New approaches for the bioassay of oximes by means of LC-ESI/MS/MS

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13th International Congress of the Romanian Society of Clinical Pharmacology, Therapeutics and Toxicology

SOCIETATEA ROMÂNĂ
DE FARMACOLOGIE, TERAPEUTICĂ
ȘI TOXICOLOGIE CLINICĂ

Poiana Brasov, June 11th-14th, 2012

State of the art: Oxime-type AChE Reactivators:

- ☺ Wide use as antidotes in organo phosphorous compounds poisoning;
- ☺ Synthesis of new congeners;
- 😐 Intense studies on their transfer through BBB;
- 😐 Intense studies on structure – properties relationships;
- 😐 Intense studies on dose – effects correlation;

- ☹ **About bioassay of Oximes:**
  ? New isolation alternatives from biomatrices
  ? Chromatographic separation issues
  ? Reserve on using MS or MS/MS detection

Our main goals:

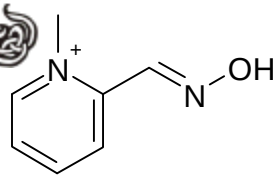
1. (ESI) - MS and MS/MS behavior of Oximes;

2. Study of chromatographic retention of Oximes through:
   - HILIC mechanism;
   - Double (duet!) cation exchange / RP mechanism;
   - Ion Pair formation mechanism with use of perfluorurated ion pair reagents;
   -

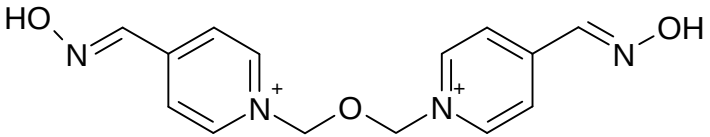
3. Isolation of Oximes from biomatrices:
   - Reevaluation of Protein Precipitation procedure through ACN addition;
   - Isolation of Oximes by means of Ionic Liquid

4. Bioassay validation (matrices: plasma and whole blood)

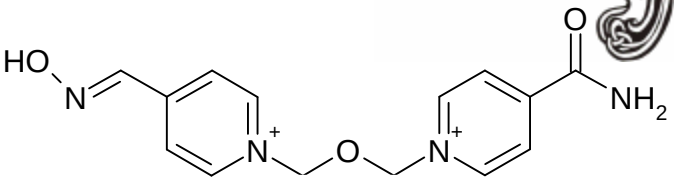
Target Compounds:



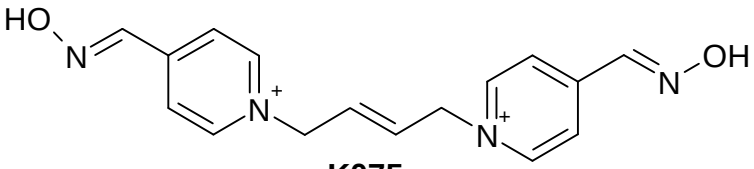
2-PAM
Mw = 137.16



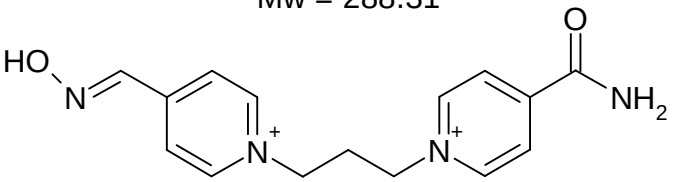
Obidoxime
Mw = 288.31



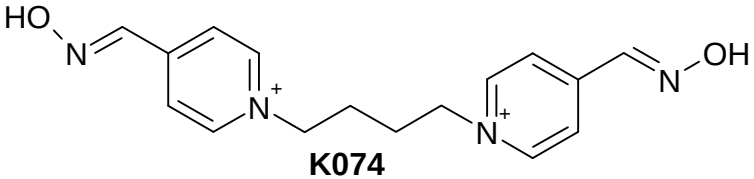
HI-6
Mw = 288.31



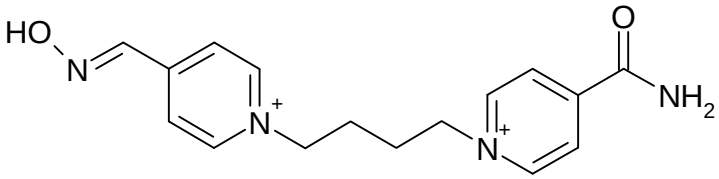
K075
Mw = 298.35



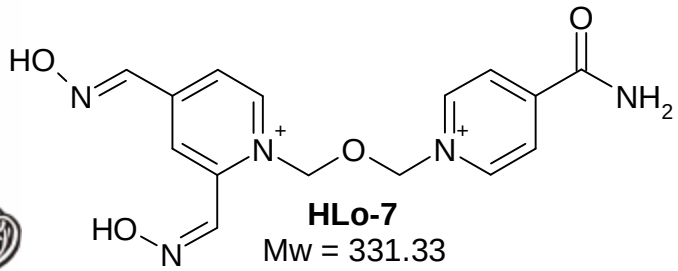
K027
Mw = 286.34



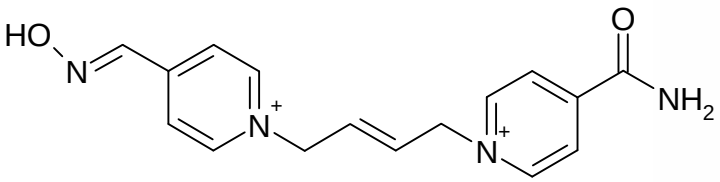
K074
Mw = 300.36



K048
Mw = 300.36



HLo-7
Mw = 331.33



K203
Mw = 298.35

Target Compounds:

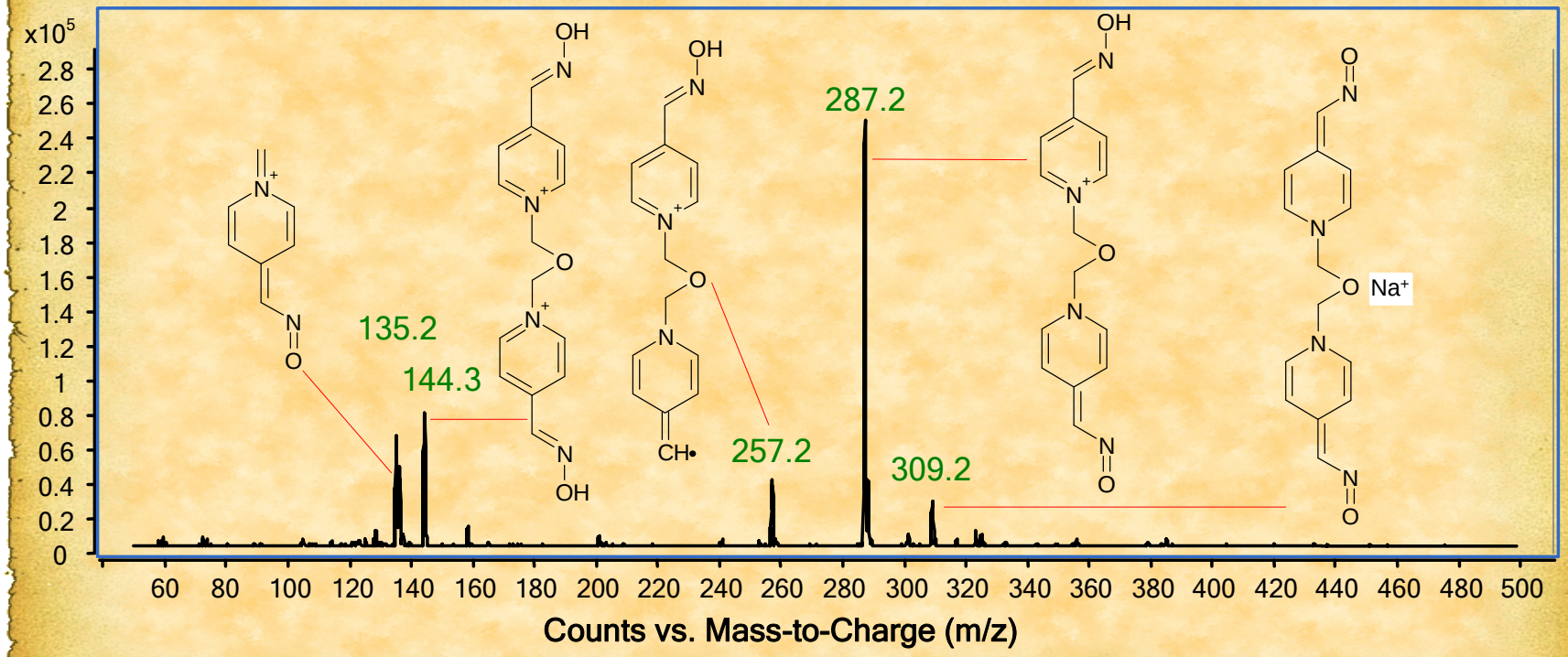
4 categories:

a. 1 pyridinium ring + 1 aldoxime moiety;
b. 2 pyridinium rings + 2 aldoxime moieties;
c. 2 pyridinium rings + 1 aldoxime moiety + 1 amide moiety;
d. 2 pyridinium rings + 2 aldoxime moieties + 1 amide moiety

