

# Introduction to Untargeted Metabolomics

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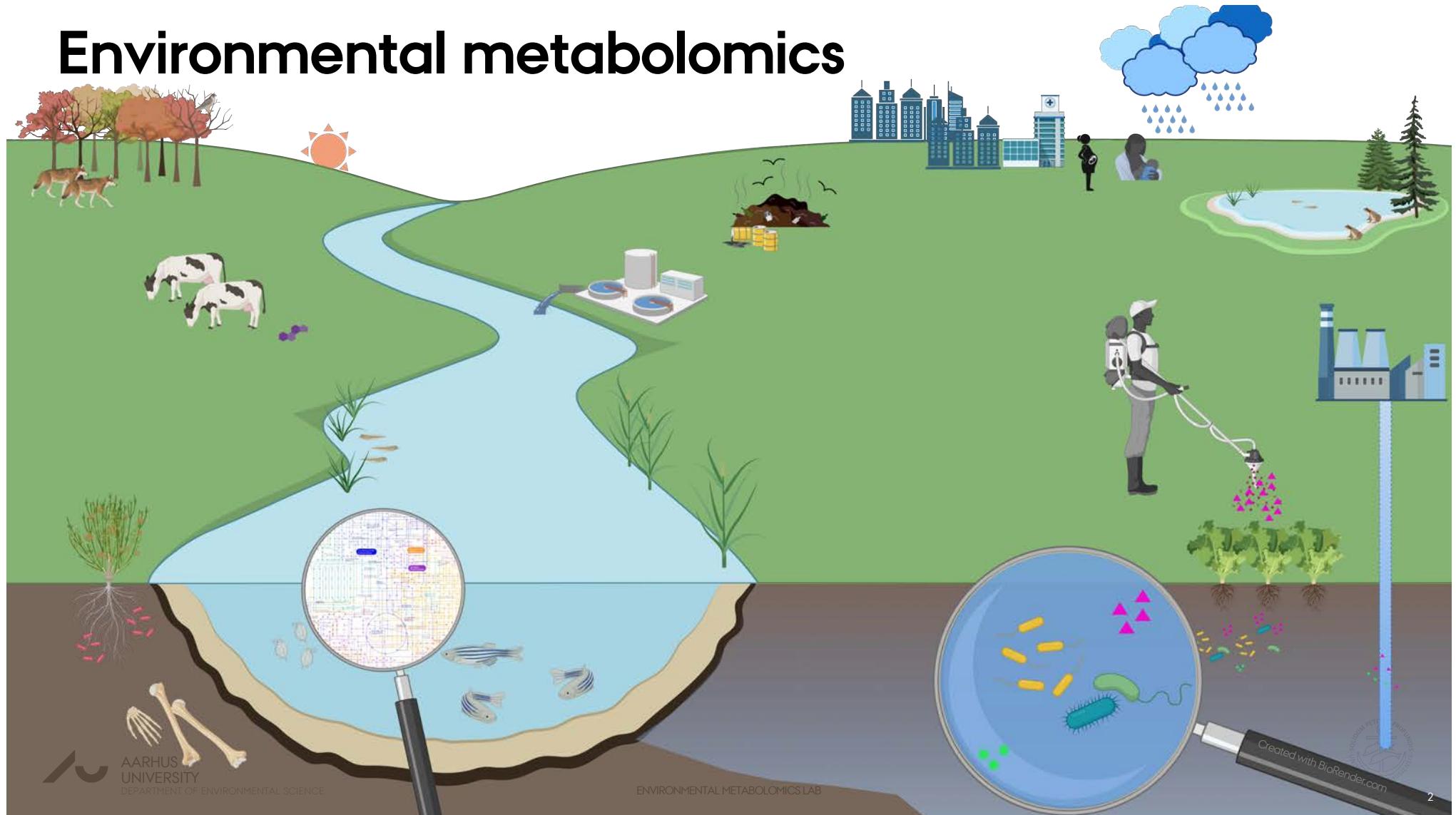
 **ERGO**  
Endocrine Guideline Optimisation

 **HoloFood**   
Horizon2020  
Research and Innovation  
Action  
2019-2022

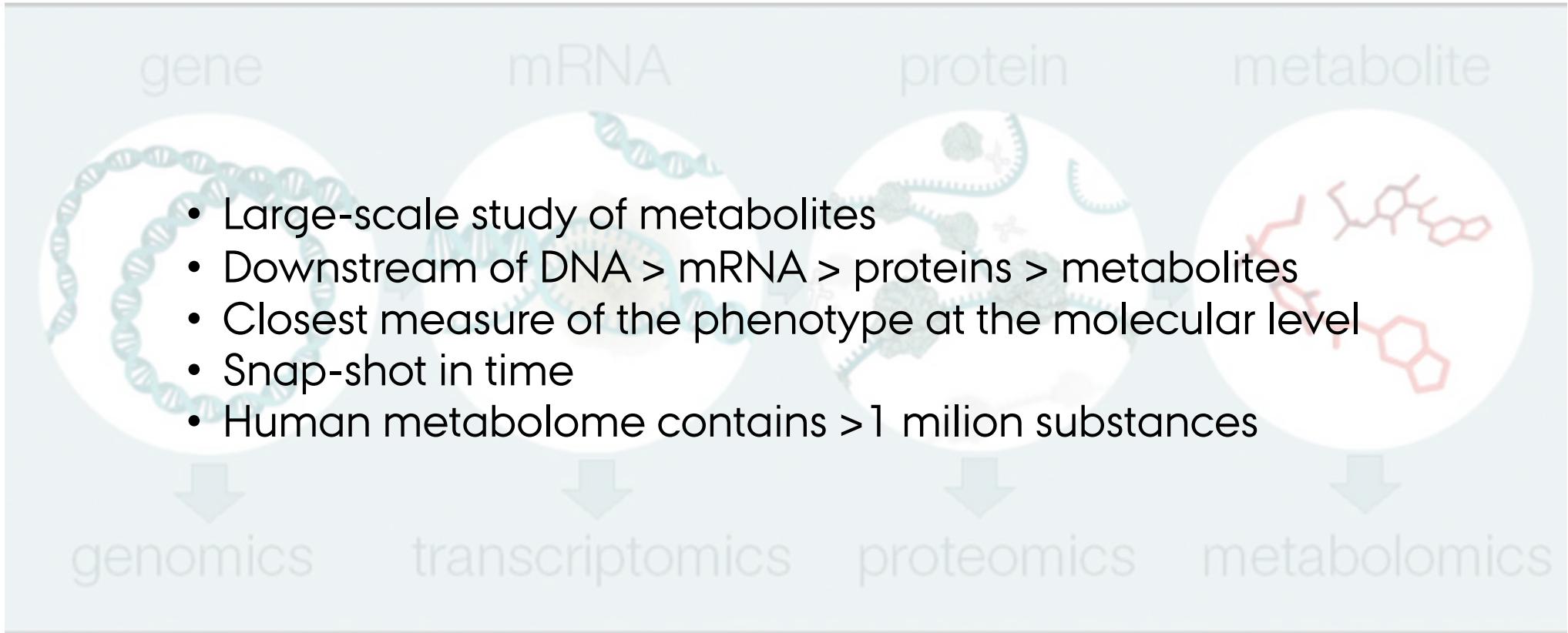
September 12, 2022

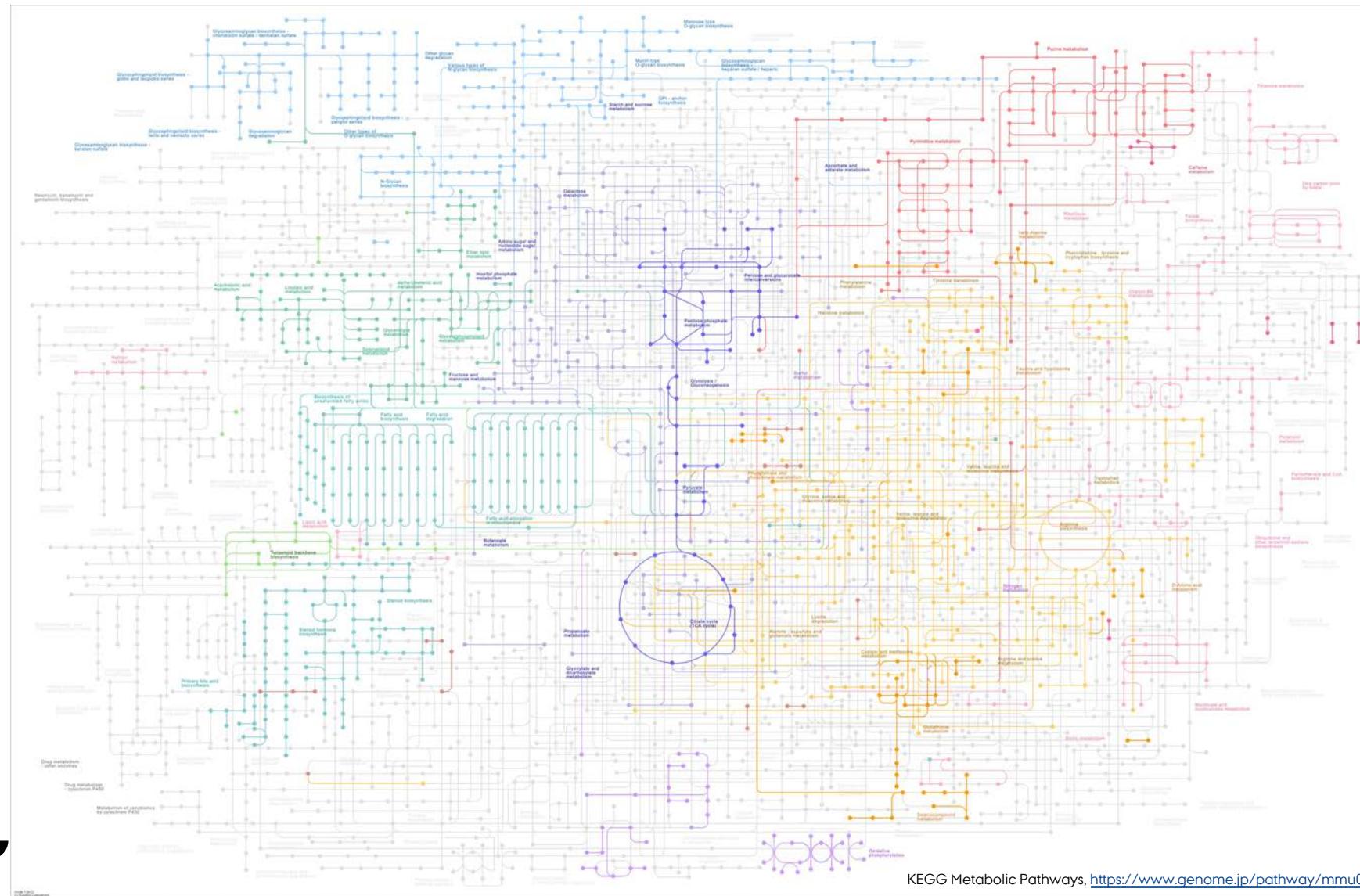


# Environmental metabolomics

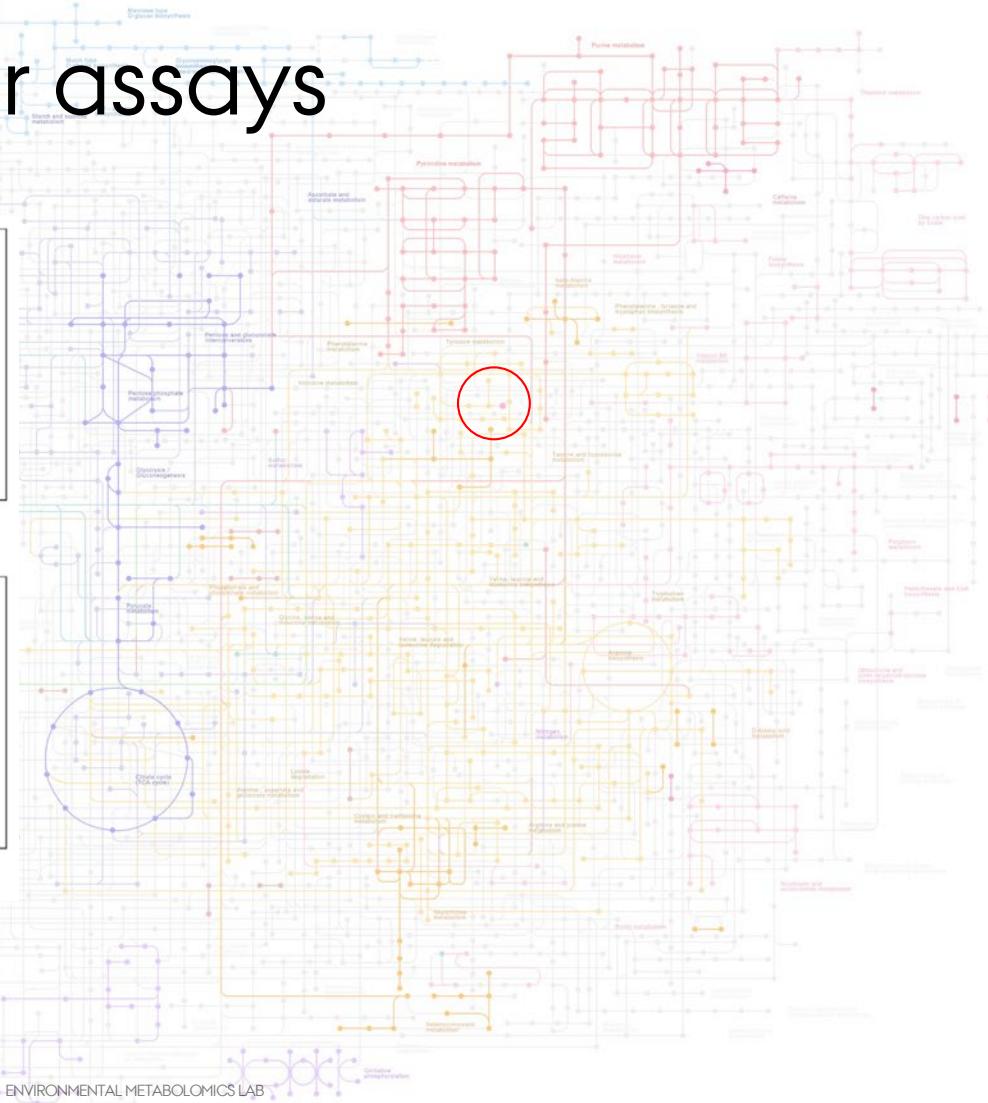
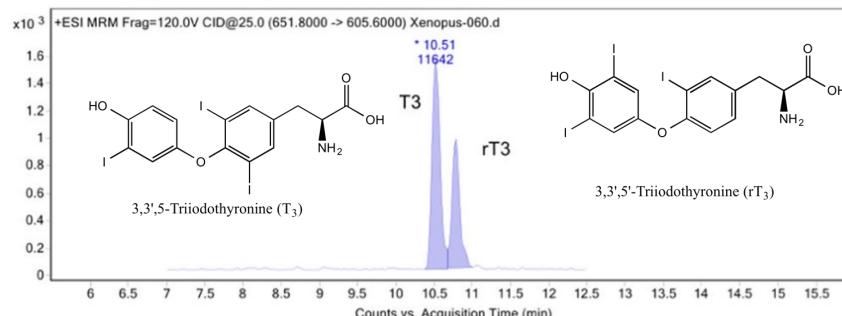
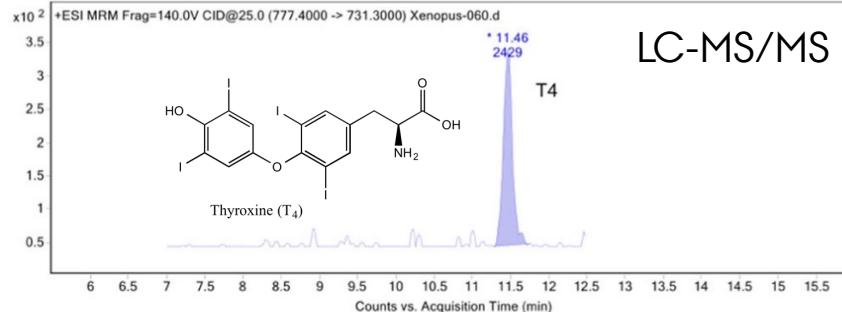


# Metabolomics





# Targeted biomarker assays

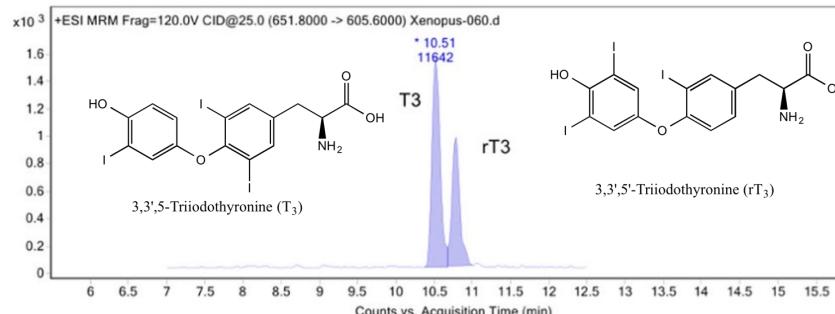
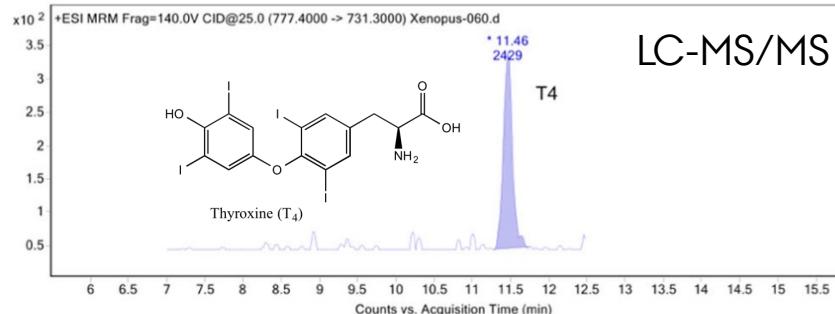


