UNIVERSITÉ DE GENÈVE

LSMS

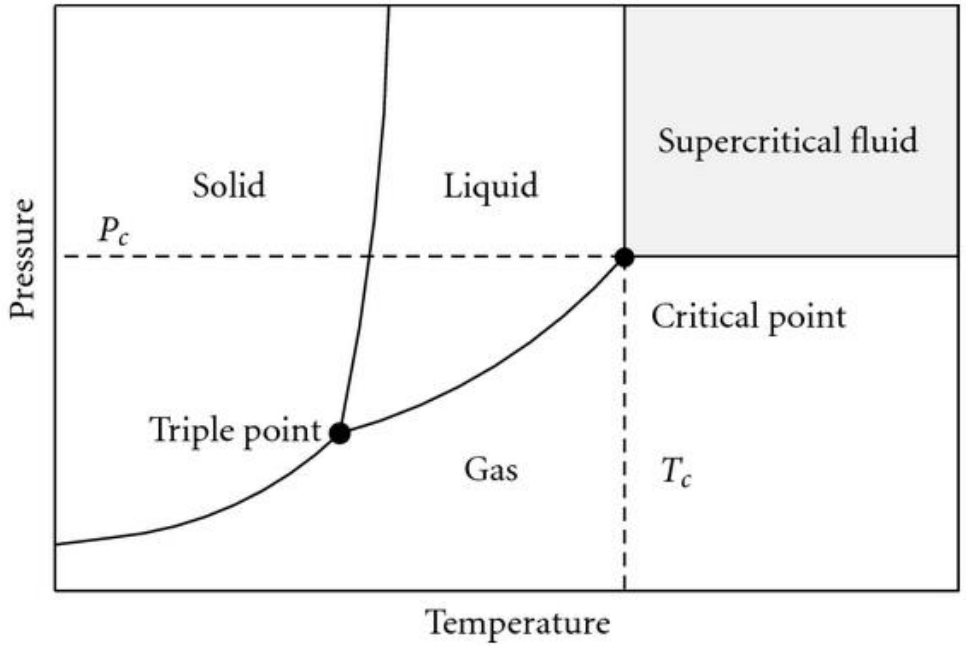
Supercritical Fluid Chromatography Coupled to Mass Spectrometry for the Analysis of Pharmaceuticals, Metabolites and Lipids in Biological Fluids

Maria Girard, Patrick Mueller, and Gérard Hopfgartner

Life Sciences Mass Spectrometry, CHIAM, University of Geneva, Switzerland

SFC User Meeting
6th – 7th of November 2024
AstraZeneca PGN Conference Center, Mölndal, Sweden

Supercritical Fluids: Facts



Fluid	T_c °C	P_c [MPa]	ρ_c [g.cm^{-3}]
CO_2	31.3	7.38	0.47
N_2O	36	7.34	0.45
nC4H10	152.0	3.80	0.23
C7H8	320.8	4.21	-
CCl_2F_2	111.8	4.12	0-56
CHF_3	25.9	4.75	0.52
NH_3	132.5	11.40	0.24

1822: Baron Charles Cagniard de la Tour

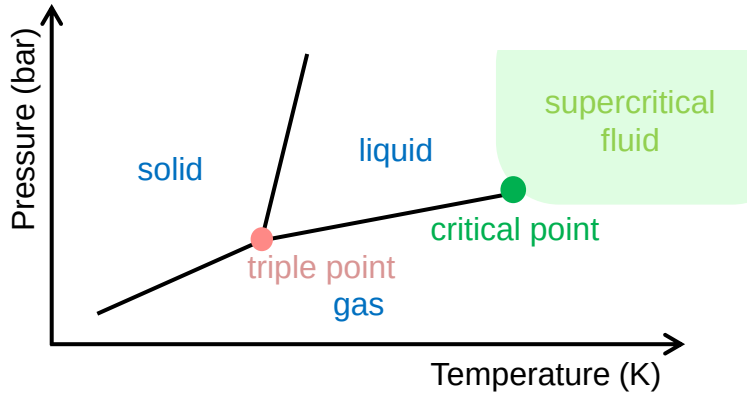
1985-1990:
Supercritical Fluid Extraction and Super Critical Fluid Chromatography

Packed and capillary open tube columns

Coupling with Mass Spectrometry with EI

Particular properties of supercritical fluids

Phase diagram of a pure substance



	ρ [g/cm³]	η [cP]	D [m²/s]
gas 1 bar, 15-30°C	(0.6 - 2.0) x 10^{-3}	(1.0 - 3.0) x 10^{-2}	(1.0 - 4.0) x 10^{-5}
supercritical fluid Tc, Pc	0.2 - 0.5	(1.0 - 3.0) x 10^{-2}	0.7 x 10^{-7}
liquid 1 bar, 15-30°C	0.6 - 1.6	0.2 - 3.0	(0.2 - 2.0) x 10^{-9}

High density
Similar to a liquid

Suitable solvent properties

Low viscosity
Similar to a gas

Low pressure drop

High diffusivity

Enhances kinetic performances
Higher optimal mobile phase velocity

Supercritical fluids and chromatographic performances

$$HEPT = A + \frac{B}{u} + C \cdot u$$

Resolving power of the column [m]

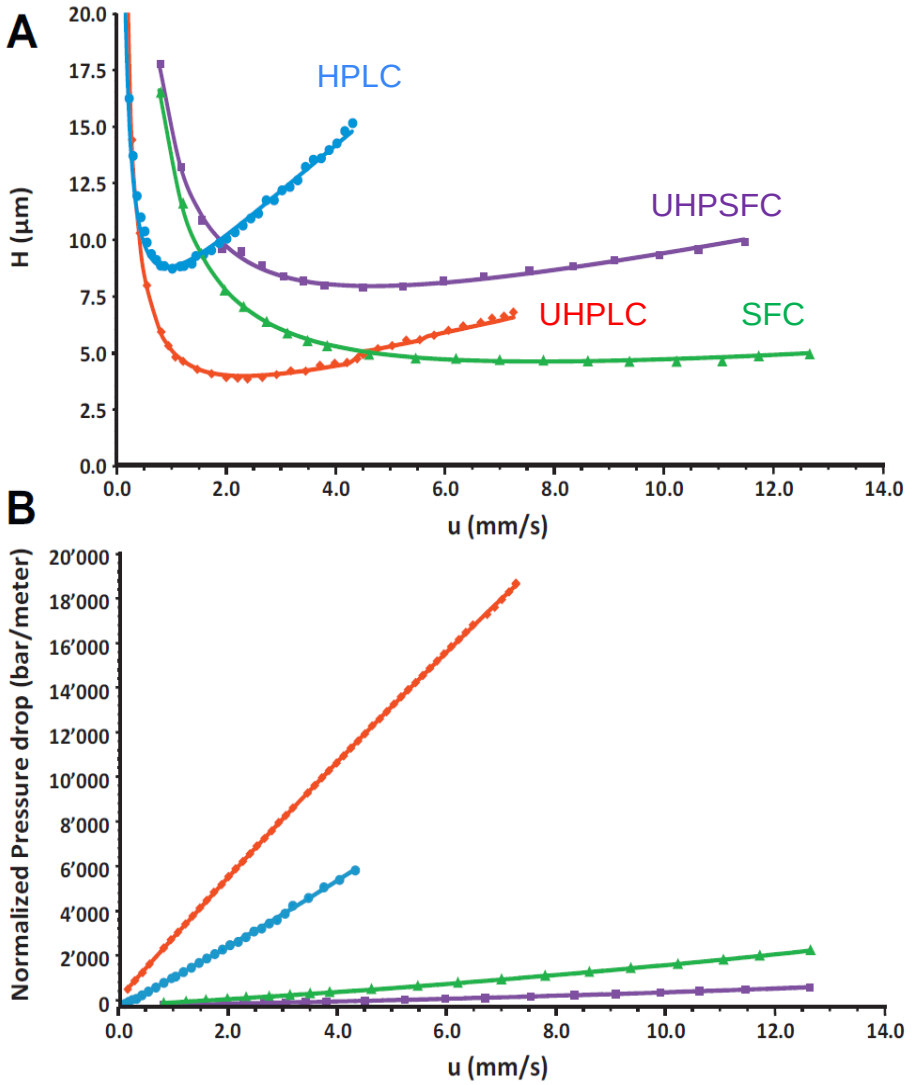
Linear velocity [m.s^{-1}]

Eddy diffusion parameter, related to channeling through a non-ideal packing [m]

Diffusion coefficient of the eluting particles in the longitudinal direction, resulting in dispersion [m^2.s^{-1}]

Resistance to mass transfer coefficient of the analyte between mobile and stationary phase [s]

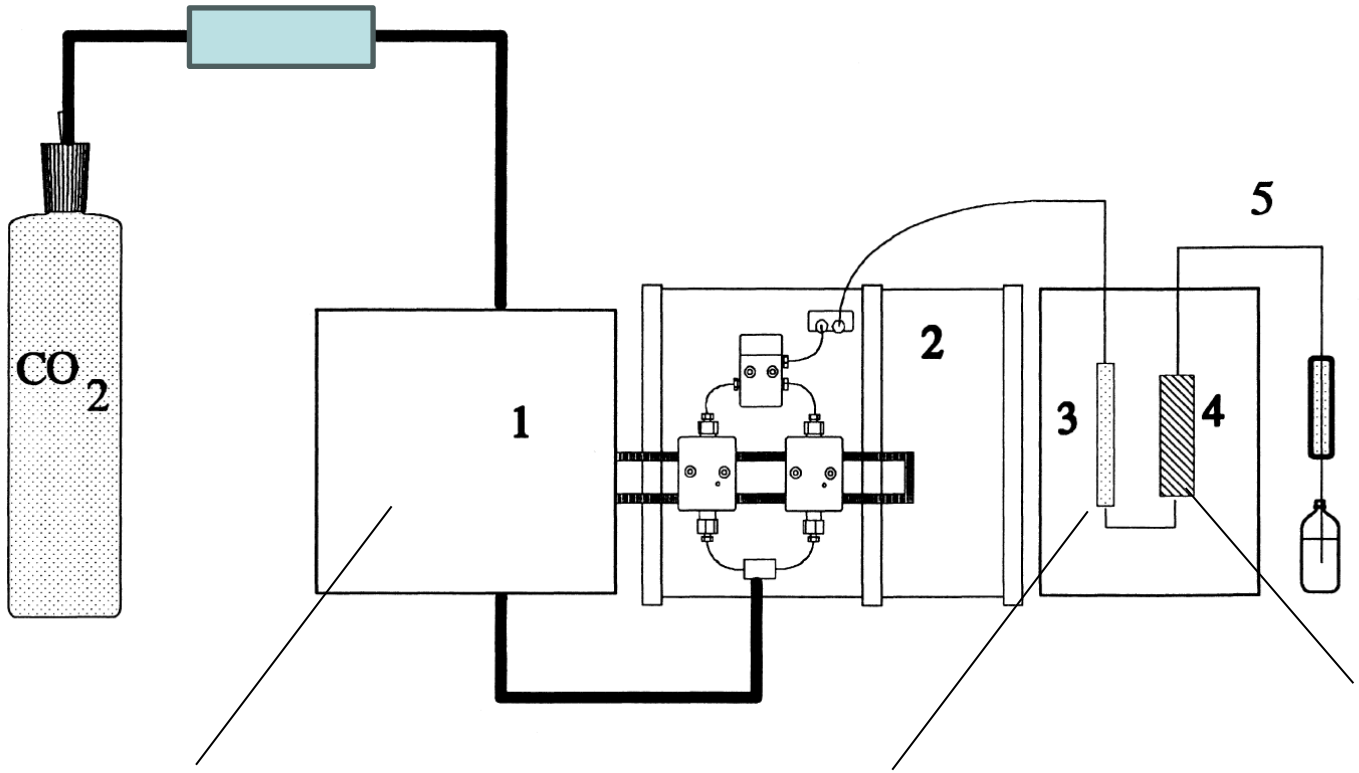
Van Deemter curves



Home-made Supercritical Fluid Systems (1990)

Charcoal Trap

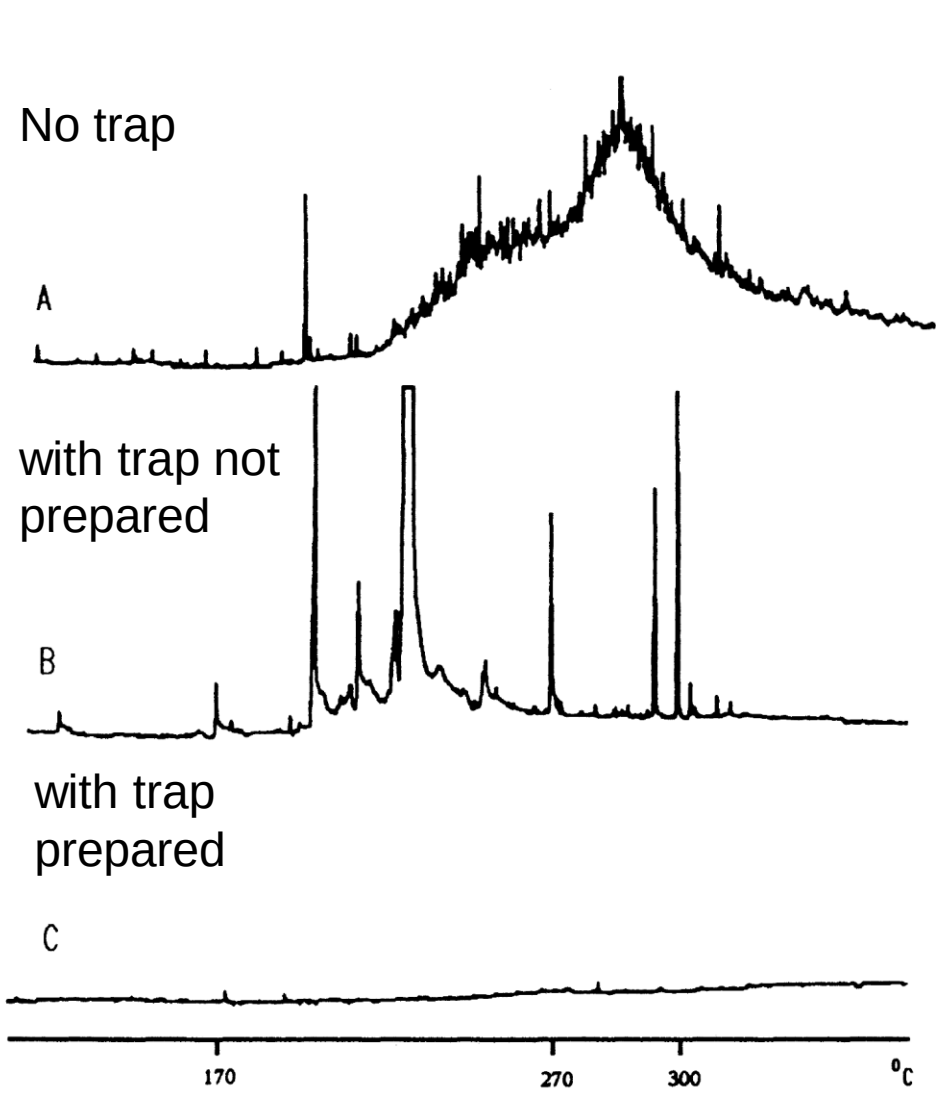
CO_2 Quality: 3.5 UV
4.5 MS

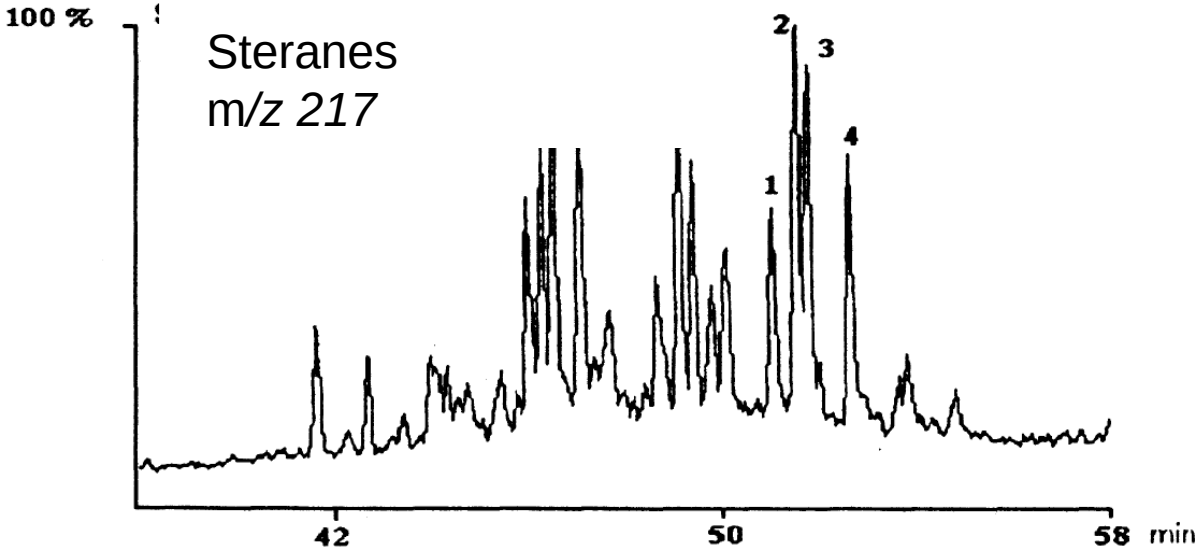


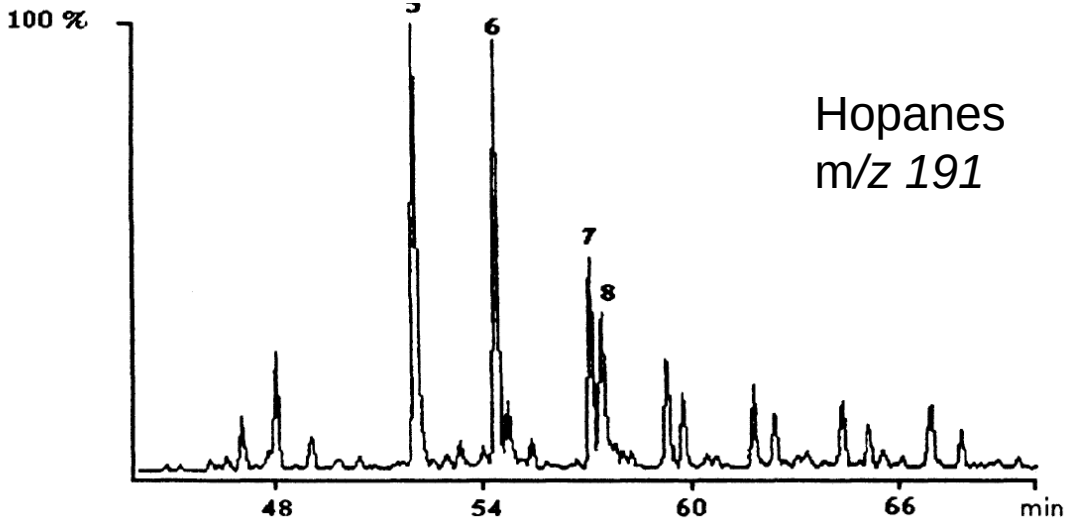
Cooling Unit T < 4 °C

Column T > 30 °C

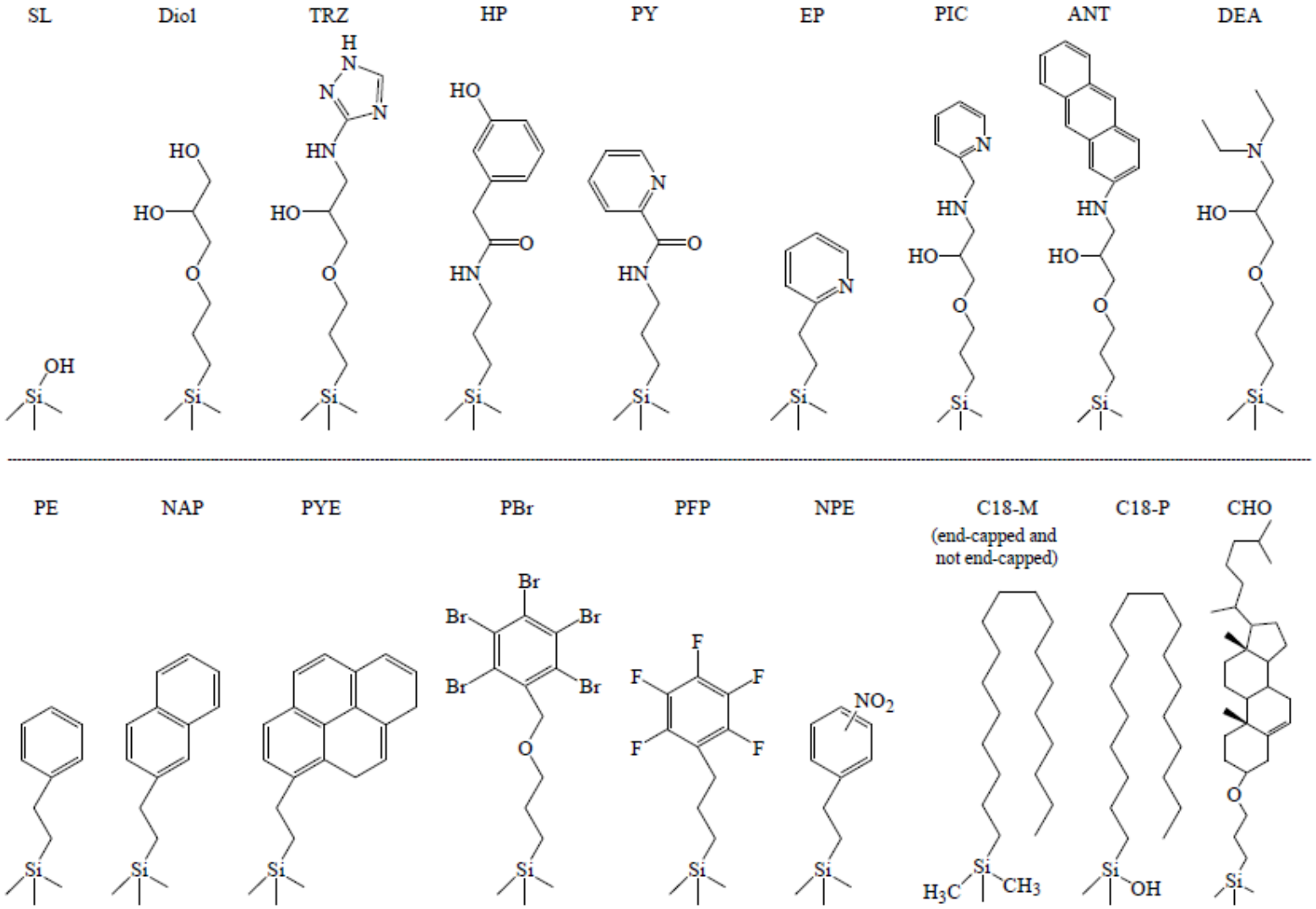
Apolar Contaminants from CO_2 by GC-FID and HRMS





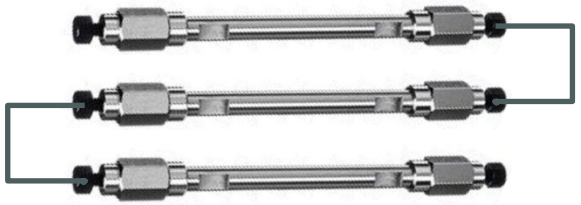


Stationnary Phases LC and SFC

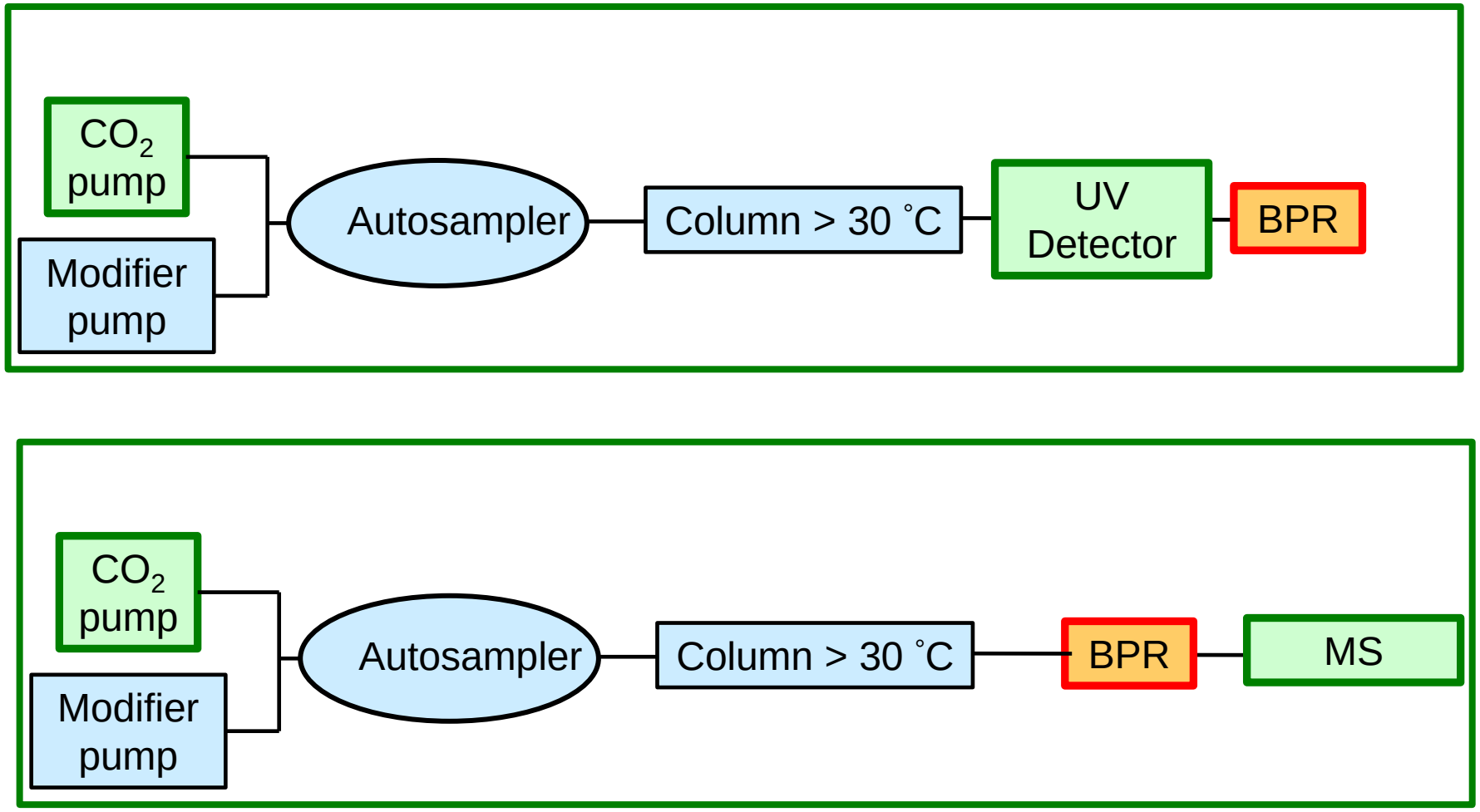


LC (Pressure)

