“From toxicity assay to metabolomics analysis”
An integrated approach to assess the toxicity of three Benzotriazoles in zebrafish (*Danio rerio*) embryos

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Faculty of Earth and Life Sciences

IVM Institute for Environmental Studies

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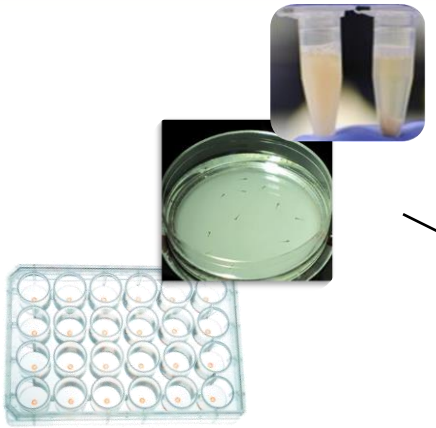
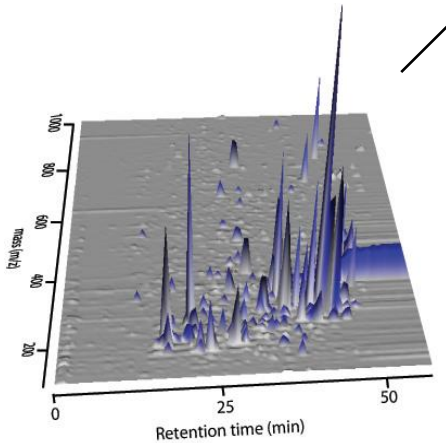
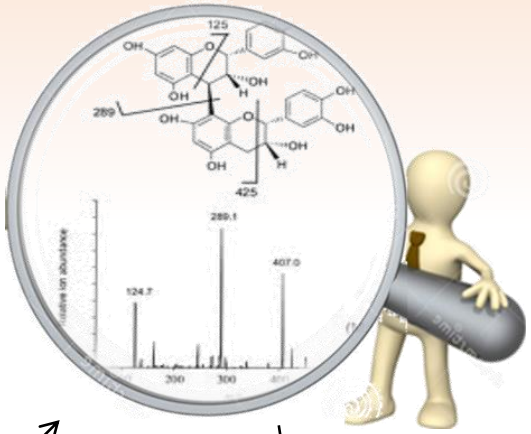


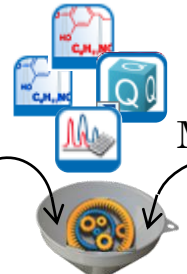


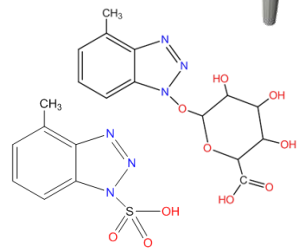


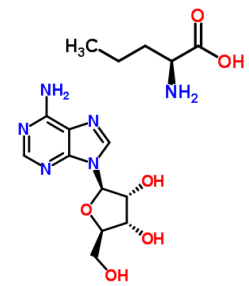
Non target screening

Suspect screening

Targeted Metabolomics



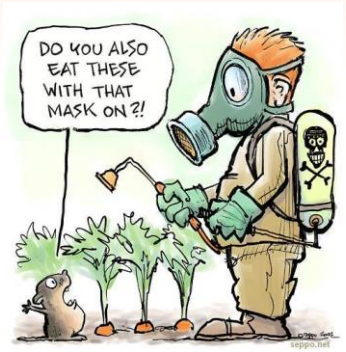


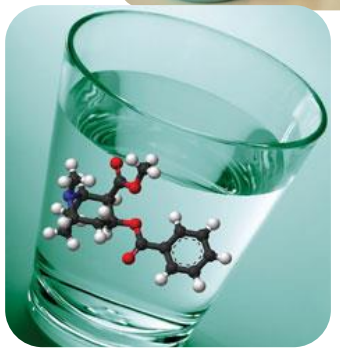


Aquatic environment contamination – Emerging pollutants (EPs)

- **Personal care products**
- **Steroids & hormones**
- **Pharmaceuticals**
- **Illicit drugs**
- **Benzotriazoles**
- **Pesticides**
- **Surfactants**
- **Perfluorinated compounds**
- **Siloxanes**

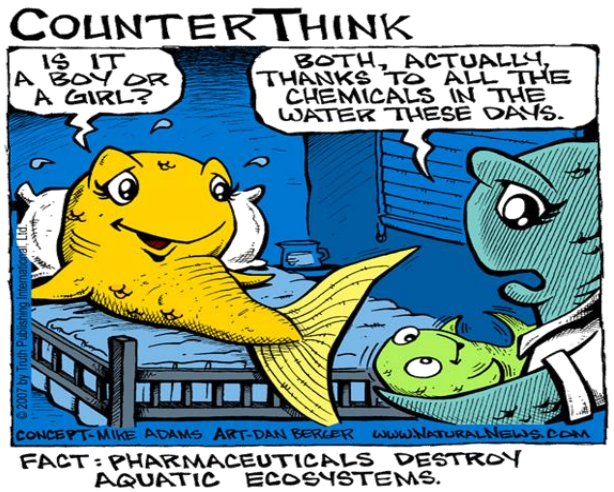
metabolites
&
Transformation products (TPs)





stimulants

antidepresants

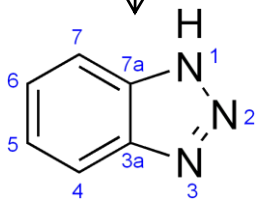


Aquatic environment contamination – Benzotriazoles (BTs)

Anticorrosives

Dishwashing detergents

Aircraft deicer & antifreeze fluids



High production volume !

WWTP

biodegradability
sorption tendency

Bio-accumulation in aquatic organisms

potential ADVERSE effects

Aim : potential **ADVERSE** effects **of BTs in aquatic organisms**

Acute toxicity **?**

LC_{50} **?**

Phenotypic abnormalities **?**

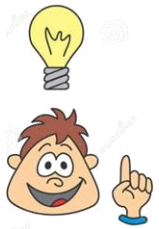
Xeno-metabolome
[xenobiotics (unmodified) + bio-TPs]

C_{int} **?**
(internal concentration)

bio- transformation **?**

Changes in **molecular** lvl **?**

Endo-metabolome
[endogenous (organism) metabolites]



lets try a **combined** approach

Toxicity assay approach !

- ☑ LC_{50} (comparable among compounds)
- ☑ toxicity **phenotype**
- ☒ focus on **specific endpoints**
- ☒ **high** concentrations

Toxicokinetic approach !

- ☑ C_{int} better estimate of **biologically effective** dose
- ☑ **Bio-transformation** assessment
- ☑ **detoxification** pathways
- ☑ ***Xeno-metabolome***

Targeted metabolomics approach !

- ☑ toxicity assessment in **molecular lvl**
- ☑ focus on **multiple endpoints**
- ☑ **metabolic pathways** affected
- ☑ ***Endo-metabolome***