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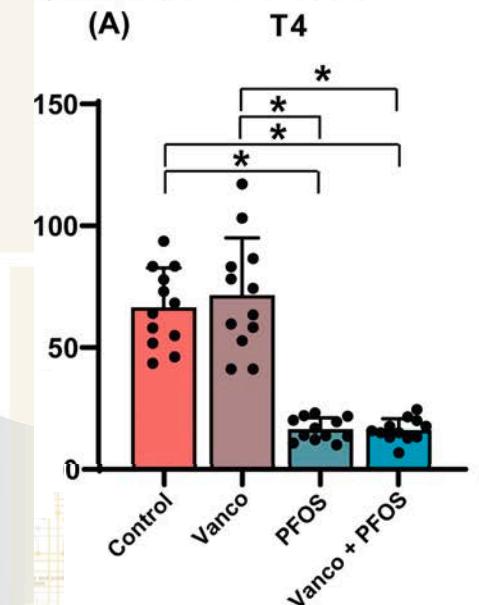
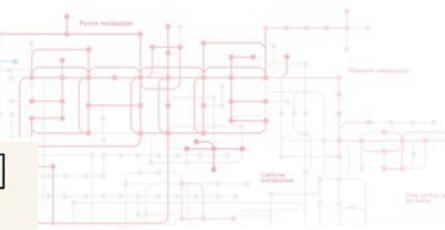
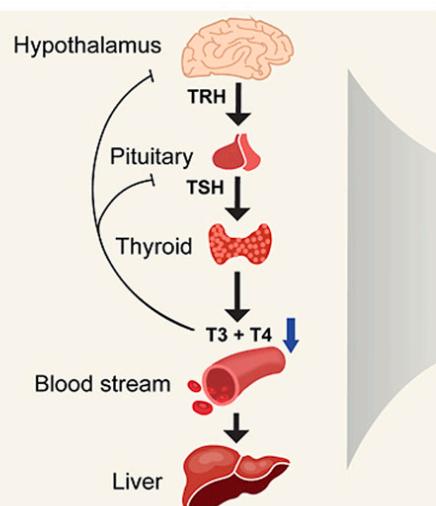
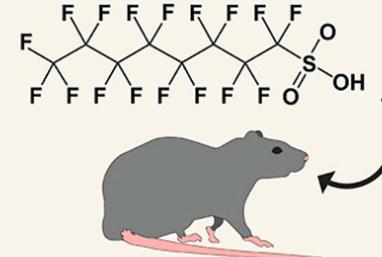
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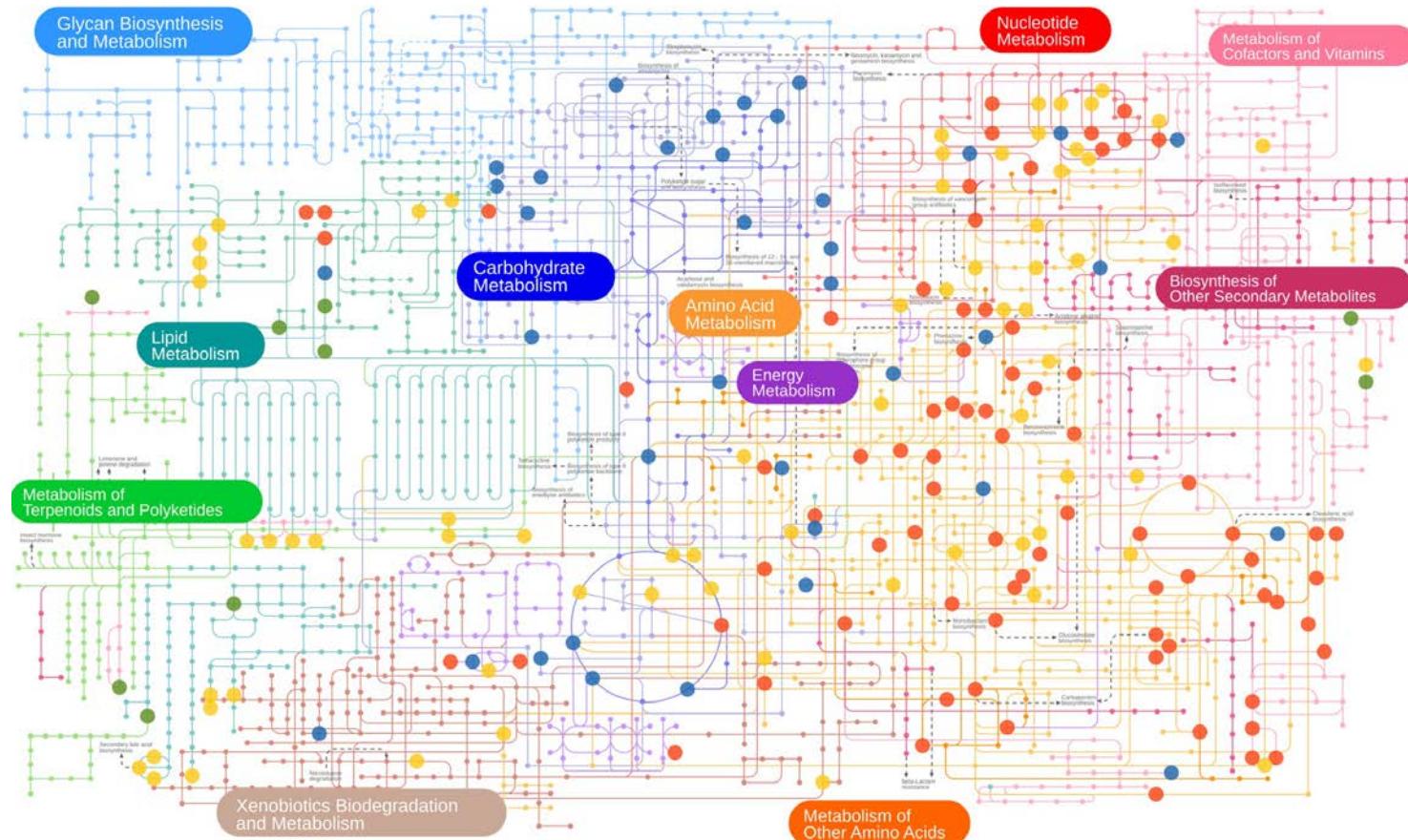
# Targeted biomarker assays



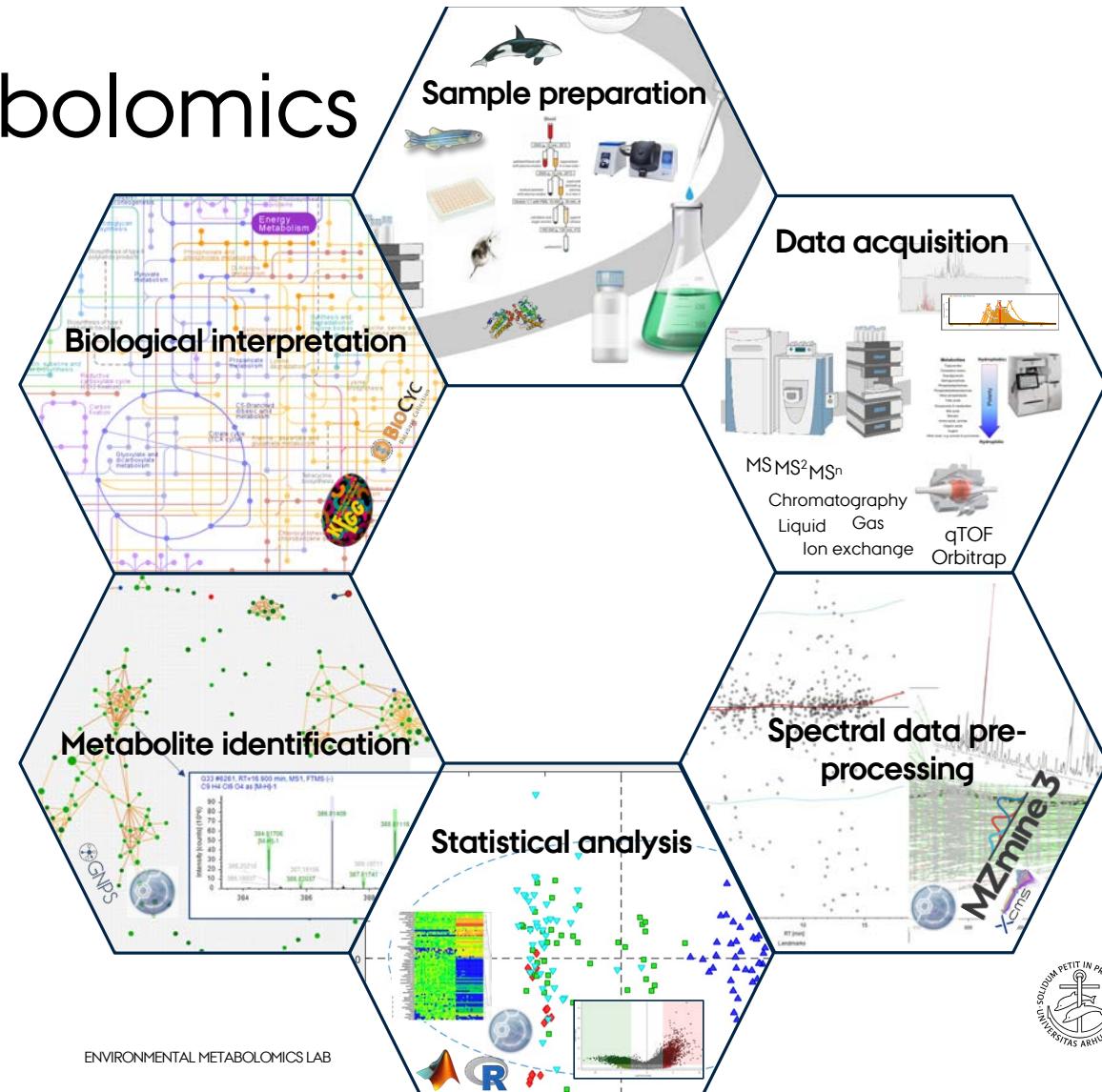
Perfluorooctanesulfonic acid (PFOS)



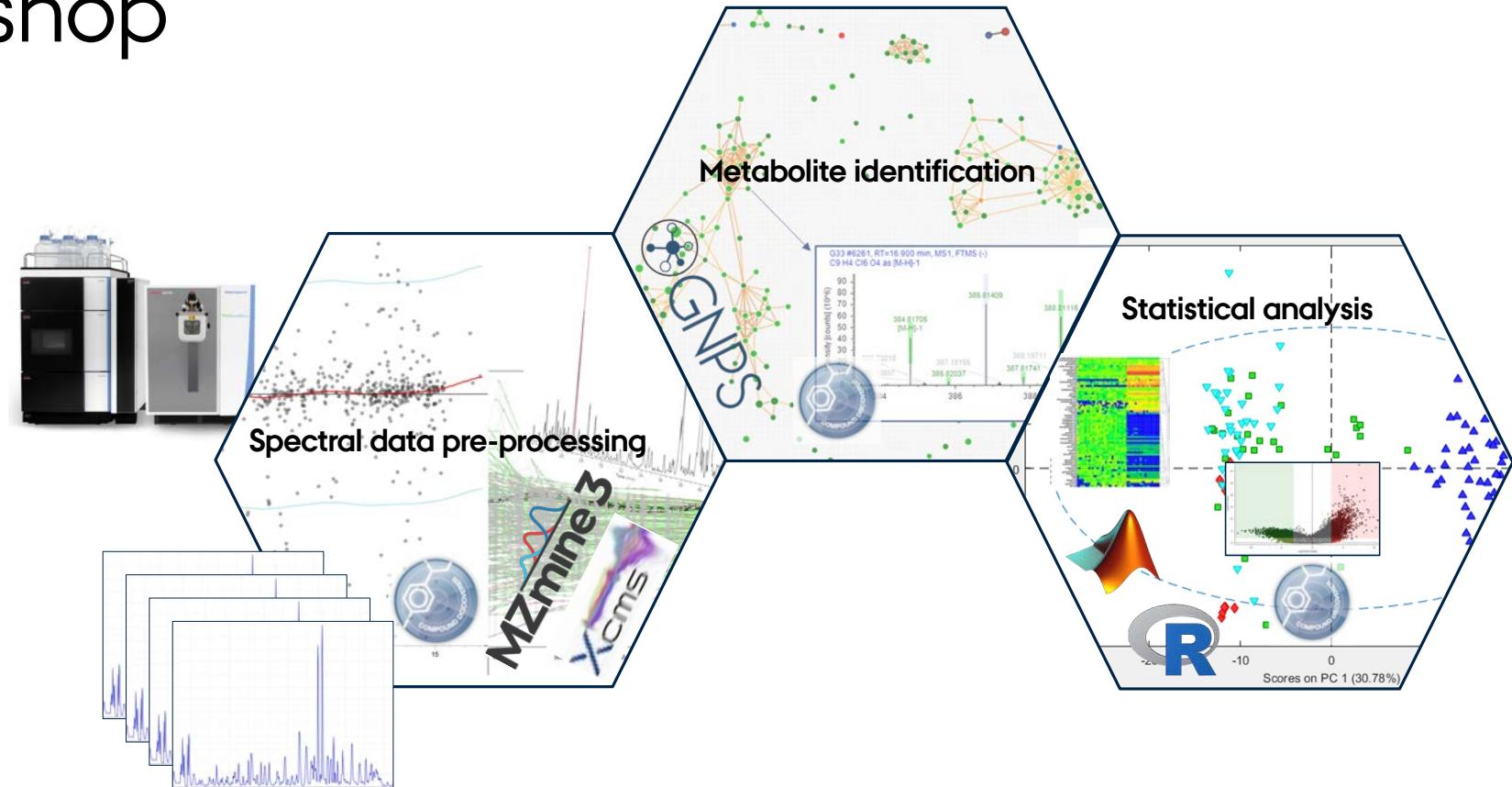
# Untargeted metabolomics



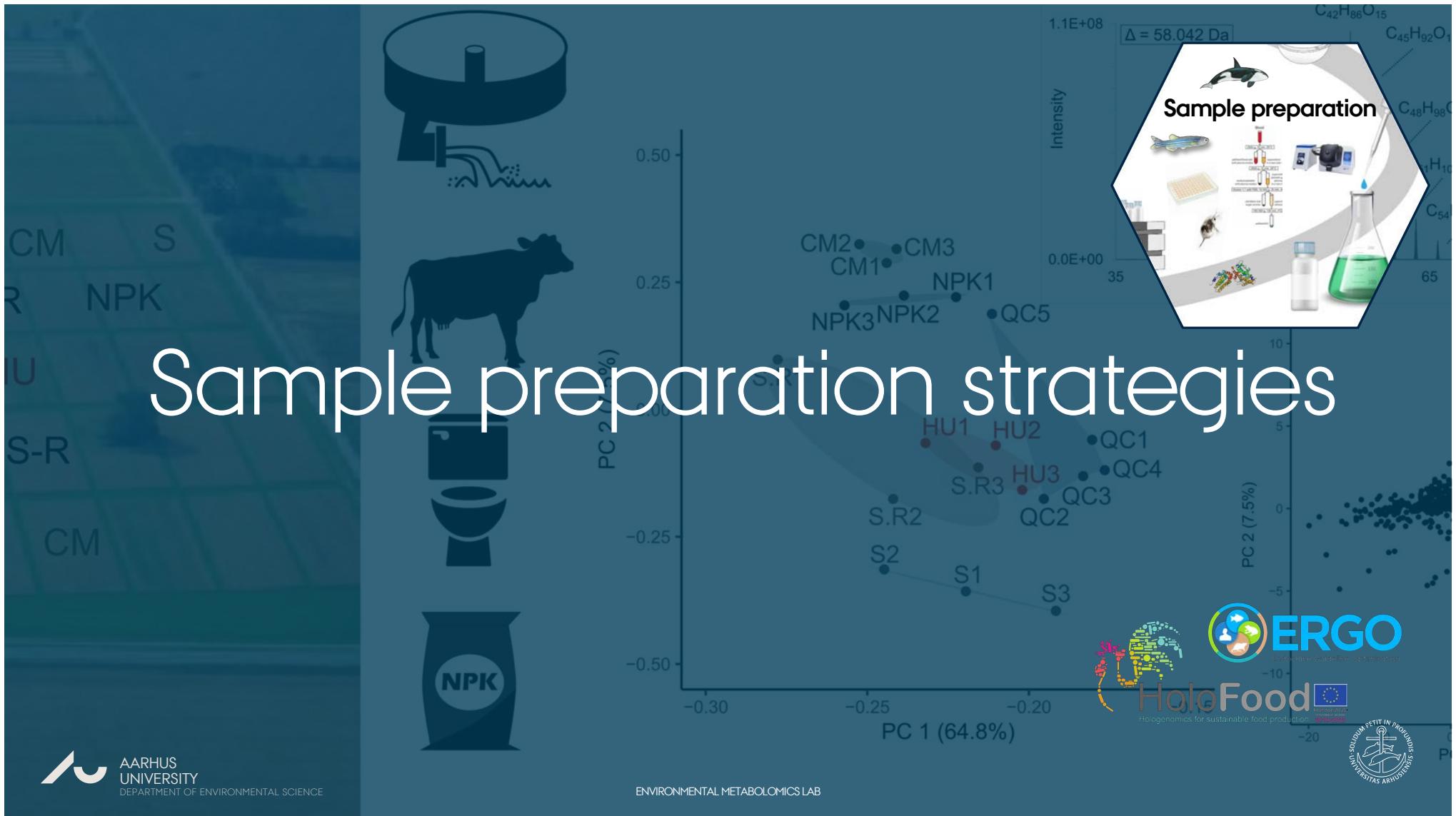
# Untargeted metabolomics workflow



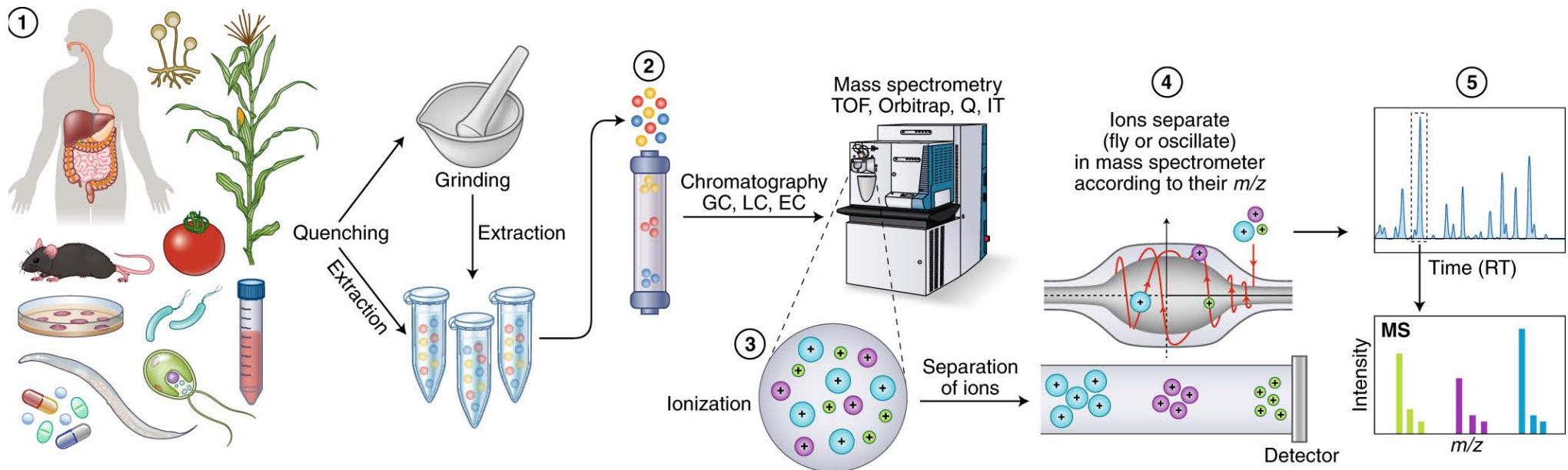
# Workshop



# Sample preparation strategies



# Metabolomics workflow



## Sample preparation and extraction

- Avoid environmental perturbation during harvesting
- Control environment: harvesting at the same time and under the same conditions
- Snap-freezing in liquid nitrogen
- Enzyme quenching: completely terminate all enzyme activities
- Standards spiked into the quenching solvent
- Grinding, isolation of cells, fast-filtration or aspiration

