Instability of biological matrices and its effect on bioanalytical method performance

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We know analytes can be unstable, but what about the matrix?

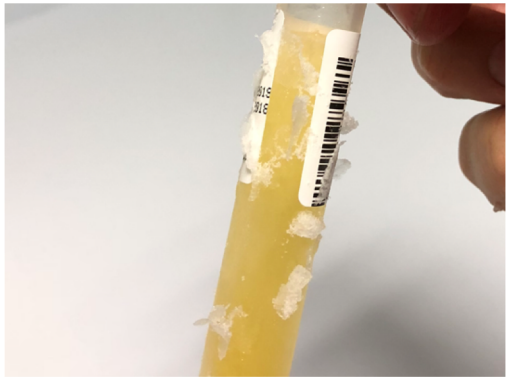
Biological matrices are not constant in composition and properties, but change as a function of time, storage temperature and other conditions

Method performance may be different in fresh than in aged matrix

Content

Effect of matrix ageing on bioanalytical method performance:

- Selectivity: formation / disappearance of endogenous compounds
- Stability: change in enzyme activity
- Recovery: change in extractability



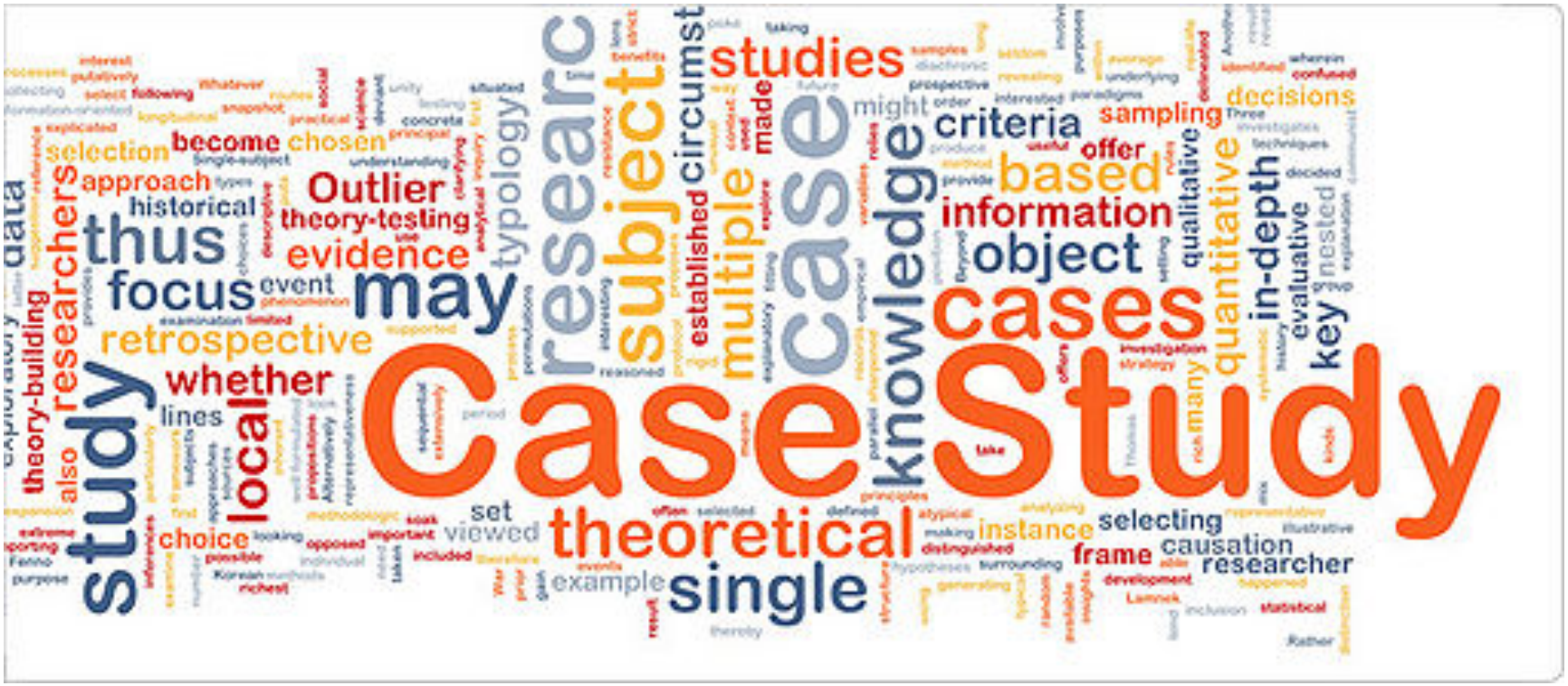
How long do we store our blank matrices?

