HBM4EU project

HBM4EU

science and policy
for a healthy future

2nd HBM4EU Training School 2018

A08 Mycotoxins and Pesticides biomarker analysis

SESSION 3:

PESTICIDE BIOMARKER ANALYSIS

Target (multi) analyte methods

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RIKILT, Wageningen, November 22nd 2018, part of
2nd HBM4EU Training School, Nijmegen, November 19th-23rd, 2018

Outline

Introduction / Pesticides in HBM4EU

Pyrethroids
Chlorpyrifos

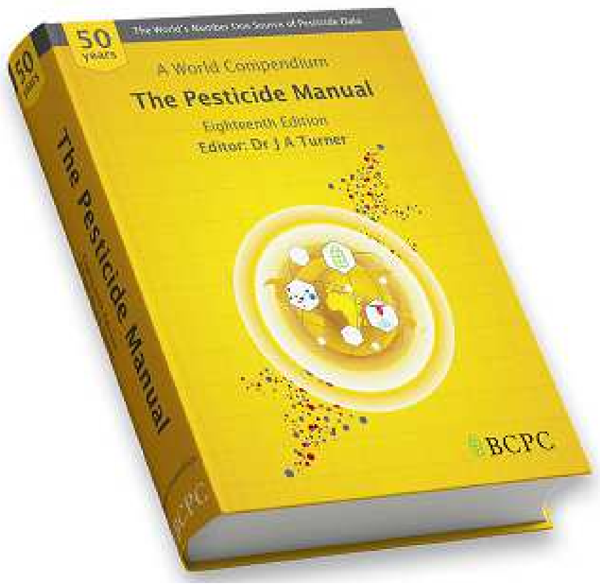
- Biomarkers
- Options for their analysis
- Example ‘development’/implementation

Note: instruments, brand names, methods etc are for information, not HBM4EU endorsements

Pesticides in HBM4EU

<u>Pesticides:</u>

>1600 known pesticides
~700 in use world-wide (other obsolete)
>450 a.i. approved in EU



Included as substance group in
2nd prioritisation round HBM4EU

Prioritisation within the substance group:

Pyrethroids
Chlorpyrifos
Glyphosate

Dimethoate
Fipronil
POE-tallow amine (POEA)

Compound group leader (CGL):
Helle Anderson, University of Southern Denmark

Currently in progress:
- Inventory of biomarker/matrices & methods
- Drafting of Scoping document

Plus: case study exposure residents in WP15 (mixtures)/WP16 non-target analysis

Exposure to pesticides

Pyrethroids
Organophosphorus pesticides
TCPy precursors

General population: food is a/the main route of exposure

Occurrence of pesticide residues in food, EU, 2016 shown are(EFSA, 2018)

https://www.efsa.europa.eu/en/efsa journal/pub/5348

Pesticide	%>LOQ	Pesticide	%>LOQ	Pesticide	%>LOQ
Dithiocarbamates	13.92	Cyhalothrin Lambda-	2.35	Pirimiphos-methyl	0.89
Boscalid	9.47	Mepiquat	2.33	**Dimethoate**	**0.57**
Chlormequat	7.67	Dimethomorph	2.25	Phosmet	0.53
Fludioxonil	6.33	Cypermethrin	2.21	Cyfluthrin	0.22
Imazalil	5.75	Metalaxyl	2.04	Acrinathrin	0.18
Acetamiprid	5.30	Trifloxystrobin	2.00	Fenvalerate	0.18
Cyprodinil	5.27	Spinosad	1.86	Malathion	0.16
Azoxystrobin	4.84	Spirotetramat	1.81	Fenpropathrin	0.15
Fluopyram	4.46	Myclobutanil	1.61	Permethrin	0.15
Chlorpyrifos	**4.42**	2-Phenylphenol	1.49	Profenofos	0.14
Tebuconazole	4.41	Thiamethoxam	1.38	Diazinon	0.12
Pyraclostrobin	4.37	Pyriproxyfen	1.27	**Fipronil**	**0.11**
Imidacloprid	4.12	Prochloraz	1.22	Tau-fluvalinate	0.10
Pyrimethanil	4.12	Deltamethrin	1.20	Fluvalinate	0.09
Thiabendazole	3.60	Etofenprox	1.17	Tefluthrin	0.04
Folpet	3.18	Propiconazole	1.16	Methidathion	0.03
Difenoconazole	3.17	Captan	1.15	**Triclopyr**	**0.03**
Glyphosate	**3.09**	**Chlorpyrifos-methyl**	**1.10**	Tetramethrin	0.02
Carbendazim	2.95	Indoxacarb	1.10	Phoxim	0.01
Chlorantraniliprole	2.86	Bifenthrin	1.08	Flumethrin	0.00
Iprodione	2.66	Pirimicarb	1.06	Phenothrin	0.00
Fenhexamid	2.64	Triadimenol	1.05	Prallethrin	0.00
Propamocarb	2.55	Buprofezin	1.03	Tralomethrin	0.00
Dithianon	2.50	Chlorpropham	0.98	Transfluthrin	0.00
Thiacloprid	2.42	Methoxyfenozide	0.96		

Shown are pesticides with detection frequency >~1%.
For pyrethroids and organophosphorus pesticides other data are also shown

HBM of pesticides

Persistent pesticides: blood, milk, adipose tissue

Non-persistent pesticides: urine

Typical biomarkers:

polar pesticides: parent compound (e.g. glyphosate)

other: phase I metabolites (e.g. hydroxylation, cleavage, ...)

phase II metabolites: glucuronides, sulfates, and more.....

Biokinetics:

Essential for interpretation

Ideally obtained through human volunteer trials

Half-lives of excretion often fast, e.g. 2-8h, after oral exposure

Substantially longer after dermal exposure

HBM of pesticides

Tabel 2.

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

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

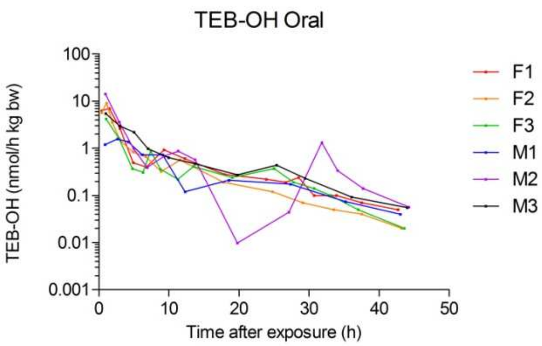
Half-lives of excretion of biomarkers of Mancozeb, Thiabendazole, Pyrimethanil, Chlorpyrifos, 2,4-D, Permethrin, Cypermethrin.

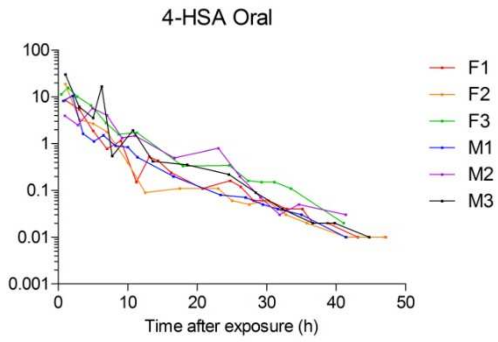
Source: Ekman, PhD Thesis, 2017, Lund University, Sweden

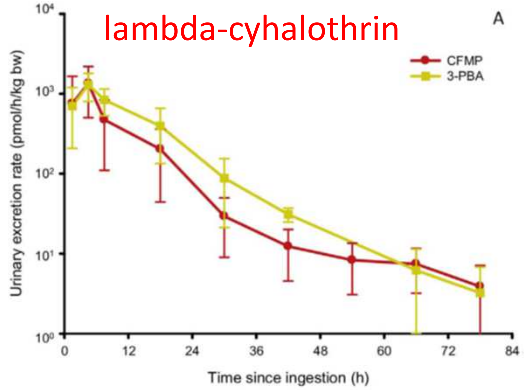
http://portal.research.lu.se/portal/en/publications/biomarkers-of-exposure-to-pesticides-in-humans(6ccb43cf-210b-4b2f-88b1-79dfff44630e).html

HBM of pesticides

Biokinetics data from human volunteer studies







Volunteer studies				Oral		Dermal	
				Excretion 48h	half-life (h)	Excretion 48h	half-life (h)
Pesticide	biomarker	n	time	median ± SD	median ± SD	median ± SD	median ± SD
Tebuconazole	Teb-OH	6	48h	0.32 ± 0.16	8.1 ± 1.0	0.0088 ± 0.0045	15 ± 4.0
Chlorpropham	4-HSA	6	48h	0.31 ± 0.14	4.7 ± 0.3	0.026 ± 0.010	9.4 ± 2.7
Prochloraz	2,4,6-TCP	6	48h	0.017 ± 0.017	24 ± 13	0.00085 ± 0.00076	Unable to determine
Asulam	asulam	6	48h	0.36 ± 0.051	2.8 ± 0.5	0.00046 ± 0.00038	13 ± 3.9
Carbendazim	carb-OH	6	48h	0.40 ± 0.18	5.0 ± 1.4	0.012 ± 0.0059	14 ± 6.2
l-cyhalothrin	3-PBA	7	84h	0.30±0.094	5.9±1.4	0.0008±0.0005	7.4±1.2
l-cyhalothrin	CFMP	7	84h	0.21±0.089	4.2±1.5	0.0012±0.0078	15.4±12.6

A. Oerlemans, R. Nijssen, H. Mol, P. Scheepers,
Volunteer studies performed as part of Project:
Exposure of residents to pesticides, NL, 2016-2018

Khemiri et al.,
Toxicology Letters 276 (2017) 115–121
Toxicology Letters 296 (2018) 132–138

3-PBA: molar excretion fractions 9%–46% (mostly ~25%), Aylward et al, 2018
Reg. Tox. Pharmacology 92:29–38.

