science and policy
for a healthy future

HBM4EU project

2nd HBM4EU Training School 2018

A08 Mycotoxins and Pesticides biomarker analysis

Biomarker discovery/verification and suspect screening of pesticide biomarkers using LC-HRMS

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

Biomonitoring of current pesticides

Matrix	Sampling	Typical target analytes	Detection window	Application
blood (serum)	invasive	wide range	min-hours	common
urine	non-invasive	polar/metabolites	hours-day(s)	common
breast milk	'non-invasive'	lipophilic&persistent / others	months / day(s)	common (lactating women)
adipose tissue	invasive	lipophilic&persistent	months	little used
hair (/nails)	non-invasive	various	weeks/months	emerging
saliva	non-invasive	polar/metabolites / others	hours-day(s)	emerging
sweat	non-invasive	polar/metabolites	hours-day(s)	emerging

This presentation: 'currently used pesticides' (non-persistent); URINE

Challenges for 'currently used pesticides'

- High numbers of pesticides
- Rapidly metabolised, parent often not present
- Lack of data on human metabolism (suitable biomarkers of exposure) / toxicokinetics
- Analytical reference standards biomarkers not (readily) available

Workflows for biomarker identification/verification

Three scenarios:

1. Exposure to single pesticide in volunteer study
2. Dietary exposure to multiple pesticides. Analyse food for pesticides and find biomarkers of those pesticides in urine
3. Analyse urine sample; additional experiments needed for verification

Three data analysis procedures:

1. Target = database of suspects = known metabolites (known exact m/z)
2. Semi-targeted = common fragments, isotope-patterns
3. Non-target = differential peaks control vs treated

Mix and match: Combine scenario with relevant data analysis workflow

Platform used: LC-Q-Orbitrap

Q-Exactive

- High resolution / accurate mass measurement
- Scan range m/z 50-6000
- Several resolving powers (scan speeds)
  17,5K (12 Hz); 35K (6 Hz); 70K (3 Hz); 140K (1.5 Hz)
- Variable precursor ion isolation width
  0.4 to Da to full mass range

Range of acquisition options

Non-targeted measurement

- Without fragmentation (Full Scan)
- With fragmentation in HCD cell

AIF = all-ion-fragmentation

vDIA = variable Data Independent Acquisition

Targeted measurement

- Without fragmentation (SIM)
- With fragmentation

  ddMS/MS, t-MS/MS, PRM

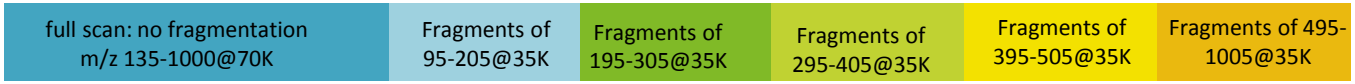
Platform used: LC-Q-Orbitrap

<u>LC-Q-Orbitrap MS (Q Exactive):</u>
Injection: 5 µL
Column: 100×3 mm ID, 3 µm Atlantis T3;
T=35°C
Gradient: water/methanol, 2 mM NH_4HCOO
Flow: 0.30 mL/min

<u>Analysis workflow known exposure:</u>

Four injections (with/without deconjugation; ESI pos/ESI neg)

FS+vDIA
Cycle time 978 ms



HCD: 30 and 80 NCE, ACG: 10^6

<u>Analysis workflow screening:</u>

Two injections (ESI pos/ ESI neg)

FS (140K)

Additional experiments include ddMS2, targeted MS/MS

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Volunteer study

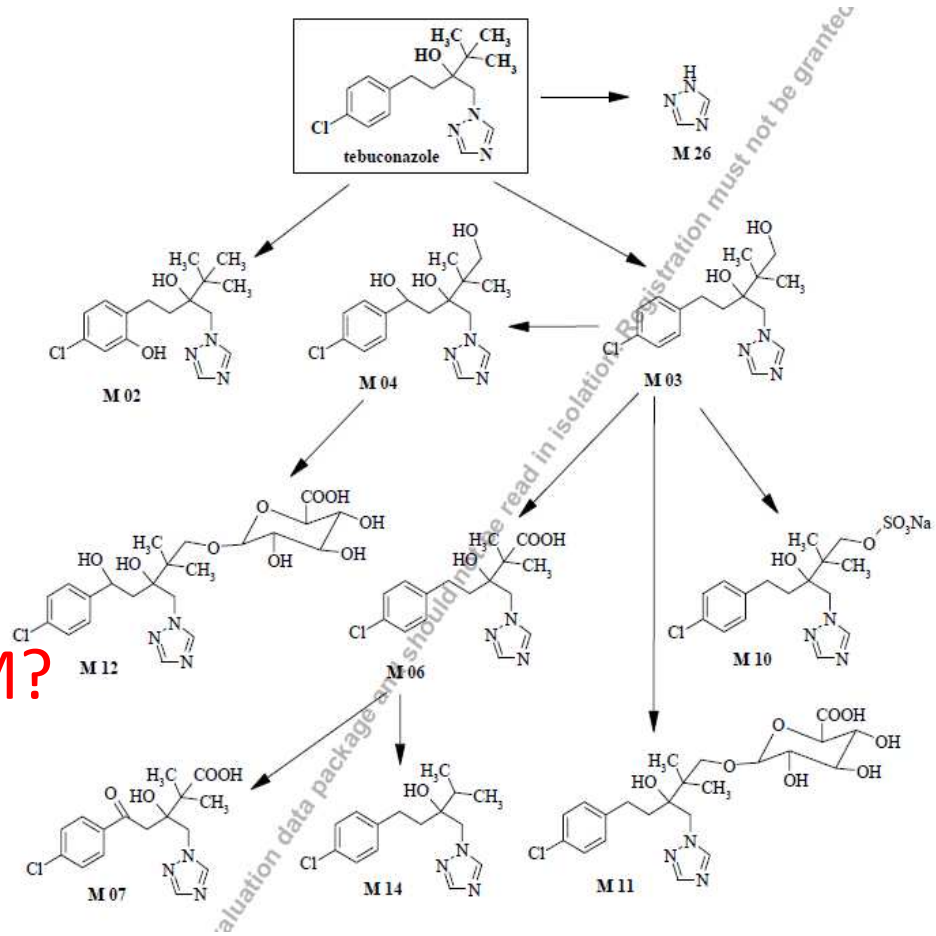
<u>Human volunteer study (collaboration Radboud University Medical Centre):</u>

Single oral administration @ <ADI to 3 males & 3 females (tebuconazole 1.5 mg)
Collection of urine before administration
Collection of all voids up to 48 hours after administration

