Atmospheric-pressure chemical ionization; 4) Electrospray ionization

**Table 3** Summary of the limit of detection (LOD), between-run precisions reported as the coefficient of variation (CV) for pesticide exposure biomarkers determined from the QC-samples in 24 batches and the between-batch precision for the pesticide biomarkers determined from duplicate sample analysis.

| Compound | LOD µg/L(n) | Between-run precision CV (%) | | Between-batch precision | | |
|---|---|---|---|---|---|---|
| | | Low level (n) | High level (n) | Range (µg/L) | (n) | (CV %) |
| ETU | 0.08 (32) | 18 (57) | 15 (57) | 0.24-22 | 827 | 11 |
| 5-OH-TBZ | 0.03 (85) | 5.8 (95) | 3.7 (96) | 0.08-132 | 172 | 8 |
| OH-PYR | 0.06 (82) | 11 (96) | 8.7 (95) | 0.16-29 | 301 | 8 |
| TCP | 0.05 (92) | 6.7 (96) | 4.2 (84) | 0.11-32 | 387 | 10 |
| 2,4-D | 0.02 (92) | 7.8 (96) | 7.6 (68) | 0.04-7.2 | 323 | 13 |
| DCCA | 0.04 (92) | 8.7 (96) | 7.2 (88) | 0.10-13 | 323 | 15 |
| 3PBA | 0.03 (92) | 8.8 (80) | 7.0 (80) | 0.06-8.1 | 394 | 14 |
| 4F3PBA | 0.01 (92) | 8.4 (96) | 13 (46) | 0.03-1.4 | 126 | 19 |

**Table 4a.** Distribution and variability of urinary, density corrected pesticide metabolite concentrations (µg/L) (N=909, women=445).

| Variable | Biomarker | % > LOD | Mean (SD) | GM (GSD | Percentile | | | | | | | ICC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | min | 10th | 25th | 50th | 75th | 90th | 97.5th | max | |
| Chlorpyrifos | TCP | 100 | 2.6 (5.1) | 1.8 (2.1) | 0.3 | 0.7 | 1.1 | 1.6 | 2.5 | 4.3 | 10 | 91 | 0.36 |
| Mancozeb | ETU | 100 | 4.3 (8.9) | 3.2 (2.0) | 0.2 | 1.5 | 2.1 | 3.1 | 4.8 | 7.1 | 14 | 251 | 0.16 |
| Pyrimethanil | OH-PYR | 86.6 | 3.8 (42) | 0.4 (5.1) | 0.03 | 0.03 | 0.2 | 0.4 | 1.1 | 2.7 | 12 | 934 | 0.28 |
| Thiabendazole | 5-OH-TBZ | 64.5 | 4.2 (39) | 0.1 (8.2) | 0.01 | 0.01 | 0.01 | 0.06 | 0.3 | 1.7 | 18 | 678 | 0.43 |
| Synthetic | DCCA | 99.2 | 1.9 (3.0) | 1.1 (2.8) | 0.04 | 0.3 | 0.6 | 1.1 | 2.1 | 3.8 | 7.9 | 46 | 0.27 |
| Pyrethroids | 3PBA | 99.8 | 1.2 (2.0) | 0.7 (2.5) | 0.04 | 0.2 | 0.4 | 0.7 | 1.3 | 2.2 | 5.0 | 33 | 0.29 |
| | 4F3PBA | 78.9 | 0.1 (0.1) | 0.03 (3.0) | 0.01 | 0.01 | 0.02 | 0.03 | 0.06 | 0.1 | 0.3 | 1.2 | 0.30 |
| 2,4D | 2,4-D | 99.8 | 0.9 (5.9) | 0.3 (2.6) | 0.03 | 0.1 | 0.2 | 0.3 | 0.5 | 1.1 | 3.4 | 159 | 0.28 |

**Table 4b.** Distribution and variability of urinary, creatinine corrected pesticide metabolite concentrations (µg/g creatinine) (N=908, women=445).