A little survey of expiry dates:

- Plasma: 2-5 years at -20°C
- Urine: 2-5 years at -20°C
- Blood: 1-2 weeks at 4°C

Is there a rationale?

- Not really..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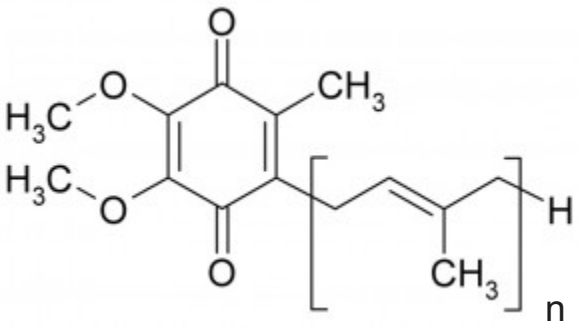
Selectivity

Formation of matrix constituents: Coenzyme Q10

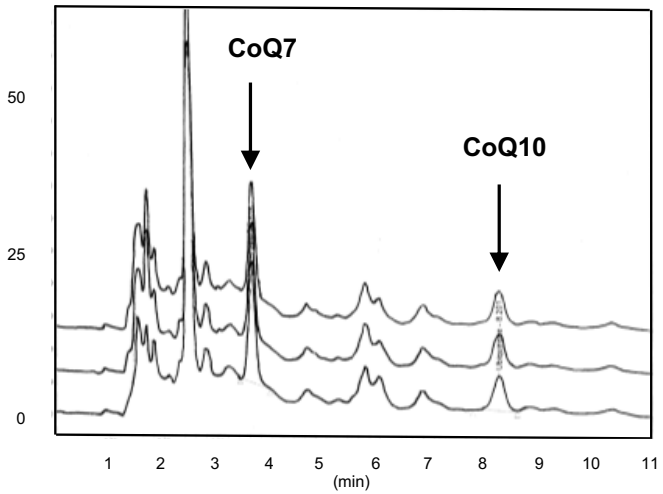
- Endogenous benzoquinone, antioxidant
- Sample preparation: oxidation with Cu(II), liquid extraction with hexane
- LC on C18 column with hexane/IPA (8:2, v/v) mobile phase
- UV detection at 275 nm



n=7: CoQ7 (internal standard)
n=10: CoQ10 (analyte)

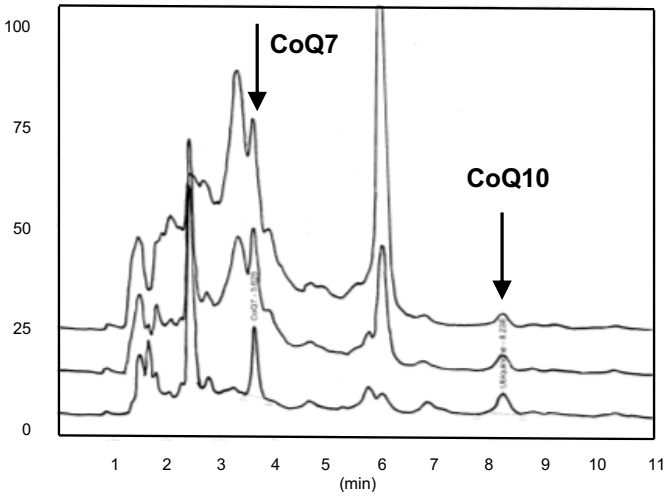
Selectivity

Formation of matrix constituents: Coenzyme Q10



-70°C

9 months
6 months
t=0



-20°C

Internal standard obscured by endogenous plasma components, formed upon storage at -20°C

