Matrix effects in lipemic plasma

practical solutions to additional issues in bioanalytical method development and validation

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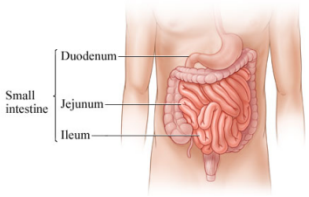
Lipemic plasma experiments…….

- Prove reliability of analytical method when applied to lipemic samples
- Analysis of spiked lipemic plasma.
- Evaluation of precision and accuracy.
- As part of bioanalytical method validation.

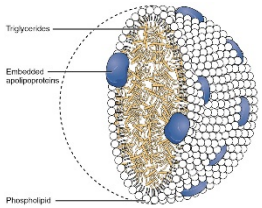
→To be addressed in method development.

- What kind of issues can be expected?
- Predictable on forehand?
- Related to analyte properties?
- How to solve / circumvent?

Bulk material and test sample

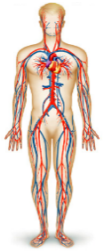


dietary fat

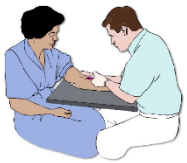


chylomicrons

lymph

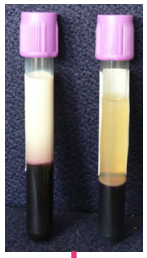


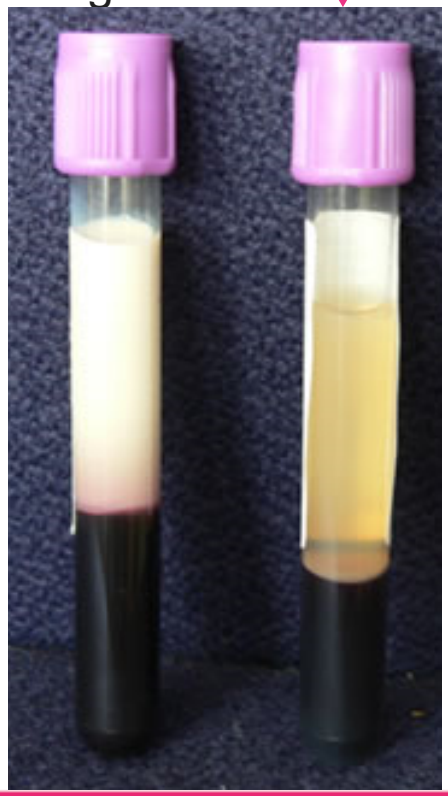
blood



tube

Centrifuge





- Commercially available.
- High triglyceride content: > 150 mg/dL ; > 1.69 mM.
- Aliquoting blank plasma in ~ 1.5 mL portions.
  - Store at -20°C until use.
- Prepare sample.
  - Spike analyte to plasma.
  - Store at -20°C/-70°C until use.

Where to expect issues?

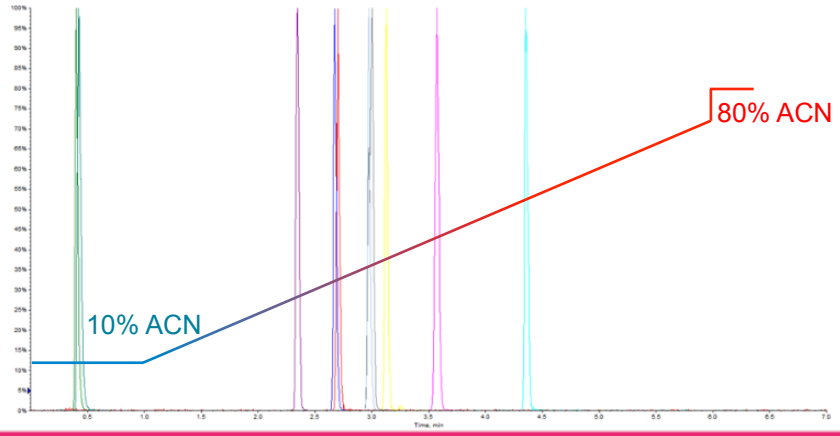
Analytical procedures

Process	Possible issue
Aliquoting bulk material	Non-representative matrix
Sampling (pipetting)	Inhomogeneous sample
Sample preparation	Recovery
Chromatography	Selectivity
Mass spectometry	Ionization suppression

