HBM4EU project

HBM4EU

science and policy
for a healthy future

2nd HBM4EU Training School 2018

A08 Mycotoxins and Pesticides biomarker analysis

Hair; an alternative matrix for HBM of pesticides?

Rosalie Nijssen

Biomonitoring of current pesticides

Matrix	Time window of exposure	Issues
Serum	short-term	invasive
Urine	short-term	metabolites
Milk	short-term	metabolites
Adipose tissue	less applicable to modern pesticides	invasive
Hair	long-term	not yet established

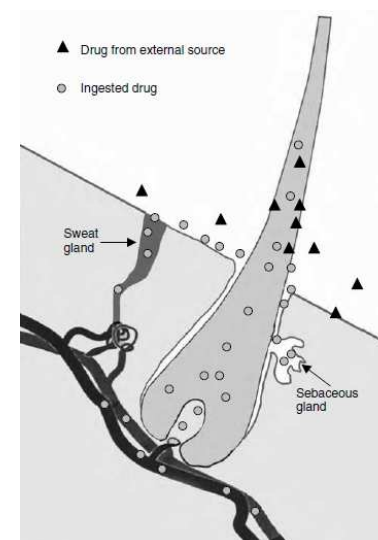Incorporation routes*:
Internal: directly from blood supply
        indirectly from sweat and sebum
External: contamination

Rate of incorporation is compound dependent

Cumulative exposure over months

* Kintz et al, Hair Analysis in
Clinical and Forensic Toxicology, 2015

Biomonitoring using hair

Hair:

Established in forensic analysis/clinical toxicology
(drugs of abuse, doping, illegal treatment of livestock)
Emerging for exposure to (food) contaminants

Advantages:

- Sampling, easy non-invasive, protocols exist*
- Easy storage (RT/dark, stable)
- Many compounds incorporated as such (analytical standards available)
- Exposure history through analysis of hair segments
- Average growth: 1 cm/month

Challenges:

- Incorporation rates of compounds are unknown
- Possible of contamination on outside of hair

* Cooper et al,
Forensic Science Int. 218 (2012) 20–24

Recent publications

Long-term occupational and environmental exposure to penconazole and tebuconazole by hair biomonitoring

Rosa Mercadante[a,*], Elisa Polledri[a], Angelo Moretto[b], Silvia Fustinoni[a]

[a] EPIGET – Epidemiology, Epigenetics, and Toxicology Lab, Department of Clinical Sciences and Community Health, Università degli Studi di Milano and Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy
[b] Dipartimento di Scienze Biochimiche e Cliniche, Università degli Studi di Milano, and International Centre for Pesticides and Health Risks Protection (ICPS), ASST Fatebenefratelli Sacco, Ospedale "Luigi Sacco", Via GB Grassi 74, 20157 Milano, Italy

Pesticide Exposure of EU Population
PILOT PROJECT - Hair Biomonitoring Campaign – 6 Countries
The Greens / European Free Alliance in the European Parliament

Determination of farm workers' exposure to pesticides by hair analysis

Claude Schummer[a,c], Guillaume Salquèbre[a], Olivier Briand[b], Maurice Millet[c], Brice M.R. Appenzeller[a,*]

[a] Laboratory of Analytical Human Biomonitoring – CRP-Sante, Université du Luxembourg, 162A avenue de la Faïencerie, L-1511, Luxembourg
[b] French Agency for Food, Environmental and Occupational Health and Safety (ANSES) - Risk Assessment Department – 27-31 avenue du Général Leclerc F-94700 Maisons-Alfort, France
[c] Equipe de Physico-Chimie de l'Atmosphère – LMSPC (UMR 4515 CNRS-Université de Strasbourg) – 1 rue Blessig – F-67084 Strasbourg Cedex, France

Analysis of House Dust and Children's Hair for Pesticides: A Comparison of Markers of Ongoing Pesticide Exposure in Children

Enrique M. Ostrea Jr.[1,*], Esterlita Villanueva-Uy[2], Dawn Bielawski[1], Sarah Birn[1] and James J. Janisse[3]

[1] Department of Pediatrics, Hutzel Women's Hospital, the Carman and Ann Adams Department of Pediatrics, Children's Hospital of Michigan, Wayne State University, Detroit, MI, USA
[2] Institute of Child Health and Human Development, University of the Philippines Manila National Institutes of Health, Manila, Philippines
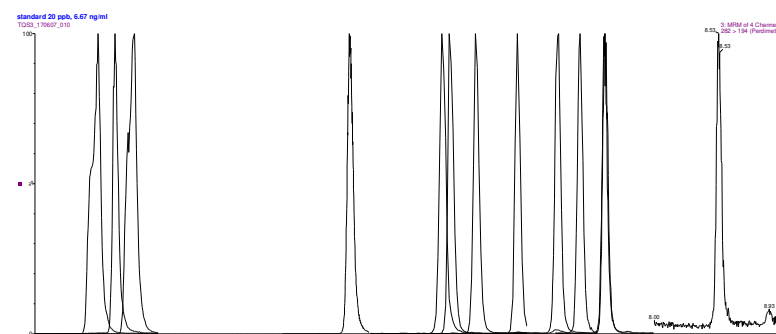[3] Department of Family Medicine and Public Health Sciences, Wayne State University, Detroit, MI, USA

