(U)HPLC for Hyphenation

Prepared by
Gerard Rozing, Karlsruhe, Germany

Delivered by
Jan Preisler, Masaryk University, Brno, Czech Republic

About the Author

- Undergraduate and graduate studies at University of Amsterdam, 1964-1976. Majors in Organic Chemistry and Chemical Engineering
- Post-doctoral research at State University of Ghent, Belgium, 1977 and post-doctoral training Analytical Chemistry, University of Amsterdam, 1978-1979
- R&D Chemist, group & project Leader, R&D section manager, HPLC column and HPLC system development at Hewlett-Packard, Waldbronn, Germany, 1979-'99
- Since 2000, Agilent Technologies University Relations and External Scientific Collaborations Manager
- Since 2006 Agilent Research Fellow
- Retired September 1, 2012. Since then, working on freelance basis. Visit my website at http://www.rozing.com e-mail: gerard@rozing.com

Acknowledgements

- Monika Dittmann, Stephan Buckenmaier, Udo Huber, Christian Scholz, Konstantin Choikhet all at Agilent Technologies, Waldbronn, Germany
- Paul Goodley, Chris Miller, Agilent Technologies, Santa Clara, USA
- Oliver Schmitz, University of Duisburg-Essen, Germany
- Achille Cappiello, Univ. Urbino, Italy
- Remco van Soest, Eksigent part of AB Sciex, Dublin, USA

Hyphenation in Separation Science

- Coupling of a separation method with a spectroscopic detection method resulting in **3 (or more) dimensions of information**

Separation System (w/w.o. detector)	Detection Technique
HPLC GC CE	UV-VIS NMR IR Mass Spectrometry

- Detection Technique is coupled "on-line" with the separation system
- Detection Technique is coupled with the Separation System through an interface in case of incompatibility between the phases or systems

Hyphenation in Separation Science

- Is not Multidimensional Separation Techniques
- In this case the "x" is used
  examples **LCxLC** coupling, **LCxGC** coupling etc.

Focus on LC-MS

- Important aspects of LC-MS
  - Detector response type
  - Ionization mechanisms and interfaces
- HPLC separation factors influencing ESI and APCI process and mass detection
- HPLC Column Technology, Special Techniques and New Developments

Concentration Sensitive Detector in HPLC

- Response proportional to concentration (e.g. UV detection)
  $Abs_{i,\lambda} = \varepsilon_{i,\lambda} \cdot c_i \cdot L_{cell}$ *Lambert Beer's law*
- Response independent of flow rate (infusion experiment!)
- Chromatographic peak height does not change with flow rate (e.g in FIA or neglecting any dispersion)
- Chromatographic peak area is given by:

  $A_i = \int c_i(v)dv = F\int c_i(t)dt$

  in case flow rate is constant ($V_{R,i} = F.t_{R,i}$) and inversely proportional with flow rate (peak width decreases in time domain)
- In almost all cases, non-destructive

Mass Flow Sensitive Detector in HPLC

- Response is proportional to mass/time (or cps) $R = a.\frac{\partial m_i}{\partial t}$
- Response increases with flow rate (infusion experiment!)
- Chromatographic peak height increases with flow rate (e.g. in FIA neglecting any dispersion) and the peak width decreases
- Chromatographic peak area is given by:

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

  and is independent of flow rate (peak width decreases in volume domain)
- In most cases a destructive detection method (FID, MS, ELSD, ICP/MS

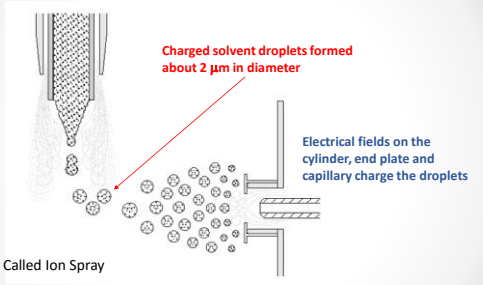
Important Aspects of LC-MS

- Detector response
- Ionization mechanisms and interfaces

Ionization Techniques in LC-MS

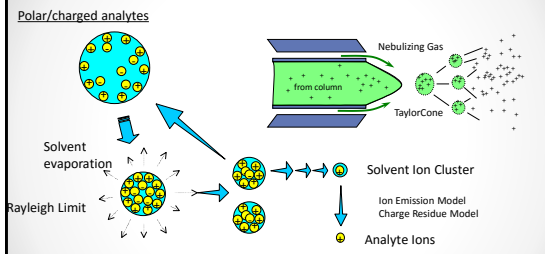
- Soft, Atmospheric Pressure (in principle no fragmentation)
  - Electrospray Ionization- ESI
  - Chemical Ionization - APCI
  - Photo Ionization – APPI
  - Laser Ionization - APLI
  - Surface Ionization (MALDI, DART)
- Soft, Vacuum Ionization
  - Matrix assisted laser desorption – MALDI
- Hard, Vacuum Ionization (with fragmentation)
  - Particle Beam
  - Direct Electron Impact
  - Supersonic Molecular Beam