SFC (Pressure)

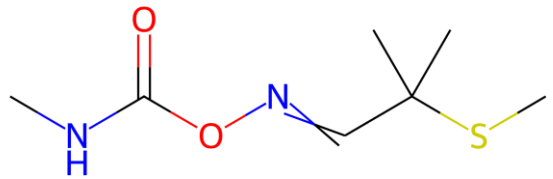


Supercritical Fluid Chromatography (SFC) with Ultra-Violet or MS Detection: Instrumental Setup

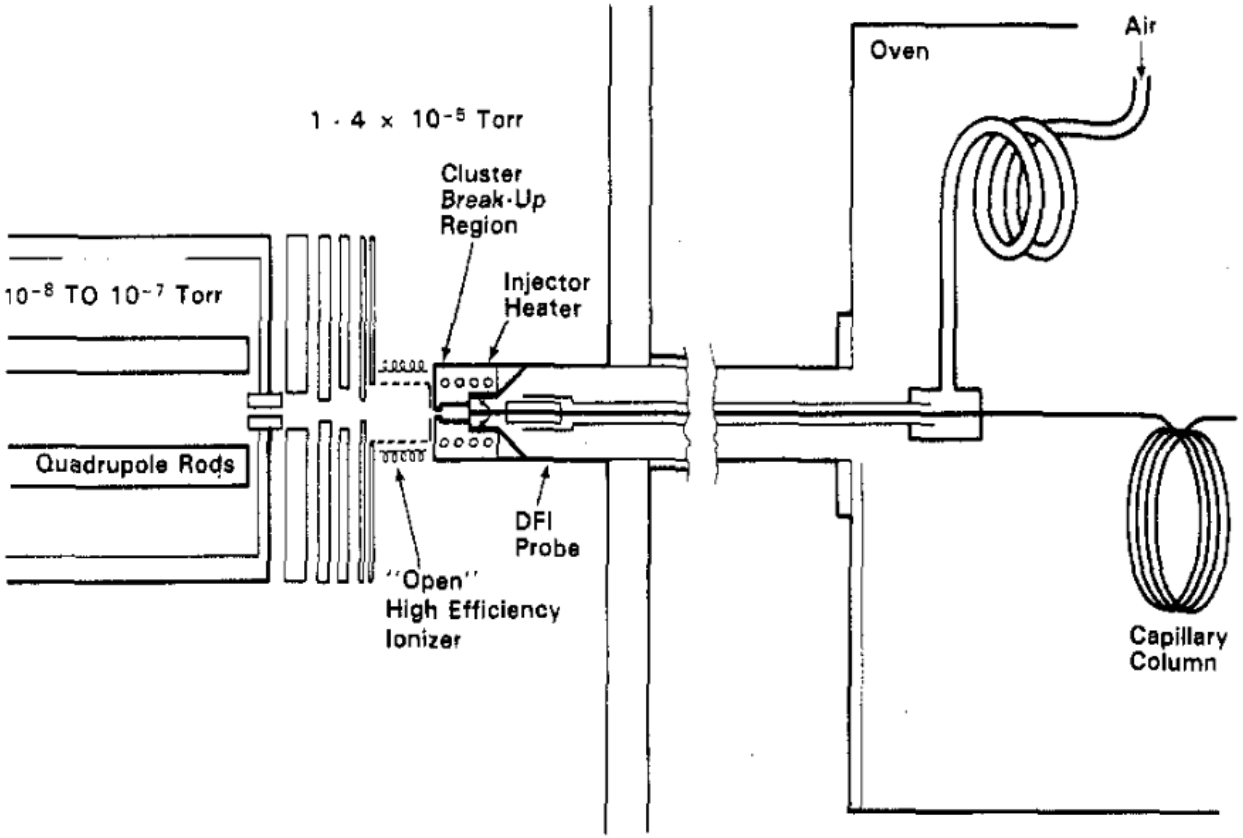


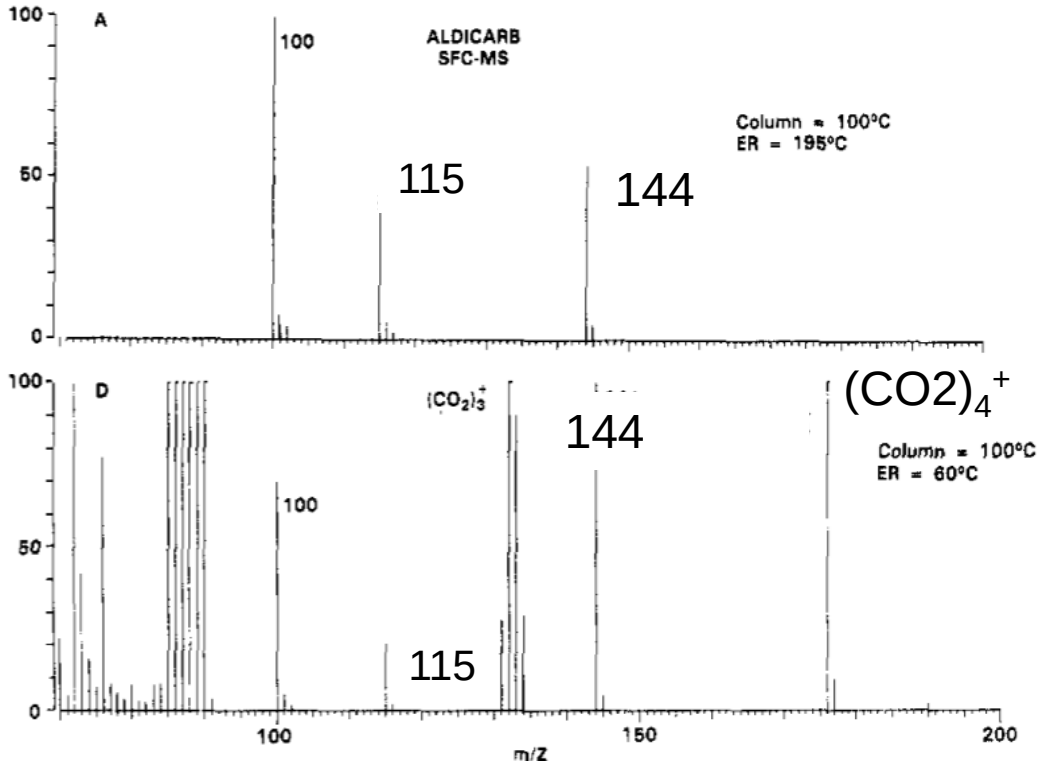
BPR: Back Pressure Regulator > 10MPa

SFC-MS with packed columns and Electron Ionisation



Aldicarb MW = 190 Da



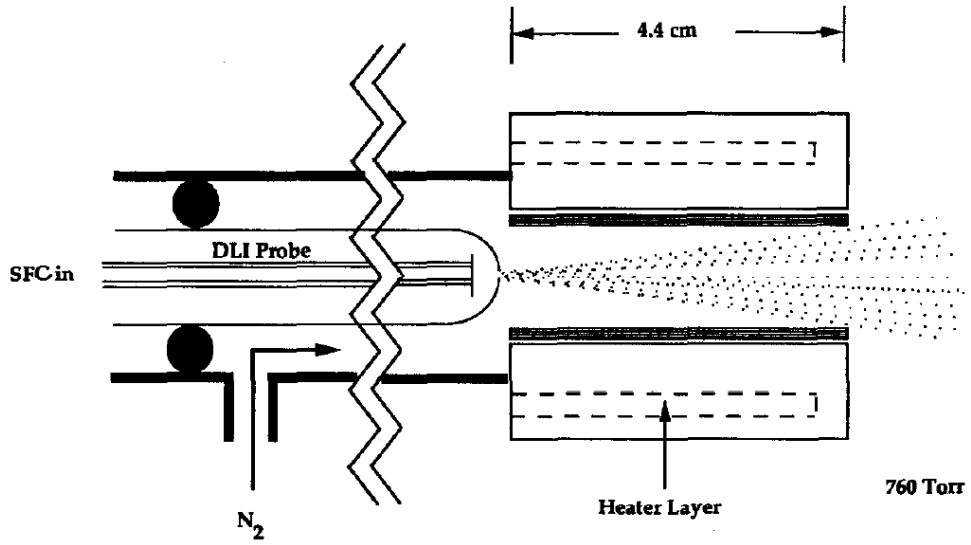


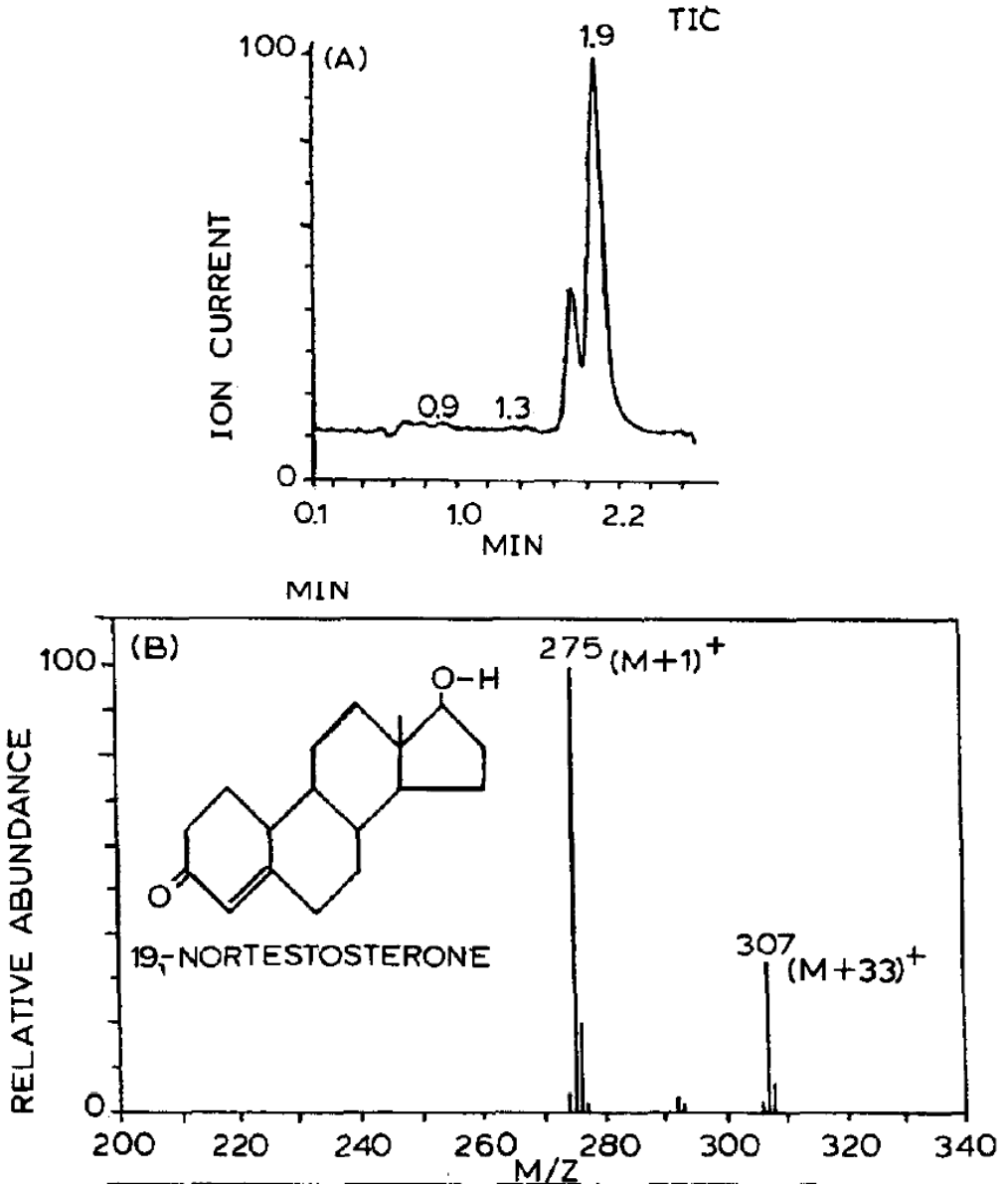
Journal of Chromatography, 511 (1990) 257–270
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 412

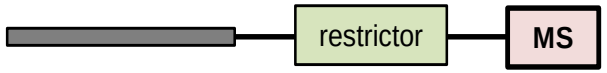
Packed-column supercritical fluid chromatography–mass spectrometry and supercritical fluid chromatography–tandem mass spectrometry with ionization at atmospheric pressure[a]

ERIC HUANG and JACK HENION*
Drug Testing and Toxicology, Cornell University, 925 Warren Dr., Ithaca, NY 14850 (U.S.A.)
and
THOMAS R. COVEY
Sciex Inc., 55 Glen Cameron Rd., Thornhill, Ontario L3T 1P2 (Canada)
(Received December 12th, 1989)

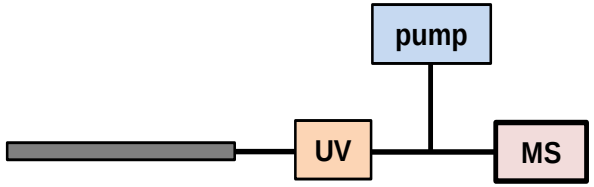




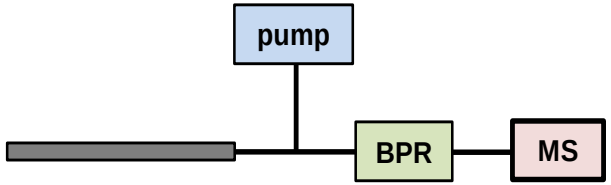
Coupling of Supercrticital Fluid Chromatography and Mass Spectrometry



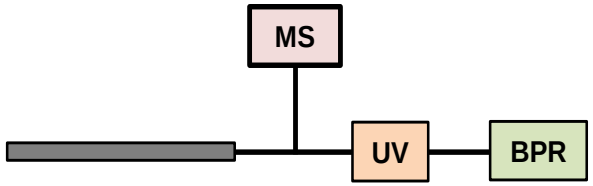
A: Direct fluid introduction



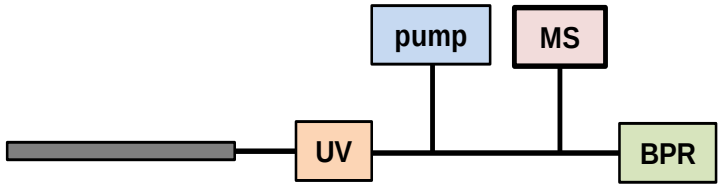
B: Pressure regulation through pumping



C: Full flow through BPR with make-up pump



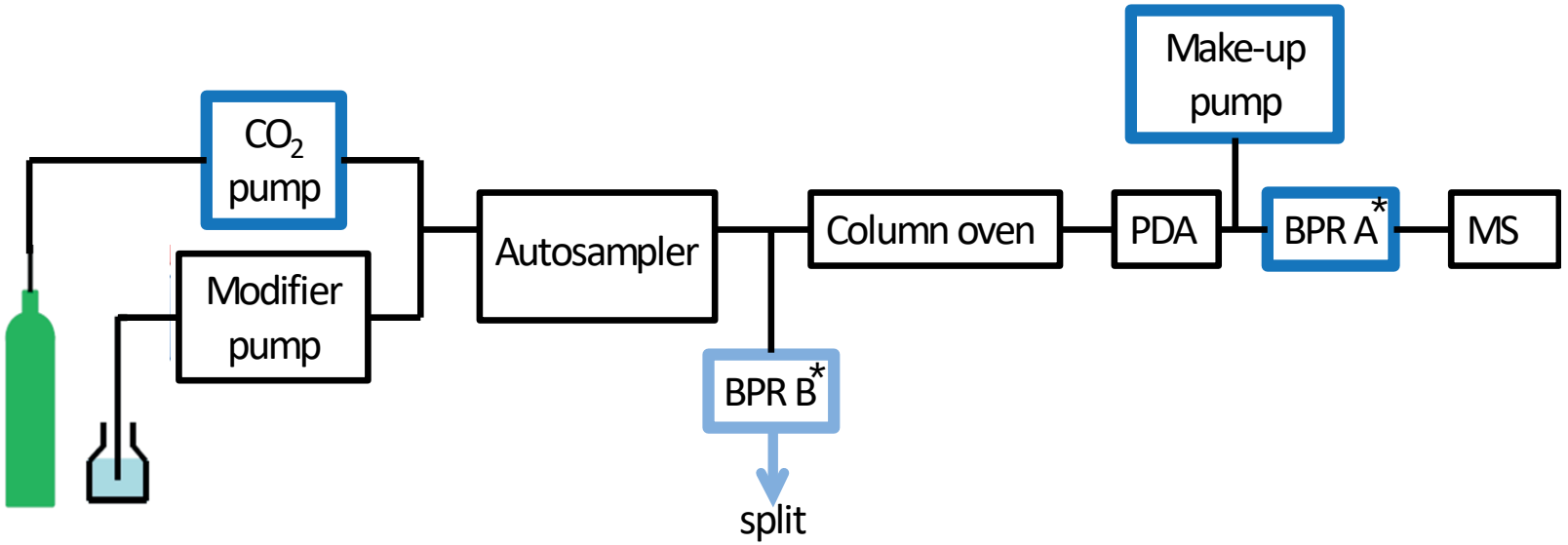
D: MS split before UV without make-up pump.



E: MS split after UV with make-up pump

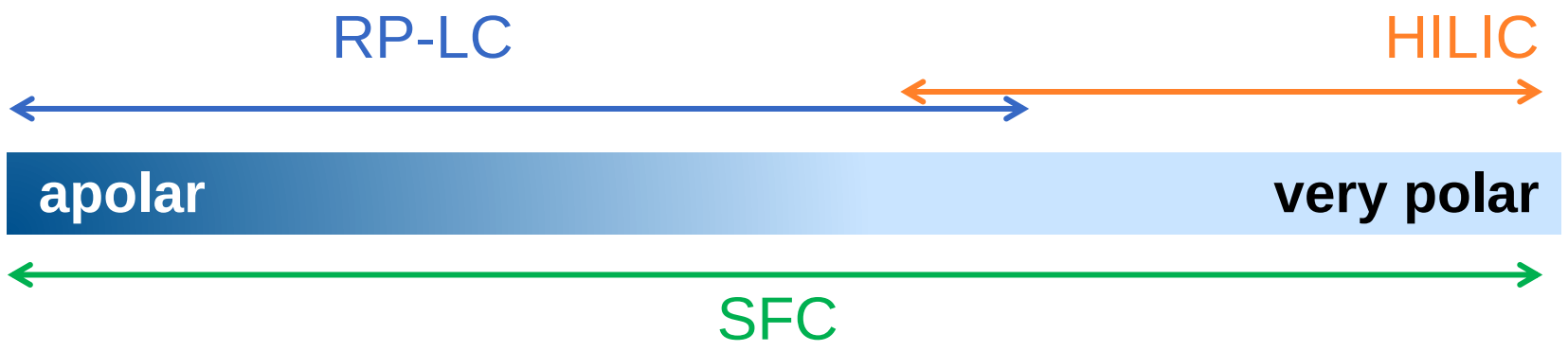
https://doi.org/10.1007/s00216-020-02715-4

Interfacing Supercritical Fluid Chromatography and Electrospray Mass Spectrometry

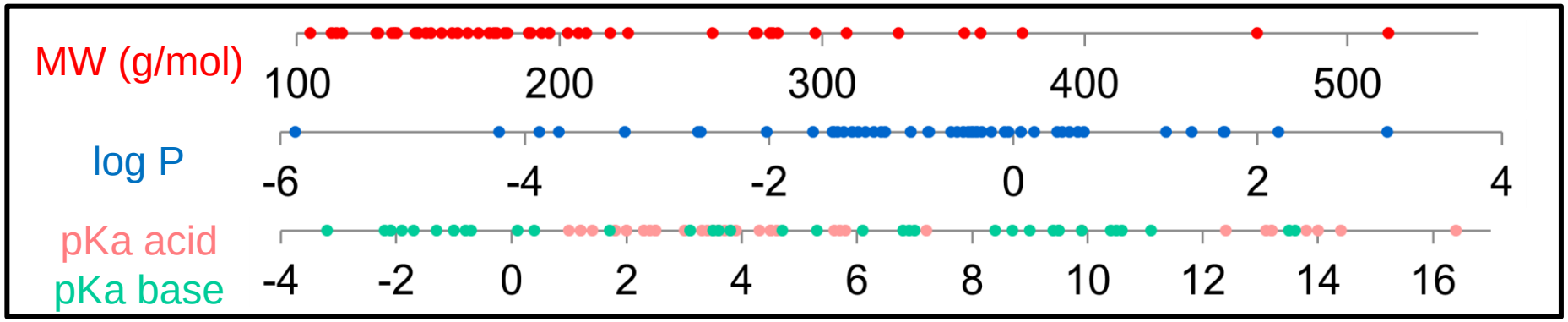


Comparison of Chromatographic Separation Modes

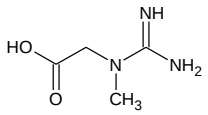
	RP-LC	***HILIC***	***SFC***
Stationary phase	**C18**	**amide**	**diol**
Mobile phase gradient	**H_2O / MeOH**	**H_2O / ACN**	**CO_2 / MeOH**

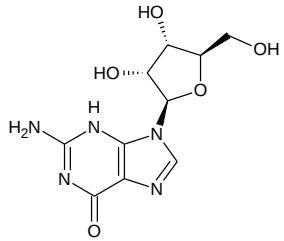


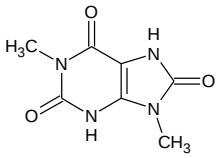
Test Mix of 51 Analytes Representative of Chemical Diversity of HMDB Metabolites

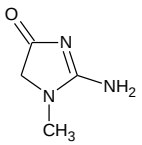


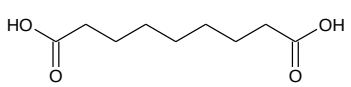
- Amino acids, peptides and analogues
- Nucleosides, nucleotides and analogues
- Aromatic heteromonocyclic compounds
- Aliphatic compounds
- Organic acids and derivatives
- Steroids and steroid derivatives
- Lipids
- Alkaloids and derivatives
- Carbohydrates and their conjugates

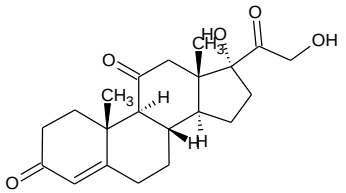

creatine

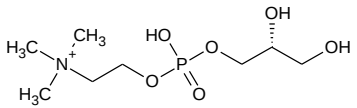

guanosine

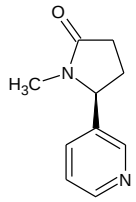

1,9-dimethyluric acid

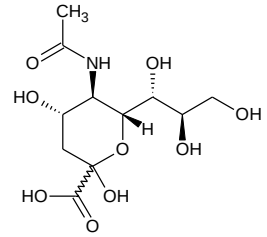

creatinine


azelaic acid


cortisone

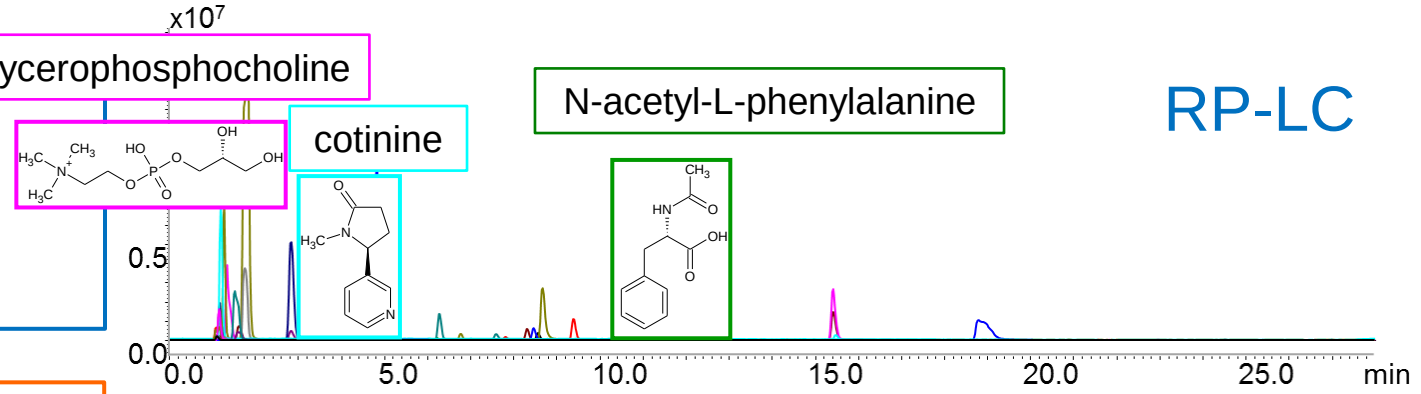

glycerophosphocholine


cotinine

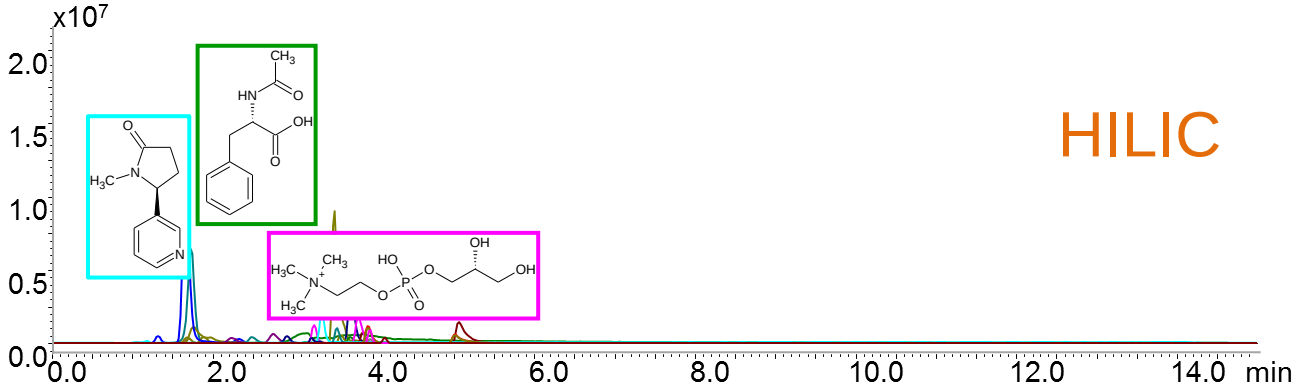

N-acetylneuraminic acid

Comparison of Chromatographic Separation Modes

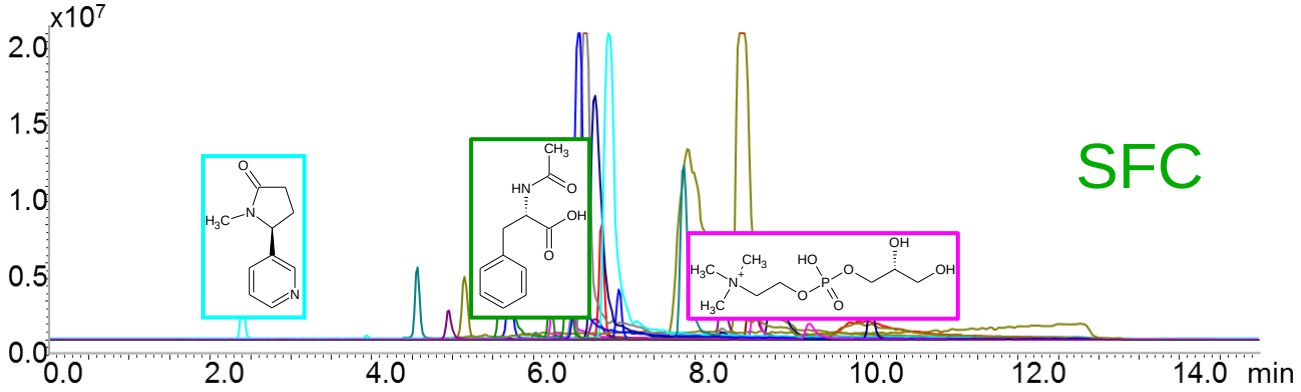
X select HSS T3 XP
2.1x150mm, 2.5 µm
A: *H_2O + 5 mM NH_4FA, pH = 3 (with FA)*
B: *MeOH 5 - 95% in 28 min*
Flow: *0.3 mL/min,* ***T°C*** *40°C*



