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LSMS

Quantification of endogenous and exogenous metabolites in small samples using parallel narrow bore to capillary LC with fast polarity switching MRM

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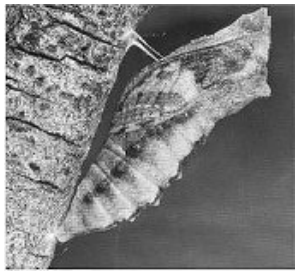
Life is a Dynamic System !

Metabolome A(t1)
Lipidome A(t1)
Proteome A(t1)

Aging

Metabolome B(t2)
Lipidome B(t2)
Proteome B(t2)

Disease *or*
Toxicological exposure

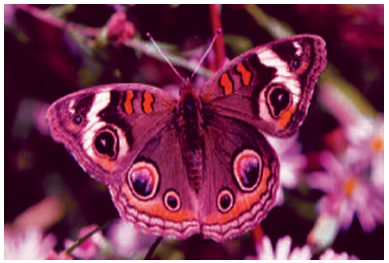


Metabolome D
Lipidome D
Proteome D

Cure
or
Change in
Lifestyle

Metabolome A'
Lipidome A'
Proteome A'

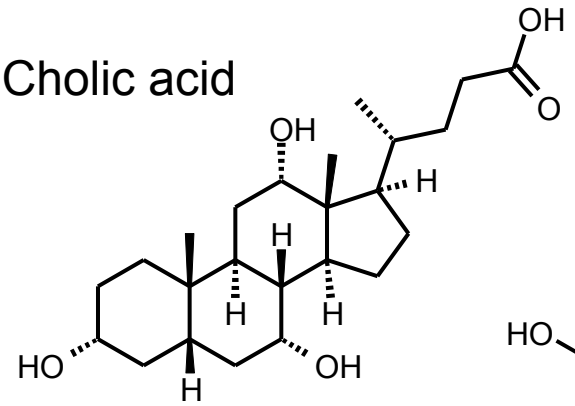
Aging
Death

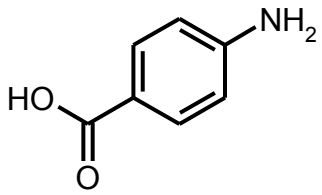


Metabolome Y
Lipidome Y
Proteome Y

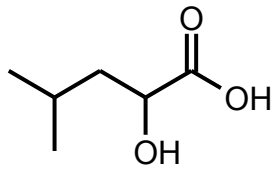
Metabolites: Large Chemical Space, Large Dynamic Range, Different MS Response Factors!

Cholic acid

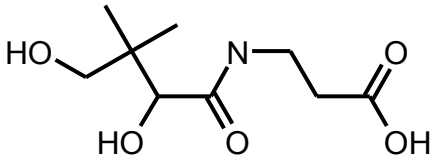




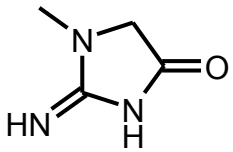
p-Aminobenzoic acid



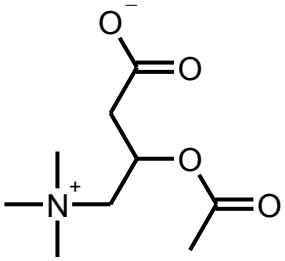
Pantothenic acid



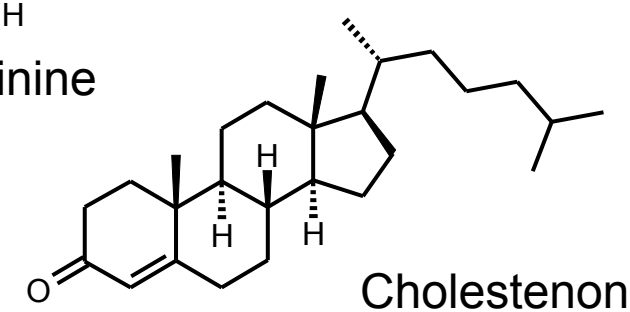
Leucinic acid



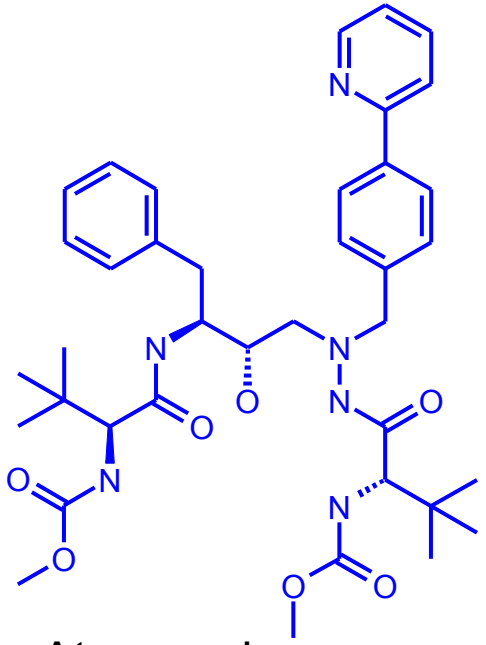
Creatinine



L-Acetylcarnitine



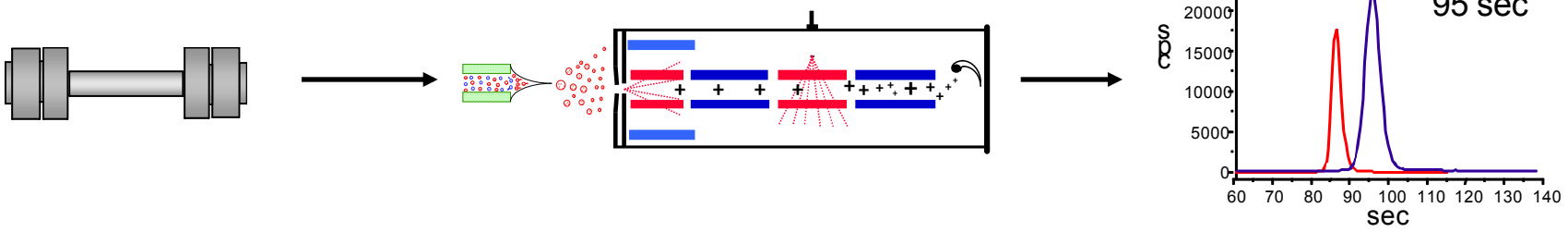
Cholestenone



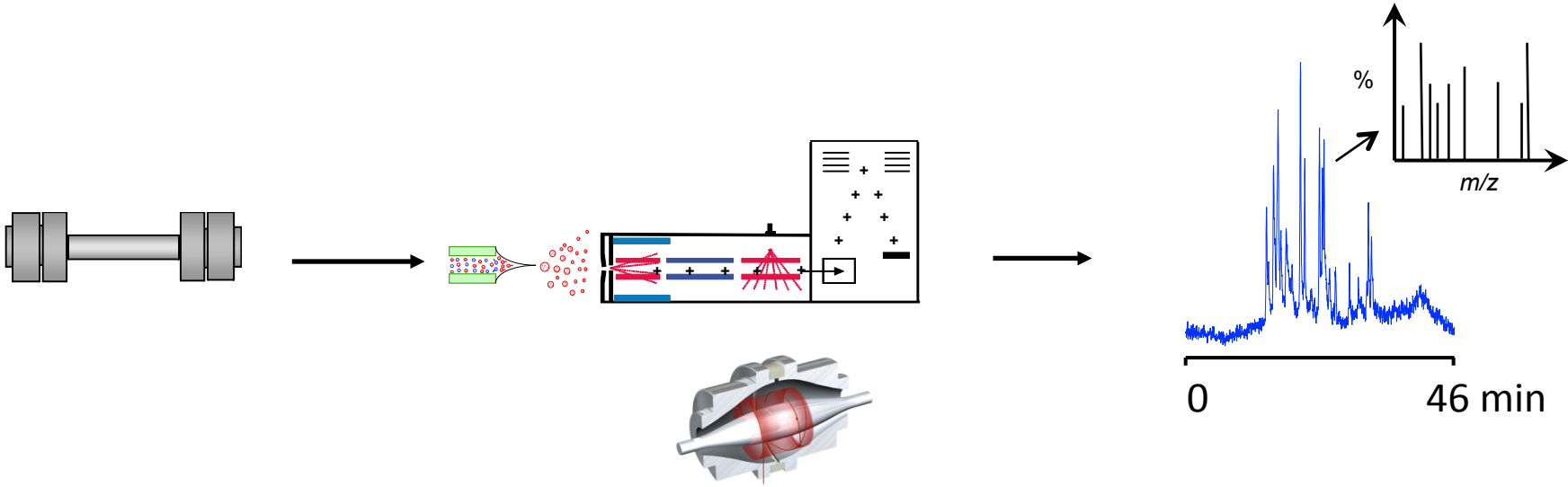
Atazanavir

MS: Qualitative and Quantitative Approaches

QUAN: Multiple Reaction Monitoring Mode (SRM/MS)

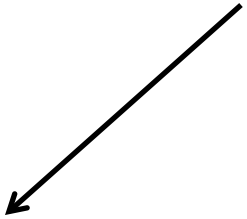


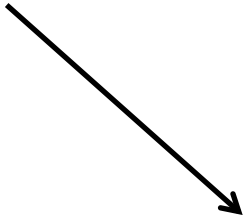
QUAL/QUAN Data Idependant Acquisition Mode (DIA/MS)



Challenges with Pharmacometabolomics

- Multi-components assays for drugs, endo and exo metabolites
- Limited samples volumes
- Large dynamic range
- Large number of sample to be analyzed





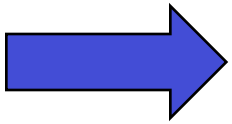
Use of parallel LC systems

Tune Sensitivity with different LC column internal diameter

LC-SRM/MS Assay for multiple Metabolites

The constrains:

- Need of different mechanisms of retention
- Multiple types of biological matrices (plasma, tissue,..)
- ESI in positive and negative mode



Parallel LC systems with column-switching
QqQ and fast polarity switching

The Analytes (n= 75)

