

**Développement d'outils de surveillance biologique pour  
l'évaluation des risques à la santé de travailleurs en  
arboriculture et en viticulture exposés aux fongicides**

par  
Aurélie Berthet

Thèse de doctorat effectuée en cotutelle  
au  
Département de Santé Environnementale et Santé au Travail  
Faculté de Médecine  
Université de Montréal  
et  
Faculté de Biologie et Médecine  
Université de Lausanne

Thèse présentée à la Faculté des études Supérieures de l'Université de Montréal  
en vue de l'obtention du grade de Philosophiæ Doctor (Ph.D.)  
en Santé Publique, option Toxicologie  
et à  
Université de Lausanne en vue de l'obtention du grade de Docteur ès sciences de la vie

Mars, 2011

Université de Montréal  
Faculté des études supérieures et postdoctorales

et

Faculté de Biologie et Médecine  
Université de Lausanne

Cette thèse intitulée :

Développement d'outils de surveillance biologique pour l'évaluation des risques à la santé de  
travailleurs en arboriculture et en viticulture exposés aux fongicides

Présentée et soutenue à l'Université de Lausanne par :  
Aurélié Berthet

a été évaluée par un jury composé des personnes suivantes :

Prof. Antoine Guisan, président-rapporteur  
Prof. Michèle Bouchard, directrice de recherche (Université de Montréal)  
Prof. Brigitta Danuser, directrice de recherche (Université de Lausanne)  
Dr Marc Baril, membre du jury (Université de Montréal)  
Dr Nancy Hopf, membre du jury (Université de Lausanne)  
Dr Olivier Viret, examinateur externe

## Sommaire

De nombreux travailleurs utilisent le captan et le folpet comme fongicides en agriculture, mais leur exposition n'est pas toujours mesurée de manière spécifique et précise. La surveillance biologique est un excellent outil à cet effet puisqu'elle permet de quantifier l'exposition réelle. Toutefois, la majorité des connaissances toxicologiques pour ces fongicides proviennent d'études sur les animaux, et les données chez l'humain sont limitées.

Le but du présent projet est donc de développer des outils de surveillance biologique pour évaluer l'exposition de travailleurs au captan et au folpet. Dans cette perspective, le projet a été subdivisé en trois parties complémentaires, soit i) de développer des méthodes analytiques spécifiques pour quantifier les biomarqueurs d'intérêt du captan, à savoir le tétrahydrophthalimide (THPI), et du folpet, à savoir le phthalimide (PI) et l'acide phthalique, dans le plasma et l'urine; ii) de déterminer la toxicocinétique des deux fongicides en exposant des volontaires de façon aiguë à de faibles doses de captan ou de folpet par voie orale et cutanée dans des conditions semi-contrôlées et en quantifiant les biomarqueurs dans chacune des deux matrices, excepté l'acide phthalique qui a été mesuré seulement dans l'urine; iii) de valider les biomarqueurs d'exposition sélectionnés et d'évaluer l'exposition réelle des travailleurs et les voies prédominantes d'exposition au captan et au folpet en collectant des données biologiques chez des travailleurs en arboriculture et en viticulture lors d'activités de traitement et d'effeuillage pendant sept jours consécutifs.

Selon ces travaux, le THPI et le PI sont deux biomarqueurs valides et spécifiques pour quantifier l'exposition au captan et au folpet, respectivement, chez l'humain. En effet, les méthodes développées pour ces deux métabolites sont robustes avec des limites de détection plus sensibles que celles rapportées dans la littérature, un taux de recouvrement de 90% pour le THPI et de 75% pour le PI, une très bonne linéarité ( $R^2 > 0,99$ ) et une bonne stabilité avec des variations intra- et inter-journalières faibles ( $RSD < 15\%$ ). Elles ont permis de déterminer les profils cinétiques des deux métabolites chez les volontaires et chez les travailleurs. Ces derniers indiquent d'ailleurs une élimination rapide, avec une demi-vie d'élimination dans l'urine de 11,7 h et 18,7 h pour le THPI et de 27,3 h et 28,8 h pour le PI, respectivement après une absorption par voie orale et cutanée, ainsi qu'une faible absorption cutanée lorsque les valeurs sont comparées pour les deux voies d'exposition. Des profils parallèles sont aussi observés entre le PI et l'acide phtalique pour les volontaires et les agriculteurs, mais le folpet se retrouve davantage métabolisé sous forme d'acide phtalique que de PI. Quant à l'étude des agriculteurs, elle montre que la voie principale d'exposition de ces travailleurs est la voie cutanée. Il est aussi souligné qu'il est important 1) de favoriser les collectes d'urines complètes sur 24 h au urines ponctuelles, 2) de mesurer plusieurs métabolites, et 3) d'associer les données de surveillance biologique à la toxicocinétique. Ainsi, les connaissances acquises par cette étude peuvent s'appliquer à d'autres fongicides, voire d'autres substances.

**Mots-clés** : captan, folpet, surveillance biologique, THPI, PI, acide phtalique, cinétique chez l'humain, viticulteurs, arboriculteurs.

## **Abstract**

Several workers use captan and folpet as fungicides in agriculture, but their exposure has yet to be measured specifically and precisely. Biomonitoring is an excellent tool for this purpose since it allows to quantify internal exposure. However, the majority of toxicological data on these fungicides come from animal studies and data in humans are limited.

The aim of this project was thus to develop biological monitoring tools in order to assess exposure to captan and folpet in humans. In this perspective, the project was divided into three complementary parts: i) to develop specific and accurate analytical methods in order to quantify captan and folpet metabolites in urine and blood, namely tetrahydrophthalimide (THPI) for captan and phthalimide (PI) and phthalic acid for folpet; ii) to determine the toxicokinetics of the two fungicides in humans by exposing volunteers acutely to low-doses of captan or folpet by oral and dermal routes under semi-controlled conditions and by quantifying the biomarkers in plasma and urine, except phthalic acid which was only measured in urine; iii) to validate the use of the selected biomarkers of exposure to captan and folpet and estimate actual exposures of workers and main exposure routes to these fungicide in the context of a field biomonitoring study in farmers during treatment and harvest activities over seven consecutive days.

This study showed that THPI and PI are both valid and specific biomarkers of exposure to captan and folpet, respectively, in humans. Indeed, the developed methods for these two metabolites are accurate showing more sensitive detection limits than those reported in the literature, good recovery rate (90% for THPI and 75% for PI), linearity ( $R^2 > 0.99$ ) and stability (RSD < 15% for intra- and inter-day precision and accuracy). They allowed determining the kinetic profiles of the two metabolites in healthy volunteers and in workers. These profiles indicate a rapid elimination of both metabolites, since the urinary elimination half-life of THPI was 11.7 h and 18.7 h following an oral and dermal absorption, respectively, and 27.3 h and 28.8 h for PI. They also evidence a low dermal absorption for both fungicides when oral and dermal route are compared. In addition, parallel profiles were observed between PI and phthalic acid, but the administered dose of folpet was mostly recovered as phthalic acid rather than PI. As for the study of farmers, it showed that the dermal route was the main route of exposure. It also pointed out that it is important 1) to perform 24-h complete urine collections rather than collect spot urines, 2) to measure several metabolites to better assess actual exposure, and 3) to rely on the toxicokinetics to help interpret biomonitoring data. Overall, knowledge acquired from this study may be applied to other fungicides or even to other substances.

**Keywords** : captan, folpet, biomonitoring, THPI, PI, phthalic acid, kinetics, human data, field workers.

## Table des matières

Sommaire .....	i
Abstract .....	iii
Table des matières.....	v
Liste des figures .....	vii
Liste des abréviations.....	viii
Remerciements .....	xi
1. Introduction .....	1
1.1. Propriétés antifongiques du captan et folpet .....	2
1.1.1. Propriétés anti-fongiques du captan .....	3
1.1.2. Propriétés anti-fongiques du folpet .....	4
1.2. Les effets sur la santé du captan et du folpet.....	4
1.2.1. Effets du captan.....	5
1.2.2. Effets du folpet.....	8
1.3. La toxicocinétique du captan et du folpet .....	10
1.3.1. Toxicocinétique du captan .....	11
1.3.2. Toxicocinétique du folpet .....	15
1.4. L'exposition au captan et folpet chez les travailleurs .....	18
1.4.1. Les voies d'expositions .....	19
1.4.1.1. L'absorption cutanée.....	20
1.4.1.2. L'absorption orale .....	21
1.4.1.3. L'inhalation .....	22
1.4.2. Les techniques d'application.....	23
1.5. Les outils de mesure de l'exposition privilégiés .....	27
1.5.1. Surveillance biologique et surveillance environnementale.....	28
1.5.2. Les modèles toxicocinétiques .....	30

2. Objectifs .....	33
3. Méthodologie.....	34
4. Résultats .....	38
4.1. Méthodes analytiques.....	38
4.1.1. THPI et PI (Article I) .....	38
4.1.2. Acide phtalique (Article II).....	39
4.2. Étude chez les volontaires exposés en conditions semi-contrôlées.....	39
4.2.1. Exposition orale (Article III).....	39
4.2.2. Exposition cutanée (Article IV) .....	40
4.3. Étude chez les arboriculteurs et les viticulteurs (Article V).....	42
Article I .....	43
Article II .....	86
Article III.....	121
Article IV .....	163
Article V .....	205
5. Discussion .....	254
5.1. Le THPI, PI et acide phtalique comme biomarqueurs d'exposition au captan et folpet (Articles I, II) .....	254
5.2. Toxicocinétiques du THPI, PI et acide phtalique (Articles III et IV) .....	255
5.3. Exposition au captan et folpet des travailleurs (Article V).....	259
5.4. Recommandations et perspectives .....	262
6. Conclusion.....	263
Bibliographie.....	i



## Liste des figures

Figure 1: Structure chimique du captan .....	3
Figure 2: Structure chimique du folpet .....	4
Figure 4: Le métabolisme du captan selon des études in vivo chez des animaux après administration de doses de captan marquées avec du $^{14}\text{C}$ ou du $^{15}\text{S}$ .....	13
Figure 5: Le métabolisme du folpet d'après des études chez l'animal et des études in vitro .....	16
Figure 3: Les principales techniques d'épandage de pesticides dans les vignes en Suisse..	27

## Liste des abréviations

<b>ACGIH</b>	American Conference of Governmental Industrial Hygienist
<b>AUC</b>	Area under the concentration-time curve
<b>AUMC</b>	Area under the first moment of concentration-time curve
<b>CL</b>	Clairance du plasma
<b>CS<sub>2</sub></b>	Disulfure de carbone
<b>DL<sub>50</sub></b>	Dose létale 50
<b>GC-MS</b>	Chromatographie en phase gazeuse couplée à une spectrométrie de masse
<b>GI</b>	Tractus gatro-intestinal
<b>IARC</b>	International Agency for Researc on Cancer
<b>LC/APCI-MS/MS</b>	Chromatographie liquide couplée à la spectrométrie de masse en mode ionisation chimique à pression atmosphérique
<b>MRT</b>	Mean residence time
<b>NIOSH</b>	National Institute of Occupational Safety and Health
<b>3-OH-THPI</b>	3-hydroxy-4,5-cyclohexène-1,2-dicarboximide
<b>PBPK</b>	Physiological-Based Pharmacokinetic model
<b>PBS</b>	Phosphate buffer saline
<b>PI</b>	Phtalimide
<b><i>t</i><sub>1/2</sub></b>	Temps de demi-vie
<b>THPI</b>	Tétrahydrophthalimide

<b>TTCA</b>	Acide thiazolidine-2-thione-4-carboxylique
<b>V<sub>d</sub></b>	Volume de distribution apparent
<b>US EPA</b>	United States Environmental Protection Agency

*À Pierre-Olivier Droz qui a permis la  
réalisation de cette cotutelle*

## Remerciements

Je remercie ma directrice Michèle Bouchard pour son soutien, ses commentaires constructifs, ses conseils et ses recommandations toujours pragmatiques, son partage des connaissances et de son expérience qui m'ont beaucoup apporté tout au long de mon doctorat. Je remercie également ma directrice de l'IST, Brigitta Danuser pour son support, son encouragement et son aide.

J'aimerais aussi remercier Michèle Berode pour son soutien, son aide et le développement analytique de la méthode de l'acide phtalique, Cong Khahn Huynh pour ses conseils pratiques et son aide précieuse pour le développement analytique des méthodes, et Grégory Plateel ainsi que tous les techniciens du laboratoire de l'IST (Christine Arnoux, Christine Kohler, Patrick Schüpfer, Patricia Stephan, Ferdinand Storti, Monique Strebel) qui ont participé au projet et qui m'ont aidé par leur savoir-faire au niveau des analyses et du terrain. Au niveau de l'Université de Montréal, je remercie Ross Thuot pour ses conseils et son aide au laboratoire ainsi que Isabelle Rheault de l'Université du Québec à Montréal (UQAM) pour son temps, ses conseils et son aide dans le développement des méthodes analytiques.

Je souhaite remercier également Gérald Chouinard de l'Institut de Recherche et de Développement en Agroenvironnement (IRDA) pour ses réflexions et son influence vers ce sujet de thèse ainsi que Olivier Viret, non seulement pour son partage des connaissances sur l'exposition des arboriculteurs et des viticulteurs en Suisse, mais aussi pour avoir accepté d'être expert externe sur ce projet et pour tous ses commentaires constructifs et son aide.

Je remercie aussi tous les volontaires, étudiants et agriculteurs, sans qui le projet n'aurait pas pu être réalisé et qui ont vraiment été très consciencieux, attentifs aux protocoles des collectes et soucieux du bon déroulement du projet.

Enfin, je remercie mes collègues de l'IST pour leur soutien, particulièrement Nancy Hopf et David Vernez pour leurs conseils scientifiques et Marie-Cécile Monin pour sa relecture, ainsi que mes collègues de l'Université de Montréal, mais aussi ma famille et mes amis qui m'ont beaucoup supporté moralement.

Ce projet a été financé par l'Agence française de sécurité sanitaire de l'environnement et du travail (AFSSET), et l'Institut de recherche Robert-Sauvé en santé et sécurité du travail du Québec (IRSST) ainsi que la Faculté des études supérieures de Montréal et l'Institut de recherche en santé publique de l'Université de Montréal (IRSPUM) m'ont attribué des bourses pour ce projet de recherche.

# 1. Introduction

Les fongicides constituent une classe de substances chimiques largement répandue en agriculture, notamment en production fruitière. C'est la première classe de pesticides utilisée dans plusieurs pays, dont la Suisse (1), et elle est également largement utilisée au Québec (2). De nombreux travailleurs agricoles sont par conséquent exposés à ces produits. Cependant, il n'est pas toujours facile de mesurer leur exposition de manière spécifique et représentative. La surveillance biologique peut alors s'avérer être un excellent outil lorsque la cinétique des agents étudiés est connue.

Cette approche consiste notamment à mesurer dans des liquides biologiques (ex.: sang, urine) des indicateurs biologiques d'exposition, soit la substance elle-même soit un ou plusieurs de ses métabolites, et permet d'identifier et de quantifier l'exposition avant l'apparition de maladies professionnelles (3). Elle est de plus en plus employée pour mesurer l'exposition des travailleurs aux pesticides. En effet, comme ces produits peuvent s'introduire dans l'organisme par plusieurs voies simultanément et que leur exposition peut provenir de différentes sources et varier selon leur usage, l'utilisation de biomarqueurs permet d'inclure toutes les voies d'exposition en déterminant la quantité de substance qui a été absorbée dans l'organisme (4). La mesure de l'exposition est ainsi plus précise qu'avec la surveillance environnementale.

Le captan et le folpet comptent parmi les fongicides les plus communément utilisés dans le monde et sur de larges variétés de culture (5-29). Toutefois, il n'existe que quelques études qui évaluent les risques chez les travailleurs, ou de façon plus large chez l'humain, pour le captan (11-13;15;16;19;20;22;24;27;28;30-37) et seulement trois pour le folpet (12;30;31).

Afin d'expliquer une recherche sur l'évaluation des risques de travailleurs exposés aux fongicides, et plus spécifiquement au captan et au folpet, à l'aide de la surveillance biologique, les principales caractéristiques du captan et du folpet seront brièvement décrites, puis les effets sur la santé et la toxicocinétique connus pour chacun des fongicides seront présentés avant de définir l'exposition des travailleurs et d'expliquer les outils de mesure privilégiés pour quantifier l'exposition aux deux fongicides.

## **1.1. Propriétés antifongiques du captan et folpet**

Les fongicides peuvent être divisés en trois catégories : protecteurs (ou de contact), curatifs (ou pénétrants) ou inhibiteurs (ou systémiques). Les fongicides protecteurs préviennent les infections fongiques en agissant comme des sporicides ou en modifiant l'environnement physiologique de la surface de la feuille; ils sont donc appliqués avant l'apparition de champignons phytopathogènes. Les curatifs sont utilisés lorsque l'infection a déjà affecté une partie de la plante. Ils préviennent le développement de l'hyphe, ou jeune mycélium fongique, qui croît dans l'épiderme de la plante en pénétrant dans la cuticule de celle-ci

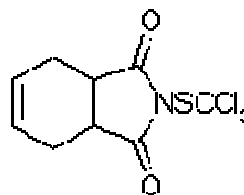


pour le détruire. Quant aux fongicides inhibiteurs, ils contrôlent le développement des infections fongiques après l'apparition des symptômes, qui ont lieu généralement après la sporulation, en tuant les nouveaux spores et le mycélium, et en pénétrant dans la cuticule de la plante à un niveau sous-cutané (38).

Le captan et le folpet appartiennent au groupe des phthalimides. Ils sont considérés comme des fongicides protecteurs et non systémiques, c'est-à-dire qu'ils agissent par contact (39).

### 1.1.1. Propriétés anti-fongiques du captan

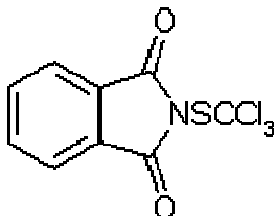
Le captan (CAS n°133-06-2), ou N-trichlorométhylthio-4-cyclohexène-1,2-dicarboximide, est caractérisé par un anneau cyclohexane (voir Figure 1), c'est ce qui le différencie du folpet qui possède un anneau aromatique. C'est un réactif non spécifique au groupement thiol qui peut inhiber la respiration de plusieurs espèces de champignons et bactéries (40;41). Plus spécifiquement, il induit une dépression de l'activité de l'enzyme glutamine deshydrogénase et une stimulation de la diphosphopyridine nucléotide oxydase chez les champignons (42;43). Il est considéré comme étant un fongicide protecteur et curatif pour les plantes.



*Figure 1: Structure chimique du captan*

### 1.1.2. Propriétés anti-fongiques du folpet

Le folpet (CAS n°133-07-3), ou N-(trichlorométhylthio)phtalimide, est quant à lui un fongicide protecteur de contact à large spectre. Il est utilisé pour contrôler le mildiou ou d'autres dommages occasionnés par des champignons sur les plantes en interagissant avec les groupes thiols présents dans les protéines des champignons afin de les dénaturer (44). Sa structure chimique est présentée à la Figure 2.



*Figure 2: Structure chimique du folpet*

## 1.2. Les effets sur la santé du captan et du folpet

Les effets sur la santé produits par le captan et le folpet ne sont pas très bien connus chez l'humain et il existe encore des incertitudes concernant les études chez les animaux. Aucune toxicité directe n'a été reportée chez l'humain, néanmoins, ces deux produits sont considérés comme des sensibilisants et des irritants sévères pour les yeux et la peau (10;40;44-51).

### 1.2.1. Effets du captan

Le captan a une toxicité aiguë orale faible et il n'y a pas d'autres signes de toxicité directe rapportés que des dermatites allergiques et des conjonctives chez l'humain. En effet, la dose létale 50 (DL<sub>50</sub>) orale est élevée chez le rat. Elle est de l'ordre de 10 000 mg/kg (38;45;51).

Néanmoins, il est reconnu pour être sensibilisant suite à un contact cutané ou une exposition par inhalation (R43), pour provoquer des dommages sévères aux yeux (R41) ou encore être toxique par inhalation (R23), plus particulièrement lorsqu'il est inhalé sous forme de poussière (48;52).

Il a été classé B2 par l'Agence environnementale américaine (USEPA), c'est-à-dire cancérigène probable pour l'humain avec preuve d'effet cancérigène chez les animaux mais pas d'étude ou de résultat exploitable chez l'humain, suite aux résultats d'études chez des souris exposés chroniquement au captan à des fortes doses, constatant une augmentation de l'incidence de tumeurs du duodénum. Toutefois, en 2004, sa classification a été révisée et modifiée pour « not likely » considérant que les doses favorisant la prolifération des tumeurs et des hyperplasies cellulaires par cytotoxicité étaient beaucoup plus élevées que celles auxquelles les travailleurs étaient exposés (51;53). Il est également classé groupe 3 par l'Agence Internationale de Recherche sur le Cancer (IARC), à savoir

non cancérigène pour l'humain (54) et A3 ou cancérigène pour les animaux par l'American Conference of Governmental Industrial Hygienist (ACGIH) (52).

Toutes ces agences se basent sur une étude qui a démontré une augmentation de l'incidence de tumeurs (adénomes et adénocarcinomes) au niveau de la partie proximal du duodénum pour les deux sexes chez la souris suite à une administration chronique de grande quantité de captan dans leur alimentation, à savoir 120 mg/kg/jour chez les femelles et 900 mg/kg/jour chez les mâles, ce qui excède la dose maximale tolérée (41;51;55). D'autres études montrent cependant que le captan n'est pas génotoxique *in vivo* du fait qu'il existe pour toutes les études sur la cancérigénicité chez la souris une dose seuil sous laquelle il n'y a pas d'apparition de tumeurs. Ces études suggèrent alors un mécanisme plutôt épigénétique qui induit la production de tumeurs (56-58).

Selon quelques études (51;59;60), le groupe trichlorométhylthio pourrait être à l'origine de la toxicité du captan puisqu'il réagit très rapidement avec les groupes thiols. Toutefois, il semble que ce ne soit qu'avec les thiols insolubles qu'une réaction toxique apparaisse alors qu'une interaction avec des thiols solubles conduirait à des processus de détoxification (61).

Quant aux études sur la tératogénicité et les effets sur la reproduction, elles ont relaté des résultats contradictoires. Ainsi, aucun effet tératogène n'a pu être identifié chez la souris, le

rat, le chien ou les primates. Cependant, quelques effets tératogènes ont été remarqués chez le lapin (déficience au niveau du squelette du fœtus, diminution du poids du fœtus et altération de la croissance) et le hamster (déficience du système nerveux central) lorsque les femelles étaient exposés durant une certaine période de la gestation seulement (41;51;62). Sur la base de ces données, il est difficile de conclure que le captan est tératogène ou peut induire des effets néfastes sur la reproduction, d'autant plus qu'aucune donnée n'est disponible pour l'humain.

Sur la base de ces dernières données, une dose journalière acceptable (DJA) chez l'humain de 0,1 mg/kg de poids corporel a été établie pour le captan (valeur estimée sur un NOAEL de 12,5 mg/kg de poids corporel / jour avec un facteur de sécurité de 100 selon une étude de reproduction chez les rats (41).

Finalement, d'autres effets sur les enzymes sont rapportés par quelques études (55;63). Par exemple, le captan pourrait inhiber l'utilisation de glucose ainsi que la phosphorylation oxydative des mitochondries hépatiques chez le rat et l'activité de la  $\text{Ca}^{2+}$ -transport-ATPase des érythrocytes humains. Cependant, ces résultats sont issus d'études *in vitro* ou animales et n'ont pas été clairement confirmés.

En résumé, très peu de données sont disponibles pour l'humain et elles sont encore précaires ou controversées en ce qui concerne les études sur les animaux. De plus, il n'y a pas de preuves évidentes que le captan est cancérigène, mutagène ou tératogène.

### **1.2.2. Effets du folpet**

Les données concernant les effets du folpet sont encore plus limitées que pour le captan. Néanmoins, il est aussi reconnu pour entraîner une sensibilité par contact avec la peau (R43) et considéré comme moyennement toxique pour ses effets sur les voies respiratoires (toxique de catégorie II ou R23, à savoir une irritation possible de la gorge et des voies respiratoires), ainsi que pour ses effets néfastes possibles aux yeux (R36) (44). Par ailleurs, comme pour le captan, la dose létale 50 (DL<sub>50</sub>) orale est élevée chez le rat. Elle est aussi de l'ordre de 10 000 mg/kg (38;45;51).

Le folpet n'est pas inscrit dans la liste des substances de l'ACGIH ni de l'IARC, mais il est classé B2 par l'US EPA. Cette classification s'appuie sur une étude mettant en évidence une augmentation de l'incidence d'adénomes et de carcinomes dans le duodénum de souris des deux sexes (44;64). Des études chez le rat ont également montré des effets cancérigènes puisqu'une augmentation de l'incidence d'adénomes et de carcinomes des cellules C de la thyroïde ainsi que des tumeurs des cellules interstitielles des testicules ont été observées. Toutefois, aucune augmentation d'incidence de tumeurs intestinales n'a été constatée chez le rat même si il peut y avoir un accroissement de la sévérité

d'hyperkératose de l'œsophage et de l'estomac (44;64). Ceci est également mis en évidence dans l'étude de Cohen *et al.* (64) pour démontrer que le folpet n'est pas un cancérigène chez l'humain, tout comme le captan.

Quant aux effets mutagènes, le folpet peut se comporter comme un composé génotoxique *in vitro* et induire des mutations géniques et des dommages à l'ADN de certaines bactéries (*E. coli*, *S. typhimurium*) (44;58). Ces résultats ne sont toutefois pas documentés *in vivo*, et il devient difficile de conclure sur l'effet mutagène du folpet.

Par ailleurs, une application cutanée répétée du fongicide chez le rat a induit de l'hyperkératose, un épaissement anormal de l'épiderme et des ulcères au niveau local alors que les seuls effets systémiques observés ont été une réduction du gain de poids corporel chez les mâles et les femelles (44). Ces résultats montrent également que le folpet est difficilement absorbé par la peau.

Au niveau des effets tératogènes, il a été prouvé que le folpet pouvait produire des effets sur le développement lors d'études chez des lapins et chez des rats (44). De même, certaines études chez les animaux, notamment chez le rat, la souris, le lapin et le hamster, constatent un effet sur la reproduction après une exposition au fongicide, mais les résultats restent incertains (44).

Sur la base de ces données, la dose journalière acceptable a été établie à 0,16 mg/kg de poids corporel (valeur estimée sur un NOEL de 10 mg/kg/jour avec un facteur de sécurité de 100 pour une étude de un an sur la toxicité chez les chiens, une étude de deux ans sur la toxicité et la cancérogénicité chez des rats et des études de toxicité sur la reproduction chez des rats et des lapins (44).

Comme pour le captan, il est difficile de conclure sur les effets confirmés et vérifiés d'une exposition au folpet chez l'humain, les informations disponibles étant encore plus restreintes.

### **1.3. La toxicocinétique du captan et du folpet**

Le métabolisme du captan et du folpet chez les plantes n'est presque pas connu, mais certains auteurs (65;66) suggèrent que la dégradation de ces composés se fait principalement par hydrolyse. Chez l'animal, plusieurs voies de métabolisme existent et pourraient induire une toxicité cellulaire (38). D'ailleurs, malgré leurs structures chimiques similaires, il existe des différences confirmées entre le métabolisme du captan et du folpet (10).



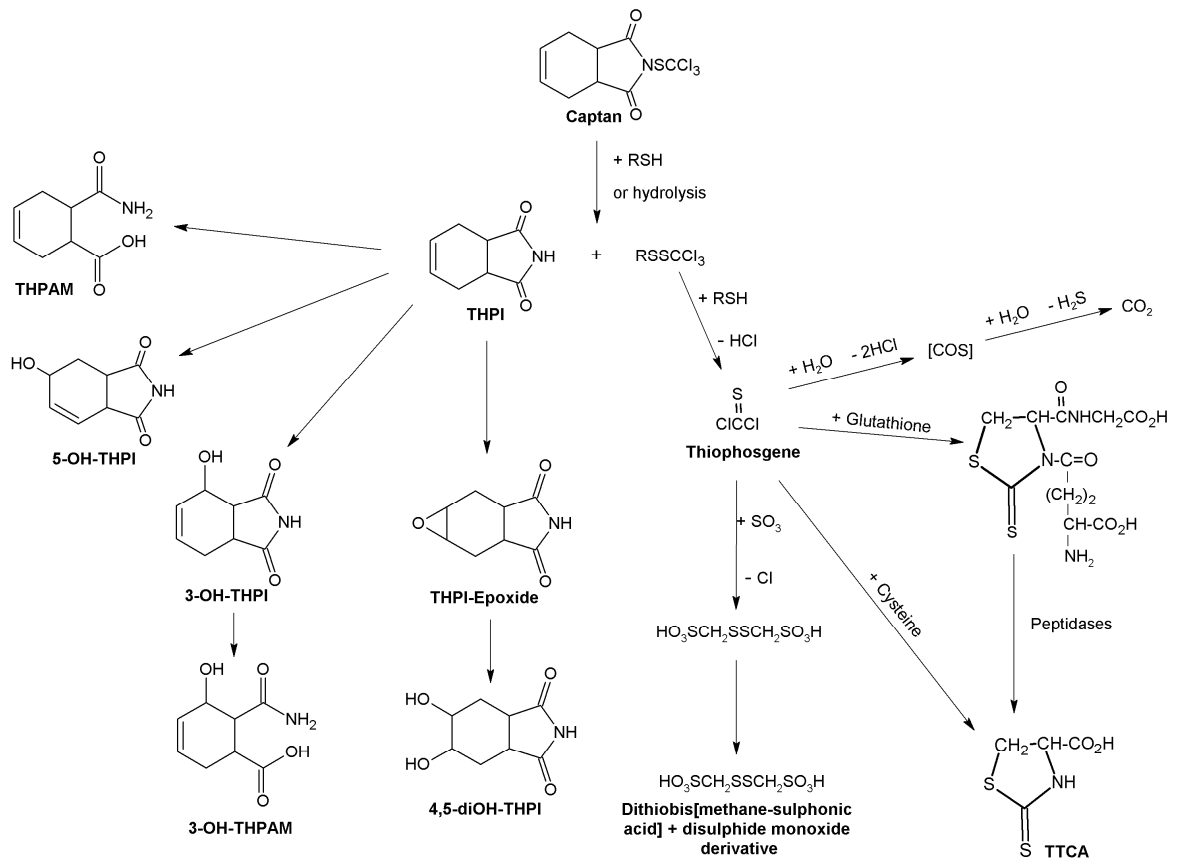
### 1.3.1. Toxicocinétique du captan

La majorité des connaissances toxicologiques sur le captan provient d'études sur les animaux, et en particulier sur les rats et les souris. En se basant sur ces études animales, il semble que le captan soit dégradé en plusieurs métabolites, dont deux métabolites urinaires connus chez l'humain, à savoir le tetrahydrophthalimide (THPI ou 4,5-cyclohexene-1,2-dicarboximide) et l'acide thiazolidine-2-thione-4-carboxylique (TTCA), ainsi qu'un métabolite très réactif avec les groupements thiols et d'autres groupements fonctionnels, le thiophosgène (15;25). Ce dernier est commun au folpet et s'avère avoir une très courte demi-vie et un métabolite instable qui peut réagir avec les enzymes comprenant le radical sulfhydryl-, amino- et hydroxyl- (10;38;67;68). Ces résidus peuvent donc être difficilement mesurables dans les matrices biologiques.

La première étape du métabolisme du captan est l'association du captan avec des thiols, ce qui conduit au clivage de la liaison N-S du captan pour former le THPI et le chlorure de thiocarbonyle, un dérivé du groupe trichlorométhylthio (10;67;69). Ce clivage a lieu généralement au niveau des régions alcalines du tractus gastro-intestinal après absorption orale ou dans le sang lors d'une exposition cutanée. Le chlorure de thiocarbonyle peut lui aussi réagir avec des thiols pour former le thiophosgène en perdant son acide chlorhydrique (HCl). Puis, à partir du thiophosgène, trois métabolites peuvent être formés (40;50;67;68). Ainsi, il peut interagir avec la cystéine ou le glutathion pour former le TTCA, se lier avec un sulfite (d'origine endogène) pour conduire à la formation du dithiobis (ou acide

méthanesulfonique), ou être oxydé ou hydrolysé et produire du dioxyde de carbone (CO<sub>2</sub>). Quant au THPI, c'est un métabolite relativement stable qui peut être métabolisé en sept composés distincts selon qu'il subit une hydroxylation, une époxidation, une scission ou encore un réarrangement : le 3-hydroxy-4,5-cyclohexène-1,2-dicarboximide (3-OH-THPI), le 5-hydroxy-3,4-cyclohexène-1,2-dicarboximide (5-OH-THPI), le 6-hydroxy-1-amido-2-carboxy-4,5-cyclohexène (acide 3-OH-THPI-amique), le 1-amido-2-carboxy-4,5-cyclohexène (THPAM), le 4,5-dihydroxy-1,2-dicarboximide (4,5-diOH-THPI), et le 4,5-époxy-1,2-dicarboximide (THPI-époxyde) (39;70;71). La figure 4 résume le métabolisme du captan chez le rat, et qui pourrait être vraisemblablement généralisé à l'humain (15).

Concernant l'absorption, la distribution et l'élimination du captan, des études chez le rat ont montré que suite à une administration orale de <sup>35</sup>S-captan, plus de 90% du produit radioactif a été éliminé dans les urines et les fèces sous la forme de métabolites dans les 24 h suivant l'administration alors que très peu (entre 0.01 et 0.05 %) est resté incorporé aux protéines et aux acides nucléiques dans le corps (67). Ceci suggère un métabolisme rapide dans l'intestin (61;72). Quant à l'étude avec une application de captan radioactif (C<sup>14</sup>) sur la peau de rats, elle a établi que le captan pénètre rapidement par la peau, malgré un faible taux d'absorption, et est aussi éliminé en peu de temps et principalement par les urines (73-75).



**Figure 3: Le métabolisme du captan selon des études *in vivo* chez des animaux après administration de doses de captan marquées avec du <sup>14</sup>C ou du <sup>15</sup>S (15;70).**

Chez l'humain, les seules données disponibles proviennent de quelques études ponctuelles utilisant la surveillance biologique chez des travailleurs (13;16;19;25-28;32-34) et d'une étude de Krieger et Thongsinthusak (15) sur deux volontaires exposés par voie orale et par voie cutanée. Cette dernière étude a montré qu'après une administration orale, le TTCA était présent en un peu plus grande quantité dans l'urine que le THPI, même si l'ordre de grandeur était relativement similaire. De plus, les deux métabolites urinaires ont été

détectés dans les urines après une période de 12 h suivant une dose orale de 0.1 mg/kg de poids corporel et après une période de 12 à 24 h suivant une dose orale de 1 mg/kg de poids corporel. De 1 à 3% de la dose administrée a été retrouvée sous forme de THPI et de 4 à 9% sous forme de TTCA. Suite à l'application cutanée, Krieger et Thongsinthusak (15) n'ont pas pu détecter les métabolites urinaires. La dose appliquée, soit une solution de captan de 40  $\mu\text{g}/\text{cm}^2$  appliquée sur les mains et l'avant bras, était peut-être trop faible pour obtenir des résultats.

Ces résultats suggèrent que la cinétique du captan est dépendante de la voie d'exposition. Ils indiquent également que d'autres métabolites seraient peut-être à considérer en plus du TTCA et du THPI pour obtenir une meilleure évaluation de l'exposition. Toutefois, beaucoup d'auteurs s'accordent à favoriser le THPI comme biomarqueur du captan en raison de sa stabilité bien établie et démontrée tant chez l'animal que chez les travailleurs (16;19;25;32). Par ailleurs, il est important de préciser que le TTCA est considéré comme un métabolite non spécifique au captan du fait qu'il est également un métabolite urinaire reconnu du disulfure de carbone ( $\text{CS}_2$ ) (25;76-78).

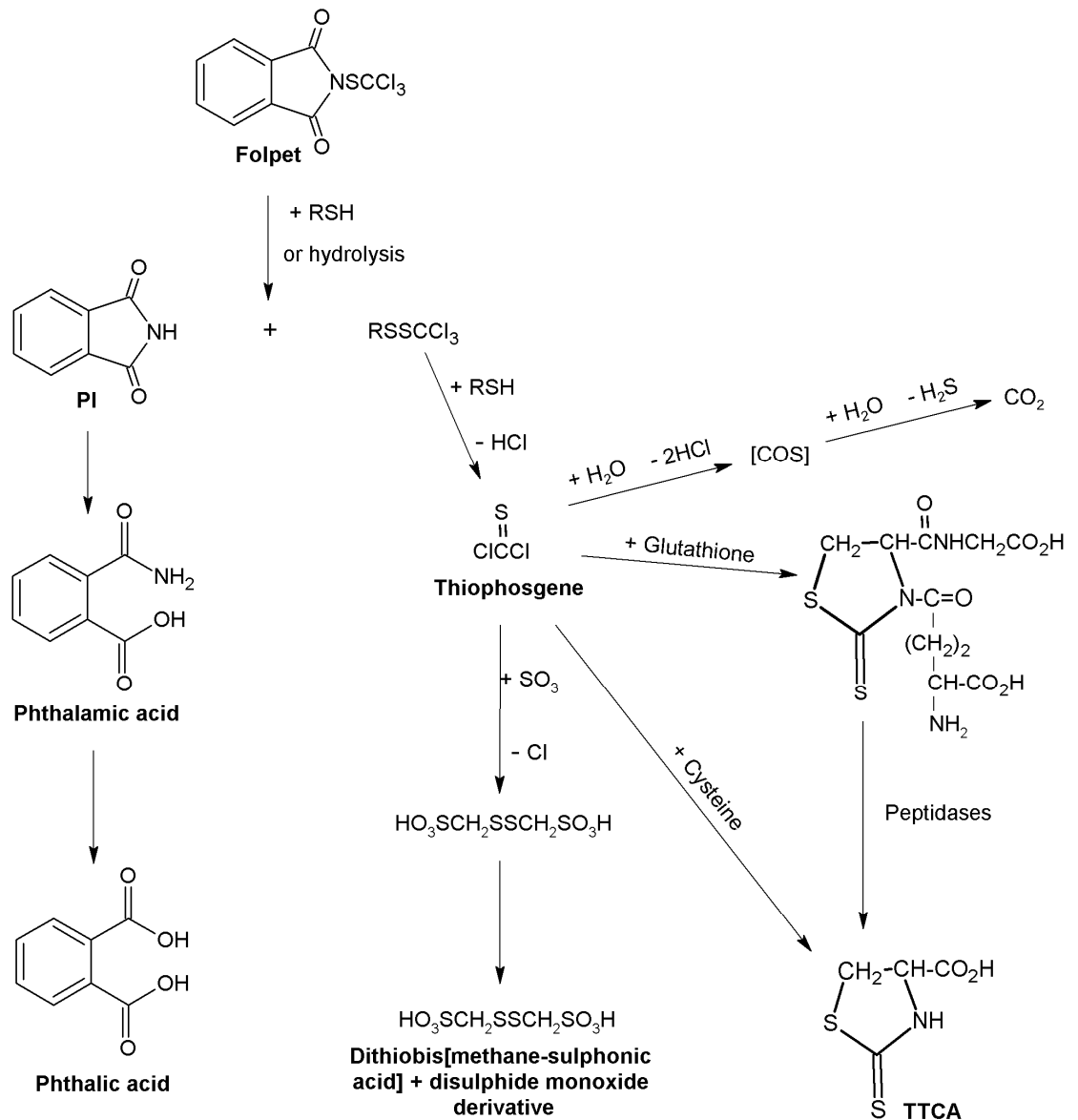
Finalement, il est important de noter qu'il n'existe presque pas de données sur la voie respiratoire chez les animaux ni chez l'humain.

### 1.3.2. Toxicocinétique du folpet

Tout comme pour le captan, la majorité des connaissances toxicocinétiques sur le folpet provient d'études sur les animaux, et en particulier sur les rats et les souris. Néanmoins, les informations concernant la biotransformation ainsi que l'absorption, la distribution et l'élimination de ce produit sont très limitées chez l'animal et presque inexistantes pour l'humain. La littérature mentionne toutefois quelques métabolites pour le folpet, notamment le thiophosgène, le phtalimide, l'acide phtalamique et des dérivés de la chaîne trichlorométhylthioside (6;10;44;69;79-81). La Figure 5 résume le métabolisme connu pour ce produit.

Ainsi, comme le met en évidence la Figure 5, le folpet est d'abord hydrolysé en phtalimide et en chlorure de thiocarbonyle, un dérivé du groupe trichlorométhylthio. Puis, le phtalimide est aussi rapidement hydrolysé pour former premièrement l'acide phtalamique, puis l'acide phtalique et de l'ammoniac ( $\text{NH}_3$ ) (6;10;44;69;79-81). Quant à la formation du thiosphogène, certains auteurs (10;44;80-82) suggèrent que ce métabolite se forme en présence de composés sulfhydryles, tels que la cystéine ou le glutathion. En conséquence, il est possible d'envisager que le groupe réactif trichlorométhylthio se clive en ions chlorures pour former le thiophosgène en présence d'un groupe sulfhydryle (thiols sanguins ou tissulaires) en milieu alcalin ou neutre (44). Ces mêmes auteurs (10;44;80-82) mentionnent que les métabolites formés à partir du groupement trichlorométhylthio pour le captan sont

semblables pour le folpet puisque la chaîne trichlorométhylthioside est similaire pour les deux produits.



**Figure 4: Le métabolisme du folpet d'après des études chez l'animal et des études *in vitro* (6;10;44;69;79-81).**

D'autre part, il semble que ni le folpet ni ses métabolites ne s'accumulent dans les tissus et qu'ils sont rapidement excrétés dans l'urine principalement, selon les données animales disponibles (75). En effet, il a été montré qu'en présence de thiols sanguins ou tissulaires, le folpet est métabolisé très rapidement et que les mêmes métabolites que ceux produits par hydrolyse sont formés (69). De même, il semble que la demi-vie du folpet dans le sang soit d'approximativement une minute du fait que sa liaison sulphénimide soit rapidement clivée pour former le phtalimide (6;10;69;79). D'ailleurs, Gordon *et al.* (69), dans leur étude effectuée à partir de sang humain, ont révélé qu'il était dégradé en phtalimide avec une demi-vie calculée de 4.9 secondes. Cependant, il ne semble pas exister d'études chez des volontaires ou des travailleurs pour corroborer ces résultats.

En ce qui attrait à l'absorption, la distribution et l'élimination du folpet, des études chez des rats démontrent qu'après administration orale, le folpet est facilement absorbé et très rapidement éliminé par les urines, même si de l'acide phtalimique a été retrouvé dans les fèces (10;80). De même, une étude sur des souris et des rats chez lesquels le produit radioactif ( $C^{14}$ ) a été administré oralement rapporte que toute la radioactivité a été éliminée au cours des 24 h suivant l'administration (81). Quant à l'administration par voie cutanée, une étude chez des rats dénote que le folpet est très peu absorbé par cette voie, soit 2.7% de la dose administrée se retrouve dans l'urine et les fèces sur une période de 72 h (75). En ce qui a trait à la voie respiratoire, il n'existe pas de données.

En somme, étant donné qu'il n'existe que très peu d'études sur le folpet et le captan chez l'humain, les données à disposition pour développer des indicateurs biologiques sont donc très limitées pour ces deux fongicides. Il manque notamment des connaissances détaillées sur leur comportement cinétique chez l'homme.

#### **1.4. L'exposition au captan et folpet chez les travailleurs**

En Suisse, les traitements utilisant des fongicides sont assez fréquents en viticulture puisque l'épandage des fongicides, surtout le folpet et le captan, commence en avril et se termine mi-août, à raison d'une application tous les 10 à 15 jours. Ceci fait une moyenne de 8 applications par année (83). En arboriculture, le captan est aussi un des fongicides les plus utilisés et le folpet est surtout épandu en fin de saison pour prévenir les maladies de conservation (communication de la station de recherche fédérale Agroscope Changins, Suisse; 2006). Au Québec, le captan est également très utilisé en arboriculture et est appliqué entre 4 à 6 fois par saison (communication de l'Institut de Recherche et de Développement en Agroenvironnement (IRDA), Canada; 2006).

Cependant, les travailleurs exposés aux fongicides ne procèdent pas toujours qu'à l'épandage et à la préparation de la bouillie. De nombreux autres travaux dans les cultures peuvent aussi conduire à des expositions par contact direct avec la végétation traitée, tels que le ramassage des fruits ou l'entretien des plants (i.e. ébourgeonnage, élagage,



éclaircissage). De plus, les travailleurs sont plus ou moins bien protégés selon les techniques d'application qu'ils emploient et ont tendance à ne porter que très peu voire aucune protection lorsqu'ils font des travaux d'entretien de la végétation entre deux épandages (84).

Par ailleurs, le rapport du Comité fédéral-provincial-territorial de l'hygiène du milieu et du travail (85) mentionne que la concentration atmosphérique des fongicides est plus élevée durant la pulvérisation par air comprimé mais surtout qu'ils sont encore décelés en concentrations appréciables dans l'air au cours des 6 à 12 h suivant l'épandage dans un verger de citronniers, soit de l'ordre de  $0,043 \mu\text{g}/\text{m}^3$  à  $0,5 \mu\text{g}/\text{m}^3$  pour le captan (aucune donnée n'est disponible pour le folpet). Quant à la distance au sol, des résidus de fongicides ont été mesurés jusqu'à une distance de 30 mètres de la zone d'épandage, toutefois il n'a pas été précisé s'il y avait présence de vent. Il faut donc prendre en compte ces possibilités d'exposition dans l'estimation de l'exposition chez les travailleurs.

#### **1.4.1. Les voies d'expositions**

La plupart des études sur les pesticides s'entendent pour considérer la voie cutanée comme la voie d'absorption majeure pour les expositions professionnelles (7-9;21;22;32;86-91). Plus précisément, selon Fenske *et al.* (9), Ngo *et al.* (88), Vermeulen *et al.* (92), la contribution relative serait environ de 10% pour les voies respiratoires et de 90% pour la voie cutanée.

#### **1.4.1.1. L'absorption cutanée**

L'estimation de l'absorption cutanée à partir de la dose externe d'exposition s'avère souvent complexe et difficile (91). En effet, la relation entre l'exposition et l'étendue de l'absorption est parfois non linéaire et par conséquent difficile à mesurer. De plus, l'étendue à laquelle les résidus de pesticides sont absorbés au point de contact de la peau n'est pas très bien caractérisée étant donné que plusieurs facteurs peuvent influencer la pénétration des composés, tels que les propriétés chimiques du pesticide, les conditions environnementales, le véhicule dans lequel la substance est mélangée pour son application, les parties du corps exposées, la nature et l'hydratation de la peau, l'occlusion de la peau, la variabilité interindividuelle, l'âge, l'hygiène personnelle (fréquence de lavage des mains), les habitudes de travail, la nature du travail, la dose ainsi que la durée de contact avec la peau (3;7;8;32;88;91;93-112).

Il faut également considérer que la peau peut absorber directement la substance lorsqu'elle est présente sous forme de particule dans l'air, mais elle peut aussi être transférée seulement quand la peau est en contact avec des surfaces contaminées ainsi que lorsque qu'une partie du corps est submergée dans la substance (89). Le contaminant peut également être en contact avec la peau sans pour autant être absorbé dans l'organisme, soit en raison de l'évaporation soit par d'autres processus tels que le lavage ou le frottement (9;86).

Le port de vêtements ou de matériels de protection peut aussi modifier le taux d'absorption des contaminants par la peau, d'autant plus que ces moyens de protection ne réduisent pas toujours totalement l'exposition de manière efficace selon Baldi *et al.* (5), Benford *et al.* (113) et Lebailly *et al.* (114). En effet, ces vêtements peuvent être contraignants à porter (surtout en période de chaleur), leur perméabilité n'est pas toujours assurée, une partie des contaminants peut quand même se déposer sur les parties non protégées du corps (notamment lorsque ces protections sont enlevées) et une certaine contamination de la couche intérieure des vêtements de protection peut avoir lieu (5;89;113;114).

#### **1.4.1.2. L'absorption orale**

L'absorption orale chez les travailleurs provient essentiellement d'un manque d'hygiène après avoir manipulé les produits. Notamment lorsque les personnes mangent ou fument avec des mains contaminées (90;113).

Toutefois, l'absorption pourrait aussi résulter d'une ingestion d'aliments contaminés car comme le montre certaines études, des résidus de captan et de folpet sont présents sur les fruits et les légumes prêts à être consommés (115-119). Dans ce cas, l'absorption ne provient pas du milieu professionnel mais elle contribue à un niveau de base d'exposition.

### 1.4.1.3. L'inhalation

L'absorption par inhalation est une voie d'exposition qui semble très mineure chez les travailleurs exposés aux pesticides par rapport à la voie cutanée. Selon une étude de Baldi *et al.* (5) chez des travailleurs en viticulture, l'exposition par les voies respiratoires représentait environ 2,9% de la contamination totale alors que l'exposition cutanée journalière contribuait en moyenne pour 51,7% de la contamination totale lors des activités de préparation et de 22,5% lors de l'application. De même, Dowling et Seiber (120) rapportent que les contaminants peu volatils comme les pesticides ne sont pas absorbés de manière significative par les voies respiratoires chez les applicateurs.

Néanmoins, le risque d'inhaler des pesticides peut être plus grand chez les travailleurs dans certaines conditions particulières. Ainsi, lorsque la préparation de la bouillie s'effectue sur des lieux mal ventilés ou si la technique d'application engendre des nuages de fines particules ou gouttelettes dispersées dans l'air, comme lors de l'utilisation de l'atomiseur à dos ou d'un tracteur sans cabine, l'exposition par les voies respiratoires peut être plus importante (120;121). De même, l'étude de de Cock *et al.* (8) montre que l'exposition par inhalation s'avère semblable pour tous les applicateurs, qu'ils aient accès à un tracteur avec ou sans cabine. Toutefois, certains facteurs ou comportements pourraient avoir une influence sur l'estimation de l'exposition par cette voie d'absorption, notamment la façon de travailler, les attitudes face à l'hygiène ou encore l'équipement utilisé (8;114).

D'autre part, bien que l'absorption des contaminants par les voies respiratoires varie selon leur taille, il reste que la loi de Henry est un paramètre très important pour les pesticides, comme le captan et le folpet, qui sont appliqués dans une solution aqueuse (5;120). Cette loi permet de calculer pour un contaminant donné, le ratio entre sa pression à l'état de vapeur et sa fraction molaire soluble dans l'eau afin de déterminer la concentration maximale de ce composé en solution en équilibre avec l'atmosphère qui contient ce composé (119). La valeur estimée pour le captan est donc de  $6 \times 10^{-6}$  atm m<sup>3</sup>/mol pour une solubilité dans l'eau de 0,5 g/ m<sup>3</sup> et une pression à l'état de vapeur de  $1 \times 10^{-8}$  atm à 20°C (120).

#### **1.4.2. Les techniques d'application**

Les traitements au captan et folpet en arboriculture et viticulture sont effectués soit par lance (gun), soit par atomiseur à dos, soit à l'aide d'un pulvérisateur tracté (tracteur) ou perlé (chenillard), soit encore par hélicoptère. Cependant, les moyens de traitement utilisés sont essentiellement choisis en fonction de la morphologie du terrain, en particulier pour les vignes.

La lance, ou gun (Figure 3a), est un système utilisé seulement pour traiter les vignes en pente très escarpée. Il s'agit d'un tuyau à haute pression (50 bar) relié à un réservoir. La bouillie contenue dans le réservoir est alors amenée par un tuyau fixe jusqu'à la zone à traiter grâce à une pompe. Il est possible de connecter le tuyau aux différents robinets

placés tout au long des vignes (Communication de l'École d'Agriculture de Châteauneuf, Suisse; 2006). C'est un système qui tend à disparaître en raison de son manque de facilité à utiliser et des importantes manipulations à effectuer avec les produits.

Le chenillard (Figure 3b) est lui aussi essentiellement utilisé dans les vignes situées dans des endroits plus ou moins escarpés, ou en pente modérée. Il consiste en un réservoir fixé sur des chenillettes afin de faciliter le transport de la bouillie et par conséquent de réduire l'effort des travailleurs. Cet appareil offre également une mobilité relativement bonne, mais demande beaucoup de dextérité. Afin d'éviter de parcourir de trop grandes distances avec le chenillard, le remplissage du réservoir s'effectue par transvasement de la préparation à partir du réservoir d'un tracteur. Le produit est par la suite projeté sur les plantes par injection du liquide dans un courant d'air produit par une hélice (communication de l'École d'Agriculture de Châteauneuf, Suisse; 2006). Le travailleur utilisant cet appareil doit porter une combinaison de protection ainsi que des gants et un casque.

Le tracteur (Figure 3c) est utilisé tant dans les vignes que dans les vergers. C'est d'ailleurs la principale technique d'application employée en arboriculture en Suisse et au Québec. La plupart des tracteurs en Suisse comprennent une cabine, mais au Québec, il reste environ 30 % des tracteurs utilisés pour épandre des pesticides qui ne sont pas munis de cabine (communication de Gérald Chouinard, IRDA, Canada; 2006).

Les tracteurs sont équipés d'un réservoir de volume variable (300 – 500 L). Une turbine, aussi appelée buse, est connectée au réservoir et permet l'épandage du produit liquide sous forme d'aérosol, tout comme le chenillard. En Suisse, le débit des buses selon la surface foliaire à traiter est adapté à la culture afin de contrôler et d'optimiser la quantité de produit pulvérisée et de limiter la propagation du produit dans l'environnement (122). Cette technique n'est pas encore appliquée au Québec. Toutefois, ce moyen d'application des pesticides n'est utilisé que pour des terrains relativement plats, mais pas pour les terrains pentus en raison des dimensions de l'engin, du manque d'espace manœuvrable et de l'instabilité du sol après de fortes pluies (Communication de l'École d'Agriculture de Châteauneuf, Suisse; 2006).

Par ailleurs, un tracteur à cabine fermée protège les travailleurs de l'exposition aux pesticides durant l'épandage, même si cela n'a pas été clairement observé dans l'étude de Cock *et al.* (120), puisqu'un filtre à charbon actif est situé sur l'ouverture d'aération. Ce filtre est changé approximativement chaque année (Communication de l'École d'Agriculture de Châteauneuf, Suisse; 2006). En conséquence, les travailleurs employant cette technique ne portent presque pas de protection à l'exception de gants et d'un masque lors de la préparation de la bouillie et du versement du mélange dans le réservoir.

Quant à l'atomiseur à dos (Figure 3d), il permet un traitement plus localisé et est parfois utilisé en complément d'un traitement aérien dans les vignes afin d'augmenter son

efficacité. Il s'agit d'un réservoir d'une dizaine de litres qui se porte sur le dos auquel est relié à un canon qui projette la bouillie sous forme d'aérosol comme pour les autres moyens de traitement (communication de l'École d'Agriculture de Châteauneuf, Suisse; 2006). C'est certainement la technique d'application qui présente le plus de risque d'exposition au produit pour le travailleur. Il faut donc que celui-ci porte un équipement de protection complet.

Finalement, lorsque les terrains sont trop escarpés, il arrive qu'il faille recourir à l'hélicoptère. C'est alors une entreprise privée qui procède à l'épandage à la demande de l'agriculteur. Le pilote est relativement bien protégé puisqu'il se trouve dans une cabine fermée, par contre l'exposition est beaucoup plus importante pour les personnes demeurant à proximité de la zone traitée. Ce moyen de traitement est désormais interdit au Québec.





a) Épandage à l'aide de la lance



b) Épandage à l'aide du chenillard



c) Épandage par tracteur avec cabine



d) Épandage à l'aide de l'atomiseur à dos

**Figure 5: Les principales techniques d'épandage de pesticides dans les vignes en Suisse**

## 1.5. Les outils de mesure de l'exposition privilégiés

Cette section a pour but d'expliquer les méthodes privilégiées dans ce projet et reconnues par la communauté scientifique pour évaluer précisément l'exposition des travailleurs au captan ou au folpet. Ainsi, la surveillance biologique est préférée à la surveillance environnementale du fait qu'elle mesure plus spécifiquement l'exposition à une substance. De plus, à partir des données biologiques obtenues, des modèles mathématiques peuvent être élaborés pour reconstituer l'exposition et la dose absorbée, ce qui est plus difficile à effectuer avec certaines méthodes traditionnelles.

### **1.5.1. Surveillance biologique et surveillance environnementale**

Traditionnellement la surveillance de l'exposition des travailleurs aux substances chimiques se fait par mesure de celles-ci dans l'air à la place de travail. Cependant, ces mesures ne permettent pas toujours de déterminer de façon fiable la dose absorbée et souvent l'exposition est surestimée. L'approche la plus appropriée pour évaluer la dose réellement absorbée s'avère souvent être la surveillance biologique (91;123;124).

La surveillance biologique a été définie par Woollen (91) et le National Research Council (125) comme étant une méthode permettant d'évaluer l'exposition humaine ou l'absorption de produits chimiques en mesurant le produit mère ou ses métabolites dans les tissus humains ou des matrices biologiques (i.e. le sang, l'urine, l'air expiré, la salive, les cheveux ou les poils, le lait maternel et le méconium). Le choix de la matrice dépend de la substance étudiée, et plus précisément de sa persistance, de son affinité et de sa demi-vie dans ce milieu biologique (126). L'urine et le sang sont toutefois les deux matrices les plus fréquemment utilisées comme elles ne sont pas spécifiques à certains produits chimiques ou à certaines populations, à la différence du lait maternel ou du méconium, et beaucoup de méthodes analytiques valides et de valeurs de référence pour ces contaminants ont été développées pour ces deux liquides matrices (124; 125).

