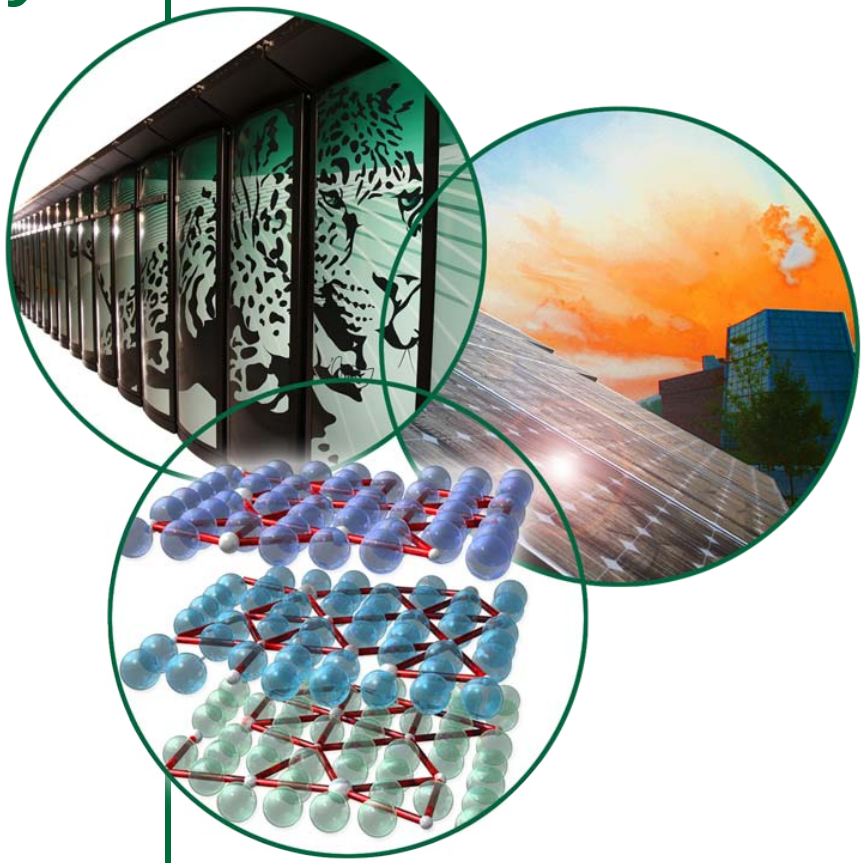
Coupling HPTLC and Mass Spectrometry: How and Why



Vilmos Kertesz
Matthew J. Walworth
Gary J. Van Berkel
Organic and Biological Mass Spectrometry Group

Presented at
HPTLC 2011

Basel, Switzerland
July 6-8, 2011

U.S. DEPARTMENT OF ENERGY

OAK RIDGE NATIONAL LABORATORY
MANAGED BY UT-BATTELLE FOR THE DEPARTMENT OF ENERGY

Why to couple (HP)TLC and MS?

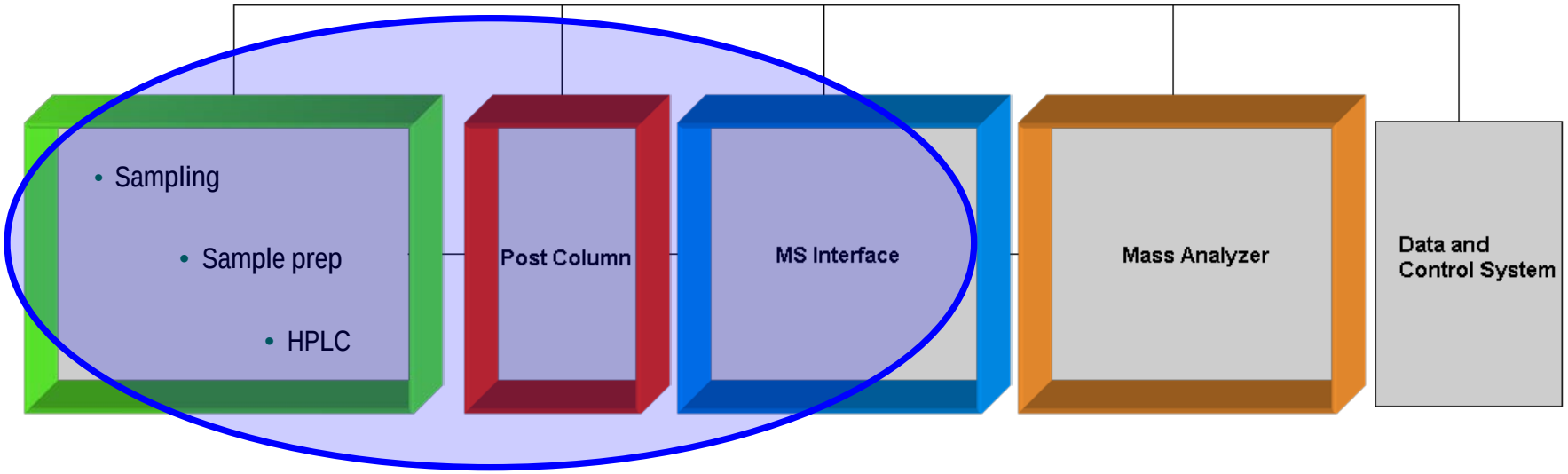
From the Detection Point-of-View: Advantages of using Mass Spectrometry in the TLC Community

- Sensitive
  - Femtomols routinely, but zeptomols (10^{-21}) possible
- Selective
  - 0.1 mDa differences, elemental composition
- Wide variety of structure elucidation methods
  - Exact mass
  - Fragmentation (MS/MS)
- Wide variety of ionization methods
  - Practically all chemicals are detectable
- In general: another dimension of separation based on mass
  - (IMS, DMS: + another dimension of separation in a couple of milliseconds!)

Mass Spectrometry – Why it is Not Used Widely in the TLC Community?

- Lack of expertise
- ~~Horrible start up costs~~
- 15-30k USD: Used triple-quad or iontrap MS with warranty

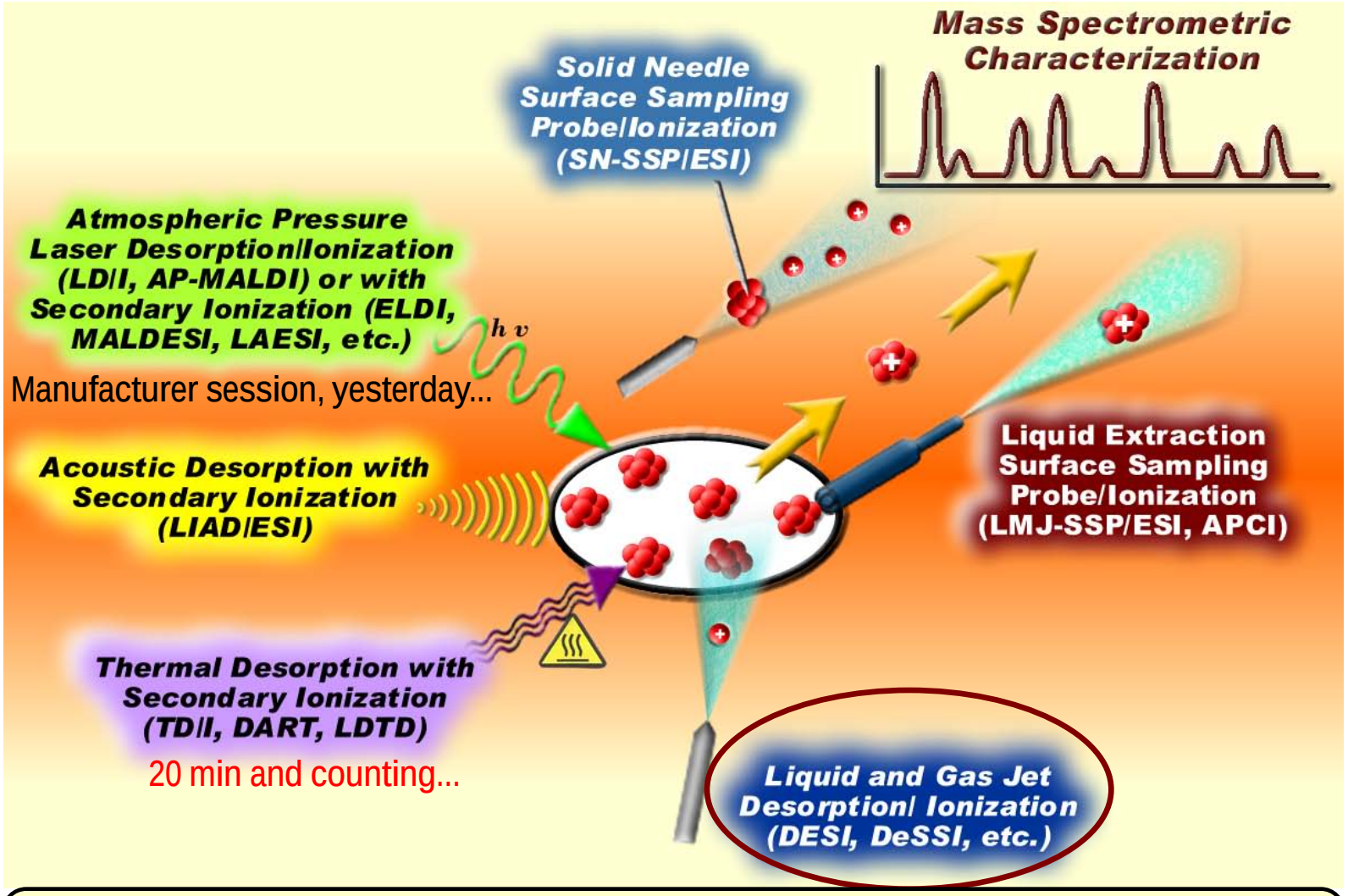
From the Scientific Point-of-View: Advantages using TLC over HPLC in the MS Community



- Simplicity, all compounds are stored on the plate, parallel development, etc.
- If simple comparison needed: quick evaluation of results
  - (Best suited when looking for DIFFERENCES in samples)

How to couple (HP)TLC and MS?

Established and Emerging Atmospheric Pressure Surface Sampling/Ionization Techniques

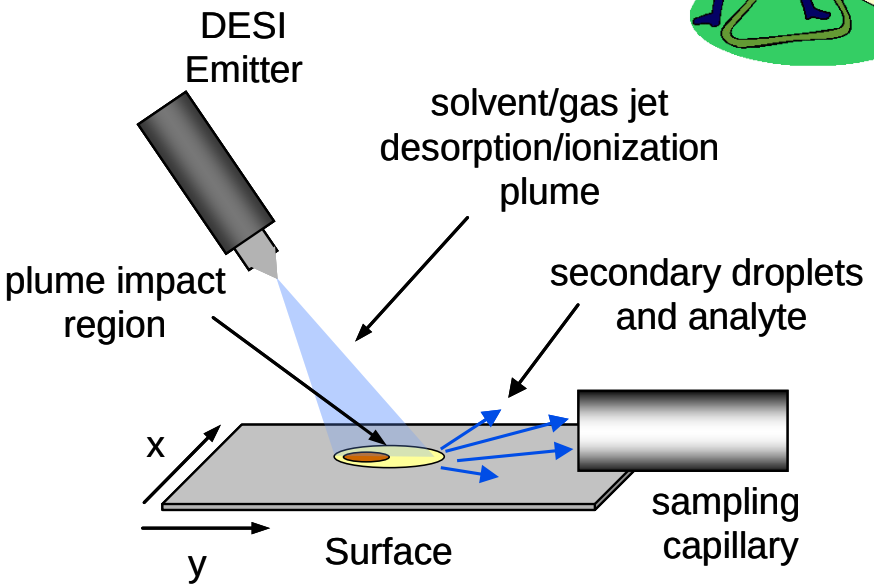


Addressing the Challenges to Enable Spatially Resolved Molecular Chemical Analysis of Interfaces Under Real World Conditions

Liquid and Gas Jet Desorption/Ionization

Desorption Electrospray Ionization (DESI)

- DESI is a multiple-step process
  - liquid-solid extraction
  - transfer of extract into gas phase/ionization



Liquid-solid extraction

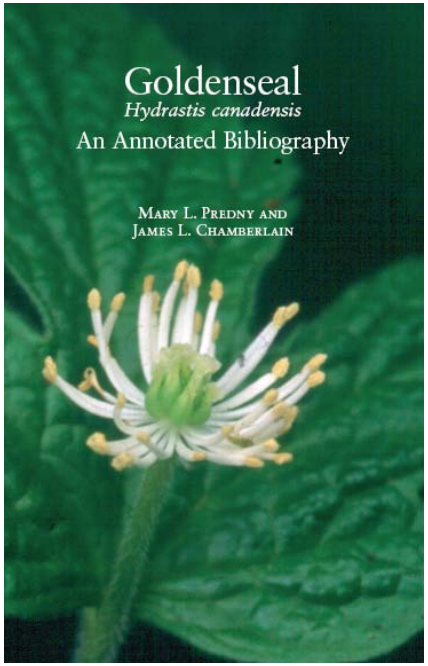
- surface/analyte characteristics
- DESI impact plume characteristics
- solvent, solvent flow rate, gas flow rate
- extraction time (surface scan rate when scanned)

Transfer into gas phase/ionization

- droplet/ion transfer to gas phase
- droplet/ion transfer in the sampling capillary
- ion identity, charge state
- analyte modification (e.g., oxidation)

