Is Ion Exchange Chromatography the gold standard method for amino acid analysis?

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Overview

- Define ‘gold standard’ method
- Initial vote
- Discussion of potential candidate methods
- Advantages/disadvantages of each
- Other things to consider?
- Summary and final voting

What do we mean by 'Gold Standard' method?

- Sometimes, *gold standard* is used to refer to the most accurate and specific test possible
- *Gold standard* is also used to refer to a diagnostic test that is the best available under reasonable conditions
- For a routine clinical laboratory, the gold standard test generally means the best performing method for a given scenario
- The gold standard test may not be the perfect test, but merely the best available one that has a standard with known results and more importantly known limitations
- The accepted gold standard test can change with time
    - new technologies become available
    - changes in clinical practice
    - standardisation

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Is Ion Exchange Chromatography the gold standard for amino acid analysis?

Why do we analyse Amino Acids?

- Inherited defects of amino acid catabolism, biosynthesis or transport can occur
- Consider amino acid analysis in a range of clinical situations
    - Hyperammonaemia
    - Lethargy, coma, seizures, vomiting
    - Metabolic acidosis/lactic acidosis
    - Metabolic decompensation
    - Unexplained developmental delay
    - Follow up of NBS
    - Dietary monitoring of patients with known IMD
- Analysis of amino acids needs to be able to detect increased and decreased concentrations of amino acids in biological fluids
- Clinical question - relevant to choice of gold standard method

What do clinicians want from AA analysis?

"Always interested in timely result, especially same day in acutely unwell patient. Needs to be accurate. Cost is secondary"

"TAT and a rapid screen for treatable amino acidopathies"

Analytical requirements?

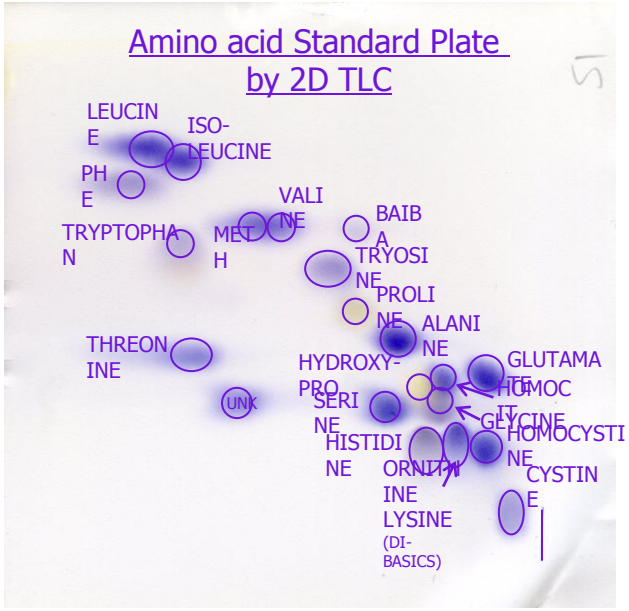
No formal acceptance criteria
Precision
Accuracy
Traceability
Robust system
Capable of rapid TAT
Clear, unambiguous interpretation of results for clinicians

Candidate Methods?

Qualitative Methods

- Cyanide/nitroprusside spot test (reacts with sulphur containing AA, homocystinuria, cystinuria)
- 1D TLC
- 2D TLC
- High voltage Electrophoresis

- Evidently not the gold standard



Candidate Methods?

Quantitative methods

- GCMS
- HPLC with UV detection
- Ion Exchange Chromatography with UV detection
- Flow injection analysis Tandem Mass Spectrometry (FIA-MSMS)
- Liquid Chromatography Mass Spectrometry (LCMS)
- Liquid Chromatography Tandem Mass Spectrometry (LCMSMS)

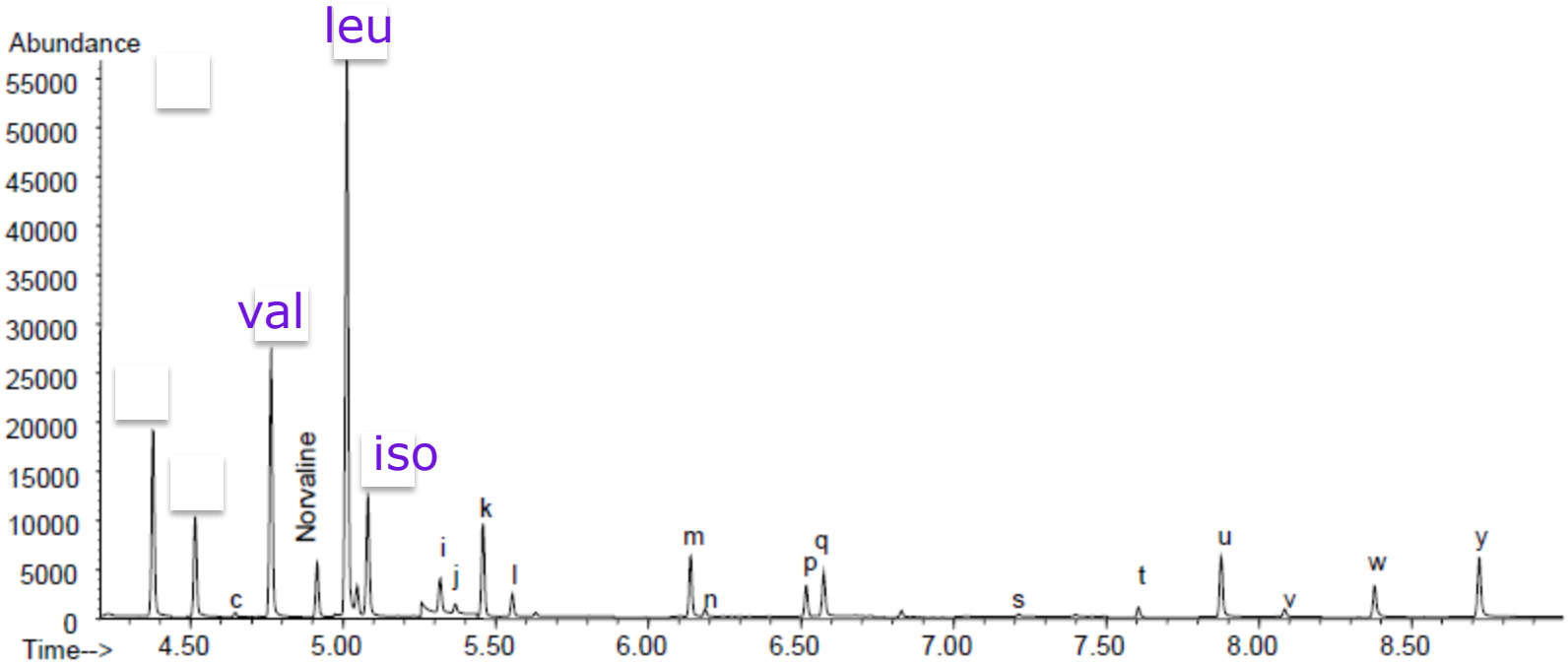
- All suitable for analysis of plasma, urine, CSF and bloodspot
- With the exception of FIA-MSMS, all potential candidates for the gold standard