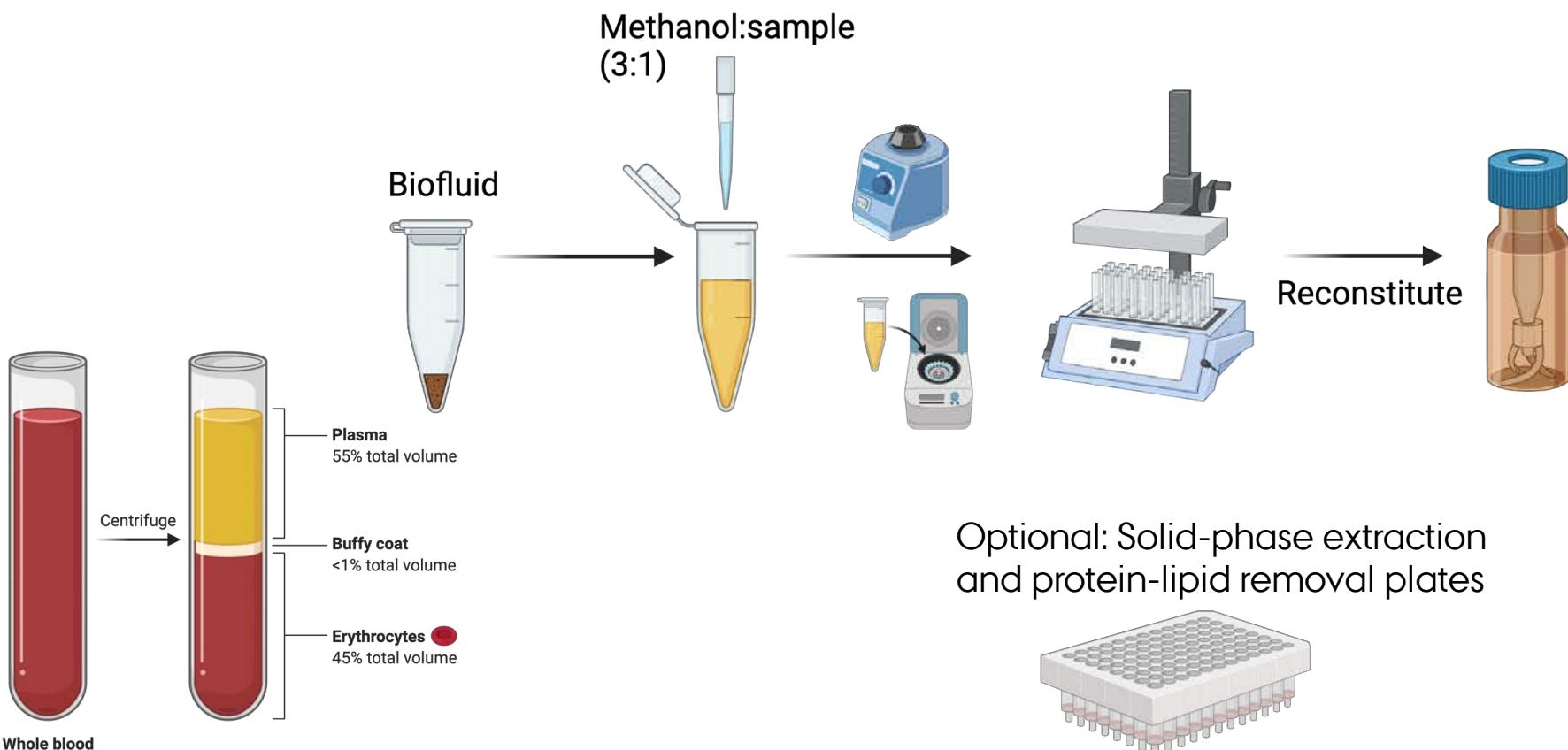
## Sample replication and randomization

- At least four biological replicates, preferably more
- Technical and analytic replicates are worthy of consideration
- Randomization of samples throughout workflows is essential
- In large-scale studies, quality-control samples and batch correction are essential

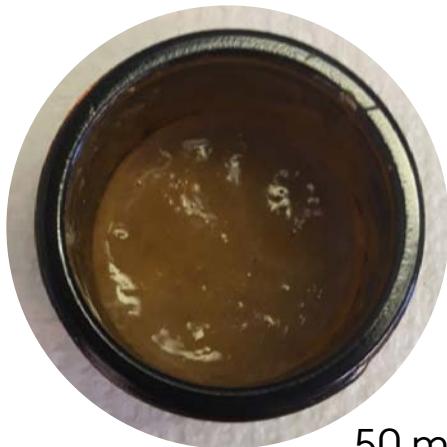
## Chromatography–mass spectrometry

- Separation methods, composition of the mobile phase, column properties and injection volume
- Metabolites are within their range of detection
- Avoid ion suppression: dilution of extracts, sonication, filtration or centrifugation, recovery test
- Choosing ionization source and type of detection mode, MS method, scan number and speed, MS/MS and energy for fragmentation

# Metabolite extraction: biofluids



# Metabolite extraction: Cells/tissue



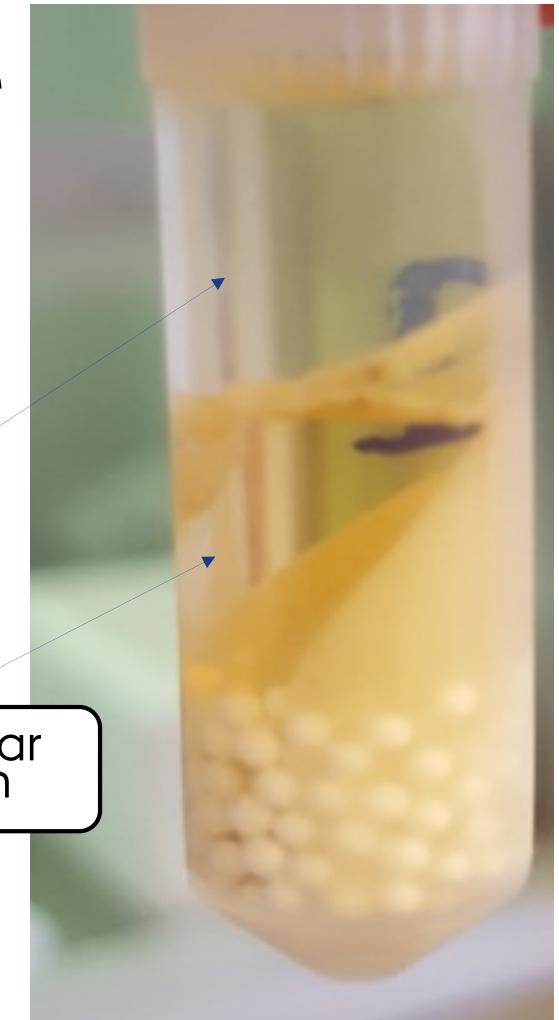
Cryo bead-beating  
Dichloromethane : Methanol : Water  
(2:1:1)

Polar  
fraction

Non-polar  
fraction

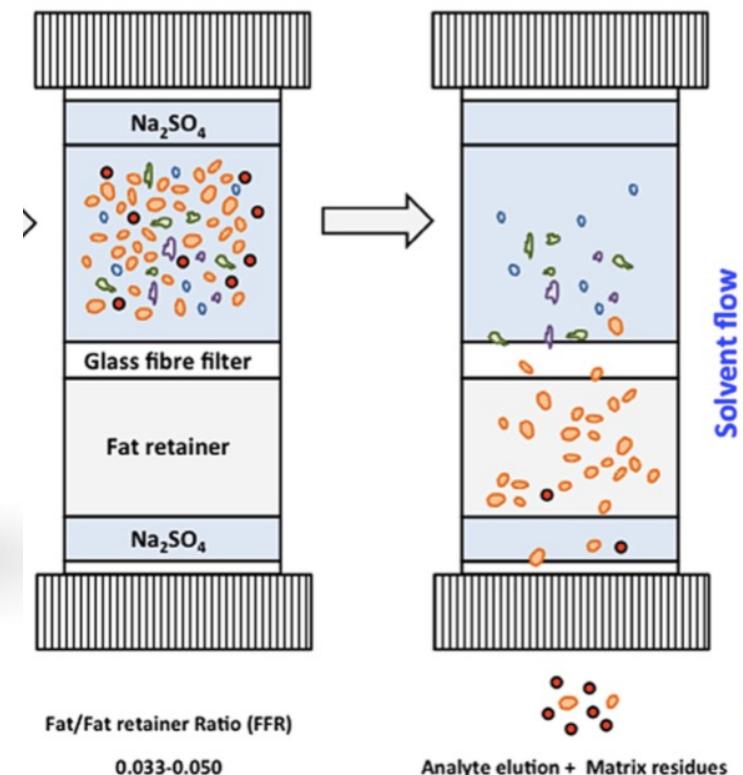


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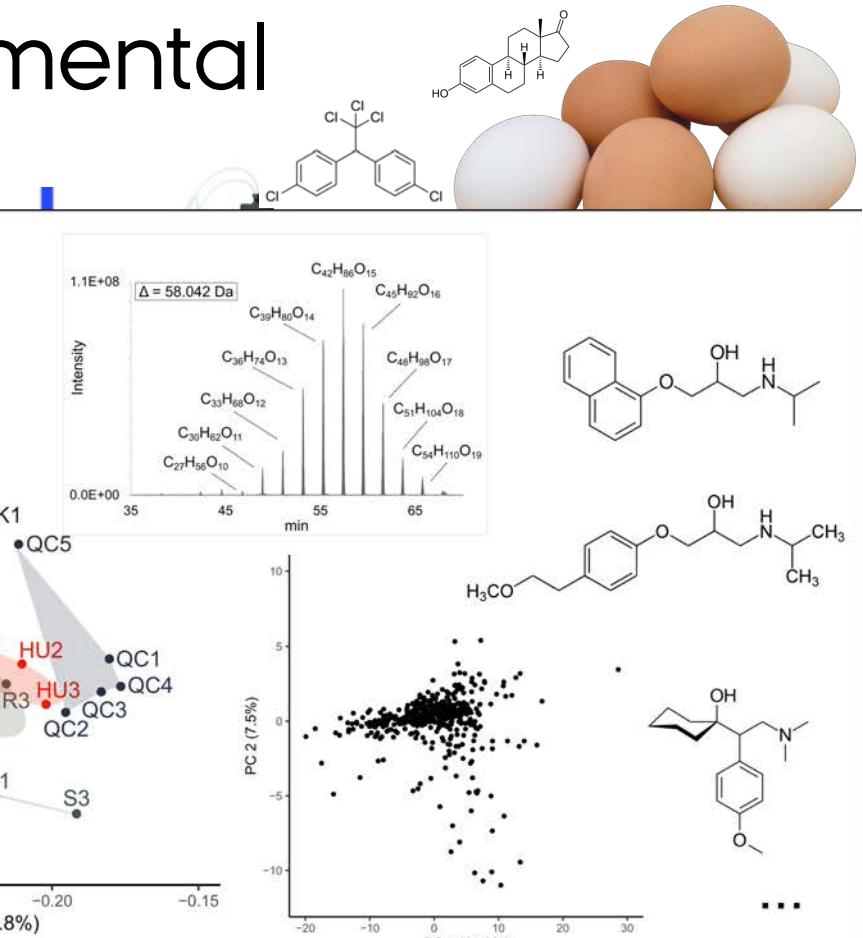
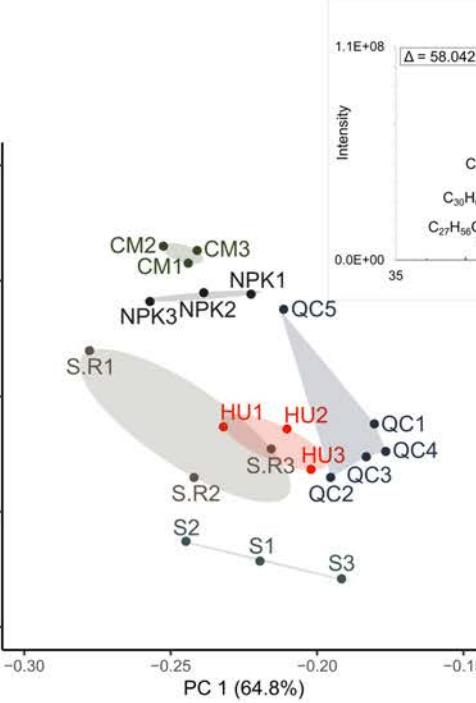
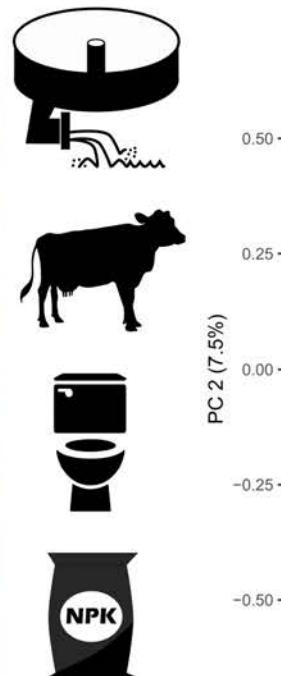
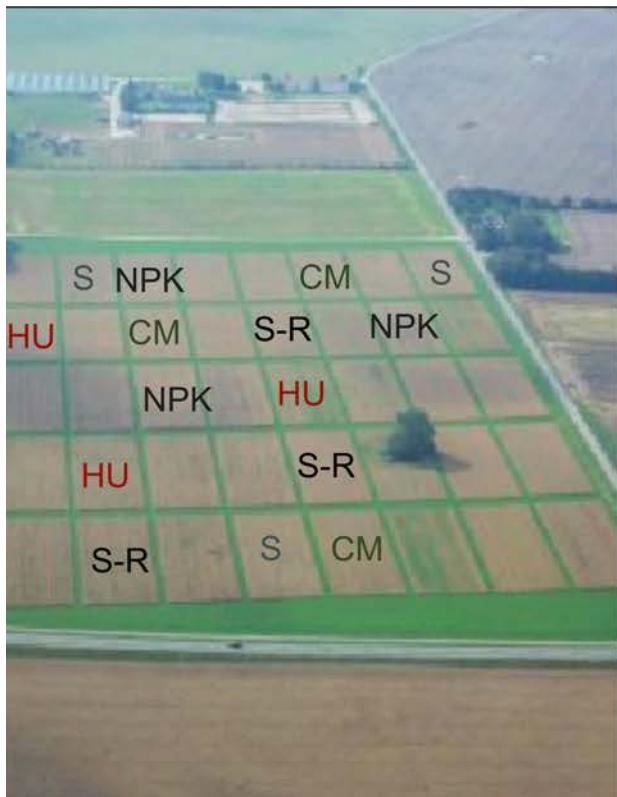
# Extraction: Tissue/environmental

## Pressurized liquid extraction



# Extraction: Tissue/environmental

## Pressurized liquid extraction



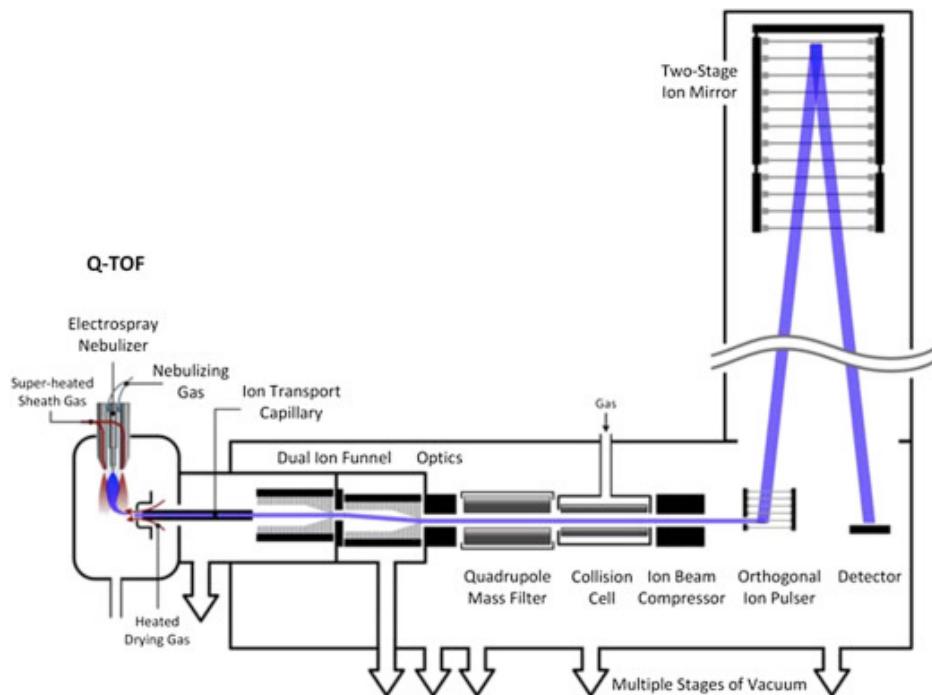
# Data acquisition Mass spectrometry



# High resolution mass spectrometry



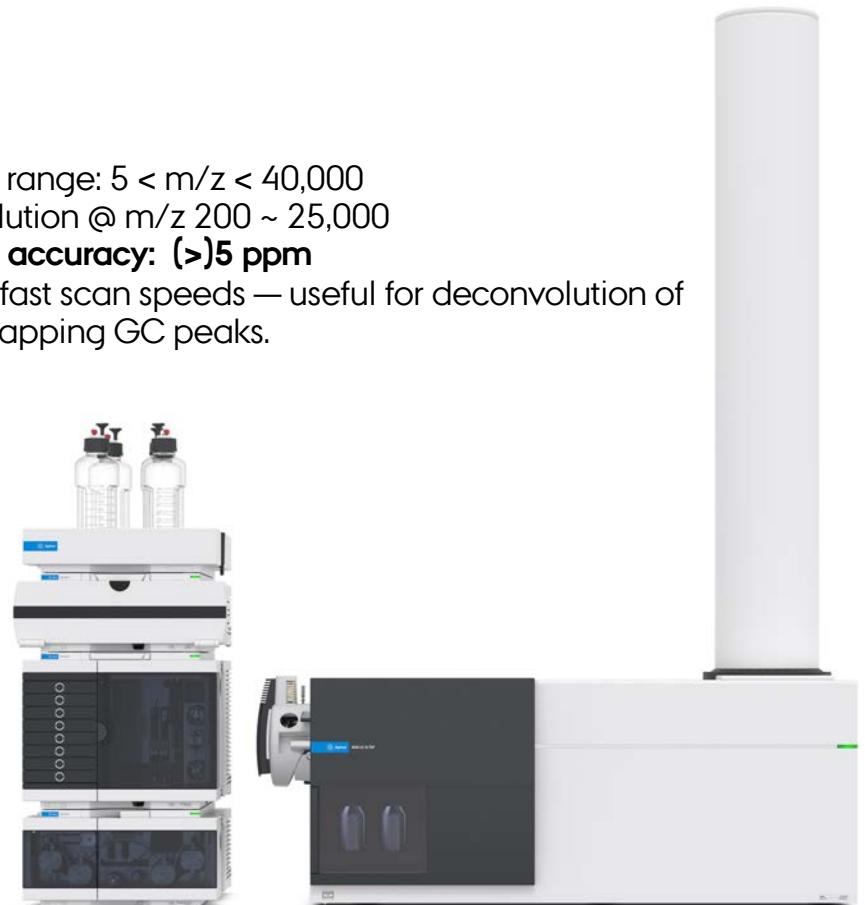
# Time-of-flight (TOF)



