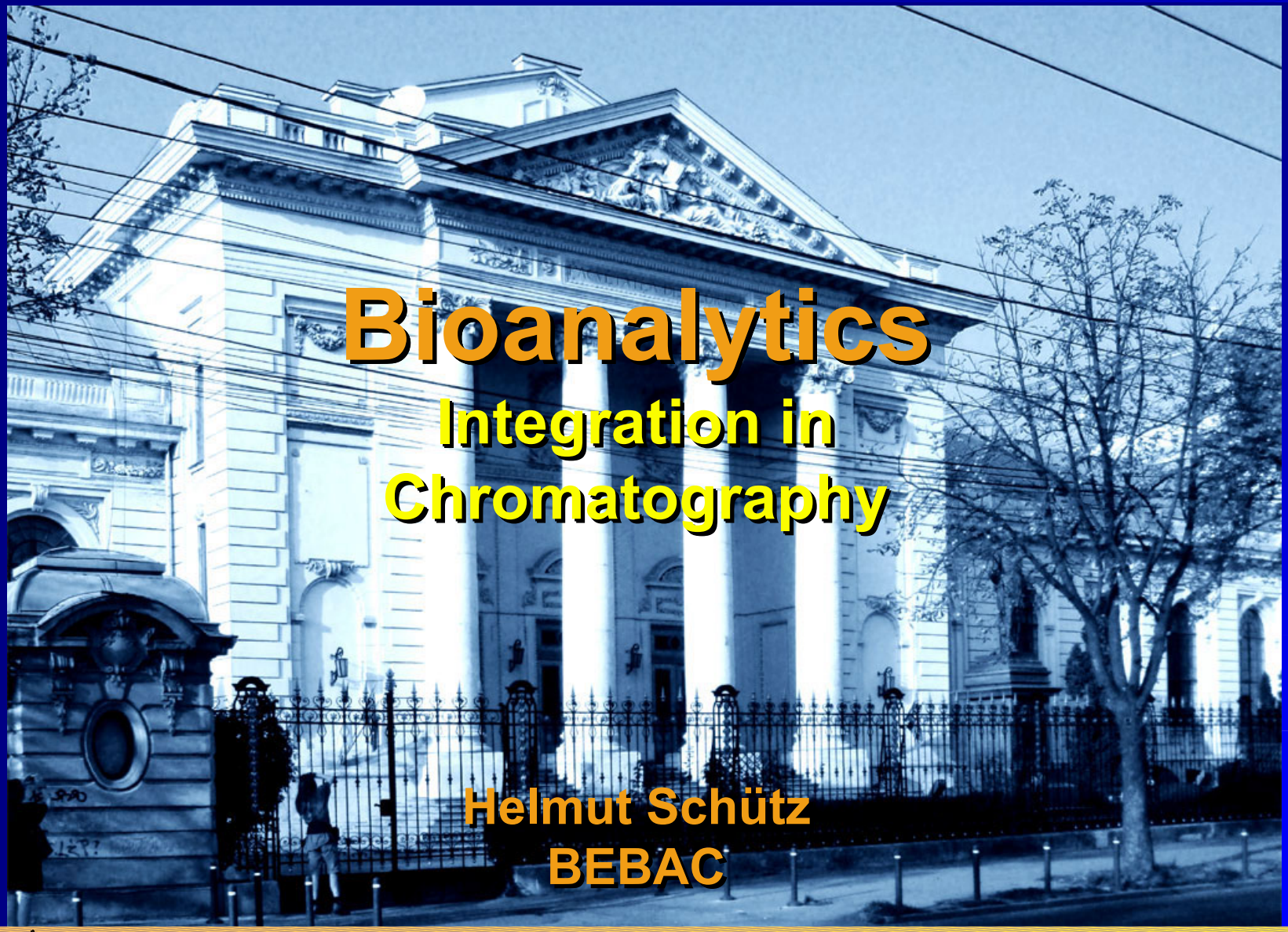


Bioanalytics

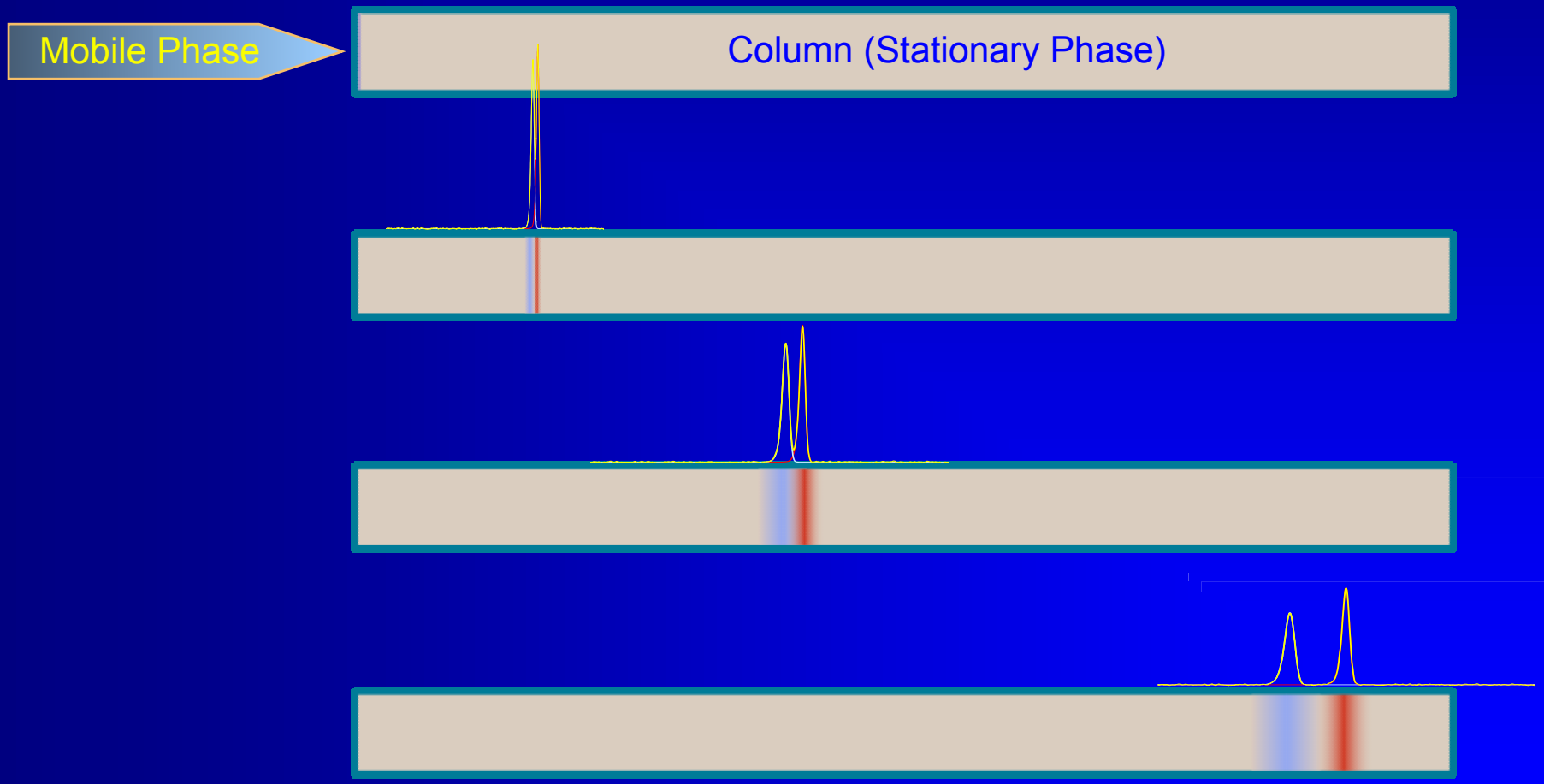
Integration in Chromatography

Helmut Schütz
BEBAC

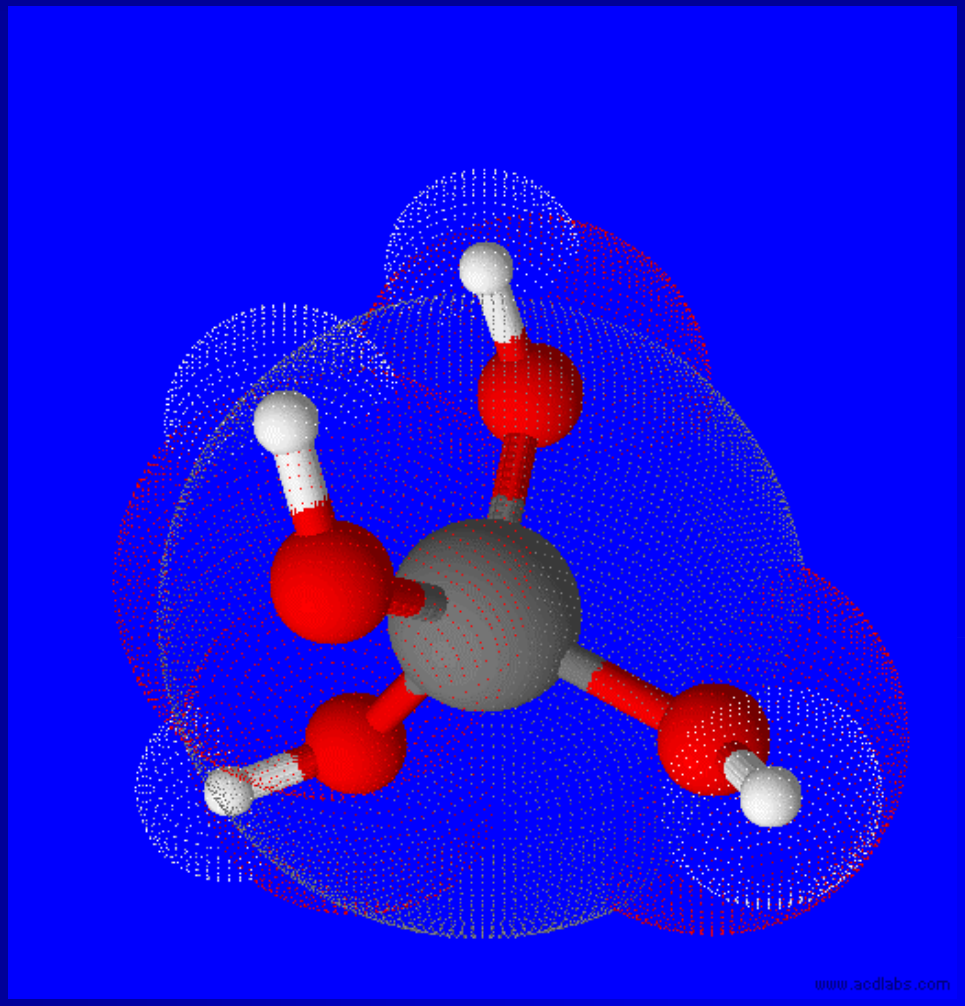
Chromatography

- Substances (analytes & interferences) continuously