Selectivity

Formation of matrix constituents: Coenzyme Q10

- Root cause: lipid oxidation products? Known to be formed upon Cu(II) treatment, when endogenous antioxidants are depleted

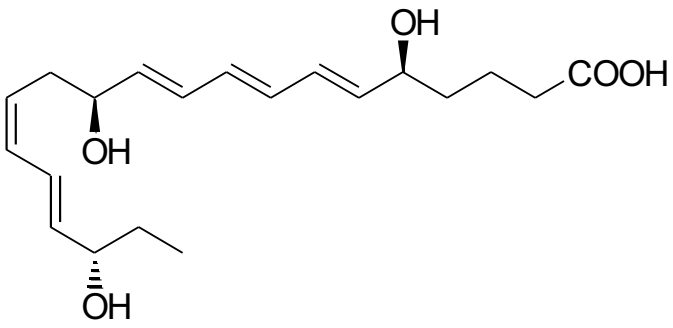
- Method development was performed with relatively fresh plasma
- Formation of interfering matrix components was not observed during accelerated stress test
- Effect could have been picked up with older plasma lots

- Method is insufficiently selective for analysis of samples stored at -20°C for more than three months

Selectivity

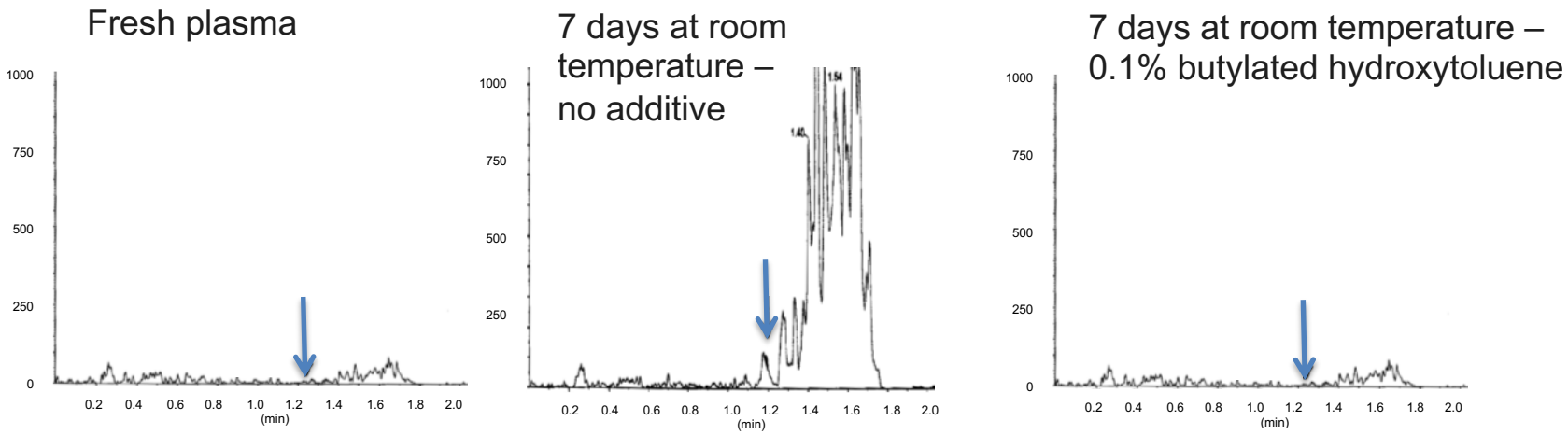
Formation of matrix constituents: Resolvin E1

- Omega-3 fatty acid
- Sample preparation: liquid extraction with acetonitrile/n-chlorobutane (3:7, v/v)
- LC on C18 column with acetonitrile / 0.05% formic acid gradient
- Negative ESI-MS/MS detection (m/z 349.1 -> 107.0)



