AMRランチョンセミナー

セミナー内容

- **全血直接インジェクション/ドライブラッドスポット法**
  完全自動化などの最新の前処理自動化及び小動物/マイクロドージング対応超高感度LC/MSの紹介

エーエムアール株式会社　　板東泰彦

- **CTCPALを用いたSPE/LC/MSのオンライン化の実際**
  ITSPを利用したヒト血漿中Atorvastatinとその代謝物における定量解析分析法の検討

株式会社　東レリサーチセンター　　櫻井　周　様

第25回日本薬物動態学会2010
2010年10月8日ランチョンセミナー

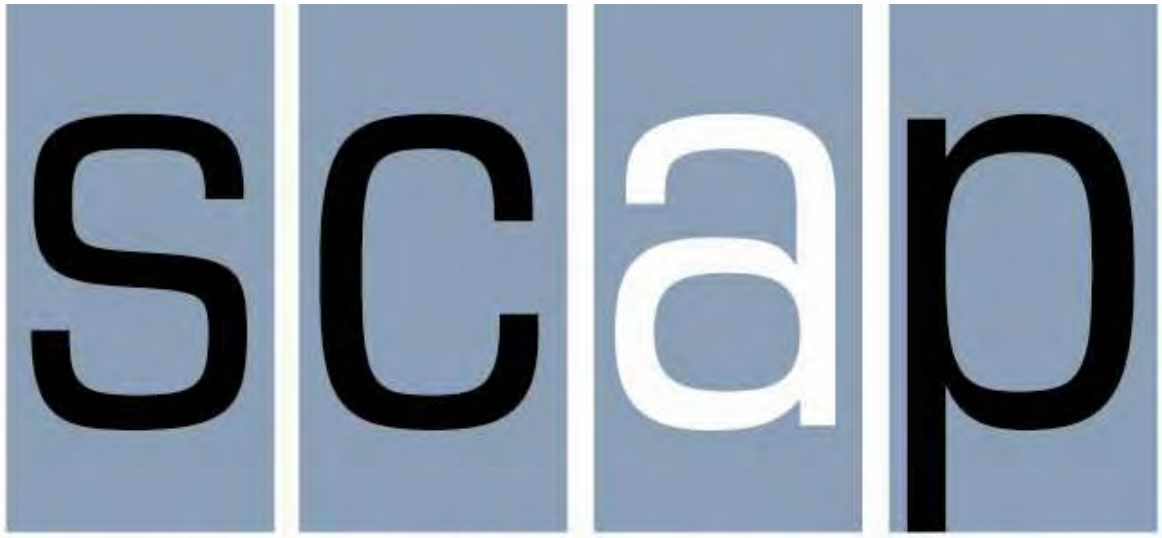
高感度分析で必要なこと

- 計測機器（MS)が高感度
- MSが高分解能、高選択性
- 物質に対するイオン化
- マトリックス効果の検討

複雑系の中から微量成分を検出するには前処理や分離が必要！

- 適切なサンプル前処理
- クロマトグラフィでの分離
- S/N比のよいイオン化

目的物がそれぞれ分離されPureになったものを解析装置に導入することにより高感度分析が実現する！！


scap
sample cartridge and prep system

scap

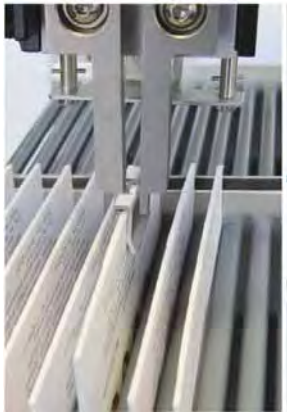
Automated sample preparation platform for online LC-MS/MS bioanalyis

PLS

Pipette & Liquid Sampling

DBS

Dry Blood Spot



scap

sample cartridge and prep system

PLS

SCAP System Consumables

SCAP System Rack, 54 Tips and Caps

Numbered SCAP Tips and Caps

SCAP System Sampling I

Attach SCAP Tip to Multistep electronic Pipette

Aspirate ISTD, sample (whole blood or plasma), anticoagulant and esterase inhibitior

Seal loaded SCAP tip with cap

SCAP System Sampling 2

Prepared SCAP cartridge, clean and easy process

Loaded SCAP cartridge containing ISTD, sample (whole blood or plasma), anticoagulant and / or esterase inhibitior

SCAP cartridge inserted in SCAP rack. Samples can be analyzed immediately or stored in freezer.

SCAP Extraction Process I

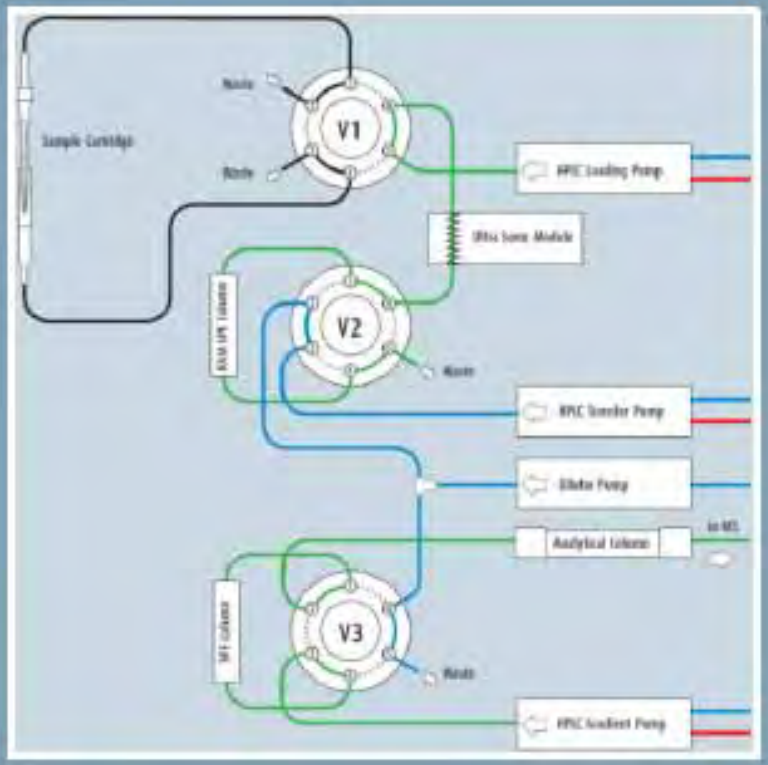
SCAP cartridge pick-up at cooled sample stack and transportation to clamp module

SCAP cartridge inserted into clamp station

Tip clamped into flow path, sample flushed out with mobile phase for sample preparation

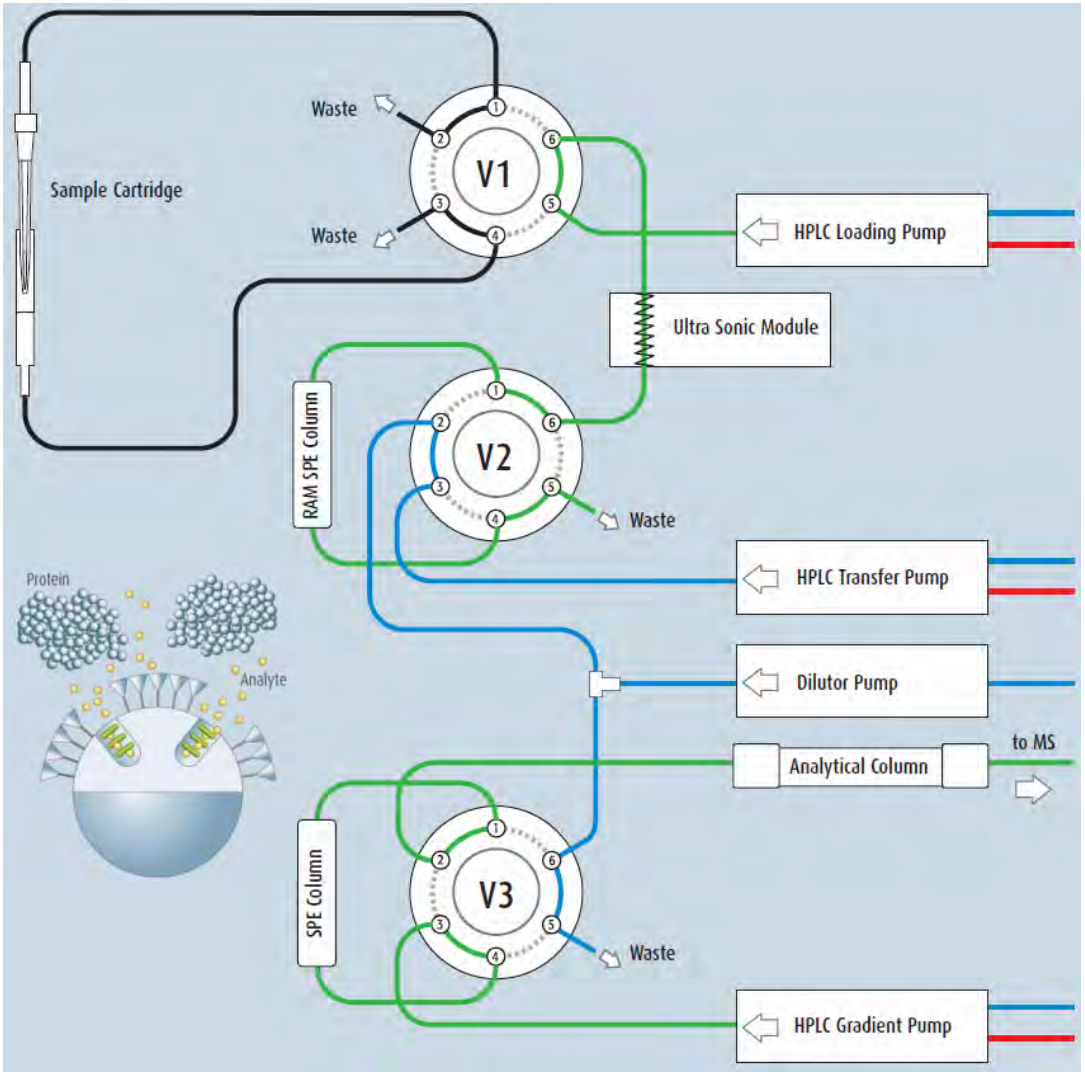
SCAP Extraction Process II

SCAP clamp module connected to Valve Module and Ultrasonic Module



Valve Switching diagram

Column and Valve Switching

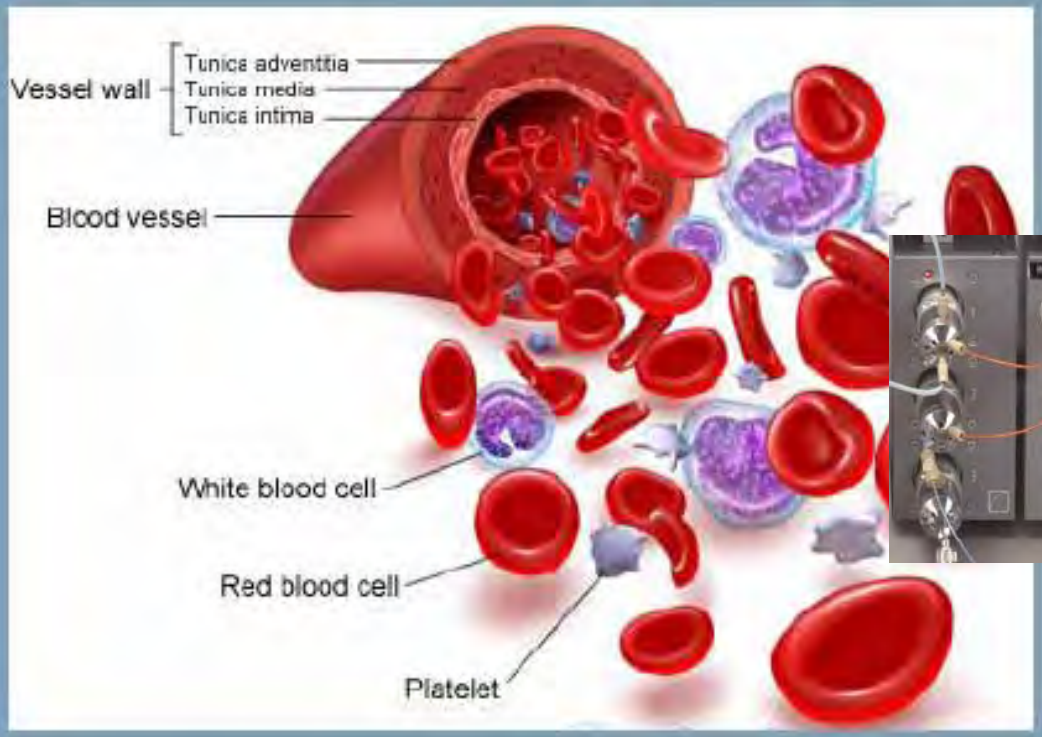


Valve 1
Sample loading out of Tip and Sonication

Valve 2
Extraction on RAM column and transfer to analytical column

Valve 3
Optional refocusing on SPE pre-column and separation on analytical column

SCAP Extraction Process Ultrasonic Step



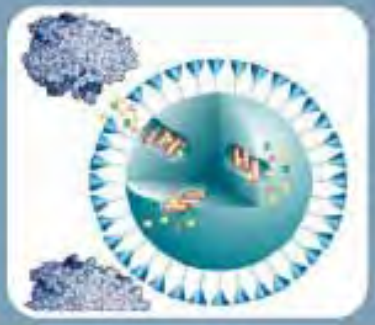
- Ultrasonic homogenizes the whole matrix to extend lifetime of the Extraction column (RAM or SPE pre-column)

SCAP Ultrasonic Step

- Used to disrupt blood cells
- Generates external sonic pressure waves into sample stream
- Pressure waves cause microbubbles formation which grow and collapse violently
- Implosion generates shock waves to break cell membranes and even break covalent bonds.

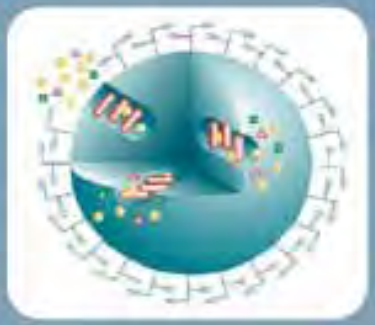
RAM Extraction Column

- Extraction and fractionation with RAM material is based on the simultaneous performance of two chromatographic processes: reversed phase/ion-pair chromatography and size exclusion chromatography.



Extraction of high molecular compounds

- Allows the direct extraction and enrichment of hydrophobic, low molecular analytes from untreated bio-fluid samples such as haemolysed blood, plasma, serum, supernatants of cell cultures and tissue.



Elution of low molecular sample compounds

- Fully automated preparation of the sample prior to the analytes being separated in the column. Untreated bio-fluid directly injected without negative effects on either the HPLC column or the separation results.

EXPERIMENTAL CONDITIONS

Calibration and Quality Control Samples:

Stock solutions at 1.00 mg/mL: all compounds were dissolved in acetonitrile and DMSO (9 + 1) and stored at – 80°C. Working solutions (stored at 6°C) were prepared in a mixture of methanol and water (1 + 1) and spiked to whole blood. Calibration ranges of 1.00 ng/mL to 4096 ng/mL for Bosentan and 2.00 ng/mL to 512 ng/mL for the three measured metabolites were used. Bosentan and the three measured metabolites were obtained from Toronto Research Chemicals.

Mass Spectrometry:

Mass spectrometer: MDS SCIEX API 4000
Positive electrospray ionization (needle voltage 5000 V, orifice 60 V and collision energy 40 V)
Curtain gas: 20, Gas 1: 40, Gas 2: 50, ion source temperature: 500°C
Analyst software, version 1.4.1 (all HLPC components were controlled by contact closure)

The following SRM transitions were used for the experiments:
Bosentan and the three metabolites

Q1 mass	Q3 mass	Dwell time	
552.3	202.1	80 msec	(Bosentan)
538.3	189.1	80 msec	(Desmethyl Bosentan)
568.3	202.1	80 msec	(Hydroxy Bosentan)
554.3	189.2	80 msec	(Hydroxy Desmethyl Bosentan)

ISTDs for Bosentan and the three metabolites (d4-labeled)

556.3	202.1	80 msec
542.3	189.1	80 msec
572.3	202.1	80 msec
558.3	189.2	80 msec

HPLC Conditions:

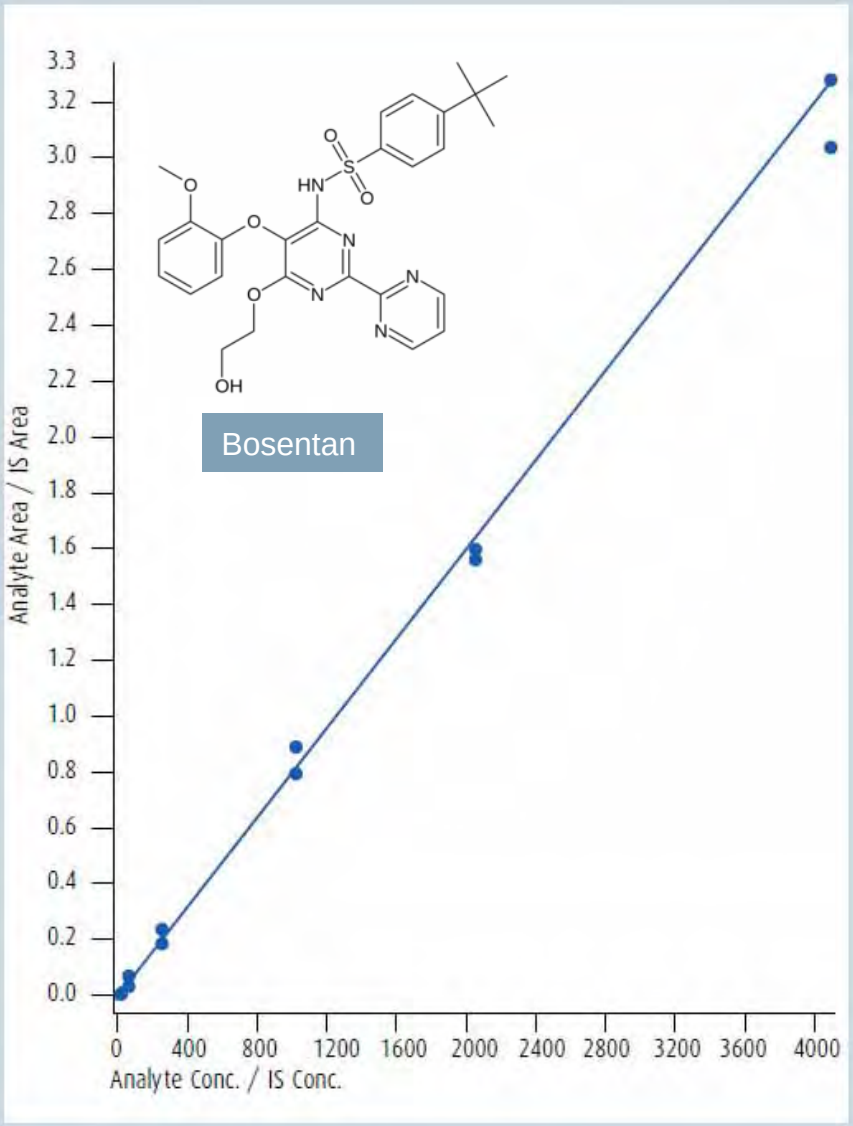
HPLC: Shimadzu 10ADvp pumps for the high pressure gradient on the analytical column and Merck 6200 and 6000 pumps for injecting / loading, trapping and transferring the samples.
Mobile Phase A: H_2O + HCOOH + NH_3(aq.), (100 + 1 + 0.05)
Mobile Phase B: MeCN + MeOH + HCOOH + NH_3(aq.), (50 + 50 + 1 + 0.05)
HPLC column 1: 2.0 x 25 mm, LiChrospher ADS (RAM), RP-18, 25 um, Merck
HPLC column 2: 2.0 x 20 mm, C18, 5 um, Clipeus, Higgins Analytical
HPLC column 3: 2.0 x 50 mm, C6-Phenyl, 3um, Phenomenex

WORKFLOW

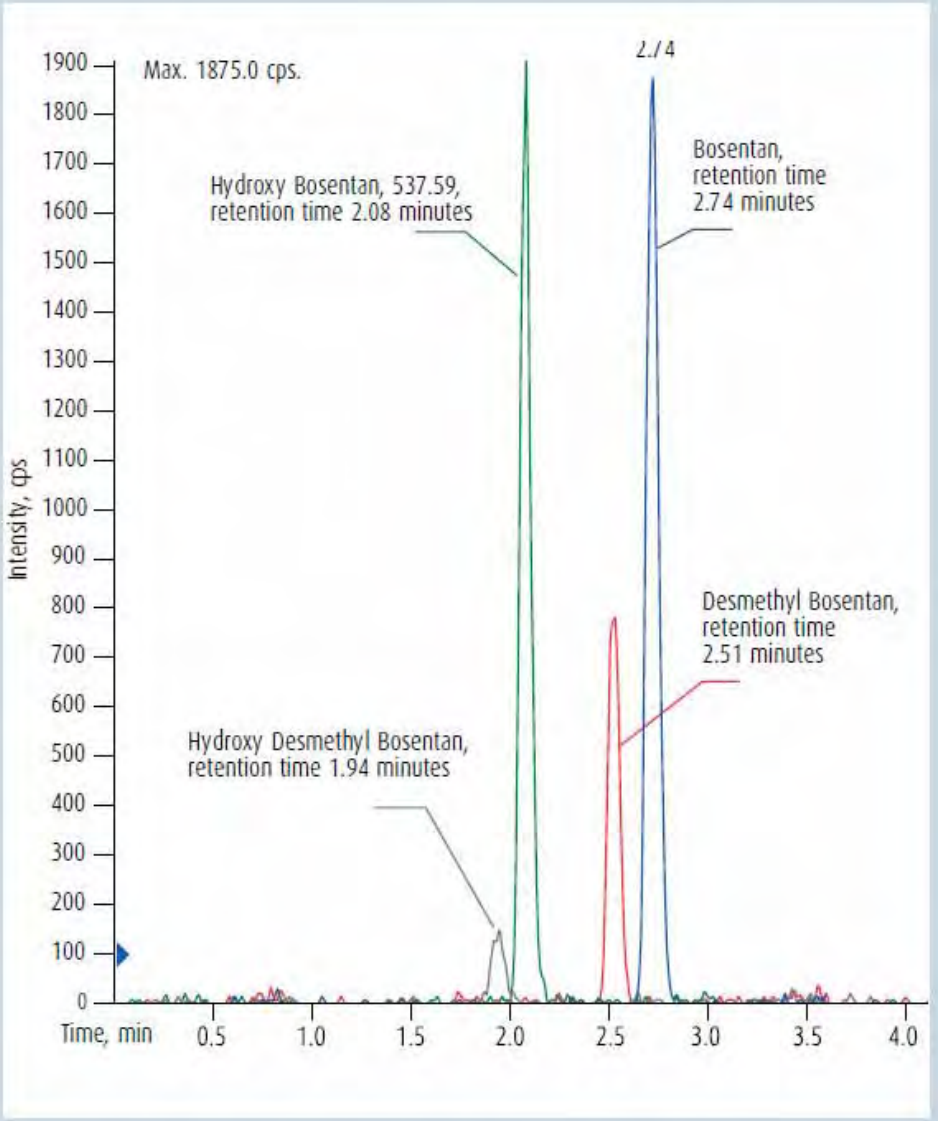
A modified electronic pipette (RAININ EDP 3) which is capable of multiple aspiration steps is used for the following procedure:

1) Take a 10 uL sample tip with the multiple aspiration pipette
2) Aspirate 1 uL of internals standard solution containing EDTA
3) Aspirate 5 uL of calibration or quality control sample
4) Aspirate 1 uL of internal standard solution containing EDTA
5) Close the sample tip with a cap and store in autosampler or freezer

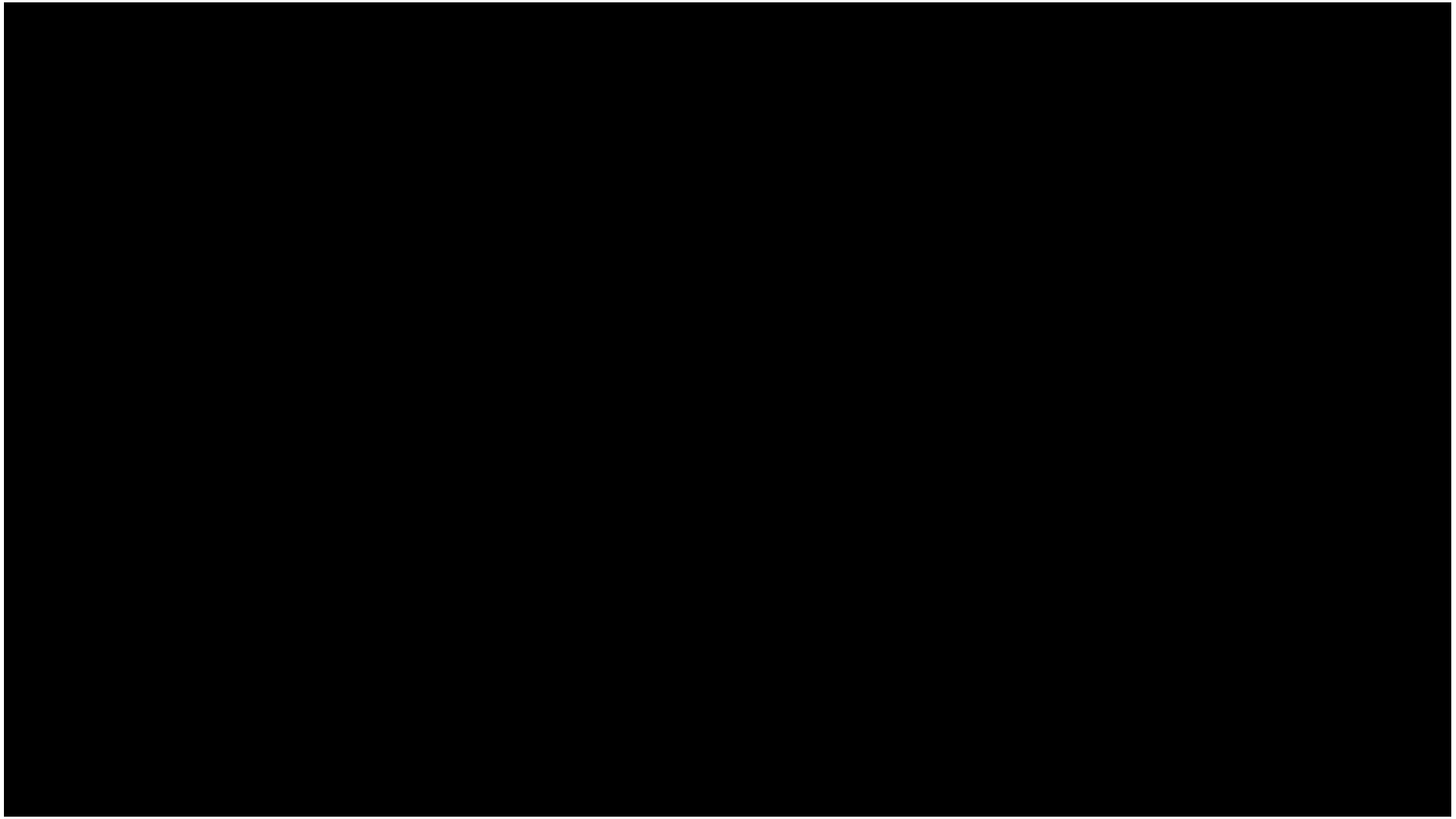
SCAP Application Example



Calibration curve for Bosentan (r = 0.9979)



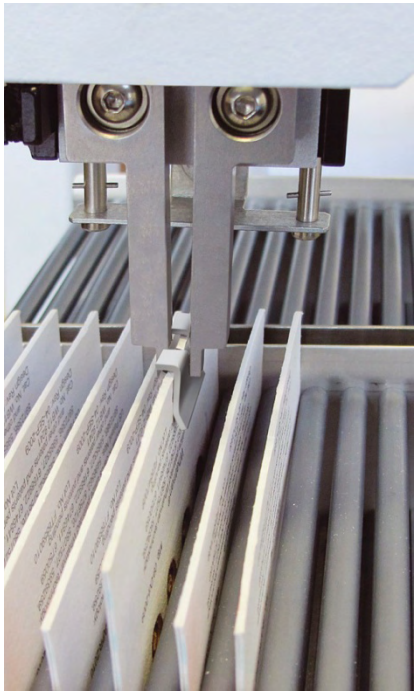
LLOQ for Bosentan and metabolites

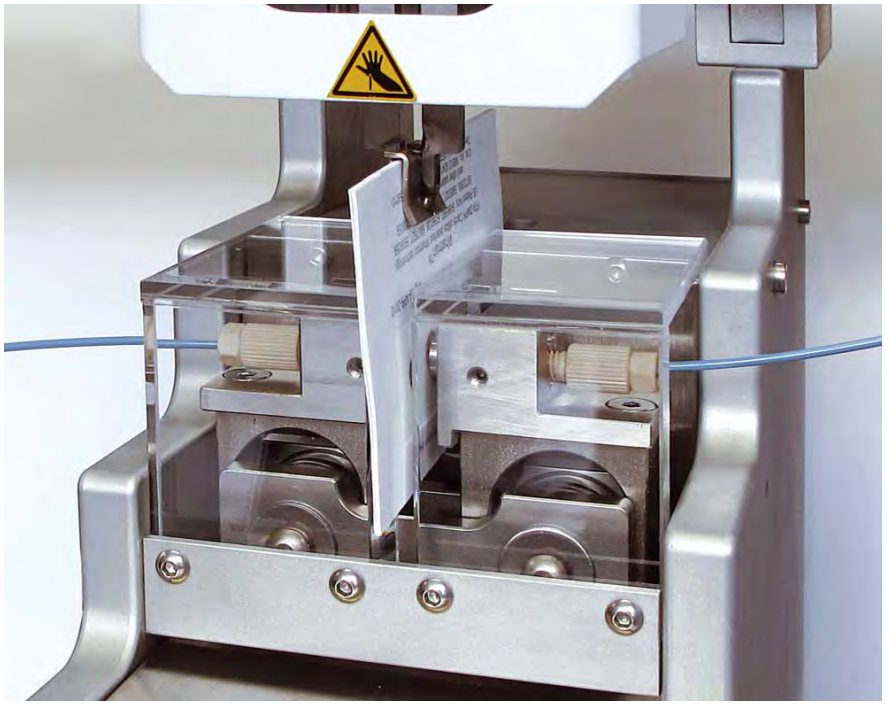


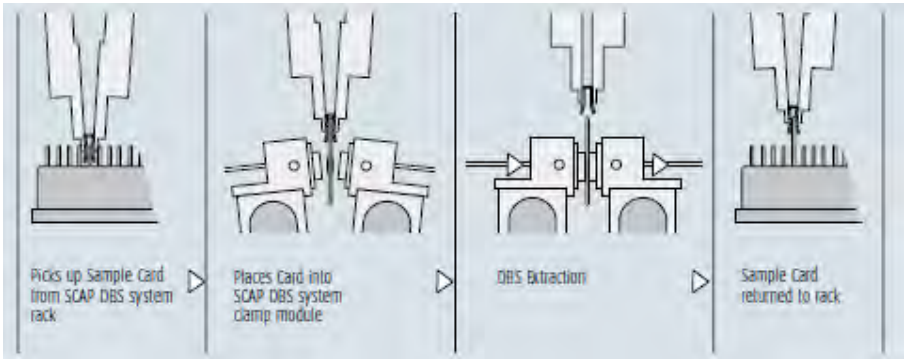
scap

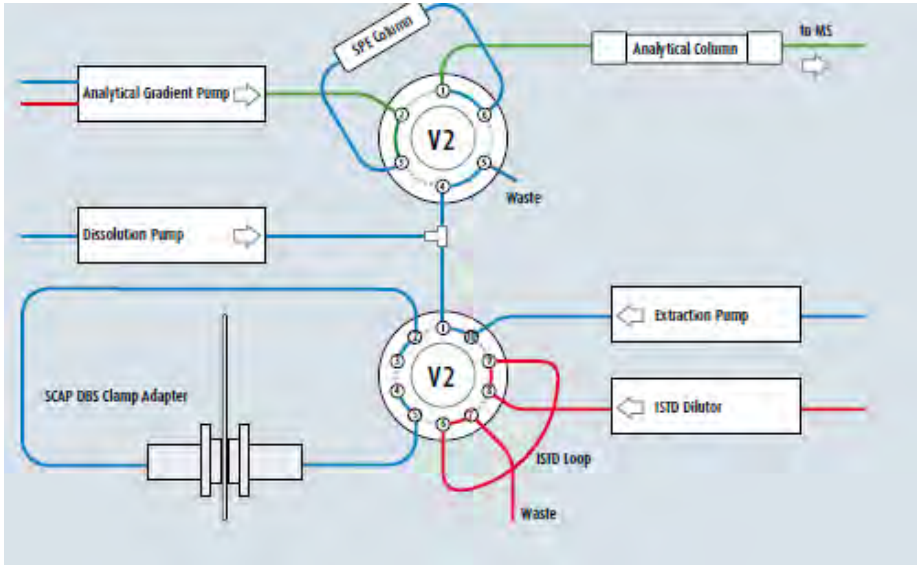
sample cartridge and prep system

DBS

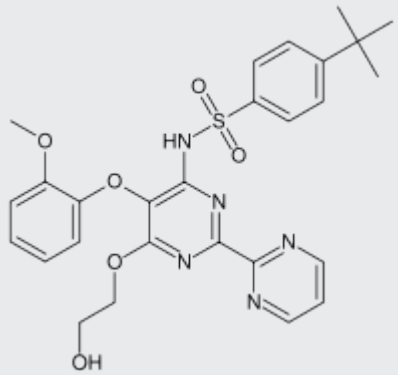





Picks up Sample Card from SCAP DBS system rack
Places Card into SCAP DBS system clamp module
DBS Extraction
Sample Card returned to rack


SPE Column
Analytical Column
to MS
Analytical Gradient Pump
V2
Waste
Dissolution Pump
Extraction Pump
SCAP DBS Clamp Adapter
V2
ISTD Dilutor
ISTD Loop
Waste

Fully-Automated LC-MS/MS-Based Quantification of Bosentan and its Metabolites in Dried Blood Spots using the DBS SCAP System



Inovalab
Advanced Technology Services Laboratory

Straightforward Sample Preparation Procedure

- Pipetting blood spots (25 µL) containing test items onto FTA DMPK-A / C Cards (Whatman)
- 2 hours of drying at room temperature
- Samples are ready for analysis (no manual punching and extraction necessary)

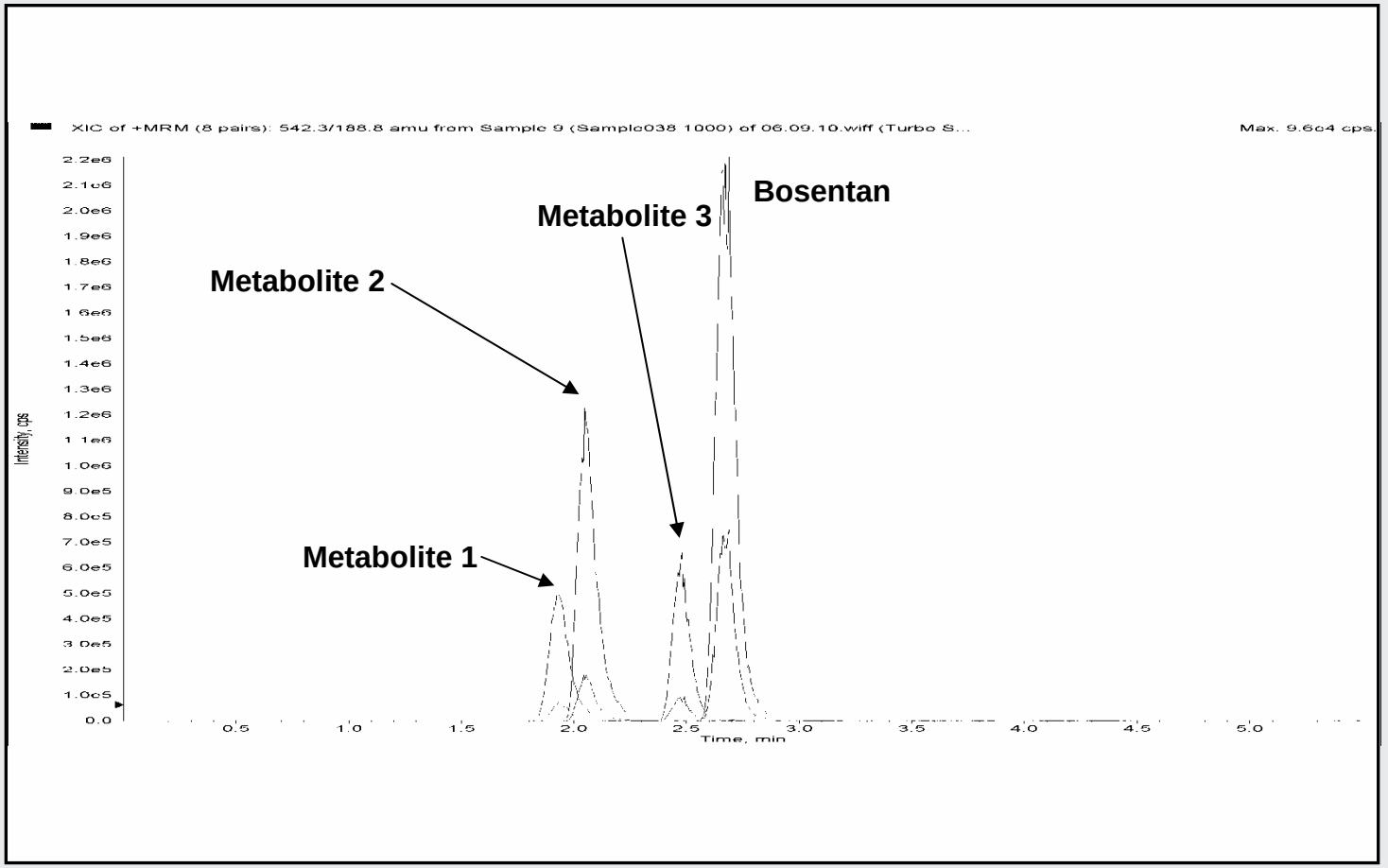
Inovalab
Advanced Technology Services Laboratory

LC-MS/MS Analysis

- DBS cards are stored in the autosampler rack
- Fully-automated sequential introduction of DBS cards into the LC flow path by robotics of the DBS SCAP System
- Online extraction of analytes and automated addition of ISTD
- Trapping of the analytes on the pre column
- Elution of analytes onto the main column and subsequent chromatographic separation
- MS detection of analytes (MRM mode) using a MDS Sciex API 5000

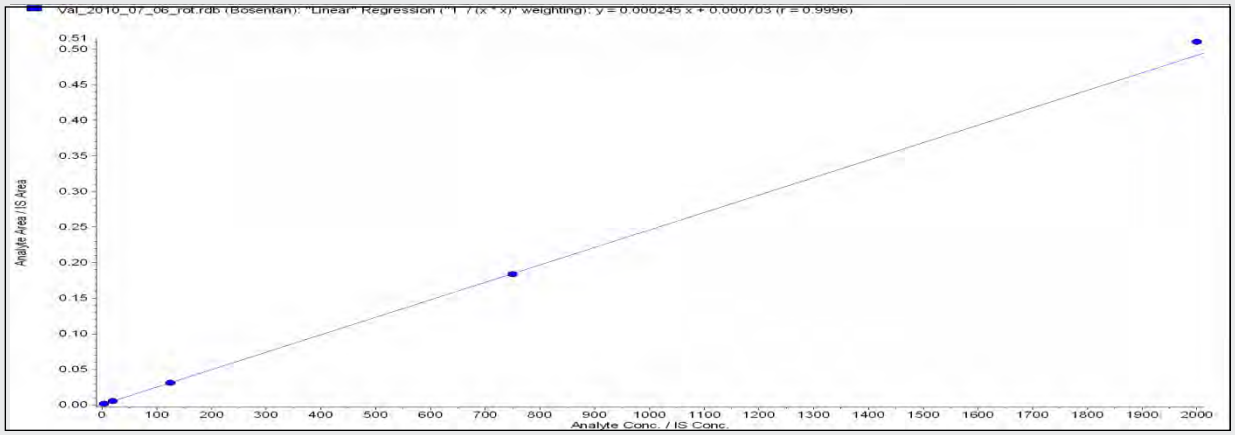
Inovalab
Advanced Technology Services Laboratory

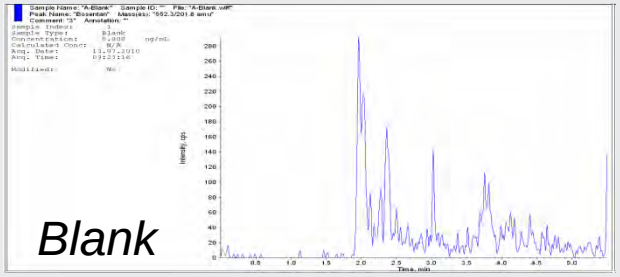
LC-MS/MS Chromatograms of Analytes and ISTDs

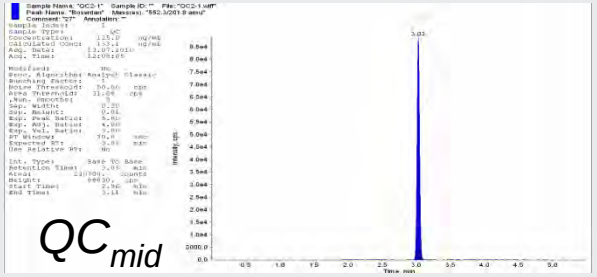


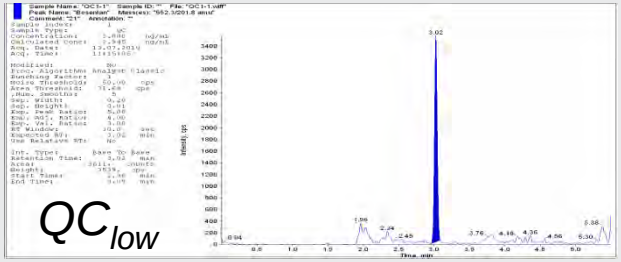
Inovalab
Advanced Technology Services Laboratory

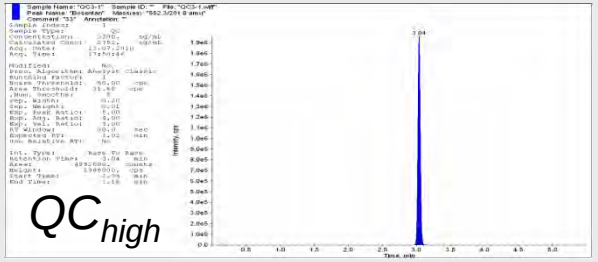
Linearity - Calibration Curve of Bosentan