Scouting experiments

Test meterial

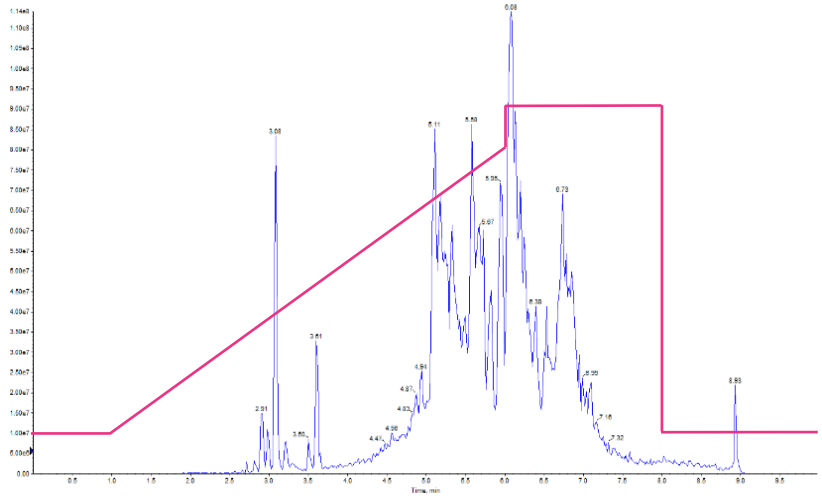
- Matrix : heparin plasma (Seralabs)
  - Lipemic: 859 mg/dL = 9.7 mM triglycerides.
  - Normal: 77.9 mg/dL = 0.9 mM triglycerides.
- Test compounds
  - Wide polarity range (LogP: 0.4 – 6).
  - Neutral and ionizable (bases).
  - Spiked to lipemic and normal plasma.
  - Combined RP-LC-MS method.



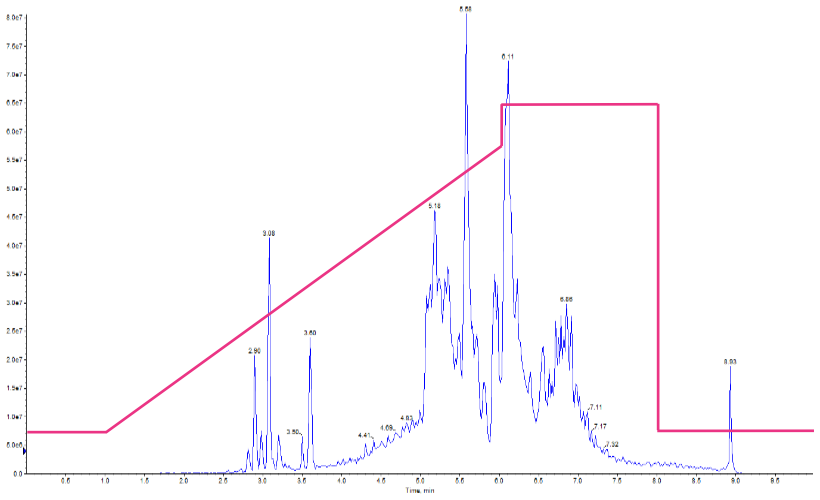
compound	lipophilicity	LogP
Miconazole		6.0
Amitriptyline		4.8
Maprotiline		4.4
Donepezil		4.2
Reserpine		3.5
Carbamazepine		2.7
Warfarin		2.7
Phenytoin		2.1
Ranitidine		1.0
Atenolol		0.4

