4th Workshop on Holistic Analytical Methods for Systems Biology Studies

Seminar
Targeted metabolomics LC-MS/MS

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Method Development
Basic Steps

- Selection of analytes
- Preparation of Stock standard solutions
- Grouping analytes
- Calibration standards

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Manual or automated MS tuning for each analyte

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Inlet method development

↓

Method Validation

↓

Application to real samples

Selection of analytes

- Ability of selection usually 80- 150 polar metabolites for a multi targeted analysis

- Analyte's logD, pkA

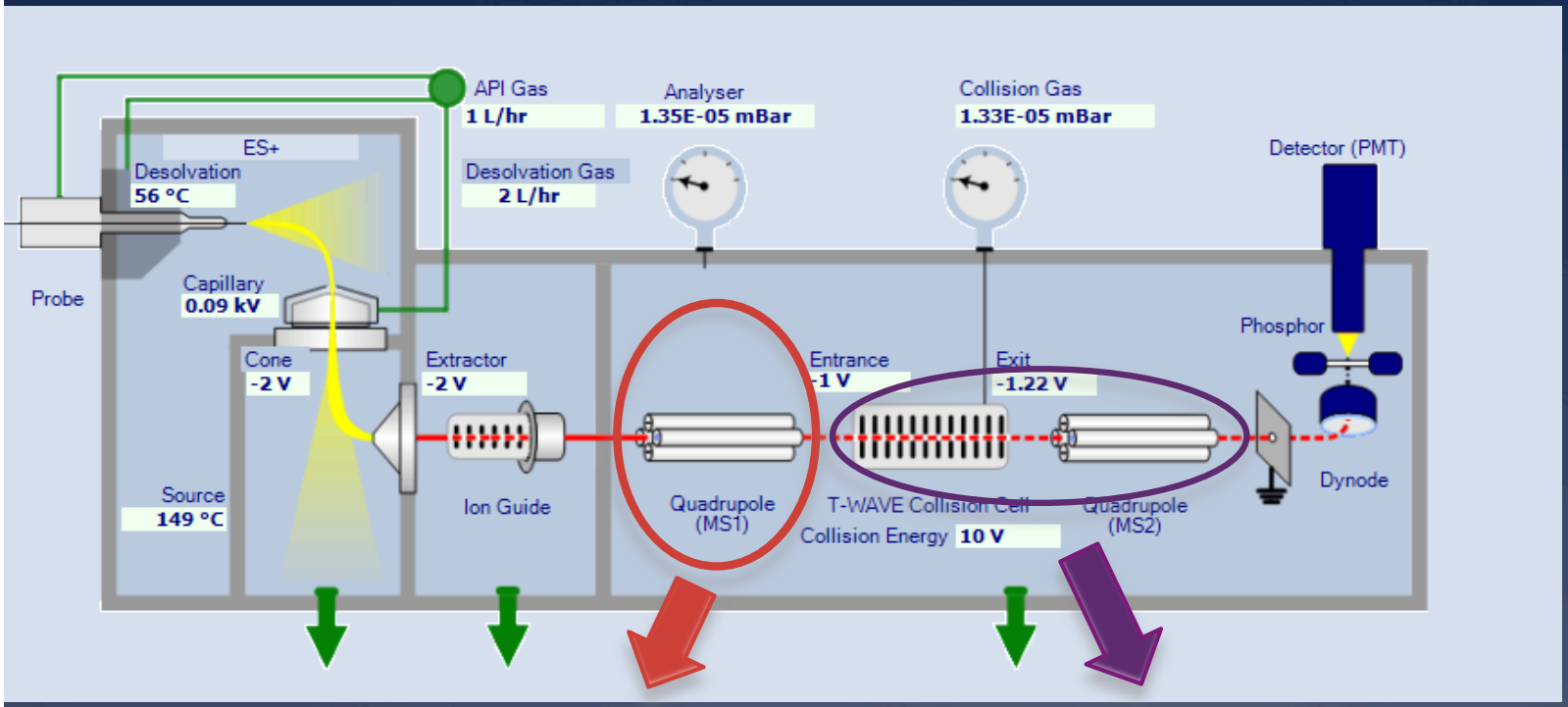
- Stock solutions min.1000 max .10000 ppm
  - Usually in H_20:MeOH, 50:50 v/v increasing lifetime of standards
  - Need a small volume of HCl or NaOH depending on analytes solubility in aqueus and/or organic solutions
  - Working standards in ACN:H_2O, 95:5 v/v (common initial conditions for HILIC separation)

- For calibration standards
  - Grouping standards in concentrations according to literature for the study matrix
  - Final composition of standards:

	MeOH %	H2O%	ACN%
STD1	0.1	5.1	94.7
STD2	0.3	5.3	94.4
STD3	1.2	6.1	92.7
STD4	3.0	7.7	89.3
STD5	7.5	11.7	80.8
STD6	15.0	18.5	66.6
STD7	18.0	21.2	60.9
STD8	22.4	25.2	52.4
STD9	29.9	31.9	38.2

Manual or automated MS tuning for each analyte

Manual tuning



1st step: SIR optimisation, MS1, always look for adducts

- ESI mode
- Cone Energy
- Capillary Voltage
- Desolvation temperature and gas flow L/h

2nd step: MRM optimisation,

- Collision Energy

(low=10, medium=20, high=30)

- Daughter Ion with the highest intensity
- Find Optimum collision energy

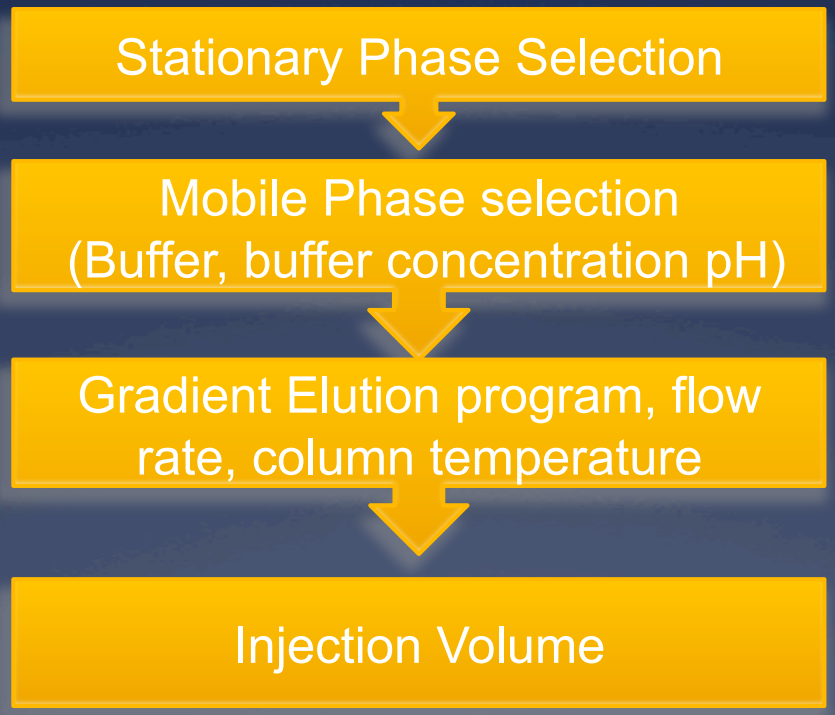
Automated tuning

- For MassLynx software use Intellistat

Final MS method

- Parent and daughter ion for MRM transitions
- Optimum cone and colission energy
- Optimum dwell time to increase sensitivity
- For MassLynx : All the other dependant parameters from tune page

Inlet method development



Inlet method development

Stationary phase Selection

- Excellent alternative to common RP-LC for the separation of polar compounds
- HILIC stationary phases should be characterized for:
  - I. degree of hydrophilicity
  - II. selectivity for hydrophilic-hydrophobic groups
  - III. selectivity for positional and conformational isomers
  - IV. evaluation of electrostatic interactions
  - V. evaluation of the acidic-basic nature of the stationary phases

Column 1: Triart Diol-HILIC 1.9 µm, 2.0 x 150mm
Column 2: Nucleodur HILIC 1.8 µm, 2 x 150mm
Column 3: BEH HILIC Amide 1.7 µm, 2.1 x 150mm,