X Bridge Amide (Waters)
2.1x100mm, 3.5 µm
A: *H_2O/ACN, 95:5, v/v , + 20 mM NH_4OH + 20 mM NH_4Ac*
B: *ACN 85 - 2% in 17 min*
Flow: *0.3 mL/min,* ***T°C*** *40°C*

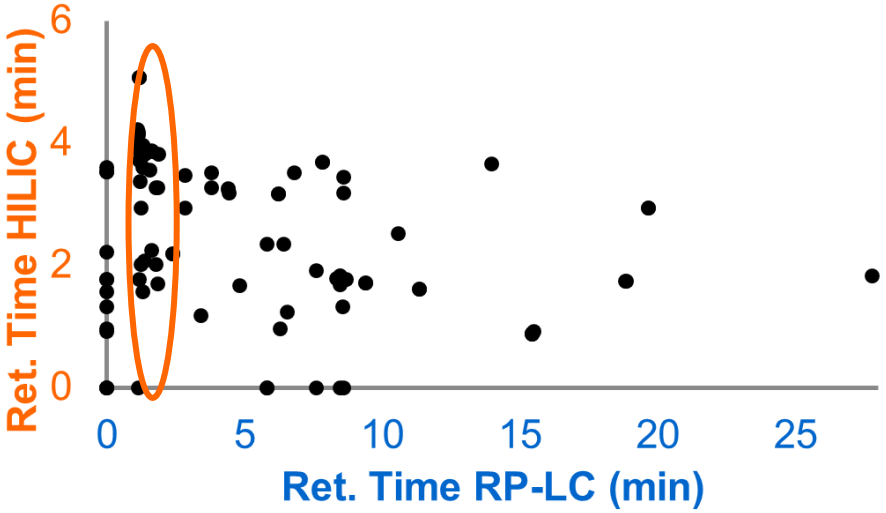


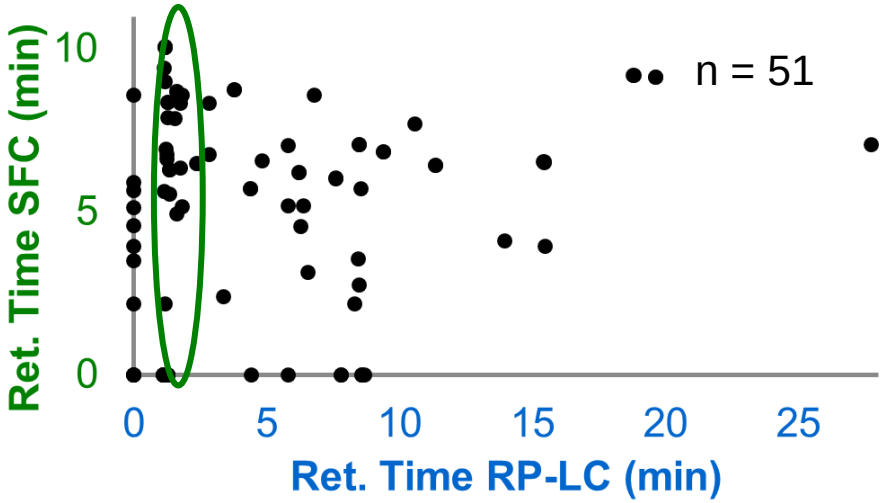
Kromasil Diol (AkzoNobel)
3.0x150mm, 2.5 µm
A: *CO_2*
B: *MeOH + 20 mM NH_4OH 5 - 50% in 15 min*
Flow: *1.5 mL/min*
P: *150 bars,* ***T°C*** *40°C*
Make-up: *0.3 mL/min MeOH/H_2O, 95:5, v/v + 25 mM NH_4Ac*

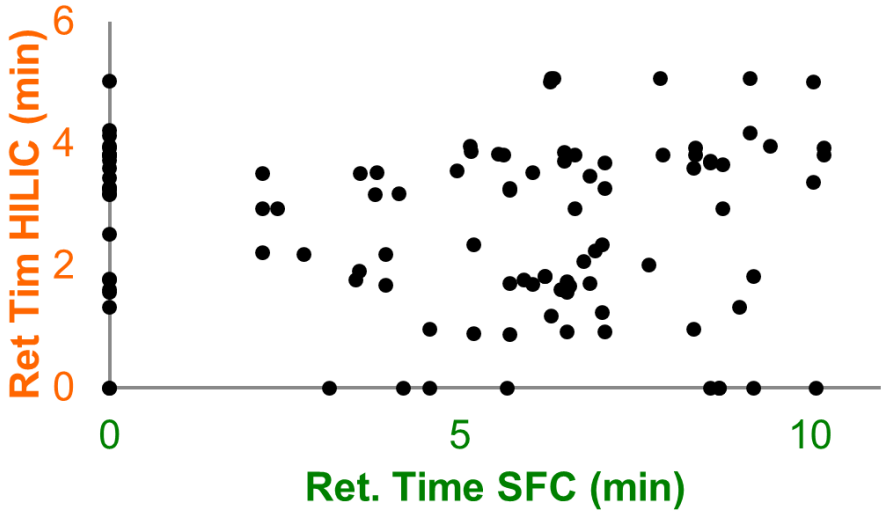


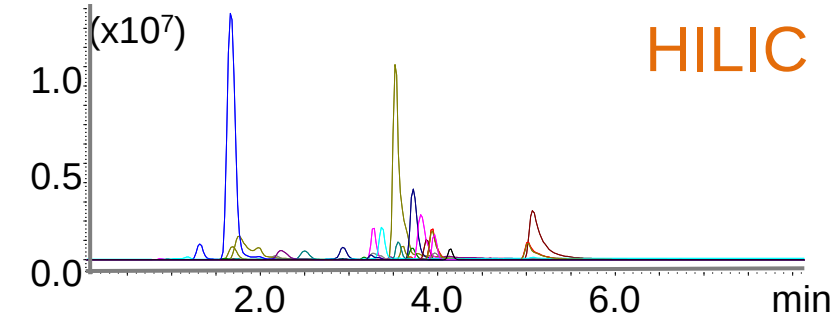
DOI: 10.1007/s00216-021-03808-x

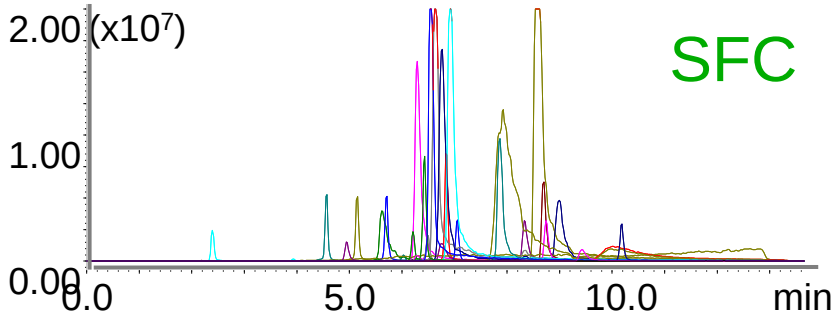
Comparison of Chromatographic Separation Modes



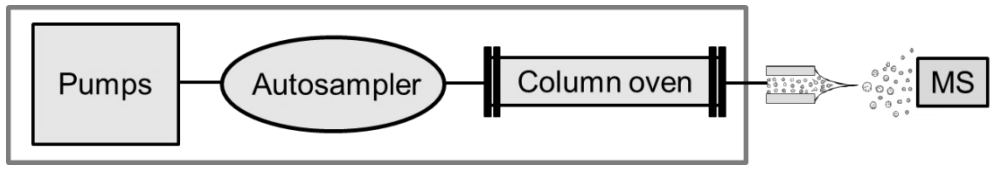




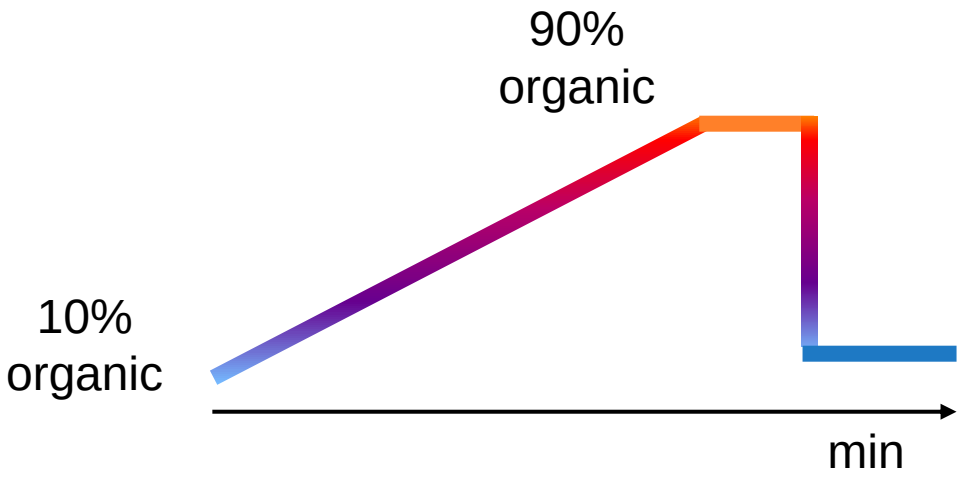




Ionization Constraints in LC-MS



Typical LC gradient



Rayleigh stability limit :

$$q_{Ry} = 8\,\pi\,\sqrt{\varepsilon_0\,\gamma\,R^3}$$

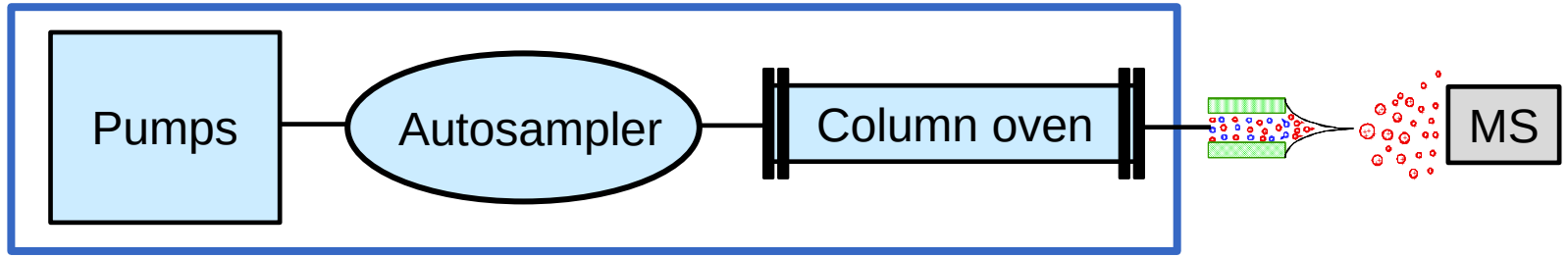
q_{Ry} = droplet charge at Rayleigh limit, C
γ= surface tension of the solvent, N/m
ε_0 = vacuum permitivity, $8.8 \cdot 10^{-12}$ C^2/Nm^2
R = droplet radius, m

solvent	H_2O	MeOH	ACN
γ (N/m)	0.073	0.0226	0.030

- Ionization conditions → dictated by mobile phase composition
  → are changing during the gradient

Liquid and Supercritical Fluid Chromatography and Mass Spectrometry Hyphenation

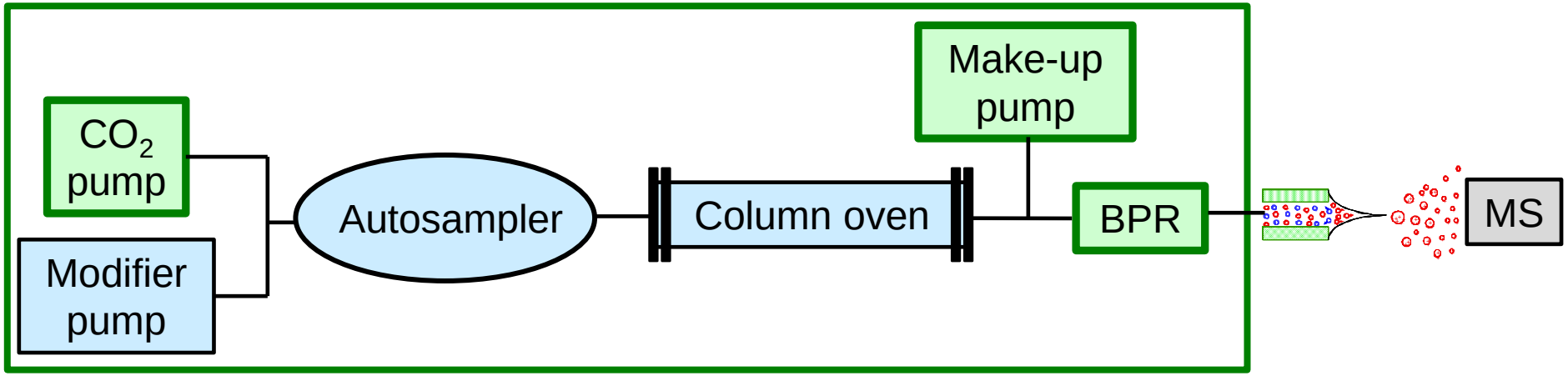
LC



MS response
LC conditions
dependent

Gradient

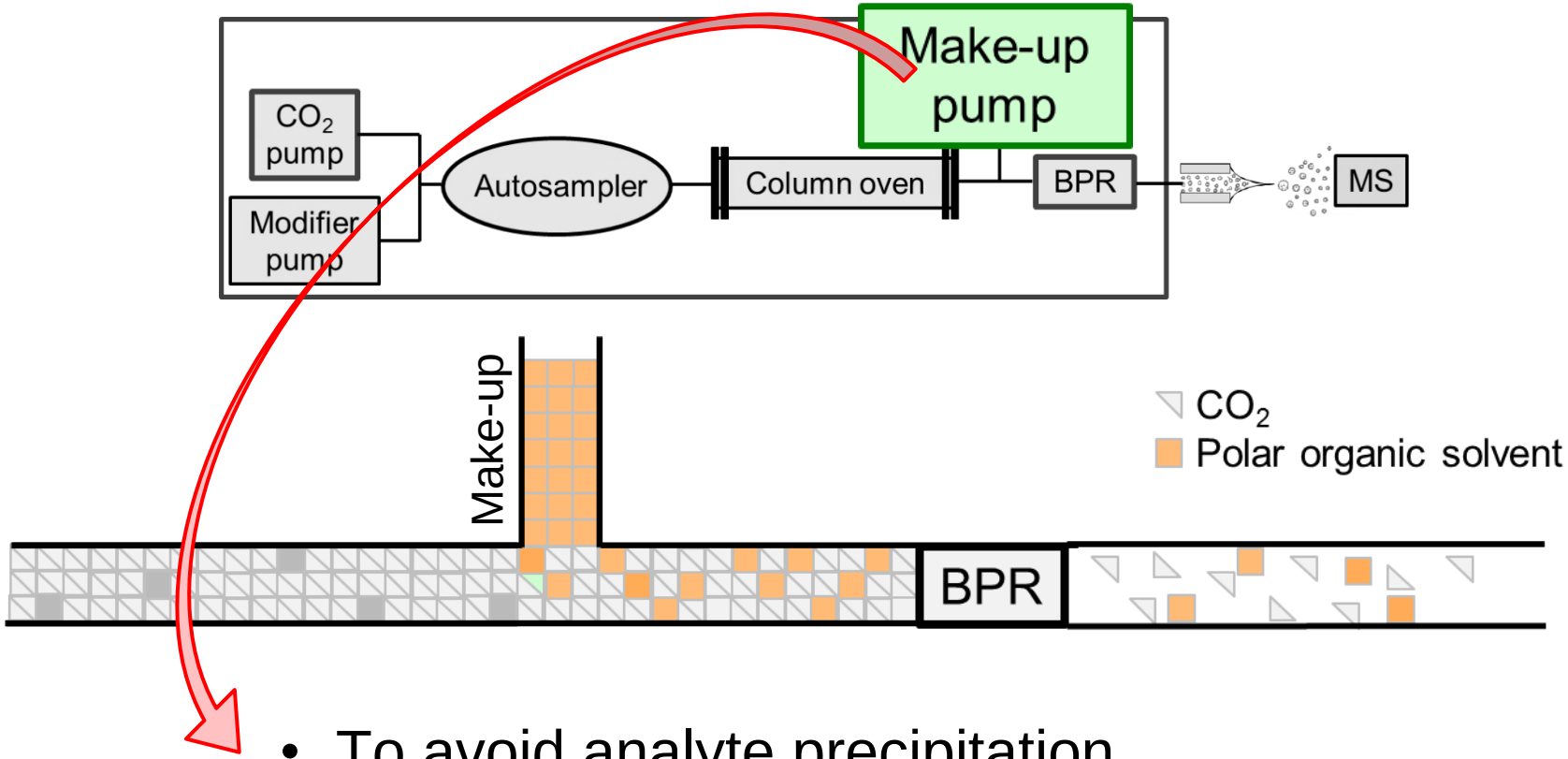
SFC



MS response
Make-up
dependent

Constant

SFC-ESI-MS: Coupling Constraints

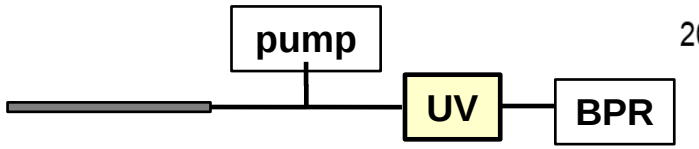


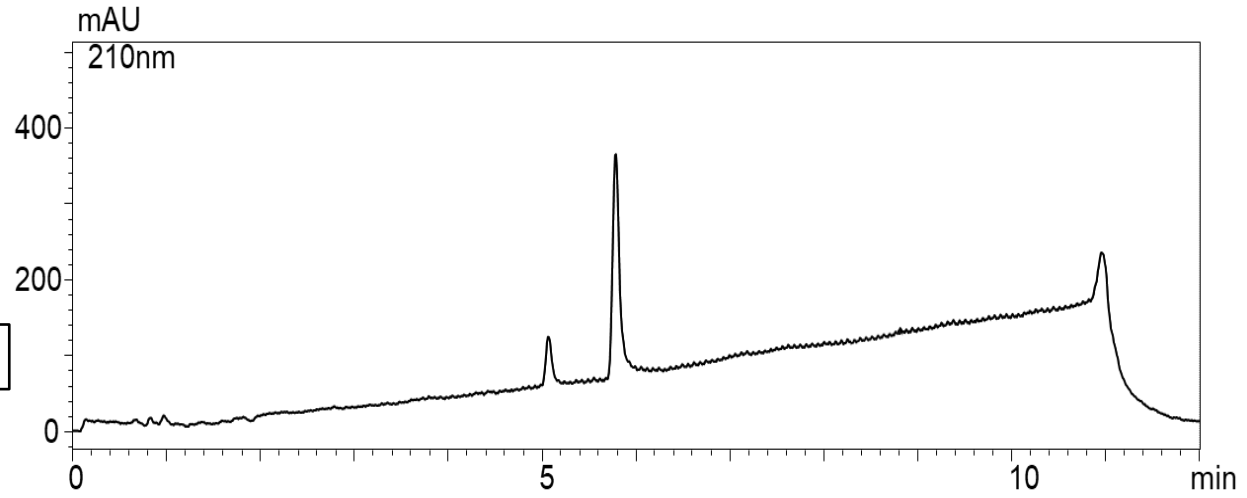
- To avoid analyte precipitation
- To tune ionization

What is the influence of make-up on the ionization ?

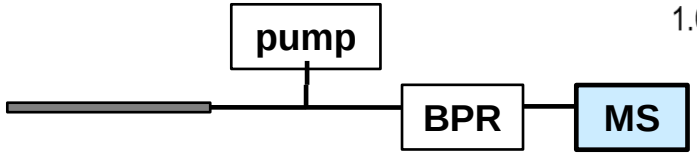
Full-flow introduction and low dead volume BPR

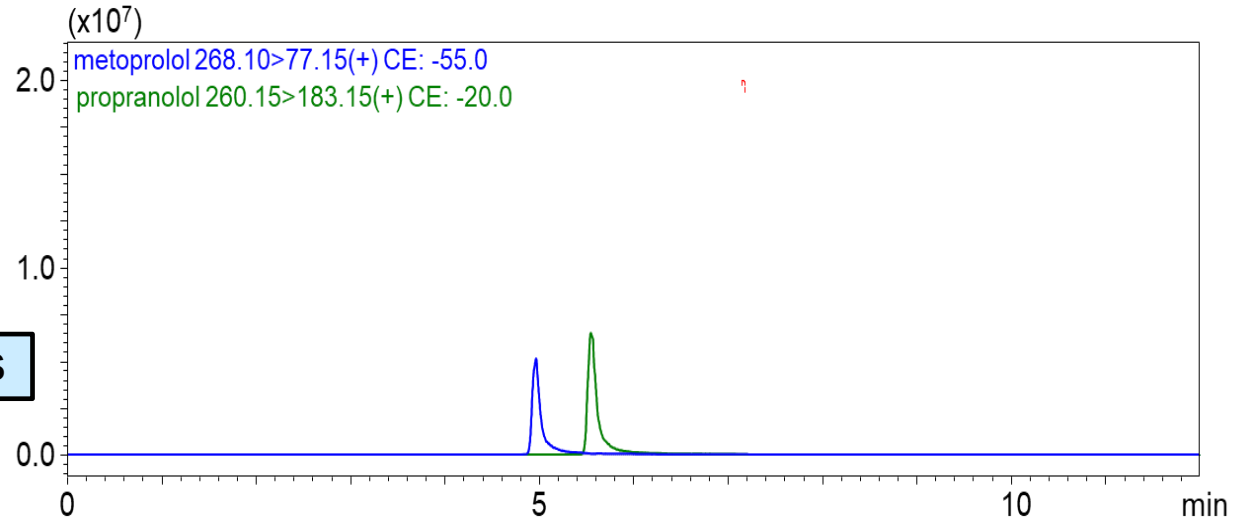
UV
(before BPR)





MS
(after BPR)

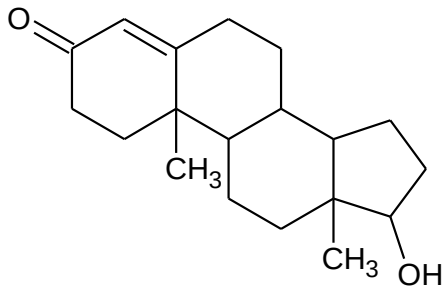




Separation of β-blockers on a diol column

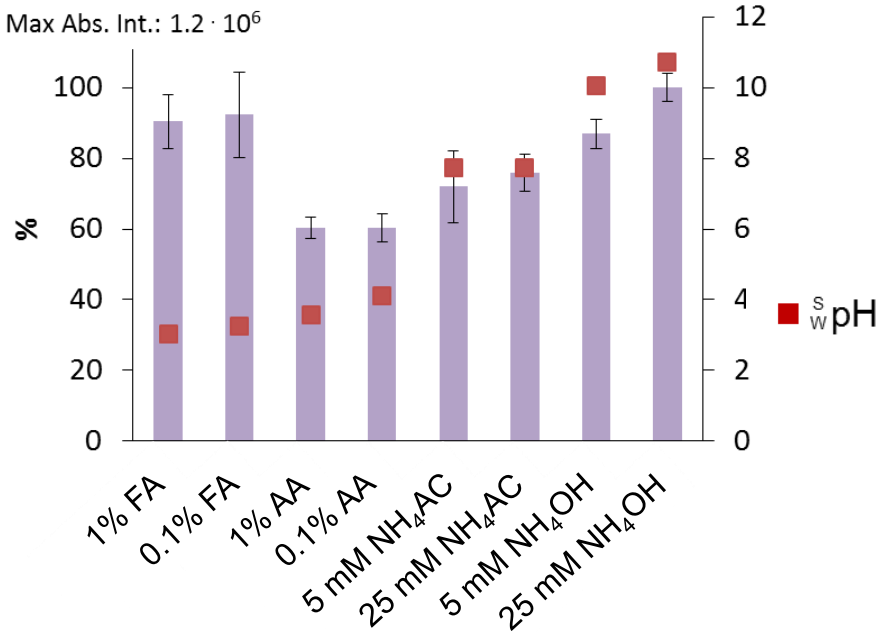
Influence of the Make-up Composition

SRM pos. mode
Stand. Dev., n = 10
% Int.

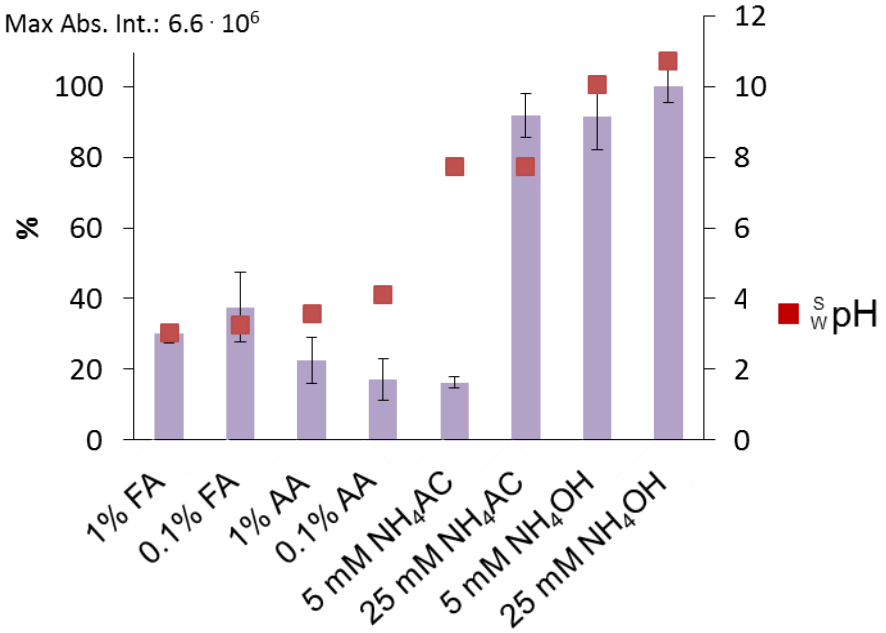


Testosterone, 289.3 > 97.2

Mobile phase: ***CO_2 / MeOH + 10 mM NH_4FA***



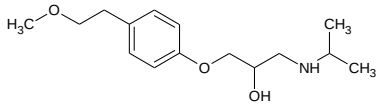
Mobile phase: ***CO_2 / MeOH***



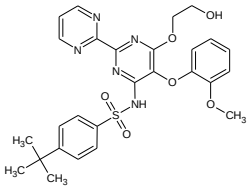
Influence of the Make-up Composition

SRM pos. Mode
Stand. Dev., n = 10
% Int.

