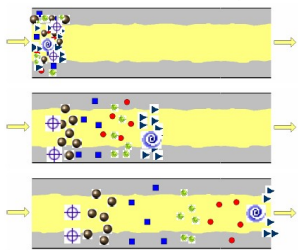


12/09	Introdução às Técnicas Cromatográficas
16/09	Cromatografia Líquida de Alta Eficiência
19/09	Cromatografia Líquida de Alta Eficiência
23/09	Cromatografia Líquida de Alta Eficiência
26/09	Cromatografia em Fase Gasosa
30/09	Cromatografia em Fase Gasosa
03/10	Preparo de Amostras
07/10	Preparo de Amostras
10/10	Validação Analítica
14/10	Prova

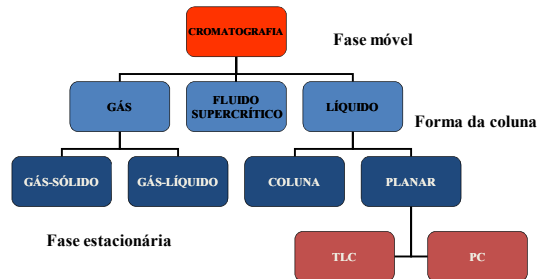
CROMATOGRAFIA

- ❖ Técnica de separação na qual os componentes de uma amostra são distribuídos entre duas fases.
- ❖ Fase **estacionária** (grande área superficial)
- ❖ Fase **móvel**

SEPARAÇÃO CROMATOGRÁFICA

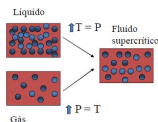
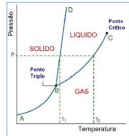


CLASSIFICAÇÃO DAS TÉCNICAS CROMATOGRÁFICAS



CROMATOGRAFIA COM FLUIDO SUPERCRÍTICO

- CO₂ (31,3°C, 72,9 atm)



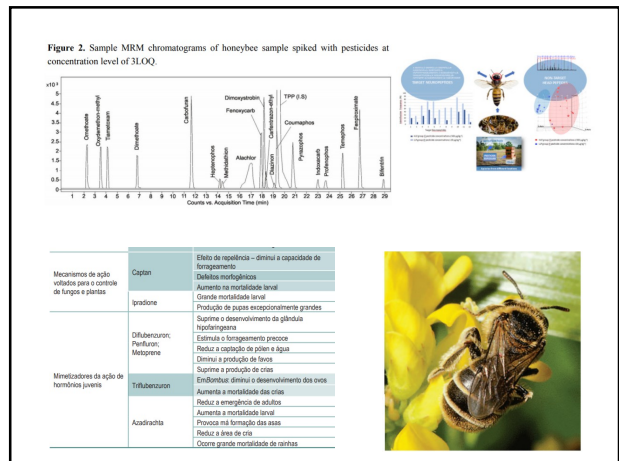
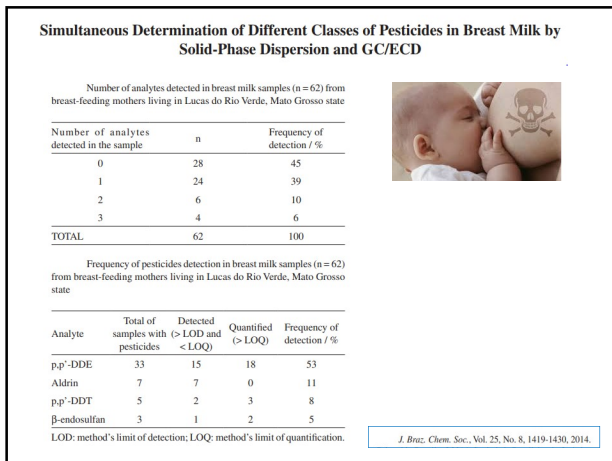
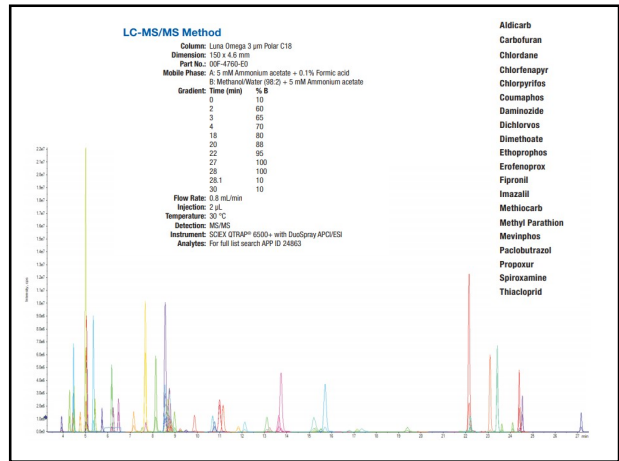
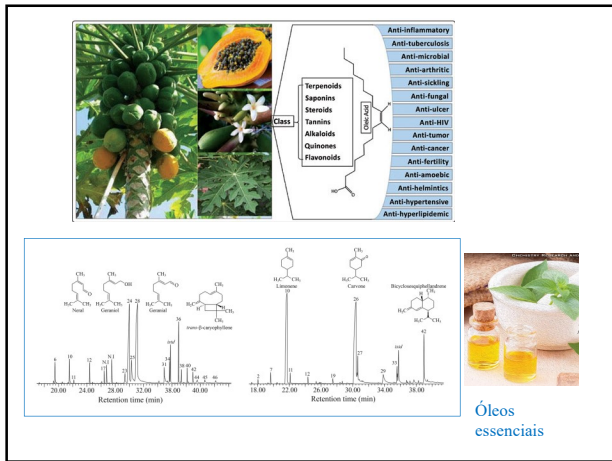
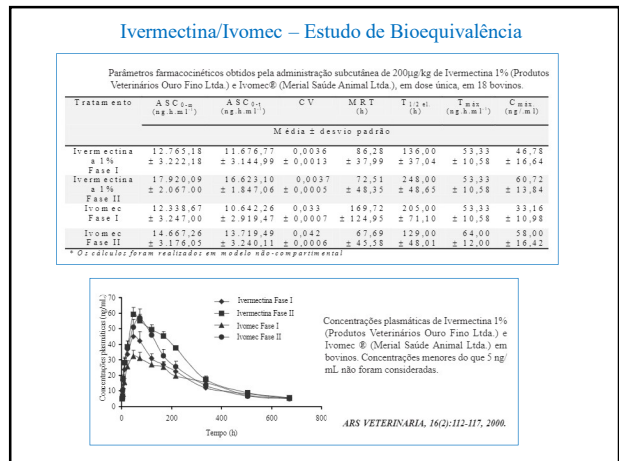
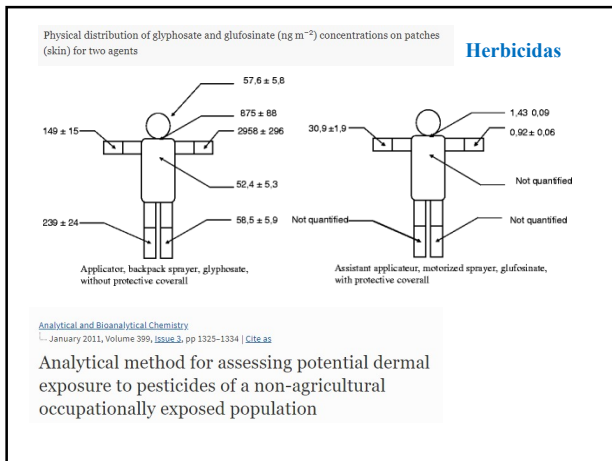
	Gas	Fluido Supercrítico	Líquido
densidade g/cm ³	(0.6 - 2) x 10 ⁻³	0.2 - 0.5	0.6 - 2
coef. de difusão (cm ² /s)	(1 - 4) x 10 ⁻⁵	10 ⁻⁴ - 10 ⁻⁴	(0.2 - 2) x 10 ⁻⁵
viscosidade (gcm ⁻¹ s ⁻¹)	(1 - 3) x 10 ⁻⁴	(1 - 3) x 10 ⁻⁴	(0.2 - 3) x 10 ⁻²

- alto solvatação (líquido) - baixa viscosidade (gás)

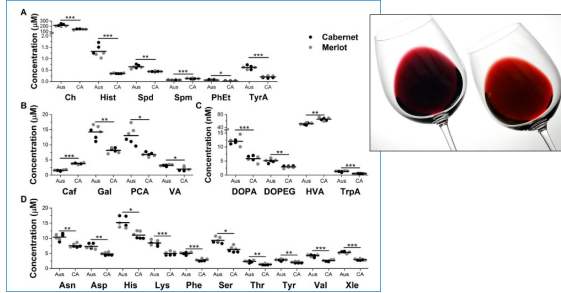
Quim. Nova, Vol. 24, No. 4, 509-515, 2001

Agricultura, pecuária e veterinária



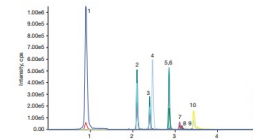


Composição ácidos fenólicos e aminos biogênicas Propriedades organolépticas (cor, odor, sabor) Variedades das uvas



Metabolites showing significant differences between locations of production. A. Biogenic amines. B. Phenolic acids. C. Trace metabolites. D. Amino acids. Unpaired, two-tailed Student's *t*-tests were performed and the false-discovery rate was used. Data shown is uncorrected for dilution. * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001. Asn: Asparagine; CA: California; Ch: choline; Hist: histamine; Spd: spermidine; Spm: spermine; PHEI: phenylethylamine; TyrA: tyramine; Caf: caffeic acid; Gal: gallic acid; PCA: p-coumaric acid; VA: vanillic acid; DOPA: 3,4-dihydroxyphenylamine; DOPEG: 3,4-dihydroxyphenylethanol; HVA: homovanillic acid; TtpA: tryptamine; Asn: asparagine; Asp: aspartic acid; His: histidine; Lys: lysine; Phe: phenylalanine; Ser: serine; Thr: threonine; Tyr: tyrosine; Val: valine; Xle: leucine/isoleucine.

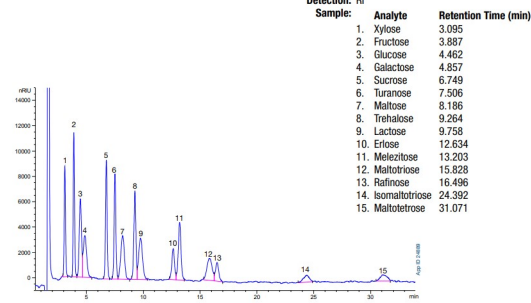
Vinho Tinto Polifenóis



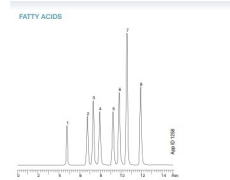
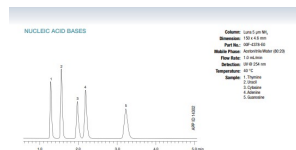
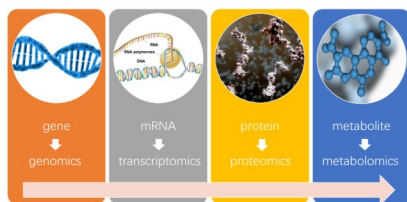
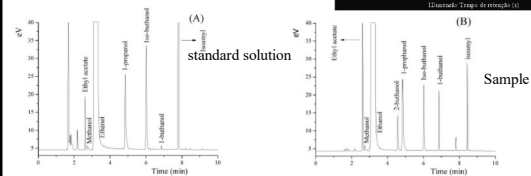
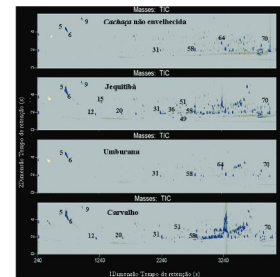
LC/MS/MS Conditions
 Column: Luna® 3 µm C8(2) 100 Å
 Dimensions: 50 x 2.0 mm
 Part No.: 008-4248-00
 Mobile Phase: A: 5 mM Ammonium acetate in Water with 0.5% Acetic acid
 B: 5 mM Ammonium acetate in Methanol with 0.5% Acetic acid
 Flow Rate: 0.1 mL/min
 Gradient: Time (min) %B
 0.00 0.00
 3.00 80
 5.00 80
 5.01 2
 8.00 2
 Injection Volume: 5 µL
 Temperature: Ambient
 MS/MS Detection: API 4000 MS/MS, ESI (negative (ESI-))

Peak No.	Analyte	RT	CI	RF (min)
1	Gallic acid	100.0	120716.0	6.59
2	Catechin	289.0	245200.0	2.08
3	Epigallocatechin	289.0	245200.0	2.08
4	Caffeic acid	179.0	130117.0	2.46
5	p-Coumaric acid	163.1	119193.0	2.85
6	p-Coumaric acid (ESI)	165.8	1287119.0	2.85
7	trans-Resveratrol	227.0	143119.0	3.09
8	trans-Resveratrol (ESI)	225.0	1901766.8	3.09
9	Myrictin	317.0	151179.0	3.15
10	cis-Resveratrol	227.0	143119.0	3.38
	Quercetin	301.0	151179.0	3.43

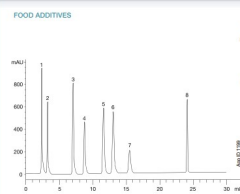
Column: Luna Omega 3 µm SUGAR
 Dimensions: 150 x 4.6 mm
 Part No.: 00F-4775-EO
 Elution Type: Isocratic
 Mobile Phase: Acetonitrile/Water (80:20)
 Flow Rate: 1.5 mL/min
 Temperature: 25 °C
 System: Agilent® 1100
 Detection: RI



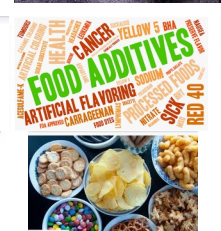
Cachaças orgânicas brasileiras: perfil físico-químico e cromatográfico

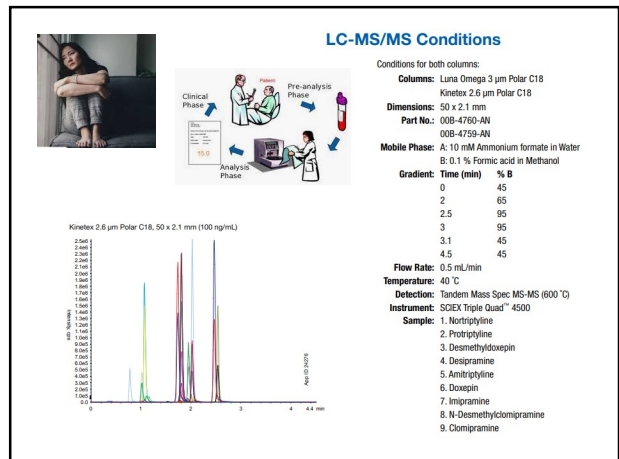
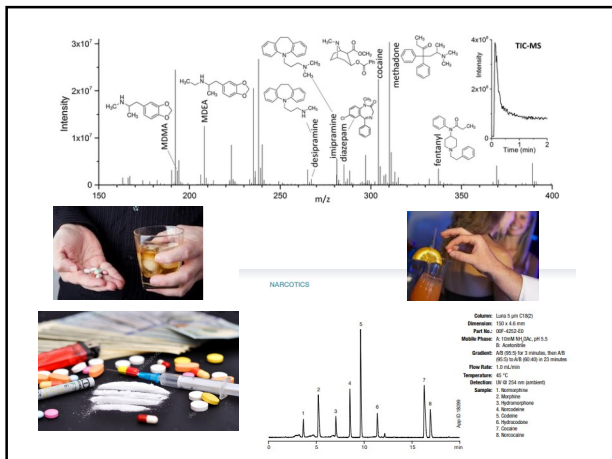