need for a model organism

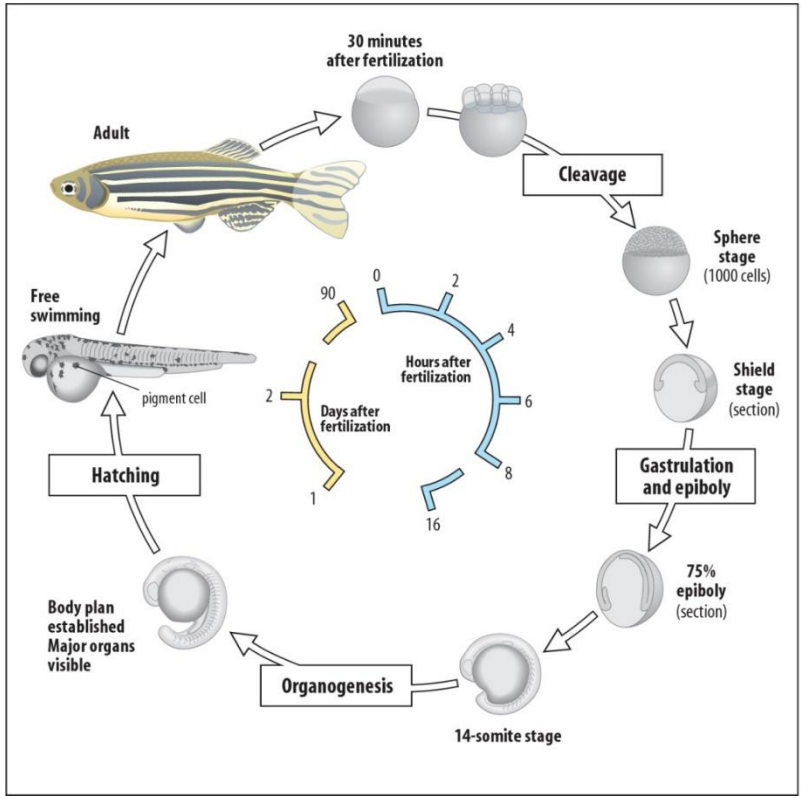
... and this is...
zebrafish

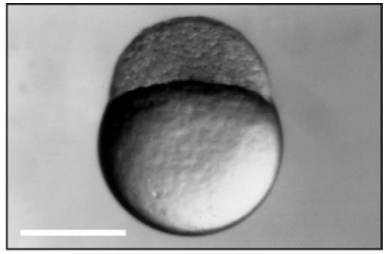
Ideal for keeping in the lab

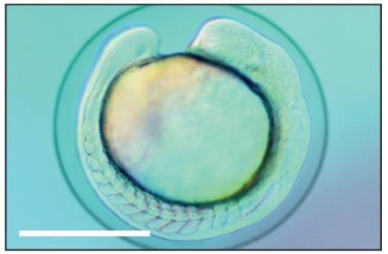
- ✓ Embryo and larval **small size**
- ✓ **Rapid generation** of large number of embryos
- ✓ Husbandry **costs**

Suitable for all 3 approaches

- ✓ Recommended by the OECD for **fish toxicity** testing (TG 236)
- ✓ Thoroughly studied organism in **all developmental stages**
- ✓ Similar **detoxification pathways** with mammals (phase I & II enzymes)
- ✓ **Metabolic pathways** are known

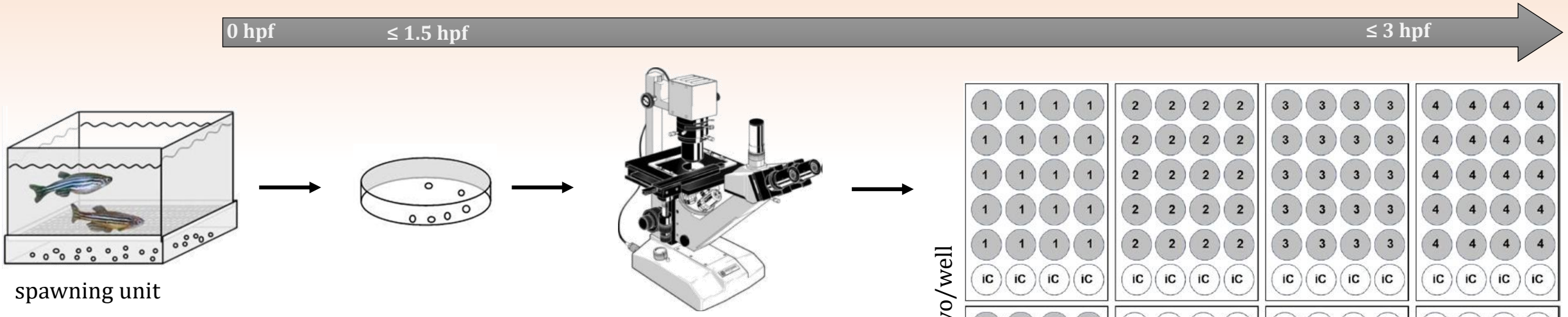




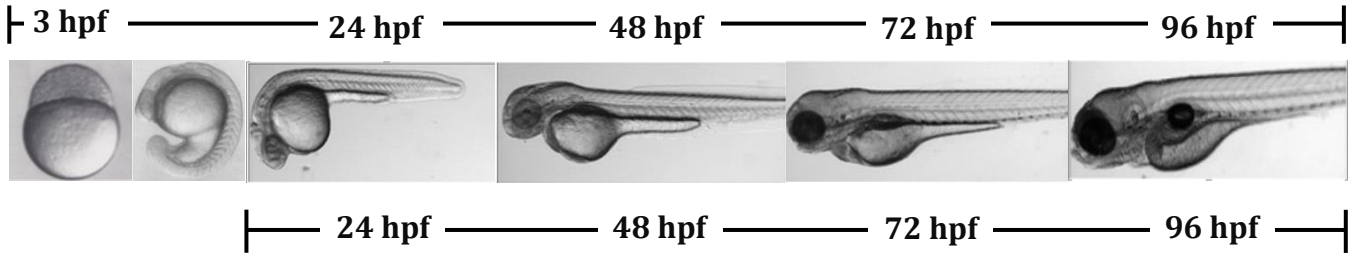


Experimental part – Toxicity assay approach

Fish **E**mbryo Acute **T**oxicity test – **FET TG 236**



(a) $\mathbf{LC_{50}}$ calculation



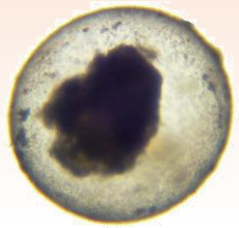
(b) morphological **phenotyping**

nC = negative control (dilution water)
iC = internal plate control (dilution water)
pC = positive control (3,4-DCA 4mg/L)
sC = solvent control

Experimental part – Toxicity assay approach

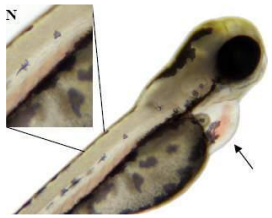
(a) LC_{50} calculation - Indicators of LETHALITY