Pneumatically Assisted Electrospray Ionization



Courtesy of Agilent Technologies Kundenschulung

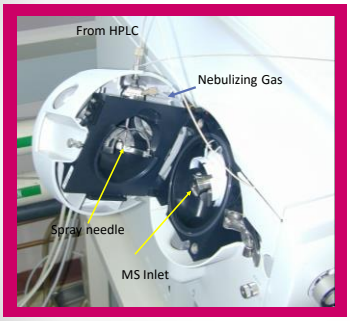
Pneumatically Assisted Electrospray Ionization



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

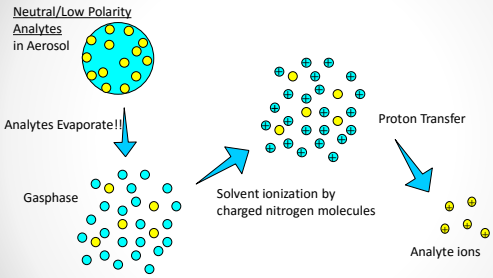
Courtesy of Agilent Technologies Kundenschulung

Pneumatically Assisted Electrospray Ionization



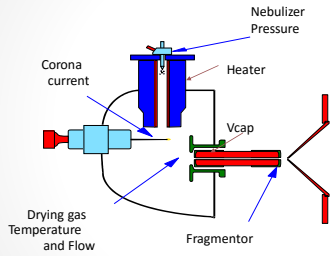
Courtesy of Agilent Technologies Kundenschulung

Atmospheric Pressure Chemical Ionization



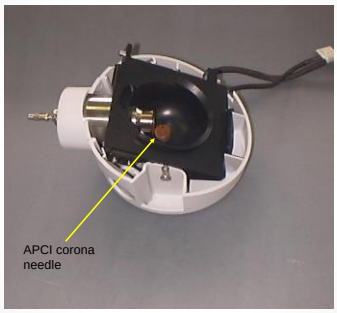
Courtesy of Agilent Technologies Kundenschulung

APCI Detailed Mechanism- Gas Phase Ionization



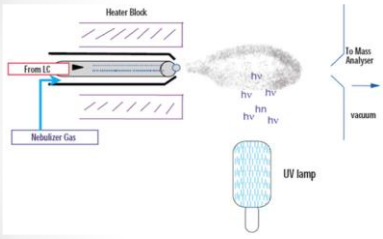
Courtesy of Agilent Technologies Kundenschulung

APCI-Interface



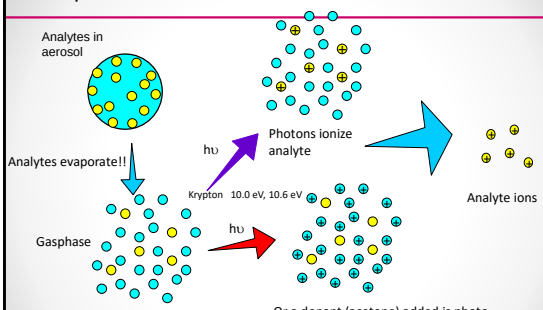
Courtesy of Agilent Technologies Kundenschulung

Atmospheric Pressure Photo Ionization



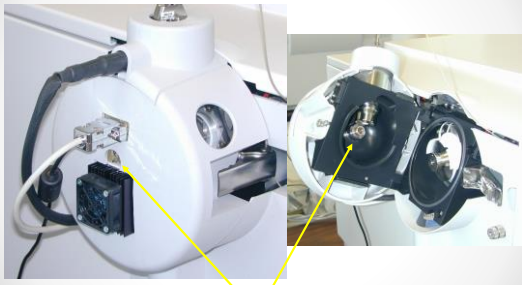
Courtesy of Oliver Schmitz, Univ. Duisburg/Essen, Germany

Atmospheric Pressure Photo Ionization



Courtesy of Agilent Technologies Kundenschulung

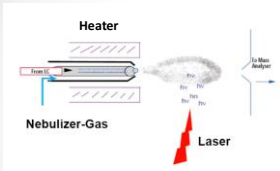
APPI-Interface



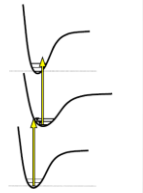
Lamp Source instead of Discharge Needle

Courtesy of Agilent Technologies Kundenschulung

Atmospheric Pressure Laser Ionization



dual photon absorption



Alternative for High MW substances:
Combine ESI with APLI!!