En pratique, la surveillance biologique est utilisée pour la prévention ou l'évaluation d'une contamination passée dans une population particulière. Elle peut également aider à

déterminer la contribution de plusieurs sources de contamination ou de voies d'exposition afin d'estimer l'importance de l'absorption, de la distribution, de la biotransformation, de l'accumulation et de l'élimination des contaminants dans l'organisme (3). Cette approche a l'avantage d'offrir une estimation réelle de l'exposition pour toutes les voies d'exposition, qu'elles soient orales, cutanées ou respiratoires. Dans un contexte d'évaluation des risques sur la santé, c'est la relation entre les biomarqueurs d'exposition et les biomarqueurs d'effet qui est utilisée pour prédire la probabilité d'apparition des effets sur la santé (3;91;123;127).

En ce qui concerne les fongicides, les données sur l'exposition des travailleurs sont limitées. Il est difficile de prédire la quantité de substance absorbée par la peau à partir des données de l'exposition externe à cause de certains facteurs comme l'hygiène (fréquence de lavage des mains), la nature du travail et les différences physiologiques individuelles qui existent au niveau du métabolisme et de l'élimination du produit (3; 91; 100; 128). Un moyen de contrer ce problème est d'une part de concevoir un protocole adapté à l'étude en sélectionnant une population appropriée et un biomarqueur pertinent pour évaluer l'exposition, et d'autre part, de procéder à une étude chez des volontaires afin de connaître la cinétique corporelle du biomarqueur d'intérêt et l'étendue de la variabilité interindividuelle (91; 100; 125).

La surveillance biologique a été utilisée dans certaines études pour évaluer l'exposition réelle des travailleurs exposés aux fongicides. Certaines limites sont néanmoins à

considérer en plus de celles déjà mentionnées, comme notamment le fait que certaines méthodes de collecte d'échantillonnage sont invasives, ou qu'il est souvent complexe d'obtenir de l'information sur les expositions passées à l'aide des biomarqueurs (ex. demi-vie trop courte) (127; 129).

Dans le cas du captan et du folpet, le manque de données toxicocinétiques est notoire. Toutefois, les grandes différences interespèces au niveau du métabolisme et les plus hautes doses utilisés dans les études animales comparées à l'exposition humaine rendent difficile l'extrapolation de la cinétique humaine à partir des études animales (88; 91; 130; 131). Ainsi, selon Woollen (91), une étude correctement conçue chez des volontaires humains et effectuée de manière sécuritaire, donne une indication de l'étendue de la variabilité individuelle au niveau du métabolisme et de l'élimination du composé chimique.

### **1.5.2. Les modèles toxicocinétiques**

Pour déterminer la relation entre la quantité de biomarqueurs mesurée dans les matrices étudiées et la dose absorbée, les modèles toxicocinétiques sont une approche de plus en plus utilisées pour l'estimation de l'exposition chez les travailleurs (132). Trois catégories de modèles peuvent être distinguées, mais pour simuler les voies physiologiques des substances étudiées et de leurs métabolites dans l'organisme, ainsi que caractériser une représentation réelle de l'exposition, les modèles toxicocinétiques à base physiologique

(« Physiological Based Pharmacokinetic » ou PBPK) sont la meilleure approche selon plusieurs auteurs (132-134).

Cependant, même si ces modèles permettent d'effectuer de nombreuses extrapolations, pour les développer, il est important de connaître la cinétique des substances étudiées pour les différentes voies d'exposition chez l'humain. Une fois conçu, le modèle doit ensuite être validé avant de pouvoir être appliqué et estimer l'exposition des travailleurs, c'est-à-dire qu'il doit prédire adéquatement les données mesurées lors d'une étude chez des travailleurs à partir des paramètres définis par les données obtenues chez des volontaires exposés de façon contrôlée (91;132). Cette approche demande également de quantifier plusieurs biomarqueurs pour différents temps donnés et sur une assez longue période de temps car les mesures répétées de plusieurs biomarqueurs permettent de déterminer plus précisément le scénario d'exposition ainsi que les doses absorbées (91;132-134).

Pour être les plus représentatifs de la réalité, les modèles PBPK requièrent donc des informations détaillées sur la cinétique des substances étudiées. Ces données sont presque inexistantes chez l'humain pour le captan et le folpet. L'utilité des modèles pour estimer les doses absorbées chez des travailleurs à partir de mesures de biomarqueurs doit ensuite être vérifiée.

Il ressort donc des éléments de la littérature sur le captan et le folpet qu'il existe des données fragmentaires difficilement utilisables directement pour une application quantitative des techniques de surveillance biologique, mais qui indiquent un bon potentiel de développement.

## 2. Objectifs

L'objectif principal de ce projet de recherche était de développer des outils de surveillance biologique pour évaluer l'exposition aux fongicides captan et folpet dans une population de travailleurs en milieu arboricole et viticole. Plus spécifiquement, les objectifs des différentes étapes du projet étaient de :

1. Développer des méthodes analytiques robustes et sensibles pour quantifier les métabolites d'intérêt du captan et du folpet, à savoir le THPI, le PI et l'acide phtalique (Articles I et II).
2. Documenter la cinétique sanguine et urinaire des métabolites du captan et du folpet chez des volontaires exposés de façon aiguë à de faibles doses de ces fongicides dans des conditions semi-contrôlées (sous surveillance médicale) (Articles III et IV).
3. Étudier l'exposition au captan et au folpet chez des travailleurs en arboriculture et en viticulture à partir de mesures répétées biologiques au cours d'une semaine suivant deux périodes d'exposition distinctes (Article V) et déterminer l'importance de la voie d'exposition chez les travailleurs exposés aux fongicides, selon le type de travail effectué, à partir des modèles toxicocinétiques développés sur la base des données biologiques recueillies chez les volontaires (Article V).

### 3. Méthodologie

Dans un premier temps, deux méthodes analytiques ont été développées pour caractériser et quantifier les biomarqueurs d'intérêt, à savoir le THPI pour l'exposition au captan et le PI, l'acide phtalamique acide et l'acide phtalique pour l'exposition au folpet. Une revue de la littérature a permis de répertorier quelques articles sur des méthodes d'analyses du THPI urinaire (13;15;16;25;135) ainsi que du THPI et du PI dans le plasma (6;136) par chromatographie en phase gazeuse couplée à une spectrométrie de masse (GC-MS). Toutefois, en raison de la faible sensibilité et des difficultés à détecter le THPI et le PI par GC-MS, c'est une méthode par chromatographie en phase liquide couplée à la spectrométrie de masse en mode ionisation chimique à pression atmosphérique (LC/APCI-MS/MS) qui a été développée. Les détails de la méthode sont décrits dans l'Article I. De nombreuses méthodes étaient également disponibles pour l'acide phtalique du fait qu'il est surtout considéré comme un métabolite des phtalates (137-145). Quant à l'acide phtalamique, seule une étude chez le rat quantifie ce métabolite dans le plasma (6). Mais, en raison de son instabilité, l'acide phtalamique n'a finalement pas été mesuré dans ce projet, mais la quantification de l'acide phtalique comme la totalité des métabolites dérivés de la moitié phtalimide, soit le PI, l'acide phtalamique et l'acide phtalique, a été préférée. La méthode est détaillée dans l'Article II.



Ces méthodes analytiques ont ensuite permis de déterminer la cinétique sanguine et urinaire des biomarqueurs sélectionnés suite à une exposition aiguë à une faible dose orale et cutanée de captan ou de folpet chez des volontaires sains. Ainsi, dix étudiants volontaires de l'Université de Lausanne ont été sélectionnés après une visite médicale d'inclusion. Tous les étudiants étaient de sexes masculins et âgés entre 20 et 30 ans. Ils ont été séparés en deux groupes de cinq individus, soit un groupe pour chaque produit étudié. Un même volontaire a participé à l'exposition par voie orale et par voie cutanée du même fongicide. Chaque exposition a été espacée par une semaine de repos minimum afin de s'assurer de l'élimination du produit dans l'organisme entre chaque administration. L'exposition a d'ailleurs débuté par l'administration par la voie orale du fait que l'absorption est en générale plus rapide que par la voie cutanée. Chaque volontaire a absorbé une dose orale (ingrédient actif pur) de 1 mg/kg de poids corporel dissoute dans du jus d'orange et reçu une dose de 10 mg/kg de poids corporel dissoute dans de l'acétone et appliquée sur une surface de 80 cm<sup>2</sup> de l'avant bras pendant 24 h sans occlusion. Les sessions d'exposition étaient spécifiques à un produit; il n'y a jamais eu d'utilisation de captan le jour d'exposition au folpet et inversement. Pour les deux scénarios d'exposition, 15 mL de sang, la première urine avant le traitement (pour établir le niveau de base) et les urines complètes de 24 h ont été collectées à des moments prédéterminés suivant le traitement pendant une période de 96 h. Le déroulement de l'étude et le protocole de collectes sont précisés dans les Articles III et IV. C'est à partir de ces données que les deux modèles cinétiques ont pu être développés.

Enfin, le profil d'excrétion urinaire détaillé des métabolites du captan et du folpet a été déterminé sur sept jours consécutifs chez des travailleurs selon deux scénarios d'exposition distincts, soit pendant des activités de préparation et d'application d'un des deux pesticides ainsi que durant des activités de suivi des cultures. Plusieurs moyens d'application (chenillettes, tracteurs avec ou sans cabine) ont été sélectionnés, en favorisant les techniques ainsi que les moments de la saison qui engendraient les pires scénarios d'exposition. Cette partie du projet visait donc à valider la spécificité des biomarqueurs sélectionnés pour estimer l'exposition au captan et au folpet chez les travailleurs ainsi qu'évaluer l'influence des unités d'expression (i.e. créatinine, urines ponctuelles, urines cumulatives sur plusieurs périodes de temps) des mesures biologiques sur l'interprétation de l'exposition et déterminer la voie d'exposition prédominante à l'aide des modèles toxicocinétiques développées pour chacun des fongicides. Elle est expliquée dans l'Article V.

### **Considérations éthiques**

Le protocole expérimental pour l'étude chez les volontaires sains et les agriculteurs ainsi que les formulaires de consentement pour les deux études ont été approuvés par le Comité d'Éthique Permanent pour la Recherche Clinique de la Faculté de Biologie et Médecine de l'Université de Lausanne et le Comité d'Éthique de la Recherche de la Faculté de Médecine de l'Université de Montréal. Tous les participants ont donné leur consentement par écrit et ont été informés des risques en participant à l'étude ainsi que leur droit de se désister en

tout temps. Du fait de l'aspect très contraignant de l'étude, les volontaires sains ont reçu une compensation financière pour leur temps et tous les inconvénients engendrés, comme le suggérait le Comité d'Éthique. En revanche, les agriculteurs n'ont reçu aucune compensation financière.

## 4. Résultats

### 4.1. Méthodes analytiques

#### 4.1.1. THPI et PI (Article I)

Les méthodes LC/APCI-MS/MS développées pour analyser le THPI et le PI dans le sang et les urines se sont avérées simples d'utilisation, sensibles, spécifiques et adaptées aux deux métabolites. Elles ont été validées à l'aide d'échantillons de plasma et d'urine des volontaires exposés par voie orale et par voie cutanée au captan ou au folpet dans des conditions semi-contrôlées et des agriculteurs. La limite de détection déterminée pour le THPI est de 1,47 µg/L (9,76 nmol/L) pour le plasma et de 0,58 µg/L (3,82 nmol/L) pour l'urine. Pour le PI, elle est de 2,17 µg/L (14,8 nmol/L) pour le plasma et de 1,14 µg/L (7,72 nmol/L) pour l'urine. Les méthodes ont montré une bonne linéarité avec un coefficient de détermination ( $R^2$ ) supérieur à 0,99, un bon taux de recouvrement pour les deux matrices avec une moyenne de 90% pour le THPI et de 75% pour le PI, une précision intra- et inter-journalière acceptable (écart type relatif (RSD) entre 15 et 20%) et une grande stabilité. Elles ont permis de déterminer précisément la cinétique des deux métabolites dans le sang et l'urine des volontaires ainsi que d'estimer la dose interne de ces métabolites chez les agriculteurs pour les deux scénarios d'exposition.

### **4.1.2. Acide phtalique (Article II)**

La méthode GC-MS décrite pour quantifier l'acide phtalique comme métabolite final des métabolites phtalimides du folpet s'est aussi révélée fiable et robuste pour déterminer la cinétique de l'acide phtalique dans l'urine des volontaires ainsi que pour évaluer l'exposition au folpet des viticulteurs. La préparation des échantillons a été adaptée de la méthode de Mettang *et al.* (146) à partir des observations de l'étude de Bray *et al.* (147). Cette méthode est sensible avec une limite de détection définie à 10 ng/mL (60,2 nmol/L), mais elle est également précise avec un écart type relatif inférieur à 13% pour les variations intra- et inter-journalières, a une bonne linéarité ( $R^2 > 0,98$ ) et un excellent taux de recouvrement d'en moyenne 97%. De plus, elle permet de transformer tout l'acide phtalimide en acide phtalique (taux de recouvrement de 100%), mais seulement 50% du PI. Toutefois, en raison des faibles quantités de PI retrouvées dans les urines des personnes exposées, il peut être assumé que la méthode permet de quantifier tous les métabolites dérivés du phtalimide.

## **4.2. Étude chez les volontaires exposés en conditions semi-contrôlées**

### **4.2.1. Exposition orale (Article III)**

Les résultats documentent la cinétique du THIPI, PI et de l'acide phtalique chez l'humain exposé par voie orale à 1 mg/kg de poids corporel au captan ou au folpet. Une différence cinétique entre le captan et le folpet a été observée. Sur la période d'étude (96 h), le

THPI est complètement éliminé avec une demi-vie ( $t_{1/2}$ ) de 15,7 h dans le plasma et 11,7 h dans l'urine, alors qu'il faut plus de temps pour que le PI et l'acide phtalique soit éliminé, avec  $t_{1/2}$  de 31,5 h dans le plasma et 27,3 h dans l'urine pour le PI et de 27,6 h dans l'urine pour l'acide phtalique. Malgré une cinétique plus rapide pour le THPI comparé au PI et l'acide phtalique, la cinétique de ces biomarqueurs peut être considérée comme rapide chez l'homme. D'autre part, 3,5% de la dose de captan administrée se retrouve sous forme de THPI dans les urines, mais 0,03% seulement de la dose de folpet se retrouve sous forme de PI et 25,2% sous forme d'acide phtalique dans les urines. Les résultats pour le THPI correspondent aux valeurs reportées par Krieger et Thongsinthusak (15) dans son étude chez deux volontaires masculins exposés oralement à une dose équivalente à celle administrée dans notre étude (1 mg/kg). Quant au PI, la très faible quantité récupérée dans les urines montrent qu'il doit être rapidement transformé en un autre métabolite, dont l'acide phtalique, dans le tractus gastro-intestinal (GI) ou dans le sang. Finalement, même si le THPI et le PI ne semblent pas être les principaux métabolites du captan et du folpet respectivement, d'un point de vue de surveillance biologique, ces métabolites sont valides et stables. Quant à l'acide phtalique, il vient renforcer et appuyer les résultats obtenus pour le PI.

#### **4.2.2. Exposition cutanée (Article IV)**

La cinétique des principaux biomarqueurs d'exposition au captan et au folpet a été déterminée après une exposition de 24 h à une dose de 10 mg/kg sur une surface de 80 cm<sup>2</sup> de l'avant-bras. Des différences cinétiques entre les deux fongicides sont également

apparentes. Les demi-vies ( $t_{1/2}$ ) d'élimination du PI et de l'acide phtalique dans l'urine sont très semblables à celles calculées pour l'exposition orale ; elles sont de 28,8 h et 29,6 h respectivement. En revanche, la  $t_{1/2}$  d'élimination du THPI dans l'urine est plus longue que pour l'exposition orale, soit 18,7 h alors qu'elle était de 11,7 h pour l'exposition orale. Les  $t_{1/2}$  dans le plasma pour le THPI et le PI sont du même ordre de grandeur que pour l'urine, avec des valeurs de 24,7 h et de 29,7 h pour les deux métabolites, respectivement. Par ailleurs, seulement 0,02% de la dose de captan appliquée est retrouvée sous forme de THPI dans les urines et 0,002% de la dose de folpet sous forme de PI. Quant à l'acide phtalique, seulement 1,8% de la dose de folpet appliquée se retrouve sous la forme de ce métabolite dans l'urine. Ces fractions pourraient expliquer, outre une faible fraction d'absorption cutanée, une transformation et donc une élimination de ces produits en d'autres métabolites comme le suggère Ackerman *et al.* et Krieger et Thongsinthusak (15;79). D'autre part, en comparant les pentes d'élimination du THPI du plasma pour les voies orales et cutanées (période 24 h – 72 h), il apparaît que celle de la voie cutanée est plus lente. Ceci implique que le taux d'élimination est influencé par le taux d'absorption cutanée dans ce cas. Quant au PI, les pentes d'élimination du plasma pour la voie orale et la voie cutanée sont similaires, ce qui indique que le taux d'absorption cutané a un effet négligeable sur le taux d'élimination du PI dans le plasma. D'un point de vue surveillance biologique, même si la dose absorbée semble très faible, le THPI et le PI restent des biomarqueurs substantiels et intéressants pour l'exposition cutanée au captan et au folpet. De plus, comme pour l'exposition par voie orale, les résultats obtenus avec l'acide phtalique viennent soutenir et valider les résultats du PI.

### **4.3. Étude chez les arboriculteurs et les viticulteurs (Article V)**

Les profils d'excrétion urinaire des biomarqueurs spécifiques et non spécifiques à l'exposition au captan et au folpet ont été définis à partir des données des travailleurs exposés au captan ou au folpet pour deux périodes d'activités distinctes : une période d'application et une période d'effeuillage. Ces profils indiquent un niveau d'exposition plus élevé lors des périodes de traitement que pendant les travaux d'effeuillage. Par ailleurs, à partir des collectes de toutes les urines ponctuelles sur sept jours consécutifs, l'influence des unités de mesure urinaire telles que l'ajustement à la créatinine ou le regroupement des urines ponctuelles par période de temps (8, 12 ou 24 h) a pu être évaluée. Ce sont les urines cumulatives sur 24 h et sans ajustement par la créatinine qui présentent la meilleure estimation de l'exposition des travailleurs aux fongicides pour cette étude, et plus particulièrement pour estimer les doses absorbées et les principales voies d'exposition à l'aide des modèles cinétiques. Ainsi, les simulations des modèles suggèrent une absorption limitée des fongicides et une exposition principalement par voie cutanée, ce qui confirme les résultats de plusieurs études chez les travailleurs exposés aux pesticides (9;21;22;32;33;36;37;88;91;148-152). Enfin, les résultats soulignent l'importance de mesurer plusieurs biomarqueurs d'exposition pour un même produit afin de caractériser l'exposition avec plus d'exactitude et de précision.



## Article I

### **Liquid chromatography - tandem mass spectrometry (LC/APCI-MS/MS) methods for the quantification of captan and folpet phthalimide metabolites in human plasma and urine**

Aurélie Berthet, Michèle Bouchard\*, Patrick Schüpfer, David Vernez, Brigitta Danuser,  
Cong Khanh Huynh

A. Berthet · P. Schüpfer · D. Vernez · B. Danuser · C. K. Huynh

Institute for Work and Health, Bugnon 21, 1011 Lausanne, Switzerland

A. Berthet · M. Bouchard (\*)

Department of Environmental and Occupational Health, School of Public Health,  
Université de Montréal, P.O. Box 6128, Main Station, Montreal, Quebec, Canada, H3C  
3J7

\* corresponding author

Analytical and Bioanalytical Chemistry (2011) 399:2243-255

DOI 10.1007/s00216-010-4601-z

## Abstract

Captan and folpet are fungicides largely used in agriculture. They have similar chemical structures, except that folpet has an aromatic ring unlike captan. Their half-lives in blood are very short, given that they are readily broken down to tetrahydrophthalimide (THPI) and phthalimide (PI), respectively. Few authors measured these biomarkers in plasma or urine and analysis was conducted either by gas chromatography coupled to mass-spectrometry (GC-MS) or liquid chromatography with UV detection (LC-UV). The objective of this study was thus to develop simple, sensitive and specific liquid chromatography - atmospheric pressure chemical ionization-tandem mass spectrometry (LC/APCI-MS/MS) methods to quantify both THPI and PI in human plasma and urine. Briefly, deuterated THPI was added as an internal standard and purification was performed by solid phase extraction followed by LC/APCI-MS/MS analysis in negative ion mode for both compounds. Validation of the methods was conducted using spiked blank plasma and urine samples at concentrations ranging from 1 to 250  $\mu\text{g/L}$  and 1 to 50  $\mu\text{g/L}$ , respectively, along with samples of volunteers exposed to captan or folpet. The methods showed a good linearity ( $R^2 > 0.99$ ), recovery (on average 90% for THPI and 75% for PI), intra- and inter-day precision (RSD < 15%) and accuracy (< 20%), and stability. The limit of detection was 0.58  $\mu\text{g/L}$  in urine and 1.47  $\mu\text{g/L}$  in plasma for THPI, and 1.14 and 2.17  $\mu\text{g/L}$ , respectively, for PI. The described methods proved to be accurate and suitable to determine the toxicokinetics of both metabolites in human plasma and urine.

**Keywords** Tetrahydrophthalimide · Phthalimide · LC/APCI-MS/MS · Plasma · Urine

## Introduction

Many winegrowers and tree farmers apply captan (1,2,3,6-tetrahydro-*N*-(trichloromethyl thio) phthalimide) or folpet (*N*-(trichloromethyl thio) phthalimide) to treat fungal diseases. These two common fungicides belong to the family of thiophthalimide pesticides. They have a very similar chemical structure, except that folpet has an aromatic ring while captan has a cyclohexene ring. Both compounds have very short half-lives in biological matrices, as assessed by Gordon *et al.* [1;2]. Captan was found to degrade with a half-life of 0.97 seconds in blood and folpet with a half-life of 4.9 seconds when <sup>14</sup>C-captan was directly added at 33.2 nmol/L and <sup>14</sup>C-folpet at 33.7 nmol/L to 1 mL of human blood [1]. Therefore, they are almost instantaneously metabolized to tetrahydrophthalimide (THPI) for captan and to phthalimide (PI) for folpet (Fig. 1). Unlike the two parent compounds, THPI and PI metabolites can be quantified in human plasma and urine [3-12]. Other metabolites of captan and folpet have also been identified in animal metabolism studies, namely 2-thiothiazolidine-4-carboxyl acid (TTCA) as a trichloromethyl thio derivative metabolite of captan, THPI derivatives, and phthalamic and phthalic acids as derivatives of PI [2;13-16]; nonetheless, THPI is the metabolite of captan most measured in the published human biomonitoring studies [5-12] while human biomonitoring data on the metabolites of folpet are limited [3;17].

The published analytical methods for the measurement of THPI and PI in human plasma or urine used either gas chromatography with mass spectrometry detection (GC-MS) [3;6;7;10] or liquid chromatography with UV detection (LC-UV) [4]. Liquid

chromatography – mass spectrometry (LC-MS) methods with atmospheric pressure chemical ionization (APCI) have yet to be developed for these biomarkers. LC-APCI-MS analysis is known to be very specific to one analyte and to improve sensitivity, especially for the detection of more polar or low-concentration compounds [18]. The objective of this work was thus to develop such analytical methods for the quantification of THPI and PI in human plasma and urine to determine the toxicokinetics of these biomarkers in volunteers as well as assess worker exposure to captan and folpet through biomonitoring.

## Materials and methods

### Chemicals and reagents

Reference standards of cis-1,2,3,6-tetrahydrophthalimide (THPI) and phthalimide (PI) (>99% purity) were obtained from Sigma-Aldrich (Buchs, St Gallen, Switzerland), while deuterated cis-1,2,3,6-tetrahydrophthalimide (THPI-d) (99% purity) was purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). HPLC grade acetonitrile, methanol and dichloromethane were also obtained from Sigma-Aldrich (Buchs, St Gallen, Switzerland). Ammonium sulphate was purchased from Merck (Zug, Switzerland). Water was purified using a TKA GenPure water treatment system obtained from TKA Wasseraufbereitungssysteme GmbH (Niederelbert, Germany).

### Standard preparation

#### *Stock standard solutions*

Individual stock standard solutions were prepared by dissolving 100 mg of THPI or PI in 100 mL of methanol and 20 mg of THPI-d in 100 mL of methanol. They were stored at -20° C in a glass container until used.

#### *Calibration curves*

To prepare the calibration curves, stock standard solutions were diluted to obtain working solutions of THPI and PI standards at 10, 100, and 1 000 mg/L. These working standard solutions were kept at 4°C and were used daily to prepare calibration curves in urine and plasma, hence to spike blank urine from nonexposed persons at six

concentration levels and blank plasma from nonexposed persons at six concentration levels. The concentration levels were the same for both THPI and PI, thus 0.5, 1, 2, 5, 10 and 50 µg/L in urine and 1, 5, 10, 50, 100 and 250 µg/L in plasma.

Similarly, the stock internal standard solution was diluted to obtain a working solution at 250 µg/L. A constant volume of 125 µL of this solution was added to the calibration curve samples, which translates into a concentration of 62.5 µg/L in the 500 µL urinary and plasma extracts after sample processing.

Simultaneously, calibration points were also prepared in methanol from the same working solutions of THPI, PI and THPI-d standards. The six concentration levels were 1, 2, 5, 10, 15 and 20 µg/L for THPI and PI. Since final volume of each level was 1 ml, 250 µL of THPI-d working solution at 250 µg/L were thus added in this case, which corresponds to a concentration of 62.5 µg/L.

### Sample treatment

#### *Analysis of THPI and PI in urine*

THPI and PI in urine were analyzed using liquid chromatography - atmospheric pressure chemical ionization-tandem mass spectrometry (LC/APCI-MS/MS) methods, after solid phase extraction (SPE). More specifically, 3-mL aliquots of urine of exposed individuals (workers or volunteers) along with that of non-occupationally exposed subjects were transferred into glass tubes and spiked with 125 µL of THPI-d internal standard (250 µg/L). Extraction of THPI and PI was then performed using Oasis® SPE cartridges

(Waters, Montreux, Switzerland). The cartridges were first conditioned with 8 mL of dichloromethane, followed by 8 mL of methanol and 12 mL of water. The urines were passed through the column and discarded. The cartridges were washed with 1.5 mL of 5% methanol: 95% water (v/v) and left to dry for 15 min under vacuum (10 inHg). The analytes were then eluted from the column with 4 mL of dichloromethane into 5 mL glass tubes. The solvent was evaporated to dryness under a gentle nitrogen flow at 40°C. The residues were resuspended in 500 µL of methanol and transferred to vials for LC/APCI-MS/MS analysis.

#### *Analysis of THPI and PI in plasma*

Analysis of THPI and PI in blood of exposed individuals was performed as described for urine, except that a 2-mL aliquot of plasma was used and a step for the denaturation of proteins was added, prior to SPE extraction. This step consisted of adding 4 mL of saturated ammonium sulphate to the plasma sample, which was then centrifuged at 3400 rpm for 20 min at 15°C.

#### Instrumental analysis

##### *Apparatus*

Analysis of THPI and PI was performed using a Varian Model 212-LC Binary Gradient LC system (Les Ulis, France) connected to a Prostar model 410 autosampler (Varian, Les Ulis, France) and coupled to a Model 1200 L quadrupole MS (Varian, Les Ulis, France) operating in APCI mode. The APCI interface was operated in the negative ion

mode and its specific APCI-MS parameters were manually optimized and identical for both analytes.

#### *Analytical conditions*

The compounds were separated using a C<sub>18</sub> Zorbax Eclipse Plus column (4.6 x 150 mm, 3.5 μm) from Agilent (Morges, Switzerland). The temperature of the column was maintained at 30°C. The mobile phase consisted of: eluent A composed of 90% water and 10% acetonitrile (9:1), and eluent B of 10% water and 90% acetonitrile (1:9). For THPI analysis, elution was performed in 14 min using a solvent gradient, at a flow rate of 0.8 mL/min. The following solvent program was used: 90% eluent A for 3 min, followed by a linear gradient to 5% eluent A from 3 to 10 min, maintained at 5% eluent A from 10-13 min before returning to initial conditions of 90% eluent A in 1 min. For PI analysis, elution was performed in 21 min at a flow rate of 0.8 mL/min using the following sequence: 90% eluent A for 15 min and then ramping to 5% eluent A in 30 sec for a 4 min clean up at 5% eluent A prior to returning to initial conditions in 1 min. Several gradients were tested to obtain a chromatographic run as short as possible while allowing separation from interfering peaks and aiming at the best sensitivity for both compounds. In addition, we opted for 12-min re-equilibration period between runs. The samples were kept at 8°C on the injection tray and 10 μL were injected.

Once analytes and mobile phase reached the ionization source, they were subjected to a needle voltage of -10 μA, a spray shield voltage of -600 V and a spray chamber temperature of 60°C. Then, air as nebulizer gas was set to a pressure of 60 psi followed



by vaporizing gas of 12 psi with a temperature of 500°C. Lastly, a drying gas (N<sub>2</sub>) was set to 35 psi with a temperature of 350°C in the hexapole before product ions were transmitted in quadrupoles. In the collision cell, ions were fragmented with argon at a pressure of 1.45 mTorr on average. Collision energy (CE) was 19.5 V for THPI, 22.5 V for THPI-d and 5V for PI. The precursor and product ion analyzed were *m/z* 149.4/95.6 for THPI, *m/z* 156.1/95.6 for THPI-d and *m/z* 145.8/145.8 for PI (PI not fragmented). Fragment ions were then detected by the electron multiplier voltage at 1455 V. Identification and quantification were performed in multiple reaction monitoring (MRM) mode.

#### Quantification of THPI and PI

The quantification of THPI and PI was obtained from standard calibration curves in urine or in plasma. These latter curves were established by plotting the response factors as a function of the concentrations levels, over a maximum range of 1 to 250 µg/L for both analytes depending on the matrix. The response factors corresponded to the peak-area ratios of each compound ion to the internal standard ion.

#### Methods validation

Since no commercial quality control samples was available for THPI and PI, the criteria used to validate the four methods were specificity, sensitivity, linearity, intra- and inter-day precision, accuracy, recovery and stability.

### *Specificity*

Specificity was determined by analyzing ten urine and plasma samples from unexposed individuals and by verifying the absence of endogenous interferences on the chromatograms for these blank matrices.

### *Sensitivity*

Sensitivity of the analytical methods was estimated by the limit of detection (LOD) and the limit of quantification (LOQ). LOD was calculated from calibration curves (6 for urines and 10 for plasma) using the definition:

$$y - y_B = 3s_B$$

where  $y$  was the response factor,  $y_B$  was the blank response or the intercept of regression line, and  $s_B$  the standard deviation of the blank [19]. The LOD of each compound was determined for each calibration curve, and then means were calculated and considered as LODs. As for the LOQ, it was defined as equivalent to 3.3 times the LOD.

### *Linearity*

Linearity of the methods was evaluated by calculating regression parameters of calibration curves in urine or in plasma for both compounds by the least square fit method. Results were expressed using the average coefficient of determination ( $R^2$ ) of eight calibration curves in urine and ten calibration curves in plasma over the studied ranges.

*Intra- and inter-day precision and accuracy*

Intra-day and inter-day variations were assessed by the precision and the accuracy of replicates of three different levels of spiking of blank urine with THPI and PI and of five levels of spiking of blank plasma. The intra-day variation was measured by the repeatability of each level prepared in two replicates and analyzed during the same day ( $n = 6$ ), and the inter-day variation was assessed by the repeatability of each level (daily prepared) on eight consecutive days for urine ( $n = 8$ ) and twenty consecutive days for plasma ( $n = 20$ ). Precision was expressed as a percentage of relative standard deviation (% RSD) and defined as the ratio of standard deviation to mean of the response factor for each level of spiking multiplied by 100. Accuracy (expressed in percentage) was estimated by the following equation:

$$\frac{(\text{average measured amount} - \text{known spiking amount})}{\text{known spiking amount}} \times 100$$

*Recovery*

To determine the recovery of each compound in both studied matrices after extraction, the ratio of measured amount of THPI and PI in extracts to the theoretical spiked amount in blank urine or plasma was calculated for each level. The recovery was expressed as a percentage.

*Stability*

To establish stability of compounds in urine and in plasma, pools of blank urine or plasma were spiked at different concentration levels defined above for calibration

curves, and then separated in aliquots and stored at  $-20^{\circ}\text{C}$ . Every day, over an 8-day period for urine and a 20-day period for plasma, an aliquot of each concentration level was thawed and analyzed.

The stability of urinary samples from volunteers exposed to captan or folpet was also tested. Two samples thawed and analyzed for THPI or PI in a given run were kept at  $4^{\circ}\text{C}$  and processed and reanalyzed during the following run.

#### Application of the methods

The use of the analytical methods for the quantification of THPI and PI in human plasma and urine samples of exposed individuals was then tested. THPI and PI concentrations were measured in the urine and plasma collected repeatedly over a 96- and 72-h period respectively following an oral and dermal exposure to captan in ten volunteers. These metabolites were also measured in urine samples repeatedly collected over a typical workweek in applicators exposed to captan and folpet.

The Permanent Ethical Committee of Clinical Research of the Faculty of Biology and Medicine of the University of Lausanne and of the Research Ethical Committee of the Faculty of Medicine of the University of Montreal approved the protocol, and all participants gave written informed consent, and were acquainted with the risks of participating and their right to withdraw from the study at all time.

## Results and discussion

The analytical methods developed allowed to accurately, specifically and sensitively quantify THPI and PI in both human urine and plasma by LC/APCI-MS/MS.

### Methods development

In developing the methods, several tests were performed to determine the best sample treatment and analysis conditions for a precise, reliable, easy and quick procedure. First, liquid-liquid extraction of THPI and PI analytes in plasma and urine using acetonitrile or dichloromethane solvents were tested, but solid phase extraction (SPE) was shown to provide noticeably improved processing recoveries and reproducibility, less interfering peaks on PI chromatograms and simpler and faster processing of samples (data not shown). Once chromatographic conditions were properly set, it was also evaluated whether acid or enzymatic hydrolyses were needed to deconjugate metabolites in human plasma and urine since interactions between thiol- and non-thiol-containing proteins and captan or folpet were reported [20-22]. This procedure was found unnecessary and even lead to some degradation of THPI and PI, as assessed on urine samples from volunteers orally exposed to captan or folpet after incubation at 37°C with  $\beta$ -glucuronidase/arylsulfatase or heating at 100°C in the presence of HCl 12 N during different time periods (2, 4, 6 and 16 h) (data not shown). Analysis was thus performed without any hydrolysis step.

For LC-MS separation and quantification of THPI and PI, different polar solvents and mixed solutions were also tested as mobile phases. Acetonitrile was finally selected instead of methanol because sensitivity was significantly increased and peak separation was better defined for PI, which fragmentation was not achieved. Several analytical columns were further tested (*i.e.*, Luna<sup>®</sup> C<sub>18</sub>, Luna<sup>®</sup> NH<sub>2</sub> columns from Phenomenex, Spherisorb ODS2 from Waters and C<sub>18</sub> Zorbax Eclipse Plus from Agilent), but since THPI is a weak base (pK<sub>a</sub> = 9.65), PI a weak acid (pK<sub>a</sub> = 6.96) and both are polar molecules, the C<sub>18</sub> Zorbax Eclipse Plus column was found to be the most appropriate to retain these two metabolites and the internal standard. Thus, short retention times were obtained with a very good repeatability for all analytes: 5.8 minutes for THPI, 8.7 minutes for PI and 5.6 minutes for THPI-d. In addition, electrospray ionization (ESI) was initially selected prior to APCI mode, but no peak was observed for PI and the sensitivity for THPI was poor.

### Chromatography

Figures 2-5 show chromatograms of plasma and urine samples of non-occupationally exposed individuals along with blank plasma and urine samples spiked with different concentrations of THPI or PI following treatment of samples by solid phase extraction (see Materials and Methods). Typical chromatograms of plasma and urine samples of volunteers treated with captan and folpet are also represented. For THPI and THPI-d quantification, clean chromatograms were observed with limited background interference, implying that clean up treatment of samples was efficient and analytical conditions were suitable for the methods to be specific. Moreover, the formation of

fragment ions (Fig. 2 and Fig. 4) facilitated identification of both analytes, which were also used for quantification. On the other hand, for PI quantification, chromatograms contained several peaks other than those of PI and THPI-d used as an internal standard (Fig. 4 and 5) and fragmentation of PI was not achieved such that only the parent ion was quantified. To separate interference peaks from that of PI, an optimization of LC separation was performed and a high percentage of water in the eluent phase was needed. Different analytical conditions were thus required to analyze PI and THPI. However, both conditions were accurate for analyzing THPI-d, although THPI elution conditions provided narrower peaks and better sensitivity.

Analysis of chromatograms of plasma and urine samples of individuals of the general population non-occupationally exposed to captan or folpet also shows the presence of a baseline level of exposure in some cases. As illustrated in Fig. 2b, Fig. 4a and Fig. 5a, THPI and PI was detectable in some urine samples of non exposed individuals and also in plasma for PI, but in very small concentrations (about 10 nmol/L for THPI in urine, and on average 5.5 nmol/L in plasma and 4 nmol/L in urine for PI). Barr *et al.* [3] detected also THPI in 43% of plasma samples from 70 non-exposed women living in an urban environment, and 51% of samples contained PI.

Chromatograms of plasma and urine samples of volunteers orally exposed to captan and folpet, with and without spiking with reference standards, were further compared (Fig. 2c, Fig. 3c, Fig. 4c and Fig. 5b). For both THPI and PI, no chromatographic differences were noted between spiked and non-spiked matrices; retention times were exactly the

same, peaks had the same shape and no interference peaks appeared. These methods thus appeared specific for the biomonitoring of exposure in individuals.

### Quantification

To quantify THPI or PI, peak area ratio of the analyte to the internal standard was calculated for the various concentration levels of the calibration curves; this adjustment by internal standard peak area allowed to account for analyte loss in extraction and analysis, as well as eliminate potential variations due to the apparatus and reduce errors associated with measurements. The use of a deuterated form of THPI as an internal standard also provided a molecule structurally related to captan and folpet biomarkers, but distinguishable by its mass. This increased the precision and specificity of the methods, even for PI, which had several similarities with THPI despite its aromatic core.

### Methods validation

The methods developed were evaluated using the following criteria described in Materials and Methods section: sensitivity, linearity, intra- and inter-day precision and accuracy, recovery and stability.

Table 1 presents the estimated LODs and LOQs of THPI and PI in urine and plasma. They were established within the concentration range of 0.5 to 50  $\mu\text{g/L}$  for THPI and PI in urine and 1 to 100  $\mu\text{g/L}$  for THPI and PI in plasma. LOD was 0.58 and 1.47  $\mu\text{g/L}$  for THPI in urine and plasma respectively, and corresponding LOQ was 1.90 and 4.87  $\mu\text{g/L}$ . In comparison, LOD was 1.14 and 2.17  $\mu\text{g/L}$  for PI in urine and in plasma respectively,



and LOQ was 3.75 and 7.19  $\mu\text{g/L}$ . The sensitivity was however better for THPI than PI given that the latter molecule could not be fragmented in MS and elution conditions had to be adjusted to provide a better separation of PI from interfering peaks on the chromatograms. The developed methods for both THPI and PI were at least as sensitive as those published in the literature (Table 2). For instance, our values were comparable to the method of Barr *et al.* [3] and Hines *et al.* [6].

As for the linearity of standard calibration curves in methanol, plasma and urine, they are displayed in Fig. 6 for THPI and in Fig. 7 for PI. All curves were linear over the studied concentration range for THPI and PI, and resulted in a coefficient of determination greater than 0.99. Furthermore, matrix effects were not tested *per se* since APCI mode is less susceptible than ESI mode [18; 20; 21]. However, for THPI, calibration curves prepared in methanol had similar slopes to those prepared in urine, indicating the absence of urinary matrix effect for this analyte. On the other hand, matrix effects were obvious for calibration curves of THPI and PI prepared in plasma as well as those of PI in urine. Calibration was thus performed using standard curves in plasma and urine for both analytes (with more spiking levels for plasma given the observed wider concentration range of THPI and PI in volunteers and more calibration curves for validation given plasma matrix effects).

Precision and accuracy of the methods of analysis of THPI and PI in plasma and urine are further presented in Table 3. Intra-day and inter-day precision and accuracy were

satisfactory with % relative standard deviation (RSD) less than 15% (except for the first urinary level of THPI and PI) and % relative error (RE) of less than 20%.

Table 3 gives also an overview of recoveries of THPI and PI in spiked plasma and urine samples, after solid-phase extraction and processing. They were quite good for both compounds in these matrices, except for PI at low concentrations given matrix effects. The mean ( $\pm$  SD) recoveries of THPI (i.e. all spiked sample results combined) were  $90.4 \pm 9.9\%$  ( $n = 20$ ) in urine and  $90.9 \pm 6.7\%$  ( $n = 100$ ) in plasma; corresponding values for PI were  $72.9 \pm 18.6\%$  ( $n = 20$ ) in urine and  $79.6 \pm 11.3\%$  ( $n = 100$ ) in plasma (as plasma was not collected for workers, more runs with urine samples were performed than with plasma samples). These results were similar to those reported in the literature. Indeed, several authors found a recovery rate of THPI in human urine varying between 82 to 90% over the same concentration range [6-10]; Barr *et al.* [3] obtained a recovery rate of 91% for THPI in human plasma, and of 89% for PI in human plasma like Canal-Raffin *et al.* [4] in rat plasma (Table 2). To our knowledge, no published methods are available for the analysis of PI in human urine.

Furthermore, stability of THPI and PI standards in plasma and urine samples kept at  $-20^{\circ}\text{C}$  was tested over an 8-day period for urine and over a 20-day period for plasma. Since intra-day precision and accuracy values were similar to those of inter-day precision and accuracy, both analytes were thus considered stable in plasma and urine over the studied period (Table 3). The same observations were made by Canal-Raffin *et*

*al.* [4] for the stability of PI in rat plasma, and Barr *et al.* [3] reported that THPI and PI in human plasma were stable over a period of four months.

THPI and PI in urine samples of volunteers treated with captan or folpet were also relatively stable, when kept at 4°C and re-analyzed in the following analytical run. A coefficient of variation ranging between 2.98 and 6.57% were obtained for THPI ( $n = 12$ ) and between 1.51 and 7.56% for PI ( $n = 12$ ). The same tests could not be performed with plasma samples because of the limited amount of matrix available.

#### Application

With the developed analytical methods, it was verified that THPI and PI could easily be quantified in urine and plasma of volunteers exposed to captan or folpet at 1 mg/kg of body weight by oral route and at 10 mg/kg of body weight by dermal route; Fig. 8 presents the mean concentration-time profiles of THPI and PI in urine and plasma following both an oral and dermal exposure. The methods were found to be sensitive enough to document the toxicokinetics of THPI in human plasma and urine at equivalently realistic daily absorbed doses in workers.

On the other hand, PI concentrations in plasma and urine were much lower than those of THPI for the same exposure dose. When PI was administered to rats, Chasseaud *et al.* [23] observed that about 80% of the dose was metabolized and excreted in urine as phthalamic acid and 7% as phthalic acid, showing that PI is a minor metabolite of folpet in urine. Moreover, Chasseaud *et al.* [24] established that when labelled  $^{14}\text{C}$ -folpet was

administered to rats, 80% was recovered in the urine as phthalamic acid, and Canal-Raffin *et al.* [4] observed that PI was rapidly hydrolyzed to phthalamic acid in plasma following an intratracheal administration of folpet in rats. PI is however more specific to folpet exposure than the phthalic acid, which is also a phthalate metabolite [25; 26].

Table 4 presents pre-and post-shift levels of THPI and PI in urine during the course of a workweek in applicators exposed to captan and folpet, respectively. This table shows that the metabolites were easily measured in the urine of workers following a one-day exposure episode, as compared to pre-seasonal measurements or, in the case of folpet, baseline values observed a few days after application. According to our results, THPI appears as a sensitive and specific biomarker of captan exposure in exposed individuals; PI can also be considered as a specific biomarker of folpet exposure but is less sensitive than THPI.

## **Conclusion**

The LC/APCI-MS/MS methods proved to be simple and reliable to quantify THPI and PI in human plasma and urine. Their good sensitivity, specificity, linearity, accuracy and precision were also validated and found at least equivalent to methods reported in the literature. These methods thus appear suitable for the biomonitoring of exposure to captan and folpet in exposed individuals.

## **Acknowledgements**

We thank Gregory Plateel and Ferdinand Storti for their technical assistance. The project was funded by the ANSES (Agence Nationale de Sécurité Sanitaire de l'alimentation, de l'environnement et du Travail). Aurélie Berthet also received a scholarship from the *Institut de recherche Robert-Sauvé en santé et sécurité du travail du Québec*.

## References

1. Gordon EB, Ehrlich T, Mobley S, and Williams M (2001) *Toxicology Methods* 11:209-223
2. DeBaun JR, Miaullis JB, Knarr J, Mihailovski A, and Menn JJ (1974) *Xenobiotica* 4:101-119
3. Barr DB, Barr JR, Maggio VL, Whitehead RD, Sadowski MA, Whyatt RM, and Needham LL (2002) *J Chromatogr B Analyt Technol Biomed Life Sci* 778:99-111
4. Canal-Raffin M, Receveur M, Martinez B, Titier K, Ohayon C, Baldi I, Molimard M, Moore N, and Brochard P (2008) *J Chromatogr B Analyt Technol Biomed Life Sci* 865:106-113
5. Dinoff TM, Bernard CE, Oliver M, Williams RL, and Krieger RI (2001) *Abstracts of Papers of the American Chemical Society* 221:U59
6. Hines CJ, Deddens JA, Jaycox LB, Andrews RN, Striley CAF, and Alavanja MCR (2008) *Ann Occup Hyg* 52:153-166
7. Krieger RI and Thongsinthusak T (1993) *Drug Chem Toxicol* 16:207-225
8. Krieger RI and Dinoff TM (2000) *Arch Environ Contam Toxicol* 38:398-403
9. Schoen SR and Winterlin WL (1982) *J Assoc Offic Anal Chem* 65:1382-1384
10. van Welie RT, van DP, Lamme EK, Jager P, van Baar BL, and Vermeulen NP (1991) *Int. Arch Occup Environ Health* 63:181-186
11. Winterlin WL, Kilgore WW, Mourer CR, and Schoen SR (1984) *J Agric Food Chem* 32:664-672
12. Winterlin WL, Kilgore WW, Mourer CR, Hall G, and Hodapp D (1986) *Arch Environ Contam Toxicol* 15:301-311

13. Ackermann H, Faust H, Kagan YS, and Voronina VH (1978) Arch Toxicol 40:255-261
14. Gordon E (2007) J Appl Toxicol 27:519-526
15. Lappin GJ, and Havell LM (1990) Captan: Biotransformation study in the rat. Unpublished report No CTL/P/2951. ICI Central Toxicology Laboratory, Macclesfield, England
16. Wood SG, Chasseaud LF, Cheng K, Hall M, Fitzpatrick K, Iqbal S, and Barlett A (1991) Metabolic fate of <sup>14</sup>C-folpet in Sprague-Dawley rats. Unpublished report No HRC/MBS 41/91499. Huntingdon Life Sciences Limited, Huntingdon, England
17. Whyatt RM, Barr DB, Camann DE, Kinney PL, Barr JR, Andrews HF, Hoepner LA, Garfinkel R, Hazi Y, Reyes A, Ramirez J, Cosme Y, and Perera FP (2003) Environ Health Perspect 111:749-756
18. Maurer HH (2005) Anal Bioanal Chem 381:110-118
19. Miller JC and Miller JN (2005) Calibration methods: regression and correlation. In: Statistics and chemometrics for analytical chemistry, 5th edition. Pearson Education Limited, England
20. Dams R, Murphy CM, Lambert WE, and Huestis MA (2003) Rapid Commun Mass Spectrom 17: 1665–1670
21. Müller C, Schäfer P, Störtzel M, Vogt S, Weinmann W (2002) J Chromatogr B Analyt Technol Biomed Life Sci 773: 47-52
22. Gordon EB (2010) In: Krieger RI (ed) Hayes Handbook of pesticide toxicology, 3<sup>rd</sup> edn. Elsevier, New York



23. Chasseaud L, Hawkins DR, Franklin ER, and Weston KT (1974) The metabolic fate of  $^{14}\text{C}$ -Folpet (Phaltan) in the rat (Folpet). Unpublished report N° CHR1-74482. Huntingdon Research Centre Ltd , Huntingdon, England
24. Chasseaud L (1980) (Carbonyl- $^{14}\text{C}$ ) Folpet metabolism in rats. Unpublished report N°DPBP 51202. 1980. Huntingdon Research Centre Ltd, Huntingdon, England
25. Blount BC, Milgram KE, Silva MJ, Malek NA, Reidy JA, Needham LL, and Brock JW (2000) *Anal Chem* 72:4127-4134
26. Silva MJ, Samandar E, Preau JL Jr., Reidy JA, Needham LL, and Calafat AM (2007) *J Chromatogr B Analyt Technol Biomed Life Sci* 860:106-112

**Table 1**

Performance parameters of the methods

Analyte	Matrix	LOD <sup>a</sup>	LOQ <sup>b</sup>	Working range
		(mean ± SD) (µg/L)	(mean ± SD) (µg/L)	
THPI	Urine ( <i>n</i> = 6)	0.58 ± 0.28	1.9 ± 0.92	0.5 – 50
	Plasma ( <i>n</i> = 10)	1.47 ± 0.69	4.87 ± 2.28	1 – 100
PI	Urine ( <i>n</i> = 6)	1.14 ± 0.22	3.75 ± 0.72	0.5 – 50
	Plasma ( <i>n</i> = 10)	2.17 ± 0.39	7.19 ± 1.29	1 – 100

<sup>a</sup> Limit of detection (LOD) was calculated from calibration curves (6 for urine and 10 for plasma) using the definition:  $y - y_B = 3s_B$ , where  $y$  was the response factor,  $y_B$  was the blank response or the intercept of regression line, and  $s_B$  the standard deviation of the blank [19]. The LOD of each compound was determined for each calibration curve, and then means were calculated and considered as LODs.

<sup>b</sup> Limit of quantification (LOQ) was defined as 3.3 times the detection limit.

**Table 2**

Comparison of performance parameters of available analytical methods for THPI and PI analysis in human plasma and urine.

Methods	Detection technique	LOD <sup>a</sup>	Spiking	Recovery (%) (mean ± SD)	Extraction type
THPI in urine					
Shoen <i>et al.</i> [9]	GC/NPD/MS	165 nmol/L	198 nmol/L	82 ± 10.5 ( <i>n</i> = 9)	Solvent extraction
			331 nmol/L	87 ± 5.2 ( <i>n</i> = 5)	
			3307 nmol/L	86 ± 5.8 ( <i>n</i> = 5)	
van Welie <i>et al.</i> [10]	GC/CI/MS	17.9 nmol/L	17.9 – 360 nmol/L	54 ± 5.0 ( <i>n</i> = 4)	Solvent extraction
Krieger and Thongsinthusak [7]	GC/NPD/MS	33.1 – 66.1 nmol/L	66.1 nmol added	90	Solvent extraction
			661 nmol added	95	
Krieger and Dinoff [8]	GC/ECD/MS	33.1 nmol/L	33.1 – 13230 nmol/L	78 ± 5.0	Solvent extraction
Hines <i>et al.</i> [6]	GC/MS	11.2 nmol/L	33.1 – 265 nmol/L	86 ± 9.1 ( <i>n</i> = 179)	Solvent extraction
Our study	LC/APCI-MS/MS	3.82 nmol/L	3.31 – 33.1 pmol added	94.3 ± 7.2 ( <i>n</i> = 24)	Solid phase extraction
THPI in plasma					
Barr <i>et al.</i> [3]	GC/HR/MS	1 pg/g	1.58 – 2646 nmol/L	91 ± 8.0 ( <i>n</i> = 6)	Solid phase extraction
Our study	LC/APCI-MS/MS	9.76 nmol/L	16.5 – 827 pmol added	90.9 ± 6.7 ( <i>n</i> = 100)	Solid phase extraction

Methods	Detection technique	LOD <sup>a</sup>	Spiking	Recovery	Extraction type
				(%) (mean ± SD)	
PI in Plasma					
Barr <i>et al.</i> [3]	GC/HR/MS	20 pg/g	1.58 – 2646 nmol/L	89 ± 6.0 ( <i>n</i> = 6)	Solid phase extraction
Canal-Raffin <i>et al.</i> [4]	HPLC-UV/DAD	33.1 nmol/L	67.9 – 679.7 nmol/L	90 ± 6.9 ( <i>n</i> = 12)	Solid phase extraction
Our study	LC/APCI-MS/MS	7.72 nmol/L	17.0 – 850 pmol added	79.6 ± 11.3 ( <i>n</i> = 100)	Solid phase extraction

*n*: Number of samples.

GC/CI/MS: gas chromatograph equipped with a chemical ionization and a mass spectrometer; GC/ECD/MS: gas chromatograph with an electrolytic conductivity detector and a mass spectrometer; GC/HR/MS: gas chromatography-high resolution mass spectrometry; GC/NPD/MS: gas chromatograph equipped with nitrogen-phosphorus detector and a mass spectrometer; HPLC-UV/DAD: high-performance liquid chromatography with ultraviolet diode array; LC/APCI-MS/MS: liquid chromatography with a mass spectrometer and atmospheric pressure chemical ionization.

<sup>a</sup> Limit of detection (LOD) was defined as the concentration with a signal-to-noise ratio of at least 3, except for Barr *et al.* [3] and our study, where the LOD was calculated as  $3s_0$  where  $s_0$  was estimated as the *y*-intercept of a linear regression analysis of a plot of the absolute standard deviation versus the concentration.

**Table 3**

Recovery, intra- and inter-day precision and accuracy of THPI and PI at three different spiking levels (pmol added) of blank human urine and at five different spiking levels (pmol added) of blank human plasma.

Analytes	Matrix	Amount added (pmol) <sup>c</sup>	Recovery <sup>d</sup> (%)	RSD <sup>e</sup> (%)	Intra-day variation <sup>a</sup>			Inter-day variation <sup>b</sup>		
					Amount found <sup>f</sup> (pmol) (mean ± SD <sup>g</sup> )	Precision (% RSD <sup>e</sup> )	Accuracy <sup>h</sup> (%)	Amount found <sup>f</sup> (pmol) (mean ± SD <sup>g</sup> )	Precision (% RSD <sup>e</sup> )	Accuracy <sup>h</sup> (%)
THPI	Urine		( <i>n</i> = 8)		( <i>n</i> = 6)			( <i>n</i> = 8)		
		3.31	103.6	15.06	3.58 ± 0.42	11.74	8.13	3.66 ± 0.61	16.69	10.55
		13.2	87.35	13.35	11.50 ± 0.38	3.28	-13.07	11.56 ± 1.54	13.35	-12.65
		33.1	93.47	9.68	31.58 ± 1.46	4.62	-4.52	30.92 ± 2.99	9.67	-6.53
	Plasma		( <i>n</i> = 20)		( <i>n</i> = 10)			( <i>n</i> = 20)		
		16.5	83.02	13.67	13.78 ± 0.97	7.03	-16.71	13.73 ± 1.88	13.67	-16.98
		33.1	82.92	13.28	27.46 ± 2.75	10.00	-16.98	28.28 ± 4.44	15.70	-14.51
165		93.44	10.81	155.05 ± 9.07	5.85	-6.25	154.53 ± 16.70	10.81	-6.56	
	331	95.32	7.99	316.14 ± 19.23	6.08	-4.42	315.30 ± 25.18	7.99	-4.67	

		827	99.49	7.77	818.61 ± 38.07	4.65	-1.01	822.72 ± 63.95	7.77	-0.51
PI	Urine		(n = 8)		(n = 6)			(n = 8)		
		3.40	42.65	6.54	1.42 ± 0.38	21.92	----- <sup>i</sup>	1.24 ± 0.36	28.92	----- <sup>i</sup>
		13.6	82.32	15.4	12.06 ± 0.36	3.03	-11.26	12.9 ± 1.06	8.19	-4.51
		34.0	88.73	14.29	31.01 ± 7.09	14.4	-8.73	33.89 ± 8.18	8.18	-0.26
	Plasma		(n = 20)		(n = 10)			(n = 20)		
		17.0	67.3	15.2	11.42 ± 0.84	7.35	----- <sup>i</sup>	9.80 ± 0.85	8.67	----- <sup>i</sup>
		34.0	73.4	14.3	25.24 ± 2.01	9.41	----- <sup>i</sup>	25.82 ± 2.23	8.65	----- <sup>i</sup>
		170	87.3	13.5	151.84 ± 14.30	11.41	-10.64	148.38 ± 17.16	11.56	-12.68
		340	85.1	10.1	277.48 ± 12.96	4.67	-18.35	277.60 ± 15.67	5.64	-18.31
		850	90.4	12.8	800.23 ± 74.62	9.32	-5.81	748.81 ± 96.99	12.95	-11.86

*n*: Number of samples for each spiking level.