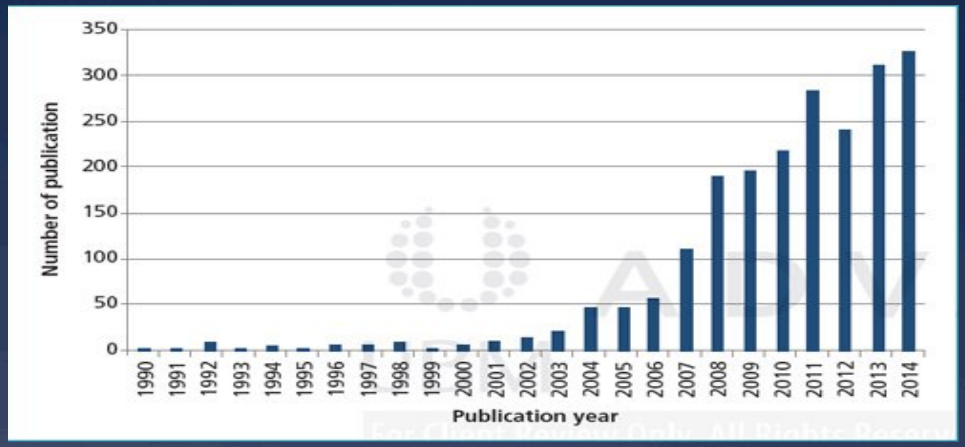
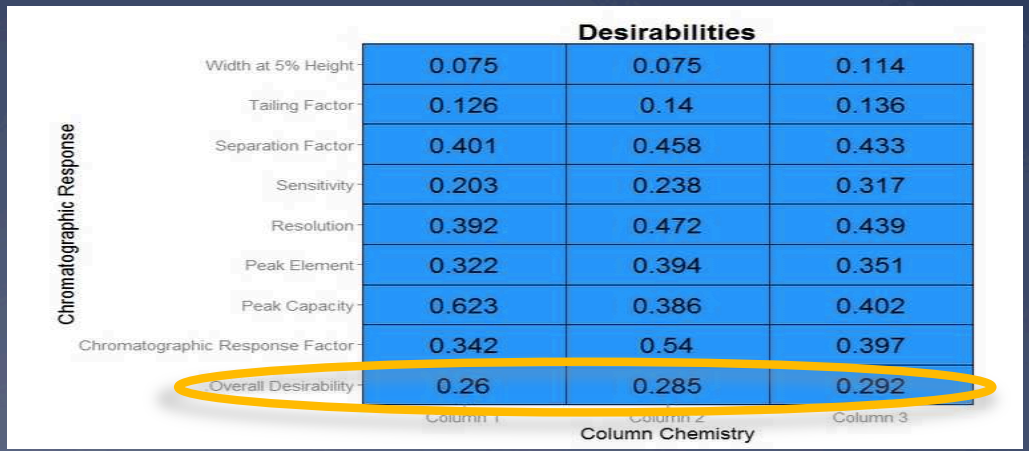
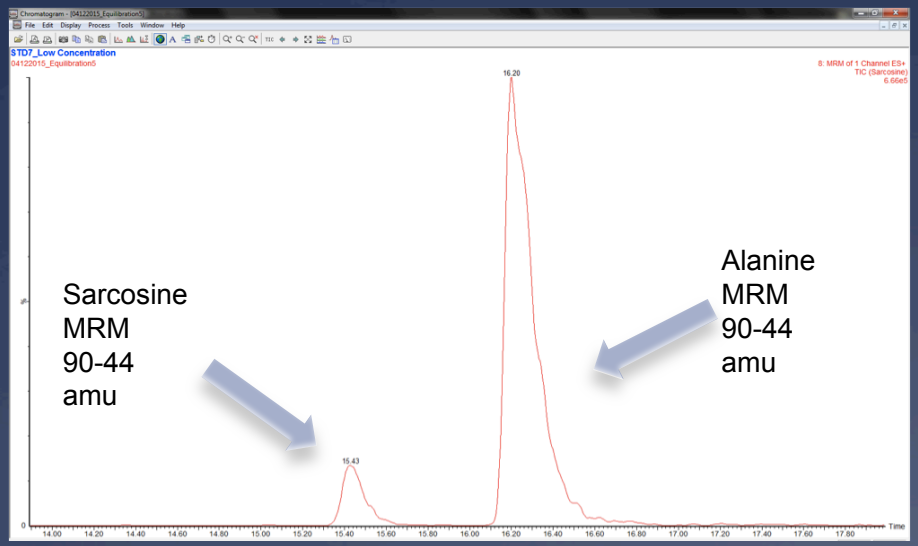


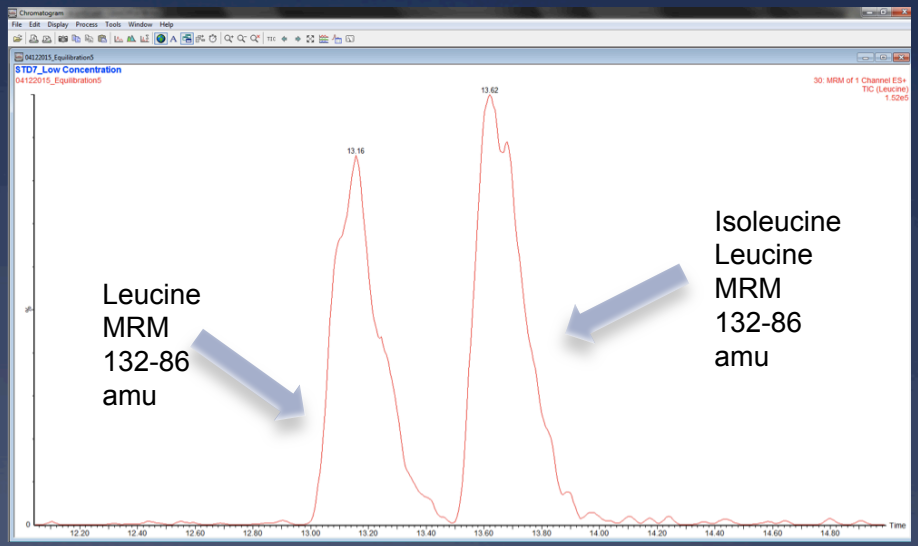
Figure. Number of publications on HILIC 1990-2014



Inlet method development

Enhance Resolution: Optimum stationary phase, column temperature, gradient program, flow rate, ms tuning

