Development of Bioanalytical Methods for BE Studies

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Which Analyte?
(Chiral *vs.* achiral)

- EMA GL on BE (2010, Section 4.1.5)
  - Achiral methods generally acceptable
  - Chiral methods, if *all* conditions are met *or are unknown*:
    1. Enantiomers exhibit different pharmacokinetics.
    2. Enantiomers exhibit pronounced differences in pharmacodynamics.
    3. The exposure (*AUC*) ratio of enantiomers is modified by a difference in the rate of absorption.
  - If only one enantiomer is active and the other is inactive or has low contribution to activity, it is sufficient to demonstrate BE for the active only.

Which Analyte?
(Parent *vs.* Metabolite)

- EMA GL on BE (2010, Section 4.1.5)
  - Generally parent drug.
  - Concerns, that C_{max} of metabolite does not reflect difference between formulations.
  - Does not distinguish between active and inactive metabolites.
  - If method too insensitive consider higher SD.
  - Metabolite acceptable *'in exceptional cases'*:
    - Present any available data supporting the view that the metabolite exposure will reflect parent drug.
    - Metabolite formation is not saturated at therapeutic doses.

Which Analyte?
(Pro-drugs)

- EMA GL on BE (2010, Section 4.1.5)
  - Generally inactive pro-drug.
  - Active does not need to be measured.
  - If pro-drug has low concentrations and is quickly eliminated it is acceptable to demonstrate BE of active metabolite (pro-drug not measured).
  - Parent compound can be considered to be an inactive pro-drug if it has no or very low contribution to clinical efficacy.

Basics of Bioanalytics

- Biological matrices
  - Whole Blood
  - Serum
  - Plasma
  - Urine
  - Liquor, Saliva, Synovia, Tissue, Faeces, Sputum,…
- Sample Preparation (Extraction / Trace Enrichment Techniques)
  - Dilution
  - Protein Precipitation
  - Liquid-Liquid Extraction (LLE)
  - Solid Phase Extraction (SPE)

Basics of Bioanalytics (cont'd)

- Separation & the 'Workhorses' in Bioanalytics
  - for 'Small Molecules': Chromatographic Methods
    - Gas Chromatography (GC)
    - High Performance Liquid Chromatography (HPLC/UHPLC)
    - High Performance Thin Layer Chromatography (HPTLC)
    - Capillary Electrophoresis (CE)
    - Supercritical Fluid Chromatography (SFC)
    - Hyphenated Techniques
      - Column Switching (GC/GC, LC/LC, SFC/GC)
      - MS-MS, TOFMS
  - for Biologics: Ligand Binding Assays
    - Enzyme Linked Immunosorbent Assay (ELISA), Enzyme Multiplied Immunoassay Technique (EMIT)
    - Radioimmunoassay (RIA)

Basics of Bioanalytics (cont'd)

- Detection
  - for GC
    - Flame Ionization Detection (FID)
    - Nitrogen-Phosporus Detector (NPD)
    - Electron Capture Dector (ECD)
    - Mass Spectrometry (MS)
    - Thermal Conductivity (TCD)
  - for HPLC
    - Ultraviolet / Visible (UV/Vis), Diode-Array (DAD)
    - Fluorescence (FL)
    - Electrochemical (EC; coloumetric and amperometric)
    - Mass Spectrometry (MS)
    - Conductivity, Diffraction (Evaporative Light Scattering, ELS), Refractive Index (RI), Radioactivity (RA),…

Basics of Bioanalytics (cont'd)

- Integration
  - Peak 'recognition'
  - Automatic *vs.* manual
  - Chromatography Data Systems (CDS)
- Calibration
  - Model Selection
  - Weighting Schemes
  - Working Range

Basics of Bioanalytics (Assumptions)

- Assumptions should – possibly – be justified in method development, *e.g.*,
  - Absence of co-eluting compounds (MS-MS < MS < FL < EC < UV-DAD < UV)
  - Lack of Matrix Effects (LC/MS-MS, Ligand Binding Assays)

$$MF = \frac{\text{peak response in presence of matrix ions}}{\text{peak response in mobile phase}}$$

Matrix Factor ~1

  - Protein-binding (generally only total concentration [free+bound] measured)

Biological matrices

- Besides clinical / PK requirements…
  - It is the job of the bioanalyst to
    - Perform pretests of stability (from sampling until thawing).
    - Recommend the type of anticoagulat.
    - Recommend a stabilizer – and the procedure!
    - Recommend storage conditions.
- It's an urban myth that – at least for BE studies – free (unbound) drug should be measured, even for highly protein bound drugs. Common sample preparation techniques lead *always* to the total concentration.

Biological matrices

- Whole Blood
  - Should be avoided if ever possible…
  - Mandatory if drug binds strongly to erythrocytes (*i.e.*, no plasma concentrations can be measured – example: chlorthalidone).
  - Recommended if drug is routinely in Therapeutic Drug Monitoring (TDM) in whole blood, even if measurement in plasma is possible.
    Examples: tacrolimus; ciclosporin? Bortezomib?

Biological matrices

- Serum
  - Only if no stability issues (sufficient time for clotting – no cooling allowed).
  - Possible problems after thawing (turbidity requiring centrifugation – does the lab have a *cooled* centrifuge?).
  - Sometimes problems become evident only in multiple freeze-thaw-cycles (part of validation, but not of method development).
  - Therefore most bioanalysts opt for…

Biological matrices

- Plasma
  - Better choice than serum, because whole blood can be cooled immediately after drawing until centrifugation.
  - Numerous anticoagulants available: heparin, citrate, different EDTA salts (Na, K, Li).
  - Type of anticoagulant must no interfere with the extraction/separation/detection!
    It is the responsibility of the bioanalyst to require the most suitable anticoagulant from the clinical site.

Biological matrices

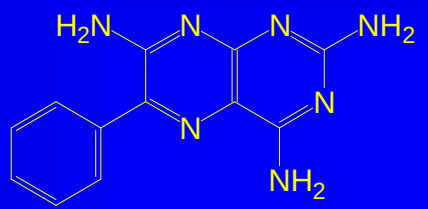
- Urine
  - Hint: Quite often clinical CROs fill urine sample vials ‘up-to-the-top’ (why?) and forget the 9% increase in volume (water → ice)…
  - All compounds must be dissolved after thawing! Sometimes it’s necessary to increase the temperature to 37 ℃ and go for a shaker or ultrasonicifaction! Sometimes dilution with H_20 helps.

Biological matrices

- Tissue
  - Only for experts! Ask!
  - Soft tissue (brain, liver, etc.): defined sample in ethanol or aceton, 30” – 60” Ultraturax, supension diluted to ~10% protein content, further treatment like plasma.
  - Everything else (including soft tissue with fibers): 30” Dismembrator in liquid nitrogen.
  - Tensides may improve poor recovery (but must be separated from the extract before HPLC).

Sample Preparation
(Extraction / Trace Enrichment)

- Dilution
  - For urine samples – with a few exceptions:
  - Simple example: triamterene in plasma
    - Solubility 48 mg/mL
    - Hydrophobicity logP
      0.3 (measured), 1.21 (predicted): extremely hydrophilic!
      LLE not possible; SPE almost not possible as well…
    - Acidity pKa 6.2
    - Protein binding 97%
    - 200µL plasma + 600µl H_20 → 20µl HPLC
      column NH_2
      Fluorescence 360nm ex. / 436nm em.
      LOQ 1ng/mL



Sample Preparation
(Extraction / Trace Enrichment)

- Protein Precipitation
  - ‘Rough treatments’ (perchloric acid, trichoroacetic acid) should be avoided: analyte will be trapped in denaturized protein clots.
  - Suitable (specially for LC/MS): acetonitrile, methanol or ethanol (CH_3CN recommended).
  - All other low molecular mass compounds (incl. endogenous ones) remain in solution. Only the best choice for high concentrations, good separation and selective detection!
  - Sometimes called ‘point-and-shoot’ – led to many disasters in LC/MS-MS (matrix effects)!

Sample Preparation
(Extraction / Trace Enrichment)

- Extraction
  - Liquid-Liquid Extraction (LLE)
  - Solid Phase Extraction (SPE)

Sample Preparation
(Liquid-Liquid Extraction)

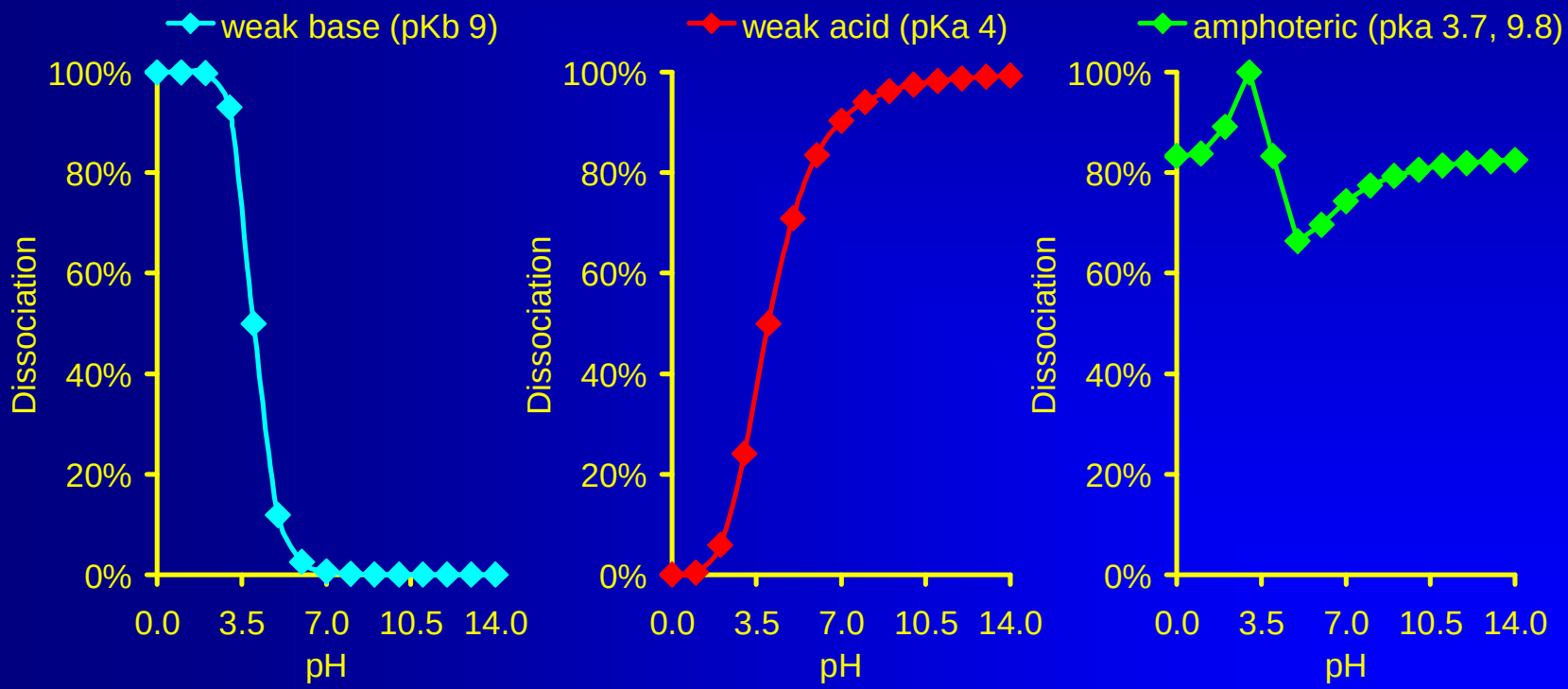
- Based on distribution between an aqueous phase (plasma, urine) and an organic phase.
  - Driven by lipophilicity of the analyte. Octanol-water partition coefficients are part of pharmacopoeial information, but only useful for neutral molecules.
  - Given as the decadic logarithm *log P*; the higher, the more lipophilic. Examples:

drug	*log P*
caffeine	-0.23
paracetamol	0.51
diclofenac	4.98
montelukast	7.26

Sample Preparation
(Liquid-Liquid Extraction)

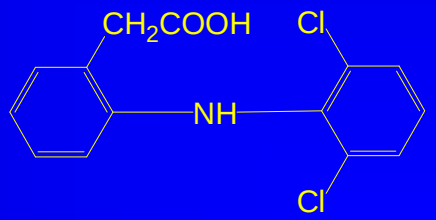
- If the analyte consists of an organic core and at least one acidic/basic group, the pH of the the aqeous phase drives the process.
  - Dissociated → dissolved salt → hydrophilic
  - Not dissociated → organic core → lipophilic
  - Amphoterics (containing acidic and basic group) are problematic. Multiple steps at different pHs may be necessary. Sometimes it's not possible to find a pH which gives sufficient recovery.
  - However, 100% recovery is not a requirement for a valid method. If the method is sensitive, accurate, and precise enough – *any* recovery is acceptable.