- Coagulation of fertilized eggs



- Lack of detachment of the tail-bud from the yolk sac

- Lack of heartbeat

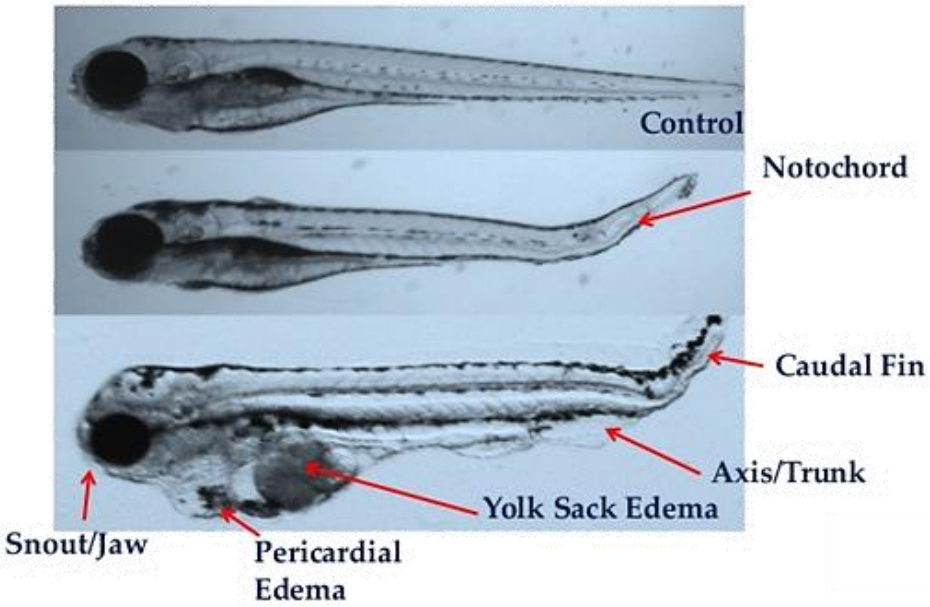


- Lack of somite formation

(b) Morphological phenotyping

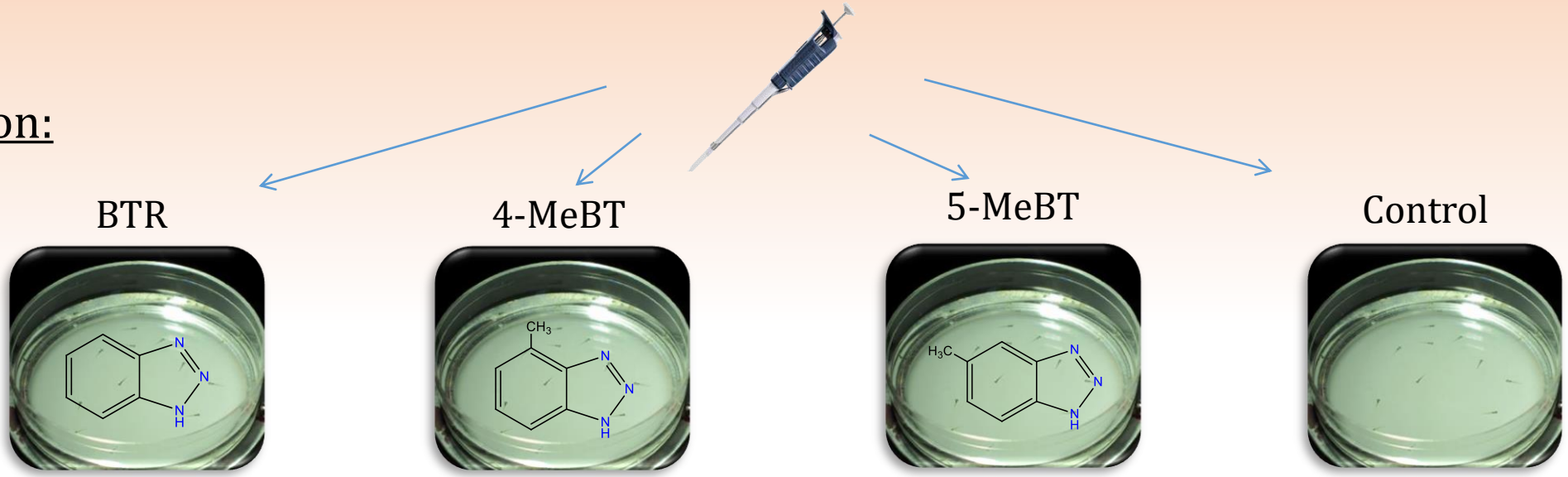
morphological endpoints

heart, spinal cord, somite, notochord, brains, arches, jaws, tail, fins, face, stomach, liver

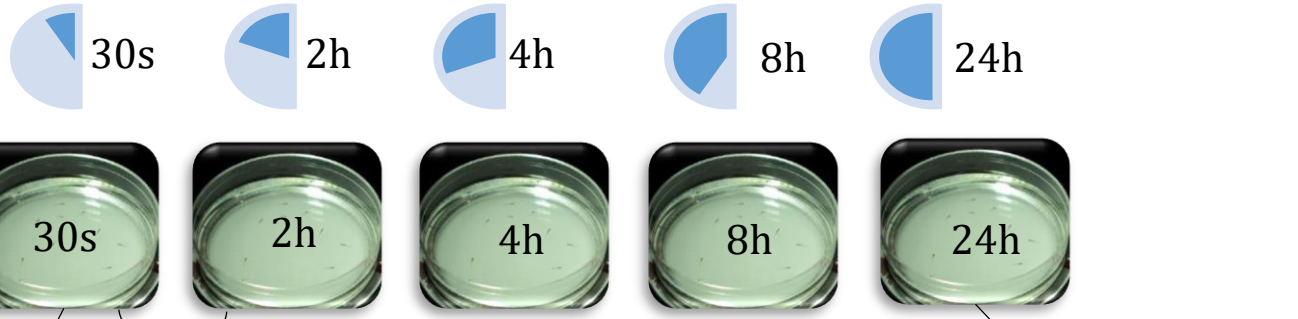


Experimental part – Toxicokinetic & Metabolomic approaches

Incubation:



for each "exposure"
5 time intervals :



time profile of :

- Xenobiotics
- Bio-TPs
- Metabolites

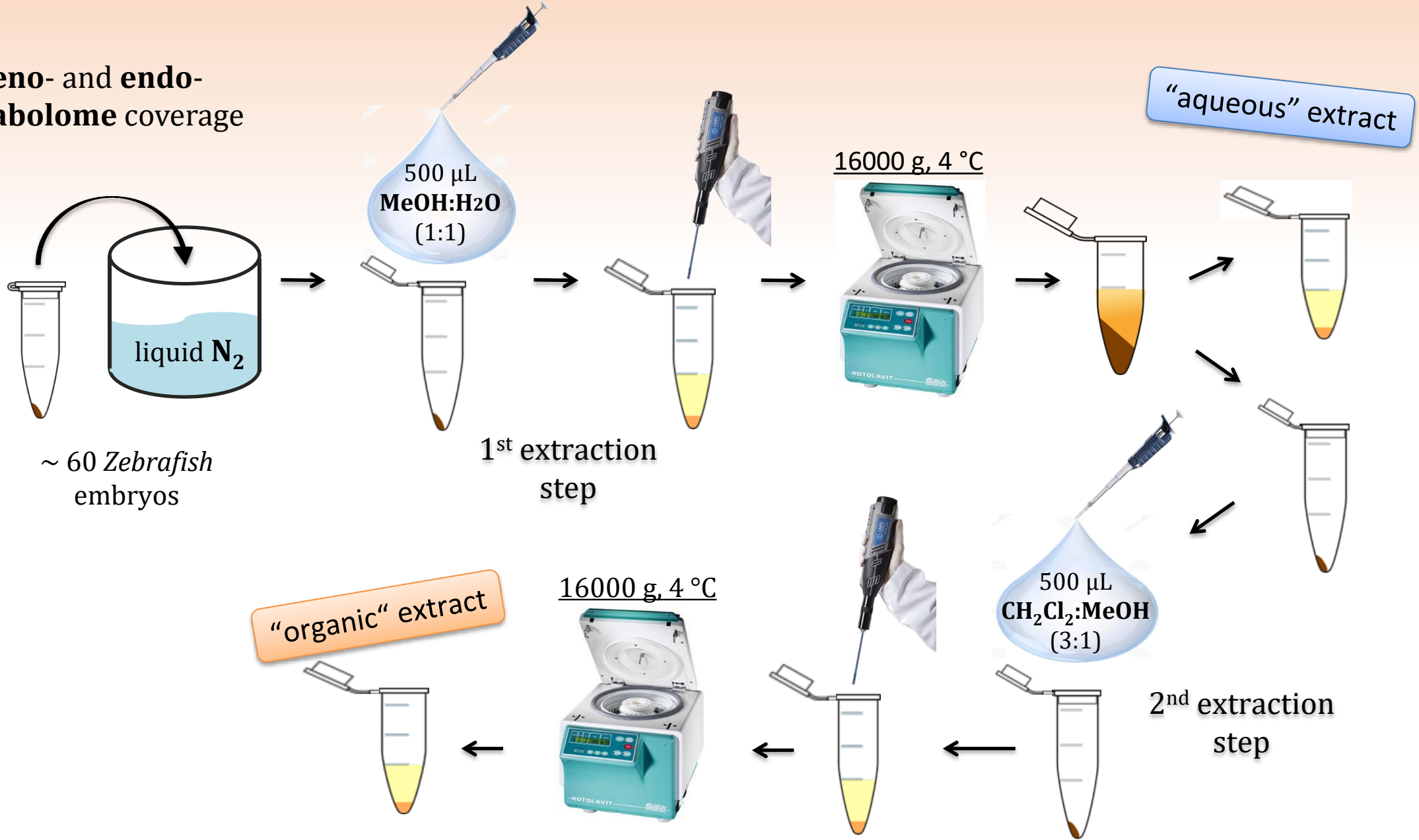
Estimation of skin adsorption

Short term effects

Long term effects

Sample preparation – Toxicokinetic & Metabolomic approaches

☑ **xeno**- and **endo**-**metabolome** coverage



Evaluation part – Toxicokinetic & Metabolomic approaches

Analysis - UHPLC-Q-TOF MS/MS

- RPLC (+/- ESI) (Acclaim C18)
- HILIC (+/- ESI) (BEH amide)

Data Acquisition modes

- Data-Independent (bb-CID)
- Data-Dependent (Auto-MS)

