University of Bucharest
Faculty of Chemistry
Department of Analytical Chemistry

Green approach in bioassays through replacement of acetonitrile from sample preparation and separation stages

Andrei Medvedovici, Florin Albu

1st INTERNATIONAL CONFERENCE ON ANALYTICAL CHEMISTRY
Analytical Chemistry for a Better Life
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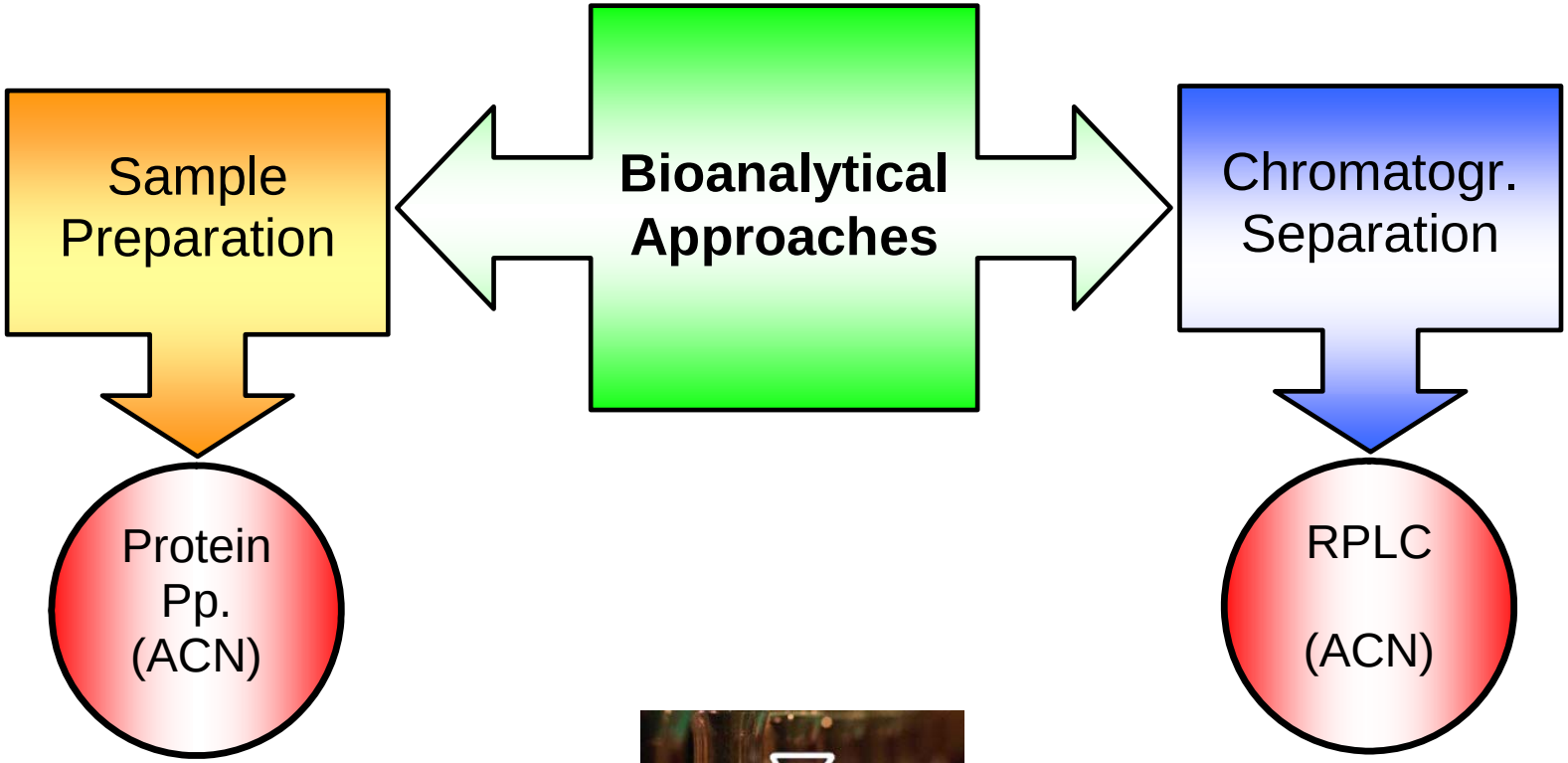
Green Analytical Chemistry

"The use of analytical chemistry techniques and methodologies that reduce or eliminate solvents, reagents, preservatives and other chemicals that are hazardous to human health or the environment and that may also enable faster and more energy-efficient analyses without compromising performance criteria"

J. Namiesnik, Green analytical chemistry – some remarks, J. Sep. Sci. 24 (2001) 151.

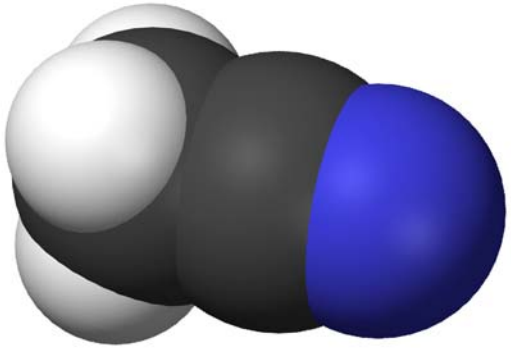
L.H. Keith, et al. Green analytical methodologies, Chem. Rev. 107 (2010) 2695.

The “classic” scenario:



ACN: Obviously, it is not a green approach!

Acetonitrile



- Listed as VOC
- Toxic
- Flammable
- Acetonitrile shortage (economic crisis)

Possible solutions:

Protein precipitation:
ACN addition
Vol. ratio > 2:1

LLE

Environ. friendly alternatives!

No solvent evaporation!

Large Volume Injection (LVI) in diluents non-miscible with the mobile phase

RPLC elution:
ACN from 5% to 100% as organic modifier in the mobile phase

ACN free elution

Environ. friendly alternatives!

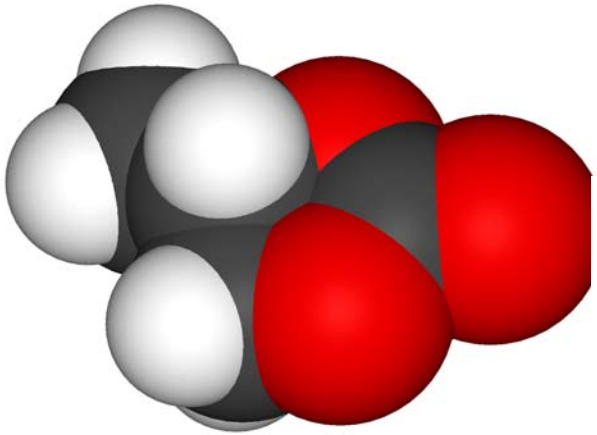
Alternatives for ACN as organic modifier!

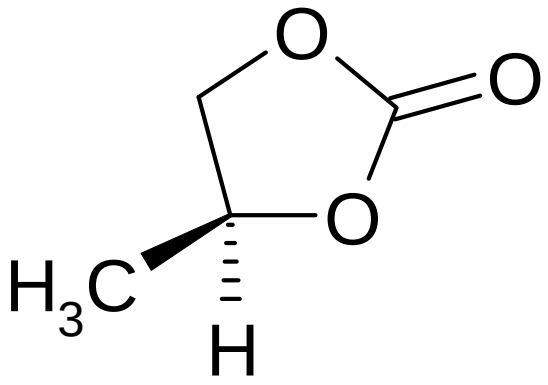
Propylene carbonate as green aprotic polar organic solvent

Alternatives:

Characteristics	ACN	PC	1-Octanol	EtOH
CAS no.	75-05-8	108-32-7	111-87-5	64-17-5
Type of solvent	Polar aprotic	Polar aprotic	Apolar	Polar protic
Molecular weight	41.04	102.09	130.23	46.07
Molecular dipole moment (D)	3.92	4.90	2.0	1.69
Dielectric constant	35.94	64.90	10.3	24.55
Hansen solubility parameters – Dispersive - δD	15.3	20.0	17.0	15.8
Polar - δP	18.0	18.0	3.3	8.8
Hydrogen bonding - δH	6.1	4.1	11.9	19.4
Boiling point (^{o}C)	81.6	241.7	194.5	78.0
Melting point (^{o}C)	-45.0	-49.2	-16.5	-113.9
Density g/cm^3	0.781	1.205	0.827	0.789
Experimental Log K_{ow}	-0.34	-0.41	3.00	-0.31
Water solubility	Fully miscible	240 g/L	0.3 mg/L	Fully miscible
Viscosity / 25 ^{o}C (cP)	0.36	2.4	7.21	1.1
Vapor pressure kPa/20 ^{o}C	9.7	0.017	0.19	5.83
Flash point ^{o}C	2	132	81.1	13
Autoignition temperature (^{o}C)	524	455	270	365
Lower flammable limit (LFL) - %	4	1.7	1.1	3.5
Upper flammable limit (UFL) - %	16	32.5	7.4	19
Oral LD50 rat mg/kg	2462.7	34596.0	4718.1	7055.3
Dermal LD50 rabbit mg/kg	980	20001	2000	19999
Acute LC50 Daphnia (48 hrs) - mg/mL (c*)	15.5	54.4	169	1627.7
NFPA Rating Health	2	1	1	0
NFPA Rating Fire	3	1	2	3
NFPA Rating Safety	2	0	1	0
Exempt VOC (acc. 40 CFR 51.100)	No	Yes	No	No
Biodegradability	~ 40% / 10 days	> 80% / 10 days	78 % / 28 days readily biodegradable	readily biodegradable

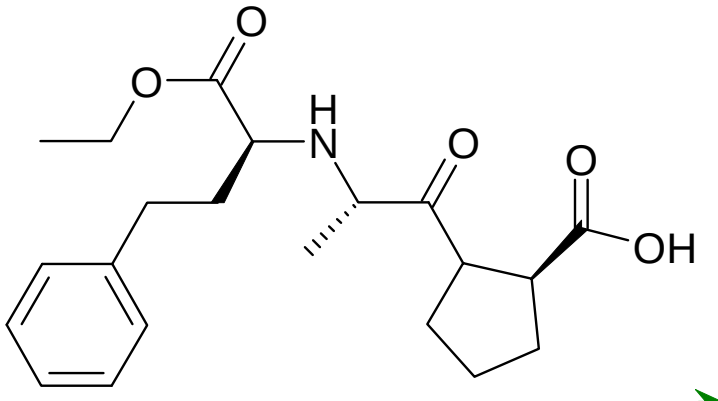
Propylene carbonate: a greener approach!



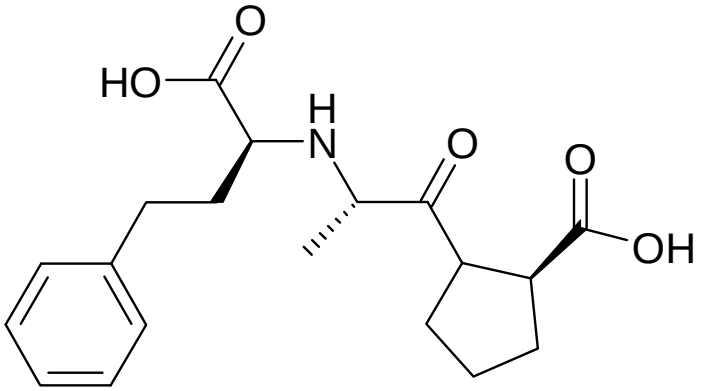


- Not listed as VO
- Readily biodegradable
- Not flammable
- Available as highly purified solvent
- Chiral (potential as chiral selector in the M.Ph.?

An application (bioassay): a possible scenario!



