System and Method Troubleshooting

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Troubleshooting
(Αντιμετώπιση Προβλημάτων)

There is no standard troubleshooting procedure.

General Pattern:

- Locate the problem by ranking (κατάταξη) possible causes.
- Verify the presence of the most probable cause.
- If present – fix the problem, otherwise verify the existence of the next possible cause.

First try to distinguish

| System problem | or | Method problem |

Method vs. System Troubleshooting

System Parameters

- Flow stability
- Backpressure (οπισθοπίεση)
- Clogging (απόφραξη)
- Detector problems
- Injection suitability
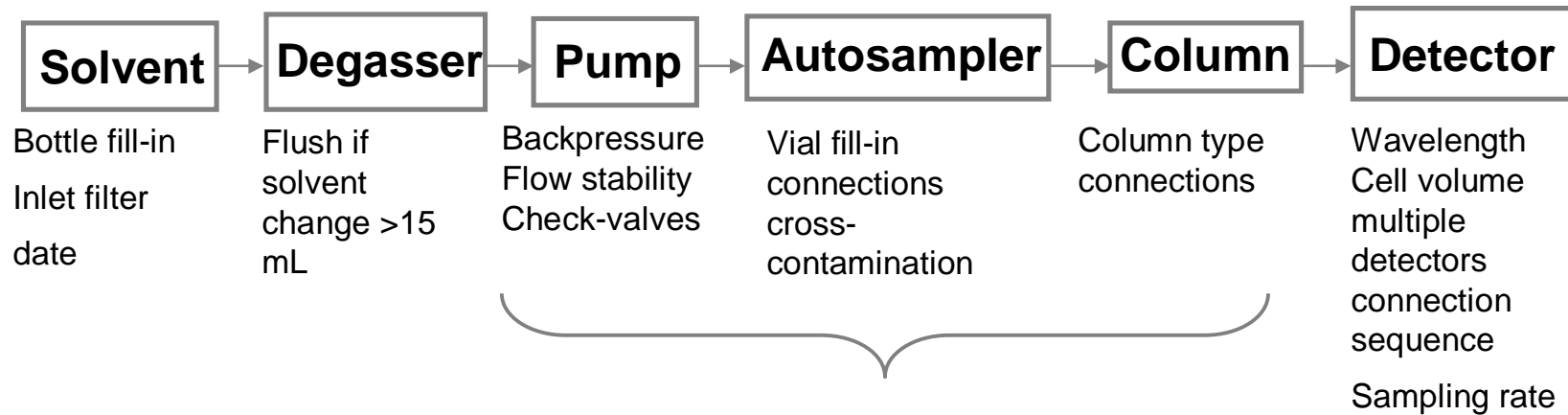- Injection volume
- Temperature

Method Parameters

- Flow rate
- Eluent (εκλουστικό) type
- Eluent composition
- pH
- pH modifier (τροποποιητής) (type)
- Injection volume
- Temperature
- Gradient profile

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System Parameters

- Simple preliminary verification of system setup can save time.

| Solvent | → | Degasser | → | Pump | → | Autosampler | → | Column | → | Detector |

Bottle fill-in

Inlet filter

date

Flush if
solvent
change >15
mL

Backpressure
Flow stability
Check-valves

Vial fill-in
connections
cross-
contamination

Column type
connections

Wavelength
Cell volume
multiple
detectors
connection
sequence

Sampling rate

**Critical connections. Minimize
tubing length**

System Suitability

Available HPLC system set margins (περιθώρια) for column selection.

- 20 µl detector flow-cell incompatible with <3 mm I.D. columns
- 10 µl sample loop incompatible with <1 mm I.D. columns.
- 0.2 µl micro-injector is useless for conventional columns.

Suitability Rule

Injection volume **<** Cell volume

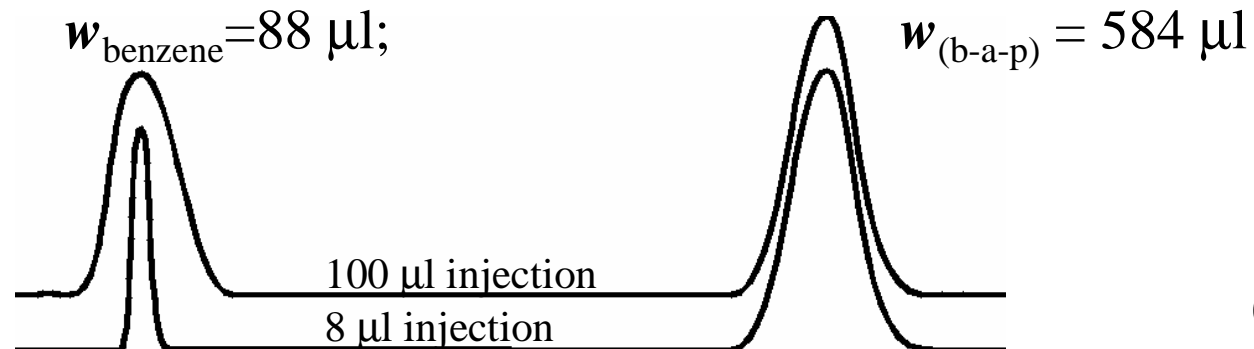Column Dead Volume ≈ 0.65 of the empty column volume

System Suitability
(Injection Volume)

Column: 150 x 4.6 mm (C18), V_o = 1.7 ml

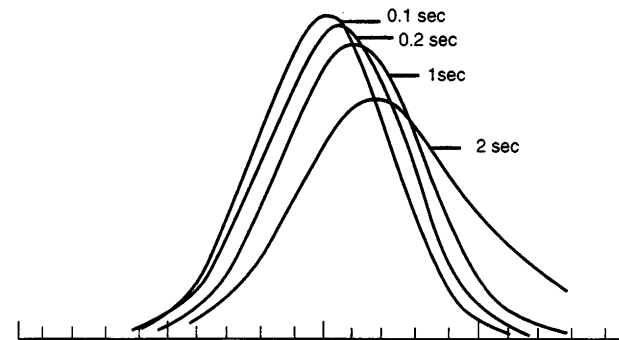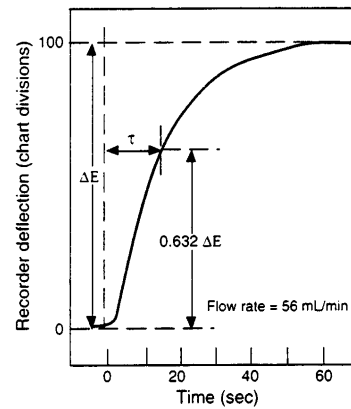**Efficiency**: 10,000 t.p. **Eluent**: MeCN/Water 70/30