Column: Luna 3 µm C8(2)
 Dimensions: 100 x 4.6 mm
 Part No.: 00F-4248-00
 Mobile Phase: A: Acetonitrile
 B: Water
 Gradient: AB (20:80) AB (20:80) in 10 min, hold for 4 min, AB (80:20) AB (80:20) in 10 min, hold for 4 min
 Flow Rate: 1.0 mL/min
 Detection: Evaporative Light Scattering (ELS)
 Temperature: 21 °C
 Sample: 1. Lauric acid
 2. Myristic acid
 3. Palmitic acid
 4. Stearic acid
 5. Oleic acid
 6. Linoleic acid
 7. Arachidonic acid
 8. Docosahexaenoic acid



Column: Luna 3 µm C8(2)
 Dimensions: 100 x 4.6 mm
 Part No.: 00F-4248-00
 Mobile Phase: A: Acetonitrile
 B: Water
 Gradient: AB (20:80) AB (20:80) in 10 min, hold for 4 min, AB (80:20) AB (80:20) in 10 min, hold for 4 min
 Flow Rate: 1.0 mL/min
 Detection: ELS
 Temperature: 21 °C
 Sample: 1. Saccharin
 2. p-Toluenesulfonic acid
 3. p-Toluenesulfonic acid methyl ester
 4. Citric acid
 5. Malic acid
 6. p-Toluenesulfonic acid ethyl ester
 7. p-Toluenesulfonic acid
 8. Propyl p-toluenesulfonate





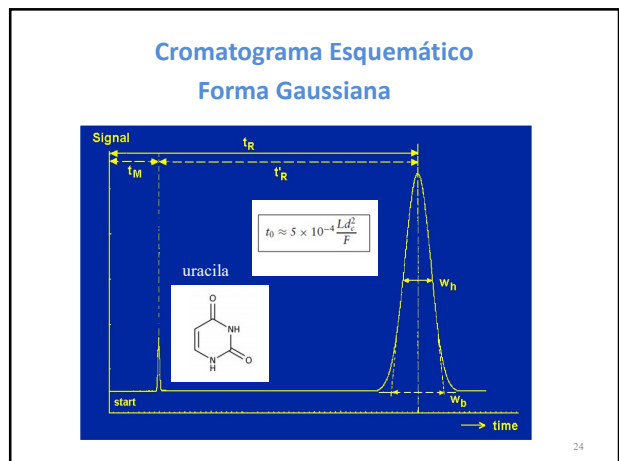
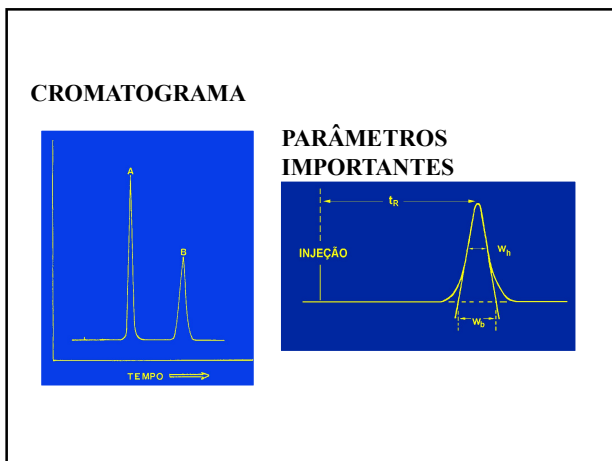
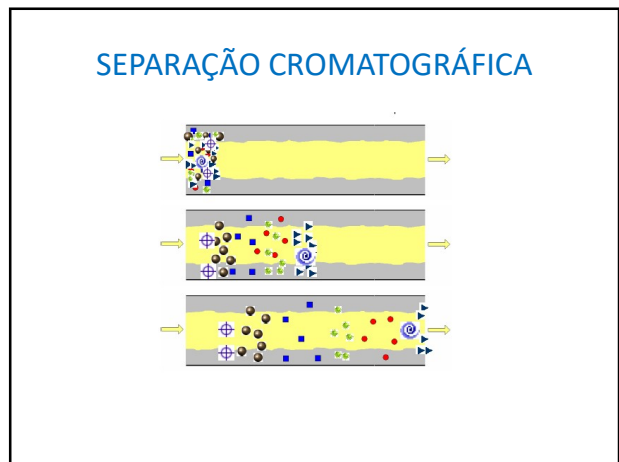
Anal. Bioanal. Chem. (2017) 499:3587–3596
 DOI 10.1007/s00216-017-0306-3

RESEARCH PAPER **Queiroz et al. 2017**

A column switching ultrahigh-performance liquid chromatography-tandem mass spectrometry method to determine anandamide and 2-arachidonoylglycerol in plasma samples

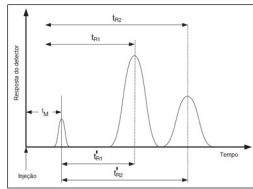
Camilla Marchioni¹ · Israel Donizeti de Souza² · Caroline Fernandes Greco² · José Alexandre Crippa³ · Vitor Tumas² · Maria Eugênia Costa Queiroz²

Amostra biológica	Grupo estudados	n	AEA (ng mL ⁻¹) (média ± DP)	2-AG (ng mL ⁻¹) (média ± DP)
Plasma	Controle	33	0,265 ± 0,145	0,368 ± 0,227
	Parkinson	52	0,256 ± 0,157	0,323 ± 0,277
	Alzheimer	32	0,428 ± 0,117	0,342 ± 0,266
	Comprometimento cognitivo leve	40	0,226 ± 0,126	0,454 ± 0,254
	Huntington	47	0,473 ± 0,243	0,291 ± 0,193
Líquido cefaloroquimiano (LCR)	Controle para Huntington	32	0,553 ± 0,162	0,234 ± 0,125
	Controle	29	0,682 ± 0,173	0,468 ± 0,163
	Parkinson	46	0,816 ± 0,226	0,383 ± 0,249
	Alzheimer	31	0,685 ± 0,291	0,477 ± 0,213
	Comprometimento cognitivo leve	28	0,691 ± 0,155	0,465 ± 0,162

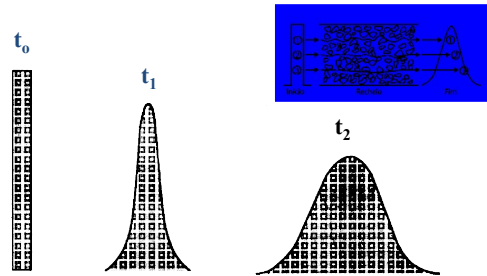


Parâmetros Cromatográficos

- ✓ Tempo de retenção (t_R)
- ✓ Tempo de retenção de um composto não-retido (t_M)
- ✓ Tempo de retenção ajustado $\rightarrow t_{R'} = t_R - t_M$

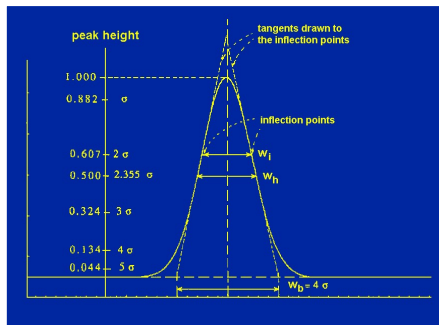


ALARGAMENTO DE UMA BANDA CROMATOGRÁFICA



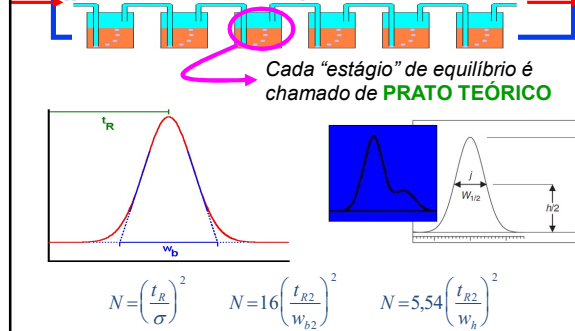
26

Pico Gaussiano - difusão longitudinal



27

1. EFICIÊNCIA DA COLUNA (picos mais estreitos)



28

1. Eficiência da Coluna

Outra maneira de medir-se a eficiência é através da Altura Equivalente a um Prato, H :

$$H = \frac{L}{N} \text{ (mm)}$$

Comparação entre diferentes colunas

$h = H/d_p$ - altura do prato reduzido

H é o comprimento necessário de uma coluna para gerar um prato teórico. Quanto maior N menor será H e mais eficiente a coluna.

29

A chromatographic analysis for the chlorinated pesticide Dieldrin gives a peak with a retention time of 8.68 min and a baseline width of 0.29 min. What is the number of theoretical plates? Given that the column is 2.0 m long, what is the height of a theoretical plate in mm?

A chromatographic analysis for the chlorinated pesticide Dieldrin gives a peak with a retention time of 8.68 min and a baseline width of 0.29 min. What is the number of theoretical plates? Given that the column is 2.0 m long, what is the height of a theoretical plate in mm?

Solution

Using equation 12.15, the number of theoretical plates is

$$N = 16 \frac{t_R^2}{w^2} = N = 16 \frac{(8.68 \text{ min})^2}{(0.29 \text{ min})^2} = 14300 \text{ plates}$$

Solving equation 12.10 for H gives the average height of a theoretical plate as

$$H = \frac{L}{N} = \frac{2.0 \text{ m}}{14300 \text{ plates}} \times \frac{1000 \text{ mm}}{\text{m}} = 0.14 \text{ mm/plate}$$

2. Resolução, R_S

$$R_S = \frac{t_{R2} - t_{R1}}{\frac{1}{2}(w_{b1} + w_{b2})}$$

R_S = Resolução entre dois picos; t_{R1} = Tempo de retenção do pico 1; t_{R2} = Tempo de retenção do pico 2; w_{b1} = Largura do pico 1 na base; w_{b2} = Largura do pico 2 na base.

2. Resolução, R_S

Para 2 picos adjacentes $w_{b1} \approx w_{b2}$, podendo-se escrever:

$$R_S = \frac{t_{R2} - t_{R1}}{w_{b2}} = \frac{\Delta t_R}{w_{b2}} \quad R_S = 1,25 \quad (\text{fins quanti})$$

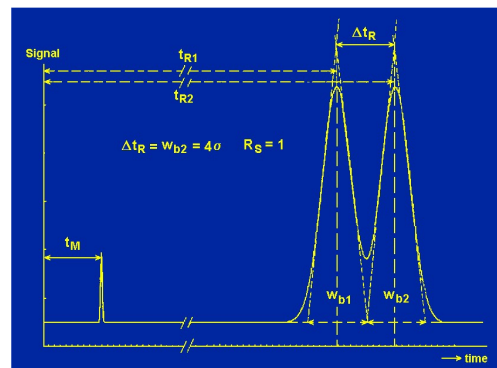
R_S valerá 1 quando $\Delta t_R = w_{b2}$

$R_S > 1,5$
(completa)

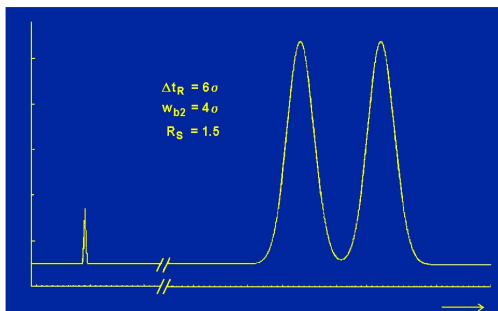
% Separação em função de R_S

R_S	0,50	0,90	1,00	1,10	1,25	1,50	2,00	2,50
%	68,28	92,82	95,44	97,22	98,76	99,73	99,99	100,00
Δt_R (em σ)	2,0	3,6	4,0			6,0		

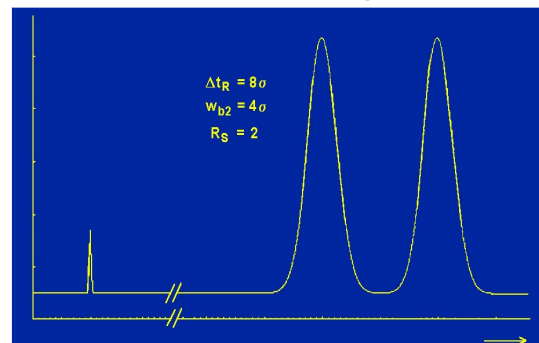
2. Resolução, R_S



2. Resolução, R_S



2. Resolução, R_S



In a chromatographic analysis of lemon oil a peak for limonene has a retention time of 8.36 min with a baseline width of 0.96 min. γ -Terpinene elutes at 9.54 min with a baseline width of 0.64 min. What is the resolution between the two peaks?

Solution

Using equation 12.1 we find that the resolution is

$$R_{10} = \frac{2\Delta t_R}{w_B + w_A} = \frac{2(9.54 \text{ min} - 8.36 \text{ min})}{1.64 \text{ min} + 0.96 \text{ min}} = 1.48 \quad (12.2.2)$$

2. Fator de Retenção, k

$$k = \frac{t_R - t_M}{t_M} \qquad k = \frac{t'_R}{t_M}$$

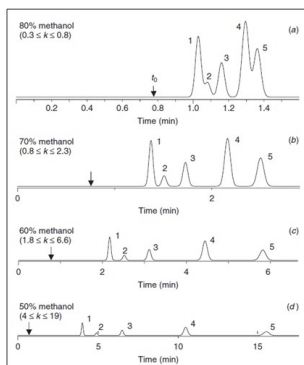
t_M = tempo que uma molécula da fase móvel passa na coluna; denominado de "hold-up time", tempo morto ou tempo de retenção de um soluto não retido.

t'_R = tempo de retenção ajustado ou tempo que o soluto passa na fase estacionária.

FATOR DE RETENÇÃO ($1 \leq k \leq 10$)

Análise de múltiplos componentes ($0,5 \leq k \leq 20$)

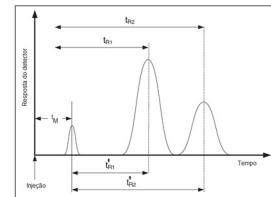
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3. Fator de separação, α

O fator de separação, α , é a relação existente entre o tempo que dois picos permanecem na fase estacionária.

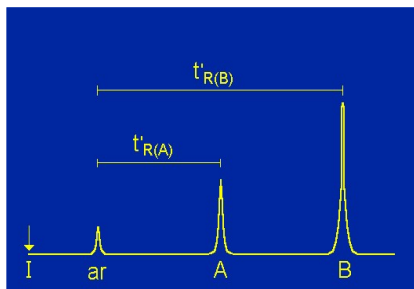
$$\alpha = \frac{t'_{R2}}{t'_{R1}} = \frac{k_2}{k_1}$$



Quanto maior o valor de α , mais seletiva será a fase estacionária para aquele par de compostos.

