Challenges in Analytical Development: The Need in Orthogonal Chromatographic Methods for a Small Molecule Project in Innovative Drug Development

Vladimir Ioffe, Ph. D.
Analytical Development

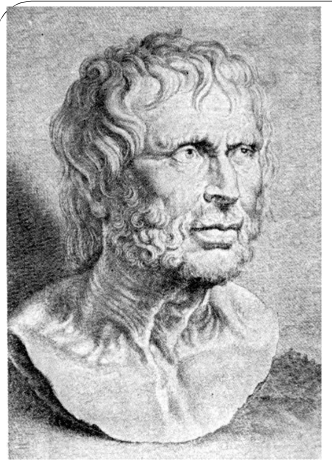
…Sometimes "simple" analytical objects are the most challenging in the development of reliable chromatographic procedures…

The aim of this workshop

The aim of this workshop is to provide an example of a mindset of the developer of HPLC methods and to explain the need in "orthogonal" complementary chromatographic procedures and the use of diverse detection techniques to ensure the complex coverage of all the possible characteristics of a pharmaceutical, biologically active material.

THERE IS NO SUBSTITUTE FOR HARD WORK

Thomas Edison



Longum iter est per praecepta, breve et efficax per exempla

(The journey is long through advice, but fast and efficient through examples)

Seneca

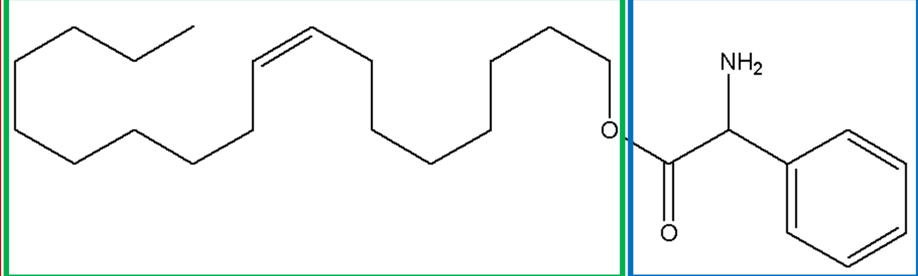
The Key Idea of Creativity in Analytical Development

- We are all so “compressed” being engaged in the production of data and firefighting that sometimes we don’t see, that there are better ways to do things.
- Creative thinking is really just making connections between what is already known.
- Most creative people feel guilty when asked where their ideas come from, because the vast majority are observations of what already exists, but connected differently.

Part I: Starting with Classics

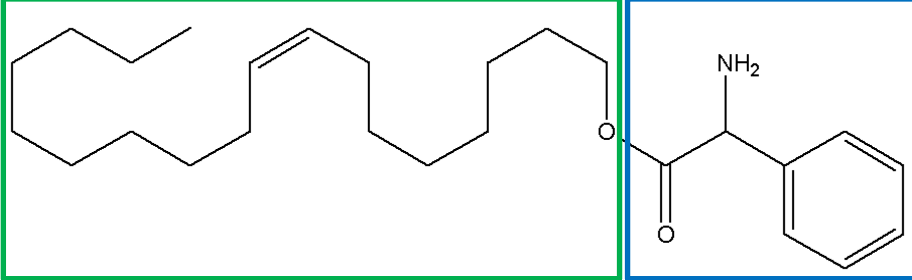
Conventional Reverse Phase Chromatography:
for Main Analyte and Closely Related Compounds

The molecule – our object



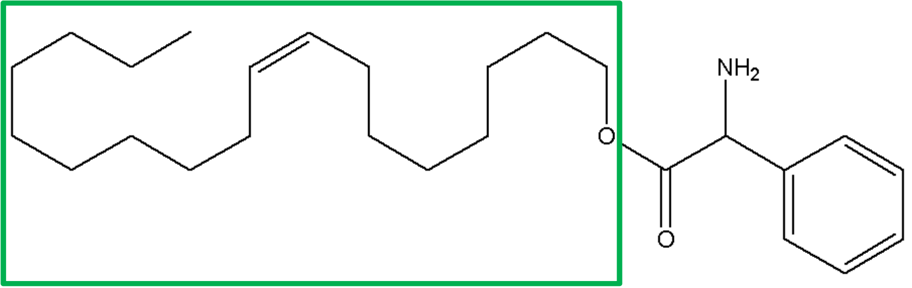
- The molecule of **TV-3813** is comparatively simple: it is an **Oleyl Ester of Phenylglycine**
- It consists of two main parts: **Phenylglycine** and **Oleyl Alcohol**

The molecule – our object

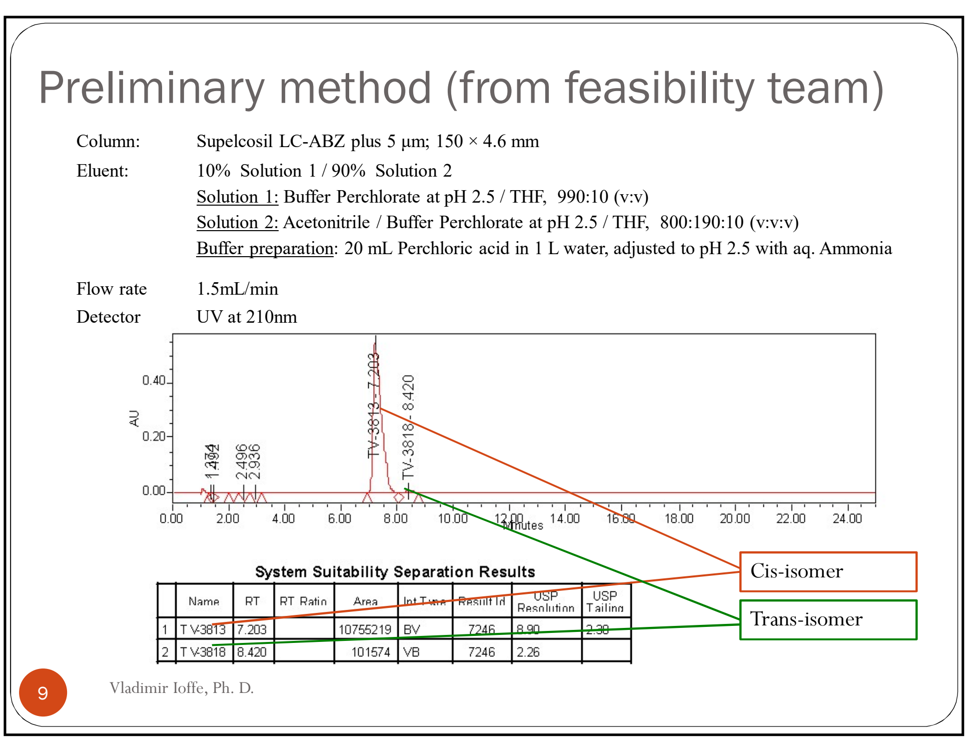


- In its manufacturing, **TV-3813** is also arranged from these two parts, therefore, **Phenylglycine** and **Oleyl Alcohol** are its main process impurities
- And the same two molecules are also the main potential degradation products

The molecule – our object



- Most common impurities of **TV-3813** originate from the impurities of the starting material – **Oleyl Alcohol**
- Those may be: **trans-isomer** (**Elaidyl alcohol**), **saturated** (**Stearyl alcohol**) or various **homologues**



Preliminary method (from feasibility team)

Column: Supelcosil LC-ABZ plus 5 µm; 150 × 4.6 mm

Eluent: 10% Solution 1 / 90% Solution 2

Solution 1: Buffer Perchlorate at pH 2.5 / THF, 990:10 (v:v)

Solution 2: Acetonitrile / Buffer Perchlorate at pH 2.5 / THF, 800:190:10 (v:v:v)

Buffer preparation: 20 mL Perchloric acid in 1 L water, adjusted to pH 2.5 with aq. Ammonia

Flow rate 1.5mL/min

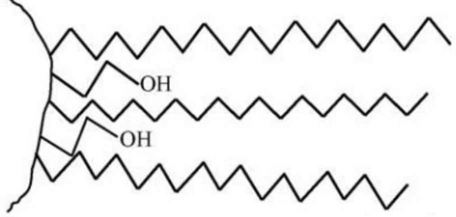
Detector UV at 210nm

System Suitability Separation Results

	Name	RT	RT Ratio	Area	Int Type	Result Id	USP Resolution	USP Tailing
1	T V-3813	7.203		10755219	BV	7246	8.90	2.30
2	T V-3818	8.420		101574	VB	7246	2.26	

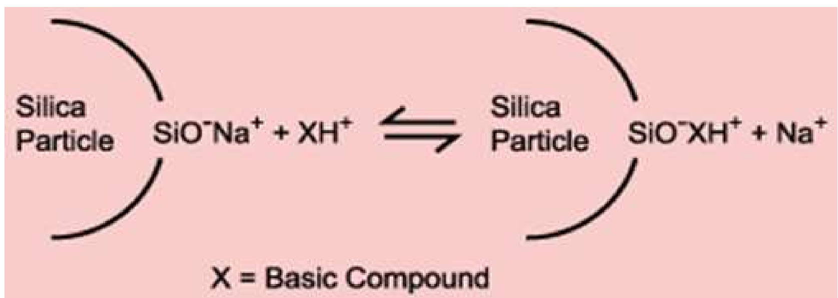
Preliminary method (from feasibility team)

- Method advantages – the use of stationary phase with **imbedded polar group** and use of the **low pH buffer**
- A common cause of peak tailing in reversed phase HPLC is the **secondary retention** that occurs when an **ion-exchange interaction** takes place between a positively charged solute (amine) and an acidic silanol on the surface of silica stationary phase support particles:
- Acidic silanols on the surface of silica stationary phase supports can form ion-exchange sites that interact with basic compounds



Preliminary method (from feasibility team)

- This **ion-exchange interaction** will often contribute to peak retention (secondary retention) and cause peak tailing when separating amines by reversed phase HPLC



- Operating at a **pH below 3** protonates silanol groups on the silica stationary phase support (pKa of silanol is ~ 3.5) and thereby makes the silanols less available for interacting with solutes

Preliminary method (from feasibility team)

- To solve the problem of interaction with residual silanols, a **new type of stationary phase** was developed, **with polar groups, such as amides or carbamates, "embedded" in the bonded phase**
  - These **"polar embedded" phases** provide polar selectivity without the poor chromatographic performance associated with stationary phases that have high silanol activity
  - The amide or carbamate group shields the silica surface and prevents solutes from directly interacting with silanol groups
  - The effect is similar to adding an amine modifier to the mobile phase, thus, there is no need of adding "ion-pair reagents" when developing separations on the **phases with embedded polar groups**

Preliminary method (from feasibility team)

- Method drawback – **Perchlorate buffer**:
  - There is no reason of using an **ion-pair reagent** with the RP column having an **imbedded polar group**
  - Use of **non-volatile buffer** for a method to be run within an innovative project: it is non-compatible with LC-MS for identification of unknown impurities

Column: **Supelcosil LC-ABZ plus** 5 µm, 150 × 4.6 mm

Eluent: 10% Solution 1 / 90% Solution 2

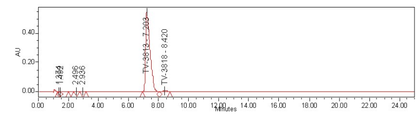
Solution 1: **Buffer Perchlorate** at pH 2.5 / THF, 990:10 (v:v)

Solution 2: Acetonitrile / **Buffer Perchlorate** at pH 2.5 / THF, 800:190:10 (v:v:v)

Buffer preparation: 20 mL Perchloric acid in 1 L water, adjusted to pH 2.5 with aq. Ammonia

Flow rate 1.5 mL/min

Detector UV at 210nm



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Our developed method: First version

Column: Symmetry Shield C18 5 µm; 250 × 4.6 mm

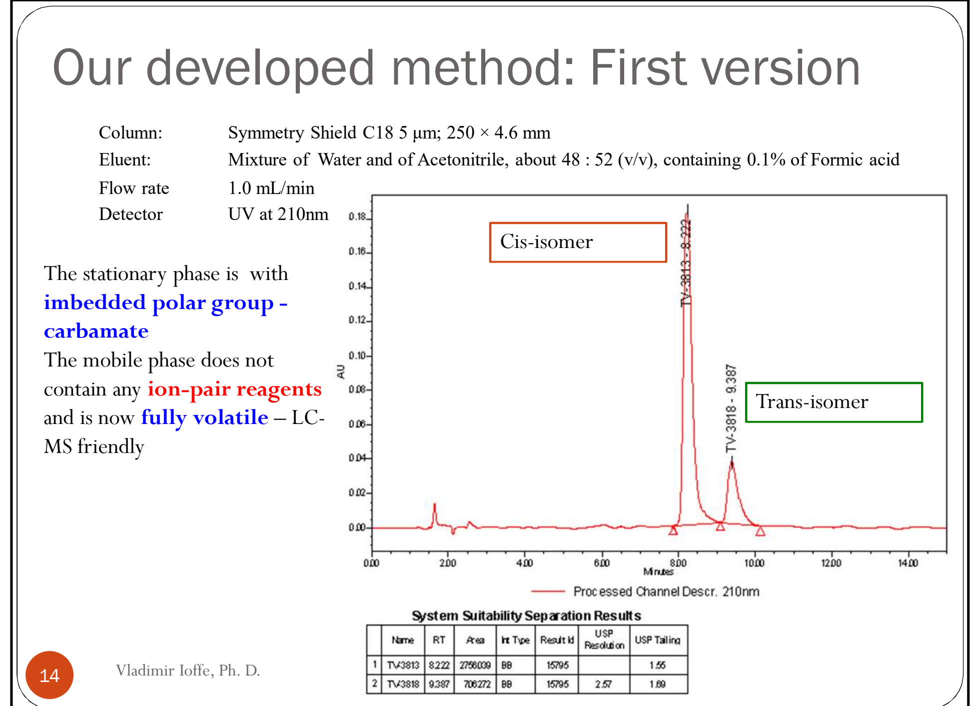
Eluent: Mixture of Water and of Acetonitrile, about 48 : 52 (v/v), containing 0.1% of Formic acid

Flow rate 1.0 mL/min

Detector UV at 210nm

The stationary phase is with **imbedded polar group - carbamate**

The mobile phase does not contain any **ion-pair reagents** and is now **fully volatile** – LC-MS friendly



System Suitability Separation Results

	Name	RT	Area	Int Type	Result Id	USP Resolution	USP Tailing
1	TV-3813	8.222	2756039	BB	15795		1.56
2	TV-3818	9.387	706272	BB	15795	2.57	1.69

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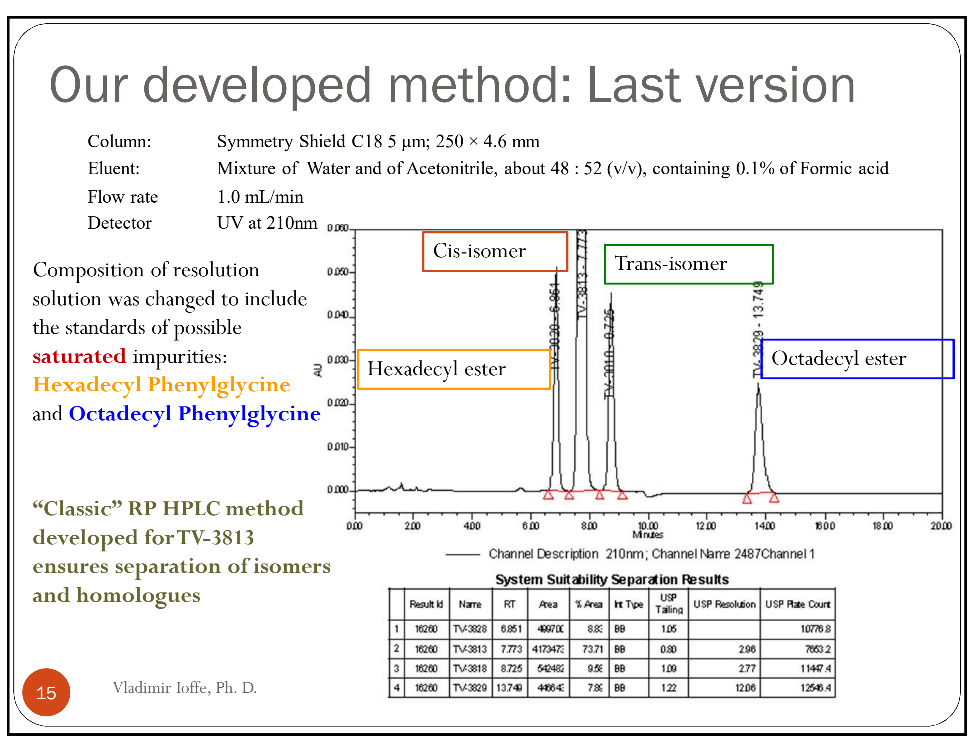
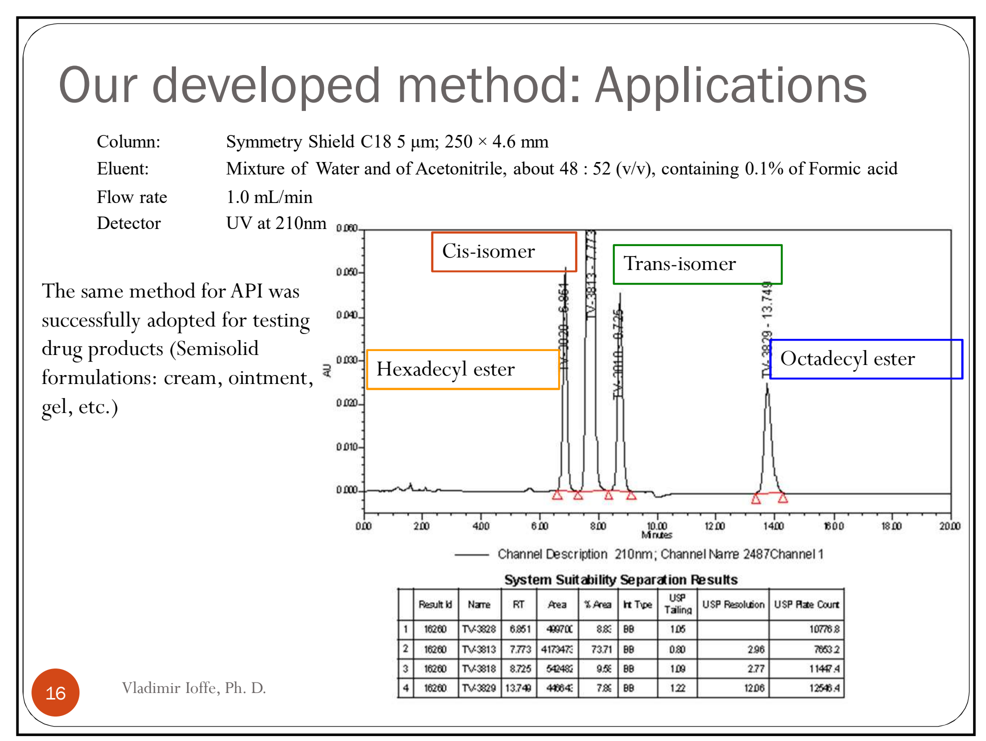


	Result Id	Name	RT	Area	% Area	Int Type	USP Tailing	USP Resolution	USP Plate Count
1	16260	TV-3828	6.851	49970	8.8	BB	1.05		10776.8
2	16260	TV-3813	7.773	417347	73.71	BB	0.80	2.96	7653.2
3	16260	TV-3818	8.725	54248	9.5	BB	1.09	2.77	11447.4
4	16260	TV-3829	13.749	44664	7.8	BB	1.22	12.06	12546.4



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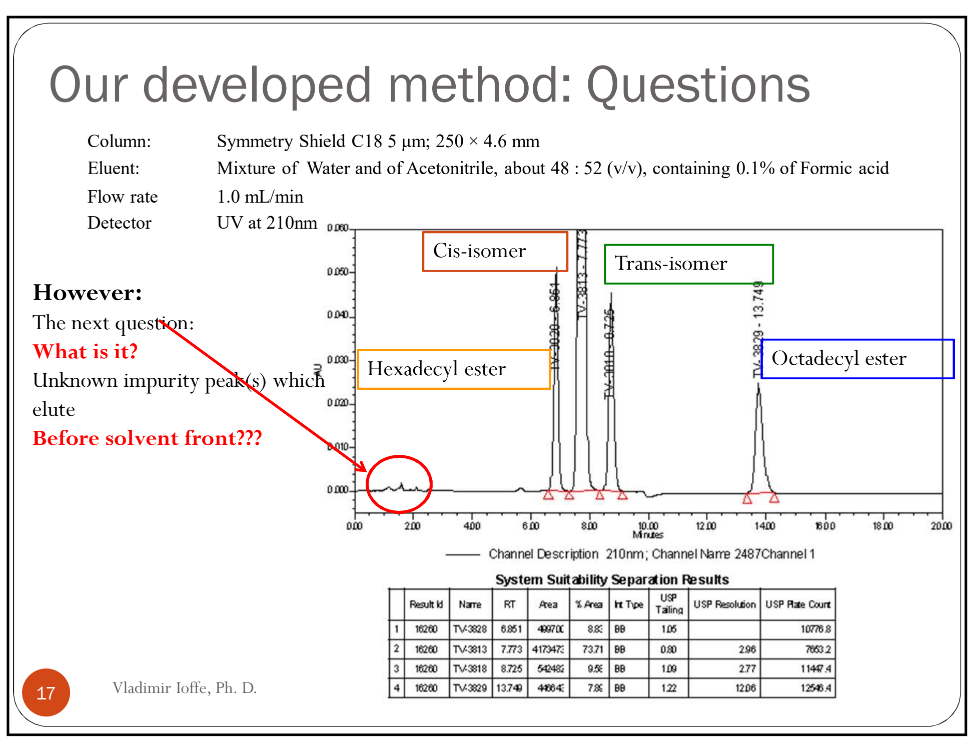

System Suitability Separation Results

	Result Id	Name	RT	Area	% Area	Int Type	USP Tailing	USP Resolution	USP Plate Count
1	16260	TV-3828	6.851	49970[illegible]	8.8[illegible]	BB	1.05		10776.8
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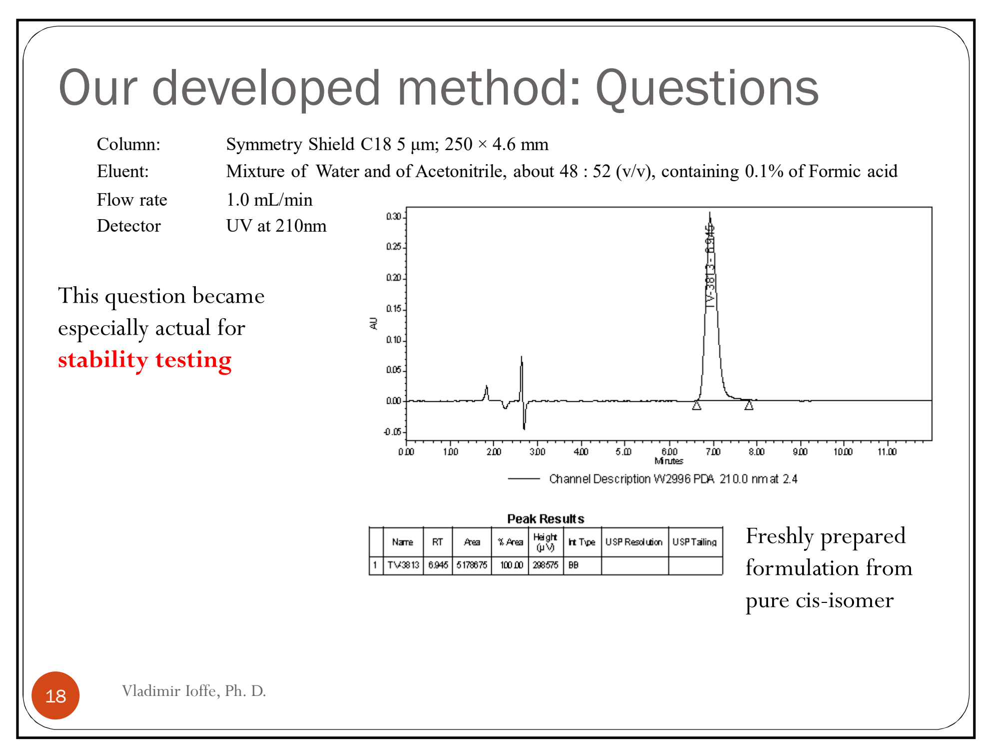
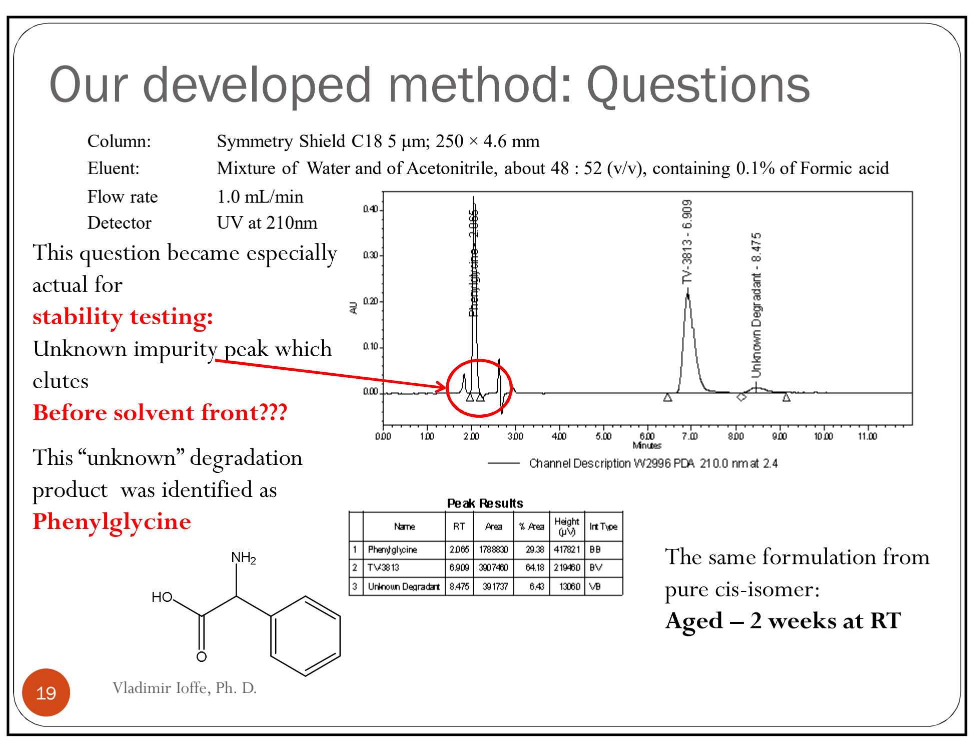
3	16260	TV-3818	8.725	54248[illegible]	9.5[illegible]	BB	1.09	2.77	11447.4
4	16260	TV-3829	13.749	44664[illegible]	7.8[illegible]	BB	1.22	12.06	12546.4



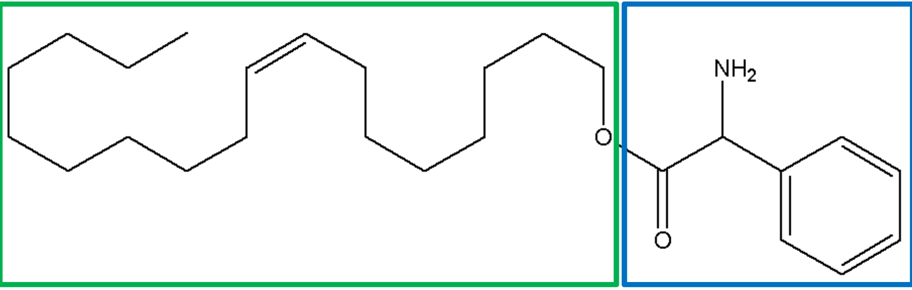
Peak Results

	Name	RT	Area	% Area	Height (µV)	Int Type	USP Resolution	USP Tailing
1	TV-3813	6.945	5178675	100.00	298575	BB		



Peak Results

	Name	RT	Area	% Area	Height (µV)	Int Type
1	Phenylglycine	2.065	1788830	29.38	417821	BB
2	TV-3813	6.909	3907460	64.18	219460	BV
3	Unknown Degradant	8.475	391737	6.43	13060	VB