$V_{R(benzene)}$=2.2 ml; $\qquad$ $V_{R(benz\text{-}a\text{-}pyrene)}$=14.6 ml

$$N = 16\left(\frac{V_R}{w_b}\right)^2 \qquad Þ \qquad w_b = \frac{4V_R}{\sqrt{N}}$$

$w_{benzene}$=88 μl; $\qquad\qquad$ $w_{(b\text{-}a\text{-}p)}$ = 584 μl
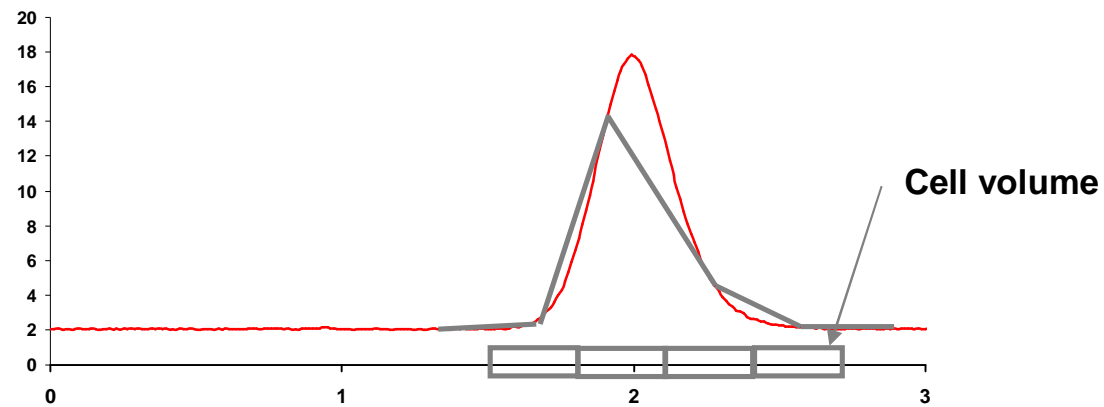

100 μl injection

8 μl injection

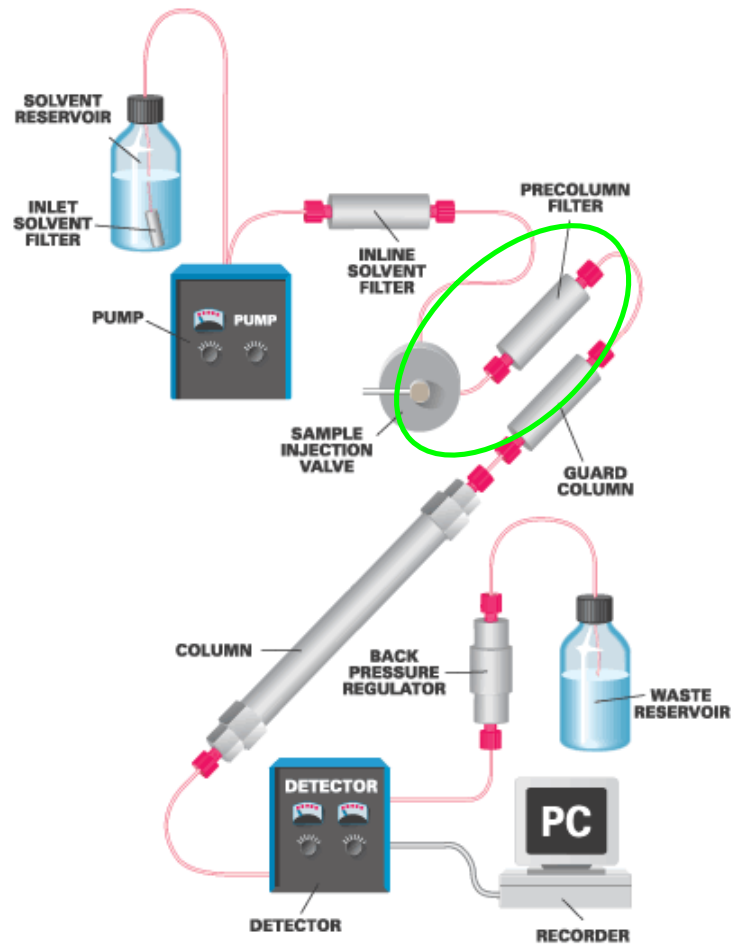# Effect of flow-cell volume and sampling rate

Response time (σταθερά απόκρισης) effect

Flow cell volume

HPLC System set up

- Minimize the volume and connections between autosampler, column, and detector.
- No guard (προστασία), no prefilter
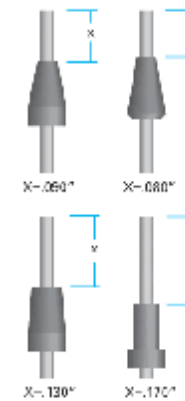
Tubing & connections

1560	.0025" (65µm) ID	Natural	7,000 psi (483 bar)*
1561	.004" (100µm) ID	Black	7,000 psi (483 bar)*
1535	.005" (125µm) ID	Red	7,000 psi (483 bar)*
1562	.006" (150µm) ID	Purple	7,000 psi (483 bar)*
1536	.007" (175µm) ID	Yellow	7,000 psi (483 bar)*
1531	.010" (.25mm) ID	Natural	7,000 psi (483 bar)*
1531B	.010" (.25mm) ID	Blue	7,000 psi (483 bar)*
1565	.015" (.40mm) ID	Gray	7,000 psi (483 bar)*
1532	.020" (.50mm) ID	Orange	7,000 psi (483 bar)*
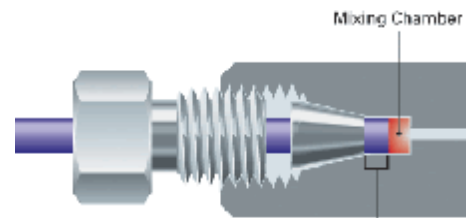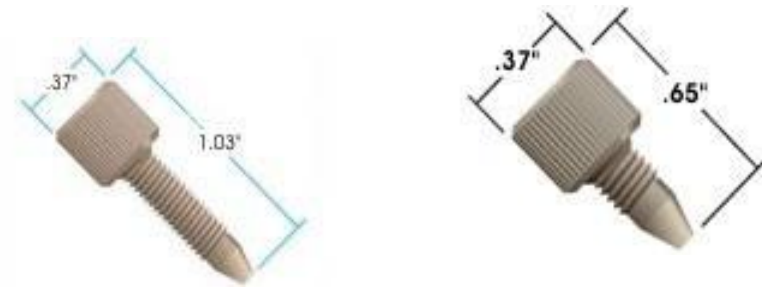
Mixing Chamber