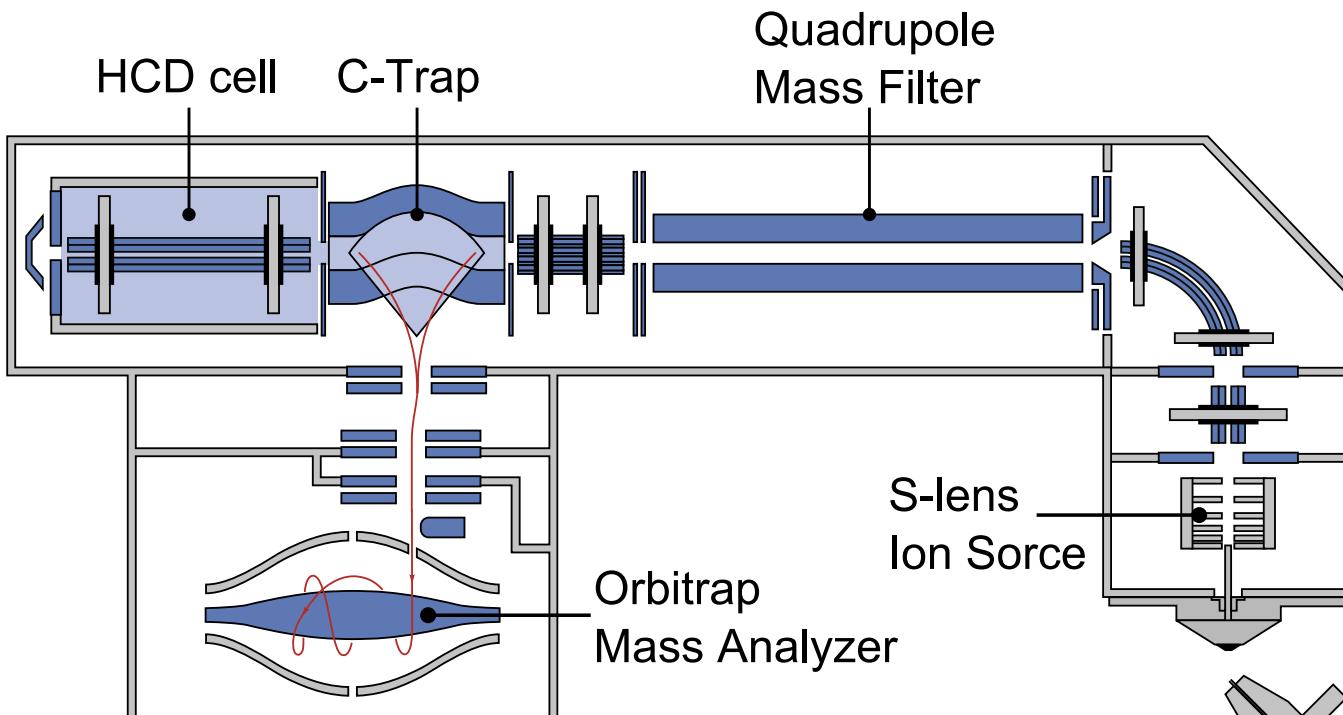
Mass range:  $5 < m/z < 40,000$   
Resolution @  $m/z 200 \sim 25,000$

**Mass accuracy: (>)5 ppm**

Very fast scan speeds — useful for deconvolution of overlapping GC peaks.



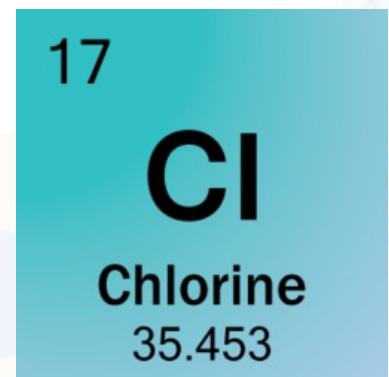
# Orbitrap



Mass range:  $50 < m/z < 6000$   
Resolution @  $m/z 200$ : **240,000** (1.5 Hz)  
**Mass accuracy: <1 ppm**  
Slow scan speed

<https://youtu.be/fqfyrravJkA>

# Exact mass

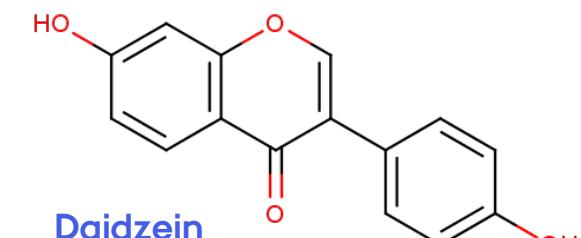
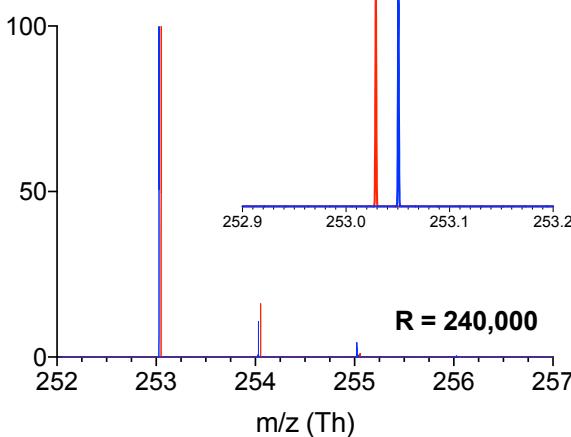
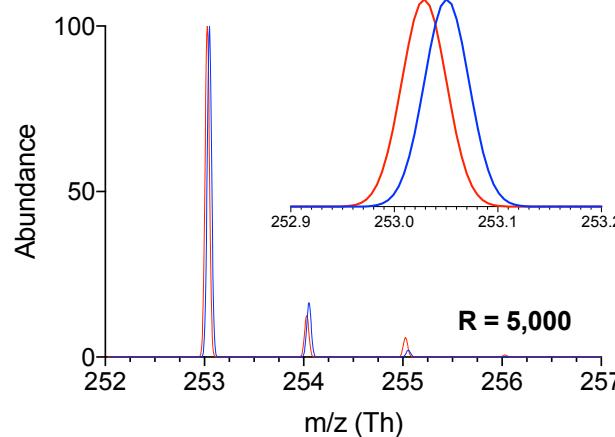
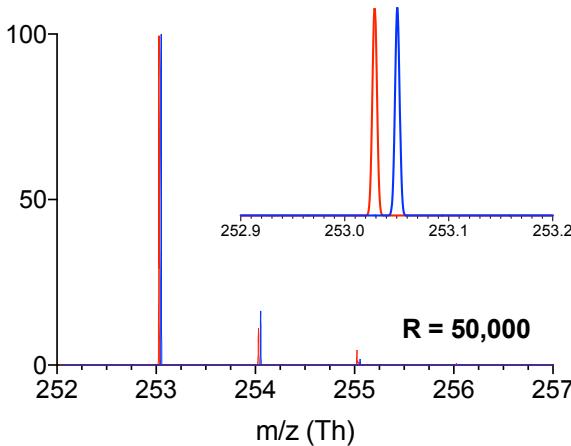
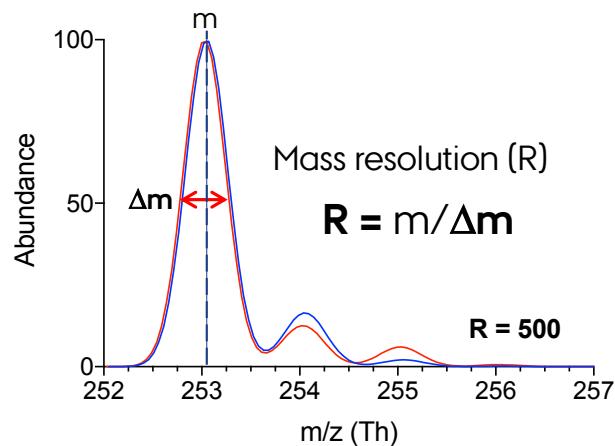


Mass  $[H_2O + H^+] =$   
 $2 * 1.007825 \text{ Da}$   
 $+ 15.9949915 \text{ Da}$   
 $+ 1.007277 \text{ Da}$   
 $19.017919 \text{ Da}$

Charge (z) = 1

m/z ( $[H_3O]^+$ ) = 19.017919

		Nominal Mass	Exact Mass (Da)	Abundance (%)
Hydrogen	H 1	1	1.007825	99.989
	$^2H$	2	2.014102	0.012
Carbon	$^{12}C$	12	12.000000	98.930
	$^{13}C$	13	13.003355	1.070
Nitrogen	$^{14}N$	14	14.003074	99.636
	$^{15}N$	15	15.000109	0.364
Oxygen	$^{16}O$	16	15.994915	99.757
	$^{17}O$	17	16.999132	0.037
	$^{18}O$	18	17.999160	0.205
Chlorine	$^{35}Cl$	35	34.968853	75.760
	$^{37}Cl$	37	36.965903	24.240
Electron	e <sup>-</sup>	Very low	0.000549	
Proton	H <sup>+</sup>	1	1.007277	



Daidzein

Monoisotopic Mass:  
253.0506323 Da

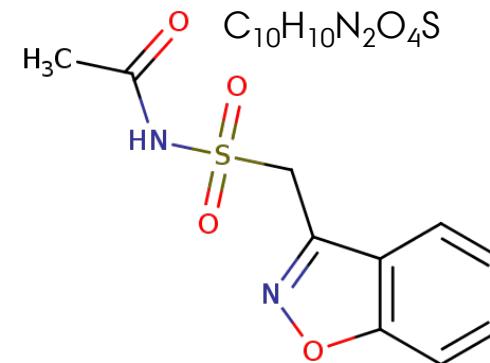
Chemical Formula:  
 $C_{15}H_{10}O_4$

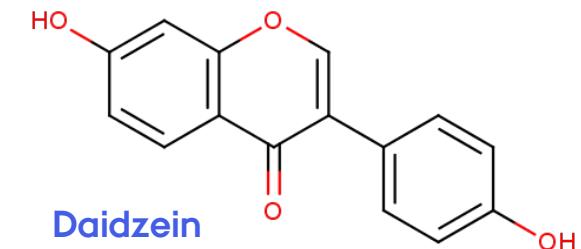
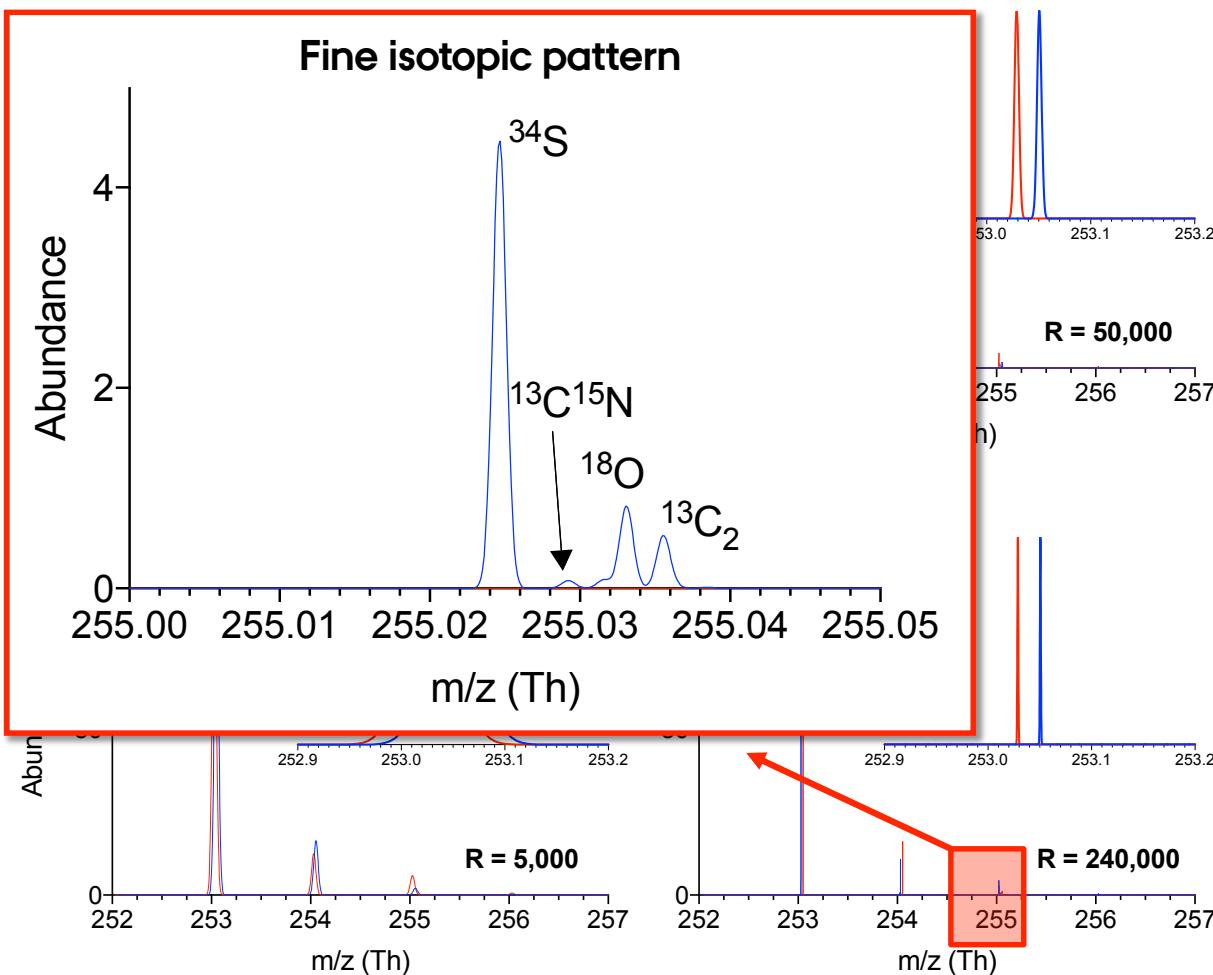
$$\Delta m = 21.8 \text{ mDa}$$

### N-acetyl zonisamide

Monoisotopic Mass:  
253.0288511 Da

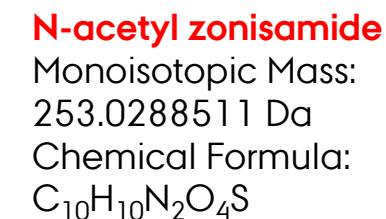
Chemical Formula:  
 $C_{10}H_{10}N_2O_4S$





Monoisotopic Mass:  
253.0506323 Da  
Chemical Formula:  
 $C_{15}H_{10}O_4$

$$\Delta m = 21.8 \text{ mDa}$$



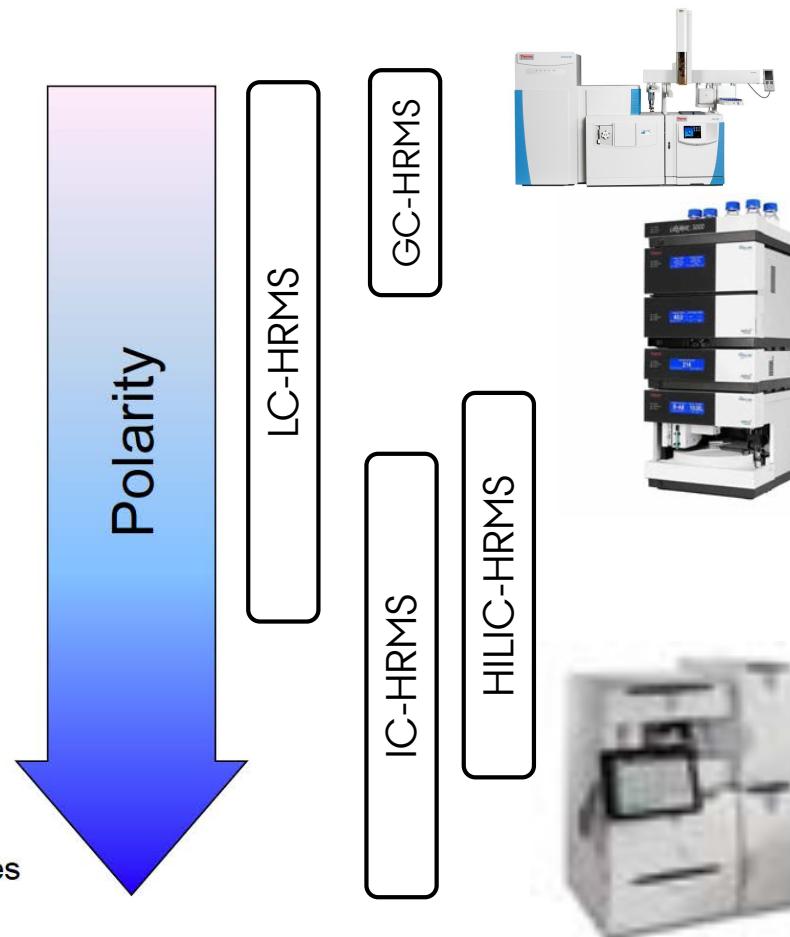
# Data acquisition Chromatography

## Data acquisition



## Metabolites

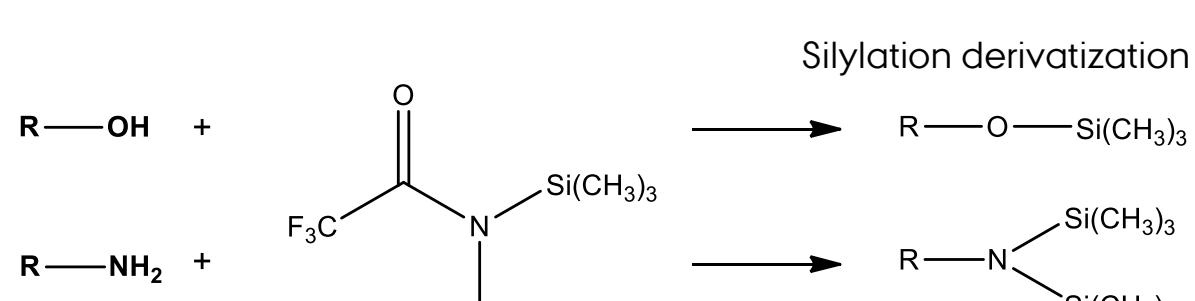
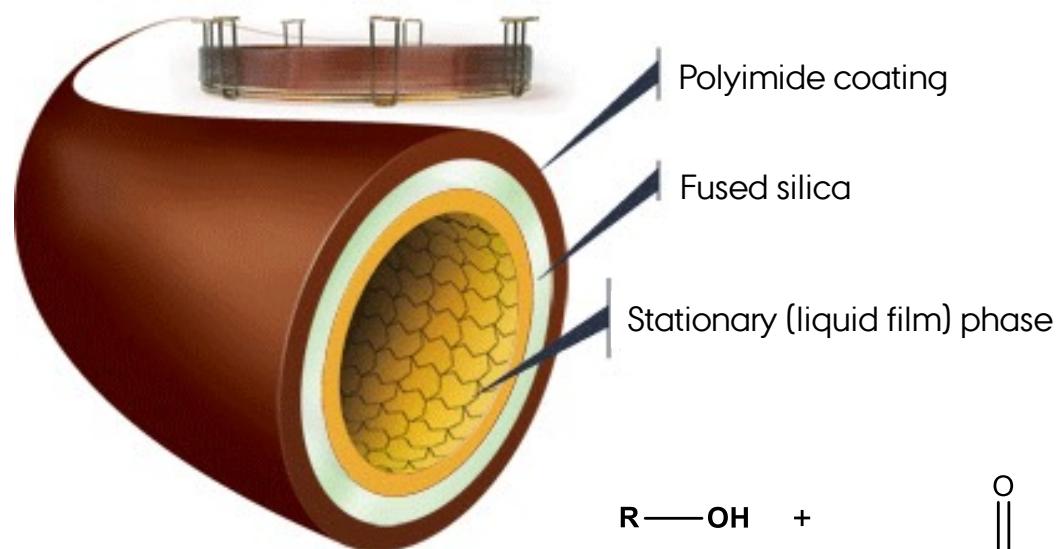
- Triglycerides
- Cholesterol esters
- Diacylglycerols
- Sphingomyelines
- Phosphatidylcholines
- Phosphatidylethanolamines
- Other phospholipids
- Fatty acids
- Eicosanoids & metabolites
- Bile acids
- Bilirubin
- Amino acids, amines
- Organic acids
- Sugars
- Other polar: e.g. purines & pyrimidines