Linkers between pyridinium rings:

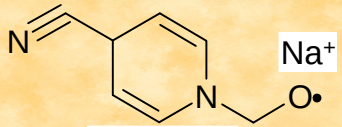
a. Ether
b. Hydrocarbonate saturated (different lengths)
c. Hydrocarbonate unsaturated (different lengths)

MS spectra: i.e. Obidoxime

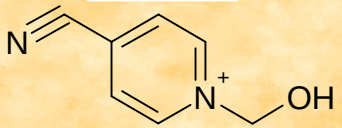


MS/MS spectra: Obidoxime

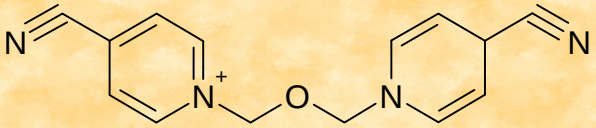
m/z = 309
[M - 2H⁺]Na⁺



m/z = 158
100

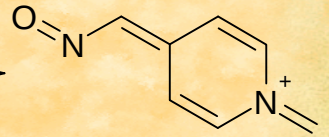


m/z = 135
22

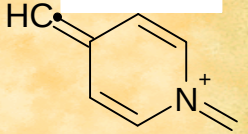


m/z = 253
8

m/z = 287
[M - H⁺]⁺



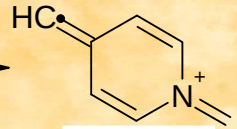
m/z = 135
100



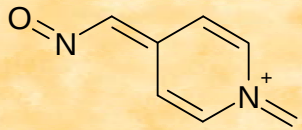
m/z = 105
30

MS/MS spectra: Obidoxime

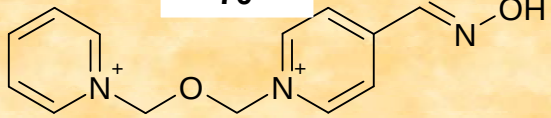
m/z = 257
$[M - H^+ - NO\cdot]^{+\cdot}$



m/z = 105
100

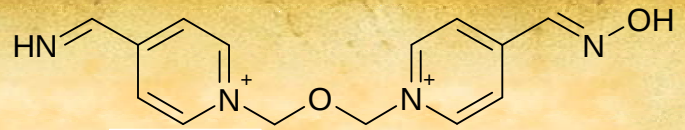


m/z = 135
70

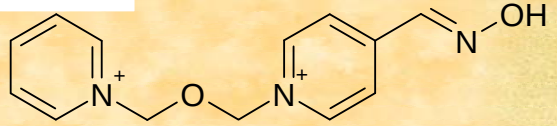


m/z = 123
28

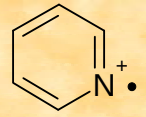
m/z = 144
$[M]^{2+}$



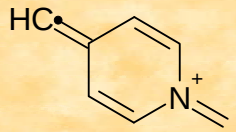
m/z = 136
100



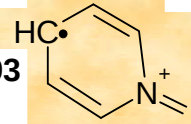
m/z = 123
80



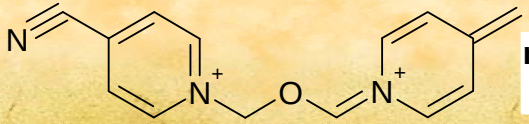
m/z = 79
80



m/z = 105
45



m/z = 93
45



m/z = 119
28

MS spectra

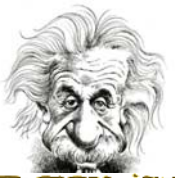
Group	AChE reactivator	$[M^{2+}]$	$[M\text{-}H^{+}]^{+}$	$[M\text{-}H^{+}\text{-}NO^{\cdot}]^{+\cdot}$	$[M\text{-}NO^{\cdot}\text{-}H_2]^{+\cdot}$	$[M\text{-}PymCHNOH]^{+}$	$[M\text{-}PymCONH_2]^{+}$
A	PAM	-	-	-	-	-	-
B	Obidoxime	30	100	15	-	-	-
	K074	100	60	-	-	-	-
	K075	40	-	-	-	100	-
C	Hi-6	6	2	100	-	25	-
	K027	45	100	-	-	-	-
	K048	80	100	-	-	-	-
	K203	25	-	-	-	100	100
D	HLo-7	20	10	-	100	-	45

Group	AChE reactivator	$[M\text{-}OCH_2PymCHNOH]^{+}$	$[M\text{-}2H^{+}]Na^{+}$	$[M\text{-}H^{+}]Na^{+}3H_2O$
A	PAM	-	-	100
B	Obidoxime	25	10	-
	K074	-	-	-
	K075	-	-	-
C	Hi-6	-	-	-
	K027	-	-	-
	K048	-	-	-
	K203	-	-	-
D	HLo-7	-	-	-

MS/MS spectra

Analyte	Precursor Ion (m/z)	Product Ions [m/z(R.A.)]						
PAM	213	91 (100)						
Obidoxime	287	135 (100)	105 (30)					
	257	135 (70)	123 (28)	105 (100)				
	144	136 (100)	123 (80)	119 (28)	105 (45)	93 (45)	79 (80)	
	309	253 (8)	158 (100)	135 (22)				
K074	299	177 (100)	160 (70)	133 (37)				
	150	128 (90)	119.5(100)	136 (22)	106 (30)	79 (18)		
K075	175	157 (10)	131 (100)	53 (15)				
	149	175 (60)	159 (20)	118.5 (83)	127 (90)	105 (33)	78 (100)	
HI-6	257	135 (15)	118 (100)	107 (11)				
	144	136 (100)	123 (50)	107 (80)	79 (70)			
K027	285	241 (6)	163 (100)	149 (60)				
	144	163 (60)	149 (10)	121 (100)	136 (60)	106 (63)	93 (25)	79 (30)
K048	299	177 (100)	160 (30)	133 (10)	106 (8)			
	150	177 (15)	163 (6)	136 (30)	128 (100)	105 (18)	93 (4)	79 (8)
K203	175	157 (14)	131 (100)	117 (10)	53 (95)			
	149	175 (40)	127 (100)	106 (25)	78 (70)	54 (60)		
HLo-7	330	208 (57)	178 (27)	161 (90)	148 (22)	121 (100)		
	300	178 (25)	161 (100)	150 (10)	123 (8)			
	208	121 (100)						
	165.5	150 (50)	136.5 (100)	122 (75)	106 (48)			

MS/MS spectra



CONCLUSIONS ON MS (ESI) IONIZATION & FRAGMENTATION – the Decalogue

1. **First rule: there are no rules!**
2. **Linkers between pyridinium rings are influencing ionization more than the substitution of the rings.**
3. **Molecular ions [M^{2+}] are always conserved / transferred from the source, but with significant differences in yields.**
4. **Pseudo molecular ions [$M\text{-}H^{+}]^{+}$ are generally formed in the ion source (exception made when a double bond is present in the linker).**
5. **Stable adducts may be formed within the source (PAM, Obidoxime).**
6. **CID preferentially occurs on [M^{2+}] precursor ions.**
7. **CID of [M^{2+}] precursor ions may produce a charge loss (m/z of product ions higher than the m/z of the precursor).**
8. **CID is less intense when pseudo molecular ions [$M\text{-}H^{+}]^{+}$ are chosen as precursors.**
9. **CID fragmentation generally arises at higher extent (low m/z are produced).**
10. **Surprisingly, conservation and transfer of ionized structures occurs with relatively low yields.**

MS spectra

MRM transitions									
Compound Name	Precursor Ion	MS1 Res	Product Ion	MS2 Res	Dwell	Fragmentor	Collision Energy	Cell Accelerator Voltage	Polarity
PAM	213.3	Wide	91.2	Wide	200	100	15	7	Positive
Obidoxime	287.2	Wide	135.1	Wide	200	100	15	7	Positive
K074	150.4	Wide	119.9	Wide	200	100	15	7	Positive
K075	149.4	Wide	127.4	Wide	200	100	15	7	Positive
HI-6	257.3	Wide	118.2	Wide	200	100	15	7	Positive
K027	285.2	Wide	163.3	Wide	200	100	15	7	Positive
K048	299.3	Wide	177.2	Wide	200	100	15	7	Positive
K203	149.3	Wide	78.2	Wide	200	100	15	7	Positive
HLo-7	300.4	Wide	161.1	Wide	200	100	15	7	Positive