If Dimension X is too short, a dead-volume or mixing chamber will occur.
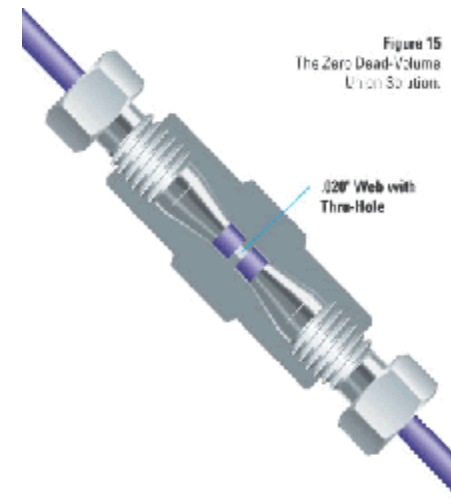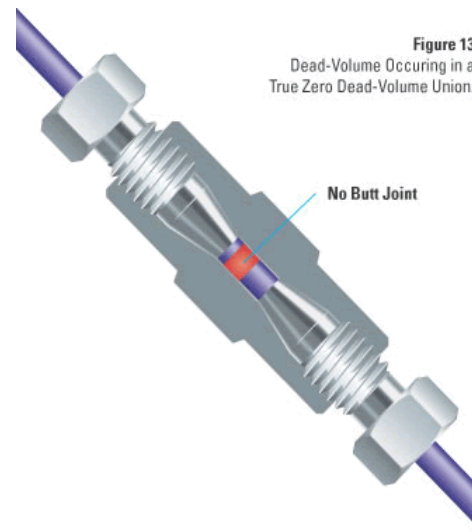
Figure 3b

X=.060" X=.080"

X=.130" X=.170"

Dimension X can range from .060" to .170" among various manufacturers.

Figure 2

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Unions (Butt Joint = σύνδεσμος αρμού)

Figure 12
True Zero Dead-Volume connection difficulties.

Butt Joint

Butt Joint

Figure 13
Dead-Volume Occuring in a
True Zero Dead-Volume Union.

No Butt Joint

Figure 15
The Zero Dead-Volume Union Solution.

.020" Web with
Thru-Hole
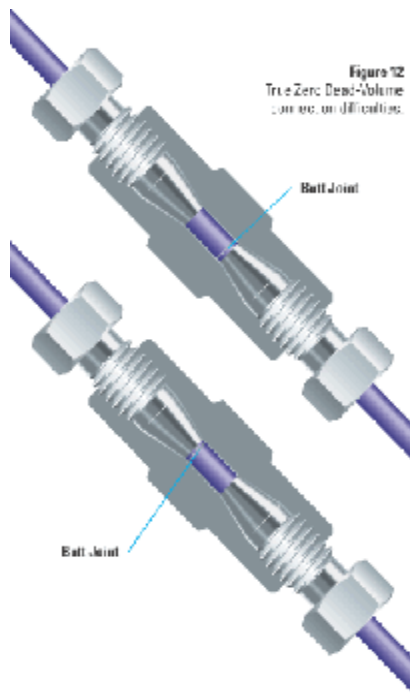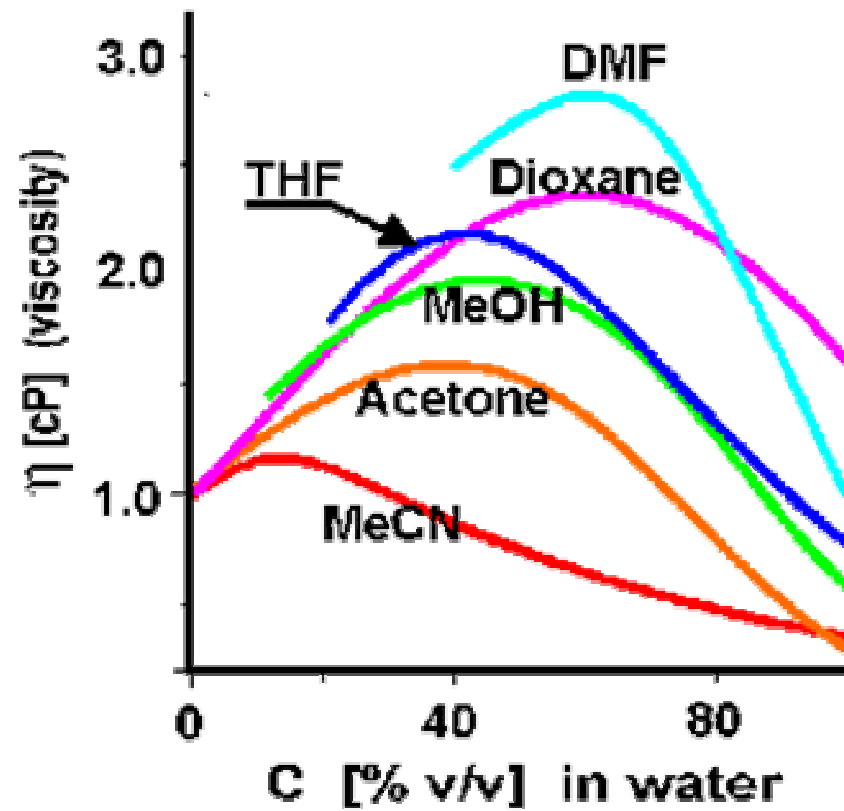
Critical Connections

Injector - Column, Column - Detector

No unions, in-line filters, guard columns
Single piece tubing (0.007" and smaller I.D.)