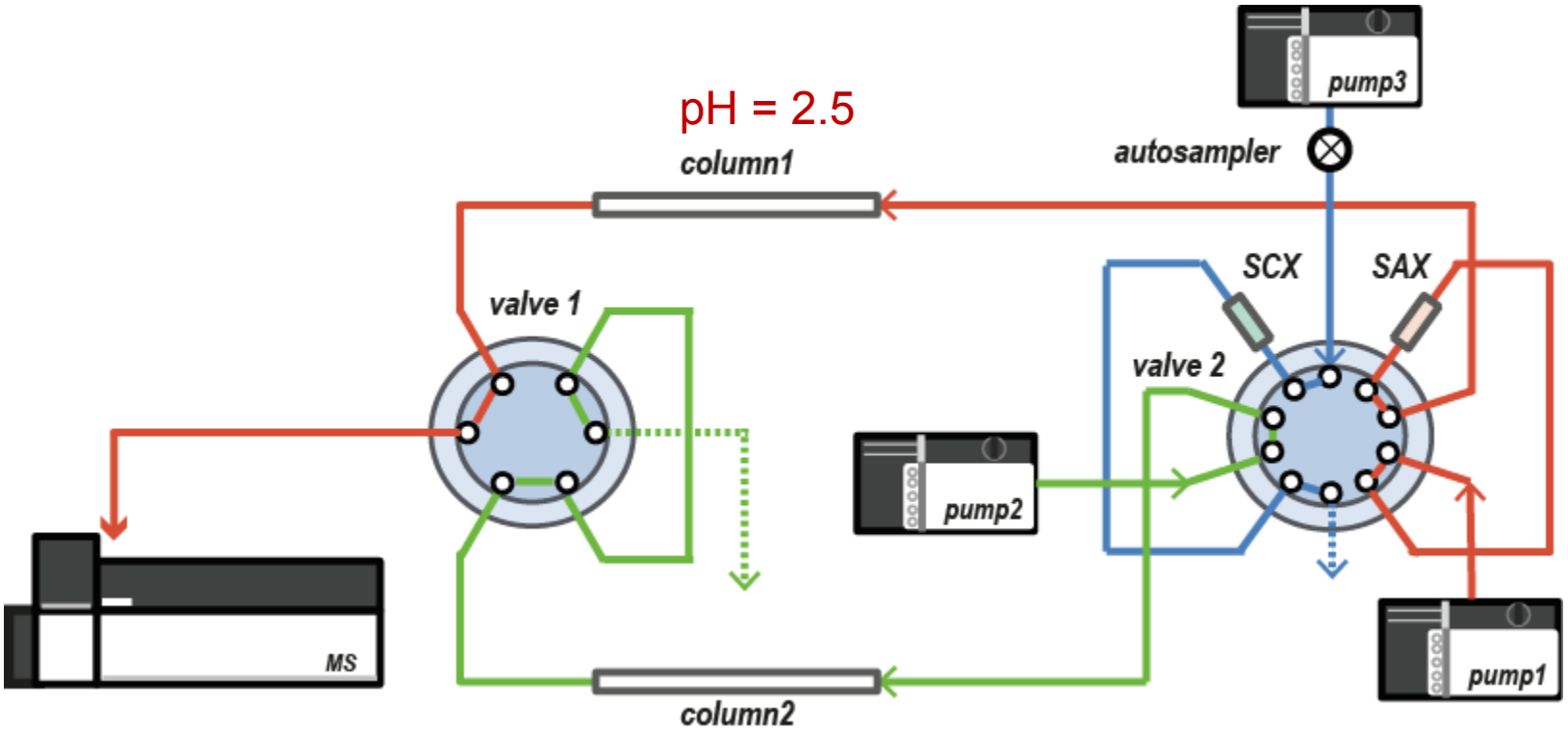
#	Compound for positive	Exact mass	Precursor ion	Product ion	CE
1	Urea	60.0	60.9	44.1	-22
2	Alanine	89.0	89.9	44.1	-11
3	Aniline	93.1	94.0	77.1	-22
4	γ-Aminobutyric acid	103.1	104.0	87.0	-15
5	Dimethylglycine	103.1	104.0	58.1	-15
6	2-Aminophenol	109.1	110.0	65.1	-23
7	Cytosine	111.0	112.0	95.1	-22
8	Histamine	111.1	112.0	95.1	-18
9	Creatinine	113.1	114.0	44.1	-16
10	Proline	115.1	115.9	70.1	-16
11	Valine	117.1	118.0	72.1	-13
12	Threonine	119.1	120.0	74.1	-10
13	Nicotinamide	122.0	123.0	80.1	-22
14	Nicotinic acid	123.0	124.0	80.1	-23
15	Creatine	131.1	132.0	90.1	-14
16	Leucine	131.1	132.0	86.2	-14
17	Asparagine	132.1	133.0	74.1	-15
18	Aspartic acid	133.0	134.0	74.1	-16
19	2-Deoxyribose	134.1	135.1	94.1	-10
20	Spermidine	145.2	146.1	72.1	-16
21	Glutamine	146.1	147.0	84.1	-19
22	Lysine	146.1	147.1	84.1	-18
23	Methionine	149.1	150.0	104.1	-14
24	Guanine	151.0	152.0	134.8	-22
25	Acetaminophen	151.1	152.0	110.1	-18
26	Histidine	155.1	156.0	110.1	-15
27	mono-Methyl adipate	160.1	161.0	143.1	-11
28	Carnitine (2Ch)	162.1	163.1	103.1	-20
29	Nicotine	162.1	163.1	130.1	-22
30	Phenylalanine	165.1	166.0	120.0	-14
31	Arginine	174.1	175.1	70.1	-22
32	Citrulline	175.1	176.1	70.2	-24
33	Theophylline	180.1	181.1	124.1	-21
34	Theobromine	180.1	181.0	138.1	-20
35	Tyrosine	181.1	182.1	136.0	-12
36	4-Pyridoxic acid	183.1	184.0	148.1	-20
37	5-Hydroxyindole-3-acetic acid	191.1	192.0	146.0	-15
38	Dimethyl phthalate	194.1	195.0	163.1	-12
39	Caffeine	194.1	195.0	138.0	-20
40	Tryptophan	204.1	205.1	188.1	-12
41	Pantothenic acid	219.1	220.1	90.0	-14
42	Diethyl phthalate	222.1	223.0	149.0	-17
43	Adenosine	267.1	268.0	136.1	-18
44	Riboflavin	376.1	377.1	243.0	-22
45	Hypoxanthine	136.0	137.0	110.2	-23
46	Tryptamine	160.1	161.1	144.2	-14
47	Glutamic acid	147.1	148.0	84.1	-16
48	Adenine	135.1	136.0	119.0	-25
49	Guanosine	283.1	284.0	152.1	-14
50	Aspartic acid-13C		135.1	73.7	-14
51	γ-Aminobutyric acid-d6		110.0	93.1	-16
52	Caffeine-13C3		198.1	140.0	-20
53	Nicotine-d4		167.1	134.1	-23

#	Compound for negative	Exact mass	Precursor ion	Product ion	CE
54	Pyruvic acid	88.0	87.0	43.1	11
55	Lactic acid	90.0	89.1	43.1	13
56	Malonic acid	104.0	103.0	59.0	11
57	Uracil	112.0	111.0	42.1	15
58	Fumaric acid	116.0	115.1	71.1	12
59	2-Hydroxy-3-methylbutyric acid	118.1	117.2	71.1	15
60	Benzoic acid	122.0	121.2	77.1	14
61	Taurine	125.0	124.2	80.0	22
62	Malic acid	134.0	133.1	115.0	16
63	Adipic acid	146.1	145.1	83.1	15
64	p-Anisic acid	152.0	151.1	107.0	15
65	3,5-Dihydroxybenzoic acid	154.0	153.1	109.1	16
66	3-Methyladipic acid	160.1	159.2	97.2	15
67	Pimelic acid	160.1	159.2	97.1	16
68	3-Phenyllactic acid	166.1	165.1	147.1	16
69	Suberic acid	174.1	173.1	111.2	17
70	N-Acetyl-aspartic acid	175.0	174.1	88.0	16
71	Ascorbic acid	176.0	175.0	115.0	14
72	Hippuric acid	176.0	178.1	134.1	14
73	Mannitol	176.0	181.1	89.0	14
74	Citric acid	192.0	191.1	111.0	13
75	5-Sulfosalicylic acid	218.0	217.0	199.0	15
76	Sucrose	342.1	341.1	179.1	14
77	Cholic acid	408.3	407.3	343.3	34
78	Maleic acid	116.0	115.2	71.0	13
79	Succinic acid	118.0	117.0	73.1	15
80	Succinic acid-d4		121.2	77.1	15

49 analytes detected in positive mode

36 analytes detected in negative mode

Dual UHPLC Column-Switching System with SCX and SAX trapping columns



LC-MS 8050

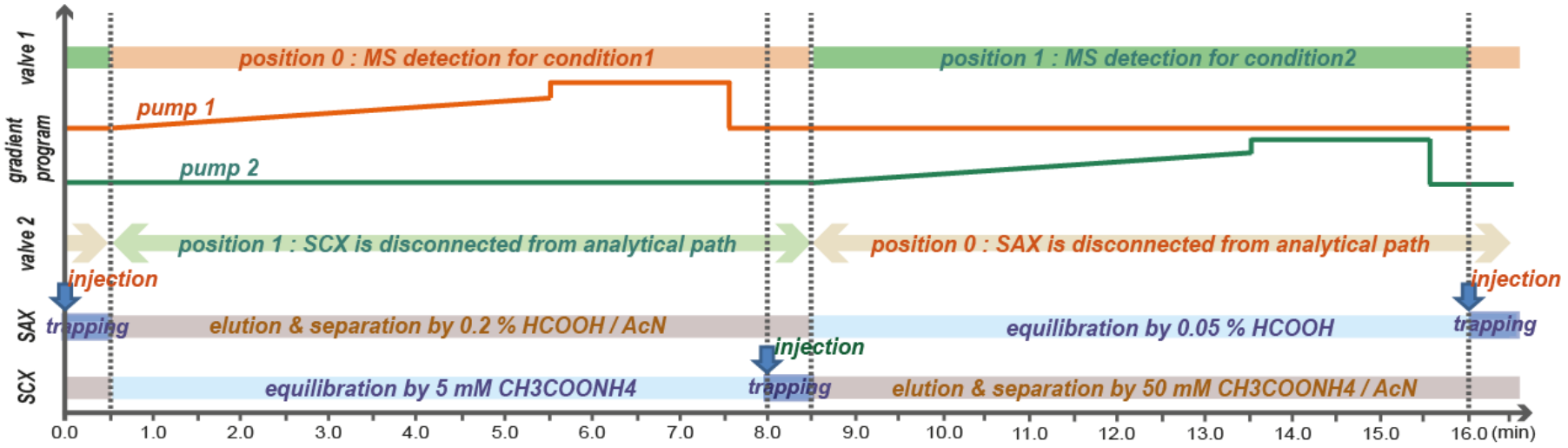
Analytical Sequence

Parallel UHPLC condition 1 (pH 2.5)