Enalapril



Enalaprilat

Recovery concerns:
Log K_{ow} 1-octanol = 3

Gradient elution

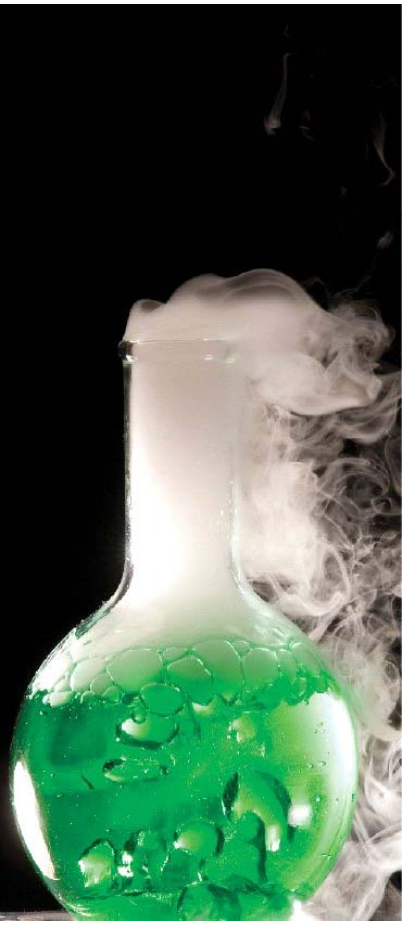
Known pharmacokinetics LC-MS/MS assay

Relatively high temperature chromatographic driven conditions

Relatively inactive pro-drug
Log K_{ow} = 0.59
Pharmacokinetic conc. range:
0.1 to 150 ng/mL
Over-selectivity:
Chromatographic separation of rotamers

Active metabolite
Log K_{ow} = -1.05
Pharmacokinetic conc. range:
0.1 to 100 ng/mL

The worst case!



“Greening” stages:

Protein Precipitation Plasma : ACN 1 : 2 → ACN based gradient elution — Method 1

1-Octanol based LLE → ACN based gradient elution — Method 2

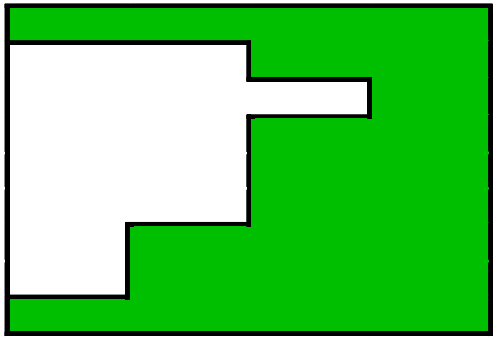
1-Octanol based LLE → PC based gradient elution — Method 2

Accommodating PC based elution → Optimizing LLE in 1-Octanol → Accommodating LVI in 1-Octanol → Accommodating MS detection with PC

Evaluation of recoveries → Evaluation of matrix effects → Quality characteristics through validation → Quality characteristics through incurred samples analysis

First concern: PC miscibility with water

Solv. A = EtOH:H_2O Solv. A / PC
1:9
1:4
3:7
2:3
1:1
3:2
7:3
4:1
9:1

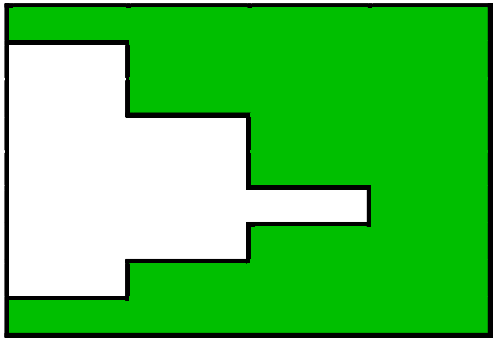


EtOH | 0% | 10% | 20% | 30% |

fully miscible

immiscible

Solv. B = EtOH:PC H_2O / Solv. B
1:9
1:4
3:7
2:3
1:1
3:2
7:3
4:1
9:1



EtOH | 0% | 10% | 20% | 30% |

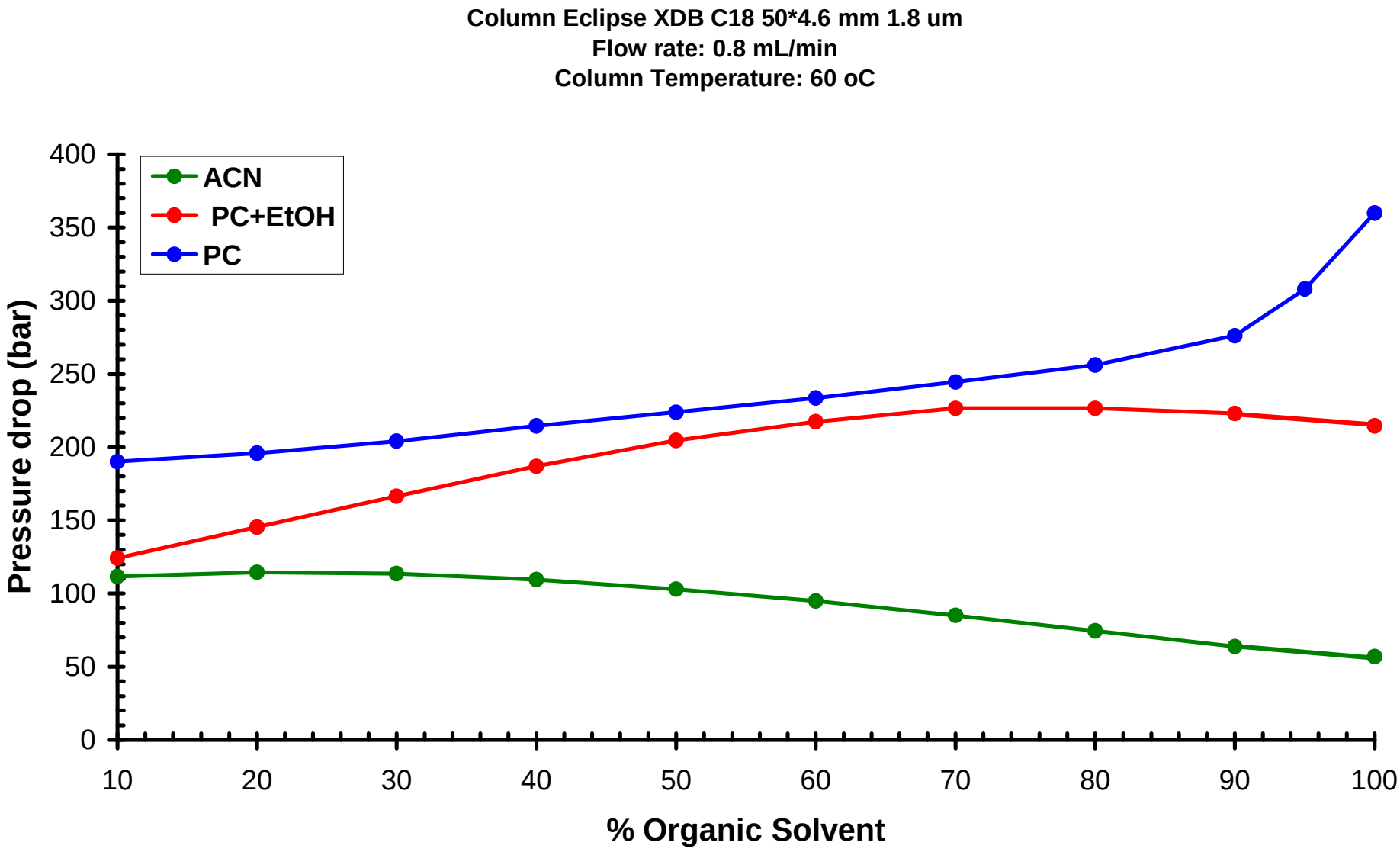
fully miscible

immiscible

a) H_2O / EtOH = 7/3 (v/v) and PC

b) PC / EtOH = 7/3 (v/v) and H_2O

Second concern: pressure drop on the column



Chromatographic conditions:

Chromatographic Column: Zorbax SB-C18 Rapid Resolution 50 mm L x 4.6 mm i.d. x 1.8 μm d.p.

Guard Column: Phenomenex C18 4 mm L x 2 mm i.d. x 5 μm d.p.

Column temperature: 50 ^{o}C

Solvent A: 0.1% HCOOH in ACN; **Solvent B:** aq. HCOOH 0.1%;

Gradient profile: (Time – min / Solvent A - % / Flow rate – mL/min): 0.0 / 10 / 0.8 → 5.0 / 70 / 0.8 → 5.01 / 100 / 0.8 → 5.50 / 100 / 0.8 →6.0 / 100 / 1.2 → 6.01 / 10 / 1.2 → 8.0 / 10 / 1.2.

Injection volume: 5 μL of water/acetonitrile solutions or 75 μL of 1-octanol solutions

Chromatographic Column: Zorbax SB-C18 Rapid Resolution 50 mm L x 4.6 mm i.d. x 1.8 μm d.p.

Guard Column: Phenomenex C18 4 mm L x 2 mm i.d. x 5 μm d.p.

Column temperature: 50 ^{o}C

Solvent A: 0.1% HCOOH in PC/EtOH (7:3 v/v); **Solvent B:** aq. HCOOH 0.1%;

Gradient profile: (Time – min / Solvent A - % / Flow rate – mL/min): 0.0 / 5 / 0.8 → 5.0 / 70 / 0.8 → 5.01 / 100 / 0.8 → 5.50 / 100 / 0.8 →6.0 / 100 / 0.8 → 6.01 / 5 / 0.8 → 10.0 / 5 / 1.2.

Injection volume: 75 μL of 1-octanol solutions

Chromatographic results:

Organic elution solvent	Analyte	ACN			PC/EtOH			PC/EtOH		
% Org. solv. at the start of the gradient		10%			10%			5%		
Injection conditions		k	N	Symm.	k	N	Symm.	k	N	Symm.
1 uL Solv.: ACN	EAT	3.9	3206	0.4	1.5	1773	0.8	3.7*	-	-
	IS2	4.9	18502	1.0	3.8	6754	0.8	5.1	18350	0.8
	E	5.8	8807	1.3	5.1	3610	1.2	5.9	6136	1.2
	IS1	7.3	36145	0.9	7.3	36244	0.8	7.7	22463	1.0
75 uL Solv.: 1-Octanol	EAT	3.7	2860	0.7	**	-	-	3.5	2708	1.3
	IS2	4.6	16386	1.0	3.5	5911	1.5	4.9	10322	1.3
	E	5.4	7859	1.0	4.9	2611	1.5	5.8	6012	1.2
	IS1	6.6	30968	0.8	6.2	27316	1.0	6.8	31640	0.7
75 uL Solv.: 1-Octanol (extraction from acidic aqueous phase)	EAT	3.7	2778	0.8	-	-	-	3.5	2668	0.9
	IS2	4.6	17126	1.0	-	-	-	4.9	16138	1.1
	E	5.4	7776	1.2	-	-	-	5.8	7745	1.2
	IS1	6.6	30152	1.0	-	-	-	6.8	30842	1.1

