Green Chemistry

Quantitative Bio-Analysis Using micro-LC TOF and micro-LC MS/MS

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Twelve Principles of Green Chemistry *

1. **Prevention**
   It is better to prevent waste than to treat or clean up waste after it has been created.
2. **Atom Economy**
   Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product.
3. **Less Hazardous Chemical Syntheses**
   Wherever practicable, synthetic methods should be designed to use and generate substances that possess little or no toxicity to human health and the environment.
4. **Designing Safer Chemicals**
   Chemical products should be designed to effect their desired function while minimizing their toxicity.
5. **Safer Solvents and Auxiliaries**
   The use of auxiliary substances (e.g., solvents, separation agents, etc.) should be made unnecessary wherever possible and innocuous when used.
6. **Design for Energy Efficiency**
   Energy requirements of chemical processes should be recognized for their environmental and economic impacts and should be minimized. If possible, synthetic methods should be conducted at ambient temperature and pressure.
7. **Use of Renewable Feedstocks**
   A raw material or feedstock should be renewable rather than depleting whenever technically and economically practicable.
8. **Reduce Derivatives**
   Unnecessary derivatization (use of blocking groups, protection/ deprotection, temporary modification of physical/chemical processes) should be minimized or avoided if possible, because such steps require additional reagents and can generate waste.
9. **Catalysis**
   Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.
10. **Design for Degradation**
    Chemical products should be designed so that at the end of their function they break down into innocuous degradation products and do not persist in the environment.
11. **Real-time analysis for Pollution Prevention**
    Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.
12. **Inherently Safer Chemistry for Accident Prevention**
    Substances and the form of a substance used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions, and fires.

GS 2010

* Anastas, P. T.; Warner, J. C.; Green Chemistry: Theory and Practice, Oxford University Press: New York, 1998.


Green carbon dioxide
Cycle time 1, 80, 500 year
Straw, wood, peat etc
VS
Black carbon dioxide
Cycle time 1000-100000 year
Brown coal, coal, oil
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LCMS *vs* Green Chemistry

Prevent waste

Safer Solvents and Auxiliaries

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Real-time analysis for Pollution Prevention

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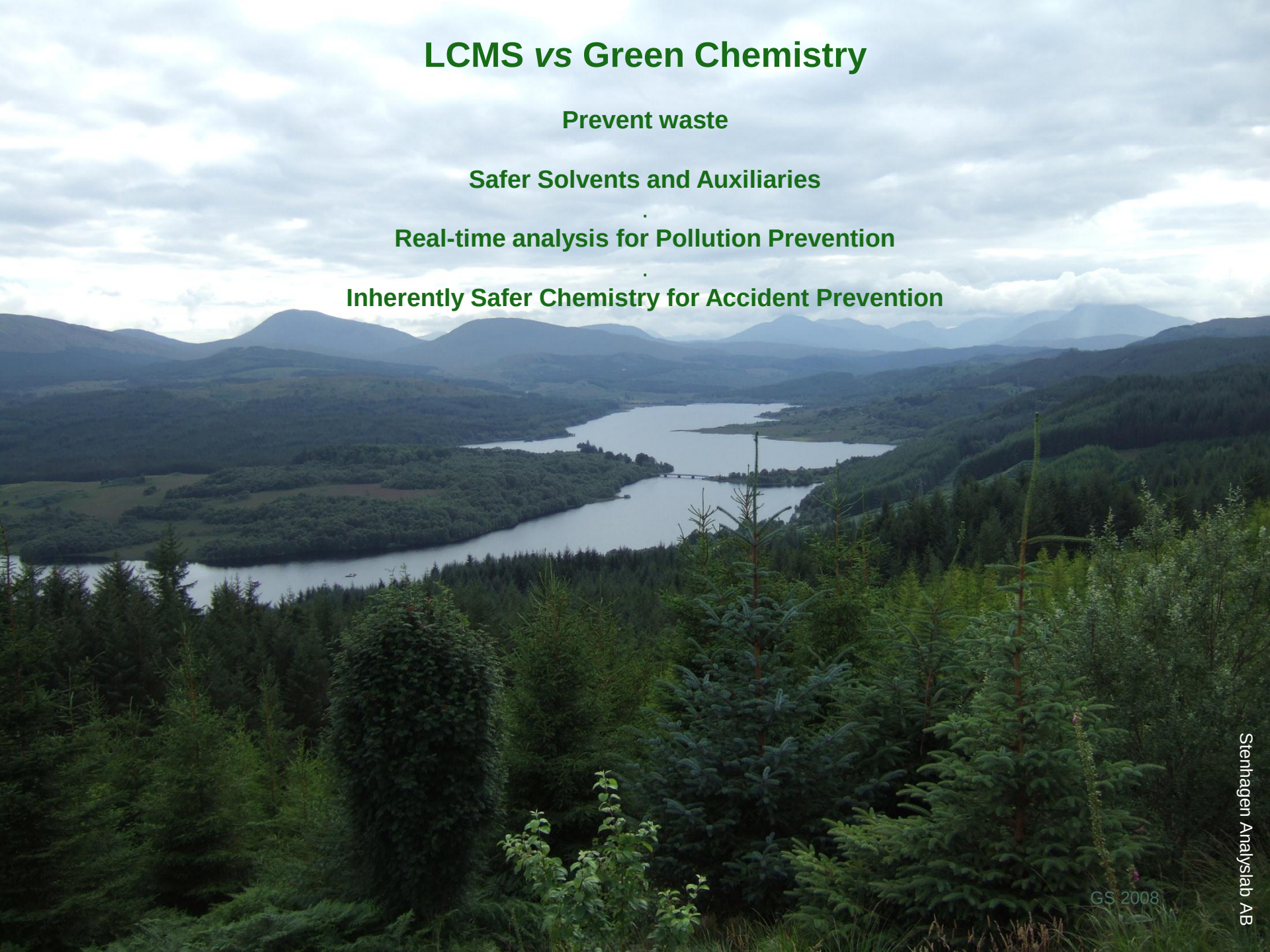
Inherently Safer Chemistry for Accident Prevention



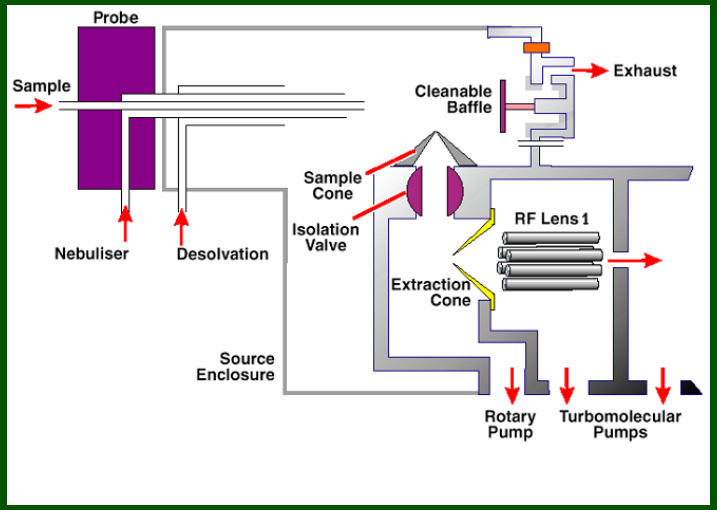
Conventional LC-ESI mass spectrometers

(flow rates from 0.5 mL/min to 2 mL/min)

To handle these high flow rates, most commercial ESI sources for mass spectrometers use pneumatic assistance for aerosol generation.

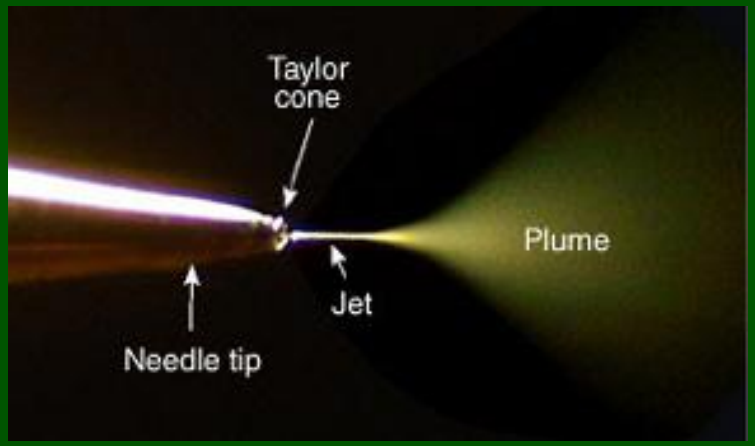
At these high flow rates, the mass spectrometer inlet samples typically less than 0.1% of the total aerosol produced.

A great deal of the sample is "wasted" because it does not contribute to ion current.



When the flow rate is **reduced** to much lower flow rate:

- Droplet formation occurs more readily, requiring only the applied voltage to generate spray.
- No sheath gas or additional heat is required.
- The stability of spray, and therefore the signal, at the lower flow rates is typically improved for aqueous or "salty" mobile phases.
- Low flow ESI is especially tolerant to a wide range of liquid compositions
- Can even spray "pure" water with a high degree of stability.



Electrospray ion current

$$I = \beta(\varepsilon)\left(\frac{Qk\,\gamma}{\varepsilon}\right)^{1/2}$$

Derived through a dimensional analysis of the charge transport process

I total spray current or total excess charges in the electrospray process
k electric conductivity of the liquid
Γ surface tension of the liquid
ε dielectric constant of the liquid
$\beta(\varepsilon)$ experimentally determined coefficient
Q liquid flow rate.

$$I_J = eA_V Q \sum_{z=1}^{i(J)} z\, f_{z,J} X_J$$

When all molecules of a compound J are completely ionized

I_J total ion current corresponding to compound J
X_J molar concentration
e electron charge (1.6x10−19 Coulomb)
A_V Avogadro's number
$f_{z,J}$ fraction of compound J molecule ions carrying z charges
$i(J)$ maximum charge carrying capacity of the compound J ions.

Charge Competition and the Linear Dynamic Range of Detection in Electrospray Ionization Mass Spectrometry
Keqi Tang, Jason S. Page, and Richard D. Smith
J Am Soc Mass Spectrom. 2004 October ; 15(10): 1416–1423.

$$I_A = eA_V Q \sum_{J=1}^{N} \left(\sum_{z=1}^{i(J)} z f_{z,J} X_J \right)$$

When all analytes are ionized, represents an ideal condition in the electrospray ionization process

$$I = \beta(\varepsilon)\left(\frac{Qk\gamma}{\varepsilon}\right)^{1/2}$$

total spray current or
total excess charges in the electrospray process

Power Supply

ESI capacity model

$$C_i = \frac{I}{I_A} = \frac{\beta(\varepsilon)}{eA_V \sum_{J=1}^{N} \left(\sum_{z=1}^{i(J)} z f_{z,J} X_J \right)} \cdot \left(\frac{k\gamma}{\varepsilon Q} \right)^{\frac{1}{2}}$$

C_i electrospray ionization charge capacity for a solution

Ci >>1, the sample solution is sufficiently dilute compared to the available excess electrospray charge. Here, ESI charge competition is expected to be negligible and the ***ESI-MS response is linear with concentration for each compound in the mixture.***

Ci ~1, the total excess charge becomes comparable to the total number of analyte ions formed by ESI. ESI **charge competition** in this case is expected to be important. Compounds with different ionization efficiencies will have different MS responses. This can result in the ***different dynamic ranges for compounds in mixtures***, as discussed below, and even the failure to detect some species in mixtures.

Ci<<1, the MS response is expected to be substantially ***independent of sample concentration*** (i.e. saturated).

ESI capacity model

$$C_i = \frac{I}{I_A} = \frac{\beta(\varepsilon)}{eA_V \sum_{J=1}^{N} \left(\sum_{z=1}^{i(J)} z f_{z,J} X_J \right)} \cdot \left(\frac{k\gamma}{\varepsilon Q} \right)^{\frac{1}{2}}$$

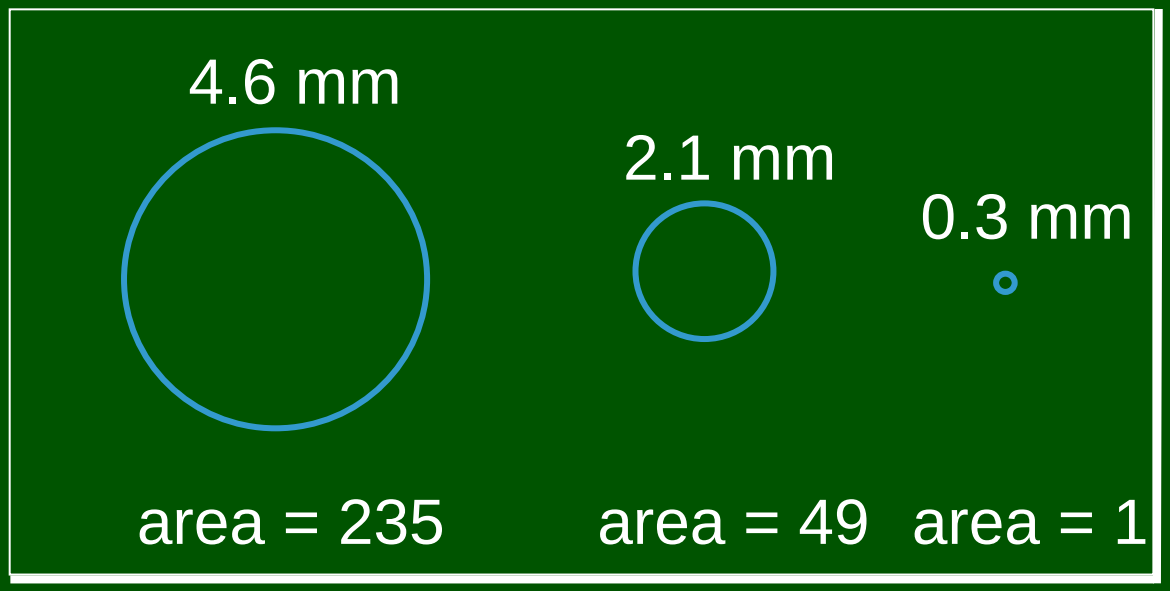
C_i is inversely proportional to the square root of the electrospray flow rate Q.

This implies that a smaller electrospray flow rate can provide a **wider linear dynamic range.**

Operating in the low flow electrospray mode will not only **increase the upper limit** of the linear dynamic range, as indicated by eq., but also **decrease the lower concentration limit**, which is essentially determined by the detection capabilities of the mass spectrometer.