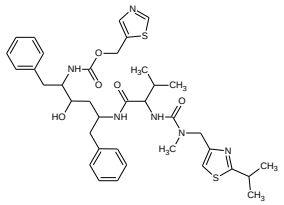
metoprolol



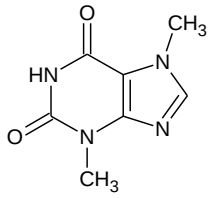
bosentan



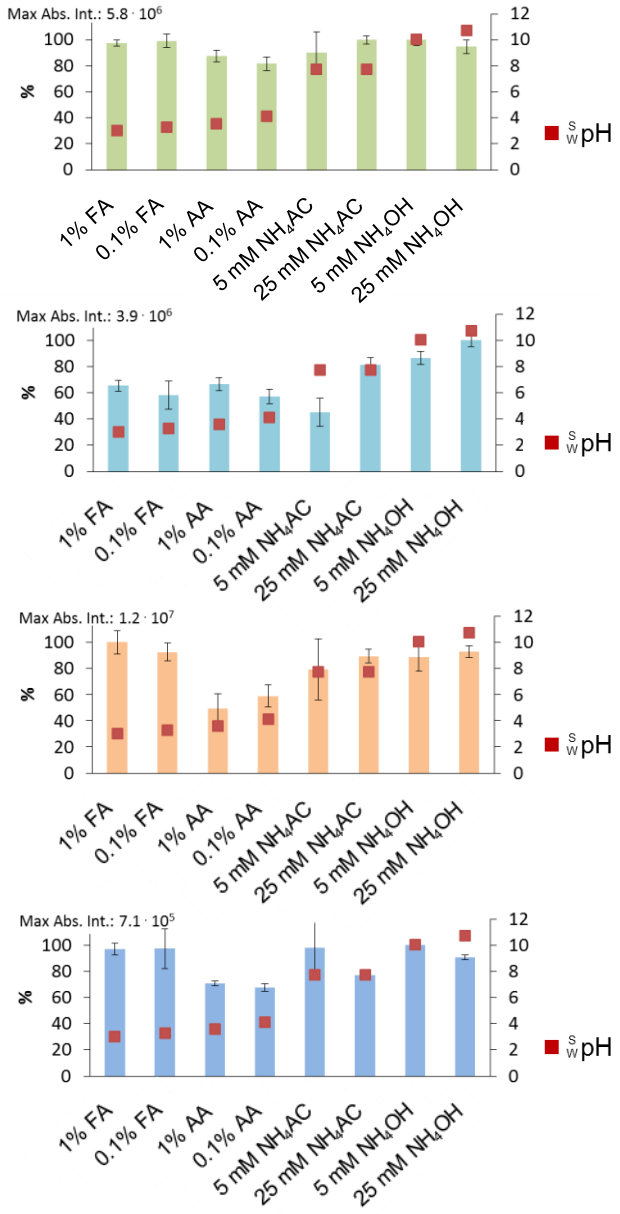
ritonavir



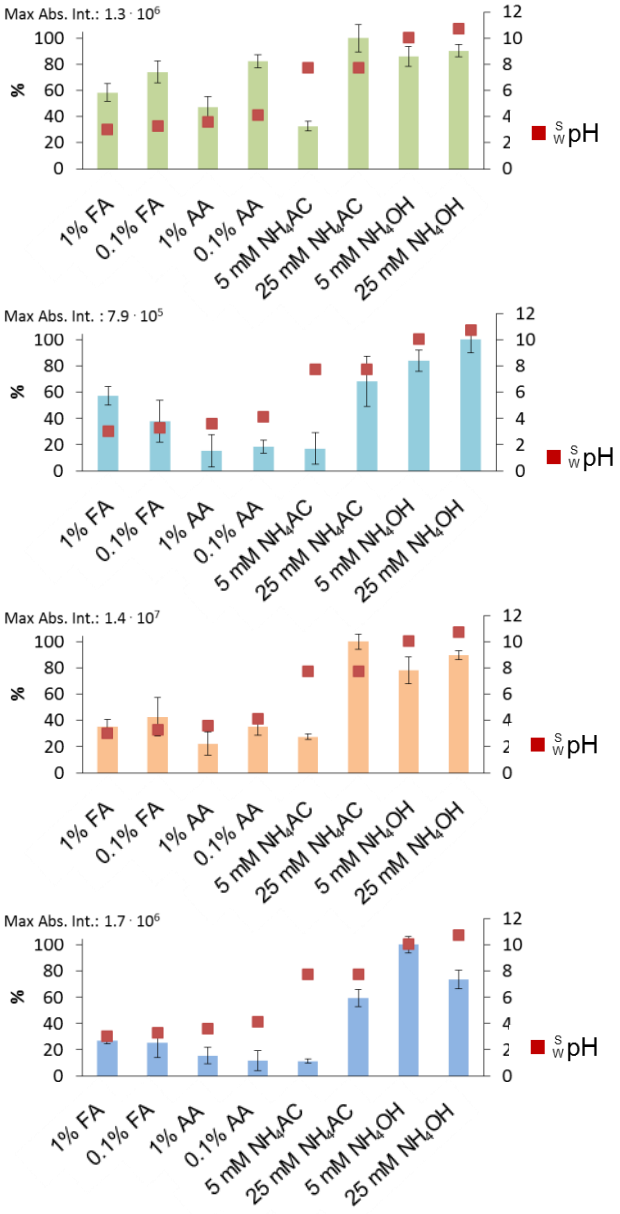
theobromine



MP: CO_2 / MeOH + 10 mM NH_4FA



MP: CO_2 / MeOH



Wrong-Way-Round Ionization Mechanism (pH = 10.8)

$[NH_4OH] = 2.5 \cdot 10^{-4}$ mol/L

→ $pH_{calc} = 10.8$

$[H^+] = 10^{-pH} = 10^{-10.8} = 1.58 \cdot 10^{-11}$ mol/L

Why do we see a strong $[M+H]^+$ ion ?

Wrong-Way-Round Ionization Mechanism

$[NH_4OH] = 2.5 \cdot 10^{-3}$ mol/L $[NH_4]^+ + [OH]^-$

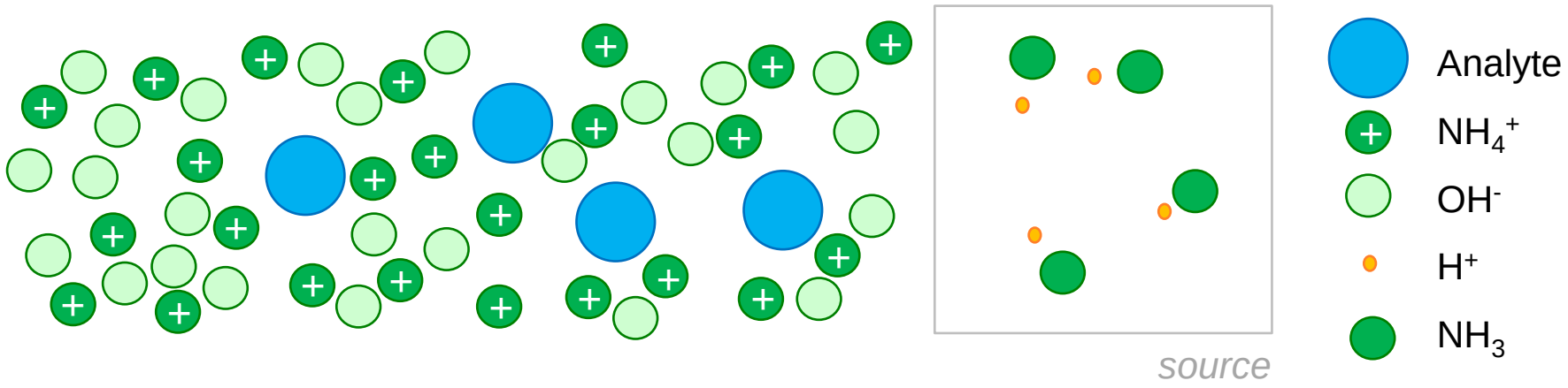
→ $pH_{calc} = 10.8$

$[H^+] = 10^{-pH} = 10^{-10.8} = 1.58 \cdot 10^{-11}$ mol/L

NH_4^+ presence in **excess**

Up-front fragmentation

$(M\text{-}NH_3\text{-}H^+)_{(g)} \longrightarrow NH_{3\,(g)} + MH^+_{(g)}$



Wrong-Way-Round Ionization Mechanism

$[NH_4OH] = 25 \cdot 10^{-3}$ mol/L

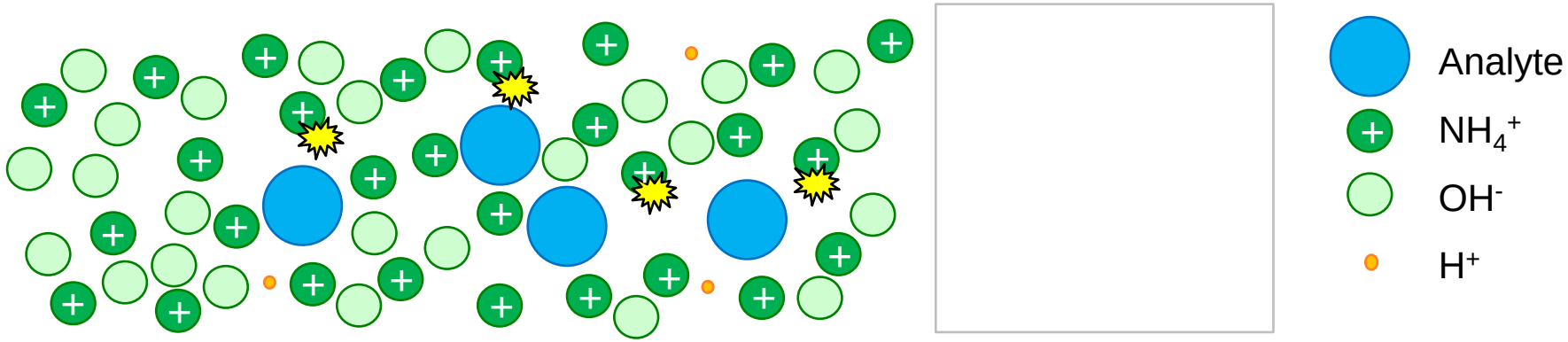
→ $pH_{calc} = 10.8$

$[H^+] = 10^{-pH} = 10^{-10.8} = 1.58 \cdot 10^{-11}$ mol/L

NH_4^+ excess

Up-front fragmentation

$(NH_3\text{-}H^+\text{-}M)_{(g)} \longrightarrow NH_{3\,(g)} + MH^+_{(g)}$



source

Wrong-Way-Round Ionization Mechanism

$[NH_4OH] = 25 \cdot 10^{-3}$ mol/L

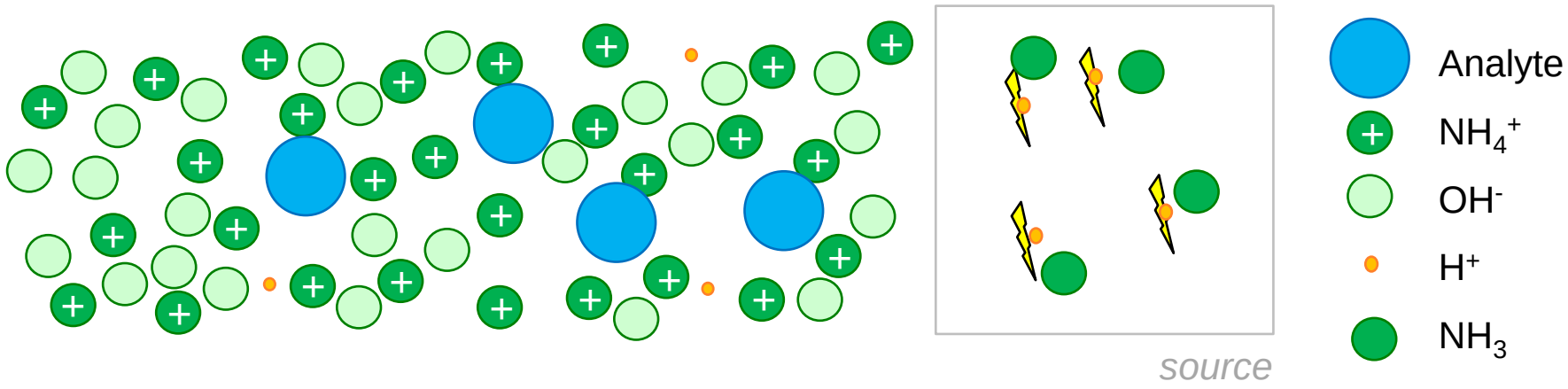
→ $pH_{calc} = 10.8$

$[H^+] = 10^{-pH} = 10^{-10.8} = 1.58 \cdot 10^{-11}$ mol/L

NH_4^+ presence in **excess**

Up-front fragmentation

$(NH_3\text{-}H^+\text{-}M)_{(g)} \longrightarrow NH_{3\,(g)} + MH^+_{(g)}$

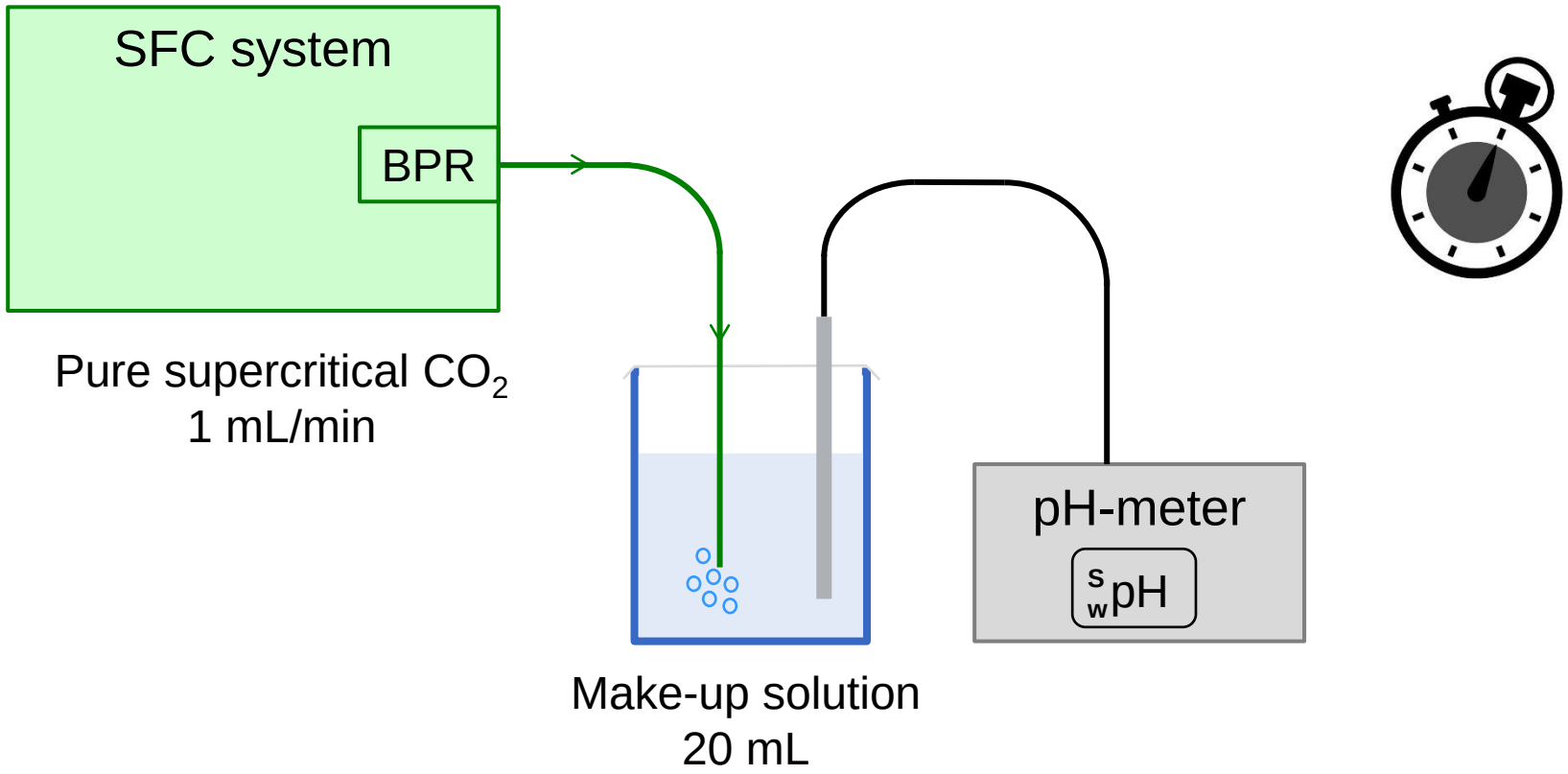


source

Effect of Carbon Dioxide

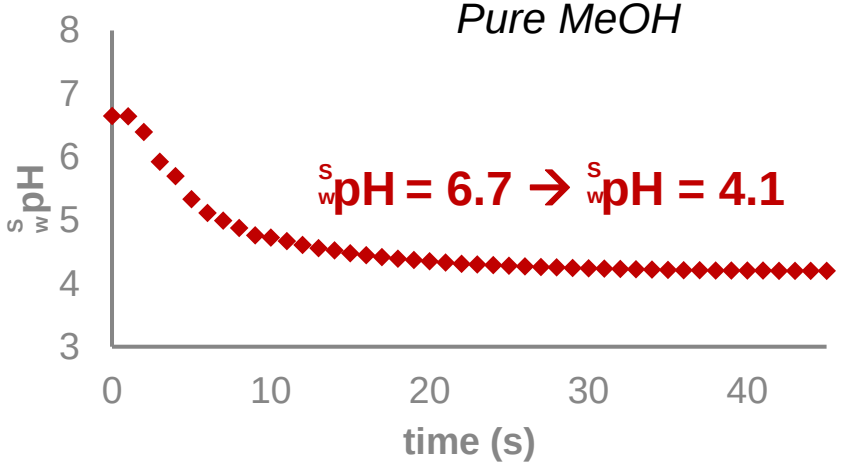
$$CO_{2\ (aq)} + H_2O_{\ (l)} \rightarrow H_2CO_{3\ (aq)}$$

$pKa_1 = 6.35$; $pKa_2 = 10.3$

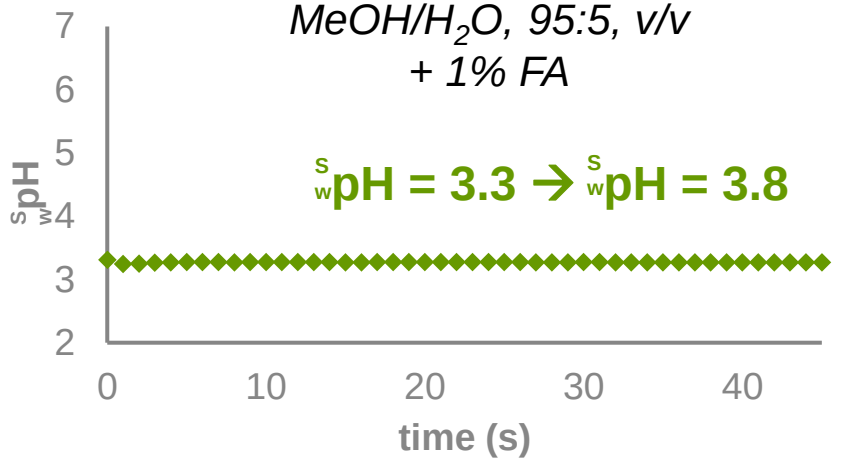


Evaluation of Acidic Power of CO_2

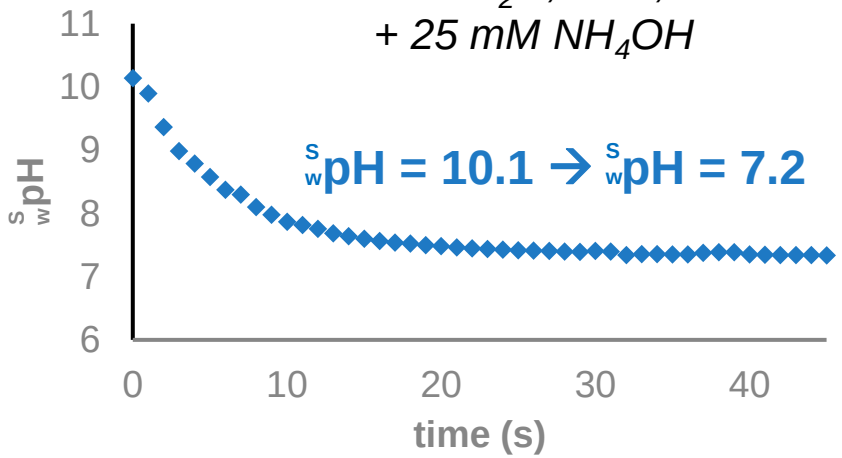
Pure MeOH



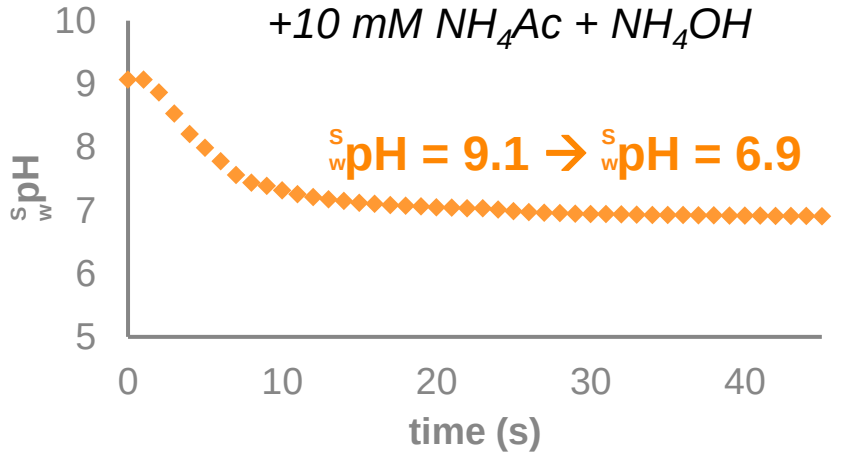
Acidic solution
MeOH/H_2O, 95:5, v/v
+ 1% FA



Basic solution
MeOH/H_2O, 95:5, v/v
+ 25 mM NH_4OH



Buffered solution
MeOH/H_2O, 95:5, v/v
+10 mM NH_4Ac + NH_4OH



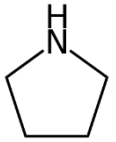
Effect of CO_2 in ESI Negative Mode Detection