$M + m\,h\nu \rightarrow M^*$

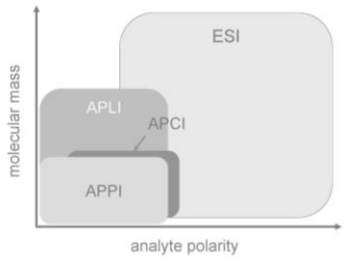
$M^* + n\,h\nu \rightarrow M^{+\cdot}$

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

Atmospheric Pressure Laser Ionization

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

Summary



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

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)

HPLC Separation Factors Influencing ESI and APCI Process and Mass Detection

- Interface Parameters (voltage(s), gases used)
- Eluent Solvent Properties
  - Flow rate
  - Composition, volatility, viscosity, conductivity
  - Mobile phase additives, pH
  - 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		

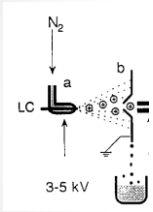
R. King et al., J. Am. Soc. Mass Spectrom., 2000, 11, 942–950

Eluent Solvent Properties

• • •

Electrospray Ionization – Influence of Flow Rate

- Pneumatically assisted electrospray



Initially electrospray was not pneumatically assisted – no direct countercurrent drying gas, no nebulizer gas

Only working with very low LC flow rates.

Bruins and Henion introduce pneumatically assisted electrospray (ion spray) *

*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)

Influence of Flow Rate on Response in LC-MS*

Pneumatically Assisted ESI (Ion Spray)

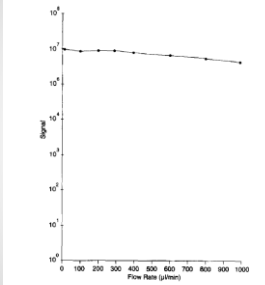


Fig. 1. Ion signal from the direct infusion of a 10 pmol/µl solution of methionine enkephalin as a function of sample flow-rate.

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

This ESI 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)

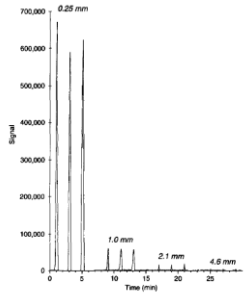


Fig. 2. TICs from methionine enkephalin injections (50 pmol each) on columns with different diameters.

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

APCI

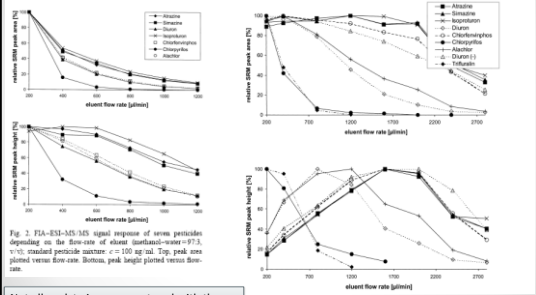


Fig. 2. FIA–ESI–MS/MS signal response of seven pesticides depending on the flow-rate of eluent (methanol–water = 97.3, v/v); standard pesticide mixture: $c = 100$ ng/ml. Top, peak area plotted versus flow-rate. Bottom, peak height plotted versus flow-rate.

Not all analyte ions are captured with the same efficiency

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

Nano-electrospray Ionization

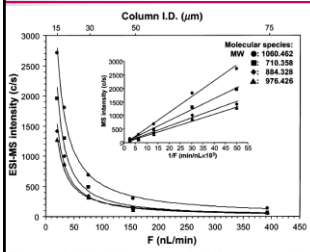
Developed by Matthias Mann & Matthias Wilm*

- Smaller droplets → generation of more ions
- No orthogonal design – sprayer is 1 – 2 mm from MS entrance
- Higher sampling rate of ions into MS

Result: dramatically higher sensitivity than standard ESI

*M. Wilm & M. Mann, International Journal of Mass Spectrometry and Ion Processes, 136, 167 (1994)