- In its manufacturing, **TV-3813** is arranged from these two parts, therefore, **Phenylglycine** and **Oleyl Alcohol** are the **starting materials**, but – not only:
- They are **main process impurities**
- And the same two molecules are also the **main potential degradation products**

TV-3813 – Analytical Methods – Next Challenges

Main challenges for further development:

- **Extremely different polarity and lipophilicity of TV-3813 and its main degradation products**
- **Absence of chromophore for Oleyl Alcohol – impossible to use UV-detection**

TV-3813 – Analytical Methods – Next Challenges

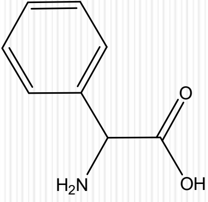
- Within chromatography, it is often advisable to use a combination of columns with different selectivities, that is, to apply **orthogonal methods**
- **"Orthogonal"** means relying on significantly differing separation mechanisms (such as: **RP LC** [various modes], hydrophilic interaction chromatography [**HILIC**], **CE**, etc.) to minimize the chances of missing impurities which may potentially coelute, elute without retention, or not elute at all
- Based on the estimated properties of potential impurities, a choice can be made for an array of separation and detection techniques

TV-3813 – Analytical Methods – Next Challenges

- Another way of finding all the impurities in a sample is to combine a separation technique with a **variety of detectors**
- Each type of detector can highlight different types of compounds because the detector response depends on the chemical structure of the compound:
  - **Does it have a UV chromophore?**
  - **Does it exhibit good ionization in an MS probe?**
  - **Does it show good conductivity?**
- **The answers to these questions point to different detectors**

Part II: Challenges of High Polarity

Mixed-Mode Chromatography:
for Polar Impurities – and Polar Starting Materials



A Complementary Method for Polar Impurities

- A need in complementary "orthogonal" HPLC technique: difficulties with retention of very polar compounds in a "classic" reverse phase chromatography
- What can be **"orthogonal"** and **complementary** to **Reverse Phase HPLC**?
  - **Normal Phase Chromatography?** – Maybe, but requires different sample preparation, in non-aqueous diluent, which evokes issues of recovery
  - **HILIC?** – Was not yet familiar at the time of development and is problematic from the point of view of "full control"
  - Emerging technology of **"Mixed Mode" Chromatography**

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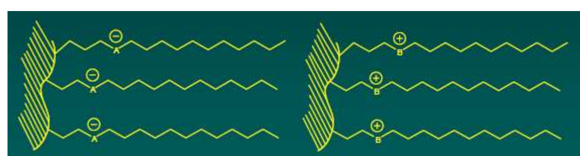
Mixed-Mode Chromatography as an Option

- **Mixed-Mode chromatography:**
  - Ensures retention of polar compounds in reverse phase system
  - Improves shape of early eluted peaks and strong bases
  - Allows replacement of complicated gradient methods for compounds of different polarity with a complementary isocratic method
- Mixed-Mode chromatography combines two (or more) retention mechanisms in one column



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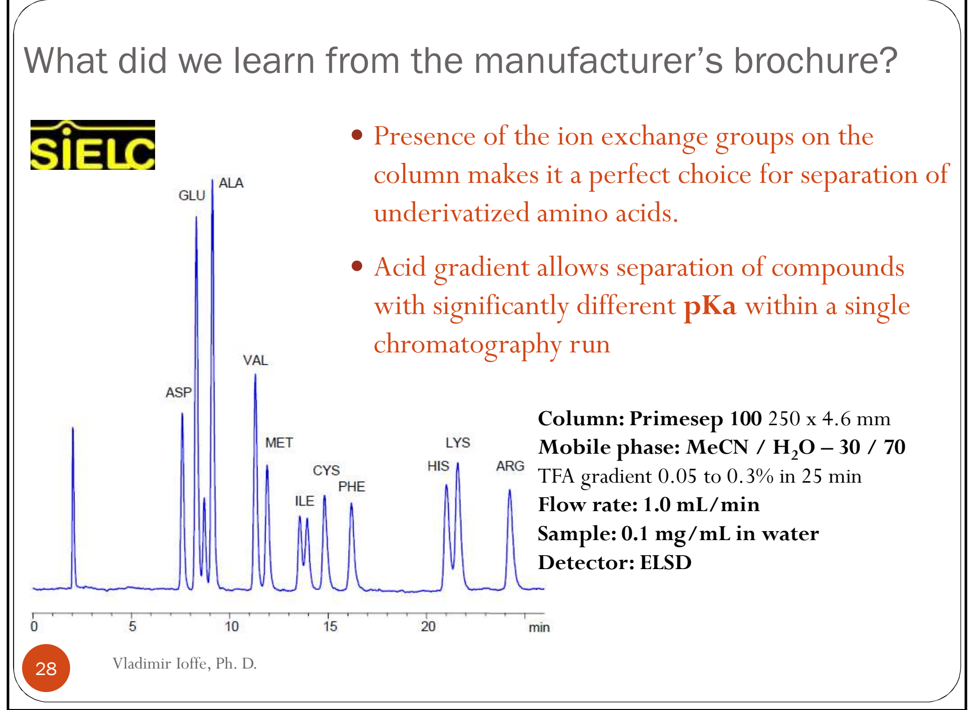
Mixed-Mode Chromatography as an Option

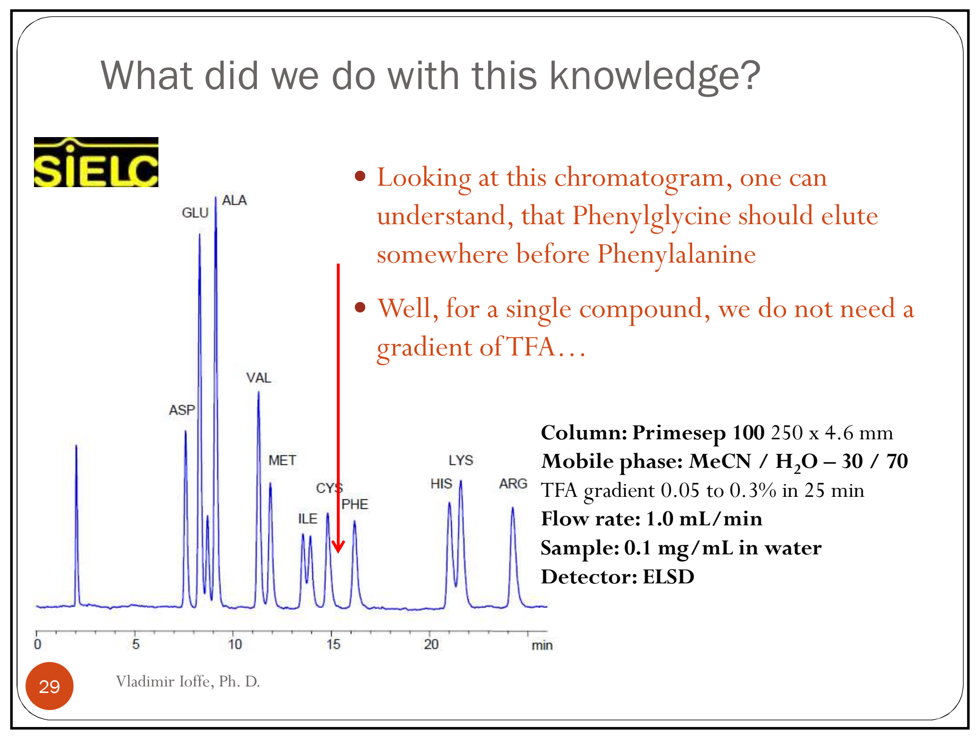
- **Primesep™ columns** (SIELC) for mixed-mode chromatography combine two independent modes of retention:
  - **Controlled ion-exchange sites to interact with ionic species of the analyte**
  - **Hydrophobic chains of the stationary phase to interact with hydrophobic "portion" of the analyte**

What did we learn from the manufacturer's brochure?



- Presence of the ion exchange groups on the column makes it a perfect choice for separation of underivatized amino acids.
- Acid gradient allows separation of compounds with significantly different **pKa** within a single chromatography run

Column: Primesep 100 250 x 4.6 mm
Mobile phase: MeCN / H_2O – 30 / 70
TFA gradient 0.05 to 0.3% in 25 min
Flow rate: 1.0 mL/min
Sample: 0.1 mg/mL in water
Detector: ELSD


What did we do with this knowledge?
SIELC
Looking at this chromatogram, one can understand, that Phenylglycine should elute somewhere before Phenylalanine
Well, for a single compound, we do not need a gradient of TFA…
ALA
GLU
VAL
ASP
MET
CYS
PHE
ILE
LYS
HIS
ARG
0
5
10
15
20
min
Column: Primesep 100 250 x 4.6 mm
Mobile phase: MeCN / H_2O – 30 / 70
TFA gradient 0.05 to 0.3% in 25 min
Flow rate: 1.0 mL/min
Sample: 0.1 mg/mL in water
Detector: ELSD
29
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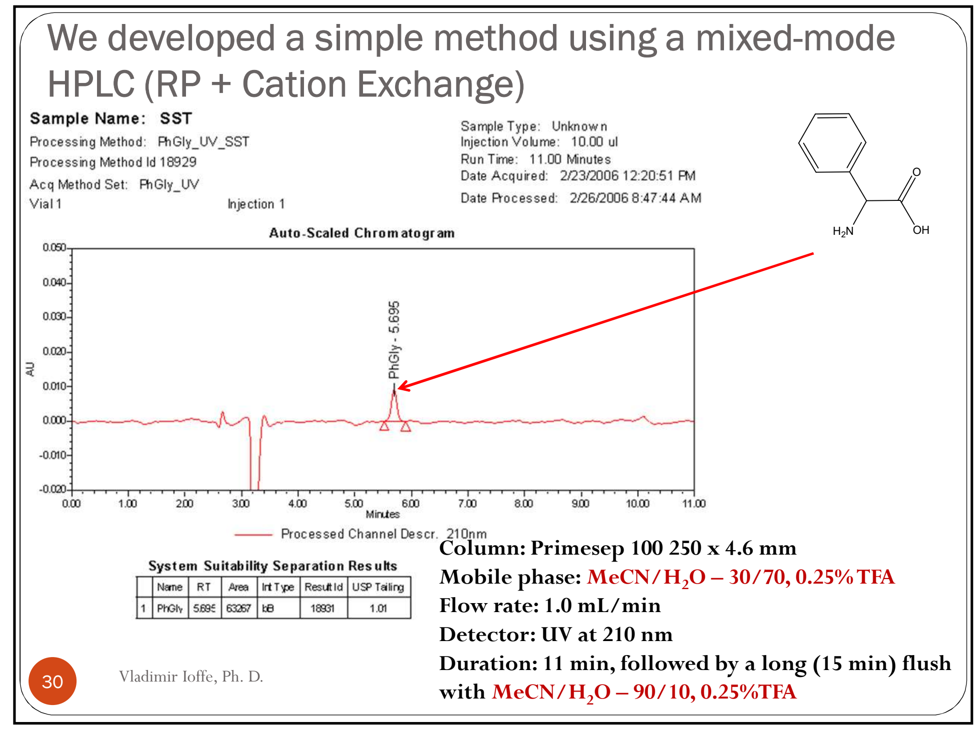
We developed a simple method using a mixed-mode HPLC (RP + Cation Exchange)
Sample Name: SST
Processing Method: PhGly_UV_SST
Processing Method Id 18929
Acq Method Set: PhGly_UV
Vial 1
Injection 1
Sample Type: Unknown
Injection Volume: 10.00 ul
Run Time: 11.00 Minutes
Date Acquired: 2/23/2006 12:20:51 PM
Date Processed: 2/26/2006 8:47:44 AM
Auto-Scaled Chromatogram
AU
PhGly - 5.695
Minutes
Processed Channel Descr. 210nm
H_2N
OH
O
System Suitability Separation Results
Name | RT | Area | Int Type | Result Id | USP Tailing
1 | PhGly | 5.695 | 63267 | bB | 18931 | 1.01
Column: Primesep 100 250 x 4.6 mm
Mobile phase: MeCN/H_2O – 30/70, 0.25% TFA
Flow rate: 1.0 mL/min
Detector: UV at 210 nm
Duration: 11 min, followed by a long (15 min) flush with MeCN/H_2O – 90/10, 0.25%TFA
30
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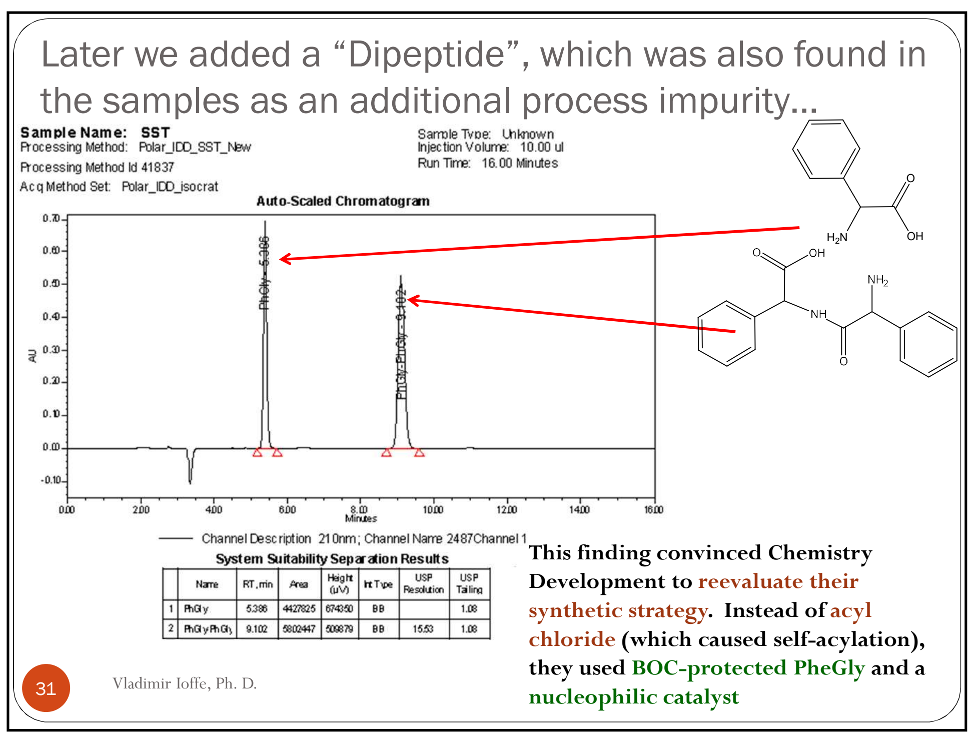
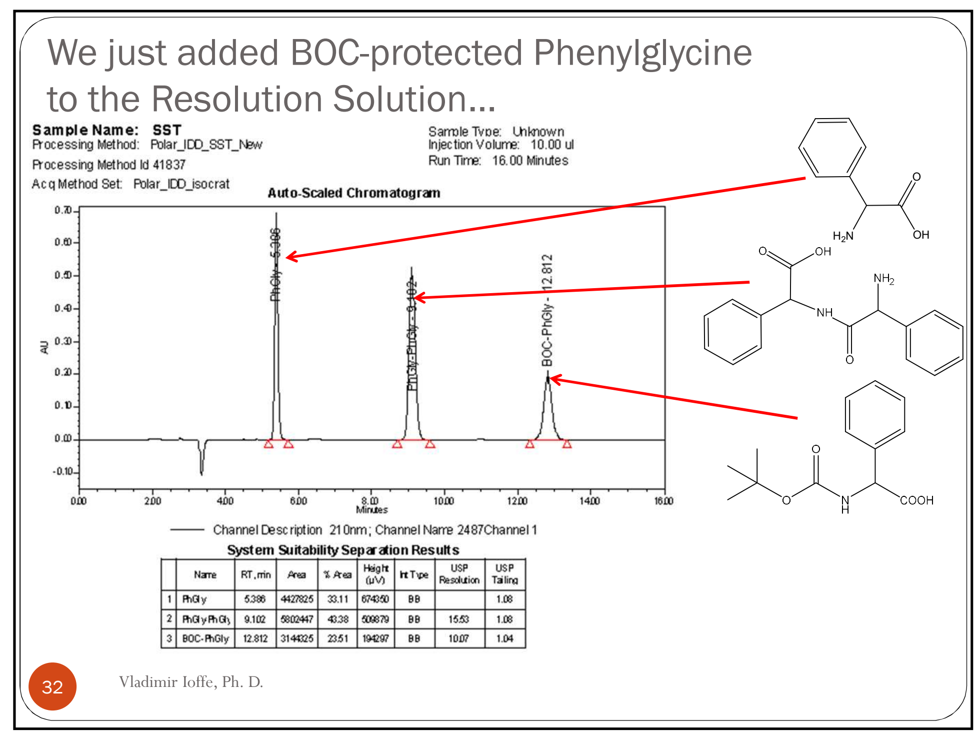


	Name	RT, min	Area	Height (µV)	Int Type	USP Resolution	USP Tailing
1	PhGly	5.386	4427825	674350	BB		1.08
2	PhGly-PhGly	9.102	5802447	509879	BB	15.53	1.08



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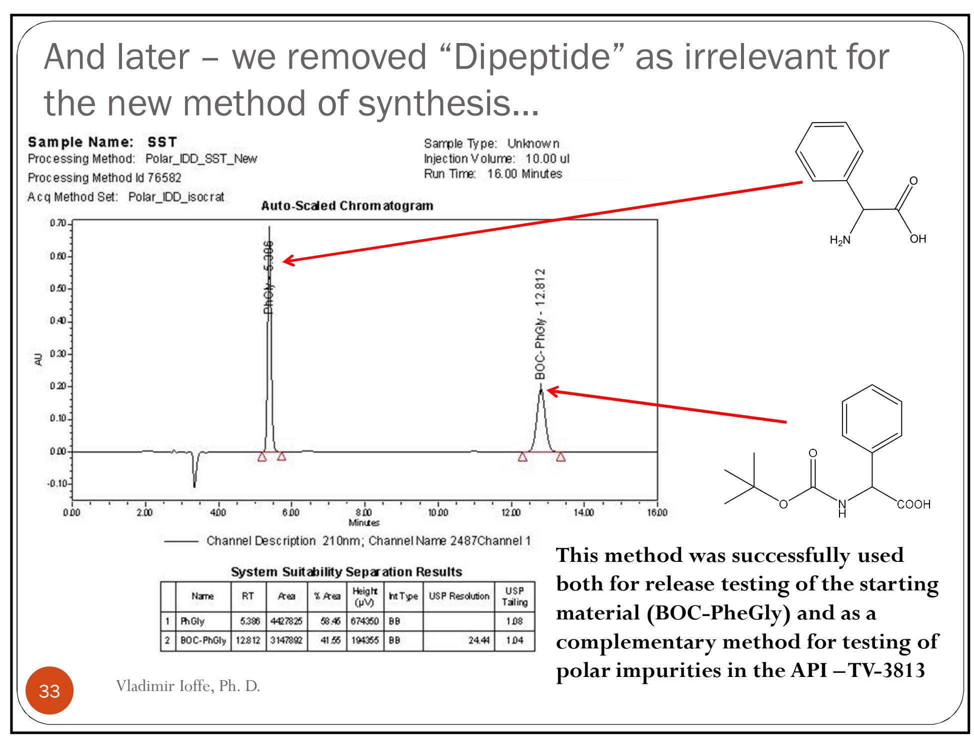
2	PhGly-PhGly	9.102	5802447	43.38	509879	BB	15.53	1.08
3	BOC-PhGly	12.812	3144325	23.51	194297	BB	10.07	1.04



System Suitability Separation Results

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2	BOC-PhGly	12.812	3147892	41.55	194355	BB	24.44	1.04

Summary

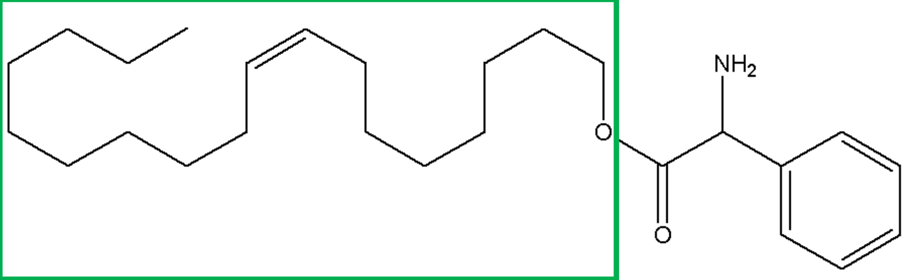
- Very simple, robust and **user-friendly** methods were developed for very polar analytes using a novel type of stationary phase:
  - A mixed mode phase, combining at least two different retention mechanisms: **Reversed Phase** and **Ion Exchange** (or **Ion Substitution**)
  - A good reason to learn something new about modern stationary phases – and how to deal with a combination of complementary retention mechanisms, smartly merged in one column
- **At the end – a facile analytical technique**

Part III: Challenges of High Lipophilicity and Lack of Chromophore

RP HPLC Using Non-Silica Based Columns
and Evaporative Detectors

OH

The molecule – our object (remember?)



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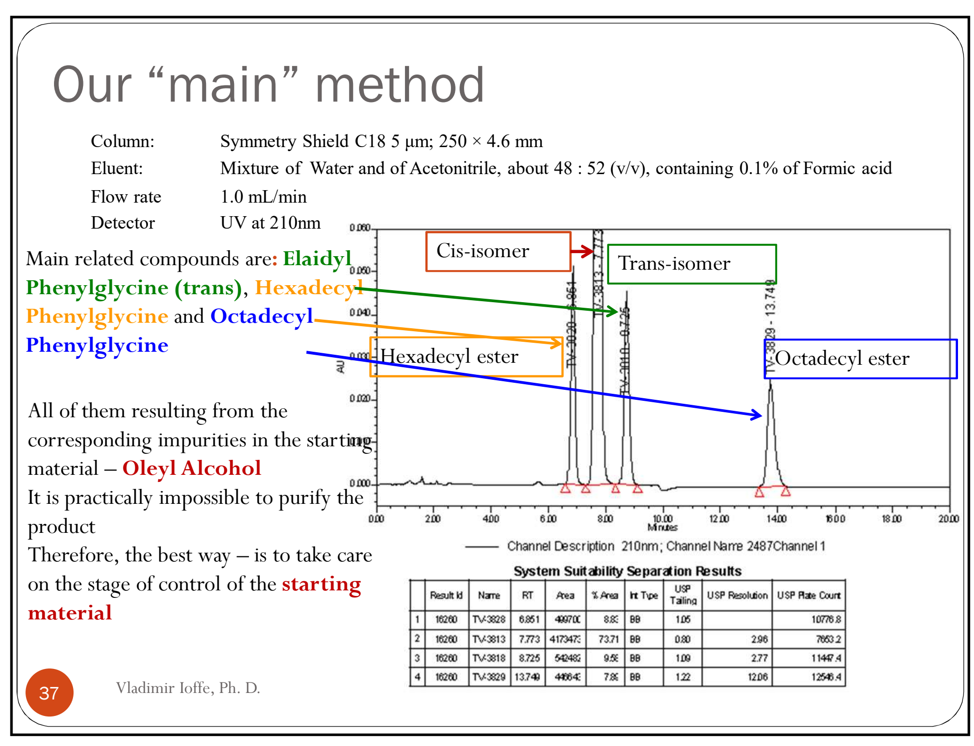
- Those may be: **trans-isomer** (**Elaidyl alcohol**), **saturated** (**Stearyl alcohol**) or various **homologues**



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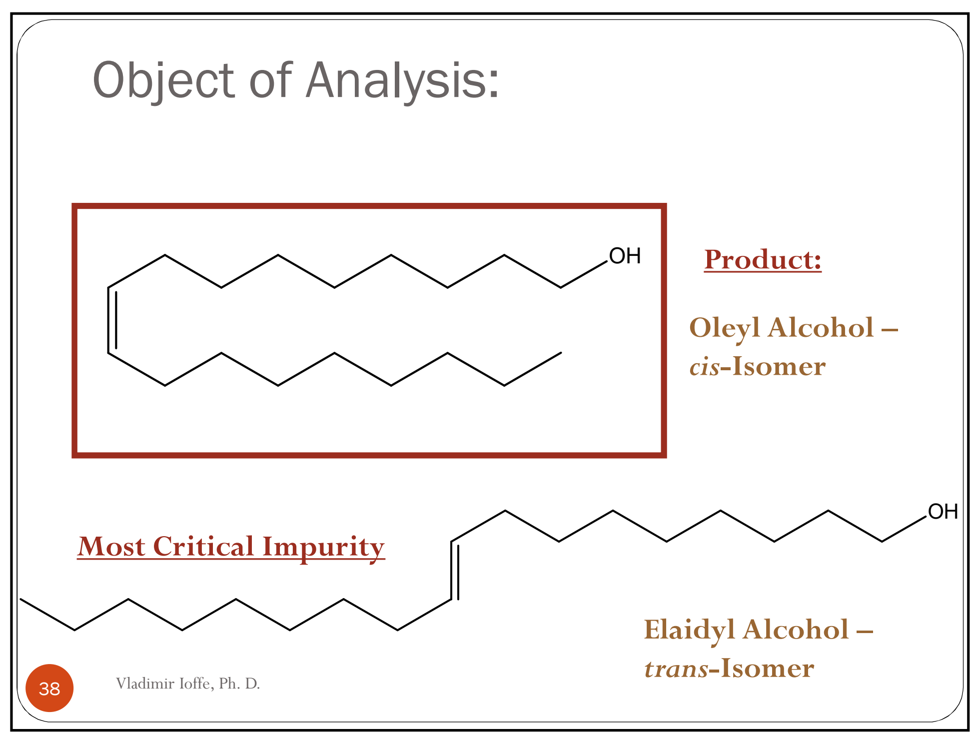
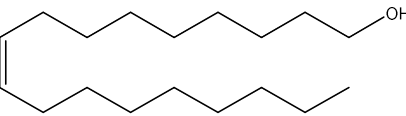


What are Our Challenges for HPLC?



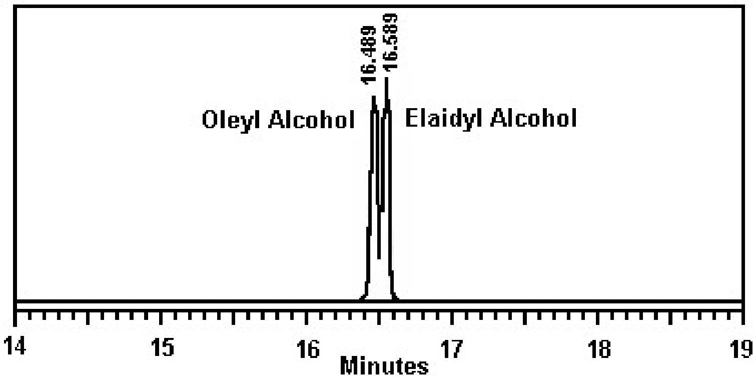
- **No** pronounced functional groups to invest into a retention mechanism…
- **No** polar functional groups to allow the use of buffers and pH-adjustments…
- **No** strong chromophore to facilitate detection in UV…
- **No** distinct differences (for chromatographers) from the critical impurities…
- **Conclusion:** Very unfriendly case for a chromatographer

What do we have for a separation method?

- **What are the differences between two isomers?**
- **Isomers** have a slight difference in **boiling point…**
- **Which** provides a chance to separate them on GC…
- **They are Flammable**, which allows detection on FID…
- Attempts to develop separation method on GC lasted for about 3 weeks of intensive work, including consulting with all available specialists in Gas Chromatography, trying different types of columns…
- **So**: What did we get after such an intensive work?

What did we get in GC after 3 weeks of development?

- **GC column:** AT Silar, 0.50 µm, 30 m X 0.53 mm
- **Temperature gradient:** from 100°C to 190°C in 20 min



RT difference – only 0.1 min (at ~16.5 min)
Separation: peaks overlap at ~1/3 height

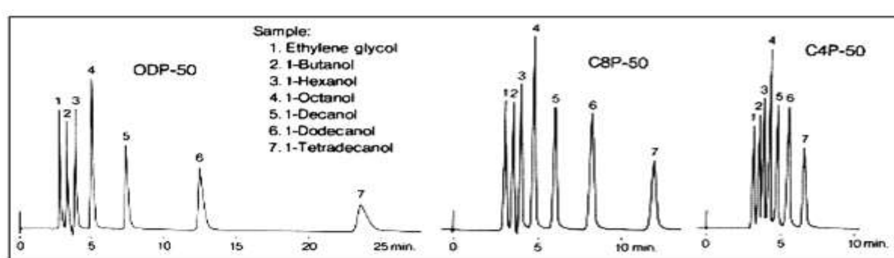
Well, what are still our options in HPLC?