- ✓ **Xeno-**metabolome (low-medium polarity)
- ✓ **Endo-**metabolome (medium-high polarity)

bio-TPs Identification

- **Suspect** Screening
  (knowledge based approach)

DataAnalysis TargetAnalysis Metabolite Predict

- **Non-target** Screening
  (treated/control comparison based)

Metabolite Detect DataAnalysis

Metabolic profiling

- **Targeted** metabolomics Database
  > 600 metabolites - RPLC (+,-) & HILIC (+,-)

TargetAnalysis DataAnalysis

- **Wide-scope** Targeted metabolomics screening

TASQ

Suspect screening

suspect list compilation

Metabolite Predict (*in-silico* drug metabolism prediction tool)

.csv

A	B	C	D
m/z	RT	sum formula	name
		C7 H7 N3	4 Me-BTR
		C7 H5 N3 O	4 Me-BTR-147
		C7 H7 N3 O	4 Me-BTR-149
		C8 H9 N3 O	4 Me-BTR-163
		C7 H7 N3 O2	4 Me-BTR-165

TargetAnalysis DataAnalysis

Identification Workflow

Ion intensity & Peak area thresholds
- (+ESI) : 1000 & 4000
- (-ESI) : 500 & 2000

Mass accuracy threshold
- 2 mDa/ 5ppm

Isotopic fit
- 250 mSigma

Absence from Control
- Rt ± 0.2 min
- m/z ± 2 mDa

Peak score (Area/Intensity)
- > 4 (Preferably in the range of 8-32)

Time trend
- Meaningful time trend between time interval samples

Filtering criteria

MS/MS spectral interpretation
- Common fragments with parent compound
- Characteristic neutral loss

Additional experimental evidence
- RPLC
- HILIC
- + ESI
- - ESI

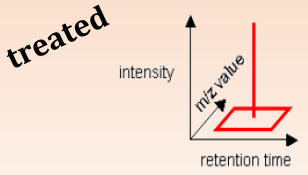
Confirmatory criteria

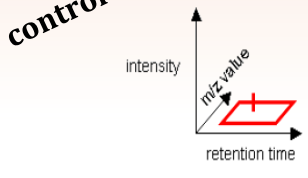
Confidence enhancement

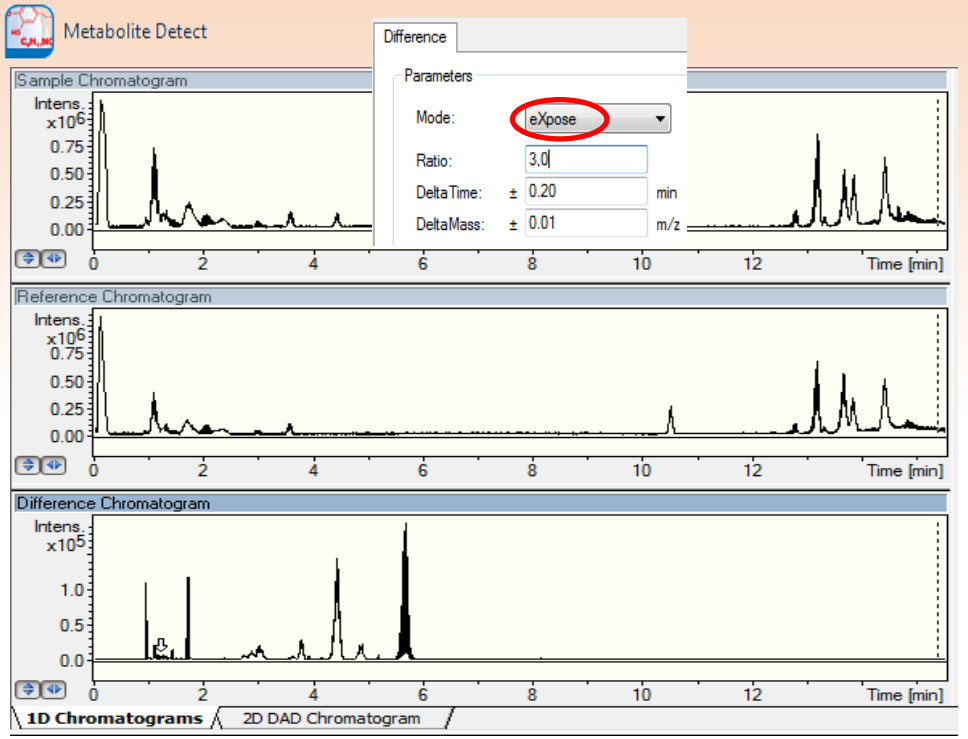
Xenobiotic Metabolism relevance
- Phase I (hydroxylation, oxidation etc)
- Phase II (glucuronidation, sulfation etc)

