Importance of sample preparation

S2004
Methods for characterization of biomolecular interactions
– classical versus modern

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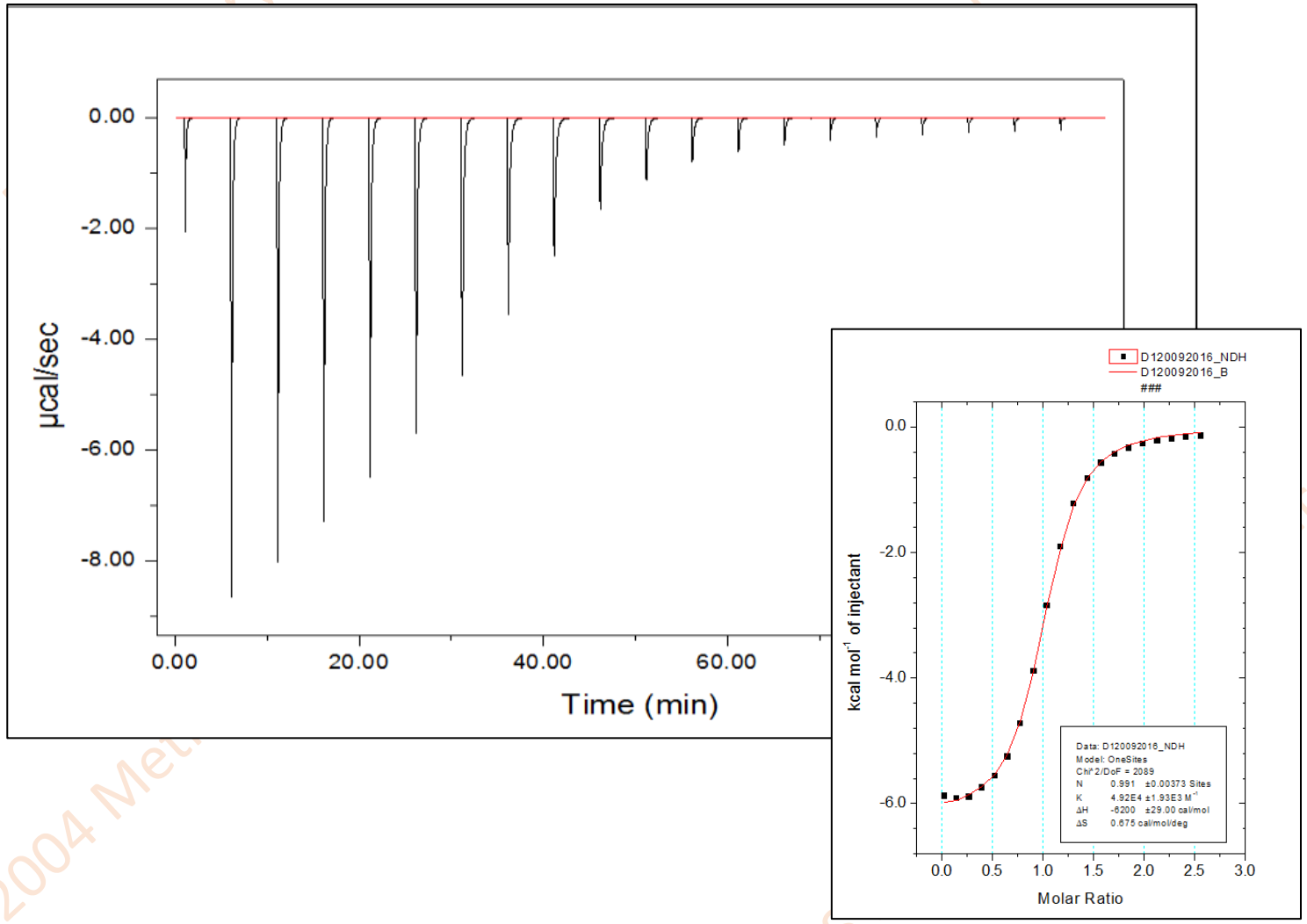
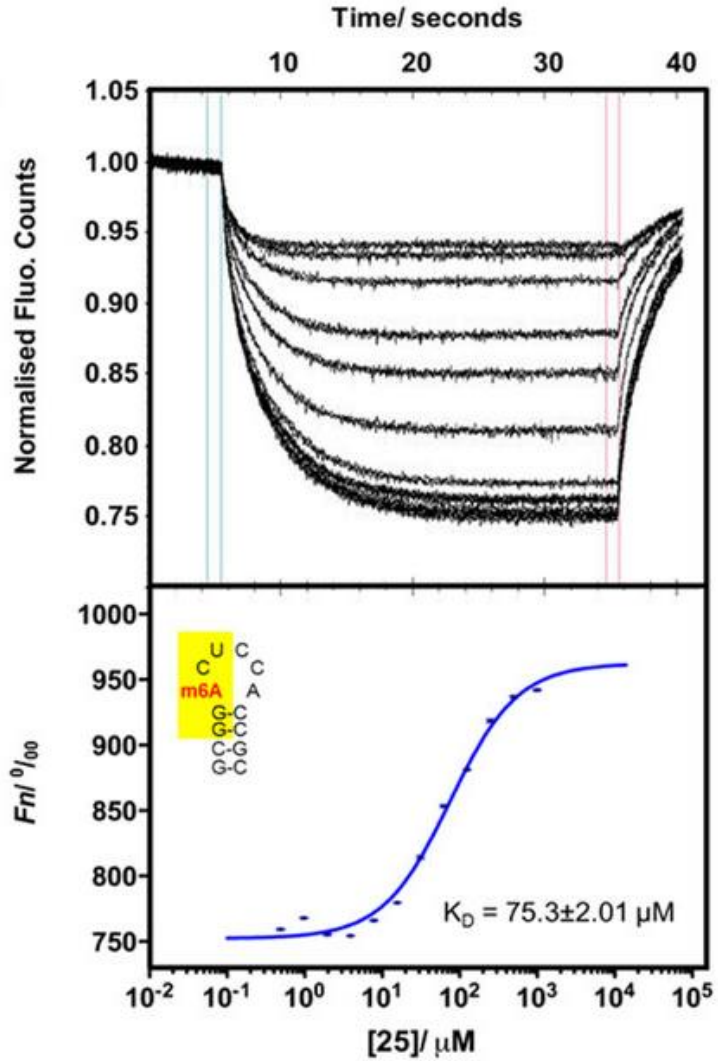
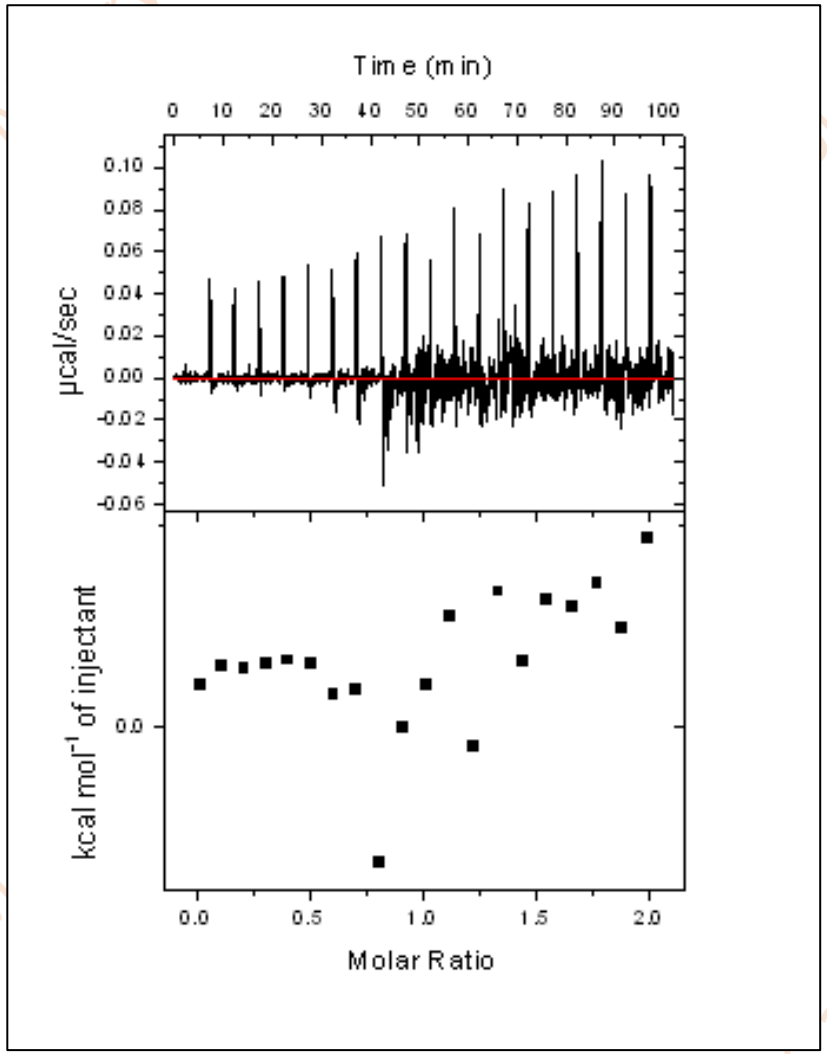
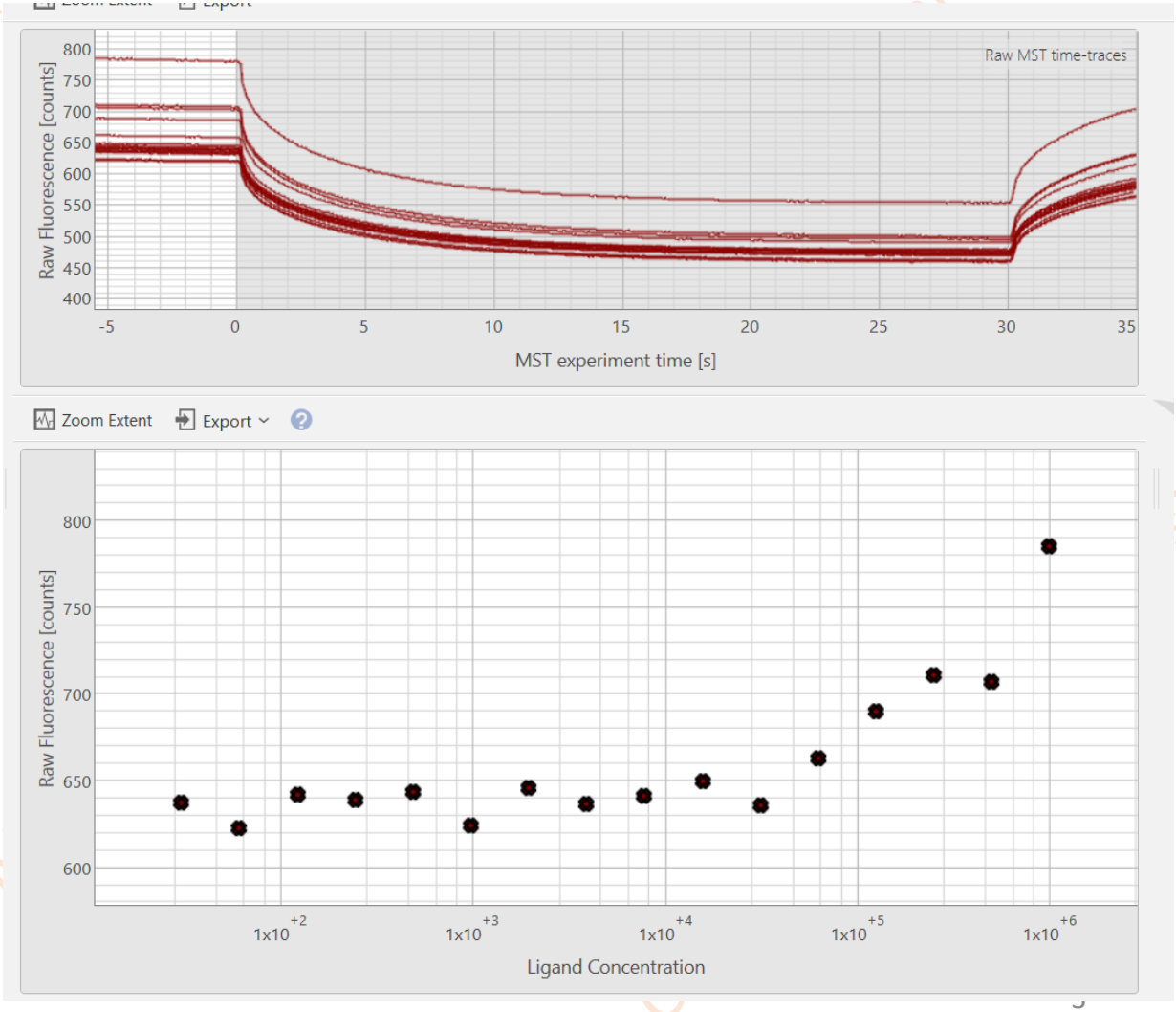
Ideal sample – ideal data





Real data – not always that ideal





Reproducibility crisis

- Based on 2016 poll with > 1500 scientists included:

  70 % were not able to repeat an experiment !

  50 % were not able to repeat at least one of their own experiments !!!

- Possible causes:
  - Selective choice of data (cherry picking)
  - Unsuitable experimental desing
  - Inappropriate data evalueation (statistics)

- It’s probable that partial problem is **insufficient characterization** of input material and procedures.