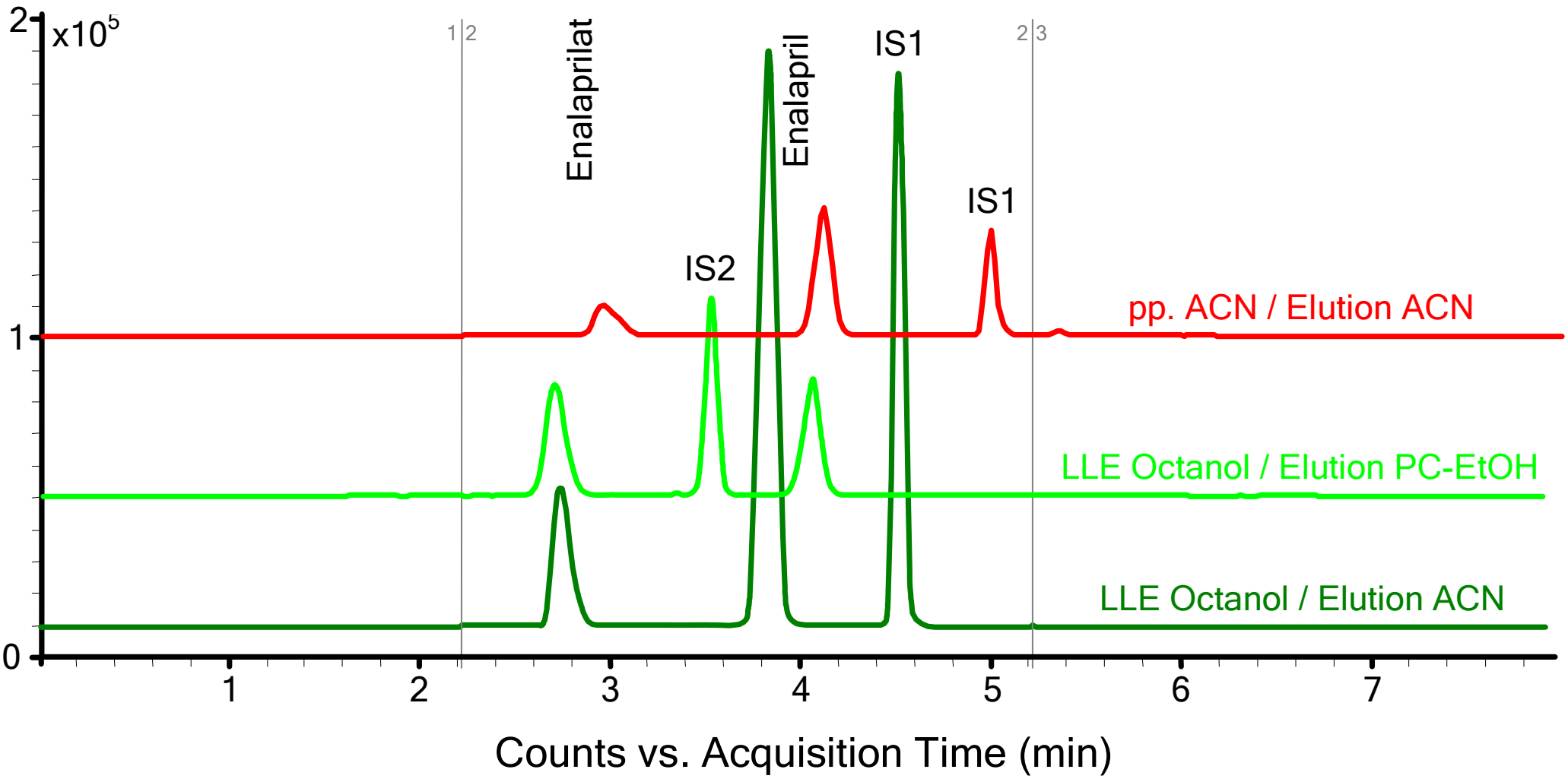
Analytes: EAT – enalaprilat; E – enalapril; IS1 – internal standard 1; IS2 – internal standard 2.

k – Retention factor; N – Efficiency; Symm. – Peak symmetry as given by integrator (a/b at 5% from peak height). These are apparent values, as long as gradient elution was used according to conditions in Experimental Section.

* *Approximate value, as the peak suffers from strong solvent focusing effects on injection.*

** *Peak splitting appears due to effects on injection; k is placed within the interval 0.8 – 1.1*

Chromatographic results:



Third concern: LVI of samples in 1-Octanol

1. Log K_{ow} diluent > Log K_{ow} analytes
2. Mobile phase composition forces diluent to saturate the Stationary Phase
3. Viscous fingering effects may arise if η diluent is significantly different from η mobile phase
4. Diluent plug is eluted from column before a consequent run

Some resulting effects:

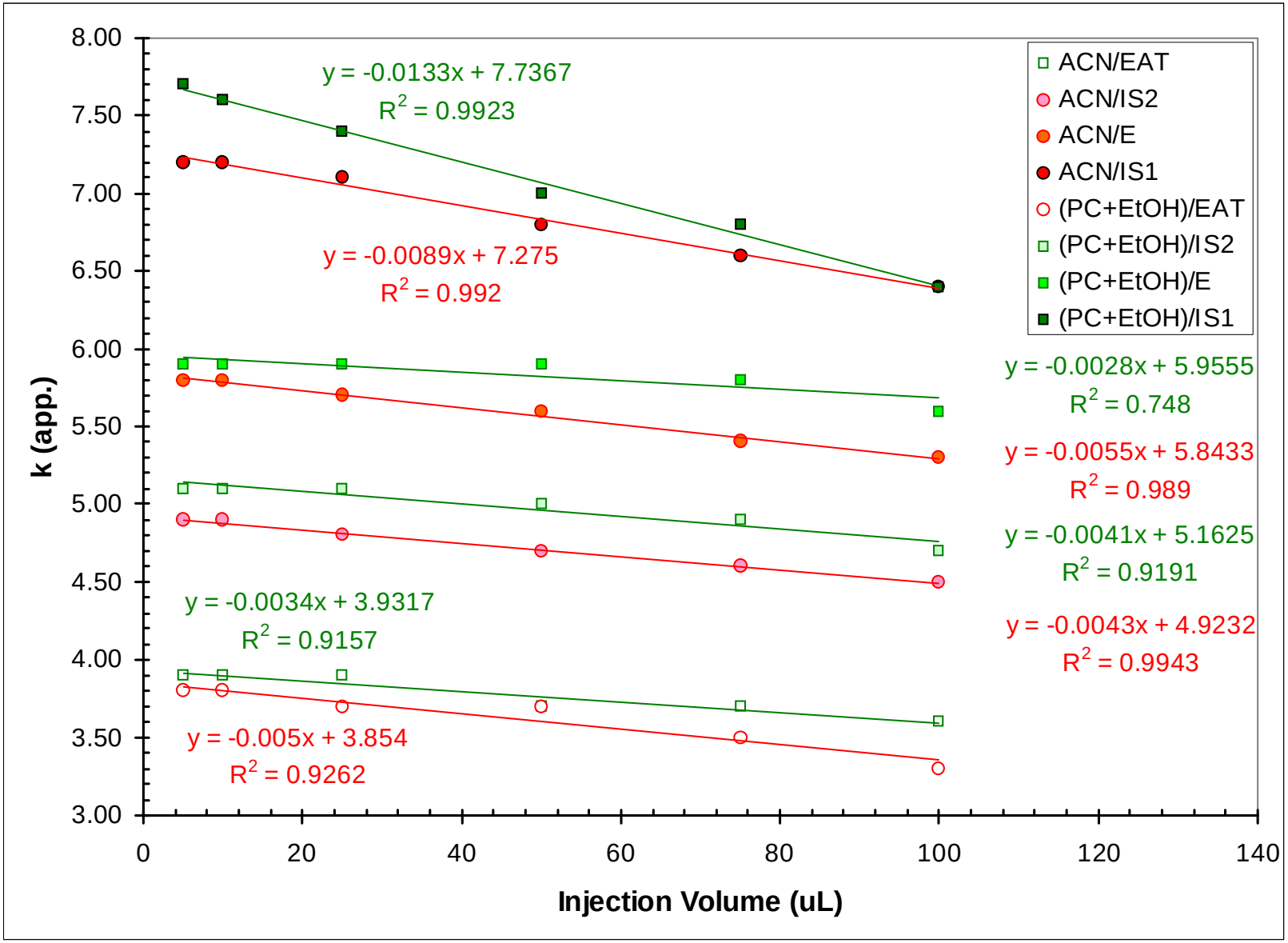
A. Retention decreases with the increase of the injected volume as the available Stationary Phase volume is lower!

B. Efficiency decreases with the increase of the injected volume!

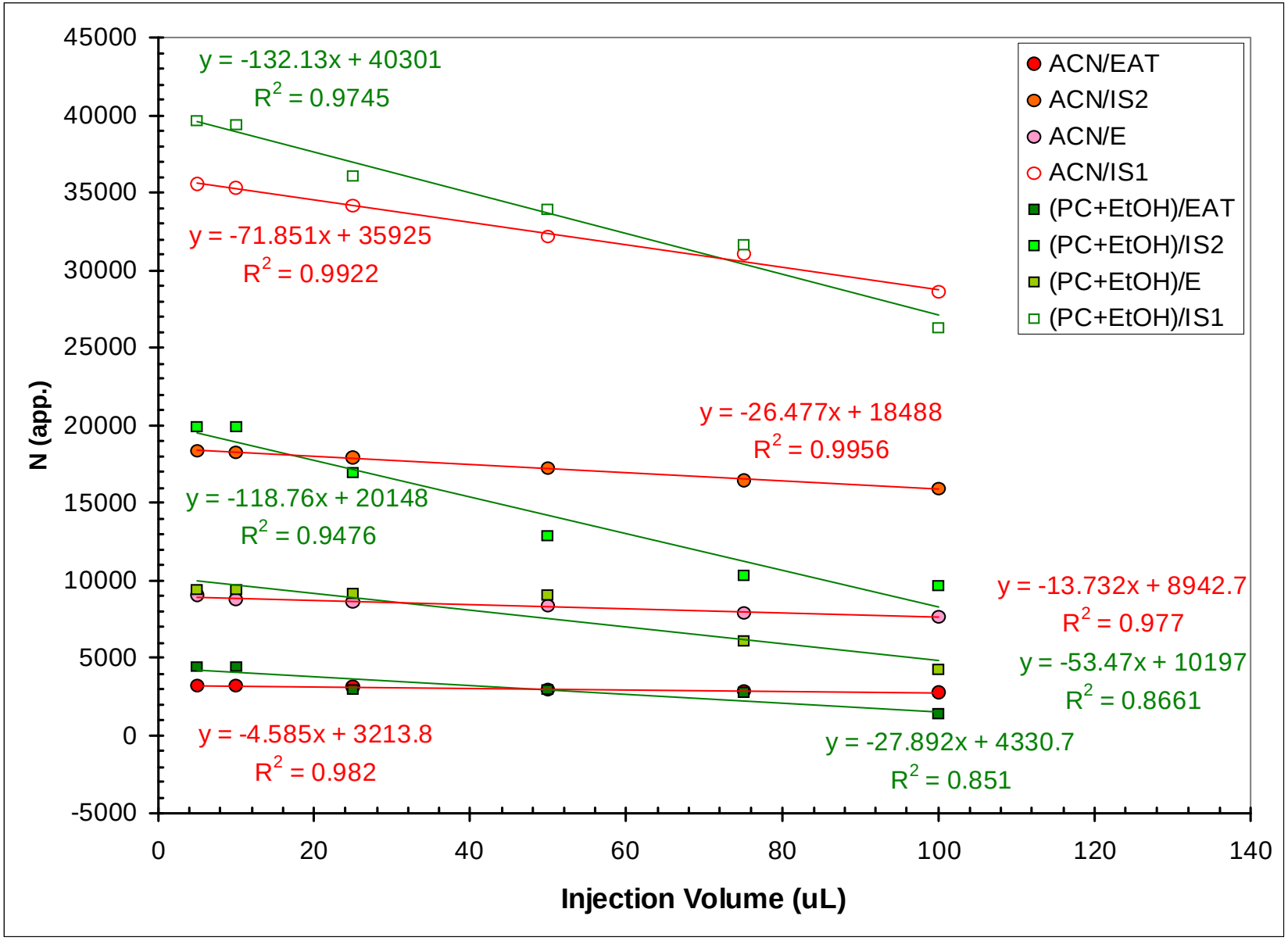
C. Peak symmetry depends on the injected volume!