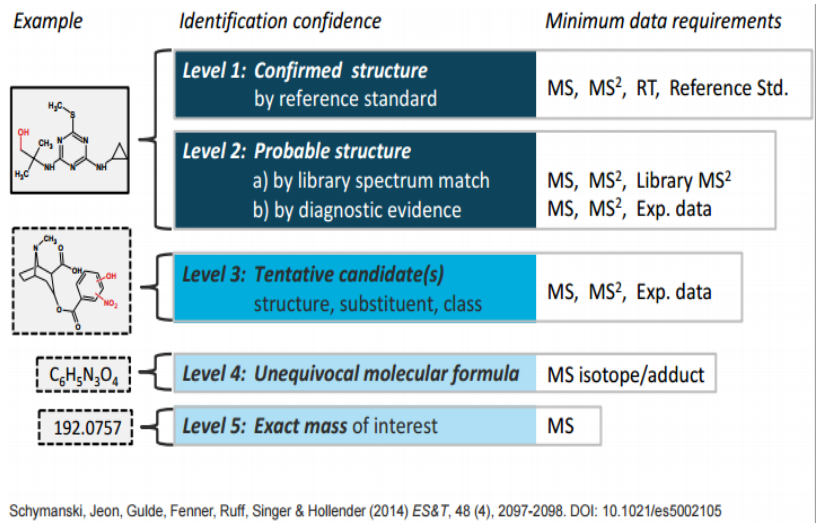
Non-target workflow ONLY

Non-target screening



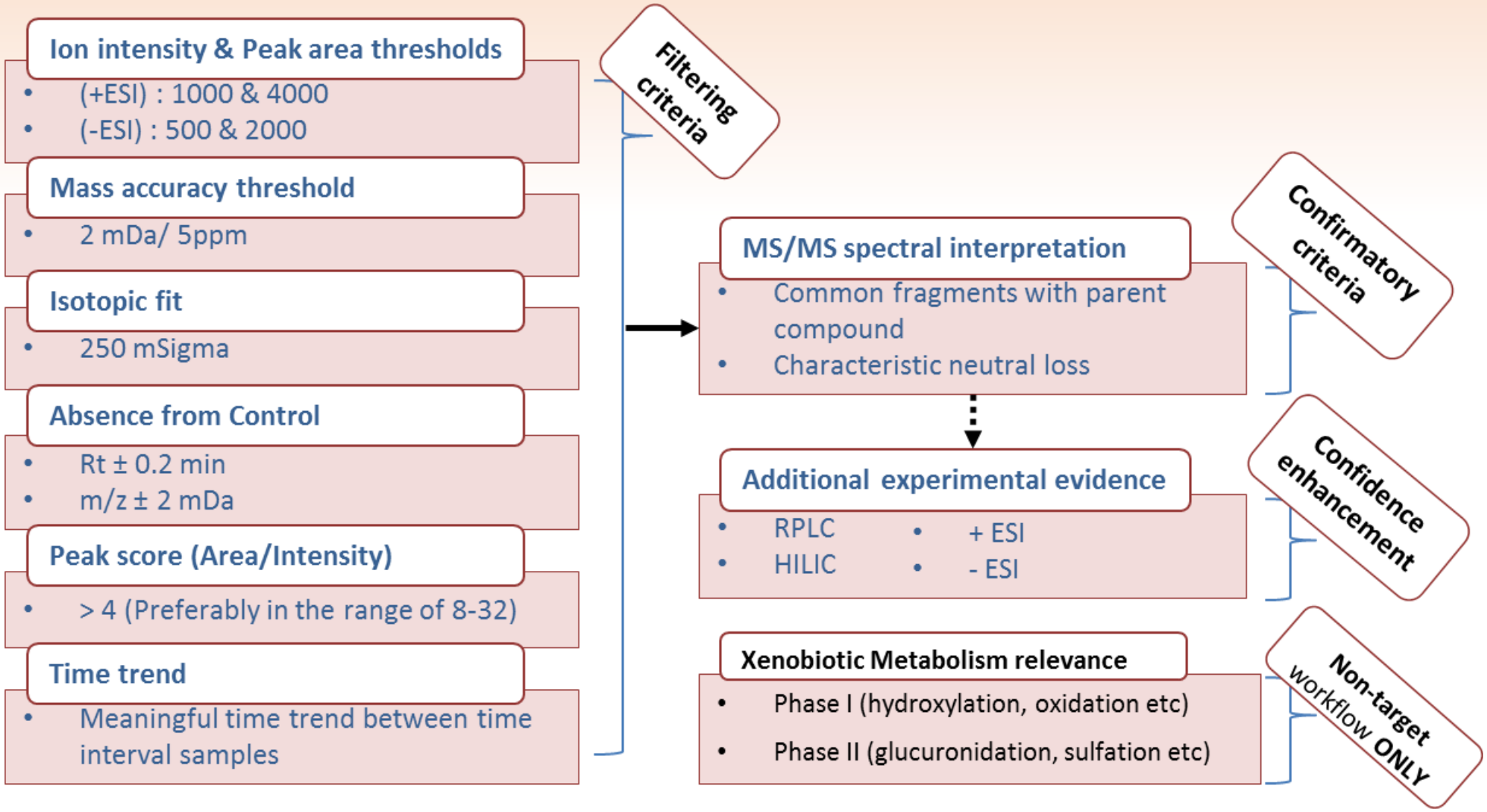






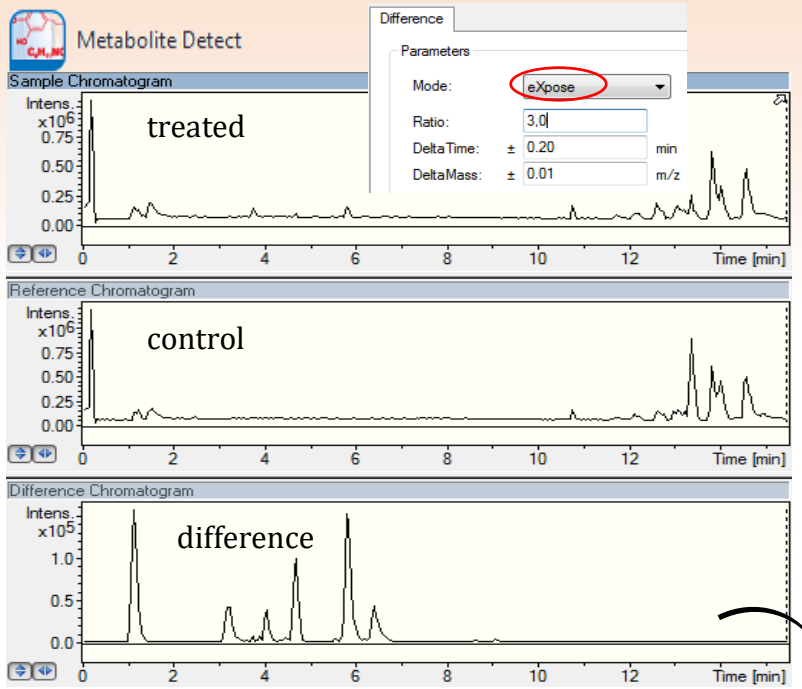
Schymanski, Jeon, Gulde, Fenner, Ruff, Singer & Hollender (2014) *ES&T*, 48 (4), 2097-2098. DOI: 10.1021/es5002105

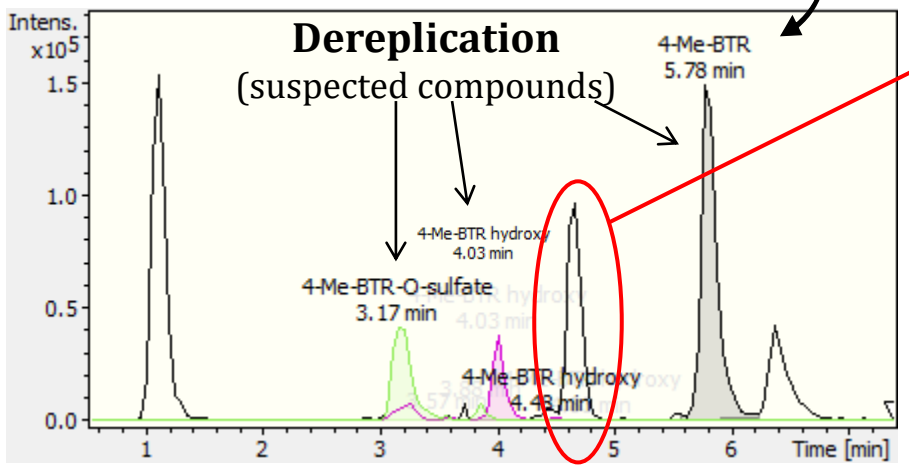
Identification Workflow



Non-target screening (example)

