LVI of non-miscible diluents in RPLC

or simply

On-line RP-SLE / RPLC ?

Principles & Applications

Andrei Medvedovici, Paul Lazar, Stefan Udrescu

University of Bucharest
Faculty of Chemistry
Department of Analytical Chemistry

December 13th, 2013

Why LVI?

↗ V_{inj} for a given C_A ⇒ ↗ m_A ⇒ ↗ $C_A^{M.Ph.}$ ⇒ ↗ D.R.

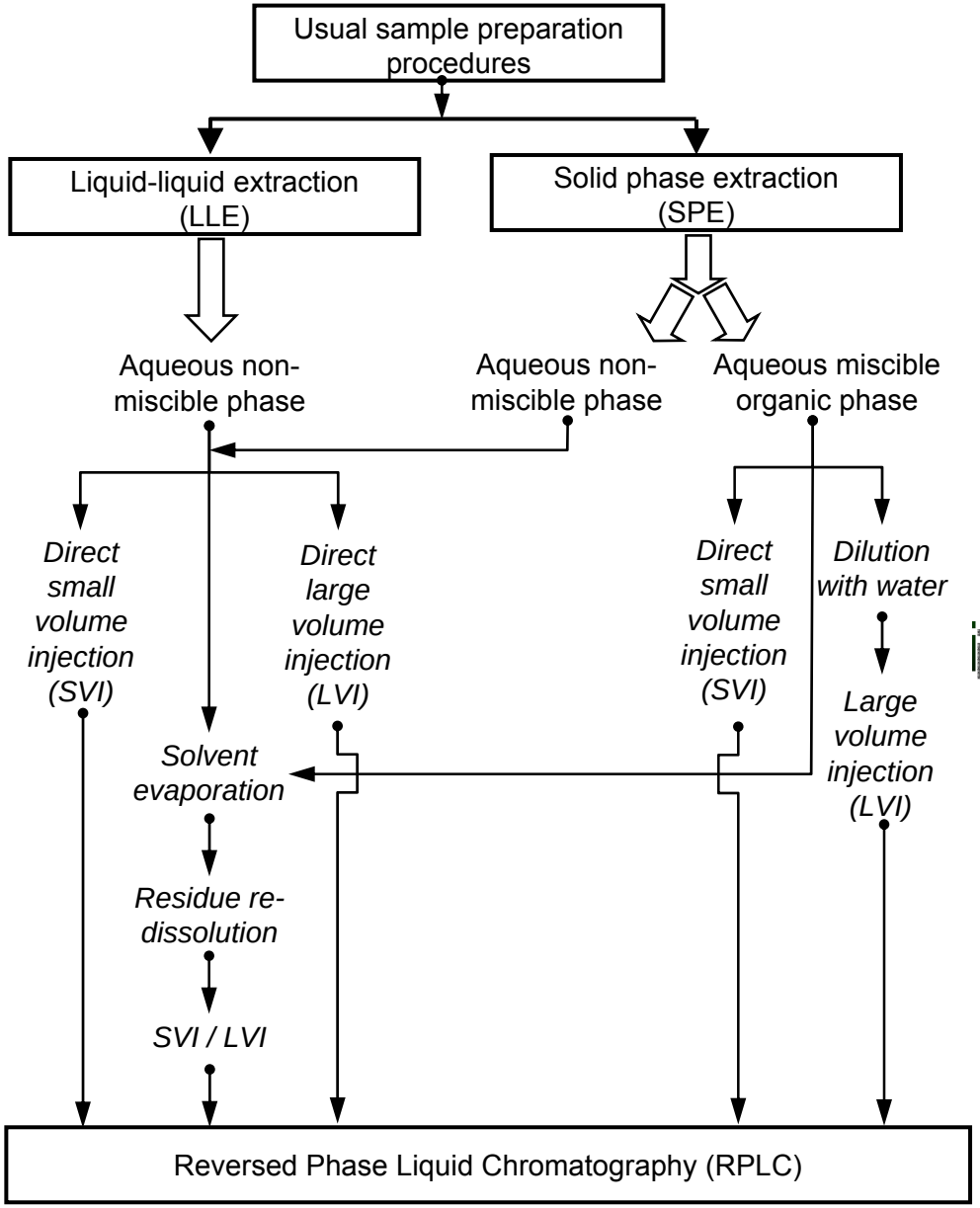
↗ D.R. ⇒ ↗ Sensitivity

thus

↗ V_{inj} ⇒ ↗ Sensitivity

The simplest way to enhance on Sensitivity is to increase Vinj !

Why using diluents non-miscible with the mobile phase?



Most of the sample preparation techniques provide target compounds in water non-miscible organic solvents!

The golden rule of the thumb in RPLC injection!

For achieving Large Volume Injection (LVI) in liquid chromatography the sample diluent should be **entirely miscible to** and **weaker than** the mobile phase composition at the beginning of the separation process.

Alternative opinions inviting to additional investigations!

Injecting Organic Solvents in Reversed-Phase

A reader wrote me recently asking what would happen if he injected his sample, which came dissolved in hexane, into a reversed-phase mobile phase of methanol-buffer. The first answer is that this is not a good habit, but is it possible? We'll see.

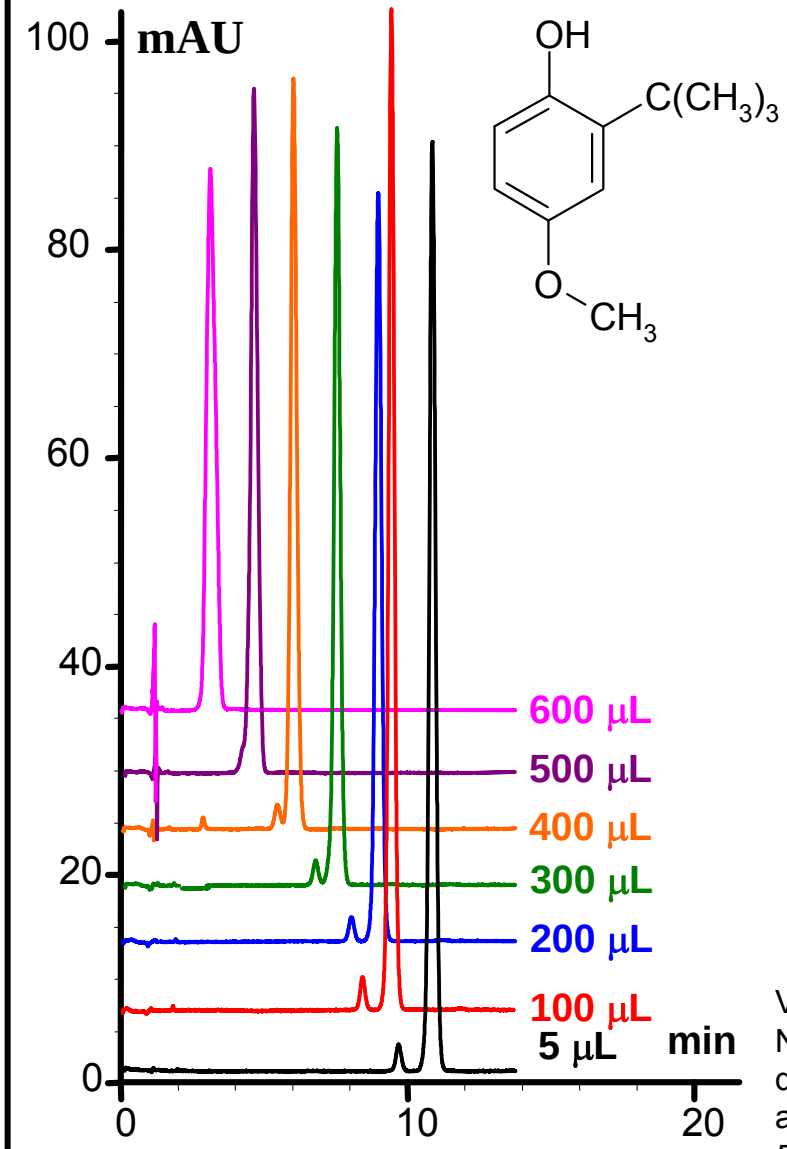
is the injection solvent much stronger than the mobile phase, but it is not miscible either. This sounds like a disaster waiting to happen – and it may be. But not so fast! If the injection volume is small enough, it may be possible to disperse the injection solvent in the mobile phase sufficiently that acceptable peak shape results. This will be a matter of trial and error. I would start by making a solution of my sample in hexane at a high enough concentration that I get a good response, even at very small volumes. First, inject the sample dissolved in mobile phase as a reference. Then inject 1 µL, 2 µL, 5 µL, 10 µL, and 20 µL of the hexane solution and see what happens. I expect that 1 µL will be acceptable, but at some volume, the peaks will start coming out too early and will be distorted, as in Figure 1(a). No, it's not ideal, but it may work. I remember doing exactly this with a sample we received that was dissolved in toluene. We already had a method for the same compound as a reversed-phase method. If I recall correctly, we were able to run with 5-µL injections and obtain acceptable results.

My favorite chemistry quote is from Izaak Kolthoff, considered by many to be the father of analytical chemistry: "Theory guides, experiment decides." Here is a good example of that – no, hexane is not compatible with a methanol-water mobile phase, but under the right conditions, you just might get away with it.

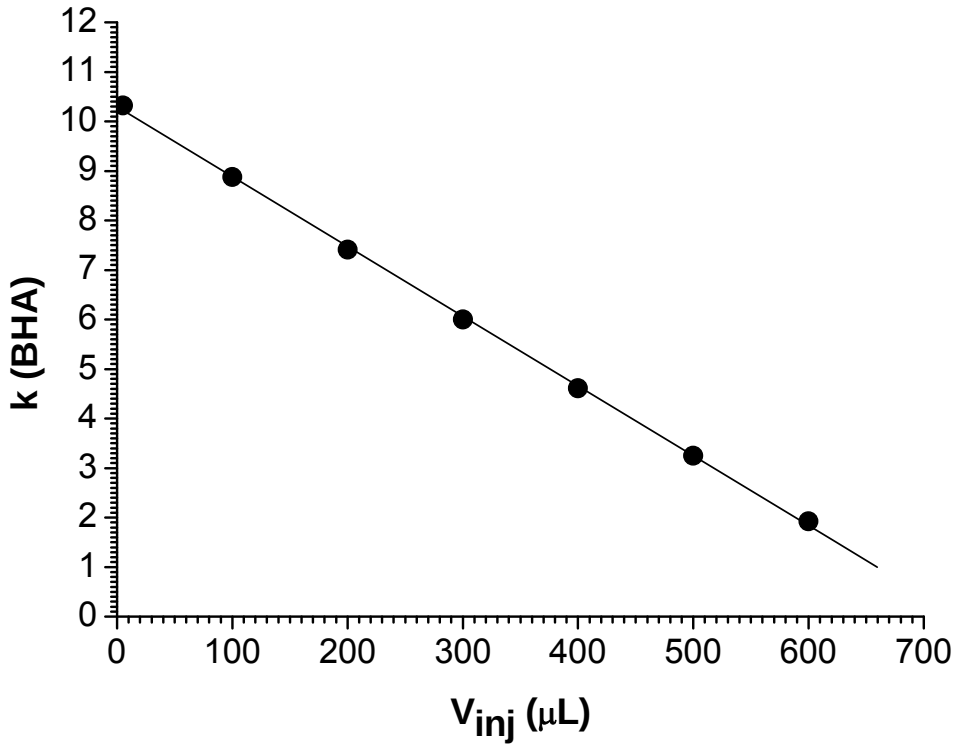
John Dolan's
HPLC Solutions

separation | science
new horizons in chromatography and detection techniques

Experimental facts!



Column: Zorbax Eclipse C-8 150 mm x 4.6 mm x 5 µm;
Mobile Phase: ACN : aq. 0.1% H_3PO_4 = 4/6 (v/v);
Flow Rate: 1 mL/min; Detection: UV – 291 nm;
Diluent (D) = *i*-octane; V_{inj} = 5 – 600 µL



V. David, C. Barcutean, C. Georgita, A. Medvedovici, Non-miscible solvent LVI-HPLC/DAD method for determination of butylated hydroxyanisole in lovastatin and simvastatin pharmaceutical formulations, *Rev. Roum. Chim.*, 5, 445-451 (2006).

Retention linearly decreases with the injection volume increase!

Possible explanations: version 1 - the partition model

k_A *(Retention Factor)* = K_A *(Partition Constant)* x $V_{S.Ph.} / V_{M.Ph.}$

D is practically totally partitioned in the S.Ph. and exhibits similar properties; consequently:

$$V_{S.Ph.}^{real} = V_{S.Ph.} + V_{inj}^{Diluent}$$

$$k_A = K_A \times (V_{S.Ph.} + V_{inj}^{Diluent}) / V_{M.Ph.}$$

$V_{inj}^{Diluent}$ ↗ *than* k_A ↗

The model does not fit to experimental results!

Possible explanations: version 2 - the adsorption model

$A_{(D)} \leftrightarrows A_{(M.Ph.)}$ [1]

$A_{(M.Ph.)} + L_{(S.Ph.)} \leftrightarrows A^*L_{(S.Ph.)}$ [2]

if assuming [D] >> [A] and log P^D > log P^A

$i\ D_{(M.Ph.)} + L_{(S.Ph.)} \leftrightarrows D_i^*L_{(S.Ph.)}$ [3]

[1] ⟷ ; [2] ⇄ ; [3] ⟷ ;

$k_A = K_A \times V'_{S.Ph.}/V_{M.Ph.}$

the $V'_{S.Ph.}$ available for A is a fraction of $V_{S.Ph.}$,

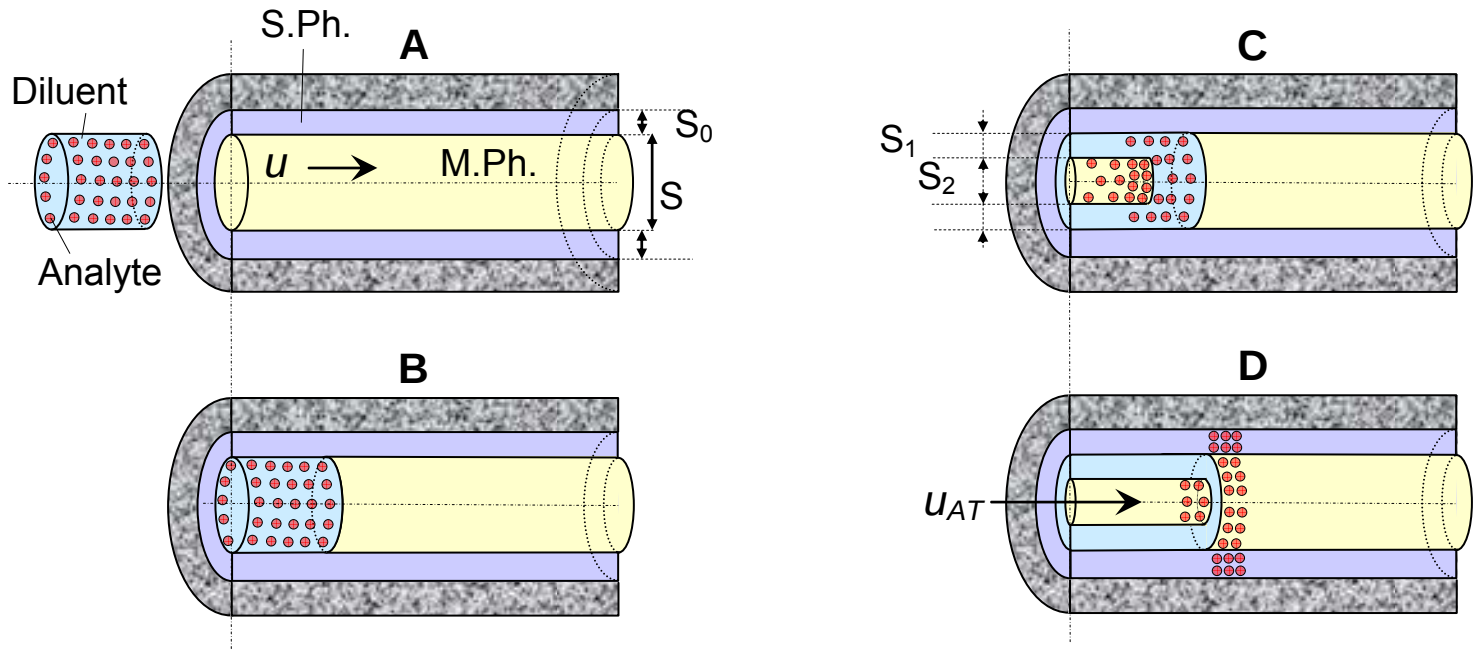
more precisely ($V_{S.Ph.} - \Delta V$), where $\Delta V = \zeta \times V_{inj}^D$, where ζ is a constant

$$k_A = K_A \times V_{S.Ph.}/V_{M.Ph.} - (K_A \times \zeta/V_{M.Ph.}) \times V_{inj}^D$$

A. Medvedovici, Victor David, Vasile David, C. Georgita, Retention phenomena induced by LVI of solvents non-miscible with the mobile phase in RPLC, *J. Liq. Chromatogr. Relat. Technol.*, 30, 199-213 (2007).