Influence of Flow Rate on Response in NanoESI



Emitter tip orifice diameter proportionally reduced

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

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

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

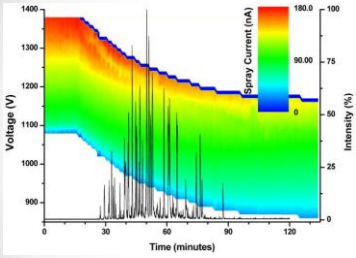
column (μm i.d. × cm)	d_p (μm)	p_i (Å)	u (cm/s)	F (nL/min)	γ	ϵ
porous						
74.5 × 87.0	3.6	300	0.19	393	20.7	0.78
47.1 × 87.0	3.6	300	0.19	155	13.1	0.79
29.7 × 87.0	3.6	300	0.22	76	8.3	0.81
19.8 × 87.0	3.6	300	0.22	33	5.5	0.81
14.9 × 87.0	3.6	300	0.23	20	4.1	0.83

Preliminary Conclusions – Flow Dependence ESI

- The pneumatically assisted ESI interface (IF) tolerates maximally 1 mL/min
- Pneumatically assisted ESI IF behaves largely like a concentration 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 (<20 nL/min)
- At low flow rate nor mass flow sensitive or concentration sensitive detector because more ions reach the MS inlet
- Ion suppression is much reduced (vide infra)

Influence of Gradient Elution on Response in LC-MS*

Nano-electrospray Ionization

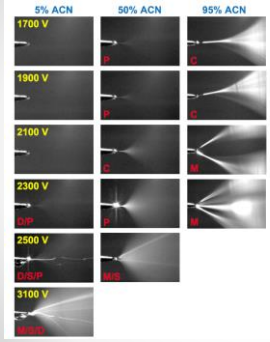


Column 75 μm i.d.
Solvent 0.2% AcOH 0.1% TFA in water vs. 0.1% TFA in ACN, 0-100%

R.D. Smith et al, J. Amer. Soc. Mass Spectrometry, 20, 682 (2009)

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

HPLC Separation Factors Influencing ESI and APCI Process and Mass Detection

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

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

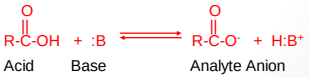
Solution Chemistry is Important

ESI mandates the formation of analyte ions in the eluent solution

Positive Mode

Base Acid Analyte Cation

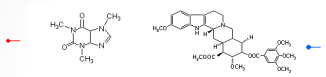
Negative Mode

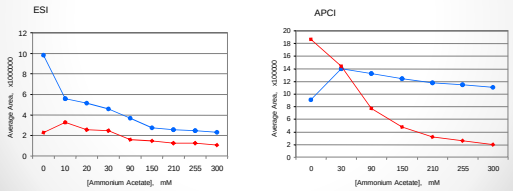


Acid Base Analyte Anion

Influence of Additive Concentration on Response

Pneumatically Assisted ESI

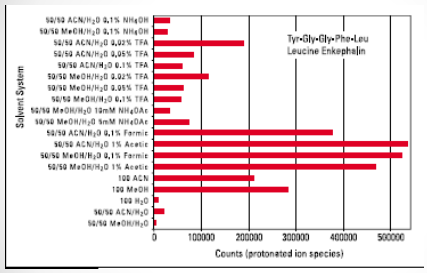




Courtesy of Agilent Technologies Kundenschulung

Influence of Additive Concentration on Response

Pneumatically Assisted ESI



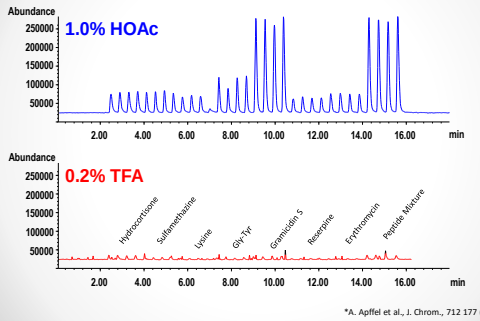
Taken from: HPLC Analysis of Biomolecules, Technical Guide
Thermo Electron Corporation

TFA Containing Solvents for Tryptic Peptides LC-MS

- Ideally suited for RP LC since trypsine cleaves at lysine or arginine leaving a basic peptide. With TFA is ion-pair separation on RP column possible.
- TFA neutralizes "hot" sites on the silica surface
- TFA forms a strong ion pair with basic peptides
- But spray instability due to high conductivity and high surface tension of the solution has been reported
- Strong signal reduction observed