| Variable | Biomarker | % > LOD | Mean (SD) | GM (GSD | Percentile | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | min | 10th | 25th | 50th | 75th | 90th | 97.5th | max |
| Chlorpyrifos | TCP | 100 | 2.4 (4.6) | 2.0 (1.0) | 0.3 | 0.8 | 1.0 | 1.5 | 2.2 | 3.6 | 8.5 | 74 |
| Mancozeb | ETU | 100 | 4.1 (7.3) | 2.1 (1.0) | 0.07 | 1.3 | 1.9 | 2.9 | 4.5 | 6.8 | 14 | 197 |
| Pyrimethanil | OH-PYR | 86.6 | 2.9 (26) | 0.3 (5.4) | 0.02 | 0.02 | 0.1 | 0.4 | 1.0 | 2.3 | 10 | 570 |
| Thiabendazole | 5-OH-TBZ | 64.5 | 4.3 (41) | 0.1 (9.9) | 0.01 | 0.01 | 0.01 | 0.05 | 0.3 | 1.5 | 16 | 754 |
| Synthetic | DCCA | 99.2 | 1.7 (2.6) | 1.0 (2.6) | 0.04 | 0.3 | 0.6 | 1.0 | 1.8 | 3.4 | 7.5 | 48 |
| Pyrethroids | 3PBA | 99.8 | 1.1 (1.9) | 0.7 (2.4) | 0.04 | 0.2 | 0.4 | 0.6 | 1.1 | 2.0 | 3.7 | 34 |
| | 4F3PBA | 78.9 | 0.1 (0.1) | 0.03 (3.2) | 0.01 | 0.01 | 0.01 | 0.03 | 0.06 | 0.1 | 0.3 | 0.8 |
| 2,4D | 2,4-D | 99.8 | 0.8 (5.6) | 0.3 (2.7) | 0.02 | 0.1 | 0.2 | 0.3 | 0.5 | 1.0 | 3.4 | 156 |

**Table 4c.** Distribution and variability of urinary, unadjusted pesticide metabolite concentrations (µg/L) (N=909, women=445).

| Variable | Biomarker | % > LOD | Mean (SD) | GM (GSD | Percentile | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | min | 10th | 25th | 50th | 75th | 90th | 97.5th | max |
| Chlorpyrifos | TCP | 100 | 2.7 (5.6) | 1.6 (2.6) | 0.06 | 0.5 | 0.9 | 1.6 | 2.7 | 4.9 | 11 | 111 |
| Mancozeb | ETU | 100 | 4.2 (7.9) | 2.9 (2.2) | 0.2 | 1.1 | 1.8 | 2.9 | 4.6 | 7.6 | 15 | 207 |
| Pyrimethanil | OH-PYR | 86.6 | 4.3 (55) | 0.4 (4.9) | 0.04 | 0.04 | 0.1 | 0.4 | 1.0 | 2.8 | 12 | 1225 |
| Thiabendazole | 5-OH-TBZ | 64.5 | 3.5 (32) | 0.1 (7.1) | 0.02 | 0.02 | 0.02 | 0.06 | 0.3 | 1.7 | 19 | 718 |
| Synthetic | DCCA | 99.2 | 2.0 (3.3) | 1.0 (3.4) | 0.03 | 0.2 | 0.5 | 1.1 | 2.2 | 4.4 | 9.6 | 44 |
| Pyrethroids | 3PBA | 99.8 | 1.3 (2.2) | 0.6 (3.2) | 0.02 | 0.1 | 0.3 | 0.6 | 1.5 | 2.7 | 6.2 | 36 |
| | 4F3PBA | 78.9 | 0.1 (0.1) | 0.03 (2.8) | 0.01 | 0.01 | 0.01 | 0.03 | 0.06 | 0.1 | 0.3 | 1.7 |
| 2,4D | 2,4-D | 99.8 | 0.8 (5.0) | 0.3 (2.9) | 0.01 | 0.09 | 0.2 | 0.3 | 0.5 | 1.2 | 3.3 | 131 |