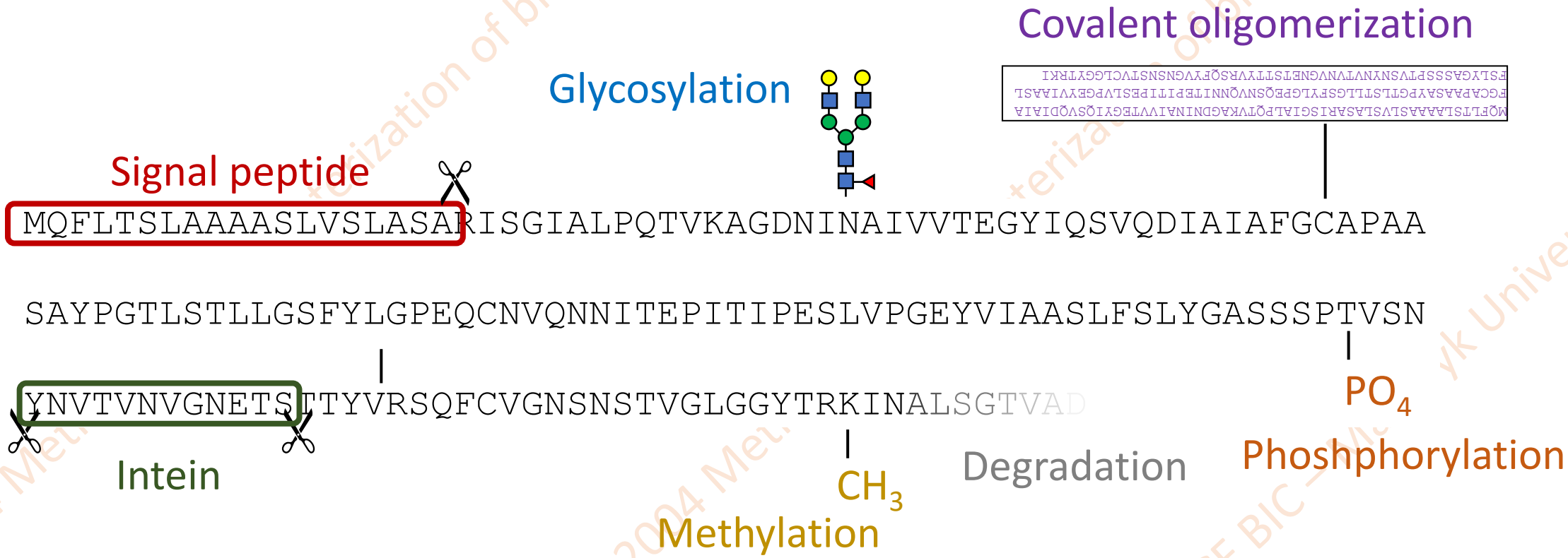
Source: nature.com

Ideal sample properties

- **Defined** (chemically, biologically, conformationally)
- **Pure** (contamination by small molecules, macromolecules)
- **Homogeneous** (micro-/macro- heterogeneity)
- **Stable** (storage, time-demanding analysis)

Sample identity

- Exact composition of sample (sequence, modifications, cleavage)
- Influence on MW, pI, interactions

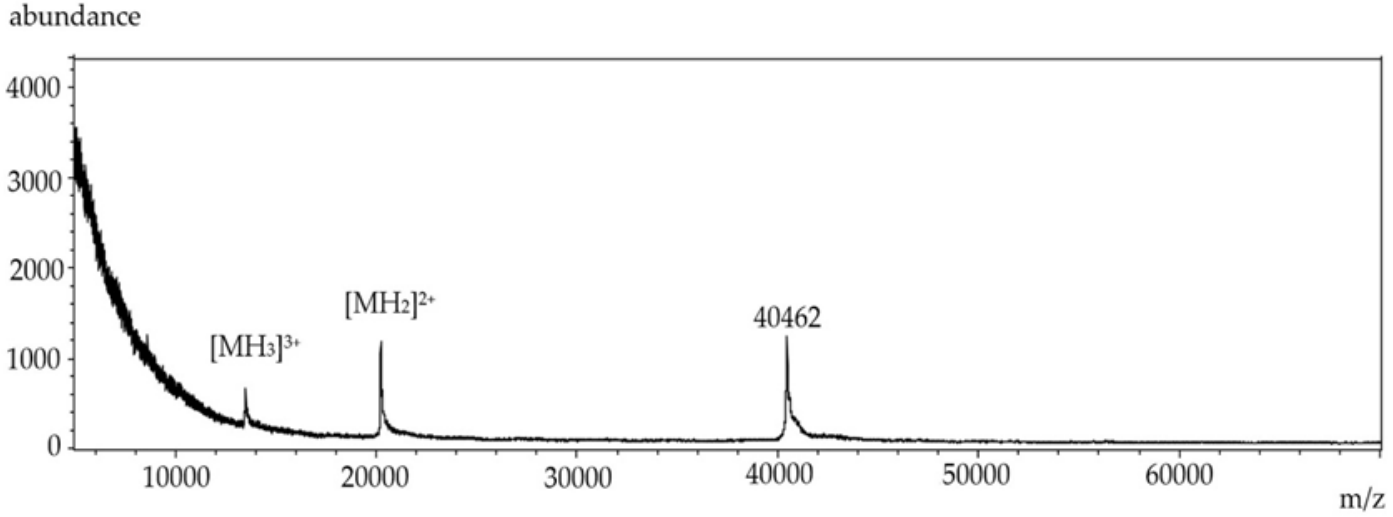


Sample identity

- MS identification

```
  1 MKKESINTSG PDNTKSSISD EIEISNEISW TALSGVISAA NNADGRLEVF
 51 GVGTNNAVWH NWQTVPNTGS SWSGWHSLNE GATSKPAVHI NSDGRLEVFV
101 RGTDNALWHN WQTVPGAGWS GWQSLGGQIT SNPVVYINSD GRLEVFARGA
151 DNALWHIWQT APHAGPWSNW QSLNGVLTSD PTVYVNASGR PEVFARSNDY
201 SLWYIKQTAS HTYPWTNWQS LSGVITSNPV VISNSDGRLE VFARGSDNAL
251 WHIWQVAPNA GWTNWRSLSG IITSDPAVHI NADGRLEVFA RGPDNALWHI
301 WQTATSDAWS EWTSLSGVIT SAPTVAKNSD GWLEVFARGA NNALCHIQQT
351 TSSWSTWTSL GGNLIDASAI K
```

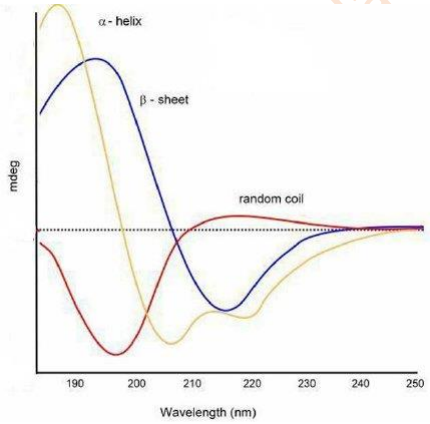
- MS intact mass analysis



Post-translational modifications
Isotope labeling
Matrix adducts

Folding – direct evidence of 2D structure

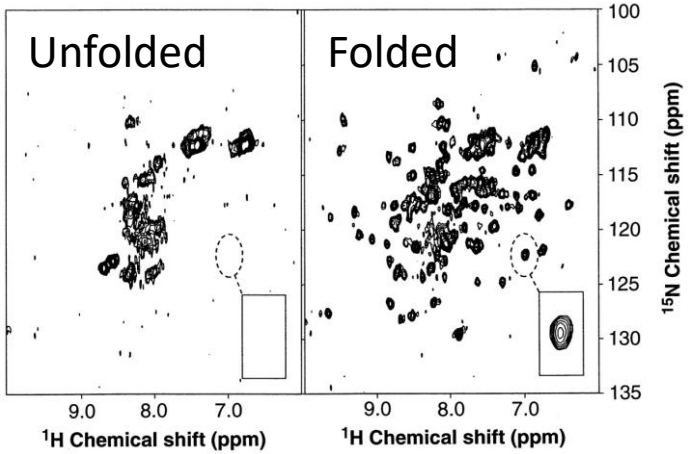
- **Circular dichroism** (CD)
  - Difference in absorption of left and right circularly polarized light by chiral compounds
  - Specific shape of spectra for 2D structural elements



Dodero 2011

- **Infrared spectroscopy** (FTIR)
  - Specific absorption bands for 2D elements

- **Nuclear magnetic resonance** (NMR)
  - Behavior of atom nuclei in magnetic field
  - Presence of defined structure results in distinguished peaks in spectrum



Balbach 1996

Sample purity

Contaminants – co-purified molecules

- Small molecules
  - Co-factors
  - Ligands
  - Salts, imidazole
  - Lipids
  - Saccharides
- Macromolecules
  - Protein isoforms
  - Proteins
  - Nucleic acids
  - Polysaccharides
  - Binding partners

Sample purity – methods

- **SDS-PAGE**
- UV-VIS spectroscopy
- SEC (SEC-MALS)
- FFF (FFF-MALS)
- Mass spectrometry

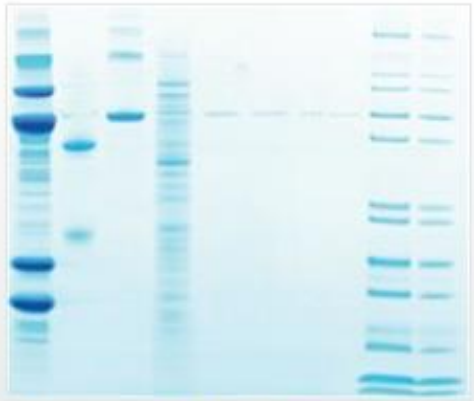
small molecules
- Co-factors
- Ligands
- Salts, imidazole
- Lipids
- Saccharides

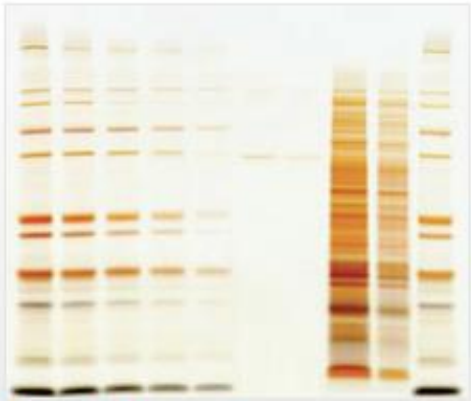
macromolecules
- Protein isoforms
- Proteins
- Nucleic acids
- Polysaccharides
- Binding partners

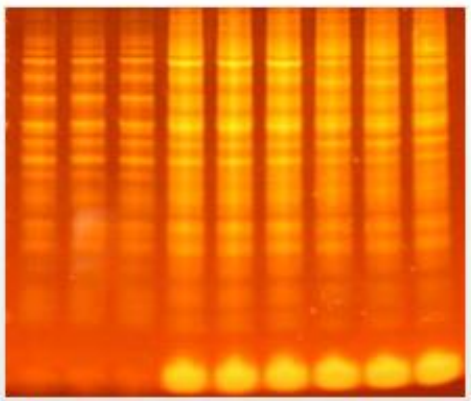
SDS-PAGE

- **Polyacrylamide** gel (8 – 20 %)
- **SDS** – uniform (?) protein charge (composition dependent)
- **Reducing agent** (optional) – βME
- **Staining** – CBB, Silver, Fluorescent, Radiological

	Coomassie staining	Silver staining	Fluorescent protein staining
Sensitivity	5-25 ng	0.25-0.5 ng	0.25-0.5 ng







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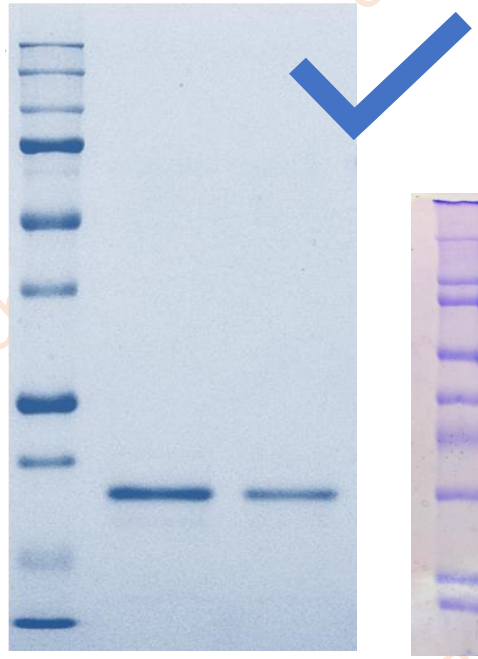
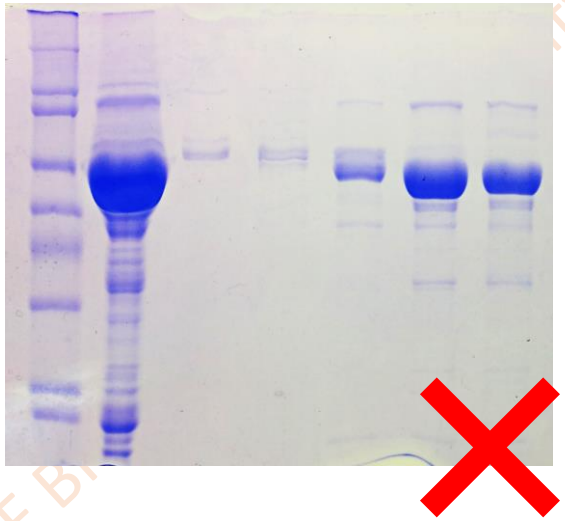
SDS-PAGE

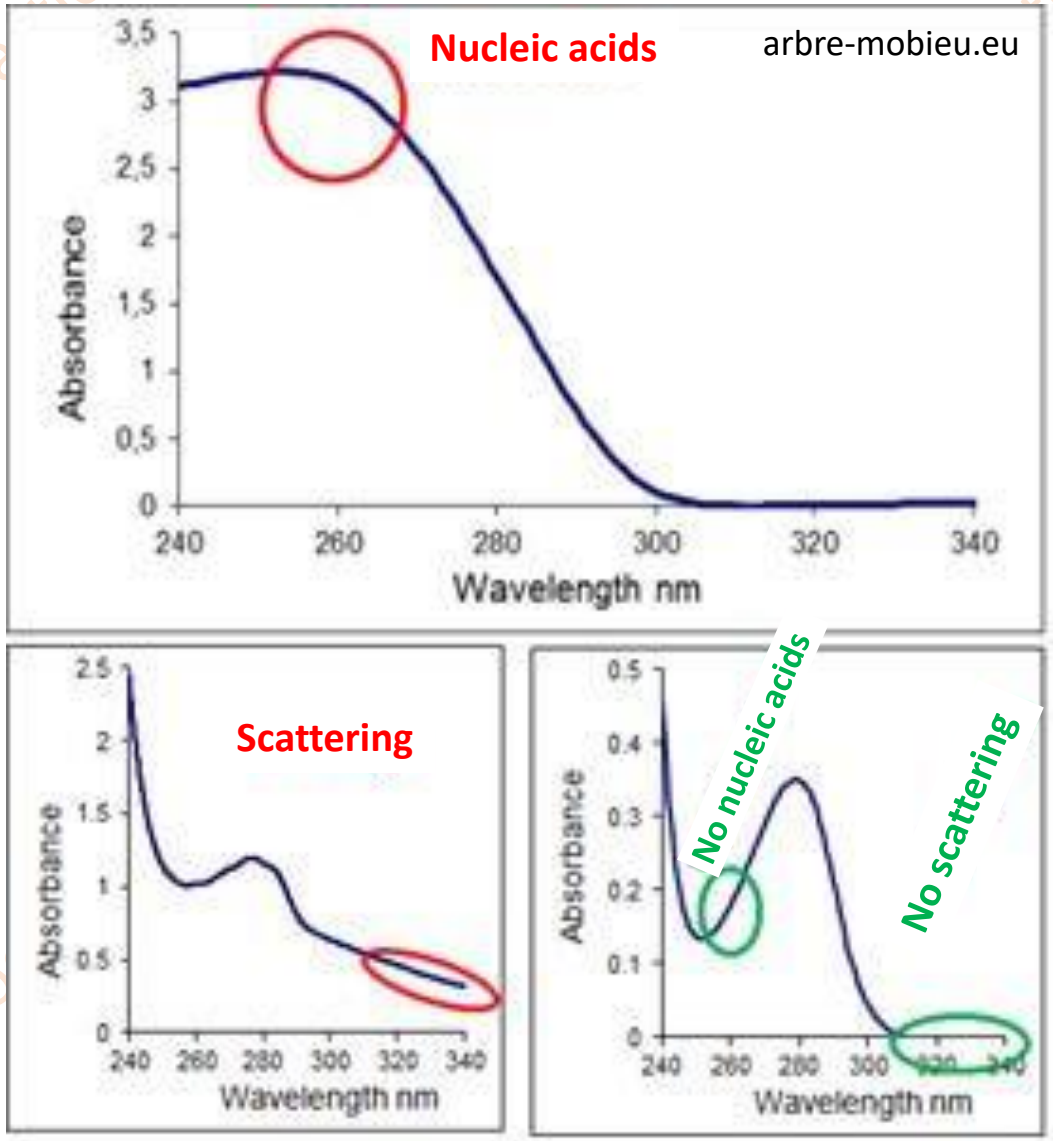
- Check overloaded as well as underloaded sample





