



**CEST 2015**

# A workflow for the orthogonal identification of biotransformation products by HILIC-QTOFMS

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- ✓ Determine the degradation rate of emerging contaminants (pharmaceuticals, illicit drugs) in the aquatic environment under aerobic conditions
- ✓ Study the occurrence and formation of transformation products
- ✓ Develop an integrated workflow for suspect and non-target screening for the identification of biotransformation products
- ✓ Study the use of HILIC as a complementary technique for the identified TPs
- ✓ Further investigation for suspect and unknown TPs which may be eluted better in HILIC conditions



Samples of activated sludge from active bioreactors and effluent waste water from the WWTP of Athens (Psitalia)



Batch reactors seeded with activated sludge under aerobic conditions in room temperature



Spike in a concentration of 2 mg/L



Sampling immediately after spiking and after several time intervals (depending on the compound)



Filtration through glass fiber syringe and then through 0.2  $\mu\text{m}$  RC filter



LC-HRMS (qTOF) both RP and HILIC



Prior to the spiking pH and TSS, were determined.

Check for sorption to the suspended sludge particles

Check for abiotic degradation processes (hydrolysis, volatilization)

- Check for contamination between sampling
- Subtraction of the background

Aerobic biodegradation

All reactors were covered with perforated caps and placed on a magnetic stirrer to aerate the sludge.







UltiMate 3000™

RSLC (Dionex) UHPLC System

Column: Thermo Acclaim RSLC C18, 2.2 $\mu$ m 120 Å, 2.1x100 mm  
Mobile Phase & gradient program: Bruker Pesticide screener

Inj. Volume: 5  $\mu$ L

RP

Column: Waters BEH Amide Acquity 1.7  $\mu$ m, 2.1x100 mm

Inj. Volume: 5  $\mu$ L

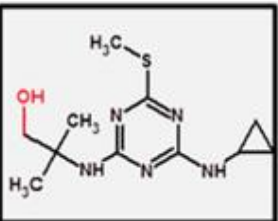
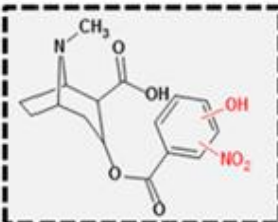
HILIC

Bruker Maxis Impact™  
Quadrupole-Time-of-Flight

Scan: 50-1000 m/z  
Spectra rate: 2Hz  
MS mode: bbCID &  
Auto MS with  
inclusion list





Example	Identification confidence	Minimum data requirements
	<b>Level 1: Confirmed structure</b> by reference standard	MS, MS <sup>2</sup> , RT, Reference Std.
	<b>Level 2: Probable structure</b> a) by library spectrum match b) by diagnostic evidence	MS, MS <sup>2</sup> , Library MS <sup>2</sup> MS, MS <sup>2</sup> , Exp. data
$C_6H_5N_3O_4$	<b>Level 3: Tentative candidate(s)</b> structure, substituent, class	MS, MS <sup>2</sup> , Exp. data
192.0757	<b>Level 4: Unequivocal molecular formula</b>	MS isotope/adduct
	<b>Level 5: Exact mass of interest</b>	MS



**Compilation** of suspect list: Metabolite prediction tools+ literature

**Screening** all the time interval chromatograms both in RP and HILIC (+ESI/-ESI)  $\Rightarrow$  XIC

**Evaluation of candidates** (Tentative): meet the set criteria, absence in the blank, chromatographic retention time plausibility (time trend)

**Acquire MS/MS** spectra with inclusion list both in RP and HILIC: Interpretation of the fragmentation pathway

**Confirmation**, if it's possible with reference standard (RT and MS/MS spectra)

 MassBank

 Metrag

 MetFusion





# Suspect screening-Workflow



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Screening all the time interval chromatograms both in RP and HILIC (+ESI/-ESI)



Bruker Compass  
Target Analysis 1.3

Compile the suspect list  
Save as .csv file

## Identification Criteria

- Mass Accuracy: Error  $\leq$  5 ppm
- Distinctive Isotopic fit:  $\leq$  100 mSigma
- Intensity  $>$  1000 (+ESI) / 200 (-ESI)

- Run Bruker Compass Target Analysis 1.3
- Set appropriate thresholds (m/z tolerance and mSigma)
- Consider mass accuracy and isotopic pattern to identify plausible TPs

A1	m/z [detected ion POS]	RT POS	sum formula	name	CAS	comment	comment	relativeRe	minimum	indivSigm	indivMass
1	m/z [detected ion POS]	RT POS	sum formula	name	CAS	comment	comment	relativeRe	minimum	indivSigm	indivMass
2			C17H27NO2	Venlafaxine							
3			C16H25NO2	N-DESMETHYLVENLAFAXINE							
4			C15H20O3	2-(1-hydroxycyclohexyl)-2-(4-methoxyphenyl)acetaldehyde							
5			C16H25NO2	O-Desmethylvenlafaxine							
6			C17H27NO3	1-[2-(dimethylamino)-1-hydroxy-1-(4-methoxyphenyl)ethyl]cyclohexan-1-ol							
7			C15H23NO2	N,N-Didesmethylvenlafaxine							
8			C15H23NO2	N,O-Didesmethyl Venlafaxine							
9			C15H19O4	2-(1-hydroxycyclohexyl)-2-(4-methoxyphenyl)acetate							
10			C16H25NO3	4-[2-(dimethylamino)-1-(1-hydroxycyclohexyl)ethyl]benzene-1,2-diol							
11			C16H25NO3	1-[1-hydroxy-1-(4-methoxyphenyl)-2-(methylamino)ethyl]cyclohexan-1-ol							
12			C14H18O3	2-(1-hydroxycyclohexyl)-2-(4-hydroxyphenyl)acetaldehyde							
13			C16H25NO3	4-[2-(dimethylamino)-1-hydroxy-1-(1-hydroxycyclohexyl)ethyl]phenol							

