IVM Institute for Environmental Studies

VU UNIVERSITY AMSTERDAM

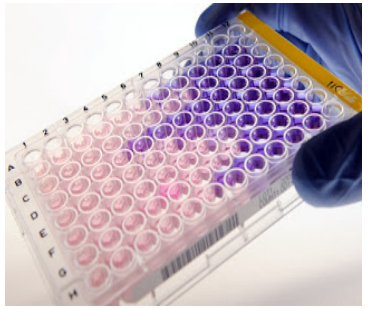
Sara Tufi

HILIC coupled to mass spectrometry for targeted and non-targeted metabolomics

Marja Lamoree

Why metabolomics for environmental quality assessment?

Limitations of (in vitro) bioassays:



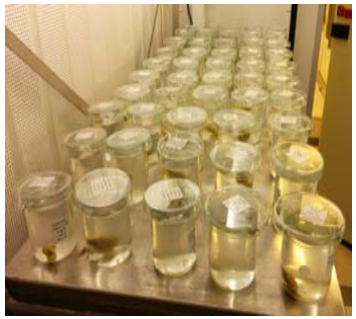
> Focus on one specific endpoint

> Limited sensitivity

Metabolomics

❖ Focus on multiple endpoints (metabolites or sets of metabolites) simultaneously

❖ Includes *in-vivo* metabolism



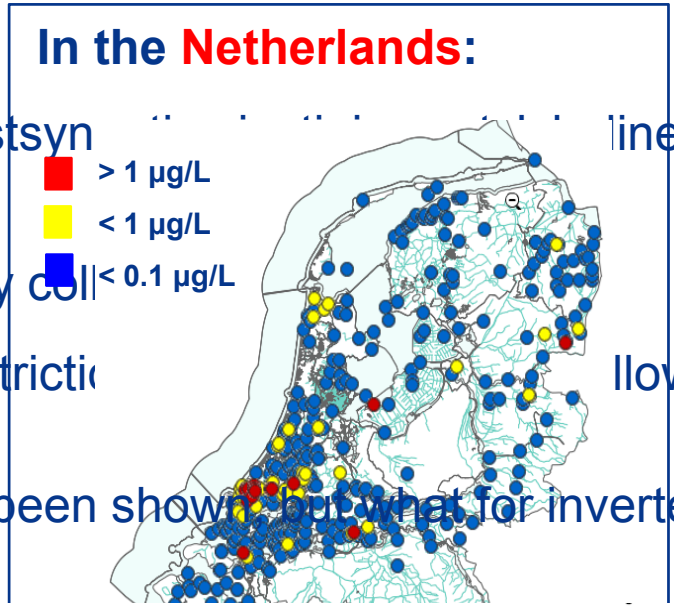
Metabolomics

Aim: study sub-lethal neurotoxic effects in non-target invertebrate species

- Non-targeted metabolomics: method development to investigate changes in (non-target) hydrophilic and hydrophobic metabolites
- Targeted metabolomics: method development for neurotransmitter profiling of *L. stagnalis*
- Apply both approaches to snails exposed to
  - i) imidacloprid
  - ii) a surface water extract

Background - Imidacloprid

- Neonicotinoids - novel generation of neuro-active insecticides
- High water solubility (610 mg/L in 20°C H_2O; log K_{ow} = 0.57)
- High environmental occurrence
- Neonicotinoid insecticides probably affect postsyn[illegible]line receptors (nAChRs)
- Evidence of a connection to honey bee colony coll[illegible]
- EU imposed a number of (temporary) use restricti[illegible]llowed in greenhouses
- Low affinity for the nAChRs of mammals has been shown, but what for invertebrates and aquatic species?