The model explains reduction of retention versus the increase of the injection volume!

Possible explanations: version 3 - the supported liquid extraction model



$$k_A = k_A^{th} \times (1 - V_{inj}^D / V_0 \times S_0/(S_0 - S_1))$$

The model is based on a "reversed supported liquid extraction" scenario, on-line created in the column's head!

The model mathematically supports the linear reduction of retention versus injected volume!

New rules governing the process:

Rule of five for „on-line RSLE”

1D exhibits an increased chromatographic retention compared to target compounds ($k_D^{front} > k_A$);

2Solubility of D in the M.Ph. should be as low as possible;

3The initial chromatographic resolution supports the “apparent” reduction of the column length (affecting selectivity).

4D plug from a previous injection is already eliminated from the column before starting a new separation process;

5Fingering effects due to different viscosities (D vs. M.Ph.) need attention and should be controlled;

Verifying the theory:

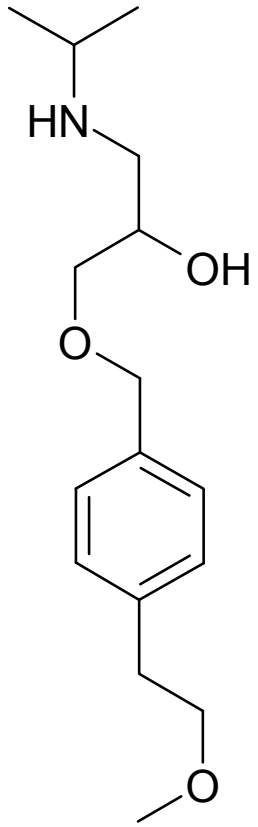
Solute: Metoprolol (log P = 1.88)

Diluents:
Butyl acetate (log P = 1.78)
Carbon tetrachloride (log P = 2.83)
1-Octanol (log P = 3.00)
Cyclohexane (log P = 3.44)
n-Hexane (log P = 3.9)

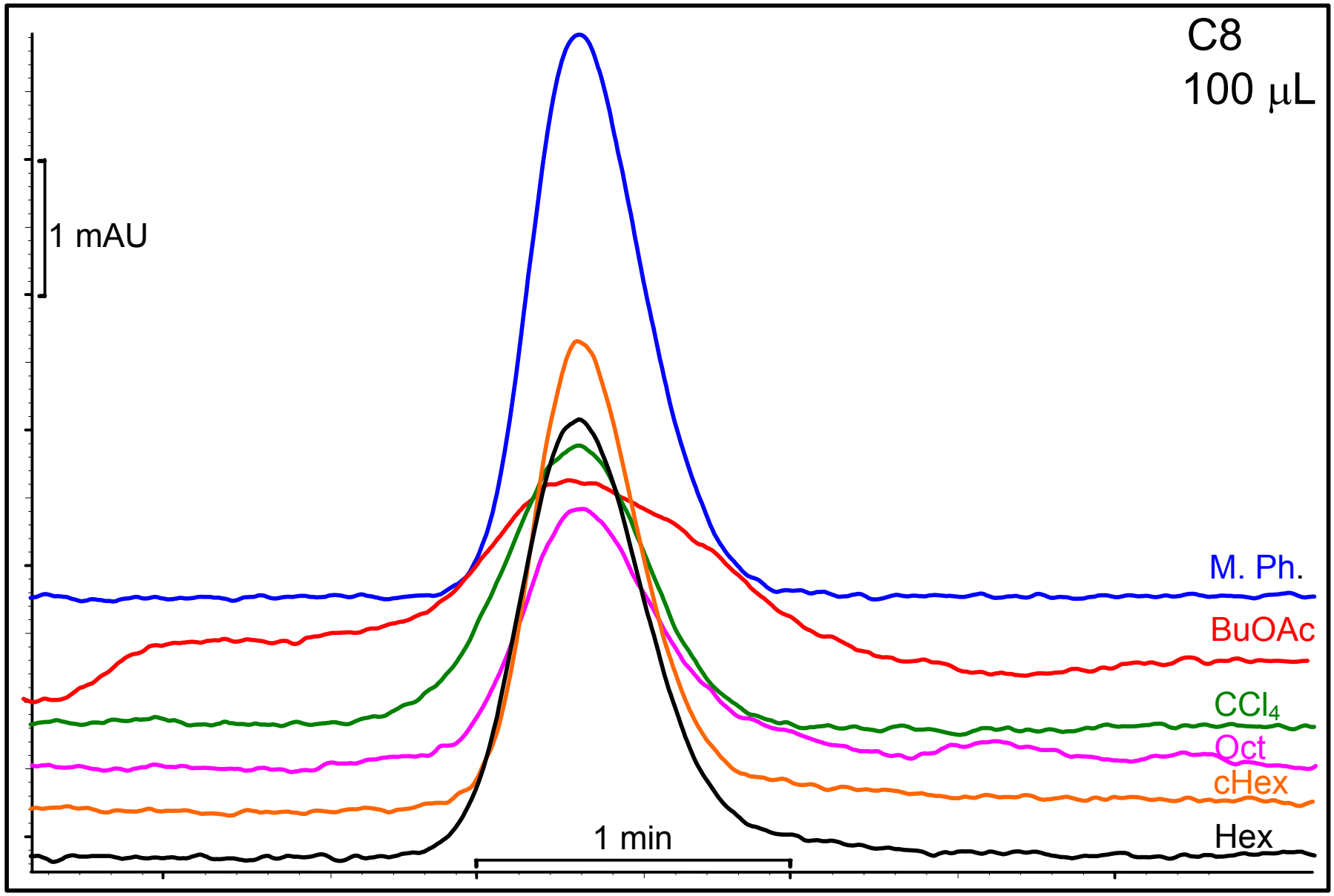
Injection Volumes: 1, 5, 10, 20, 50, 75, 100 □L

Chromatographic Columns:
Zorbax Eclipse XDB C-8 (150 mm x 4.6 mm x 5 μm);
Zorbax Eclipse XDB C-18 (150 mm x 4.6 mm x 5 μm);
Zorbax C-18 Stable Bond AQ (150 mm x 4.6 mm x 5 μm);
Chromolith Performance C-18 (100 mm x 4.6 mm);
Betasyl Phenyl (150 mm x 4.6 mm x 5 μm);
Luna PFP (100 mm x 4.6 mm x 3 μm);

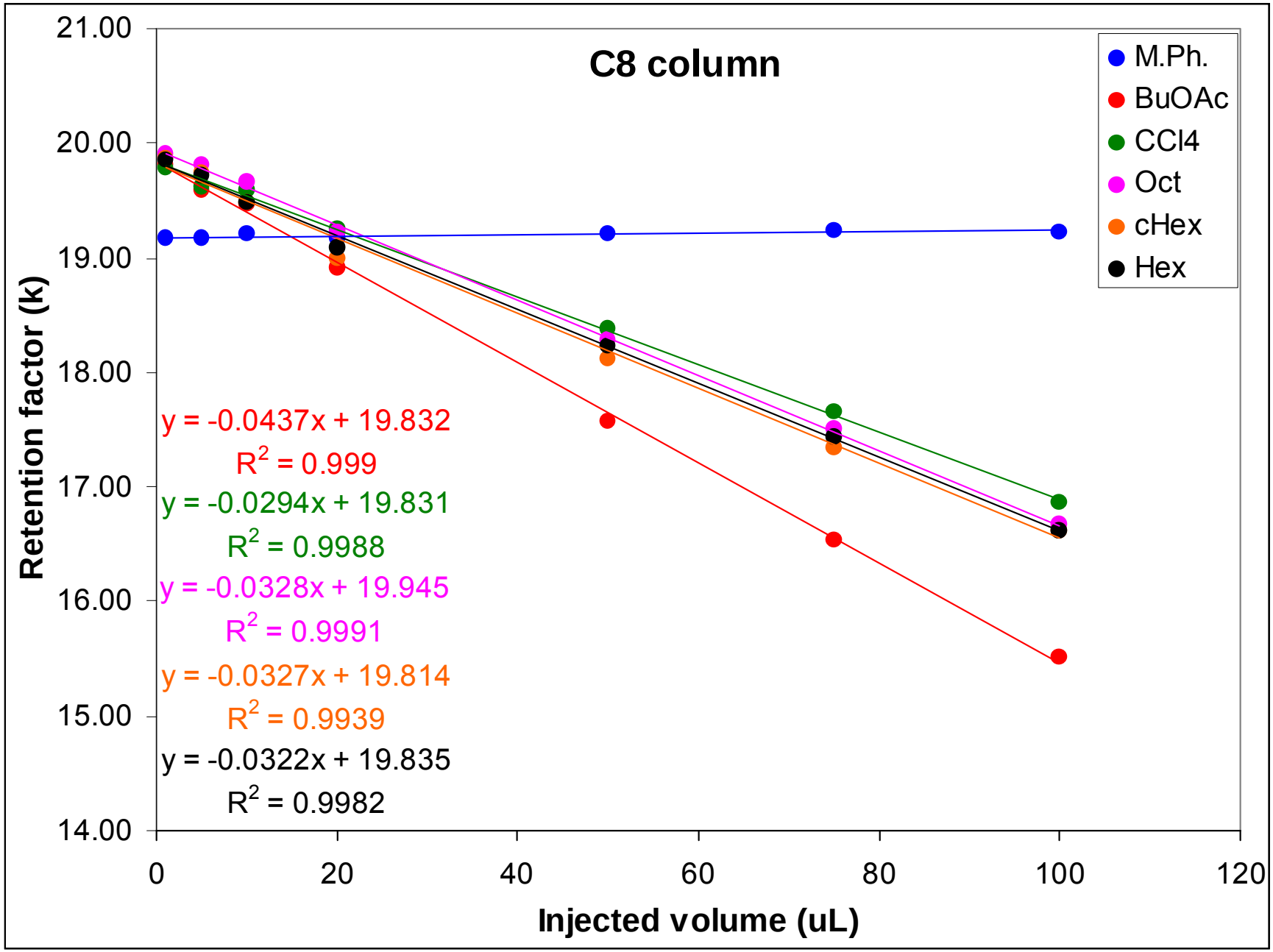
Mobile Phase:
Isocratic Elution
Organic Solvent: ACN
Aqueous Solvent: 50 mM HCOONa + 0.2% TEA at pH = 3.5 with HCOOH
Composition: Organic / Aqueous Solvents = 10/90 (v/v)



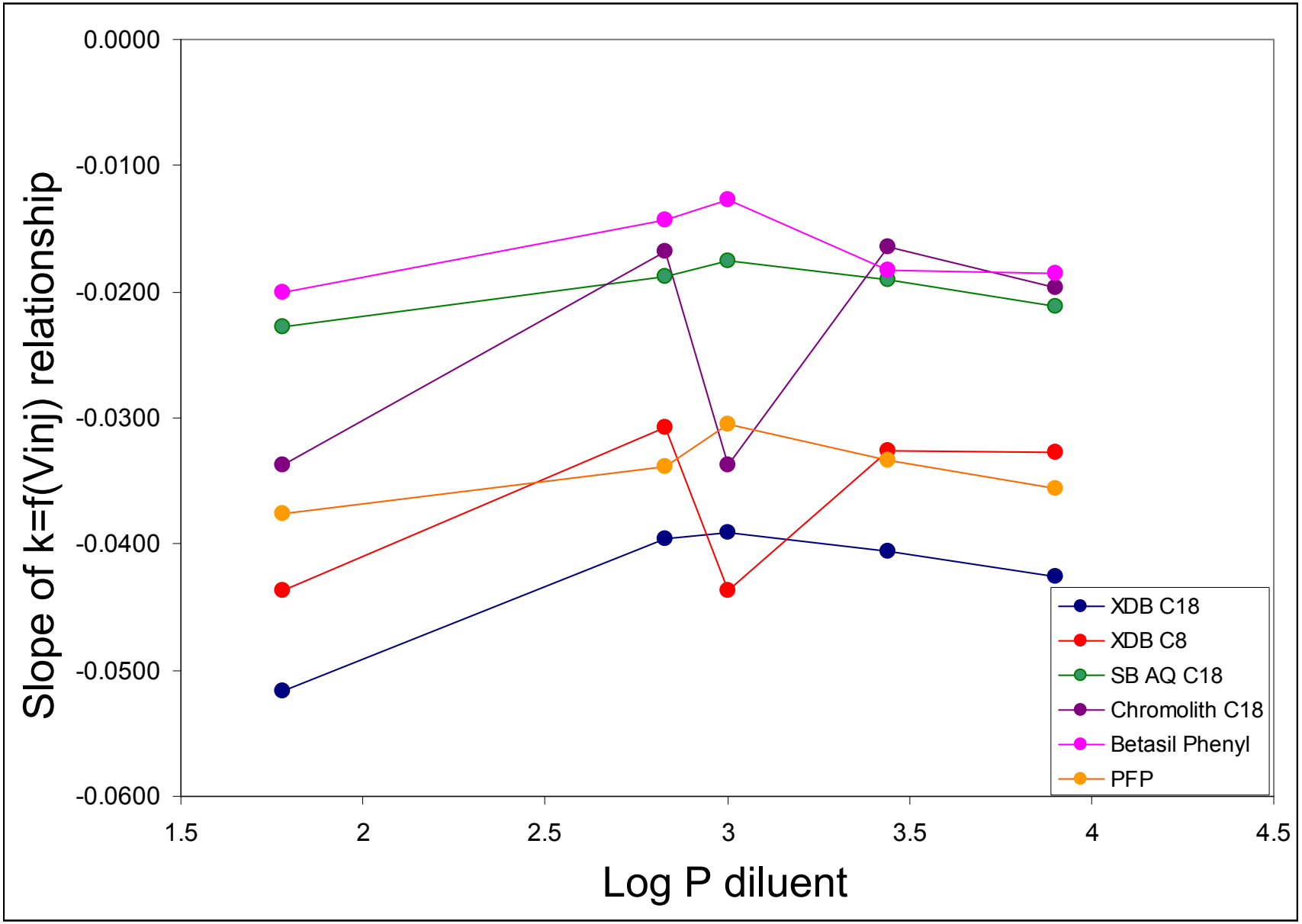
An example on the C8 column:



Retention always linearly decreases with injection volume increase



The slopes of k=f(Vinj) do not correlate with log P of the diluent on different stationary phases.