- It looks like, that for such non-polar molecules, most obvious technique should be the **Normal Phase HPLC**
- However, it does not provide a reliable mechanism for retention and for separation between cis- and trans- isomers (being the only "slight" structural difference…)
- Other alternative types of LC (such as size exclusion, ion exchange / ion substitution, etc.) were also helpless
- **Can we return to an idea about RP HPLC?**

Well, what are still our options in HPLC?

- Using reverse phase HPLC can allow separation between these isomers, based on differences in hydrophobic interactions (due to steric effects for different "geometry")
- However, using silica-based columns, there is a prevailing interaction with residual silanol groups, even for highly endcapped columns which even does not allow to achieve reliable peak shape…
- Possible direction – non-silica based (polymeric?) columns. However, using the famous **Hamilton's PRP-1,** alcohols with C18 carbon chain are hardly released from it – **the column is too lipophilic for this substrate**
- **Are there less lipophilic polymer-based RP columns?**

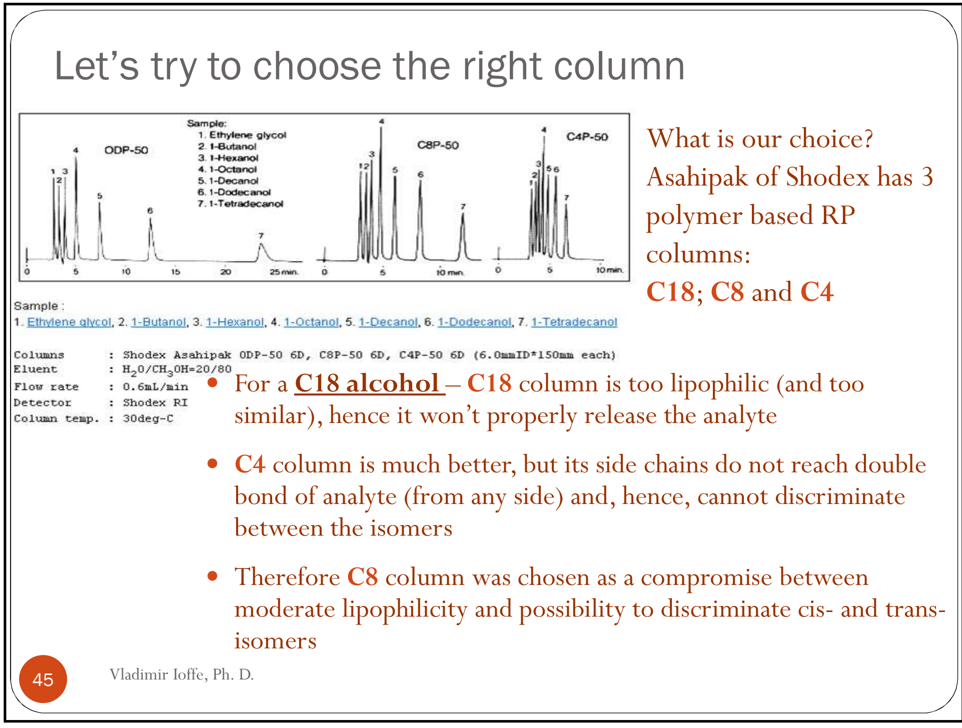
RP HPLC example found in the literature



Sample :
1. Ethylene glycol, 2. 1-Butanol, 3. 1-Hexanol, 4. 1-Octanol, 5. 1-Decanol, 6. 1-Dodecanol, 7. 1-Tetradecanol

```
Columns      : Shodex Asahipak ODP-50 6D, C8P-50 6D, C4P-50 6D (6.0mmID*150mm each)
Eluent       : H2O/CH3OH=20/80
Flow rate    : 0.6mL/min
Detector     : Shodex RI
Column temp. : 30deg-C
```

- Only one, but a very optimistic case of RP HPLC separation of aliphatic alcohols
- **The last alcohol in this example is C14**


Let's try to choose the right column
Sample:
1. Ethylene glycol
2. 1-Butanol
3. 1-Hexanol
4. 1-Octanol
5. 1-Decanol
6. 1-Dodecanol
7. 1-Tetradecanol
ODP-50
C8P-50
C4P-50
Sample :
1. Ethylene glycol, 2. 1-Butanol, 3. 1-Hexanol, 4. 1-Octanol, 5. 1-Decanol, 6. 1-Dodecanol, 7. 1-Tetradecanol
Columns : Shodex Asahipak ODP-50 6D, C8P-50 6D, C4P-50 6D (6.0mmID*150mm each)
Eluent : H2O/CH3OH=20/80
Flow rate : 0.6mL/min
Detector : Shodex RI
Column temp. : 30deg-C
What is our choice? Asahipak of Shodex has 3 polymer based RP columns:
C18; C8 and C4
For a C18 alcohol – C18 column is too lipophilic (and too similar), hence it won't properly release the analyte
C4 column is much better, but its side chains do not reach double bond of analyte (from any side) and, hence, cannot discriminate between the isomers
Therefore C8 column was chosen as a compromise between moderate lipophilicity and possibility to discriminate cis- and trans-isomers
45
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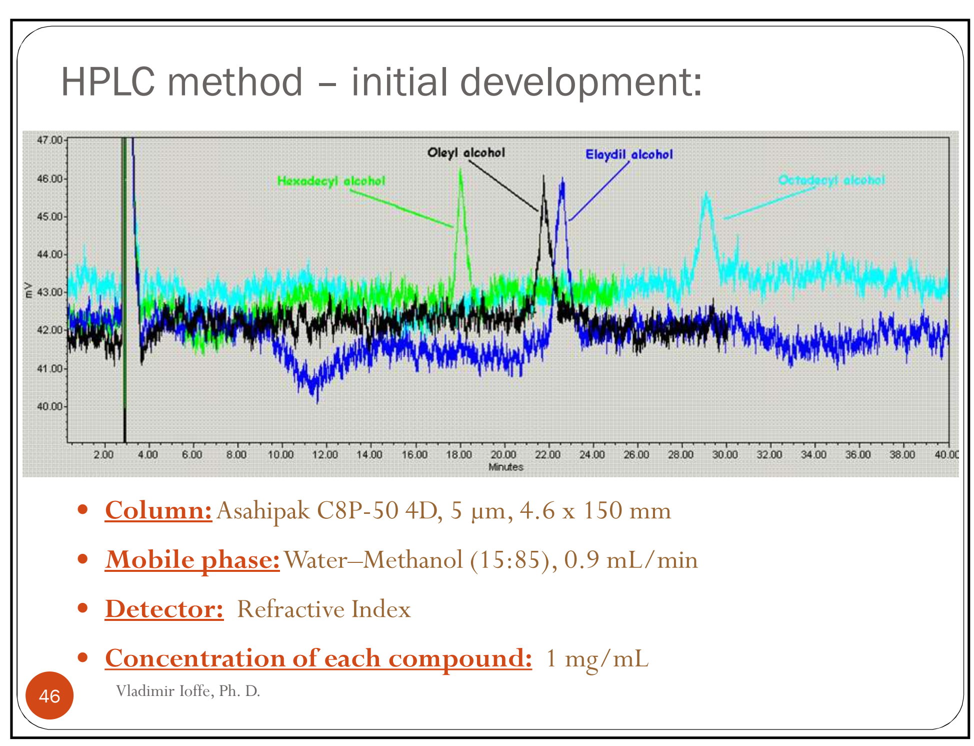
HPLC method – initial development:
Oleyl alcohol
Elaydil alcohol
Hexadecyl alcohol
Octadecyl alcohol
mV
Minutes
Column: Asahipak C8P-50 4D, 5 µm, 4.6 x 150 mm
Mobile phase: Water–Methanol (15:85), 0.9 mL/min
Detector: Refractive Index
Concentration of each compound: 1 mg/mL
46
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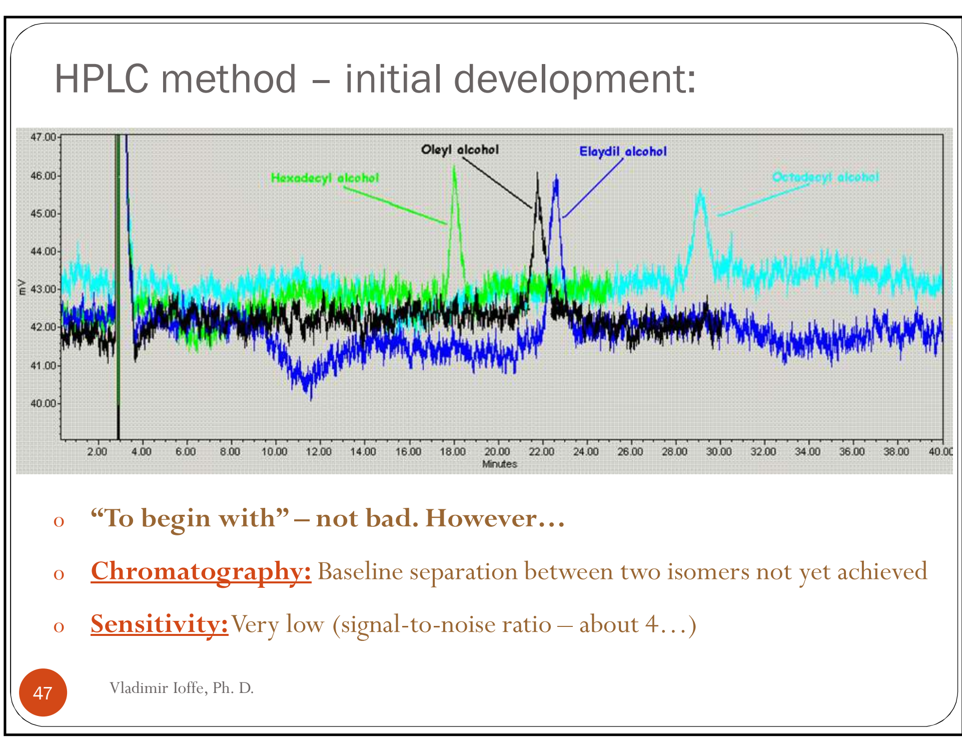
HPLC method – initial development:



- **"To begin with" – not bad. However…**
- **Chromatography:** Baseline separation between two isomers not yet achieved
- **Sensitivity:** Very low (signal-to-noise ratio – about 4…)

Part III-a: Challenges of Non-Linear Detection in HPLC

Evaporative Light Scattering Detector (ELSD) – a Universal HPLC Detector

Introduction: ELSD

- As we know, UV detection is the most widely used, but is not sufficient for many compounds of interest, especially those, which have no strong chromophore. Therefore, the alternative, so-called universal detectors were developed
- These detectors are also called "Integral" because their detection is not based on spectral characteristics and, therefore, does not allow spectral identification
- The most widespread today integral detector in HPLC is the **Evaporative Light Scattering Detector (ELSD)**

Introduction: ELSD

ELSD Principles

Detection comprises three stages:

- **Nebulization** – with a flow of air or nitrogen to produce aerosol or droplets
- **Mobile phase evaporation** – in a heated drift tube to evaporate mobile phase and to leave a particulate form of target compound
- **Detection** – by measuring intensity of the light, scattered by micro particles of analyte at a fixed angle from the incident light

Column effluent
Nebulizer
Nebulizer gas (air or nitrogen)
Nebulizer chamber
Drift tube (heated-zone evaporation stage)
Analyte
Photomultiplier tube or photodiode
Signal output
Light source
Light scattering cell
Amplifier

Introduction: ELSD

- **ELSD is not a spectroscopic detector** – and does not obey Beer's law
- The law is: $A = a^m$ where: **A** – peak area, **m** – quantity of analyte, **a** – response factor (slope)
- Otherwise (following Raley's law of scattered light): $A = am^2 + bm + c$
- One of the "limitations" of routinely running the methods on **ELSD** is the concern about non-linearity of this detection technique
- However, a modern software available today with most of the instruments allows calculations using non-linear calibration curves

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HPLC method – improving sensitivity:

- **Column:** Asahipak C8P-50 4D, 5 µm, 4.6 x 150 mm
- **Mobile phase:** Water–Methanol (15:85)
- **Flow rate:** 0.9 mL/min
- **Detector:** ELSD at 30°C
- **Concentration of each compound:** 1 mg/mL

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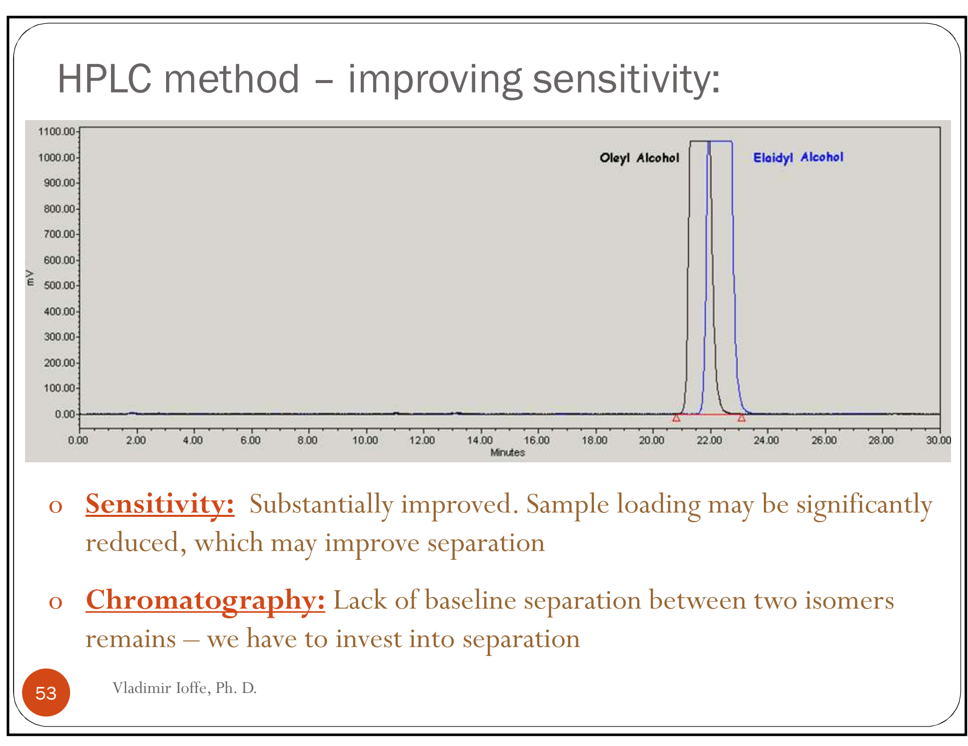
HPLC method – improving sensitivity:
Oleyl Alcohol
Elaidyl Alcohol
mV
Minutes
Sensitivity: Substantially improved. Sample loading may be significantly reduced, which may improve separation
Chromatography: Lack of baseline separation between two isomers remains – we have to invest into separation
53
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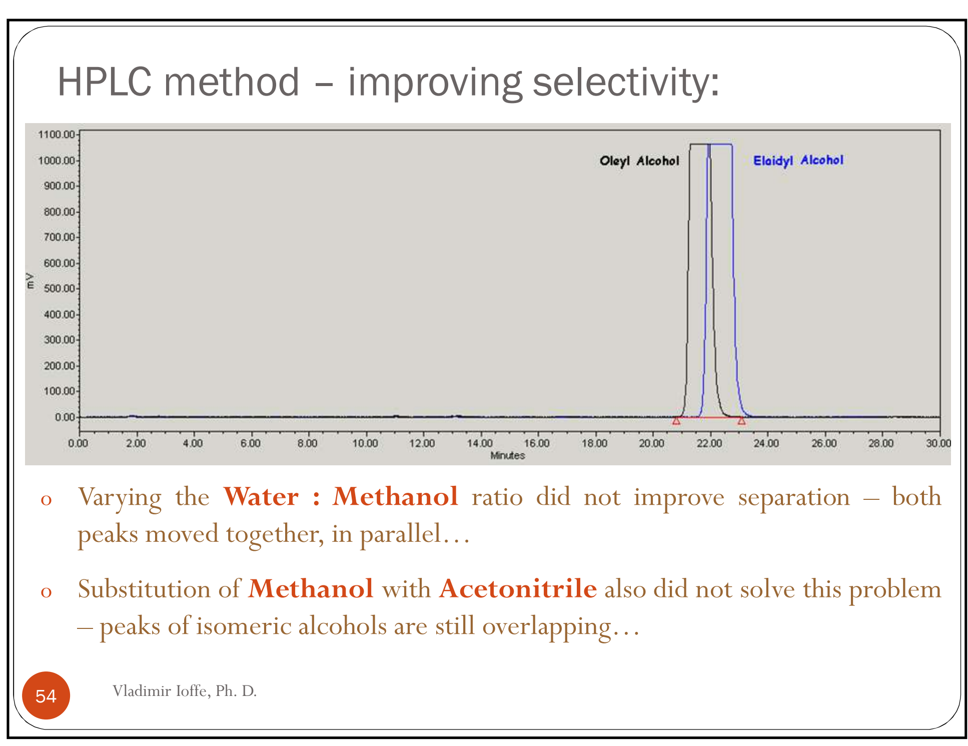
HPLC method – improving selectivity:
Oleyl Alcohol
Elaidyl Alcohol
mV
Minutes
Varying the Water : Methanol ratio did not improve separation – both peaks moved together, in parallel…
Substitution of Methanol with Acetonitrile also did not solve this problem – peaks of isomeric alcohols are still overlapping…
54
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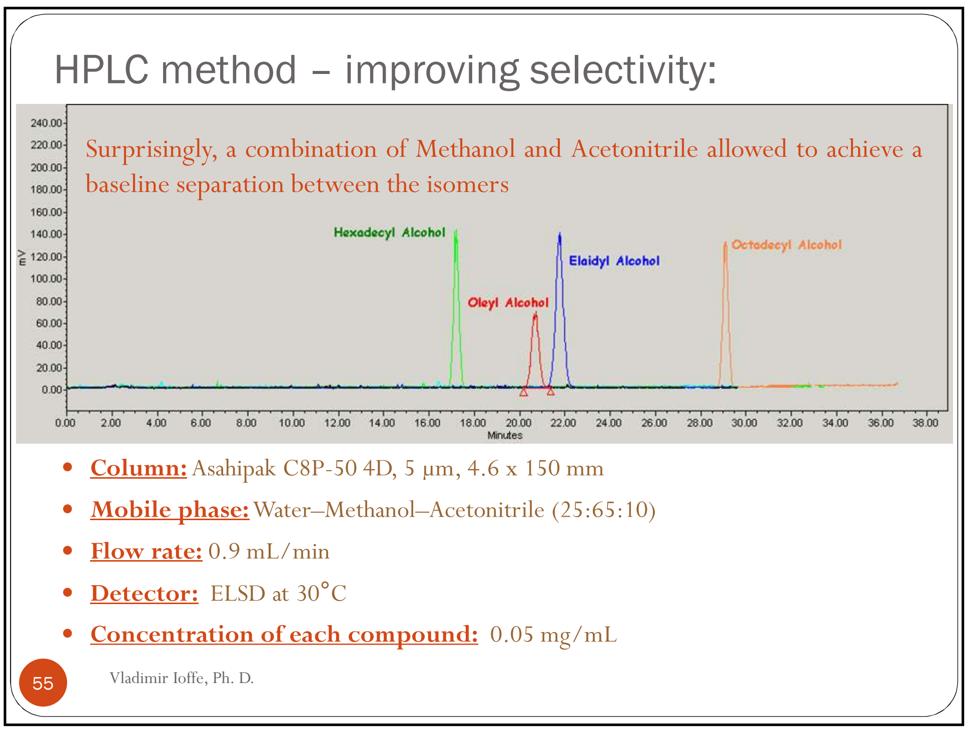
HPLC method – improving selectivity:
Surprisingly, a combination of Methanol and Acetonitrile allowed to achieve a baseline separation between the isomers
Hexadecyl Alcohol
Elaidyl Alcohol
Octadecyl Alcohol
Oleyl Alcohol
mV
Minutes
Column: Asahipak C8P-50 4D, 5 µm, 4.6 x 150 mm
Mobile phase: Water–Methanol–Acetonitrile (25:65:10)
Flow rate: 0.9 mL/min
Detector: ELSD at 30°C
Concentration of each compound: 0.05 mg/mL
55
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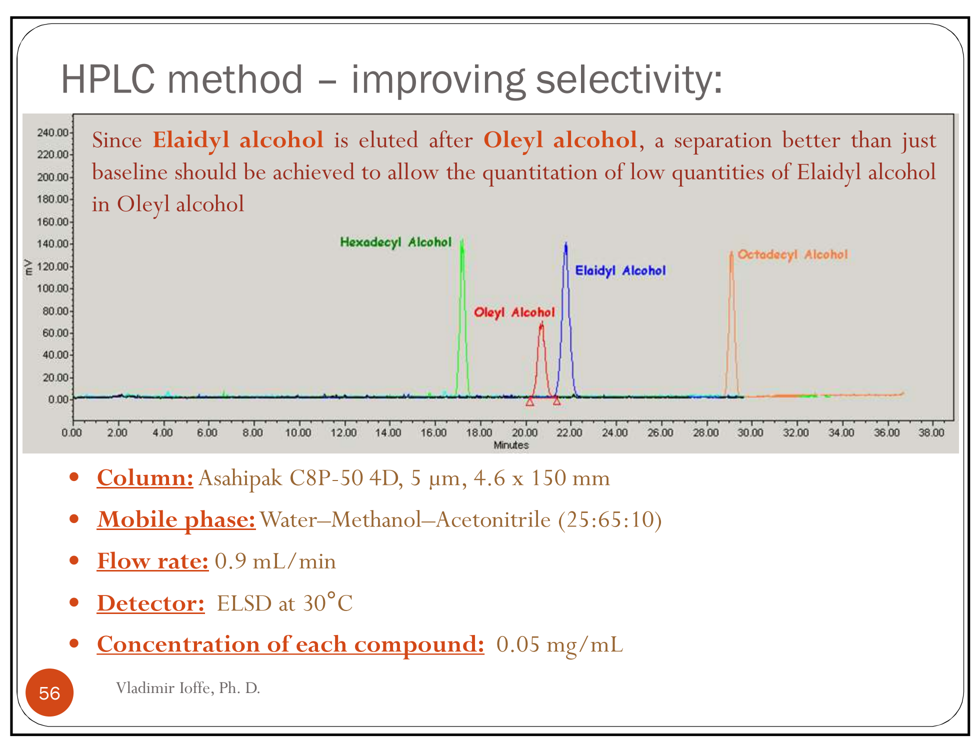
HPLC method – improving selectivity:
Since Elaidyl alcohol is eluted after Oleyl alcohol, a separation better than just baseline should be achieved to allow the quantitation of low quantities of Elaidyl alcohol in Oleyl alcohol
Hexadecyl Alcohol
Elaidyl Alcohol
Octadecyl Alcohol
Oleyl Alcohol
mV
Minutes
Column: Asahipak C8P-50 4D, 5 µm, 4.6 x 150 mm
Mobile phase: Water–Methanol–Acetonitrile (25:65:10)
Flow rate: 0.9 mL/min
Detector: ELSD at 30°C
Concentration of each compound: 0.05 mg/mL
56
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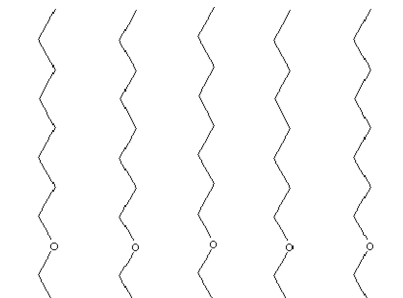
Ideas about Retention Mechanism: Driving Force of Separation

- Polymer RP column with C8 side chains - Asahipak C8P:
- Conformation of C8 chains in a moderate polarity eluent (no globulization in solvent having a low water content)



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Ideas about Retention Mechanism: Driving Force of Separation

- Conformation of isomeric alcohols in a moderate polarity eluent (no globulization in solvent having a low water content)