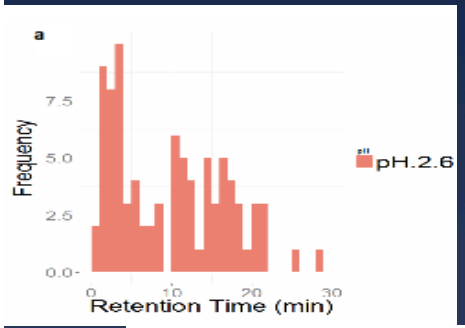


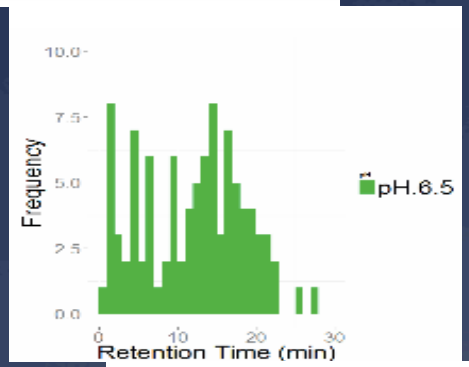


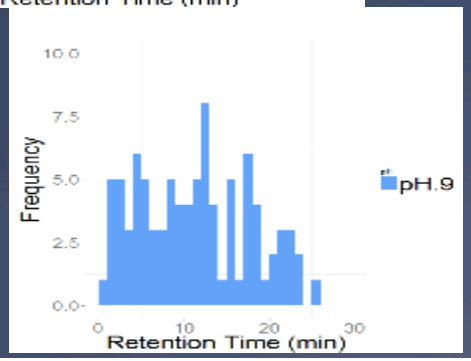
Inlet method development

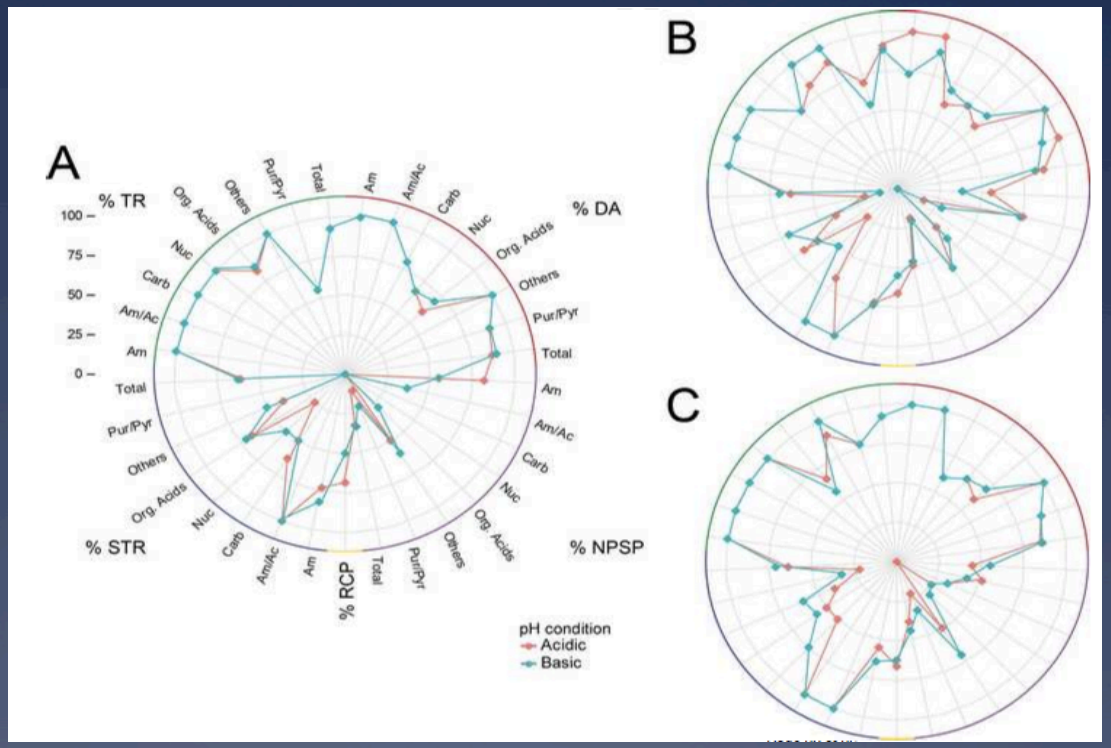
Mobile phase Selection

Buffer Concentration and pH



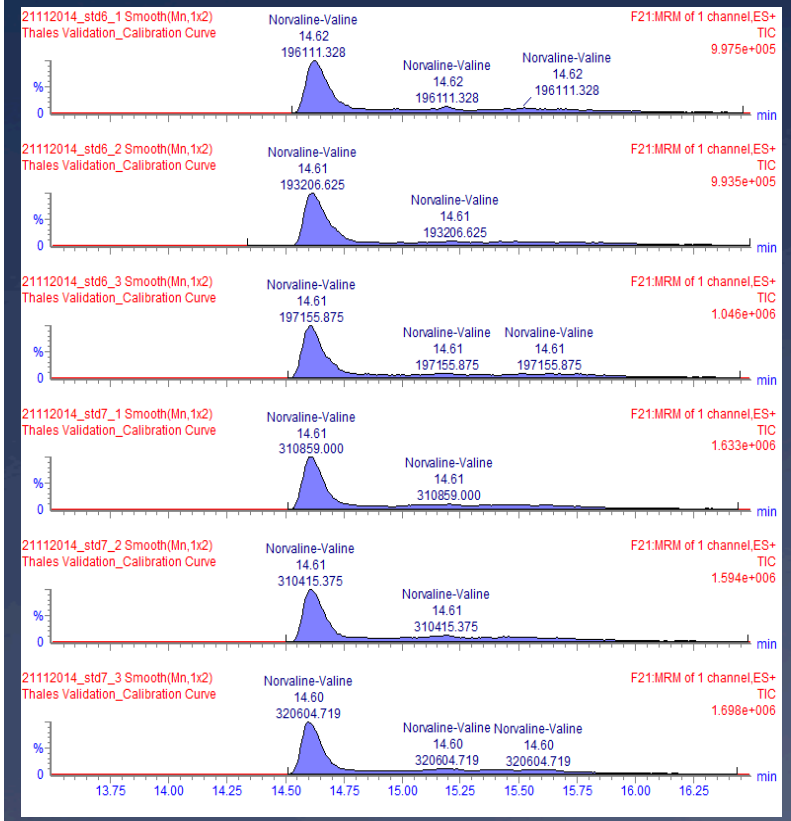


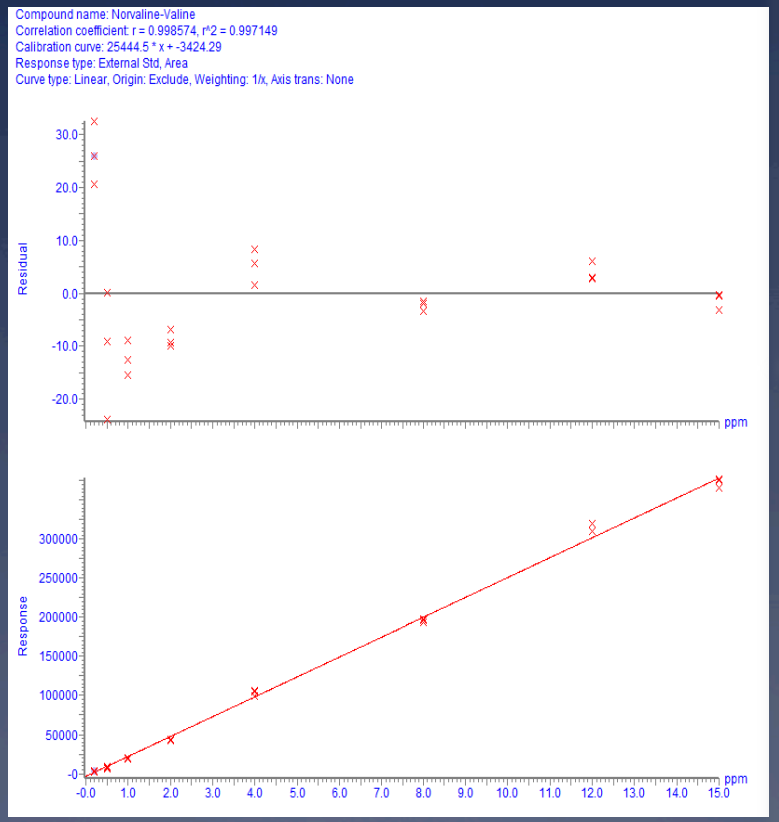




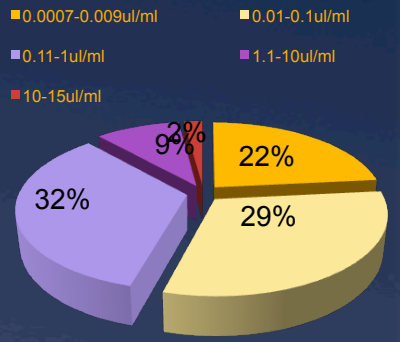
Method Validation

Linearity, LOD, MDL and LOQ was determined using 9 calibration standards, after 5 replicate injections, **LOD, MDL**: 3* SD, $t_{value=3,747}$*SD

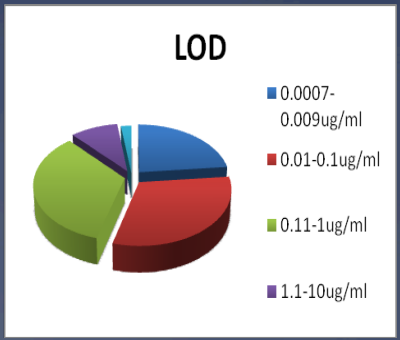




Distribution of linear range

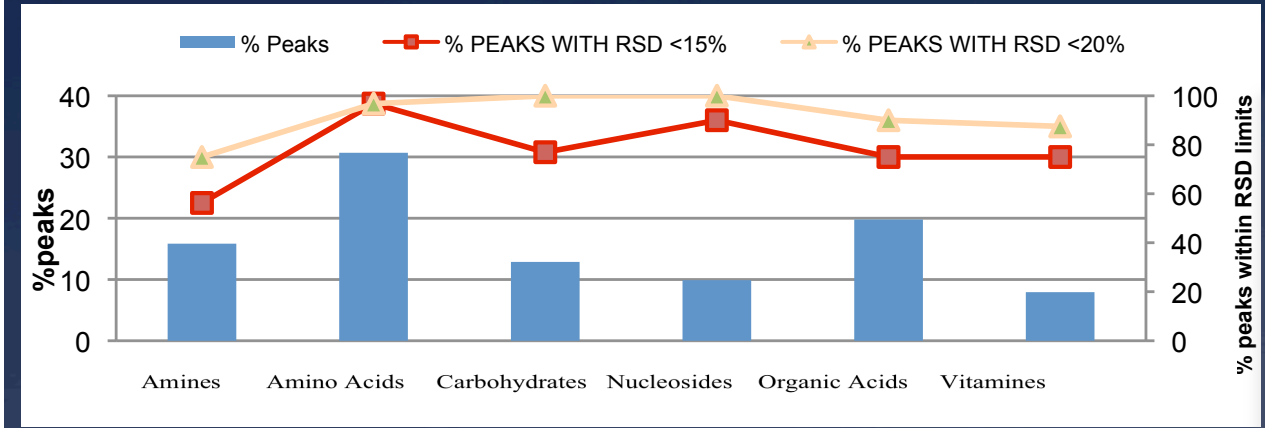


Distribution of LOD

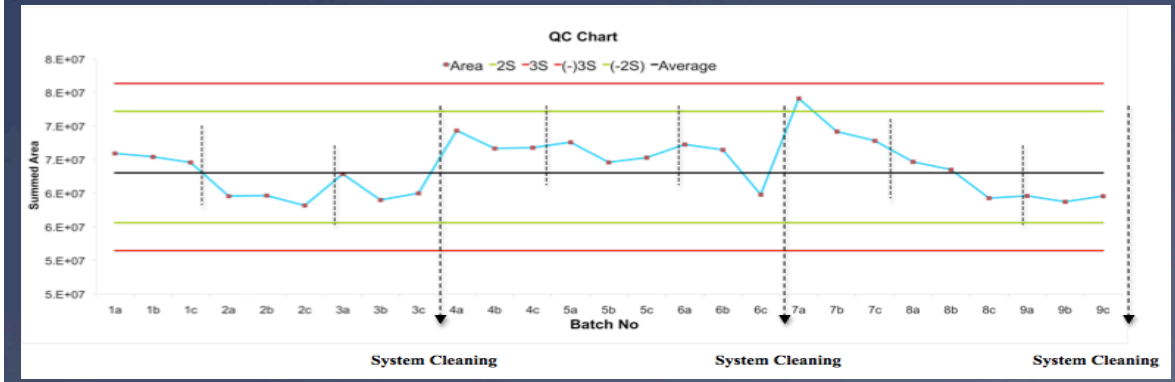


Method Validation

Precision: Repeatable analysis of std mix
Intra batch precision (std mix run every after 10 samples in a batch of 40 real samples)

