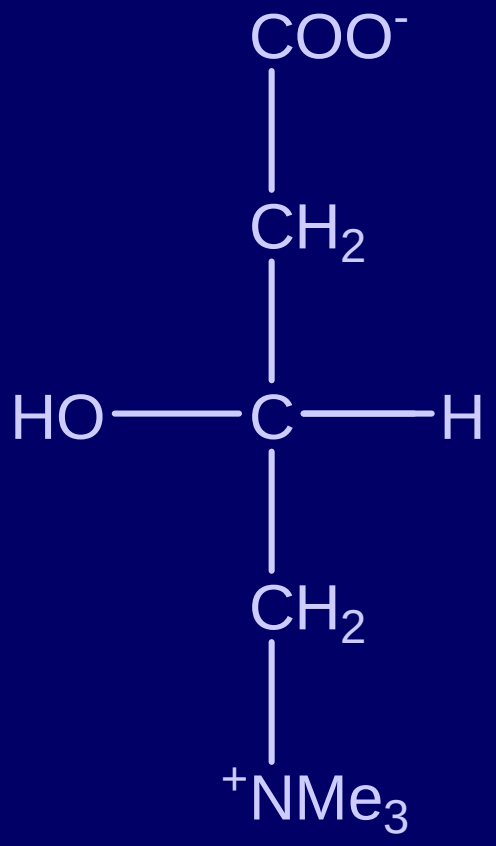
Acylcarnitines And Inherited Metabolic Disease

David Hardy

Overview

- Free Carnitine and Acylcarnitines
  - Role in fatty acid oxidation
  - Appearance in disease
- Measurement by tandem MS
- Examples of use in diagnosis

Carnitine

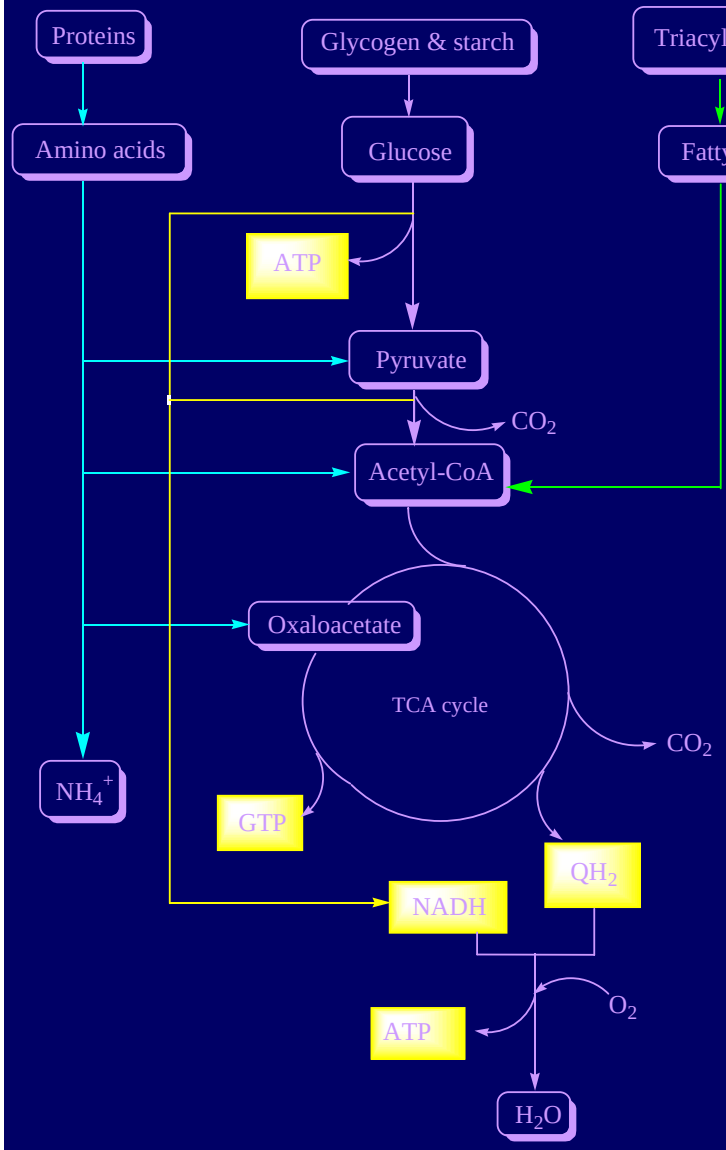


- Essential component of fatty acid oxidation
- Deficiency leads to impaired long chain fatty acids

Glucose, Fats and Energy

- Glucose is a primary fuel
  - Glycogen reserves are exhausted in 24 – 48 h
  - Additional glucose comes from gluconeogenesis
    - Occurs concurrently with glycogenolysis, but also on its own when glycogen exhausted
    - Gluconeogenesis from pyruvate (via oxaloacetate) provides glucose to organs that cannot use other fuels
- Fatty acid oxidation provides alternative source of ATP, and fuel (ketones) to some other organs
  - Fatty acids are better fuels than amino acids and carbohydrates,
    - 1 g fat generates 37.7 kJ
    - 1 g carbohydrate generates 16.7 kJ
  - Energy may be used directly (heat) or stored chemically
  - Also promotes gluconeogenesis

Catabolism: A Bird's-eye View



Acetyl CoA common intermediate – feeds into TCA to complete oxidation process

Abundance of acetyl-CoA stimulates gluconeogenesis

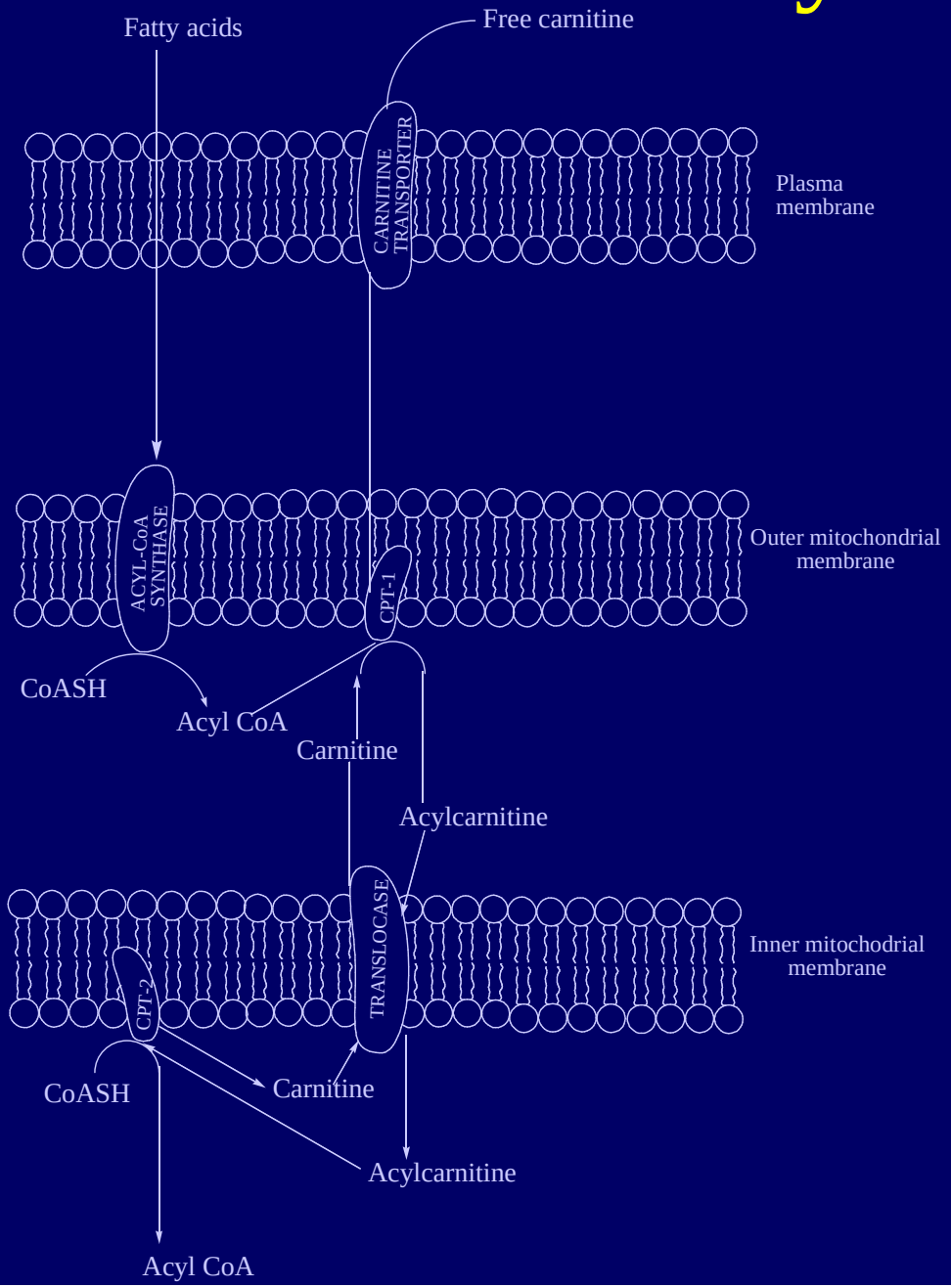
Energy produced by catabolism stored as ATP, GTP, NADH, QH_2