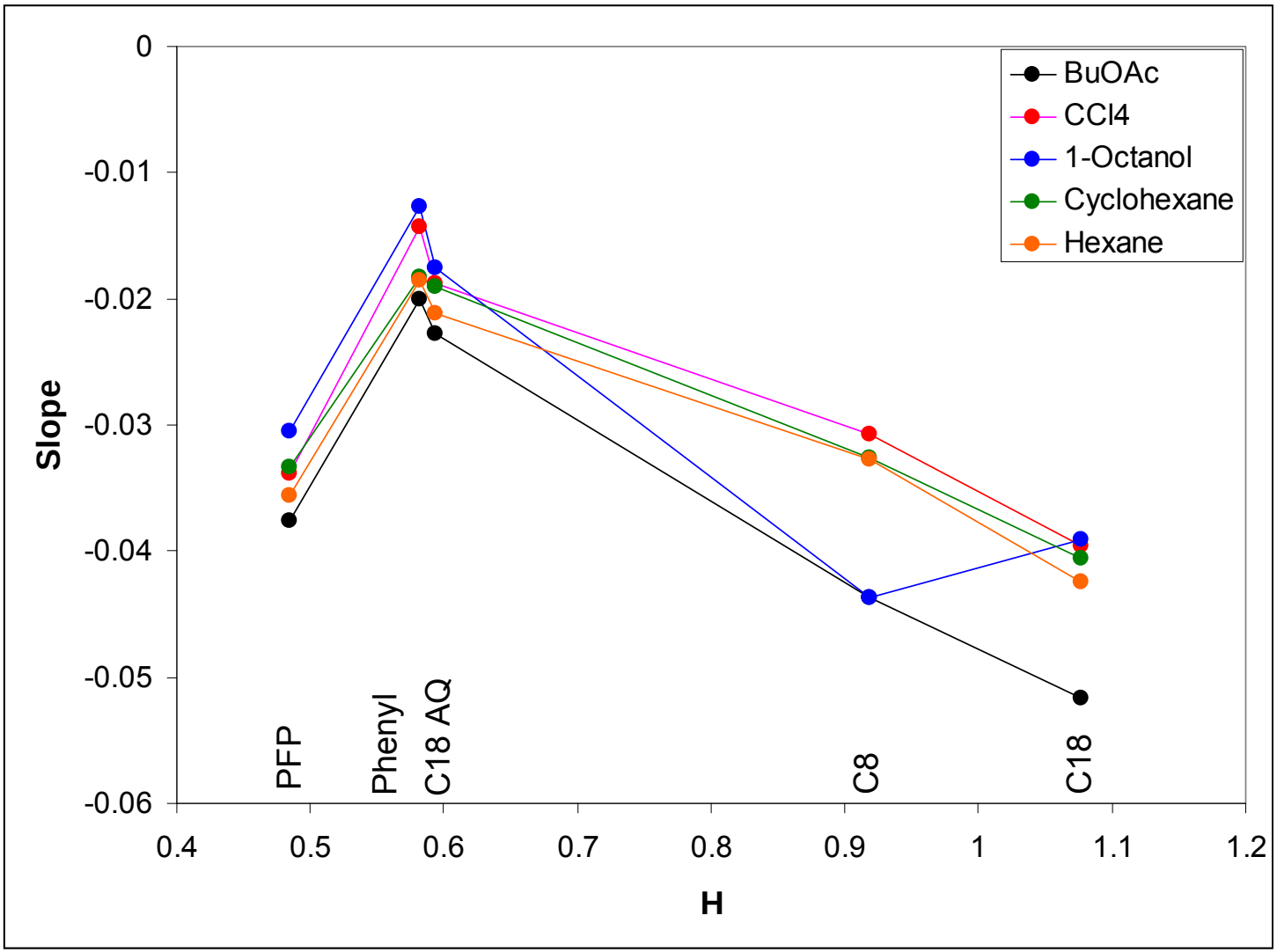


The slopes of k=f(Vinj) are not influenced by the flow rate.

Diluent		Slopes of the regression k = f(V_{inj})					
	Column/ Flow rate (mL/min)	**XDB C18**	**XDB C8**	**SB AQ C18**	**Chromolith C18**	**Betasil Phenyl**	**PFP**
1-Octanol	0.5	-	-	-	-	-	-0.0267
	0.75	-	-	-	-	-	-0.0323
	1	-0.0408	-0.047	-0.0181	-0.0342	-0.0133	-0.0304
	1.25	-	-	-	-	-	-0.0317
	1.5	-0.0364	-0.0465	-0.0173	-0.0358	-0.0132	-0.0315
	2	-0.0374	-0.0452	-0.0183	-0.0319	-0.0125	-
	2.5	-0.0397	-0.0402	-0.0161	-0.0328	-0.0127	-
	3	-0.0412	-0.0398	-0.0177	-0.0338	-0.012	-
	Mean	***-0.0391***	***-0.0437***	***-0.0175***	***-0.0337***	***-0.0127***	***-0.0305***
	St. Dev.	0.0021	0.0035	0.0009	0.0015	0.0005	0.0022
	RSD%	***5.4***	***8.0***	***5.0***	***4.4***	***4.2***	***7.3***
Cyclohexane	0.5	-	-	-	-	-	-0.0316
	0.75	-	-	-	-	-	-0.0345
	1	-0.0370	-0.0306	-0.0183	-0.0178	-0.0147	-0.0342
	1.25	-	-	-	-	-	-0.0326
	1.5	-0.0406	-0.0345	-0.0189	-0.0169	-0.0167	-0.0341
	2	-0.0400	-0.0327	-0.0188	-0.0158	-0.0181	-
	2.5	-0.0426	-0.0315	-0.0196	-0.0156	-0.0182	-
	3	-0.0430	-0.0336	-0.0198	-0.0160	-0.0201	-
	Mean	***-0.0406***	***-0.0326***	***-0.0191***	***-0.0164***	***-0.0176***	***-0.0334***
	St. Dev.	0.0024	0.0016	0.0006	0.0009	0.0020	0.0012
	RSD%	***5.9***	***4.8***	***3.2***	***5.6***	***11.4***	***3.7***

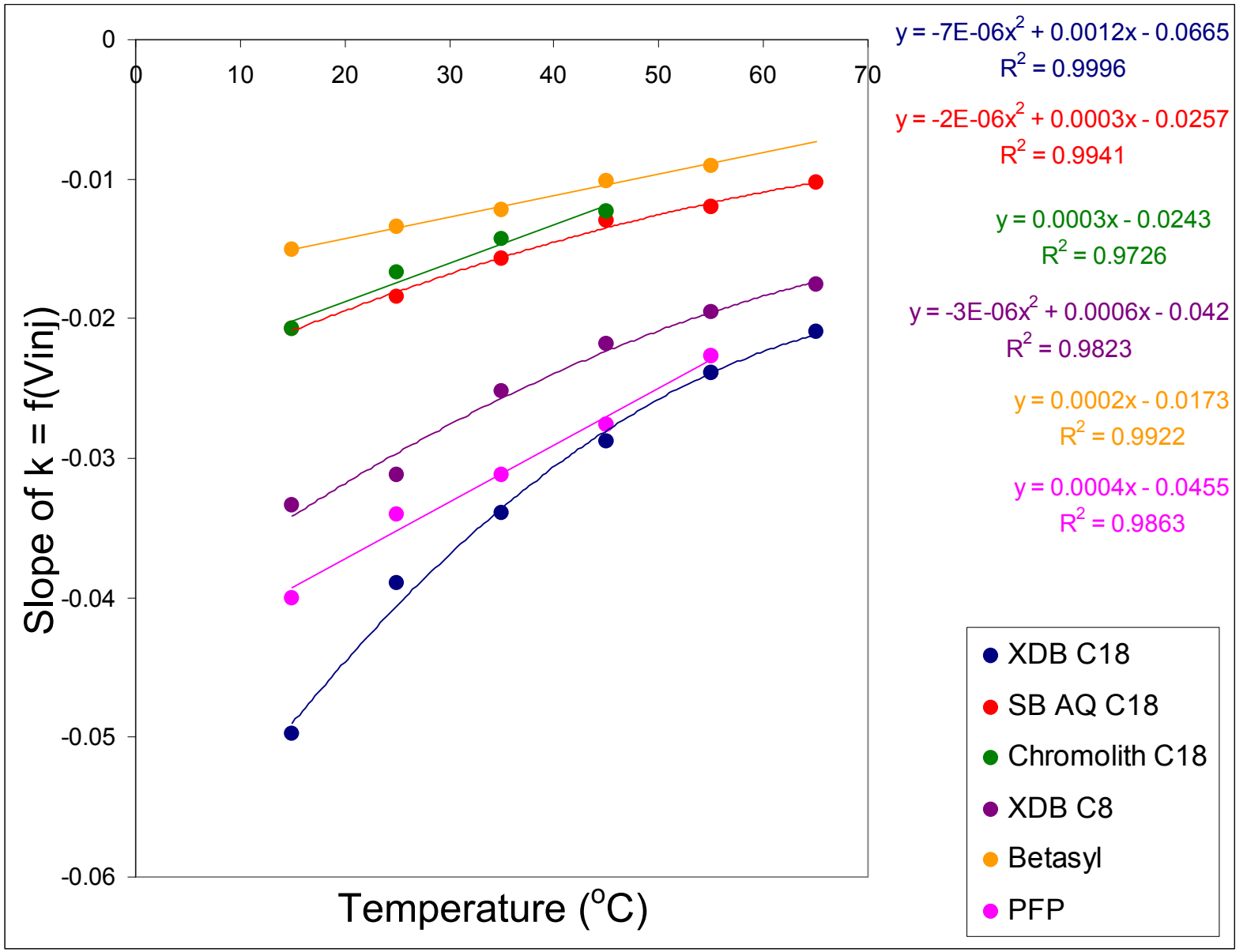
Difficult to explain through the Adsorption model!

The slopes of k=f(Vinj) according to the H (hydrophobicity) character of the stationary phase



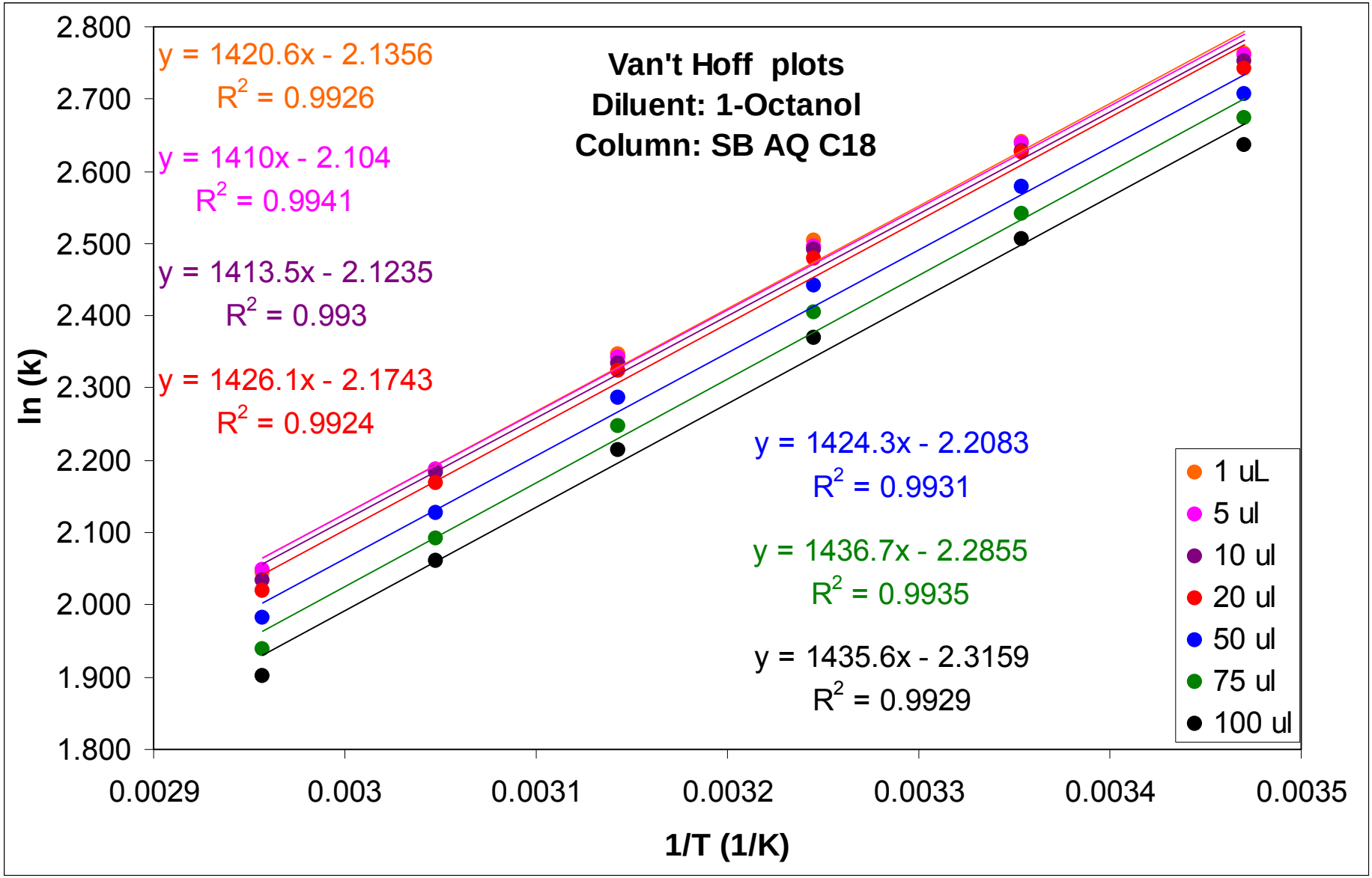
PQRI ct. = Hydrophobicity (H) - Steric interaction (S) + H bond acidity (A) + H bond basicity (B) + Ion exchange capacity at pH= 2.8 (C)

The process is favored by temperature!