<sup>a</sup> Average variation between *N* replicates of the same level of spiking prepared and analyzed the same day.

<sup>b</sup> Average variation between *N* replicates of the same level of spiking prepared and analyzed on different days over an 8-day period for urine and a 20-day period for plasma.

<sup>c</sup> Amount of THPI or PI (expressed in pmol) added to blank urine or plasma at the different concentration levels.

<sup>d</sup> Percent recovery of THPI or PI amounts (different levels) added to blank urine or plasma samples and processed as described in Materials and Methods.

<sup>e</sup> Precision or RSD: relative standard deviation for *N* replicates calculated as  $(SD/mean) \times 100$ .

<sup>f</sup> Recovered amount of THPI or PI after sample processing and calculated from calibration curves.

<sup>g</sup> SD: standard deviation of *N* replicates.

<sup>h</sup> Accuracy, or percent relative error, calculated using the following equation:  $(\text{amount found} - \text{amount added})/\text{amount added} \times 100$ .

<sup>i</sup> These accuracy values are not reported since matrix effects were apparent for PI at these low spiking levels contrary to THPI-d used as an internal standard.

**Table 4**  
Concentrations of THPI and PI in applicators exposed to captan and folpet, respectively, during the course of a typical workweek along with pre-seasonal concentrations

Metabolite analyzed	Applicator	Concentration (nmol/L)										
		Pre-seasonal	Workday 1 <sup>a</sup>		Workday 2		Workday 3		Workday 4		Workday 5	
			Pre-shift	Post-shift	Pre-shift	Post-shift	Pre-shift	Post-shift	Pre-shift	Post-shift	Pre-shift	Post-shift
THPI												
	Applicator 1	<LOD <sup>b</sup>	4.24	14.17	23.61	43.18	10.01	11.27	12.07	12.93	10.62	5.83
	Applicator 2	<LOD	<LOD	21.53	54.17	43.09	43.71	17.55	15.38	14.16	8.55	10.17
PI												
	Applicator 1	<LOD <sup>c</sup>	16.95	11.41	8.42	17.01	8.87	<LOD	<LOD	<LOD	<LOD	17.74
	Applicator 2	≈LOD	21.71	42.43	42.61	31.51	33.97	26.19	≈LOD	<LOD	13.86	21.70
	Applicator 3	<LOD	<LOD	13.71	15.06	<LOD	<LOD	<LOD	<LOD	<LOD	8.96	≈LOD

<sup>a</sup> Captan and folpet were sprayed on workday 1 only.

<sup>b</sup> LOD of THPI in urine = 3.82 nmol/L.

<sup>c</sup> LOD of PI in urine = 7.72 nmol/L.



## Figure captions

**Fig. 1** Chemical structures of (a) captan and its metabolite THPI and (b) folpet and its metabolite PI.

**Fig. 2** Representative chromatograms of THPI and THPI-d in human urine: (a) blank urine spiked with 10  $\mu\text{g/L}$  of THPI (1) and 75  $\mu\text{g/L}$  of THPI-d (2); (b) urine from an unexposed subject (3) and blank urine spiked with 1  $\mu\text{g/L}$  of THPI (4); (c) blank urine spiked with 10  $\mu\text{g/L}$  of THPI (1) and 75  $\mu\text{g/L}$  of THPI-d (2) along with a urine sample of a volunteer exposed orally to captan (5) spiked with 75  $\mu\text{g/L}$  of THPI-d (6). Chemical structures of THPI, THPI-d and their measured ion fragment are also represented.

**Fig. 3** Representative chromatograms of PI and THPI-d in human urine: (a) blank urine spiked with 1  $\mu\text{g/L}$  of PI (1) and 26.5  $\mu\text{g/L}$  of THPI-d (2); (b) urine from an unexposed subject (3) and blank urine spiked with 10  $\mu\text{g/L}$  of PI (4), 50  $\mu\text{g/L}$  of PI (5) and 100  $\mu\text{g/L}$  (6); (c) blank urine spiked with 1  $\mu\text{g/L}$  of PI (1) and 26.5  $\mu\text{g/L}$  of THPI-d (2) along with a urine sample of a volunteer exposed orally to folpet (7) spiked with 26.5  $\mu\text{g/L}$  of THPI-d (8). Chemical structures of PI, THPI-d and its measured ion fragment are also represented.

**Fig. 4** Representative chromatograms of THPI and THPI-d in human plasma: (a) blank plasma spiked with 50  $\mu\text{g/L}$  of THPI (1) and 79.5  $\mu\text{g/L}$  of THPI-d (2); (b) plasma sample from an unexposed subject (3) and blank plasma spiked with 1  $\mu\text{g/L}$  of THPI (4); (c) blank plasma spiked

with 50 µg /L of THPI (1) and 79.5 µg /L of THPI-d (2) along with a plasma sample of a volunteer exposed orally to captan (5) spiked with 79.5 µg /L of THPI-d (6).

**Fig. 5** Representative chromatograms of PI and THPI-d in human plasma: **(a)** plasma sample from an unexposed subject (1) spiked with 79.5 µg /L of THPI-d (2) and blank plasma spiked with 1 µg /L of PI (3) and 79.5 µg /L of THPI-d (4); **(b)** plasma from an unexposed subject (5) and blank plasma spiked with 10 µg /L of PI (6); **(c)** blank plasma spiked with 1 µg /L of PI (3) and 79.5 µg /L of THPI-d (4) along with a plasma sample of a volunteer exposed orally to folpet (7) spiked with 79.5 µg /L of THPI-d (8).

**Fig. 6** Calibration curves of THPI over the concentration range of 0.003 to 0.66 µmol/L (0.5 to 100 µg/L) prepared in methanol (▼), blank urine (○) and blank plasma (●). The linearity of curves is described by the coefficient of determination ( $R^2$ ) and equations are presented.

**Fig. 7** Calibration curves of PI over the concentration range of 0.5 to 50 µg/L prepared in methanol (▼), blank urine (○) and blank plasma (●). The linearity of curves is described by the coefficient of determination ( $R^2$ ) and equations are presented.

**Fig. 8** **(a)** Concentration-time profiles of THPI (mean ± SD) in volunteers exposed to captan orally (1 mg/kg) or dermally (10 mg/kg) and **(b)** concentration-time profiles of PI (mean ± SD) in volunteers exposed orally and dermally to folpet (1 and 10 mg/kg, respectively). (-●-) Urinary excretion profile following oral exposure; (-○-) urinary excretion profile following dermal

exposure; (-▼-) plasma profile following oral exposure; (-Δ-) plasma profile following dermal exposure.

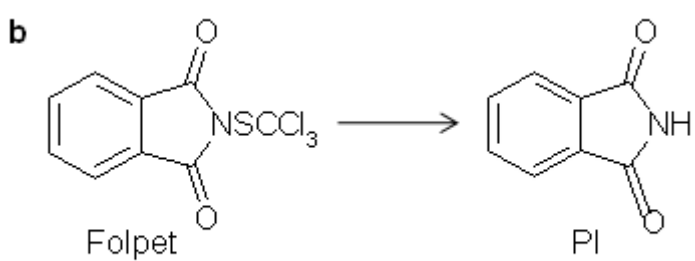
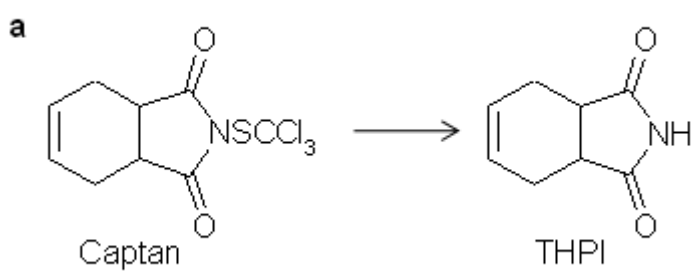
**Figure 1**

Figure 2

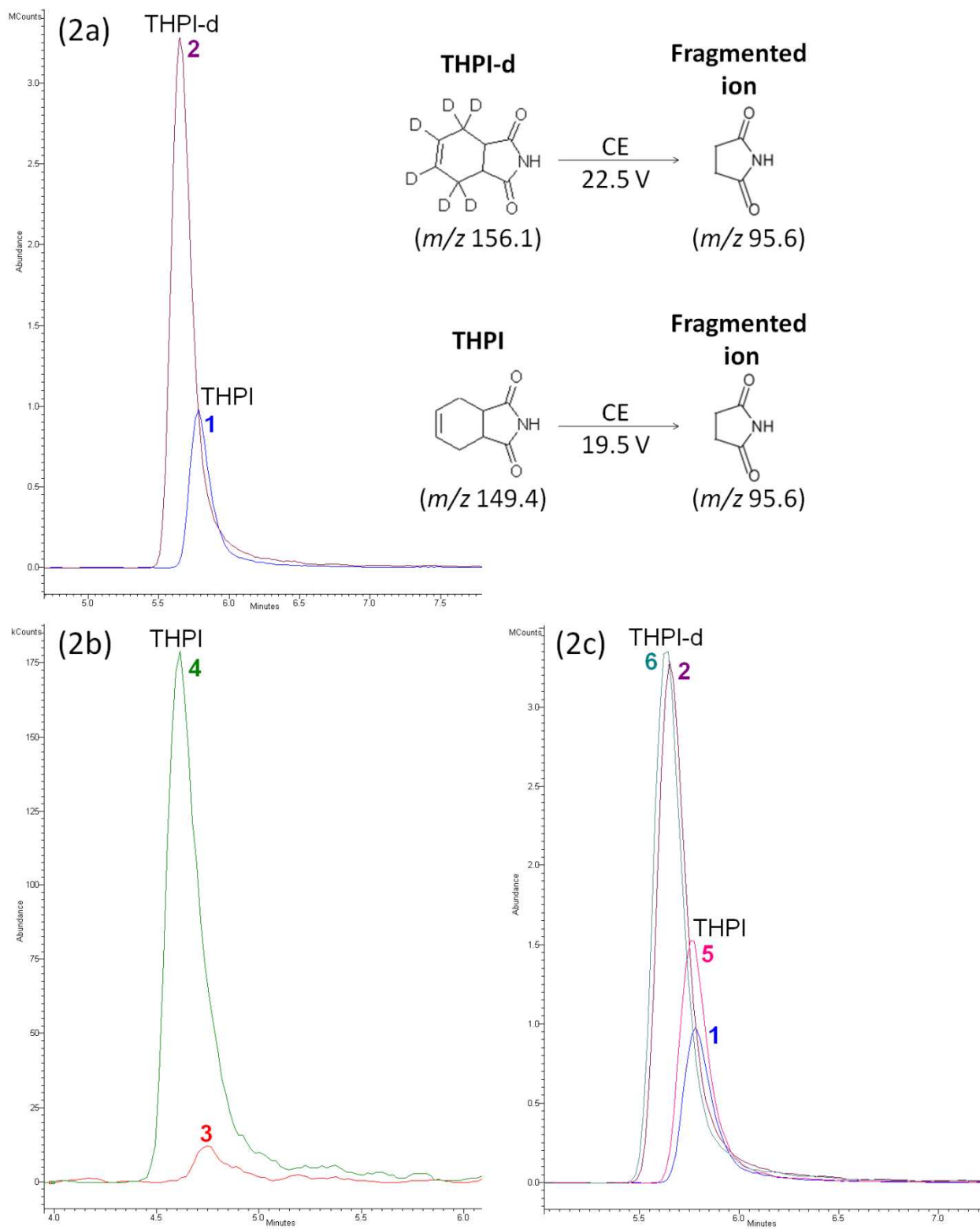


Figure 3

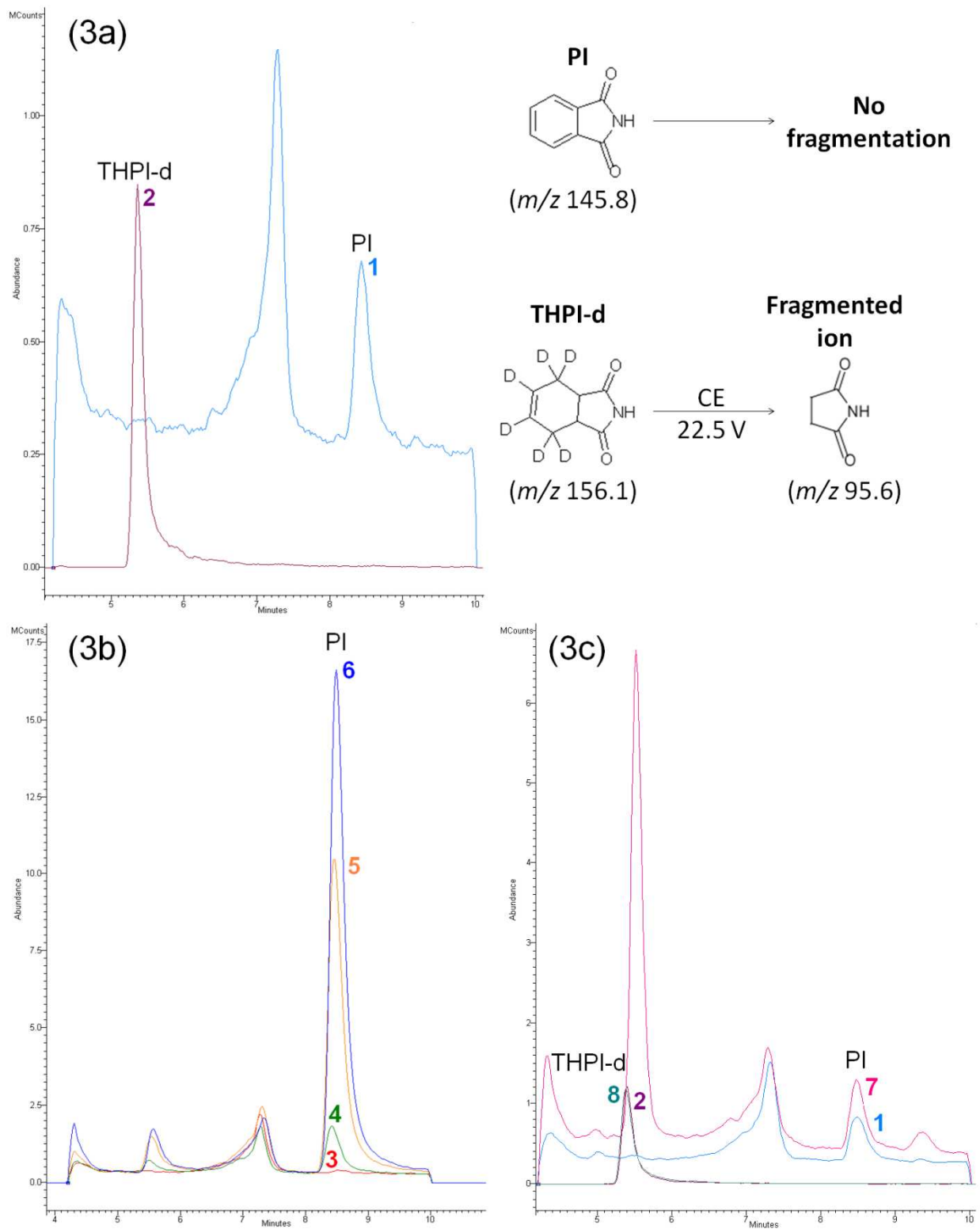


Figure 4

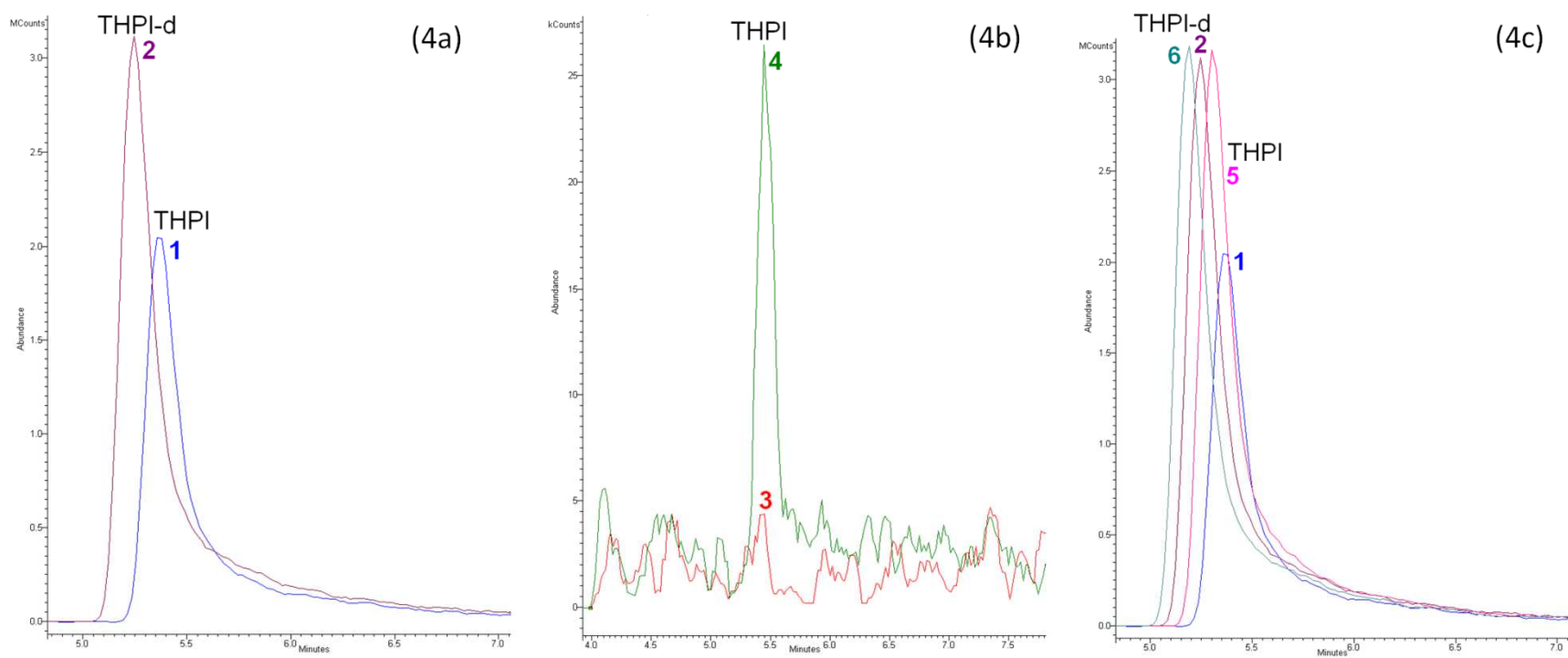


Figure 5

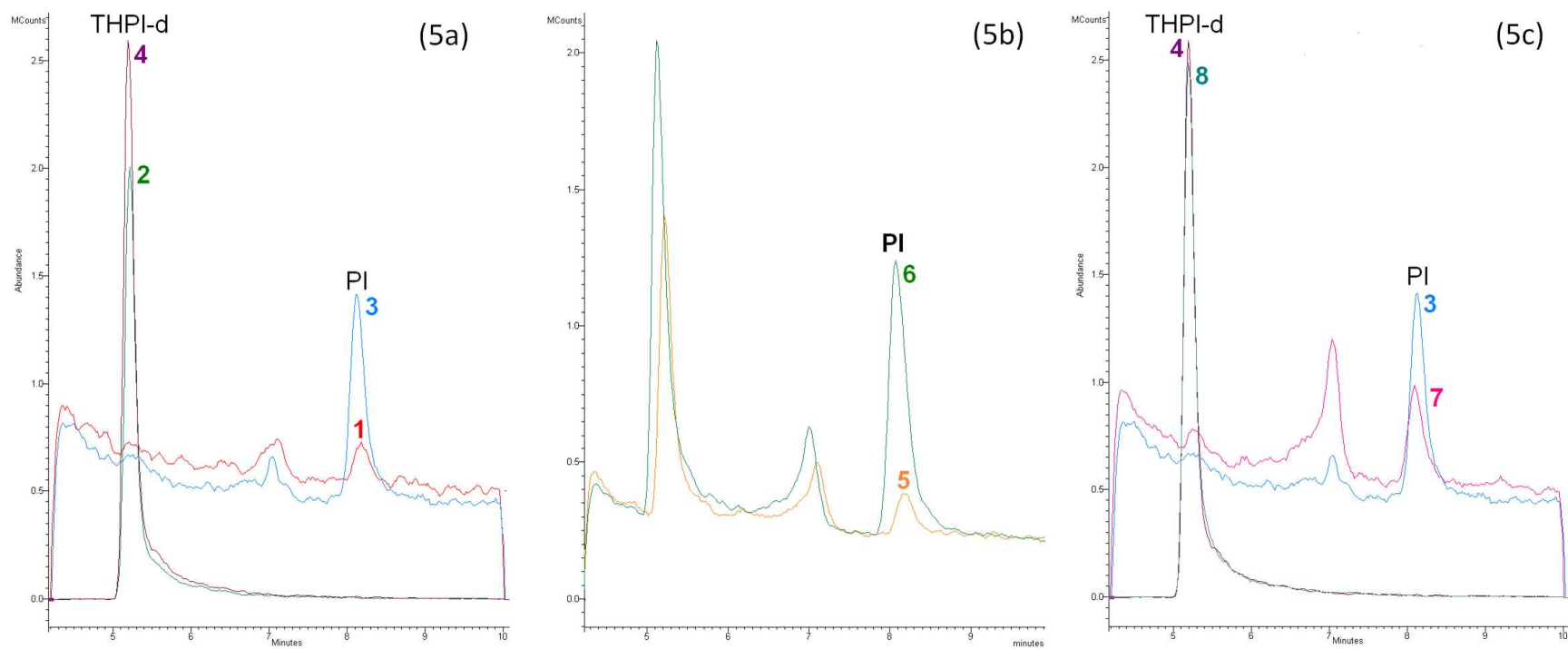




Figure 6

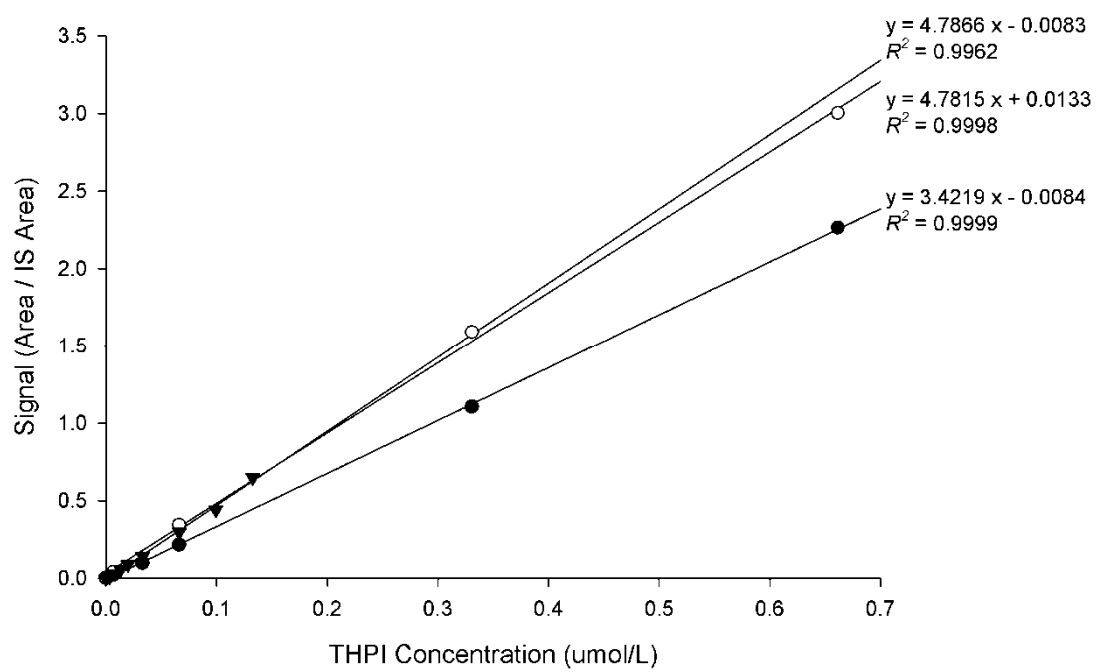


Figure 7

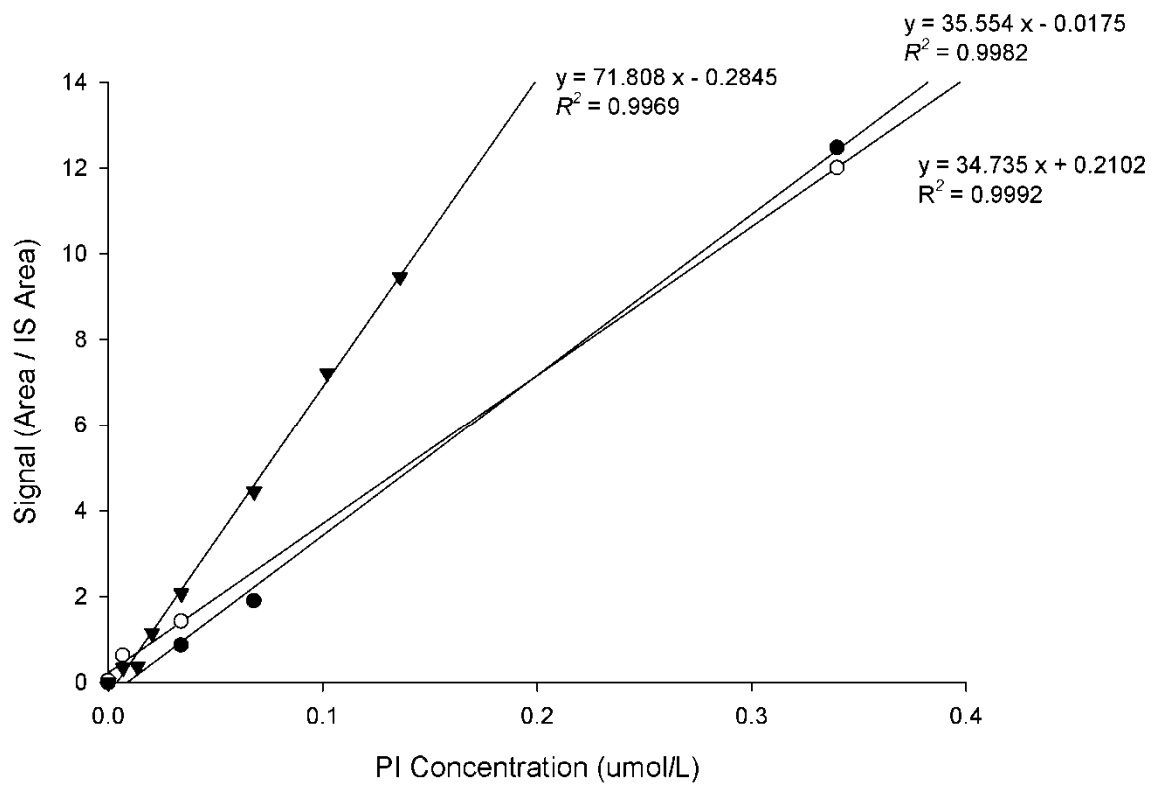
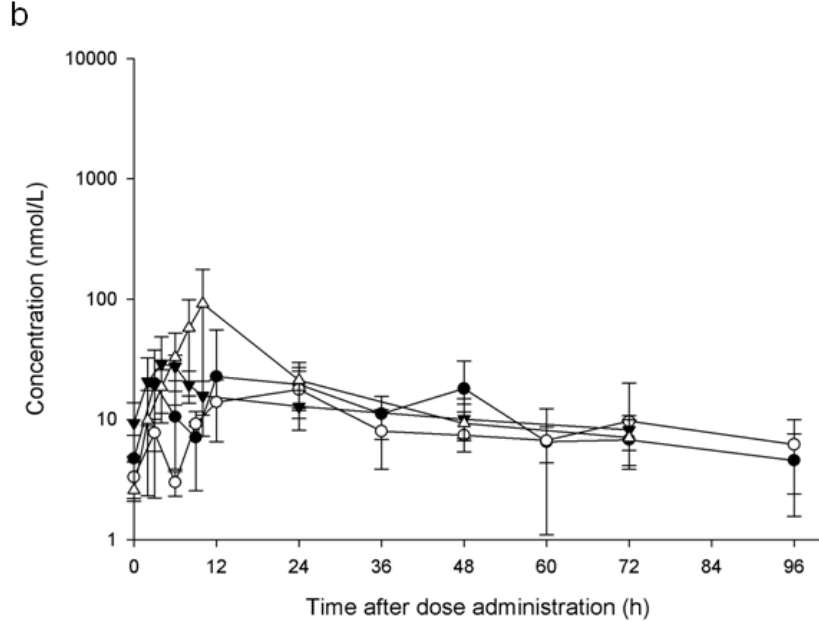
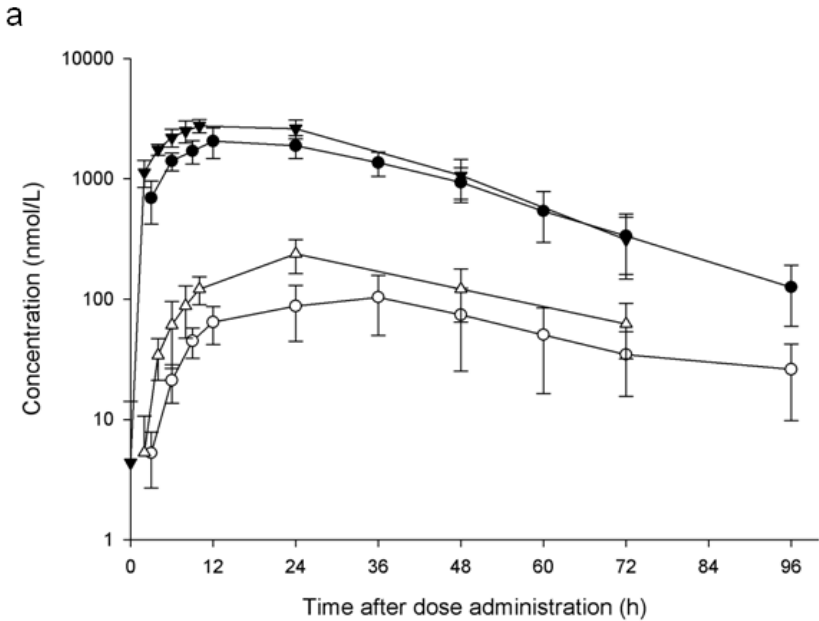


Figure 8



## Article II

### **Gas-chromatography mass-spectrometry determination of phthalic acid in human urine as a biomarker of folpet exposure**

Aurélie Berthet, Michèle Berode, Michèle Bouchard\*

A. Berthet · M. Berode

Institute for Work and Health, Bugnon 21, 1011 Lausanne, Switzerland

A. Berthet · M. Bouchard (\*)

Department of Environmental and Occupational Health, School of Public Health,  
Université de Montréal, P.O. Box 6128, Main Station, Montreal, Quebec, Canada,  
H3C 3J7

\*corresponding author

Analytical and Bioanalytical Chemistry

Article soumis (Novembre 2010)

## Abstract

Agricultural workers are exposed to folpet but biomonitoring data are limited. Phthalimide (PI), phthalamic acid (PAA) and phthalic acid (PA) are the ring metabolites of this fungicide according to animal studies but they have not yet been measured in human urine as metabolites of folpet, only PA as a metabolite of phthalates. The objective of this study was thus to develop a reliable gas chromatography – tandem mass spectrometry (GC-MS) method to quantify the sum of PI, PAA and PA metabolites ring-metabolites of folpet in human urine. Briefly, the method consisted of adding *p*-methylhippuric acid as an internal standard (IS), performing an acid hydrolysis at 100°C to convert ring-metabolites into PA, purifying samples by ethyl acetate extraction and derivitizing with N,O-bis(trimethylsilyl)trifluoro acetamide (BSTFA) prior to GC-MS analysis. The method had a detection limit of 60.2 nmol/L (10 ng/mL); it was found to be accurate (mean recovery of 97%), precise (inter- and intra-day percentage relative standard deviations < 13%) and with a good linearity ( $R^2 > 0.98$ ). Validation was conducted using unexposed person urines spiked at concentrations ranging from 4.0 to 16.1  $\mu\text{mol/L}$ , along with urine samples of volunteers dosed with folpet and of exposed workers. The method proved to be i) suitable and accurate to determine the kinetic profile of PA equivalents in the urine of volunteers orally and dermally administered folpet and ii) relevant for the biomonitoring of exposure in workers.

**Keywords** Phthalic acid · Phthalamic acid · Folpet · Human urine · Biological monitoring

## Introduction

Folpet, or N-trichloromethyl-thio-phthalimide (CAS number 133-07-3), has extensive application as a contact fungicide in a variety of fruit and vegetable crops or vineyards. Although a large number of workers use or are in contact with folpet, there is a paucity of data on the extent of occupational exposure to this compound. Some environmental measurements have been conducted [1] but only one study performed a biomonitoring of exposure to folpet in pregnant women through phthalimide (PI) measurements [2].

Folpet metabolism is however well characterized in animals and *in vitro* studies [3-7]. According to these studies, folpet is rapidly metabolized to PI and thiosphogene, an unstable metabolite which reacts with cysteine or glutathione to form thiazolidine-2-thione-4-carboxylic acid (TTCA). Phthalimide is also rapidly hydrolyzed to phthalamic acid (PAA), and in turn to phthalic acid (PA). The chemical structures of folpet and of its ring metabolites are displayed in Fig. 1.

According to Chasseaud *et al.* [4;5;8] and Canal-Raffin *et al.* [3], the main ring-metabolite of folpet is PAA in rats following an oral, intratracheal or intraperitoneal administration. More specifically, Chasseaud *et al.* [5;8] observed that 80% of a labeled  $^{14}\text{C}$ -folpet dose orally administered to rats was recovered as PAA in urine. Canal-Raffin *et al.* [3] reported that PI was rapidly metabolized to PAA in plasma when folpet was intraperitoneally and intratracheally administered to rats.

To quantify PAA, Canal-Raffin *et al.* [3] analyzed rat plasma by high-performance liquid chromatography with UV detection (HPLC-UV) after specific solid-phase extraction, but they did not measure the metabolite in urine. Nevertheless, as determined by Bray *et al.* [9], PAA is a very unstable compound in urine, which has to be precisely extracted at pH 8.3 and is transformed to PA in acidic conditions (pH 2-3). Namely, under electron impact ionization (EI) for GC-MS analysis, water loss is induced and the neutral compound PAA can rearrange to PI, phthalic anhydride or phthalic acid [10;11]. It is thus more convenient to transform PI and PAA metabolites of folpet to PA in acid conditions, and to measure total PA equivalents in urine as a biomarker of exposure.

Analytical methods have already been developed to quantify PA as a urinary metabolite of phthalates, because it is also the final hydrolysis product of phthalates [12-16], but no method has been published for the quantification of total PA equivalents as a biomarker of folpet exposure to our knowledge. Therefore, the objectives of this study were 1) to measure total PA equivalents in urine as a biomarker of folpet exposure by adapting existing gas chromatography - mass spectrometry [GC-MS] methods after oxidation of PI and PAA and trimethylsilylation of PA molecule, and 2) to use this method to quantify the urinary excretion of total PA equivalents in volunteers exposed to folpet as well as 3) to assess worker exposure to this fungicide through biomonitoring.

## Materials and methods

### Chemicals and reagents

Phthalic acid (1,2-Benzenedicarboxylic acid), phthalamic acid (benzoic acid,2-aminocarbonyl), phthalimide and *p*-methylhippuric acid used as reference standards (>99% purity) were obtained from Sigma-Aldrich (Buchs, St Gallen, Switzerland). Other chemicals and reagents such as HPLC-grade acetonitrile, dimethylformamide, ethyl acetate, N,O-bis(trimethylsilyl)trifluoro acetamide (BSTFA), hydrochloric acid and sodium chloride (NaCl) were also purchased from Sigma-Aldrich (Buchs, St Gallen, Switzerland). Purified tap water by a TKA GenPure (Niederelbert, Germany) was used.

### Standard preparation

#### *Stock standard solutions*

A stock standard solution of 130 mg/L (0.8 mmol/L) of PA was prepared by dissolving 13 mg of PA in 100 mL of 1% (v/v) dimethylformamide in acetonitrile. Similarly, a stock standard solution of the internal standard (IS) *p*-methylhippuric acid of 100 mg/L (0.5 mmol/L) was prepared by dissolving 10 mg of *p*-methylhippuric acid in 100 ml of 1% (v/v) dimethylformamide in acetonitrile. In addition, to measure the fraction of PI and PAA converted to PA with this analytical method, two individual stock standard solutions of 112 mg/L (0.8 mmol/L) of PI and 114 mg/L (0.7 mmol/L) of PAA were prepared by dissolving 11.2 mg of PI and 11.4 mg of PAA, respectively, in 100 mL of 1% (v/v) dimethylformamide in acetonitrile. The stock solutions were stored at -20°C in glass containers until used.



### *Calibration curves*

Calibration curves were prepared by spiking aliquots of pooled urine from unexposed persons at different concentrations of PA and a constant concentration of IS. Working solutions of PA at 26 mg/L (0.16 mmol/L) and IS at 20 mg/L (0.1 mmol/L) were thus prepared daily from stock solutions diluted 5-fold in water. Calibration curves of PA consisted of urine from unexposed persons spiked at six concentration levels prepared in duplicates: 0, 0.67, 1.3, 2.0, 2.3 and 2.7  $\mu\text{g/mL}$  (0, 4.0, 8.1, 12.1, 14.1 and 16.1  $\mu\text{mol/L}$ ). For each level, 2 mL of urine were spiked with a constant volume of 100  $\mu\text{L}$  of IS working solution as well as defined volumes of PA working solution and completed with water to obtain a final volume of 2.3 mL.

### *Sample treatment*

The analytical approach used to quantify total urinary PA equivalents consisted of an acid hydrolysis, solvent extraction and derivatization with BSTFA followed by gas chromatography – mass spectrometry (GC-MS) analysis. Sample preparation was adapted from method of Mettang *et al.* [14]. Two-mL aliquots of urine were transferred into screw-cap reaction vials and spiked with 100  $\mu\text{L}$  of internal standard working solution (20 mg/L or 0.1 mmol/L) and 200  $\mu\text{L}$  of water. An acid hydrolysis was then performed by adding HCl (300  $\mu\text{L}$  at 12 N) and heating at 100°C for 12 h. After cooling, samples were extracted twice with 2 mL of ethyl acetate by agitating for 15 min and centrifuging for 10 min at 2500 rpm. Upper organic layers were transferred into glass tubes. Extracts were evaporated to dryness under a gentle nitrogen flow at 40°C to concentrate samples. Residues were resuspended in 300  $\mu\text{L}$  of ethyl acetate; 30  $\mu\text{L}$  of BSTFA were added and samples were heated at 60°C for 60 min to convert total PA equivalents and *p*-methylhippuric acid IS into

trimethylsilyl phthalic acid (TMS-PA) and trimethylsilyl *p*-methylhippuric acid (TMS-IS), respectively, by replacement of the labile hydrogen of the hydroxy groups. Before transferring derivatized extracts to vials for GC-MS analysis, extracts were cooled for a 2-h period.

#### Instrumental analysis

Analyses were carried out on a HP 5973 gas chromatograph equipped with a mass selective detector Agilent MSD-G1098A and a CP-SIL 8 CB fused silica capillary column (60 m length, 1  $\mu$ m film thickness, 250  $\mu$ m I.D.). Helium was used as a carrier gas at a 27 cm/s linear velocity. The injector was set to a temperature of 260°C and a constant column flow rate was held at 1.0 mL He/min. The transfer line was set at 250°C and the ion source at 230°C. The GC oven temperature program was set initially at 200°C for 3 min; it was then increased to 260°C at 30°C/min, held for 11 min, and finally increased to 280°C at 35°C/min and held for 4 min. The mass detector was operated in single ion monitoring mode for quantification and fragment ions were generated by electron impact ionization at 70 eV. The fragments analyzed were  $m/z$  295 for TMS-PA (qualitative ions were  $m/z$  221 and 147) and  $m/z$  220 (qualitative ions were  $m/z$  177 and 119) for TMS-IS. Two  $\mu$ L were injected onto the GC with an Agilent auto-sampler using a 5 mL/min split. Under these conditions, retention times were 10.6 min for TMS-PA and 16.0 min for TMS-IS.

#### Quantification of PA

Quantification of PA was carried out using standard calibration curves in urine. This was achieved by plotting the response factors as a function of the six standard concentration levels of PA (0, 0.67, 1.3, 2.0, 2.3 and 2.7  $\mu$ g/mL or 0, 4.0, 8.1, 12.1,

14.1 and 16.1  $\mu\text{mol/L}$ ). The response factors were the peak-height ratios of TMS-PA to TMS-IS.

#### Estimation of the fraction of PI and PAA converted to PA

The fraction of PI and PAA converted to PA with the analytical processing used was also determined. Aliquots of urine from an unexposed person were independently spiked with standard solutions of 22.5 mg/L of PI or 22.8 mg/L of PAA (stock standard solutions diluted 5-fold) at six concentration levels prepared in duplicates: 0, 0.56, 1.12, 1.69, 1.97 and 2.25 g/L (0, 3.8, 7.6, 11.5, 13.4 and 15.3 mmol/L) for PI and 0, 0.57, 1.14, 1.71, 1.99 and 2.28 g/L (0, 3.4, 6.9, 10.3, 12.1 and 13.8 mmol/L) for PAA. These samples were then treated using the method described previously for the quantification of TMS-PA. The fraction of PI and PAA converted to total PA equivalents (expressed as a percentage) was calculated using the following equation:

$$\begin{aligned} & \text{Fraction of PI and PAA converted to PA (\%)} \\ &= \frac{\text{Amounts of PA in extracts of spiked samples (mol)}}{\text{Added amounts of PI or PAA in urine samples (mol)}} \times 100 \end{aligned}$$

#### Method validation

In order to validate the method, the following criteria were verified: limit of detection (LOD), limit of quantification (LOQ), linearity, intra- and inter-day precision, accuracy, recovery and stability. In addition, internal quality control (QC) urinary samples were prepared from a pool of urine from unexposed individuals spiked at two levels of concentrations (1 and 2.5  $\mu\text{g/mL}$  or 6 and 15  $\mu\text{mol/L}$ ). From this pool, 6-mL aliquots were prepared and stored at  $-20^\circ\text{C}$ . During each daily run, QC samples were analyzed in triplicates, as unknown samples.

Specificity of the method was confirmed by verifying the absence of interferences on the chromatograms obtained from urine samples of unexposed individuals ( $n = 22$ ). The LOD was established as three times the signal-to-noise ratio for the specific ions  $m/z$  295 and  $m/z$  220 and the LOQ as ten times the signal-to-noise ratio. Linearity was calculated from regression parameters of 22 calibration curves, by the least square fit method. Results were expressed using the mean of coefficient of determination ( $R^2$ ).

To assess intra- and inter-day variations, the precision and accuracy were calculated from replicate analysis of previously prepared QC samples and of daily prepared aliquots of a pool of urine from unexposed individuals spiked with PA at five different levels. The intra-day variation was estimated by the repeatability of triplicates of QC samples and of duplicates of each spiking level prepared daily and analyzed the same day. The inter-day variation was estimated by the reproducibility of these previous samples on 22 consecutive days.

Precision, expressed as percentage of relative standard deviation (% RSD), was determined as the ratio of standard deviation to mean of the response factor for each spiking level (QC samples and prepared daily spiked samples) multiplied by 100.

Accuracy, expressed in percentage, was calculated as follows:

$$\frac{(\text{average measured amounts of PA} - \text{known spiking amounts of PA})}{\text{known spiking amounts of PA}} \times 100$$

Recovery of PA in urine after extraction and derivatization was calculated for each spiking level by the ratio of measured amounts of PA in extracts to the theoretical spiked amounts in urine of unexposed individuals and expressed as a percentage.

The stability of PA in urine was ascertained by analyzing every day over a 22-day period an aliquot of QC samples kept at  $-20^{\circ}\text{C}$ , and thawed daily and processed. The stability of PA equivalents in urinary samples of workers exposed to folpet was also determined. More specifically, the effect of preservation of urine samples at  $4^{\circ}\text{C}$  over a 24-h-period was tested. Two urine samples of workers thawed, processed and analyzed for PA equivalents in a given run were thus kept at  $4^{\circ}\text{C}$ , processed and reanalyzed during the following run.

#### Application of the method

The method was used to determine total urinary amounts of ring-metabolites of folpet in exposed individuals. First, concentrations of total PA equivalents were determined in all urines collected repeatedly and at predetermined times over a 96-h period following an oral and dermal administration of 1 and 10 mg/kg of folpet, respectively, in five volunteers (the same five volunteers were used for the oral and dermal dosing).

Secondly, concentrations of PA equivalents were measured in all complete urine voids collected over a 7-day period following a folpet treatment and sequences of harvesting in grapegrowers ( $n = 3$ ). Each worker collected i) a pre-seasonal complete first-morning void, hence a urine sample prior to folpet spraying and harvesting season, ii) all complete urine voids prior to and during a typical workweek following a folpet spraying, and iii) all complete urine voids prior to and during a typical workweek involving harvest activities. During spraying period, the three workers applied folpet only on the first workday; during harvest activities period, workers 1

and 3 were in the treated area only on workday 1 while worker 2 harvested from workdays 1 to 3.

The Permanent Ethical Committee of Clinical Research of the Faculty of Biology and Medicine of the University of Lausanne and of the Research Ethical Committee of the Faculty of Medicine of the University of Montreal approved the protocol, and all participants gave their written informed consent, and were acquainted with the risks of participating and their right to withdraw from the study at all time. The volunteers administered folpet received a small monetary compensation for their time and any inconvenience caused, as suggested by the Ethics Committee who considered the study as restrictive. Conversely, workers were not compensated.

## Results and discussion

### Method development

To optimize the method of Mettang *et al.* [17] for the specific analysis of total PA equivalents as a biomarker of exposure to folpet, several preliminary tests were performed: 1) the verification of potential contamination of laboratory materials by phthalates, 2) the selection of a proper IS, 3) the effect of NaCl addition prior to extraction and 4) the establishment of the most efficient type of hydrolysis. Firstly, to verify if that laboratory materials (i.e. vials, caps, pipettes) were a contamination source of PA during sample processing, triplicate controls with water and ethyl acetate were subjected to the processing procedure; no PA peak was observed after analysis. Secondly, potential internal standards were also tested. Four deuterium phthalic acids (PA-d) were initially considered, but the separation of PA and PA-d on the GC column was insufficient to differentiate both peaks on chromatograms. *Para*-methylhippuric acid was then assayed because the molecule comprised a carboxylic acid and an amine group, and finally selected. Thirdly, to improve extraction, the effect of adding NaCl to urine samples prior to extraction and analysis was compared with results obtained after analysis of the same urine samples without NaCl addition. The results are presented in Table 1 and show that extraction efficiency was better without NaCl addition.

Lastly, efficiency of acid hydrolysis as compared to enzymatic hydrolysis was tested. This latter step was described by several authors for the quantification of phthalates [14;17-23]. To perform enzymatic hydrolysis, 20  $\mu$ L of  $\beta$ -glucuronidase-arylsulfatase were added to 2 mL of four urine samples from one person administered folpet and

samples were heated at 37°C for 12 h; for acid hydrolysis, 500 µL of HCl 2N were added and samples were heated either at 37°C or 100°C for 12 h. As shown in Table 2, higher concentrations of total PA equivalents were observed after acid hydrolysis at 100°C than at 37°C, while enzymatic hydrolysis appeared inadequate and insufficiently strong to transform PI and PAA into PA. Pfäffli [24] previously reported that enzymatic hydrolysis was unnecessary because PA appears to be mainly excreted as free acid and not partly conjugated and excreted as glucuronide. Table 3 also shows that when spiking urine samples with PAA and PI, PAA is virtually all converted to TMS-PA (100%) and half of PI was converted to TMS-PA (≈50%) following an acid hydrolysis at 100°C. Thus, acid hydrolysis was selected for the quantification of total PA equivalents. Even if PI was not completely converted to TMS-PA by acid hydrolysis, it is a very minor metabolite of folpet as compared to PAA according to *in vivo* studies in rats exposed to folpet [3-8].

Representative chromatograms of TMS-PA and TMS-IS in urine from an unexposed individual spiked with 12.1 µmol/L of PA and 6.72 µmol/L of IS, as well as the mass spectra and the molecule structure of both derivatives are presented in Figure 2. These chromatograms were obtained with the optimized method after acid hydrolysis, solvent extraction and derivatization. Chromatograms show very limited background interference. Clean chromatograms were observed not only for spiked and non-spiked urines from unexposed individuals, but also for samples from volunteers dosed with folpet and from workers, as displayed in Fig. 3. Therefore, analytical conditions were specific to quantify TMS-PA as the sum of total ring-metabolites folpet.



It is to be noted that TMS-PA was detectable in urine samples of unexposed persons, as illustrated in Fig. 3b. Hence, an average PA concentration ( $\pm$  SD) of  $285 \pm 150$  nmol/mmol creatinine ( $492 \pm 275$  ng/mL) was obtained in the urine of unexposed individuals ( $n = 10$ ) and  $186 \pm 79.7$  nmol/mmol creatinine ( $507 \pm 280$  ng/mL) in pre-seasonal urines of workers exposed to folpet ( $n = 3$ ). These values are equivalent to PA concentrations determined by Pfäffli [24] in pre-shift urines of workers exposed to phthalic anhydride (490 nmol/mmol creatinine) and in urines of occupationally unexposed people (0.34  $\mu$ mol/mmol creatinine). Similarly, in biomonitoring studies of phthalates, Jönsson *et al.* [25], Kato *et al.* [19] and Vermeulen *et al.* [26] reported also relatively high PA baseline levels in unexposed people or in workers during a non-exposure period. These levels were on average lower than those found in our study; however, large interindividual variability was found. Namely, Jönsson *et al.* [25] reported a mean PA concentration of 94 nmol/mmol creatinine (95<sup>th</sup> percentile) in urine samples from 234 Swedish men, with a maximum concentration of 2100 nmol/mmol creatinine; Kato *et al.* [19] measured a median PA concentration of 6.19 ng/mL in 43 unexposed and anonymous adults with a range of 1.54 to 44.4 ng/mL (creatinine was not measured in this study); Vermeulen *et al.* [26] obtained a median PA concentration of 77 ng/mL (47 nmol/mmol creatinine) in Sunday urine samples of rubber workers with a range of 4 to 2449 ng/mL (2.44 to 1495 nmol/mmol creatinine). This baseline is unavoidable because phthalates are ubiquitous in the environment and exposure can occur from various sources [12;14-31]; exposure to folpet in the general population can also occur through the diet, thus also contributing to baseline levels. Biomonitoring results should thus be interpreted with caution in non-occupationally exposed individuals. However, these studies

observed higher PA concentrations when people were occupationally exposed to phthalates.

#### Method validation

To validate the robustness of the current method, we evaluated its linearity, LOD, LOQ, intra- and inter-day precision and accuracy, recovery and stability using a pool of urine from unexposed individuals spiked at five different levels and two QC samples.

All calibration curves ( $n = 22$ ) were linear within the spiking range in urine of 0.67 to 2.7  $\mu\text{g/mL}$  (4.0 to 16.1  $\mu\text{mol/L}$ ) with a mean equation of  $y = 0.957x + 0.088$  and a coefficient of determination of 0.98. The LOD and LOQ of PA in urine were 10  $\text{ng/mL}$  (60.2  $\text{nmol/L}$ ) and 33  $\text{ng/mL}$  (199  $\text{nmol/L}$ ), respectively. In comparison with methods developed for the analysis of PA as a biomarker of phthalate exposure, our method had a better LOD than the one obtained with the liquid chromatography – tandem mass spectrometry (LC/MS/MS) method described by Jönsson *et al.* [25] (LOD = 15  $\text{ng/mL}$  or 90.3  $\text{nmol/L}$ ) or the GC-MS method of Pfäffli [24] (LOD = 100  $\text{ng/mL}$  or 602  $\text{nmol/L}$ ) and a LOD similar to the one reported by Lim *et al.* [13] with a HPLC/UV method (LOQ = 100  $\text{ng/mL}$  or 602  $\text{nmol/L}$ ). However, Kato *et al.* [19], Silva *et al.* [22] and Vermeulen *et al.* [26] reported LC/MS/MS methods for the quantification of PA metabolites of phthalates with a slightly better LOD (LOD of 0.42, 1.59 and 5  $\text{ng/mL}$  or 2.53, 9.57 and 30.1  $\text{nmol/L}$ , respectively). Nonetheless, to determine phthalate exposure to date, specific metabolites (i.e. monoesters and oxidation products) are preferably measured instead of PA, which is a common final metabolite of many phthalates [13; 17-18; 28-29].

Results for precision and accuracy of the method are summarized in Table 4 for each of the five daily prepared spiking levels of aliquots of a pool of urine from unexposed individuals as well as for two pre-prepared QC samples. Results indicate that urinary PA was reliably measured in urine because the intra- and inter-day precision expressed by the percent relative standard deviation (% RSD) was less than 13% overall (less than 10% for spiking amounts higher than 2 nmol), and accuracy established by the percent relative error (% RE) was less than 10%.

Table 4 also reveals an excellent recovery of PA in extracts after acid hydrolysis, solvent extraction and derivatization of urine samples spiked with PA. Hence, the mean ( $\pm$  SD) recovery of PA (all spiked levels combined) was  $97.5 \pm 8.8\%$ , indicating that very little PA was lost during processing.

QC samples kept at  $-20^{\circ}\text{C}$  and thawed daily and processed over a 22-day period also appeared stable; this is evident when comparing mean intra-day variation in recovered PA amounts to mean inter-day variation (Table 4). Likewise, PA was stable in urine samples of workers exposed to folpet. A coefficient of variation ranging from 6.9 to 13.3% was obtained when two aliquots of the same urine collection of two workers kept at  $4^{\circ}\text{C}$  for 24 h were processed and analyzed during the following run.

#### Application of the method to biomonitoring

To further validate the method and verify that total ring-metabolites of folpet (*i.e.* total PA equivalents) could be efficiently quantified in the urine of individuals

exposed to folpet, urines of volunteers orally and dermally dosed with folpet as well as urines of grapegrowers during a typical work week were analyzed.

The mean concentration-time profiles of total PA equivalents in the urine of volunteers following an oral and dermal administration are presented in Fig. 4. Although there was a background PA level, it was easy to document the human toxicokinetics of this biomarker of exposure [32-33]. Briefly, PA had a relatively short elimination half-life in urine and it was similar for both exposure routes, hence 27.6 h and 29.6 h for oral and dermal routes, respectively. Similarly, Table 5 displays pre- and post-shift urinary levels of total PA equivalents in workers exposed to folpet during and following spraying or harvest activities. PA was measurable in all urine samples of workers and an increase in excretion was observed for most workers following spraying or harvest activities. According to these results, workers were more exposed during spraying activities than during harvest activities. Consequently, the method proved to be suitable to quantify actual daily exposures to folpet in workers.

## Conclusion

In summary, the method was found to have a LOD of 60.2 nmol/L, an excellent recovery (mean of 97%), a good linearity with a  $R^2$  superior to 0.98. It is also precise with an inter- and intra-day percentage relative standard deviations inferior to 13%. In addition, with this method, only a small urine volume is needed (2 mL) and sample treatment is straightforward. Moreover, our results evidenced that the current GC-MS method was reliable and accurate to quantify total PA equivalents in the urine of individuals dosed with folpet as well as relevant to assess worker exposure using biomonitoring.

## Acknowledgements

We thank Gregory Plateel for his assistance in method adjustment and Patricia Stephan, Christine Arnoux and Christine Kohler for their technical assistance. The project was funded by the ANSES (*Agence Nationale de Sécurité Sanitaire de l'alimentation, de l'environnement et du Travail*). Aurélie Berthet also received a scholarship from the *Institut de recherche Robert-Sauvé en santé et sécurité du travail du Québec*.