Pyrethroids biotransformation

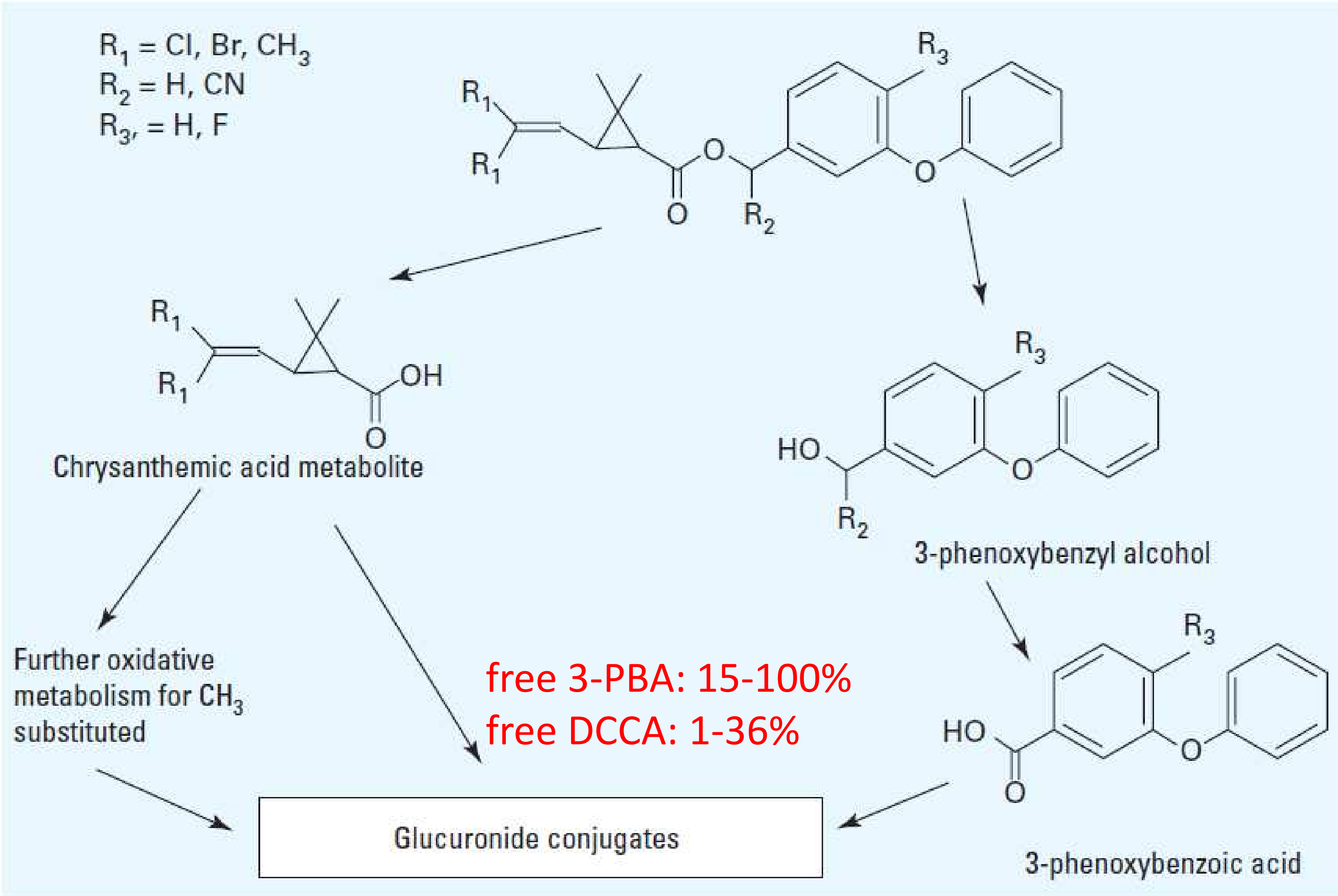
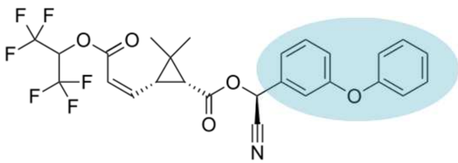
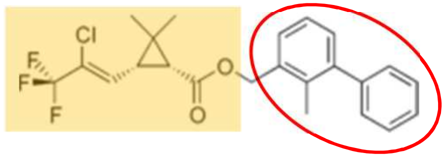


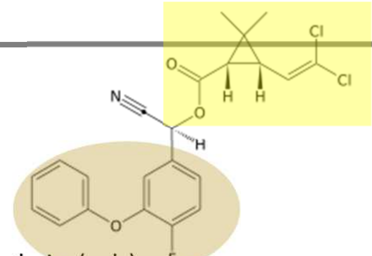
Figure 1. General metabolism of both type I and type II pyrethroid insecticides. Type I insecticides have an R_2 substitution of H, and type II insecticides have an R_2 substitution of CN (cyano group).

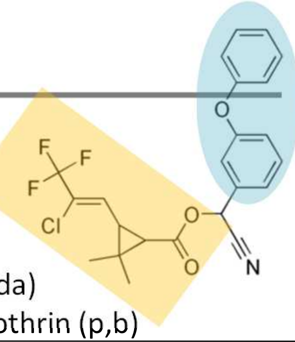
Sources: Barr et al (2010), Env. Health Perspectives 118 :742-748
Baker et al 2004 Arch. Environ. Contam. Toxicol. 46, 281–288.

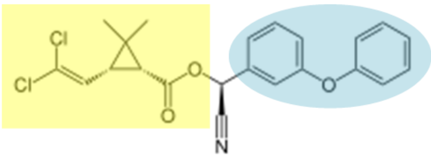
Pyrethroids

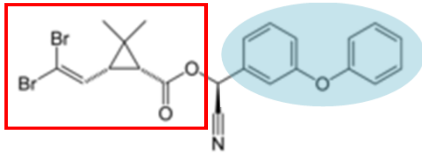

Acrinathrin (p)

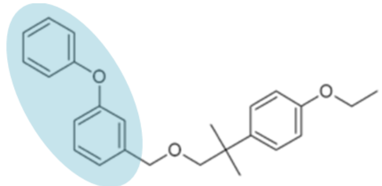

Bifenthrin (p,b)

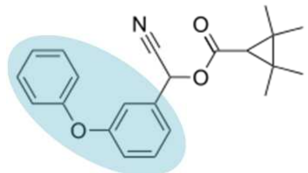

Cyfluthrin (p,b)

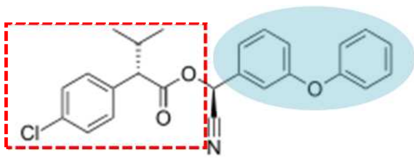

(lambda) Cyhalothrin (p,b)

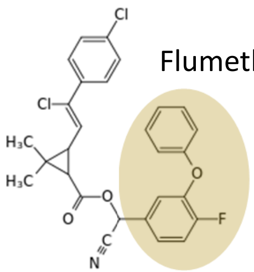

Cypermethrin (p,b,vl)

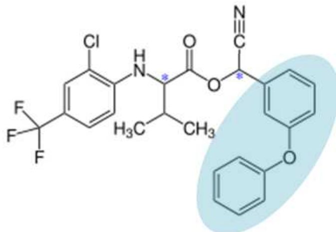

Deltamethrin (p,b,vl,vp)

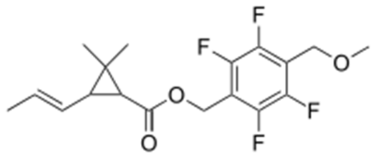

Etofenprox (p,b)

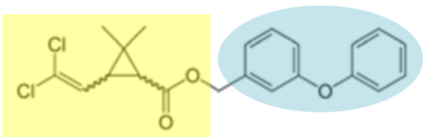

Fenpropathrin (p*)

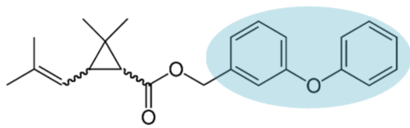

(es)fenvalerate (p)

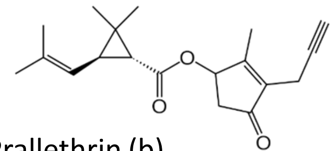

Flumethrin (vl,vp)

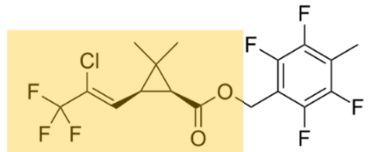

(tau)Fluvalinate (p,vl)


Metofluthrin (b)


Permethrin (b,vl,vp)

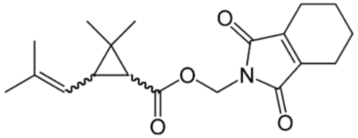

Phenothrin (b)

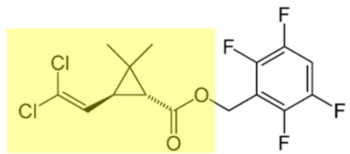

Prallethrin (b)


Tefluthrin (p)

