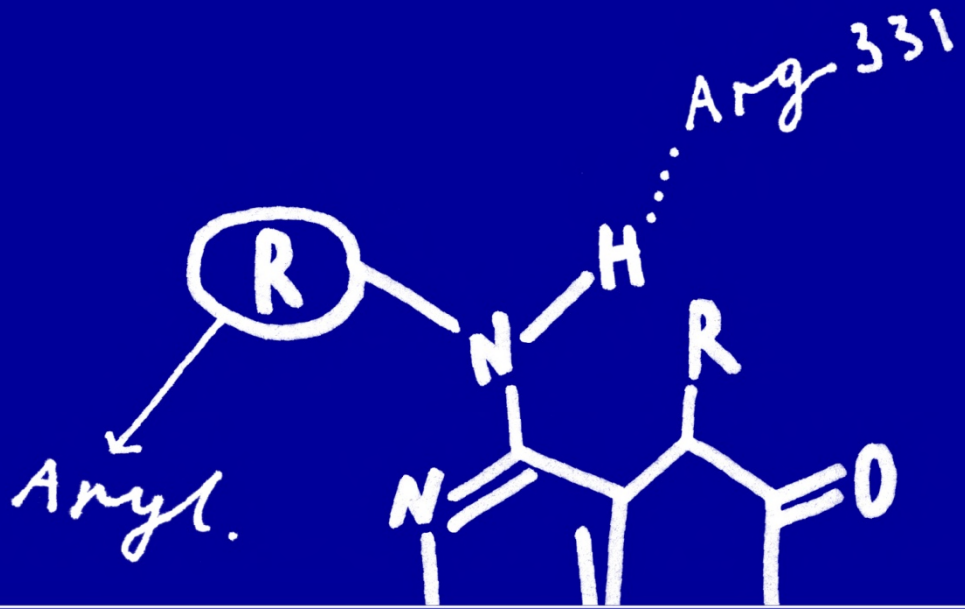
evotec

#RESEARCHNEVERSTOPS

Identification of metabolites: analytical challenges for conducting *in vitro* metabolism characterisation of pesticides



Workshop on "*In vitro* comparative metabolism studies in Regulatory Pesticide Risk Assessment"
Parma, EFSA, 15-16 November 2018

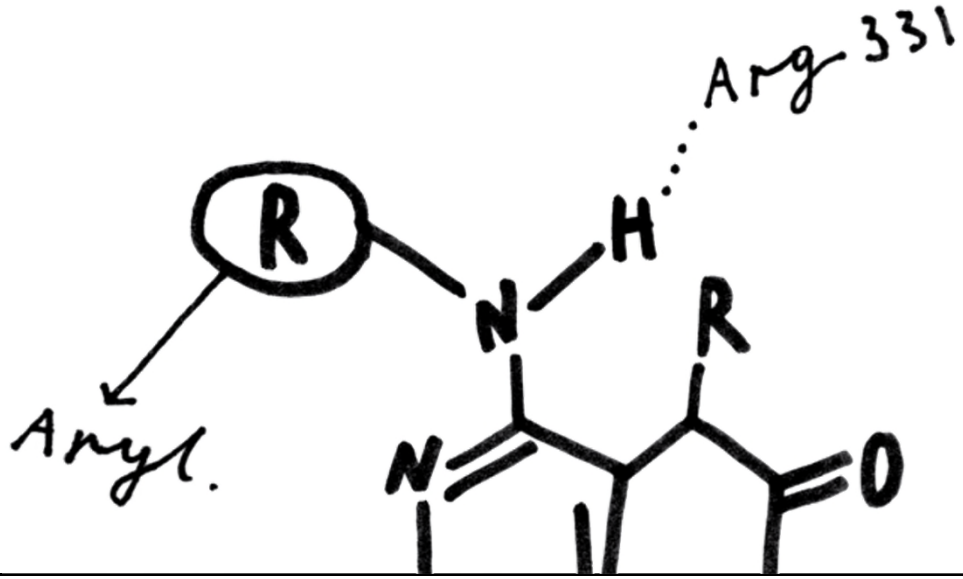
Outline

Introduction

Analytical aspects

Data processing

Conclusions



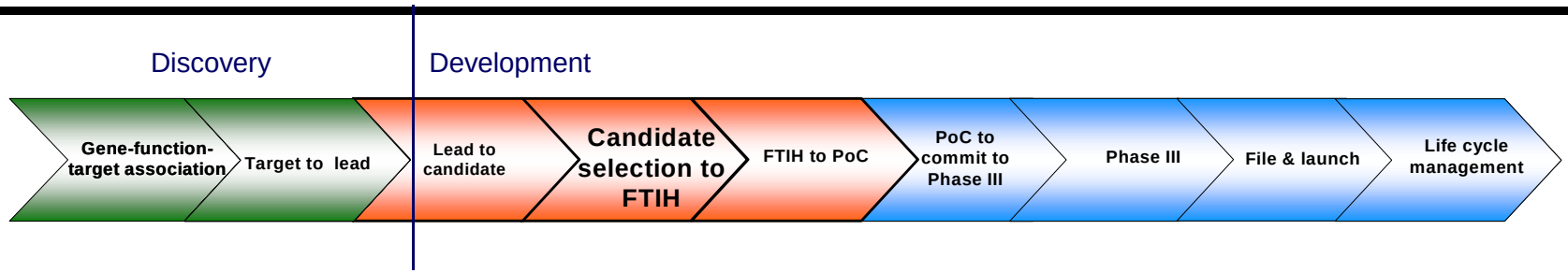
Why do we need to identify metabolites?

Metabolites can affect the efficacy and safety of potential drugs

- Efficacy
  - The metabolites modulate the efficacy of drugs in the treatment of disease
  - Metabolites may possess pharmacological activity
- Safety
  - Metabolite may be toxic (bioactivation)
  - Active metabolites and reactive metabolites may impact on safety

Pharmaceutical Companies are mandated
by Regulatory Agencies to identify metabolites of NCEs

What is the stage-based need for metabolite identification?



- In discovery stage:
  - Address clearance issues (metabolic hot-spots) leading to short half-life
  - Provide biotransformation pathway information for candidate selection as well as during lead optimisation
  - Generate potential new leads
  - Eliminate compounds that produce potentially reactive metabolites

- In development stage:
  - Determine metabolic pathways in preclinical species and in humans
  - Attempt to model metabolism in humans
  - Ensure that the preclinical species chosen for safety evaluation are adequate
  - Ensure all major metabolites are monitored

Metabolite identification workflow

Continuous and iterative process

- Multiple species/Time points
- Procedural Blank sample
- Generic extraction procedure