Figure 3a

Ferrule cannot seat properly

If Dimension X is too long, leaks will occur

Figure 3b

Mixing chamber

If Dimension X is too short, a dead-volume, or mixing chamber, will occur
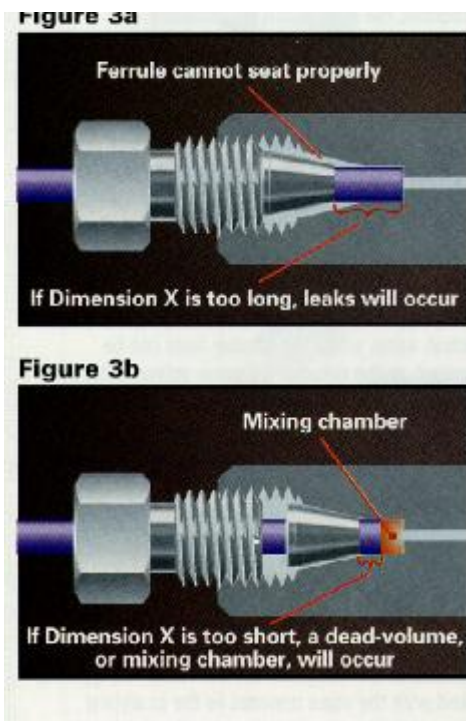
Eluent Composition Effects on the Column Back Pressure

Guard Columns
Στήλες Προστασίας

Purpose - trapping retentive impurities (παγίδευση συγκρατούμενων ακαθαρσιών)
Disadvantage - introduces extra-connections in critical zone

Sample has 1% impurity. How many injections will kill 1% of column
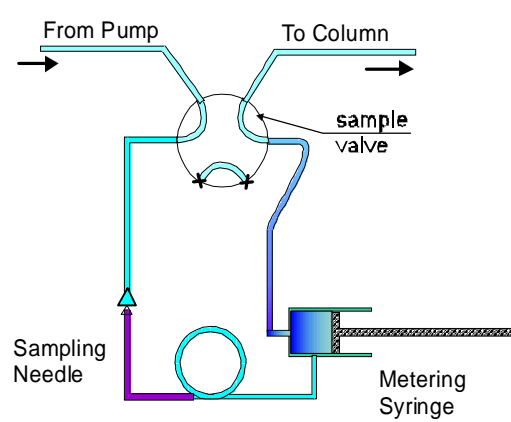surface with 1% sample solution and 10 μl injection volume?
1% column surface ~ 2-3 m^2, it could adsorb ~ 0.1 μMole
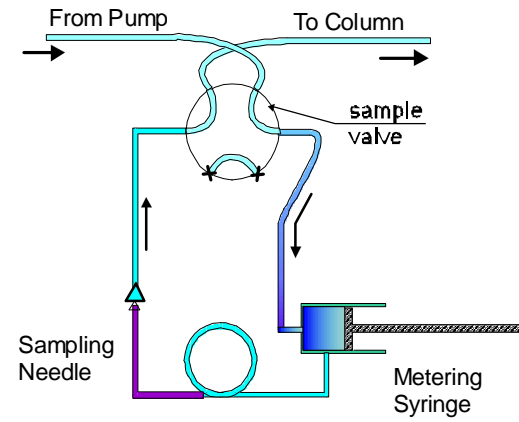**300** injections will reach this level.

Guard column always decreases system efficiency

	Retention time		W1/2		Theortical Plates	
	Guard	No Guard	Guard	No Guard	Guard	No Guard
aniline	2.743	2.696	.1047	0.083	3802	5845
Methyl aniline	3.898	3.734	0.0865	0.0832	11250	11159
NN-dimethyl aniline	4.274	4.188	0.0952	0.0879	11166	12576