Clear Results

Screen

General Unknown

Save

Print

DataAnalysis

Print with Excel

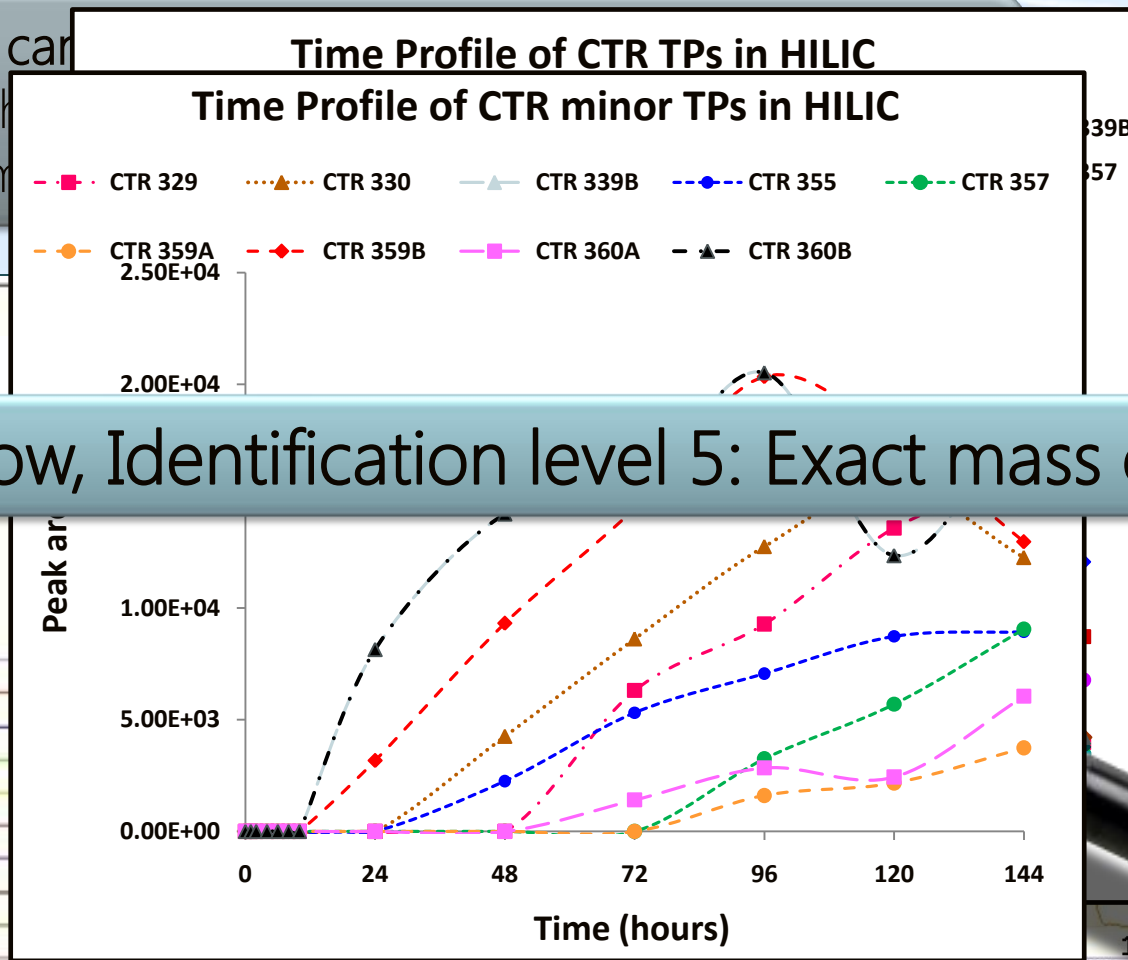
Comment:

Multi Target Screening with 'C:\Users\Katerina\Desktop\Databases\Venlafaxine\_TPs.csv'

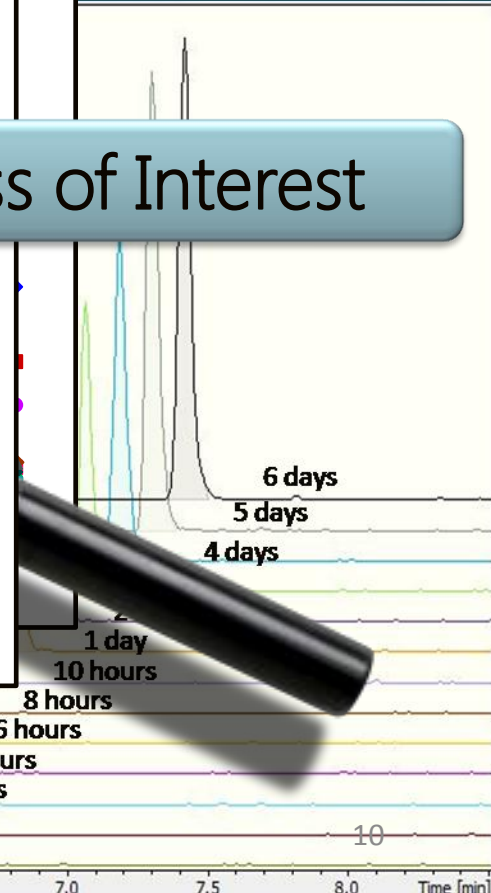
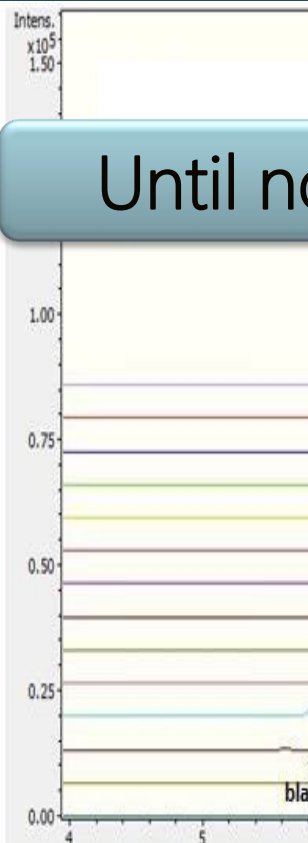
#	Id	Cmpd.Name	Formula	PMI	m/z calc.	m/z meas.	Err [ppm]	Err [mDa]	mSigma	RT exp. [min]	RT meas. [min]	deltaRT [min]
8	+	N-DESMETHYLVE...	C 16 H 25 N 1 O 2	[...]	264.1958	264.1943	5.6	1.5	6.3	0.00	7.48	-7.48
9	+	O-Desmethylven...	C 16 H 25 N 1 O 2	[...]	264.1958	264.1943	5.6	1.5	6.3	0.00	7.48	-7.48
11	+	VEN 294	C 17 H 28 N 1 O 3	[...]	295.2142	295.2137	-1.6	-0.5	13.8	0.00	10.51	-10.51
5	+	N	C 15 H 23 N 1 O 2	[...]	250.1802	250.1788	5.5	1.4	13.8	0.00	6.46	-6.46
6	+	N	C 15 H 23 N 1 O 2	[...]	250.1802	250.1788	5.5	1.4	13.8	0.00	6.46	-6.46
46	+	1-[2-(dimethylami...	C 17 H 27 N 1 O 3	[...]	294.2064	294.2055	-2.8	-0.8	13.8	0.00	13.63	-13.63
47	+	1-[2-(dimethylami...	C 17 H 27 N 1 O 3	[...]	294.2064	294.2055	-2.8	-0.8	13.8	0.00	13.63	-13.63
1	+	N-DESMETHYLVE...	C 16 H 25 N 1 O 2	[...]	264.1958	264.1950	3.0	0.8	14.6	0.00	6.16	-6.16
2	+	O-Desmethylven...	C 16 H 25 N 1 O 2	[...]	264.1958	264.1950	3.0	0.8	14.6	0.00	6.16	-6.16