LC: liquid chromatography, IC: ion exchange chromatography,  
GC: gas chromatography, HILIC: hydrophilic interaction chromatography

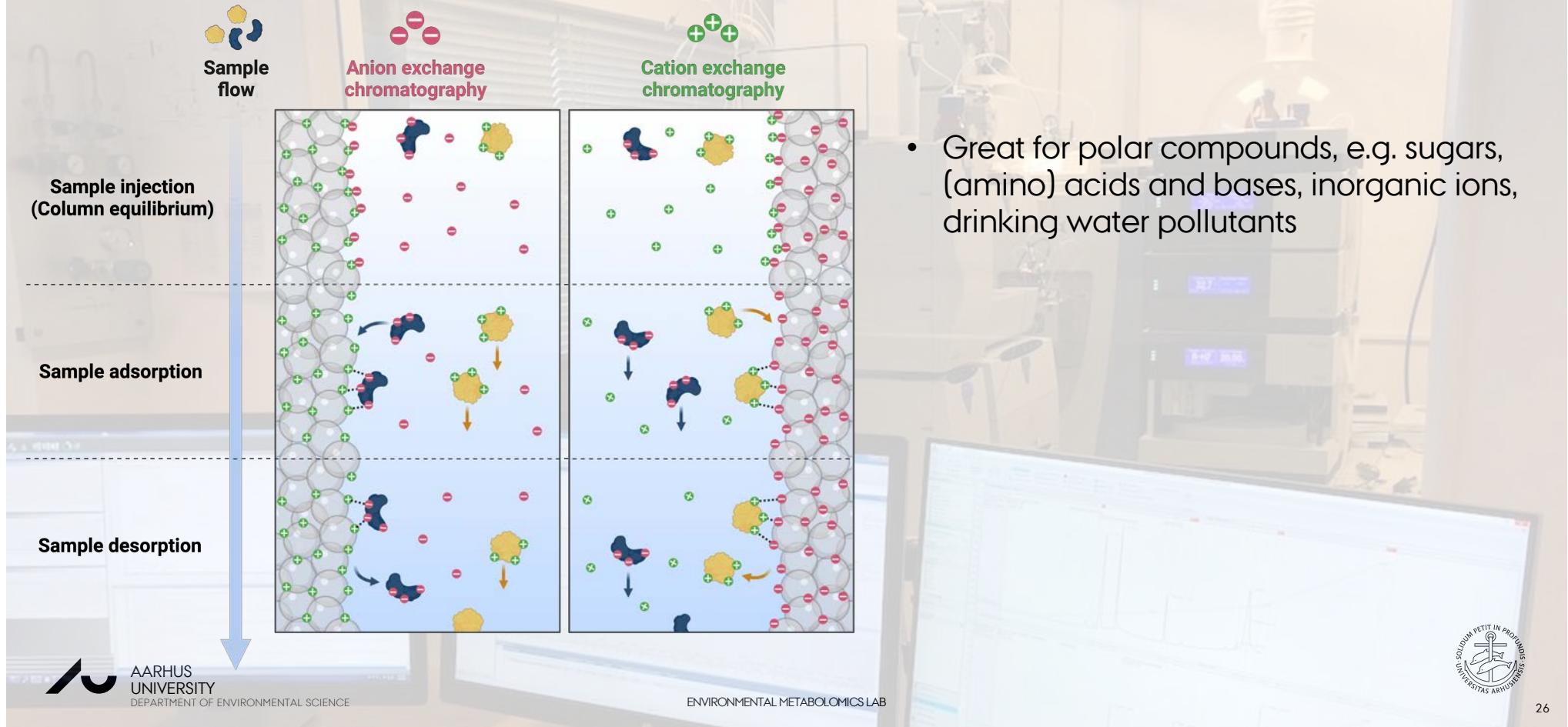
# Gas chromatography

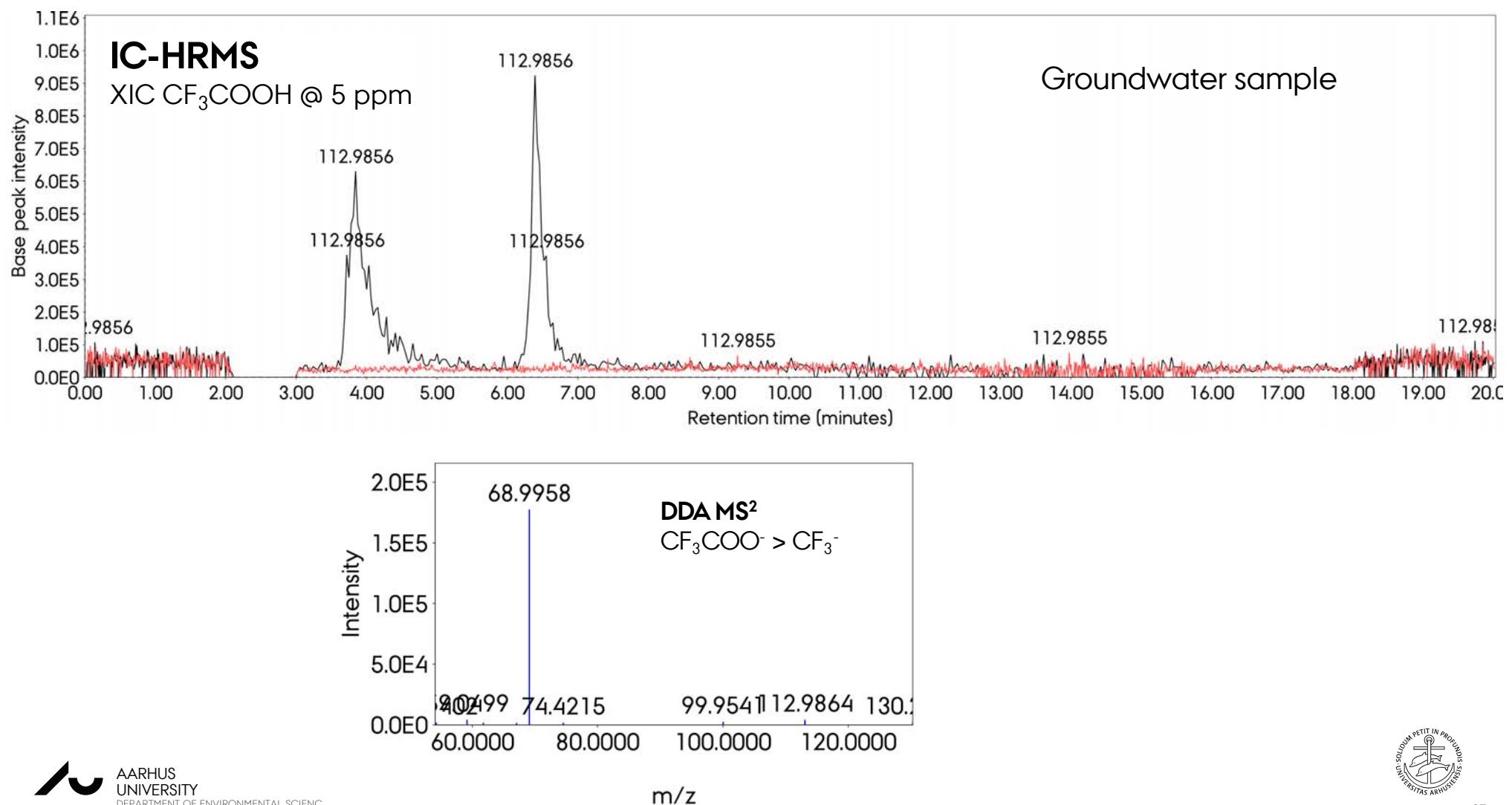
- For substances that can be vaporized without decomposition
- Derivatize to improve volatility, thermal stability (polar  $\rightarrow$  less polar)
- Electron impact (hard) ionization



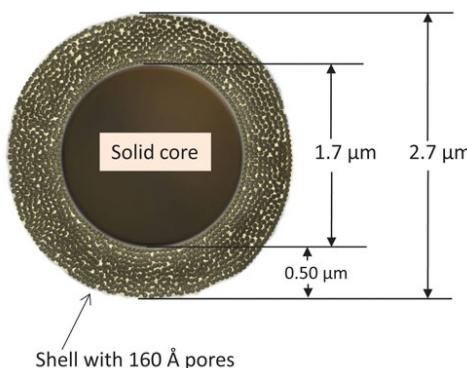
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# Ion exchange chromatography

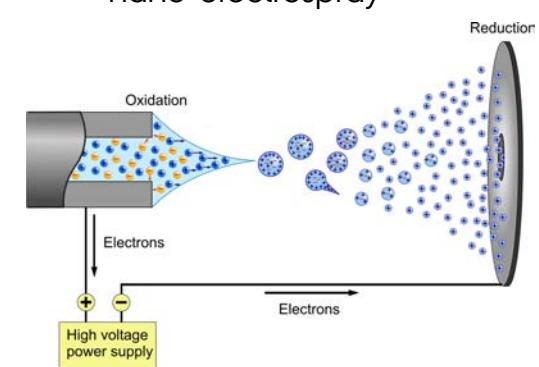
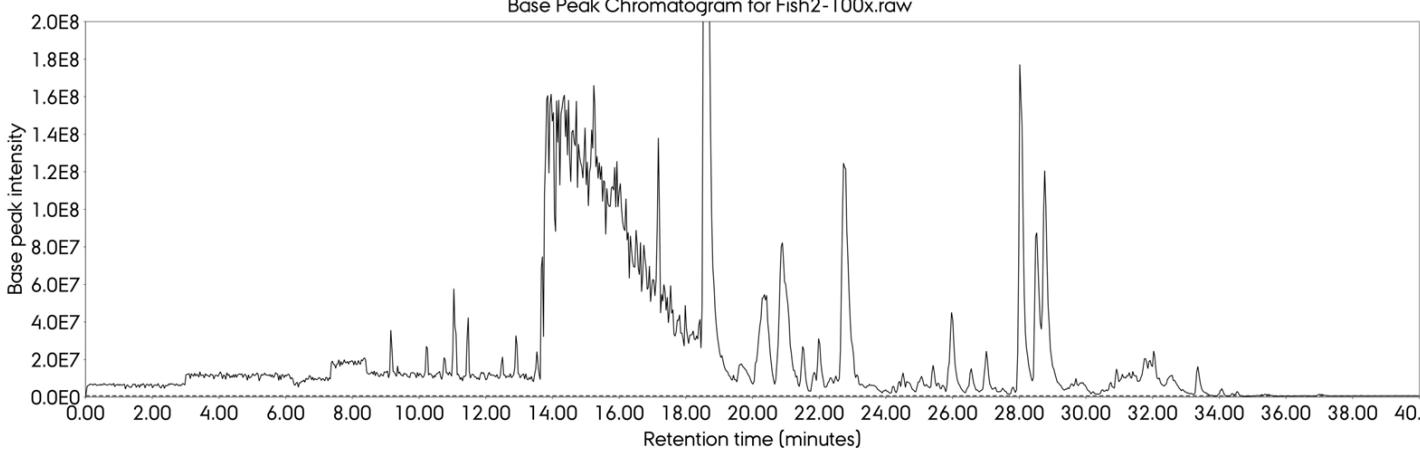
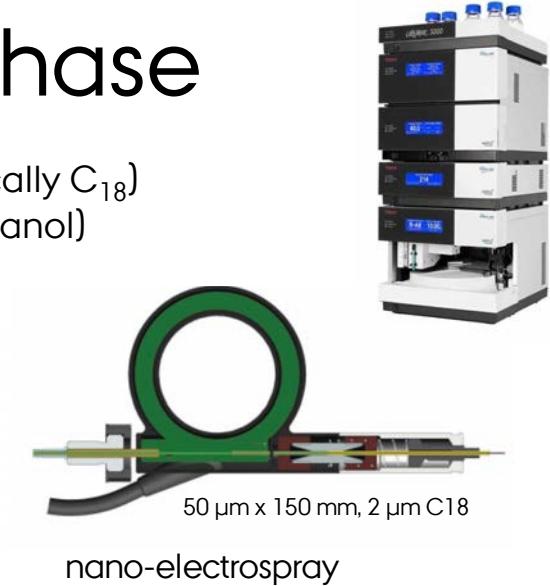
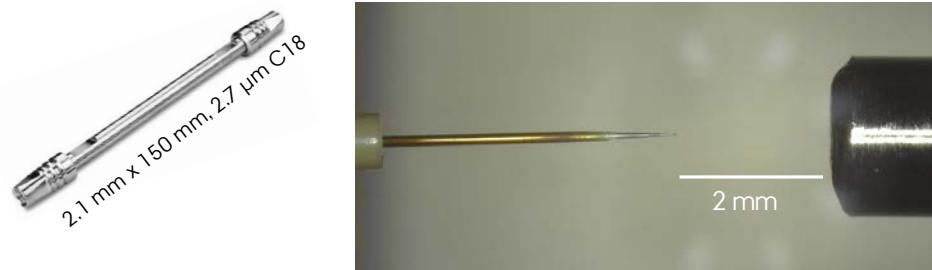




