How to optimize your (U)HPLC separation for optimal coupling with mass spectrometry: Part-1: “do-and-donot-do”

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Strategies for Efficient Coupling of (U)HPLC with MS

Quoted from Marja-Liisa Riekkola, Helsinki, Finland*

"Many important advances in column materials and technology have contributed to improve the resolution of analytes in liquid chromatography. As is well known, liquid chromatographic separations critically depend on column type, choice of stationary phase, and type and composition of the eluent employed as mobile phase. The selectivity of separations can be enhanced by adjusting the stationary or mobile phase. The best separations are achieved through careful optimization of conditions.

Liquid chromatography–mass spectrometry (LC–MS) has become increasingly popular in recent years. Although three atmospheric pressure ionization (API) techniques (electrospray ionization, atmospheric pressure chemical ionization and atmospheric pressure photoionization) are available to facilitate the coupling of LC to MS, the MS detection is not always compatible with the solvents and additives required in the preceding LC separation. Compromises must be accepted between the best LC separation conditions, especially eluent composition, and the best ionization conditions if highest selectivity and sensitivity are to be achieved."

*J. Chromatography, 1216, 684 (2009)

Aim of this Tutorial

Given the strongly different environments in which separation ((U)HPLC, liquid phase) and detection (MS, vacuum) take place they are in principle incompatible methods. When coupling, keep the following in mind:

- The mass spectrometer does not have much room for compromises.
- An (U)HPLC method may have to be compromised in order to be coupled with MS.
- An interface is needed that accepts the eluent from the column, transfers the eluent from the liquid into gas phase and provides a way to charge the solutes. The interface may cause further compromises in the (U)HPLC method

- It is the aim of this tutorial to explain how (U)HPLC parameters affect the ionization processes and vice versa limit the separation conditions that can be selected.
- Provide you a set of guidelines that allows one to get LC-MS work efficiently.

Strategies for Efficient Coupling of (U)HPLC with MS

Part 1:

(U)HPLC method development for LC–MS.

- Is the mass spectrometer a concentration sensitive or a mass (flow) sensitive detector?
- Very brief review of ionization techniques and interfaces
- MS response vs. flow rate for different ionization methods
- Influence of mobile phase properties on MS detection
- Use of inorganic buffers, role of pH adjustment and mobile phase additives

Part 2:

Optimal (U)HPLC Column Technology and Systems for LC-MS

Concentration Sensitive Detection in HPLC

- Response proportional to concentration (e.g. UV detection)

$$Abs_{i,\lambda} = \varepsilon_{i,\lambda} \cdot c_i \cdot L_{cell}$$ *e.g. Lambert Beer's law*

- Response (Abs/conc.) is independent of flow rate (e.g. infusion!)
- Peak height does not change with flow rate (e.g. flow injection analysis, neglecting (chromatographic) zone broadening)
- Chromatographic peak area in a concentration sensitive detector is given by:

$$A_i = \int c_i(V)dV = F\int c_i(t)dt$$

in case flow rate is constant ($V_{ret} = F.t_{ret}$) and is inversely proportional with flow rate (peak width is reduced in time domain)

- Concentration sensitive detectors are mostly non-destructive and preserving the eluting zone (preparative chromatography!)

Mass Flow Sensitive Detection in HPLC

- Response is proportional to mass/time (or cps)

$$R = a.\frac{\partial m_i}{\partial t}$$

- Response increases with flow rate (infusion!)
- Chromatographic peak height increases with flow rate (e.g. flow injection analysis, neglecting (chromatographic) zone broadening)
- Chromatographic peak area is given by:

$$A_i = \int \frac{m_i}{t} dt$$

and is independent of flow rate

- In most cases a destructive detection method (FID, ELSD, ICP/MS)

Concentration Sensitive versus Mass Flow Sensitive Detection

Why is it important to realize the difference between these modes of detection?

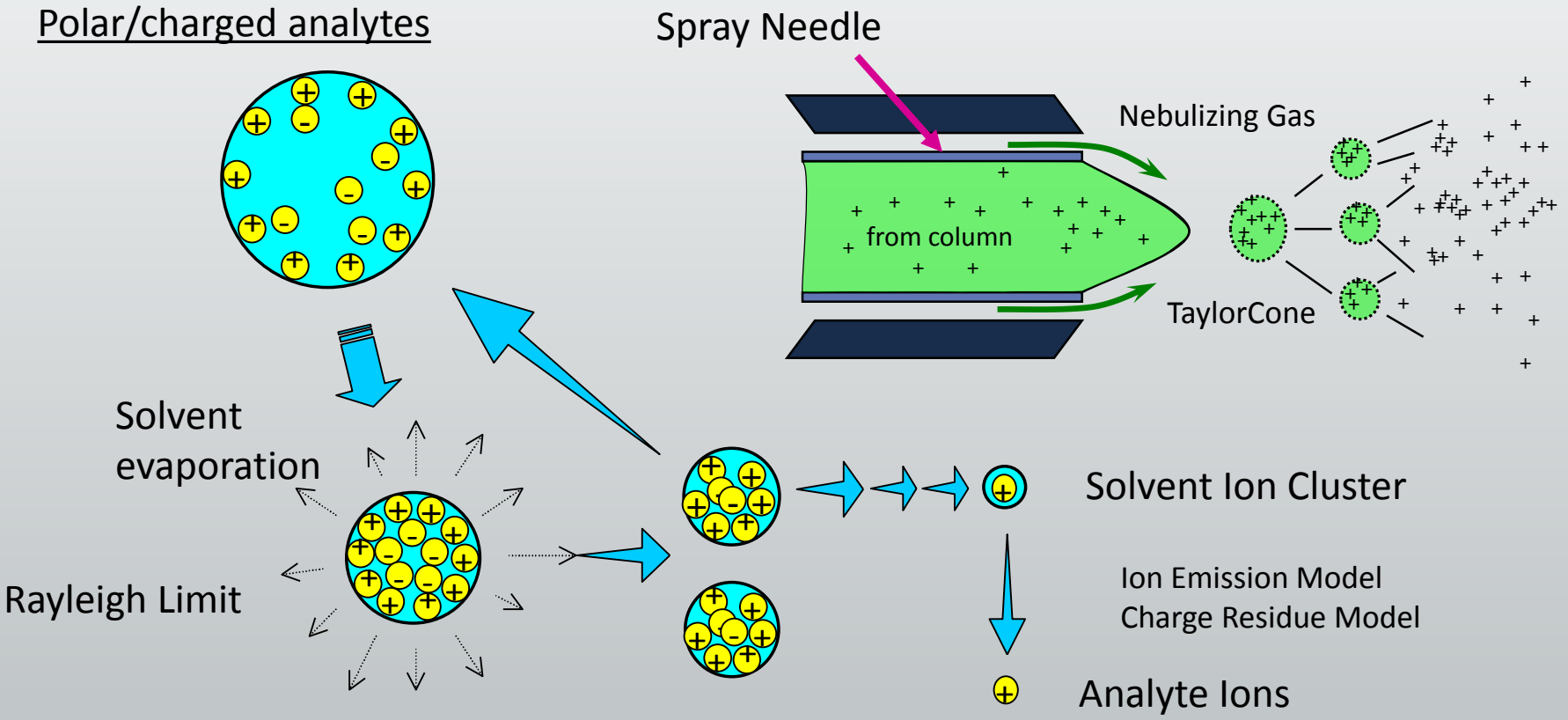
- Since in case one detector works in a mass flow sensitive mode, the usage of narrower columns will not improve the detection limits
- LC-MS interfaces will differ in this property

One needs to ask the question whether the LC-MS interface (IF) that is used renders the detector a concentration sensitive or a mass (flow) sensitive detector when coupled with HPLC?

Ionization Techniques for LC-MS

- Soft, Atmospheric Pressure Ionization (API, in principle no fragmentation)
  - Electrospray Ionization- ESI
  - Chemical Ionization - APCI
  - Photo Ionization – APPI
  - Laser Ionization - APLI
  - Surface Ionization (MALDI, DART)
- Soft, Vacuum Ionization
  - Matrix assisted laser desorption – MALDI (not online coupled)
- Hard, Vacuum Ionization (with fragmentation)
  - Particle Beam
  - Direct Electron Impact
  - Supersonic Molecular Beam

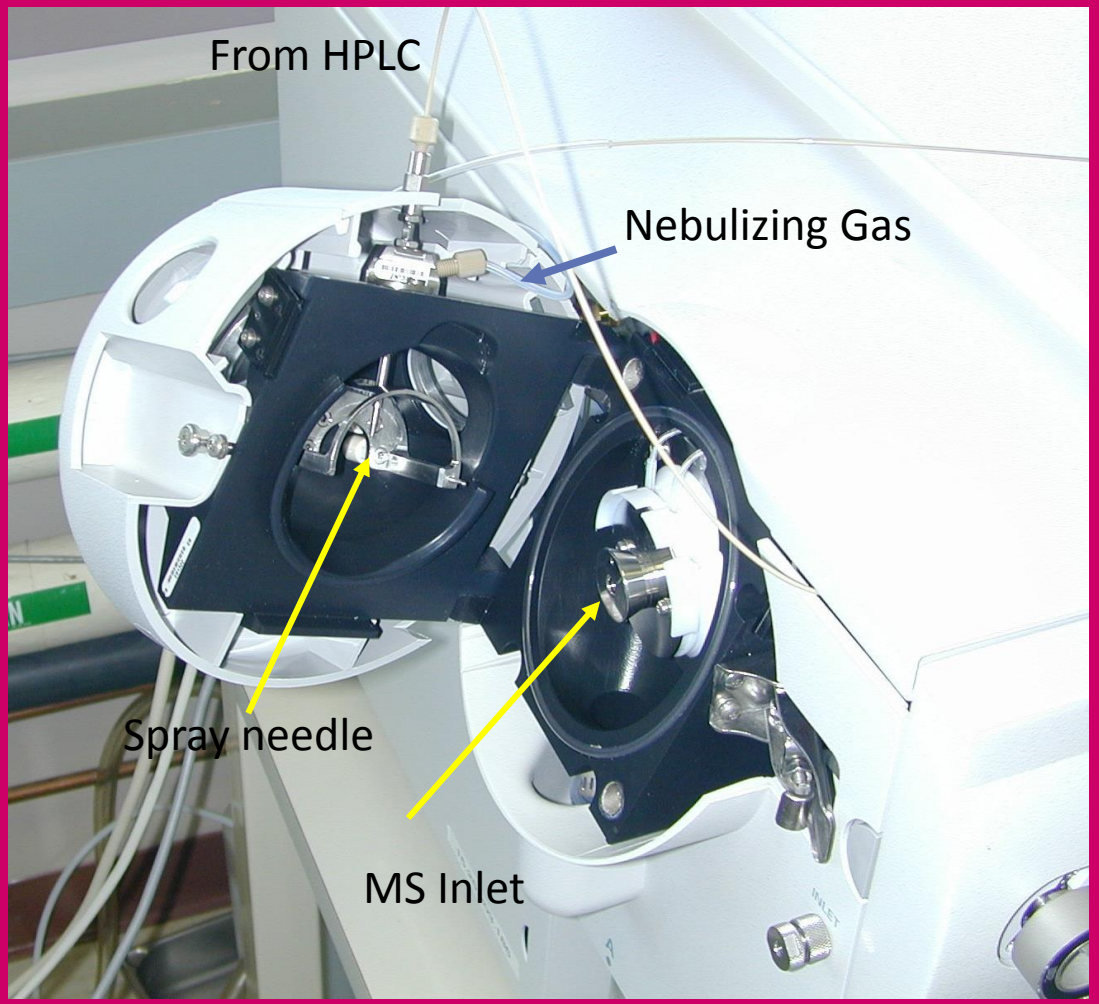
Pneumatically Assisted Electrospray Ionization



An detailed explanation of the electrospray ionization process can be found at:
http://www.mcponline.org/content/early/2011/05/19/mcp.R111.009407/suppl/DC1