(p) = pesticide
(b) = biocide
(vl, vp) vet drug livestock/pets

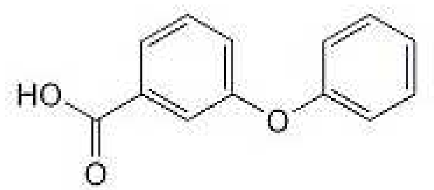
Tetramethrin (b)



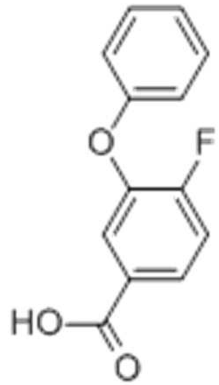

Transfluthrin (b)

Pyrethroids biomarkers

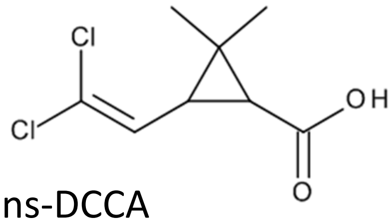
Established urinary biomarkers



3-PBA (common for many pyrethroids

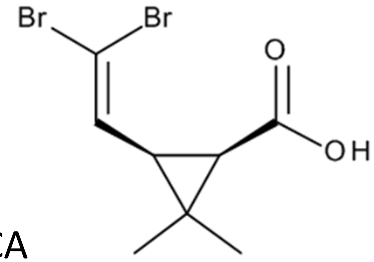


4-F-3-PBA
(for: cyfluthrin, flumethrin)



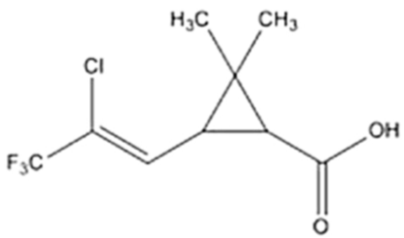
Conjugates!

cis/trans-DCCA
(for: cyfluthrin, cypermethrin, permethrin, transfluthrin?)

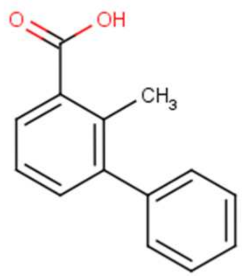


cis-DBCA
(for: deltamethrin)

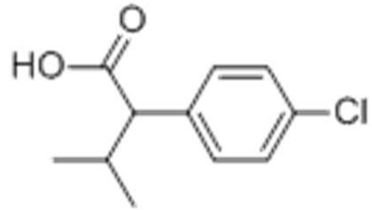
'New'/optional additional biomarkers



CFMP
(for: bifenthrin, cyhalothrin, tefluthrin?



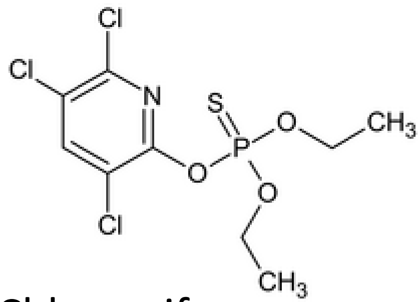
2-methyl-3-phenylbezoic acid (MPA)
(for: bifenthrin)



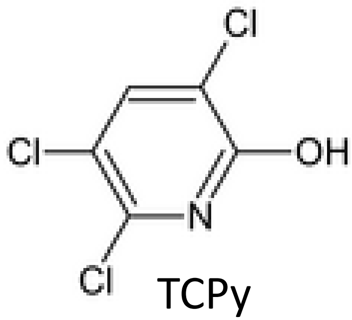
CPBA(?)
For (es)fenvalerate

Chlorpyrifos biomarkers

Established urinary biomarkers



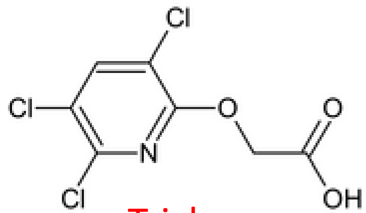
Chlorpyrifos



TCPy
3,5,6-trichloro-2-pyridinol

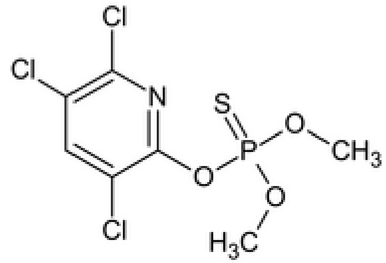
Conjugates!

but also

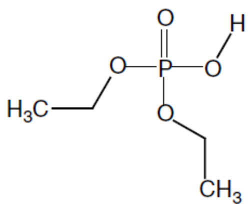


Triclopyr

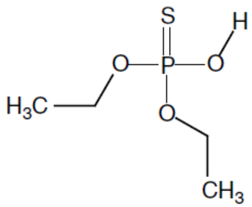
Chlorpyrifos-methyl



Diethyl(thio)phosphates



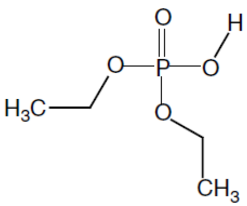
Diethylphosphate (DEP)



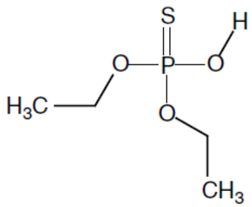
Diethylthiophosphate (DETP)

Non specific biomarkers organophosphorus pesticides

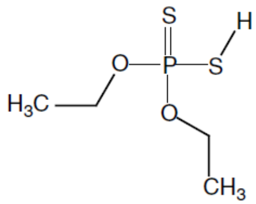
DAPs: dialkylphosphates



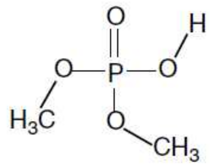
Diethylphosphate (DEP)



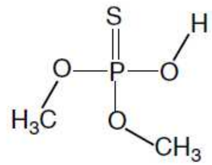
Diethylthiophosphate (DETP)



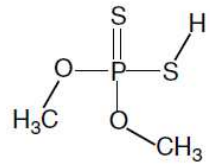
Diethyldithiophosphate (DEDTP)



Dimethylphosphate (DMP)



Dimethylthiophosphate (DMTP)



Dimethyldithiophosphate (DMDTP)

DAPs for example organophosphorus pesticides registered/found in the EU

	ethyl phosphates			methyl phosphates		
	DEP	DETP	DEDTP	DMP	DMTP	DMDTP
chlorpyrifos (p)	x	x				
chlorpyrifos-methyl (p)				x	x	
diazinon (vl, vp)	x	x				
dimethoate (p,b)				x	x	x
malathion (p,b,h)				x	x	x
methidathion				x	x	x
phosmet (p)				x	x	x
phoxim (vl)	x	x				
pirimiphos-methyl (p)				x	x	x
tolclofo-methyl (p)<f>				x	x	

b = biocide
h = human medicine
p = pesticides
vl/vp = vet drug livestock/pets
<f> fungicide

Options for biomarker analysis

Focus on / limitation to:

Pyrethroids: 3-PBA, DCCA, DBCA, 4-F-3-PBA

Chorpyrifos: TCPy

Next slides: options from literature
Question: which one would you select (and why?)

Pyrethroids: 3-PBA, DCCA, DBCA, 4F-3-PBA

GC-based options

<u>Schettgen 2002</u>
10 ml urine + ILIS
1 ml 37% HCl, 1h, 90°C
chemical deconj

Extr. 2x 5 ml hexane
Take hexane phase
Add 2 ml 0.1 N NaOH
Remove hexane
Acidify with 0.1 ml 37% HCl
Re-extr. with 2 ml hexane
Take 1.8 ml hexane
Evaporate to dryness
Acid/base partitioning

Reconst. 50 µl toluene
+10 µl MTBSTFA
45 min, 70°C
derivatisation

1 µl injection (eq to 0.15 ml urine)
GC-EI-MS (SIM mode)
LOD 0.05 ng/ml for all
EI single MS

<u>Fortin 2008</u>
5 ml urine + ILIS
10 µl β-glucuronidase (Helix Pomatia)
5 ml NaAc buffer, pH~5)
16h -overnight 37°C
1 ml 37% HCl
enzymatic deconj

5 ml DCM 2x
Dry down organic phase

Reconst. 250 µl ACN
30 µl F6-IPA/20 µl DIPC 10 min
Stop with 1 ml NaHCO3
Extract with 250 µl hexane > 150 µl
derivatisation

2 µl injection (eq 0.04 ml urine)
GC-NCI-MS (CH_4), SIM
LOD 0.01, 0.008, 0.006, 0.005 ng/ml
NCI single MS

Pyrethroids: 3-PBA, DCCA, DBCA, 4F-3-PBA

LC-based options

Olssen 2004 (CDC)
2 ml urine + ILIS
1.5 ml 0.2 M NaAc pH 4.5
β-gluc./sulf. (Helix Pomatia H-1)
17h, 37°C

enzymatic deconj

SPE OASIS HLB (3 ml)
Cond. 1 ml water, 1 ml MeOH, 1 ml 1% HAc
Wash 5% MeOH/1%Hac water
Elute 1.5 ml MeOH
Evap 50°C/N2
Reconst. 50 µl ACN

Generic SPE cleanup

2 µl injection (eq to 0.08 ml urine)
LC-MS/MS API4000
isocrat 49/51 ACN/water 0.1%Hac
100 mm x 1 mm ID 5 µm Betasil C18
LOD 0.1, 0.2, 0.1, 0.2 ng/ml