Gas Chromatography Mass Spectrometry

- GC methods have been available since the 1960's
- Variety of detectors can be used – flame ionisation, electron capture, nitrogen-phosphorous, flame photometry and **mass spec**

- Columns – typically use 15m column, non-polar (BP-1) or a slightly polar stationary phase
- Internal standards – traditionally pipecolic acid, norleucine
- Sample derivitisation is required to convert the AA to stable volatile derivatives (eg propyl chloroformate, N-acyl amino acid alkyl esters)

GCMS Plasma Amino Acid Chromatogram - MSUD (propyl chloroformate derivatives)



Advantages of GCMS

- Rapid analysis time ~15 minutes
- Sensitive
- Specific
- Advantage that structural information provides unequivocal identification of AA
- Stable isotope IS are available
- Commercially available reagent kits (EZfaast)
- Automated sample extraction/derivitisation eg Gerstel MPS prep station
- System can be used for other assays

Disadvantages of GCMS

- Sample preparation – derivitisation is required, some derivitisation reagents require anhydrous conditions
- Derivitisation can result in formation of multiple derivatives which makes chromatogram complex
- Depending on which derivitisation method is used certain AA may or may not be detected eg serine, threonine, arginine
- Arginine derivative are unstable and decompose to ornithine
- Glutamate rearranges to pyroglutamate
- Argininosuccinic acid is not detected
- Not routinely used in clinical laboratories

RP-HPLC with UV detection

- Norleucine or 4-nitrophenylalanine typically used as internal standard
- Protein removal with SSA containing IS
- Requires pre-column derivitisation of sample to produce a stable chromophore prior to analysis
- Commonly used derivitisation reagents include PITC (phenylisothiocyanate) and AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate)
- PITC reacts with amino and carboxyl group to produce cyclic ring structure
- Derivatives separated by reverse phase HPLC, column at 40ºC
- Acetate buffer, increasing gradient organic solvent
- Post column UV detection at 254nm

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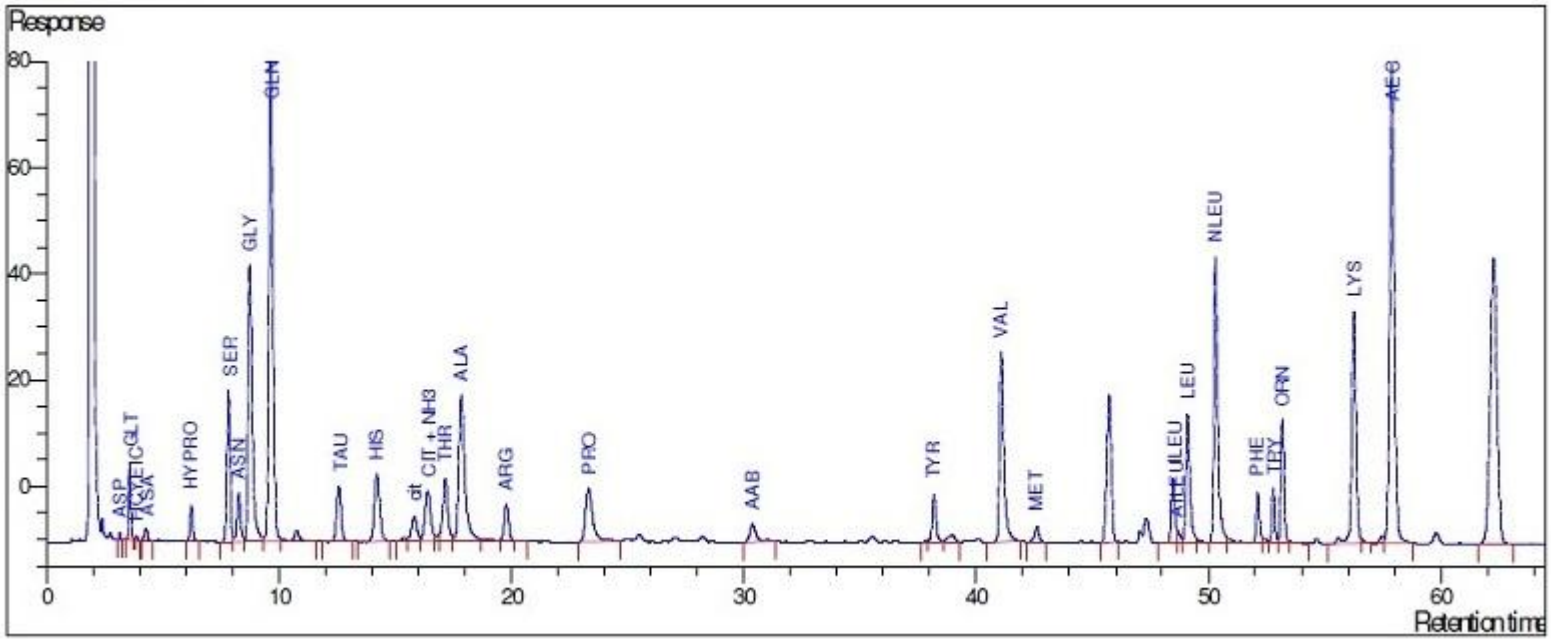
Example HPLC plasma amino acid chromatogram