A. Medvedovici et al. J. Liq. Chrom. & Rel. Technol. 30 (2007) 199.
A. Medvedovici et al. J. Sep. Sci. 31 (2008) 2939.

LVI in 1-octanol: Retention



LVI in 1-octanol: Efficiency



Sample preparation procedures

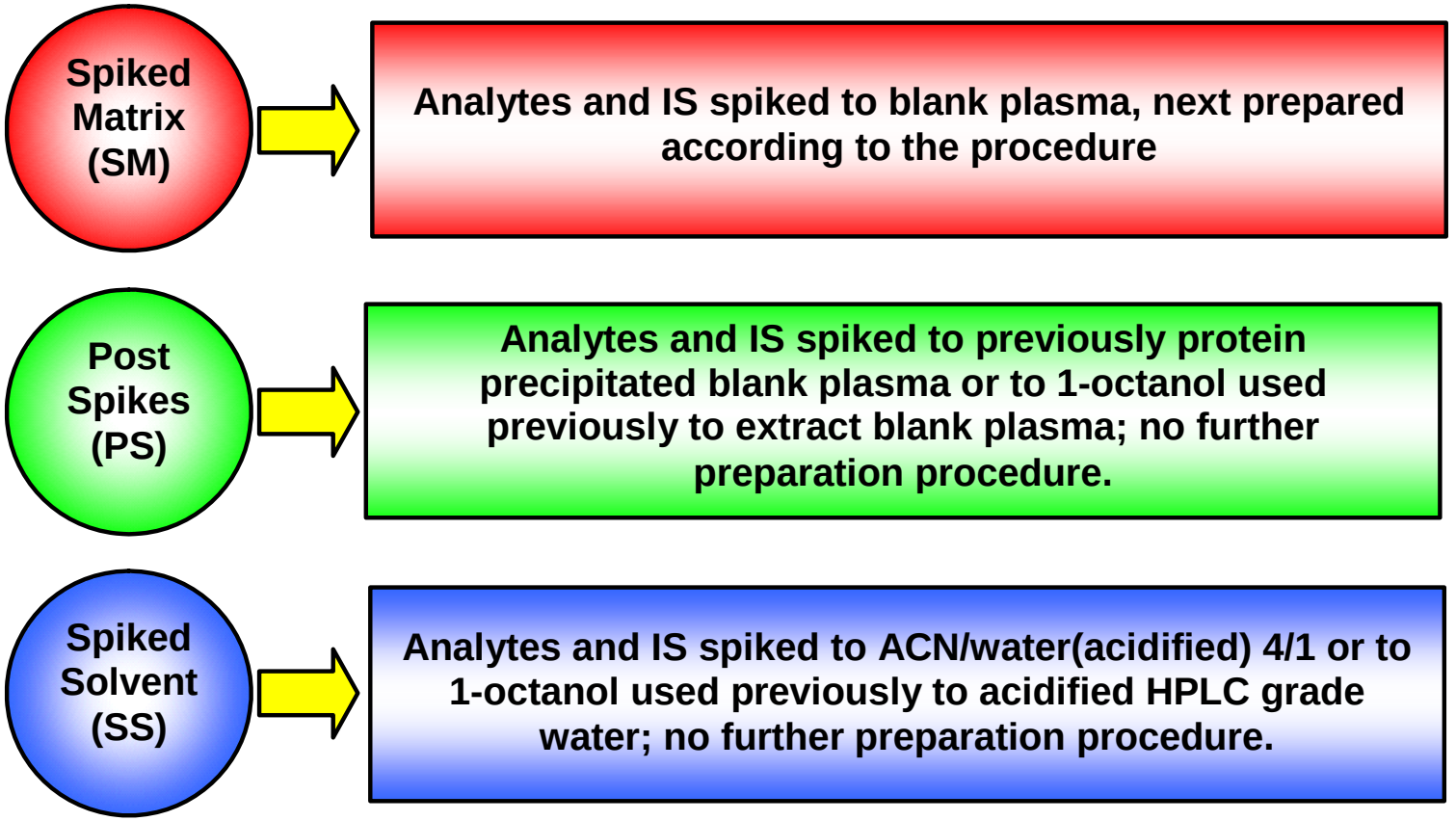
0.4 mL of IS (50 ng/mL) solution in ACN → 0.2 mL Plasma Sample → Vortex (10 min) → Centrifuge (9000 x g; 5 min; 25 °C) → Inject 5 mL from supernatant

0.3 mL of IS (50 ng/mL) solution in 1-Octanol → Vortex (10 min) → Centrifuge (9000 x g; 5 min; 25 °C) → Inject 75 mL from 1-Octanol layer

20 μL HCOOH conc. → 0.2 mL Plasma Sample → Vortex (5 min) → Acidified Plasma sample

A. Medvedovici et al. J. Pharm. Biomed. Anal. 54 (2011) 1163.
A. Medvedovici et al. Bioanalysis 3 (2011) 1935.

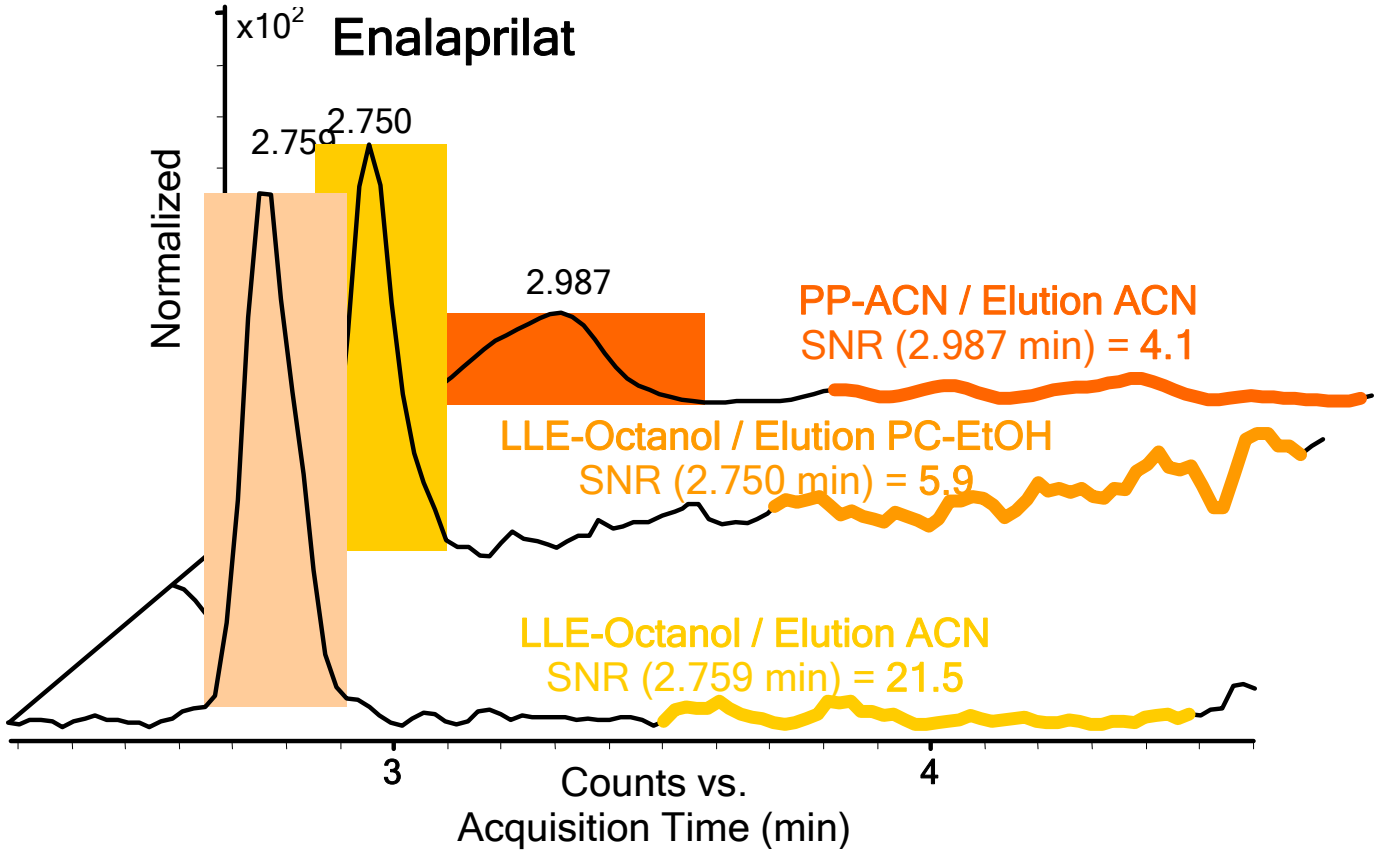
Matrix effects & Recoveries (1)



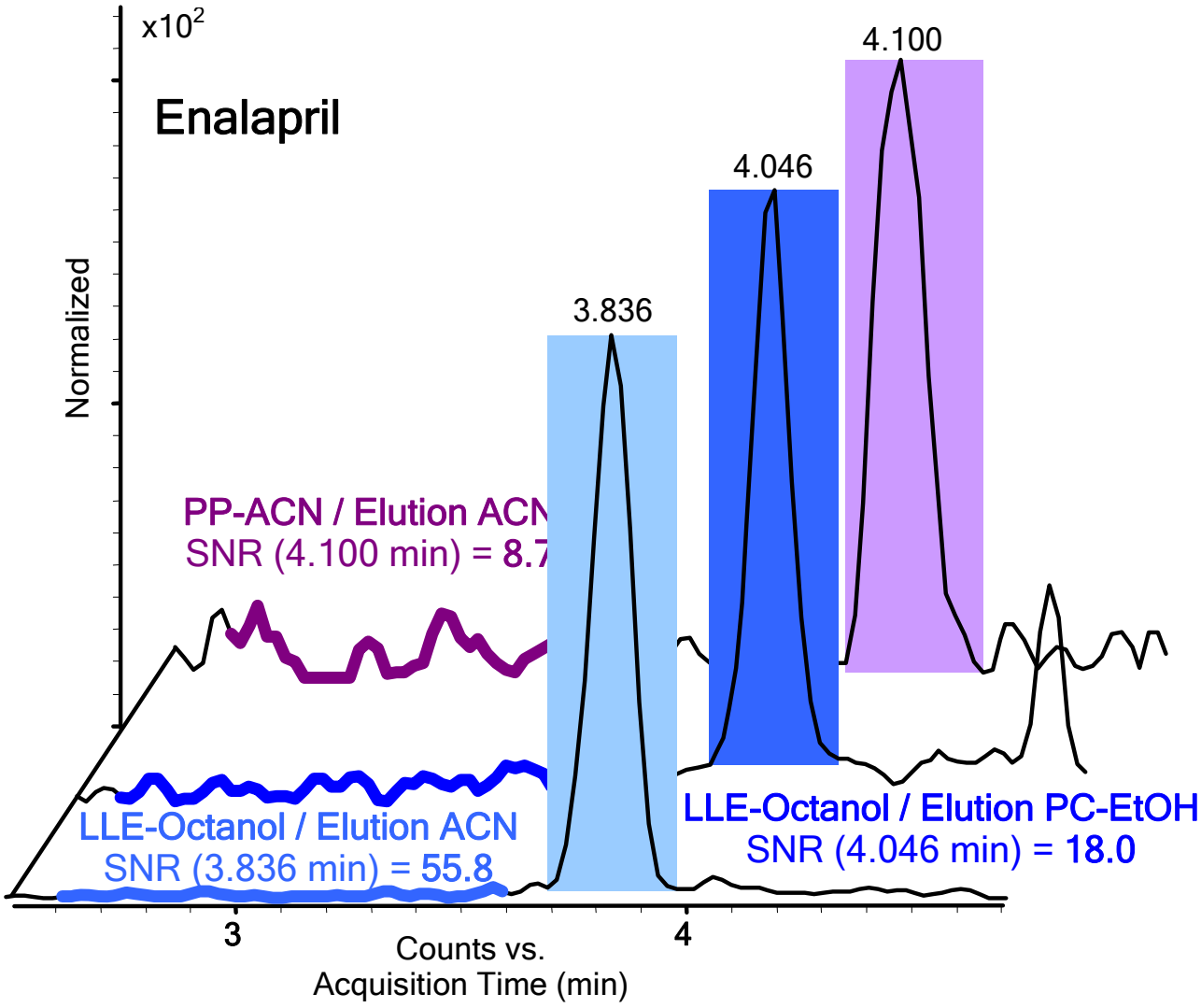
Matrix effects & Recoveries (2)

