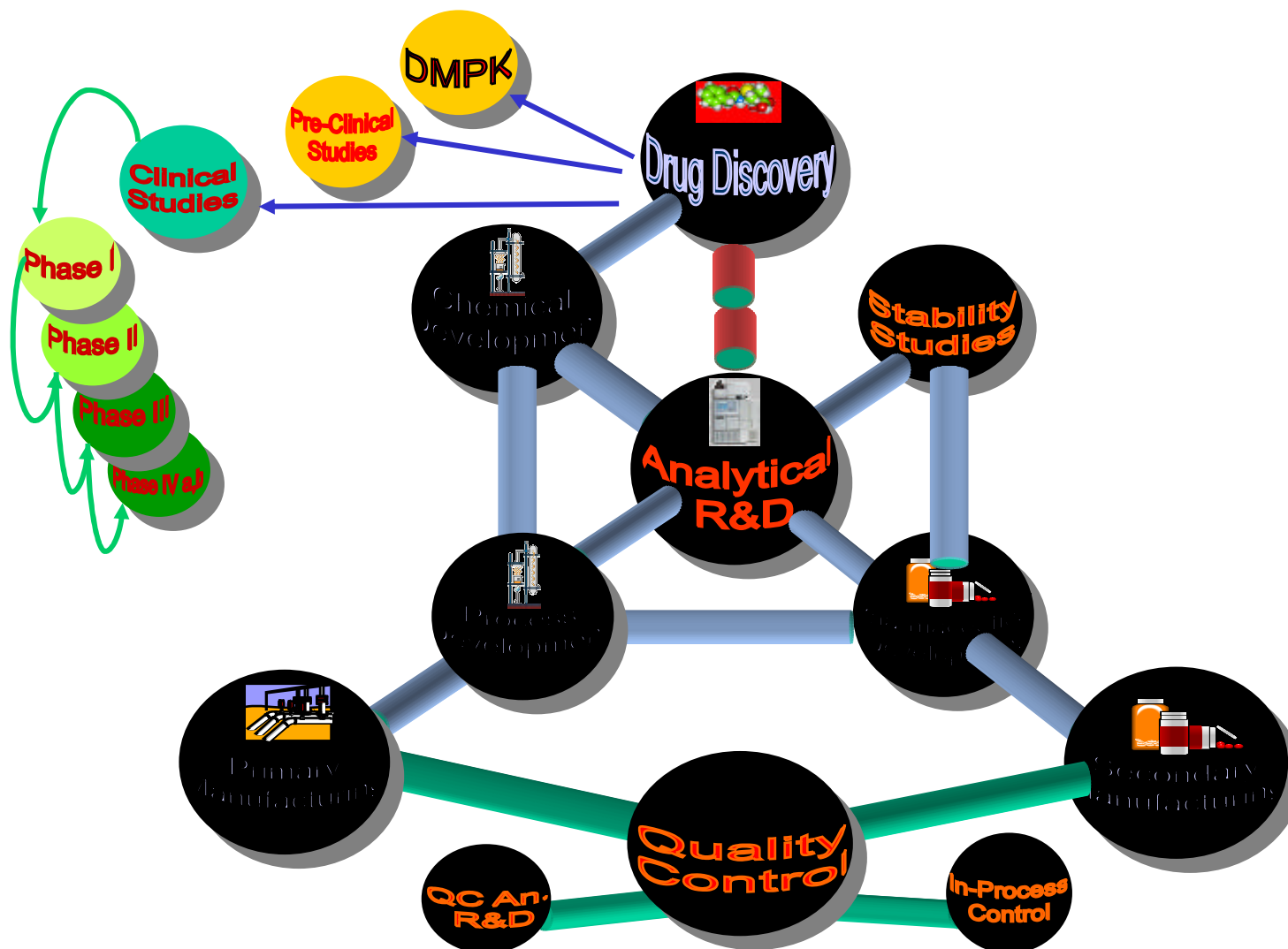


# **Unexpected Peaks in Chromatograms - Are They Related Compounds, System Peaks or Contaminations?**

## **From the Diary of an HPLC Detective**

SHULAMIT LEVIN

# HPLC in Pharmaceuticals



# Stability Indicating Methods

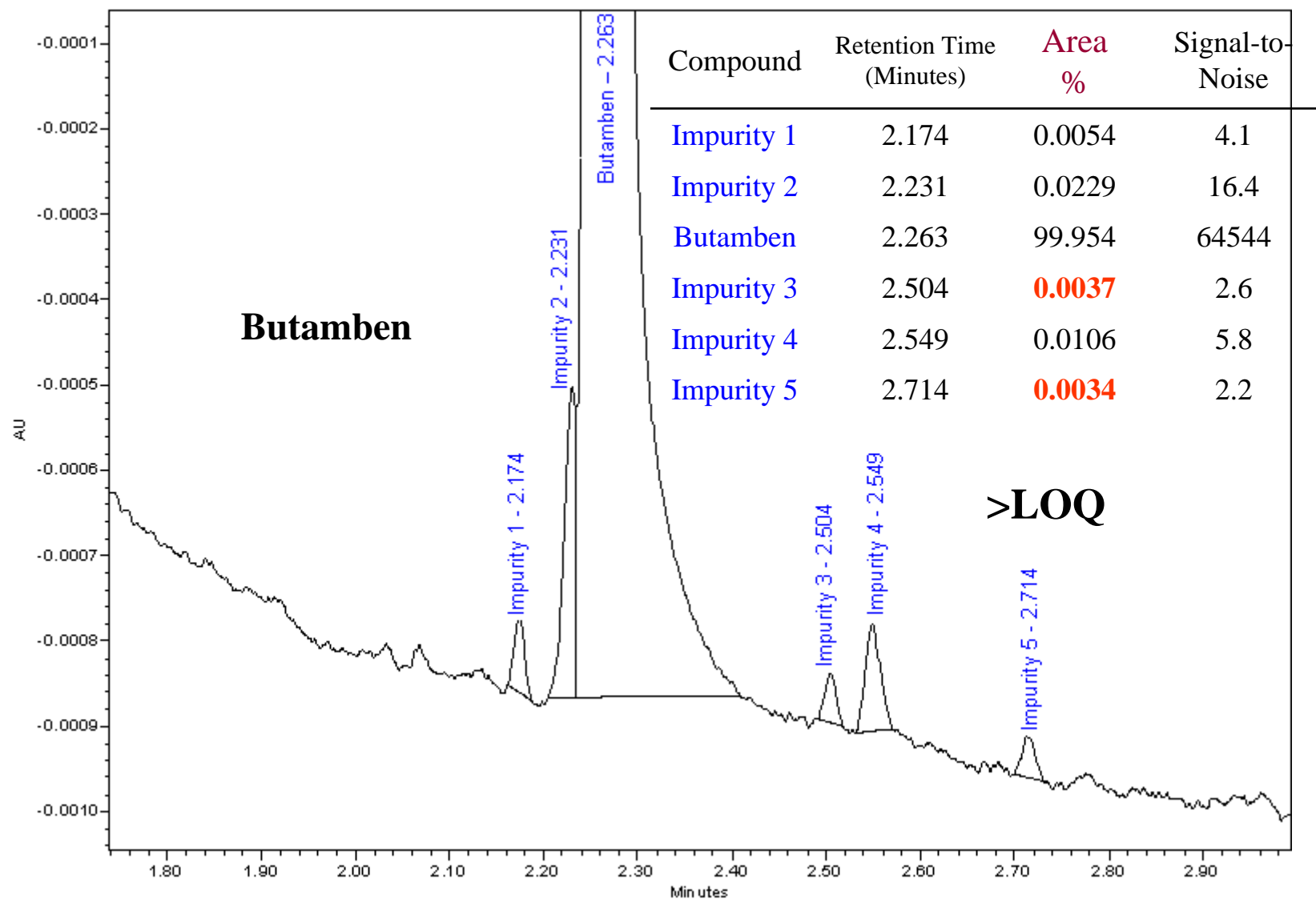
Extra Sensitive to Extraneous Peaks

- **Accurately quantifies the active pharmaceutical ingredient without interference from:**
  - Impurities
  - Degradation products
  - Excipients
  - Other potential impurities
  - Other active ingredients

## FDA Guidelines:

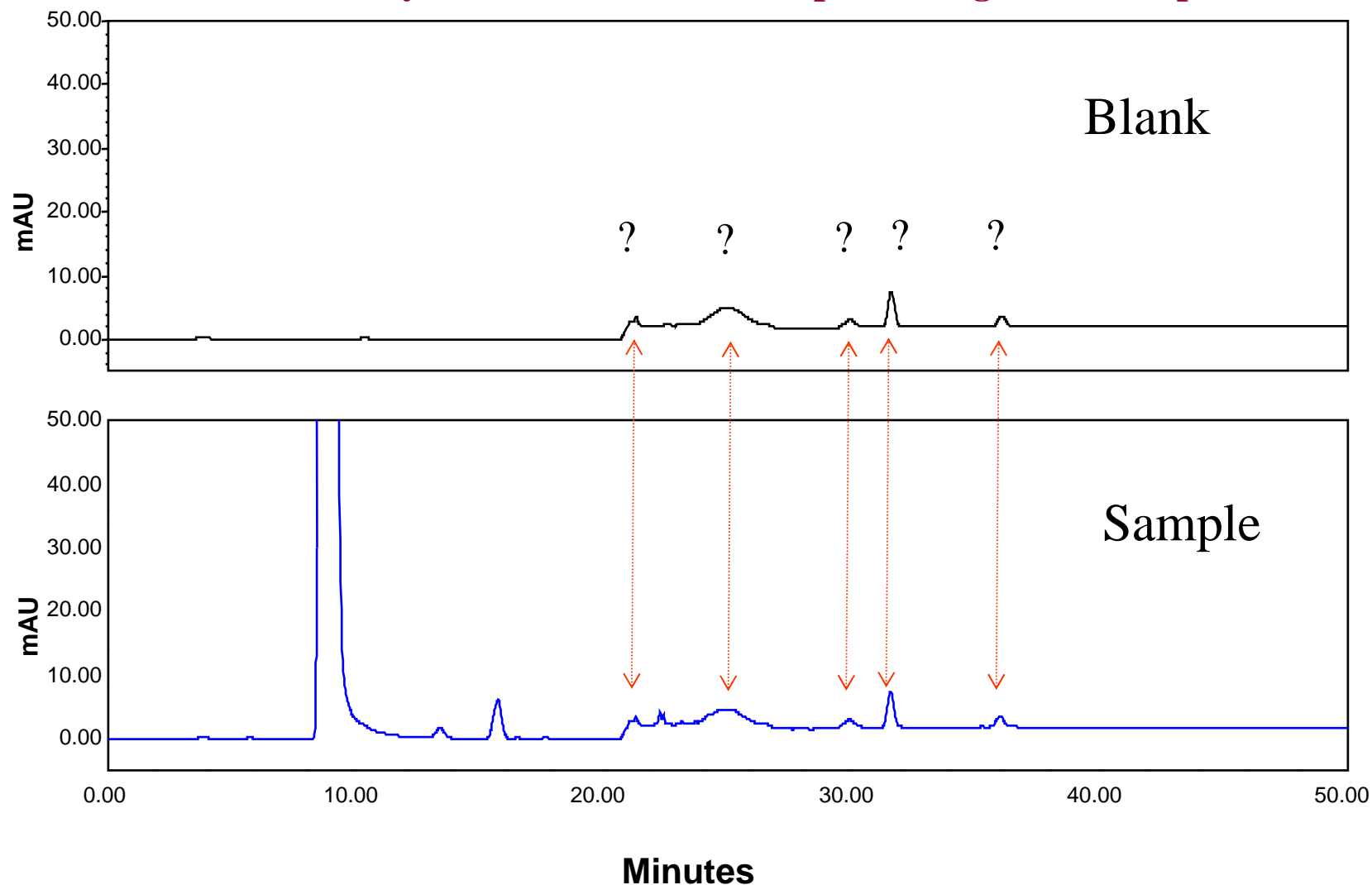
Where an analytical method reveals the presence of impurities in addition to the degradation products (e.g., impurities arising from the synthesis of the drug substance), **the origin of these impurities should be discussed.**

## Accounting for Every Peak in the Related Compounds' Profile - Even Down to 0.003 %Area

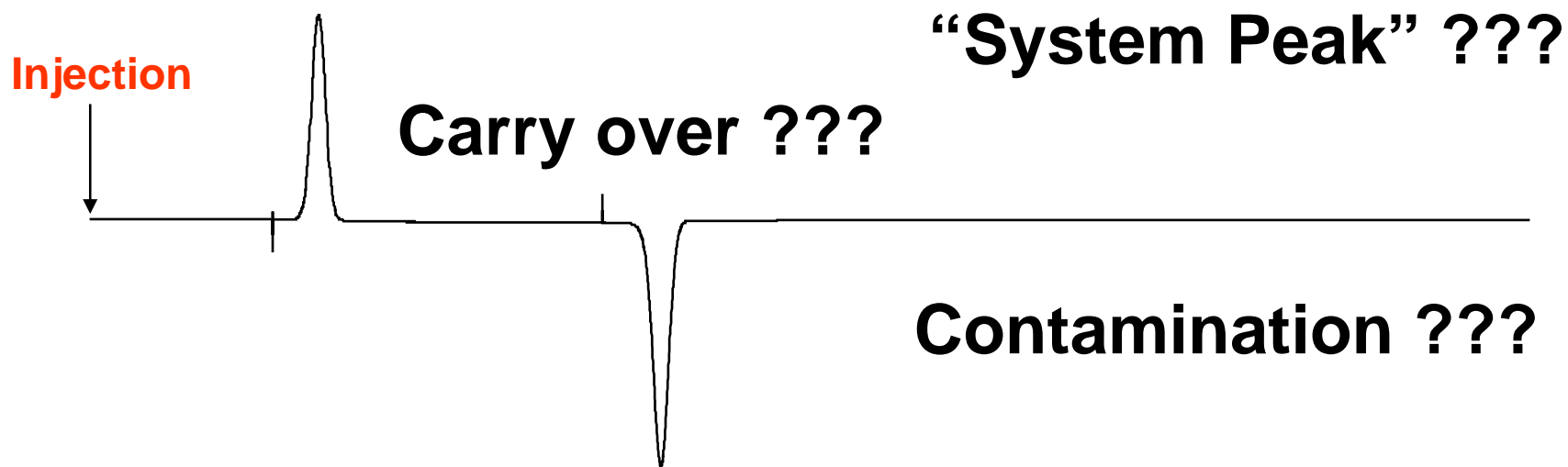


# Sample vs. Blank (Diluent)

Unexpected peaks can appear in blanks and in all following injections  
In this case they are excluded from the processing of the sample