Sample Preparation
(Liquid-Liquid Extraction)



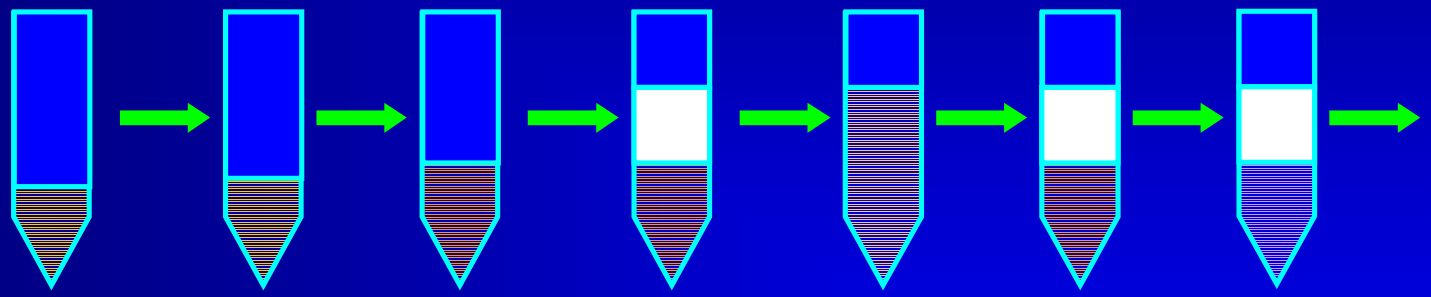
Sample Preparation
(Liquid-Liquid Extraction)

- Simple example: diclofenac
  - Solubility:
    50 mg/mL (sodium salt, measured)
    4.5 µg/mL (free acid, predicted)
  - Hydrophobicity logP
    3.9 (measured), 4.98 (predicted)
  - Acidity pKa 4.15
  - Protein binding >99%

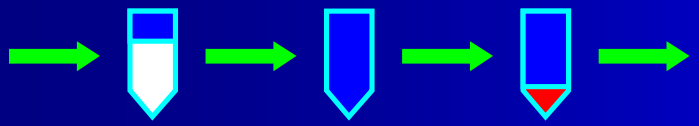


Sample Preparation
(Liquid-Liquid Extraction)

- Simple example: diclofenac

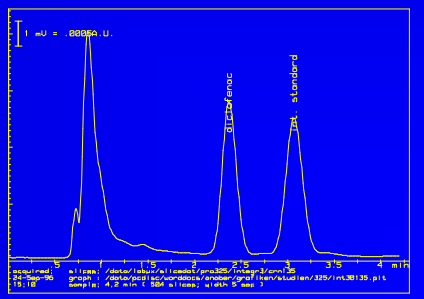


1mL plasma | +100µL i.St. | +100µL 38% HCl | +1mL hexane/CH_2Cl_2 | 30" Vortex | 20' centr. at 5°C | 10' freeze at -70°C



transfer to autosampler vial | vacuum-centr. to dryness | diss. in 500µl 40% CH_3OH

50µl HPLC
RP18
UV 285nm
LOQ 10ng/mL



Sample Preparation
(Liquid-Liquid Extraction)

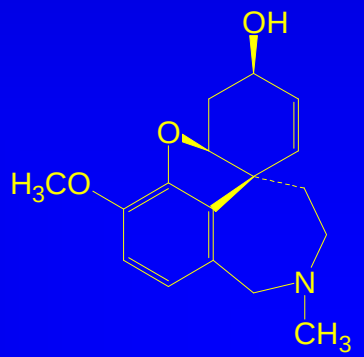
- Extreme example: lipophilic base
  - Plasma + HCl: analyte dissociates (becomes hydrophilic) $\rightarrow$ 1st LLE, acidic and neutral interferences $\rightarrow$ organic phase, disposed
  - Remaining plasma + KOH: analyte does not dissociate (becomes lipophilic) $\rightarrow$ 2nd LLE, analyte in organic phase
  - Organic phase + aqueous acid: analyte dissociates (becomes hydrophilic again) $\rightarrow$ 3rd LLE, analyte and remaining lipophilic basic interferences in aqueous phase…

Sample Preparation
(Solid Phase Extraction)

- Based on distribution between an aqueous phase (plasma, urine) and chemically bonded solid phase matrix.
  - Most common reversed phase silica: C18, C8, C4, C2, Phenyl
  - Rarely normal phase: NH_2, OH, CN
  - Selective washing steps possible
  - Can be automated – or even linked to the main HPLC-system (column switching)

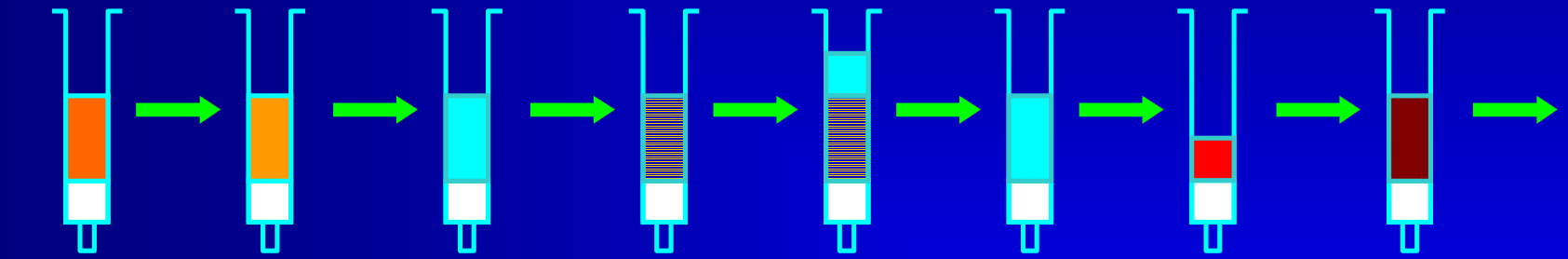
Sample Preparation
(Solid Phase Extraction)

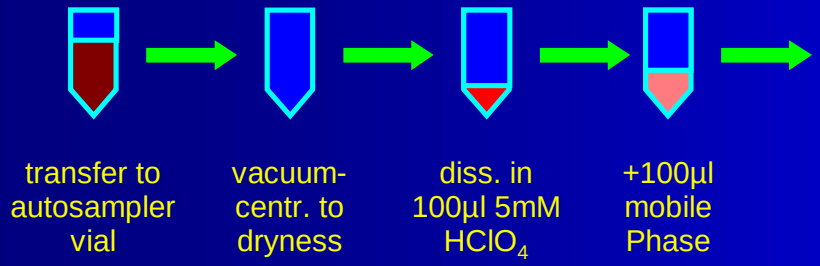
- Advanced example: galanthamine
  - Solubility:
    10 mg/mL (HBr salt, measured)
    1.7 µg/mL (predicted)
  - Hydrophobicity logP
    1.8 (measured), 1.39 (predicted)
  - Acidity pKa ?
  - Protein binding 18%



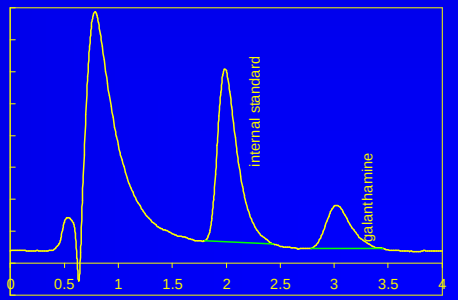
Sample Preparation
(Solid Phase Extraction)

- Advanced example: galanthamine





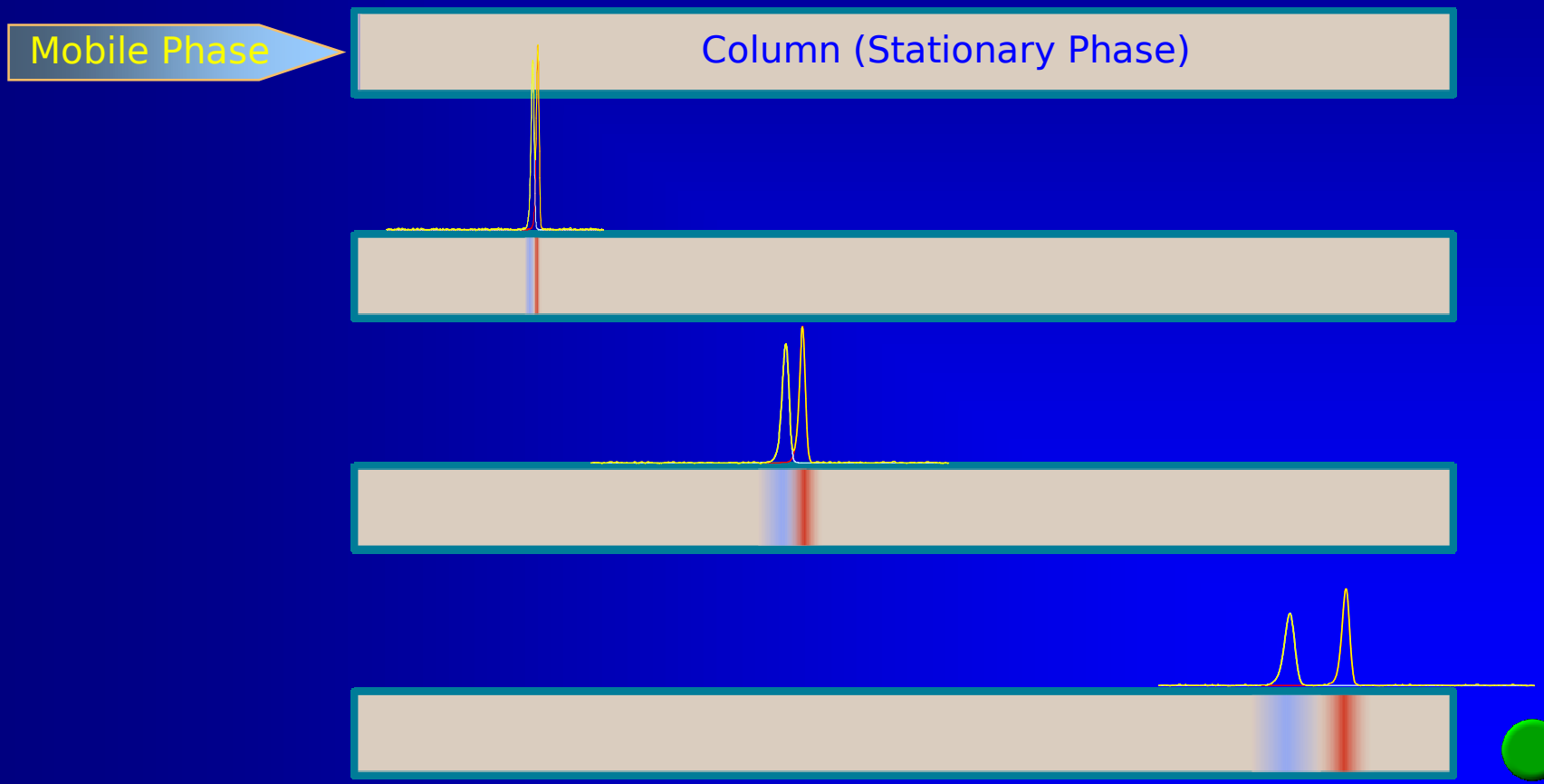
50µl HPLC
SCX Cat. Exch.
FL 230nm/310nm
LOQ 15ng/mL



Chromatography

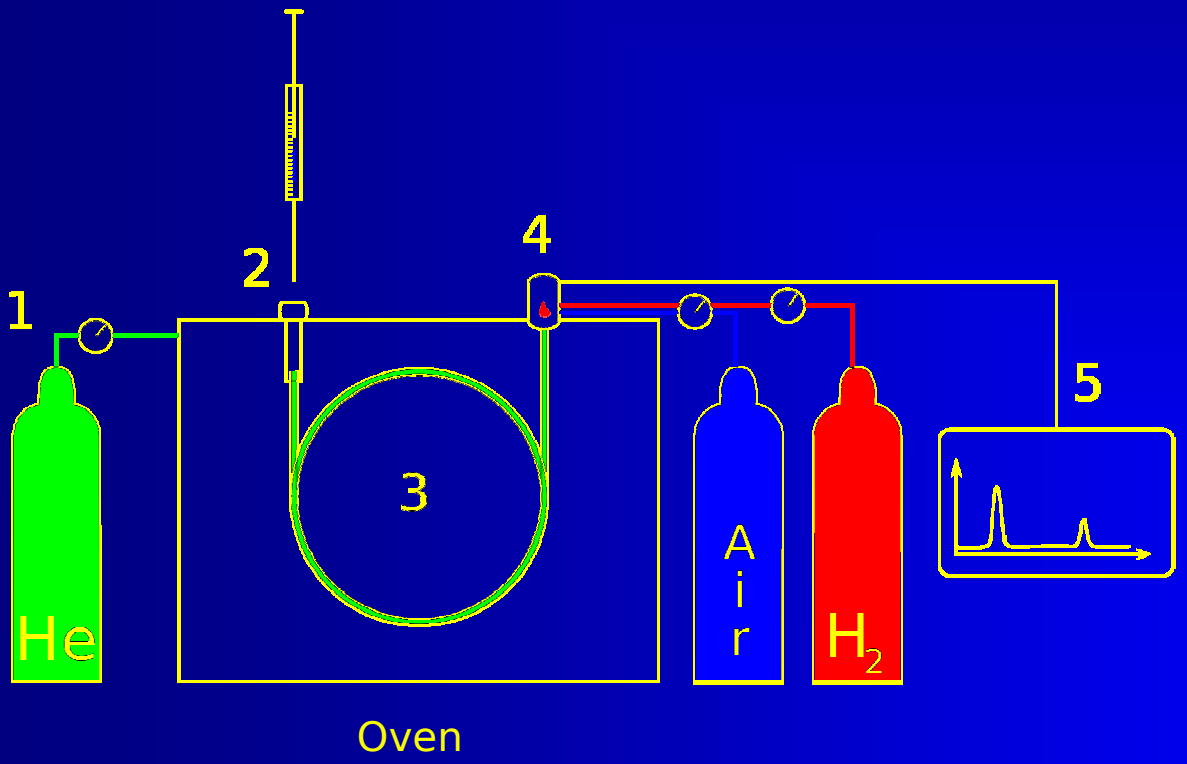
- Substances (analytes & interferences) continuously exchange between Mobile and Stationary Phase
  - Different Mechanism in parallel (solubility, lipophilicity, ionization,…)
  - Retention influenced by type of Stationary Phase, column lenggth, composition of Mobile Phase (type and % organic modifier, gradient, pH, buffer), temperature, pressure, flow rate,…

Chromatography



Chromatography

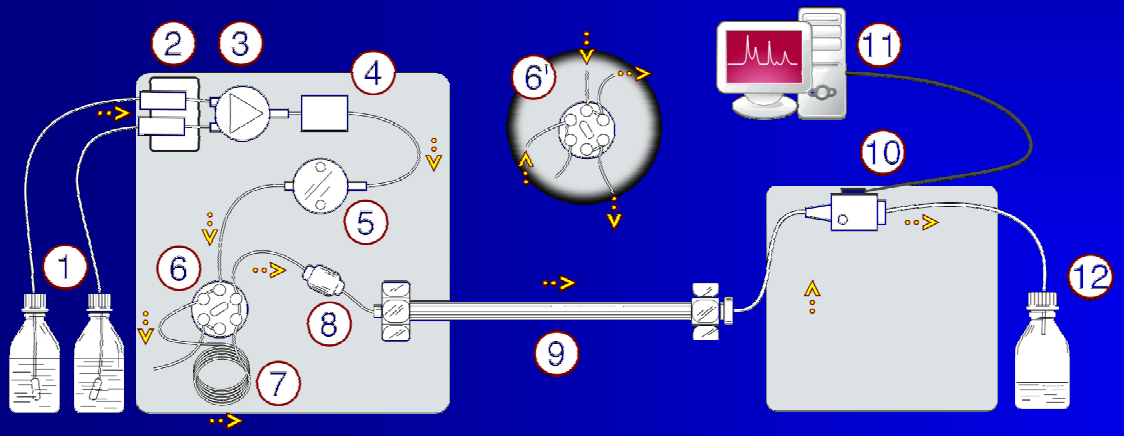
- Gas Chromatography



1. Mobile Phase (Carrier Gas)
2. Injector
3. Column (Stationary Phase)
4. Detector (here: FID)
5. Data System (A/D Converter, Integrator, Storage → LIMS)

Chromatography

- High Performance Liquid Chromatography



1. Mobile Phase Reservoir
2. M.Ph. Degasser
3. Gradient Valve
4. Mixer
5. Pump
6. Injector
7. Sample Loop
8. Pre-Column
9. Column (St.Ph.)
10. Detector
11. Data System
12. Waste