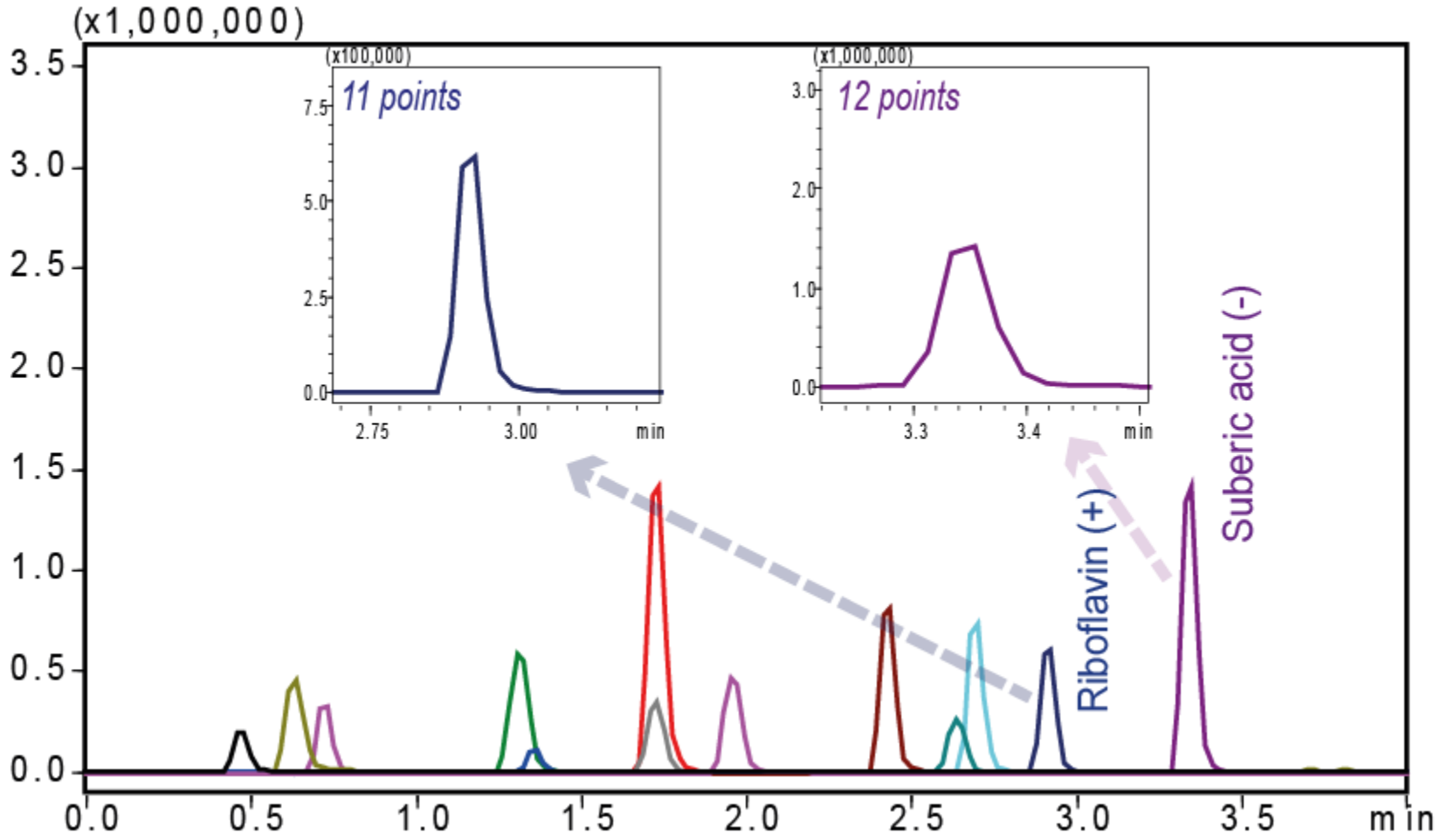
Trapping column	: EPX SAX (3 µm, 2.1 mm i.d. x 5 mm)
Trapping mobile phase	: 0.05 % formic acid aq.
Trapping flow rate	: 0.4 mL/min
Analytical column	: Kinetex XB-C18 (1.7 µm, 2.1 mm i.d. x 150 mm)
Mobile phases	: A) 0.2 % formic acid aq. (pH 2.5), B) AcN
Flow rate	: 0.4 mL/min
Gradient	: B conc. (%) = 0 -> 60 (0.00 - 5.00 min), 90 (5.01 - 7.00 min), 5 (7.01 - 8.00 min)
Columns temperature	: 45 °C
Injection volume	: 1-50 µL

Parallel UHPLC condition 2 (pH 6.9)

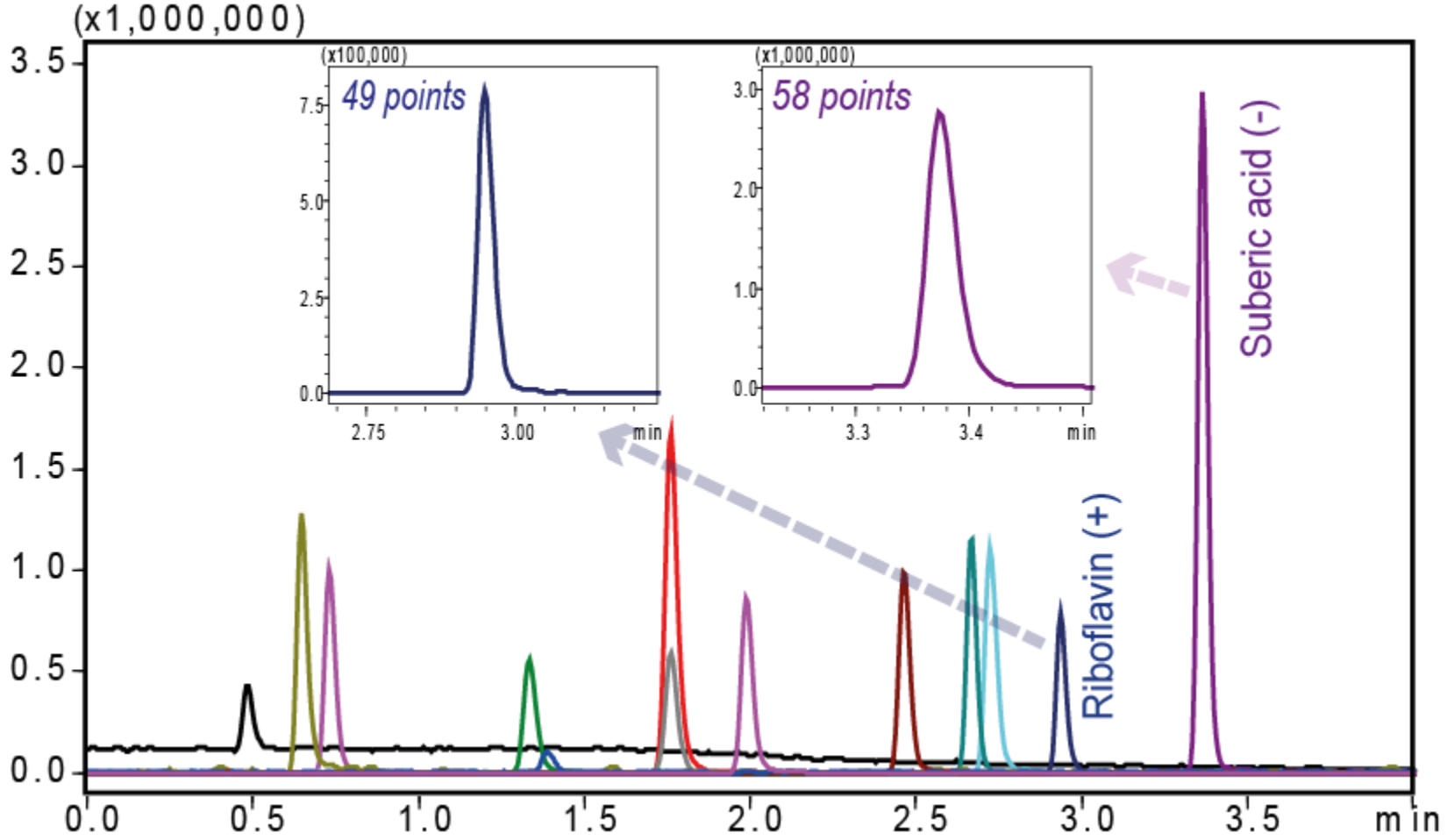
Trapping column	: EPX SCX (3 µm, 2.1 mm i.d. x 5 mm)
Trapping mobile phase	: 5 mmol/L Ammonium acetate aq.
Trapping flow rate	: 0.4 mL/min
Analytical column	: Hypersil GOLD C18 (1.9 µm, 2.1 mm i.d. x 150 mm)
Mobile phases	: A) 50 mmol/L Ammonium acetate aq. (pH 6.9), B) AcN
Flow rate	: 0.4 mL/min
Gradient	: B conc. (%) = 0 -> 60 (0.00 - 5.00 min), 90 (5.01 - 7.00 min), 5 (7.01 - 8.00 min)
Columns temperature	: 45 °C
Injection volume	: 1-50 µL



LC-SRM/MS Pause time 10 msec, dwell time 15 msec (LCMS 8050 - 50 transitions)

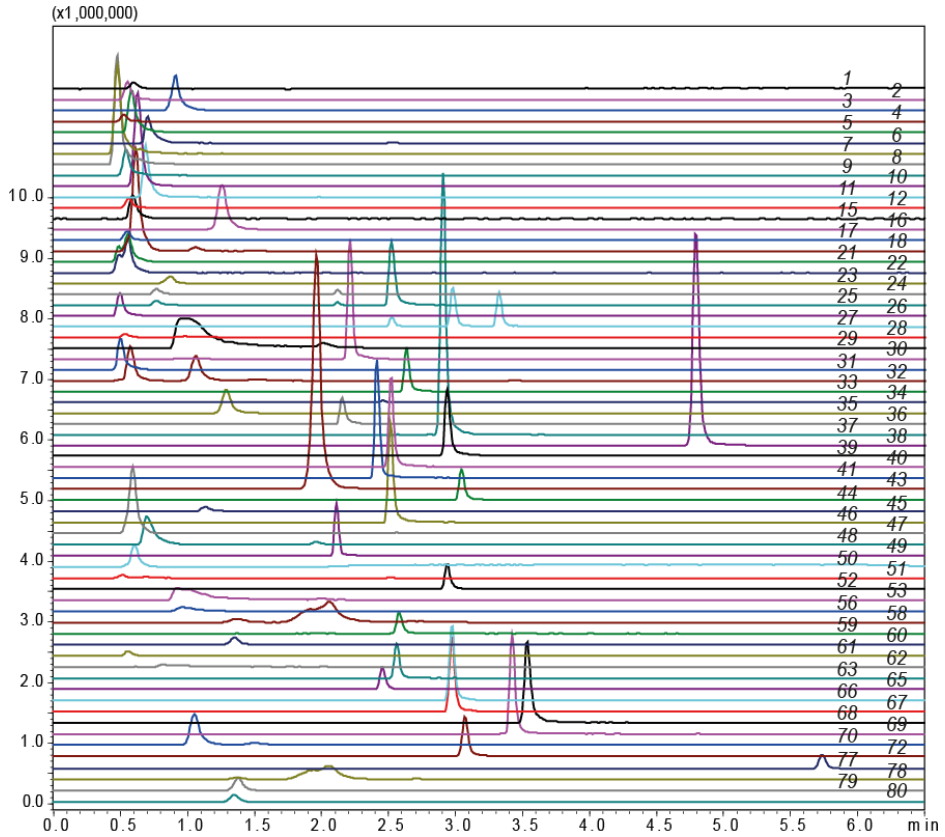


LC-SRM/MS Pause time 2 msec, dwell time 2 msec (LCMS 8050 - 50 transitions)