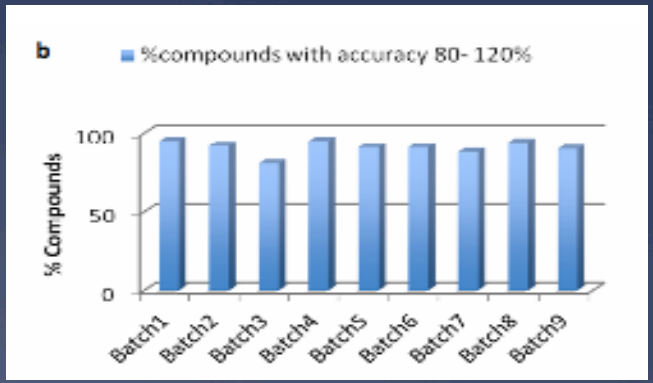


Systems performance to a matrix **System equilibration** after cone and column cleaning (system cleaning every after 3 batches, minimum of 4 injections at the beginning of each batch for column equilibration)



Inter batch precision (assess of std mix in 9 batches)

Accuracy : % Recovery of std mix at medium concentration



Limitations Method Validation

Due to not available analyte free matrix

- Selectivity
- Recovery
- Matrix effects

OUR APPROACH

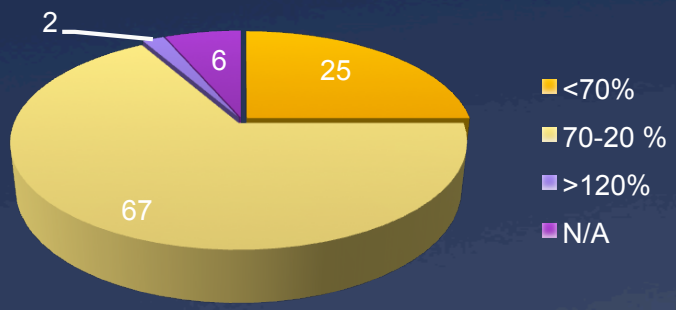
Calculation of Matrix Effect (ME)
Selection of 2 Standard mixtures (low and high concentration)
Evaporation under stream of N_2
Reconstitution in samples' extract
ME= [(Area(post extrction spiking QC)- Area(QC) /Area(standard) -1) *100]

Calculation of Recovery (Re)
Selection of 2 Standard mixtures (low and high concentration)
Evaporation under stream of N_2
Reconstitution in samples' extract + MeCN for extraction
ME=(Area(prior extraction spikeing QC)- Area(post extrction spiking QC))* 100%.

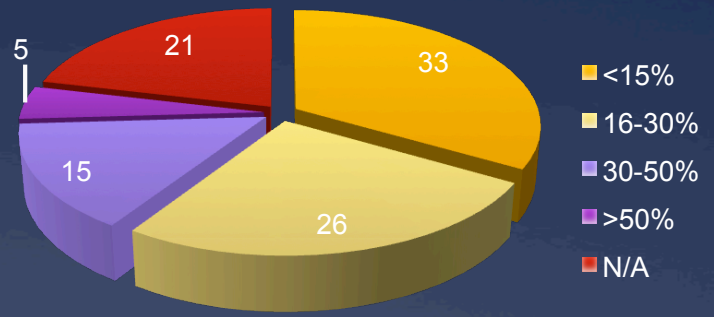
Quantitation

Recovery and Matrix Effect distribution of 108 endogenous polar metabolites

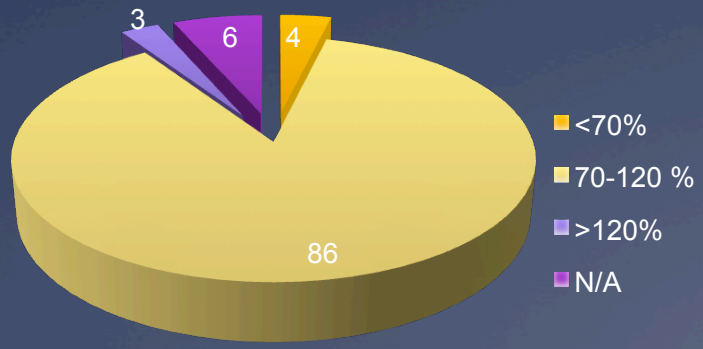
Recovery
Low Concentration standard



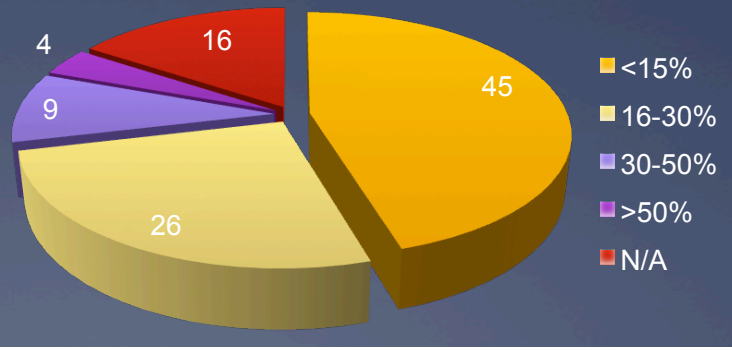
Matrix Effect
Low standard concentration



