QUALITY ASSURANCE IN LABORATORY TESTING FOR IEM

Qualitative Organic Acid

ERNDIM Workshop, 21st & 22nd November 2017, Manchester, UK

Mrs Camilla Scott
Dr Claus-Dieter Langhans
Dr Clothilde Roux

Slide title

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- Overview of Sheffield current (CS)
- Overview of Heidelberg current (CL)
- Why do we measure organic acids (clinical reasons) (CS)
- Analytical preparation of the samples (CL)
- Analytical pitfalls and problems (CL)
- (when to use Quantitative- CR)
- Interpretations and scoring (pitfalls and tricks to look for) (CS)
- Scoring and appeals (CS & CD)
- Problem Cases : (CS & CD)

Further chromatograms for the cases, interpretation and analytical problems discussion time permitting

Sheffield Team

Scientific Advisors – meet the team

- Mrs Camilla Scott
- Dr Jane Dalley (retd)

Deputy Scientific Advisor

- Miss Sharon Colyer

Administrator

Mrs Lynne Wolstenholme

Heidelberg Team

Scientific Advisors – meet the team

- Dr Claus-Dieter Langhans
- Dr Verena Peters
- Prof. Dr Georg Hoffmann

Deputy Scientific Advisor

- Prof. Dr Nenad Blau

Scheme overview

- Nine heat treated samples per year
- To be analysed in 3 batches of 3
- Results submitted currently by paper – hopefully for 2018 by website
- Include normal and abnormal samples
- Wide range of disorders or abnormalities commonly observed in the laboratory
- We are severely restricted by quantity, crisis samples, quality of samples

Participants

- Number of participants in Sheffield 107 with one educational participant
- Number of participants in Heidelberg …
- Wide geographical representation
- Educational participants are participants who wish to participate in an EQA scheme to assist in setting up a new test who are not currently offering a clinical service for that test. Educational participation is limited to 2 years.

Workshop

- Why do we measure organic acids in urine?

Workshop to answer…

Clinical

- As a general screening tool to rule out metabolic causes of a common dysfunction, e.g. developmental delay, epilepsy, autism, hypoglycaemia
- To prove the hypothesis that metabolic disease is the cause of the clinical presentation e.g. hyperammonaemia, severe metabolic acidosis, hyperalkalotic states.
- To confirm abnormalities in other investigations, e.g. NMR, burst suppression EEG abnormal MRI
- To monitor known patients
- To investigate families of known disorders

1. Analytical

Procedure for sample preparation

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1. Determine creatinine in urine sample (Jaffe method)
2. Use an aliquot equivalent to 1µmol creatinine
3. Add: 100 µl 2-oxocaproic acid (1.25 mmol/l)
   100 µl 4-nitrophenol d_4 (1.25 mmol/l)
   100 µl PFBHA (50 mg/l) for oximation
   300 µl 5N HCl
   500 µl NH_4Cl (saturated)
4. Reaction time for oximation: 60 min at RT
5. Extract twice with 5 ml ethyl acetate
   Centrifuge for 5 min at 3500 rpm
6. Add solid sodium sulfate to the ethyl acetate phase
   After 30 min centrifuge for 5 min at 3500 rpm
7. Remove ethyl acetate under a gentle stream of nitrogen at 40 °C
8. Dissolve in 200 µl dry ethanol
   Transfer ethanol in 1 ml GC vial
9. Remove ethanol under a gentle stream of nitrogen at 40 °C
10. Add 50 µl MSHFBA for silylation
11. Reaction time for silylation: 60 min at 60°C
12. Add 50 µl n-hexane
13. Use 1 µl for injection into GCMS

Procedure for sample preparation

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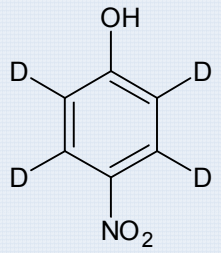
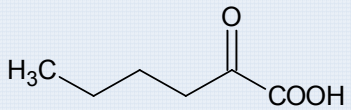
- Urine volume equivalent to 1 µmol creatinine
- Internal standards:

  100 µl 4-nitrophenol-d_4 (1.25 mM)

  100 µl 2-oxocaproic acid (1.25 mM)



4-Nitrophenol-d_4



2-Oxocaproic acid

Procedure for sample preparation

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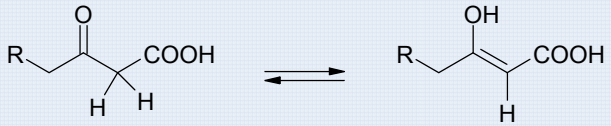
- Oximation of oxoacids:

  100 µl PFBHA (50 mg/l)

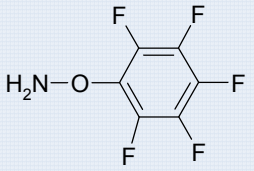
  Reaction time: 60 minutes

- improve recovery of oxoacid
- Keto-Enol-tautomery

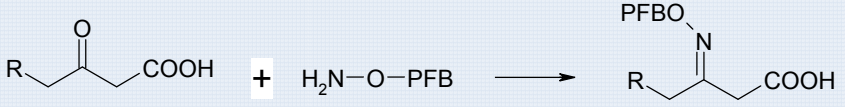


„Keto“ Enol

- Reagent: Pentafluorobenzylhydroxylamine **(PFBHA)**



- Oxime formation:



Procedure for sample preparation

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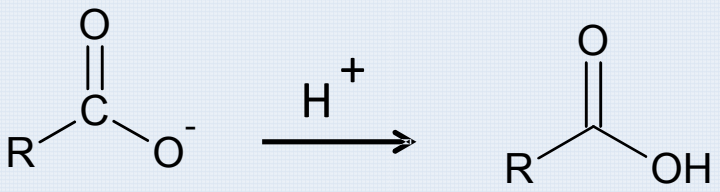
- Acidification:

  300 µl HCl (5N)



➢ Converting polar carboxylats to non-polar, more lipophilic carboxylic acids

Procedure for sample preparation

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- Increasing ion strength:

  500 µl NH_4Cl (saturated solution)

- Large amounts of inorganic salts „bind“ water
- Salting-out effect: decreasing solubility of organic metabolites in aqueous solution

Procedure for sample preparation

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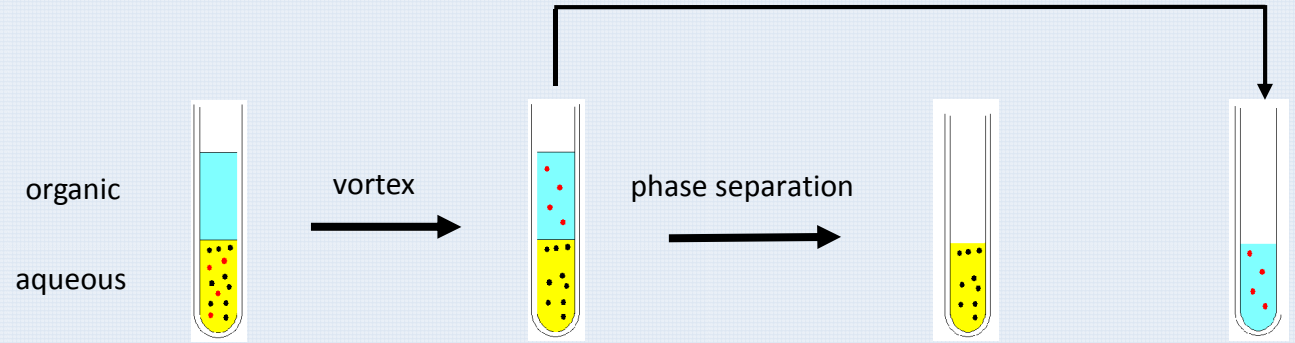
- Liquid-liquid extraction: = solvent extraction

  2 × 5 ml Ethylacetate

Prerequisite 2 immiscible liquids

Distribution according to the partition coefficient



Procedure for sample preparation

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- Solvent drying:

  solid anhydrous Na_2SO_4

- Removing of water
- Anhydrous sodium sulphate binds water

	Solubility of water
Ethylacetate	2.9%
Diethylether	1.5%
Methylacetate	8.0%

Procedure for sample preparation

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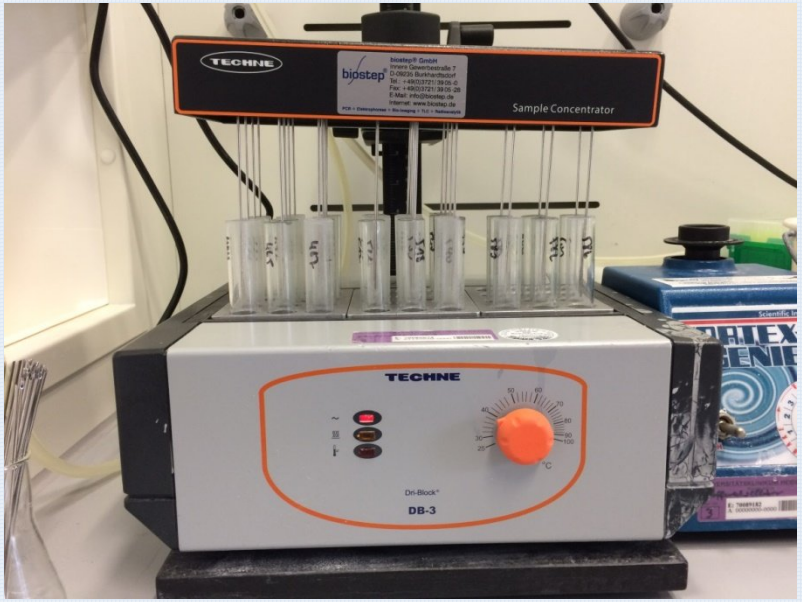
- Solvent evaporation:

  with a stream of nitrogen at 40°C

➢ Sample concentrator



Procedure for sample preparation

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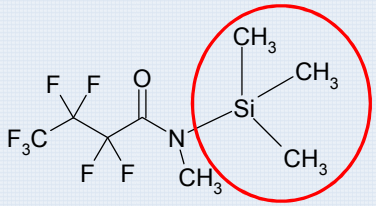
- Derivatization:

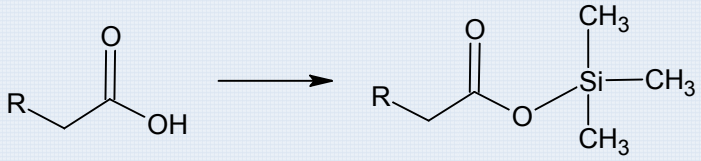
  50 µl MSHFBA
  Reaction time: 60 minutes

- Synthesis of volatile derivatives
- Masking of the polar parts in the molecules (OH groups)

- Reagent: N-methyl-N-trimethylsilyl-heptafluorobutyramid **(MSHFBA)**



TMS



TMS ester

Gas Chromatography Mass Spectrometry (GCMS)