After Horton et al. Principles of Biochemistry 3e

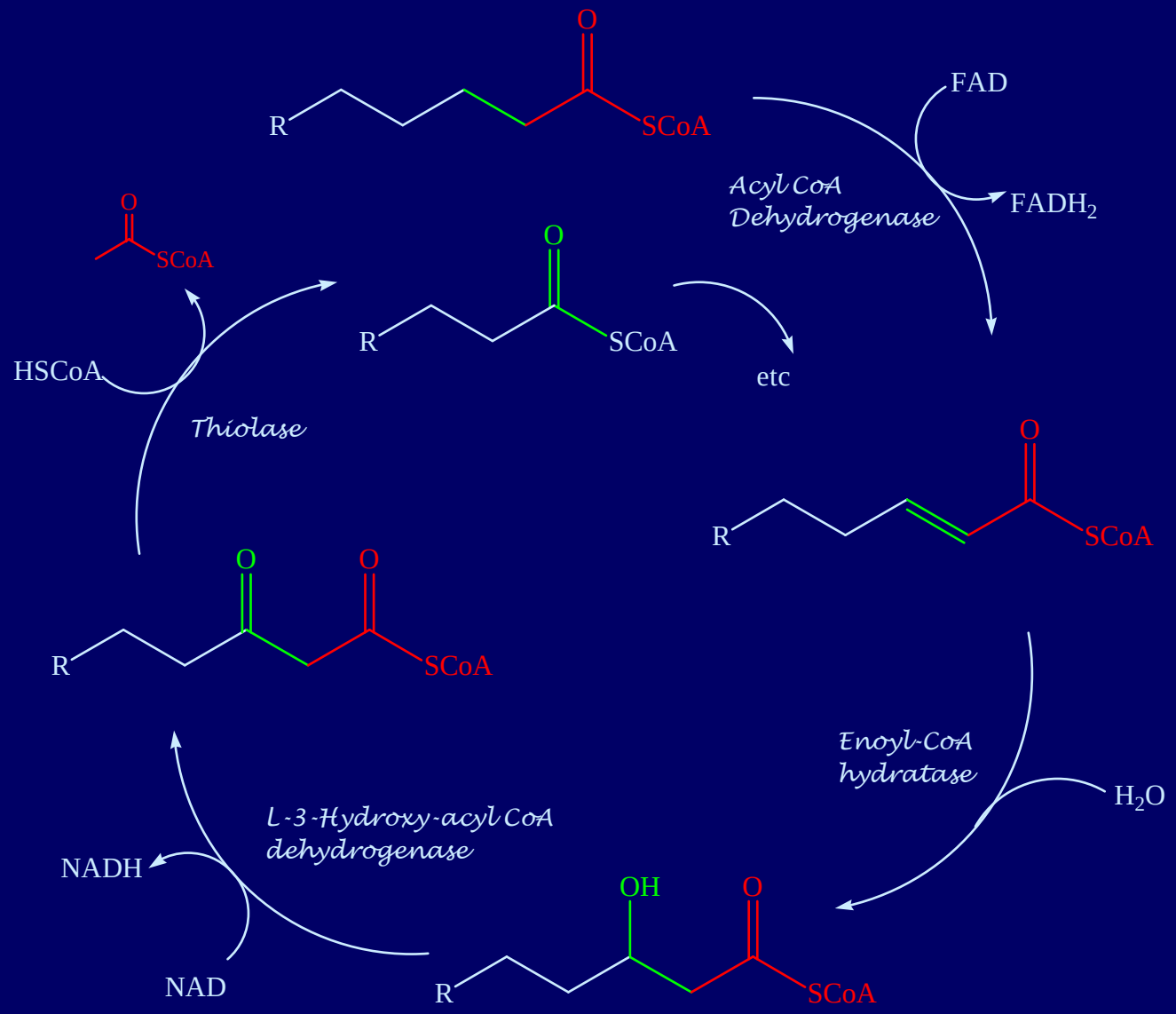
Mitochondrial Fatty Acid Oxidation

- Mitochondrial fatty acid oxidation requires:
  - Carnitine shuttle
    - Active transport mechanism
    - Facilitates entry of long chain fatty acids acyl CoA species into mitochondrion
    - Not required for medium/short chain species (< C12) - free passage
  - β-oxidation spiral
    - For acids with 20 or less carbons
    - Series of reactions that sequentially shorten the carbon skeleton
    - Generate acetyl-CoA as end product

The Carnitine Cycle



β-Oxidation Spiral

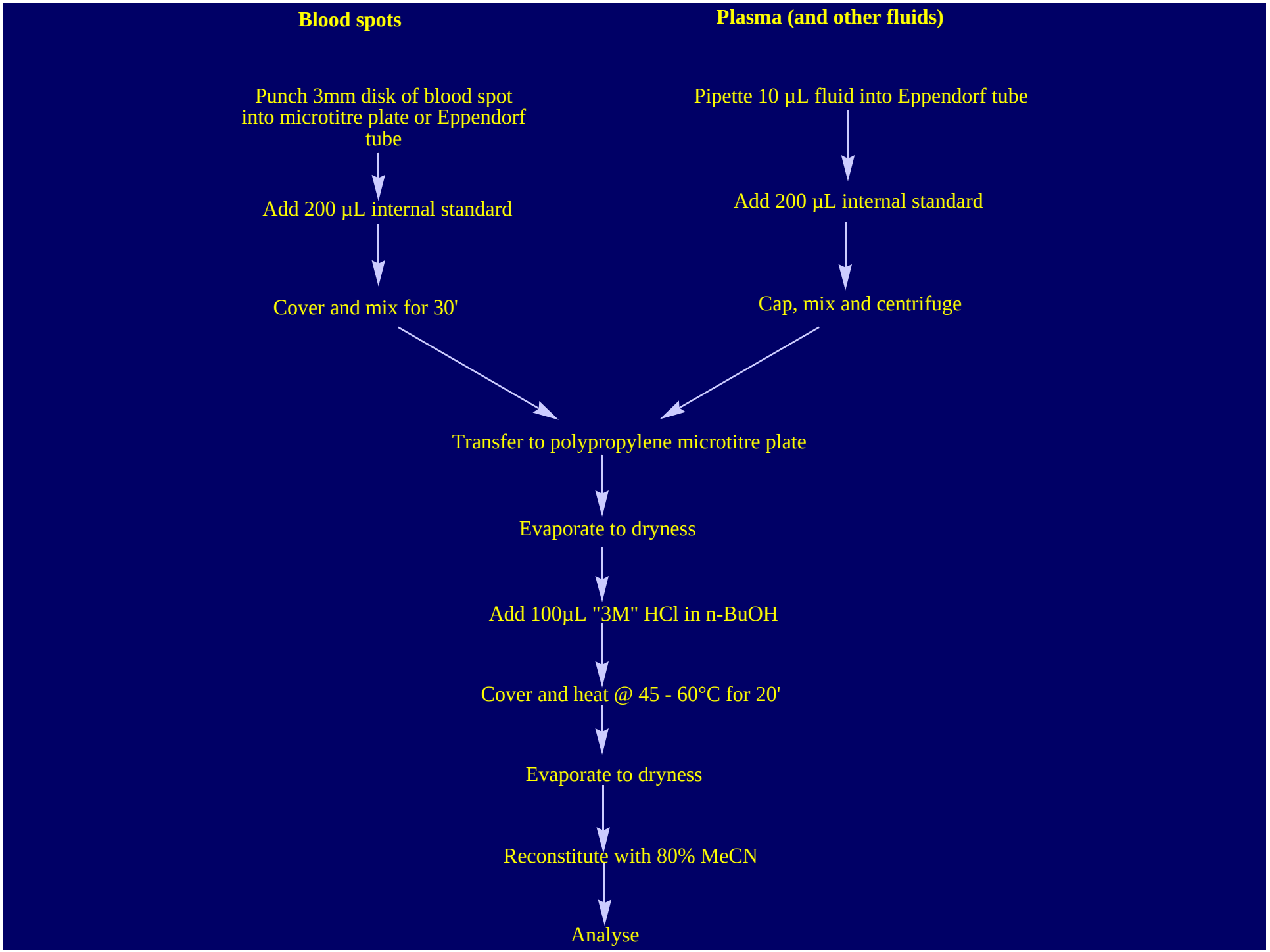


Acyl Carnitines in Fatty Acid Oxidation & Organic Acid Disorders

- Acyl carnitines are intermediates in normal long chain fatty acid oxidation.
- A defect in long chain fatty acid oxidation might be expected to lead to secondary acyl carnitine formation.
- Acyl carnitines are formed from acyl CoA species; defects in any other pathway involving acyl Co A species can lead to secondary accumulation of acyl carnitines
  - Fatty acid oxidation defects after the carnitine shuttle
  - Organic acidaemias

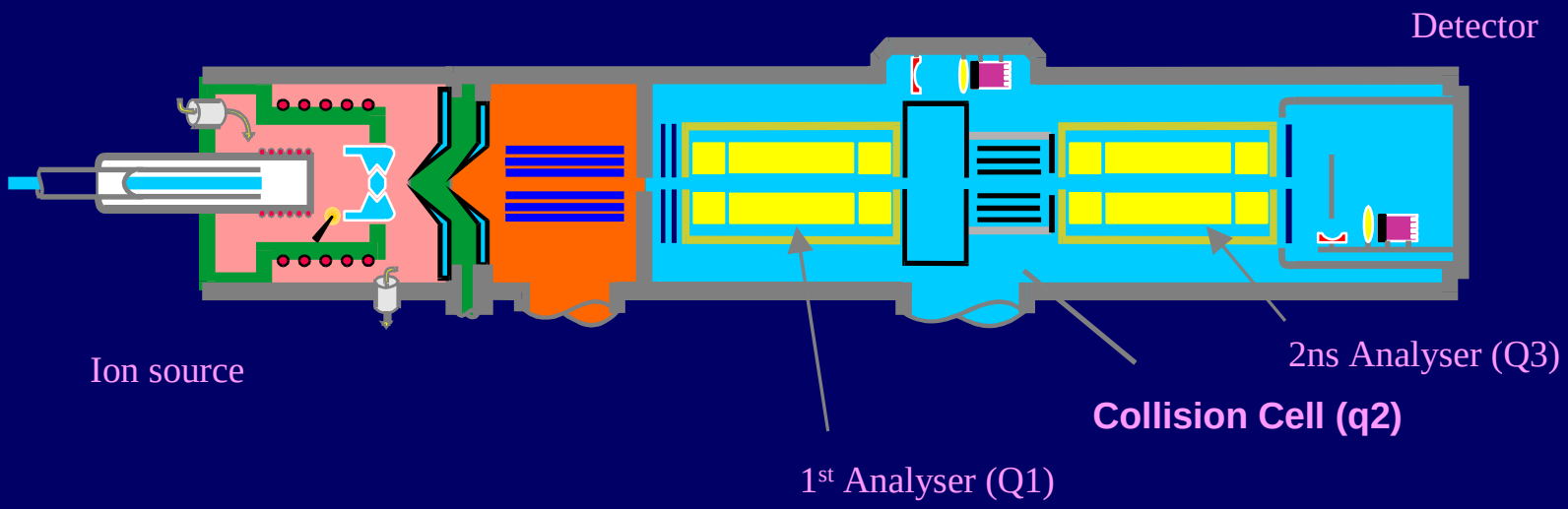
Analytical Aspects

- Sample requirements
  - Dried blood spot (3mm disc punched)
  - Plasma/serum – 100µL (10 µL used)
    - Early literature suggested problems with EDTA, but LiHep, FlOx and EDTA OK in personal experience
  - Urine – 100µL (10 µL used)
  - Bile – 100µL (10 µL used)
- Essentially the same for PKU screening assay


Blood spots
Plasma (and other fluids)
Punch 3mm disk of blood spot into microtitre plate or Eppendorf tube
Pipette 10 µL fluid into Eppendorf tube
Add 200 µL internal standard
Add 200 µL internal standard
Cover and mix for 30'
Cap, mix and centrifuge
Transfer to polypropylene microtitre plate
Evaporate to dryness
Add 100µL "3M" HCl in n-BuOH
Cover and heat @ 45 - 60°C for 20'
Evaporate to dryness
Reconstitute with 80% MeCN
Analyse