# INJECTION OF PURE SOLVENT: Why would there be peaks?



# System Peaks

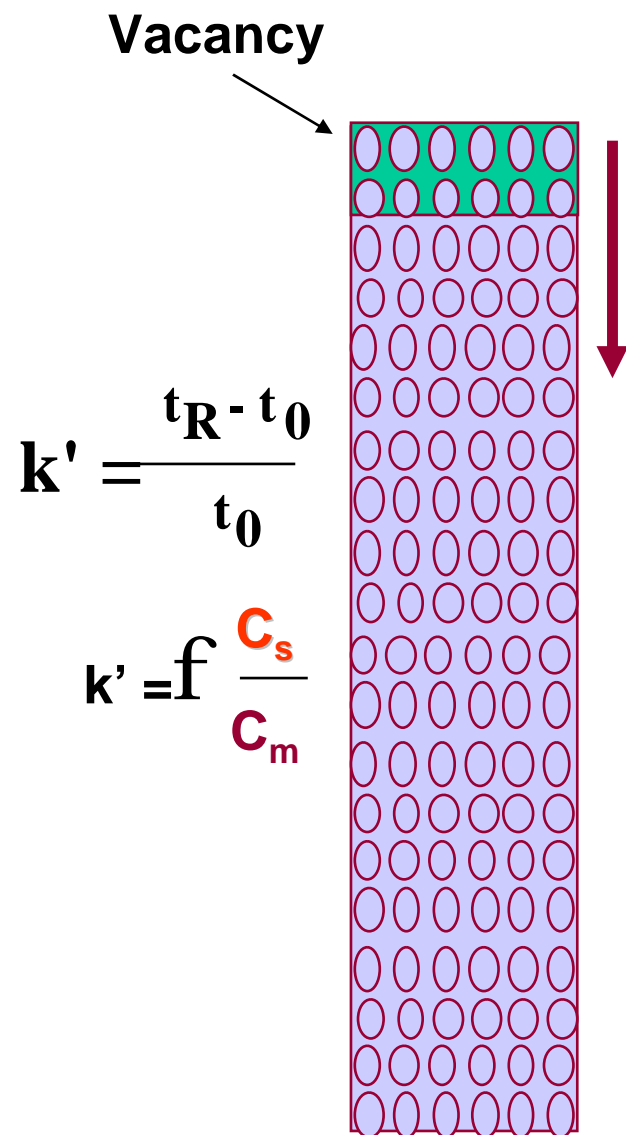
“Legitimate” System Peaks  
Originate from the Mobile Phase Components Going  
Through Re-Equilibration

# **CONDITIONS FOR APPEARANCE OF REAL SYSTEM PEAKS**

- **Mobile phase is multi-component ( $n \geq 2$ )**
- **Mobile phase contains adsorbable components**
- **Mobile phase's components respond to the detector (high background)**
- **Sample or sample-diluent is different from the mobile phase, enough to create equilibrium perturbation.**



# Mechanism of System Peaks Formation



**EXAMPLE:**  
Two additives in the mobile phase

**Example:  $k'(1) = 1$**

Step 1:

**Equilibrium:**

**$C_s=1$ ;  $C_m = 1$**

Step 2:

**Injection of  
Vacancy:**

**$C_s=1$ ;  $C_m=0$**

Step 3:

**Re-equilibration:**

**$C_s=0.5$ ;  $C_m=0.5$**

**Example:  $k'(2) = 2$**

Step 1:

**Equilibrium:**

**$C_s=2$ ;  $C_m = 1$**

Step 2:

**Injection of  
Vacancy:**

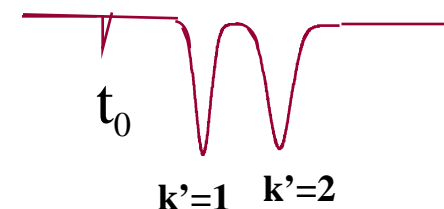
**$C_s=2$ ;  $C_m=0$**

Step 3:

**Re-equilibration:**

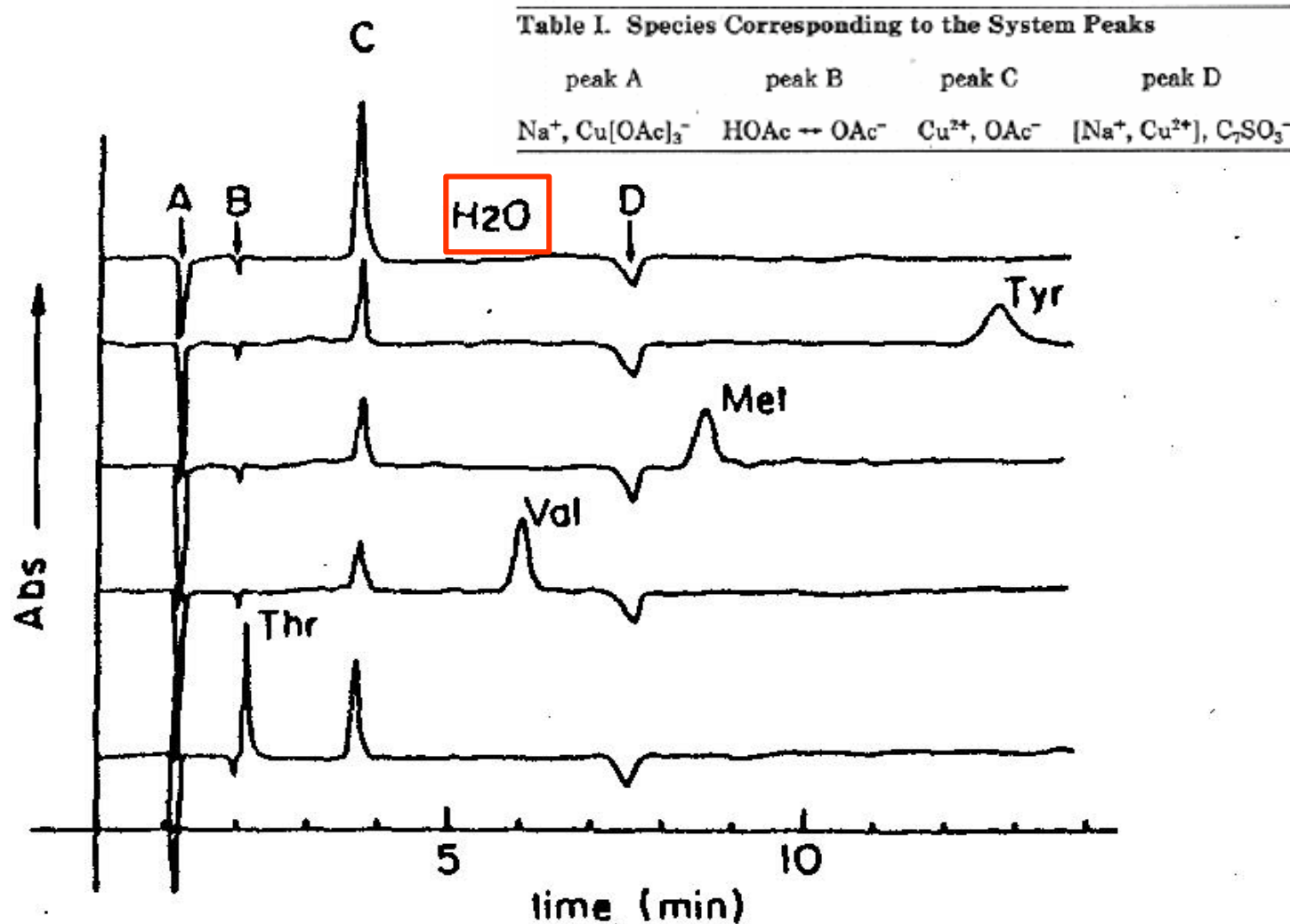
**$C_s=1.33$ ;  $C_m=0.67$**

**CHROMATOGRAM**



**Injection of free amino acids into mobile phase containing:  
Ac buffer, CuAc, and Heptsulfonate.**

**Diluent: Water**



## Legitimate System Peaks Result from Mobile phase components due to their absence in the injected sample

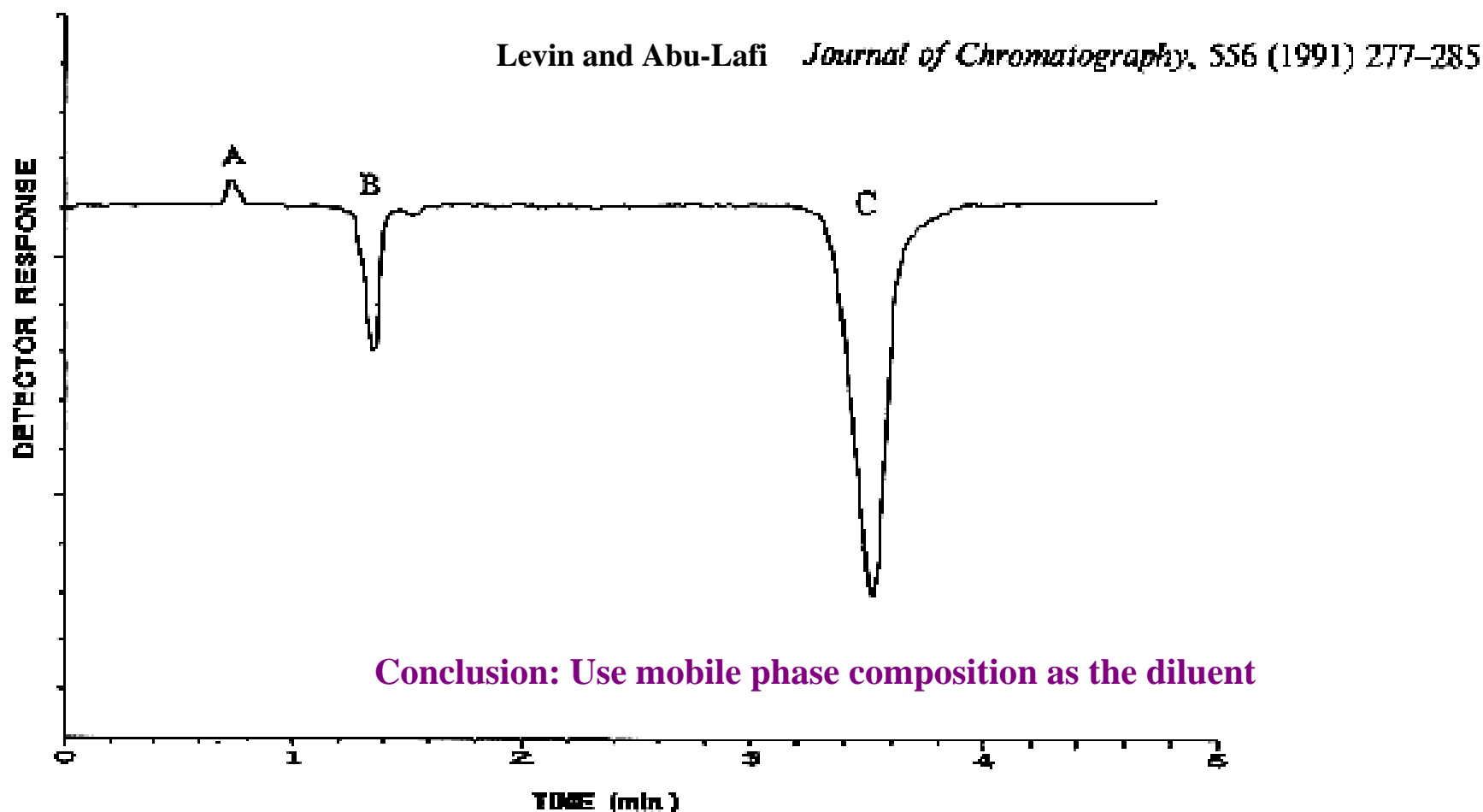
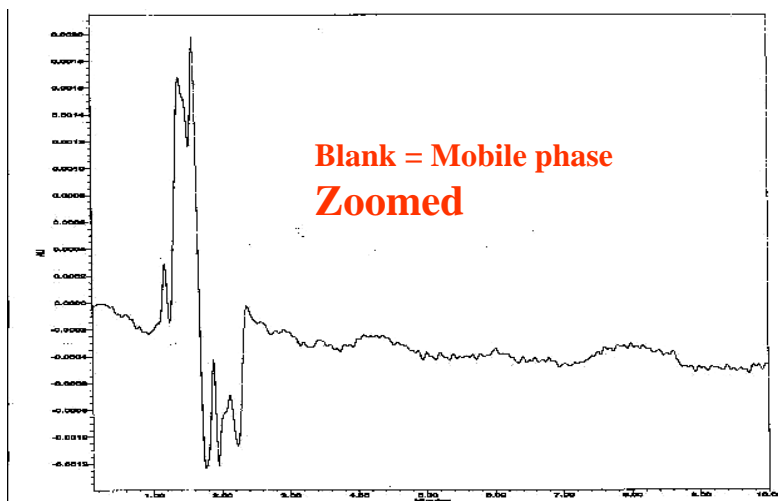
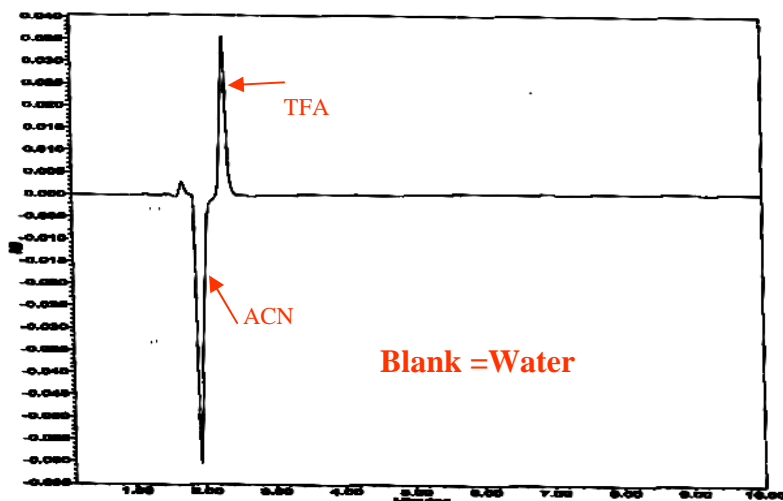
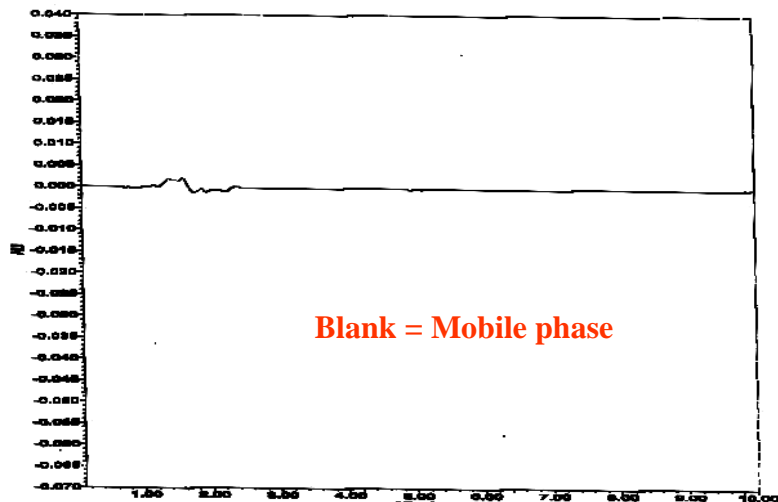
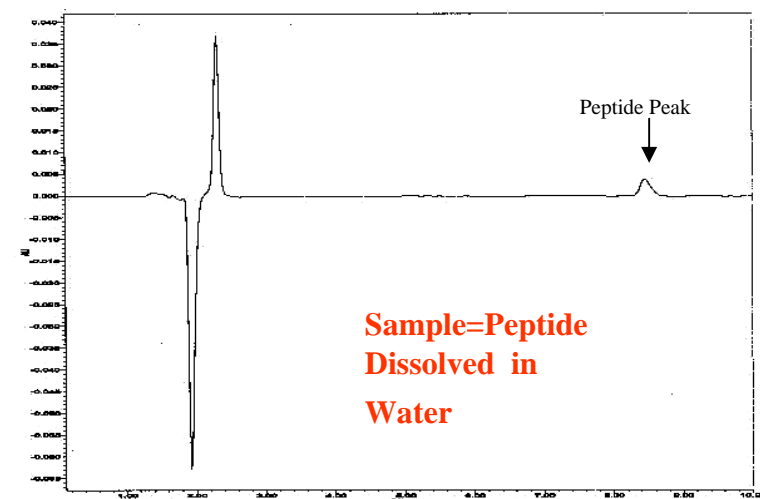