## References

1. Burroughs GE and Hora J (1982) Health hazard evaluation report. Report No. HETA-80-147-1076 Calhio Chemical Company, Perry, Ohio. National Institute for Occupational Safety and Health (NIOSH), Cincinnati, Ohio
2. Whyatt RM, Barr DB, Camann DE, Kinney PL, Barr JR, Andrews HF, Hoepner LA, Garfinkel R, Hazi Y, Reyes A, Ramirez J, Cosme Y, and Perera FP (2003) *Environ Health Perspect* 111:749-756
3. Canal-Raffin M, Receveur M, Martinez B, Titier K, Ohayon C, Baldi I, Molimard M, Moore N, and Brochard P (2008) *J Chromatogr B Anal Technol Biomed Life Scie* 865:106-113
4. Chasseaud L, Hawkins DR, Franklin ER, and Weston KT (1974) The metabolic fate of  $^{14}\text{C}$ -Folpet (Phaltan) in the rat (Folpet). Unpublished report No CHR1-74482. Huntingdon Research Centre Ltd, Huntingdon, England
5. Chasseaud L, Wood SG, Cheng K, Hall M, Fitzpatrick K, Iqbal S, and Barlett A (1991) Metabolic fate of  $^{14}\text{C}$ -folpet in Sprague-Dawley rats. Unpublished report No HRC/ MBS 41-91499. Huntingdon Research Centre Ltd, Huntingdon, England
6. Gordon EB, Ehrlich T, Mobley S, and Williams M (2001) *Toxicol Met* 11:209-223
7. Gordon EB (2010) Captan and folpet. In: Krieger RI (ed) *Hayes Handbook of pesticide toxicology*, 3<sup>rd</sup> edn. Elsevier, New York
8. Chasseaud L (1980) (Carbonyl- $^{14}\text{C}$ ) Folpet metabolism in rats. Unpublished report No DPBP 51202. Huntingdon Research Centre Ltd, Huntingdon, England

9. Bray HG, Hybs Z, and Thorpe WV (1951) *Biochem J* 48:192-199
10. Selva A, Traldi P, Ventura P, and Valle VD (1982) *Org Mass Spectrom* 17:524-528
11. Selva A, Traldi P, Ventura P, and Servadio V (1983) *Org Mass Spectrom* 18:278-281
12. Albro PW, Jordan S, Corbett JT, and Schroeder JL (1984) *Anal Chem* 56:247-250
13. Lim DS, Shin BS, Yoo SD, Kim HS, Kwack SJ, Alm MY, and Lee BM (2007) *J Toxicol Environ Health Part A* 70:1344-1349
14. Mettang T, Alscher DM, Pauli-Magnus C, Dunst R, Kuhlmann U, and Rettenmeier AW (1999) *Adv Perit Dial* 15:229-233
15. Samandar E, Silva MJ, Reidy JA, Needham LL, and Calafat AM (2009) *Environ Res* 109:641-646
16. Williams DT and Blanchfield BJ (1974) *Bull Environ Contam Toxicol* 12:109-112
17. Baird DD, Saldana TM, Nepomnaschy PA, Hoppin JA, Longnecker MP, Weinberg CR, and Wilcox AJ (2010) *J Expo Sci Environ Epidemiol* 20:169-175
18. Blount BC, Milgram KE, Silva MJ, Malek NA, Reidy JA, Needham LL, and Brock JW (2000) *Anal Chem* 72:4127-4134
19. Kato K, Silva MJ, Needham LL, and Calafat AM (2005) *Anal Chem* 77:2985-2991
20. Koch HM, Gonzalez-Reche LM, and Angerer J (2003) *J Chromatogr B Analyt Technol Biomed Life Sci* 784:169-182



21. Preuss R, Koch HM, and Angerer J (2005) *J Chromatogr B Analyt Technol Biomed Life Sci* 816:269-280
22. Silva MJ, Slakman AR, Reidy JA, Preau JL, Herbert AR, Samandar E, Needham LL, and Calafat AM (2004) *J Chromatogr B Analyt Technol Biomed Life Sci* 805:161-167
23. Silva MJ, Samandar E, Preau JL, Jr., Reidy JA, Needham LL, and Calafat AM (2007) *J Chromatogr B Analyt Technol Biomed Life Sci* 860:106-112
24. Pfaffli P (1986) *Int Arch Occup Environ Health* 58:209-216
25. Jonsson BAG, Richthoff J, Rylander L, Giwercman A, and Hagmar L (2005) *Epidemiology* 16:487-493
26. Vermeulen R, Jonsson BA, Lindh CH, and Kromhout H (2005) *Int Arch Occup Environ Health* 78:663-669
27. Frederiksen H, Skakkebaek NE, and Andersson AM (2007) *Mol Nutr Food Res* 51:899-911
28. Hines CJ, Hopf NBN, Deddens JA, Calafat AM, Silva MJ, Grote AA, and Sammons DL (2009) *Ann Occup Hyg* 53:1-17
29. Latini G (2005) *Clin Chim Acta* 361:20-29
30. Meeker JD, Sathyanarayana S, and Swan SH (2009) *Philos Trans R Soci Lond B Biol Sci* 364:2097-2113
31. Wormuth M, Scheringer M, Vollenweider M, and Hungerbuhler K (2006) *Risk Analysis* 26:803-824
32. Berthet A, Bouchard M, and Danuser D (2011) Toxicokinetics of captan and folpet biomarkers in orally exposed volunteers. *J Appl Toxicol*. In press.
33. Berthet A, Bouchard M, and Vernez D (2011) Toxicokinetics of captan and folpet biomarkers in dermally exposed volunteers. *J Appl Toxicol*. In press.

**Table 1**

Comparison of PA concentration (nmol/mL) measured in four urine samples collected at different time points in a same individual experimentally administered folpet by the oral route, with or without the addition of sodium chloride prior to extraction.

Urine sample <sup>b</sup>	PA concentrations (nmol/mL) <sup>a</sup>	
	Extraction	
	without NaCl	with NaCl <sup>c</sup>
Urine 1	4.60	3.91
Urine 2	33.6	27.2
Urine 3	122	103
Urine 4	36.8	35.4

<sup>a</sup> Urinary concentrations observed following sample processing as described in Materials and Methods but with or without addition of sodium chloride prior to extraction.

<sup>b</sup> Urine samples collected at four different time periods in one volunteer orally administered folpet (1 mg/kg dose).

<sup>c</sup> 250 mg of sodium chloride (NaCl) were added to 2 mL of urine prior to first extraction.

**Table 2**

Comparison of PA concentration (nmol/mL) measured in four urine samples collected at different time points in a same individual experimentally administered folpet by the oral route following an enzymatic hydrolysis at 37°C or an acid hydrolysis at 37°C or 100°C.

Urine sample <sup>b</sup>	PA concentrations (nmol/mL) <sup>a</sup>		
	Enzymatic Hydrolysis <sup>c</sup>	Acid hydrolysis at 37°C <sup>d</sup>	Acid hydrolysis at 100°C <sup>e</sup>
Urine 1	2.15	2.26	4.03
Urine 2	2.62	12.5	13.7
Urine 3	2.62	16.5	17.2
Urine 4	2.82	24.2	24.3

<sup>a</sup> Urinary concentrations observed following sample processing as described in Materials and Methods but with either enzymatic hydrolysis at 37°C or acid hydrolysis at 37°C or 100°C.

<sup>b</sup> Urine samples collected at four different time periods in one volunteer orally administered folpet (1 mg/kg dose). Urines were different from those used to test the effect of addition of NaCl on extraction efficiency (Table 1).

<sup>c</sup> 20 µL of glucuronidase-arylsulfatase were added to 2 mL of urine and samples were heated at 37°C for 12 h.

<sup>d</sup> 500 µL of HCl 2N were added to 2 mL of urine and samples were heated at 37°C for 12 h.

<sup>e</sup> 500  $\mu$ L of HCl 2N were added to 2 mL of urine and samples were heated at 100°C for 12 h.

**Table 3**

Conversion of PAA and PI as TMS-PA (expressed as a percentage) after sample processing (acid hydrolysis, extraction and derivatization) of aliquots of a same urine from an unexposed subject spiked at five different levels (nmol added).

Spiked metabolite	Amount added <sup>a</sup> (nmol)	Amount of TMS-PA found <sup>b</sup> (nmol)	Conversion <sup>c</sup> (%)
PAA	6.90	6.42	93.1
	13.8	14.3	104
	20.7	20.7	99.8
	24.1	24.6	102
	27.6	27.9	101
PI	7.65	4.79	62.6
	15.3	7.99	52.3
	22.9	10.3	44.9
	26.8	13.2	49.5
	30.6	16.4	53.5

PAA : phthalamic acid ; PI : phthalimide ; TMS-PA : trimethylsilyl-phthalic acid.

<sup>a</sup> Amount of PAA or PI (expressed in nmol) added to the urine of an unexposed subject and processed as described in Materials and Methods. Duplicates were prepared for each spiking level.

<sup>b</sup> Amount of total PA equivalents recovered (as TMS-PA) after sample processing of a urine spiked with PAA or PI and after subtracting baseline amounts of PA found in urine of the unexposed subject, which was 0.6025 nmol (calculated from the ratio of the intercept to slope of the calibration curve).

<sup>c</sup> Percent of PAA or PI converted to TMS-PA after sample processing, as described in Materials and Methods, of a urine spiked with different amounts of PAA and PI.

**Table 4**

Recovery of PA, intra- and inter-day precision and accuracy from replicate analysis of daily prepared aliquots of a pool of urine from unexposed individuals spiked with PA at five different levels and previously prepared QC samples spiked at two levels (n = 20 replicates for each spiking level).

Amount added (nmol) <sup>c</sup>	Recovery <sup>d</sup> (%)	RSD <sup>e</sup> (%)	Intra-day variation <sup>a</sup>				Inter-day variation <sup>b</sup>			
			Amount found <sup>f</sup> (nmol) (mean ± SD <sup>g</sup> )	Precision (% RSD <sup>e</sup> )	Overall RSD <sup>h</sup> (%)	Accuracy  i (%)	Amount found <sup>f</sup> (nmol) (mean ± SD <sup>g</sup> )	Precision (% RSD <sup>e</sup> )	Overall RSD <sup>h</sup> (%)	Accuracy  y <sup>i</sup> (%)
<i>Spiked urine from unexposed persons</i>										
1.33	95.8	11.3	1.28 ± 0.14	11.3	8.65	-4.22	1.28 ± 0.11	8.33	6.09	-4.00
2.66	99.5	9.04	2.65 ± 0.24	9.04		-0.48	2.66 ± 0.18	6.65		-0.08
3.99	97.6	7.08	3.90 ± 0.28	7.08		-2.41	3.90 ± 0.21	5.39		-2.27
4.66	96.3	8.28	4.49 ± 0.37	8.28		-3.73	4.49 ± 0.28	6.31		-3.65
5.33	98.6	9.29	5.25 ± 0.49	9.29		-1.41	5.24 ± 0.31	5.99		-1.64

*QC spiked samples*

1.98	91.9	12.5	1.83 ± 0.23	12.5	10.14	-8.06	1.83 ± 0.16	8.64	6.87	-7.94
4.96	98.2	9.26	4.91 ± 0.45	9.26		-1.18	4.91 ± 0.32	6.51		-1.16

---

<sup>a</sup> Average variation between *N* replicates of the same level of spiking prepared and analyzed the same day.

<sup>b</sup> Average variation between *N* replicates of the same level of spiking prepared and analyzed on different days over a 22-day period.

<sup>c</sup> Amount of PA (expressed in nmol) added at the different concentration levels to aliquots of a pool of urine from an unexposed individual.

<sup>d</sup> Percent recovery of total PA amounts as TMS-PA (different levels) after addition of PA to urine from an unexposed individual and processed as described in Materials and Methods.

<sup>e</sup> Precision or RSD: relative standard deviation for 20 replicates.

<sup>f</sup> Recovered amount of total PA (as TMS-PA) after sample processing and calculated from calibration curves by subtracting baseline amounts of PA found in urine of the unexposed subject (calculated from the ratio of the intercept to slope of the calibration curve).

<sup>g</sup> SD: standard deviation of *N* replicates.

<sup>h</sup> Overall RSD: mean relative standard deviation with all spiked urine from unexposed persons combined (*n* = 100) and all QC samples combined (*n* = 40).

<sup>i</sup> Accuracy, or percent relative error.



**Table 5**

Creatinine adjusted urinary concentrations of total PA equivalents in workers during the course of a typical workweek following a spraying episode of folpet or harvest activities in a previously treated area, along with pre-seasonal concentrations.

Workers <sup>a</sup>	Activities <sup>b</sup>	Shift Length <sup>c</sup>	Urinary PA concentration (nmol/mmol creatinine)										
			Pre-seasonal	Workday 1 <sup>d</sup>		Workday 2		Workday 3		Workday 4		Workday 5	
				Pre-shift	Post-shift	Pre-shift	Post-shift	Pre-shift	Post-shift	Pre-shift	Post-shift	Pre-shift	Post-shift
Worker 1	Spraying	4 h	268	655	2448	1152	2790	1557	3508	1252	1528	1120	1779
	Harvest	6 h	268	846	1244	1293	1486	913	2496	1604	ns	ns	ns
Worker 2	Spraying	6 h	108	152	312	588	561	636	435	195	162	135	246
	Harvest	36 h	108	237	230	197	256	345	234	311	259	598	214
Worker 3	Spraying	3 h	181	334	845	406	334	357	174	312	242	227	292
	Harvest	38 h	181	126	216	219	155	130	409	275	771	310	124

ns: no sample collected.

<sup>a</sup> Each worker collected their urine void during a typical workweek involving spraying of folpet and harvest activities in a previously treated area.

<sup>b</sup> Each worker performed both spraying and harvest activities, which were separated by a minimum of two weeks.

<sup>c</sup> Total duration of spraying and harvest activities for each worker.

<sup>d</sup> Folpet were sprayed and harvest activities were performed only on workday 1, except for worker 2, who harvested from workdays 1 to 3.

**Figure captions**

**Fig. 1** Chemical structure of (a) folpet and its main ring-metabolites: (b) phthalimide, (c) phthalamic acid and (d) phthalic acid.

**Fig. 2** Representative chromatograms of TMS-PA and TMS-IS in blank human urine spiked with 12.1  $\mu\text{mol/L}$  of PA and 6.72  $\mu\text{mol/L}$  of *p*-methylhippuric acid (IS), along with the mass spectra and structure of both TMS derivatives.

**Fig. 3** Representative chromatograms of TMS-PA and TMS-IS in human urine: (a) blank urine spiked with 12.1  $\mu\text{mol/L}$  of PA and 6.72  $\mu\text{mol/L}$  of methylhippuric acid (IS); (b) urine from a non-occupationally exposed person spiked with 6.72  $\mu\text{mol/L}$  of IS; (c) urine sample of a volunteer orally dosed with folpet (9 h following absorption) spiked with 6.72  $\mu\text{mol/L}$  of IS; (d) urine sample of a volunteer dermally dosed with folpet (24 h following application) spiked with 6.72  $\mu\text{mol/L}$  of IS; (e) urine sample of a farmer spraying folpet (13 h following the beginning of spraying).

**Fig. 4** Concentration-time profiles of total PA equivalents (mean  $\pm$  SD) in the urine of volunteers administered folpet by the oral (1 mg/kg) (-●-) or dermal (10 mg/kg) (-○-) route.

Figure 1

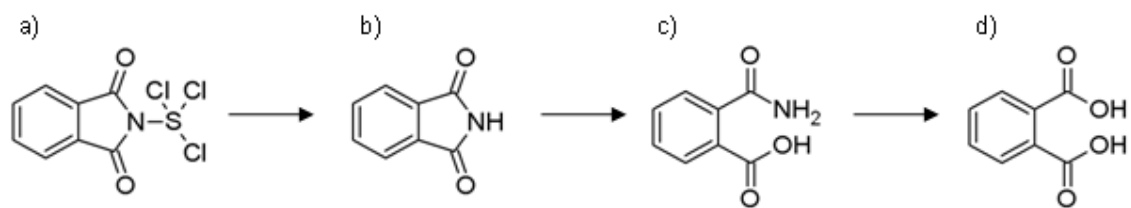


Figure 2

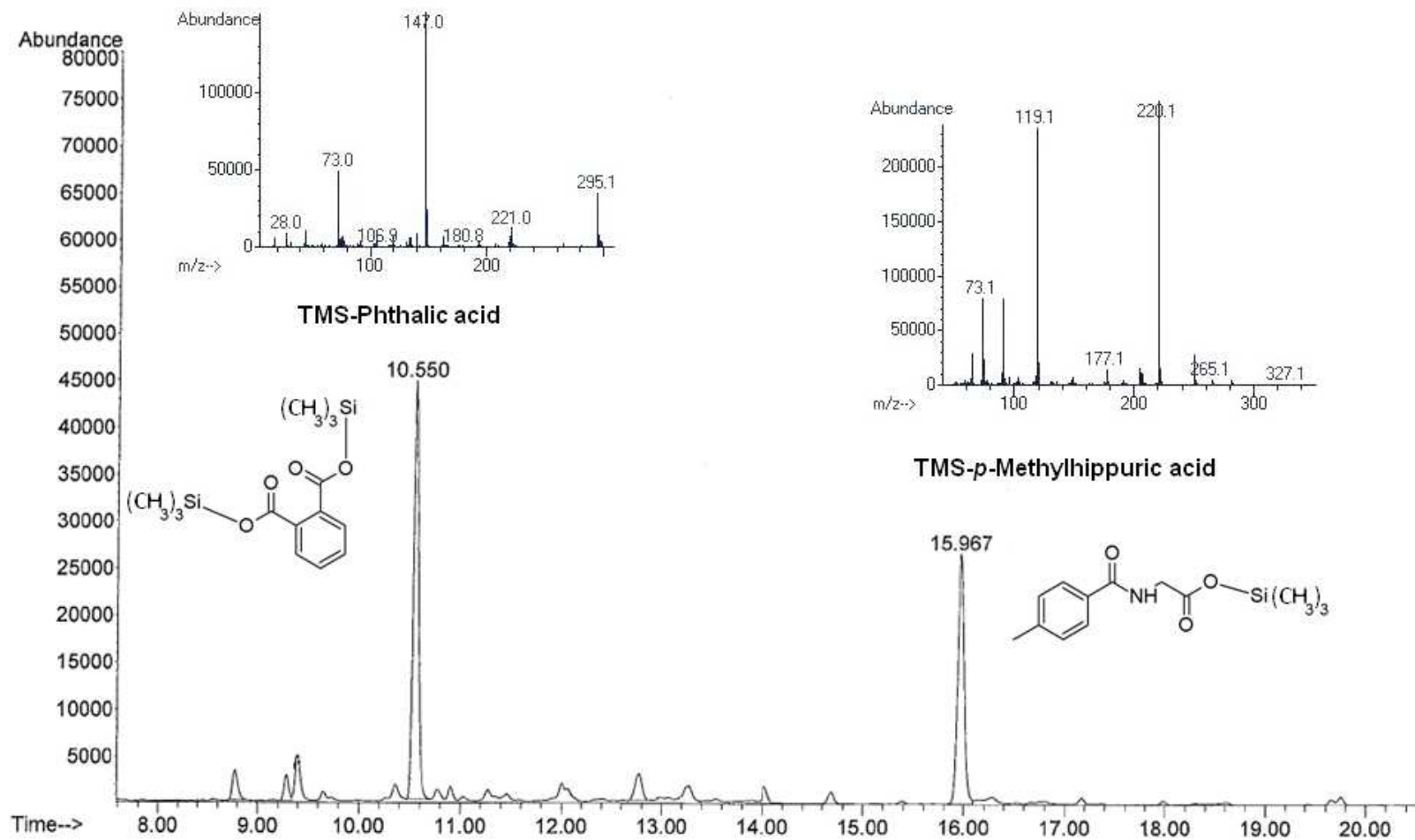


Figure 3

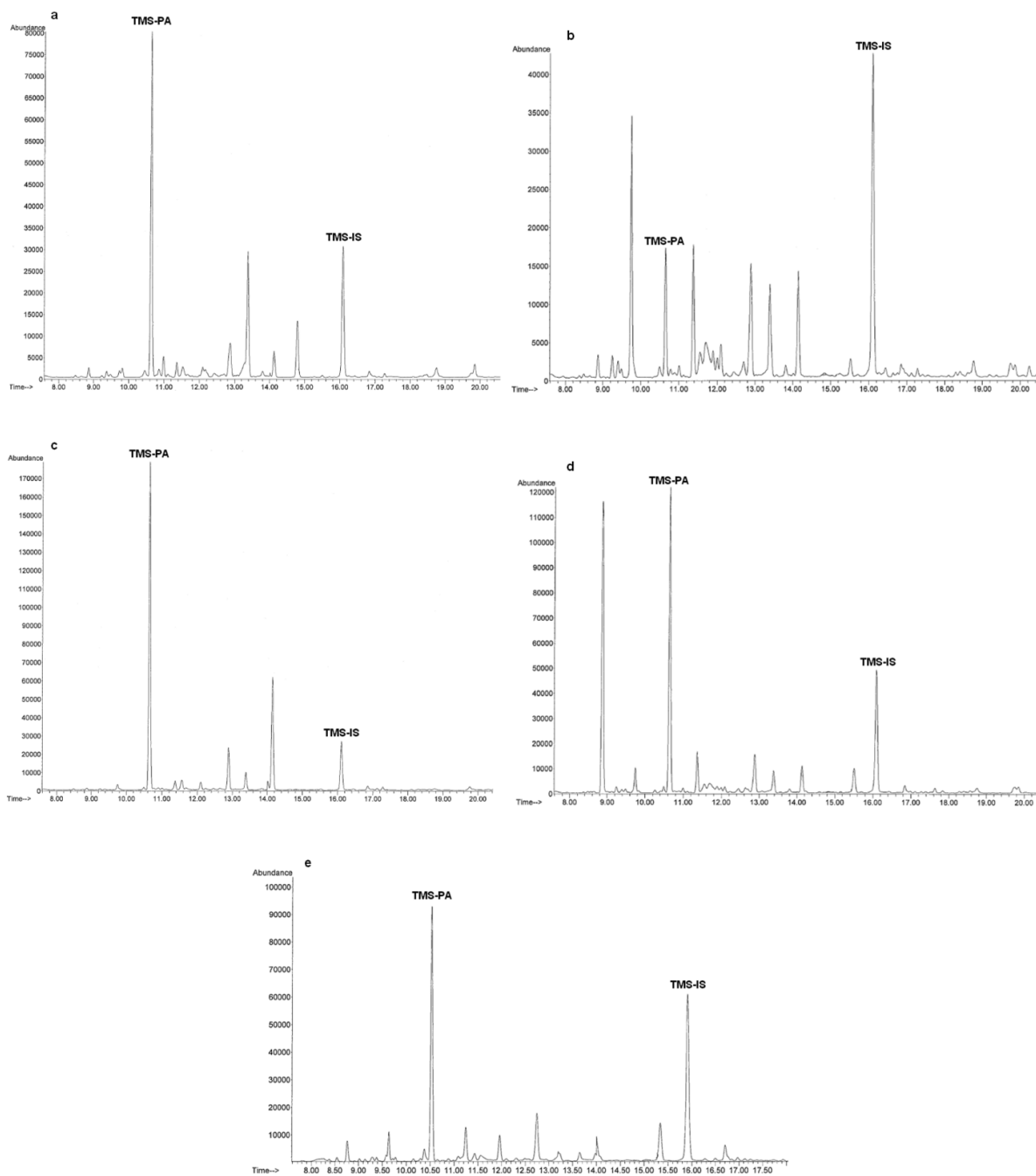
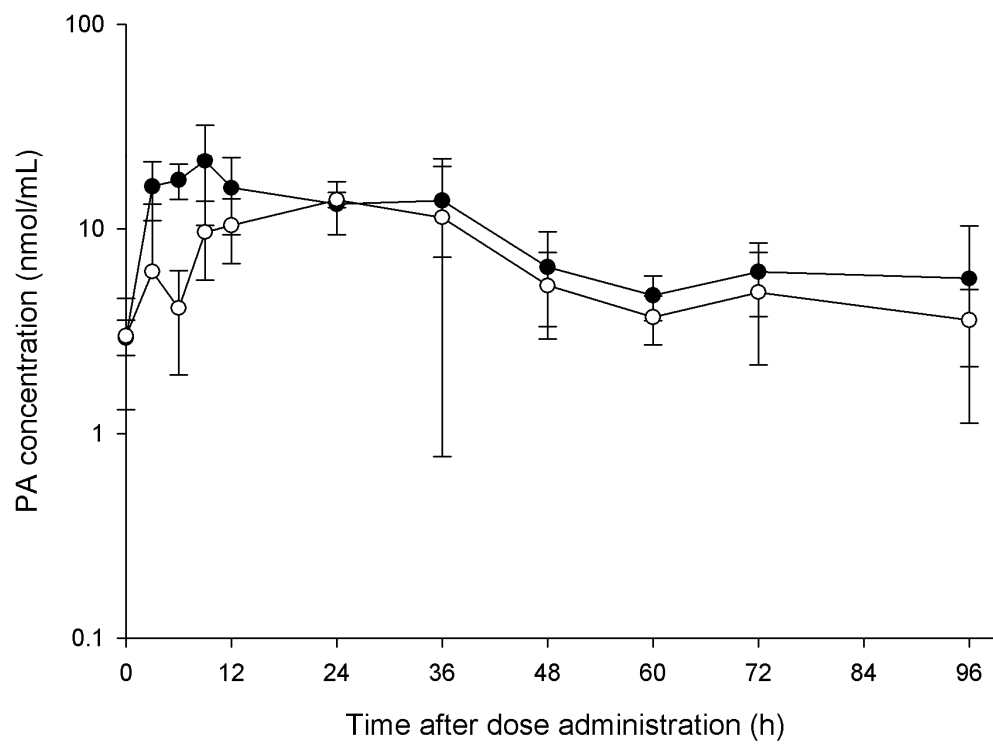


Figure 4



## Article III

### **Toxicokinetics of captan and folpet biomarkers in orally exposed volunteers**

Aurélie Berthet,<sup>ab</sup> Michèle Bouchard<sup>a\*</sup> and Brigitta Danuser<sup>b</sup>

<sup>a</sup> Département de santé environnementale et santé au travail, Chaire d'analyse et de gestion des risques toxicologiques and Institut de recherche en santé publique de l'Université de Montréal (IRSPUM), Faculté de Médecine, Université de Montréal, C.P. 6128, Succursale Centre-ville, Montréal, Québec, Canada, H3C 3J7

<sup>b</sup> Institute for Work and Health, Rue du Bugnon 21, 1011 Lausanne, Switzerland

\*corresponding author

Journal of Applied Toxicology

Article accepté (Décembre 2010) – ID 795151-668048

**ABSTRACT**

The time courses of key biomarkers of exposure to captan and folpet was assessed in accessible biological matrices of orally exposed volunteers. Ten volunteers ingested 1 mg kg<sup>-1</sup> body weight of captan or folpet. Blood samples were withdrawn at fixed time periods over the 72 h following ingestion and complete urine voids were collected over 96 h post-dosing. The tetrahydrophthalimide (THPI) metabolite of captan along with the phthalimide (PI) and phthalic acid metabolites of folpet were then quantified in these samples. Plasma levels of THPI and PI increased progressively after ingestion, reaching peak values ≈10 and 6 h post-dosing, respectively; subsequent elimination phase appeared monophasic with a mean elimination half-life ( $t_{1/2}$ ) of 15.7 and 31.5 h, respectively. In urine, elimination rate time courses of PI and phthalic acid evolved in parallel, with respective  $t_{1/2}$  of 27.3 and 27.6 h; relatively faster elimination was found for THPI, with mean  $t_{1/2}$  of 11.7 h. However, phthalic acid was present in urine in 1 000-fold higher amounts than PI. In the 96-h period post-treatment, on average 25% of folpet dose was excreted in urine as phthalic acid as compared to only 0.02% as PI. Corresponding value for THPI was 3.5%. Overall, THPI and PI appear as interesting biomarkers of recent exposure, with relatively short half-lives; their sensitivity to assess exposure in field studies should be further verified. Although not a metabolite specific to folpet, the concomitant use of phthalic acid as a major biomarker of exposure to folpet should also be considered.

**Keywords:** : toxicokinetics; captan; folpet; tetrahydrophthalimide; phthalimide; phthalic acid; oral exposure; human; biomarker



**Table of Contents – Short abstract**

To determine the kinetics of ring metabolites of captan and folpet in human matrices following an oral exposure, volunteers ingested  $1 \text{ mg kg}^{-1}$  of these two largely used fungicides. Blood samples and complete urine voids were collected at fixed time periods respectively over 72 h and 96 h following ingestion. The results show a relatively short half-life for the three studied metabolites as well as their sensitivity as biomarkers of exposure to captan and folpet.

## INTRODUCTION

Captan and folpet are widely used in different crops to treat fungal diseases. Health effects were documented mostly from animal toxicity studies (US EPA, 1975, 1999). No direct toxicity was reported in humans, except allergic dermatitis, severe eye irritations and irritation of the nose and throat (Hayes, 1982; Lisi *et al.*, 1987; ACGIH, 1991; Edwards *et al.*, 1991; Trochimowicz *et al.*, 1991; Guo *et al.*, 1996; Tomlin, 1997; Costa, 2008; Gordon, 2010). Nonetheless, folpet was classified B2, or probable human carcinogen, by the U.S. Environmental Protection Agency (US EPA, 1975, 1999), based on an increased incidence of duodenum tumors in mice chronically exposed to high doses by gavage. Captan was also found to induce the same type of tumors in mice through the same mode of action and was thus initially categorized B2. However, in 2004, the US EPA revised the classification of captan and changed it to “not likely” (US EPA, 2004; Gordon, 2007), given that cancers were observed at doses several orders of magnitude higher than those encountered in occupational settings, which promoted proliferation of nascent tumors through cytotoxicity and cell hyperplasia.

The US EPA (1975, 1999) has also derived a reference dose (RfD) to prevent chronic effects of ingested captan and folpet in the general population. It was respectively established at  $0.1 \text{ mg kg}^{-1}$  of body weight (body wt) for captan (value estimated on the basis of a no-observed adverse effect level (NOAEL) of  $12.5 \text{ mg kg}^{-1}$  of body wt per day in a three-generation reproduction study in rats to which a safety factor of 100 was applied), and  $0.16 \text{ mg kg}^{-1}$  body wt per day for folpet (value assessed on the basis of a

no-observed effect level (NOEL) of 10 mg kg<sup>-1</sup> of body wt per day from a one-year toxicity study on dogs and a two-years toxicity and carcinogenicity study in rats to which a safety factor of 100 was applied) (Larsen, 1996).

With regard to captan and folpet metabolism (Fig. 1 and 2, respectively), it has been fairly well documented in *in vivo* studies in animals usually exposed to the radiolabelled compounds as well as *in vitro* studies, but human data are limited (Gordon, 2010). According to these studies, captan is broken down to 1,2,3,6-tetrahydrophthalimide (THPI) and thiophosgene (Wolfe *et al.*, 1976). Thiophosgene is an unstable metabolite with a very short half-life that reacts *in vivo* with thiol groups or several functional groups to form thiazolidine-2-thione-4-carboxylic acid (TTCA) (Wolfe *et al.*, 1976). THPI and TTCA have been proposed as biomarkers of captan exposure (DeBaun *et al.*, 1974; Krieger and Thongsinthusak, 1993). Both these metabolites can be quantified in human urine as evidenced by the only published urinary excretion time course study in two volunteers exposed to captan by the oral route (Krieger and Thongsinthusak, 1993). However, only THPI was repeatedly used as a biomarker in the few cross-sectional biomonitoring studies reported among workers exposed to captan (Winterlin *et al.*, 1984; Winterlin *et al.*, 1986; van Welie *et al.*, 1991; de Cock *et al.*, 1998; Krieger and Dinoff, 2000; Hines *et al.*, 2008). TTCA may also appear as a potential biomarker of captan exposure, but it is not specific to this fungicide and only van Welie *et al.* (1991) quantified it in the urine of exposed workers.

On the other hand, in *in vivo* experiments in animals and *in vitro* studies, folpet was shown to be first hydrolyzed to phthalimide (PI) and thiophosgene (Gordon *et al.*, 2001; Zainal and Que Hee, 2003; Canal-Raffin *et al.*, 2008; Gordon, 2010). Thiophosgene forms TTCA in the same way as captan, and PI is rapidly hydrolyzed in animals to unstable phthalamic acid, but also to phthalic acid (Gordon, 2010). However, to our knowledge, there are no reported kinetic studies in humans for folpet to date.

Overall, there is a lack of detailed knowledge of the toxicokinetics of captan and folpet in humans necessary to interpret biomarker data in field studies and to relate biomarker data to health effects. Since interspecies differences in the kinetics of chemical substances are likely to occur (Krieger and Thongsinthusak, 1993; Poet and McDougal, 2002; Ngo *et al.*, 2010) and given the high doses administered in the animal studies on captan and folpet compared to occupational and environmental exposure levels, it is difficult to extrapolate *a priori* animal time course data to humans. As mentioned by Finley and Paustenbach (1997) and Woollen (1993), information obtained directly in humans will avoid some factors of uncertainty that are present in animal studies, and may give indications on the individual variability in metabolism and elimination of the studied substances. The aim to this study was thus to determine kinetics of captan and folpet metabolites in accessible biological matrices of orally exposed volunteers to help better assess biomonitoring data.

## **MATERIALS AND METHODS**

### **Study design**

A controlled kinetic time course study in accessible biological matrices of healthy subjects was conducted following an acute oral exposure to captan and folpet. Captan and folpet metabolites were measured in urine and blood samples collected prior to treatment to obtain pre-test values, and then at predetermined time points post-dosing (amounting to a total of 9 samples for plasma and 11 samples for urine).

The experimental protocol and consent forms were approved by the Permanent Ethics Committee for Clinical Research of the Faculty of Biology and Medicine of the University of Lausanne and the Research Ethics Committee of the Faculty of Medicine of the University of Montreal. All the participants gave their written consent, and were informed of the risks of participating and their right to withdraw from the study at anytime. As suggested by the Ethics Committee who considered the study as very restrictive, the participants received a monetary compensation for their time and any inconvenience caused.

### **Subjects studied**

Participants were recruited on a voluntary basis among the medical students of the University of Lausanne, Switzerland. The volunteers were ten male students aged from

20 to 30 years old, weighing 60 to 85 kg and measuring 169 to 184 cm in height. They were healthy, non-smokers and did not take medication or drugs. They underwent a medical examination by an occupational physician prior to enrolment. They had never been exposed to captan or folpet, except perhaps through their diet. During the study period and the two-days prior to dosing, they were asked not to eat fruits and vegetables in order to limit ingestion of contaminated food. They were also requested to avoid consumption of alcohol during this period, since it is known to affect the metabolism of some chemical compounds.

### **Dosing and sampling**

The experimental dosing and sampling was conducted at the Institute for Work and Health of Lausanne, Switzerland. The morning of study onset, each participant was asked to collect his complete first morning urine void, to obtain pre-test values; they then spent the first day of the study in a room at the Institute for dosing and the first 12-h sampling. On the following 3-day sampling period, participants visited the Institute for their daily morning blood sampling and handing-in urine collections.

The volunteers ingested a dose of captan or folpet equivalent to  $1 \text{ mg kg}^{-1}$  body wt ( $n = 5$  per group). Fungicides used to prepare the exposure dose of volunteers were Captan PESTANAL<sup>®</sup> (assay (HPLC) area 99.1 to 99.4%) and Folpet PESTANAL<sup>®</sup> (assay (HPLC) area 99.5 to 99.9%). Both were purchased from Fluka – Sigma-Aldrich (Buchs, St Gallen, Switzerland). The single dose was weighted in a labelled plastic cup and

adjusted to the weight of each volunteer. The fungicide dose was mixed with 25 ml of orange juice and administered to the participants. The cup used for dosing was then rinsed with another 25 ml of orange juice and also administered to the participants.

Complete micturitions were then collected at pre-determined times over the 96-h period post-dosing, that is at around 0, 3, 6, 9, 12, 24, 36, 48, 60, 72, 84 and 96 h post-dosing. Each timed-void was collected in separate polypropylene Nalgene<sup>®</sup> bottles of 1 l. To assist participants, they received a schedule specifying the date and the required time of urine collection. They then only had to complete the sheet with the actual time of collection and to indicate whether or not there were any urine losses. They also had to identify their plastic bottles with distinct pre-printed labels indicating the code, and mark on them the date and time of urine collection.

Once collected, urine samples were kept in the refrigerator prior to measurement of total urine volume per void. To allow repeated analysis while avoiding possible degradation due to freezing and defreezing of samples, each urine collection then was aliquoted in 4 labelled tubes of 15 ml and one bottle of 120 ml before storage at -20°C until analysis of captan or folpet metabolites.

Blood samples of 15 ml were also collected at specific times, that is at  $t = 0, 2, 4, 6, 8, 10, 24, 48$  and 72 h post-dosing. To facilitate collection, a catheter was installed by a nurse prior to dosing along with a drip system of physiological saline the first day of sampling. Blood was withdrawn by the nurse into vacutainers pre-labelled with a code,

the date and time of sampling. Immediately after collection, blood samples were centrifuged to precipitate red blood cells and isolate the plasma. The plasma samples were then split into 3 labelled aliquots and stored at -20°C until analysis of captan and folpet metabolites.

During the study period, volunteers were also asked to fill a questionnaire to document personal information (weight, height), life habits (i.e. physical activities, smoking), medication intake (including ibuprofen), alcohol consumption during the 3 days prior to treatment, consumption of fruits, vegetables and cereals during the 4 days prior to treatment and possible dosing-related symptoms.

### **Chemicals and reagents**

Reference standards (>99% purity) were obtained from Sigma-Aldrich (Buchs, St Gallen, Switzerland), except for deuterated cis-1,2,3,6-tetrahydrophthalimide (THPI-d) (99% purity), which was purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). HPLC grade acetonitrile, methanol, ethyl acetate and dichloromethane were also obtained from Sigma-Aldrich (Buchs, St Gallen, Switzerland), along with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), the reagent used to derivatize the phthalic acid molecule. HCl and ammonium sulphate were purchased from Merck (Zug, Switzerland). Water was purified using a TKA GenPure water treatment system obtained from TKA Wasseraufbereitungssysteme GmbH (Niederelbert, Germany).



### **Analysis of THPI and PI in plasma and urine**

A liquid chromatography - atmospheric pressure chemical ionization-tandem mass spectrometry (LC/APCI-MS/MS) method was developed to analyze THPI and PI in urine and plasma and is described elsewhere (Berthet *et al.*, 2011a). Briefly, THPI and PI were isolated by adding 125  $\mu\text{l}$  of THPI-d internal standard ( $1.59 \mu\text{mol l}^{-1}$ ) in 3 ml of urine or 2 ml of plasma, conditioning the Oasis® solid phase extraction cartridge (Waters, Montreux, Switzerland) with 8 ml of dichloromethane followed by 8 ml of methanol and 12 ml of water, and lastly by loading aliquots on SPE cartridges (in the case of plasma, following a denaturation of proteins). The analytes were then eluted from the column with 4 ml of dichloromethane. The solvent was evaporated to dryness under a gentle nitrogen flow at 40°C. The residues were resuspended in 500  $\mu\text{l}$  of methanol and analyzed using a Varian Model 212-LC Binary Gradient LC system (Les Ulis, France) connected to a Prostar model 410 autosampler (Varian, Les Ulis, France) and coupled to a Model 1200 L quadrupole MS (Varian, Les Ulis, France) operating in APCI mode. The APCI interface was operated in negative ion mode. The compounds were separated using a C<sub>18</sub> Zorbax Eclipse Plus column (4.6 x 150 mm, 3.5  $\mu\text{m}$ ) from Agilent (Morges, Switzerland). The mobile phase consisted of: eluent A composed of 90 % water and 10% acetonitrile (v/v), and eluent B of 10% water and 90% acetonitrile (v/v). For THPI analysis, 10  $\mu\text{l}$  of the sample were injected and elution was performed in 26 min at a flow rate of 0.8 ml min<sup>-1</sup> using a solvent gradient starting at 90% eluent A for 3 min, followed by a linear gradient to 5% eluent A from 3 to 10 min, maintained at 5% eluent A from 10 to 13 min before running to initial conditions of 90% eluent A in 1

min for a 12-min re-equilibration of the column prior to subsequent injection. As for PI analysis, 10  $\mu\text{l}$  of the sample were also injected and elution was performed in 33 min at a flow rate of  $0.8 \text{ ml min}^{-1}$  using an isocratic mode at 90% eluent A for 15 min and then ramping to 5% eluent A in 30 sec for a 4 min clean up at 5% eluent A prior to returning to initial conditions in 1 min to allow 12 min re-equilibration of the column. The fragments analyzed were  $m/z$  149.4/95.6 for THPI with a collision energy (CE) of 19.5 V,  $m/z$  156.1/95.6 for the internal standard THPI-d (CE of 22.5 V) and  $m/z$  145.8 for PI (no fragmentation). The analytical limit of detection for THPI was  $3.82 \text{ nmol l}^{-1}$  of urine and  $9.76 \text{ nmol l}^{-1}$  of plasma and, for PI,  $7.72 \text{ nmol l}^{-1}$  of urine and  $14.8 \text{ nmol l}^{-1}$  of plasma. The quantification of THPI or PI was obtained from standard calibration curves prepared in urine or plasma adjusted by the THPI-d internal standard peak area.

### **Analysis of phthalic acid in urine**

A gas chromatography mass spectrometry (GC-MS) method was developed for the analysis of phthalic acid in urine, as previously described (Berthet *et al.*, 2011b). In short, urine samples were subjected to an acid hydrolysis prior to liquid-liquid extraction with ethyl acetate and derivatization with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). Analysis was then performed using an Agilent Mass Selective Detector (MSD) G1098A (Agilent Technologies Inc, Waldbronn, Germany) coupled with a HP 5973 mass spectrometer (Agilent Technologies Inc, Waldbronn, Germany). Separation of the analytes was performed using a 60 m CP-SIL 8 CB column (1  $\mu\text{m}$  film thickness, 250  $\mu\text{m}$  I.D.) (Varian, Les Ulis, France). The initial column temperature was  $200^\circ\text{C}$  for

3 min, then it was increased to 260°C at 30°C min<sup>-1</sup>, held for 11 min, and finally increased to 280°C at 35°C min<sup>-1</sup> and held for 4 min. For the analysis, 2 µl were injected using a 5 ml min<sup>-1</sup> split. The ions monitored were trimethylsilyl phthalic acid (TMS phthalic acid) with *m/z* 295 and the internal standard TMS methylhippuric acid with *m/z* 220. The quantification was obtained from standard calibration curves of phthalic acid prepared in urine and adjusted by the methylhippuric acid internal standard peak area. The analytical limit of detection was 60.2 nmol l<sup>-1</sup> urine.

### Calculations

The molar fraction of captan or folpet administered dose recovered in urine as THPI, PI or phthalic acid was calculated from the following equation:

$$\left[ \frac{\left( \frac{Q_{\text{urinary metabolite}}}{MW_{\text{metabolite}}} \right)}{\left( \frac{Dose_{\text{parent compound}}}{MW_{\text{parent compound}}} \right)} \right] \times 100$$

where  $Q_{\text{urinary metabolite}}$  corresponds to total amounts of THPI, PI or phthalic acid in urine over the 96-h urine collection period (mg),  $MW_{\text{parent compound}}$  is the molecular weight of captan or folpet,  $MW_{\text{metabolite}}$  is the molecular weight of THPI, PI or phthalic acid and  $Dose_{\text{parent compound}}$  is the orally administered dose of captan or folpet (mg).

### Toxicokinetic analysis

To determine elimination rate constants of THPI and PI in plasma and in urine, we considered that, following peak levels, elimination was monophasic and a first-order reaction. The elimination rate constant ( $k$ ) was thus calculated from the slope of the linear terminal phase of the plasma or urine time profile (Hayes, 2008). The apparent elimination half-life ( $t_{1/2}$ ) was then defined using the equation  $t_{1/2} = 0.693 k^{-1}$  (Hayes, 2008). All calculations were performed using MS Excel<sup>®</sup> 2007 software.

From plasma concentration (C) - time profile, we also calculated the area under the concentration-time curve (AUC), the area under the first moment of concentration-time curve (AUMC), the mean residence time (MRT), the plasma clearance (CL) and the apparent volume of distribution ( $V_d$ ). Equations used to calculate these parameters are:

$$AUC = \frac{1}{2} \sum_{\forall i} (t_i - t_{i+1}) [C(t_i) + C(t_{i+1})]$$

$$AUMC = \frac{1}{2} \sum_{\forall i} (t_i - t_{i+1}) [t_i C(t_i) + t_{i+1} C(t_{i+1})]$$

$$MRT = \frac{AUMC}{AUC}$$

$$CL = \frac{\text{Absorbed dose}}{AUC}$$

$$V_d = \frac{CL}{k} \quad \text{where } k \text{ is the overall elimination rate from blood}$$

## RESULTS

### Time courses of THPI and PI in plasma

The time courses of THPI and PI in plasma of volunteers over the 72-h period following ingestion of captan or folpet ( $1 \text{ mg kg}^{-1}$  body wt) are presented in Fig. 3. In control samples taken prior to captan or folpet ingestion ( $t_0$ ), concentrations of THPI were below the analytical limit of detection and those of PI were very low, in the order of  $9.3 \text{ nmol l}^{-1}$ . Following ingestion, plasma levels of THPI and PI increased progressively with peak levels of THPI being observed on average at time 10 h post-dosing as compared to 6 h post-dosing for PI. Subsequently, elimination phase of THPI and PI from plasma appeared monophasic (6-10 h to 72-h post-dosing) with a mean apparent elimination half-life of 15.7 and 31.5 h, respectively (Table 1). Table 2 presents the toxicokinetic parameters calculated from the THPI and PI time profiles in plasma and shows that THPI had a greater bioavailability and faster clearance rate than PI. As also shown in Table 2, PI metabolite in plasma represented only a small fraction of folpet dose. Nonetheless, mean residence time (MRT) of both THPI and PI was similar and in the order of one day, which is relatively short compared to other chemical compounds. THPI and PI also had similar relatively small apparent volume of distribution ( $V_d$ ).

### **Time courses of THPI, PI and phthalic acid in urine**

The time courses of THPI, PI and phthalic acid excretion rate in the urine of volunteers over a 96-h period following ingestion of  $1 \text{ mg kg}^{-1}$  body wt of captan or folpet are presented in Fig. 4. Peak levels were observed on average 9 h post-dosing for THPI, and between 3 and 12 h for PI and phthalic acid. Following peak excretion, elimination rate time courses evolved in parallel for PI and phthalic acid and was relatively slower than that of THPI, with mean apparent elimination half-lives calculated for the 24- to 96-h period post-dosing being 27.3, 27.6 and 11.7 h, respectively. Although the time courses of PI and phthalic acid was similar, phthalic acid was present in urine in 1 000-fold higher amounts than PI as shown in Fig. 4, and in Fig. 5 depicting the cumulative urinary excretion time courses of these metabolites. From the cumulative urinary excretion of PI and phthalic acid over the 96-h collection period post-dosing, it was calculated that 25% of the ingested dose of folpet was excreted in urine as phthalic acid as compared to only 0.02% as PI. Corresponding value for THPI was 3.5%.

Figure 6 shows that the time course of THPI in urine obtained in our study is similar to that obtained by Krieger and Thongsinthusak (1993) in volunteers orally exposed to the same dose of captan. In the current study, cumulative urinary excretion of THPI over the 96-h collection period post-treatment represented 3.5% of the administered dose, which is compatible with the value of 2.2% over the 72-h collection period in the study of Krieger and Thongsinthusak (1993). Similar to Krieger and Thongsinthusak (1993), elimination of THPI in urine was almost complete 96 h post-dosing.

### **Comparison of plasma and urinary time courses of THPI and PI**

As expected for both THPI and PI, comparison of the plasma and urinary rate time courses in the studied volunteers (Fig. 3 and 4) shows that profiles evolved in parallel in the 10- to 72-h period post-dosing. However, for both THPI and PI, plasma levels (nmol) were about 50-fold higher than urinary excretion rates ( $\text{nmol h}^{-1}$ ), indicating that the transfer rate of THPI and PI from plasma to urine was approximately 0.02 per hour, corresponding to a half-life of  $\approx 30$  h.

## DISCUSSION

This study allowed a better understanding of the kinetics of key biomarkers of exposure to captan and folpet. It provided novel human data on the kinetics of THPI in plasma, and PI and phthalic acid in plasma and urine, while confirming THPI urinary data of Krieger and Thongsinthusak (1993). Interestingly, several similarities were observed with available toxicokinetics of total radioactivity measured in rats orally exposed to labelled captan or folpet.

Results of the current study showed that the metabolite of captan, THPI, and the metabolites of folpet, PI and phthalic acid, had a rapid kinetics in humans after a single oral dose of  $1 \text{ mg kg}^{-1}$  body wt, since these metabolites were almost completely excreted over a 96-h period post-treatment. Peak levels in plasma were observed on average 10 h post-ingestion for THPI and 6 h post-dosing for PI while elimination half-life of THPI from plasma was in the order  $\approx 15$  h as compared to  $\approx 30$  h for PI.

Results of the current study also show negligible storage in tissues. Indeed, from the plasma time course, similar relatively small volume of distribution ( $V_d$ ) were calculated for THPI and PI, suggesting these compounds remain mainly in the circulation and have limited distribution in body tissues, implying a low storage of these compounds in tissue. This is in line with animal studies showing negligible accumulation of THPI (Piccirillo, 2001) or phthalimide moiety (Couch *et al.*, 1977; Ackermann *et al.*, 1978) in tissues following oral or intraperitoneal administration of labelled captan or folpet.



Furthermore, although the time courses of PI and phthalic acid evolved in parallel, PI metabolite in plasma represented only a small fraction of folpet dose and only 0.03% of the folpet dose was recovered in urine as PI while 25% of the folpet dose was excreted in urine as phthalic acid over the 96-h period post-dosing. This is consistent with a rapid site-of-entry biotransformation of PI into phthalimic acid and phthalic acid once formed (Ackermann *et al.*, 1978; Canal-Raffin *et al.*, 2008), thus limiting the amounts of PI available for absorption in blood. It also shows that the acids formed in the GI following oral exposure are effectively absorbed.

Results obtained in the current study on the fraction of ingested dose recovered in urine as folpet metabolites is in accordance with mass-balance studies conducted in animals exposed to labelled folpet. When PI was orally administered to rats, about 80% of the administered dose was metabolized and excreted in urine as phthalamic acid while 7% was found as phthalic acid, and less than 1% of the dose was recovered as PI in urine (Chasseaud *et al.*, 1974). Phthalamic acid also represented the main metabolite (i.e. 80%) when labelled <sup>14</sup>C-folpet was orally administered to rats (Chasseaud, 1980). They however reported that this metabolite was unstable in urine.

In comparison with the folpet metabolites assessed in our study, on average 3.5% of the administered oral dose of captan was excreted as THPI in the urine of volunteers over the 96-h collection period. As shown in Fig. 6, these findings are similar to those obtained by Krieger and Thongsinthusak (1993) where 2-3% of the same orally administered dose of captan was recovered as THPI in the urine of a human volunteer

over the 72-h period post-dosing. These human values are in the same range as those reported in a unique published animal study on the excretion of specific metabolites following non-labelled administration (van Welie *et al.*, 1991). Namely, van Welie *et al.* (1991) reported that on average 0.7 to 5.4% of a single oral dose of 400, 815 or 1250 mg kg<sup>-1</sup> of captan in Wistar rats were recovered in urine as THPI over the 0-24 h period post-dosing as compared to 2.8 to 9.1% over the 0-48 h period post-dosing, with lowest percentages observed at the highest dose. When non-labelled THPI itself was administered orally to rats, van Welie *et al.* (1991) also observed that on average only 4% of dose was recovered as THPI in urine over the 0-48 h period post-dosing.

It is to be noted that although a small percentage of orally administered captan in volunteers and rats was observed as THPI in urine, following oral administration of ring-labelled captan in animals, the majority of the radioactivity was recovered in urine (75 to 85% of administered dose) over a 24-h to 96-h period post-dosing, while only 7 to 12% of dose were excreted in feces over the same period of time. More precisely, Lappin and Havell (1990) reported that 81% of an oral dose of 10 mg kg<sup>-1</sup> of <sup>14</sup>C-ring-captan were recovered in urine as labelled equivalents as compared to 8-9% in feces over a 96-h period post-dosing, and 97% of the dose were eliminated at this time. Similarly, Trivedi (1990) reported that, following an oral administration of 10 mg kg<sup>-1</sup> of <sup>14</sup>C-ring-labelled captan, 75% of the administered dose were excreted in urine as <sup>14</sup>C-equivalents and 6.5% in feces over the 24-h period post-dosing.

Similarly to captan, following oral or intraperitoneal administration of ring-labelled folpet, between 90 and 100% of the administered dose were excreted in the urine of rats over a 24-h period post-dosing. This is based on the studies of Wood *et al.* (1991) showing that 92% of an orally administered dose of 10 mg kg<sup>-1</sup> of <sup>14</sup>C-labelled folpet in rats were recovered in urine as <sup>14</sup>C equivalents as compared to 6% in feces, and on the study of Couch *et al.* (1977) indicating that virtually 100% of an intraperitoneal dose of 6 mg kg<sup>-1</sup> of <sup>14</sup>C-folpet were recovered in urine as <sup>14</sup>C equivalents and 1.7% in feces over the 24-h period post-dosing.

This confirms that, in humans as observed in animals (Lappin and Havell, 1990; van Welie *et al.*, 1991), studied PI and THPI metabolites are not the main metabolites of captan and folpet following oral exposure. As shown by our results and reported by van Welie *et al.* (1991), other metabolites of captan might be more representative than THPI, although the analytical method developed by our team for this metabolite was specific, accurate and sensitive. Following an oral administration of 10 or 500 mg kg<sup>-1</sup> of <sup>14</sup>C-ring-labelled captan in Sprague-Dawley rats, Lappin and Havell (1990) identified six metabolites other than THPI in urine: 3-hydroxy-4,5-cyclohexene-1,2-dicarboximide (3-OH-THPI) (representing 42% of total urinary metabolites), 5-hydroxy-3,4-cyclohexene-1,2-dicarboximide (5-OH-THPI) (6%), 6-hydroxy-1-amido-2-carboxy-4,5-cyclohexene (3-OH-THPI-amic acid) (13%), 1-amido-2-carboxy-4,5-cyclohexene (THPAM) (7%), 4,5-dihydroxy-1,2-dicarboximide (4,5-diOH-THPI) (6%), and 4,5-epoxy-1,2-dicarboximide (THPI-epoxide) (5%), and two unidentified metabolites accounting for 4 and 2% of total urinary metabolites.

Overall, in a perspective of biomonitoring of exposure, THPI and PI appear as interesting biomarkers of recent exposure given that they are metabolites specific to captan and folpet, respectively, with relatively short half-lives. However, they represent only a small percentage of the orally administered dose in our study, such that their sensitivity as biomarkers of exposure in workers and even more in the general population remains to be more extensively verified. Other metabolites of captan and folpet appear quantitatively more important in human urine than THPI or PI. The most important urinary metabolite of captan in rats was found to be 3-OH-THPI (Lappin and Havell, 1990); it would be interesting to quantify this metabolite in human urine since the current study indicates that the metabolism and excretion of captan metabolites may be similar in rats and humans. Furthermore, this human study in line with the rat studies of Chasseaud *et al.* (1974, 1980) showed that phthalamic or phthalic acid appear as quantitatively more important biomarkers than PI while urinary excretion time courses are similar for phthalic acid and PI. Nonetheless, phthalic acid is not a metabolite specific to folpet; it is also a derivative of phthalates, which are ubiquitous molecules in our environment (Blount *et al.*, 2000; Silva *et al.*, 2007). Given these considerations, perhaps the best biomonitoring strategy, to assess occupational or environmental exposure, would be to measure multiple metabolites of captan or folpet and to perform repeated measurements in time.

## **Acknowledgements**

Authors wish to thank Dr. Michèle Berode and the technicians P. Stefan, M. Nobel and C. Kohler (Institute for Work and Health) for their help in organizing the study. We also thank Dr. Khanh Huynh (Institute for Work and Health) for his help with the LC/MS/APCI system. We also extend our gratitude to Robert Krieger for providing the data published in his work in 1993. The study was funded by the ANSES (Agence Nationale de Sécurité Sanitaire de l'alimentation, de l'environnement et du Travail) and Aurélie Berthet received a Ph.D. scholarship from the *Institut de recherche Robert-Sauvé en santé et sécurité du travail du Québec* for this research.

**REFERENCES**

Ackermann H, Faust H, Kagan YS, Voronina VH. 1978. Metabolic and Toxic Behaviors of Phthalimide Derivatives in Albino-Rat .2. Placental Passage of Chloromethyl Phthalimide, Oxymethyl Phthalimide, and Phthalimide - Their Fetal Metabolism. *Arch. Toxicol.* **40**: 255-261.

American Conference of Governmental Industrial Hygienists (ACGIH). 1991. Documentation of the threshold limit values and biological exposure indices, 6<sup>th</sup> edition. Volumes I, II, III. In American Conference of Governmental Industrial Hygienists: Cincinnati; 214.

Berthet A, Bouchard M, Schüpfer P, Vernez D, Danuser B, Huynh CK. 2011a. A liquid chromatography - tandem mass spectrometry (LC/APCI-MS/MS) methods for the quantification of captan and folpet phthalimide metabolites in human plasma and urine. *Anal. Bioanal. Chem.* **339**: 2243-2255. DOI: 10.1007/s00216-010-4601-z.

Berthet A, Berode M, Bouchard M. 2011b. Gas-chromatography mass-spectrometry determination of phthalic acid in human urine as a biomarker of folpet exposure. (In press).

Blount BC, Milgram KE, Silva MJ, Malek NA, Reidy JA, Needham LL, Brock JW. 2000. Quantitative detection of eight phthalate metabolites in human urine using HPLC-APCI-MS/MS. *Anal. Chem.* **72**: 4127-4134.

Canal-Raffin M, Receveur M, Martinez B, Titier K, Ohayon C, Baldi I, Molimard M, Moore N, Brochard P. 2008. Quantification methods of folpet degradation products in plasma with HPLC-UV/DAD: Application to an in vivo toxicokinetic study in rats. *J. Chromatogr. B-Anal. Technol. Biomed. Life Sci.* **865**: 106-113. DOI: 10.1016/j.jchromb.2008.02.011.

Chasseaud L, Hawkins DR, Franklin ER, Weston KT. 1974. The metabolic fate of  $^{14}\text{C}$ -Folpet (Phaltan) in the rat (Folpet). Huntingdon Research Centre Ltd.: Huntingdon. Unpublished report No CHR1-74482.