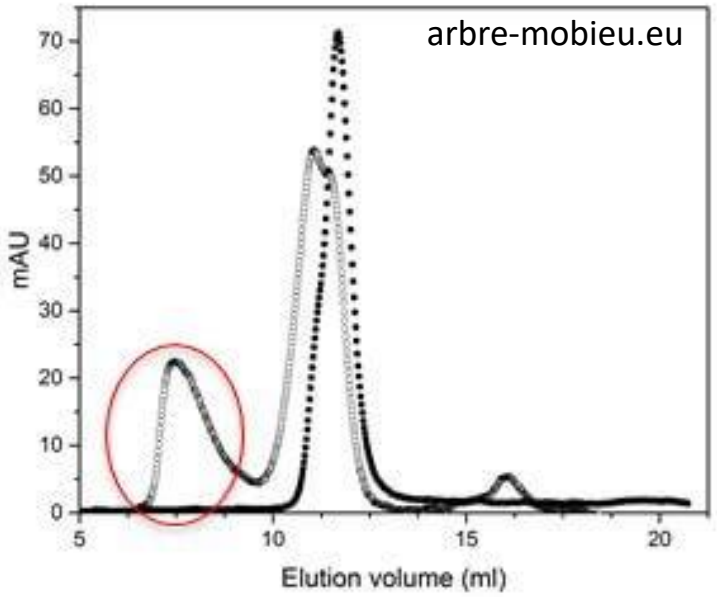
UV-VIS spectroscopy

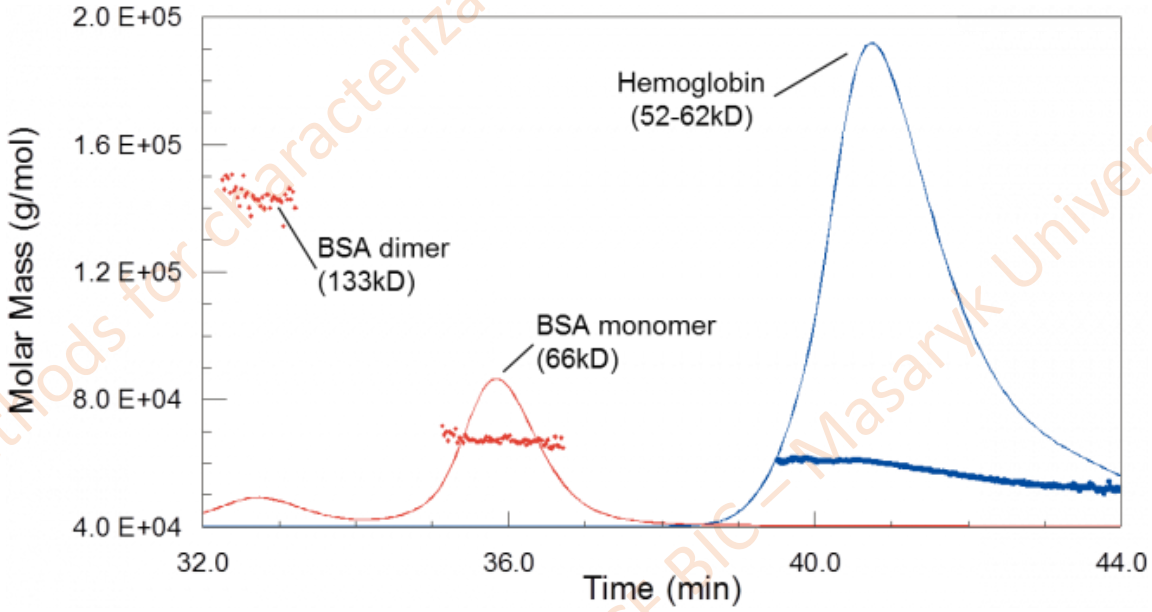
- (200-) 240 – 340 nm
- Trp (and Tyr) has absorption peak around 280 nm
- Detection of:
  - **Nucleic acid** contamination
  - **Aggregation** (scattering)
  - **UV-absorbing contaminants**



Size exclusion chromatography

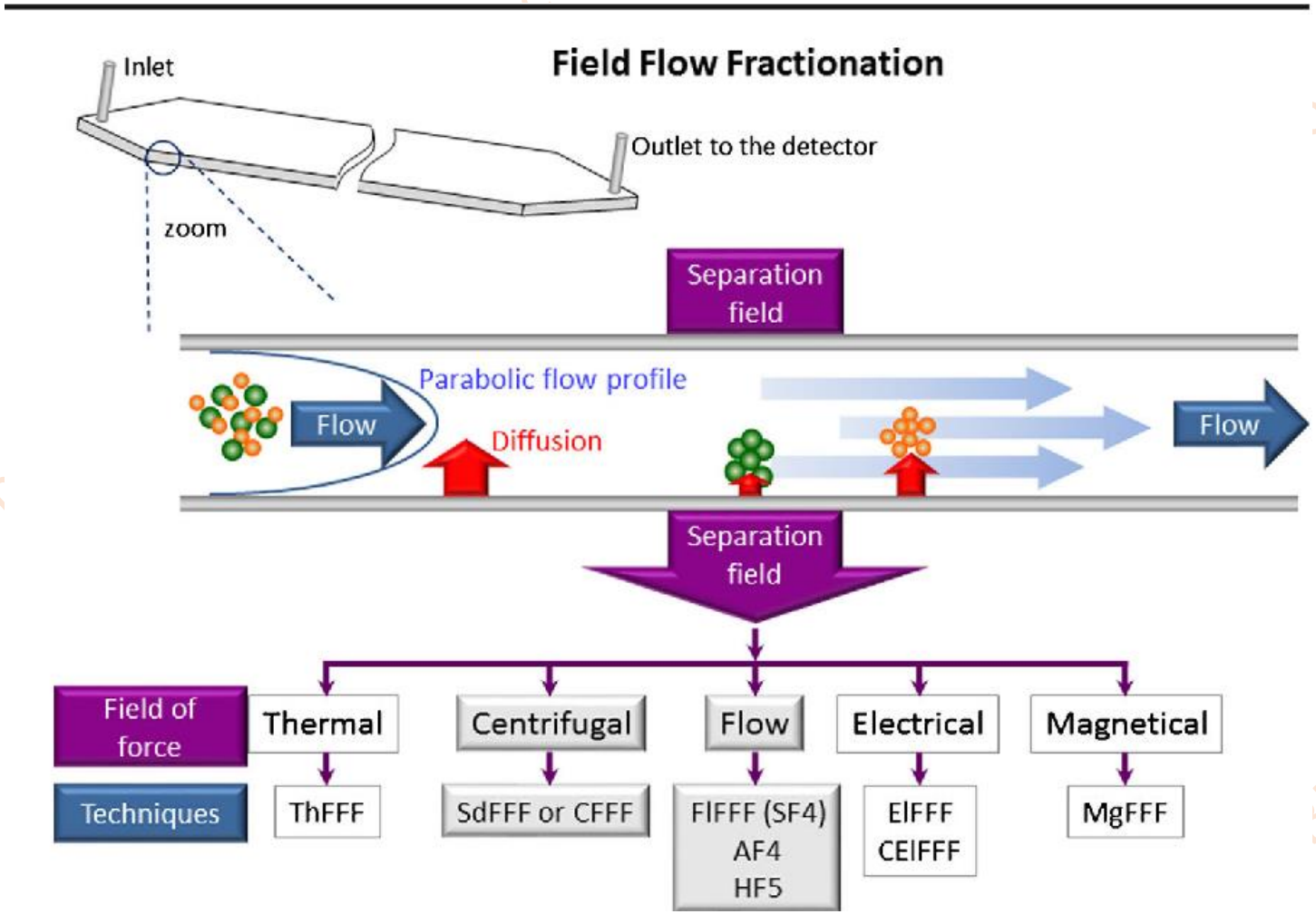
- Separation of particles based on “size”
- Interaction with matrix possible (!)
- Usually coupled to multiple detectors
  (UV, MALS, viscosity)





Field flow fractionation

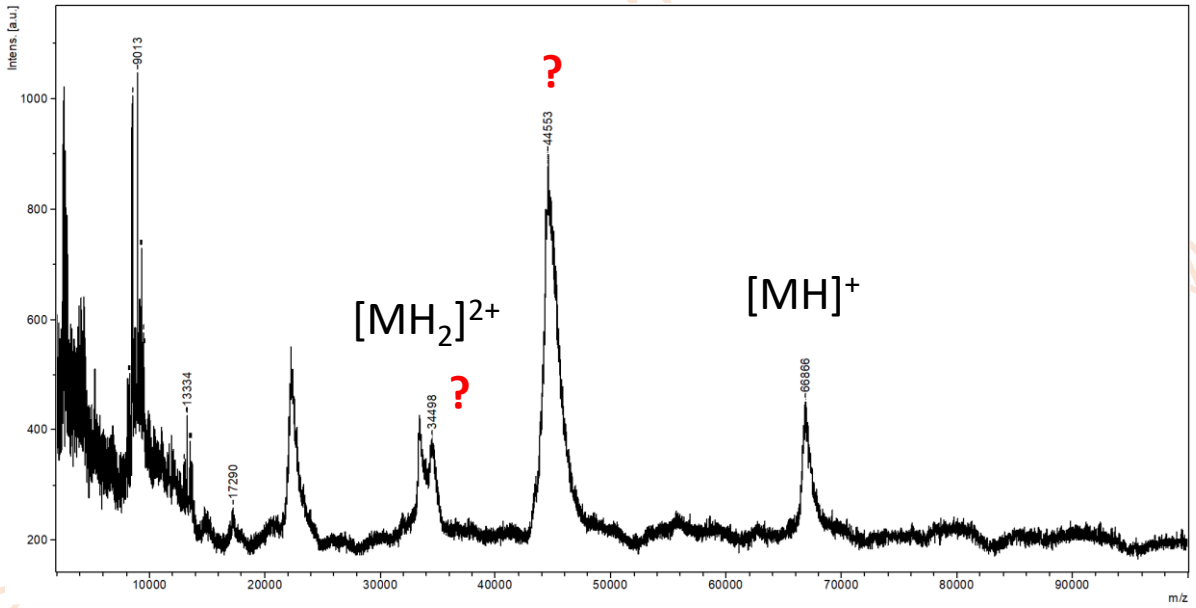
- Separation of particles in solution by external force



Mass spectrometry

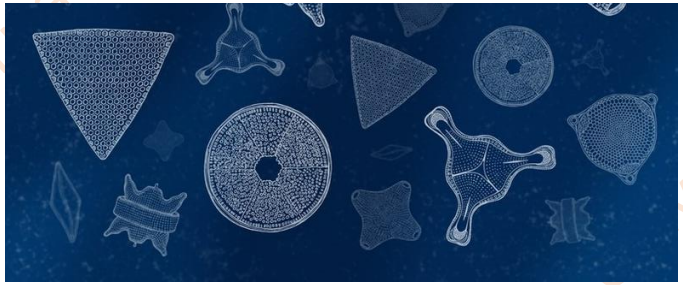
- Detecting of exact mass of particles
- Various applications based on set-up

- **Intact mass analysis** – protein and non-protein contaminants



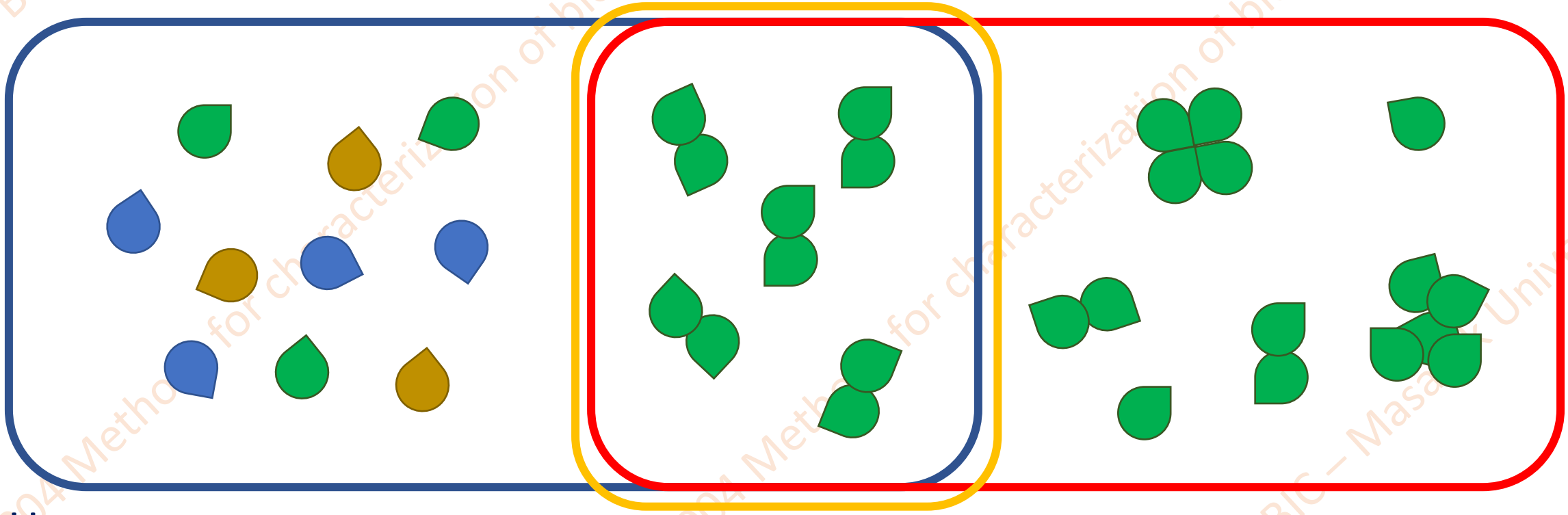
Sample homogeneity

- **Macroscopic** – precipitation – visual detection
- **Microscopic** – oligomeric states, folding states, microheterogeneity – biophysical methods