FIA with 1% AcOH/0.25% TFA*

Pneumatically Assisted ESI



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

Remedy in Practice

- Post-column addition of the "TFA-fix"
  - No compromise on chromatography
  - Additional hardware required (cost, reliability, mixing efficiency)
- New stationary phases that have low silanophilic interactions allowing good peptide separations without compromising chromatograph by using formic acid etc.
  - Dionex Acclaim Pepmap
  - Waters CSH130 C^{18}
  - Thermo BioBasic columns
  - Agilent AdvanceBio Peptide Mapping columns

Pneumatically Assisted API

ESI

- In principle concentration sensitive
- In many cases more sensitive
- Wide flow rate range → nanobore – normal bore columns
- Solvent composition (gradient) OK
- Mobile phase additives compromise response
- Tolerates low concentration of inorganic buffers
- High matrix effect

APCI

- In principle mass flow sensitive
- More selective, non-polar substances
- No advantage at low flow rates
- Organic solvent may have a large influence on response
- Chlorinated solvents will assist ionization
- Tolerate up to 100 mmol inorganic buffers
- Low matrix effect

HPLC Separation Factors Influencing ESI and APCI Process and Mass Detection

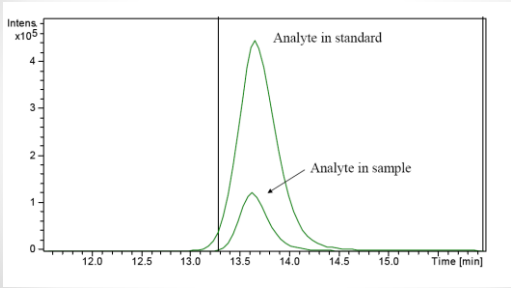
- Interface Parameters (voltage(s), gases used)
- Eluent Solvent Properties
  - Flow rate
  - Composition, volatility, viscosity, conductivity
  - Mobile phase additives, pH
  - Ion Suppression/Matrix effects in ESI/MS
- Practice of LC-MS
  - Use of inorganic buffers
  - Common background ions & contaminants

What is 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
- Standards are clean solutions
- Different response for the same analyte concentration in sample solution than in standard solution
- Matrix effect depends on analyte concentration

*Annelie Kruve, Univ. of Tartu, Estonia

Example of Matrix Effect in LC-ESI/MS*



*Annelie Kruve, Univ. of Tartu, Estonia

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
- As with TFA ion pair formation renders 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
- Address in the whole procedures the probable mechanism of the matrix effect
- Smaller droplets → nanoelectrospray!
- Use another ionization method e.g APCI or Direct Election Impact LC-MS interface (vide infra)

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

HPLC Separation Factors Influencing ESI and APCI Process and Mass Detection

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

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

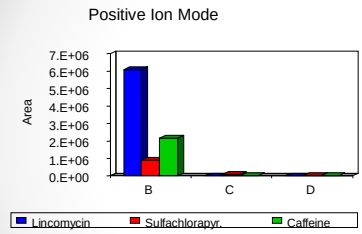
MS Conditions:

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

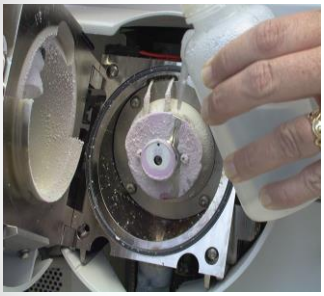
Using Non-volatile Buffers in the Mobile Phase

Influence on Response



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

API-Spray Chamber after using a 25 mM Phosphate Buffer



No comment needed

HPLC Separation Factors Influencing ESI and APCI Process and Mass Detection

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

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
130	[M+Na+CH3CN]+	DMSO
122	[M+H]+	Tris
123	[M+H]+	Dimethylaminopyridine
130	[M+H]+	Diisopropylethylamine
146	[M+H]+	Tripropylamine
145/147	[2M+Cu]+	Acetonitrile
146	[2M+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]+	Dioctylethexylurea
239/241	[M-HCl]2-Cl]+	Triethylamine
242	M+	Tetrabutylammonium
243	M+	Trityl
257	[2M+H]+	DMSO
267	[M+H]+	Tributylphosphate
271	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
448	[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

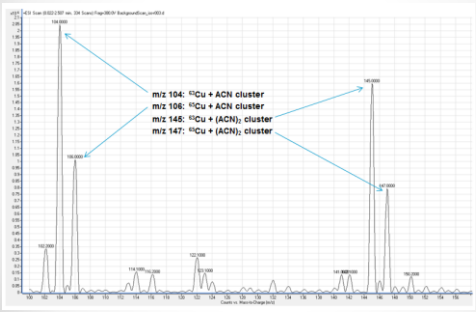
Other sources
New Objective Inc.
http://www.newobjective.com/downloads/technotes/PV-3.pdf

