

**Quantitative Quality Control (QQC) Validation
Studies: A comparative Evaluation of ELSD and NMR
Quantification Results**

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OUTLINE

- Introduction
- Quantification by ELSD
- Quantification by NMR
- Calculations of physicochemical properties of study compounds
- An attempt to correlate the different ELSD responses of study compounds with some of their corresponding physicochemical properties
- Conclusions

INTRODUCTION

Why? (are we doing this)

- Accurate and precise quantification are essential for purified libraries, Discovery Automated Sample Handling (DASH) process and IC50 determination.

What? (is the objective)

- A validated QQC method without a requirement for specific analyte standards for rapid, cheap and readily automated quantification

How? (detector choices)

ELSD



CLND

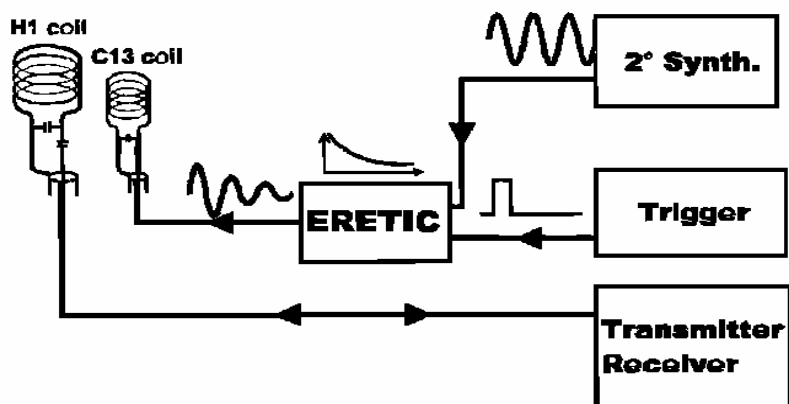


CoronaCAD



Others

ERETIC NMR



Barantin et al, *Magn. Reson. Med.* **1997**, 38, 179-182

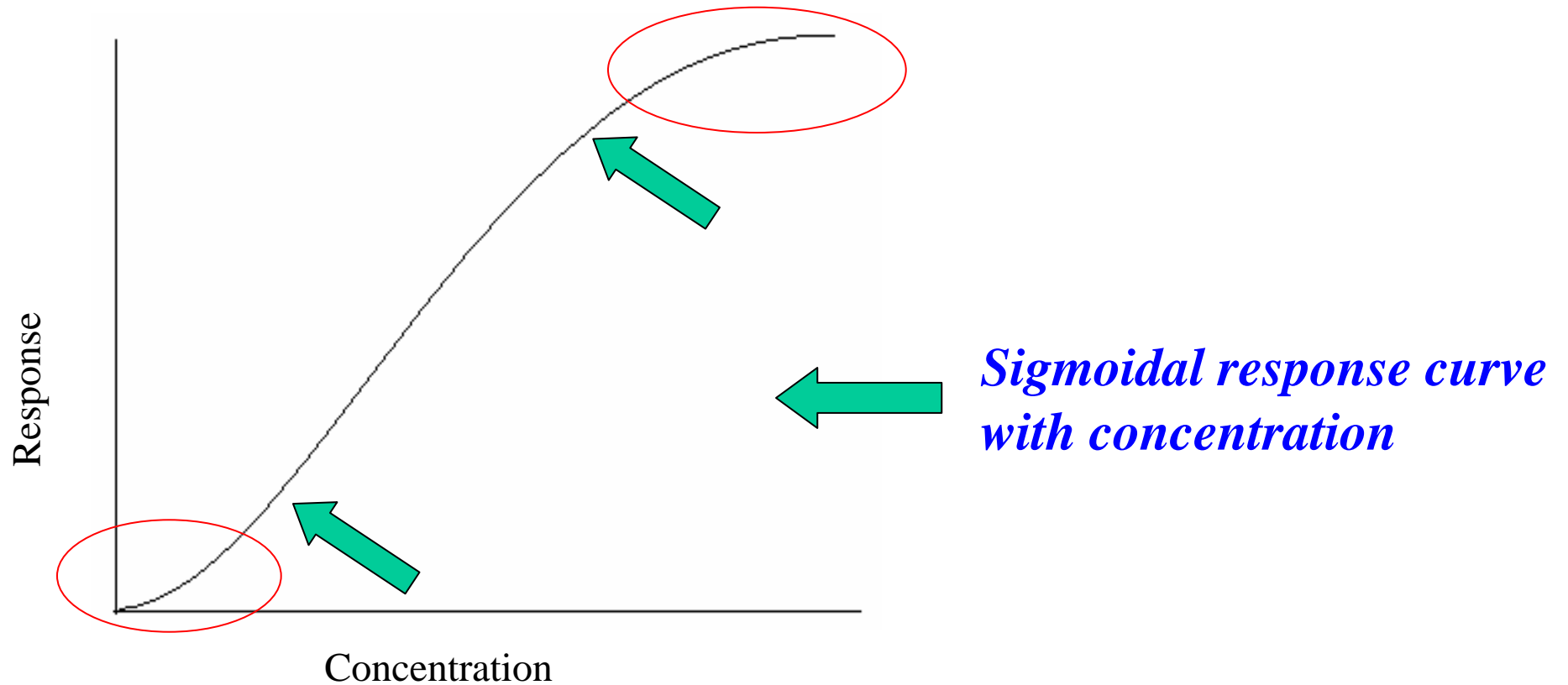
- Best quantification results are obtained with NMR. The ERETIC method in proton NMR has been utilized as a “Gold Standard” to compare single-calibrant ELSD and CLND based quantifications (*A. Chem.* (2005), 77(14), 4354-4365).