Ionization suppression

Protein Precipitation



Phospholipids in normal plasma

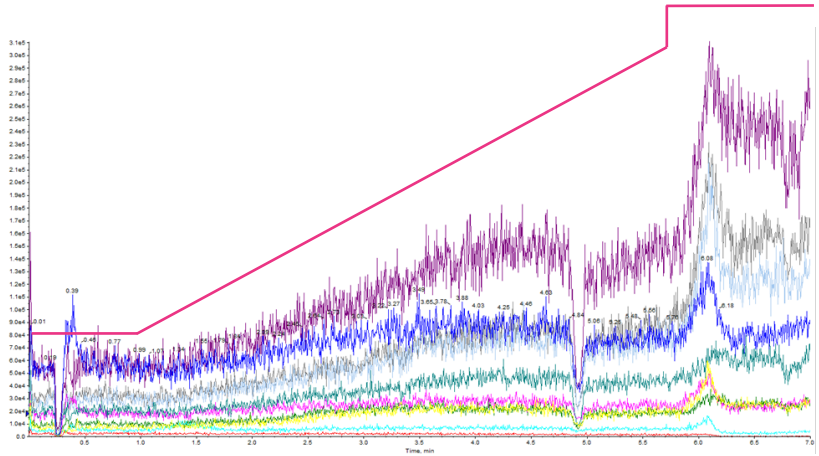


Phospholipids in lipemic plasma

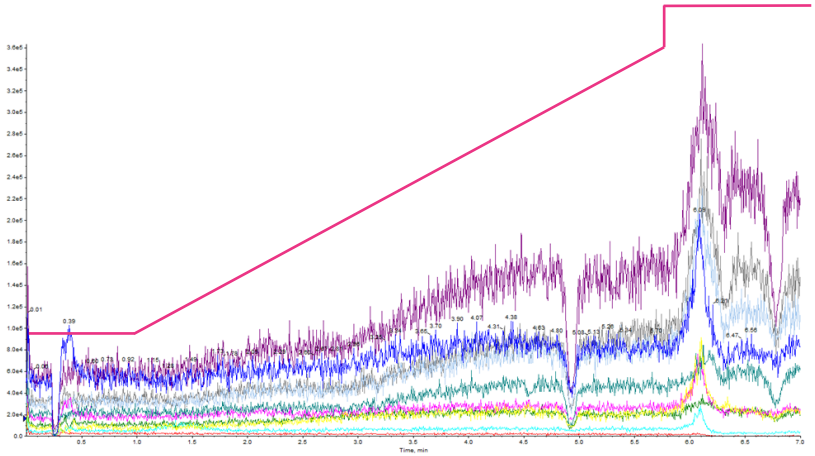
- No clear increased phospholipid peaks in lipemic plasma extract.
  - Precursor scan of m/z 184; Q1 scan range: 400 – 1000.

Ionization suppression

Protein Precipitation



Suppression test of normal plasma



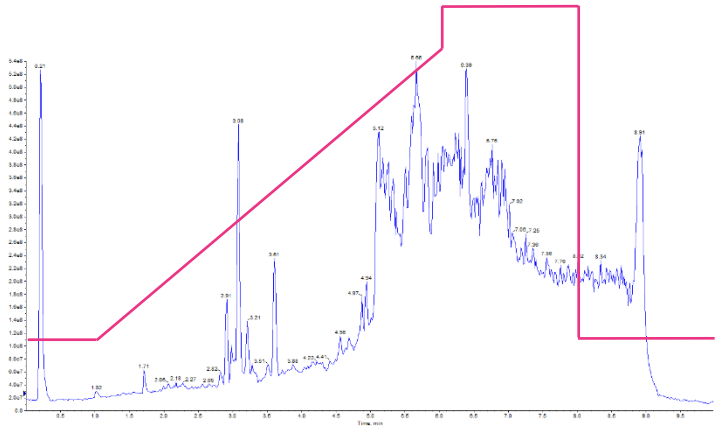
Suppression test of lipemic plasma

- No additional suppression zones.

Selectivity

Protein Precipitation

- 50 of sample + 150 µL of methanol.
  - Supernatant diluted with 0.1% FA in water.



Tic of Q1 scan of normal plasma

Tic of Q1 scan of lipemic plasma

- No additional large peaks in lipemic plasma extract.
  - Q1 scan range: 400 – 1200.
- Triglycerides elute at very high modifier concentrations: >90% ACN (lit.).