Hair, the matrix

65-95% proteins (keratins)
1-9% lipids (triglycerides, free fatty acids, wax, cholesterol-esters, squalene, paraffins)
0.1-5% pigments (melanine)
Trace elements, polysaccharides, water, …

- Regularly detected in food 1 multi-residue method
- High application rates in NL agriculture $\Rightarrow$ LC-ESI$^+$-MS/MS

Acetamiprid	I	Imazalil	F
Azoxystrobin	F	Imidacloprid	I
Boscalid	F	Kresoxim-methyl	F
Carbendazim	F	Metolachlor	H
Cyproconazole	F	Pendimethalin	H
Cyprodinil	F	Prochloraz	F
Difenoconazole	F	Pyraclostrobin	F
Diflufenican	H	Pyrimethanil	F
Epoxiconazole	F	Tebuconazole	F
Ethofumesate	H	Thiabendazole	F/P
Flonicamid	I	Thiabendazole-5OH	met
Fludioxonil	F	Thiacloprid	I
Fluopyram	F	Trifloxystrobin	F

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Aim: remove surface residues
            standardize the sample

Experimental outline:
Hair sample with incurred residues
Take 'equivalent' segments
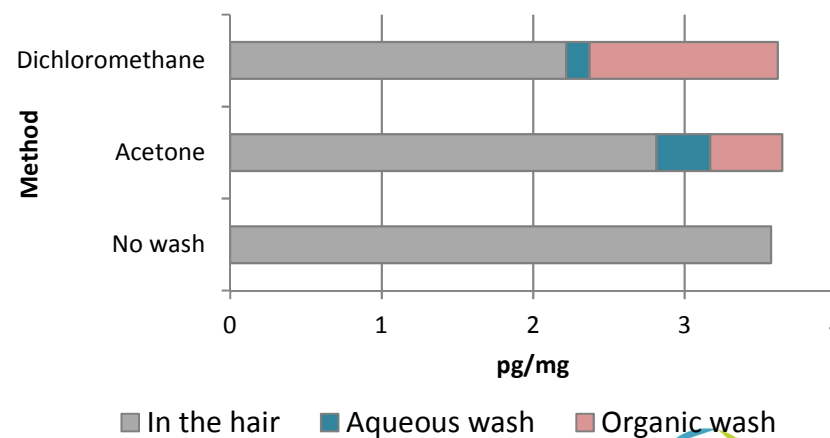Wash with water/organic solvent | dry | pulverize

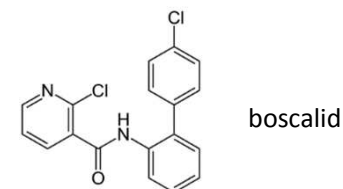analyse   analyse   analyse

Azoxystrobin
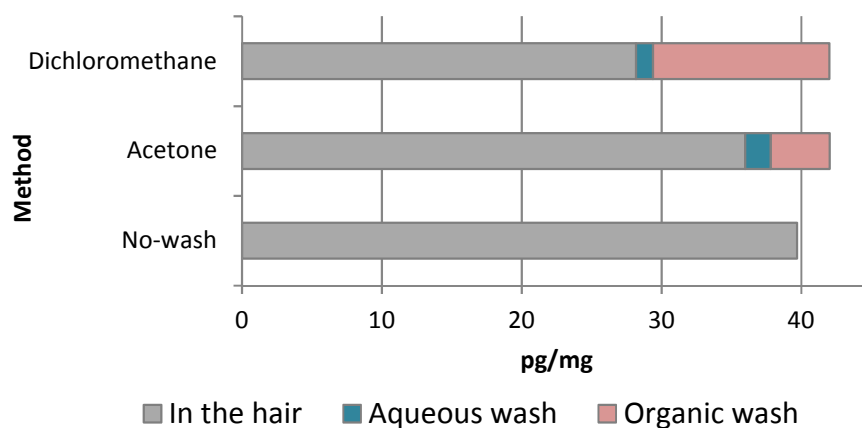
Boscalid
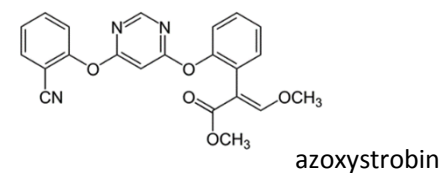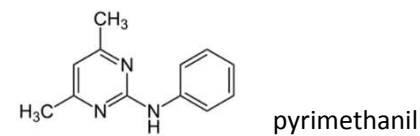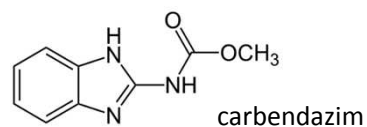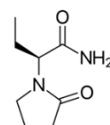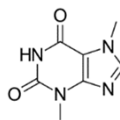
Experimental set up