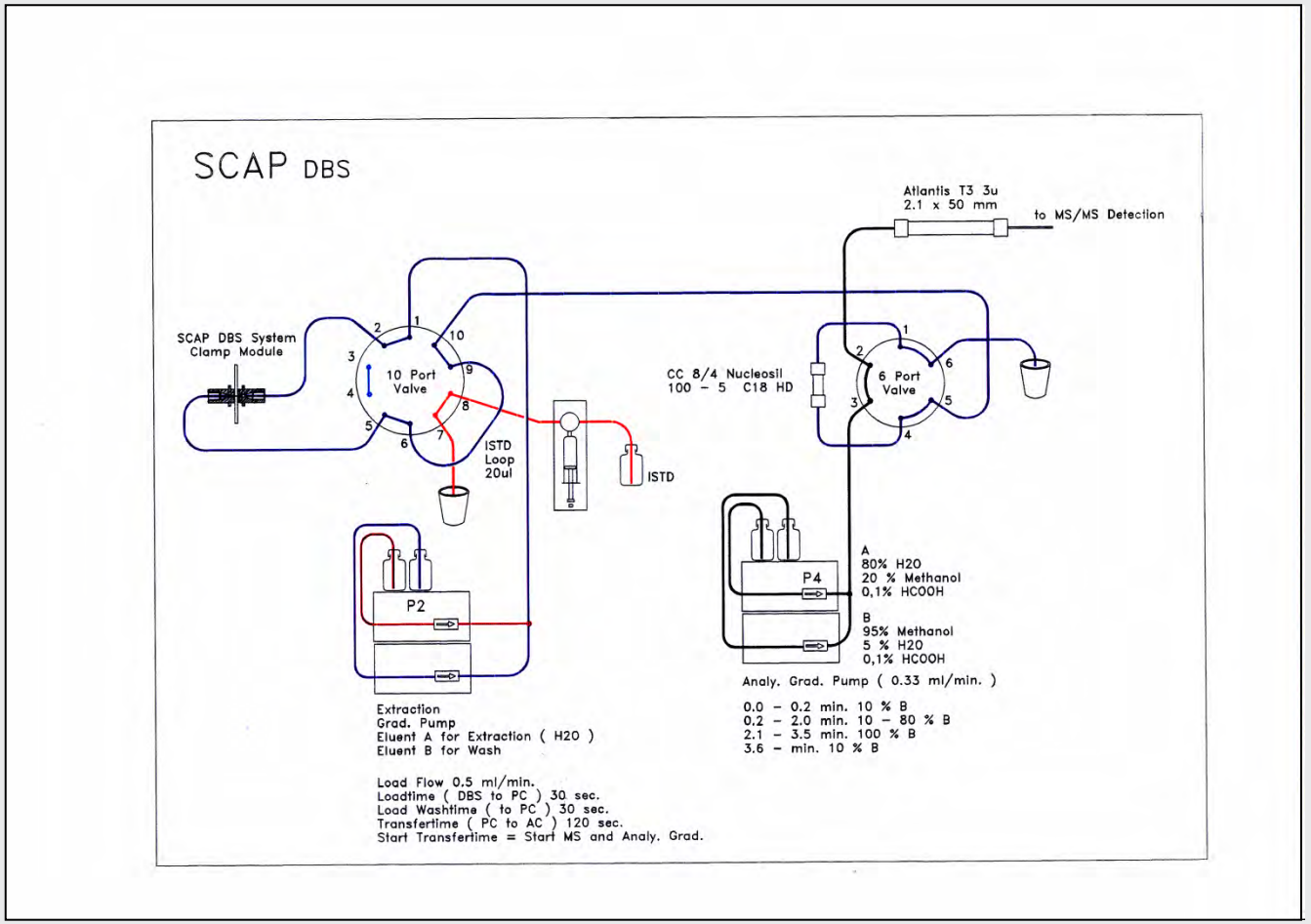






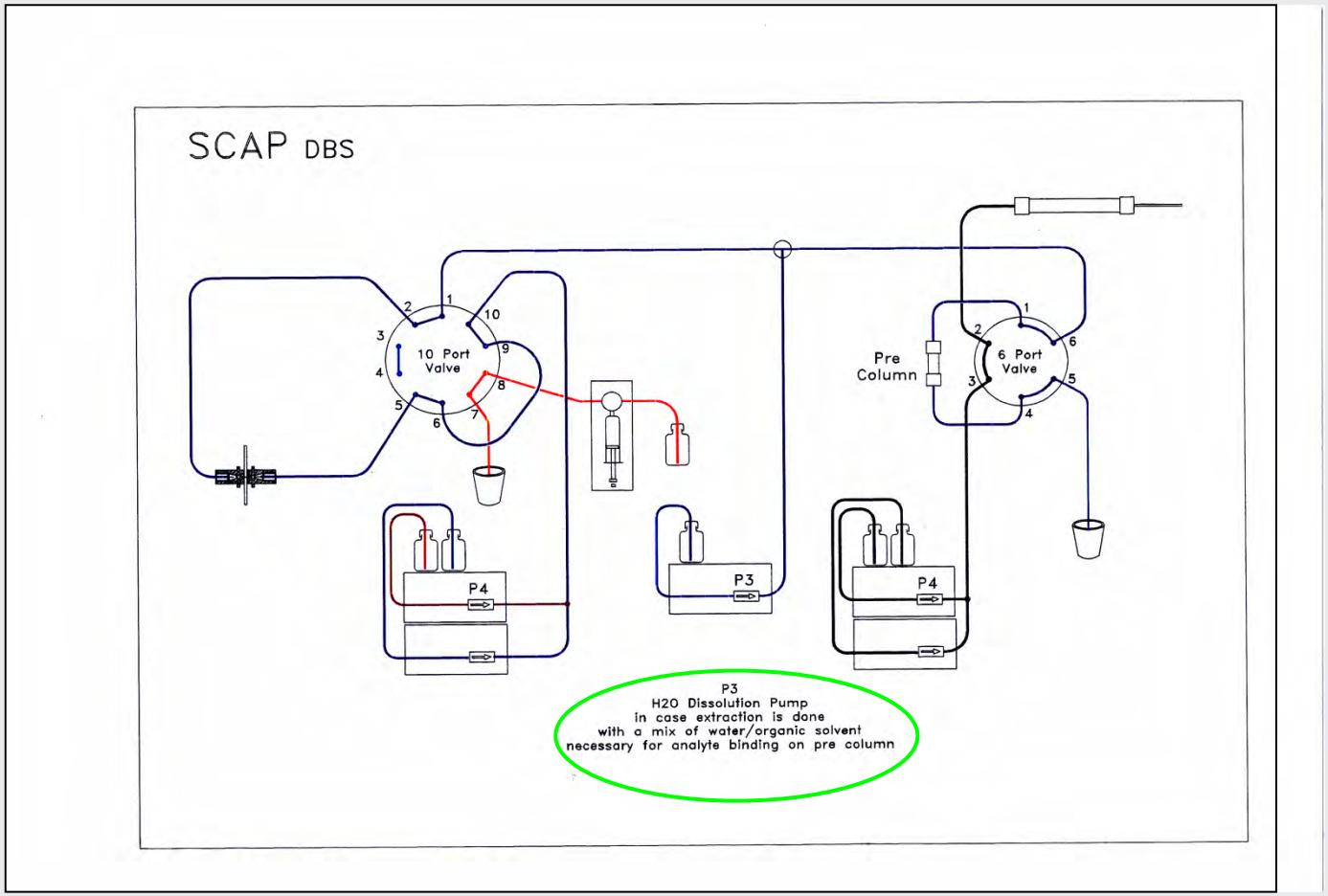
Inovalab
Advanced Technology Services Laboratory

SCAP DBS Flow Path



Inovalab
Advanced Technology Services Laboratory

SCAP DBS Flow Path with Dissolution Pump (P3)



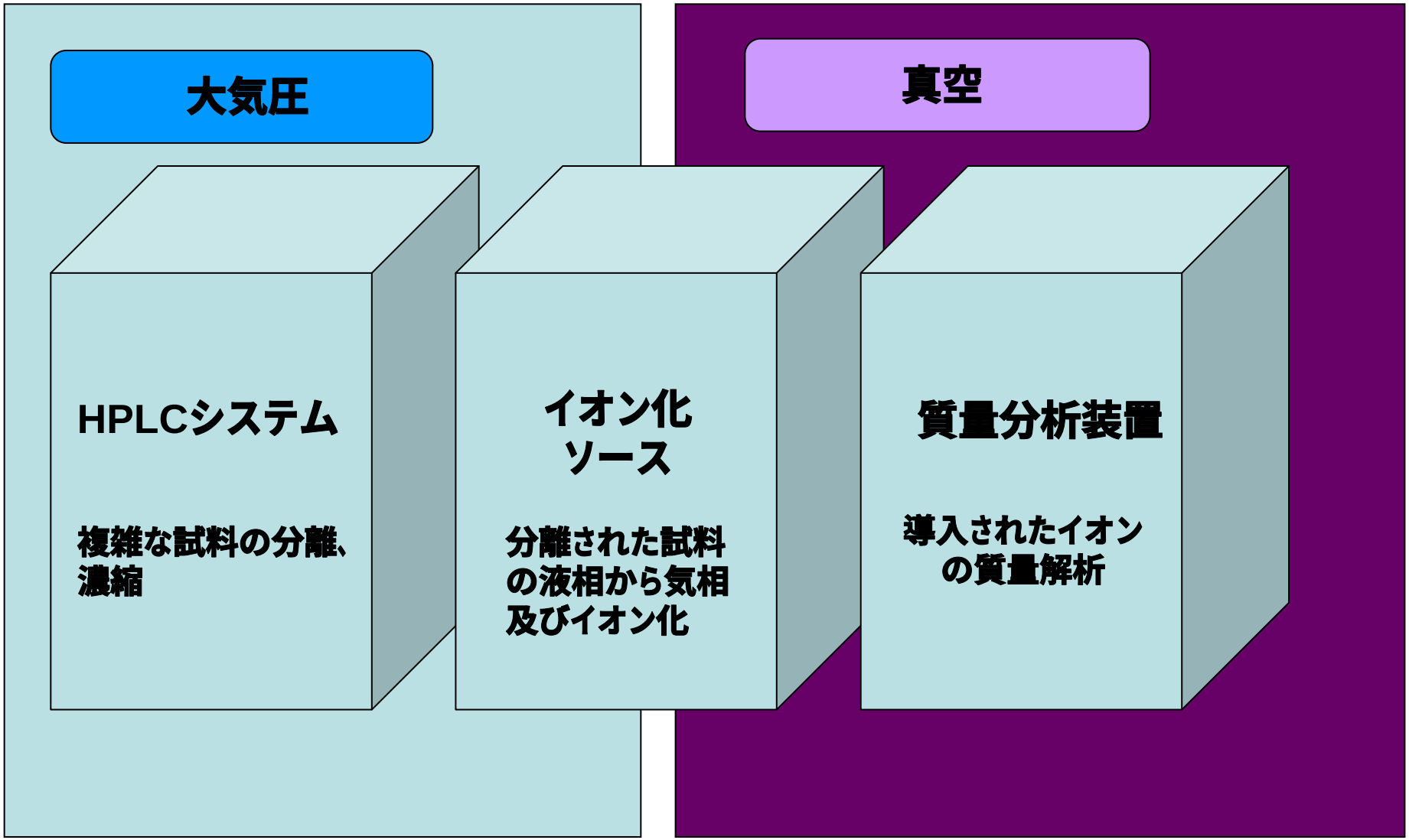
Inovalab
Advanced Technology Services Laboratory

Summary

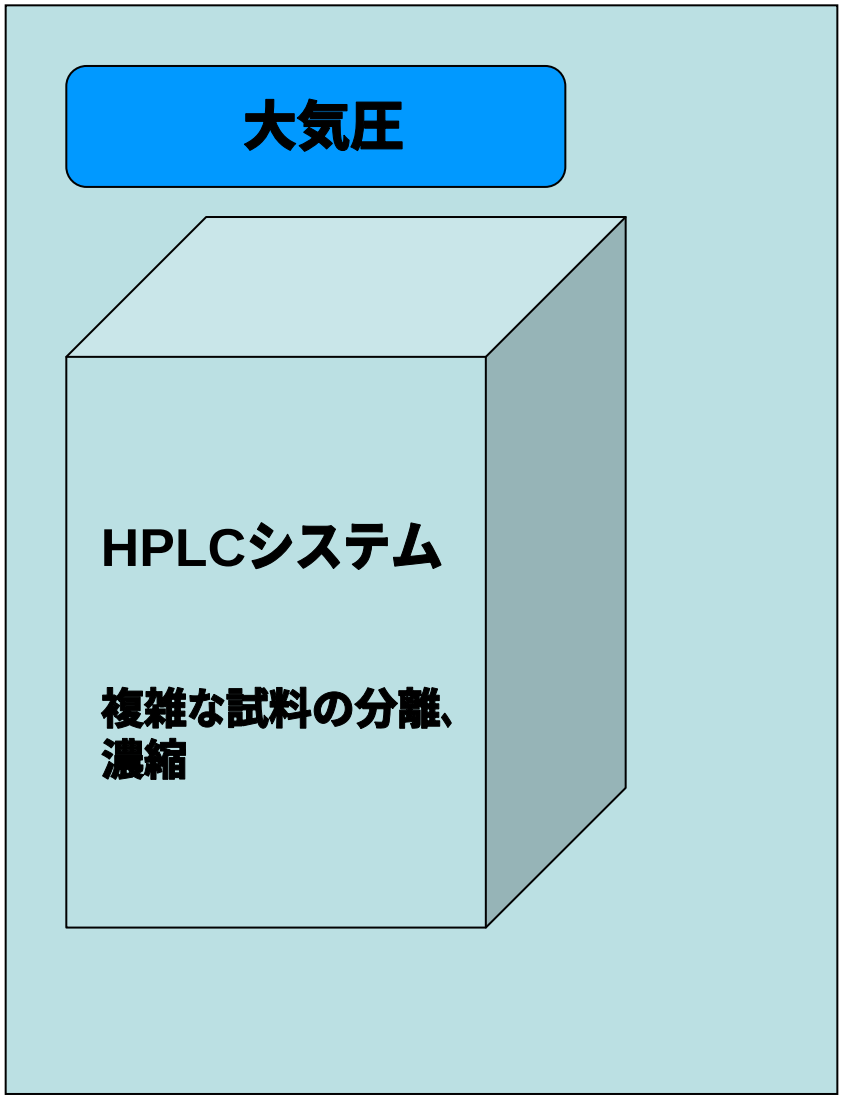
- Very simple sample prep compared to traditional DBS analysis
- DBS SCAP System enables **fully-automated** analysis of dried blood spots
- Linear range from 2.00 (5.00) ng/mL to 1500 ng/mL for Bosentan and its metabolites