Sample homogeneity vs. purity

- Various methods may evaluate sample in different way

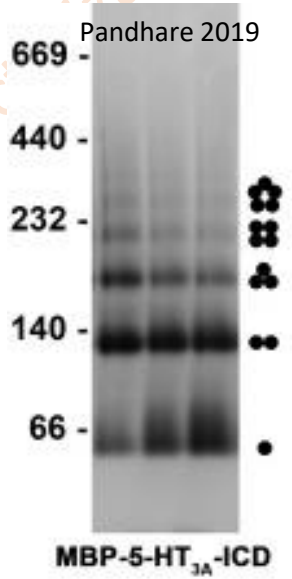


Sample homogeneity – methods

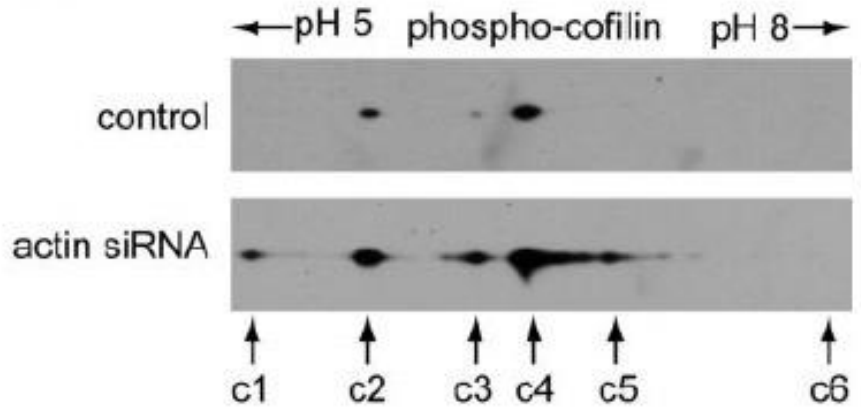
- **SEC-MALS, FFF**
- **Native electrophoresis**
- **Light scattering**
- **Analytical ultracentrifuge**

Native electrophoresis

- Possibility to observe various **oligomers** (relatively imprecise and unreliable) and **isoforms** (2D PAGE preferred)
- Not efficient for **aggregation** detection

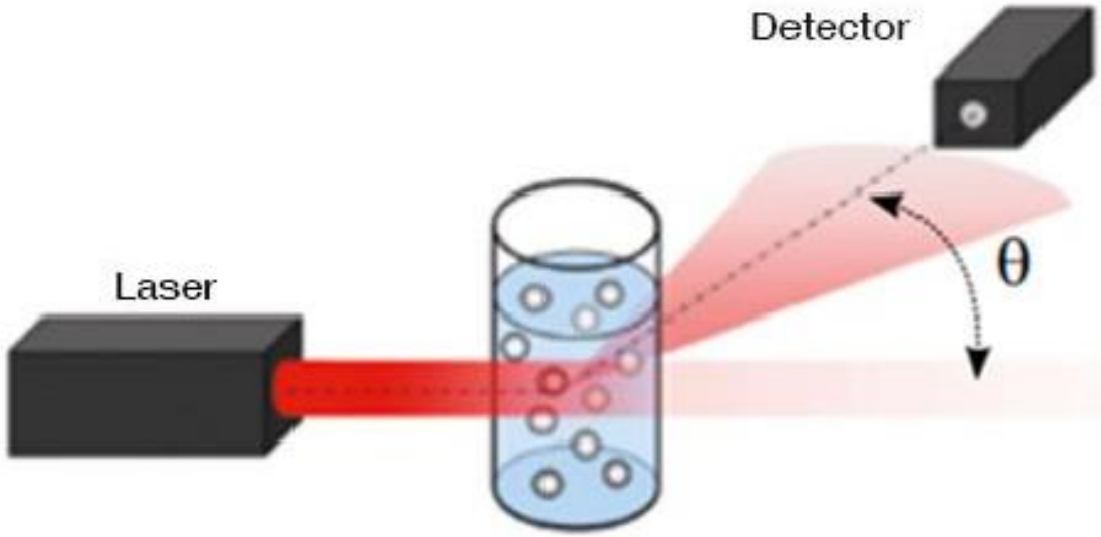


Pandhare 2019



Light scattering

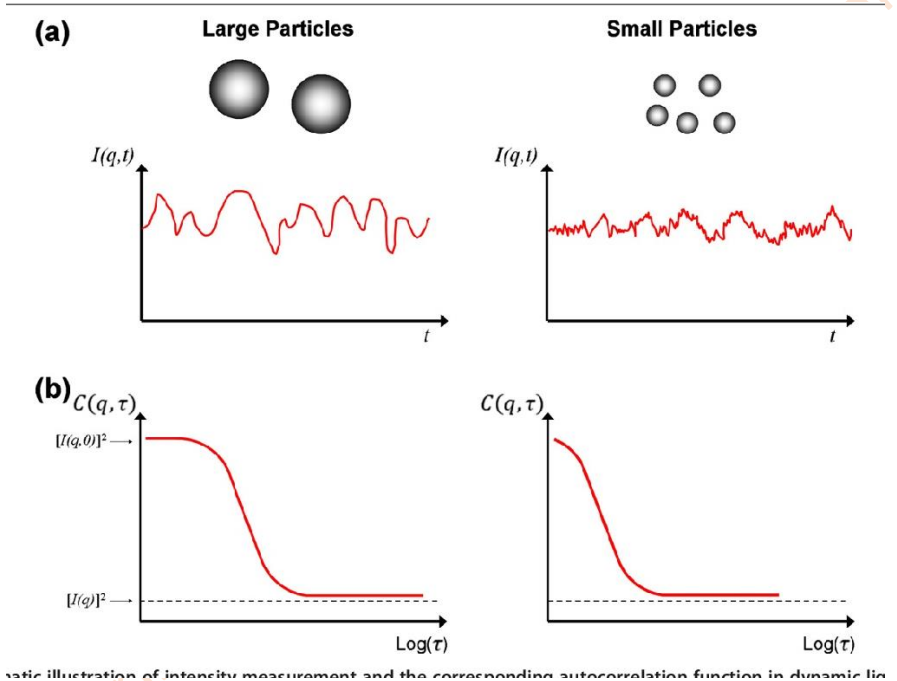
- Interaction of incident light with particles in solution
- Intensity of light at given
- Typically red/infrared light

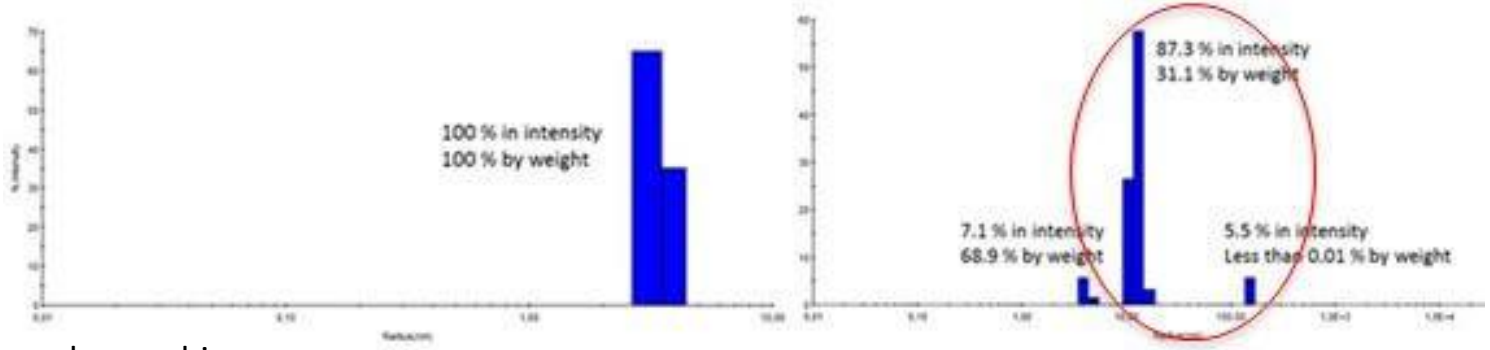


Light scattering

- **Dynamic light scattering**
  – size of particles
  – sensitive to **aggregation**

- **Static light scattering**
  – mass of particles
  – averaged value, separation required



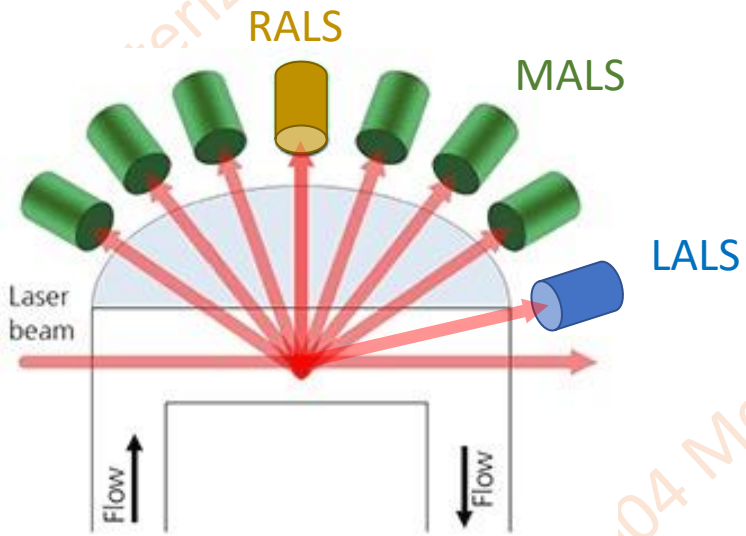


arbre-mobieu.eu

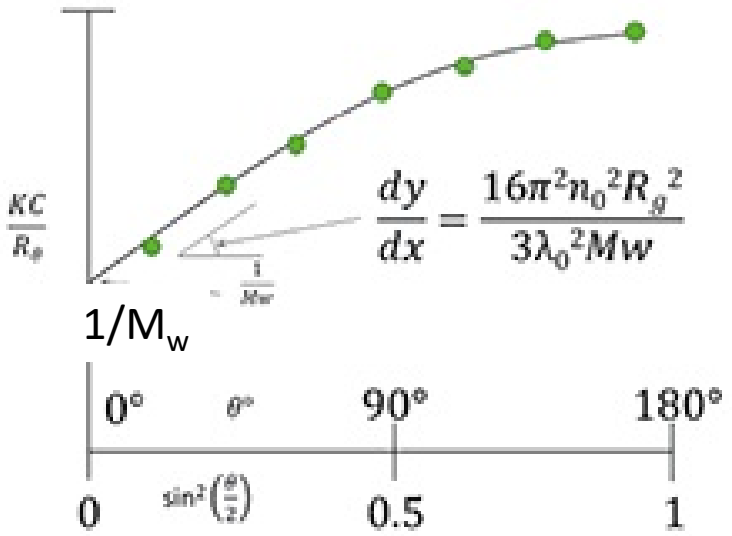
Static light scattering (SLS)

Low-angle light scattering (LALS) – big molecules
Right-angle light scattering (RALS) – small molecules
Multi-angle light scattering (MALS) – M_w and R_g

- Intensity of scattered light
- **Mass** of the particle (molecular weight)

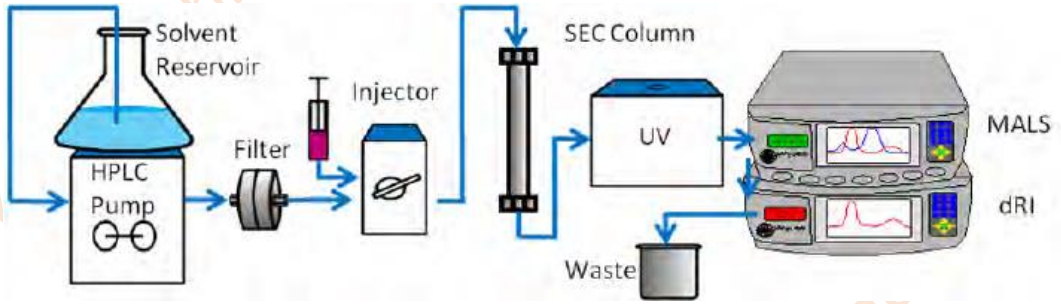


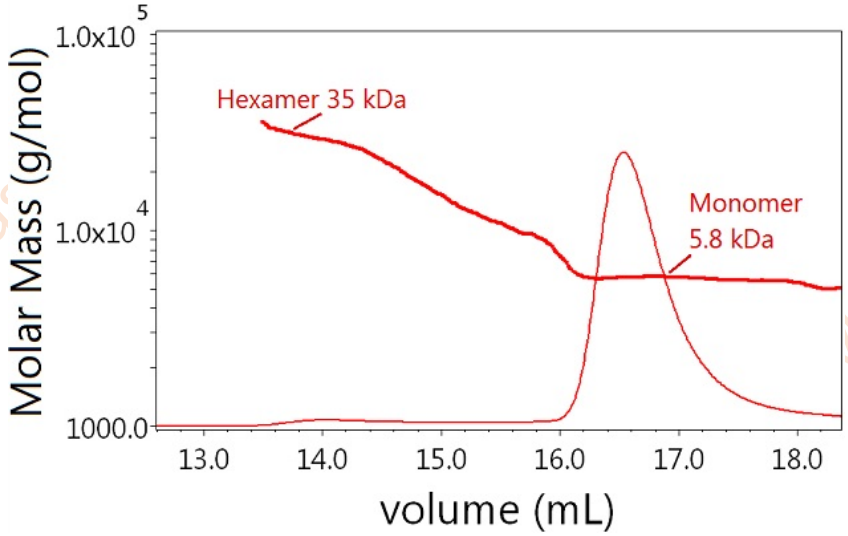
www.malvernpanalytical.com

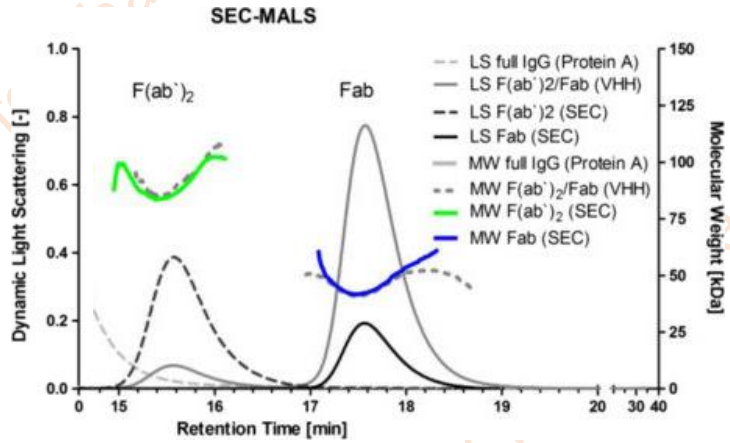


Static light scattering

- Average of all sample particles !
- Typically coupled to separation (SEC, FFF)