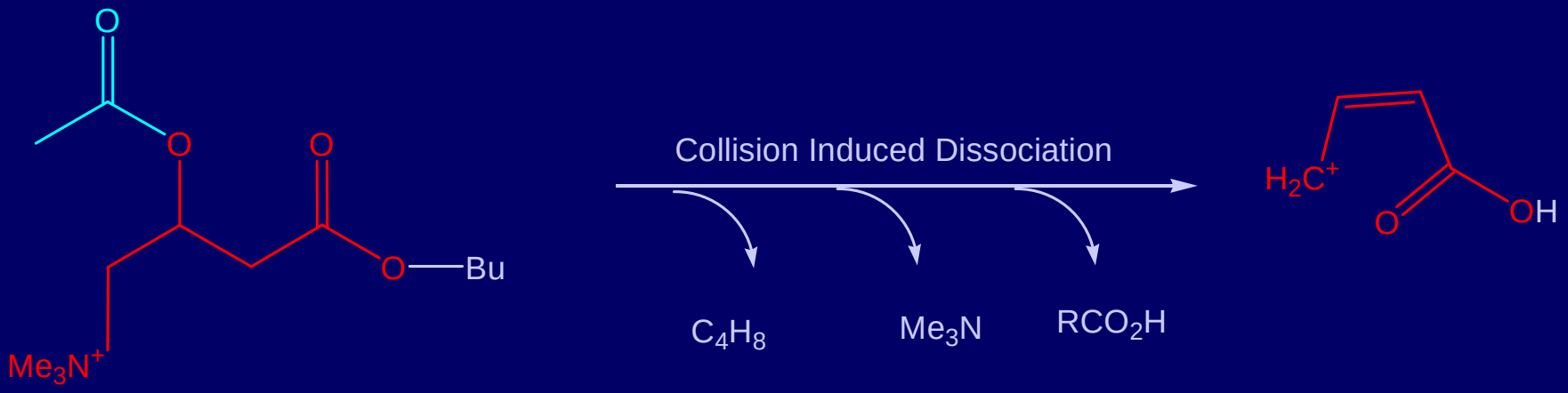
MS/MS 1

–Generic triple quadrupole tandem mass spectrometer



MS/MS 2

- Parents of m/z 85 – acyl carnitines as butyl esters



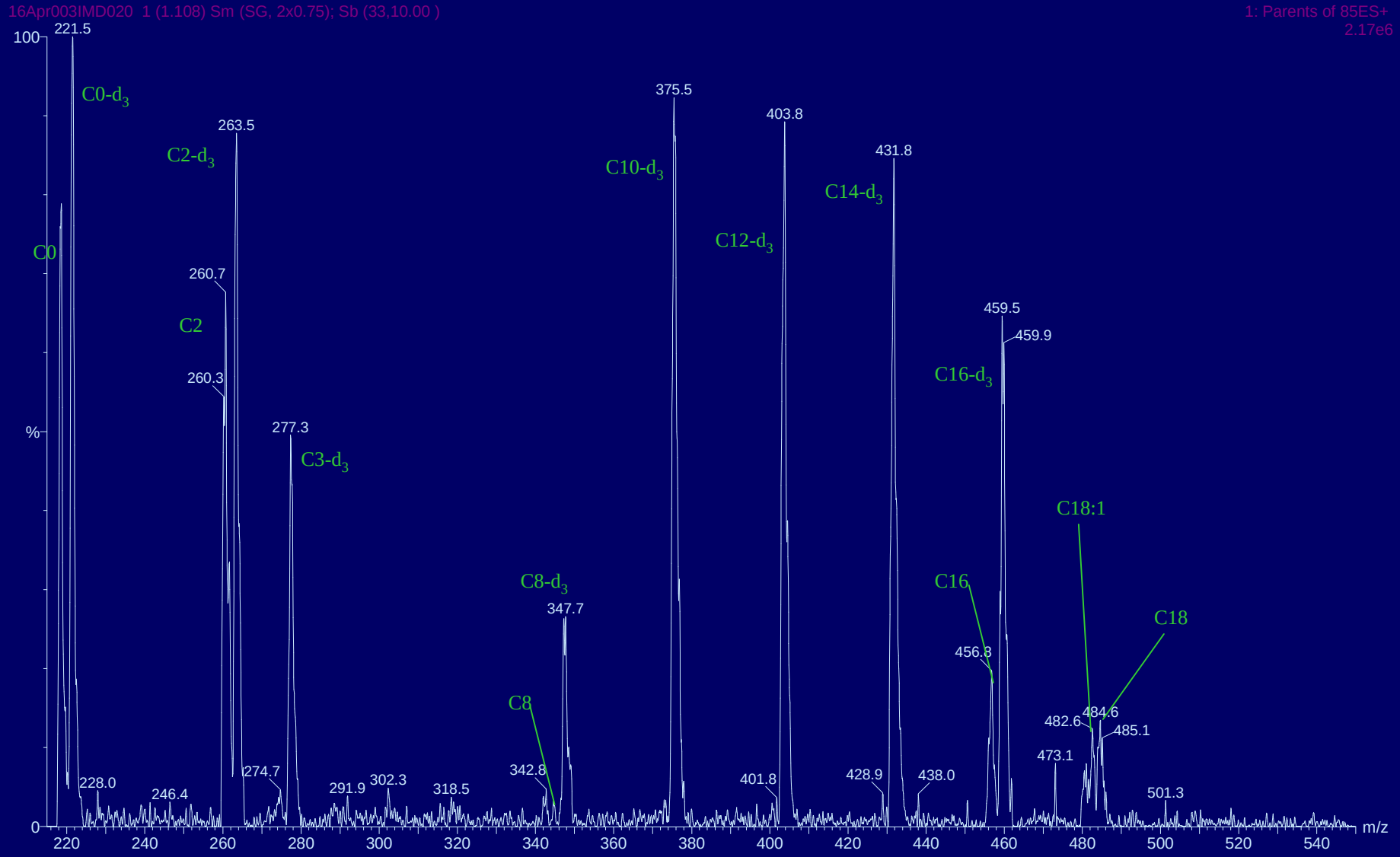
- First quadrupole scans m/z 215 – 550
- Second quadrupole – gas cell – collision induced dissociation
- Third quadrupole static at m/z 85

MS/MS 3

- Allows quantitation of plasma free carnitine
- Allows identification of disease-specific patterns
- Quick and easy – “stat” results in ca. 1h

- Butylation methods result in slight hydrolysis effect
  - Free carnitine slightly higher than true value (few µM)
- Detects anything that gives a m/z 85 fragment
  - ***NOT*** specific for acyl carnitines, but good enough most of the time

Normal Acylcarnitine Pattern



Acylcarnitines in Health

- C0 – free carnitine
- C2 – acetyl carnitine
- C3 – propionyl carnitine – small amount
- C4 – butyryl carnitine – small amount
- C8 – octanoyl carnitine – trace
- C16 – palmitoyl carnitine
- C18:2 – linoleyl carnitine
- C18:1 – oleyl carnitine
- C18:0 – stearoyl carnitine

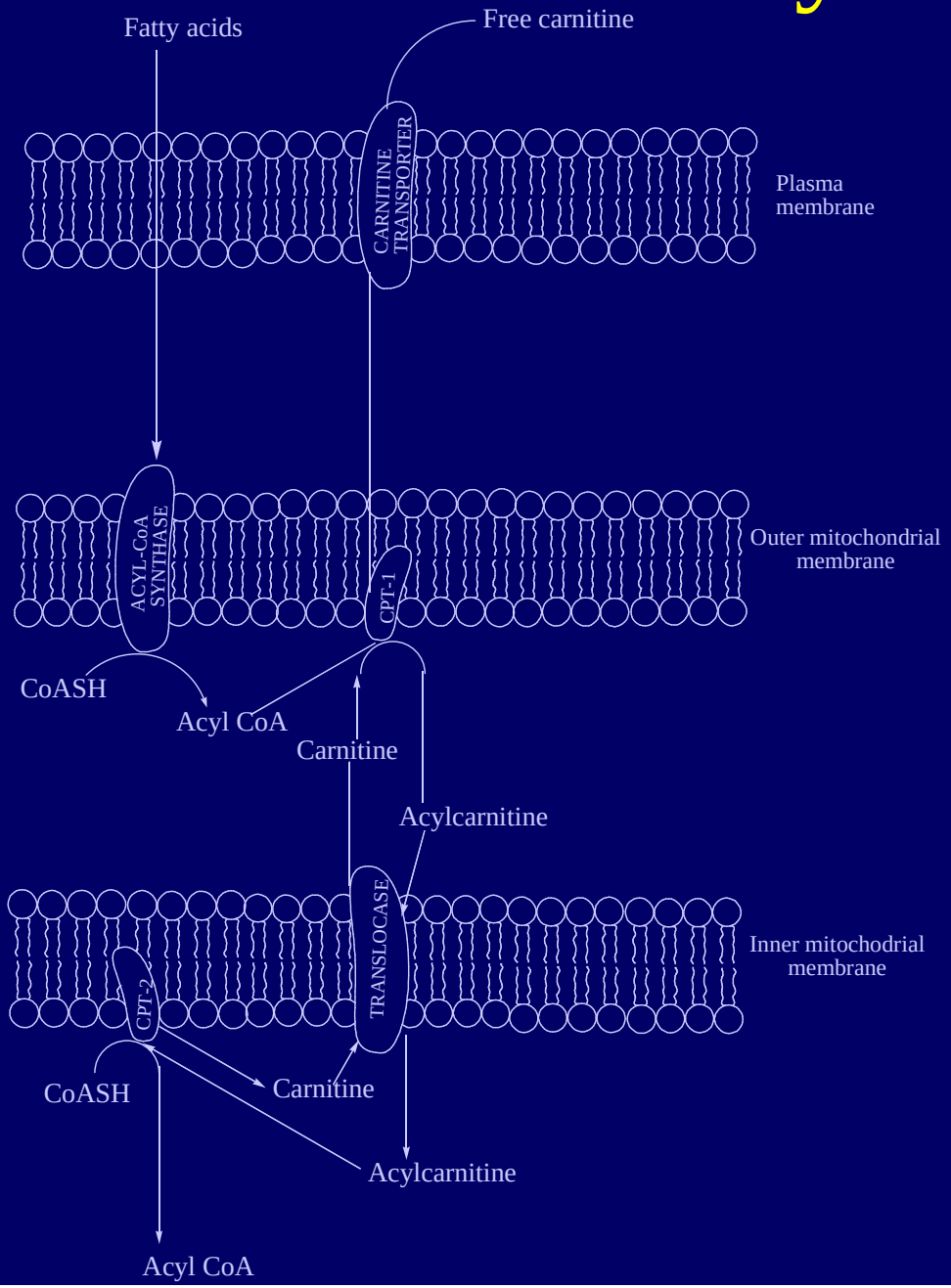
Why Numbers?

- Acylcarnitines are referred to by the number of carbon atoms present in the acyl group
- Structural isomers exist for several acyl groups
  - These have the same m/z ratio
  - Definitive identification is not possible from simple parents of 85 experiment
  - Using C numbers overcomes this
- Some acylcarnitines are derived from hydroxylated acyl groups and are denoted, e.g. C5-OH
- Those from dicarboxylic acyl groups are denoted, e.g. C5-DC
- Unsaturated species are denoted, e.g. C16:1

Diagnostic Uses

- In principle can detect any disorder resulting in the accumulation of acyl-CoA species.
- In practice, about 24 conditions can be detected
  - PA, MMA & B12 deficiency, malonic aciduria, 3-methylcrotonyl-CoA carboxylase deficiency, IVA, GA-1, biotinidase deficiency, holocarboxylase synthase deficiency, 2-methyl-3-hydroxybutyryl CoA dehydrogenase deficiency, isobutyryl-CoA dehydrogenase deficiency, β-ketothiolase deficiency, HMG-CoA lyase deficiency, carnitine transporter deficiency, CPT-1, translocase, CPT-2, VLCADD, TFP/LCHADD, MCADD, SCADD, SCHADD, MADD