LC-MS/MS

Le Grand 2010
5 ml urine + ILIS
1.25 ml 1 M NaAc pH 4.8
20 µl β-gluc. (Helix Pomatia type HP-2)
16 h, 37°C

enzymatic deconj

1 ml 37% HCl
Extr. 2x 6 ml hexane
Take hexane phase
Add 3 ml 0.1 N NaOH
Remove hexane
Acidify with 0.2 ml 37% HCl
Re-extr. with 6 ml hexane
Evaporate hexane to dryness
Reconst. 80 µl 30% MeOH/water 0.1% FA

Acid/base partitioning

10 µl injection (eq to 0.625 ml urine)
LC-MS/MS API5000
LOD 0.015 ng/ml for all

LC-MS/MS

Chlorpyrifos: TCPy, GC-MS based

<u>Schmidt 2015 TCPy (and phenolic EDCs)</u>
1 ml urine + ILIS
0.5 0.4 M NaAc, pH 5)
10 µl β-gluc/AS (Helix Pomatia) — enzymatic deconj
3h, 37°C

SPE Isolut-101 25 mg/1 ml (PS-DVB)
- condition 0.5 ml MeOH, 0.25 ml ACN, 2x 0.5 ml buffer pH 5
- load sample
- wash 0.5 ml buffer, 0.75 ml water, 0.5 ml <u>50% MeOH</u>/water
- elute 0.8 ml ACN — dedicated SPE cleanup

Add 0.2 ml toluene, evaporate to 0.2 ml

+ 30 µl MTBSTFA 30 min RT,
Evap. to 100 µl — derivatization

1 µl injection (eq 0.1 ml urine)
GC-EI-MS/MS Agilent 7000 — EI MS/MS
LOD 0.07-0.2 ng/ml (depending on way of determination)

<u>Morgan 2005</u> — chemical deconj
0.1 ml 37% HCl/ml urine 80°C 1h
LLE (Cl-butane) instead of SPE — LLE
MTBSTFA
GC-EI-MS — EI Single MS

<u>Ormand 1999</u> — chemical deconj
37% HCl/ml urine 80°C 2h
LLE (toluene) instead of SPE — LLE
MTBSTFA
GC-NCI-MS — NCI Single MS

Pyrethroids and chlorpyriphos biomarkers

Davis 2013 (also incl. TCPy)
1 ml urine + ILIS
0.75 ml enzyme/buffer (0.2 M NaAc, pH~5)
β-glucuronidase (Helix Pomatia type H-1)
>6h -overnight 37°C

enzymatic deconj

SPE OASIS HLB 30 mg (96 well format)
condition 0.5 ml acetone, 0.5 ml 1% Hac
wash with 25% MeOH/1% Hac
elute 750 μl acetone
evaporate 40°C/N2
reconstitute 120 μl 25% MeOH/water

Semi-generic SPE cleanup

30 μl injection (eq 0.25 ml urine)
LC-MS/MS Thermo Scientific QuantumUltra
Gradient water 5% MeOH 1% Hac, / ACN
100 mm × 2.1 mm, 3 μm Betasil C18
LOD 0.03, 0.4, 0.4, 0.03 (TCPy 0.1) ng/ml
3-PBA, DCCA, DBCA, 4F-3-PBA

LC-MS/MS

Which method(s) would you choose for determination of Pyrethroid biomarkers and the Chlopyrifos biomarker?

Pyrethroids and chlorpyriphos biomarkers

Davis 2013 (also incl. TCPy)
1 ml urine + ILIS
0.75 ml enzyme/buffer (0.2 M NaAc, pH~5)
β-glucuronidase (Helix Pomatia type H-1)
>6h -overnight 37°C

enzymatic deconj

SPE OASIS HLB 30 mg (96 well format)
condition 0.5 ml acetone, 0.5 ml 1% Hac
wash with 25% MeOH/1% Hac
elute 750 µl acetone
evaporate 40°C/N2
reconstitute 120 µl 25% MeOH/water

Semi-generic SPE cleanup

30 µl injection (eq 0.25 ml urine)
LC-MS/MS Thermo Scientific QuantumUltra
Gradient water 5% MeOH 1% Hac, / ACN
100 mm × 2.1 mm, 3 µm Betasil C18
LOD 0.03, 0.4, 0.4, 0.03 (TCPy 0.1) ng/ml
3-PBA, DCCA, DBCA, 4F-3-PBA

LC-MS/MS

Methods based on Olsson 2004/Davis 2013 used in number of studies and extended with one or more of the following:

IMPY (diazinon)
DEAMPY (pirimiphos-methyl)
PNP (parathion/-methyl)
MDA (malathion)
2,4-D
2,4,5-T
Acetochlor mercapturate
Metolachlor mercapturate
Alachlor mercapturate
Atrazine mercapturate
TEB-OH (tebuconazole)
TBZ-OH (thiabendazole)
PYR-OH (pyrimethanil)

Pyrethroids & chlorpyrifos biomarkers

Example method 'development'/implementation
3-PBA, DCCA, DBCA, 4-F-3-PBA, TCPy

LC-MS or GC-MS?

LC-MS shorter chromatographic run times
More robust / less instrument maintenance
No derivatisation required

MS/MS optimization
(U)HPLC
Extraction/cleanup
Deconjugation
Validation

Pyrethroids & chlorpyrifos biomarkers

MS/MS optimization (infusion)

Pyrethroids biomarkers, organic acids,
no obvious protonation moieties => ESI neg: $[M-H]^-$

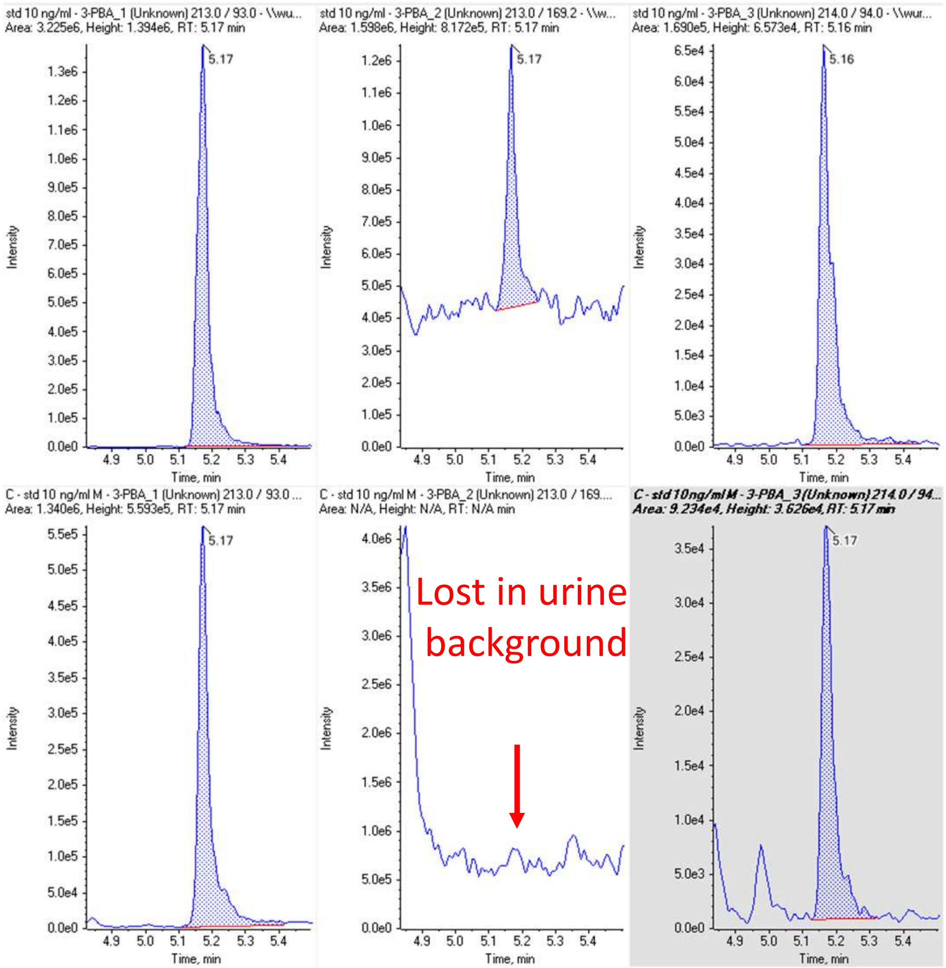
TCPy also ESI neg: $[M-H]^-$

<u>MS/MS issues:</u>
3-PBA: only one 1 sensitive/selective product ion

Pyrethroids & chlorpyrifos biomarkers

3-PBA transitions

Quan m/z 213>93 | Qual m/z 213>169 | m/z 214>94 M+1, ^{13}C-isotope



10 ng/ml in solvent

10 ng/ml in urine extract

Non-sensitive/selective qualifier
13C-isotope not a real alternative qual
but provides ID support

Pyrethroids & chorpyrifos biomarkers

MS/MS optimization

MS/MS fragmentation issues:

3-PBA: only one 1 sensitive/selective product ion

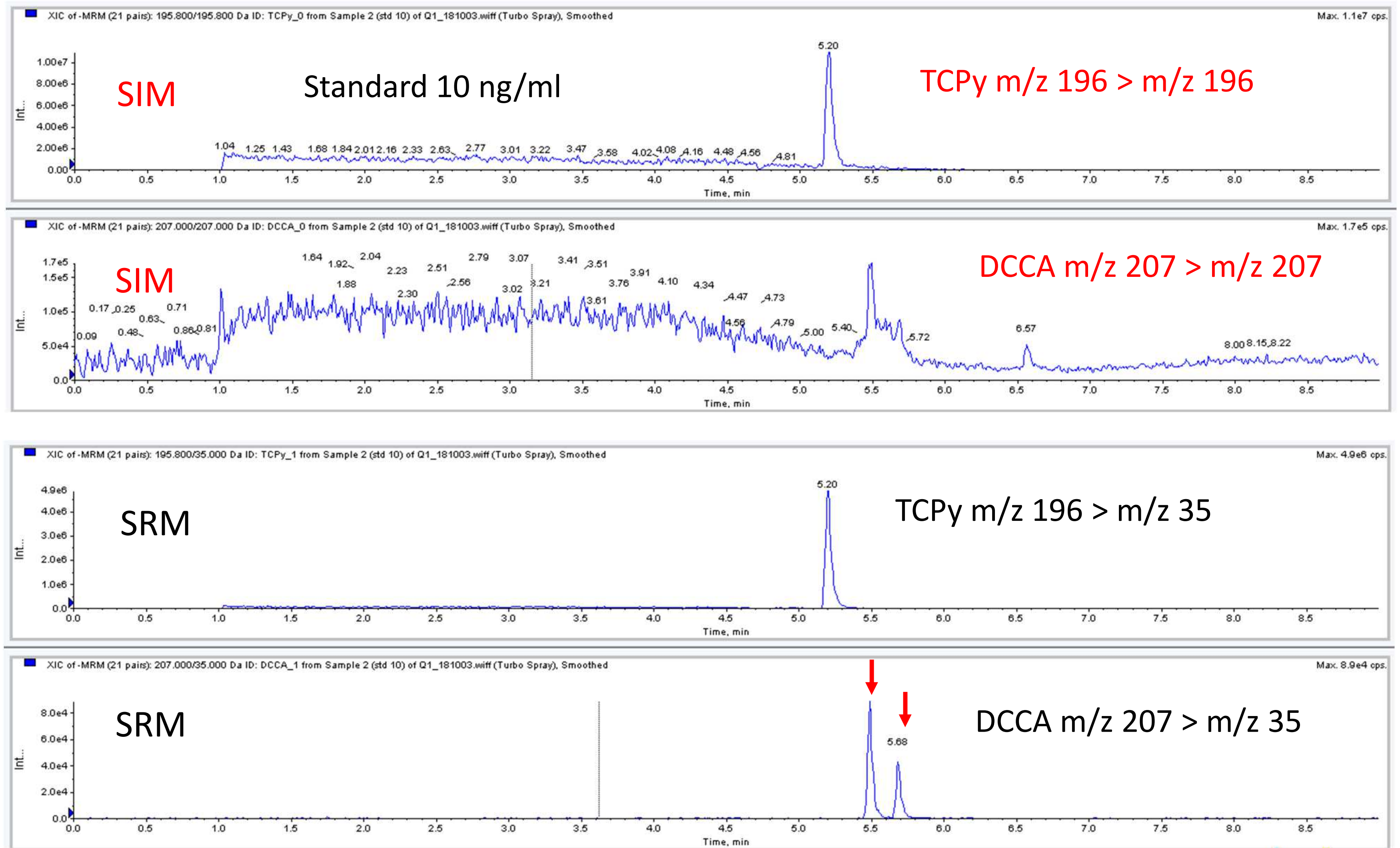
DCCA, DBCA and TCPy: fragmentation yields only Cl or Br
no suitable fragments, only Cl^- (m/z 35, 37) for DCCA and TCPy

Some LC-MS/MS instruments have difficulties to detect Cl^- (m/z 35 & 37)

Issue det. Cl in MS/MS:	MS/MS det. Cl successful with e.g.
Thermo Quantum Ultra [Davis 2013]	API3000 [Baker 2004]
Thermo TSQ 7000 [Olssen 2004]	API4000 [Olssen 2004],
Waters TQ-S [Gari 2018]	API5000 [Le Grand 2010]
Sciex Qtrap 6500 [RIKILT]	Sciex 5500 Qtrap [Ekman 2017] [RIKILT]
Waters TQ-(X)S [RIKILT]	Sciex Qtrap 6500+ (?)

Pyrethroids & chorpyrifos biomarkers

Alternative: SIM instead of SRM (CE ~5)



Sciex 5500 Qtrap

Pyrethroids & chorpyrifos biomarkers

SIM instead of SRM (CE low) m/z 207 > m/z 207

Improvement of S/N

Option 1: narrow down precursor-ion window of Q1 from ±0.7 to ±0.2

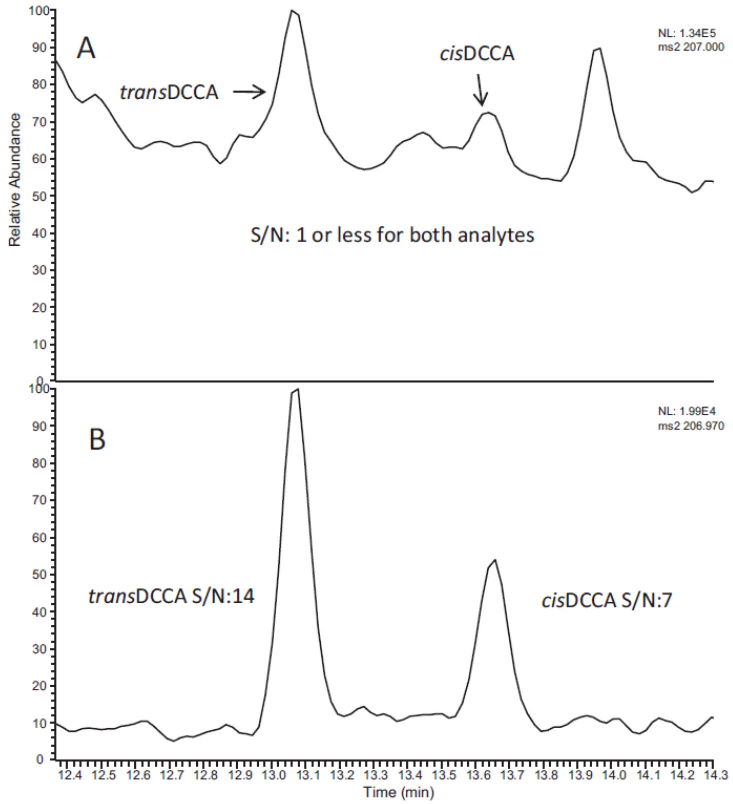


Fig. 3. Comparison of (A) low resolution analysis to (B) high resolution analysis of DCCA isomers in the same injection.

Q1 m/z 206.97 ±0.2

Q1 m/z 207 ±0.7

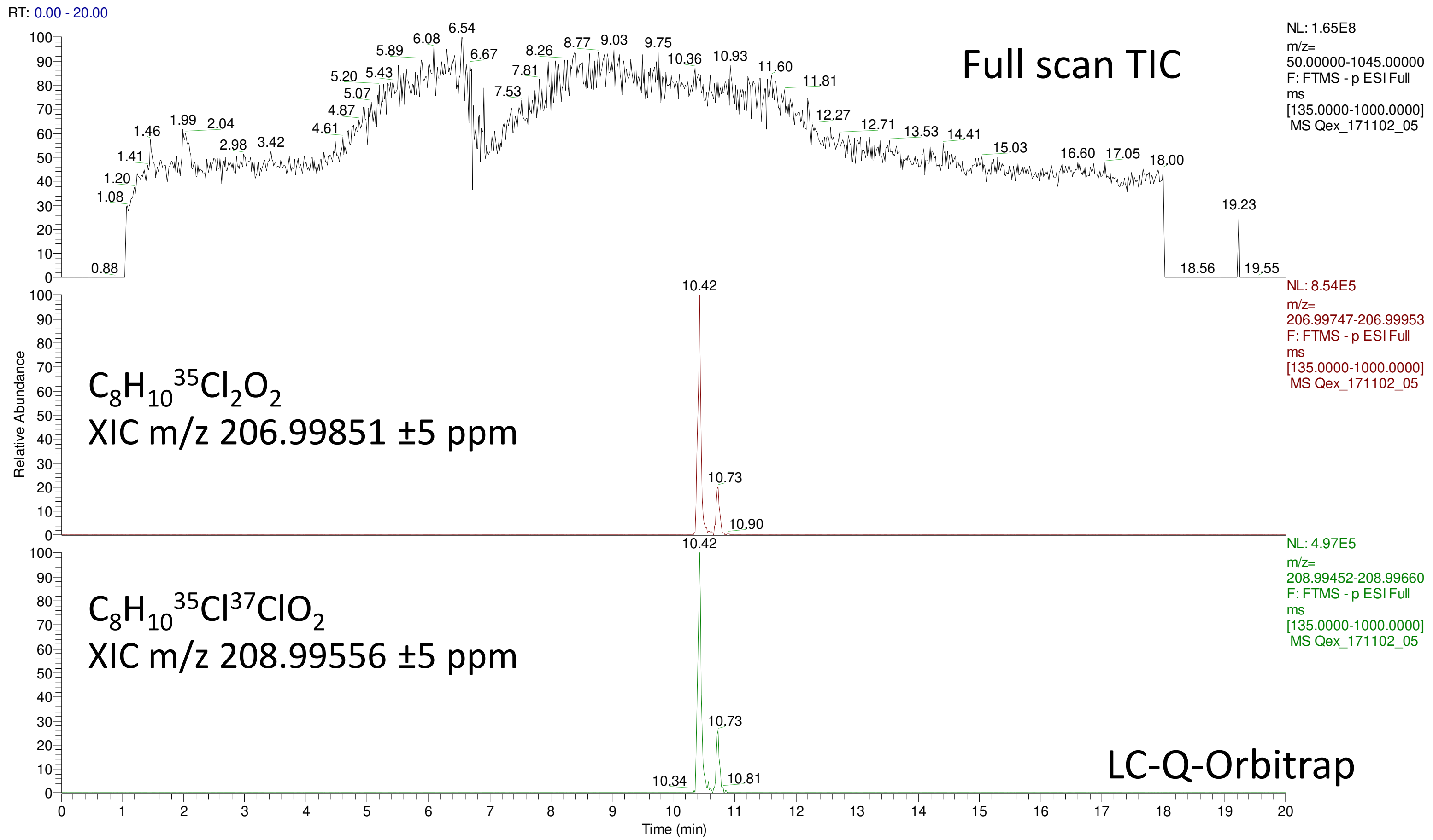
Thermo Quantum Ultra triple quad

Davis et al, J. Chromatogr. B, 929 (2013) 18– 26.