# Liquid chromatography: reverse phase

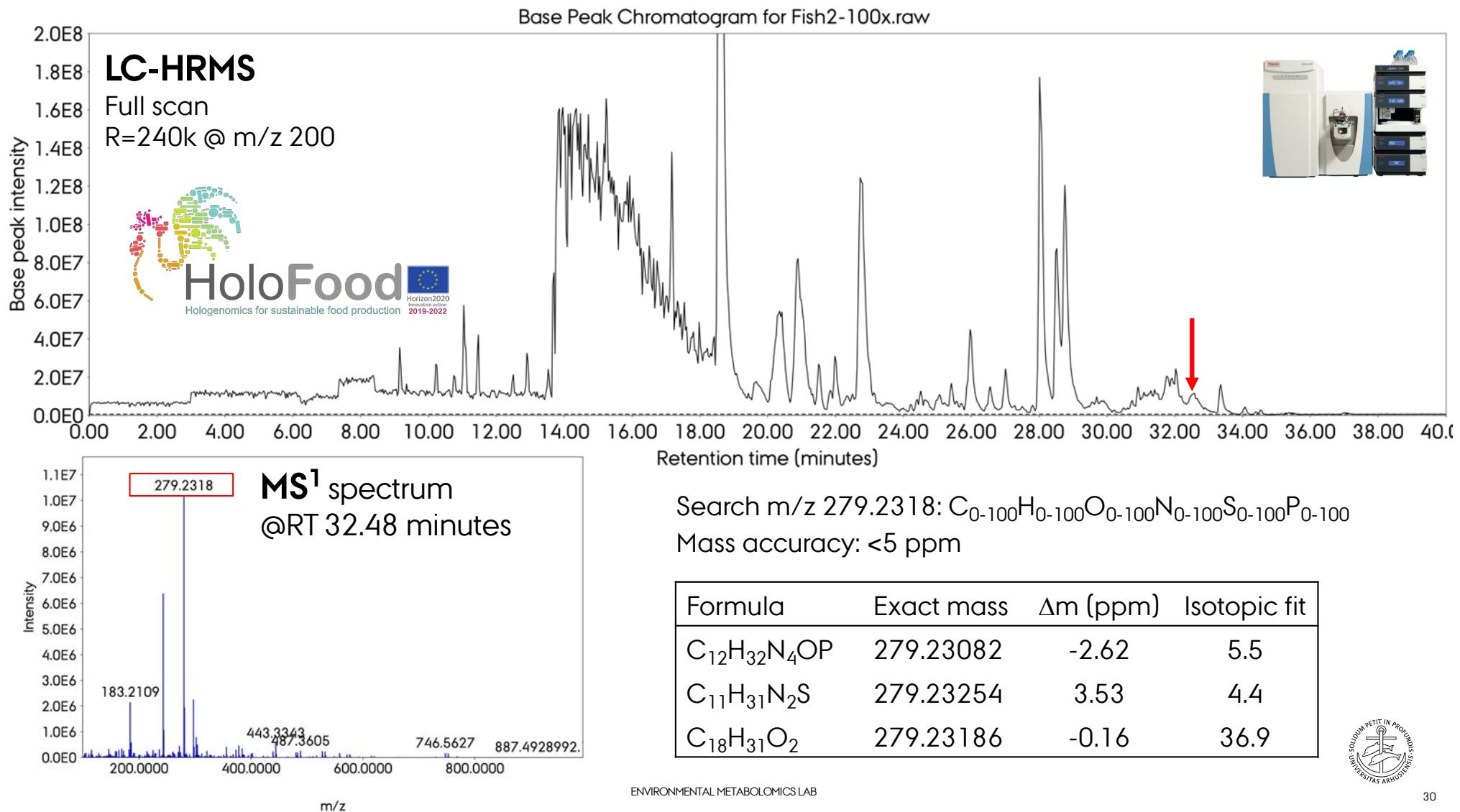


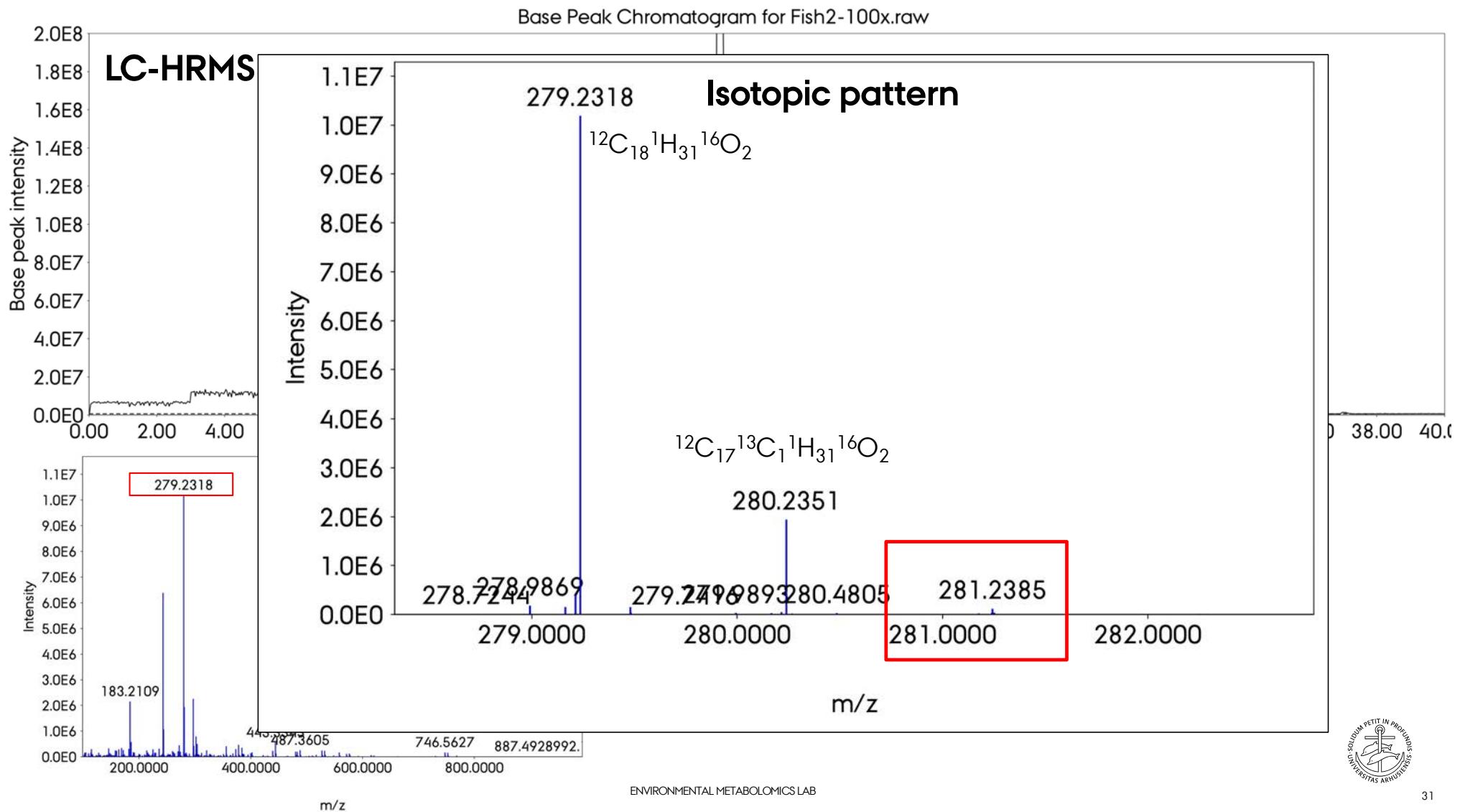
- Reverse phase chromatography
- Large selection of mobile and stationary phases (typically C<sub>18</sub>)
- Organic solvents have high elution strength (e.g. methanol)
- Possible to separate a wide molecular space



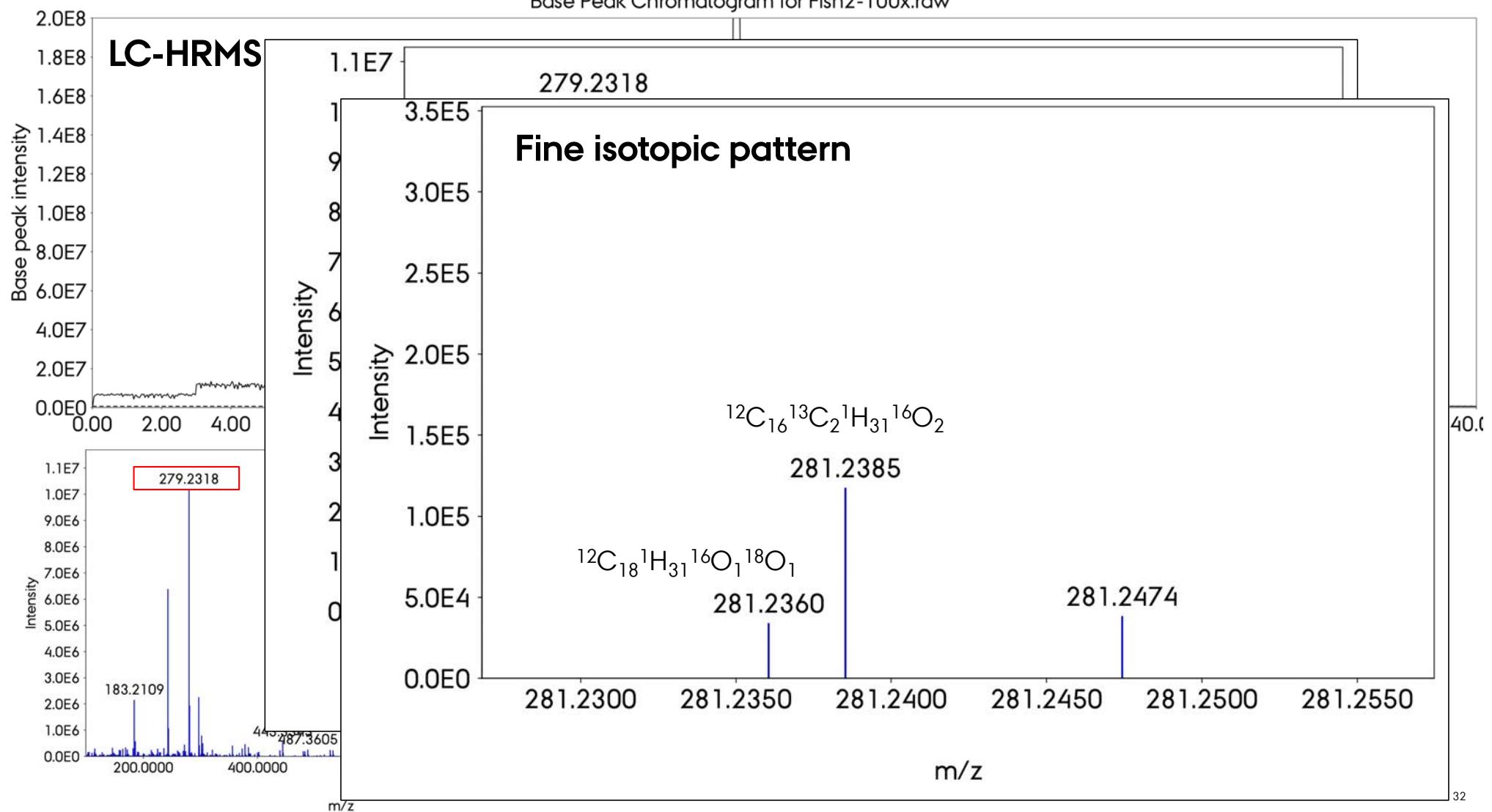
# Data acquisition Hyphenation: LC-HRMS



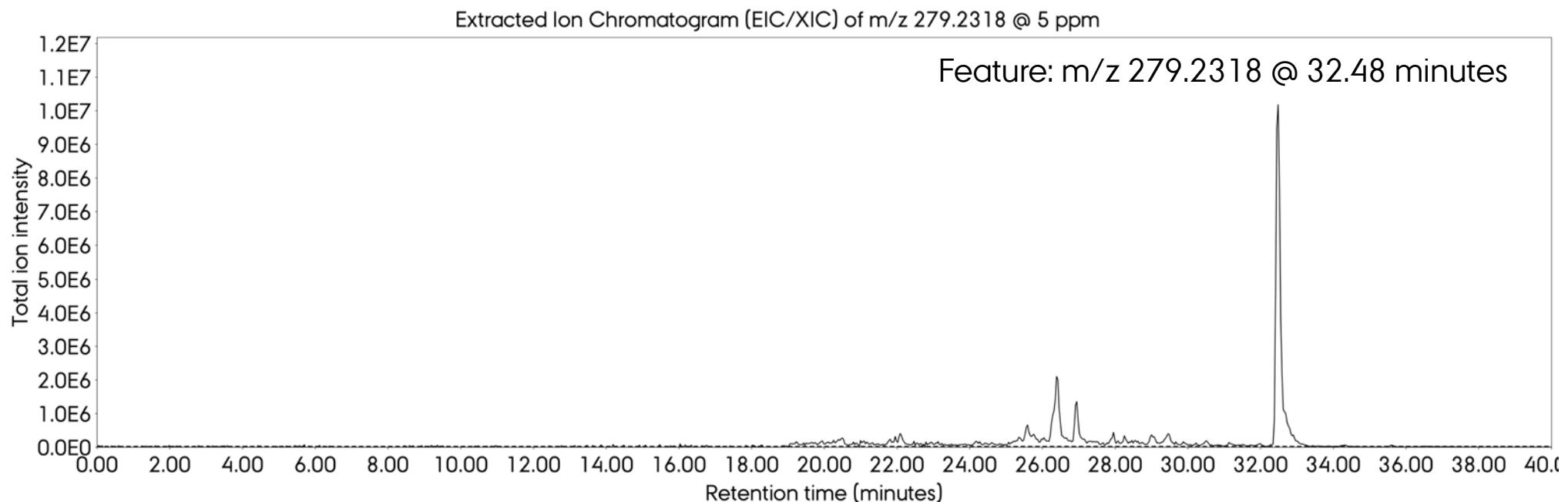




Base Peak Chromatogram for Fish2-100x.raw

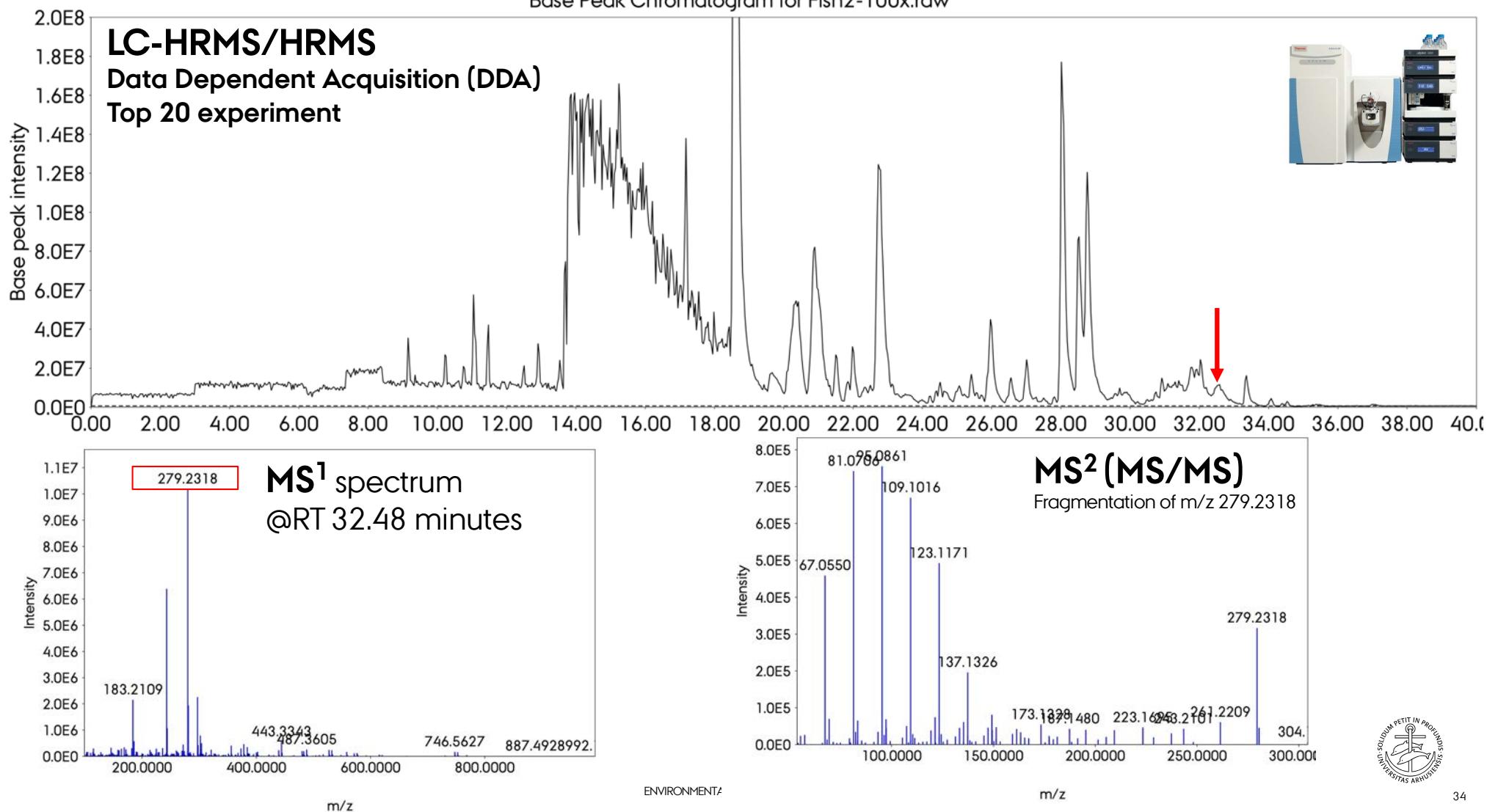


# Feature



Peak area: relative quantification of substance !

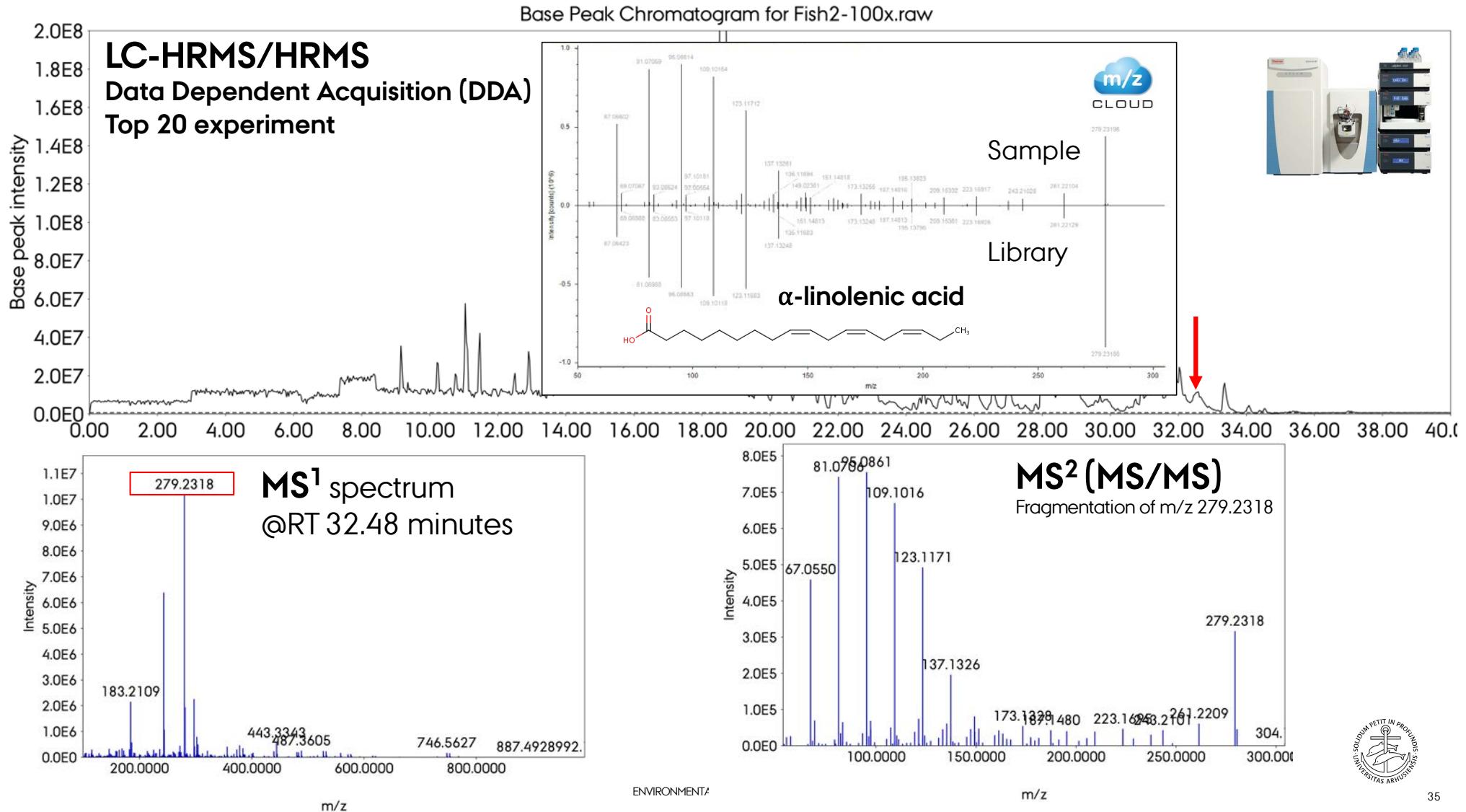
Base Peak Chromatogram for Fish2-100x.raw



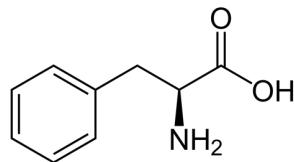
# LC-HRMS/HRMS

## Data Dependent Acquisition (DDA)

### Top 20 experiment



# Metabolite identification levels



Phenylalanine

Amino acids

$\text{C}_9\text{H}_{11}\text{NO}_2$

## Metabolomics Standards Initiative Metabolomics Society

### Level 1 - Identified metabolites

- Two or more orthogonal properties of authentic standard (e.g. RT, m/z, fragment pattern)