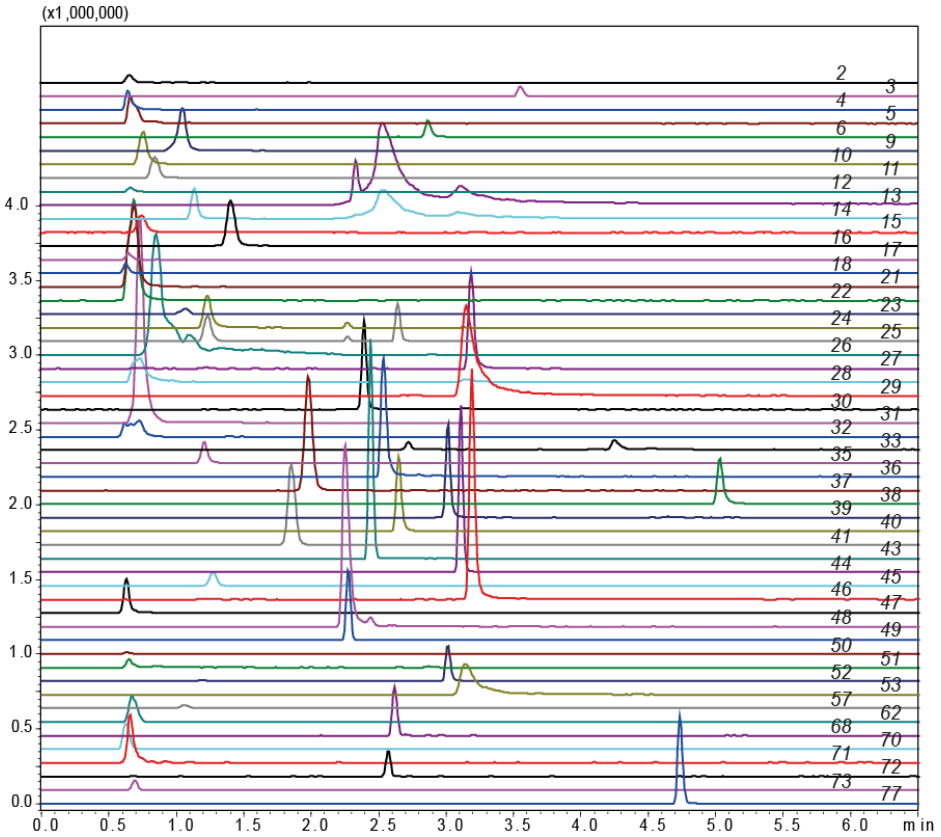


Standard LC-SRM/MS Chromatograms

Column 1: C18 pH 2.5

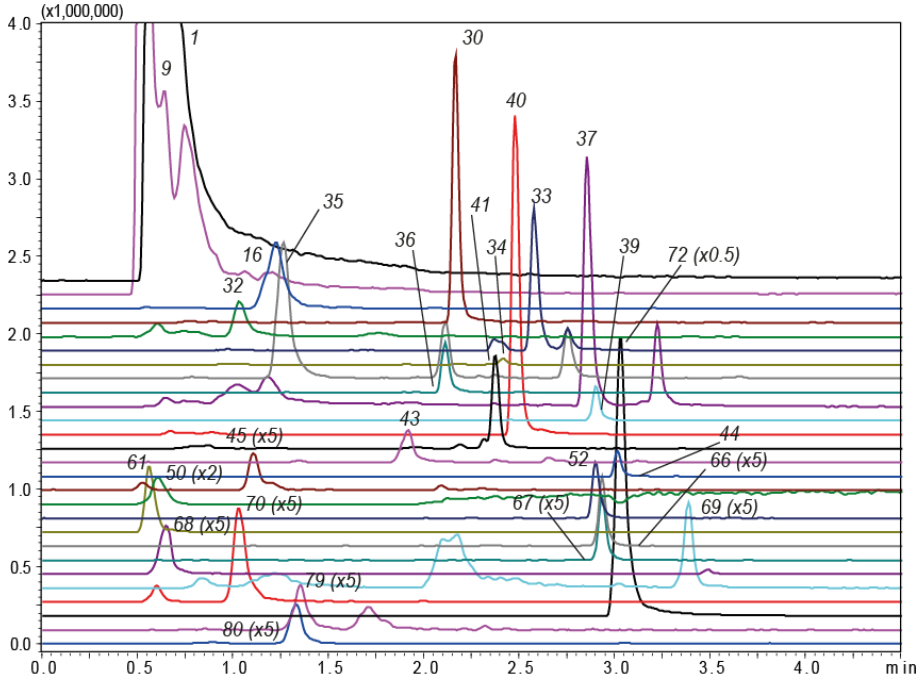


Column 2: C18 pH 6.9

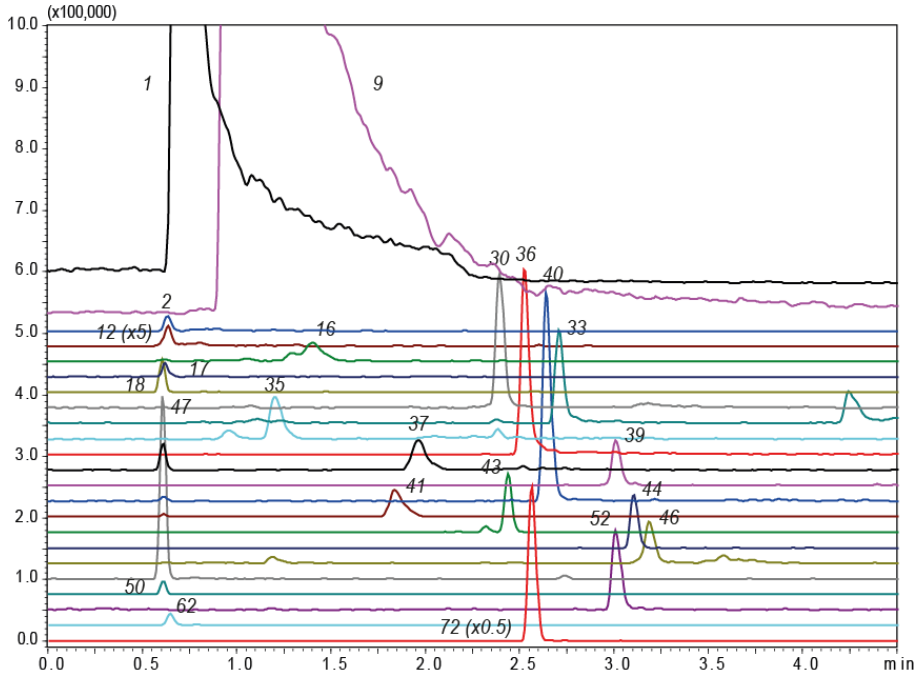


Urine Sample

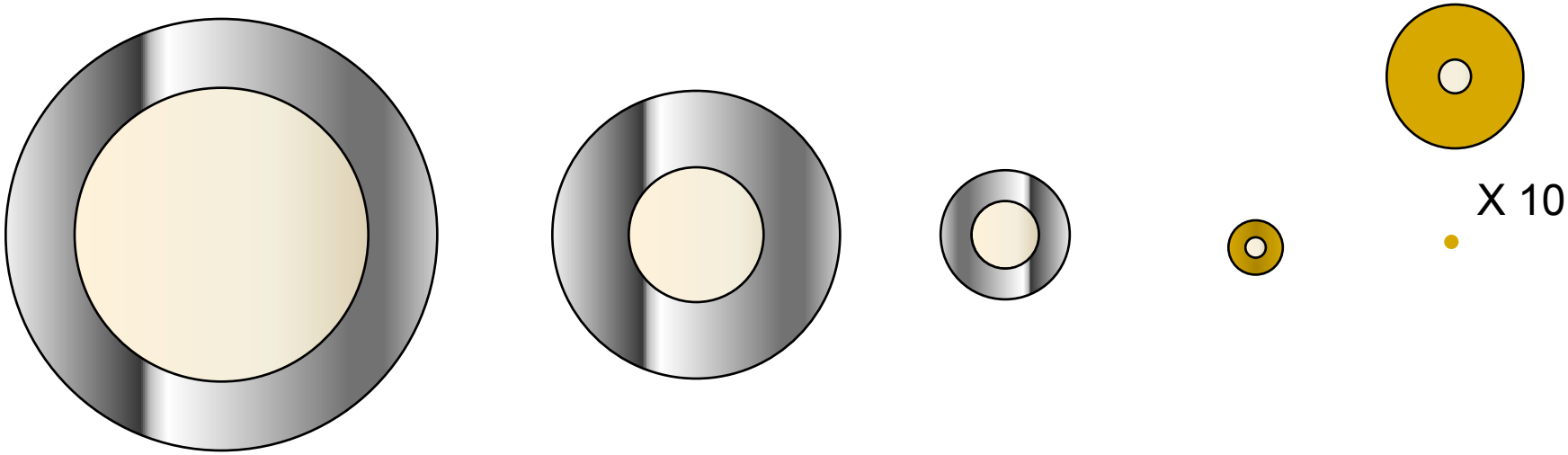
Column 1: C18 pH 2.5



Column 2: C18 pH 6.9



ESI as a Concentration Sensitive Detector
LC column miniaturisation



Definition	Column inner diameter	Flowrate	Injection volume
Regular LC	4.0 / 4.6 mm	1.0 ml/min.	**25 µl**
Narrowbore LC	2.0 / 2.1 mm	200 µl/min.	**5 µl**
Microbore LC	0.8 - 1.0 mm	40 µl/min.	**1.25 µl**
Capillary LC	180 - 300 µm	2-4 µl/min.	**125 nl**
Nano-LC	50 - 100 µm	150-250 nl/min.	**3 nl**

Downscaling factor

Downscaling factor (f) Sensitivity gain for the same injected amount

$$f = \left(\frac{\text{i.d.}_{\text{conv.}}}{\text{i.d.}_{\text{miniat.}}} \right)^2$$

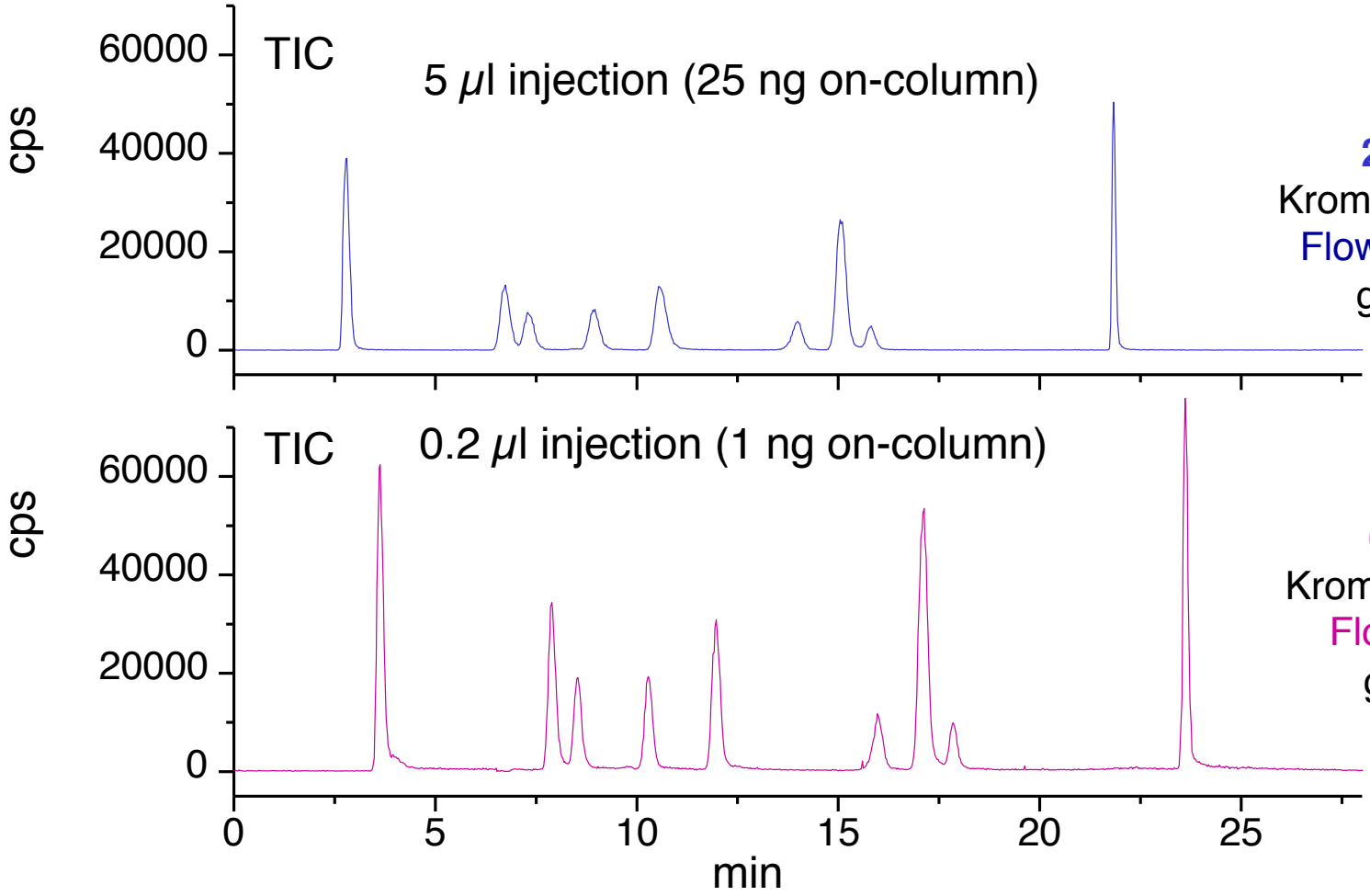
Micro-LC	$(4.6/1)^2 \cong 20$
Cap-LC	$\mathbf{(2.1/0.3)^2 \cong 49}$