Evaluation of car  
absence in the  
plausibility (time

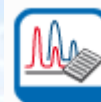


Until now, Identification level 5: Exact mass of Interest



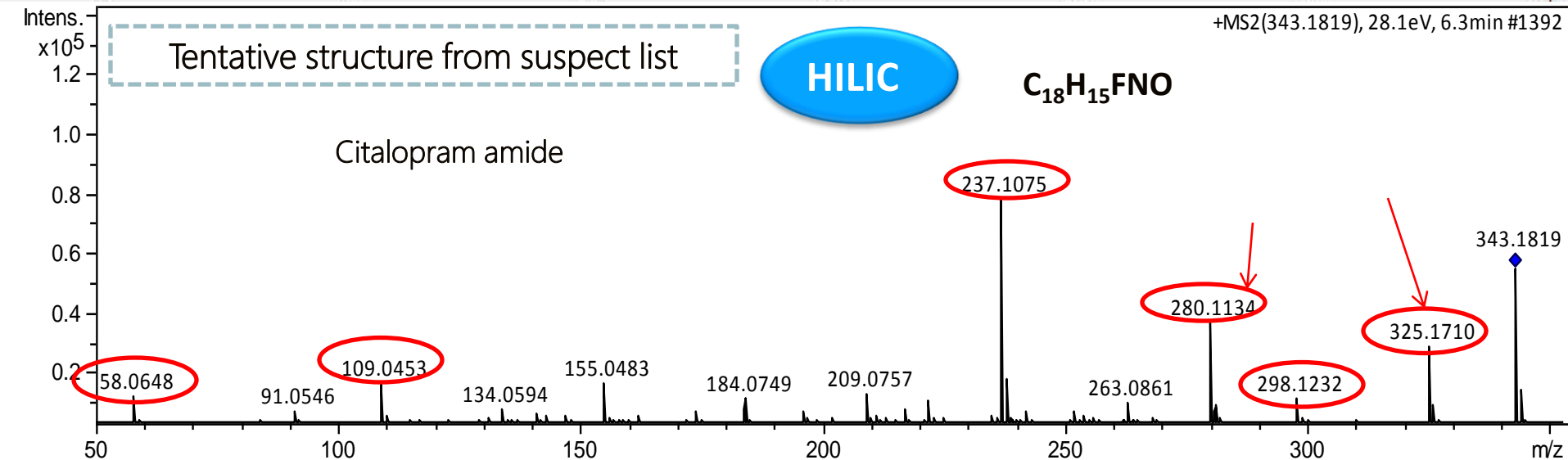
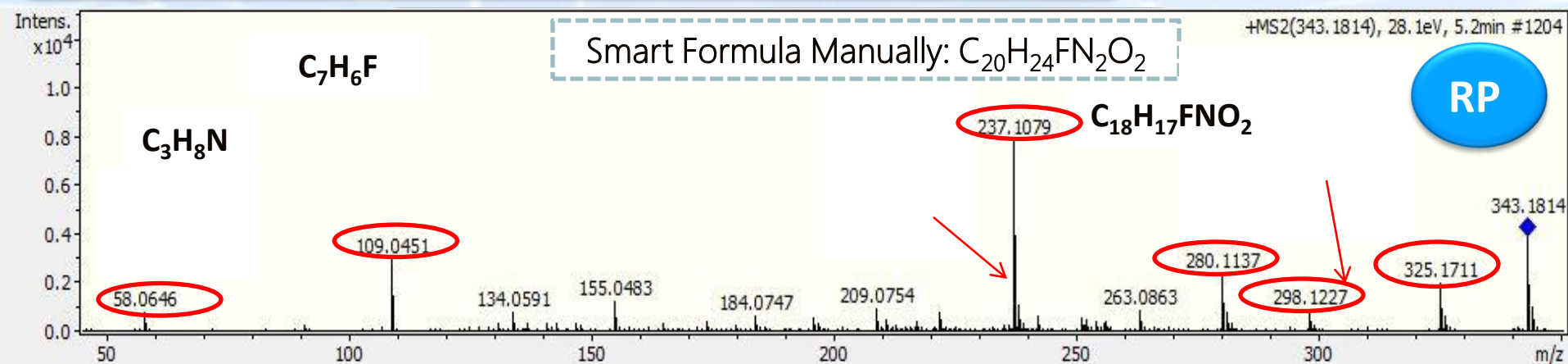


Acquire MS/MS spectra with inclusion list both in RP and HILIC: Interpretation of the fragmentation pathway



Data analysis

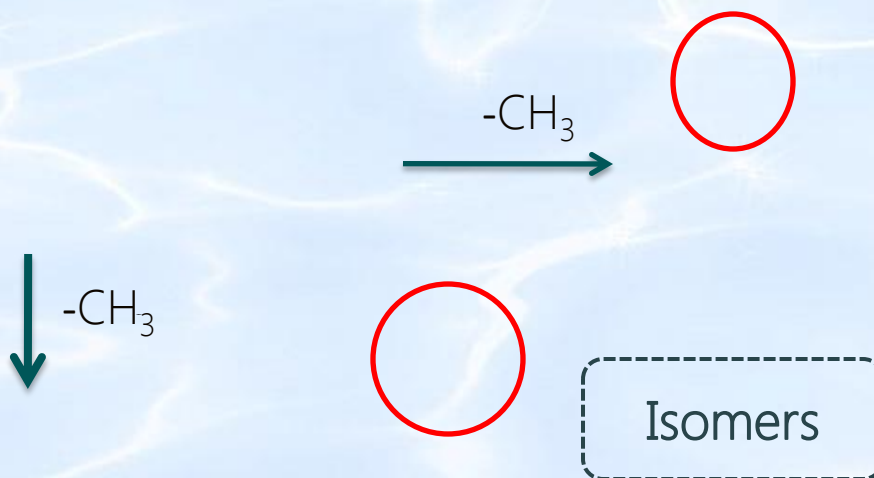
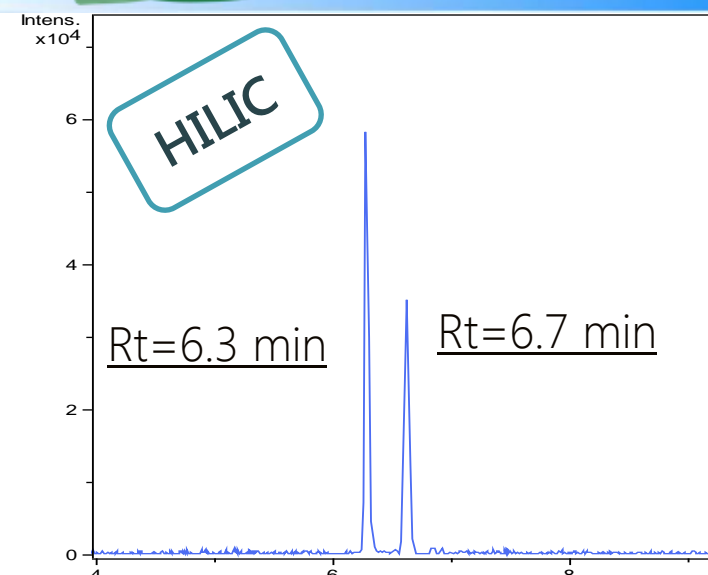
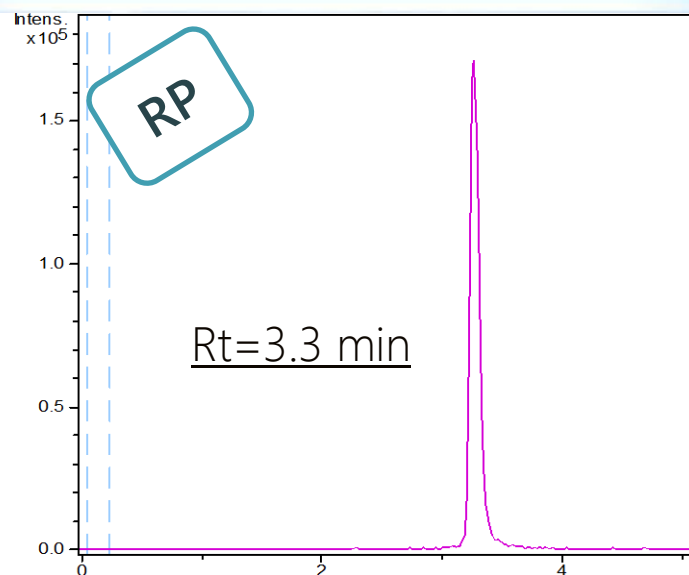
CTR 343