Micro-LC

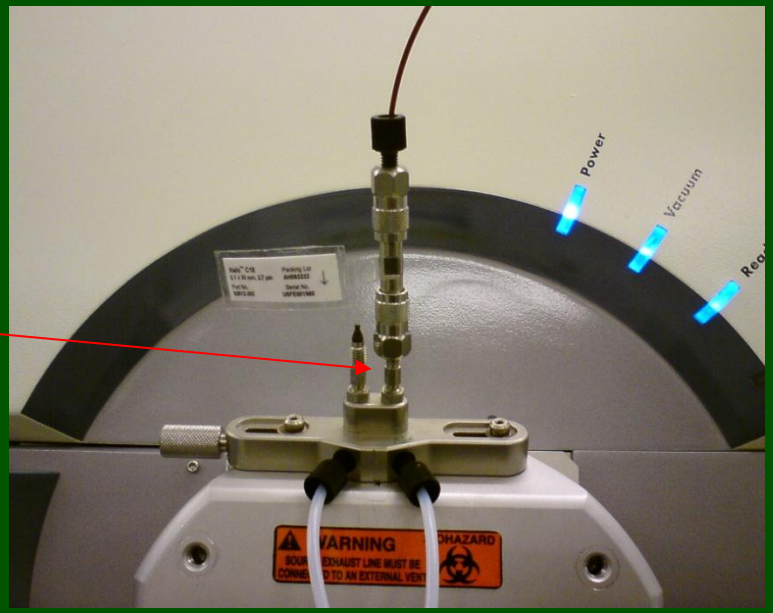
- Micro-LC refers to LC using columns whose inner diameter is ≤ 0.5 mm, commonly 0.3 mm.
- Conventional LC uses columns that are 2.1–4.6 mm in diameter.



Plumbing

To take advantages of the inherent benefits of micro-LC the instrument must have very little dispersion, i.e. extra volume

- Injector
- Interconnecting tubing from injector to column
- Column
- Interconnecting tubing from column to ion source
- Fitting and frits
- Ion souce

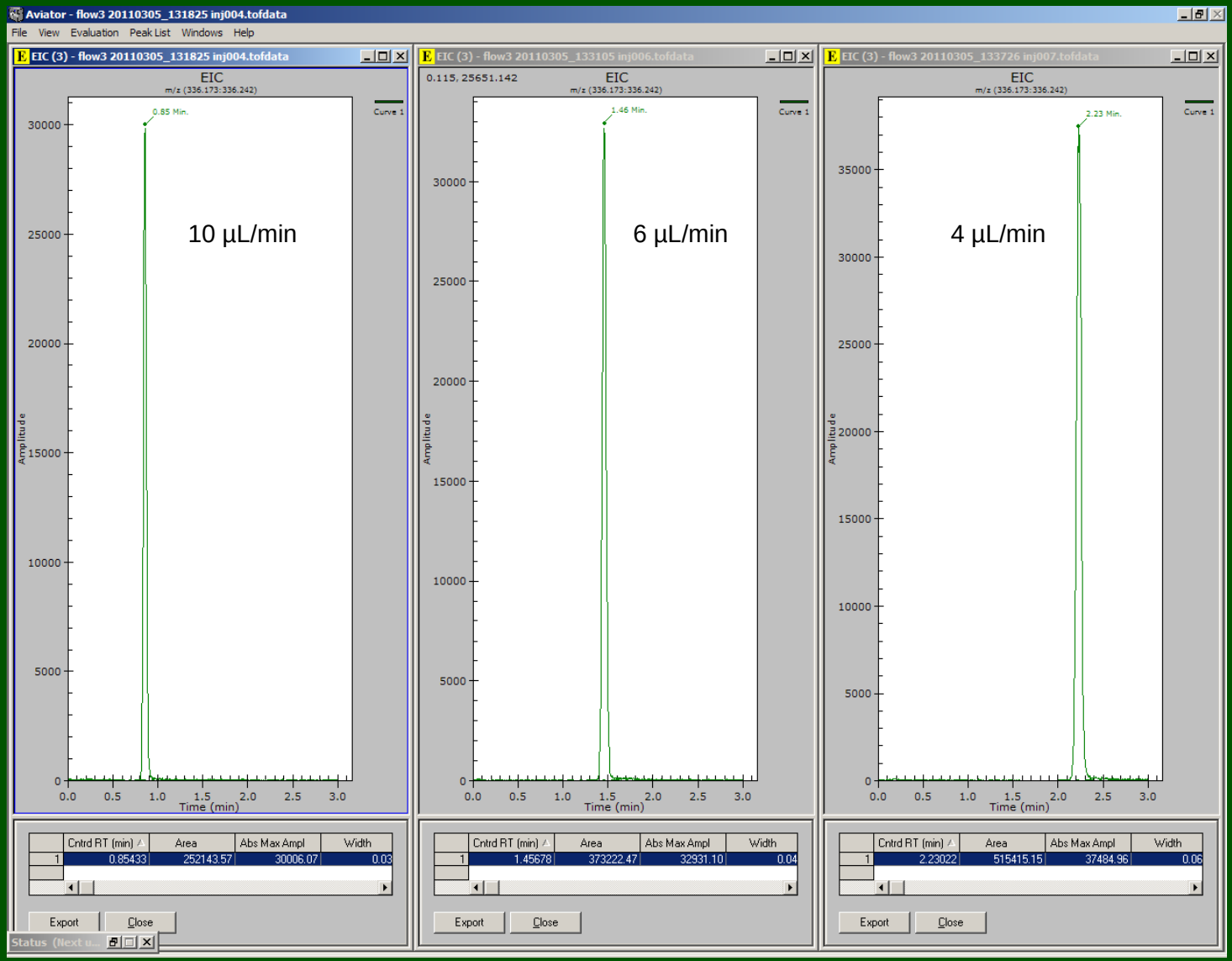


Band-broadening are independent of each other and the peak variance:

$$\sigma^2 = \sigma_{inj}^2 + \sigma_{col}^2 + \sigma_{tubing}^2 + \sigma_{fittings}^2 + \sigma_{ion\text{-}source}^2$$

Detector response at different flow rates

100 nM AZcomp 1 µL inj on Halo C18 0.3x50mm column
Mobile phase: 75% Acn/H2O



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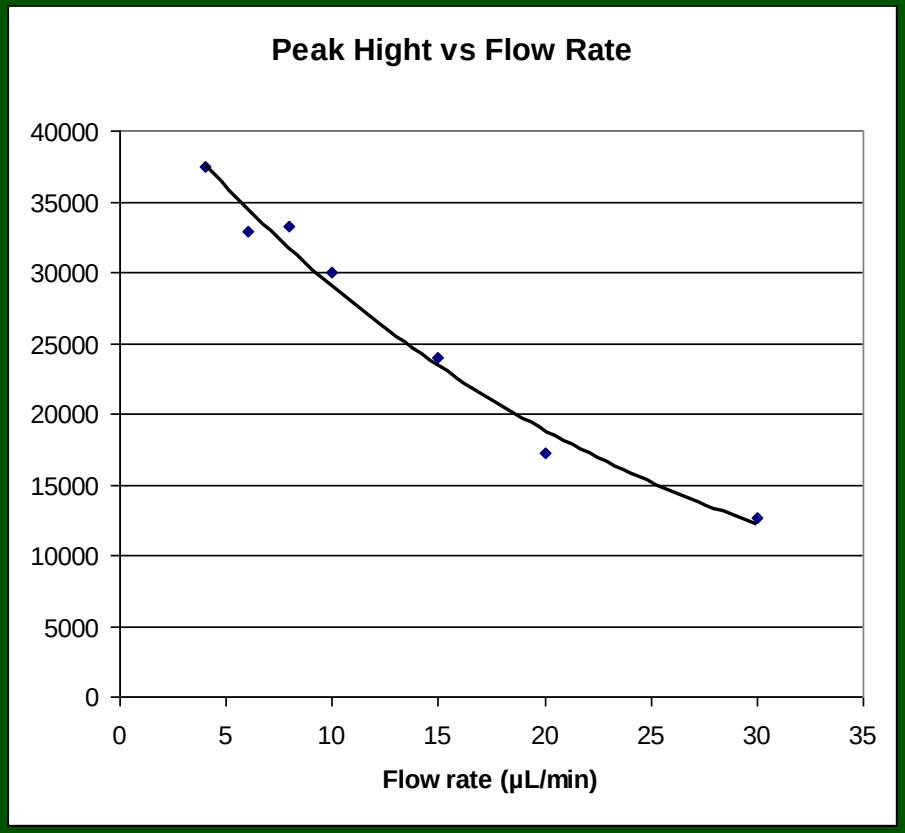
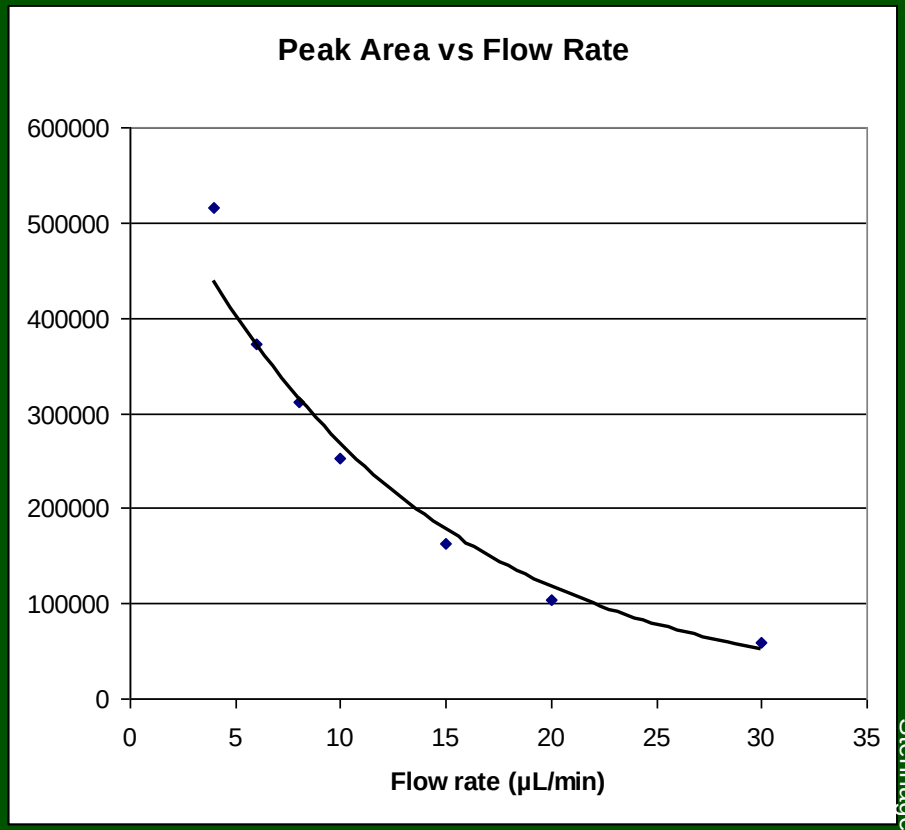
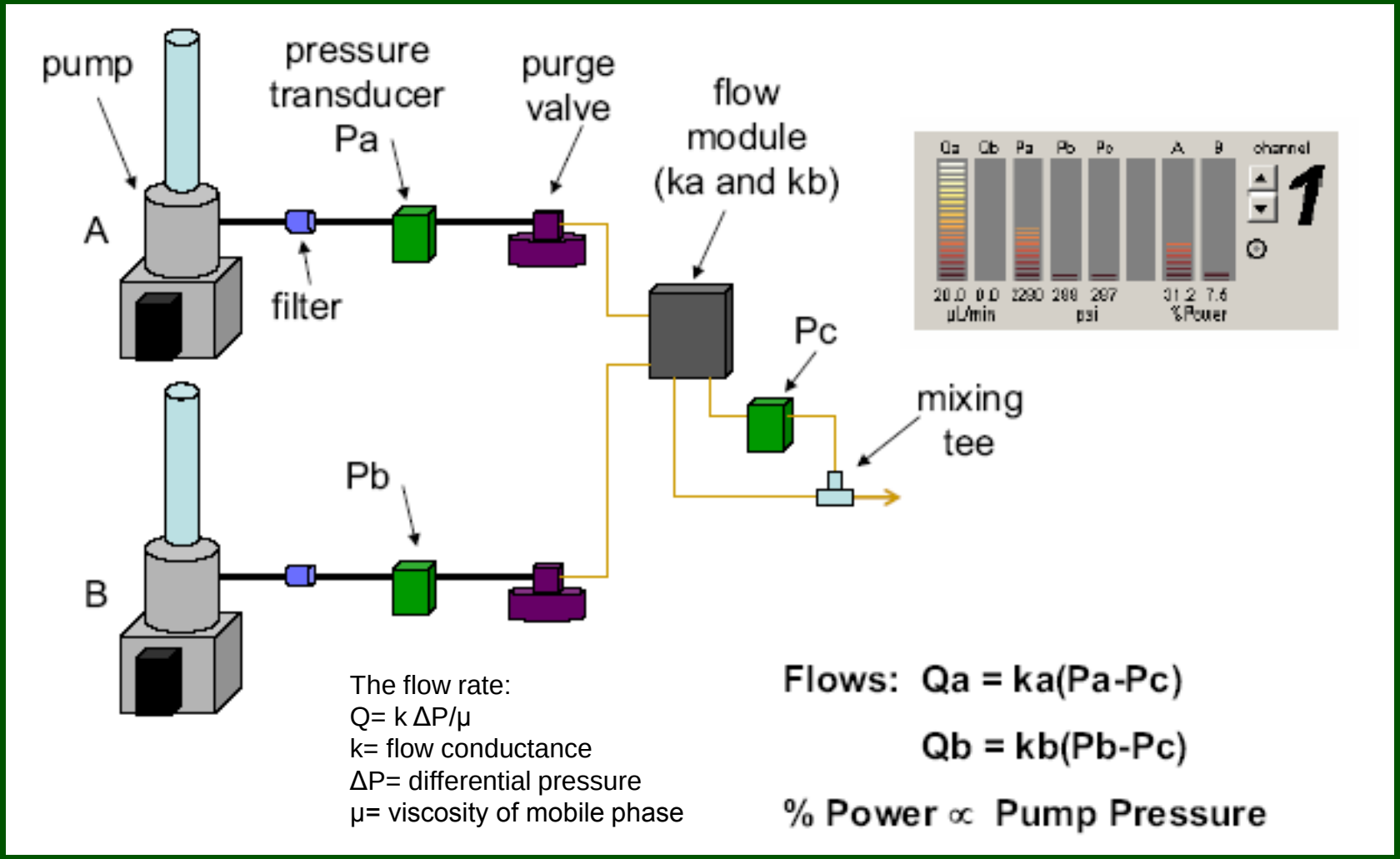