exchange between Mobile and Stationary Phases
  - Different Mechanisms 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,…

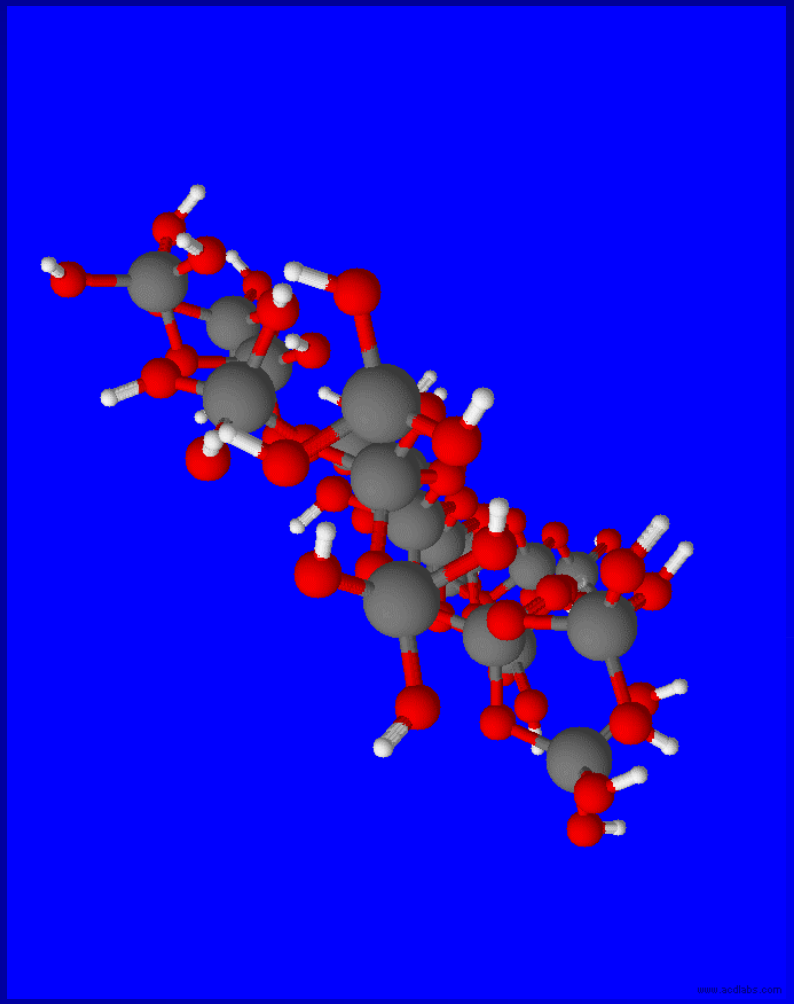
Chromatography



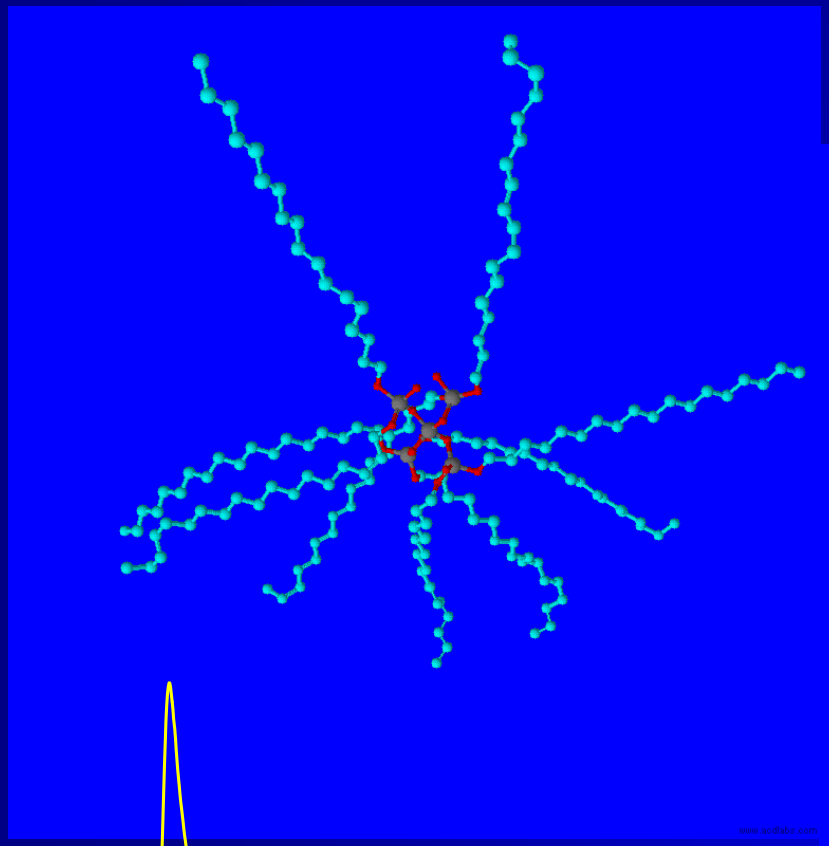
Orthosilic Acid (H_4SiO_4)