In vitro Assay

LC MS Analyses

- Shortest LC run time
- Both polarity

- Untargeted analysis
- Don't miss any "unexpected" metabolite

Data processing

- Reduce number of injection
- Limited amount of sample

LC-MS^n Analyses

Structural Assignment

Identification confidence

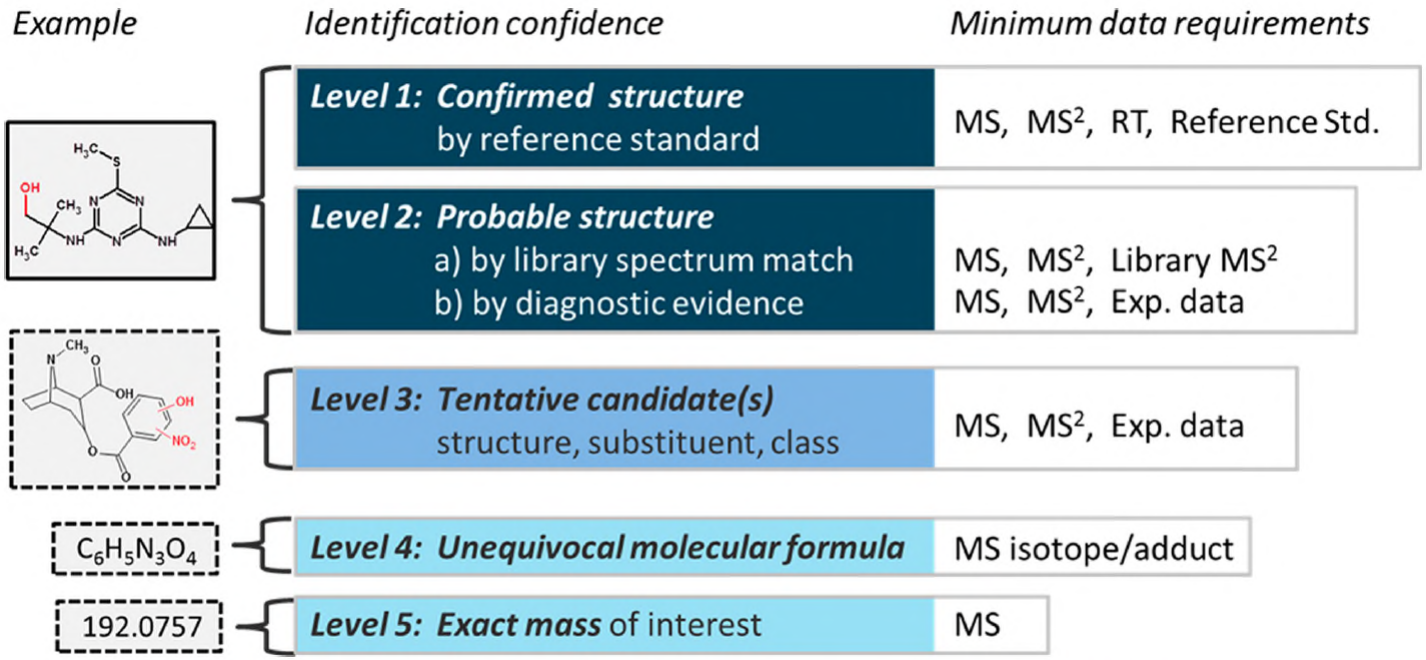


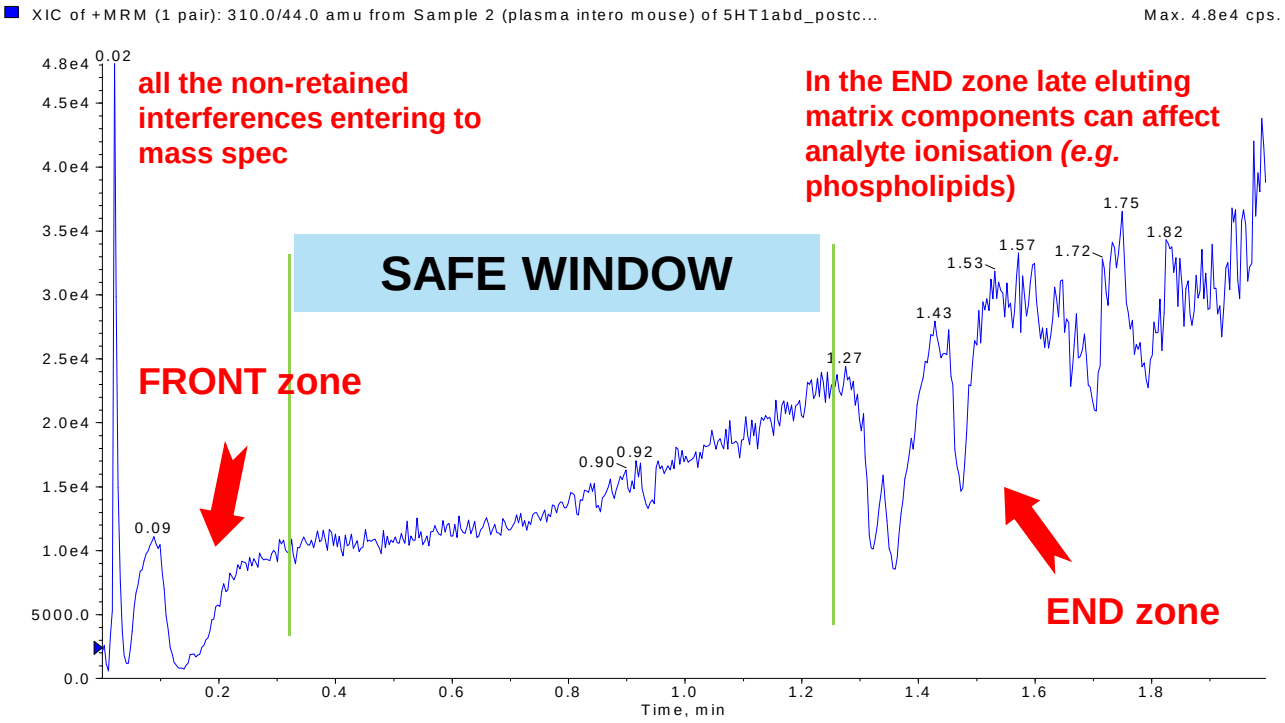
Figure 1. Proposed identification confidence levels in high resolution mass spectrometric analysis. Note: MS^2 is intended to also represent any form of MS fragmentation (e.g., MS^e, MS^n).

Schymanski et al, dx.doi.org/10.1021/es5002105 | Environ. Sci. Technol. 2014, 48, 2097–2098

LC separation

Samples should be analysed using generic LC conditions to balance adequate retention and reasonable elution times of various metabolites

Co-elution of metabolites should be avoided case of isobaric species and as well as liquid chromatography coupled to UV/radio detection



(Matuszewsky at al., Anal. Chem. 1998; Fu, Woolf, Matuszewsky, J. Pharm. Biomed. Anal, 1998; Murphy et al., Rapid Commun. Mass Spectrom., 2002)

Metabolite characterisation: Mass spectrometry

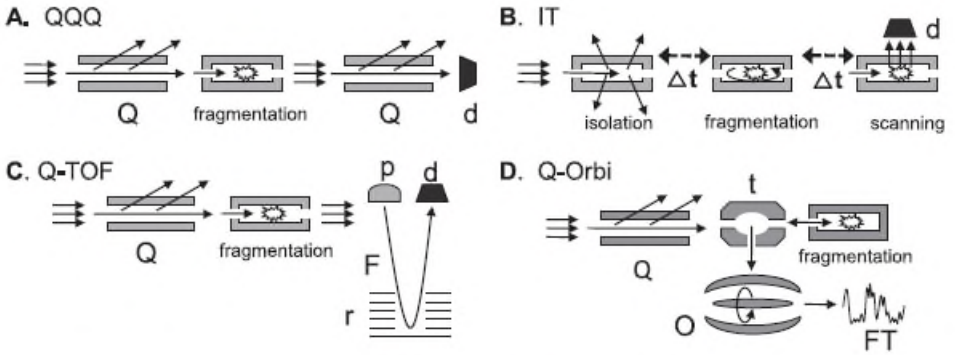
Tandem Mass Spectrometry

technique to identify metabolites in complex biological matrices

Equipment

- Triple Quadrupole (QQQ)
- Ion Traps (IT and LIT)

- Q-Orbitrap
- Q-Time Of Fly (TOF)



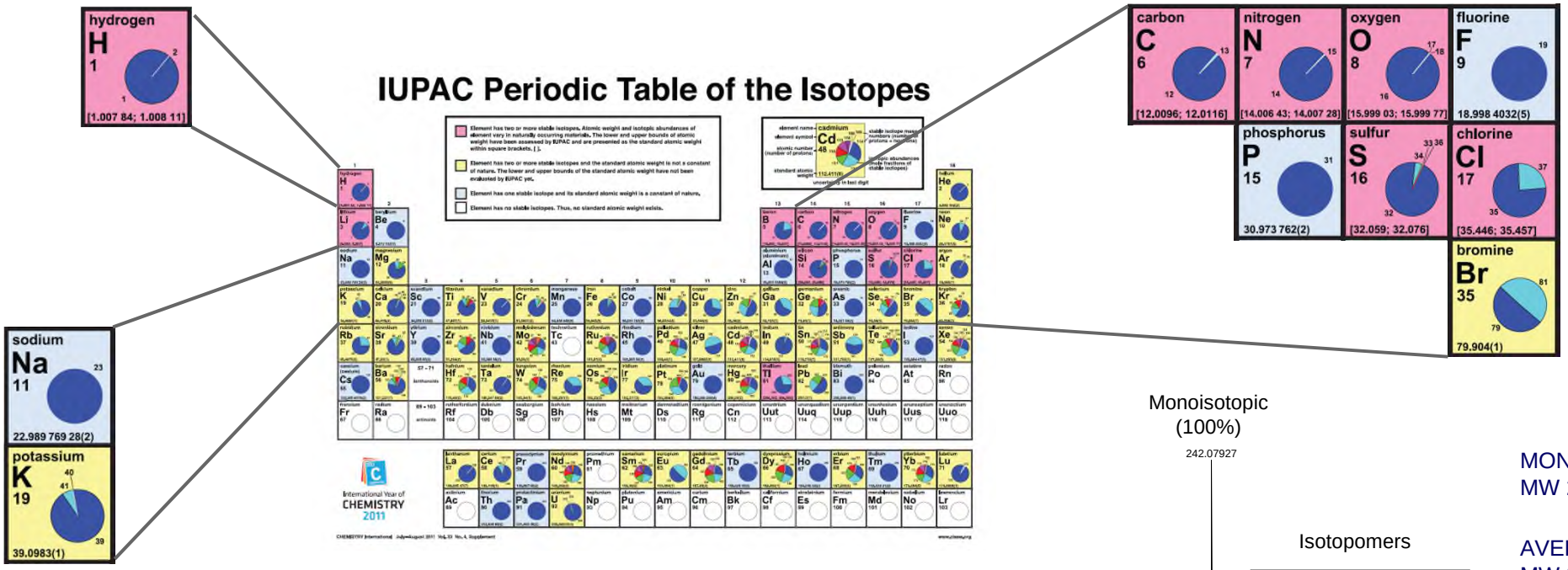
		A. QQQ-MS	B. IT-MS	C. Q-TOF-MS	D. Orbi-MS
	Mass Resolution	low	low	high	high/ultra-high
Sensitivity	Global acquisition	1	2	3	3
	Targeted acquisition	3 (4)	2 (3)	(2) 3	(2) 3
Specificity	Global acquisition	1	1	(2) 3	3 (4)
	Targeted acquisition	3	(3) 4	(3) 4	4 (5)
Acquisition rate	Global acquisition	1	3	(3) 4	3
	Targeted acquisition	3	2	(2) 3	2
Sum	Global acquisition	3	6	**8-10**	**9-10**
	Targeted acquisition	**9-10**	7-9	**7-10**	**8-10**

Fig. 2. Scheme of the 4 most used LC-MS technologies: 2 low resolution technologies, triple quadrupole (A: QQQ-MS) and ion trap MS (B: IT-MS) and 2 high resolution technologies, quadrupole-time-of-flight-MS (C: Q-TOF) and quadrupole-orbi-MS (D: Q-orbi); adapted from [12]. **The table below allocates a grade according to global or targeted MS performance (1 to 5 points for poor to excellent, respectively). Global acquisition corresponds to high resolution full scan (HR-FS) or data-independent-acquisition (DIA).** Targeted acquisition corresponds to SIM (single ion monitoring), SRM (selected reaction monitoring) or product ion scan. Sensitivity, specificity and acquisition rate (scan speed) are considered and points are summed in the last row. **Abbreviations.** CC: collision cell; d: detector; F: flight; FT: Fourier transform; IT: ion trap; O: orbitrap; p: pusher; Q: quadrupole; r: reflectron; Δt : delta of time.