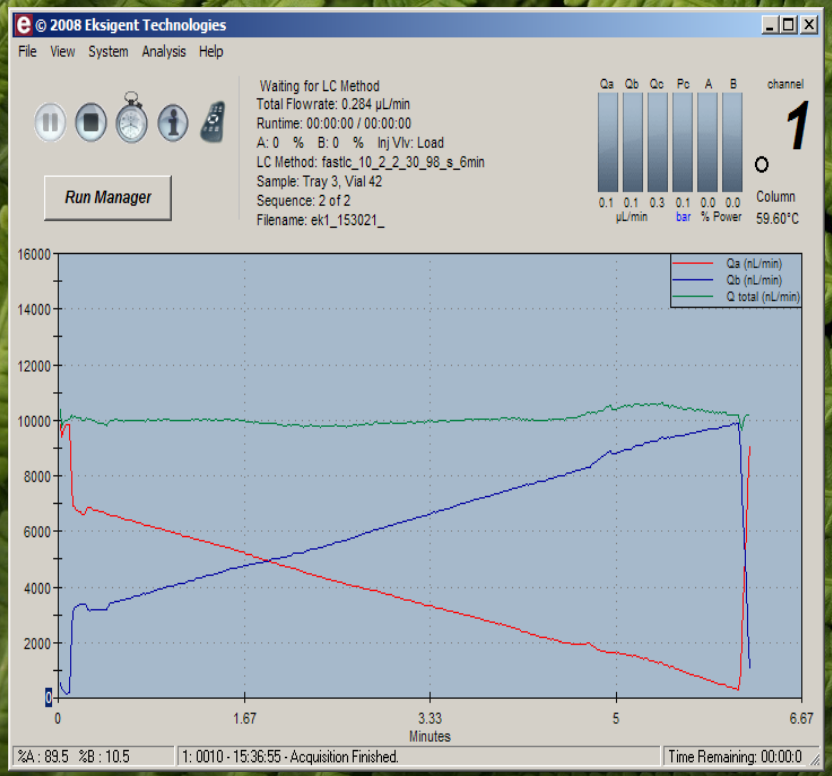


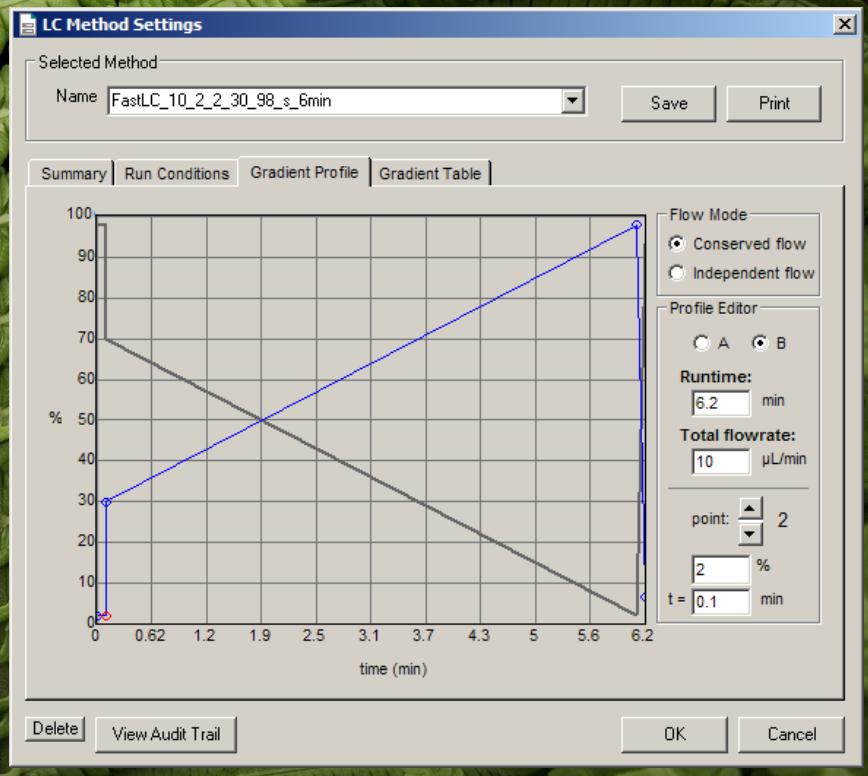
Need accurate gradient at low flow rate



LC gradient system Eksigent ExpressHT

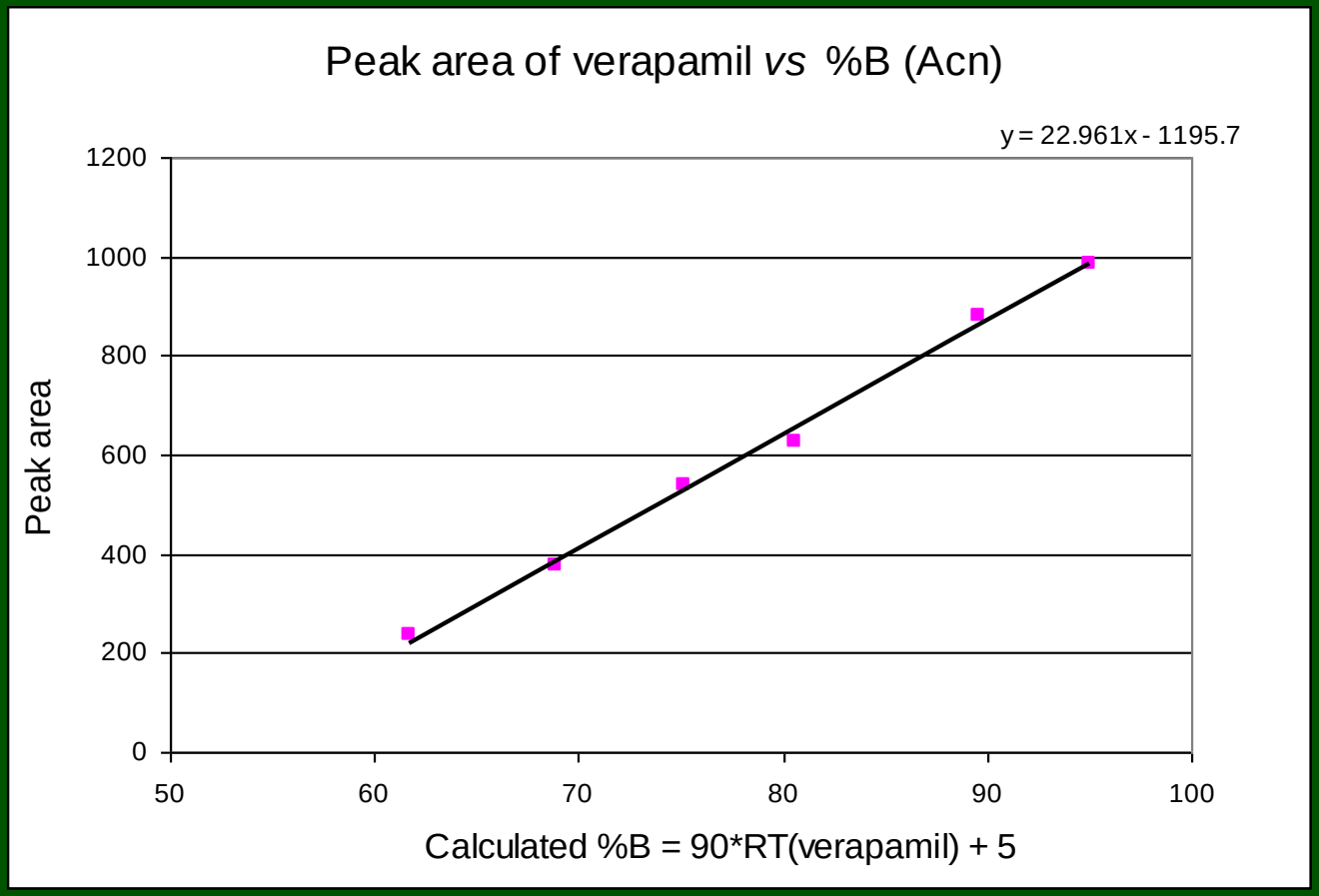
Gradient control





Sensitivity dependent on eluent composition and flow rate

Acquity BEH C18 1.7µm 1x50 mm, flow rate 70-200µL/min, gradient 5-95% Acetonitril/water in 2 min

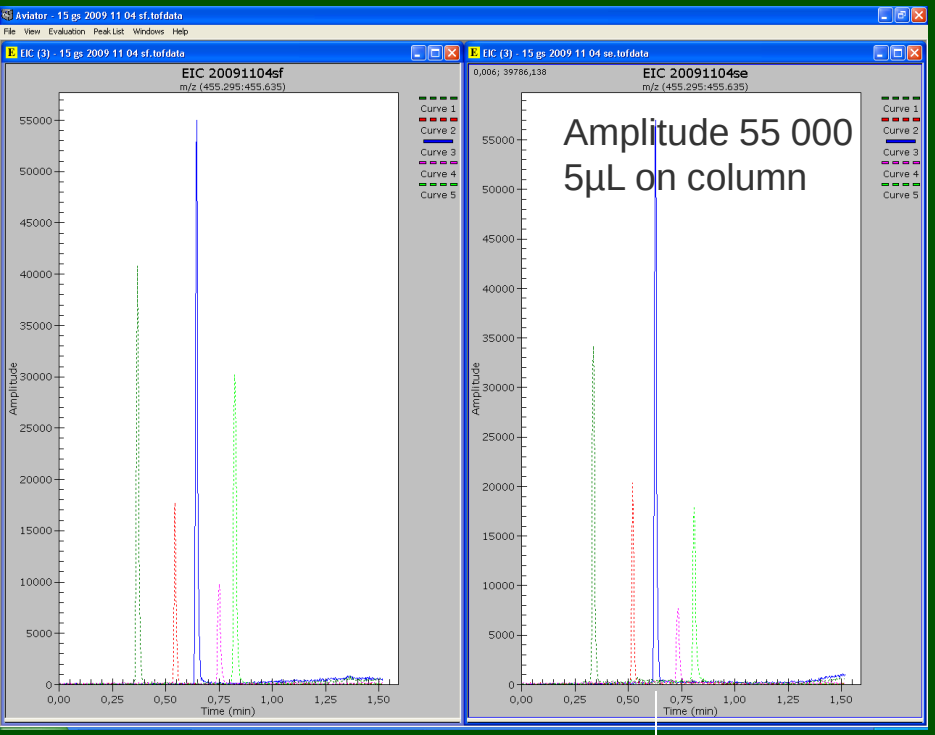


1% change in acetonitril/water composition give 5% larger peak area
Gradient performance are important!

Mass chromatogram (repeated) of control substances

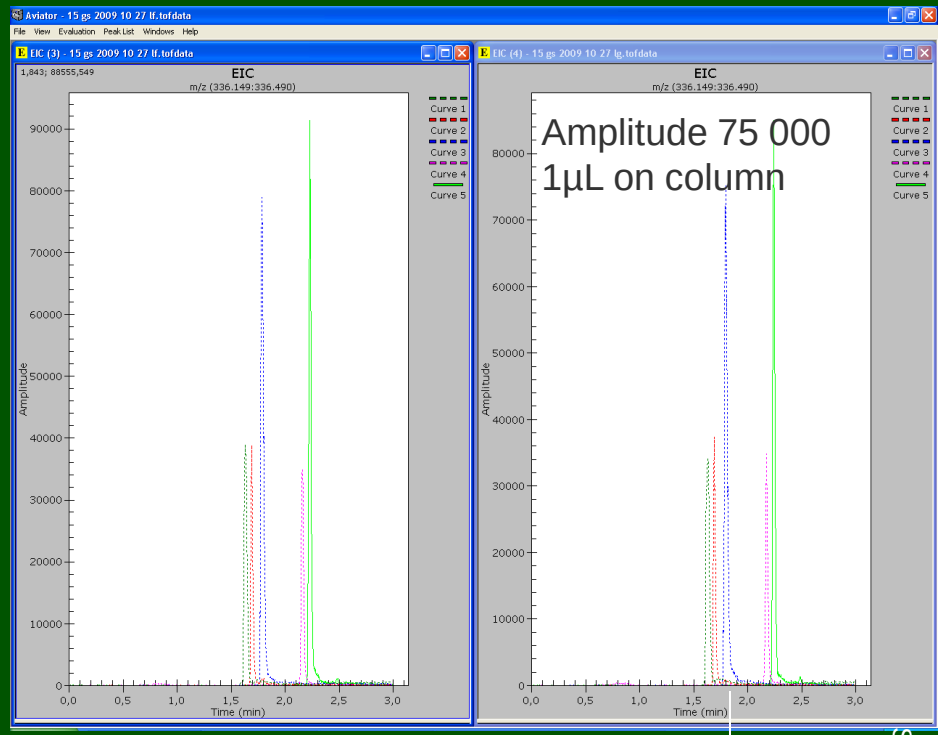
Different eluent flow rates, same solvent composition

Xbridge C18 2.5µm 2.1x20 mm, Flow 700µL/min, Inj: 5µL



Gradient: %B = 90*RT+ 5 77% B

Halo C18 0.3x50 mm, Flow 20µL/min, Inj: 1µL



Gradient: %B = 23*RT+ 30 71% B

More signal with less sample!

Sample preparation

Green Finch

Sample preparation

Protein Precipitation (PPT)

- Aliquot of sample
- Add cold acetonitrile spiked with internal standard
- Vortex
- Centrifuge (20min)
- Remove supernatant
- Reconstitute
- Transfer to plate
- Inject onto LC column

Micro-LC *vs* LC on QQQ

µLC analytical method

Mass spectrometer:	Waters Quattro Premier
LC gradient system:	Eksigent ExpressHT
Gradient:	2-70% Acetonitrile/H2O in 2min
Auto sampler:	CTC/PAL
LC column:	Eclips XDB -C18 3.5µm 1x50mm
Column temp.	20 C
Mobile phase flow:	**40 µl/min**
Injection volume:	1 µl