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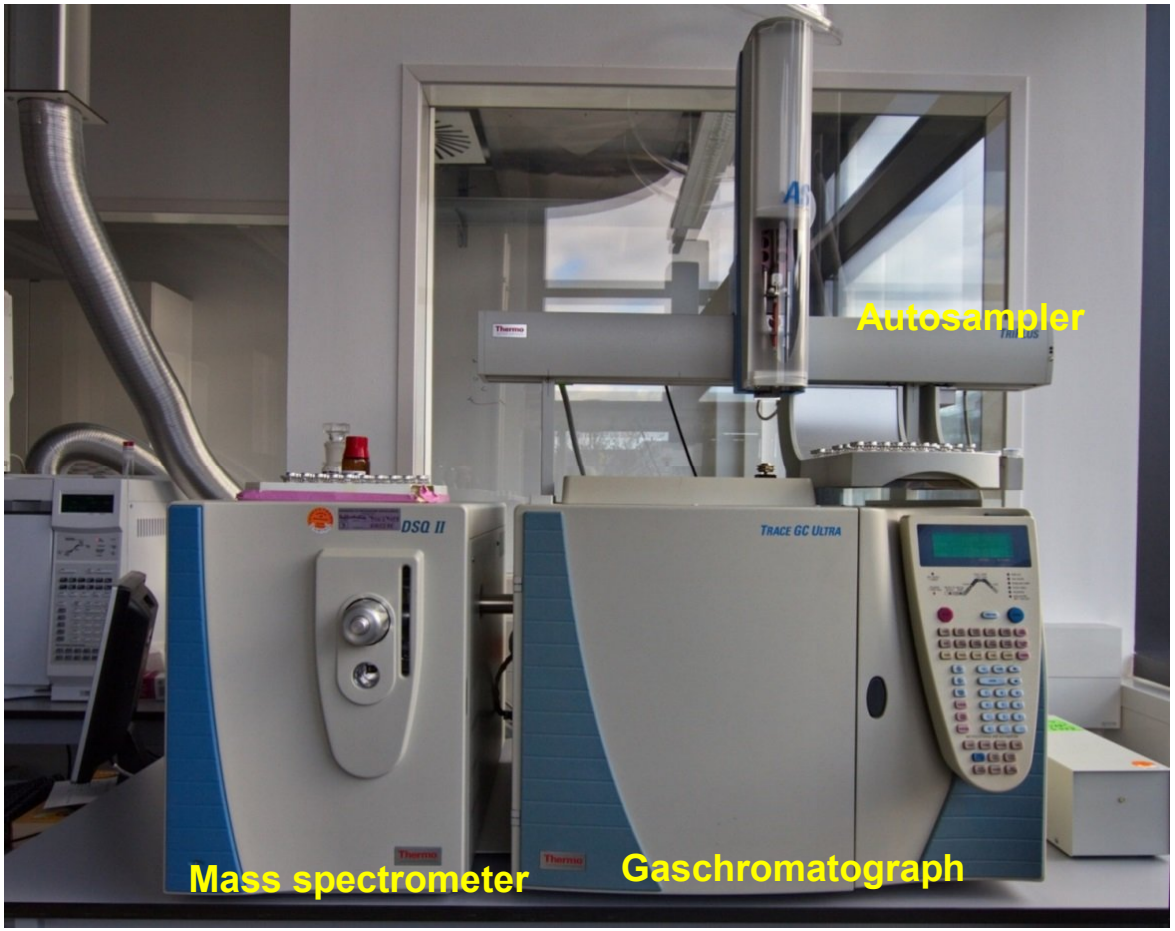

TRACE GC Ultra

DSQ II Quadrupol mass spectrometer

Thermo Fisher Scientific

Gas Chromatography Mass Spectrometry (GCMS)

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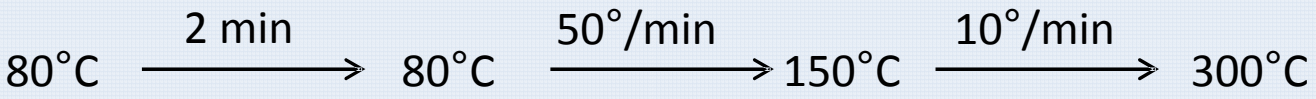
- Gas chromatography - Parameters:

Column: DB-5 MS, 30 m × 0.25 mm ID, 0.25 µm df (J&W Scientific)

Injector temperature:	260°C
Transfer line:	280°C
Split injection:	1 µl sample



Gas Chromatography Mass Spectrometry (GCMS)

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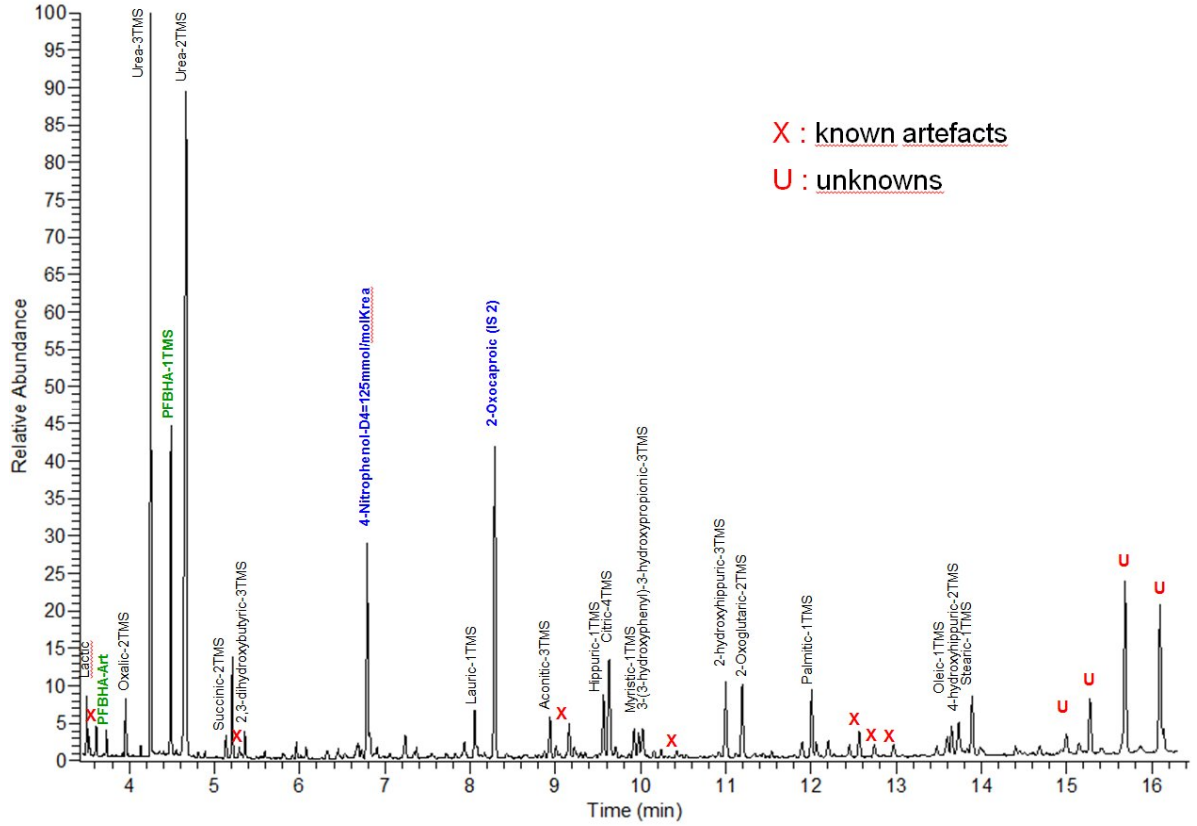
- Normal pattern of organic acids:



Reproducibility

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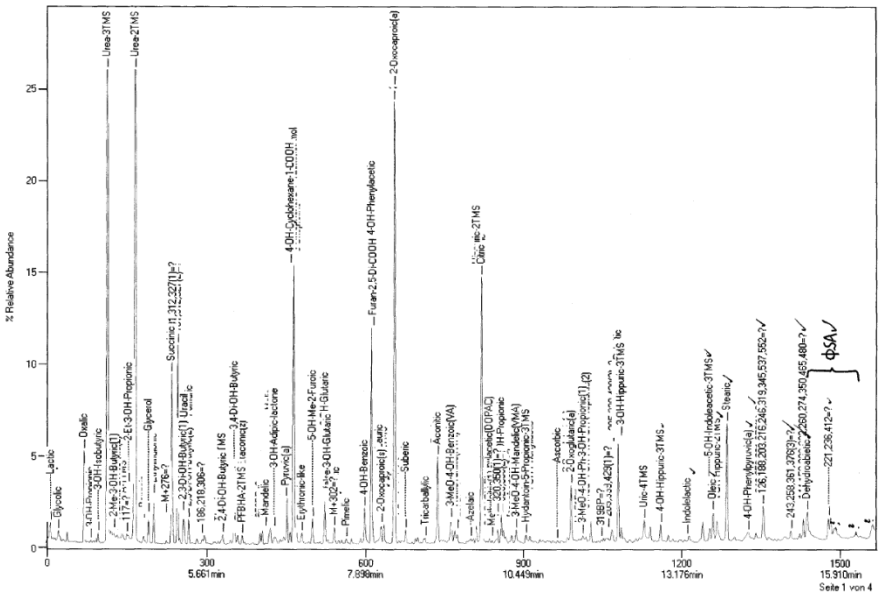
Untargeted ⟹ Targeted



Material	Beschreibung		+/-	Ergebnis	St.	t.Vorw.1	NormV	NormB	Einheit	Vorwert
Urin	3-OH-Isovalerat	§		5	:		0	214	mmol/molKrea	
Urin	2-Methyl-3-OH-Butyrat	§		10	:		0	33	mmol/molKrea	
Urin	Malonat	§		5	:		0	14	mmol/molKrea	
Urin	Methylmalonat	§		11	:		0	18	mmol/molKrea	
Urin	2-Ethyl-Hydracylat	§		15	:		0	21	mmol/molKrea	
Urin	2-OH-Isocapronat	§		1	:		0	1	mmol/molKrea	
Urin	2-OH-3-Methylvalerat	§		1	:		0	2	mmol/molKrea	
Urin	4-OH-Butyrat	§		1	:		0	14	mmol/molKrea	
Urin	Glycerol	§		25	:		0	225	mmol/molKrea	
Urin	Ethylmalonat	§		10	:		0	19	mmol/molKrea	
Urin	Phenylacetat	§		2	:		0	2	mmol/molKrea	
Urin	Succinat		+	500	:		0	142	mmol/molKrea	
Urin	Methylsuccinat	§		3	:		0	5	mmol/molKrea	
Urin	Glycerat	§		10	:		0	12	mmol/molKrea	
Urin	Uracil		+	75	:		0	29	mmol/molKrea	
Urin	Fumarat	§		12	:		0	21	mmol/molKrea	
Urin	Propionylglycin	§		1	:		0	2	mmol/molKrea	
Urin	2-Methyl-2,3-DiOH-Butyrat	§		4	:		0	6	mmol/molKrea	
Urin	Mevalonolacton	§		3	:		0	6	mmol/molKrea	
Urin	Thymin	§		1	:		0	1	mmol/molKrea	
Urin	Glutarat	§		5	:		0	8	mmol/molKrea	
Urin	3-Methylglutarat	§		5	:		0	10	mmol/molKrea	
Urin	3-Methylglutaconat	§		6	:		0	15	mmol/molKrea	
Urin	Mandelat	§		1	:		0	1	mmol/molKrea	
Urin	Malat	§		10	:		0	44	mmol/molKrea	
Urin	Dihydrouracil	§		1	:		0	17	mmol/molKrea	
Urin	Dihydrothymin	§		1	:		0	1	mmol/molKrea	
Urin	Isovalerylglycin	§		1	:		0	17	mmol/molKrea	
Urin	Adipat	§		16	:		0	30	mmol/molKrea	
Urin	Pyruvat	§		10	:		0	118	mmol/molKrea	
Urin	5-Oxoprolin	§		75	:		0	80	mmol/molKrea	
Urin	2-Oxoisovalerat	§		2	:		0	3	mmol/molKrea	
Urin	Tiglylglycin	§		1	:		0	6	mmol/molKrea	
Urin	3-Methylcrotonylglycin	§		2	:		0	2	mmol/molKrea	
Urin	2-OH-Phenylacetat	§		2	:		0	2	mmol/molKrea	
Urin	2-OH-Glutarat		+	60	:		0	30	mmol/molKrea	
Urin	3-OH-Glutarat	§		3	:		0	8	mmol/molKrea	
Urin	Acetoacetat	§		75	:		0	150	mmol/molKrea	

