Optimization of chromatographic method

Examples from LC&GC Europe

Aims?

- "Develop the method until it is good enough for your aims, then stop!"
  - Every method can be improved
  - Method developments takes a lot of time

Factors to think about?

- Number of samples
- Run length
- Number of analytes
- Number of matrices
- Sensitivity
- Repeatability
- Reproducibility
- Trueness
- Concentration range
- Limitations of apparatus
- Sample preparation
- Validation

Type of chromatography?

- Reversed phase
- Normal phase
- Ion chromatography
- Ion-pair chromatography
- HILIC
- Size exclusion chromatography

Parameters for check

- Resolution (R_s)
  - Retention factor (k)
  - Factor of asymmetry (A_s)
  - Column efficiency as plate number (N)

Separation depends on?

$$R_s = \frac{1}{4}\frac{k}{k+1}(\alpha - 1)\sqrt{N}$$

$I \quad II \quad III$

- Retention
- Selectivity
- Efficiency (plate number)

Which method parameter you could change?

Table 2: Chromatographic variables.

Variable	Change in α	Universal	Convenient	Low-UV/ LC–MS	Robustness	Equilibration
%B	0	+	+	+	+	+
Temperature	–	+	+	+	+	+
Solvent type	++	+	+	0	+	0
Ion pair	+	–	+	0	–	–
pH	+++	–	0	0	–	+
Column type	+	+	0	+	+	+

7

Starting point

- C18 or C8 column
  - 150 mm × 4.6 mm 5 µm
  - 100 mm × 4.6 mm, 3 µm
  - 1–2 mL/min
- In case of LC/MS
  - 50 mm × 2.1 mm 3 µm
  - 0.2–0.5 mL/min

It is assumed that LC/MS does not require so much resolution. It is not always true!

8

Starting point

- Silica based columns
  - 2 < pH < 8
  - for UV detection phosphate buffer 15-25 mM pH = 2.0...3.0
  - MS requires volatile buffer
    - 0.1% formic acid
  - Basic compound may need a specific column pH > 8.0

9

Starting point

- Organic solvent
  - MeCN
  - MeOH
    - Not for UV detection <220 nm
  - THF
    - Not with PEEK capillary
    - Not for UV detection <240 nm

10

1. Retention

- Good if $2 < k < 10$
  - acceptable $1 < k < 20$
- $k < 2$
  - Separation is very sensitive to small changes in the mobile phase
  - Interrupting dead time peak

11

1. Retention

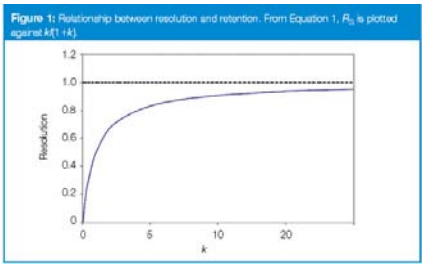


Figure 1: Relationship between resolution and retention. From Equation 1, R_S is plotted against $k/(1+k)$.

12

1. Retention

- Increasing $k = 0.5$ to $k = 2$?
  - Separation improves 3 times
  - Large dead time peak
- Increasing $k = 10$ to $k = 20$?
  - Separation improves ca 5%
  - Run time increases 2 times

13

1. Retention and analyses time

- Increasing k also increases run time
- Time can be decreased with increasing flow rate
  - HPLC pressure limit is 400 bar
  - UHPLC/UPLC pressure limit is 1600 bar
  - Most columns today do not give less separation at higher flow rates

14

1. Optimizing retention (isocratic elution)

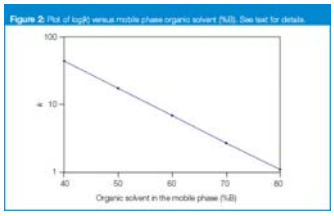
- Start with 100% organic eluent
- Add water phase with small steps (e.g. 10%)
- If suitable retention is found small changes in mobile phase can be made