Chasseaud L. 1980. (Carbonyl- $^{14}\text{C}$ ) Folpet metabolism in rats. Huntingdon Research Centre Ltd.: Huntingdon. Unpublished report No DPBP 51202.

Costa LG. 2008. Toxic effects of pesticides: the basic science of poisons. In *Casarett and Doull's Toxicology: the Basic Science of Poisons*, Casarett LJ, Doull J, Klaassen CD (eds). McGraw-Hill: New York; pp 883-930.

Couch RC, Siegel MR, Dorough HW. 1977. Fate of Captan and Folpet in Rats and Their Effects on Isolated Liver Nuclei. *Pest. Biochem. Physiol.* **7**: 547-558.

de Cock J, Heederik D, Kromhout H, Boleij JSM, Hoek F, Wegh H, Ny ET. 1998. Exposure to captan in fruit growing. *Am. Ind. Hyg. Assoc. J.* **59**: 158-165.

DeBaun JR, Miaullis JB, Knarr J, Mihailovski A, Menn JJ. 1974. The fate of N-trichloro( $^{14}\text{C}$ )methylthio-4-cyclohexene-1,2-dicarboximide ( $^{14}\text{C}$ -captan) in the rat. *Xenobiotica* **4**: 101-119.

Edwards R, Ferry DG, Templ WA. 1991. Fungicides and related compounds. In *Handbook of Pesticide Toxicology*, Hayes WJ, Laws ER (eds). Academic Press: San Diego, CA; 1409-1470.

Finley BL, Paustenbach DJ. 1997. Using applied research to reduce uncertainty in health risk assessment: Five case studies involving human exposure to chromium in soil and groundwater. *J. Soil Contam.* **6**: 649-705.

Gordon EB, Ehrlich T, Mobley S, Williams M. 2001. Measurement of the reaction between the fungicides captan or folpet and blood thiols. *Toxicol. Meth.* **11**: 209-223.

Gordon EB. 2007. Captan: transition from 'B2' to 'not likely'. How pesticide registrants affected the EPA Cancer Classification Update. *J. Appl. Toxicol.* **27**: 519-526.

Gordon EB. 2010. Captan and folpet. In *Handbook of pesticide toxicology*, Krieger RI (ed). Elsevier: New York; 1915-1949.

Guo YLL, Wang BJ, Lee CC, Wang JD. 1996. Prevalence of dermatoses and skin sensitisation associated with use of pesticides in fruit farmers of southern Taiwan. *Occup. Environ. Med.* **53**: 427-431.

Hayes WJ. 1982. Fungicides and related compounds. In *Pesticides studied in man*, Hayes WJ (ed). Williams and Wilkins: Baltimore; 578-622.

Hayes AW. 2008. Toxicokinetics. In *Principles and Methods of Toxicology, 5<sup>th</sup> edition*, Hayes AW (ed). Taylor and Francis Group: Boca Raton; 179-230.

Hines CJ, Deddens JA, Jaycox LB, Andrews RN, Striley CAF, Alavanja MCR. 2008. Captan exposure and evaluation of a pesticide exposure algorithm among orchard pesticide applicators in the agricultural health study. *Ann. Occup. Hyg.* **52**: 153-166.

Krieger RI, Thongsinthusak T. 1993. Captan Metabolism in Humans Yields 2 Biomarkers, Tetrahydrophthalimide (THPI) and Thiazolidine-2-Thione-4-Carboxylic Acid (TTCA) in Urine. *Drug Chem. Toxicol.* **16**: 207-225.

Krieger RI, Dinoff TM. 2000. Captan fungicide exposures of strawberry harvesters using THPI as a urinary biomarker. *Arc. Environ. Contam. Toxicol.* **38**: 398-403.

Lappin GJ, Havell LM. Captan: Biotransformation study in the rat. ICI Central Toxicology Laboratory: Macclesfield. Unpublished report.



Larsen J-J. 1996. Captan. In *Pesticide Residues in Food: Evaluations Part II - Toxicological and Environmental*, World Health Organization: Geneva.

Lisi P, Caraffini S, Assalve D. 1987. Irritation and Sensitization Potential of Pesticides. *Contact Dermatitis* **17**: 212-218.

Ngo MA, O'Malley M, Maibach HI. 2010. Percutaneous absorption and exposure assessment of pesticides. *J. Appl. Toxicol.* **30**: 91-114. DOI: 10.1002/jat.1505.

Piccirillo V. 2001. Analysis of the toxicology and mechanistic data supporting the cancer reclassification of captan. VJP Consulting, INC., for the Captan Task Force, comprised of Markhteshim-Agan of North America, Inc. and Tomen Agro. Unpublished report.

Poet TS, McDougal N. 2002. Skin absorption and human risk assessment. *Chem. Biol. Interact.* **140**: 19-34.

Silva MJ, Samandar E, Preau JL Jr., Reidy JA, Needham LL, Calafat AM. 2007. Quantification of 22 phthalate metabolites in human urine. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **860**: 106-112. DOI: 10.1016/j.jchromb.2007.10.023.

Tomlin C. 1997. *The Pesticide Manual - World Compendium, 10<sup>th</sup> edition*. British Crop Protection Council: Thornton Heath.

Trivedi S. 1990. Captan: Excretion and tissue retention of a single oral dose (10 mg/kg) in the rat. ICI Central Toxicology Laboratory: Macclesfield. Unpublished report No CTL/P/2820.

Trochimowicz HJ, Kennedy GL, Krivanek ND. 1991. Heterocyclic and miscellaneous nitrogen compounds. In *Patty's Industrial Hygiene and Toxicology, 4<sup>th</sup> edition, Vol. 2, part E*, Clayton GD, Clayton FE (eds). Wiley: New York; 3285-3521.

United States Environmental Protection Agency (US EPA). Initial scientific and mini-economic review of captan. In Office of Pesticide Programs, Criteria and Evaluation Division: Washington. Report No EPA/540-1-75-012.

United States Environmental Protection Agency (US EPA). Registration Eligibility Decision (RED) - Folpet. US Environmental Protection Agency: Washington. Report No EPA 738-R-99-011.

United States Environmental Protection Agency (US EPA). Amendment to the 1999 captan Registration Eligibility Decision (RED). Captan: cancer reclassification. US Environmental Protection Agency: Washington. Report No Fed. Reg. 68357-68360.

van Welie RT, van Duyn P, Lamme E K, Jager P, van Baar BL, Vermeulen NP. 1991. Determination of tetrahydrophthalimide and 2-thiothiazolidine-4-carboxylic acid, urinary metabolites of the fungicide captan, in rats and humans. *Int. Arch. Occup. Environ. Health* **63**: 181-186.

Winterlin WL, Kilgore WW, Mourer CR, Schoen SR. 1984. Worker Reentry Studies for Captan Applied to Strawberries in California. *J. Agric. Food Chem.* **32**: 664-672.

Winterlin WL, Kilgore WW, Mourer CR, Hall G, Hodapp D. 1986. Worker Reentry Into Captan-Treated Grape Fields in California. *Arch. Environ. Contam. Toxicol.* **15**: 301-311.

Wolfe NL, Zepp RG, Doster JC, Hollis RC. 1976. Captan Hydrolysis. *J. Agric. Food Chem.* **24**: 1041-1045.

Wood SG, Chasseaud LF, Cheng K, Hall M, Fitzpatrick K, Iqbal S, Barlett A. Metabolic fate of <sup>14</sup>C-folpet in Sprague-Dawley rats. Huntingdon Life Sciences Limited: Huntingdon. Unpublished report No HRC/MBS 41-91499.

Woollen BH. 1993. Biological monitoring for pesticide absorption. *Ann. Occup. Hyg.* **37**: 525-540.

Zainal H, Que Hee SS. 2003. Folpet permeation through nitrile gloves. *Appl. Occup. Environ. Hyg.* **18**: 658-668.

**Table 1.** First-order apparent elimination half-lives of THPI and PI in human plasma and of THPI, PI and phthalic acid in human urine following ingestion of 1 mg kg<sup>-1</sup> of captan or folpet

Metabolites	Matrices	Mean first-order elimination half-life (h) <sup>a</sup> (n=5)	Coefficient of determination (R <sup>2</sup> )
THPI	Plasma	15.7	0.99
	Urine	11.7	0.98
PI	Plasma	31.5	0.84
	Urine	27.3	0.86
Phthalic acid	Urine	27.6	0.82

<sup>a</sup> The elimination half-life ( $t_{1/2}$ ) was calculated using the equation  $t_{1/2} = 0.693 k^{-1}$ , where  $k$  is the elimination rate constant obtained from the slope of the linear terminal phase of the plasma profile (10- to 72-h period) or urine excretion rate time course (24- to 96-h period) for each metabolite

**Table 2.** Toxicokinetic parameters for THPI and PI in plasma of volunteers following ingestion of 1 mg kg<sup>-1</sup> of captan or folpet

Model parameters	First order toxicokinetic values			
	THPI		PI	
	Mean (n=5)	SD	Mean (n=5)	SD
AUC [(nmol x h l <sup>-1</sup> ) kg <sup>-1</sup> ]	1 652	289	13.4	3.3
AUMC [(nmol x h <sup>2</sup> l <sup>-1</sup> ) kg <sup>-1</sup> ]	40 647	13 533	397	117
MRT (h)	23.9	4.0	29.4	2.2
CL (l h <sup>-1</sup> )	0.18	0.03	0.09	0.02
V <sub>d</sub> (l)	3.4	0.6	4.3	1.1

### Captions to figures

**Figure 1.** Captan metabolism according to *in vivo* studies in animals administered  $^{14}\text{C}$ - or  $^{15}\text{S}$ - radiolabelled doses (Lappin and Havell, 1990; Krieger and Thongsinthusak, 1993)

**Figure 2.** Folpet metabolism according to *in vivo* studies in animals and *in vitro* studies (Ackermann *et al.*, 1978; Wood *et al.*, 1991; US EPA, 1999; Canal-Raffin *et al.*, 2008; Gordon, 2010)

**Figure 3.** Time courses of THPI and PI in plasma of volunteers (expressed as nmol) over a 72-h period following ingestion of  $1 \text{ mg kg}^{-1}$  of captan or folpet. Each point represents mean and vertical bars are standard deviations ( $n = 5$ ).

**Figure 4.** Time courses of THPI, PI and phthalic acid excretion rate in urine (expressed as nmol/h) of volunteers over 96-h period following ingestion of  $1 \text{ mg kg}^{-1}$  of captan or folpet. Each point represents mean and vertical bars are standard deviations ( $n = 5$ ).

**Figure 5.** Time courses of THPI, PI and phthalic acid cumulative excretion in urine (expressed as nmol) of volunteers over a 96-h period following ingestion of  $1 \text{ mg kg}^{-1}$  of captan or folpet. Each point represents mean and vertical bars are standard deviations ( $n=5$ ).

**Figure 6.** Comparison of the time course of THPI urinary excretion rate (expressed as a % dose/h) in volunteers of the present study with that obtained by Krieger and Thongsinthusak (1993) following ingestion of  $1 \text{ mg kg}^{-1}$  of captan. Each point represents mean and vertical bars are standard deviations ( $n = 5$ ).

Figure 1.

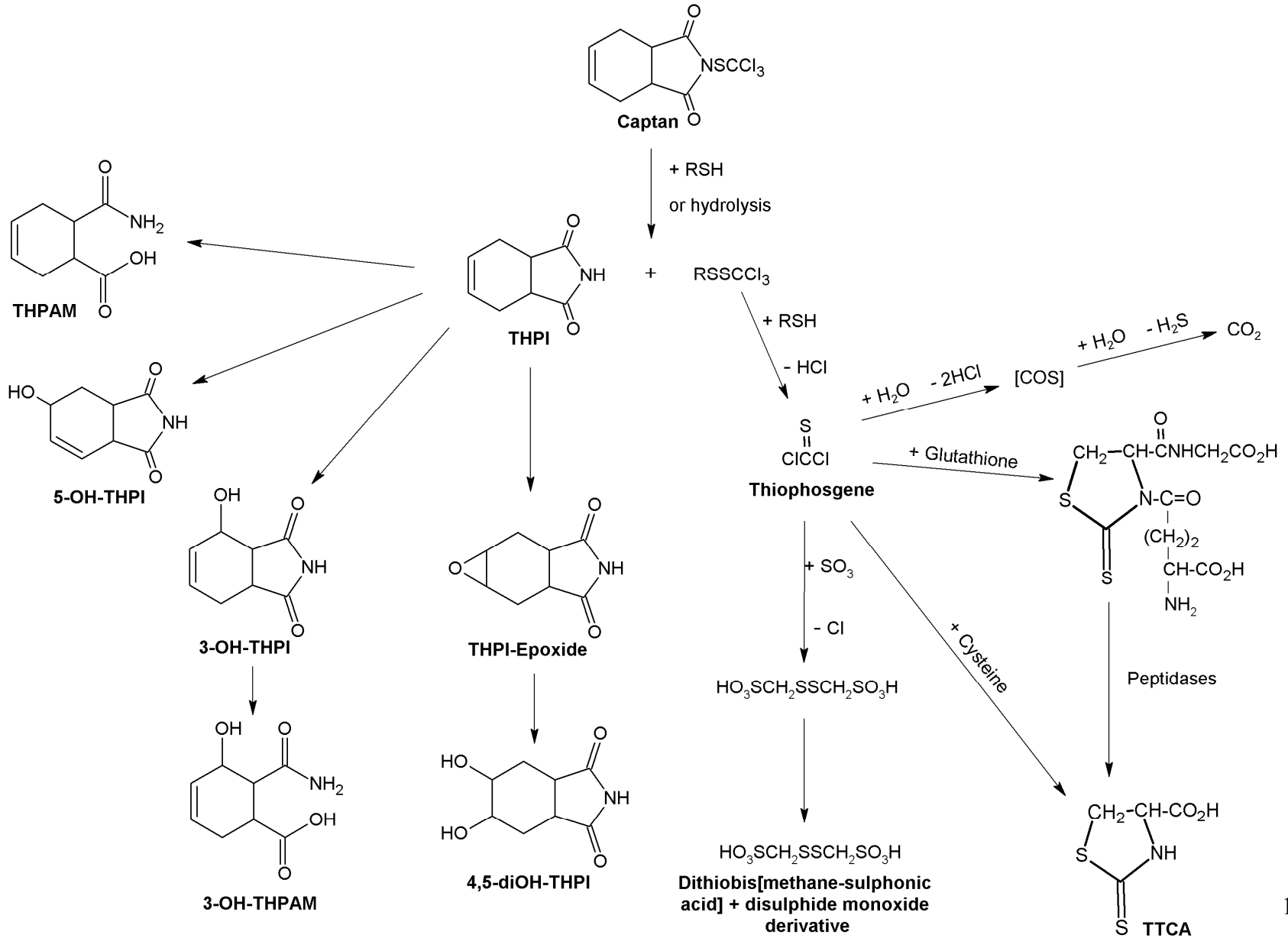


Figure 2.

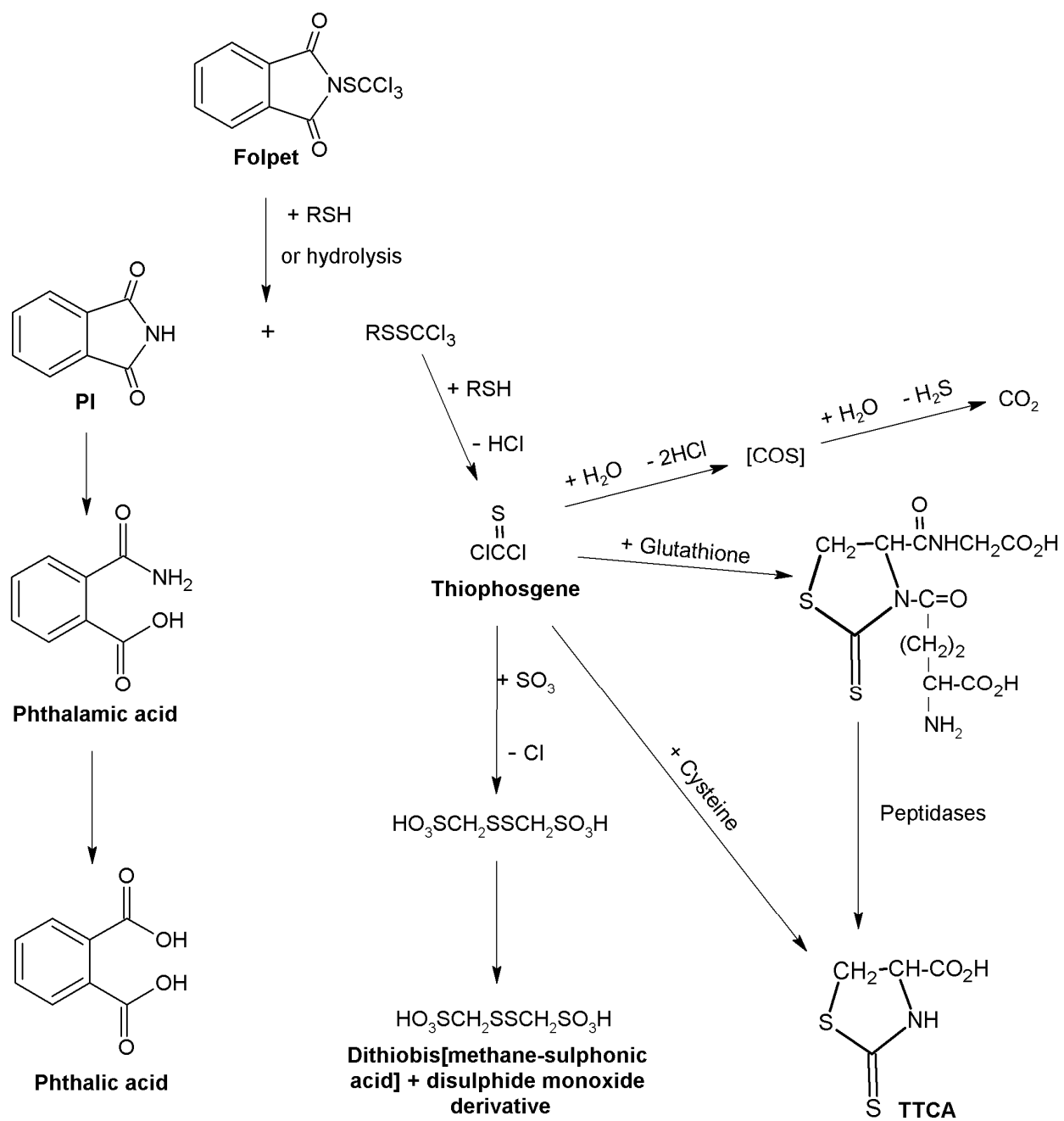




Figure 3.

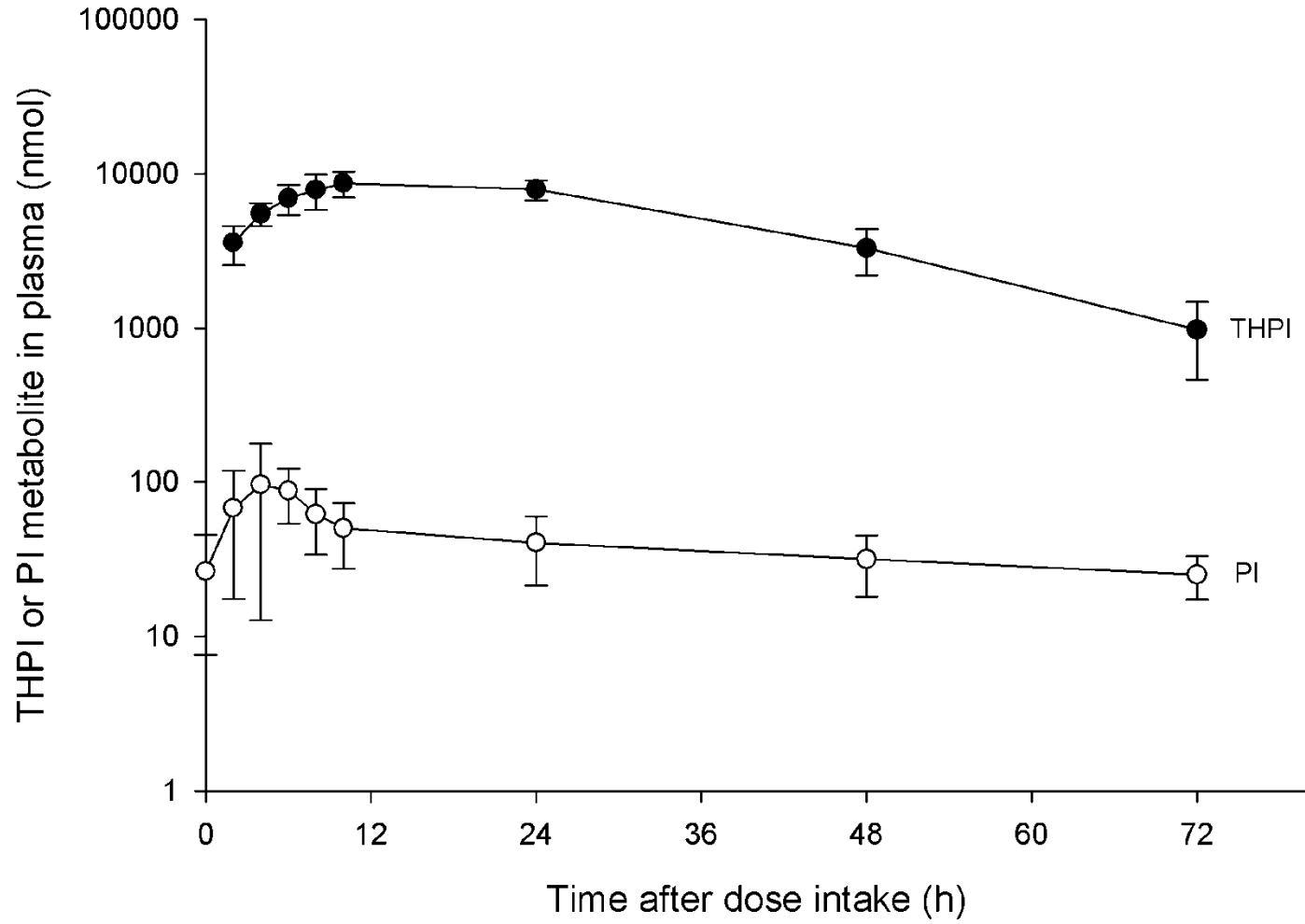


Figure 4.

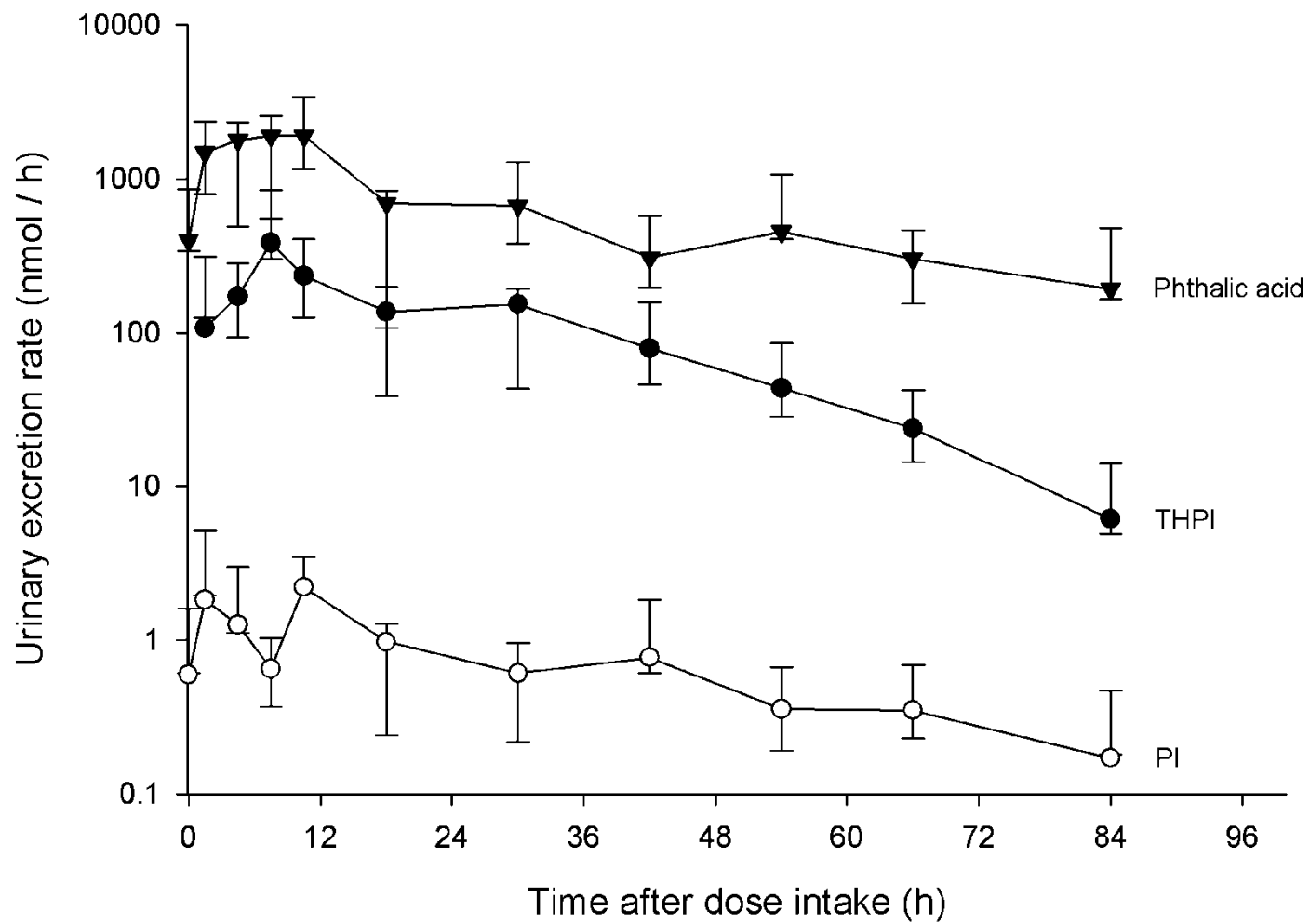


Figure 5.

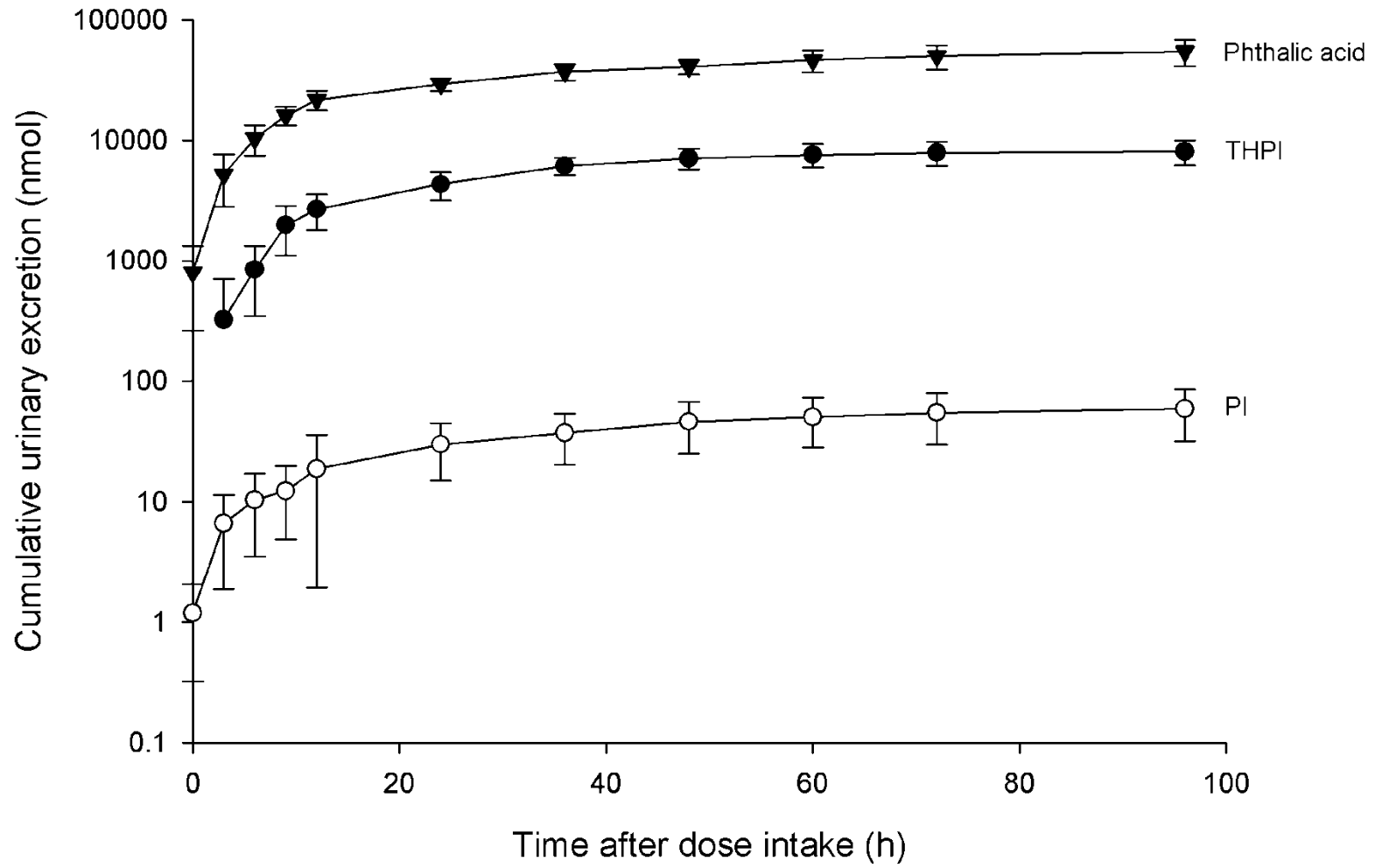
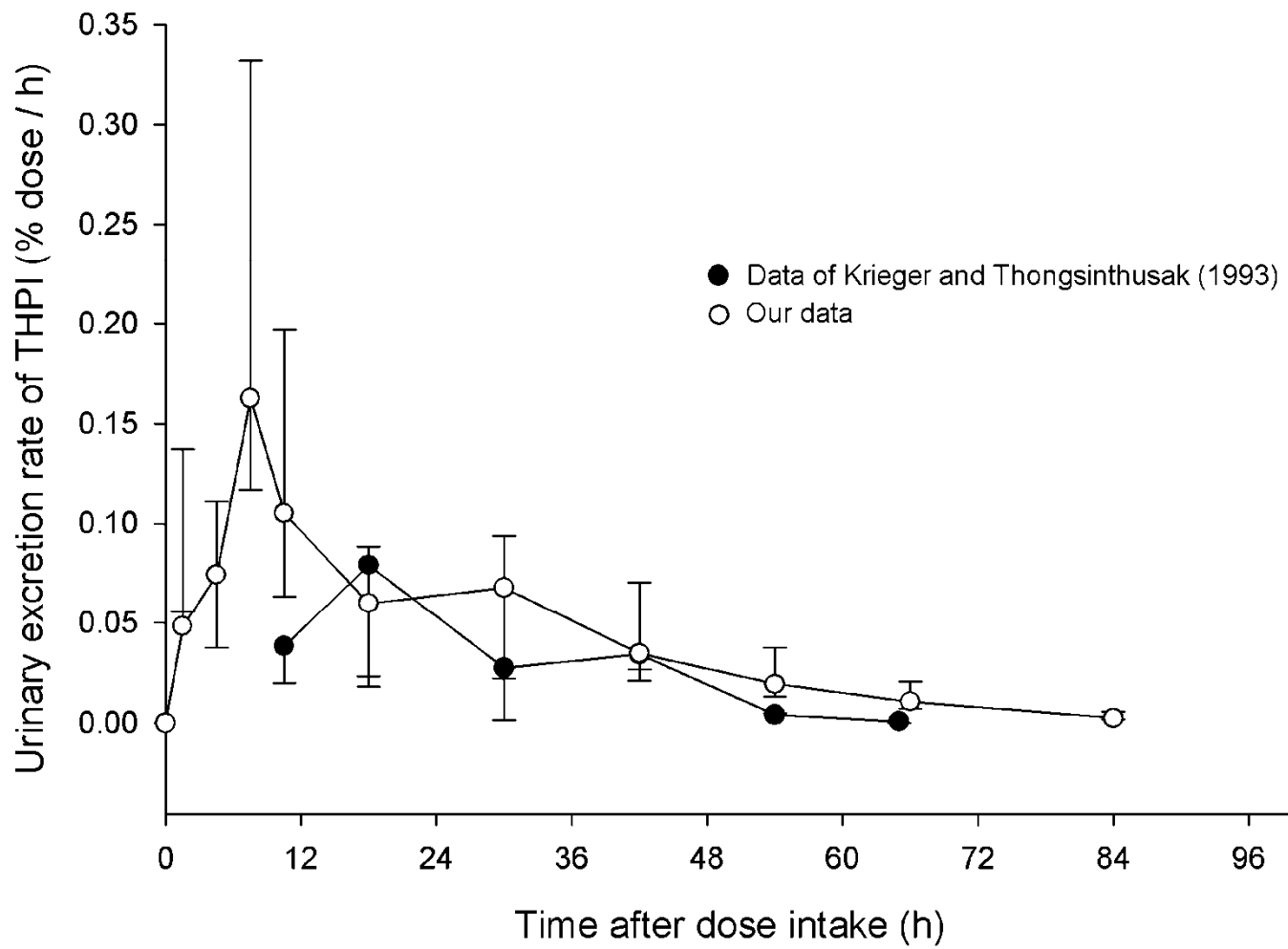


Figure 6.



**Supplemental table**

Average levels of THPI, PI and phthalic acid quantified in plasma and in urine at different points time in volunteers orally exposed to 1 mg kg<sup>-1</sup> of captan or folpet.

Time (h) <sup>b</sup>	Concentrations (nmol ml <sup>-1</sup> ) <sup>a</sup>				
	THPI <sup>c</sup> (mean ± SD)		PI <sup>d</sup> (mean ± SD)		Phthalic acid <sup>e</sup> (mean ± SD)
	Plasma	Urine	Plasma	Urine	Urine
0	0.004 ± 0.01	0	0.009 ± 0.004	0.005 ± 0.003	2.96 ± 1.65
2	1.14 ± 0.29		0.021 ± 0.012		
4	1.76 ± 0.19		0.029 ± 0.020		
3		0.69 ± 0.27		0.020 ± 0.018	16.06 ± 5.09
6	2.21 ± 0.38	1.41 ± 0.24	0.027 ± 0.007	0.011 ± 0.007	17.27 ± 3.40
8	2.51 ± 0.52		0.019 ± 0.006		
9		1.70 ± 0.37		0.007 ± 0.005	21.42 ± 11.0
10	2.77 ± 0.34		0.016 ± 0.005		
12		2.06 ± 0.59		0.023 ± 0.033	15.82 ± 6.42
24	2.62 ± 0.47	1.89 ± 0.41	0.013 ± 0.005	0.019 ± 0.008	13.17 ± 3.79
36		1.36 ± 0.31		0.011 ± 0.004	13.69 ± 6.37
48	1.07 ± 0.39	0.94 ± 0.30	0.010 ± 0.003	0.018 ± 0.013	6.51 ± 3.15
60		0.54 ± 0.24		0.007 ±	8.67 ± 8.81

				0.002	
72	$0.31 \pm 0.17$	$0.34 \pm 0.18$	$0.008 \pm 0.003$	$0.007 \pm 0.003$	$6.18 \pm 2.41$
96		$0.13 \pm 0.07$		$0.005 \pm 0.003$	$5.74 \pm 4.62$

---

<sup>a</sup> Urinary concentrations observed following sample processing as described in Materials and Methods.

<sup>b</sup> Fixed time periods (expressed in hour) of urine and blood collections following fungicide ingestion.

<sup>c</sup> Metabolite of captan.

<sup>d</sup> Metabolite of folpet.

<sup>e</sup> Metabolite of folpet.

**Supplemental table**

Average levels of THPI, PI and phthalic acid quantified in plasma and in urine at different points time in volunteers orally exposed to 1 mg kg<sup>-1</sup> of captan or folpet.

Time (h) <sup>b</sup>	Concentrations (nmol ml <sup>-1</sup> ) <sup>a</sup>				
	THPI <sup>c</sup>		PI <sup>d</sup>		Phthalic acid <sup>e</sup>
	(mean ± SD)		(mean ± SD)		(mean ± SD)
	Plasma	Urine	Plasma	Urine	Urine
0	0.004 ± 0.01	0	0.009 ± 0.004	0.005 ± 0.003	2.96 ± 1.65
2	1.14 ± 0.29		0.021 ± 0.012		
4	1.76 ± 0.19		0.029 ± 0.020		
3		0.69 ± 0.27		0.020 ± 0.018	16.06 ± 5.09
6	2.21 ± 0.38	1.41 ± 0.24	0.027 ± 0.007	0.011 ± 0.007	17.27 ± 3.40
8	2.51 ± 0.52		0.019 ± 0.006		
9		1.70 ± 0.37		0.007 ± 0.005	21.42 ± 11.0
10	2.77 ± 0.34		0.016 ± 0.005		
12		2.06 ± 0.59		0.023 ± 0.033	15.82 ± 6.42
24	2.62 ± 0.47	1.89 ± 0.41	0.013 ± 0.005	0.019 ± 0.008	13.17 ± 3.79
36		1.36 ± 0.31		0.011 ± 0.004	13.69 ± 6.37
48	1.07 ± 0.39	0.94 ± 0.30	0.010 ± 0.003	0.018 ± 0.013	6.51 ± 3.15
60		0.54 ± 0.24		0.007 ± 0.002	8.67 ± 8.81
72	0.31 ± 0.17	0.34 ± 0.18	0.008 ± 0.003	0.007 ± 0.003	6.18 ± 2.41
96		0.13 ± 0.07		0.005 ± 0.003	5.74 ± 4.62

<sup>a</sup> Urinary concentrations observed following sample processing as described in Materials and Methods.

<sup>b</sup> Fixed time periods (expressed in hour) of urine and blood collections following fungicide ingestion.

<sup>c</sup> Metabolite of captan.

<sup>d</sup> Metabolite of folpet.

<sup>e</sup> Metabolite of folpet.



## Article IV

### **Toxicokinetics of captan and folpet biomarkers in dermally exposed volunteers**

Aurélie Berthet,<sup>a,b</sup> Michèle Bouchard<sup>a\*</sup> and David Vernez<sup>b</sup>

<sup>a</sup> Département de santé environnementale et santé au travail, Chaire d'analyse et de gestion des risques toxicologiques and Institut de recherche en santé publique de l'Université de Montréal (IRSPUM), Faculté de Médecine, Université de Montréal, C.P. 6128, Succursale Centre-ville, Montréal, Québec, Canada, H3C 3J7

<sup>b</sup> Institute for Work and Health, Rue du Bugnon 21, 1011 Lausanne, Switzerland

\*corresponding author

Journal of Applied Toxicology

Article accepté (Décembre 2010) - DOI 10.1002/jat.1659

**ABSTRACT**

To better assess biomonitoring data in workers exposed to captan and folpet, the kinetics of ring metabolites (tetrahydrophthalimide (THPI), phthalimide (PI) and phthalic acid) were determined in urine and plasma of dermally exposed volunteers. A 10 mg kg<sup>-1</sup> dose of each fungicide was applied on 80 cm<sup>2</sup> of the forearm and left without occlusion or washing for 24 h. Blood samples were withdrawn at fixed time periods over the 72 h following application and complete urine voids were collected over 96 h post-dosing, for metabolite analysis. In the hours following treatment, a progressive increase in plasma levels of THPI and PI was observed, with peak levels being reached at 24 h for THPI and 10 h for PI. The ensuing elimination phase appeared monophasic with a mean elimination half-life ( $t_{1/2}$ ) of 24.7 and 29.7 h for THPI and PI, respectively. In urine, time courses PI and phthalic acid excretion rate rapidly evolved in parallel, and a mean elimination  $t_{1/2}$  of 28.8 and 29.6 h, respectively, was calculated from these curves. THPI was eliminated slightly faster, with a mean  $t_{1/2}$  of 18.7 h. Over the 96-h period post-application, metabolites were almost completely excreted, and on average 0.02% of captan dose was recovered in urine as THPI while 1.8% of the folpet dose was excreted as phthalic acid and 0.002% as PI, suggesting a low dermal absorption fraction for both fungicides. This study showed the potential use of THPI, PI and phthalic acid as key biomarkers of exposure to captan and folpet.

**Keywords:** toxicokinetics; captan; folpet; tetrahydrophthalimide; phthalimide; phthalic acid; dermal exposure; human; biomarker

**Table of Contents – Short abstract**

To better assess biomonitoring data in workers exposed to captan and folpet, the kinetics of ring metabolites were determined in urine and plasma of dermally exposed volunteers. Each fungicide was applied on 80 cm<sup>2</sup> of the forearm for 24 h. Blood samples and complete urine voids were collected respectively over 72 h and 96 h following application. Overall, the studied metabolites appeared as key biomarkers of exposure to captan and folpet despite the low dermal absorption fraction.

## INTRODUCTION

The skin is in many instances the primary route of pesticide exposure for agricultural workers, as demonstrated by several studies (Ritcey *et al.*, 1987; Fenske, 1990; Woollen, 1993;; Ngo *et al.*, 2010). It was estimated by a number of authors to account for 90% of exposure whatever the activity performed (i.e. preparation of mixture, spraying, material washing, harvesting) (Fenske, 1990; Vermeulen *et al.*, 2002; Ngo *et al.*, 2010). However, numerous factors influence dermal penetration of compounds and affect exposure measurements: physicochemical properties of compounds (solubility, chemical structure, partition coefficient), environmental conditions (humidity, temperature, wind), vehicle used to apply pesticides (physicochemical properties, solubility, pH, concentration), skin properties and cutaneous metabolism (skin condition, skin hydration, type, thickness, enzyme activities, wounds), shunt diffusion, anatomical site of exposure (skin thickness, hair follicle size and density), inter-individual variability (age, gender, race), dose, duration and occlusion (Lotte *et al.*, 1993; Kromhout and Vermeulen, 2001; Tsai *et al.*, 2001; Mangelsdorf *et al.*, 2006). Therefore, biological monitoring becomes a reliable means of estimating dermal exposure in humans as underlined by several authors (de Cock *et al.*, 1995; Ngo *et al.*, 2010; van Welie *et al.*, 1991).. Nonetheless, to be accurate, biomonitoring data need to be supported by adequate kinetic studies in human volunteers (Wester and Maibach, 1983; Woollen, 1993; Ngo *et al.*, 2010).

Captan and folpet are considered as two commonly used fungicides in various crops of many countries (Guo *et al.*, 1996; Fenske *et al.*, 1998; Tielemans *et al.*, 1999; Carden *et al.*, 2005; Baldi *et al.*, 2006; Juraske *et al.*, 2007; Greenburg *et al.*, 2008). However, only a limited number of studies are reported in the literature that use biomonitoring to assess captan exposure in workers (Hansen *et al.*, 1978; Mcjilton *et al.*, 1983; Winterlin *et al.*, 1984; Winterlin *et al.*, 1986; Verberk *et al.*, 1990; Krieger and Thongsinthusak, 1993; Lavy *et al.*, 1993; de Cock *et al.*, 1995, 1998; Krieger and Dinoff, 2000; Hines *et al.*, 2008). Moreover, to our knowledge, no reports are available that describe biomonitoring of folpet. As for kinetic studies in human volunteers, one is described in the literature following an oral exposure to captan (Krieger and Thongsinthusak, 1993) while such data is lacking for folpet.

On the other hand, the metabolism of both fungicides is well documented in *in vivo* studies in animals usually exposed to the radiolabelled compounds and in *in vitro* studies. According to these data, the first step in the metabolism of captan is its breakdown at the N-S link when it is in contact with thiol groups (e.g. glutation, cysteine, proteins, etc.), and the reaction is enhanced in alkaline medium (Wolfe *et al.*, 1976). From this nonenzymatic process a tetrahydrophtalimide (4,5-cyclohexene-1,2-dicarboximide; THPI) and a thiocarbonyl chloride are formed, the latter being a derivative of the trichloromethylthio group. This thiocarbonyl chloride further reacts with thiols to form thiophosgene, a transient metabolite, which readily reacts with cysteine or glutathione hence leading to the formation of thiazolidine-2-thione-4-carboxylic acid (TTCA); two other metabolites can also be formed, the dithiobis (or

methanesulfonic acid) and its disulphide monoxide derivative, arising from the reaction with sulphite ions (DeBaun *et al.*, 1974). From animal studies and two occupational studies carried out by Winterlin *et al.* (1984, 1986) in strawberry pickers and in grape field workers, THPI was suggested as possible biomarker of captan exposure by the Environmental Protection Agency (US EPA) (1975).

Similar to captan initial metabolism step, folpet is also broken down at the N-S link when it is in contact with thiol groups; this leads to the formation of a phthalimide (PI) and a thiocarbonyl chloride, but unlike for captan, the reaction is enhanced in acid conditions (Gordon *et al.*, 2001; Gordon, 2010). From the thiocarbonyl chloride, the same metabolic reactions as captan occur and the same thiol metabolites are formed. On the other hand, the ring metabolite of folpet, PI, is rapidly hydrolyzed to phthalamic acid mainly but also to phthalic acid, according to animal studies (Gordon *et al.*, 2001; Zainal and Que Hee, 2003; Canal-Raffin *et al.*, 2008; Gordon, 2010). For the biological monitoring of exposed individuals, no biomarker was proposed for this fungicide since no human study was reported.

Overall, although some metabolism data are available from animal studies for these two fungicides, there is a paucity of kinetic data in humans following dermal exposure. These human kinetic data are especially needed given the well known interspecies differences in dermal absorption (Feldmann and Maibach, 1974; Wester and Maibach, 1983; Kao and Carver, 1990; Fiserova-Bergerova, 1993; Fenske *et al.*, 1998, Poet and

McDougal, 2002). In addition, the dermal route is in many circumstances the main route of exposure to pesticides in workers.

The aim to this study was thus to determine the kinetics of captan and folpet ring metabolites in accessible biological matrices of dermally exposed volunteers to better assess biomonitoring data in workers. It also aimed to compare the toxicokinetics of captan and folpet metabolites, given the related chemical structure of the parent compound and initial breakdown metabolites.

## **MATERIALS AND METHODS**

### **Study design**

The study design was similar to the one previously described for volunteers orally exposed to the same fungicides and reported in Berthet *et al.* (2011a). Thus, a controlled kinetic time course study in accessible biological matrices of healthy subjects was conducted following a dermal exposure to captan and folpet. Captan and folpet metabolites were measured in urine and blood samples collected prior to treatment to obtain pre-test values, and then at predetermined time points post-dosing (amounting to a total of 9 samples for plasma and 11 samples for urine).

The experimental protocol and consent forms were approved by the Permanent Ethics Committee for Clinical Research of the Faculty of Biology and Medicine of the University of Lausanne and the Research Ethics Committee of the Faculty of Medicine of the University of Montreal. All the participants gave their written consent, and were informed of the risks of participating and their right to withdraw from the study at anytime. As suggested by the Ethics Committee who considered the study as very restrictive, the participants received a monetary compensation for their time and any inconvenience caused.

### **Subjects studied**

Participants were recruited on a voluntary basis among the medical students of the University of Lausanne, Switzerland. The volunteers were nine male students aged from 20 to 30 years old, weighing 60 to 85 kg and measuring 169 to 184 cm. They were healthy, non-smokers and did not take medication or drugs. They underwent a medical examination by an occupational physician prior to enrolment. They had never been exposed to captan or folpet, except an acute oral dose of  $1 \text{ mg kg}^{-1}$  two weeks before, and perhaps through their diet. During the study period and the two-days prior to dosing, they were asked not to eat fruits and vegetables in order to limit ingestion of contaminated food. They were also requested to avoid consumption of alcohol during this period, since it is known to affect the metabolism of some chemical compounds.



## Dosing and sampling

The experimental dosing and sampling was also conducted at the Institute for Work and Health of Lausanne, Switzerland. The morning of study onset, each participant was asked to collect his complete first morning urine void, to obtain pre-test values; they then spent the first day of the study in a room at the Institute for dosing and the first 12-h sampling. On the following 3-day sampling period, participants visited the Institute for their daily morning blood sampling and handing-in urine collections.

A dose of captan or folpet equivalent to  $10 \text{ mg kg}^{-1}$  body wt was applied on  $80 \text{ cm}^2$  of one of the forearm of each volunteer ( $n = 5$  for captan and  $n = 4$  for folpet). Fungicides used to prepare the exposure dose of volunteers were Captan PESTANAL<sup>®</sup> (assay (HPLC) area 99.1 to 99.4%) and Folpet PESTANAL<sup>®</sup> (assay (HPLC) area 99.5 to 99.9%). Both were purchased from Fluka – Sigma-Aldrich (Buchs, St Gallen, Switzerland). The single dose was weighted in a labelled glass container and adjusted to the weight of each volunteer. The fungicide dose was dissolved in 30 mL of acetone, mixed and applied on the skin. To ensure that the dose was applied on  $80 \text{ cm}^2$ , a  $20 \times 4$  cm cardboard frame was fixed on the forearm of each volunteer to delimit the area. To avoid injuring the participant, a plastic pipette was used to apply the dose. The participants washed their arm only 24 h following application. The exposed area was not occluded to avoid influencing absorption (Feldmann and Maibach, 1974; Wester and Maibach, 1983; Curdy *et al.*, 2004; Carden *et al.*, 2005), except some of the participants wore long sleeve shirts since the study was performed during the winter season.

Complete micturitions were then collected at pre-determined times over the 96-h period post-dosing, that is at around 0, 3, 6, 9, 12, 24, 36, 48, 60, 72, 84 and 96 h post-dosing. Each timed-void was collected in separate polypropylene Nalgene® bottles of 1 l. To assist participants, they received a schedule specifying the date and the required time of urine collection. They then only had to complete the sheet with the actual time of collection and to indicate whether or not there were any urine losses. They also had to identify their plastic bottles with distinct pre-printed labels indicating the code, and mark the date and time of urine collection.

Once collected, urine samples were kept in the refrigerator prior to measurement of total urine volume per void. To allow repeated analysis while avoiding possible degradation due to freezing and defreezing of samples, each urine collection then was aliquoted in 4 labelled tubes of 15 ml and one bottle of 120 ml before storage at -20°C until analysis of captan or folpet metabolites.

Blood samples of 15 ml were also collected at specific times, that is at  $t = 0, 2, 4, 6, 8, 10, 24, 48$  and 72 h post-dosing. To facilitate collection, a catheter was installed by a nurse prior to dosing along with a drip system of physiological saline the first day of sampling. Blood was withdrawn by the nurse into vacutainers pre-labelled with a code, the date and time of sampling. Immediately after collection, blood samples were centrifuged to precipitate red blood cells and isolate plasma. The plasma samples were then split into 3 labelled aliquots and stored at -20°C until analysis of captan and folpet metabolites.

During the study period, volunteers were also asked to fill a questionnaire to document personal information (weight, height), life habits (i.e. physical activities, smoking), medication intake (including ibuprofen), alcohol consumption during the 3 days prior to treatment, consumption of fruits, vegetables and cereals during the 4 days prior to treatment and possible dosing-related symptoms.

### **Chemicals and reagents**

Reference standards (>99% purity) were obtained from Sigma-Aldrich (Buchs, St Gallen, Switzerland), except for deuterated cis-1,2,3,6-tetrahydrophthalimide (THPI-d) (99% purity), which was purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). HPLC grade acetonitrile, methanol, ethyl acetate and dichloromethane were also obtained from Sigma-Aldrich (Buchs, St Gallen, Switzerland), along with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), the reagent used to derivatize the phthalic acid molecule. HCl and ammonium sulphate were purchased from Merck (Zug, Switzerland). Water was purified using a TKA GenPure water treatment system obtained from TKA Wasseraufbereitungssysteme GmbH (Niederelbert, Germany).

### **Analysis of THPI and PI in plasma and urine**

A liquid chromatography - atmospheric pressure chemical ionization-tandem mass spectrometry (LC/APCI-MS/MS) method was developed to analyze THPI and PI in

urine and plasma, and is described elsewhere (Berthet *et al.*, 2011b). Briefly, THPI and PI were isolated by solid phase extraction (SPE) (in the case of plasma, following a denaturation of proteins), eluted in dichloromethane and analyzed using a Varian Model 212-LC Binary Gradient LC system (Les Ulis, France) connected to a Prostar model 410 autosampler (Varian, Les Ulis, France) and coupled to a Model 1200 L quadrupole MS (Varian, Les Ulis, France) operating in APCI mode. The APCI interface was operated in negative ion mode. The compounds were separated using a C<sub>18</sub> Zorbax Eclipse Plus column (4.6 x 150 mm, 3.5 μm) from Agilent (Morges, Switzerland). The mobile phase consisted of: eluent A composed of 90% water and 10% acetonitrile (9:1), and eluent B of 10% water – 90% acetonitrile (1:9). For THPI analysis, 10 μl of the sample were injected and elution was performed in 26 min using a solvent gradient at a flow rate of 0.8 ml min<sup>-1</sup>, and for PI analysis, 10 μl of the sample were also injected and elution was performed in 33 min in isocratic mode at a flow rate of 0.8 ml min<sup>-1</sup>. The fragments analyzed were *m/z* 149.4/95.6 for THPI with a collision energy (CE) of 19.5 V, *m/z* 156.1/95.6 for the internal standard THPI-d (CE of 22.5 V) and *m/z* 145.8 for PI (no fragmentation). The analytical limit of detection for THPI was 3.82 nmol l<sup>-1</sup> of urine and 9.76 nmol l<sup>-1</sup> of plasma and, for PI, 7.72 nmol l<sup>-1</sup> of urine and 14.8 nmol l<sup>-1</sup> of plasma. The quantification of THPI or PI was obtained from standard calibration curves prepared in urine or plasma adjusted by the THPI-d internal standard surface area.

### **Analysis of phthalic acid in urine**

A gas chromatography mass spectrometry (GC-MS) method was developed for the analysis of phthalic acid in urine, as previously described (Berthet *et al.*, 2011c). In short, urine samples were subjected to an acid hydrolysis prior to liquid-liquid extraction with ethyl acetate and derivatization with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). Analysis was then performed using an Agilent Mass Selective Detector (MSD) G1098A (Agilent Technologies Inc, Waldbronn, Germany) coupled with a HP 5973 mass spectrometer (Agilent Technologies Inc, Waldbronn, Germany). Separation of the analytes was performed using a 60 m CP-SIL 8 CB column (1  $\mu\text{m}$  film thickness, 250  $\mu\text{m}$  I.D.) (Varian, Les Ulis, France). The initial column temperature was 200°C for 3 min, then it was increased to 260°C at 30°C  $\text{min}^{-1}$ , held for 11 min, and finally increased to 280°C at 35°C  $\text{min}^{-1}$  and held for 4 min. For the analysis, 2  $\mu\text{l}$  were injected using a 5  $\text{ml min}^{-1}$  split. The ions monitored were trimethylsilyl phthalic acid (TMS phthalic acid) with  $m/z$  295 and the internal standard TMS methylhippuric acid with  $m/z$  220. The quantification was obtained from standard calibration curves of phthalic acid prepared in urine and adjusted by the methylhippuric acid internal standard surface area. The analytical limit of detection was 0.6  $\mu\text{mol l}^{-1}$  urine.

### **Calculations**

The molar fraction of captan or folpet administrated dose recovered in urine as THPI, PI or phthalic acid was calculated from the following equation:

$$\left[ \frac{\left( \frac{Q_{\text{urinary metabolite}}}{MW_{\text{metabolite}}} \right)}{\left( \frac{Dose_{\text{parent compound}}}{MW_{\text{parent compound}}} \right)} \right] \times 100$$

where  $Q_{\text{urinary metabolite}}$  corresponds to total amounts of THPI, PI or phthalic acid in urine over the 96-h urine collection period (mg),  $MW_{\text{parent compound}}$  is the molecular weight of captan or folpet,  $MW_{\text{metabolite}}$  is the molecular weight of THPI, PI or phthalic acid and  $Dose_{\text{parent compound}}$  is the orally administered dose of captan or folpet (mg).

### Toxicokinetic analysis

To determine elimination rate constants of THPI and PI in plasma and in urine, we considered that, following peak levels, elimination was monophasic and a first-order reaction. The elimination rate constant ( $k$ ) was thus calculated from the slope of the linear terminal phase of the plasma or urine time profile. The apparent elimination half-life ( $t_{1/2}$ ) was then defined using the equation  $t_{1/2} = 0.693 k^{-1}$  (Hayes, 2008).