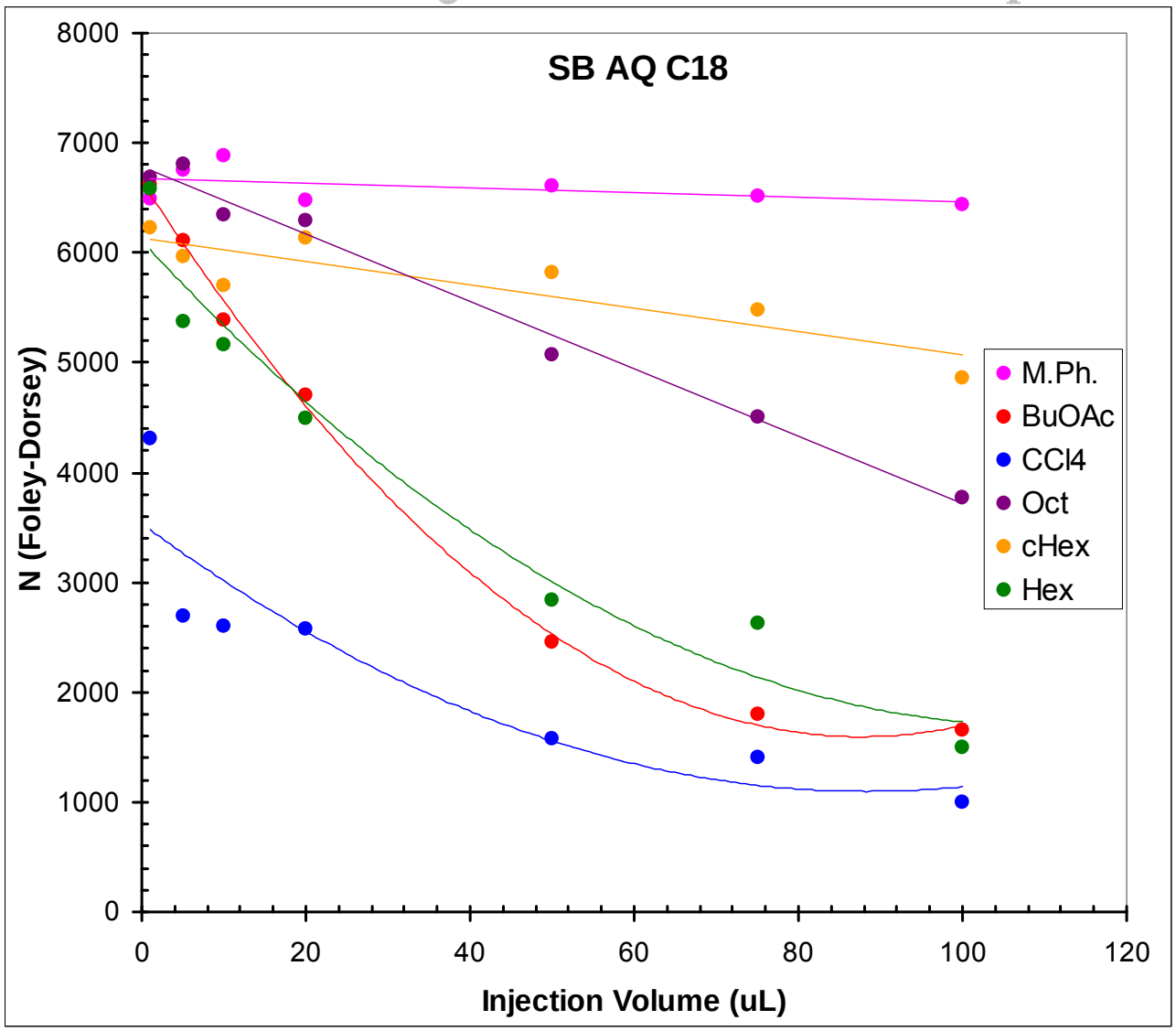


Hard to explain through the Adsorption model!

No enthalpy changes could be observed!



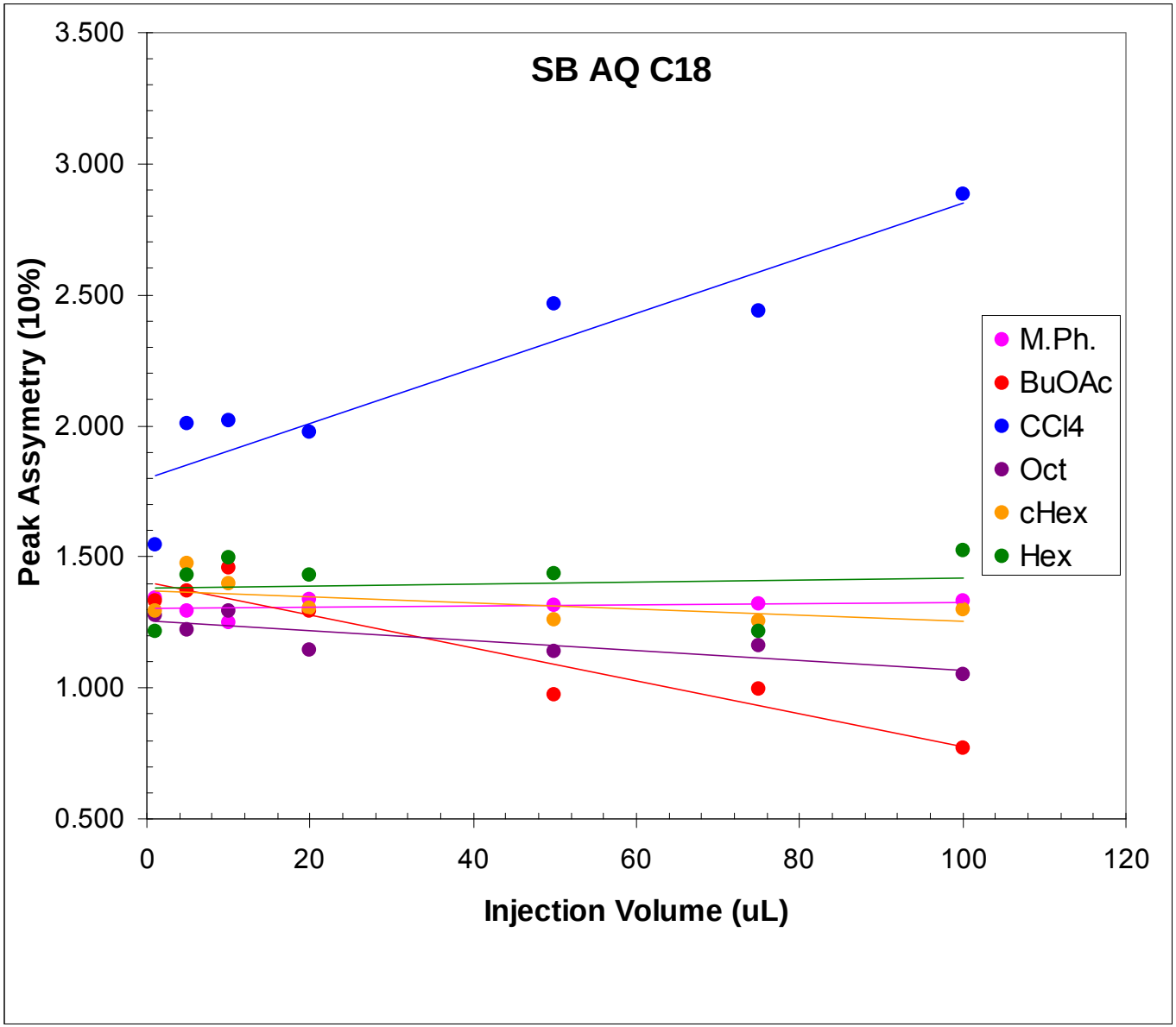
Some inherent dissadvantages: reduction of the separation efficiency!



Efficiency decreases with the increase of the injection volume.

Efficiency loss is higher than expected (due to column shortening).

Some inherent dissadvantages: peak symmetry variation!



To conclude:

1. The on-line RP-SLE model fits better to experimental observations compared to the adsorption model.

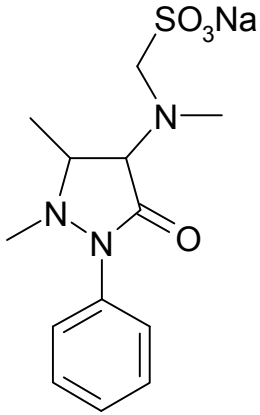
2. The non-miscibility of the diluent with the mobile phase seems to play the most important role compared to the relationship between the hydrophobic characteristics of the diluent and analytes.

3. The kinetic of the LLE process is less important for analytes having an increased hydrophobic character, as long as the “free” stationary phase will refocus them.

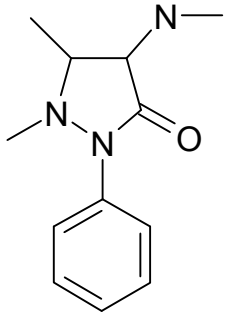
4. The kinetic of the LLE process becomes important for analytes having hydrophilic character, as long as the “free” stationary phase will not refocus them.

5. The process will be better controlled by means of a gradient elution scenario: rich aqueous composition fix the diluent front, next the increase of the organic modifier produces LLE and control refocusing.

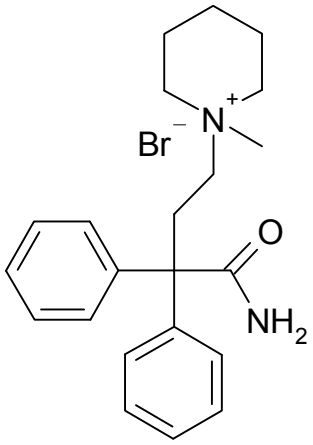
Applications: (1) Resolving "difficult" pharmaceutical combinations



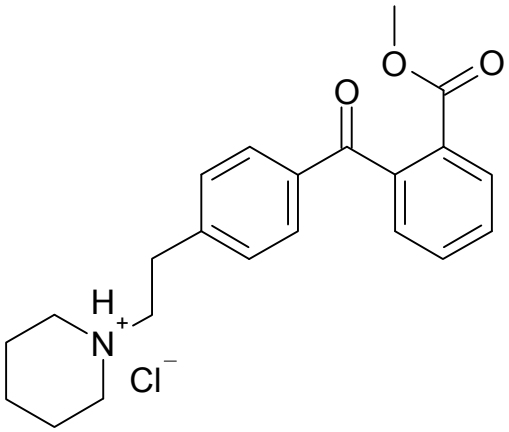
Metamizole sodium
(MTZ)
log Dow (pH=3)
-2.24
500 mg/mL
500 X dilution



Metamizole Imp. C
(MTC)
Log Dow (pH=3)
0.76
(max. 3.5% from MTZ)
(17.5 mg/mL)
25 X dilution



Fenpiverine Bromide
(FPB)
Log Dow (pH=3)
-0.56
20 ug/mL
IP-LLE+RP-SLE



Pitofenone Hydrochloride
(PTF)
Log Dow (pH=3)
0.66
2 mg/mL
25 X dilution

Polar Compounds!

Opposite ion pairing characteristics!

Tailing favored by increased interaction to residual silanols!

Quantitatively uncompensated mixture:
(MTZ/FPB = 1/25,000; PTF/FPB = 1/100; MTZ/PTF = 1/250)

T. Galaon, M. Radulescu, V. David, A. Medvedovici, use of an immiscible diluent in ionic-liquid / ion-pair LC for the assay of an injectable analgesic, *Cent.Eur. J. Chem.*, 10(4), 1360-1368 (2012).

Applications: (1) Resolving "difficult" pharmaceutical combinations

Column: Luna C8(2)

150 mm x 4.6 mm x 5 μm;

T °C = 25 °C;

Organic modifier: MeOH;

Aqueous component: aq. 10 mM SHS + 10 mM BMP-TFB at pH=3 with H_3PO_4 ;

Isocratic elution mode: Org./Aq. 48/52 (v/v)

Detection: UV 290 nm (MTZ, MTC, PTF)

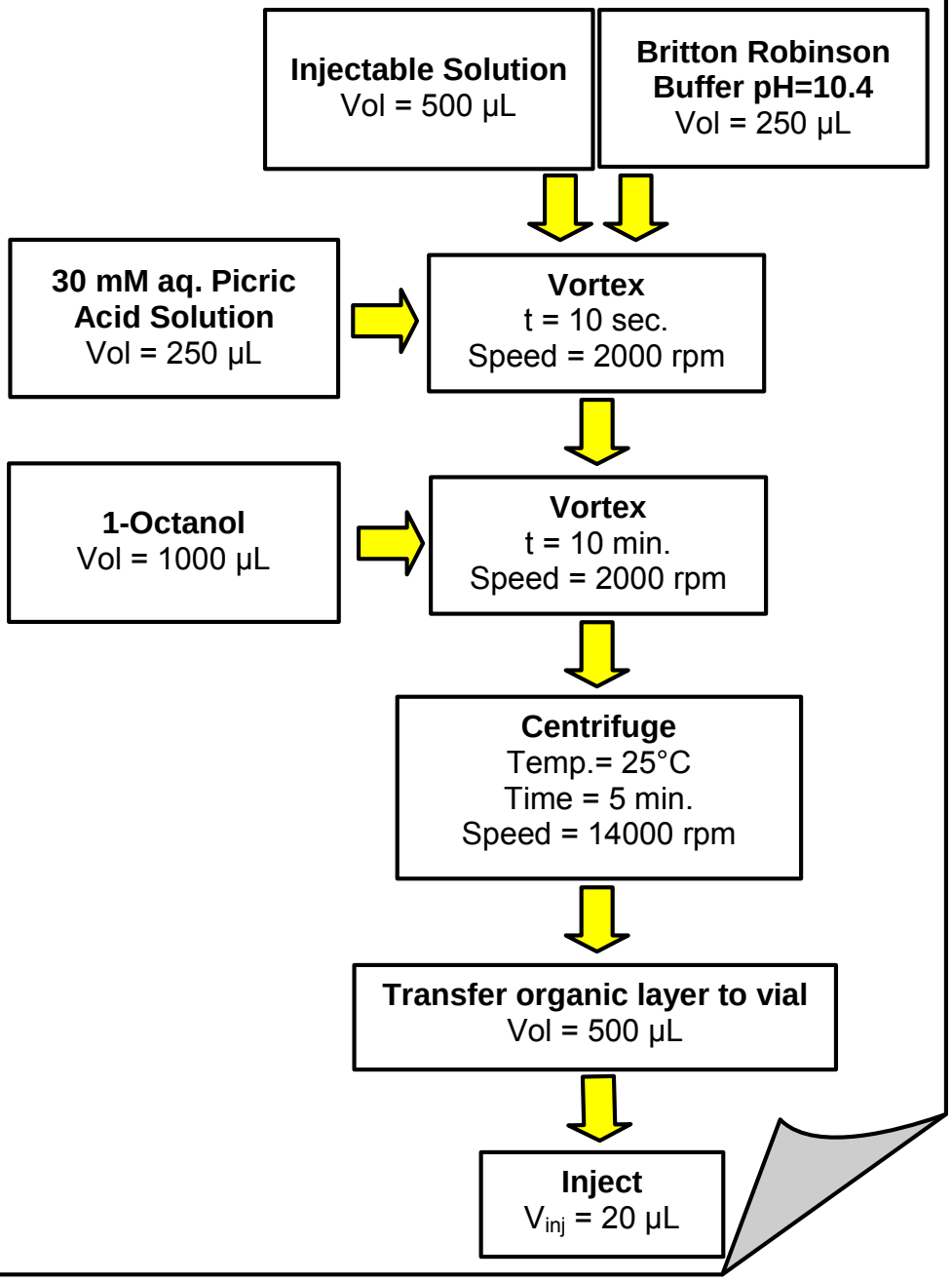
UV 220 nm (FPB)