**RAN301  
(desmethyl  
ranitidine)**

Probable structure  
by diagnostic  
evidence





# Suspect screening-Workflow



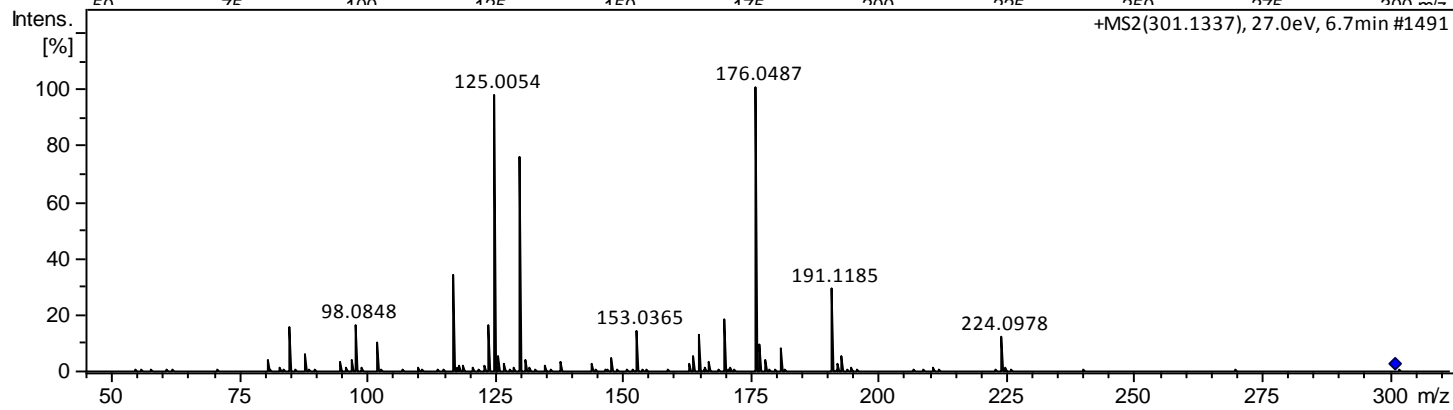
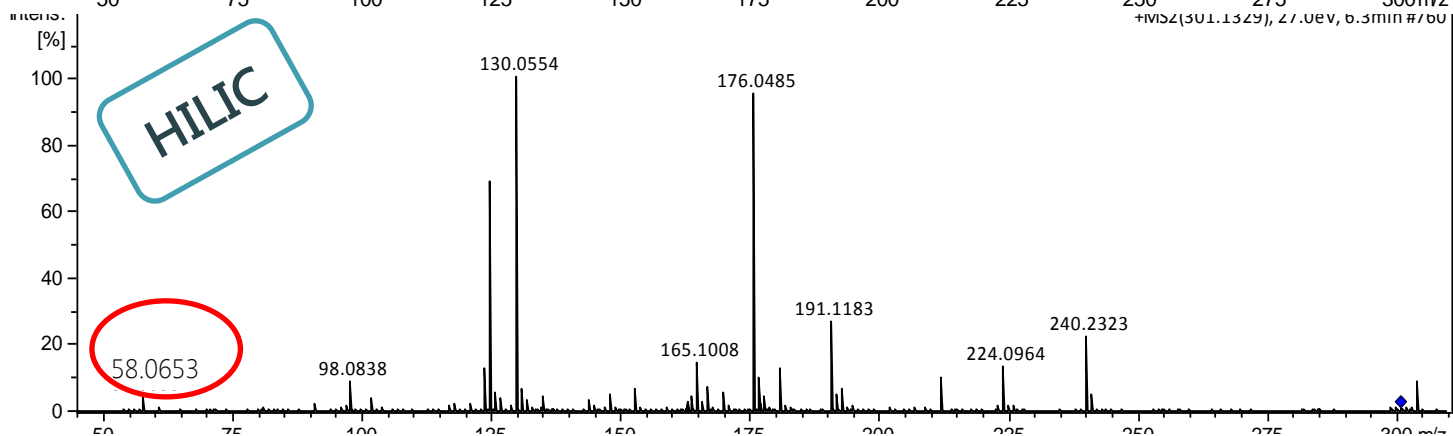
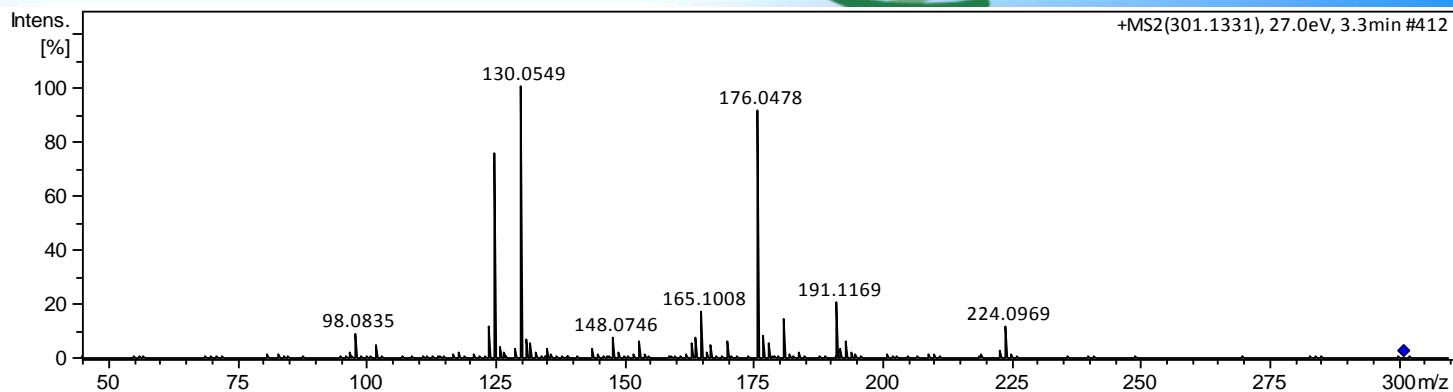
CEST 2015