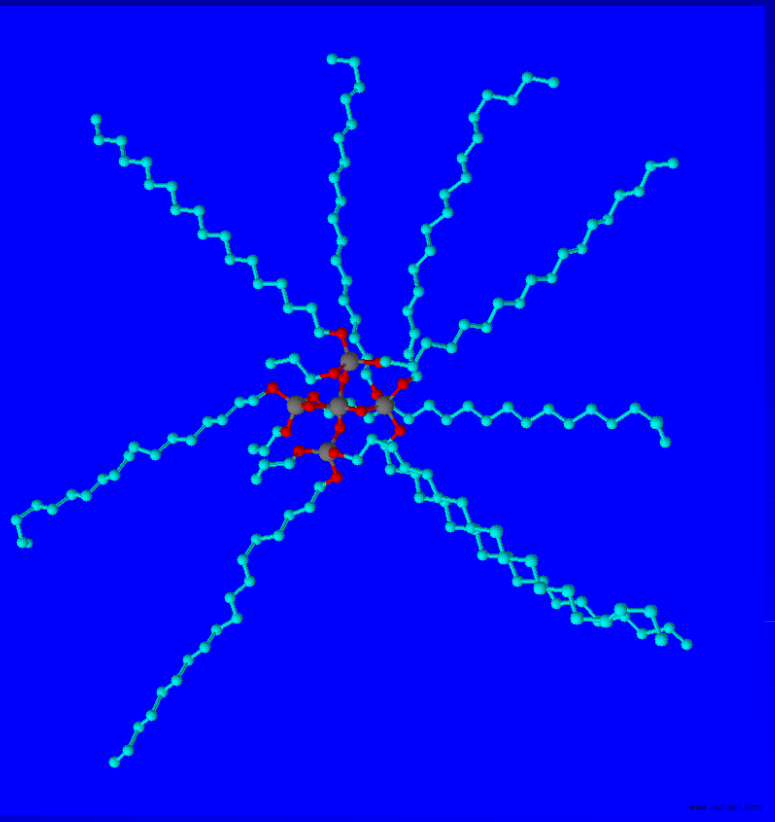
Polysilic Acid



C18 Reversed Phase

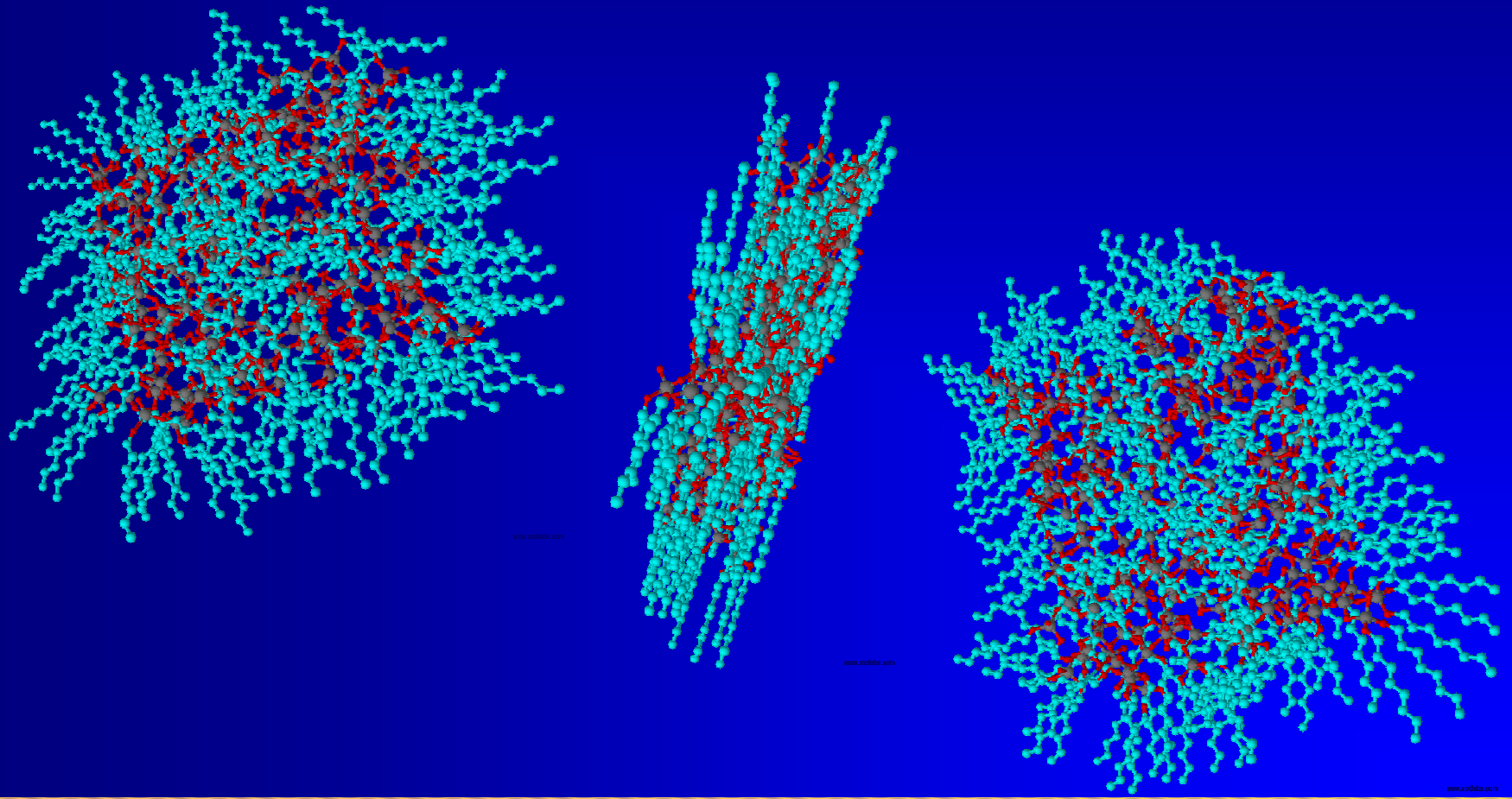


Residual OH-Groups lead to ‘Peak Tailing’ of Basic Compounds

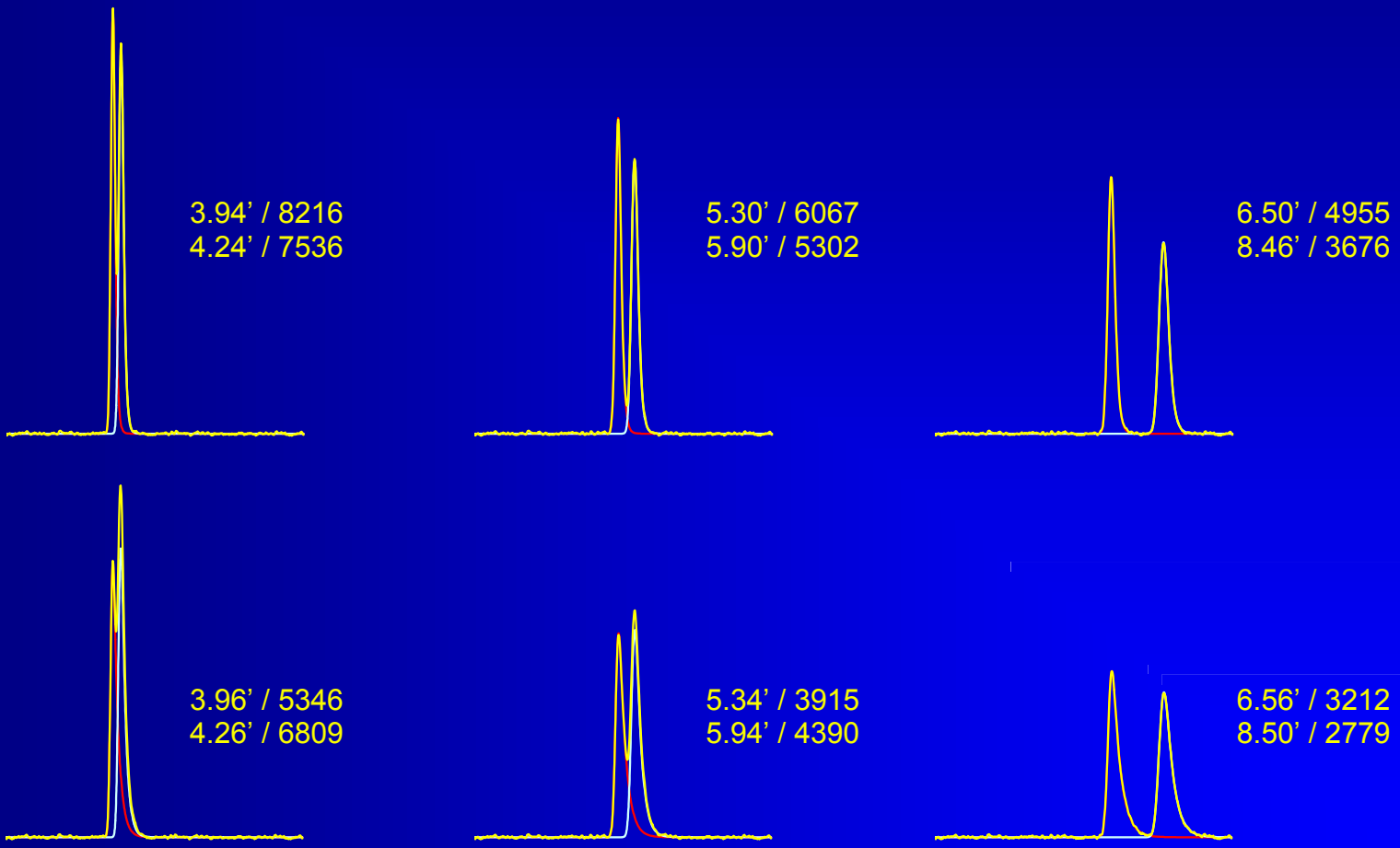


Resolved by ‘Encapping’ with C4

C8 Reversed Phase

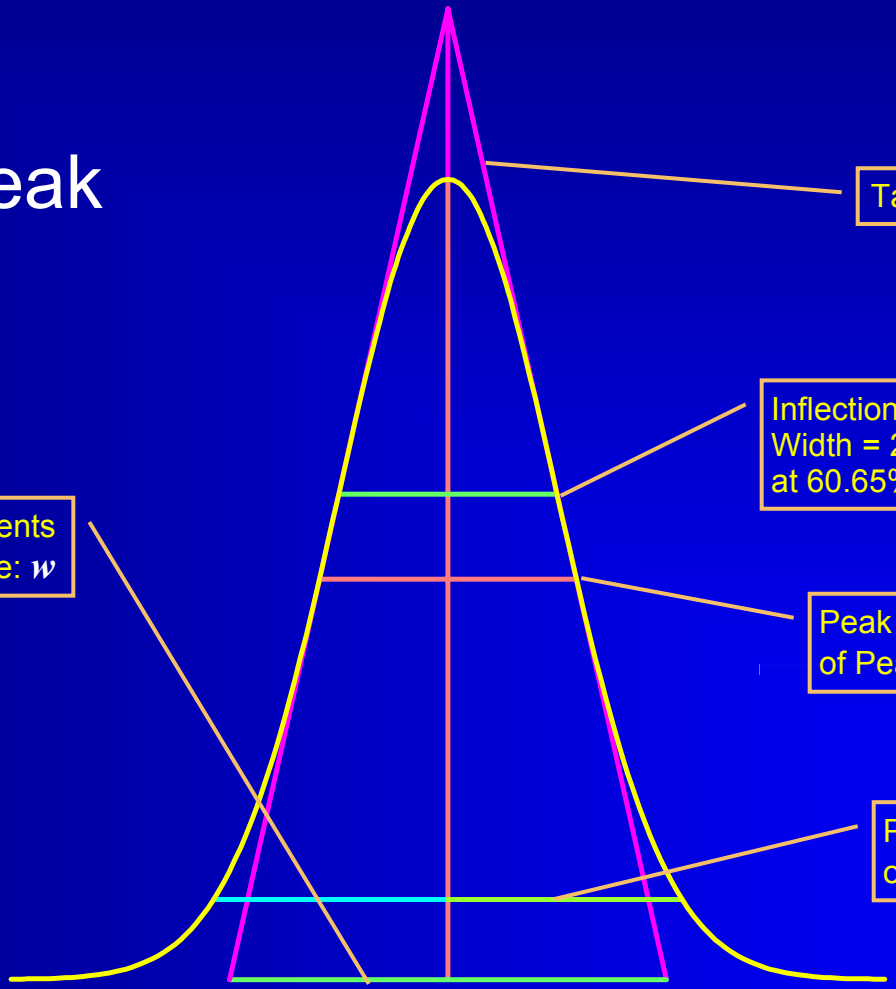


Retention Time and Tailing

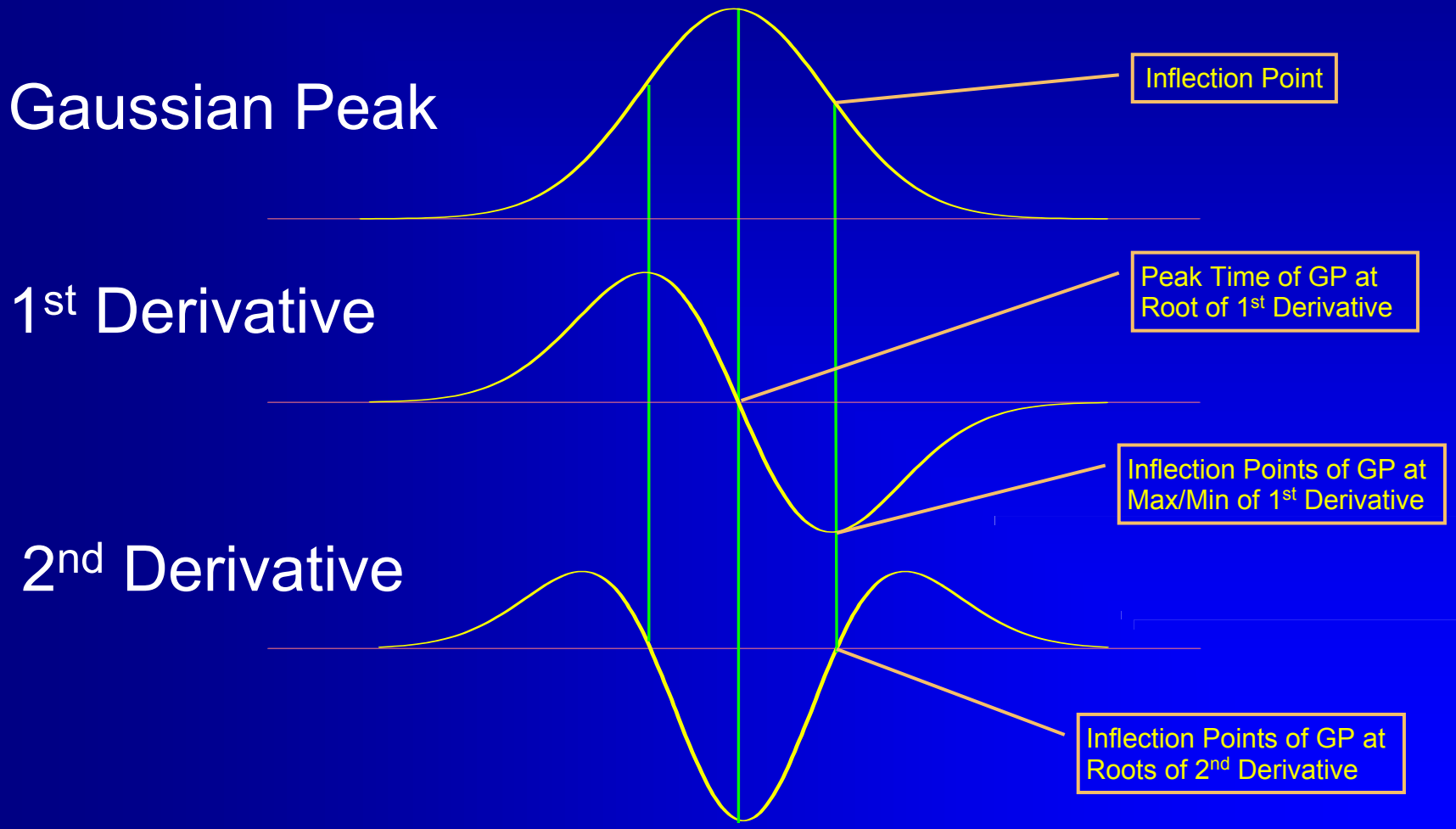


Characteristics of Peaks