V_{inj} = 20 μL (for FPB);

Diluent : 1-Octanol

SHS = sodium hexane sulfonate

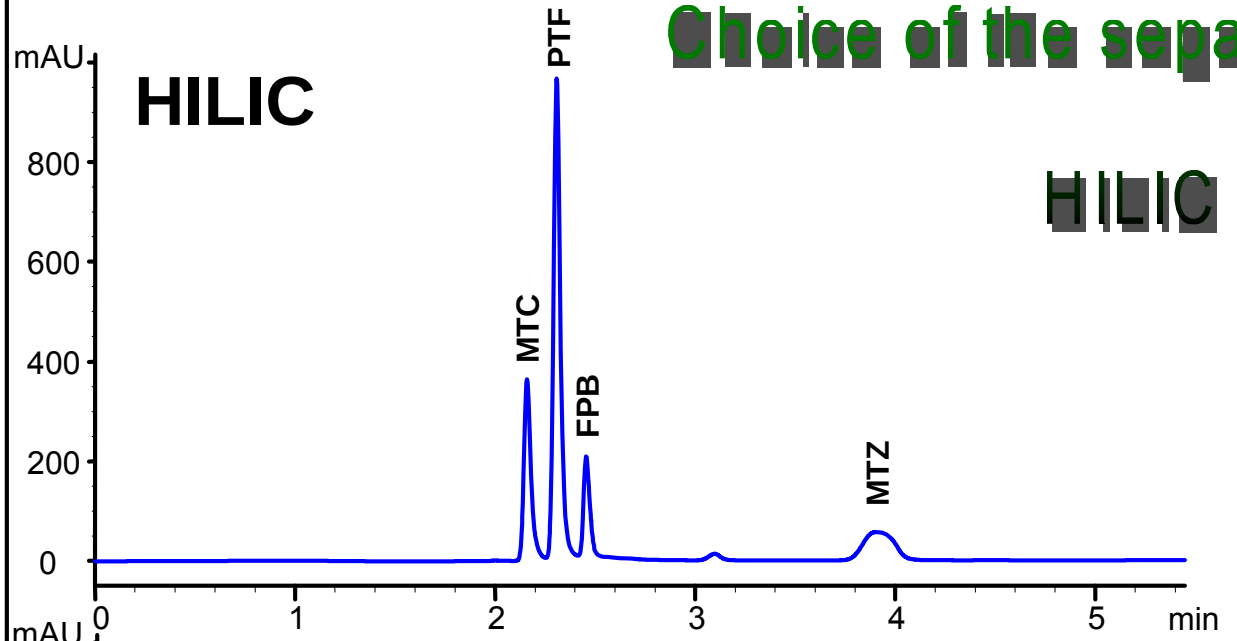
BMP-TFB = 1-butyl 1-methyl pyrrolidinium tetrafluoroborate

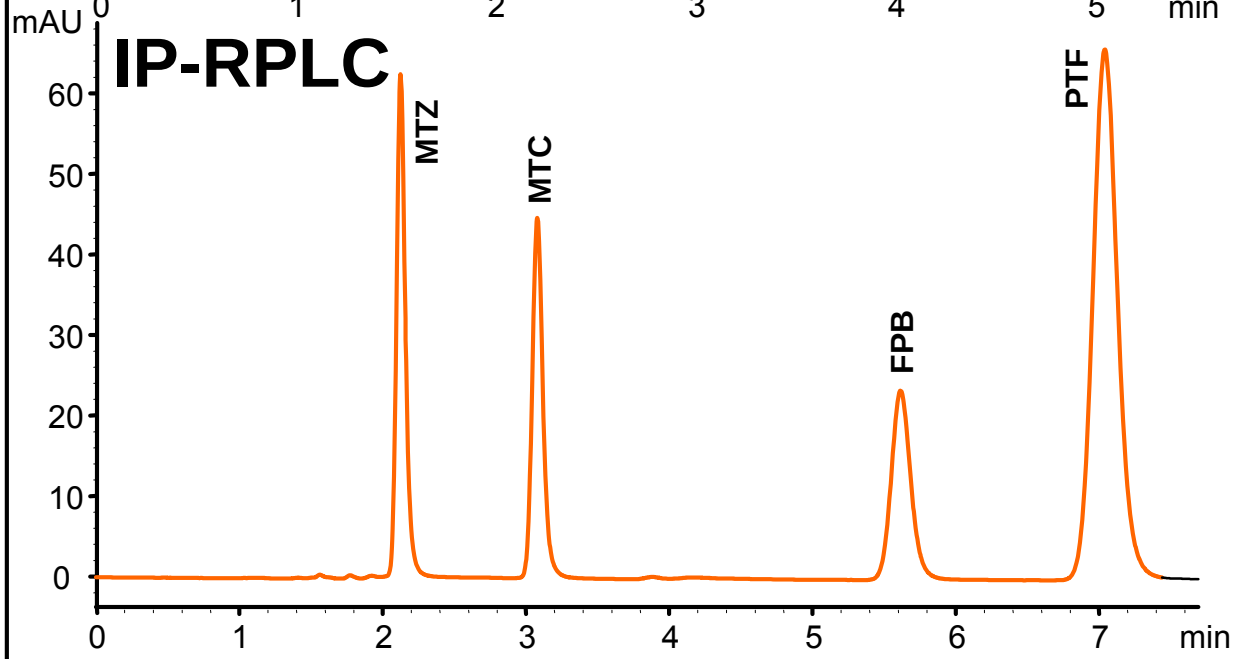


Applications: (1) Resolving "difficult" pharmaceutical combinations

Choice of the separation mechanism!

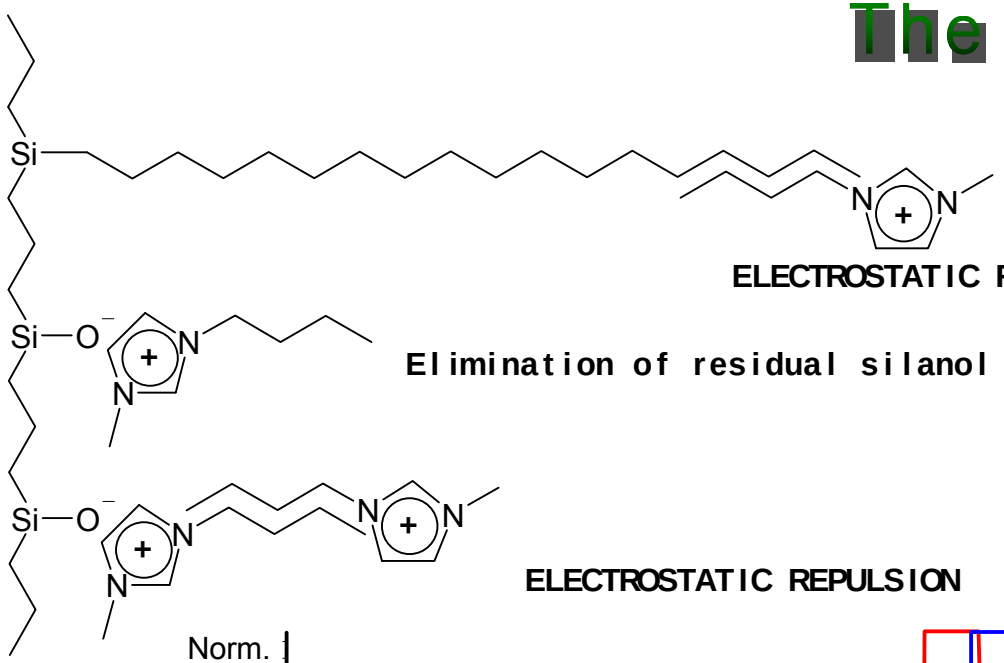
HILIC vs. RP

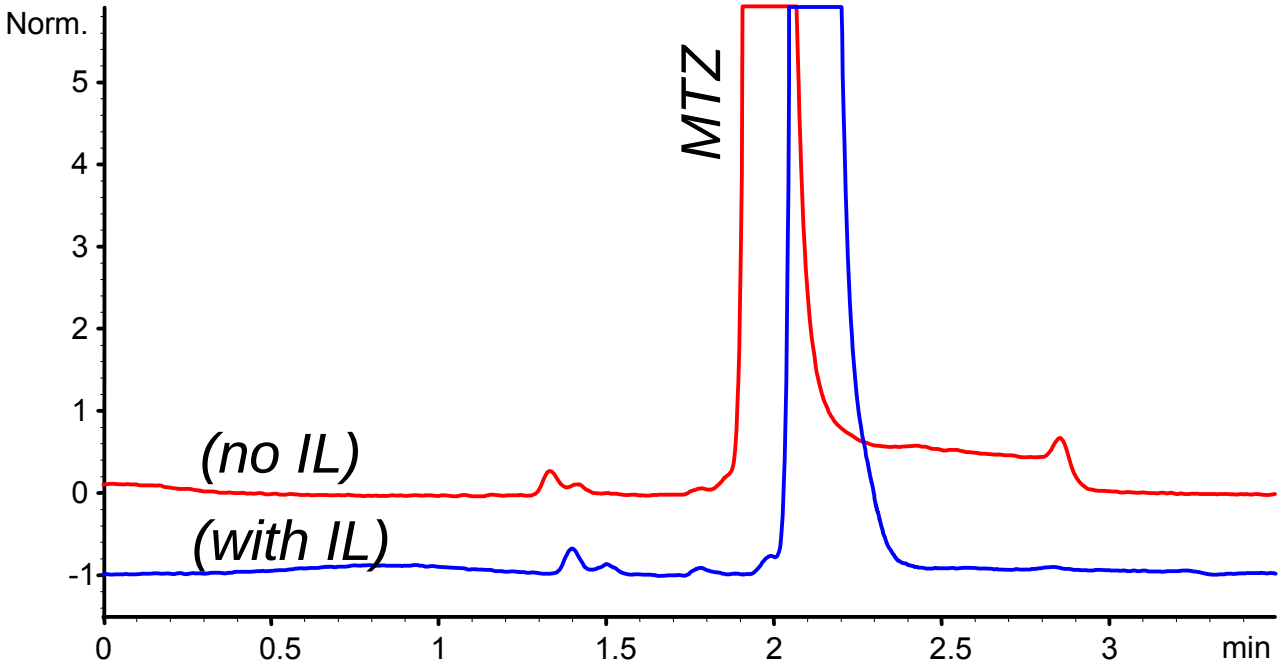




Applications: (1) Resolving "difficult" pharmaceutical combinations

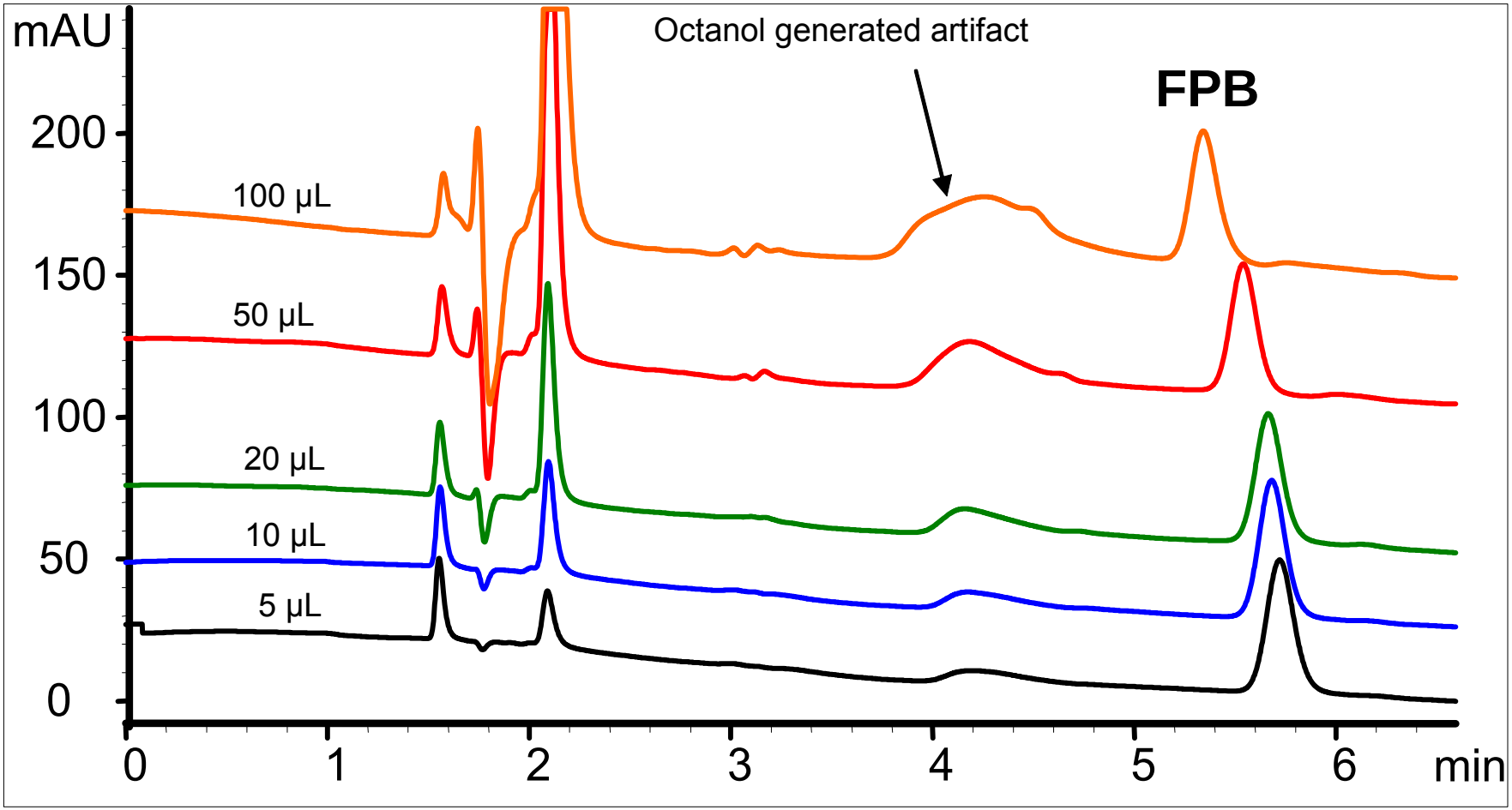
The role of the Ionic Liquid!