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

Alberta University
www.chem.ualberta.ca/~massspec/es_ions.pdf

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 background 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 only after checking manufacturer literature

Focus on LC-MS

- Important aspects of LC-MS
- Factors Influencing ESI Process and Mass Detection
- HPLC Column Technology, Special Techniques and New Developments
  - Is separation prior to MS needed?
  - HPLC instrumental factors
  - What column diameter to use
  - HPLC Chip column technologies for LC-MS
  - Direct EI LC-MS

HPLC Column Technology, Special Techniques and New Developments

- Is separation prior to MS needed?
- HPLC instrumental factors
- What column diameter to use
- HPLC Chip column technologies for LC-MS
- Direct EI LC-MS

"Chromatographic separation is not required when using MS. Extract individual m/z values, do SIM or choose precursor ions for MS/MS."

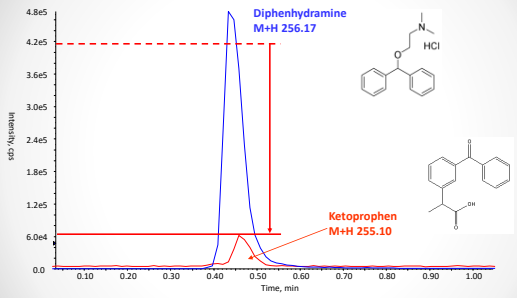
• • •

Is separation prior to MS needed?

Why is Separation Needed?

- MS will not/barely differentiate isomeric substances
  (same MW but different structure of stereoisomers)
- MS will not/barely differentiate isobaric substances
  (same molecular formula but different molecules)
- May mitigate matrix effect

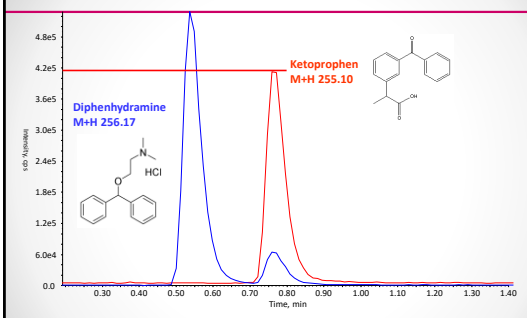
No Separation before MS



5:95 Water/Methanol, 0.2 % Acetic Acid

Courtesy Udo Huber, Agilent Technologies Waldbronn

Separation before MS



30:70 Water/Methanol, 0.2 % Acetic Acid

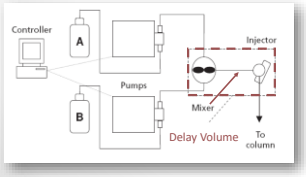
Courtesy Udo Huber, Agilent Technologies Waldbronn

HPLC Column Technology, Special Techniques and New Developments

- Is separation prior to MS needed?
- HPLC instrumental factors
- What column diameter to use
- HPLC Chip column technologies for LC-MS
- Direct EI LC-MS

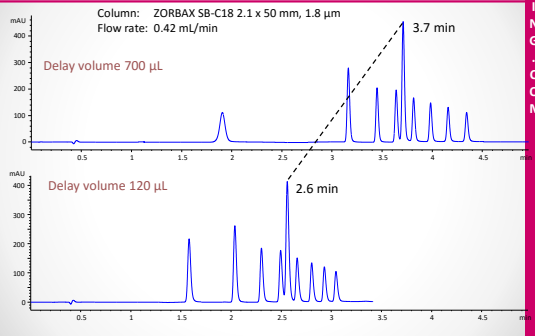
HPLC Instrumental Factors – System Dwell Volume*

- Volume from the point of mobile phase mixing to the column head
- Delays the arrival of eluent composition change (gradient)



*J.W. Dolan LCGC 2006, Vol 24, 458-466

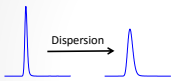
Influence of Dwell Volume



Courtesy Udo Huber, Agilent Technologies Waldbronn

HPLC Instrumental Factors : Extra Column Dispersion

- *"Dispersion is the sample bandspreading or dilution which occurs in connecting tubing, sample valves, flow cells and in column end-fittings."*