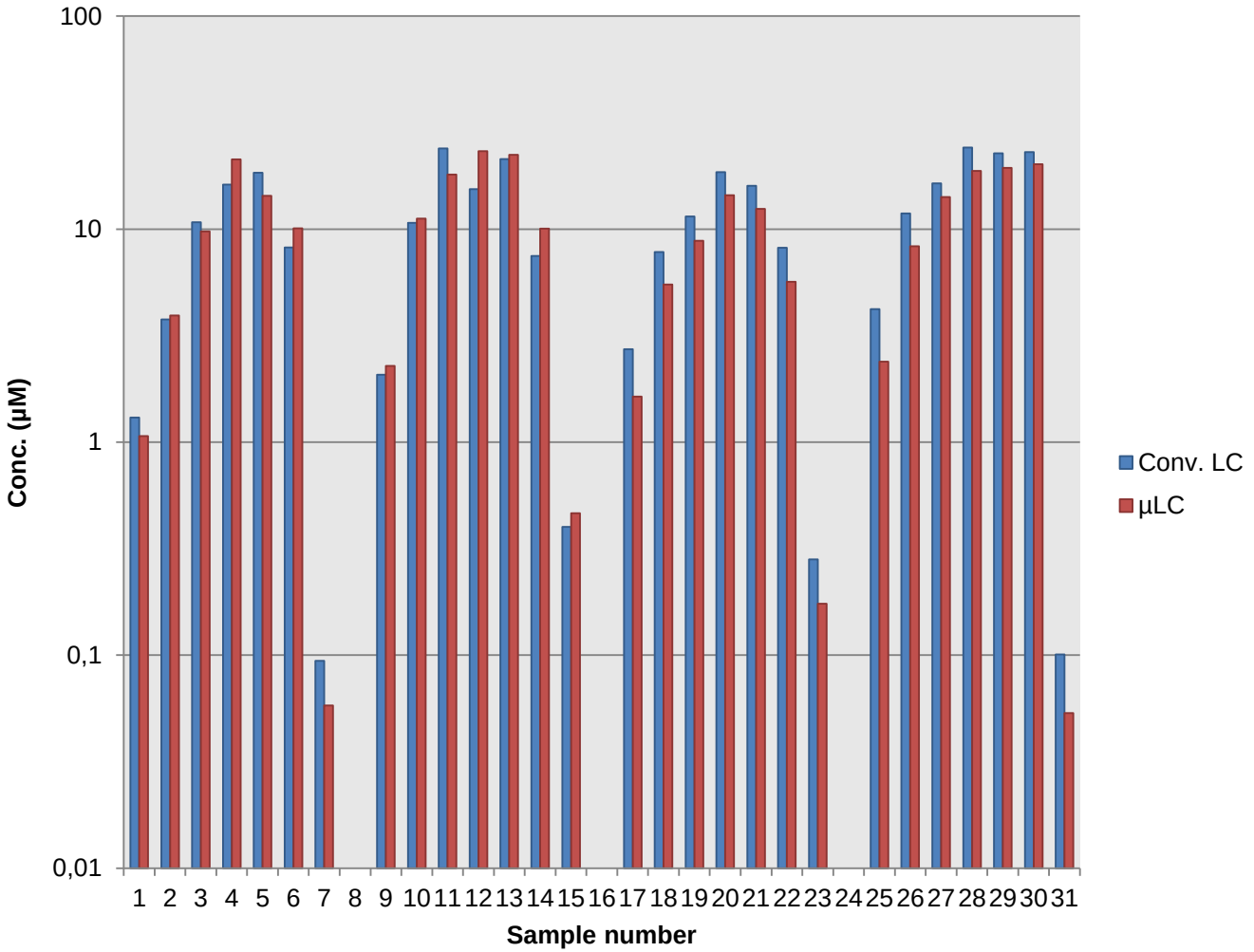
Conventional LC analytical method

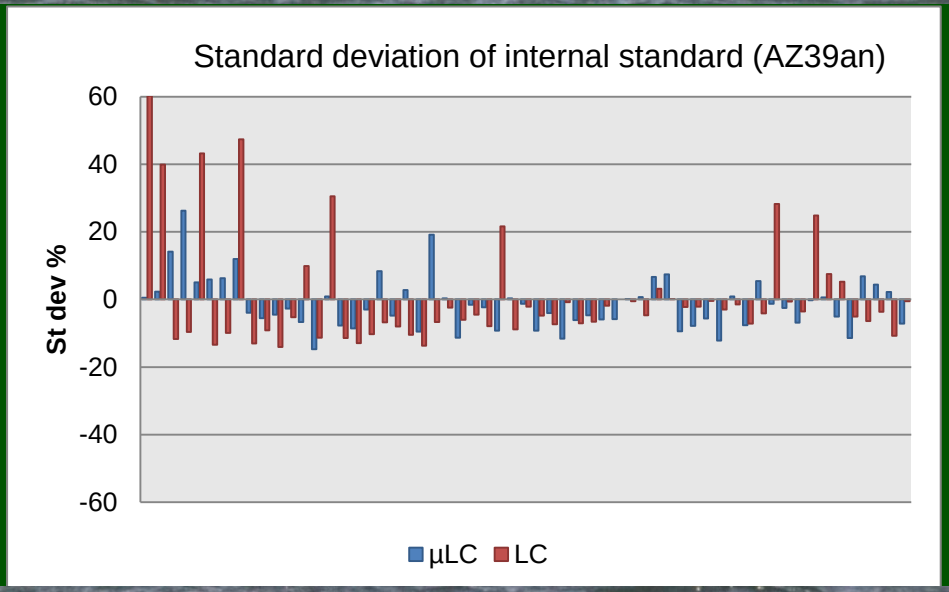
Mass spectrometer:	Waters Quattro Premier
LC gradient system:	Shimadzu LC10 AD
Gradient:	5-95% Acetonitrile/H2O in 1.5 min
Auto sampler:	CTC/PAL
LC column:	Halo C18 2.7µm C18 2.1x30mm
Column temp.	20 C
Mobile phase flow:	**700 µl/min**
Injection volume:	1 µl

Same QQQ tune settings

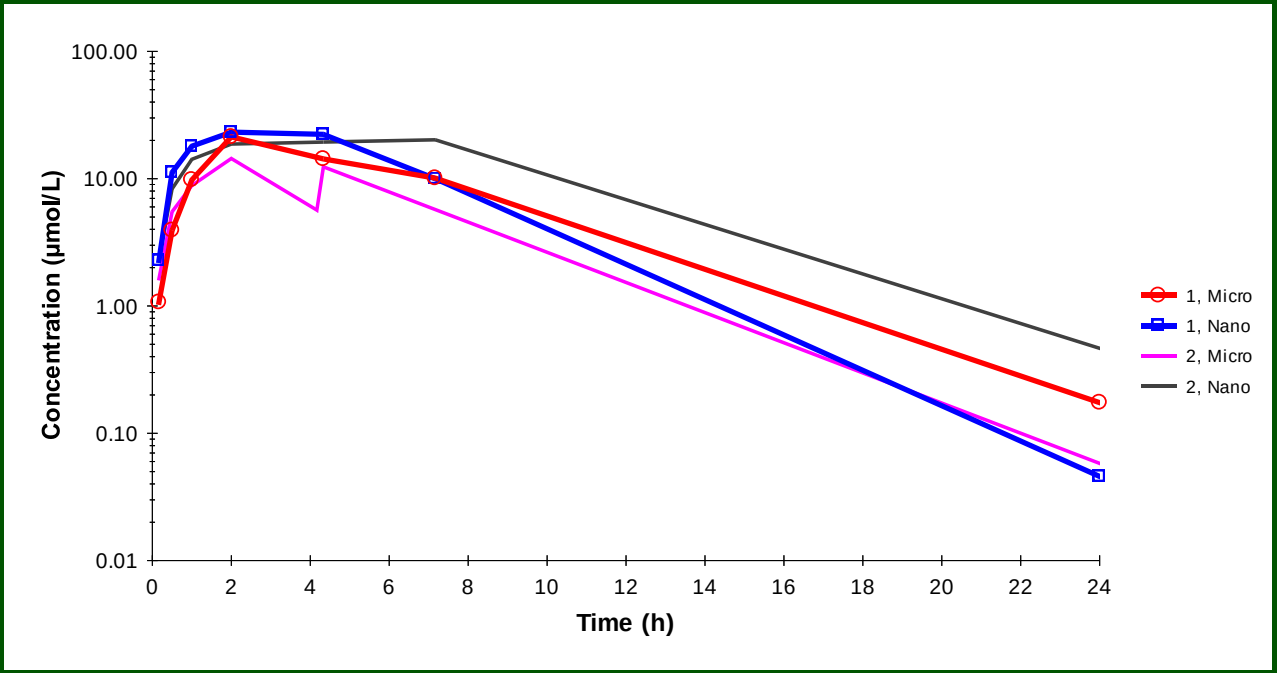
Comparison of samples run on µLC- and conv.LC-systems connected to the same QQQ

Plasma concentrations (µM) after oral dose (rat) AZcomp





Individual plasma concentration after oral dose in rats wit micro- and nano suspension



Quantitative Bio-Analysis Using Micro-LC and Time of Flight MS

GS 2010

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GS 2010

William Penn on top of Philadelphia city hall

Pen-TOF (PerkinElmer)

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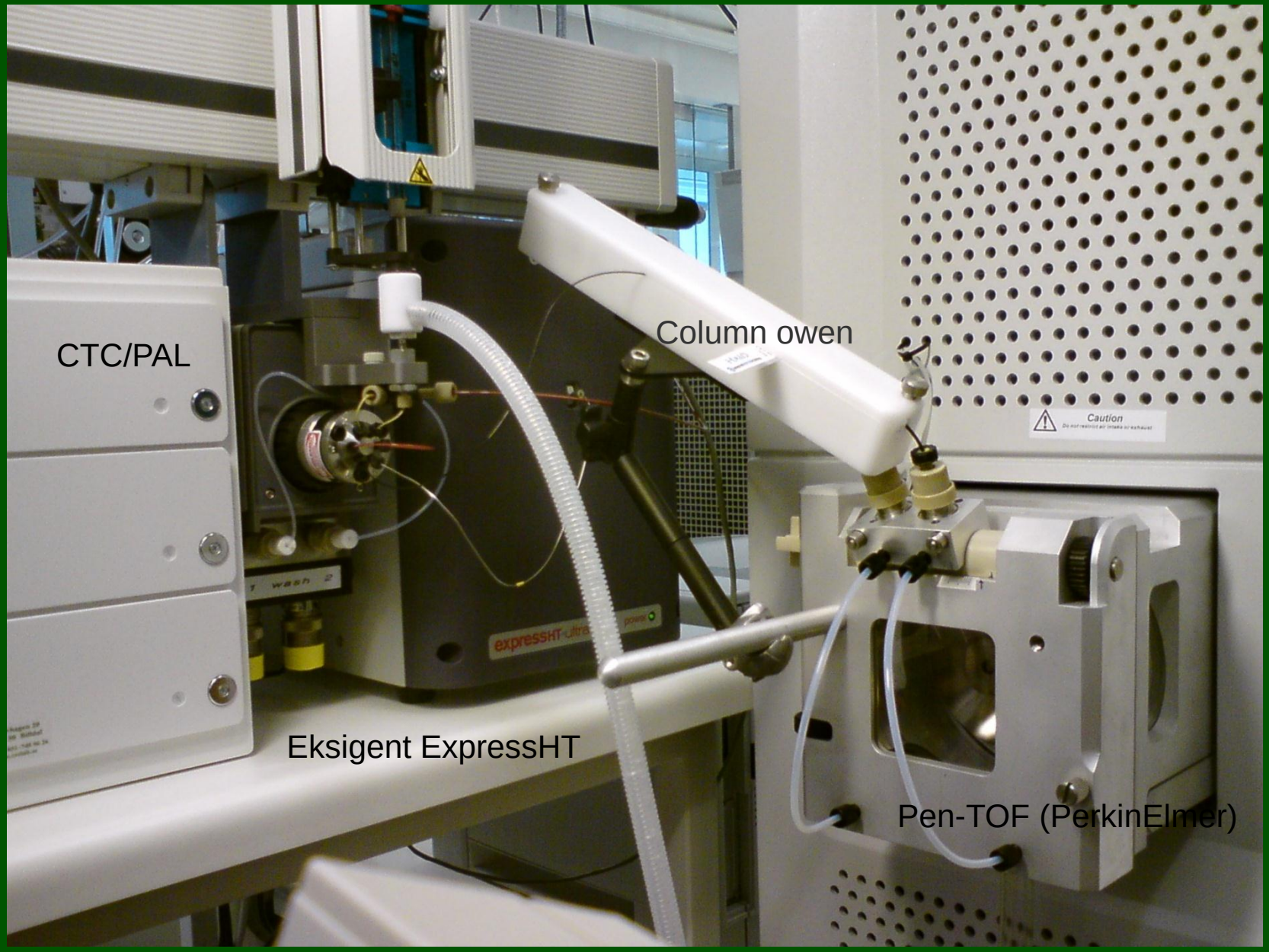
PenTof with microLC



Resolution

Quarters for the night, remote birds 2 km Nikon 500/4 1/100s

GS 2011

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Selectivity
GS 2000
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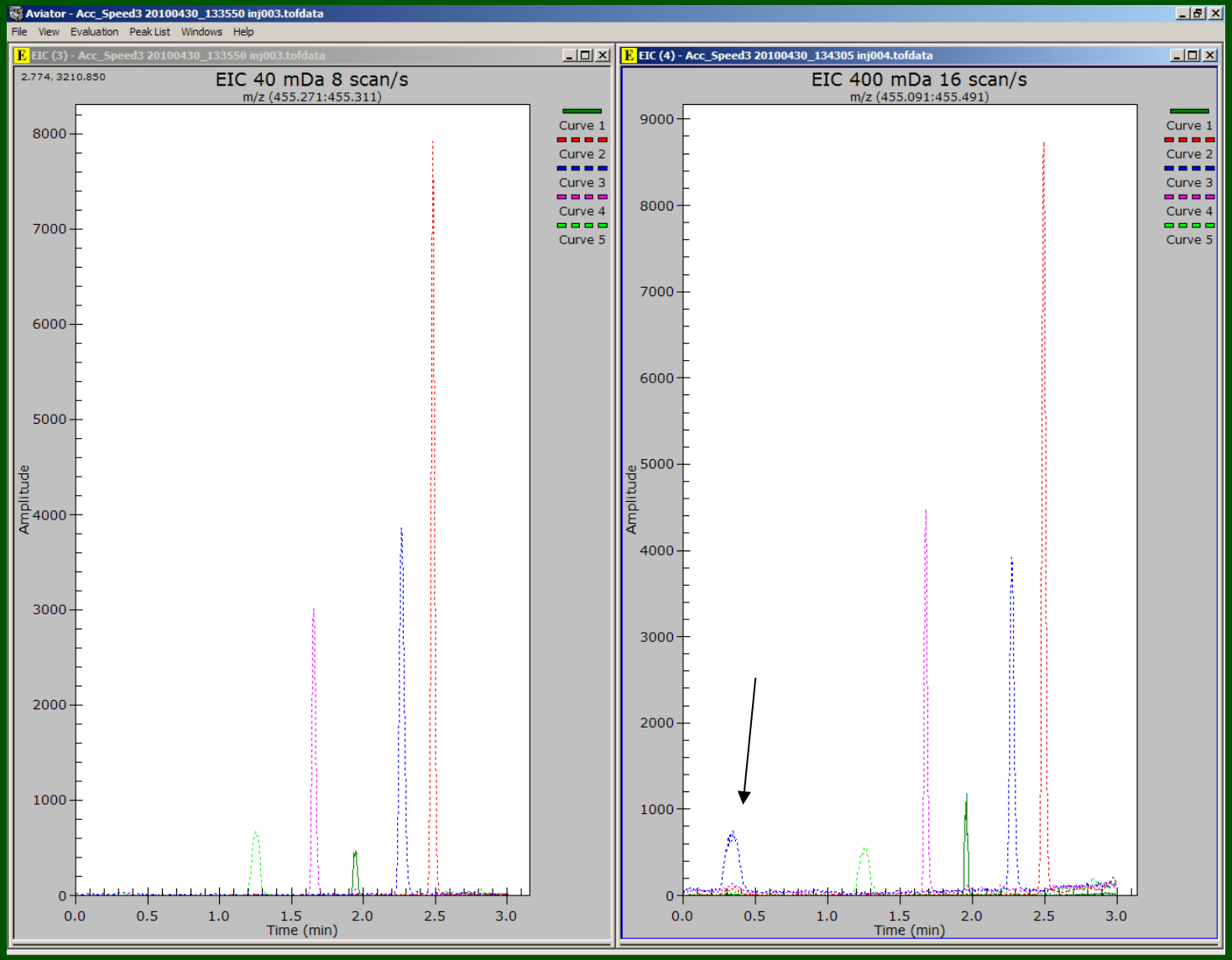
Selectivity by narrow mass window