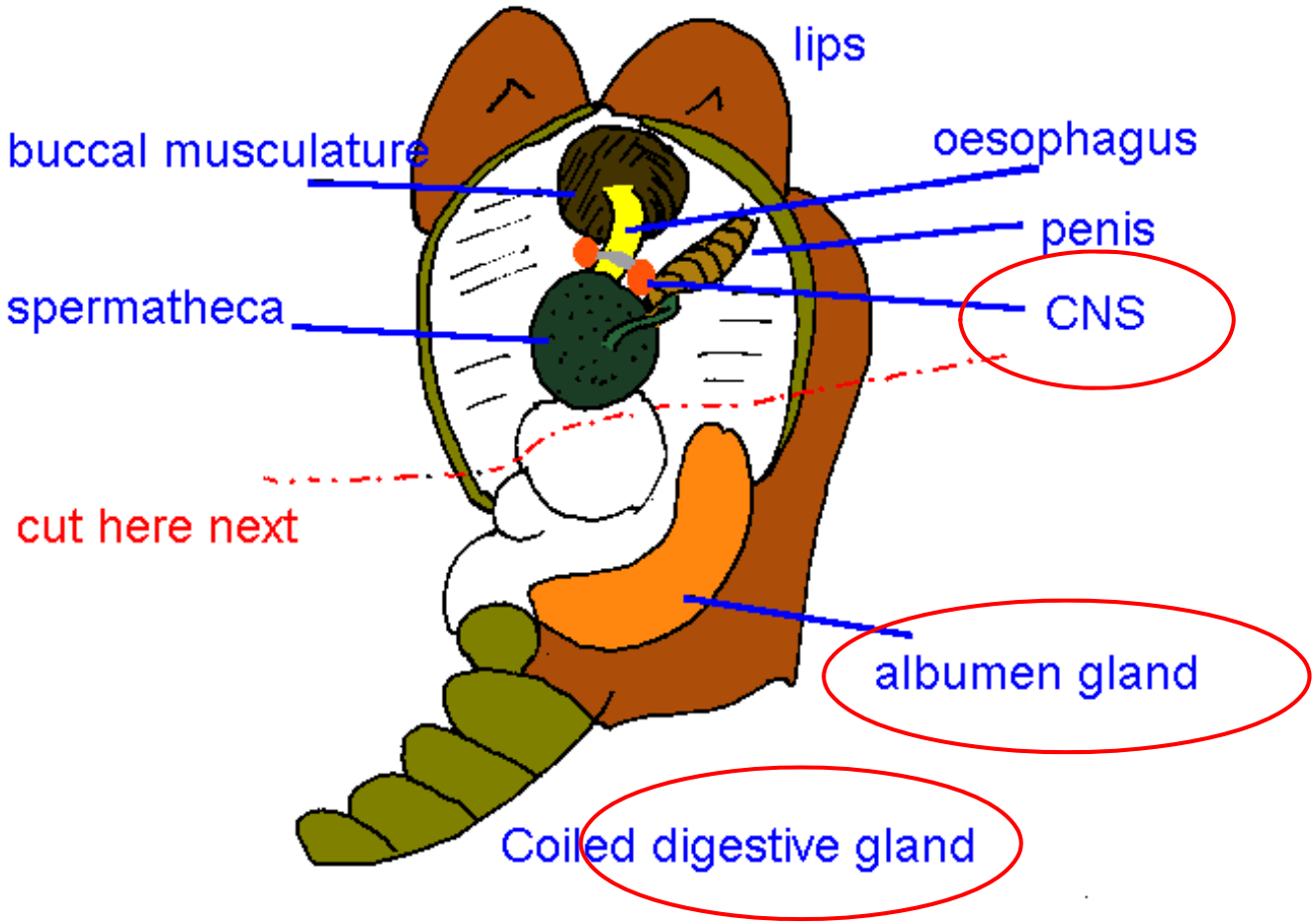


Macro-Invertebrate Decline in Surface Water Polluted with Imidacloprid

Tessa C. Van Dijk, Marja A. Van Staalduinen, Jeroen P. Van der Sluijs*

PLOSone 2013

Lymnaea stagnalis organ dissection



Sample pretreatment:

Snap freezing in liquid nitrogen to quench the metabolic activity

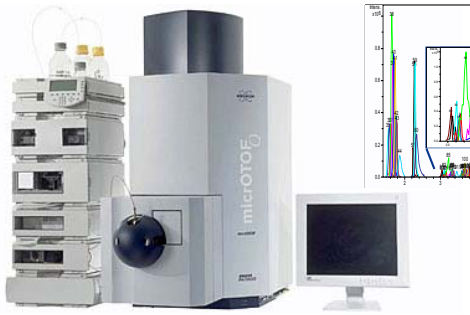
16 week old snails,

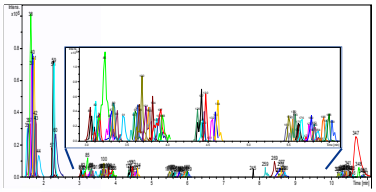
± 2.85 cm shell length

Metabolomics workflow

Study design

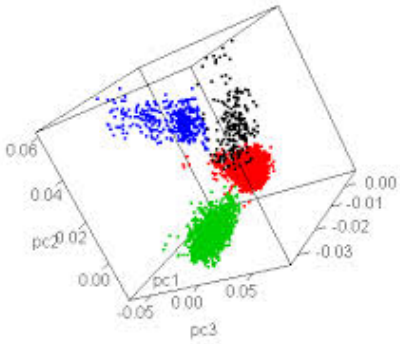
Extraction of metabolites



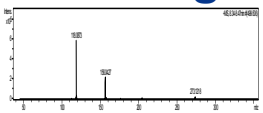


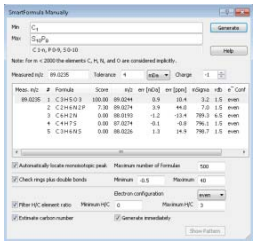
Chemical analysis

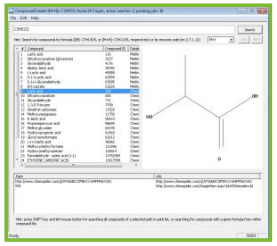
Data analysis



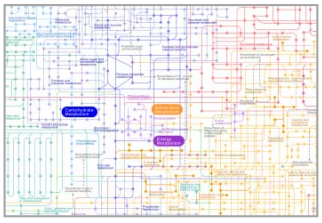
Metabolite screening/identification



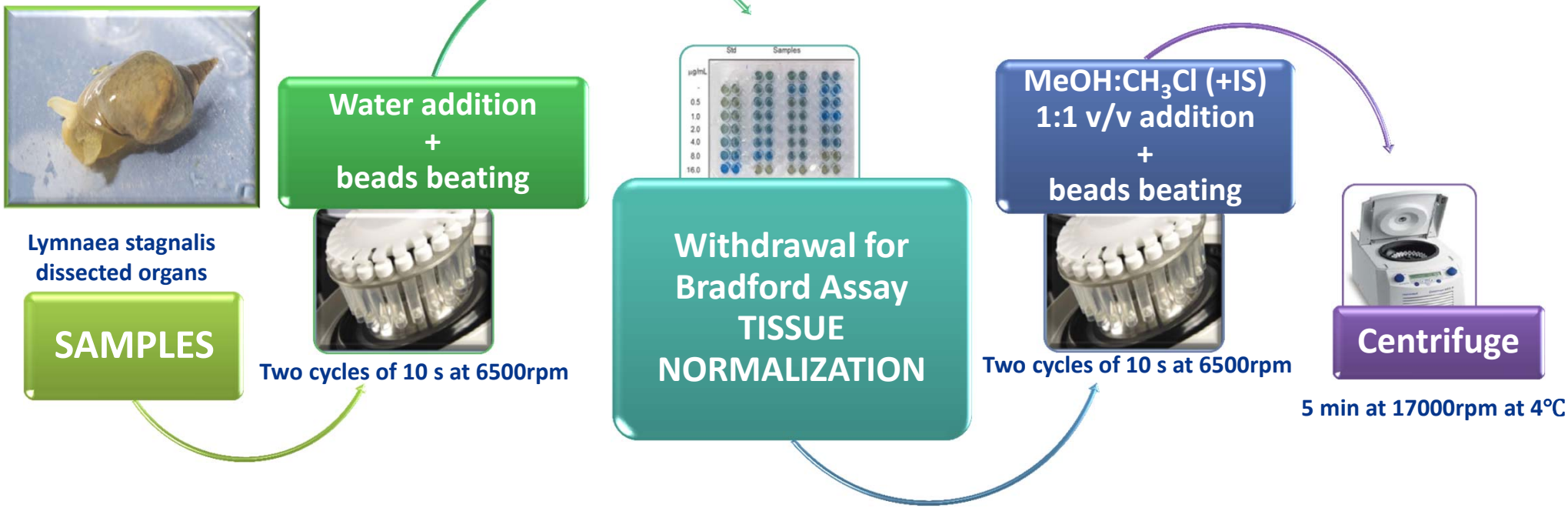




Biological interpretation

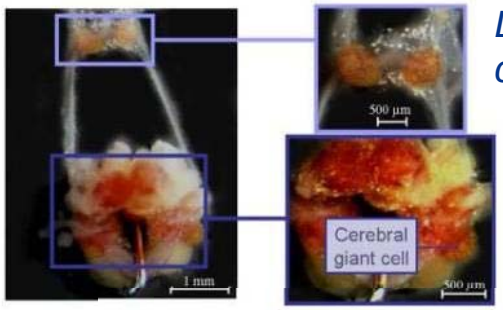


Sample preparation

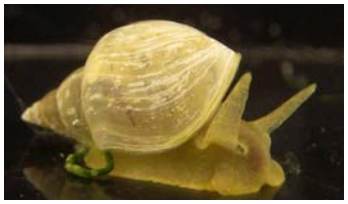


- A tissue normalization step was included to normalize data
- A mix of internal standards of different metabolite classes was added to normalize the signal intensities and to align retention times

Analytical strategy



Lymnaea stagnalis
central nervous system



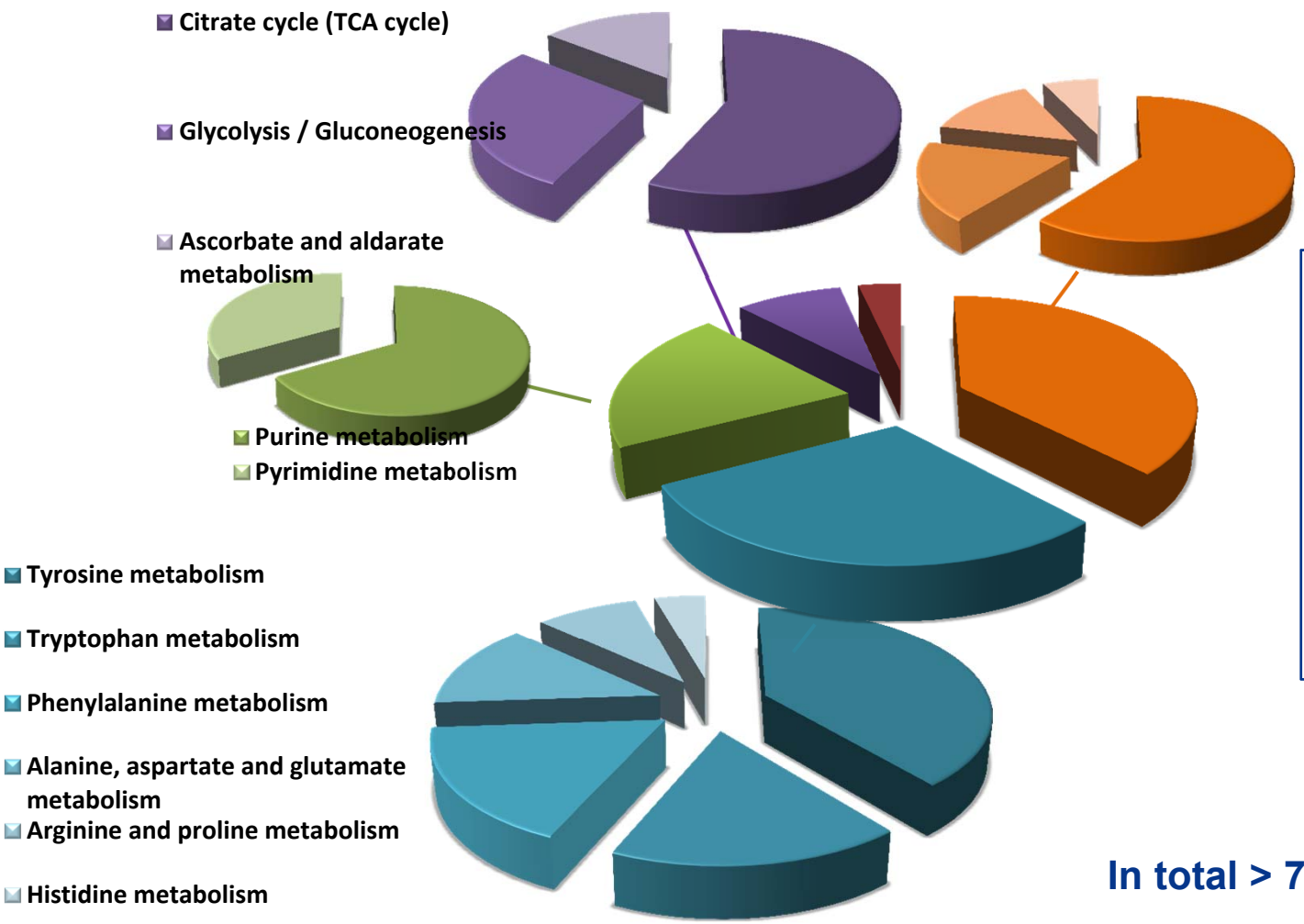
Samples — **SNAIL CNS**

$MeOH/H_2O$ fraction | **Chloroform fraction** — **EXTRACTION**

TARGETED ANALYSIS neurotransmitters | **NONTARGETED ANALYSIS** polar metabolites | **NONTARGETED ANALYSIS** nonpolar metabolites — **ANALYTICAL STRATEGY**