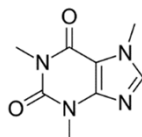**A)** Hair (1 subject): dietary/high level <u>incurred</u> substances (**caf/theobr/lev**)
B) Hair (mix multiple subjects) with <u>incurred</u> **pesticides** (low pg/mg range)

carbendazim

pyrimethanil

imidacloprid

azoxystrobin

thiabendazole

imazalil

boscalid

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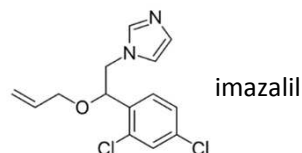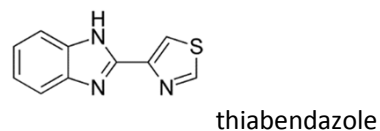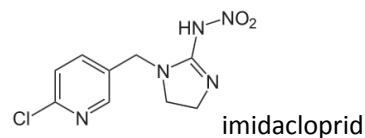
Experimental set up

1 'bulk' sample (hair A ~2 g; hair B ~5 g)
Decontaminate and pulverize into powder (ball mill, 25 Hz, 4 min)
5 Extraction methods; triplicates + 1 reagent blank
Isotopic labels added to sample to exclude procedural losses/matrix effects

MeOH	ACN	ACN/H2O (8/2)	Protease VIII*	TCEP**	~~Acid~~	~~Alkaline~~
A) 50 mg hair (caf/theob/lev); B) 100 mg hair (pesticides)						
add isotope labels for each analyte: A @ 250 pg/mg, B @10 pg/mg						
2 mL	2 mL	2 mL	2 mL	2 mL		
40°C, overnight, ultrasonic			37°C, 1 hour B: + 2 mL MeOH	ambient, 1 hour B: + 2 mL MeOH		
centrifuge, take out supernatant						
		SPE (only B)	SPE	SPE		
evaporate to dry						
Reconstitute in 300 µL: A in eluent; B in ACN/water 1/1						
Inject into LC-MS/MS A 5 µL, B 10 µL						

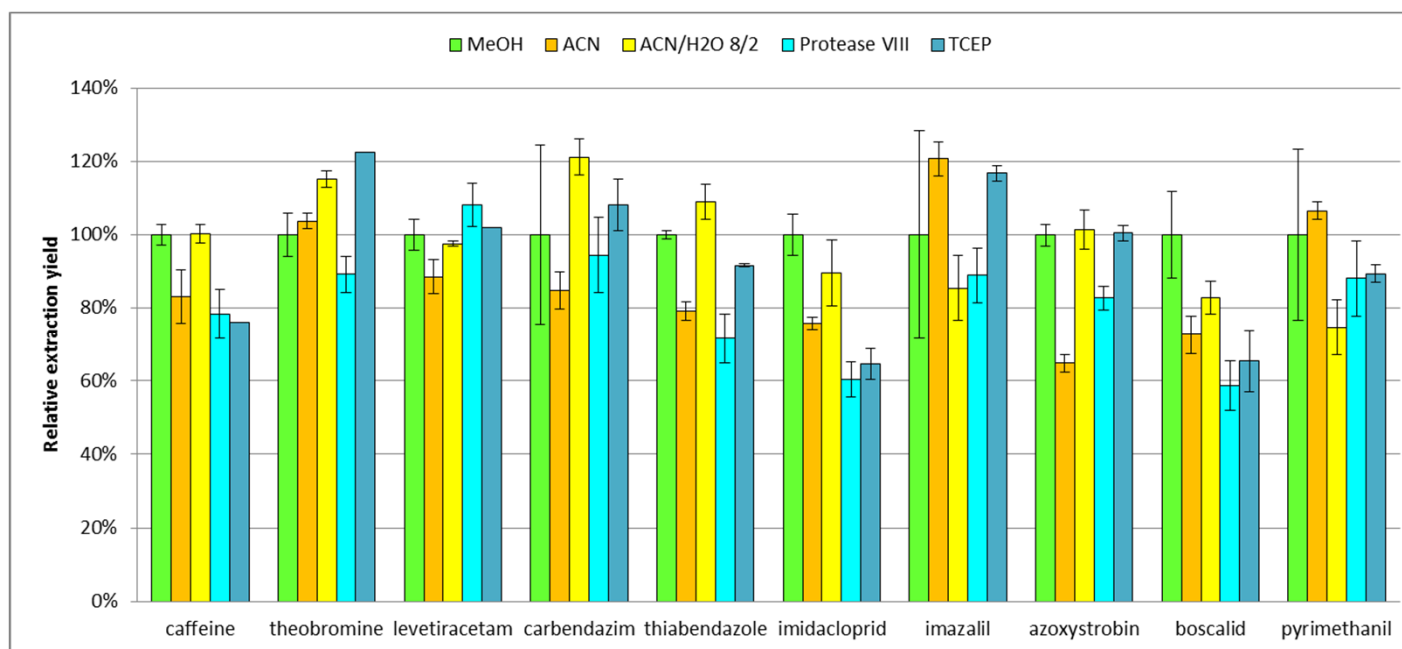* based on De Kesel et al, Talanta 144 (2015) 62–70
** based on Stolker et al, Anal Bioanal Chem (2009) 395:1075–1087
     TCEP = tris(2-carboxyethyl)phosphine hydrochloride

Results

Calculate relative response native extracted vs label

Results normalised to methanol extraction:

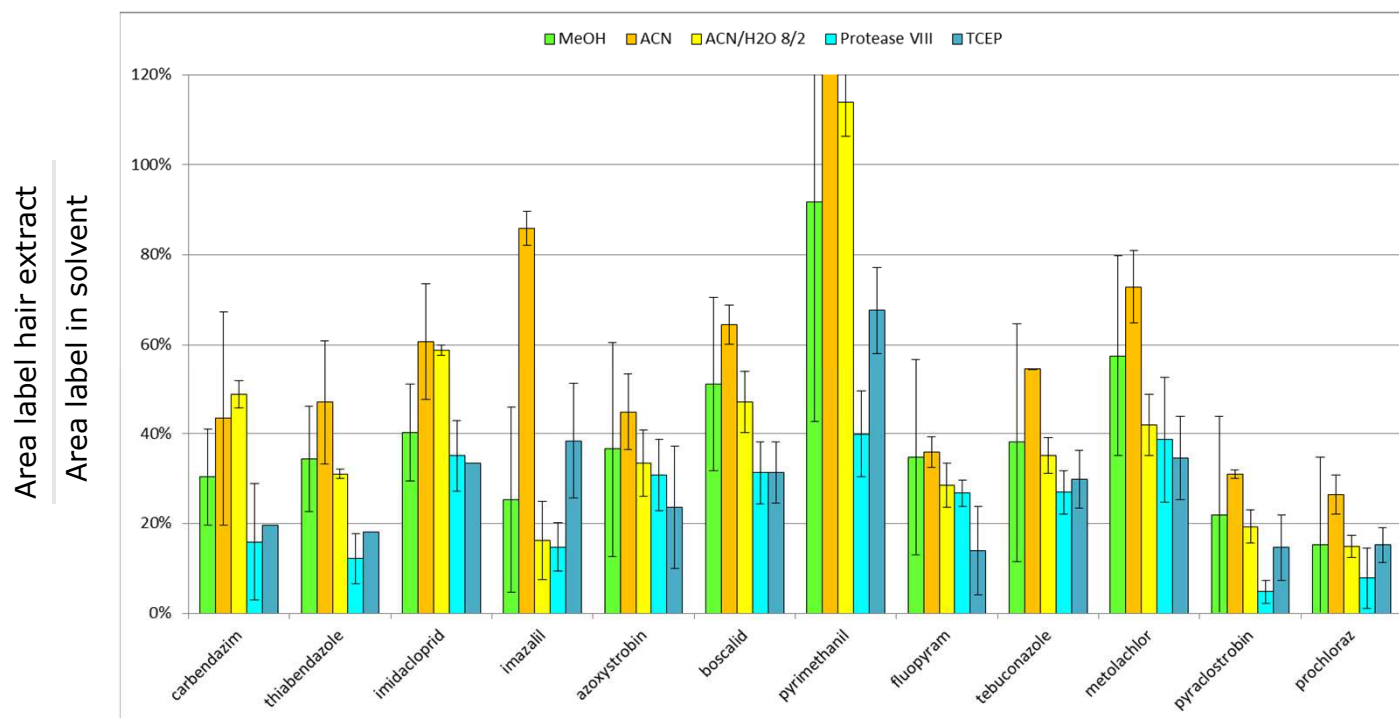

$\Rightarrow$Optimum extraction method compound dependent
$\Rightarrow$MeOH > ACN/H$_2$O~TCEP > ACN > Protease
$\Rightarrow$Differences up to 40%, but mostly <20-30%

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Results

Extraction method also affects co-extractants

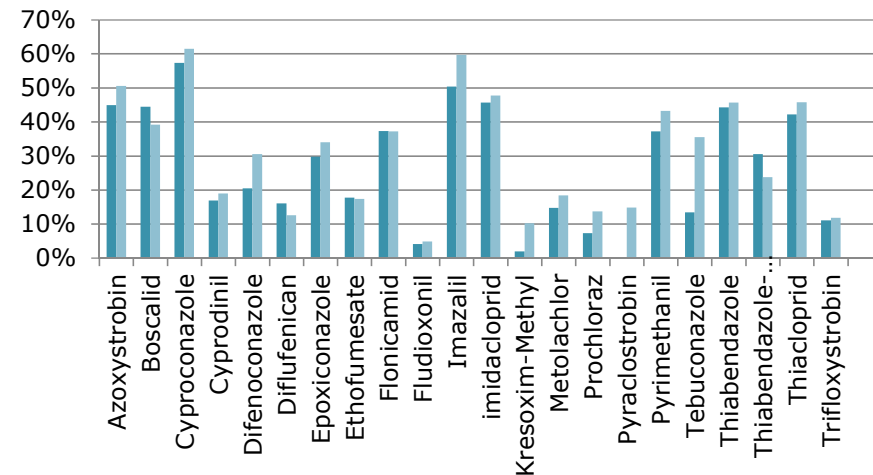$\Rightarrow$ ion suppression in LC-MS; selectivity