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Advantages of HPLC

- Analysis time ~60 minutes
- UPLC can reduce the run time further ~ 30 min
- Commercial kits are available e.g. Waters MassTrak AAA kit
- Quantitation of 42 amino acids
- Good precision CV<5%

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Disadvantages of HPLC

- Sample preparation (precipitation followed by derivitisation)
- Lacks specificity
- Interferences from drugs and any compound that reacts with PITC to give products which absorb at 254nm
- Does not identify all analyes of interest
- Citrulline co-elutes with ammonia
- Alloisoleucine is not separated
- Not suitable for analysis of homocysteine or mixed disulphide

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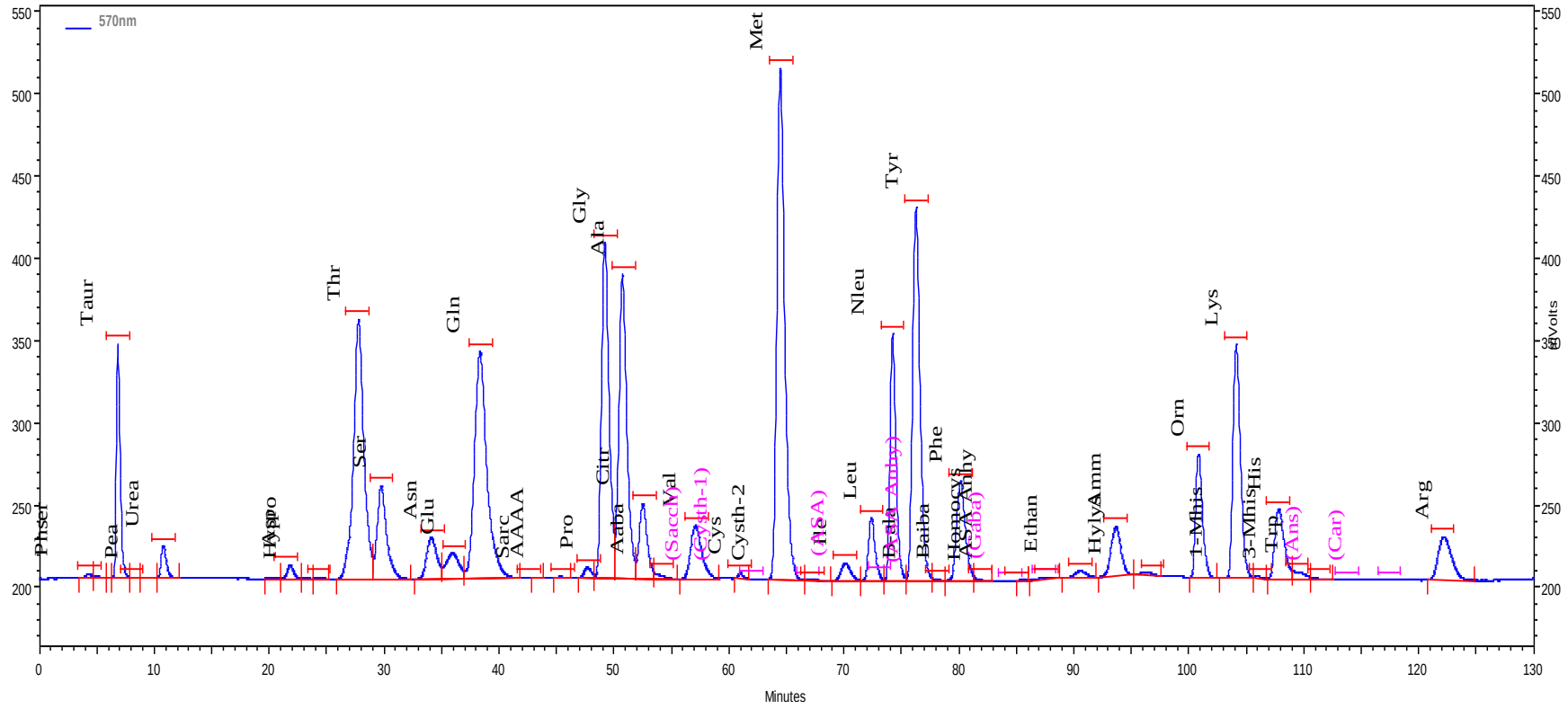
Ion exchange chromatography

- Traditionally IEC is referred to as the gold standard methodology
- Routinely used in the clinical laboratory
- Cation exchange chromatography with post column ninhydrin detection
- Stepwise elution of a series of lithium citrate buffers
- Start with acidic buffer – amino acids retained by the resin
- Then increase the pH and ionic strength with each step
- More acidic species elute first
- Post column derivitisation – eluent from the column is mixed with ninhydrin in the reaction coil at 135ºC
- Dual detection at 440nm (yellow) and 570 nm (purple)

Example IEC plasma amino acid chromatogram



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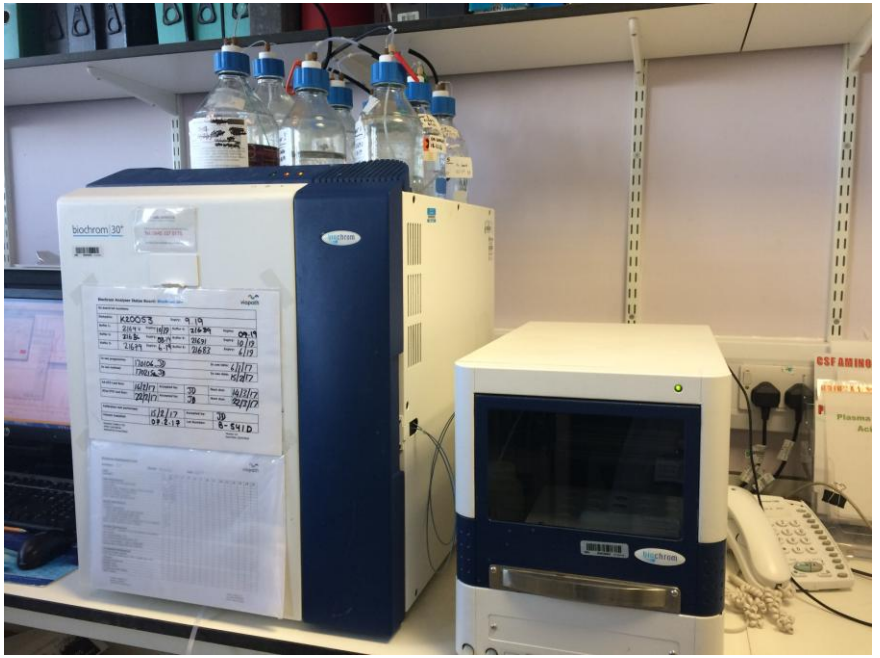
Out with the old.....