Stand. Dev.
n = 5

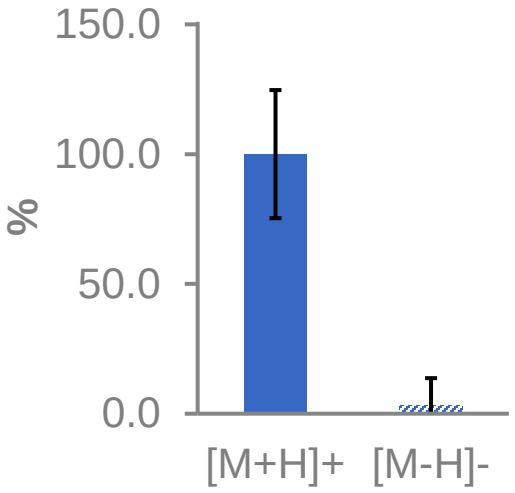
Formic acid
$^{s}_{w}$pH = 3.0

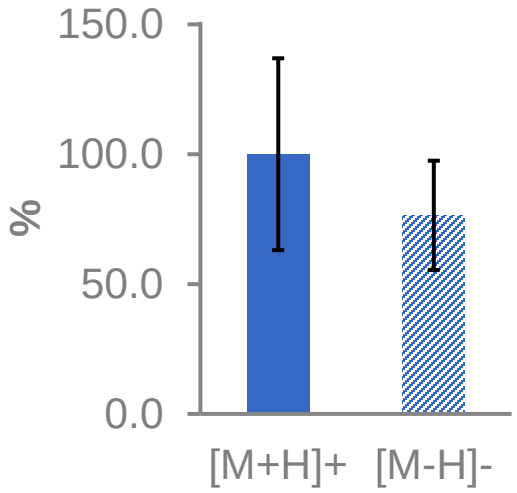
Ammonium hydroxide
$^{s}_{w}$pH = 10.3

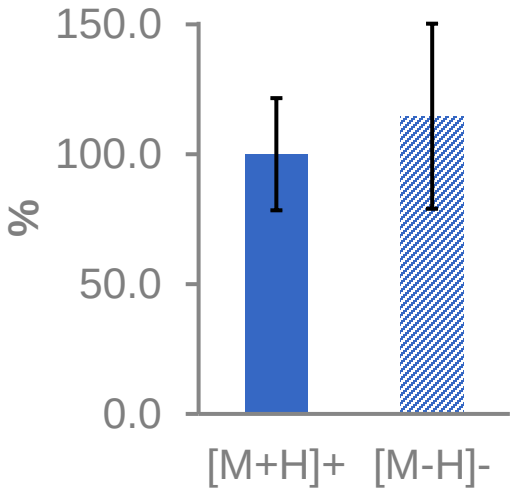
Pyrrolidine
$^{s}_{w}$pH = 11.1



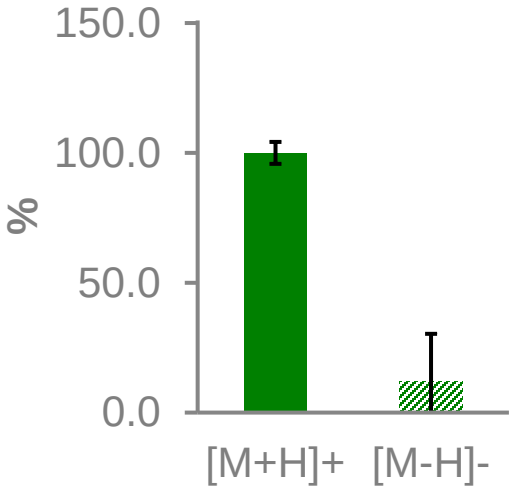
infusion

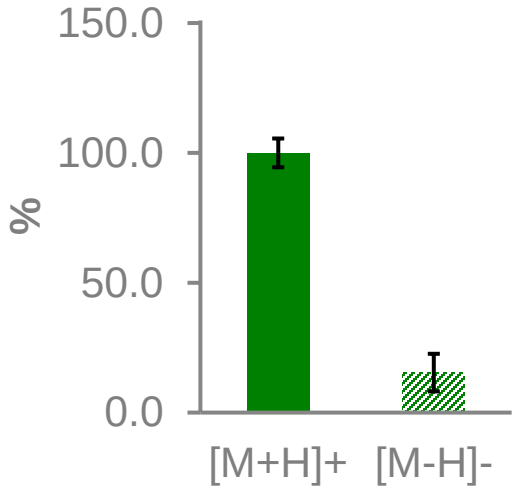


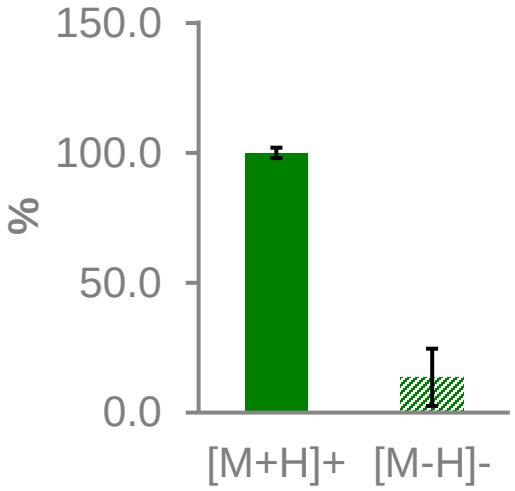


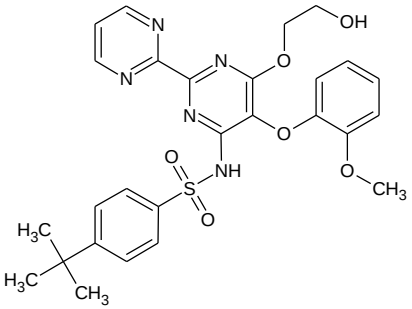


SFC





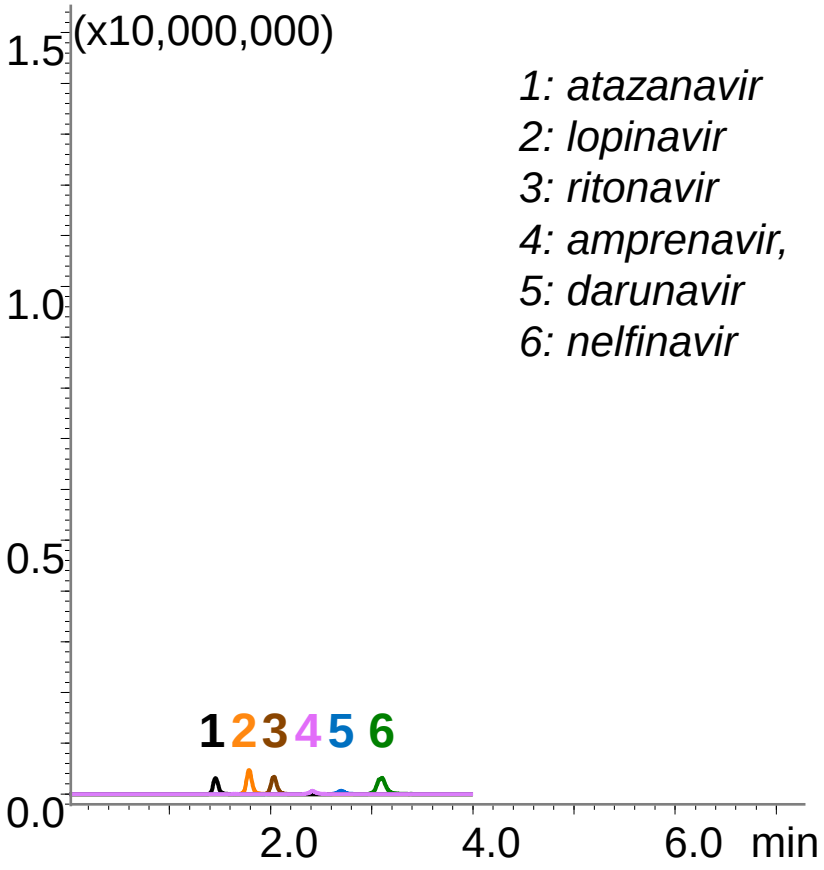




Make-Up SFC for Assays Performance Improvement Antiviral Drugs (Dried Plasma Spot)

SFC

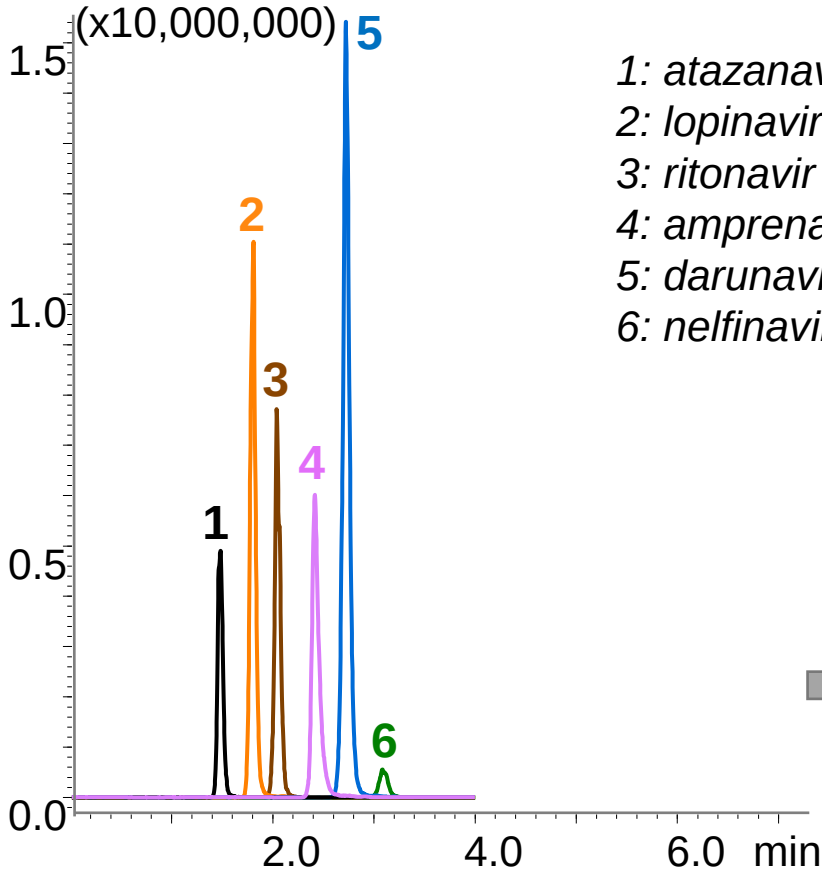
Make-up:
MeOH/H_2O, 95:5, v/v + 0.1% FA



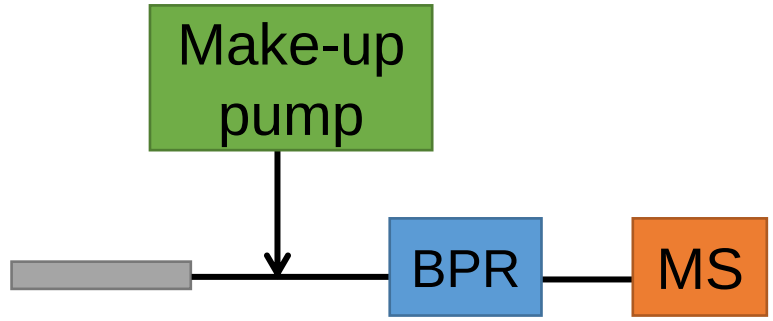
1: atazanavir
2: lopinavir
3: ritonavir
4: amprenavir,
5: darunavir
6: nelfinavir

SFC

Make-up:
MeOH/H_2O, 95:5, v/v + 25 mM NH_4Ac

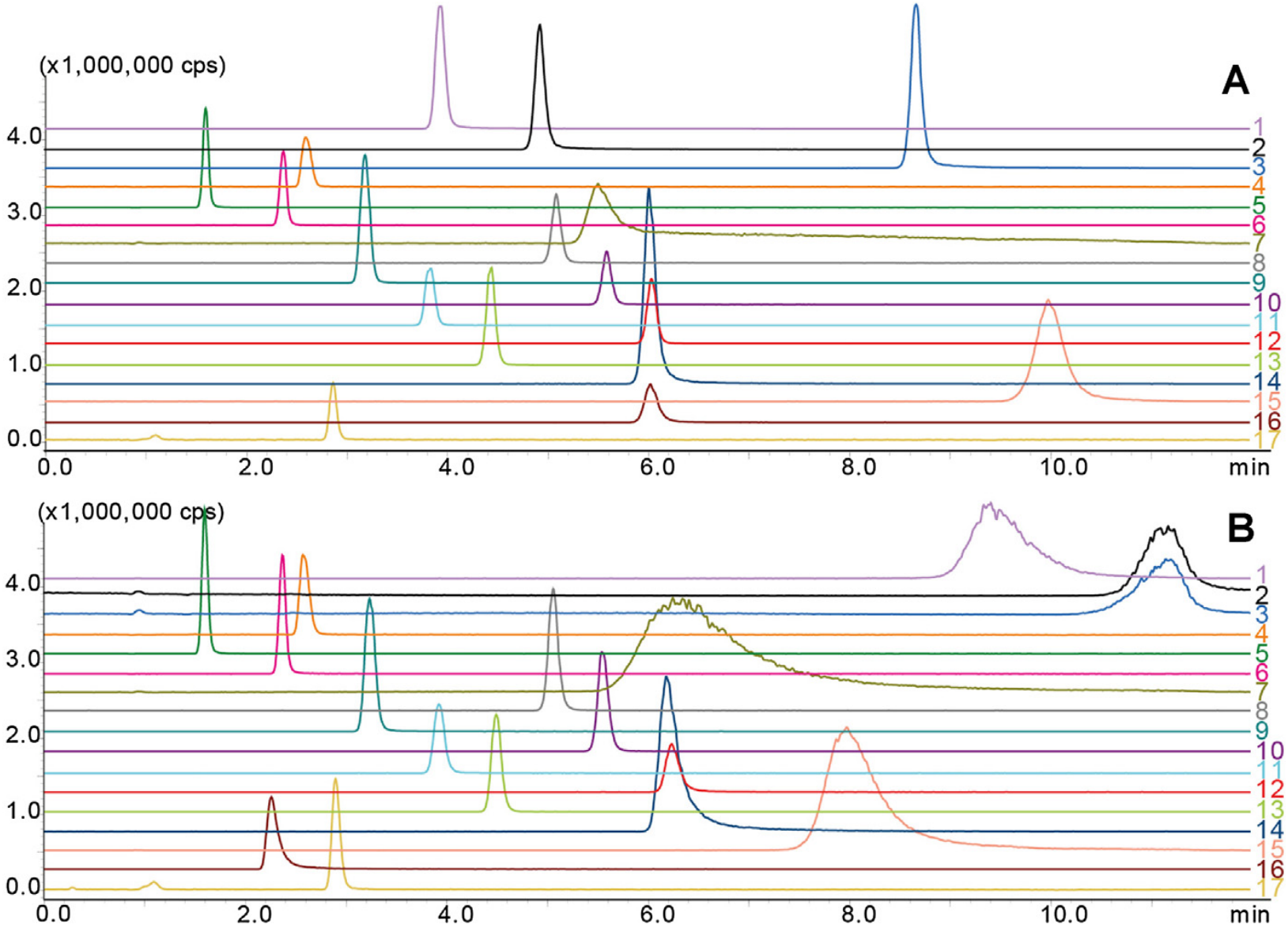


1: atazanavir
2: lopinavir
3: ritonavir
4: amprenavir,
5: darunavir
6: nelfinavir



wrong way round mechanism

Chromatograms with (A) or without (B) 10 mM NH_4FA as additive in the mobile phase

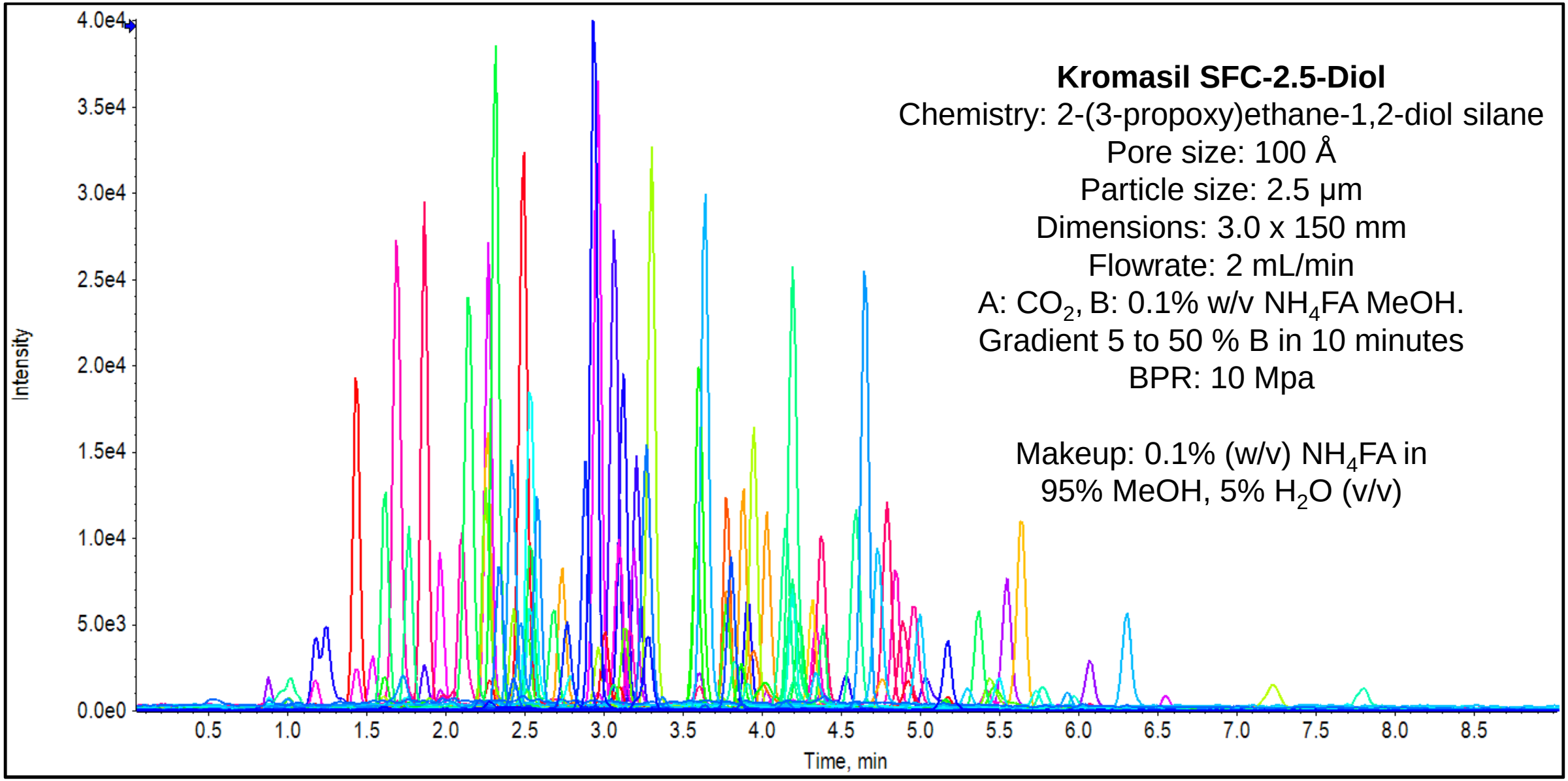


make-up: H_2O/MeOH, 5/95, v/v, + 10 mM NH_4Ac at 0.3 mL/min.

1:metoprolol,
2: propranolol,
3: pindolol,
4: etrone,
5: progesterone,
6: testosterone,
7: bosentan,
8: amprenavir,
9: atazanavir,
10: darunavir,
11: lopinavir,
12: nelfinavir,
13: ritonavir,
14: adenine,
15: guanosine,
16: nicotinic acid,
17: theobromine,

[C] = 100 ng/mL

Extracted Ion Current Profile of Large Mix : SFC-MS on Diol Column



Kromasil SFC-2.5-Diol

Chemistry: 2-(3-propoxy)ethane-1,2-diol silane

Pore size: 100 Å

Particle size: 2.5 µm

Dimensions: 3.0 x 150 mm

Flowrate: 2 mL/min

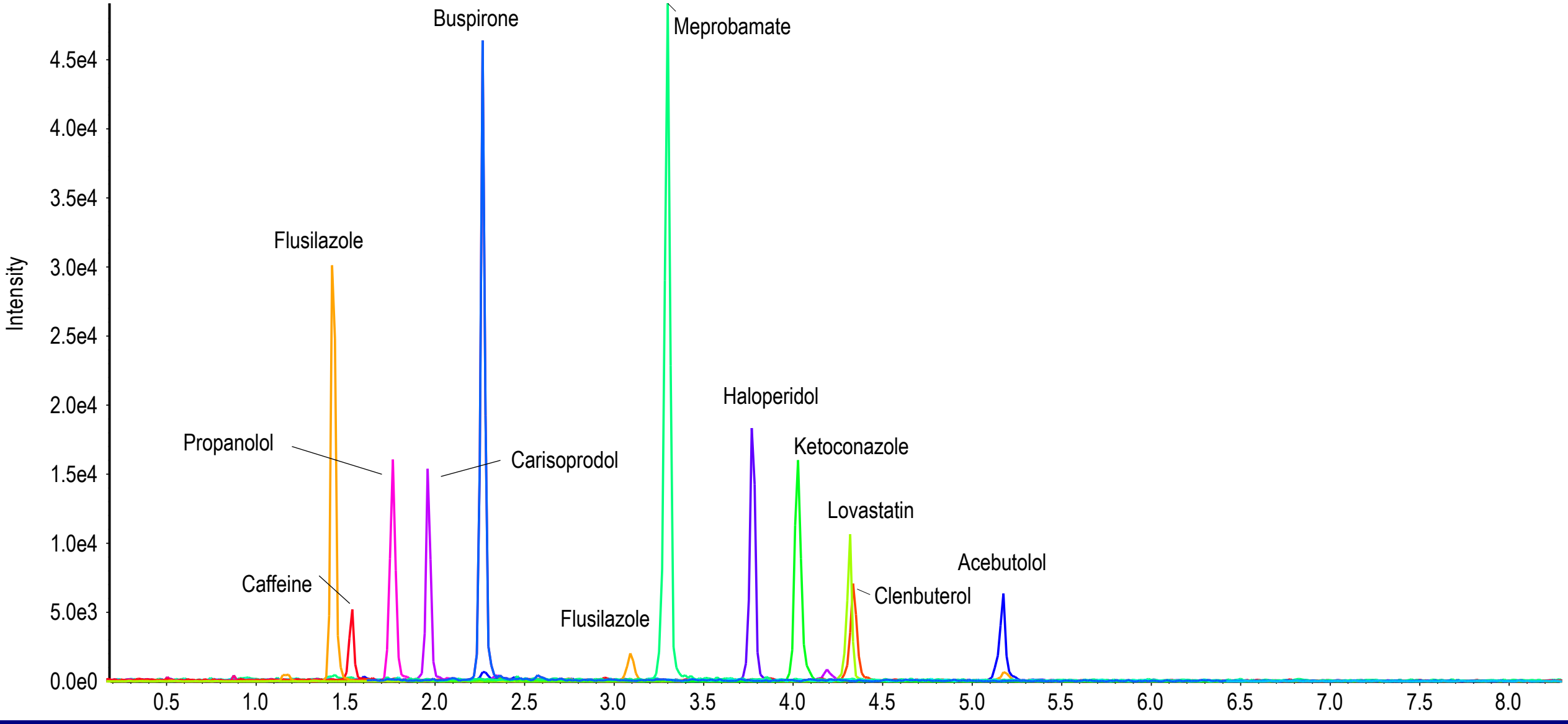
A: CO_2, B: 0.1% w/v NH_4FA MeOH.

Gradient 5 to 50 % B in 10 minutes

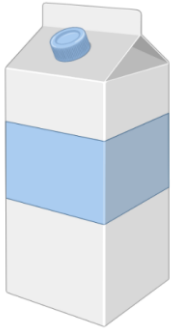
BPR: 10 Mpa

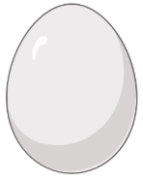
Makeup: 0.1% (w/v) NH_4FA in 95% MeOH, 5% H_2O (v/v)

Extracted Ion Current Profile : SFC-MS on Diol Column

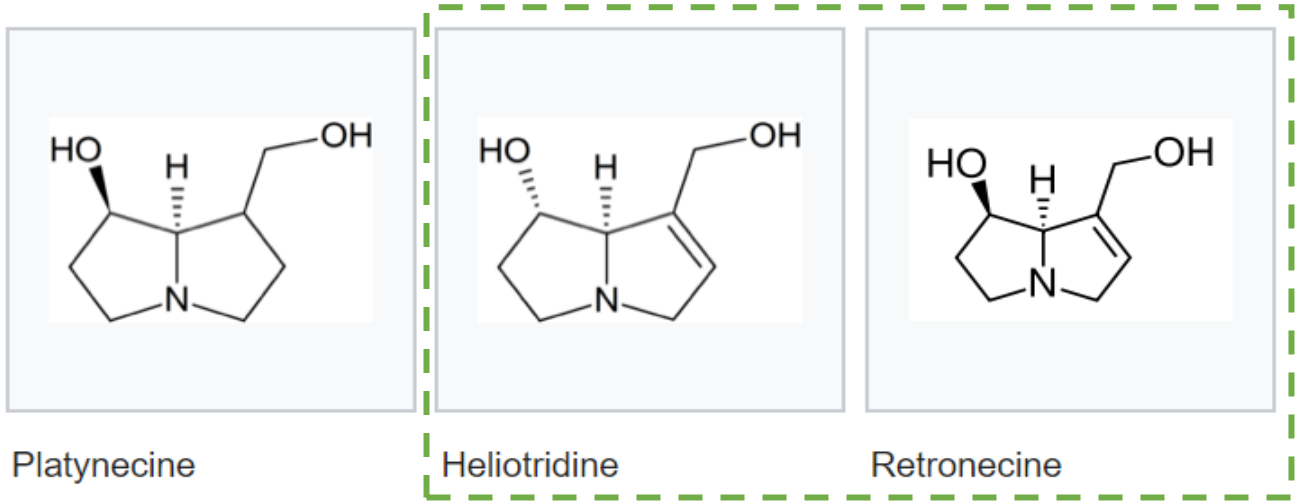


Pyrrolizidine alkaloids (PAs)





- >600 PAs in >6000 plants with many structural isomers
- Phytotoxic 1,2-unsatured PAs

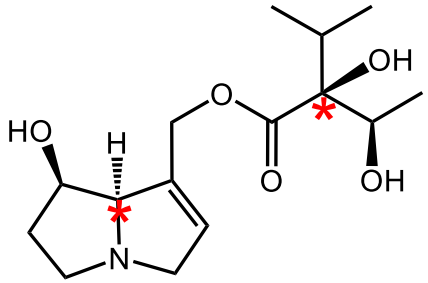


Structures of the three most common families of PAs

- The European Food Safety Authority (EFSA) has proposed a list of 28 PAs to be monitored in food products.