Prepare composite 24-hr urine samples for each subject

Volunteer study: Tebuconazole

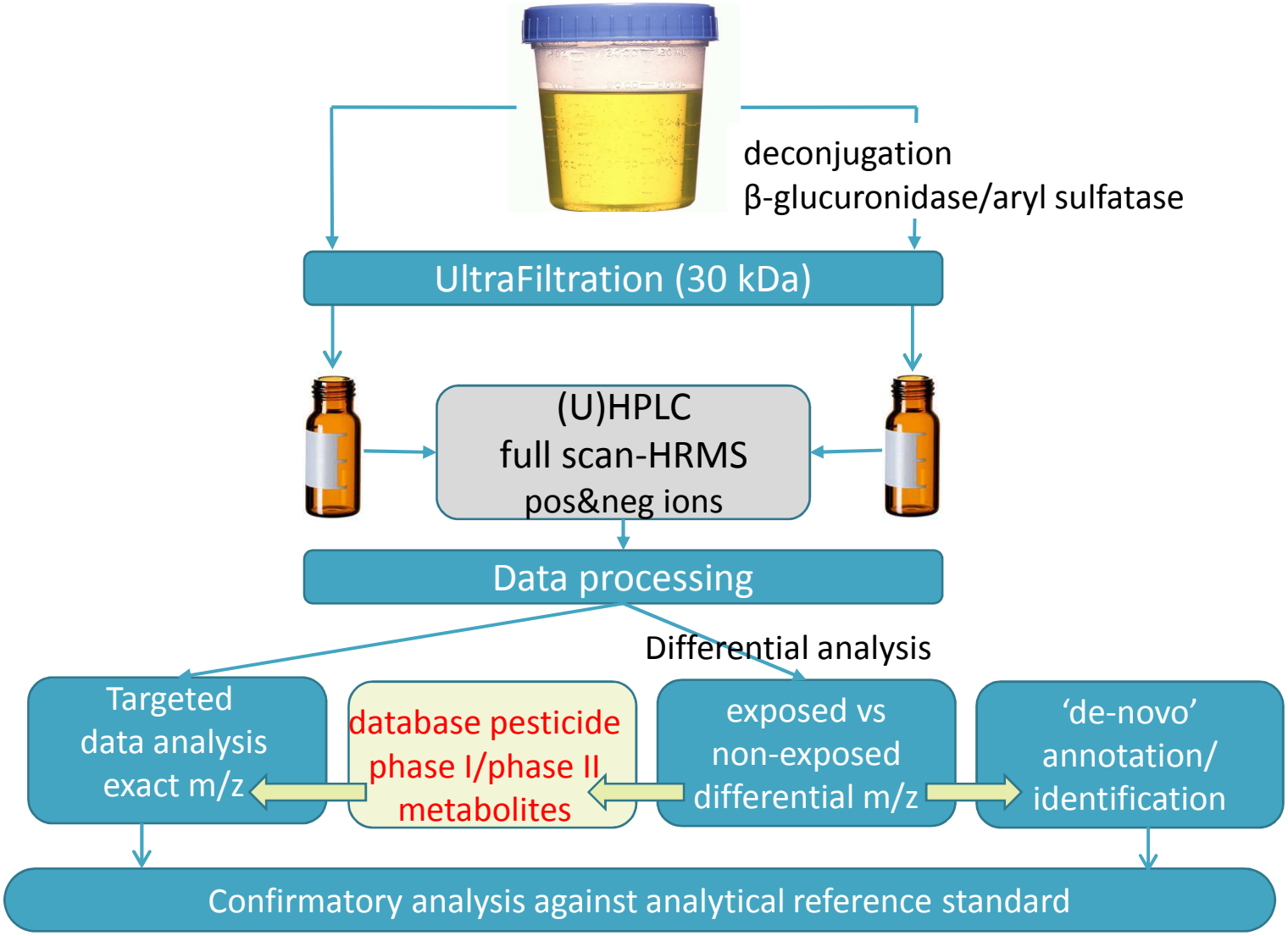
Fungicide
Seed dressing + spray applications
World-wide / wide range of crops
Frequent residues in food



Are humans like rats?

Which is best one for HBM?

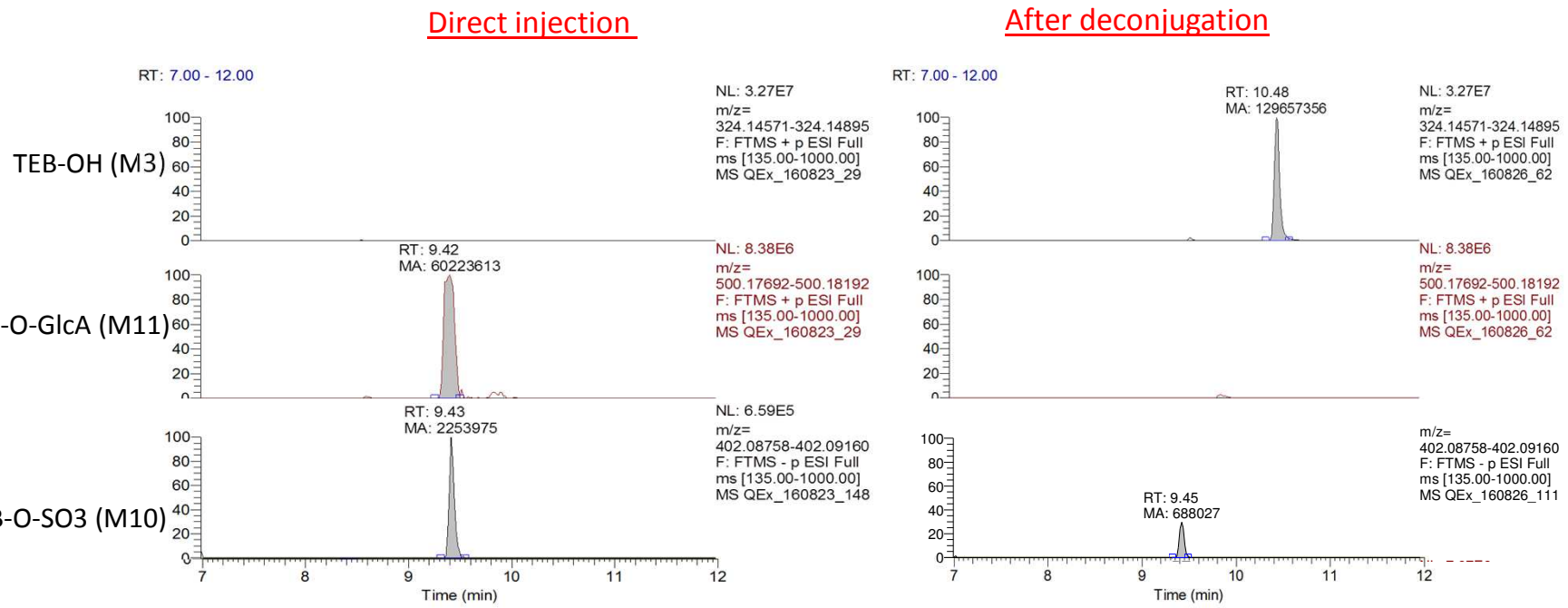
Volunteer study: Workflow



Volunteer study: Tebuconazole metabolites

Targeted data-analysis workflow:

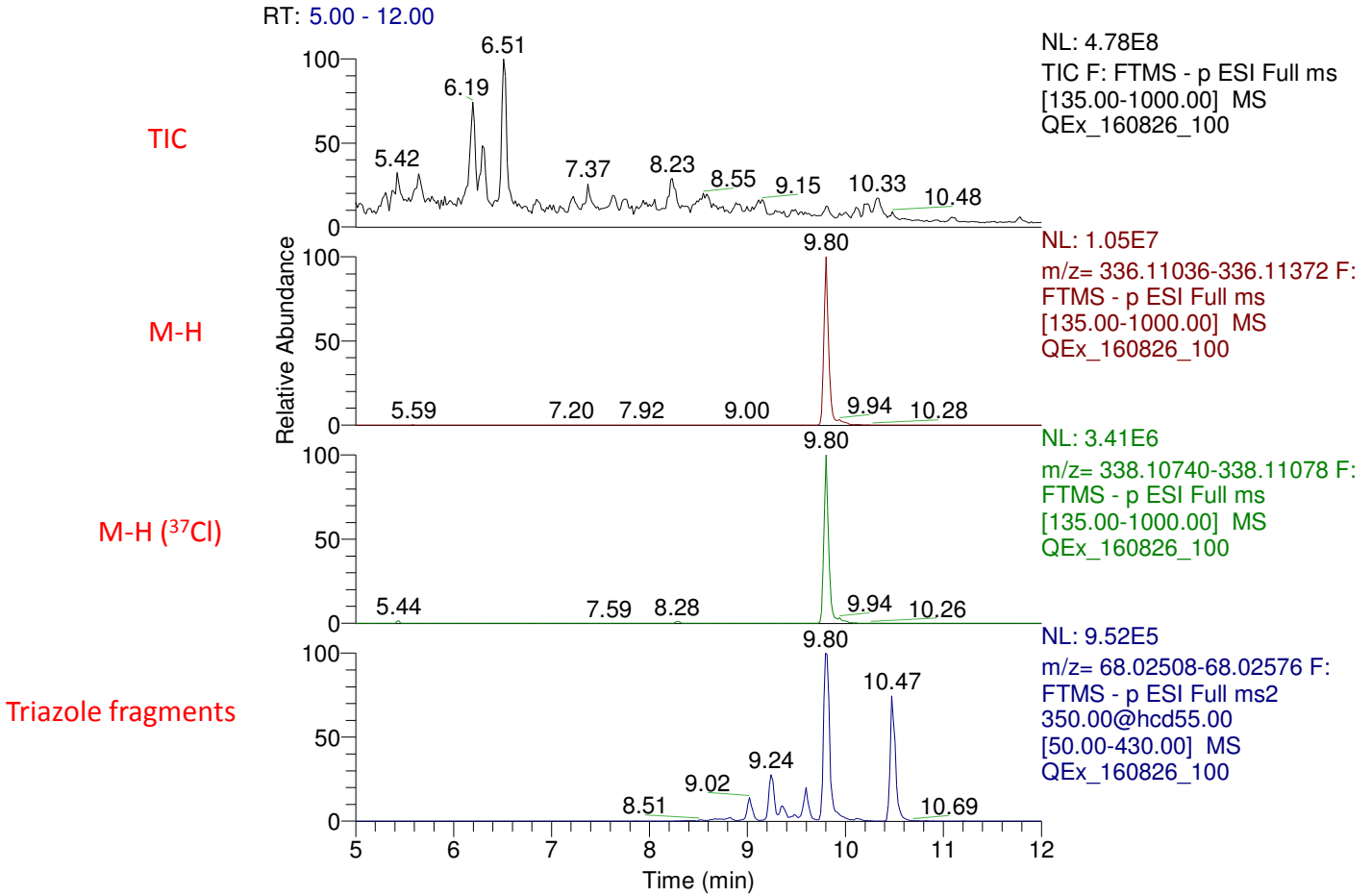
Known tebuconazole metabolites; example tebuconazole-OH + conjugates



Volunteer study: Tebuconazole metabolites

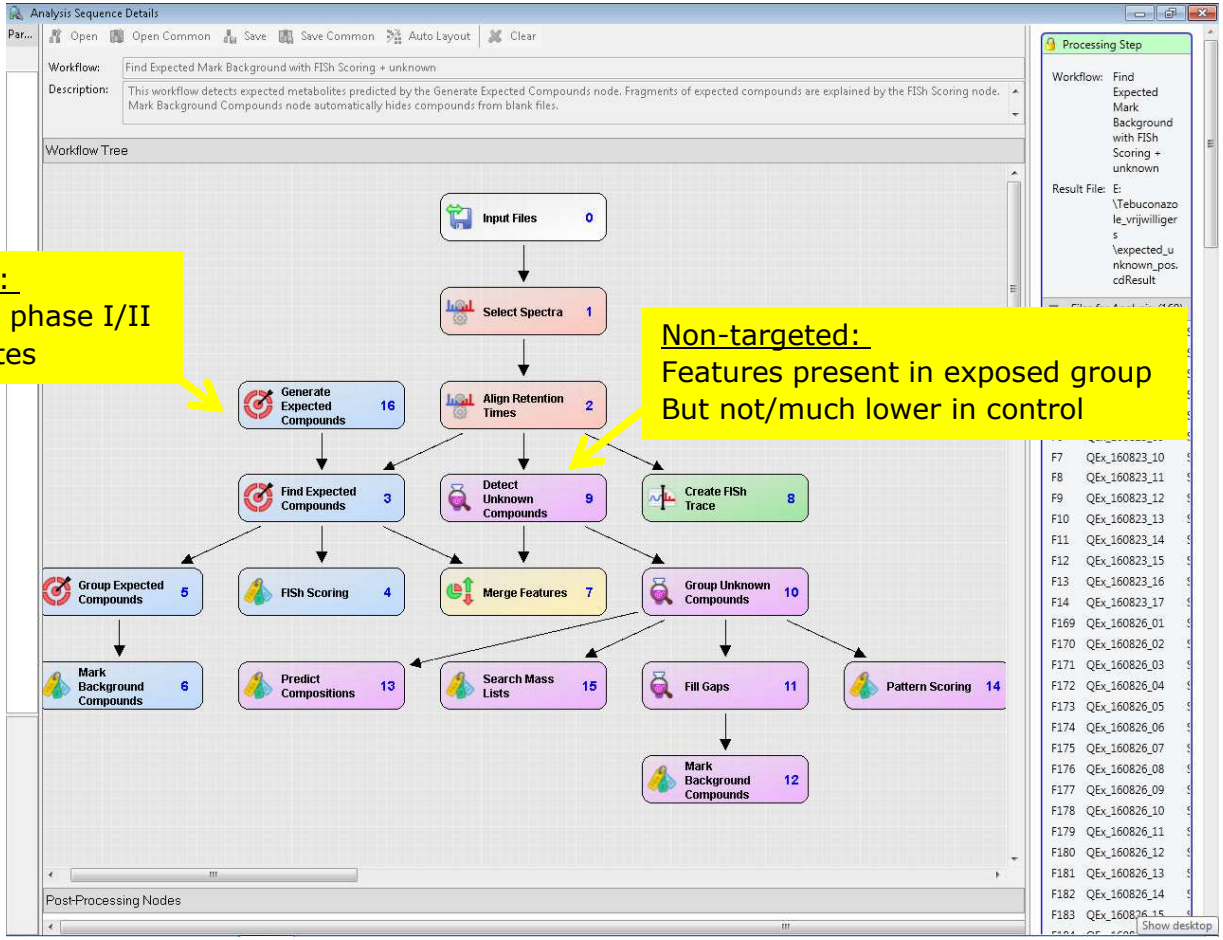
Semi-targeted data-analysis workflow:

Common fragment: example tebuconazole-acid metabolite



Volunteer study: Data-processing

Data processing by dedicated software: Compound Discoverer



Targeted:
Expected phase I/II metabolites

Non-targeted:
Features present in exposed group
But not/much lower in control

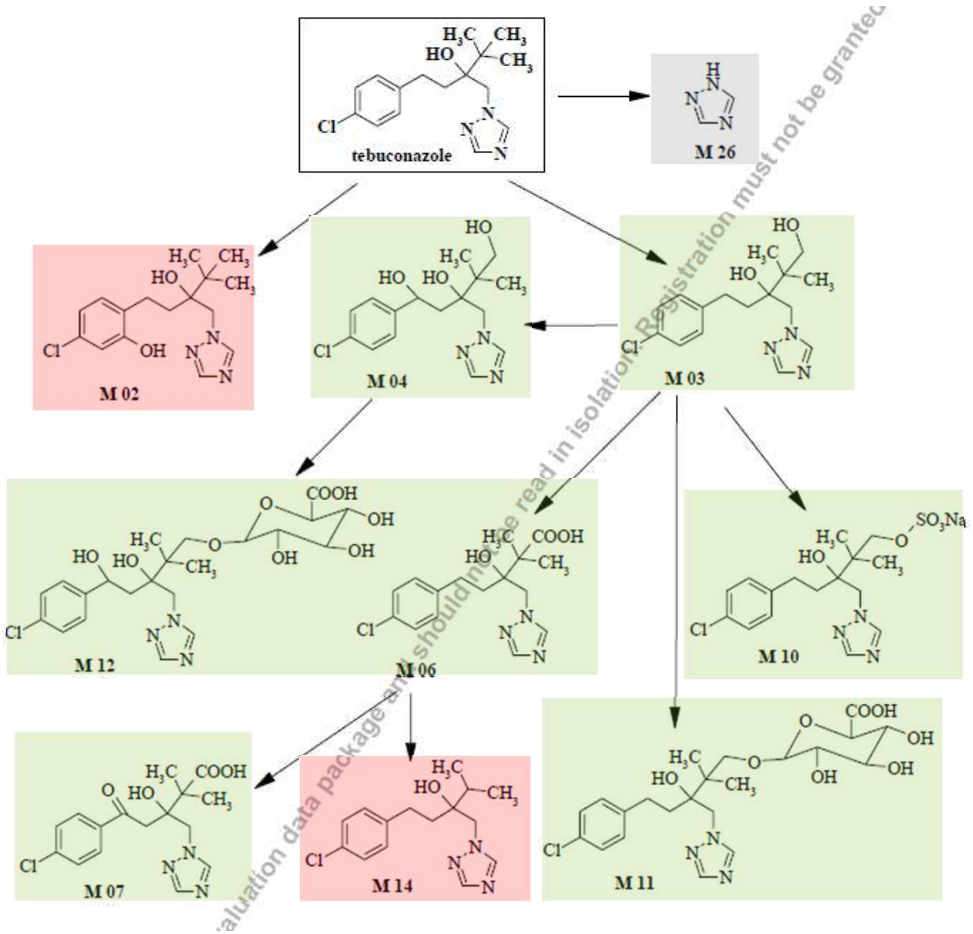
Volunteer study: Results Tebuconazole

Are humans like rats?

⇒ To a certain extend yes

Additionally found (tentative):

- M6-gluc
- M4(2)
- M4(2)-gluc



Volunteer study: Results Tebuconazole

Evaluation of detectability

	without deconjugation			with deconjugation		
Metabolite/biomarker	tr (min)	precursor	rel. resp.	tr (min)	precursor	rel. resp.
Tebuconazole M03 hydroxy				10.49	M+H	1.00
Tebuconazole M10 M03 SO3	9.41	M-H	0.02			
Tebuconazole M11 M03 gluc	9.4	M+H	0.26			
Tebuconazole M04 OH OH				9.6	M-H	0.12
Tebuconazole M04 OH OH				9.8	M-H	0.23
Tebuconazole M12 M04-gluc	8.44	M+H	0.03			
Tebuconazole M12 M04-gluc	8.7	M+H	0.05			
Tebuconazole M06 acid	9.79	M-H	0.18	9.83	M-H	0.70
Tebuconazole M06 acid -gluc	9.03	M+H	0.16			
Tebuconazole M07 acid keto	9.25	M-H	0.08			

$\Rightarrow$ M03 and/or M06 after deconjugation

M03 confirmed through reference standard (kind gift Bayer Crop Science)

Workflows for biomarker identification/verification

Scenarios:

1. Exposure to single pesticide in volunteer study
2. Dietary exposure to multiple pesticides. Analyse food for pesticides and find biomarkers of those pesticides in urine
3. Analyse urine sample; additional experiments needed for verification

Three data analysis procedures:

1. Target = database of suspects = known metabolites (known exact m/z)
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Dietary exposure: Example strawberries

Dietary exposure
2 kg of strawberries from the market
1 kg => analysis for residues
1 kg => consumption by volunteer
Collection of 24 hr urine

Residues in strawberries:

Pesticide	mg/kg
Boscalid	0.86
Cyprodinil	0.24
Fenhexamid	0.28
Fludioxonil	0.18
Fluopyram	0.17
Pyraclostrobin	0.43
Trifloxystrobin	0.27

⇒Oral intake of 0.17-0.86 mg
⇒Compilation of target list of known metabolites
⇒Targeted search

<u>Follow up on hits found:</u>

- isotope signature
- plausibility retention time
- fragment ions
- conjugates: compare with/without deconjugation
- signal in control urine if available

Dietary exposure: Workflow

deconjugation
β-glucuronidase/aryl sulfatase

UltraFiltration (30 kDa)