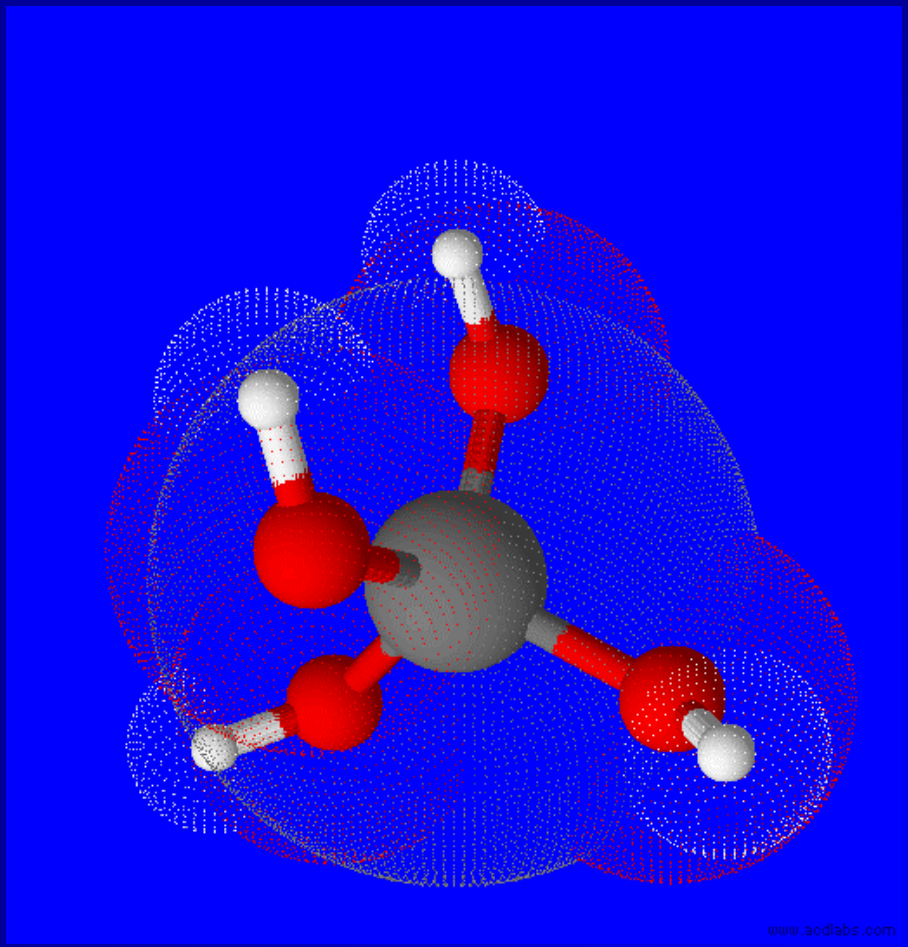
Chromatography

- GC *vs.* HPLC
  - GC
    - + For volatile and temperature-insensitive compounds (example: valproic acid)
    - + Easy coupling to MS (analyte already in gas phase)
    - + High separation power (capillary length up to 50m)
    - + Fast analyses possible (with short columns only restricted by the detector and data system)
    - + Low matrix effects in MS (small absolute amounts injected and high selectivity)
    - - Non volatiles require derivatization (majority of drugs; needs expertise)
    - - Chiral columns expensive and delicate

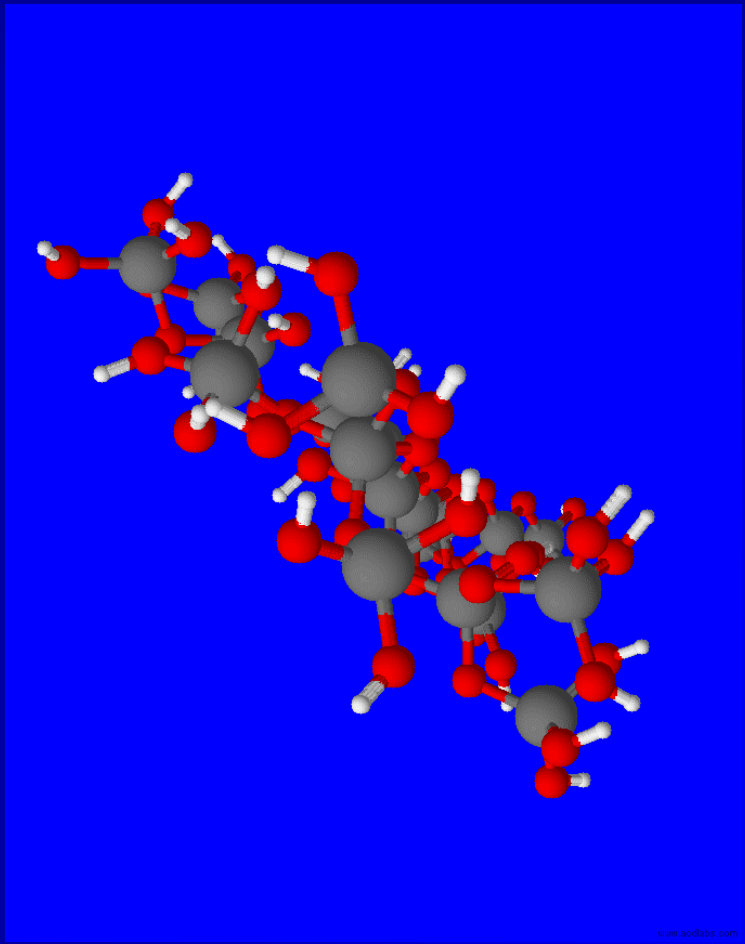
Chromatography

- GC *vs.* HPLC
  - HPLC
    - + Method of choice for non-volatile and/or temperature-sensitive compounds
    - + Most common method nowadays
    - ± Fast analysis times (UHPLC, particle size <3µm)
    - ± Coupling to MS (800× more volume than in GC)
    - ± Matrix effects in MS (*may* require sophisticiated sample preparation)
    - - Highly hydrophilic basic/acidic compounds problematic (ion exchange instead of RP; needs expertise)
    - - Highly hydrophilic neutral compounds require derivatization
    - - Chiral columns expensive and delicate

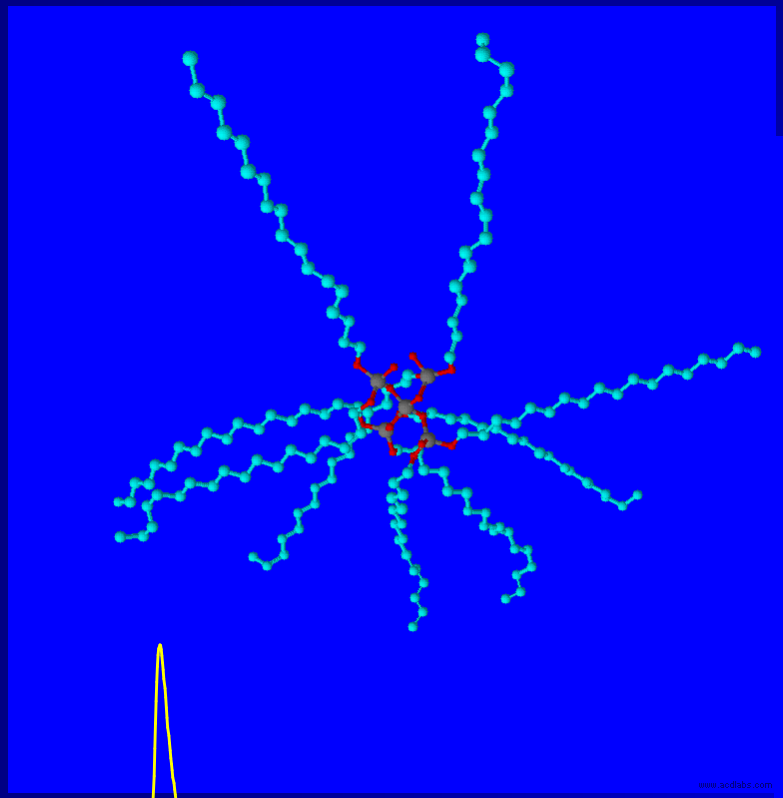
Orthosilic Acid (H_4SiO_4)