### Level 2 - Putatively annotated compounds

- Compare to MS2 spectral libraries

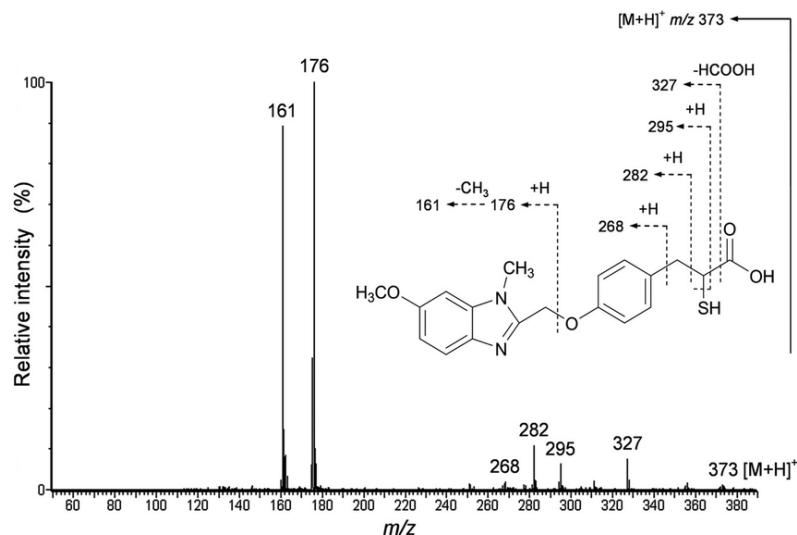
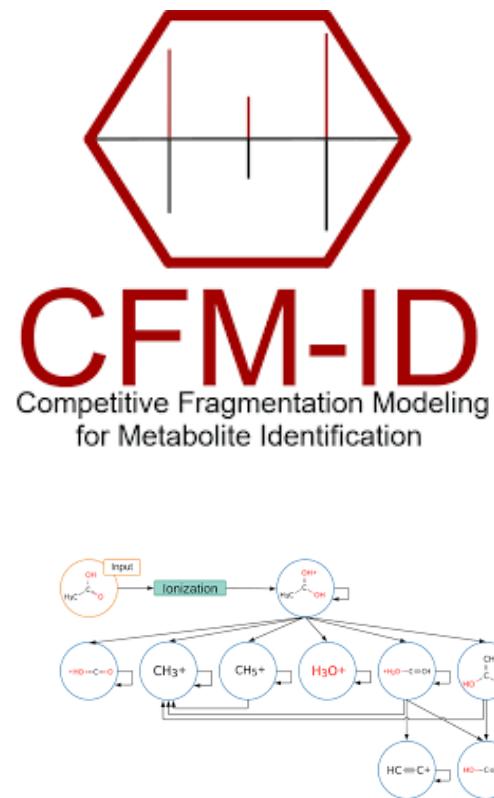
### Level 3 - Putatively characterised compound classes

- Spectral similarities to class

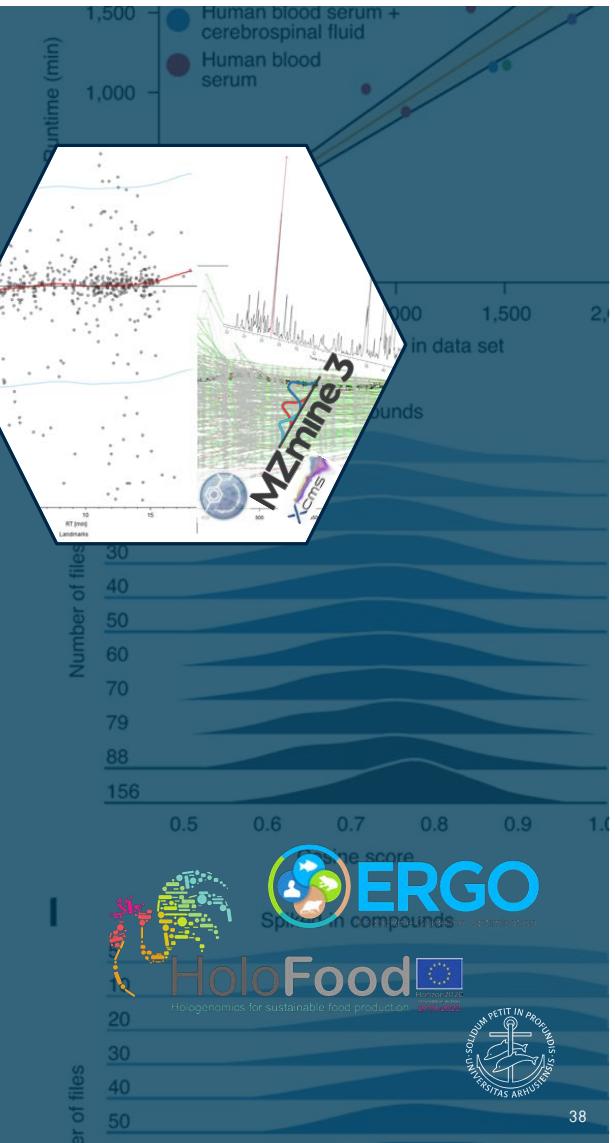
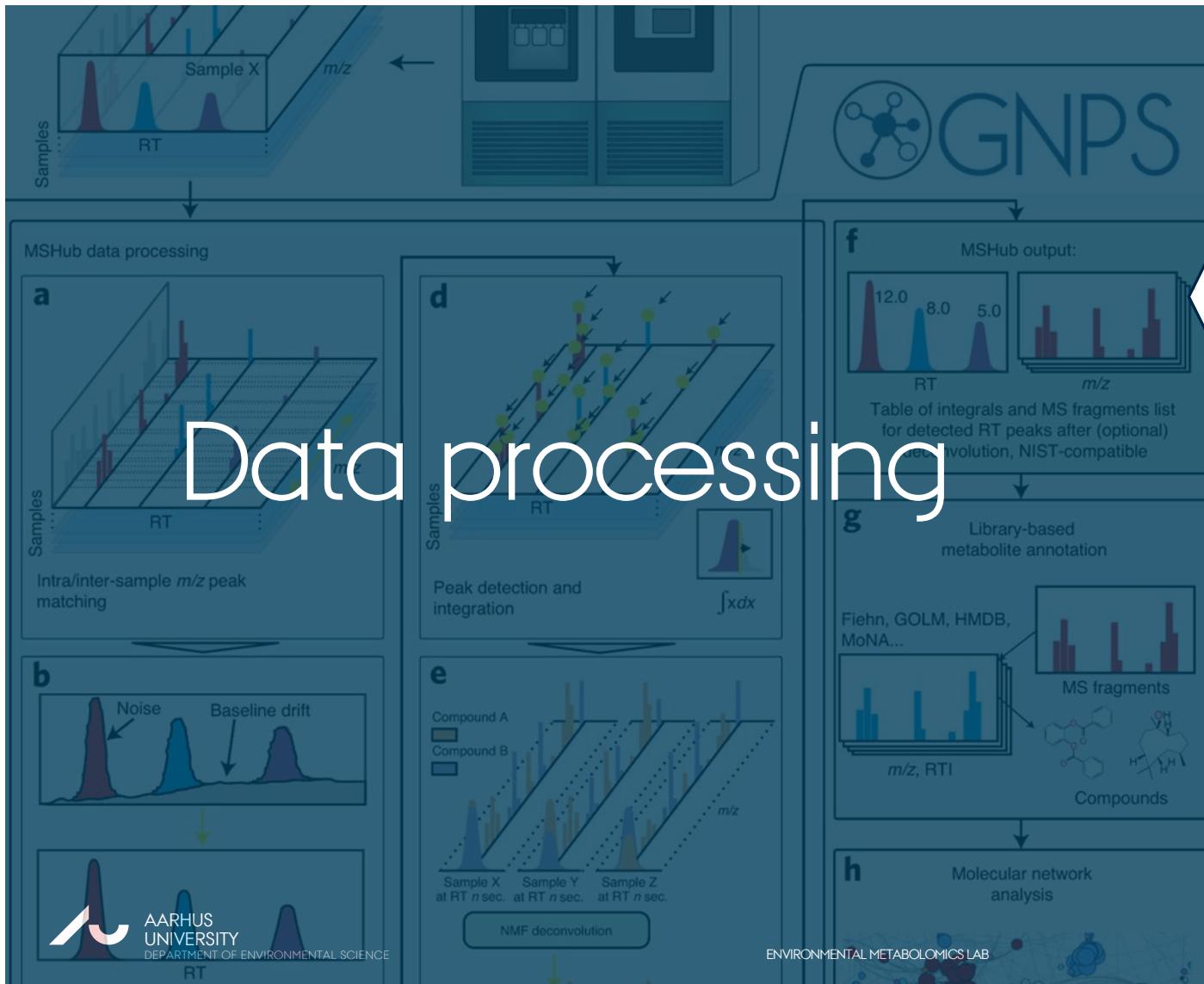
### Level 4 - Unknown compounds

- Everything else

# Metabolite annotation



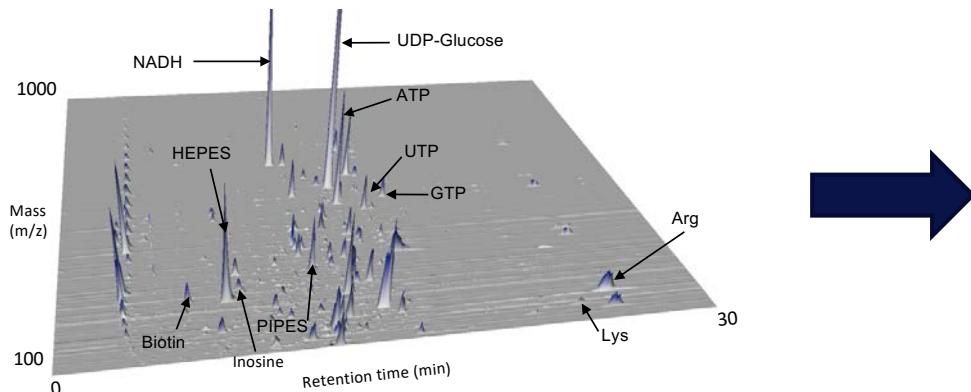
# Data processing



# The goal of raw MS data processing in metabolomics

## Raw data

*“what comes from the machine”*

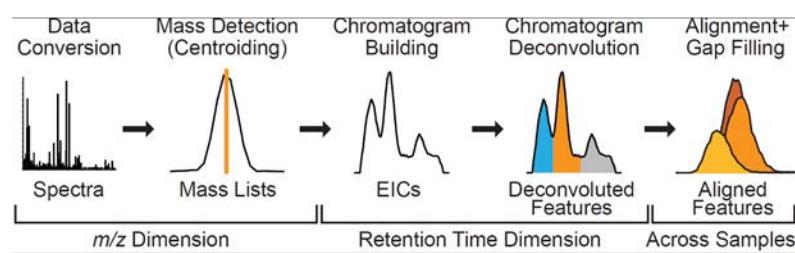


## Metabolite list

*“what the biologist understands”*

Sample/ metabolite	S1	S2	S3	S4
Leucine	32	33	43	54
Adenosine	231	23	234	12
Metabolite X	234	243	343	321
Metabolite Y	5	4	7	3
...	...	...	...	...

**MZmine 3**



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Courtesy of Robin Schmid & Steffen Heuckeroth



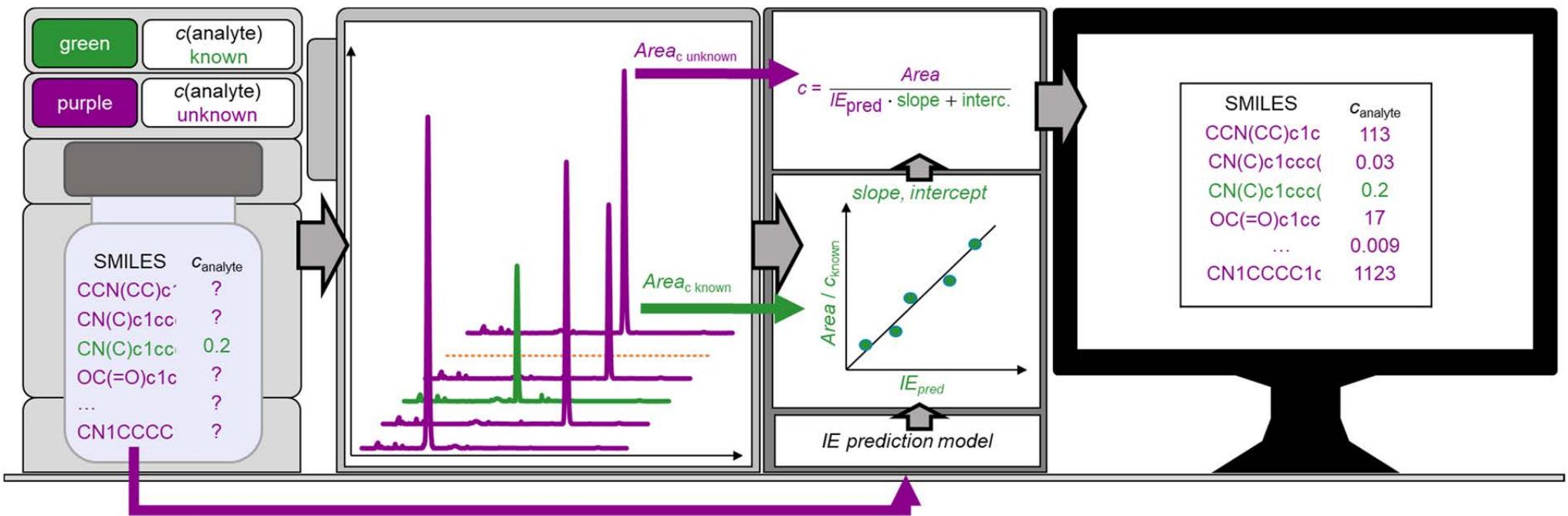
# Data repositories

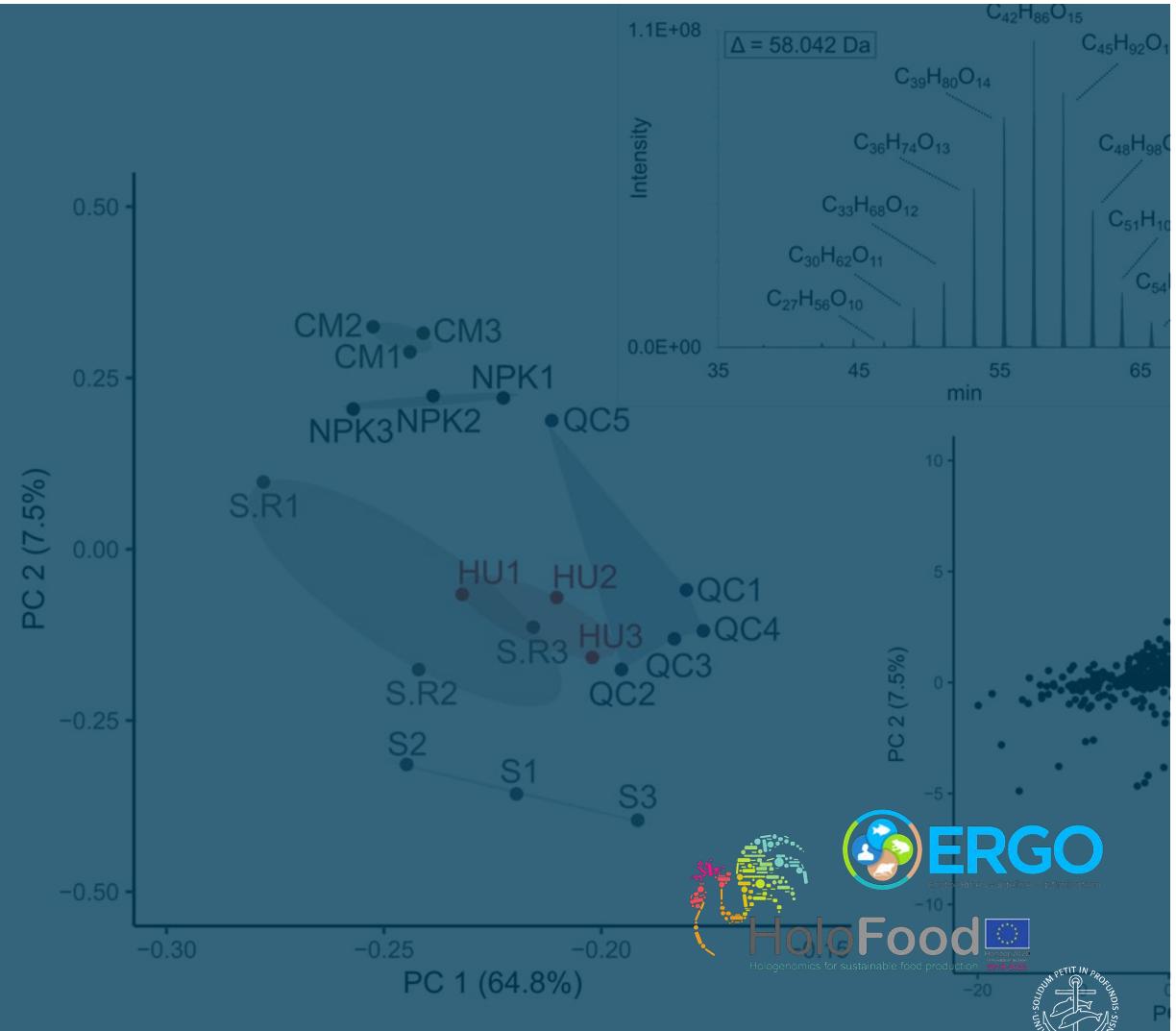


The homepage of the Metabolomics Workbench. It has a dark header with the title "METABOLOMICS WORKBENCH". Below the header is a search bar and a navigation menu with links like "Home", "Data Repository", "Databases", "Protocols", "Tools", "Training / Events", "About", and "Search". The main content area features a "National Metabolomics Data Repository" section with a table showing study statistics. There are also sections for "Recently released studies on NMDR" and "Tweets by @MetabolomicsWB".