(U)HPLC
full scan-HRMS
pos&neg ions

Data processing

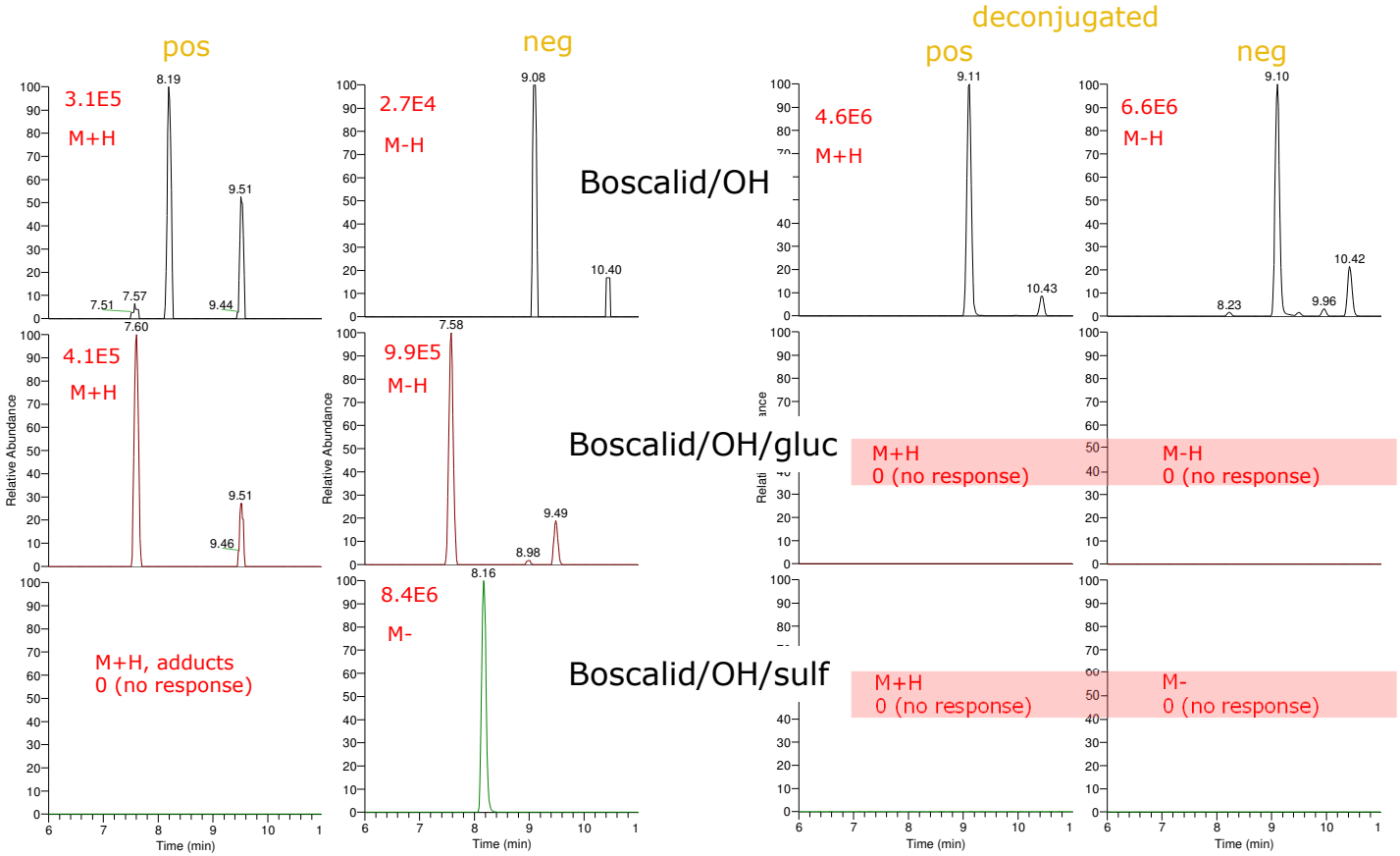
Targeted
data analysis
exact m/z

database
phase I/phase II
metabolites

Confirmatory analysis against analytical reference standard

Dietary exposure: Boscalid metabolites

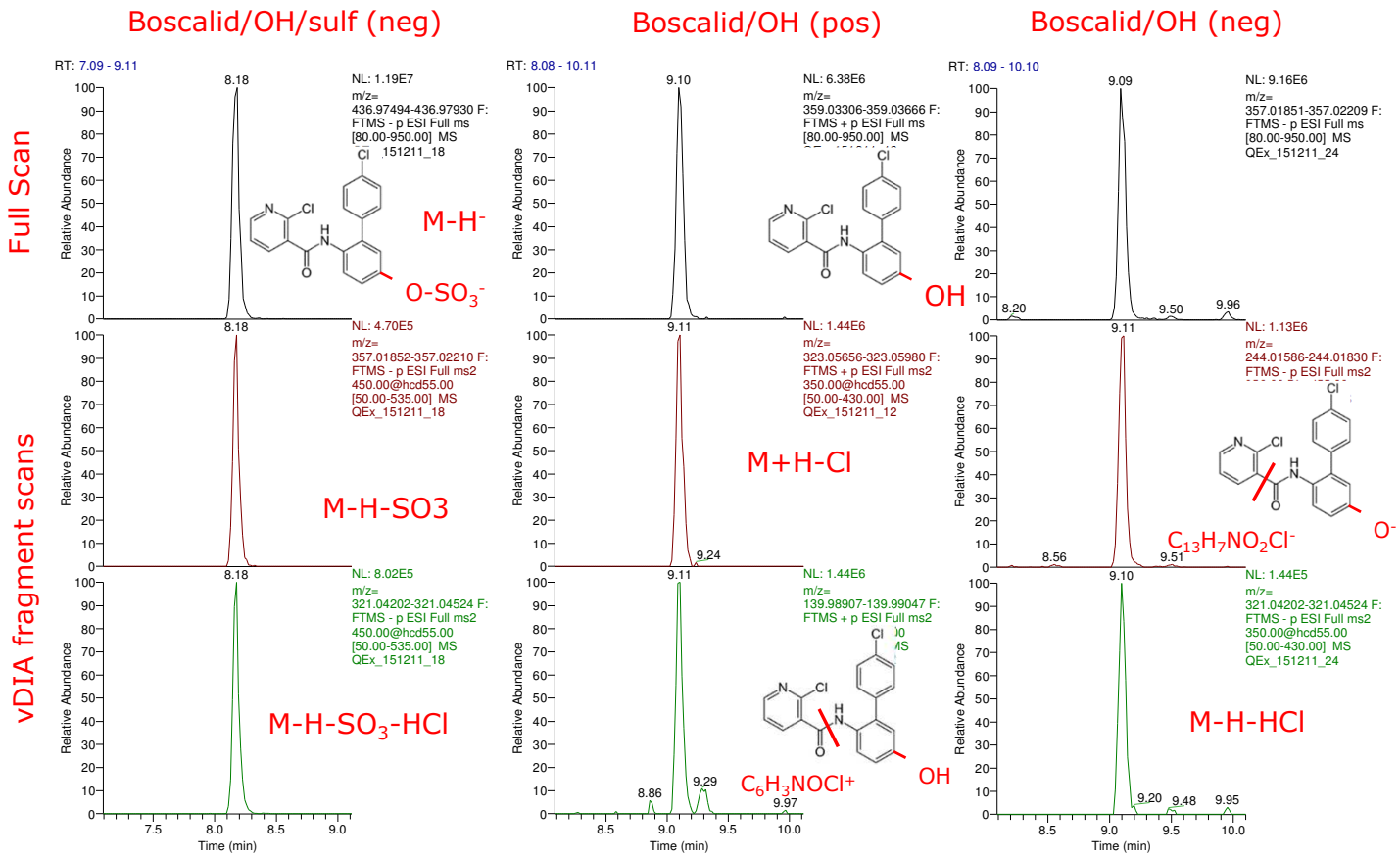
Boscalid metabolites tentatively detected



XICs exact m/z ±5 ppm

Dietary exposure: Boscalid metabolites

Identification using vDIA fragmentation



Dietary exposure: Results example strawberries

Metabolites detected in 24hr urine after 1 kg strawberry consumption

Biomarker	Detectability in 24-hour urine (x*LOD) w/o deconjugation	with deconjugation
Boscalid M510F01[1(a)] | Glucuronide[2] | Sulfate[2]	- | 66 | 522	715 | - | -
Cyprodinil CGA 304076[2] | Sulfate[2]	- | 3	1700 | -
Cyprodinil 6U (1U-sulfate)[2]	15	-
Cyprodinil 7U[3]	29	23
Fenhexamid [M03/M06/M16][3] | Glucuronide[2]	- | 12	129 | -
Fluopyram AE C656948-benzamide[1] | Gluc.[2]	- | 3350	110 | 918
Fluopyram AE C656948-[7/8]-hydroxy[3] | Gluc.[2]	- | 113	137 | -
Pyraclostrobin 500M07[3]	4	-
Pyraclostrobin 500M04[3] | Sulfate (500M05)[2]	11 | 35	159 | -
Pyraclostrobin 500M51[3]	166	172
Trifloxystrobin CGA321113[1]	48	195

[1] identified by reference standard; [2] tentative identification by matching isotope and diagnostic fragment (e.g. [M+H-176]); [3] tentative by matching isotopic pattern; - = not detected; [(a)] kind gift CVUA Stuttgart

⇒ Biomarker(s) (tentatively) detected for 6 out of 7 strawberry pesticides

⇒ Responses obtained indicate feasibility of detection at lower exposure levels

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In HBM4EU a similar workflow is being developed/used in WP 16

Suspect screening: Database

Suspect screening of 'random' urine samples
Suspect = pesticide urinary metabolites
Database not available, needed to be created

<u>First initiation:</u>
Prioritised ~125 pesticides
- Commonly found in food in NL
- Frequent/high kg agricultural use in NL
- Dual use (biocides, vet drug in pets)