15

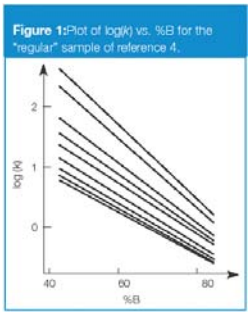
1. Optimizing retention

$$\log(k) = \log(k_0) - S \cdot \%B$$

- k_0 corresponds to organic solvent %B concentration of 0%
- S is the slope
  - For most compounds $S \approx 3$



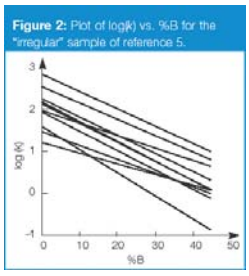
2.1 Optimizing selectivity



- Homologs
- Similar log(k) vs %B graph
- If %B changes the relative position (selectivity) of peaks does not change

17

2.1 Optimizing selectivity



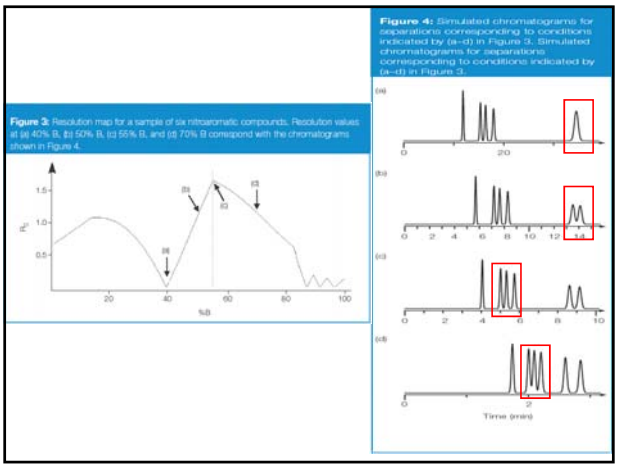
- For compounds with different functional groups the case is more complicated
  - But changes in %B do alter selectivity

18

2.1 Optimizing selectivity

- Based on the previous graph we can calculate the α and evaluate the resolution R_S for the critical pair
  - Lets assume $N = 10\,000$ (see Starting point)

19



2.1 Selectivity

- Similar graphs can be made for
  - temperature
  - pH
  - type of solvent

21

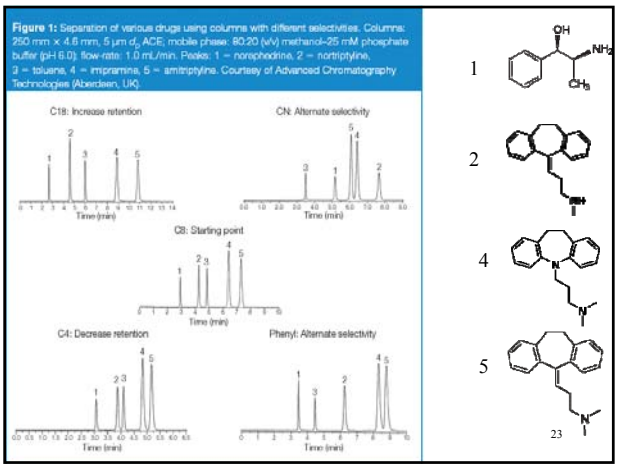
2.2 Selectivity of the column

- Particles
  - Silica, aluminum oxide, polymer
- Bonded phase
  - C_{18}, C_8, phenyl, CN

Two C_{18} columns from different producers are different!!!

  - In case of optimizing column phase same suppliers should be used – it is easier to predict changes

22



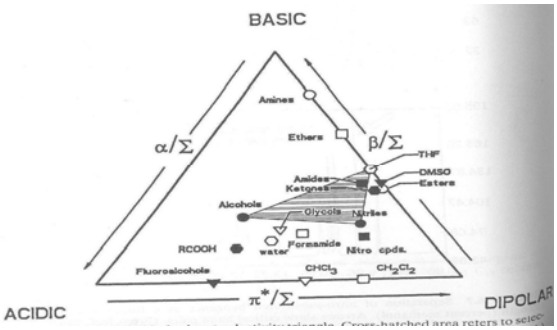
23

Organic solvent

- Changes in peak order and retention
- Selectivity is influenced by acidity, basicity and polarity of the solvent
- Solvent selectivity triangle
- For the critical pair it is usually sufficient to alter selectivity by 2-5%
- While choosing the organic solvent keep in mind:
  - UV absorption
  - Column pressure
  - Solvent purity
  - Solvent stability