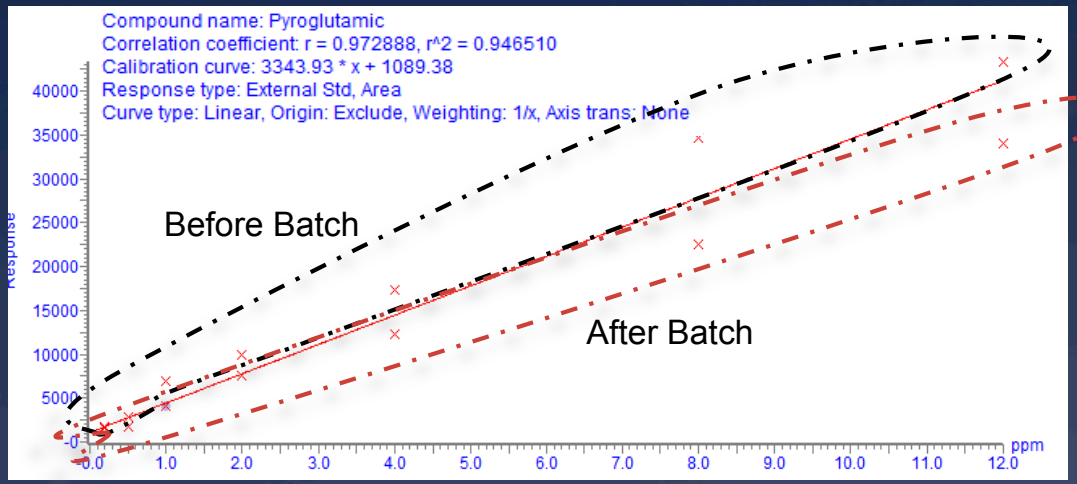
Recovery
High Standard Concentration

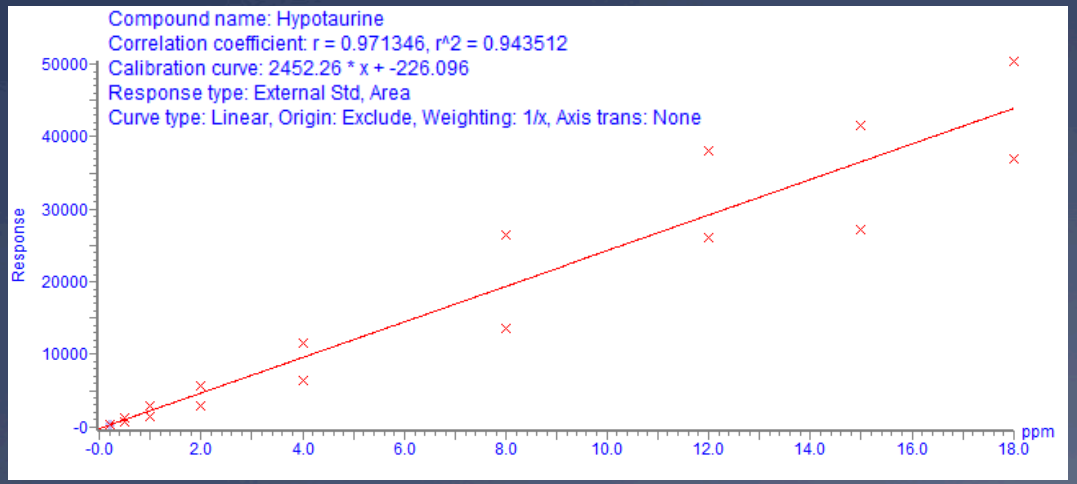


Matrix Effect
High standard concentration



Quantitation





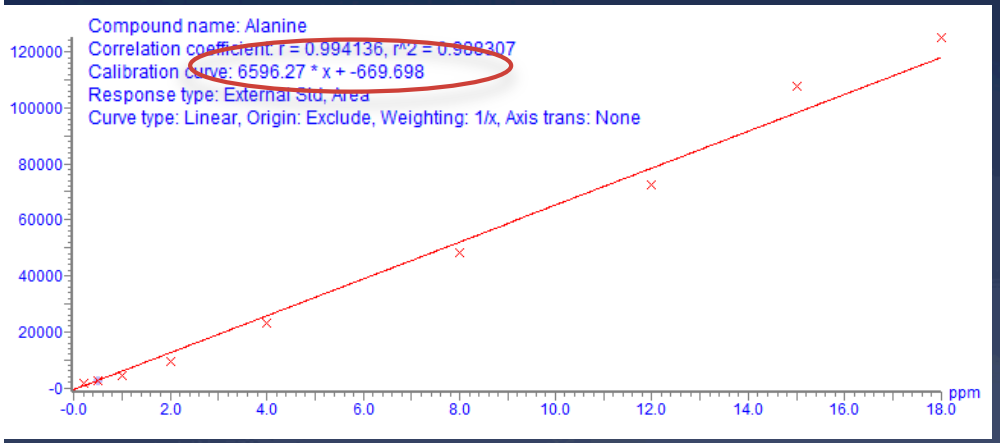
Where external calibration curve is used for quantification:
run a calibration curve before and after test samples and use mean calibration for analytes quantification

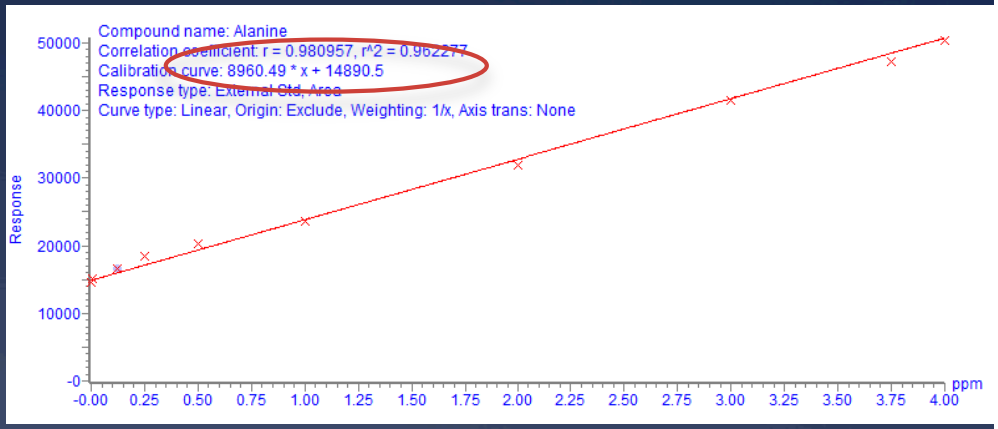
- a mean of normalisation in case of large sample sets where decrease in signal can be observed

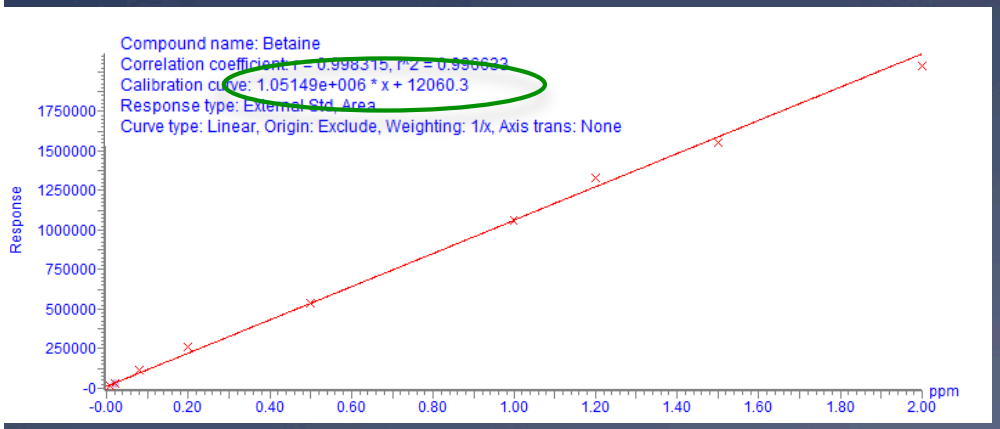
- In TargetLynx this can be done automatically
- Otherwise Python script