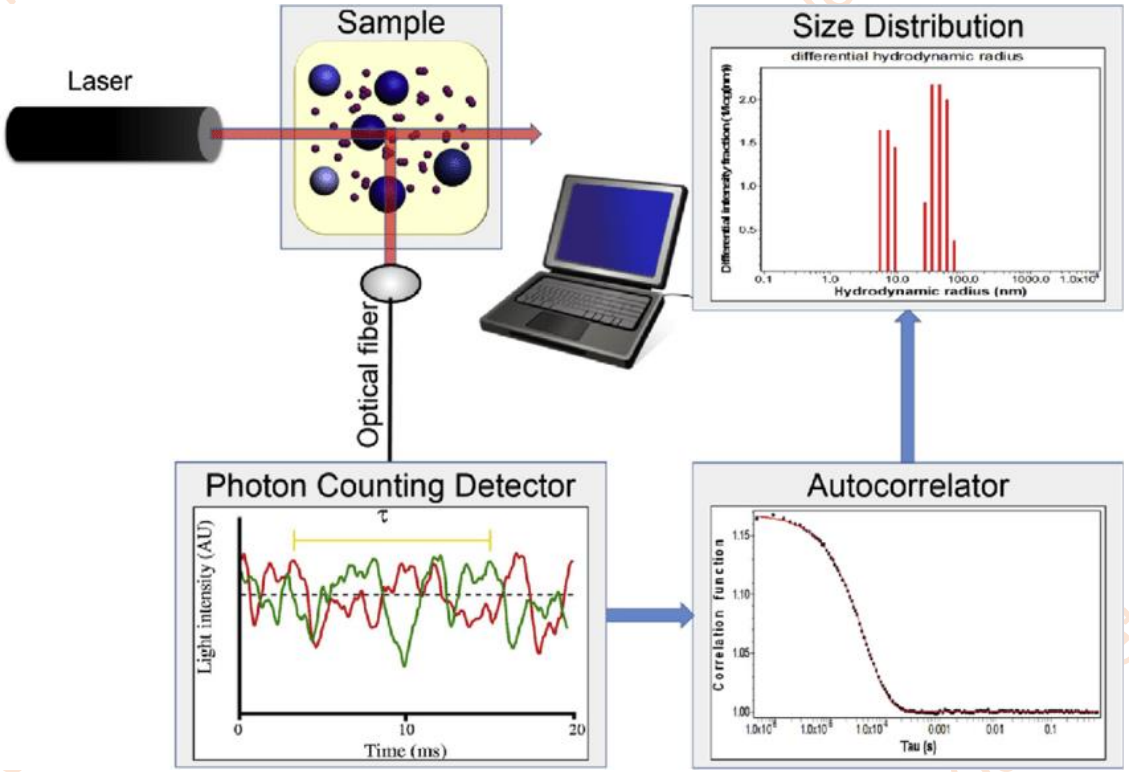


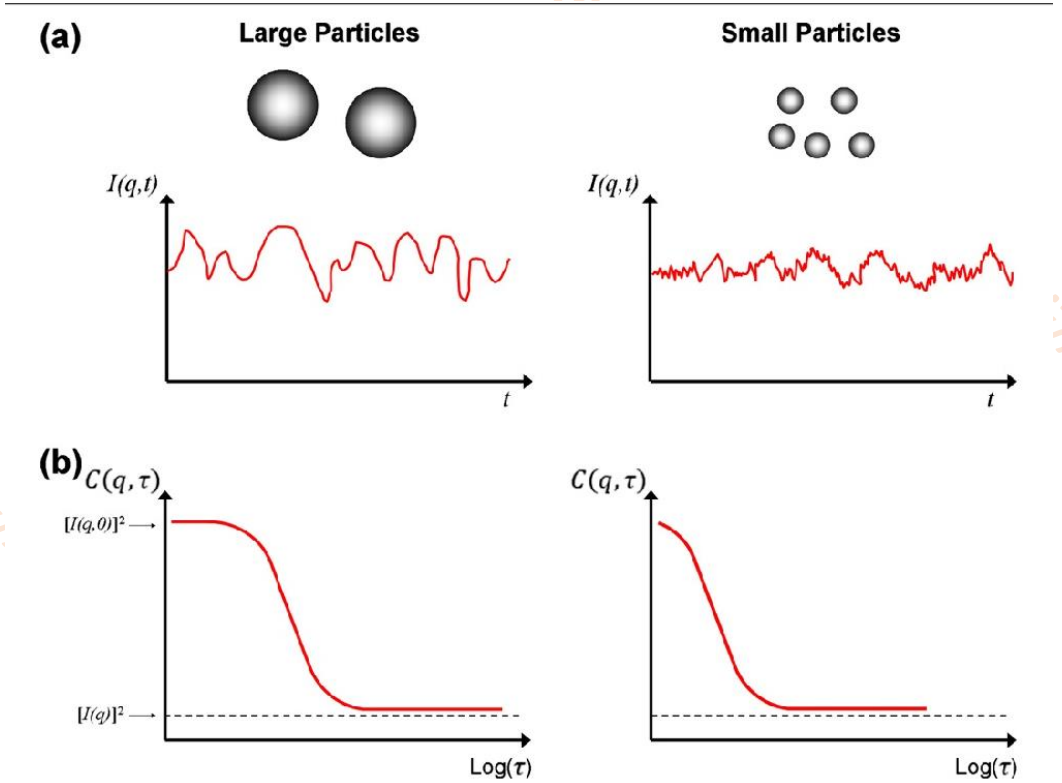




Dynamic light scattering (DLS)

- Time-dependent fluctuations in scattered light
- **Size** of the particle (hydrodynamic radius)

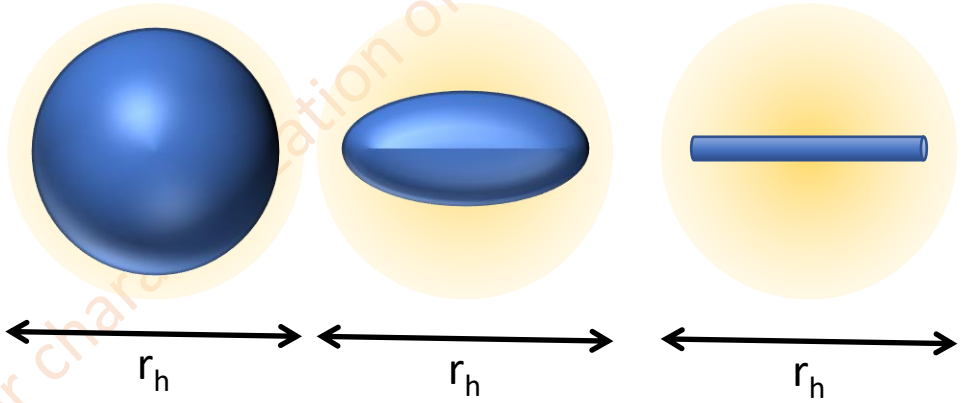


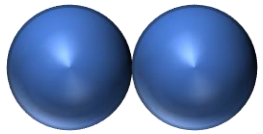


Allen P Minton,
DOI: 10.1016/j.ab.2016.02.007

Dynamic light scattering (DLS)

- Shape dependent
- Low resolution





r_h(dimer) ~ 2 × r_h(monomer)

r_h(dimer) ~ r_h(monomer)

For ideal sphere:

$$M \sim V = 4/3\ \pi\ r^3$$

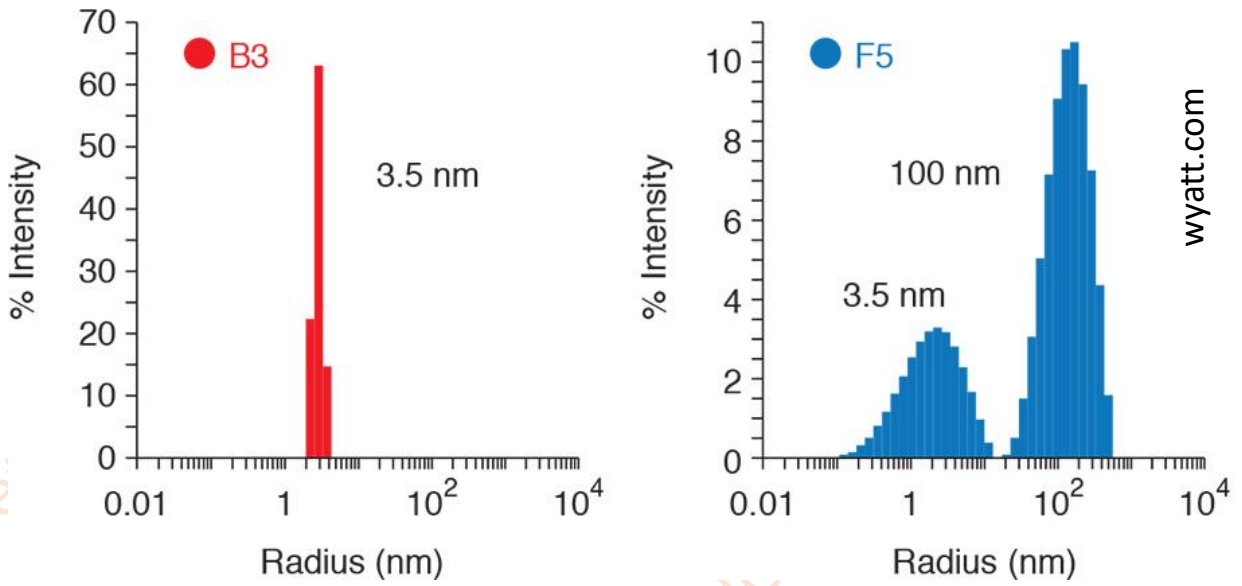
$$M_2 = 2 \times M_1$$

$$r_2 = \sqrt[3]{2} \times r_1 = 1.26\ r_1$$

Dynamic light scattering (DLS)

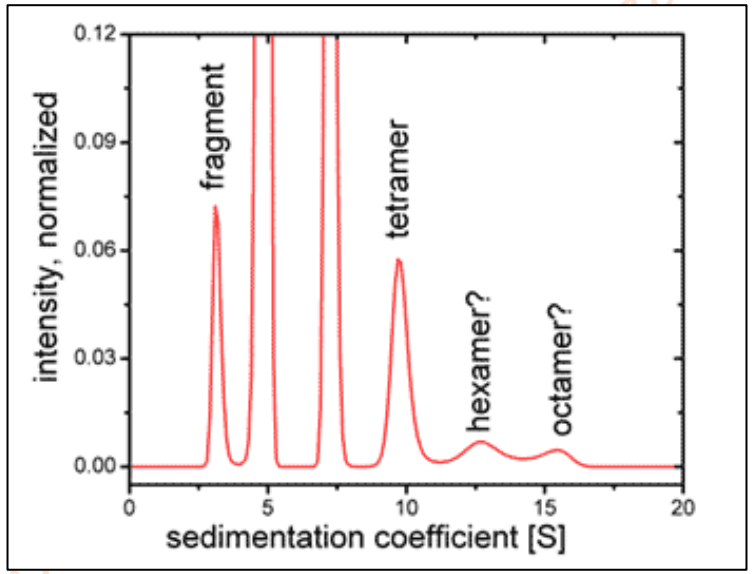
– Microheterogeneity reflects in **polydispersity** – peak width

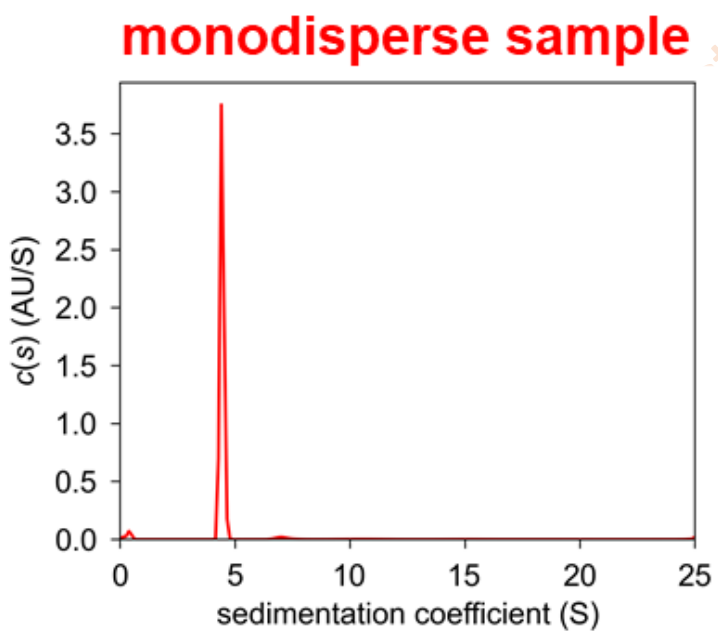
– Large particles scatter light with much higher intensity – sensitive to **aggregation**

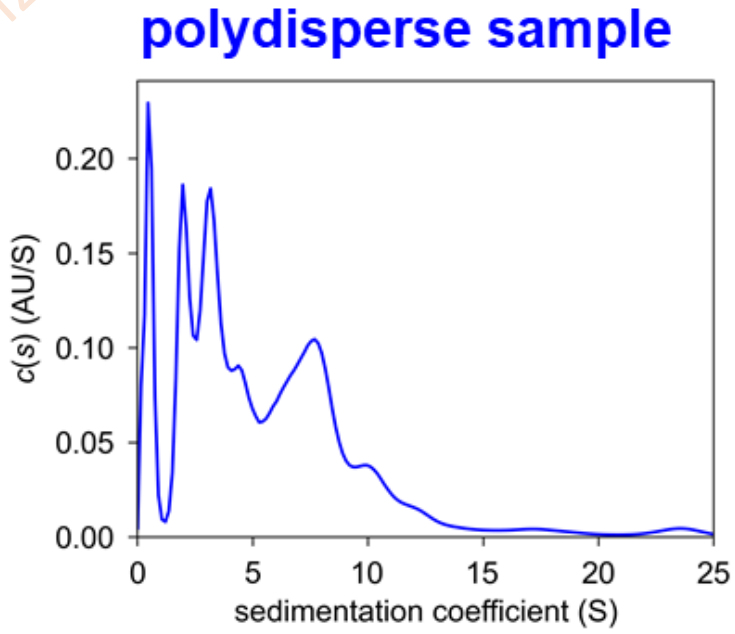


Analytical ultracentrifugation (AUC)