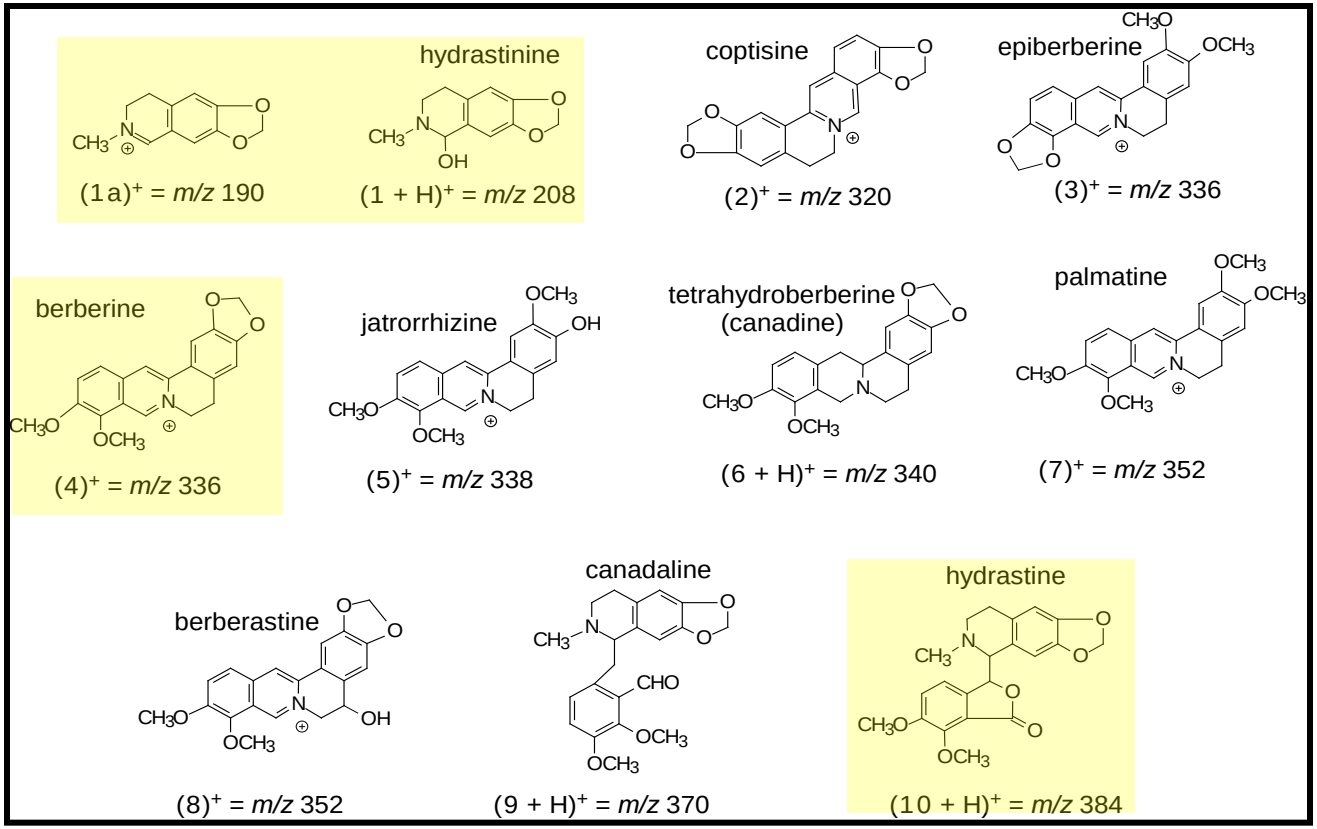
Desorption Sonic Spray Ionization (DeSSI)

Turn off the high voltage and turn up nebulizing gas velocity



TLC/DESI-MS: Goldenseal

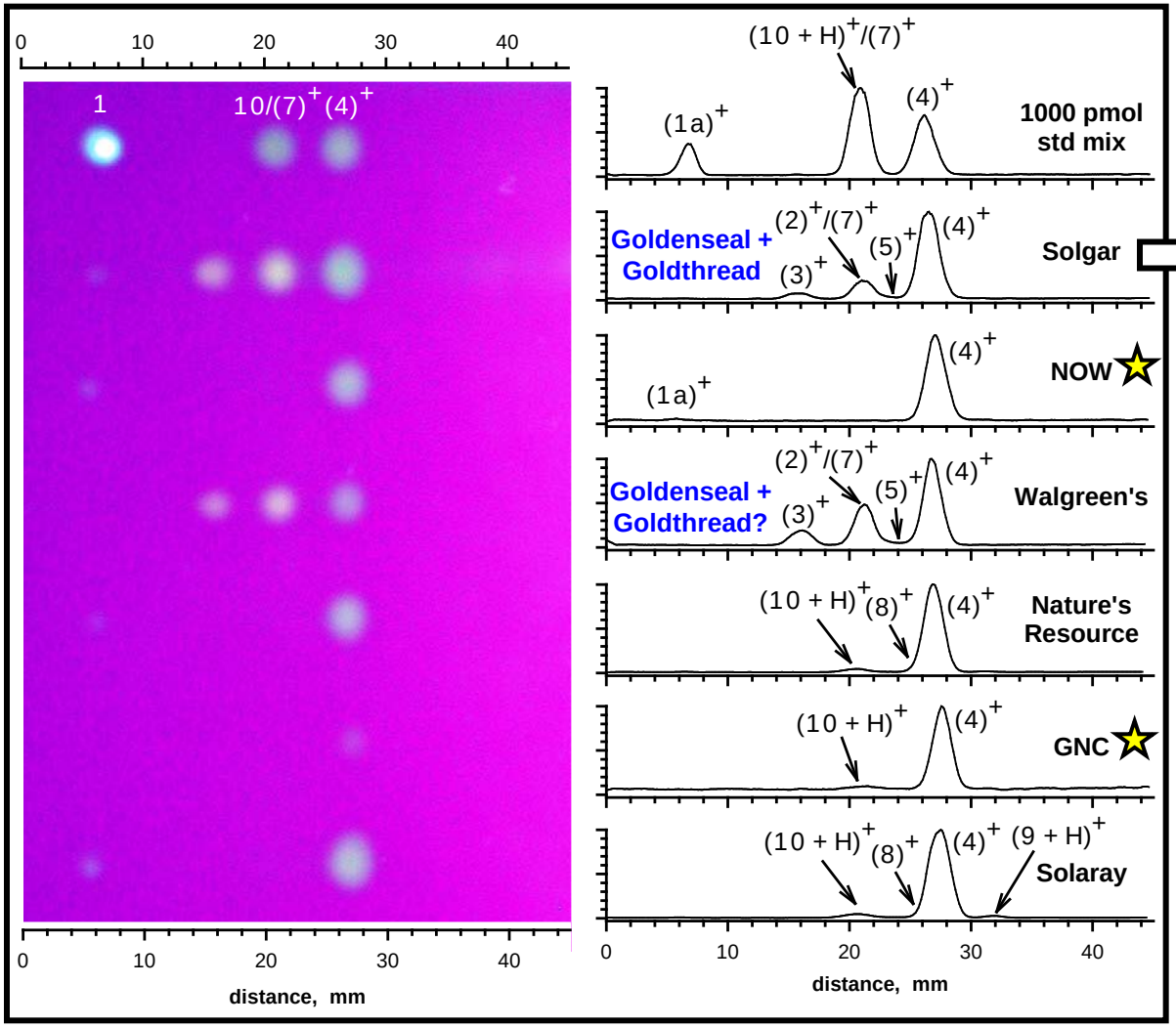
- A top-selling herbal product in USA
- Berberine, hydrastine, hydrastinine
- Substitute/admix with other alkaloid containing herbs (e.g., Goldthread)



Van Berkel, et al., *Anal. Chem.* 79, 2778 -2789, 2007

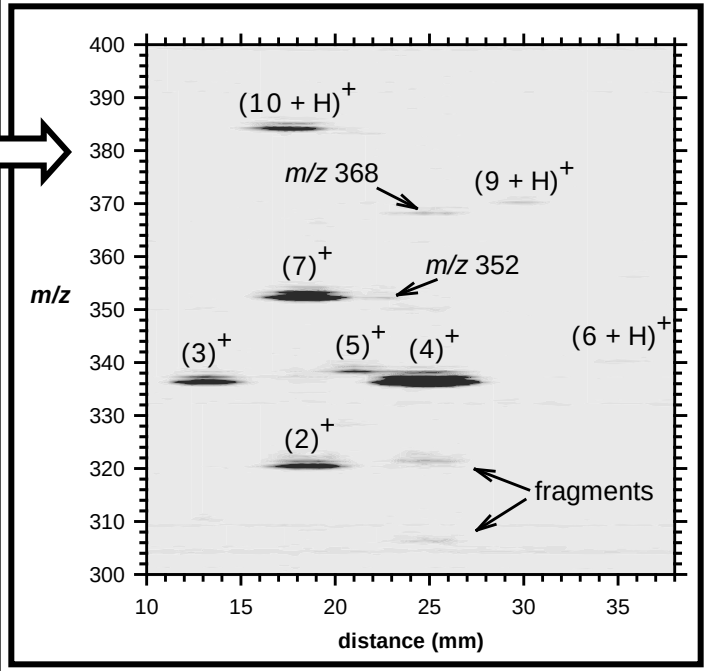
TLC/DESI-MS: Goldenseal

Analysis of six commercial "Goldenseal" products



NP-glass back HPTLC plate
50/10/6/3 v/v/v/v ethyl acetate/methanol/formic acid/water

Solgar sample ion map



- Sample complexity revealed in ion map
- Considerable component overlap in separation lane
  - Detection and quantitation by simple optical techniques compromised

TLC/DESI-MS: Goldenseal

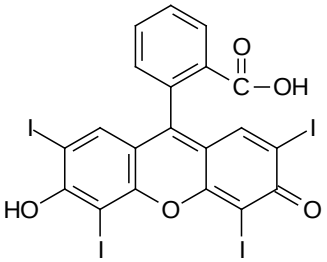
Quantification Results for Goldenseal Alkaloids in Two Commercially-Available Brands Determined Using TLC/DESI-MS and Fluorescence Spectroscopy, and Compared with Label Values

		Calculated Mass of Alkaloid per Capsule, mg[1]		
	Method of Quantitation	Berberine	Palmatine	Hydrastinine[2]
Solgar	TLC/DESI-MS	16 ± 2.3; n = 4	2.2 ± 0.37; n = 4	<0.24
	Fluorescence	19 ± 0.86; n = 3	8.4 ± 0.47; n = 3	< 0.24
	Label Value[3]	15		
Nature's Resource	TLC/DESI-MS	12 ± 0.91; n = 4	not detected	<0.24
	Fluorescence	14 ± 1.2; n = 3	not detected	< 0.24
	Label Value[4]	13.4		

[1] Reporting convention is mean ± standard deviation based on "n" replicates.
[2] Hydrastinine was observed, but at a mass below its calculated detection limit of 0.24 mg/capsule for both DESI-MS and fluorescence.
[3] Estimated total alkaloid content is 15 mg/capsule, based on the label values. This value has been assigned to berberine for comparison purposes.
[4] Label values also includes 10.7 mg hydrastine/capsule.

TLC/DESI-MS: Wettable RP C18

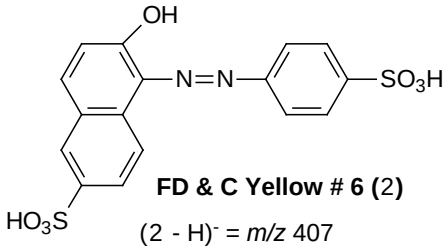
Food Dyes



FD & C Red # 3 (1)

$(1 - H)^-$ = *m/z* 834

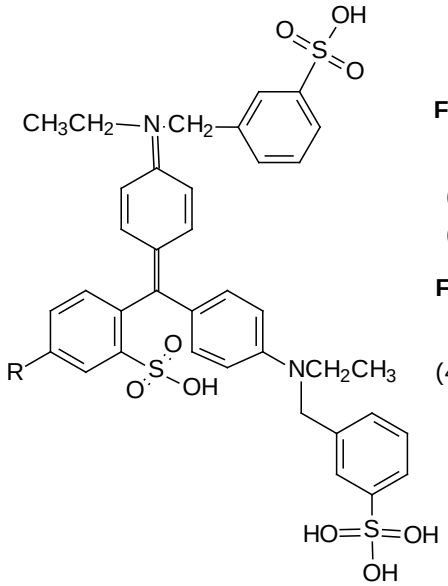
240 ng



FD & C Yellow # 6 (2) **320 ng**

$(2 - H)^-$ = *m/z* 407

$(2 - 2H + Na)^-$ = *m/z* 429



FD & C Green # 3 (3) **250 ng**

R = OH

$(3 - 2H)^{2-}$ = *m/z* 381

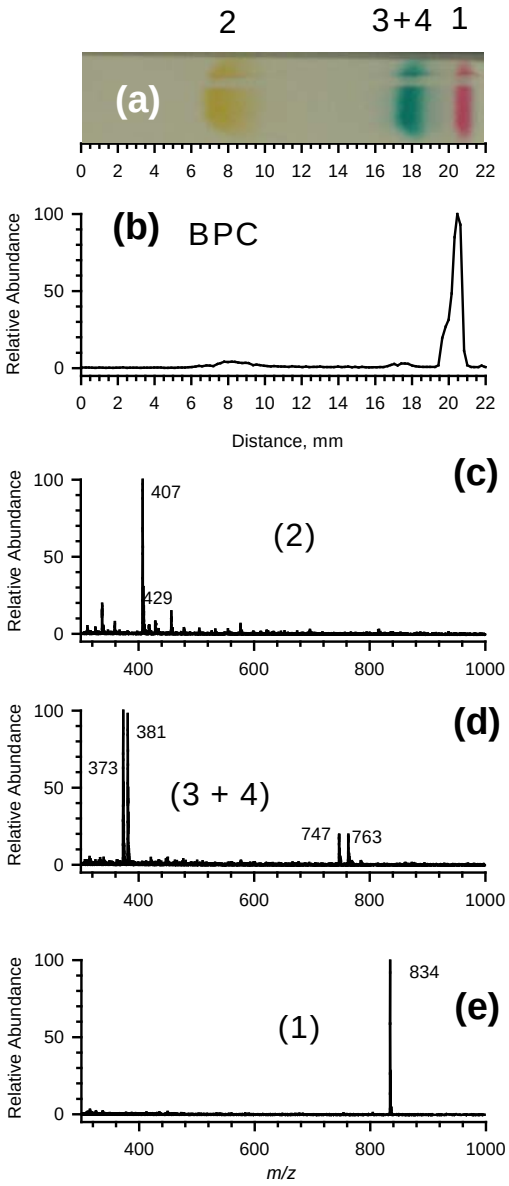
$(3 - H)^-$ = *m/z* 763

FD & C Blue # 1 (4) **260 ng**

R = H

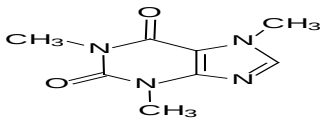
$(4 - 2H)^{2-}$ = *m/z* 373

- full scan (EMS) negative ion mode data
- 190 µm/s surface scan rate
- 10 µL/min methanol spray solvent



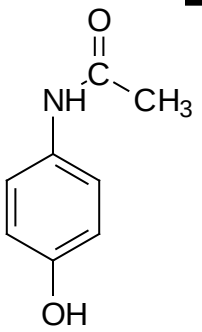
TLC/DESI-MS: Normal Phase

Pharmaceuticals



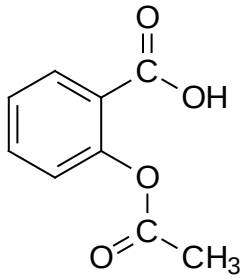
Caffeine (1)
$(1 + H)^+ = m/z$ 195
$(1 + Na)^+ = m/z$ 217