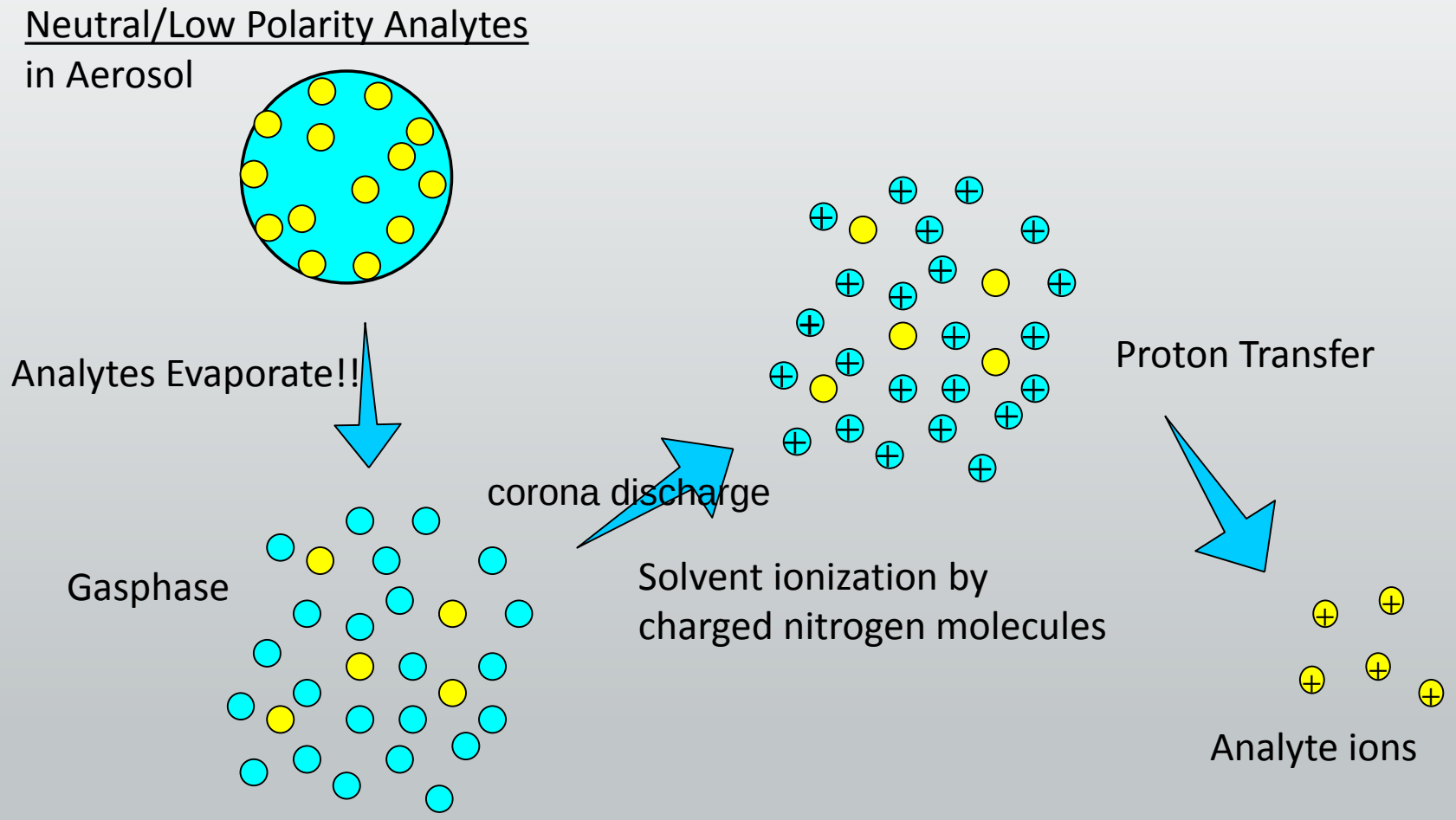
Courtesy of Agilent Technologies Kundenschulung

Pneumatically Assisted Electrospray Ionization



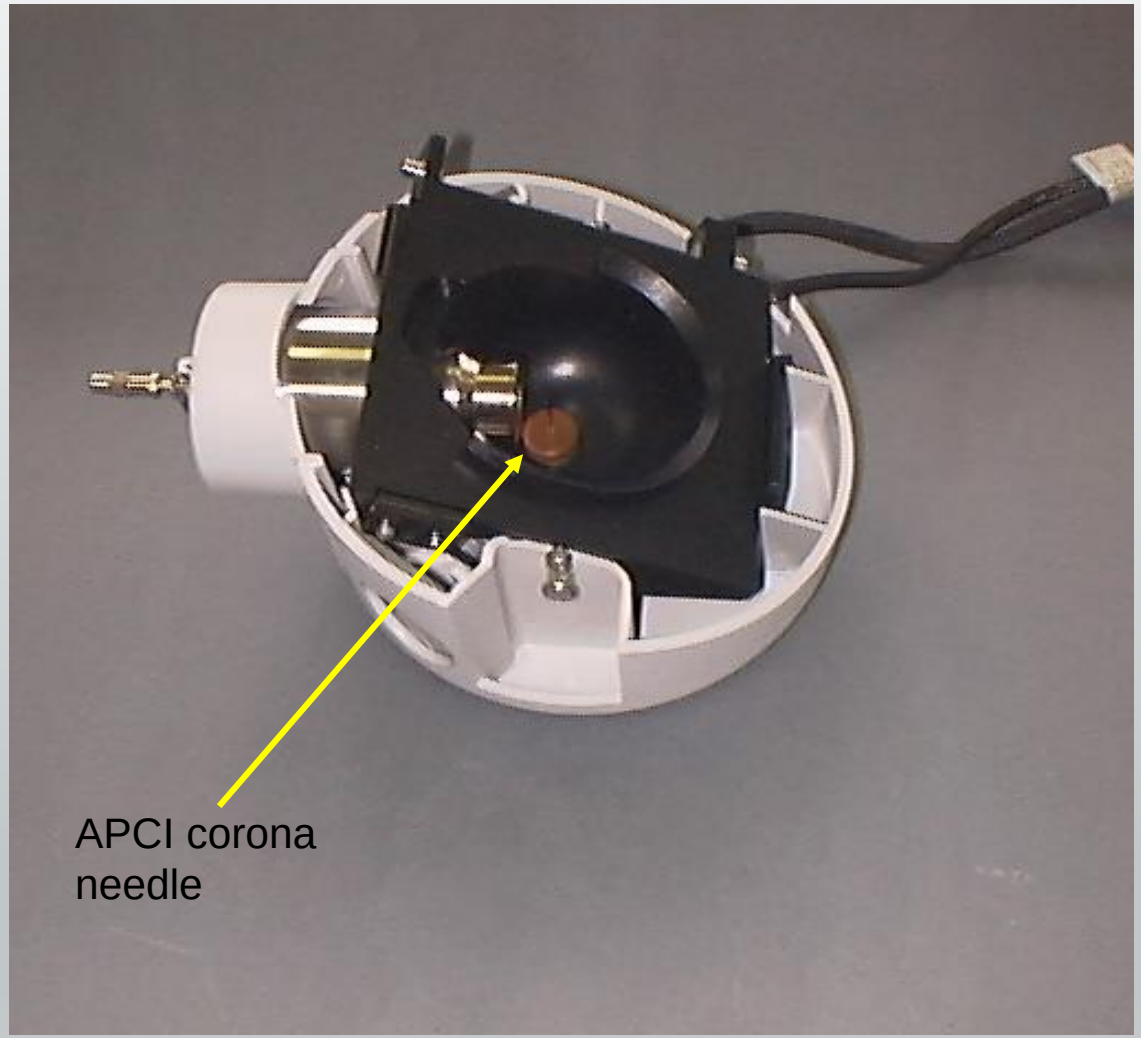
Courtesy of Agilent Technologies Kundenschulung

Atmospheric Pressure Chemical Ionization



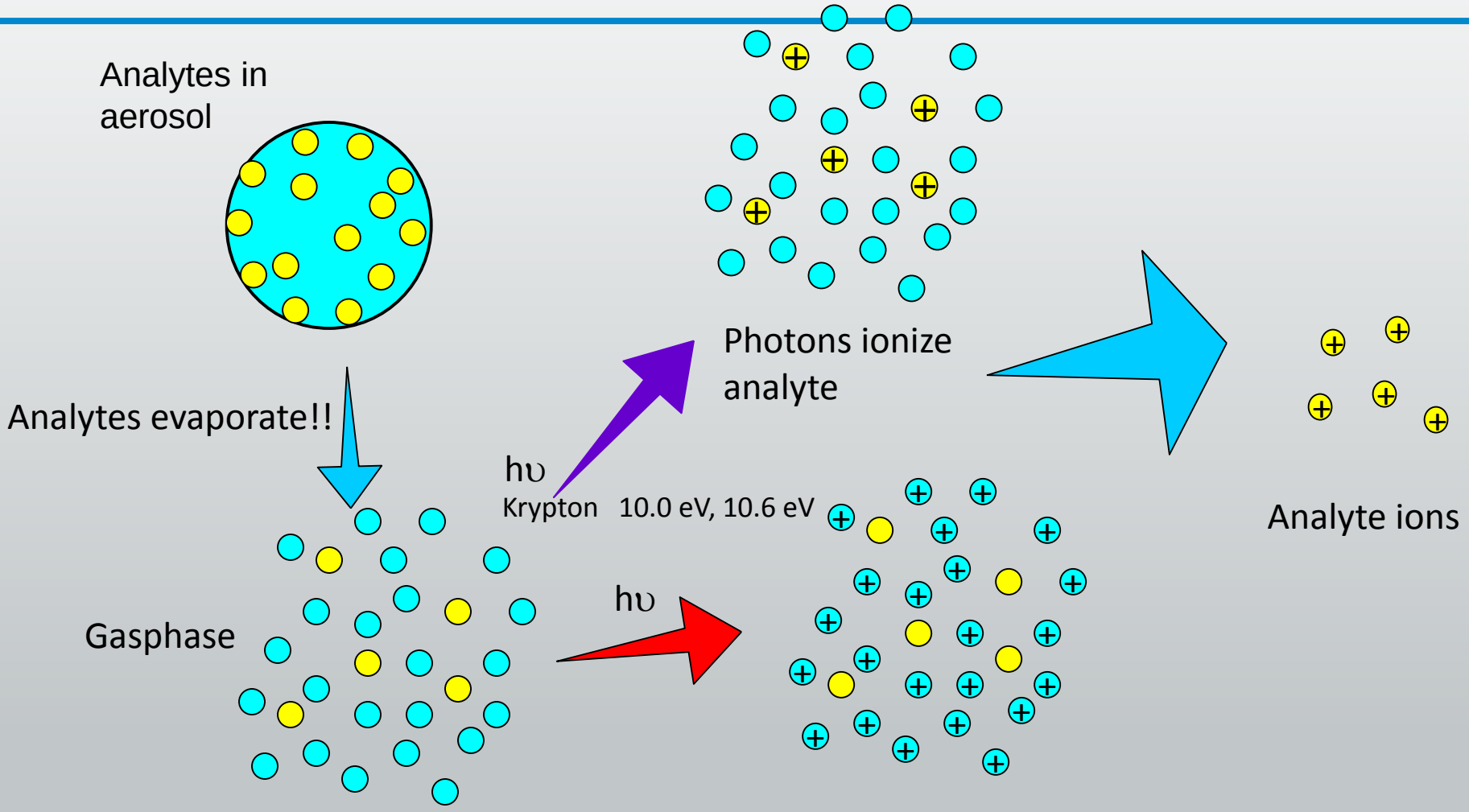
Courtesy of Agilent Technologies Kundenschulung

APCI-Interface



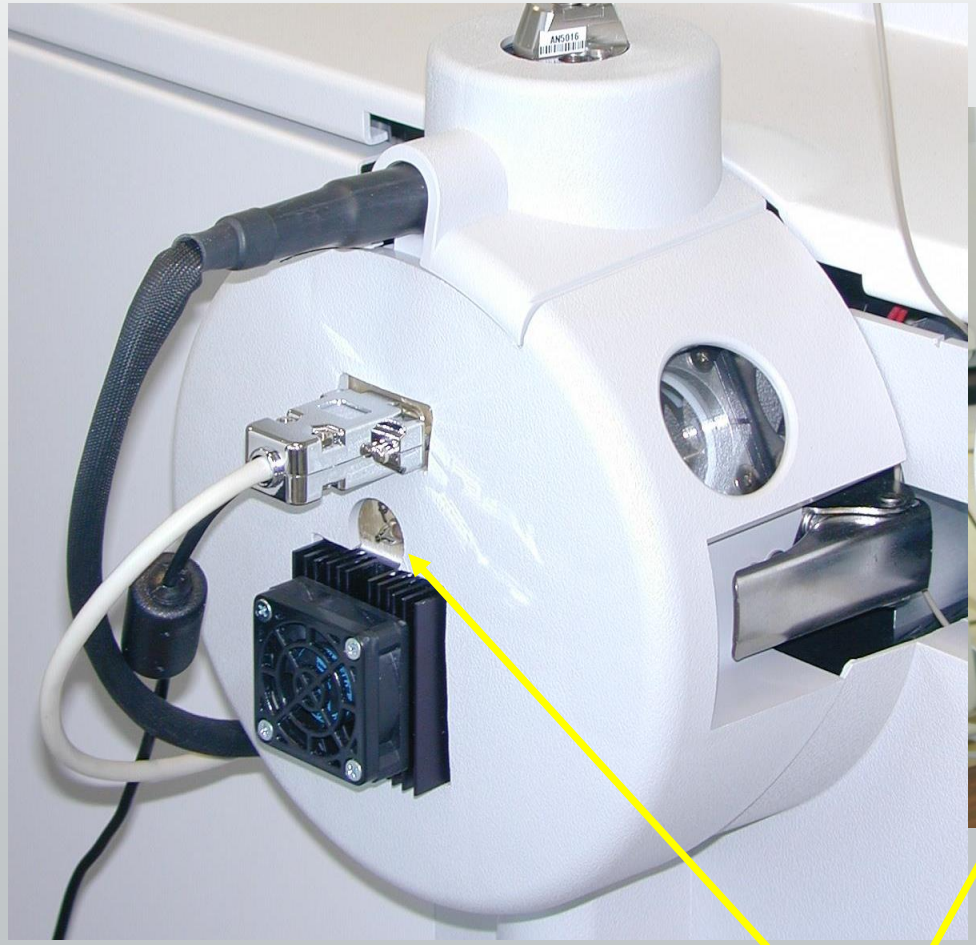
Courtesy of Agilent Technologies Kundenschulung