Peakheight: Reduced sensitivity
Peakwidth: Resolution loss

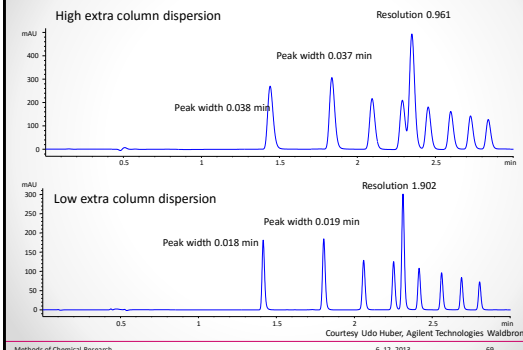
- Connection capillaries (I.D. Length)

$$\sigma_v^2 = \frac{\pi \cdot d^4 \cdot F \cdot L}{96 \cdot D_m}$$

Aris-Taylor Gleichung

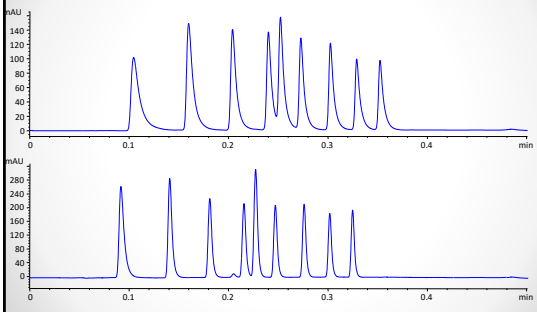
Courtesy Udo Huber, Agilent Technologies Waldbronn

HPLC Instrumental Factors : Extra Column Dispersion



Courtesy Udo Huber, Agilent Technologies Waldbronn

Influence of Poor Connections



Courtesy Udo Huber, Agilent Technologies Waldbronn

Recommendations for Sample Preparation

Positive ion ESI

- Dissolve samples in acid
- Basic sites (N and O) bind to proton to give the molecule a positive charge
- Other cat ions (Na+, or K+) may also be used to form positive ions
- Anions (Cl-) may be removed from a molecule to form a positive ions

- ESI works best when the samples are free of salt
- Samples that contain salt can be desalted in many ways (divert valve)

Negative ion ESI

- Dissolve samples in base
- Acidic sites (acids) give up a proton to form a negative ion.

HPLC Column Technology, Special Techniques and New Developments

- Is separation prior to MS needed?
- HPLC instrumental factors
- What column diameter to use
- HPLC Chip column technologies for LC-MS
- Direct EI LC-MS

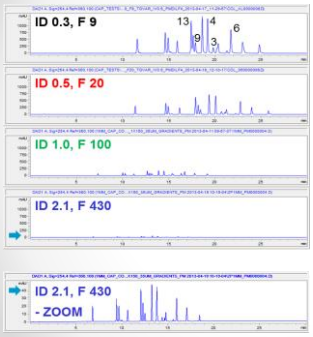
What Column Diameter to Use?

For a chromatographic separation

$$c_{i,\max} = \frac{m_{inj,i}}{\sqrt{2\pi} \cdot \sigma_{v,col}} = \frac{c_{i,0} V_{inj.}}{\sqrt{2\pi} \cdot \sigma_{v,col}} \qquad c_{i,\max} \propto \frac{m_i}{d_c^2}$$

When the column diameter is reduced:
For a concentration sensitive detector, response increases with the square root of the diameter ratio in case the same amount of analyte is injected.

Influence of Column Diameter – UV response

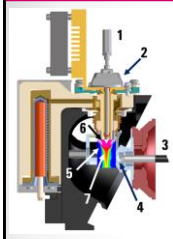


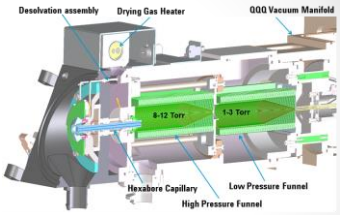
Mobile phase: Water/CAN, 0.1% FA
gradients from 5 – 90 %B in 15'
Inj. vol. 0.5 µL

Peak height increase

Courtesy Stephan Buckenmaier,
Agilent Technologies, Waldbronn

Jetstream IF, Ion Funnel





1. Nebulizer w/ 50 µm needle
2. Nebulizing gas (35 psi)
3. Sampling capillary (Vcap 4 kV)
4. Drying gas (200°C, 13 L/min)
5. Sheath gas (400°C, 12 L/min)
6. Nozzle voltage (1500 V)
7. Thermal gradient focussing region