2 µg



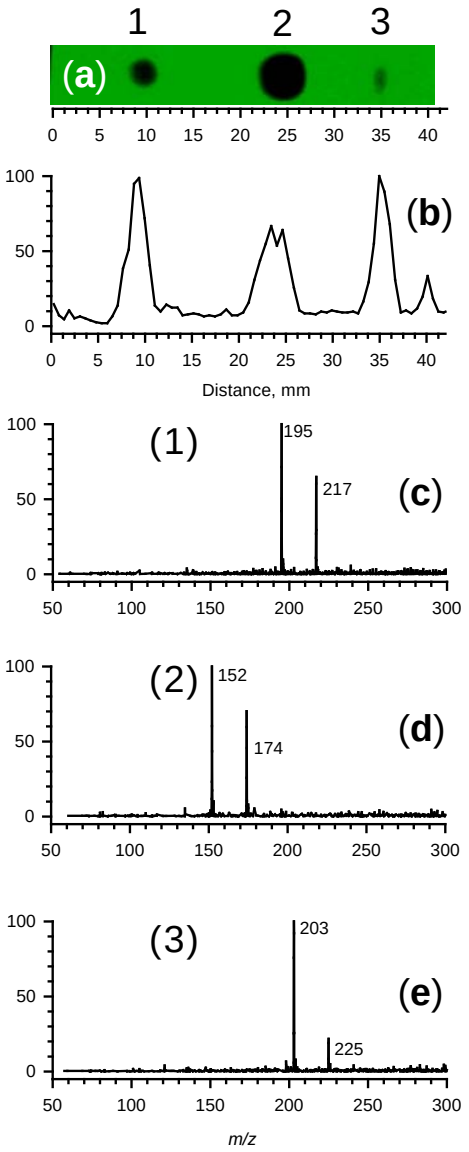
Acetaminophen (2)
$(2 + H)^+ = m/z$ 152
$(2 + Na)^+ = m/z$ 174

10 µg



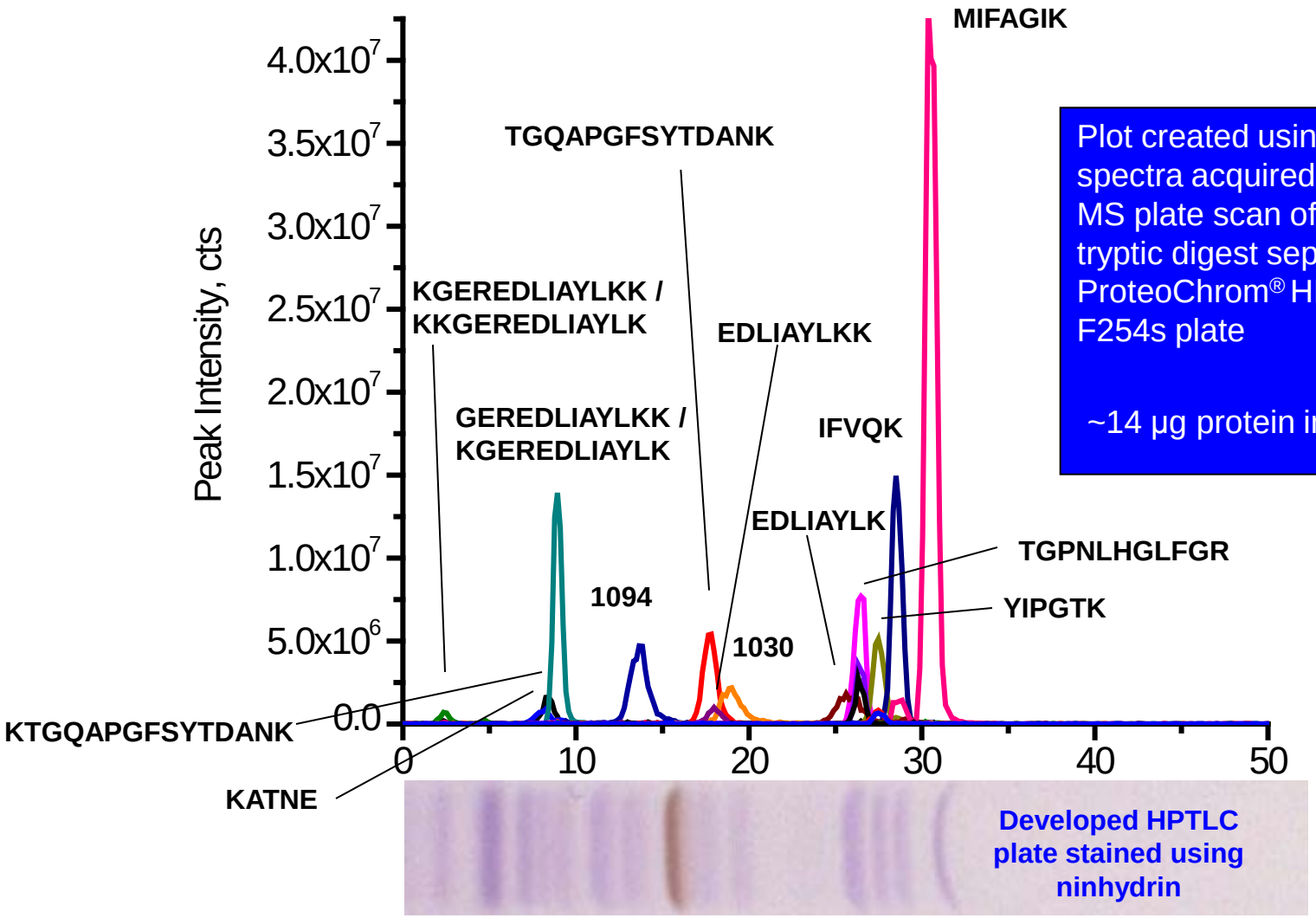
Aspirin (3)
$(3 + Na)^+ = m/z$ 203
$(3 + 2Na - H)^+ = m/z$ 225

10 µg



- aspirin, acetaminophen and caffeine in Excedrin tablets
- **190 µm/s surface scan rate**
- **10 µL/min methanol spray solvent**
- Desorption/ionization efficiency lower compared to reversed phase plates

Using HPTLC/DESI-MS for Peptide Identification in 1D Separation of a Tryptic Protein Digest



Plot created using full scan mass spectra acquired from a DESI-MS plate scan of cytochrome c tryptic digest separated on a ProteoChrom® HPTLC Silica 60 F254s plate

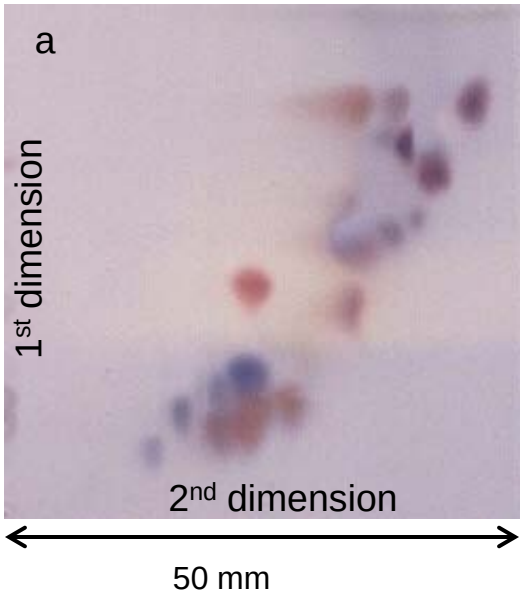
~14 µg protein in original band

Pasilis, S. P., et al., *Anal. Bioanal. Chem.* 2008, *391*, 317-324

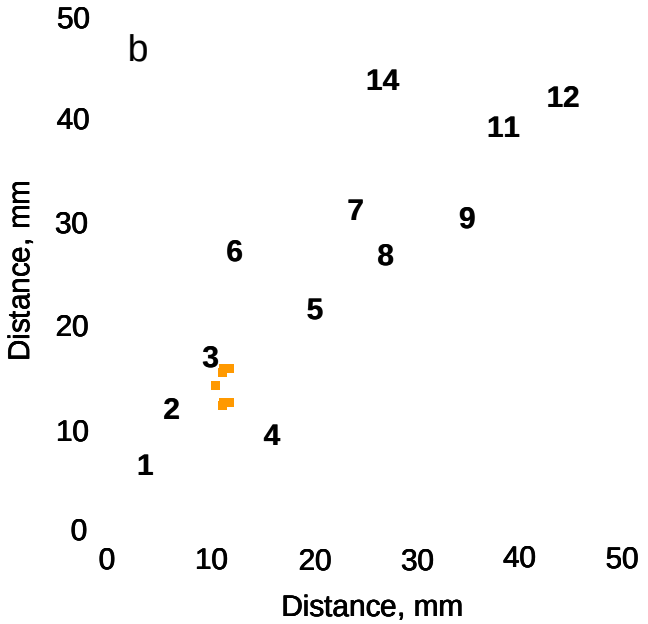
HPTLC/DESI-MS Imaging of a Tryptic Protein Digest Separated in 2D

Peptide distribution for a cytochrome c tryptic digest separated on a ProteoChrom® HPTLC Cellulose sheet.

Stained HPTLC sheet (ProteoChrom® Color Peptide Stain)



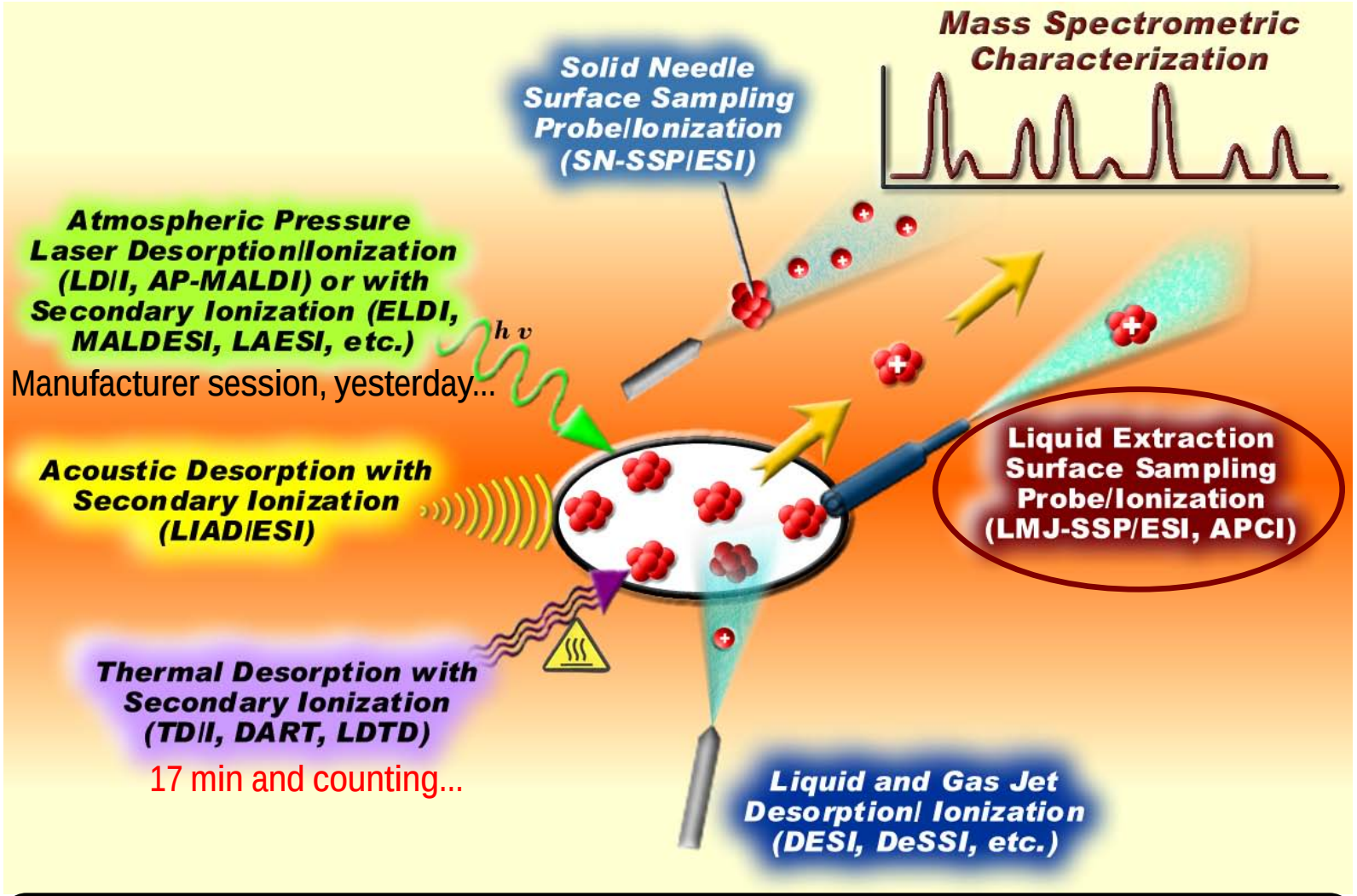
2D map created from MS/MS spectra acquired during sequential plate scans (imaging)



Peptide	ID
KKGER	1
KGER	2
KATNE	3
KGK	4
KTGQAPGFSYTDANK	5
KKGEREDLIAYLK	6
KGEREDLIAYLK	7
KYIPGTK	8
KIFVQK	9
TGPNLHGLFGR	10
IFVQK	11
MIFAGIK	12
EDLIAYLK	13
GITWGEETLMEYLENPK	14
Sequence coverage, from MS/MS data	80.0%

Pasilis, S. P., et al., *J. Mass Spectrom.* 2008

Established and Emerging Atmospheric Pressure Surface Sampling/Ionization Techniques



Addressing the Challenges to Enable Spatially Resolved Molecular Chemical Analysis of Interfaces Under Real World Conditions

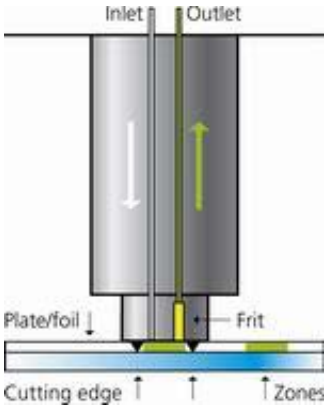
Liquid Extraction Based Surface Sampling

Sealing Surface Sampling Probes

Liquid Extraction Surface Sampling Probe/Ionization

Sealing Surface Sampling Probe (SSSP)

- **Available from CAMAG**
- **Couples between HPLC pump and MS**
  - **ESI, APCI, APPI, etc.**
- **Designed for on-line extraction from TLC plates to MS**
- **Probe knife edge seals against suitable surface types, e.g.,**
  - **TLC phase on glass or aluminum**
  - **Blood spots on paper**
  - **Thin tissue sections on paper**
- **Semi-automatic**
- **Blank spot washout required to eliminate sample-to-sample carryover**