Fig. 1. System peaks A, B and C detected at 254 nm when 20  $\mu$ l of water were injected into a mobile phase of 0.025 M phenylalanine in 0.1 M acetate buffer (pH 3.7).

## An Example for Real System Peaks in Gradients with Trifluoroacetic Acid (TFA) in the Mobile Phase

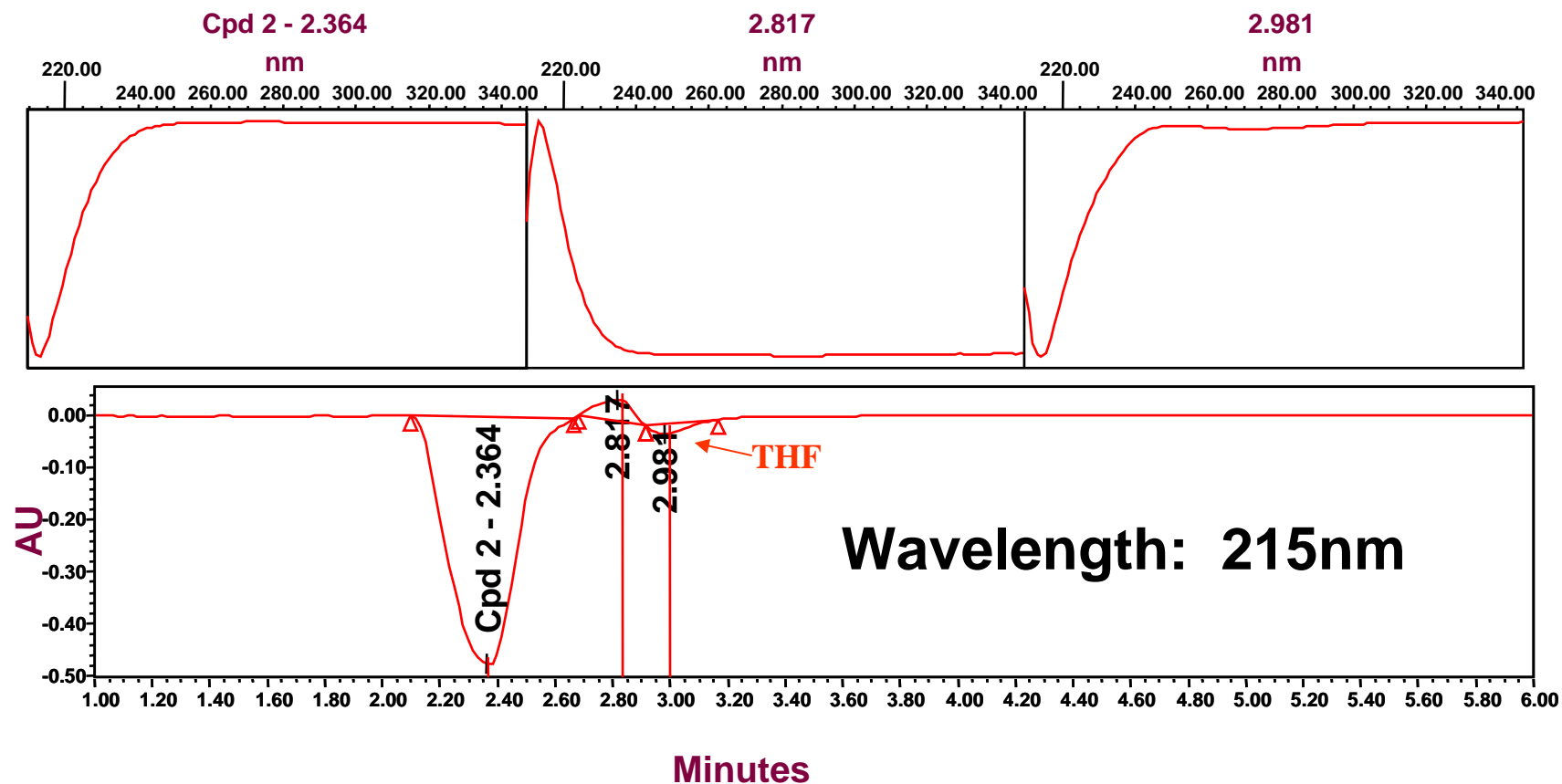


System peaks appear because diluent does not contain TFA

# Real System Peaks in Multi-component Mobile Phase

## H<sub>2</sub>O, MeCN, MeOH, THF

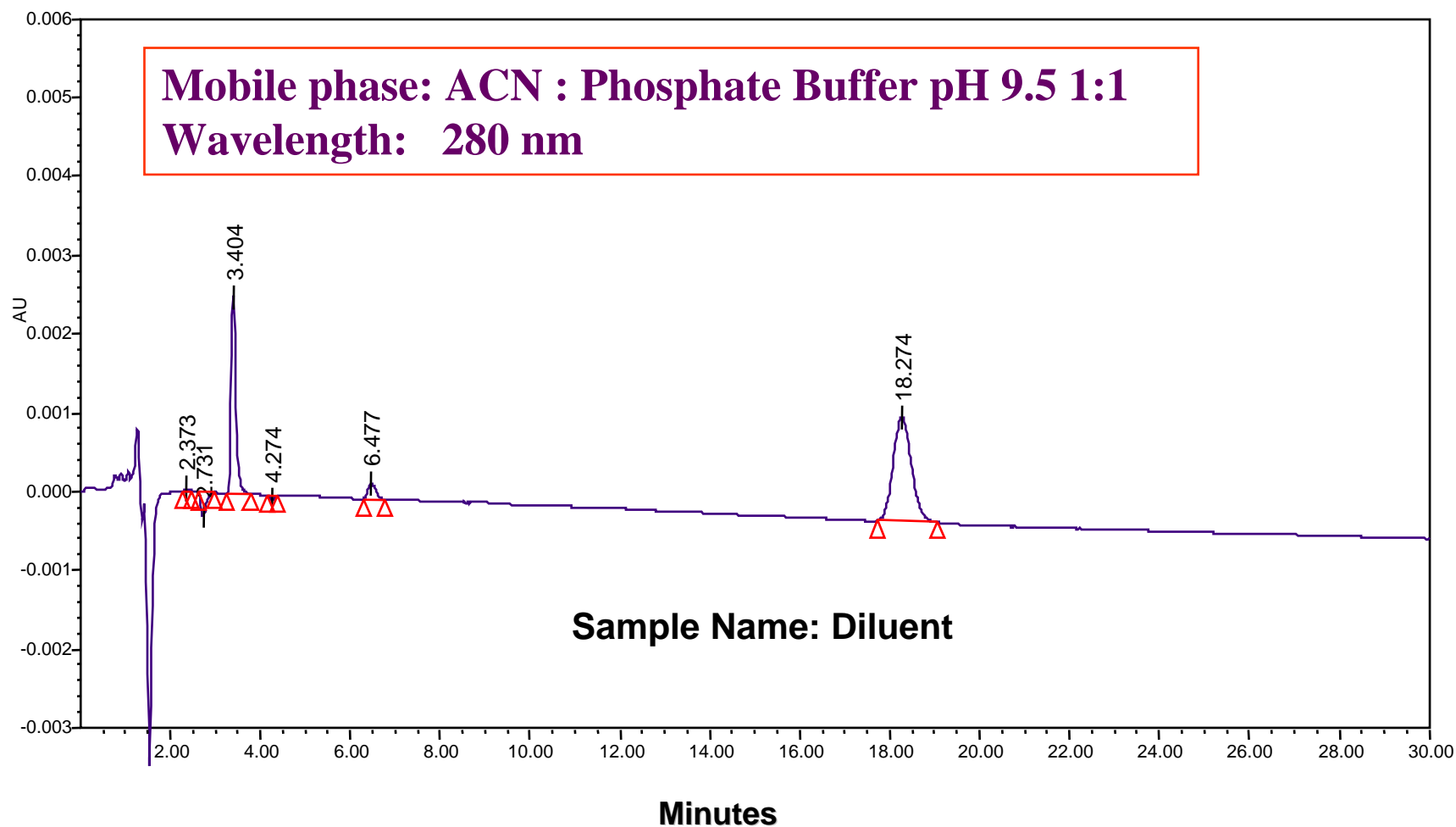
### Chromatogram and Peaks' UV-VIS Spectra



**Diluent does not contain all mobile phase components**

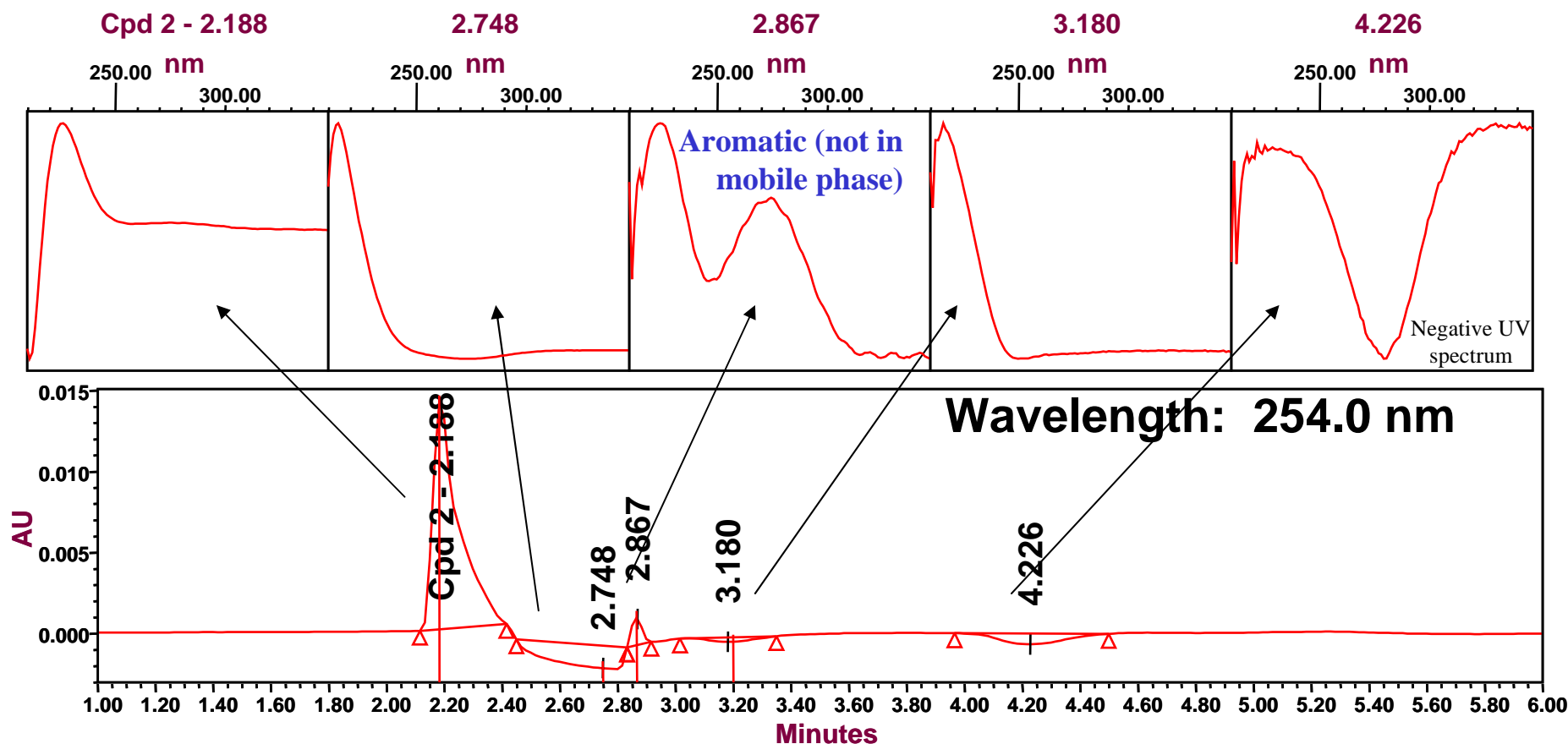
# Contaminations Peaks - **Not** System Peaks!