The Carnitine Cycle



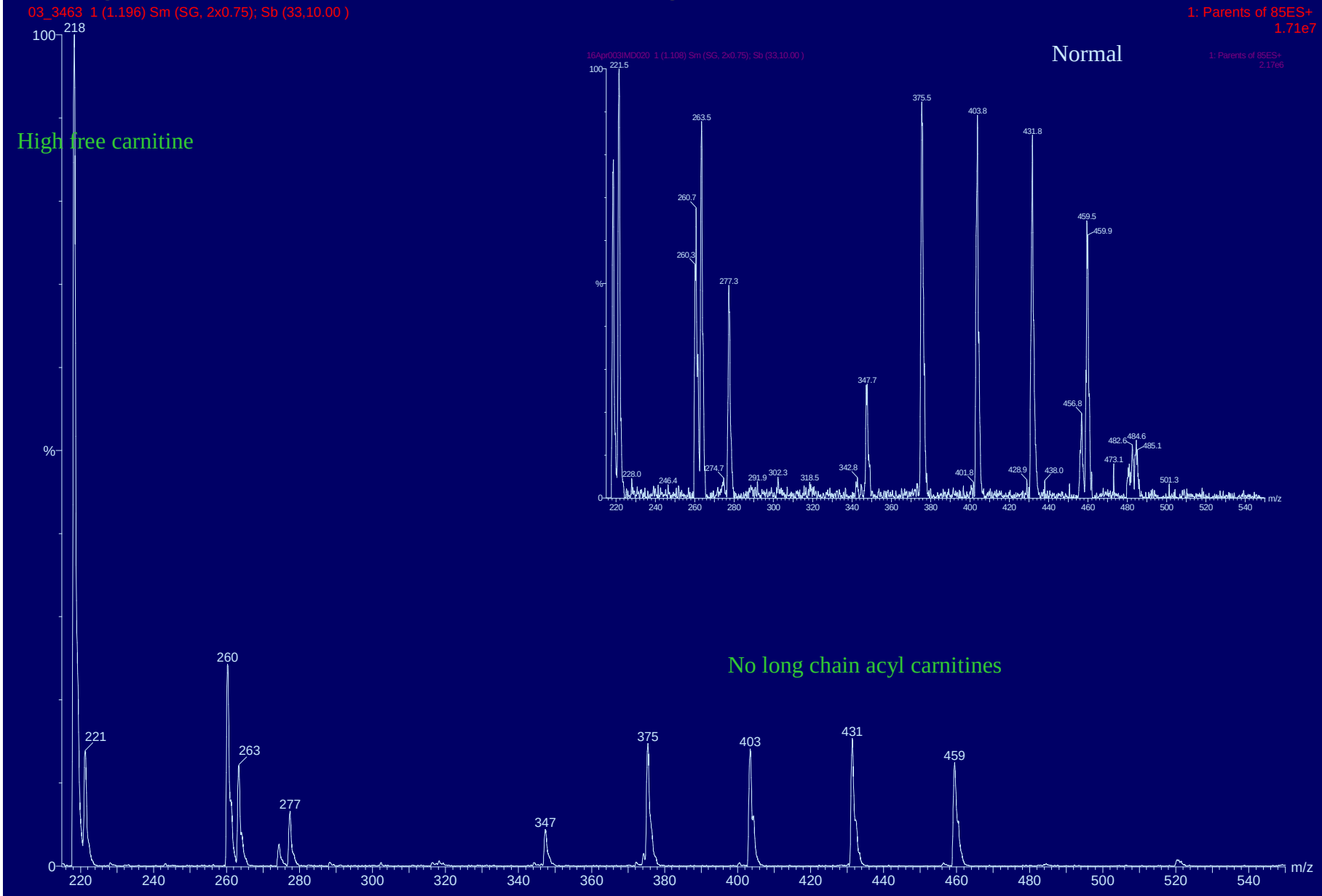
CPT-I Deficiency 1

- Blocked formation of long chain acyl carnitines from acyl-CoA esters
  - Long chain acyl-CoA species accumulate – toxic!
  - Other pathways metabolise them (peroxisomes) to medium chain species which are free to enter β-oxidation
- Presentation – largely hepatic
  - Coma, seizures, hepatomegaly, hypoketotic hypoglycaemia (often set off by fasting)
  - Some cases have increased CK(MM) – not all
  - No chronic muscle weakness or cardiomyopathy

CPT-1 Deficiency 2

- Deficiency means long chain acyl carnitines are not synthesised
- High free carnitine and virtually undetectable long-chain acyl carnitines is diagnostic pattern

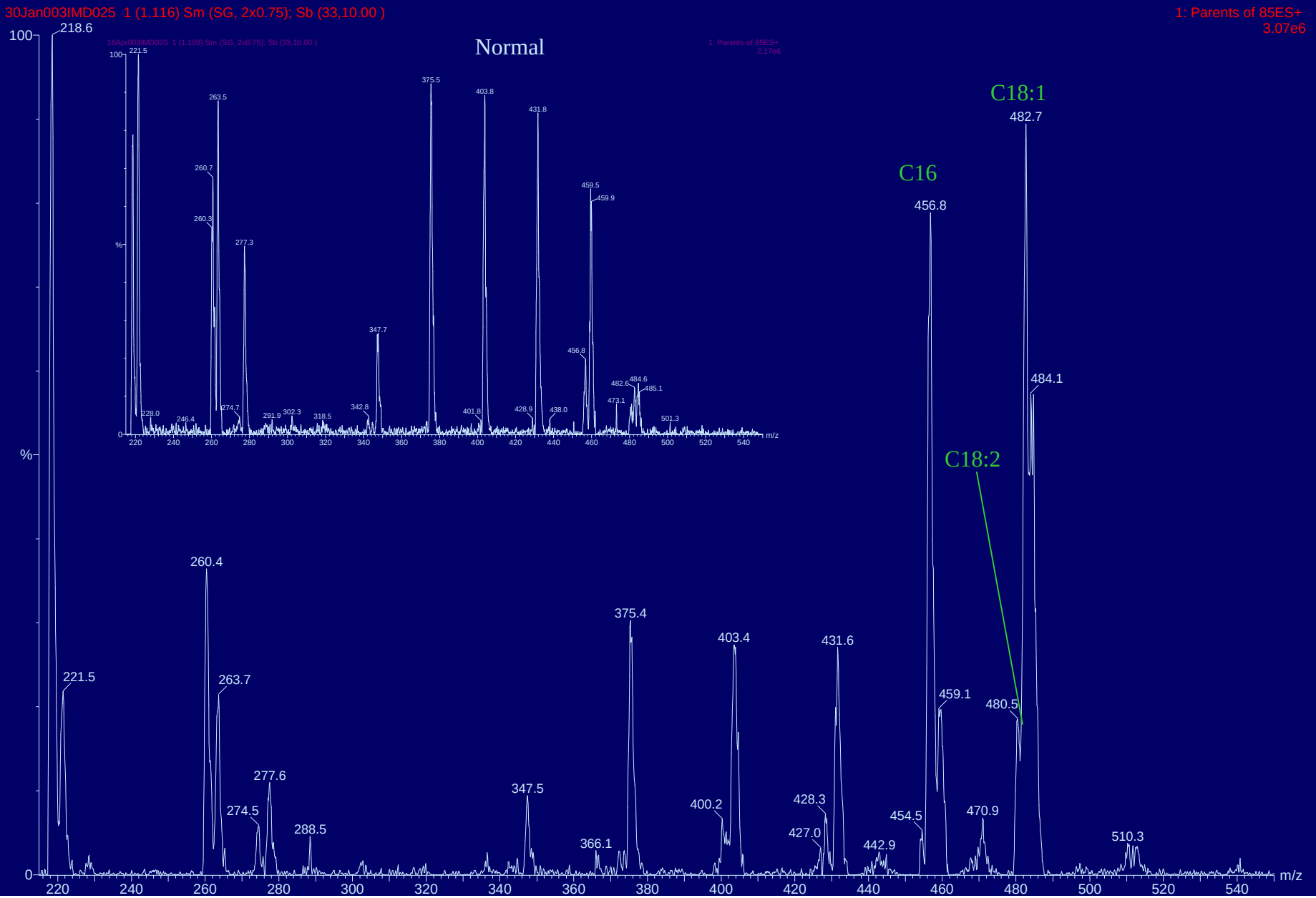
Typical CPT-I Acyl Carnitine Pattern



CPT-II Deficiency

- Defect in regenerating acyl-CoA from acyl carnitine
- Consequences
  - Toxic long chain acyl carnitines accumulate
- Presentation
  - Classical muscular form
    - Adult presentation, myoglobinuria and muscle weakness on exercise
    - CK may be normal between attacks
  - Neonatal (severe/fatal) form – more hepatic
    - Coma, hypoketotic hypoglycaemia
    - Hepatomegaly, cardiomegaly, cardiac arrythmias

Typical CPT-II/ Translocase Acyl Carnitine Pattern

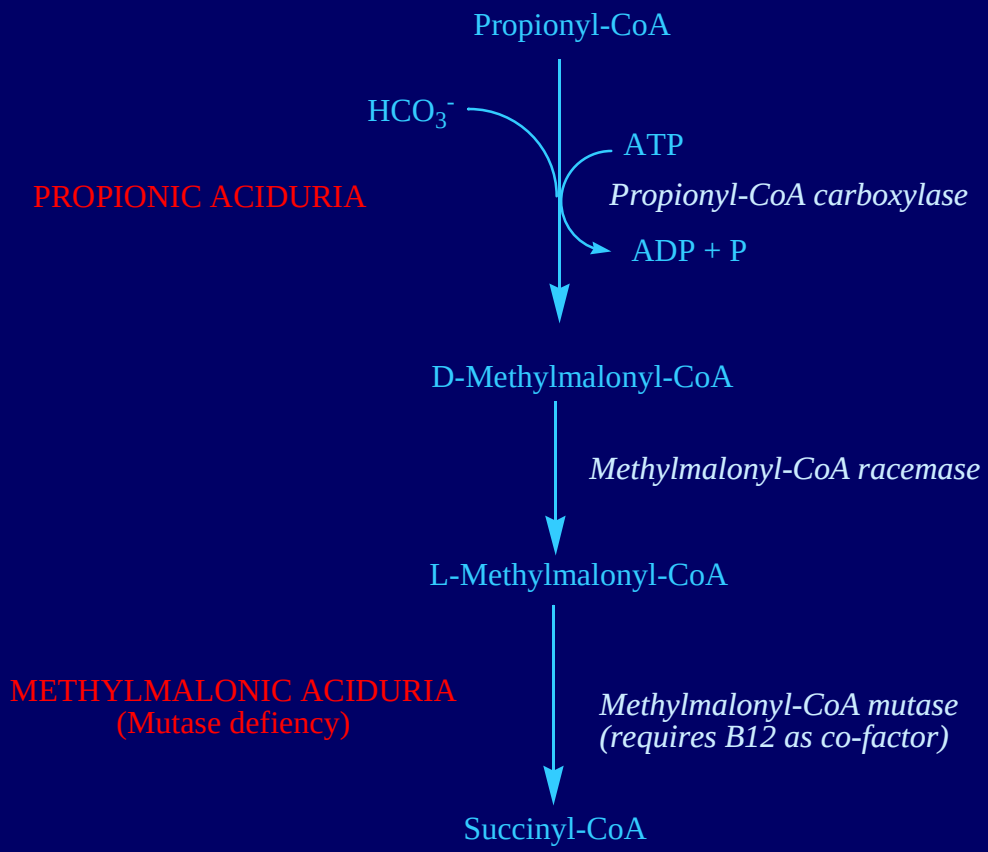


Propionic & Methylmalonic Acidurias 1

- Clinical findings
  - Moderate hepatomegaly
  - Acidosis, ketosis
  - Hyperammonaemia (may be confused with urea cycle defect if NH_3 > 800 µM)
  - Hypocalcaemia and hyperlactic acidaemia common
  - Glucose may be low, normal, high
  - Neurological complications due to basal ganglia necrosis
  - Renal damage, possibly leading to renal failure in MMA