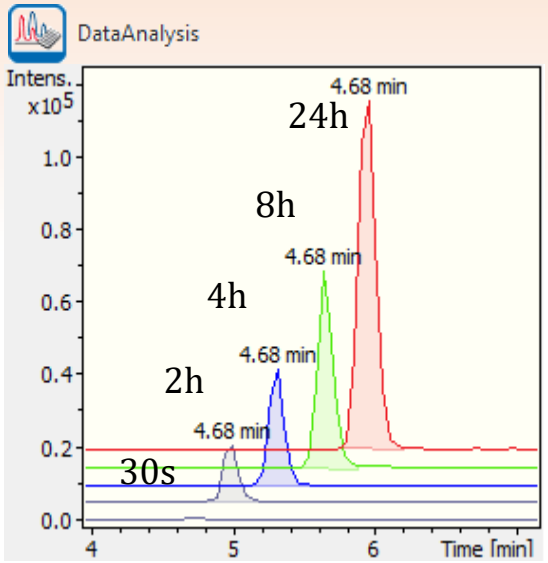
4-Me-BTR treated sample: 24h



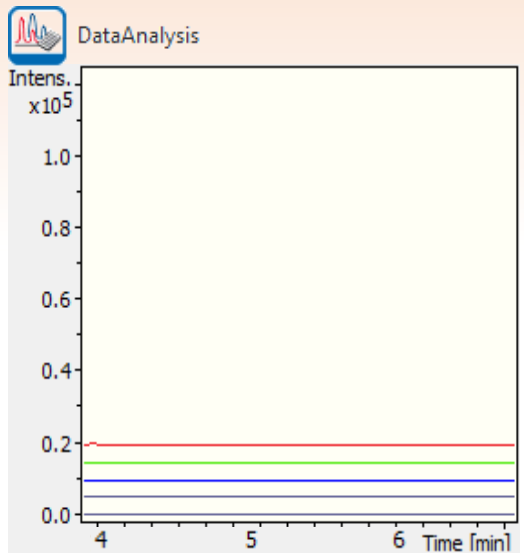


Unequivocal Mol. Form.: $C_7H_6N_3O_3S$

time profile ✓

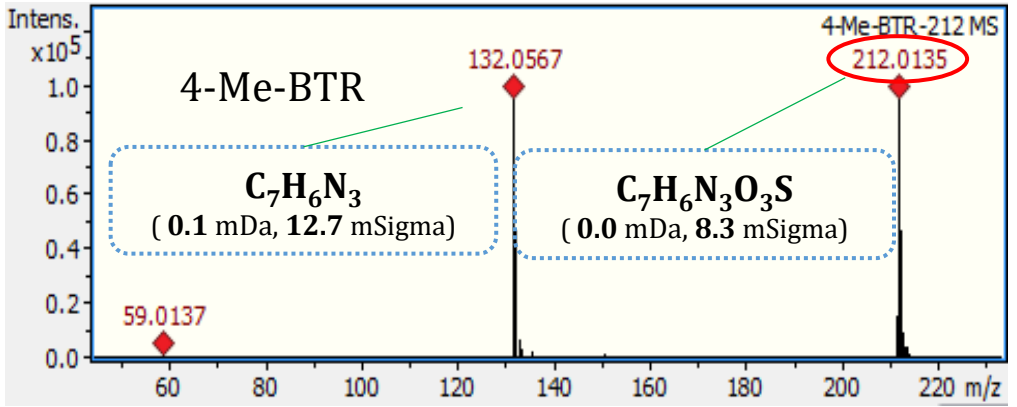


Absence from control ✓



m/z of interest: 212.0135

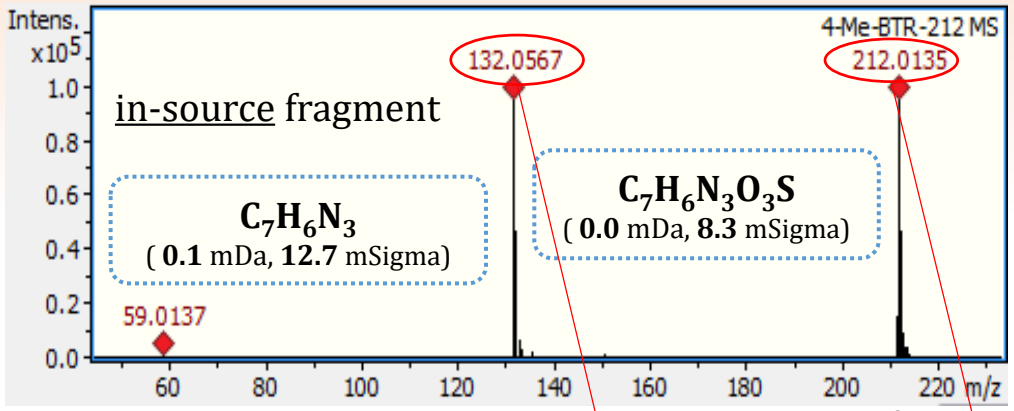
mass error ✓ isotopic fit ✓



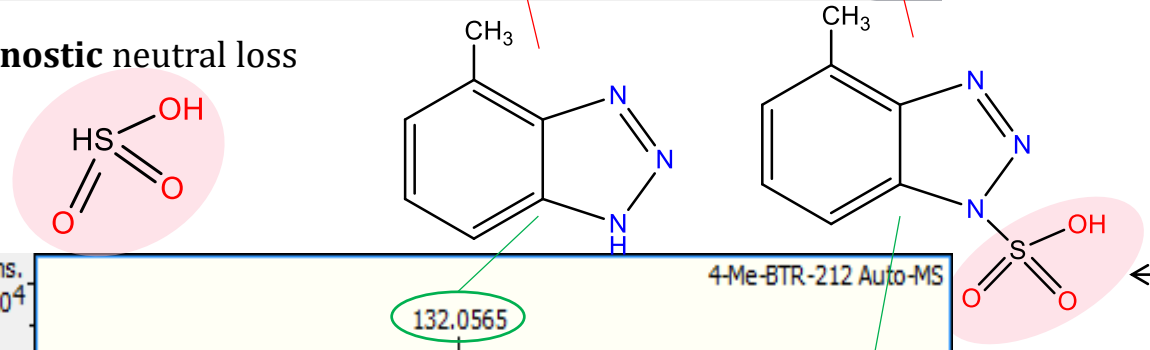
Non-target screening (example)

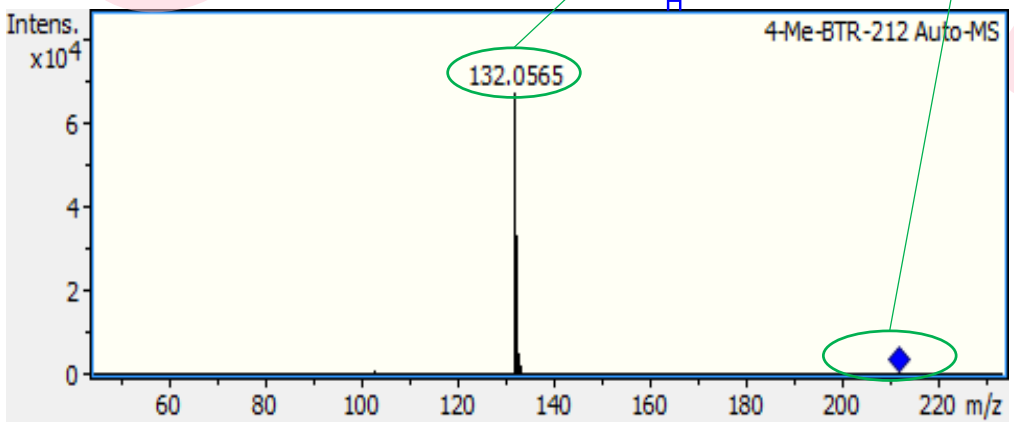
Identification

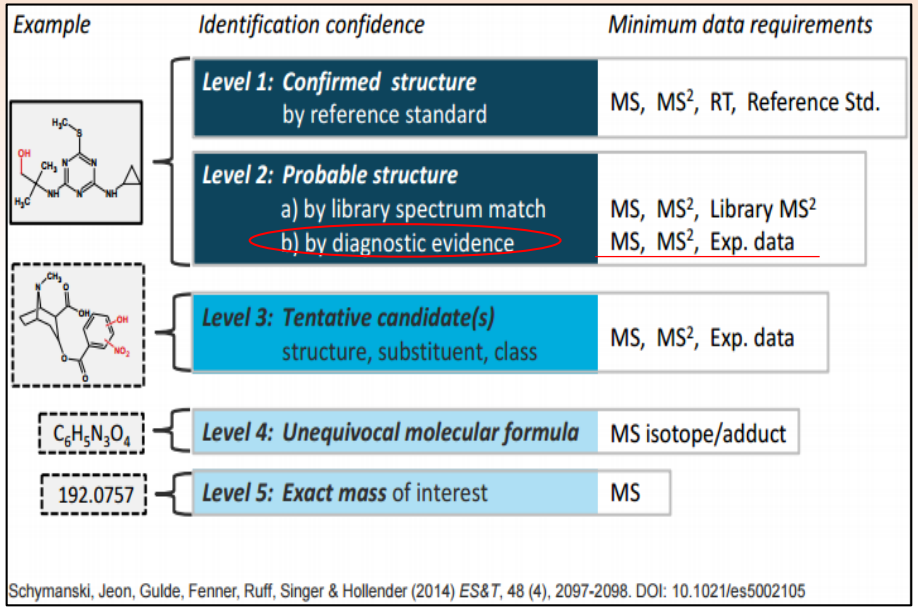
Unequivocal Mol. Form.: $C_7H_6N_3O_3S$



diagnostic neutral loss

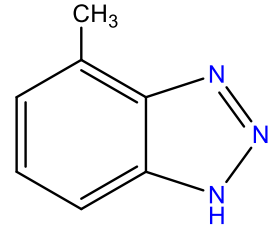






SULTs

Sulfate conjugation



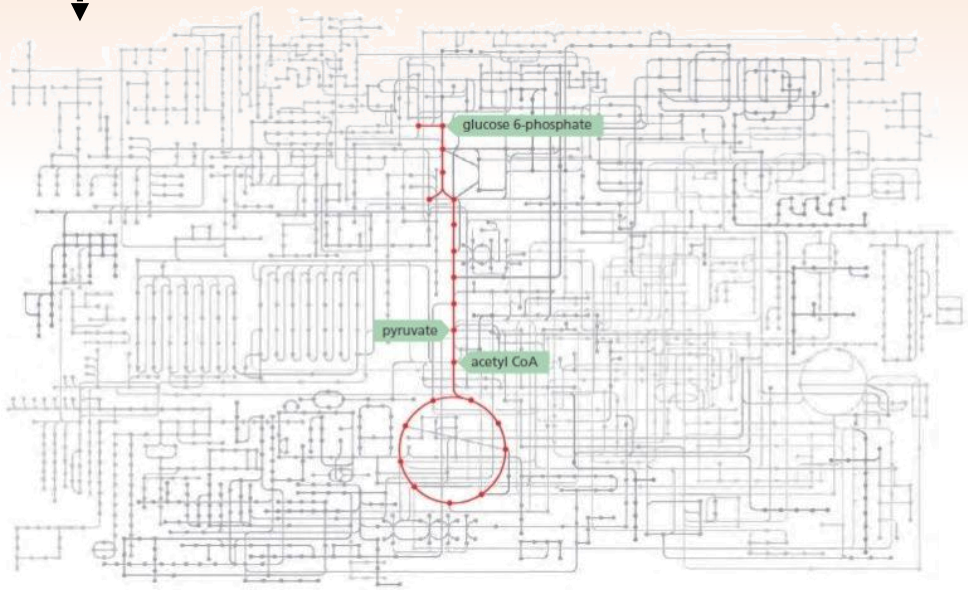
additional
experimental evidence

RPLC (+) – (-)
HILIC (+) – (-)

explained by
xenobiotic metabolism
rules

“Classic” Targeted Metabolomics

only **a few** pathways or metabolites



“http://reasonandscience.heavenforum.org/t1996-biosynthesis-and-metabolism”