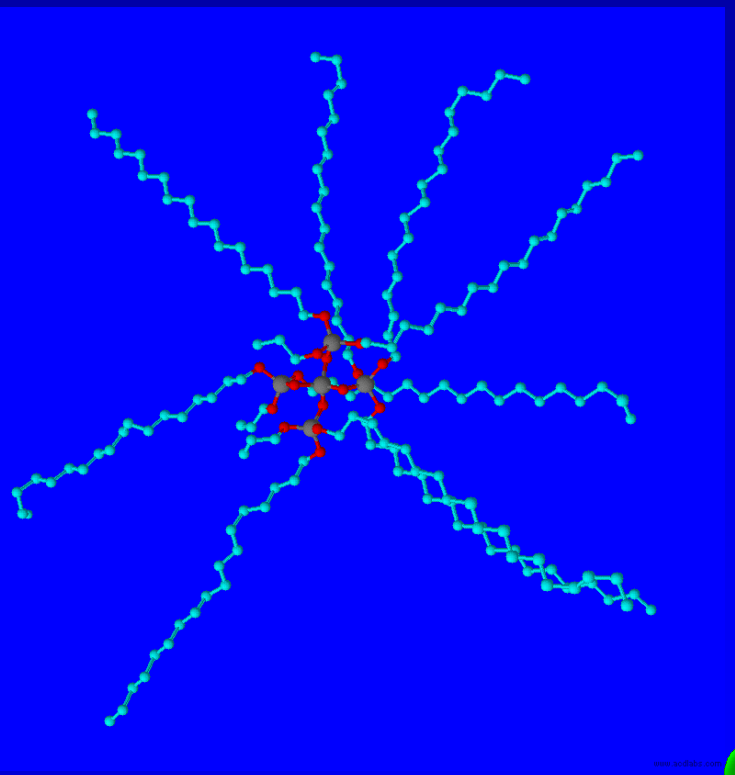
Polysilic Acid



C18 Reversed Phase

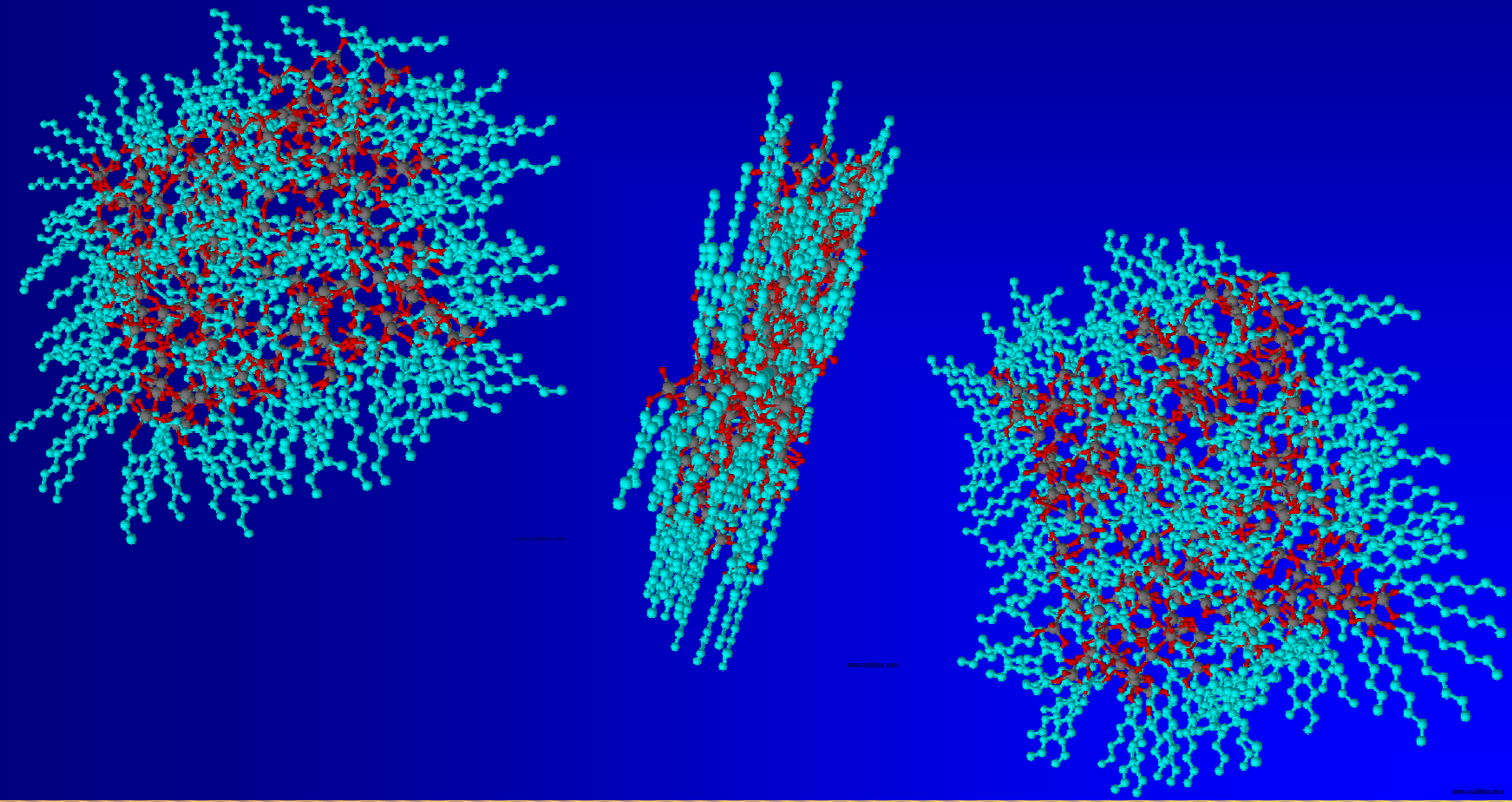


Residual OH-Groups lead to ‘Peak Tailing’ of Basic Compounds

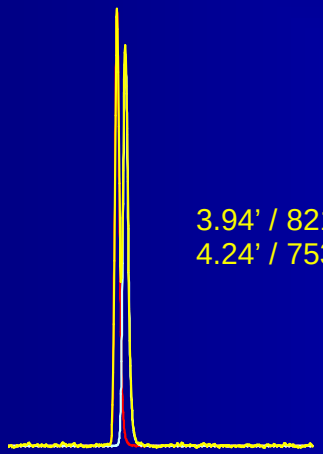


Resolved by ‘Encapping’ with C4

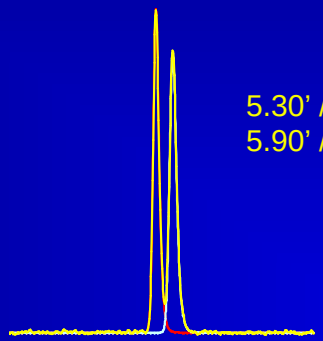
C8 Reversed Phase



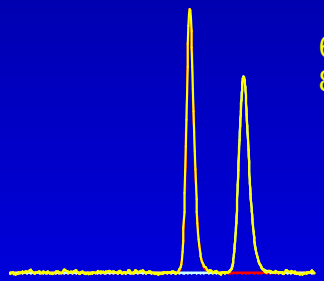
Retention Time and Tailing



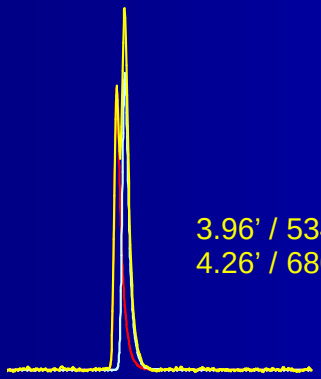
3.94’ / 8216
4.24’ / 7536



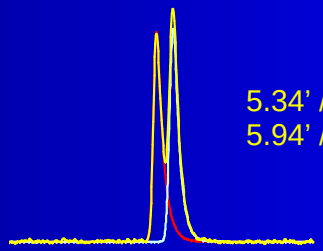
5.30’ / 6067
5.90’ / 5302



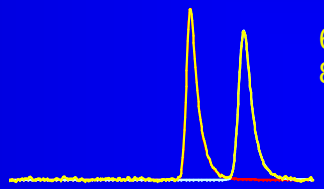
6.50’ / 4955
8.46’ / 3676



3.96’ / 5346
4.26’ / 6809



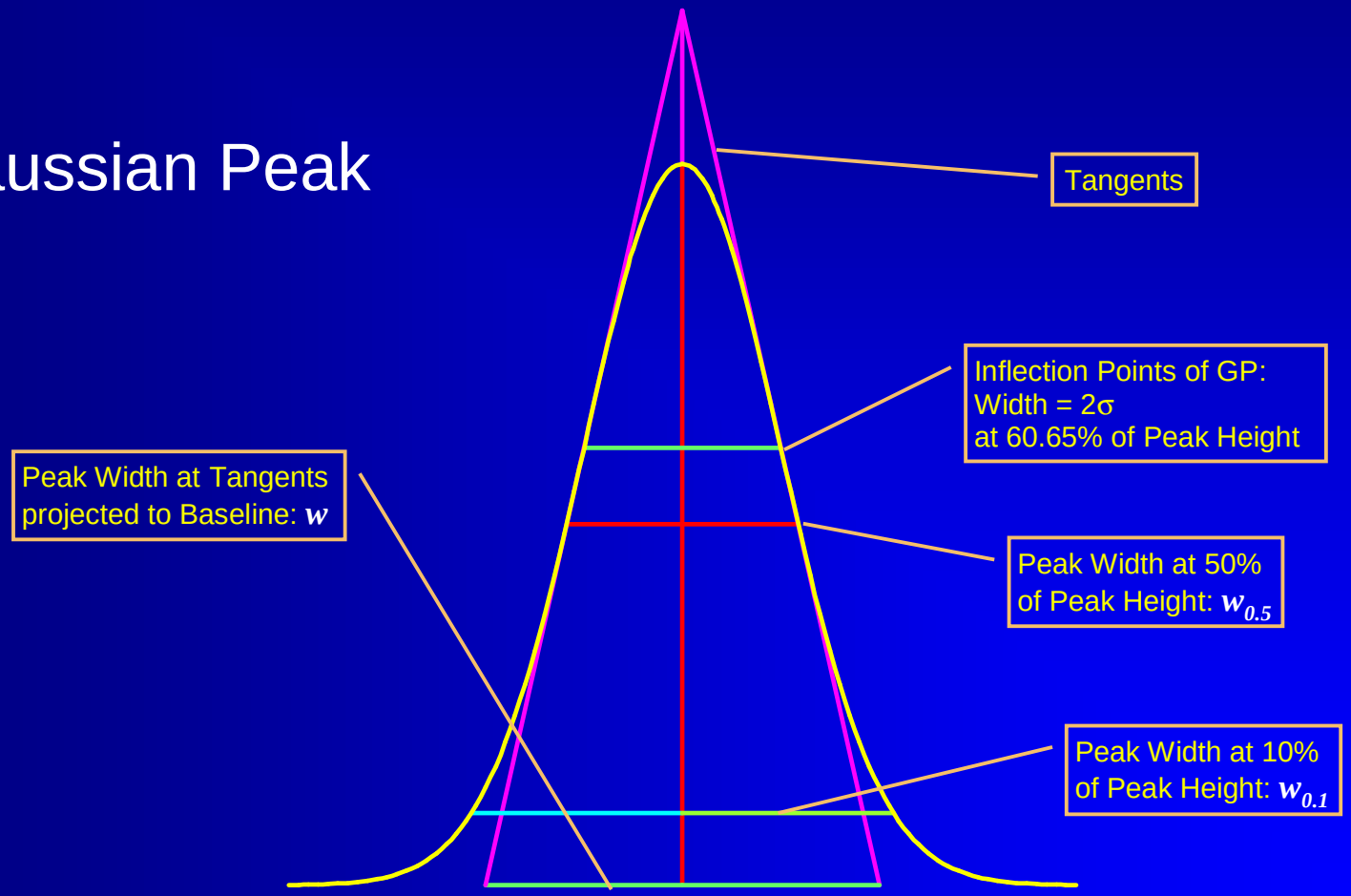
5.34’ / 3915
5.94’ / 4390



6.56’ / 3212
8.50’ / 2779

Characteristics of Peaks

Gaussian Peak



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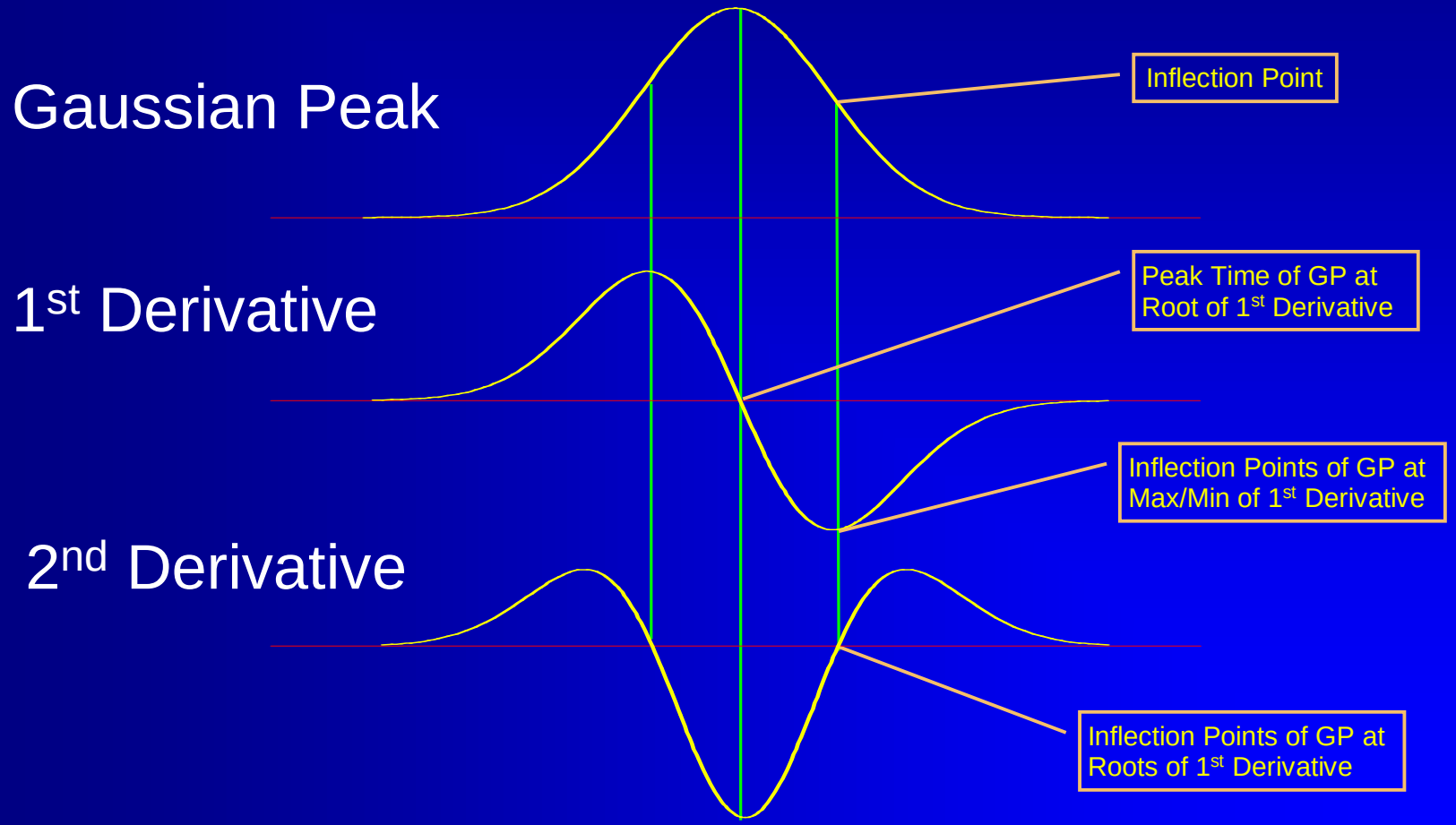

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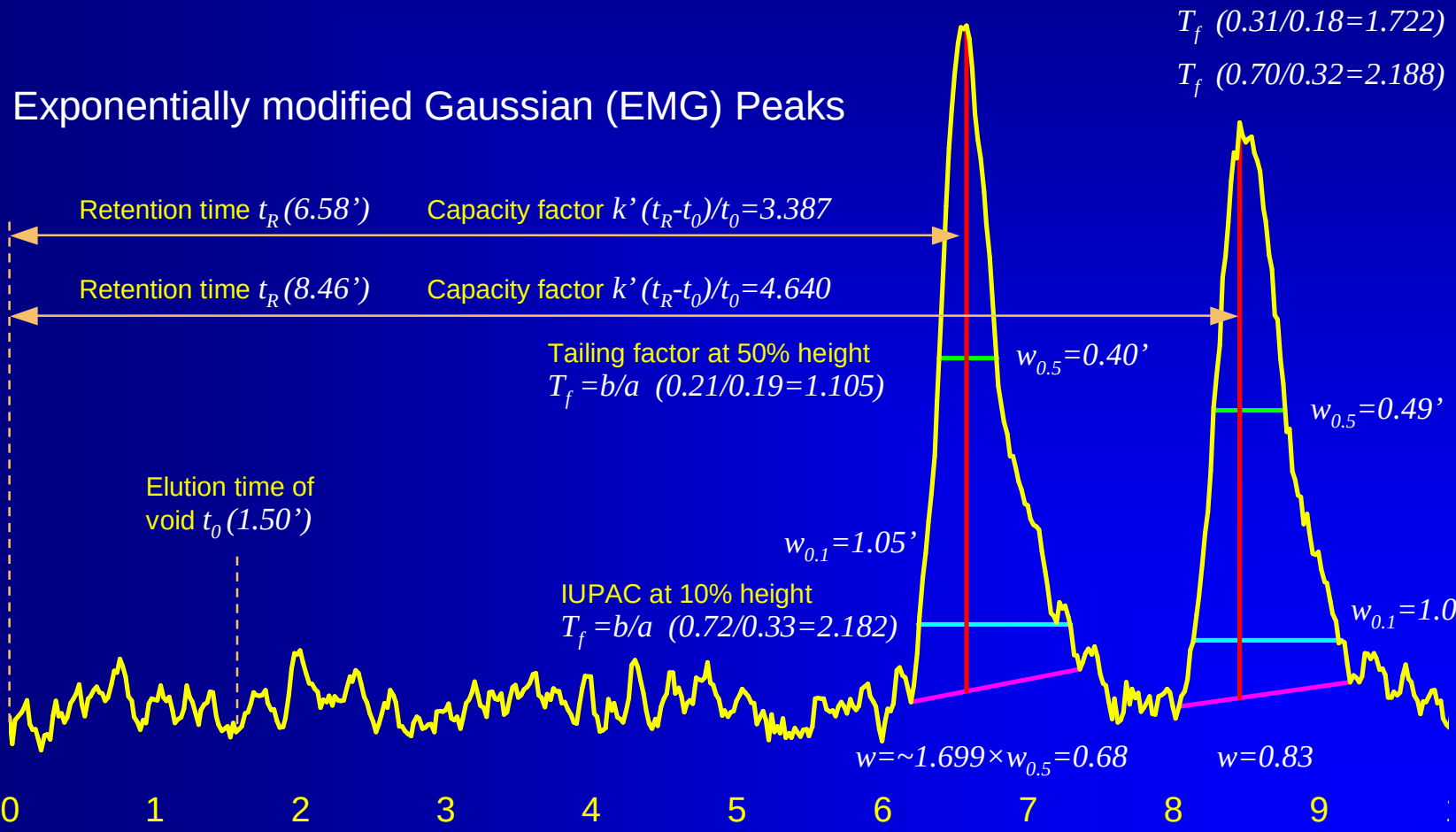
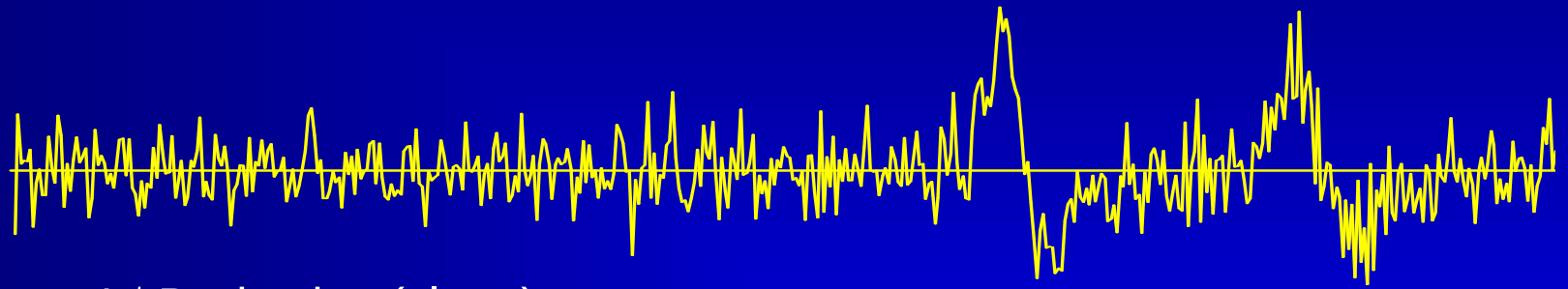
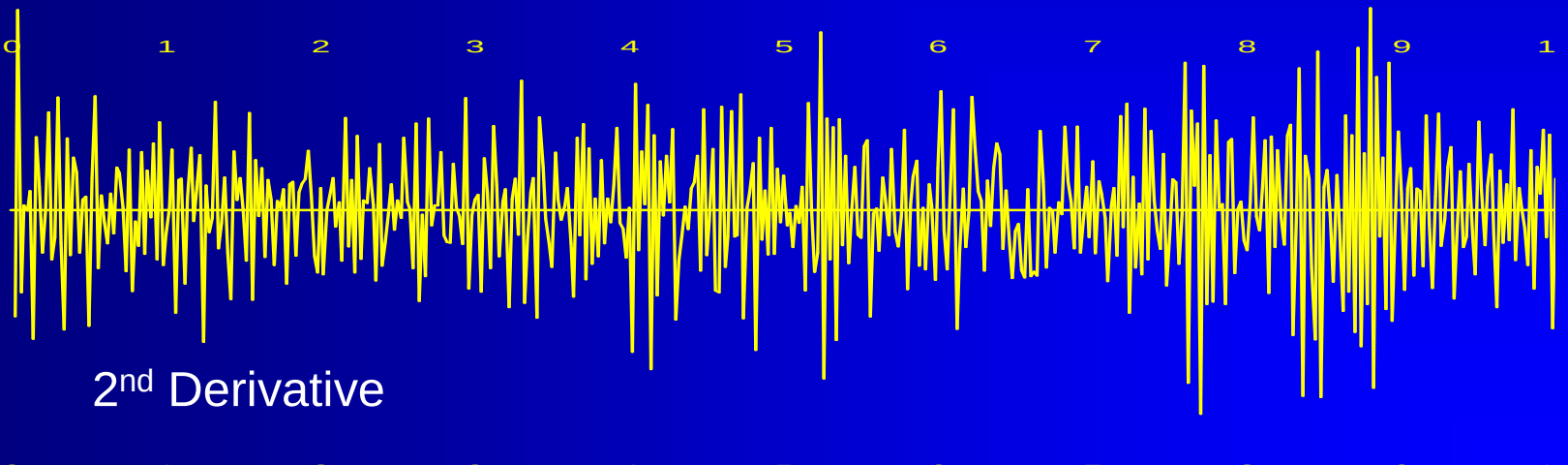