Pyrethroids & chorpyrifos biomarkers

Option 2: LC-HRMS

Standard DCCA 100 ng/ml



5500 Qtrap MS/MS more sensitive than LC-Q-Orbitrap

Pyrethroids & chorpyrifos biomarkers

(U)HPLC-MS/MS

Ultra Aqueous C18, 3 µm, 2.1 x 100 mm
Flow rate 0.4 ml/min, 40°C

Eluent can affect:
Chromatography
MS sensitivity

Slightly better peak shapes
for acetonitrile as modifier
Little effect on sensitivity

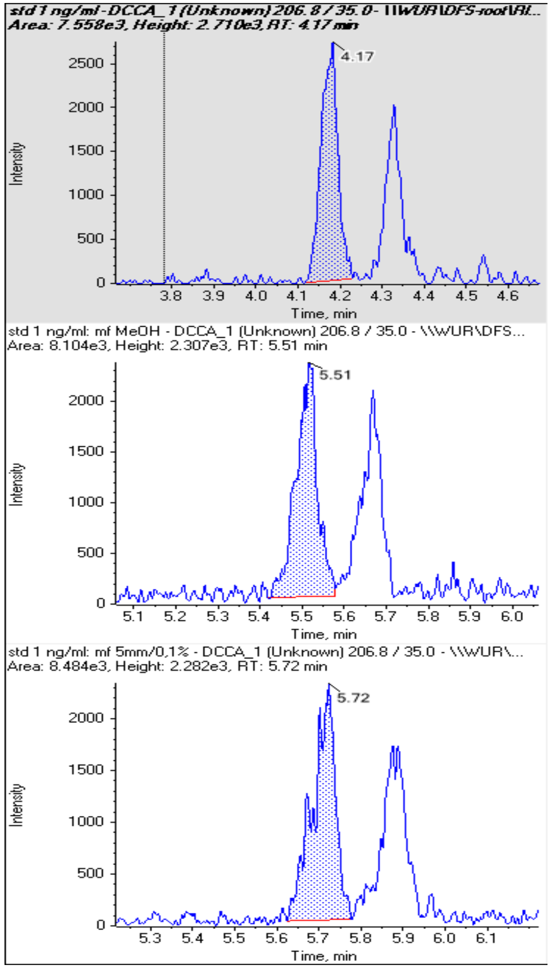
Injection volume:
Standards/extracts in ACN/water 20/80,
up to 50 µl no peak distortion

trans & cis DCCA (1 ng/ml)

0.1% FA/ACN

0.1%FA/MeOH

5mM NH_4Form
0.1% FA/MeOH



Pyrethroids & chorpyrifos biomarkers

Extraction/cleanup:

<u>Options:</u>

LLE

SPE
- OASIS HLB
- OASIS MAX
- Strata-X
- ......

} all non-polar, functionalised, max: also anion exchange

<u>Aim: 10x concentration & cleanup</u>

Load/wash: trap target analytes (but not matrix)

Elute: release target analytes (but not matrix)

Wash: water, increasing %MeOH/water,

Elution: different organic solvents/pH

Evaluate: recovery target analytes

matrix effect

Pyrethroids & chorpyrifos biomarkers

Extraction/cleanup, example results:

OASIS MAX	3-PBA		DCCA		DBCA	
Elution	rec%	ME	rec%	ME	rec%	ME
0.1% Acetic acid	0%	67%	75%	71%	77%	75%
1% acetic acid	17%	45%	83%	49%	90%	38%
2% formic acid	103%	27%	90%	20%	100%	20%

ME = matrix effect, ion suppression expressed here as: area cleaned urine extract/area solvent)

OASIS MAX: ⇒ Good recovery / high matrix effects
⇒ Low matrix effect / poor recovery

	3-PBA	DCCA	DBCA
	matrix effect		
OASIS HLB			
Urine 1	20%	19%	19%
Strata-X			
Urine 1	41%	54%	59%
Urine 2	56%	62%	62%
Urine 3	36%	41%	38%
Urine 4	45%	61%	50%

Good recovery for both OASIS HLB and Strata-X

Cleaner extracts/lower matrix effects with Strata-X

Pyrethroids & chorpyrifos biomarkers

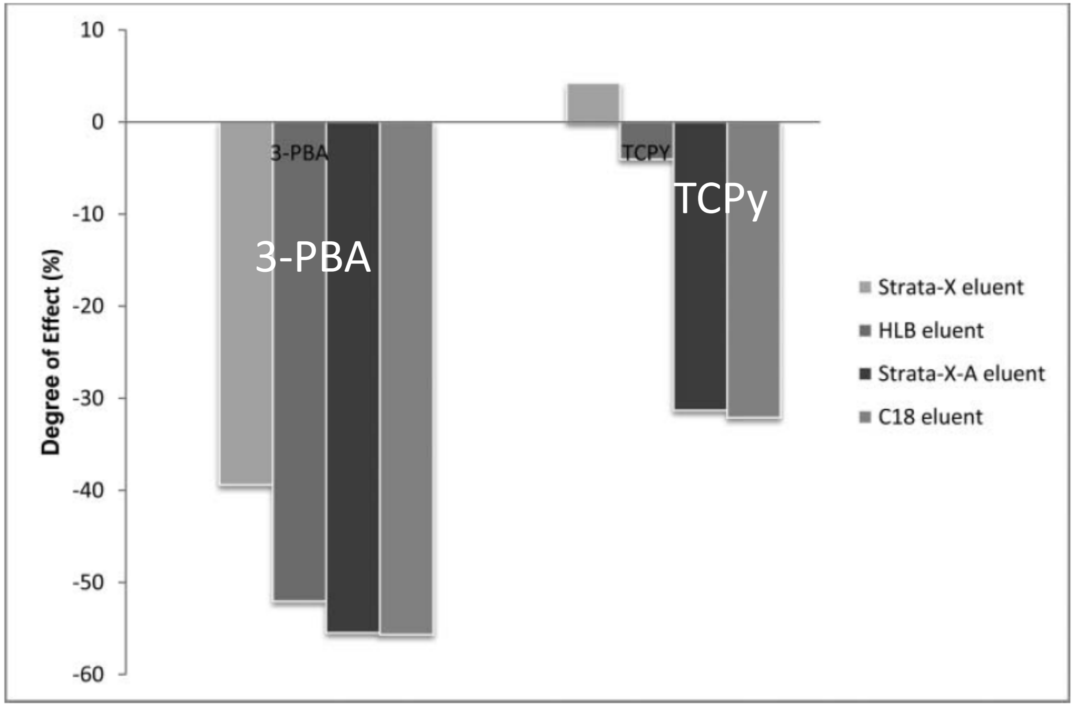


Figure 5. Potential matrix effects associated with type of cartridge used. Matrix effects were investigated using a post-extraction spike matrix comparison. Experiments began with extracting 1 mL of urine (three replicates) using four different cartridges: Oasis HLB (3 cc, 60 mg; Waters Corp., Milford, MA), Strata-X (3 cc, 200 mg; Phenomenex, Torrance, CA), Strata-XA (3 cc, 60 mg; Phenomenex), and C18 (6 cc, 500 mg; JT Baker, Center Valley, PA). Generic extraction methods were followed per each manufacturer. Prior to evaporation, the eluents were spiked with standard containing 10 ppb of target compounds. Dried residues were reconstituted with 100 μL of 30:70 MeOH:water (v/v), and 10 μL was injected into a QQQ 6490 LC-MS/MS system (Agilent Technologies, Waldbronn, Germany) coupled with a negative ESI interface. The LC and MS modules were programmed and controlled using Mass Hunter Software version B.04.01 (Agilent Technologies). The chromatographic and MS/MS parameters were set the same as those mentioned in Figure 3. An average response of quantitative MS/MS transition for 3-PBA (m/z 213→99 @ 15 V CE) and quantitative pseudo-MS/MS transition for TCPY (m/z 207→207 @ 1 V CE) was calculated. The degree of matrix effects (%) was calculated in a manner similar to that in Figure 3.