✓ Separation  
of isomers

**RAN301  
(desmethyl  
ranitidine)**

Probable  
structure by  
diagnostic  
evidence

$m/z = 58.0651$   
characteristic  
fragment for  
N-demethylation





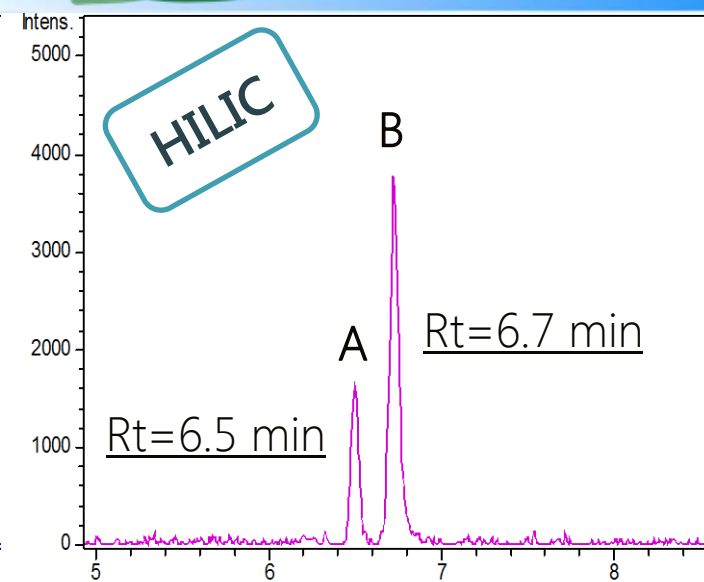
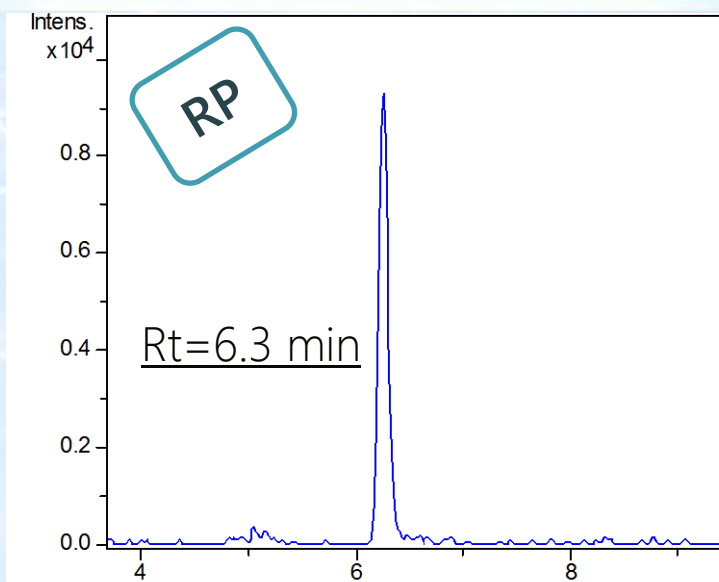
✓ Separation of isomers