Characteristics of Peaks



1st Derivative (slope)



2nd Derivative

Recommendations

- Capacity factor k' for analytes >2
  - Example:
    $(6.58–1.50)/1.50=3.39$ ✓
    $(8.46–1.50)/1.50=4.64$ ✓
- Resolution between two adjacent peaks
  - $R_s = 2 \times (t_{R2} - t_{R1}) / (w_1 + w_2)$
    Baseline width w not easily accessible; for a Gaussian [*sic*] peak $w \sim 1.699 \times w_{0.5}$ holds.
  - Desirable >2
  - Example: $2\times(8.46–6.58)/(0.68+0.83)=5.69$ ✓

Recommendations

- Tailing factor T_f for analytes <2
  - Example (IUPAC at 10% of peak height):

    $0.72/0.33=2.18$ ✗

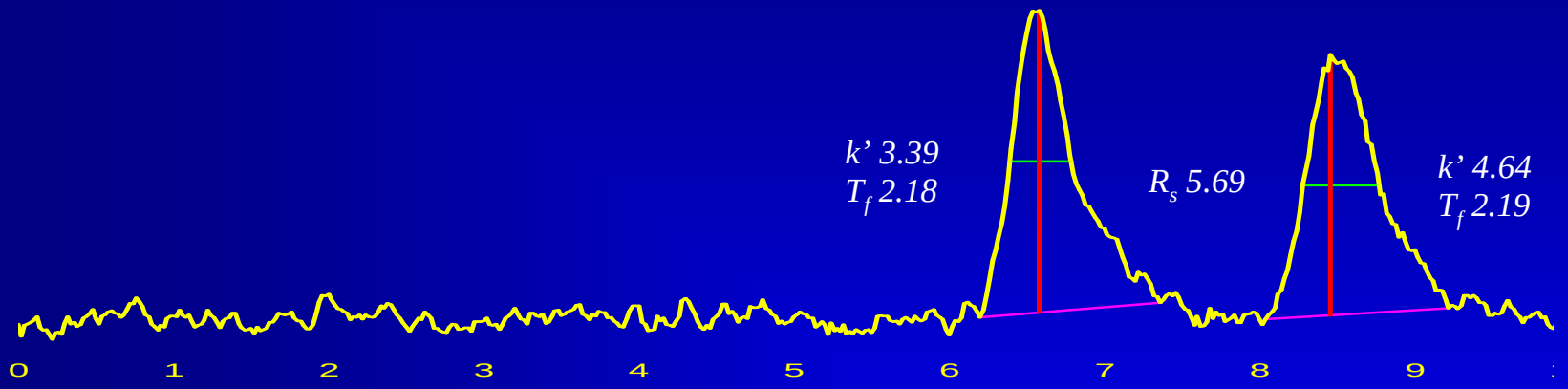
    $0.70/0.32=2.19$ ✗

    >2, but acceptable for an enantiomeric separation, where columns show ‘bad separation’ in general.

Recommendations

- Run times
  - The longer, the better the separation – but
  - Peak heights will decrease (band broadening → higher LOQ)
- Run times are decreased by
  - Type of stationary phase C18 → C8
  - ↓ Column length
  - ↑ Particle size 3 µm → 5 µm
  - ↑ Flow rate
  - Type of organic modifier in mobile phase CH_3OH → CH_3CN
  - ↑ % of organic modifier in MP
  - ↑ Temperature

Hurry up!

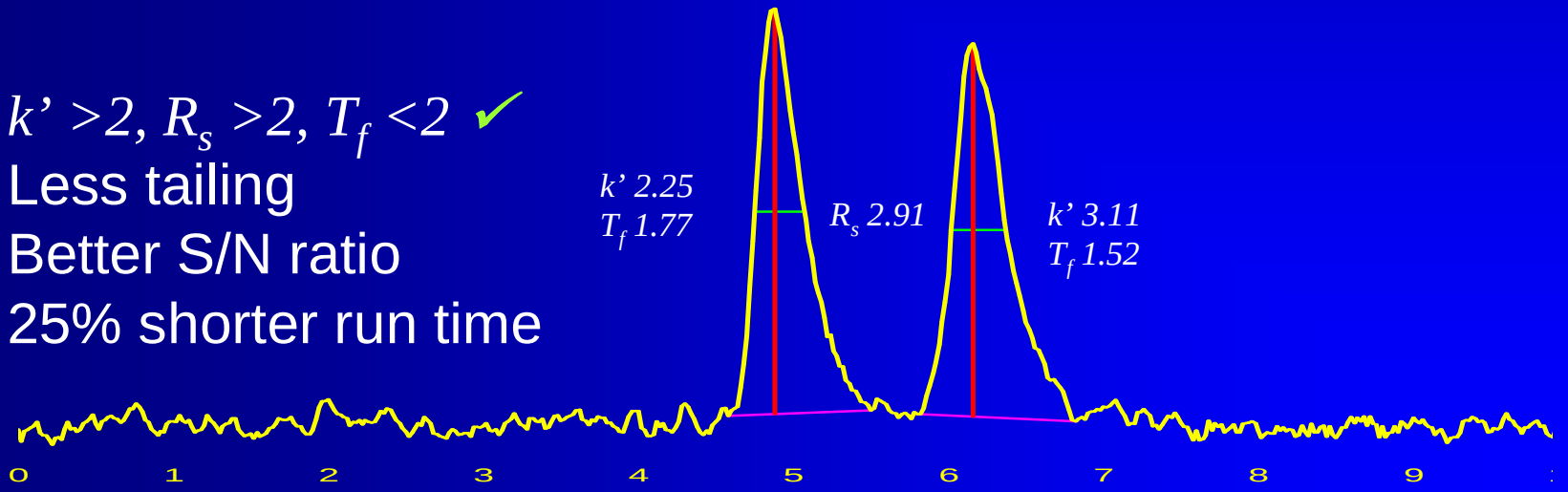


$k' > 2$, $R_s > 2$, $T_f < 2$ ✔

Less tailing

Better S/N ratio

25% shorter run time

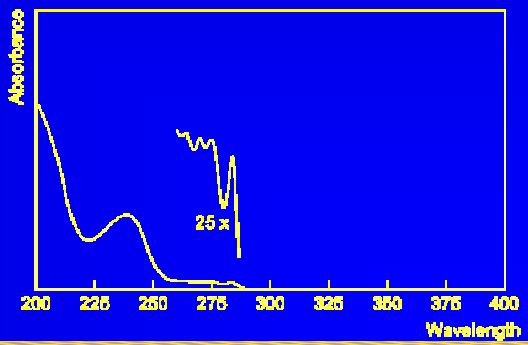


Detection

- Flame Ionization Detector (GC)
  - Applicable to all organic compounds (response linear to carbon content)
  - Analytes are pyrolyzed by a hydrogen-air flame
  - Ionized by high voltage
  - Induced current is measured
  - In ‘pharma’ almost of historical interest only

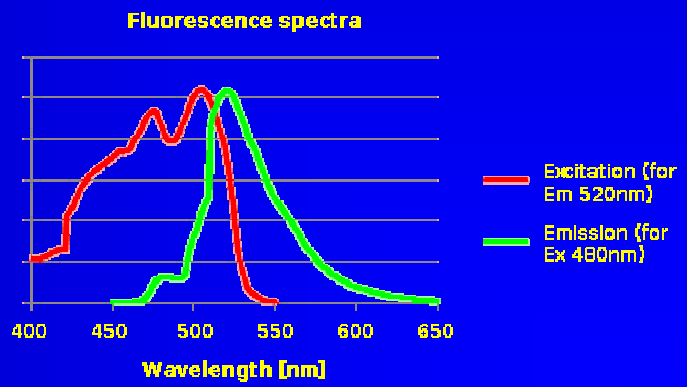
Detection

- Ultraviolet/Visible Detector (UV/Vis; HPLC)
  - Applicable to all compounds with a chromatophor (response linear to concentration)
  - Not very selective (at <200nm even dissolved O_2 shows absorbance); selectivity may be enhanced by Diode Array Detection (DAD)
  - Useful in method development (shows inter-ferences, where the MS is 'blind' due to high selectivity)
  - In 'pharma' almost of historical interest only



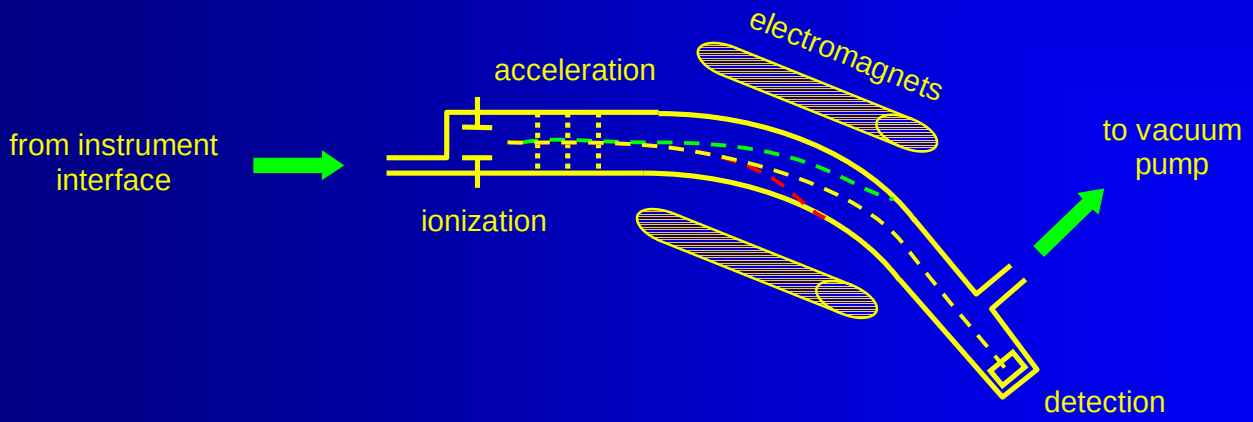
Detection

- Fluorescence Detector (FL; HPLC)
  - Applicable to most compounds with a conjugated system of double bonds
  - Pre- and post-column derivatization for many functional groups possible
  - Very selective (unlikely that two compounds show the same excitation and emission wavelengths)
  - For some compounds still the method of choice



Detection

- Mass Spectrometry (MS; GC, HPLC)
  - Applicable to all compounds
  - Very selective
  - Not very sensitive *per se*, but due to high selectivity (lacking interferences)

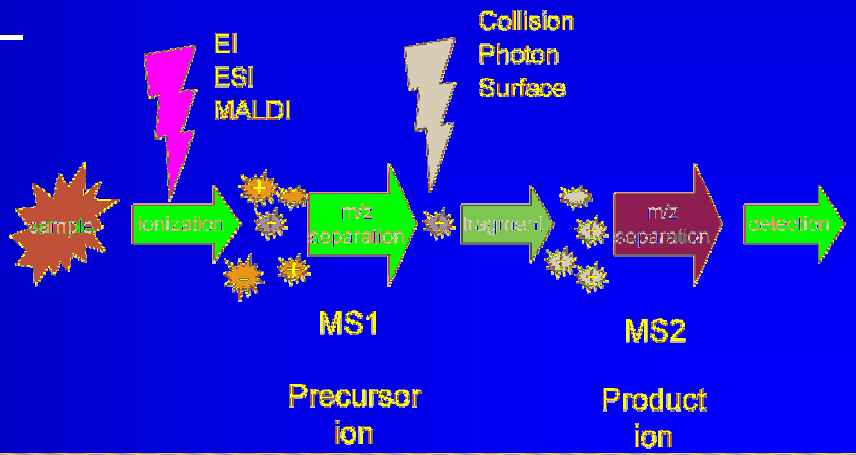


Detection

- Mass Spectrometry (MS; GC, HPLC)
  - MS operates in high vacuum (<10^{-6} mbar); mobile phase must be removed in the instrument/MS interface
  - Analytes are ionized. Common methods:
    - Electron impact (EI)
    - Thermospray
    - Electrospray (ESI)
    - Atmospheric pressure chemical ionization (APCI)
    - Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS)
    - …

Detection

- Mass Spectrometry (MS; GC, HPLC)
  - Analytes are deflected in the magnetic field based on their m/z (mass/charge ratio)
    - Ions with lower mass are more deflected
    - Ions with higher charge are more deflected
  - Quadrupoles (four magnetic rods) are commonly used, mostly in series – acting as an ion trap
  - MS in series: Tandem MS (or LC/MS-MS)



Matrix Effects (LC/MS-MS)

- Problems with LC/MS(-MS)
  - In the past (?) ‘strange’ PK results were seen after the transition from a conventional HPLC method to LC/MS, mainly with protein precipitation as the sample preparation
  - Due to the high selectivity, the MS is ‘blind’ for interferences – the chromatogram looks ‘clean’, but coeluting compounds may supress or enhance the ionization of the analyte
  - With a less selective detection these interferences would be visible…