- Sedimentation of particles in centrifugal field by **hydrodynamic properties**
- Two modes:
  - Sedimentation equilibrium – mass determination
  - Sedimentation velocity – size distribution





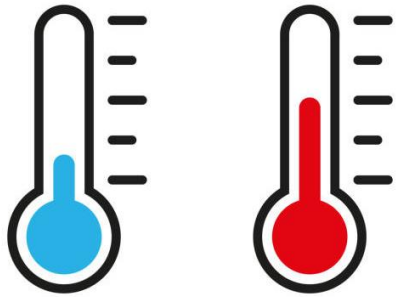


Comparison

	Light scattering	Analytical ultracentrifugation
Sample volume	0.5-30 ul (DLS) 1-50 ul (SLS, SEC-MALS)	150 – 450 ul
Sample concentration	0.1 – 200 mg/ml	0.1 – 1 mg/ml
Particle size	1 nm – 10 µm	1 – 300 nm
Resolution and accuracy	Low – Average	Average – High
Speed of analysis	1 min (DLS, SLS) 30 mins (SEC-MALS)	4 hrs (SV) 3-4 days (SE)

Sample stability

- **Temperature** stability



- **Chemical** stability
  - pH
  - Ionic strength
  - Oxidizing agents
  - Protein-specific compounds

- Long-term stability – **storage**

Temperature

- Affects stability and interaction parameters

$$\ln K_A = -\frac{\Delta G_0}{R\,T} \qquad k = A\,e^{\frac{-E_a}{R\,T}}$$ Arrhenius equation

- Typical temperatures:

  –80 °C, –20 °C, 4 °C, 20 °C, 25 °C, 37 °C

- **Room temperature** (RT) – vaguely defined

  mostly 20 – 25 °C, but varies from 15 – 30 °C

  usually means that temperature was not set (!)

pH

$$pH = -\log [H_3O^+]$$

Typical range: 4 – 9, specific proteins 1 – 12

pH of **pure water**: 7 (theor.), 5.8 (due CO_2 absorption)

Buffers: dissociable compounds with defined pK_a
various pH ranges – typically (pK_a–1) – (pK_a+1)

pH – buffers

- Organic/Inorganic
- Universal buffers – mixtures with broad pH range

Good's Buffer	pKa (20 °C)	pH
MES	6.15	5.5-7.0
Bis-Tris	6.46	5.7-7.3
ADA	6.60	5.8-7.4
PIPES	6.80	6.1-7.5
ACES	6.90	6.0-7.5
MOPSO	6.95	6.2-7.4
BES	7.15	6.6-8.0
MOPS	7.20	6.5-7.9
TES	7.50	6.8-8.2
HEPES	7.55	6.8-8.2
TAPSO	7.70	7.0-8.2
POPSO	7.85	7.2-8.5
HEPPSO	7.90	7.4-8.6
EPPS	8.00	7.5-8.5
Tricine	8.15	7.8-8.8
Bicine	8.35	7.7-9.1
TAPS	8.40	7.7-9.1
CHES	9.50	8.6-10.0
CAPS	10.40	9.7-11.1

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http://www.aimspress.com/

Letter

Universal buffers for use in biochemistry and biophysical experiments

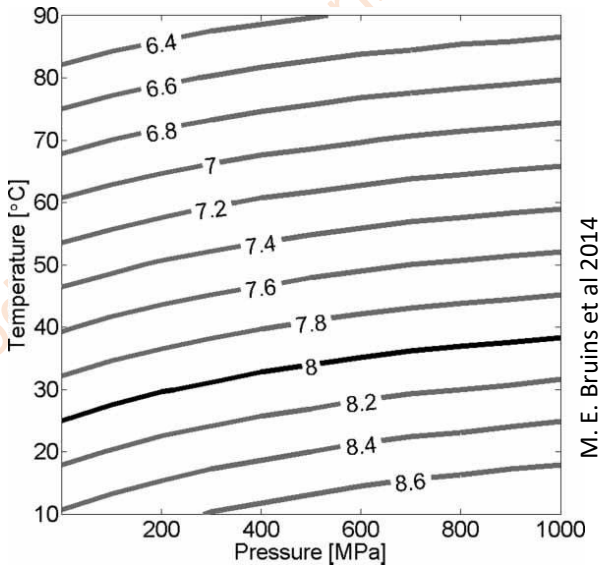
Dewey Brooke [S], Navid Movahed [S], and Brian Bothner *

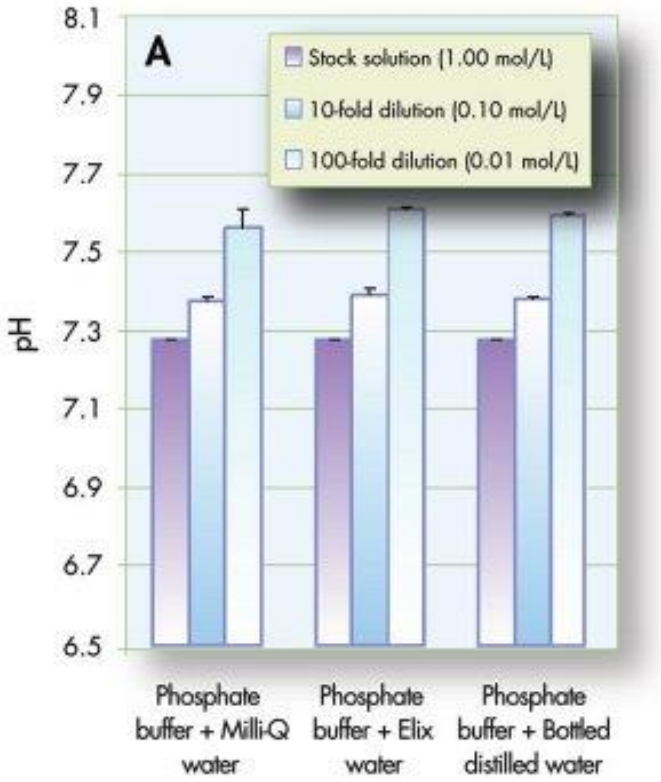
Department of Chemistry and Biochemistry, Montana State University, Bozeman MT 59717, USA

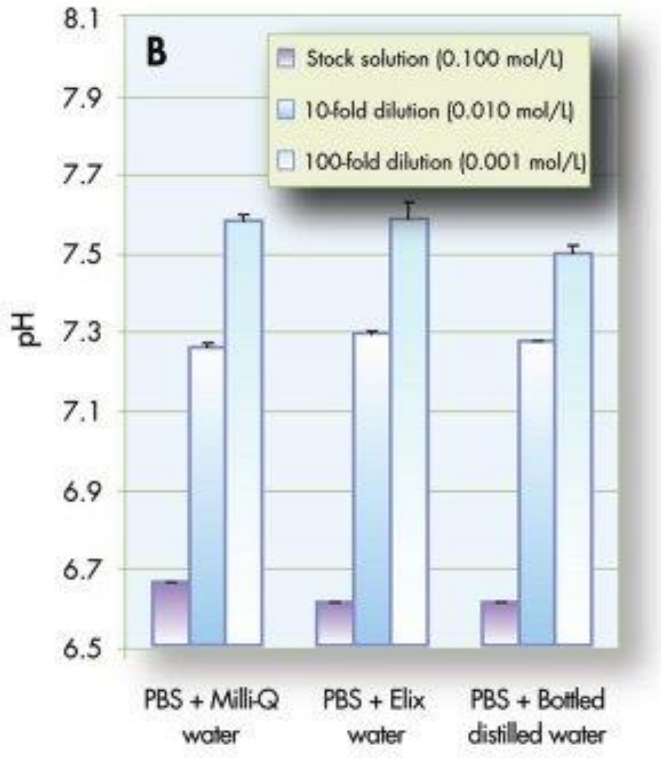
pH

- is **temperature** dependent
- changes with **dilution**
- changes in **time**

Tris buffer pH set to 8.0 at 25°C



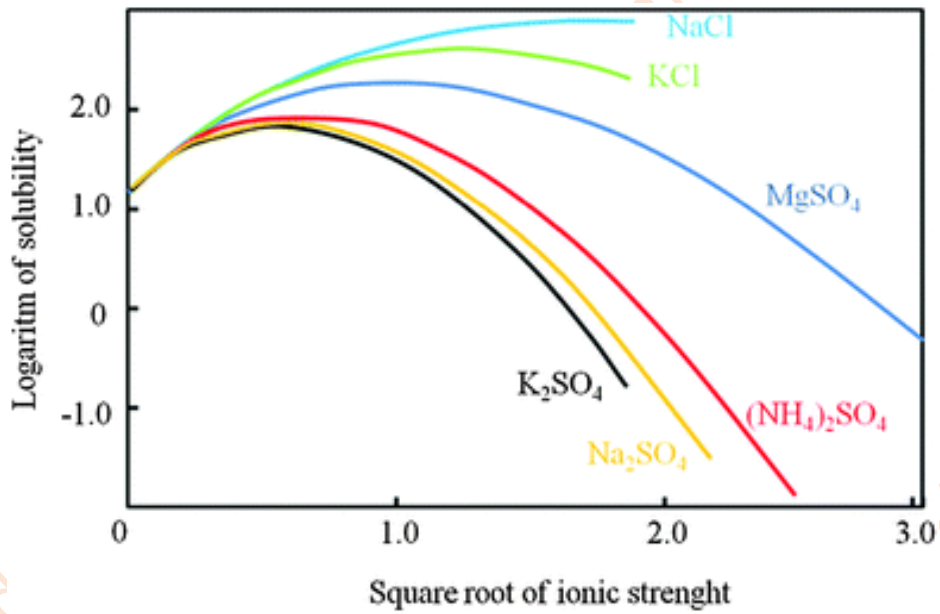




Ionic strength

Ionic strength, *I*, is a measure of the concentration of electrically charged species in solution

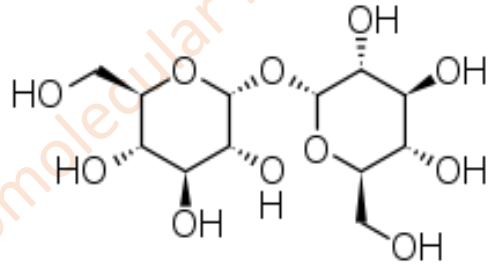
$$I = \frac{1}{2} \sum_i c_i Z_i^2$$



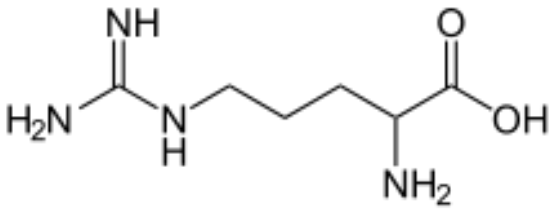
- Protein solubility changes with **ionic strength** as well as with **solute composition**

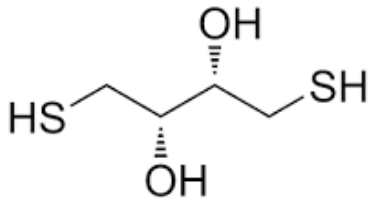
Impurities/Additives

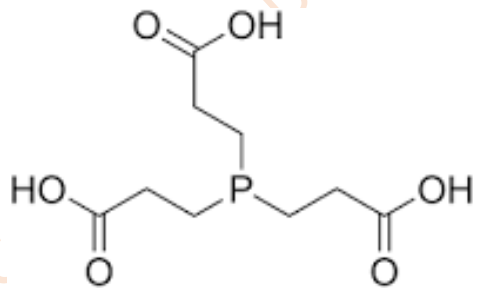
- Various compounds affect protein stability/solubility

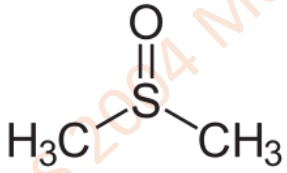


- **Saccharides** – saccharose, trehalose
- **Amino acids** – Arg, Glu, Pro
- **Reducing/oxidizing** agents – βME, DTT, TCEP
- **DMSO**
- Protein-specific compounds (ligands)