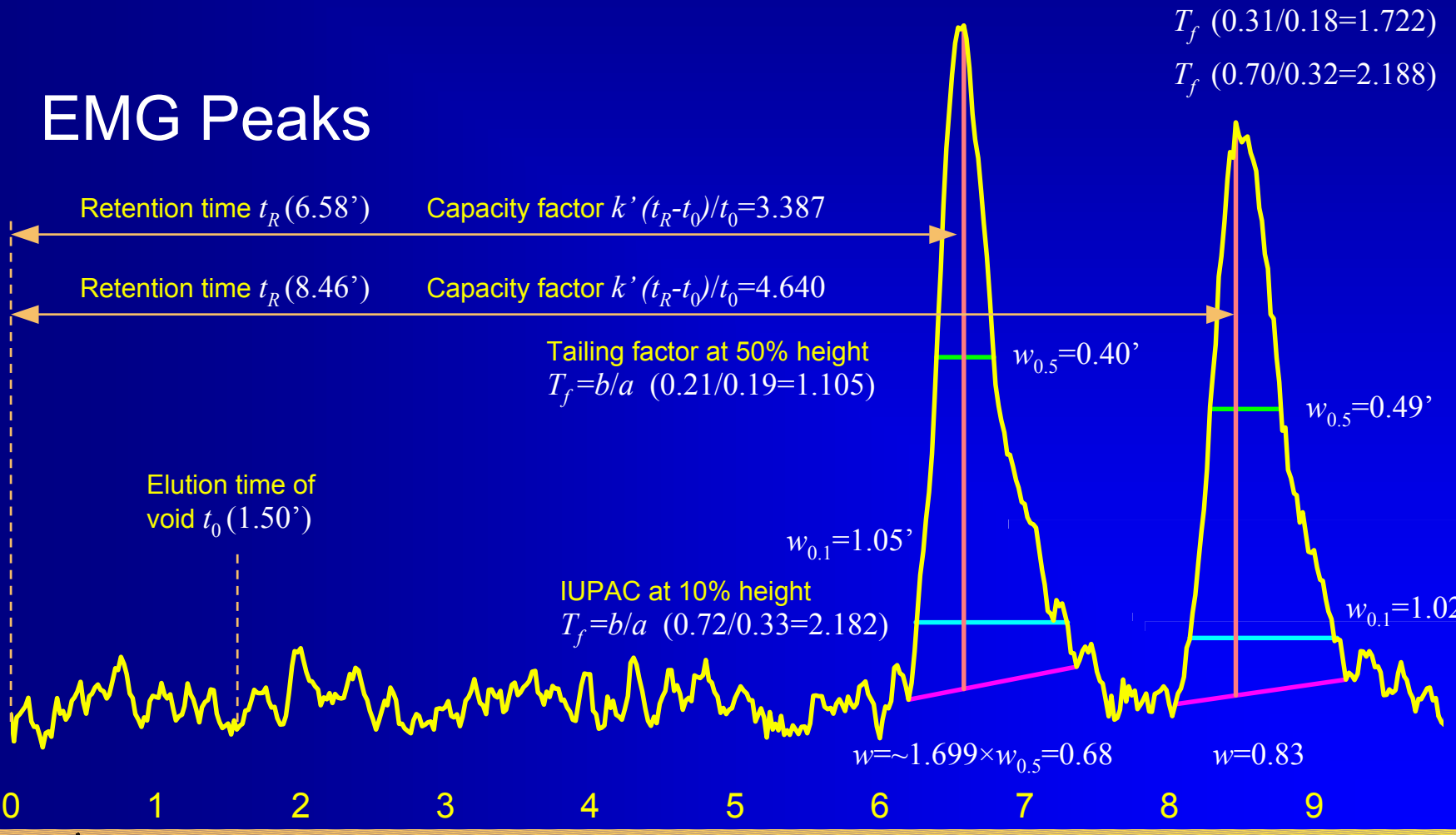
Gaussian Peak



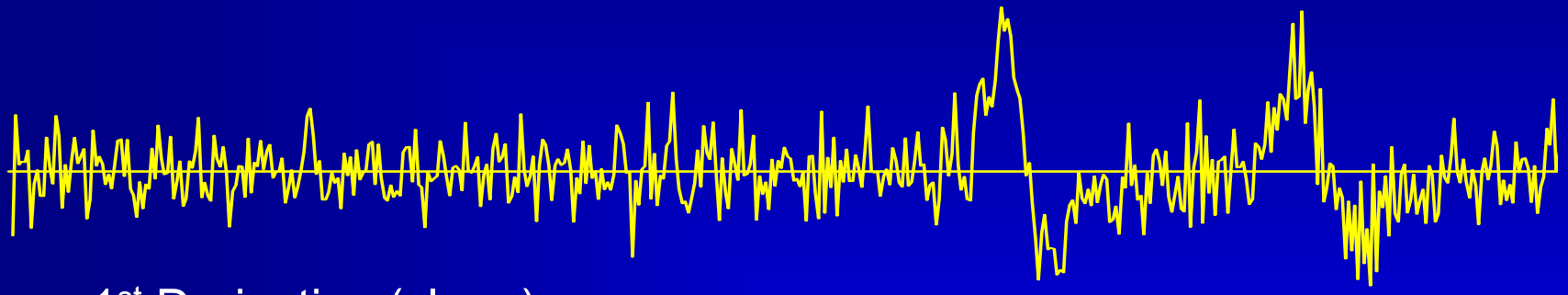
Characteristics of Peaks



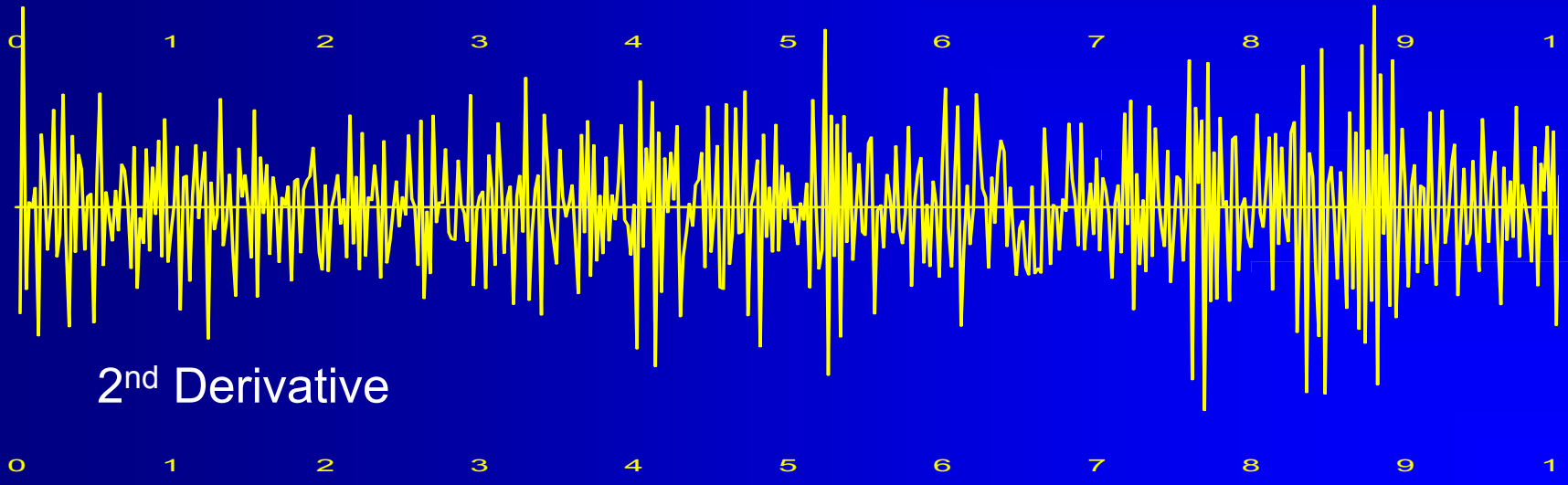
Characteristics of Peaks



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
  - $R_s = 2 \times (t_{R_2} - t_{R_1}) / (w_1 + w_2)$
    Baseline width w not 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
  - IUPAC at 10% of peak height:

    0.72/0.33=2.18 ✗

    0.70/0.32=2.19 ✗