LC-QQQ | **HILIC - (ESI) TOF** | **GC – (APCI) TOF** — **ANALYTICAL METHOD**

Metabolic pathways investigated



- Lipid Metabolism
- Amino Acids Metabolism
- Nucleotide metabolism
- Carbohydrate Metabolism
- Other

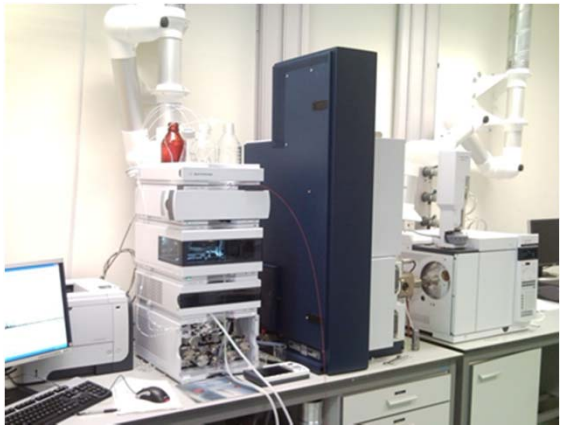
In total > 70 metabolites covered

HILIC, RP-LC and GC analysis

Waters **XBridge Amide** column, 150×2.1 mm, 3.5 µm; Amide guard column 10×2.1mm, 3.5 µm Gradients of ACN/H2O 30:70 v/v NH4HCOOH 10 mM (A) and ACN/H2O 95:5 v/v ammonium acetate 10 mM (B); for **pH 9.0** adjustment with ammonium hydroxide and for **pH 2.8** with HCOOH to pH 2.8. For both LC approaches, the separation occurred at 30 °C, the flow rate was set to 0.25 ml/min, and the injection volume was 5 µL

Waters **C18** Symmetry column, 150×2.1 mm, 3.5 µm; C18 guard column 10×2.1 mm, 3.5 µm Gradient of H_2O and ACN with 0.1 % formic acid.

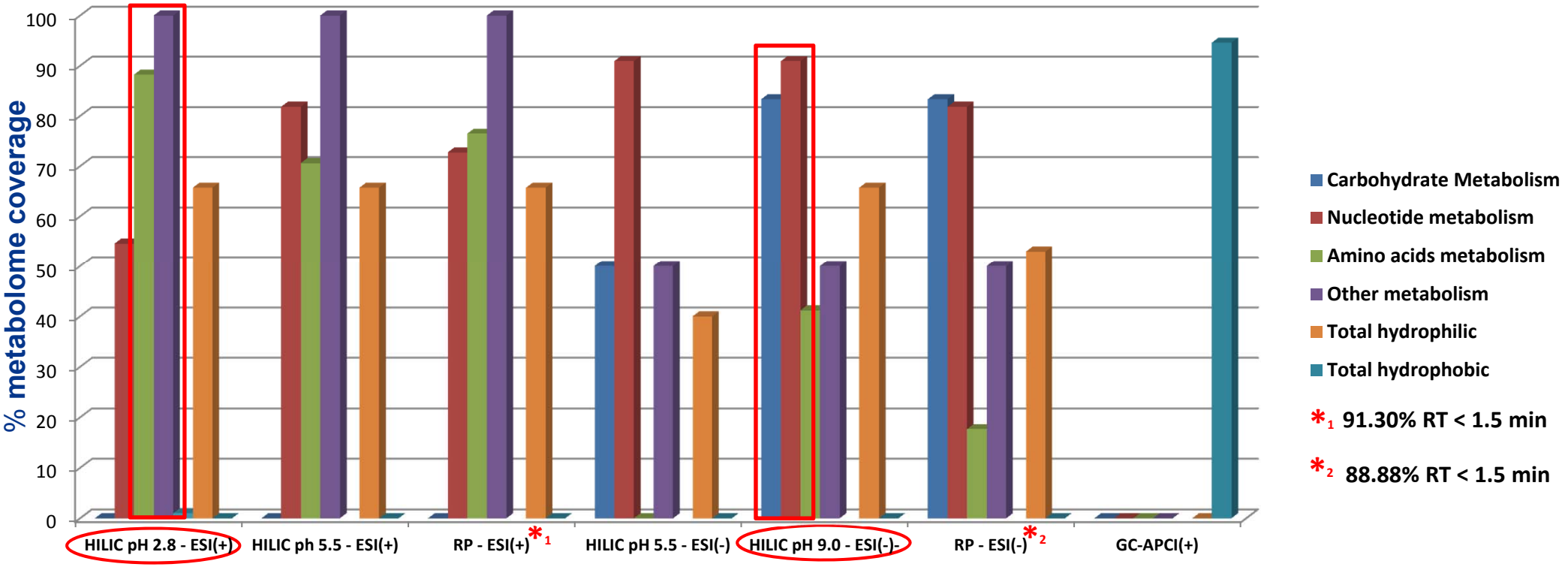
GC-APCI Derivatization of chloroform fractions with 500 µl of a methanolic BF_3 solution, kept for 30 min at 80 °C, with subsequent liquid/liquid extraction with hexane