24

2.3 Solvent selectivity triangle



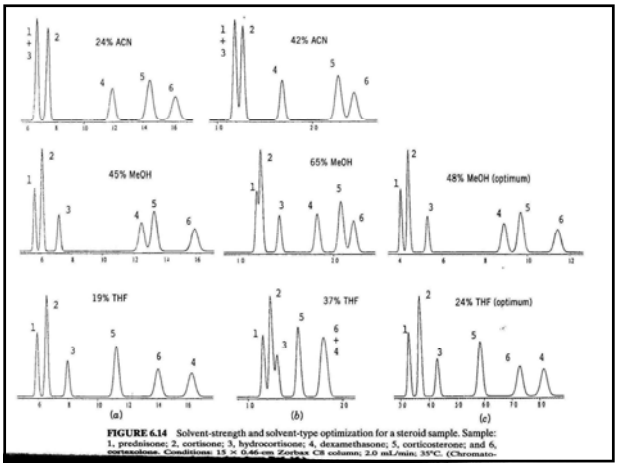
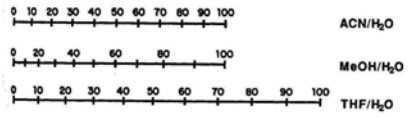


FIGURE 6.14 Solvent-strength and solvent-type optimization for a steroid sample. Sample: 1, prednisone; 2, cortisone; 3, hydrocortisone; 4, dexamethasone; 5, corticosterone; and 6, cortexolone. Conditions: 15 × 0.46-cm Zorbax C8 column; 2.0 mL/min; 35°C. (Chromato-

2.3 Organic solvent

- In practice 3 solvents – MeCN, MeOH and THF – are used
- These solvents can be mixed to achieve suitable selectivity

Comparison of solvnets elution power:



27

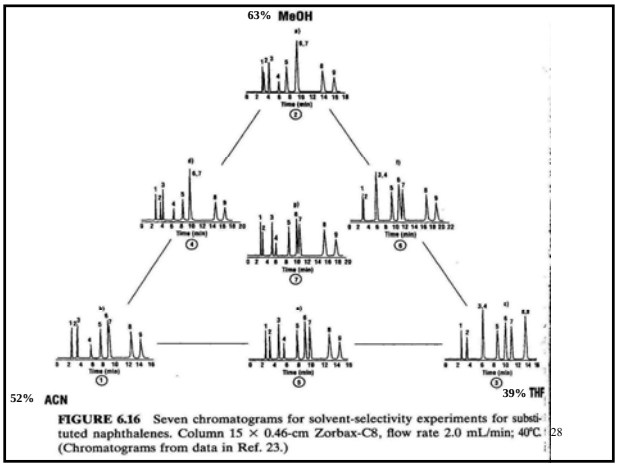


FIGURE 6.16 Seven chromatograms for solvent-selectivity experiments for substituted naphthalenes. Column 15 × 0.46-cm Zorbax-C8, flow rate 2.0 mL/min; 40°C. (Chromatograms from data in Ref. 23.)

28

2.4 pH

- In case of ionizable compounds pH may result in *k* change 10 times
- For neutral compounds *k* does not change
- Selectivity α changes as well
- Keep in mind the robustness!
- pH may also changes analytes UV absorption
- Silica columns usually stand 2<pH<8