Autosampler – Column/Pump Connections

From Pump   To Column

sample valve

Sampling Needle

Metering Syringe

Wrong connection

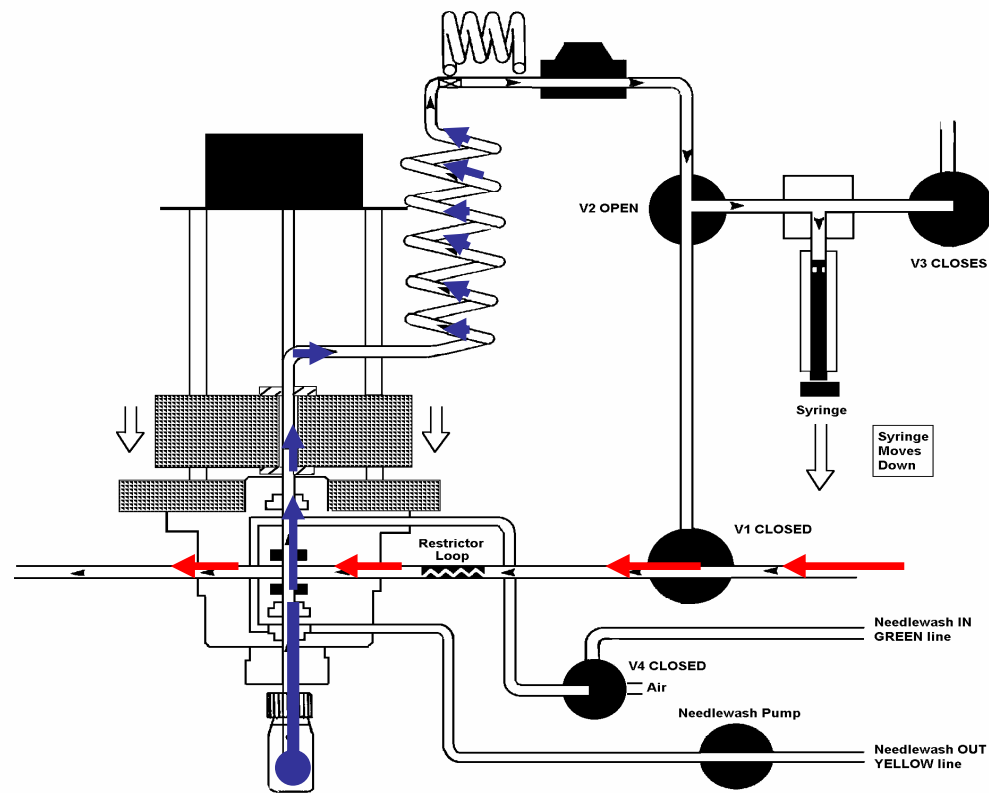From Pump   To Column

sample valve
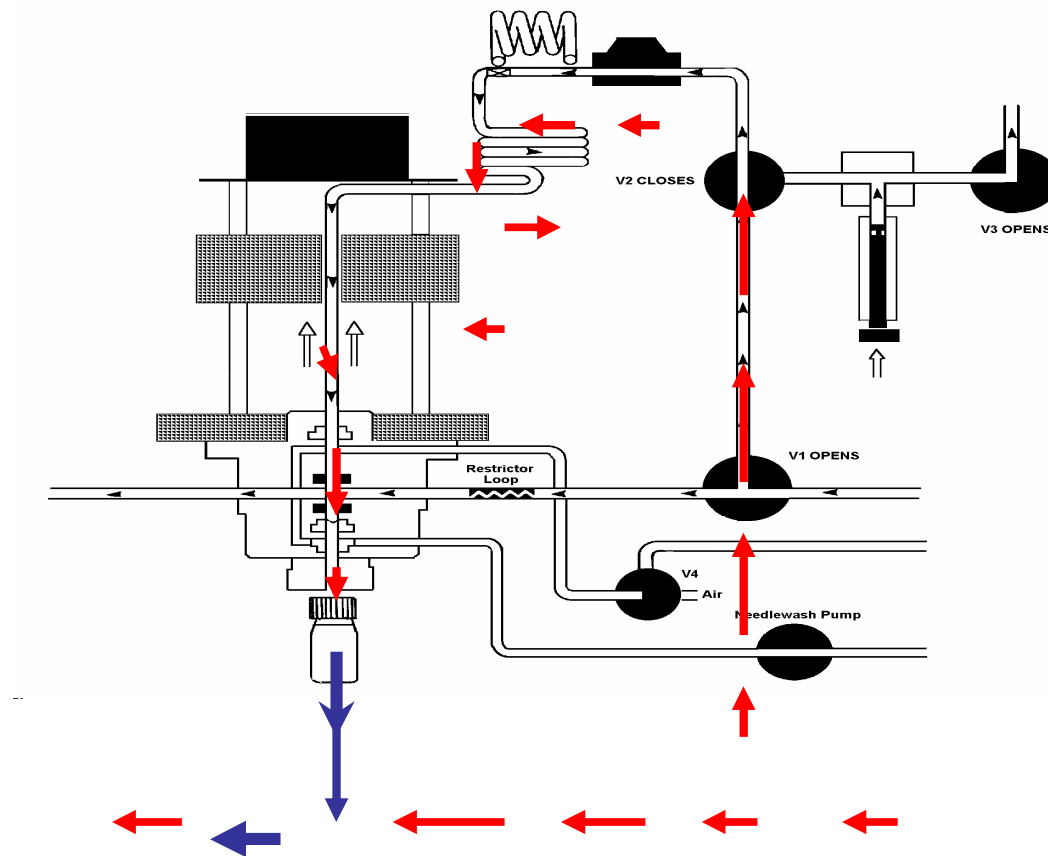
Sampling Needle

Metering Syringe

Correct connection

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Waters system (Injection, Drawing Sample)

Waters system (Injection, Injecting Sample)
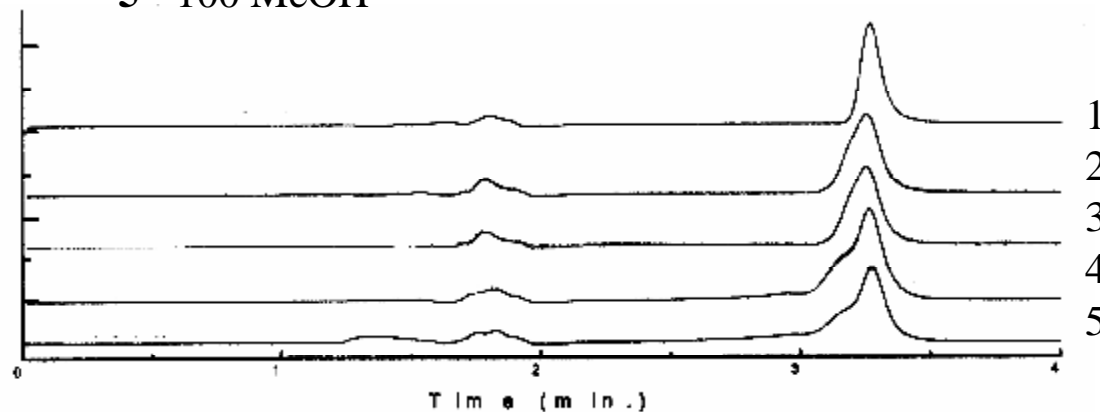
Sample Diluent (Αραιωτής) Effect

Sample diluent:

    1- 50/50 MeOH/Water

    2 - 80/20 MeOH/Water

    3 - 90/10 MeOH/Water

    4 - 95/5 MeOH/Water

    5 - 100 MeOH

50/50 Buffer/MeOH

Buffer: 20 mM Citrate, pH=4.6

Incompatible solvents may cause sample precipitation and column clogging

Different eluent pH and composition may cause peak splitting

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Column Length

- Column length is a compromise (συμβιβασμός) between the efficiency and backpressure
- Column efficiency is proportional to the column length
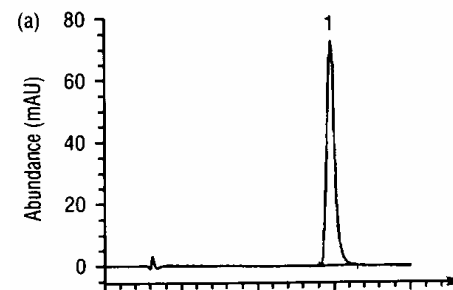- Specific efficiency (# of particles per one plate) decreases with length increase.