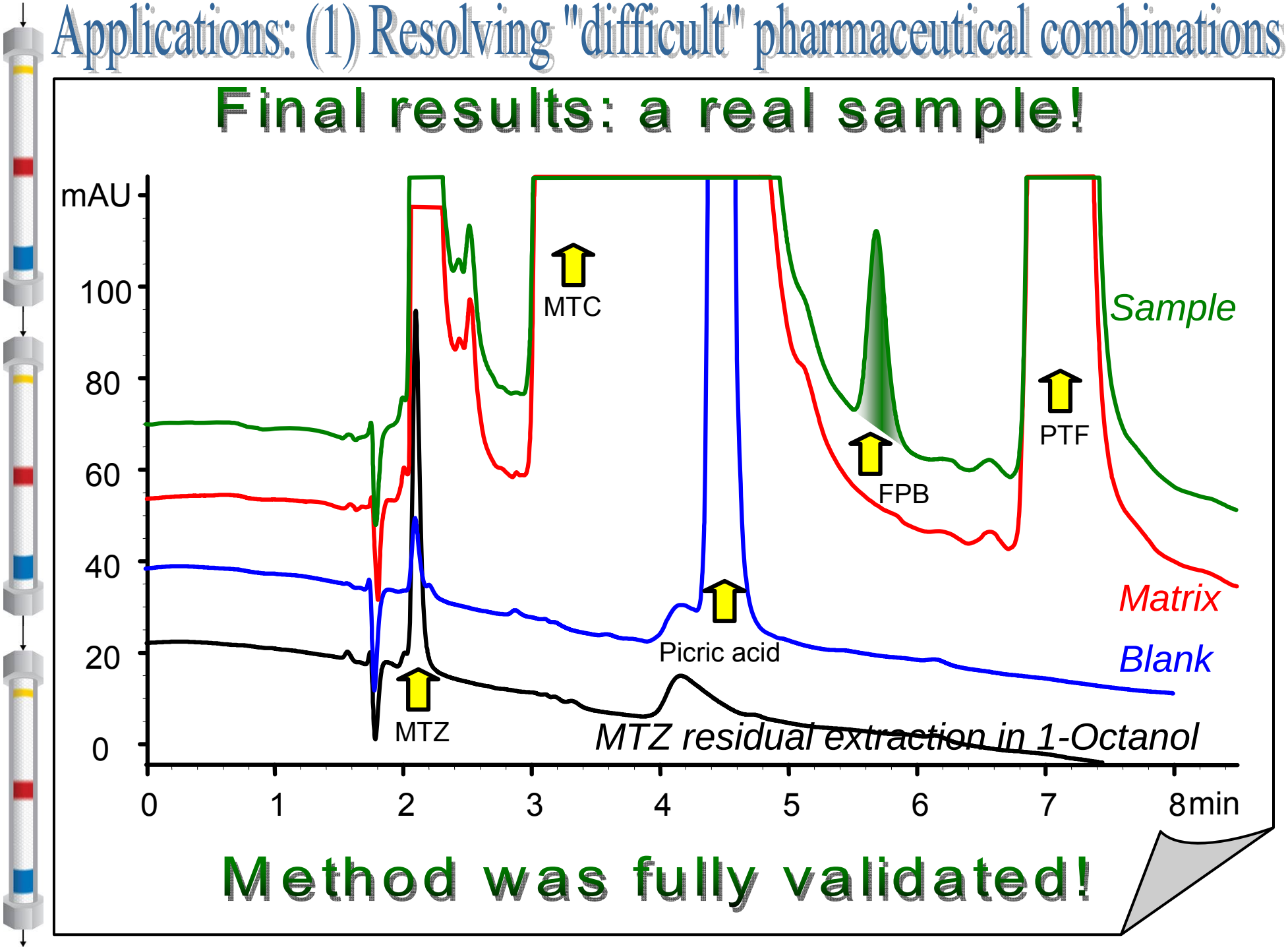


Applications: (1) Resolving "difficult" pharmaceutical combinations

Compatibility of the on-line RP-SL(back)E!



Applications: (1) Resolving "difficult" pharmaceutical combinations

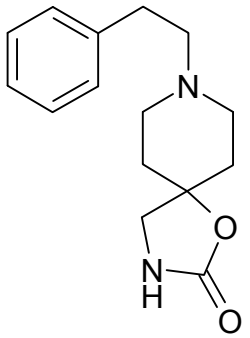


Applications: (2) Assay of fenspiride in human plasma for bioequivalence purposes.

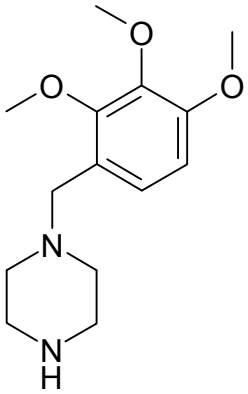
Column: Zorbax SB C18 RR
50 mm x 4.6 mm x 1.8 μm;
T °C = 50 °C;
Organic modifier: ACN;
Aqueous component: aq. 0.1% HCOOH ;
Gradient profile :

Time (min.)	ACN (%)	Flow rate (mL/min)
0	2	0.8
5	30	0.8
5.01	100	0.8
5.50	100	0.8
6.0	100	1.2
6.01	2	1.2
7.0	2	1.2

V_{inj} = 75 μL ;
Diluent : 1-Octanol



FEN



TMZ (IS)

5% Na_2CO_3 aq. solution
Volume = 50 µL

Plasma
Volume = 500 µL

Vortex
Time = 2 min.
Speed = 2000 rpm

IS working solution

Conc. Trimetazidine = 20 ng/mL
Volume = 750 µL
Solvent = 1-octanol

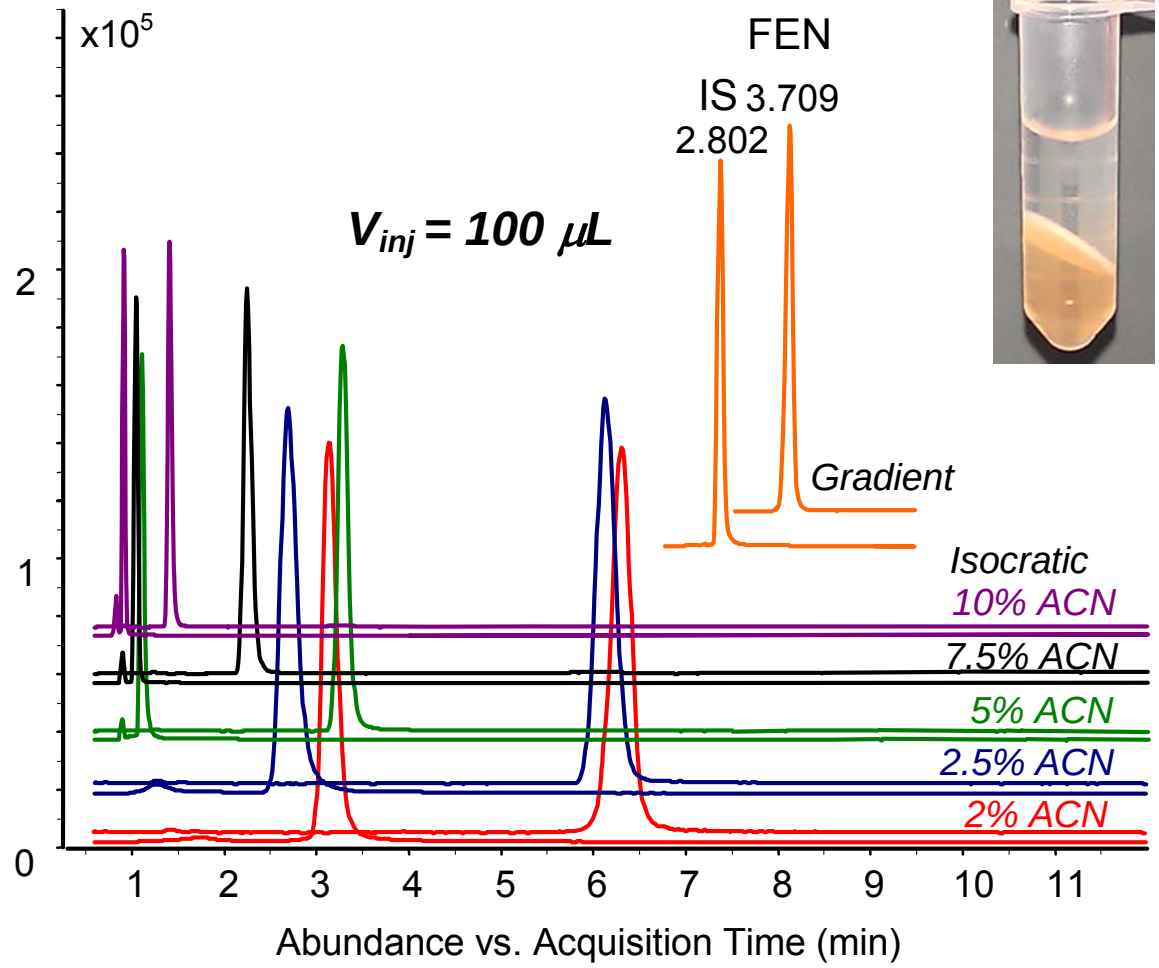
Vortex
Time = 10 min.
Speed = 2000 rpm

Centrifuge
Temperature = 25 °C
Time = 5 min.
Speed = 14000 rpm

Quantitatively transfer the supernatant to vial

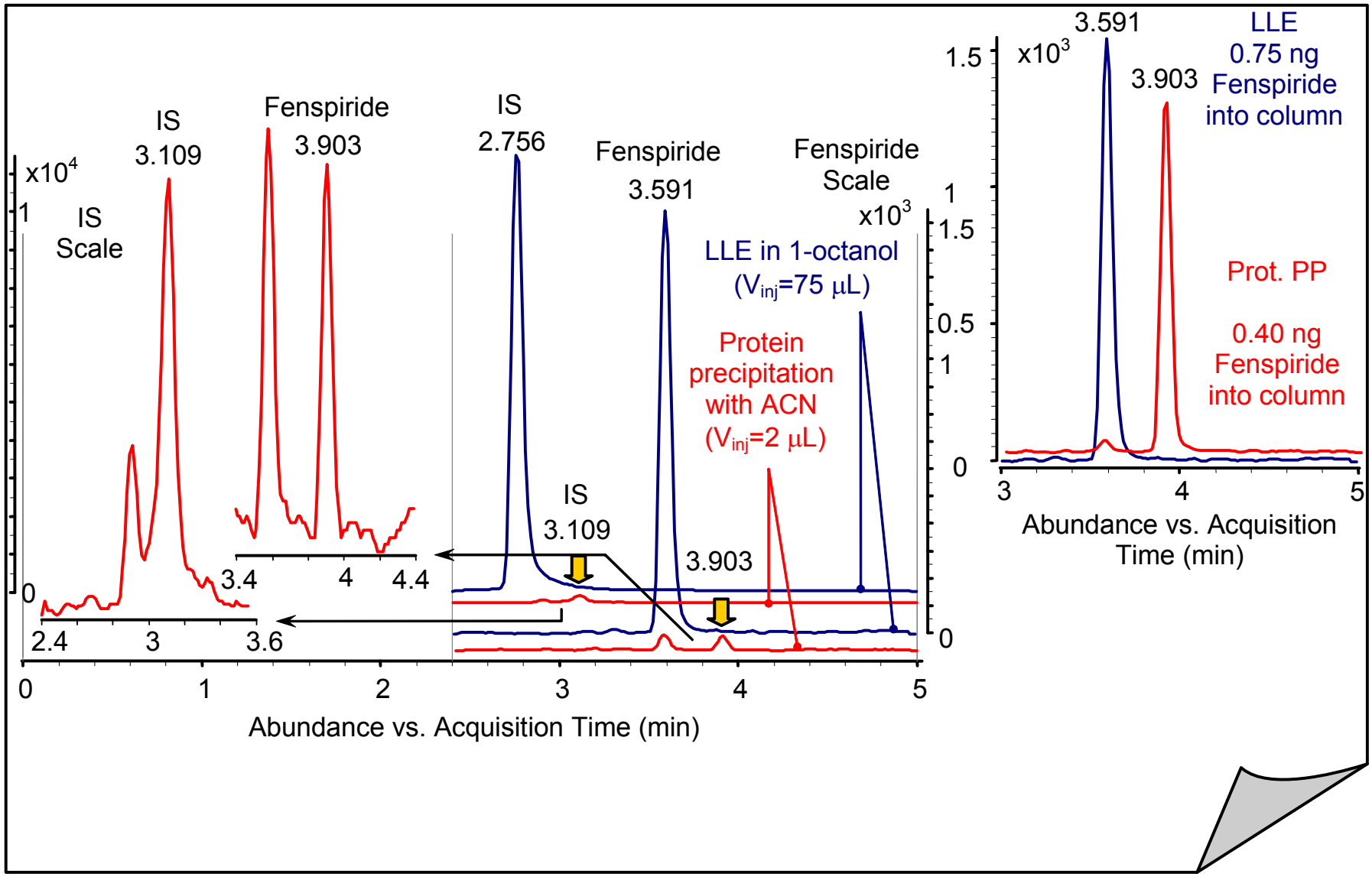
Injection
V_{inj} = 75 µL

Applications: (2) Assay of fenspiride in human plasma for bioequivalence purposes.

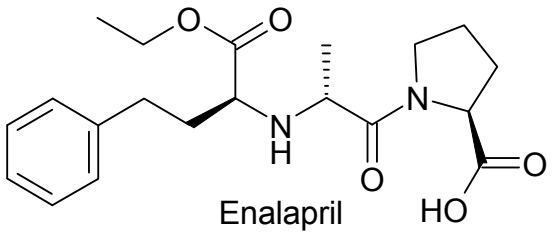


A. Medvedovici, S. Udrescu, F. Albu, F. Tache, V. David, LVI of sample diluents not miscible with the mobile phase as an alternative approach in sample preparation for bioanalysis: An application for fenspiride bioequivalence, *Bioanalysis*, 3(17), 1935-1947 (2011).

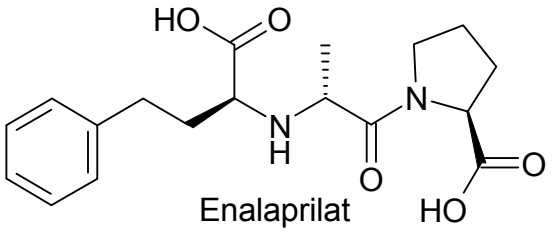
Applications: (2) Assay of fenspiride in human plasma for bioequivalence purposes.



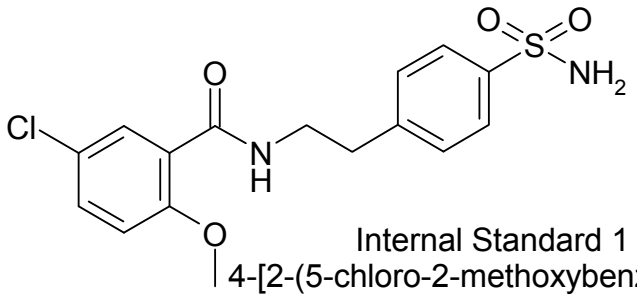
Applications: (3) Greening bioanalytical approaches: Enalapril & Enalaprilat in human plasma



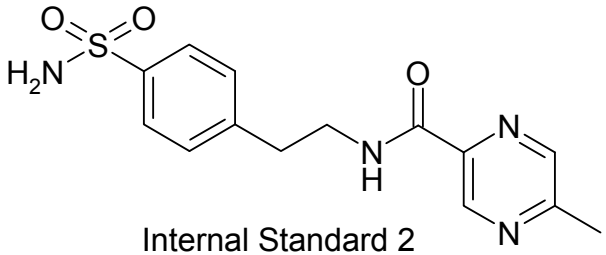
Enalapril



Enalaprilat



Internal Standard 1
4-[2-(5-chloro-2-methoxybenzamido) ethyl]benzene-sulphonamide



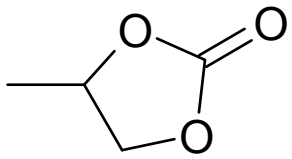
Internal Standard 2
4-[2-(5-methyl-pyrazine-2-carboxamido) ethyl]benzene-sulphonamide

Greening steps:

1Shifting from protein precipitation through ACN addition to LLE in 1-octanol followed by on-line RP-SLE.