Chromatographic Separation
HILIC mode

Chromatographic Column:

Luna 3u HILIC (lot 5540-13; S.N. 459421-1; P.N. 00F-4449-B0)
150 mm L x 2 mm i.d. x 3 μm d.p.
Temperature: 25 ^{o}C

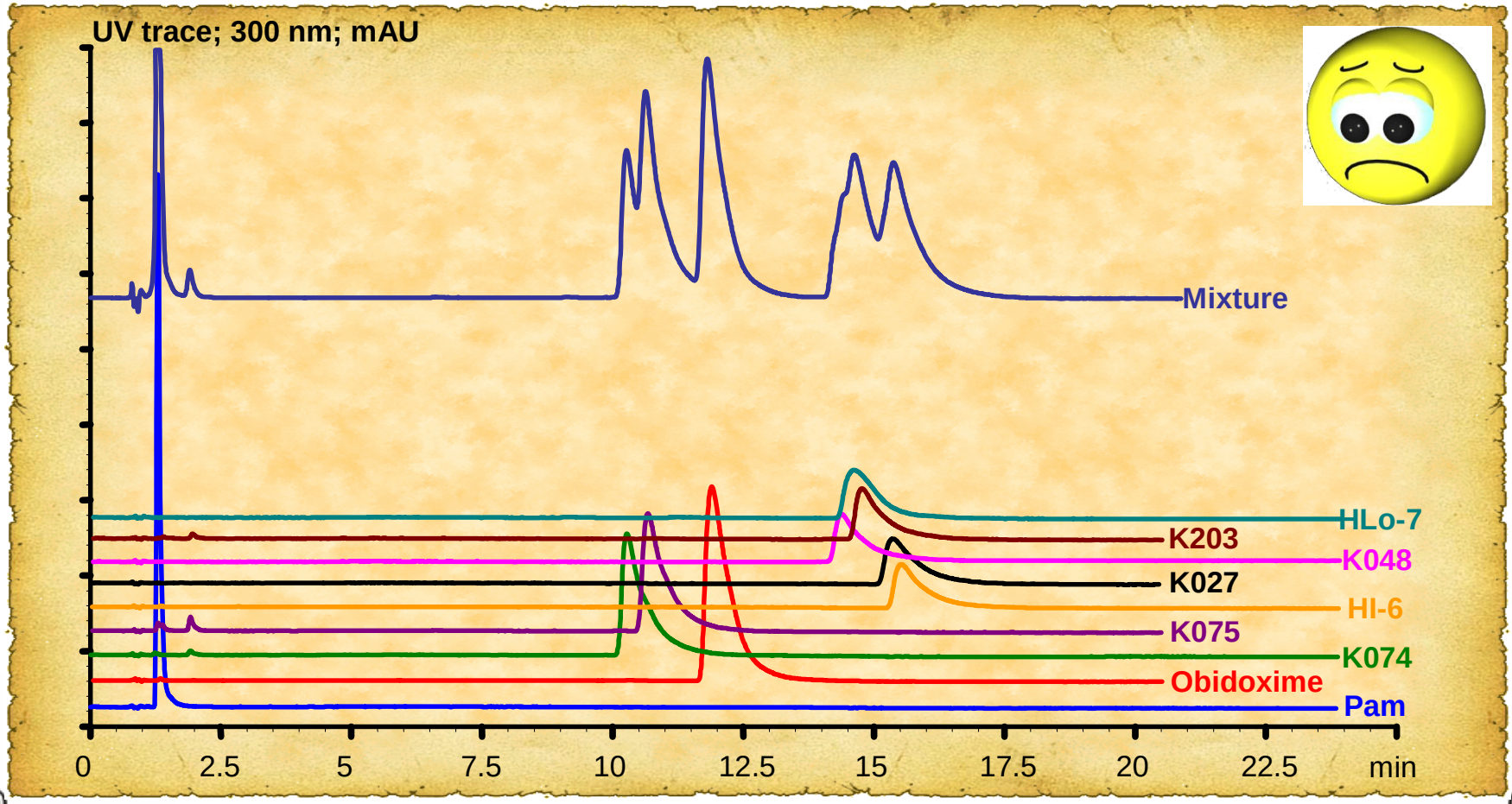
Mobile Phase:

Solvent A: 50 mM aqueous ammonium formate buffer pH = 3.2
Solvent B: Acetonitrile
Elution: Gradient
Gradient profile: 0 – 2 min: 95% B; 12 min: 90% B
Flow rate: 0.8 mL/min

Injection volume: 1 μL

Optimized conditions!

Chromatographic Separation
HILIC mode

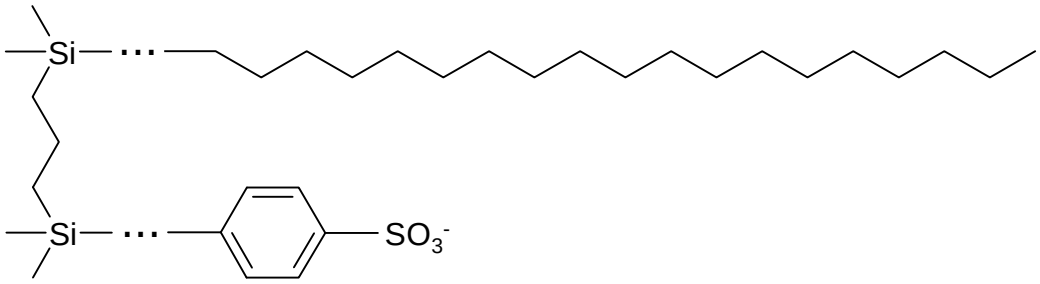


Chromatographic Separation
Mixed mode: SCX / RP (Duet!)

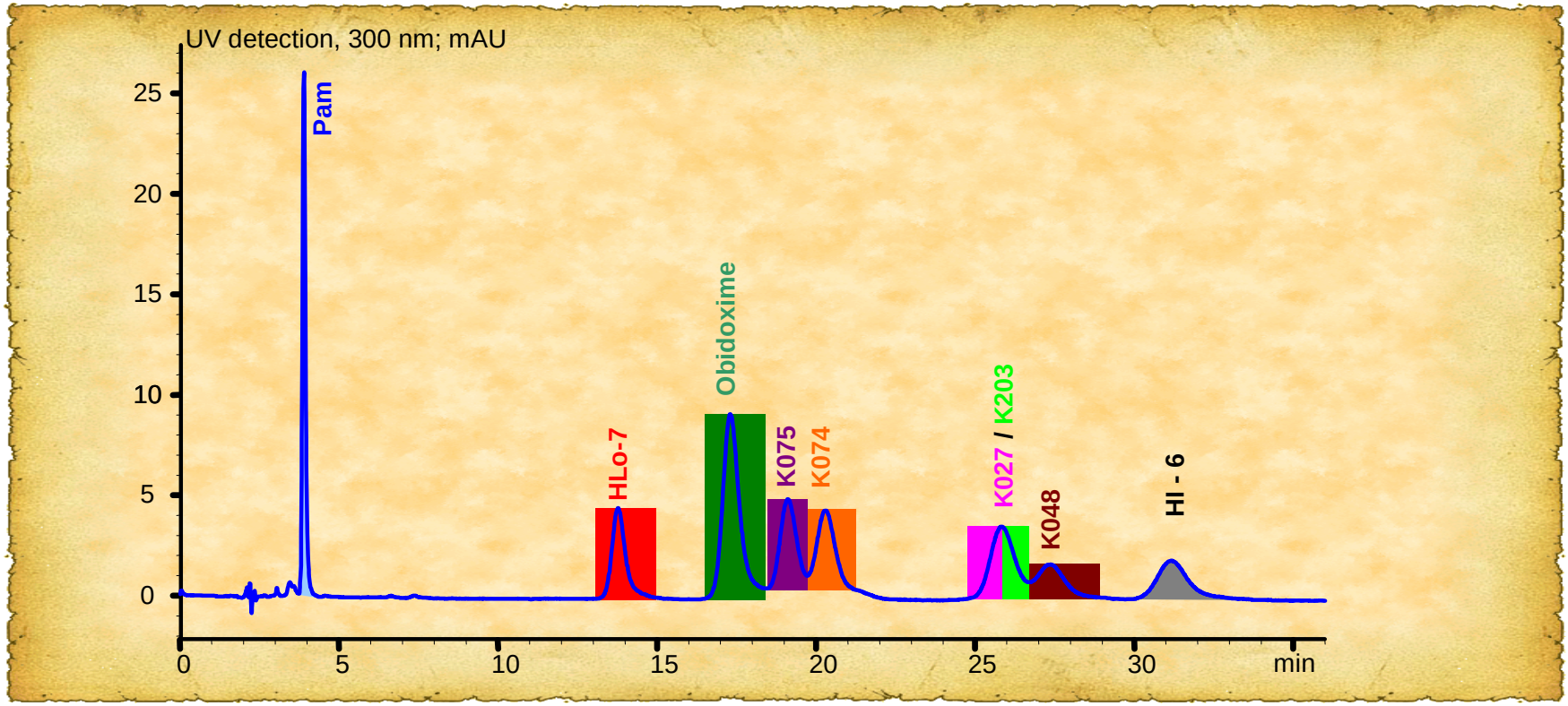
Chromatographic Column:
Hypersil Duet C18/SCX (lot 150/11054; S.N. 12100899G4; P.N. 34405-154630)
150 mm L x 4.6 mm i.d. x 5 μm d.p.
Temperature: 25 °C

