

## **HPLC:**

- 1. PORAZDELITVENA ( liquid-liquid )**
- 2. IONSKA**
- 3. IZKLJUČITVENA oziroma GELSKA**
- 4. ADSORPCIJSKA oziroma LIQUID-SOLID**  
majhni delci ( 3-10  $\mu\text{m}$ )  $\rightarrow$   $\uparrow$ št. teoret. podov

**INJICIRANJE (20 – 50  $\mu\text{l}$ , max. 500  $\mu\text{l}$ ) preko ZANKE.**

**KOLONE (10 – 30 cm), več 10.000 podov/m, predkolone**

**POLNILA za KOLONE:**

**silikati – ponavadi obdelani (prevlečeni s filmi)**

**DETEKTORJI**

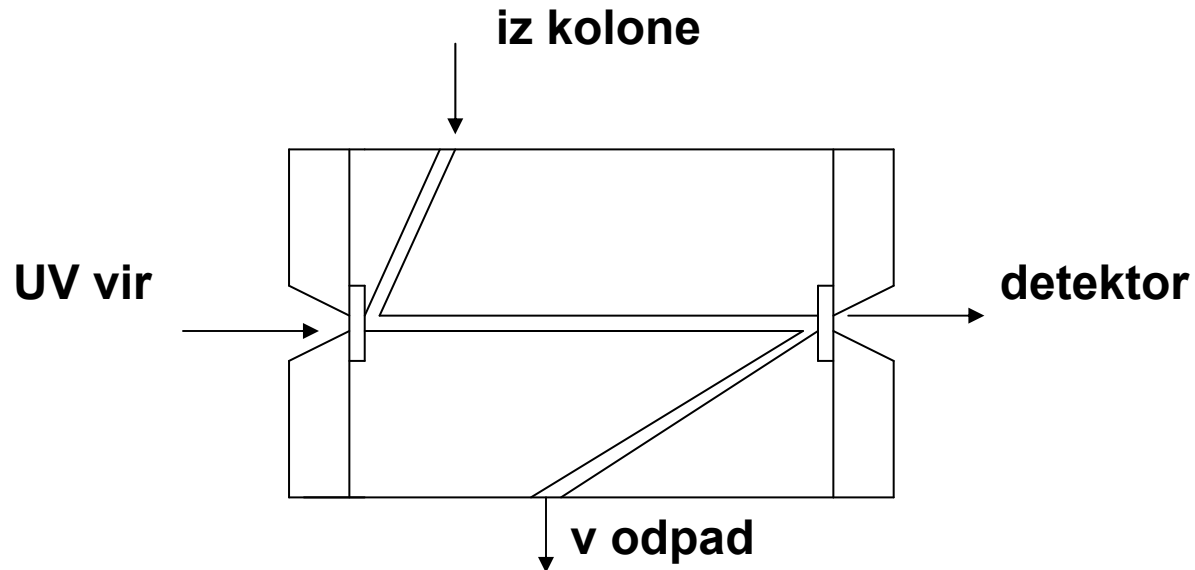
**občutljivi na “bulk property”**

**občutljivi na lastnosti topljenca**

## UV detektor

a) s filtri

b) z monokromatorji



IR detektor:

- Fourier transform

Fluorescenčni detektor:

- eksitacija, emisija

Detektor na refraktivni indeks

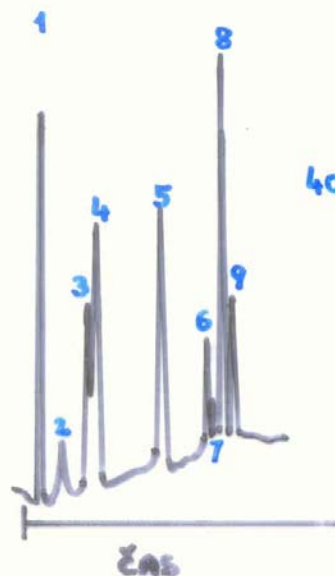
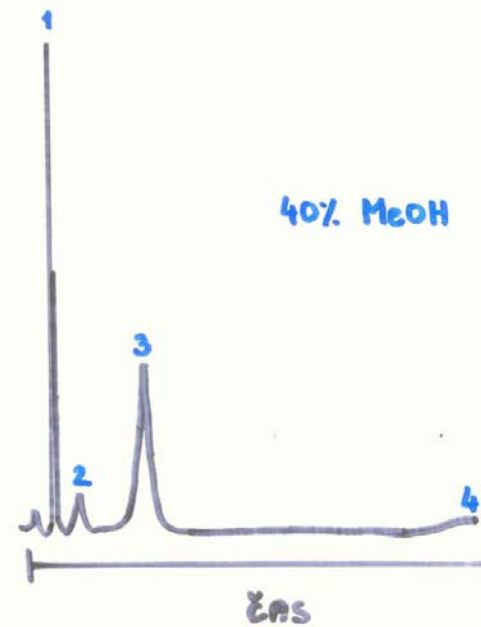
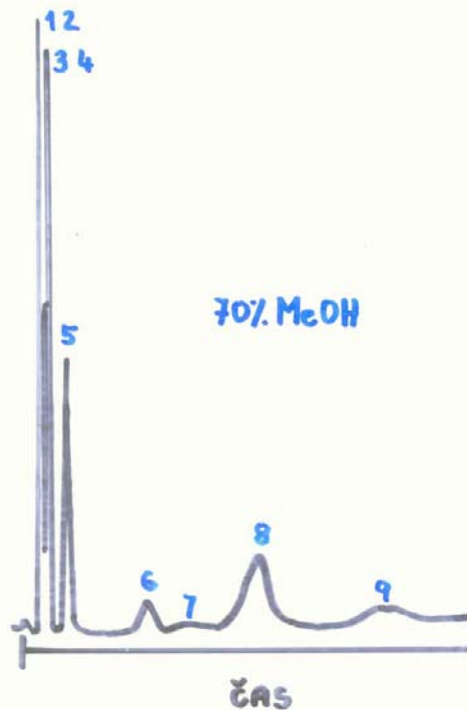
Elektrokemijski detektor