Inovalab
Advanced Technology Services Laboratory

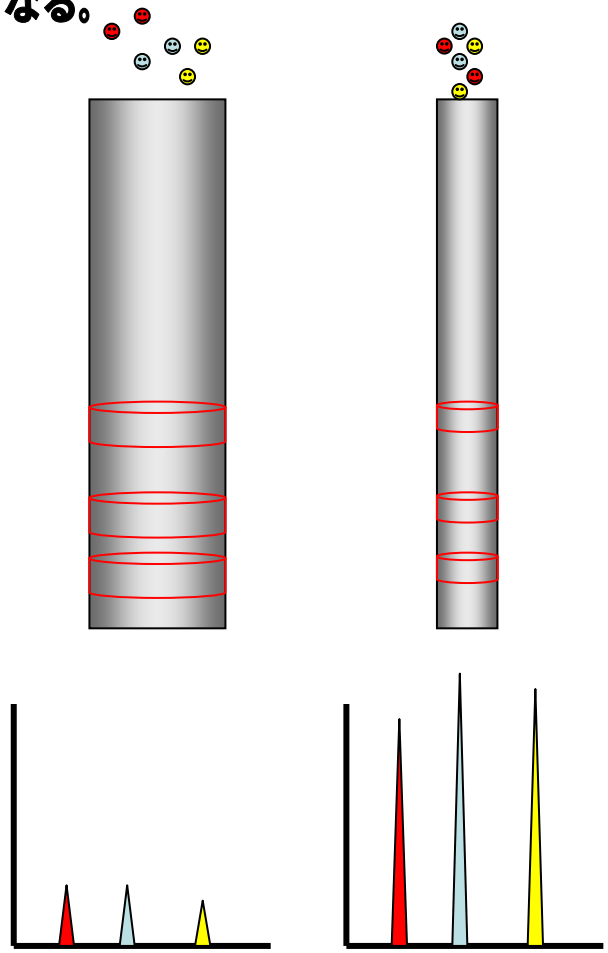
LC/MSのシステム構成



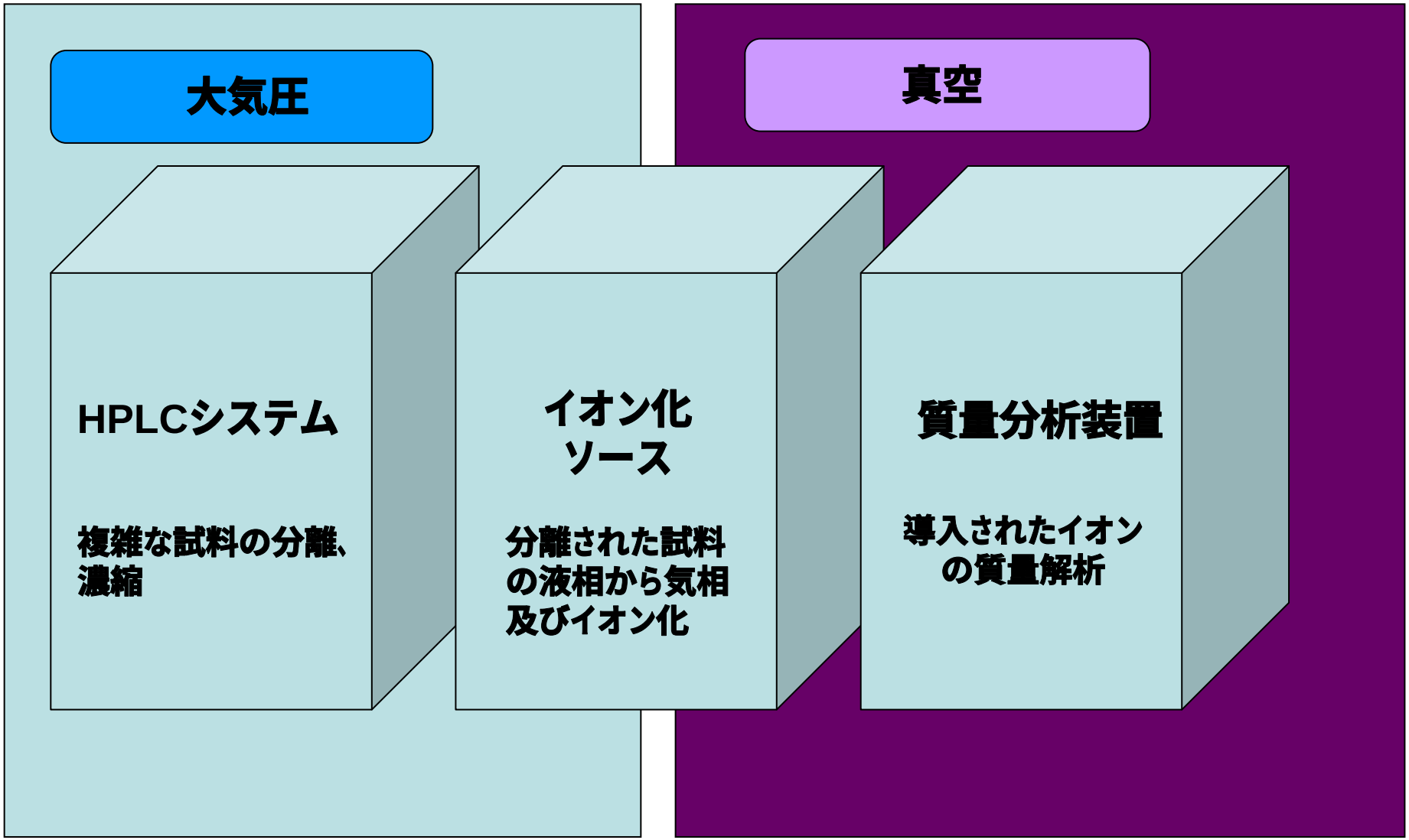
超高感度にするには？



カラムから溶出されるサンプルの検出はその濃度に依存する。単位面積当たりの流速（線速）を統一するとカラム断面積の細い方が濃度が高くなる。



LC/MSのシステム構成

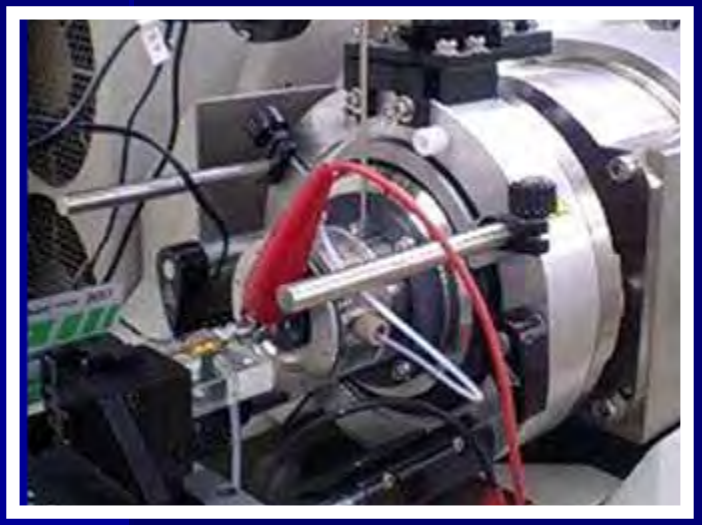


Conventional-type

Unbiased ESI インターフェース

- カラムダイレクト接続
- シンプル構造
- 密閉構造

Closed type



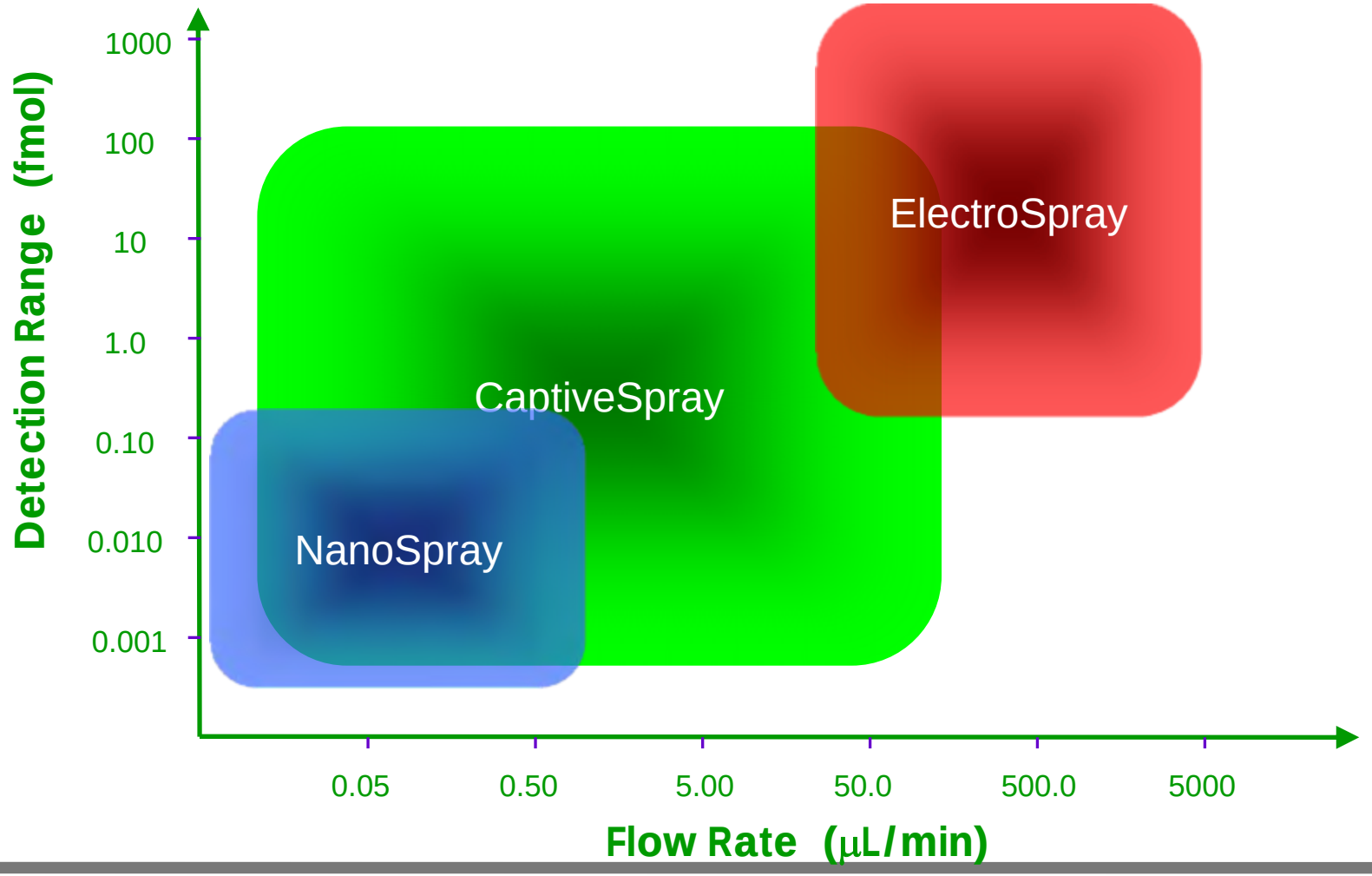
AD-H6(AMR, Inc.)

Thermo Scientific

AB Sciex

Captive Spray™(Michrom Bioresources, Inc.)

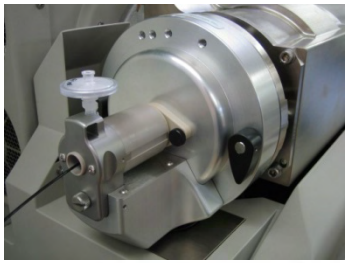
CaptiveSpray Bridges the Gap



Advance CaptiveSpray Source

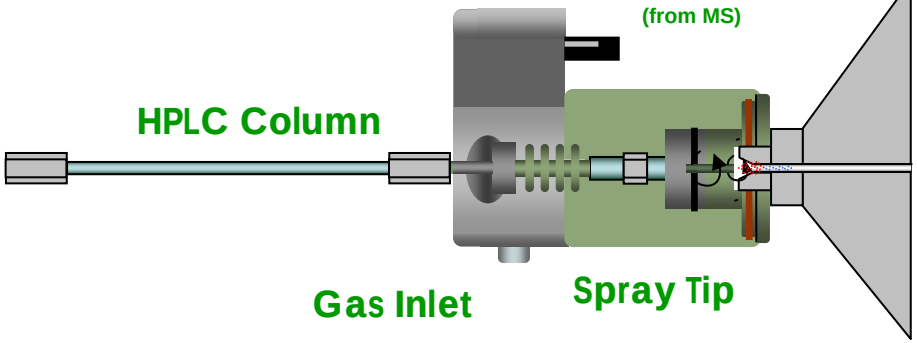
イオンソース

SCIEX

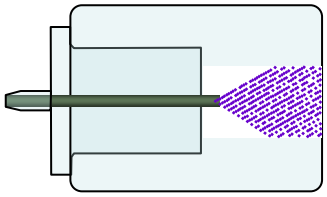


THERMO

BRUKER

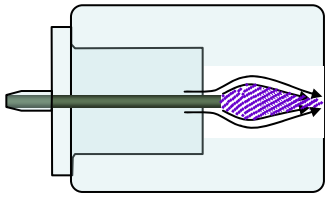


CaptiveSpray Operation



Conventional Spray

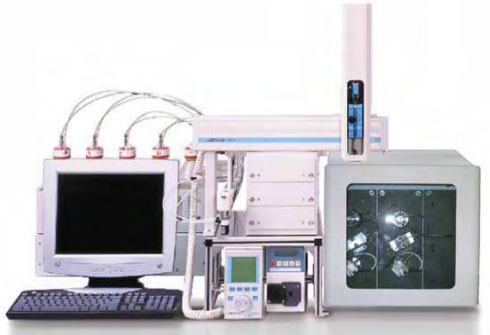
Unfocused spray from the emitter allows some ions into the MS.



CaptiveSpray

Gas vortex around the spray concentrates and focuses ions into the MS.

ADVANCE Nanoflow UHPLC system



10000psiまで対応する高圧仕様のナノHPLC

ダイレクトシリンジポンプによるスプリットレス送液

フローセンサーによる流量制御

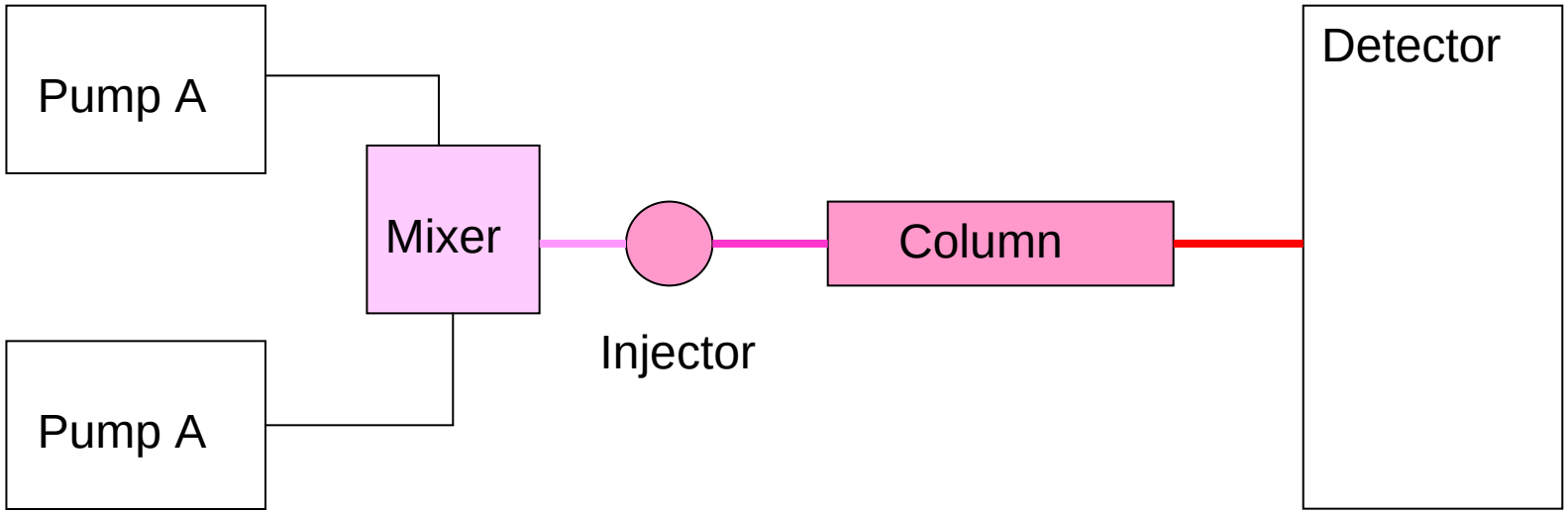
デッドボリュームが少ないのでグラジエントディレイが少ない

さまざまなカラムスイッチングが高圧仕様でも可能

高圧でもキャリーオーバーの少ないオートサンプラー仕様

2-Pump Binary
2-Pump + 1 Valve Binary
2-Pump + 2 Valves Binary
2-Pump + 1 Valve + Loading Pump Binary
3-Pump Ternary

Gradient Elution System Volume

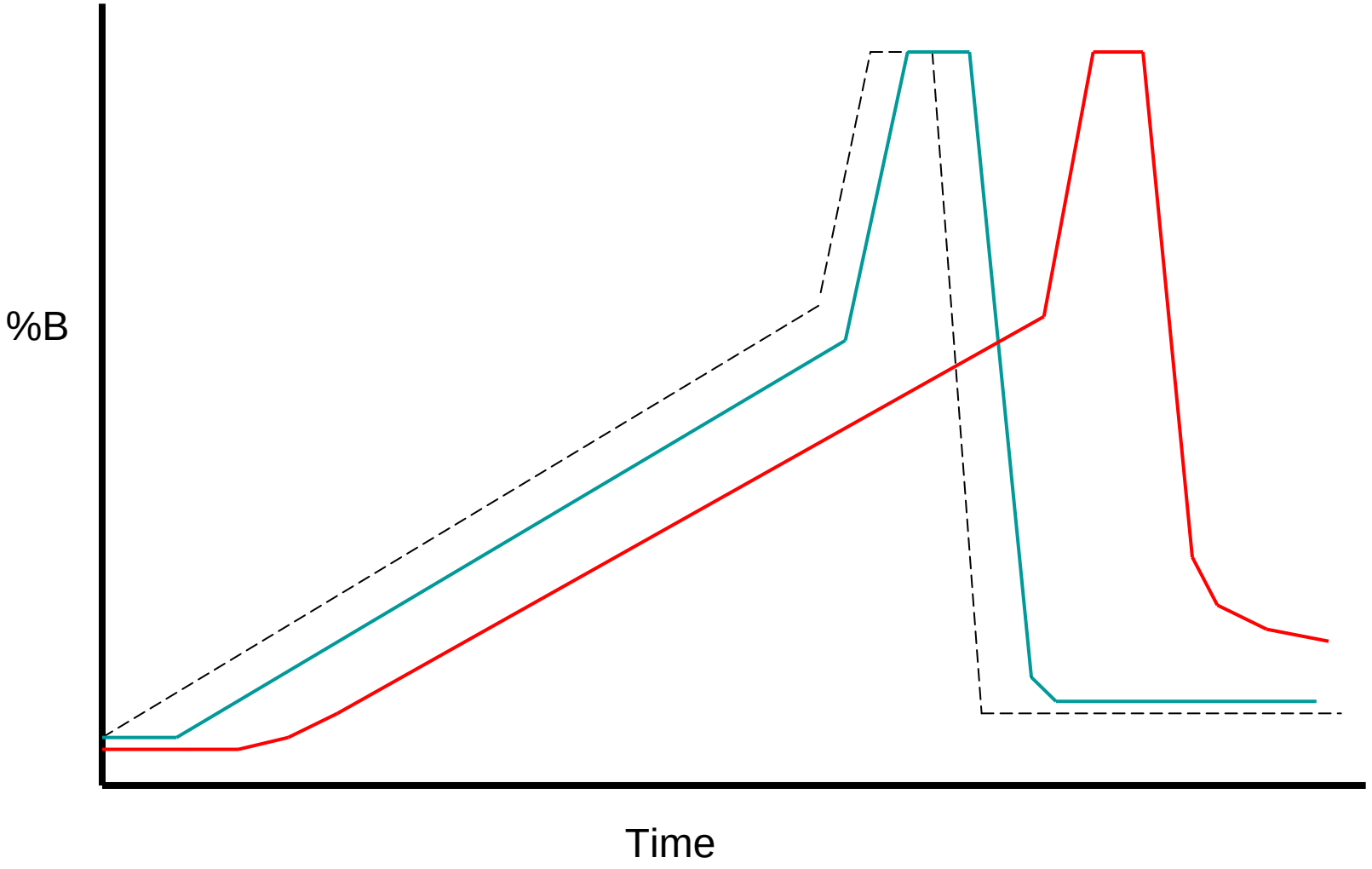


Critical Volume (Must be as low as possible and well swept)

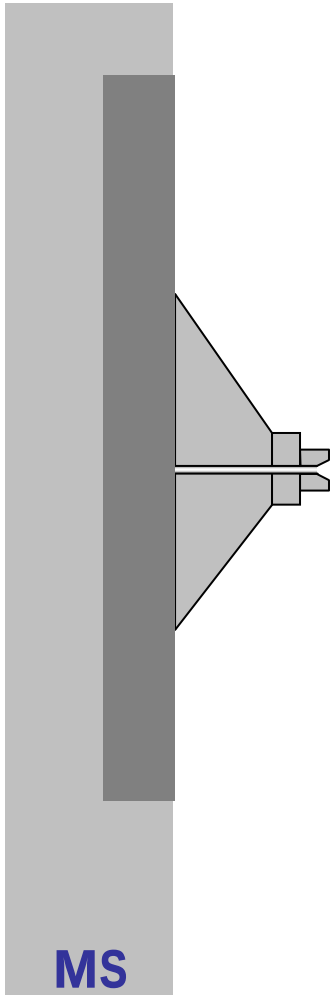
T_0 Volume (Defines the gradient run time required)

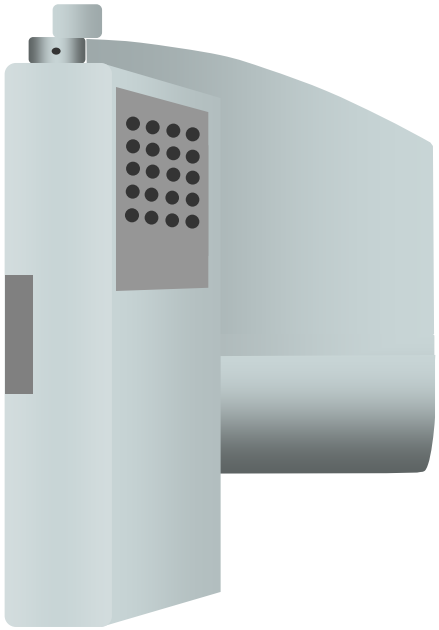
Gradient Delay Volume (Impacts throughput and MS DC)