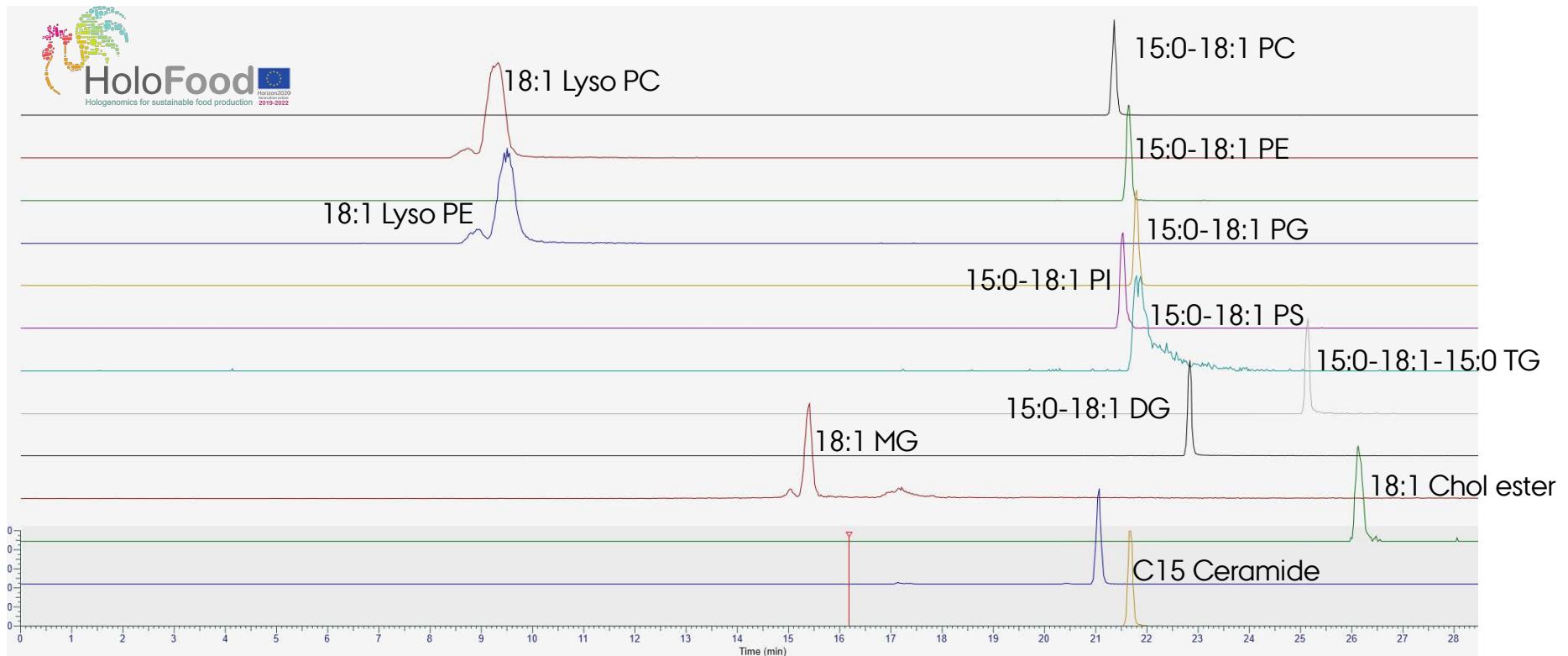
The homepage of MetaboLights. The header includes the EMBL-EBI logo and a search bar. The main title "MetaboLights" is displayed with a bar chart icon. Below the title is a navigation menu with links to "Home", "Browse Studies", "Browse Compounds", "Browse Species", "Download", "Help", "Give us feedback", and "About". On the right side, there is a sidebar for "Tweets by @Metabolights" and a "Submit Study" and "Login" button. The central content area features three main sections: "Study" (with "BROWSE" and "ORCID SEARCH" options), "Compound Library" (with "COMPOUNDS" and "SPECIES" options), and "Training" (with "METABOLOMICS TRAIN ONLINE" and "METABOLIGHTS QUICK TOUR" options). A large blue button at the bottom says "Submit to MetaboLights".

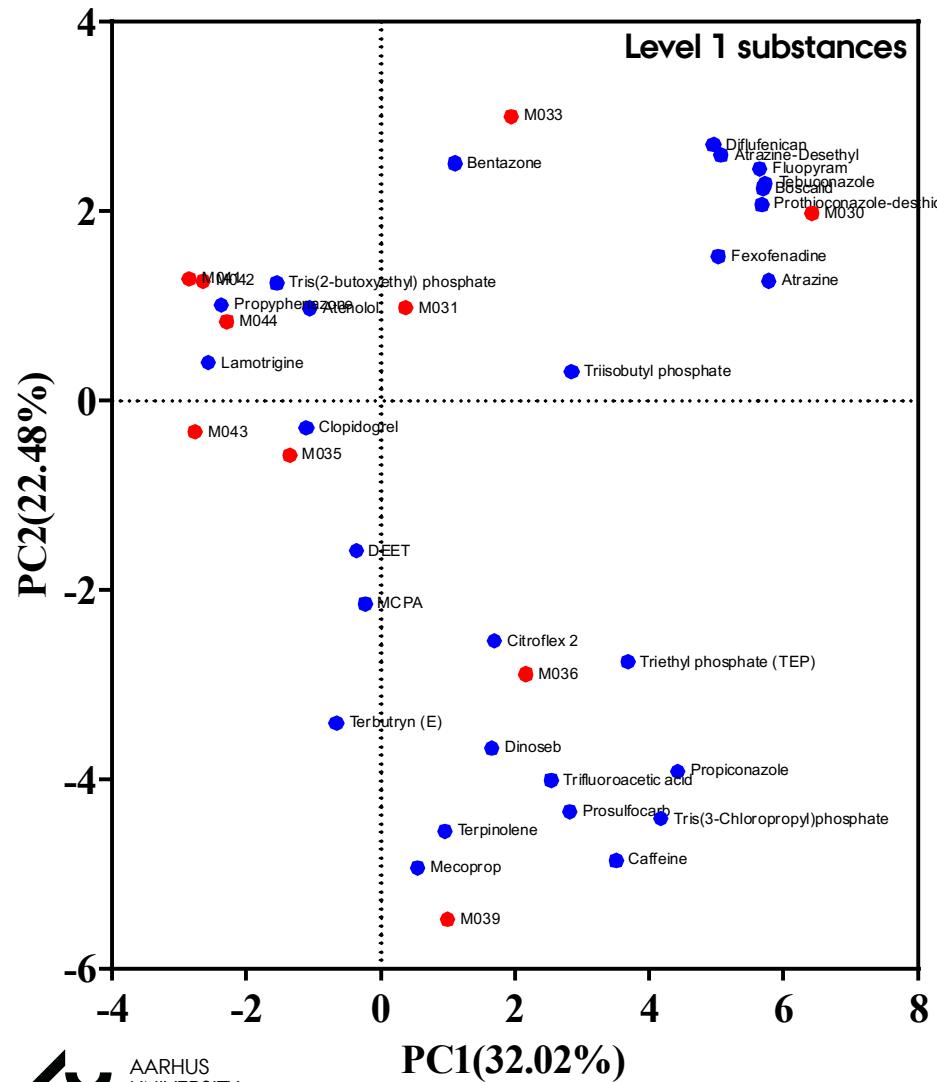
# In silico: concentration prediction





# Lipidomics

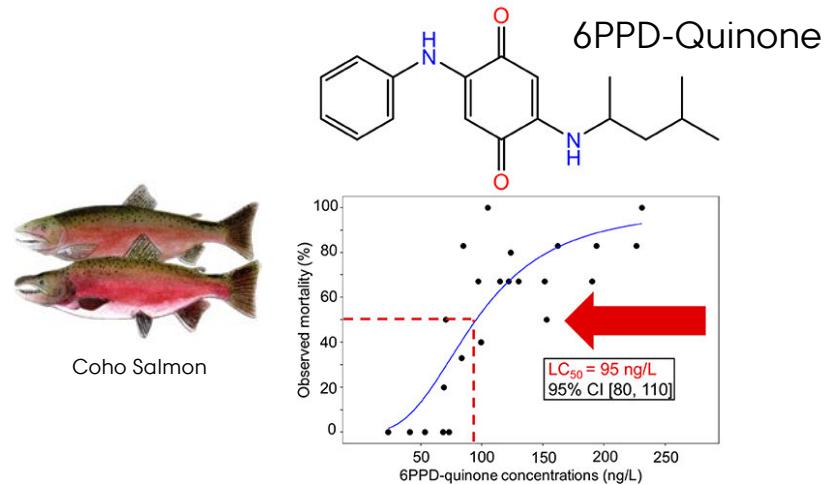
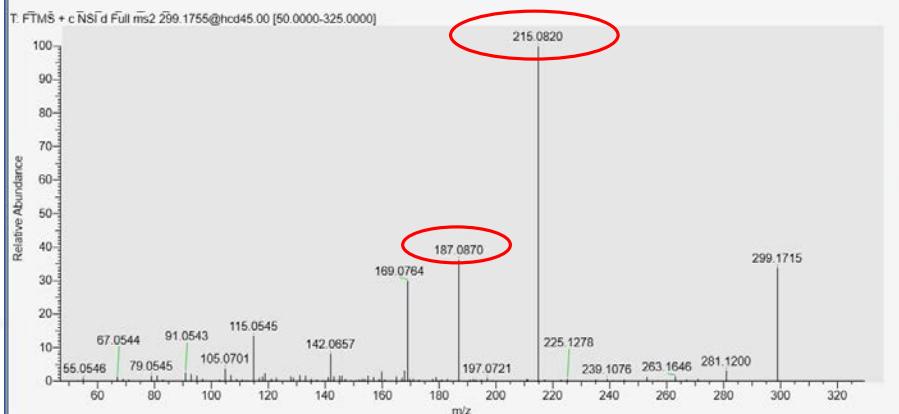
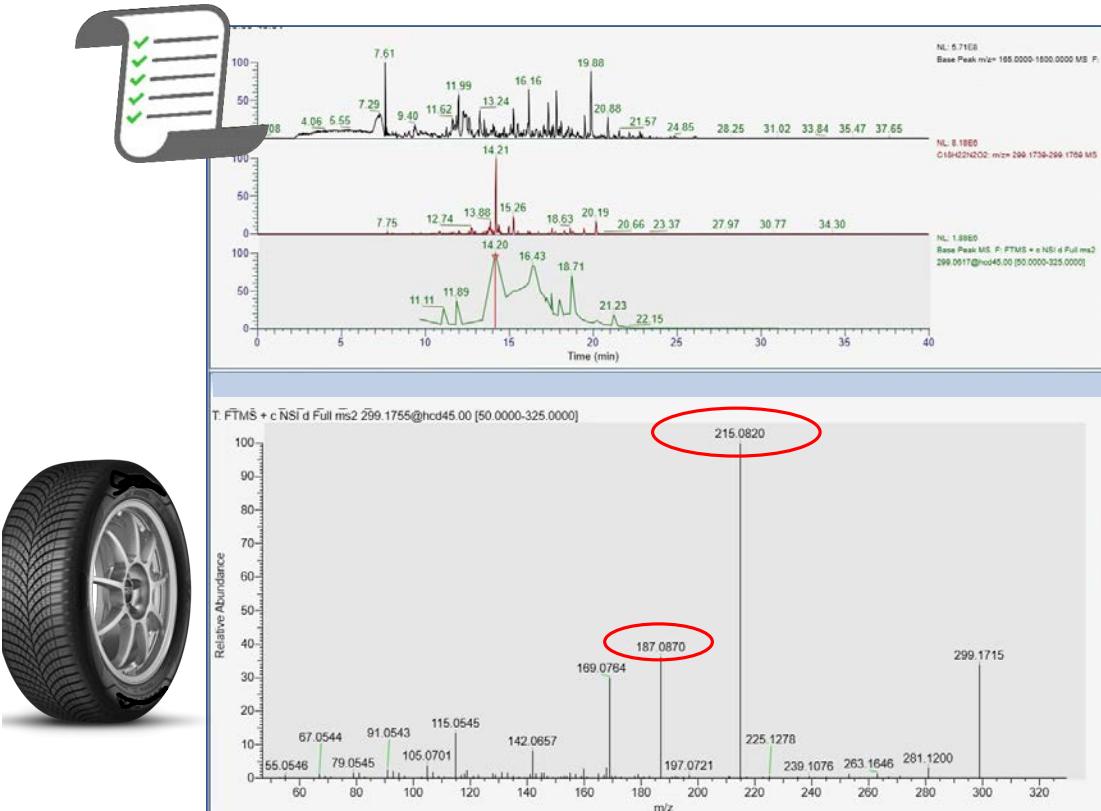




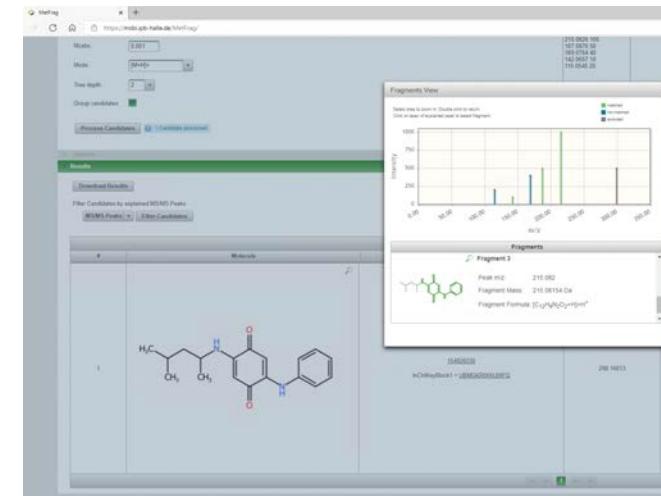
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# Retrospective analysis



Tian et al., Environ. Sci. Technol. Lett. 2022, 9, 2, 140–146

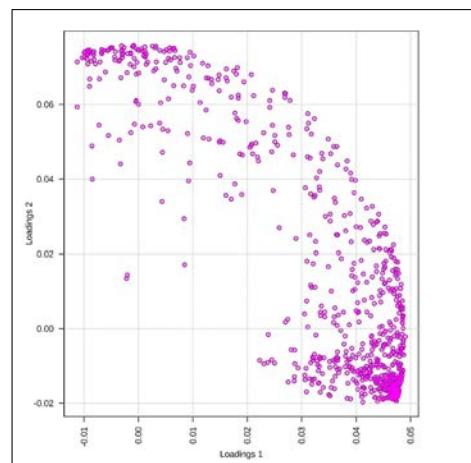
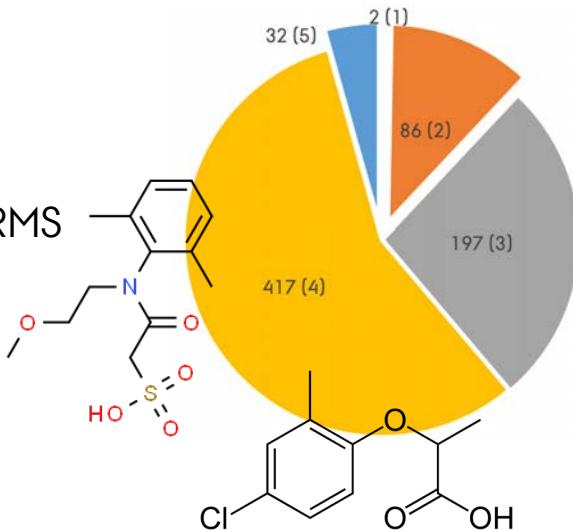
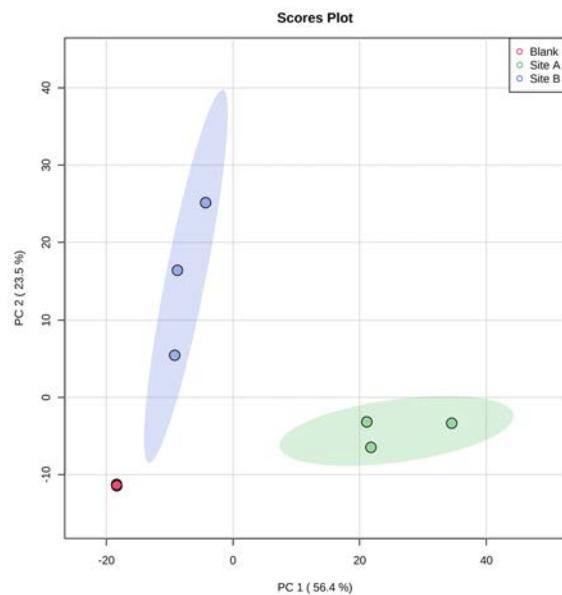


# Drinking water

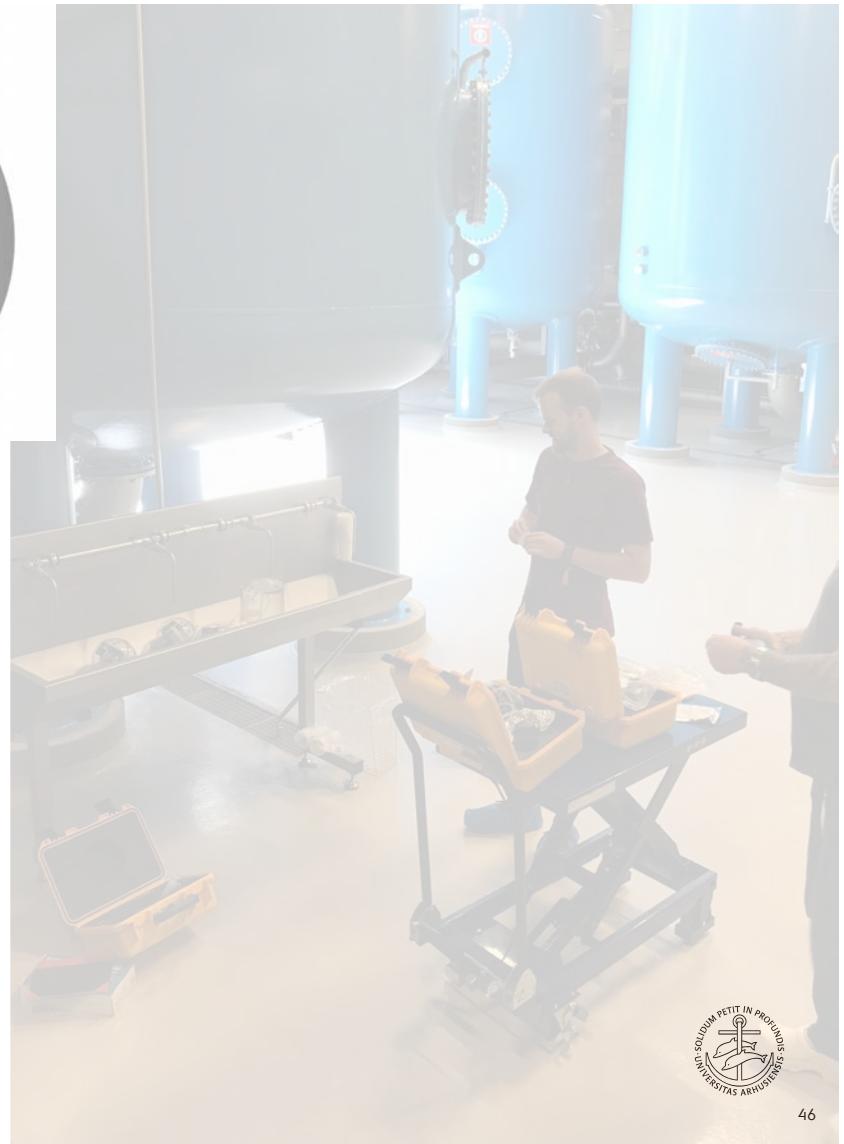


Enriched >300 L water  
Ion chromatography HRMS  
>700 substances

Dimethachlor ESA  
Mecoprop  
Trifluoroacetic acid



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Funded by the Horizon 2020  
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Ministry of Environment  
of Denmark

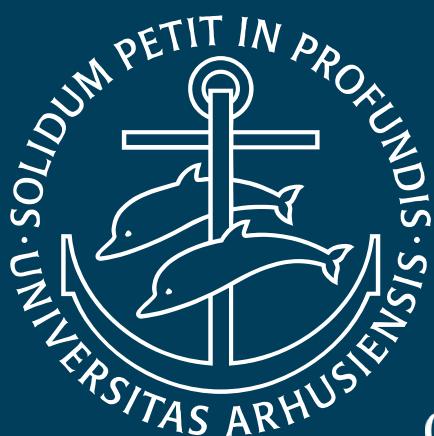
Environmental  
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Ministry of Higher  
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