Selectivity

Formation of matrix constituents: Resolvin E1



Analyte obscured by endogenous plasma components, formed upon storage at RT

Prevention in the presence of anti-oxidant BHT

Selectivity

Formation of matrix constituents: Resolvin E1

- Root cause: fatty acid oxidation

- Interferences present in many lots of blank plasma, correlated with age
- Formation can be prevented by adding BHT
- BHT added to fresh blank matrix and study samples

- No further consequences for method performance

Stability

Modulation of enzyme activity: proprietary compound

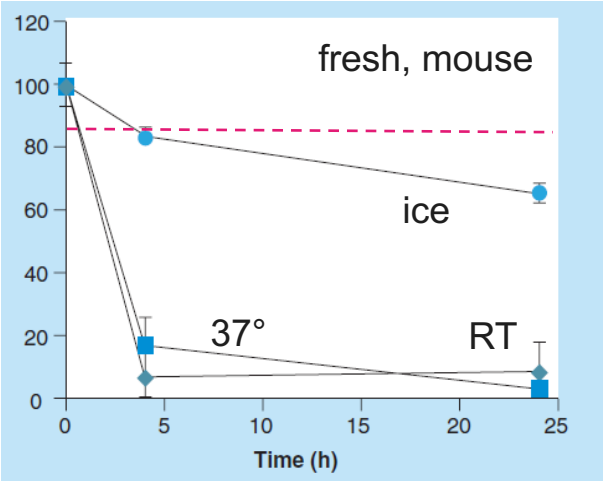
- Some instability seen, but acceptable when plasma is handled on ice
- Sample preparation: liquid extraction with tBME
- LC on C18 column with methanol/acetonitrile and 0.1% formic acid gradient
- Positive ESI-MS/MS detection

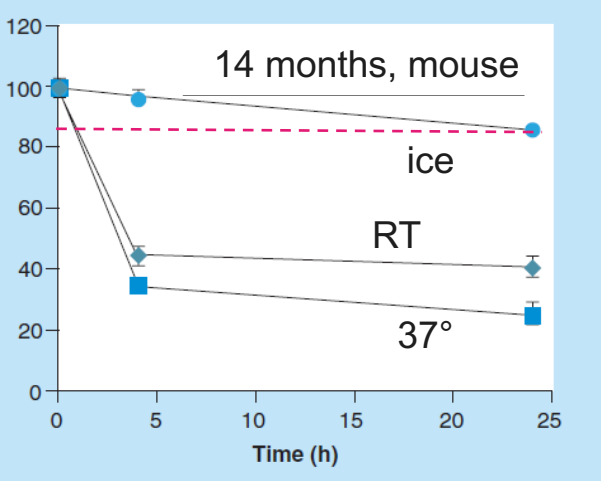
- ISR failed in mouse plasma, second result 39-73% (average 58%) lower than first result

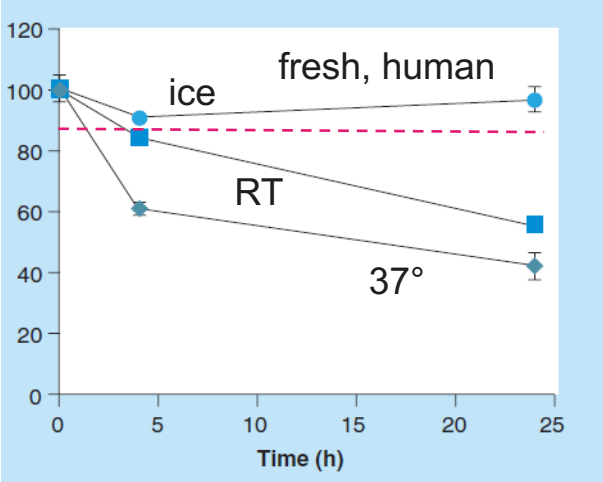
van de Merbel & de Vries, Bioanalysis 5(19), 2013, 2393-2407