Luftmann, *Anal. Bioanal. Chem.* 2004, *378*, 964: basic technique, ESI

Luftmann, et al. *RCM* 2007, *21*, 3772: semi-automation

TLC-MS Interface

Sample extractor

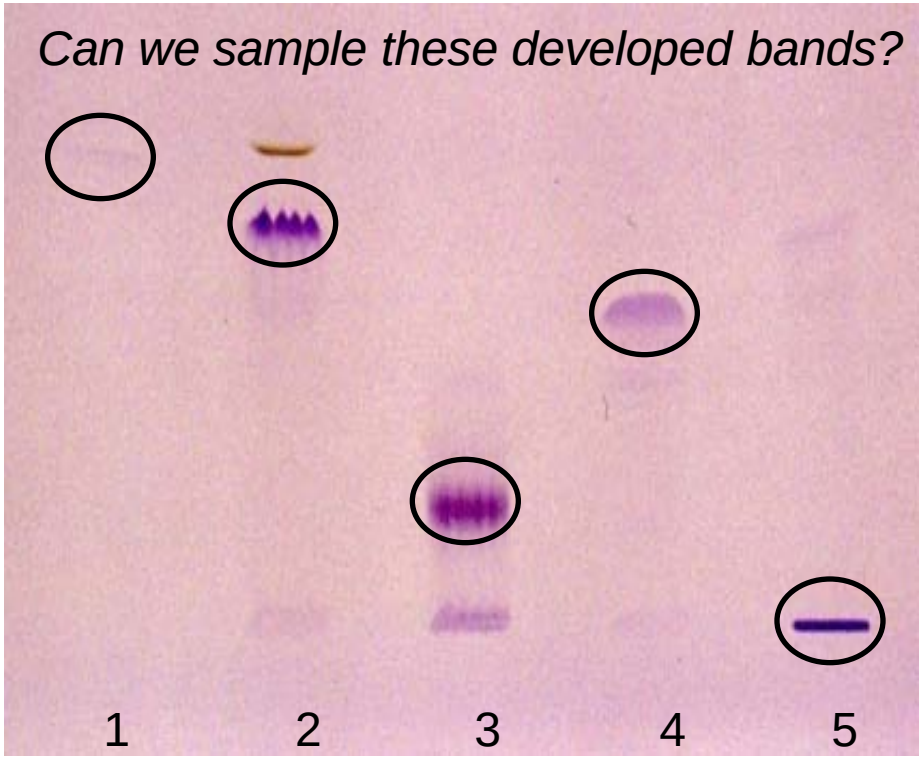
Surfaces to be analyzed:

TLC, Dried blood spots, Animal/plant tissues, etc.

Detector to be used:

MS, NMR, UV/VIS, etc.

Intact Protein Development on HPTLC Plates



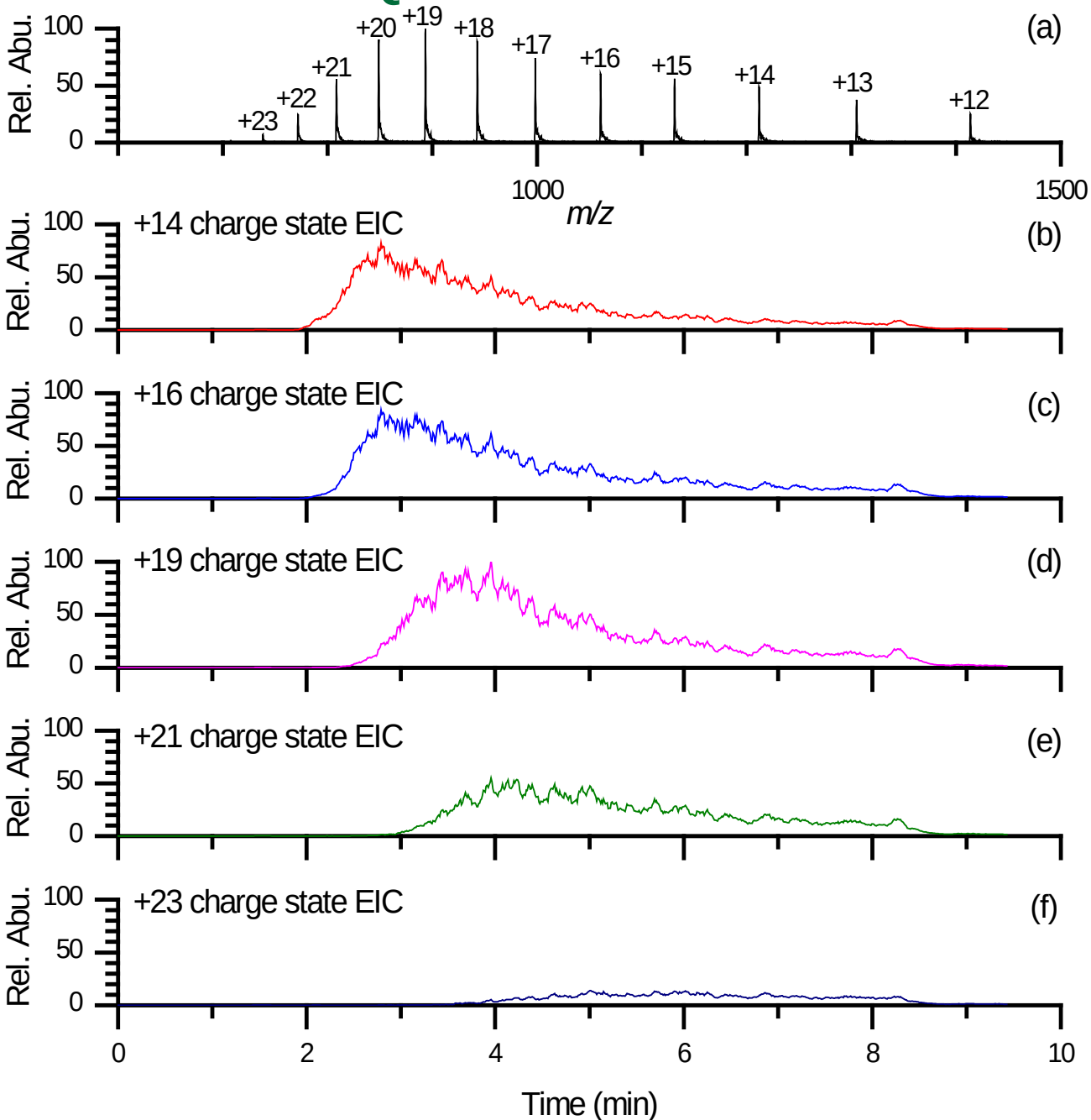
Track	Sample	Conc.	Loaded Mass (µg)
1	Glucagon	0.35mg/ml	2.45
2	Myoglobin	4mg/ml	12
3	Cytochrome C	4mg/ml	12
4	ß-Casein	4mg/ml	16
5	Ubiquitin	1.35mg/ml	9.45

- Ninhydrin stained bands indicates proteins are moving

HPTLC plate:
ProteoChrom® HPTLC Silica gel 60 F254s

Plate Development:
1-butanol/pyridine/NH3/water 39/20/10/31 (v/v/v/v) in an unsaturated glass twin trough TLC tank

S-SSP LTQ-XL MS Readout - Myoglobin

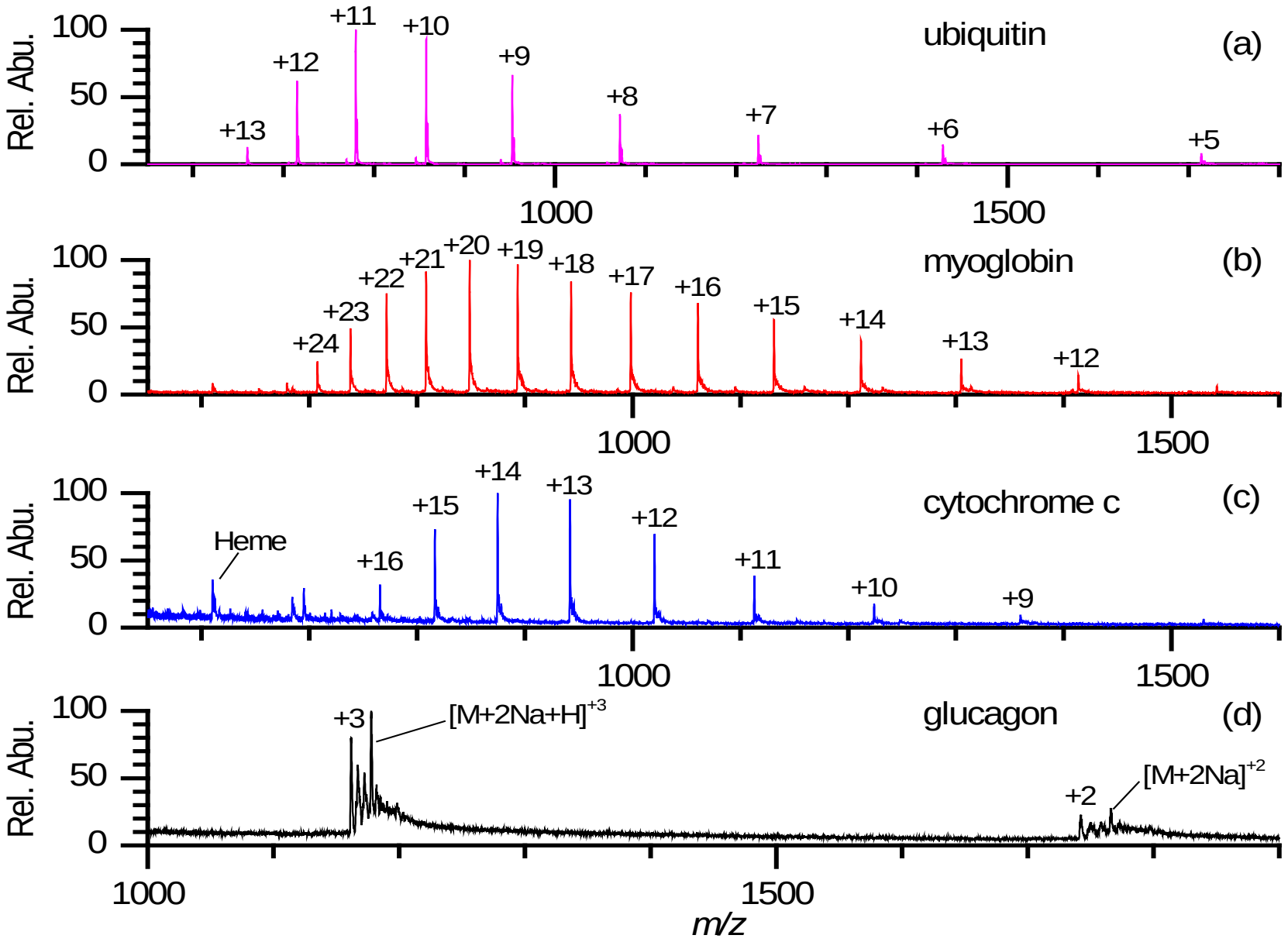


Can we sample these developed bands? ***YES***

Elution Solvent: 70/30/0.1 water/ACN/formic acid (v/v/v) at 50 µL/ min

Electrospray Ionization

S-SSP LTQ-XL MS Readout - Proteins



CAMAG TLC-MS
Dried Blood Spots

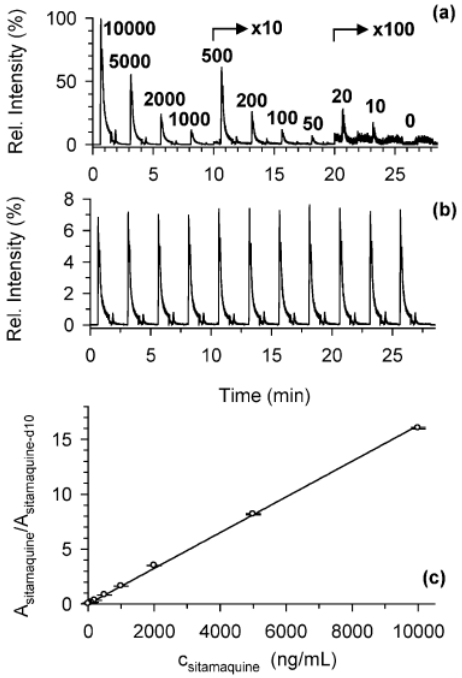
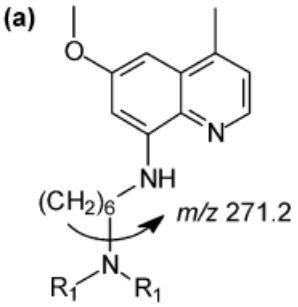


Figure 1. SRM ion current chronograms of (a) sitamaquine and (b) sitamaquine-d_{10} obtained from the analysis of dried rat blood spot calibration standards using positive ion mode ESI. The concentration of sitamaquine is shown in part a. (c) Calibration curve constructed using calibration standards (line) and average of QC samples (○) with error bars (CV) using the ratio of background corrected integrated (over the 60 s sampling period) SRM signal of sitamaquine (10−10000 ng/mL) and that of sitamaquine-d_{10} (570 ng/mL) ($A_{sitamaquine}/A_{sitamaquine\text{-}d_{10}}$) as a function of sitamaquine concentration ($c_{sitamaquine}$) in the blood spotted onto the paper substrate. The calibration data was analyzed using a least-squares regression with a $1/c_{sitamaquine}$ weighting and fit the model of $A_{sitamaquine}/A_{sitamaquine\text{-}d_{10}} = (1.63 \times 10^{-3})c_{sitamaquine} - (1.12 \times 10^{-2})$ ($r^2 = 0.999$). Statistical results obtained for the QC samples are summarized in Table 2.