HR-TOF-MS on MICROTOF II, Bruker

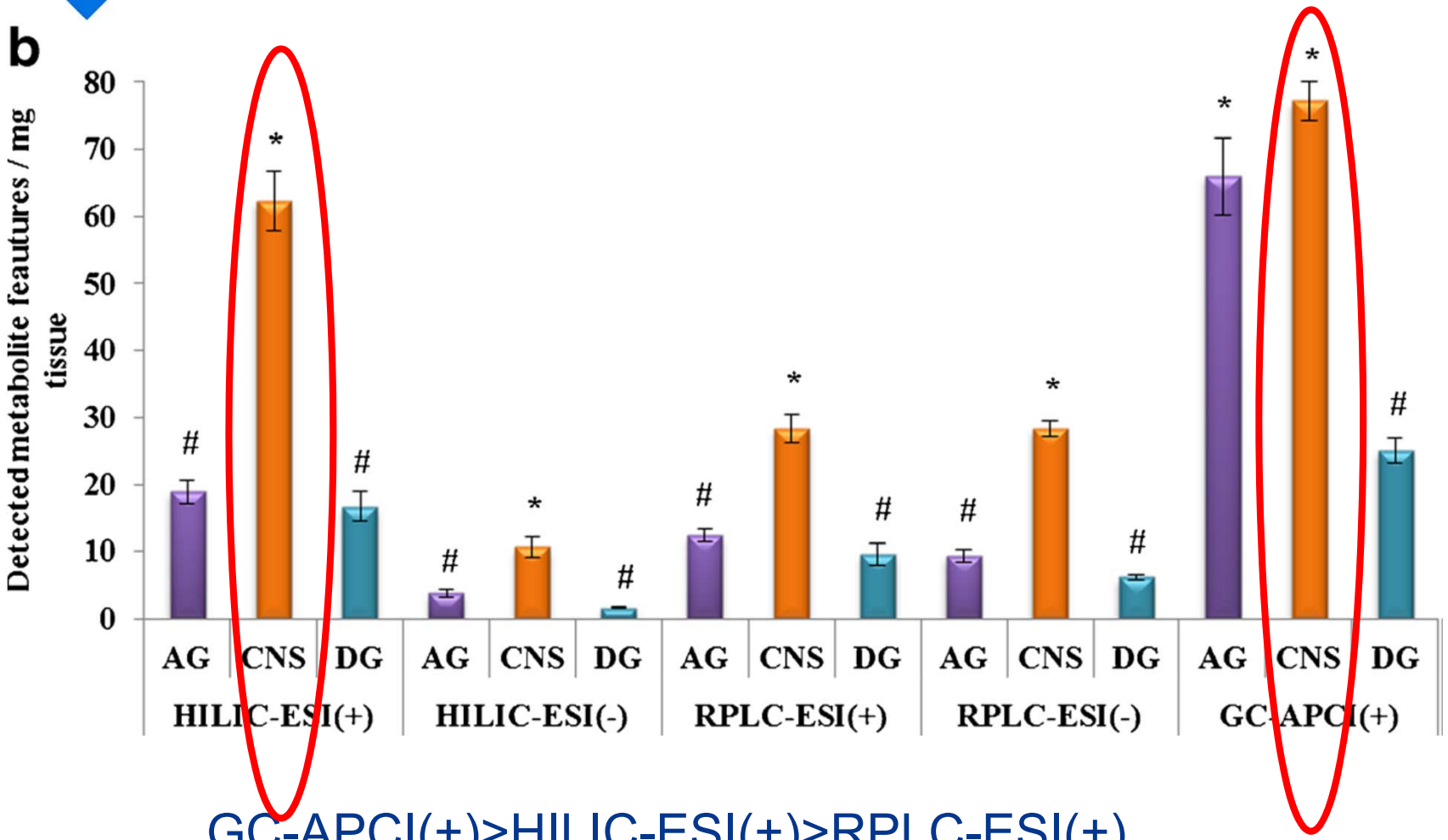
Platform performance - metabolome coverage

Hydrophilic and hydrophobic metabolite fractions



- Each analytical platform has its own specific performance for the different metabolite classes
- HILIC represents an important chromatographic method for polar metabolites

Molecular features



GC-APCI(+)>HILIC-ESI(+)>RPLC-ESI(+)