    Although >2, acceptable for a chiral method where columns show limited 'separation power' in general.
  - at 50% of peak height:

    0.21/0.19=1.11 ✓

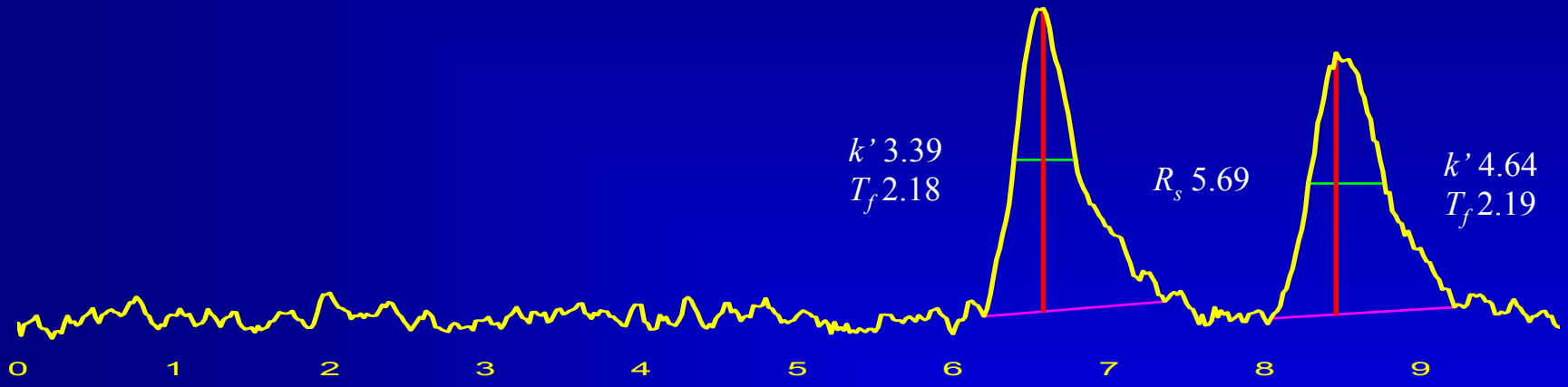
    0.31/0.18=1.72 ✓

    <2 – avoid IUPAC's method!

Recommendations

- Run times
  - The longer, the better the separation – but
  - Peak heights will decrease (band broadening → worse LLOQ)
- 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!