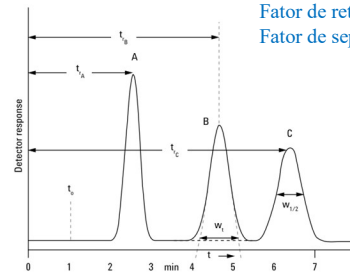
40

3. Fator de separação, α

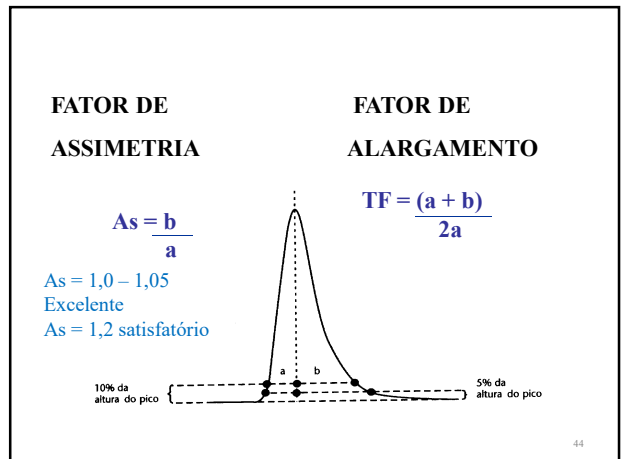
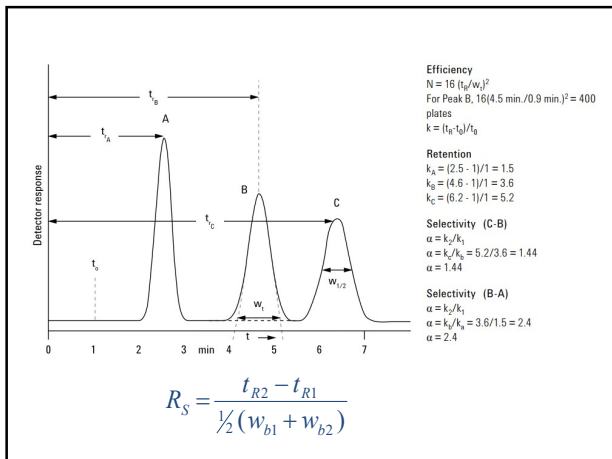


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Eficiência
Resolução
Fator de retenção
Fator de separação



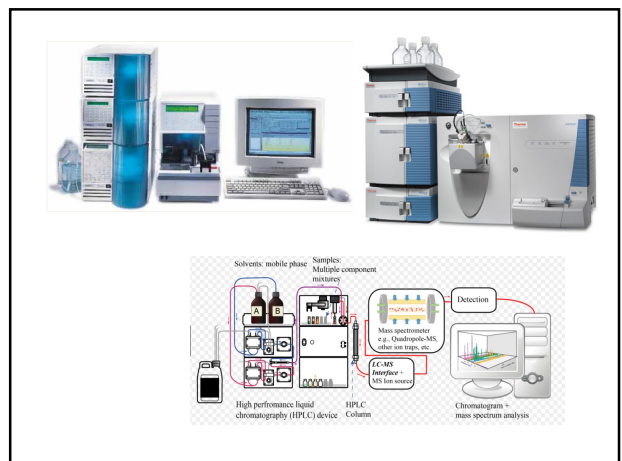
$$t_{RA} = 2,5 \text{ min} \quad t_{RB} = 4,6 \text{ min} \quad t_{RC} = 6,2 \text{ min}$$



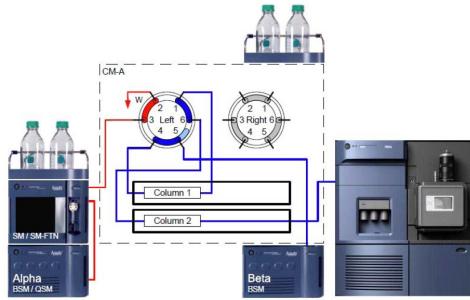
CROMATOGRAFIA LÍQUIDA DE ALTA EFICIÊNCIA

Prof. Dra. Maria Eugênia Queiroz Nassur

- ### HPLC- ANALITOS
- AMOSTRA SOLÚVEL NA FASE MÓVEL
 - ESPÉCIES IÔNICAS
 - MACROMOLÉCULAS
 - COMPOSTOS DE ALTA MASSA MOLAR
 - COMPOSTOS TERMICAMENTE INSTÁVEIS
 - A MAIORIA DAS SEPARAÇÕES OCORRE EM TEMPERATURA AMBIENTE
 - PERMITE POSTERIOR CARACTERIZAÇÃO ESTRUTURAL



Sistema Bidimensional



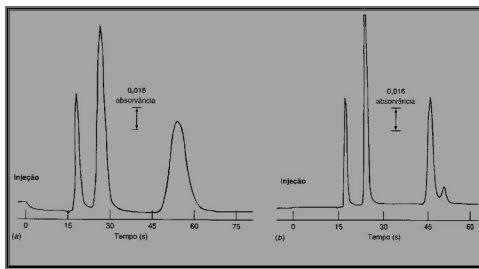
COLUNAS

Colunas	Comprimento (cm)	Diâmetro interno (mm)	Vazão	Tamanho de partícula (µm)
Análitica HPLC	5-30	2-6	1 - 5 mL/min	3, 5, 10
Análitica UHPLC	5-15	1-2,1	< 1 mL/min	< 2,0
Capilar recheada	20-200	0,25 - 0,5	0,1 - 2 µL/min	1, 3
Capilar aberto	100 - 10.000	0,01 - 0,075	0,05 - 2 µL/min	a
Preparativa	≥ 20	≥ 10	≥ 1 mL	> 10

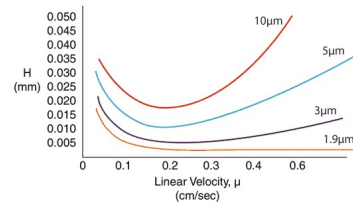
a: filme líquido ligado às paredes



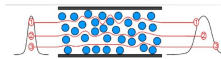
Colunas recheadas com partículas de sílica de diâmetros iguais a) 10 µm b) 5 µm



ALTURA DO PRATO EM FUNÇÃO DA VAZÃO PARA TAMANHOS DE PARTÍCULAS DA FASE ESTACIONÁRIA

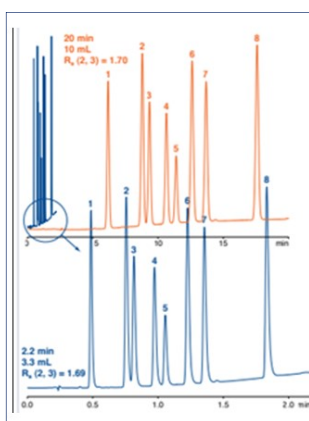


$$H = \frac{L}{N}$$



Years	Particle size	Plates/15 cm
1950s	100 µm	200
1967	50 µm	1000
1972	10 µm	6000
1985	5 µm	12 000
1992	3-3.5 µm	22 000
1996 ^{a)}	1.5 µm	30 000
2000	2.5 µm	25 000
2004	1.7 µm	30 000

a) Nonporous silica or resins.



Fármacos ácidos
 Colunas: 50 x 4 mm NUCLEOSHELL RP 18, 2.7 µm
 150 x 4 mm fully porous C18, 5 µm
 Eluente: A) acetoniitrila, B) 25 mmol/L KH₂PO₄, pH 7, 25-40% A in 2.2 min, 25-40% A in 20 min
 Fluxo: 1.5 mL/min, 0.5 mL/min
 Pressão: 219 bar, 92 bar
 Temp.: 20 °C
 Detecção: UV, 215 nm
Picos:
 1. Ketoprofeno
 2. Fenoprop
 3. Fenoprofeno
 4. Flurbiprofeno
 5. Ibuprofeno
 7. Carprofeno
 8. Diclufenaco
 9. Ácido meclonâmico

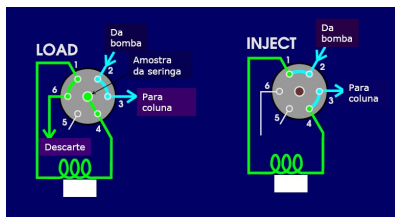
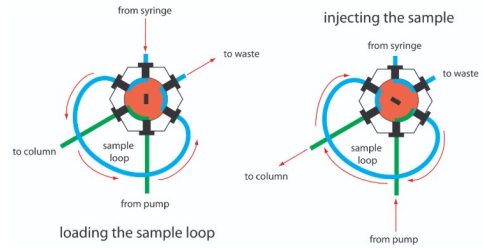
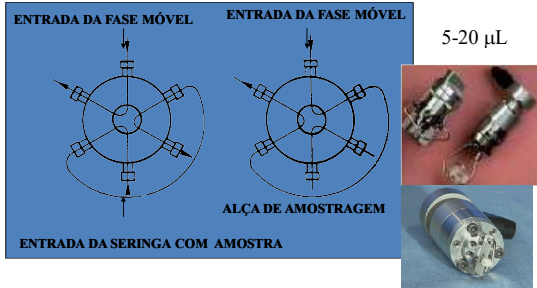
INTRODUÇÃO DA AMOSTRA



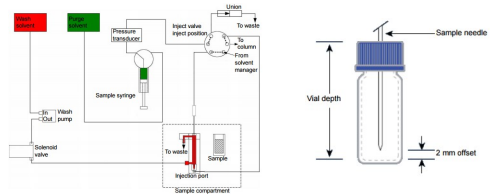
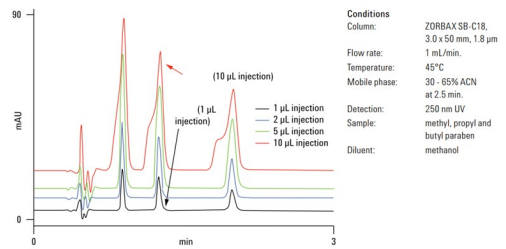
Diluir a amostra em fase móvel ou em um dos componentes
 Evitar - precipitação no injetor ou coluna

INJETOR – ALÇA DE AMOSTRAGEM

- POSIÇÃO CARREGAR - POSIÇÃO INJETAR



Volume da amostra: 1% do volume da coluna vazia
 $V_c = \pi r^2 L$



Sample syringe assembly components:



PROTEÇÃO DA COLUNA

- COLUNA DE GUARDA (PRÉ - COLUNA)
- FASE ESTACIONÁRIA - ANÁLOGA A COLUNA ANALÍTICA
- 1 A 4 cm DE COMPRIMENTO
- ADSORÇÃO IRREVERSÍVEL DE IMPUREZAS

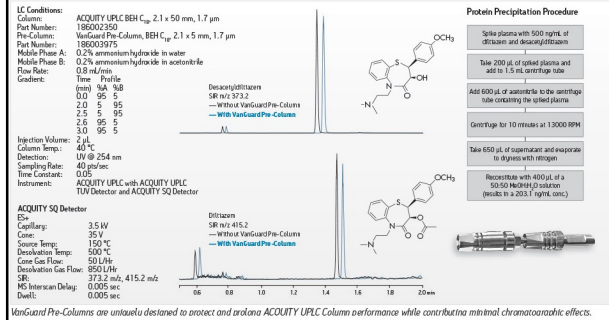


Cost effective column protection



Minimal Chromatographic Effects with VanGuard Pre-Columns

VanGuard Pre-columns are uniquely designed to protect and prolong ACQUITY UPLC Column performance while contributing minimal chromatographic effects



RESERVATÓRIO FASE MÓVEL



Desgaseificação da fase móvel



FASE MÓVEL

- MISTURA DUAS OU MAIS SUBSTÂNCIAS MISCÍVEIS
- ALTO GRAU DE PUREZA – sensibilidade analítica (filtrar e eliminar gases dissolvidos)
- SOLUBILIZAR A AMOSTRA - SEM DECOMPOSIÇÃO DOS CONSTITUINTES
- NÃO SOLUBILIZAR OU DECOMPOR A FASE ESTACIONÁRIA
- BAIXA VISCOSIDADE E PONTO DE EBULIÇÃO
- AQUECIMENTO DA COLUNA
- Diminui tr, melhor resolução, menor viscosidade, aumento da difusão, melhor repetitividade tr
- BAIXA ABSORÇÃO NA REGIÃO UV
- ISOCRÁTICA
- ELUIÇÃO POR GRADIENTE



Filtros e purificadores de água

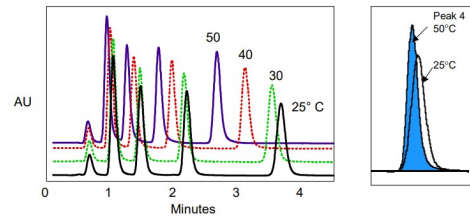


0.22 μm Millipak membrane filter

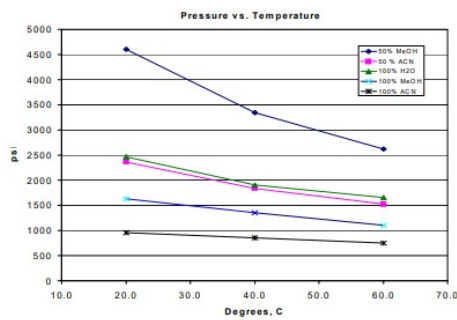
ÁGUA ULTRAPURA

Resistividade	18.2 MΩ.cm - 25 °C
TOC	< 10 ppb
Micro-organismos	< 0.1 cfu/ml after Millipak or BioPak (membrana filtradora de 0,22 µm)
Vazão	1.5 L/min

Cfu: Colony Forming Unit

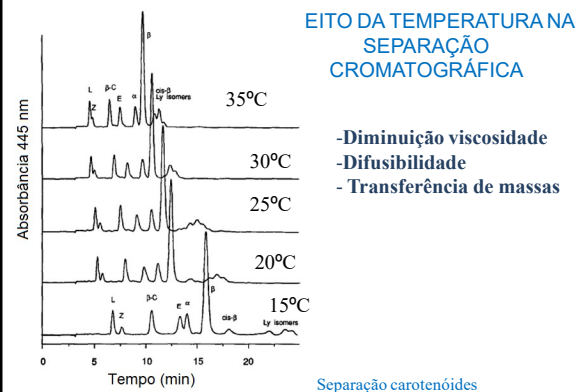


Effect of temperature on retention time and peak shape
Isocratic separation of acetone and C2-C4 alkylphenones in 60:40 methanol-water on a Symmetry® C₁₈ 2.1x30mm column