**Phosphate Buffer and Acetonitril Do Not Produce Such System Peaks!**



## Use of Diode-Array Detector for Troubleshooting of Contamination Peaks in Multicomponent Mobile Phase

### Chromatogram and Peaks' UV-VIS Spectra



# Carry Over:

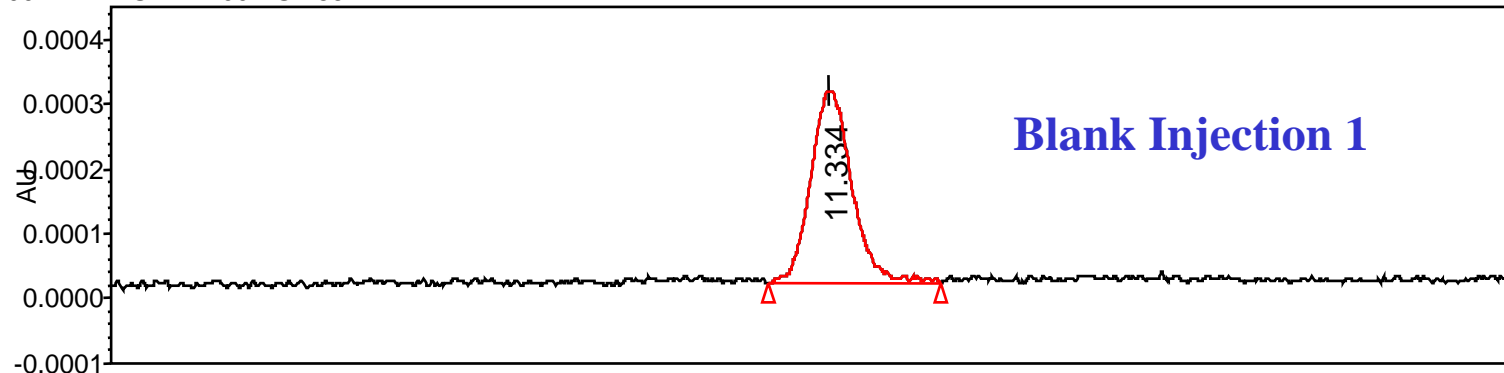
## Chemical Residues in the system

- ☐ Residues in the injector
- ☐ Non-specific irreversible adsorption on column
- ☐ Accumulation on system's surfaces

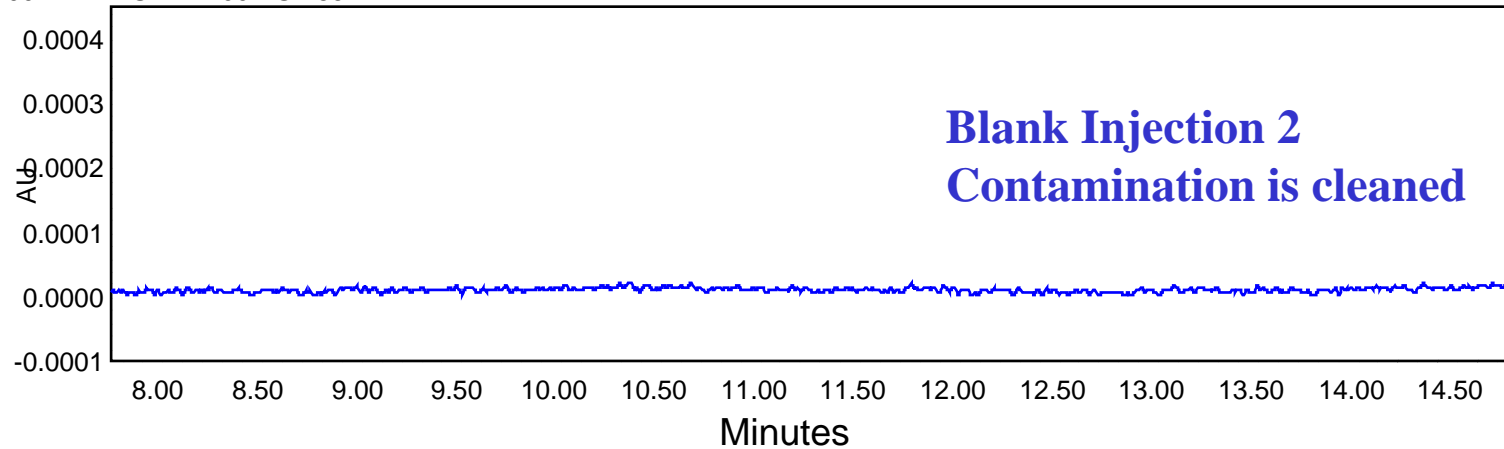


# Carry Over in the Injector Washed by Blank Injection

A1100 VWD AU - A1100 AU 286 nm

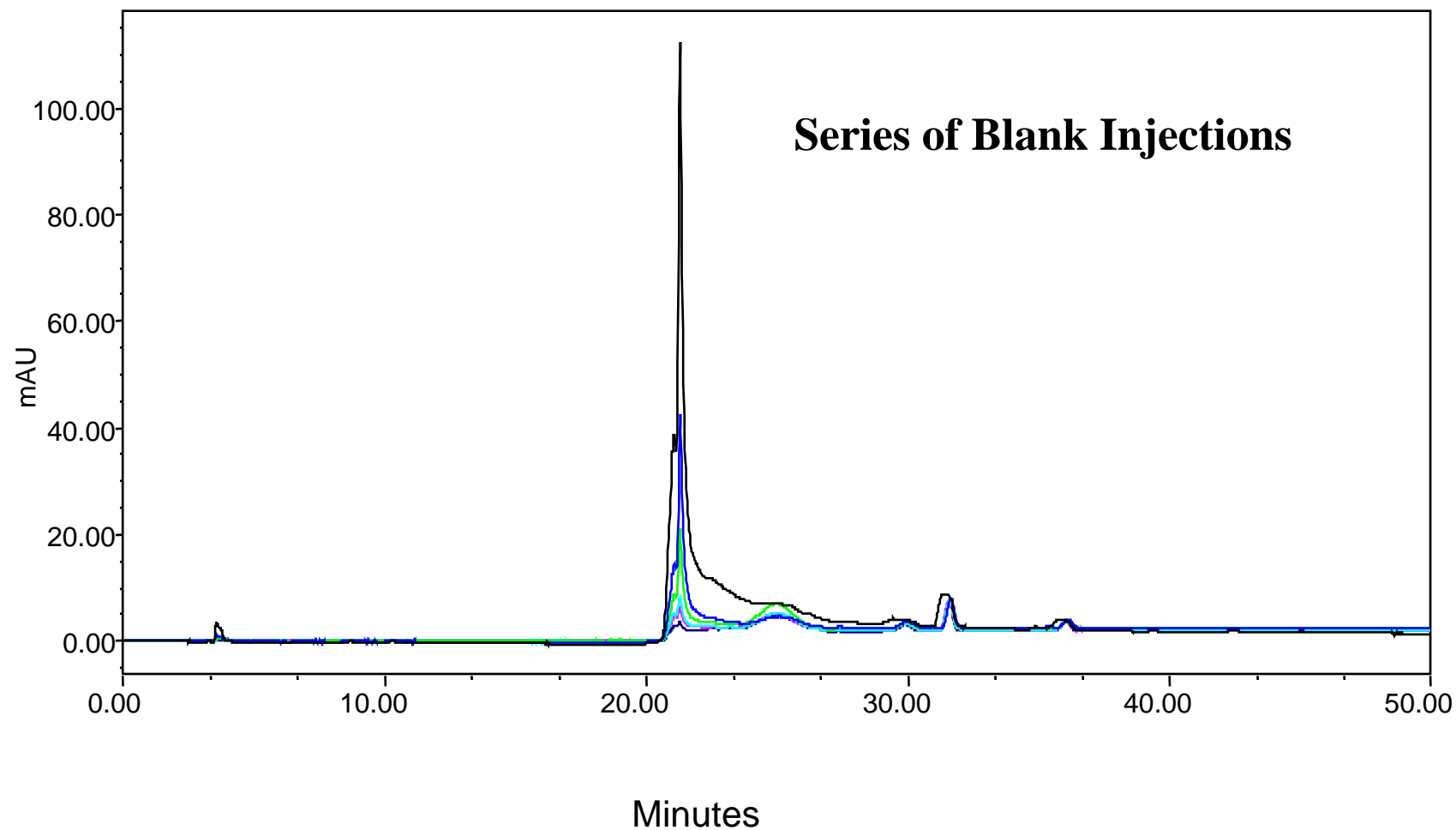


A1100 VWD AU - A1100 AU 286 nm



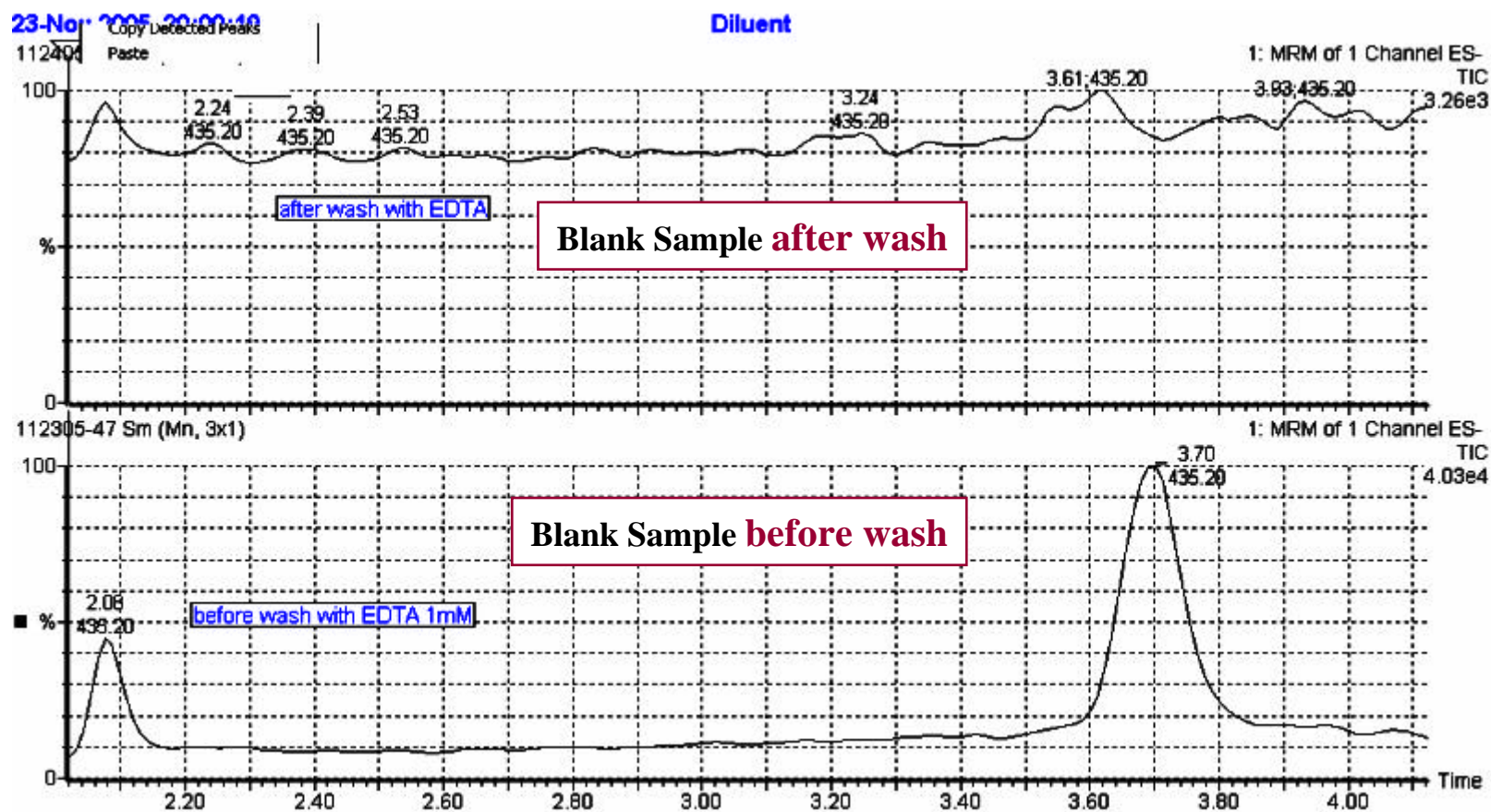
# Blank Injections

Signals are reduced from Injection to Injection indicate carry over in the injector