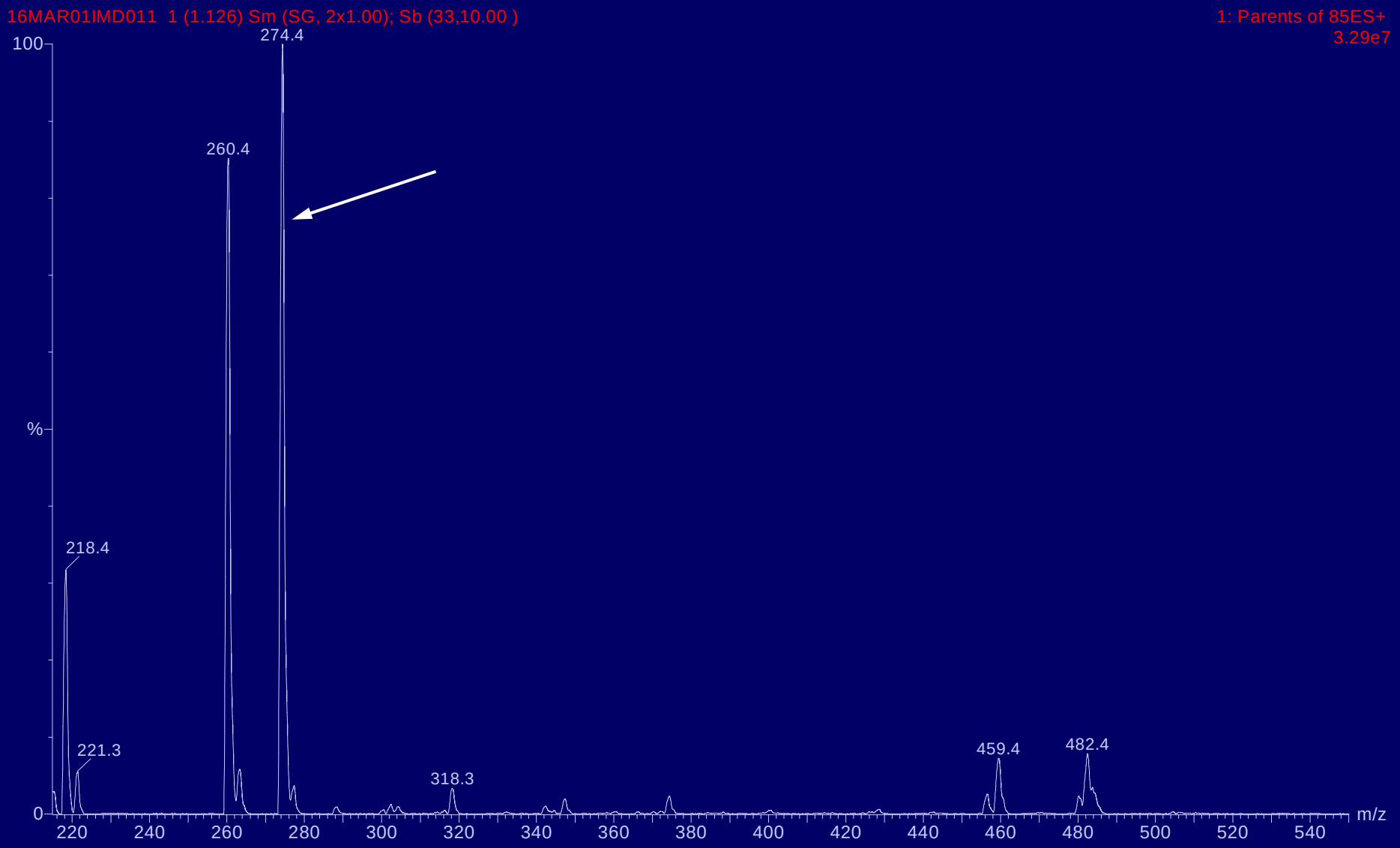
Propionic & Methylmalonic Acidurias 2

- Finding: Elevated C3 (sometimes slightly increased C4DC in MMA)



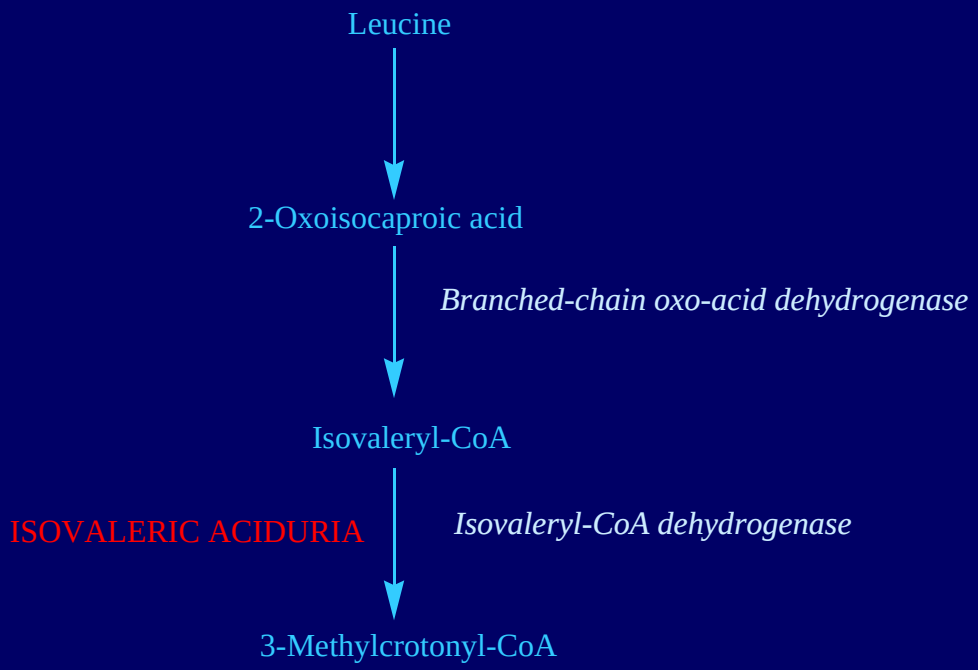
Requirement for vitamin B12 means B12 deficiency may mimic MMA!

Propionic Aciduria

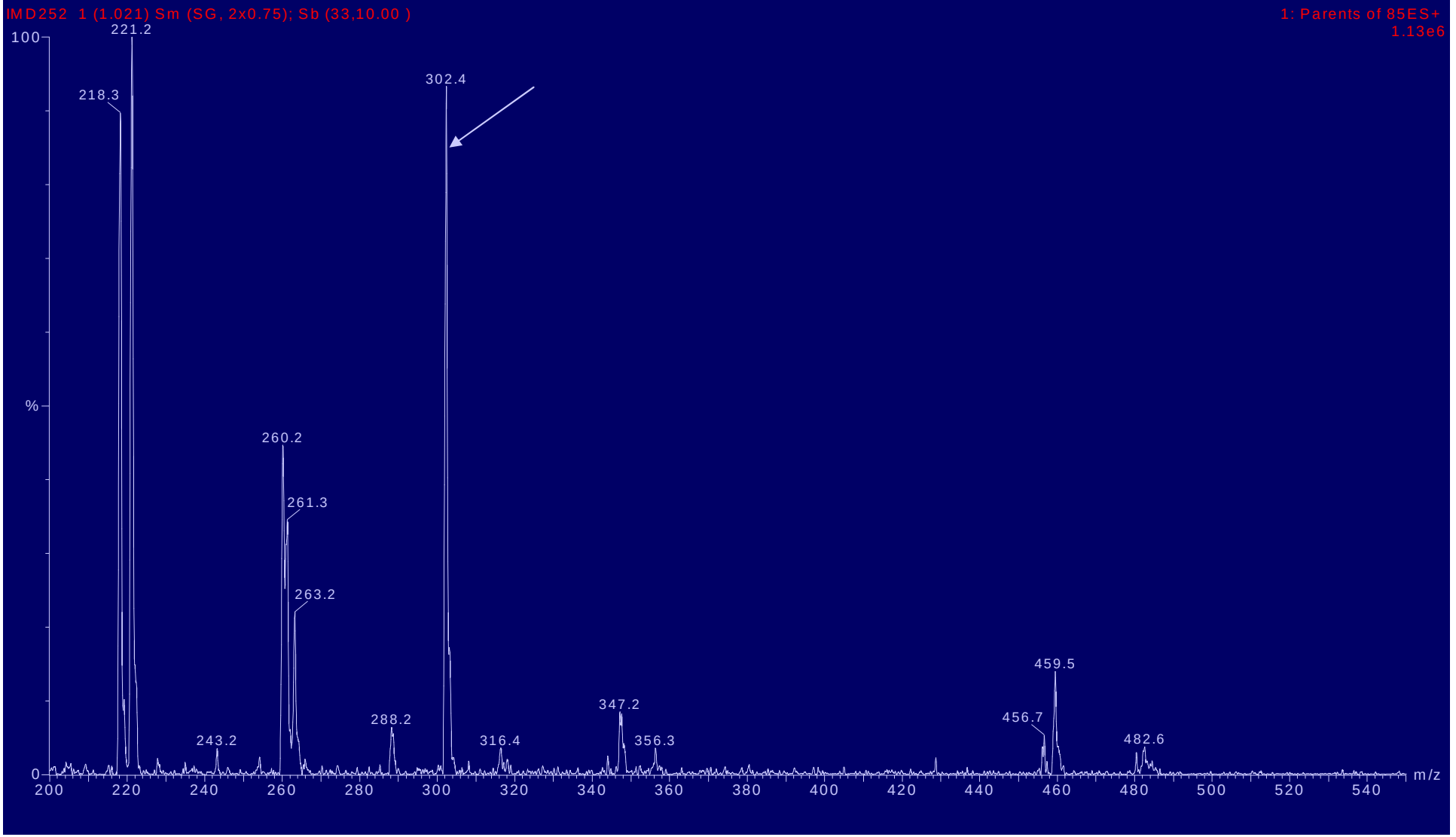


Isovaleric Aciduria

- Clinical features
  - Essentially the same as PA/MMA
  - Characteristic odour of “sweaty feet”