Pressure vs. Temperature

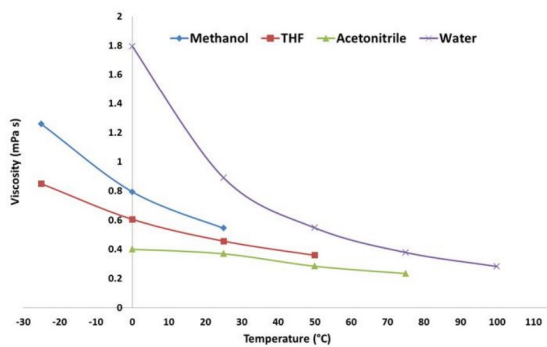
Column: Symmetry® C18, 2.1x 30mm
Flow rate: 1.0 mL/min
Temperature: 20°C to 60°C



EITO DA TEMPERATURA NA SEPARAÇÃO CROMATOGRÁFICA

- Diminuição viscosidade
- Difusibilidade
- Transferência de massas

Separação carotenóides
Coluna C18, 5% THF em metanol



TEMPERATURA DA COLUNA (30-60°C)

- DIMINUI A VISCOSIDADE DA FASE MÓVEL
- DIMINUI O TEMPO DE ANÁLISE
- MAIOR REPETITIVIDADE TR
- PONTO DE EBULIÇÃO DOS SOLVENTES

DETECTOR UV

Comprimento de onda limite

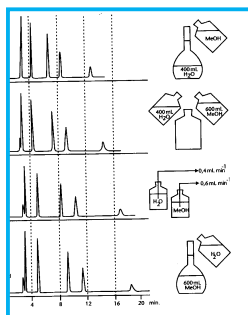
FASE MÓVEL	UV nm
ACETONITRILA	190
METANOL	205
ÁGUA	190
THF	210
ACETATO DE ETILA	250
N-HEXANO	200
1-PROPANOL	200
TAMPÃO ACETATO	210 (10mmol/L)
TAMPÃO FOSFATO	< 200 (0,1%)

DETECTOR UV

Comprimento de onda limite

Solvent	UV cut off (nm) ¹	Viscosity (mPa s) ²	B.P. (°C) ³	Polarity (P) ²
Acetone	330	0.306	56.2	5.1
Acetonitrile	190	0.369	81.6	5.8
Chloroform	245	0.542	61.2	4.1
DMSO	265	1.987	189	7.2
Dioxane	215	1.177	101.2	4.8
Ethanol	210	1.074	78.3	5.2
Hexane	210	0.300	68.7	0.1
IPA	210	2.04	82.4	3.9
Methanol	210	0.544	64.7	5.1
THF	220	0.456	66.0	4.0
Water	191	0.89	100	10.2

Influência dos diferentes modos de preparar a fase móvel na resolução cromatográfica



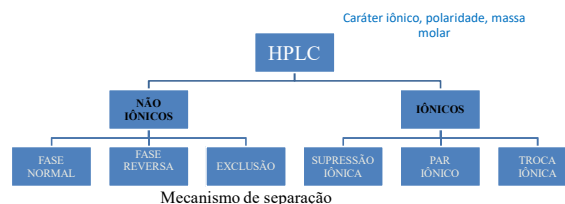
Contração do volume

MEDIR VOLUMES SEPARADAMENTE

DIARIAMENTE
-EVAPORAÇÃO DO SOLVENTE

CLASSIFICAÇÃO MECANISMOS DE SEPARAÇÃO

HPLC



CL EM FASE NORMAL

CROMATOGRAFIA LÍQUIDO SÓLIDO

- FASE ESTACIONÁRIA: **POLAR**
- FASE MÓVEL: **MENOS POLAR FE**
- MECANISMO DE SEPARAÇÃO : **ADSORÇÃO**

- LIGAÇÕES DE HIDROGÊNIO
- DIPOLO – DIPOLO
- DIPOLO INDUZIDO –DIPOLO (moléculas polarizáveis – moléculas aromáticas)
- LIGAÇÕES π

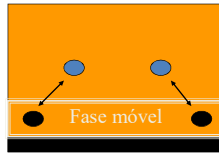
CROMATOGRAFIA LÍQUIDA EM FASE NORMAL

- CONFIGURAÇÃO ESPACIAL (*cis-trans*)
- *Isômeros estruturais* (diferem na posição dos átomos ou grupos)
- ELETRONEGATIVIDADE
- DIFERENTES GRUPOS FUNCIONAIS
- NÚMEROS DE GRUPOS FUNCIONAIS
- (ácidos graxos e álcoois)

Mecanismos de separação

FM apolar ou moderadamente polar – fraca interação dipolo

Competição – solvente

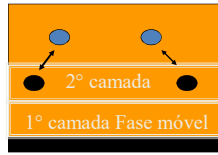


Superfície adsorvente

● Analito ● Fase móvel

FM polar

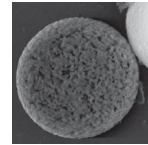
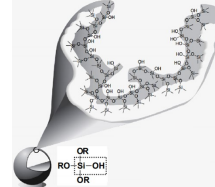
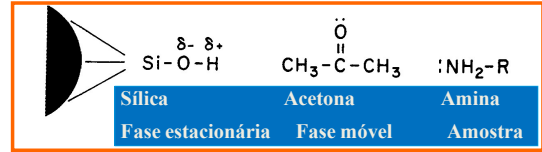
Interação/competição - solvente



Superfície adsorvente

adsorção

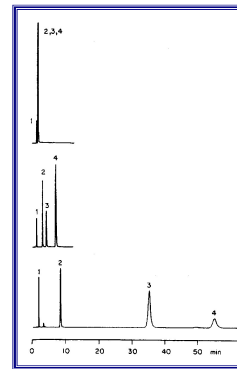
CROMATOGRAFIA LÍQUIDA EM FASE NORMAL



PARÂMETRO DE POLARIDADE – SNYDER CROMATOGRAFIA POR ADSORÇÃO

SOLVENTE	(FORÇA ELUENTE) ϵ°
HEXANO, HEPTANO	0,00
CLOROFÓRMIO	0,26
CLOROFORMO DE METILENO	0,32
ÉTER METIL t-BUTIL	0,35
ÉTER ETÍLICO	0,38
ACETATO DE ETILA	0,48
DIOXANO	0,51
ACETONITRILA	0,52
THF	0,53
1 OU 2-PROPANOL	0,60
METANOL	0,70

1 = ρ -xileno, 2= nitrobenzeno, 3 = acetofenona, 4 = 2,6 dinitrotolueno



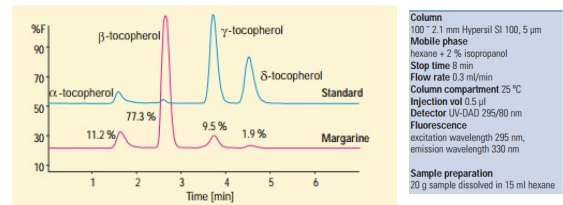
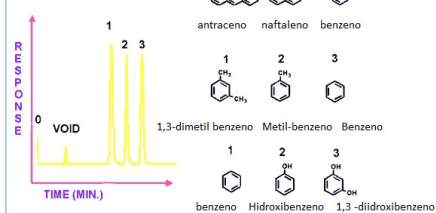
Éter metil-t-butil $\epsilon^\circ = 0,48$

Hexano: éter metil-t-butil 9:1 v/v $\epsilon^\circ = 0,14$

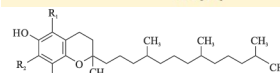
Hexano $\epsilon^\circ = 0,0$

LiChrosorb Si 60 5 μm

ELUTION ORDER IN NORMAL PHASE

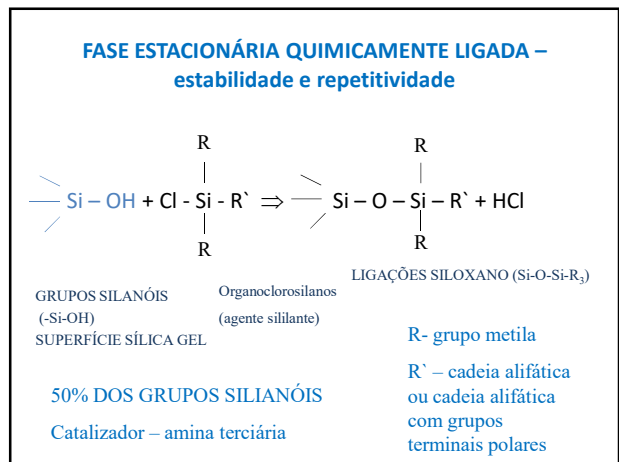
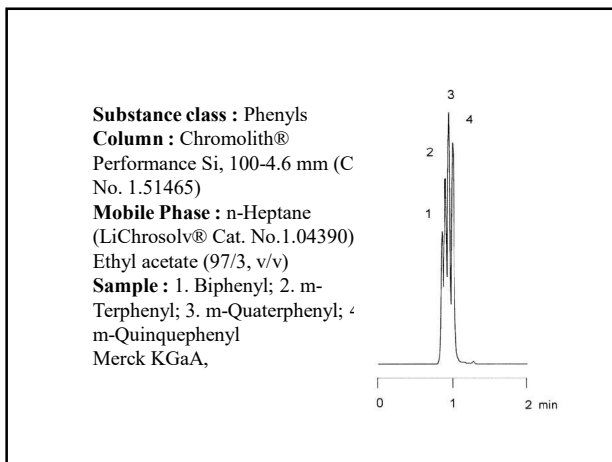
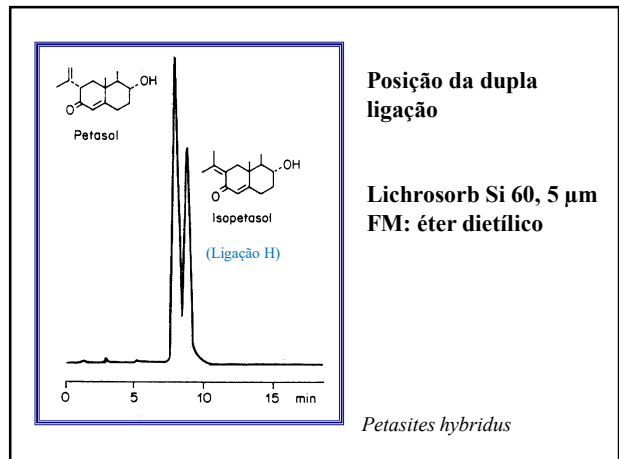
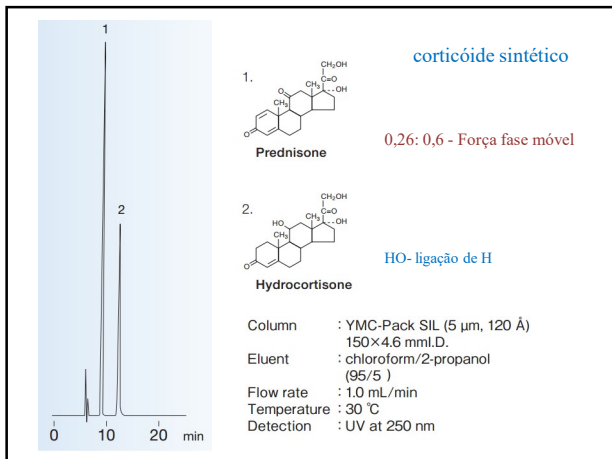


Column
 100 x 2.1 mm Hypersil SI 100, 5 μm
 Mobile phase
 hexane + 2% isopropanol
 Stop time 8 min
 Flow rate 0.3 ml/min
 Column compartment 25 °C
 Injection vol 0.5 μl
 Detector UV-DAD 295/60 nm
 Fluorescence
 excitation wavelength 295 nm,
 emission wavelength 330 nm
 Sample preparation
 20 g sample dissolved in 15 ml hexane



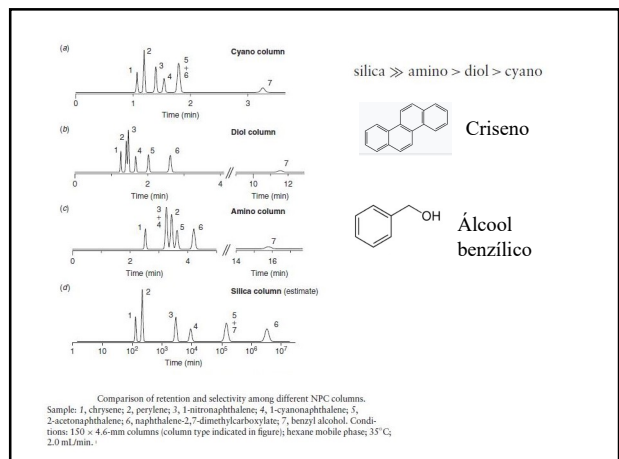
Alfa, beta, gama e delta

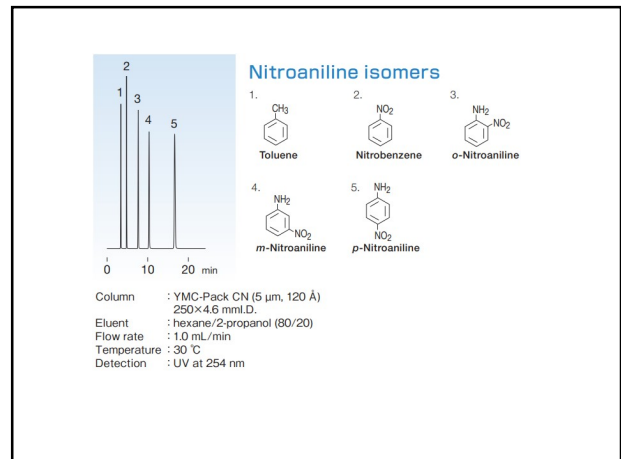
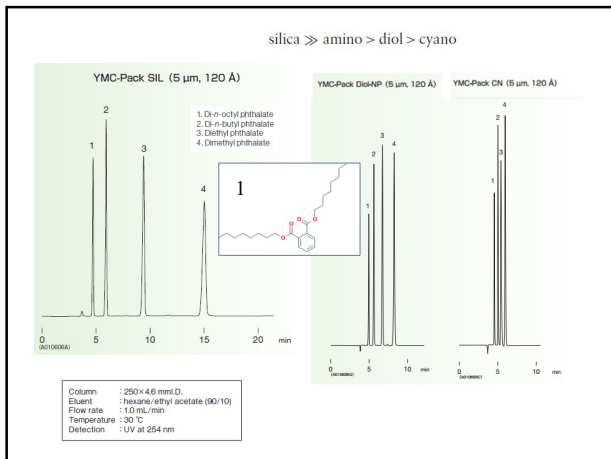
α - tocopherol: $R_1 = R_2 = R_3 = \text{CH}_3$
 β - tocopherol: $R_1 = R_2 = \text{CH}_3$; $R_3 = \text{H}$
 γ - tocopherol: $R_1 = \text{H}$; $R_2 = R_3 = \text{CH}_3$
 δ - tocopherol: $R_1 = R_2 = \text{H}$; $R_3 = \text{CH}_3$