Table 2. Nominal ($c_{sitamaquine}$) and Calculated Mean ($c_{calcd,sitamaquine}$) Concentrations, Precision (% CV, $n = 6$), and Accuracy (% Bias) of Sitamaquine Quality Control DBS Samples Based on a Linear Fit of Calibration Standards

$c_{sitamaquine}$ (ng/mL)	$c_{calcd,sitamaquine}$ (ng/mL)	precision (% CV)	accuracy (% bias)
10000	9851.9	0.4	−1.5
5000	5030.0	0.6	0.6
2000	2159.4	0.3	8.0
1000	1012.0	0.7	1.2
500	513.6	0.3	2.7
200	208.7	0.9	4.4
100	100.0	0.7	0.0
50	55.4	0.6	10.8
20	24.5	1.1	22.6
10	15.6	3.4	55.7



sitamaquine (R_1=C_2H_5)

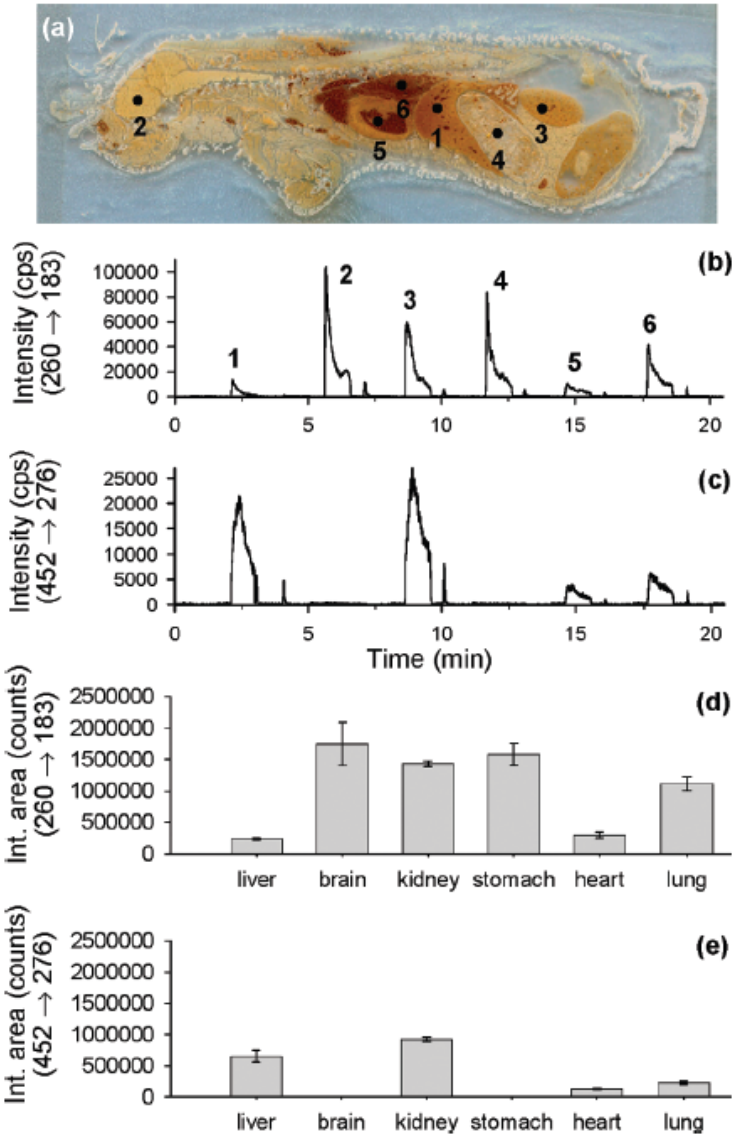
$(M + H)^+$ = *m/z* 344.4

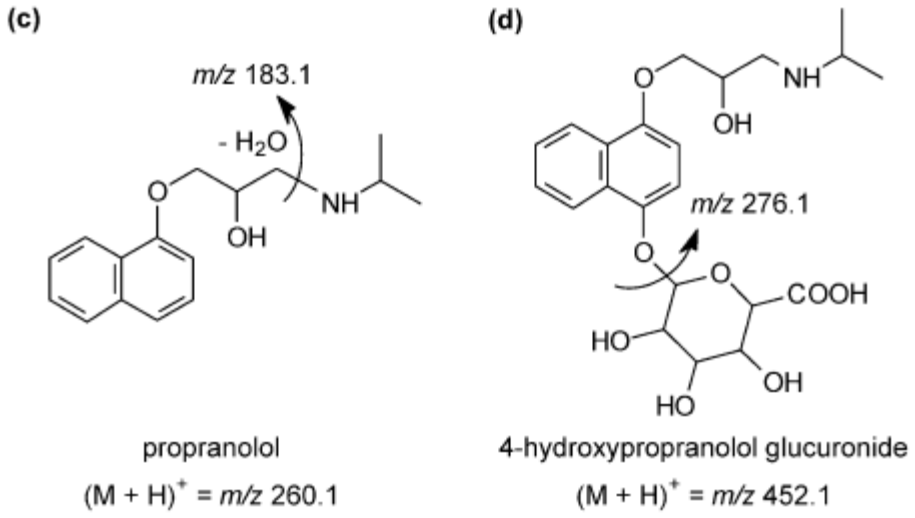
sitamaquine-d10 (R_1=C_2D_5)

$(M + H)^+$ = *m/z* 354.4

Van Berkel and Kertesz, *Anal. Chem.* 2009, 81, 9146–9152.

CAMAG TLC-MS
Tissue – drug and metabolite distribution





propranolol

$(M + H)^+$ = *m/z* 260.1

4-hydroxypropranolol glucuronide

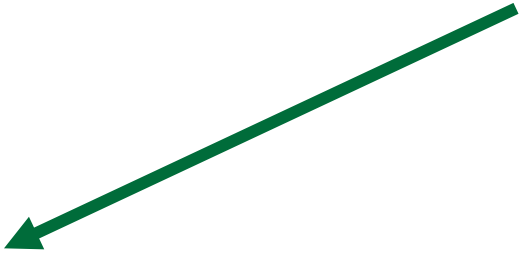
$(M + H)^+$ = *m/z* 452.1

Figure 3. (a) Photograph of a propranolol dosed mouse whole-body thin tissue section on adhesive tape. The six discrete points analyzed are annotated: 1 = liver; 2 = brain; 3 = kidney; 4 = stomach/contents; 5 = heart; 6 = lung. SRM ion current chronograms for (b) propranolol and (c) hydroxypropranolol glucuronide recorded during a 60 s sampling period at each point using positive ion mode ESI. Average integrated area with error bars (CV) of the SRM signals of (d) propranolol and (e) hydroxypropranolol glucuronide for organs analyzed in two separate tissue sections.

Liquid Extraction Based Surface Sampling

Sealing Surface Sampling Probes

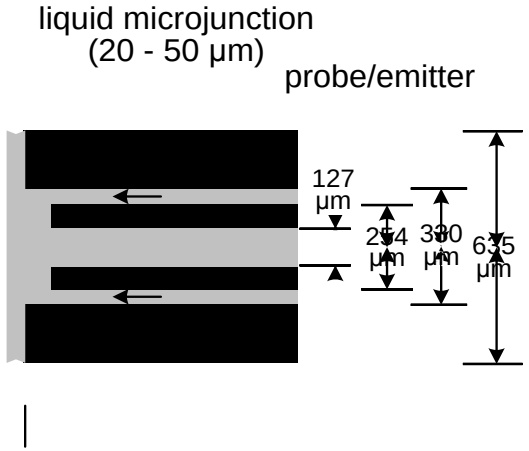
Liquid Microjunction Probes



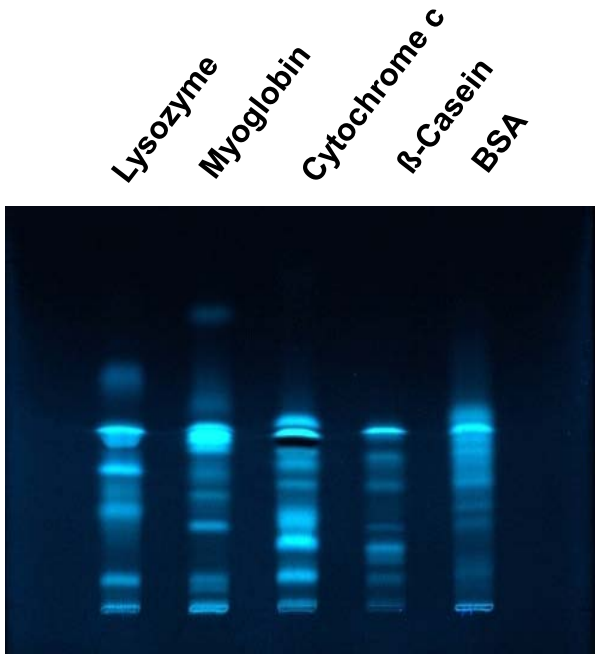
Two Concentric Capillaries

Liquid Extraction Surface Sampling Probe/Ionization

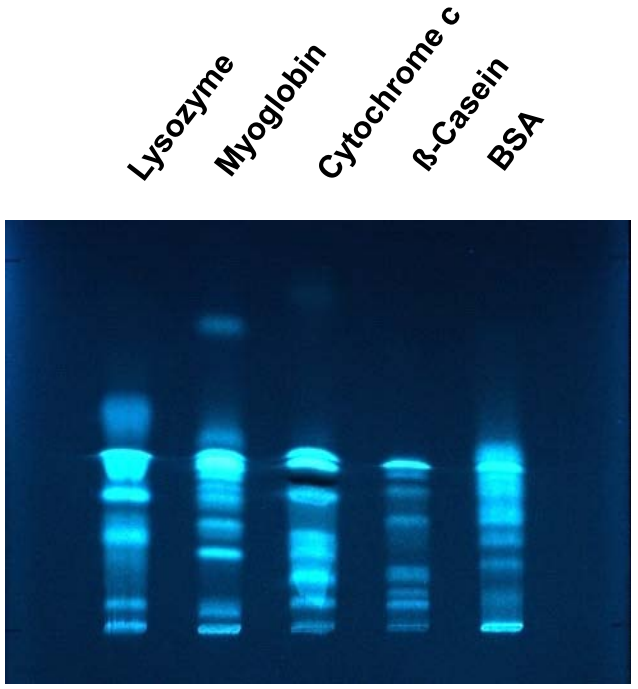
- **Eluting solvent pumped towards the surface through the annulus of the sampling and solvent delivery capillaries**
- **Solvent forms liquid micro-junction with surface**
- **Material from surface dissolved in solvent is aspirated from the surface through inner sampling capillary and sprayed**
- **Local pressure drop from pneumatic nebulizer used to aspirate solvent from the surface through inner sampling capillary**



1D HPTLC Separation of Tryptic Petides*



Stationary Phase: HPTLC RP-8 F254s
Mobile Phase: methanol/water 70/30 (v/v)
+ 0.1 M ammonium acetate
Stain: Fluorescamin

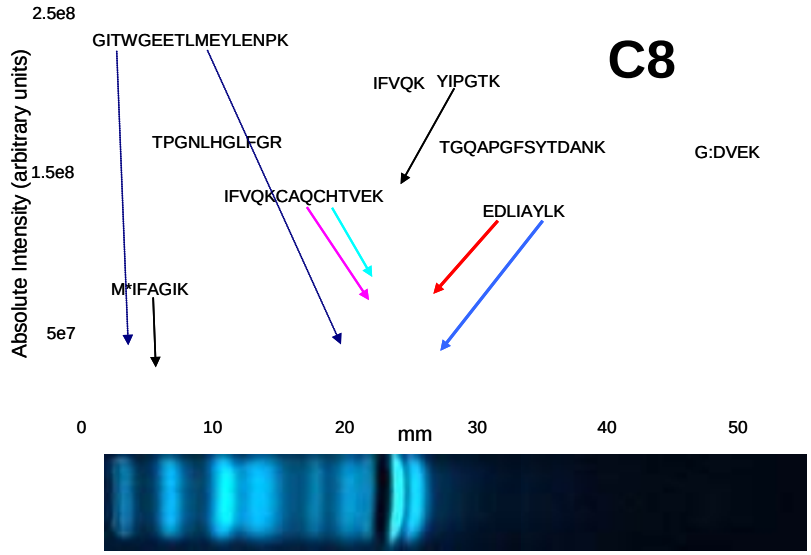


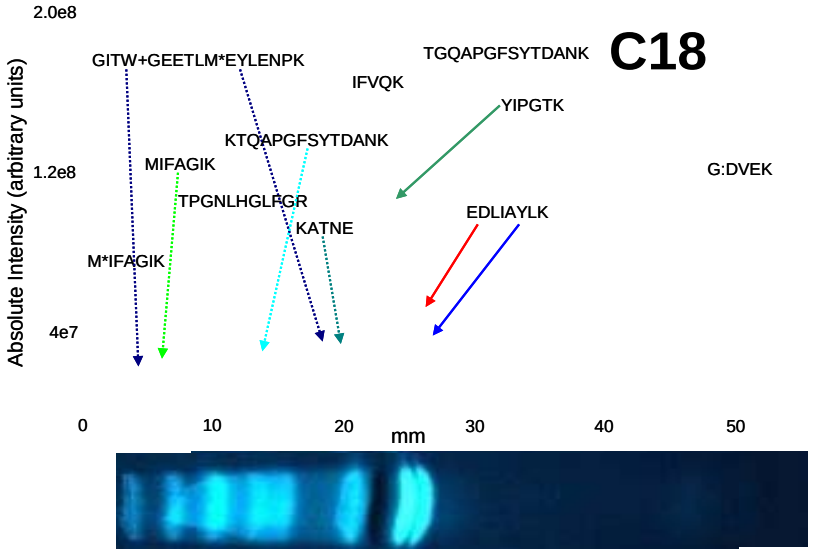
Stationary Phase: HPTLC RP-18 F254s
Mobile Phase: methanol/water 70/30 (v/v)
+ 0.1 M ammonium acetate
Stain: Fluorescamin

Hydrophobic RP HPTLC
14 µg of material/band (applied using spraying technique)

*** Courtesy of Michael Schulz, Merck KGaA**

Ion Current Profiles for Identified Cytochrome c Peptides





- **Data obtained using LCQ - IDs via MS/MS and data base searching**
- **Lanes scans at 27 or 45 µm/s**
- **70/30 (v/v) water/acetonitrile with 0.1% by volume formic acid at 10 µL/min**
- **Overall the RP-8 plates provided better separations than RP-18**
- **N-terminal acetylation prevents ac-GDVEK from being visible as a stained band**
- **A small degree of band overlap is observed in the cytochrome c separations**
- **Both unoxidized and oxidized versions of the peptide GITWGEETLMEYLENPK were observed**

LCQ Deca - Data Dependent Scans of HPTLC Plates

Sequence coverages obtained for five protein tryptic digests analyzed by LMJ-SSP/ESI-MS/MS for the RP-8 and RP-18 HPTLC plates and by DESI-MS/MS for the silica gel (NP) and cellulose HPTLC plates

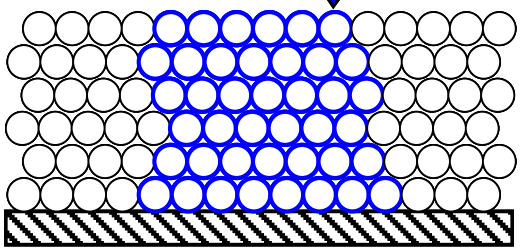
	LMJ-SSP#		DESI*	
Protein	RP-C8 HPTLC	RP-C18 HPTLC	Silica gel HPTLC	Cellulose HPTLC
BSA (66 kDa, 21 pmol)	5.3%	8.6%	14.3%	8.6%
Beta Casein (25 kDa, 560 pmol)	12.1%	12.1%	17.4%	14.7%
Cytochrome C. (12 kDa, 1132 pmol)	62.5%	59.6%	59.6%	69.2%
Myoglobin (17 kDa, 824 pmol)	58.2%	54.2%	66.0%	60.8%
Lysozyme (14 kDa, 979 pmol)	45.7%	34.1%	27.1%	50.4%