Mass window 40mDa

Mass window 400mDa



Sensitivity

Admiral butterfly

GS 2010

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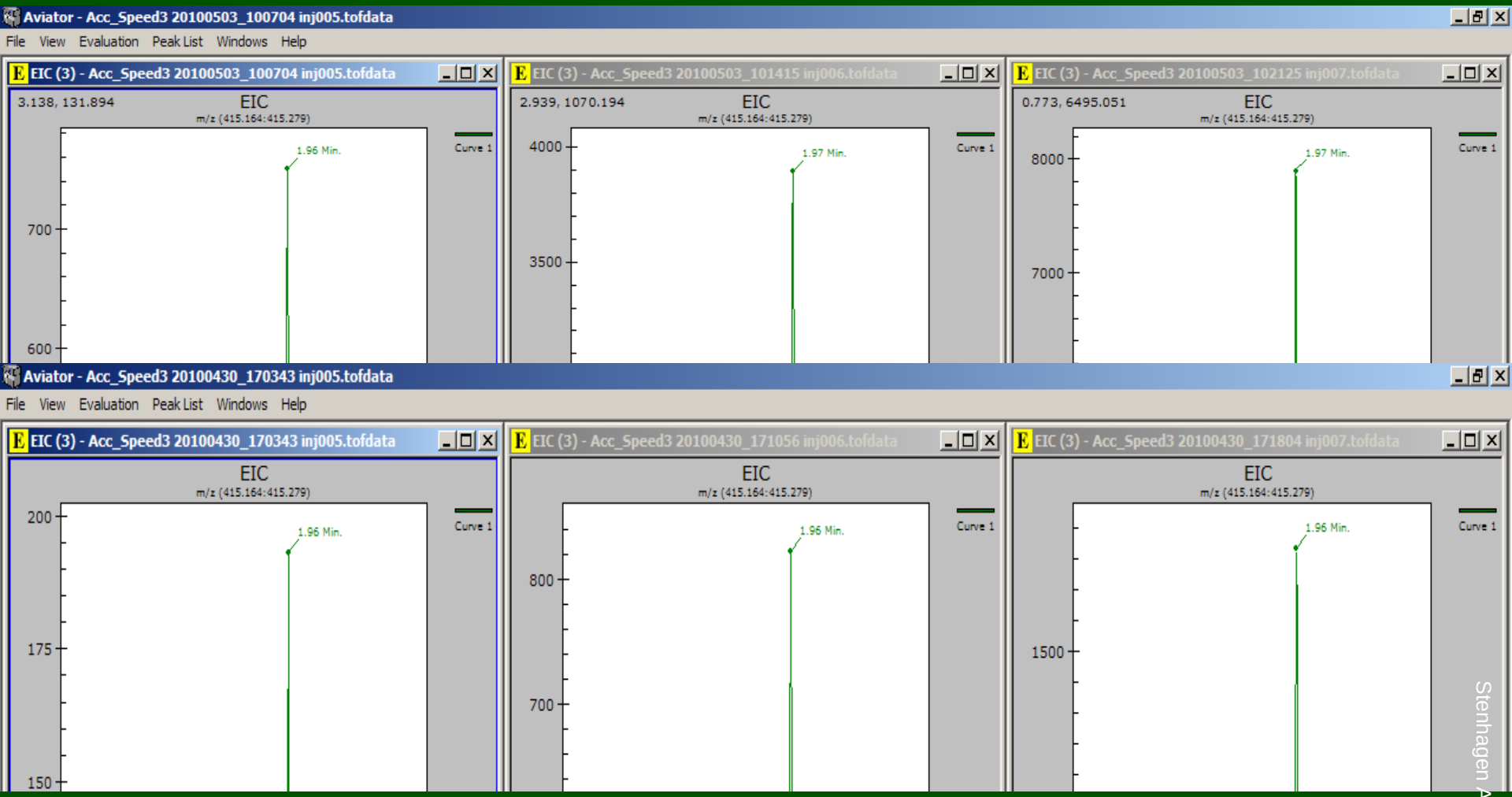
Sensitivity by trap enhencement, 5 times



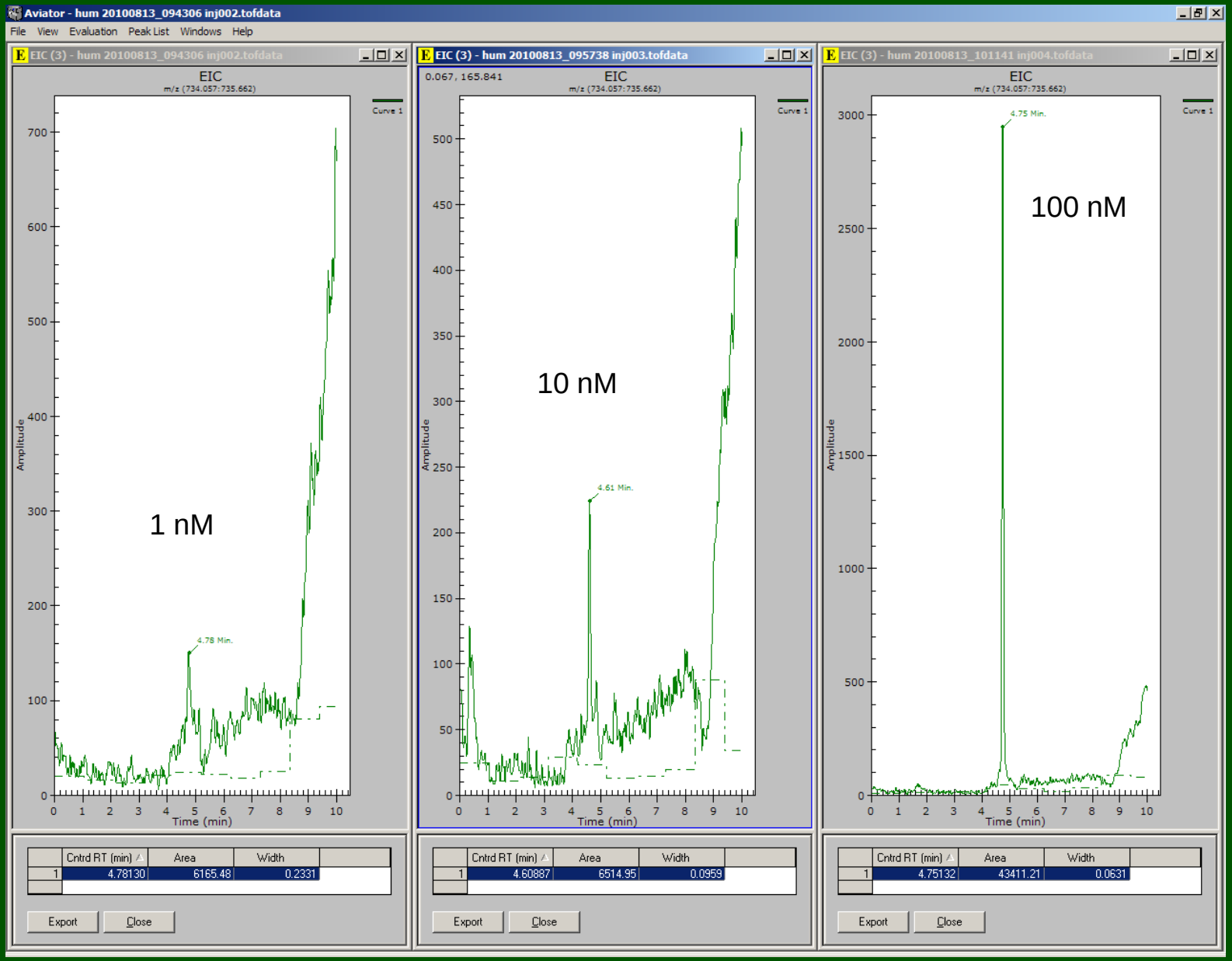
1k nM

5k nM

10k nM

24-Peptide in buffert

Halo C18 p 0.3x150mm, **10µl/min**, 39-95% Acn/H2O 0.2% FA 10min, 1µl inj



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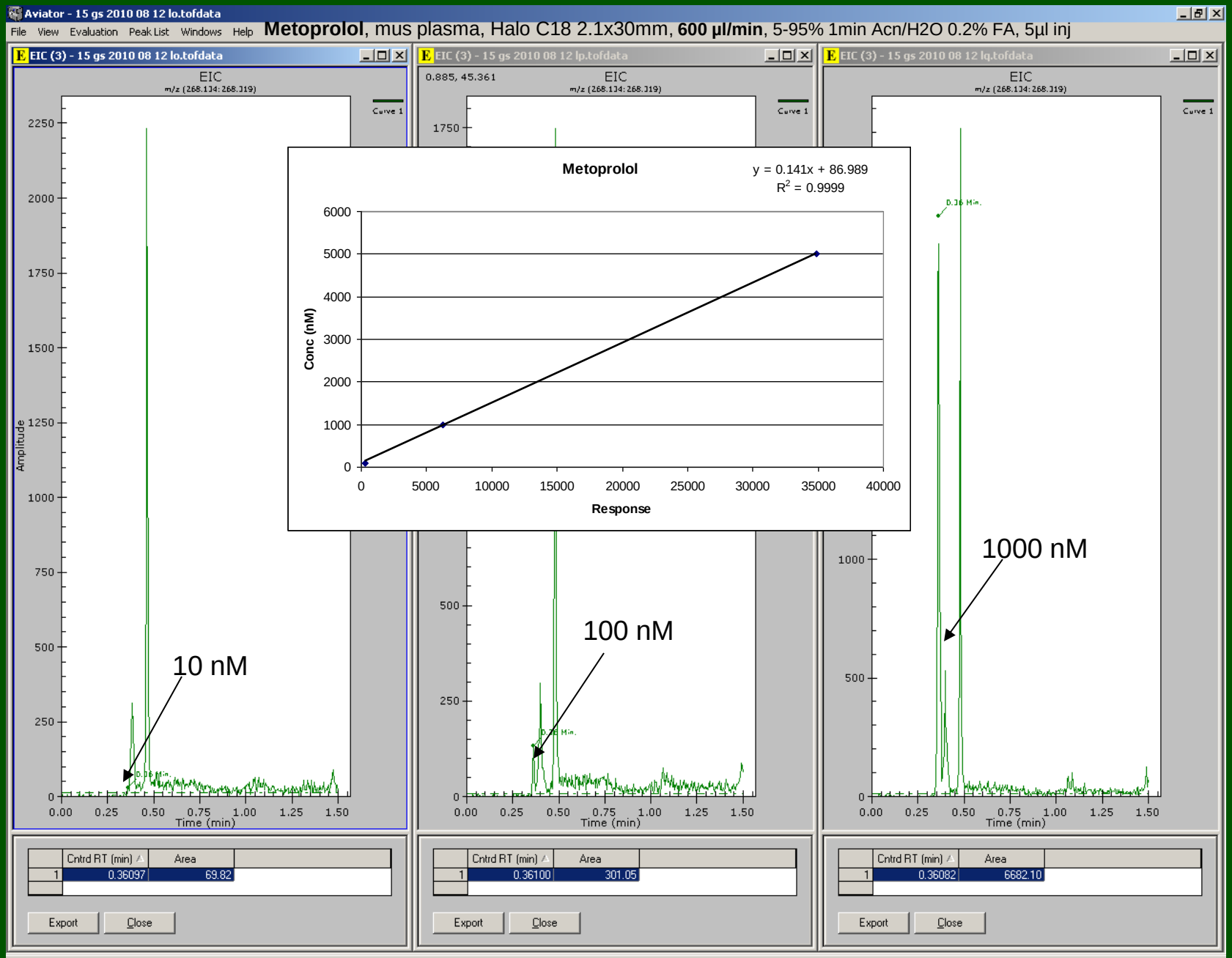
Conventional LC 0.6 mL/min TOF-MS



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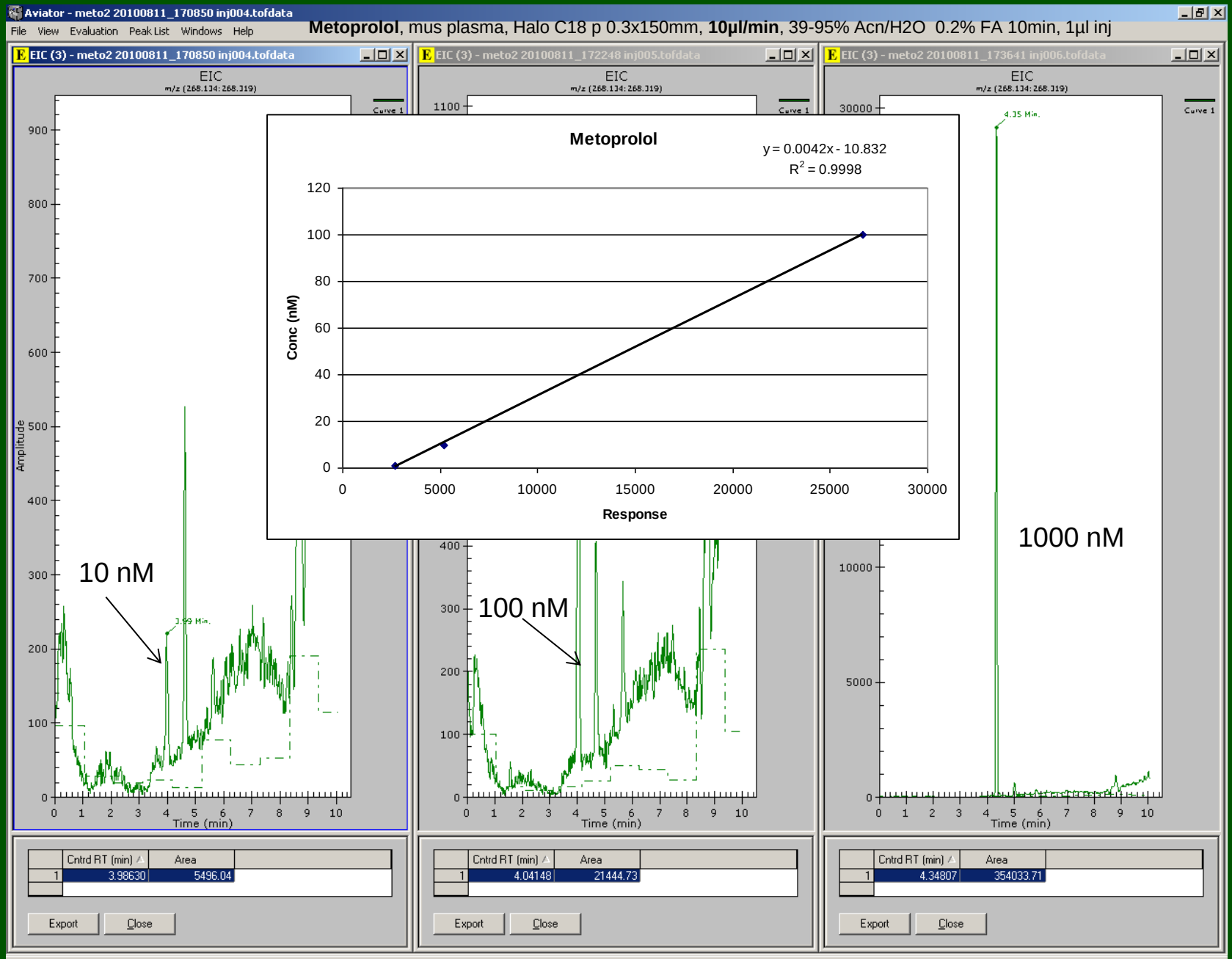
Micro LC 10µL/min TOF MS