How? (Study Design and Strategy)

- Develop and validate a rugged/robust quantification method using HPLC/ELSD
- Optimize and implement the ERETIC method in proton NMR for quantification
- Apply HPLC/ELSD and ERETIC methods for the quantitative determination of std set samples and purified libraries
- Compare ELSD results to those from NMR
- Calculate selected physicochemical properties of study compounds and apply statistical methods analysis and integrate data to establish, if possible, any physicochemical properties correlation with ELSD response

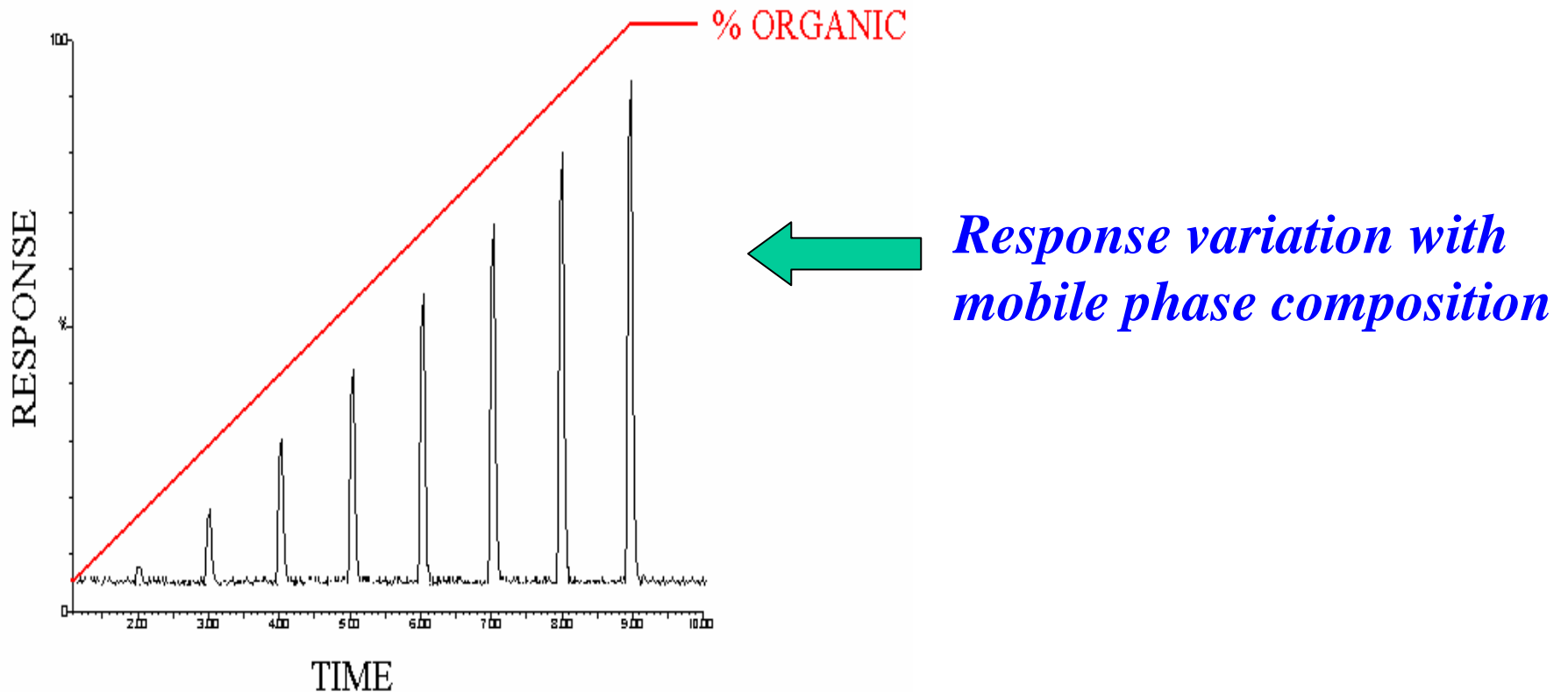
Quantification By ELSD

- Linear dynamic range (0.16- 0.8 mg/mL).....**VERY CRUCIAL**

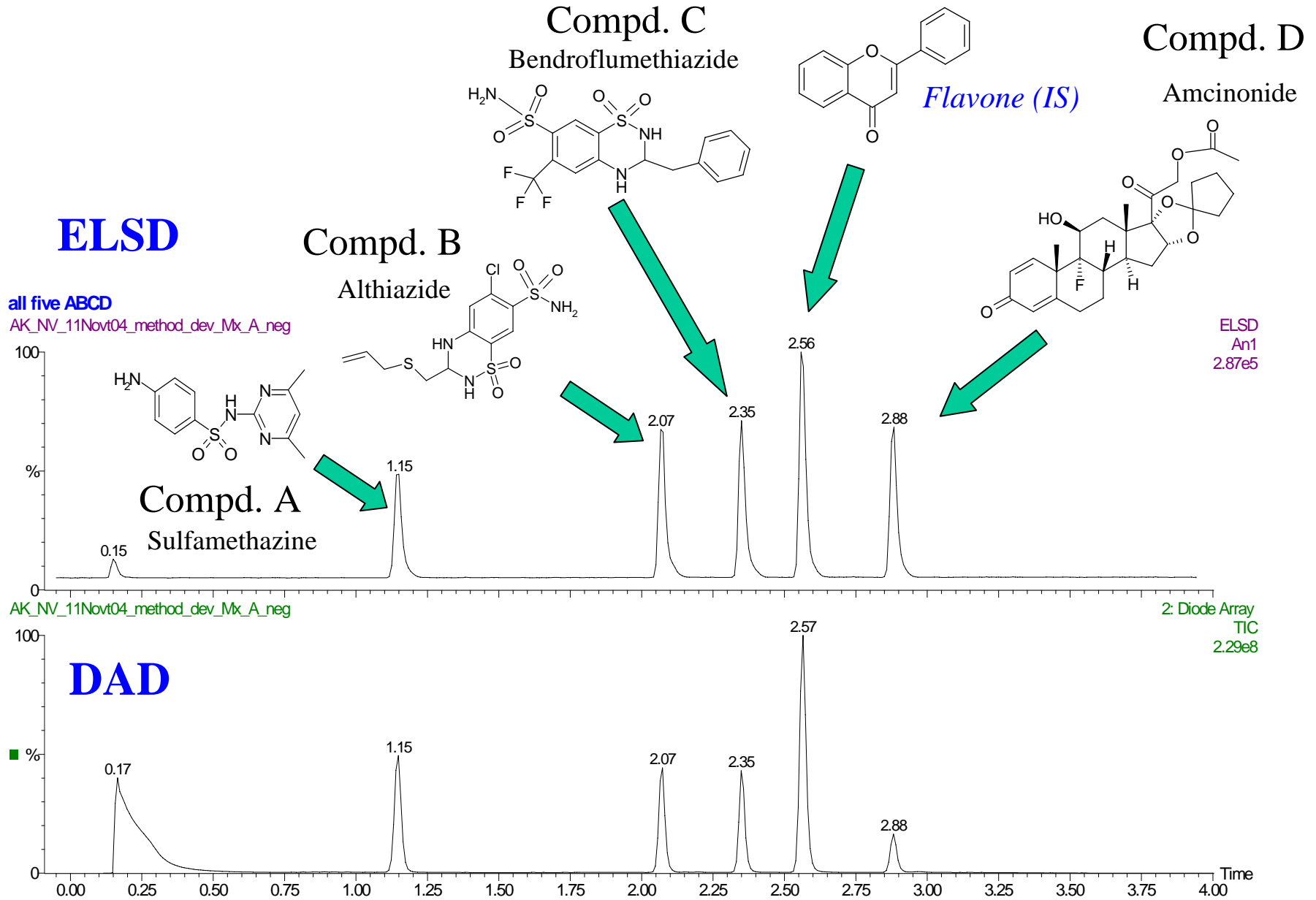


Quantification By ELSD (cont'd)

- Internally standardized, 4-component calibration curve run in duplicate and quantification using a “nearest calibrant” approach