Advantages of Ion Exchange Chromatography

- Minimal sample prep required- no derivitisation step
- Stable and precise
- Large dynamic range, approx 3 -4 orders magnitude
- Suitable for sulphur containing amino acids
- Separates citrulline from ammonia
- Identifies all analytes of interest
- Identifies atypical Aas
- Commercial kit (reagent rental)

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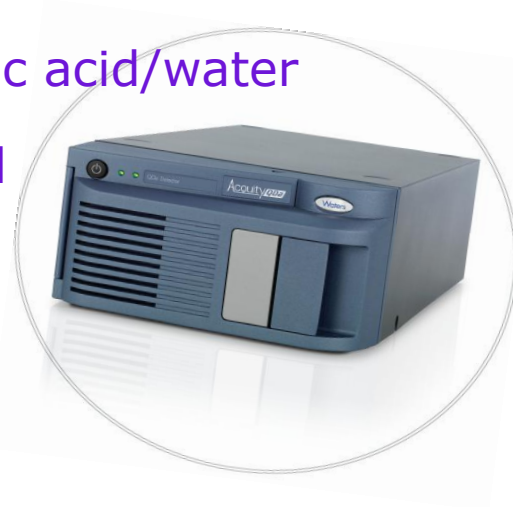
Disadvantages of Ion Exchange Chromatography

- Long analysis time ~ 130 min for standard profile
- Dedicated instruments, often running at capacity
- Internal standard is structural analogue
- Method lacks specificity
    - identification based on Rt alone
    - co-eluting substances (eg homocitrulline & methionine)
    - interferences from drugs and any ninhydrin positive compound
    - 570/440 ratio of limited use
- Poor resolution of sulphocysteine at front end
- Operator experience
- Identifies atypical AA
- Manufacturer now promotes ‘accelerated’ method which is 90 minutes long but ? Lacks robustness

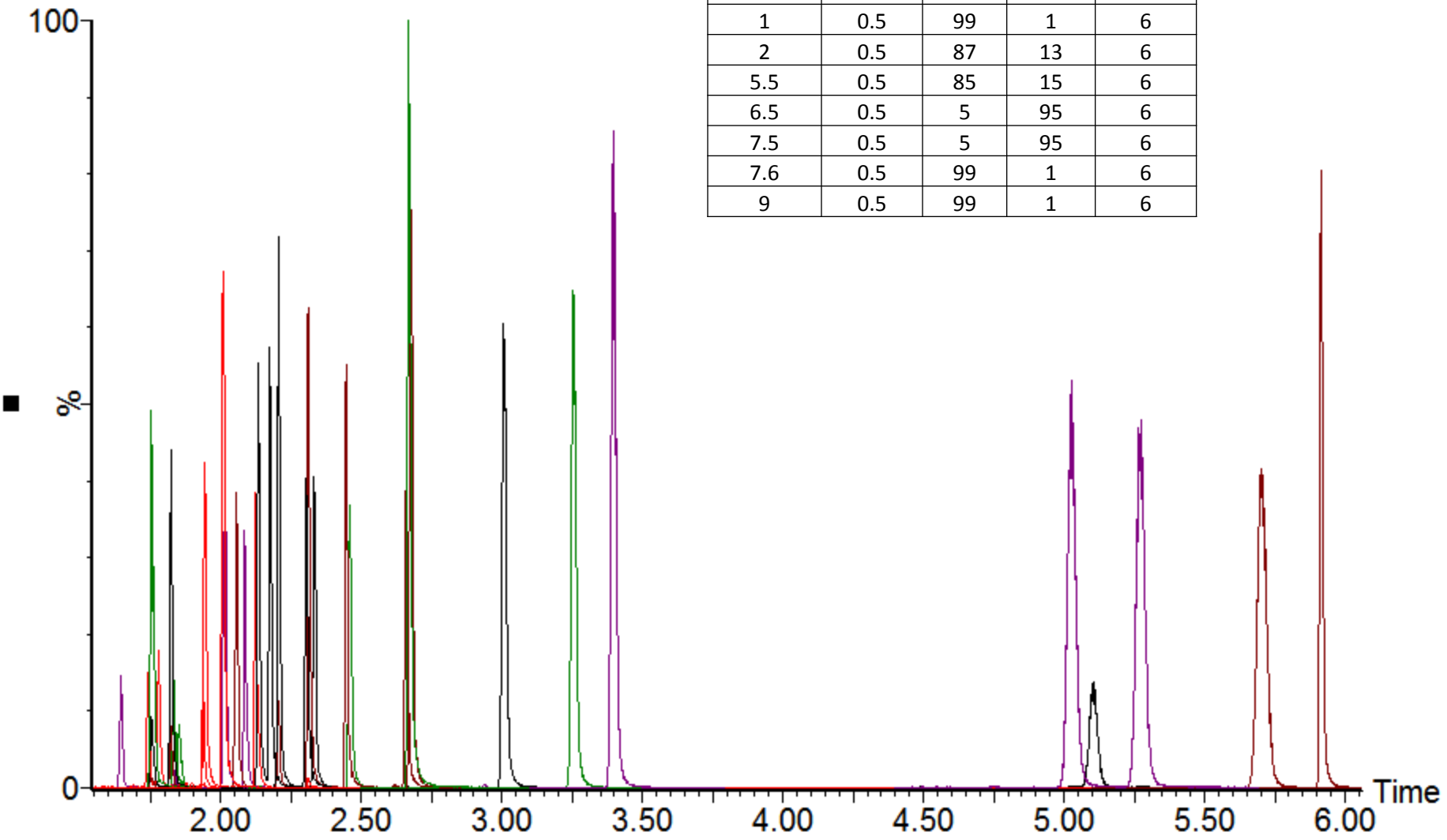
Liquid chromatography mass spectrometry

- Protein removal with SSA containing stable isotope IS
- Derivitisation of sample to produce a stable and well retained product which ionises well e.g. 6-aminoquinolone, heat at 55°C, 10 minutes
- Derivatives separated by reverse phase UPLC
- C18 column (2.1x100x1.6um)
- Gradient elution
- Mobile phase is ACN/formic acid/water
- Detection with single quad