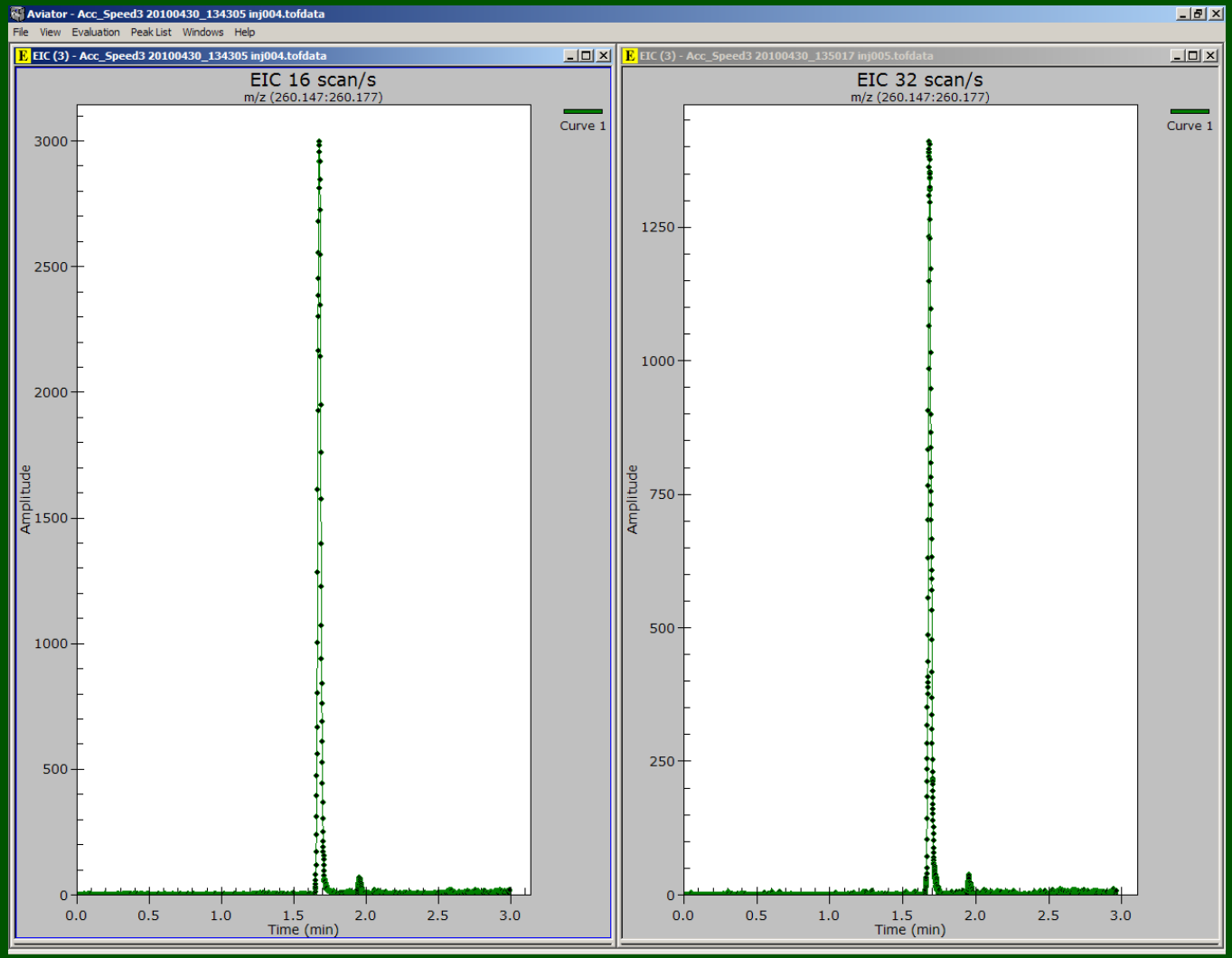


Scan speed

M.G. TD 2-seat Sports 1952
British racing green or BRG

Scan speed

Column: Halo C18 50x0.3mm, 20µl/min




Linear Dynamic Range
GS 2009
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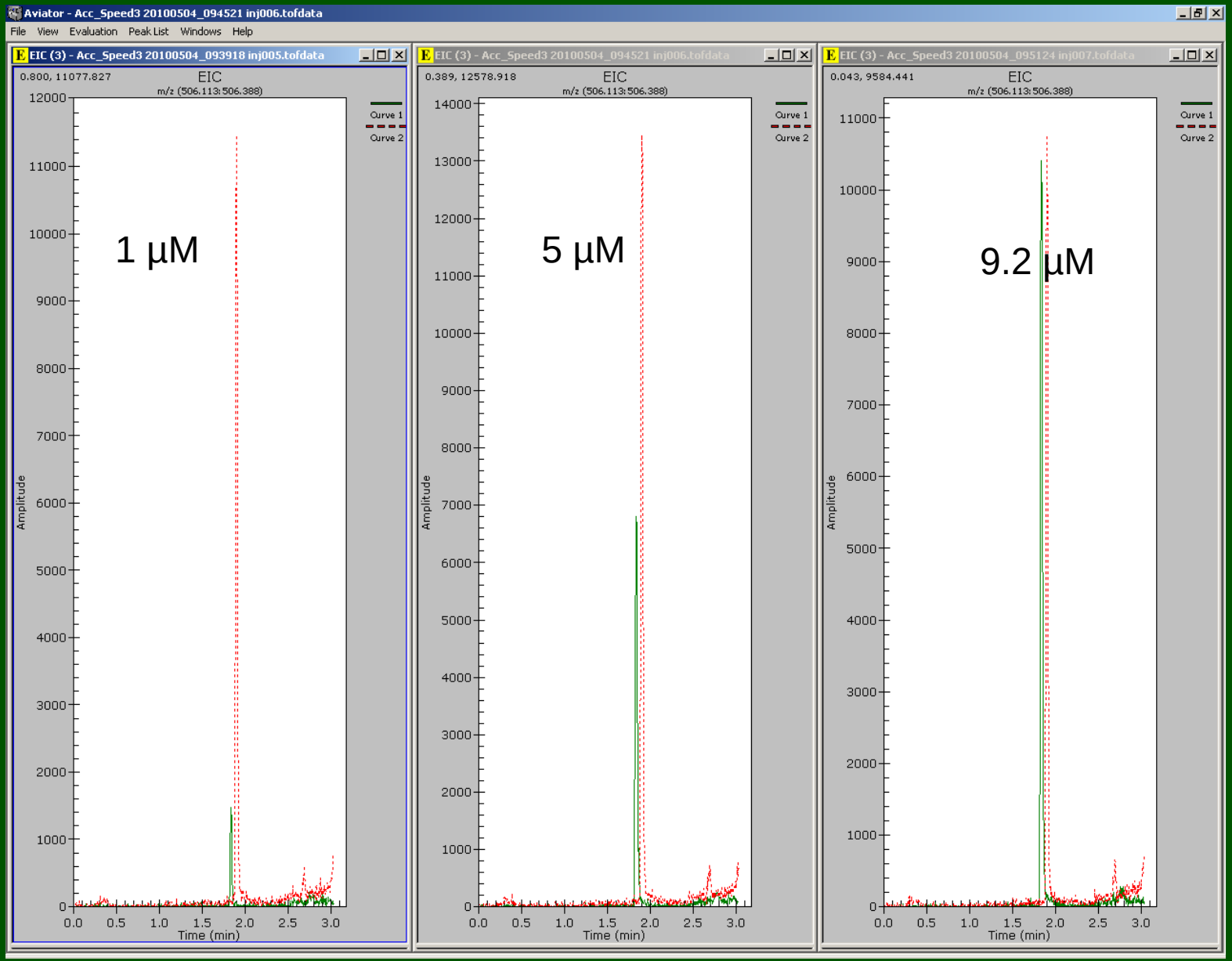
AZComp (brain) Halo C18 50x0.3mm 20µL/min



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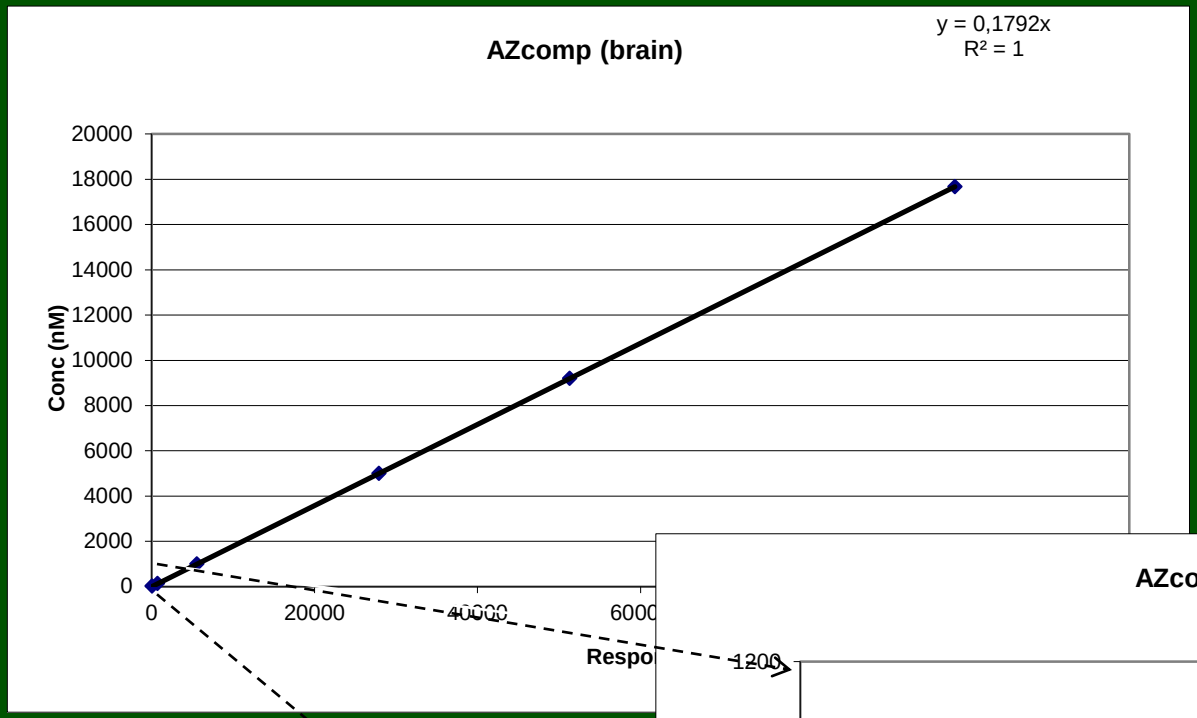
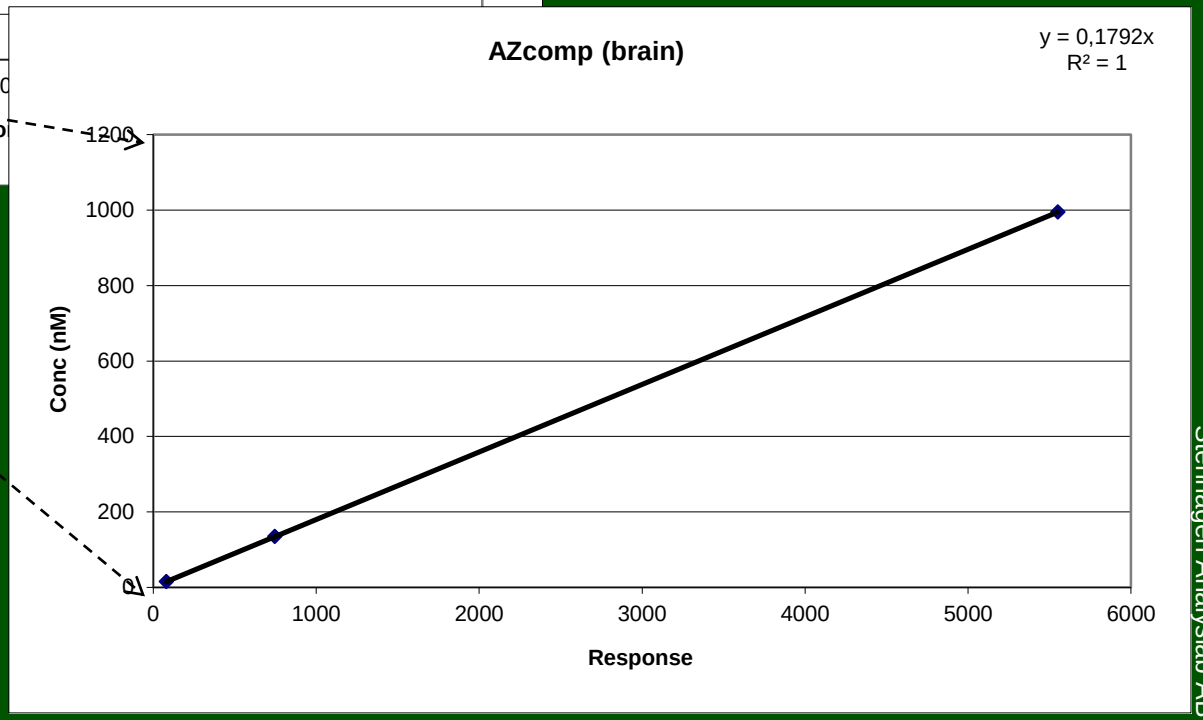
Linear Dynamic Range





Sample preparation 2

squirrel

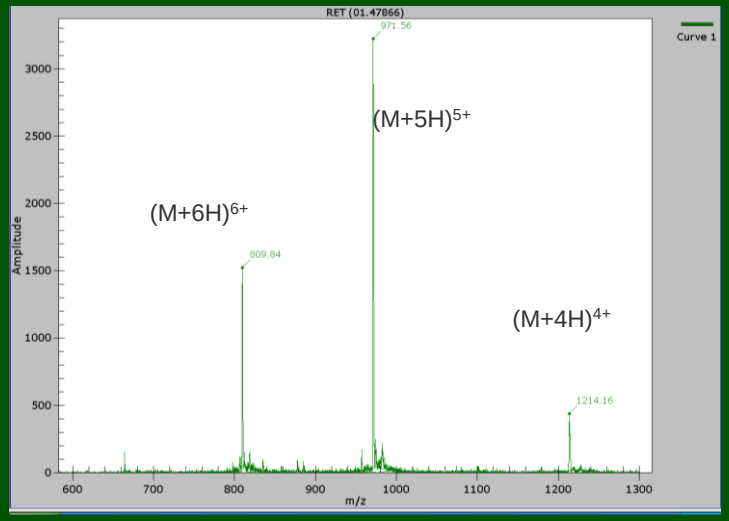
Peptide Sample Preparation

Protein Precipitation (PPT)