HPLC/UV/ELSD chromatograms for 4 component calibration standard + Flavone (a total of 2 µg on column of each standard)



Quantification By ELSD (cont'd)

- 5 μ l injection volume
- Three QC concentrations at 0.3, 0.5 and 0.7 mg/mL
- External QC std CP-122,817 at concentration of 0.5 mg/mL
- All samples were dissolved in DMSO and run in **triplicate**.
- Sample dilution was made in DMSO as needed.
- Quantification was performed using QuanLynx software with 1/x weighted curve.

Quantification by NMR

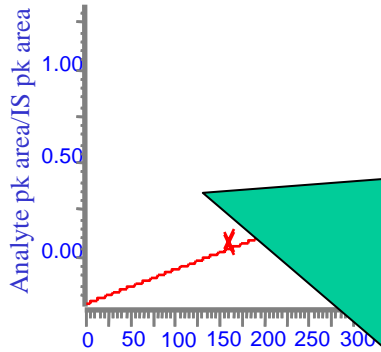
- 10 μ l sample in regular DMSO using 1mm sample robot
- DMSO signal suppression using WET
- No sample shimming and locking
- ERETIC signal at -1 ppm in every spectrum
- Two samples with known concentration (30mM) were used to calibrate ERETIC signal strength for each run
- Data collected on Bruker DRX500 equipped with 1mM microprobe
- Automatic spectra processing using ACD software
- Manual analysis (auto-analysis in development)