OH

Oleyl Alcohol (cis-isomer): **"Bended" Chain**

OH

Elaidyl Alcohol (trans-isomer): **Straight Chain**

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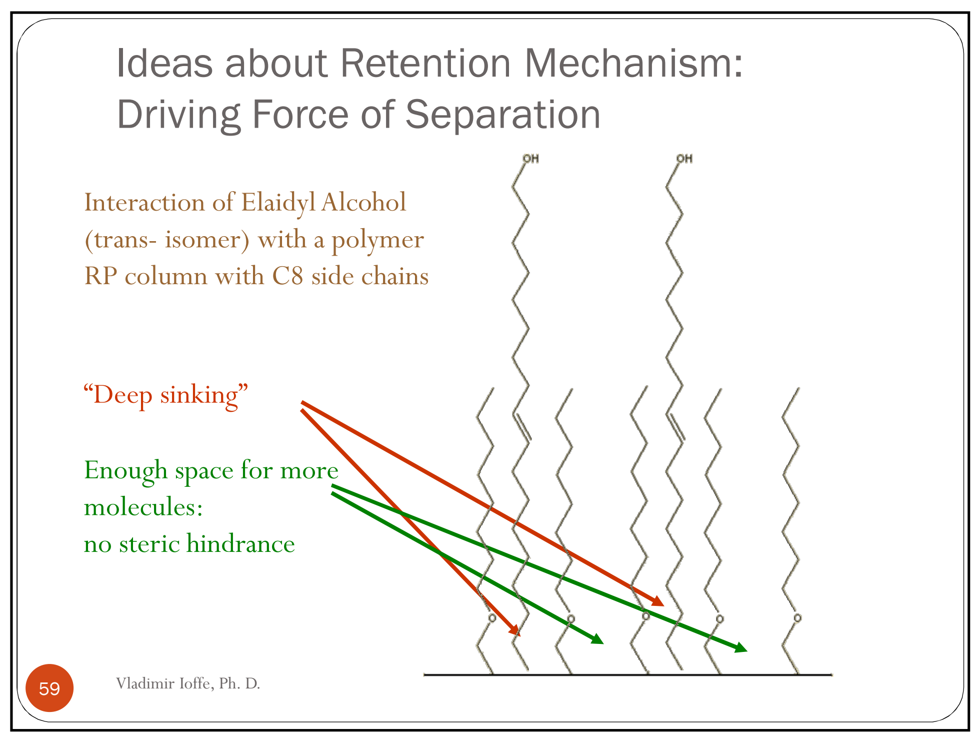
Ideas about Retention Mechanism:
Driving Force of Separation
Interaction of Elaidyl Alcohol
(trans- isomer) with a polymer
RP column with C8 side chains
"Deep sinking"
Enough space for more
molecules:
no steric hindrance
OH
OH
59
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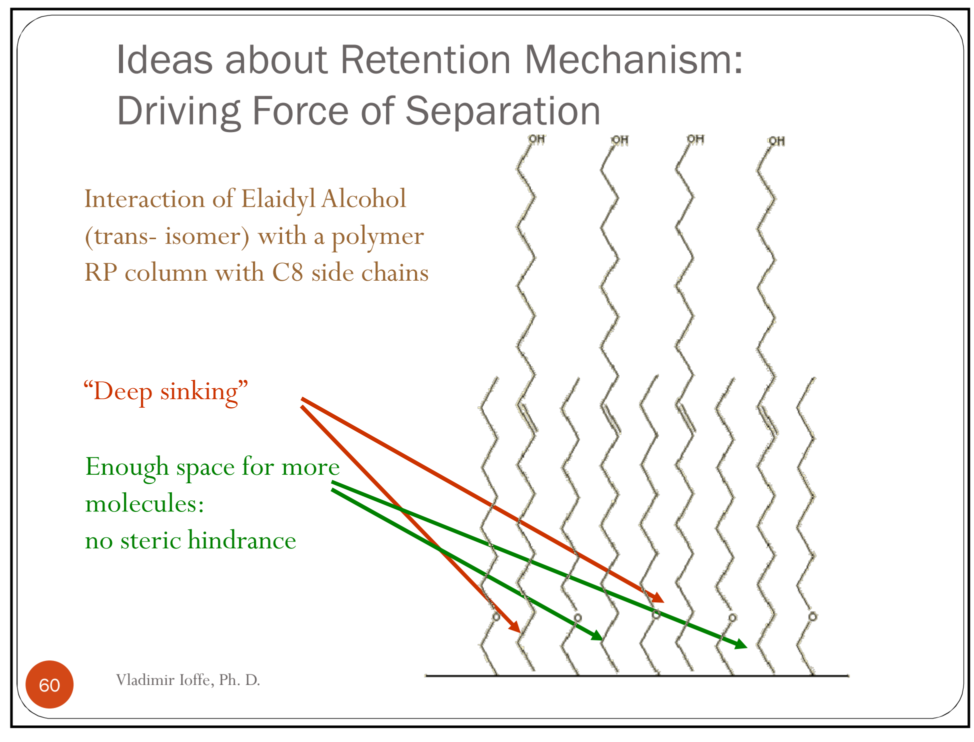
Ideas about Retention Mechanism:
Driving Force of Separation
Interaction of Elaidyl Alcohol
(trans- isomer) with a polymer
RP column with C8 side chains
"Deep sinking"
Enough space for more
molecules:
no steric hindrance
OH
OH
OH
OH
60
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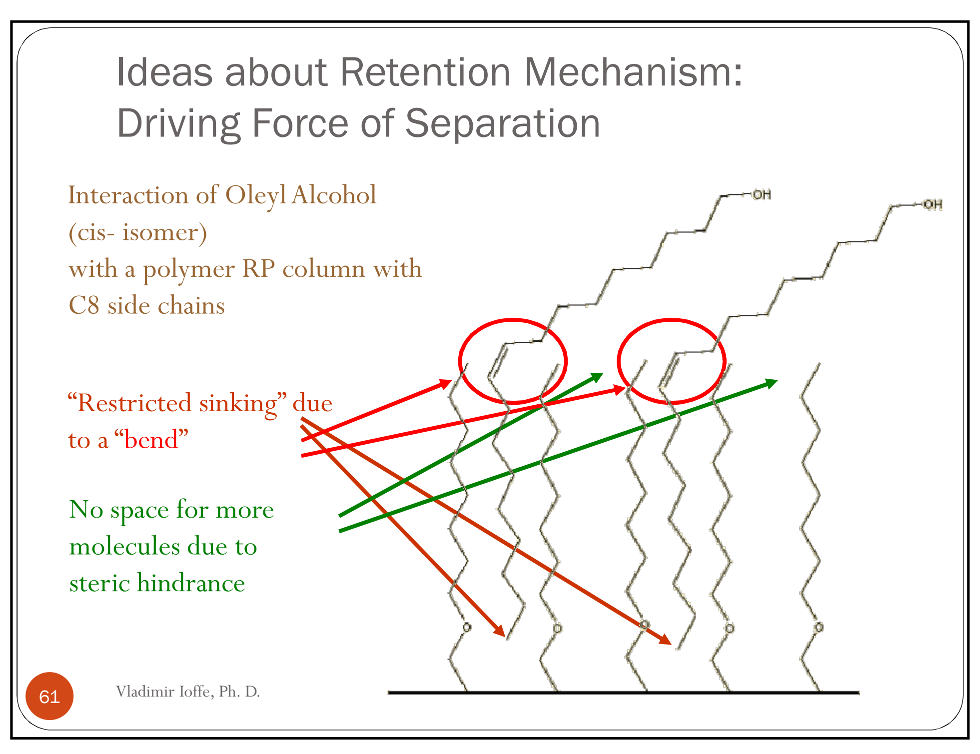
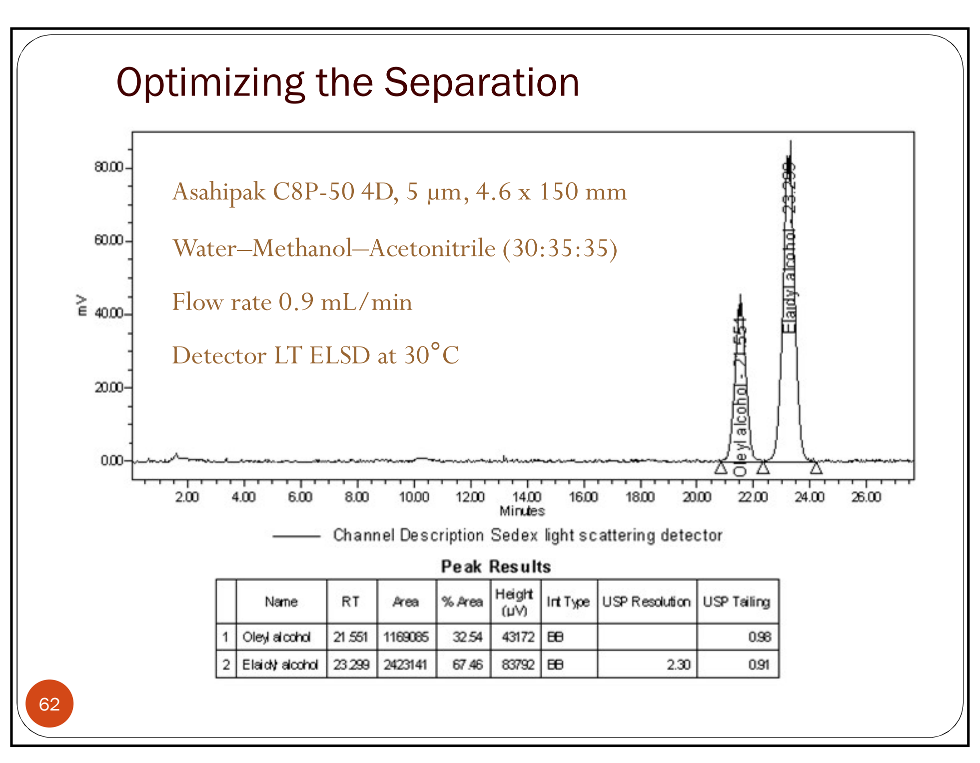
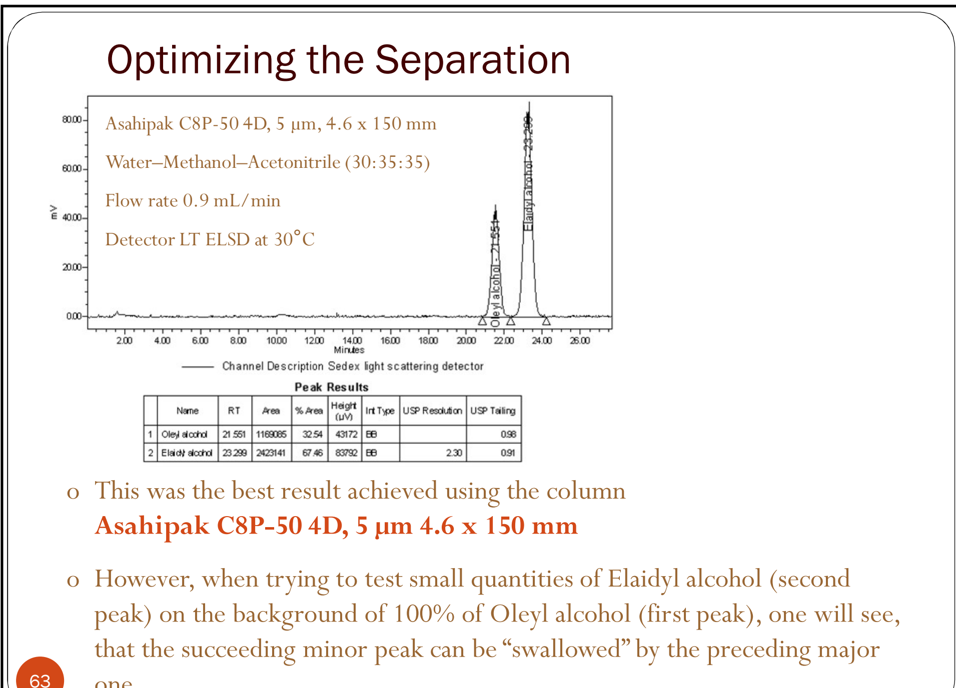


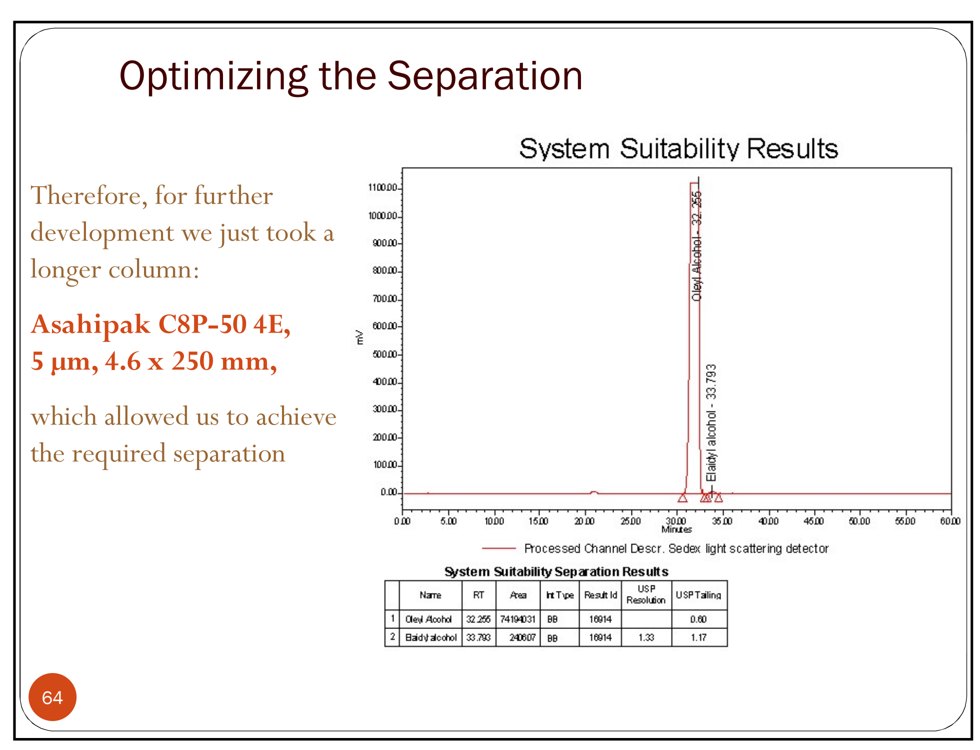


	Name	RT	Area	% Area	Height (µV)	Int Type	USP Resolution	USP Tailing
1	Oleyl alcohol	21.551	1169085	32.54	43172	BB		0.98
2	Elaidyl alcohol	23.299	2423141	67.46	83792	BB	2.30	0.91



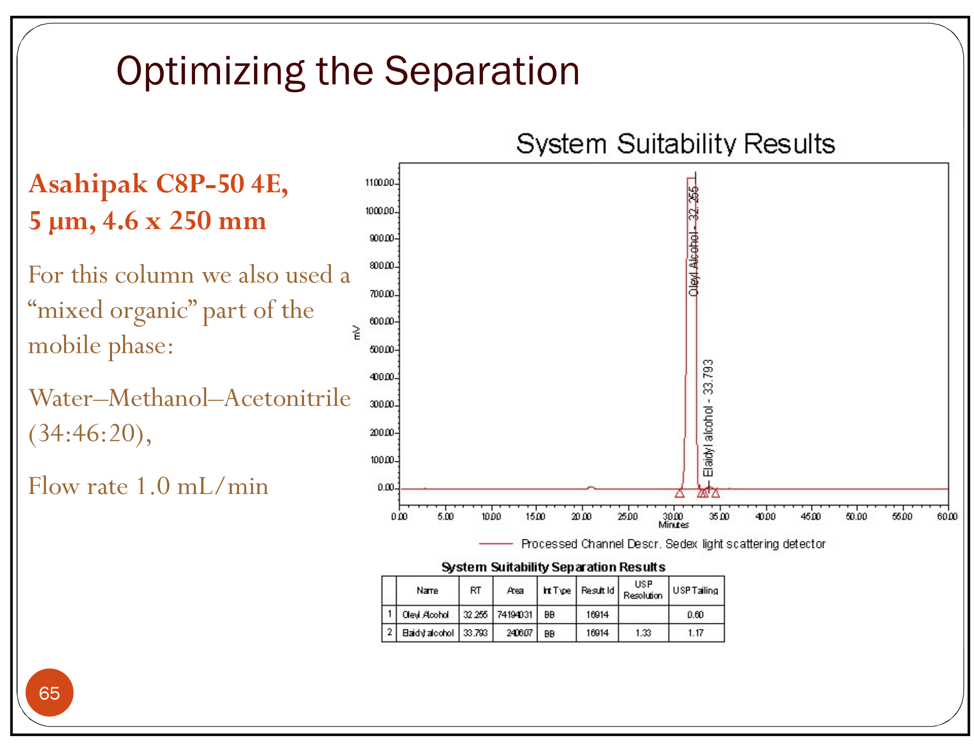
Peak Results

	Name	RT	Area	% Area	Height (µV)	Int Type	USP Resolution	USP Tailing
1	Oleyl alcohol	21.551	1169085	32.54	43172	BB		0.98
2	Elaidyl alcohol	23.299	2423141	67.46	83792	BB	2.30	0.91



System Suitability Separation Results

	Name	RT	Area	Int Type	Result Id	USP Resolution	USP Tailing
1	Oleyl Alcohol	32.255	74194031	BB	16914		0.60
2	Elaidyl alcohol	33.793	240607	BB	16914	1.33	1.17



System Suitability Separation Results

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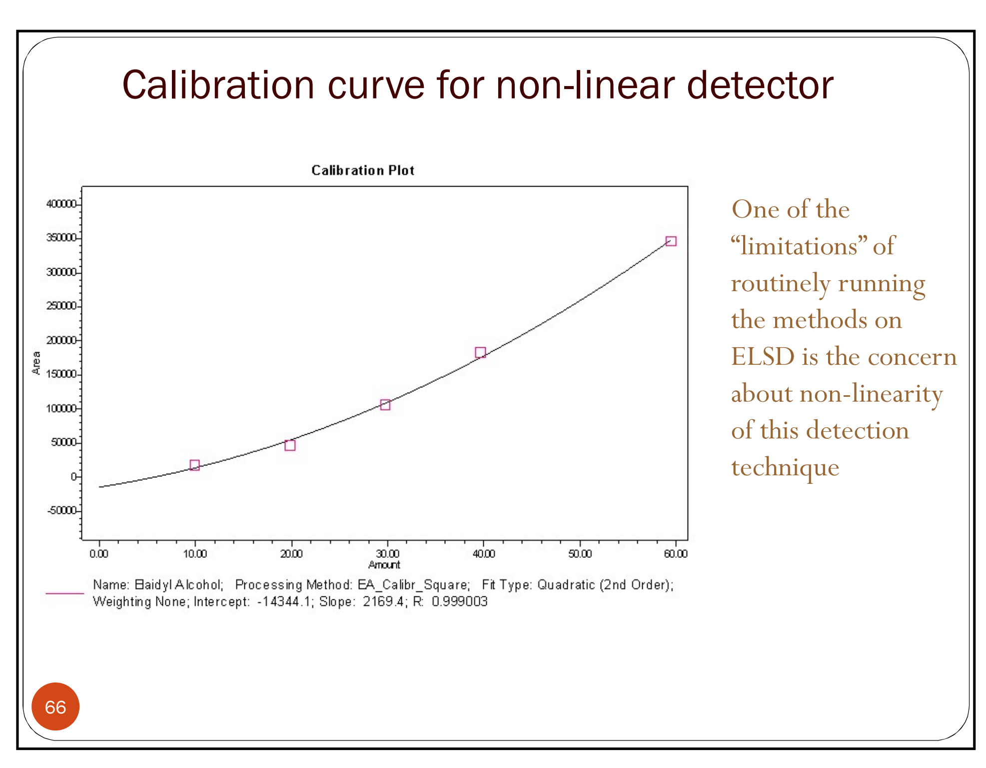
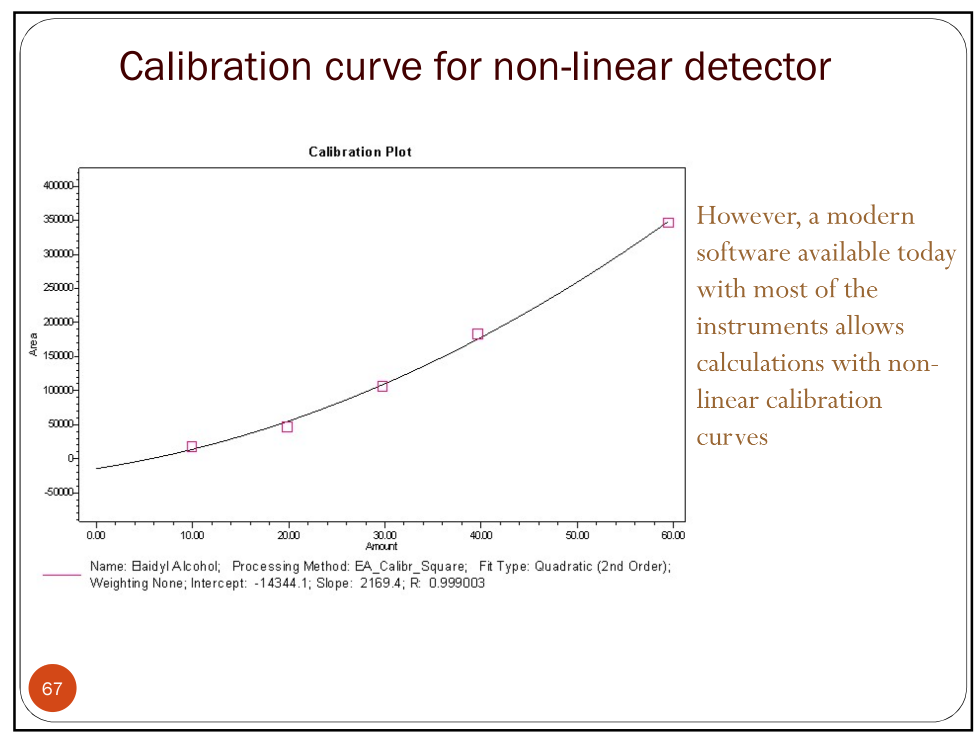
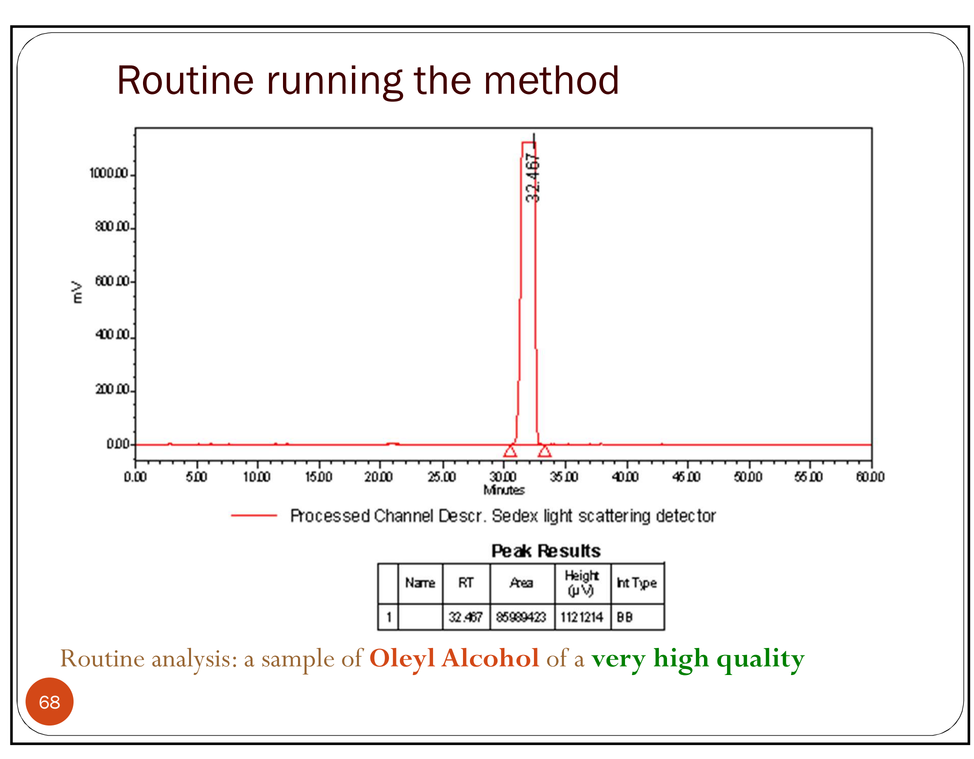
1	Oleyl Alcohol	32.255	74194031	BB	16914		0.60
2	Elaidyl alcohol	33.793	240607	BB	16914	1.33	1.17

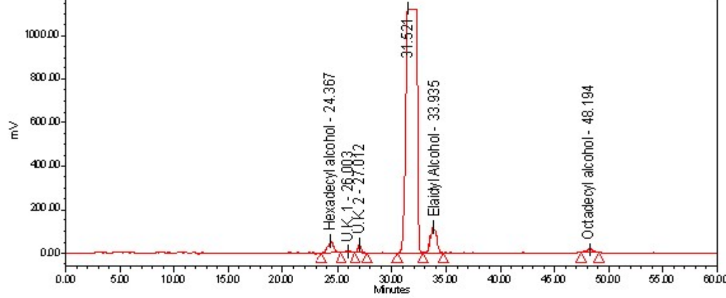






	Name	RT	Area	Height (µV)	Int Type
1		32.467	85989423	1121214	BB

Routine running the method



Peak Results

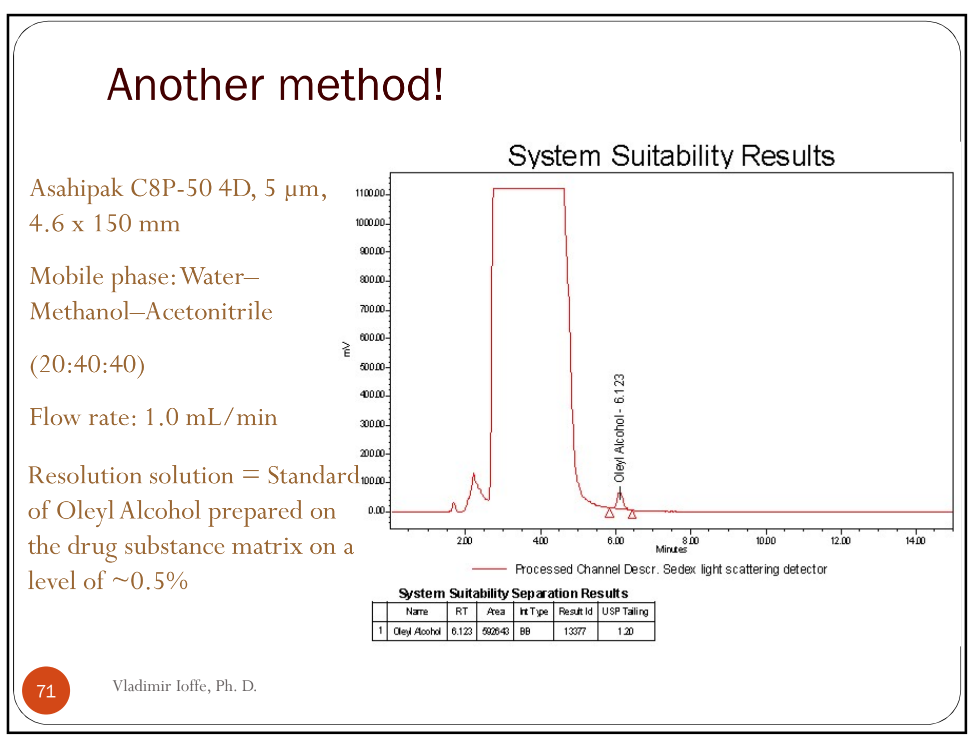
	Name	RT	Area	Height (µV)	% Area	Int Type
1	Hexadecyl alcohol	24.367	1943442	56729	2.25	BB
2	U.K 1	26.003	225538	6989	0.26	BB
3	U.K 2	27.012	834308	37200	0.97	BB
4		31.521	75264605	1124295	87.14	BB
5	Elaidyl Alcohol	33.935	4128528	117538	4.78	BB
6	Octadecyl alcohol	48.194	716517	17487	0.83	BB

Routine analysis: a sample of **Oleyl Alcohol** of a **very bad quality**

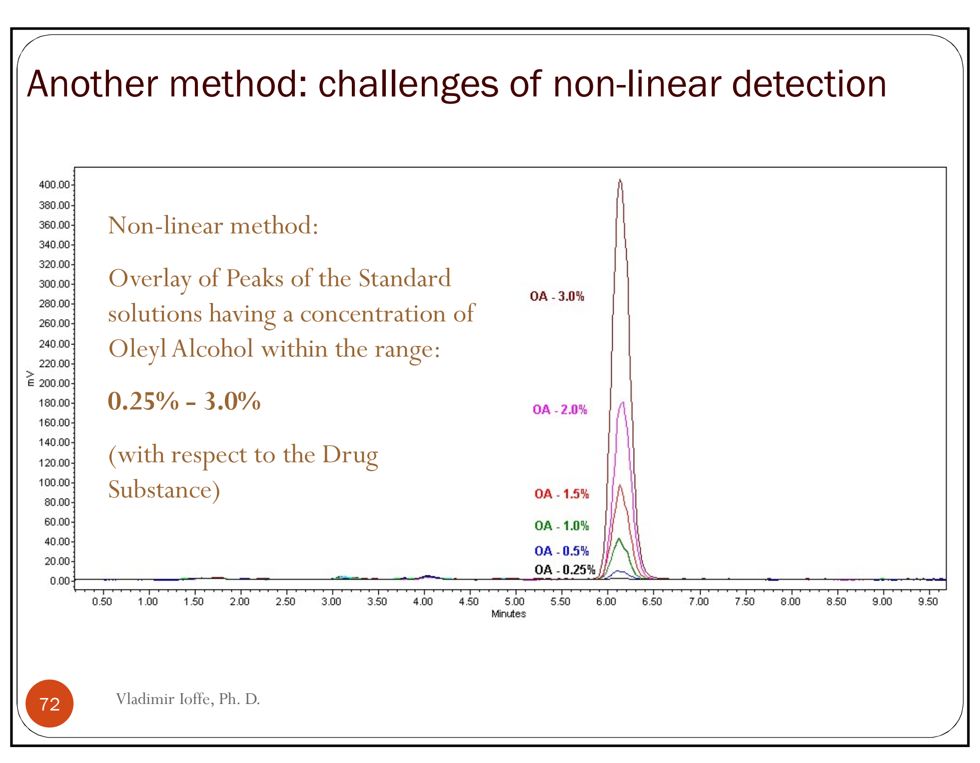
Another method?