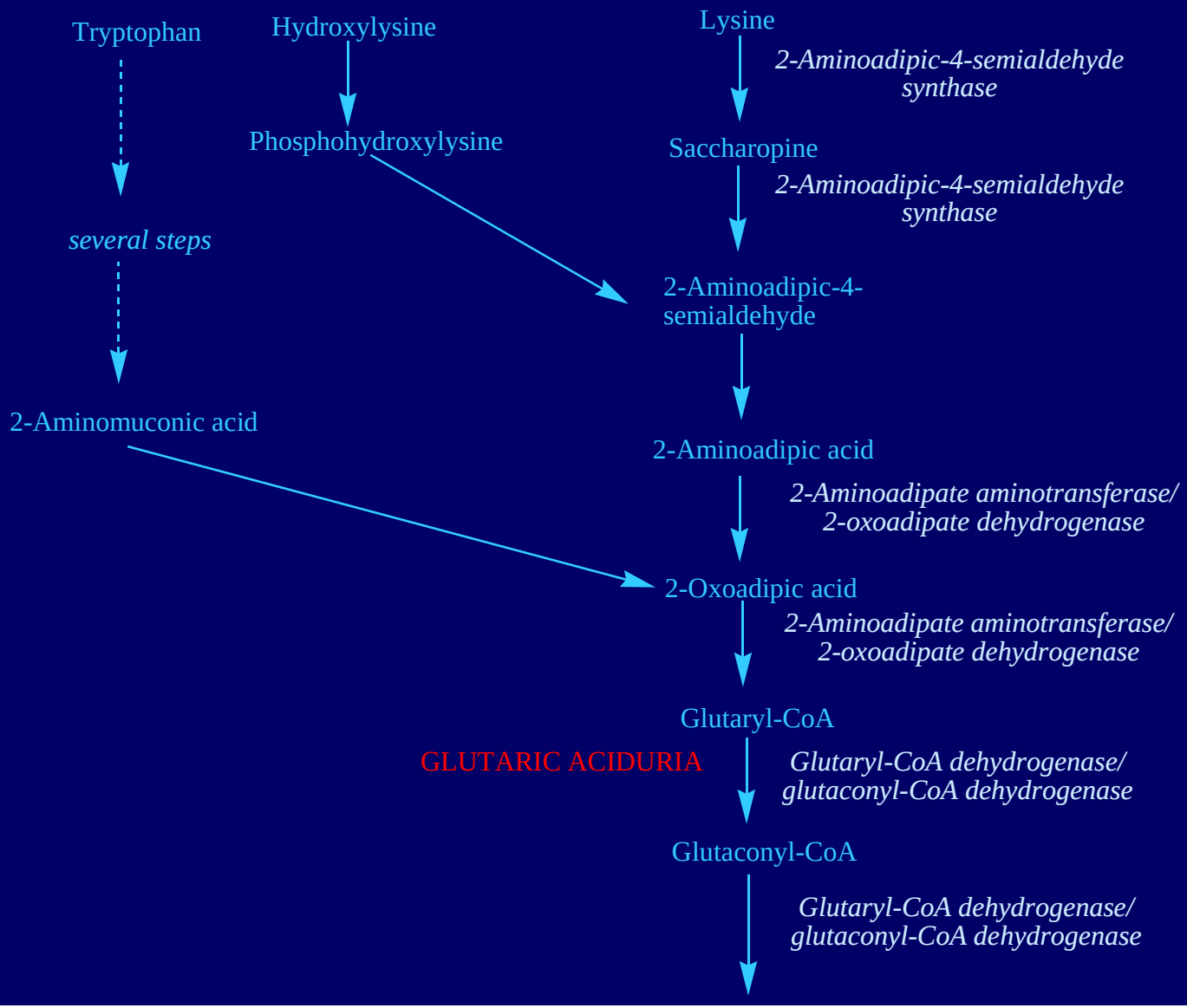
Isovaleric Aciduria



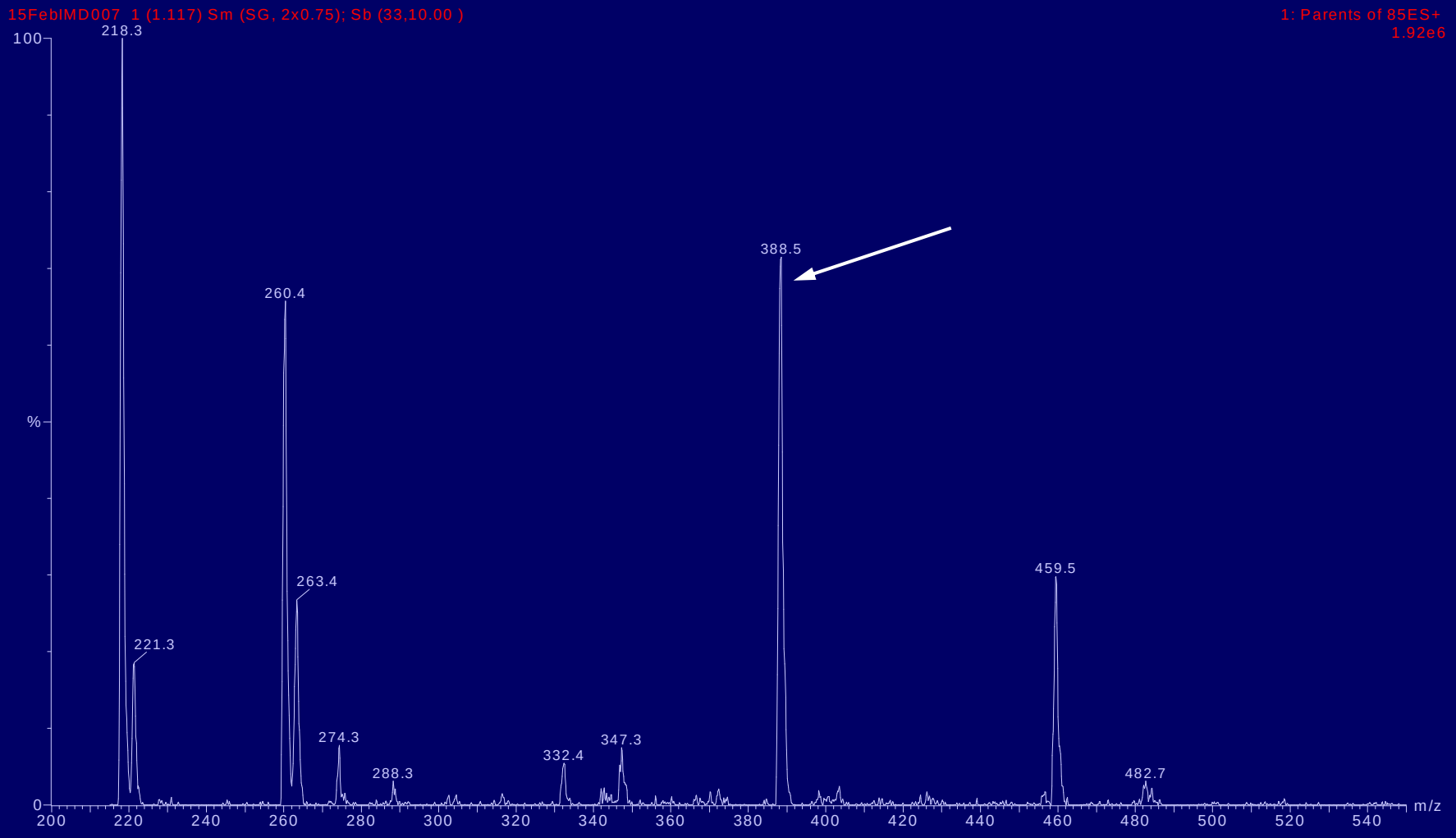
Glutaric Aciduria Type 1

- Clinical Feature
  - Macrocephalic at birth, preceding severe neurological crisis
  - Hypotonia, head-lag, irritability and jitteriness
  - Feeding problems
  - First febrile illness, or immunisation, leads to increased (but reversible) hypotonia
  - Neuroimaging reveals fronto-temporal atrophy and delayed myelination
  - Subdural haemorrhaging common when starting to walk
  - Illness and fasting may precipitate neurological crises
  - Brain damage results in loss of motor and posture, but intelligence is preserved – damage not reversible at this stage