Panuwet et al. (2016) Crit. Rev. in Anal. Chem. 46:93–105

Pyrethroids & chorpyrifos biomarkers

Deconjugation:
Chemical or enzymatic?
β-glucuronidase/arylsulfatase Helix Pomatia or β-glucuronidase E. coli

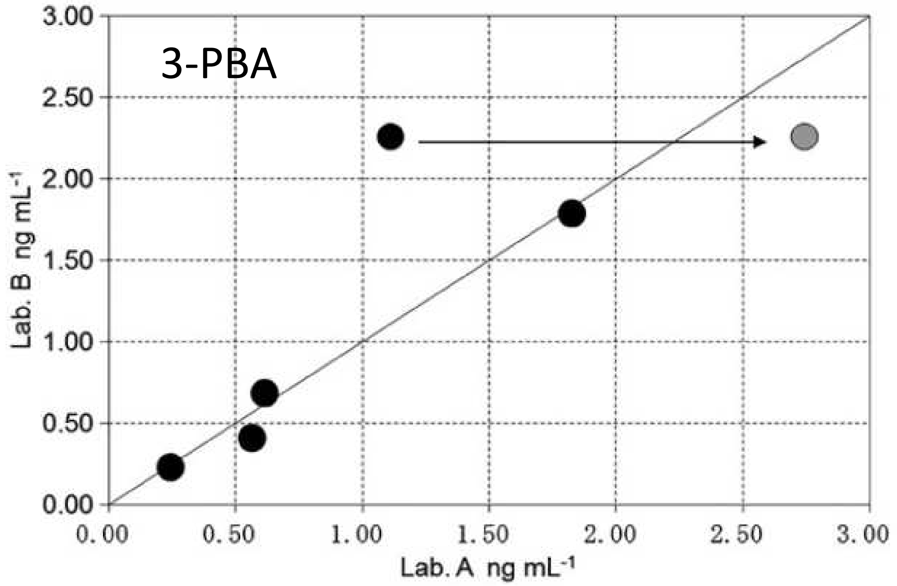


Figure 1. Comparison of urinary 3-PBA concentration determined in Lab A (LC–MS-MS) and Lab B (GC–MS). Gray mark denotes 3-PBA concentration in urine of ID. 3 determined in Lab A using acidic deconjugation while others (black circles) were determined using enzymatic deconjugation in Lab A. Arrow indicates change of 3-PBA concentration from enzymatic to acidic deconjugation. In Lab B acidic deconjugation was used for all of the samples.

Lab A: enzymatic deconjugation (Helix Pomatia, pH 4.5) (LC-MS/MS)

Lab B: chemical deconjugation (HCl, 100°C) (GC-MS)

3-PBA in 42 urine samples
Some higher with chemical deconj.

Toshima et al, J. Anal Toxicol. 2015;39:133–136

Pyrethroids & chorpyrifos biomarkers

Implemented method:

5 ml urine + ILIS ($^{13}C_6$-3-PBA, $^{13}C_6$-4-F-3-PBA, D6-t-DCCA @ 10 ng/ml)
2.5 ml 0.2 M NaAc, pH 4.5
25 µl β-glucuronidase/arylsulfatase (Helix Pomatia)
overnight, 37°C

SPE Strata-X 200 mg/6 ml
- condition 5 ml MeOH, 5 ml water
- load sample
- wash 10 ml 1% formic acid, 10 ml 50%MeOH/1%FA/water
- dry 60 s, elute 6 ml acetone

Evaporate 40°C/N2

Reconstitute in 0.5 ml ACN/water 20/80
50 µl injection (eq. 0.5 ml urine) into LC-MS/MS

Pyrethroids & chorpyrifos biomarkers

LC-MS/MS conditions

Ultra Aqueous C18, 3 µm, 2.1 x 100 mm
Flow rate 0.4 ml/min, 40°C
Eluent A: 0.1% formic acid in water
Eluent B: 0.1% formic acid in acetonitrile
Gradient: 30% B (0.5 min) to 100% B in 6 min (2 min), back to 30%/recondition (10 min run)

ESI negative mode

Analyte	Quan	Qual
DCCA	207>35	209>35
t-DCCA-D6	213>35	
DBCA	297>79	297>81
3-PBA	213>93	213>169
13C6_3-PBA	219>99	
4-F-3-PBA	231>93	231>187
13C6_4-F-3-PBA	237>99	99
TCPy	196>35	198>35
13C3_TCPy	199>35	

Optionally other isotope transitions

Pyrethroids & chorpyrifos biomarkers

<u>Initial validation</u>

reagent blank

6 urines (as blank as possible)

3 concentrations

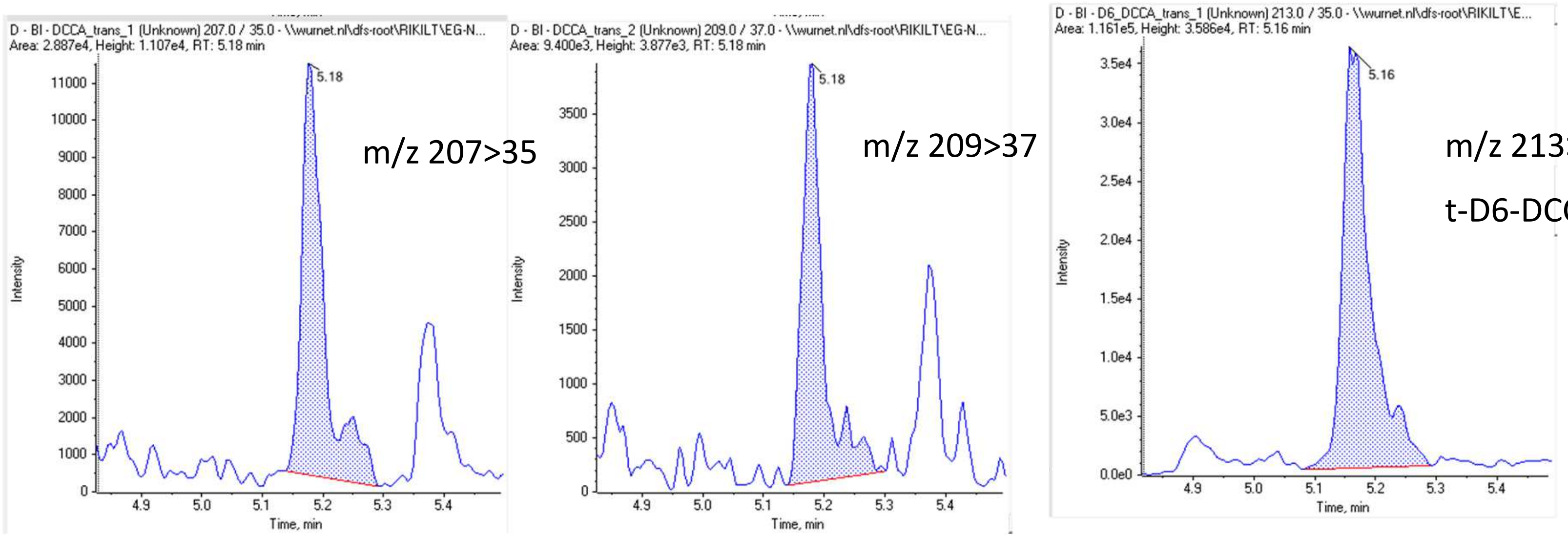
Cal. Standards 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5 ng/ml

	0.1 ng/ml		0.5 ng/ml		2.5 ng/ml	
	rec%	RSD	rec%	RSD	rec%	RSD
c/t-DCCA	100%	7%	90%	11%	97%	8%
*c-DBCA**	*81%*	*11%*	*77%*	*10%*	*85%*	*8%*
3-PBA	86%	14%	88%	10%	98%	10%
4-F-3-PBA	106%	5%	115%	9%	118%	5%
TCPy	pos		108%	9.1%	101%	5.8%

