Gradient elution

Basics

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Concept

- Gradient elution – Mobile phase content changes during elution
- Isocratic elution – Mobile phase is all the time the same during elution

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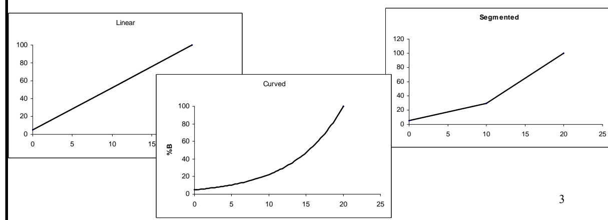
How?

- Concentration of component with stronger eluting power increases during elution.
- Linear, curved of segmented gradient.



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Why?

- For a lot of analytes it may be impossible to achieve $0.5<k<20$
- Sample contains large molecules (M>1000 Da)
- Samples that contain late-eluting matrix components.
- Suitable for dilute samples that are dissolved in a weak solvent
- In method development gradient elution is very good to gain information about the sample

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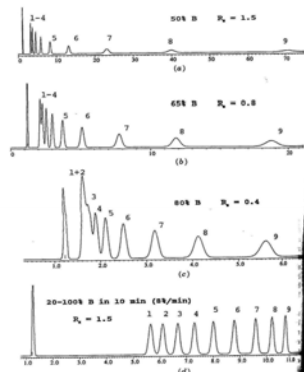

FIGURE 8.2 Separation of dialkylphthalate homologs by reversed-phase

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Problems?

- Lab does not have suitable apparatus
- More complicated then isocratic elution
- Some detectors can not be used
- Column need stabilization time after each run
- Transfer from one instrument to another is complicated
- Possible fall-out of salts.

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Problems?

- Additives with strong retention hinder usage of gradient elution. Equilibration may take too long and resolving may be unreproducible
  - Amine additives
  - Ion-pair reagents

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Alternatives

- If $0.5<k<20$ is not achievable for sample compounds
  - Use a different stationary phase, eg –CN
  - Use THF instead of MeCN or MeOH
  - Use two-dimentional chromatography

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Method development for gradient elution

- Useful also if isocratic method is preferred for the final method
- We can get information about the retention of sample components
  - Do we need to use gradient or is isocratic suitable?
  - Can decide if samples are suitable for reversed phase analyses

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Method development for gradient elution

- If isocratic method can be used we can determine the average organic content that should be used.
- If gradient elution need to be used we can determine the range of organic solvent content that should be used.

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Method development for gradient elution

- Gradient elution in method development allows to separate more peaks in first test -> time saving.
- Less likely compounds with small concentration are overlooked if they elute very early or late.

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First gradient

- 15 x 0.46 cm column
- Gradient from 5% to 100% of MeCN with 60 min
- If all peaks are in a beginning of the chromatogram -> sample is too hydrophilic for reversed phase.
- If no peaks occur -> 1. detector is not suitable for these compounds or 2. sample is too hydrophobic

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Isocratic or gradient?

- Retention time of the first t_{Ra} and the last t_{Rz} peak
- Retention time difference $\Delta t_R = t_{Rz} - t_{Ra}$
- Time of the gradient t_G
- $\Delta t_R / t_G < 0.4$ isocratic elution is suitable
- Gradient speed affects selectivity very effectively (more then organic modifier content in isocratic elution), therefore sometimes for $\Delta t_R / t_G > 0.15$ gradient is preferred

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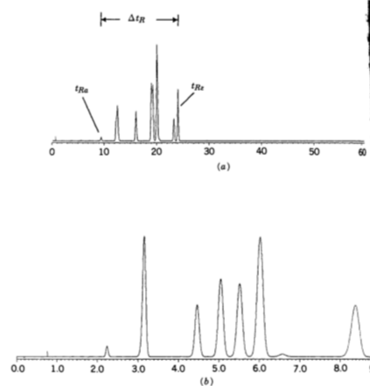

FIGURE 8.6 Use of an initial gradient run in HPLC method development. Subs tuted aniline sample [14,15]; conditions: 15 × 0.46-cm column; 2.0 mL/min; 35°C (a) 5 to 100% acetonitrile–water gradient in 60 min; (b) isocratic separation wi 37% acetonitrile–water. See the text for details.

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TABLE 8.2 Estimation of % B (ACN) for the First Isocratic Run, Based on the Retention Time t_{Rz} of the Last Peak in the Gradient Run[a]

	$(\% B)_{est}$ to Give Indicated k for Last Band in Isocratic Run		
t_{Rz} (min)	$k = 5$	$k = 10$	$k = 20$
5	6	0	—
10	19	12	5
15	29	22	14
20	37	30	22
25	45	38	30
30	53	46	38
35	61	54	46
40	69	62	54
45	77	70	62
50	85	78	70
55	93	86	78
60	100	94	86
65	—	100	94

Source: Refs. 14 and 15.