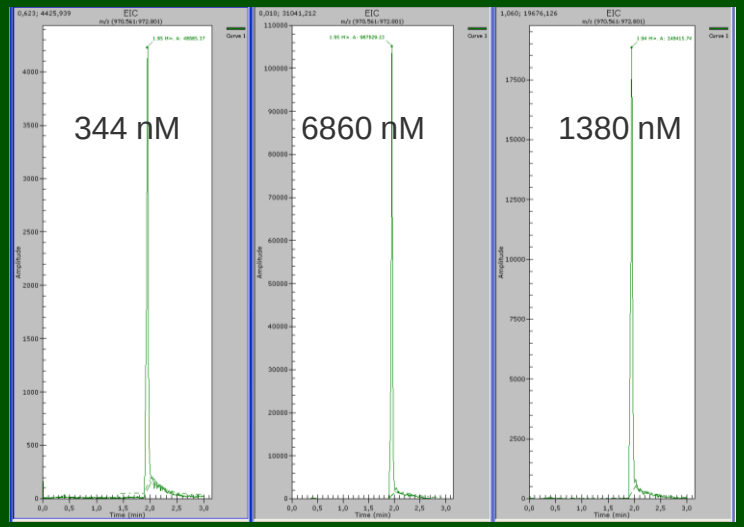
- Aliquot of sample
- Add cold ethanol spiked with internal standard
- Vortex
- Centrifuge (20min)
- Remove supernatant
- Reconstitute
- Transfer to plate
- Inject onto LC column

4850 Da Peptide

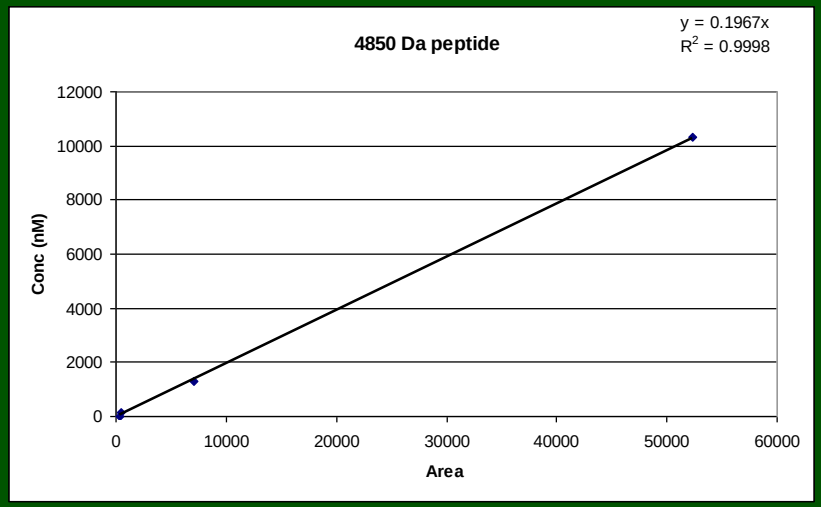
Mass spectrum



Mass chromatogram (0.3x50mm LC column)



Calibration curve (CSF)



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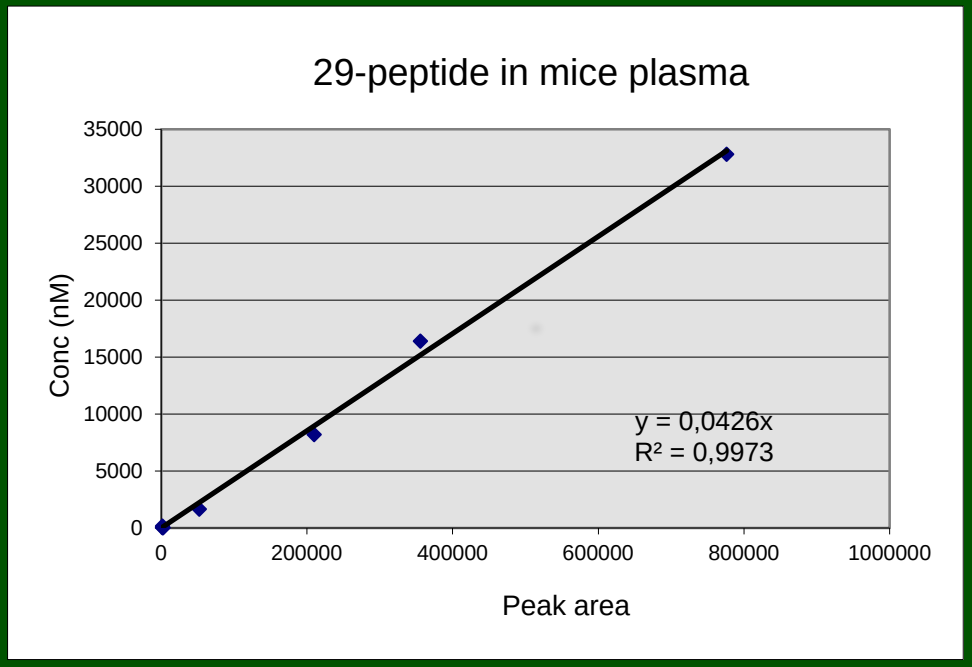
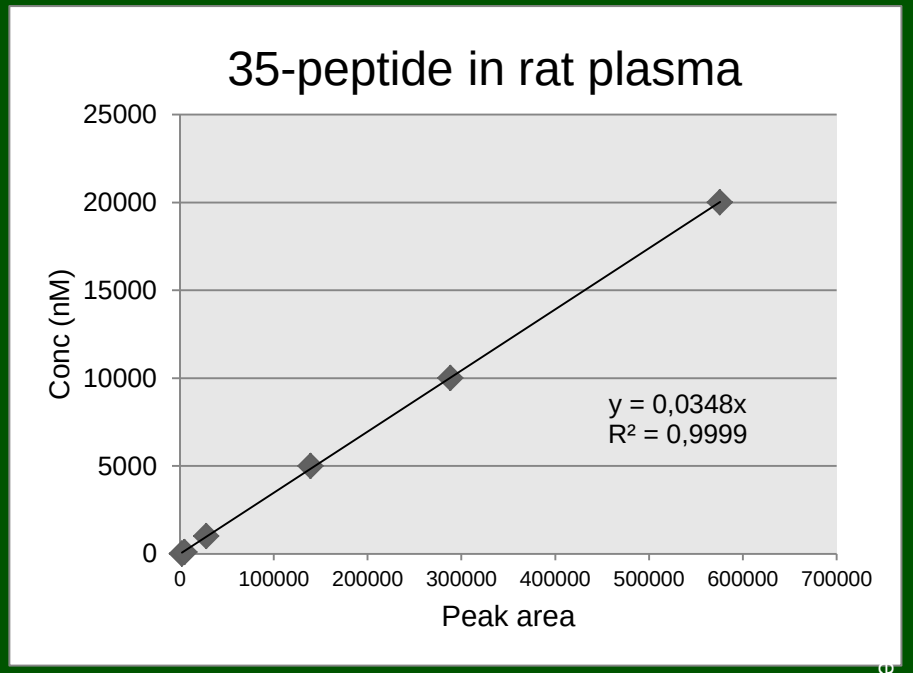
Linear Dynamic Range

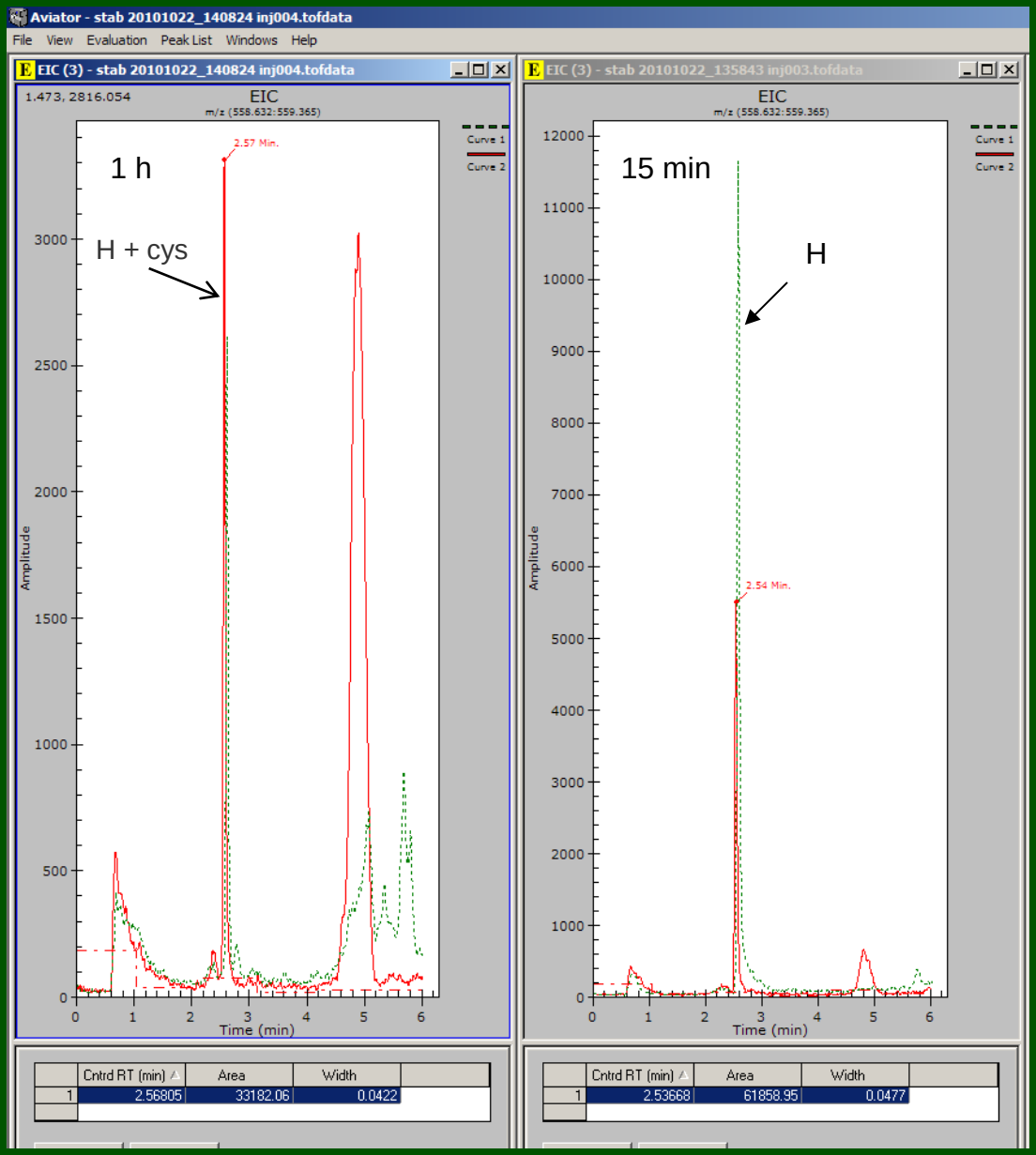


5 – 32 000 nM

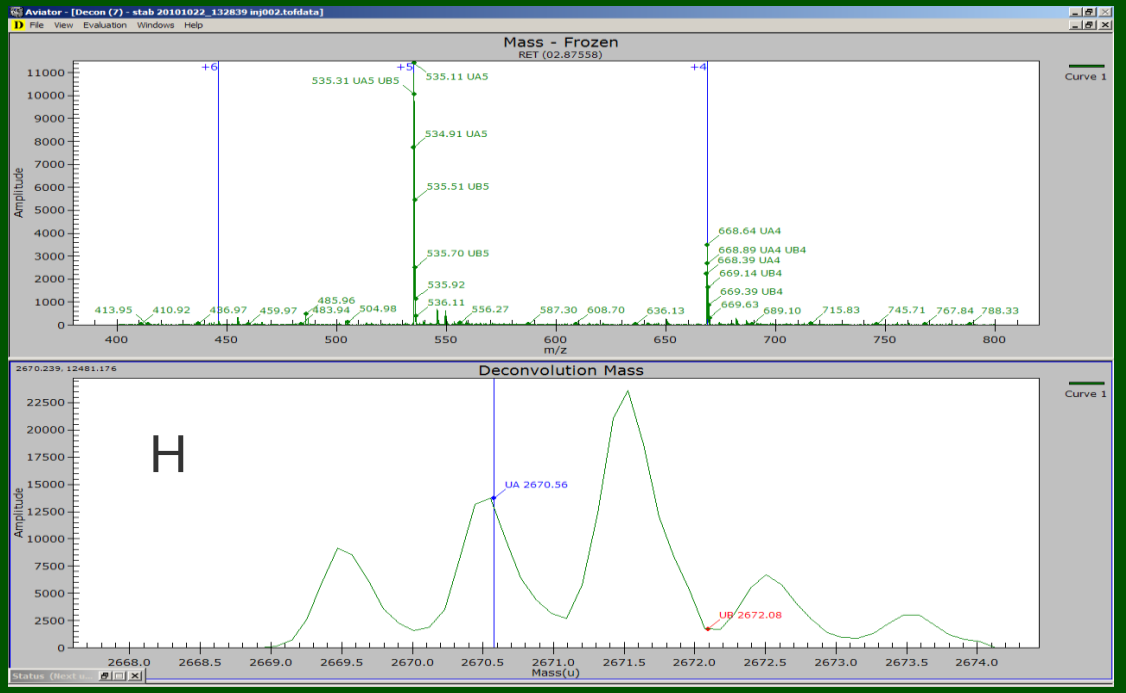


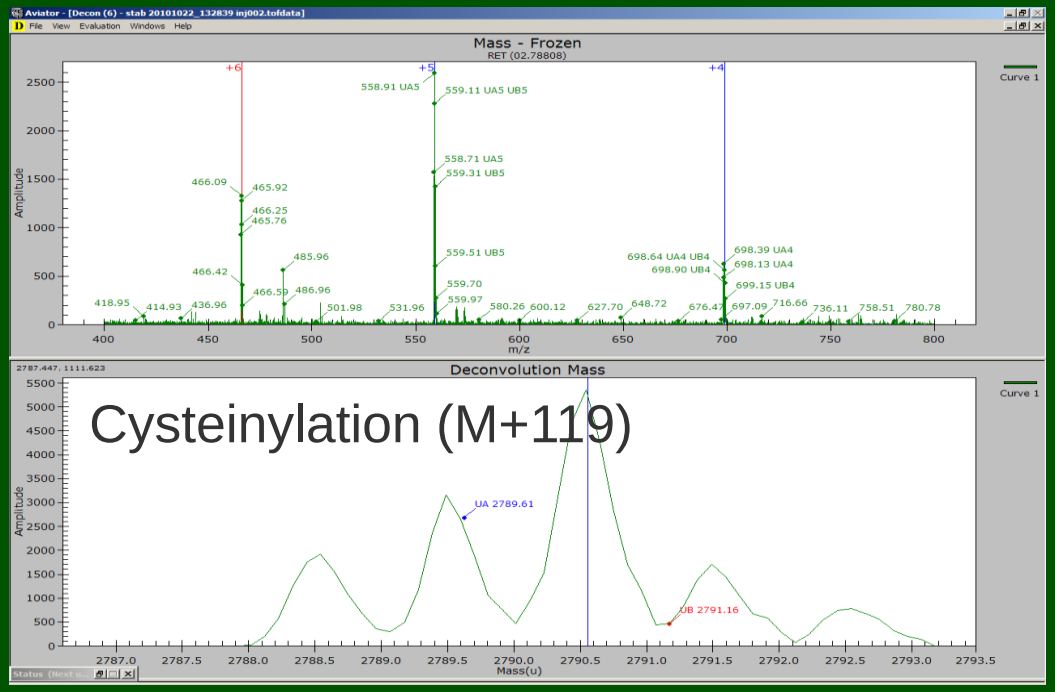
10 -20 000 nM

Mass chromatogram of H-peptide (stability in plasma)