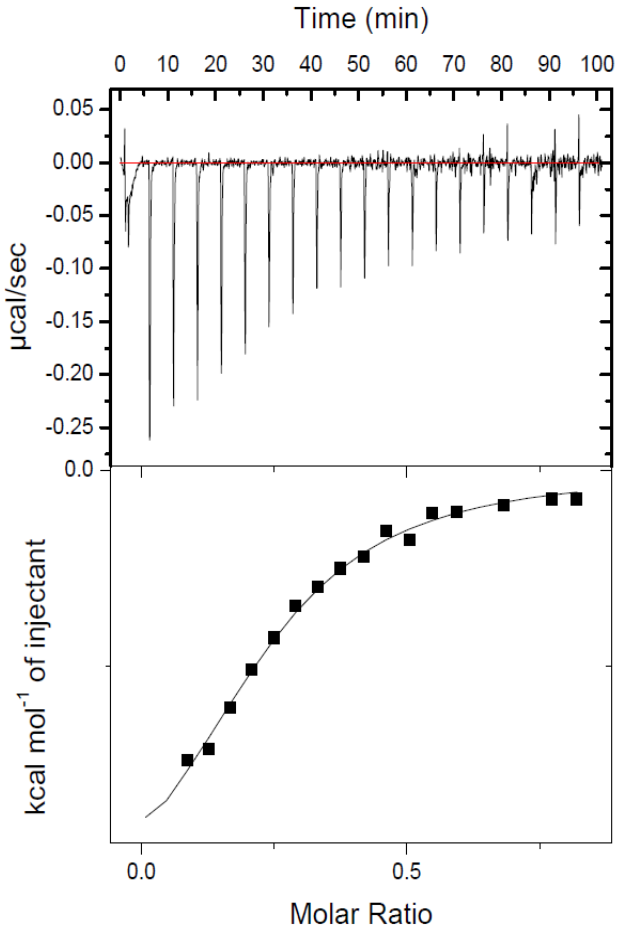


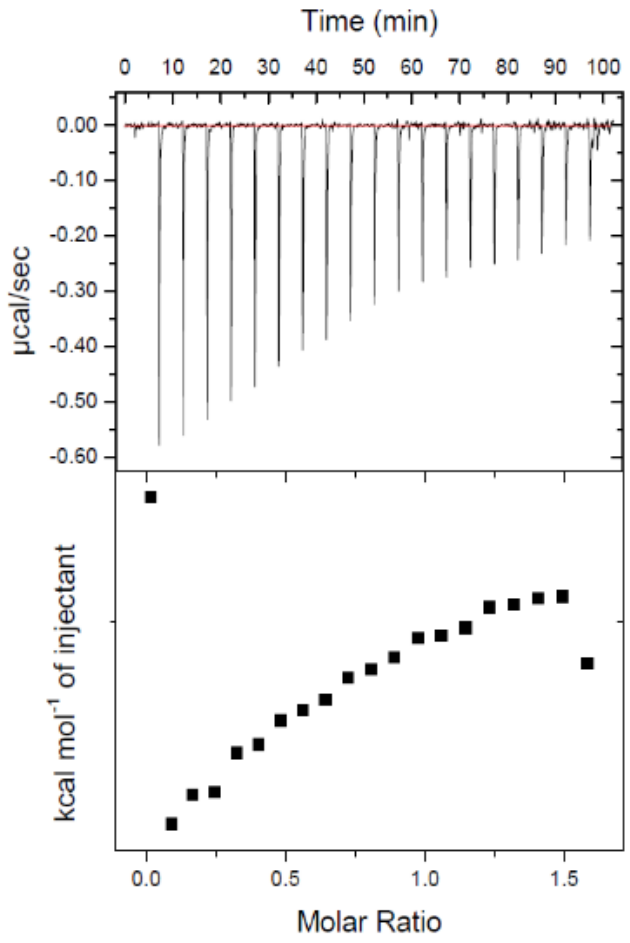


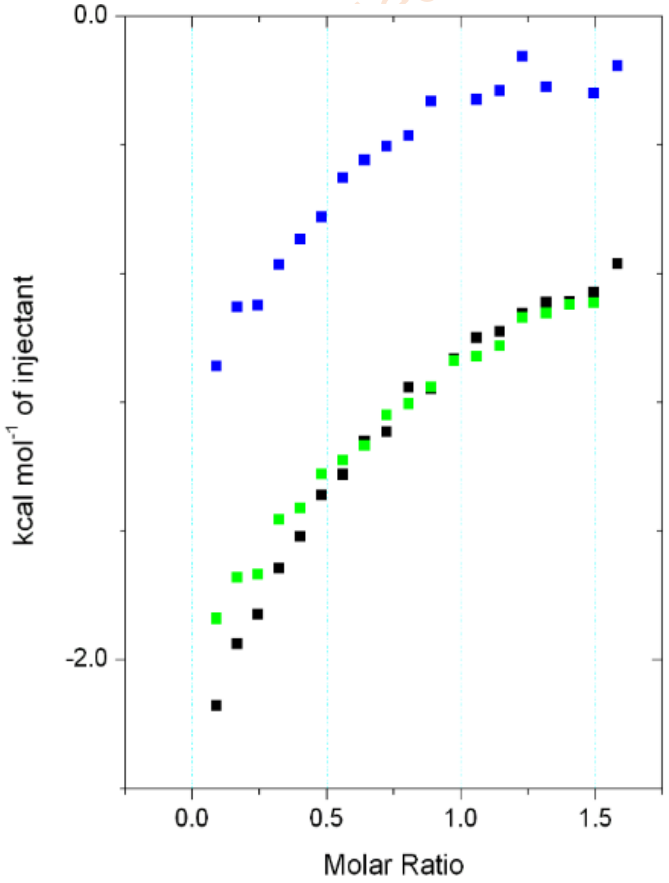




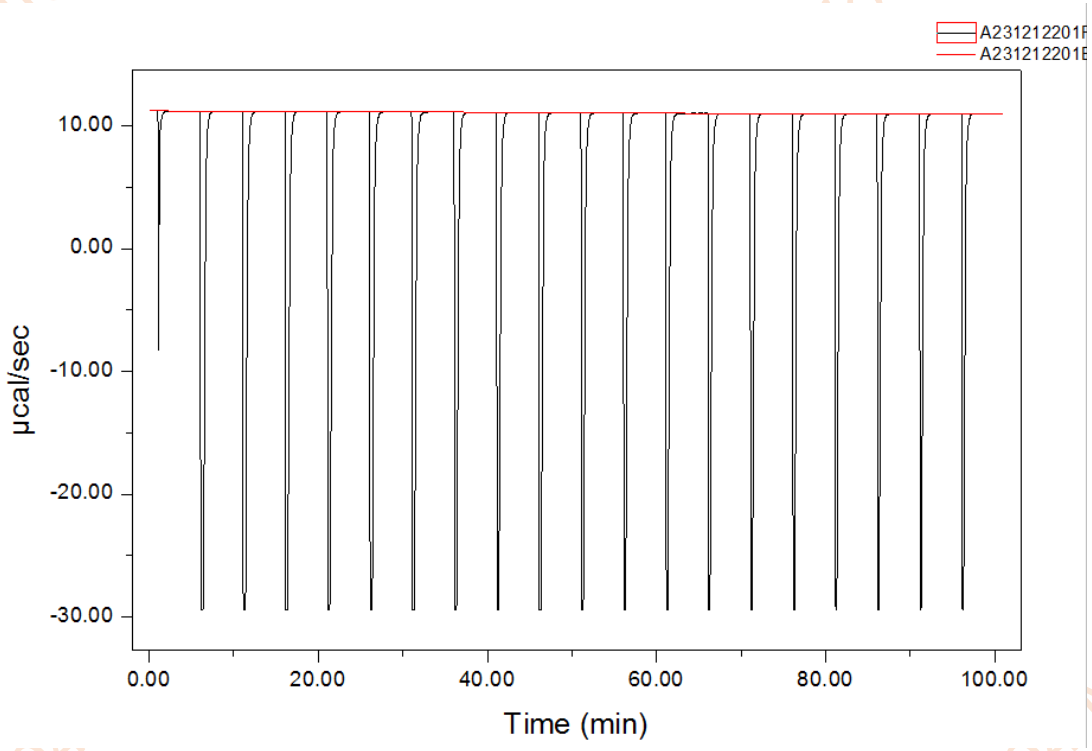
Effect of impurities on ITC

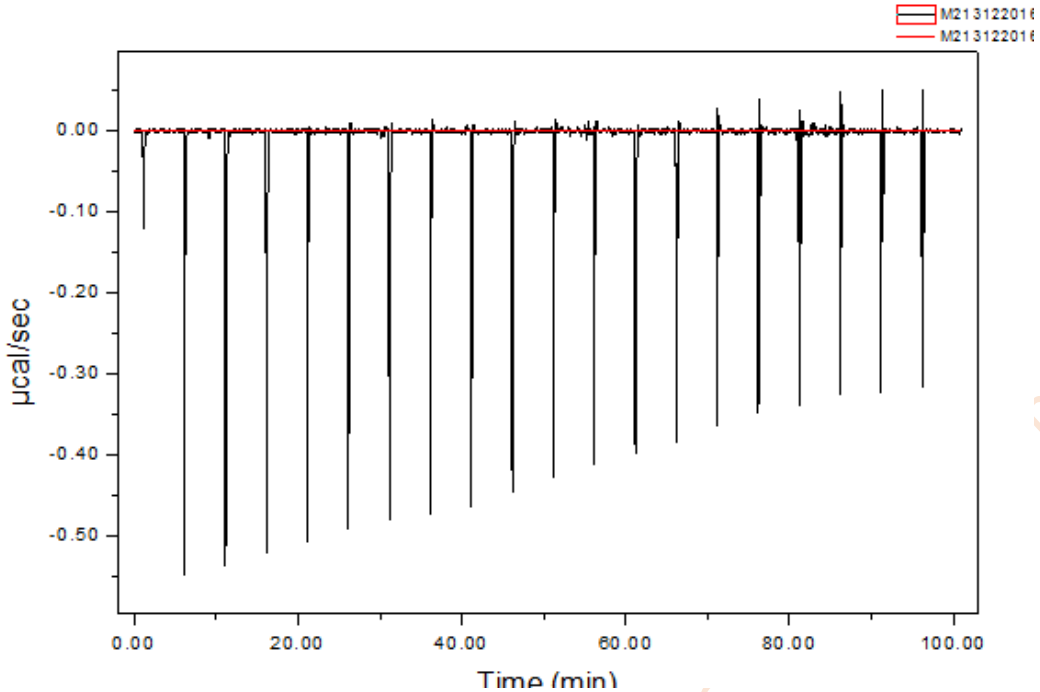




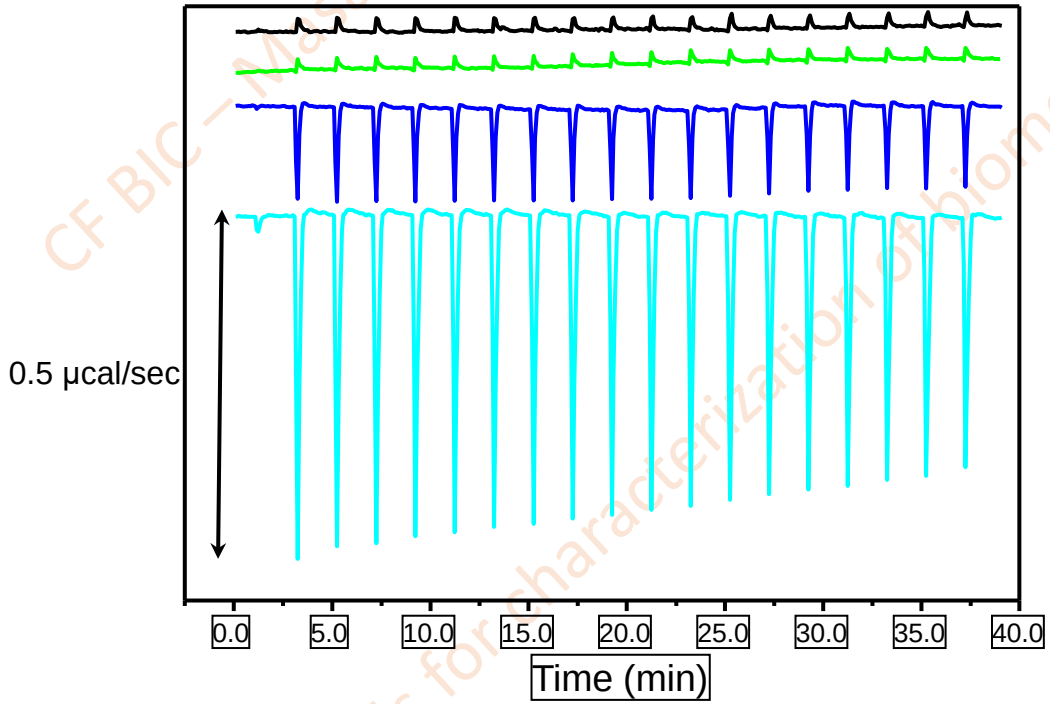


Effect of impurities on ITC – DMSO





DMSO buffer mismatch

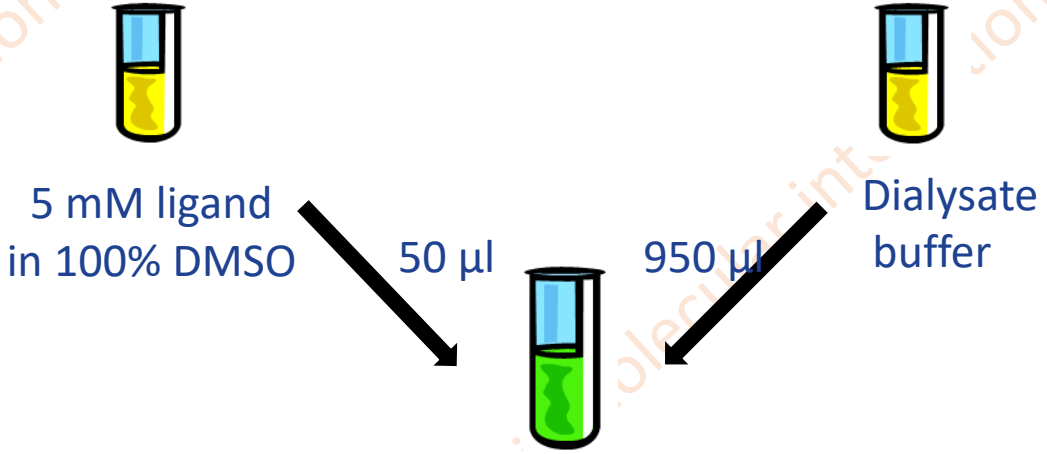


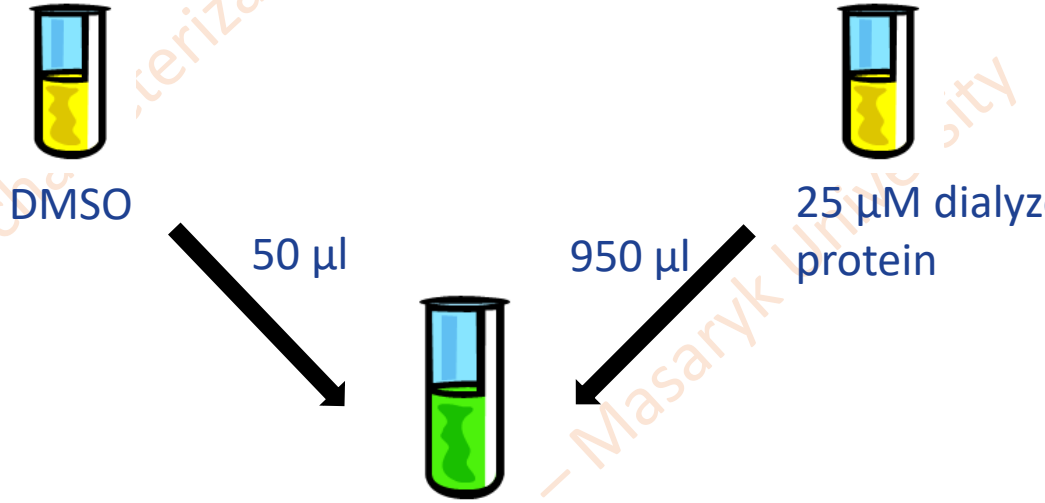
Buffer into buffer

5% DMSO into 5% DMSO

5% DMSO into 4.5% DMSO

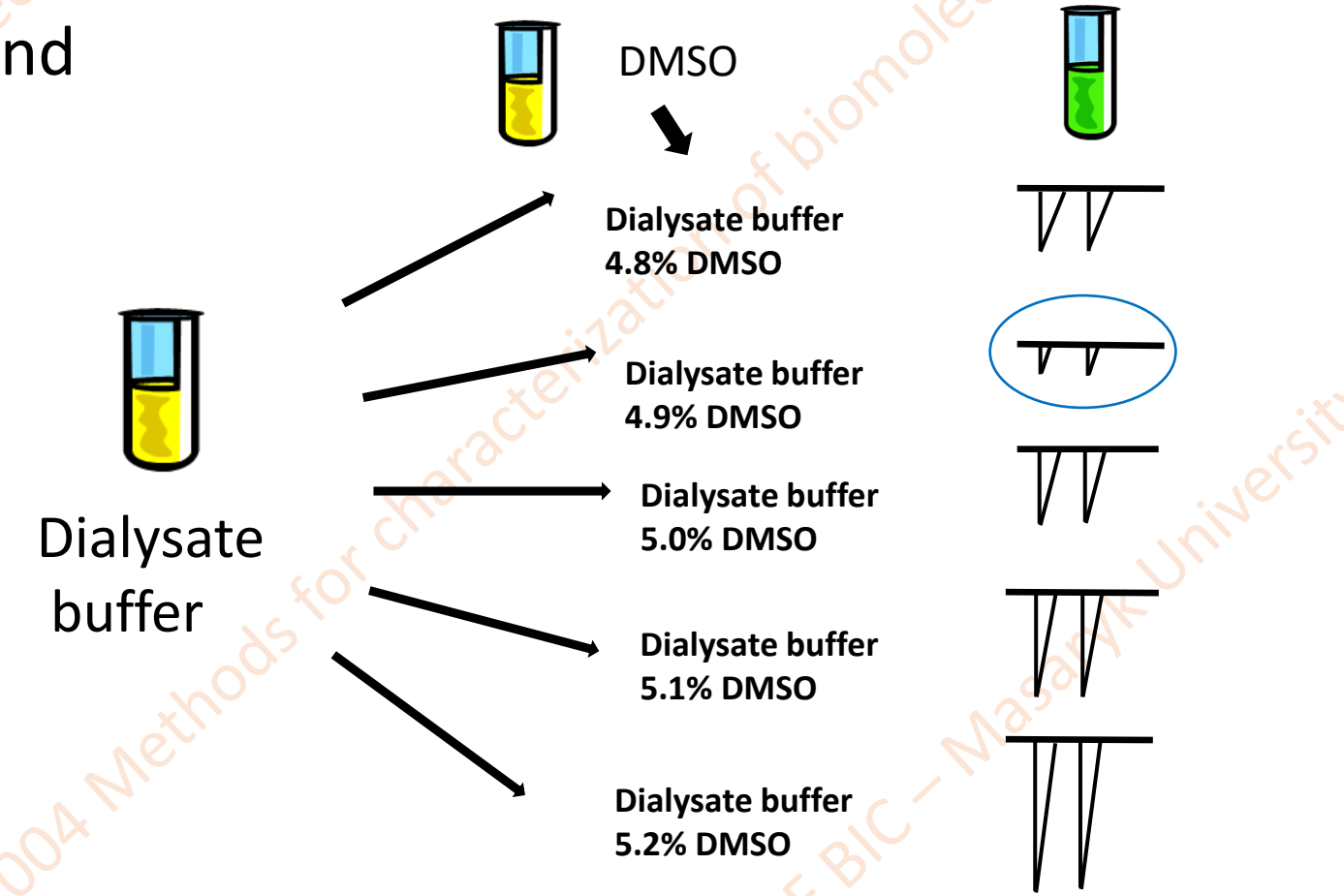
5% DMSO into 4 % DMSO





DMSO buffer mismatch

- Determine DMSO concentration
- Match for protein and ligand



Buffer optimization

- Buffer affects:
  - Stability
  - Activity (interactions)
  - Storage

- Many buffers do not meet all requirements

Buffer optimization desired

Buffer optimization

- Various **commercial screens** available

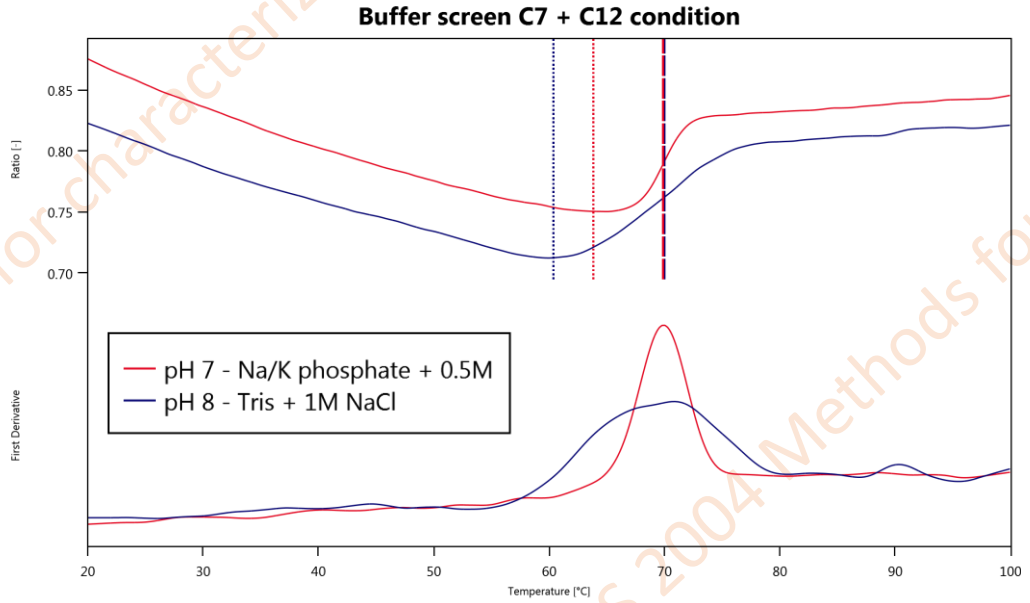
- Differences in composition, number of conditions

Example: buffer screen designed by CF BIC, CEITEC MU

	1	2	3	4	5	6	7	8	9	10	11	12
A	H_2O	pH 2-12										
B	pH 4-9.5 (alternate buffers from A row)											
C	Ionic strength (for pH 6-8)											
D	Pre-defined buffers					Additives						

Buffer optimization

	1	2	3	4	5	6	7	8	9	10	11	12
A	59.2°C	-	43.6°C	37.7°C	55.0°C	61.3°C	59.8°C	62.1°C	55.5°C	59.0°C	33.4°C	33.2°C
B	36.5°C	42.1°C	48.3°C	52.2°C	55.0°C	58.5°C	66.2°C	66.4°C	58.7°C	59.4°C	63.1°C	63.3°C
C	57.2°C	59.2°C	62.7°C	62.1°C	67.0°C	68.1°C	69.9°C	66.5°C	60.2°C	61.8°C	66.5°C	70.0°C
D	60.6°C	58.5°C	69.4°C	63.4°C	46.2°C	55.2°C	58.2°C	54.5°C	59.2°C	59.5°C	-	59.2°C



Original buffer **59.2**

vs.

Best buffer **69.9**

> 10°C difference !!!

Sample storage

- Depends on sample stability
- Freezing (phase transition) may decrease protein stability in solution

Avoid repeated freeze-thaw cycles !

- Fridge: 4 °C
- Freezer: – 20 °C, – 80 °C (cryo-protectants addition – glycerol)