MS detektor

# HPLC

High pressure liquid chromatography

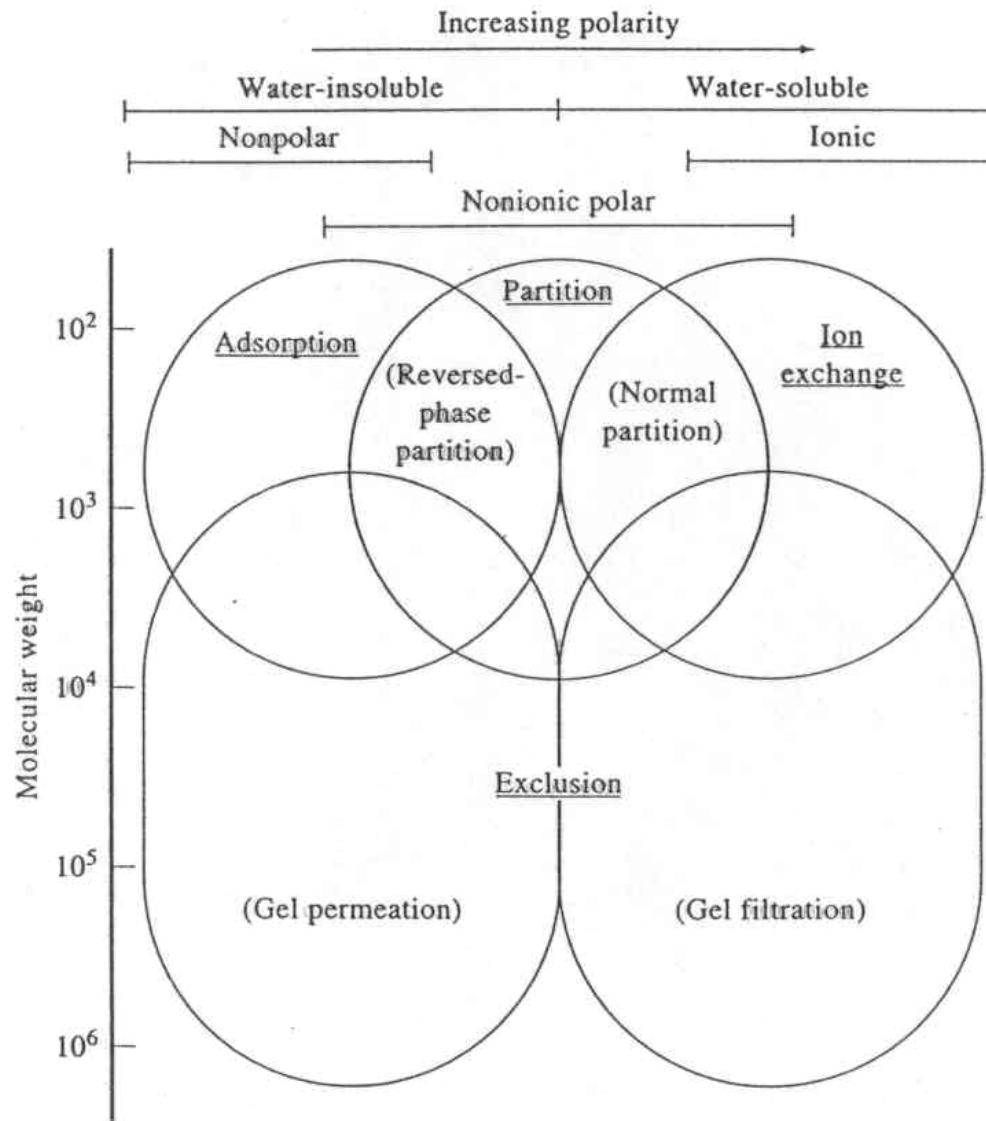
High performance liquid chromatography



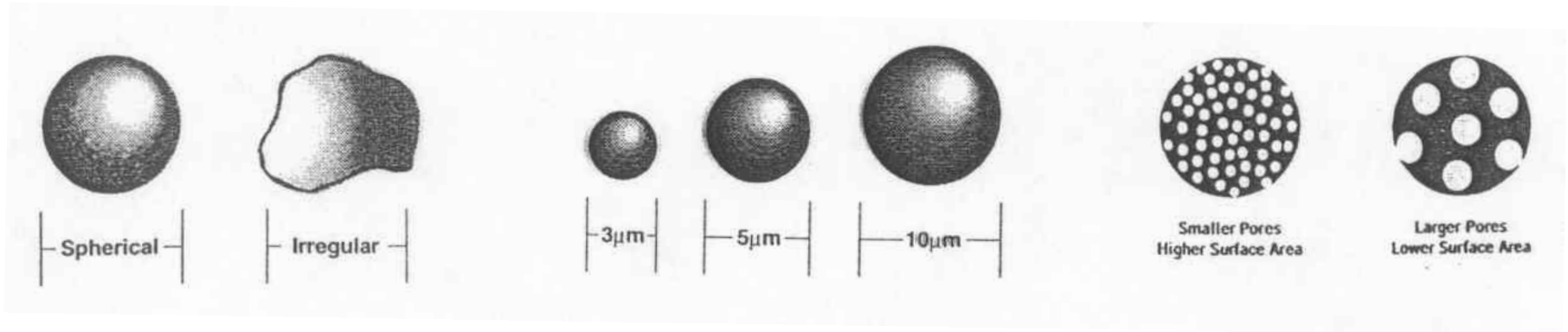
IZOKRATSKO

GRADIENTNO

# Področja uporabe tekočinske kromatografije



## Oblika, velikost in poroznost delcev stacionarne faze



### Velikost kolon:

❖ *dolžina*

10 cm; 12,5 cm; 15 cm; 25 cm; 30 cm

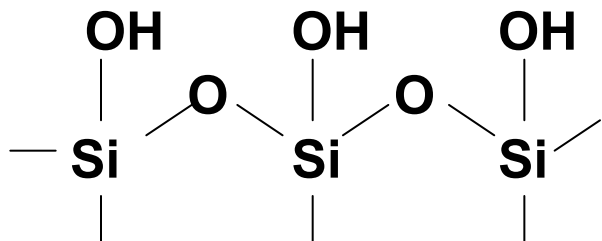
❖ *notranji premer*

3 mm; 4,3 mm; 4,6 mm; 6,2 mm

# PORAZDELITVENA KROMATOGRAFIJA

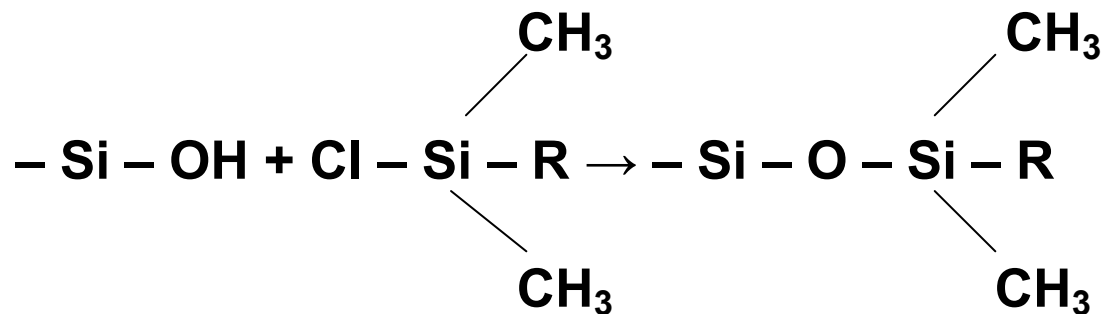
1. liquid-liquid + 2. vezana stacionarna faza

Površina hidroliziranega (0.1 M HCl, ↑T, 1-2 dni) silikata:



8  $\mu\text{mol SiOH}$  skup./m<sup>2</sup>

S silanizacijo (z organoklorsilanom) dobimo siloksane:



R – alkil (C<sub>2</sub>, C<sub>8</sub>, C<sub>18</sub>)



**Sterični efekti → silanizira se le 4  $\mu\text{mol}/\text{m}^2$**

**nezreagirane silanolne skupine → tailing (baze!); capped – blokada prostih OH s  $\text{ClSiMe}_3$  (klorotrimetilsilan)**

**Reverzne faze:**

- nepolarne**
- mobilna faza – polarna**

**Normalne faze:**

- polarne**
- mobilna faza – nepolarna**

# PRIMERJAVA NORMALNE IN REVERZNE HPLC

Faktor	Normalna	Reverzna
<b>Stacionarna faza</b>	polarna silica, diol (-C <sub>2</sub> H <sub>4</sub> CN), (-C <sub>3</sub> H <sub>6</sub> NH <sub>2</sub> )	nepolarna silica prekrita s hidrofobnim slojem: C <sub>2</sub> , C <sub>6</sub> , C <sub>8</sub> , C <sub>18</sub> silan
<b>Mobilna faza</b>	nepolarna heksan + modifikator (CHCl <sub>3</sub> (IPA/MeOH) CH <sub>2</sub> Cl <sub>2</sub> , Et – eter	polarna MeOH (MeCN, THF)/H <sub>2</sub> O pH modifikacija
<b>Topljenec</b> <b>POLAREN</b> ↑ polarnost topila ↓ polarnost topila	dolga retencija krajša daljša	kratka retencija krajša daljša
<b>NEPOLAREN</b> ↑ polarnost topila ↓ polarnost topila	kratka retencija daljša krajša	dolga retencija daljša krajša

# IZBIRA MOBILNE FAZE

“Močno” topilo – interagira s topljencem

Topnostni parameter (  $\delta$  )

$$\delta = ( \Delta E / V )^{1/2}$$

$\Delta E$  = energija uparitve

$V$  = molarni volumen

## MOBILNA FAZA

Topilo	Tip	Parameter $\delta$	Topljenec	Parameter $\delta$
n-heksan	N	7,3	naftalen	9,64
benzen	N	9,1	testosteron	10,90
dietileter	B	7,7	salicilna kislina	10,80
kloroform	A	9,3	Pr-p-hidroksibenzoat	11,84
THF	B	9,5	Et-p-aminobenzoat	12,04
dioksan	B	10,0	fenobarbiton	12,54
acetonitril	B	11,9	sulfatiazol	13,10
2-propanol	AB	11,5	kofein	14,04
metanol	AB	14,5	p-hidroksibenz. kislina	15,30
voda	AB	23,4		

$$\delta_i = \frac{1}{2}(\delta_m + \delta_s) \quad \delta_m - \delta_s = 2(\delta_i - \delta_s) \quad \text{selektivnost}$$

$$\frac{\delta_s + 7,3}{2} < \delta_i < \frac{\delta_s + 23,4}{2}$$

**INDEKS POLARNOSTI (P') (-Synder) – VPLIV NA k'**

**Topnost v :**        **dioksanu (šibak dipol proton akceptor)**  
                          **nitrometanu (močan dipol proton akceptor)**  
                          **etanolu (močan dipol proton donor)**

**– 2 < P' < 10.2 (voda)**

**Zmes dveh topil:         $P'_{AB} = \Phi_A P'_A + \Phi_B P'_B$**

**normal. faza:         $k'_2/k'_1 = 10^{(P'_1 - P'_2)/2}$**

**reverz. faza:         $k'_2/k'_1 = 10^{(P'_2 - P'_1)/2}$**

## VPLIV MOBILNE FAZE NA SELEKTIVNOST ( $\alpha$ ):

- spreminjamo kemijsko naravo topil pri ohranitvi  $k'$  na isti vrednosti

topila pri reverz. fazi: metanol, CH<sub>3</sub>CN, tetrahidrofuran / voda

topila pri normal. fazi: etileter, CH<sub>2</sub>Cl<sub>2</sub>, CH<sub>3</sub>Cl / n – heksan