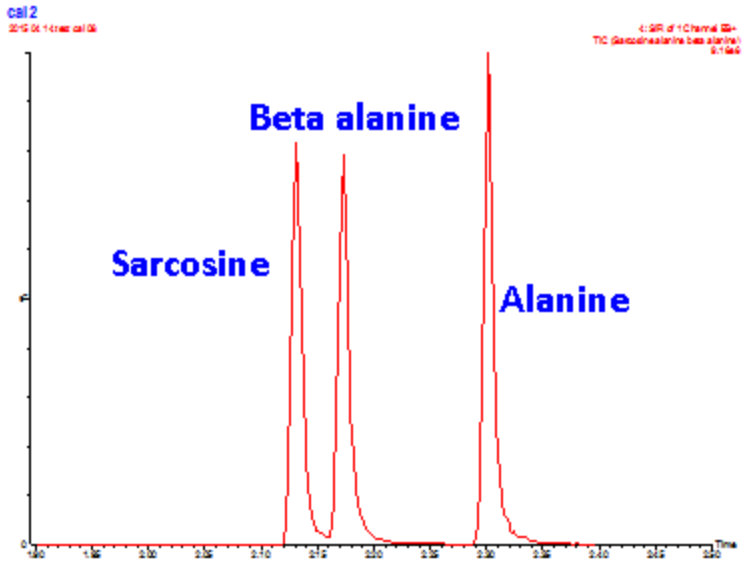


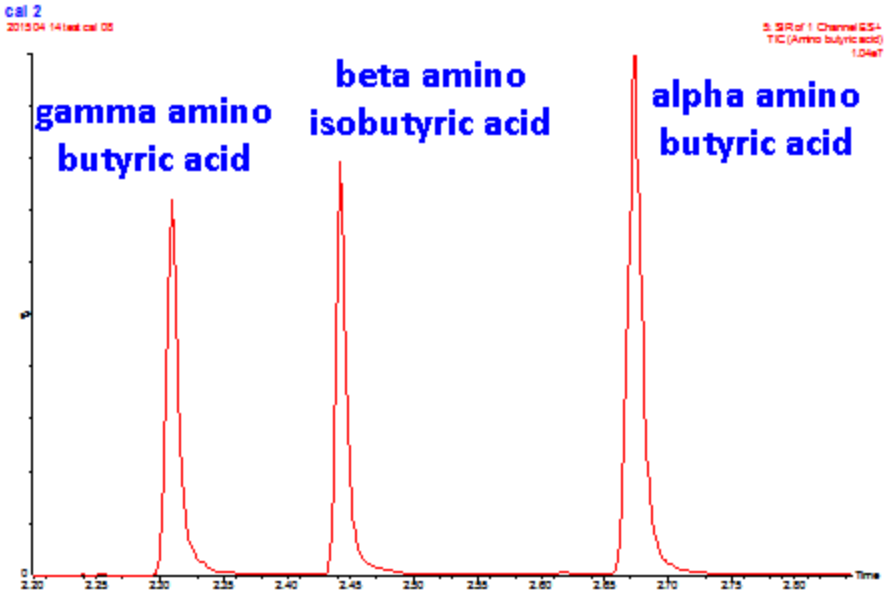
LCMS Chromatogram

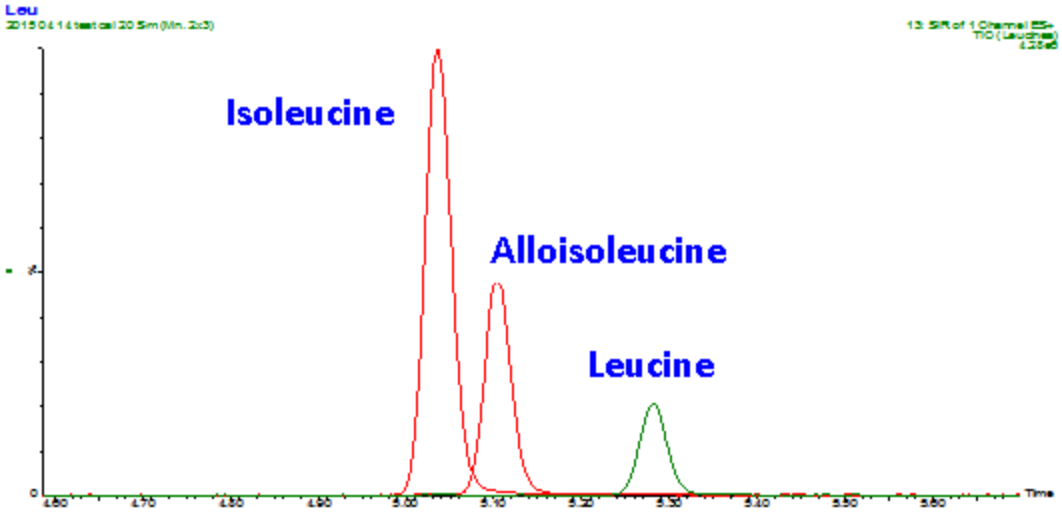
Time(min)	Flow (mL/min)	%A	%B	Curve
Initial	0.5	99	1	Initial
1	0.5	99	1	6
2	0.5	87	13	6
5.5	0.5	85	15	6
6.5	0.5	5	95	6
7.5	0.5	5	95	6
7.6	0.5	99	1	6
9	0.5	99	1	6



Separation of isobaric amino acids







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Advantages of LCMS

- Rapid analysis time ~10 mins
- Stable and precise
- Stable isotope IS
- Specific
- Chromatographic separation of isobaric compounds (iso, leu, allo)
- Suitable for sulphur containing amino acids
- Commercial kits are available eg Waters AccQTAG kit with Acquity UPLC and QDa mass detector, cost comparable to IEC
- Quantitation of 49 analytes of interest
- System can be used for other applications