Reproducibility

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Method evaluation

For 73 most relevant metabolites

Intraday with N = 10

Interday with N = 10

Reproducibility

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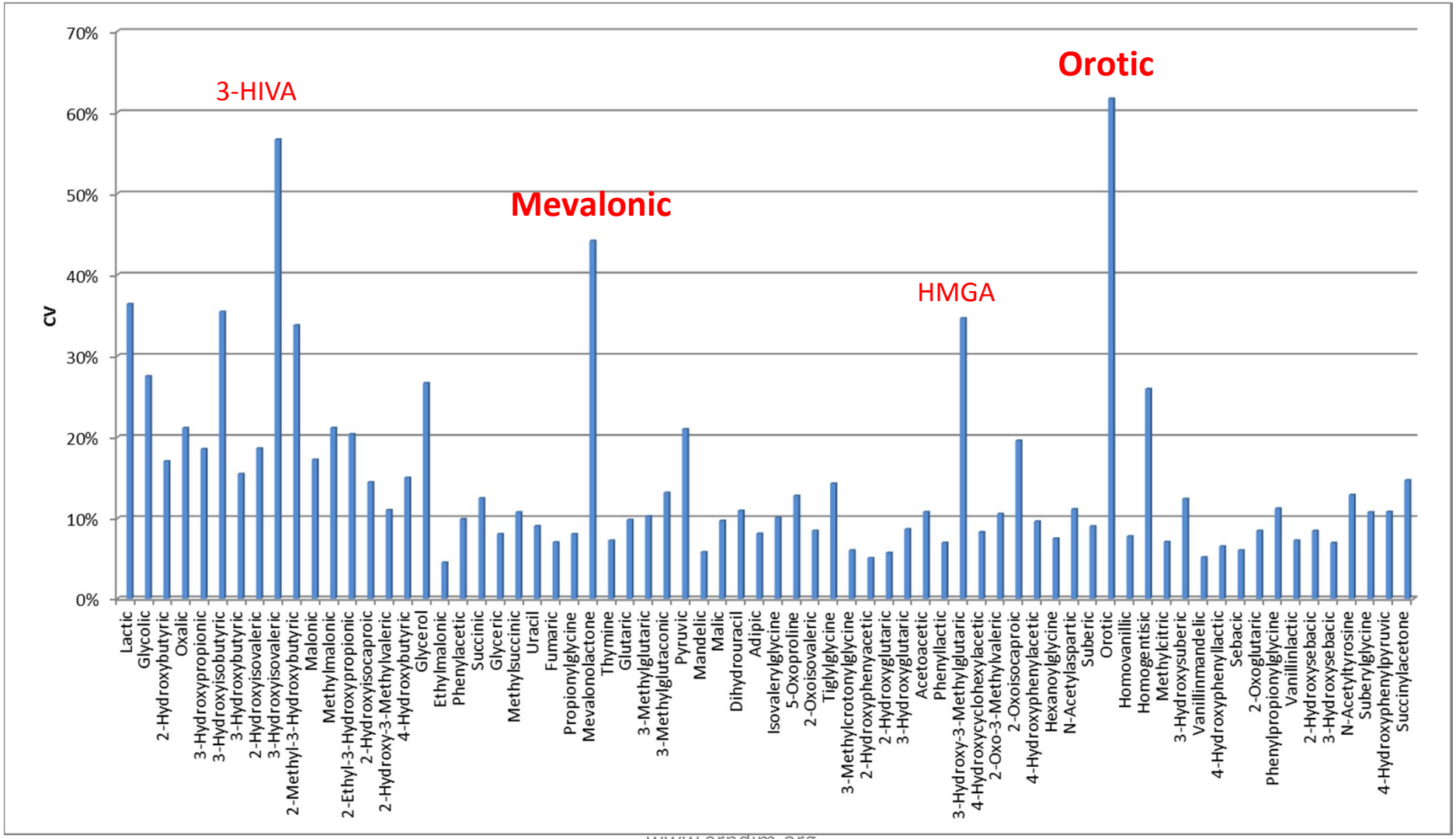

Reproducibility

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Product certificate Special Assays in Urine MCA

Product name Control Special Assays in Urine

Product code

Level	Product code	Colour cap
1	SAU-01.1	Green
2	SAU-01.2	Red

Date of issue 12-12-2016

Batch numbers and Expiry date

Level	Batch number	Exp. date stored at +2°C to +8°C
1	LOT 2016.0091	**2020-09**
2	LOT 2016.0092	**2020-09**

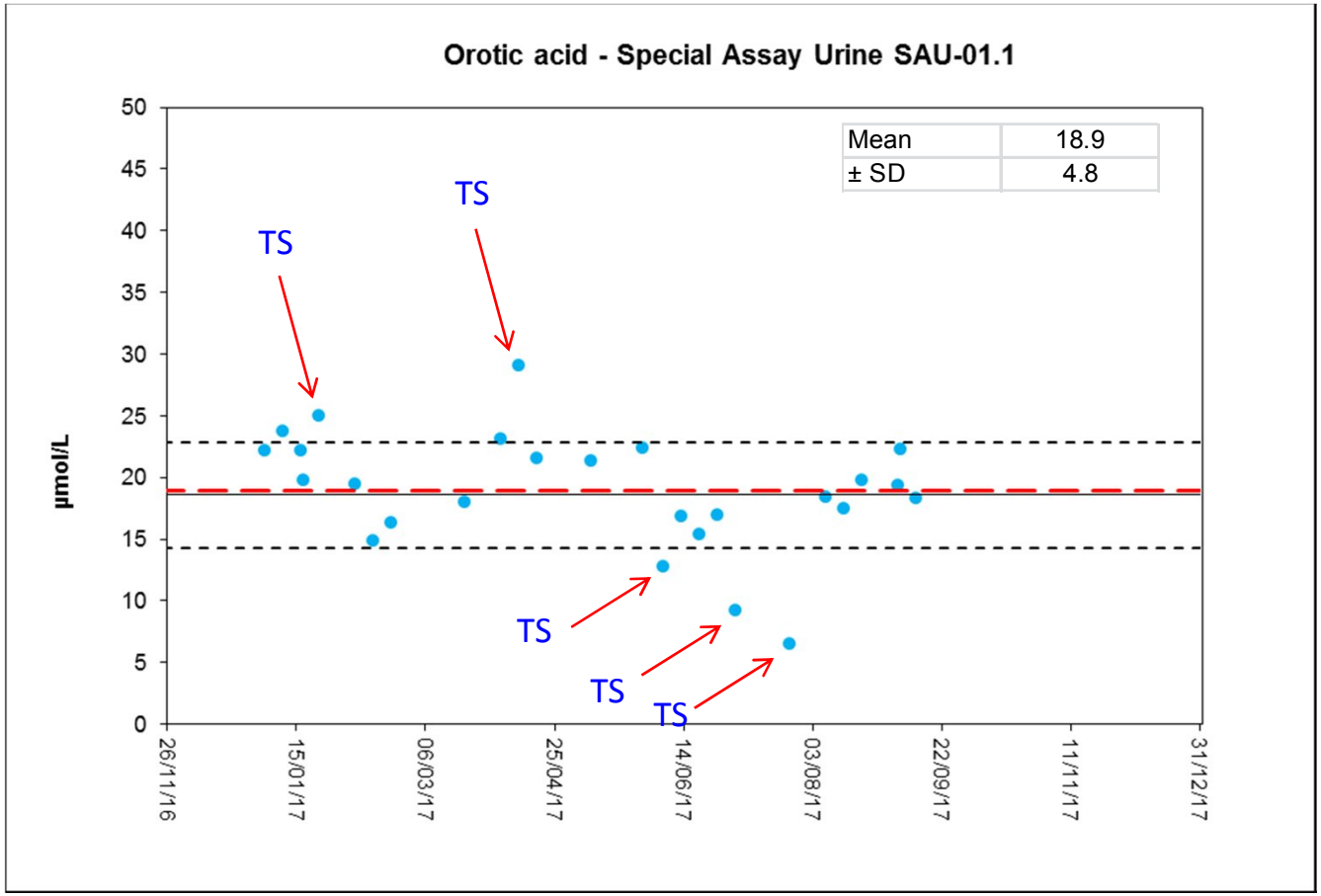
Calculated Consensus Values

Analyte	Unit	Level 1		Level 2	
		Calculated Consensus Value *	Range Laboratories *	Calculated Consensus Value *	Range Laboratories *
Orotic acid	µmol/L	18.6	14.3 - 22.8	87.0	72.6 - 101

= 4.7 mmol/mol crea

Cut off (orotic acid): < 3 mmol/mol crea

Reproducibility



TS = technical service

- ✓ Cleaning ion source
- ✓ Change column
- ✓ Change inlet liner

Reproducibility

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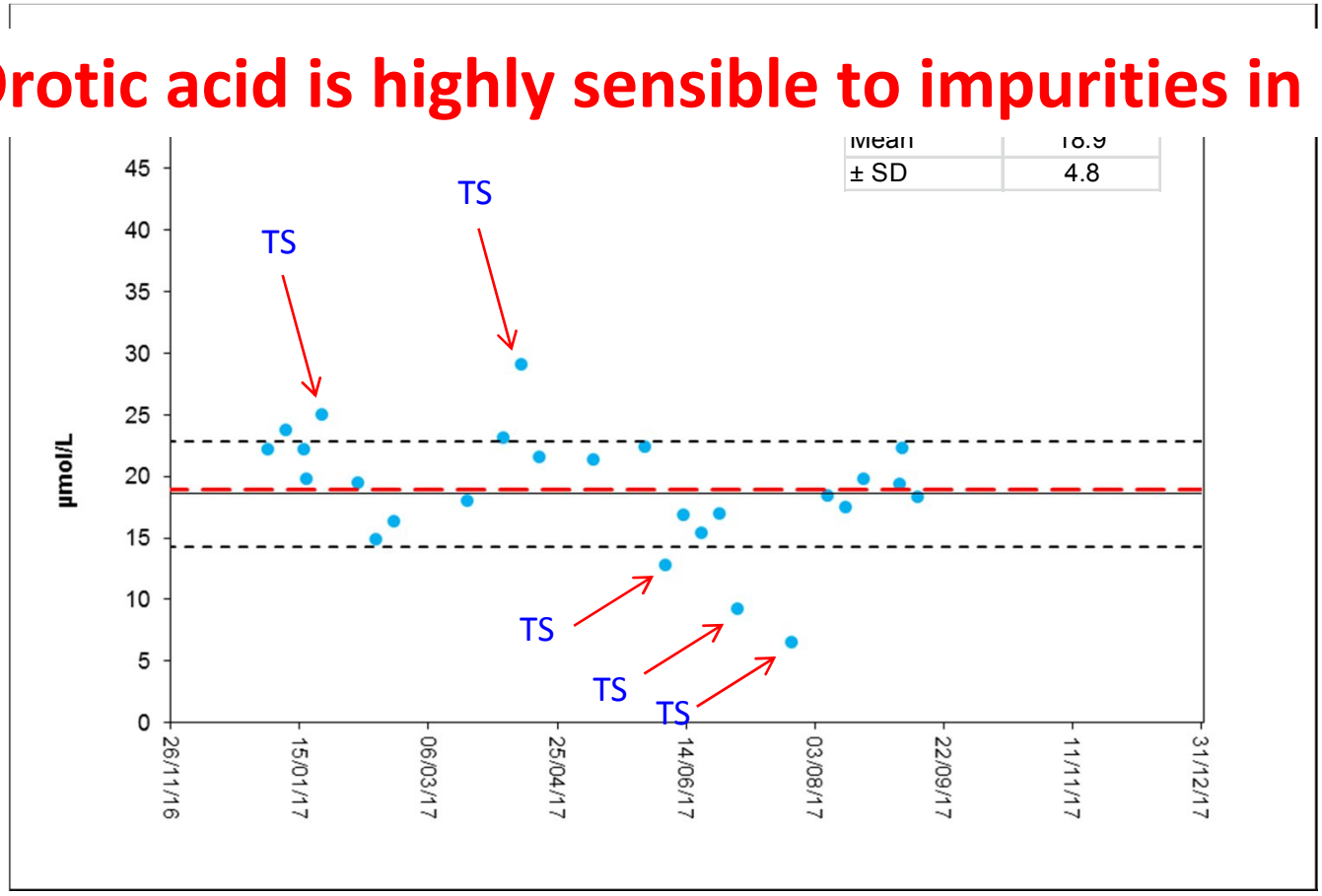
Orotic acid is highly sensible to impurities in GCMS