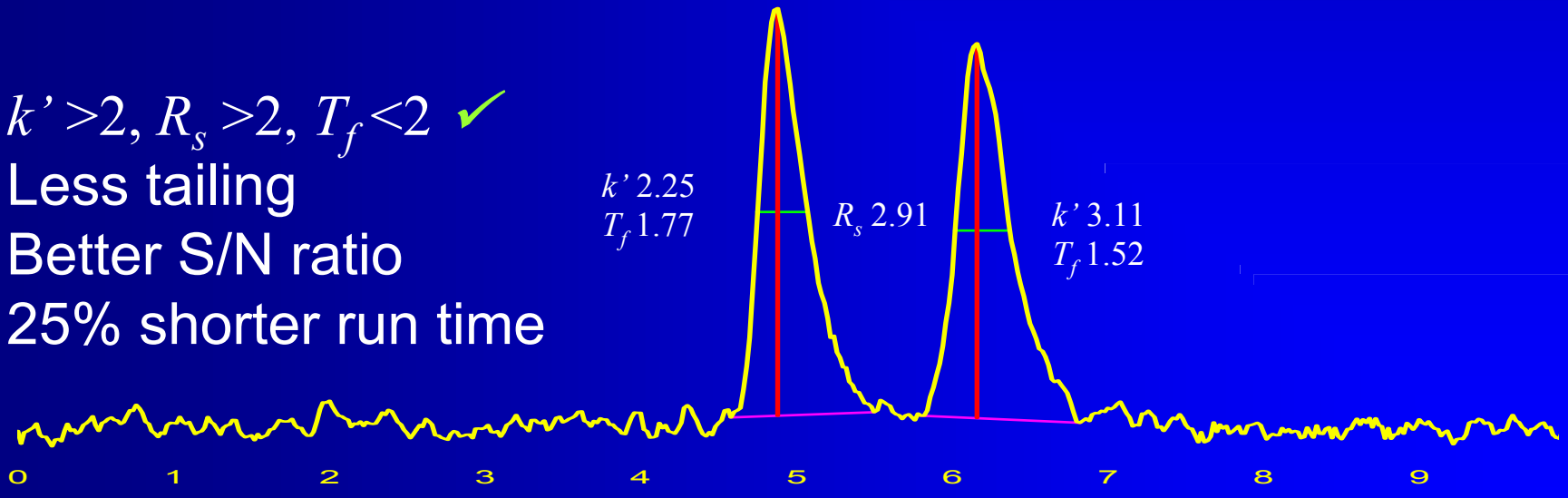


$k' > 2$, $R_s > 2$, $T_f < 2$ ✔

Less tailing

Better S/N ratio

25% shorter run time



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 → aquisition rate of 0.5–1” (60–120 Hz).
  - Peak start and end depends on:
    - Noise threshold
    - Baseline drift (mainly important for gradient elution)
    - Area threshold (peaks below this value are not integrated)