FASES QUIMICAMENTE LIGADAS SELETIVIDADE

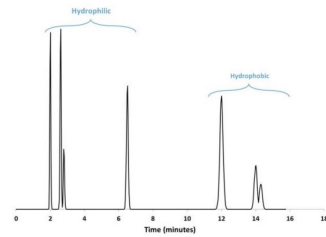
Modo	Grupo químico	Estrutura
Fase Normal	Amino	-NH ₂
	Ciano	-CN
	Diol	-Si-O-CH ₂ -CH(OH)-CH ₂ -OH
Fase reversa	Dimetil	-Si(CH ₃) ₂ -
	Octil	-Si-CH ₂ -(CH ₂) ₆ -CH ₃
	Octadecil	-Si-CH ₂ -(CH ₂) ₁₆ -CH ₃
	Fenil	-Si-C ₆ H ₅





CROMATOGRAFIA LÍQUIDA EM FASE REVERSA - RPLC

- **MAIS UTILIZADA EM LC - 75% SEPARAÇÕES LC (ANALITOS NÃO IÔNICOS)**
- **FASE ESTACIONÁRIA – APOLAR**
- **Grupos apolares quimicamente (ligações covalente-ESTABILIDADE QUÍMICA) ligados aos grupos silanóis da sílica**
- **GRUPOS OCTIL (-C₈H₁₇), OCTADECIL (-C₁₈H₃₇), FENIL (-C₆H₅)**
- **FASE MÓVEL AQUOSA – MENOS APOLAR FE**



ORDEM DE ELUIÇÃO

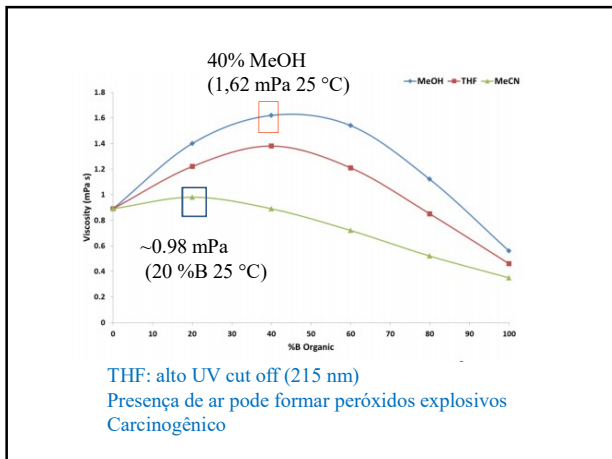
MECANISMOS DE INTERAÇÃO EM FASE REVERSA

- **PARTIÇÃO** - interação hidrofóbica
- **FASE ESTACIONÁRIA** (sítios específicos) – seletividade
- **ADSORÇÃO** (partículas sólidas modificadas)

CARACTERÍSTICAS DOS SOLVENTES

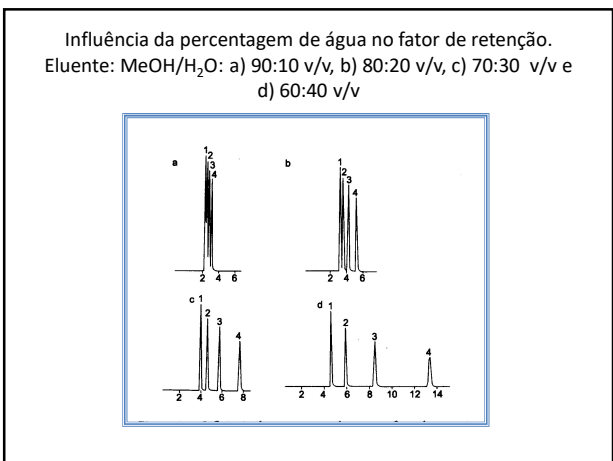
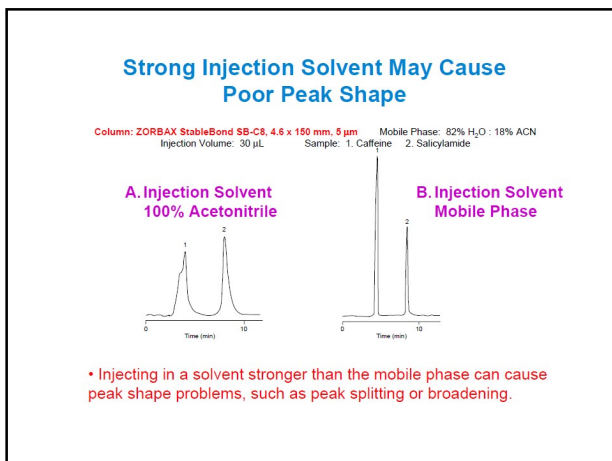
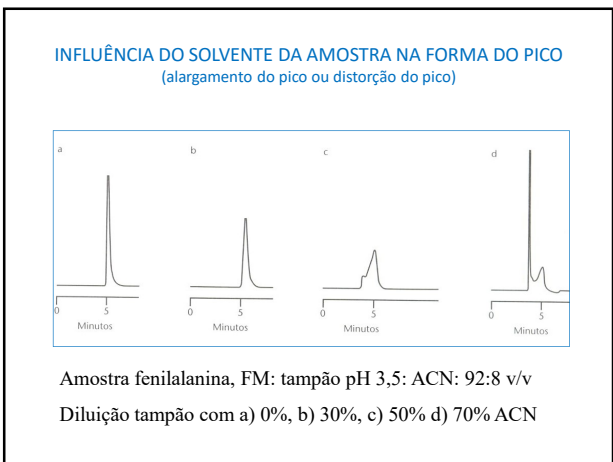
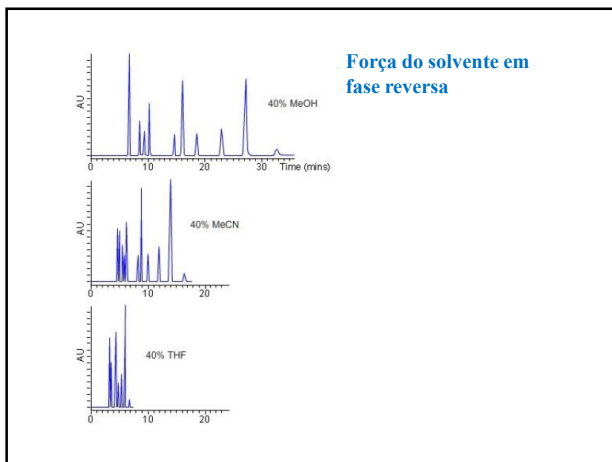
SOLVENTE	FATOR FORÇA PESO (S)	
	FASE REVERSA	FASE NORMAL
ACETONITRILA	3,2	5,8
ÁGUA	0	10,2
METANOL	2,6	5,1
TETRAIDROFURANO	4,5	4,0
CLOROFÓRMIO		4,1
DICLOROMETANO		3,1
ÉTER ETÍLICO		2,8
ÉTER METIL TERC-BUTÍLICO		2,5
HEXANO		0

Forte – polaridade semelhante à fase estacionária

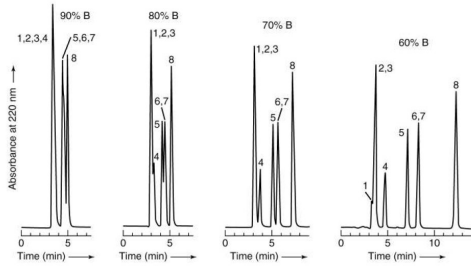


CROMATOGRAFIA LÍQUIDA EM FASE REVERSA - RPLC

- FASE MÓVEL AQUOSA
- ACETONITRILA /ÁGUA – BAIXA VISCOSIDADE
- METANOL, THF
- SOLVENTES ORGÂNICOS MISCÍVEIS EM ÁGUA
- MAIOR QUANTIDADE ÁGUA – MAIOR RETENÇÃO
- ÁGUA (SOLVENTE MAIS FRACO) < METANOL < ACETONITRILA < TETRAIDROFURANO

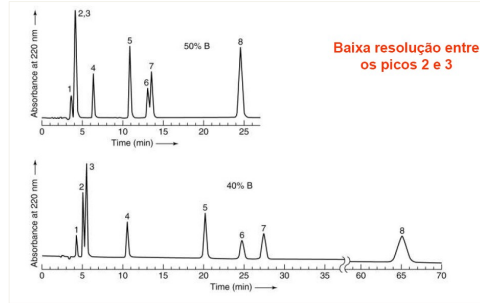


SEPARAÇÃO HPLC ISOCRÁTICA



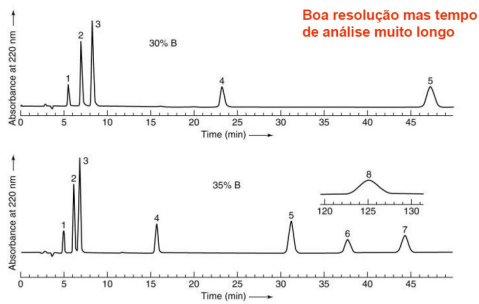
Separação de álcool benzílico (1), fenol (2), 3,4-dimetoxiacetofenona (3), benzoina (4), benzoato de etila (5), tolueno (6), 2,6-dimetoxitolueno(7), o-metoxibifenila (8). B refere-se ao solvente orgânico Acetonitrila

SEPARAÇÃO HPLC ISOCRÁTICA



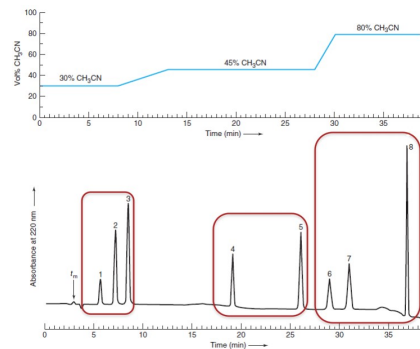
Baixa resolução entre os picos 2 e 3

SEPARAÇÃO HPLC ISOCRÁTICA



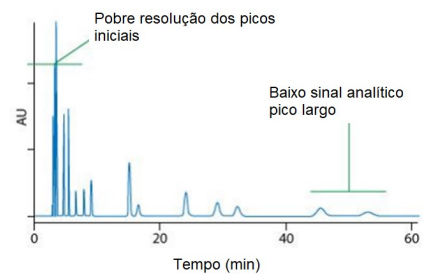
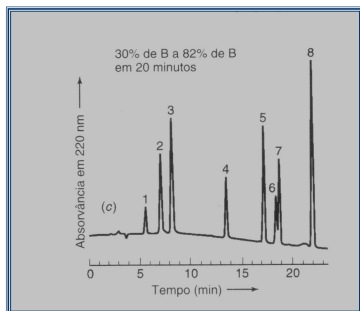
Boa resolução mas tempo de análise muito longo

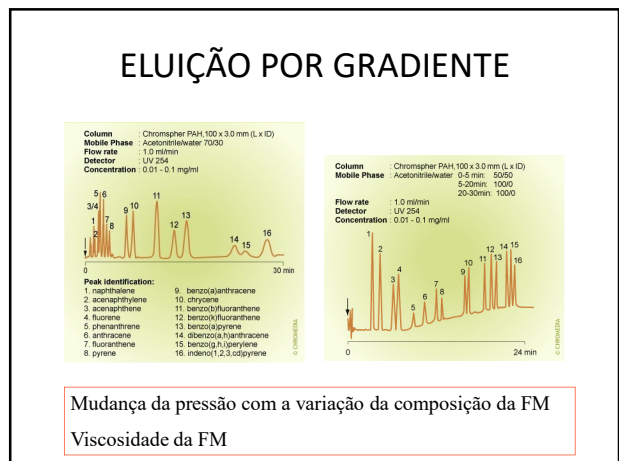
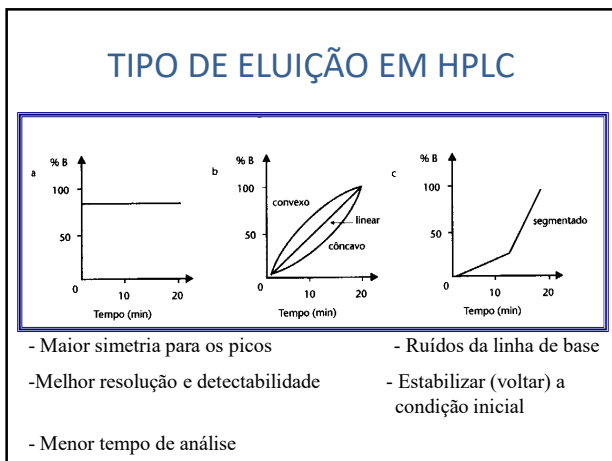
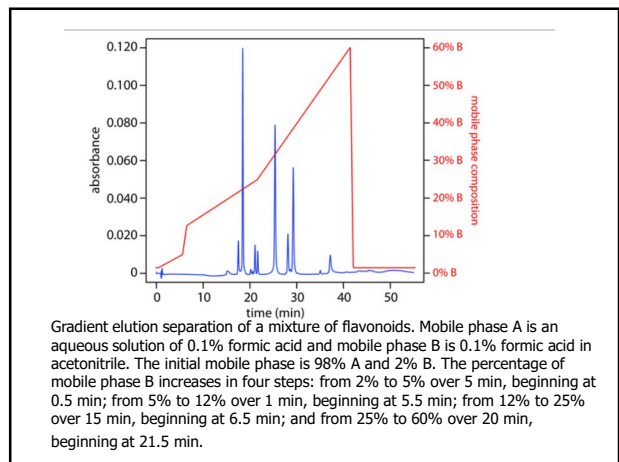
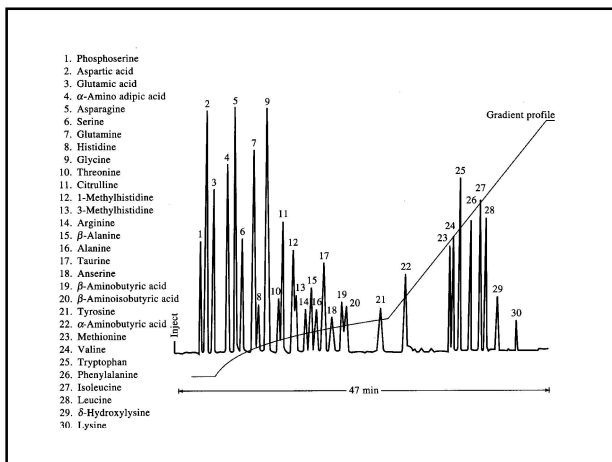
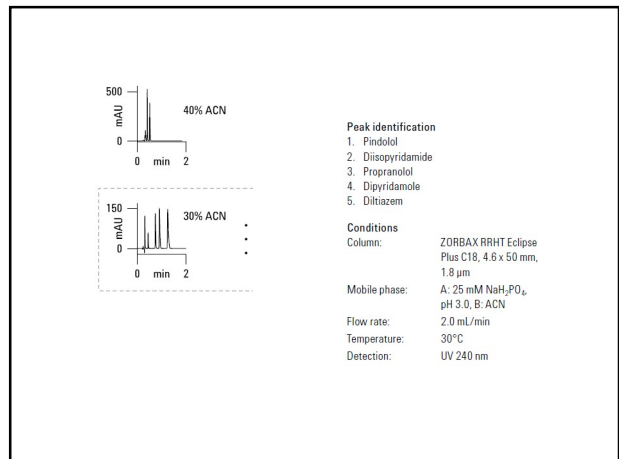
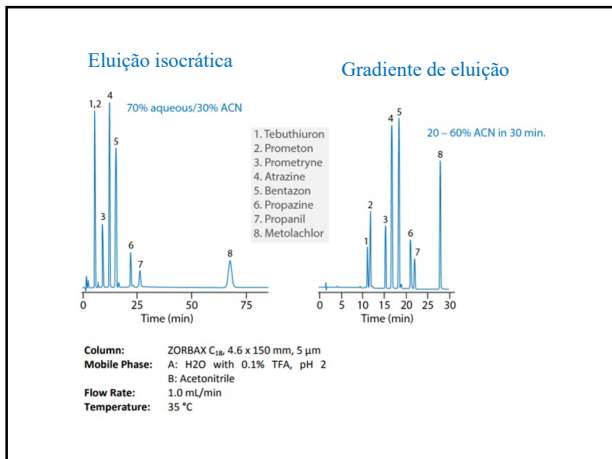
ELUIÇÃO POR GRADIENTE