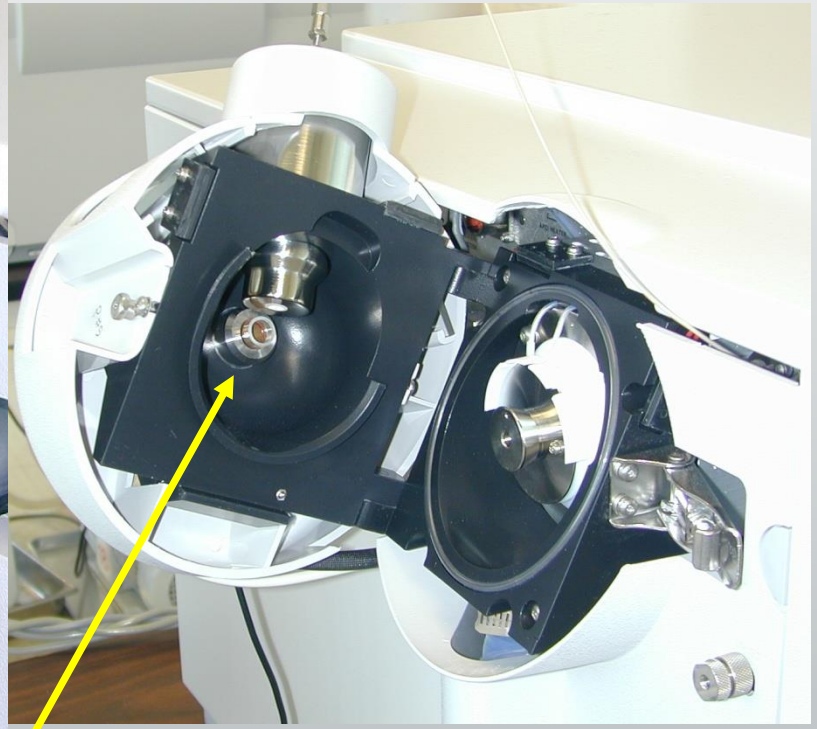
Atmospheric Pressure Photo Ionization



Courtesy of Agilent Technologies Kundenschulung

APPI-Interface

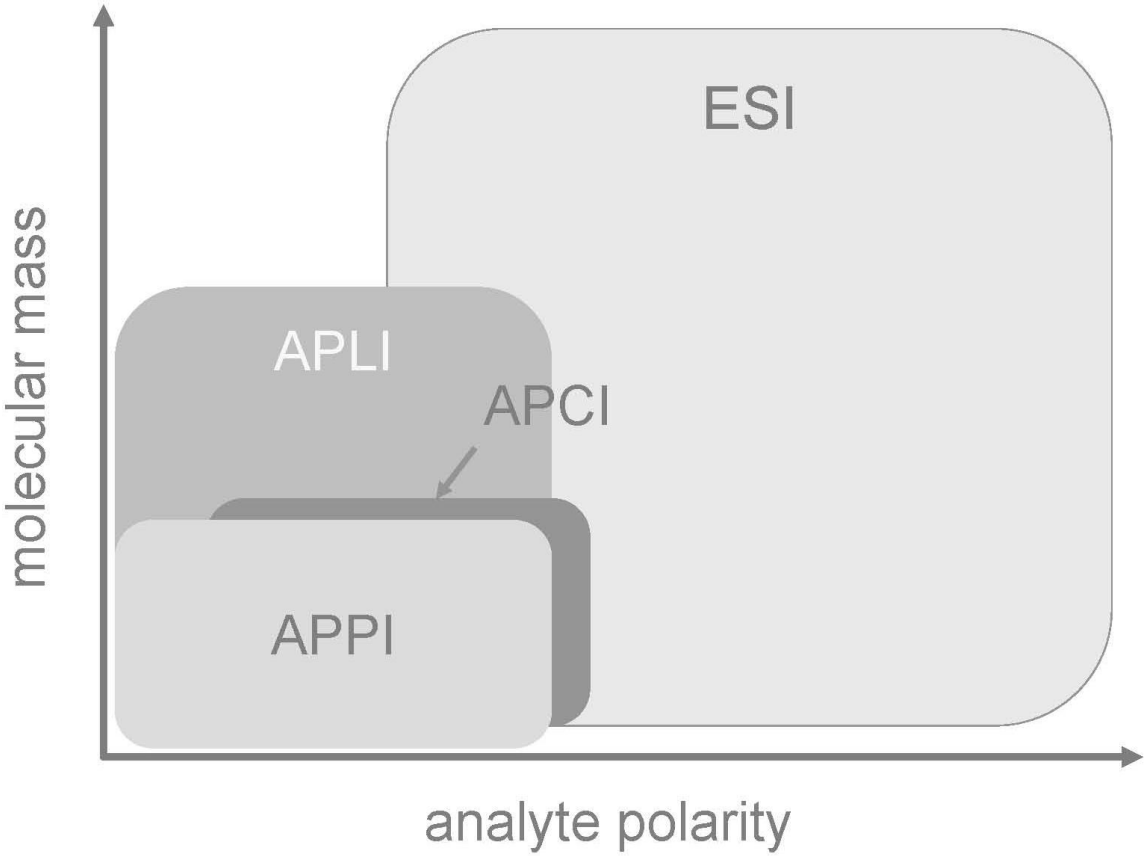




Lamp Source instead of Discharge Needle

Courtesy of Agilent Technologies Kundenschulung

Summary: Atmospheric Pressure Ionization for LC-MS



Courtesy of Oliver Schmitz, Univ. Duisburg/Essen, Germany

Factors Influencing API Process and Mass Detection

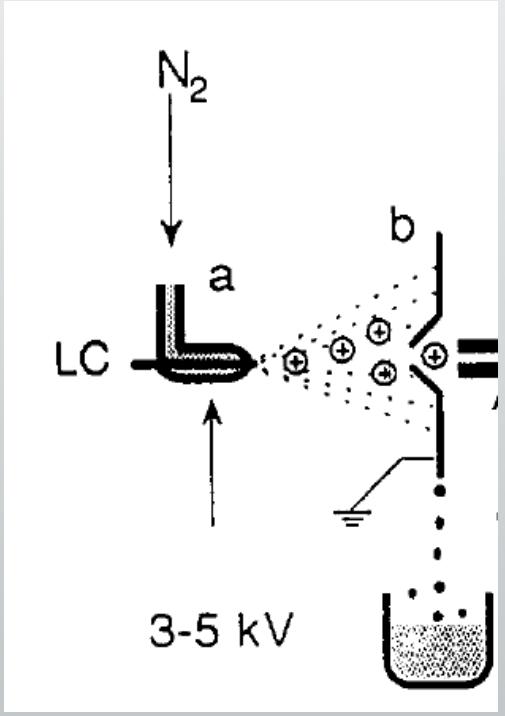
- Interface Parameters (voltage(s), gases used)
- Solute Properties
- Eluent Solvent Properties
  - Flow rate
  - Composition, volatility, viscosity, conductivity
  - Mobile phase additives (buffers, surfactants, pH modifiers)
  - Ion Suppression/Matrix effects
- Practice of LC-MS
  - Use of inorganic buffers
  - Common background ions & contaminants

System variables	Compound variables	Method variables
Electric field	Surface activity	Flow rate
ES–capillary diameter	Proton affinity	Electrolyte concentration
ES–capillary voltage	pKa	pH
Distance to counter electrode	Solvation energy	Solvent properties (boiling point, surface tension, etc.)
Heat capacity of ambient gas		
Solvent saturation level of ambient gas		

R. King et al., J. Am. Soc. Mass Spectrom., 2000, 11, 942–950

Electrospray Ionization – Influence of Flow Rate

Pneumatically assisted electrospray ionization



Initially (early 80ties) electrospray ionization only worked with very low LC flow rates (<10 µL/min) mandating very low i.d. HPLC columns (< 0.3 mm) for separation.

In order to cope with higher flow rates used with higher i.d. HPLC columns (2.1 mm i.d.), Henion et al. introduced pneumatically assisted electrospray (ion spray)*

Today everybody calls it:
Electrospray Ionization (ESI)

*A.P. Bruins , Th. R. Covey , J. D. Henion, Anal. Chem., 1987, 59 (22), pp 2642–2646

Picture taken from G. Hopfgartner et al., J. Chrom. A, 647, 51 (1993)

LC-MS Detection:

Concentration- or a mass-flow sensitive device?*

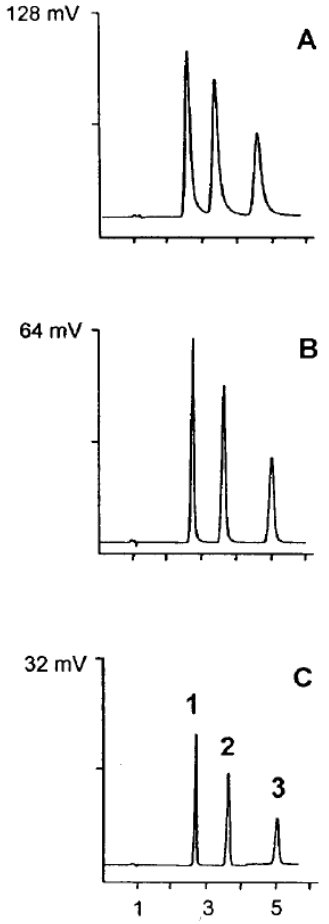


Fig. 2. LC–UV traces of alkyl benzoate esters on three different column I.D.s: (A) 1 mm I.D., flow-rate = 65 μl/min; (B) 2.0 mm I.D., flow-rate = 210 μl/min; (C) 4.6 mm I.D., flow-rate = 1000 μl/min. UV Detector with a 0.5-μl cell, 254 nm, 5-μl injection. Peaks: 1 = ethyl benzoate; 2 = propyl benzoate; 3 = butyl benzoate.

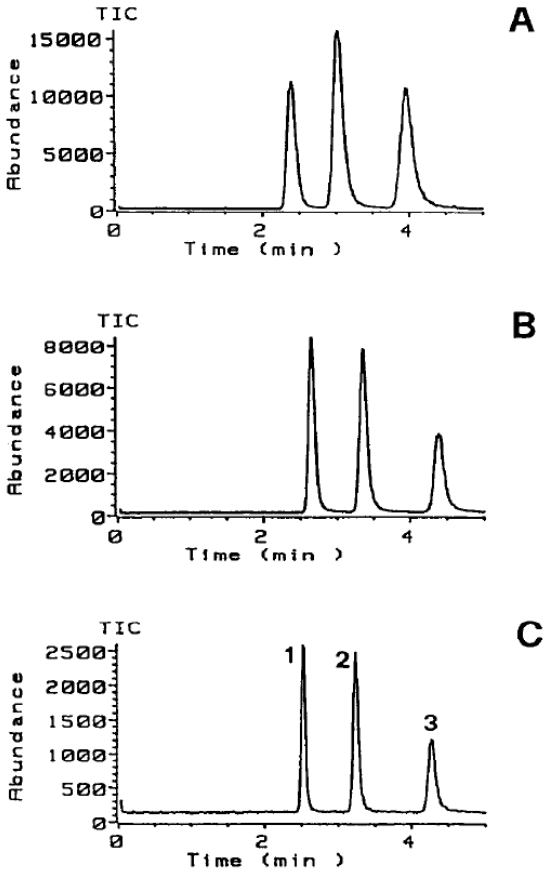


Fig. 3. LC–IS-MS traces of alkyl benzoate esters on three different column I.D.s with post-column splitting. (A) 1 mm I.D., flow-rate = 65 μl/min; (B) 2.0 mm I.D., flow-rate = 210 μl/min, splitting flow-rate to mass spectrometer 65 μl/min; (C) 4.6 mm I.D., flow-rate = 1000 μl/min, splitting flow-rate to mass spectrometer 65 μl/min, injection volume 5 μl. TIC = Total ion current.

UV Detection

Peak height increase below theory:

- too much ext. column bandspreading
- poor column packing

MS Detection with post column flow split

Peak height increase like is expected with concentration sensitive detector but below theory.

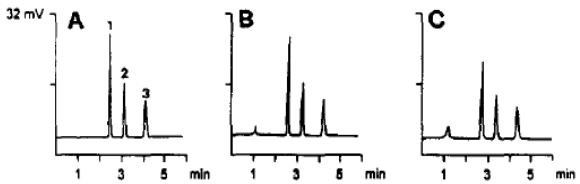


Fig. 4. LC–UV response with post-column splitting. The separation of the alkyl benzoates was performed on a 100 × 4.6 mm column, UV detector with a 2.8-μl cell, 254 nm. (A) No split, 1000 μl/min to the detector: (B) split 5:1, 200 μl/min to the detector; (C) split 20:1, 50 μl/min to the detector.

UV Detection with post column flow split

- Peak height does not depend on flow rate.

Same amount injected on all columns

*G. Hopfgartner et al., J. Chrom. A, **647**, 51 (1993)

Influence of Flow Rate on Response in LC-MS*

Pneumatically Assisted ESI (Ion Spray)

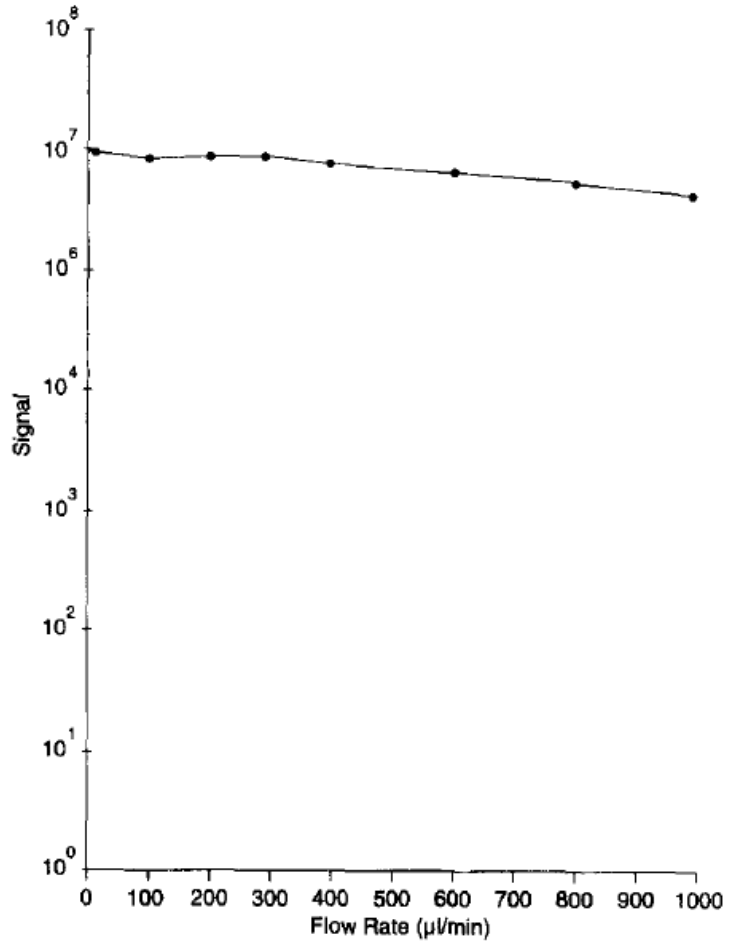


Fig. 1. Ion signal from the direct infusion of a 10 pmol/μl solution of methionine enkephalin as a function of sample flow-rate.