Integration

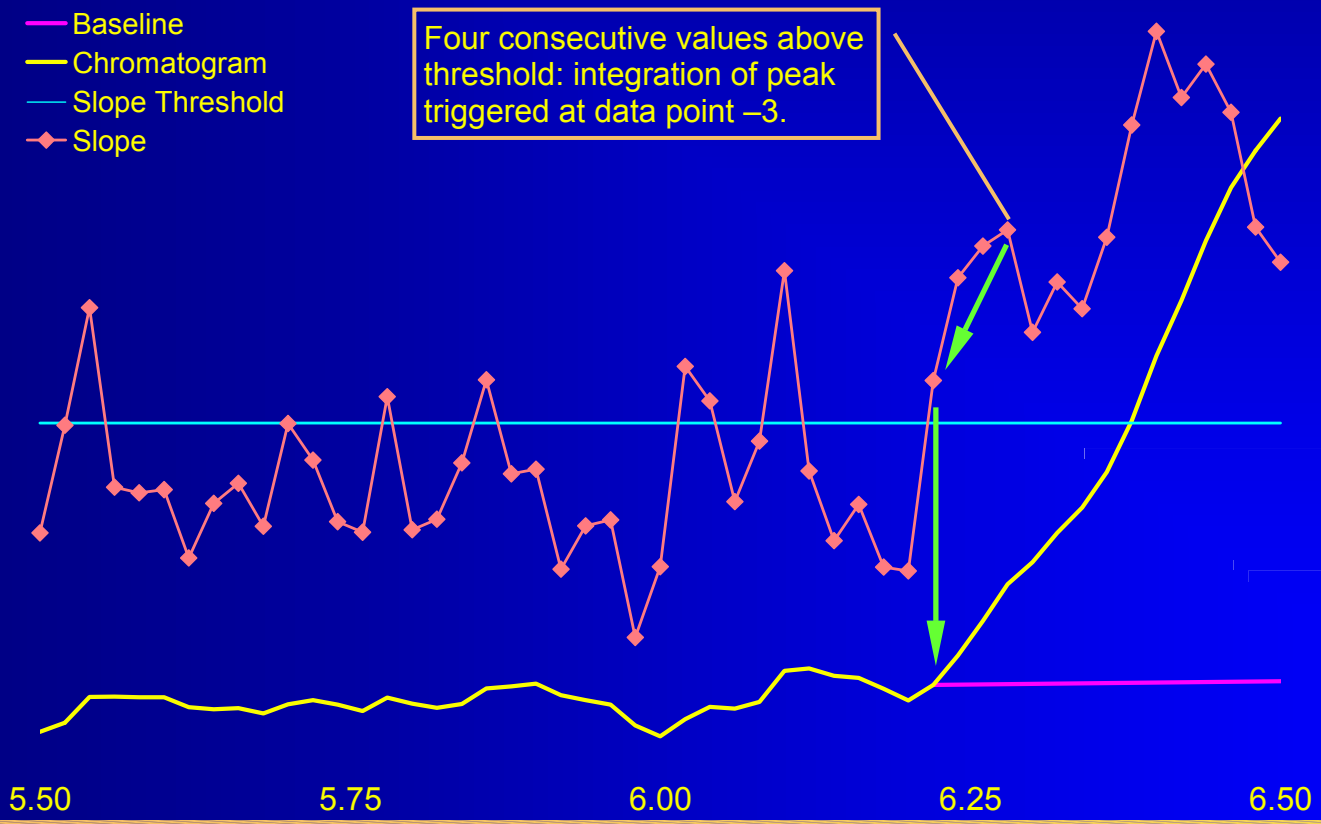
- Peak ‘recognition’
  - Peak start and end triggered by:
    - Upward-/downward slope detection:
      The data system fits a couple of data points to a function (moving average, polynomial, smoothing spline, Savitzky-Golay, …) and calculates the first derivative at each time point.
      If the derivative is positive and ≥ the threshold
      → start of peak;
      if the slope is negative and ≤ the threshold
      → end of peak.

Integration

- Peak ‘recognition’
  - Peak start and end triggered by:
    - Upward-/downward slope detection:
      For a Gaussian peak upward- / downward thresholds are the same, but in chromatography peaks are always 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

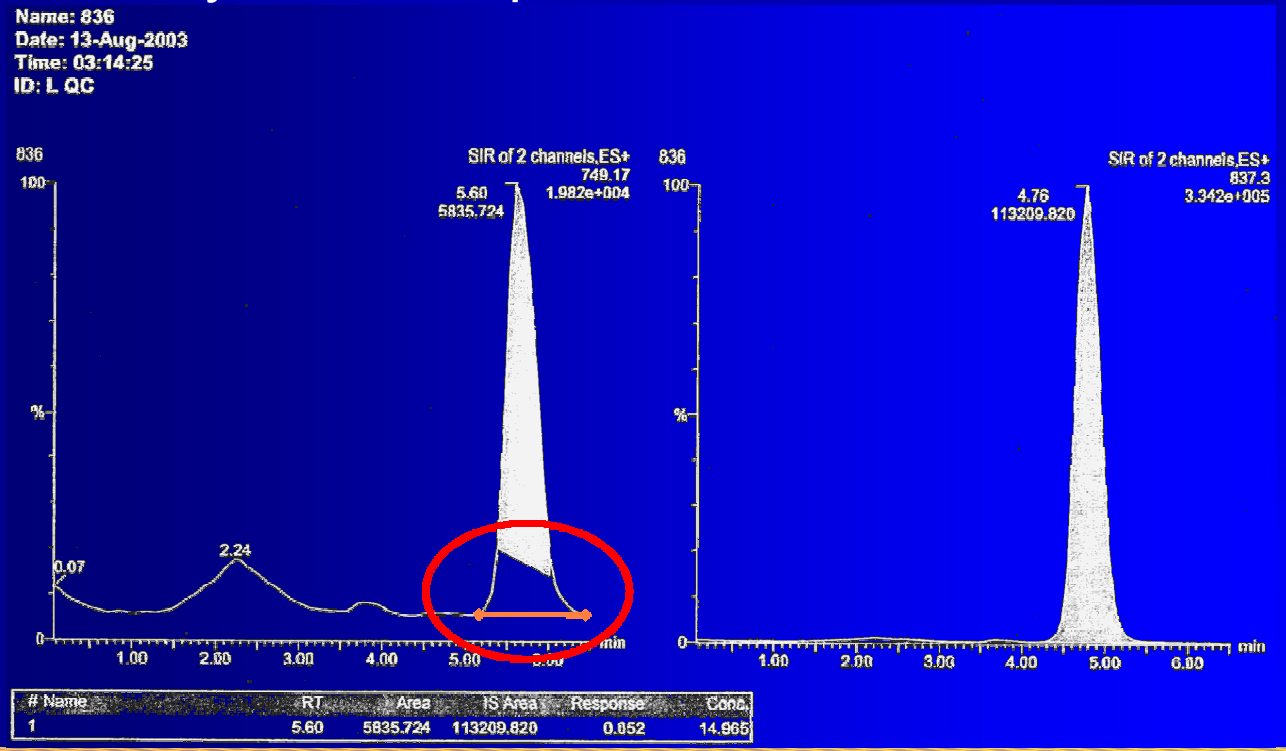
- Automatic *vs.* manual
  - Integration parameters are saved in the CDS' method and work in the background
  - The automatic integration may fail:
    - Mainly for small peaks close to the LLOQ
    - But also (rarely) for high peaks,
      if a series of positive random noise triggers an 'end of peak' too early or
      negative random noise draws the baseline too late.
    - There is no *'correct'* integration for any given peak!
      Identical raw data most likely will result in different values if evaluated by another CDS.