Mobile Phase:
Solvent A: 200 mM aqueous ammonium formate buffer pH = 3
Solvent B: Acetonitrile
Elution: Isocratic
Composition: Solvent A / Solvent B
Flow rate: 0.8 mL/min

Injection volume: 1 μL

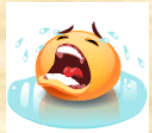


Chromatographic Separation
Mixed mode: SCX / RP (Duet!)

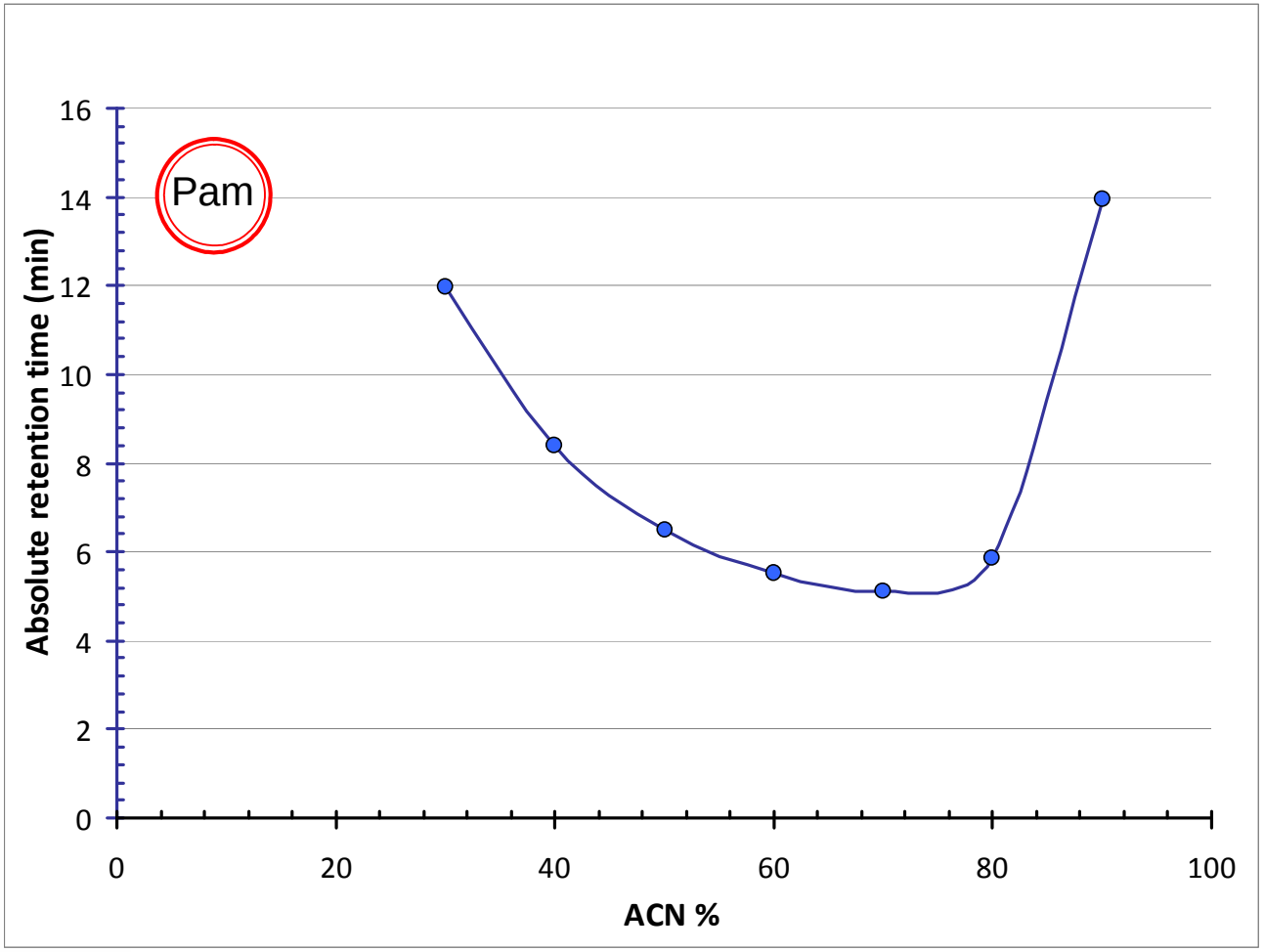


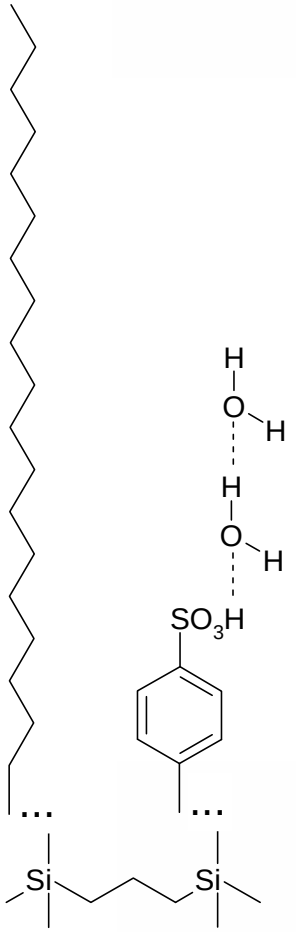
Better selectivity compared with HILIC!

Long chromatographic run!
The pair K027/K203 unresolved!