Stability

Modulation of enzyme activity: proprietary compound







Stability on ice in old mouse plasma just acceptable

Stability on ice in fresh mouse plasma fails

Stability on ice in fresh human plasma acceptable

Stability

Modulation of enzyme activity: proprietary compound

- Root cause: reduced enzyme activity in old mouse plasma, that was used for method development and validation

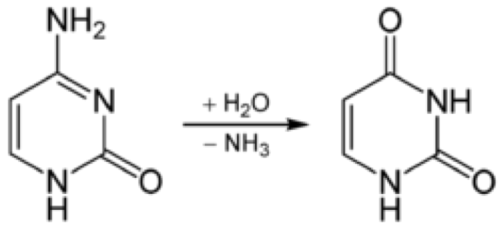
- Stored blank mouse plasma not representative for fresh study samples
- Analyte appears more stable than it actually is

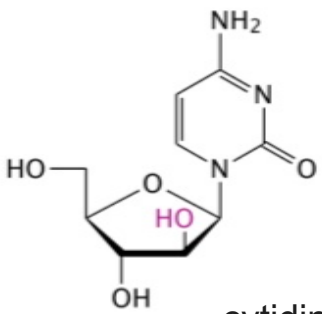
- Original bioanalytical results potentially compromised

Stability

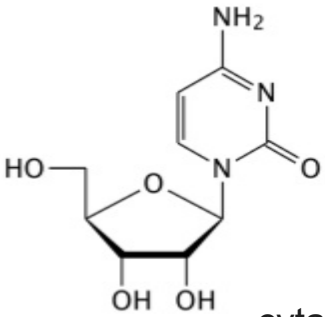
Modulation of enzyme activity: cytarabine

- Pyrimidine nucleoside analogue
- Sensitive to degradation by cytidine deaminase
- Blood collection in tubes containing inhibitor tetrahydrouridine (THU)
- Sample preparation: mixed-mode cation-exchange SPE
- LC on C18 column with methanol and 0.1% formic acid gradient
- Positive ESI-MS/MS detection (m/z 224.0 -> 112.0)