Impact of Different System Volumes



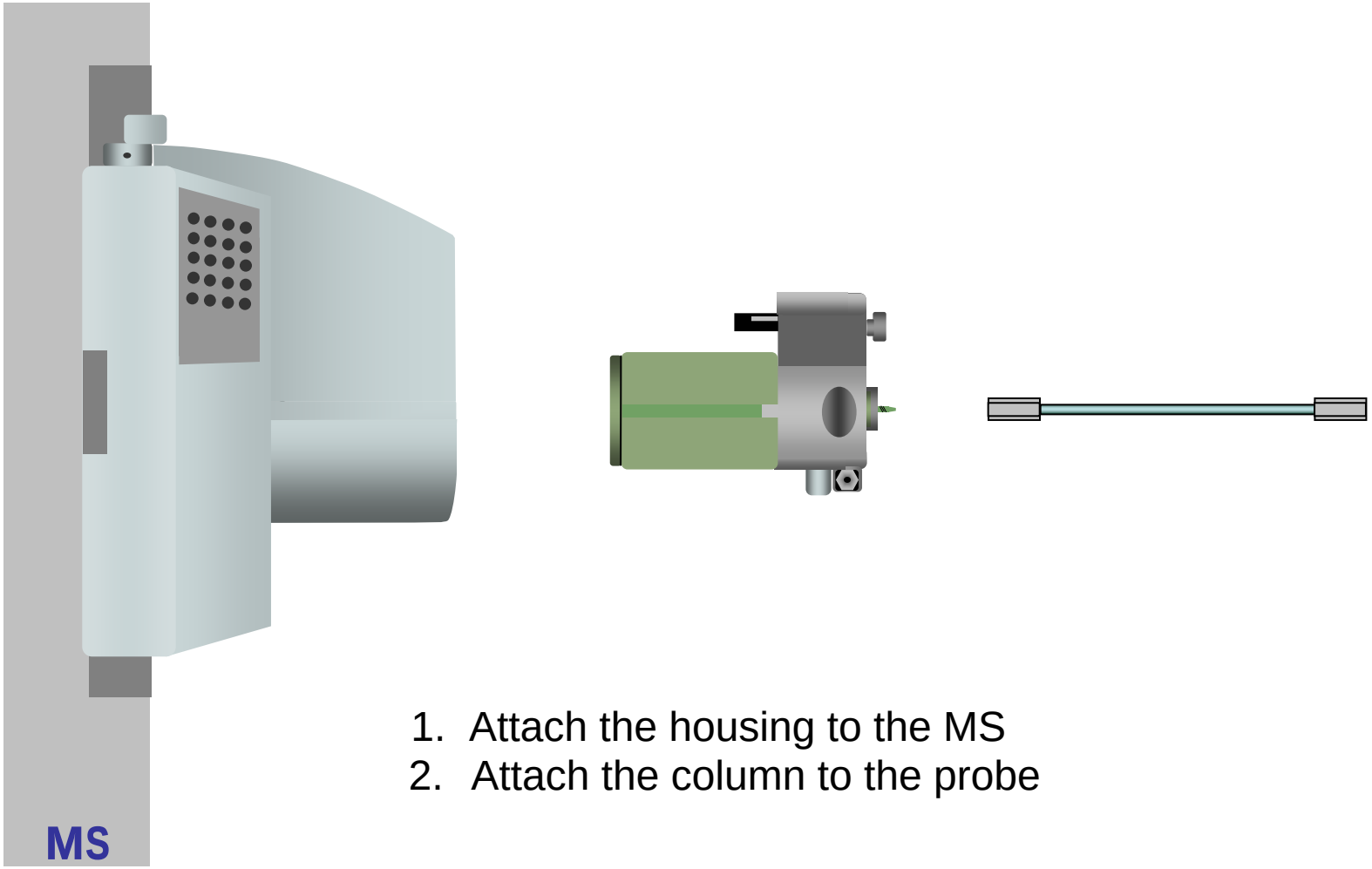
CaptiveSpray Installation





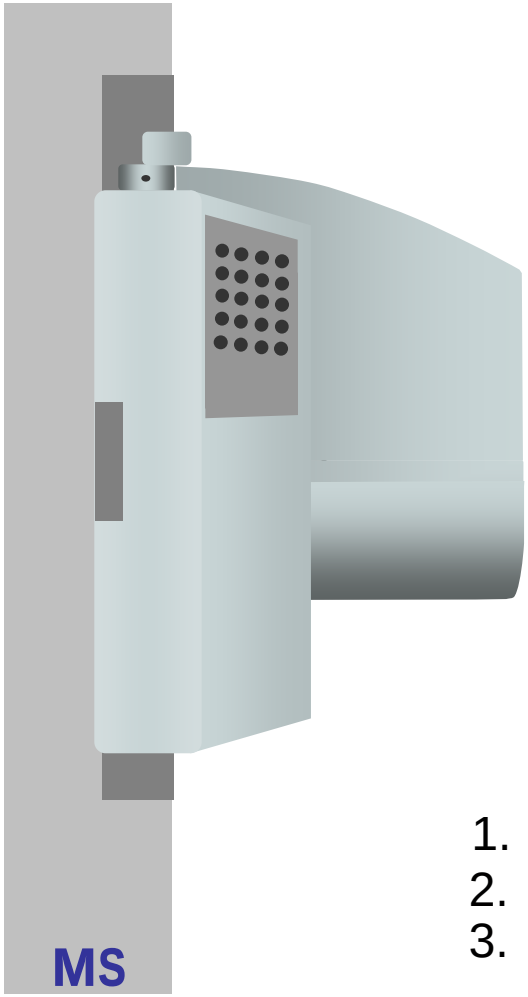
1. Attach the housing to the MS

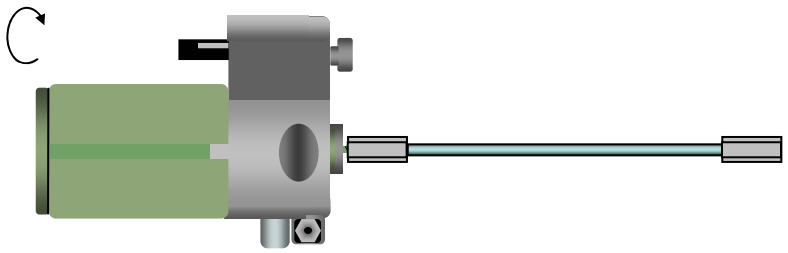
CaptiveSpray Installation



1. Attach the housing to the MS
2. Attach the column to the probe

CaptiveSpray Installation

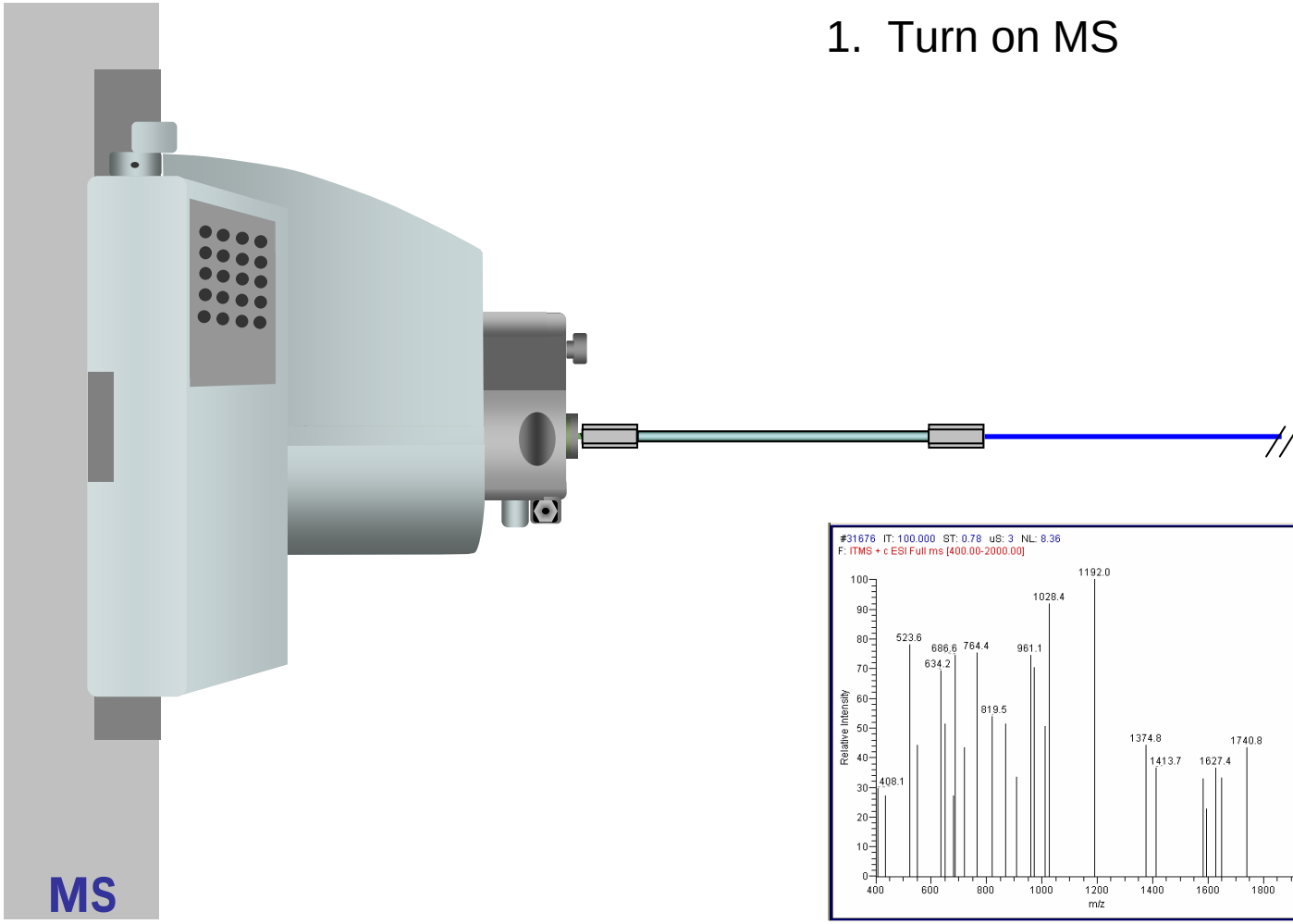


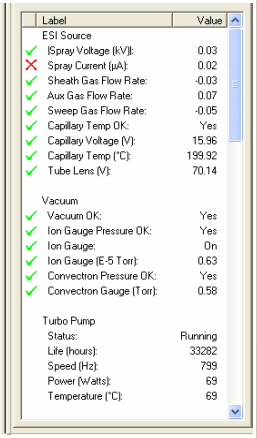


1. Attach the housing to the MS
2. Attach the column to the probe
3. Insert probe into housing, and secure with set screw

CaptiveSpray Operation

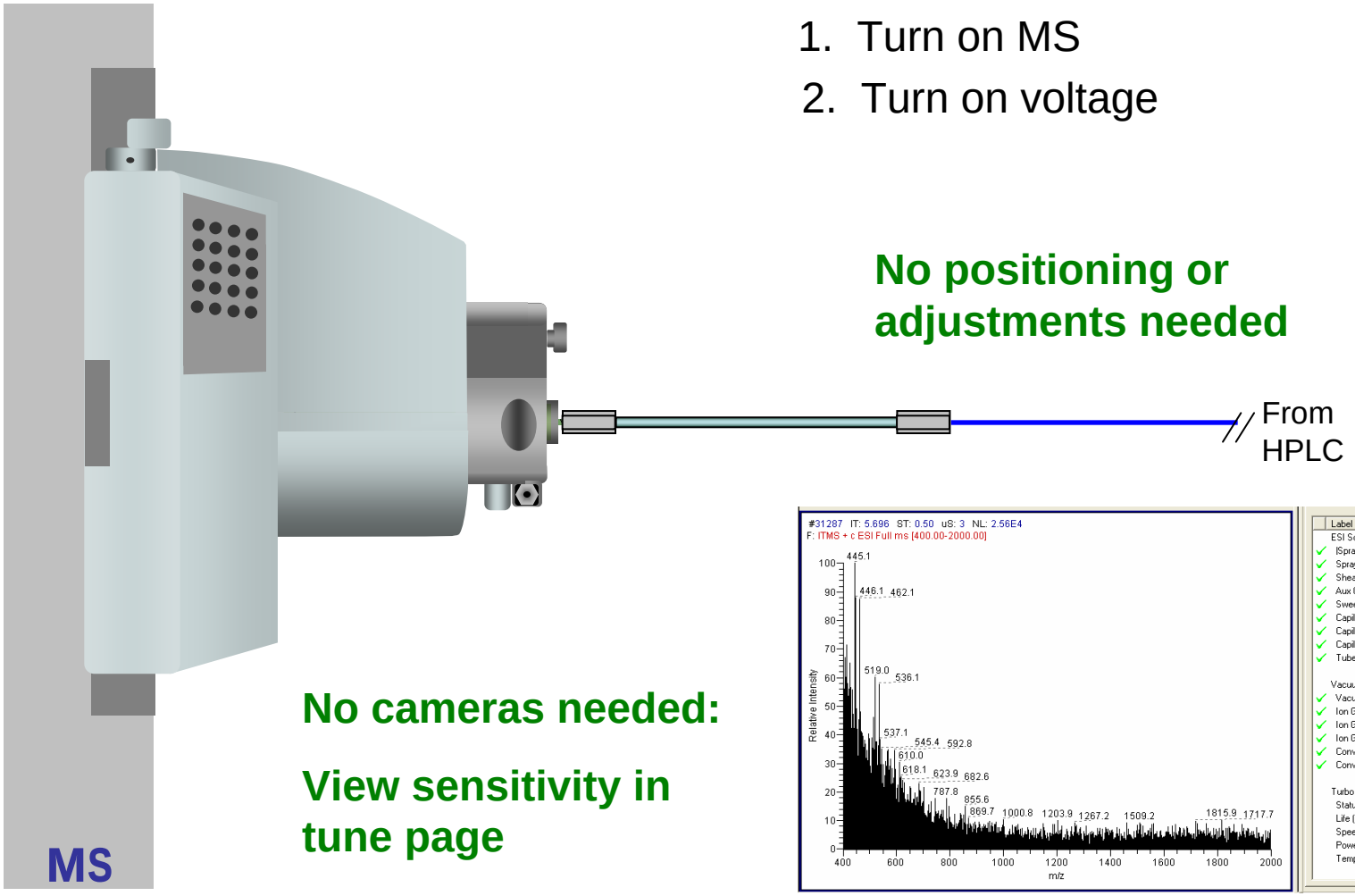
1. Turn on MS





CaptiveSpray Installation

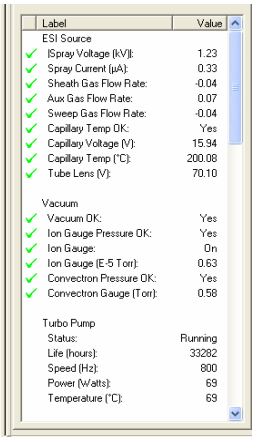
1. Turn on MS
2. Turn on voltage



No positioning or adjustments needed

No cameras needed:

View sensitivity in tune page



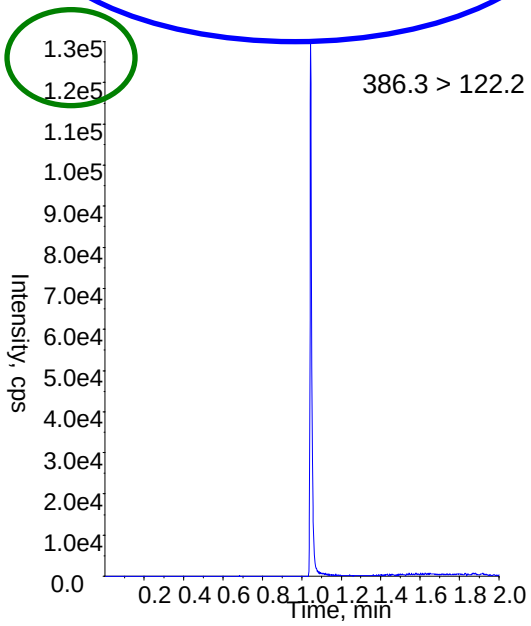
Chromatographic Conditions

Parameter	Analytical Flow Runs	Micro Flow Runs	Capillary Flow Runs
LC System	Paradigm MS2-MA	Paradigm MS2-NC	Advance UHPLC
LC Autosampler	Advance Bio-Cool AS	Advance Bio-Cool AS	Advance Bio-Cool AS
LC Column	2x50mm Halo C18	0.5x50mm Halo C18	0.2x50mm Halo C18
Flow (Pressure)	500 µl/min (3350 psi)	32 µl/min (3500 psi)	5 µl/min (3450 psi)
Linear Velocity	2.5 mm/sec	2.5 mm/sec	2.5 mm/sec
Solvent A	0.1% HCOOH in H_2O	0.1% HCOOH in H_2O	0.1% HCOOH in H_2O
Solvent B	Acetonitrile	Acetonitrile	Acetonitrile
Gradient	10-90%B in 90 sec	10-90%B in 90 sec	10-90%B in 90 sec
Total Run Time	2.5 minutes	2.5 minutes	2.5 minutes
MS System	4000 Q-Trap	4000 Q-Trap	4000 Q-Trap
MS Source	Turbo-V ESI	µ Turbo-V ESI	Advance CSI
Buspirone MRM	386.3 → 122.2	386.3 → 122.2	386.3 → 122.2
Spray Voltage	4500	4500	1400
Source Temp	350 ºC	350 ºC	N.A.
Gas Flows	10/20/2020	10/20/2020	0/0/0

CaptiveSpray Increases Sensitivity

ElectroSpray Ionization (ESI)

Sensitivity = 130 cps/pg



1000 pg Buspirone – ESI

100 µL injection

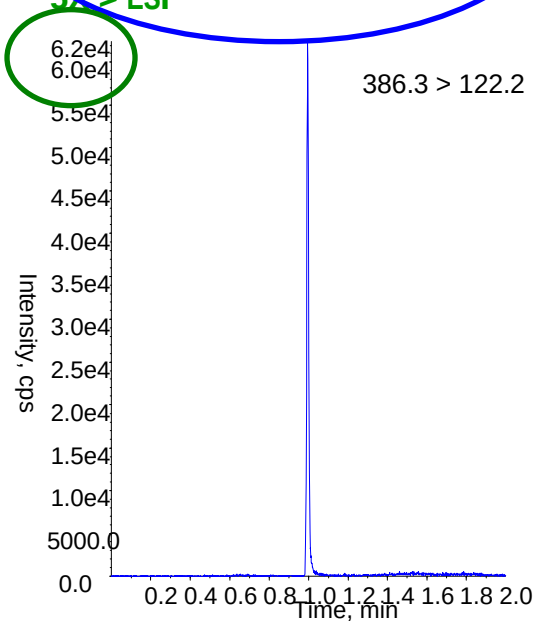
2 x 50 mm HALO C18

2.5 mm/second

Micro-Capillary Spray (µESI)

Sensitivity = 620 cps/pg

5X > ESI



100 pg Buspirone – µESI

10 µL injection

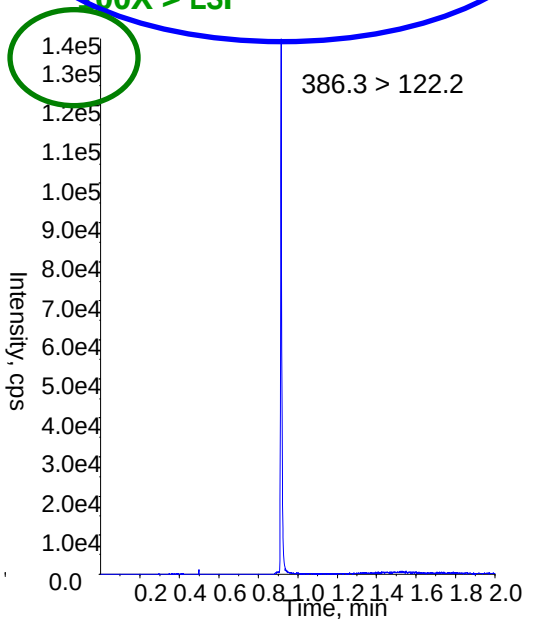
0.5 x 50 mm HALO C18

2.5 mm/second

CaptiveSpray (CSI)

Sensitivity = 14,000 cps/pg

100X > ESI



10 pg Buspirone – CSI

1 µL injection

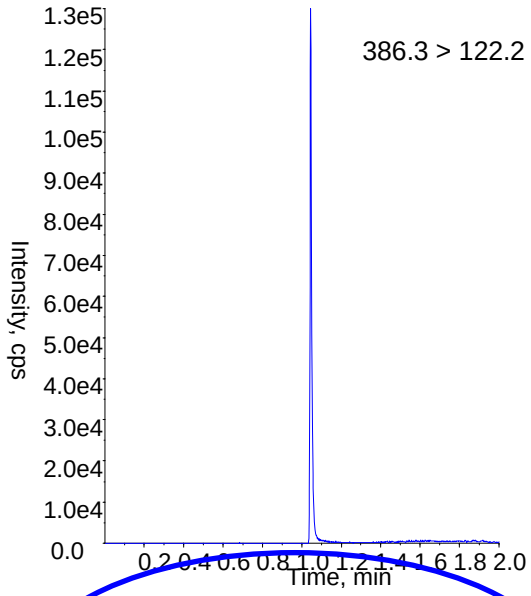
0.2 x 50 mm HALO C18

2.5 mm/second

CaptiveSpray Requires Less Sample

ElectroSpray Ionization (ESI)

Sensitivity = 130 cps/pg



1000 pg Buspirone – ESI

100 µL injection

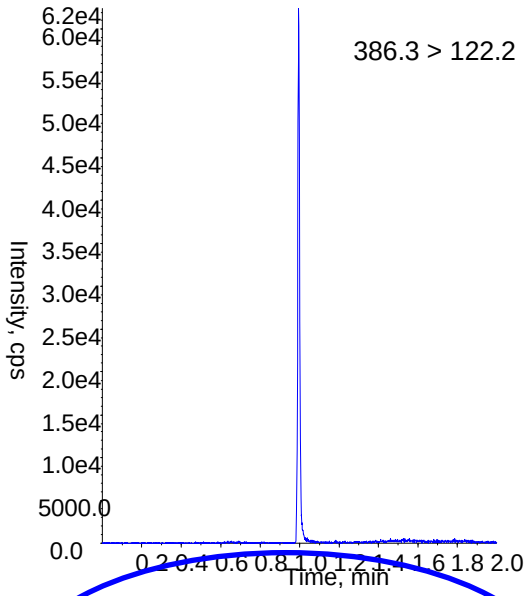
2 x 50 mm HALO C18

2.5 mm/second

Micro-Capillary Spray (µESI)

Sensitivity = 620 cps/pg

5X > ESI



100 pg Buspirone – µESI

10 µL injection

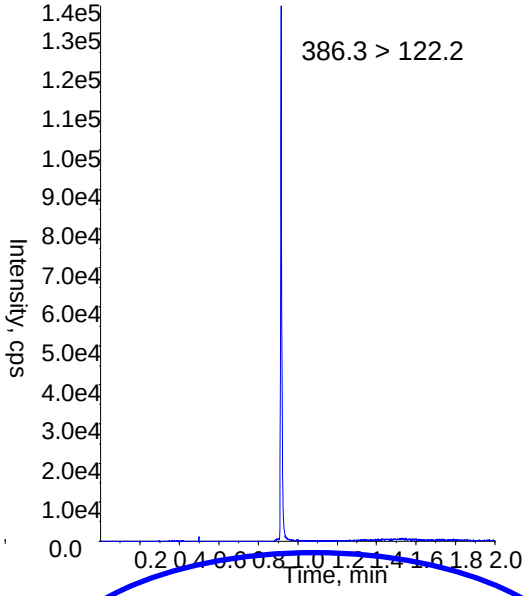
0.5 x 50 mm HALO C18

2.5 mm/second

CaptiveSpray (CSI)

Sensitivity = 14,000 cps/pg

100X > ESI



10 pg Buspirone – CSI

1 µL injection

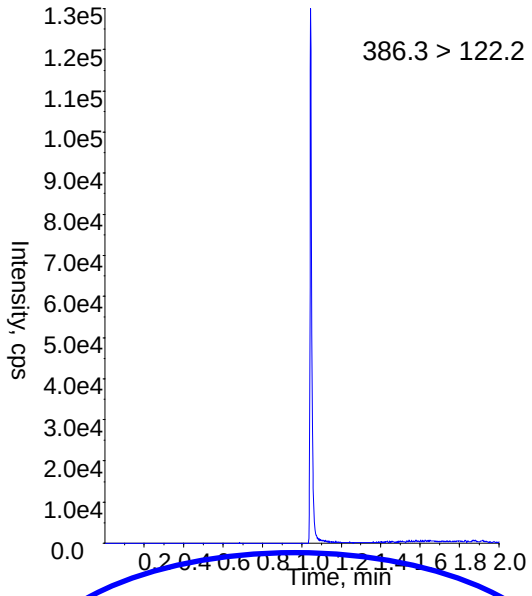
0.2 x 50 mm HALO C18

2.5 mm/second

CaptiveSpray Saves Solvent

ElectroSpray Ionization (ESI)

Sensitivity = 130 cps/pg



1000 pg Buspirone – ESI

$310.06 solvent costs

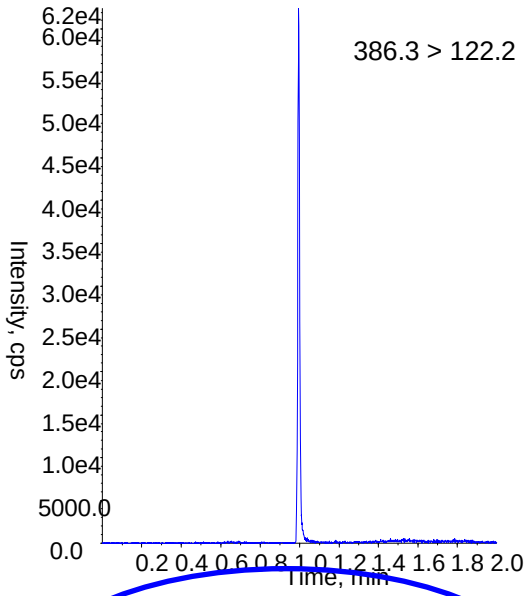
4100 inj 500 µL/min

7.1 days = 5.13L mobile phase

Micro-Capillary Spray (µESI)

Sensitivity = 620 cps/pg

5X > ESI



100 pg Buspirone – µESI

$19.85 Solvent Costs

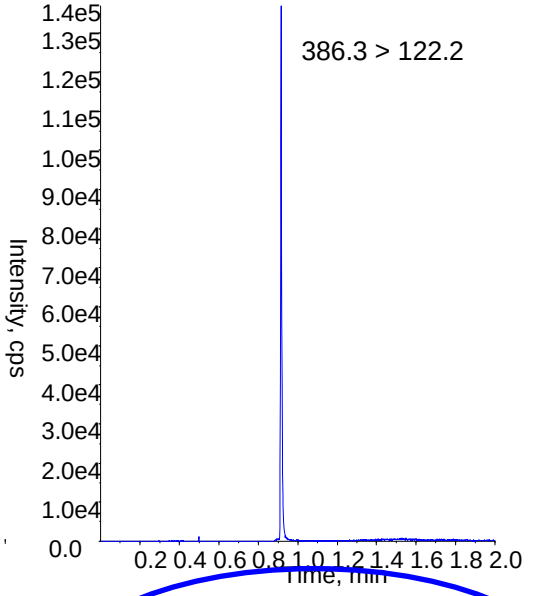
4100 inj 32 µL/min

7.1 days = 0.33L mobile phase

CaptiveSpray (CSI)

Sensitivity = 14,000 cps/pg

100X > ESI



10 pg Buspirone – CSI

$3.10 solvent costs

4100 Inj 5 µL/min

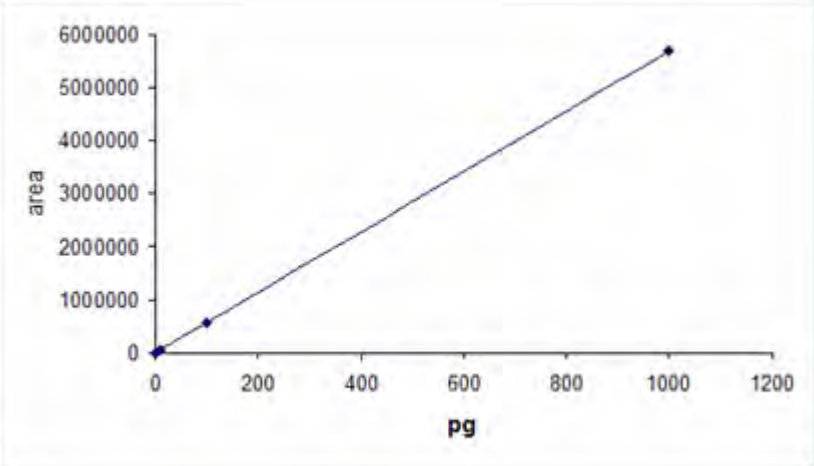
7.1 days = 0.05L mobile phase

- 01/01/10 Purchase price $77/L MS grade ACN
- 01/01/10 Purchase price $44/L MS grade Water
- $2.10/L CA Disposal costs

CaptiveSpray Linearity & Robustness

CaptiveSpray Linearity

4 Orders of magnitude



CaptiveSpray Robustness

1pg buspirone in 1ul Human Plasma extract

24hours/day 1 week run

4032 runs in total

150 sec : Injection to injection interval

Back pressure 10% up

Retention Time : CV 0.27%

Peak area : CV 12.8%

Reprinted from *American Laboratory e-Supplement* March 2010

Technical Article

by Kerry Nugent, Lori Ann Upton, Yixin Zhu, and Chris Loran

Capillary UHPLC Coupled with MRM-MS for Quantitative, High-Sensitivity Bioanalysis

Over the past 20 years, liquid chromatography-multiple reaction monitoring-mass spectrometry (LC-MRM-MS) has become the primary tool for the bioanalysis of drugs and their metabolites in physiological fluids.[1] Advances in genomics, proteomics, combinatorial chemistry, and high-throughput screening have all contributed to an increase in the number of potential drug candidates in the development pipeline of pharmaceutical companies. These advances place a continuous demand on pharmaceutical scientists to develop high-sensitivity, high-throughput, and robust assays for qualitative and quantitative bioanalysis.[2] These requirements have also prompted HPLC and MS vendors to make continued improvements to LC-MS instruments, consumables, and methods to help their customers meet the growing demands.