29

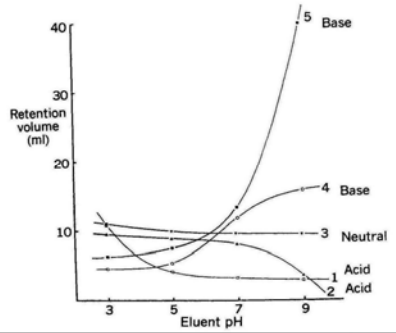
7.2 ACIDIC AND BASIC SAMPLES 299

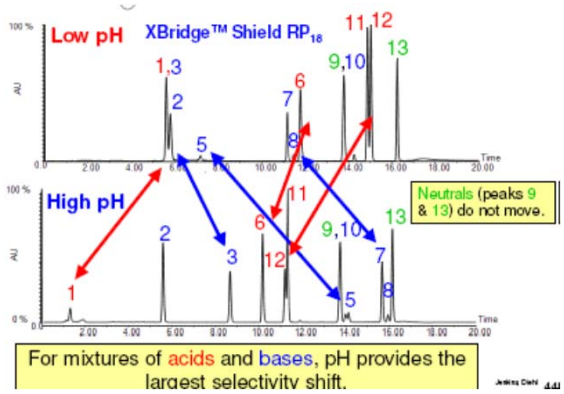
TABLE 7.1 Buffers for Use in HPLC Separation

Buffer	pK_a	Buffer Range[a]	UV Cutoff[b]
Trifluoracetic acid	>>2	1.5–2.5	210 nm (0.1%)
Phosphoric acid/mono- or di-K phosphate	2.1	< 3.1	< 200 nm (0.1%)
	7.2	6.2–8.2	
	12.3	11.3–13.3	< 200 nm (10 mM)
Citric acid/tri-K citrate	3.1		
	4.7	2.1–6.4	230 nm (10 mM)
	5.4		
Formic acid/K-formate	3.8	2.8–4.8	210 (10 mM)
Acetic acid/K-acetate	4.8	3.8–5.8	210 nm (10 mM)
Mono-/di-K carbonate	6.4	5.4–7.4[c]	< 200 nm (10 mM)
	10.3	9.3–11.3	< 200 nm (10 mM)
Bis-tris propane[e] · HCl/Bis-tris propane	6.8	5.8–7.8	215 nm (10 mM)
	9.0	8.0–10.0	225 nm (10 mM)
Tris[d] · HCl/tris	8.3	7.3–9.3	205 nm (10 mM)
Ammonium chloride/ammonia	9.2	8.2–10.2	200 nm (10 mM)
1-Methylpiperidine · HCl/1-Methylpiperidine	10.1	9.1–11.1	215 nm (10 mM)
Triethylamine · HCl/triethylamine	11.0	10.0–12.0	< 200 nm (10 mM)

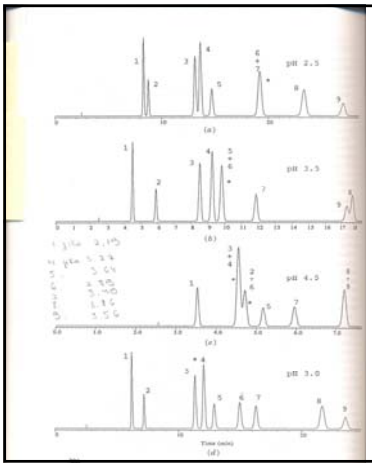
[a] pH range allowed with this buffer (conservative estimate).
[b] Absorbance <0.5 A; from Ref. 7.
[c] Requires addition of an acid (e.g., acetic or phosphoric).
[d] Tris(hydroxymethyl)aminomethane.
[e] 1,3-bis [Tris(hydroxymethyl)methylamino] propane.

2.4 Retention dependence on pH





32



Derivates of benzoic acid

1. 2-nitro
2. phthalic acid
3. additive
4. 2-fluoro
5. 3-cyano
6. 2-chloro
7. 3-nitro
8. 3-fluoro
9. 2,6-dimethyl

33

3. Efficiency

- Alter efficiency (plate number) if:
  - Resolution is not good enough
  - selectivity is ok
- If resolution is sufficient we can make the method faster
- Starting coulmn150 mm × 4.6 mm column with particle size 5 µm
  - ca 10 000 plates

34

3. Efficiency is altered by

- Eluent flow rate
- Column length
- Particle size

35

3.1 Eluent flow rate

- Van Deemter plot
- Columns with different particle size show different lines on the plot
  - Optimal flow rate for particle size 5 µm is 1-1.2 mL/min
    - Increasing flow rate-> decreasing efficiency
  - Smaller particles allow using higher flow rates