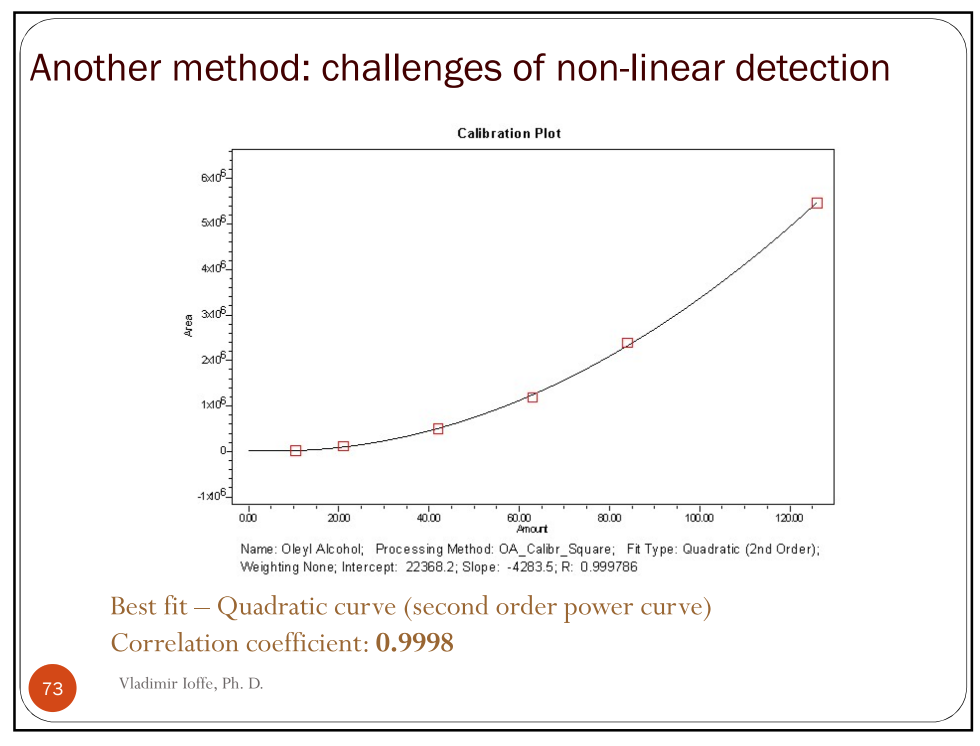
- And what to do with the earlier purchased shorter column?
- We may try to use it **for determination of Oleyl Alcohol in the drug substance and drug product as a process impurity and / or degradation product**
  (for release and stability studies)
- And for testing of **residual Oleyl Alcohol in the reaction mixture** (for in-process control)

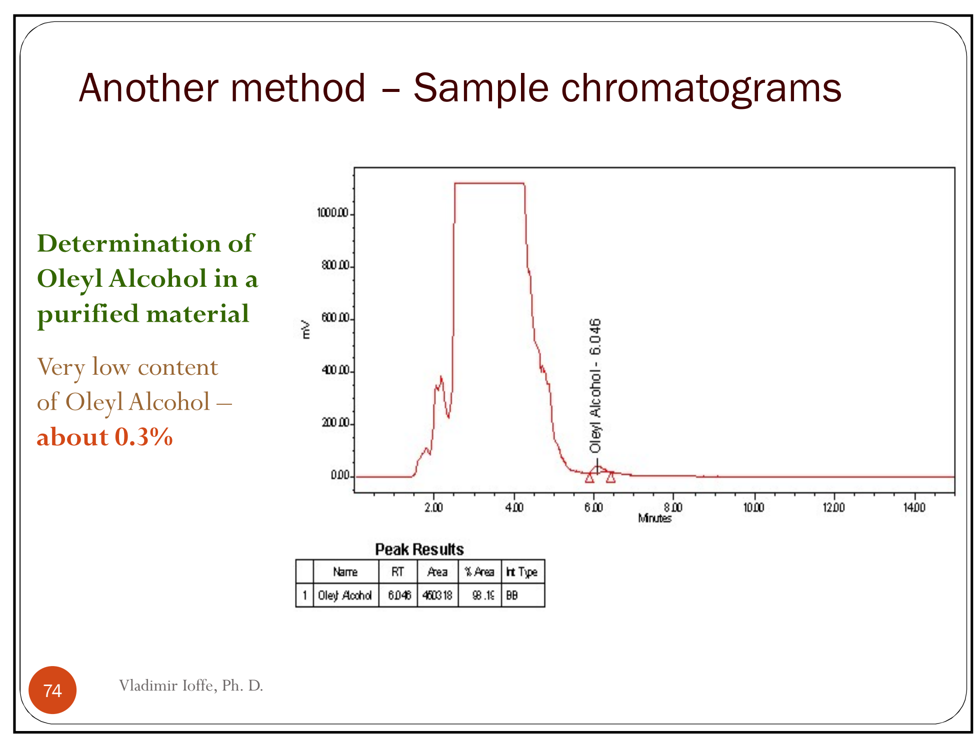
OH



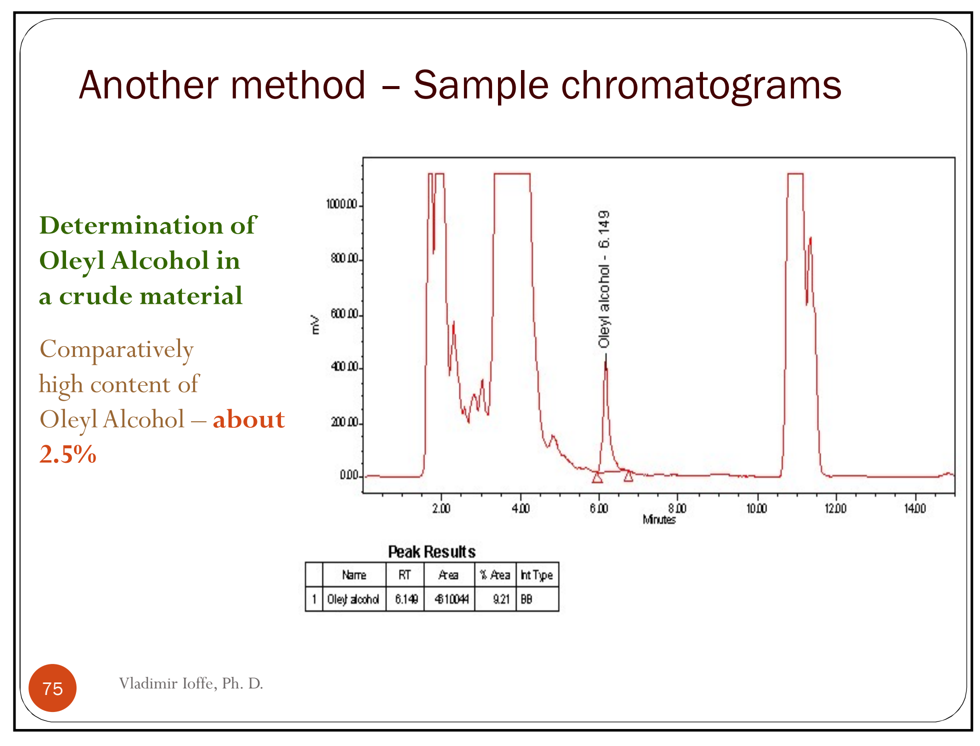
	Name	RT	Area	Int Type	Result Id	USP Tailing
1	Oleyl Alcohol	6.123	592643	BB	13377	1.20







	Name	RT	Area	% Area	Int Type
1	Oleyl Alcohol	6.046	460318	98.19	BB


Another method – Sample chromatograms
Determination of Oleyl Alcohol in a crude material
Comparatively high content of Oleyl Alcohol – about 2.5%
Oleyl alcohol - 6.149
mV
1000.00
800.00
600.00
400.00
200.00
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
Minutes
Peak Results
Name RT Area % Area Int Type
1 Oleyl alcohol 6.149 4810044 9.21 BB
75
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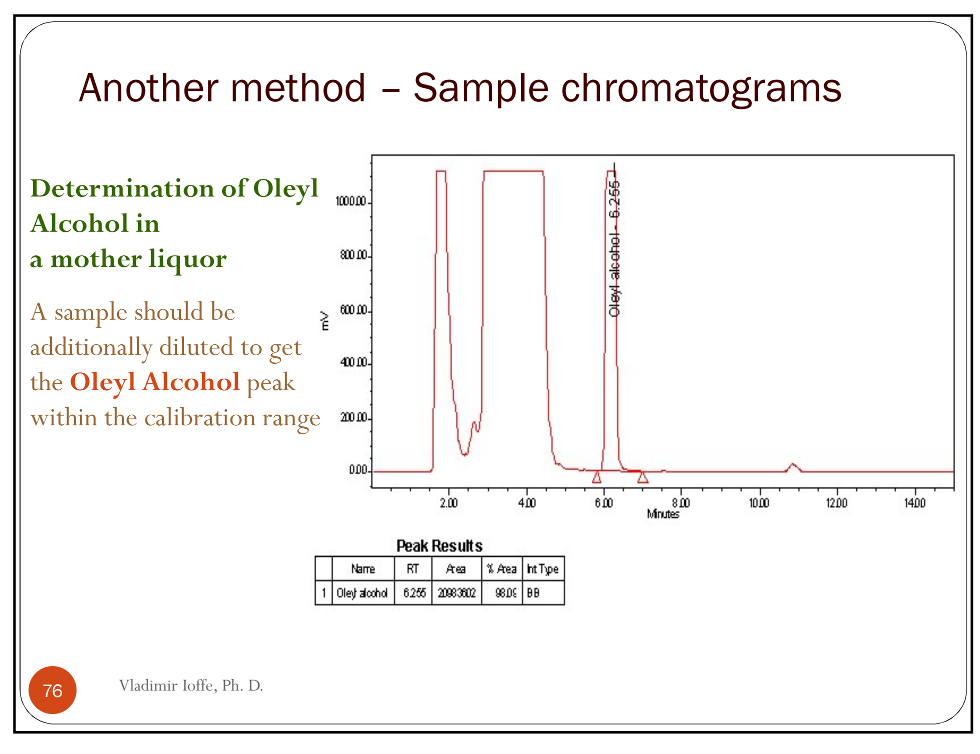
Another method – Sample chromatograms
Determination of Oleyl Alcohol in a mother liquor
A sample should be additionally diluted to get the Oleyl Alcohol peak within the calibration range
Oleyl alcohol - 6.255
mV
1000.00
800.00
600.00
400.00
200.00
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
Minutes
Peak Results
Name RT Area % Area Int Type
1 Oleyl alcohol 6.255 20983602 98.09 BB
76
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Summary

- Useful routine methods were developed for very "analytically unfriendly" analytes using:
  - Non-routine polymer-based real RP columns
  - A comparatively non-routine detection technique – evaporative light scattering
  - An unusually looking non-linear calibration curve
- **At the end – a facile analytical technique**

Part III-b: "Upgrading" the Methods for Oleyl Alcohol

Corona CAD:

A Novel Universal Detection Technique for HPLC and a New Type of Detection Technique

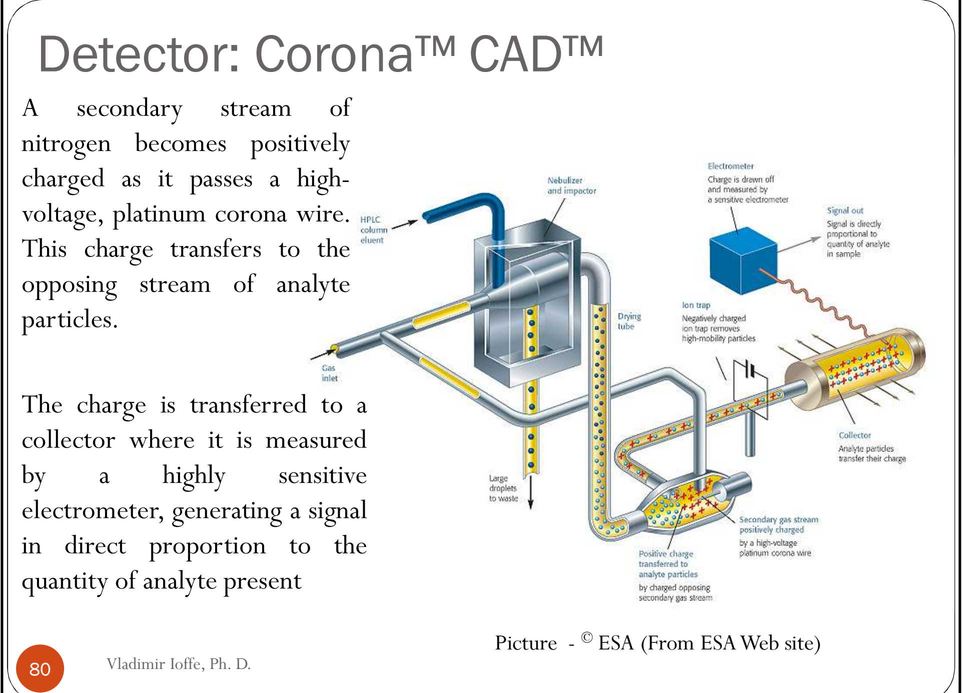
Detector: Corona™ CAD™

- In the recent years a novel type of HPLC detector was developed and appeared on the market – **Corona Charged Aerosol Detector (Corona™ CAD™)**
- Based on an innovative detection principle, it intends to offer significant performance benefits
- As the ELSD, it is an evaporative (and, as a result, a "destructive") detector. Therefore, main limitations of ELSD apply also to **Corona™ CAD™**
- **Corona™ CAD™** is a novel technology, in which the eluent is first nebulized with nitrogen and the droplets are dried to remove mobile phase, producing analyte particles (as it begins also in ELSD, but here the similarity of these detectors ends)

Detector: Corona™ CAD™

A secondary stream of nitrogen becomes positively charged as it passes a high-voltage, platinum corona wire. This charge transfers to the opposing stream of analyte particles.

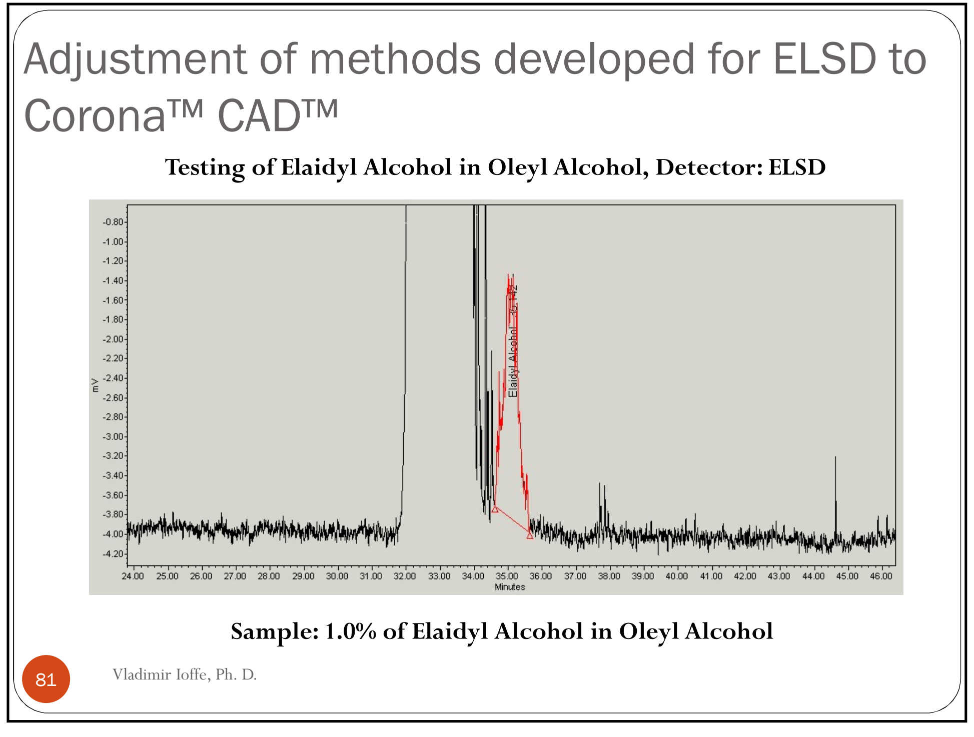
The charge is transferred to a collector where it is measured by a highly sensitive electrometer, generating a signal in direct proportion to the quantity of analyte present



Picture - © ESA (From ESA Web site)

Adjustment of methods developed for ELSD to Corona™ CAD™

Testing of Elaidyl Alcohol in Oleyl Alcohol, Detector: ELSD



Sample: 1.0% of Elaidyl Alcohol in Oleyl Alcohol

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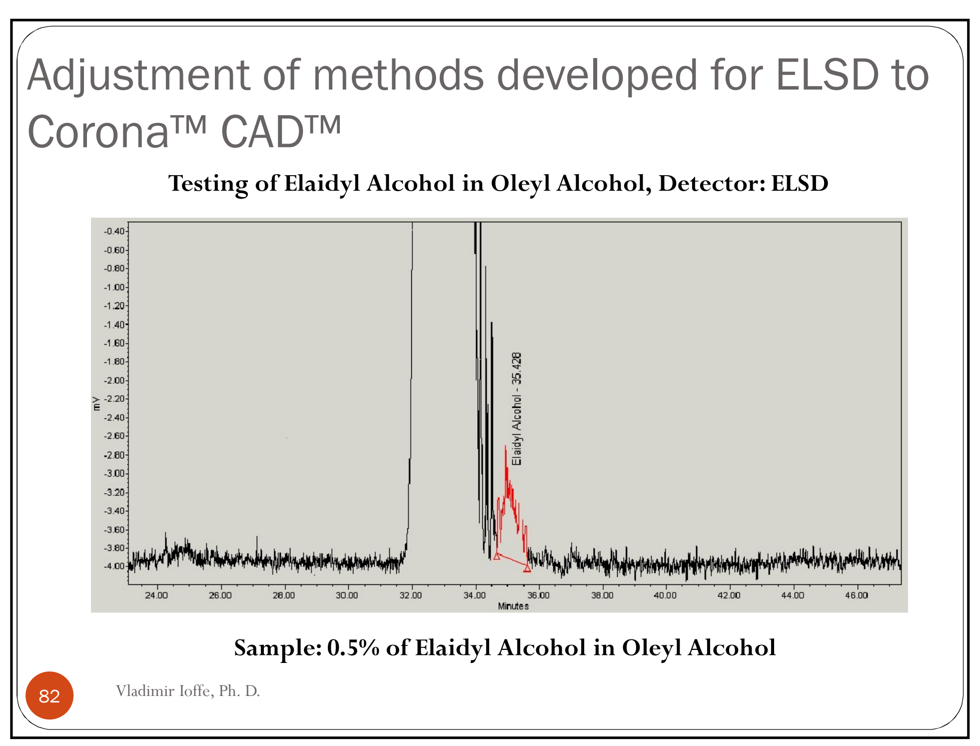
Adjustment of methods developed for ELSD to Corona™ CAD™

Testing of Elaidyl Alcohol in Oleyl Alcohol, Detector: ELSD



Sample: 0.5% of Elaidyl Alcohol in Oleyl Alcohol

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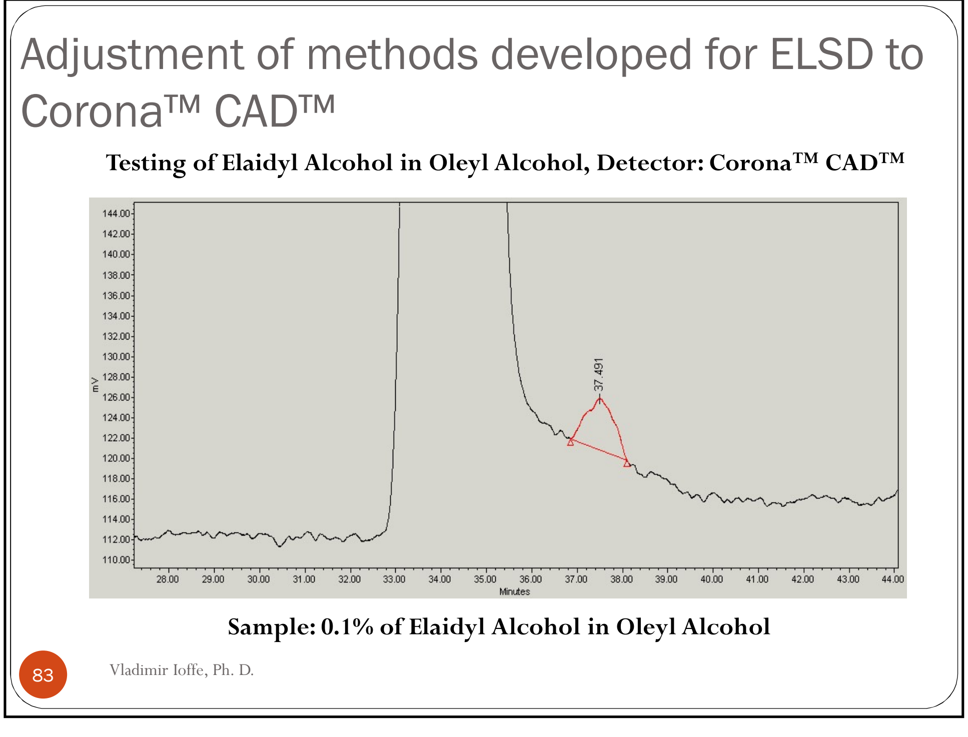
Adjustment of methods developed for ELSD to Corona™ CAD™

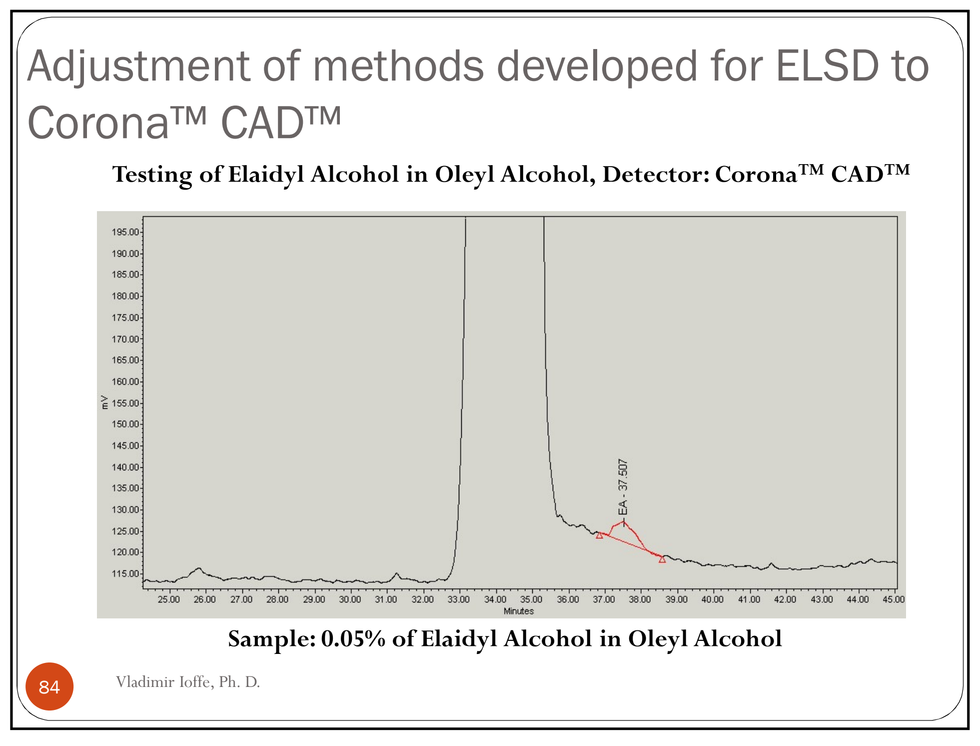
Testing of Elaidyl Alcohol in Oleyl Alcohol, Detector: Corona™ CAD™



Sample: 0.1% of Elaidyl Alcohol in Oleyl Alcohol

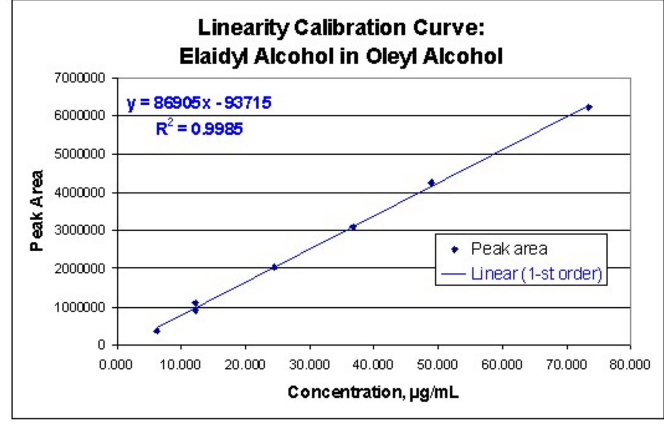
Adjustment of methods developed for ELSD to Corona™ CAD™

Testing of Elaidyl Alcohol in Oleyl Alcohol, Detector: Corona™ CAD™



Sample: 0.05% of Elaidyl Alcohol in Oleyl Alcohol

Adjustment of methods developed for ELSD to Corona™ CAD™



Detector Corona™ CAD™

Calibration curve for Elaidyl Alcohol in Oleyl Alcohol, Range: 0.10% – 3.0%

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Adjustment of methods developed for ELSD to Corona™ CAD™

Method	Testing of EA in OA		Testing of OA in Drug Substance	
Detector	ELSD	Corona™ CAD™	ELSD	Corona™ CAD™
Quantitation Limit	0.25%	0.05%	0.50%	0.10%
Detection Limit	0.10%	0.02%	0.25%	0.05%
Correlation Curve	Quadratic	Linear	Quadratic	Linear

Summary of method performance characteristics

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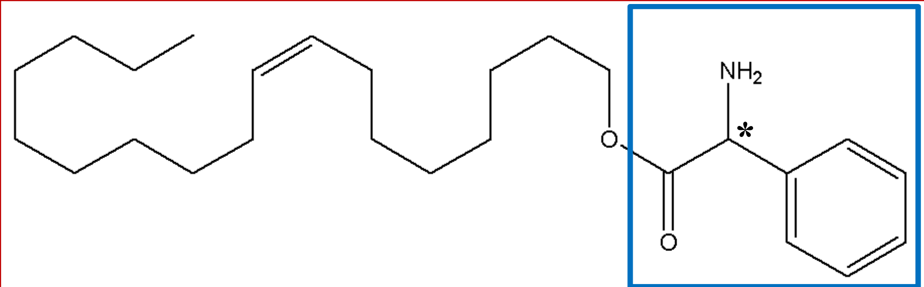
Summary:

- Sometimes an industrial analyst has to step off the beaten track…
- Working under high pressure, we are often using only the methods which we have always used…
- Today we are exposed to such a diversity of instruments and techniques…
- **Trying something new – always pays (at the end)…**

Part IV: Challenges of Chiral Separation

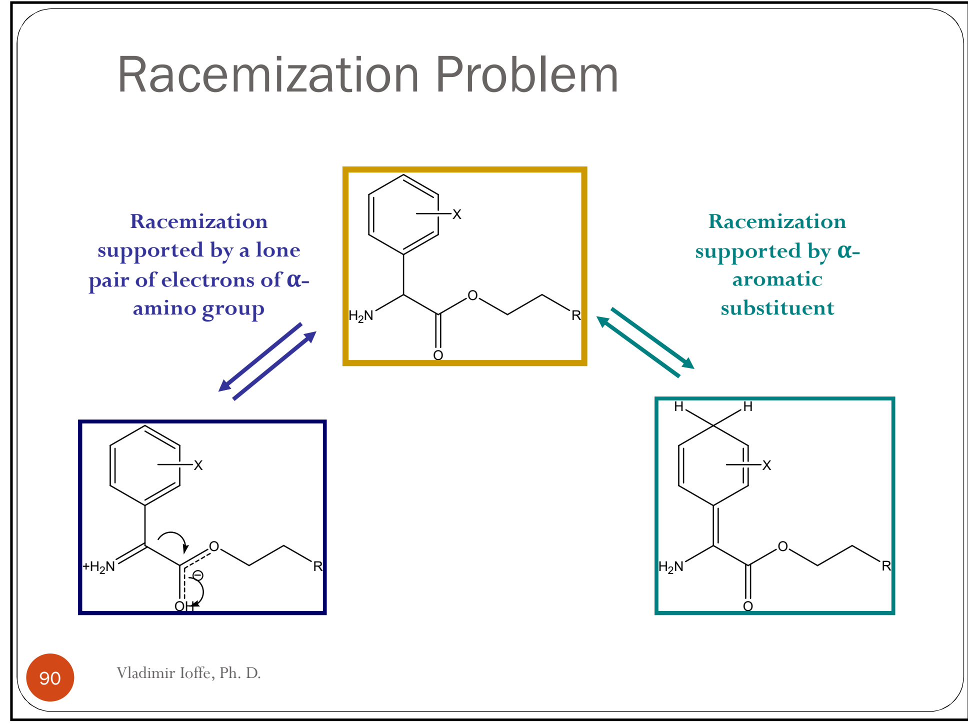
Polysaccharide Based Enantioselective Columns

One more degradation product



- Additional source of impurities of **TV-3813** are degradation products related to **Phenylglycine**, bearing a chiral centre
- In presence of water, one of the most critical processes is **racemization**, which causes formation of a degradation product – the **opposite enantiomer**

Racemization Problem



Racemization Problem

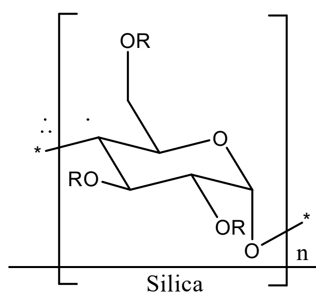
Taking into account the enantiomeric instability of **α-aryl substituted α-amino acids**, as well as their potential hydrolytic lability, chiral separation using Reverse Phase mode is very problematic.

Chiral RP columns work in the range of neutral to moderately basic pH and require the use phosphate or borate buffers. As we have found, such conditions are crucial for retention of configuration of this type of analytes.

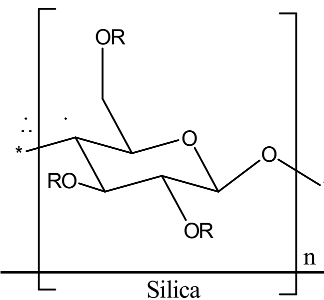
Therefore, only the Normal Phase chromatographic conditions may be relevant to obtain the reliable picture of enantiomeric composition.