TS = technical service

- ✓ Cleaning ion source
- ✓ Change column
- ✓ Change inlet liner

Analytical problems

- Workshop to answer

Possible Pitfalls

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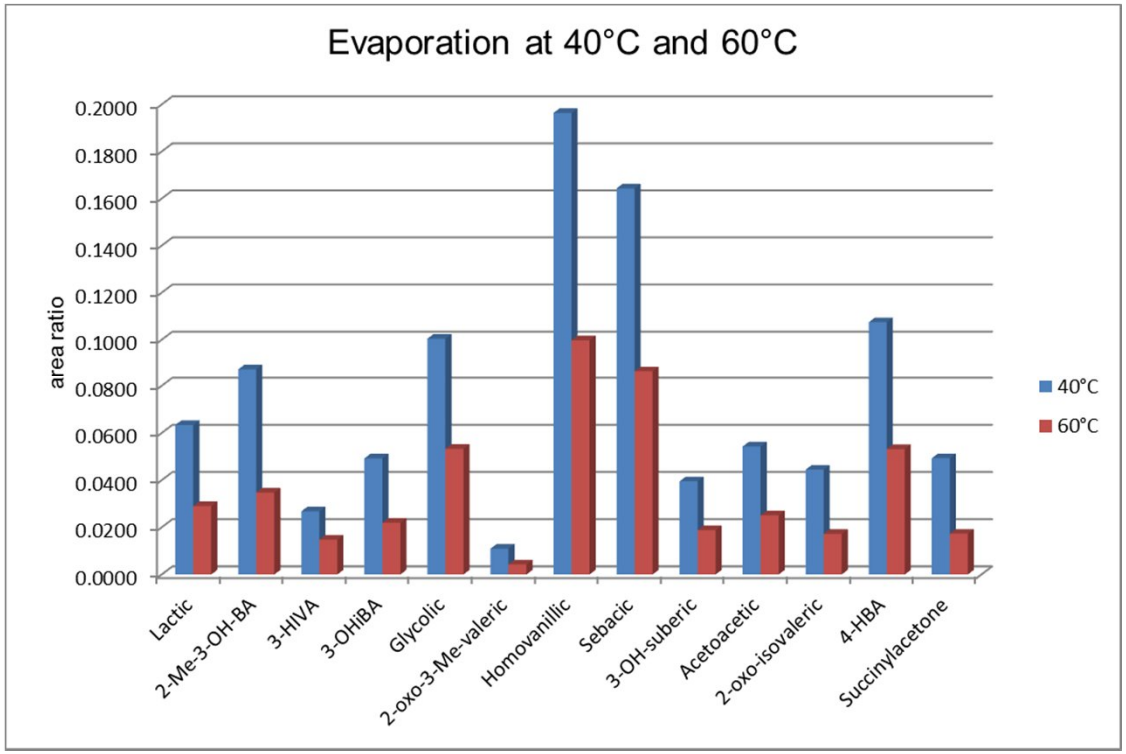
- Solvent evaporation I
  - ➢ loss of metabolites at higher temperature



Possible Pitfalls

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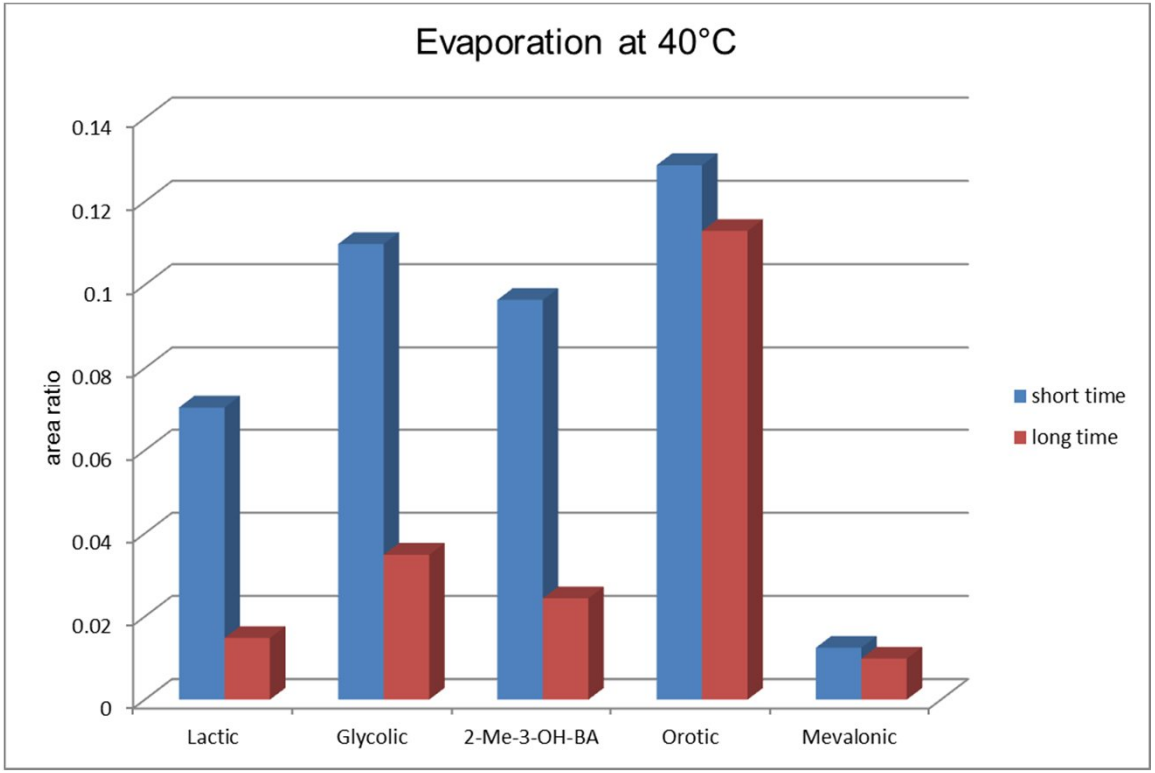
- Solvent evaporation II

➢ blowing nitrogen on dry samples might result in sample loss



Short time:
- Samples just dry
- Nitrogen flow stopped on time

Long time:
- Samples just dry
- Nitrogen flow continues for longer time

Possible Pitfalls

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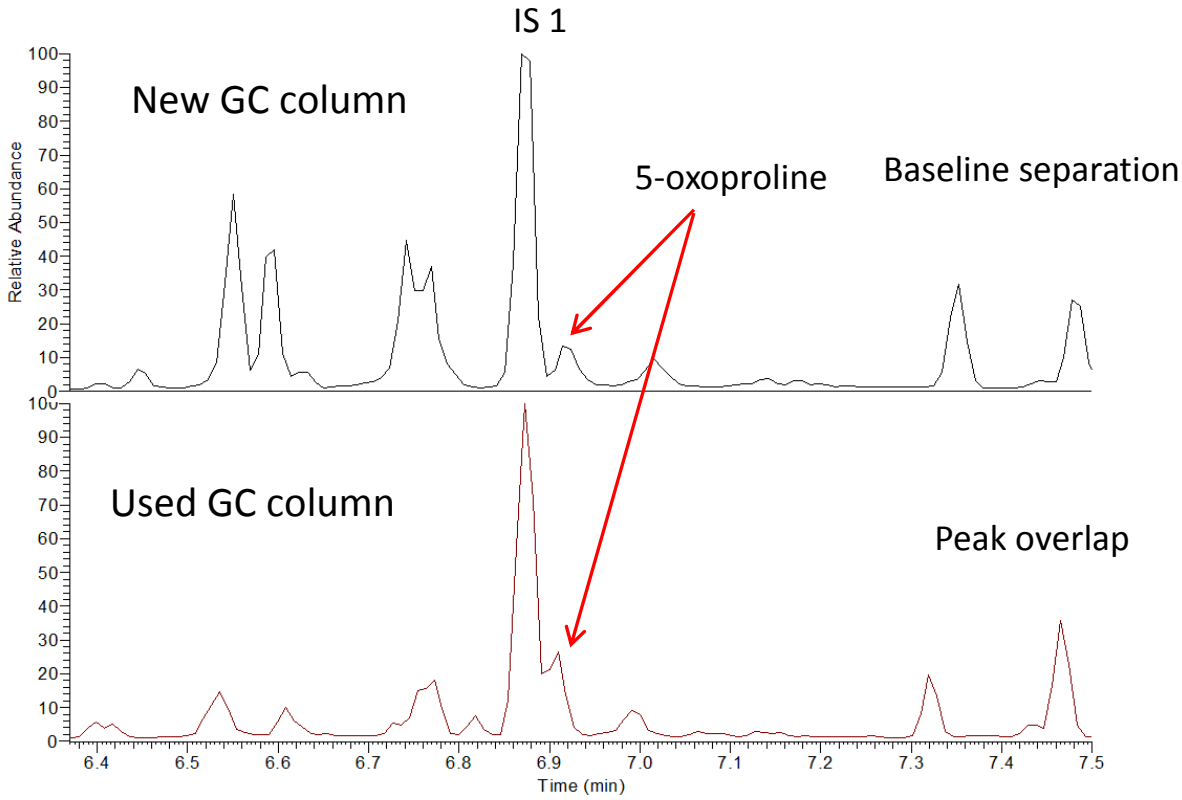
- GC column deterioration

➢ Wall-coated GC column deteriorate with time due to contamination and phase bleeding



Possible Pitfalls

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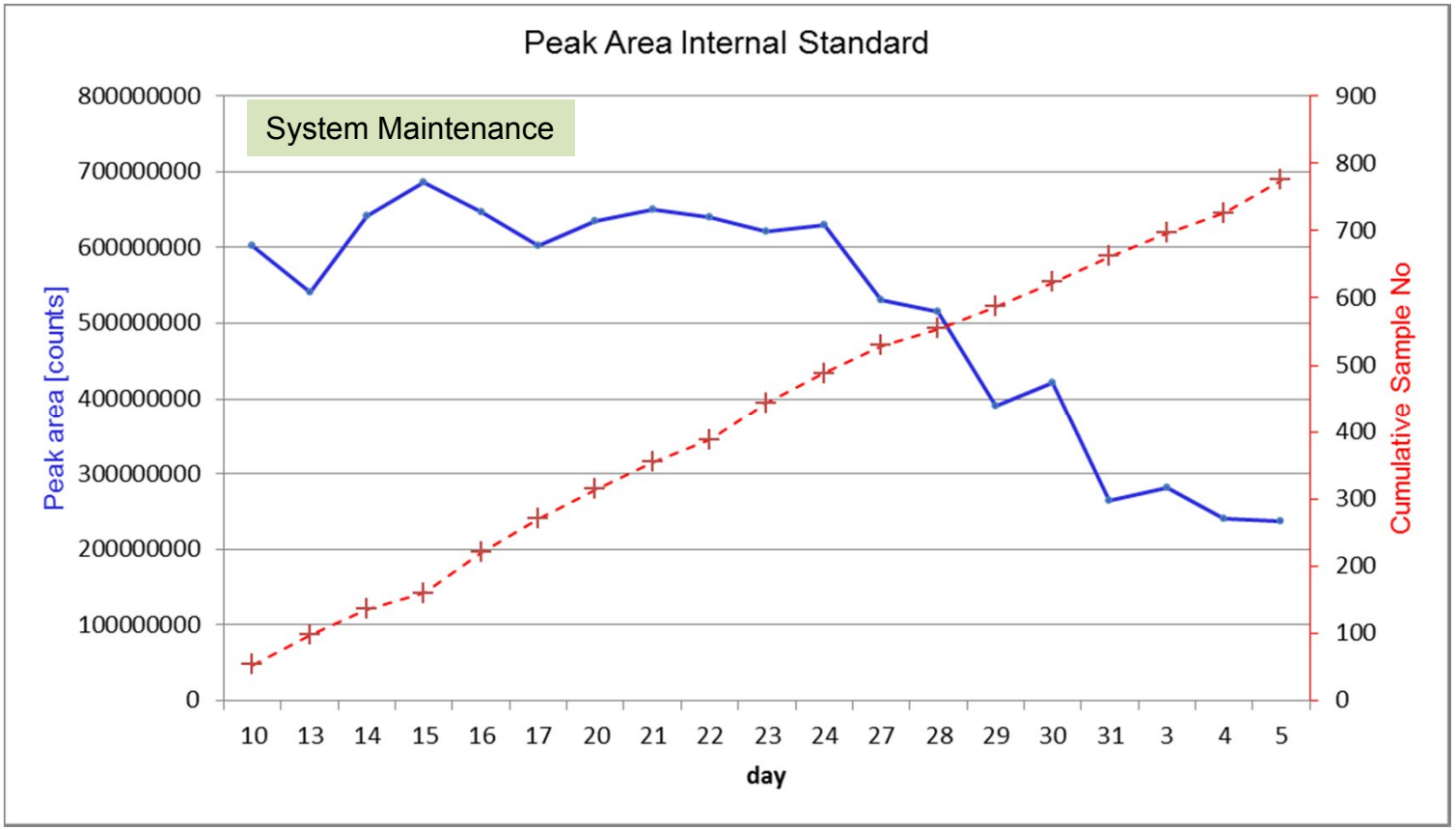
- GCMS performance



Recommendations I

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- GCMS requires regularly scheduled maintenance
  - ✓ Ion source
  - ✓ GC column
  - ✓ Inlet liner
  - ✓ Leak check
- Method re-evaluation
  - ✓ Careful examining of all crucial steps in sample work-up
  - ✓ Solvent evaporation
  - ✓ Silylation (reagent, derivatization time)
  - ✓ In GC: inlet liner style and packing

Recommendations II

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- Define your own criteria for system performance

- Monitor performance of
  - ✓ Gas chromatograph
  - ✓ Mass spectrometer
  - ✓ Sample work-up

When to Quant

Interpretation

- Analytical issues must be checked first
- Check abundance
- Check internal standards