Targeted analysis of neurotransmitters, precursors and metabolites

choline

normetanephrine

glutamate

epinephrine

L-Dopa

serotonin

5-HIAA

histidine

tyramine

glutamate

dopamine

acetylcholine

3-MT

glutamine

5-hydroxy-L-tryptophan

GABA

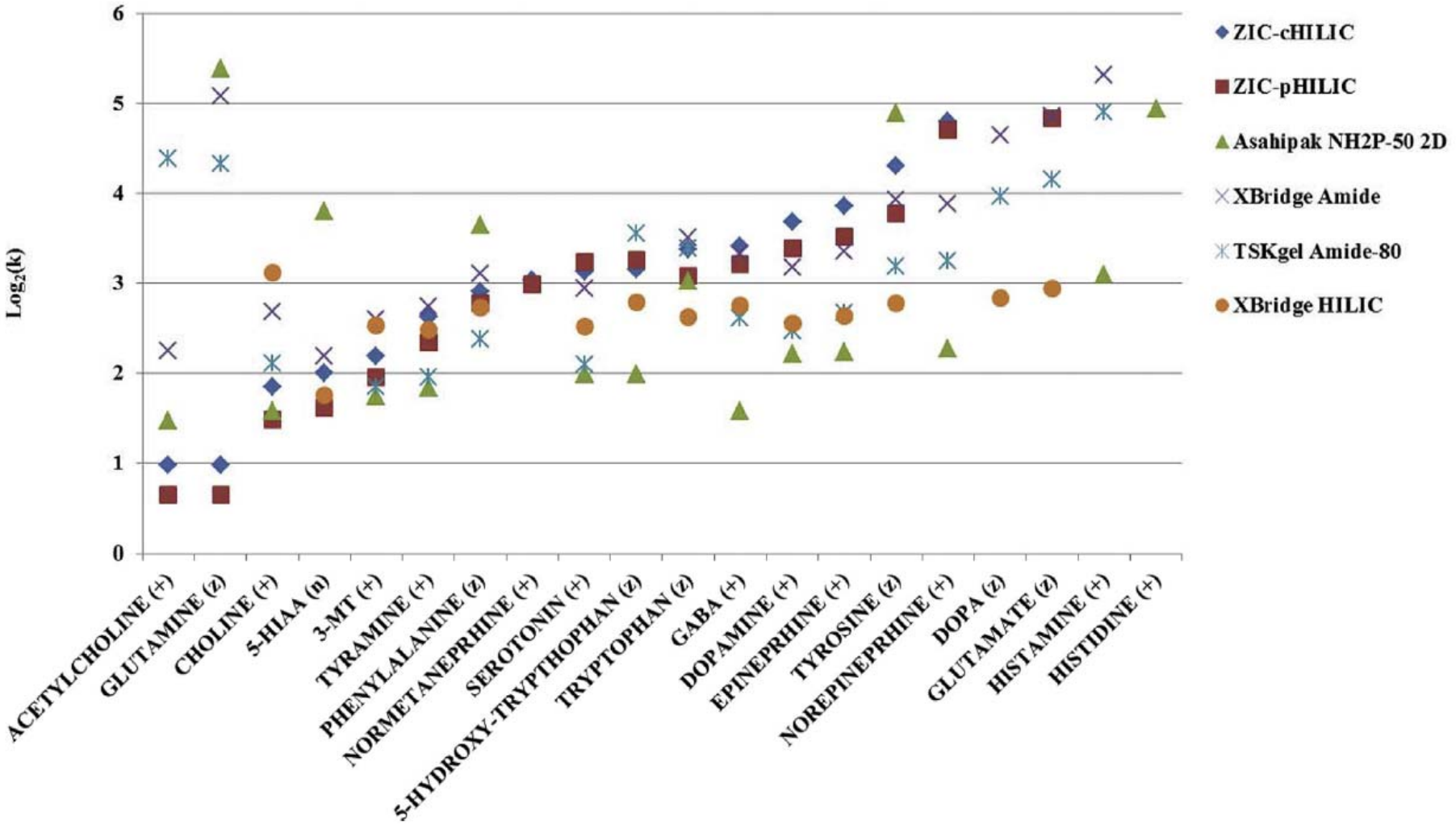
histamine

HILIC columns tested

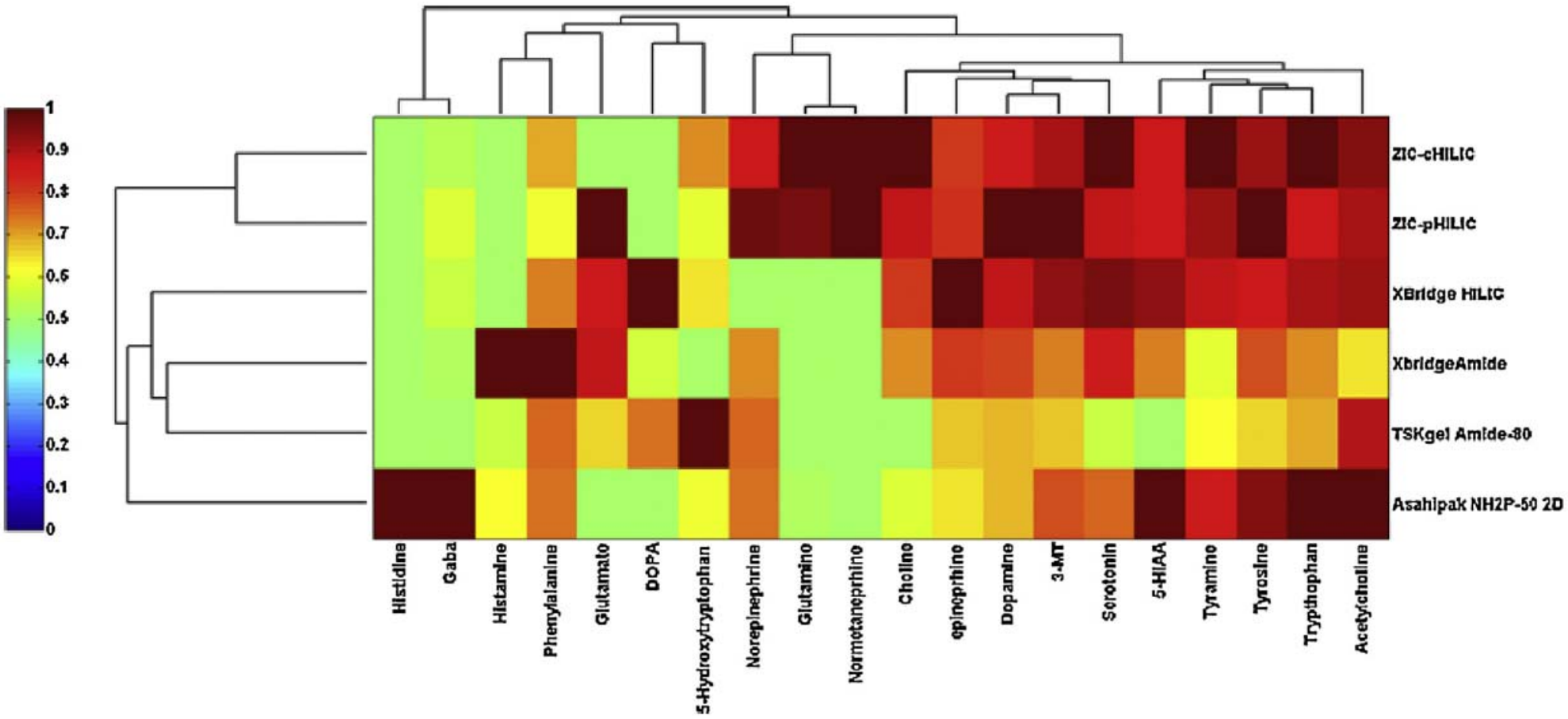
Physico-chemical parameters of the HILIC columns tested evaluated using a ToF-MS.