*matrix matched calibration

Pyrethroids & chorpyrifos biomarkers

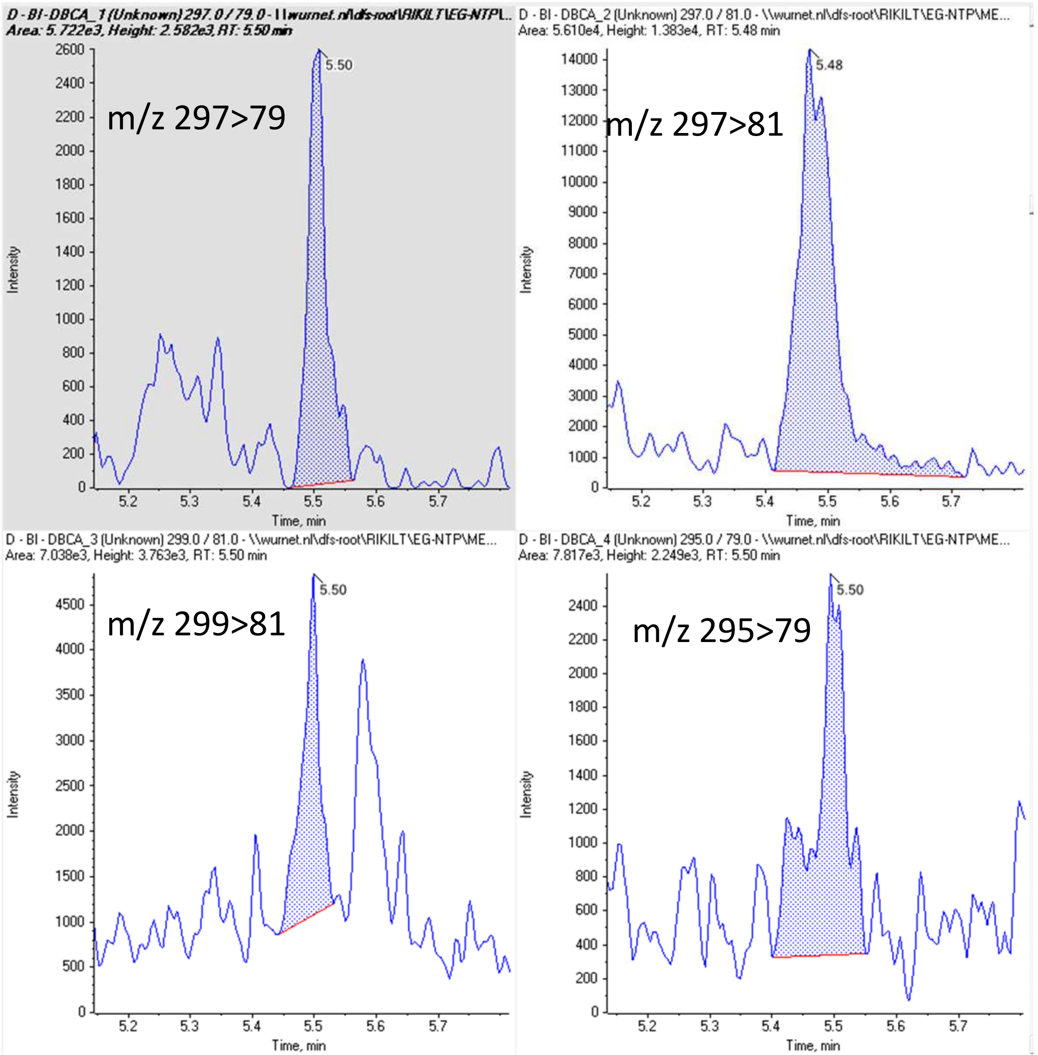
Example chromatograms



Urine sample containing DCCA 0.25 ng/ml

Pyrethroids & chorpyrifos biomarkers

Example chromatograms

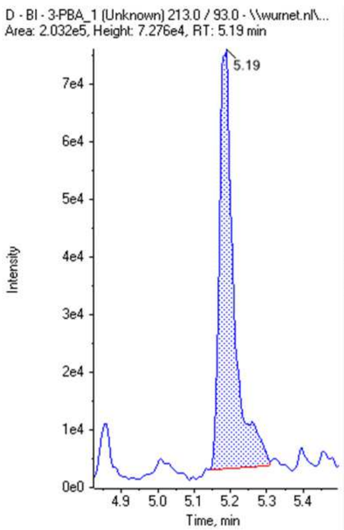


Urine sample
cis-DBCA 0.19 ng/ml

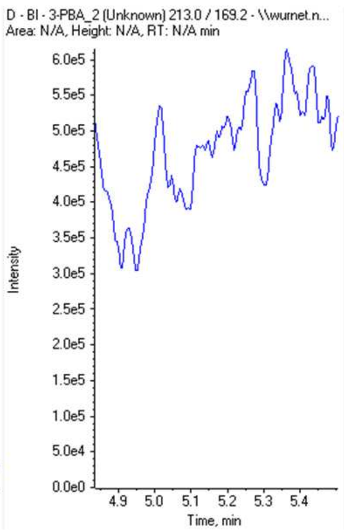
Pyrethroids & chorpyrifos biomarkers

Example chromatograms

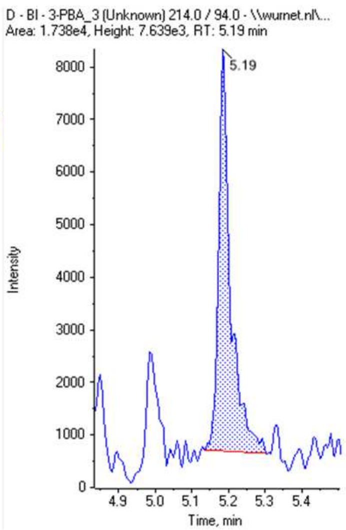
Quan
m/z 213>93



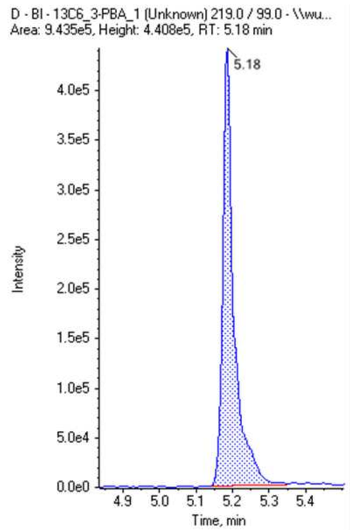
Qual
m/z 213>169



m/z 214>94
M+1, ^{13}C-isotope



$^{13}C_6$-label
m/z 219>99



Urine sample
3-PBA 0.18 ng/ml

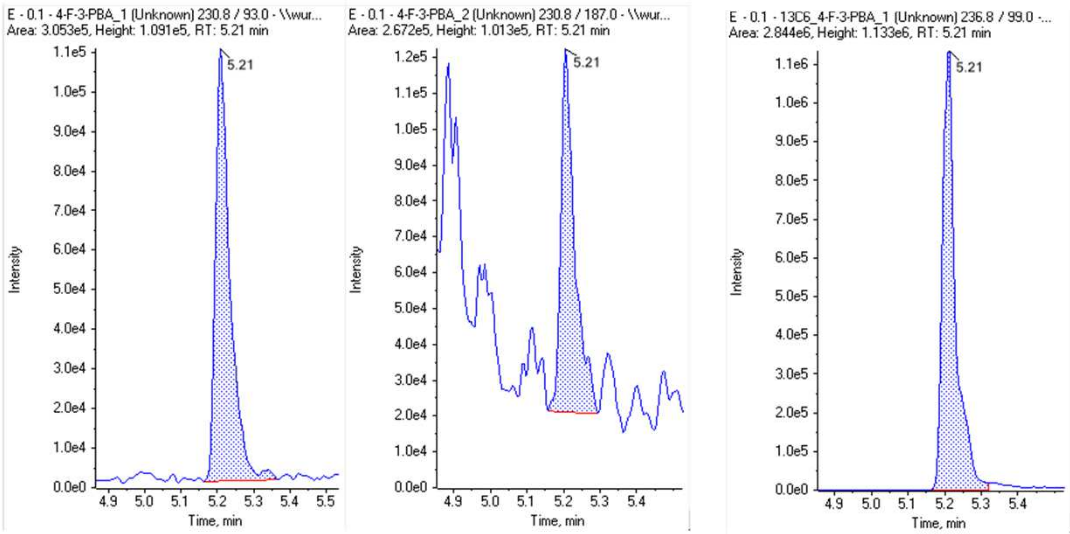
Pyrethroids & chorpyrifos biomarkers

Example chromatograms

Quan
m/z 231>93

Qual
m/z 231>187

$^{13}C_6$-label
m/z 237>99



Urine sample spiked with 4-F-3-PBA @ 0.1 ng/ml

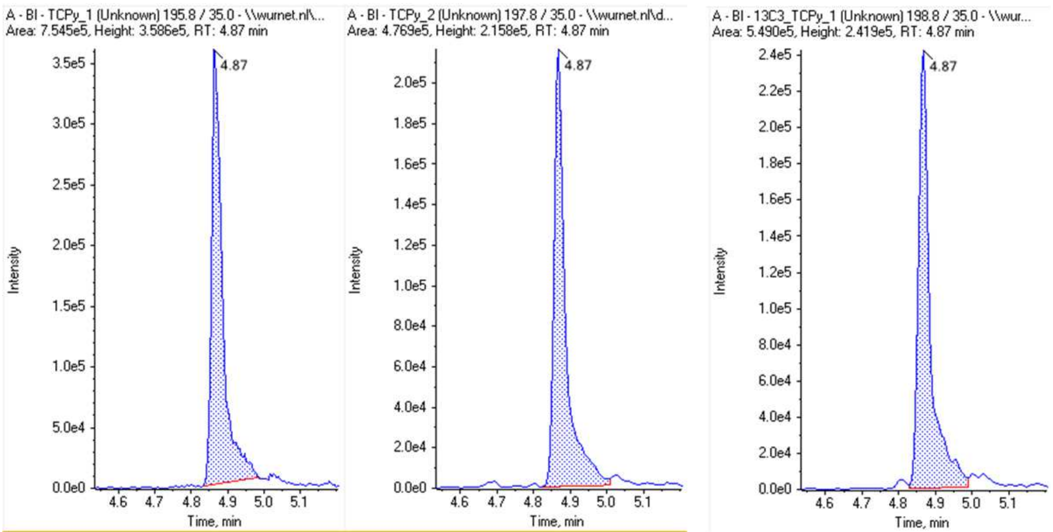
Pyrethroids & chorpyrifos biomarkers

Example chromatograms

Quan
m/z 196>35

Qual
m/z 198>35

$^{13}C_3$-label
m/z 199>35



Urine sample containing TCPy 0.22 ng/ml

Extension to other (pesticide) biomarkers?

Possibilities:

Inclusion of several other pesticide biomarkers already demonstrated
In principle further extension possible, even beyond pesticides...

Requires (even) more generic extraction/cleanup procedures

Generic SPE PS-DVB Weak wash	'QuEChERS'* approach ACN extraction + salt-out

Limitations:

Compatibility deconjugation procedure enzyme/pH, ...?
More generic cleanup => dirtier extracts, more matrix effects
Limited availability of isotopic labels of 'new' biomarkers

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Speaker's information

Hans Mol is senior scientist at the department of Natural Toxins and Pesticides, RIKILT, Wageningen, The Netherlands. He is an analytical chemist with more than 20 years of experience in determination of pesticides, mycotoxins, and their metabolites in food, environmental and biological samples.

In HBM4EU he is involved in WP9 (9.4 Quality Assurance, organisation of ICI/EQUAS, 9.1 biomarker/method inventory)

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