- Essentials to interpreting organic acid chromatograms
  - Good laboratory technique
  - Good library
  - Knowledge of:
    - Exogenous Compounds
    - Ambiguous results
    - Pitfalls and problems
- Characteristic patterns aid diagnosis, however diagnosis can rarely be made without additional tests

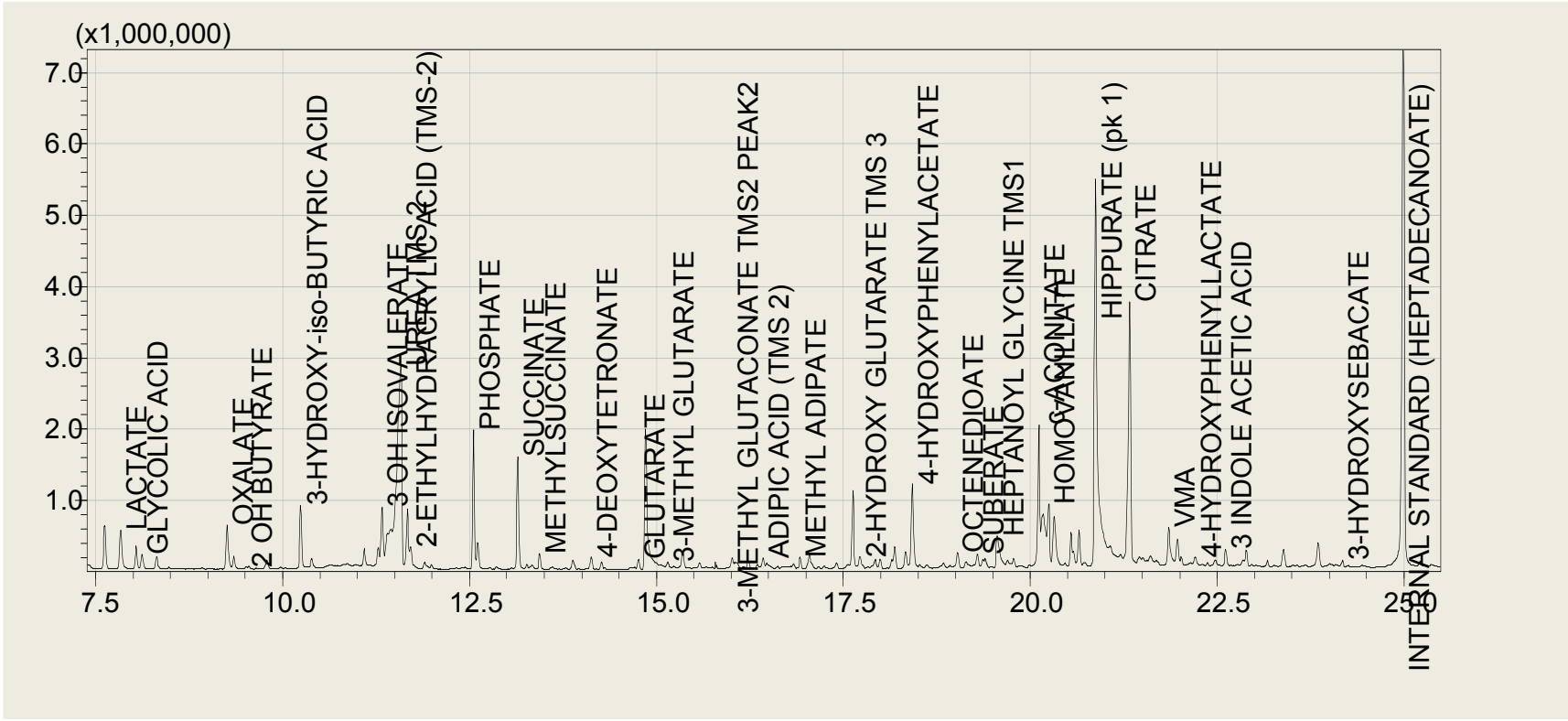
Key points to pattern recognition

- Look at the whole chromatogram
- Carry out initial checks by looking at macros or extract ions for low excretion compounds e.g. acylglycines
  - Note that small peaks will be present in normal urines. Look closely at anything >10K
- Be aware of
  - Drug interactions
  - Post mortem changes
  - Bacterial degradation
  - Feeds

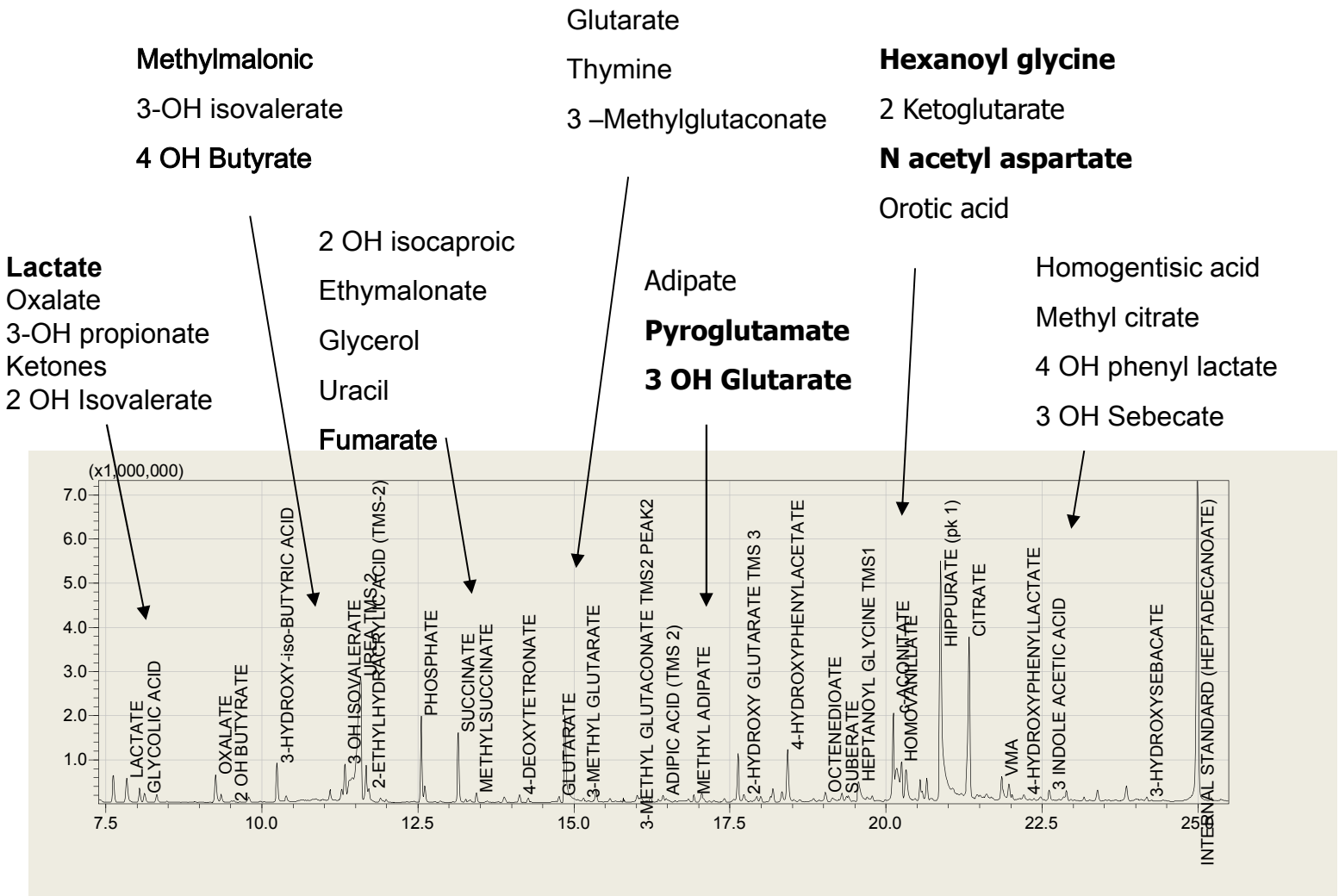
  Unusual patterns due to these factors are much more common then ‘inherited metabolic diseases’ – always act the detective and look for clues in the chromatogram

Pattern Recognition

- Essential to know what's normal



Pattern Recognition – Working through the Chromatogram



Problems and pitfalls with interpretation

- Workshop to discuss

Problems and Pitfalls

- Missed peaks – due to low excretion (mevalonolactone)
- Miss identified peaks ‘look alike peaks’ (example citramalate, 2-hydroxyglutarate and 3-hydroxyglutarate)
- Incomplete pattern recognition
- Unknown compounds/incomplete library (e.g. Pyrrole 2 carboxylic acid glycine)
- Inadequate interpretation – scientific knowledge e.g. MCT/GA2
- Mixed peaks

Problems observed in the over the last few distributions

- Orotic Acid – mainly an analytical problem
- Mevalonic Acid – low excretion/small peaks
- Hyperprolinaemia type 2 – inadequate library/unknown compound
- MCT – inadequate knowledge of exogenous compounds

Orotic Acid – Mass spectrum

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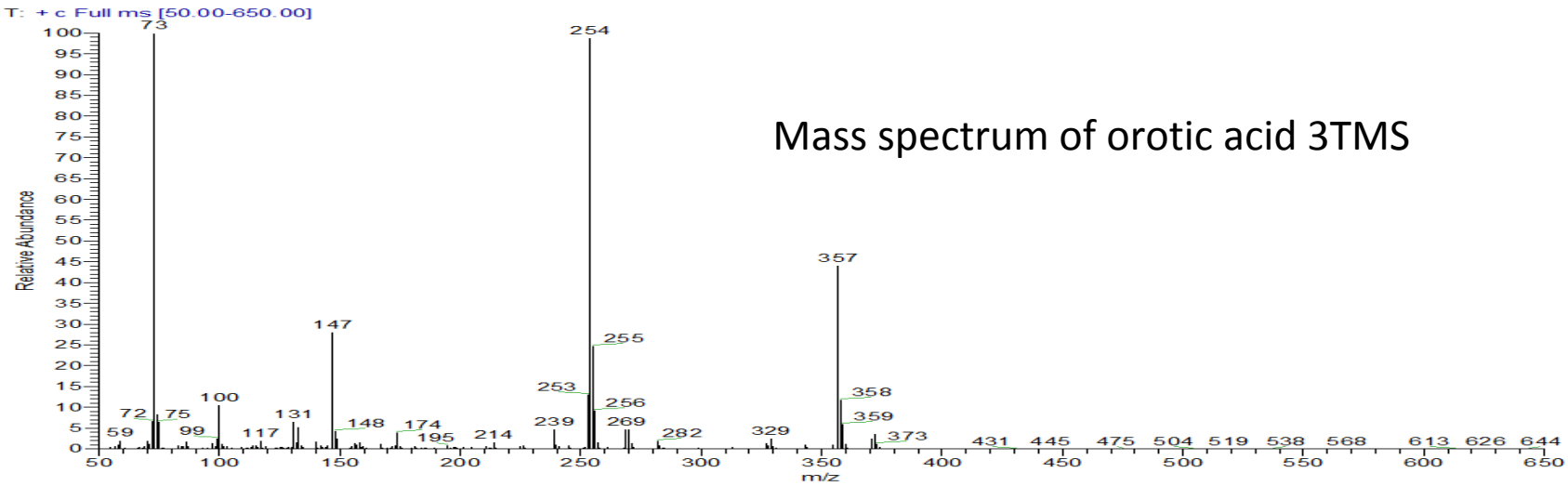
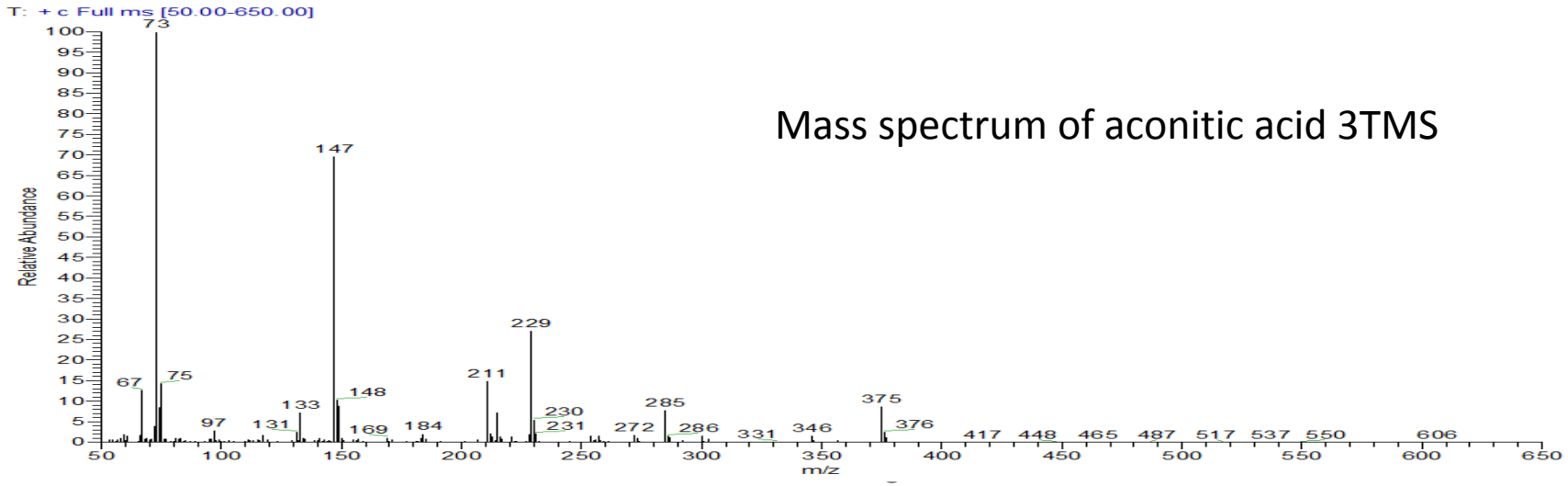