**CTR360B**

Probable structure by diagnostic evidence

**CTR360A**

Tentative candidate



Isomers

Carboxylic acid of CTR N-oxide

Hydroxylated derivative of CTR carboxylic acid



✓ Separation of isomers

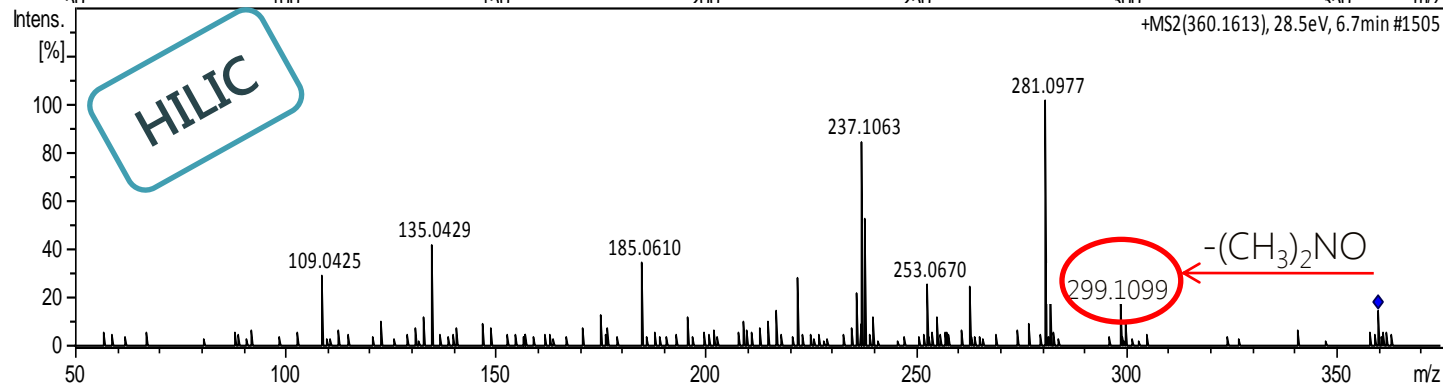
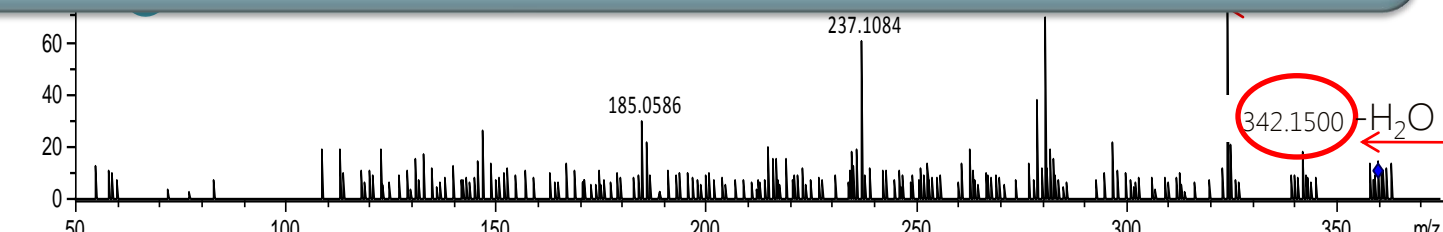
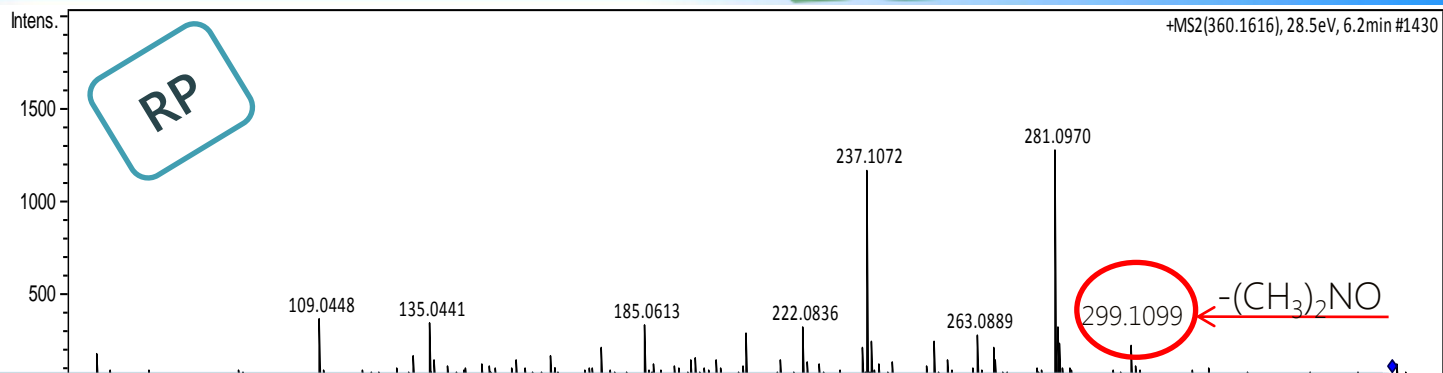
**CTR360**

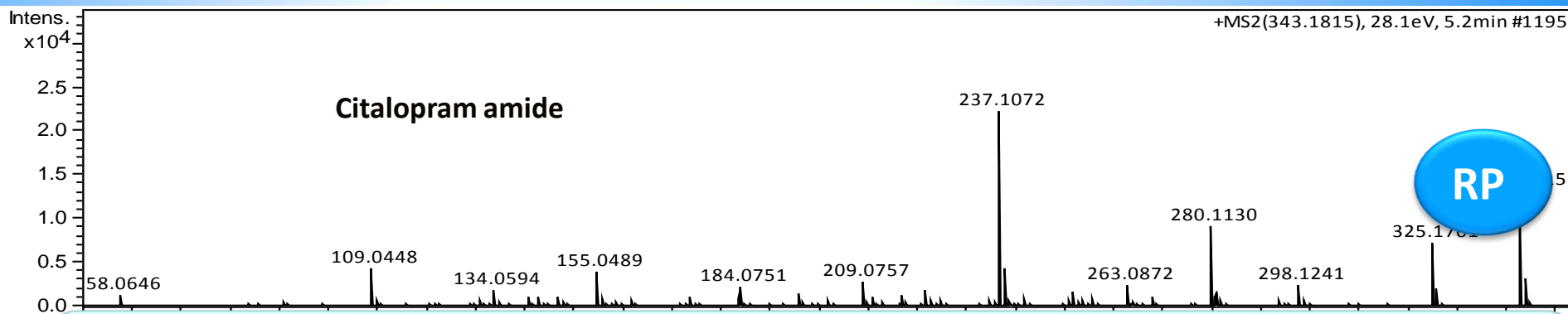
Probable structure by diagnostic

Identification level 2b: Probable structure by diagnostic evidence

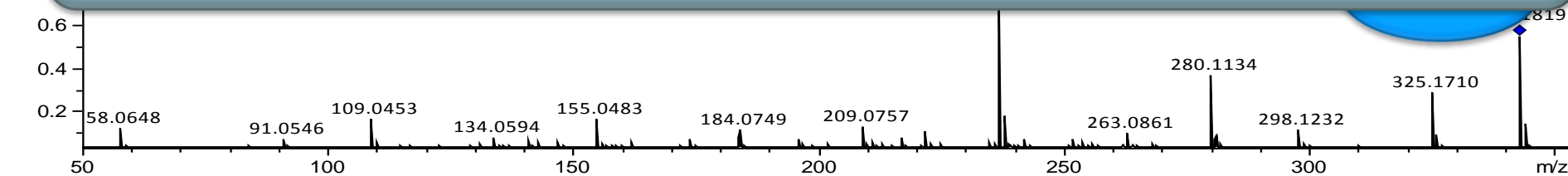
characteristic fragmentation pattern that not occur in CTR N-oxide

$m/z = 299.1099$   
Elimination of  $(\text{CH}_3)_2\text{NO}$ : characteristic fragmentation pattern only in CTR N-oxide



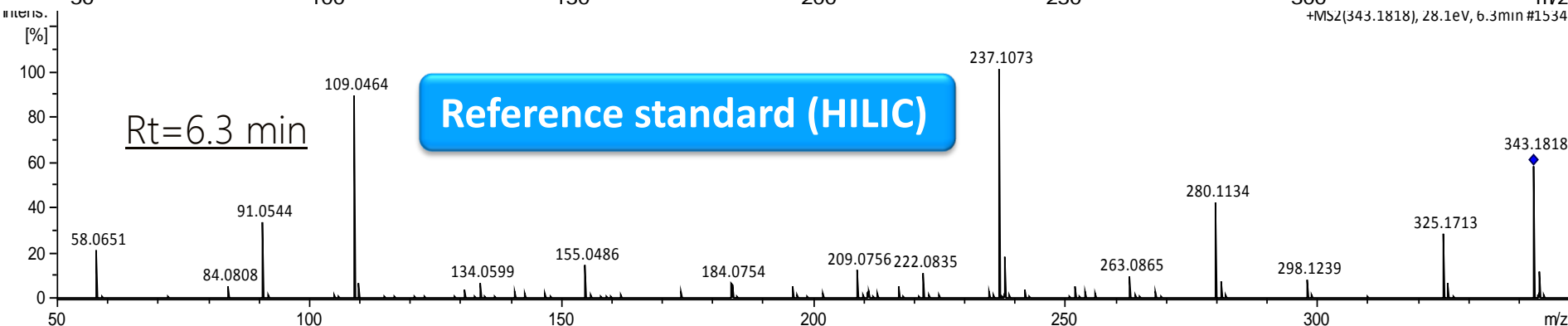


## Identification level 1: Confirmation with Reference Standard



Rt=6.3 min

Reference standard (HILIC)