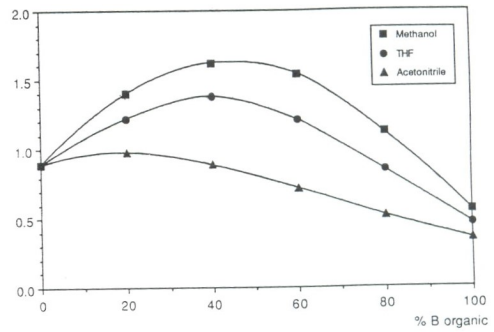


Força cromatográfica aumenta gradativamente

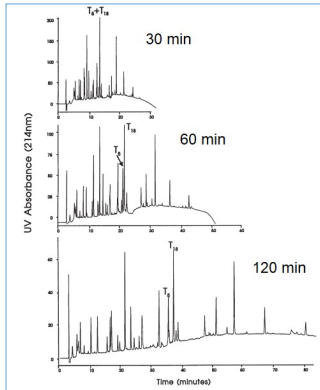
ELUIÇÃO POR GRADIENTE





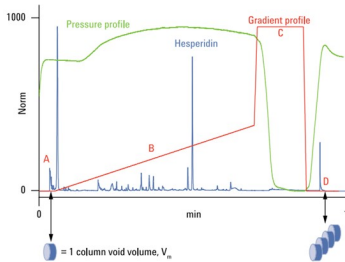


Viscosity of mixtures of water and organic solvents at 25 °C. (Numeric values after J. W. Dolan and L. R. Snyder, *Troubleshooting LC Systems*, Humana Press, Clifton, 1989, p. 85.)



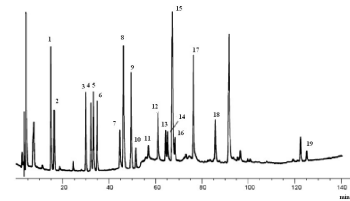
Coluna: RP-C4
25 cm x 4,6 mm di, 5 µm

Gradiente linear
0-90% ACN
1 mL/min
25°C



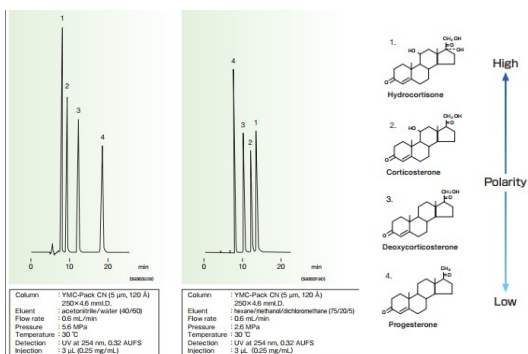
Time	%B	mL/min
0	0	0.8
0.5	0	0.8
7	40	0.8
7.1	100	0.8
8.6	100	0.8
8.7	0	0.8
10	0	0.8

Conditions
LC: Agilent 1290 Infinity with DAD
Column: Agilent RRHD SB-C18 2.1 x 150 mm, 1.8 µm PN 859700-902
Injection: 1 µL of 0.2 µm filtered grapefruit juice
Temperature: 40 °C
Detection: UV, 276 nm
Solvent A: Water
Solvent B: Acetonitrile



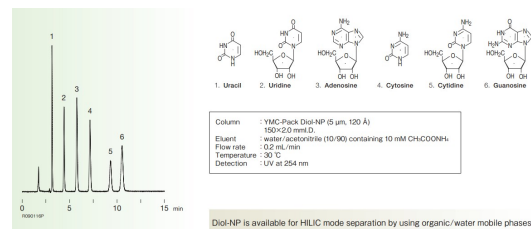
Separação de herbicidas em fase reversa (Supelcosil LC-ABZ, 25 cm x 4,6 mm) com gradiente de eluição de 10 a 90% de acetonitrila em água, aumento 0,5% de acetonitrila por minuto. 1- metamiton, 2-fenuron, 3-mitonuron, 4-simazina, 5-bromacil, 6-cianazina, 7-atrazina, 8-carbaril, 9-isoproturon, 10-promacil, 11-propazina, 12-terbutilazina, 13-linuron, 14-propanil, 15-prometrina, 16-fenamifós, 17-fenitrotion, 18-paration, 19-permetrina.

Normal and reverse phase – cyanopropyl group – medium polarity



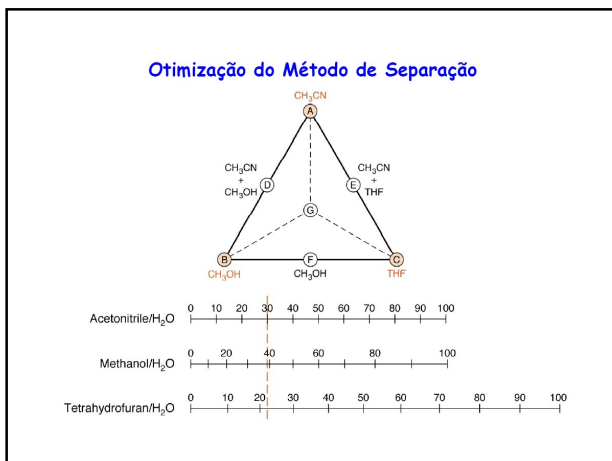
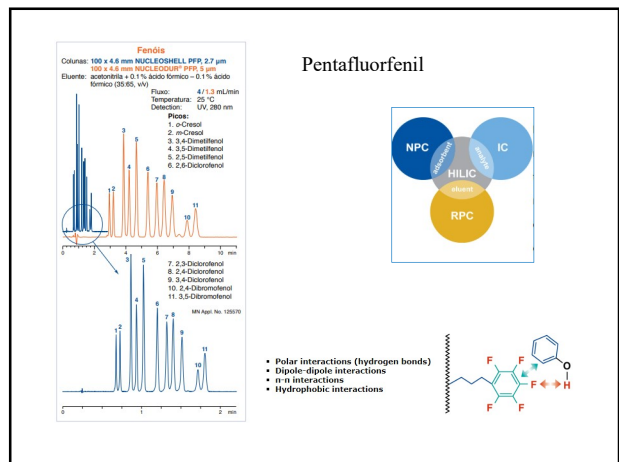
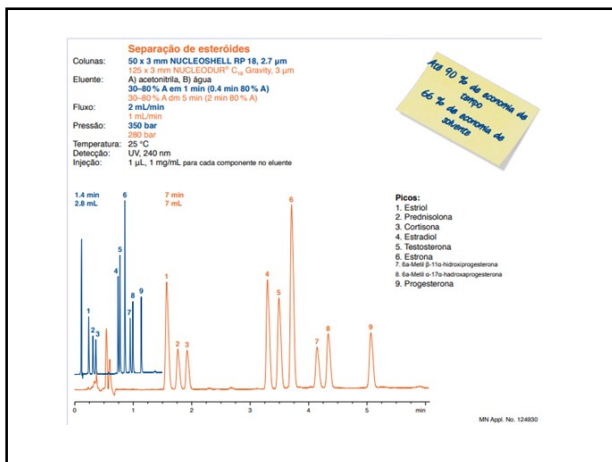
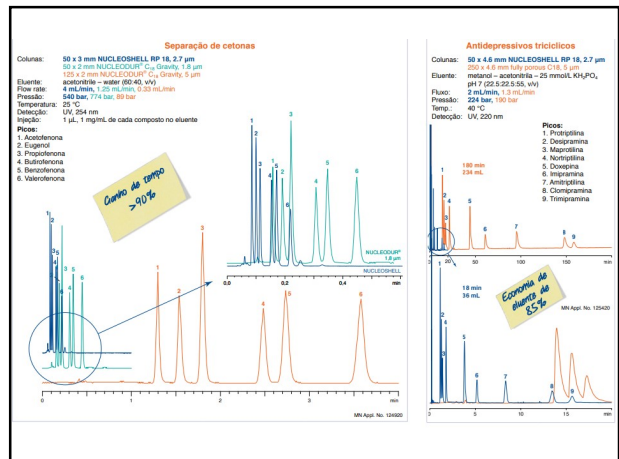
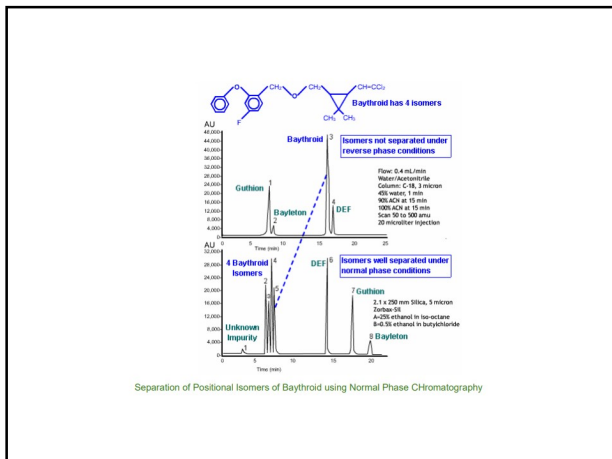
Column: YMC-Pack CN (5 µm, 120 Å)
Eluent: 250x4.6 mm I.D. acetonitrile/water (40/60)
Flow rate: 0.6 mL/min
Pressure: 3.2 MPa
Temperature: 30 °C
Detection: UV at 254 nm, 0.32 AUFS
Injection: 3 µL (0.25 mg/mL)

Column: YMC-Pack CN (5 µm, 120 Å)
Eluent: 250x4.6 mm I.D. hexane/methanol/dichloroethane (75/20/5)
Flow rate: 0.6 mL/min
Pressure: 2.2 MPa
Temperature: 30 °C
Detection: UV at 254 nm, 0.32 AUFS
Injection: 3 µL (0.25 mg/mL)



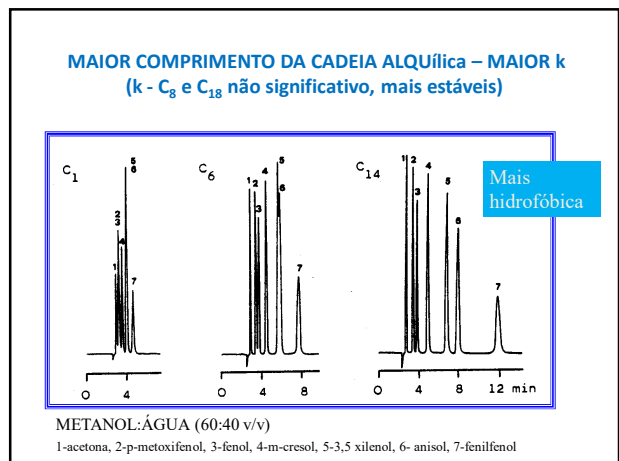
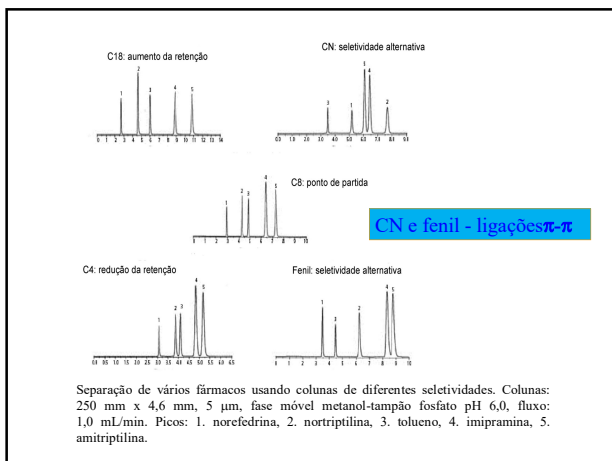
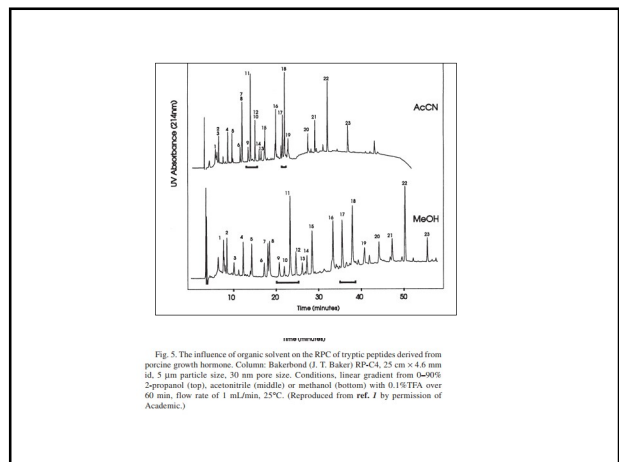
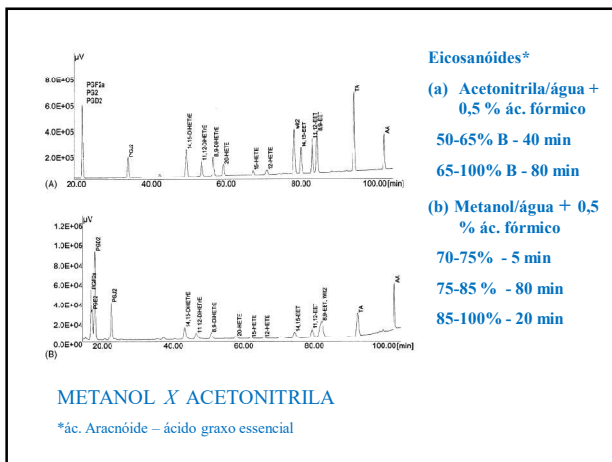
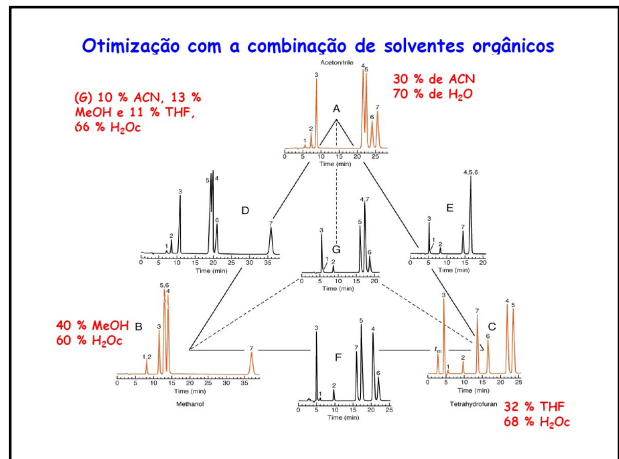
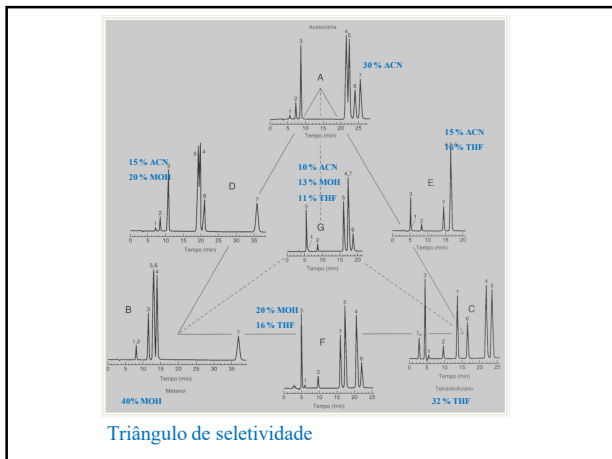
Column: YMC-Pack Diol-NP (5 µm, 120 Å)
150x3.0 mm I.D.
Eluent: water/acetonitrile (10/90) containing 10 mM CH3COONH4
Flow rate: 0.2 mL/min
Temperature: 30 °C
Detection: UV at 254 nm

Diol-NP is available for HILIC mode separation by using organic/water mobile phases.