Emory, J. F., et al. *Eur. J. Mass Spectrom.* 2009, *16,* 21-23
* Pasilis, S. P., et al., *Anal. Bioanal. Chem.* 2008, *391*, 317-324

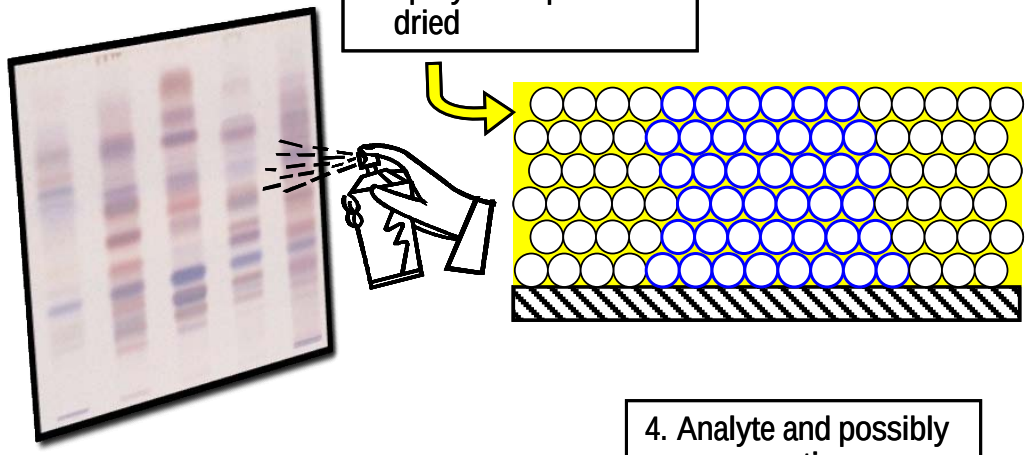
- **Despite lower quality separations, RP HPTLC plates provided comparable sequence coverage to NP HPTLC plates**
- **RP-8 plates generally provide slightly higher sequence coverage than RP-18 plates**
- **Larger proteins were spotted in lower molar amounts and provided lower sequence coverage than smaller proteins spotted in higher molar amounts**

Enabling Liquid Microjunctions on Wettable Surfaces: Silicone Treatment

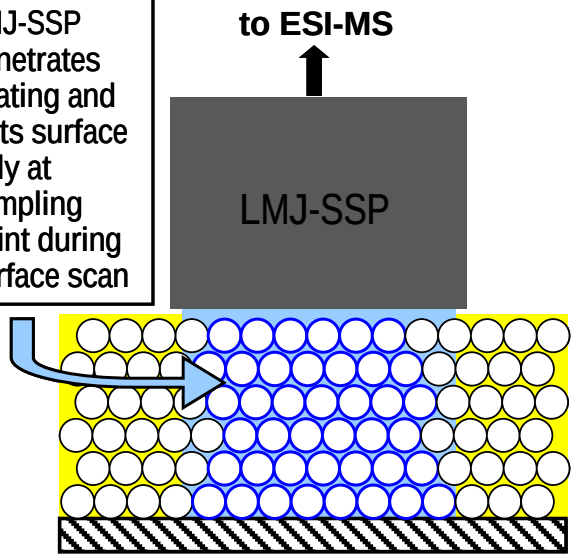
1. Analyte band on wettable TLC plate



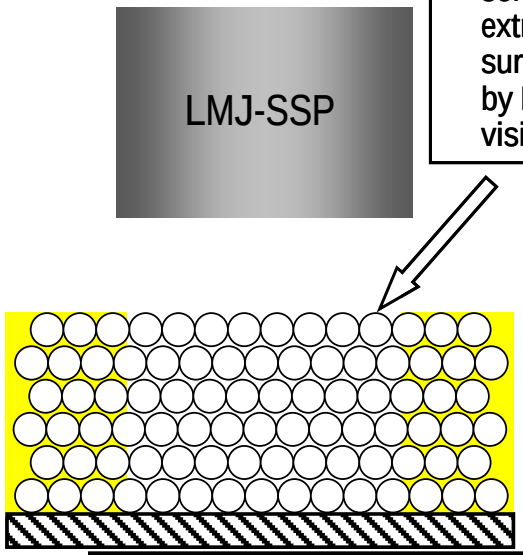
2. Hydrophobic coating sprayed on plate and dried



3. Extraction solvent from LMJ-SSP penetrates coating and wets surface only at sampling point during surface scan



4. Analyte and possibly some coating extracted from surface as evidenced by MS signal and visible track

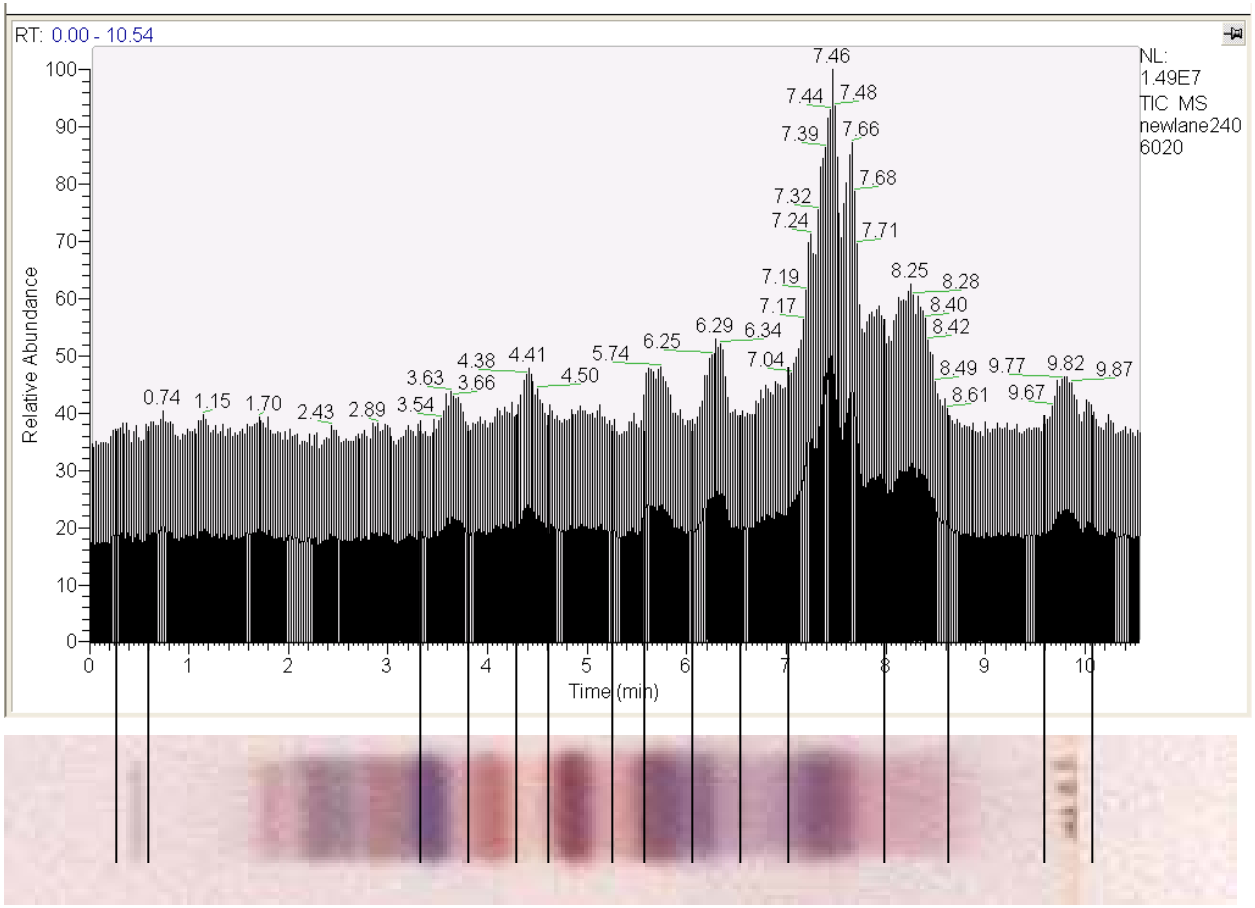


Walworth, et al., *Anal. Chem.* 2011, *83*, 591-597

Treatment used on all but hydrophobic RP C8 and RP C18 plates

LTQ - Data Dependent Scans of Tryptic Peptides Separated on Wettable NP Cellulose HPTLC Plates

Now possible to analysis wettable surfaces with LMJ-SSP

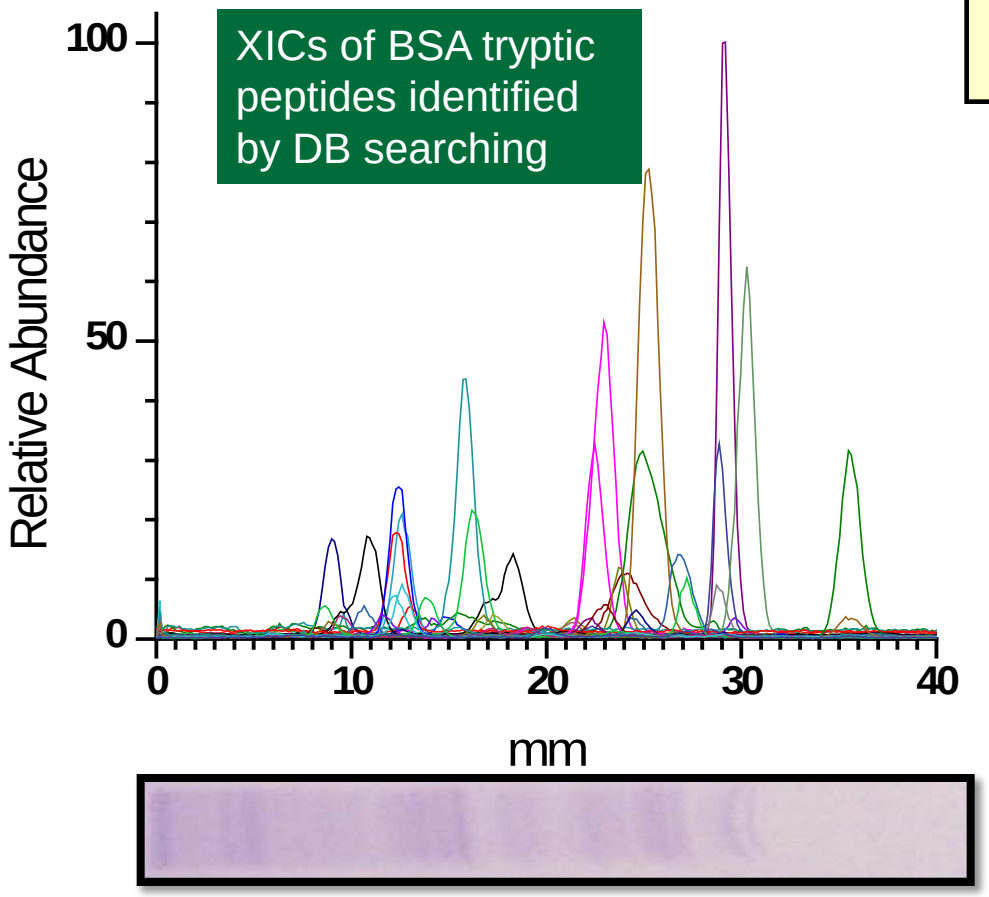


Myoglobin tryptic peptides separated on cellulose

- **Data obtained using LTQ - IDs via MS/MS and data base searching**
- **Lanes scans at 100 µm/s**
- **60/40 (v/v) water/acetonitrile with 0.1% by volume formic acid at 10 µL/min**
- **Cellulose plate provided better separations than RP-18 or RP-8**

LMJ-SSP TLC NP-HPTLC Development Lane Scan of Tryptic Peptides

BSA Lane Scan on Silica Gel HPTLC plate



Number of peptides identified and protein sequence coverage achieved superior to that for LMJ-SSP with RP plates and DESI with normal phase plates

Protein sequence coverages

Protein	Cellulose*	Silica gel*
BSA	40.5%	60.5%
Beta Casein	22.3%	29.5%
Cytochrome C	90.4%	89.4%
Myoglobin	98.0%	85.6%
Lysozyme	75.2%	88.4%

***Treated with silicone aerosol spray prior to analysis**

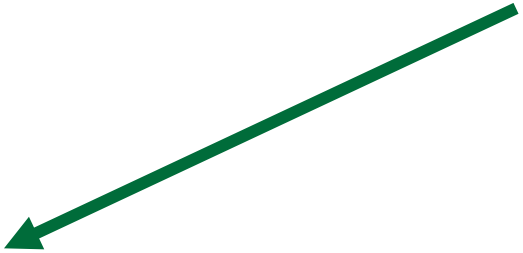
- 41 peptides identified in single lane scan

Walworth, et al., *Anal. Chem.* 2011, *83*, 591-597

Liquid Extraction Based Surface Sampling

Sealing Surface Sampling Probes

Liquid Microjunction Probes

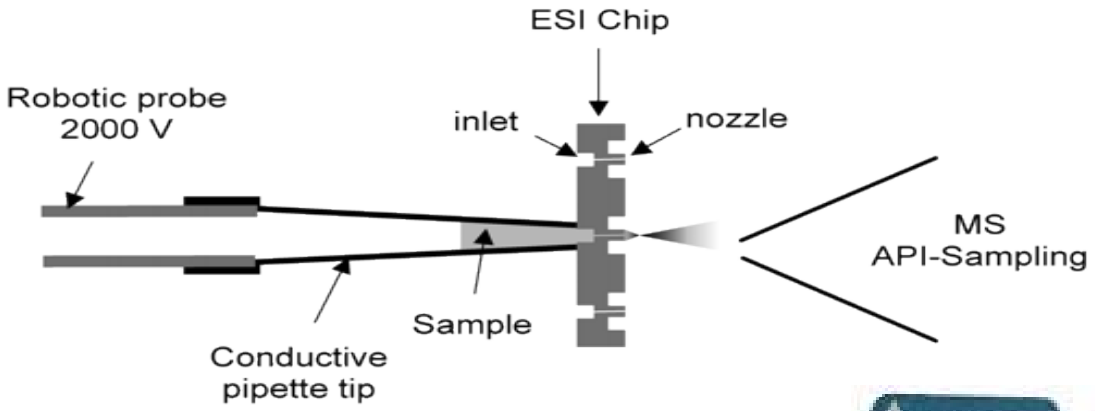


Two Concentric Capillaries

Autonomous Syringe

Liquid Extraction Based Surface Sampling with the TriVersa NanoMate System

Robotic nanospray platform utilizing a microfluidics chip.