Disadvantages of LCMS

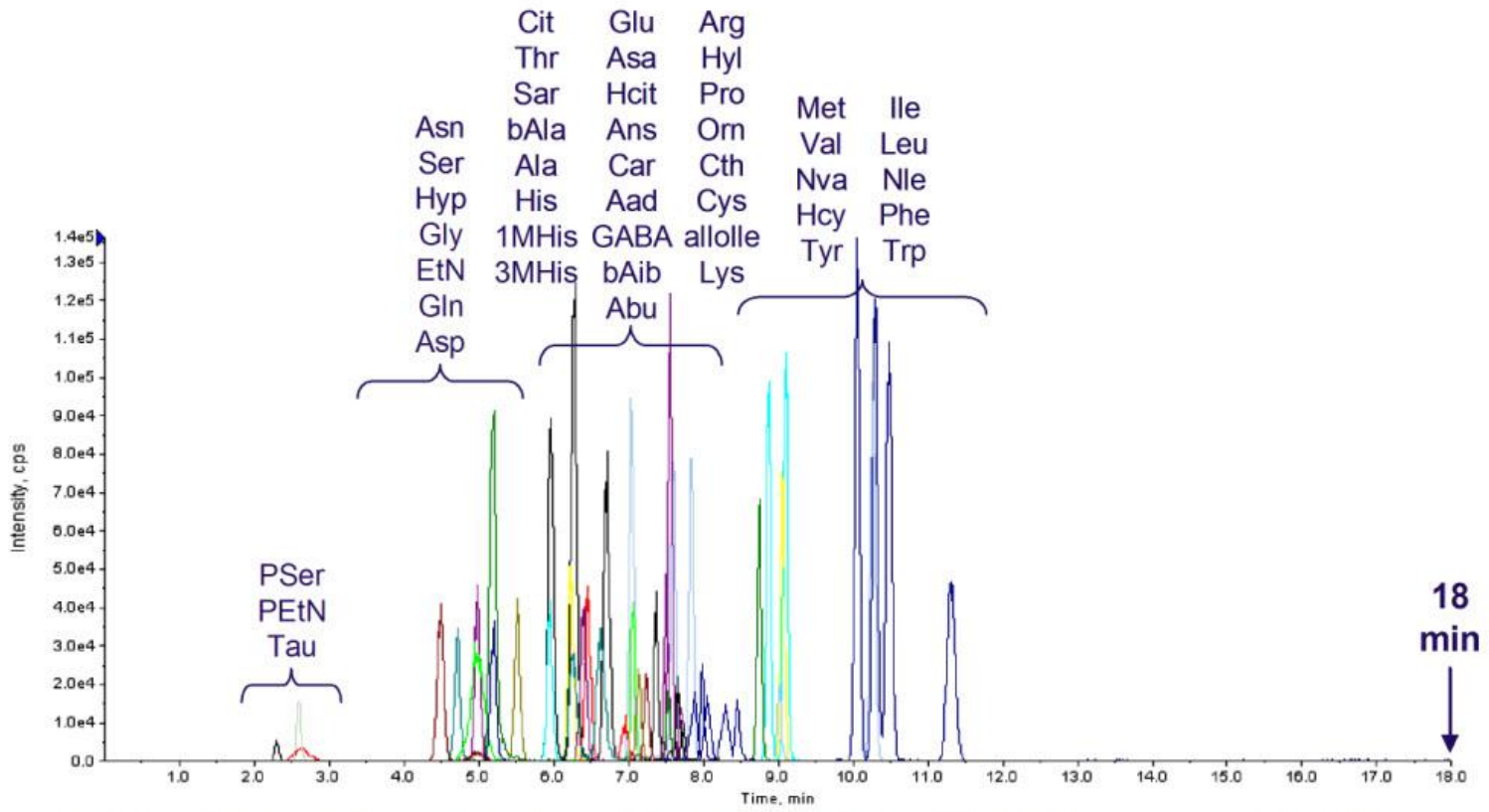
- Derivitisation required
- Sample prep takes additional 20 minutes

- Only see what you ask to see (SIM)

Liquid chromatography tandem mass spectrometry

- Derivitisation of sample to produce a stable and well retained product which ionises well e.g. Applied Biosystems iTRAQ kit
- Derivatives separated by reverse phase HPLC C18 column (2.1x100x1.6um)
- Gradient elution
- Mobile phase is ACN/formic acid/water with ion pair reagent
- Detection with triple quad MS
- Ability to quantitate 45 amino acids

Example of full profile amino acid chromatogram by LCMSMS



Representative chromatogram showing analysis of 45 amino acids using aTRAQ™ reagents and LC/MS/MS analysis. Total run time is 18 minutes.

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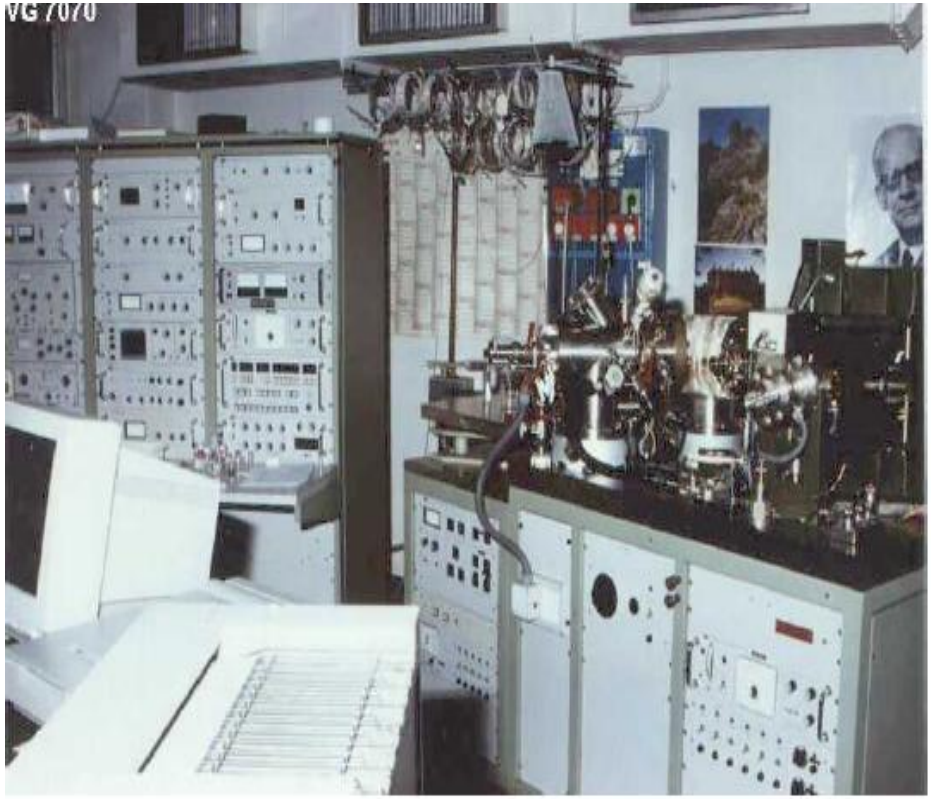