$$C_{max} = \frac{m\sqrt{N}}{\sqrt{2\pi}\,\underbrace{V_0\,(1+k')}_{V_R}} = \frac{m\sqrt{N}}{(\pi r_c^2 L)\,\varepsilon\,\sqrt{2\pi}\,(1+k')}$$

$$C_{max} = \frac{m}{d_c^2} \times \frac{4\sqrt{N}}{\sqrt{2\pi^3}\;\varepsilon\;L\,(1+k')}$$

m = injected amount
d_c = column diameter
N = column plates
L = column length
V_0 = dead volume
ε = column porosity factor
k' = retention factor

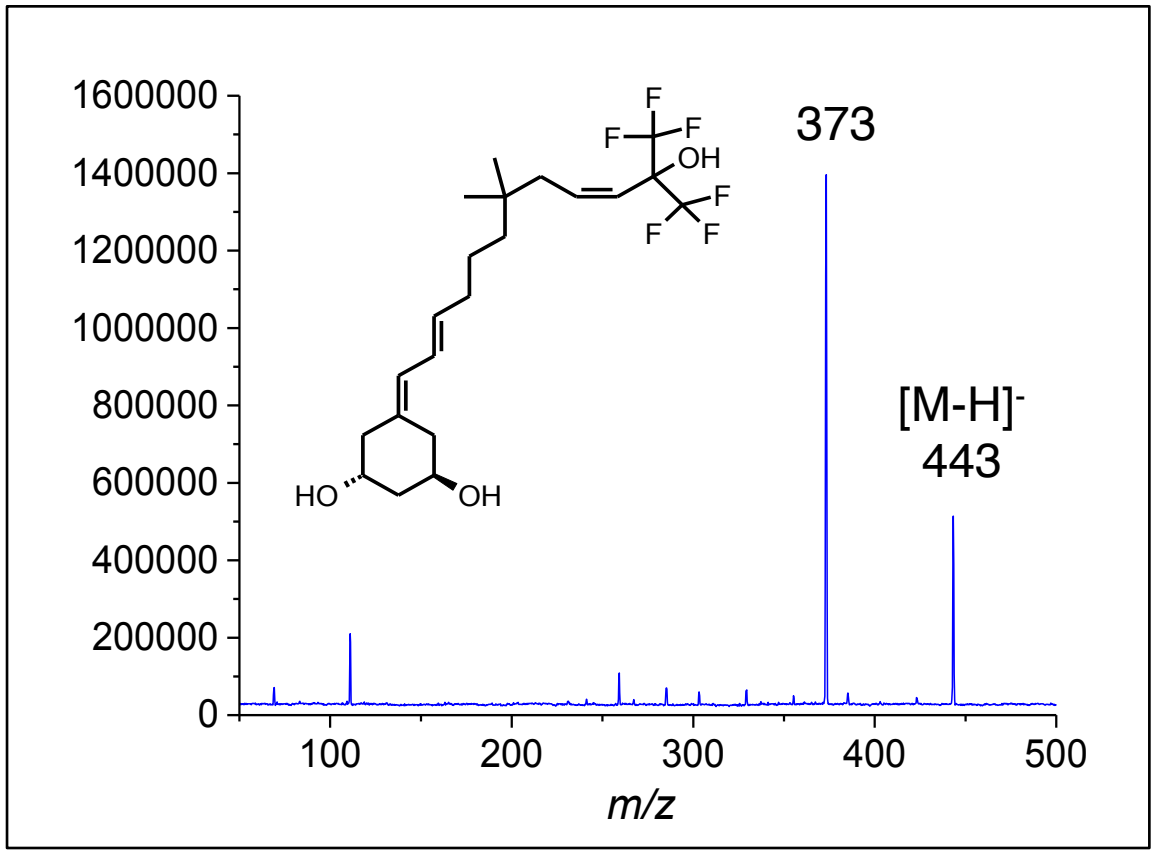
The “Concentration Sensitive” behavior of Ion Spray LC-MS reducing column i.d.

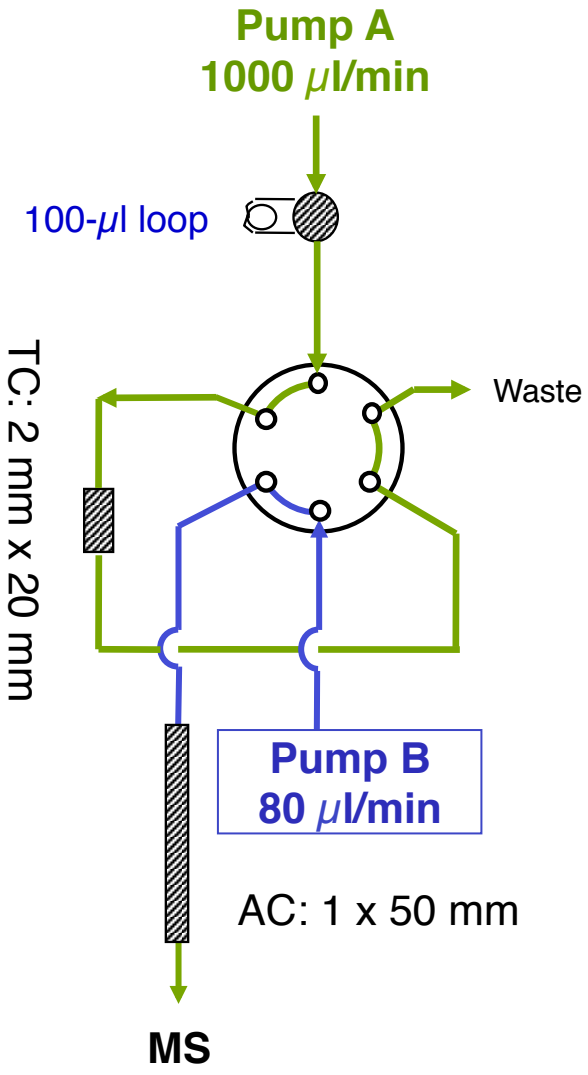


Narrow-bore
API III+
2.0 x 125 mm
Kromasil 100 C18 5 µm
Flow rate: 200 ul/min
gradient mode

Microbore
API III+
0.3 x 150 mm
Kromasil 100 C18 5 µm
Flow rate: 5 µl/min
gradient mode

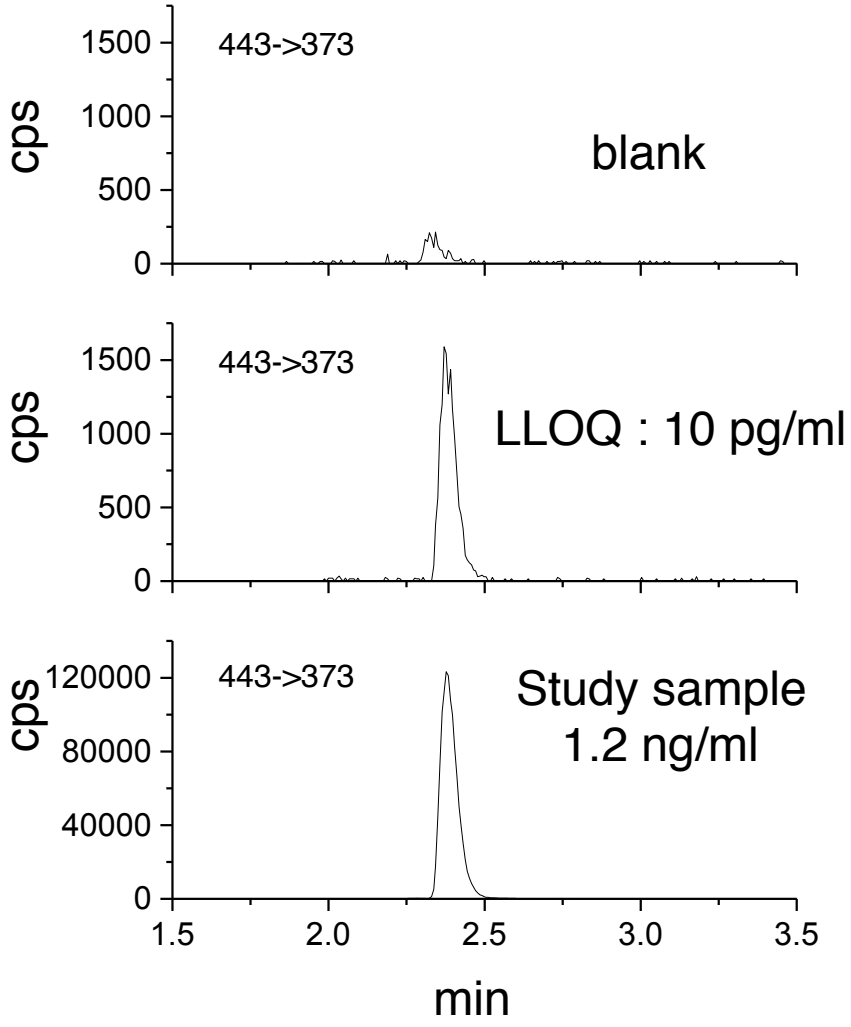
LC-MS/MS Analysis of a Cyclohexanediol Derivative down to 1 pg/ml with column-switching





Microbore LC-MS/MS assay

Calibration Range 10-50'000 pg/ml



Sample preparation

200 µl human plasma

LLE with hexane/ethyl acetate

50/50 v/v

reconstituted in 120 µl

methanol/1% formic acid (30/70, v/v)

100 µl injection

deuterated IS

Liquid chromatography

column-swichting

TC : Kromasil 100 C18, 5 µm

2 mm x 20 mm, 1000 µl/min

AC: Symmetry Shield RP18, 3.5 µm

1 mm x 50 mm, 80 µl/min

A: MeOH/1% HCOOH (85/15, v/v)

B: MeOH/1% HCOOH (95/15, v/v)