# Carry-Over in the Injector LC-MS/MS

1. Peak elutes at RT of main component
2. MRM of the main component



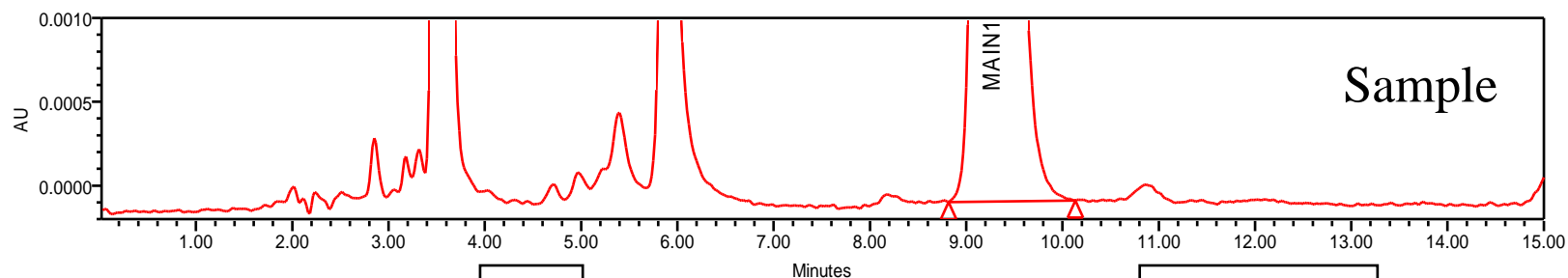
**Injection-Clean-up with EDTA solved the problem**

# **Carry Over:**

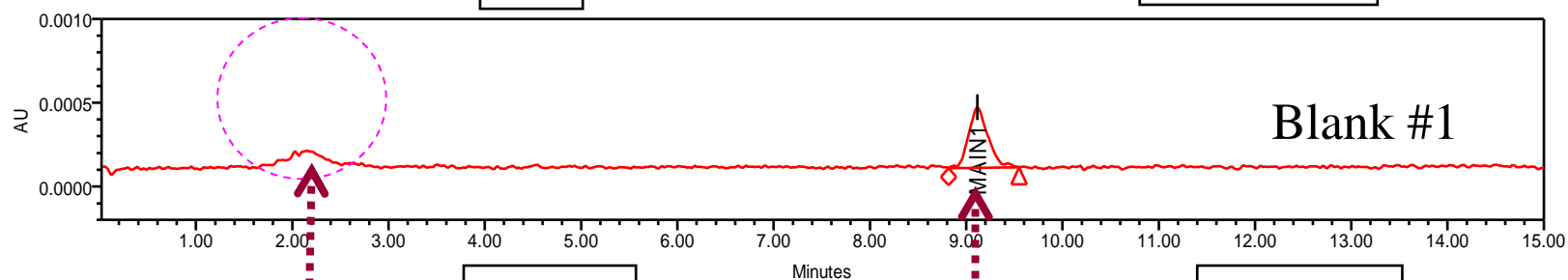
## **Residues in the system**

- ☐ **Residues in the injector**
- ☐ **Non-specific irreversible adsorption on column**
- ☐ **Accumulation on system's surfaces**

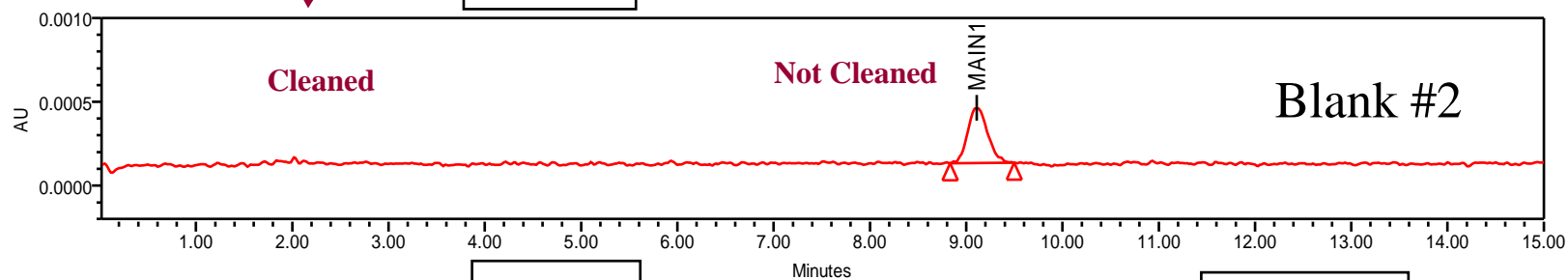
# Accumulation on Column



— SampleName: SST; Vial: 1; Injection: 1; Name: MAIN1; Area: 833913



— SampleName: DILUENT; Vial: 2; Injection: 1; Name: MAIN1; Area: 5063

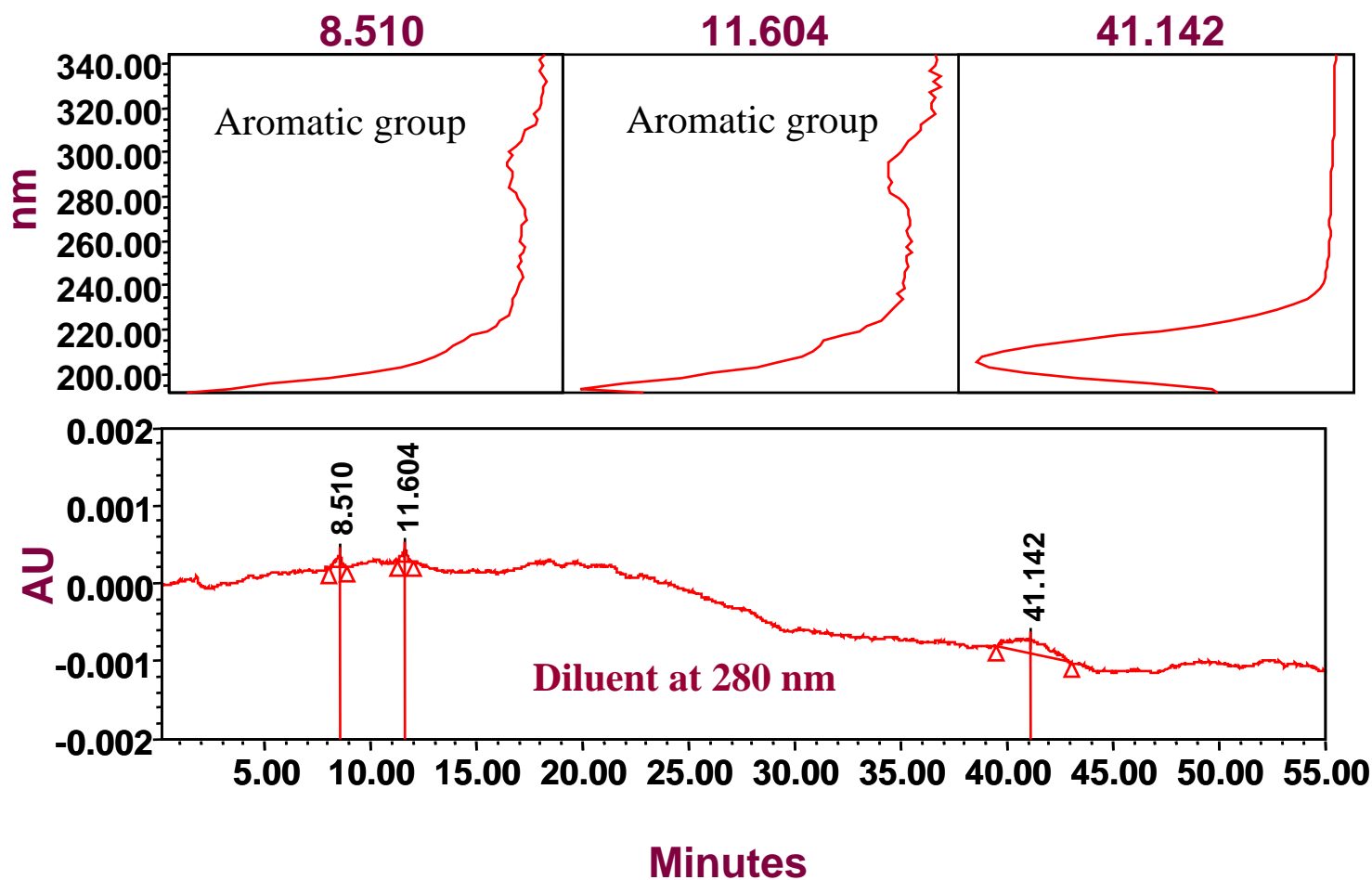


— SampleName: DILUENT; Vial: 2; Injection: 2; Name: MAIN1; Area: 4784

# Contamination Peaks

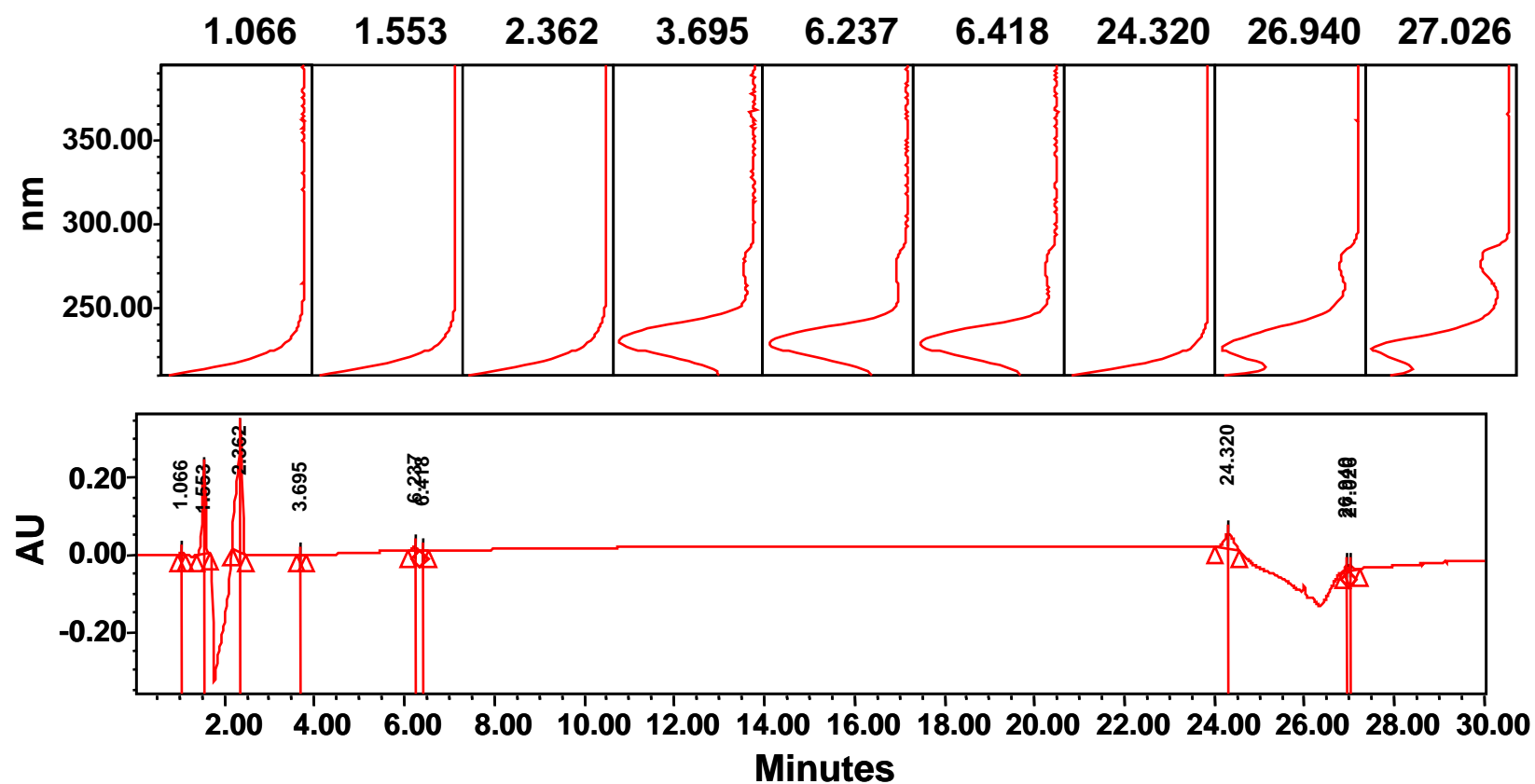
**TFA Containing Mobile Phase: TFA, H<sub>2</sub>O, MeCN - Do Not Contain Aromatic Components!**