MANTER POLARIDADE
MUDANÇA SELETIVIDADE (MeOH:H₂O 70:30 v/v)

- $\phi c = \phi b \times Sb / Sc$
- ϕc : fração em volume do novo solvente
- ϕb : fração em volume do solvente inicial
- Sb : Fator força peso do solvente B
- Sc : Fator força do solvente C
- $\phi_{ACN} = 0,76 \times 2,6 / 3,2 = 0,617$



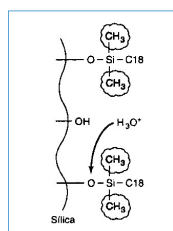
CROMATOGRAFIA LÍQUIDA COM SUPRESSÃO IÔNICA

- EXTENSÃO CLRP
- COMPOSTOS IONIZÁVEIS

CROMATOGRAFIA LÍQUIDA EM FASE REVERSA - ANALITOS IONIZÁVEIS

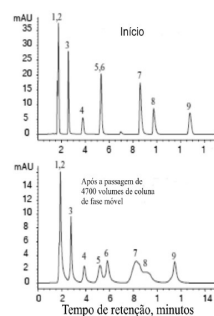
- SUPRESSÃO DA IONIZAÇÃO
- ADIÇÃO SOLUÇÃO TAMPÃO A FASE MÓVEL – 5 a 25 mM (capacidade tamponante – solubilidade em solventes orgânicos)
- ANALITOS ÁCIDOS FRACOS OU BASES FRACAS $pK_a = 2$ a 8
- SOLUÇÃO TAMPÃO pH 3-8
- FASE MÓVEL pH ≥ 8 – SÍLICA SOLÚVEL
- Composto não ionizado ou parcialmente ionizado – retenção em FR

Hidrólise da ligação siloxano da sílica (fase reversa), em pH abaixo de 2



$2 < \text{pH} < 8$ estável

pH > 8
Sílica solúvel



ESTABILIDADE DA FASE ESTACIONÁRIA

- Meio alcalino ataca silanóis – dissolução da sílica
- Degradação da fase estacionária

Teste de estabilidade da fase estacionária Phenomenex Luna C18 (2) (19% de carbono): Fase móvel: 45:55 (v/v) acetonitrila-fosfato de potássio (pH10: 20 mmol/L). Vazão: 1,0 mL/min. Temperatura: 30 °C. Solutos: 1 = labetalol; 2 = atenolol; 3 = acetabotol; 4 = metoprolol; 5 = oxprenolol; 6 = quinidina; 7 = lidocaína; 8 = alprenolol; 9 = propranolol.

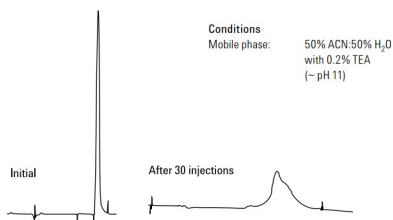
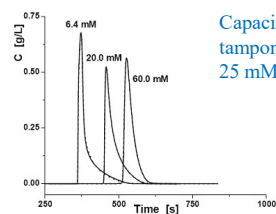


Figure 50. The effect of operating a silica column at high pH



Capacidade tamponante – 25 mM

Efeito da concentração da solução tampão na separação de propranolol. Coluna em fase reversa X terra – C18, fase móvel metanol: tampão succinato, pH 5,61 (40:60 v/v).

CROMATOGRAFIA LÍQUIDA EM FASE REVERSA - ANALITOS IONIZÁVEIS

- COMPOSTOS ÁCIDOS
- -TAMPÃO ACETATO OU FOSFATO
- -ácido acético ou fosfórico pH 2-5 (pH = pKa - 1,0)
- COMPOSTOS BÁSICOS -TAMPÃO FOSFATOS ALCALINOS
- -NH₄OH, trietilamina, dibutilamina - pH 7-8 (pH = pKa + 1,0)

- Mudanças de retenção pKa ± 1,0
- 5 - 10 mmol/L

Equação de Henderson-Hasselbalch

$$\text{pH} = \text{pKa} + \log \frac{[\text{A}^-]}{[\text{HA}]}$$

$$\text{pH} = \text{pKa} + \log 1/10$$

$$\text{pH} = \text{pKa} - 1$$



- Hidrofóbica Hidrofílica
- Maior retenção em FR Menor retenção em FR

Table 1. Properties of Common Buffers

Common Buffers	pK _a	Useful pH Range
Phosphate	pK ₁	2.1
	pK ₂	7.2
	pK ₃	12.3
Citrate	pK ₁	3.1
	pK ₂	4.7
	pK ₃	5.4
Formate	3.8	2.8-4.8
Acetate	4.8	3.8-5.8
Tris	8.3	7.3-9.3
Ammonia	9.2	8.2-10.2
Borate	9.2	8.2-10.2
Diethylamine	10.5	9.5-11.5

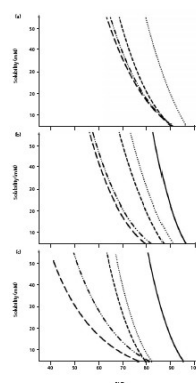
buffer	pH range	LC-MS compatible
phosphate (pK ₁)	1.1 - 3.1	X
phosphate (pK ₂)	6.2 - 8.2	X
phosphate (pK ₃)	11.3 - 13.3	X
acetate ¹	3.8 - 5.8	YES
citrate (pK ₁)	2.1 - 4.1	X
citrate (pK ₂)	3.7 - 5.7	X
citrate (pK ₃)	4.4 - 6.4	X
trifluoroacetic acid (0.1%)	2.0	YES
phosphoric acid (0.1%)	2.0	X
formic acid (0.1%)	2.7	YES
ammonium formate	2.7 - 4.7	YES
ammonium bicarbonate	6.6 - 8.6	YES
borate	8.3 - 10.3	YES

¹ suitable for LC-MS as ammonium acetate

LC-MS interface - vaporizar a fase móvel

Solubility of Potassium Phosphate, pH 7.0, in Common HPLC Solvents

%B	MeOH	ACN	THF
50 ²	>50 mM	>50 mM	25 mM
60	>50	45	15
70	35	20	10
80	15	5	<5
90	5	0	0



Solubility of five buffers in mixtures with (a) methanol, (b) acetonitrile, and (c) tetrahydrofuran, where — represents ammonium acetate at pH 5.0, •••• represents ammonium phosphate at pH 3.0, --- represents potassium phosphate at pH 3.0, -••- represents ammonium phosphate at pH 7.0, and -- represents potassium phosphate at pH 7.0.

solubility of cations in water (NH₄⁺ > K⁺ > Na⁺).

Solubility of Various Buffers in Acetonitrile

%B	ammonium acetate pH 5.0	ammonium phosphate pH 3.0	potassium phosphate pH 3.0	ammonium phosphate pH 7.0	potassium phosphate pH 7.0
60 ²	>50 mM	>50 mM	>50 mM	50 mM	45 mM
70	>50	>50	>50	25	20
80	>50	35	20	5	0
90	25	5	0	0	0

Tabela 1. Variação do pH em função do tipo de tampão, do tipo de modificador orgânico e da percentagem de modificador orgânico. Todos os valores foram normalizados a 20 °C (valores retirados das refs. 62-64)

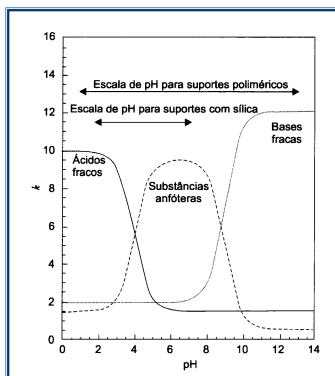
% de metanol na fase móvel	pH					
	0%	20%	40%	50%	60%	80%
H ₂ PO ₄ -HPO ₄ ⁻	2.11	2.63	3.09	3.35	3.68	4.3
H ₂ PO ₄ -HPO ₄ ²⁻	7.20	7.55	8.04	8.36	8.75	9.58
H ₂ Cl ⁺ -H ₂ Cl ⁻	3.13	3.44	3.84	4.07	4.30	4.77
H ₂ Cl ⁺ -Cl ⁻	4.76	5.12	5.53	5.8	6.10	6.64
HCl ⁺ -Cl ⁻	6.40	6.83	7.39	7.66	7.96	8.60
HAc ⁺ -Ac ⁻	4.76	5.05	5.43	5.66	5.92	6.46
NH ₄ ⁺ -NH ₃	9.24	9.11	8.97	8.89	8.82	8.63
% de acetonitrila na fase móvel	0%	20%	40%	50%	60%	80%
H ₂ PO ₄ -HPO ₄ ⁻	2.11	2.28	2.55	2.54		
H ₂ PO ₄ ⁻ -HPO ₄ ²⁻	7.2	7.48	7.66	8.01		
H ₂ Cl ⁺ -H ₂ Cl ⁻	3.13	3.15	3.43	3.64		
H ₂ Cl ⁺ -Cl ⁻	4.76	4.32	4.65	5.01		
HCl ⁺ -Cl ⁻	6.4	6.55	6.84	7.28		
HAc ⁺ -Ac ⁻	4.76	5.39	5.8	6.47		
NH ₄ ⁺ -NH ₃	9.24	9.79	9.39	9.25		
H ₂ CO ₃ -HCO ₃ ⁻	3.72	3.96	4.4	4.87		
Piperazina(pK _a)	5.37	5.26	5.19	5.06		
Piperazina(pK _a)	9.76	9.62	9.53	9.42		
Tris	8.08	7.94	7.85	7.72		
Borato	9.23	9.85	10.43	11.00		
HCO ₃ ⁻ -CO ₃ ²⁻	10.35	10.82	11.31	11.82		

pKa Values of Bases in Methanol–Water and Acetonitrile–Water

Compound	Water	20% MeOH	30% MeOH	40% MeOH	60% MeOH	70% MeOH
Nortriptyline	10.2	10.0	9.8	9.7	9.4	9.3
Diphenhydramine	9.2	9.0	8.9	8.8	8.5	8.2
Codeine	8.2	8.1	7.9	7.8	7.6	ND
Procainamide	9.3	9.2	9.1	8.9	8.7	8.5
Benzylamine	9.5	9.3	9.2	9.2	9.0	8.9
Protriptyline	10.7	10.4	10.3	10.2	9.9	9.7
Amitriptyline	9.3	9.3	9.1	8.9	8.5	8.3

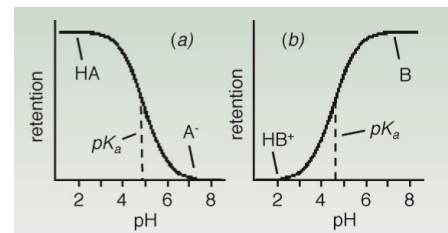
Compound	Water	20% ACN	40% ACN	60% ACN
Nortriptyline	10.2	10.1	9.9	9.7
Diphenhydramine	9.2	9.2	8.9	8.7
Codeine	8.2	8.1	7.8	7.5
Procainamide	9.3	9.3	9.2	9.0
Benzylamine	9.5	9.4	9.1	8.8

ND = not determined.



Relação teórica entre o fator de retenção (k) e o pH da fase móvel

Retention vs. pH for a hypothetical acid (a) and base (b).

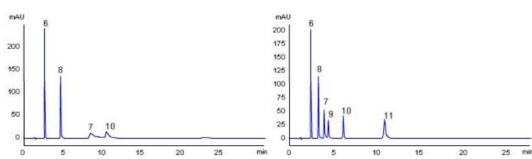
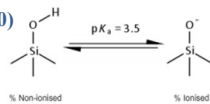


bases, ionizáveis, e compostos iônicos

Surface Silanol Equilibrium

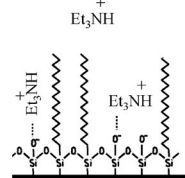
$$(pH = pK_a - 1,0)$$

Silanol surface $\approx 8 \mu\text{mol}/\text{m}^2$
Chemical modification $> 4 \mu\text{mol}/\text{m}^2$