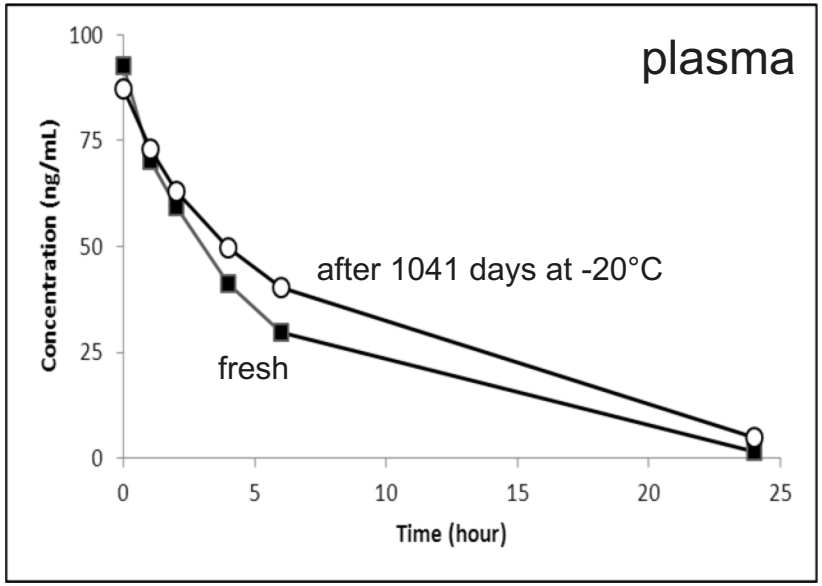
cytidine

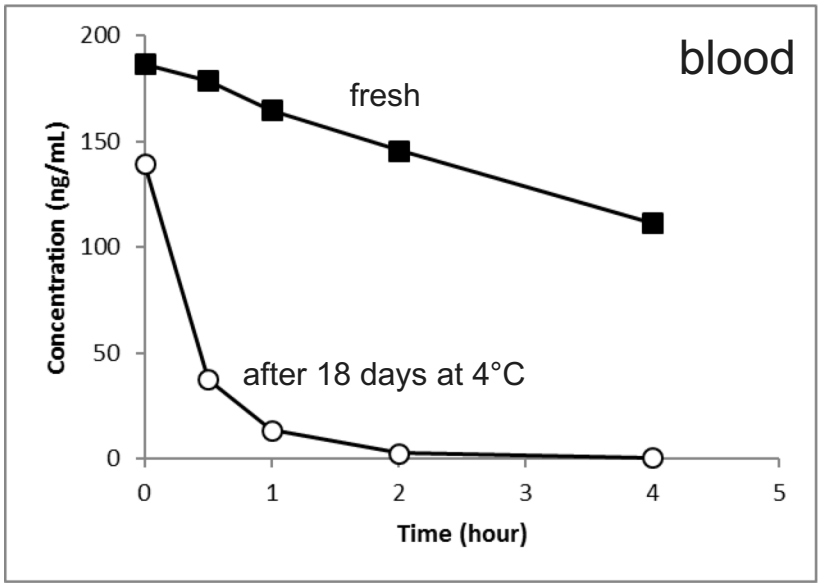


cytarabine

Stability

Modulation of enzyme activity: cytarabine





Rapid degradation in untreated plasma and whole blood

Slightly slower in aged plasma

A lot faster in aged whole blood

Stability

Modulation of enzyme activity: cytarabine

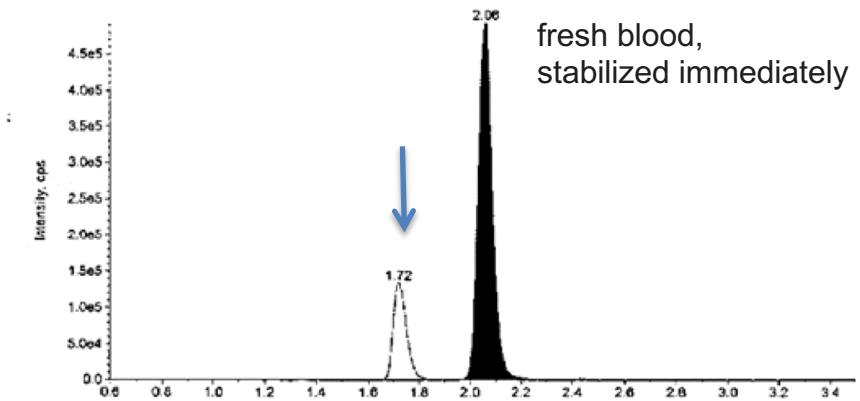
- Root cause: higher enzyme activity in old blood because of enzyme leakage from erythrocytes upon storage

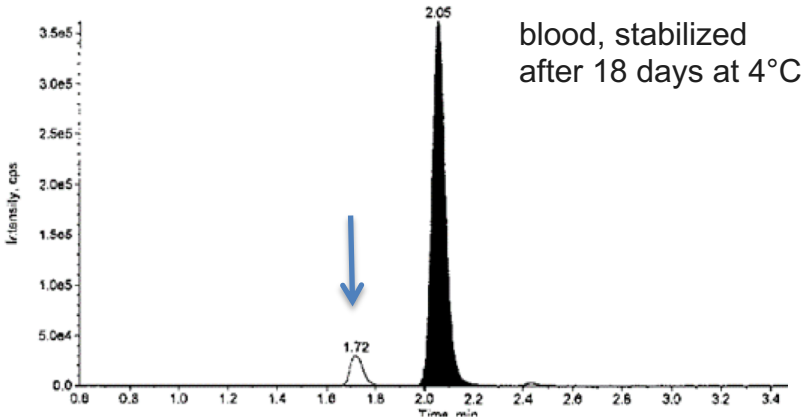
- Stored whole blood not representative for fresh blood
- Analyte appears more unstable than it actually is