ACN > MeOH~ACN/H_2O > TCEP > Protease

Matrix effects

$$\frac{\text{slope in matrix}}{\text{slope in solvent}} \times 100\%$$

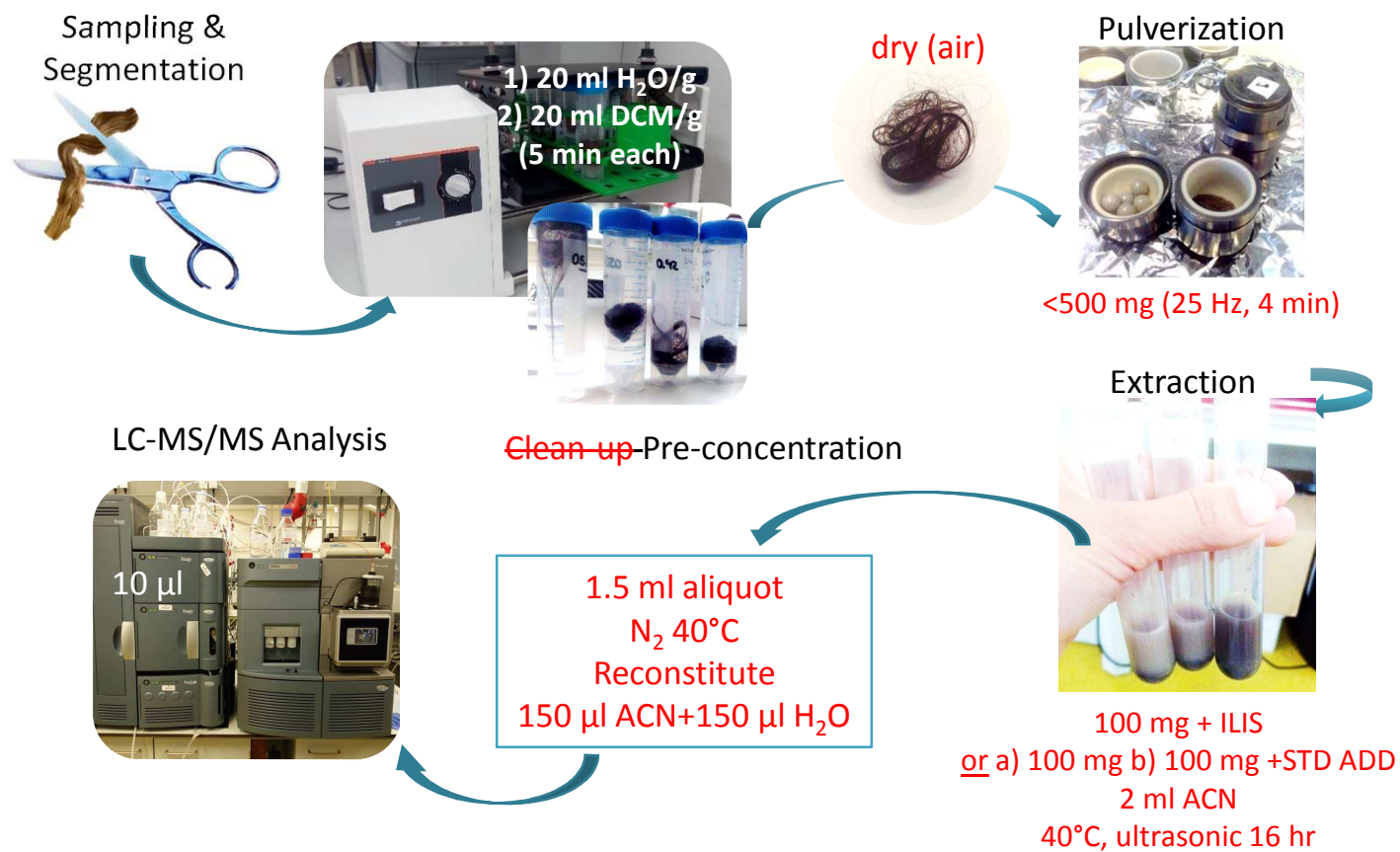Matrix effects for 2 hair samples LC-ESI-MS/MS

Solutions:
- Clean-up of extract
- Matrix-matched calibration
- Addition of isotopically labelled internal standards
- Standard addition (to sample or extract)

Final method

Sampling & Segmentation

1) 20 ml H_2O/g
2) 20 ml DCM/g
(5 min each)

dry (air)

Pulverization

<500 mg (25 Hz, 4 min)