Mass spectrum of orotic acid 3TMS



Mass spectrum of aconitic acid 3TMS

Orotic Acid – normal control

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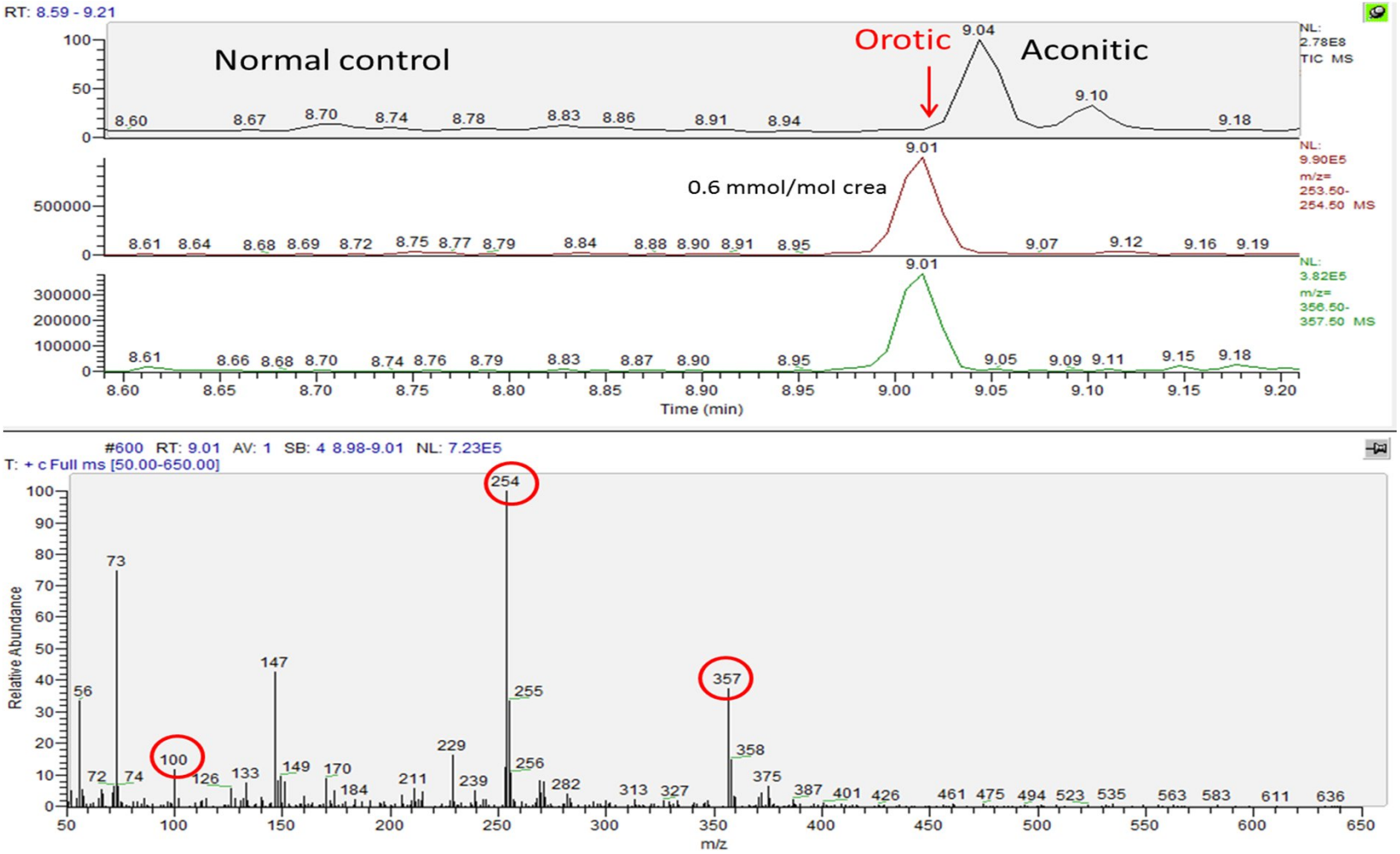

Orotic Acid – QOA H234

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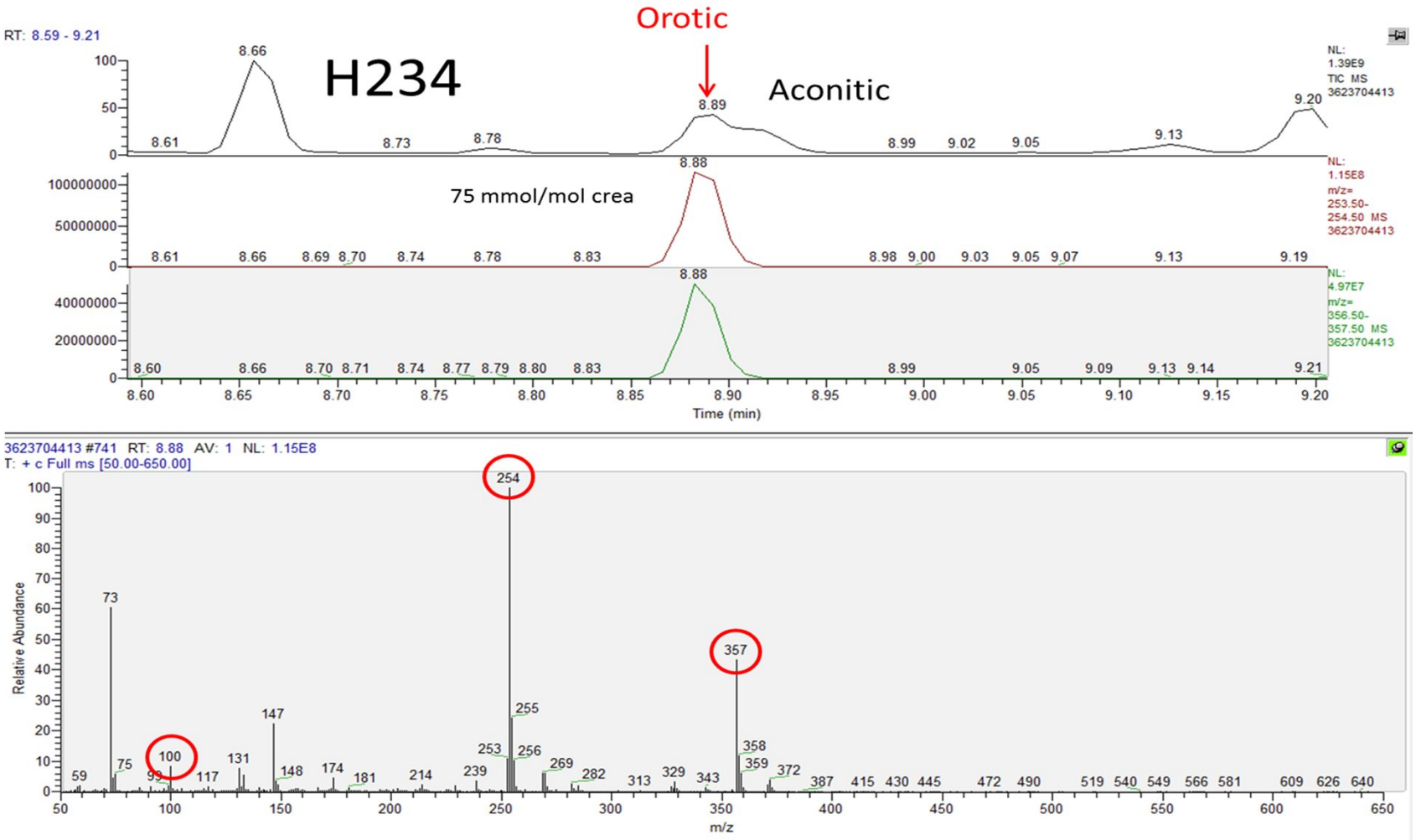

Orotic Acid – QOA S238

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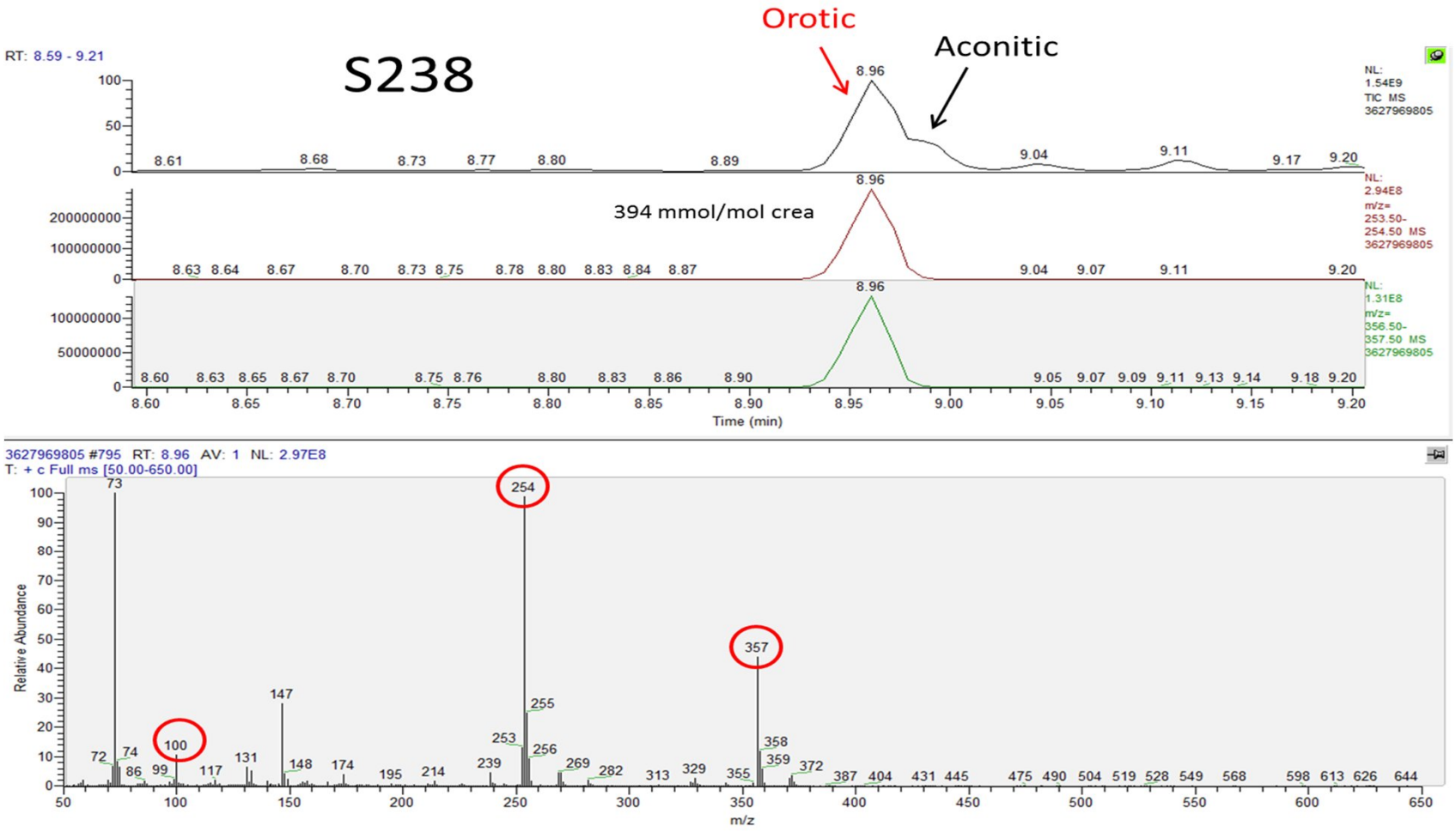