- Lyophylization = Freeze-drying: water sublimation

Check sample quality BEFORE and AFTER storage !

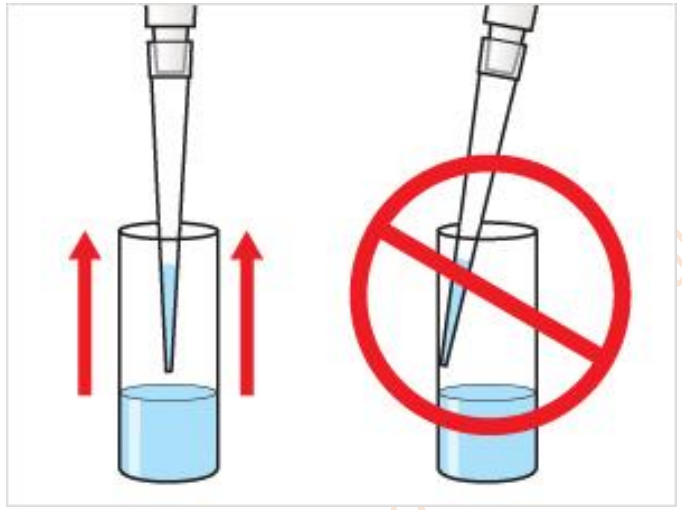
Practical aspects

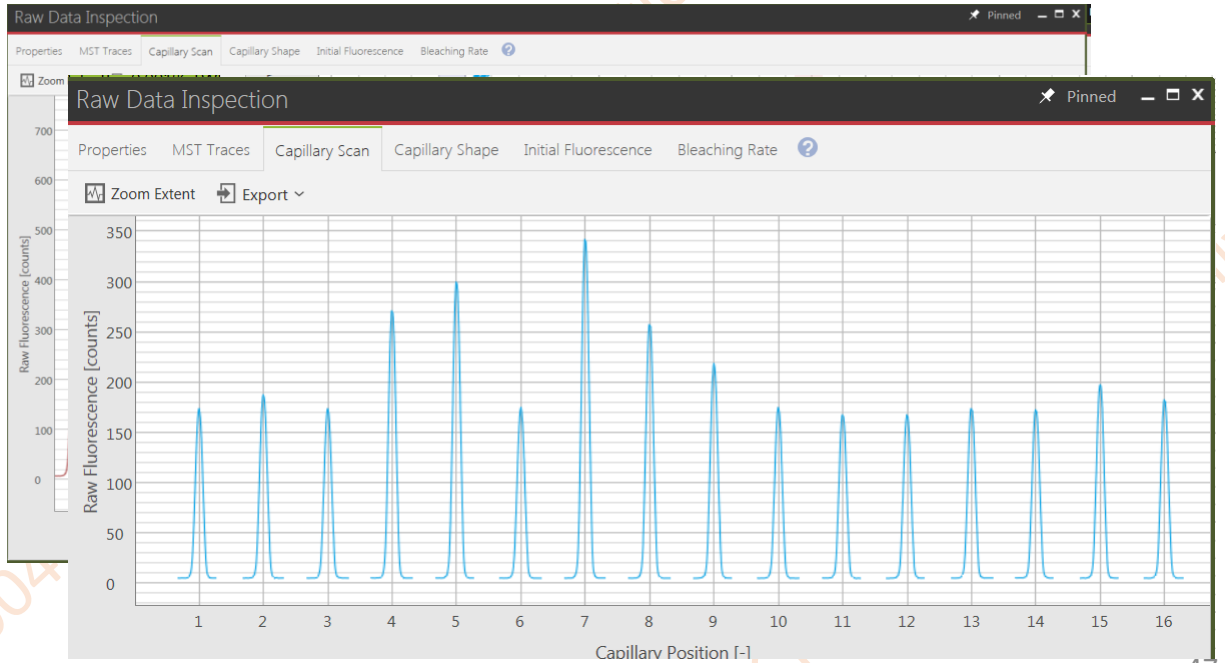
Concentration

Method	+	–
Nitrogen content (e.g. Kjeldahl)	Absolute (golden standard)	Time, sample and equipment demanding
UV absorbance at 280 nm	Fast, easy, low sample consumption, no calibration	Sequence dependent, buffer influence, (inaccuracy in l, ε)
Bradford (Coomassie Brilliant Blue)	Easy, fast	Standard dependent (calibration), sequence dependent, buffer influence
Bicinchoninic acid	Less buffer dependent	Standard dependent (calibration), more time demanding
UV absorbance at 205 nm	Less sequence dependent, + the same as A_{280}	Buffer absorbance

Pipetting

- Pipetting is a science
- Many **variables**
  - Viscosity
  - Type of tip/pipette
  - Immersion depth
  - Angle
  - Tip-pipette match
  - Pipette holding
  - Moisture
  - …





Sample aging

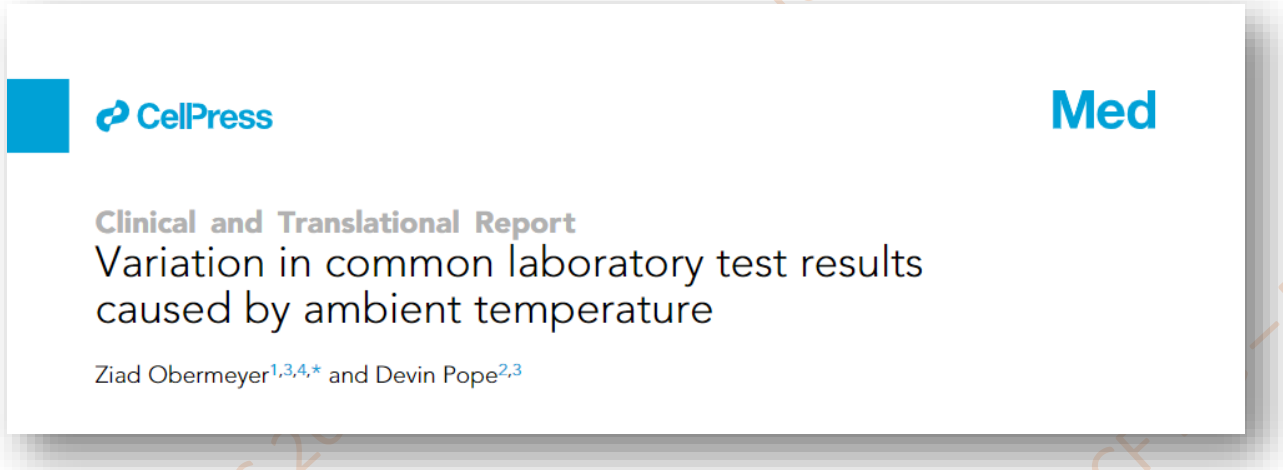
- Check **storage** conditions
- Avoid **freeze-thawing** cycles as much as possible
- Check buffer pH – use **freshly prepared buffers**
- **Batch-to-batch** verification

Batch to batch quality check

- Enormous amount of variables in preparation process
- Two sample batches may not be the same
- Minimal tests desired to **verify sample quality**

Temperature

- Many machines keep specific temperature
- **Check settings**
- **Room temperature** varies over day, week, seasons
- Avoid “bad spots” in lab – heating, direct sun, air conditioning



Methods are not identical

- **Results** from different methods usually vary
- Ideal match of values (e.g. Kd) is unlikely
- Some methods require **specific sample preparation** and conditions
- **Know method principles and limitations !!!**
- **Know your sample !!!**

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