Bertrand Rochat, From targeted quantification to untargeted metabolomics: Why LC-high-resolution-MS will become a key instrument in clinical labs, Trends in Analytical Chemistry (2016), doi: 10.1016/j.trac.2016.02.009

Accurate mass measurement

Metabolite Characterization using Mass Spectrometry

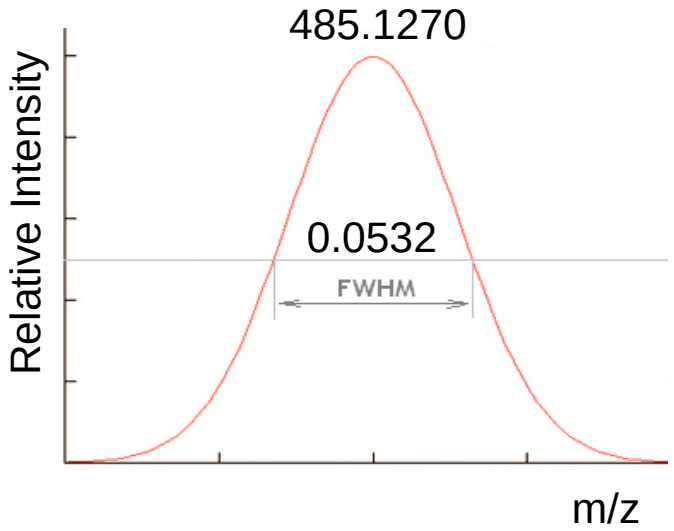


Take advantage of Nature’s imperfection!

Resolution and accuracy

Metabolite characterisation using Mass Spectrometry

Resolution in mass spectrometry, is a measure of the ability to distinguish between two peaks of different mass-to-charge ratio (m/z) in a mass spectrum.



Full Width at Half Maximum (**FWHM**)
is a way to define resolution in mass spectrometry

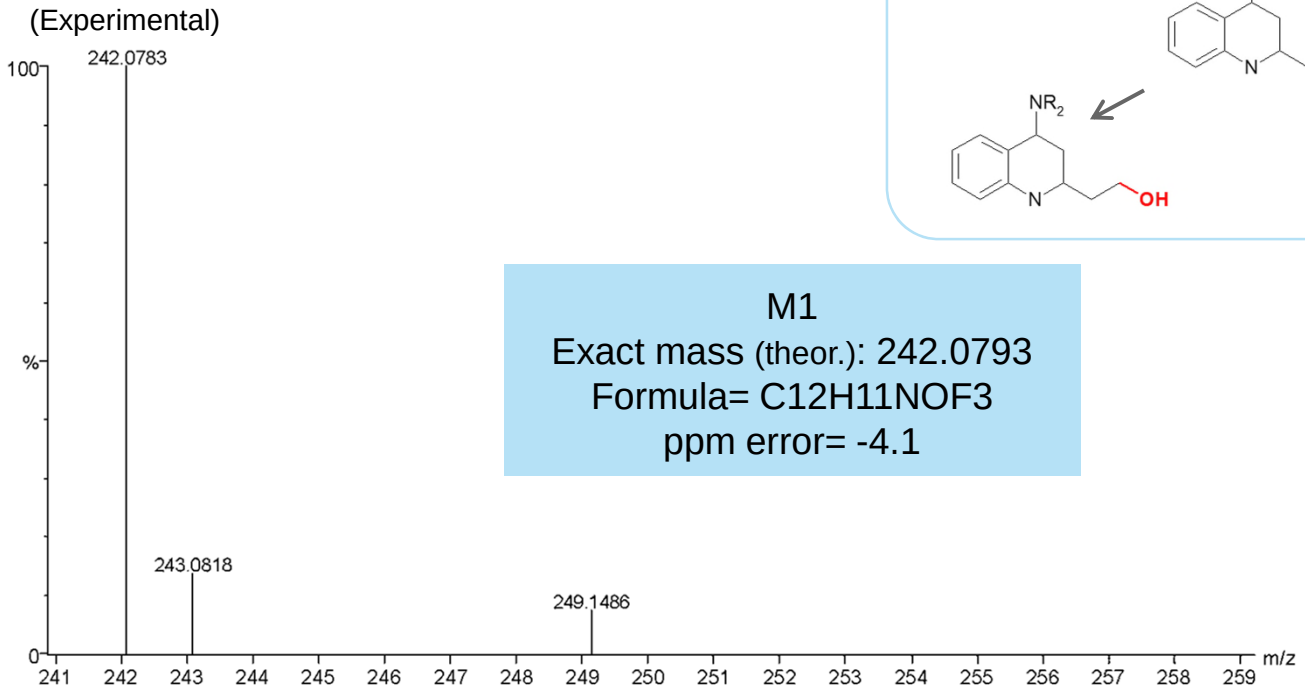
$$\text{FWHM} = \frac{485.1270}{0.0532} \sim 9000$$

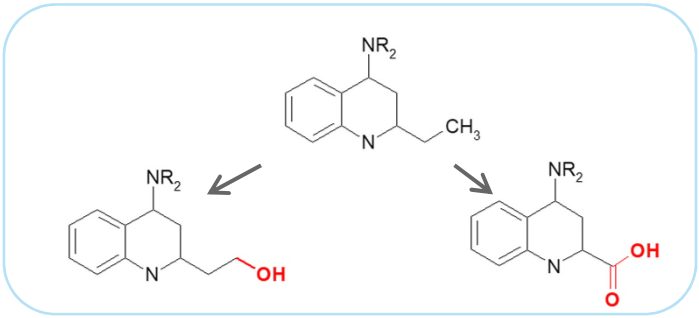
Accurate Mass Measurement

$$\text{ppm} = \frac{m/z\_Observed - m/z\_Theorethical}{m/z\_Theorethical}(10)^6$$

Accurate mass measurement

Exact Mass can confirm and distinguish
elemental composition for metabolites with the same nominal mass





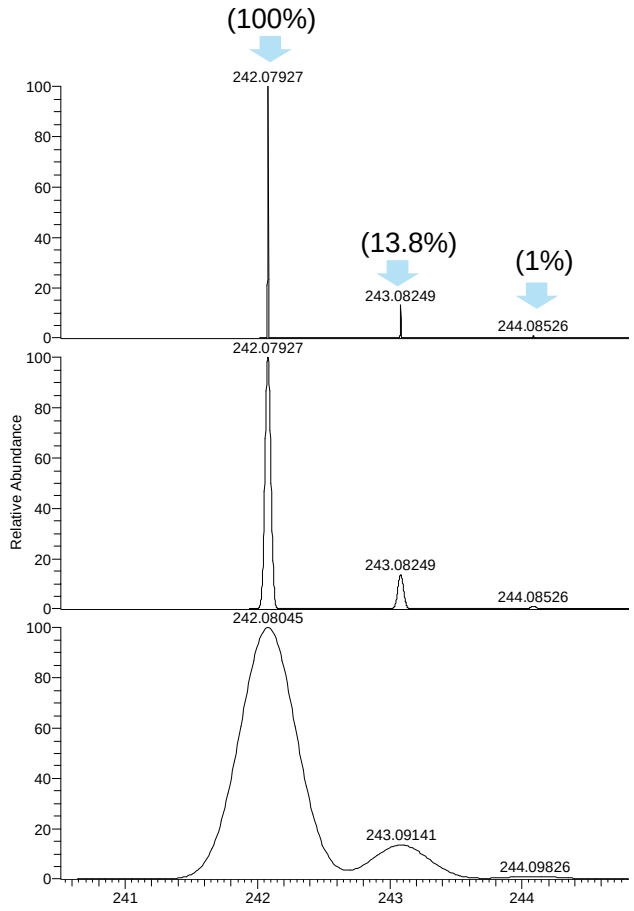
M1
Exact mass (theor.): 242.0793
Formula= C12H11NOF3
ppm error= -4.1

M2
Exact mass (theor.): 242.0429
Formula= C11H ✔ 7NO2F3
ppm error= 146.2

Obtained using QToF II

Resolution and isotopic distribution

Metabolite characterisation using mass spectrometry



FWHM= 50000

FWHM= 5000

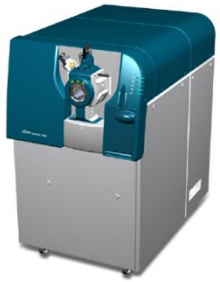
FWHM= 500

Even highest mass accuracy and resolution, however, is not sufficient to determine the unique chemical formula of each ion

Isotope pattern evaluation can be useful to reduce the search space and determine the molecular formula

HR mass spectrometer

ThermoFisher SCIENTIFIC



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SHIMADZU

Agilent Technologies

HRMS	Q-Orbitrap	QTOF	QTOF	Q-TOF and IT-TOF	Q-TOF
Software	**Compound Discoverer™**	**MetabolitePilot™**	**UNIFI™**	**MetID solution**	**Mass MetaSite**