[a] Required conditions: 15 × 0.46-cm column, 5 to 100% ACN in 60 min, 2 mL/min.

Which gradient parameters?

- Calculate at which %B the first and last peak elute from the column and later optimize gradient between these values.

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Principles of gradient elution

- Eluting power of the eluent increases during chromatographic run
- For each peak retention *k* changes during evolution in column
- Compounds with shorter retention times start moving in the column at lower %B
- Late eluting compounds do not move at all in the beginning

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Principles of gradient elution

- Effective k^* is determined by the k of the peak at half distance through the column
- In case of linear gradient the k^* values are approximately the same for all peaks
- Therefor all peaks have similar width and resolution does not change over the chromatogram

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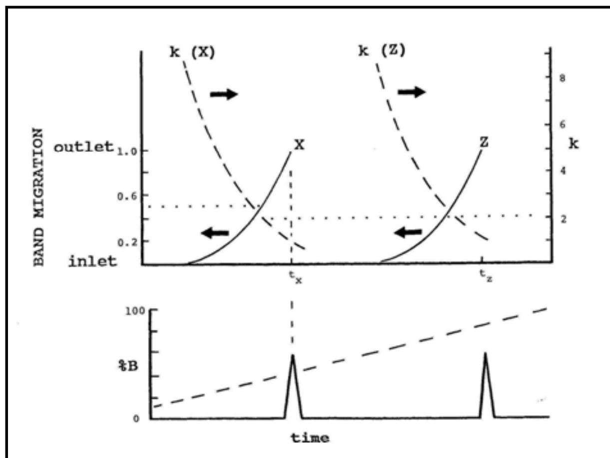

Gradient speed

- Gradient speed changes k^* value
- Higher k^* results in
  - Resolution R_S increases
  - Peaks become wider and decrease in height
  - Run time increases

- %B/min change is similar to %B change in isocratic elution
- k^* change is similar to k change in isocratic elution

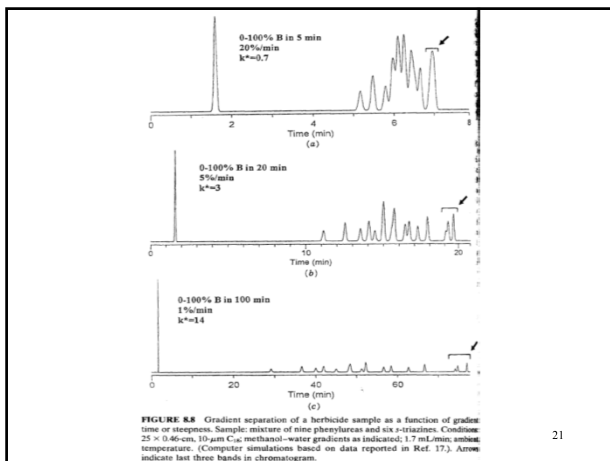

FIGURE 8.8 Gradient separation of a herbicide sample as a function of gradient time or steepness. Sample: mixture of nine phenylureas and six s-triazines. Conditions: 25 × 0.46-cm, 10-μm C_{18}; methanol–water gradients as indicated; 1.7 mL/min; ambient temperature. (Computer simulations based on data reported in Ref. 17.). Arrows indicate last three bands in chromatogram.

Influence of gradient starting point

- Do not start gradient with 100% of water phase, it may ruin the column
- First peaks should not elute very late – it waists time!
- Too high %B in the gradient starting point decreases resolution

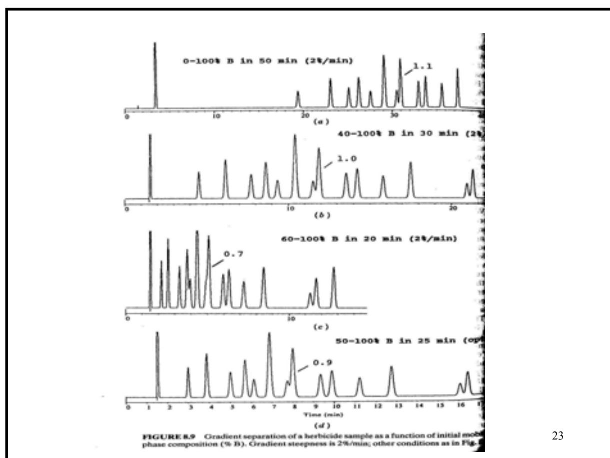

FIGURE 8.9 Gradient separation of a herbicide sample as a function of initial mobile phase composition (% B). Gradient steepness is 2%/min; other conditions as in Fig.