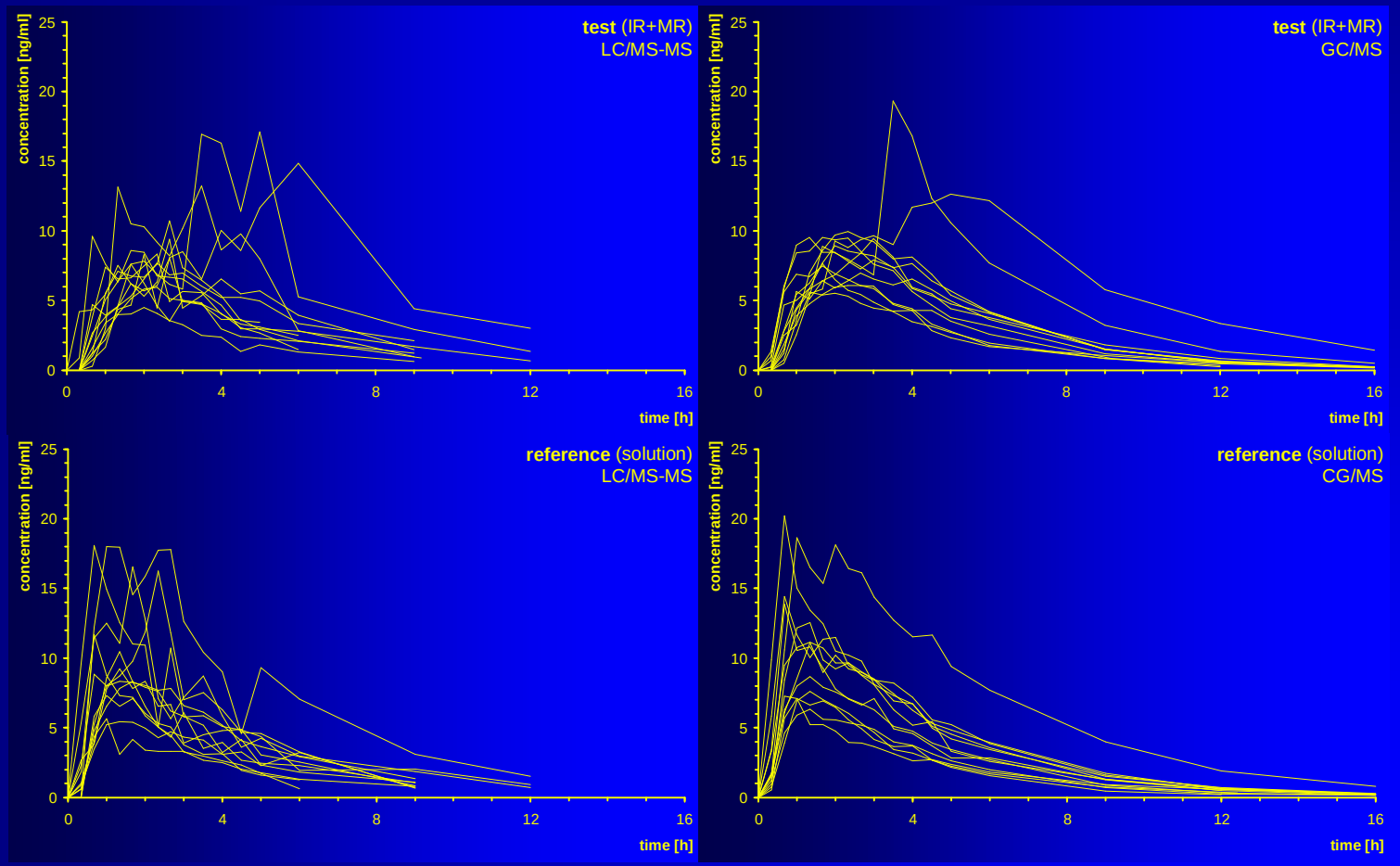
Matrix Effects (LC/MS-MS)

- Example: central stimulant
  - Development of a formulation combining IR and DR characteristics
  - 24 m+f subjects, fasting
  - test 10 mg IR + 10 mg DR
  - reference 20 mg oral solution
- Bioanalytics
  - Validated LC/MS-MS method
  - LLOQ 200 pg/mL

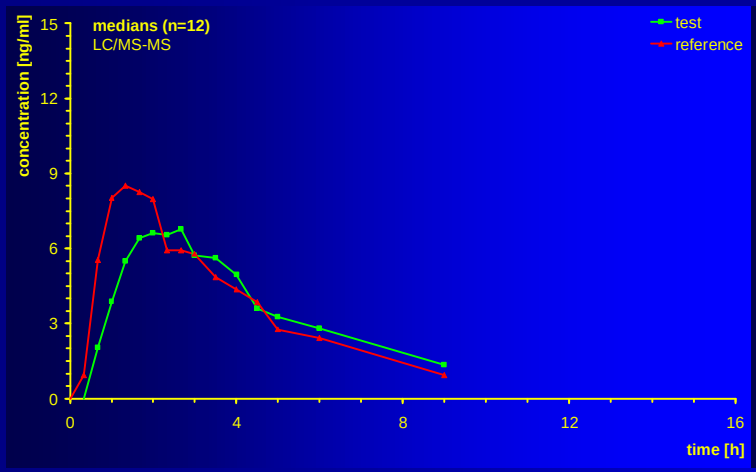
Matrix Effects (LC/MS-MS)

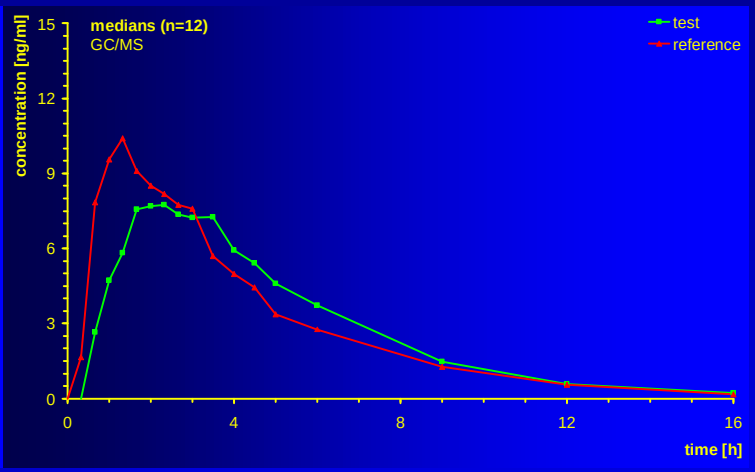
- Plausibility Review
  - LLOQ *really* 200 pg/mL?
  - in some subjects presumed to be >1.5 ng/mL
- LC/MS-MS stopped after 12 subjects due to suspected matrix effects
- Development of a GC/MS-method
  - stable isotope internal standardization
  - LLOQ 143 pg/mL

Matrix Effects (LC/MS-MS)



Matrix Effects (LC/MS-MS)





LC/MS-MS						
	statistic	CL-lo	CL-hi	PE	CV	CI
AUC	ANOVA	92.6%	114.4%	102.7%	14.1%	21.8%
	WMW	91.2%	116.2%	103.0%	–	24.9%
C_{max}	ANOVA	78.6%	99.8%	88.4%	16.5%	21.2%
	WMW	76.8%	97.4%	86.9%	–	20.6%
t_{max}	WMW	+0.58	+2.50	+1.33	–	1.92

GC/MS						
	statistic	CL-lo	CL-hi	PE	CV	CI
AUC	ANOVA	93.8%	110.3%	101.6%	10.8%	16.5%
	WMW	93.2%	112.3%	102.6%	–	19.1%
C_{max}	ANOVA	71.1%	96.4%	82.5%	20.8%	25.3%
	WMW	72.6%	97.8%	81.4%	–	25.2%
t_{max}	WMW	+0.50	+2.17	+1.00	–	1.67

Matrix Effects (LC/MS-MS)

- Approaches to examine Matrix Effects
  - Extract various lots of blank matrix, add a constant amount of analyte and internal standard and plot the ratio for each lot. If the ratio remains constant, the matrix effect is insignificant.
  - Compare the slopes of calibration curves prepared in different sources of matrix.
  - Infuse low levels of analyte post column while injecting reconstituted extracted matrix on the LC. This allows the observation of matrix effects under various chromatographic conditions.

Matrix Effects (LC/MS-MS)

- Minimization of Matrix Effects
  - Use internal standard of similar structure (preferably stable isotope – labeled; recommended in EMA 2011 GL)
  - Avoid *'precipitate and shot'*–methods
  - Conduct sufficient sample cleanup – especially to remove phospholipids
  - Use new chromatographic methods (UPLC, Rapid Resolution LC) to enhance separation
  - Use weak acid wash solution for on-line SPE negative ion methods to break up Na^+/analyte ion pairs

Matrix Effects (LC/MS-MS)

- Minimization of Matrix Effects
  - Maintain a clean interface / MS source!
  - Consider APCI or FAIMS
  - No need to change from an *established conventional method* to LC/MS-MS out of fashion.
  - If everything fails, consider GC/MS!

Other Problems (LC/MS-MS)

- Metabolites
  - Some metabolites dissociate in the ion-source resulting in the parent drug's m/z ion.
  - If these metabolites *are not separated* from the parent drug in chromatography, one can not distinguish between parent + metabolite in MS.
  - Examples of documented back-conversion: acyl-glucuronides, esters, N-oxides, lactone-rings
  - Since metabolites are often not commerically available, short run-times should be avoided for such drugs. BTW, metabolites may be extracted from urine…

Integration

- Peak ‘recognition’
- Automatic *vs.* manual
- Chromatography Data System (CDS)

Integration

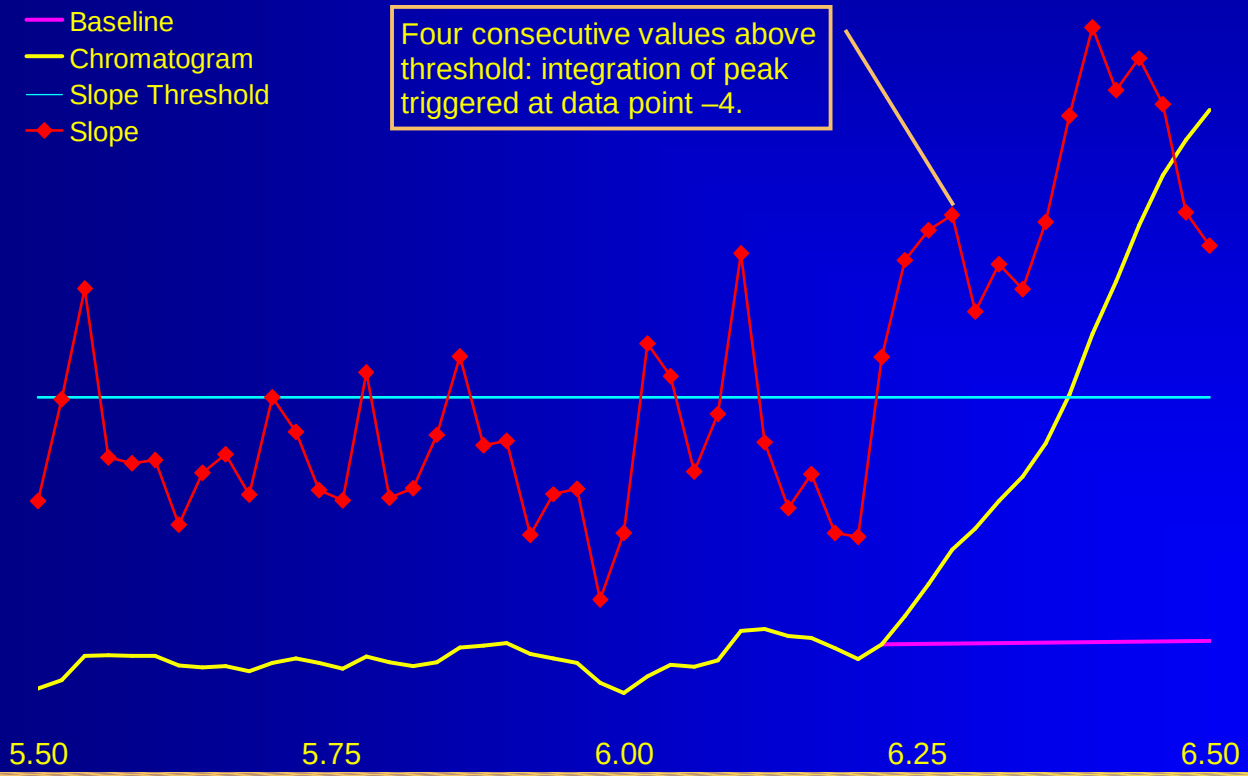
- Peak ‘recognition’
  - Detector delivers signal at high data rates
  - Raw signal is bundled to ‘peak slices’ based on an appropriate time constant. Rule of thumb: $w_{0.5}$ of the narrowest peak divided by 10–20. 10” peak $\rightarrow$ aquisition rate of 0.5 ”–1” (60–120 Hz).
  - Peak start and end ‘recognized’ by:
    - Noise threshold
    - Baseline drift: mainly important for gradient elution
    - Area threshold: values below this value are not followed

Integration

- Peak ‘recognition’
  - Peak start and end ‘recognized’ by:
    - Upward-/downward slope detection: The data system fits a couple of data points to a function (Polynomial, smoothing spline, Savistzky-Golay, …) and calculates the first derivative at each time point. If the derivative is positive and above the threshold = start of peak; if the slope is negative and below the threshold = end of peak. For a Gaussian peak upward- / downward thresholds would be the same, but in chromatography peaks are asymmetrical. Some data systems correct for that by using more slices if the slope is negative or even change to a different fitting algorithm.

Integration

- Peak ‘recognition’



Integration

- Automatic *vs.* manual
  - Integration parameters are saved in the CDS's method and work in the background
  - The automatic integration may fail:
    - Mainly for small peaks close to the LOQ
    - But also (rarely) for high peaks, when a series of positive random noise may trigger a 'end of peak' too early or negative random noise draws the baseline too late.
    - There is no *'correct'* integration for any given peak! The same raw data most likely will result in different values if exported to another CDS.
  - All chromatograms should be reviewed and the integration corrected if necessary

Integration

- Automatic *vs.* manual
  - The review has to be done before (!) concentrations are calculated. Changing integration of a peak in order to bring a calibrator / QC to the expected value (*e.g.*, make a batch valid which would be rejected otherwise) or a pre-dose concentration <LLOQ would be clear evidence of fraud.
  - Acceptable by current GLs (FDA 2001, EMA 2011). SOP in place; report which chromatograms were reintegrated (why, by whom, when: all the usual stuff needed for an audit trail).