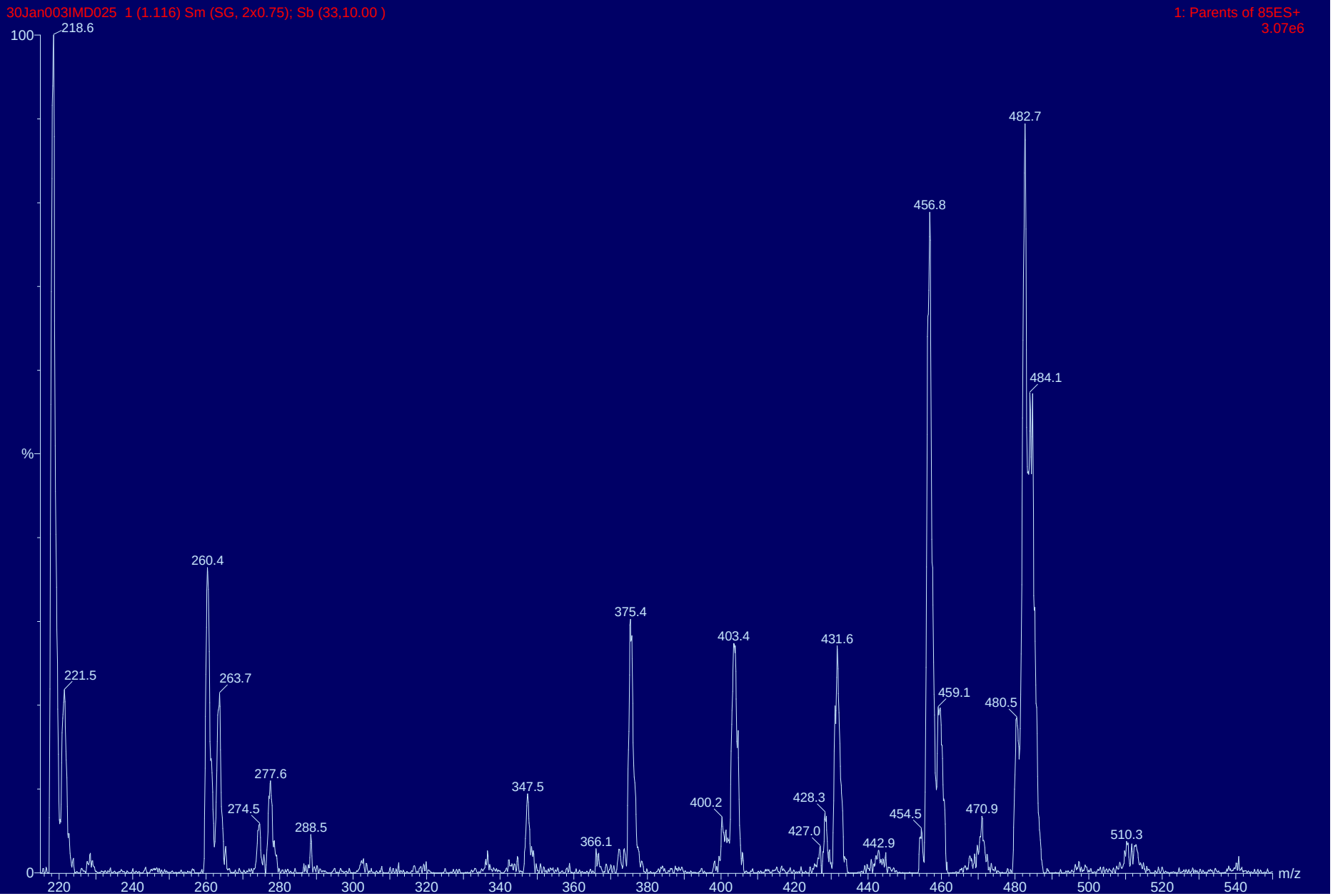
Glutaric Aciduria Type 1



Glutaric Aciduria Type 1



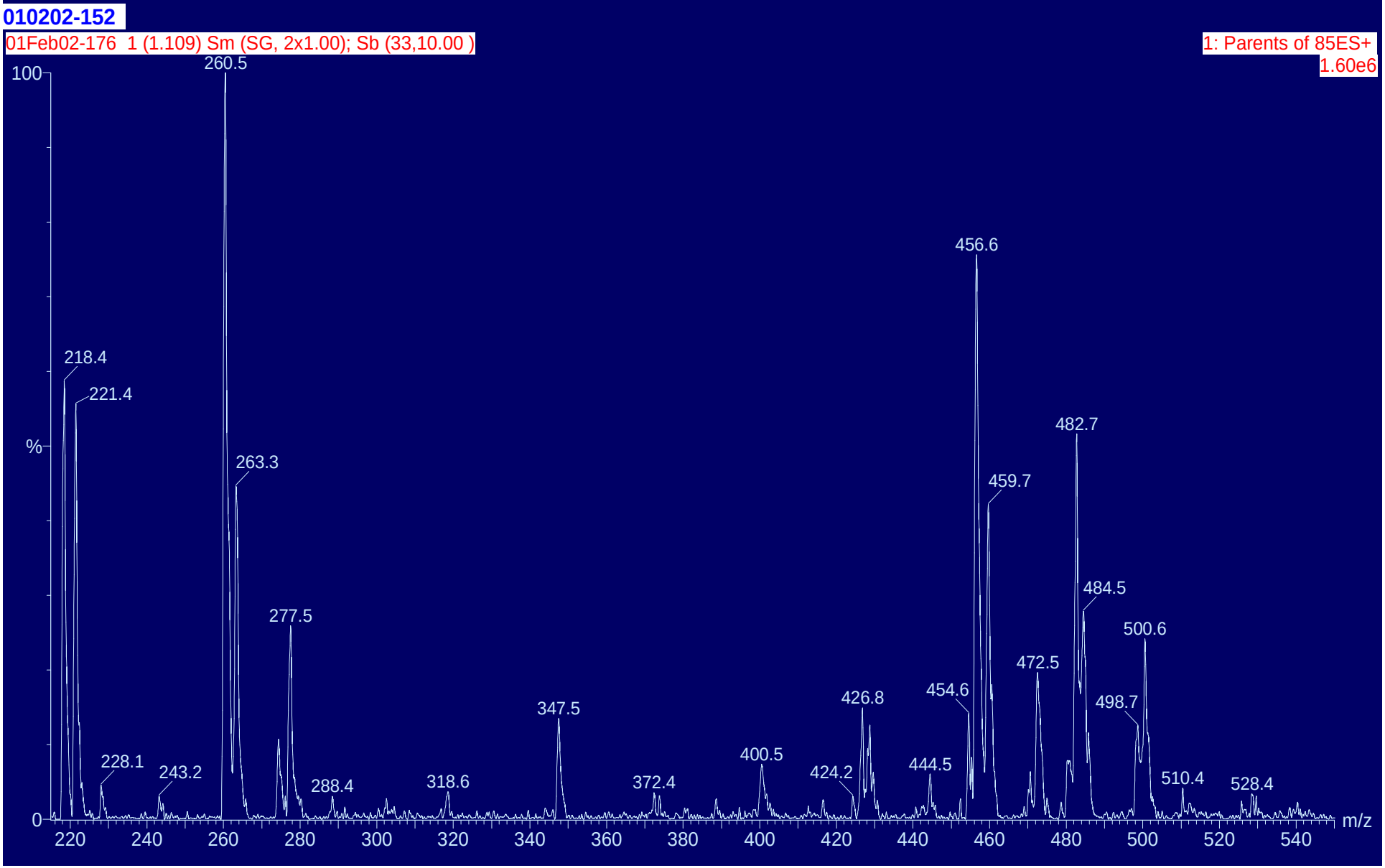
Worked Example 1



Translocase Deficiency

- Defect in transporting acyl carnitines across inner mitochondrial membrane & antiporting free carnitine
- Presentation – may be severe or mild
  - Hypoglycaemia, hyperammonaemia, muscle weakness
  - Cardiomyopathy
- Lab findings
  - C16:0, C18:1 and C18:2 acyl carnitines predominate
  - Low free carnitine (most converted to esters)
  - Short chain species may be seen (esp. in urine) showing peroxisomal oxidation still occurs
- Diagnosis by fibroblast enzyme activity

Worked Example 2



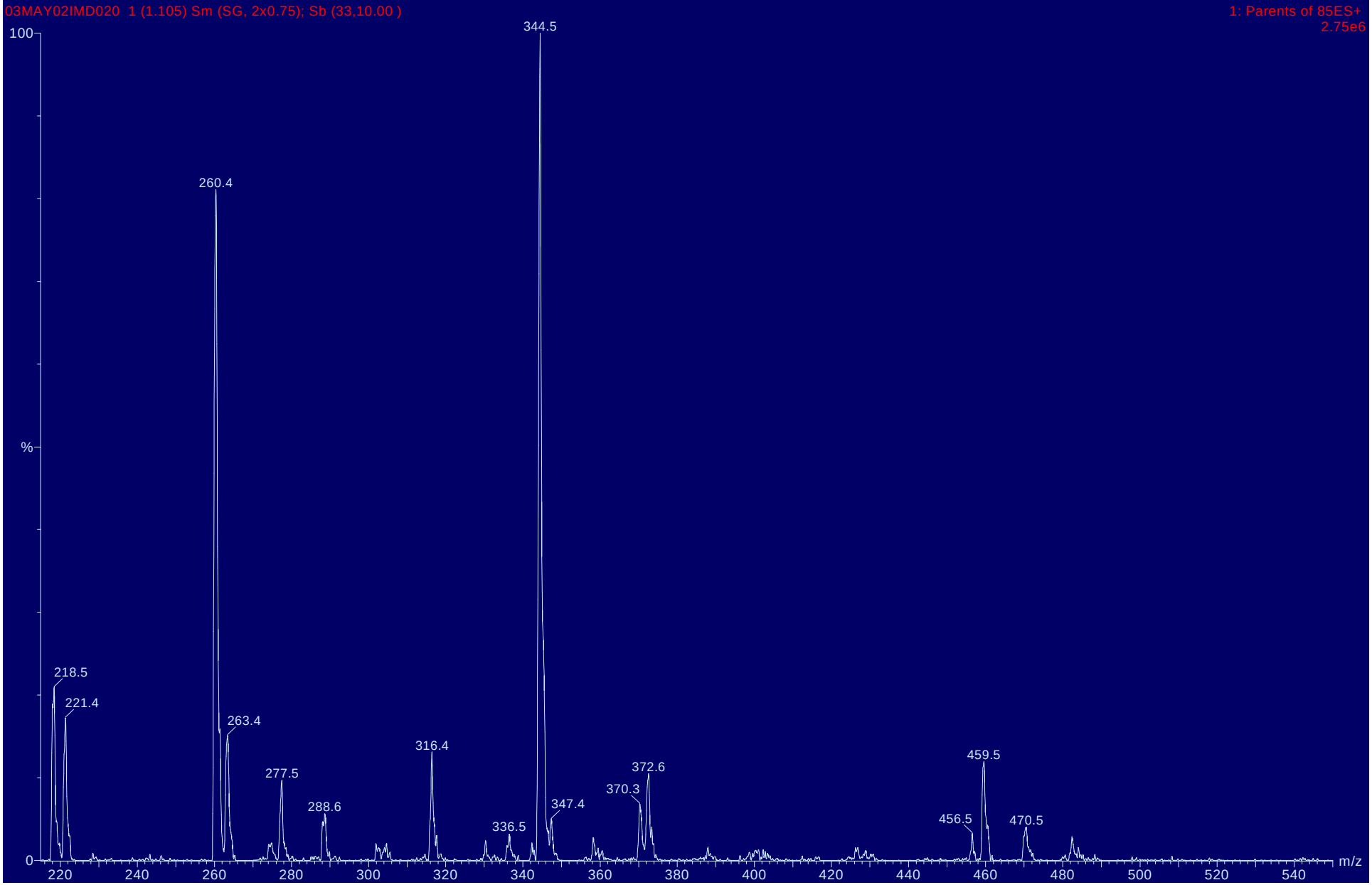
LCHADD 1

- Defective metabolism of long chain 3-hydroxyacyl-CoA
- Consequences
  - Build-up of long chain 3-hydroxyacyl-CoA species
  - Evidence of toxicity
    - Cardiotoxic
    - Other effects on metabolism
- Replacement of long chain fats with medium chain species and carbohydrate rich feeds is means of Rx

LCHADD 2

- Presentation
  - Very heterogeneous
    - Fulminant liver disease – liver disease may be very severe in LCHADD
    - Hypertrophic cardiomyopathy
    - Occasionally progressive neuropathy/ pigmenting retinopathy (neuropathy uncommon in other FAODs)
  - Most have fasting-induced hypoketotic hypoglycaemia
  - Some have cardiomegaly (LVH)
  - Some have muscle weakness – elevated CK and myoglobinuria may be seen during attacks

Worked Example 3



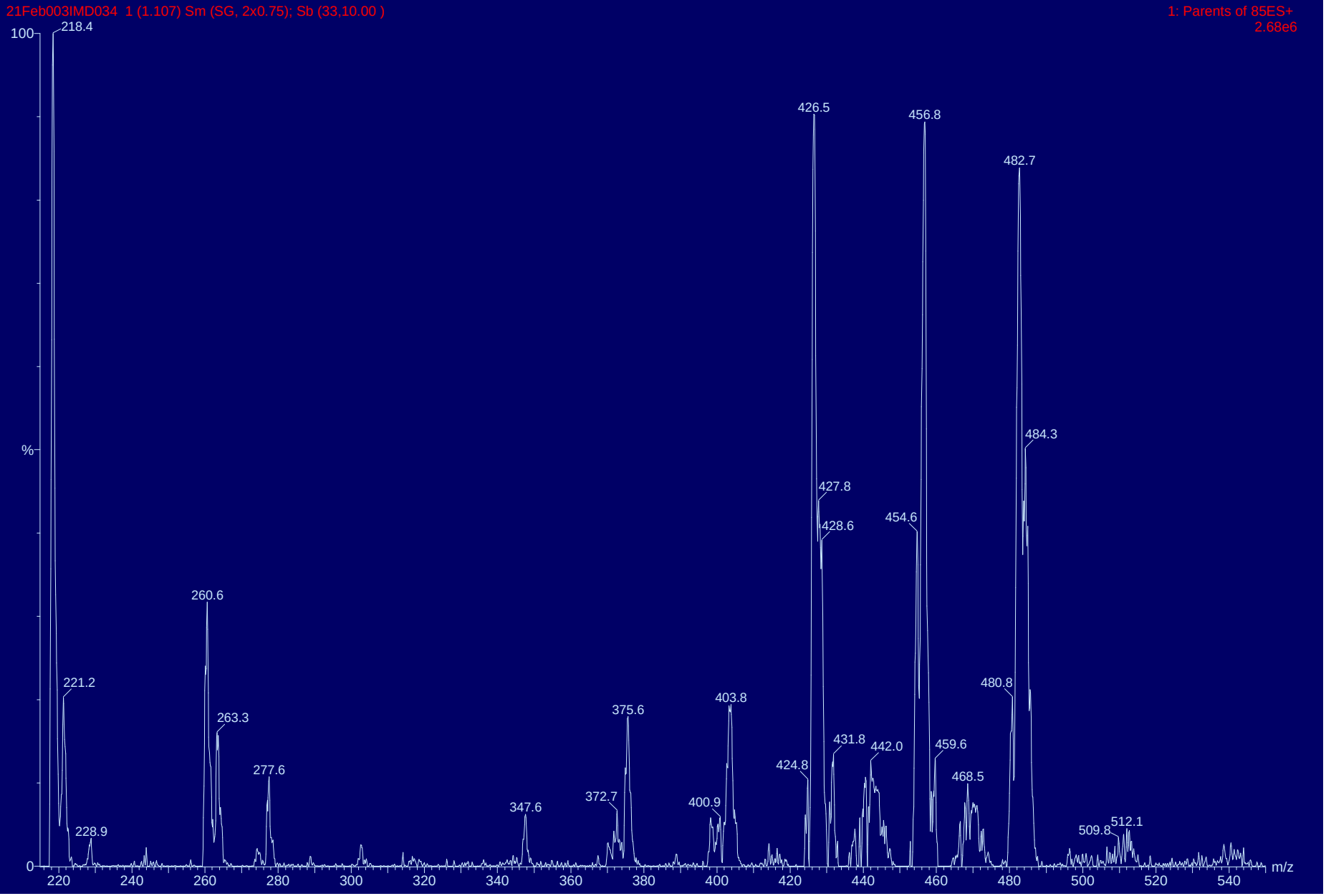
Medium Chain Acyl-CoA Dehydrogenase Deficiency 1

- Defective oxidation of C6 – C10 acyl CoA
- Clinical
  - Commonest fatty acid oxidation defect but easily treated!
  - Ca. 1:15000 incidence in NW Europe
  - 25% of patients die at first episode
  - 25% remain unsymptomatic for life
  - Hypoketotic hypoglycaemia during acute attacks
  - Liver dysfunction
  - Lethargy/coma
  - Cardiomyopathy, respiratory arrest

MCADD 2

- Lab findings
  - Organic acids
    - Characteristic metabolites present during acute crises
    - Profile may be normal when well
  - Acyl carnitines
    - Profiles preserved in well-states
- Molecular Biology
  - 80 - 90% of caucasian cases have G985A mutation
  - Several other mutations know
    - A799G, T157C, A447G, C730T, C1124T etc
    - Some are clinically silent, but not biochemically so
      - Incidence of MCADD may change when MS/MS screening routine