**Table 5a.** Median of urinary, creatinine corrected pesticide metabolite concentrations (µg/g creatinine)

| Variable | Biomarker | ISA | NewYork | Generation R | | |
|---|---|---|---|---|---|---|
| country | | Costa Rica | USA | Netherlands | Japan | China |
| Women(N) | | 445 | 386 | 100 | 231 | 1149 |
| Author | | This study | Berkowitz et.al 2003 | Ye et al 2009 | Zhang et al. 2013 | Qi et al. 2012 |
| Chlorpyrifos | TCP | 1.46 | 11.3 | 1.6 | - | - |
| Synthetic Pyrethroids | DCCA | 1.01 | - | - | - | 2.55 |
| | 3PBA | 0.63 | 19.3 | - | 0.34 | 1.55 |

**Table 5b.** Geometric mean of urinary, creatinine corrected pesticide metabolite concentrations (µg/g creatinine)

| Variable | Biomarker | ISA | NHANES | Generation R | MoBa | | |
|---|---|---|---|---|---|---|---|
| country | | Costa Rica | USA | Netherlands | Norway | Japan | China |
| Women(N) | | 445[a] | 87 | 100[b] | 110[c] | 231 | 1149 |
| Author | | This study | Ye et al 2009 | Ye et al 2009 | Ye et al 2009 | Zhang et al. 2013 | Qi et al. 2012 |
| Chlorpyrifos | TCP | 2.02 | 1.60 | 1.85 | 1.47[a] | - | - |
| Synthetic Pyrethroids | DCCA | 1.02 | - | - | - | - | 2.64 |
| | 3PBA | 0.66 | - | - | - | 0.36 | 1.53 |

a) ISA 908 repeated samples from 445women; b
b) Generation R 100 random samples from a population of 8880 pregnant women;
c) MoBa 10 pooled samples from 110 women, estimated geometric mean due to the pooled samples.

**Table 5c.** Median of urinary, unadjusted pesticide metabolite concentrations (µg/L)

| Variable | Biomarker | ISA | NHANES | Generation R | | | CHAMACOS |
|---|---|---|---|---|---|---|---|
| country | | Costa Rica | USA | Netherlands | Japan | China | USA |
| Women(n) | | 445 | 342 | 100 | 231 | 1149 | 601 |
| Author | | This study | Castorina et al. 2010 | Ye et al 2008 | Zhang et al. 2013 | Qi et al. 2012 | Castorina et al. 2010 |
| Chlorpyrifos | TCP | 1.61 | 1.6 | 1.2 | - | - | 2.1-3.2 |
| Mancozeb | ETU | 2.91 | - | - | - | - | <LOD |
| Synthetic Pyrethroids | DCCA | 1.13 | <LOD | - | - | 2.55 | <LOD |
| | 3PBA | 0.64 | <LOD | - | 0.35 | 1.55 | <LOD |
| | 4F3PBA | 0.03 | <LOD | - | - | - | <LOD |
| 2.4D | 2.4-D | 0.27 | <LOD | - | - | - | <LOD |

**Table 5d.** Geometric mean of urinary, unadjusted pesticide metabolite concentrations (µg/L)

| Variable | Biomarker | ISA | NHANES | Generation R | MoBa | | | | PROTECT |
|---|---|---|---|---|---|---|---|---|---|
| country | | Costa Rica | USA | Netherlands | Norway | Japan | China | Carabian Islands | PuertoRico |
| Women(n) | | 445[a] | 87 | 100 | 110 | 231 | 1149 | 295 | 54 |
| Author | | This study | Ye et al 2009 | Ye et al 2009 | Ye et al 2009 | Zhang et al. 2013 | Qi et al. 2012 | Dewailly et al. 2014 | Lewis et al. 2014 |
| Chlorpyrifos | TCP | 1.56 | 1.6 | 1.2 | 0.99 | - | - | - | 0.4 |
| Synthetic Pyrethroids | DCCA | 0.99 | - | - | - | - | 1.69 | 2.4 | <LOD |
| | 3PBA | 0.64 | - | - | - | 0.36 | 0.97 | 3.5 | 0.2 |
| | 4F3PBA | 0.03 | - | - | - | - | - | 0.1 | <LOD |
| 2.4D | 2.4-D | 0.30 | - | - | - | - | - | - | 0.4 |