Analyte	Concentration	Recovery % SM / PS		Matrix effects PS / SS		IS normalized MF	
		%	RSD%	MF	RSD%	IS-MF	RSD%
pp. ACN / Elution ACN							
Enalapril	0.3 ng/mL	95.3	7.0	1.193	7.7	**0.86**	10.3
	150 ng/mL	90.1	4.5	1.013	1.2		
	Average	**92.4**	6.3	**1.103**	10.2		
Enalaprilat	0.3 ng/mL	98.7	5.2	1.231	6.6	**0.88**	10.2
	100 ng/mL	110.8	2.6	1.038	1.2		
	Average	**105.3**	7.0	**1.134**	10.1		
IS1	100 ng/mL	**82.0**	**3.3**	**1.291**	**3.2**	-	-
LLE Octanol / Elution ACN							
Enalapril	0.3 ng/mL	36.6	5.4	1.251	1.5	**1.38**	11.6
	150 ng/mL	44.1	5.4	1.001	1.2		
	Average	**40.4**	11.1	**1.126**	11.7		
Enalaprilat	0.3 ng/mL	58.1	3.6	1.023	2.4	**1.24**	3.3
	100 ng/mL	53.6	2.7	1.003	1.4		
	Average	**55.8**	5.2	**1.013**	2.2		
IS1	75 ng/mL	**112.1**	2.6	**0.819**	2.9	-	-
LLE Octanol / Elution PC-EtOH							
Enalapril	0.3 ng/mL	52.2	2.8	0.739	3.0	**0.91**	7.1
	150 ng/mL	45.4	8.5	0.740	5.2		
	Average	**48.8**	9.3	**0.740**	4.0		
Enalaprilat	0.3 ng/mL	47.3	10.7	0.633	8.6	**0.77**	9.5
	100 ng/mL	55.1	5.4	0.606	6.7		
	Average	**51.2**	11.1	**0.619**	7.7		
IS2	75 ng/mL	**65.1**	8.7	**0.812**	6.2	-	-

LLOQ (1)


x10²
Enalaprilat
2.750
2.759
2.987
Normalized
PP-ACN / Elution ACN
SNR (2.987 min) = 4.1
LLE-Octanol / Elution PC-EtOH
SNR (2.750 min) = 5.9
LLE-Octanol / Elution ACN
SNR (2.759 min) = 21.5
3
4
Counts vs.
Acquisition Time (min)

LLOQ (2)


x10^2
Enalapril
Normalized
4.100
4.046
3.836
PP-ACN / Elution ACN
SNR (4.100 min) = 8.7
LLE-Octanol / Elution ACN
SNR (3.836 min) = 55.8
LLE-Octanol / Elution PC-EtOH
SNR (4.046 min) = 18.0
3
4
Counts vs.
Acquisition Time (min)

Validation: Response Function

Quality characteristics	Method 1	Method 2	Method3
Studied concentration domain (ng/mL) (E/EAT)	0.1 (LLOQ) to 150 (ULOQ) / 0.1 (LLOQ) to 100 (ULOQ)		
No. of calibration levels/(conc. in ng/mL) (E/EAT)	8 / (L1-0.1 / L2-0.5 / L3-1 / L4-10 / L5-25 / L6-50 / L7-100 / L8-150) 8 / (L1-0.1 / L2-0.5 / L3-1 / L4-10 / L5-25 / L6-50 / L7-75 / L8-100)		
Regression fit (E/EAT)	linear linear	linear linear	linear linear
Weighing regression (E/EAT)	$1/x^2$ $1/x^2$	$1/x^2$ $1/x^2$	$1/x^2$ $1/x^2$
Regression parameters (y = B x + A) (E/EAT)	B=0.0299; A=0.00189 B=0.0127; A=0.0000036	B=0.0269; A=0.00069 B=0.0087; A=0.00025	B=0.0206; A=0.00105 B=0.0269; A=0.00036
Correlation coefficient (r_{xy}) (E/EAT)	0.9905 0.9942	0.9941 0.9928	0.9937 0.9938
S/N ratio (at 0.1 ng/mL) (E/EAT)	8.7 4.1	55.8 21.5	18.0 5.9
Minimum RSD% per concentration level (level) (E/EAT)	1.1% (L6) 1.1% (L8)	0.8% (L3) 0.45% (L7)	1.6% (L8) 2.6% (L8)
Maximum RSD% per concentration level (level) (E/EAT)	5.4% (L1) 8.9% (L1)	11.5% (L1) 8.1% (L1)	14.75% (L1) 9.7% (L1)
Minimum % Bias per concentration level (conc.) (E/EAT)	-9.7% (L2) -4.98% (L3)	-10.1% (L2) -8.9% (L4)	-6.1% (L3) -7.1% (L2)
Maximum % Bias per concentration level (conc.) (E/EAT)	13.4% (L8) 10.9% (L8)	9.5% (L8) 10.8% (L8)	5.4% (L7) 6.0% (L6)

Validation: Precision

Quality characteristics	Method 1	Method 2	Method3
No. QC levels (number /concentration in ng/mL) (E/EAT)	5 (QC1-0.1/QC2-0.3/QC3-5/QC4-30/QC5-75) 5 (QC1-0.1/QC2-0.3/QC3-5/QC4-30/QC5-60)		
Intraday minimum RSD% (QC level) (n=10) (E/EAT)	1.02% (QC5) 0.63% (QC5)	0.57% (QC4) 0.79% (QC4)	0.95% (QC4) 3.15% (QC3)
Intraday maximum RSD% (QC level) (n=10) (E/EAT)	4.9% (QC2) 6.9% (QC1)	8.2% (QC1) 6.2% (QC1)	5.8% (QC1) 7.0% (QC1)
Intraday minimum averaged % bias (QC level) (E/EAT)	-12.6% (QC3) -6.7% (QC3)	-7.5% (QC1) -1.5% (QC3)	-7.3% (QC2) -8.5% (QC3)
Intraday maximum averaged % bias (QC level) (E/EAT)	7.7% (QC5) 6.3% (QC5)	8.9% (QC4) 12.9% (QC 5)	1.3% (QC4) 1.1% (QC1)
Interday minimum RSD% (QC level) (n=6) (E/EAT)	4.2% (QC5) 4.5% (QC4)	1.9% (QC4) 2.96% (QC5)	3.2% (QC4) 3.0% (QC3)
Interday maximum RSD% (QC level) (n=6) (E/EAT)	12.9% (QC1) 11.4% (QC1)	6.3% (QC1) 8.2% (QC1)	15.5% (QC1) 9.8% (QC1)
Interday minimum averaged % bias (QC level) (E/EAT)	-8.2% (QC3) -4.2% (QC3)	-9.2% (QC1) -7.2% (QC2)	-11.9% (QC2) -7.9% (QC2)
Interday maximum averaged % bias (QC level) (E/EAT)	7.8% (QC5) 7.2% (QC5)	4.2% (QC5) 8.01% (QC1)	4.3% (QC4) 4.8% (QC5)

Comparison of results from incurred samples

Bioequivalence Study: Single dose, randomized, open-label, 2 periods, 2 treatments, 2 sequences, cross-over trial with 14 days wash-out between periods I and II, fasting conditions.

Pharmaceutical formulations: Tablets

Dose: 10 mg

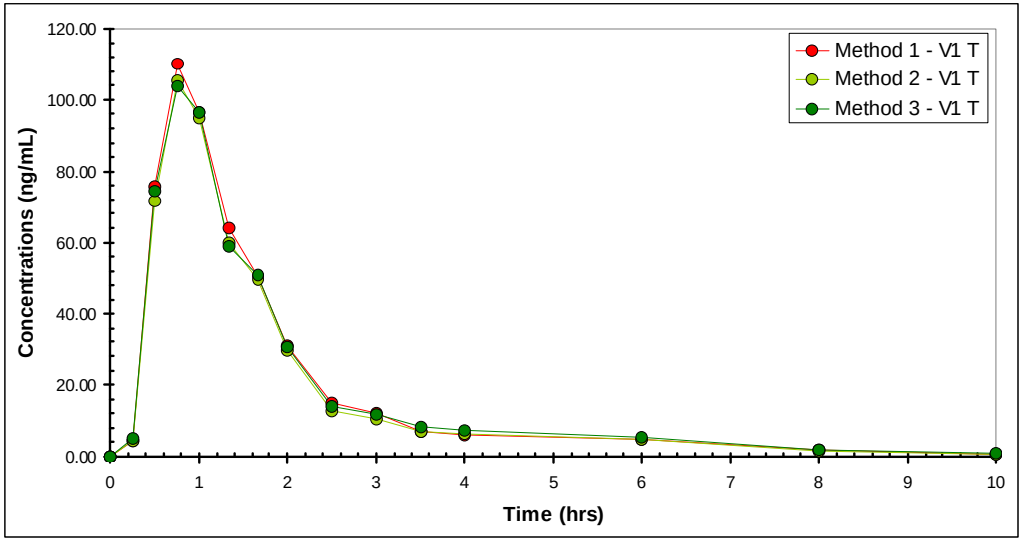
Number of Healthy Volunteers: 26

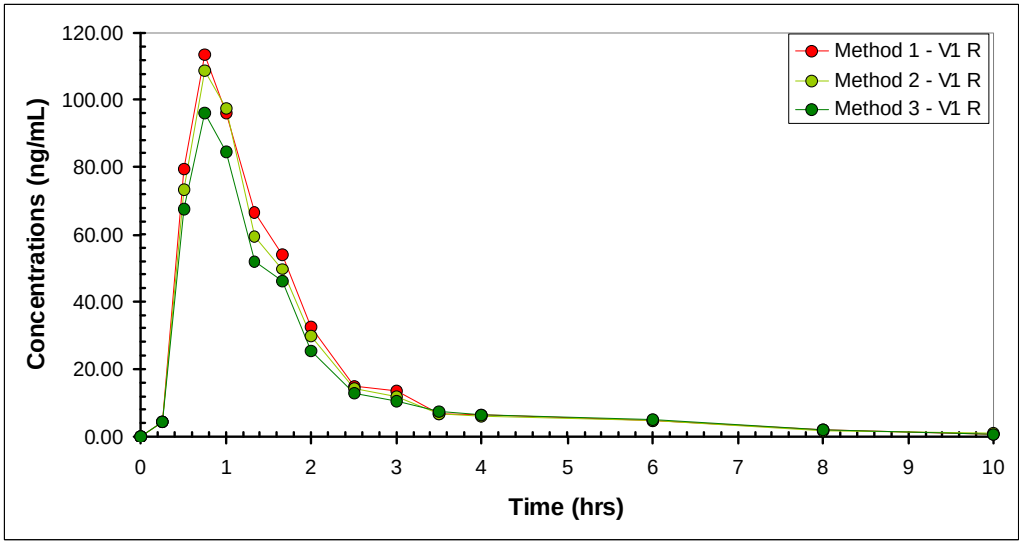
Selected volunteers: 2 (both phases, Reference & Tested)

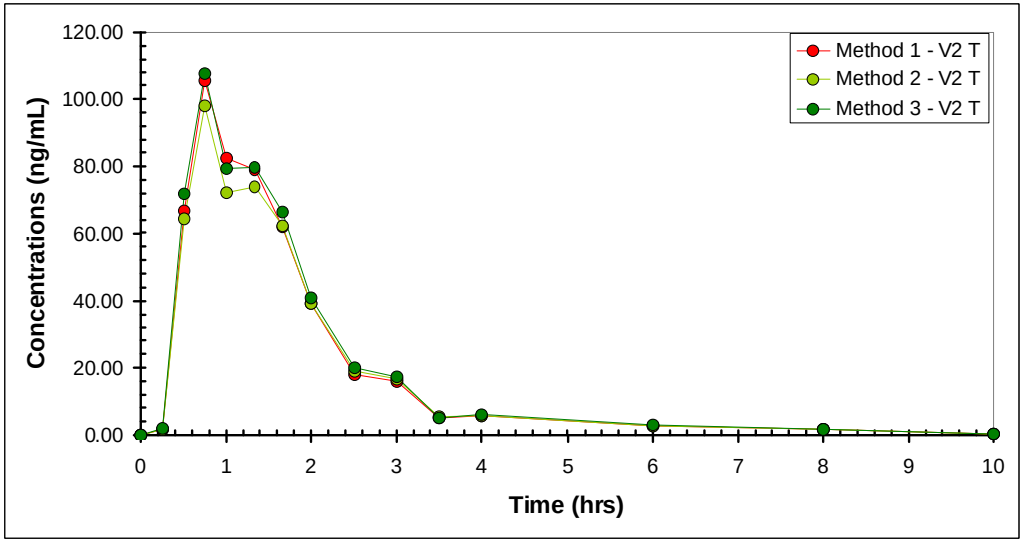
Sample withdrawal: before the oral administration and 0.25 – 0.5 – 0.75 - 1 – 1.33 – 1.66 - 2 – 2.5 - 3 – 3.5 - 4 – 6 – 8 – 10 – 12 – 16 - 24 – 48 - 72 hours after administration