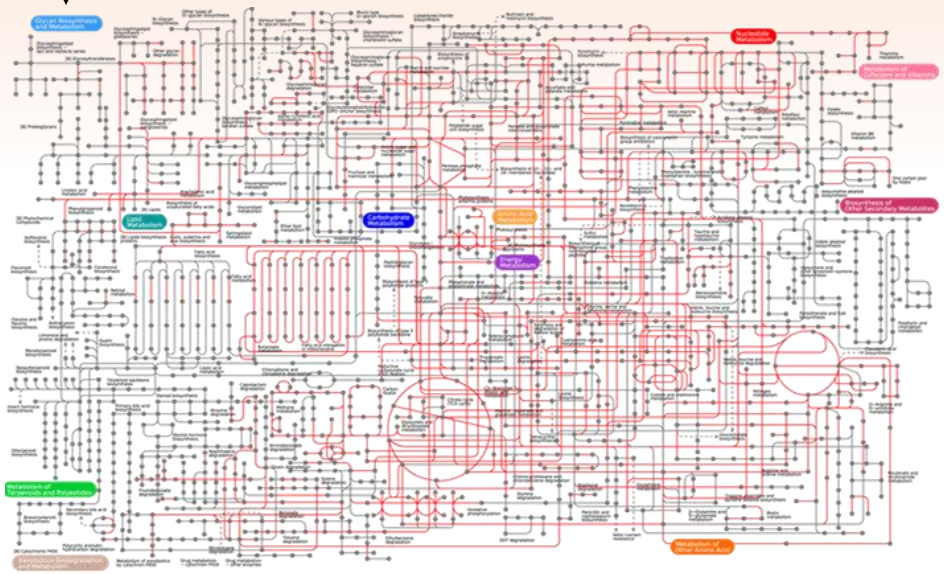
low resolution MS (QqQ)

low metabolite **coverage** & **throughput** capacity

hypothesis-**driven** approach

Wide-scope Targeted Metabolomics

broad range of primary metabolism



http://userweb.eng.gla.ac.uk/umer.ijaz/bioinformatics/Metaproteomics.html

high resolution MS (q-TOF, Orbitrap)

extended metabolite **coverage** – no need for **identification**

vs “**classic**” targeted approach

vs **UN**-targeted approach

hypothesis-**generating** approach

Wide-scope Targeted Metabolomics – Database Compilation

IROA - MSMLS

carboxylic acids, aminoacids, nucleotides, vitamins, sugars, biogenic amines, fatty acids etc

std mix preparation →

~ 75 std/mix

analysis →

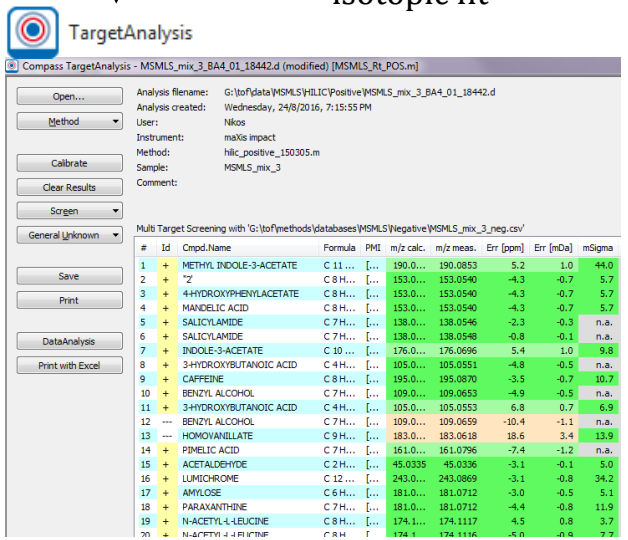
RPLC (+/- ESI)

HILIC (+/- ESI)

target screening →

Criteria

- peak area
- peak intensity
- mass accuracy
- isotopic fit



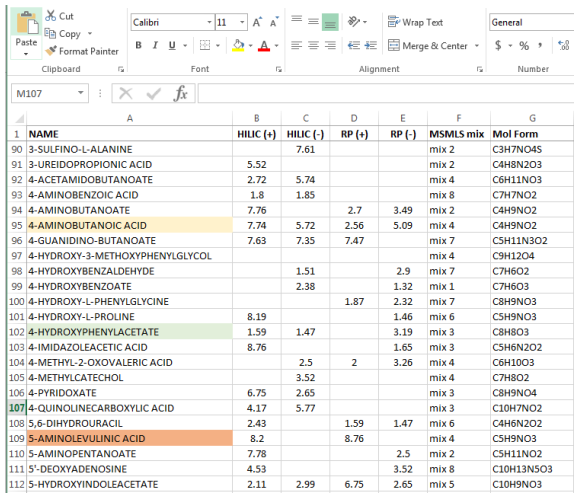
#	Id	Cmpd.Name	Formula	PMI	m/z calc.	m/z meas.	Err [ppm]	Err [mDa]	mSigma
1	+	METHYL INDOLE-3-ACETATE	C 11 ...	[...	190.0...	190.0853	5.2	1.0	44.0
2	+	"2'	C 8 H...	[...	153.0...	153.0540	-4.3	-0.7	5.7
3	+	4-HYDROXYPHENYLACETATE	C 8 H...	[...	153.0...	153.0540	-4.3	-0.7	5.7
4	+	MANDELIC ACID	C 8 H...	[...	153.0...	153.0540	-4.3	-0.7	5.7
5	+	SALICYLAMIDE	C 7 H...	[...	138.0...	138.0546	-2.3	-0.3	n.a.
6	+	SALICYLAMIDE	C 7 H...	[...	138.0...	138.0548	-0.8	-0.1	n.a.
7	+	INDOLE-3-ACETATE	C 10 ...	[...	176.0...	176.0696	5.4	1.0	9.8
8	+	3-HYDROXYBUTANOIC ACID	C 4 H...	[...	105.0...	105.0551	-4.8	-0.5	n.a.
9	+	CAFFEINE	C 8 H...	[...	195.0...	195.0870	-3.5	-0.7	10.7
10	+	BENZYL ALCOHOL	C 7 H...	[...	109.0...	109.0653	-4.9	-0.5	n.a.
11	+	3-HYDROXYBUTANOIC ACID	C 4 H...	[...	105.0...	105.0553	6.8	0.7	6.9
12	---	BENZYL ALCOHOL	C 7 H...	[...	109.0...	109.0659	-10.4	-1.1	n.a.
13	---	HOMOVANILLATE	C 9 H...	[...	183.0...	183.0618	18.6	3.4	13.9
14	+	PIMELIC ACID	C 7 H...	[...	161.0...	161.0796	-7.4	-1.2	n.a.
15	+	ACETALDEHYDE	C 2 H...	[...	45.0335	45.0336	-3.1	-0.1	5.0
16	+	LUMICHROME	C 12 ...	[...	243.0...	243.0869	-3.1	-0.8	34.2
17	+	AMYLOSE	C 6 H...	[...	181.0...	181.0712	-3.0	-0.5	5.1
18	+	PARAXANTHINE	C 7 H...	[...	181.0...	181.0712	-4.4	-0.8	11.9
19	+	N-ACETYL-L-LEUCINE	C 8 H...	[...	174.1...	174.1117	4.5	0.8	3.7