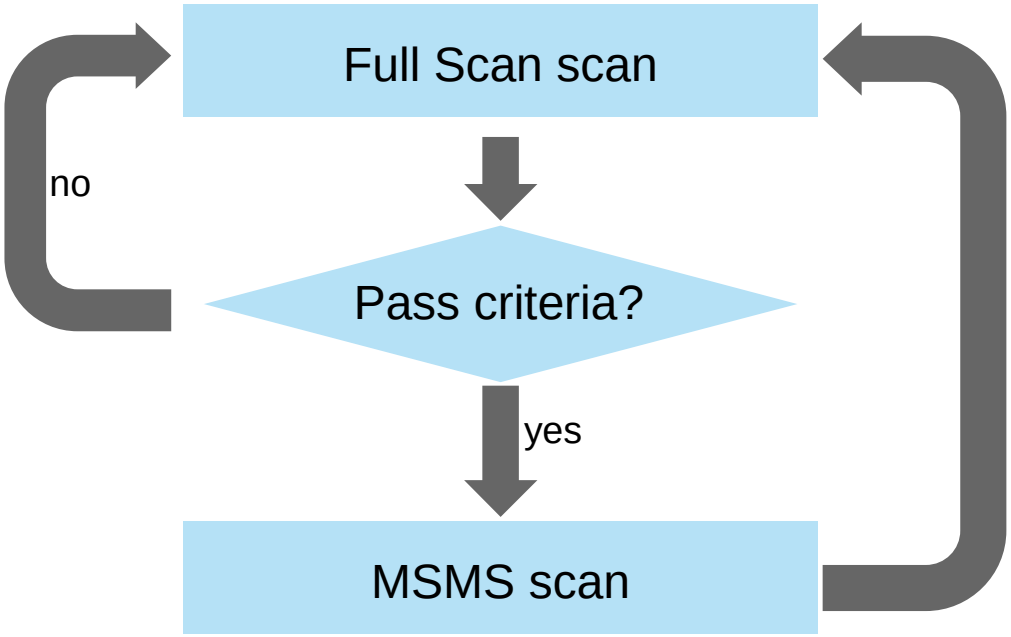
Data Elaboration is the bottleneck of Metabolite Identification

IT : Ion Trap, TOF : Time of Flight, Q: Quadrupole

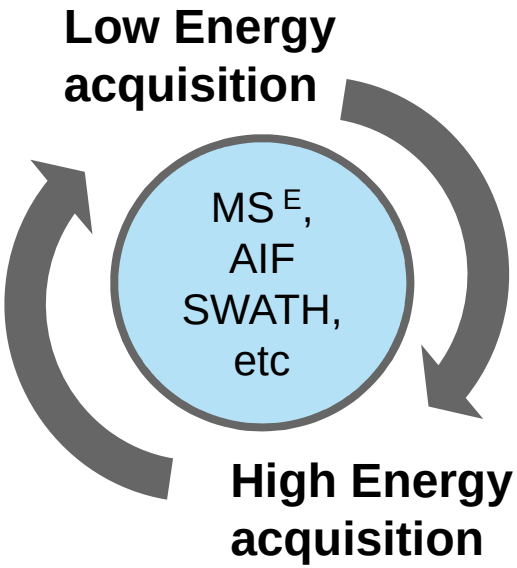
Metabolite characterisation: Mass spectrometry

Acquisition process

Data Dependent Analyses



Data Independent Analyses



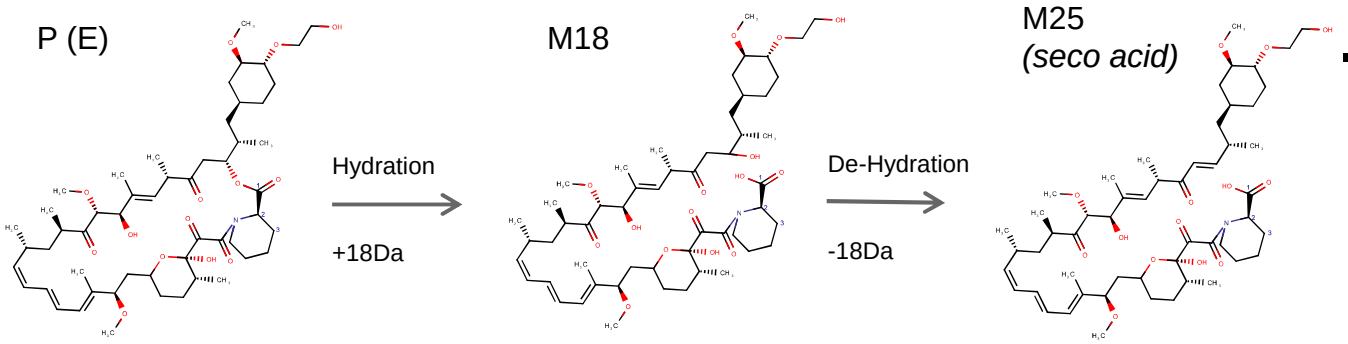
Correlation of Product and Precursor Ion based on retention time and peak shape

Data Processing

Missed MSMS scan

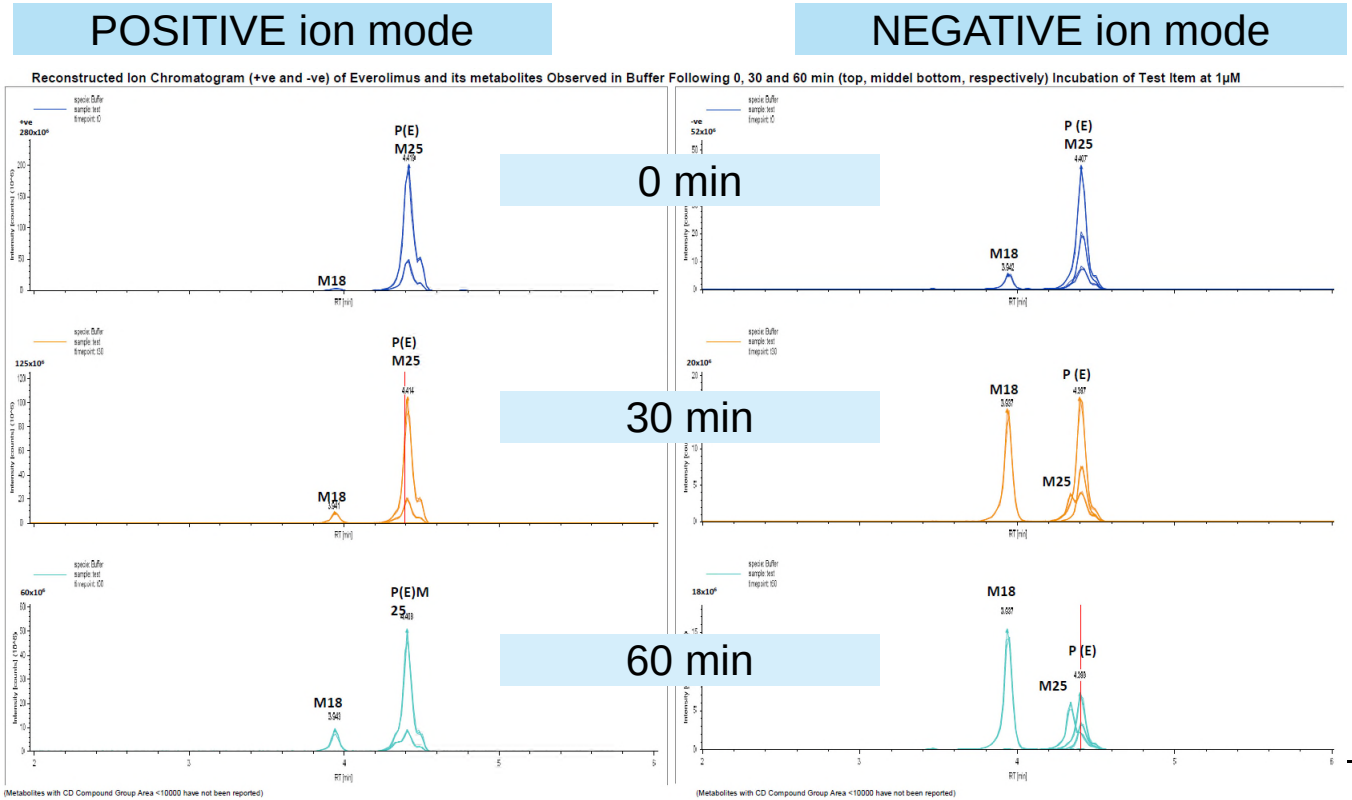
MSMS scan

Polarity switching in metabolite profiling



It can be advantageous to screen for metabolites using both positive and negative ionization modes.