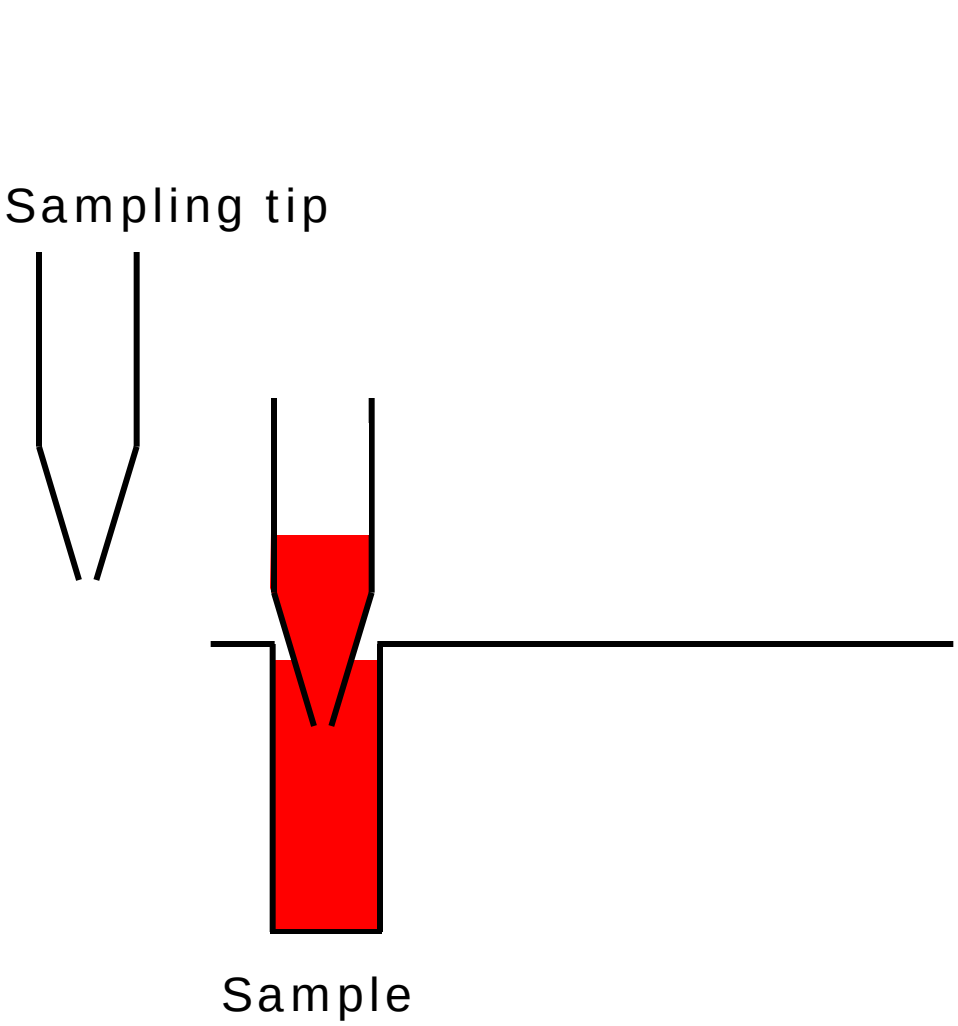
The microfluidics chip contains an array of nanoelectrospray nozzles etched in a silicon wafer.

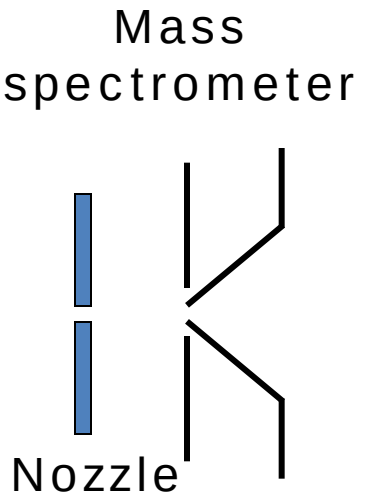
Liquid Extraction Surface Analysis (LESA):

Fully automated, liquid extraction-based surface sampler
One sample, one tip, one nozzle, no carryover

Kertesz and Van Berkel, *J. Mass Spectrom.* 2010, *45*, 252-260

Normal Operation using the NanoMate System



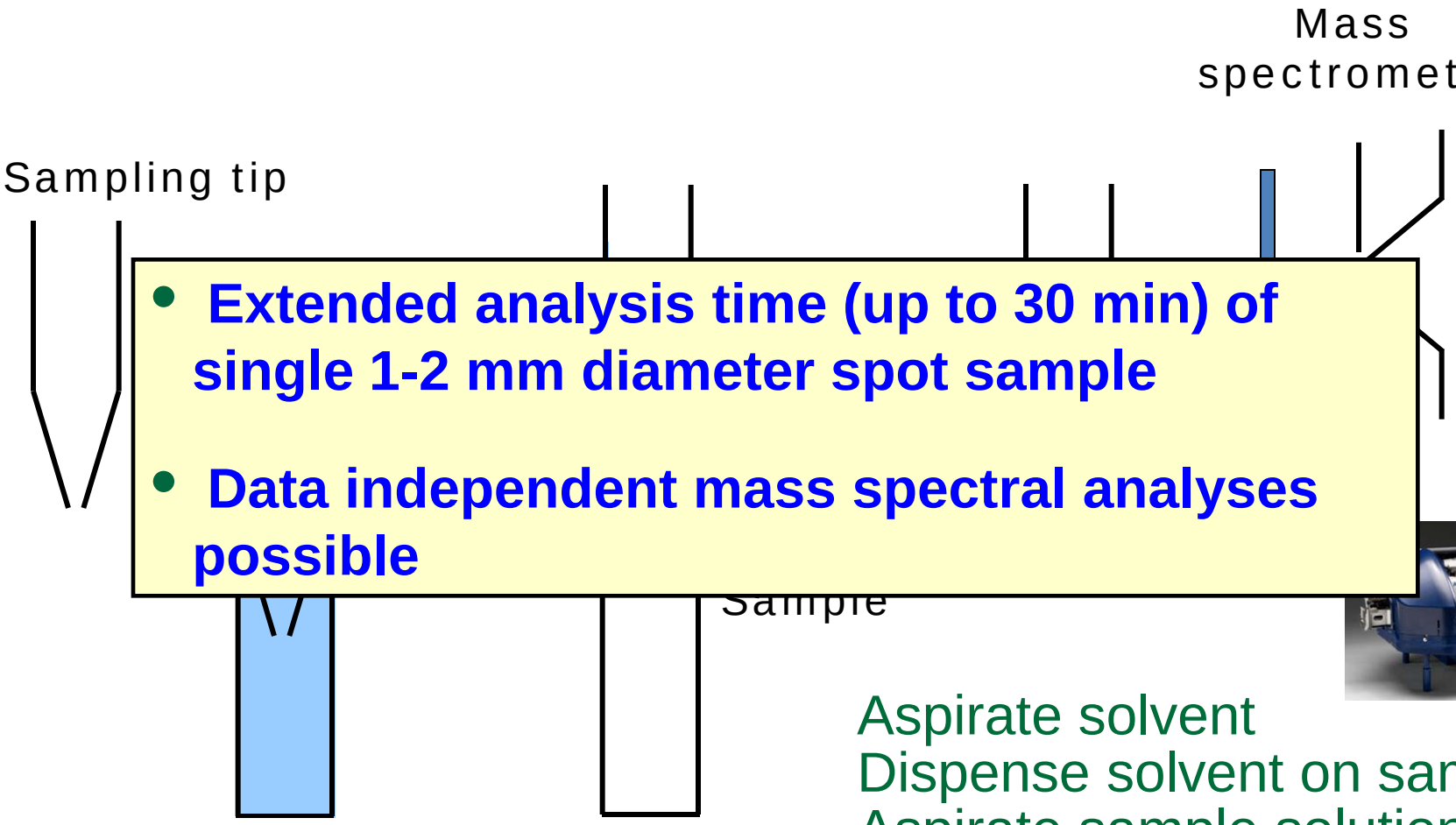


Aspirate sample

Transfer sample

Apply HV, spray sample

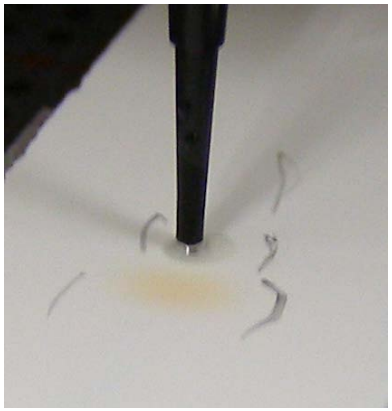
Operation using the NanoMate System for Surface Sampling



- **Extended analysis time (up to 30 min) of single 1-2 mm diameter spot sample**
- **Data independent mass spectral analyses possible**

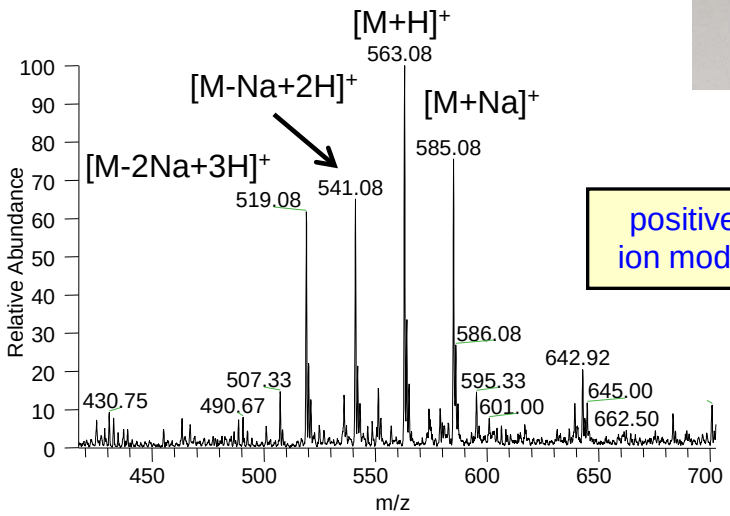
Aspirate solvent
Dispense solvent on sample
Aspirate sample solution
Transfer sample
Spray sample

LESA: RP C8 HPTLC Plate



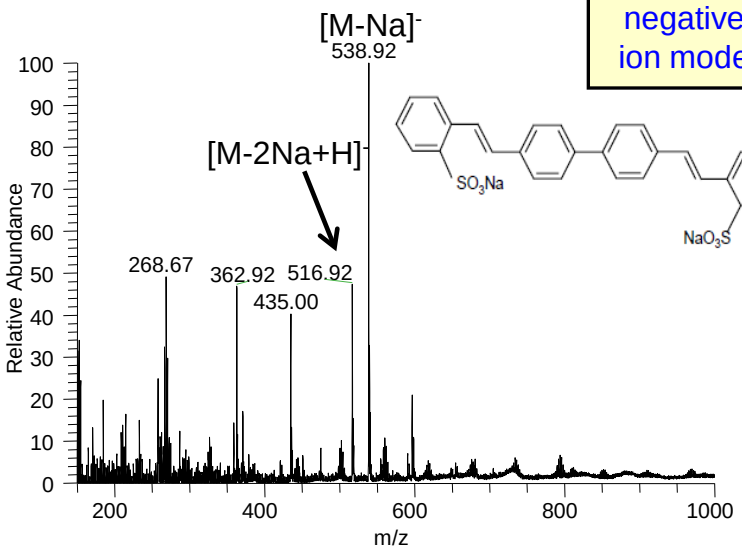
- Dyes separated on hydrophobic RP C-8 HPTLC plate
- Developed using 60:40 MeOH:THF, 100mM ammonium acetate
- 50:50:0.1 ACN:H_2O:FA as an extraction/nanospray solvent