2Shifting from ACN based elution gradient to PC/EtOH/Water RP separation

PC = propylene carbonate



M. Cheregi, F. Albu, S. Udrescu, N. Raducanu, A. Medvedovici, Greener bioanalytical approach for LC-MS/MS assay of enalapril and enalaprilat in human plasma with total replacement of acetonitrile througout all analytical stages, *J. Chromatoghgr. B*, 927, 124-132 (2013).

Applications: (3) Greening bioanalytical approaches: Enalapril & Enalaprilat in human plasma

Column: Zorbax SB-C18 RR
50 mm x 4.6 mm x 1.8 μm;
T °C = 50 °C;
Organic modifier: ACN;
Aqueous component: aq. 0.1% HCOOH ;
Gradient profile :

Time (min.)	ACN (%)	Flow rate (mL/min)
0.00	10	0.8
5.00	70	0.8
5.01	100	0.8
5.50	100	0.8
6.00	100	1.2
6.01	10	1.2
8.00	10	1.2

Time (min.)	%PC/EtOH (7/3)	Flow
0.00	5	0.8
5.00	70	0.8
5.01	100	0.8
6.00	100	0.8
6.01	5	0.8
10.00	5	0.8

V_{inj} = 5 μL ;

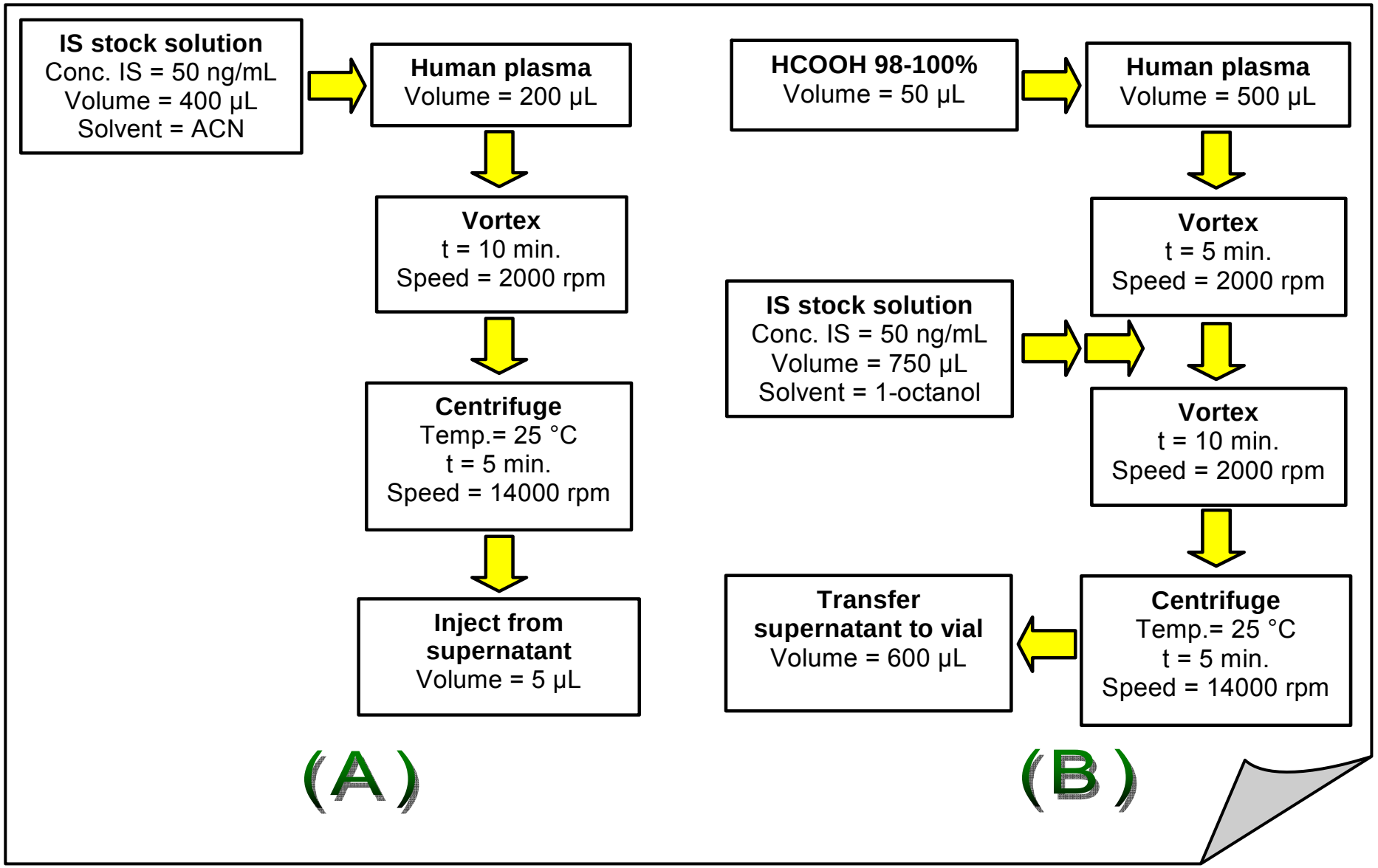
Diluent : ACN

V_{inj} = 75 μL ;

Diluent : 1-Octanol

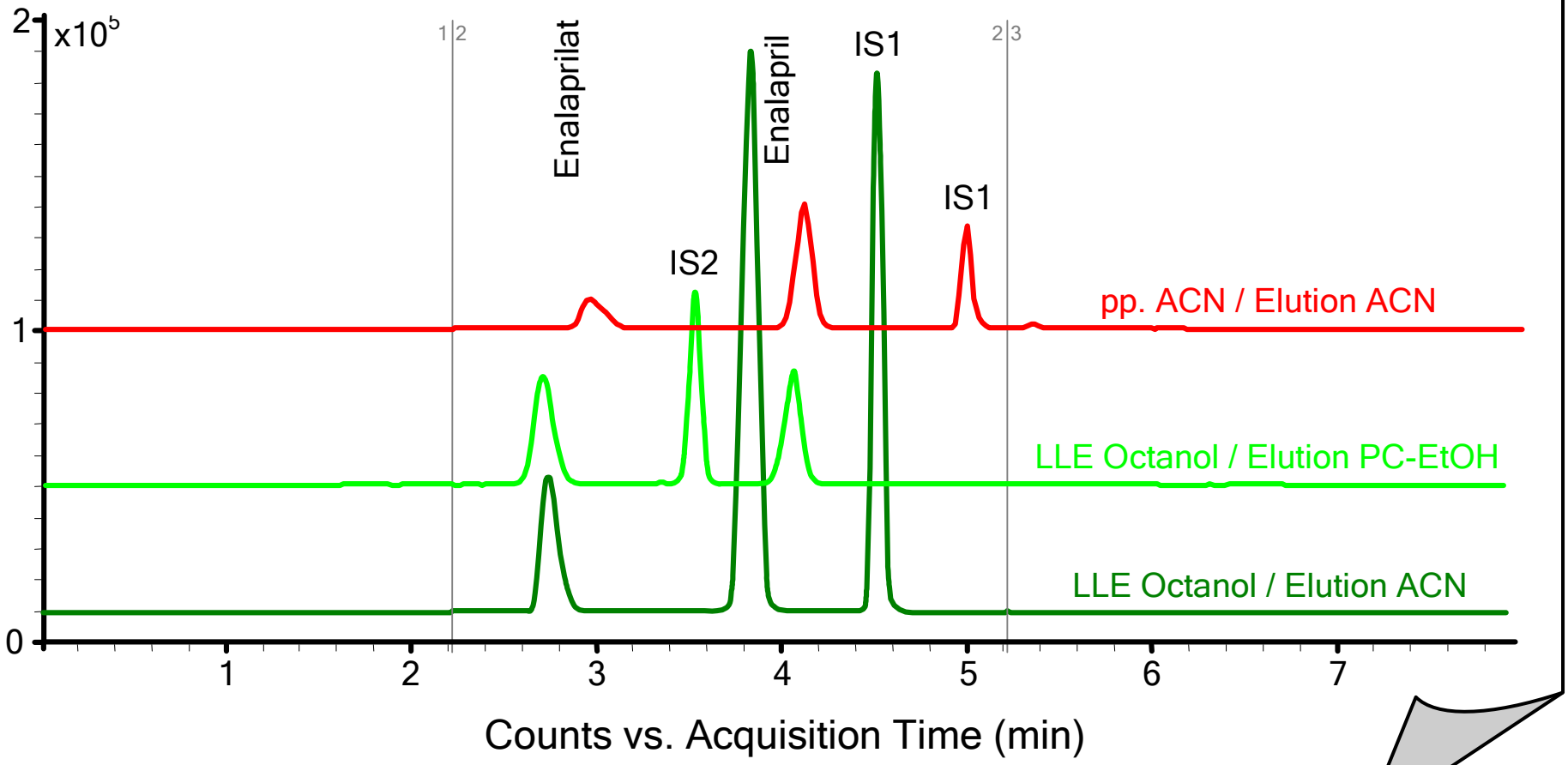
Chromatographic conditions:

Applications: (3) Greening bioanalytical approaches: Enalapril & Enalaprilat in human plasma

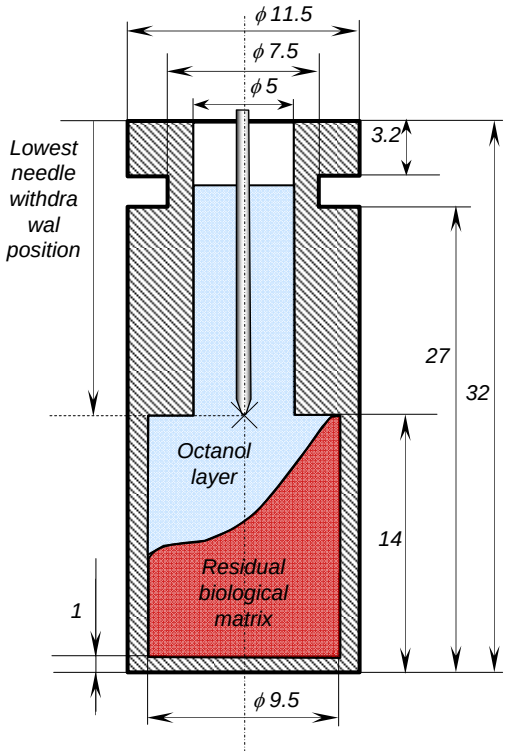


Applications: (3) Greening bioanalytical approaches: Enalapril & Enalaprilat in human plasma

Results:



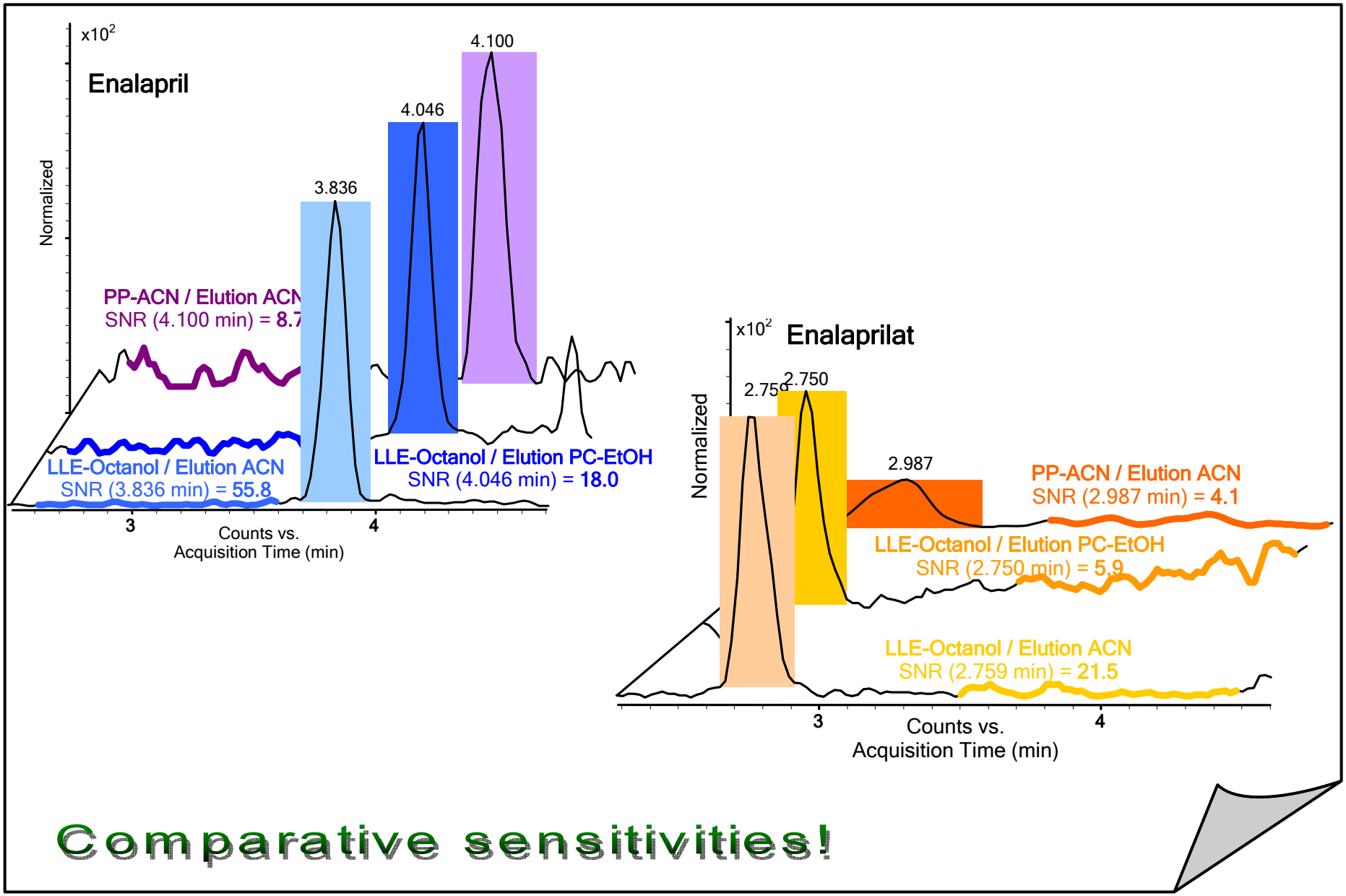
Applications: (3) Greening bioanalytical approaches: Enalapril & Enalaprilat in human plasma



Sample preparation & injection from a single vial!