Orotic Acid – Overlay

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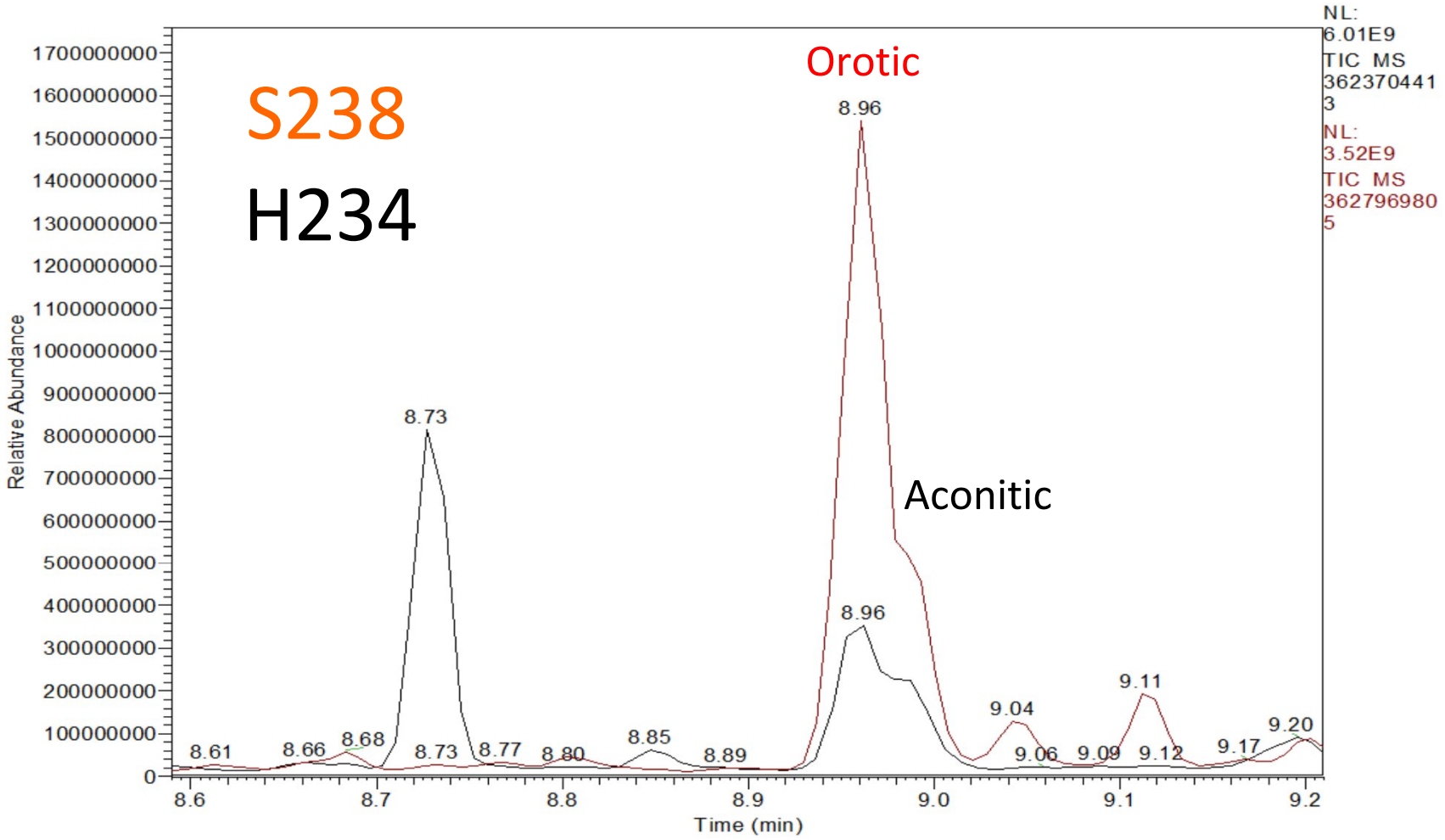
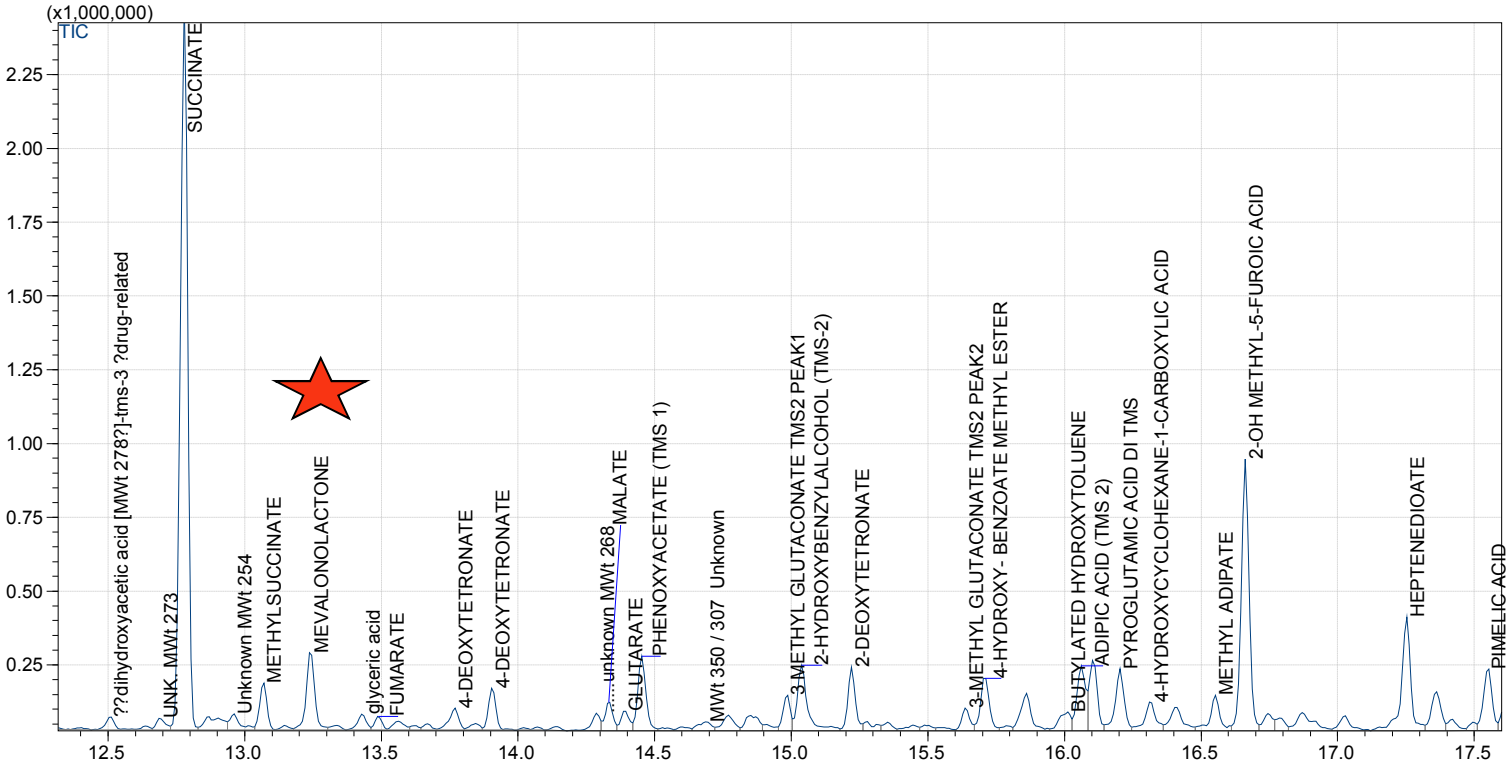
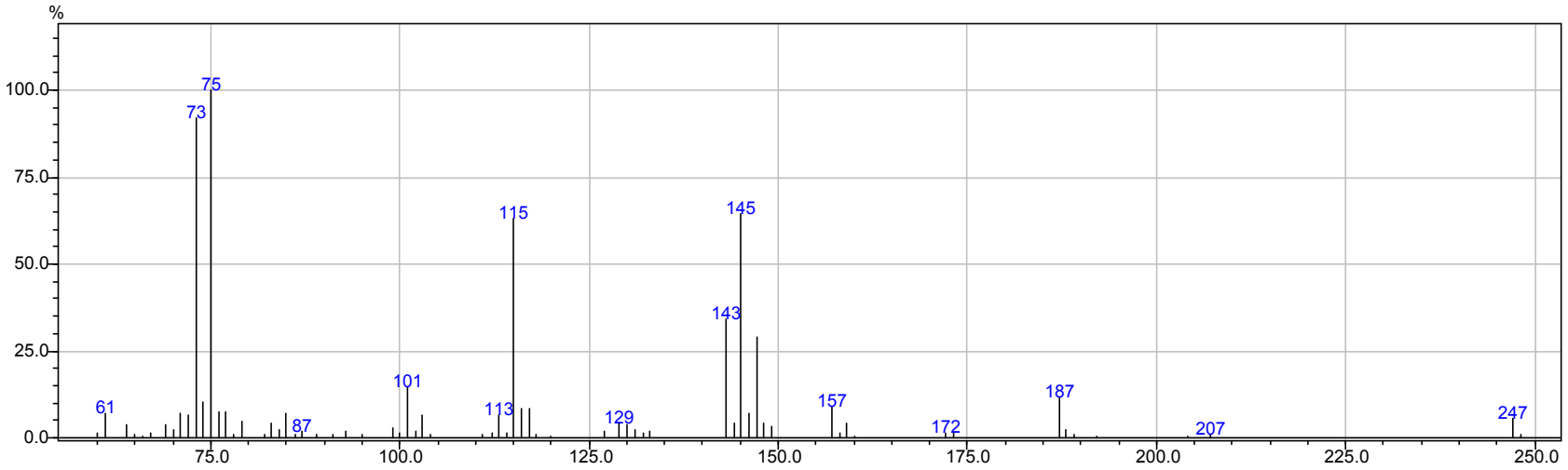