Diastereomeric Pyrrolizidine Alkaloids

m/z 300



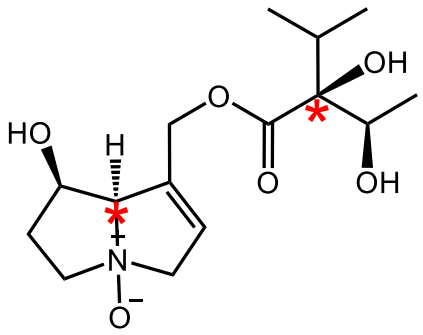
Intermedine

Lycopsamine

Echinatine

Indicine

m/z 316



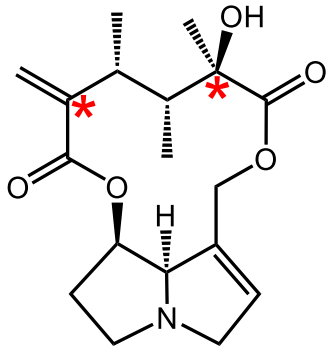
Intermedine-N-oxide

Lycopsamine-N-oxide

Echinatine-N-oxide

Indicine-N-oxide

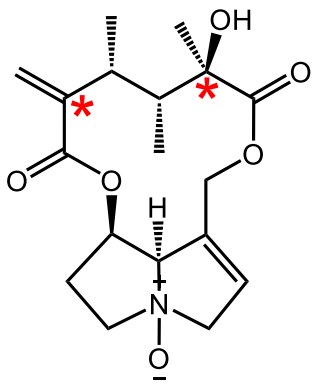
m/z 336



Senecivernine

Senecionine

m/z 352



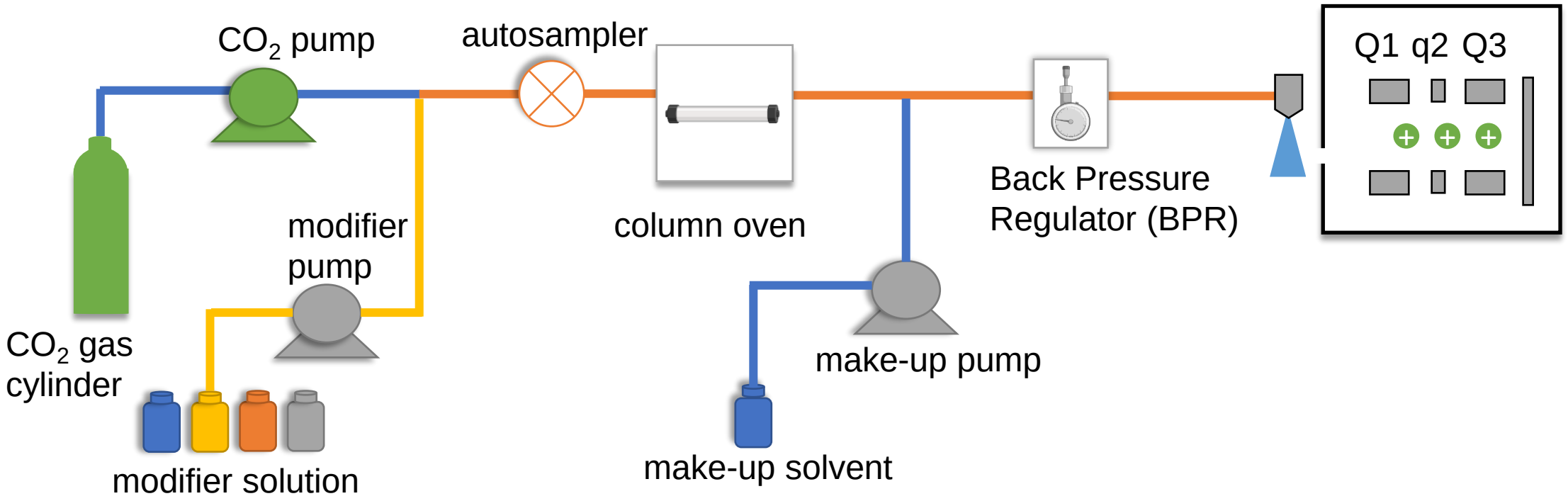
Senecivernine-N-oxide

Senecionine-N-oxide

Jacobine

Retrorsine

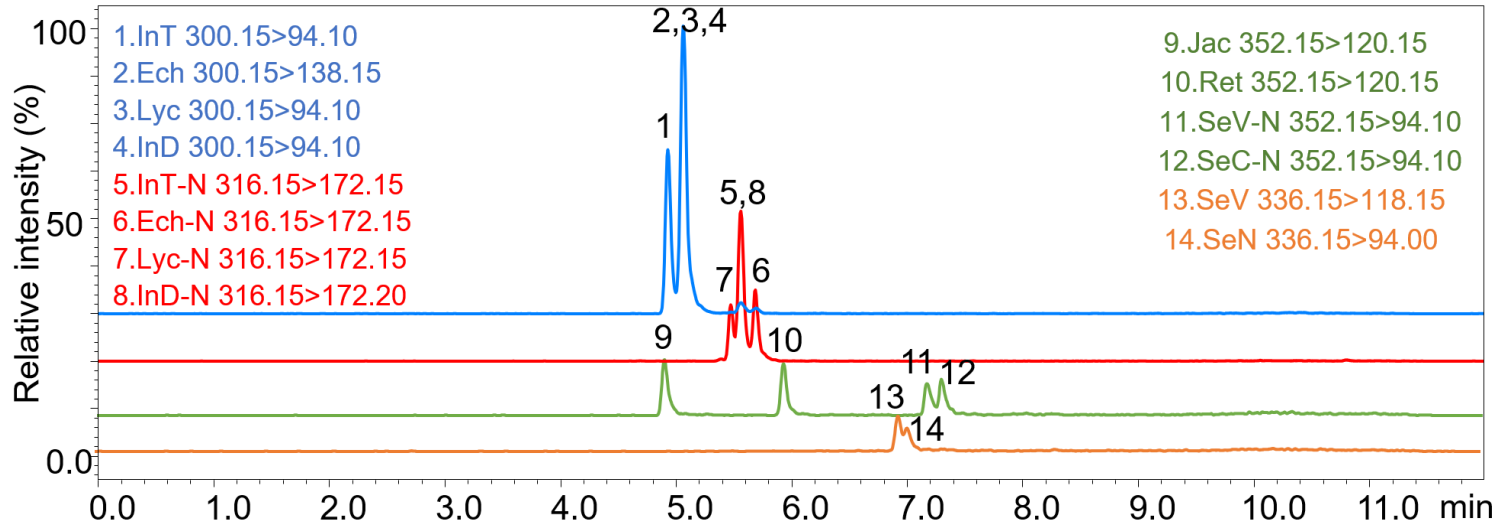
Hyphenation of SFC to MS/MS for PA's



Separation challenges for current LC-MS and SFC-MS methods

LC-MS

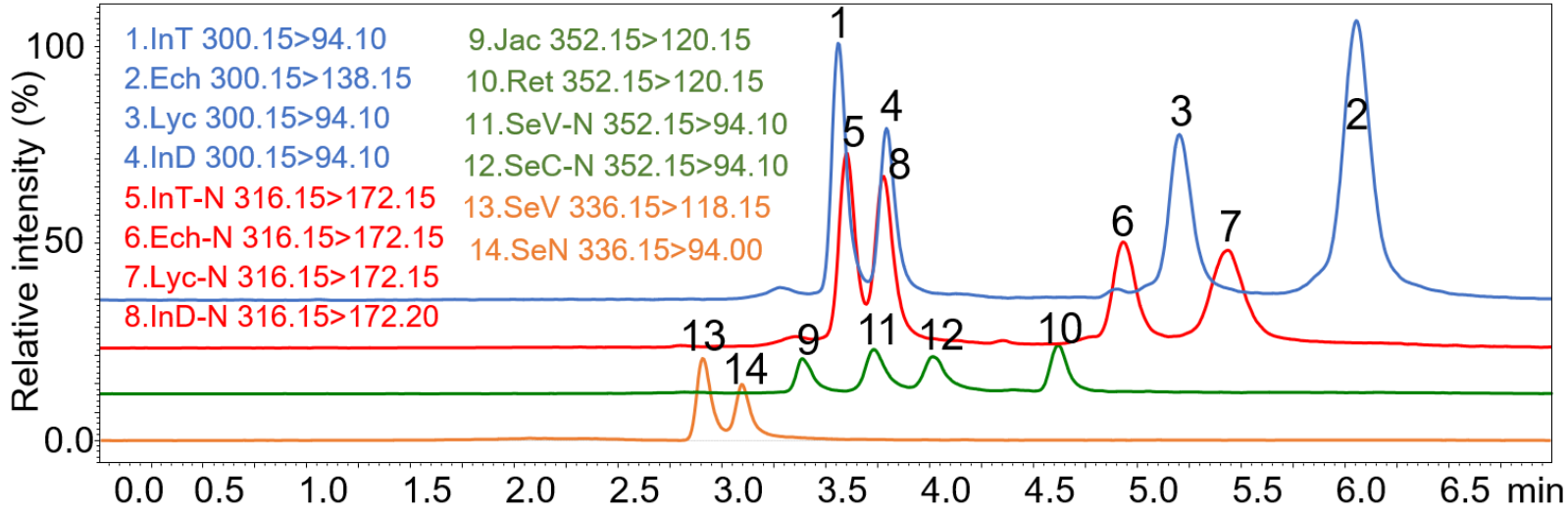
Kinetex XB-C18
2.1 ×150 mm
1.7 µm
Phenomenex



Some diastereomers coelute in LC

SFC-MS

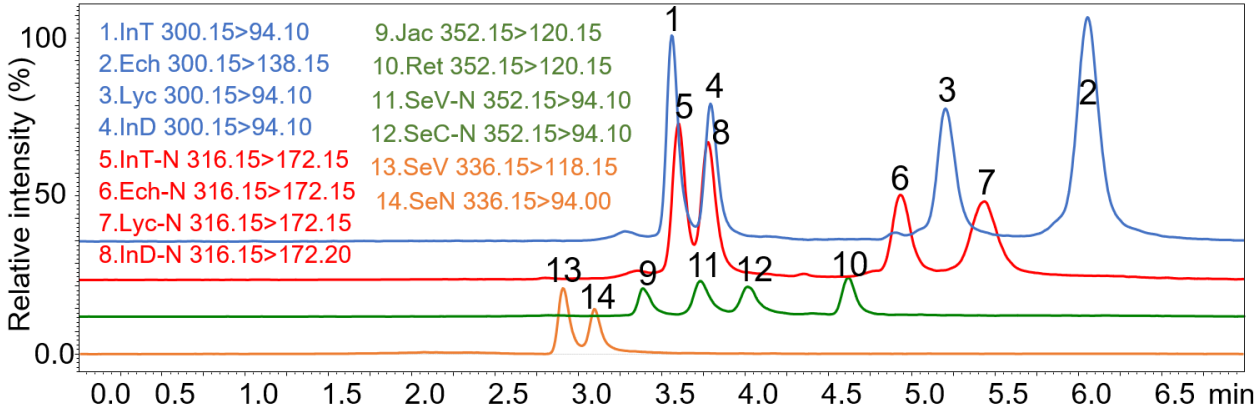
CHIRALPAK®IG-3
3.0 ×100 mm, 3 µm
SFC column
Daicel, France



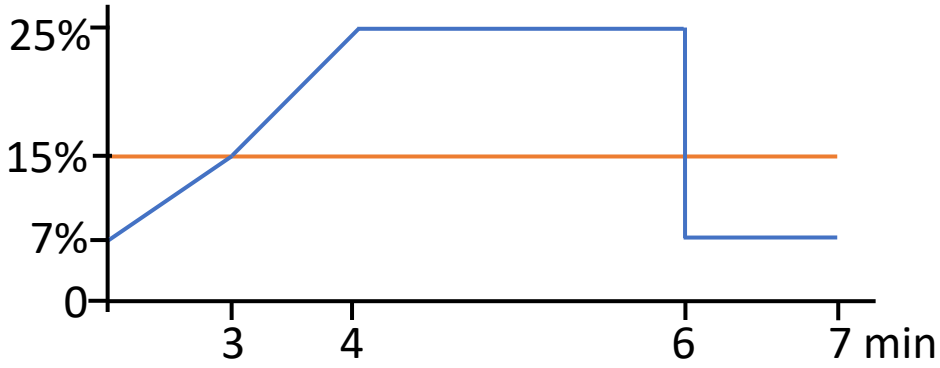
All diastereomers separated in SFC

SFC-MRM/MS methods for PAs

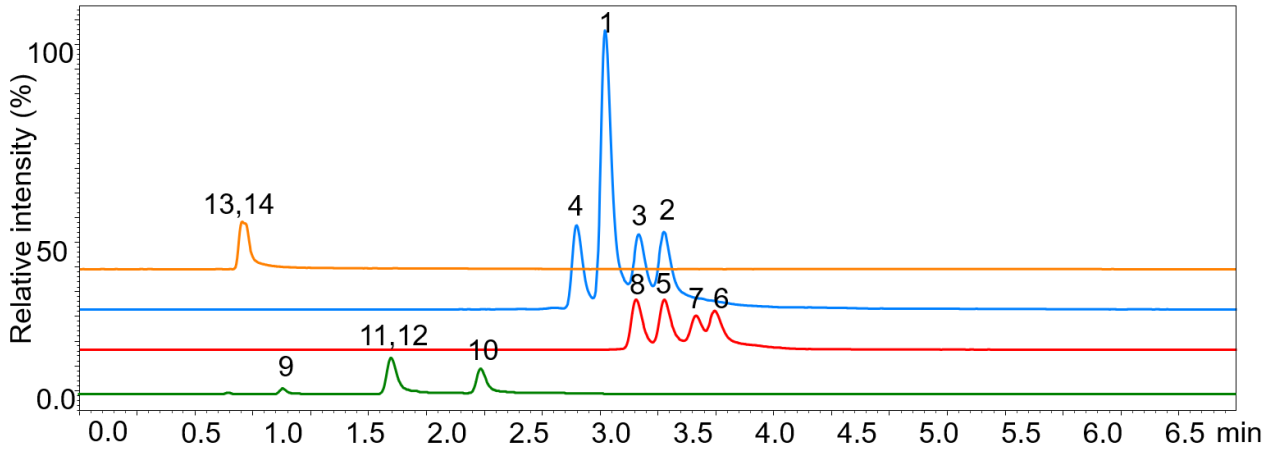
SFC-MRM/MS (chiral column) 14/14



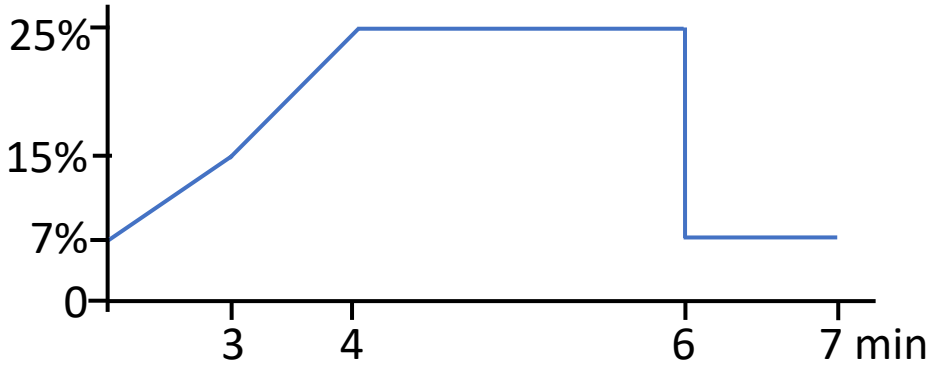
CHIRALPACK IG-3, 100 x 3 mm, 3 µm SFC, Diacel
Mobile phase **A**: CO2 **B:** 50 mM NH4F in MeOH **C:** MeOH
Make-up: MeOH + 0.1% FA (0.2 mL/min)
Flow rate: 1.0 ml/min



SFC-MRM/MS (achiral column) 10/14

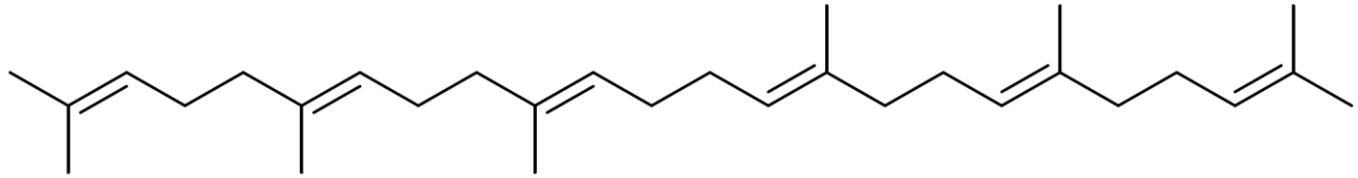


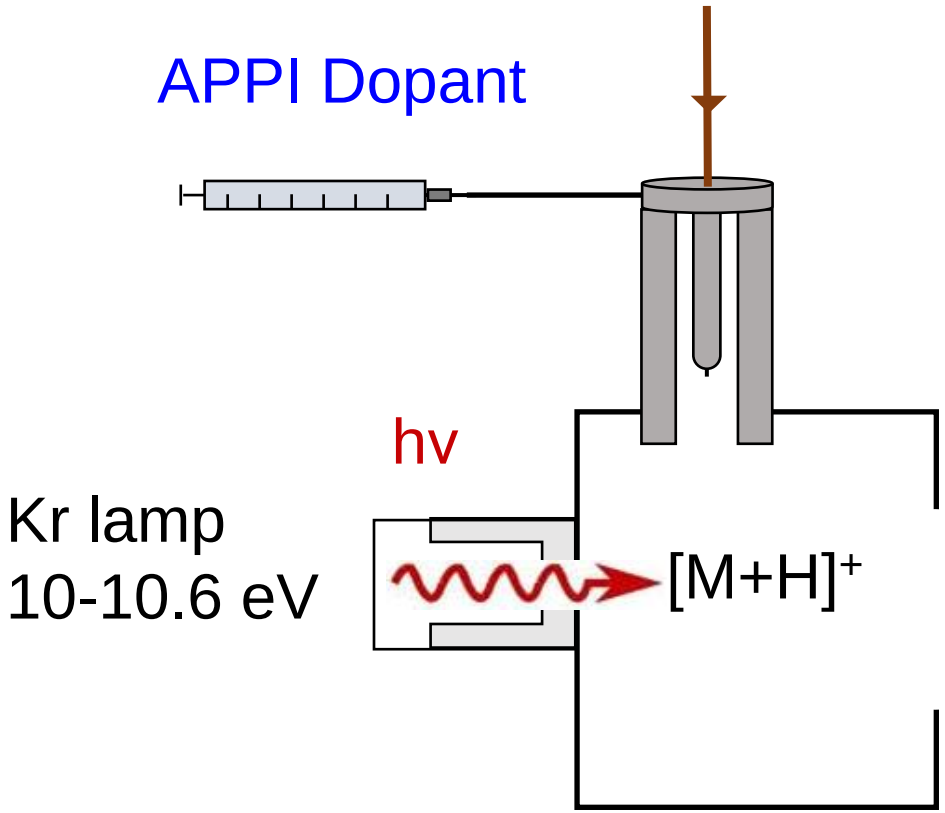
Torus-2-PIC, 5µm, 3.0 x 250 mm
Mobile phase A: CO2 **B:** 50 mM NH4F in MeOH
Make-up: MeOH + 0.1% FA (0.2 mL/min)
Flow rate: 3.0 ml/min

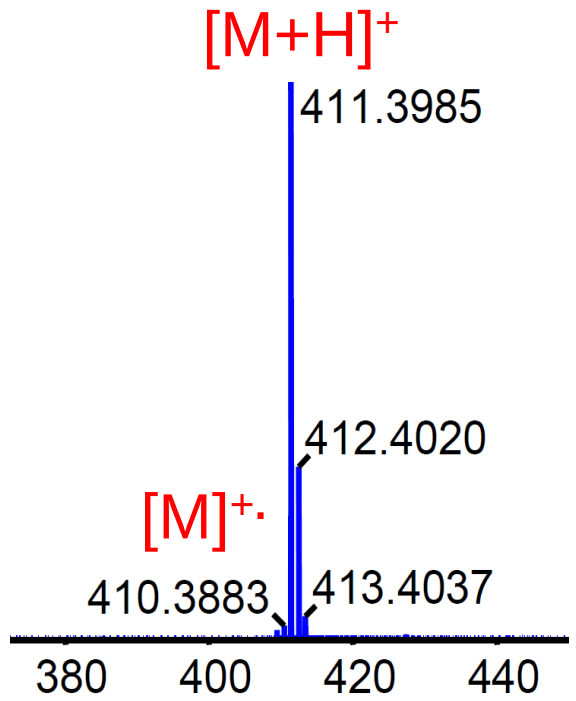


Revisiting Atmospheric Pressure Photoionization (APPI)

Liquid Chromatography
(300- 400 µl/min)

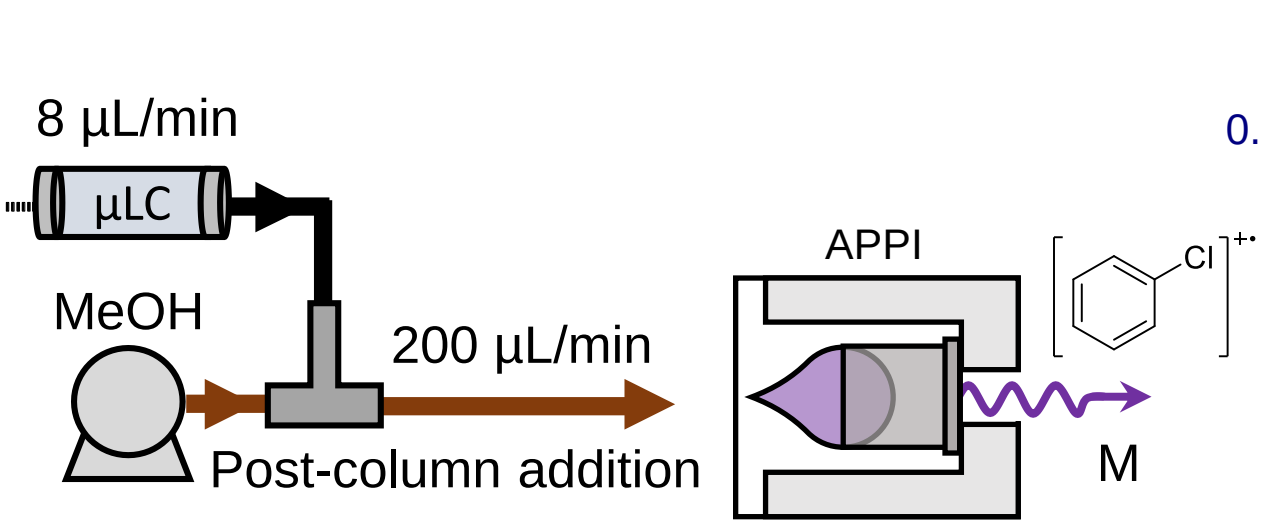




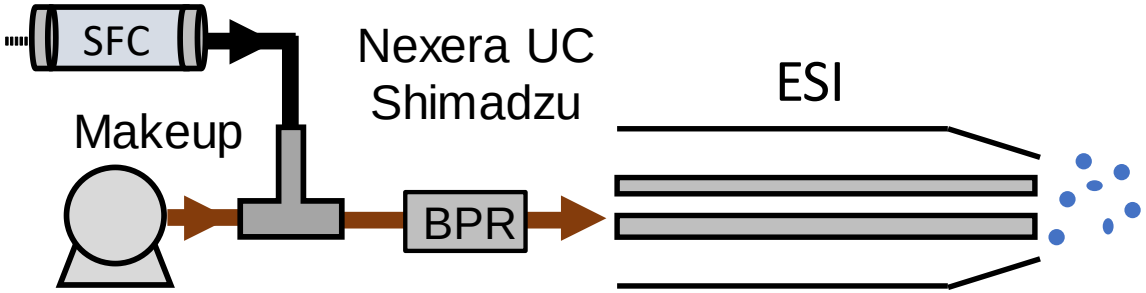


µLC and SFC Interfacing to Mass Spectrometry

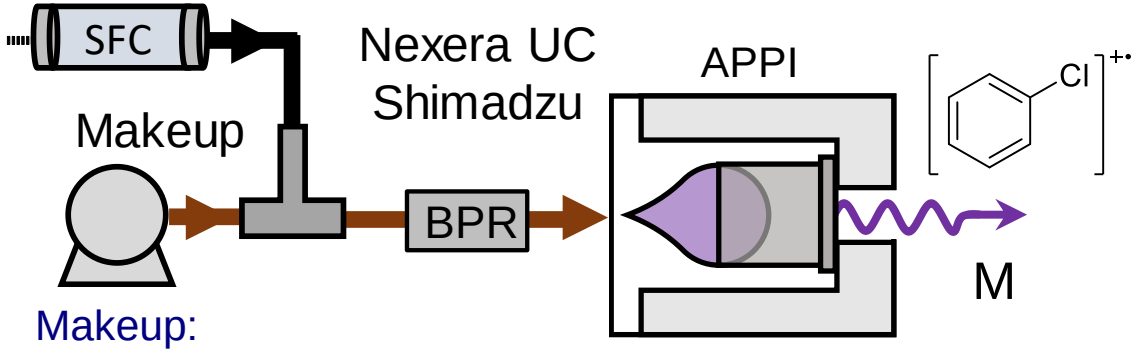
Controlled Ionization Conditions



Makeup:
95% MeOH, 5% H_2O
0.1% FA, 0.1 NH_4FA (w/v)



Generation of odd electron Ion Precursors $M^{+\cdot}$
CID-> Electron Impact like Spectra
Use of EI Library