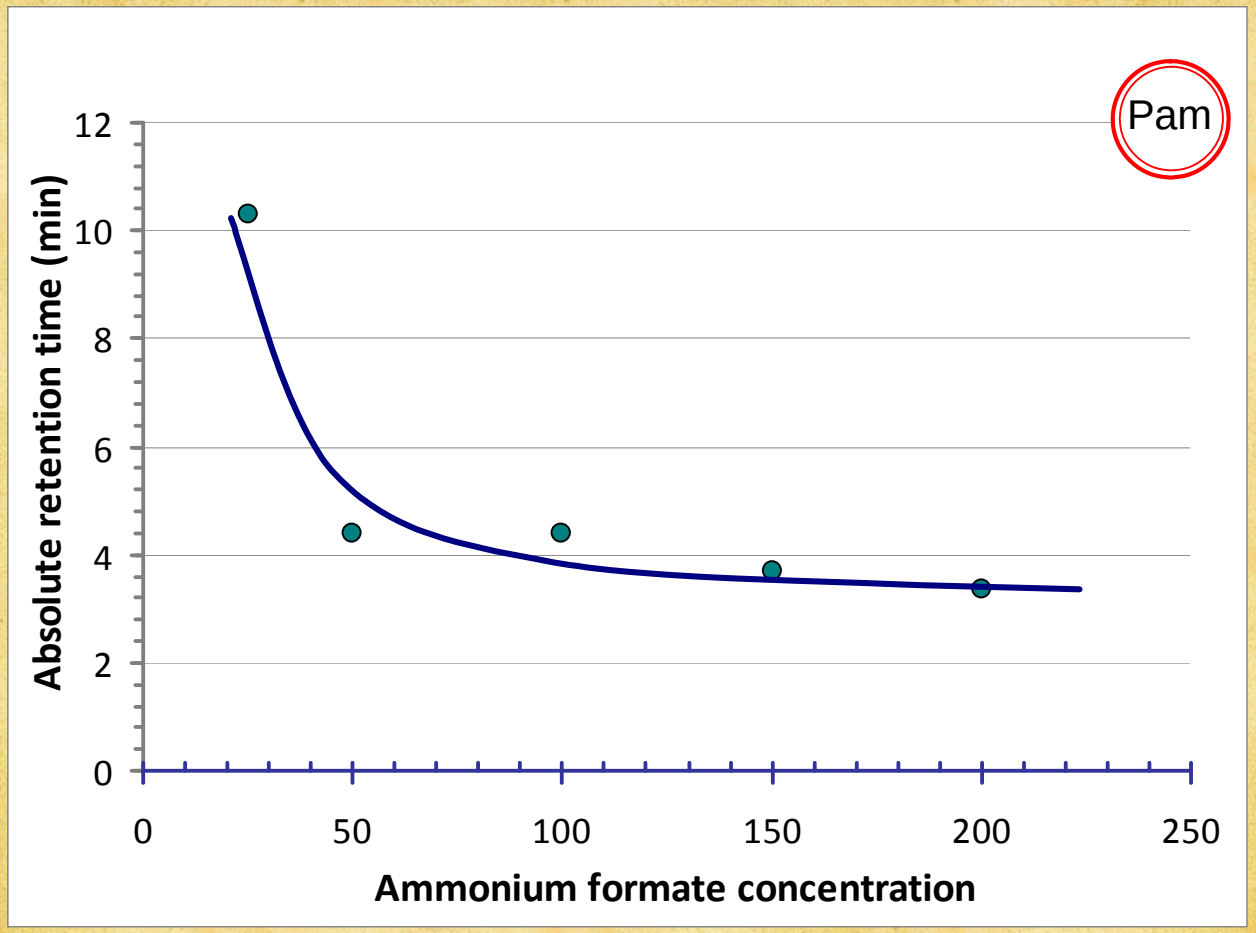


Insights about the mixed retention mechanism: In fact we get HILIC modulated with RP!

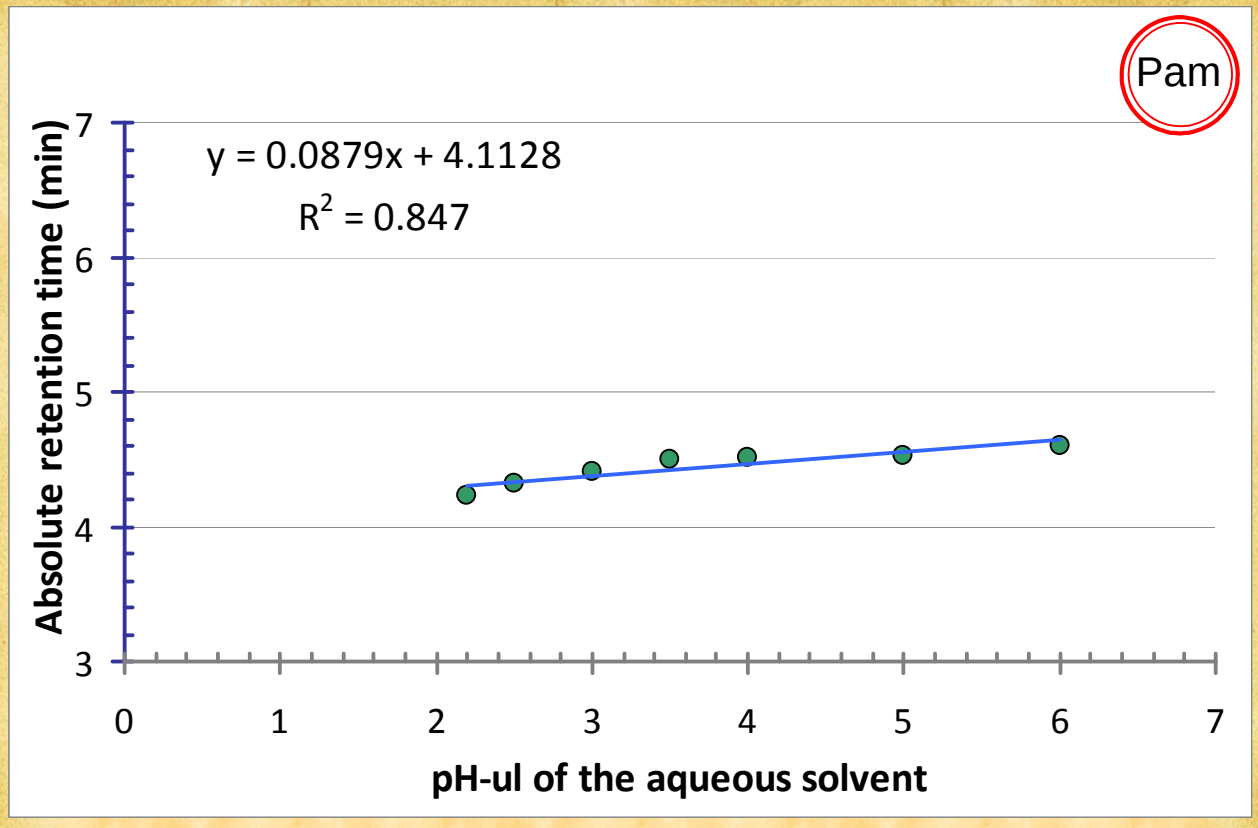




Insights about the mixed retention mechanism
Influence of the buffer concentration:



Insights about the mixed retention mechanism
Influence of the pH:



unusual!

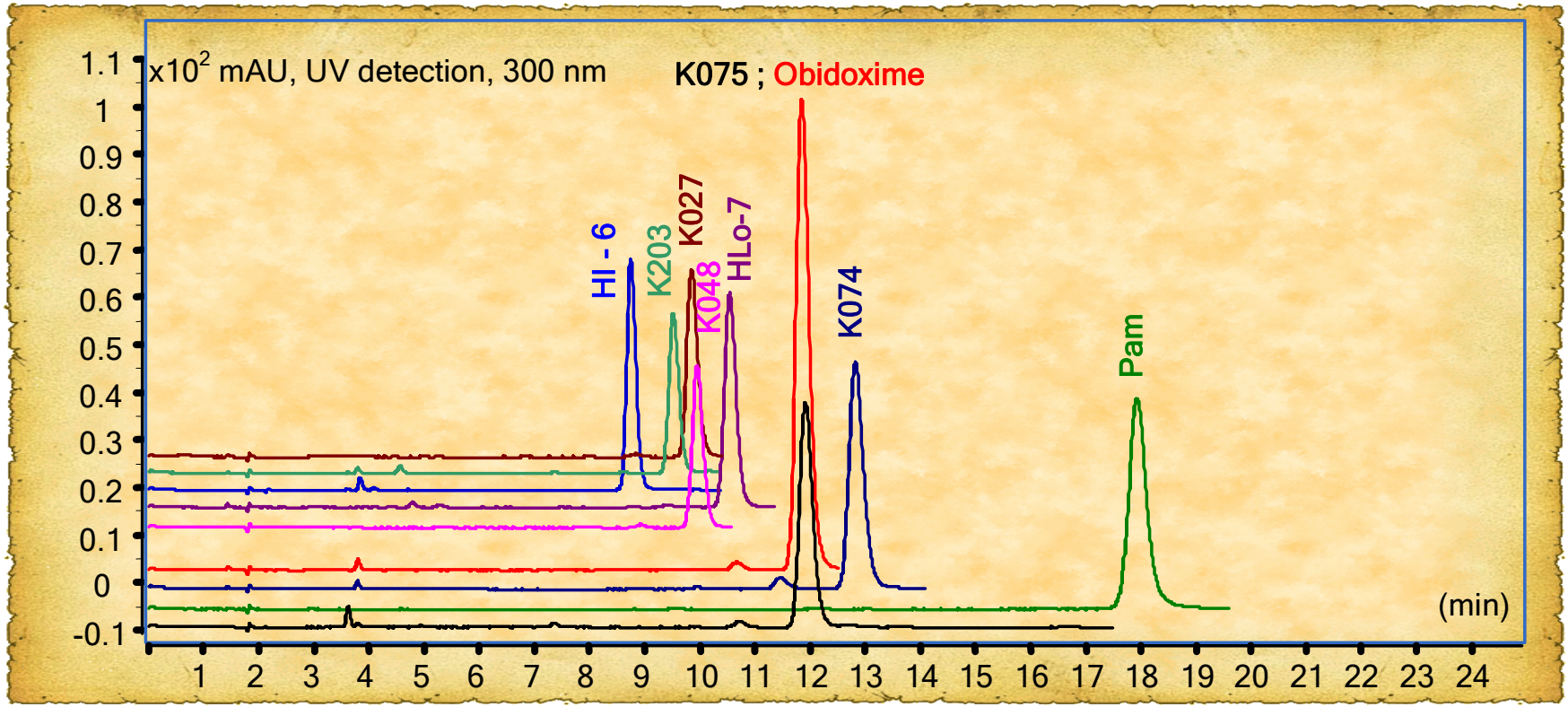
Chromatographic Separation
Perfluorinated Ion Pair RP