Column	Provider	Functionality/Support	Size (mm)	Particle size (μm)
XBridge HILIC	Waters	Silica/Silica	4.6 × 100	2.5
XBridge Amide	Waters	Amide/Silica	2.1 × 100	3.5
TSKgel Amide-80	Tosoh	Amide/Silica	2.0 × 100	5
Asahipak NH2P-50 2D	Shodex	Amino/Polyvinyl alcohol Amino	2.0 × 150	5
ZIC-cHILIC	Merck-Sequant	Zwitterionic/Silica	2.1 × 150	3
ZIC-pHILIC	Merck-Sequant	Zwitterionic/Polymeric	2.1 × 150	5

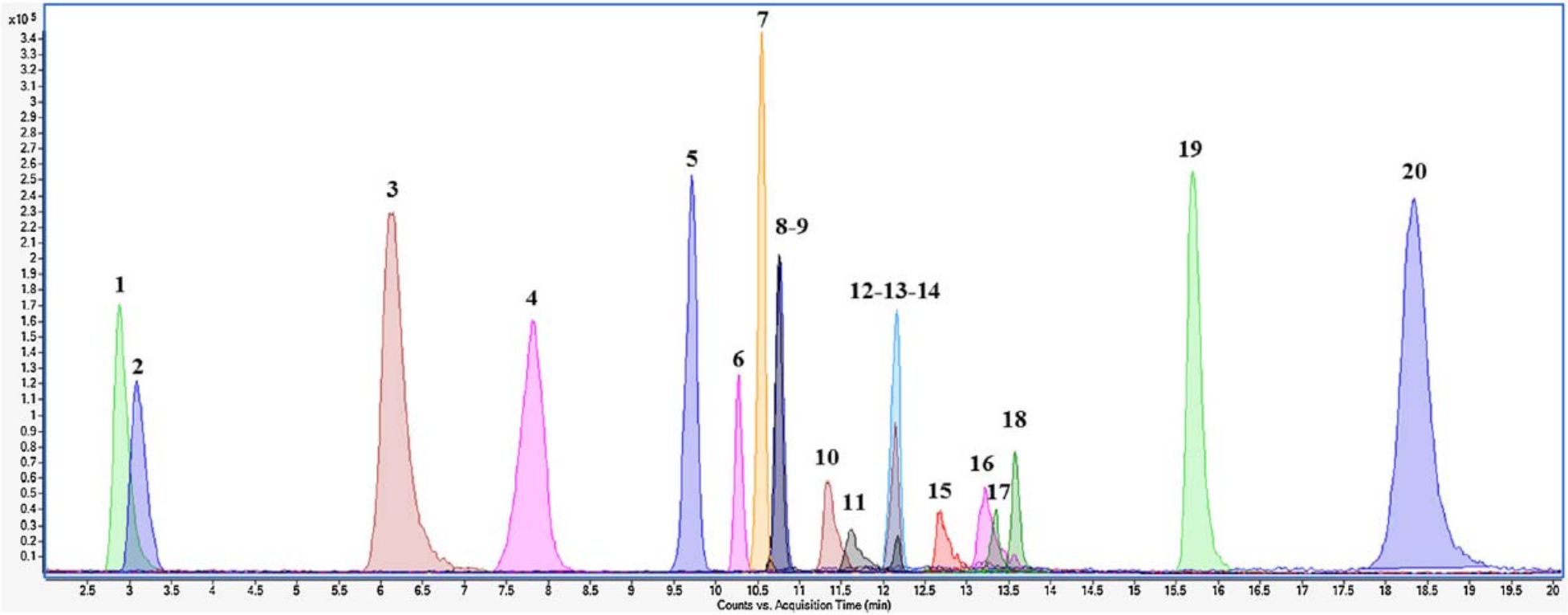
Separation factor k (log_2) distribution



Heat map and hierarchical clustering analysis of peak areas



HILIC-QqQ chromatogram of 20 neurotransmitters, precursors and metabolites



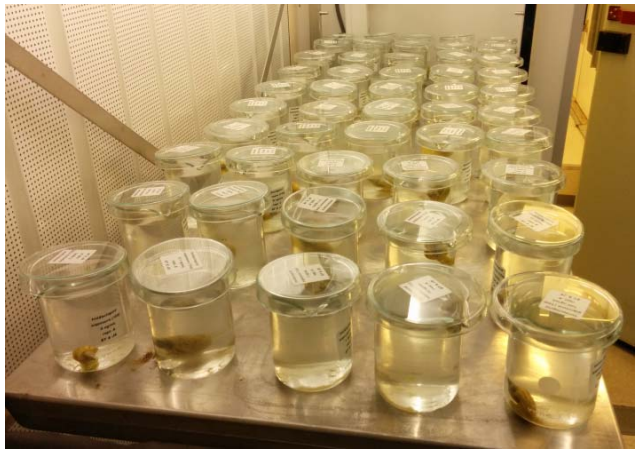
0.3 ml/min; gradient of 100% H_2O and 90:10 ACN:H_2O (v/v)

Method validation parameters

Analyte	Labeled IS	RT	RSD %; RT Intraday	RSD %; RT Interday	Linear range (ng/mL)	R^2	LOD (ng/mL)	LOQ (ng/mL)	R %	RSD % Method
3-MT	3-MT-d_4	7.7	1.0	6.2	5–3000	0.996	1.11	3.3	95	2.2
5-HIAA	5-HIAA-d_5	3.4	1.6	4.1	10–2500	0.990	3.85	12	90	2.3
5-Hydroxy-L-tryptophan	5-Hydroxy-L-tryptophan-d_4	12.2	1.0	2.6	20–3000	0.975	3.68	11	73	6.8
Acetylcholine	Acetylcholine-d_4	3.0	0.5	4.8	0.05–1000	0.998	0.02	0.1	92	1.1
Choline	Choline-d_{13}	6.3	0.9	5.4	1–1000	0.990	0.35	1.0	85	2.1
L-DOPA	DOPA-d_3	13.3	0.5	4.7	100–3000	0.902	19.5	58	84	9.4
Dopamine	Dopamine-d_4	11.4	0.4	3.8	20–2500	0.995	5.50	17	86	2.7
Epinephrine	Epinephrine-$^{13}C_2$ ^{15}N	11.6	0.3	4.4	20–2500	0.986	5.56	17	87	3.3
GABA	GABA-d_6	12.1	0.3	3.5	40–3000	0.979	19.3	58	81	4.2
Glutamate	Glutamate-d_5	13.6	0.8	2.2	20–3000	0.960	2.47	7.4	63	18
Glutamine	Glutamine-^{13}C ^{15}N	13.3	0.3	2.5	5–3000	0.942	1.51	4.5	79	1.7
Histamine	L-Tryptophan-d_3	18.3	0.5	1.5	100–3000	0.973	25.2	76	89	1.7
Histidine	L-Tryptophan-d_3	15.8	0.4	2.5	20–400	0.930	0.31	0.9	89	1.7
L-Tryptophan	L-Tryptophan-d_3	10.7	0.4	3.2	5–2500	0.993	0.54	1.6	89	1.7
L-Tyrosine	L-Tyrosine-d_4	12.1	0.5	3.2	40–3000	0.948	8.22	25	72	3.0
Norepinephrine	Norepinephrine-d_6	12.7	0.5	4.2	40–2500	0.942	13.2	40	89	4.2
Normetanephrine	Norepinephrine-d_6	10.7	0.2	3.6	1–1000	0.995	0.28	0.9	89	4.2
Phenylalanine	Epinephrine-$^{13}C_2$ ^{15}N	10.3	1.0	3.2	0.1–1500	0.988	0.03	0.1	87	3.3
Serotonin	Serotonin-d_4	10.5	0.8	3.9	0.5–2500	0.994	0.10	0.3	89	1.6
Tyramine	3-MT-d_4	9.7	0.5	4.8	1–1000	0.997	0.35	1.0	95	2.2