Sample preparation

PP and liquid liquid extraction

Protein Precipitation:

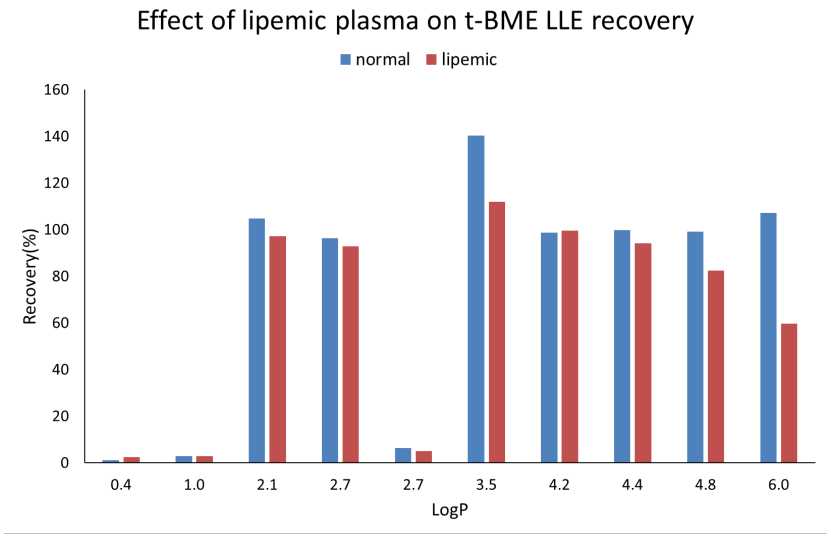
- 50 µL of sample + 50 µL of methanol + 150 µL of acetonitrile.
  - Supernatant diluted with 0.1% FA in water.

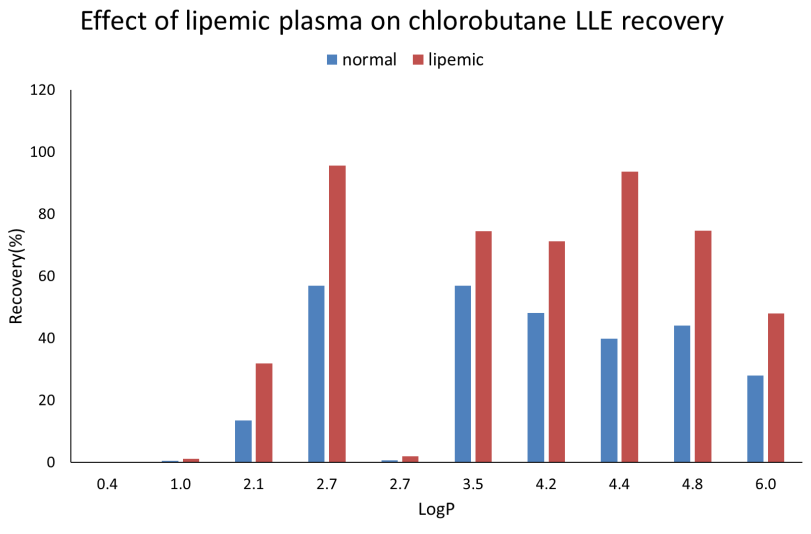
➢No recovery difference between control and lipemic plasma.

Sample preparation

liquid liquid extraction

- t-BME or n-chlorobutane; 50 µL of sample.
- Basified with 0.5% ammonia.
- Organic phase dried under N_2 and reconstituted.





- For t-BME lower recovery for lipophyllic analytes
- Consistent higher recovery with chlorobutane.
- Effects are corrected by STIL

Sample preparation

Solid shase extraction

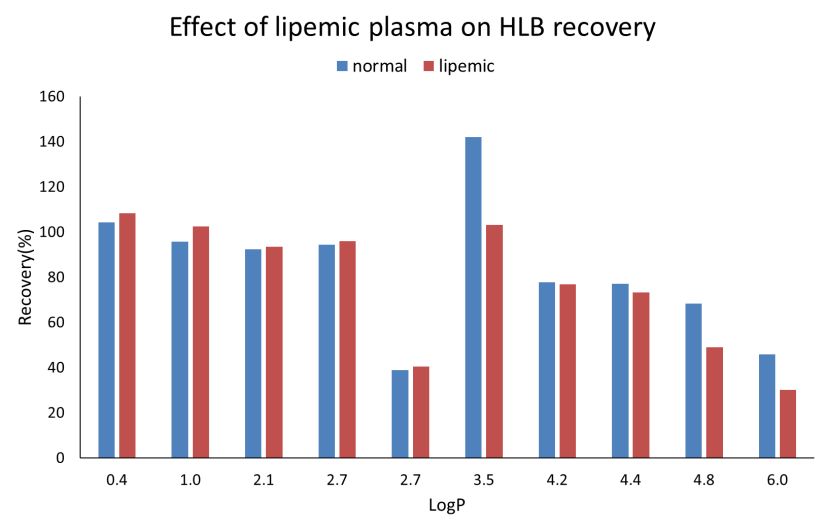
- 50 µL of sample on Waters µElution plate:

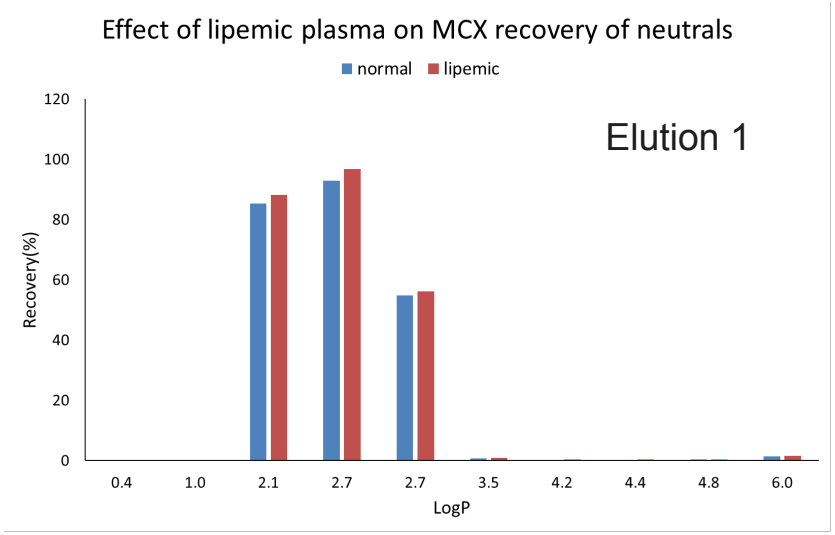
Oasis HLB (RP)	Oasis MCX
Sample: basified with 0.5% ammonia	Sample: acidified with 0.1% formic acid
Wash with 0.5% ammonia	Wash with 0.1% formic acid
Elute with 100% methanol	Elute with methanol (neutrals)
Dilute with 0.1% formic acid	Elute with 2% ammonia methanol (bases)
	Dilute with 0.1% formic acid

➢ Some lipemic samples lead to obstructed µElution plate columns.