Chromatographic Column:
Zorbax Eclipse XDB-C18, Rapid Ressolution
(lot B07083; S.N. USWA008251; P.N. 963967-902)
150 mm L x 4.6 mm i.d. x 3.5 μm d.p.
Temperature: 25 ^{o}C

Mobile Phase:
Solvent A: 0.15% aqueous Heptafluorobutyric Acid
Solvent B: Methanol
Elution: Isocratic
Composition: Solvent A / Solvent B = 70 / 30 (v/v)
Flow rate: 0.8 mL/min

Injection volume: 5 μL

Chromatographic Separation
Perfluorinated Ion Pair RP



Conclusions of chromatographic separation mechanisms

Separation Mechanism	k of the first eluting peak	Duration (min)	Co-eluted pairs	Pairs with $R_s<1$	Recommended for bioassay	Recommended for QC
HILIC	0.63	20	1 (HI-6 / K027)	5	-	-
Duet	0.70	35	1 (K027 / K203)	1	-	✓
RP-PFIP	3.88	20	1 (Obidoxime / K075)	2	✓	✓

Sample preparation / Protein Precipitation
Human Plasma

Acetonitrile
600 µL

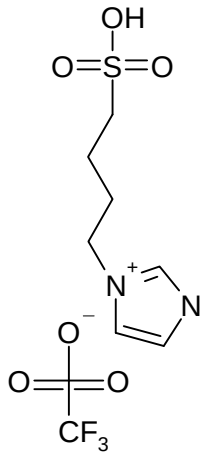
Human Plasma
200 µL

Vortex
10 min/2000 rpm

Centrifuge
10 min/9000 x g

Supernatant

Inject
Up to 5 µL



Human Plasma
400 µL

Ionic Liquid
200 µL

Vortex
10 min/2000 rpm

Centrifuge
20 min/9000 x g

Supernatant

Inject
Up to 20 µL

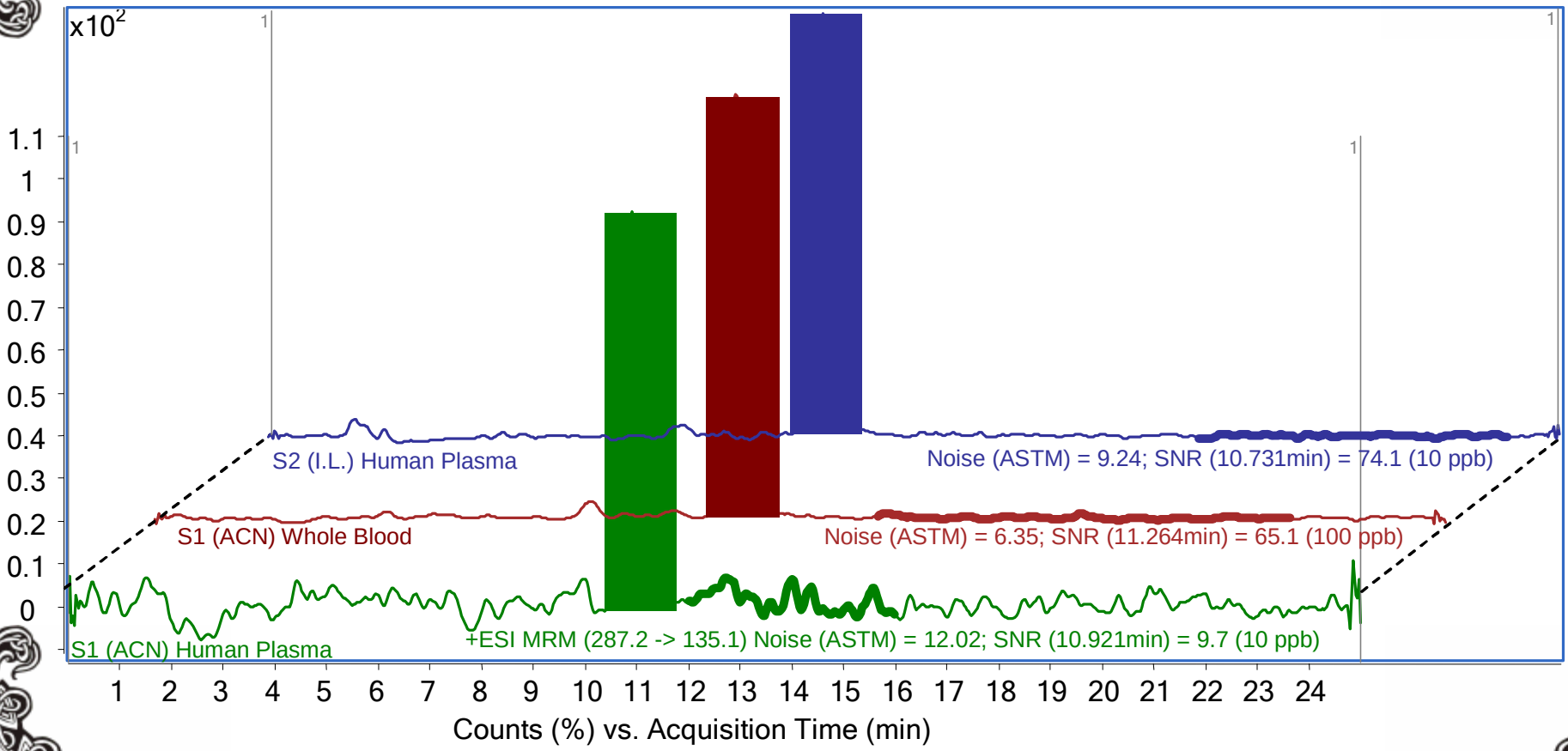
Characteristics of the Sample Preparation Methods

Matrix	Sample Preparation Technique	Characteristics	HLo-7	K048	Obidoxime	K027	HI-6	Pam	K074	K203
Whole Blood	S1	Recovery	35.3	37.7	55.3	30.7	40.5	87.6	45.5	40.4
		RSD% (n=10)	9.8	8.3	6.4	8.3	7.0	3.2	6.1	9.7
		Matrix Effect	101.9	78.8	94.1	72.0	99.0	92.6	81.9	130.5
		RSD% (n=6)	0.9	0.7	1.2	0.9	0.6	0.6	0.8	4.1
Plasma	S1	Recovery	46.1	49.2	89.3	40.8	47.6	119.1	75.3	33.0
		RSD% (n=5)	4.4	5.2	4.0	9.1	3.8	3.0	5.7	10.8
		Matrix Effect	144.8	143.1	127.8	139.1	111.0	113.2	120.7	187.3
		RSD% (n=6)	0.5	0.2	0.8	1.1	1.1	0.5	0.8	1.7
Plasma	S2	Recovery	14.8	23.5	42.5	30.6	22.8	57.2	43.0	34.4
		RSD% (n=5)	0.0	1.6	0.9	2.7	2.7	6.9	1.3	0.2
		Matrix Effect	86.8	80.4	86.2	78.5	64.3	81.4	82.9	101.6
		RSD% (n=5)	2.2	2.5	2.0	3.0	4.4	0.5	2.1	14.4