One major area of improvement over the past few years has been the introduction of ultrahigh-performance liquid chromatography (UHPLC) using smaller particle and column technology to provide significant improvements in chromatographic resolution and throughput, with modest gains in sensitivity versus conventional HPLC.[3] MS vendors have also improved instrumentation to make bioanalysis more sensitive and robust, reducing the extent of physiological fluid sample cleanup and enhancing overall sample throughput as well. Despite these LC-MS advances, optimum performance is currently achieved using a 2 × 50 mm LC column at flows from 200 to 800 µL/min using conventional electrospray ionization (ESI) sources with MRM-MS on triple quadrupole mass spectrometers. These conditions provide high sample loading capacity (10–1000 µL), robust operation with quantitative results, and high throughput using fast gradient separations in 2–5 min.

As newer drug candidates are developed with higher potency (resulting in lower dosages), the need for improvements in sensitivity for bioanalysis continues to challenge pharmaceutical scientists.[4] Since ESI-MS sources are concentration dependent, many attempts have been made to run at lower LC flow rates using smaller i.d. columns with nanospray ionization (NSI) or microspray ionization (MSI) sources in place of a conventional ESI source to improve sensitivity. Nano-LC (100–1000 nL/min) coupled with NSI-MS offers the highest possible sensitivity for LC-MS; however, this technique requires long run times (>30 min inject-to-inject) and significant user intervention to achieve the desired results. Micro-LC (10–100 µL/min) coupled with MSI-MS offers a modest gain in sensitivity over analytical LC- (200–2000 µL/min) ESI-MS, but the decreased loading capacity makes these gains difficult to realize on physiological fluid samples.

This article describes a nano-capillary UHPLC system coupled with a CaptiveSpray™ ionization- (CSI) MS source (**Michrom Bioresources**, Auburn, CA) that offers significant gains in sensitivity for bioanalysis without compromising sample throughput, method robustness, or quantitation. The nano-capillary UHPLC provides splitless gradient flows from 0.1 to 10 µL/min at pressures up to 10,000 psi with optimized fluidics so that gradients as fast as 1%/sec can be achieved with minimal extracolumn volume. The system was applied to the determination of buspirone in human plasma samples, and the results show that this technology offers up to a 100-fold sensitivity improvement over conventional LC-ESI-MS with 150-sec inject-to-inject times.

Experimental

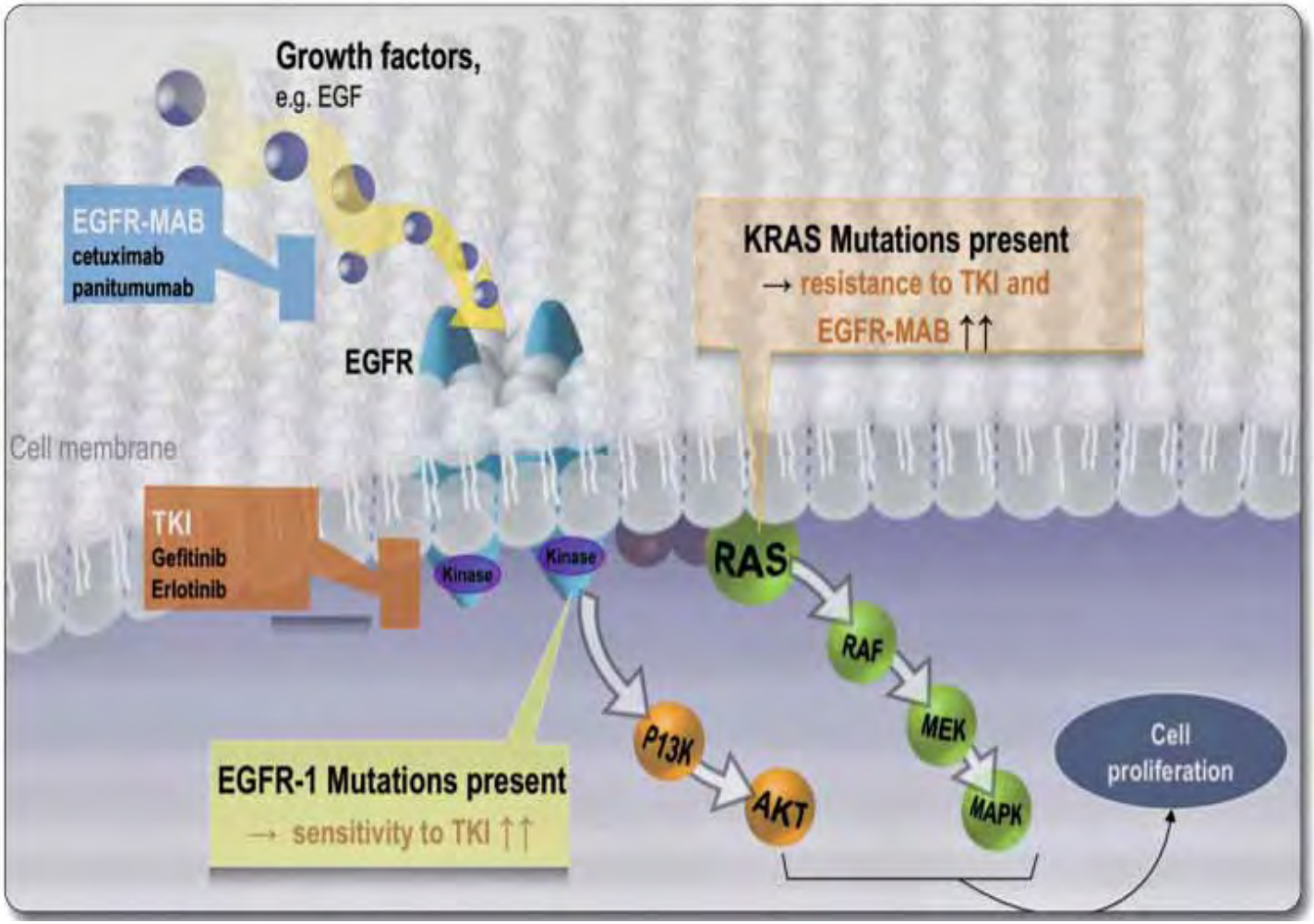
Materials

Buspirone and lyophilized human plasma were obtained from **Sigma-Aldrich** (St. Louis, MO). All solvents were high-purity Burdick & Jackson brand purchased from **Honeywell** (Muskegon, MI). Acrylate immobilized liquid extraction

Proteomic Analysis of Formalin Fixed Tissue and Frozen tissue by Mass Spectrometry

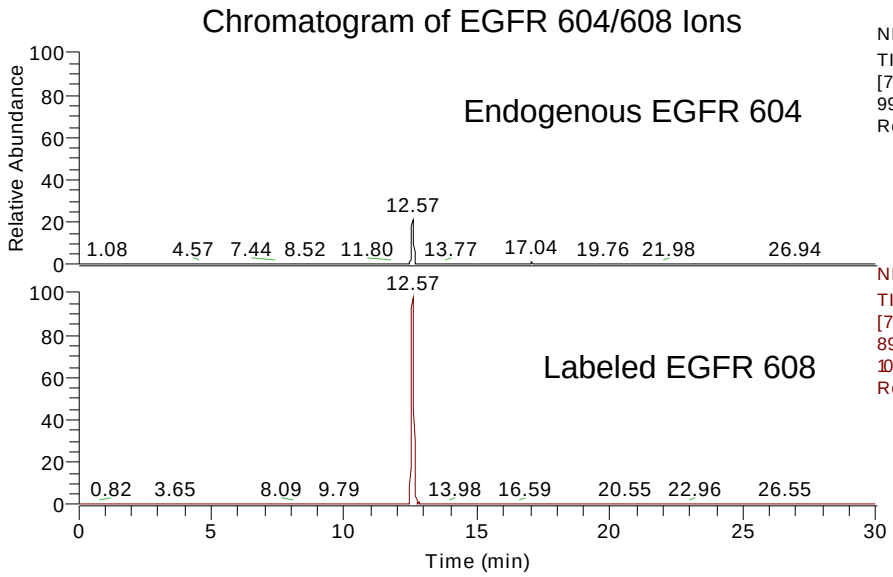
Design of EGFR Rx SRM Assay

Is there a better way to direct EGFR therapy decisions?



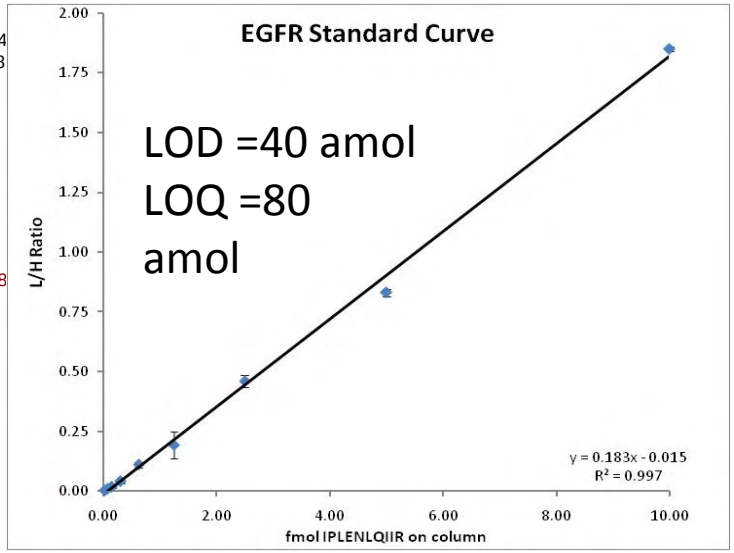
Detection and Quantitation of Total EGFR

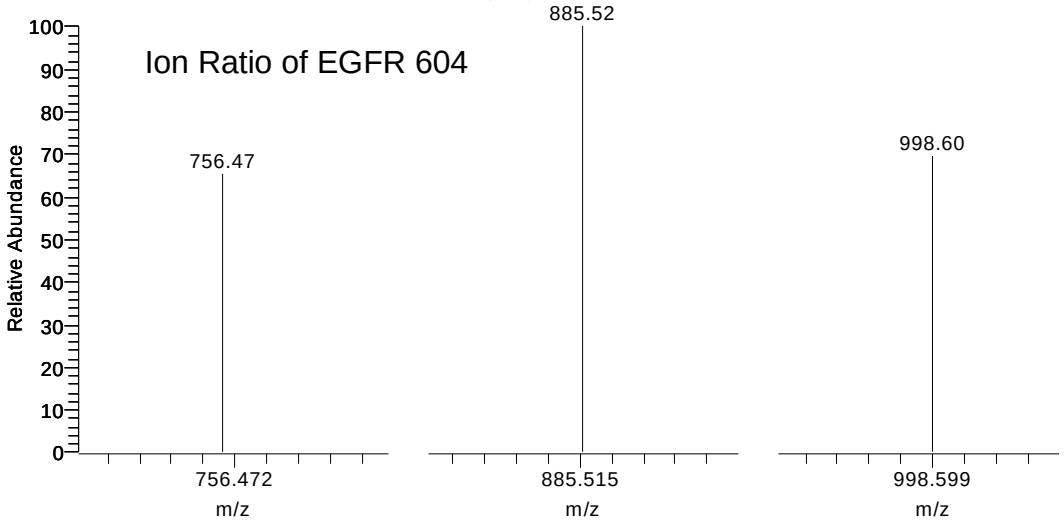
SRM Assay Development

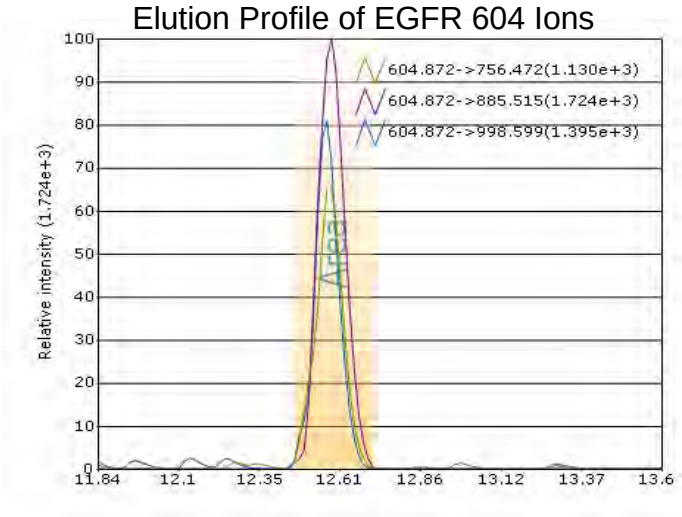


NL: 2.00E4
TIC F: + c NSI SRM ms2 604 [756.471-756.473, 885.514-885.516, 998.598-998.600] MS Rep1_100617JA_01

NL: 2.00E4
TIC F: + c NSI SRM ms2 608 [763.488-763.490, 892.531-892.533, 1005.615-1005.617] MS Rep1_100617JA_01

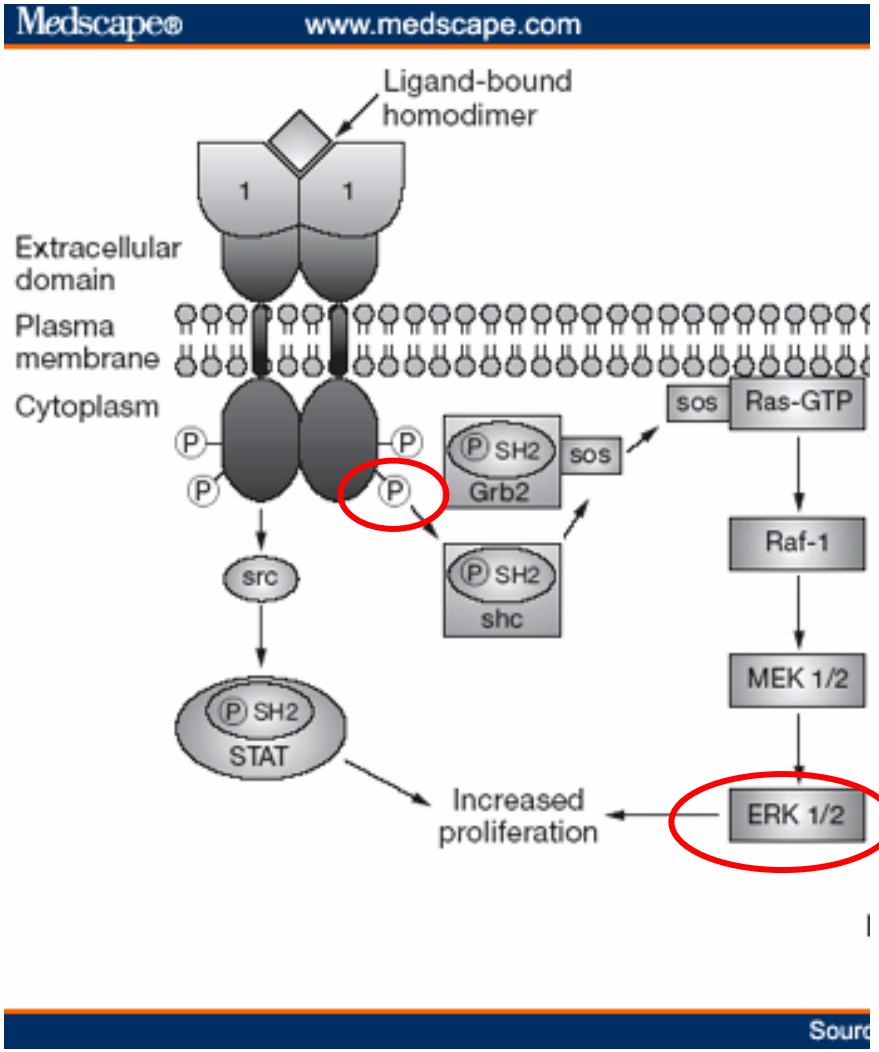


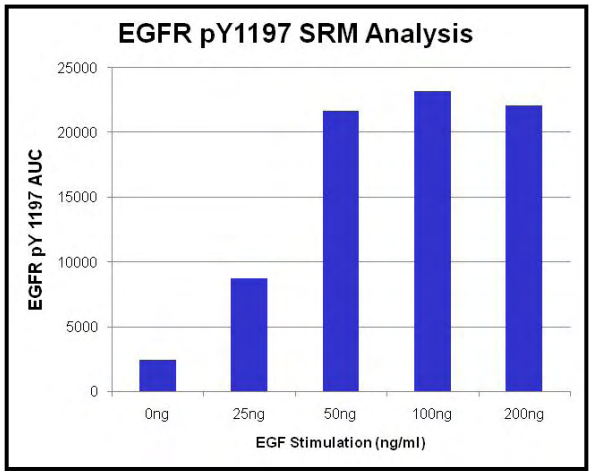


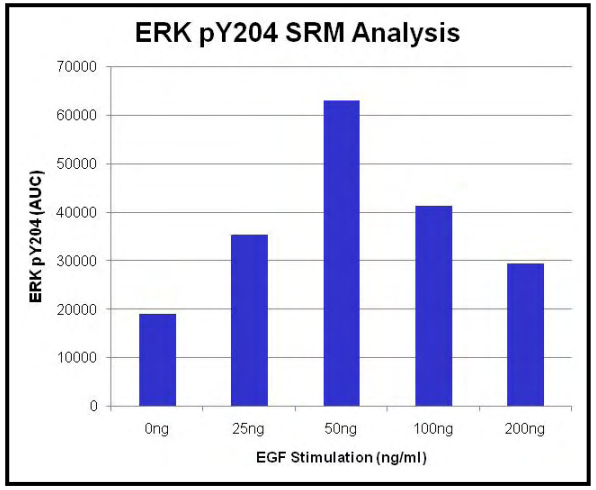


SRM Analysis of EGFR Signaling Pathway

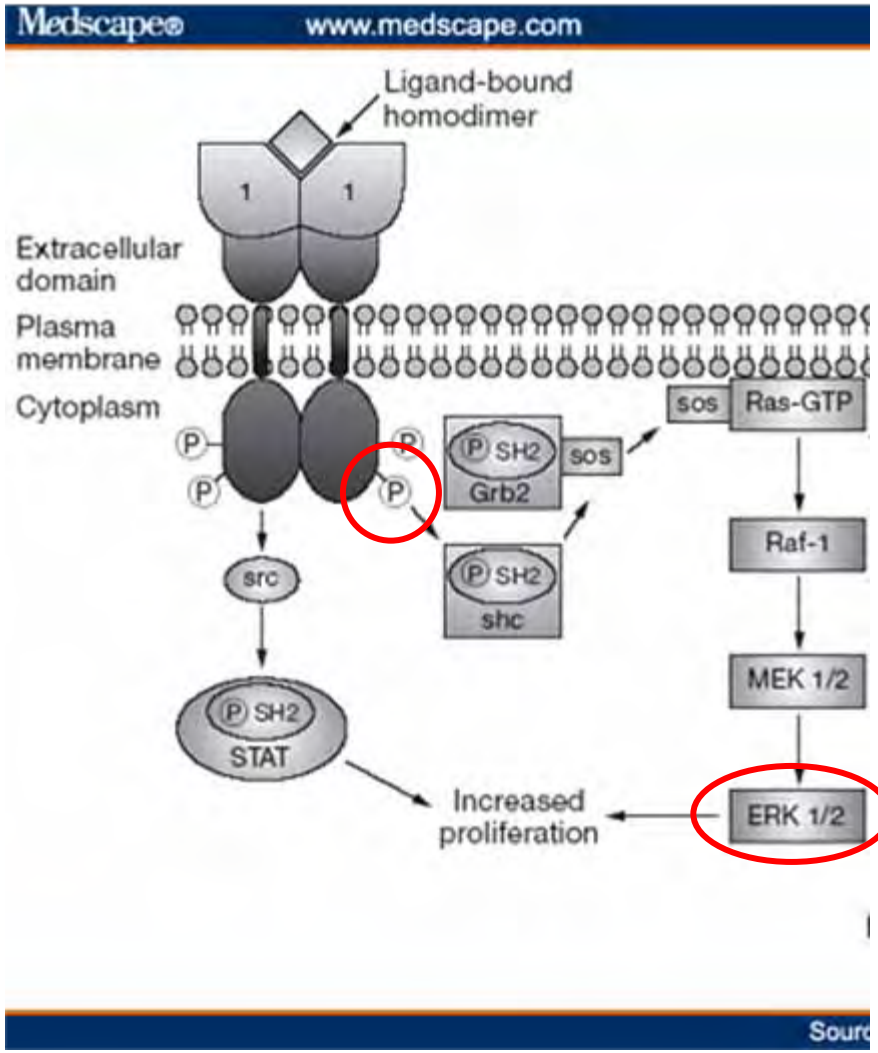
Measurement of EGFR pY1197 and ERK pY204 in FFPE A431 Cells