## TVORBA DERIVATOV:

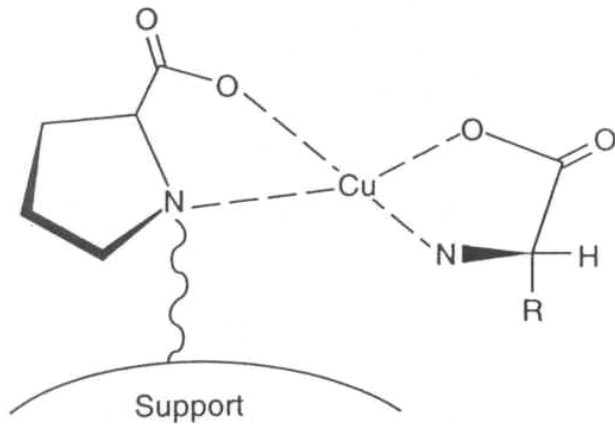
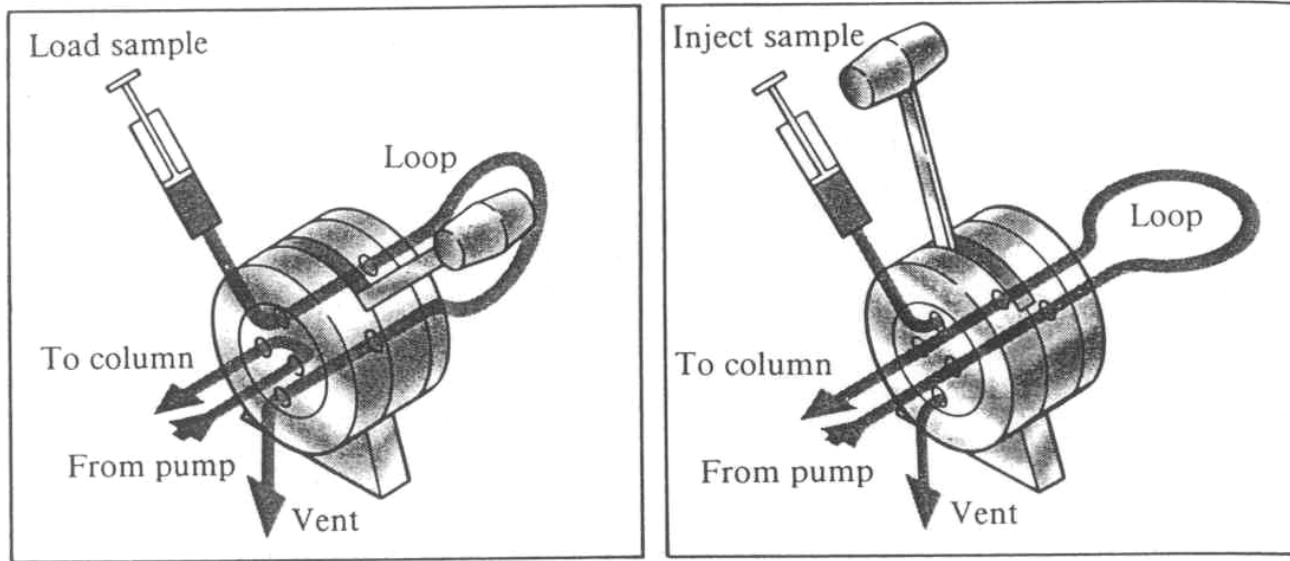
- ↓ polarnost
- boljši odziv detektorja za vse komponente ali
- selektivno boljši odziv detektorja le za nekatere komponente

## TVORBA IONSKIH PAROV

KIRALNE STACIONARNE FAZE – *silica-polimer-optično aktivni izomer*

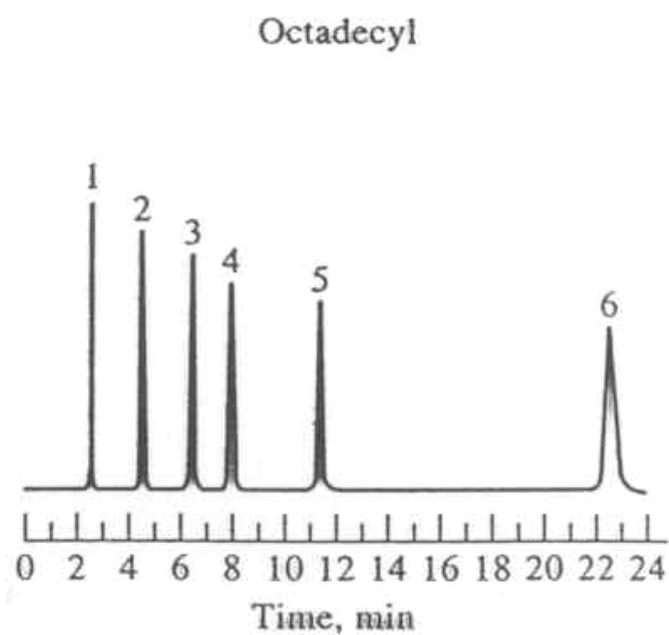
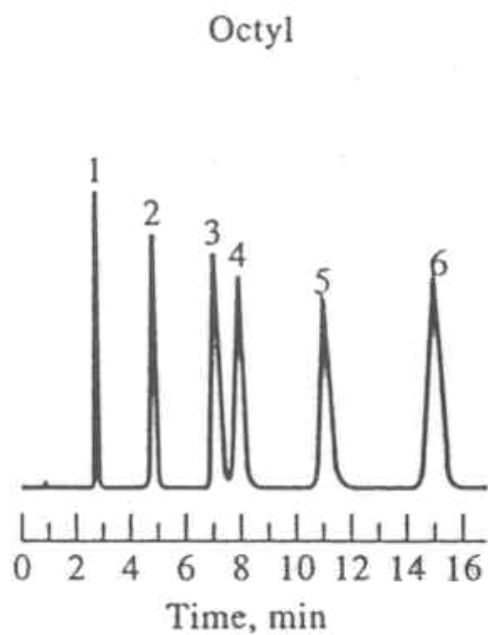
npr. *L-amino kisl.prolin-polistiren-p-divinilbenzen + Cu kompleksi*

**Figure 1: A sampling loop for liquid chromatography.**

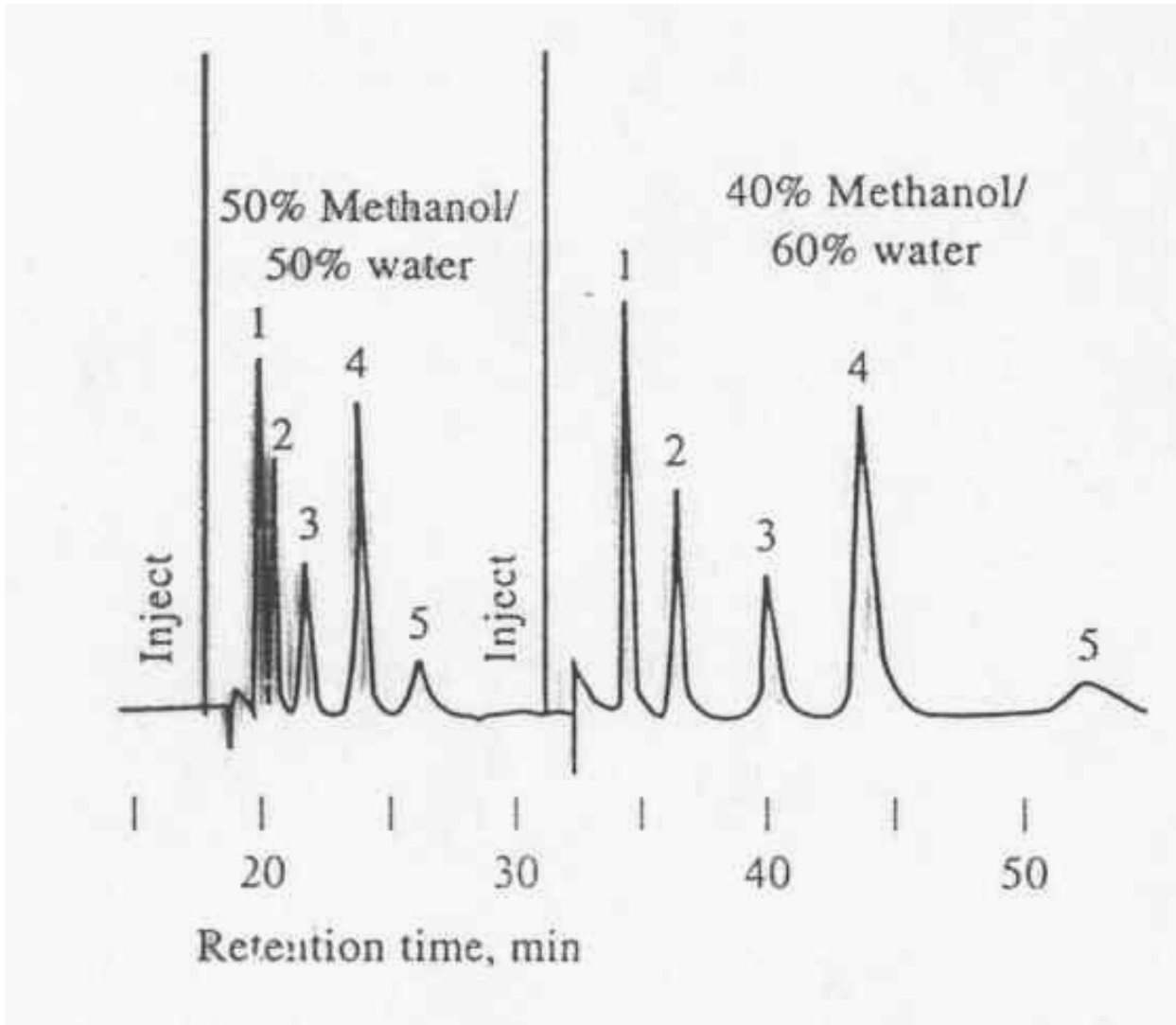


**Figure 2: Schematic of the ternary complex formed between an L-proline bonded-phase, an analyte amino acid and copper(II) ion.**