Tufi et al., J. Chromatogr. A 2015

Exposure to imidacloprid



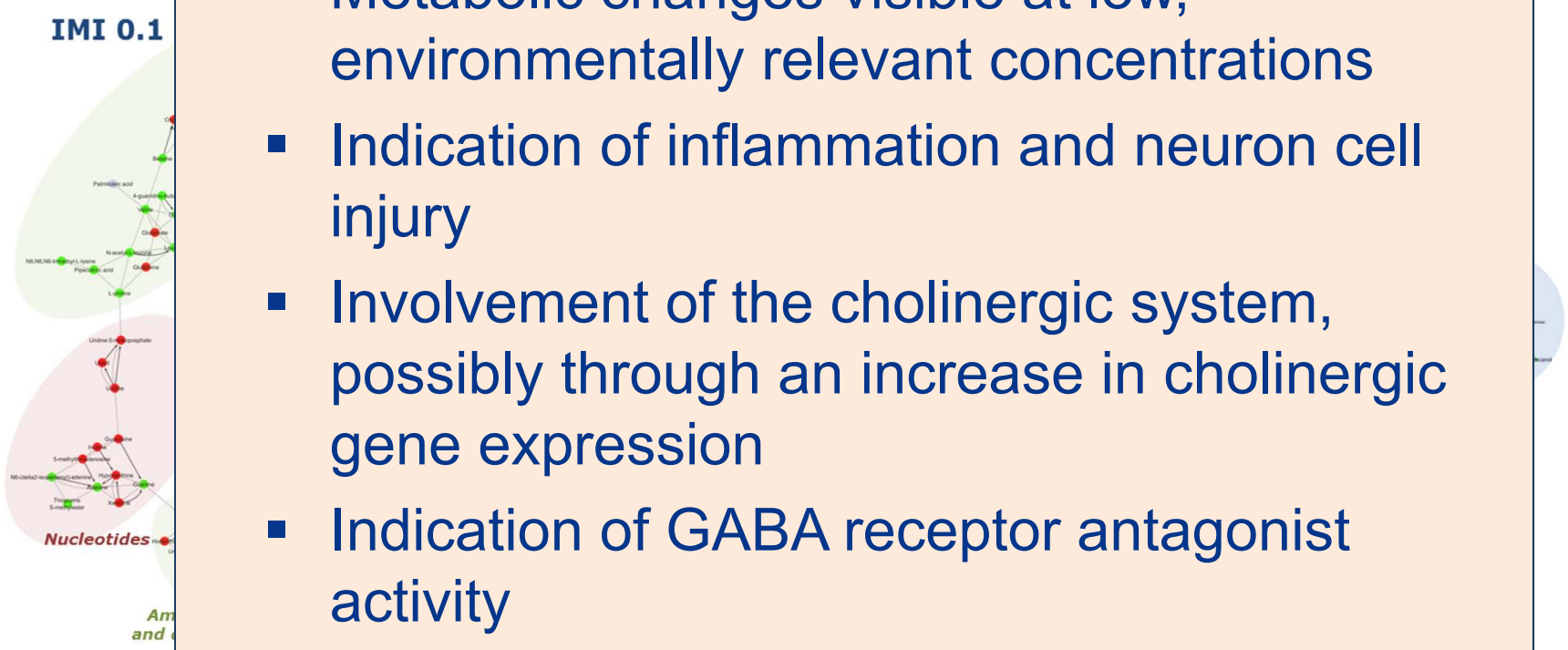
10 DAYS
CTRL GROUP
0.1 µg/L
1 µg/L
10 µg/L
100 µg/L

Conditioning period of 2 days for adaptation

Refreshing and egg counting every second day

Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
Selection	Condition	Condition	Exposure	Exposure	Exposure	Exposure	Exposure	Exposure	Exposure	Exposure	Exposure	Exposure	Dissection
			Egg counting		Egg counting		Egg counting		Egg counting		Egg counting		Egg counting
Food			Food		Food		Food		Food		Food		Food
Refreshing			Refreshing		Refreshing		Refreshing		Refreshing		Refreshing		Refreshing

Data analysis workflow



- Metabolic changes visible at low, environmentally relevant concentrations
- Indication of inflammation and neuron cell injury
- Involvement of the cholinergic system, possibly through an increase in cholinergic gene expression
- Indication of GABA receptor antagonist activity
- Insight in the involvement of different or unexpected metabolic pathways

Down-regulated
Up-regulated
Not-detected

Acknowledgment

More info: sara.tufi@vu.nl
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- Pim Leonards
- Jacob de Boer

BRUKER

EDA EMERGE

THANK YOU

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