Extraction

LC-MS/MS Analysis

10 μl

~~Clean up~~ Pre-concentration

1.5 ml aliquot
N_2 40°C
Reconstitute
150 μl ACN+150 μl H_2O

100 mg + ILIS
or a) 100 mg b) 100 mg +STD ADD
2 ml ACN
40°C, ultrasonic 16 hr

Sample collection (2016-2017):
- 52 samples
- 36 subjects (males/females, 4-71 year)
- Segments analysed separately for 7 subjects
- 1 subject occupationally exposed
- In most cases undefined hair segment
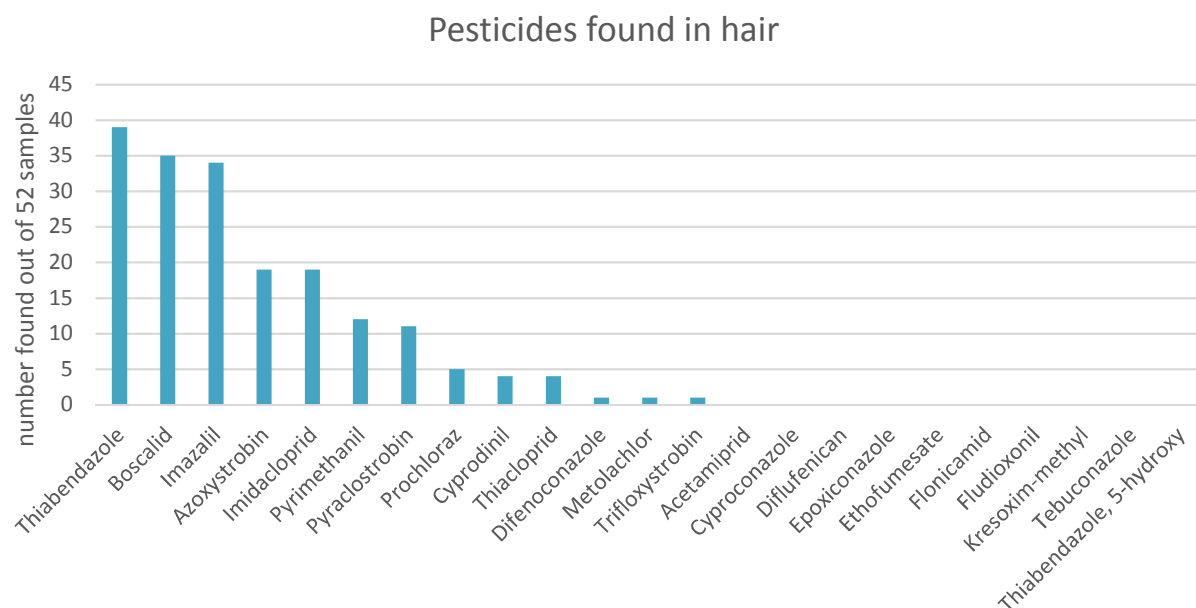- Various hair colours (blond, brown, red)

13 out of 23 pesticides were found
In 50 of 52 samples at least 1 pesticide was detected, up to 7 in 1 sample

Pesticides found in hair

Pesticide	Count
Thiabendazole	39
Boscalid	35
Imazalil	34
Azoxystrobin	19
Imidacloprid	19
Pyrimethanil	12
Pyraclostrobin	11
Prochloraz	5
Cyprodinil	4
Thiacloprid	4
Difenoconazole	1
Metolachlor	1
Trifloxystrobin	1
Acetamiprid	0
Cyproconazole	0
Diflufenican	0
Epoxiconazole	0
Ethofumesate	0
Flonicamid	0
Fludioxonil	0
Kresoxim-methyl	0
Tebuconazole	0
Thiabendazole, 5-hydroxy	0

Detected levels of pesticides — Sample analysis

Levels found in hair samples
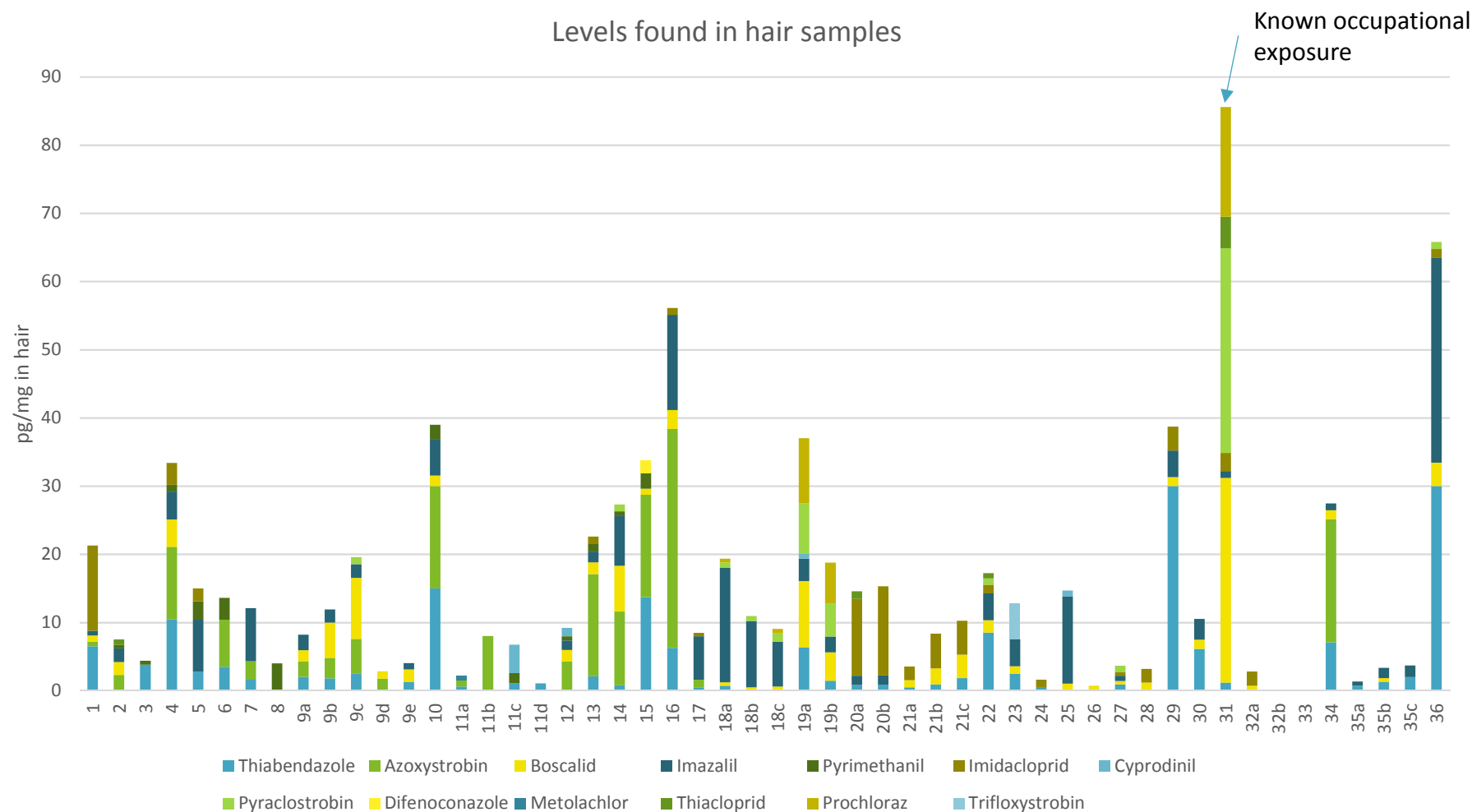
Link with food residues?