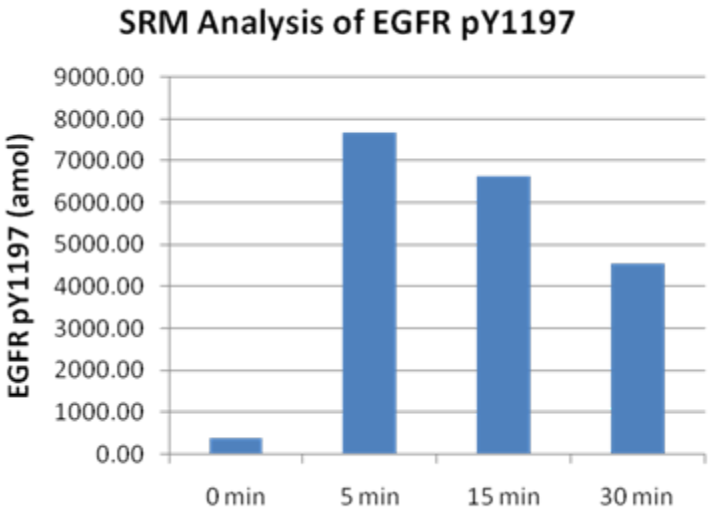


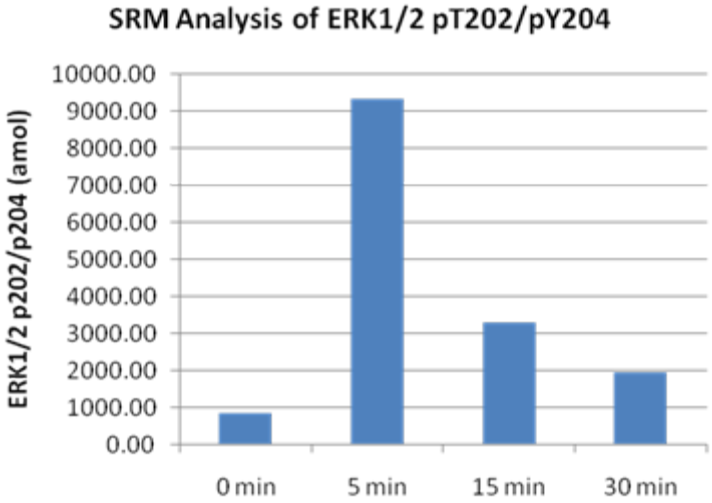




Timecourse Analysis of EGFR/ERK Pathway







3連四重極型質量分析計による SRM定量解析の悩み

- メソッドが複雑では？
- どのペプチドをターゲットに選ぶか？
- データの解析はどうすればよいか？
- ESIは安定性は？

解析のすべてをサポート

解析の流れ

SRMペプチドデザイン

目的の候補蛋白質に対してSRMによる定量を行うターゲットペプチドのデザインを行う。ペプチドの選択、SRMでのトランジッションの設計など。

実験による検証

すべてのサンプルからプロジェクトコントロールを作成し実際のLC/MS分析を行い、分析条件の最適化及びデザインされたペプチドが解析できるかを検証。

実サンプルのSRMによるLC/MS解析

1検体につきn=3で測定を行い、すべてのサンプルをＬＣ／ＭＳ解析する。

データ解析及びレポート作成

測定データの解析を行い、定量結果からレポートを作成する。

Stabilize from the moment of sampling

Maintainor ™ Tissue

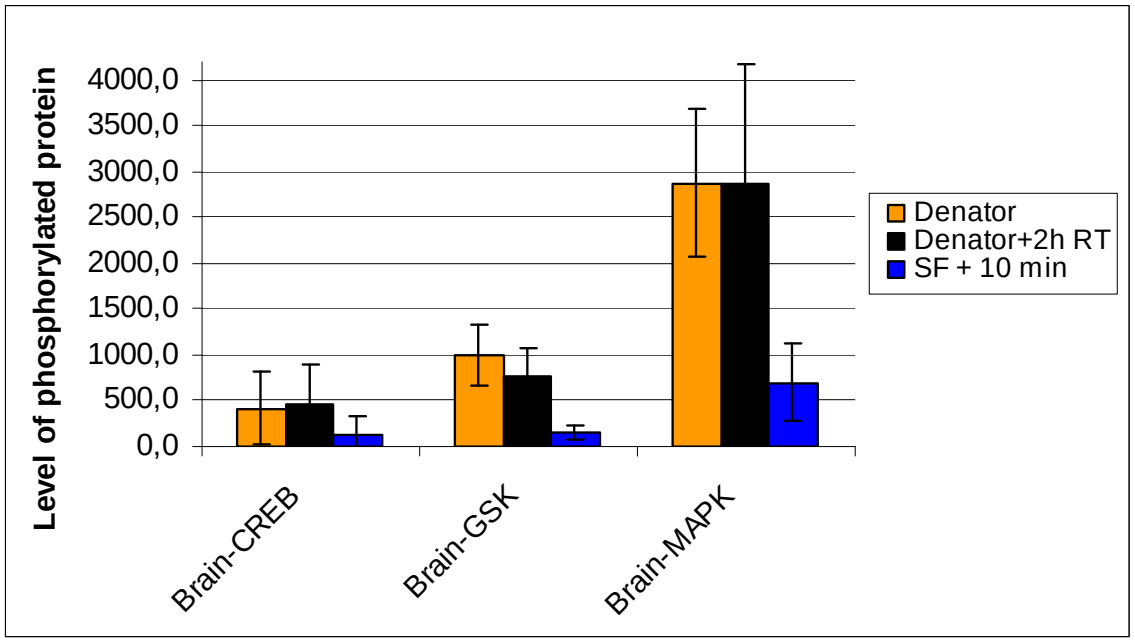
Treatment/Storage

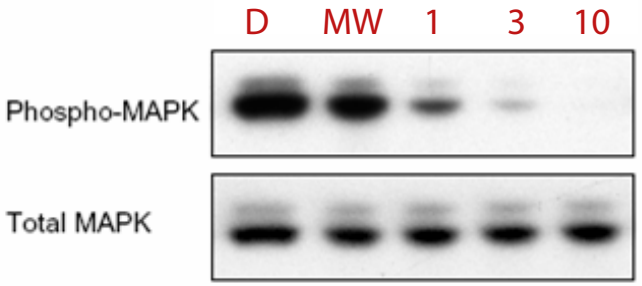
Stabilizor ™ T1

Stabilization

Stabilize - maintain the *in vivo* state

Phosphorylated proteins

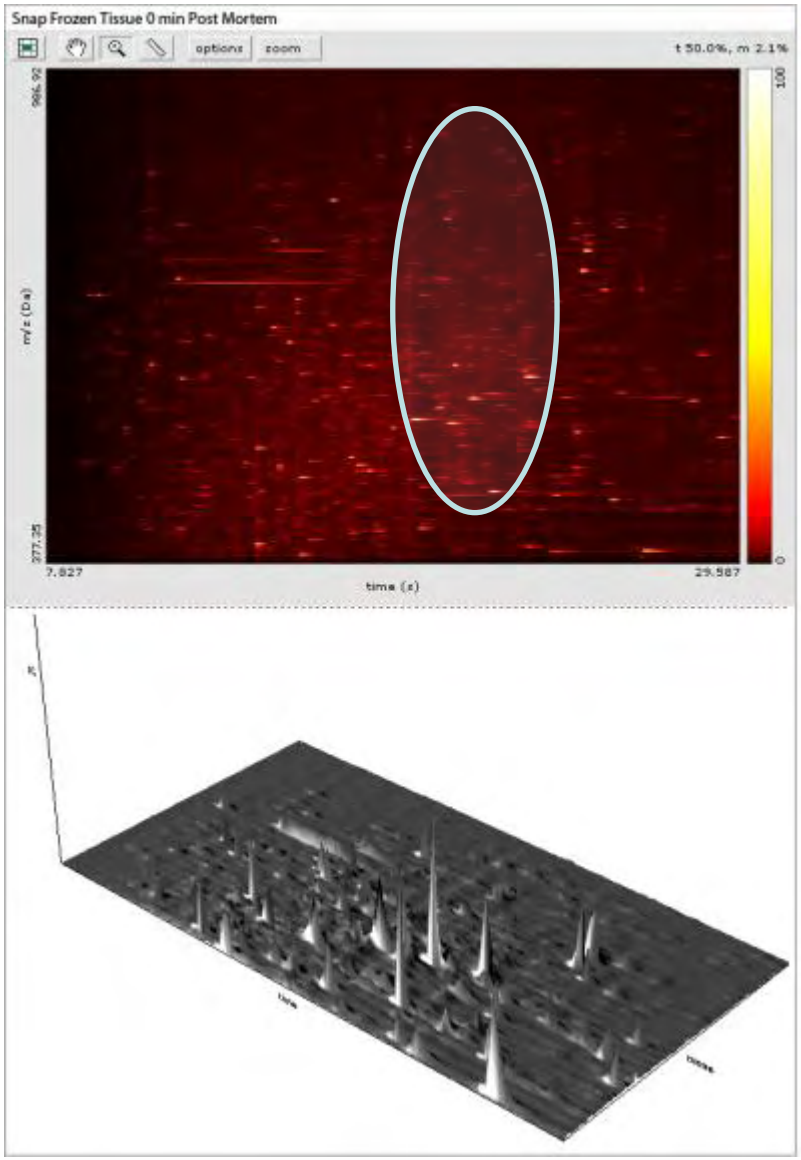


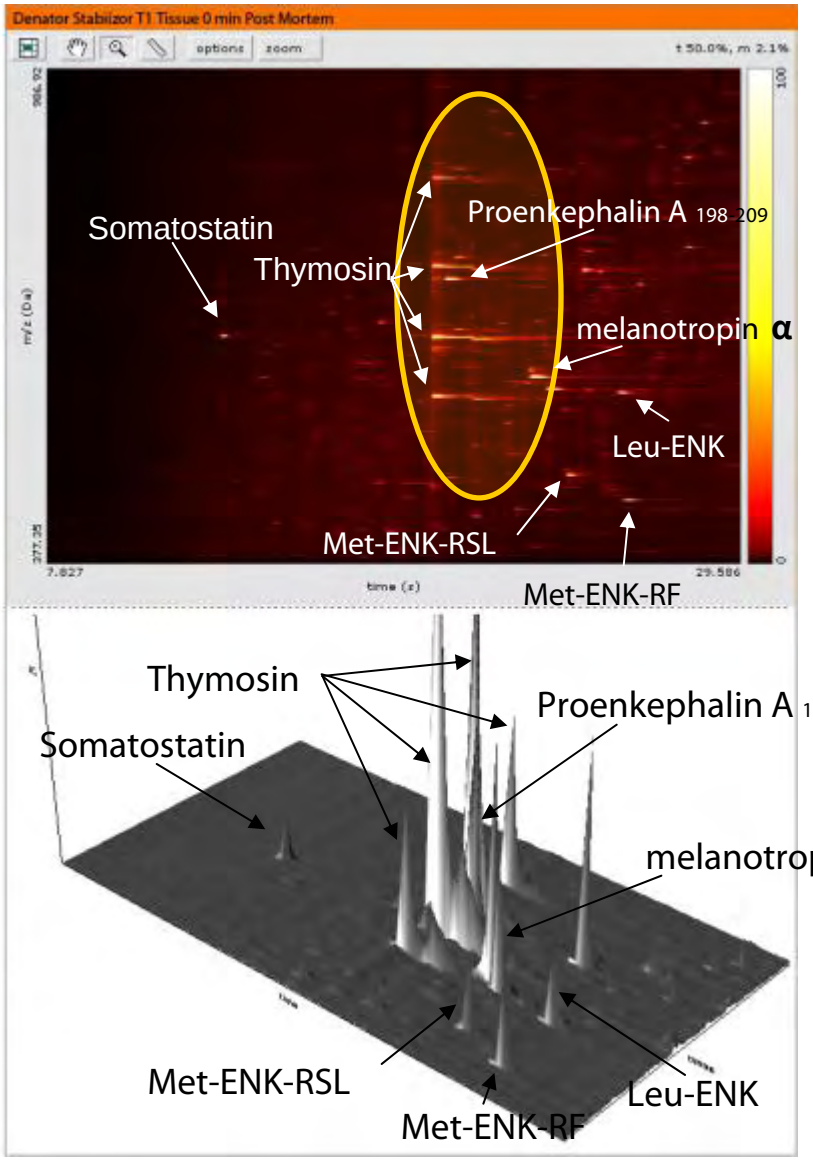


Comparison between focused MW and Denator in combination with a time course study.

In collaboration with Karolinska Institutet

Nano-LC-ESI MS

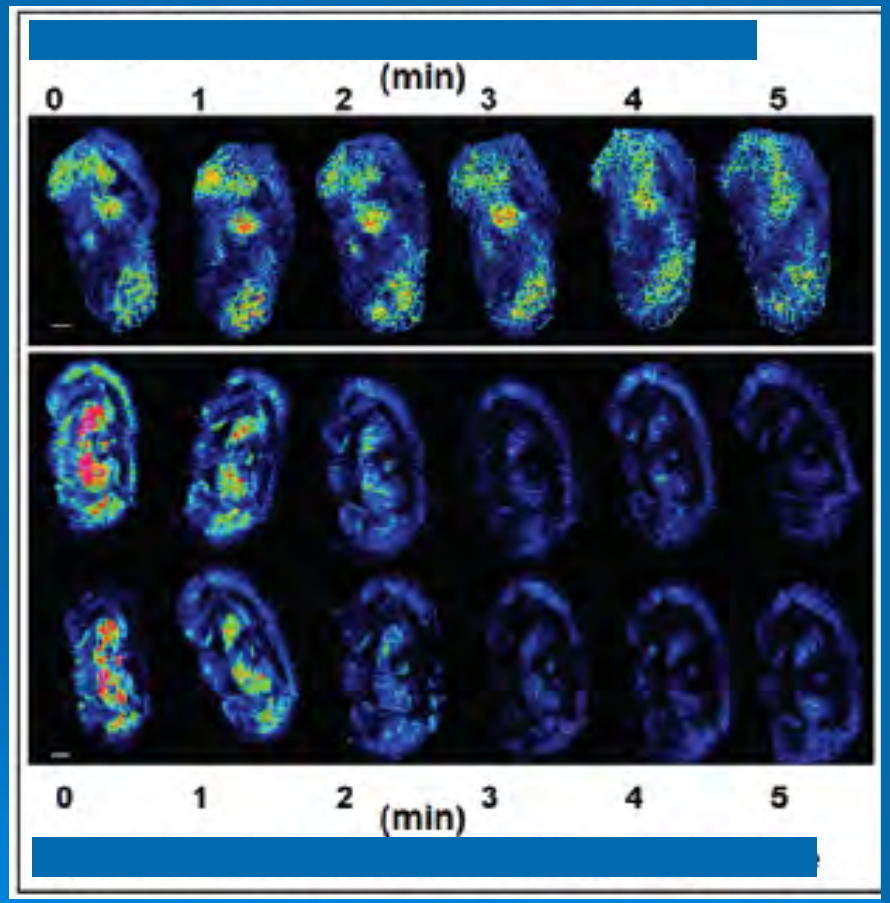




Snap frozen

Denator treated

Mouse brain: Peptidomics on MALDI-IMS



Stabilized

Untreated

Mw 6723,5

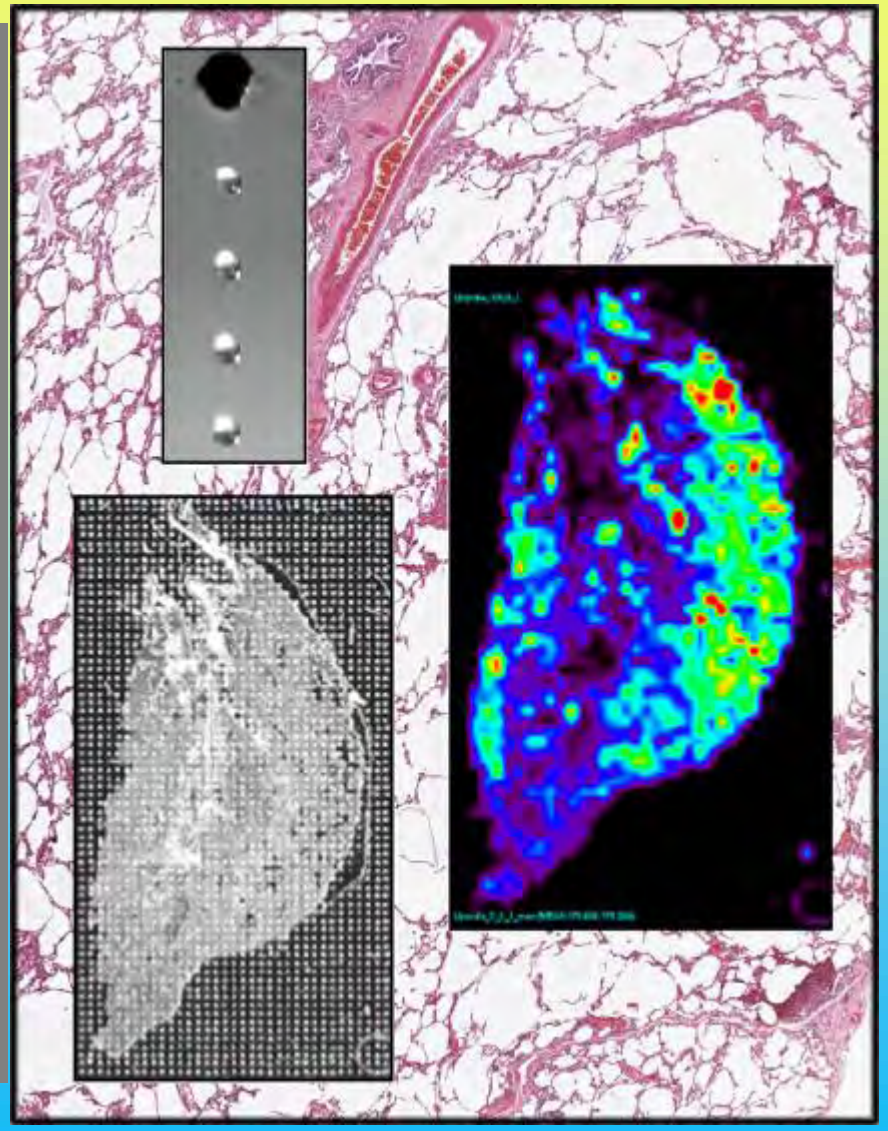
Data courtesy of:
Professor Andrew Pitt at Glasgow University

Tissue imaging for compound and metabolite distribution in tissues

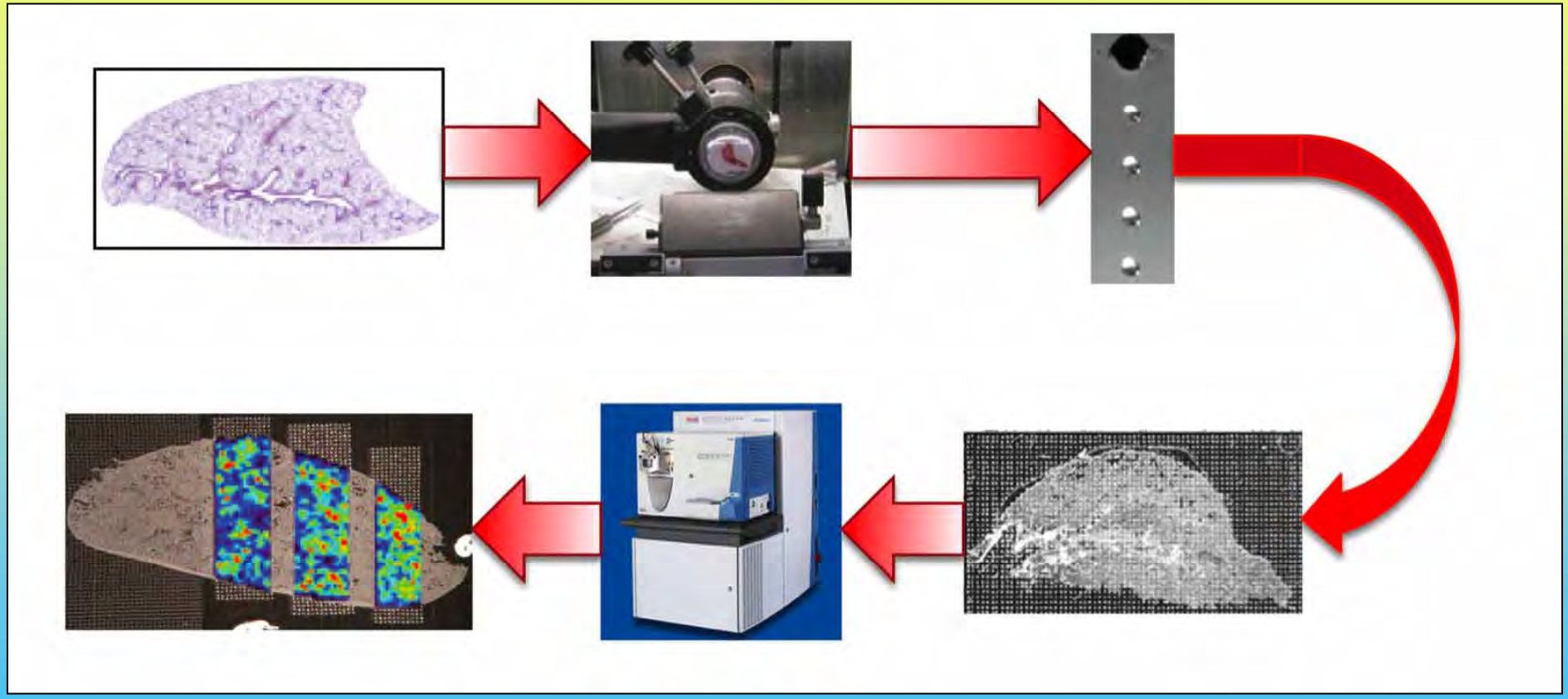
Today
Optimise lung retention and correlate drug PK in lung with effect and toxicity when only total concentration of drug in lung tissue homogenate can be measured.