Solv.: MeOH/Water 50/50, 0.1% AcOH
Source: Analytica of Branford
MS: SQ HP89A, 100-1000 m/z p.s.

This ESI interface works as an Concentration Sensitivity Detector

*F. Banks Jr., J. Chrom. A, **743**, 99, 1996

Influence of Flow Rate on Response in LC-MS*

Pneumatically Assisted ESI (Ion Spray)

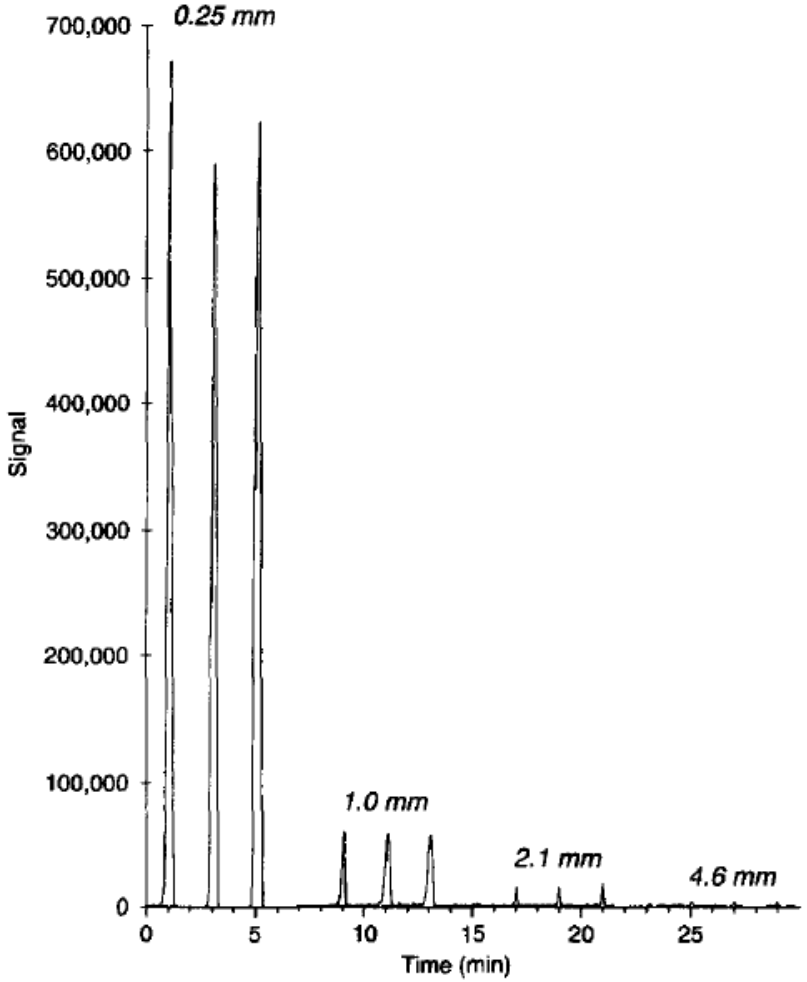


Fig. 2. TICs from methionine enkephalin injections (50 pmol each) on columns with different diameters.

Injection of equal amounts (50 pmol) of methionine enkephaline on columns with different i.d.

Signal height increase is 163x short of 339x by column diameter ratio^2
Attributed to poor packing of the microbore column

*F. Banks Jr., J. Chrom. A, **743**, 99, 1996

Influence of Flow Rate on Response in LC-MS*

Pneumatically Assisted ESI vs. APCI

ESI

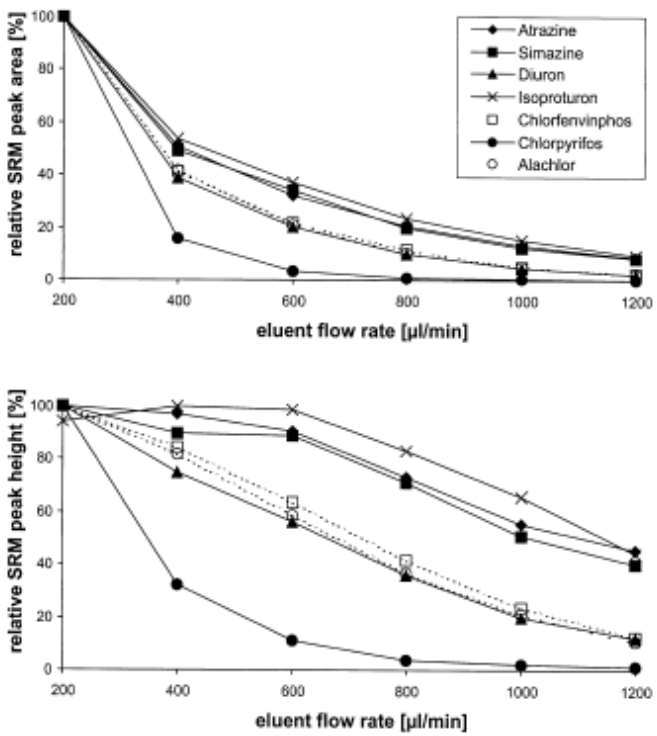
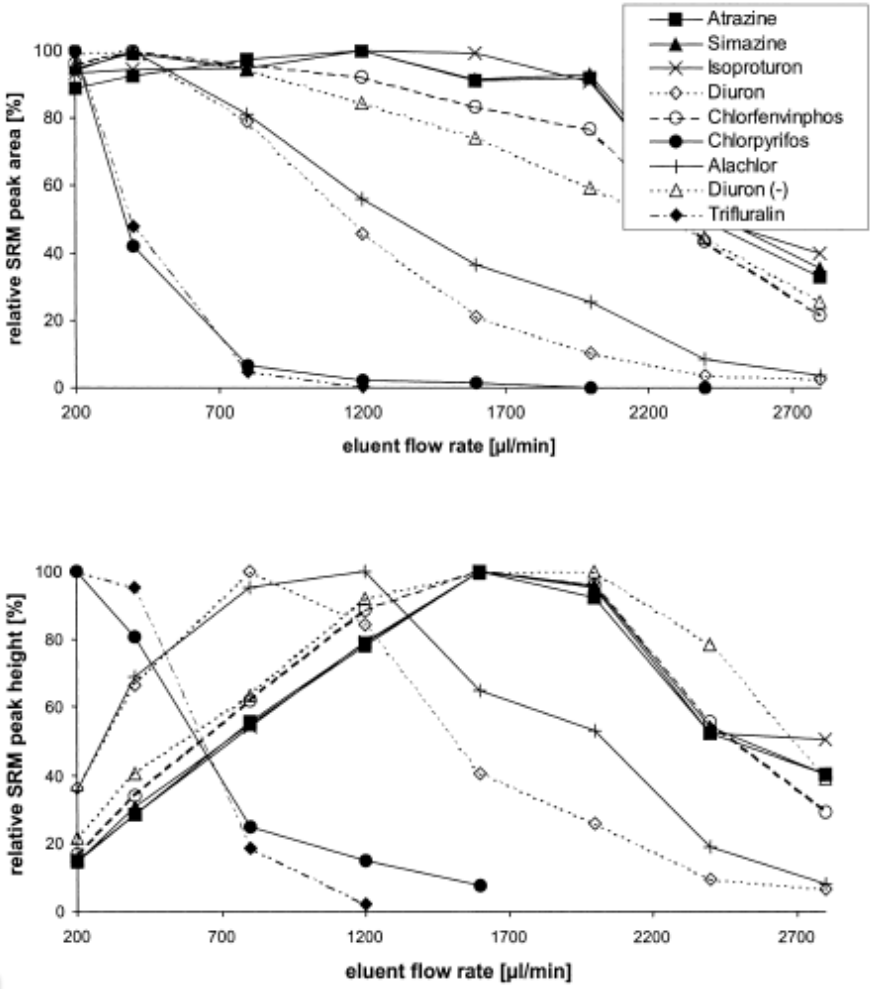


Fig. 2. FIA–ESI–MS/MS signal response of seven pesticides depending on the flow-rate of eluent (methanol–water = 97:3, v/v); standard pesticide mixture: $c = 100$ ng/ml. Top, peak area plotted versus flow-rate. Bottom, peak height plotted versus flow-rate.

APCI



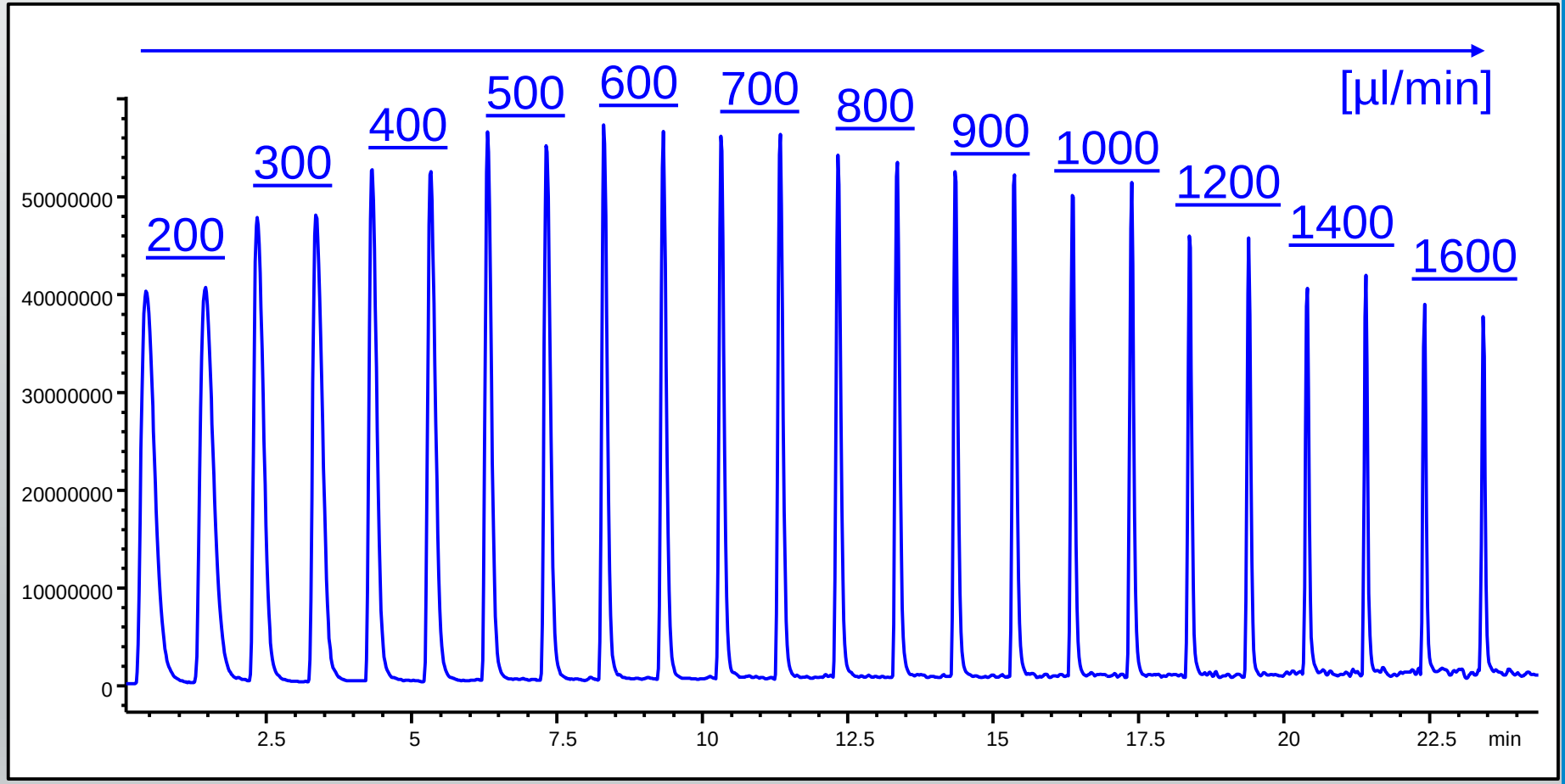
Not all analyte ions are captured with the same efficiency

*W. Engewald et al., Journal of Chromatography A, 937 (2001) 65–72

Influence of Flow Rate on Response in LC-MS*

Pneumatically Assisted ESI

Flow Injection of PEG400 in water



***Slide Courtesy Agilent Kundenschulung**

ElectroSpray Ionization (ESI) Brief Summary

- Initially electrospray ionization, tolerated maximum flow rate of 10 µL/min to mobile phases with low aqueous content in order to allow for a stable electrospray[1]
- In 1987 Henion et al. introduced pneumatically assisted spray formation and called it “ion spray”[2] which has been commercialized by Sciex and other manufacturers
- Nowadays, in practice ESI for LC-MS is in fact exclusively “ion spray”
- An ESI interface behaves largely like a concentration sensitive detector
- APCI and APPI interfaces largely behave like a mass flow sensitive detector