Chemistry of Enantioselective Columns

Polysaccharide-Derived Coated Normal Phase Chiral Stationary Phases (DAICEL)



Amylose-Derived Chiral Stationary Phases (CHIRALPAK®, A-series)



Cellulose-Derived Chiral Stationary Phases (CHIRALCEL®, O-series)

Chemistry of Enantioselective Columns

Derivatizing Groups (Hydroxyl Substituents) of Polysaccharide-Derived Chiral Stationary Phases

R = –C(=O)–Me

For the columns OA

R = –C(=O)–C₆H₅

For the columns OB and OB-H

R = –C(=O)–C₆H₄–Me

For the columns OJ and OJ-H

R = –C(=O)–CH=CH–C₆H₅

For the columns OK

Esters: **Acetate, Benzoate, 4-Methylbenzoate, Cinnamate**

Vladimir Ioffe, Ph. D.

Chemistry of Enantioselective Columns

Derivatizing Groups (Hydroxyl Substituents) of Polysaccharide-Derived Chiral Stationary Phases

R = –C(=O)–HN–C₆H₅

For the columns OC

R = –C(=O)–HN–C₆H₄–Cl

For the columns OF

R = –C(=O)–HN–C₆H₄–Me

For the columns OG

Carbamates:

Phenylcarbamate,
4-Chlorophenylcarbamate,
4-Methylphenylcarbamate

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Chemistry of Enantioselective Columns

Derivatizing Groups (Hydroxyl Substituents) of Polysaccharide-Derived Chiral Stationary Phases

R = (C=O)–NH–(3,5-dimethylphenyl) [structure: Me, HN, O, Me]

For the columns OD and OD-H
and the columns AD and AD-H

Carbamates:

3.5-Dimethylphenylcarbamate

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First approach to a chiral method

- Hexane or Heptane with added ~1 – 5% of Isopropanol (2-Propanol; IPA) was taken as a "first choice" mobile phase
- A "first choice column" was selected using the smartest scientific choice option: the only one which was available in the lab…
- The first runs were made on the column **CHIRALCEL® OJ**

R = (C=O)–(4-methylphenyl) [structure: Me, O]

For the columns OJ and OJ-H

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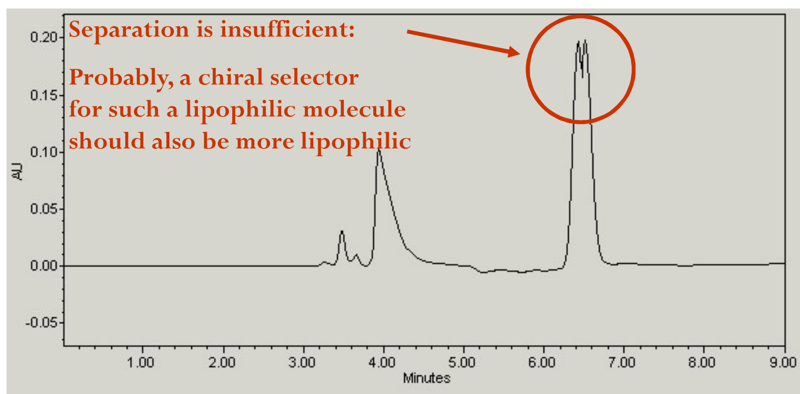
First approach to a chiral method



Column: CHIRALCEL® OJ

Eluent: Heptane – IPA (97:3)

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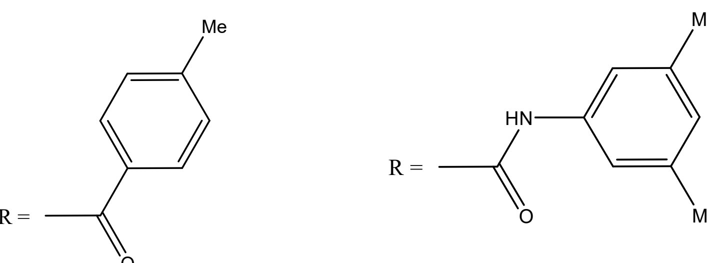
Improving separation – a different phase



For the columns OJ and OJ-H

For the columns OD and OD-H and the columns AD and AD-H

What are the differences between these stationary phases?

1. **Higher hydrolytic stability** (Carbamate Vs. Ester)
2. **Higher lipophilicity**
3. **Higher steric restrictions** (more bulky substituent)
4. **Higher π-electron density**

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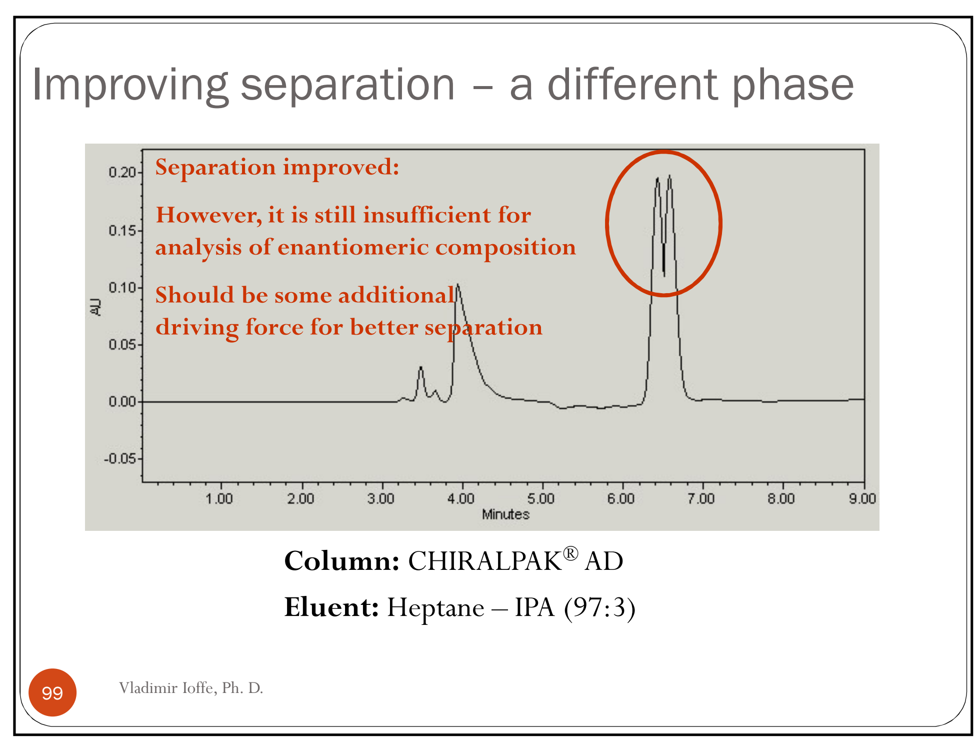
Improving separation – a different phase
Separation improved:
However, it is still insufficient for analysis of enantiomeric composition
Should be some additional driving force for better separation
0.20
0.15
0.10
0.05
0.00
-0.05
AU
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
Minutes
Column: CHIRALPAK® AD
Eluent: Heptane – IPA (97:3)
99
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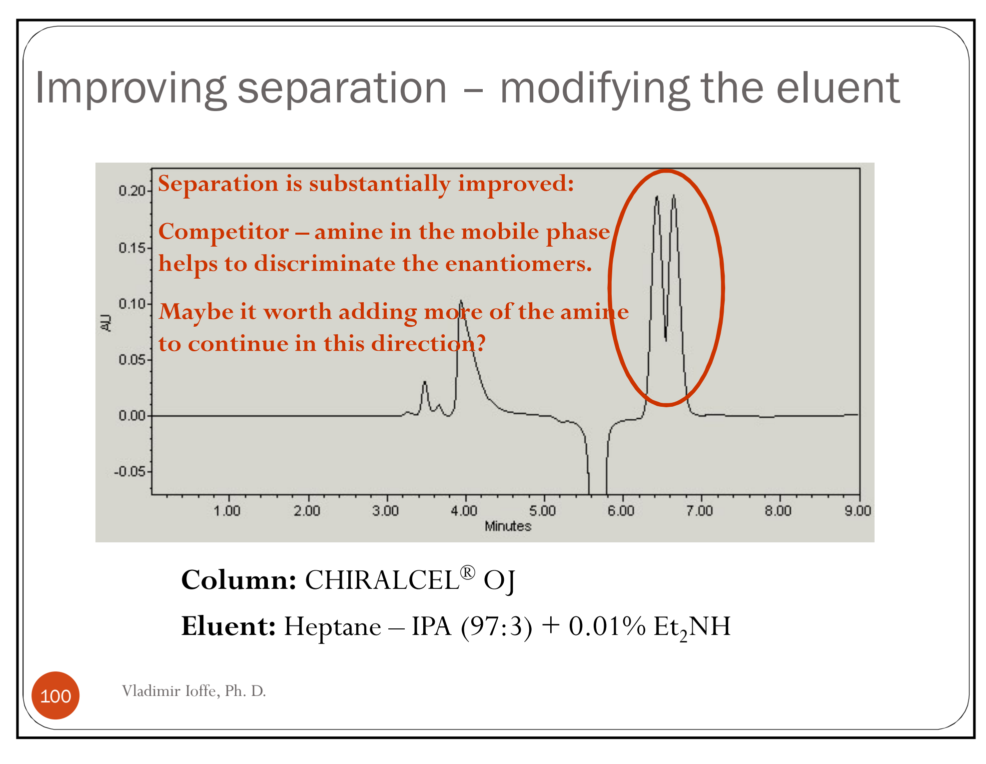
Improving separation – modifying the eluent
Separation is substantially improved:
Competitor – amine in the mobile phase helps to discriminate the enantiomers.
Maybe it worth adding more of the amine to continue in this direction?
0.20
0.15
0.10
0.05
0.00
-0.05
AU
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
Minutes
Column: CHIRALCEL® OJ
Eluent: Heptane – IPA (97:3) + 0.01% Et_2NH
100
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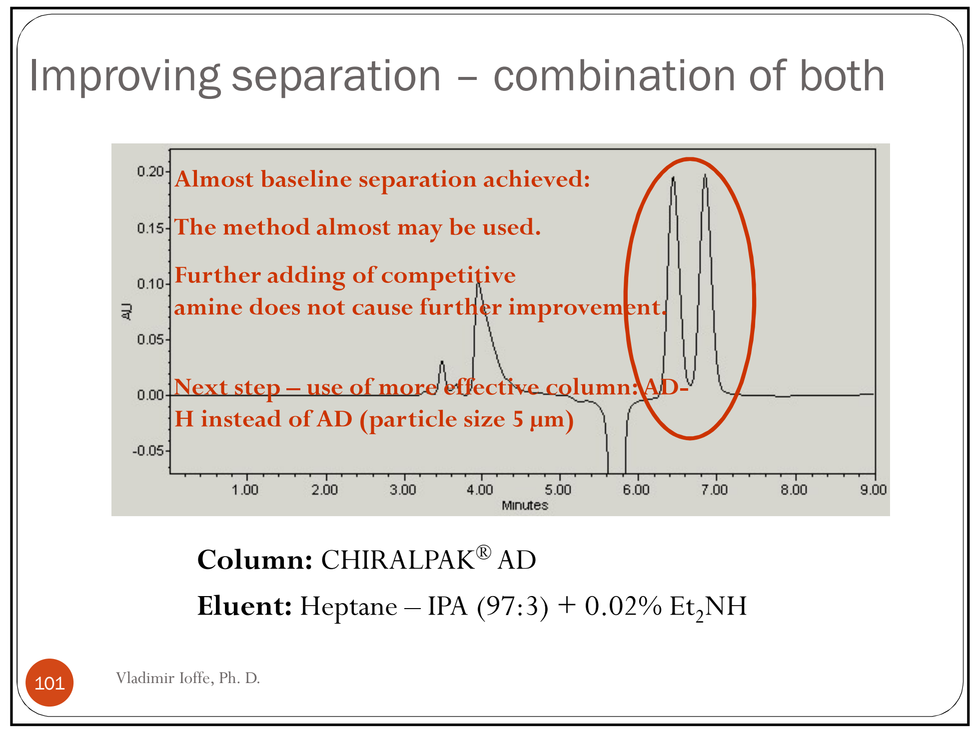
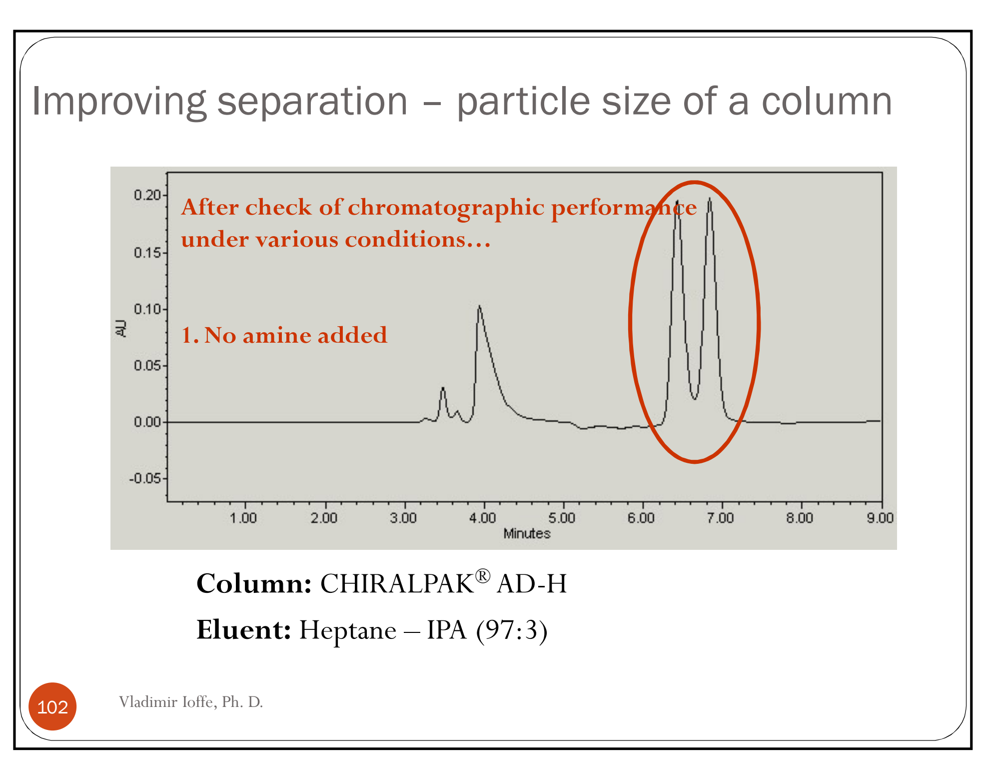
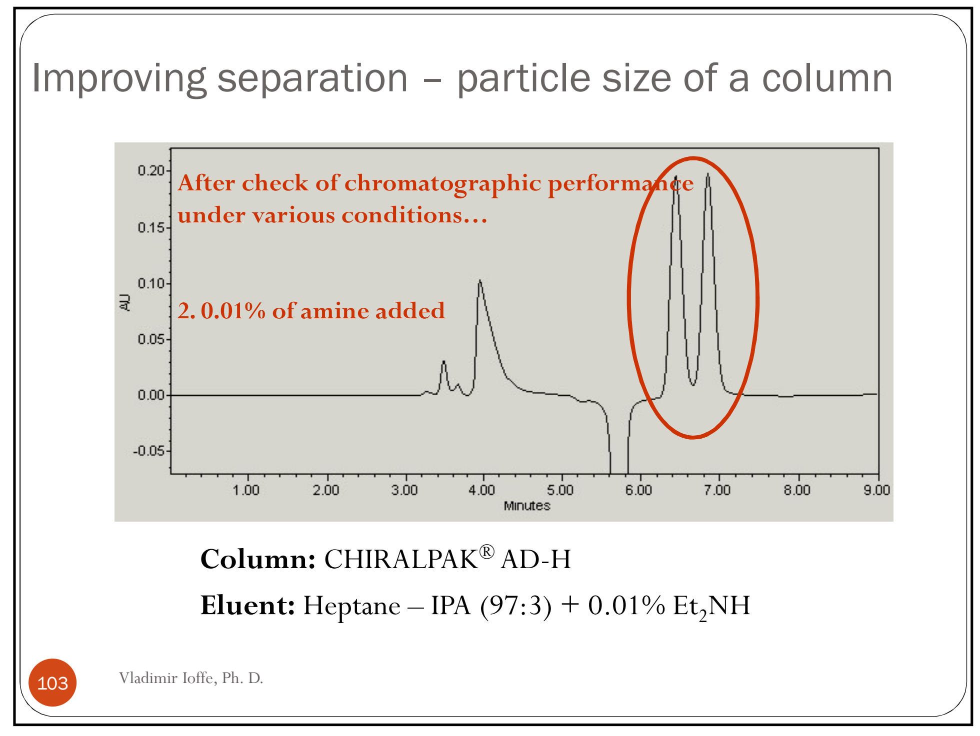
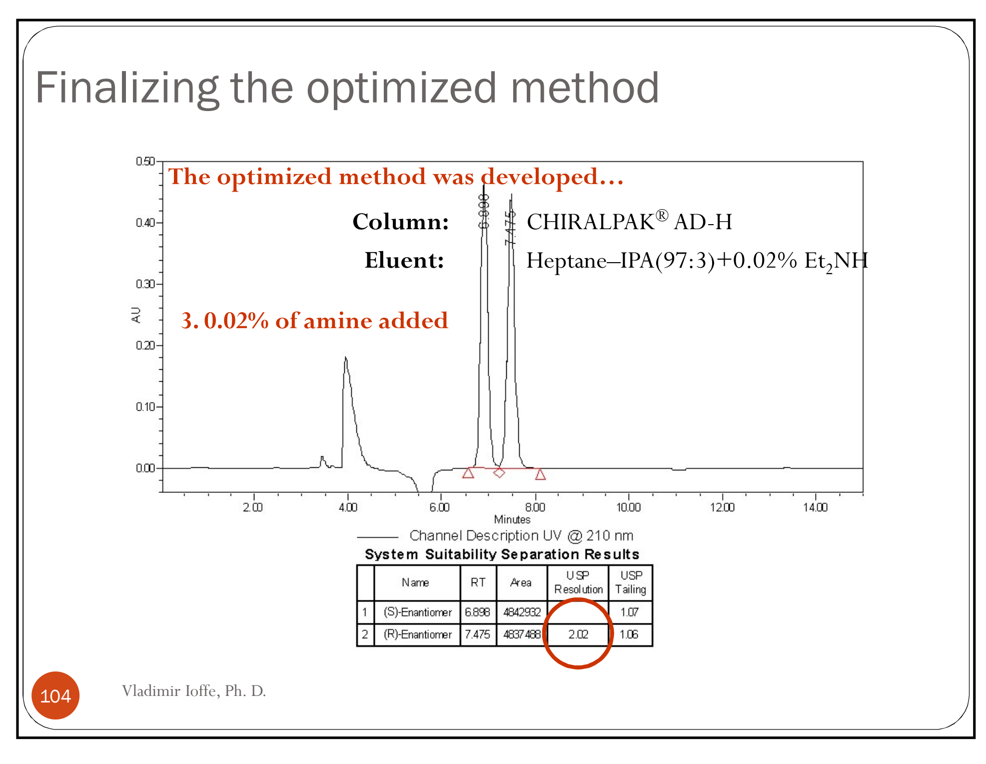


Improving separation – combination of both
Almost baseline separation achieved:
The method almost may be used.
Further adding of competitive
amine does not cause further improvement.
Next step – use of more effective column: AD-
H instead of AD (particle size 5 µm)
AU
0.20
0.15
0.10
0.05
0.00
-0.05
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
Minutes
Column: CHIRALPAK® AD
Eluent: Heptane – IPA (97:3) + 0.02% Et_2NH
101
Vladimir Ioffe, Ph. D.



Improving separation – particle size of a column
After check of chromatographic performance
under various conditions…
1. No amine added
AU
0.20
0.15
0.10
0.05
0.00
-0.05
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
Minutes
Column: CHIRALPAK® AD-H
Eluent: Heptane – IPA (97:3)
102
Vladimir Ioffe, Ph. D.



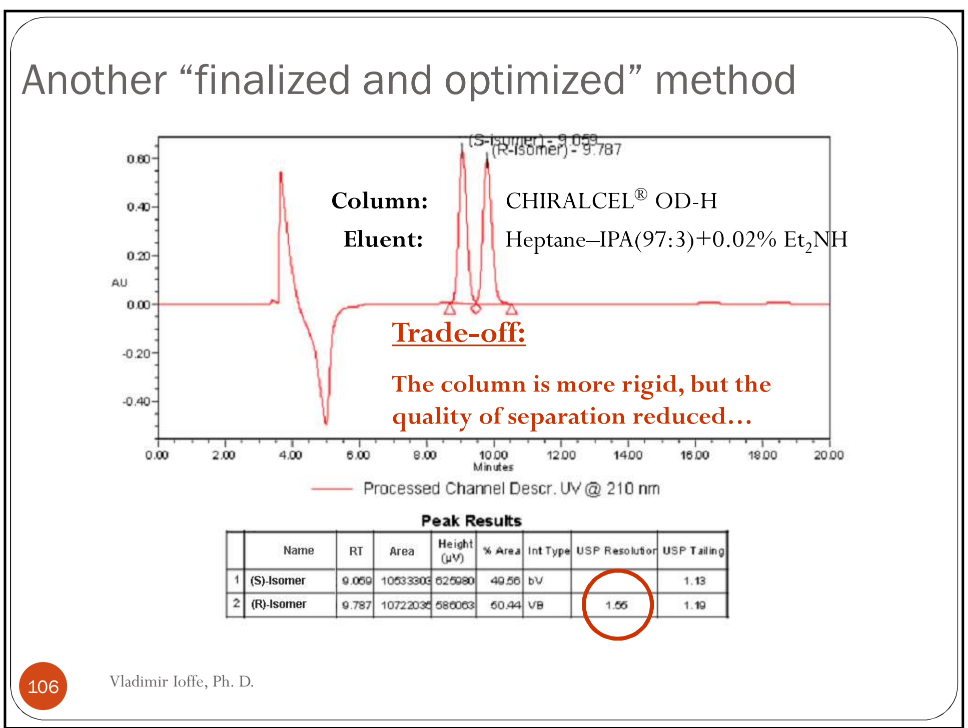


	Name	RT	Area	USP Resolution	USP Tailing
1	(S)-Enantiomer	6.898	4842932		1.07
2	(R)-Enantiomer	7.475	4837488	2.02	1.06

Was the method really finalized and optimized?

- Actually, this quality of chromatographic separation is sufficient for issuing the method
- Unfortunately, it was found, that the column **CHIRALPAK® AD-H** undergoes rapid aging, especially if the testing of enantiomeric composition is performed not for API, but for a formulated drug product or for bioanalytical purposes
- Therefore we tried to run the method using the column **CHIRALCEL® OD-H**, which appears to be more stable (Cellulose Vs. Amylose basis)

Another “finalized and optimized” method



Peak Results

	Name	RT	Area	Height (µV)	% Area	Int Type	USP Resolution	USP Tailing
1	(S)-Isomer	9.059	10633303	625980	49.56	bV		1.13
2	(R)-Isomer	9.787	10722035	586063	50.44	VB	1.56	1.19

What is the price for such an optimization?

- In fact, the column **CHIRALCEL® OD-H (based on derivatized cellulose coating)** is more stable, than **CHIRALPAK® AD-H (based on derivatized amylose coating)**
- However, this came on account of weaker separation: The resolution factor decreased from more than **2** to only **1.55**
- Such a low resolution is not a problem when the content of minor enantiomer is comparatively high, but it is not sufficient for monitoring of enantiomeric stability for pure enantiomers, especially in biological matrices

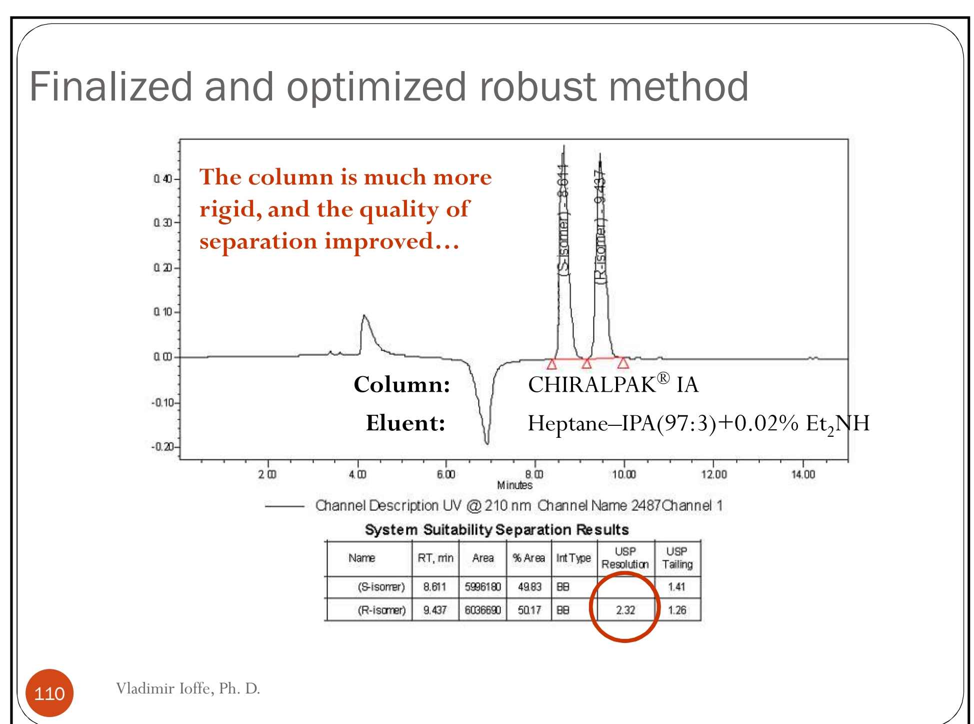
More options for method optimization?

- Fortunately for us, a new generation of polysaccharide based chiral columns has been developed: **CHIRALPAK® IA** (based on modified Amylose) and **CHIRALPAK® IB** (based on modified Cellulose)
- Chemically they correspond to **CHIRALPAK® AD-H** and **CHIRALCEL® OD-H**, respectively, and have the same derivatizing group
- The difference is, that in all the columns of previous generation (AD and OD series) silica is **physically coated with the chiral selector**, whilst in the columns of new generation **the chiral selector is chemically immobilized** to silica

More options for method optimization?