36

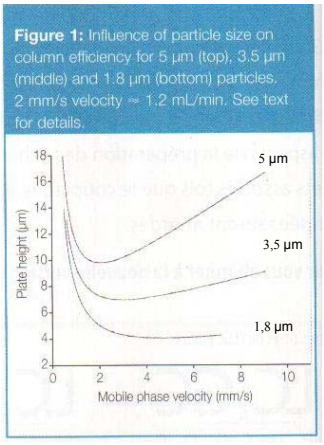


Figure 1: Influence of particle size on column efficiency for 5 µm (top), 3.5 µm (middle) and 1.8 µm (bottom) particles. 2 mm/s velocity ≈ 1.2 mL/min. See text for details.

37

3.2 Column length

- Replacing 150 mm column with 100 mm column
  - Time saved 33%
  - Resolution decreases 6%
- If resolution is sufficient it is possible to make the method faster with using a shorter column

38

3.3 Particle size

- Same length column with particle size 1.7–1.8 µm
  - 3 times higher N
  - 1.7 times better resolution
  - Higher flow rates possible (without loss of efficiency)
  - Faster analyses possible
- Same length column with particle size 5 µm
  - Lower pressure

39

Table 1: Influence of particle size on resolution, plate number, and pressure*

Parameter:	R_s	N	Pressure (psi)
Proportional to:	$1/d_p^{1/2}$	$1/d_p$	$1/d_p^2$
10 µm	1.08	6950	105
5 µm	1.62	3,850	425
3.5 µm	1.92	19,500	865
3.0 µm	2.04	22,200	1180
1.7 µm	2.46	32,250	3660

* Calculated values, 150 mm × 4.6 mm column, 65% acetonitrile–water, 35 °C, 1.0 mL/min

Table 2: Examples of column parameter changes

L (mm)	d_p (µm)	N	R_s	Pressure (psi)	t_R (min)
150	5	12000	2.0	2000	15
100	3	13300	2.1	3700	10
50	1.7	11750	2.0	5750	5
150	5	12000	1.7	2000	15
150	3	20000	2.2	5550	15
75	1.7	17650	2.1	8650	7.5

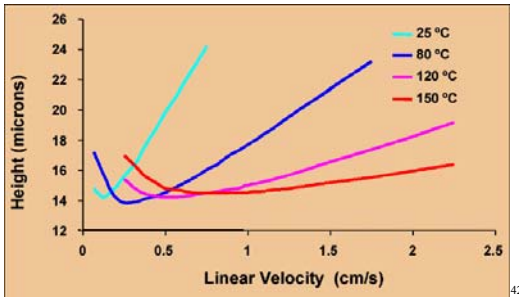
40

3.4 N increases ...

- ....if....
  - Column is well packed
  - Column is long
  - Flow rate is optimal
  - Packing particles are small
  - Mobile phase viscosity is low and temperature high
  - Analyte molecules are small
  - Dead volume is minimal

41

3.4 Plate height at different temperatures



42

Coefficient of uniformity of SP particles

Capacity factor functions

Average diameter of SP particles

Thickness of SP layer on on the sorbent particle

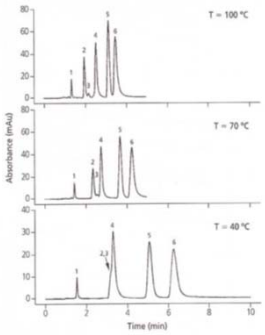
$$H = 2\lambda d_p + \frac{2\gamma D_M}{u} + \frac{f_S(k')d_f^2}{D_S}u + \frac{f_M(k')d_p^2}{D_M}u$$

Resistance of sorbent to diffusion

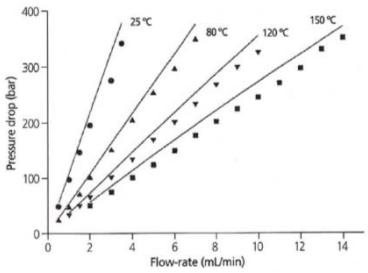
Diffusion coefficient of the analyte in the MP

Diffusion coefficient of analyte in the SP

Example



Effect on pressure



How to perform high temp HPLC?