1. M. Yamashita, J.B. Fenn, J. Phys. Chem., 56, 2590 (1984)
2. A.P. Bruins, Th. R. Covey, and J. D. Henion, Anal. Chem., 59, 2642 7 (1987)

Nano-electrospray Ionization

Developed by Matthias Mann & Matthias Wilm[1]

- Ultra low flow rate (sub µL/min!!)
- Smaller droplets → generation of more ions
- Sprayer needle is 1 – 2 mm from MS entrance
- Higher sampling efficiency of ions into MS

Result:
dramatically higher sensitivity of Nano-ESI than standard ESI

1. M. Wilm & M. Mann, Anal. Chem. 68, 1 (1995)

Influence of Flow Rate on Response in NanoESI

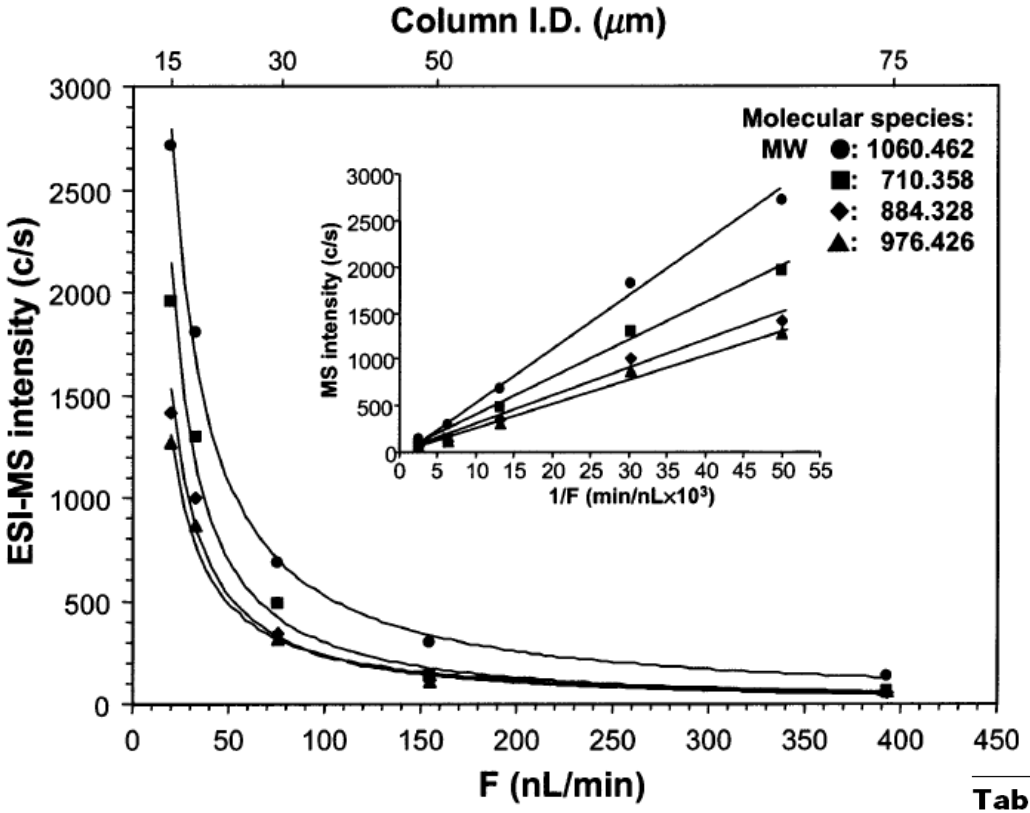


Figure 8. Relationship between nanoESI-MS response and mobile-phase flow (or inner diameters) for individual species in yeast soluble protein digest. Conditions: the flow rates were r

Emitter tip orifice diameter proportionally reduced

Table 1. NanoLC Packed Capillary Parameters Operated at 10 000 Psi[a]

columns (μm i.d. × cm)	d_p (μm)	p_d (Å)	u (cm/s)	F (nL/min)	γ	ϵ
porous						
74.5 × 87.0	3.6	300	0.19	393	20.7	0.78
47.1 × 87.0	3.6	300	0.19	155	13.1	0.79
29.7 × 87.0	3.6	300	0.22	76	8.3	0.81
19.8 × 87.0	3.6	300	0.22	33	5.5	0.81
14.9 × 87.0	3.6	300	0.23	20	4.1	0.83

R.D. Smith et al., Anal. Chem. 74, 4235 (2002)

What have we learned – Flow Dependence ESI

- Limit the flow rate with pneumatically assisted ESI interfaces to maximally 1 – 2 mL/min
- Pneumatically assisted ESI IF behaves largely like a concentration sensitive detector; APCI behaves largely like a mass sensitive detector
- Reduction of column i.d. demands very low extra column dispersion and well packed columns in order to exploit sensitivity gain with concentration sensitive detection
- Nano-ESI response increases dramatically at very low flow rates (<50 nL/min)
- At very low flow rate ESI is nor mass flow sensitive or concentration sensitive detector because more ions reach the MS inlet
- Ion suppression is much reduced (vide infra)

Influence of Solvent Composition on ESI

- Quoted from Garcia*:
  "A suitable eluent for electrospray ionization should contain an organic modifier (methanol or acetonitrile) and a volatile buffer whose concentration could also be critical; concentrations that are too high may result in the suppression of the analyte signal, while concentrations that are too low may lead to poor peak shape and efficiency"

*M.C. Garcia, J. Chromatography B, **825**, 111 (2005)
Further reading R. Kostiainen et al., J. Chrom. A, **1216**, 685 (2009)

Good Solvents for Atmospheric Pressure Ionization

Formation of analyte ions in solution is essential to achieving good electrospray.

- Use volatile buffers in the mobile phase to avoid the buildup of precipitates in the ion source
- Adjust mobile phase pH along the pK_a/pK_b of all solutes
- Use solvents that have low heats of vaporization and low surface tensions to enhance ion desorption

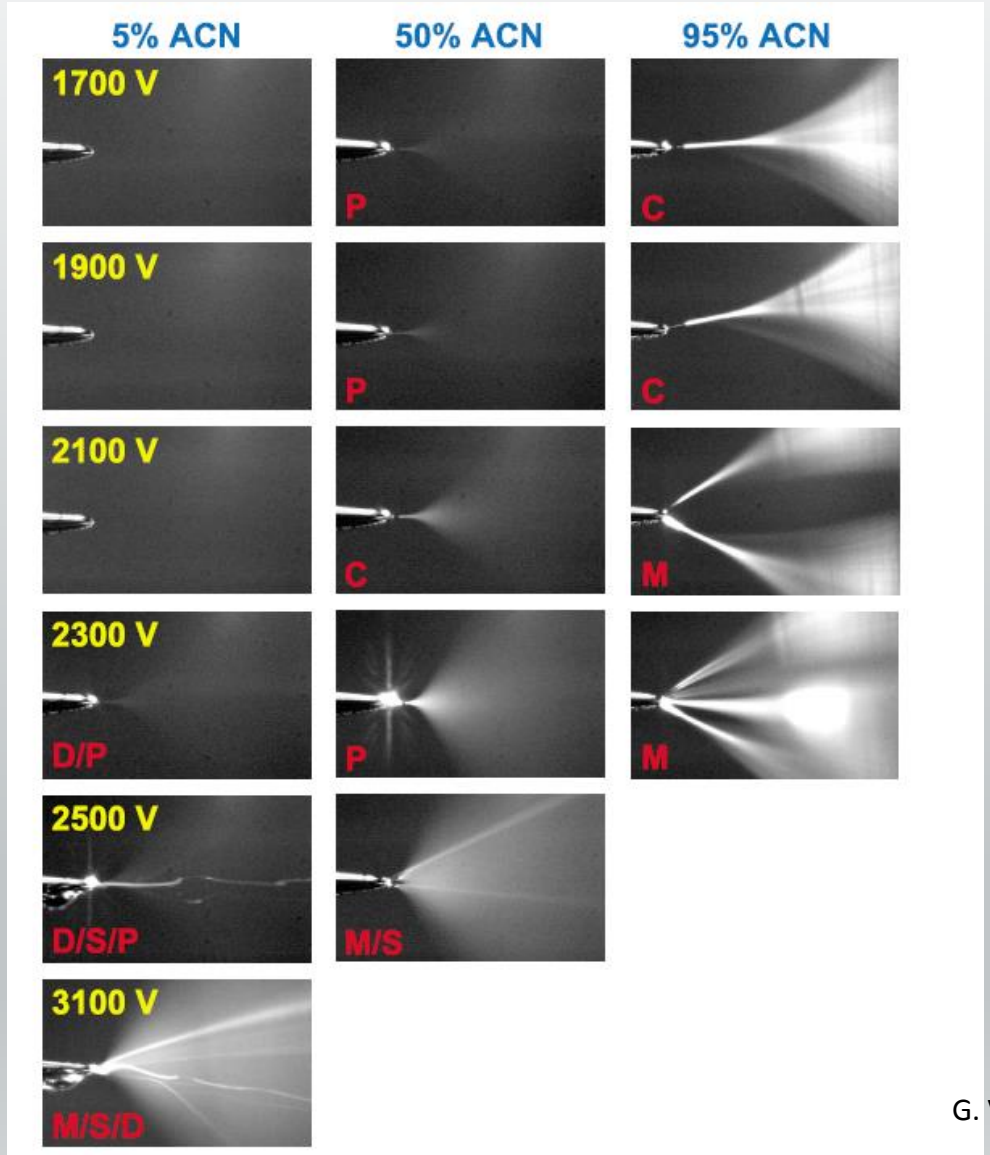
ESI, APCI	APCI
Alcohols	Aliphatic solvents
Acetonitrile	Aromatic solvents
Tetrahydrofurane	Carbondisulfide
Water	Tetrachloromethane
Acetone	
Dimethylformamide (<10% vv)	

Further reading see:
http://hplctips.blogspot.de/2014/06/popular-hplc-volatile-mobile-phase.html

Slide Courtesy Agilent Kundenschulung

Influence of Gradient Elution on Response in LC-MS*

Nano-electrospray Ionization



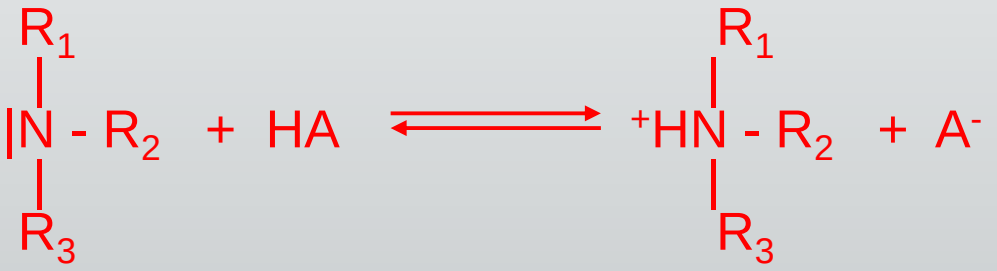
C = cone jet
P = pulsed cone jet
M = multi jet
D = dripping
S = spindle

G. Valaskovic, J Am. Soc. Mass Spectrom., 2004, 15, 1201–1215

Solute Ionization

ESI mandates the formation of analyte ions in the eluent solution

Positive Mode



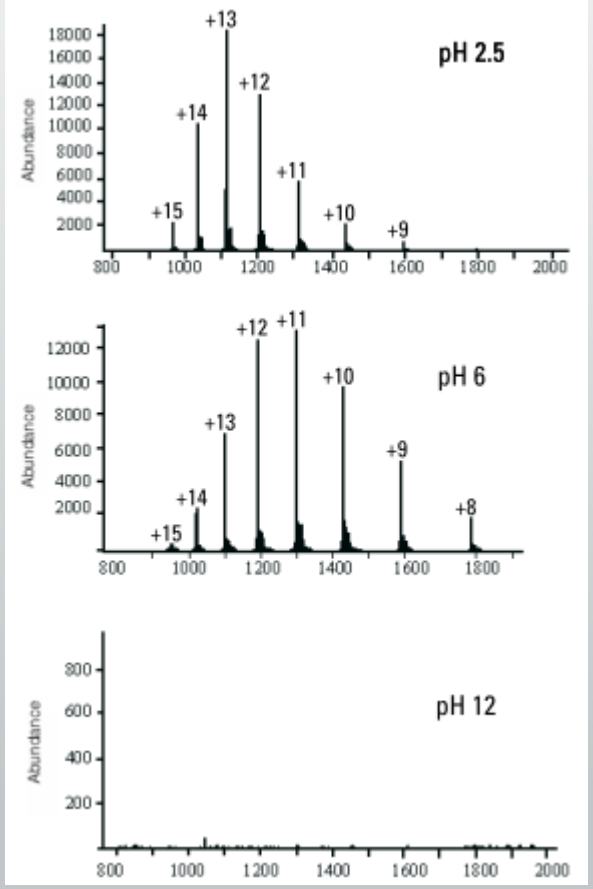
Base Acid Analyte Cation

Negative Mode

Acid Base Analyte Anion

With weak acidic and/or basic solutes in your sample,
adjust mobile phase pH to 0% resp. 100% dissociation