- As expected, these columns of new generation (**CHIRALPAK® IA** and **CHIRALPAK® IB**) appeared to be significantly more durable and chemically stable than **CHIRALPAK® AD (AD-H)** and **CHIRALCEL® OD (OD-H)**, but not only this
- Due to **chemical immobilization**, these columns have much more uniform covering of silica surface by chiral selector thus ensuring **higher quality of enantiomeric separations**
- These columns also allow **higher solvent flexibility**: any miscible organic solvent may be used

Finalized and optimized robust method



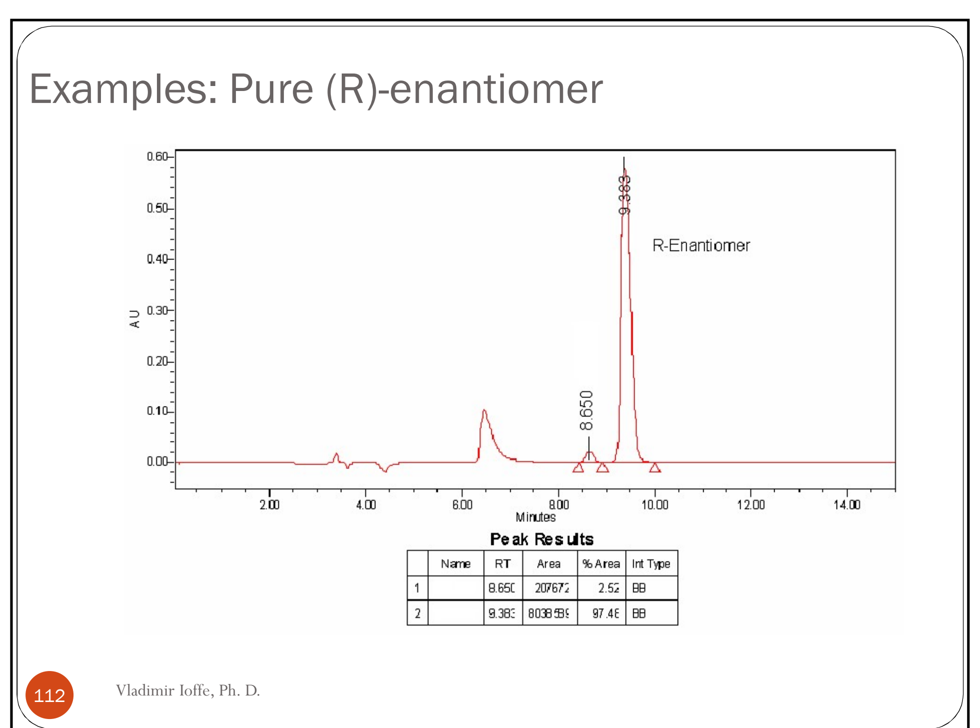
System Suitability Separation Results

Name	RT, min	Area	% Area	Int Type	USP Resolution	USP Tailing
(S-isomer)	8.611	5996180	49.83	BB		1.41
(R-isomer)	9.437	6036690	50.17	BB	2.32	1.26

Finalized and optimized robust method

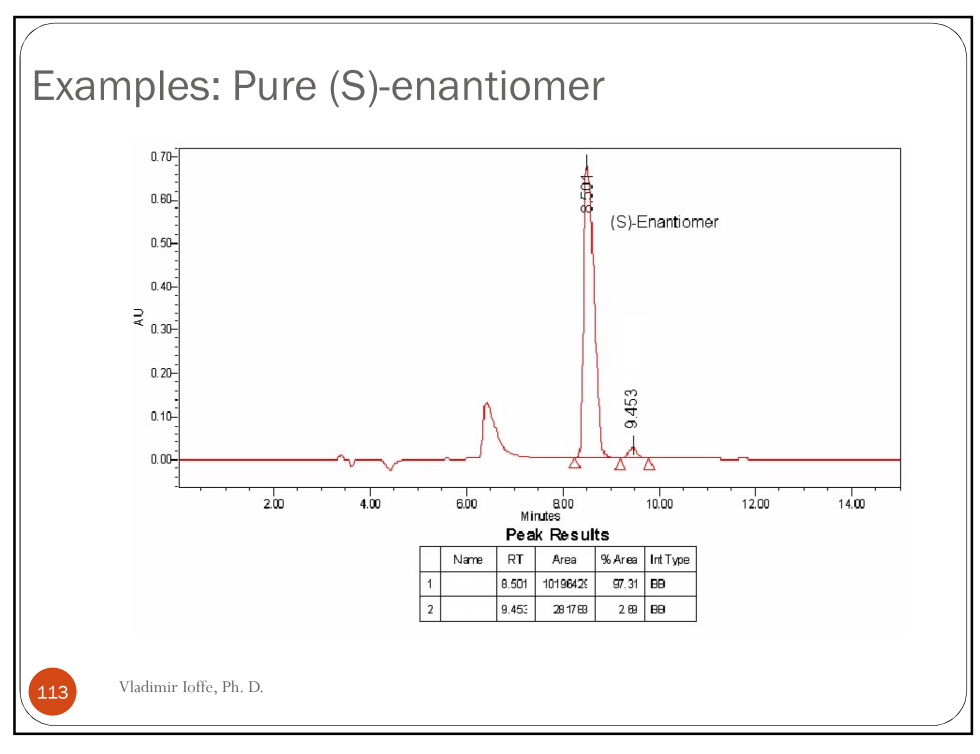
- Running the same analytical procedure on the column **CHIRALPAK® IA:**
  - Improves Resolution factor (from **2.0** to **2.3**)
  - Improves peak width (from **0.7** to **0.5** min)
  - Increases column life:
    - From 500 to 2000 sample injections in analysis of semisolid formulations with low potency
    - From 200 to 1500 sample injections in bioanalytical applications

Examples: Pure (R)-enantiomer



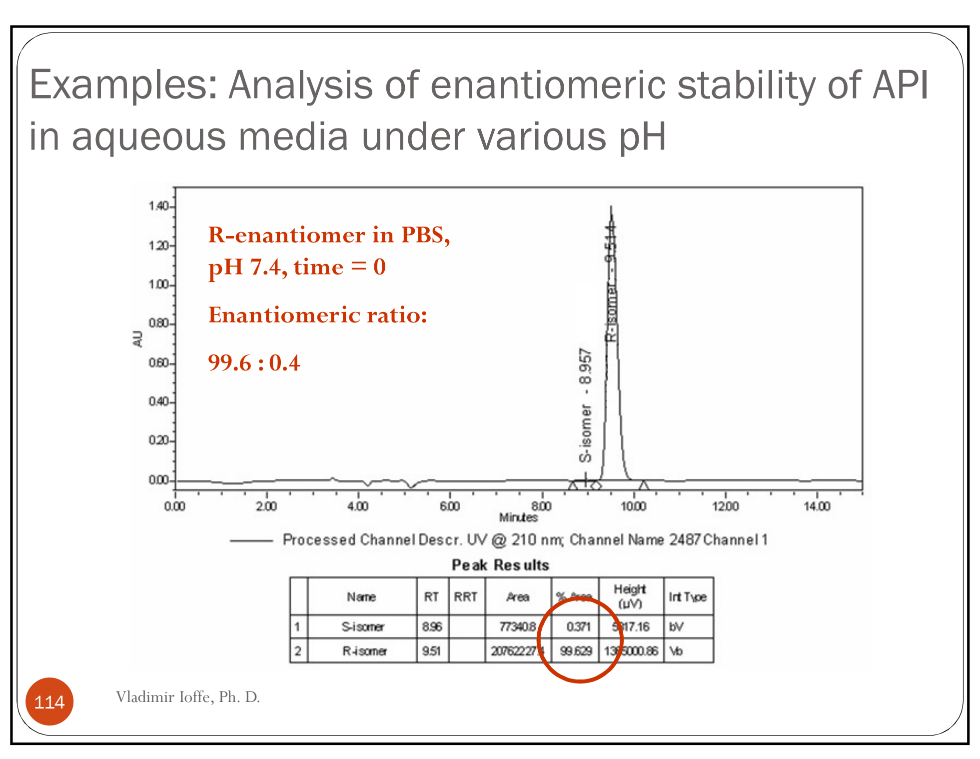
	Name	RT	Area	% Area	Int Type
1		8.650	207672	2.52	BB
2		9.383	8038589	97.48	BB

Examples: Pure (S)-enantiomer

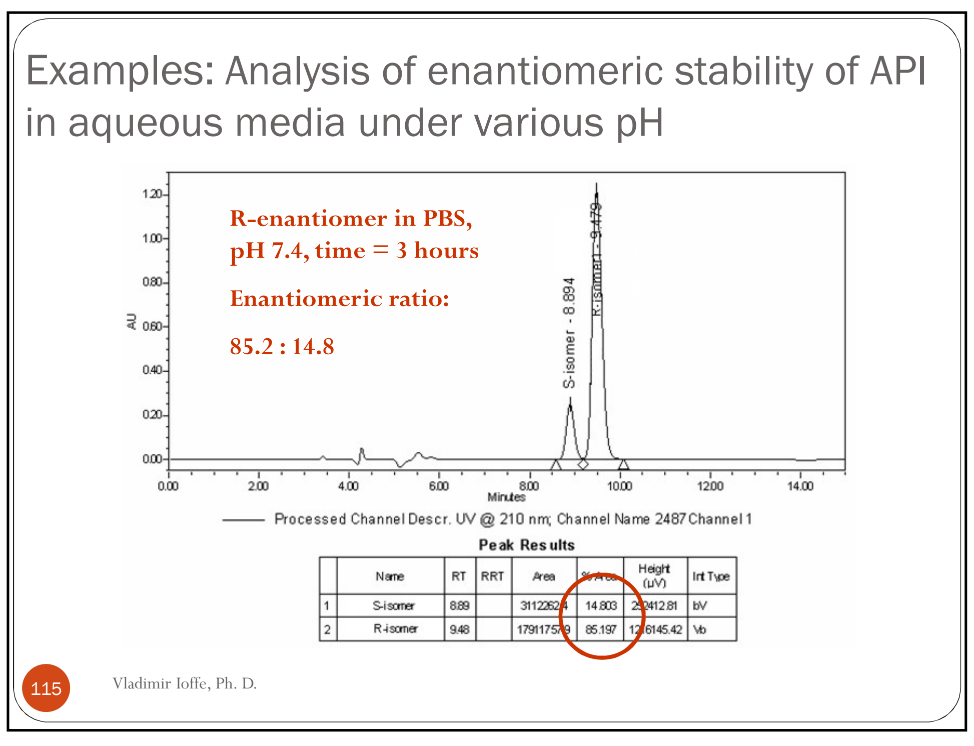


	Name	RT	Area	% Area	Int Type
1		8.501	10196429	97.31	BB
2		9.453	281783	2.69	BB

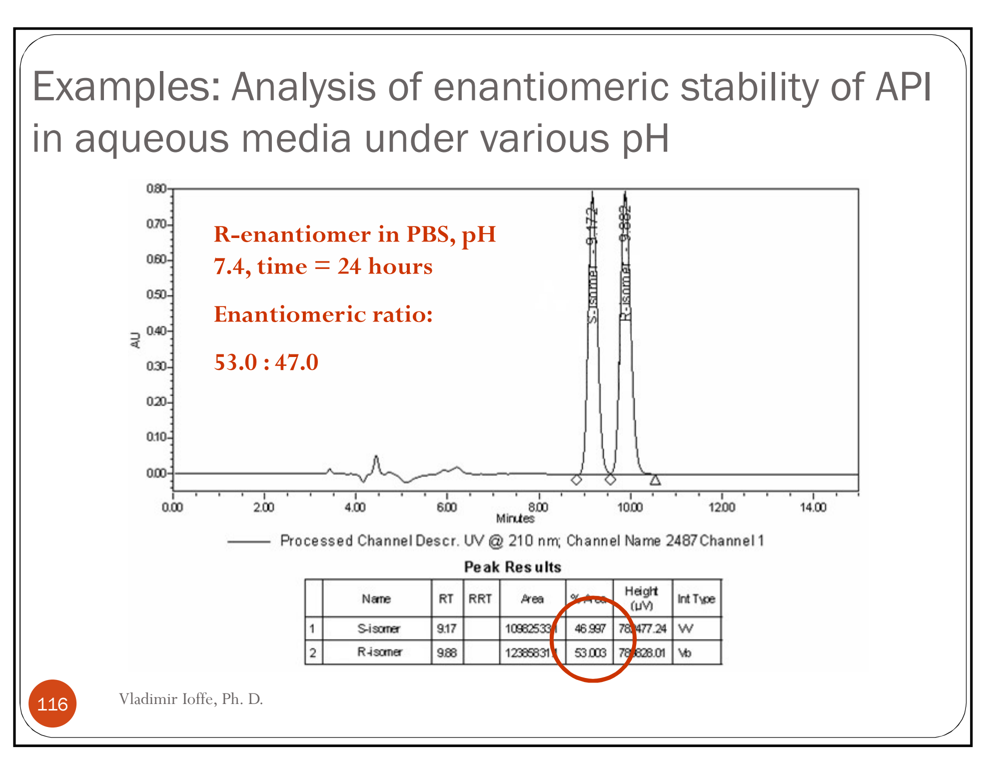
Examples: Analysis of enantiomeric stability of API in aqueous media under various pH



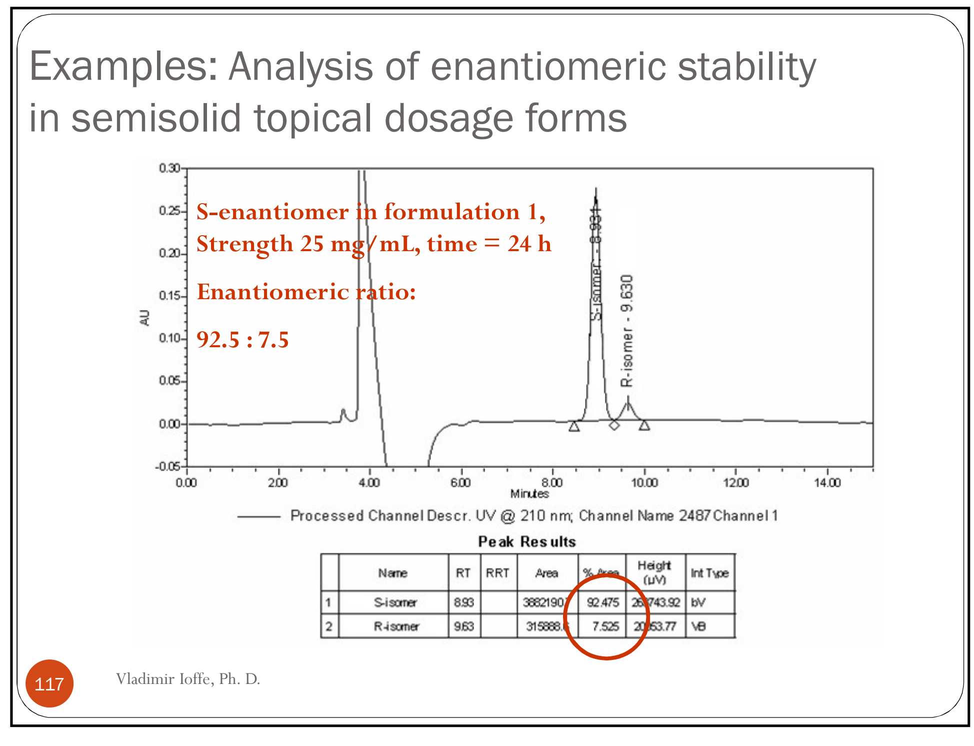
	Name	RT	RRT	Area	% Area	Height (µV)	Int Type
1	S-isomer	8.96		773408	0.371	5[illegible]17.16	bV
2	R-isomer	9.51		20762227[illegible]	99.629	13[illegible]5000.86	Vb



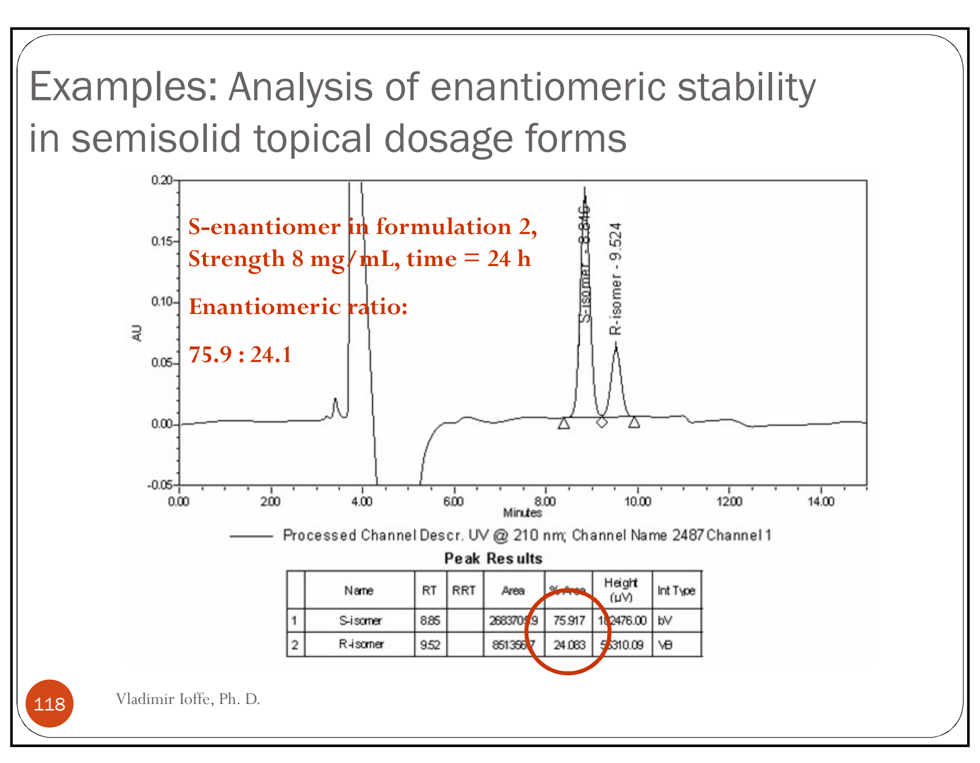
	Name	RT	RRT	Area	% Area	Height (µV)	Int Type
1	S-isomer	8.89		3112262[illegible]4	14.803	2[illegible]2412.81	bV
2	R-isomer	9.48		179117[illegible]9	85.197	12[illegible]6145.42	Vb



	Name	RT	RRT	Area	% Area	Height (µV)	Int Type
1	S-isomer	9.17		1098253[illegible]	46.997	78[illegible]477.24	VV
2	R-isomer	9.88		1238583[illegible]	53.003	78[illegible]828.01	Vb



	Name	RT	RRT	Area	% Area	Height (µV)	Int Type
1	S-isomer	8.93		3882190[illegible]	92.475	26[illegible]743.92	bV
2	R-isomer	9.63		315888.[illegible]	7.525	20[illegible]63.77	VB



	Name	RT	RRT	Area	% Area	Height (µV)	Int Type
1	S-isomer	8.85		268370[illegible]9	75.917	1[illegible]2476.00	bV
2	R-isomer	9.52		85135[illegible]7	24.083	5[illegible]310.09	VB

Diversity of Detection Techniques

- All the chromatograms illustrating the method were obtained using a "simple" UV detector
- Due to a weak chromophore of the molecules under research, one needs to apply alternative detection techniques to enhance method sensitivity
- Fortunately, mobile phase does not contain any non-volatile components
- This allows using evaporative detectors: **ELSD** or **Corona® CAD®** for low concentrations of API in drug product and **LC-MS(MS)** for bioanalytical purposes

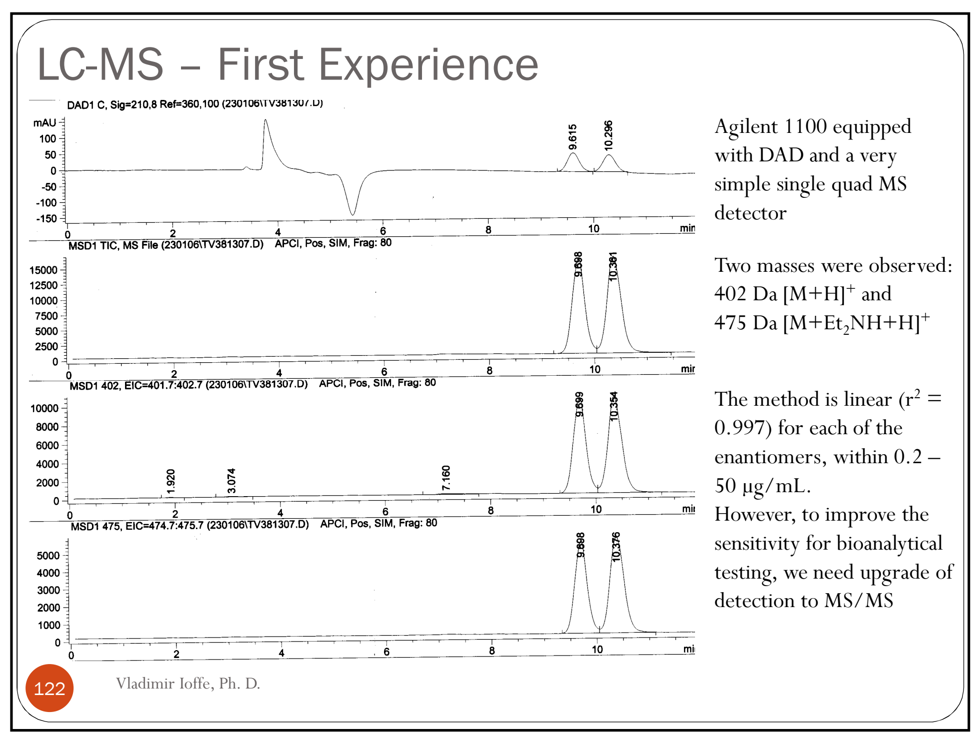
Towards LC-MS – Barriers?

- Now – what about **LC-MS** for bioanalytical purposes?
- "Mentality" barriers in mindset:
  - LC-MS for normal phase? – **Are you kidding me?**
  - Will it be sufficiently ionized in the medium which does not support ionization?
  - Well, ES doesn't work… Will it be safe enough to apply APcI (with its corona discharge!) for such a flammable (and explosive!) eluent? – **Is the ion source explosion-proof? (!!!)**
  - And – last but not least – how to **"downgrade"** the chromatography for separation of compounds having exactly the same mass (and fragmentation!)?

Towards LC-MS – overcoming the barriers

- Breaking through "Mentality" barriers in mindset:
  - LC-MS for normal phase? – **Why not?**
  - Will it be sufficiently ionized in the medium which does not support ionization? – **Maybe it worth trying first?**
  - Well, ES doesn't work… Will it be safe enough to apply APcI (with its corona discharge) for such an inflammable (and explosive!) eluent? Is the ion source explosion-proof? (!!!) – **OMG! I will be in another room!** ☺
  - And – last but not least – how to "downgrade" the chromatography for separation of compounds having exactly the same mass (and fragmentation!)? – **No way to "downgrade"… Well, if the run time is all what I pay for this!..**

LC-MS – First Experience



Agilent 1100 equipped with DAD and a very simple single quad MS detector

Two masses were observed: 402 Da $[M+H]^+$ and 475 Da $[M+Et_2NH+H]^+$

The method is linear (r^2 = 0.997) for each of the enantiomers, within 0.2 – 50 µg/mL.
However, to improve the sensitivity for bioanalytical testing, we need upgrade of detection to MS/MS

LC-MS/MS – First Upgrade

Type of ion	Structure	Molecular mass, Da
Protonated TV-3813 (parent ion)		**402.0**
Fragment ion		**106.0**
Fragment ion		**117.0**

Detector: Sciex AB API 300 LC MS/MS

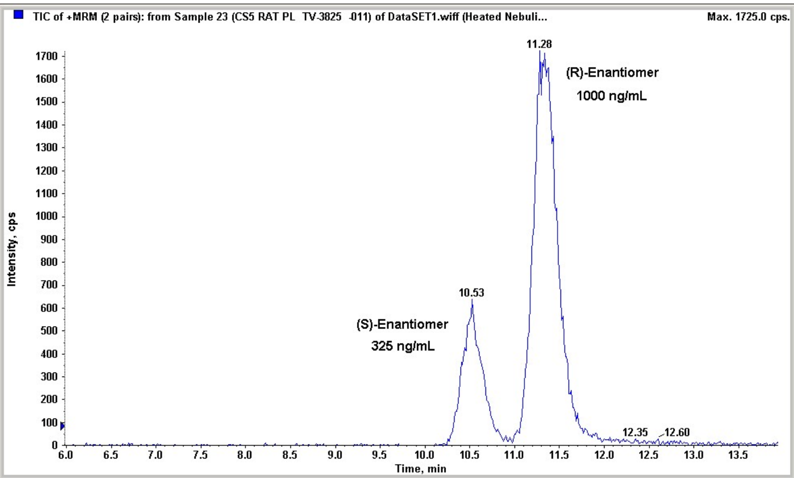
Concentration range: **20 to 1000 ng/mL** (for each enantiomer), based on the levels of TV-3813 observed in plasma samples.

Mode	Accuracy (%)	Correlation Coefficient
SIM (Q1)	**91.1-107.3**	**0.9982**
MRM (Q3)	**94.0-107.7**	**0.9984**

However, further method improvement was required for metabolic studies in animals and humans

LC-MS/MS – First Upgrade

Typical Chromatogram of Calibration Standards Solution Prepared on Rat Plasma



Detection: Dual Mass Mode Q1 → Q3 = 402.0 →117.0 + 402.0→ 106.0

LC-MS/MS – Further Improvement

Fragmentation of Analyte and Internal Standard:

Compound	Q1 (Da)	Q3 (Da)
(S)- or (R)- TV-3813	402.0	106.0 117.0
All-^{13}C-TV-3813	426.7	112.0 138.0

Detectors:
Sciex AB API 5000
Sciex AB Qtrap 4000

Use of all-^{13}C-labeled TV-3813 (racemate) as internal standard

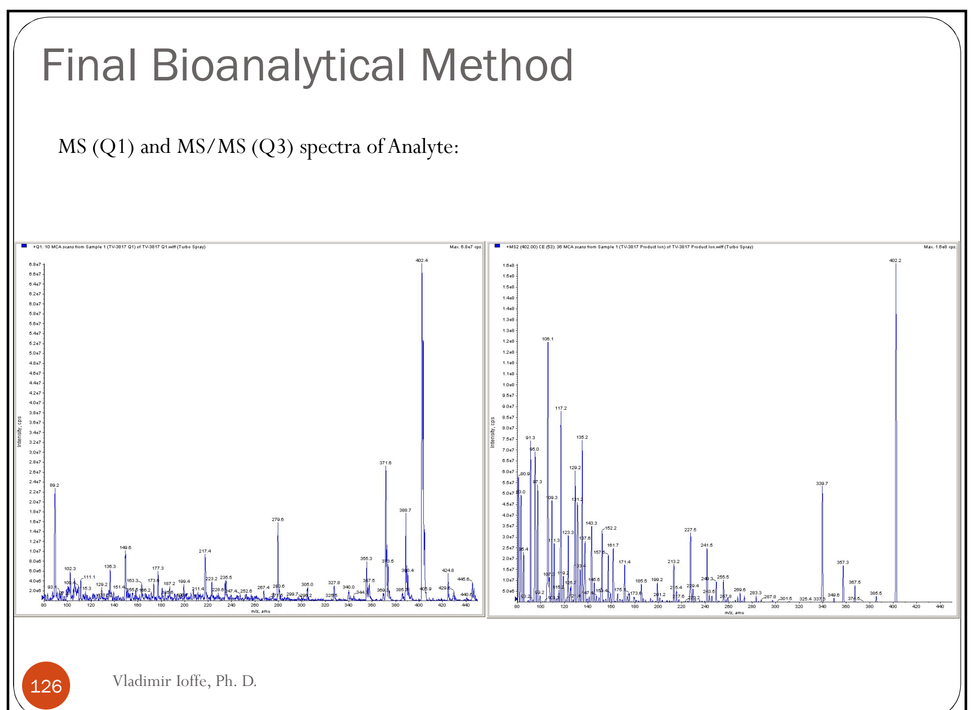
Concentrations Range	Max CV (%)	Correlation Coefficient
QL Level	7.3	$\geq$ 0.9972
Other Concentrations	5.2	

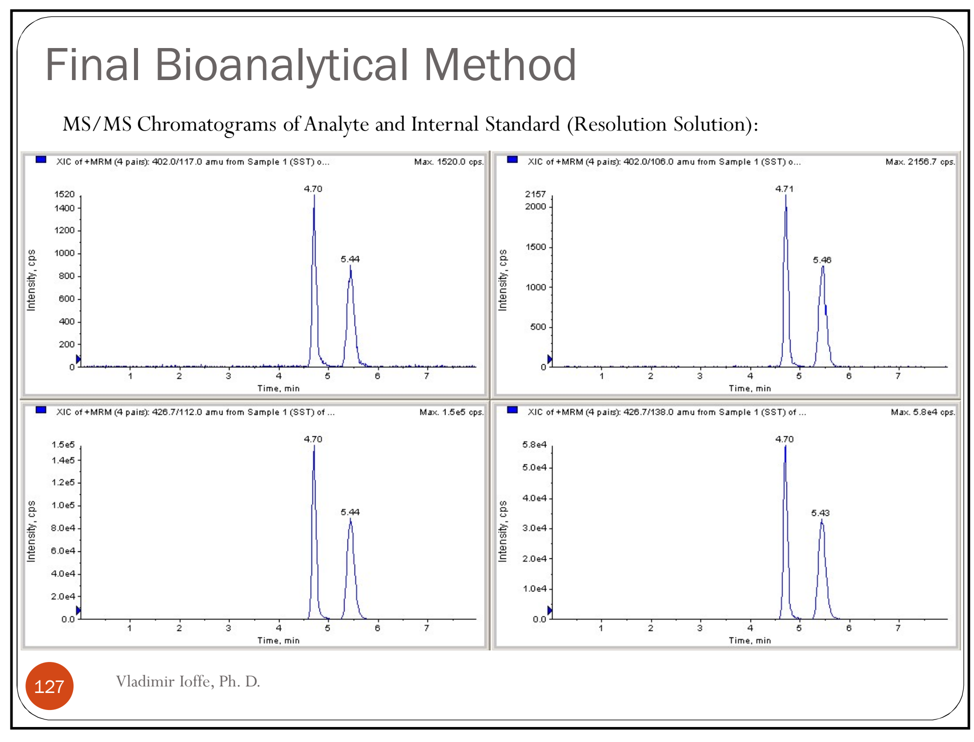
Concentration range: **10 to 2000 ng/mL** (for each enantiomer), based on the levels of TV-3813 observed in plasma samples.