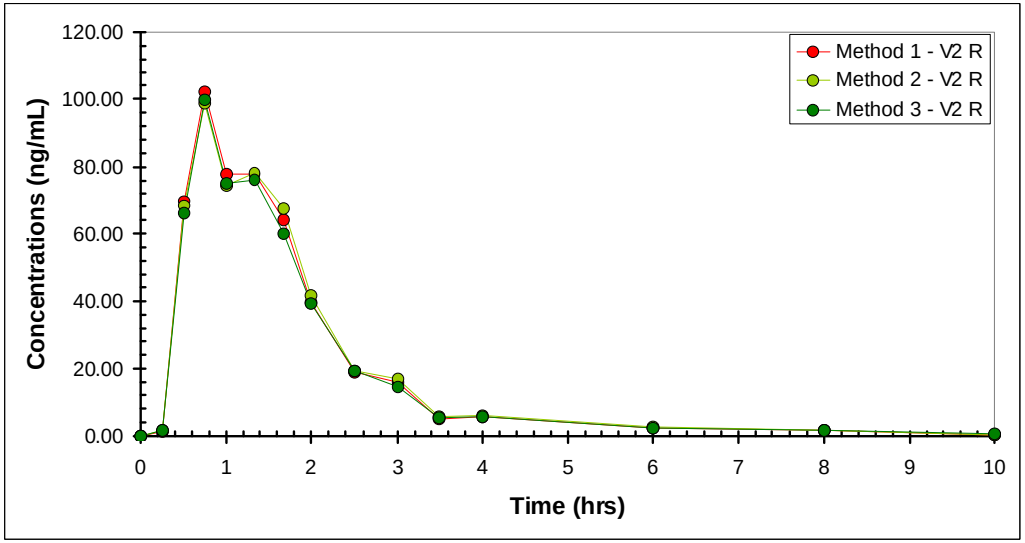
Protocol: acceptance from the Romanian Drug Agency & National Ethics Committee.

Comparison: Visual (Enalapril)

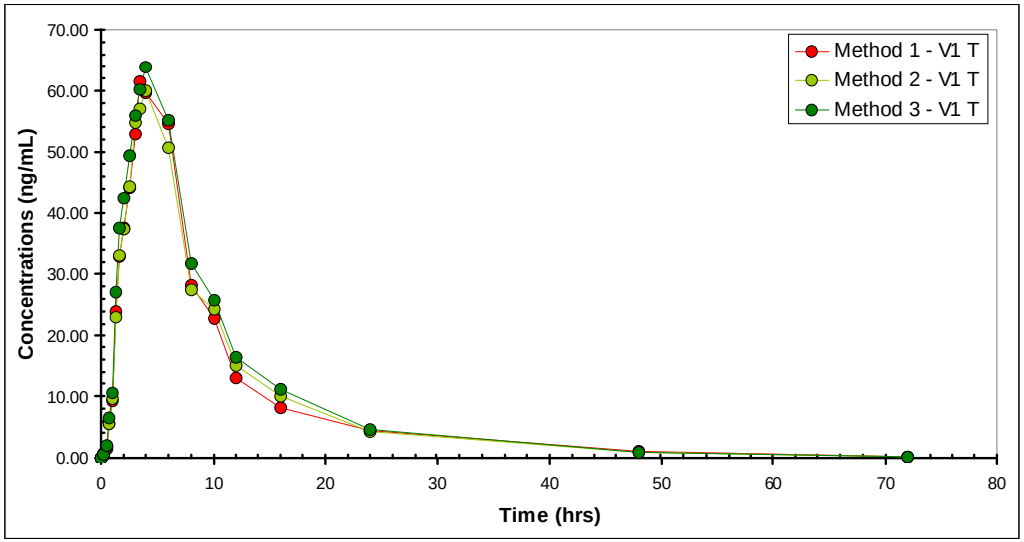


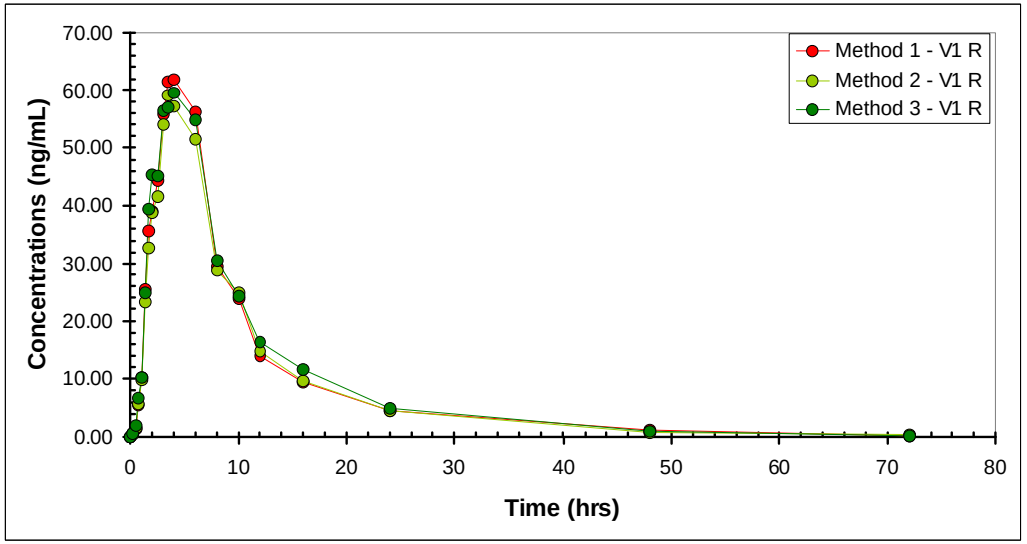


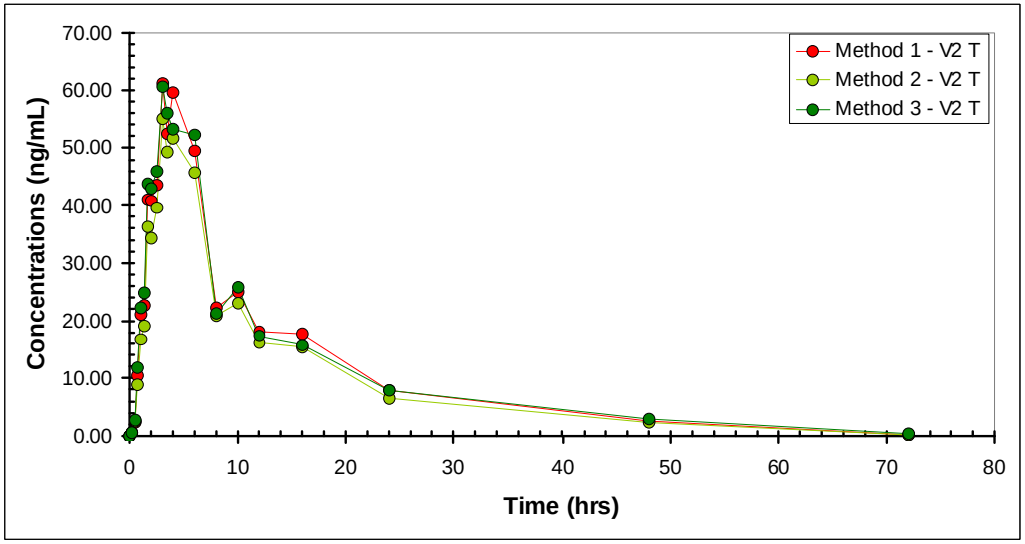


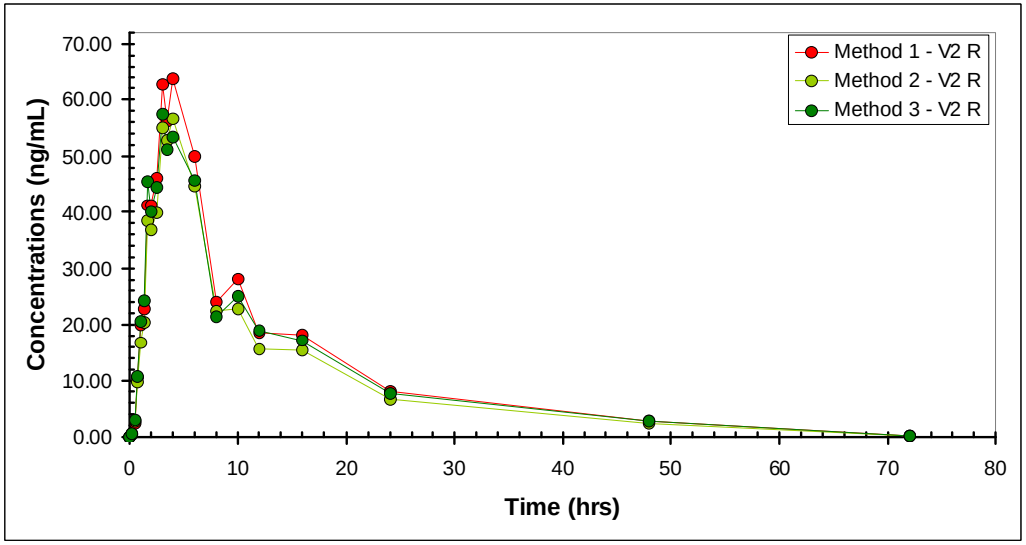


Comparison: Visual (Enalaprilat)