Chemistry is Important! – Mobile phase pH



Example Lysozyme, pI 9.35

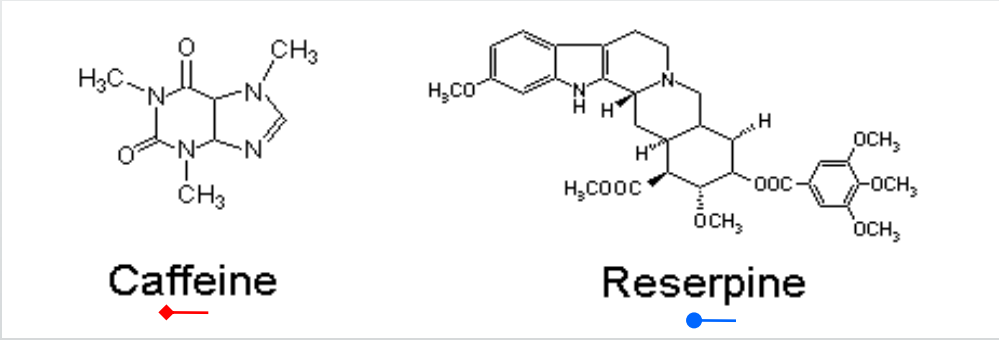
Frequently Used Mobile Phase Additives in ESI/MS

Positive Mode	Negative Mode
Ammonium Acetate	Ammonium Acetate
Ammonium Formate	Ammonium Formate
Acetic Acid (pH 3-4)	Ammonia/Ammonium Hydroxide (pH>7)
Formic Acid (pH 2-3)	Triethylamine (pH >7)
Trifluoro-acetic Acid (pH 1-2)	N-Methylmorpholin

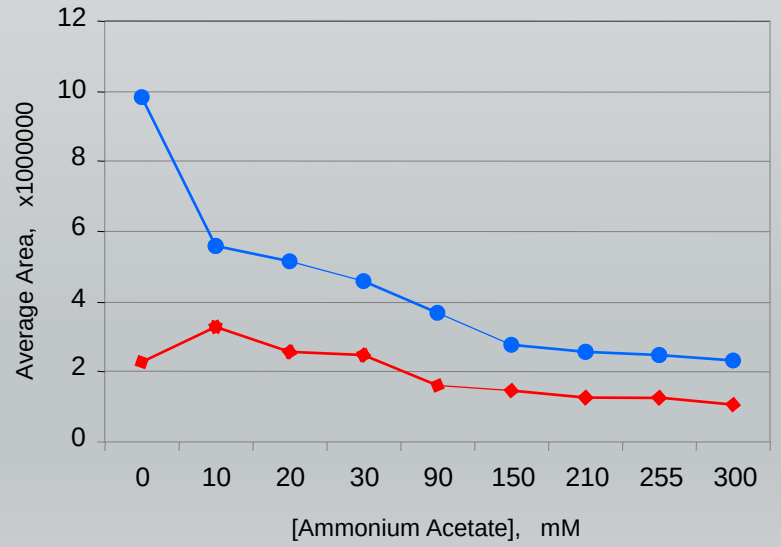
- As additive or by post-column addition in case the solvent pH for optimal separation differs from the pH for optimal ionization.
- Additives will cause an high background signal (TFA (m/z 113) in negative mode, TEA (m/z 102) in positive mode), increase conductivity of the solvent and may cause ion suppression

Courtesy of Agilent Technologies Kundenschulung

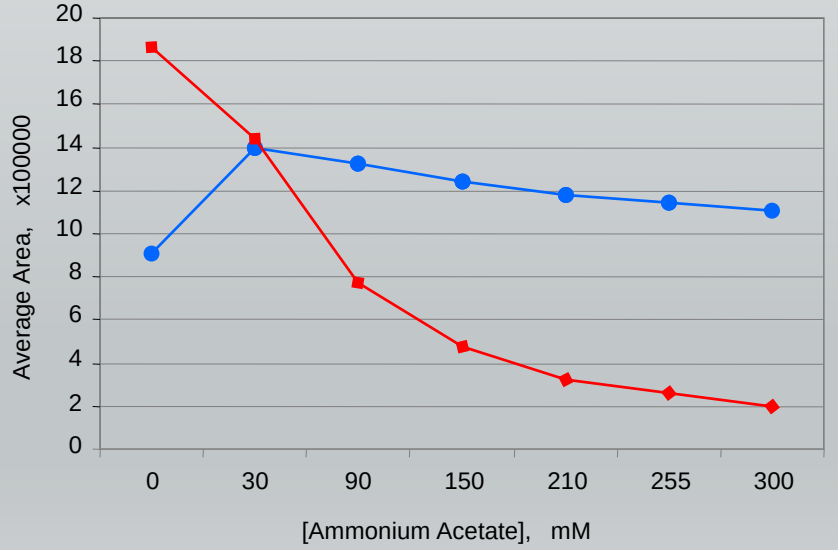
Influence of Additive Concentration on Response



ESI



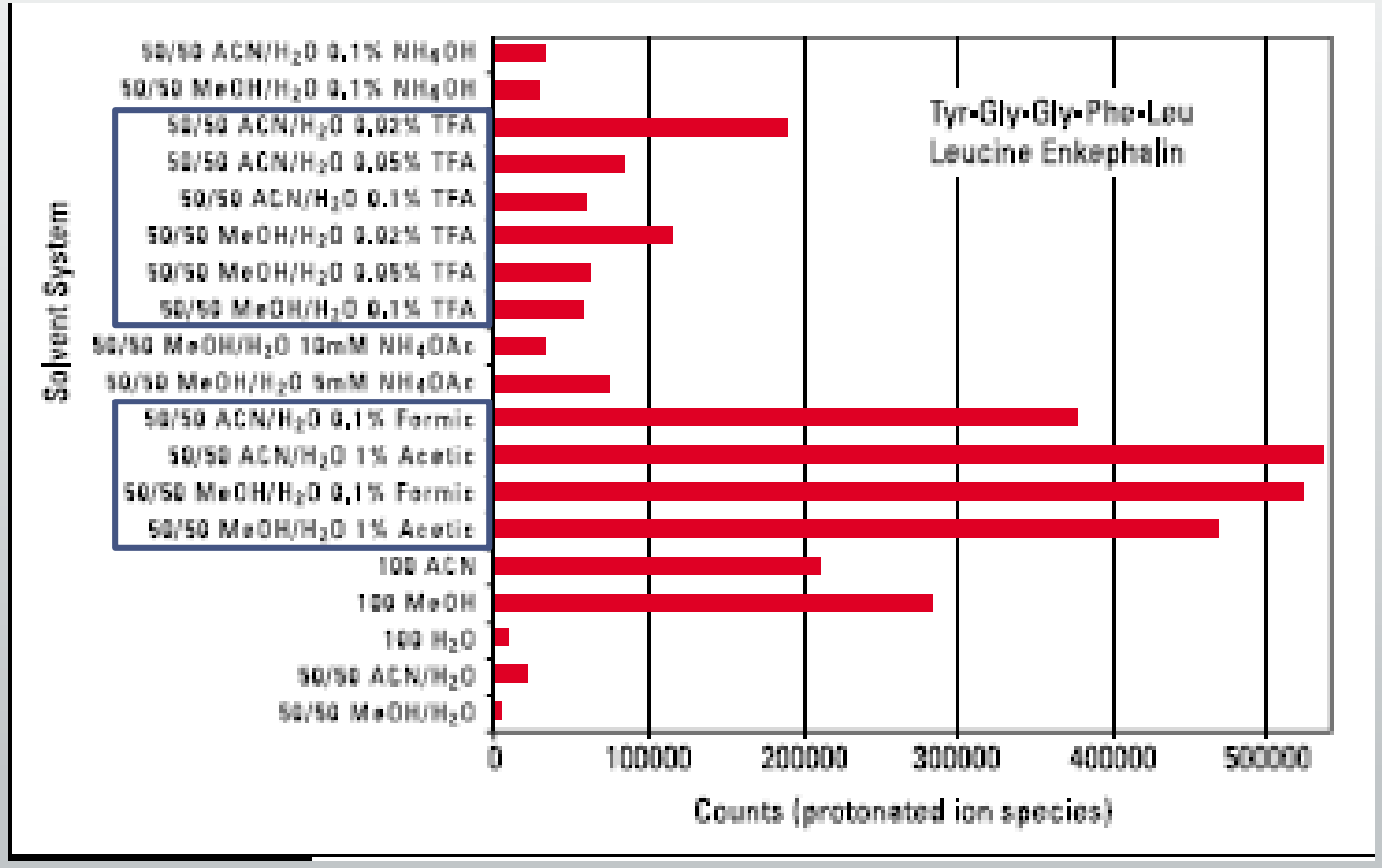
APCI



Courtesy of Agilent Technologies Kundenschulung

Influence of Additive Concentration on Response

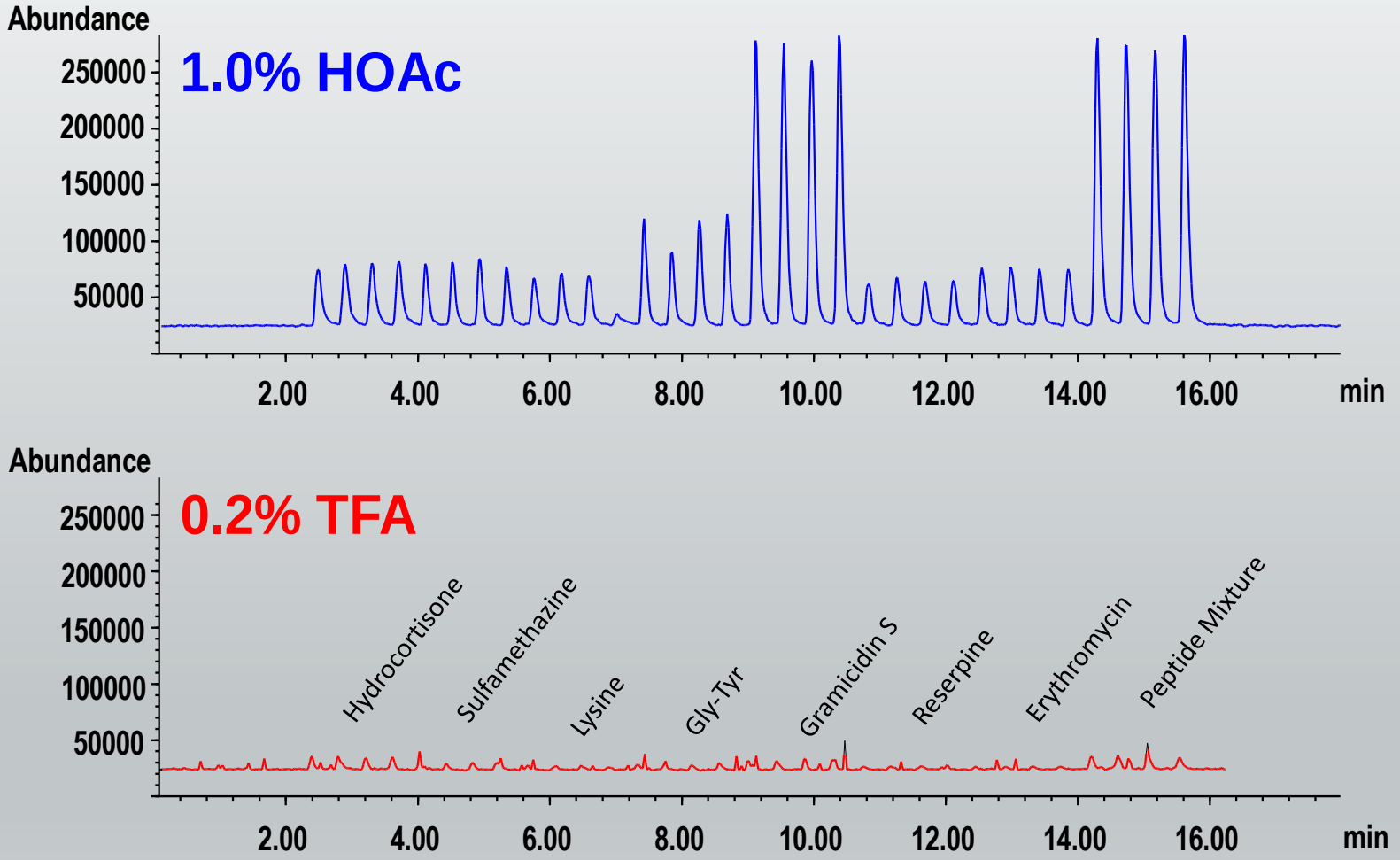
Pneumatically Assisted ESI



Taken from: HPLC Analysis of Biomolecules, Technical Guide
Thermo Electron Corporation