This is especially true for phase II metabolism which tends to make molecules more polar and often more acidic



Functionality provided by Met ID profiling tools

- Identification of drug and related metabolites as chromatographic peaks from the complex TIC trace
- Assignment of chemical structures for each identified metabolite

Comparison with Control	Biotransformation List	Isotope filtering
Mass Defect Filtering	MSMS acquisition method creation	Formula Prediction
MS/MS Data Interpretation	Linkage to specific Database	Statistical Analyses

Biotransformation list

- Parent
- Set Biotransformation
- Generate List
  Expected Metabolite
- Output
  Expected Metabolites
- Comparison with Control
- List of putative metabolites

Table 1 Effects of common phase I metabolic reactions on the mass-spectrometric behavior and retention in comparison with the parent drug

Nominal mass shift (ΔDa)	Metabolic reaction (elemental composition change)	Exact mass shift (mDa)	Examples of relative retention shift[a]	References
–	Azo reduction to amines ($R_1N=NR_2$ to $R_1NH_2 + R_2NH_2$)	–	–	[145]
−14x[b]	Hydrolysis of esters to carboxylic acid (-C_xH_{2x})	−15.7x	–	[106]
−90	Reductive debenzylation (-C_7H_6)	−47.0	0.74 [134]	[134]
−78	Reductive debromination (-Br+H)	+89.5	–	[113]
−74	Oxidative debenzylation (-C_7H_6+O)	−52.0	–	[180]
−68	Loss of CF_3 (-CF_3+H)	+12.6	–	[107]
−62	Oxidative debromination (-Br+OH)	+84.4	0.39 [17]	[17]
−56	Debutylation (-C_4H_8)	−62.6	–	[107]
−45	Hydrolysis of nitrate to alcohol (-NO_2+H)	+14.9	–	[113]
	Reductive loss of nitro group (-NO_2+H)	+14.9	1.37 [46]	[46, 106]
−44	Decarboxylation (-CO_2)	+10.2	–	[106]
−42	Depropylation (-C_3H_6)	−47.0	0.73—isopropyl [131]; 0.85, 1.09—propyl [24]	[24, 131]
−36	Loss of HCl (-HCl)	+23.3	–	[148]
−34	Reductive dechlorination (-Cl+H)	+39.0	–	[148]
−30	Nitro reduction to amine (-O_2+H_2)	+25.8	0.75 [144]	[144]
−28	Deethylation (-C_2H_4)	−31.3	0.87 [76]	[76]
−25	Reductive loss of nitrile group (-CN+H)	+4.8	0.88 [45]	[45, 106]
−18	Alcohol dehydration (-H_2O)	+10.6	2.08[c] [17]	[17]
	Oxidative dechlorination (-Cl+OH)	+33.9	0.73 [146]	[146]
	Reductive defluorination (-F+H)	+9.4	–	[147]
−16	Desulfuration (-S+O)	+22.8	0.54 [68]	[68]
	Reduction of sulfoxide to thioether (-O)	+5.1	–	[113]
	Reduction of hydroxylamine to amine (-O)	+5.1	–	[113]
−14	O-demethylation (-CH_2)	−15.7	0.88, 0.93 [19]; 0.56, 0.64 [40]; 0.59 [129]; 0.87, 0.88, 0.90 [130]	[19, 25, 40, 46, 108, 129–131]
	N-demethylation (-CH_2)	−15.7	0.82 [52]; 0.84 [135]; 0.87 [51]; 0.88 [19]; 0.90 [40]; 0.92 [133]; 0.95 [130]; 0.99 [45]	[19, 40, 45, 47, 50–52, 130, 133, 135, 136]
	S-demethylation (-CH_2)	−15.7	–	[132]
−6	Aromatization of saturated ring (-H_6)	−47.0	–	[152]
−2	Alcohol oxidation to ketone/aldehyde (-H_2)	−15.7	1.01 [83]	[83, 106]
	Ring formation (-H_2)	−15.7	1.20 [112]	[112]
	Oxidative defluorination (-F+OH)	+4.3	–	[77, 147]
−1	Oxidative deamination to ketone/aldehyde (-NH_3+O)	−31.6	–	[142]
+1	Oxidative deamination to alcohol (-NH+O)	−16.0	–	[113]
	Hydrolysis of amide to carboxyl (-NH+O)	−16.0	–	[143]
+2	Ketone/aldehyde reduction to alcohol (+H_2)	+15.7	0.72 [56]; 0.70 [40]	[33, 40, 56, 126]
	Hydrogenation (+H_2)	+15.7	1.13 [124]	[17, 124]
	Ring opening (+H_2)	+15.7	0.59 [149]	[47, 149–151]
+14	Methyl/methylene oxidation to aldehyde/ketone (+O-H_2)	−20.7	0.92, 1.02 [102]; 1.05 [45]	[45, 102]
	Alcohol oxidation to carboxylic acid (+O-H_2)	−20.7	–	[106]
	Hydroxylation and cyclization (+O-H_2)	−20.7	–	[106]
+16	Hydroxylation (+O)	−5.1	0.38 [51]; 0.40 [131]; 0.46 [137]; 0.46, 0.50, 0.62 [124]; 0.51, 0.73, 0.82, 0.89, 0.96 [76]; 0.56 [40]; 0.58 [135]; 0.60, 0.62 [112]; 0.61 [45]; 0.69 [130]; 0.80 [52]; 0.82 [138]; 0.85 [102]	[23, 24, 40, 45, 47, 51, 52, 76, 83, 102, 108, 112, 124, 130, 131, 135–139]
	Epoxidation (+O)	−5.1	0.56 [124]; 0.72 [140]; 0.89 [77]	[77, 124, 140, 141]
	N-oxidation (+O)	−5.1	0.93 [40]; 1.04 [45]; 1.09 [76]	[40, 45, 51, 76, 131, 136]
	S-oxidation of thioether to sulfoxide or sulfoxide to sulfone (+O)	−5.1	0.34 (S-SO) [141]; 0.82 (S-SO) [138]; 1.02 (SO-SO_2) [143]	[138, 141, 143]
	Aldehyde oxidation to carboxyl (+O)	−5.1	–	[126]
	Oxidation and ring formation (+O)	−5.1	0.46, 0.51 [137]; 0.53, 0.64 [80]	[80, 137]
+18	Ring opening by water addition (+H_2O)	+10.6	1.10 [18]	[18, 19]
	Hydrolysis of nitrile to amide (+H_2O)	+10.6	–	[45]
+30	Methyl oxidation to carboxylic acid (+O_2-H_2)	−25.8	0.24 [167]; 0.34 [40]	[40, 50, 167]
+32	Dihydroxylation (+O_2)	−10.2	0.49 [54]; 0.39 [124]; 0.80, 0.81 [160]; 0.84 [83]; 0.63, 0.67, 0.71, 0.79, 0.94 [76]	[54, 76, 83, 124, 160]
	S-oxidation of thioether to sulfone (+O_2)	−10.2	0.79 [138]	[138]
+34	Epoxidation and hydration (+H_2O_2)	+5.5	0.39 [124]; 0.47 [140]; 0.51, 0.68 [141]	[124, 140, 141]
+48	Trihydroxylation (+O_3)	−15.3	–	[113]
	S-oxidation of thiol to sulfonic acid (+O_3)	−15.3	–	[113]

[a] The parent drug has a relative retention shift (RRS) of 1.00.
[b] The value of x corresponds to the length of the alkyl chain.
[c] RRS is related to another metabolite.