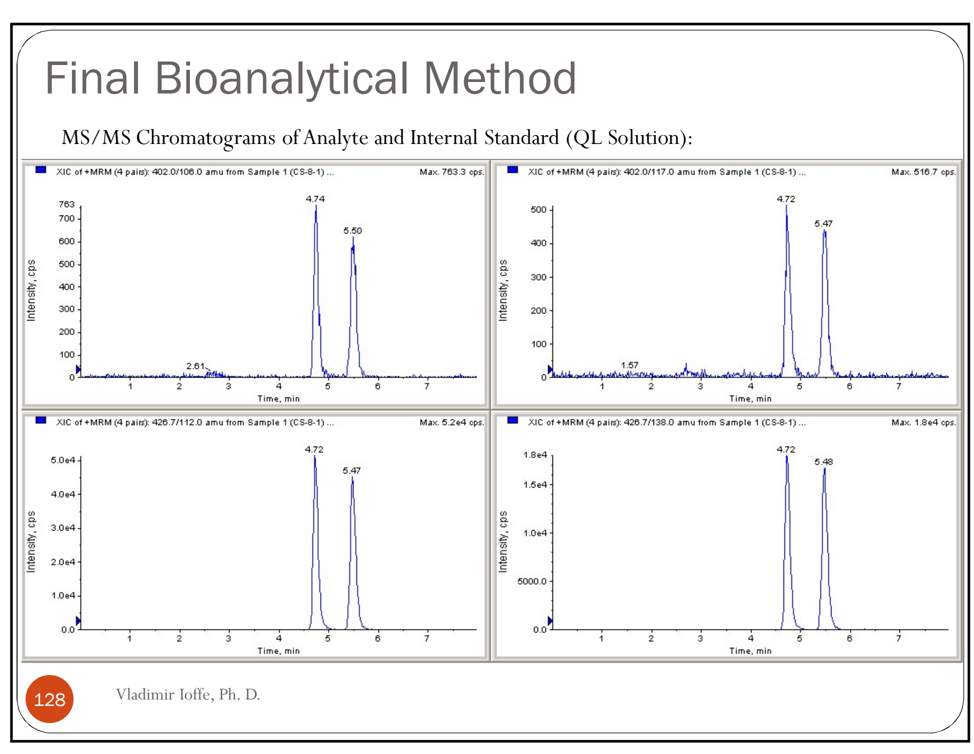
The improved method is illustrated on the following slides:

Final Bioanalytical Method

MS (Q1) and MS/MS (Q3) spectra of Analyte:




Final Bioanalytical Method
MS/MS Chromatograms of Analyte and Internal Standard (Resolution Solution):
XIC of +MRM (4 pairs): 402.0/117.0 amu from Sample 1 (SST) o...
Max. 1520.0 cps.
XIC of +MRM (4 pairs): 402.0/106.0 amu from Sample 1 (SST) o...
Max. 2156.7 cps.
XIC of +MRM (4 pairs): 426.7/112.0 amu from Sample 1 (SST) of ...
Max. 1.5e5 cps.
XIC of +MRM (4 pairs): 426.7/138.0 amu from Sample 1 (SST) of ...
Max. 5.8e4 cps.
Intensity, cps
Time, min
4.70
5.44
4.71
5.46
4.70
5.44
4.70
5.43
127
Vladimir Ioffe, Ph. D.


Final Bioanalytical Method
MS/MS Chromatograms of Analyte and Internal Standard (QL Solution):
XIC of +MRM (4 pairs): 402.0/106.0 amu from Sample 1 (CS-8-1) ...
Max. 763.3 cps.
XIC of +MRM (4 pairs): 402.0/117.0 amu from Sample 1 (CS-8-1) ...
Max. 516.7 cps.
XIC of +MRM (4 pairs): 426.7/112.0 amu from Sample 1 (CS-8-1) ...
Max. 5.2e4 cps.
XIC of +MRM (4 pairs): 426.7/138.0 amu from Sample 1 (CS-8-1) ...
Max. 1.8e4 cps.
Intensity, cps
Time, min
4.74
5.50
2.61
4.72
5.47
1.57
4.72
5.47
4.72
5.48
128
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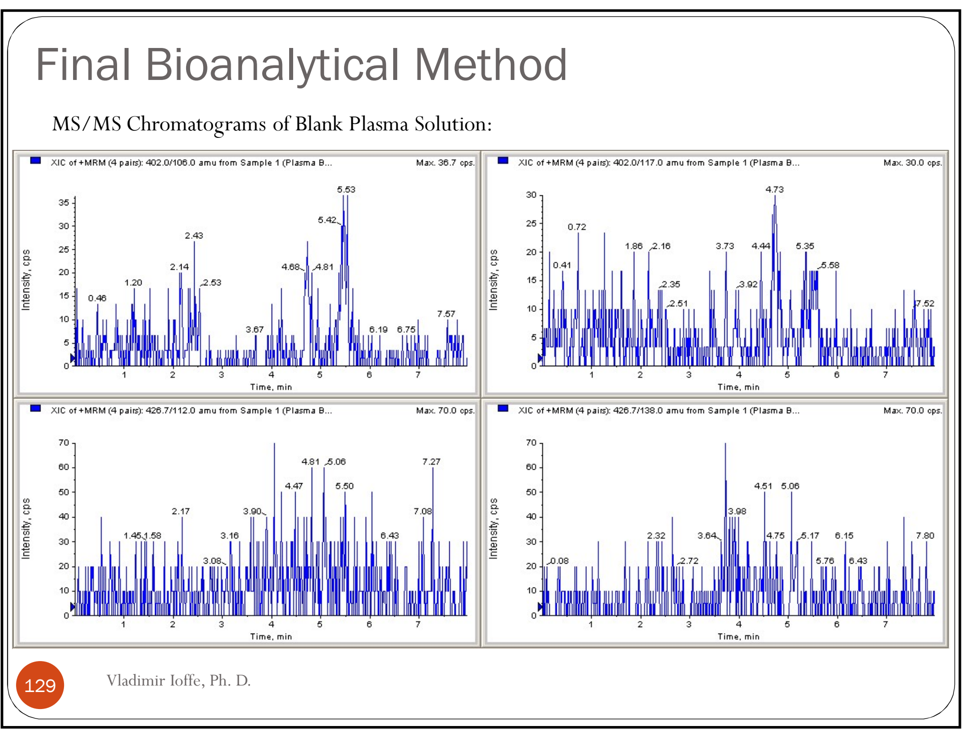
Final Bioanalytical Method

MS/MS Chromatograms of Blank Plasma Solution:



Summary

- A feasible HPLC method using an enantioselective stationary phase has been developed under normal phase (NP) chromatographic conditions for testing the enantiomeric composition of long-chain aliphatic esters of α-amino acids having an aromatic substituent in α-position.
- Due to aromatic substituent in α-position, the analytes may undergo a noticeable racemization in the aqueous media at pH close to and above pKa, which avoids using a reverse phase (RP) enantioselective chromatography.
- The method comprises silica-based NP chromatographic columns physically coated with the polymeric chiral selector (amylose or cellulose derivatives) or, alternatively, a silica support onto which the polymeric chiral selector (polysaccharide derivatives) has been immobilized.
- Optimized column selection and optimization of the mobile phase composition to achieve reliable separation between (R)- and (S)- isomers are discussed.
- The method was upgraded to be run with MS and MS/MS detectors equipped with APCI ion source.
- The developed LC-MS method has been used for investigating in-vivo racemization in metabolic studies.

Part V: Challenges of Compatibility of HPLC Method with HPLC Instrument

Unexpected Troubleshooting Session, or:

What Happened to Our Chromatogram?

At the beginning everything was fine…

Determination of **Oleyl Alcohol** in the drug substance and drug product as a process impurity or a degradation product

Asahipak C8P-50 4D, 5 µm, 4.6 x 150 mm

Water–MeOH–AcN 20:40:40 (v/v), 1.0 mL/min

Oleyl Alcohol - 6.123

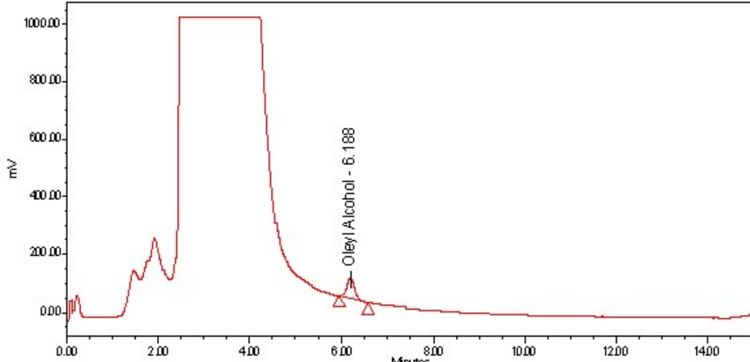
Minutes

Processed Channel Descr. Sedex light scattering detector

	Name	RT	Area	Int Type	Result Id	USP Tailing
1	Oleyl Alcohol	6.123	592643	BB	13377	1.20

Vladimir Ioffe, Ph. D.

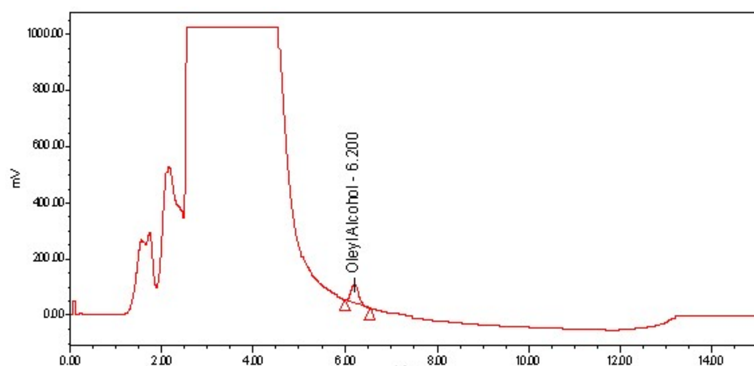
We were very surprised when the problem occurred…



Then, after a long period of work with this method without any problems, we began to pay attention, that the peak of the main substance (which is in overrange) began to broaden

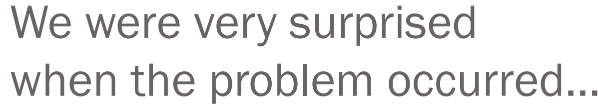
Vladimir Ioffe, Ph. D.

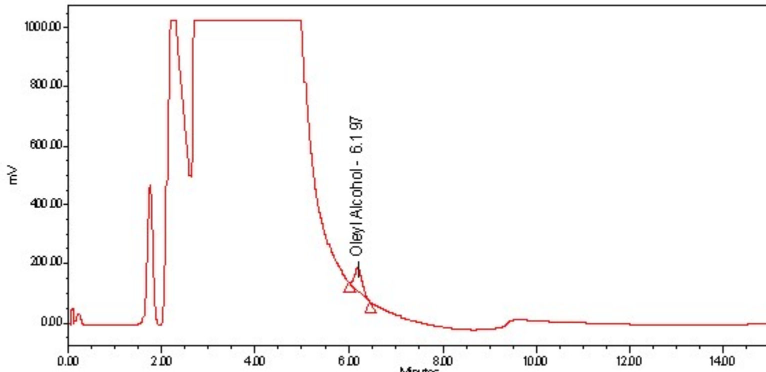
We were very surprised when the problem occurred…



Then, after a long period of work with this method without any problems, we began to pay attention, that the peak of the main substance (which is in overrange) began to broaden

Vladimir Ioffe, Ph. D.





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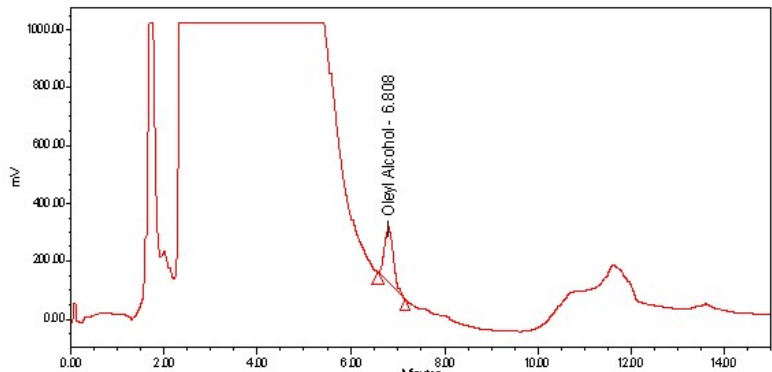

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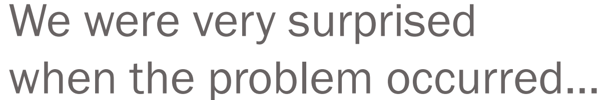

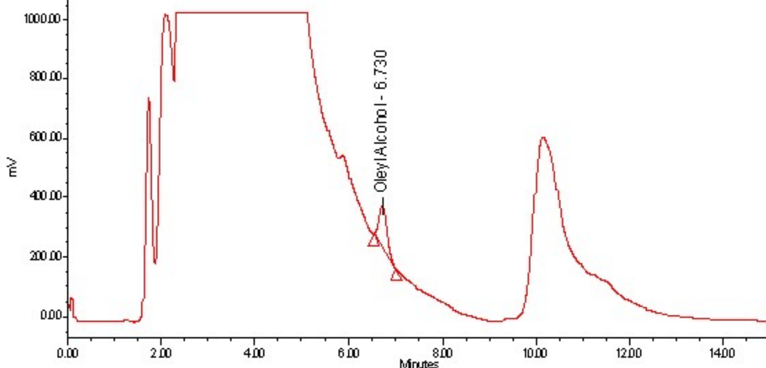

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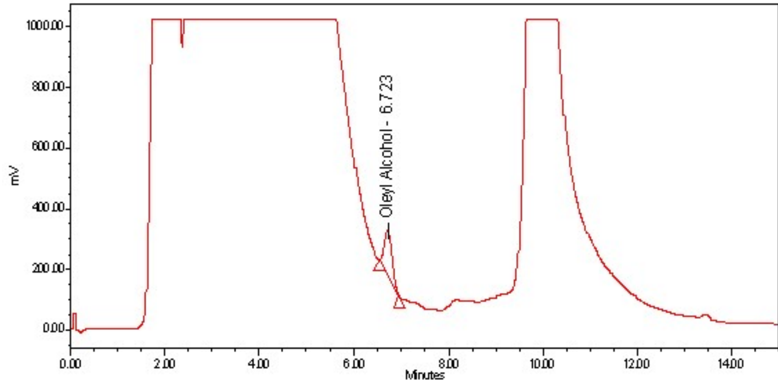

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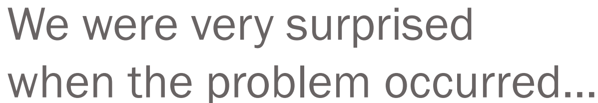

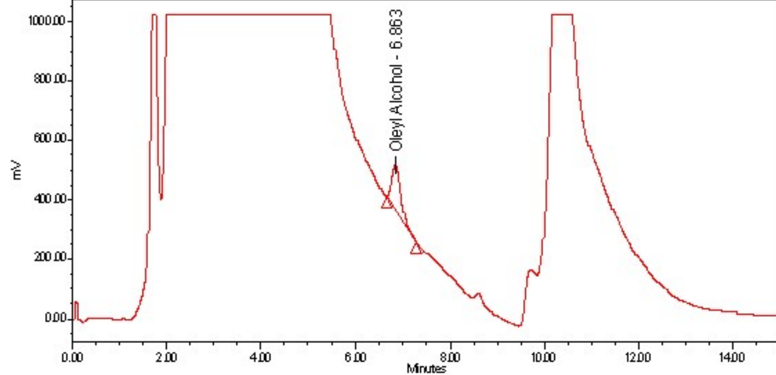

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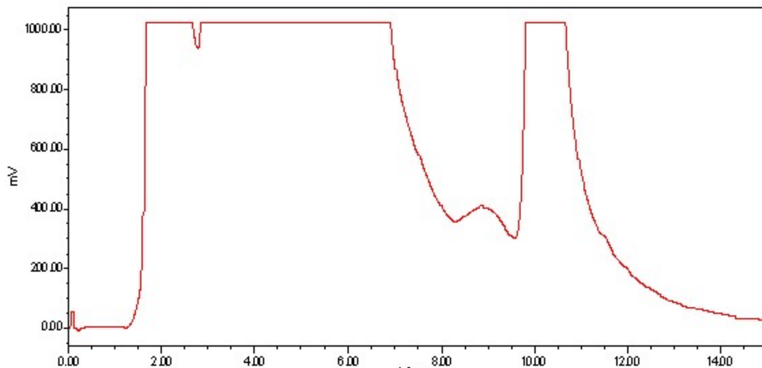
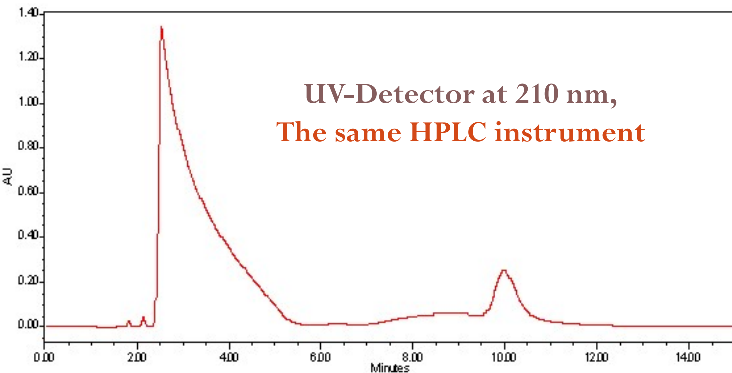


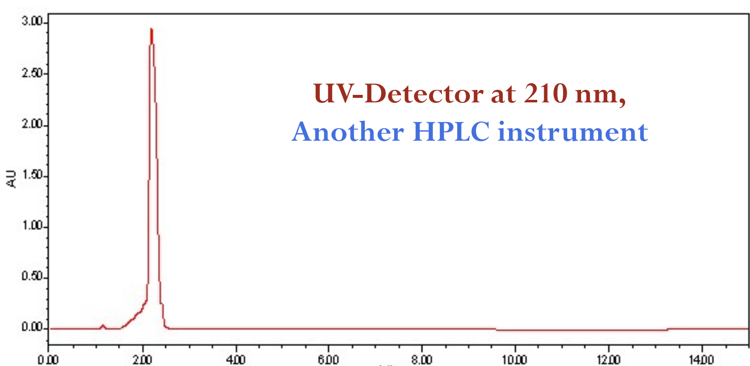
Then, after a long period of work with this method without any problems, we began to pay attention, that the peak of the main substance (which is in overrange) began to broaden. Later an additional peak (as a "second injection", with a delay of about 10 min) appeared, which persistently grew and, finally, the main peak swallowed the peak of Oleyl Alcohol…

A troubleshooting session began…



We have proven, that there is **no problem with detector, neither with column or mobile phase**. The run on UV-detector on the same HPLC instrument (although unable to detect Oleyl alcohol) has also shown a deteriorated main peak and an evidence of a "double injection" – an additional peak after about 10 min

A troubleshooting session began…



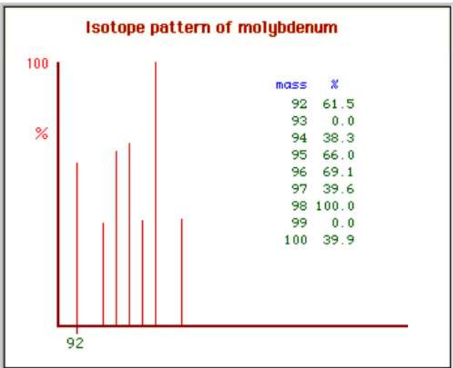
When we performed this run on another HPLC instrument, which was never exposed to this method, the picture looked quite different, as it was at the early stages on the "old" instrument

Assumptions from troubleshooting session

- Troubleshooting of this problem provided us with an evidence, that the cause for such peak deterioration is the materials of injection system of HPLC, mainly, the stainless steel parts: needle and diffusers of injector valves
- The first problem is the stickiness of the main analyte to metal surfaces due to its high lipophilicity, supported by low content of water in the mobile phase
- The additional problem is presence in the molecule of main analyte of the elements of **α-amino acids**, which can form complexes with one of the metals of this type of stainless steel – **Molybdenum**, and thus affix to the surface of the firmware of the HPLC system, namely, injector assembly
- As a result, the peak of the main compound broadened, and an effect of "second injection" also appeared

Confirmation of assumptions

- As an evidence of this "complexation" in the injector, we found a mass-spectrum of Molybdenum while analyzing our chromatograms on the LC-MS instrument
- In the mass-chromatogram of the main compound under RP conditions, we found, next to the main substance peak, a multiplet corresponding to the isotope pattern of the natural abundance of isotopes of Molybdenum



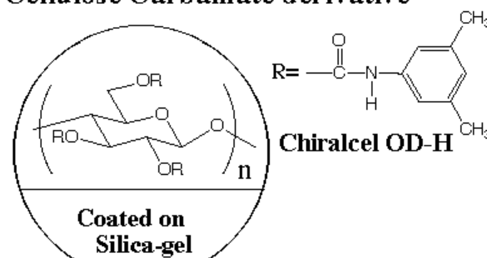
Redevelopment of the method?

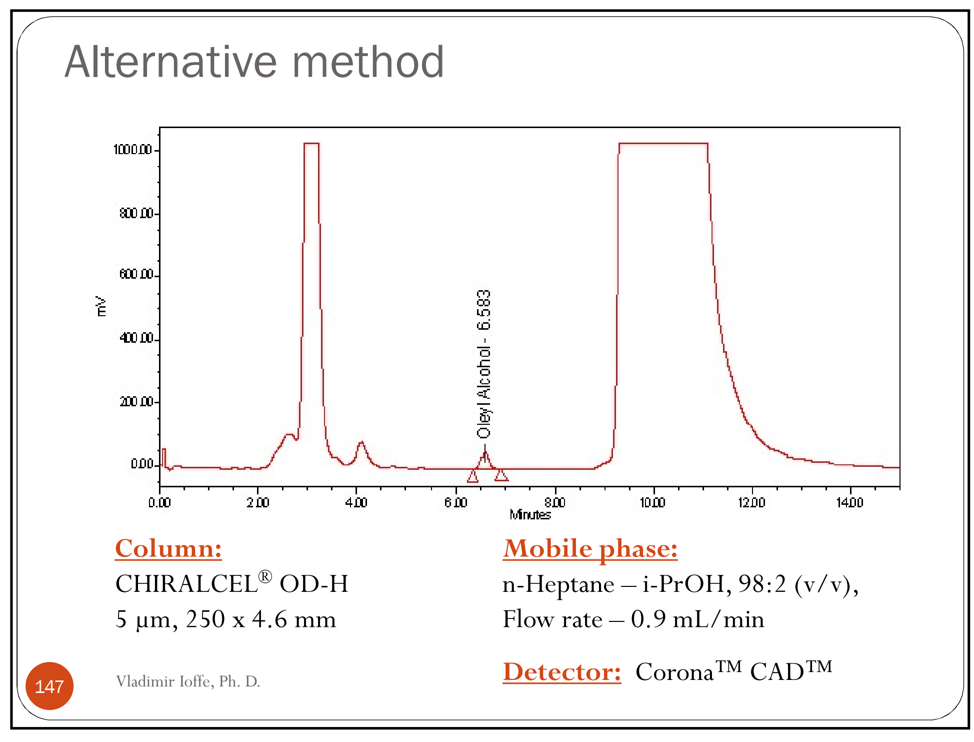
- The main idea for redevelopment was: to **invert the order of elution** of the peaks of the main analyte and Oleyl Alcohol and to exclude water and acidic pH from the mobile phase – the factors that support Molybdenum complex formation
- Such an inversion, generally, could be achieved if, instead of **Reverse Phase**, one applies **Normal Phase** separation
- Unfortunately, none of the usual ("classic") Normal Phase columns (Silica, Cyano, Amine, etc.) could supply a retention mechanism for these very lipophillic compounds
- An element of **hydrophobic interaction** is missing in these phases, to ensure reliable retention and separation of such molecules, bearing a long aliphatic chain

Redevelopment of the method!

- Therefore, a most lipophilic column based on modified polysaccharides, used for normal phase enantioselective separations, Chiralcel® OD-H of Daicel (JP) was chosen
- Its side chains, containing very lipophilic substituent, should provide the required hydrophobic interactions to ensure the desired retention mechanism

Cellulose Carbamate derivative





Alternative method
1000.00
800.00
600.00
400.00
200.00
0.00
mV
Oleyl Alcohol - 6.583
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
Minutes
Column:
CHIRALCEL® OD-H
5 µm, 250 x 4.6 mm
Mobile phase:
n-Heptane – i-PrOH, 98:2 (v/v),
Flow rate – 0.9 mL/min
Detector: Corona™ CAD™
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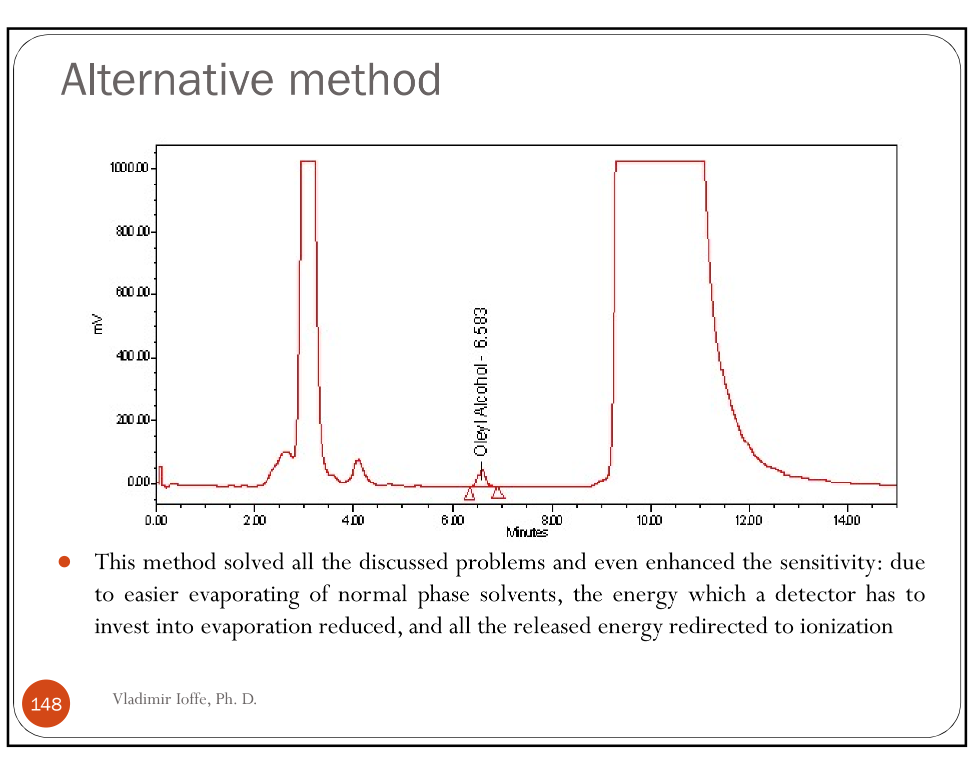

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Minutes
This method solved all the discussed problems and even enhanced the sensitivity: due to easier evaporating of normal phase solvents, the energy which a detector has to invest into evaporation reduced, and all the released energy redirected to ionization
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Instead of conclusions…

- ***Sometimes an industrial analyst has to step off the beaten track…***
- ***Working under high pressure, we are often using only the methods which we have always used…***
- ***Today we are exposed to such a diversity of instruments and techniques…***
- ***Trying something new and non-trivial – always pays (at the end)…***

Closing Remarks

A short lesson in Gematria:

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26

K	N	O	W	L	E	D	G	E
11	14	15	23	12	5	4	7	5

= **96%**

H	A	R	D	W	O	R	K
8	1	18	4	23	15	18	11

= **98%**

A	T	T	I	T	U	D	E
1	20	20	9	20	21	4	5

= **100%**

Thank You For Your Attention And Interest