Subtraction of the background (Bruker's MetaboliteDetect 2.0)

Generation of a peak list of unknown

Treat further as "suspect peaks"

Background Subtraction Parameters

Difference

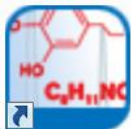
eXpose mode, Ratio: 3

Detect -Mass spectrum

Parameters

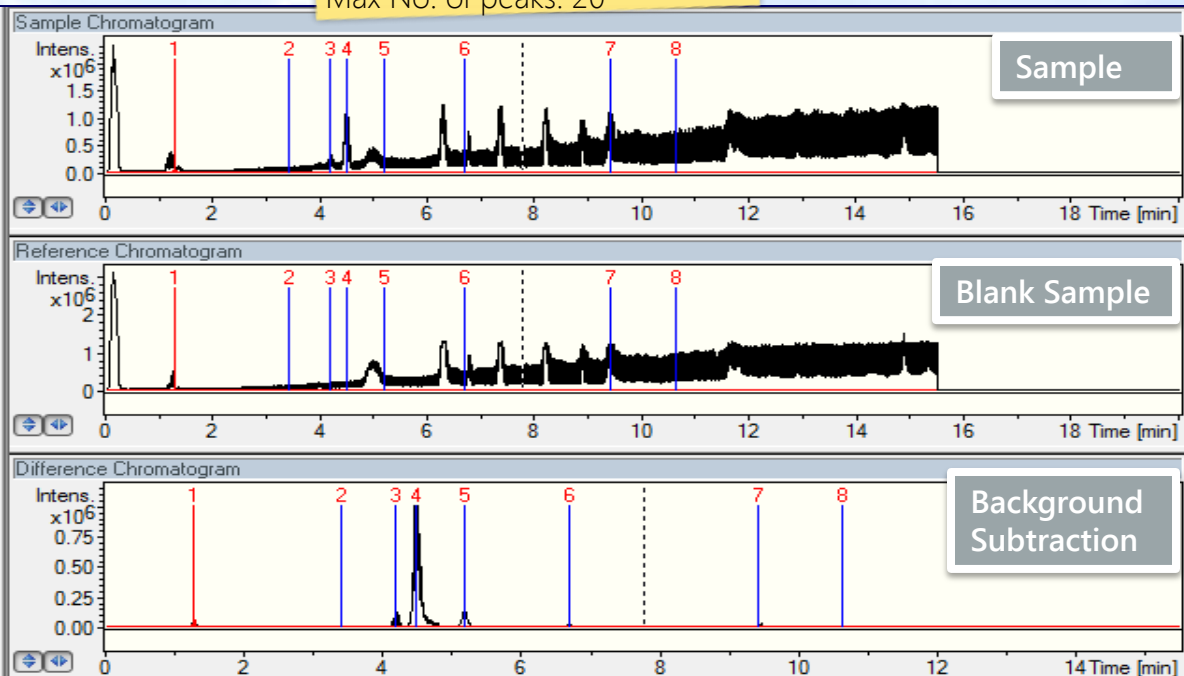
Int. threshold: 30%

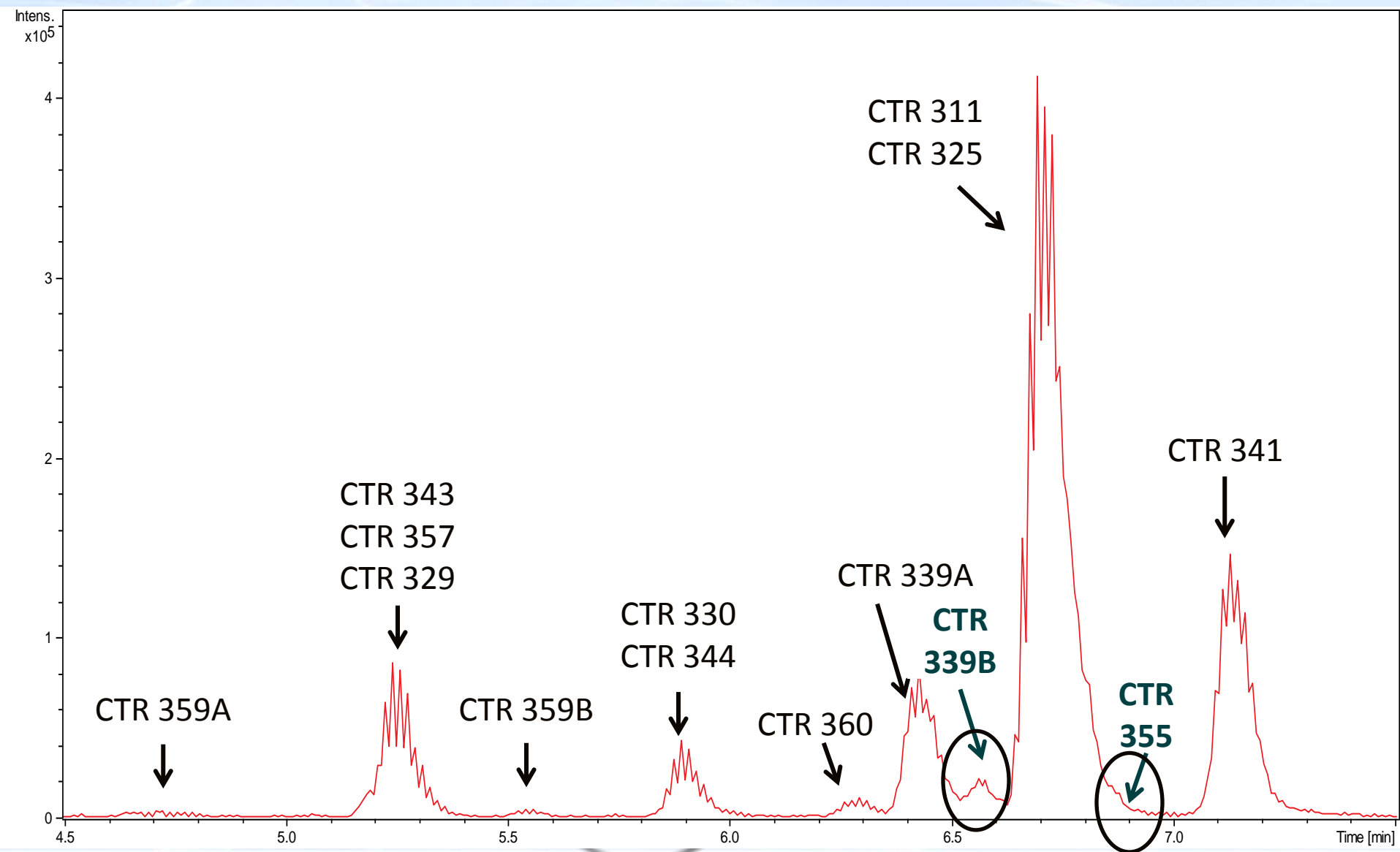
Max No. of peaks: 20



Metabolite Detect

detected		expected		Generation of peak list			
No.	Chrom.	Gp.	RT-S[min]	m/z-det.	MS/MS	Comment	
	A	-	Base Peak	-	-	Base Peak Chi	
	B	+	BPC Int.	-	-	Base Peak Chi	
	C	-	TIC	-	-	Total Ion Curr	
	D	+	EIC	-	-	Extracted Ion	
1		1*	1.3	107.9681	(yes)	Det. Trace 1	
2	+	1	1.3	123.9411	(yes)	Det. Trace 2	
3	+	1	1.3	139.9146	(yes)	Det. Trace 3	
4	+	2*	3.4	216.1231	(yes)	Det. Trace 4	
5	+	3a1*	4.2	207.1489	(yes)	Det. Trace 5	
6	+	3b1	4.2	266.1975	(yes)	Det. Trace 6	
7	+	4*	4.5	86.0973	(yes)	Det. Trace 7	
8	+	5*	5.2	86.0963	(yes)	Det. Trace 8	
9	+	6*	6.7	219.1491	(yes)	Det. Trace 9	
10	+	7*	9.4	652.2653	(yes)	Det. Trace 10	
11	+	7	?	694.2812	(yes)	Det. Trace 11	
12	+	8*	10.6	910.5027	(yes)	Det. Trace 12	





# Results



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PC`	TP	Mass [M+H] <sup>+</sup>	Molecular Formula	Appearance of max peak area	Time trend	Id. Level	Name
Lidocaine	LDC207	207.1504	C12H18N2O	7d	↗↘	1	MEGX
	LDC251	251.1756	C14H22N2O2	2d	↗↘	1	Lidocaine-N-oxide
	LDC219	219.1485	C13H18N2O	1d	↗↘	2b	
	LDC233	233.1282	C13H16N2O2	7d	↗→	4	
	LDC265	265.1538	C14H20N2O3	9d	↗→	4	
Citalopram	CTR311	311.1554	C19H19FN2O	6 d	↗	1	Desmethyl CTR
	CTR329	329.1660	C19H21FN2O2	6 d	↗	2b	Desmethyl CTR amide
	CTR330	330.1500	C19H20FNO3	6 d	↗	2b	Desmethyl CTR carboxylic acid
	CTR339A	339.1503	C20H19FN2O2	6 d	↗	1	3-Oxo-CTR
	CTR339B	339.1880	C21H23FN2O	6 d	↗	2b	Methylated derivative of CTR
	CTR341	341.1660	C20H21FN2O2	3 d	↗↘	2a	CTR-N-oxide
	CTR343	343.1816	C20H23FN2O2	6 d	↗	1	CTR amide
	CTR344	344.1656	C20H22FNO3	5 d	↗	1	CTR carboxylic acid
	CTR355	355.1457	C20H19FN2O3	6 d	↗	3	Hydroxylated derivative of 3-Oxo-CTR
	CTR357	357.1609	C20H21FN2O3	6 d	↗	2b	Amide of 3-Oxo-CTR
	CTR359A	359.1765	C20H23FN2O3	6 d	↗	3	Hydroxylated derivative of CTR amide