Anal Bioanal Chem (2008) 391:59–78

Biotransformation list

M1
Cleavage to Epicatechin

M2-M6
Conjugation of Epicatechin

M7-M9
C-Ring Cleavage of Epicatechin

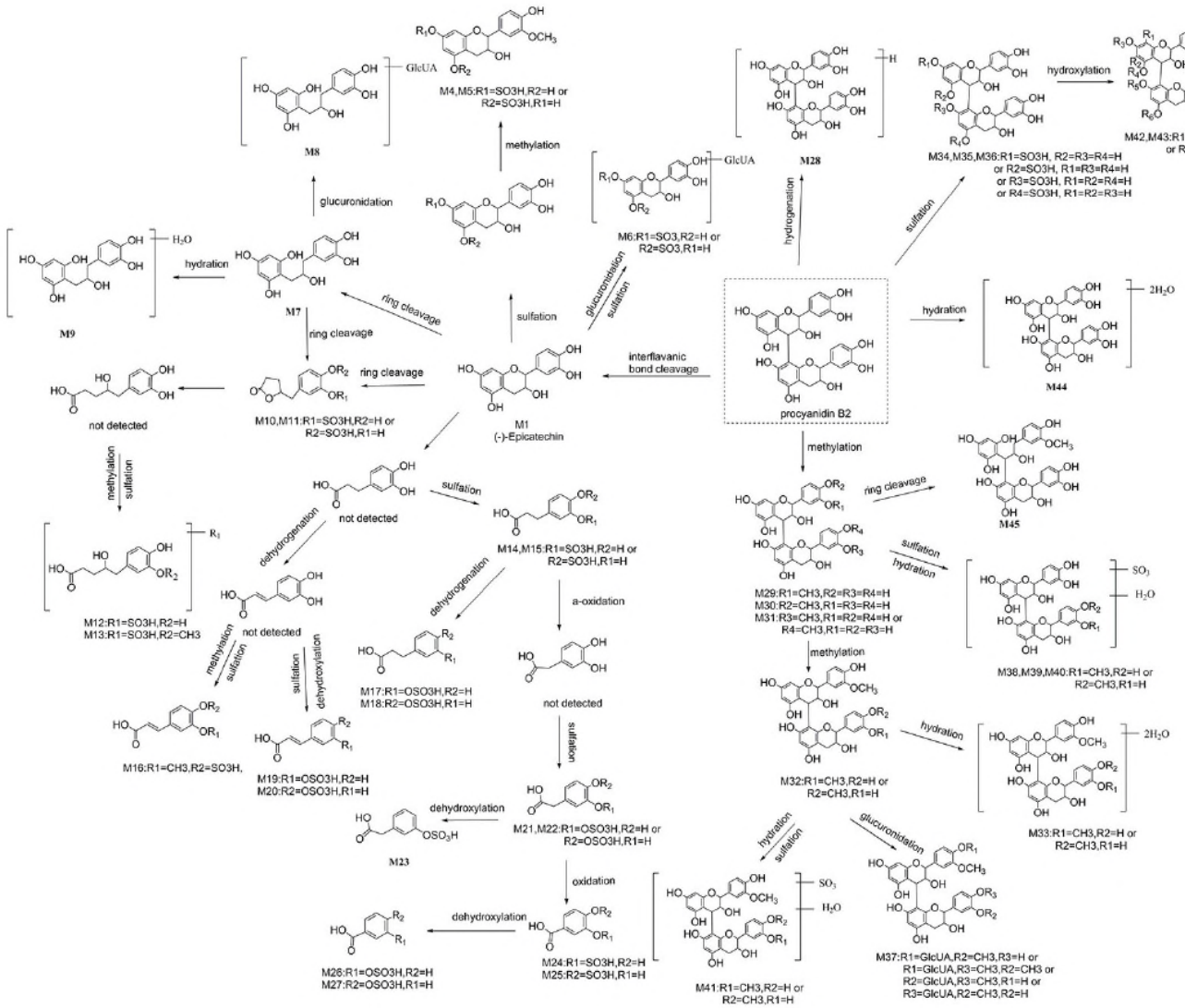
M10-M11
Phenylvalerolactone Metabolites

M12-M13
Phenylvalenic Acid Metabolites

M14-M20
Phenylpropionic Acid Metabolites

M21-M23
Phenylacetic Acids Metabolites

M24-M27
Benzoic Acid Metabolites



M28
Hydrogenation

M29–M33
Methylation

M34–M36
Sulfation

M37
Methylation and Glucuronidation

M38–M41
Methylation and Sulfation

M42–M43
Hydroxylation and Sulfation

M44
Hydration

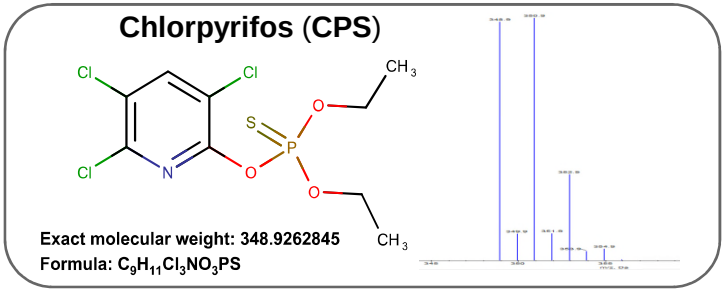
M45
C-Ring Cleavage in Upper Epicatechin Unit of Procyanidin B2

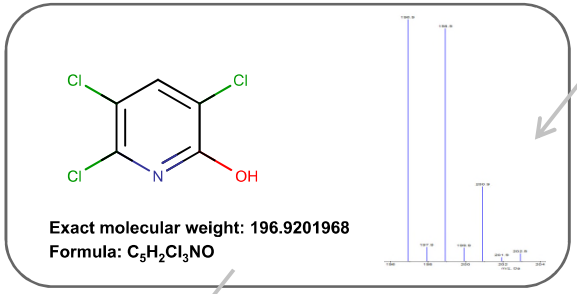
FIGURE 5 | The proposed metabolic pathway of 45 identified metabolites of procyanidin B2 in mice.

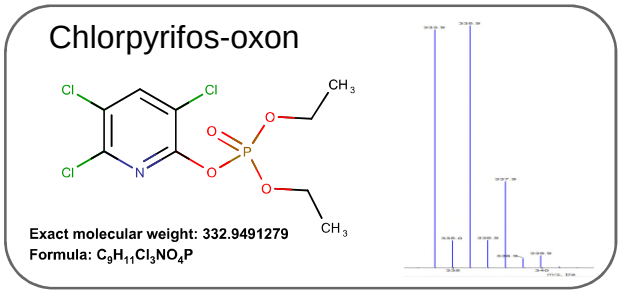
Xiao Y, Hu Z, Yin Z, Zhou Y, Liu T, Zhou X and Chang D (2017) Profiling and Distribution of Metabolites of Procyanidin B2 in Mice by UPLC-DAD-ESI-IT-TOF-MSn Technique. Front. Pharmacol. 8:231. doi: 10.3389/fphar.2017.00231

Isotopic filtering

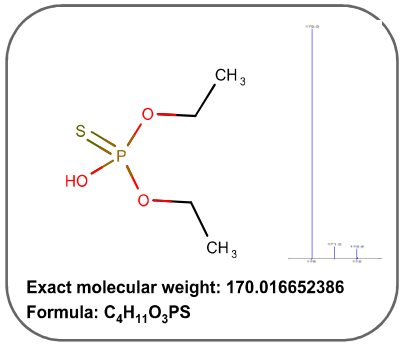
Discriminate metabolites based on characteristic isotopic pathway

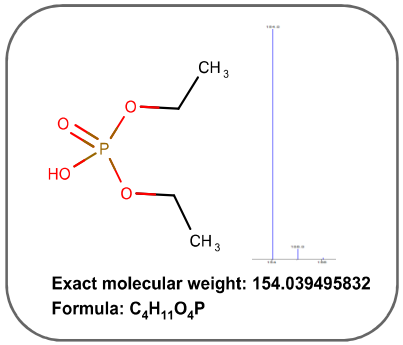






Conjugated metabolites





- Used as post-acquisition data processing tool
- Less recommended triggering DDA experiments

Mass defect filter

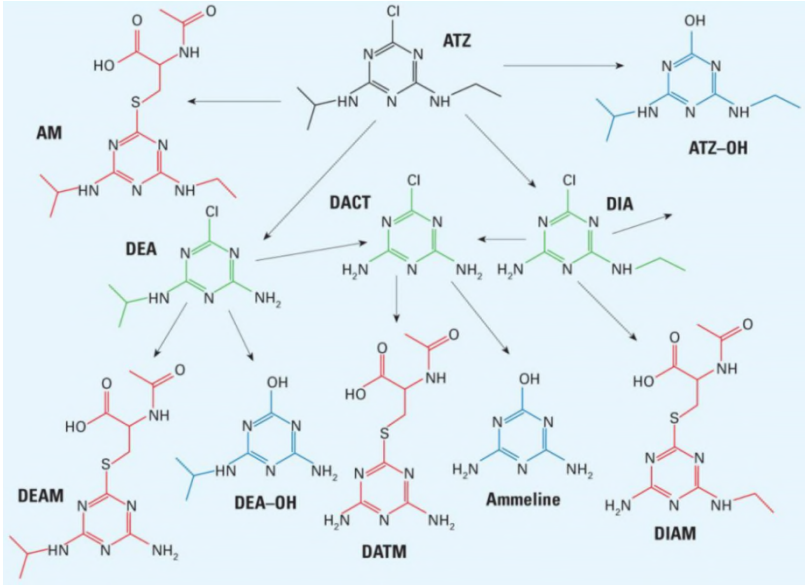
Proposed metabolites of Atrazine (ATZ)

DEALKYLATED METABOLITES
DACT, diaminochlorotriazine
DEA, desethylatrazine
DIA, desisopropyl atrazine

HYDROXYLATED METABOLITES
ATZ-OH, hydroxyatrazine
DEA-OH, hydroxydesethylatrazine
Ammieline

GLUTATHIONE-DERIVED MERCAPTURIC ACID
AM, atrazine mercapturate
DEAM, desethylatrazine mercapturate
DIAM, desisopropylatrazine mercapturate
DATM, diaminotriazine mercapturate

ID	Description of the Biotransformation	Nominal Mass shift (Da)
AM	Dechlorination Mercapturic acid conj.	127
DEAM	Dechlorination Mercapturic acid conj. N-dealkylation	99
DIAM	Dechlorination Mercapturic acid conj. N-dealkylation	85
DATM	Dechlorination Mercapturic acid conj. N-dealkylation	57
DEA	N-dealkylation	-28
DIA	N-dealkylation	-42
DACT	N-dealkylation	-70
ATZ-OH	Oxidative dechlorination	-18
DEA-OH	N-dealkylation & Oxidative dechlorination	-46
Ammieline	N-dealkylation & Oxidative dechlorination	-88



Mass defect filter

Mass Defect Filter (MDF)

Difference between the exact mass of an element (or a compound) and its closest integer value