From plasma concentration (C) - time profile, we also calculated the area under the concentration-time curve (AUC), the area under the first moment of concentration-time curve (AUMC), the mean residence time (MRT), the plasma clearance (CL) and the apparent volume of distribution ( $V_d$ ). Equations used to calculate these parameters are:

$$AUC = \frac{1}{2} \sum_{\forall i} (t_i - t_{i+1}) [C(t_i) + C(t_{i+1})]$$

$$AUMC = \frac{1}{2} \sum_{\forall i} (t_i - t_{i+1}) [t_i C(t_i) + t_{i+1} C(t_{i+1})]$$

$$MRT = \frac{AUMC}{AUC}$$

$$CL = \frac{\textit{Absorbed dose}}{AUC}$$

$$Vd = \frac{CL}{k} \quad \text{where } k \text{ is the overall elimination rate from}$$

blood

## RESULTS

### Time courses of THPI and PI in plasma

The time courses of THPI and PI in plasma of volunteers over the 72-h period following an application of captan or folpet ( $10 \text{ mg kg}^{-1}$  body wt) are presented in Fig. 1. In control samples taken prior to captan or folpet ingestion ( $t_0$ ), concentrations of THPI were below the analytical limit of detection and those of PI were very low, in the order of  $3.9 \text{ nmol l}^{-1}$ . Following application, a progressive increase in plasma levels of THPI and PI was observed, with peak levels being reached 24 h post dosing for THPI and 10 h post-treatment for PI. Subsequent elimination phase of THPI and PI from plasma over the 24- to 72-h period post-dosing appeared monophasic with an apparent elimination half-life of 24.7 and 29.7 h, respectively (Table 1). From the THPI and PI time profiles in plasma, various toxicokinetic parameters were calculated and are presented in Table 2. These show that following dermal application, THPI had a greater bioavailability and slightly faster clearance rate than PI; on the other hand, they had similar, relatively small, volume of distribution ( $V_d$ ).

### Time courses of THPI, PI and phthalic acid in urine

Figure 2 presents the time courses of THPI, PI and phthalic acid excretion rate in the urine of volunteers over a 96-h period following application of  $10 \text{ mg kg}^{-1}$  body wt of captan or folpet. As expected, these profiles evolved in unison with the blood profiles



(see Figure 1 for comparison). It is readily observed from Fig. 2 that, following peak excretion, elimination rate time courses of PI and phthalic acid were parallel, with mean apparent elimination half-lives of 28.8 and 29.6 h, respectively, calculated for the 36- to 96-h period post-dosing (Table 1). THPI elimination was slightly faster with an apparent elimination half-life of 18.7 h calculated for the same period (Table 1).

Figure 3 displays the corresponding cumulative urinary excretion time courses of the three metabolites over the 96-h collection period post-dosing. From these data, it was calculated that 1.8% of the applied dose of folpet was recovered in urine as phthalic acid as compared to only 0.002% for PI. This indicates that phthalic acid was present in urine in 1 000-fold higher amounts than PI despite the fact that they had similar urinary time-profiles and thus elimination half-lives. By comparison, the percentage of applied captan dose recovered in urine as THPI was 0.02%, which is 10-fold higher than the percentage of applied folpet dose found as PI in urine.

## DISCUSSION

### **Kinetics of biomarkers of exposure to captan and folpet in dermally exposed volunteers**

The present study provided novel data on the toxicokinetics of key biomarkers of exposure to captan and folpet following dermal exposure in humans. Such dermal kinetic data were lacking for both fungicides, not only in humans but also in animals. The studied biomarkers were found to be rapidly formed and eliminated from the body, with elimination half-lives following dermal application in the order of 20-25 h for THPI and 30 h for folpet ring-metabolites. No built up in the body was apparent; elimination from the body was complete 96 h post-treatment. The small percentage of dose recovered in urine as the studied biomarkers also suggests a low dermal absorption fraction of both captan and folpet.

Although dermal kinetic data on these specific biomarkers were not available in the literature for comparison purpose, the dermal absorption, tissue distribution and excretion of total radioactivity has been documented in animals dermally exposed to labelled captan or folpet (Grissom *et al.*, 1985; Shah *et al.*, 1987; Fisher *et al.*, 1992). These animal data are in accordance with current results, showing a rapid elimination of labelled dose following dermal exposure and a low dermal absorption fraction. In particular, in one of the few published dermal kinetic studies of captan in animals, Fisher *et al.* (1992) observed that, on average, 82% of a dermally applied dose of  $^{14}\text{C}$ -ring-

captan ( $286 \text{ nmol cm}^{-2}$ ) were recovered in the analyzed tissues and fluids (kidneys, liver, total skin, carcass, urine, feces and blood) of aging Fischer 344 female rats (33 and 82 days) over the 0-120 h collection period. After 120 h, on average, 10.8 and 11.5% of the applied dose had penetrated young and adult skin, respectively, and 9% of dose in young rats and 10% of dose in adults were recovered as total radioactivity in urine. Similar to published oral experiments in rats exposed to ring-labelled captan, this implied that most of the absorbed dose was excreted in urine while very little was excreted in feces.

Similarly, in one of the only dermal penetration study on folpet in animals, Shah *et al.* (1987) estimated a low dermal absorption fraction, as assessed from analysis of radioactivity in skin and carcasses at 72 h post-application of a low, medium and high dose of  $^{14}\text{C}$ -trichloromethyl labelled folpet in Fisher 344 rats as well as in urine and feces collected over the 72-h period post-dosing. On average 12% of the low dose of folpet were calculated to be absorbed through the skin during that time (application of  $0.1 \mu\text{mol cm}^{-2}$  on  $2.8$  and  $5.6 \text{ cm}^2$  of young and adult rats, respectively) as compared to only 3 and 1 % for the two higher doses (application of  $0.5$  and  $2.7 \mu\text{mol cm}^{-2}$ , respectively).

The study of Krieger and Thongsinthusak (1993) is the only available published time course study of specific THPI biomarker in the urine of volunteers exposed under controlled conditions. In the latter study, two volunteers were orally exposed to  $0.1$  and  $1 \text{ mg kg}^{-1}$  captan and it was observed that THPI represented 1 to 3% of the administered dose. In a preliminary attempt, captan was also applied to the hands, forearms and

inguinal region of the volunteers (15 mg on 40  $\mu\text{g cm}^{-2}$  region of the skin of volunteers weighing 150 and 84 kg) but the dose was too low to allow quantification of THPI in urine. Nonetheless, they estimated a captan dermal absorption of 0.3% per day for humans.

Furthermore, from the observed kinetic data in the current study, some essential biological determinants can be identified. In particular, the dermal kinetics of the two studied biomarkers of exposure to folpet, PI and phthalic acid, exhibited similar time profiles, indicating that they were governed by the same essential biological processes. However, phthalic acid was found to be present in much higher amounts than PI in urine, in line with the substantial metabolism of PI into acids documented by some authors following oral or intraperitoneal exposure (Ackermann *et al.*, 1978; Chasseaud *et al.*, 1991; Canal-Raffin *et al.*, 2008; Gordon, 2010). In particular, Canal-Raffin *et al.* (2008) reported an elimination half-life of on average 2.5 h for PI in plasma following a single intraperitoneal folpet dose of 10 mg kg<sup>-1</sup> in Wistar rats. Ackermann *et al.* (1978) also estimated a PI half-life of 2 h in rat fetuses following an oral administration of 2.5 mg kg<sup>-1</sup> of <sup>15</sup>N-phthalimide to pregnant Wistar-strain albino rats and observed a fast metabolism of PI into phthalamic acid. This latter metabolite is then transformed to phthalic acid, the final ring-metabolite of folpet (Williams and Blanchfield, 1974).

By comparison, current results suggest that THPI is a minor metabolite of captan, since urinary amounts were found to represent only a very small percentage (0.02%) of the dermal dose in volunteers. This is corroborated by oral mass-balance data in animals

following radiolabelled doses, with specific identification of metabolites in urine, showing in particular the presence of 3-hydroxy-4,5-cyclohexene-1,2-dicarboximide (3-OH-THPI) derivative as a more important metabolite (Lappin and Havell, 1990; Gordon, 2010).

### **Comparison of the kinetics of captan and folpet ring metabolites in dermally exposed volunteers**

This study also allowed comparing the kinetics of captan and folpet ring metabolites in dermally exposed volunteers. Although these biomarkers are rapidly eliminated from the body following dermal exposure with apparent negligible tissue accumulation, differences in the dermal kinetics of THPI ring-metabolite of captan as compared to that of PI and phthalic acid ring-metabolites of folpet were observed. THPI was eliminated slightly faster than folpet ring-metabolites. Dermal absorption fraction also appeared more important for folpet than captan when comparing amounts of metabolites recovered overall in urine (1.8% of dermal folpet dose recovered as phthalic acid in urine as compared to 0.02% of dermal captan dose found as THPI in urine with reference to 25 and 3.5% respectively after oral administration).

### **Comparison of the dermal and oral kinetics of ring-metabolites of captan and folpet**

When compared to oral kinetics, the dermal kinetics of captan and folpet ring-metabolites in humans exhibited marked differences. Indeed, the plasma time profile of THPI observed in the current study following dermal application in volunteers together with calculated toxicokinetic parameters can be compared to the ones described in Berthet *et al.* (2011a) following an oral administration in the same volunteers. The apparent elimination half-life of THPI calculated from the terminal elimination phase along with the plasma clearance indicate a somewhat slower elimination of THPI from plasma following dermal application than after ingestion (average  $t_{1/2}$  of 24.7 and 15.7 h, respectively; mean CL of 0.24 and 0.18 l h<sup>-1</sup>, respectively). THPI was also found to have a 10-fold lower area under the curve following dermal application than oral exposure (average AUC of 137.3 and 1650 (nmol x h l<sup>-1</sup>) kg<sup>-1</sup>), respectively. As compared to ingestion, dermal exposure is thus associated with a lower absorption fraction and slower absorption rate, the latter influencing the elimination rate of THPI from plasma.

Similarly, the current time courses of PI in plasma of volunteers and associated toxicokinetic parameters can also be compared to the oral time courses in the same volunteers described in Berthet *et al.* (2011a). The apparent elimination half-life and plasma clearance rate of PI were similar for both routes of exposure, showing a negligible effect of the dermal absorption rate on elimination rate of PI from plasma (average  $t_{1/2}$  of 29.7 and 31.5 h, respectively; mean CL of 0.13 and 0.09 l h<sup>-1</sup>,

respectively). On the other hand, PI was found to have a slightly higher area under the curve following dermal application than oral exposure (average AUC of 26.1 and 13.4 (nmol x h l<sup>-1</sup>) kg<sup>-1</sup>), respectively; this is compatible with a proportionally more extensive site-of-entry metabolism of PI into its derivatives following oral administration.

The urinary time course of THPI observed in this study following dermal application in volunteers can further be compared to the one reported in Berthet *et al.* (2011a) following an oral administration, again in the same volunteers. In accordance with the plasma profiles, the apparent elimination half-life of THPI calculated from urinary excretion rate time courses indicates a slightly slower elimination of THPI following dermal application than ingestion (average t<sub>1/2</sub> of 18.7 and 11.7 h, respectively). The percentage of captan dose recovered in urine as THPI was also 175 times lower following dermal exposure than after oral administration (on average 0.02 versus 3.5%), indicating a very low dermal absorption fraction.

Similarly, the dermal time courses of folpet metabolites in urine can be compared to the ones reported in Berthet *et al.* (2011a) following oral exposure in the same volunteers. In accordance with the plasma profiles, the apparent elimination half-lives of PI and phthalic acid were similar for both routes of exposure, showing a negligible effect of the dermal absorption rate on elimination rate of PI and phthalic acid (average t<sub>1/2</sub> of 28.8 and 27.3 h, respectively for PI, and 29.6 and 27.6 h for phthalic acid, respectively). On the other hand, the percentage of folpet dose recovered in urine as PI and phthalic acid was 10- and 14-fold lower, respectively, following dermal exposure than after oral

administration (on average 0.002 versus 0.02% for PI and 1.8 versus 25% for phthalic acid), indicating a low dermal absorption fraction.

Animal studies also confirm a lower absorption fraction of captan and folpet following dermal exposure as compared to an oral exposure. Indeed, as mentioned previously, Fisher *et al.* (1992) estimated a dermal absorption fraction of on average 10.8 to 11.5% in Fisher rats based on the time course in skin and excreta over a 120-h period following a dermal dose of 286 nmol cm<sup>-2</sup>. As for Grissom *et al.* (1985), they estimated a dermal penetration of 7.8% in Dublin ICR mice dermally exposed to 1 mg kg<sup>-1</sup> cm<sup>-2</sup> of <sup>14</sup>C-captan, as assessed from analysis of radioactivity in skin, tissues and excreta (urine, feces and exhaled air) over a 24-h period post-dosing. Moreover, again as mentioned previously, on average 12, 3 and 1% of folpet were absorbed through the skin over a 72-h period following a dermal application of 0.1, 0.5 and 2.7 μmol cm<sup>-2</sup> of radiolabelled folpet in Fisher rats, respectively (Shah *et al.*, 1987). By comparison, following oral exposure, Trivedi (1990) observed that about 75% of a 10 mg kg<sup>-1</sup> dose of <sup>14</sup>C-ring labelled captan in male Sprague-Dawley rats were recovered in urine as <sup>14</sup>C-equivalents over the 24-h period post-dosing as compared to 6.5% in feces. Similarly, following an oral administration of 10 mg kg<sup>-1</sup> of <sup>14</sup>C-labelled folpet in rats, Chasseaud *et al.* (1991) also showed that 92% of the dose were recovered in urine as <sup>14</sup>C equivalents as compared to 6% in feces.



**Potential use of ring-metabolites of captan and folpet as biomarkers of exposure in workers on the basis of the current dermal kinetic data**

The current dermal kinetic data in volunteers can serve to establish the potential use of ring-metabolites of captan and folpet as biomarkers of exposure in workers. Although THPI and PI in urine represented only a small fraction of the applied dose, their kinetics could easily be established in exposed volunteers. Given these data and the fact that THPI and PI are metabolites specific to captan and folpet, respectively, as well as the availability of simple and sensitive analytical methods for their detection, their use as bioindicators of exposure to captan and folpet should be further considered in worker biomonitoring studies. The measured phthalic acid is less specific since it is also a phthalate metabolite (Blount *et al.*, 2000; Vermeulen *et al.*, 2001; Kato *et al.*, 2005; Lim *et al.*, 2007, Silva *et al.*, 2007). However, it is a major biomarker of folpet and its measurement could usefully serve in combination with that of PI to assess worker exposure.

It should be mentioned that the ring-metabolites are to be used as bioindicators of exposure and thus to reflect absorbed doses of captan or folpet. These biomarkers are not implicated in the pathways leading to toxicity. Eventual assessment of metabolites derived from the thiophosgene moiety (DeBaun *et al.*, 1974) may help link biomarkers of exposure to potential health outcomes.

As compared to current results, some differences in the dermal absorption fraction and rate may be observed in the context of a dermal exposure in workers, given the use of acetone as a vehicle for dose administration in this study, which enhances permeability of the skin and thus absorption (Tsai *et al.*, 2001). Some differences in the absorption could also occur in workers depending on the anatomical site exposed (Feldmann and Maibach, 1974). However, the forearm was selected in the present study as the application site, since hands and arms are the anatomical regions most exposed in workers (Mcjilton *et al.*, 1983; Zweig *et al.*, 1985; de Cock *et al.*, 1998; Hughes *et al.*, 2006; Ngo *et al.*, 2010).

Overall, the present study provided new data of the dermal kinetics of key biomarkers of exposure to captan and folpet. It highlighted distinctions in the kinetics of captan and folpet phthalimide derivatives, but also route-to-route differences. However, THPI and PI appeared as convenient biomarkers to quantify captan or folpet exposure. As for phthalic acid, even if it is not a metabolite specific to folpet, it is a derivative of PI that can be used to corroborate results obtained for PI.

## **Acknowledgements**

Authors wish to thank Dr. Michèle Berode and the technicians P. Stefan, M. Nobel and C. Kohler (Institute for Work and Health) for their help in organizing the study. We also thank Dr. Khanh Huynh (Institute for Work and Health) for his help with the LC/MS/APCI system. The study was funded by the ANSES (Agence Nationale de Sécurité Sanitaire de l'alimentation, de l'environnement et du Travail) and Aurélie Berthet received a Ph.D. scholarship from the *Institut de recherche Robert-Sauvé en santé et sécurité du travail du Québec* for this research.

## REFERENCES

Ackermann H, Faust H, Kagan YS, Voronina VH. 1978. Metabolic and Toxic Behaviors of Phthalimide Derivatives in Albino-Rat .2. Placental Passage of Chloromethyl Phthalimide, Oxymethyl Phthalimide, and Phthalimide - Their Fetal Metabolism. *Arch. Toxicol.* **40**: 255-261.

Baldi I, Lebailly P, Jean S, Rougetet L, Dulaurent S, Marquet P. 2006. Pesticide contamination of workers in vineyards in France. *J. Expo. Sci. Environ. Epidemiol.* **16**: 115-124. DOI: 10.1038/sj.jea.7500443.

Berthet A, Bouchard M, Vernez D. 2011a. Toxicokinetics of captan and folpet biomarkers in orally exposed volunteers. *In press*. DOI: 10.1002/jat.1653.

Berthet A, Bouchard M, Schüpfer P, Vernez D, Danuser B, Huynh C K. 2011b. A liquid chromatography - tandem mass spectrometry (LC/APCI-MS/MS) method for the quantification of captan and folpet phthalimide metabolites in human plasma and urine. *Anal. Bioanal. Chem.* **339**: 2243-2255. DOI: 10.1007/s00216-010-4601-z.

Berthet A, Berode M, Bouchard M. 2011c. Development and validation of a chemical hydrolysis method and GC-MS determination for phthalamic and phthalic acids in urine samples. Application to the assessment of Folpet exposure. *In press*.

Blount BC, Milgram KE, Silva MJ, Malek NA, Reidy JA, Needham LL, Brock JW. 2000. Quantitative detection of eight phthalate metabolites in human urine using HPLC-APCI-MS/MS. *Anal. Chem.* **72**: 4127-4134.

Canal-Raffin M, Receveur M, Martinez B, Titier K, Ohayon C, Baldi I, Molimard M, Moore N, Brochard P. 2008. Quantification methods of folpet degradation products in plasma with HPLC-UV/DAD: Application to an in vivo toxicokinetic study in rats. *J.*

*Chromatogr. B-Anal. Technol. Biomed. Life Sci.* **865**: 106-113. DOI: 10.1016/j.jchromb.2008.02.011.

Carden A, Yost MG, Fenske RA. 2005. Noninvasive method for the assessment of dermal uptake of pesticides using attenuated total reflectance infrared spectroscopy. *Appl. Spectrosc.* **59**: 293-299.

Chasseaud L, Wood S G, Cheng K, Hall M, Fitzpatrick K, Iqbal S, Barlett A. Metabolic fate of <sup>14</sup>C-folpet in Sprague-Dawley rats. Huntingdon Research Centre Ltd.: Huntingdon. Unpublished Report No HRC/ MBS 41-91499.

Curdy C, Naik A, Kalia YN, Alberti I, Guy RH. 2004. Non-invasive assessment of the effect of formulation excipients on stratum corneum barrier function in vivo. *Int. J. Pharm.* **271**: 251-256.

de Cock J, Heederik D, Kromhout H, Boleij JS M, Hoek F, Wegh H, Ny ET. 1998. Exposure to captan in fruit growing. *Am. Ind. Hyg. Assoc. J.* **59**: 158-165.

de Cock J, Heederik D, Hoek F, Boleij J, Kromhout H. 1995. Urinary excretion of tetrahydrophthalimide in fruit growers with dermal exposure to captan. *Am. J. Ind. Med.* **28**: 245-256.

DeBaun JR, Miaullis JB, Knarr J, Mihailovski A, Menn JJ. 1974. The fate of N-trichloro(14C)methylthio-4-cyclohexene-1,2-dicarboximide((14C)captan) in the rat. *Xenobiotica* **4**: 101-119. DOI: 10.3109/00498257409049350.

Feldmann RJ, Maibach HI. 1974. Percutaneous penetration of some pesticides and herbicides in man. *Toxicol. Appl. Pharmacol.* **28**: 126-132.

Fenske RA. 1990. Nonuniform dermal deposition patterns during occupational exposure to pesticides. *Arch. Environ. Contam. Toxicol.* **19**: 332-337.

Fenske RA, Schuller C, Lu C, Allen EH. 1998. Incomplete removal of the pesticide captan from skin by standard handwash exposure assessment procedures. *Bull. Environ. Contam. Toxicol.* **61**: 194-201.

Fiserova-Bergerova V. 1993. Relevance of occupational skin exposure. *Ann. Occup. Hyg.* **37**: 673-685.

Fisher HL, Hall LL, Sumler MR, Shah PV. 1992. Dermal penetration of [<sup>14</sup>C]captan in young and adult rats. *J. Toxicol. Environ. Health.* **36**: 251-271.

Gordon EB. 2010. Captan and folpet. In *Handbook of pesticide toxicology*, Krieger RI (ed). Elsevier: New York; 1915-1949.

Gordon EB, Ehrlich T, Mobley S, Williams M. 2001. Measurement of the reaction between the fungicides captan or folpet and blood thiols. *Toxicol. Meth.* **11**: 209-223.

Greenburg DL, Rusiecki J, Koutros S, Dosemeci M, Patel R, Hines CJ, Hoppin JA, Alavanja MC. 2008. Cancer incidence among pesticide applicators exposed to captan in the Agricultural Health Study. *Cancer Causes Control* **19**: 1401-1407. DOI: 10.1007/s10552-008-9187-9.

Grissom RE, Brownie C, Guthrie FE. 1985. Dermal Absorption of Pesticides in Mice. *Pest. Biochem. Physiol.* **24**: 119-123.

Guo YLL, Wang BJ, Lee CC, Wang JD. 1996. Prevalence of dermatoses and skin sensitisation associated with use of pesticides in fruit farmers of southern Taiwan. *Occup. Environ. Med.* **53**: 427-431.

Hansen JD, Schneider BA, Olive BM, Bates JJ. 1978. Personnel safety and foliage residue in an orchard spray program using azinphosmethyl and captan. *Arch. Environ. Contam. Toxicol.* **7**: 63-71.

Hayes AW. 2008. Toxicokinetics. In *Principles and Methods of Toxicology*, 5<sup>th</sup> edition, Hayes AW (ed). Taylor and Francis Group: Boca Raton; 179-230.

Hines CJ, Deddens JA, Jaycox LB, Andrews RN, Striley CAF, Alavanja MCR. 2008. Captan exposure and evaluation of a pesticide exposure algorithm among orchard pesticide applicators in the agricultural health study. *Ann. Occup. Hyg.* **52**: 153-166.

Hughes EA, Zalts A, Ojeda JJ, Flores AP, Glass RC, Montserrat JM. 2006. Analytical method for assessing potential dermal exposure to captan, using whole body dosimetry, in small vegetable production units in Argentina. *Pest. Manag. Sci.* **62**: 811-818. DOI: 10.1002/ps.1232.

Juraske R, Anton A, Castells F, Huijbregts MA. 2007. Human intake fractions of pesticides via greenhouse tomato consumption: comparing model estimates with measurements for Captan. *Chemosphere* **67**: 1102-1107.

Kao J, Carver MP. 1990. Cutaneous Metabolism of Xenobiotics. *Drug Metab. Rev.* **22**: 363-410.

Kato K, Silva MJ, Needham LL, Calafat AM. 2005. Determination of 16 phthalate metabolites in urine using automated sample preparation and on-line preconcentration/high-performance liquid chromatography/tandem mass spectrometry. *Anal. Chem.* **77**: 2985-2991.

Krieger RI, Dinoff TM. 2000. Captan fungicide exposures of strawberry harvesters using THPI as a urinary biomarker. *Arch. Environ. Contam. Toxicol.* **38**: 398-403.

Krieger RI, Thongsinthusak T. 1993. Captan Metabolism in Humans Yields 2 Biomarkers, Tetrahydrophthalimide (THPI) and Thiazolidine-2-Thione-4-Carboxylic Acid (TTCA) in Urine. *Drug Chem. Toxicol.* **16**: 207-225.

Kromhout H, Vermeulen R. 2001. Temporal, personal and spatial variability in dermal exposure. *Ann. Occup. Hyg.* **45**: 257-273.

Lappin GJ, Havell LM. Captan: Biotransformation study in the rat. ICI Central Toxicology Laboratory: Macclesfield. Unpublished report.

Lavy TL, Mattice JD, Massey JH, Skulman BW. 1993. Measurements of year-long exposure to tree nursery workers using multiple pesticides. *Arch. Environ. Contam. Toxicol.* **24**: 123-144.

Lim DS, Shin BS, Yoo SD, Kim HS, Kwack SJ, Alm MY, Lee BM. 2007. Toxicokinetics of phthalic acid: The common final metabolite of phthalic acid esters in rats. *J. Toxicol. Environ. Health-Part A* **70**: 1344-1349. DOI 10.1080/15287390701432293.

Lotte C, Wester RC, Rougier A, Maibach HI. 1993. Racial differences in the in vivo percutaneous absorption of some organic compounds: a comparison between black, Caucasian and Asian subjects. *Arch. Dermatol. Res.* **284**: 456-459.

Mangelsdorf S, Otberg N, Maibach HI, Sinkgraven R, Sterry W, Lademann J. 2006. Ethnic variation in vellus hair follicle size and distribution. *Skin Pharmacol. Physiol.* **19**: 159-167. DOI: 10.1159/000093050.

Mejilton CE, Berckman GE, Deer HM. 1983. Captan Exposure in Apple Orchards. *Am. Ind. Hyg. Assoc. J.* **44**: 209-210.



Ngo MA, O'Malley M, Maibach HI. 2010. Percutaneous absorption and exposure assessment of pesticides. *J. Appl. Toxicol.* **30**: 91-114. DOI: 10.1002/jat.1505.

Poet TS, McDougal JN. 2002. Skin absorption and human risk assessment. *Chem. Biol. Interact.* **140**: 19-34.

Ritcey G, Frank R, McEwen FL, Braun HE. 1987. Captan residues on strawberries and estimates of exposure to pickers. *Bull. Environ. Contam. Toxicol.* **38**: 840-846.

Shah PV, Fisher HL, Sumler MR, Monroe RJ, Chernoff N. 1987. Comparison of the Penetration of 14 Pesticides Through the Skin of Young and Adult-Rats. *J. Toxicol. Environ. Health* **21**: 353-366.

Silva MJ, Samandar E, Preau JL Jr., Reidy JA, Needham LL, Calafat AM. 2007. Quantification of 22 phthalate metabolites in human urine. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **860**: 106-112. DOI: 10.1016/j.jchromb.2007.10.023.

Tielemans E, Louwse E, de Cock J, Brouwer D, Zielhuis G, Heederik D. 1999. Exposure to fungicides in fruit growing: Re-entry time as a predictor for dermal exposure. *Am. Ind. Hyg. Assoc. J.* **60**: 789-793.

Trivedi S. Captan: Excretion and tissue retention of a single oral dose (10 mg/kg) in the rat. ICI Central Toxicology Laboratory: Macclesfield. Unpublished report No CTL/P/2820.

Tsai JC, Sheu HM, Hung PL, Cheng CL. 2001. Effect of barrier disruption by acetone treatment on the permeability of compounds with various lipophilicities: Implications for the permeability of compromised skin. *J. Pharm. Sci.* **90**: 1242-1254.

United States Environmental Protection Agency (US EPA). Initial scientific and mini-economic review of captan. Office of Pesticide Programs, Criteria and Evaluation Division: Washington. Report No -EPA/540-1-75-012.

van Welie RT, van Duyn P, Lamme EK, Jager P, van Baar BL, Vermeulen NP. 1991. Determination of tetrahydrophthalimide and 2-thiothiazolidine-4-carboxylic acid, urinary metabolites of the fungicide captan, in rats and humans. *Int. Arch. Occup. Environ. Health* **63**: 181-186.

Verberk MM, Brouwer DH, Brouwer EJ, Bruyzeel DP, Emmen HH, van Hemmen JJ, Hooisma J, Jonkman EJ, Ruijten MW, Salle HJ. 1990. Health effects of pesticides in the flower-bulb culture in Holland. *Med. Lav.* **81**: 530-541.

Vermeulen R, Kromhout H, Bruyzeel DP, de Boer EM, Brunekreef B. 2001. Dermal exposure, handwashing, and hand dermatitis in the rubber manufacturing industry. *Epidemiology* **12**: 350-354.

Vermeulen R, Stewart P, Kromhout H. 2002. Dermal exposure assessment in occupational epidemiologic research. *Scand. J. Work Environ. Health* **28**: 371-385.

Wester RC, Maibach HI. 1983. Cutaneous pharmacokinetics: 10 steps to percutaneous absorption. *Drug Metab. Rev.* **14**: 169-205. DOI: 10.3109/03602538308991388.

Williams DT, Blanchfield BJ. 1974. Retention, excretion and metabolism of phthalic acid administered orally to the rat. *Bull. Environ. Contam. Toxicol.* **12**: 109-112.

Winterlin WL, Kilgore W W, Mourer C R, Schoen S R. 1984. Worker Reentry Studies for Captan Applied to Strawberries in California. *J. Agric. Food Chem.* **32** 664-672.

Winterlin WL, Kilgore WW, Mourer CR, Hall G, Hodapp D. 1986. Worker Reentry Into Captan-Treated Grape Fields in California. *Arch. Environ. Contam. Toxicol.* **15**: 301-311.

Wolfe NL, Zepp RG, Doster JC, Hollis RC. 1976. Captan Hydrolysis. *J. Agric. Food Chem.* **24**: 1041-1045.

Woollen BH. 1993. Biological monitoring for pesticide absorption. *Ann. Occup. Hyg.* **37**: 525-540.

Zainal H, Que Hee SS. 2003. Folpet permeation through nitrile gloves. *Appl. Occup. Environ. Hyg.* **18**: 658-668.

Zweig G, Leffingwell JT, Pependorf W. 1985. The relationship between dermal pesticide exposure by fruit harvesters and dislodgeable foliar residues. *J. Environ. Sci. Health B* **20**: 27-59.

**Table 1.** First-order elimination half-lives of THPI and PI in plasma and of THPI, PI and phthalic acid in urine following application of 10 mg kg<sup>-1</sup> of captan or folpet on the forearm of volunteers.

Metabolites	Matrices	Mean first-order elimination half-life (h) <sup>a</sup> (n = 5)	Coefficient of determination (R <sup>2</sup> )
THPI	Plasma	24.7	0.99
	Urine	18.7	0.86
PI	Plasma	29.7	0.93
	Urine	28.8	0.90
Phthalic acid	Urine	29.6	0.86

<sup>a</sup> The apparent elimination half-life ( $t_{1/2}$ ) was calculated using the equation  $t_{1/2} = 0.693 k^{-1}$ , where  $k$  is the elimination rate constant obtained from the slope of the linear terminal phase of the plasma profile (24-72 h) or urine excretion rate time course (36-96 h) for each metabolite.

**Table 2.** Toxicokinetic parameters for THPI and PI in plasma of volunteers following a dermal application of 10 mg kg<sup>-1</sup> of captan or folpet on 80 cm<sup>2</sup> of the forearm.

Model parameters <sup>a</sup>	First order toxicokinetic values			
	THPI		Phthalimide	
	Mean	SD	Mean	SD
	(n=5)		(n=4)	
AUC [(nmol x h l <sup>-1</sup> ) kg <sup>-1</sup> ]	137.3	43.38	26.01	14.36
AUMC [(nmol x h <sup>2</sup> l <sup>-1</sup> ) kg <sup>-1</sup> ]	4 772	1 929	532.2	152.5
MRT (h)	34.07	5.52	22.50	5.54
CL (l h <sup>-1</sup> )	0.24	0.07	0.13	0.06
Vd (l)	7.44	2.14	6.07	2.57

### Captions to figures

**Figure 1.** Time courses of THPI and PI in plasma of volunteers (expressed as nmol) over a 72-h period following a single application of 10 mg kg<sup>-1</sup> of captan or folpet on 80 cm<sup>2</sup> of the forearm. Each point represents mean and vertical bars are standard deviations.

**Figure 2.** Time courses of THPI, PI and phthalic acid excretion rate in urine (expressed as nmol/h) of volunteers over a 96-h period following a single application of 10 mg kg<sup>-1</sup> of captan or folpet on 80 cm<sup>2</sup> of the forearm. Each point represents mean and vertical bars are standard deviations.

**Figure 3.** Time courses of THPI, PI and phthalic acid cumulative excretion in urine (expressed as nmol) of volunteers over a 96-h period following a single application of 10 mg kg<sup>-1</sup> of captan or folpet on 80 cm<sup>2</sup> of the forearm. Each point represents mean and vertical bars are standard deviations.

Figure 1.

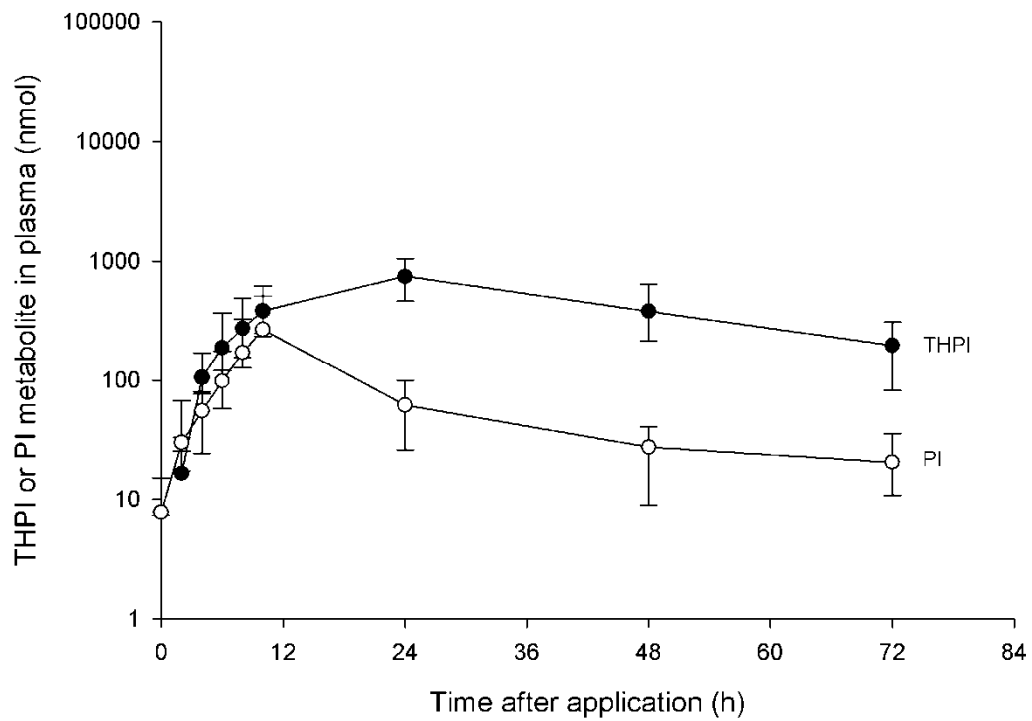


Figure 2.

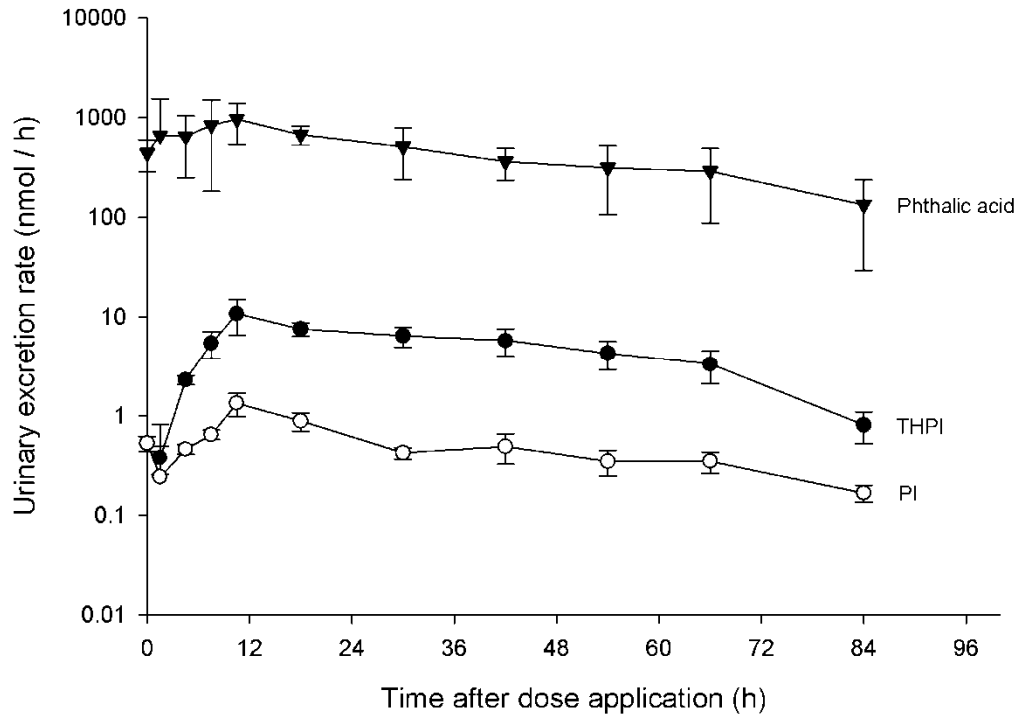
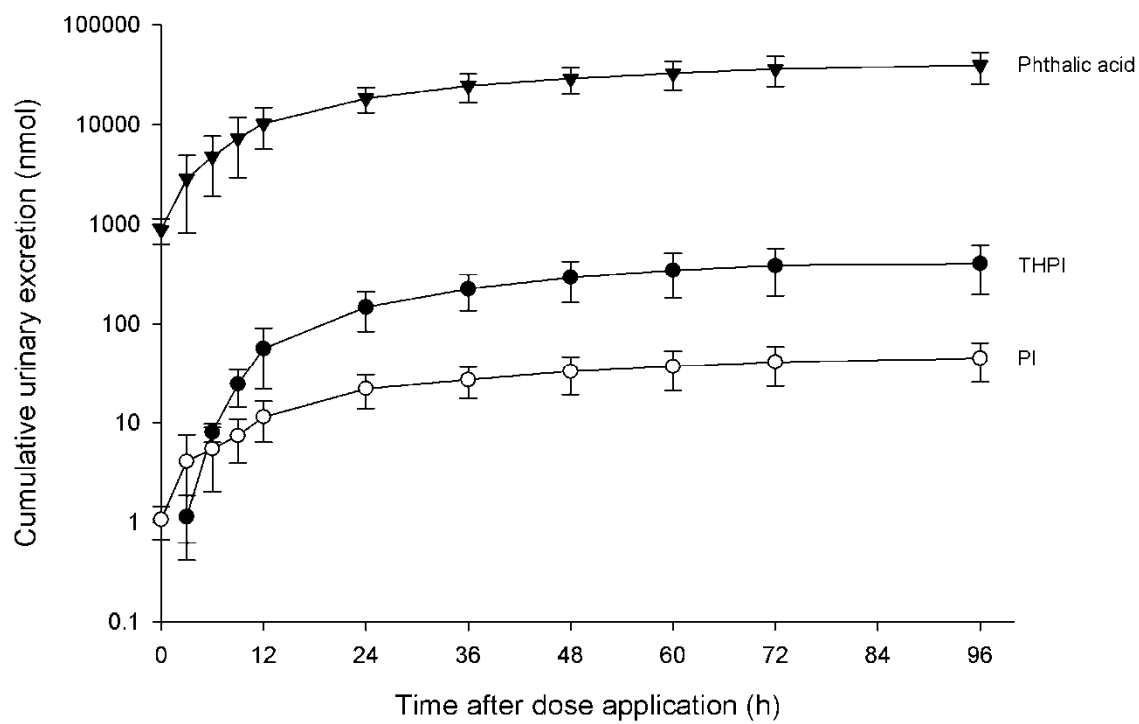
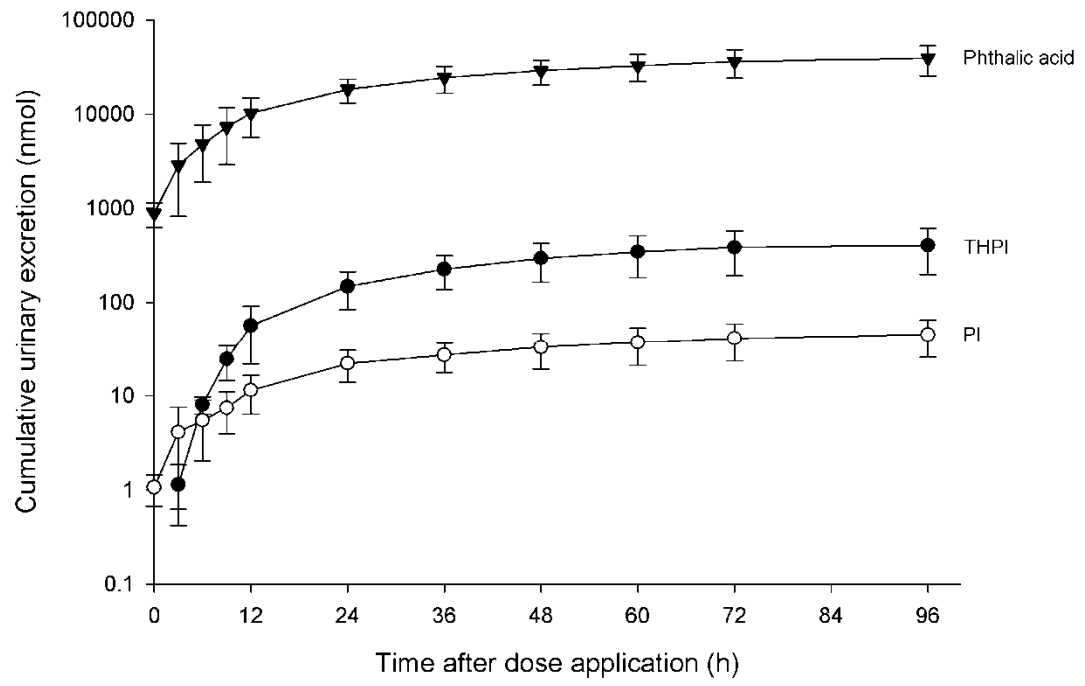




Figure 3.





## Article V

# **A Detailed Urinary Excretion Time Course Study of Captan and Folpet Biomarkers in Workers for the Estimation of Dose, Main Route-of-Entry and Most Appropriate Sampling and Analysis Strategies**

AURÉLIE BERTHET<sup>1,2</sup>, ROBERTO HEREDIA-ORTIZ<sup>1</sup>, DAVID VERNEZ<sup>2</sup>,  
BRIGITTA DANUSER<sup>2</sup> and MICHÈLE BOUCHARD<sup>1\*</sup>

<sup>1</sup> *Department of Environmental and Occupational Health, School of Public Health, Université de Montréal, P.O. Box 6128, Main Station, Montreal, Quebec, Canada, H3C 3J7;* <sup>2</sup> *Institute for Work and Health, Bugnon 21, 1011 Lausanne, Switzerland*

\*corresponding author

## ABSTRACT

Captan and folpet are two fungicides largely used in agriculture, but biomonitoring data are mostly limited to punctual measurements of urinary concentrations of captan metabolites in workers, which restricts interpretation of results in terms of internal dose estimation, daily variations according to tasks performed and most plausible routes of exposure. This study aimed at performing repeated biological measurements of exposure to captan and folpet in field workers to i) better assess internal dose along with main routes-of-entry according to tasks and ii) establish most appropriate sampling and analysis strategies. The urinary excretion time courses of specific and non-specific biomarkers of exposure to captan and folpet were established in tree farmers and winegrowers over a typical work week (7 consecutive days), including spraying and harvest activities following a re-entry delay. The effect of the units of expression of urinary measurements (excretion rate values adjusted or not for creatinine contents or cumulative amounts over given time periods (8, 12, 24 h)) was evaluated. Absorbed doses and main routes-of-entry were then estimated from the 24-h cumulative urinary amounts, through the use of a kinetic model. The time courses showed that exposure levels were higher during spraying than harvest activities (following the required re-entry delay). Model simulations also suggest a limited absorption in the studied workers and an exposure most plausibly mainly through the dermal route. It further pointed out the use of expressing biomarker values in terms of body-weight adjusted amounts in repeated timed-urine collections over the longest feasible period (i.e. 24 h in the current case) as compared to concentrations or excretion rates in spot samples, without the

necessity for creatinine corrections, as well as the advantage of performing multiple biomarkers measurements to most accurately measure worker exposure.

*Keywords:* captan; folpet; field workers; exposure assessment; biomonitoring; dose reconstruction spraying activities; re-entry activities

## INTRODUCTION

Captan (*N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide) and folpet (*N*-[Trichloromethylthio]phthalimide) are two common dicarboximide fungicides used in various crops. Captan was patented by Kittleson (1952) and first introduced in 1951, while folpet was first registered as a pesticide in 1948. Both compounds have thus been used by workers for almost 60 years, but their health effects are still controversial and mostly documented from animal toxicity studies.

The U.S. Environmental Protection Agency (1975; 1999) initially classified both fungicides as probable human carcinogens (B2) based on an increased incidence of duodenum tumors in mice chronically exposed to high doses by gavage. However, in 2004, the Agency revised the classification of captan and changed it to “not likely” considering that the doses administered to the mice were much higher than those encountered in occupational settings and induced proliferation of nascent tumors through cytotoxicity and cell hyperplasia (Gordon, 2007; US EPA, 2004). Similarly, Cohen *et al.* (2010) demonstrated in their review that folpet is not likely to be a human carcinogen for the same reasons as captan, and Greenburg *et al.* (2008) found no evidence of an increase in the incidence of cancer among applicators exposed to captan over a 9-year period. Captan has also been classified as a Group 3 carcinogen (or limited evidence of carcinogenicity in experimental animals) by the International Agency for Research on Cancer (IARC, 1987) and in Group A3 (or confirmed animal carcinogen with unknown relevance to humans) by the American Conference of Governmental

Industrial Hygienists (ACGIH, 2010). As for folpet, it is actually not listed in the index of the latter two organizations.

Even though no systemic toxicity of captan and folpet was reported in humans, both fungicides are considered as sensitizers and strong irritants of the eyes, skin and respiratory airways (ACGIH, 1991; Costa, 2008; Edwards *et al.*, 1991; Gordon, 2010; Hayes, 1982; NIOSH, 2007; Tomlin, 1997; Trochimowicz *et al.*, 1991; US EPA, 1999; US EPA, 2004). A few studies reported skin problems (i.e. allergic reactions, dermatitis) in workers exposed to captan or folpet (Burroughs and Hora, 1982; Guo *et al.*, 1996; Lisi *et al.*, 1987). Burroughs and Hora (1982) also mentioned that 48.4% of workers employed in a fungicide production plant stated having eye problems (i.e. burning, itching and tearing of eyes) and 58.1% declared respiratory problems (i.e. dry throat, sore throat, coughing, wheezing, shortness of breath, difficulty breathing) ( $n = 66$ ). As a result, occupational guidelines were proposed for captan, namely a TLV<sup>®</sup>-TWA of 5 mg/m<sup>3</sup> (ACGIH, 2010) or a Recommended Exposure Limit (REL<sup>®</sup>) of 5 mg/m<sup>3</sup> (NIOSH, 2007), but none are available to date for folpet, except the recommendation by the US EPA (1999) to wear gloves when handling the product. Therefore, risks related to occupational exposure to captan and especially folpet are not well defined.

Worker exposure and absorption may also be affected by multiple factors and conditions. For instance, in addition to frequent reported factors such as the dose, exposure duration, vehicle, skin conditions and its composition, or physicochemical characteristics of compounds, others factors such as the type of crop, meteorological

conditions, the delay of re-entry, or work habits and practices may also be important determinants of exposure and absorption (Geer *et al.*, 2004; Hughes *et al.*, 2006; Stewart *et al.*, 2001; Tielemans *et al.*, 1999; Winterlin *et al.*, 1986; Zweig *et al.*, 1985).

To identify factors or activities most likely to increase worker exposure to captan, some authors have performed environmental measurements using personal dosimeters or skin pads, while assessing the impact of wearing masks or hand washing (Burroughs and Hora, 1982; Mcjilton *et al.*, 1983; Oudbier *et al.*, 1974; Ritcey *et al.*, 1987; Stevens and Davis, 1981; Tielemans *et al.*, 1999; Zweig *et al.*, 1985). However, external exposure measurements are known to present limitations and to lead to overestimations of true absorbed doses. The best means of accurately assessing worker exposure to such type of compound is recognized to be through biological monitoring, since it allows estimating actual rather than potential absorption by workers and integrating exposure by all routes (de Cock *et al.*, 1995; He, 1993; Woollen, 1993).

Some field studies have attempted to associate environmental measurements with biomonitoring data to assess captan exposure (de Cock *et al.*, 1995; Hansen *et al.*, 1978; Hines *et al.*, 2008; Krieger and Dinoff, 2000; Lavy *et al.*, 1993; Maddy *et al.*, 1989; Winterlin *et al.*, 1984; Winterlin *et al.*, 1986), but poor correlations were obtained. These studies, as well as those of van Welie *et al.* (1991) and of Verberk *et al.* (1990) which used only biomonitoring, assessed worker exposure for a maximum three consecutive days, with incomplete collections; typical tasks involving potential exposure to captan (e.g. spraying and harvest activities) were also assessed in workers. However, according



to some authors (Ross *et al.*, 2001; Thongsinthusak *et al.*, 1999; Woollen, 1993), to accurately estimate absorption, especially through the dermal route, the optimal sampling protocol would be to collect 24-h voids for seven days, in a worker performing different tasks during a workweek and thus subjected to various exposure scenarios.

By comparison with captan, there is a paucity of data on occupational exposure to folpet, although it is also widely used in agriculture. The only available data comes from a health hazard report conducted by the NIOSH (Burroughs and Hora, 1982) to evaluate captan and folpet exposure in about 60 employees working in a fungicide production plant through environmental and medical evaluation.

There is thus a need to better assess occupational exposure to these two fungicides and this can effectively be achieved through biomonitoring. Nonetheless, such approach requires a minimum knowledge of the toxicokinetics of the compound under study, hence of major metabolites, together with a sensitive analytical method for their quantification in accessible biological matrices (Wester and Maibach, 1983; Woollen, 1993). For the biomonitoring of worker exposure to captan, tetrahydrophthalimide (THPI) was quantified in the published studies as a urinary metabolite of captan due to its stability. This was confirmed by our previous kinetic studies in volunteers orally and dermally exposed to captan in controlled conditions (Berthet *et al.*, 2010a; Berthet *et al.*, 2010b). According to the time course data of Berthet *et al.* (2010a,b), phthalimide (PI) and total ring-metabolites of folpet (expressed as phthalic acid (PA)) also proved to be two suitable biomarkers of folpet exposure.

This study thus aimed at i) better assessing agricultural worker exposure to captan and folpet through repeated biological measurements following spraying and harvest activities (internal dose and main route-of-exposure) and ii) establishing most appropriate sampling and analysis strategies.

## MATERIALS AND METHODS

### *Study design*

The detailed time profiles of key biomarkers of exposure to captan and folpet were characterized in the urine of agricultural workers subjected to different exposure scenarios, preparing/mixing/loading/spraying activities and harvest activities following the required delay of re-entry. Captan and folpet ring-metabolites were quantified in pre-seasonal urines and, for each exposure scenario, in all urines voided over seven consecutive days. From these data, the dose absorbed by workers and main route-of-entry were estimated using toxicokinetic models previously developed by our team, which allow to reconstruct absorbed doses of captan and folpet from biomarker data considering different exposure scenarios (Heredia *et al.*, 2011a,b).

The experimental protocol and consent forms were approved by the Permanent Ethics Committee for Clinical Research of the Faculty of Biology and Medicine of the University of Lausanne and the Research Ethics Committee of the Faculty of Medicine of the University of Montreal. All the participants gave their written consent, and were informed of the risks of participating and their right to withdraw from the study at anytime.

*Studied workers*

Participants were recruited on a voluntary basis among tree farmers and winegrowers living within a 100-km area from Lausanne (Switzerland). Approximately twelve workers were contacted, but only five persons accepted to participate, namely two tree farmers (exposed to captan) and three winegrowers (exposed to folpet), due to the restrictive protocol. All participants were male workers aged between 35 and 55 years of age, weighing 74 to 115 kg and measuring 178 to 192 cm. They were healthy and non-smokers, and underwent a medical examination by an occupational physician prior to enrolment.

*Urine sample collection*

Urine sample collections were conducted over seven consecutive days (or 168 h) following two different types of exposure, namely spraying activities (including preparing, mixing and loading tasks) and harvest activities following the required re-entry delay (i.e. pruning, thinning), except for one winegrower performing harvest activities who collected all his urine voided over a 72-h period. During the collection period, several spraying techniques were used by the studied workers (i.e. tractors with closed or half-opened cabins, small airblast sprayers and back air-sprayers) and sampling was conducted during the season period thought to be associated with worst exposure scenarios.

More specifically, to determine urinary baseline levels of the studied metabolites, a pre-seasonal complete first-morning void was collected for each worker; during this period, they were not occupationally exposed to captan or folpet. At the beginning of the fungicide treatment period, workers were then asked to provide all urine voided during the course of a typical workweek involving a spraying episode of captan or folpet (in general, a 168-h collection period with spraying the first sampling day). Each void was collected in separate polypropylene Nalgene<sup>®</sup> bottles of 1 l; workers were asked to indicate the date and time of urine collection on the pre-coded bottle labels.

During the high season of thinning activities and pruning of vineyards or orchards, the same workers were again asked to provide a second round of urine collection. During this period, the vegetation was dense and abundant, and workers were easily in contact with treated leaves. All urine voided during the course of a typical workweek involving harvest activities were thus collected following the required delay of re-entry (in general, a 168-h collection period with harvest activities on several days). Several weeks (at least two) separated the two exposure scenarios.

Once collected, urine samples were kept in the refrigerator and daily picked up by our team. Total urine volume per void was then measured upon arrival at the laboratory. To allow repeated analysis while avoiding possible degradation due to freezing and thawing of samples, each urine collection was then aliquoted in 4 labelled tubes of 15 ml and one bottle of 120 ml prior to storage at -20°C until analysis.

In addition, during each urinary collection period, workers were invited to complete a timesheet with the actual time of each voiding and to indicate whether or not there were any urine losses. They were also asked to fill a questionnaire to document personal factors (weight, height), information related to spraying and harvest activities (i.e. commercial product name, application days, techniques, tasks), work habits (i.e. safety equipments, decontamination tasks, hand washing), treatments (i.e. other captan/folpet treatments or other pesticides sprayed during the study period), life habits (i.e. physical activities, smoking), medication intake (including ibuprofen) and possible symptoms during workdays. Distinct questionnaires were elaborated for the two exposure scenarios and adapted to the tasks performed.

### *Sample analysis*

*THPI and PI.* THPI and PI were quantified in urine according to the method of Berthet *et al.* (2010c). In short, THPI and PI were isolated by solid phase extraction (SPE), eluted in dichloromethane and analyzed by liquid chromatography - atmospheric pressure chemical ionization-tandem mass spectrometry (LC/APCI-MS/MS), in negative ion mode. The fragments analyzed were  $m/z$  149.4/95.6 for THPI,  $m/z$  156.1/95.6 for the internal standard THPI-d, and  $m/z$  145.8 for PI (no fragmentation). The analytical limit of detection in urine was  $3.82 \text{ nmol l}^{-1}$  and  $7.72 \text{ nmol l}^{-1}$  for THPI and PI, respectively. The quantification of THPI or PI was obtained from standard calibration curves prepared in urine or plasma adjusted by the THPI-d internal standard peak area.

*Phthalic acid.* Total ring-metabolites of folpet, expressed as PA equivalents, were measured according to the method of Berthet *et al.* (2010d). Briefly, urine samples were subjected to an acid hydrolysis prior to liquid-liquid extraction with ethyl acetate and derivatization with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). Analysis was then performed using a by gas chromatography – mass spectrometry (GC-MS). The ions monitored were trimethylsilyl phthalic acid with  $m/z$  295 and the internal standard TMS methylhippuric acid with  $m/z$  220. The quantification was obtained from standard calibration curves of PA prepared in urine and adjusted by the methylhippuric acid internal standard peak height. The analytical limit of detection was 60.2 nmol l<sup>-1</sup> urine.

### *Creatinine*

Creatinine was measured in urine by an alkaline picric acid method with deproteinization, namely by the Jaffé method with deproteinization (enzymatic colorimetric test PAP from Boehringer Mannheim, Germany).

To adjust THPI, PI and PA urinary excretion rates by creatinine contents, the following equation, described by Viau *et al.* (2004), was used:

$$\left[\left(\frac{\Delta\text{metabolite}}{\Delta t}\right)\right]_{\text{Adj}} = \left[\left(\frac{\Delta\text{metabolite}}{\Delta t}\right)\right]_i \times \frac{\left[\frac{\Delta\text{Creatinine}}{\Delta t}\right]_{\text{mean}}}{\left[\frac{\Delta\text{Creatinine}}{\Delta t}\right]_i}$$

where  $\left[\left(\frac{\Delta\text{metabolite}}{\Delta t}\right)\right]_{\text{Adj}}$  is the adjusted excretion rate of the studied metabolite,

$\left[\left(\frac{\Delta\text{metabolite}}{\Delta t}\right)\right]_i$  is the excretion rate observed over a determined time interval  $i$ ,

$\left[\frac{\Delta\text{Creatinine}}{\Delta t}\right]_{\text{mean}}$  is the average creatinine excretion rate for the total study period, and

$\left[\frac{\Delta\text{Creatinine}}{\Delta t}\right]_i$  is the average creatinine excretion rate over a determined time interval  $i$ .

### *Toxicokinetic modeling*

Multi-compartment toxicokinetic models were developed to describe the time courses of captan and folpet key biomarkers in accessible biological matrices following multi-routes of exposure (Berthet *et al.*, 2010a; Berthet *et al.*, 2010b). These models were used in the current study to reconstruct the absorbed doses of these fungicides in workers from serial urinary biomarker measurements and obtain an indication of the predominant route of exposure for these workers.