Integration

- Automatic *vs.* manual
  - All chromatograms should be reviewed and the integration corrected if necessary
  - The review has to be done before concentrations are calculated.
    Changing integration of a peak in order to force a calibrator / QC towards the expected value (*e.g.*, make a batch valid which would be rejected otherwise) or a pre-dose concentration <5% C_{max} would be clear evidence of fraud.

Integration

- Automatic *vs.* manual
  - Do not try to fool inspectors!



Integration

- Automatic *vs.* manual
  - Review and manual correction acceptable according to current GLs (FDA 2001, EMA 2011)
    - SOP in place
    - Consistently across the study's chromatograms
    - Report which chromatograms were reintegrated (why, by whom, when – all the usual data 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 (at LLOQ)

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%)

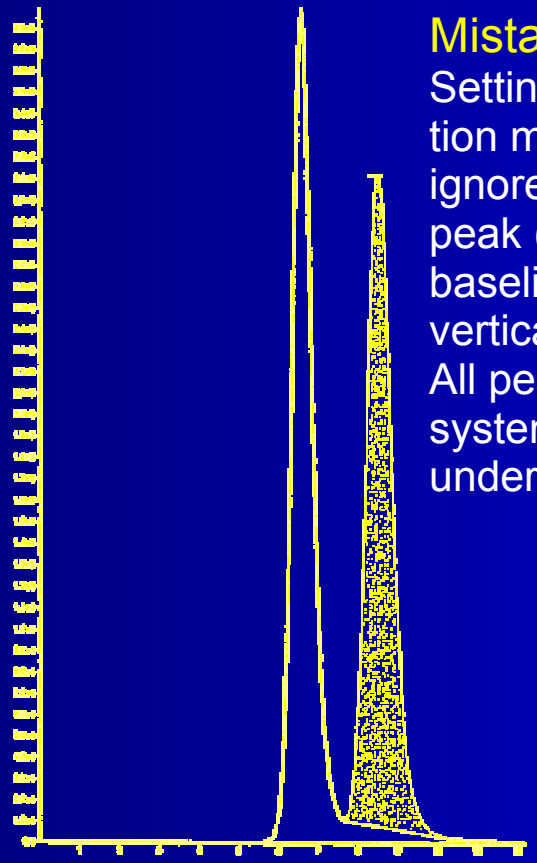
H Kirchherr, *Data Evaluation in LC-MS*
In: H-J Kuss and S Kromidas (eds.), Quantification in LC and GC, Wiley, p243-259 (2009)

Integration

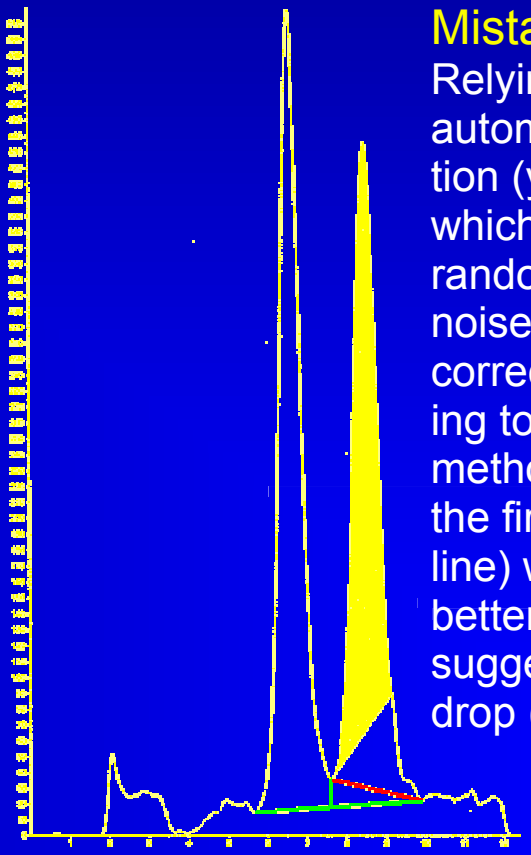
- Automatic *vs.* manual
  - Some analyst are afraid of getting problems in an inspection – believing automatic integration is the 'gold standard' and manual integration some kind of data manipulation.
    - Example:
      Fairly recent (06/2010) BE study, active *l*-enantiomer *vs.* racemate, LC/MS-MS; 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.

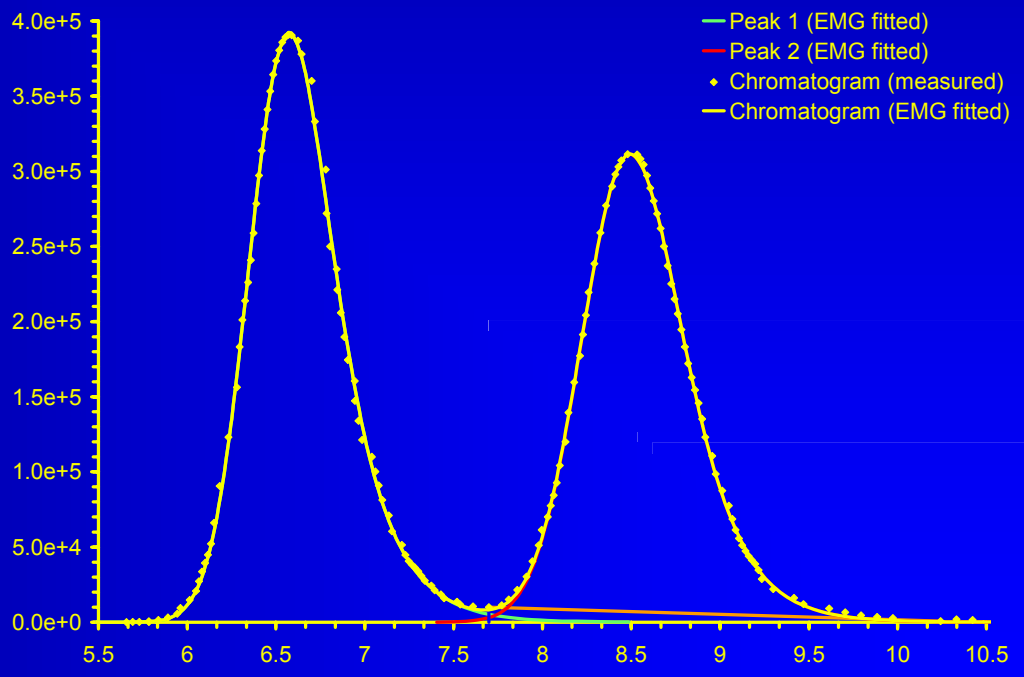


Mistake 2
Relying upon automatic integration (yellow area), which failed due to random positive noise. Even a correction according to the chosen method (ignoring the first peak – red line) would be better. I would suggest a vertical drop (green lines).

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-90s 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.
    - Provide the sponsor a DVD with raw data files.

Thank You!

Integration in Chromatography

Open Questions?

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