70% B -> 0.1 min 100% B

Mass spectrometry

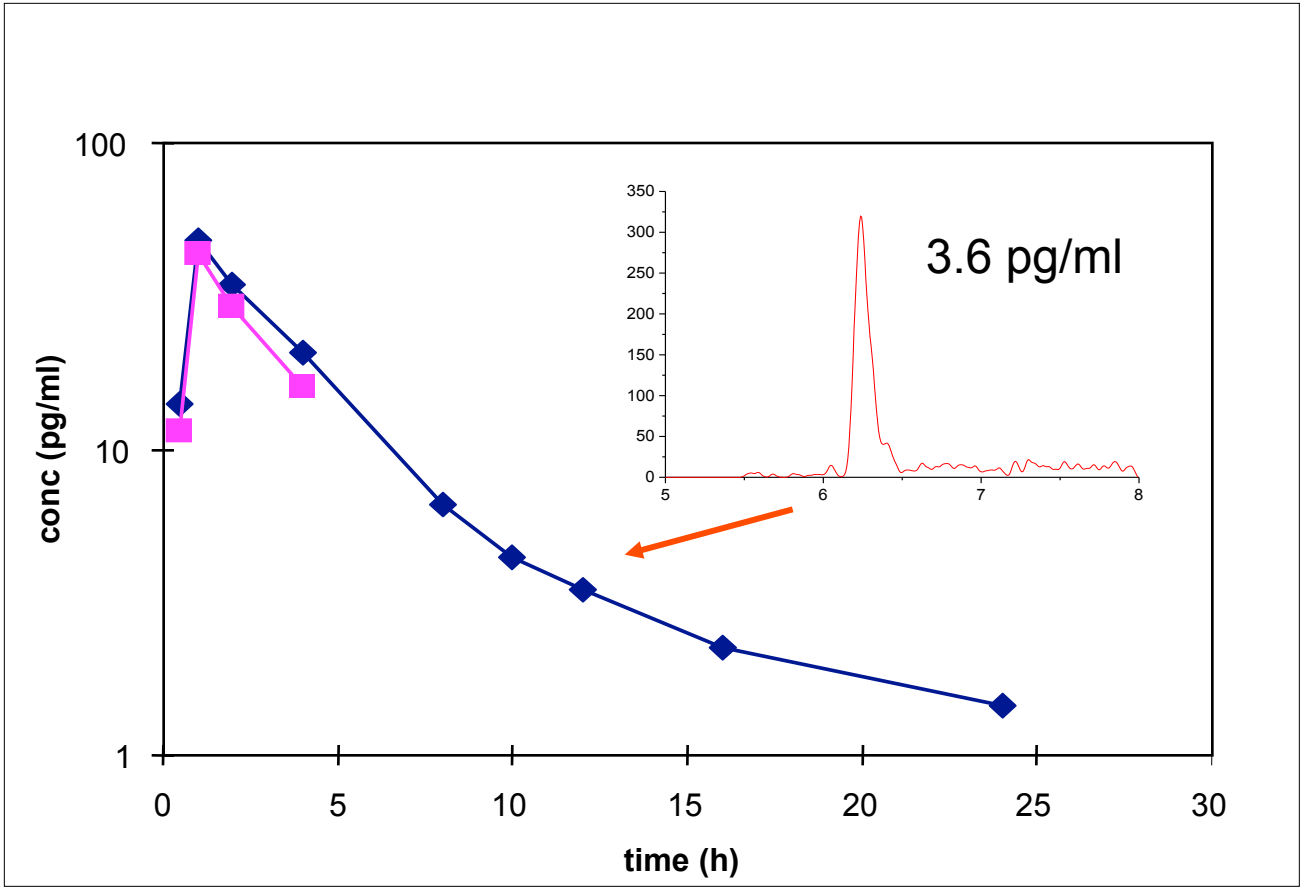
Ion spray negative mode

Selected reaction monitoring

From 1.0 to 0.3 mm capillary column-switching method

1 mm i.d.

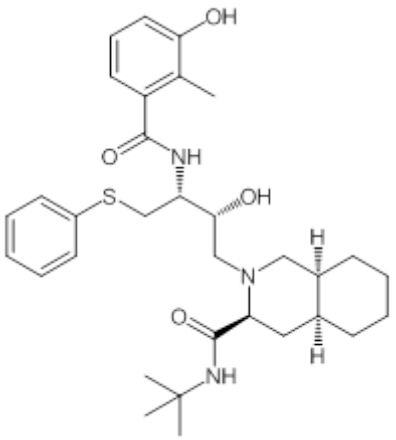
0.3 mm i.d.



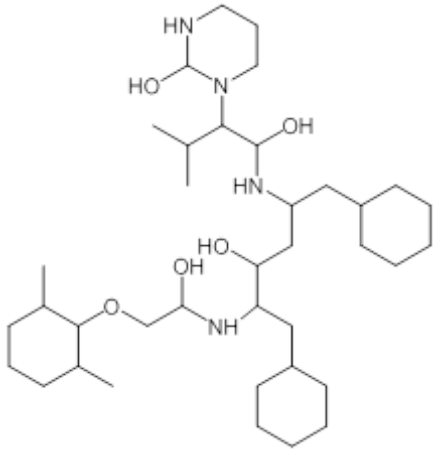
QC samples
n = 12 determinations

Conc. added (pg/ml)	Conc. found (pg/ml)	Accuracy (%)	RSD (%)
5	4.92	98.4	14
10	9.36	93.6	7.4
100	95.5	95.5	2.6
500	486	97.1	5.2

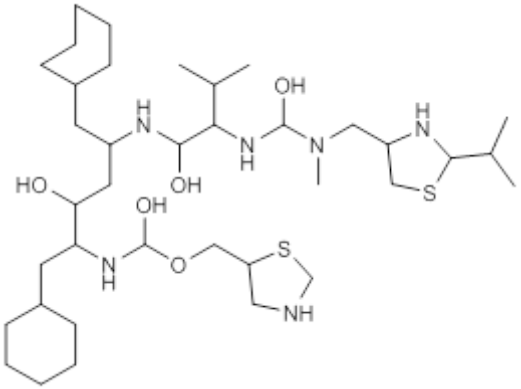
Protease Inhibitors with DBS
From 2 mm i.d. to 0.3 mm i.d. column



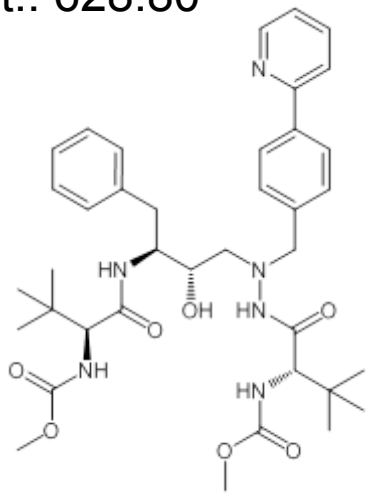
Nelfinavir $C_{32}H_{45}N_3O_4S$
Mol. Wt.: 567.78



Lopinavir $C_{37}H_{48}N_4O_5$
Mol. Wt.: 628.80



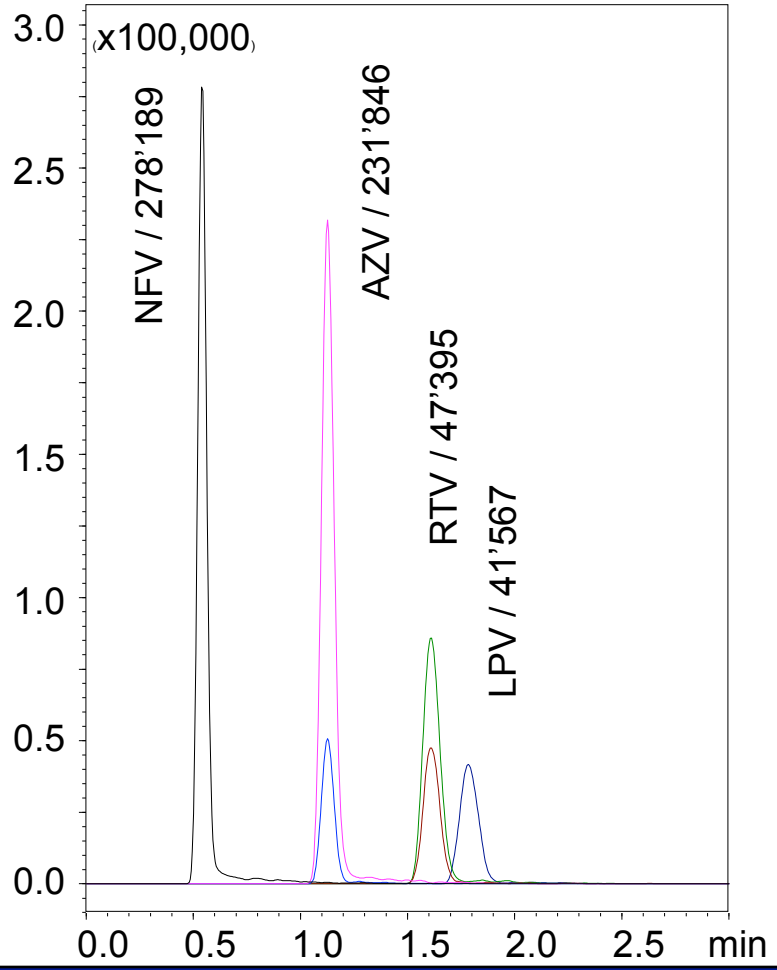
Ritonavir $C_{37}H_{48}N_6O_5S_2$
Mol. Wt.: 720.95



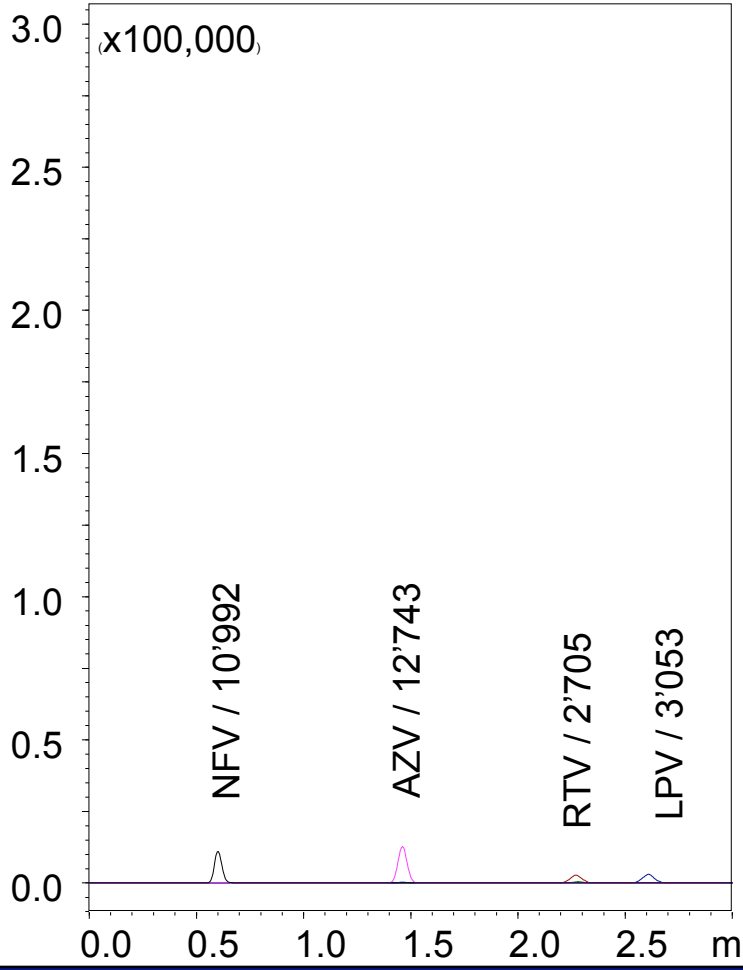
Atazanavir $C_{38}H_{52}N_6O_7$
Mol. Wt.: 704.86