Advantages of LCMSMS

- Rapid analysis time eg 25 mins
- Stable isotope IS
- Superior specificity - combination of chromatographic separation and MRM enables separation of isobarics
- Stable and precise
- Suitable for sulphur containing amino acids
- Commercial kits are available that include columns, reagents, standards and controls e.g. Applied Biosystems iTRAQ
- Ability to analyse up to 45 amino acids in a single injection
- Other analytes can also be analysed

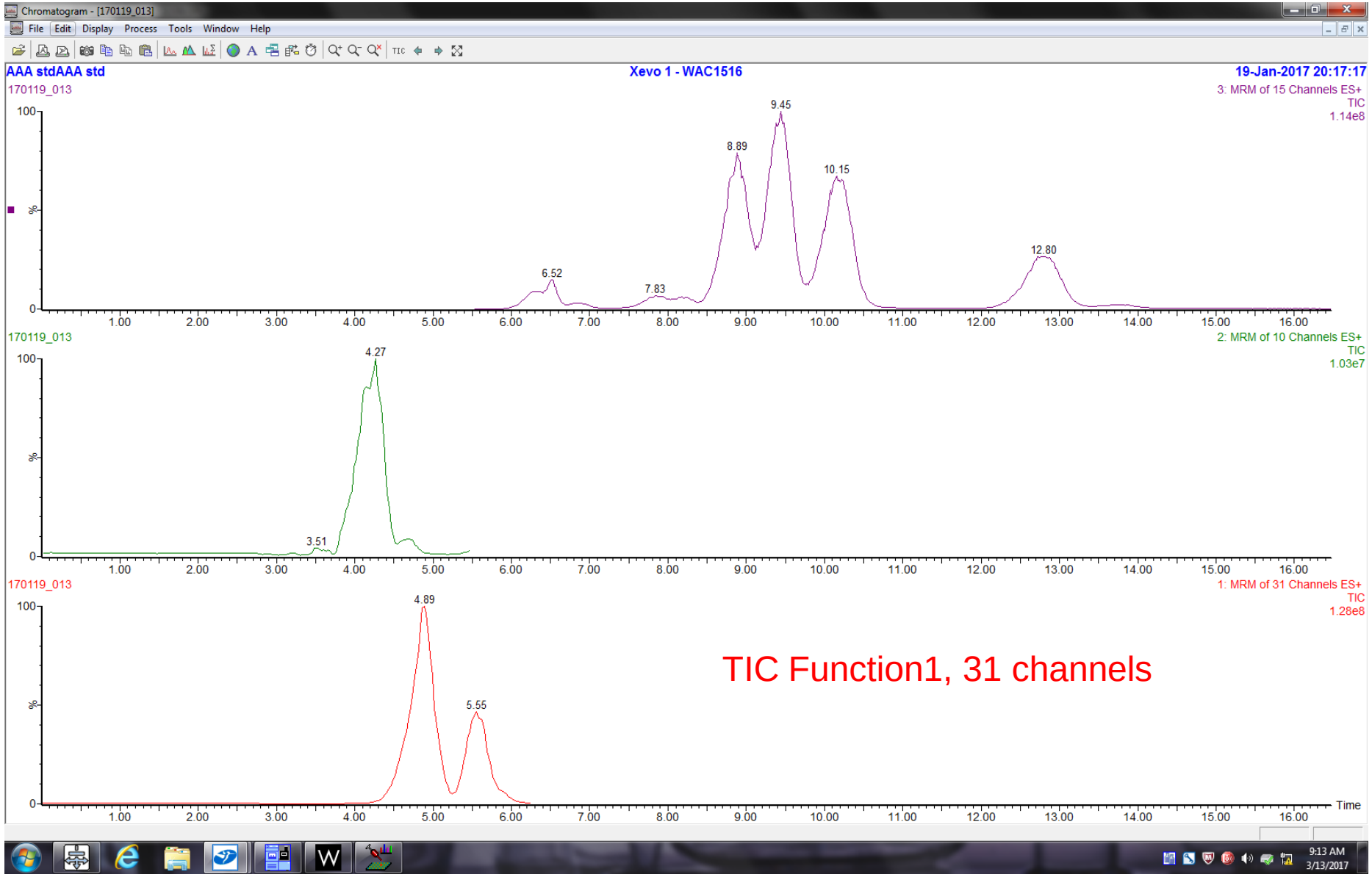
Disadvantages of LCMSMS

- Smaller linear range
- Not as precise as IED 10% cf 5%
- Derivitisation required - sample prep takes -30 minutes
- Ion pair reagent results in a dedicated LCMSMS system
- Limited number of MRMs in a given experiment – only see selected amino acids
- Operator expertise
- Cost

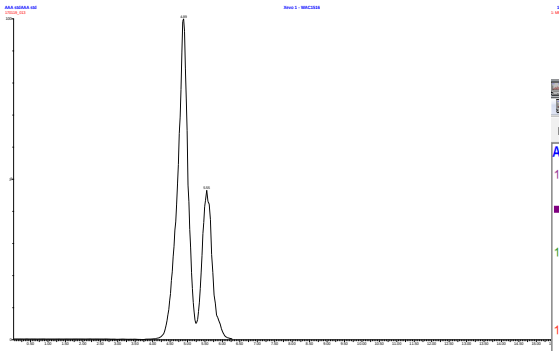
The disadvantages of LCMSMS are being overcome