**Table 6.** Description of density corrected pesticide metabolite concentrations (µg/L) of 36 women working in agriculture during pregnancy (n=77 urine samples) compared with 409 women who did not work in agriculture during pregnancy (n= 832 urine samples) estimated with mixed-effects multiple regression models.

| Variable | GM (95% CI) | | p |
|---|---|---|---|
| | Women working in agriculture | Women not working in agriculture | |
| TCP | 2.07 (1.69 – 2.53) | 1.72 (1.62 – 1.83) | 0.08 |
| ETU* | 4.21 (3.58 – 4.96) | 3.15 (2.99 – 3.31) | 0.001 |
| OH-PYR | 0.43 (0.28 – 0.65) | 0.38 (0.34 – 0.43) | 0.61 |
| 5-OH-TBZ | 0.84 (0.49 – 1.43) | 0.08 (0.06 – 0.09) | <0.0001 |
| DCCA | 1.21 (0.93 – 1.57) | 1.09 (1.01 – 1.18) | 0.48 |
| 3PBA | 0.76 (0.60 – 0.97) | 0.71 (0.66 – 0.77) | 0.62 |
| 4F3PBA | 0.03 (0.03 – 0.04) | 0.03 (0.03 – 0.03) | 0.68 |
| 2,4D | 0.29 (0.23 – 0.37) | 0.34 (0.32 – 0.37) | 0.20 |

* In part reported by van Wendel de Joode et al. 2014

**Table 7.** Pairwise Pearsons'r correlation coefficients between log10 transformed density adjusted pesticide metabolite concentrations (n=909, women=445)

| Variable | By variable | Correlation | Lower 95% | Upper 95% | P-value |
|---|---|---|---|---|---|
| TCP | ETU | 0.21 | 0.15 | 0.27 | <0.0001 |
| | OH-PYR | 0.19 | 0.12 | 0.25 | <0.0001 |
| | 5-OH-TBZ | 0.16 | 0.10 | 0.22 | <0.0001 |
| | DCCA | 0.22 | 0.16 | 0.28 | <0.0001 |
| | 3PBA | 0.19 | 0.13 | 0.25 | <0.0001 |
| | 4F-3PBA | 0.20 | 0.14 | 0.26 | <0.0001 |
| | 2.4-D | 0.04 | -0.02 | 0.11 | 0.18 |
| ETU | OH-PYR | 0.16 | 0.10 | 0.22 | <0.0001 |
| | 5-OH-TBZ | 0.10 | 0.04 | 0.17 | 0.002 |
| | DCCA | 0.14 | 0.08 | 0.21 | <0.0001 |
| | 3PBA | 0.15 | 0.08 | 0.21 | <0.0001 |
| | 4F-3PBA | 0.10 | 0.04 | 0.17 | 0.002 |
| | 2.4-D | 0.05 | -0.01 | 0.12 | <0.10 |
| OH-PYR | 5-OH-TBZ | 0.18 | 0.11 | 0.24 | <0.0001 |
| | DCCA | 0.23 | 0.16 | 0.29 | <0.0001 |
| | 3PBA | 0.17 | 0.10 | 0.23 | <0.0001 |
| | 4F-3PBA | 0.15 | 0.09 | 0.22 | <0.0001 |
| | 2.4-D | 0.07 | 0.00 | 0.13 | 0.04 |
| 5-OH-TBZ | DCCA | 0.09 | 0.03 | 0.16 | 0.005 |
| | 3PBA | 0.11 | 0.05 | 0.18 | 0.0006 |
| | 4F-3PBA | 0.12 | 0.05 | 0.18 | 0.0004 |
| | 2.4-D | -0.05 | -0.11 | 0.02 | 0.16 |
| DCCA | 3PBA | 0.81 | 0.78 | 0.83 | <0.0001 |
| | 4F-3PBA | 0.41 | 0.36 | 0.47 | <0.0001 |
| | 2.4-D | 0.08 | 0.02 | 0.15 | 0.01 |
| 3PBA | 2.4-D | 0.07 | 0.00 | 0.13 | 0.04 |
| | 4F-3PBA | 0.41 | 0.35 | 0.46 | <0.0001 |
| 4F3PBA | 2.4-D | -0.01 | -0.07 | 0.06 | 0.85 |