## Chromatogram and UV-VIS Spectra of Contamination Peaks



## Peaks of aromatic compounds in non aromatic mobile phase: Cannot be Legitimate System Peaks

### Chromatogram and UV-VIS Spectra of a Blank Run



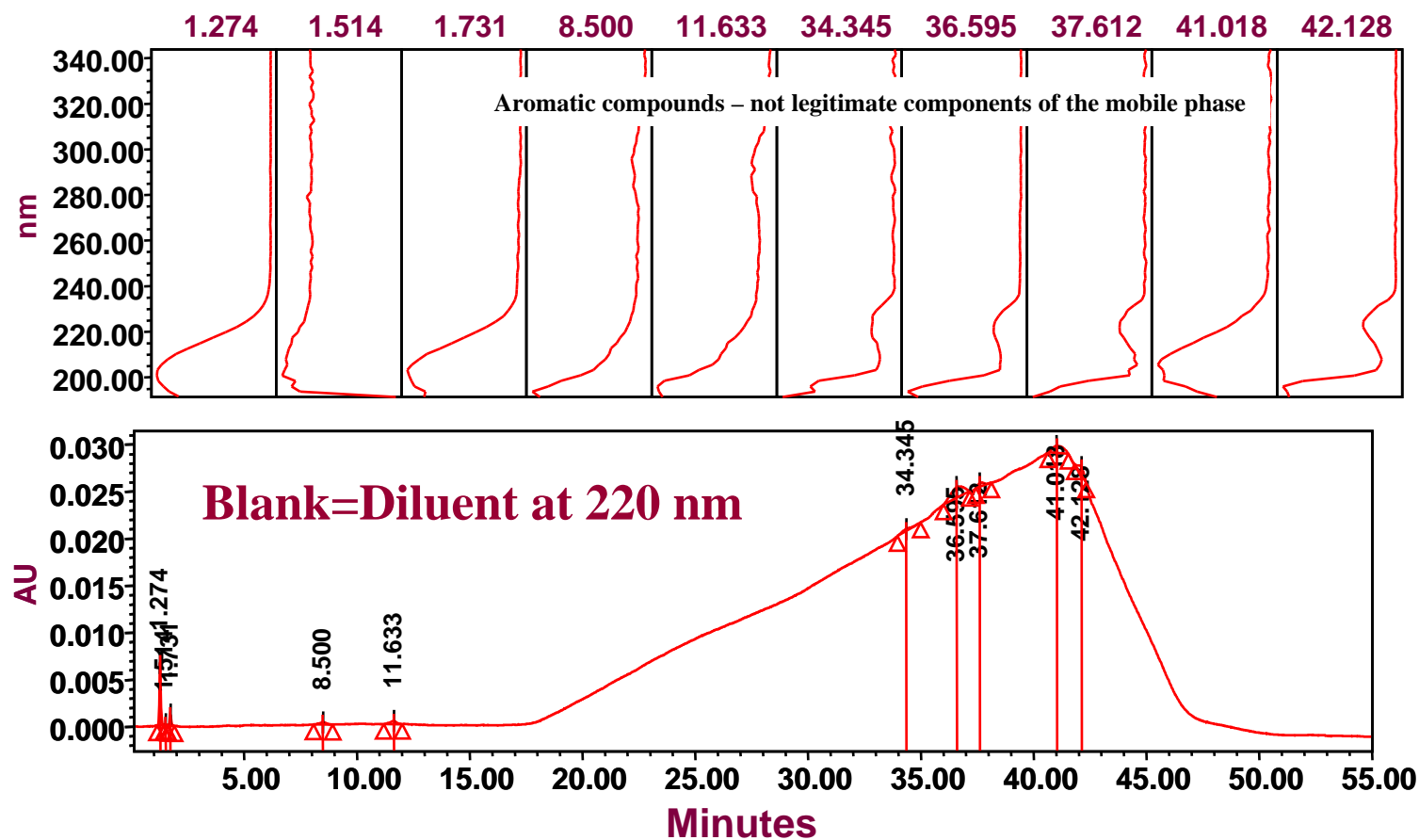
Sample Name blank ; Wavelength 214

Mobile phase: Gradient of 0.1% TFA in water with ACN

# Contamination Peaks - Residues

Contamination on-column and/or in the mobile phase

## Chromatogram and UV-VIS Spectra of Contaminations' Peaks



Gradient with TFA



# Carry Over:

## Residues in the system

- ☐ Residues in the injector
- ☐ Non-specific irreversible adsorption on column
- ☐ Accumulation on system's surfaces

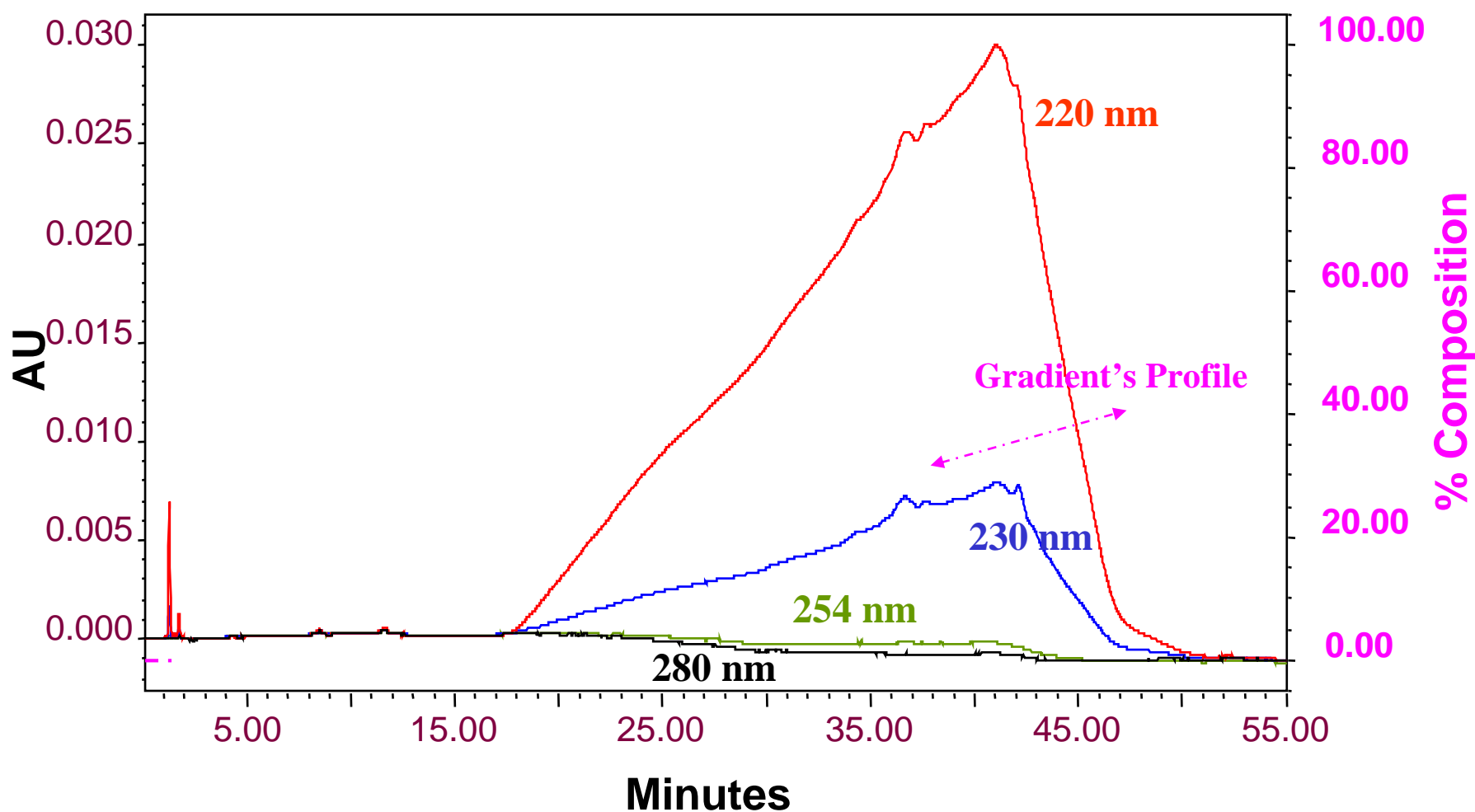
# Contaminations

## Origin – Outside the Sample

- ☐ Non-pure solvents
- ☐ Vials' leachables
- ☐ Reservoir's leachables

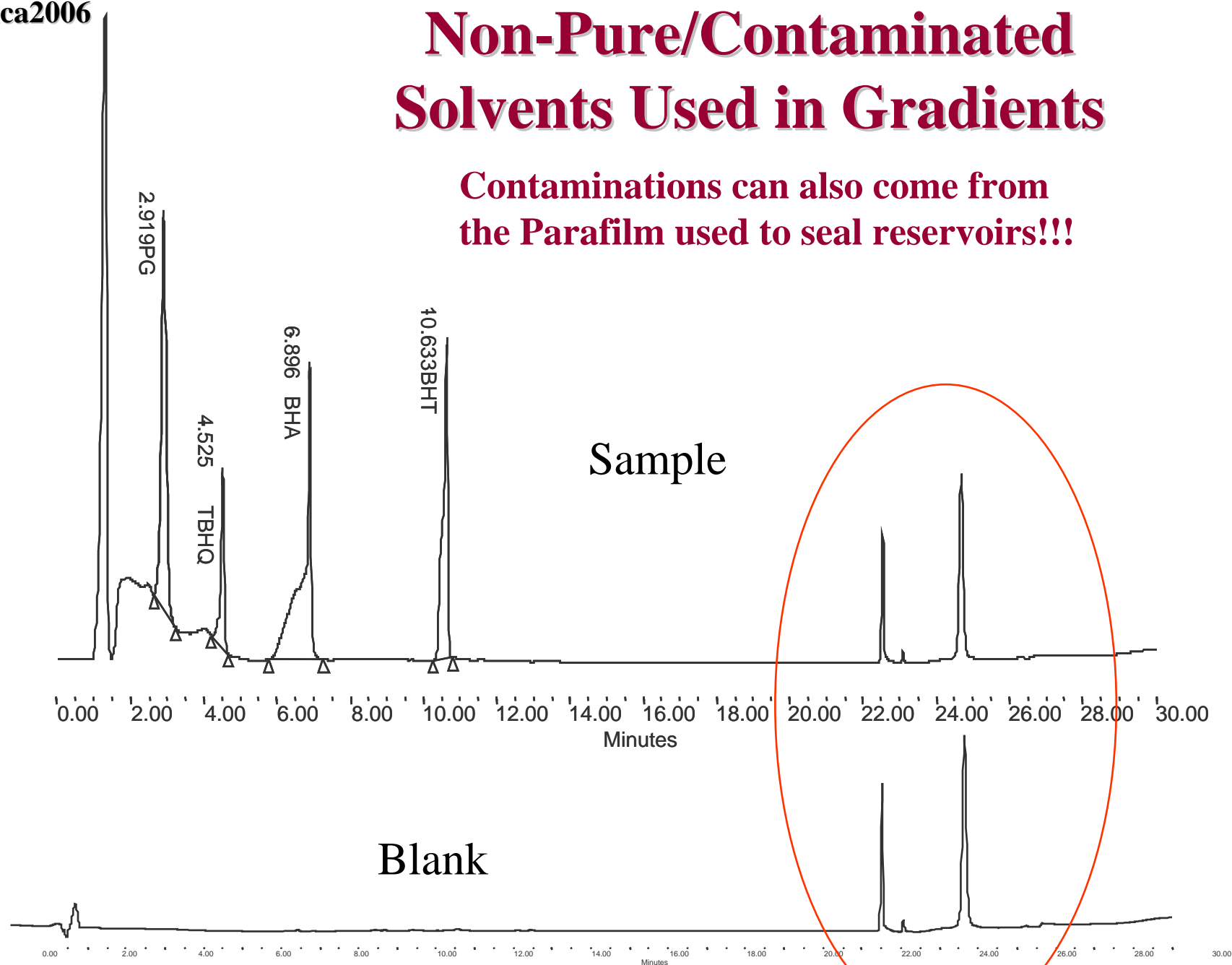
# Baseline at Gradient

Change of Baseline with Time at Various Wavelengths:  
Indication for non pure solvent



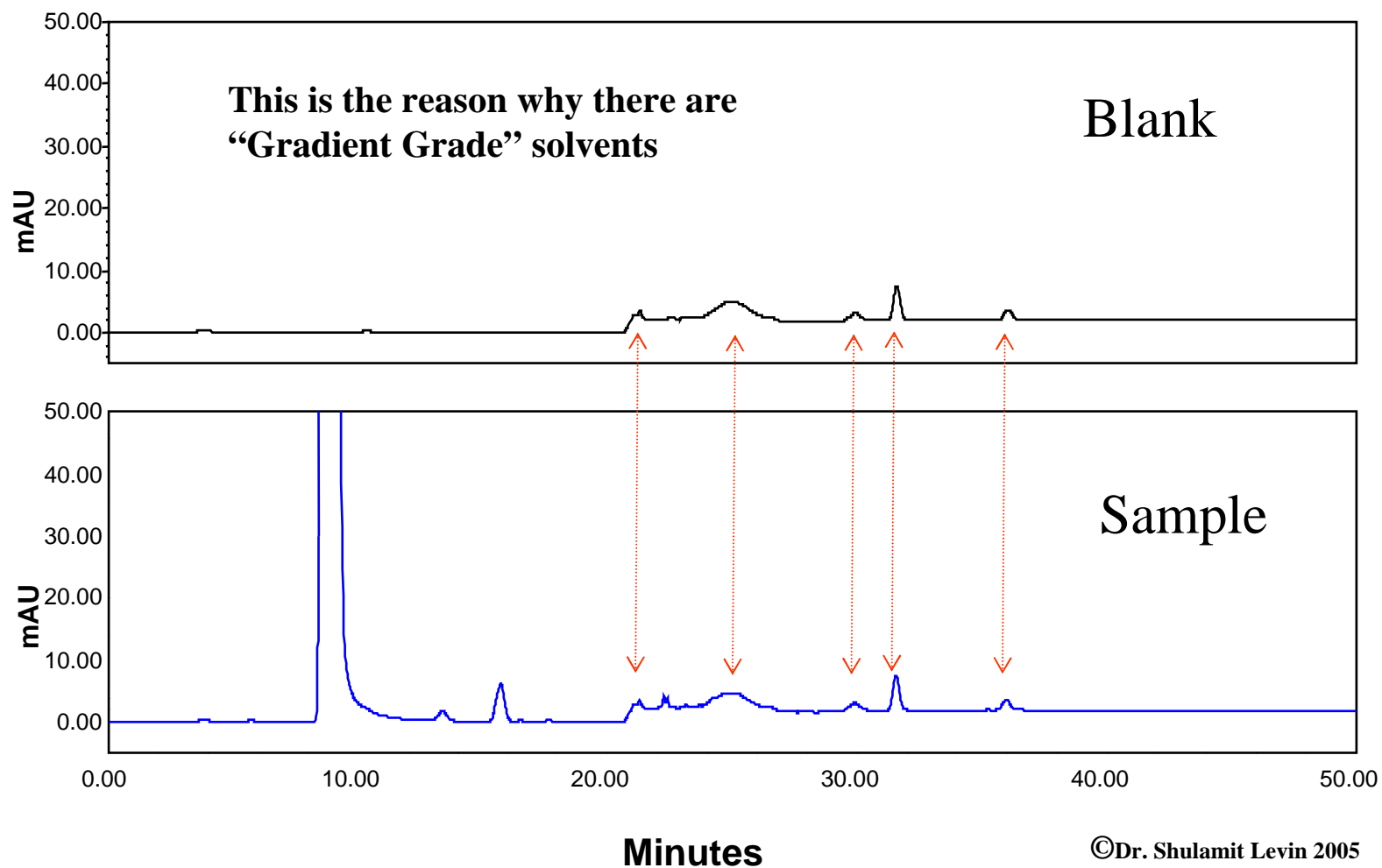
# Non-Pure/Contaminated Solvents Used in Gradients

Contaminations can also come from  
the Parafilm used to seal reservoirs!!!