- With proper enzyme inhibition no further consequences for the method

Stability

Note: endogenous cytidine levels decrease upon storage



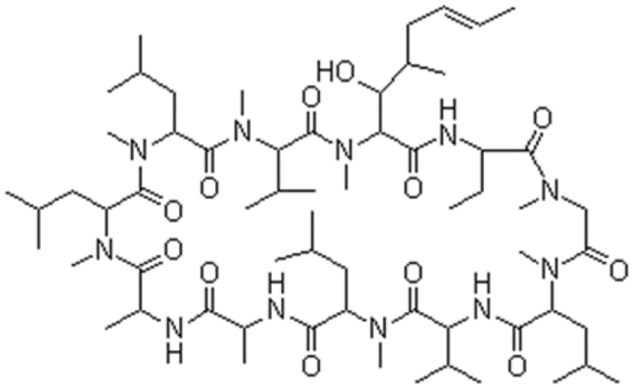


- Stored matrix not representative for fresh
- Concentration of interfering endogenous compound appears lower than it actually is

Recovery

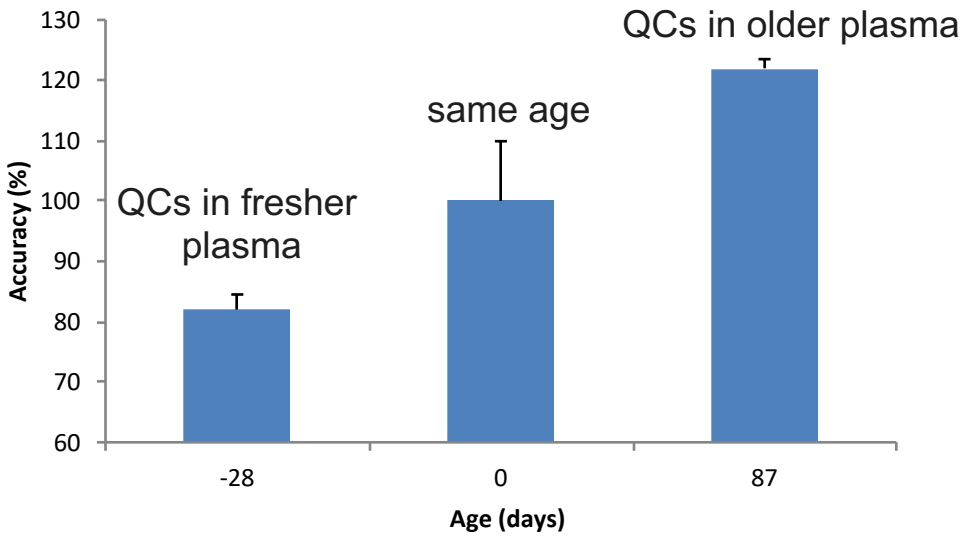
Modified extractability: cyclosporin A

- Cyclic peptide
- Quantification in whole blood, because of variable partitioning between plasma and erythrocytes
- 1:1 dilution of blood with water prior to storage to facilitate cell disintegration
- Sample preparation: liquid extraction with n-chlorobutane
- LC on PFP column with acetonitrile and 0.1% formic acid gradient
- Positive ESI-MS/MS detection (m/z 1216.9 -> 689.6)



Recovery

Modified extractability: cyclosporin A



Results for freshly spiked QCs in blank matrix of a different age than that of the corresponding freshly spiked calibration curve

Higher recovery / enhanced extractability for older matrix (only for high QC level)

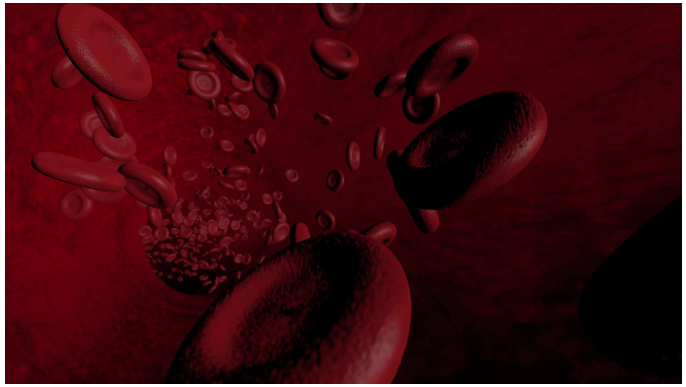
Recovery

Modified extractability: cyclosporin A

- Root cause: cellular material in older blood possibly more disintegrated and analyte more easily extractable