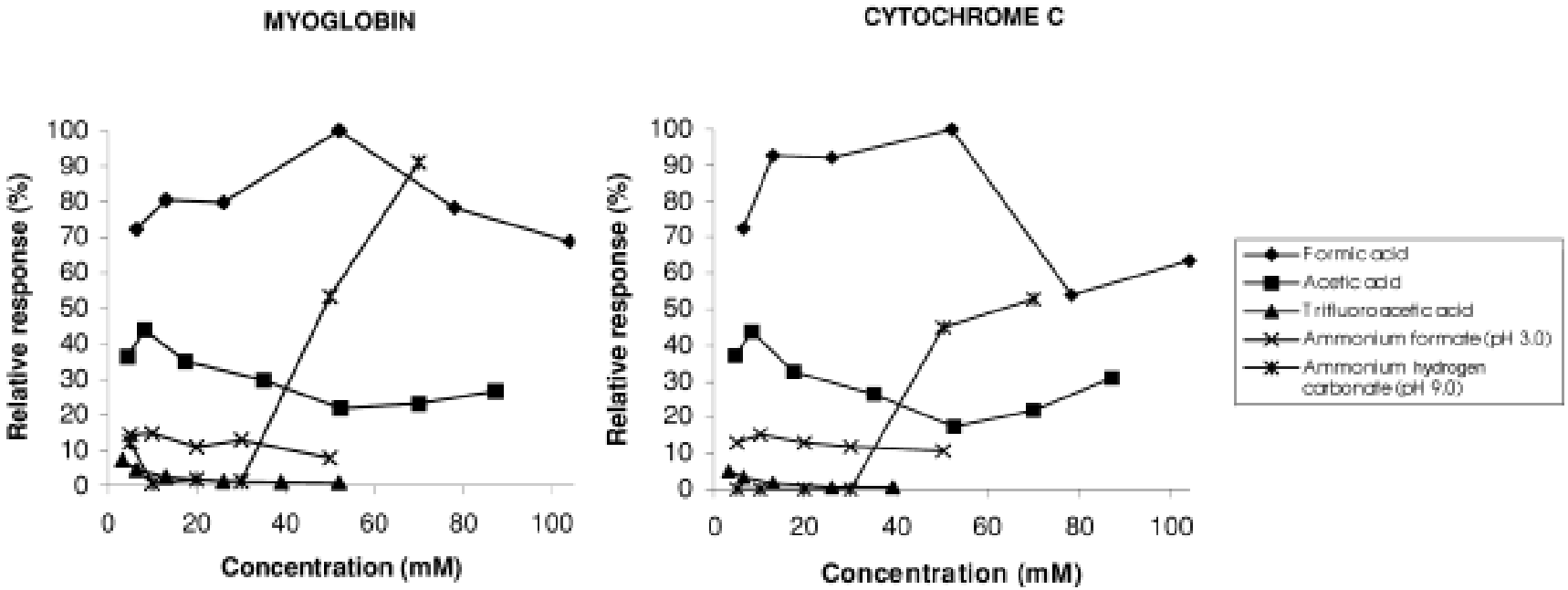
Flow Injection Analysis-ESI/MS with 1% AcOH/0.2% TFA*

Pneumatically Assisted ESI



*A. Apffel et al., J. Chrom., 712 177 (1995)

Infusion of Protein Sample*



Variation of the signal (expressed as response related to the highest signal observed) obtained in ESI-MS for 2 mM solutions of myoglobin and cytochrome c with the concentration of acetic acid, formic acid, TFA, ammonium formate (pH 3) and ammonium hydrogencarbonate (pH 9). FIA at 0.1% ml/min. Mobile phase, water–ACN (50:50).

*M. Garcia et al. J. of Chrom. A, 957 (2002) 187–199

Avoid Sensitivity Loss with TFA Containing Eluents

- Post-column addition of a “TFA-fix” *(e.g. propionic acid)
  - No compromise on chromatography
  - Additional hardware required (cost, reliability, mixing efficiency)

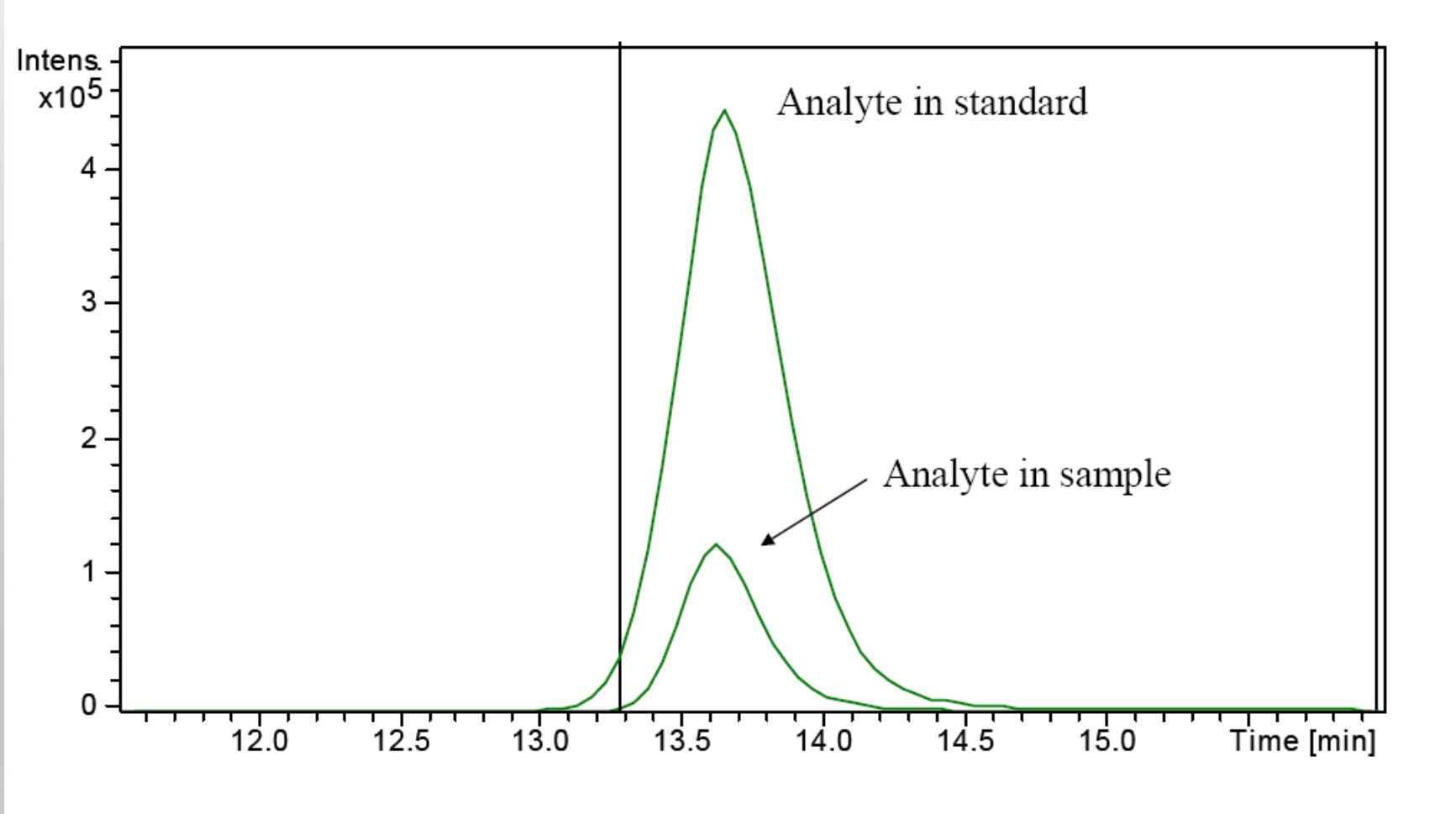
*A. Apffel et al., J. Chrom., 712 177 (1995)

Matrix Effect/Ion Suppression in LC-ESI/MS*

- Ionization efficiency of ESI depends
  - Solvent properties – mostly constant but for gradient elution
  - Source parameters
  - Compounds co-eluting with analyte
- Standard solutions are “clean”
- Lower response for the same analyte concentration in dependence of additives in the mobile phase
- Different response for the same analyte concentration in sample matrix than in standard solution

***Annelie Kruve, Univ. of Tartu, Estonia**

Example of Matrix Effect in LC-ESI/MS*



*Annelie Kruve, Univ. of Tartu, Estonia

Ion Supression/Matrix Effect - Causes

- Competition for available charges
  (Keep in mind that a very low fraction from the analytes actually make it into the MS)
- Interfering substances may cause increase of viscosity and surface tension therewith hampering the formation of droplet
- Formation of solid particles including the analyte
- Like with TFA, ion pair formation may occur, rendering the analyte neutral.

Remedies for the Matrix Effect

- Assess the scope of the effect by the post-column addition method*
- If possible prepare standard in sample matrix (e.g. serum) and run it through the sample prep procedure
- Smaller droplets will reduce the matrix/ion suppression effect➔ nanoelectrospray!
- Use another ionization method e.g APCI or Direct Electron Impact LC-MS interface

***Matuszewski et al., *Anal. Chem.* 2003, 75, 3019-3030)**

Using Non-volatile Buffers in the Mobile Phase

LC Conditions:

Mobile phase: 8% methanol in one of the following:

A: water

B: 0.2% acetic acid in water

C: 50 mM ammonium phosphate, pH 7

D: 50 mM sodium phosphate, pH 7

Flow rate: ESI - 0.3 ml/min; APCI - 0.7 ml/min

Injection: 1 µl of a mixture containing 10 ng/µl each of lincomycin, caffeine and sulfachloropyradizine

Column: Zorbax Eclipse XDB C8 2.1 mm x 50 mm @ 30 °C

MS Conditions:

SIM ions:

Positive ion mode: 195, 285 and 407 amu

Negative ion mode: 193, 283 and 405 amu

Fragmentor: Ramped 70 V for 193/195; 50 V for 283/285; 80 V for 405/407

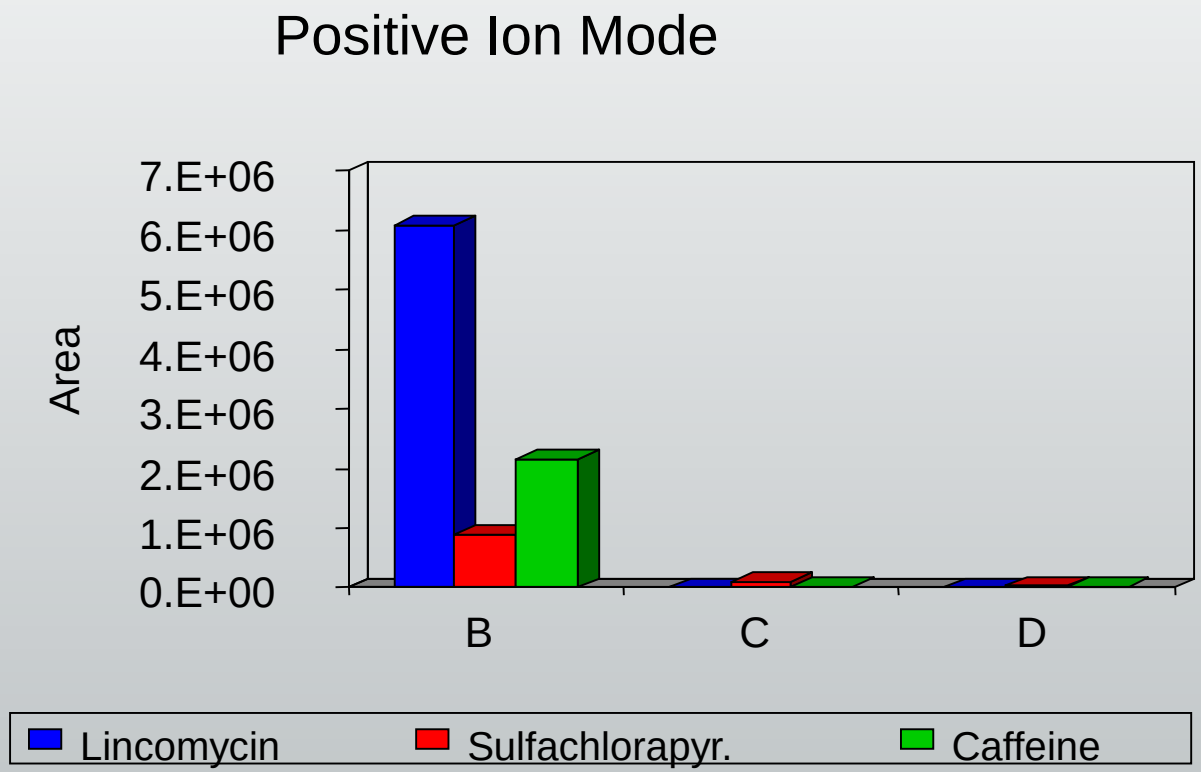
Vcap: ESI - 4000 V; APCI - 3000 V

Drying gas: ESI - 350°C, 10 l/min; APCI - 350 °C, 5 l/min

Nebulizer: ESI - 25 psig; APCI - 60 psig

Using Non-volatile Buffers in the Mobile Phase

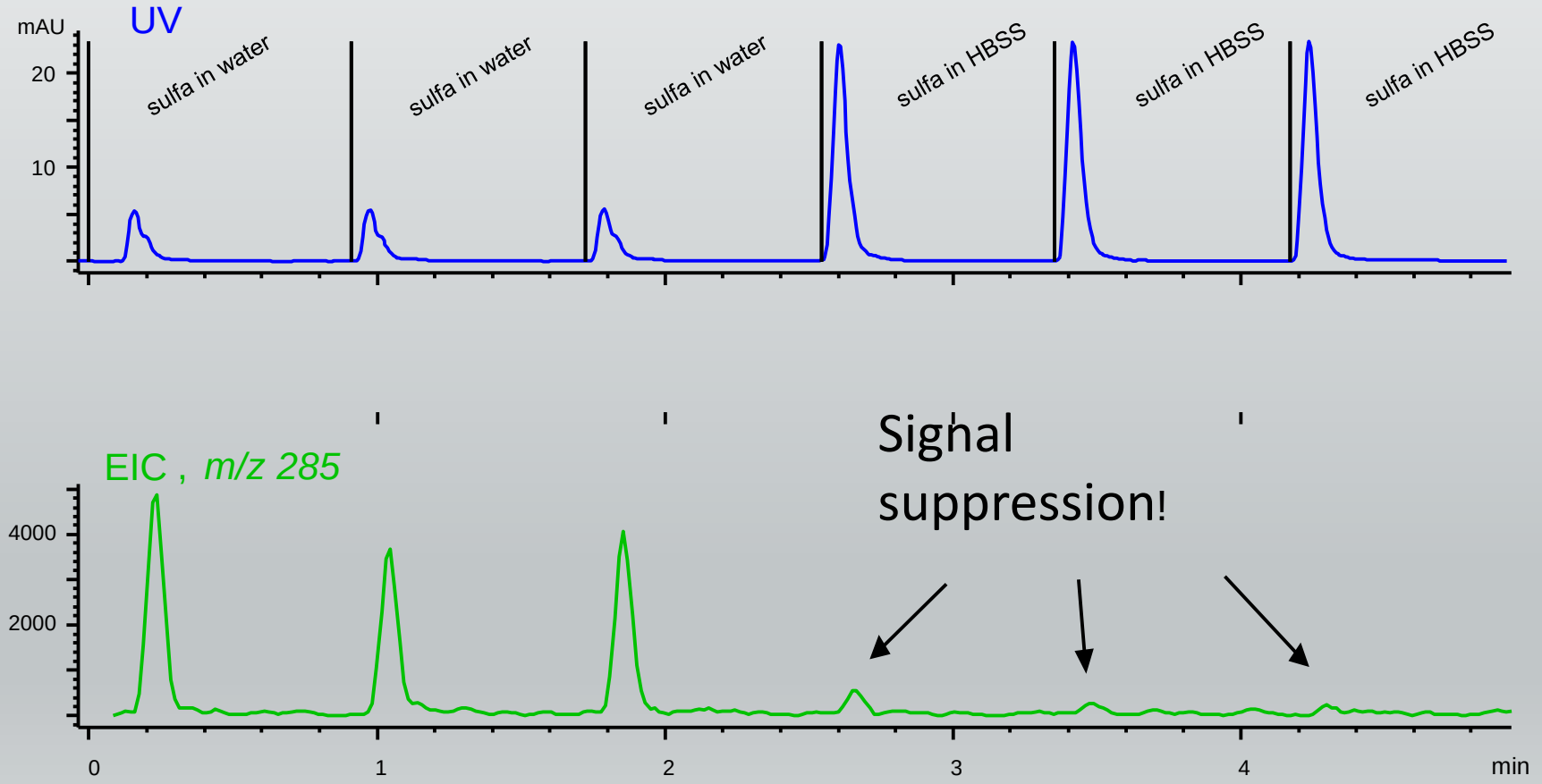
Influence on Response



Mobile Phase Conditions:
(B) 0.2% acetic acid;
(C) 50 ammonium phosphate;
(D) 50 mM sodium phosphate

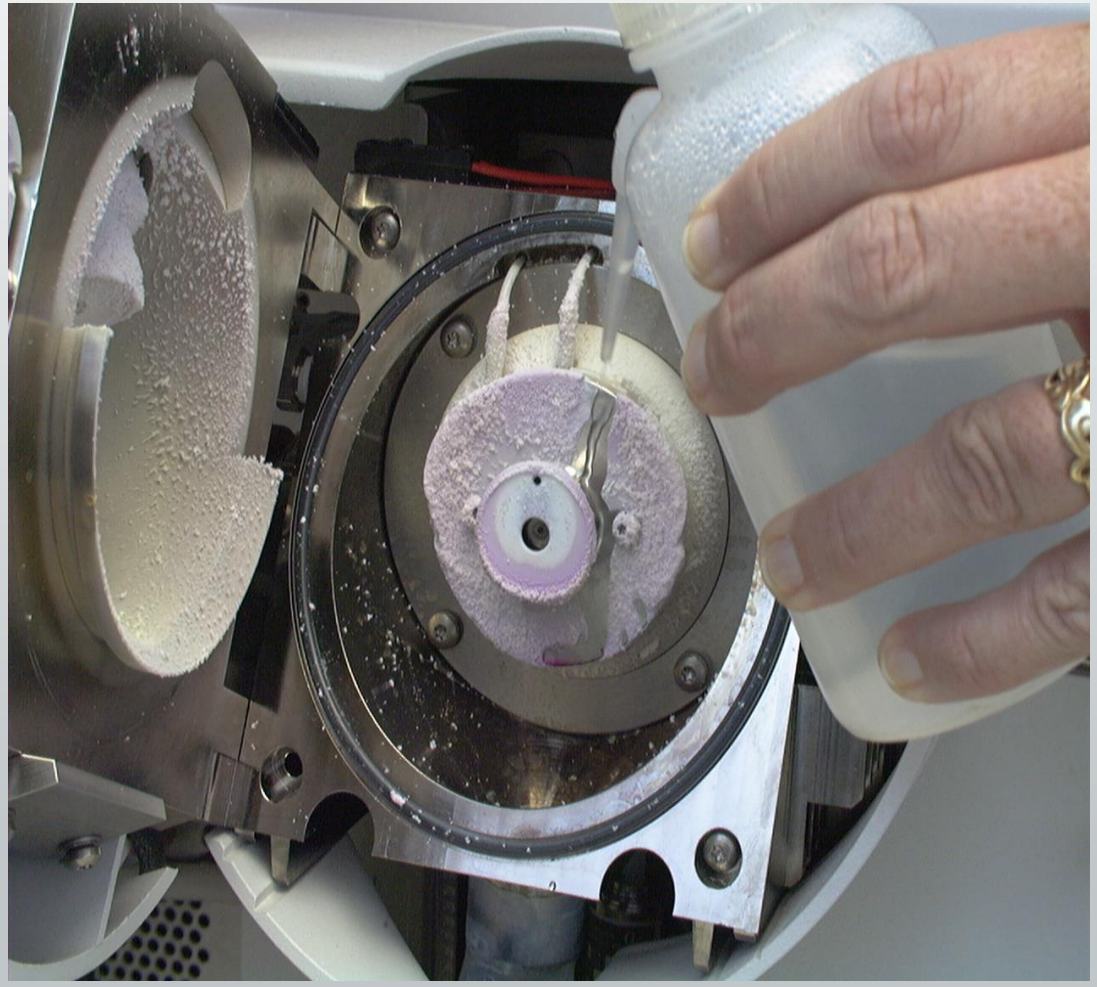
Using Non-volatile Buffers in the Mobile Phase

FIA of 5 ppm sulfachloropyridazin in water and in Hanks Balanced Salt Solution*



*http://en.wikipedia.org/wiki/Balanced_salt_solution

APCI-Spray Chamber after using a 25 mM Phosphate Buffer



No comment needed

Common Contaminant & Background Ions

m/z	Ion	Compound
101	[M+Na]+	DMSO
102	[M+H]+	Triethylamine
104/106	[M+Cu]+	Acetonitrile
105	[2M+Na]+	Acetonitrile
120	[M+Na+CH3CN]+	DMSO
122	[M+H]+	Tris
123	[M+H]+	Dimethylaminopyridine
130	[M+H]+	Diisopropylethylamine
144	[M+H]+	Tripropylamine
145/147	[2M+Cu]+	Acetonitrile
146	[3M+Na]+	Acetonitrile
150	[M+H]+	Phenyldiethylamine
153	[M+H]+	1,8-diazabicyclo[5.4.0]undec-7-ene
157	[2M+H]+	DMSO
159	[M+Na]+	Sodium trifluoroacetate
179	[2M+Na]+	DMSO
186	[M+H]+	Tributylamine
225	[M+H]+	Dicyclohexylurea
239/241	[(M.HCl)2-Cl]+	Triethylamine
242	M+	Tetrabutylammonium
243	M+	Trityl
257	[3M+H]+	DMSO
267	[M+H]+	Tributylphosphate
273	M+	Monomethoxytrityl
279	[M+H]+	Dibutylphthalate
301	[M+Na]+	Dibutylphthalate
317	[M+K]+	Dibutylphthalate
336	[M+H]+	Tributyl
371	[M+H]+	Polysiloxane,
391	[M+H]+	Diisooctyl phthalate
413	[M+Na]+	Diisooctyl phthalate
429	[M+K]+	Diisooctyl phthalate
445	[M+H]+	Polysiloxane
462	[M+NH4]+	Polysiloxane
449	[2M+H]+	Dicyclohexyl urea
798	[2M+NH4]+	Diisooctyl phthalate
803	[2M+Na]+	Diisooctyl phthalate
74 m/z units apart		polydimethylcyclosiloxane

National Mass Spectrometry Facility UK
www.nmssc.ac.uk/documents/ESI_contam_and_bg_ions.pdf

New Objective Inc.
http://www.newobjective.com/downloads/technotes/PV-3.pdf

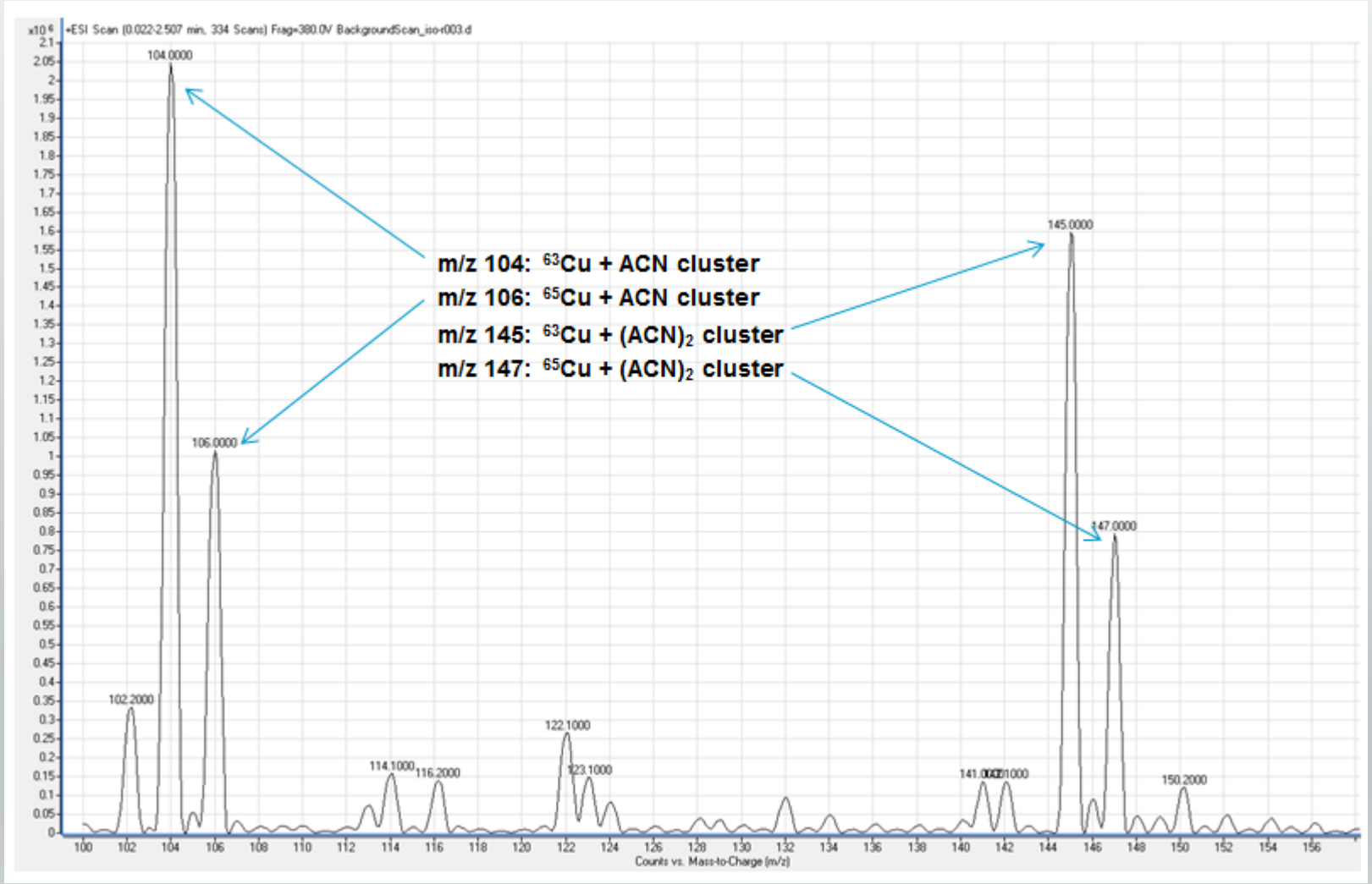
Waters
https://www.waters.com/webassets/cms/.../docs/bkgrnd_ion_mstr_list.pdf

Alberta University
www.chem.ualberta.ca/~massspec/es_**ions**.pdf

Leiden University
http://www.lc-ms.nl/

Background Ions in LC-MS

Copper/Acetonitrile Adducts



Courtesy Daniel Thielsch, Agilent Technologies

Avoid/Eliminate Contamination

- Utmost cleanliness of lab articles, solvents etc.
  - Unlike UV-VIS, remember a MS “sees” everything!
- Run solvent only – no HPLC column
  - Step gradient – monitor and identify back ground ions
  - Locate source of contamination
  - Replace parts, modules or clean system (see next page)
- Run with HPLC column
  - Step gradient – monitor and identify background ions
  - Inject a blank sample
- Use a sample divert valve to avoid sample salts and early eluting sample components enter the MS

Clean-up your HPLC System

- Flush with water (no column, bypass UV-detection cell, outlet to waste) e.g. at 3 mL/min for 15-20 minutes to remove salts
- Flush with i-propanol as above or at low flow rate overnight. Do blank sample injections with i-propanol to clean injection path
- Flush with organics cleaning solution as above
  (e.g. from Agilent (50:25:15:10 acetonitrile/isopropanol/cyclohexane/dichloromethane)
  Do blank sample injections with cleaning solution
- Change back to isopropanol and flush. Do blank injections with i-propanol to clean injection path
- Flush with 100% methanol HPLC grade
- Install column and flush with 100% methanol at elevated temperature
- Switch to mobile phase. In case of gradient analysis do a reverse gradient.
- After pumping down MS connect LC
- As an alternative, one may use a solution of a few % formic acid in acetonitrile
- Formal passivation with strong acid or alike only after checking manufacturers literature

End of Part 1