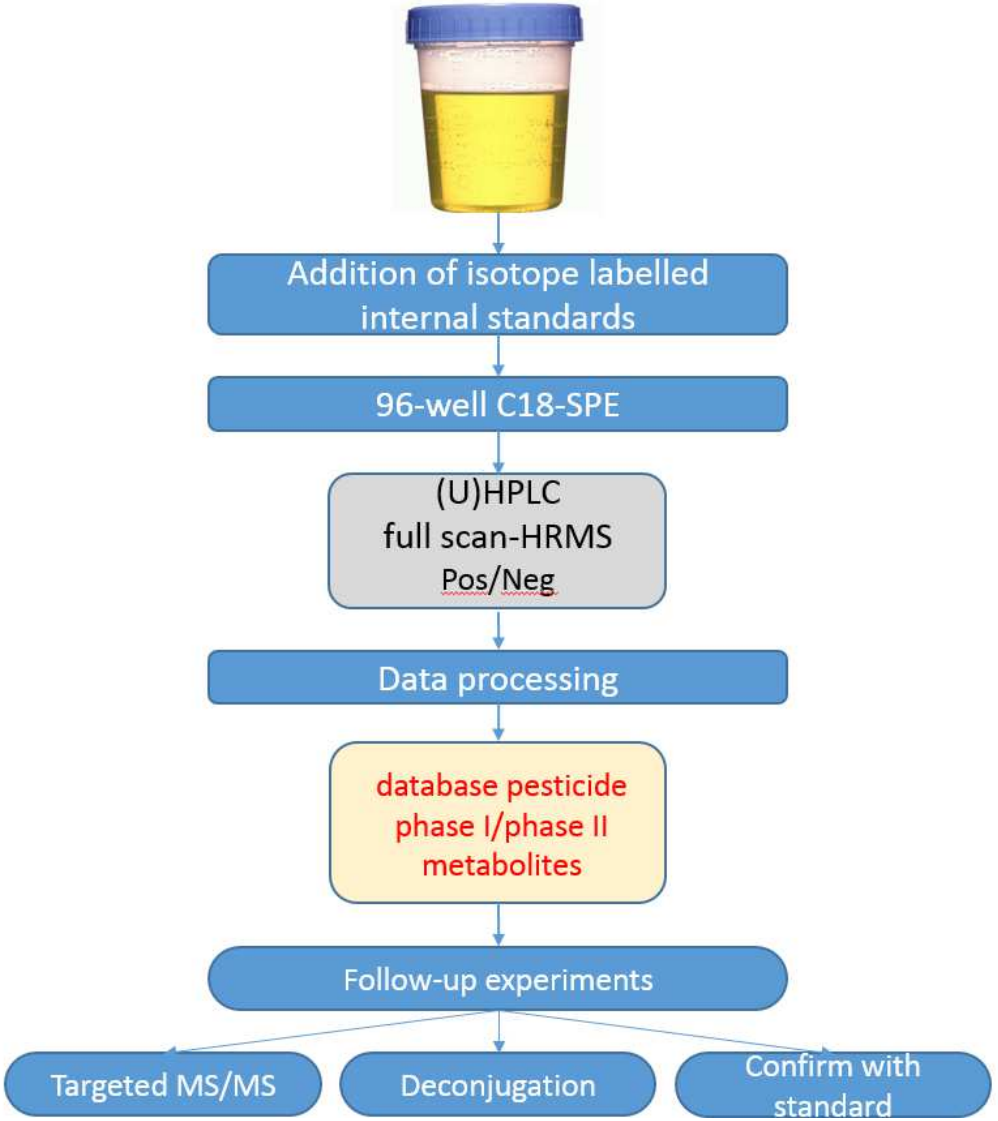
Compile all phase I/non-conjugated metabolites from DARs, JMPR, literature

<u>Result:</u>
~1500 metabolites: Name / formula / exact mass
Search ESI pos: 1500 + GlcA conjugates of these
Search ESI neg: 1500 + GlcA + SO_4 conjugates of these

Pesticide metabolite database

abamectin/ B1a	C48H72O14	872.4922
abamectin/ B1a+H2	C48H74O14	874.5078
abamectin/ B1a+CH2O	C49H74O15	902.5027
abamectin/ B1a_nCH2	C47H70O14	858.4765
abamectin/ B1a_nCH2+O	C47H70O15	874.4714
abamectin/ B1a_nCH2+CH2O	C48H72O15	888.4871
abamectin/ B1b	C47H70O14	858.4765
abamectin/ B1b+H2	C47H72O14	860.4922
abamectin/ B1b+CH2O	C48H72O15	888.4871
abamectin/ B1b_nCH2	C46H68O14	844.4609
abamectin/ B1b_nCH2+O	C46H68O15	860.4558
abamectin/ B1b_nCH2+CH2O	C47H70O15	874.4714
acetamiprid	C10H11ClN4	222.0672
acetamiprid-CH2	C9H9N4Cl1	208.0516
acetamiprid-C3H2N2	C7H9N2Cl1	156.0454
acetamiprid-C4H4N2	C6H7N2Cl1	142.0298
acetamiprid-C6H4ClN	C4H7N3	97.06399
acetamiprid-C7H6ClN	C3H5N3	83.04834
acetamiprid_IC_O	C6H4ClNO2	156.9931
acetamiprid_IC_O+C2H3NO	C8H7N2O3Cl1	214.0145
acetamiprid_IM-O	C6H6ClNO	143.0138
acetamiprid_IM-O+C3H5N	C9H11N2O1Cl1	198.056
acetamiprid_IM-O+C2H3N	C8H9N2O1Cl1	184.0403
asulam	C8H10N2O4S	230.0361
asulam+C2H2O	C10H12N2O5S1	272.0467
asulam-C2H2O2	C6H8N2O2S1	172.0306
asulam-O	C8H10N2O3S1	214.0412
azoxystrobin	C22H17N3O5	403.1168
azoxystrobin-CH2	C21H15N3O5	389.1012
azoxystrobin+O	C22H17N3O6	419.1117
azoxystrobin-C7H3N	C15H14N2O5	302.0903
azoxystrobin-C15H12N2O4	C7H5N1O1	119.0371
azoxystrobin-C11H10O3	C11H7N3O2	213.0538
azoxystrobin-C11H5N3O2	C11H12O3	192.0786
azoxystrobin+C5H7NO3S	C27H24N4O8S1	564.1315
azoxystrobin+C3H5NO2S	C25H22N4O7S1	522.1209
azoxystrobin+SCH2	C23H19N3O5S1	449.1045
azoxystrobin+S	C22H17N3O5S1	435.0889
azoxystrobin+SO	C22H17N3O6S1	451.0838
azoxystrobin+SCH2O	C23H19N3O6S1	465.0994
azoxystrobin_nC2H2O	C20H15N3O4	361.1062
azoxystrobin_nC2H2O+O	C20H15N3O5	377.1012
azoxystrobin_nC2H2O-CH2	C19H13N3O4	347.0906

Suspect screening: random urine sample



Suspect screening: Results

Compound Discoverer or MetAlign software *

Data processing: Preprocessing (noise filtering/adduct analysis/rt adjustments)

⇒ unique peaks / accurate mass

⇒ elemental compositions

Match against database of pesticide metabolites

Compounds | Compounds per File | Features | Mass List Search Results | Input Files