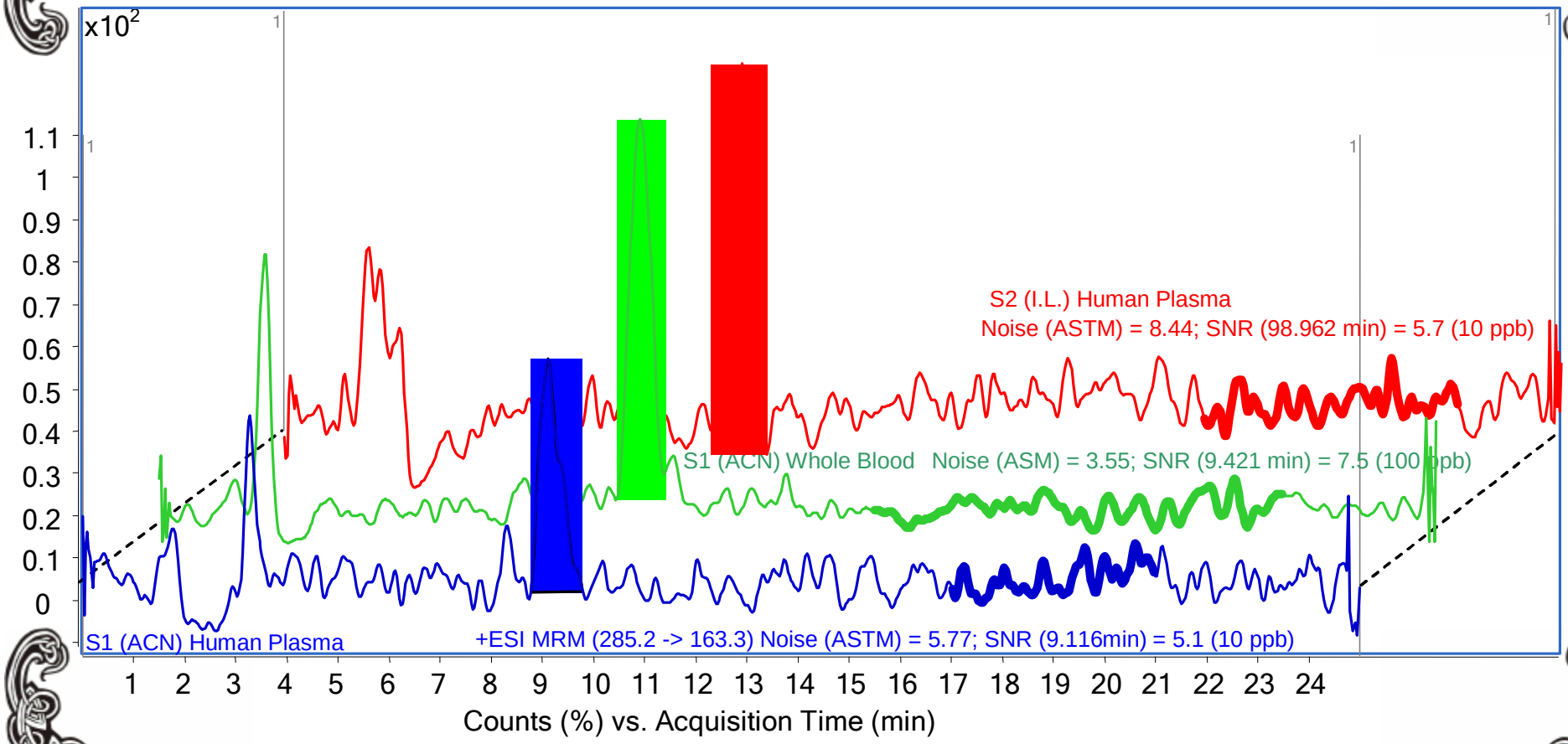
LLOQs acc. to sample prep. procedures

Compound	S/N / (concentration) Sample Prep. S1 Matrix: Human Plasma	S/N / (concentration) Sample Prep. S1 Matrix: Whole Blood	S/N / (concentration) Sample Prep. S2 Matrix: Human Plasma
HI-6	7.9 / (**10**)	89.9 / (**100**)	88.1 / (**10**)
K203	5.4 / (**100**)	5.2 / (**250**)	9.5 / (**10**)
K027	5.1 / (**100**)	7.5 / (**100**)	5.7 / (**10**)
K048	5.0 / (**100**)	6.6 / (**100**)	6.3 / (**100**)
HLo-7	10.5 / (**10**)	37.1 / (**100**)	26.4 / (**10**)
Obidoxime	9.7 / (**10**)	65.1 / (**100**)	74.1 / (**10**)
K075	5.6 / (**100**)	9.0 / (**100**)	20.9 / (**10**)
K074	5.9 / (**100**)	7.7 / (**100**)	21.7 / (**10**)
PAM	124.4 / (**10**)	677.2 / (**100**)	68.7 / (**10**)

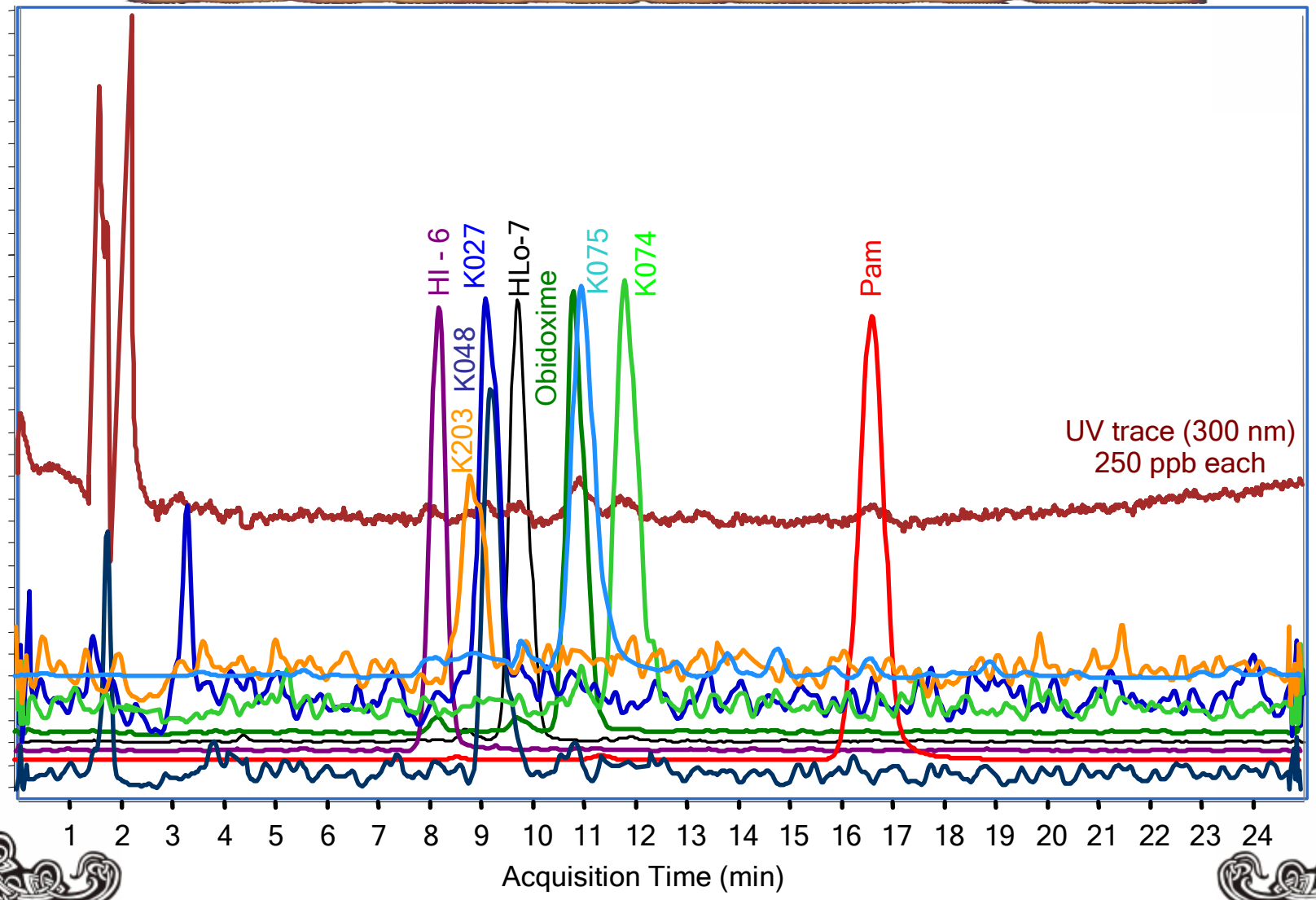
LLOQs acc. to sample prep. Procedures
Examples: Obidoxime