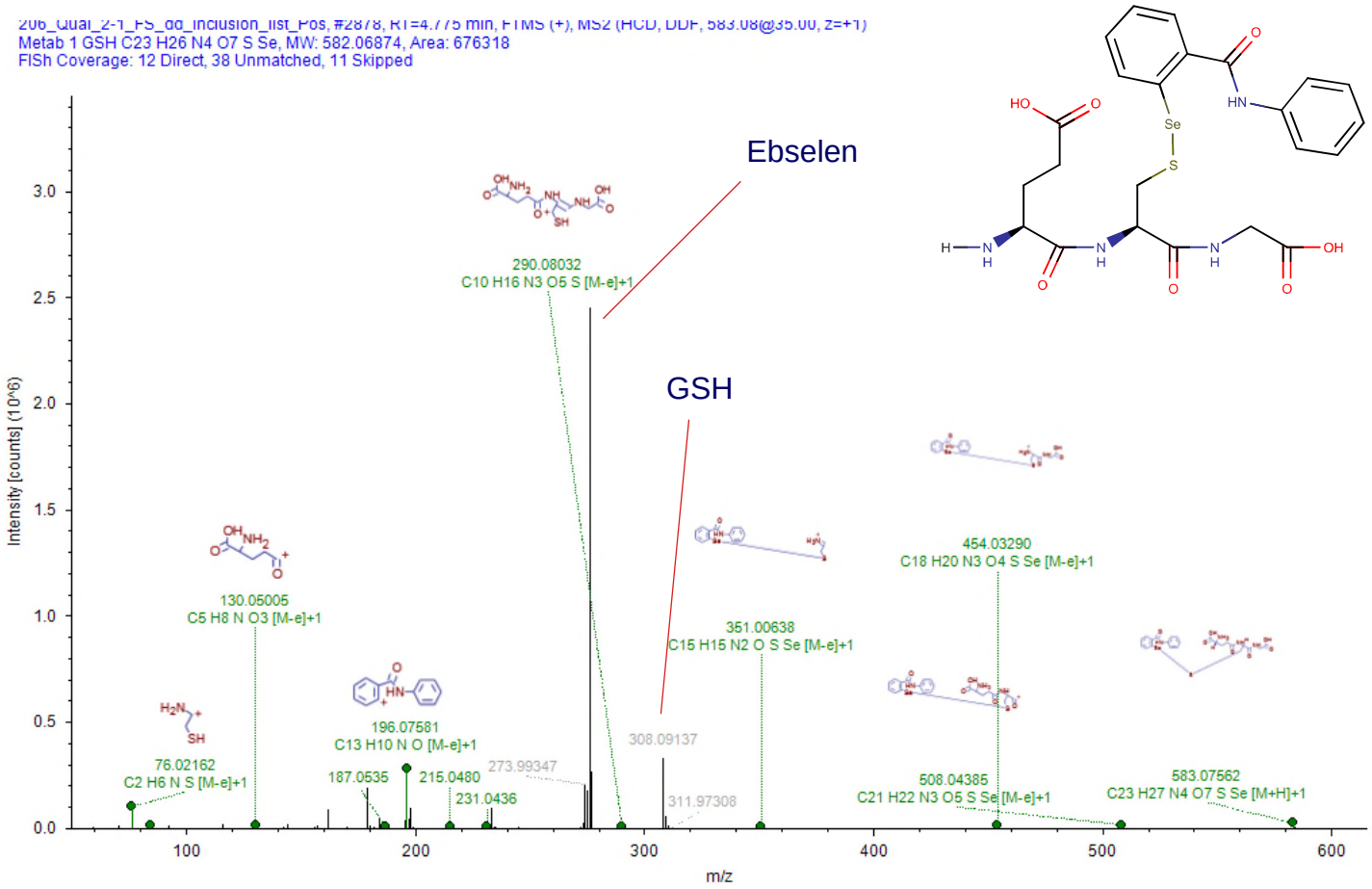
- Used as post-acquisition data processing tool
- Some software consider MDF filtering accordingly to predicted cleavage products

ID	Description of the Biotransformation	Formula Change	Nominal Mass shift (Da)	Accurate Mass Shift (Da)	Mass Defect (mDa)
AM	Dechlorination Mercapturic acid conj.	-Cl, +C5H8NO3S	**127**	127.0536	**53.6**
DEAM	Dechlorination Mercapturic acid conj. N-dealkylation	-Cl, +C3H4NO3S	**99**	99.0223	**22.3**
DIAM	Dechlorination Mercapturic acid conj. N-dealkylation	-Cl, +C2H2NO3S	**85**	85.0067	**6.7**
DATM	Dechlorination Mercapturic acid conj. N-dealkylation	-ClH2, +NO3S	**57**	56.9754	**-24.6**
ATZ-OH	Oxidative dechlorination	-Cl, +HO	**-18**	-17.9661	**33.9**
DEA	N-dealkylation	-C2H4	**-28**	-28.0313	**-31.3**
DIA	N-dealkylation	-C3H6	**-42**	-42.0470	**-47.0**
DEA-OH	N-dealkylation & Oxidative dechlorination	-ClC2H3, +O	**-46**	-45.9974	**2.6**
DACT	N-dealkylation	-C5H10	**-70**	-70.0783	**-78.3**
Ammieline	N-dealkylation & Oxidative dechlorination	-ClC5H9, +O	**-88**	-88.0444	**-44.4**

MS/MS data interpretation

While assigning the structures, some software predict the theoretical fragments for the parent drug and metabolites, and assign/compares them with experimentally obtained MS/MS results

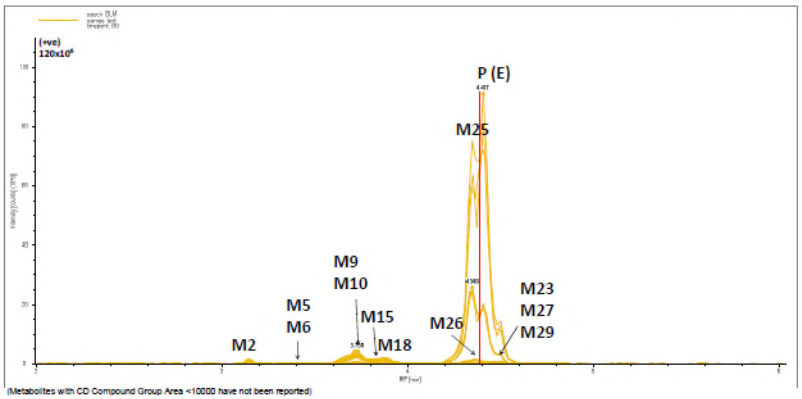
In absence of standard structural assignments are always tentative

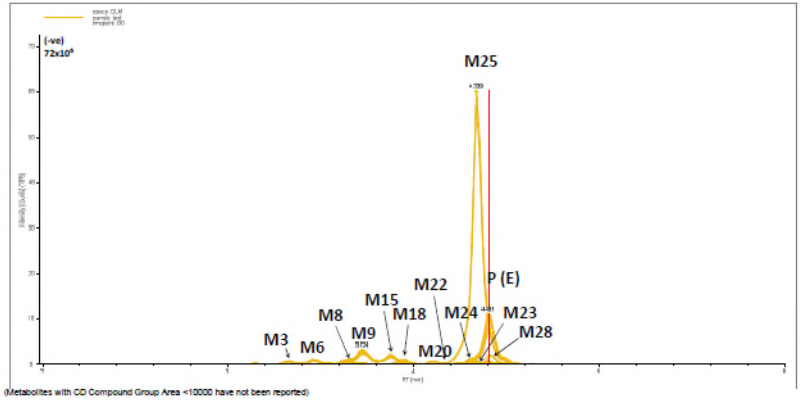


Cross species comparison

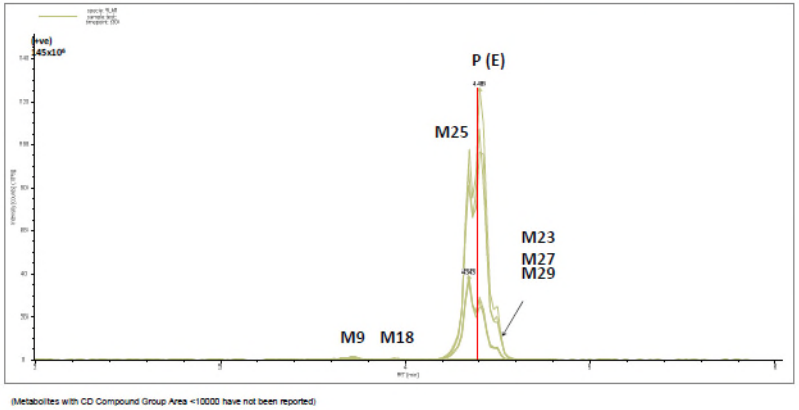
- Identify human metabolites that differ from those observed in animal models, assisting pre-clinical safety and toxicity studies
- inter-species metabolic stability assessment should be considered in study design

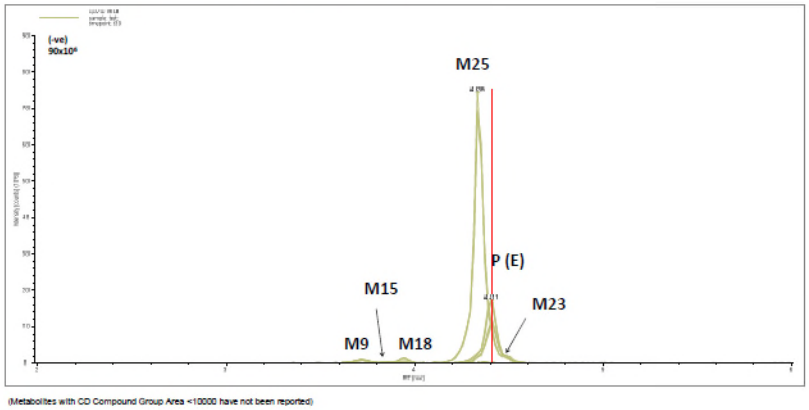
Reconstructed Ion Chromatogram (+ve and -ve) of Everolimus and its metabolites Observed in Dog live Microsome Following 30 min Incubation of Test Item at 1µM





Reconstructed Ion Chromatogram (+ve and -ve) of Everolimus and its metabolites Observed in Rat live Microsome Following 30 min Incubation of Test Item at 1µM