Cysteinylation* of peptide in plasma

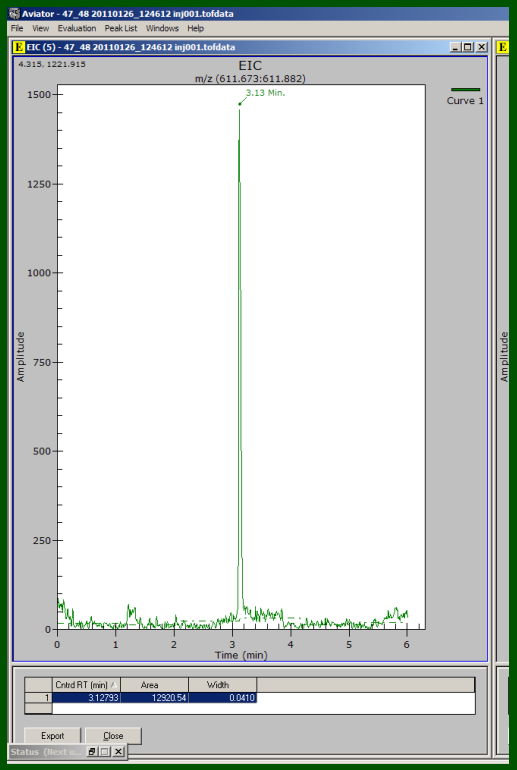




*This was confirmed by adding DTT to plasma

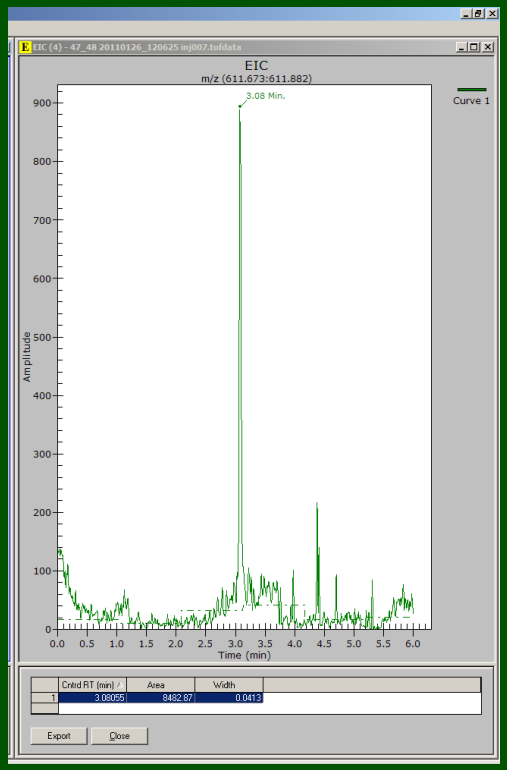
Protein Precipitation (PPT) of Peptide Sample with Heavy Metal

Buffer



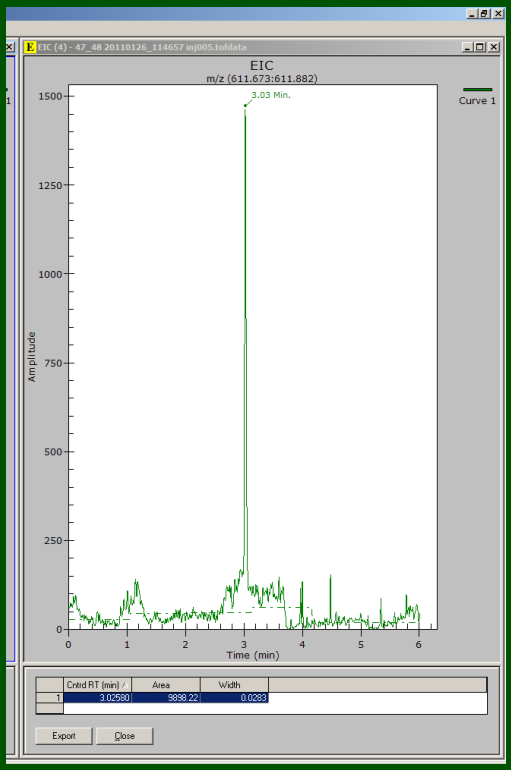
50µL Buffer 100nM peptide
Add 150µL 33% acetonitrile
Vortex
Inject onto LC column

Acetonitrile



50µL Plasma 100nM peptide
Add 150 µL cold acetonitrile
Vortex
Centrifuge (20min)
Remove supernatant
Reconstitute
Transfer to plate
Inject onto LC column

$ZnSO_4$

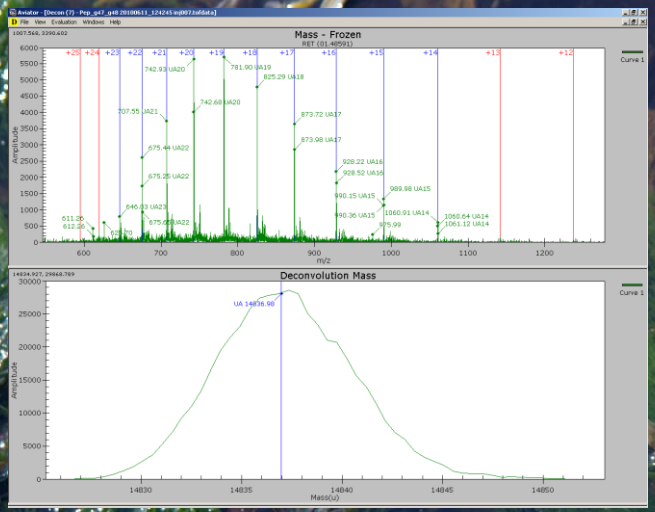


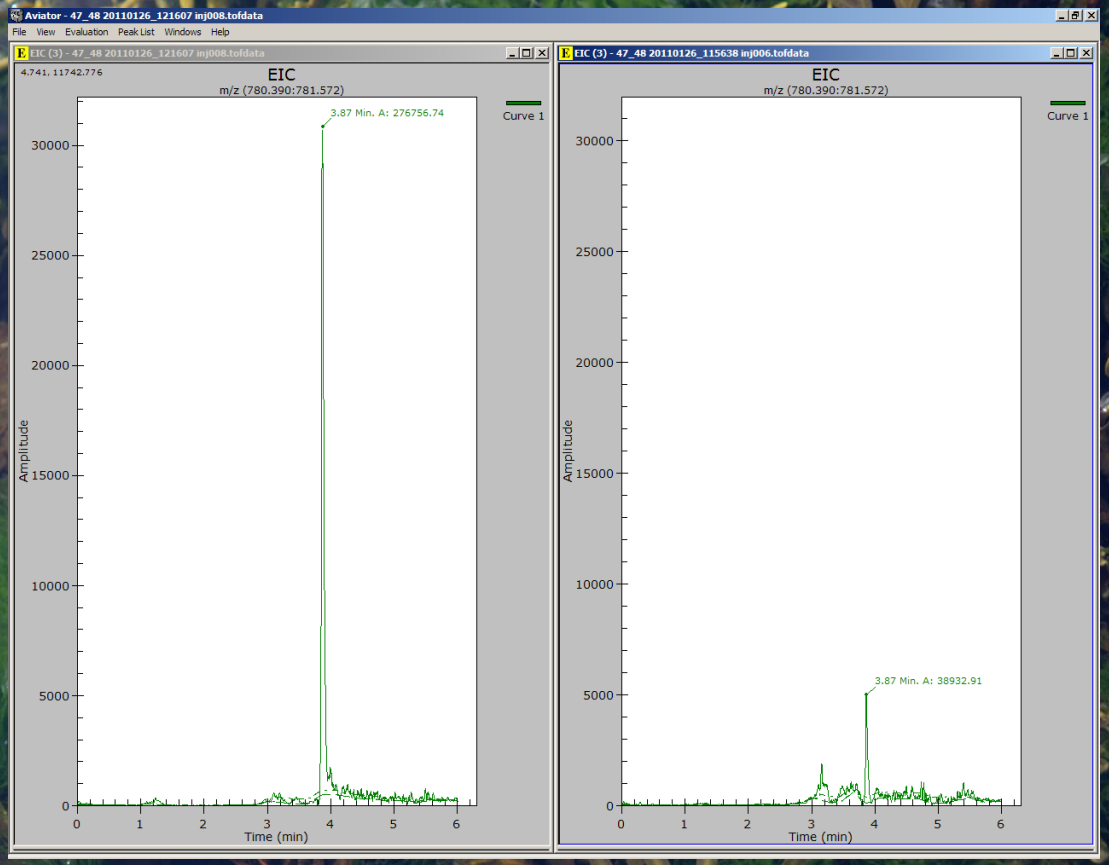
50µL Plasma 100nM peptide
Add 25µL 5% Ammonium hydroxide
and 125µL 10% ZnSO4
Vortex
Centrifuge (20min)
Remove supernatant
Reconstitute
Transfer to plate
Inject onto LC column

PPT Acetonitril

PPT $ZnSO_4$

14kDa peptide

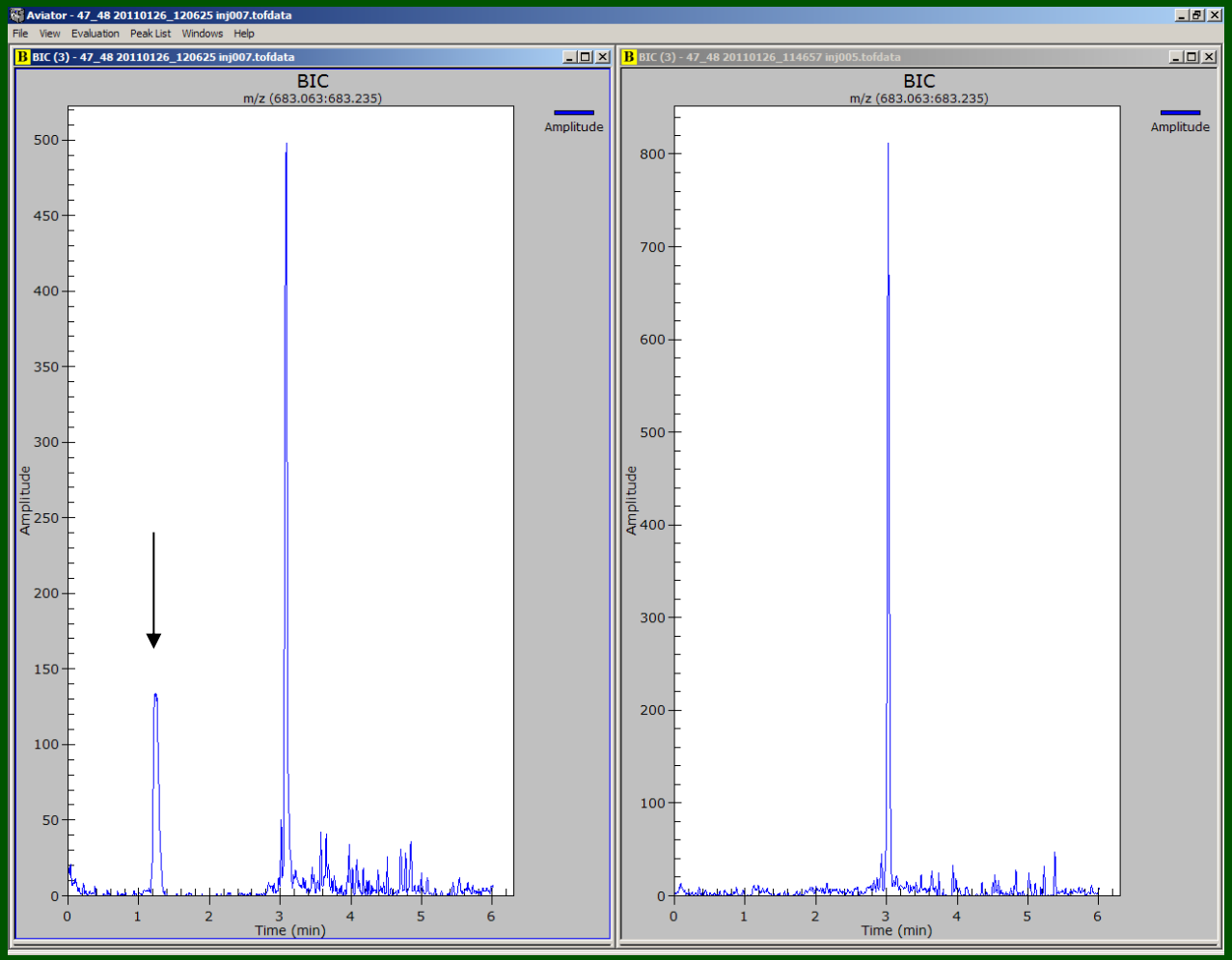




Background PPT

PPT Acetonitril

PPT ZnSO4



Less acetonitrile concentration in final sample
eliminate risk for pre-elution at relative large injection volume

Conclusion

Conclusion quantification with TOF-MS

Advantage	**Consequence**
No optimization needed	Easy to set up
High speed of analysis	High analytical capacity
Large dynamic range	Dilution of sample can be avoided
Linear calibration curves	The simplest model is preferred
Accurate mass measurement	Safe identification of component
Accurate isotopic pattern	Useful for interpretation
Mass spectral data saved	Possibility for later evaluation
Disadvantage	
Large amount of data collected	Need for efficient data handling and storage
Less selective than e.g.. MRM	Need for high chromatographic performance and reproducible retention times

Conclusion Micro-LC

Advantage	**Consequence**
High chromatographic performance	Very complex sample can be analyzed
Large dynamic range	Dilution of sample can be avoided
Rapid eluent mixing and fast gradients	Reproducible retention times
High mass sensitivity	Reduced sample size
Compact instrument set up	Less space requirements
Low eluent consumption / waste generation*	Less contamination of ion source
Protein precipitation with $ZnSO_4$	No organic solvent in sample solution
Disadvantage	
Low dead (delay) volumes critical	Short distances before and after column
Narrow tubings and fittings	Risk for clogging

*** Green HPLC**

Employing 0,3 mm ID columns saves up to 98% of the solvent compared with using 2.1 mm ID columns.

(600 L/year reduced to 12 L/year)


Stenhagen Analyslab AB