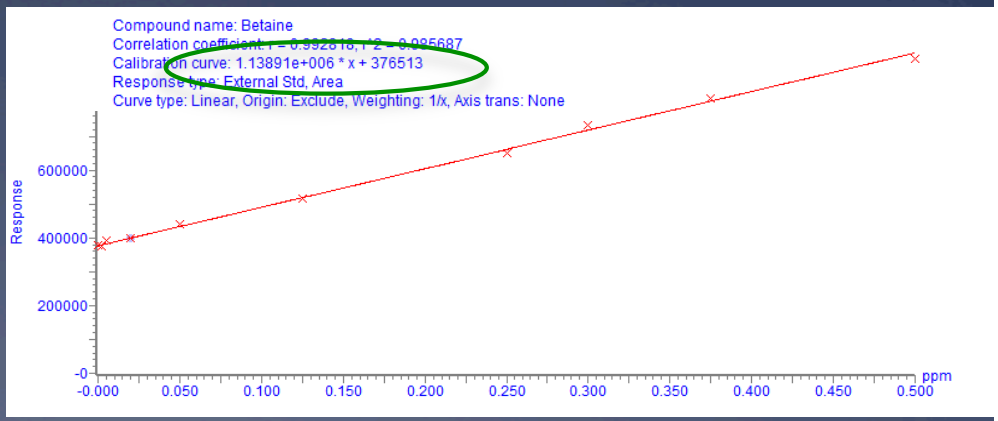
Quantitation

External Vs Standard Addition Calibration Curve



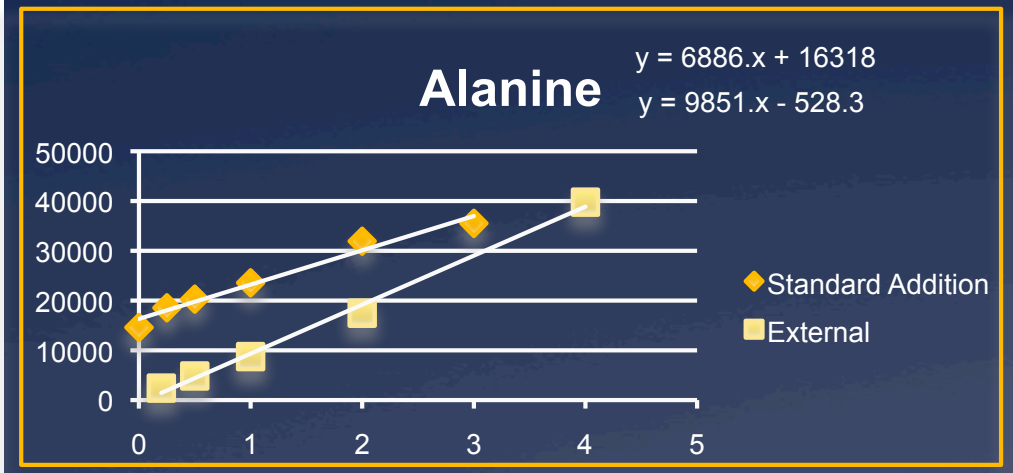


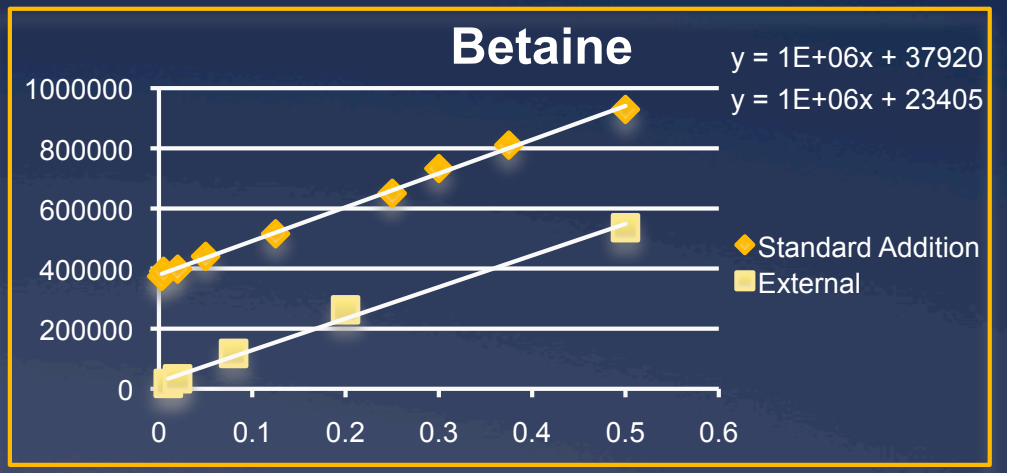


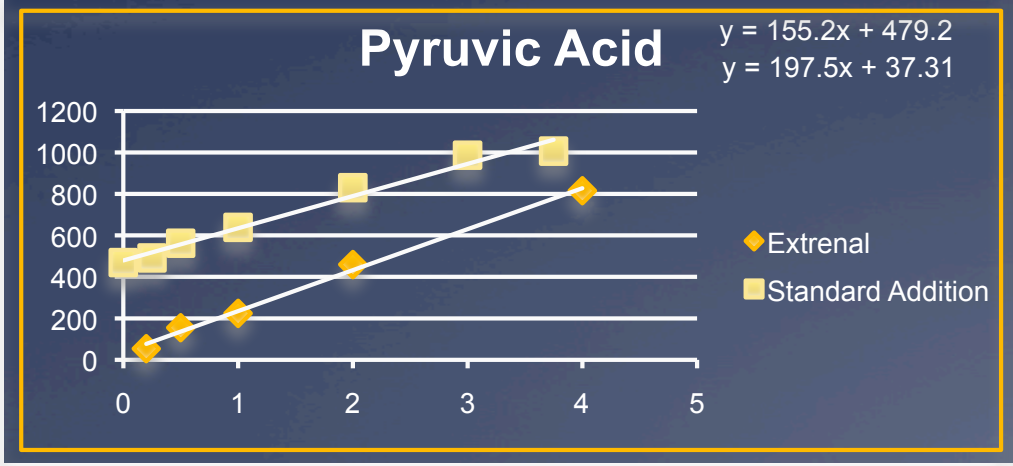


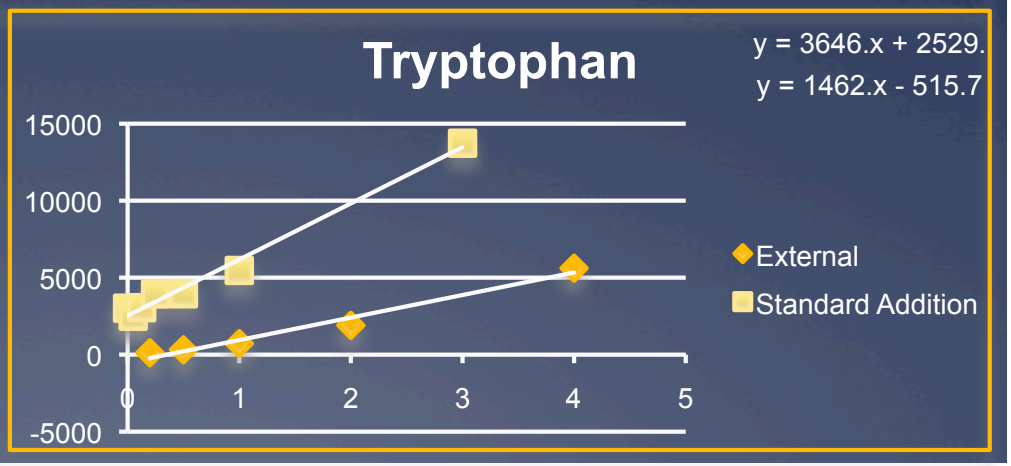
Quantitation

Standard Addition Vs External Calibration Curve



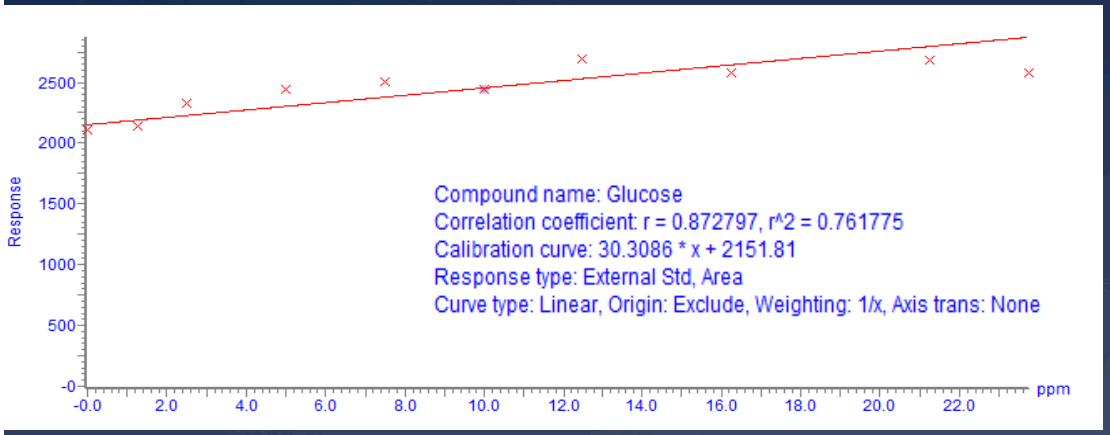


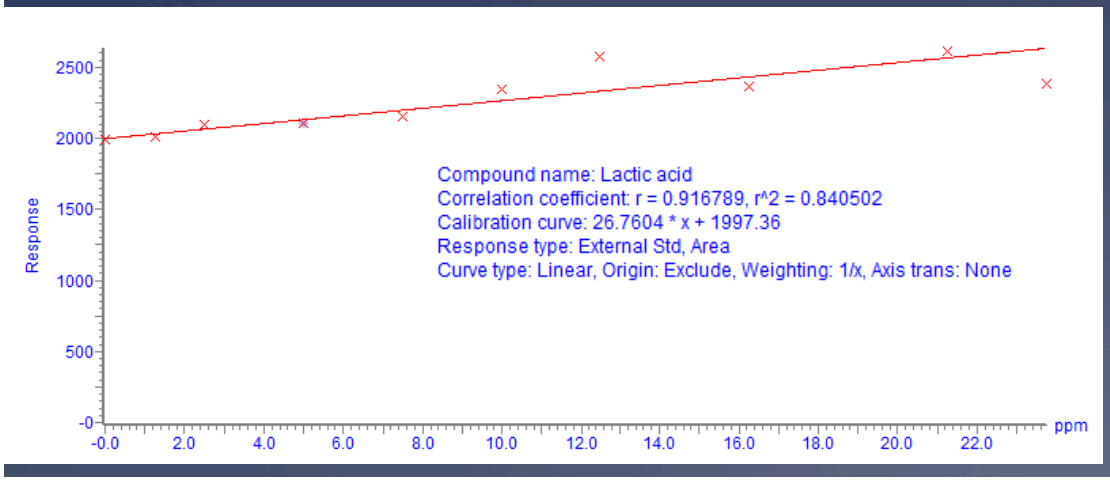




Quantitation

Linear Range after standard addition





When endogenous analyte concentrations are relatively high in a control matrix,

- The measurement of relatively small additional amounts of analyte have the potential to introduce significant error in measurement
- The measurement of relatively high amounts of analyte may extrapolates the calibration curve beyond the linear range