	CTR359B			4 d	↗↘	2b	Amide of CTR-N-oxide
	CTR360A	360.1606	C20H22FNO4	6 d	↗	3	Hydroxylated derivative of CTR carb.acid
CTR360B	↗				2b	Carboxylic acid of CTR-N-oxide	
MTF	MTF103	103.0614	C2H6N4O	2d	↗	1	Guanylurea

# Results



CEST 2015

PC	TP	Mass [M+H] <sup>+</sup>	Molecular Formula	Appearance of max peak area	Time trend	Id. Level	Name
Ranitidine	RAN286A	286.0856	C11H15N3O4S	1d	↗↘	4	-
	RAN286B			2d	↗→	4	-
	RAN286C			10h	↗↘	4	-
	RAN301A	301.1329	C12H20N4O3S	10h	↗↘	2b	desmethyl RAN
	RAN301B					2b	
	RAN302A	302.0805	C11H15N3O5S	1d	↗	3	
	RAN302B			1d	↗↘	3	
	RAN316A (2peaks)	316.1331		3d	↗	3	
	RAN316B			3d	↗	3	
	RAN317 (2peaks)	317.1278	C12H20N4O4S	3d	↗	3	Desmethyl RAN-S-oxide
	RAN331A (2peaks)	331.1435	C13H22N4O4S	1d	↗↘	1	RAN-S-oxide
	RAN331B			1d	↗↘	1	RAN-N-oxide
Atorvastatin	ATR515	515.2341	C31H31FN2O4	2d	↗	2b	β-oxidation product of ATR
	ATR471	471.2078	C29H27FN2O3	2d	↗	2b	β-oxidation product of ATR515
	ATR487A	487.2028	C29H27FN2O4	2d	↗	3	Monohydroxylation of ATR471
	ATR487B			2d	↗	3	





5 pharmaceuticals (metformin, ranitidine, lidocaine, citalopram and atorvastatine) were investigated for their TPs under aerobic biodegradation experiments and 34 TPs were identified, in total.



Nine of them were confirmed by a reference standard and the majority of the rest reached Id. Level 2: probable structure by diagnostic evidence.



HILIC was presented to be fit for the orthogonal identification of TPs and suitable for screening of more polar metabolites



HILIC provided better peak shapes and greater intensities in some of the identified TPs.



HILIC also, permitted the identification and characterisation of isomeric TPs, due to the better detection sensitivity that provided clearer spectra for interpretation.



OPERATIONAL PROGRAMME  
EDUCATION AND LIFELONG LEARNING  
*investing in knowledge society*  
MINISTRY OF EDUCATION & RELIGIOUS AFFAIRS  
MANAGING AUTHORITY

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Eleni Georgantzi

Our collaborators:

Kathrin Fenner

Rebekka Gulde



**eawag**  
aquatic research ooo

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# Thank you for your attention



<http://trams.chem.uoa.gr/>

<http://tremepol.chem.uoa.gr/>