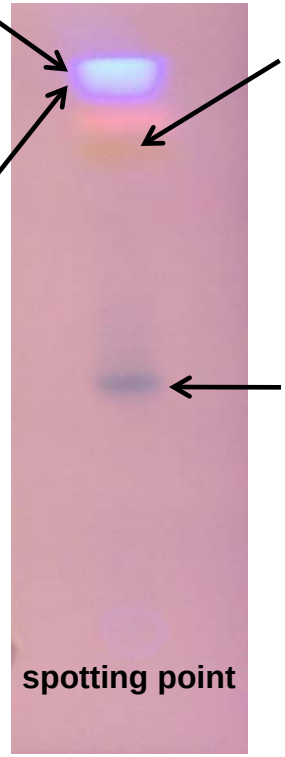
Stilbene 420



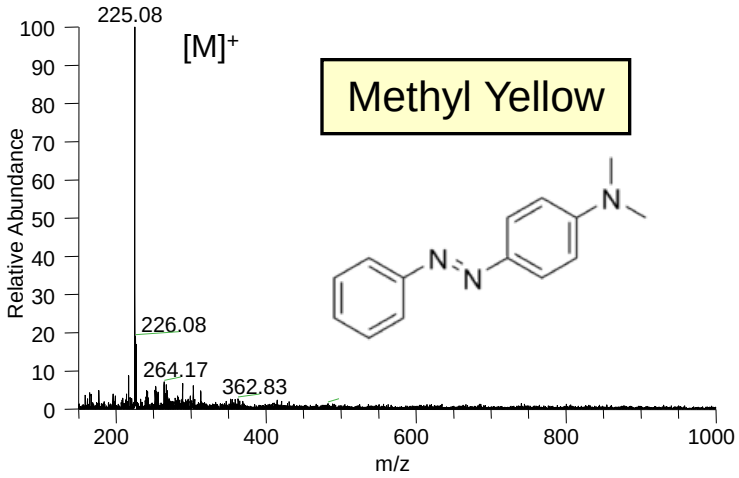
positive ion mode

negative ion mode

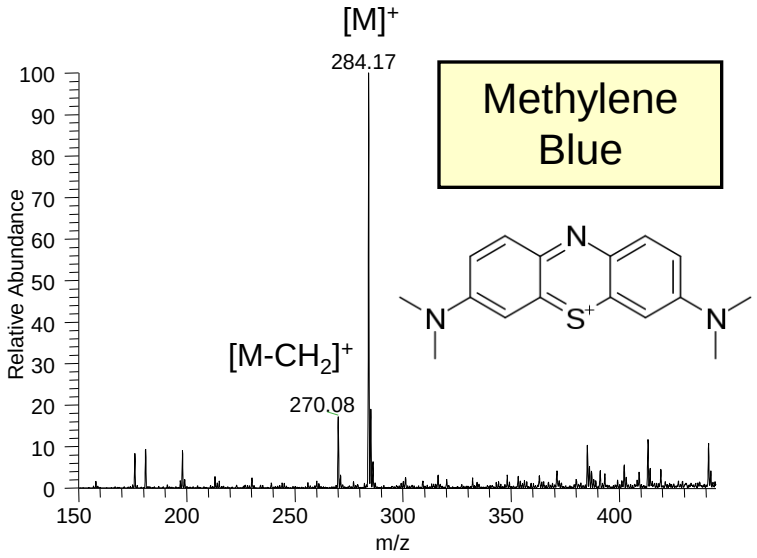




Methyl Yellow

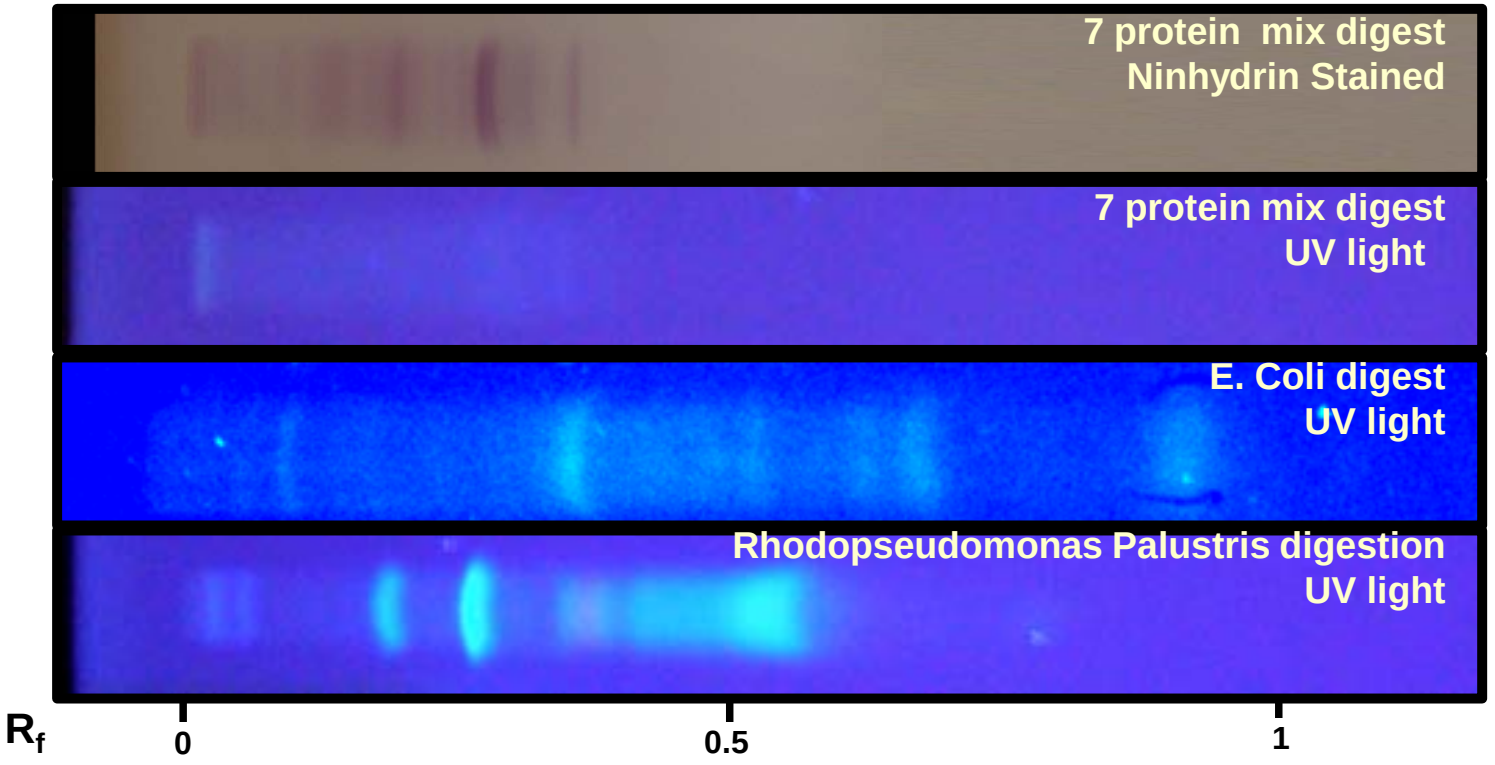


Methylene Blue



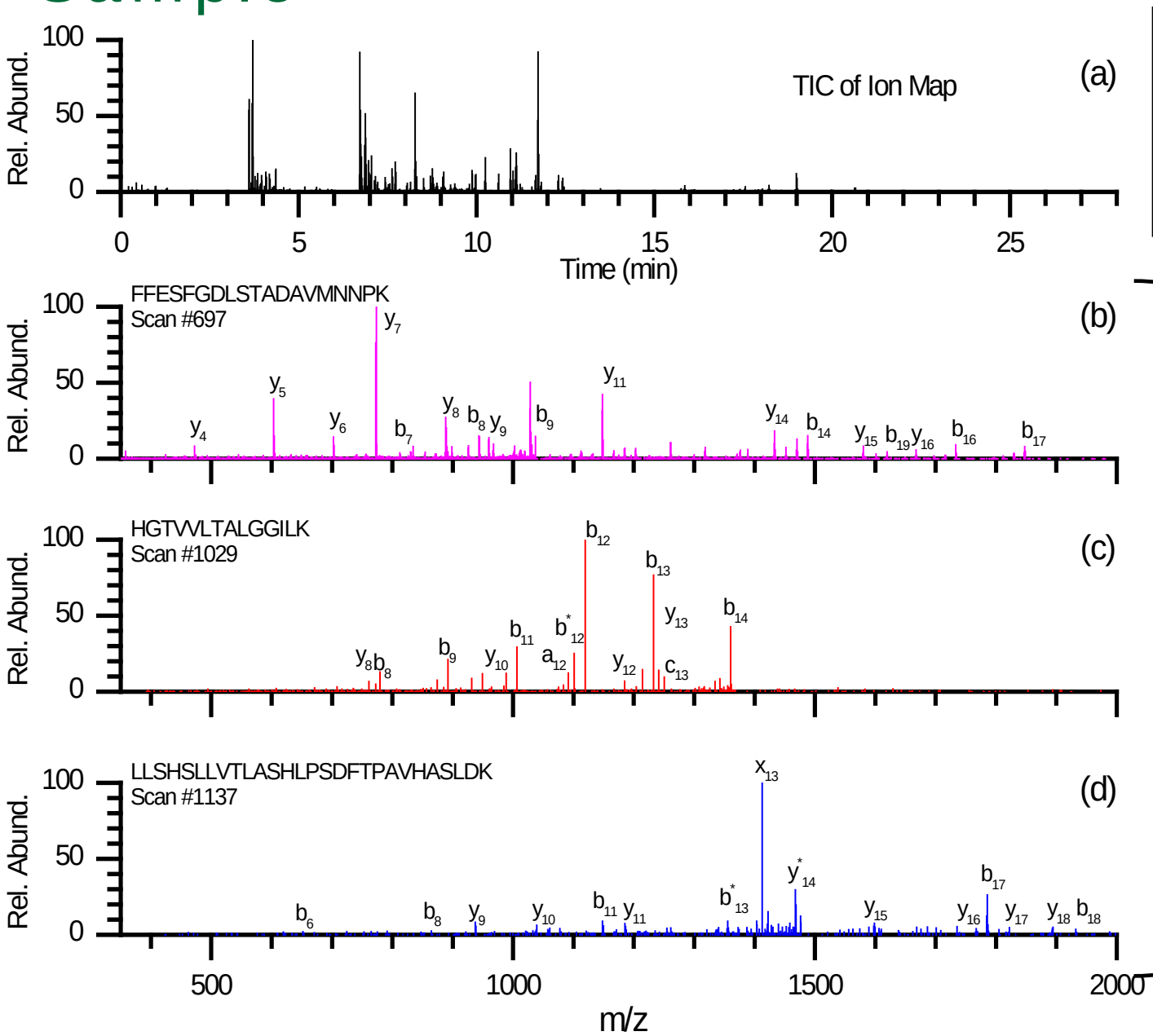
Complex Peptide Mixtures Separated on HPLTC Plates: LESA Readout Using Multiple Spot Samples and Data Independent Ion Mapping

Complex Samples Fill Development Lane with Bands



Ion Mapping Mode: MS/MS product ion spectrum obtained at each *m/z* from *m/z* 350 – 1500 for each spot sample

Ion Mapping Data from Single Spot Sample



Product ion current profile obtained by stepping through each *m/z* precursor ion in scan range

Example product ion spectra from selected precursor ion *m/z* values

LESA and Ion Mapping vs Lane Scanning: 7 Protein Mix Digest

sequence coverage

Single Lane Scan

27 Spot Samples along Lane

	CF – LMJ-SSP	LESA Ion Mapping
	5mmBand 20 µL ap	5mmBand 20 µL ap
HORSE HEART CYTOCHROME C	79	81
BOVIN CARBONIC ANHYDRASE II	7.3	22.3
BOVIN HEMOGLOBIN BETA CHAIN	44.1	55.2
BOVIN HEMOGLOBIN ALPHA CHAIN	43.3	43.3
HORSE LIVER CHAIN A ALCOHOL DEHYDROGENASE	0	7.5
HORSE MYOGLOBIN	76.5	52.9
CHICK Lysozyme C	73.9	53.5
RABBIT GLYCERALDEHYDE-3-PHOSPHATEDEHYDROGENASE	33	31.8
Proteins IDs	**6**	**7**
peptide IDs	**70**	**117**

Data Dependent Analysis

Ion Mapping – MS/MS of all m/z

Separation and Read Out of E. Coli Digest

LESA Ion Mapping	Proteins	Peptide IDs	Copies	FDR%
Redundant	924	2815	3530	
Nonredundant	909	2385	3085	0.7
CF-LMJ-SSP				
Redundant	89	313	443	
Nonredundant	85	296	426	0.3

- **100 µL of E. coli trypsin digest spotted as a 4 mm band for each analysis**
- **E. coli digest developed on ProteoChrom HPTLC Silica Gel 60 F(254S) plates**
- **Read out using data dependent scan function with CF-LMJ-SSP-MS and ion mapping scan function with LESA spot sampling mode**
- **LESA: 49, 2 mm spaced spot samples along development lane**
  - **70/30/0.1 water/ACN/formic acid (v/v/v) as extraction/nanoESI solvent**
  - **23.5 hours analysis time**
- **CF-LMJ-SSP: scan at 100 µm/s alone development**
  - **10 min. analysis time**
  - **70/30/0.1 water/ACN/formic acid (v/v/v) extraction/ESI solvent**
- **Mods- G (+42.0106), M (+15.9949), W (+31.9898), Acetylation of N terminus**

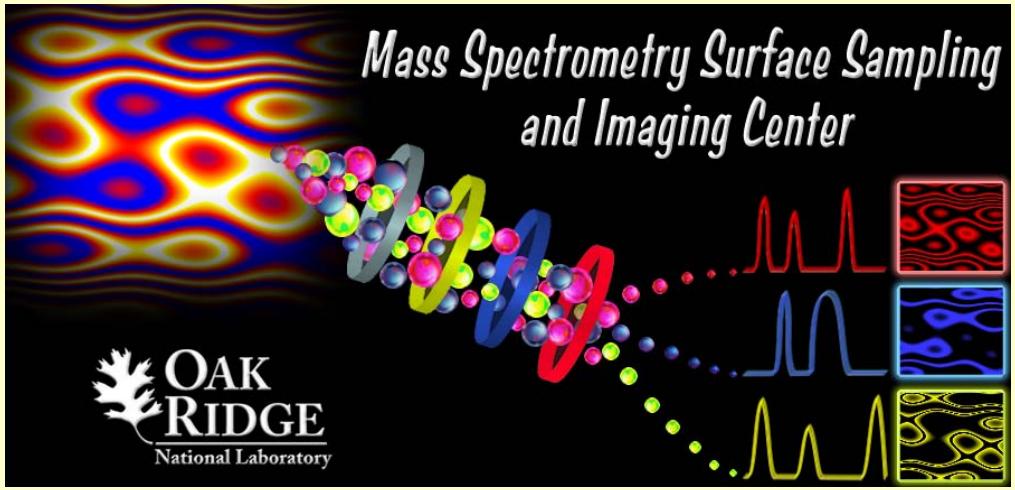
Future

- **New area: omics**
  - **Immediate: proteomics**
    - **Top-down, bottom-up approaches**
    - **Biomarkers: looking for differences, best suited for TLC**

Further development

- **Plates**
- **Methods**
- **Cooperation between TLC and MS communities**

Acknowledgement



- **DOE Basic Energy Sciences Mass Spectrometry Program**

Department of Energy
Office of Basic Energy Sciences

- **CRADA with**

AB SCIEX

State-of-the-art instrumentation, scientific collaboration

- **Instrumentation loans/donations, samples, scientific collaborations, "WFO" agreements, LDRD, IP maturation**

Thermo SCIENTIFIC

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

AstraZeneca

MERCK

PharmaCadence Analytical Services, LLC
"Analytical Excellence at the Pace of Discovery"

Advion

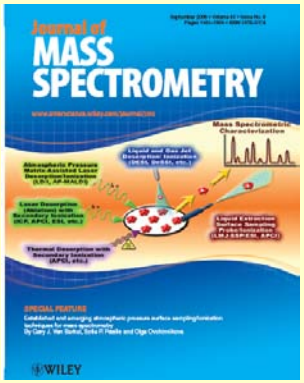
ASAP Mass Spectrometry

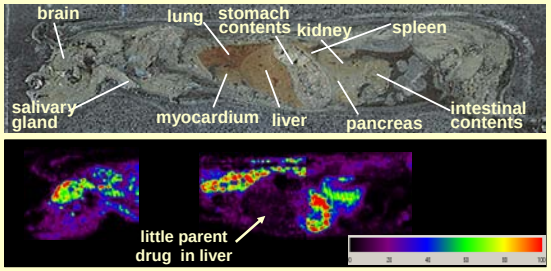
Abbott
A Promise for Life

MARS

MERCK

OAK RIDGE NATIONAL LABORATORY
TECHNOLOGY TRANSFER
AND ECONOMIC DEVELOPMENT





Laboratory Directed Research and Development Program
OAK RIDGE NATIONAL LABORATORY

Homeland Security

gsk GlaxoSmithKline

CAMAG

http://www.ornl.gov/sci/ms_imaging_center