manual inspection

↓ false positives

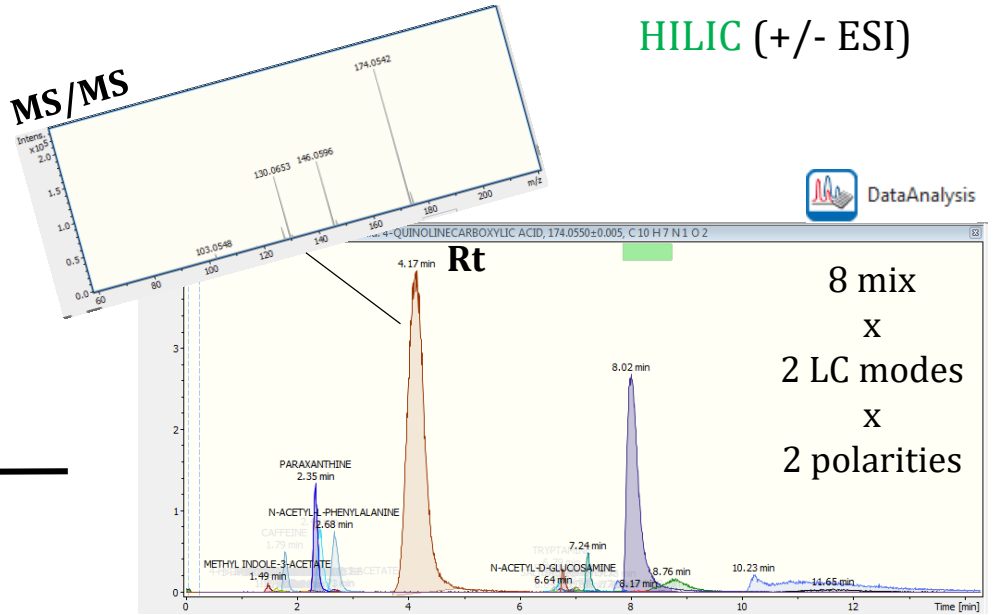
↓ false negatives

DATABASE



	NAME	HILIC (+)	HILIC (-)	RP (+)	RP (-)	MSMLS mix	Mol Form
90	3-SULFINO-L-ALANINE		7.61			mix 2	C3H7NO4S
91	3-UREIDOPROPIONIC ACID	5.52				mix 2	C4H8N2O3
92	4-ACETAMIDOBUTANOATE	2.72	5.74			mix 4	C6H11NO3
93	4-AMINOBENZOIC ACID	1.8	1.85			mix 8	C7H7NO2
94	4-AMINOBUTANOATE	7.76		2.7	3.49	mix 2	C4H9NO2
95	4-AMINOBUTANOIC ACID	7.74	5.72	2.56	5.09	mix 4	C4H9NO2
96	4-GUANIDINO-BUTANOATE	7.63	7.35	7.47		mix 7	C5H11N3O2
97	4-HYDROXY-3-METHOXYPHENYLGLYCOL					mix 4	C9H12O4
98	4-HYDROXYBENZALDEHYDE		1.51		2.9	mix 7	C7H6O2
99	4-HYDROXYBENZOATE		2.38		1.32	mix 1	C7H6O3
100	4-HYDROXY-L-PHENYLGLYCINE			1.87	2.32	mix 7	C8H9NO3
101	4-HYDROXY-L-PROLINE	8.19			1.46	mix 6	C5H9NO3
102	4-HYDROXYPHENYLACETATE	1.59	1.47		3.19	mix 3	C8H8O3
103	4-IMIDAZOLEACETIC ACID	8.76			1.65	mix 3	C5H6N2O2
104	4-METHYL-2-OXOVALERIC ACID		2.5	2	3.26	mix 4	C6H10O3
105	4-METHYLCATECHOL		3.52			mix 4	C7H8O2
106	4-PYRIDOXATE	6.75	2.65			mix 3	C8H9NO4
107	4-QUINOLINECARBOXYLIC ACID	4.17	5.77			mix 3	C10H7NO2
108	5,6-DIHYDROURACIL	2.43		1.59	1.47	mix 6	C4H6N2O2
109	5-AMINOLEVULINIC ACID	8.2		8.76		mix 4	C5H9NO3
110	5-AMINOPENTANOATE	7.78			2.5	mix 2	C5H11NO2
111	5'-DEOXYADENOSINE	4.53			3.52	mix 8	C10H13N5O3
112	5-HYDROXYINDOLEACETATE	2.11	2.99	6.75	2.65	mix 5	C10H9NO3

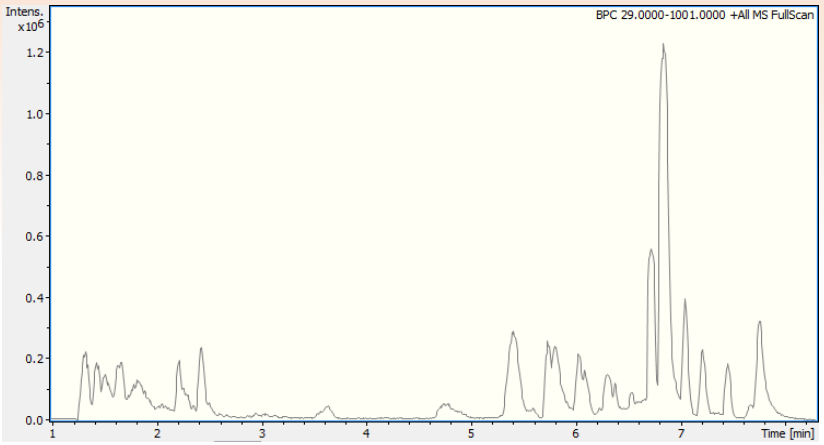
←



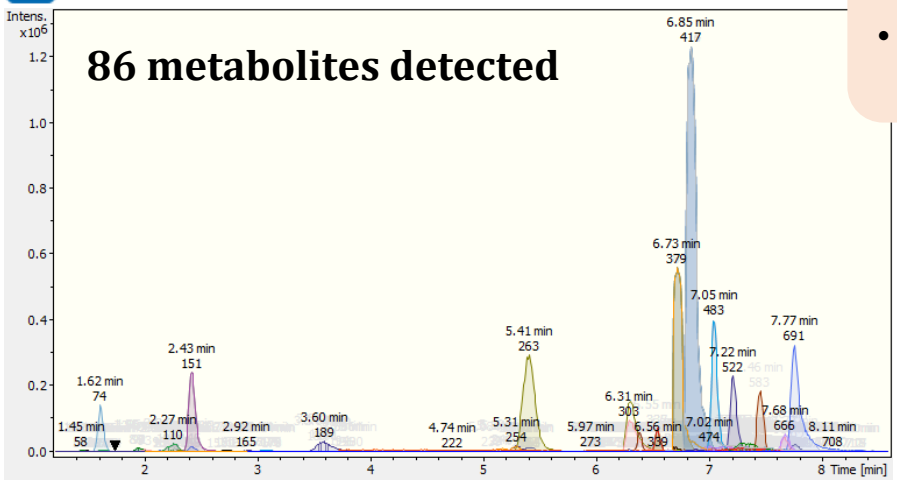
8 mix
x
2 LC modes
x
2 polarities

Wide-scope Targeted Metabolomics – screening example

4-MeBT 30s – HILIC (+)



DataAnalysis



Screening Criteria

- peak area **> 2000**
- peak intensity **> 500**
- mass accuracy **< 2mDa/ 5ppm**
- isotopic fit **< 250mSigma**
- ΔRt **< ± 0.2 min**

TASQ

Conclusion

- BTs induced **cardiotoxicity** to zebrafish embryos
- 4-MeBT appeared to be the most toxic
- Oxidative (hydroxylation) and Conjugative (glucuronide & sulfate conjugation) detoxification reactions
- N-sulfation dominated the detoxification process
- Extent of biotransformation proved informative for the interpretation of toxicity
- **Wide-scope** targeted metabobolics approach proved the hypothesis of known uknowns
- the **combined** approach covered the whole picture (xeno- and endo-metabolome)

Acknowledgment

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TrAMS
Trace Analysis and Mass Spectrometry Group

KEEP CALM AND THANKS FOR YOUR ATTENTION

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IVM Institute for Environmental Studies

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