Worked Example 4



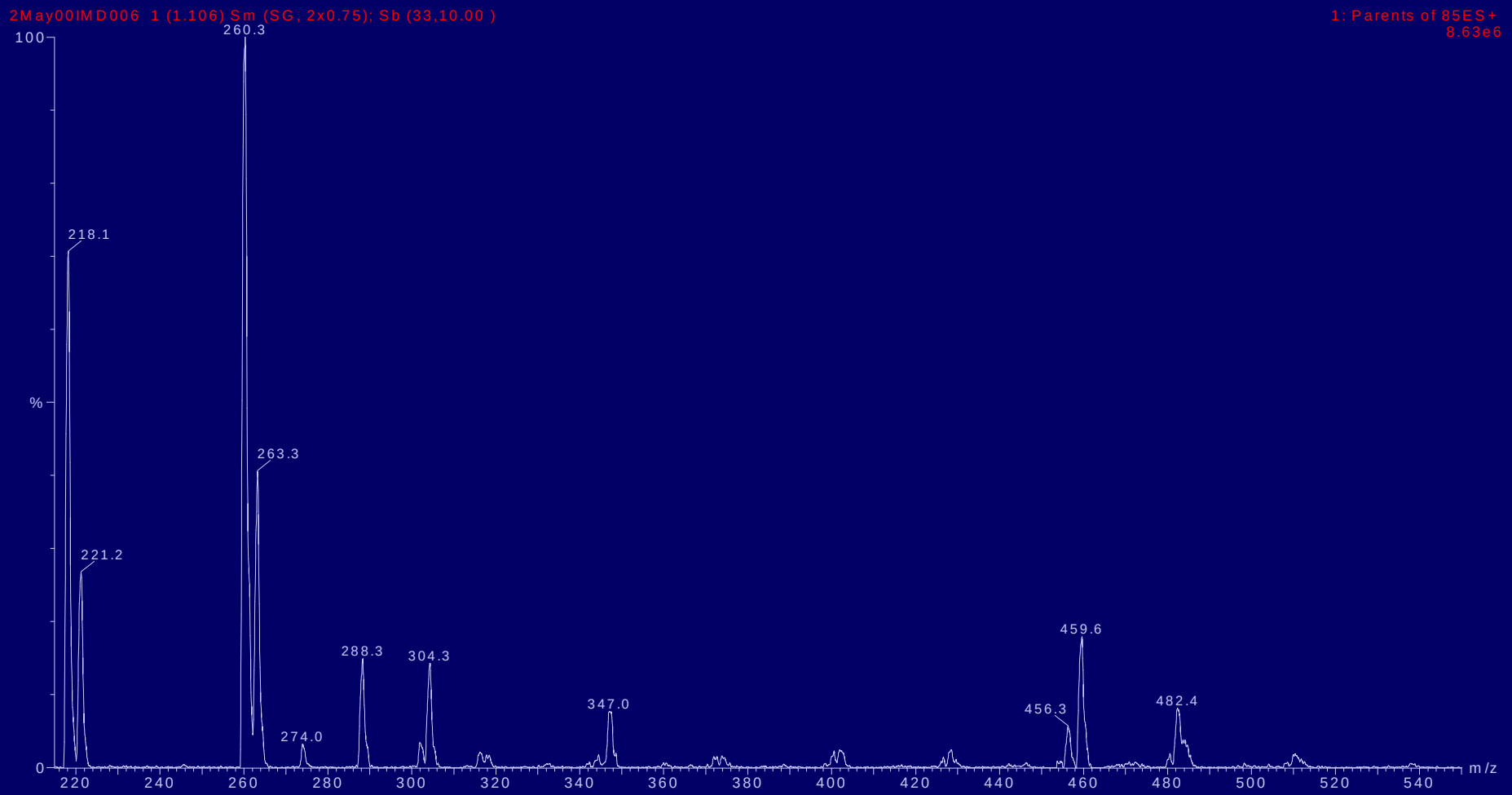
VLCADD

- Defect in metabolising C12 – C18 acyl CoAs
- Consequences
  - Long chain species accumulate
    - Toxic and metabolised by peroxisomes and microsomes to limit build-up
  - Replacing long chain fats in diet by MCT affords Rx
- Presentation – 3 forms
  - Neonatal – lethal with cardiomyopathy and hepatic involvement (hyperammonaemia, coma)
  - Childhood and adult – mostly muscular like adult CPT-II with myoglobinuria

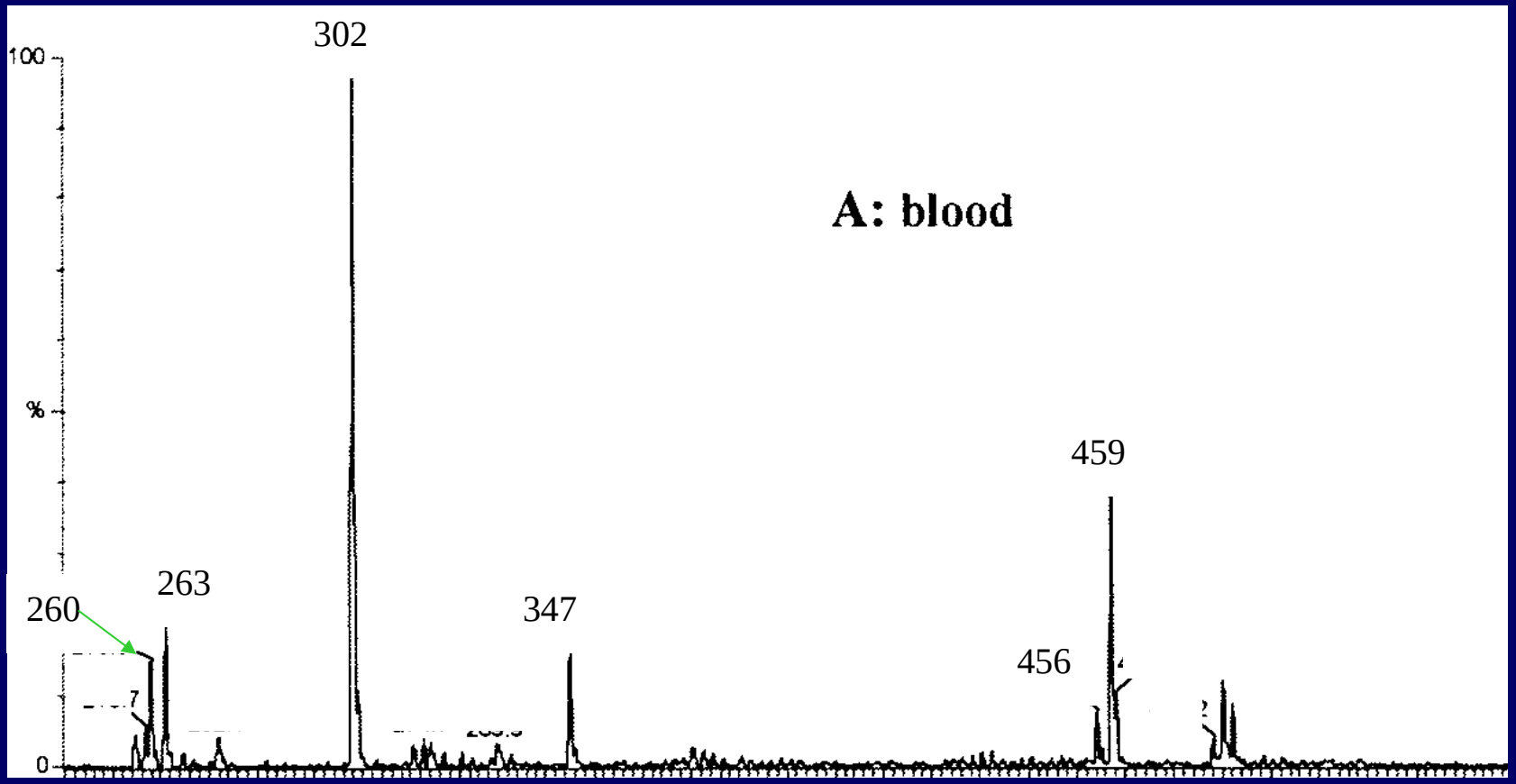
Post-Mortem Samples

- Variable presentation
- Usually see increase in free and short chain acyl carnitines
  - May have more or less abnormalities
  - Useful for investigation of SUDI
  - Statistically, most cases reveal no biochemical abnormalites other than PM changes
  - Some cases are clear cut (e.g. for MCADD)
  - Some cases are just uninterpretable

Typical Post-Mortem Profile



Diagnosis?



Wrong!

J. Inher. Metab. Dis. 21 (1998) 624–630

Diagnosis of isovaleric acidaemia by tandem mass spectrometry: False positive result due to pivaloylcarnitine in a newborn screening programme

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MS received 9.9.97 Accepted 14.1.98

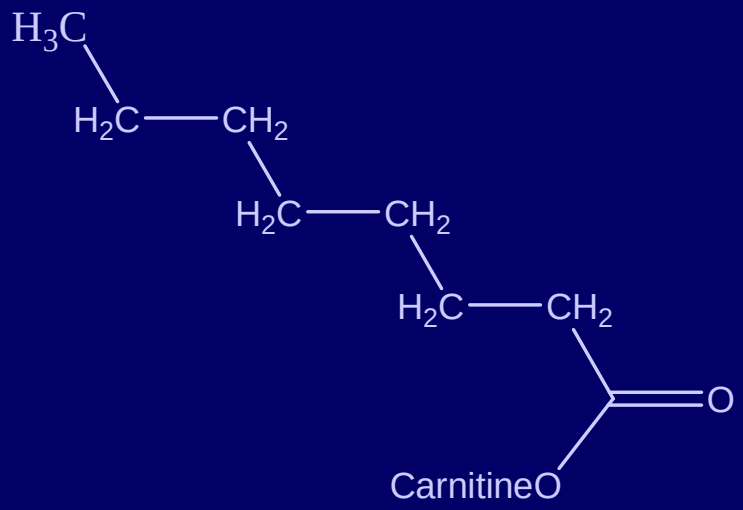
Why?

- PAR 85 experiment *not specific* to acylcarnitines
  - acylcarnitines are detected because they form a m/z 85 fragment
  - other species forming m/z 85 fragments will also be detected
  - possible diagnostic problems if other species has same mass as an acylcarnitine

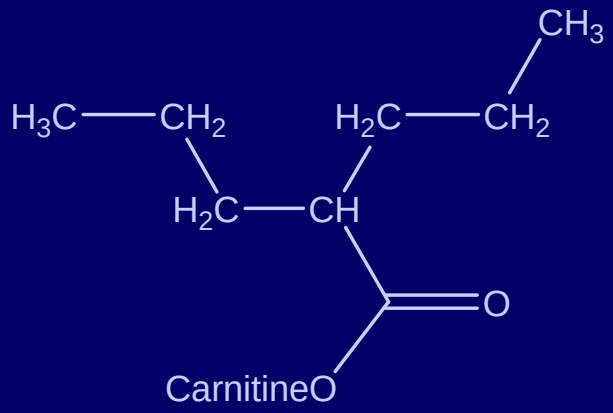
Isobaric Species

- Compounds with the same m/z ratio, but not necessarily the same chemical composition (cf. isomers)
  - Pivaloylcarnitine and isovalerylcarnitine
  - Valproylcarnitine and octanoylcarnitine

Isomers



Octanoyl carnitine



2-Propylpentanoyl carnitine
(valproyl carnitine)

Isomers & Isobars – On The Buses…

Other Considerations

- Non-derivitisation
  - PAR 85 experiment, but m/z are 56 units less than corresponding Bu esters
  - Ion counts lower – more ion suppression
  - Less sensitive for dicarboxylic species (e.g. in GA-1)
- Paired blood spots and plasma specimens?
  - Generally plasma is more sensitive, but exceptions
  - Ideal to have both (e.g. from one whole blood spec)
  - Can use either, but if only one available better to just have plasma

Summary

- Acylcarnitine profiling is easy to implement with MS/MS.
- Several diseases give diagnostic patterns, although in some cases definite diagnosis is not possible.
- Acylcarnitines usefully complement organic and amino acid profiling in diagnosis of metabolic “small molecule” disease.