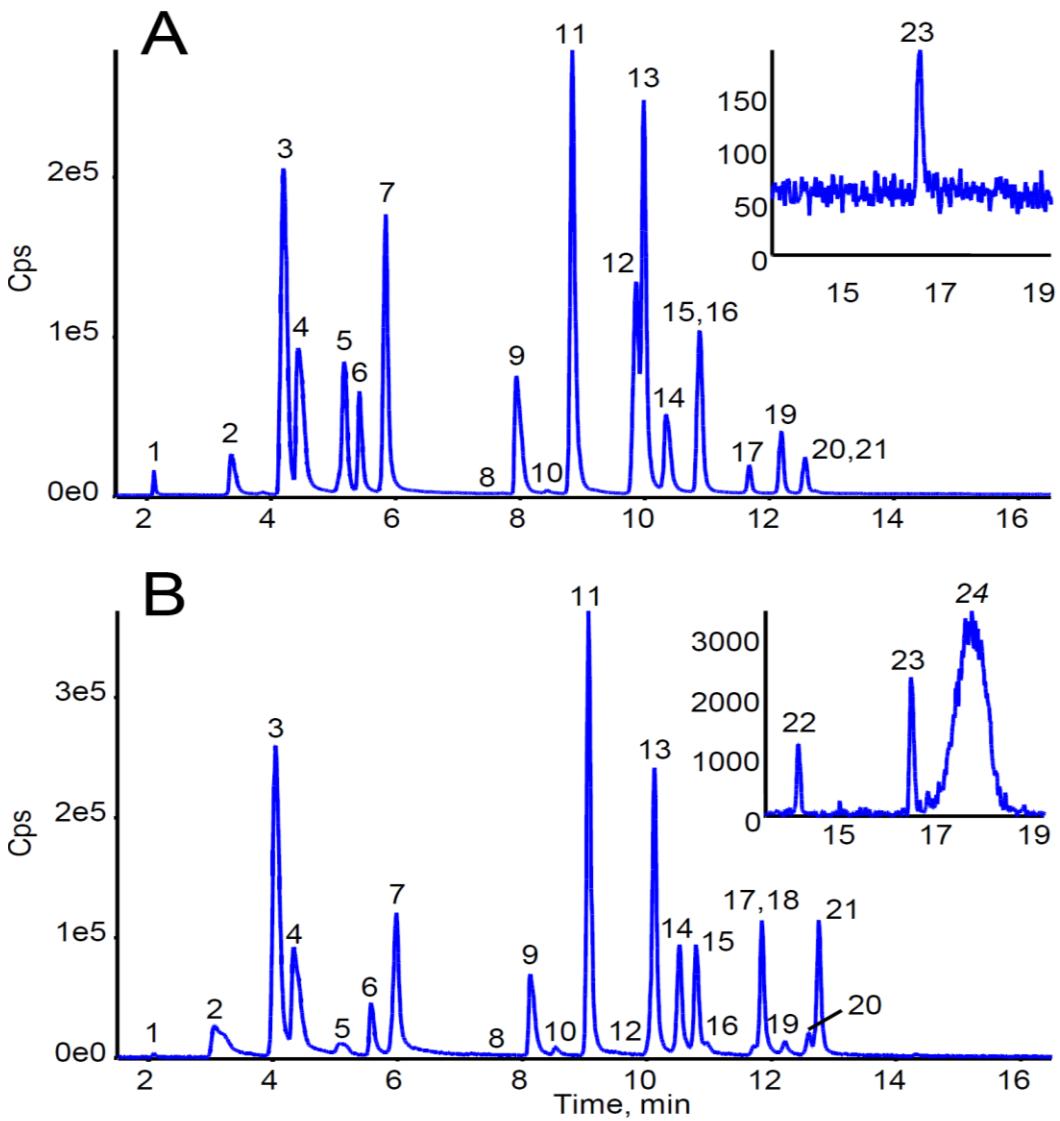


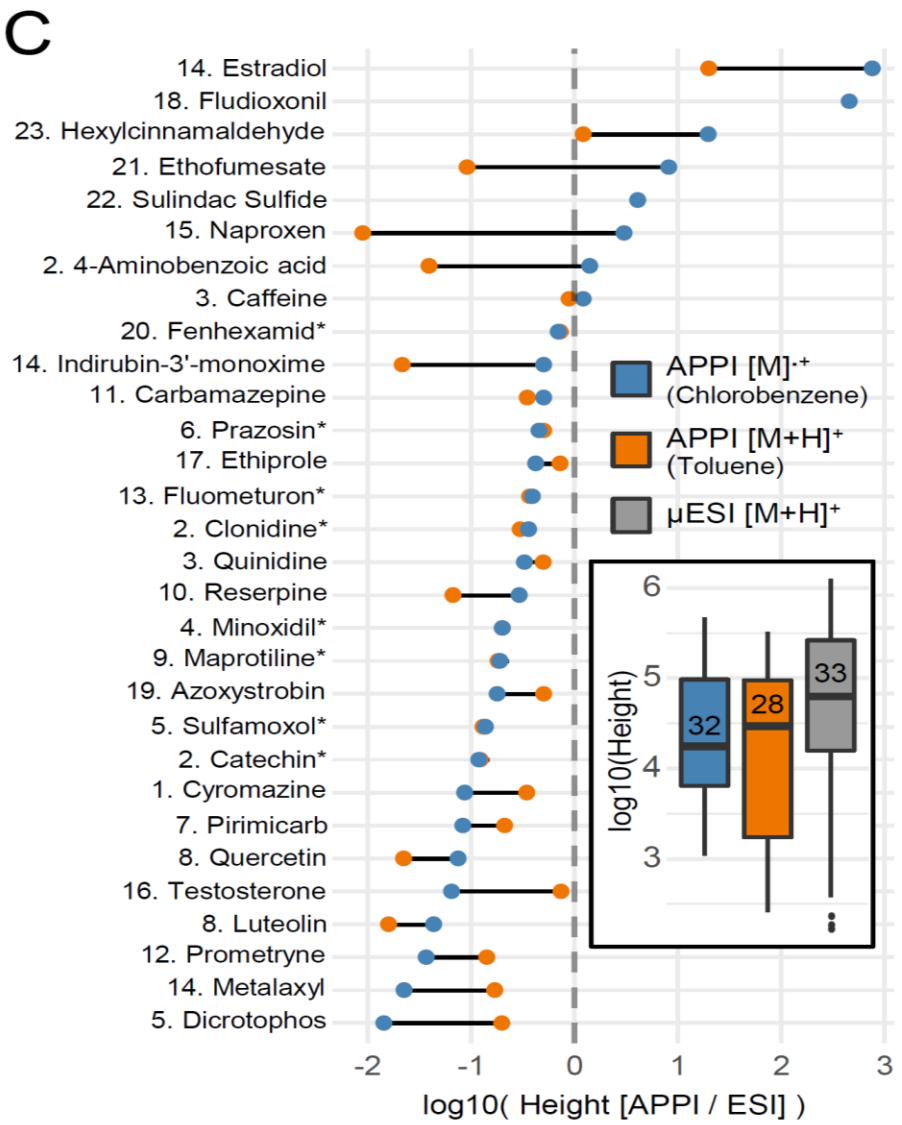
Makeup:
90% MeOH, 10% ClBz

Toluene versus Chlorobenzene to control Precursor Ions type

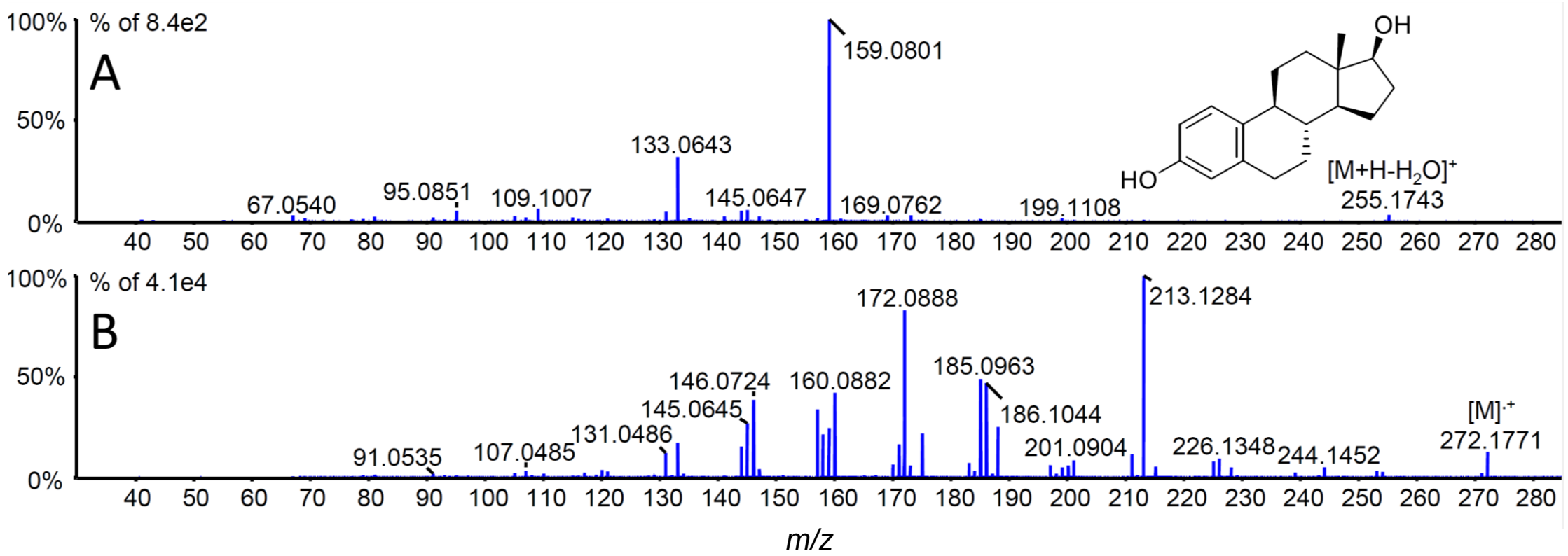
Dopant
Toluene
$[M+H]^+$

Dopant
Chlorbenzene
$M^{+\bullet}$

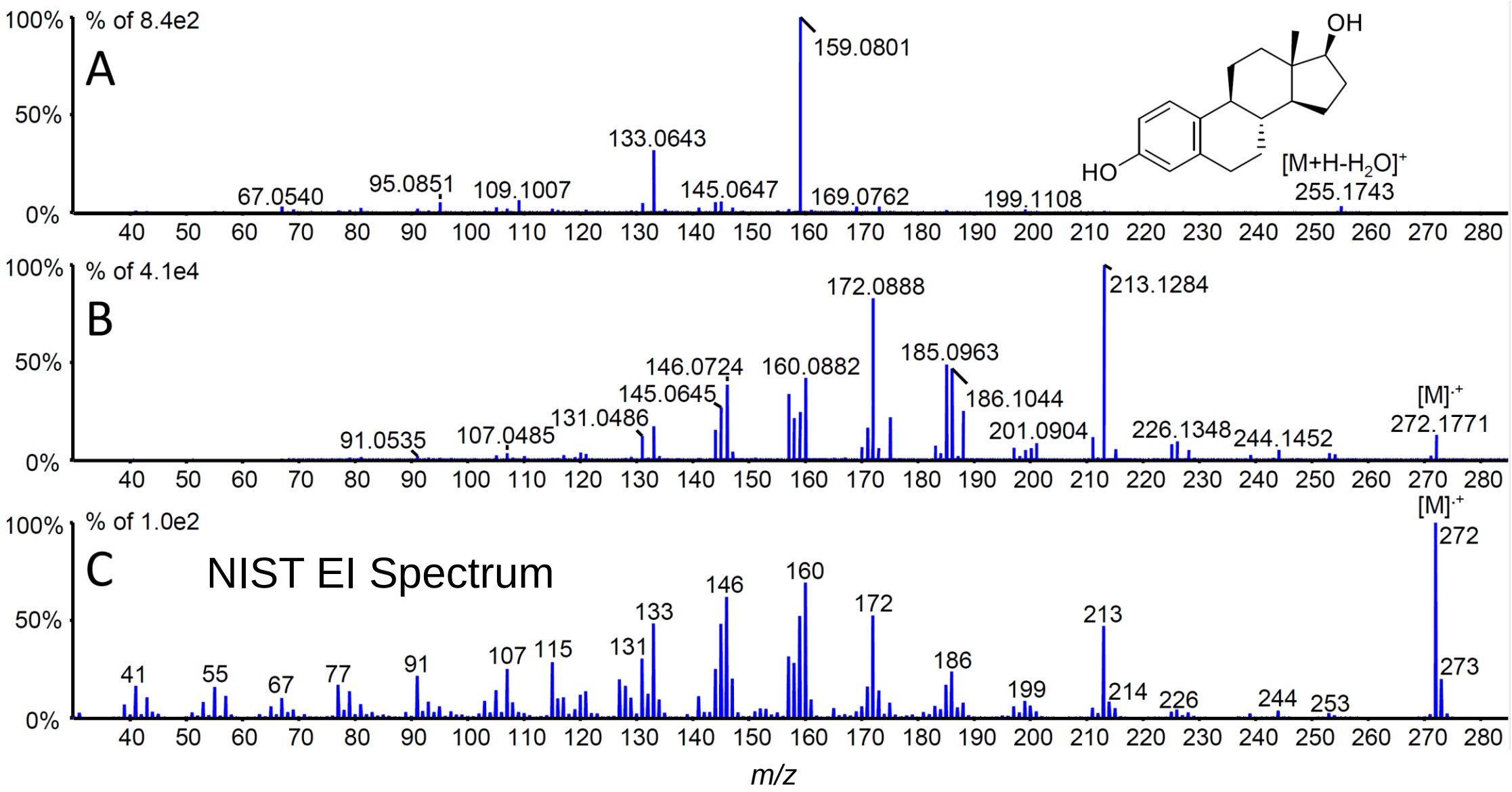




MS/MS (CE = 30 eV) of Protonated Precursor versus Radical Cation



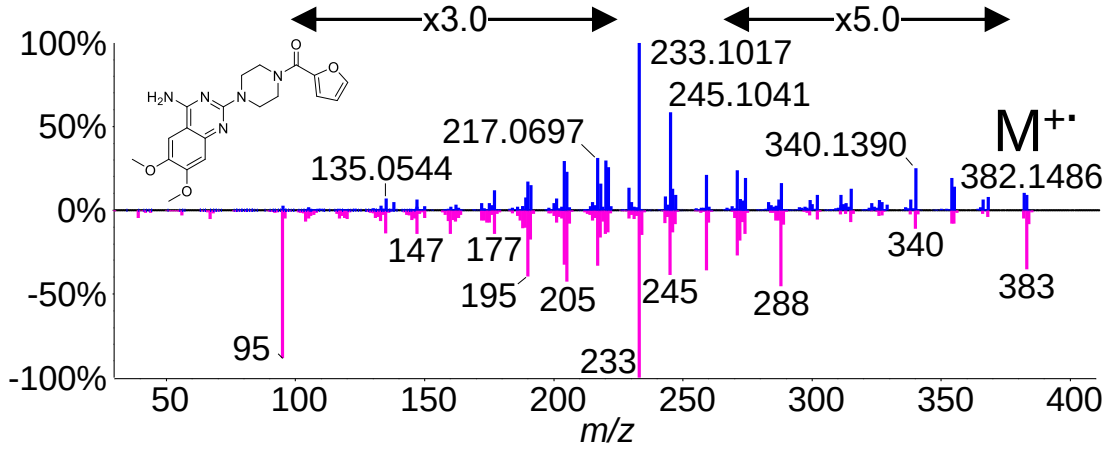
MS/MS (CE = 30 eV) of Protonated Precursor versus Radical Cation



MS/MS Fragmentation (CE 10-70 eV) of $M^{+\cdot}$ and $[M+H]^+$ Spectra and Library Searches

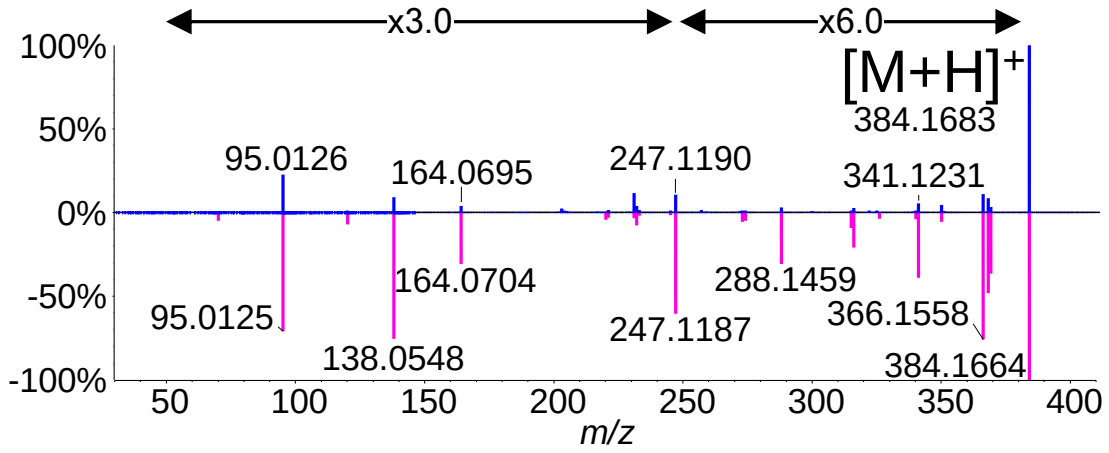
Prazosin

APPI, Dopant: Chlorobenzene, Rank: 1, Match: 753, Probability; 100%



APPI

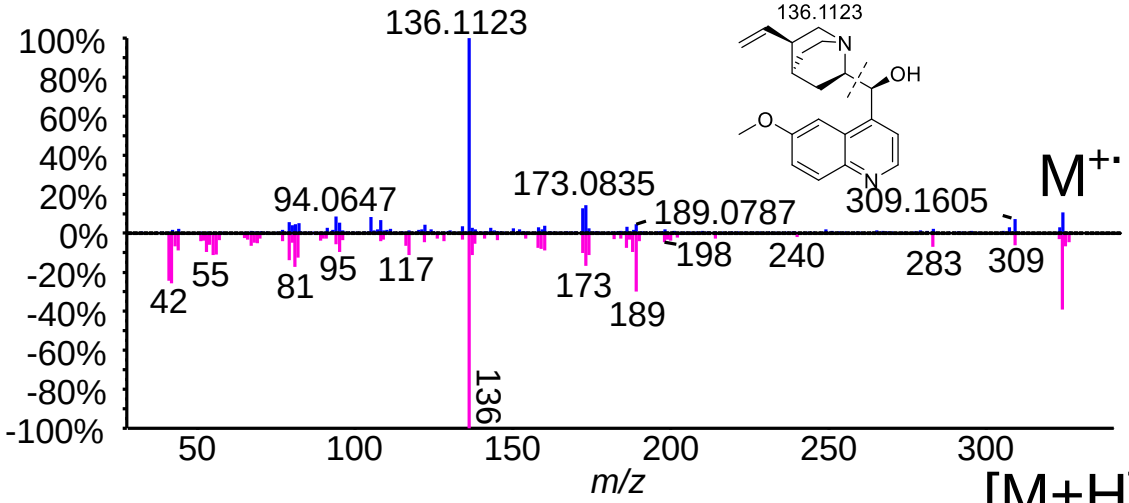
µESI, Rank: 1, Score: 874, Probability; 98.3%



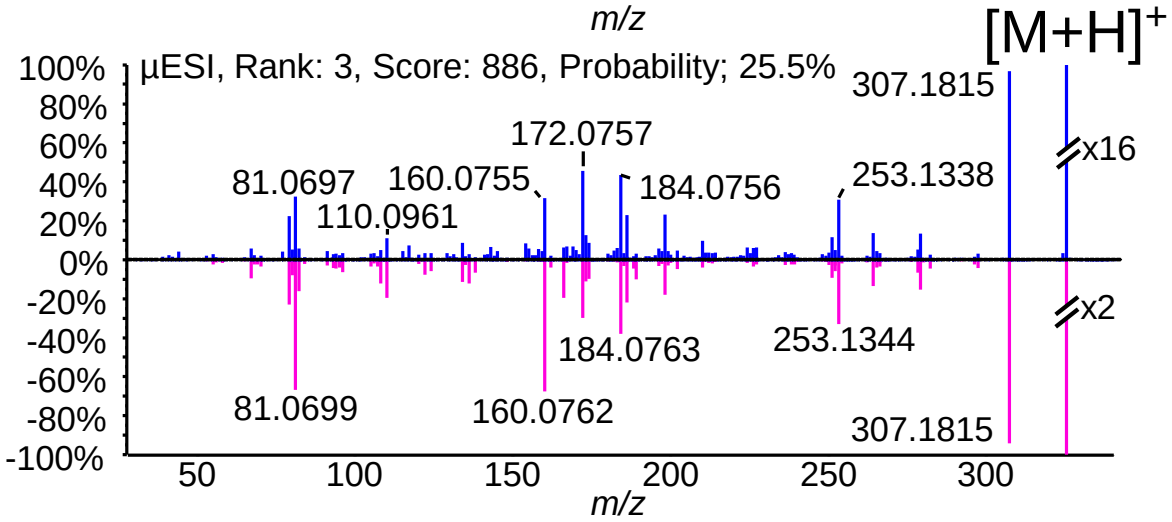
ESI

Quinidine

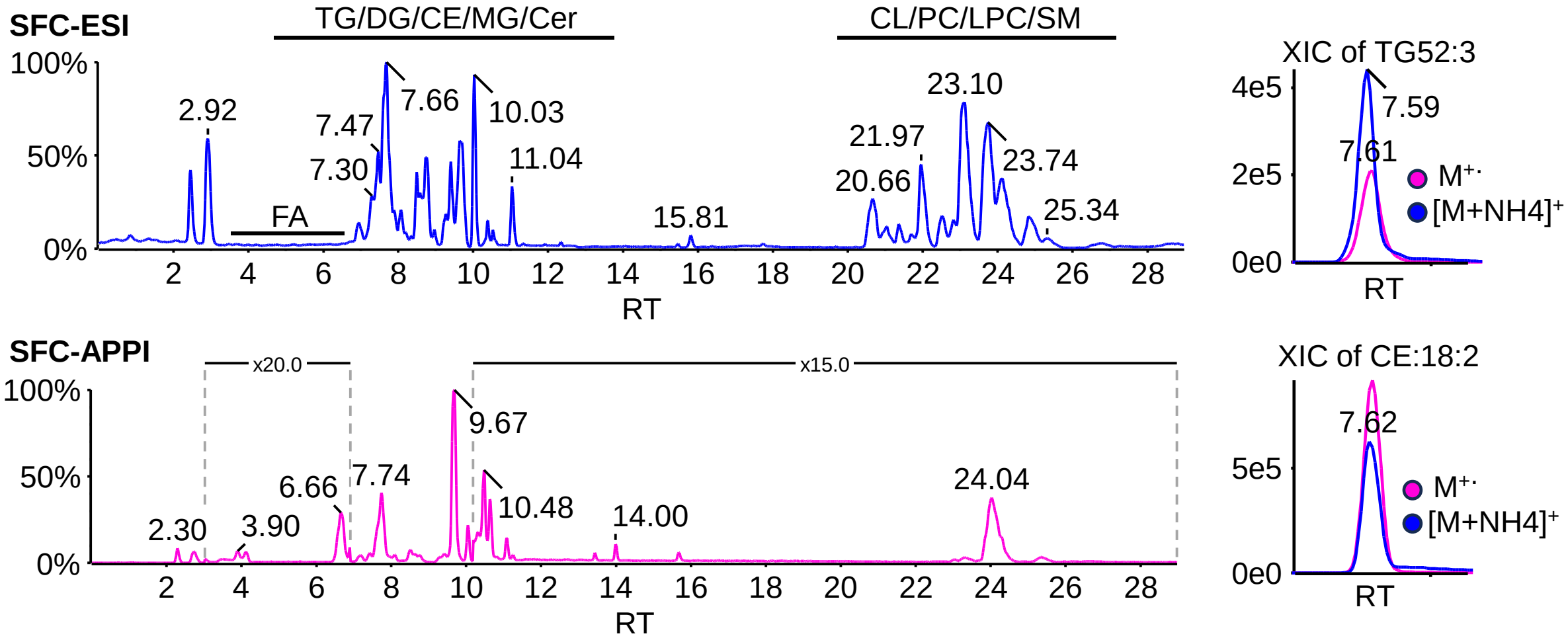
APPI, Dopant: Chlorobenzene, Rank: 1, Match: 770, Probability; 76.2%



µESI, Rank: 3, Score: 886, Probability; 25.5%

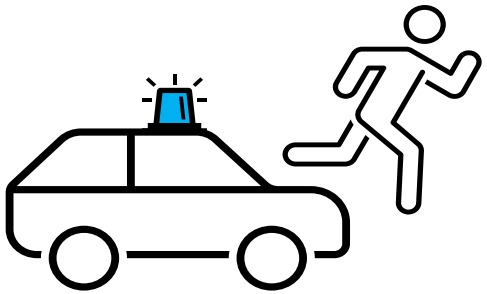


SFC-MS Lipidomic Screening of Whole Blood ESI and APPI



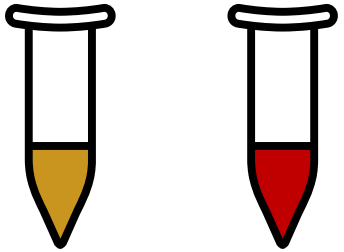
Workflow for Comprehensive Screening of Biological Fluids using SFC-ESI/APPI-SWATH

Sample acquisition



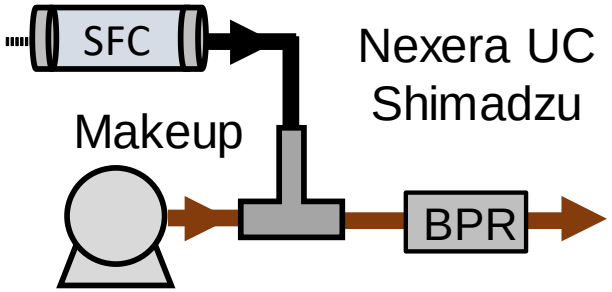
Roadside control
Traffic accidents

Urine Blood



n = 202

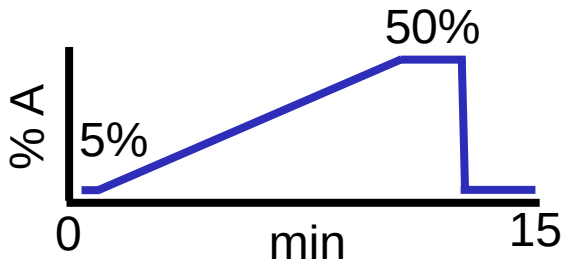
Chromatography



Nexera UC
Shimadzu

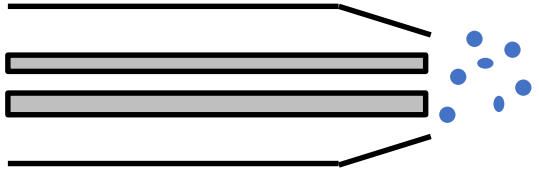
Urine & blood metabolomics

Kromasil SFC-2.5 Diol column
3.0 x 150 mm

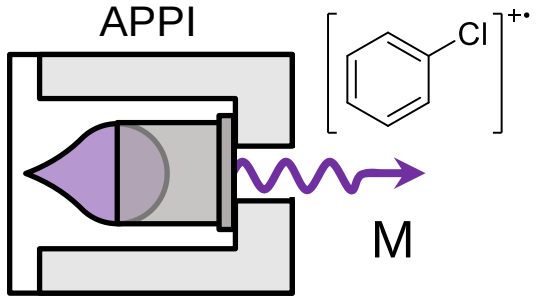


A: MeOH, 0.1% NH_4FA (w/v)

Ionization

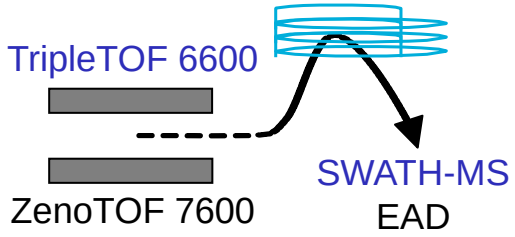


Makeup:
95% MeOH, 5% H_2O
0.1% FA, 0.1 NH_4FA (w/v)

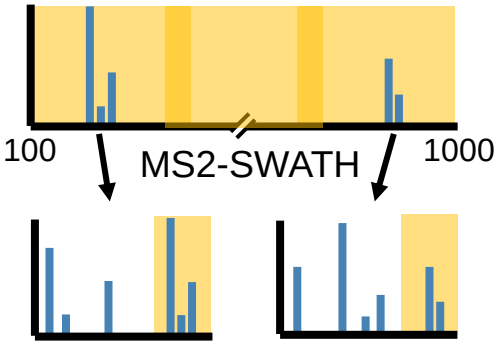


Makeup:
90% MeOH, 10% ClBz

Instrumentation

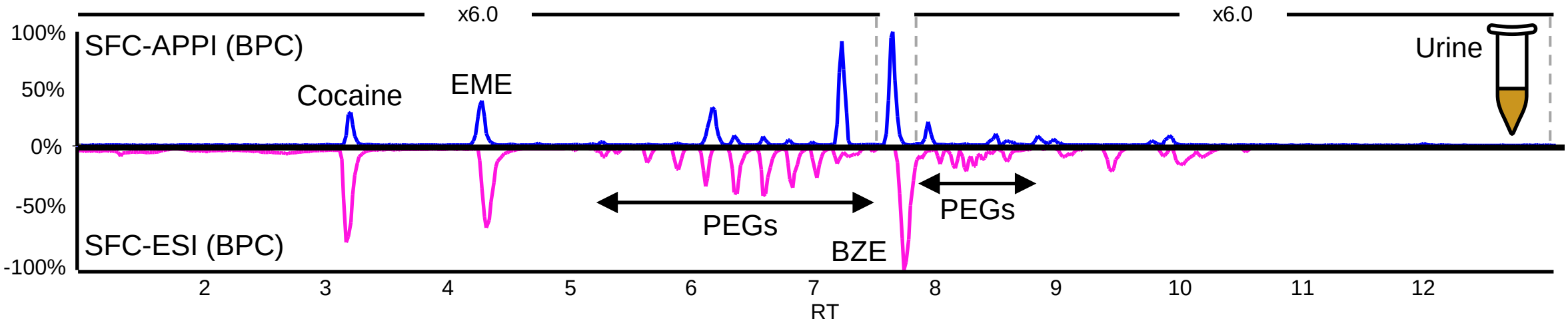


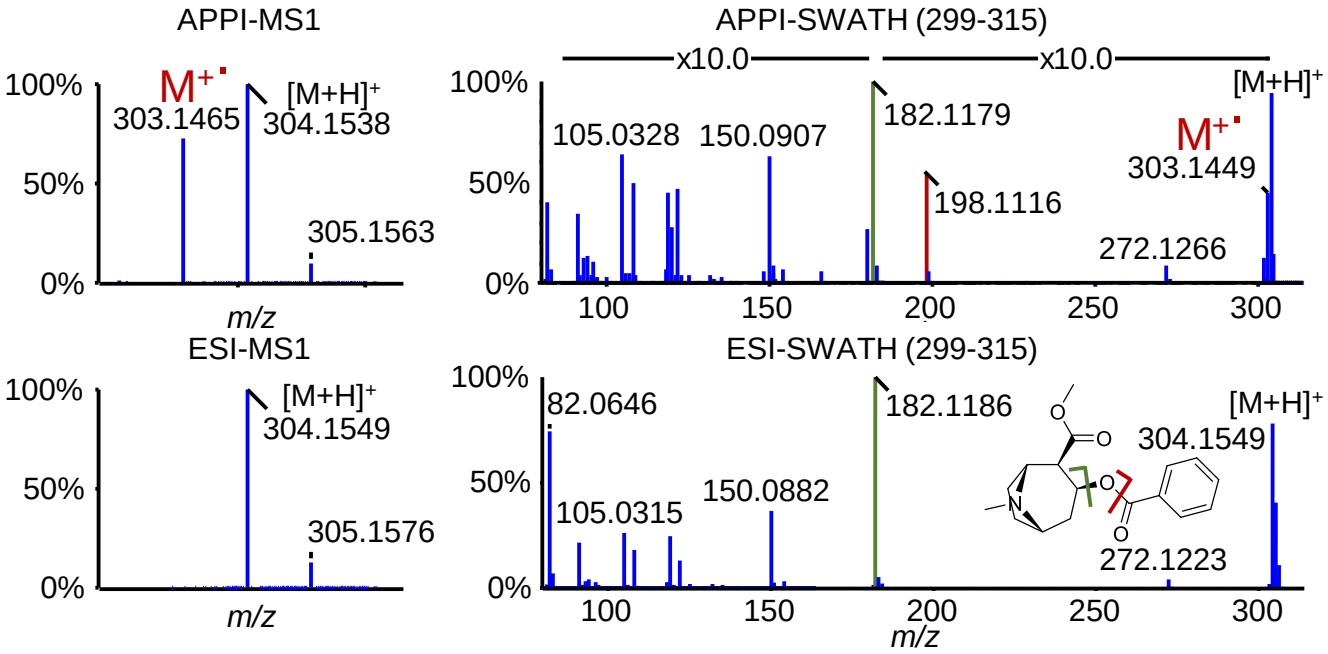
MS1-Spectra



Cycle time: ~1s

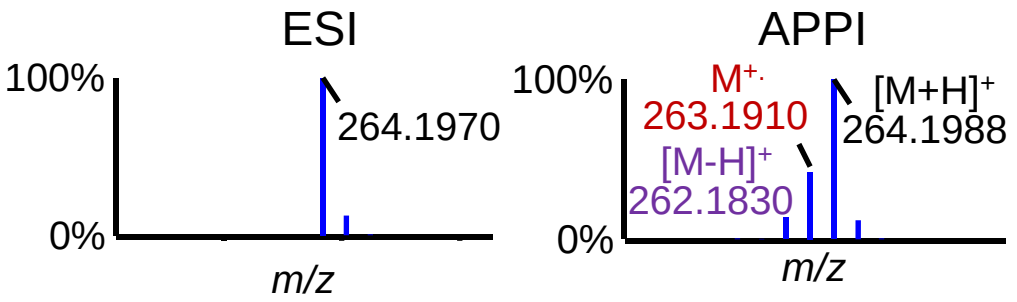
Case Study on a Cocaine Positive Subject