Protease Inhibitors
From 2 mm i.d. to 0.3 mm i.d. column

Column: HALO C18, 2.7um
0.3 mm i.d. x 100 mm
Flow rate: 15 µL/min, T = 50 °C

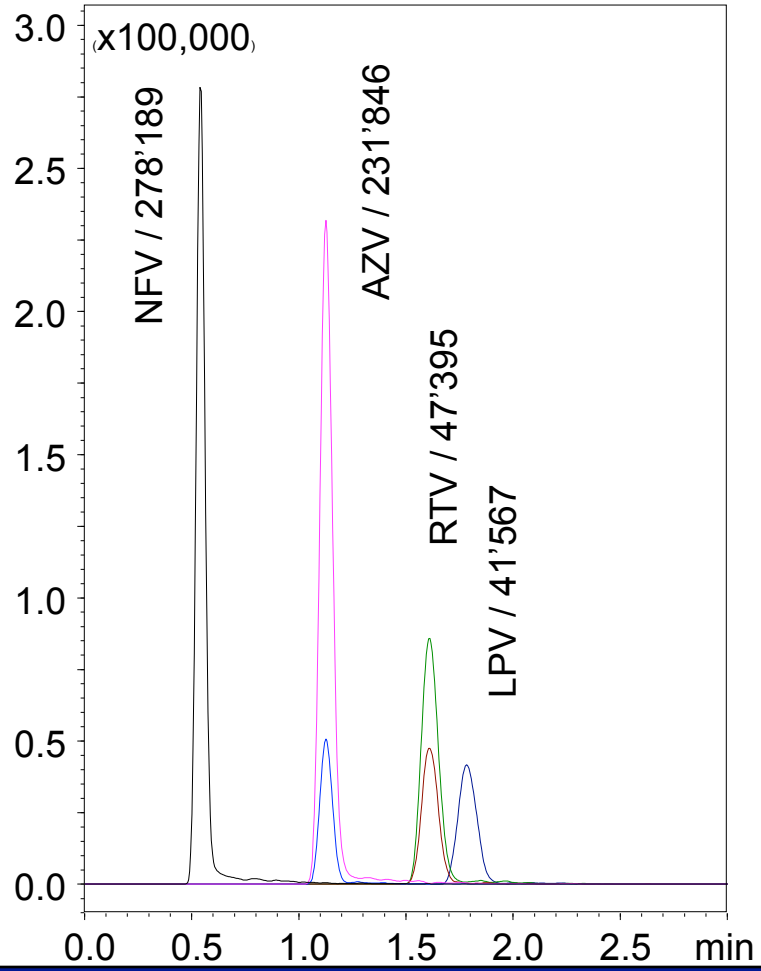


Column: HALO C18, 2.7um
2.1 mm i.d. x 100 mm
Flow rate: 450 µL/min, T = 50 °C

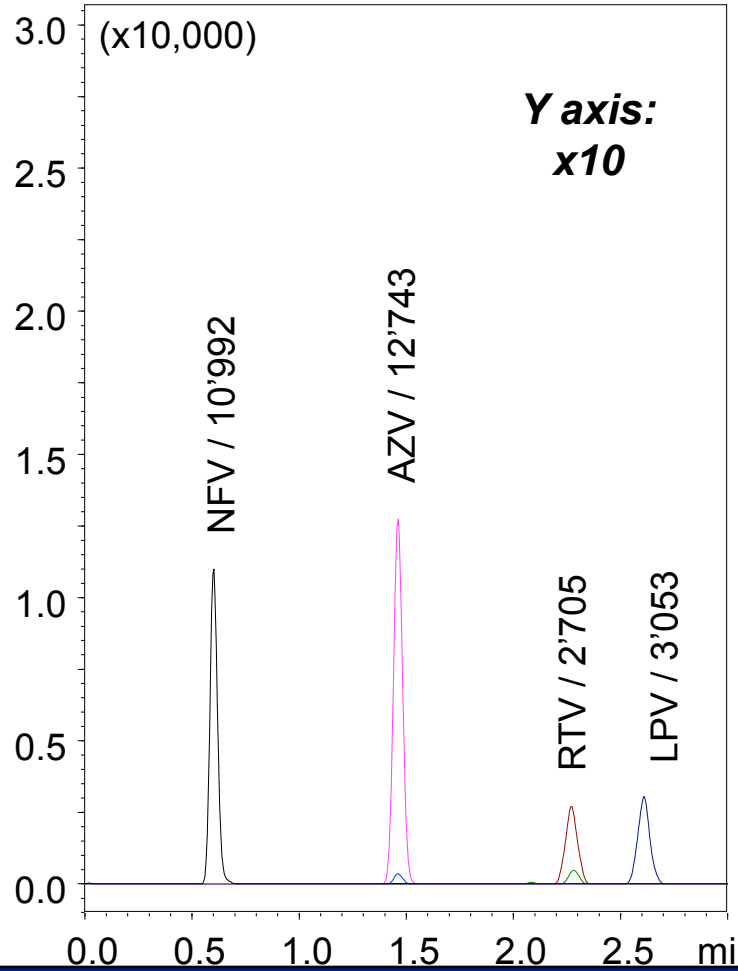


Protease Inhibitors
From 2 mm i.d. to 0.3 mm i.d. column

Column: HALO C18, 2.7um
0.3 mm i.d. x 100 mm
Flow rate: 15 µL/min, T = 50 °C

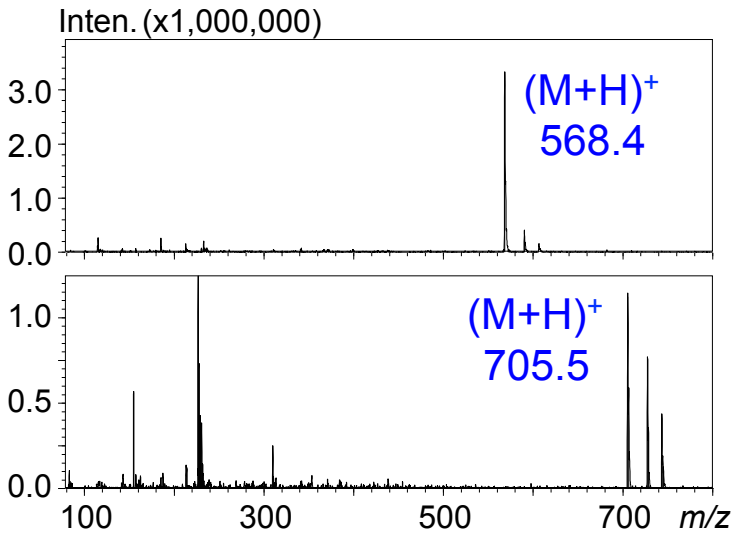


Column: HALO C18, 2.7um
2.1 mm i.d. x 100 mm
Flow rate: 450 µL/min, T = 50 °C

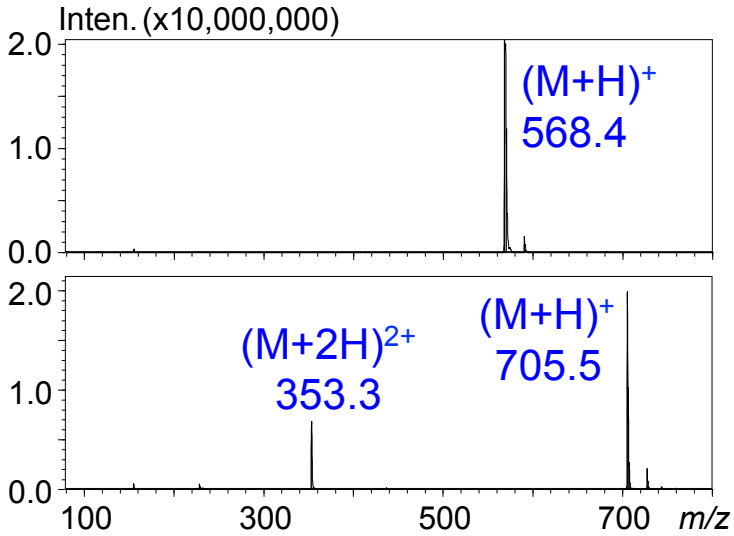


Effect of Flow Rate on ESI Charge State

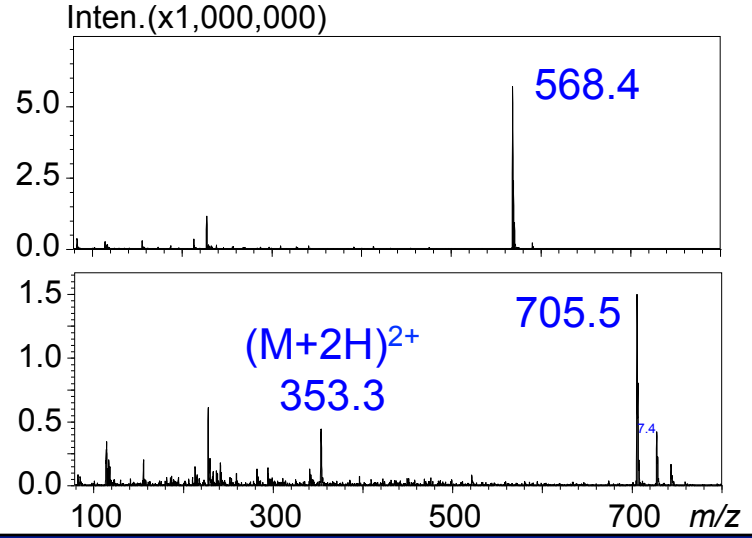
2.1mm column, 312.5ng/mL x 1µL



0.3mm column, 312.5ng/mL x 1µL



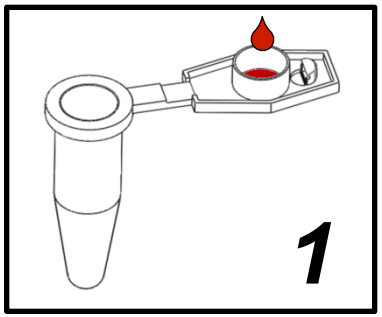
0.3mm column, 15.6ng/mL x 1µL

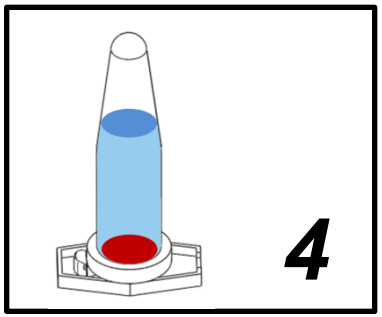


Dried Blood Spot in Tube Format

Blood deposition

15 µl



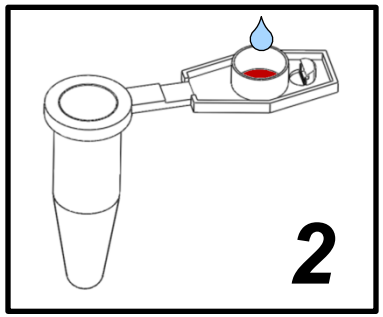


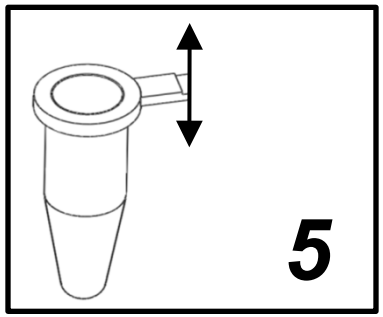
Contact

Ultrasound

Wetting phase + IS

15 µl



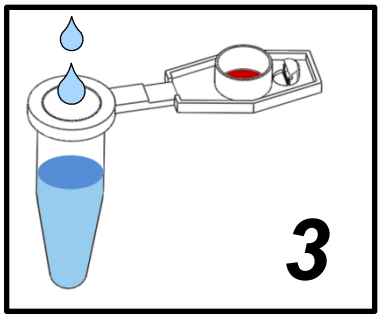


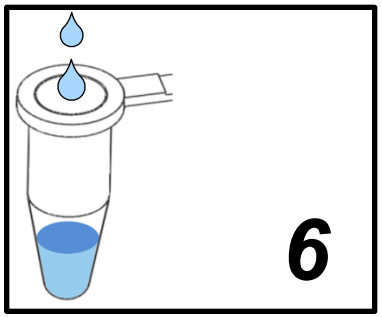
Cut the lid

Evaporation

Extraction phase

350 µl

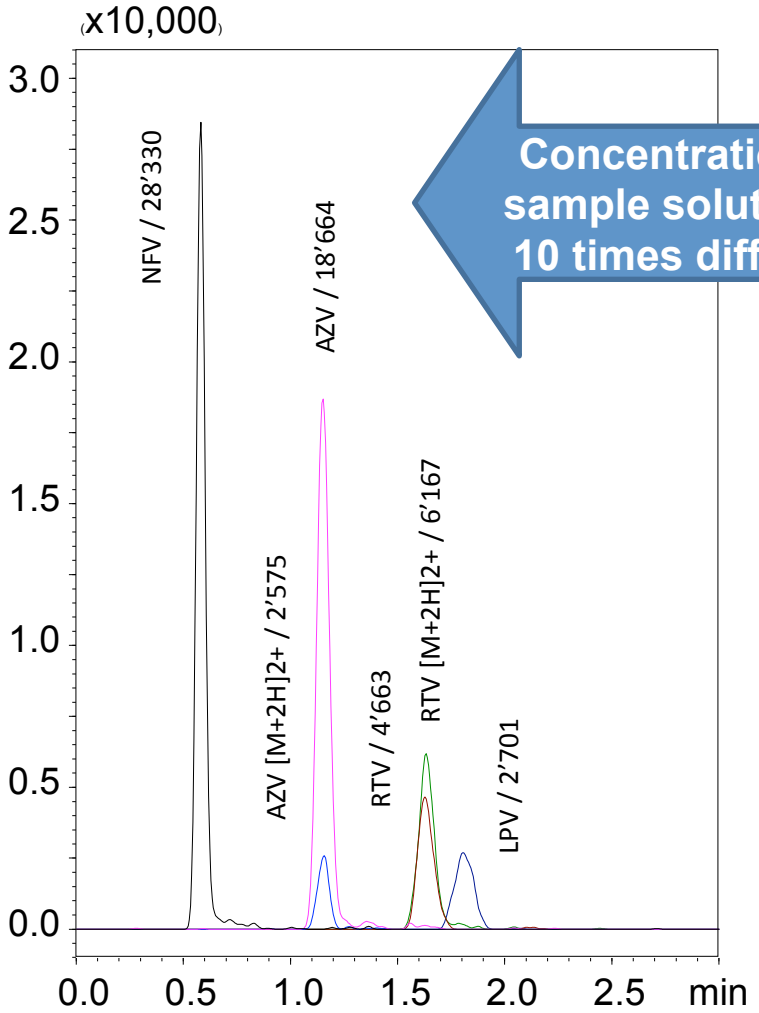




Reconstitution

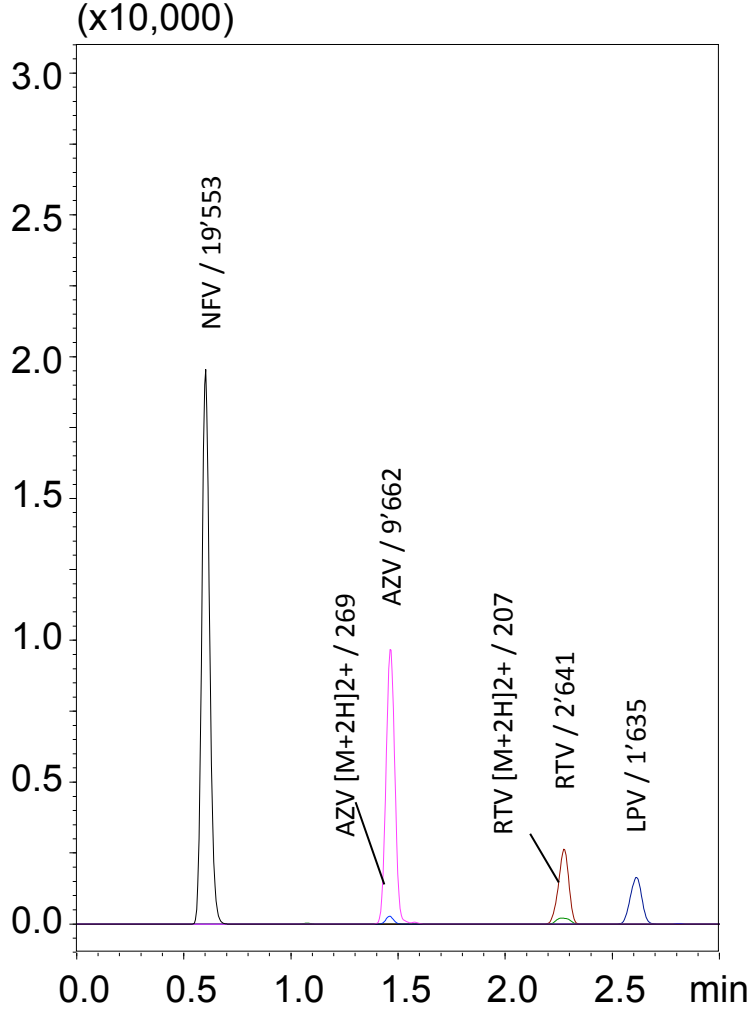
15 µL plasma sample on filter paper
(25ng/mL spiked plasma)

Column: HALO C18, 2.7um
0.3 mm i.d. x 100 mm



Concentration of sample solution is 10 times different

Column: HALO C18, 2.7um
2.1 mm i.d. x 100 mm



Gain in Sensitivity 0.3 mm versus 2.1 mm i.d. columns and Matrix Effects

Expected theoretical gain = 49 fold

Signal ratio 0.3/2.1 mm		NFV	AZV*	RTV*	LPV