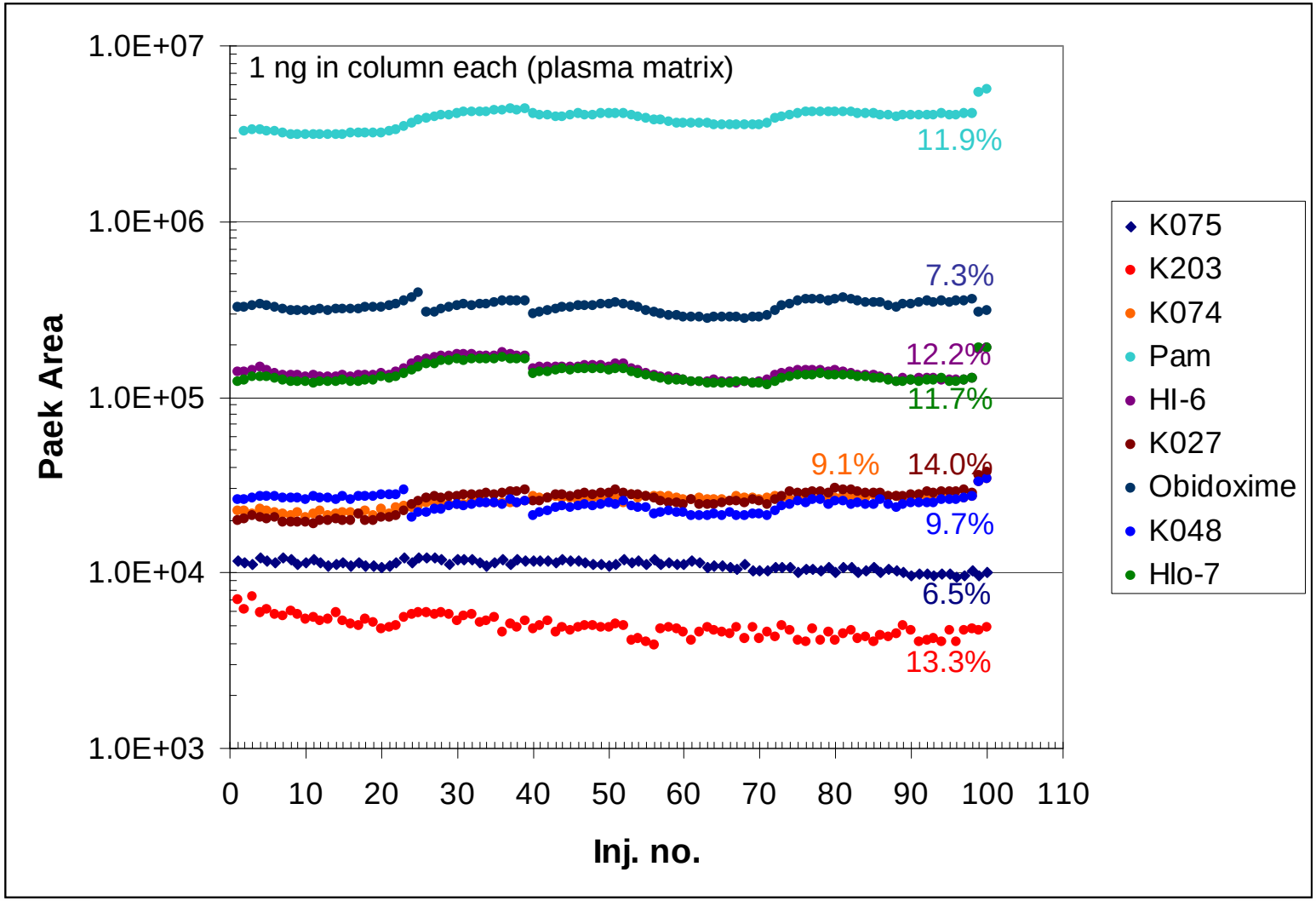
LLOQs acc. to sample prep. Procedures
Examples: K027



MS/MS and UV detection: Comparison



MS/MS response stability (PFIP-RP conditions)



Validation data:
Plasma; protein precipitation ACN

Analyte	LLOQ (ppb)	ULOQ (ppb)	B	S_B	A	S_A	r_{xy}	RSD% min.	RSD% max.	Bias% min.	Bias% max.
HI-6	10	10000	75.36	2.17	120.35	96.55	0.9952	0.95	11.19	-11.30	9.54
K203	100	10000	2.95	0.09	61.77	108.33	0.9960	2.35	6.86	-10.88	7.28
K027	100	10000	7.97	0.23	87.90	277.32	0.9966	2.24	9.30	-10.87	7.71
K048	100	10000	8.32	0.23	-16.47	272.49	0.9966	0.47	7.60	-11.13	6.94
HLo-7	10	10000	77.54	2.65	11.01	1179.25	0.9944	0.24	4.49	-14.35	19.86 (LLOQ)
Obidoxime	10	10000	118.33	4.11	1782.54	1830.71	0.9943	0.31	9.43	-12.59	11.75
K075	100	10000	4.19	0.21	-142.42	250.58	0.9908	0.25	12.39	-14.69	11.96
K074	100	10000	8.35	0.36	-118.85	432.62	0.9933	0.73	10.04	-12.65	10.30
Pam	10	10000	2266.17	79.83	18003.47	35590.87	0.9941	0.37	11.08	-13.40	10.94

Matrix: Plasma; Sample Preparation: protein precipitation with ACN; Regression model: Linear, weighted 1/x

Validation data:
Whole Blood; protein precipitation ACN

Analyte	LLOQ (ppb)	ULOQ (ppb)	B	S_B	A	S_A	r_{xy}	RSD% min.	RSD% max.	Bias% min.	Bias% max.
HI-6	100	10000	110.17	3.46	-1976.85	4149.74	0.9957	1.84	6.88	-11.79	9.6
K203	250	10000	3.24	0.12	-153.76	200.31	0.9948	1.41	12.71	-10.22	10.7
K027	100	10000	6.93	0.17	56.06	208.04	0.9958	2.85	10.79	-10.78	8.4
K048	100	10000	7.64	0.19	-164.76	223.57	0.9969	1.03	9.68	-12.68	13.2
HLo-7	100	10000	60.40	1.76	-2614.43	2106.38	0.9959	0.90	9.14	-13.98	19.6 (LLOQ)
Obidoxime	100	10000	104.27	3.07	1744.84	3688.80	0.9962	2.91	7.98	-12.89	12.2
K075	100	10000	3.33	0.11	-169.90	136.47	0.9951	1.76	15.35 (LLOQ)	-9.41	11.2
K074	100	10000	6.53	0.26	-176.98	307.81	0.9938	2.50	6.70	-12.38	17.1 (LLOQ)
Pam	100	10000	2048.48	87.14	16679.66	104564.04	0.9935	1.61	3.98	-12.50	12.5

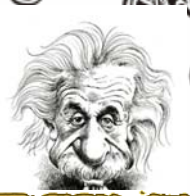
Matrix: Whole Blood; Sample preparation: Protein precipitation with ACN; Regression model: Linear, weighted 1/x

Validation data:
Plasma; protein trapping by Ionic Liquid

Analyte	LLOQ (ppb)	ULOQ (ppb)	B	S_B	A	S_A	r_{xy}	RSD% min.	RSD% max.	Bias% min.	Bias% max.
HI-6	10	10000	79.05	1.55	3785.37	691.08	0.9952	1.07	13.95	-13.7	10.1
K203	10	10000	6.24	0.19	1136.07	82.94	0.9950	0.43	9.44	-14.5	7.5
K027	10	10000	4.20	0.11	322.44	47.75	0.9956	1.41	11.21	-8.7	9.6
K048	100	10000	2.40	0.08	372.16	91.85	0.9942	1.43	11.39	-9.1	10.9
HLo-7	10	10000	24.65	0.51	2713.34	227.75	0.9966	0.63	12.16	-11.2	11.2
Obidoxime	10	10000	54.78	1.09	11776.42	485.70	0.9957	0.02	11.80	-18.7 (LLOQ)	13.1
K075	10	10000	2.40	0.06	918.09	27.10	0.9966	0.07	6.76	-17.1 (LLOQ)	11.4
K074	50	10000	3.90	0.11	2154.20	93.62	0.9956	0.41	7.33	-10.6	11.4
Pam	10	10000	7575.04	254.92	305924.50	113648.41	0.9949	0.04	2.99	-17.8 (LLOQ)	13.4

Matrix: plasma; Sample preparation: Protein trapping with the Ionic Liquid; Regression model: Linear, weighted 1/x

Final Conclusions



1. The bioassay of oximes through PFIP-RP chromatographic separation and (+) ESI-MS/MS detection is feasible.
2. Sensitivity depends on the matrix (i.e. plasma, whole blood) and sample preparation methodology (i.e. protein precipitation through ACN addition, protein trapping through ionic liquid addition).
3. Although more sensitive compared to UV detection, MS/MS detection remains surprisingly poor for oximes (low transmission yields in the ion source).
4. PFIP-RP/(+)ESI-MS/MS method for oximes were successfully validated.

I hear, I know; I see, I remember; I do, I understand.
Confucius (551-479 B.C.)

Acknowledgments:

Esteemed colleagues, Dr. J. Bajgar, K. Kuca, and K. Musilek from the Department of Toxicology of the Faculty of Military Health Sciences, University of Defense, Hradec Kralove, Czech Republic, are gratefully acknowledged for providing us the pyridine oximes K027, K048, K074, K075, K203 used during experiments.

Andrei Medvedovici acknowledges the financial support given through the Romanian project PNII_ID_PCE_2011_3_0152.