- Stored blood not representative for fresh blood
- Overestimation of analyte levels in stored samples when analysed against fresh curve

- Solution: analyse study samples always against calibrators and QCs of the same age



Recovery

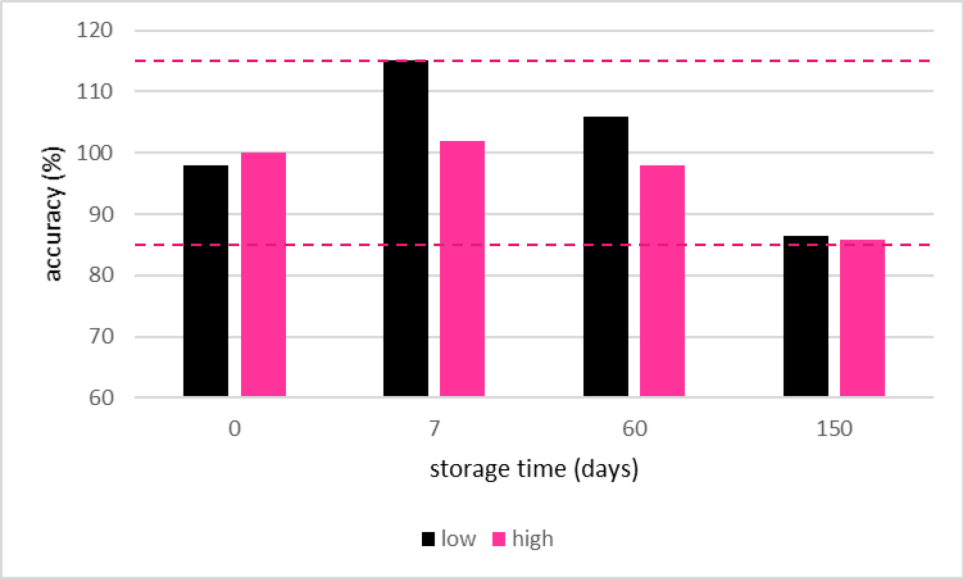
Modified extractability: proprietary compound

- Dried blood samples (VAMS)
- Rehydration of VAMS tip in water, followed by protein precipitation with acetonitrile
- LC on C18 column with acetonitrile and 0.1% formic acid gradient
- Positive ESI-MS/MS detection

Recovery

Modified extractability: proprietary compound

- Slight decrease in accuracy with storage time (ambient)



Recovery

Modified extractability: proprietary compound

- Root cause: more difficult re-solubilization of analyte from dried blood (instability unlikely)
- Stored dried blood not representative for freshly dried blood
- Slight underestimation of analyte levels when analysed against freshly dried calibrators

Consequences

Selectivity:

formation or disappearance of endogenous compounds during storage

- Through oxidation or enzyme activity
- Selectivity in fresh matrix may be better or worse than in stored matrix
- Most likely for endogenous or near-endogenous analytes

- Can be tested by screening matrix lots of different ages in MD
- If applicable: addition of anti-oxidant

Consequences

Stability:

increase or decrease of enzyme activity during storage

- Plasma: decrease most likely
- Blood: increase also possible
- Stability in fresh matrix may be better or worse than in stored matrix
- Most likely for analytes that are unstable

- Can be tested by using matrix lots of different ages for stability assessment, especially for analytes with borderline stability
- If applicable: use fresh plasma for validation of stability
- Use blood as freshly as possible

Consequences

Recovery:

increased extractability by sample disintegration or decreased extractability by drying effects during storage

- Wet samples: increase most likely
- Dried samples: decrease most likely
- Recovery in fresh matrix may be better or worse than in stored matrix
- Most likely for blood

- Can be tested by using matrix lots of different ages for recovery assessment
- Use (spiked) matrix of the same age for calibrators / QCs

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