## Vpliv polarnosti stacionarne faze na separacijo



## Vpliv polarnosti mobilne faze na separacijo



- izokratska elucija
- gradientna elucija



## Separacija polarnih spojin z RP kromatografijo

(• dodatek aminov, • prilagoditev pH, • tvorba ionskih parov)

Column: C-8 bonded phase 15 cm × 4.6 mm

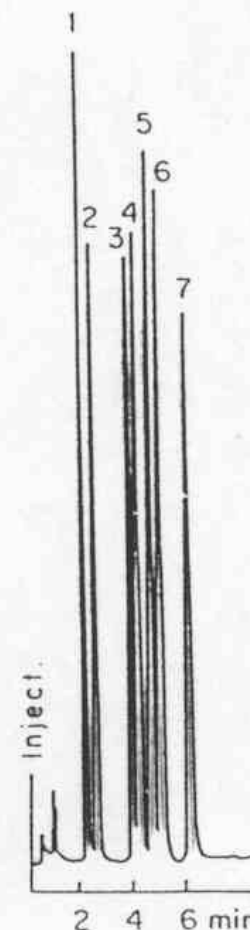
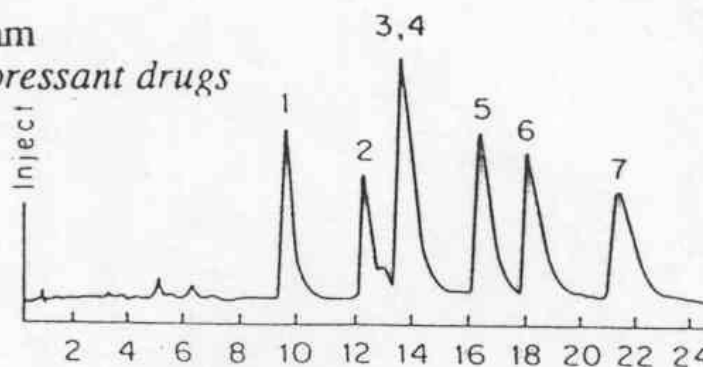
Mobile phase: (i) CH<sub>3</sub>CN/0.01 mol dm<sup>-3</sup> H<sub>3</sub>PO<sub>4</sub> adjusted to pH 2.5 with KOH

(ii) CH<sub>3</sub>CN/0.01 mol dm<sup>-3</sup> H<sub>3</sub>PO<sub>4</sub> + 0.005 mol dm<sup>-3</sup> nonylamine, pH 2.5

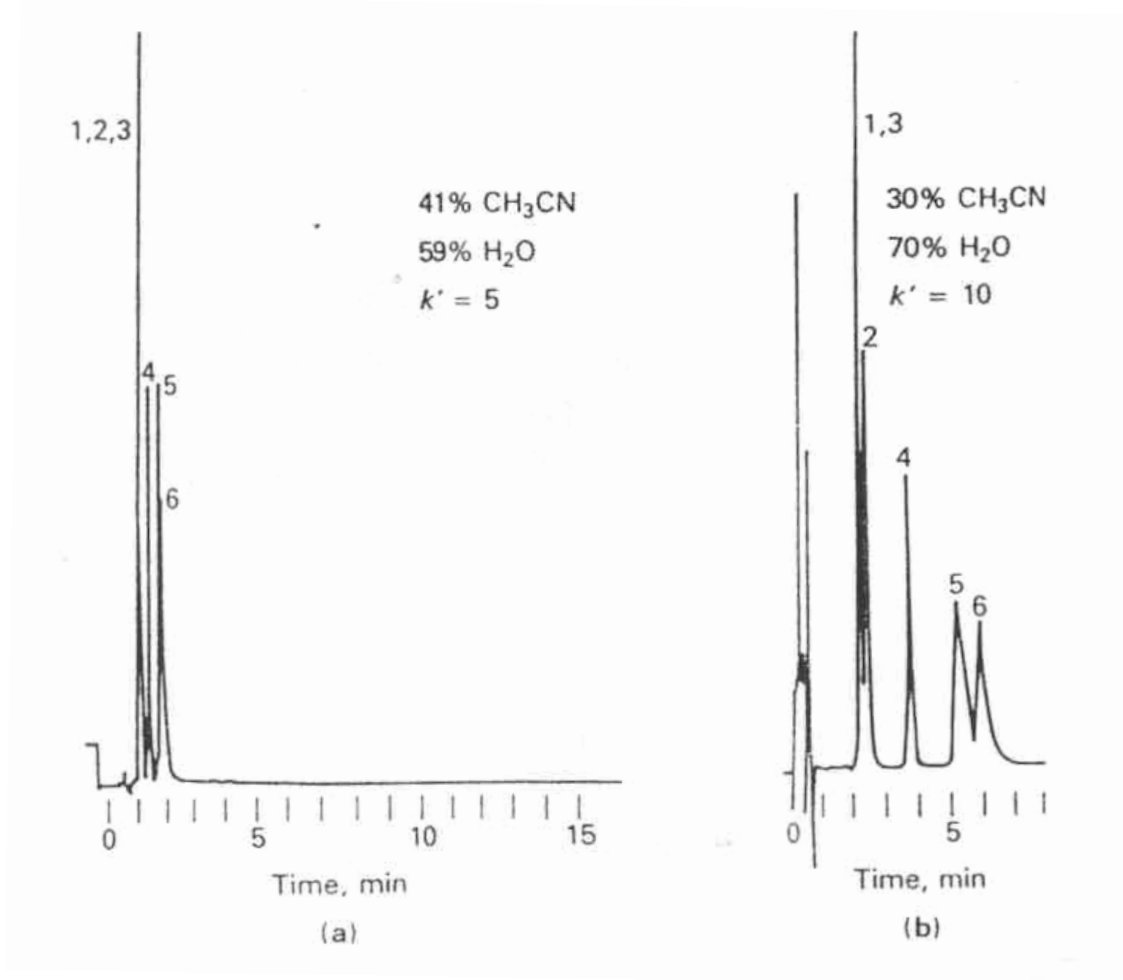
Flow rate: 2 cm<sup>3</sup> min<sup>-1</sup>

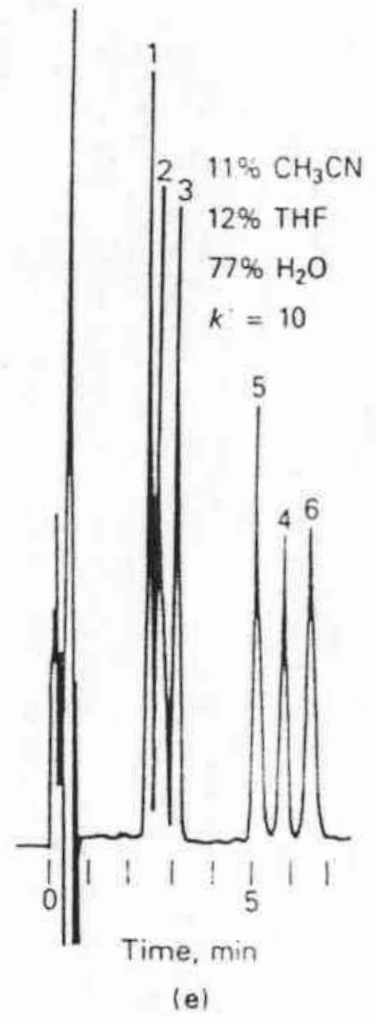
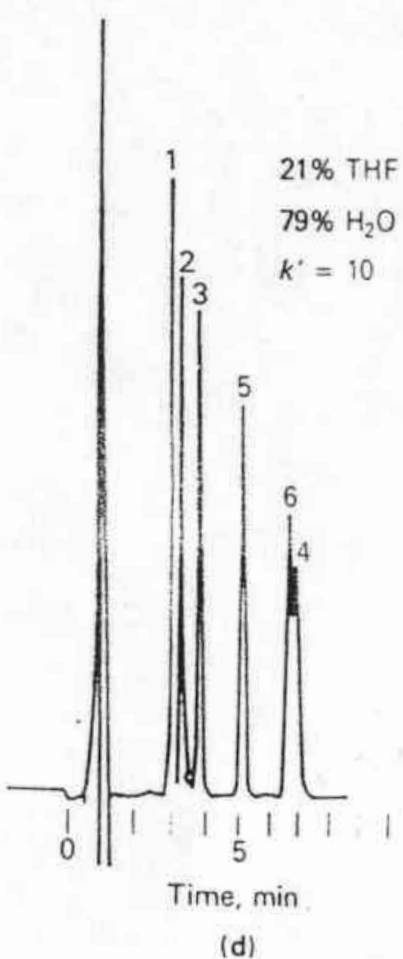
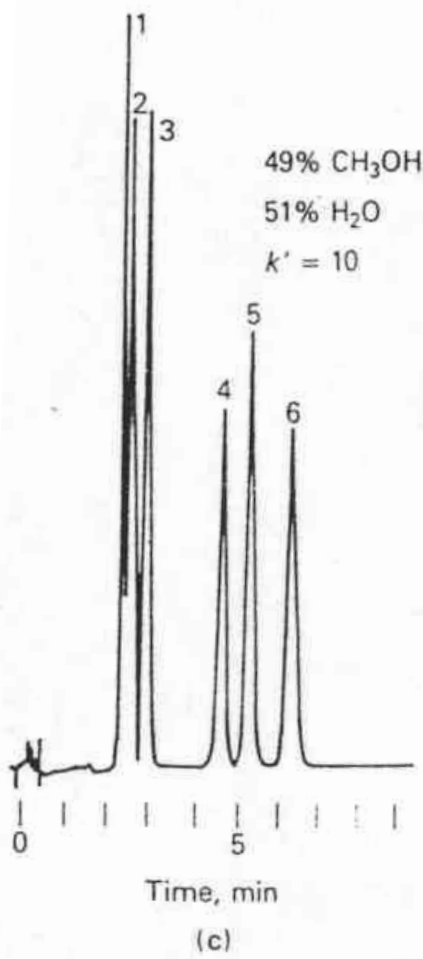
Detector: UV absorption, 254 nm