Calculations of Physicochemical Properties and Statistical Methods Analysis of Study Compounds

- Software programs such as MPBPVP, SPARC, ACD, Molecular Modeling Pro., ProPred and ChemOffice for the calculation of some physicochemical properties have been tested for their predictive ability (*Environmental Toxicology & Chemistry, 2003. Vol. 22., No. 8, 1696-1709*).
- In addition to the MW, physicochemical properties such as BP, MP, VP, pKa, LogD were calculated for study compounds using MPBPVP
- Statistical methods analysis (*Graphical presentation and Classification and Regression Tree, Friedman, J. H., Olshen, R. A. and Stone, C. J. (1984)*) were applied.

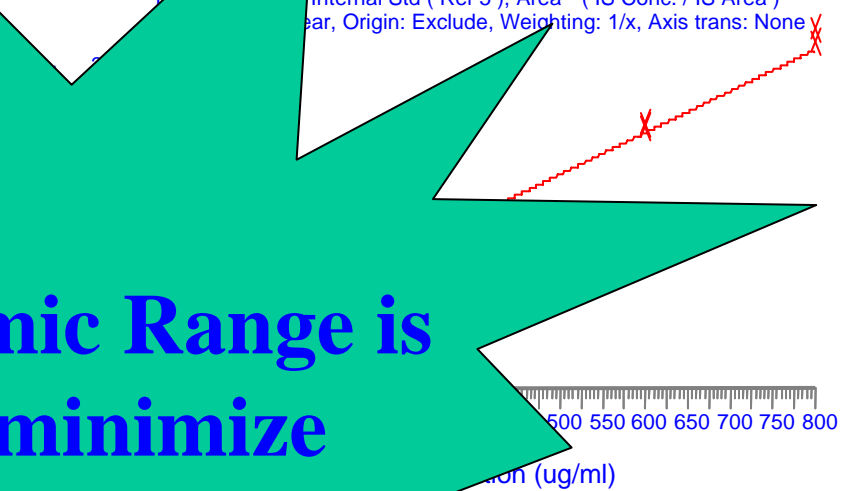
Std Curve A

Compound name: **A**
Correlation coefficient: $r = 0.988128$, $r^2 = 0.976398$
Calibration curve: $0.00182511 * x + -0.249499$
Response type: Internal Std (Ref 5), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None