25 ng/mL	std-DBS	30.1	18.0	11.4	16.8
25 ng/mL	plasma-DBS	13.2	19.7	17.5	17.6

Signal ratio 0.3/2.1 mm		NFV	AZV*	RTV*	LPV
500 ng/mL	std-DBS	32.2	17.3	14.0	15.7
500 ng/mL	plasma-DBS	18.8	20.1	17.8	17.3

*Only singly charge SRM transition
n=3, CV< 10%

Conclusions

- In mass spectrometry based pharmacometabolomics several dimensions needs to be take in account in particular the chemical space (*m/z*, -/+ detection and response factors).
- As chromatographic performance is important fast acquiring MS are mandatory with fast polarity switching
- Dual LC systems are an elegant way to increase throughput or to expend the numbers of analytes to monitor.
- Column i.d. reduction still is a promising approach when limited sample is available and to achieve good sensitivity. However, differences is MS response at different flow regimes with standards or extracts can be observed and should be further investigated.

Acknowledgments

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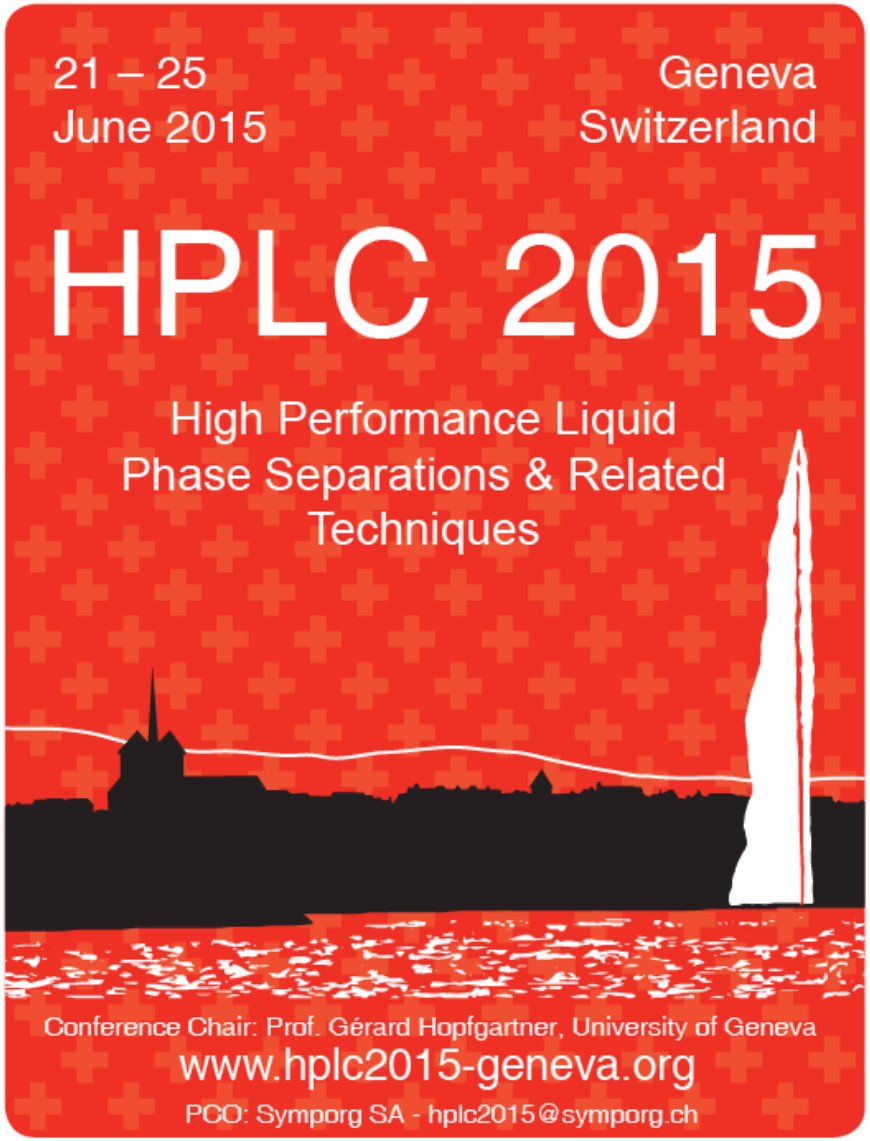
SystemsX
The Swiss Initiative in Systems Biology

LSMS
AntivirX
University of Massachusetts Medical School
SIB



SCS Swiss Chemical Society — Division of Analytical Sciences

CICG Centre International de Conférences Genève

Topics

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Bioanalysis (small molecules, proteins)

Important Dates

Opening of abstract submissions and registration:
1 Octobre 2014

Closing of abstract submissions for oral contributions
5 January 2015

Closing of poster abstract submissions and Early Bird registration
1 April 2015

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