Integration

- Automatic *vs.* manual
  - Example: LC/MS-MS, risperidone, protein precipitation, dilution factor 8, API 4000, software Analyst 1.4.1; 1 ng/mL and 0.1 ng/mL (LOQ)

integration method	1 ng/mL	0.1 ng/mL
	CV (n=10)	
automated (smoothing 1, bunching 2)	6.5%	15.1%
manual correction (one analyst)	6.3%	11.1%
manual correction (ten analysts)	5.2% (3.8% – 6.8%)	12.8% (6.9% – 16.0%)

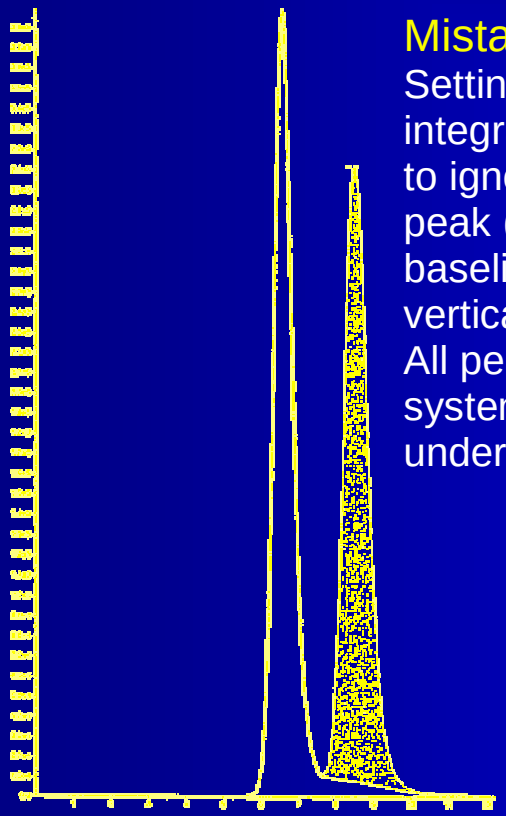
H Kirchherr, *Data Evaluation in LC-MS*
In: H-J Kuss and S Kromidas (eds.), *Quantification in LC and GC*, Wiley, p243–59 (2009)

Integration

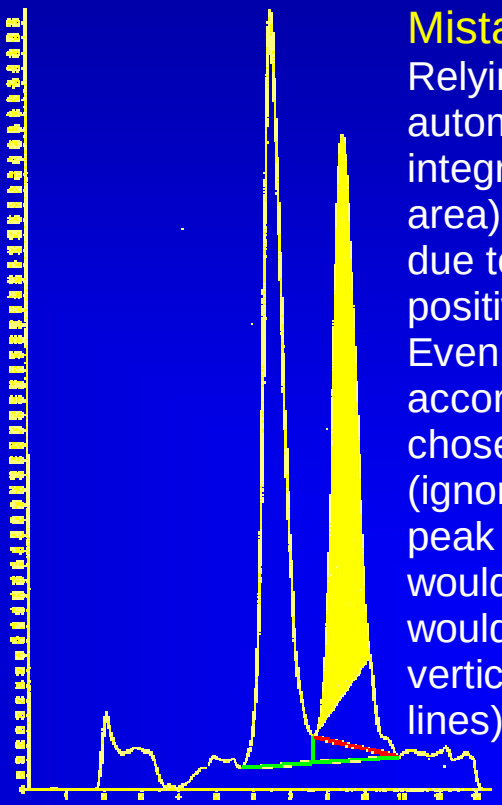
- Automatic *vs.* manual
  - Some analyst are afraid of getting problems in an inspection – believing automatic integration is the ‘gold standard’ and manual integration some kind of data manipulation.
    - Example: recent (June 2010) BE study, active *l*-enantiomer *vs.* racemate, LC/MS-MS; chromatograms of
      - high calibration standard
      - low QC sample

Integration

- Automatic *vs.* manual



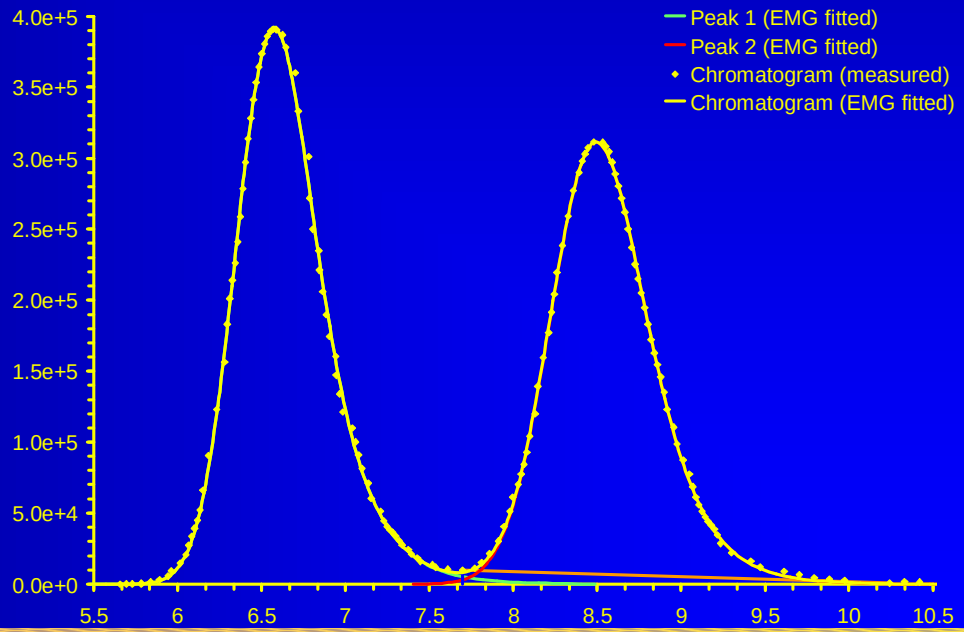
Mistake 1
Setting the integration method to ignore the first peak (tangential baseline instead of vertical drop).
All peak areas are systematically underestimated.



Mistake 2
Relying upon automatic integration (yellow area), which failed due to random positive noise.
Even a correction according to the chosen method (ignoring the first peak – red line) would be better. I would suggest a vertical drop (green lines).

Integration

- Automatic *vs.* manual
  - It would be possible to calculate peak areas by deconvolution. Not available in current CDS! Only supported by Merck / Hitachi's mid-1990ies D-7000 HPLC system manager (HSM v4.1) or external software (PeakFIT from Systat).



Integration

- Chromatography Data System (CDS)
  - Bundled with chromatograph / MS
    - Xcalibur® (Thermo Scientific)
    - Analyst® (Applied Biosystems/MDS Sciex)
    - EZChrome Elite (Agilent Technologies)
    - Empower™ (Waters)
    - Chromeleon® (Dionex)
    - LabSolutions (Shimadzu)
  - Commercial, vendor independent
    - PowerChrom® (eDAQ)
  - Cross-platform freeware
    - ezDataPowerChrom® (chemilab.net)
  - Deconvolution
    - PeakFIT® (Systat)

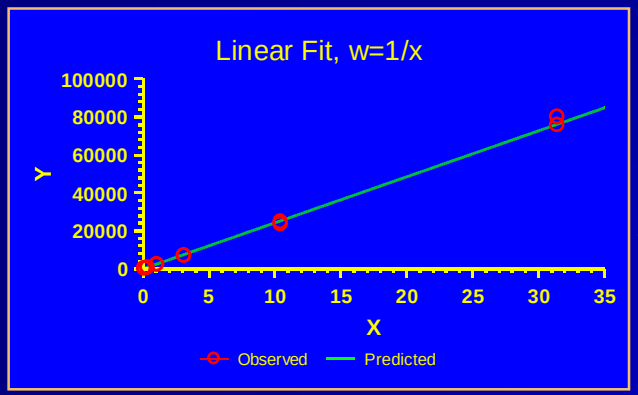
Integration

- Chromatography Data System (CDS)
  - Important points
    - Audit Trail?
    - Data transfer to LIMS?
    - Data format: Preferable not only the integration parameters, but the raw peak slices are stored.
      - ANDI/netCDF (AIA) Chromatography Data Interchange Format (ASTM standard E1947-98)
      - Last resort: CSV (Character Separated Variables)
    - FDA 21 CFR Part 11 compliant (rarely; ask!)
    - If possible data should not be stored only at the instrument's PC, but copied to a central location for secured backup.
    - Ask the CRO for a DVD with raw data files.

Calibration

- Model Selection
  - Linear $y = A + Bx$
  - Quadratic $y = A + Bx + Cx^2$
  - 4-parameter logistic (LBA) $y = D + \frac{(A - D)}{\left(1 + \left(\frac{x}{C}\right)^B\right)}$
- Weighting Schemes
  - Unweighted (simple linear regression; not recommended)
  - Weighted $w=1/x$, $w=1/x^2$, $w=1/s^2$
- Working Range (accurate and precise within LLOQ and ULOQ)

Calibration



Sum of squared residuals:
0.194707E+08

Sum of weighted squared residuals:
473468

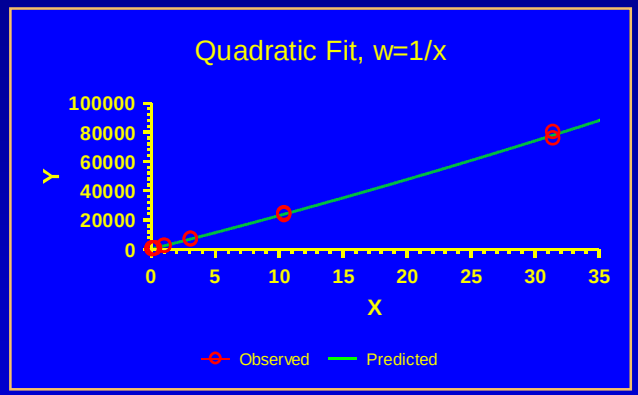
S:
217.593 (df =10)

Correlation:
0.9992

Weighted correlation:
0.9989

AIC:
160.81407

4 runs:
++--------+-



Sum of squared residuals:
0.965269E+07

Sum of weighted squared residuals:
155546

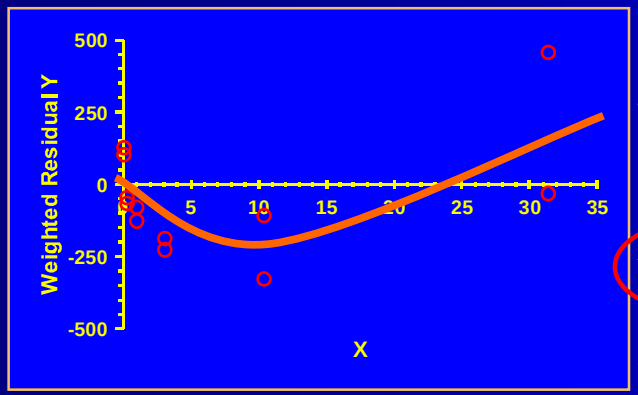
S:
131.465 (df=9)

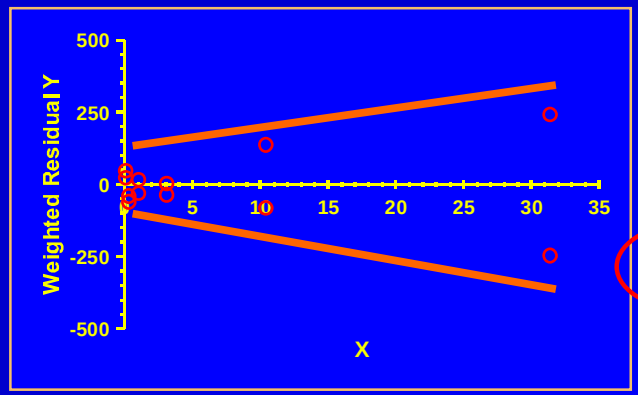
Correlation:
0.9995

Weighted correlation:
0.9996

AIC:
149.45640

6 runs:
++---++--++-



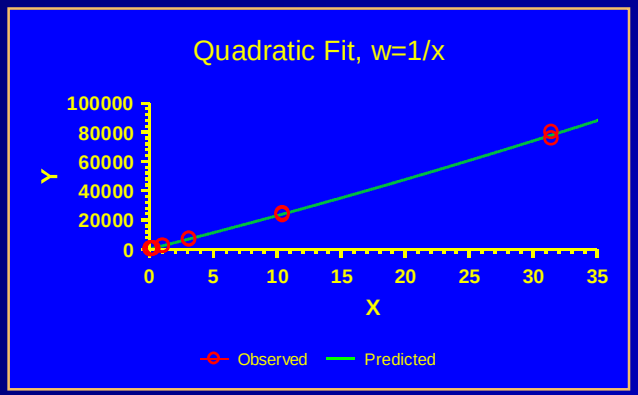


Calibration

Back-calculated standards (linear, 1/x)			
nominal	Acc [%]	mean	CV [%]
0.102	125.09	122.7	2.77
0.102	120.29		
0.313	94.58	93.4	1.78
0.313	92.23		
1.045	91.95	93.4	2.23
1.045	94.90		
3.107	93.15	92.4	1.09
3.107	91.73		
10.42	93.47	95.6	3.21
10.42	97.81		
31.42	105.19	102.4	3.84
31.42	99.62		

Back-calculated standards (quadr., 1/x)			
nominal	Acc [%]	mean	CV [%]
0.102	109.96	107.3	3.46
0.102	104.70		
0.313	94.76	93.5	1.94
0.313	92.19		
1.045	97.81	99.4	2.28
1.045	101.02		
3.107	100.06	99.3	1.08
3.107	98.54		
10.42	98.31	100.5	3.10
10.42	102.72		
31.42	102.40	100.0	3.47
31.42	97.50		

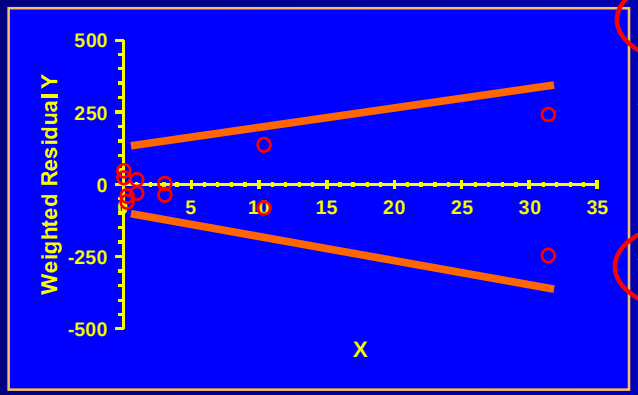
Calibration



Sum of squared residuals:
0.965269E+07

Sum of weighted squared residuals:
155546

S:
131.465 (df=9)

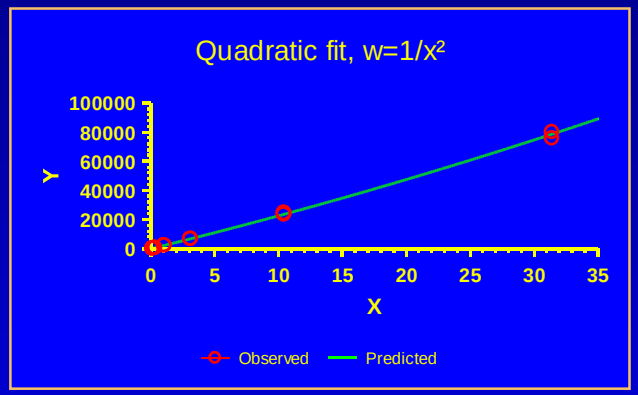


Correlation:
0.9995

Weighted correlation:
0.9996

AIC:
149.45640

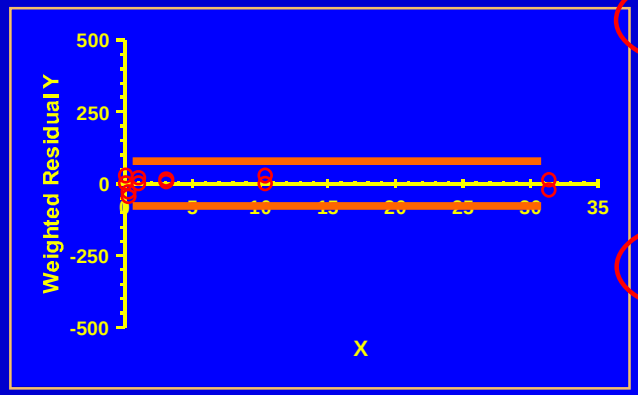
6 runs:
++---++--++-



Sum of squared residuals:
0.924405E+10

Sum of weighted squared residuals:
5751.13

S:
25.2787 (df =9)