Data representative sampling fruit/veg
2014-2016 (N=1648)*

Residues in fruit/veg
vs residues found in hair

Link?
Yes and no.....

Pesticide	#detected (in 1648 samples)	#detected (in 52 samples hair
boscalid	176	35
imazalil	172	34
fludioxonil	155	
thiabendazole	113	39
cyprodinil	111	4
fluopyram	100	
iprodione	93	
pyraclostrobin	88	11
pyrimethanil	84	12
chlorpyrifos	83	
imidacloprid	78	19
azoxystrobin	61	19
spinosad (a & d)	57	
fenhexamide	53	
prochloraz	51	5
propamocarb	51	
difenoconazole	47	1
tebuconazole	47	
trifloxystrobin	44	1

* Dutch Food and Consumer Product Safety Authority (NVWA)

Samples

Two strands ~30/40 cm; 1 brown 1 blond

Split into two sub strands.......

Sample segmentation

Segmentation
~3 cm segments

Each segment:
Decontamination
Pulverization
Duplicate analysis

scalp side

Azoxystrobin in hair strands

Azoxystrobin

pg/mg	I	II
A-0	30.6	
A-1	18.1	19.4
A-2	16.4	16.6
A-3	13.9	13.6
A-4	14.3	15.5
A-5	14.4	15.0
A-6	19.6	17.3
A-7	19.3	21.3
A-8	28.9	27.2
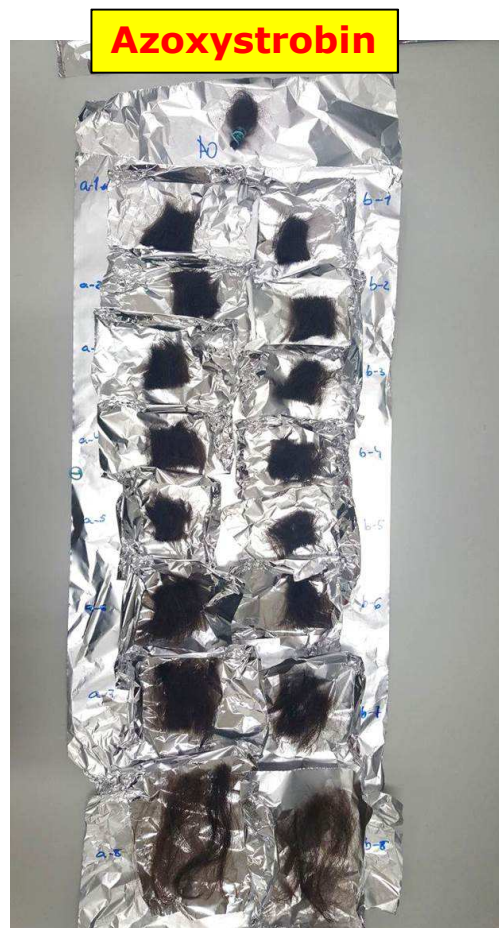
I	II	
33.6		B-0
20.7	19.6	B-1
14.6	14.5	B-2
16.4	15.7	B-3
15.5	18.1	B-4
18.5	17.6	B-5
19.6	18.6	B-6
19.6	20.0	B-7
35.3	36.0	B-8