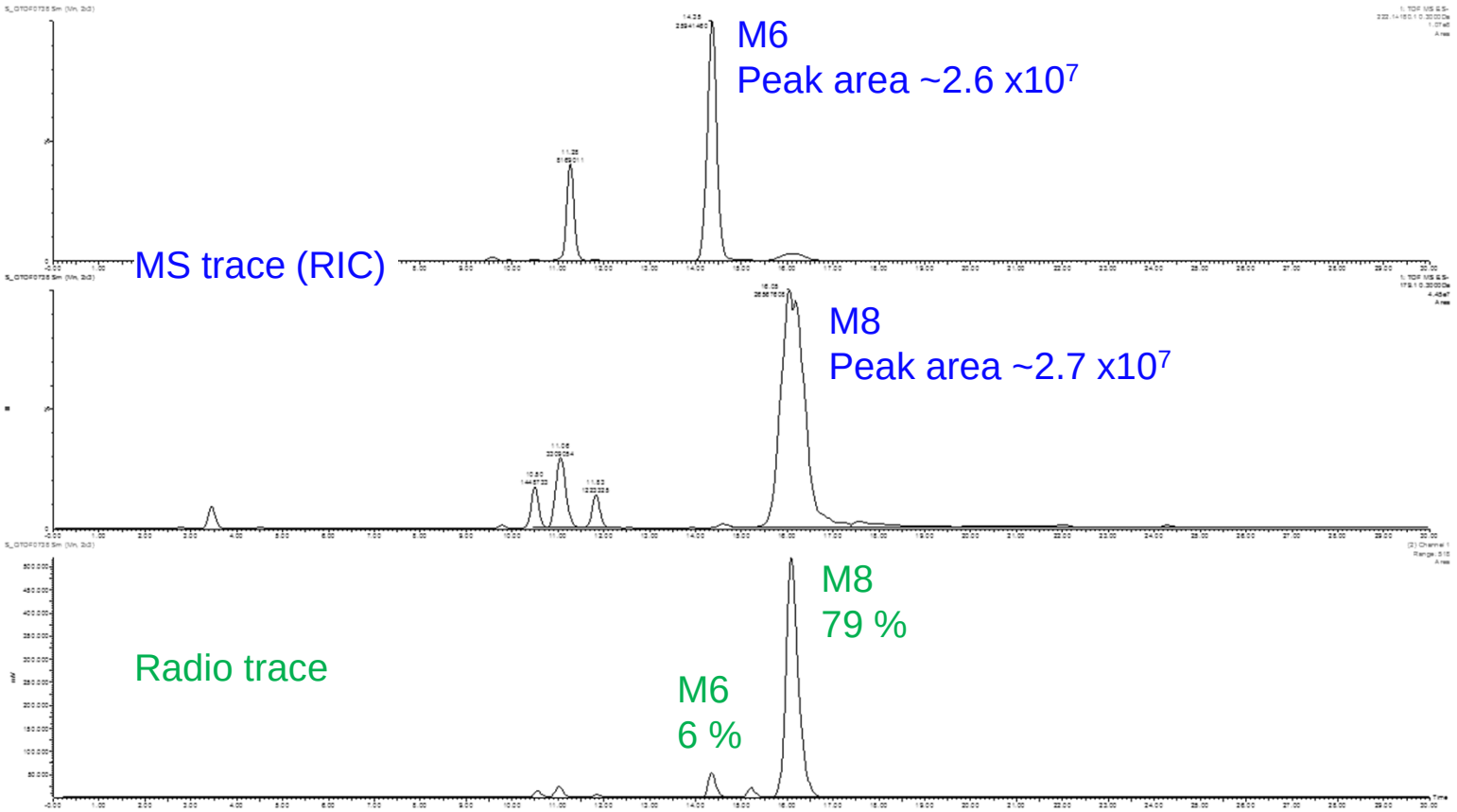




Cross species comparison

Peak Area is not necessarily proportional to analyte abundance, but it's associated to ionization efficiency of each analyte

In some cases the addition of complementary UV detection is sufficient for metabolite quantification



In Ratio Trace % is referred to total sample radioactivity

Conclusions

Take-home messages

- Study outcomes should meet stage-based needs
- LC-HRMS is the technique of choice
- Data processing is still the most demanding step
- Use customised processing workflow
- In absence of standards or complementary detection techniques mass spectrometry is not quantitative

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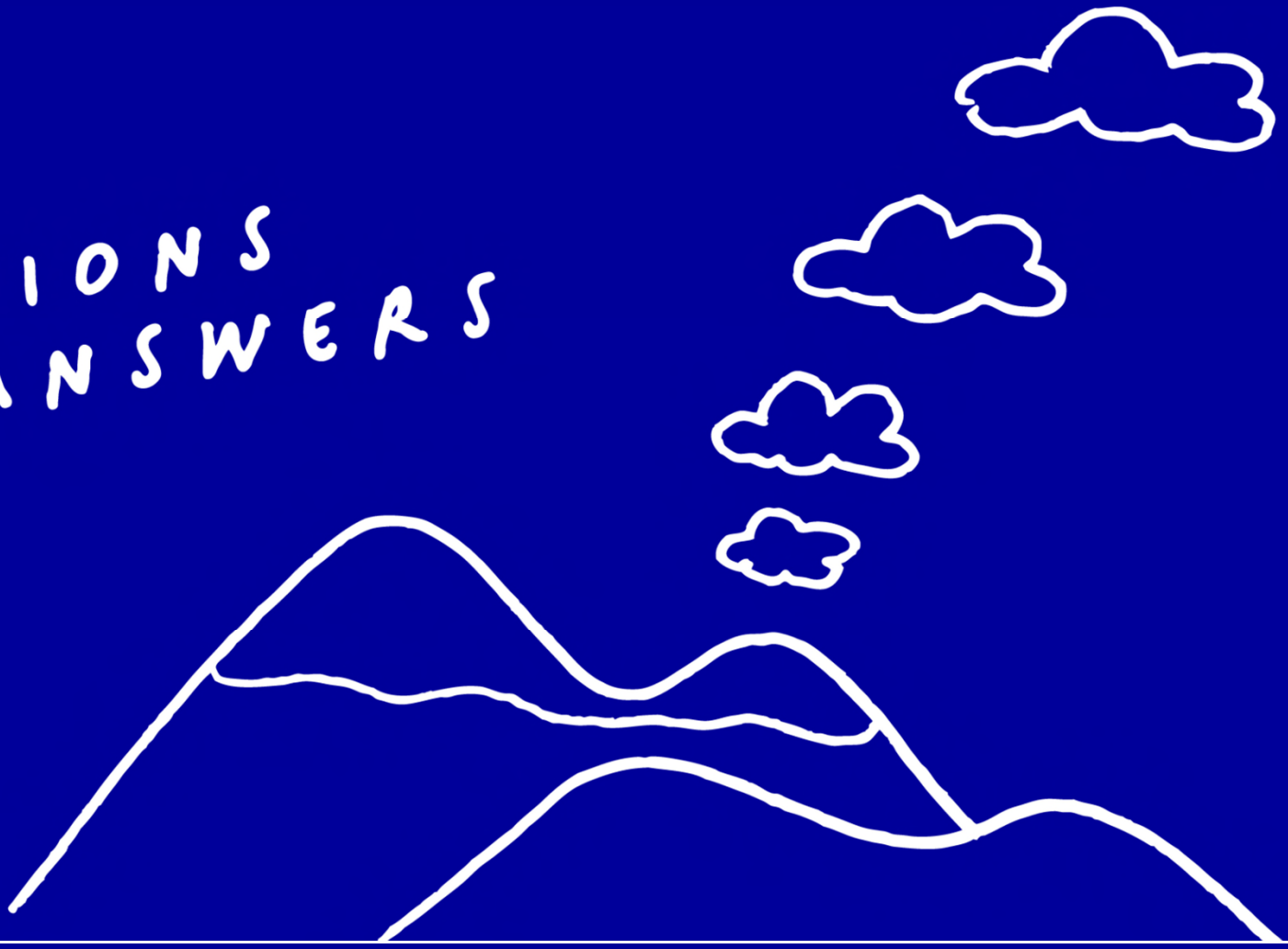
QUESTIONS AND ANSWERS



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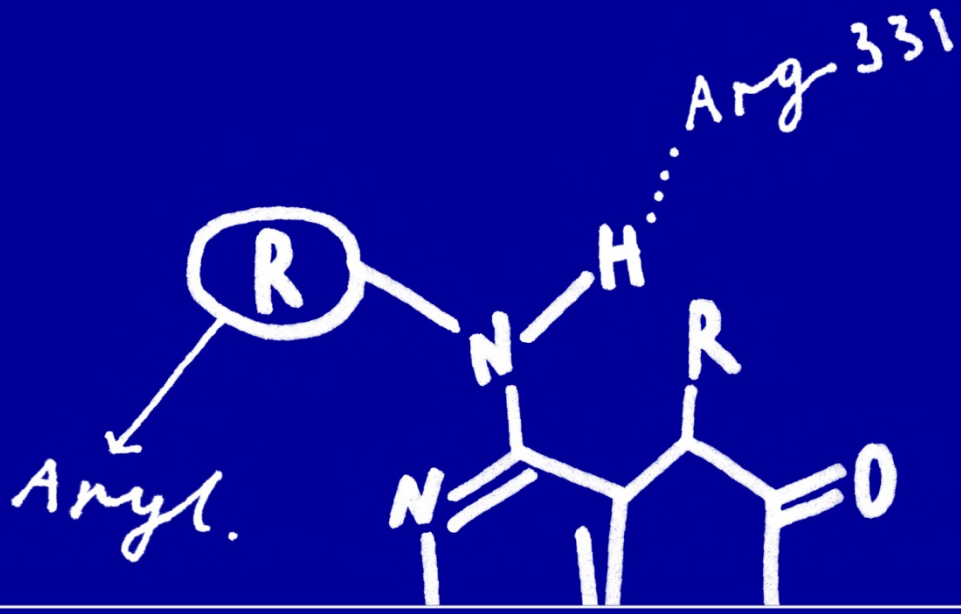

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