Small but significant peaks



Mevalonolactone

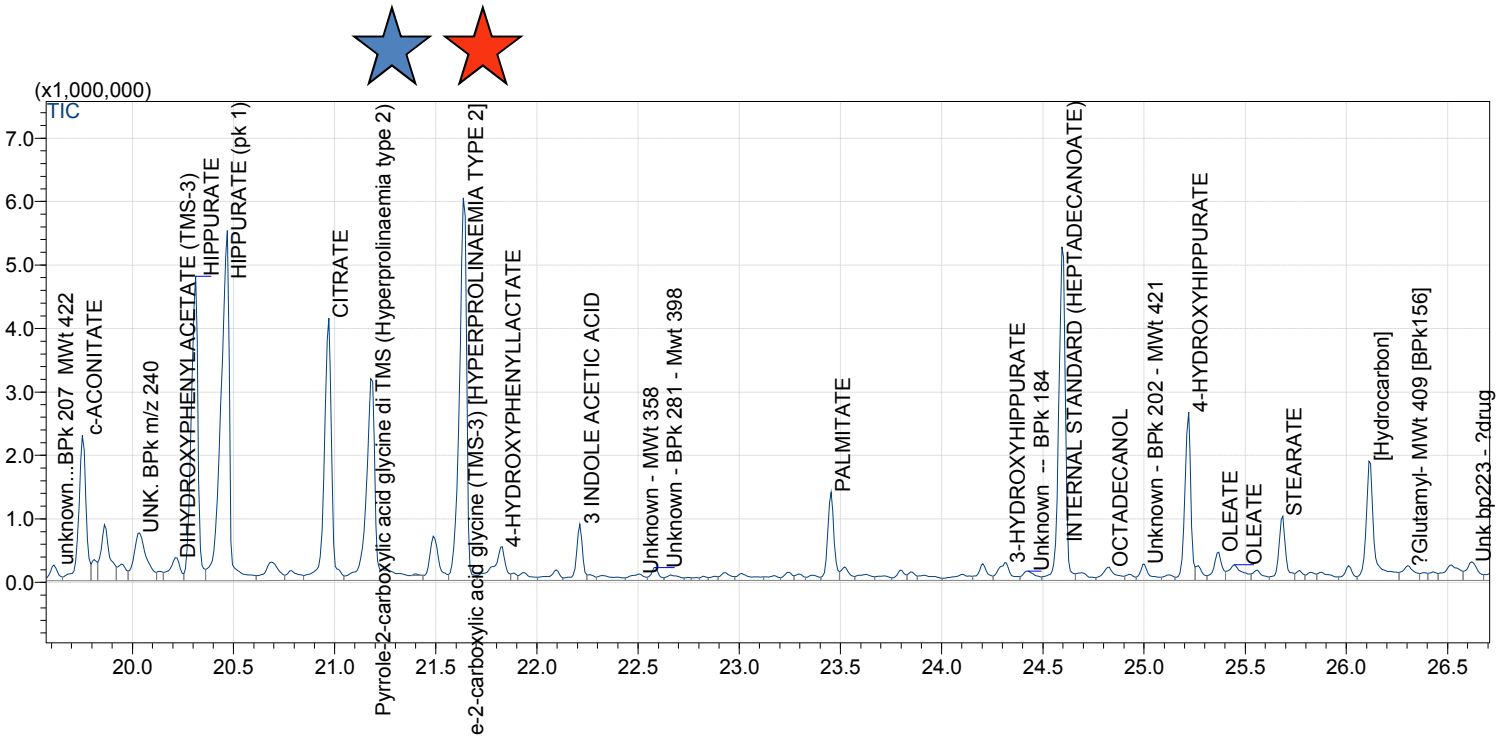
Mass ion spectra



Mevalonate Kinase deficiency

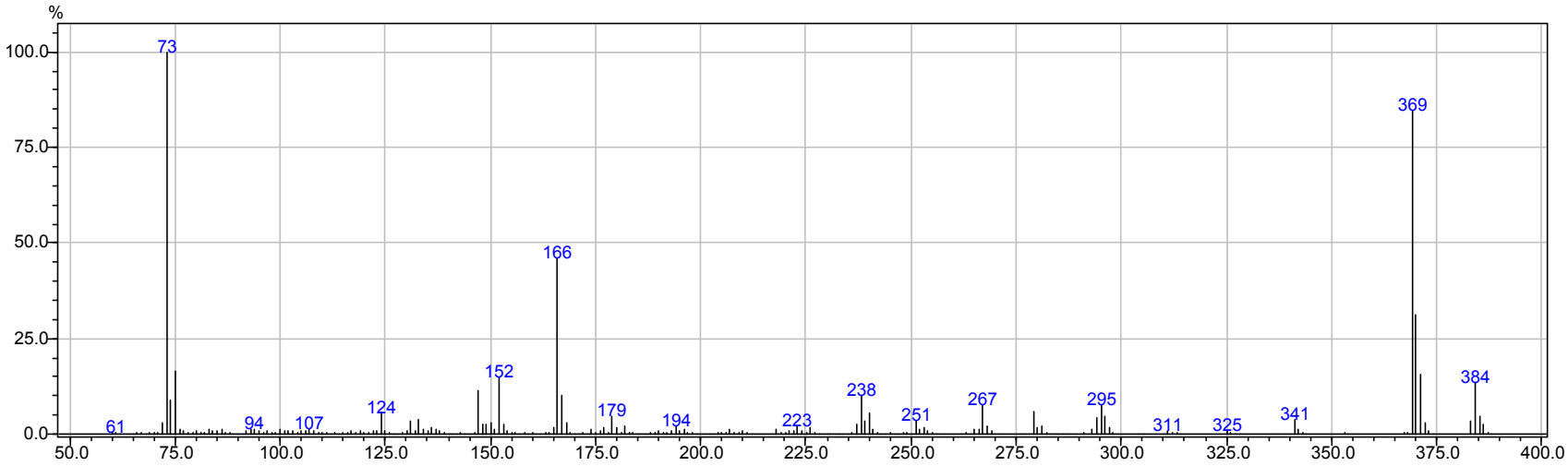
- Mevalonic aciduria: Dysmorphism, dystrophy, ataxia, recurrent crises, skin rashes, hepatosplenomegaly. Mevalonic acid, mevalonolactone (>500 mmol/mol creatinine)
- Hyper IgD syndrome: recurrent febrile attacks much lower amounts in hyper-IgD syndrome. May be normal outside of febrile attacks.

Unknown compounds/inadequate library



Hyperprolinaemia type 2
N – (pyrrole 2 carboxyl) glycine

Mass ion spectra tri TMS



Hyperprolinaemia type 2

Pyrroline-5-carboxylate (P5C) dehydrogenase deficiency

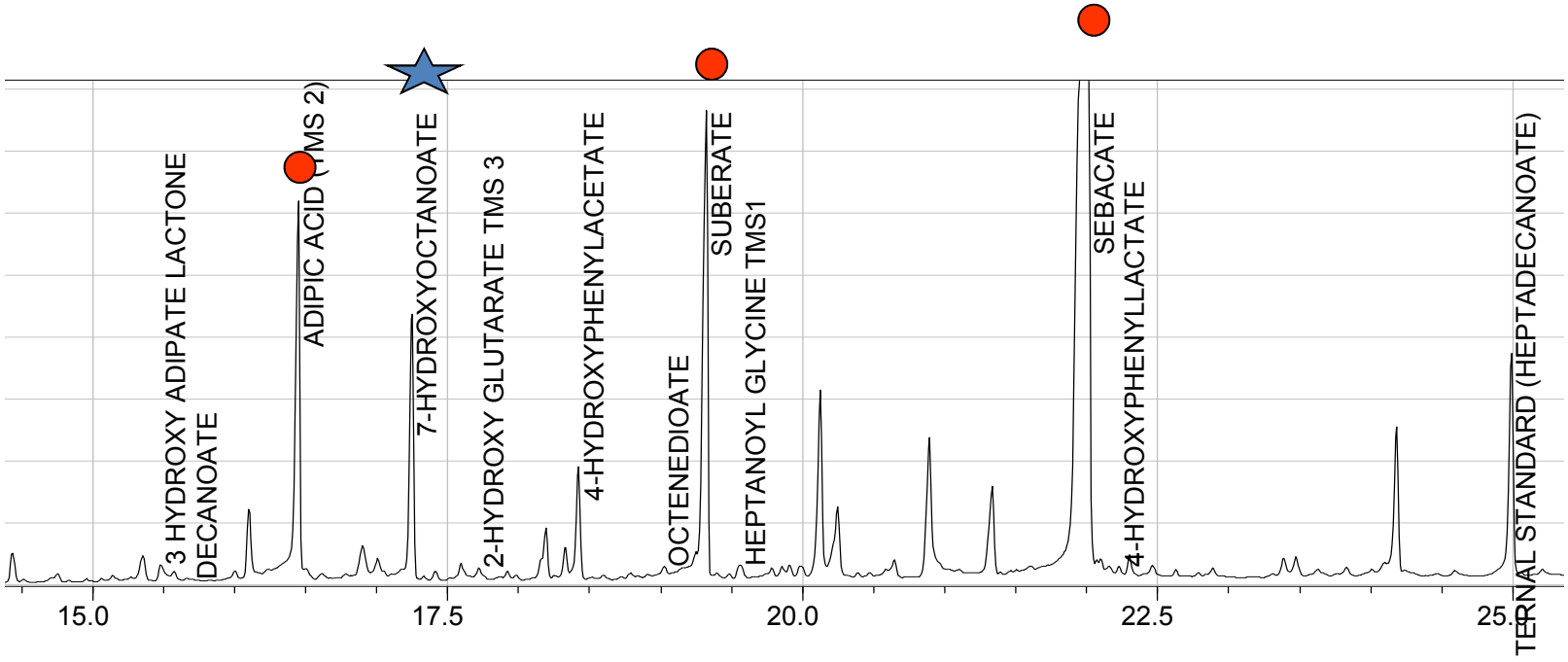
Associated with epilepsy, intellectual disability, maybe asymptomatic

ALDH4A1 gene

MCT Feeds

MCT feeds:
adipate<suberate<sebacate

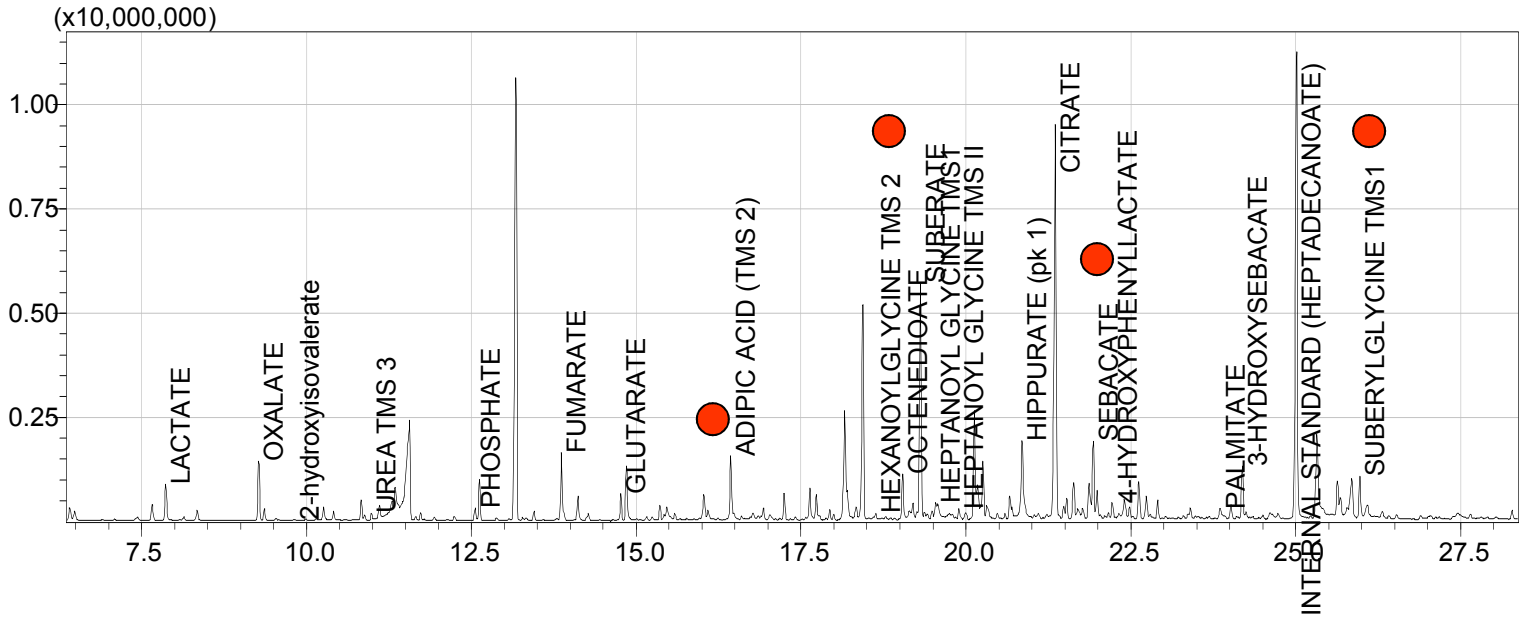
+ 7 Hydroxyoctanoate



Hexanoyl glycine

- Increased in MCADD
- Increased in ketosis
- Increased in MADD
- Valproate

Non crises samples show very low excretion



Interpretations and scoring

- Interpretation is an educated opinion based on a recognisable pattern, prior knowledge, experience and taking all factors into consideration, including clinical details.
- It Is acknowledged that in real life urine organic acid analysis form only part of the diagnosis.
- However, we do sometimes only get requested for this test and have to interpret in isolation in real life.
- Scoring is based on the Scientific Advisors experience and requires a team discussion. It is not based on an individuals knowledge or opinion.
- In tricky/controversial cases the scientific board is called upon for a collective expert opinion.

Educational Samples and Critical Error

- A sample is educational when at least 20% or greater of the participants fail to identify the abnormal metabolites or the significance of the metabolite – this is discussed on a case by case basis at the SAB
- A critical error can be made on a single sample in which the abnormalities are very clear and missing the diagnosis will have a significant effect on the patient. A critical error will generate unsatisfactory performance even when the overall score is above 70%
- Part of the role of the scheme is educational, this includes sometimes education of the SA by the participants
- This is always a two way process