Sample: Separation of antidepressant drugs



**Figure 2: Systematic approach to the separation of six steroids. The use of water to adjust  $k'$  is shown in (a) and (b). The effects of varying  $\alpha$  at constant  $k'$  are shown in (b), (c), (d) and (e). Column: 0,4 x 150 mm packed with 5- $\mu$ m C<sub>8</sub> bonded, reversed - phase particle; T: 50°C; flow rate: 3,0 cm<sup>3</sup>/min; detector: UV-254 nm; THF = tetrahydrofuran, CH<sub>3</sub>CN = acetonitrile. Compounds: (1) prednisone, (2) cortisone, (3) hydrocortisone, (4) dexamethasone, (5) corticosterone, (6) corticoexolone.**





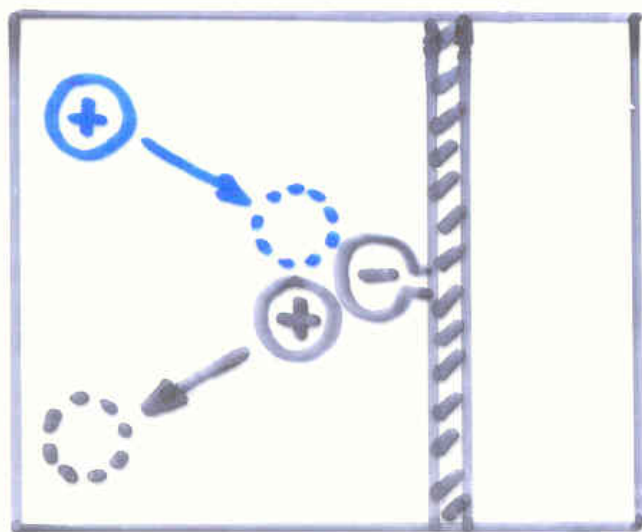
**Table 1: Systems for Reversed-Phase Ion-pair Chromatograms**

<b>Sample</b>	<b>Mobile Phase</b>	<b>Counter-ion</b>	<b>Type Stationary Phase</b>
<b>Amines</b>	<b>0.1 M HClO<sub>4</sub>/H<sub>2</sub>O/ acetonitrile</b>	<b>ClO<sub>4</sub><sup>-</sup></b>	<b>BP<sup>a</sup></b>
	<b>H<sub>2</sub>O/CH<sub>3</sub>OH/H<sub>2</sub>SO<sub>4</sub></b>	<b>C<sub>12</sub>H<sub>25</sub>SO<sub>3</sub><sup>-</sup></b>	<b>BP</b>
<b>Carboxylic acids</b>	<b>pH 7.4</b>	<b>(C<sub>4</sub>H<sub>9</sub>)<sub>4</sub>N<sup>+</sup></b>	<b>BP</b>
	<b>pH 7.4</b>	<b>(C<sub>4</sub>H<sub>9</sub>)<sub>4</sub>N<sup>+</sup></b>	<b>L<sup>b</sup></b>
<b>Sulfonic acids</b>	<b>H<sub>2</sub>O/C<sub>3</sub>H<sub>7</sub>OH</b>	<b>(C<sub>16</sub>H<sub>33</sub>)(CH<sub>3</sub>)<sub>3</sub>N<sup>+</sup></b>	<b>BP</b>
	<b>pH 7.4</b>	<b>(C<sub>4</sub>H<sub>9</sub>)<sub>4</sub>N<sup>+</sup></b>	<b>L<sup>b</sup></b>
	<b>pH 3.8</b>	<b>Bis-(2-ethylhexyl)phosphate</b>	<b>L<sup>c</sup></b>
<b>Dyes</b>	<b>pH 2–4; H<sub>2</sub>O/CH<sub>3</sub>OH</b>	<b>(C<sub>4</sub>H<sub>9</sub>)<sub>4</sub>N<sup>+</sup></b>	<b>BP</b>