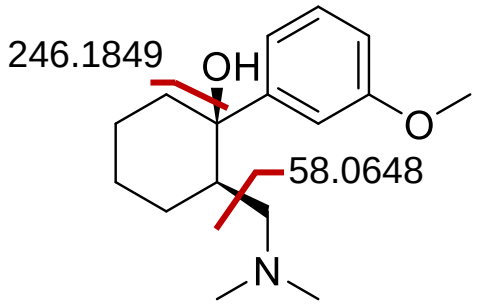


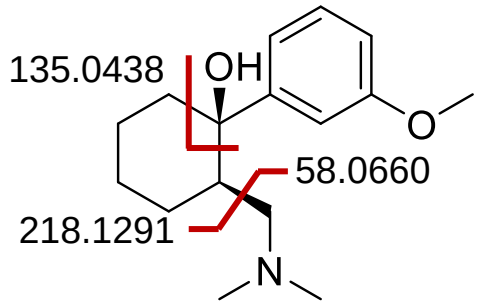
Compound	Urine ESI	Urine APPI	Blood ESI	Blood APPI
Cocaine	+	+	+	+
EME	+	+	+	+
BZE	+	+	+	+
PEGs	+	+	+	+
…	+	+	+	+

Case Study on Urine of a Tramadol Positive Subject

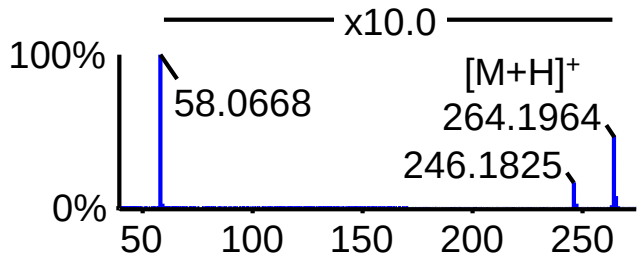


Putative Features		ESI			APPI			
Name	RT	Mass	Isotopes	Library hit	[M-H]+	M+.	[M+H]+	Library hit
Tramadol	5.48							

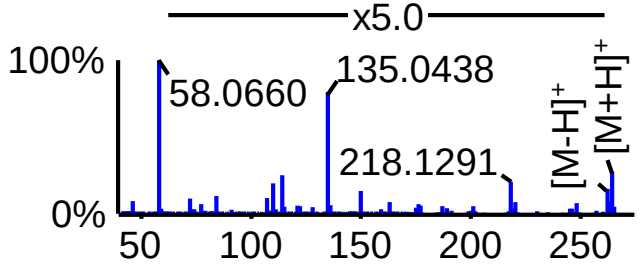




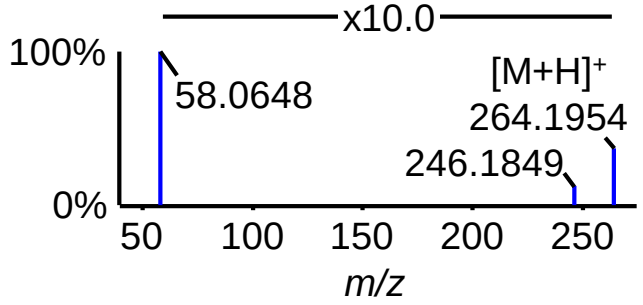
SFC-ESI-SWATH (254-270)



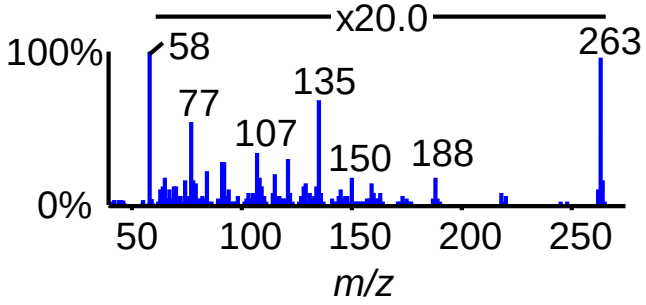
SFC-APPI-SWATH (254-270)



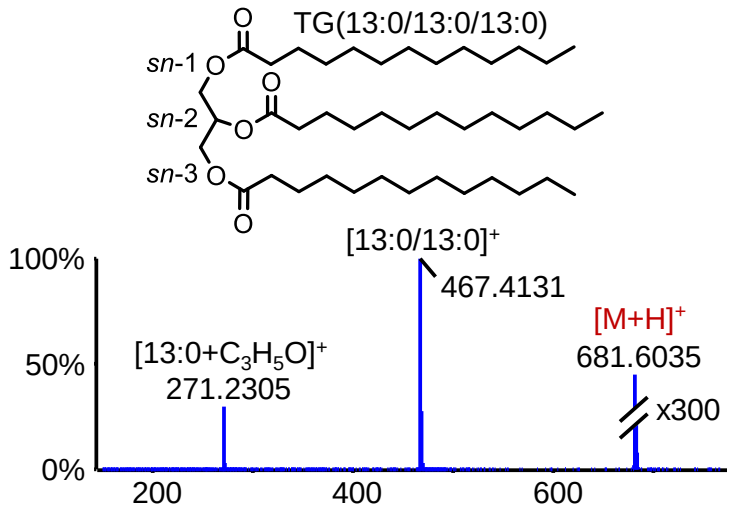
MS/MS Library
Tramadol, Rank 1, Probability 98.6%



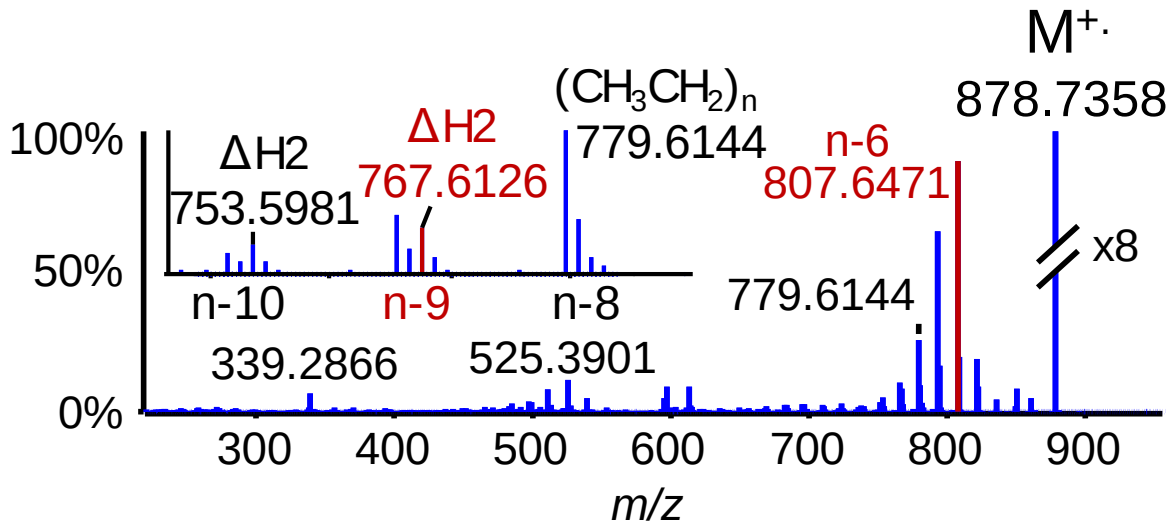
EI Library
Tramadol, Rank 1, Probability 96.5%



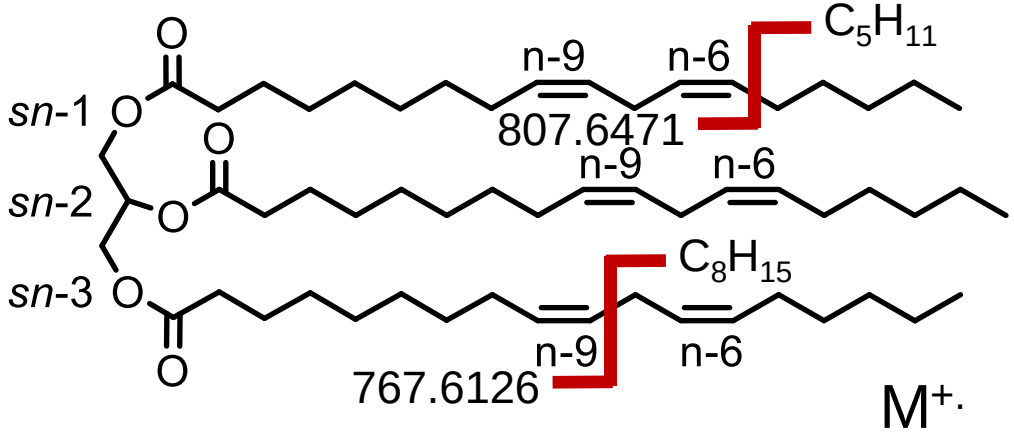
APPI: The Number of Double Bonds Influences Acylglyceride Ion Speciation



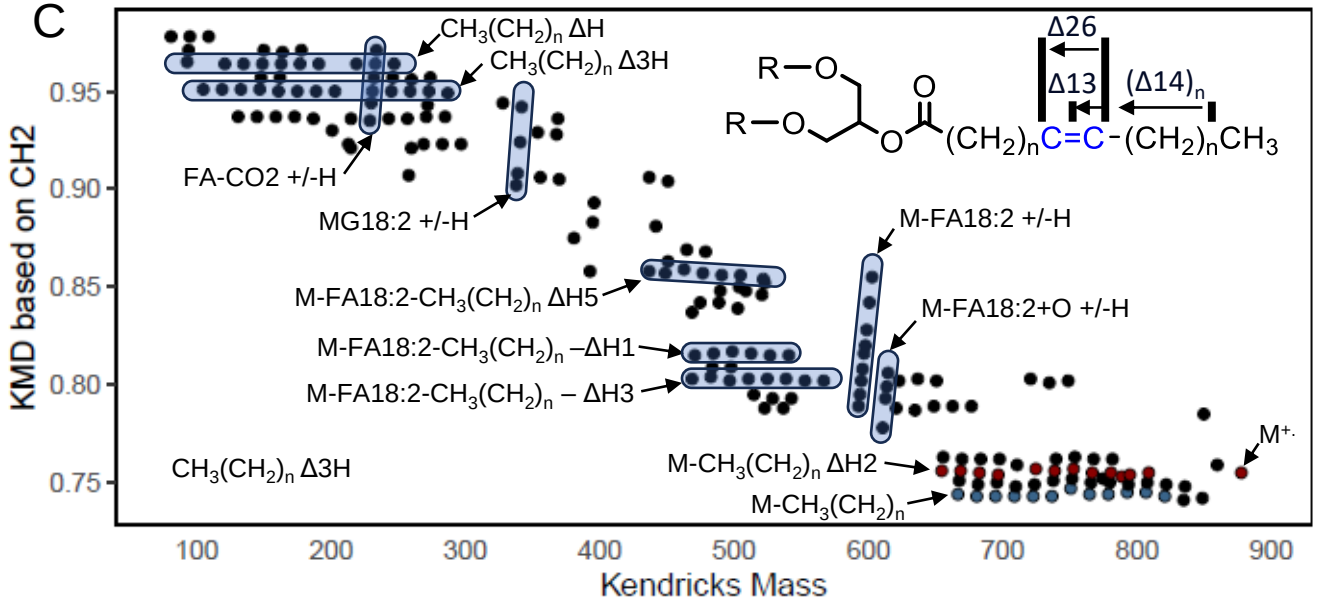
CID Spectra of TG's From Cation Radical Precursor



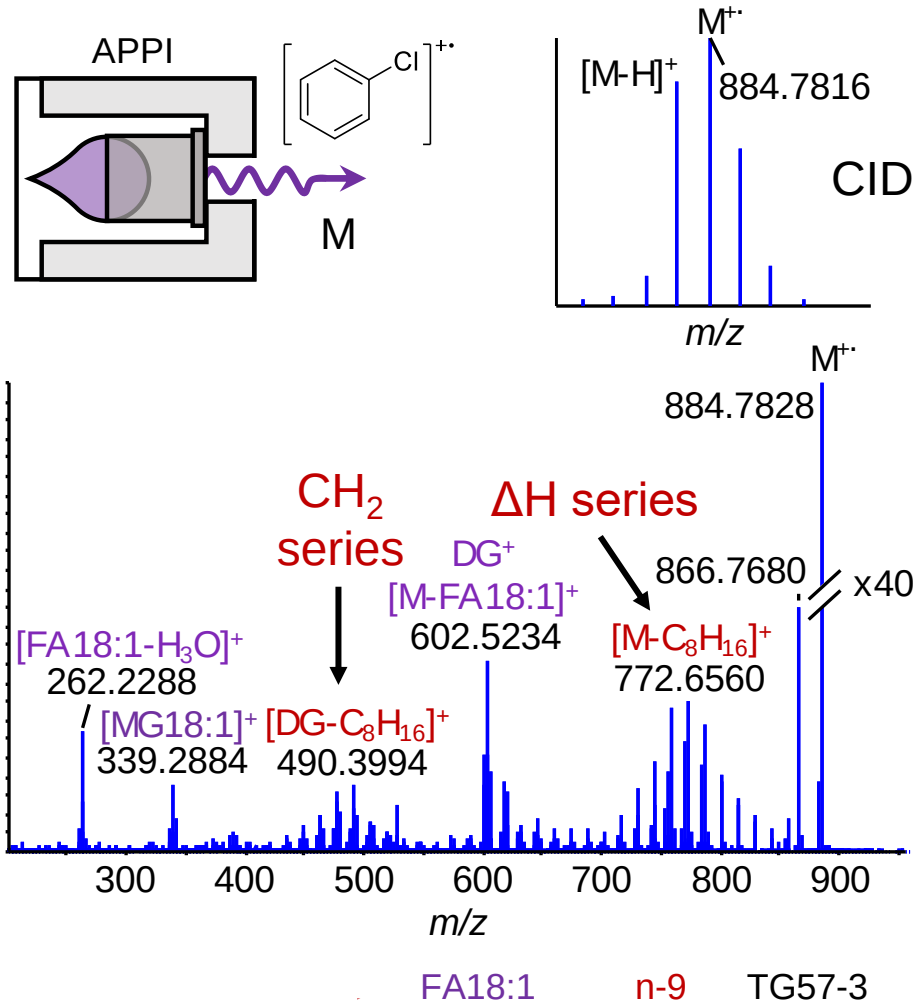
B

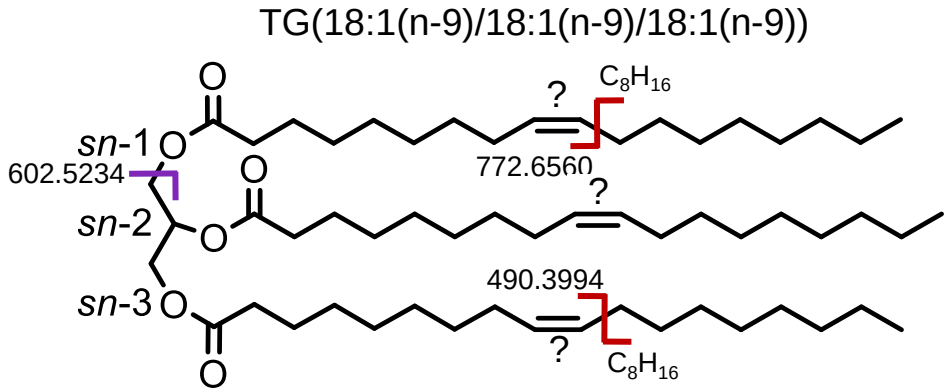


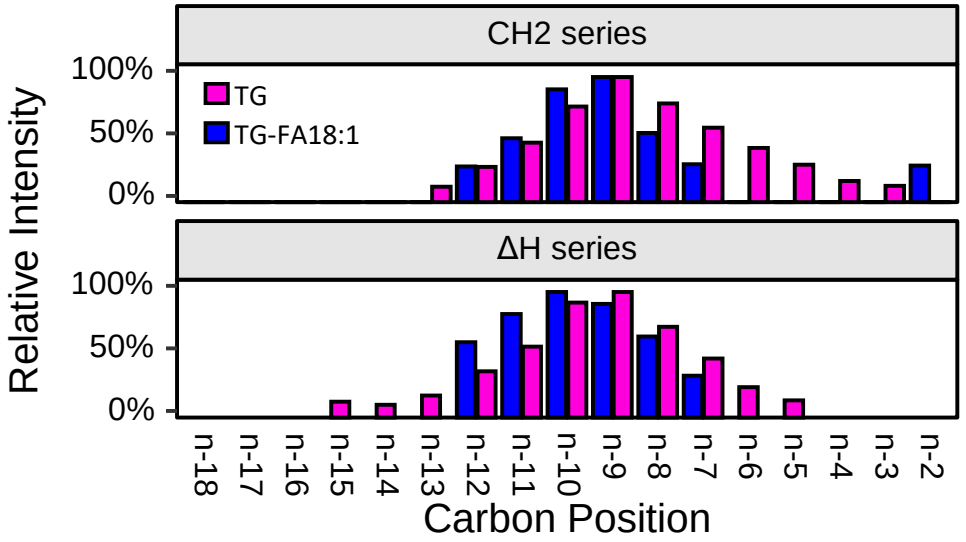
C



Collision-Induced Dissociation of Radical Cations Allows to Pinpoint Double Bond Positions Denovo

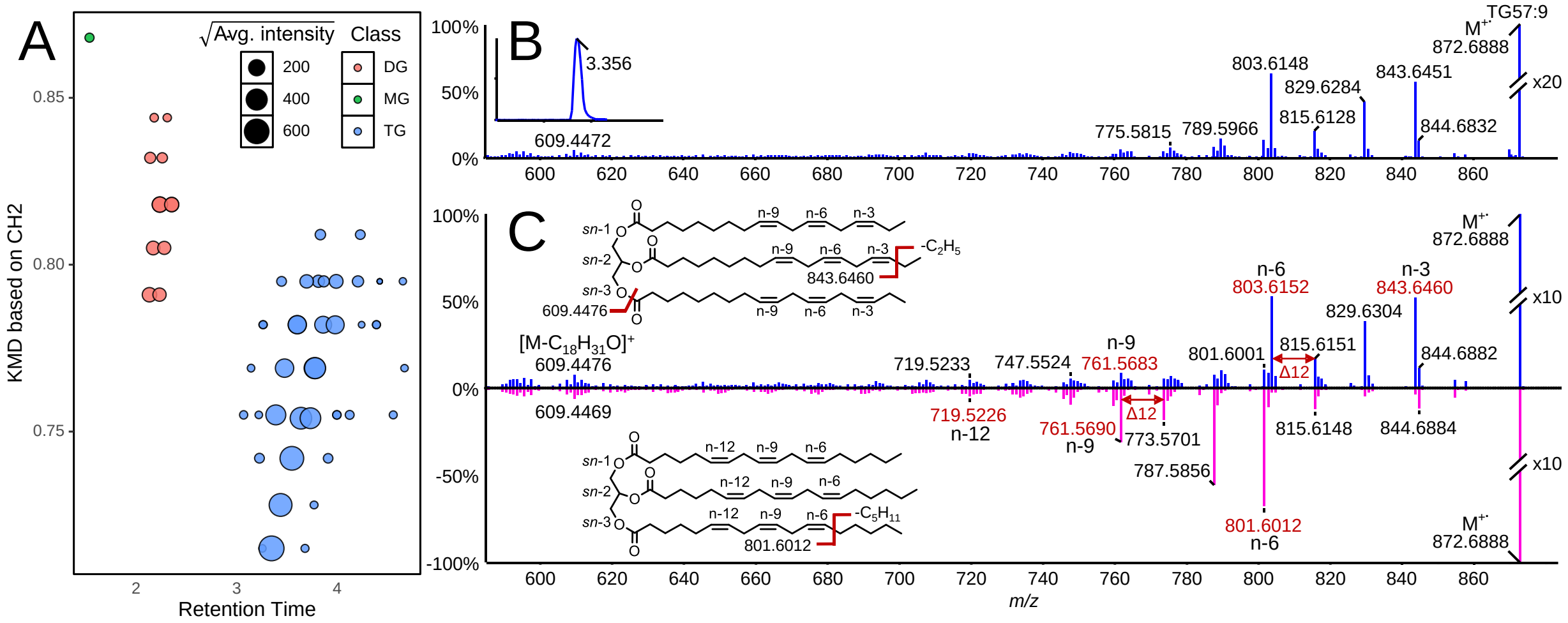






https://doi.org/10.26434/chemrxiv-2024-2xh03

SFC-APPI-MS analysis of Acylglycerols in Linseed Oil
with double bonds annotation and confirmation with standard

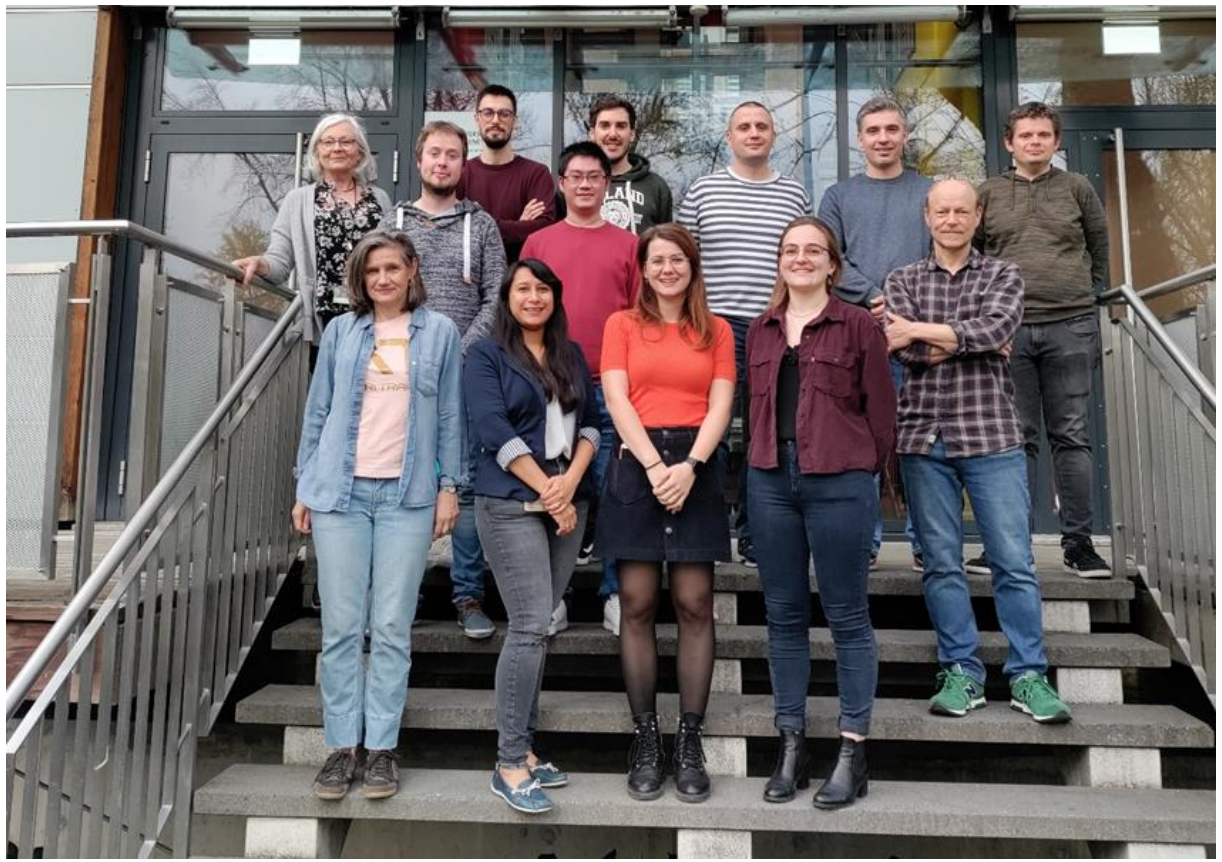


https://doi.org/10.26434/chemrxiv-2024-2xh03

Conclusions

- The Hyphenation of Supercritical Fluid Chromatography to Mass Spectrometry with ESI, APCI, APPI is straightforward when no splitting is applied.
- The use of a make-up liquid phase in SFC-MS is beneficial to control ESI ionization and allow to decouple both processes.
- Supercritical Fluid Chromatography is complementary to liquid chromatography in particular with diol or chiral columns, to reduce analysis time or to use long columns.
- With APPI-oeCID electrno impact like spectra can be obtained for LC-MS.

Acknowledgments



Laura Akbal
Eliane Kuehn
Stepan Stepanovic
Romain Giraud
Maria Fernanda Cifuentes Girard
Laura Gisela Gonzalez Iglesias
Charlotte Jacquet
Quentin Rouchon
Sandra Salinas
Xiaobo Tian

Patrick Knight

Groningen University
Frank Klont

Günter Boehm

University of Bern
Stefan König

Swiss National Science Foundation

Swiss Institute of Bioinformatics