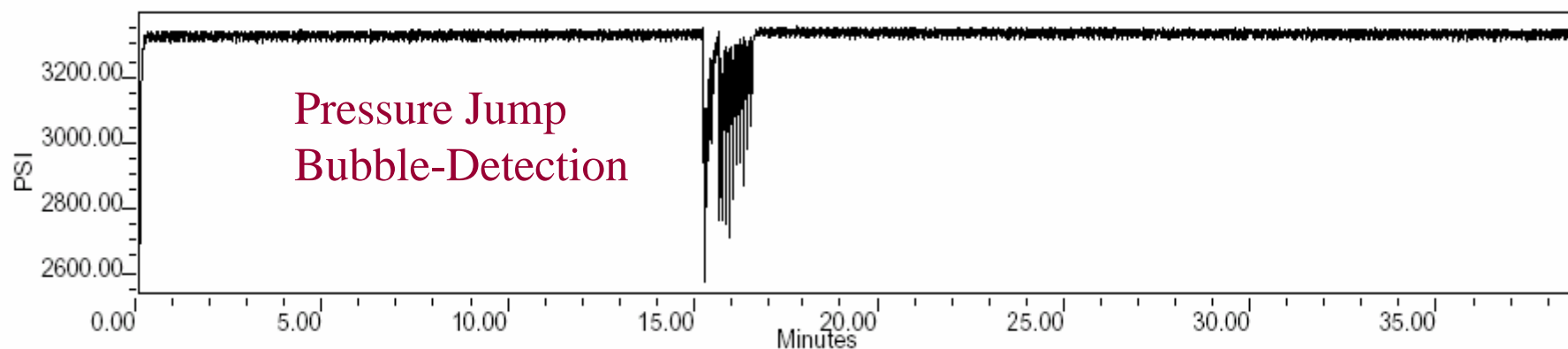


# Sample vs Blank

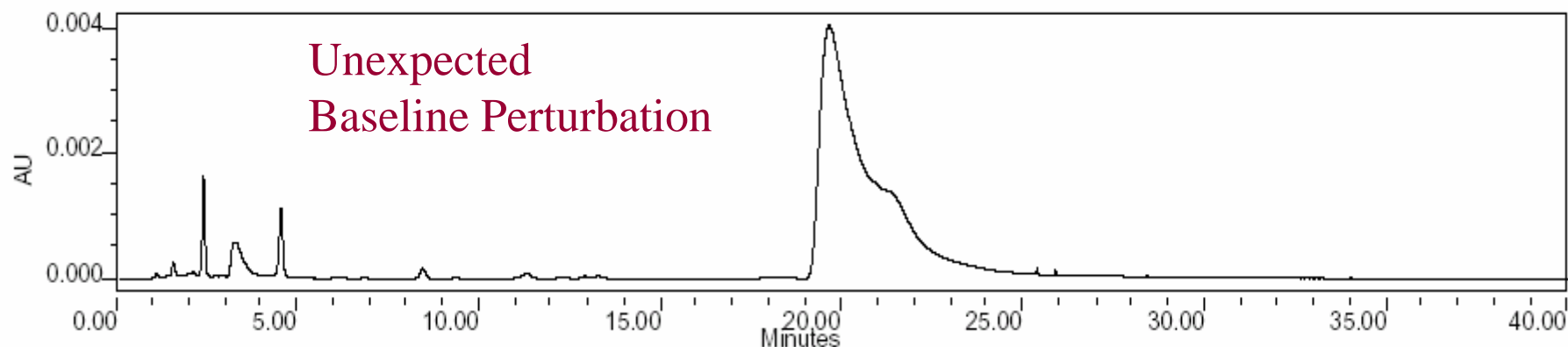
If **Solvents are not entirely pure** their impurities are adsorbed on the Stationary Phase at the beginning of the run and then elute from the column as the gradient develops.



## Peak's Origin: Contamination accumulated on pump's in-line filter



SampleName Mobile phase QC Date Acquired 03/01/02 10:32:07 Acq Method Set [REDACTED]



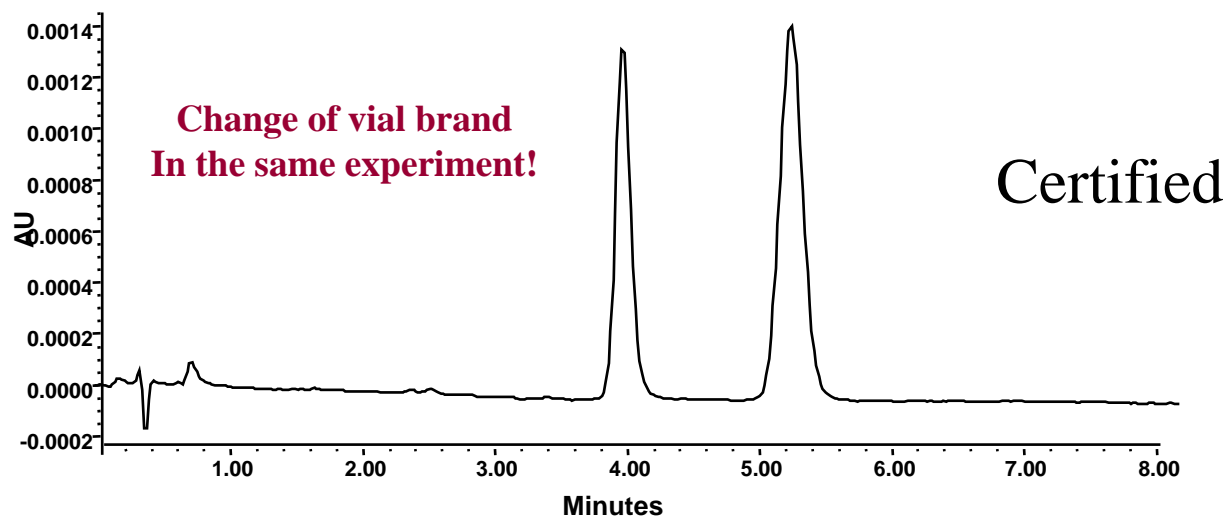
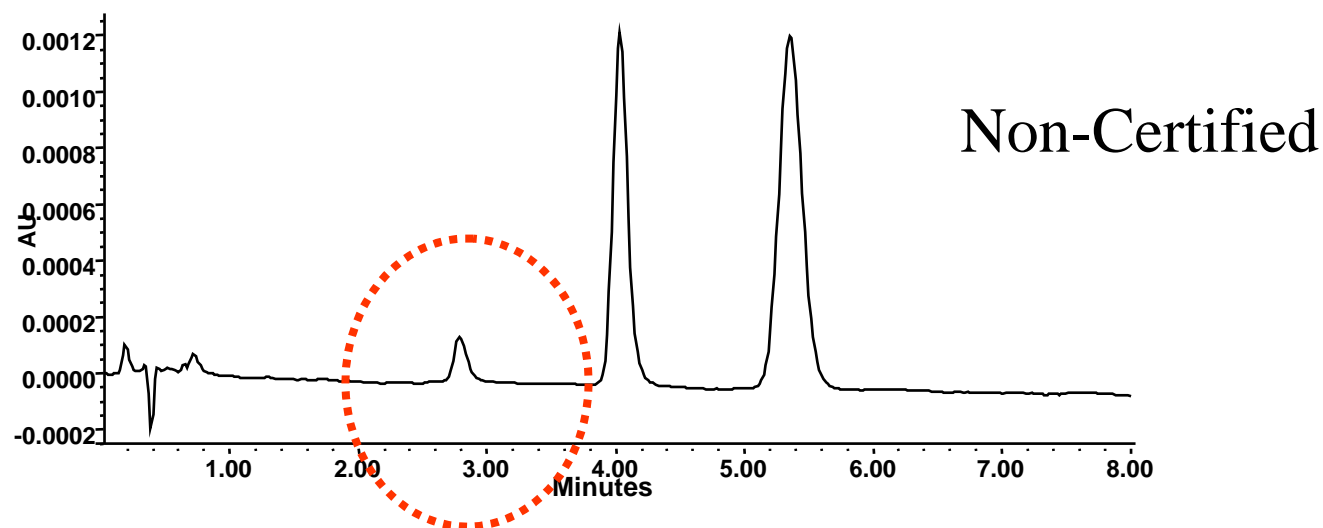
SampleName Mobile phase QC Date Acquired 03/01/02 10:32:07 Acq Method [REDACTED]

# Contaminations

Origin – Outside the Sample

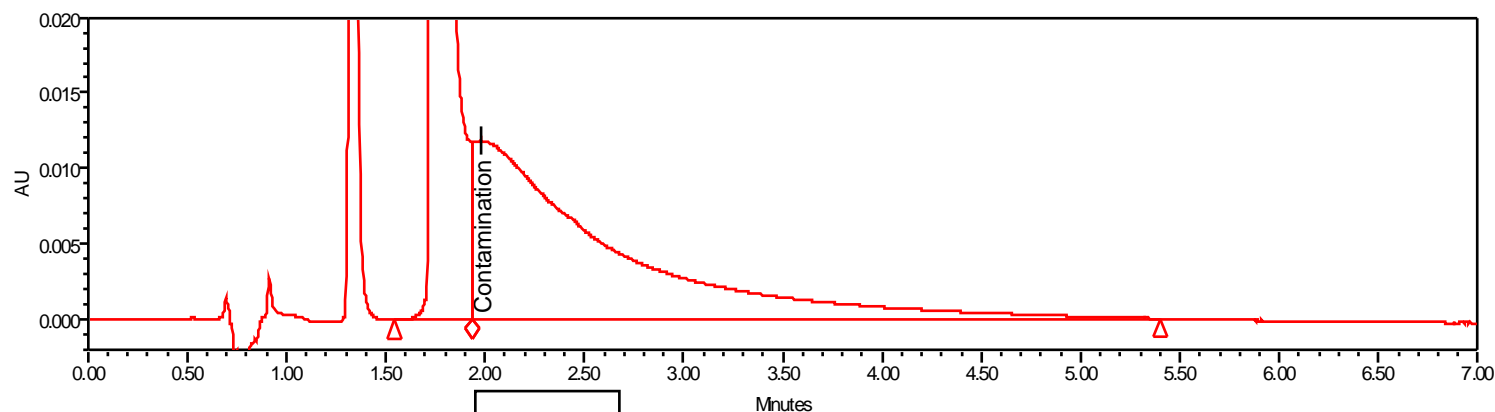
- ☐ Non-pure solvents
- ☐ Vials' leachables
- ☐ Reservoir's leachables