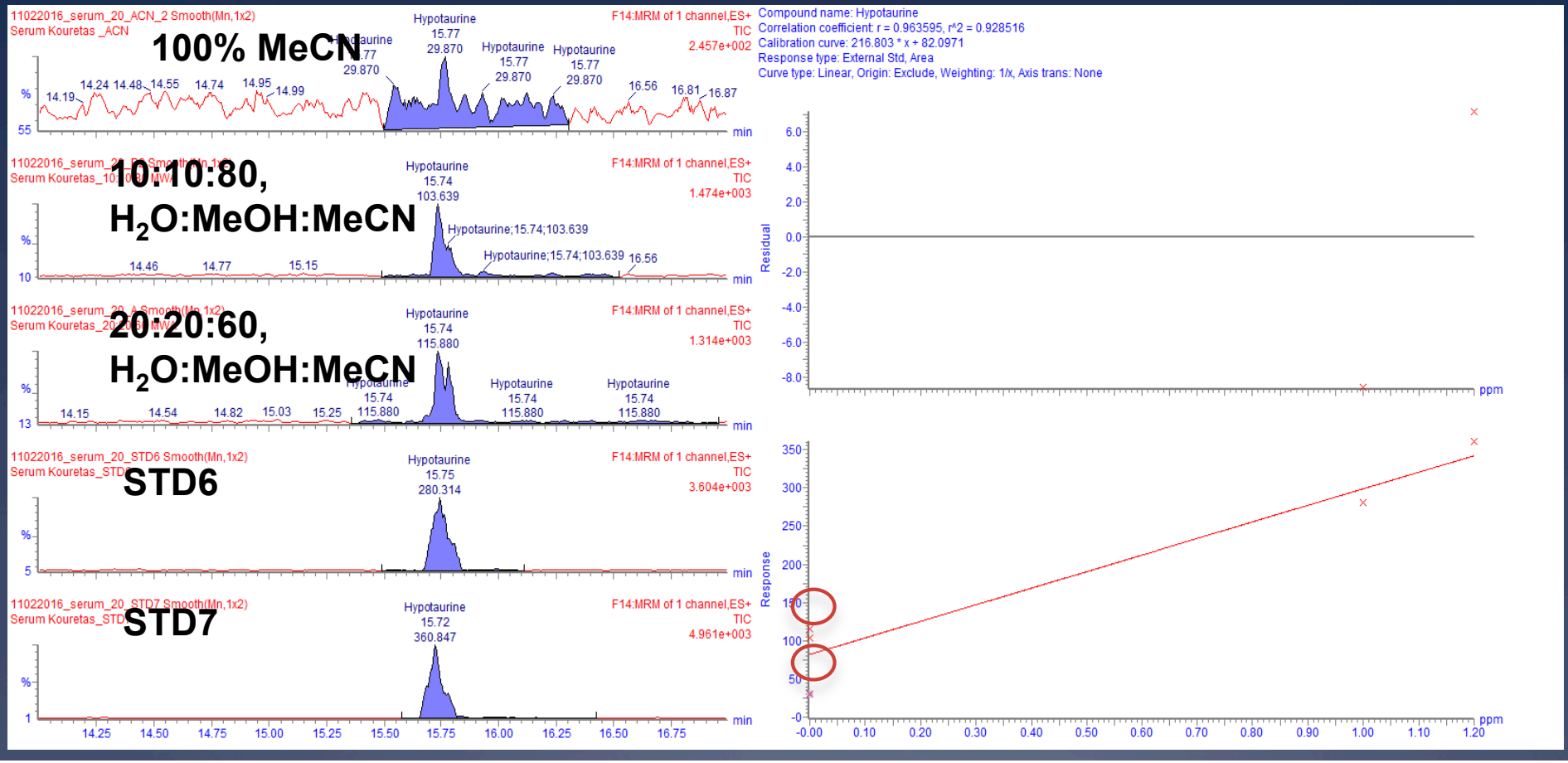
Our approach

- Detuning specific analytes
- Lower injection volume
- Optimisation of extraction protocol (addition of F.A, delipidised matrix)

Quantitation

Composition in the vial

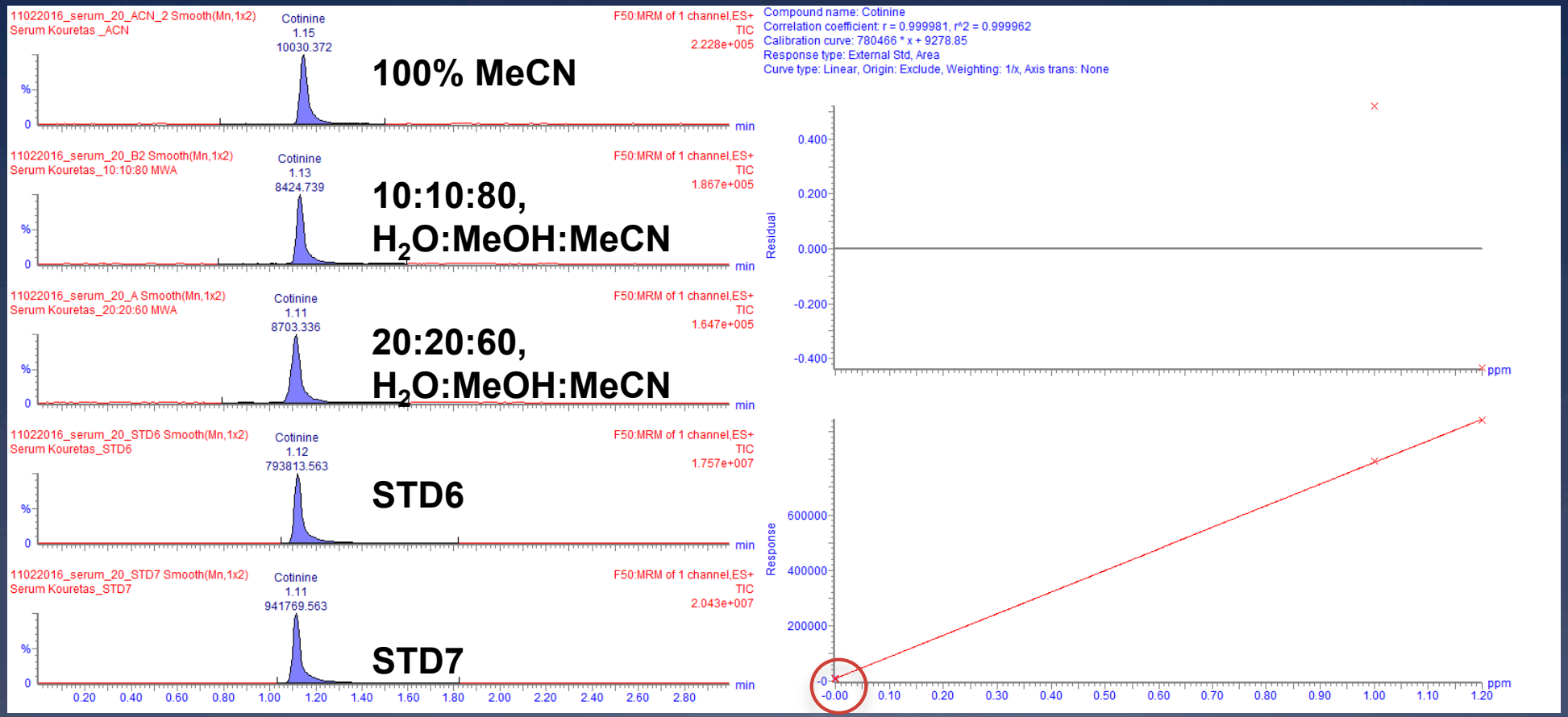
Analyte is not detected when 100% MeCN is used for extraction



Quantitation

Composition in the vial

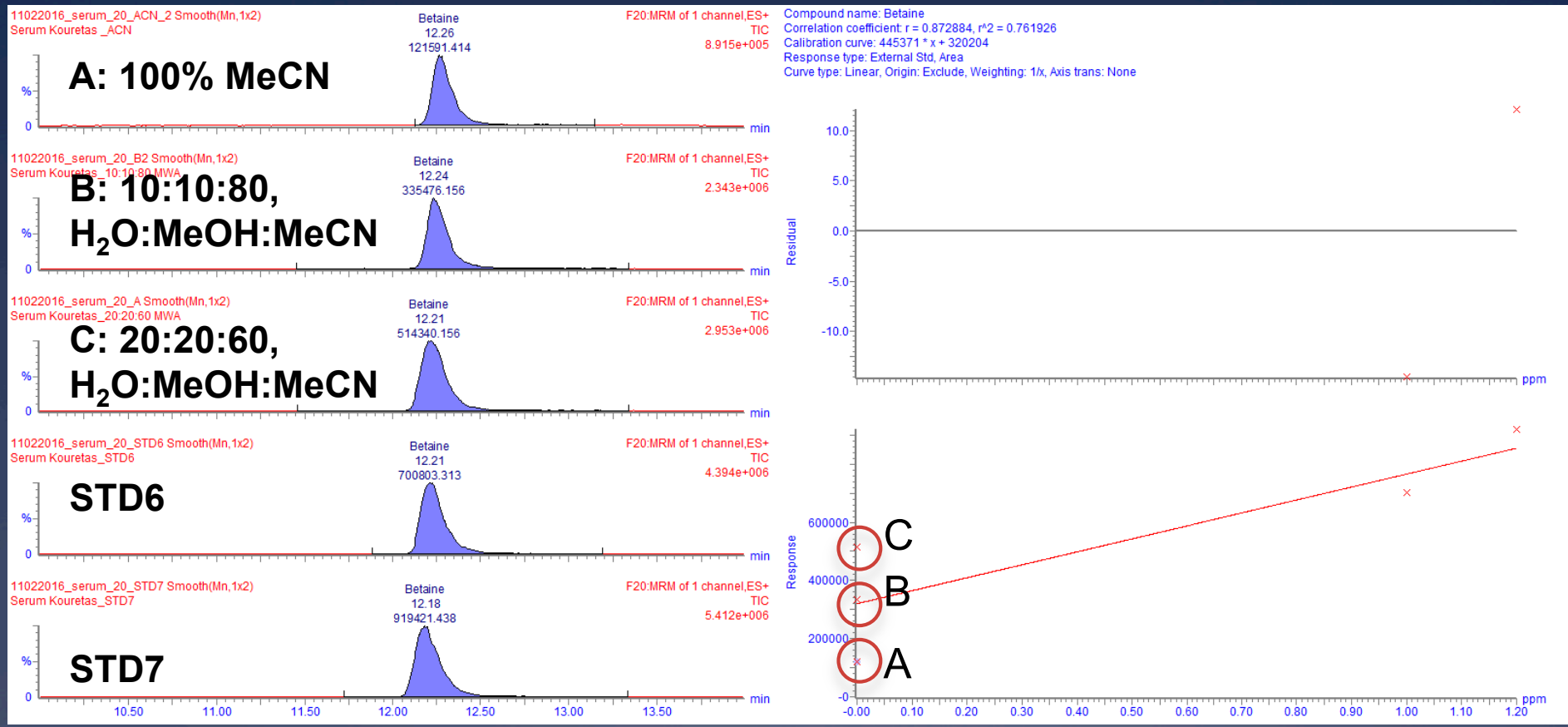
Analytes peak area is the same in all cases