Length [cm]	Particle Dia. [um]	Efficiency, N	Specific Efficiency, h
10	3	11111	3
10	5	10526	1.9
15	5	13636	2.2
25	5	15625	3.2
25	10	10000	2.5

Column Overloading
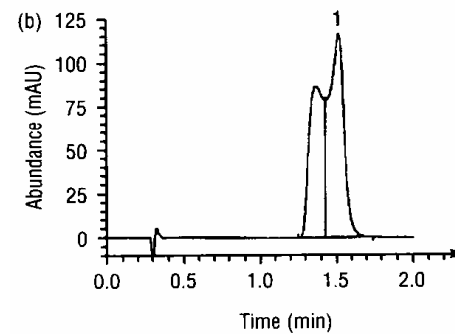## Υπερφόρτωση Στήλης

1 μl

5 μl

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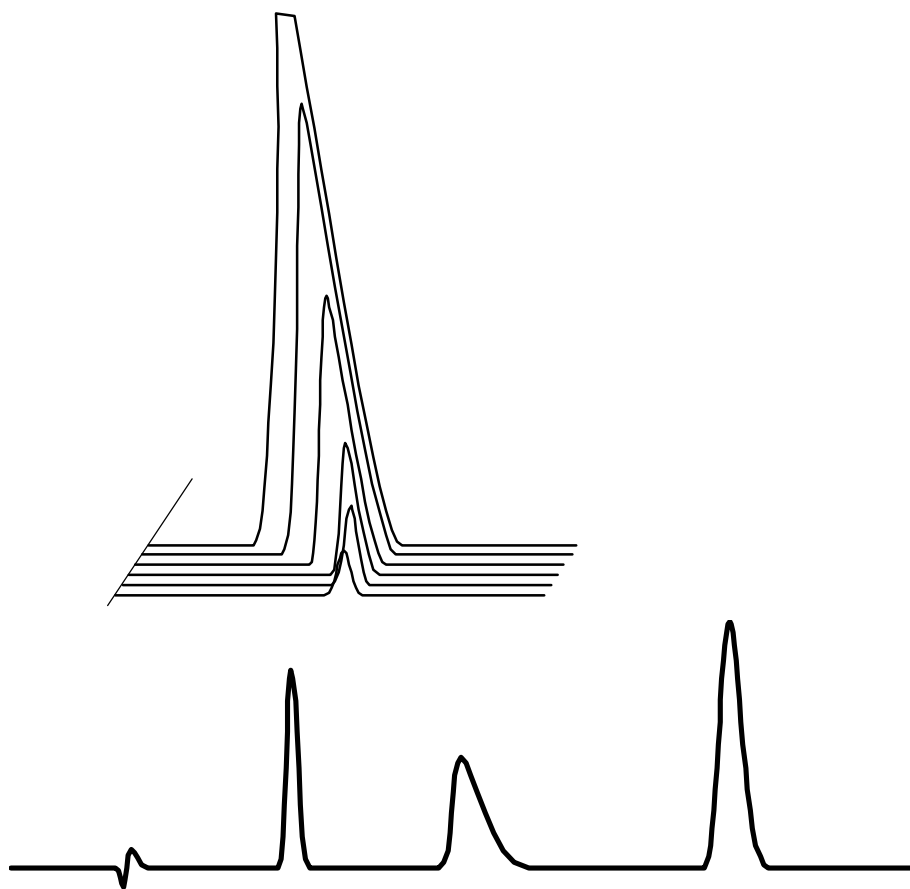
Effect of pH on Aniline (pK$_b$ = 9,42, pK$_a$ = 4,58) UV absorbance

The mobile phase pH at a constant organic composition may have an effect on an ionizable analyte's UV response. At 232 nm there is a decrease in aniline's absorbance as this analyte becomes progressively more ionized. A plot of the UV absorbance at a particular wavelength versus the ^s_spH of the mobile phase will lead to a sigmoidal dependence. The inflection point corresponds to the analyte pK$_a$.
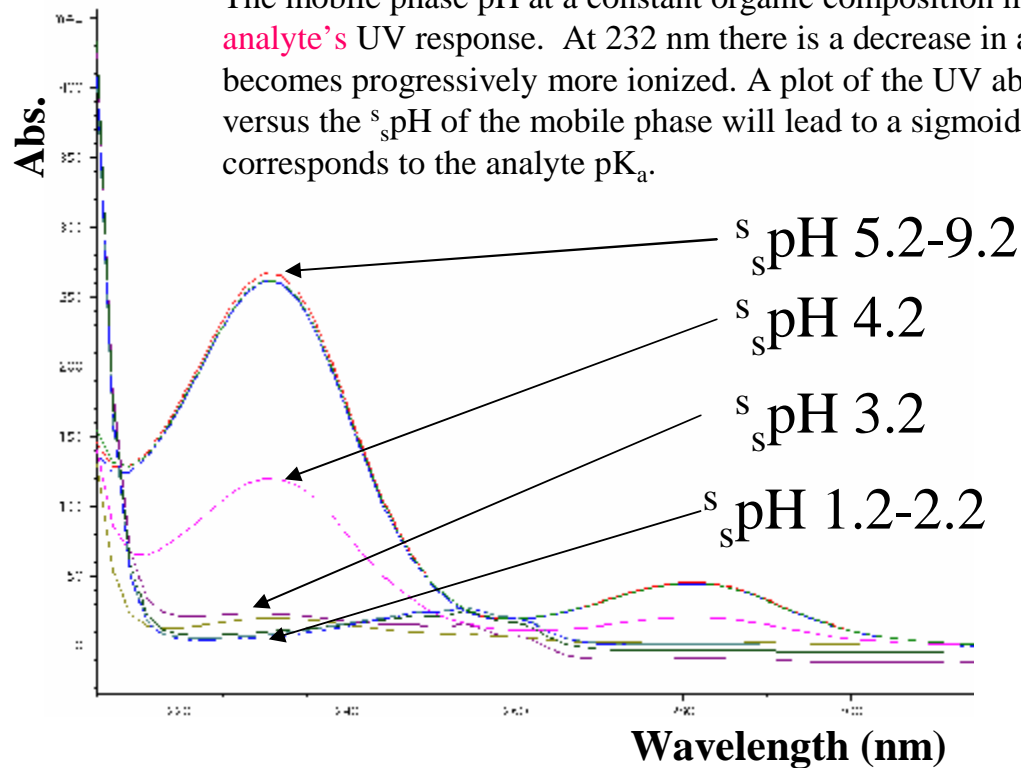
^s_spH 5.2-9.2

^s_spH 4.2

^s_spH 3.2

^s_spH 1.2-2.2

Abs.

Wavelength (nm)

Chromatographic Conditions
Column: 15 cm x 0.46 cm Luna C18(2)
Eluent: 90% Aqueous:10% MeCN
Aqueous: 15 mM K$_2$HPO$_4$•7H$_2$O adj. to
            ^w_wpH 1 - 9 with H$_3$PO$_4$
Flow rate: 1 ml/min
Temp: 25°C

Effect of pH on Aniline Retention and UV response (220 nm)

Detector Response (mV)

$^w_wpH\ 2$

$^w_wpH\ 4$

$^w_wpH\ 5$

$^w_wpH\ 6$

$^w_wpH\ 9$

Time (min.)