Briefly, in the models, the body was represented by compartments. The rates of change in the amounts of compounds or its metabolites in the different compartments were represented by a set of linear first-order ordinary differential equations. The model was first used to reproduce the time courses of captan and folpet metabolites in blood and urine of volunteers orally and dermally exposed (Berthet *et al.*, 2010a; Berthet *et al.*, 2010b), with a minimum number of parameters. Then, kinetics of fungicides and their experimentally relevant metabolites were modeled for three different exposure routes: oral, dermal and inhalation. To describe oral exposure, the considered compartments were the parent compound and its almost instantaneously generated metabolites in the gastrointestinal tract, the body burden of experimentally relevant metabolites in blood and in tissues in dynamical equilibrium with blood, the other non-monitored ring-



metabolites and thiol metabolites, and the different excretion compartments to represent the cumulative amounts of each urinary and fecal metabolite, both monitored and non-monitored. To simulate dermal exposure, the epidermis and dermis were represented by distinct compartments and a compartment for captan or folpet in blood and tissues in equilibrium with blood was also represented. Lastly, inhalation exposure was modeled with direct inputs to the blood compartment due to the rapid absorption of both fungicides through the respiratory tract. All amounts in models were initially expressed on a molar basis (see Figure 1 for model representation and parameter values).

## RESULTS

### *Worker exposure*

Table 1 summarizes the characteristics, exposure conditions and activities of the workers under study. In the case of workers exposed to captan, field spraying was conducted using tractors with a cabin; they did not wear masks or coveralls during spraying or harvest activities, and only one wore gloves during preparation, mixing, loading and cleaning tasks, but not during harvest activities. In the case of workers exposed to folpet, they tended to protect themselves better since all wore masks during preparation and spraying activities as well as gloves and pants during harvest activities. This increased protection was probably due to the fact they used airblast sprayers or back air-sprayers to apply folpet and were thus more likely to be in contact with the applied fungicide. In fact, two workers reported eye irritations following folpet spraying and one following harvest activities also, while no symptoms were mentioned by workers exposed to captan.

To assess the importance of exposure due to spraying and harvest activities considering the previously mentioned exposure conditions, THPI was measured in urine as a biomarker of exposure to captan while PI and PA were quantified to assess folpet exposure. Figure 2 depicts the urinary time profiles of THPI in the two studied workers exposed to captan over a 7-day period following spraying or harvesting; Figure 3 presents corresponding time profiles for PI and PA biomarkers of exposure to folpet in

the three studied workers. Exposure to captan was found to be higher during spraying than harvest activities following the required delay of re-entry. This is particularly apparent for worker 2 since he was barely exposed during harvest activities, with values close to pre-seasonal values. Worker 2 appeared more exposed than worker 1 over the spraying period, but he did not wear gloves during work and he manipulated larger amounts of captan given that he had to treat a broader area. Similarly, workers seemed more exposed to folpet during spraying than harvest activities. This is especially visible from the time profiles of PI and PA in worker 2. However, differences in excretion values between both activities were less noticeable than for captan. As presented in Table 1, workers exposed to folpet were better protected during spraying activities contrary to workers exposed to captan.

From Figure 3, the urinary time courses of PI and PA in workers following spraying and harvesting can also be compared. As expected, similar profiles were obtained for both biomarkers, except for worker 1 during spraying period, possibly due to a concomitant marked exposure to phthalates. For comparison purposes, the time course of PA was assessed in workers exposed to captan during spraying period, as depicted in Figure 4. This allowed pointing out a substantial baseline level of PA in workers due to an exposure other than folpet.

*Creatinine adjustments and timed collections*

Figures 5 and 6 shows the impact of creatinine adjustment on the urinary excretion time course of THPI, PI and PA along with profile variations when expressing urinary results in terms of spot or pooled measurements over 8, 12 or 24-h periods. Results show that creatinine adjustment had little effect on the time courses of all three biomarkers in spot or pooled samples, as non-adjusted and creatinine-adjusted rate profiles were found to quantitatively evolve in a similar manner. This was even more evident with pooled urines, especially 24-h urine collections. Figures 5 and 6 also show that excretion rate profiles were less variable when urines were pooled over the longest period of time, hence 24 h. In contrast with 24-h collections, it was also less obvious to infer on the main route-of-exposure from punctual urines due to the shifty variations between some data points.

*Exposure route simulations*

The models were used to reproduce the time courses of THPI, PI and PA metabolites in workers for both spraying and harvest scenarios, considering the various possible absorption routes (inhalation, dermal or oral) (see Figure 7 for an example). Simulations of a dermal exposure scenario for both captan and folpet, during spraying period as well as harvest activities, provided the closest description of the observed time courses as compared to oral and inhalation scenarios. However, contrary to workers exposed to captan, it was less obvious from PI and PA time courses that dermal absorption was the

predominant exposure route for workers exposed to folpet. Indeed, given the rapid absorption of folpet following oral, dermal and inhalation routes, time courses of PI and PA were comparable whatever the simulated exposure scenarios.

## DISCUSSION

Results of the present study show notable variations in captan and folpet biomarker levels according to field tasks, hence spraying or harvest activities. Through biomonitoring, it was thus evidenced that workers were more exposed during application than re-entry activities. Model simulations of urinary time course data considering various exposure route scenarios further indicated that captan and most plausibly also folpet were mainly absorbed through the skin following both spraying and harvesting. In addition, collections of complete voids over seven consecutive days allowed confirming that measurements of biomarkers in 24-h collections without creatinine normalization provided the most reliable assessment of worker exposure to captan and folpet. The results also highlight the usefulness of multiple biomarker measurements (PI and PA) to estimate folpet exposure in workers.

### *Comparison of exposure levels between spraying and harvest activities*

According to biomonitoring results in the studied workers (Figures 2 and 3), exposure to captan and folpet were more important during spraying period than harvest activities, although exposure was of shorter duration and less frequent, and workers were more protected (Table 1). This is probably due mostly to mixing and loading prior to spraying as suggested by de Cock *et al.* (1998a). The same observations were reported in studies assessing captan exposure through spot measurements (de Cock *et al.*, 1998a; Geer *et al.*, 2004; Krieger, 1995; Tielemans *et al.*, 1999; Winterlin *et al.*, 1986).

Thus, harvesting activities following a re-entry in treated fields resulted in limited dermal absorption according to biomonitoring results in the studied workers (Figures 2 and 3), even though half-life of captan on leaf surfaces was estimated to be between 2.5 to 24 days (US EPA, 2004). Indeed, de Cock *et al.* (1998a) reported a captan half-life on treated vegetation of 10 to 17 days, Winterlin *et al.* (1984) and Tielemans *et al.* (1999) of 5 to 11 days, and Stamper *et al.* (1987) and Phalen and Que Hee (2003) of minimum 5 days. For folpet, Cabras *et al.* (2000) estimated a half-life on grapes of 8.9 days. In addition, some studies (Alary *et al.*, 1995; Cabras *et al.*, 1997; el-Zemaity, 1988; Frank *et al.*, 1983) confirmed the persistence of captan and folpet residues on fruits (Cabras *et al.*, 2000). For folpet, however, results of the current study do not exclude the possibility that workers could be also exposed by oral or inhalation routes.

Although workers of the current study were more exposed during spraying than harvesting, urinary excretion values of THPI were in general lower than those reported in the literature and summarized in Table 2. For applicators exposed to captan, our mean 24-h excretion values following the beginning of treatment period were equivalent to those obtained by Hines *et al.* (2008), but lower than most of the other available studies. Likewise, our workers exposed to captan during harvesting exhibited lower THPI concentrations than those of other published studies (Krieger and Dinoff, 2000; Winterlin *et al.*, 1984; Winterlin *et al.*, 1986). In the other studies, larger amounts of captan were however applied and a wider treatment area was covered compared to the current study.

In addition, urinary THPI concentrations in captan workers were lower than maximum values observed in a controlled kinetic time course study in volunteers dermally applied  $10 \text{ mg kg}^{-1}$  of captan on  $80 \text{ cm}^2$  of forearm during 24 h (Berthet *et al.*, 2010b) (maximum concentration obtained for both workers of  $44.9 \text{ nmol l}^{-1}$  compared to average maximum concentration for volunteers of  $180 \text{ nmol l}^{-1}$ ). On the other hand, urinary PI and PA concentrations in two of the three studied folpet workers (workers 2 and 3) reached maximum values similar those observed in a kinetic time course study in volunteers dermally exposed to folpet (i.e. maximum of  $18.3 \text{ nmol l}^{-1}$  for PI in workers compared to  $17.7 \text{ nmol l}^{-1}$  in volunteers, and  $13.6 \text{ nmol ml}^{-1}$  for PA in workers compared to  $13.9 \text{ nmol ml}^{-1}$  in volunteers), but maximum values in the third worker (worker 1) were rather similar to those observed in a time course study in volunteers orally exposed to  $1 \text{ mg kg}^{-1}$  of folpet (i.e. maximum of  $26.91 \text{ nmol l}^{-1}$  for PI in workers compared to  $22.7 \text{ nmol l}^{-1}$  in volunteers, and  $19.92 \text{ nmol ml}^{-1}$  for PA in workers compared to  $21.4 \text{ nmol ml}^{-1}$  in volunteers).

As for the major route of exposure, in line with current results (see model simulations in Figure 7), dermal absorption was reported as the primary route-of-entry for both mixers/loaders/applicators and re-entry workers in contact with pesticides (Geer *et al.*, 2004; Gunther *et al.*, 1977; Ritcey *et al.*, 1987; Ross *et al.*, 2001; Thongsinthusak *et al.*, 1999). In particular, de Cock *et al.* (1995) and Hansen *et al.* (1978) found that respiratory exposure route to captan was minor compared to dermal absorption.



*Parameters influencing exposure assessment through biomonitoring*

Biomonitoring in field workers allows estimating doses truly absorbed in workers whatever the exposure scenario (Woollen, 1993). However, depending on tasks and activities, workers are not exposed constantly or equally during a workday or a week. When feasible, it is thus preferable to obtain complete daily collections over several days to assess most accurately worker exposure, as suggested by some authors (Ross *et al.*, 2001; Thongsinthusak *et al.*, 1999; Woollen, 1993), instead of spot urine samples. This was also particularly evident from our results, showing that an overestimation or underestimation of exposure may be induced with punctual urines, as illustrated in Figure 4, since there are significant void-to-void variations in metabolite concentrations and urinary volumes (Spencer *et al.*, 1995; Woollen, 1993). With combined 8-h urine collections, time profiles were better defined for the three studied metabolites, but it was the daily (24-h) variations in biomarker levels which allowed to reproduce most closely the time course in workers using the toxicokinetic models previously developed from data in volunteers exposed under controlled conditions (Berthet *et al.*, 2010a, b).

Creatinine normalization of metabolite excretion rates, as proposed by Viau *et al.* (2004), also appeared unnecessary in this study since adjusted values were close to non-adjusted values, especially in 24-h urine collections (Figures 5 and 6). Consequently, when feasible, using complete 24-h voids over a week, including days off, appears to be the most reliable procedure to estimate worker exposure to captan and particularly folpet, given the paucity of available biomonitoring data.

Comparison of the time courses of PI and PA metabolites of folpet also highlighted the relevance of multiple biomarker measurements to assess more accurately exposure in field work, especially for metabolites non specific to the studied compound such as PA. Although baseline levels of PA were relatively high, due to the fact that it is also a phthalate metabolite, all urinary profiles were in concordance with those of PI, except for one worker (worker 1) over the spraying period.

In summary, the present biomonitoring study used detailed repeated-measurements along with kinetic modeling tools to better assess worker exposure to captan and folpet and main route-of-entry. Despite the limited number of participants, sufficient data were obtained to confirm punctual results reported in the literature for captan and to provide new data on folpet exposure. However, more investigations are needed to further document exposure to folpet in workers and confirm main absorption route.

## **FUNDING**

Agence Nationale de Sécurité Sanitaire de l'alimentation, de l'environnement et du Travail (ANSES).

*Acknowledgements*– Authors wish to thank the five workers to their participation and their attention to respect the protocol as well as Dr. Michèle Berode, Gregory Plateel and the technicians P. Stefan, M. Nobel, C. Kohler and P. Storti (Institute for Work and Health) for their help in analytical analysis. We also thank Dr. Khanh Huynh (Institute for Work and Health) for his help with the LC/MS/APCI system. Aurélie Berthet received a Ph.D. scholarship from the *Institut de recherche Robert-Sauvé en santé et sécurité du travail du Québec* for this research.

## REFERENCES

- Alary J, Bescos D, Monge MC, Debrauwer L, Bories GF. (1995) Laboratory simulation of captan residues degradation during apple processing. *Food Chemistry*; 54 205-211.
- American Conference of Governmental Industrial Hygienists. (1991). Documentation of the threshold limit values and biological exposure indices, 6<sup>th</sup> edition. Volumes I, II, III. Cincinnati, Ohio. American Conference of Governmental Industrial Hygienists.
- American Conference of Governmental Industrial Hygienists. (2010). TLVs® and BEIs®. Based on Documentation of the Threshold Limit Values for chemical substances and physical agents and Biological Exposure Indices, 7th edition. Cincinnati, Ohio. American Conference of Governmental Industrial Hygienists.
- Berthet A, Bouchard M, Danuser B. (2010a) Toxicokinetics of captan and folpet biomarkers in orally exposed volunteers. *J Appl Toxicol*; ID 795151-668048.
- Berthet A, Bouchard M, Vernez D. (2010b) Toxicokinetics of captan and folpet biomarkers in dermally exposed volunteers. *J Appl Toxicol*; In Press.
- Berthet A, Bouchard M, Schüpfer P, Vernez D, Danuser B, Huynh CK. (2010c) A liquid chromatography - tandem mass spectrometry (LC/APCI-MS/MS) method for the quantification of captan and folpet phthalimide metabolites in human plasma and urine. *Anal Bioanal Chem*; In Press. DOI 10.1007/s00216-010-4601-z.
- Berthet A, Berode M, Bouchard M. (2010d) Gas-chromatography mass-spectrometry determination of phtalic acid in human urineas a biomarker of folpet exposure. *Anal Bioanal Chem*; In Press.
- Burroughs, G. E. and Hora, J. (1982). HETA-80-147-1076. Health Hazard Evaluation - Calhio Chemical Company, Perry, Ohio. Cincinnati, Ohio. National Institute for Occupational Safety and Health.

Cabras P, Angioni A, Caboni P, Garau VL, Melis M, Pirisi FM, Cabitza F. (2000) Distribution of folpet on the grape surface after treatment. *J Agric Food Chem*; 48 915-916.

Cabras P, Angioni A, Garau VL, Melis M, Pirisi FM, Farris GA, Sotgiu C, Minelli EV. (1997) Persistence and metabolism of folpet in grapes and wine. *J Agric Food Chem*; 45 476-479.

Cohen SM, Gordon EB, Singh P, Arce GT, Nyska A. (2010) Carcinogenic mode of action of folpet in mice and evaluation of its relevance to humans. *Critical Rev Toxicol*; 40 531-545.

Costa LG. (2008) Toxic effects of pesticides: the basic science of poisons. In Casarett LJ, Doull J, Klaassen CD, editors. *Casarett and Doull's Toxicology: the Basic Science of Poisons*. McGraw-Hill: New York. p. 883-930. ISBN 978 0 07 147051 3.

de Cock J, Heederik D, Hoek F, Boleij J, Kromhout H. (1995) Urinary excretion of tetrahydrophthalimide in fruit growers with dermal exposure to captan. *Am J Ind Med*; 28 245-256.

de Cock J, Heederik D, Kromhout H, Boleij JSM, Hoek F, Wegh H, Ny ET. (1998a) Exposure to captan in fruit growing. *Am Ind Hyg Assoc J*; 59 158-165.

de Cock J, Heederik D, Kromhout H, Boleij JSM, Hoek F, Wegh H, Ny ET. (1998b) Determinants of exposure to captan in fruit growing. *Am Ind Hyg Assoc J*; 59 166-172.

Edwards R, Ferry DG, Templ WA. (1991) Fungicides and related compounds. In Hayes WJ, Laws ER, editors. *Handbook of Pesticide Toxicology*. Academic Press: San Diego, CA. p. 1409-1470. ISBN 978 0 12 334160 0.

el-Zemaity MS. (1988) Residues of captan and folpet on greenhouse tomatoes with emphasis on the effect of storing, washing, and cooking on their removal. *Bull Environ Contam Toxicol*; 40 74-79.

Frank R, Braun HE, Stanek J. (1983) Removal of Captan from Treated Apples. *Arch Environ Contam Toxicol*; 12 265-269.

Geer LA, Cardello N, Dellarco MJ, Leighton TJ, Zendzian RP, Roberts JD, Buckley TJ. (2004) Comparative analysis of passive dosimetry and biomonitoring for assessing chlorpyrifos exposure in pesticide workers. *Ann Occup Hyg*; 48 683-695.

Gordon E. (2007) Captan: transition from 'B2' to 'not likely'. How pesticide registrants affected the EPA Cancer Classification Update. *J Appl Toxicol*; 27 519-526.

Gordon EB. (2010) Captan and folpet. In Krieger RI, editor. *Handbook of pesticide toxicology*. Elsevier: New York. p. 1915-1949. ISBN 978 0 12 374367 1.

Greenburg DL, Rusiecki J, Koutros S, Dosemeci M, Patel R, Hines CJ, Hoppin JA, Alavanja MC. (2008) Cancer incidence among pesticide applicators exposed to captan in the Agricultural Health Study. *Cancer Causes Control*; 19 1401-1407.

Gunther FA, Iwata Y, Carman GE, Smith CA. (1977) The citrus reentry problem: research on its causes and effects, and approaches to its minimization. *Residue Rev*; 67 1-132.

Guo YLL, Wang BJ, Lee CC, Wang JD. (1996) Prevalence of dermatoses and skin sensitisation associated with use of pesticides in fruit farmers of southern Taiwan. *Occup Environ Med*; 53 427-431.

Hansen JD, Schneider BA, Olive BM, Bates JJ. (1978) Personnel safety and foliage residue in an orchard spray program using azinphosmethyl and captan. *Arch Environ Contam Toxicol*; 7 63-71.

Hayes WJ (1982) Fungicides and related compounds. In Hayes WJ, editor. *Pesticides studied in man*. Williams and Wilkins: Baltimore. p. 578-622. ISBN 978 0 68 303896 5.

He F. (1993) Biological monitoring of occupational pesticides exposure. *Int Arch Occup Environ Health*; 65 S69-S76.

Hines CJ, Deddens JA, Jaycox LB, Andrews RN, Striley CAF, Alavanja MCR. (2008) Captan exposure and evaluation of a pesticide exposure algorithm among orchard pesticide applicators in the agricultural health study. *Ann Occup Hyg*; 52 153-166.

Hughes EA, Zalts A, Ojeda JJ, Flores AP, Glass RC, Montserrat JM. (2006) Analytical method for assessing potential dermal exposure to captan, using whole body dosimetry, in small vegetable production units in Argentina. *Pest Manag Sci*; 62 811-818.

International Agency for Research on Cancer. (1987). Captan. Overall evaluations of carcinogenicity: An updating of IARC Monographs. Volumes 1 to 42, [Supplement 7]. Lyon, France. World Health Organization.

Kittleson AR. (1952) A New Class of Organic Fungicides. *Science*; 115 84-86.

Krieger RI. (1995) Pesticide exposure assessment. *Toxicol Lett*; 82-83 65-72.

Krieger RI, Dinoff TM. (2000) Captan fungicide exposures of strawberry harvesters using THPI as a urinary biomarker. *Arch Environ Contam Toxicol*; 38 398-403.

Lavy TL, Mattice JD, Massey JH, Skulman BW. (1993) Measurements of year-long exposure to tree nursery workers using multiple pesticides. *Arch Environ Contam Toxicol*; 24 123-144.

Lisi P, Caraffini S, Assalve D. (1987) Irritation and Sensitization Potential of Pesticides. *Contact Dermatitis*; 17 212-218.

Maddy KT, Krieger RI, Oconnell L, Bisbiglia M, Margetich S. (1989) Use of Biological Monitoring Data from Pesticide Users in Making Pesticide Regulatory Decisions in California - Study of Captan Exposure of Strawberry Pickers. *Acs Symposium Series*; 382 338-353.

Mejilton CE, Berckman GE, Deer HM. (1983) Captan Exposure in Apple Orchards. *Am Ind Hyg Assoc J*; 44 209-210.



National Institute for Occupational Safety and Health. (2007). DHHS Publication No 2005-149, -454: 2007. NIOSH pocket guide to chemical hazards. Cincinnati, Ohio. National Institute for Occupational Safety and Health.

Oudbier AJ, Bloomer AW, Price HA, Welch RL. (1974) Respiratory Route of Pesticide Exposure As A Potential Health Hazard. *Bull Environ Contam Toxicol*; 12 1-9.

Phalen RN, Que Hee SS. (2003) Permeation of captan through disposable nitrile glove. *J Hazard Mater*; 100 95-107.

Ritcey G, Frank R, McEwen FL, Braun HE. (1987) Captan residues on strawberries and estimates of exposure to pickers. *Bull Environ Contam Toxicol*; 38 840-846.

Ross JH, Driver JH, Cochran RC, Thongsinthusak T, Krieger RI. (2001) Could pesticide toxicology studies be more relevant to occupational risk assessment? *Ann Occup Hyg*; 45 S5-S17.

Spencer JR, Sanborn JR, Hernandez BZ, Krieger RI, Margetich SS, Schneider FA. (1995) Long vs. short monitoring intervals for peach harvesters exposed to foliar azinphos-methyl residues. *Toxicol Lett*; 78 17-24.

Stamper JH, Nigg HN, Queen RM. (1987) Dislodgeable Captan Residues at Florida Strawberry Farms. *Chemosphere*; 16 1257-1271.

Stevens ER, Davis JE. (1981) Potential Exposure of Workers During Seed Potato Treatment with Captan. *Bull Environ Contam Toxicol*; 26 681-688.

Stewart PA, Prince JK, Colt JS, Ward MH. (2001) A method for assessing occupational pesticide exposures of farmworkers. *Am J Ind Med*; 40 561-570.

Thongsinthusak T, Ross JH, Saiz SG, Krieger RI. (1999) Estimation of dermal absorption using the exponential saturation model. *Regul Toxicol Pharmacol*; 29 37-43.

Tielemans E, Louwense E, de Cock J, Brouwer D, Zielhuis G, Heederik D. (1999) Exposure to fungicides in fruit growing: Re entry time as a predictor for dermal exposure. *Am Ind Hyg Assoc J*; 60 789-793.

Tomlin C. (1997) *The Pesticide Manual - World Compendium*, 10<sup>th</sup> edition. British Crop Protection Council: Thornton Heath. ISBN 978 1 90 139626 3.

Trochimowicz HJ, Kennedy GL, Krivanek ND. (1991) Heterocyclic and miscellaneous nitrogen compounds. In Clayton GD, Clayton FE editors. *Patty's Industrial Hygiene and Toxicology*, 4<sup>th</sup> edition, Vol. 2, part E. Wiley: New York. p. 3285-3521. ISBN 978 0 47 101282 5.

United States Environmental Protection Agency. (1975). EPA/540-1-75-012: 1975. Initial scientific and mini-economic review of captan. Washington, D.C.. United States Environmental Protection Agency, Office of Pesticide Programs, Criteria and Evaluation Division.

United States Environmental Protection Agency. (1999). EPA 738-R-99-011: 1999. Registration Eligibility Decision (RED) - Folpet. Washington, D.C.. US Environmental Protection Agency.

United States Environmental Protection Agency. (2004). 69 Fed. Reg. 68357-68360: 2004. Amendment to the 1999 captan Registration Eligibility Decision (RED). Captan: cancer reclassification. Washington, D.C.. US Environmental Protection Agency.

van Welie RT, van DP, Lamme EK, Jager P, van Baar BL, Vermeulen NP. (1991) Determination of tetrahydrophthalimide and 2-thiothiazolidine-4-carboxylic acid, urinary metabolites of the fungicide captan, in rats and humans. *Int Arch Occup Environ Health*; 63 181-186.

Verberk MM, Brouwer DH, Brouwer EJ, Bruyzeel DP, Emmen HH, van Hemmen JJ, Hooisma J, Jonkman EJ, Ruijten MW, Salle HJ. (1990) Health effects of pesticides in the flower-bulb culture in Holland. *Med Lav*; 81 530-541.

Viau C, Lafontaine M, Payan JP. (2004) Creatinine normalization in biological monitoring revisited: the case of 1-hydroxypyrene. *Int Arch Occup Environ Health*; 77 177-185.

Wester RC, Maibach HI. (1983) Cutaneous pharmacokinetics: 10 steps to percutaneous absorption. *Drug Metab Rev*; 14 169-205.

Winterlin WL, Kilgore WW, Mourer CR, Hall G, Hodapp D. (1986) Worker Reentry Into Captan-Treated Grape Fields in California. *Arch Environ Contam Toxicol*; 15 301-311.

Winterlin WL, Kilgore WW, Mourer CR, Schoen SR. (1984) Worker Reentry Studies for Captan Applied to Strawberries in California. *J Agric Food Chem*; 32 664-672.

Woollen BH. (1993) Biological monitoring for pesticide absorption. *Ann Occup Hyg*; 37 525-540.

Zweig G, Leffingwell JT, Pependorf W. (1985) The relationship between dermal pesticide exposure by fruit harvesters and dislodgeable foliar residues. *J Environ Sci Health B*; 20 27-59.

**Table 1**

Characteristics of captan or folpet exposure for each worker following fungicide treatment and harvest activities.

	Captan exposure		Folpet exposure		
	Worker #1 <sup>b</sup>	Worker #2 <sup>c</sup>	Worker #1 <sup>d</sup>	Worker #2 <sup>e</sup>	Worker #3 <sup>f</sup>
<i>Application activities<sup>a</sup></i>					
Active ingredient %	80% captan	80% captan	50% folpet	80% folpet	25% folpet
Amounts (kg)	4 kg (or 1kg ha <sup>-1</sup> )	nd	1kg ha <sup>-1</sup>	3.5 kg (or 1kg ha <sup>-1</sup> )	1kg ha <sup>-1</sup>
Water volume	400 l ha <sup>-1</sup>	500 l ha <sup>-1</sup>	nd	700 l (200 l ha <sup>-1</sup> )	nd
Treated area (hectares)	2.5	5	nd	3.3	nd
Spraying date	07-08/05/2009	08/08/2009	25/06/2009	07-08/05/2009	16/05/2009
Total spraying duration	2 h	8 h	4 h	6 h	3 h
Spraying technique	Airblast pulled by a tractor with an opened cabin	Airblast pulled by a tractor with a closed cabin	Airblast sprayer	Airblast sprayer	Airblast sprayer and a back air-spray
Safety equipments worn during application	- Leather shoes - Waterproof gloves	- Rubber boots - Cap	- Full-face helmet with filter - Coveralls - Waterproof gloves	- Complete forced air helmet - Tissue coveralls - Safety shoes	- Half-face helmet with filter - Tissue hat - Waterproof gloves
Symptoms	No	No	Eyes irritation	No	Eyes irritation
<i>Harvest activities</i>					

	Captan exposure		Folpet exposure		
	Worker #1 <sup>b</sup>	Worker #2 <sup>c</sup>	Worker #1 <sup>d</sup>	Worker #2 <sup>e</sup>	Worker #3 <sup>f</sup>
Active ingredient %	80% captan	80% captan	50% folpet	80% folpet	60% folpet
Harvesting date	15/06/2009	17 to 20/06/2009	25/06/2009	27 to 30/05/2009	22 to 26/06/2009
Total harvesting duration	5 h	30 h	6 h	36 h	45 h
Symptoms	No	No	No	No	Eyes irritation

nd = not available.

<sup>f</sup> All workers performed mixing, loading, and material cleaning activities in addition to spaying.

<sup>b</sup> Worker 1 (captan exposure) wore leather shoes and waterproof gloves also during preparation and cleaning. Preparation was conducted inside. No personal protective equipment was worn during harvesting period. Gloves only were decontaminated with water post-spraying. Hands were washed after spraying and harvesting, and clothes were removed at home at the end of the workday for both activities.

<sup>c</sup> Worker 2 (captan exposure) wore rubber boots and a cap also during preparation and cleaning. Preparation was conducted outside. No decontamination of equipment was performed post-spraying. No personal protective equipment was worn during harvesting period. Hands were washed after spraying and harvesting, and clothes were removed at home at the end of the workday for both activities.

<sup>d</sup> Worker 1 (folpet exposure) wore a mask, coverall and waterproof gloves also during preparation, but no personal protective equipment during cleaning. Preparation was conducted outside. Mask and gloves were decontaminated with soap and water post-spraying. For harvesting, he wore gloves and pants. Hands were washed after spraying and harvesting, and clothes were removed at home at the end of the workday for spraying activities and at work for harvesting activities.

<sup>e</sup> Worker 2 (captan exposure) wore a half-face helmet with filter, tissue coveralls, safety shoes and waterproof gloves also during preparation and cleaning (except helmet for cleaning). Preparation was conducted inside with a ventilation system. Helmet and tissue coveralls were decontaminated

with soap and water post-spraying. For harvesting, he wore gloves and pants. Hands were washed after spraying and harvesting, and clothes were removed at work at the end of the workday for spraying activities and at home for harvesting activities.

<sup>f</sup> Worker 3(captan exposure) wore a tissue hat and waterproof gloves also during preparation and cleaning. Preparation was conducted inside with a ventilation system. Mask and hat were decontaminated with soap and water post-spraying. For harvesting, he wore only rubber gloves. Hands were washed after spraying and harvesting, and clothes were removed at home at the end of the workday for both activities.

**Table 2**

Published mean concentrations of THPI in the urine of workers exposed to captan following different activities in various types of crops.

References	Mean concentration/amount of THPI				Worker exposure scenario <sup>a</sup>				
	Pre-shift urine <sup>b</sup>	Post-shift urine <sup>c</sup>	24-h urine <sup>d</sup>	<i>n</i> <sup>e</sup>	Activity <sup>f</sup>	Crops <sup>g</sup>	Duration <sup>h</sup>	Total treated area (mean) <sup>i</sup>	Captan amount <sup>j</sup>
Winterlin <i>et al.</i> (1984)									
	NE	< 30 µg l <sup>-1</sup>	NE	1	Applicator/ loader/mixer	Strawberries	8 h	0.5-2 acres	2.2 lbs of AI acre <sup>-1</sup>
	NE	58-60 µg l <sup>-1k</sup>	NE	12	Harvesters	Strawberries	8 h	0.5-2 acres	
Winterlin <i>et al.</i> (1986)									
	50 µg l <sup>-1</sup>	63 µg l <sup>-1</sup>	57 µg l <sup>-1</sup>	3	Loader/mixer/ applicators	Grapes	8 h	36-40 acres	2.0-2.5 lbs AI
	32 µg l <sup>-1</sup>	50 µg l <sup>-1</sup>	47 µg l <sup>-1</sup>	16	Harvesters	Grapes	8 h	37 acres	2.0 lbs AI
Maddy <i>et al.</i> (1989)									
	NE	NE	0.005 µg l <sup>-1l</sup>	10	Pickers	Strawberries	3 days	72 acres	4 lbs AI acre <sup>-1</sup>
Verberk <i>et al.</i> (1990)									
	8 µmol mol <sup>-1</sup> creat.	NE	20 µmol mol <sup>-1</sup> creat.	6	Dipping bulbs in captan solution	Flower-bulbs	NE	NE	NE





References	Mean concentration/amount of THPI				Worker exposure scenario <sup>a</sup>				
	Pre-shift urine <sup>b</sup>	Post-shift urine <sup>c</sup>	24-h urine <sup>d</sup>	<i>n</i> <sup>e</sup>	Activity <sup>f</sup>	Crops <sup>g</sup>	Duration <sup>h</sup>	Total treated area (mean) <sup>i</sup>	Captan amount <sup>j</sup>
	0.26 µg l <sup>-10</sup>	2.70 µg l <sup>-10</sup>	2.95 µg l <sup>-10</sup>	2	Loader/mixer/ applicators	Apple trees	2 h to 8 h	2.5 to 5 acres	1 kg AI acre <sup>-1</sup>
	(1.69 nmol l <sup>-1</sup> )	(17.8 nmol l <sup>-1</sup> )	(19.5 nmol l <sup>-1</sup> )						
	0.14 µg l <sup>-10</sup>	0.89 µg l <sup>-10</sup>	0.59 µg l <sup>-10</sup>	2	Pruning, thinning	Apple trees	5 h to 30 h	2.5 to 5 acres	1 kg AI acre <sup>-1</sup>
	(0.93 nmol l <sup>-1</sup> )	(5.88 nmol l <sup>-1</sup> )	(3.90 nmol l <sup>-1</sup> )						

NE = not estimated; AI = active ingredient; lbs = pounds.

<sup>a</sup> Information concerning worker exposure to captan: activities during exposure, type of studied crops, duration of exposure, mean total treated area and amounts of captan applied on fields during the studied period.

<sup>b</sup> Mean concentration or amounts of THPI measured in pre-shift urine of workers exposed to captan.

<sup>c</sup> Mean concentration or amounts of THPI measured in post-shift urine of workers exposed to captan.

<sup>d</sup> Mean concentration or amounts of THPI measured in 24-h complete urine collection in workers exposed to captan following a workday.

<sup>e</sup> Number of workers participating in the study.

<sup>f</sup> Activities performed by workers during the studied period of exposure to captan.

<sup>g</sup> Studied crop fields.

<sup>h</sup> Duration of worker exposure to captan during the study period.

<sup>i</sup> Mean total area (expressed in acres) treated with captan.

<sup>j</sup> Mean amounts of captan (as active ingredient) sprayed on studied area during the study period.

<sup>k</sup> Values for 3 days post-application.

<sup>l</sup> Mean THPI levels in strawberry pickers, working with or without gloves, during the 3-day study period. Complete 24-h urine collections were obtained.

<sup>m</sup> No background was available because when the study began, all workers had captan exposures during the previous 2 weeks.

<sup>n</sup> Mean THPI levels in strawberry harvesters, working with or without rubber latex gloves, for the 3-day study period. Complete 24-h urine specimens were obtained.

<sup>o</sup> Mean THPI levels calculated from data of both workers.

## FIGURE CAPTIONS

### **Figure 1**

Toxicokinetic models of captan (A) and of folpet (B) including percentage dermally absorbed and metabolite transfer half-lives between compartment.

### **Figure 2**

Time courses of THPI urinary excretion rate (expressed as nmol/h/kg of body weight) over a 168-h period in two workers (A and B) exposed to captan following both spraying and harvest activities.

### **Figure 3**

Time courses of PI (A and B) and PA (C and D) urinary excretion rate (expressed as nmol/h/kg of body weight) over a 168-h period in three workers exposed to folpet following spraying activities (A and C) and harvest activities after a delay-of-reentry (B and D). Arrows represent treatment period or harvesting period (for the three workers).

### **Figure 4**

Time courses of PA urinary excretion rate (expressed as nmol/h/kg of body weight) over a 168-h period in two workers exposed to captan following spraying activities.

**Figure 5**

Time courses of THPI excretion rates (expressed as nmol/h/kg of body weight) non-adjusted (open symbols) and adjusted by creatinine (closed symbols) in spot urines (A) or 8-h (C), 12-h (D) and 24-h (B) collections in a worker exposed to captan during spraying activities. The dermal maximum lines represent maximum values measured in the urine of volunteers exposed to captan by the dermal route (10 mg/kg) (Berthet *et al.*, 2010b; Berthet *et al.*, 2010d).

**Figure 6**

Time courses of PI and PA excretion rates (expressed as nmol/h/kg of body weight) non-adjusted (open symbols) and adjusted by creatinine (closed symbols) in spot urines (A) or 8-h (B) and 24-h (C) collections in a worker exposed to folpet during spraying activities. The dermal and oral maximum lines represent maximum values measured in the urine of volunteers exposed to folpet by the dermal (10 mg/kg) or oral route (1 mg/kg) (Berthet *et al.*, 2010b; Berthet *et al.*, 2010d).

**Figure 7**

Dermal model simulations (solid line) compared with experimental data on the time courses of THPI, PI and PA in the urine of a worker exposed to captan or folpet over a work week following spraying and harvest activity periods. Solid circles and gray bars show experimental rate values in 24-h collections, and black bars on right axis represent the corresponding simulated absorbed dose scenario.

## FIGURES

Figure 1

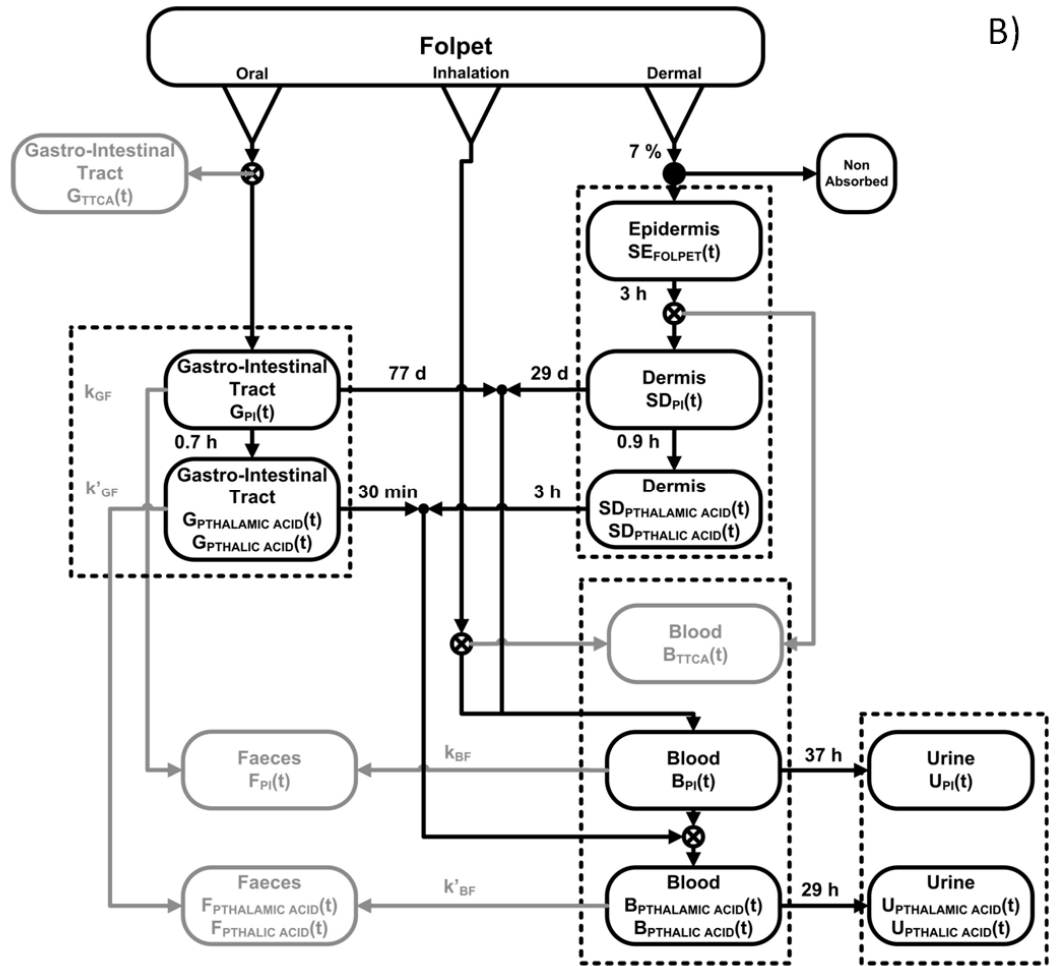
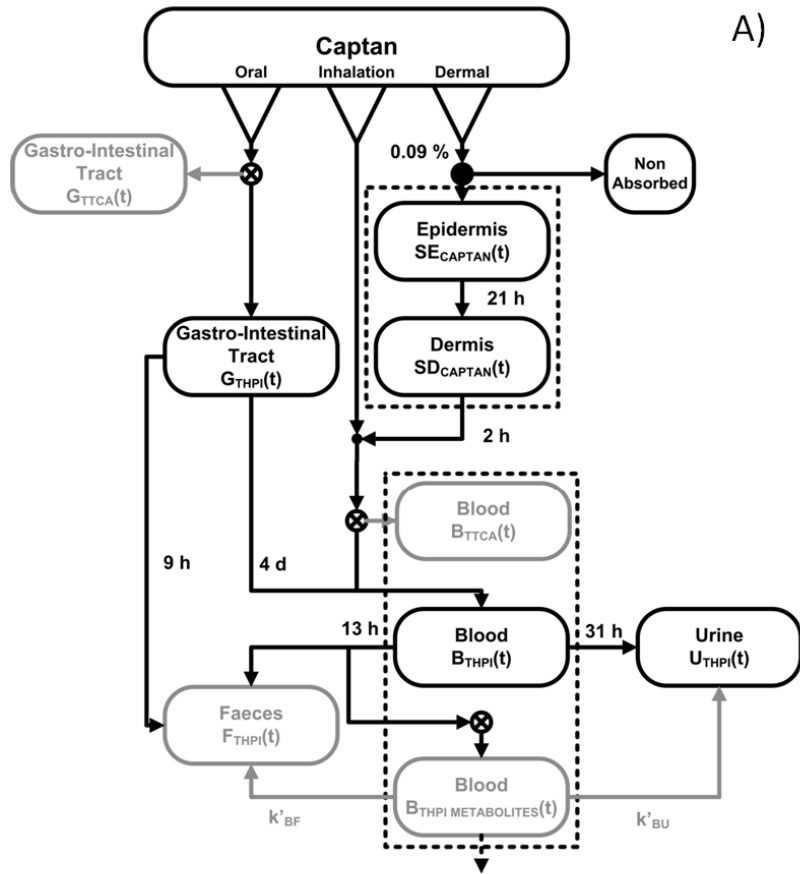


Figure 2

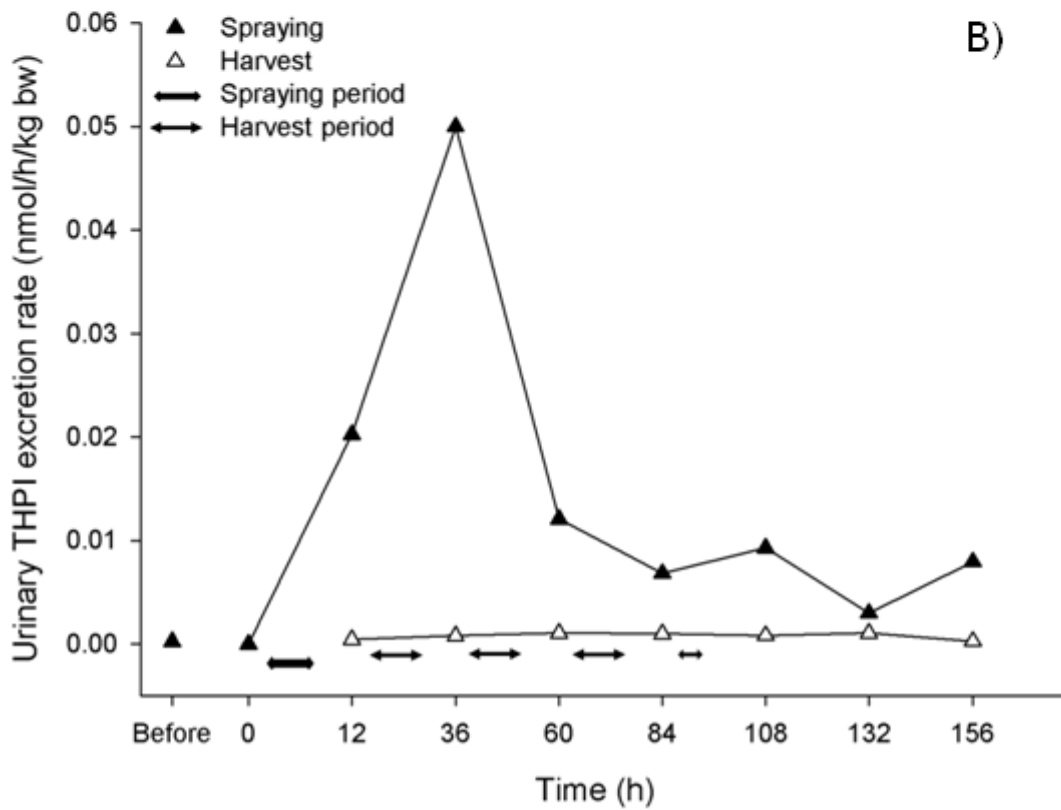
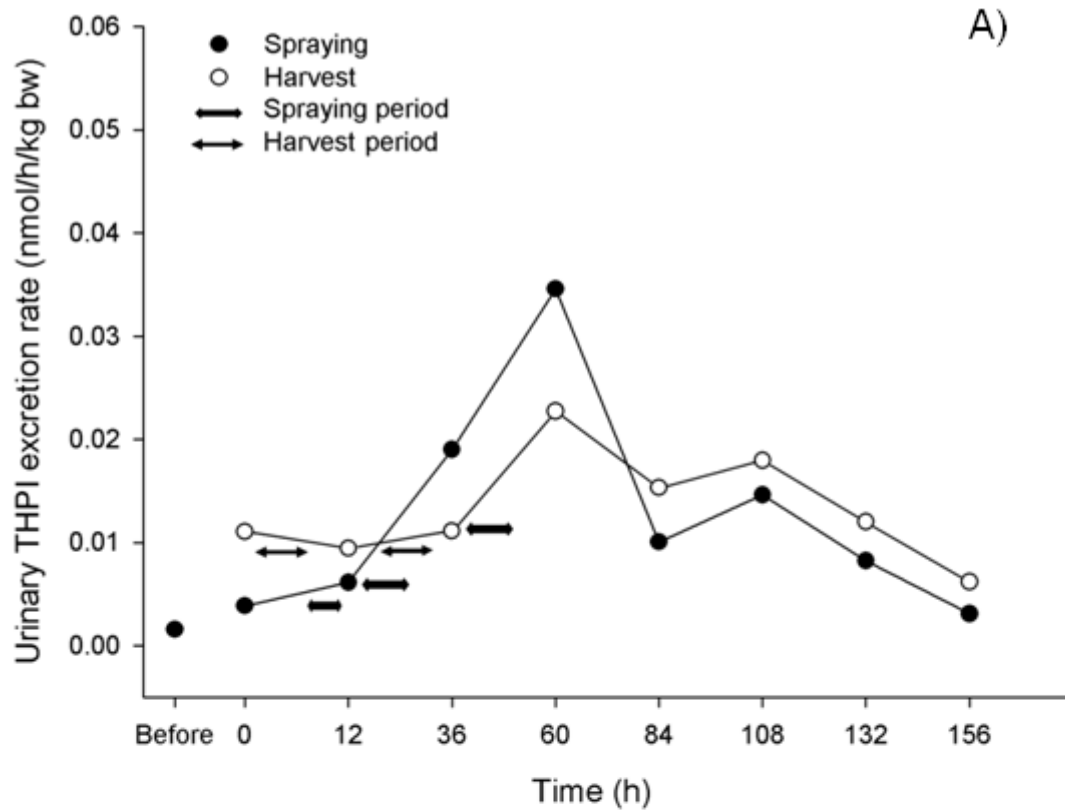


Figure 3

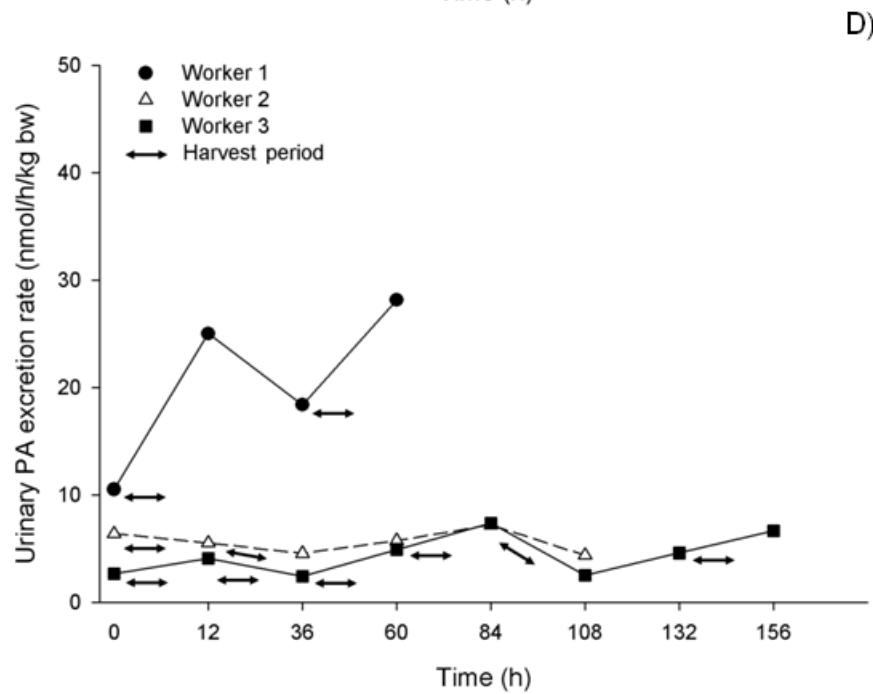
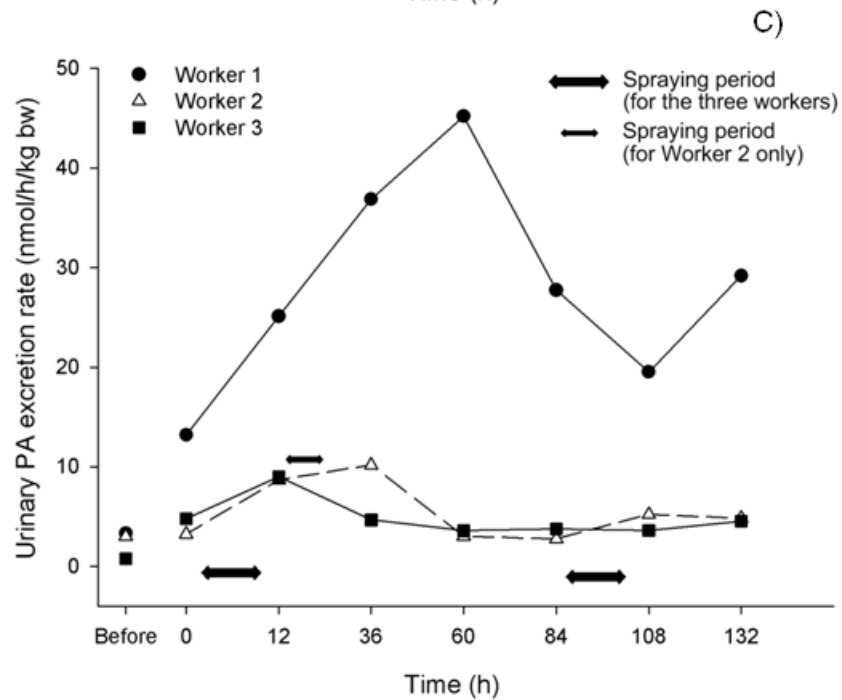
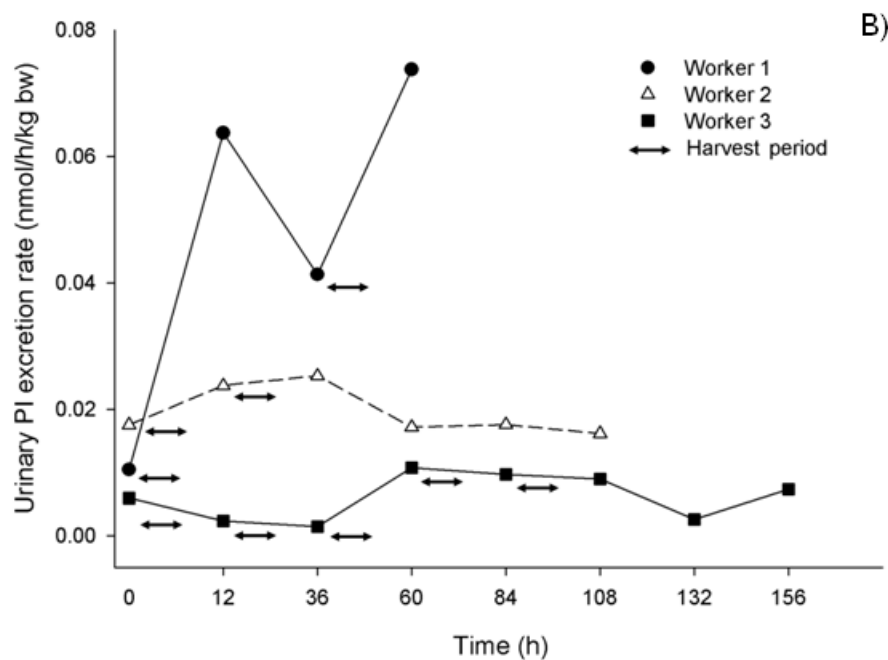