	Checked	Name	Formula	Annotation Sc	Molecular Weight	RT [min]	Area (Ma	# Usable QC	RSD QC Areas [%]	RSD corr. QC Areas [%]	Mass List Matches
11544			C21 H25 N4 O12 P S3		652.03746	6.286	44052	3	2	1	
11545		trifloxystrobin_pO2	C20 H19 F3 N2 O6		440.11922	5.533	43645	3	25	15	
11546			C14 H17 N5 O		271.14284	5.370	43139	2	23	0	
11547		Sulfaquinoxaline	C14 H12 N4 O2 S		300.06932	5.823	43008	3	16	11	
11548		N,N-Dimethyltryptamine	C12 H16 N2		188.13107	7.354	42691	3	16	10	
11549			C11 H22 N4 O9 P2 S		448.05828	6.282	42482	3	3	2	
11550			C14 H35 N10 O8 P S2		566.18316	5.926	42245	3	5	4	
11551		Terbutaline	C12 H19 N O3		225.13629	4.398	42007	3	7	5	
11552		metham_ methyldithiocarbamic acid_nSpOCH2 glucurc	C9 H15 N O7 S		281.05660	4.867	41972	3	16	16	
11553			C11 H19 N3 O2 S		257.11937	5.953	41920	2	28	0	
11554			C5 H N9		187.03563	5.611	41859	3	8	7	
11555			C9 H11 N3 O2		193.08493	5.076	40800	3	8	5	
11556			C7 H9 N9		219.09810	3.839	40551	2	23	0	
11557			C5 H12 O7 P2 S		277.97695	6.215	40211	3	6	1	
11558			C12 H18 N8 O5 P2 S		448.06127	8.046	40068	3	9	3	
11559			C11 H28 N3 O8 P3		423.10704	5.178	39355	3	21	13	
11560		Pyraclostrobin_nC10H11NO3 sulfate	C9 H7 Cl N2 O4 S		273.98189	6.237	39349	3	5	5	
11561			C8 H23 N8 O6 P S		390.11858	8.308	39031	3	9	6	
11562		N,N-di(2-hydroxyethyl)-p-toluidine	C11 H17 N O2		195.12561	6.802	38892	3	10	9	
11563			C23 H31 N9 O2 P2		527.21011	6.035	37772	3	11	5	
11564			C19 H28 O8		384.17916	5.820	36513	3	10	7	
11565			C18 H14 O5 P2 S3		467.94710	6.214	34682	3	7	3	

Problem: >10.000 peaks in total;

>3000 suspects

* Arjen Lommen, Anal. Chem. 2014, 86, 5463−5469, Ultrafast PubChem Searching Combined with Improved Filtering Rules for Elemental Composition Analysis

Suspect screening: how to select "best" hit?

Is the suspect detected in positive and negative mode? (not likely in case of sulfate-conjugate)

Are multiple suspects for a single parent compound detected in one sample?

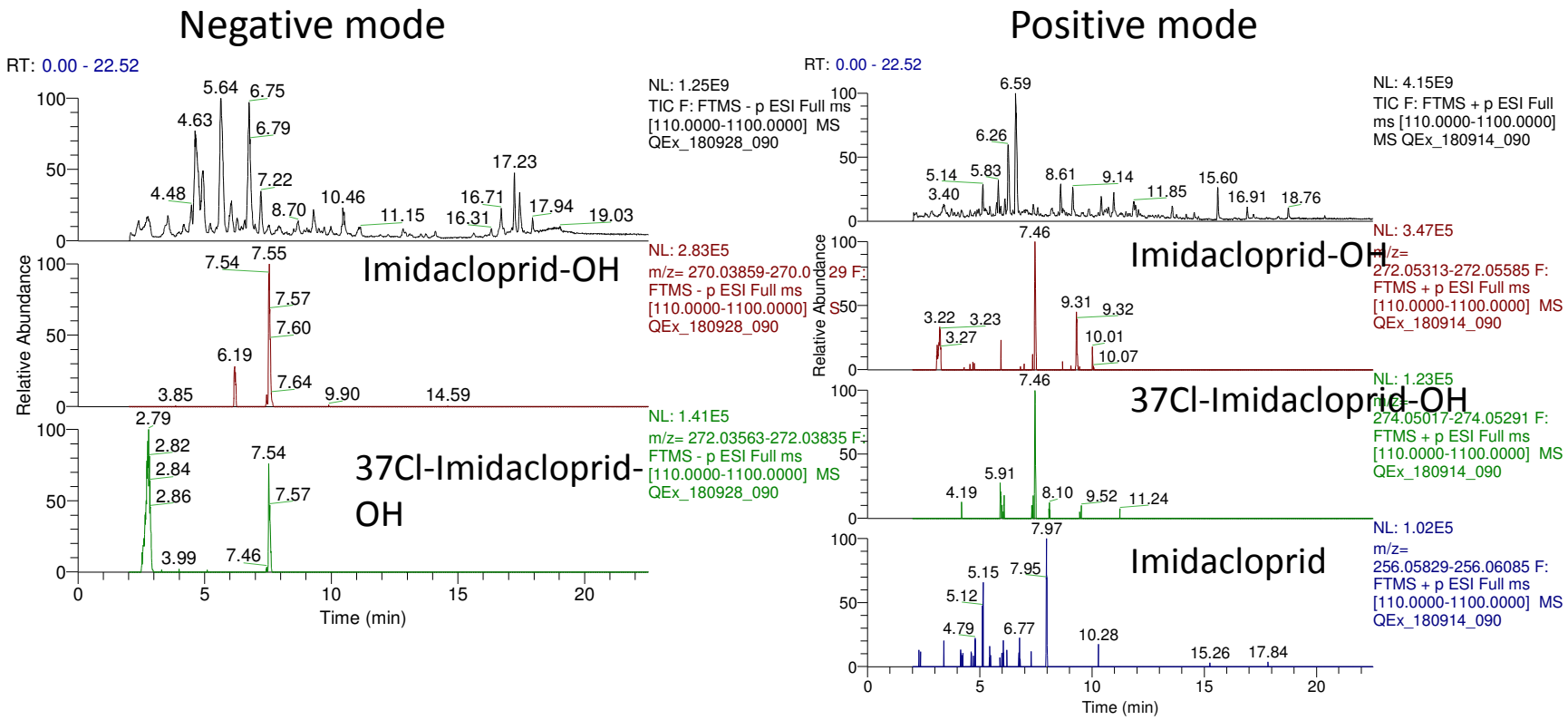
Is the detected suspect a common metabolite in animal studies?

Manually evaluate hits

- 'Uniqueness' of hit (#peaks, Cl/Br), occurrence in other samples
- anticipated t_r
- literature
- common sense