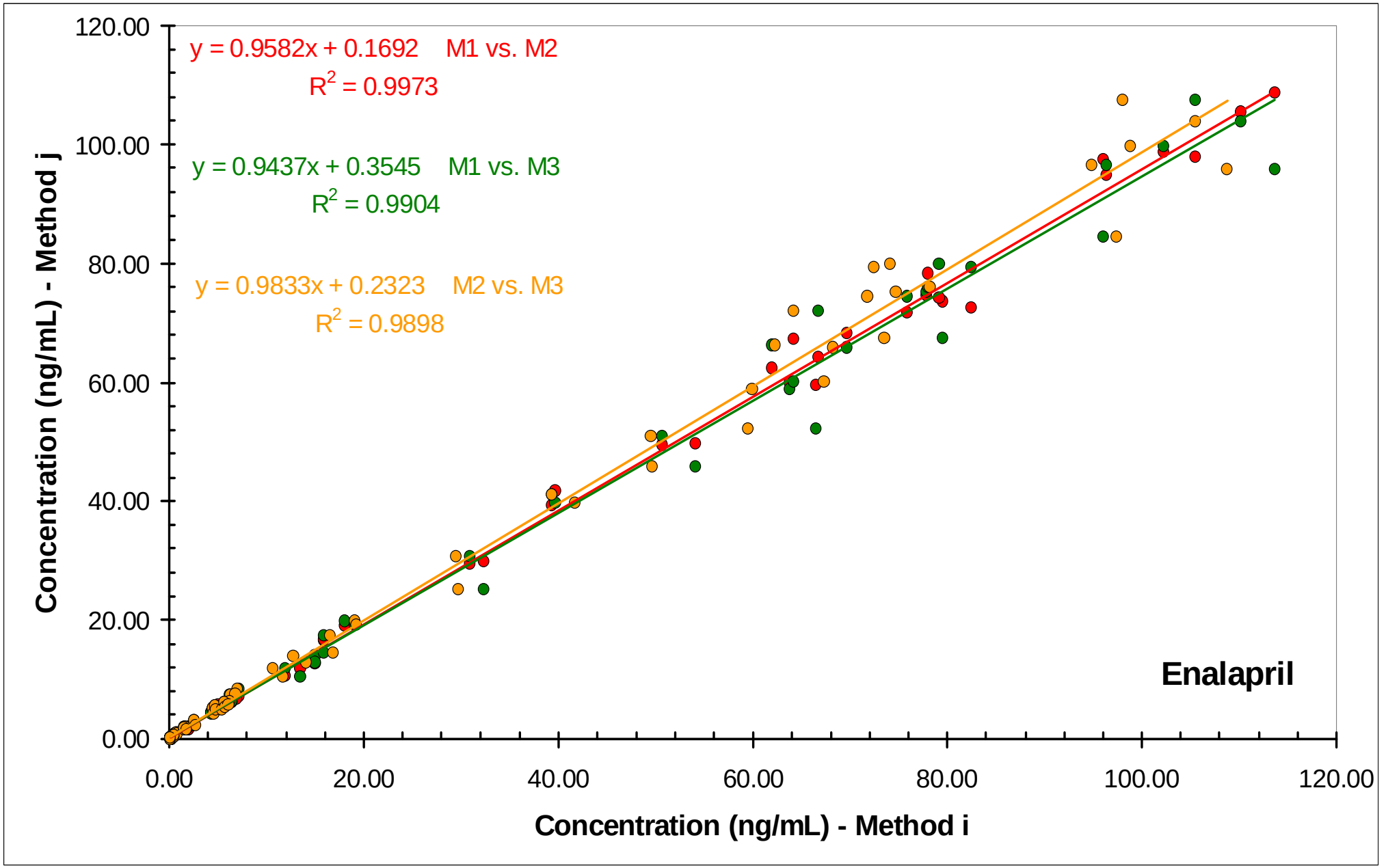




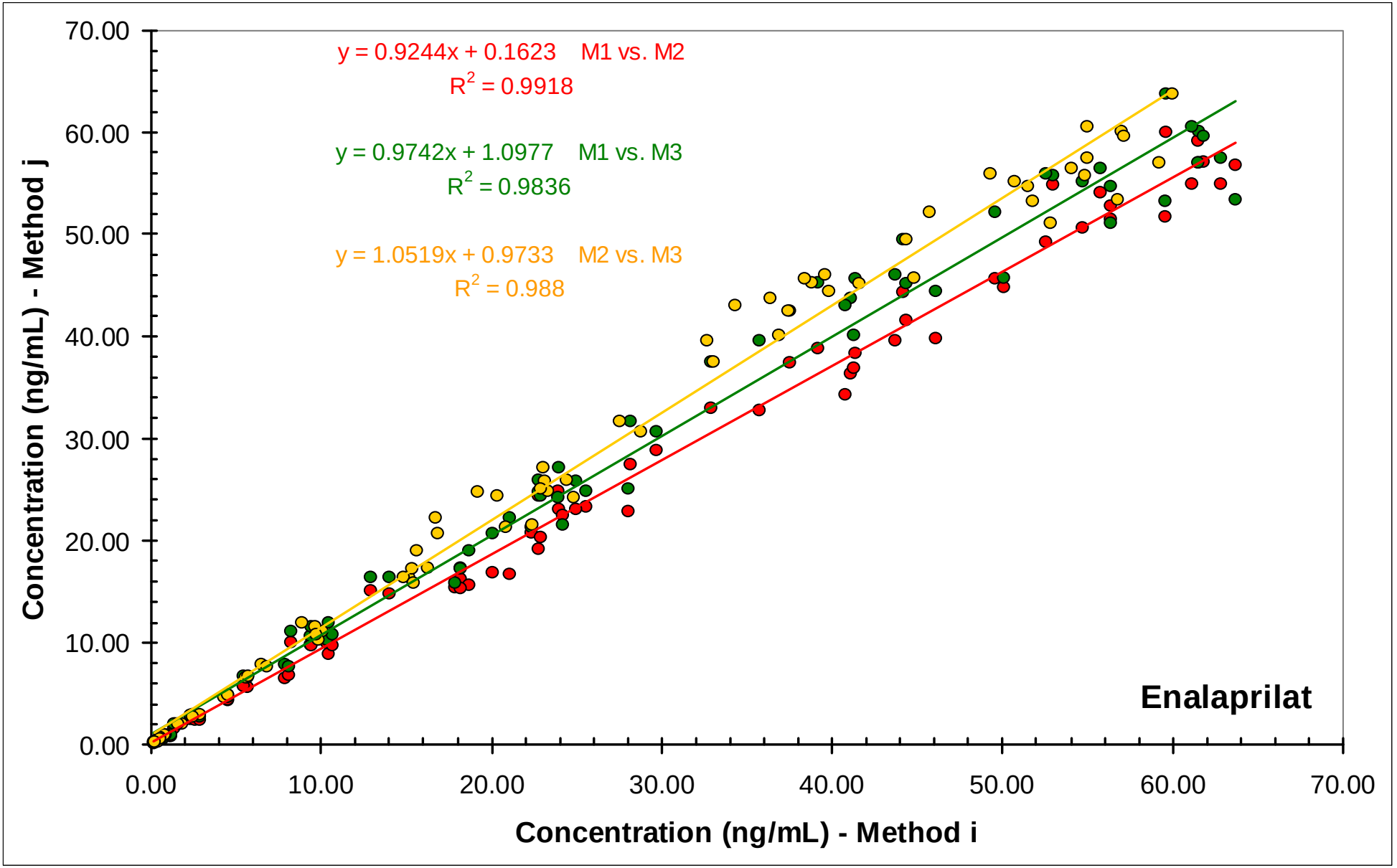




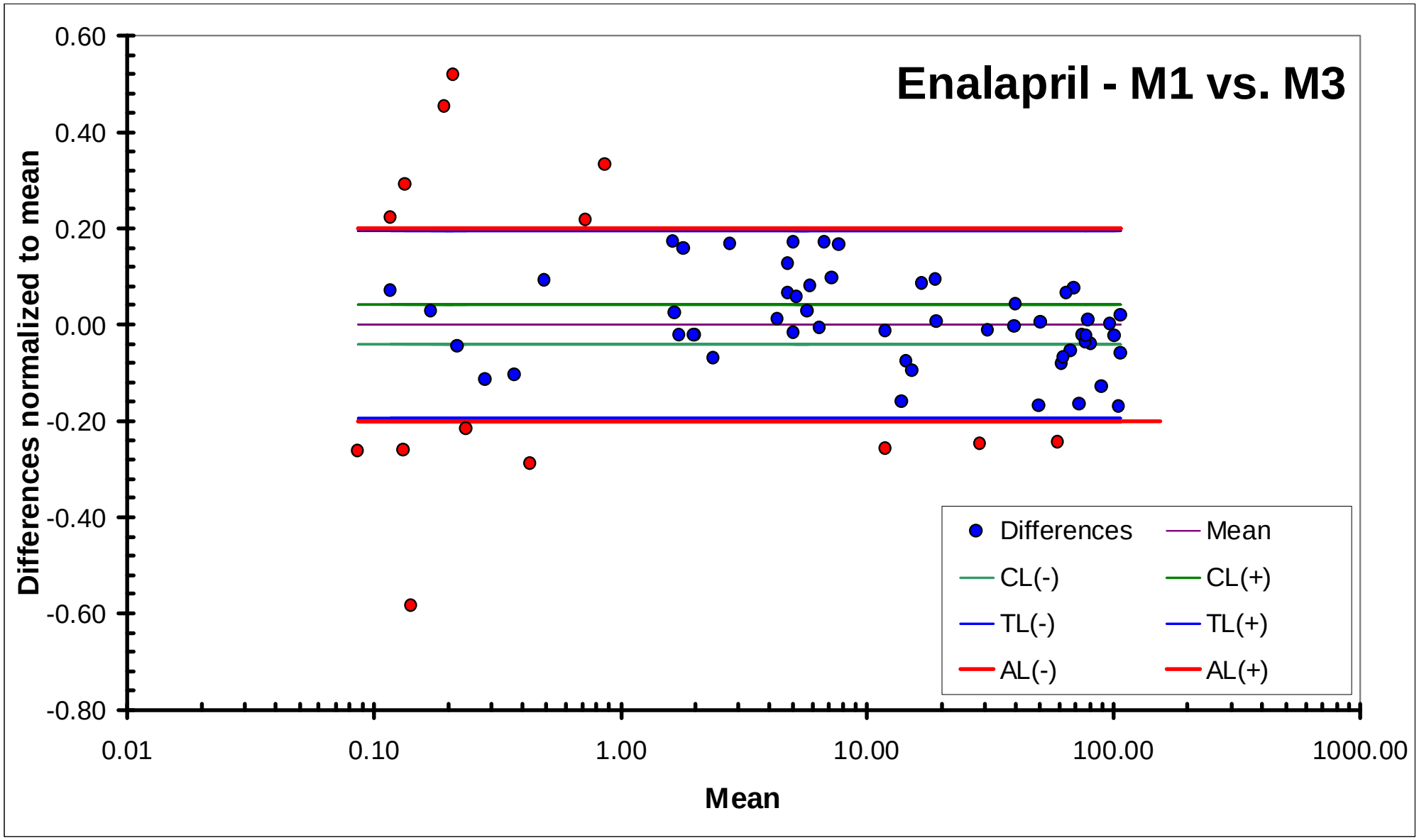
Comparison: Linear Regression (Enalapril)



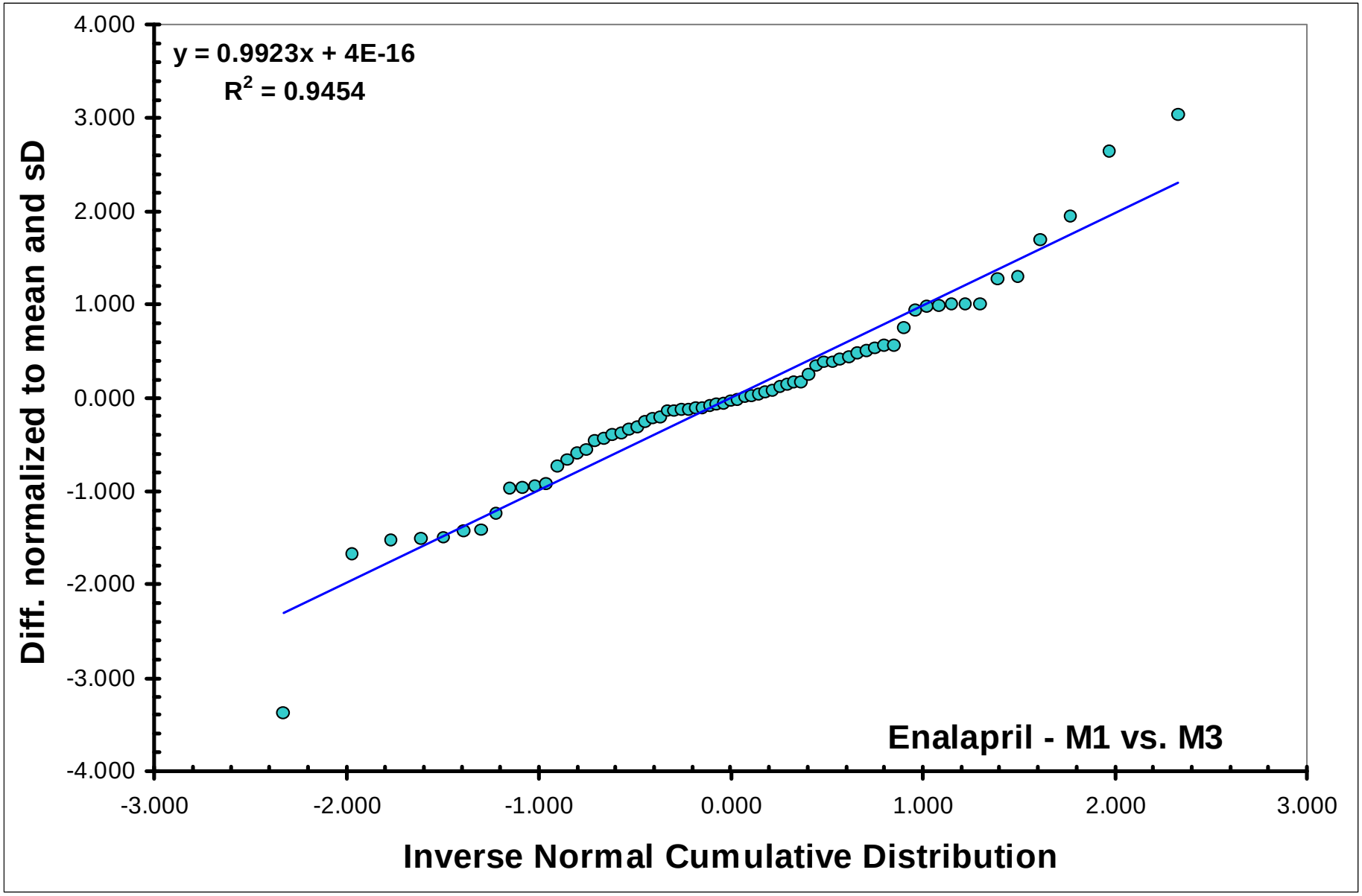
Comparison: Linear Regression (Enalaprilat)