- Technology has improved in last 5 years: instruments now have faster scan speeds/shorter dwell times/improved pos neg switching
- A given experiment can contain more MRMs and still produce quantitative data
- Analysis of full amino acid profile is possible without sample derivitisation or use of an ion pair reagent
- Can utilise simple protein crash and still achieve rapid analysis time (15 minutes)

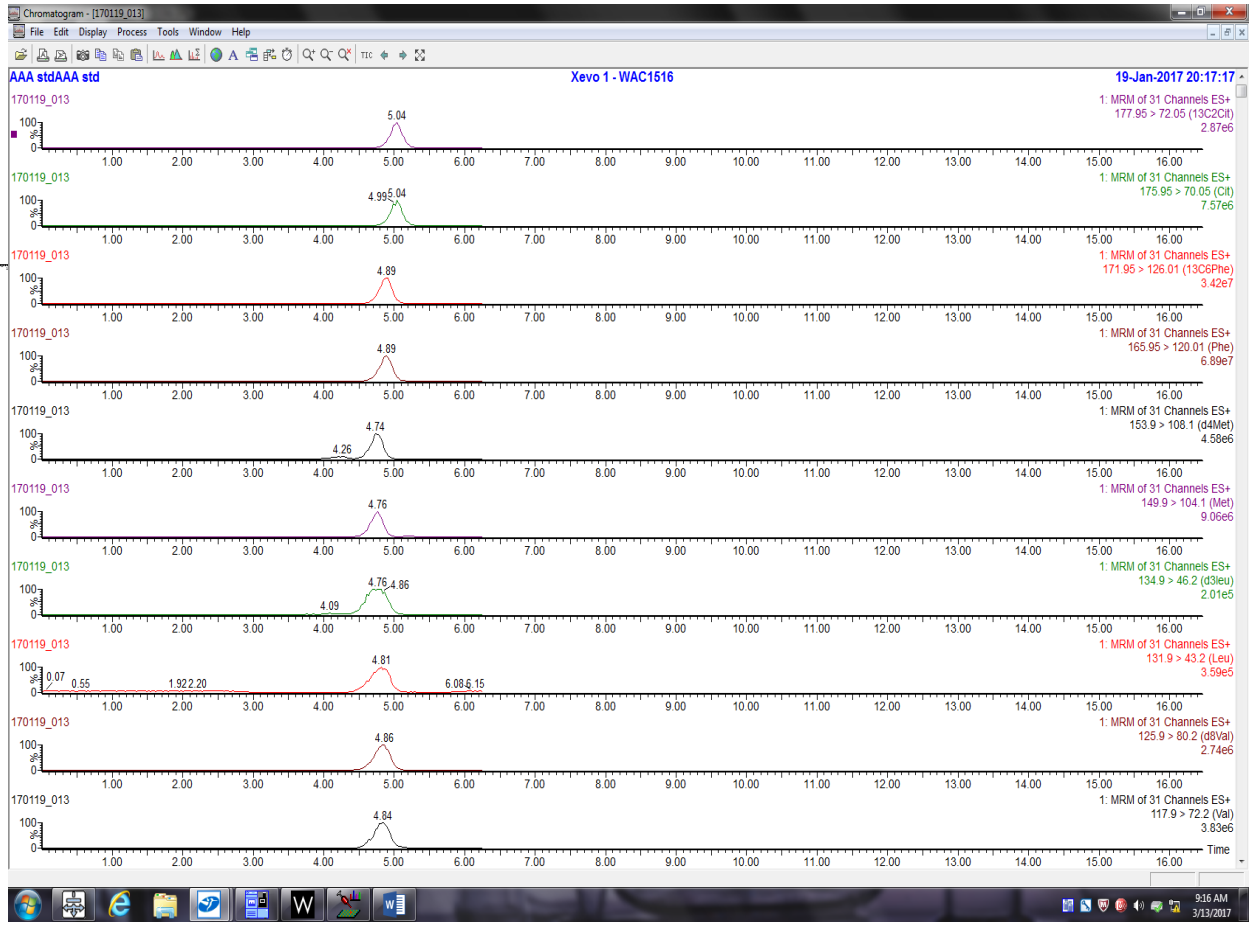
Xevo TQS Amino acid analysis - Positive ionisation, 3 time functions



TIC Function1, 31 channels



XIC Function1, 10 channels displayed



Disadvantages of LCMSMS

- More sensitive instruments = reduced linear range reduce
- Precision – jury is out?
- Upfront cost of stable isotopes for in house methods
- Cost of instrument
- Operator expertise

Advantages of LCMSMS

- Rapid analysis time eg 15 mins
- Stable isotope IS
- Superior specificity - combination of chromatographic separation and MRM enables separation of isobarics
- Open access instrument
- Other analytes can be analysed in the same injection

Performance characteristic	GC/GCMS	RP-HPLC	IEC	LCMS	LCMSMS
Analysis time (min)	15	60	180	10	15
Specificity	Good	Limited	Limited	Good	Excellent
Sensitivity	Reasonable	Good	Good	Good	Excellent
Sample prep	Derivitise	Derivitise	Simple	Derivitise	Simple
Internal standard	Stable isotopes	Structural analogue	Structural analogue	Stable isotopes	Stable isotopes
Clinical application AA disorders	Very limited	Limited	Full AA profile plus extras	Full AA profile	Full AA profile
Flexibility of system	Open	Open??	Dedicated	Open	Open
Operator expertise	XX	XX	XX	XX	XXX
Cost	££	£	££	££	£££

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QUALITY ASSURANCE IN LABORATORY TESTING FOR IEM

ERNDIM Quantitative Amino Acid Scheme

- Number of participants using a given method

Methodology	2001	2007	2015	2016
GC or GCMS	2	2	2	3
Tandem MS	0	5 (3%)	24 (10%	29 (12%)
RP-HPLC	17 (13%)	24 (13%)	34 (14%)	33 (14%)
IEC	115 (86%)	147 (82%)	172 (71%)	161 (67%)
Other	1	0	11 (4%)	13 (5%)
Total	*134*	*178*	*243*	*241*

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