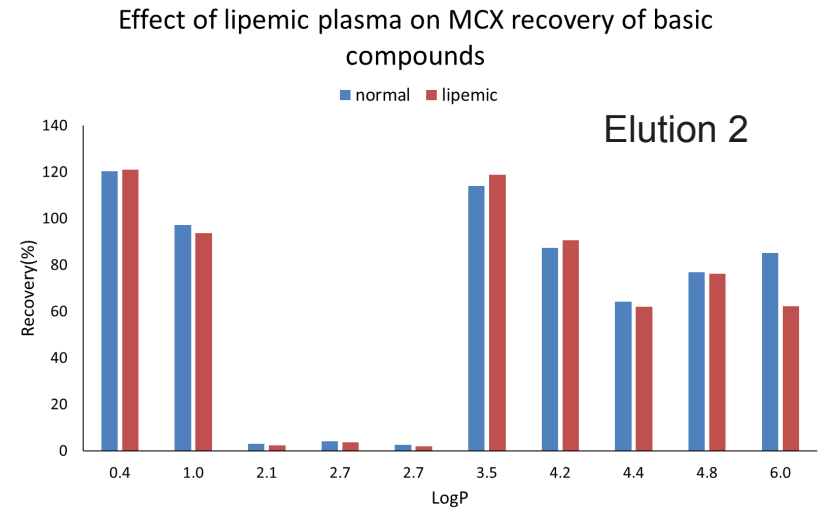
Sample preparation

Solid phase extraction





- No significant effect on SPE



Processing of bulk material

aliquoting

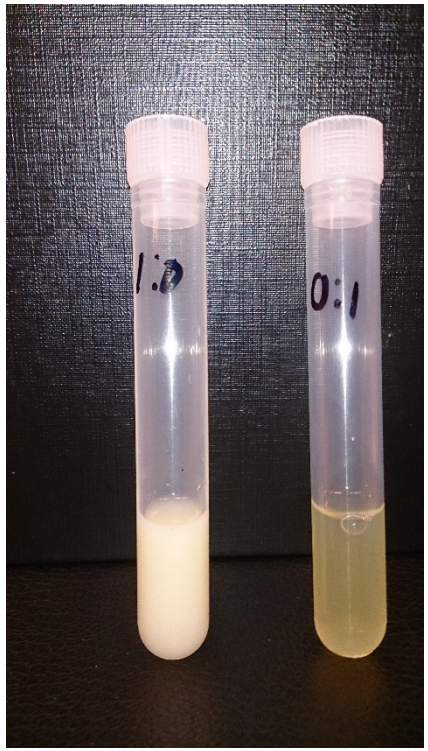
- Bulk material may be inhomogeneous upon arrival:
  - Fatty lumps.
  - Clots / fibers (heparin plasma).
- Centrifugation.
  - Fatty surface layer.
    - Remains in container upon decantation.
  - Clots / fibers as sediment.
- Homogenize at least the fat and bulk matrix.
  - Vigorous shaking (may result in fine fat particles).
  - Dispersing using an ultra turrax at 30 °C.
- Aliquot in 1.5 mL portions and store at -20 °C.

Effect on sampling (pipetting)

centrifugation

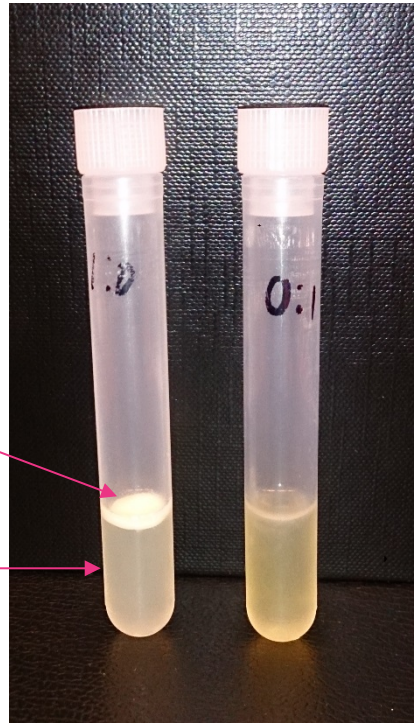
Common procedure prior to sample preparation:

1. *Thaw the samples at room temperature / on ice and homogenize.*
2. *Centrifuge the samples at 2500 x G for 10 min at +4°C.*



Before centrifugation

After centrifugation



Fatty surface layer

Pipetted matrix

Effect on sampling / pipetting

- Fatty surface layer may contain higher content of lipophilic analytes.
- Sampling / pipetting recovery test by PP method.
  - Lipemic and normal plasma mixed in different ratios.
    - Triglyceride concentrations range from 78 to 859 mg/dL.
  - Spiked with test compound mixture.
  - Analysis before and after centrifugation .

➢ Before centrifugation: comparable recoveries for all test compounds in all plasma samples over the whole triglyceride range.

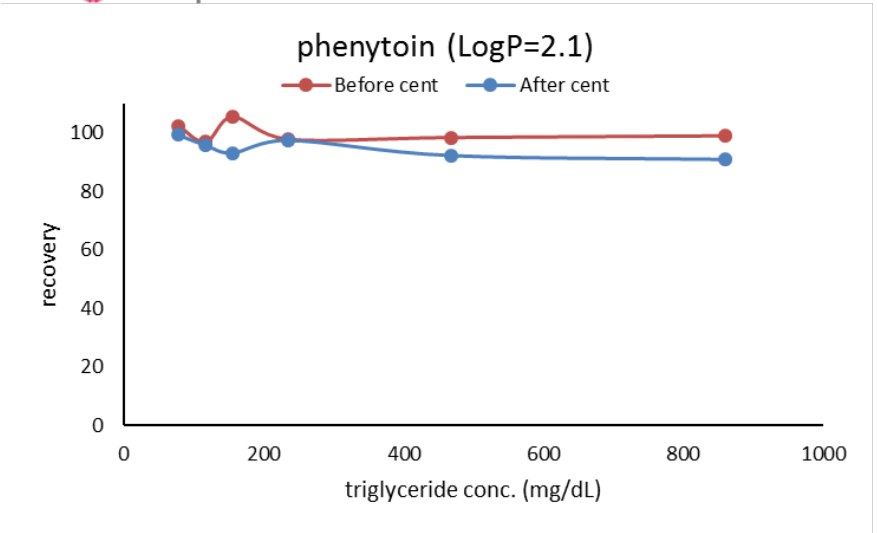
➢ After centrifugation: significant decrease in recovery for lipophyllic compounds in lipemic plasma: up to -50%!