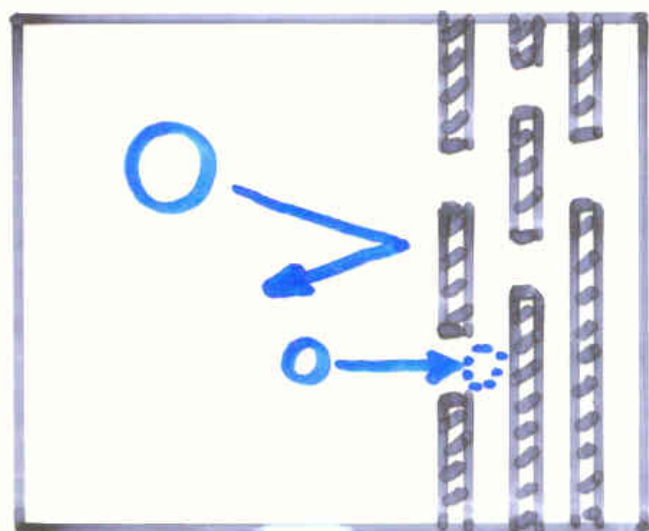
<sup>a</sup>Bonded-phase

<sup>b</sup>Absorbed 1-pentanol

<sup>c</sup>Absorbed bis-(2-ethylhexyl)phosphoric acid/CHCl<sub>3</sub>

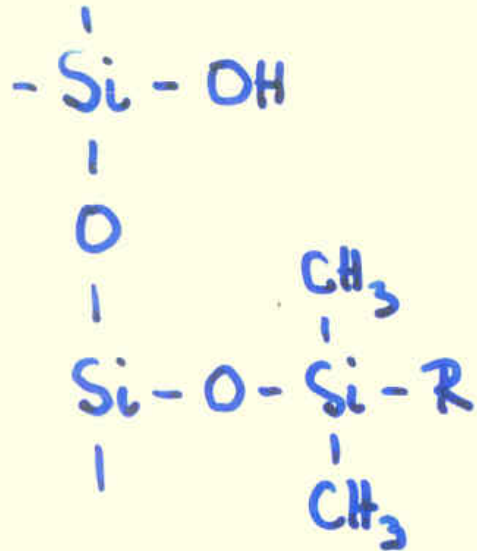


IEC



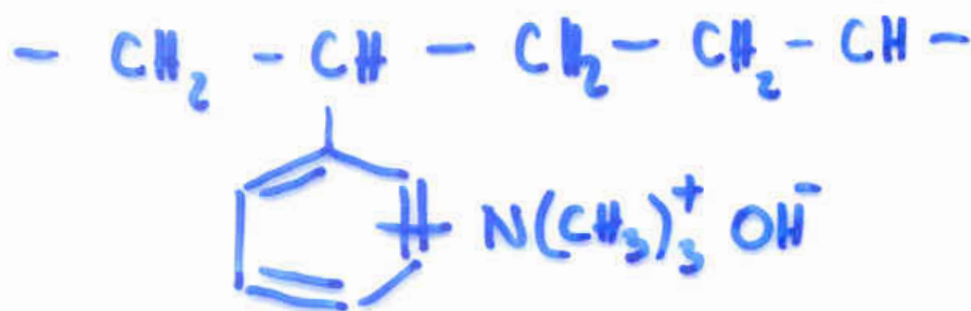
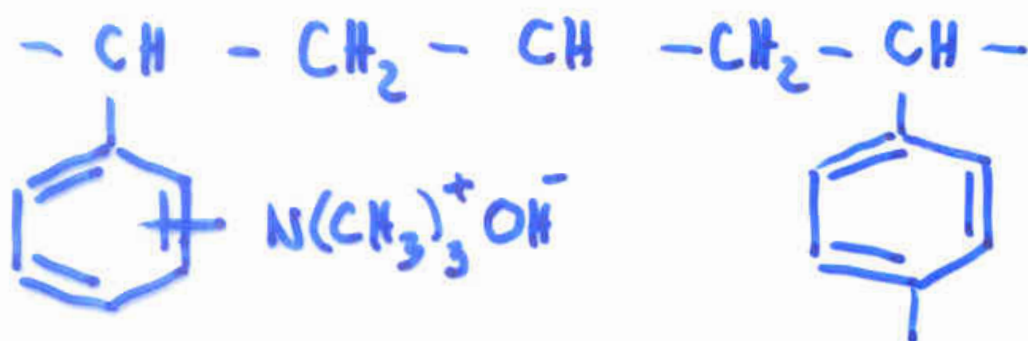
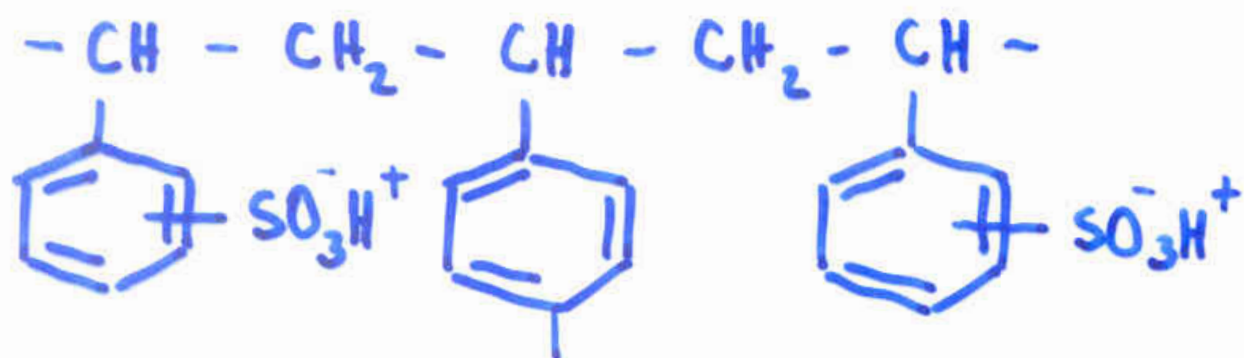
GPC

## SILANOL



## SILOKSAN





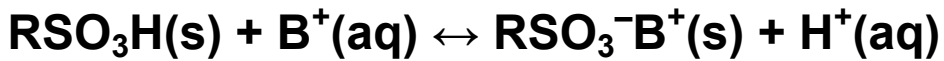
POLISTIREN 1.1.

## IONSKA KROMATOGRAFIJA:

KATIONSKI I., ANIONSKI I.

R-SO<sub>3</sub>H, R-COOH - kationski

R-N(CH<sub>3</sub>)<sub>3</sub><sup>+</sup>OH<sup>-</sup>, R-NH<sub>3</sub><sup>+</sup>OH<sup>-</sup> - anionski



spiramo (eluiramo) s HCl

$$K_{\text{aq}} = \frac{[\text{RSO}_3^-\text{B}^+]_s [\text{H}^+]_{\text{aq}}}{[\text{RSO}_3^-\text{H}^+]_s [\text{B}^+]_{\text{aq}}} \quad \left. \begin{array}{l} [\text{RSO}_3^-\text{H}^+]_s \gg [\text{RSO}_3^-\text{B}^+]_s \\ [\text{H}^+]_{\text{aq}} \gg [\text{B}^+]_{\text{aq}} \end{array} \right\} \longrightarrow$$

$$\frac{[\text{RSO}_3^-\text{B}^+]_s}{[\text{B}^+]_{\text{aq}}} = \underbrace{K_{\text{aq}} \frac{[\text{RSO}_3^-\text{H}^+]_s}{[\text{H}^+]_{\text{aq}}}}_{\text{konst.}} \Rightarrow \frac{[\text{RSO}_3^-\text{B}^+]_s}{[\text{B}^+]_{\text{aq}}} = \mathbf{K} = \frac{c_s}{c_M}$$

detektor: konduktometrija (prevodnost)



## **GELSKA (IZKLJUČITVENA) KROMATOGRAFIJA:**

**Stacionarna faza:**            *polistiren–divinilbenzen*  
   *silica (porous)*

**celokupni volumen ( $V_t$ ):**  $V_t = V_g + V_i + V_f$

$V_g$  = volumen matriksa,  $V_i$  = volumen topila v porah,  $V_f$  = prosti volumen

$V_e$ (volumen elucije) =  $V_f + KV_i \rightarrow K = (V_e + V_f) / V_i = c_s / c_m$

## **ADSORPCIJSKA KROMATOGRAFIJA (liquid–solid):**

**Stacionarna faza:** *silica, alumina*

Tu le mobilna faza vpliva na  $k$ ,  $\alpha$ ; pri reverz. f. tudi stacionarna faza na  $\alpha$

“eluent strength”  $\epsilon^0$  (adsorpcijska energija topila na enoto površine)

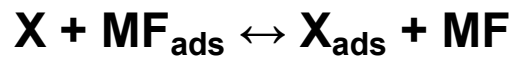
**Table 2: Properties of Common Chromatographic Mobile Phases**

<b>Solvent</b>	<b>Refractive Index (25°C)</b>	<b>Viscosity, centipoise</b>	<b>Boiling Point, °C</b>	<b>Polarity Index, P'</b>	<b>Eluent Strength,<sup>a</sup> <math>\epsilon^0</math></b>
<b>Flouroalkanes<sup>b</sup></b>	<b>1,27 – 1,29</b>	<b>0,4 – 2,6</b>	<b>50 – 174</b>	<b>&lt; – 2</b>	<b>– 0,25</b>
<b>Cyclohexane</b>	<b>1,423</b>	<b>0,90</b>	<b>81</b>	<b>0,04</b>	<b>– 0,2</b>
<b>n – hexane</b>	<b>1,372</b>	<b>0,30</b>	<b>69</b>	<b>0,1</b>	<b>0,01</b>
<b>1 – Chlorobutane</b>	<b>1,400</b>	<b>0,42</b>	<b>78</b>	<b>1,0</b>	<b>0,26</b>
<b>Carbon tetrachloride</b>	<b>1,457</b>	<b>0,90</b>	<b>77</b>	<b>1,6</b>	<b>0,18</b>
<b>i – Propyl ether</b>	<b>1,365</b>	<b>0,38</b>	<b>68</b>	<b>2,4</b>	<b>0,28</b>
<b>Toluene</b>	<b>1,494</b>	<b>0,55</b>	<b>110</b>	<b>2,4</b>	<b>0,29</b>
<b>Diethyl ether</b>	<b>1,350</b>	<b>0,24</b>	<b>35</b>	<b>2,8</b>	<b>0,38</b>
<b>Tetrahydrofuran</b>	<b>1,405</b>	<b>0,46</b>	<b>66</b>	<b>4,0</b>	<b>0,57</b>
<b>Chloroform</b>	<b>1,443</b>	<b>0,53</b>	<b>61</b>	<b>4,1</b>	<b>0,40</b>
<b>Ethanol</b>	<b>1,359</b>	<b>1,08</b>	<b>78</b>	<b>4,3</b>	<b>0,88</b>
<b>Ethyl acetate</b>	<b>1,370</b>	<b>0,43</b>	<b>77</b>	<b>4,4</b>	<b>0,58</b>
<b>Dioxane</b>	<b>1,420</b>	<b>1,2</b>	<b>101</b>	<b>4,8</b>	<b>0,56</b>
<b>Methanol</b>	<b>1,326</b>	<b>0,54</b>	<b>65</b>	<b>5,1</b>	<b>0,95</b>
<b>Acetonitrile</b>	<b>1,341</b>	<b>0,34</b>	<b>82</b>	<b>5,8</b>	<b>0,65</b>
<b>Nitromethane</b>	<b>1,380</b>	<b>0,61</b>	<b>101</b>	<b>6,0</b>	<b>0,64</b>
<b>Ethylene glycol</b>	<b>1,431</b>	<b>16,5</b>	<b>182</b>	<b>6,9</b>	<b>1,11</b>
<b>Water</b>	<b>1,333</b>	<b>0,89</b>	<b>100</b>	<b>10,2</b>	<b>Large</b>

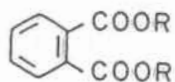
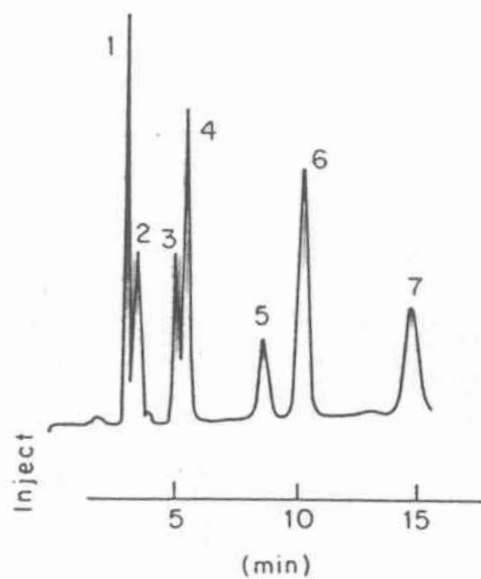
<sup>a</sup> On Al<sub>2</sub>O<sub>3</sub>. Multiplication by 0,8 gives  $\epsilon^0$  on SiO<sub>2</sub>.