Figure 4

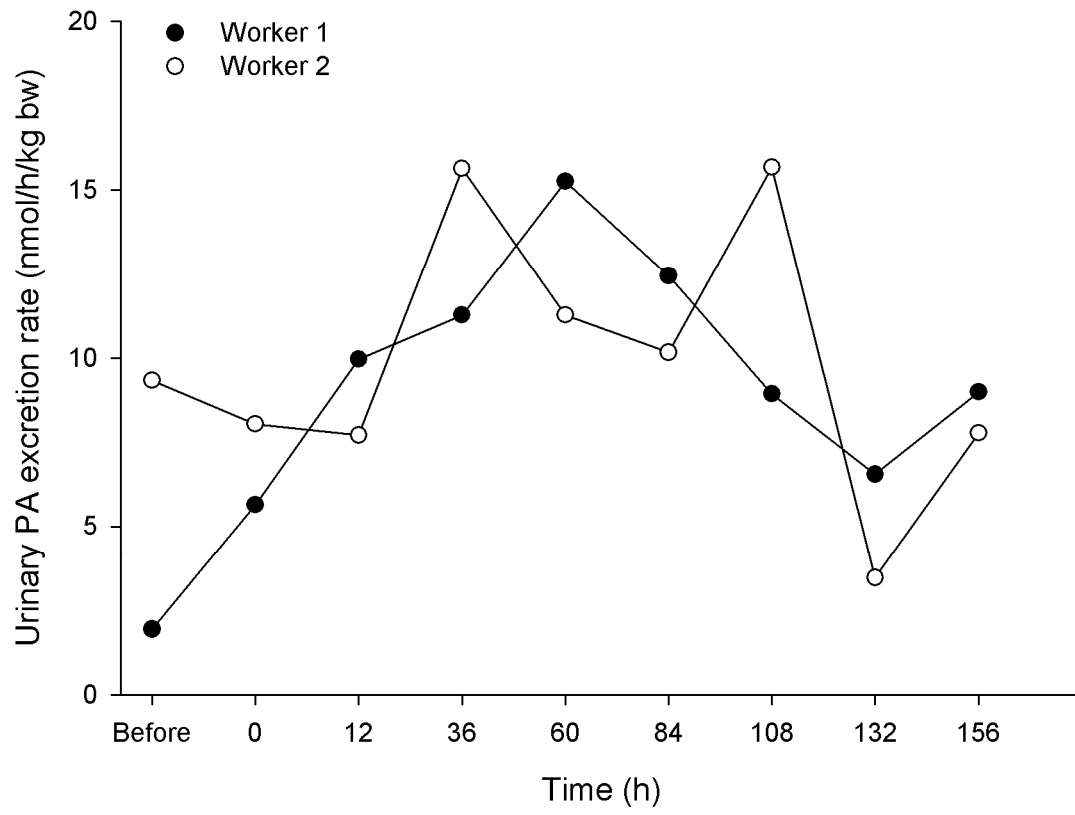




Figure 5

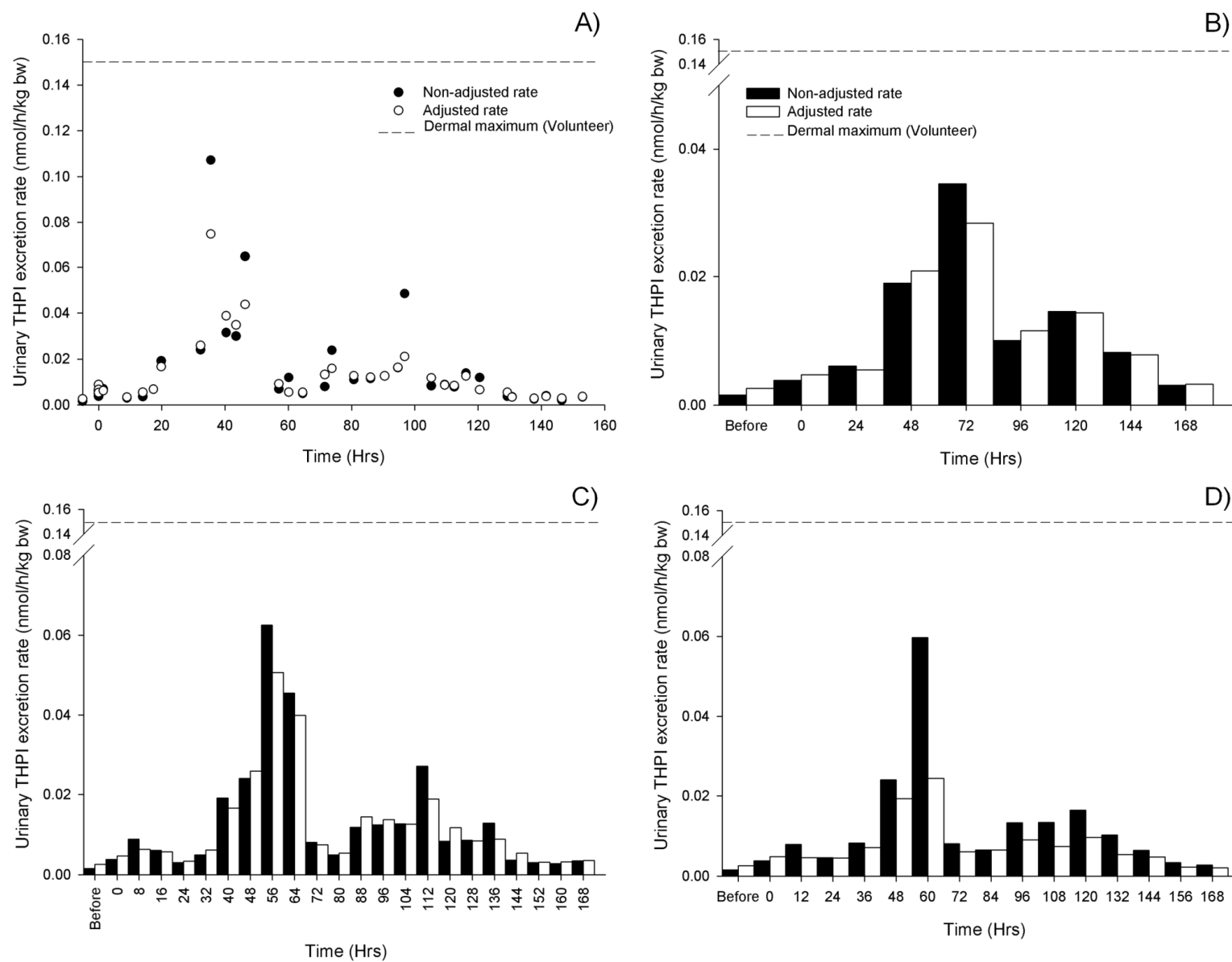


Figure 6

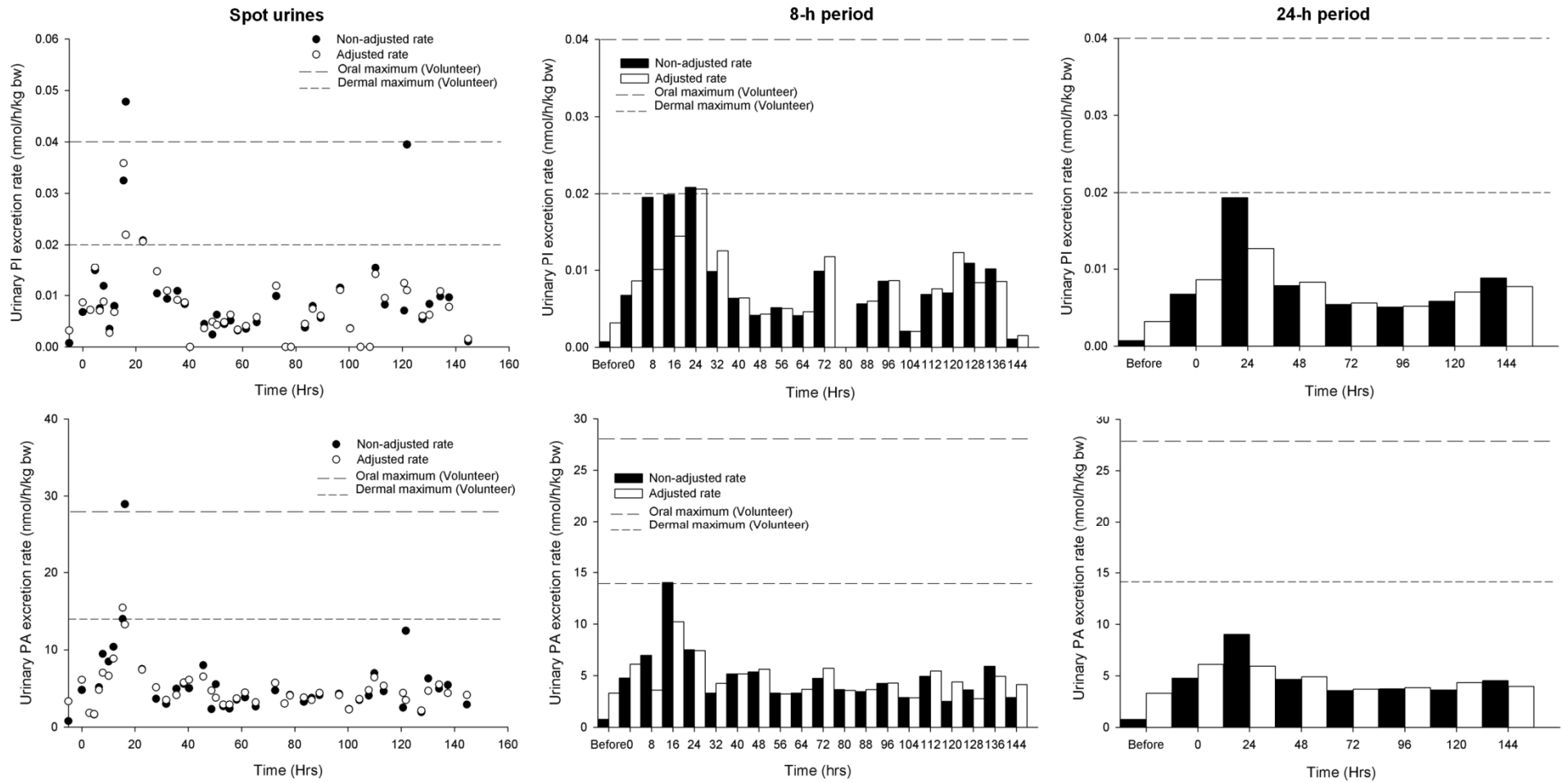
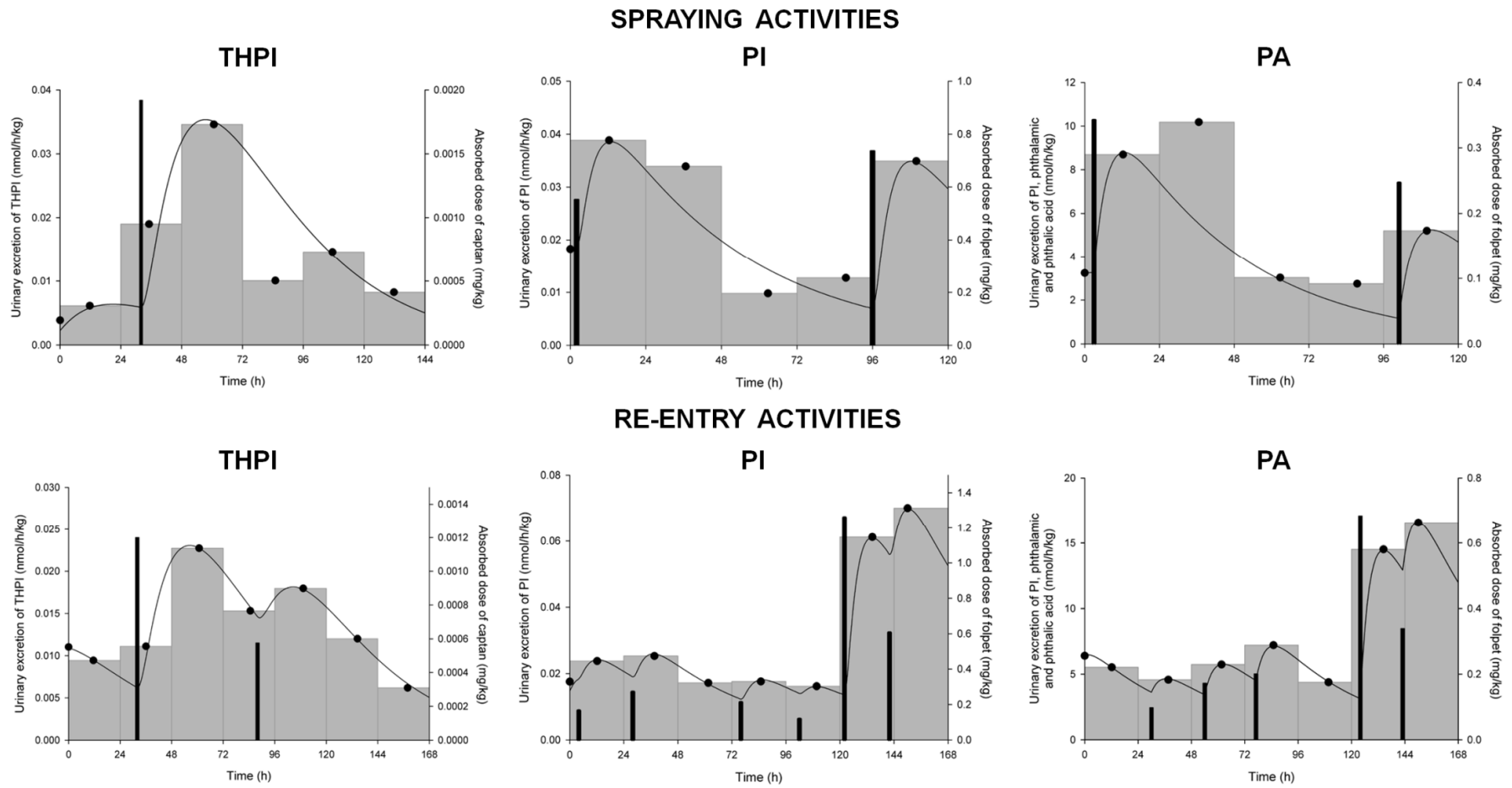


Figure 7



## **5. Discussion**

### **5.1. Le THPI, PI et acide phtalique comme biomarqueurs d'exposition au captan et folpet (Articles I, II)**

Les méthodes analytiques développées pour quantifier le THPI, le PI et l'acide phtalique se sont avérées spécifiques et très sensibles, et plus particulièrement celle du THPI et de l'acide phtalique par rapport au PI. En effet, comme le PI ne se fragmente pas, sa méthode est moins spécifique; des pics interférents doivent être séparés sur les chromatogrammes et un effet de matrice pourrait plus facilement influencer les résultats que dans le cas du THPI. Néanmoins, la méthode a permis de mesurer le PI dans le plasma des volontaires et dans l'urine des volontaires et des agriculteurs à de relativement faibles quantités et très précisément. Il peut donc être estimé comme un biomarqueur spécifique au folpet, au même titre que le THPI pour le captan.

L'acide phtalique peut lui aussi être considéré comme un biomarqueur d'exposition au folpet, mais en raison de son manque de spécificité il est surtout complémentaire au PI. Pour les travailleurs, une grande variabilité a été observée et les profils urinaires n'étaient pas toujours parallèles à ceux du PI. En raison de l'ubiquité des phtalates dans l'environnement, il devient très important de mesurer la quantité présente de ce métabolite dans les urines avant l'exposition au folpet pour déduire le niveau déjà présent. De plus, la méthode analytique de ce composé permet de transformer qu'une partie du PI. Le PI et

l'acide phtalique doivent donc être mesurés conjointement pour une estimation adéquate de l'exposition au folpet.

Comme démontré dans l'exemple du folpet, la mesure de plusieurs métabolites pour un même composé se révèle nécessaire pour améliorer l'estimation de l'exposition réelle. A priori, le TTCA pourrait également être un autre biomarqueur à étudier plus en détails, d'autant plus que ce métabolite représente la voie métabolique qui serait à l'origine de la toxicité des deux fongicides puisqu'il dérive du groupe trichlorométhylthio. Les quantités mesurées pourraient alors être comparées pour le captan et le folpet afin de mieux définir leur métabolisme. Toutefois, des essais préliminaires effectués dans le laboratoire ont montré que la détection analytique est difficile; elle doit donc être améliorée et l'extraction par méthode SPE testée avant de considérer celui-ci comme un autre biomarqueur potentiel.

## **5.2. Toxicocinétiques du THPI, PI et acide phtalique (Articles III et IV)**

L'étude chez les volontaires est la première qui documente la cinétique du folpet ainsi que du captan pour la voie cutanée chez l'humain. Les résultats sont concomitants avec ceux des études chez le rat et avec ceux de l'étude de Krieger et Thongsinthusak (15) pour l'exposition par voie orale au captan.

Des différences ont toutefois été remarquées entre le captan et le folpet. Tant le captan que le folpet sont hydrolysés non enzymatiquement pour former le THPI et PI, respectivement. Toutefois, le pH joue un rôle non négligeable dans la scission des deux fongicides, surtout lors de l'absorption orale (10). Par ailleurs, au niveau de l'exposition cutanée chez les volontaires, l'absorption apparaît plus importante pour le folpet que pour le captan, ce qui est vérifié dans l'étude des agriculteurs pour les activités d'effeuillage. La formation des métabolites apparaît aussi différente pour les deux fongicides. Pour les métabolites aromatiques du folpet, c'est une hydrolyse qui permet la métabolisation du PI en acide phtalamique puis en acide phtalique. Au contraire, plusieurs réactions enzymatiques sont impliquées pour dériver le THPI en autres métabolites au niveau du foie. Enfin, au niveau cinétique, le PI et l'acide phtalique sont éliminés plus lentement que le THPI et la vitesse d'élimination est similaire pour les voies orale et cutanée d'absorption alors que le THPI est éliminé plus rapidement après absorption orale. Toutefois, aucune accumulation de ces métabolites dans les tissus n'a lieu après une exposition au captan ou au folpet.

Pour confirmer, valider plus précisément et approfondir ces observations, l'analyse d'autres métabolites s'avérerait nécessaire. Par exemple, il serait intéressant de quantifier le 3-OH-THPI pour le captan puisqu'environ 40% de la dose de captan administré par voie orale chez le rat est retrouvée sous forme de ce métabolite dans les urines (70;71). Quant au folpet, l'analyse de l'acide phtalique dans le plasma aurait permis de vérifier d'une part qu'une quantité limitée de PI est absorbée par la paroi du GI après une absorption orale car

majoritairement transformé en acide phtalamique directement dans le GI, et d'autre part que le PI est très rapidement métabolisé dans le sang en acide phtalamique après une absorption cutanée.

Par ailleurs, la méthode analytique quantifiant le total des formes PI et acides phtalamique et phtalique (et exprimé sous forme d'équivalent acide phtalique) présente certaines limites. Selon l'étude de Chasseaud *et al.* (81), 80% de la dose de folpet marqué au C<sup>14</sup> et administrée par voie orale chez les rats se retrouvent sous forme d'acide phtalamique. Chez les volontaires, seulement 25% de la dose de folpet est retrouvée sous forme de la somme des métabolites aromatiques au lieu des 80% attendus sur la base des données animales. Or Chasseaud *et al.* (81) dans son étude observe des interconversions entre l'acide phtalamique, le PI, l'acide phtalique et l'anhydride phtalique en GC-MS après des conditions d'extraction et de dérivation similaires à la méthode utilisée dans cette étude. L'acide phtalamique par exemple pouvait se déshydrater pour former du PI ou s'hydrolyser pour former de l'acide phtalique, et en réaction avec le N,O-bis(triméthylsilyl)trifluoroacétamide (BSTFA), il tendait à se reformer en triméthylsilyle phtalimide (TMS-PI). Par conséquent, la méthode analytique pourrait être encore améliorée en détectant d'autres ions en plus de l'acide phtalique triméthylsilyle.

Il est par ailleurs à noter que l'étude chez les volontaires a impliqué un petit nombre de participants mais beaucoup de facteurs se rapportant à la variation inter-individuelle (âge, sexe, poids) ou influençant le métabolisme (consommation d'alcool, de tabac ou de médicaments, l'alimentation) ont été contrôlés. Toutefois, les origines ethniques n'ont pas été un critère de sélection, et deux volontaires exposés au captan étaient natifs du continent africain. Aucune influence n'a cependant été observée dans les résultats, leurs concentrations mesurées étaient très proches de celles des caucasiens. D'autre part, le protocole de collecte de sang et des urines était assez invasif pour les participants, mais les périodes de temps fixes et prédéterminées ont permis une bonne uniformité des résultats.

Les résultats obtenus étant très peu variables entre les volontaires pour les deux fongicides ainsi que très proches de ceux de l'étude de Krieger et Thongsinthusak (15) pour le captan, les modèles cinétiques développés pourraient donc être généralisés à la population générale ainsi qu'à d'autres travailleurs.

L'effet du choix de la population étudiée ainsi que du véhicule utilisé pour appliquer les fongicides sur la peau seraient des aspects à étudier plus spécifiquement dans des études futures. En effet, dans le présent travail, les volontaires étaient une population contrôle mais pas représentatifs de la population des travailleurs car ils étaient plus jeunes, n'avaient jamais été exposés au captan ou au folpet excepté par l'alimentation, et ne devaient pas consommer d'alcool ni de médicament. Leurs données peuvent seulement être appliquées à



la population générale chez les hommes car aucune donnée n'a été collectée pour les femmes comme il était présumé que les travailleurs en arboriculture et en viticulture étaient essentiellement des hommes. Or, beaucoup de femmes sont employées pour ramasser les fruits et les trier, et peuvent aussi être en contact avec ces fongicides. Il manque donc des données pour cette population.

De même, l'acétone utilisé dans l'étude chez les volontaires n'était pas le véhicule d'application le plus représentatif de celui utilisé par les travailleurs, qui est l'eau. De plus, l'acétone augmente la perméation de la peau et donc l'absorption (88;110). Les quantités de captan et de folpet absorbées ont donc pu être plus importantes pour les volontaires, qui ont de toute façon été exposés au produit pur et non à un produit commercial.

### **5.3. Exposition au captan et folpet des travailleurs (Article V)**

Les résultats obtenus pour l'étude des travailleurs sont équivalents à ceux rapportés dans la littérature pour le captan. Ils confirment que les activités de traitement sont plus à risque que les activités d'effeuillage et que la voie cutanée reste la voie principale d'exposition chez les travailleurs utilisant des pesticides. Ces observations sont applicables aussi aux travailleurs exposés au folpet bien qu'il soit plus difficile d'affirmer que l'absorption cutanée est plus importante que les autres voies d'absorption en raison des  $t_{1/2}$  d'élimination du PI et de l'acide phtalique dans l'urine qui sont identiques peu importe la voie d'entrée.

D'autre part, les techniques d'application ne semblent pas influencer le niveau d'exposition, mais les travailleurs étaient probablement adéquatement protégés. Une étude avec un plus grand nombre de travailleurs serait cependant nécessaire pour valider ces résultats car trois reste un nombre insuffisant de participants.

En comparaison avec l'étude de Lebailly *et al.* (17;153;154) sur des agriculteurs en France exposés au captan ou au folpet lors d'activités de traitement, la présente étude montre qu'il est important de collecter les urines complètes de 24 h et sur une période de plusieurs jours pour bien caractériser l'exposition des travailleurs. (Les urines des études de Lebailly *et al.* ont été analysées à l'IST avec les méthodes analytiques du THPI, du PI et de l'acide phtalique décrites précédemment). Dans l'étude de Lebailly *et al.* (17;153;154), seule la première miction avant la journée de travail, celle à la fin de la journée de travail et la première du lendemain ont été collectées pour le captan. Pour le folpet, des urines ponctuelles ont été collectées aux heures prédéfinies suivantes : 0, 4, 12 et 48 h. Dans les deux populations de travailleurs, plusieurs urines sont manquantes, notamment les mictions précédant la journée de travail, et les résultats ne représentent que des quantités ponctuelles qui peuvent être éventuellement comparées entre les différents agriculteurs. Il est alors difficile d'estimer une exposition et d'exploiter ces données. Les urines complètes sur 24 h sont donc à favoriser, ou autrement sur des périodes de 8 ou 12 h comme le montre l'Article V. De plus, lorsque les  $t_{1/2}$  d'élimination sont assez courtes, il est aussi intéressant d'inclure les jours de repos.

Ainsi, dans la présente étude chez les travailleurs, le nombre important de collectes urinaires sur 7 jours consécutifs et sur deux périodes d'activités distinctes a permis une bonne caractérisation de l'exposition et une augmentation considérable des valeurs disponibles en contre partie du faible nombre de participants. Cela a également permis de déterminer l'influence des différentes unités, de la normalisation par la créatinine et des quantités cumulatives sur différentes périodes de temps sur les résultats.

Les résultats pourraient s'appliquer aux travailleurs exposés au captan ou au folpet de manière plus générale, pas seulement aux arboriculteurs ou aux viticulteurs. Cependant, en considérant le peu de données disponibles pour l'exposition au folpet, une étude intégrant un plus grand nombre de participants permettrait de confirmer ces résultats et d'accroître les connaissances sur ce produit.

La considération de mesures environnementales pour compléter et corrélérer les données de surveillance biologique serait aussi à considérer dans des études futures. En effet, des applications de pads sur différentes parties du corps, la technique de lavage des mains (hand washing) et des prélèvements d'air à l'aide d'une pompe portative pourraient être effectués afin de mieux définir et estimer les quantités réelles de captan ou de folpet auxquelles les travailleurs étaient exposés.

## 5.4. Recommandations et perspectives

Quatre points importants devraient être considérés pour améliorer l'évaluation de l'exposition des travailleurs au captan et au folpet ainsi que leur protection :

- Développer une méthode valide et robuste pour quantifier le TTCA, la deuxième voie de métabolisme du captan et du folpet qui est à l'origine de la cytotoxicité, afin de corrélérer ces résultats avec le THPI pour le captan, et le PI et l'acide phtalique pour le folpet.
- Vérifier que les équipements de protection utilisés ainsi que les habitudes de travail sont appropriés considérant les propriétés sensibilisantes et irritantes des deux fongicides;
- Proposer des niveaux sécuritaires de biomarqueurs urinaires qui correspondent à des doses d'exposition limites (NOAEL);
- Définir des directives précises sur les effets sur la santé du folpet ainsi que des normes de travail telle qu'une valeur limite d'exposition (VME).

## 6. Conclusion

Ce projet de recherche a permis d'une part de déterminer et de mieux comprendre la cinétique du captan et du folpet chez l'humain et d'autre part d'estimer l'exposition de travailleurs à ces deux fongicides à l'aide de la surveillance biologique.

Plus précisément, les méthodes analytiques robustes et précises ont permis de déterminer que le THPI était un biomarqueur spécifique à l'exposition au captan et le PI à l'exposition au folpet, même si ce ne sont pas les métabolites principaux qui ressortent chez les études animales ni selon les résultats obtenus. Elles ont également montré que l'acide phtalique pouvait aussi être considéré comme un biomarqueur d'exposition au folpet, mais qu'une valeur de pré-exposition était nécessaire et qu'il devait être mesuré conjointement avec le PI. Quant à l'étude chez les volontaires, elle a permis de mieux documenter la cinétique des trois biomarqueurs d'exposition et d'établir que leurs demi-vies biologiques étaient courtes chez l'humain et que leur élimination était un peu plus lente par voie cutanée que par voie orale. Ces résultats sont d'ailleurs très similaires à ceux rapportés chez le rat et ils ont permis de développer deux modèles toxicocinétiques suffisamment robustes pour mieux comprendre les processus biologiques qui régissent la cinétique des biomarqueurs étudiés. Enfin, l'étude chez les agriculteurs s'est avérée nécessaire pour valider les modèles toxicocinétiques et les biomarqueurs d'exposition, mais également pour documenter l'exposition des travailleurs au folpet et d'établir les stratégies d'échantillonnage et d'analyse des résultats les plus appropriées.

Ainsi, les connaissances acquises lors de ce projet de recherche pourraient facilement s'appliquer à la population générale mais aussi à d'autres fongicides.

## Bibliographie

- (1) Office Fédéral de la Statistique (OFS). Agriculture Suisse - Statistiques de poche 2009. Neuchâtel, Switzerland: Office Fédéral de la Statistique; 2009.
- (2) Gorse I, Dion S. Bilan des ventes de pesticides au Québec pour l'année 2007. Québec, Canada: Ministère du Développement durable, de l'Environnement et des Parcs; 2010.
- (3) Jakubowski M, Trzcinka-Ochocka M. Biological monitoring of exposure: Trends and key developments. *J Occup Health* 2005; 47(1):22-48.
- (4) Barr DB, Needham LL. Analytical methods for biological monitoring of exposure to pesticides: a review. *J Chromatogr B Analyt Technol Biomed Life Sci* 2002; 778(1-2):5-29.
- (5) Baldi I, Lebailly P, Jean S, Rougetet L, Dulaurent S, Marquet P. Pesticide contamination of workers in vineyards in France. *J Expo Sci Environ Epidemiol* 2006; 16(2):115-24.
- (6) Canal-Raffin M, Receveur M, Martinez B, Titier K, Ohayon C, Baldi I, et al. Quantification methods of folpet degradation products in plasma with HPLC-UV/DAD: Application to an in vivo toxicokinetic study in rats. *J Chromatogr B Analyt Technol Biomed Life Sci* 2008; 865(1-2):106-13.
- (7) Carden A, Yost MG, Fenske RA. Noninvasive method for the assessment of dermal uptake of pesticides using attenuated total reflectance infrared spectroscopy. *Appl Spectrosc* 2005; 59(3):293-9.
- (8) de Cock J, Heederik D, Kromhout H, Boleij JSM, Hoek F, Wegh H, et al. Exposure to captan in fruit growing. *Am Ind Hyg Assoc J* 1998; 59(3):158-65.
- (9) Fenske RA, Schulter C, Lu C, Allen EH. Incomplete removal of the pesticide captan from skin by standard handwash exposure assessment procedures. *Bull Environ Contam Toxicol* 1998; 61(2):194-201.
- (10) Gordon EB. Captan and folpet. In: Krieger RI, editor. *Handbook of pesticide toxicology*. 3<sup>rd</sup> ed. New York: Elsevier; 2010. p. 1915-49.

- (11) Greenburg DL, Rusiecki J, Koutros S, Dosemeci M, Patel R, Hines CJ, et al. Cancer incidence among pesticide applicators exposed to captan in the Agricultural Health Study. *Cancer Causes Control* 2008; 19(10):1401-7.
- (12) Guo YLL, Wang BJ, Lee CC, Wang JD. Prevalence of dermatoses and skin sensitisation associated with use of pesticides in fruit farmers of southern Taiwan. *Occup Environ Med* 1996; 53(6):427-31.
- (13) Hines CJ, Deddens JA, Jaycox LB, Andrews RN, Striley CAF, Alavanja MCR. Captan exposure and evaluation of a pesticide exposure algorithm among orchard pesticide applicators in the agricultural health study. *Ann Occup Hyg* 2008; 52(3):153-66.
- (14) Juraske R, Anton A, Castells F, Huijbregts MA. Human intake fractions of pesticides via greenhouse tomato consumption: comparing model estimates with measurements for Captan. *Chemosphere* 2007; 67(6):1102-7.
- (15) Krieger RI, Thongsinthusak T. Captan Metabolism in Humans Yields 2 Biomarkers, Tetrahydrophthalimide (THPI) and Thiazolidine-2-Thione-4-Carboxylic Acid (TTCA) in Urine. *Drug Chem Toxicol* 1993; 16(2):207-25.
- (16) Krieger RI, Dinoff TM. Captan fungicide exposures of strawberry harvesters using THPI as a urinary biomarker. *Arch Environ Contam Toxicol* 2000; 38(3):398-403.
- (17) Lebailly P, Devaux A, Pottier D, De MM, Andre V, Baldi I, et al. Urine mutagenicity and lymphocyte DNA damage in fruit growers occupationally exposed to the fungicide captan. *Occup Environ Med* 2003; 60(12):910-7.
- (18) Lisi P, Caraffini S, Assalve D. Irritation and Sensitization Potential of Pesticides. *Contact Dermatitis* 1987; 17(4):212-8.
- (19) Maddy KT, Krieger RI, Oconnell L, Bisbiglia M, Margetich S. Use of Biological Monitoring Data from Pesticide Users in Making Pesticide Regulatory Decisions in California - Study of Captan Exposure of Strawberry Pickers. *Acs Symposium Series* 1989; 382:338-53.
- (20) Mcjilton CE, Berckman GE, Deer HM. Captan Exposure in Apple Orchards. *Am Ind Hyg Assoc J* 1983; 44(3):209-10.
- (21) Phalen RN, Que Hee SS. Permeation of captan through disposable nitrile glove. *J Hazard Mater* 2003; 100(1-3):95-107.
- (22) Ritcey G, Frank R, McEwen FL, Braun HE. Captan residues on strawberries and estimates of exposure to pickers. *Bull Environ Contam Toxicol* 1987; 38(5):840-6.



- (23) Stamper JH, Nigg HN, Queen RM. Dislodgeable Captan Residues at Florida Strawberry Farms. *Chemosphere* 1987; 16(6):1257-71.
- (24) Tielemans E, Louwerse E, de Cock J, Brouwer D, Zielhuis G, Heederik D. Exposure to fungicides in fruit growing: Re entry time as a predictor for dermal exposure. *Am Ind Hyg Assoc J* 1999; 60(6):789-93.
- (25) van Welie RT, van DP, Lamme EK, Jager P, van Baar BL, Vermeulen NP. Determination of tetrahydrophthalimide and 2-thiothiazolidine-4-carboxylic acid, urinary metabolites of the fungicide captan, in rats and humans. *Int Arch Occup Environ Health* 1991; 63(3):181-6.
- (26) Verberk MM, Brouwer DH, Brouwer EJ, Bruyzeel DP, Emmen HH, van Hemmen JJ, et al. Health effects of pesticides in the flower-bulb culture in Holland. *Med Lav* 1990; 81(6):530-41.
- (27) Winterlin WL, Kilgore WW, Mourer CR, Schoen SR. Worker Reentry Studies for Captan Applied to Strawberries in California. *J Agric Food Chem* 1984; 32(3):664-72.
- (28) Winterlin WL, Kilgore WW, Mourer CR, Hall G, Hodapp D. Worker Reentry Into Captan-Treated Grape Fields in California. *Arch Environ Contam Toxicol* 1986; 15(3):301-11.
- (29) Zainal H, Que Hee SS. Folpet permeation through nitrile gloves. *Appl Occup Environ Hyg* 2003; 18(9):658-68.
- (30) Barr DB, Needham LL. Analytical methods for biological monitoring of exposure to pesticides: a review. *J Chromatogr B Analyt Technol Biomed Life Sci* 2002; 778(1-2):5-29.
- (31) Burroughs GE, Hora J. Health Hazard Evaluation Report No. HETA-80-147-1076, Calhio Chemical Company, Perry, Ohio. Cincinnati, OH: National Institute for Occupational Safety and Health; 1982.
- (32) de Cock J, Heederik D, Hoek F, Boleij J, Kromhout H. Urinary excretion of tetrahydrophthalimide in fruit growers with dermal exposure to captan. *Am J Ind Med* 1995; 28(2):245-56.
- (33) Hansen JD, Schneider BA, Olive BM, Bates JJ. Personnel safety and foliage residue in an orchard spray program using azinphosmethyl and captan. *Arch Environ Contam Toxicol* 1978; 7(1):63-71.

- (34) Lavy TL, Mattice JD, Massey JH, Skulman BW. Measurements of year-long exposure to tree nursery workers using multiple pesticides. *Arch Environ Contam Toxicol* 1993; 24(2):123-44.
- (35) Oudbier AJ, Bloomer AW, Price HA, Welch RL. Respiratory Route of Pesticide Exposure As A Potential Health Hazard. *Bull Environ Contam Toxicol* 1974; 12(1):1-9.
- (36) Stevens ER, Davis JE. Potential Exposure of Workers During Seed Potato Treatment with Captan. *Bull Environ Contam Toxicol* 1981; 26(5):681-8.
- (37) Zweig G, Leffingwell JT, Popendorf W. The relationship between dermal pesticide exposure by fruit harvesters and dislodgeable foliar residues. *J Environ Sci Health B* 1985; 20(1):27-59.
- (38) Ecobichon DJ. Toxic effects of pesticides. In: Klaassen CD, editor. *Casarett and Doull's toxicology: the basic science of poison*. New York: McGraw-Hill Companies; 2001. p. 763-810.
- (39) Somerville L. The Metabolism of Fungicides. *Xenobiotica* 1986; 16(10-11):1017-30.
- (40) Edwards R, Ferry DG, Templ WA. Fungicides and related compounds. In: Hayes WJ, Laws ER, editors. *Handbook of Pesticide Toxicology*. San Diego, CA: Academic Press; 1991. p. 1409-70.
- (41) United States Environmental Protection Agency (US EPA). Initial scientific and mini-economic review of captan. Washington, D.C.: Office of Pesticide Programs, Criteria and Evaluation Division; 1975. Report No.: EPA/540/1-75-012.
- (42) Byrde RJW, Martin JT, Nicholas DJD. Effect of Fungicides on Fungus Enzymes. *Nature* 1956; 178(4534):638-9.
- (43) Dugger WM, Humphreys TE, Calhoun B. Influence of N-(Trichloromethylthio)-4-Cyclohexene-1,2-Dicarboximide (Captan) on Higher Plants .2. Effect on Specific Enzyme Systems. *Am J Bot* 1959; 46(3):151-6.
- (44) United States Environmental Protection Agency (US EPA). Registration Eligibility Decision (RED) - Folpet. Washington, D.C.: US Environmental Protection Agency; 1999. Report No.: EPA 738-R-99-011.
- (45) American Conference of Governmental Industrial Hygienists. Documentation of the threshold limit values and biological exposure indices, 6<sup>th</sup> edition. Volumes I, II, III. 214. Cincinnati, Ohio: American Conference of Governmental Industrial Hygienists; 1991.

- (46) Costa LG. Toxic effects of pesticides: the basic science of poisons. In: Casarett LJ, Doull J, Klaassen CD, editors. Casarett and Doull's Toxicology: the Basic Science of Poisons. 7th ed ed. New York: McGraw-Hill; 2008. p. 883-930.
- (47) Hayes AW. Toxicokinetics. In: Hayes AW, editor. Principles and Methods of Toxicology, 5<sup>th</sup> edition. 5th ed ed. Boca Raton: Taylor and Francis Group; 2008. p. 179-230.
- (48) National Institute for Occupational Safety and Health (NIOSH). NIOSH pocket guide to chemical hazards. Cincinnati, Ohio: NIOSH; 2007. Report No.: DHHS Publication No 2005-149.
- (49) Tomlin C. The Pesticide Manual - World Compendium, 10<sup>th</sup> edition. 8th ed. Thornton Heath: British Crop Protection Council; 1997.
- (50) Trochimowicz HJ, Kennedy GL, Krivanek ND. Heterocyclic and miscellaneous nitrogen compounds. In: Clayton GD, Clayton FE, editors. Patty's Industrial Hygiene and Toxicology, 4<sup>th</sup> edition, Vol. 2, part E. 4th ed. New York: Wiley; 1991. p. 3285-521.
- (51) United States Environmental Protection Agency (US EPA). Amendment to the 1999 captan Registration Eligibility Decision (RED). Captan: cancer reclassification. Washington, D.C.: US Environmental Protection Agency; 2004. Report No.: 69 Fed. Reg. 68357-68360.
- (52) American Conference of Governmental Industrial Hygienists (ACGIH). 2010 TLVs® and BEIs®. Based on Documentation of the Threshold Limit Values for chemical substances and physical agents and Biological Exposure Indices, 7th edition. Cincinnati, Ohio: ACGIH; 2010. p. 272.
- (53) Gordon E. Captan: transition from 'B2' to 'not likely'. How pesticide registrants affected the EPA Cancer Classification Update. J Appl Toxicol 2007; 27(5):519-26.
- (54) International Agency for Research on Cancer (IARC). Overall evaluations of carcinogenicity: An updating of IARC Monographs Volumes 1 to 42. [Supplement 7]. Monographs on the evaluation of carcinogenic risks to humans. Lyon, France: World Health Organization; 1987. p. 440.
- (55) Iturri SJ, Soto J. Inhibitory effect of captan in the small intestine absorption capacity of the mouse. Bull Environ Contam Toxicol 1994; 53(5):648-54.
- (56) Wilkinson FC. Scientific analysis of the data relating to the reclassification of captan under EPA'S new guidelines for carcinogen risk assessment. Captan Task Force; 2003.

- (57) Innes JR, Ulland BM, Valerio MG, Petrucelli L, Fishbein L, Hart ER, et al. Bioassay of pesticides and industrial chemicals for tumorigenicity in mice: a preliminary note. *J Natl Cancer Inst* 1969; 42(6):1101-14.
- (58) Arce GT, Gordon EB, Cohen SM, Singh P. Genetic toxicology of folpet and captan. *Crit Rev Toxicol* 2010; 40(6):546-74.
- (59) Peeples A, Dalvi RR. Toxicologic Studies of N-Trichloromethylthio-4-Cyclohexene-1,2-Dicarboximide (Captan) - Its Metabolism by Rat-Liver Drug-Metabolizing Enzyme-System. *Toxicology* 1978; 9(4):341-51.
- (60) Siegel MR. Reactions of Folpet [N-(Trichloromethylthio) Phthalimide] with Proteins. *Pest Biochem Physiol* 1971; 1(2):225-233.
- (61) Dalvi RR. Metabolism of Captan and Its Hepatotoxic Implications - A Review. *J Environ Biol* 1989; 10(1):81-6.
- (62) Food and Agriculture Organization of the United Nations (FAO). Pesticide residues in food 2007 - Joint FAO/WHO Meeting on Pesticide Residues. Rome, Italy: FAO; 2007.
- (63) Nelson BD. Action of the fungicides captan and folpet on rat liver mitochondria. *Biochem Pharmacol* 1971; 20(4):737-48.
- (64) Cohen SM, Gordon EB, Singh P, Arce GT, Nyska A. Carcinogenic mode of action of folpet in mice and evaluation of its relevance to humans. *Crit Rev Toxicol* 2010; 40(6):531-45.
- (65) Casida JE, Lykken L. Metabolism of Organic Pesticide Chemicals in Higher Plants. *Ann Rev Plant Physiol* 1969; 20:607-636.
- (66) Wolfe NL, Zepp RG, Doster JC, Hollis RC. Captan Hydrolysis. *J Agric Food Chem* 1976; 24(5):1041-5.
- (67) DeBaun JR, Miaullis JB, Knarr J, Mihailovski A, Menn JJ. The fate of N-trichloro(14C)methylthio-4-cyclohexene-1,2-dicarboximide((14C)captan) in the rat. *Xenobiotica* 1974; 4(2):101-19.
- (68) Lukens RJ, Sisler HD. Chemical Reactions Involved in the Fungitoxicity of Captan. *Phytopathology* 1958; 48(5):235-44.
- (69) Gordon EB, Ehrlich T, Mobley S, Williams M. Measurement of the reaction between the fungicides captan or folpet and blood thiols. *Toxicol Methods* 2001; 11(3):209-23.

- (70) Lappin GJ, Havell LM. Captan: Biotransformation study in the rat. Alderley Park, Macclesfield, Cheshire: from ICI Central Toxicology Laboratory; 1990. Report No.: No CTL/P/2951.
- (71) Piccirillo V. Analysis of the toxicology and mechanistic data supporting the cancer reclassification of captan. VJP Consulting, INC., for the Captan Task Force, comprised of Markhteshim-Agan of North America, Inc. and Tomen Agro, Inc.; 2001. Report No.: MRID 45391901.
- (72) Elder RL. Safety Assessment of Cosmetic Ingredients. *J Am Coll Toxicol* 1989;8(4):R6.
- (73) Fisher HL, Hall LL, Sumler MR, Shah PV. Dermal penetration of [<sup>14</sup>C]captan in young and adult rats. *J Toxicol Environ Health* 1992; 36(3):251-71.
- (74) Grissom RE, Brownie C, Guthrie FE. Dermal Absorption of Pesticides in Mice. *Pest Biochem Physiol* 1985; 24(1):119-23.
- (75) Shah PV, Fisher HL, Sumler MR, Monroe RJ, Chernoff N. Comparison of the Penetration of 14 Pesticides Through the Skin of Young and Adult-Rats. *J Toxicol Environ Health* 1987; 21(3):353-66.
- (76) Amarnath V, Amarnath K, Graham DG, Qi QP, Valentine H, Zhang J, et al. Identification of a new urinary metabolite of carbon disulfide using an improved method for the determination of 2-thioxothiazolidine-4-carboxylic acid. *Chem Res Toxicol* 2001; 14(9):1277-83.
- (77) Simon P, Nicot T, Dieudonne M. Dietary habits, a non-negligible source of 2-thiothiazolidine-4-carboxylic acid and possible overestimation of carbon disulfide exposure. *Int Arch Occup Environ Health* 1994; 66(2):85-90.
- (78) Vermeulen R, Jonsson BA, Lindh CH, Kromhout H. Biological monitoring of carbon disulphide and phthalate exposure in the contemporary rubber industry 9. *Int Arch Occup Environ Health* 2005; 78(8):663-9.
- (79) Ackermann H, Faust H, Kagan YS, Voronina VH. Metabolic and Toxic Behaviors of Phthalimide Derivatives in Albino-Rat .2. Placental Passage of Chloromethyl Phthalimide, Oxymethyl Phthalimide, and Phthalimide - Their Fetal Metabolism. *Arch Toxicol* 1978; 40(4):255-61.
- (80) Chasseaud L, Hawkins DR, Franklin ER, Weston KT. The metabolic fate of <sup>14</sup>C-Folpet (Phaltan) in the rat (Folpet). Huntingdon, England: Huntingdon Research Centre Ltd.; 1974. Report No.: CHR1/74482.

- (81) Chasseaud L, Wood SG, Cheng K, Hall M, Fitzpatrick K, Iqbal S, et al. Metabolic fate of  $^{14}\text{C}$ -folpet in Sprague-Dawley rats. Huntingdon, Cambridgeshire, England: Huntingdon Research Centre Ltd; 1991. Report No.: HRC/ MBS 41/91499, Report HRC/ MBS 41-91499.
- (82) Owens RG. Metabolism of fungicides and related compounds. *Ann N Y Acad Sci* 1969; 160(1):114-32.
- (83) Viret O, Dubuis PH, Bloesch B, Fabre AL, Dupuis D. Dosage des fongicides adapté à la surface foliaire en viticulture : efficacité de la lutte. *Revue suisse de viticulture arboriculture horticulture* 2010; 42(4):226-233.
- (84) Reeves M, Schafer KS. Greater risks, fewer rights: U.S. farmworkers and pesticides. *Int J Occup Environ Health* 2003; 9(1):30-9.
- (85) Comité fédéral-provincial-territorial de l'hygiène du milieu et du travail. Guide canadien d'évaluation des incidences sur la santé - Volume 4: Impacts sur la santé par secteur industriel. Ottawa, Canada: Santé Canada; 2004.
- (86) Fenske RA. Nonuniform dermal deposition patterns during occupational exposure to pesticides. *Arch Environ Contam Toxicol* 1990; 19(3):332-7.
- (87) Fenske RA. Dermal exposure: a decade of real progress. *Ann Occup Hyg* 2000; 44(7):489-91.
- (88) Ngo MA, O'Malley M, Maibach HI. Percutaneous absorption and exposure assessment of pesticides. *J Appl Toxicol* 2010; 30(2):91-114.
- (89) Schneider T, Cherrie JW, Vermeulen R, Kromhout H. Dermal exposure assessment. *Ann Occup Hyg* 2000; 44(7):493-9.
- (90) van Hemmen JJ, Brouwer DH. Assessment of dermal exposure to chemicals. *Sci Total Environ* 1995; 168(2):131-41.
- (91) Woollen BH. Biological monitoring for pesticide absorption. *Ann Occup Hyg* 1993; 37(5):525-40.
- (92) Vermeulen R, Stewart P, Kromhout H. Dermal exposure assessment in occupational epidemiologic research. *Scand J Work Environ Health* 2002; 28(6):371-85.
- (93) Blume U, Ferracin J, Verschoore M, Czernielewski JM, Schaefer H. Physiology of the vellus hair follicle: hair growth and sebum excretion. *Br J Dermatol* 1991; 124(1):21-8.

- (94) Cnubben NH, Elliott GR, Hakkert BC, Meuling WJ, van de Sandt JJ. Comparative in vitro-in vivo percutaneous penetration of the fungicide ortho-phenylphenol. *Regul Toxicol Pharmacol* 2002; 35:198-208.
- (95) Curdy C, Naik A, Kalia YN, Alberti I, Guy RH. Non-invasive assessment of the effect of formulation excipients on stratum corneum barrier function in vivo. *Int J Pharm* 2004; 271(1-2):251-6.
- (96) Feldmann RJ, Maibach HI. Percutaneous penetration of some pesticides and herbicides in man. *Toxicol Appl Pharmacol* 1974; 28(1):126-32.
- (97) Fiserova-Bergerova V. Relevance of occupational skin exposure. *Ann Occup Hyg* 1993; 37(6):673-85.
- (98) Guy RH, Maibach HI. Correction Factors for Determining Body Exposure from Forearm Percutaneous-Absorption Data. *J Appl Toxicol* 1984; 4(1):26-8.
- (99) Kao J, Carver MP. Cutaneous Metabolism of Xenobiotics. *Drug Metab Rev* 1990;22(4):363-410.
- (100) Kromhout H, Vermeulen R. Temporal, personal and spatial variability in dermal exposure. *Ann Occup Hyg* 2001; 45(4):257-73.
- (101) Lotte C, Rougier A, Wilson DR, Maibach HI. In vivo relationship between transepidermal water loss and percutaneous penetration of some organic compounds in man: effect of anatomic site. *Arch Dermatol Res* 1987; 279(5):351-6.
- (102) Lotte C, Wester RC, Rougier A, Maibach HI. Racial differences in the in vivo percutaneous absorption of some organic compounds: a comparison between black, Caucasian and Asian subjects. *Arch Dermatol Res* 1993; 284(8):456-9.
- (103) Mangelsdorf S, Otberg N, Maibach HI, Sinkgraven R, Sterry W, Lademann J. Ethnic variation in vellus hair follicle size and distribution. *Skin Pharmacol Physiol* 2006; 19(3):159-67.
- (104) McDougal JN, Jepson GW, Clewell HJ, III, Gargas ML, Andersen ME. Dermal absorption of organic chemical vapors in rats and humans. *Fundam Appl Toxicol* 1990; 14(2):299-308.
- (105) Oesch F, Fabian E, Oesch-Bartlomowicz B, Werner C, Landsiedel R. Drug-metabolizing enzymes in the skin of man, rat, and pig. *Drug Metab Rev* 2007; 39(4):659-98.

- (106) Otberg N, Richter H, Schaefer H, Blume-Peytavi U, Sterry W, Lademann J. Variations of hair follicle size and distribution in different body sites. *J Invest Dermatol* 2004; 122(1):14-9.
- (107) Rougier A, Dupuis D, Lotte C, Roguet R, Wester RC, Maibach HI. Regional variation in percutaneous absorption in man: measurement by the stripping method. *Arch Dermatol Res* 1986; 278(6):465-9.
- (108) Rougier A, Lotte C, Maibach HI. In vivo percutaneous penetration of some organic compounds related to anatomic site in humans: predictive assessment by the stripping method. *J Pharm Sci* 1987; 76(6):451-4.
- (109) Schwindt DA, Wilhelm KP, Maibach HI. Water diffusion characteristics of human stratum corneum at different anatomical sites in vivo. *J Invest Dermatol* 1998; 111(3):385-9.
- (110) Tsai JC, Sheu HM, Hung PL, Cheng CL. Effect of barrier disruption by acetone treatment on the permeability of compounds with various lipophilicities: Implications for the permeability of compromised skin. *J Pharm Sci* 2001; 90(9):1242-54.
- (111) Van Duzee BF. Thermal analysis of human stratum corneum. *J Invest Dermatol* 1975 Oct;65(4):404-8.
- (112) Wester RC, Maibach HI. Cutaneous pharmacokinetics: 10 steps to percutaneous absorption. *Drug Metab Rev* 1983; 14(2):169-205.
- (113) Benford DJ, Cocker J, Sartorelli P, Schneider T, van HJ, Firth JG. Dermal route in systemic exposure. *Scand J Work Environ Health* 1999; 25(6):511-20.
- (114) Lebailly P, Bouchart V, Baldi I, Lecluse Y, Heutte N, Gislard A, et al. Exposure to pesticides in open-field farming in France. *Ann Occup Hyg* 2009; 53(1):69-81.
- (115) Angioni A, Garau VL, Del Real AA, Melis M, Minelli EV, Tuberoso C, et al. GC-ITMS determination and degradation of captan during winemaking. *J Agric Food Chem* 2003; 51(23):6761-6.
- (116) Cabras P, Angioni A, Garau VL, Melis M, Pirisi FM, Farris GA, et al. Persistence and metabolism of folpet in grapes and wine. *J Agric Food Chem* 1997; 45(2):476-9.
- (117) Cabras P, Angioni A, Caboni P, Garau VL, Melis M, Pirisi FM, et al. Distribution of folpet on the grape surface after treatment. *J Agric Food Chem* 2000; 48(3):915-6.



- (118) el-Zemaity MS. Degradation behavior of captan and folpet on greenhouse tomatoes. *Bull Environ Contam Toxicol* 1988; 40(1):80-5.
- (119) el-Zemaity MS. Residues of captan and folpet on greenhouse tomatoes with emphasis on the effect of storing, washing, and cooking on their removal. *Bull Environ Contam Toxicol* 1988; 40(1):74-9.
- (120) Dowling KC, Seiber JN. Importance of respiratory exposure to pesticides among agricultural populations. *Int J Toxicol* 2002; 21(5):371-81.
- (121) de Cock J, Heederik D, Kromhout H, Boleij JSM, Hoek F, Wegh H, et al. Determinants of exposure to captan in fruit growing. *Am Ind Hyg Assoc J* 1998; 59(3):166-72.
- (122) Viret O, Werner S, Bloesch B, Taillens J, Mittaz C. Dosage des fongicides adaptés à la surface foliaire : résultats en 2006. *Revue suisse de viticulture arboriculture horticulture* 2007; 39(1):65-68.
- (123) He F. Biological monitoring of occupational pesticides exposure. *Int Arch Occup Environ Health* 1993; 65(1 Suppl):S69-S76.
- (124) Lauwerys R, Hoet P. Industrial chemical exposure: Guidelines for biological monitoring, 3<sup>rd</sup> edition. Boca Raton: Lewis Publishers; 2001.
- (125) National Research Council. Human Biomonitoring for Environmental Chemicals. Washington, D.C.: The National Academies Press; 2006.
- (126) Needham LL, Patterson DG, Barr DB, Grainger J, Calafat AM. Uses of speciation techniques in biomonitoring for assessing human exposure to organic environmental chemicals. *Anal Bioanal Chem* 2005; 381(2):397-404.
- (127) Draper WM. Biological monitoring: Exquisite research probes, risk assessment, and routine exposure measurement. *Anal Chem* 2001; 73(12):2745-60.
- (128) He F. Biological monitoring of exposure to pesticides: current issues. *Toxicol Lett* 1999; 108(2-3):277-83.
- (129) Weis BK, Balshaw D, Barr JR, Brown D, Ellisman M, Liou P, et al. Personalized exposure assessment: promising approaches for human environmental health research. *Environ Health Perspect* 2005; 113(7):840-8.
- (130) Poet TS, McDougal JN. Skin absorption and human risk assessment. *Chem Biol Interact* 2002; 140(1):19-34.

- (131) Wester RC, Noonan PK. Relevance of animal models for percutaneous absorption. *Int J Pharm* 1980; 7(2):99-110.
- (132) Andersen ME, Dennison JE. Toxicokinetic models: Where we've been and where we need to go! *Hum Ecol Risk Assesst* 2002; 8(6):1375-95.
- (133) Balant LP, Gexfabry M. Physiological Pharmacokinetic Modeling. *Xenobiotica* 1990; 20(11):1241-57.
- (134) Gerlowski LE, Jain RK. Physiologically Based Pharmacokinetic Modeling - Principles and Applications. *J Pharm Sci* 1983; 72(10):1103-27.
- (135) Schoen SR, Winterlin WL. Gas-Chromatographic Determination of the Captan Metabolite Tetrahydrophthalimide in Urine. *J Assoc Official Anal Chem* 1982; 65(6):1382-4.
- (136) Barr DB, Barr JR, Maggio VL, Whitehead RD, Sadowski MA, Whyatt RM, et al. A multi-analyte method for the quantification of contemporary pesticides in human serum and plasma using high-resolution mass spectrometry. *J Chrom B-Anal Technol Biomed Life Sci* 2002; 778(1-2):99-111.
- (137) Blount BC, Milgram KE, Silva MJ, Malek NA, Reidy JA, Needham LL, et al. Quantitative detection of eight phthalate metabolites in human urine using HPLC-APCI-MS/MS. *Anal Chem* 2000; 72(17):4127-34.
- (138) Hauser R, Meeker JD, Duty S, Silva MJ, Calafat AM. Altered semen quality in relation to urinary concentrations of phthalate monoester and oxidative metabolites. *Epidemiology* 2006; 17(6):682-91.
- (139) Jonsson BAG, Richthoff J, Rylander L, Giwercman A, Hagmar L. Urinary phthalate metabolites and biomarkers of reproductive function in young men. *Epidemiology* 2005; 16(4):487-93.
- (140) Kato K, Silva MJ, Needham LL, Calafat AM. Determination of 16 phthalate metabolites in urine using automated sample preparation and on-line preconcentration/high-performance liquid chromatography/tandem mass spectrometry. *Anal Chem* 2005; 77(9):2985-91.
- (141) Kato K, Silva MJ, Needham LL, Calafat AM. Determination of total phthalates in urine by isotope-dilution liquid chromatography-tandem mass spectrometry. *J Chrom B-Anal Technol Biomed Life Sci* 2005; 814(2):355-60.
- (142) Koch HM, Gonzalez-Reche LM, Angerer J. On-line clean-up by multidimensional liquid chromatography-electrospray ionization tandem mass spectrometry for high

throughput quantification of primary and secondary phthalate metabolites in human urine. *J Chrom B-Anal Technol Biomed Life Sci* 2003; 784(1):169-82.

- (143) Lim DS, Shin BS, Yoo SD, Kim HS, Kwack SJ, Alm MY, et al. Toxicokinetics of phthalic acid: The common final metabolite of phthalic acid esters in rats. *J Toxicol Environ Health-Part A-Current Issues* 2007; 70(15-16):1344-9.
- (144) Silva MJ, Slakman AR, Reidy JA, Preau JL, Herbert AR, Samandar E, et al. Analysis of human urine for fifteen phthalate metabolites using automated solid-phase extraction. *J Chrom B-Anal Technol Biomed Life Sci* 2004; 805(1):161-7.
- (145) Silva MJ, Samandar E, Preau JL, Reidy JA, Needham LL, Calafat AM. Quantification of 22 phthalate metabolites in human urine. *J Chrom B-Anal Technol Biomed Life Sci* 2007; 860(1):106-12.
- (146) Mettang T, Alscher DM, Pauli-Magnus C, Dunst R, Kuhlmann U, Rettenmeier AW. Phthalic acid is the main metabolite of the plasticizer di(2-ethylhexyl) phthalate in peritoneal dialysis patients. *Adv Perit Dial* 1999; 15:229-33.
- (147) Bray HG, Hybs Z, Thorpe WV. Metabolism of Derivatives of Toluene .6. Tolunitriles, Benzotrile and Some Related Compounds. *Biochem J* 1951; 48(2):192-9.
- (148) Fenske RA, Birnbaum SG, Methner MM, Soto R. Methods for assessing fieldworker hand exposure to pesticides during peach harvesting. *Bull Environ Contam Toxicol* 1989; 43(6):805-13.
- (149) Geer LA, Cardello N, Dellarco MJ, Leighton TJ, Zendzian RP, Roberts JD, et al. Comparative analysis of passive dosimetry and biomonitoring for assessing chlorpyrifos exposure in pesticide workers. *Ann Occup Hyg* 2004; 48(8):683-95.
- (150) Gunther FA, Iwata Y, Carman GE, Smith CA. The citrus reentry problem: research on its causes and effects, and approaches to its minimization. *Residue Rev* 1977; 67:1-132.
- (151) Ross JH, Driver JH, Cochran RC, Thongsinthusak T, Krieger RI. Could pesticide toxicology studies be more relevant to occupational risk assessment? *Ann Occup Hyg* 2001; 45 Suppl 1:S5-S17.
- (152) Thongsinthusak T, Ross JH, Saiz SG, Krieger RI. Estimation of dermal absorption using the exponential saturation model. *Regul Toxicol Pharmacol* 1999; 29(1):37-43.

- (153) Lebailly P, Vigreux C, Lechevrel C, Ledemeney D, Godard T, Sichel F, et al. DNA damage in mononuclear leukocytes of farmers measured using the alkaline comet assay: discussion of critical parameters and evaluation of seasonal variations in relation to pesticide exposure. *Cancer Epidemiol Biomarkers Prev* 1998; 7(10):917-27.
- (154) Lebailly P, Vigreux C, Lechevrel C, Ledemeney D, Godard T, Sichel F, et al. DNA damage in mononuclear leukocytes of farmers measured using the alkaline comet assay: modifications of DNA damage levels after a one-day field spraying period with selected pesticides. *Cancer Epidemiol Biomarkers Prev* 1998; 7(10):929-40.