Business benefit of tissue imaging
Localisation of unlabelled drugs and metabolites and peptides/proteins in tissue

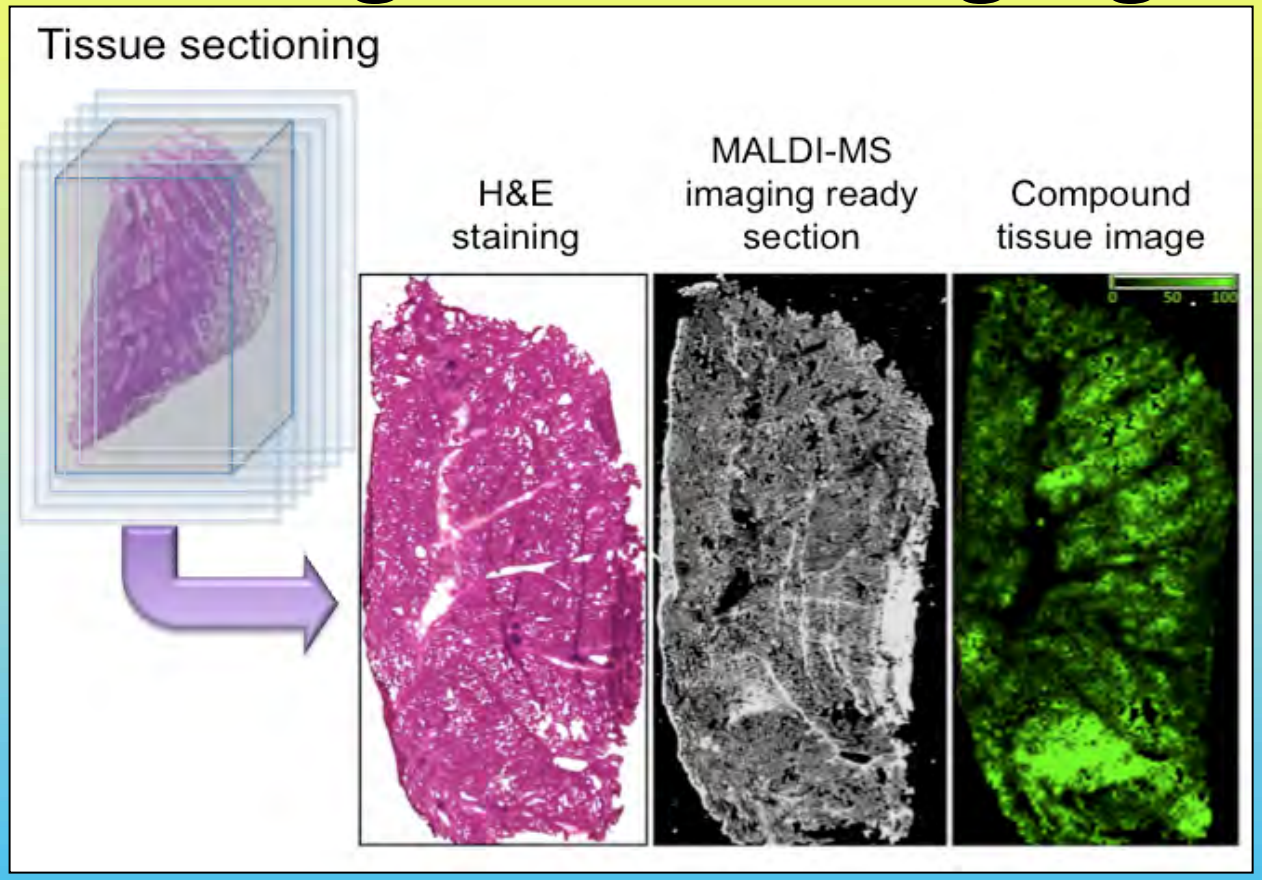
- PK/PD – does compound distribute to target site?
- Toxicity – does compound distribute specifically to affected tissue/cell type?
- **Drive chemistry towards compounds with optimal distribution – FIC & BIC**
- Applicable to **Lung/Liver/Kidney/Brain** Pathophysiology



Compound Tissue Imaging



Drug Tissue Imaging



Molecular imaging process; including, tissue sectioning, hisological staining, tissue section preparation, ready for tissue imaging, and localization of drug compound

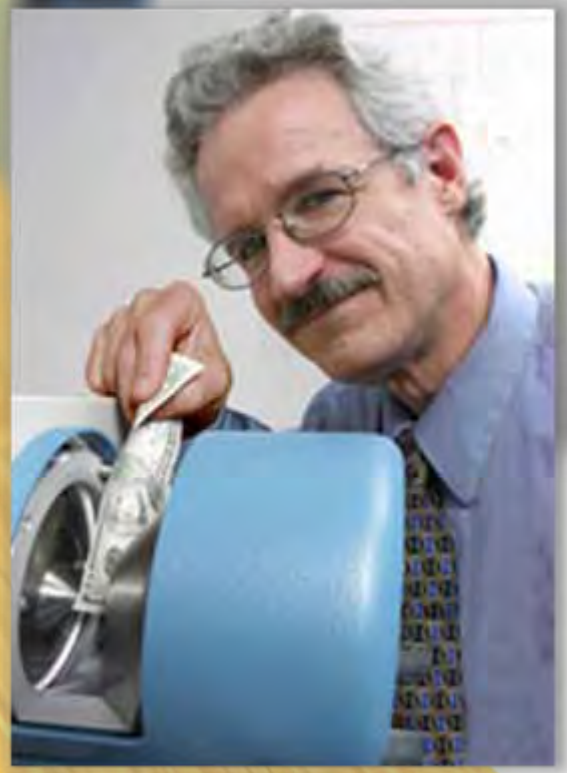
Vegvari, Marko-Varga, Chem. Rev., 2010,3278

固体、液体などサンプルをかざすだけで解析することができる最新のイオン化ソース
DART(Direct Analysis in Real Time)

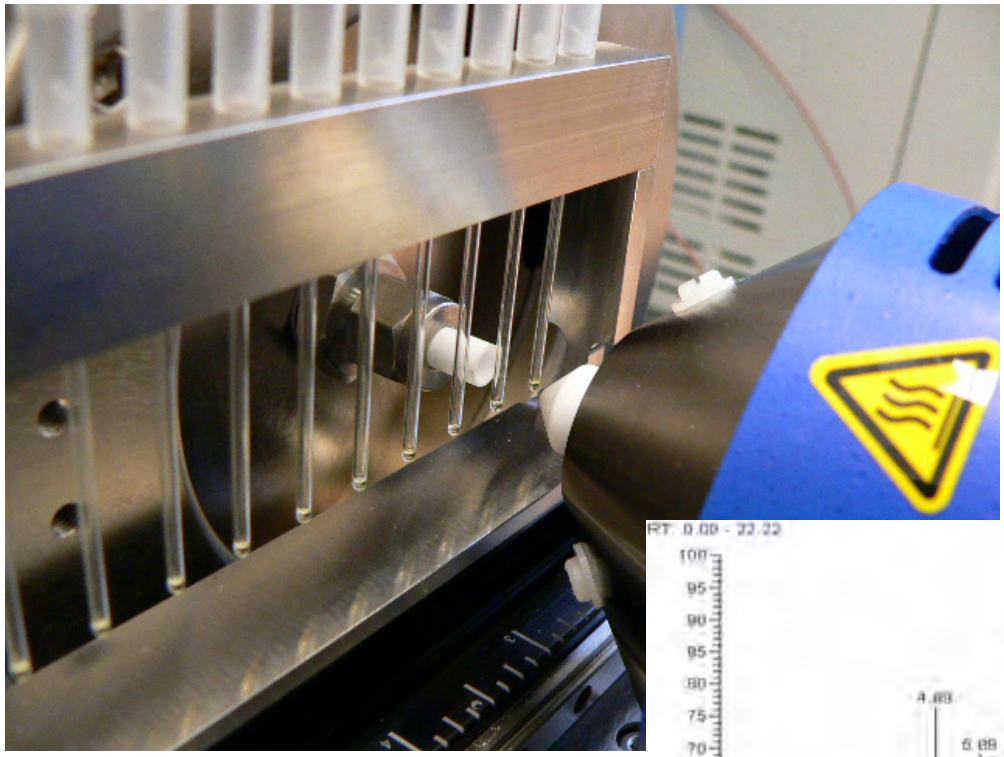
Introducing the latest in DART technology

AMR INCORPORATED

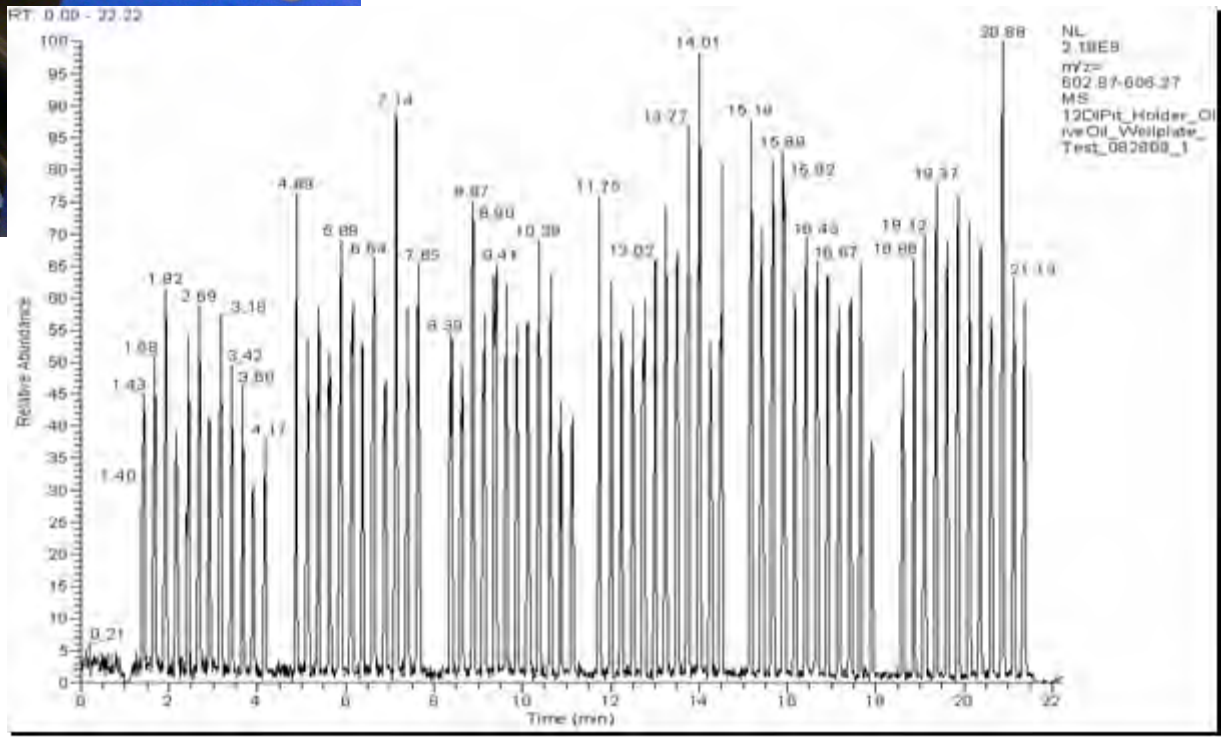
New Optionの紹介



12 Dip-it 連続分析オプション

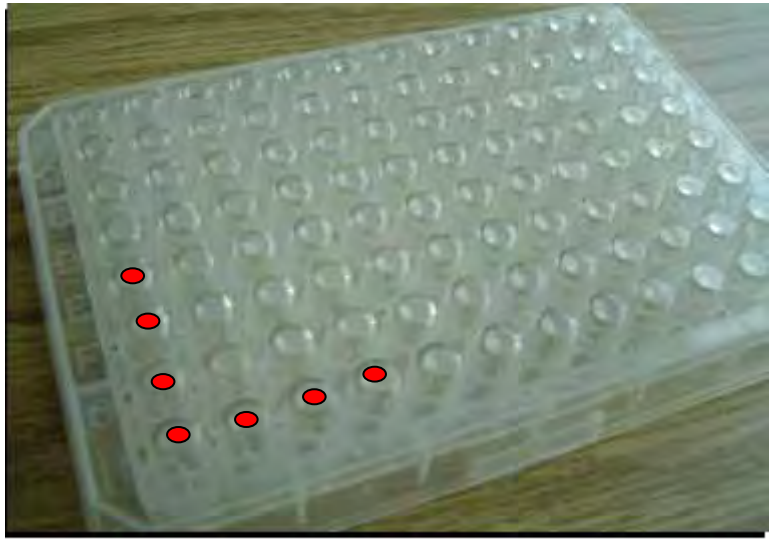


- For liquids you can analyze 12 samples per minute at high speed for qualitative analysis….or slow down the presentation speed to deliver more quantitative analysis.

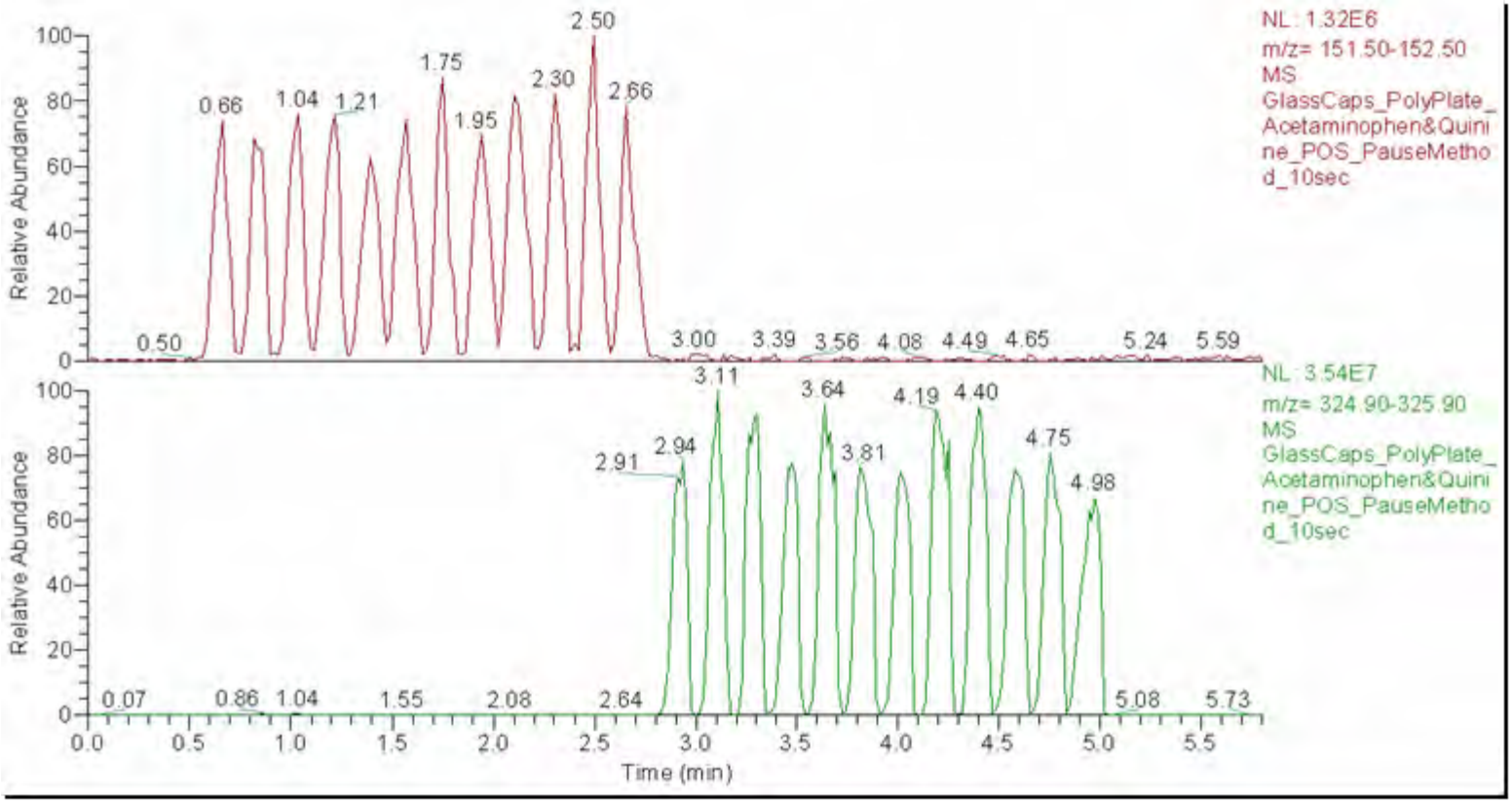


- Analysis of 72 samples of Virgin Olive Oil in 21 minutes looking for di- and tri- glyceride content.

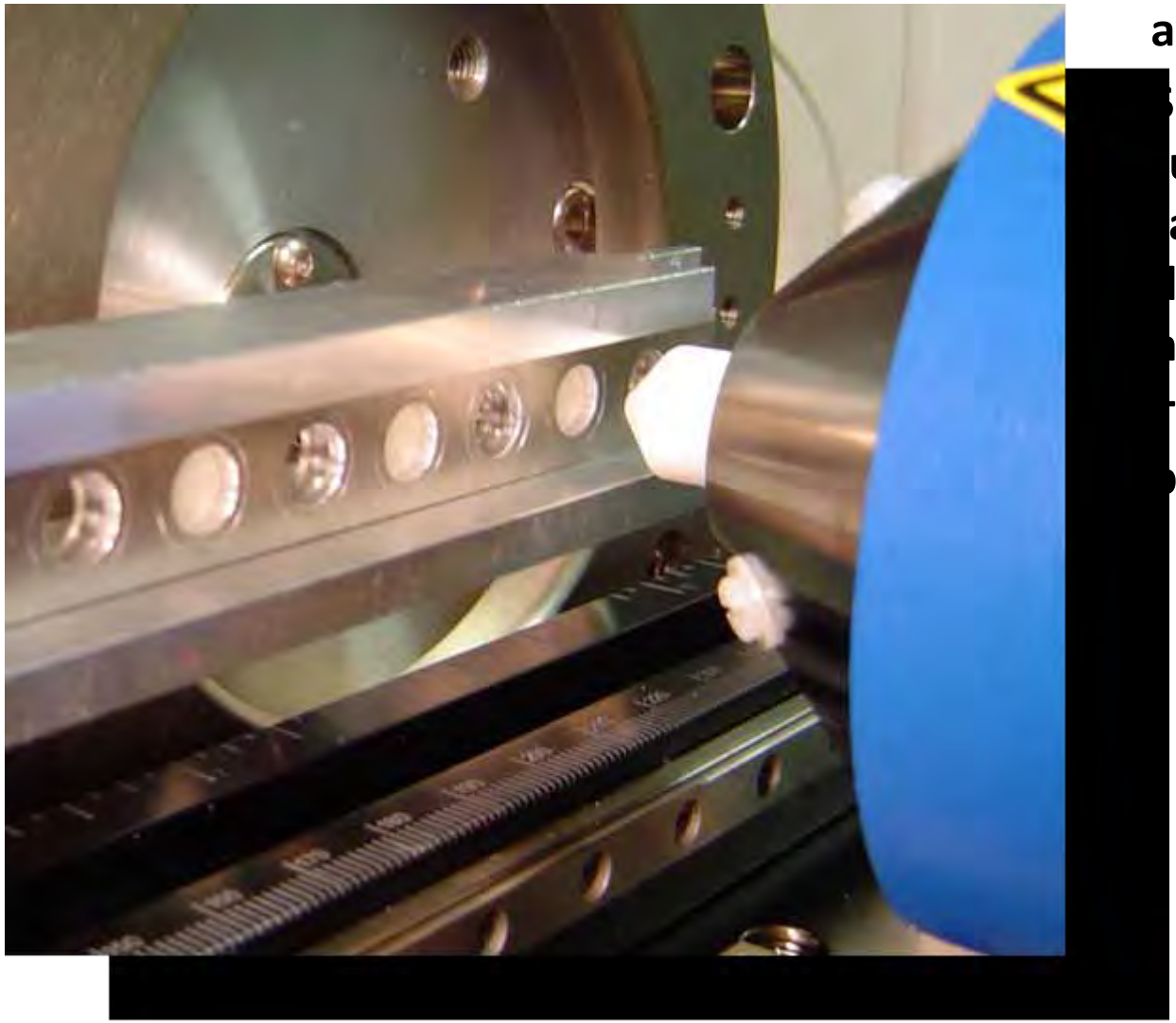
96-Well Plate Inverted Glass Inserts



- **Sampling using the 3+D scanner module.**
- **Liquid samples pipetted onto the top surface of the glass inserts.**
  - **Row A: Acetaminophen *m/z* 152**
  - **Row B: Quinine *m/z* 325**



Transmission DART Swab Scanner Module



- **Concentrate trace amount of analytes on a sponge type swab for DART analysis.**
- **Scan up to 5 swabs in one run using the temperature ramp method with user defined sampling time and heater temperatures.**
- **The heated DART gas passes through the swab.**