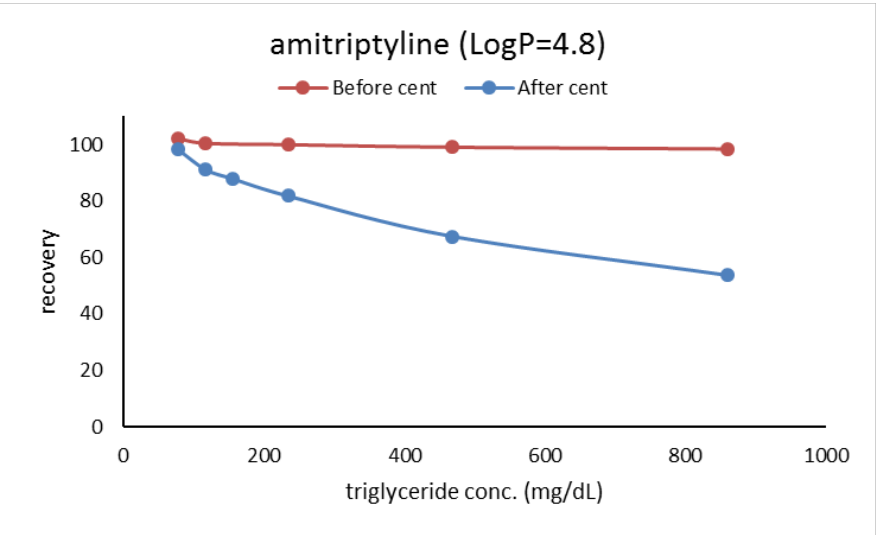
➢ Recovery of hydrophobic compounds inversely proportional with trigliceride concentration.

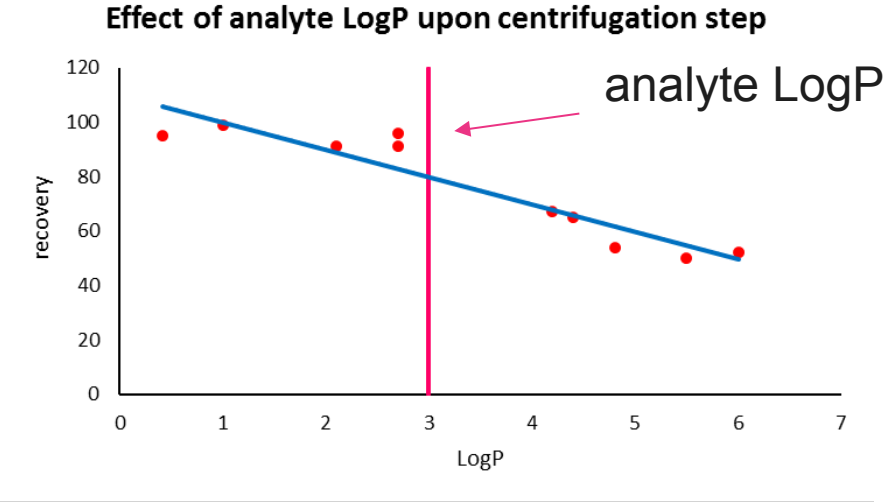
➢ Not corrected by internal standard.

Effect of centrifugation on sampling

pipetting







Recoveries: relative to mean response, before centrifugation

Conclusions

1. To obtain a lipemic matrix with specified lipid content for MD and validation experiments, the blank (bulk) lipemic plasma should be carefully homogenized prior to processing and not centrifuged.
2. No sign for additional ionization suppression in lipemic plasma.
3. No sign for additional peaks in chromatogram in lipemic plasma.
4. Recovery effect on LLE depends on extraction solvent.
5. No recovery effect on SPE.
6. The main effect of lipemic plasma on the accuracy of bioanalytical results is caused by pipetting centrifuged samples.
7. The fatty surface layer does not appear at centrifugation of freshly drawn blood to separate cells from plasma.

Questions