Influence of gradient end point

- If last peak elutes very much before the end of the gradient it is not needed to let the gradient reach 100% of organic phase
- Gradient length and overall run time are not the same (column dead time!)
- If gradient is stopped before the last peak elutes the run becomes longer and last peaks become wider

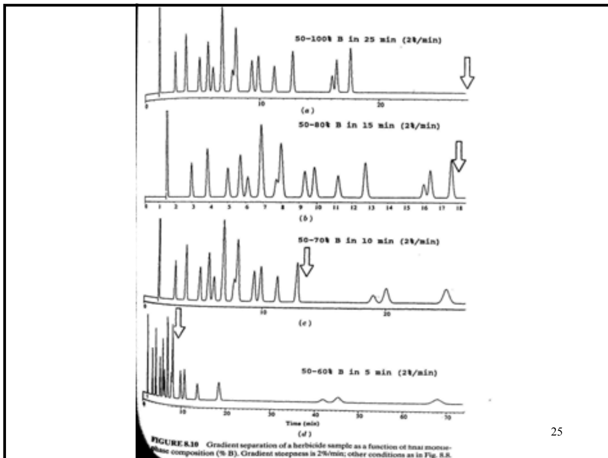

FIGURE 8.10 Gradient separation of a herbicide sample as a function of final mobile-phase composition (% B). Gradient steepness is 2%/min; other conditions as in Fig. 8.8.

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Gradient shape

- Mostly linear gradient
  - Easy to optimize
- Curved suits for oligomer separation, but resolution decreases in the end
- Segmented is needed if peaks are located in very different regions of chromatogram in the first run
- Test segmented gradient before curved gradient
  - Easier to optimize

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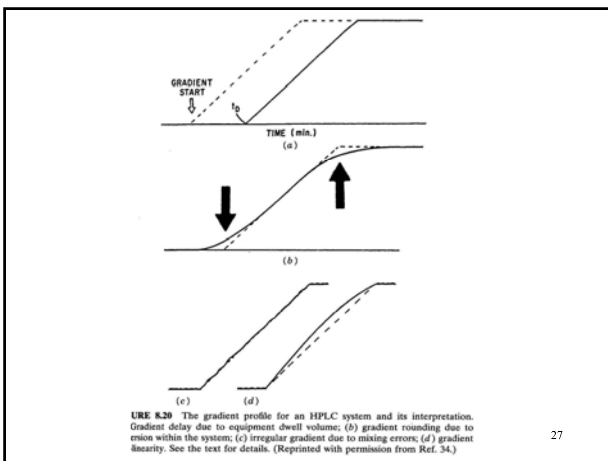

URE 8.20 The gradient profile for an HPLC system and its interpretation. Gradient delay due to equipment dwell volume; (b) gradient rounding due to ersion within the system; (c) irregular gradient due to mixing errors; (d) gradient linearity. See the text for details. (Reprinted with permission from Ref. 34.)

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Segmented gradient

- Faster gradient speed is used in regions with low peak density
- Smaller %B/min suits for regions with high peak density

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Gradient optimization

- You need to define the beginning and end of the gradient (%B) and shape of the gradient
- Selectivity and efficiency can be optimized in the same manner as in isocratic elution

- First run 5-100% of B

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Gradient optimization

- %B in the beginning influences very much first peaks but very little the last peaks
- Therefore
  - **Start optimization from the beginning of the chromatogram without changing the end before resolution for first peaks is achieved!**

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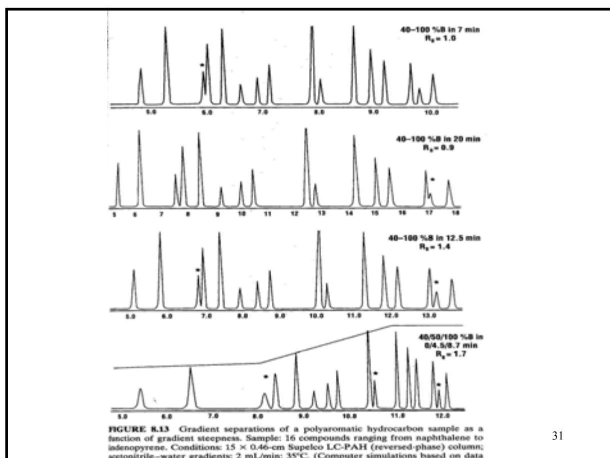
40–100 %B in 7 min
R_s = 1.0
40–100 %B in 20 min
R_s = 0.9
40–100 %B in 12.5 min
R_s = 1.4
40/50/100 %B in
0/4.5/8.7 min
R_s = 1.7
FIGURE 8.13 Gradient separations of a polyaromatic hydrocarbon sample as a function of gradient steepness. Sample: 16 compounds ranging from naphthalene to indenopyrene. Conditions: 15 × 0.46-cm Supelco LC-PAH (reversed-phase) column; acetonitrile–water gradients; 2 mL/min; 35°C. (Computer simulations based on data
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