Pesticides detected
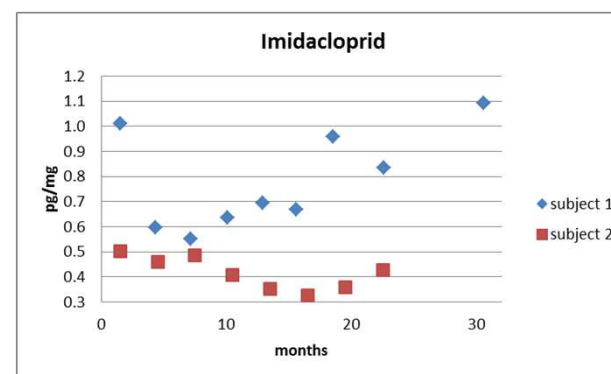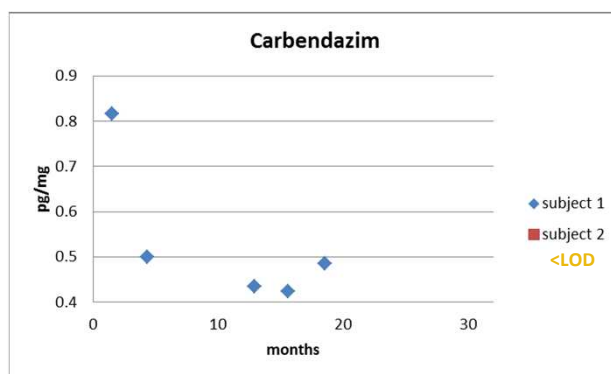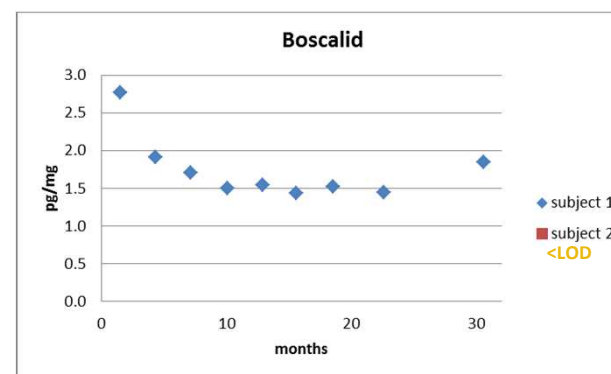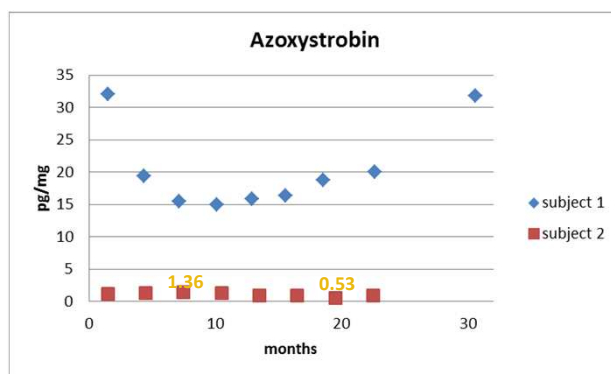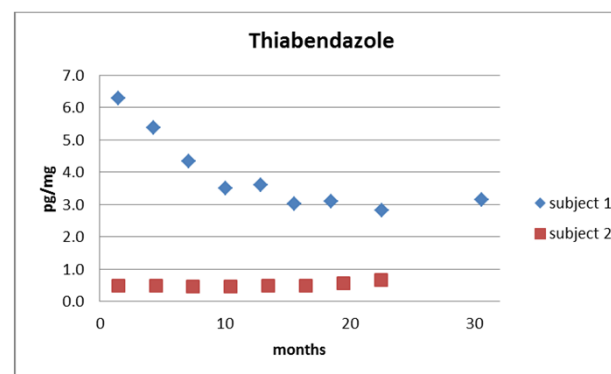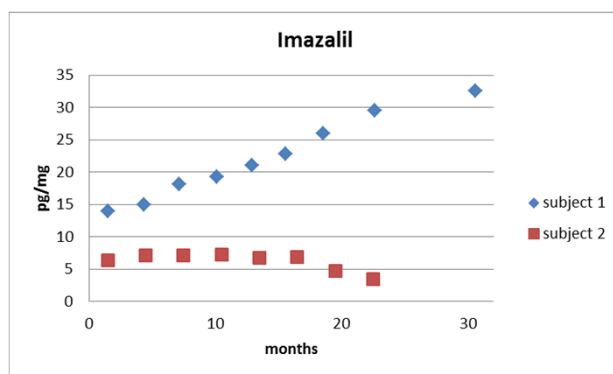
Conclusions

Analytics:

- MeOH gives best extraction yields, ACN best detectability
- LOQs for generic LC-MS/MS based method down 0.5-1 pg/mg (ppb)

First data sample analysis:

- Part of targeted pesticides found in hair of general population
- High detection rates in hair associate with high detection rates in food
- Range 0.5-40 pg/mg

Variability within hair strands:

- Good repeatability of duplicate strands/segments
- pg/mg in 3 cm-segments varies but not more than factor 2.5 over 30 cm/months

Hair analysis tips

- Hair decontamination is an important step. Test and validate the protocol
- Extraction efficiency can only be tested with incurred material
- Use either isotope labelled internal standard for each compound or a standard addition method for quantification.
- Concentrations are low in general population, be aware of contamination risks in the lab and instrumental carry-over.
- It is possible to compare population groups, but not yet possible to calculate exposure from hair analysis results

Thank you

Contacts

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RIKILT – Wageningen University & Research

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