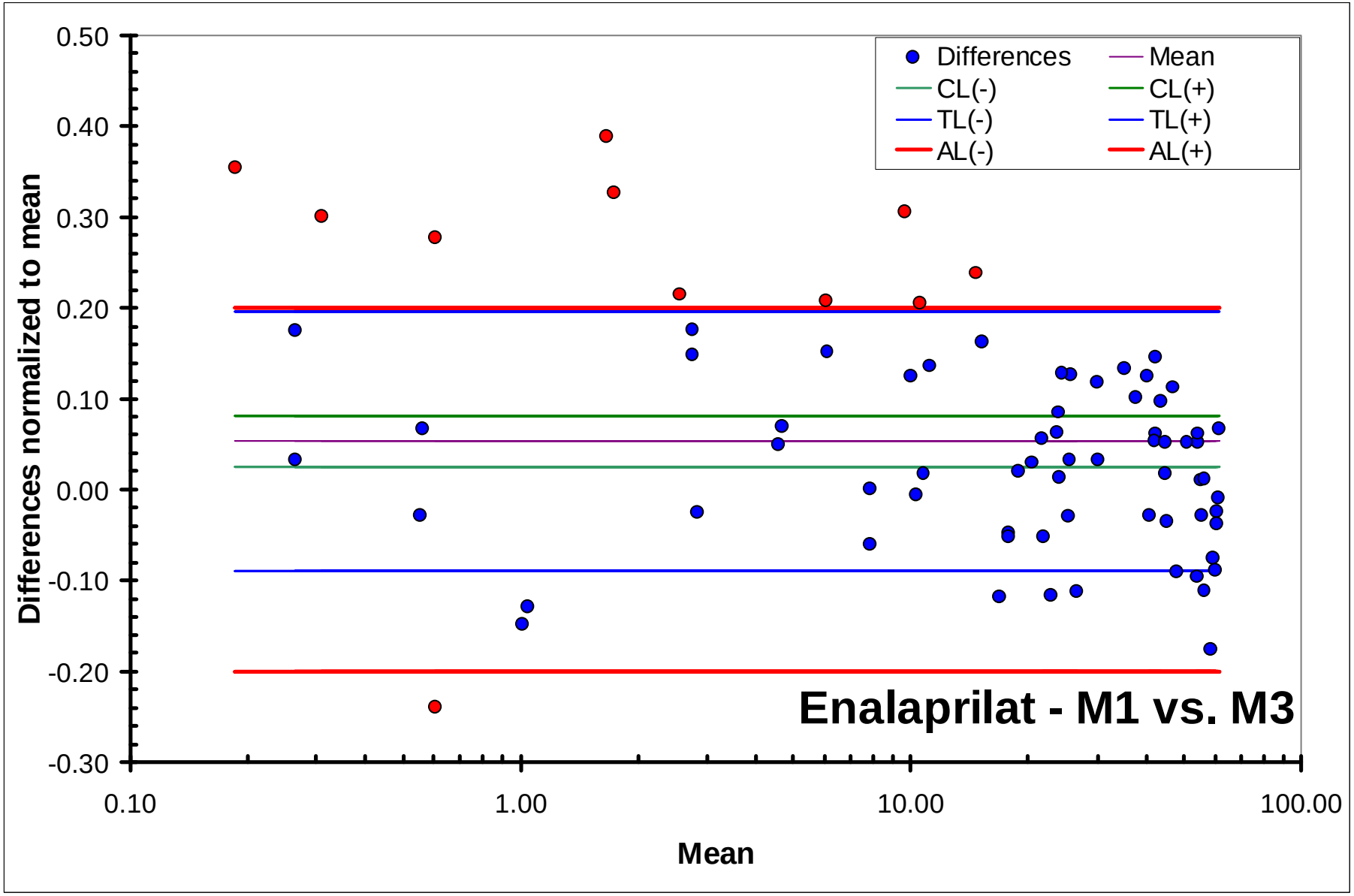
Comparison: Bland Altman (Enalapril)



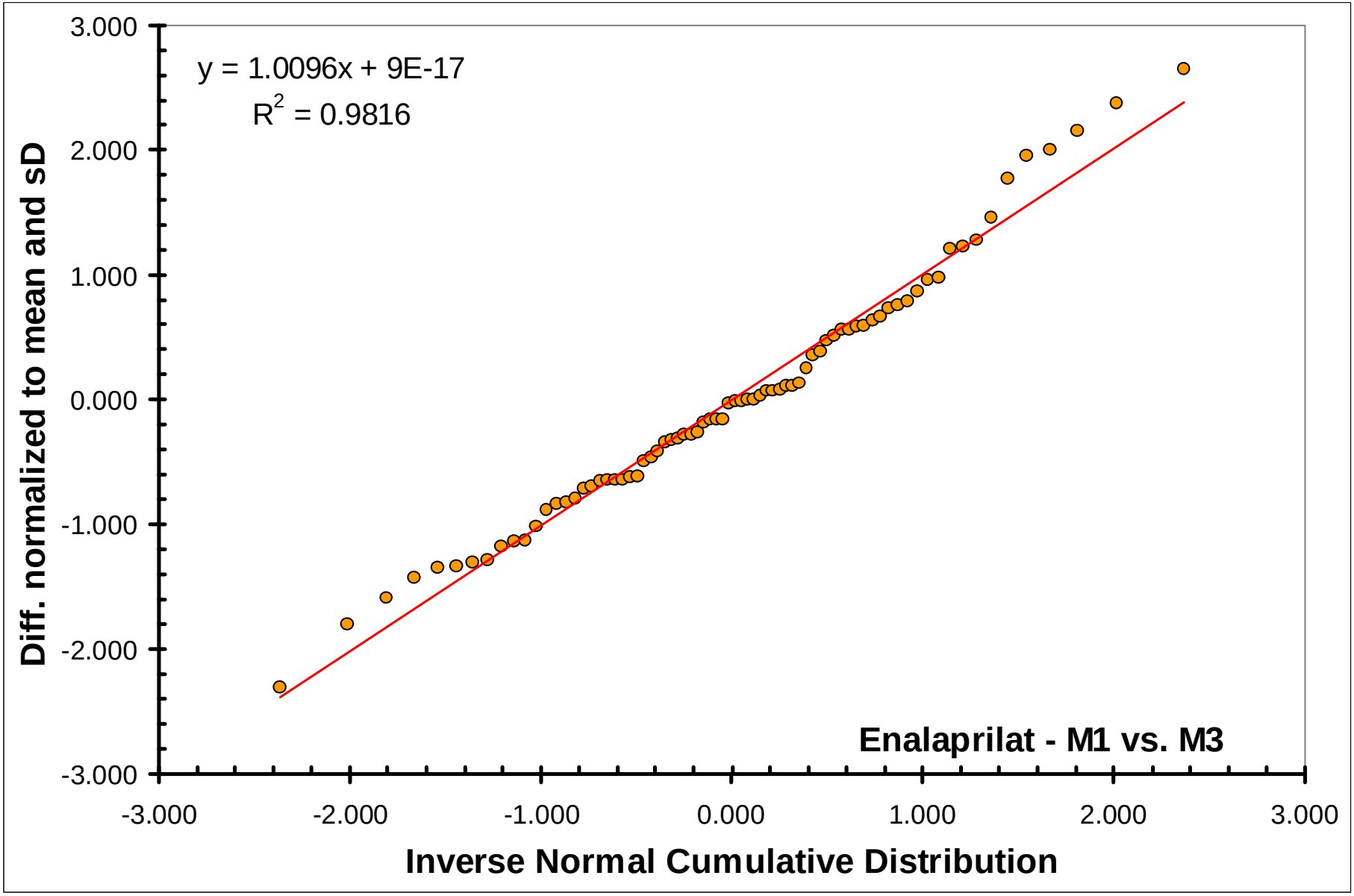
Comparison: Probability Plots (Enalapril)



Comparison: Bland Altman (Enalaprilat)



Comparison: Probability Plots (Enalaprilat)



Comparison: Bland Altman

Parameter	Method 1 vs. Method 2		Method 1 vs. Method 3		Method 2 vs. Method 3	
	Enalapril	Enalaprilat	Enalapril	Enalaprilat	Enalapril	Enalaprilat
Number of pairs	69	76	69	76	69	76
Minimum of the means	0.10	0.20	0.09	0.19	0.09	0.20
Maximum of the means	112.0	60.3	107.0	61.72	104.63	61.90
Average of the mean normalized differences	-0.0234	-0.0600	0.0001	0.0534	0.0235	0.1135
S_D	0.1323	0.1226	0.1719	0.1267	0.1394	0.0997
Confidence Limit (-)	-0.0546	-0.0875	-0.0405	0.0249	-0.0094	0.0911
Confidence Limit (+)	0.0078	-0.0324	0.0406	0.0819	0.0564	0.1359
Tolerance limit (-)	-0.1700	-0.2000	-0.1959	-0.0896	-0.1354	0.0011
Tolerance limit (+)	0.1300	0.0800	0.1961	0.1964	0.1824	0.2259
No. of pairs out of Acceptance Limits	10	7	14	11	8	13
% of pairs out of Acceptance Limits	14.5	9.21	20.3	14.5	11.6	17.1
CV%	9.35	8.67	12.16	8.96	9.86	7.05
Distribution of errors	Normal	Normal	Normal	Normal	Normal	Normal
Slope	0.9911	0.9900	0.9454	1.0096	1.0115	0.9708
R ^2	0.9430	0.9437	0.9923	0.9816	0.9822	0.9075
No. of outliers	1	2	1	0	0	1
Status of the Bland-Altman approach	Pass	Pass	Pass	Pass	Pass	Pass
No. of pairs out of 80-120% interval*	9	6	13	11	9	20
% of pairs out of 80-120% interval*	13.0	7.9	18.8	14.5	13	26.3

** when comparison is not made with respect to the mean, but with respect to the absolute values determined by the first method.*

J.M. Bland, D.G. Altman, Lancet, 327 (1986) 307.

D.J. Altman, J.M. Bland, Clin. Chem., 48 (2002) 801.

Conclusions

1. **All analytical alternatives behave similarly**.
2. **“Greening” of the bioassay is feasible:**
   - Protein precipitation with ACN was replaced by LLE in 1-Octanol
   - LVI of diluents non miscible with the M.Ph. is feasible
   - PC/EtOH (7/3) successfully replaces ACN as organic modifier
   - Small modification of the gradient profile is needed when shifting from CAN to PC/EtOH
3. **No compromise in performance criteria is made.**

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Thank you for your patience and attention!