<u>**Chromatographic Conditions**</u>
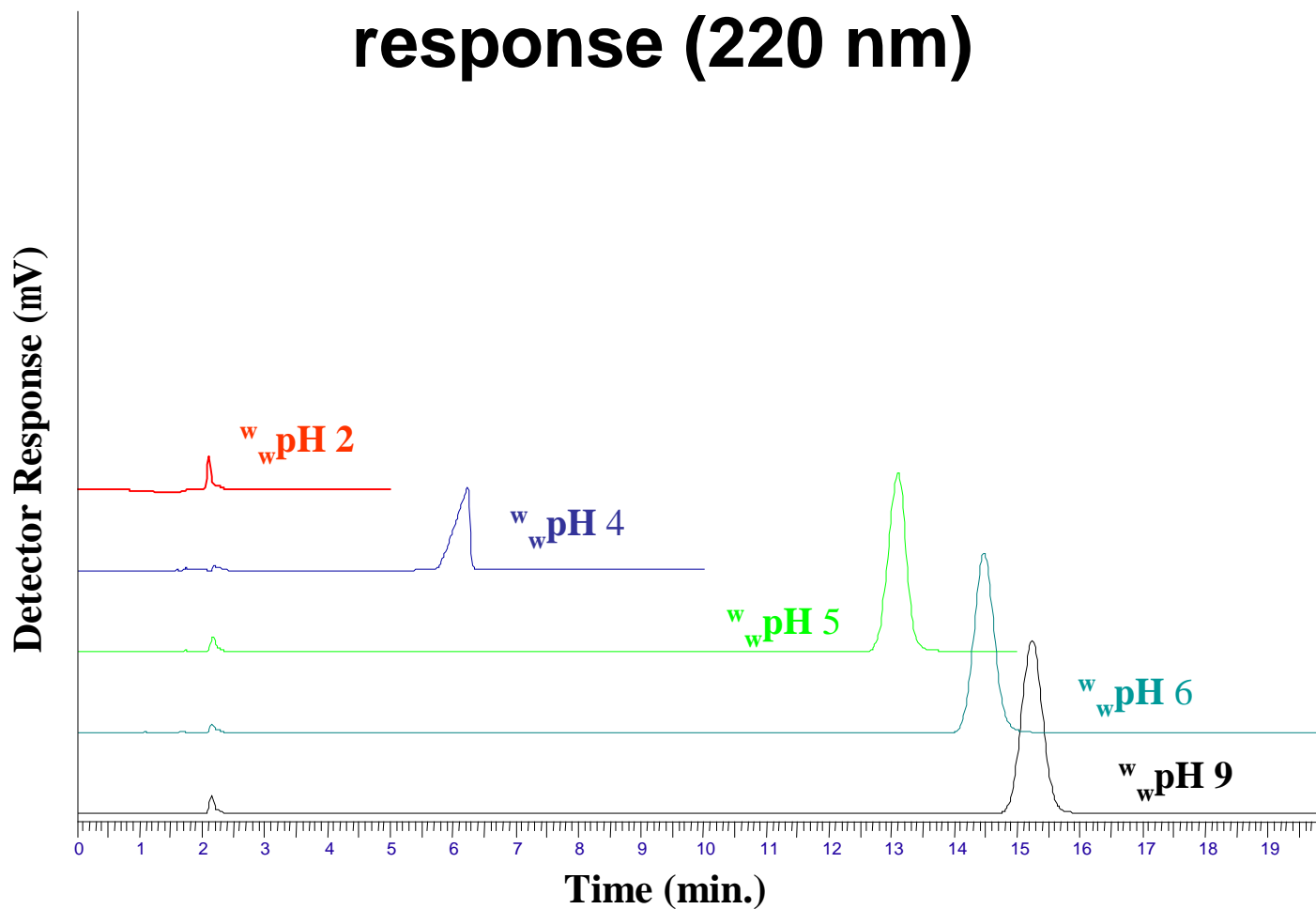Column: 15 cm x 0.46 cm Luna C18(2)   Eluent: 90% Aqueous: 10% MeCN
Aqueous: 15 mM $K_2HPO_4 \cdot 7H_2O$ adj. to ^w_wpH 1.5 - 9 with H_3PO_4
Flow rate: 1 ml/min   Temp: 25ºC

•Enhanced sensitivity is obtained by analyzing aniline in its neutral state   21

Column Equilibration

- Column equilibrates (εξισορροπεί) within 30 min in normal eluent composition range.

- Check retention time stability by injecting standard mixture 3 - 4 times.

- Very high organic (>98%) or very high aqueous (>80%) need ~1 - 2 h equilibration at 1 ml/min.

- In pure water after ~20 h equilibration all analytes elute with void volume. "Chain collapse"? - No.   After 20 h of water pumping all organic removed from adsorbent pores. Water is not wetting the alkylated hydrophobic surface. There is no flow through adsorbent particles, only around.

Solvent Purity

How much solvent (0.1 ppm total impurity) will
contaminate 10% of adsorbent surface?

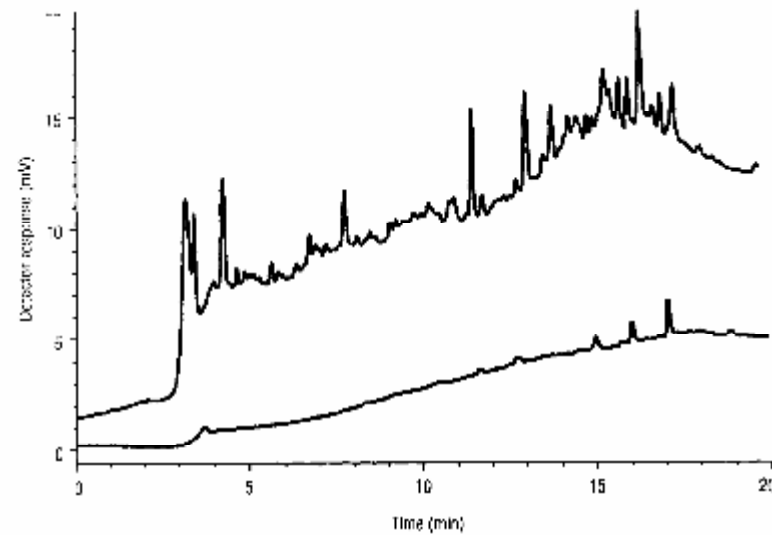Average column - 200 m^2/g
Assume molecular area of 100 Å^2

$$n_{(moles)} = \frac{S}{A \cdot N_A} = \frac{20m^2}{100\mathring{A}^2 \cdot 6 \cdot 10^{23}} \approx 30 \quad \textbf{\textit{m}}Mole$$