➔ Higher sampling from ion spray

Courtesy Stephan Buckenmaier,
Agilent Technologies, Waldbronn

Influence of Column Diameter – MS response

with Jetstream IF and Ion Funnel

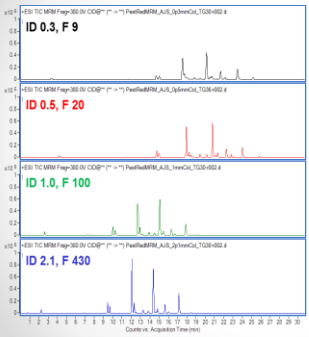


Table-2 Cpd	*H*-ratio (0.3/2.1)	*A*-ratio (0.3/2.1)
#3	0.6	1.0
#4	0.4	0.8
#6	1.5	2.7
#9	0.6	1.0
#13	0.4	0.7

Advances in ionization and sampling efficiency peak abundances are maintained, independent of column-ID, flow rates, and sample concentration.

Courtesy Stephan Buckenmaier,
Agilent Technologies, Waldbronn

Columns for NanoESI/MS

Nano-electrospray MS mandates flow rates 100 – 1000 nL/min.
For (U)HPLC to work properly the solvent has to move with a velocity of 1-10 mm/s

$$d_c = \sqrt{\frac{4.F}{\varepsilon.\pi.u}}$$

Column I.D. must be between 0.05 and 0.15 mm

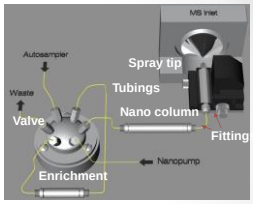
Sensitivity of NanoESI/MS increases dramatically at flow rates <50 nL/min

Proteomics research
➔ Ultra small samples
➔ Ultra high sensitivity mandated

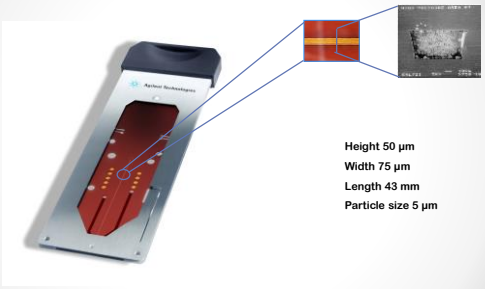
Example – NanoESI Interface Agilent

Typical Set-up of Nanoflow HPLC Electro-Spray Ionization MS System

- Sensitivity
  - 75µm ID analytical column
- Challenging to Set-up & Maintain
  - Multiple Parts
  - Possible leaks, misalignments
- Robustness & Ease-of-use
  - Clogging of spray needle
  - After part replacement system can take hours to stabilize
- Chromatographic Fidelity
  - Rel. large extra column volume leads to band broadening
  - Limited to peptide separation



Agilent HPLC-Chip Technology

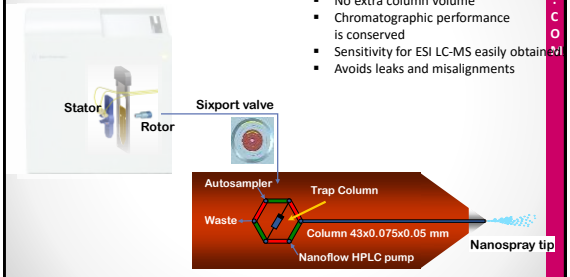


Height 50 µm
Width 75 µm
Length 43 mm
Particle size 5 µm

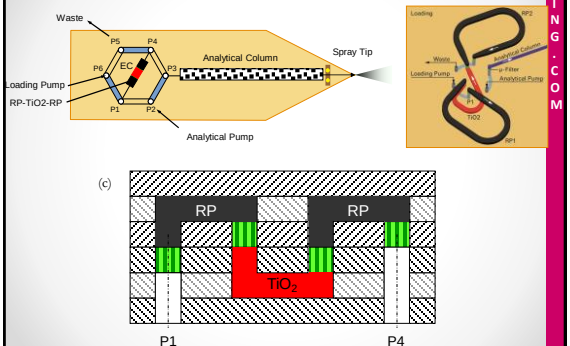
HPLC-Chip MS

Essential Components for Nanoflow HPLC are chip-integrated

- No extra column volume
- Chromatographic performance is conserved
- Sensitivity for ESI LC-MS easily obtained
- Avoids leaks and misalignments



HPLC-Chip - Phosphopeptide Enrichment Chip Design



Thanks for your attention

. . .

PDF Copy can be found at http://www.rozing.com
(registration required)