<sup>b</sup> Properties depend upon molecular weight. Range of data given.

## Adsorpcijska kromatografija



### Primer: separacija ftalatov

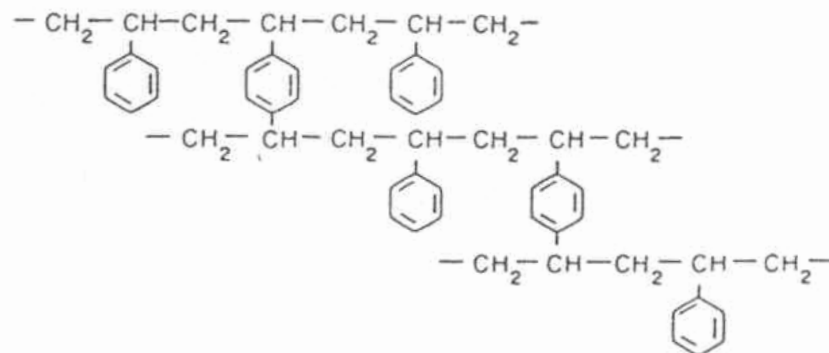
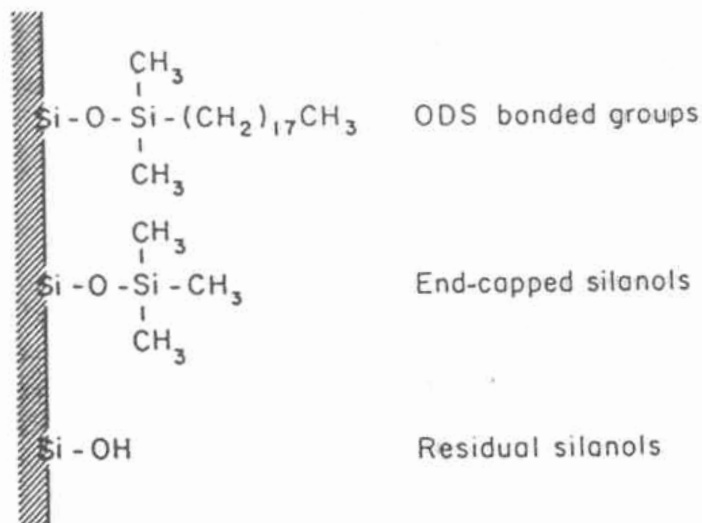
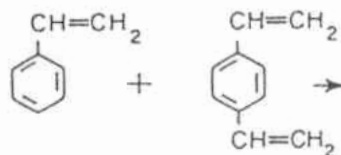
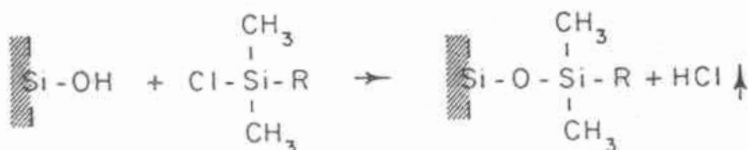


Column: 10  $\mu\text{m}$  silica 30 cm  $\times$  4 mm  
Mobile phase: butyl ethanoate/*iso*-octane 5:95  
Detector: UV absorption, 254 nm

Sample: 1 R = *n*-C<sub>8</sub>H<sub>17</sub> 2 *iso*-C<sub>8</sub>H<sub>17</sub> 3 *iso*-C<sub>4</sub>H<sub>9</sub> 4 *n*-C<sub>4</sub>H<sub>9</sub>  
5 C<sub>6</sub>H<sub>5</sub> 6 C<sub>2</sub>H<sub>5</sub> 7 CH<sub>3</sub>

# Porazdelitvena kromatografija

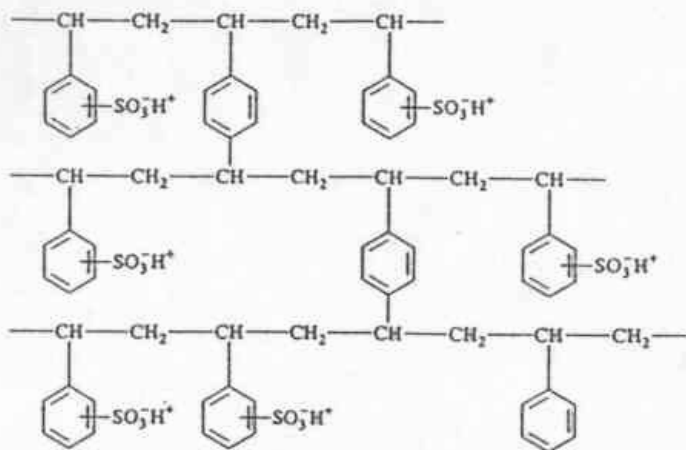
Stacionarna faza:



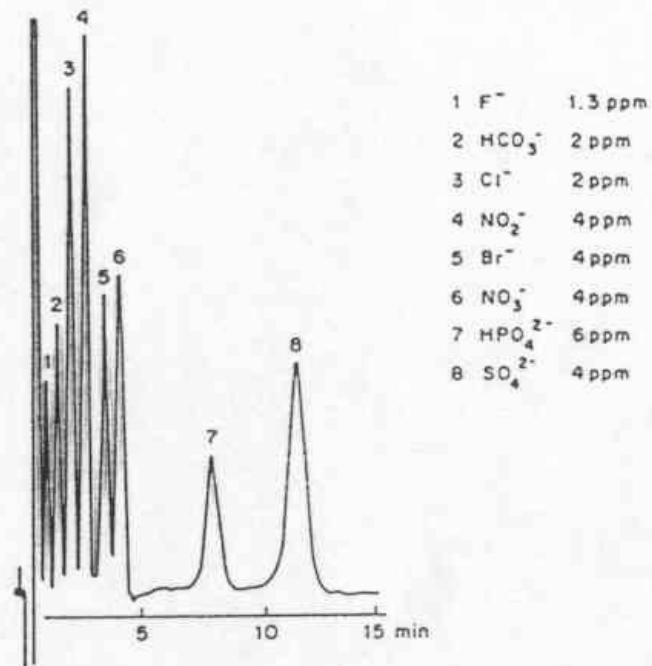
- **Normalnofazna kromatografija (NP):** diol,  $\text{NO}_2$ , CN,  $\text{NH}_2$
- **Reverznofazna kromatografija (RP):** fenil, C4, C8, C18