Correlation:
0.9994

Weighted correlation:
0.9990

AIC:
109.88582

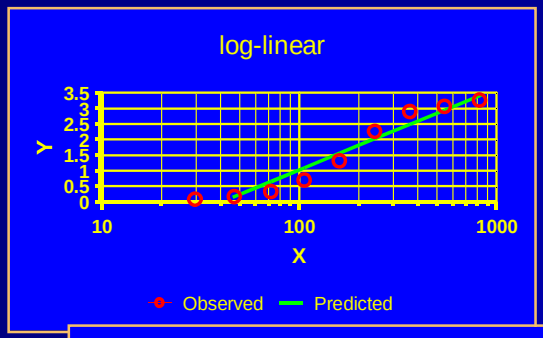
6 runs:
++--++++-++-

Calibration

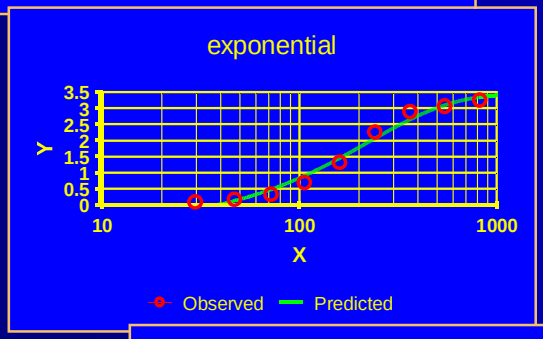
Back-calculated standards (quadr., 1/x)			
nominal	Acc [%]	mean	CV [%]
0.102	109.96	107.3	3.46
0.102	104.70		
0.313	94.76	93.5	1.94
0.313	92.19		
1.045	97.81	99.4	2.28
1.045	101.02		
3.107	100.06	99.3	1.08
3.107	98.54		
10.42	98.31	100.5	3.10
10.42	102.72		
31.42	102.40	100.0	3.47
31.42	97.50		

Back-calculated standards (quadr., $1/x^2$)			
nominal	Acc [%]	mean	CV [%]
0.102	105.60	102.9	3.74
0.102	100.16		
0.313	95.19	93.9	1.99
0.313	92.54		
1.045	100.01	101.7	2.29
1.045	103.31		
3.107	102.52	101.7	1.08
3.107	100.96		
10.42	99.91	102.1	3.06
10.42	104.33		
31.42	101.69	99.3	3.37
31.42	96.95		

Calibration (LBA Example)



Correlation:
0.9715
AIC:
1.76539



Correlation:
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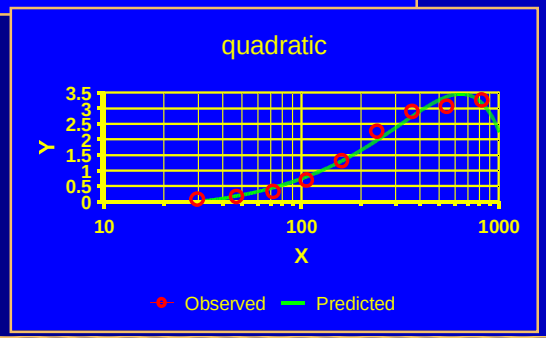
AIC:
-6.47417



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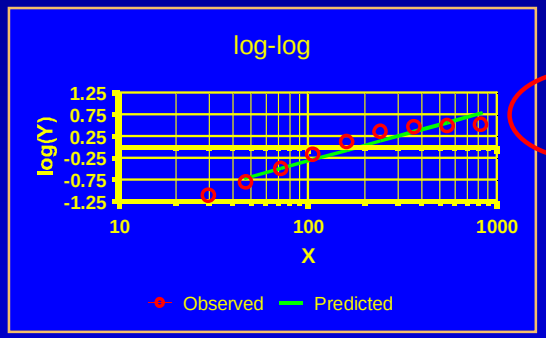

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AIC:
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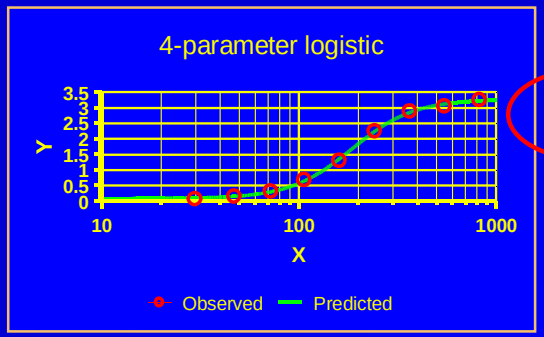

Correlation:
0.9996
AIC:
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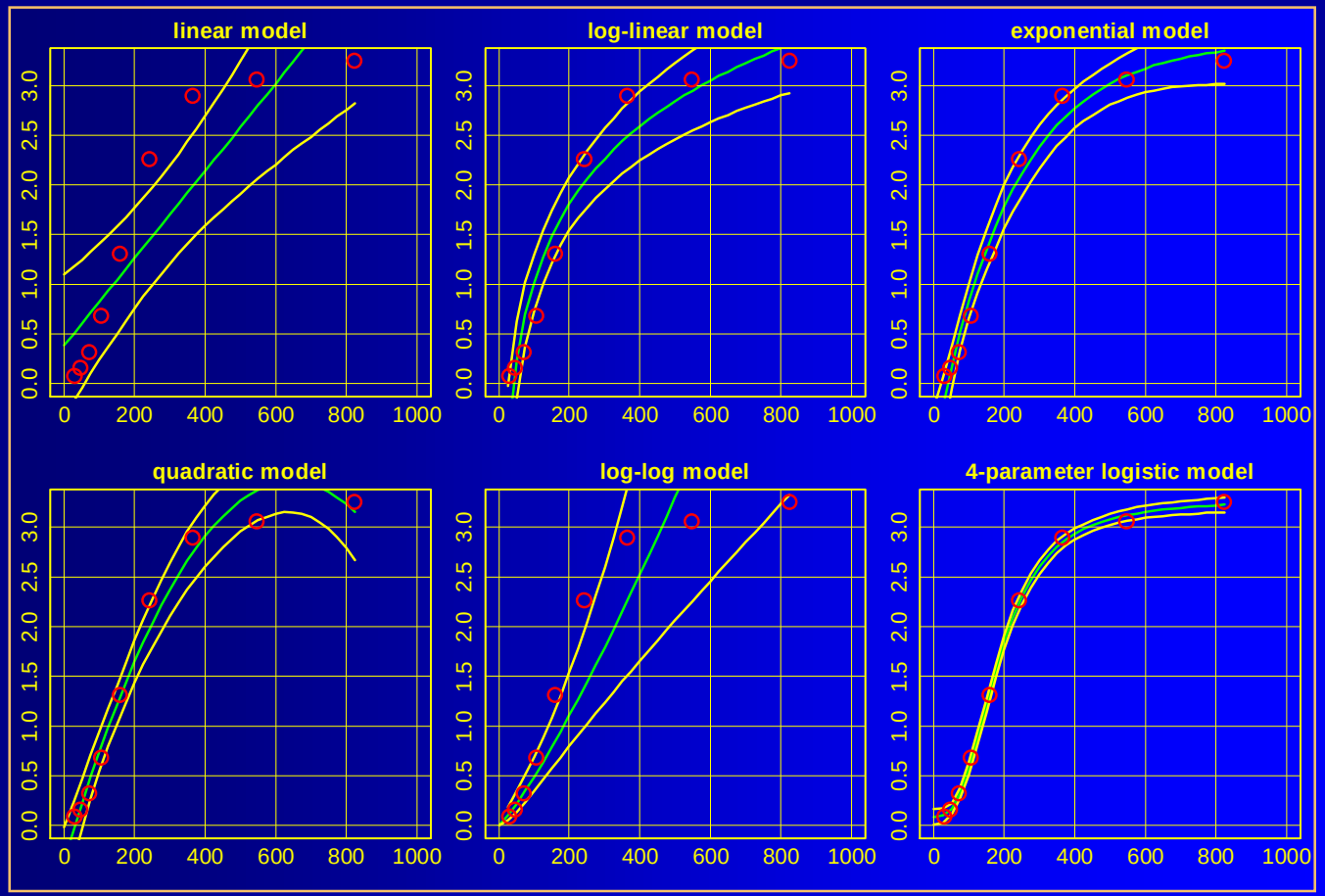
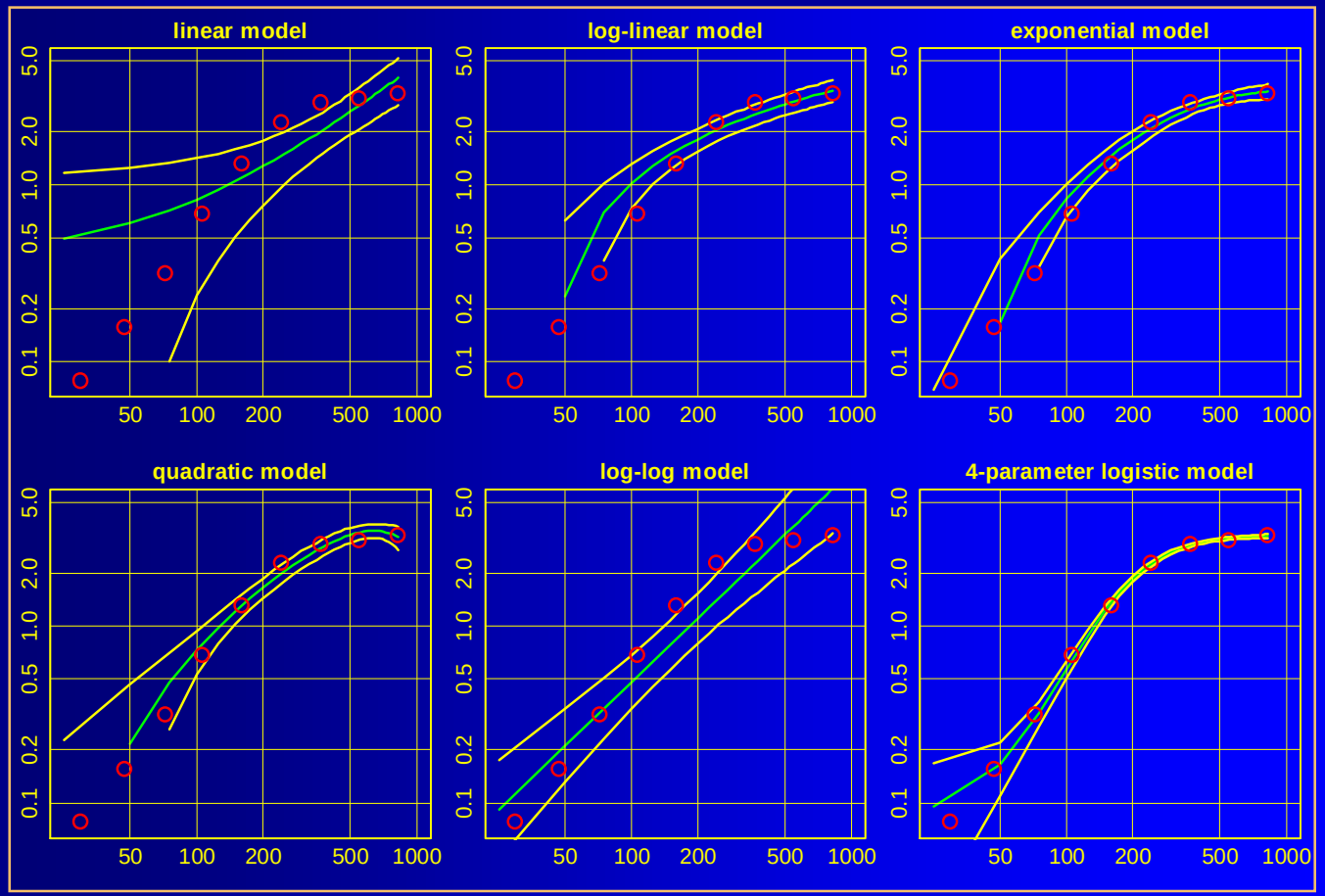
Example data (monoclonal antibody enzyme-linked immunosorbent assay) from Findlay & Dillard (2007)

Calibration (LBA Example)



Calibration (LBA Example)



Calibration (LBA Example)

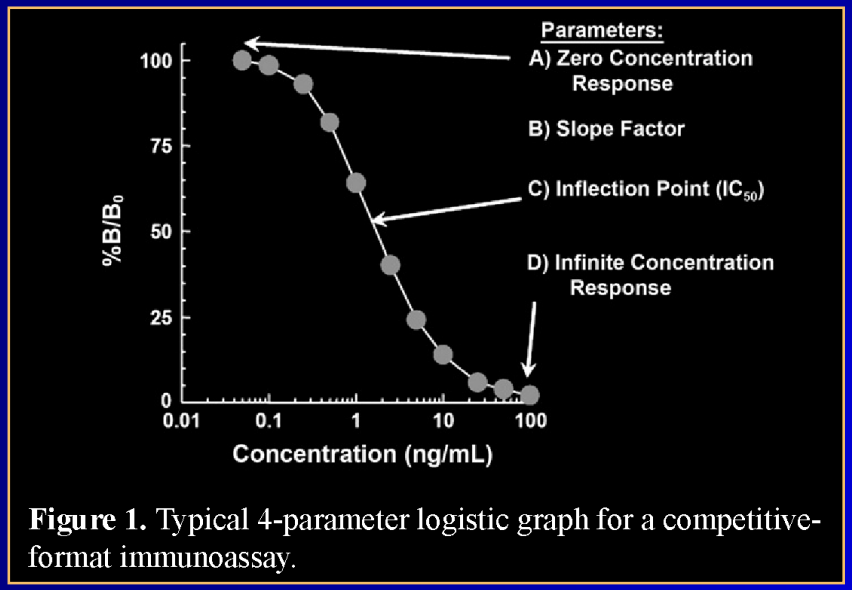


Figure 1. Typical 4-parameter logistic graph for a competitive-format immunoassay.

$$y = D + \frac{(A - D)}{\left(1 + \left(\frac{x}{C}\right)^{B}\right)}$$

Thank You!

Development of Bioanalytical Methods for BE Studies

Open Questions?

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