Suspect screening: Imidacloprid metabolites

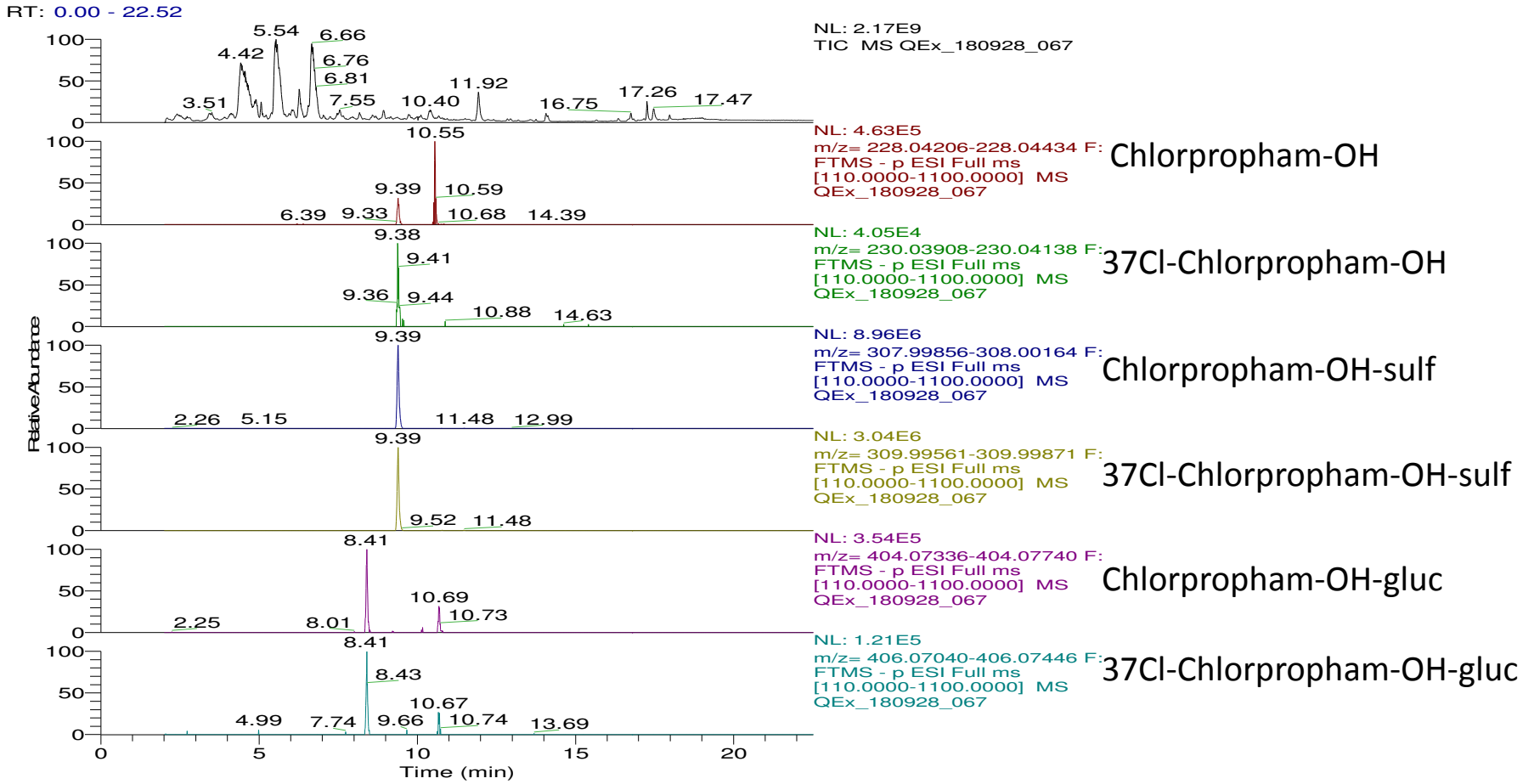
Sample 512560 -> imidacloprid metabolites



Suspect screening: Chlorpropham metabolites

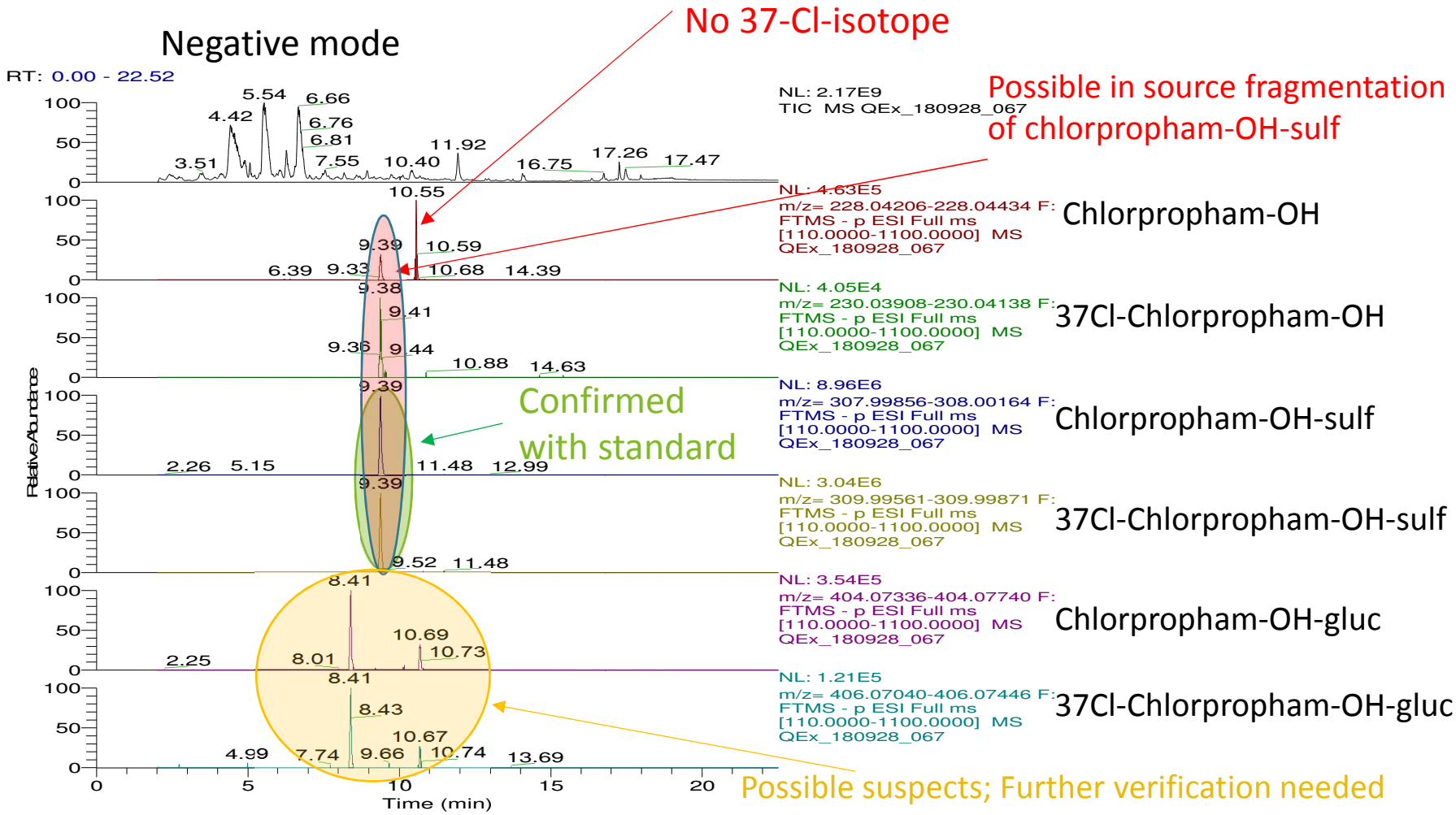
Sample 511030 -> chlorpropham metabolites

Negative mode



Suspect screening: Chlorpropham metabolites

Sample 511030 -> chlorpropham metabolites



Suspect screening: Follow-up experiments

Further experiments for identification/verification:

- In case of conjugate -> analyse after deconjugation
- Target-MS/MS for fragmentation pattern

-> confirm with reference standard

Conclusions

- Results indicate that current instruments are sensitive enough to detect metabolites from realistic (food) exposure levels
- Both (semi)-targeted and non-targeted workflows are useful, depending on the available samples
- Application of the technique facilitates in selection of best (most sensitive) biomarker to obtain/purchase for quantitative analysis

Thank you

WAGENINGEN
UNIVERSITY & RESEARCH

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RIKILT – Wageningen University & Research

Acknowledgements
Arné Oerlemans, Radboud University Medical Centre
Paul Scheepers, Radboud University Medical Centre
Arjen Lommen, RIKILT
Robin Wegh, RIKILT
Frederike van Tricht, RIKILT

RIVM: funding of volunteer study tebuconazole