# Ionsko izmenjalna kromatografija

## Stacionarne faze

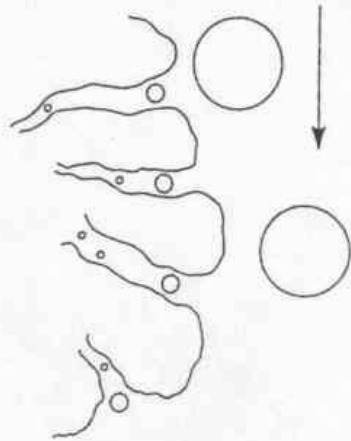


## Separacija ionov

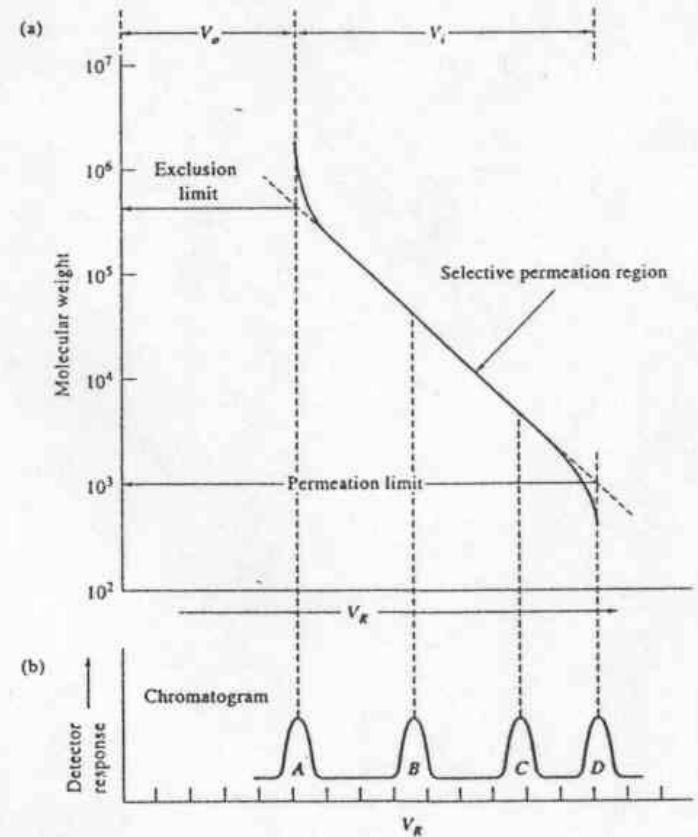


# Izključitvena kromatografija

## Princip delovanja

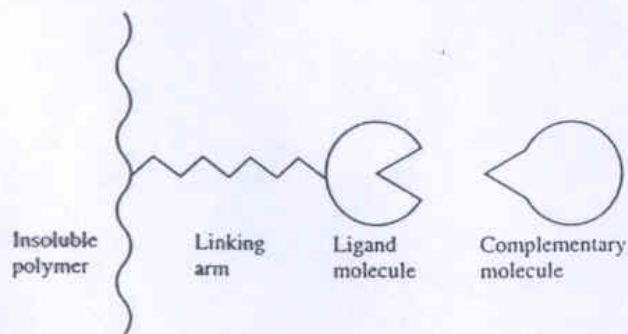


## Separacija v odvisnosti od M

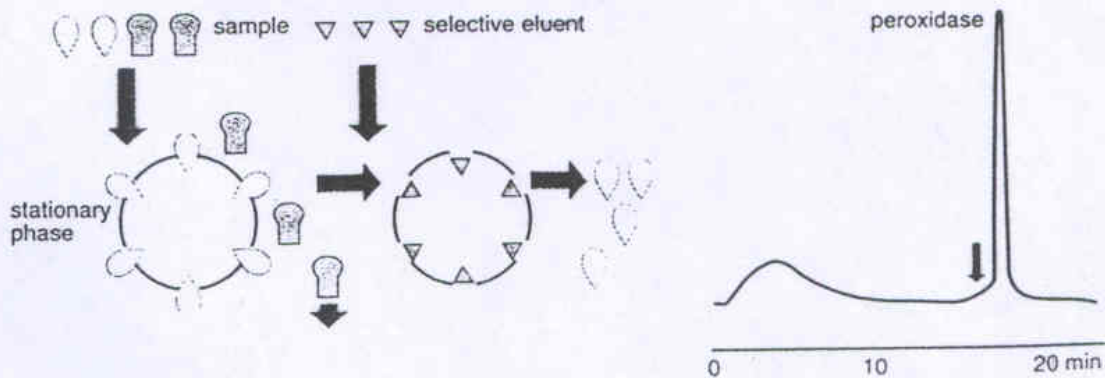


# Afinitetna kromatografija

## Stacionarne faze



## Selektivna separacija spojin



## KVANTITATIVNE MERITVE

### 1. Uporaba zunanjega standarda

$$c_x = \frac{A_x}{A_{st}} c_{st} \qquad y = a + b \cdot x$$

### 2. Uporaba notranjega standarda

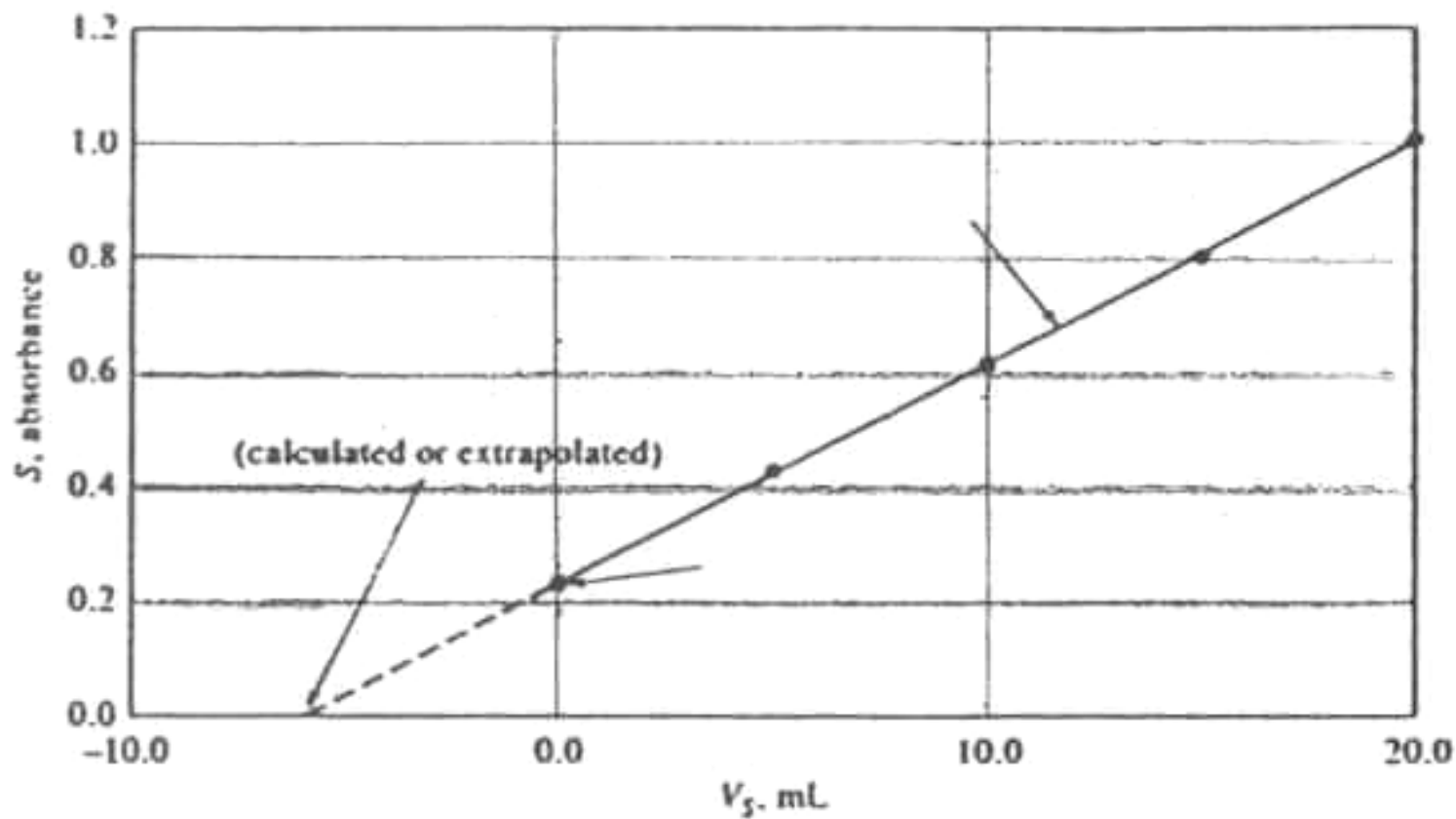
$$c_x = \frac{A_x}{A_{IS}} \cdot \frac{A_{IS}^0}{A_{st}} \cdot c_{st} \qquad y = a + b \cdot x$$



### 3. Uporaba standardnega dodatka

$$c_x = \frac{A_1}{A_2 - A_1} \cdot \frac{V_{st}}{V_x} \cdot c_{st} \quad y = a + b \cdot x$$

$$c_x = \frac{a}{b \cdot V_x} \cdot c_{st}$$



### **Primeri:**

1. Retencijski čas spojine A je pri 12,5 cm dolgi koloni 7,4 min, spojine B pa 8,7 min. Širini pikov na bazni liniji sta za A 1,2 min ter za B 1,4 min. Izračunaj a) resolucijo in povprečno število podov ter b) resolucijo in povprečno število podov v primeru, če uporabimo 25 cm dolgo kolono enake kakovosti.  
(a)  $R_s = 1,00$ ;  $N = 613$       (b)  $R_s = 1,41$ ;  $N = 1226$

2. V standardnem kromatogramu je pri conc. spojine A 1,50 mg/100 ml njen retencijski čas 4,1 min, višina pika pa 38 mm. Interni standard s conc. 2,00mg/100 ml ima v tem kromatogramu retencijski čas 6,4 min in višino 48 mm.

Izračunaj conc. spojine A po metodi a) eksternega in b) internega standarda, če dobimo v vzorčnem kromatogramu pik pri  $t_R = 4,1$  min z višino 20 mm ter pik pri  $t_R = 6,4$  min z višino 42 mm.

(a) 0,79 mg/100 ml      (b) 0,90 mg/100 ml

**3. V tri 50 ml bučke odpipetiramo po 15,0 ml vzorca, v katerem določamo conc. spojine A. Prvemu vzorcu dodamo 0,0, drugemu 5,0 in tretjemu 10,0 ml raztopine standarda A s conc. 15,0 mg/L. Po dopolnitvi do konstantnega volumna izmerimo na spektrofotometru absorbance raztopin pri 260 nm ter izračunamo umeritveno premico:  $A = 0,321 + 0,045 \cdot V_{st}$ .**

**Izračunaj conc. spojine A v vzorcu.**

**(7,13 mg/L)**

**VISOKA POLARNOST**



**NIZKA POLARNOST**

**org. kisline, baze  
alkoholi  
amini  
aldehidi, ketoni  
estri  
nenasičeni ogljikovodiki  
nasičeni ogljikovodiki**

**DOLGI RET. ČASI**



**KRATKI RET. ČASI**