pH 7,0 - amfetamina - pico 7
pH 2,5 amfetamina - pico 7

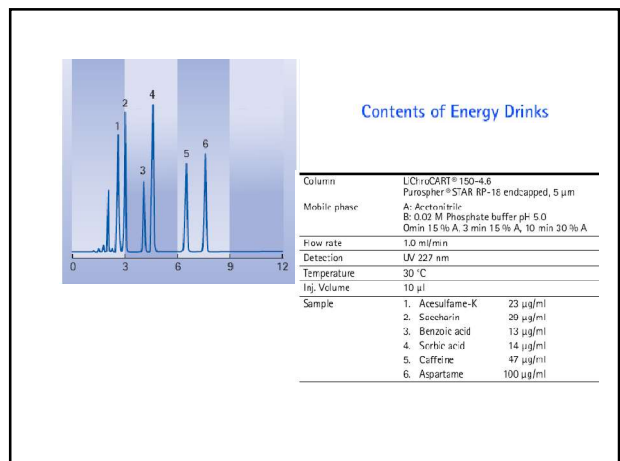
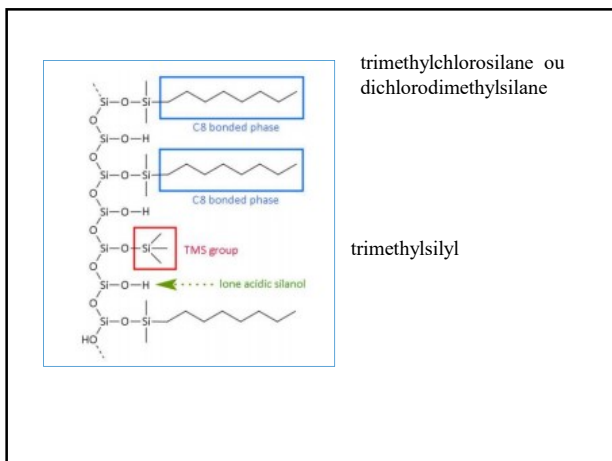
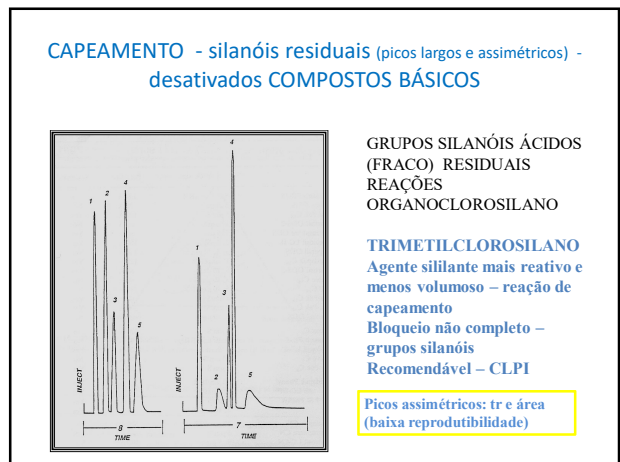
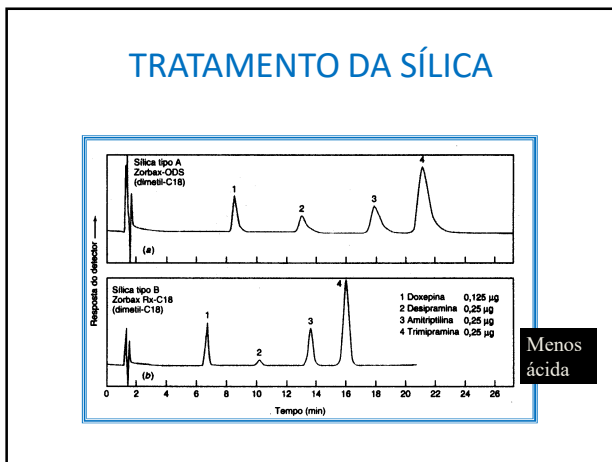
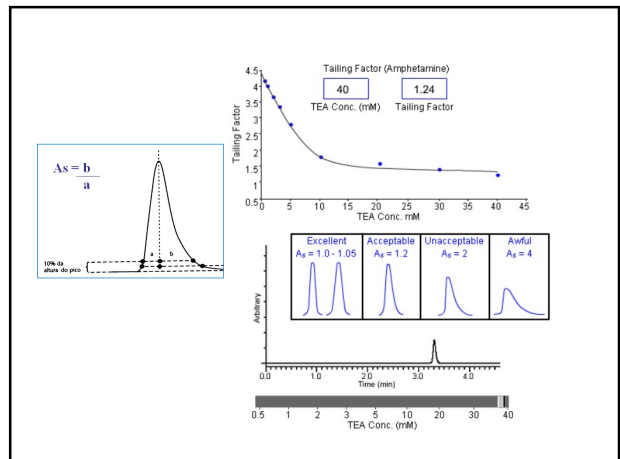
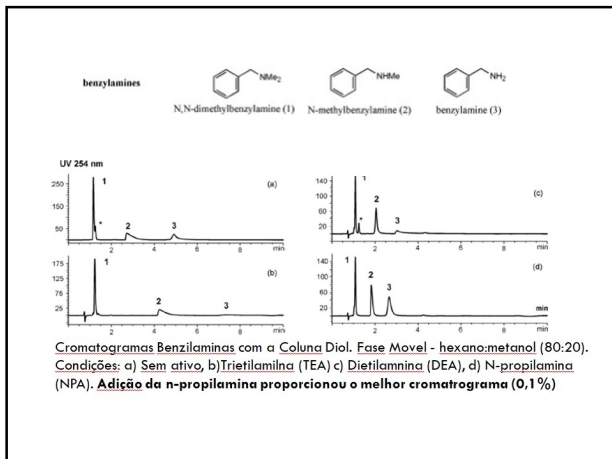
Separação de bases fracas



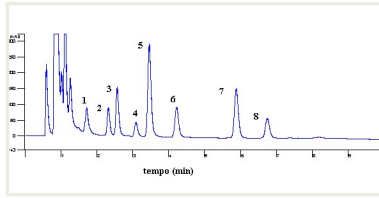
Interação de aminas (trietilamina) com grupos silanóis livres na superfície da coluna C8

Reduzir cauda de compostos básicos

Alternativa: colunas sílica mais pura menos ácida, colunas capeadas,



ANÁLISE DE ANTICONVULSIVANTES EM AMOSTRAS DE PLASMA DE PACIENTES

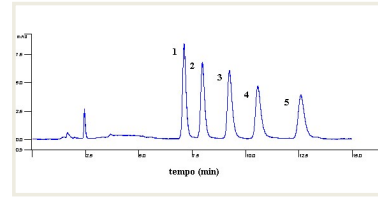


- 1: PEMA
- 2: PB
- 3: PRM
- 4: LTG
- 5: MPRM
- 6: CBZ-E
- 7: PHT – pKa = 8,3
- 8: CBZ

solução tampão fosfato de potássio (0,01 mol. L⁻¹, pH 7,5) – acetonitrila – metanol (65: 18: 17 v/v/v)

QUEIROZ, MEC *et al.* 2002

ANÁLISE DE ANTIDEPRESSIVOS EM AMOSTRAS DE PLASMA DE PACIENTES

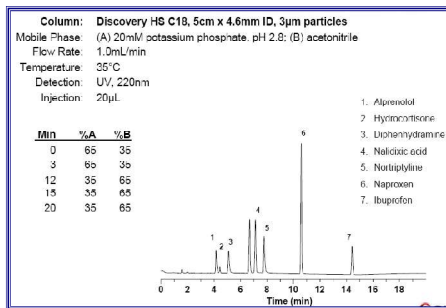


- 1: DES
- 2: NOR
- 3: IMI
- 4: AMI
- 5: CLO

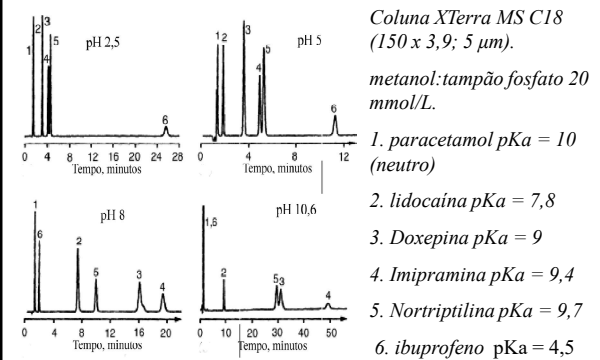
tampão acetato de sódio (0,01 mol. L⁻¹, pH 5,4) – acetonitrila (50: 50 v/v)

QUEIROZ, MEC *et al.* 2002

CROMATOGRAFIA LÍQUIDA COM SUPRESSÃO IÔNICA



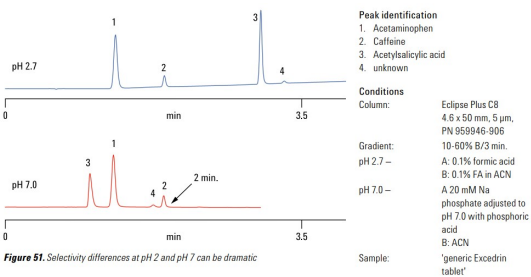
- 1. Alprazolol
- 2. Hydrocortisone
- 3. Diphenhydramine
- 4. Naloxonic acid
- 5. Nortriptyline
- 6. Naproxen
- 7. Ibuprofen



Coluna XTerra MS C18 (150 x 3,9; 5 µm).

metanol:tampão fosfato 20 mmol/L.

- 1. paracetamol pKa = 10 (neutro)
- 2. lidocaina pKa = 7,8
- 3. Doxepina pKa = 9
- 4. Imipramina pKa = 9,4
- 5. Nortriptilina pKa = 9,7
- 6. ibuprofeno pKa = 4,5



- Peak identification
- 1. Acetaminophen
 - 2. Caffeine
 - 3. Acetylsalicylic acid
 - 4. unknown
- Conditions
- Column: Eclipse Plus C8 4.6 x 50 mm, 5 µm, PN 959046-906
- Gradient: pH 2.7 – 10-60% B/3 min.
- pH 2.7 – A: 0.1% formic acid
- pH 7.0 – B: 20 mM Na phosphate adjusted to pH 7.0 with phosphoric acid
- Sample: 'generic Excedrin tablet'

Figure S1. Selectivity differences at pH 2 and pH 7 can be dramatic

CROMATOGRAFIA LÍQUIDA POR PAR IÔNICO

ANALITOS IÔNICOS

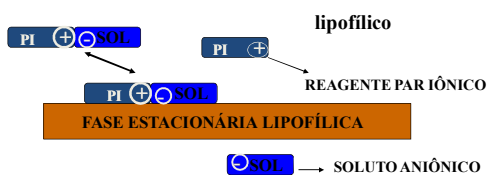
FASE REVERSA - CROMATOGRAFIA DE PAR IÔNICO

- **ÁCIDOS E BASES FORTES**
- **REAGENTE PAR IÔNICO – LIPOFÍLICO COM CARGA OPOSTA ANALITO**
- Ionizado – ampla faixa pH fase móvel
- Lipofílico – adsorção FR
- **ANALITO ANIÔNICO – TETRAALQUILAMÔNIO**
- **ANALITO CATIÔNICO – ÁCIDO ALQUILSULFÔNICOS**
- **PAR IÔNICO NEUTRO**

Ion Pair Chromatography

- Adjust pH to fully ionize analyte
- Add ion pair former of opposite charge in mobile phase
 - acids (anions): tetraalkyl ammonium salt
 - bases (cations): alkyl sulfonic acids
- Use standard reversed phase column

Atração eletrostática e hidrofóbica

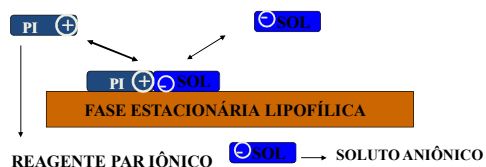


Fase Móvel – mistura de solvente orgânico e água, com ácido, base ou solução tampão – pH – promover ionização analito

• MECANISMO DE SEPARAÇÃO

• **PAR IÔNICO - F. MÓVEL - DISTRIBUIÇÃO ENTRE AS FASES**

MECANISMO DE SEPARAÇÃO CROMATOGRAFIA DE PAR IÔNICO



Muito lipofílico – equilíbrio com a fase estacionária – revestimento – fortemente ligado

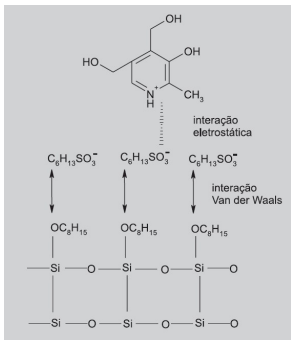
Removido do eluente – permite uso subsequente

CLFR – PAR IÔNICO analitos ionizados

- **ÂNIONS** – ácidos fortes ou fracos
- tetraalquilamônio
- $[C_{14}H_{29}(CH_3)_3N^+]$ cetiltrimetilamônio
- Tamponar fase móvel pH 7,5
- **CÁTIONS** – bases fortes e fracas
- Ácidos alquilsulfônicos
- $H_3C-(CH_2)_n - SO_3^- Na^+$ $n = 4$ a 9
- Tamponar fase móvel pH 3,5

Reagente Par iônico adicionado na fase móvel

- (separação mistura: ácidos, básicos, anfóteros e neutros) valores de pKa semelhantes
- Analitos neutros – não altera retenção
- Íons - mesma carga iônica RPI – diminui retenção
- Íons - carga iônica oposta RPI – aumenta a retenção



Soluto: cloridrato de piroxina

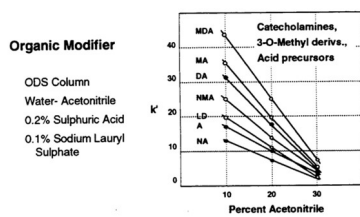
Reagente PI: hexassulfonato de sódio

Coluna: C18

MECANISMOS DE RETENÇÃO

- Íons (analito) - carga iônica oposta RPI – aumenta a retenção – aumenta concentração RPI (FM)
- Íons (analito) - carga iônica oposta RPI – aumenta a retenção – RPI mais lipofílico
- Retenção de todos analitos diminui - % modificador orgânico aumenta, menor concentração RPI na FM

Effect of Organic Modifier



J. H. Knox, et al., *J. Chromatogr.*, 125 (1976) 89-101.

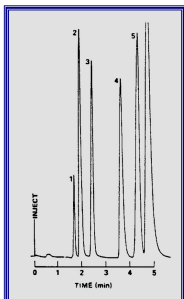
Ion Pair Reagent Chain Length



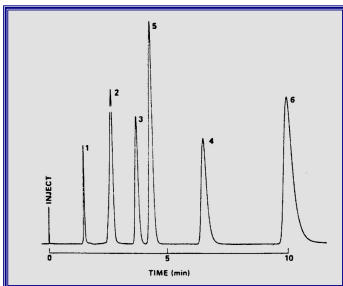
C18: 50:50 Methanol/Water; 0.005 M SQ_3^- , 1% Acetic Acid
1. Maleic Acid, 2. Phenylephrine, 3. Phenylpropanolamine, 4. Naphazoline, 5. Phenacetin, 6. Pyrilamine.

Waters Associates (1992).

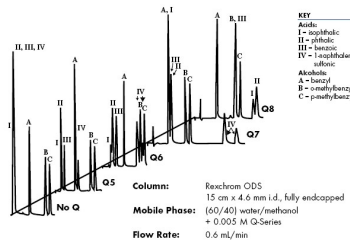
Column: μ Bondapak C18
Mobile Phase: Methanol/water with 5 mM pentane sulfonic acid and 1% acetic acid (50/50)



Column: μ Bondapak C18
Mobile Phase: Methanol/water with 5 mM heptane sulfonic acid and 1% acetic acid (50/50)



Solutes: 1 - Maleic acid, 2 - phenylephrine, 3 - phenylpropanolamine, 4 - naphazoline, 5 - phenacetin, 6 - pyrilamine



KEY
Acids:
I - isophthalic
II - o-phthalic
III - benzoic
IV - isophtalene sulfonic
Alcohols:
A - benzyl
B - cresyl/benzyl
C - p-methylbenzyl

Product

Q5 [1-pentyltriethylammonium phosphate]

Q6 [1-hexyltriethylammonium phosphate]

Q7 [1-heptyltriethylammonium phosphate]

Q8 [1-octyltriethylammonium phosphate]

Q12 [1-dodecyltriethylammonium phosphate]

Column: Reschrom ODS
15 cm x 4.6 mm i.d., fully endcapped
Mobile Phase: (60/40) water/methanol + 0.005 M G-Series
Flow Rate: 0.6 mL/min

Figure 4. In a mixture of ionic and nonionic compounds, first separate the nonionic compounds from each other (See above). Then choose the ion pair reagent that retains the ionic compounds as desired. Here, Q6 seems to be the reagent of choice since all peaks are visibly separated.