# Contamination from Vials' Surfaces

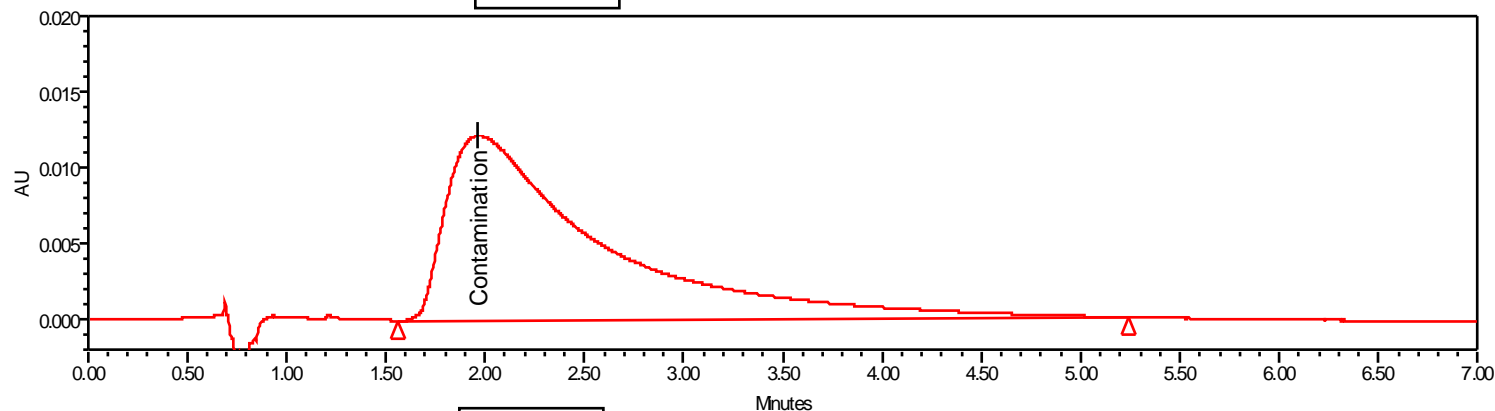




## Contamination is developed during the experiment in complex Diluents (Sample's Solvent)



— SampleName: Sample; Vial: 1:F,1; Injection: 1; Name: Contamination



— SampleName: Diluent; Vial: 1:E,2; Injection: 1; Name: Contamination

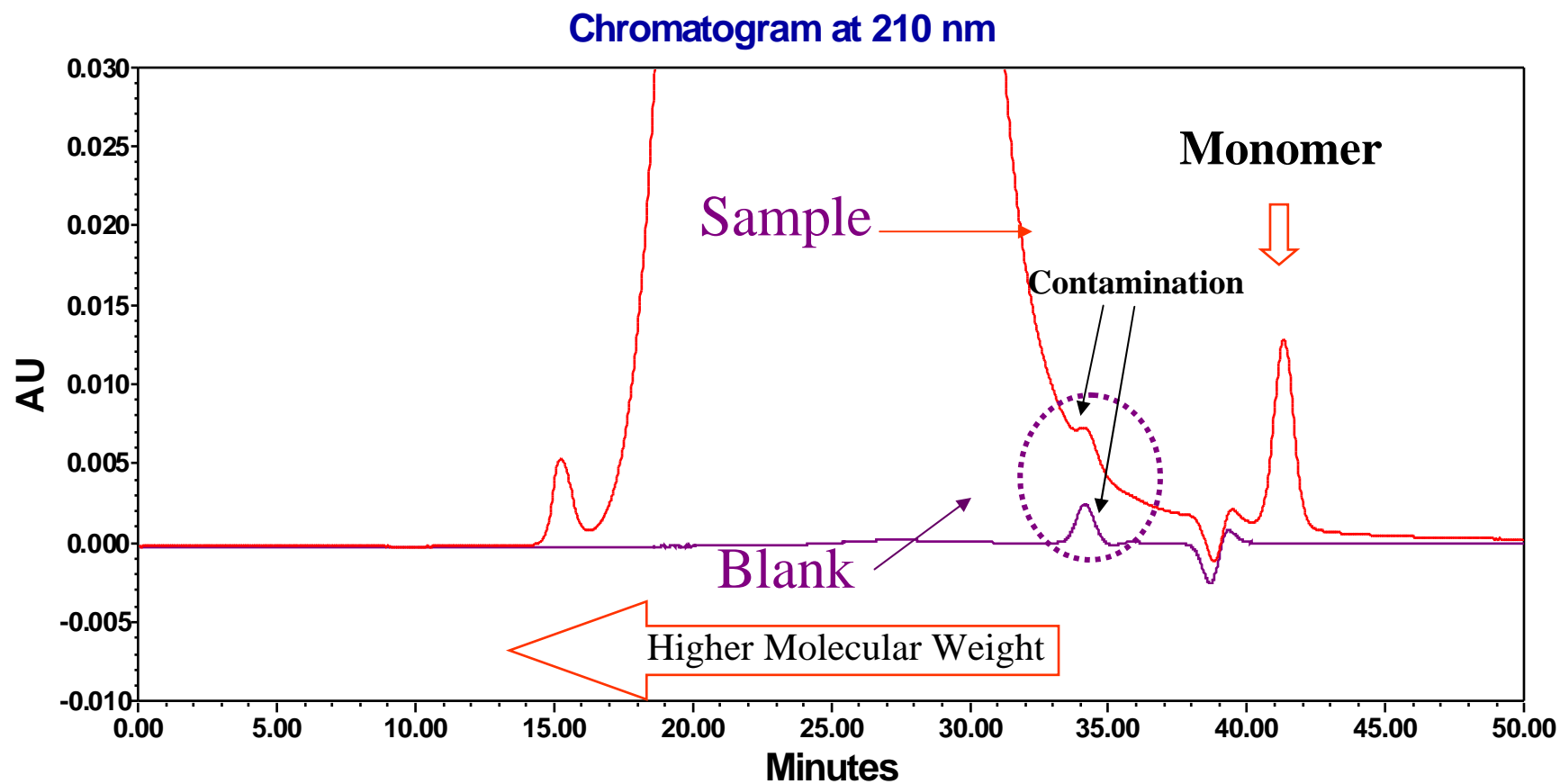
# Contaminations

Origin – Outside the Sample

- ☐ Non-pure solvents
- ☐ Vials' leachables
- ☐ Reservoir or Column's leachables

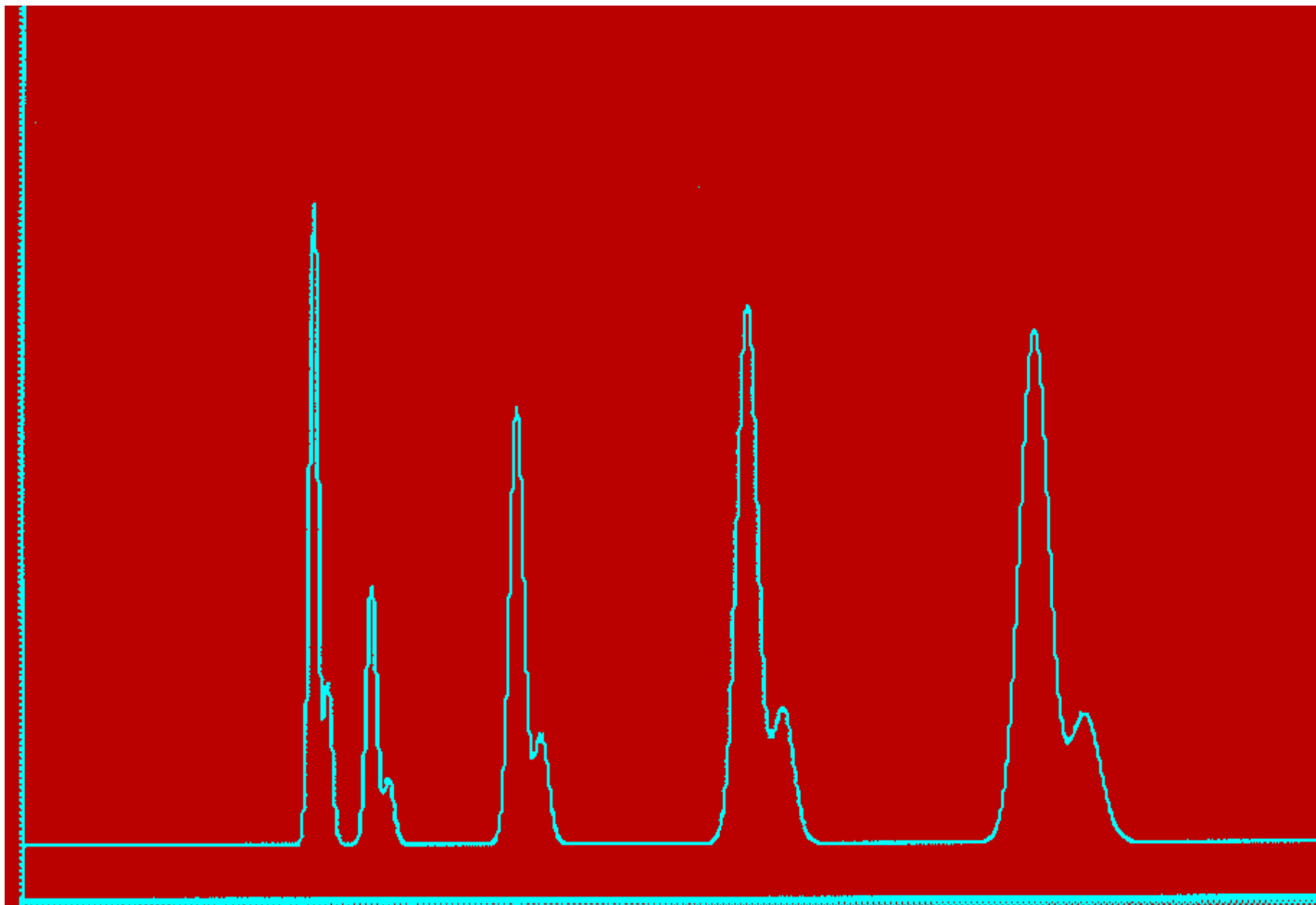
# Contamination Peaks in Size Exclusion Chromatography (SEC)

Contamination is NOT a monomer!



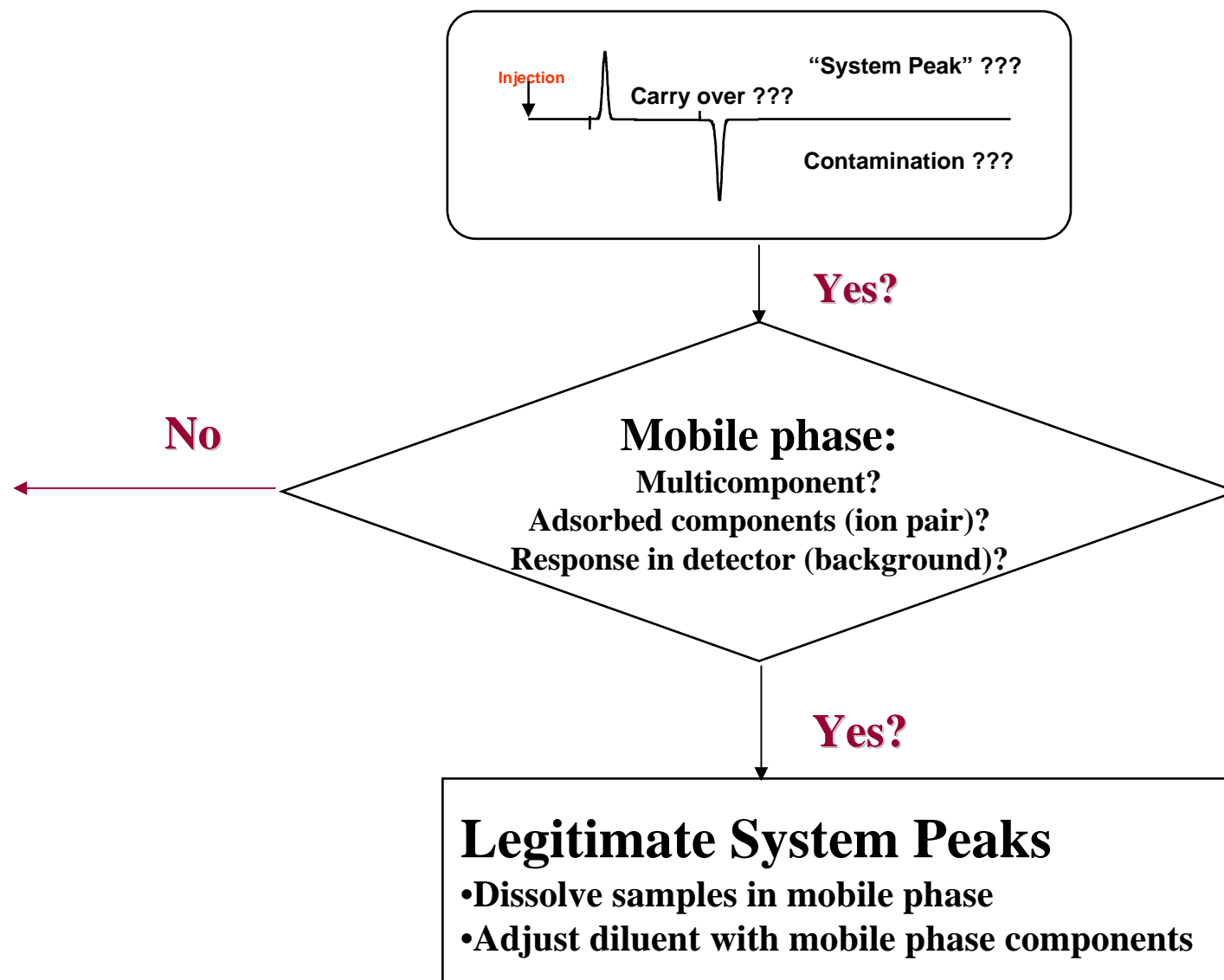
# **Other Reasons for Extraneous Peaks**

# Column's Packing Collapse

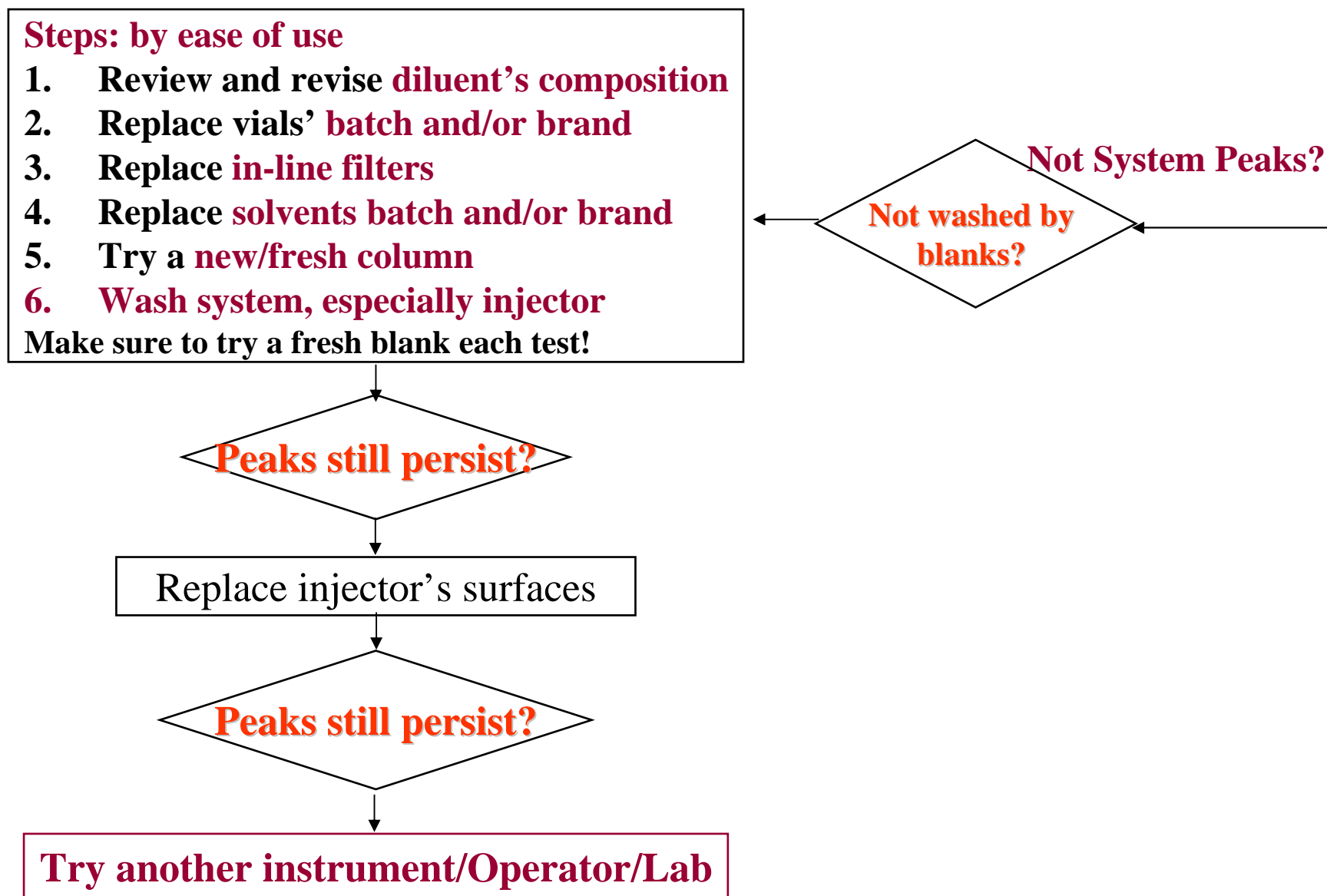


All Peaks are split

# Suggested Flow-Chart for Troubleshooting In a Regulated Environment (no change in method!)



## Suggested Flow-Chart for Troubleshooting Contd.



**Many times the extraneous  
peak disappears on its own and  
remains an unsolved  
mystery...**