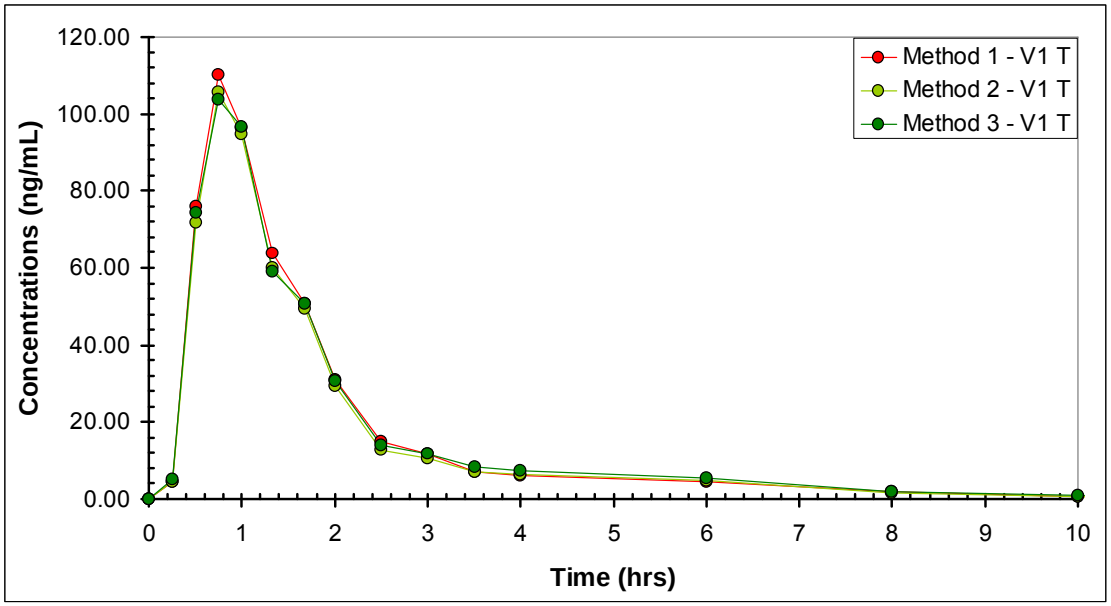
Sample preparation in a single vial: a straightforward solution.

Applications: (3) Greening bioanalytical approaches: Enalapril & Enalaprilat in human plasma



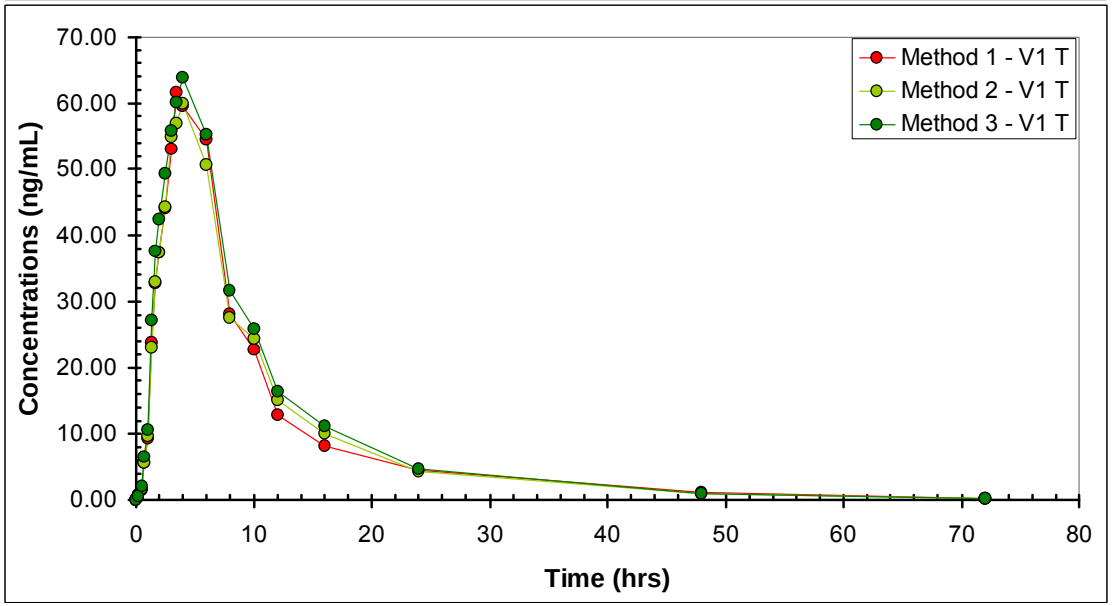
Comparative sensitivities!

Applications: (3) Greening bioanalytical approaches: Enalapril & Enalaprilat in human plasma



(Enalapril)

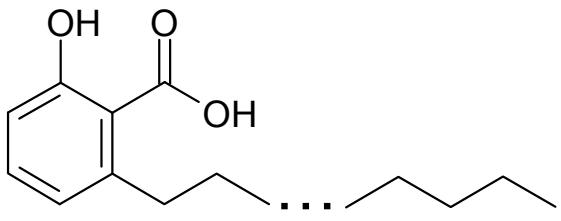
(Comparative Concentration vs. Time profiles for incurred samples from one volunteer)



(Enalaprilat)

Applications: (4) Ginkgolic acids in standardized Ginkgo Biloba extracts.

Analytes: Ginkgolic Acids (C13; C15; C17)



Diluent: Hexane

Log P (Hexane) = 3.29;
Log P (C13:GA) = 8.69;
Log P (C15:GA) = 9.45;
Log P (C17:GA) = 10.4;

$k_{SF} < k_A$

0.5 g Dry Extract + 5 mL MeOH + 0.5 mL HCOOH (98%)
up to 10 mL with H_2O

0.5 mL Hexane → **Vortex –mix** 1 min

Wait until phase separation
5 min (no centrifugation needed)

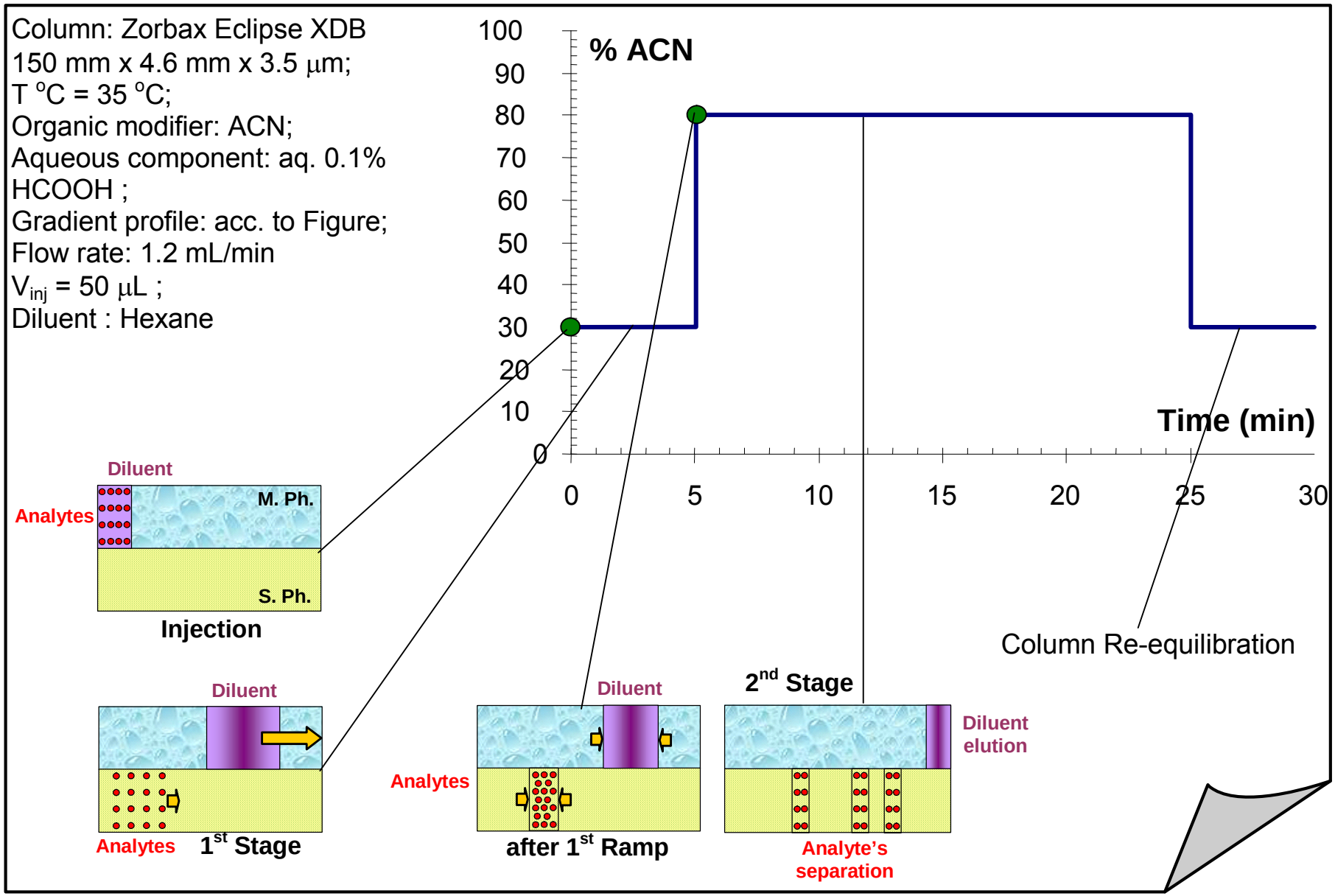
Aliquot from upper layer
~ 0.2 mL

Inject
50 µL

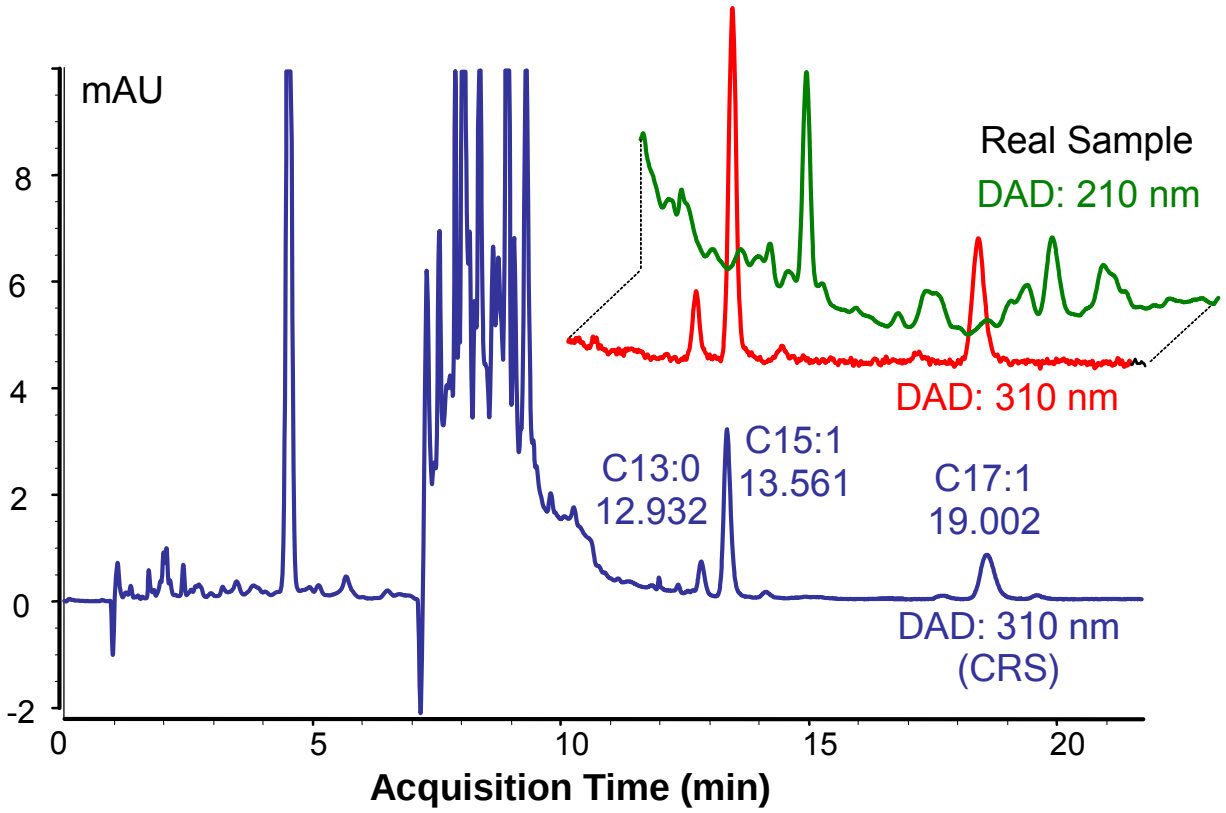
S. Udrescu, I.D. Sora, V. David, A. Medvedovici, LVI of hexane solutions in RPLC/UV to enhance on sensitivity of the assay of Ginkgolic Acids in Ginkgo Biloba standardized extracts, *J. Liq. Chromatogr. Rel. Technol.*, 33, 133-149 (2010).

Applications: (4) Ginkgolic acids in standardized Ginkgo Biloba extracts.

Column: Zorbax Eclipse XDB
150 mm x 4.6 mm x 3.5 μm;
T °C = 35 °C;
Organic modifier: ACN;
Aqueous component: aq. 0.1% HCOOH ;
Gradient profile: acc. to Figure;
Flow rate: 1.2 mL/min
V_{inj} = 50 μL ;
Diluent : Hexane



Applications: (4) Ginkgolic acids in standardized Ginkgo Biloba extracts.



(Few) General Conclusions:

LVI of M.Ph. non-miscible diluents in RPLC is in fact an on-line RP-SL(back)E. Although complex and difficult to be brought at a parametrization stage, the process may be successfully controlled (mainly through gradient elution) and used as a valuable tool for enhancing on sensitivity/selectivity.

The process logically continues sample preparation „classical" procedures, offering interesting opportunities for high throughput and/or automated approaches.

V. David, M. Cheregi, A. Medvedovici, Alternative sample diluents in bioanalytical LC-MS, *Bioanalysis*, 5(24), 3051-3061 (2013).

Acknowledgements:

The financial support (45%) given by the Romanian project PNII_ID_PCE_2011_3_0152/C. no. 310/2011.

To my colleague and friend, Prof. Dr. Victor David, for sharing the interest on the topic and continuing to develop it together.

To my past & present co-workers Corina (Barcutean / Endes), Cristina (Georgita), Iulia (Sora), Florin (Albu), Toma (Galaon) Mihaela (Cheregi), Mona (Iorgulescu) and Florentin (Tache) for their contributions (and hard work) to the topic.

Acknowledgements:

To the unknown reviewer rejecting our first manuscript on LVI of immiscible diluents, for encouraging us to continue.

"The work carried out in this area is very limited and I do not think that it will acquire a broad practical significance in the future. The work presented here seems to be original, it is an interesting combination of experiment and theory and for this reason it is publishable, however …"

Critics, you of sterile blossoms,
Driven out by pride and spell,
It's just easy to write verses,
When you have nothing to tell.

M. Eminescu
(To my critics)