Quantitation

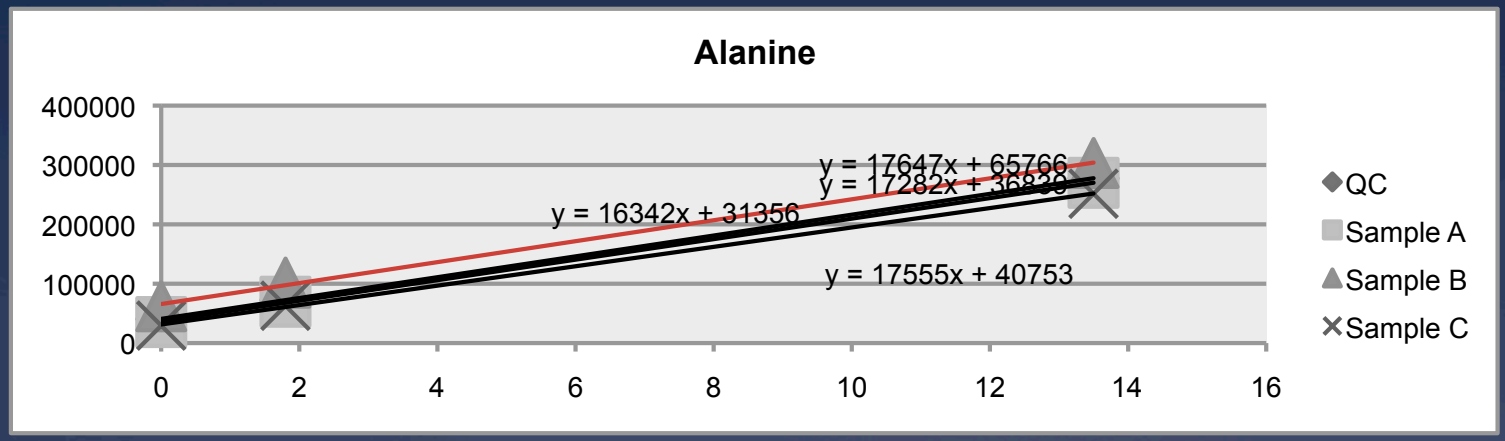
Composition in the vial

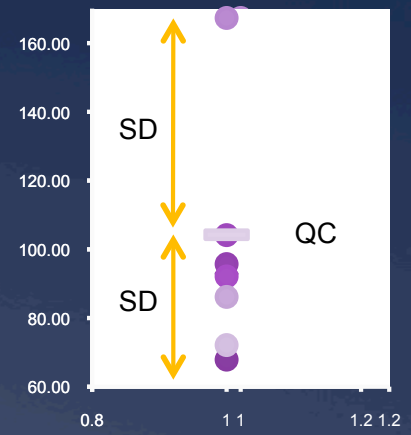
Analytes peak area differs significantly

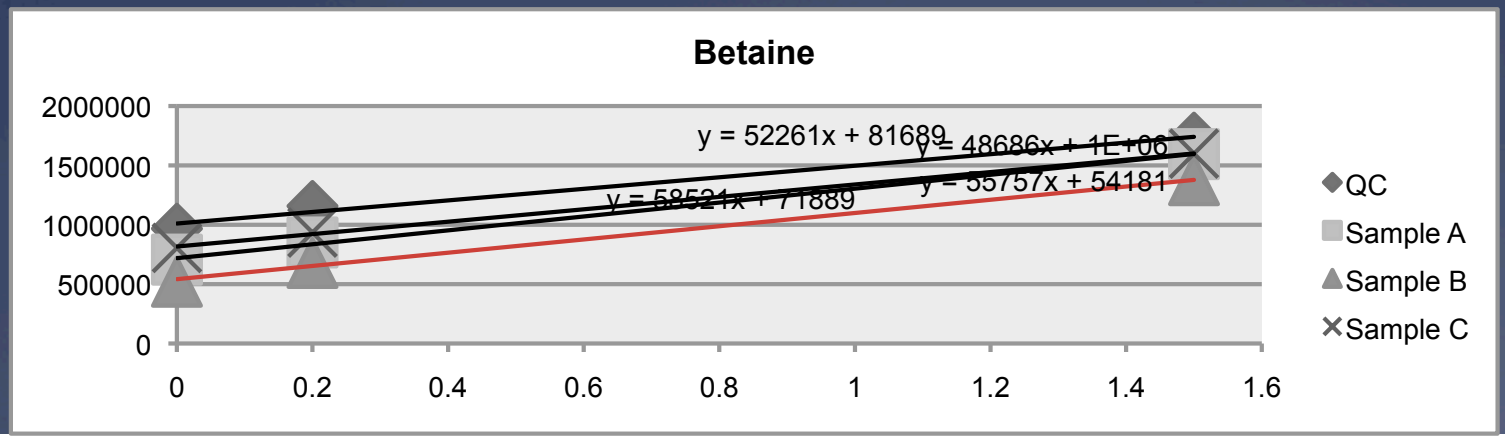


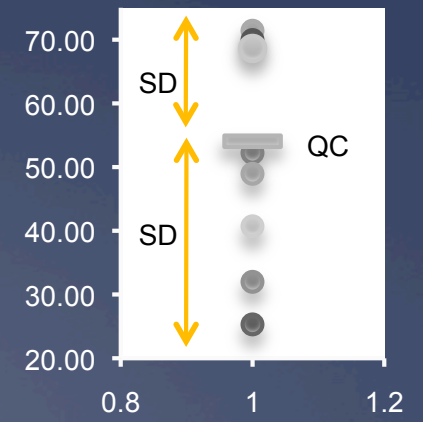
Quantitation

Standard Addition Calibration Curve: Could QC's StdAdd Calibration Curve applied for the quantitation of analytes in individual samples ?









Conclusion

Targeted approach can be applied for more than 100 metabolites, from different chemical classes, intermediates of metabolic pathways

- Quantified data
- Easy data analysis
- Uni- and multi- variate analysis underlying metabolic profile differences of study samples in metabolomics studies

For quantitation of analytes external calibration curve could be applied for analytes with the same slope in both external and standard addition calibration curve

Matrix effect and recovery is not easy to be assessed for endogenous compounds

- Evaporation of standards and reconstitution may result in non-soluble analytes

Standard addition calibration curve is applied in our lab for quantification of metabolites in test samples

- Individual standard addition calibration curve for each sample (cost, time consuming)

Sample extraction protocol is depending on composition of standards used for standard addition calibration curve

Discussion 1

Isotope labeled standards

Use of labeled isotopes in multi-targeted metabolomics based methods

- Isotope labeled standards do not exist for all the metabolites
- Increase cost of analysis
- Decrease number of MRM transitions in a single MS method in order not to lose sensitivity or
- Loose sensitivity
- Could we use a limited number of isotope labeled standards, representative for each chemical class including within method ?

Discussion 2

Authentic matrix and surrogate analyte

Quantitation of an endogenous analyte can be performed by reference to calibration curve constructed from samples of the authentic matrix spiked with a stable-isotope-labeled form of the analyte

Limitations

- Choice of a suitable stable isotope-labeled standard
- Chromatographic retention time increases as the number of deuteriums increase in the analogue
- Analyte and stable isotope-labeled standard yield unique product ions, rather than a fragment resulting from the loss of the portion of the molecule tagged with the stable isotopes to prevent cross-talk between selected monitoring channels

Discussion 3

Surrogate Matrix

The advantage of this approach is that differences in analyte response caused by matrix effects or differential recovery between calibration standards and samples are minimised due to matrix matching

Limitations

- Analyte solubility in a surrogate matrix
- Non-polar analytes normally present in lipoprotein fraction of plasma, maybe partly insoluble even in the presence of proteins.
- Analyte extractability may be different in authentic and surrogate matrices ie specific carrier proteins, testosterone and sex hormone binding globulin (SHBG)
- Check by preparing a series of calibrators in surrogate and authentic matrix and determine the slopes of the calibration curves. Similar slopes indicate a comparable extraction yield
- Derivatisation produces another problem ie reagent exhaustion. Aqueous standards Vs plasma samples. Reduced yield
- Important that stability also be demonstrated for the analyte in the surrogate matrix, as this could differ from stability in the authentic matrix.

Discussion 4

Commercial Plasma/Serum Products

- Defibrinated
- Defibrinated Delipidised
- Defibrinated Resin Treated
- Defibrinated Delipidised Resin Treated
- Defibrinated Charcoal Treated
- Defibrinated Double Charcoal Treated
- Defibrinated Dielipidised
- Charcoal Treated
- Defibrinated Delipidised Double Charcoal Treated

Discussion 5

Standard Addition Method Limitations

- Calculation of LOQ ?
- Preparation of calibration standard mixtures (final composition) and
- Optimum extraction or according to standard composition?
- Evaporation ?
- Matrix Effect and Recovery ?
- Cost ?
- Spike individual samples or only QC for standard addition calibration curve and report results with SD ?
- Linearity in case of high endogenous concentration ?