Assume average 100 g/mole - 3 mg total accumulation
this comes from **30 L** of solvent with 0.1 ppm total purity
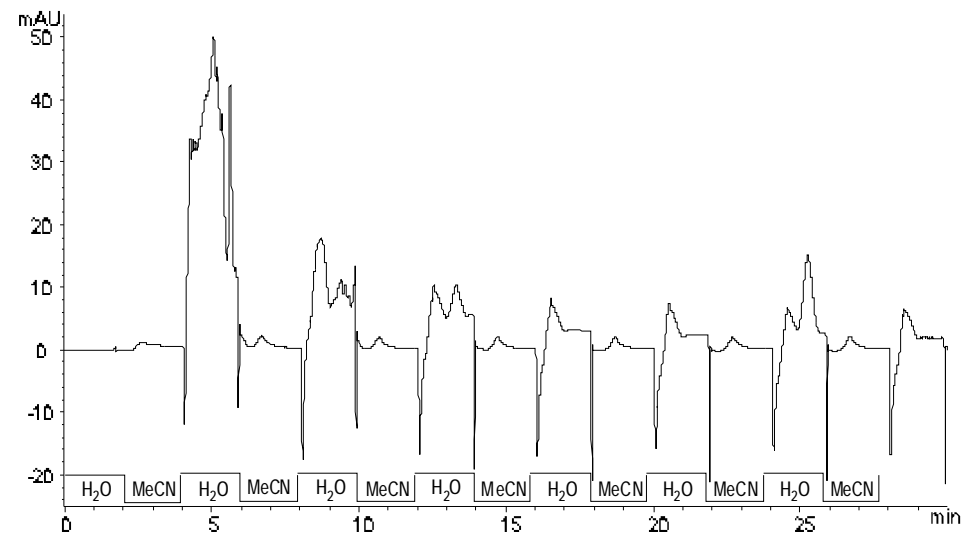
Column has to be cleaned at least once a week

Gradient

• High pressure vs. low pressure mixing
• System dwell (νεκρός) volume effect

Column Cleaning

Solvent front (μέτωπο διαλύτη) disturbs phase equilibrium
Release of trapped (παγιδευμένες) impurities

Method troubleshooting

- Problems are usually related to one of the following:

  1. **System**
  2. **Column**
  3. **Sample**
  4. **Mobile Phase**

System

- System-to-system compatibility

    - **Differences in configuration (detector sequence, etc.)**
    - **Different dwell volume**
    - **Detector sensitivity always different**
    - **Wavelength accuracy**
    - **Bandwidth**
    - **Environment effects**

Sample

Avoid particulate in the sample   Typical cause of inlet filter clogging

Filter   Sample filtration can change composition

Centrifuge   Usually cumbersome (δυσκίνητη)

Sample vials

Type of the vial cap and septa affect contamination and carry-over

Waters systems require 75% filling of 2 mL vial
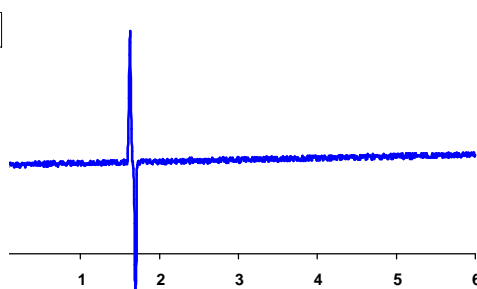
Troubleshooting sequence

- Pump
  - Any reciprocal pattern (ανάποδη εικόνα) on chromatogram
  - Pressure fluctuations
  - Baseline drift (possible contamination of the solvent)
- Autosampler
  - Injection marks (baseline disturbance)
  - Cross-contamination
  - Vial fill-in (sample level)
- Detector
  - Response (baseline noise, drift, etc.)
  - Wavelength (bandwidth, accuracy, etc.)
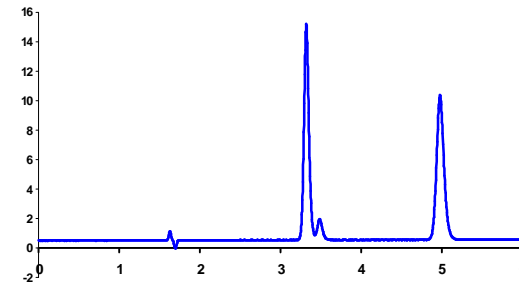
Troubleshooting sequence

- First check is always the plumbing (σωληνώσεις) (leak, flow rate, pressure)
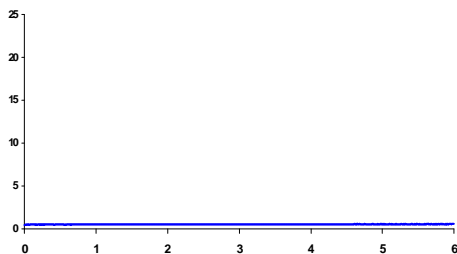- Output (chromatogram) evaluation
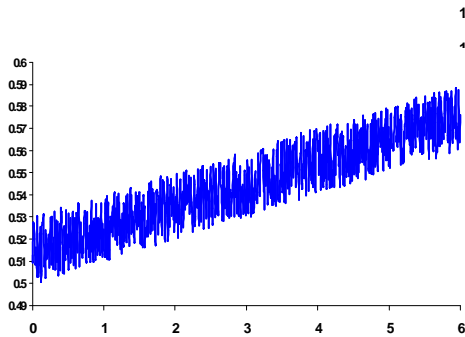
1

2

3

1- flow or detection problem
2 – possible injection problem
3 – correct chromatogram

Troubleshooting sequence

- Analysis of chromatogram
  - Compare with previous results
  - Peak tailing
  - Retention shift
  - Reverse elution