Std Curve B

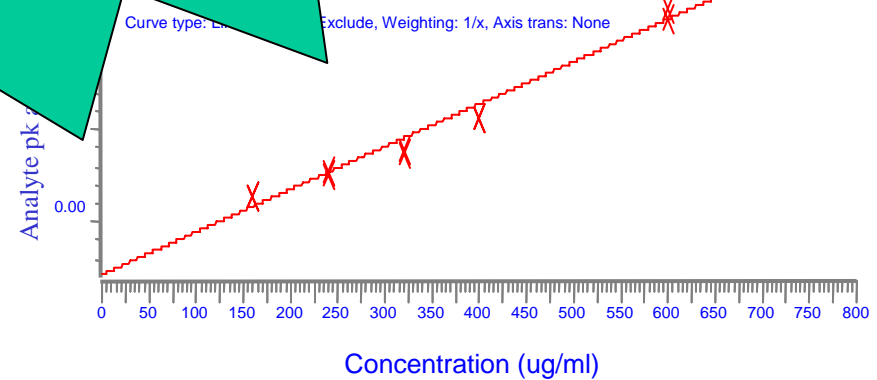
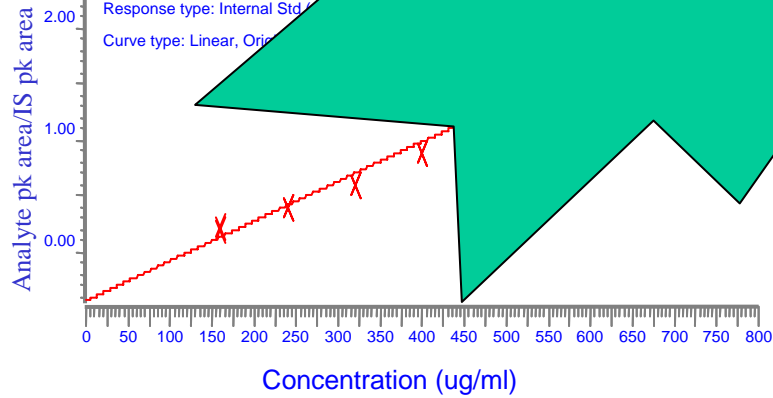
Compound name: **B**
Correlation coefficient: $r = 0.988870$, $r^2 = 0.977863$
Calibration curve: $0.00329731 * x + -0.43736$
Response type: Internal Std (Ref 5), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None



Linear dynamic Range is crucial to minimize errors in quantitative determination

Std Curve D

Compound name: **D**
Correlation coefficient: $r = 0.993$
Calibration curve: $0.00353897 * x + -0.572771$
Response type: Internal Std (Ref 5), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None



Representative accuracy of ELSD Standard Curves (A and B)

Table 1. Standard Curve accuracy (12/07/05) for compounds A, B, C, and D in DMSO					
	Nominal Conc. (ug/ml)	Calc. Conc. (ug/ml)	Accuracy (%)		
Compound A					
Standard 1	160	184	115		
Standard 1	240	240	100		
Standard 1	320	288	90		
Standard 1	400	364	91		
Standard 1	600	587	98		
Standard 1	800	882	110		
Standard 2	160	173	108		
Standard 2	240	243	101		
Standard 2	320	301	94		
Standard 2	400	363	91		
Standard 2	600	587	98		
Standard 2	800	827	103		
Compound B					
Standard 1	160	180	112		
Standard 1	240	233	97		
Standard 1	320	290	91		
Standard 1	400	368	92		
Standard 1	600	601	100		
Standard 1	800	888	111		
Standard 2	160	183	115		
Standard 2	240	234	97		
Standard 2	320	297	93		
Standard 2	400	367	92		
Standard 2	600	605	101		
Standard 2	800	793	99		

ELSD Intra-assay Precision and Accuracy for Compds A, B, C and D

Table 2. Intra-assay precision and accuracy (12/07/05) for compounds A, B, C, and D in DMSO

Analyte	Nominal conc. (ug/ml)	Calculated conc. (mean \pm SD, n=3) (ug/ml)	RSD (%)	Deviation (%)
Compound A	300	274 \pm 5	2.0	-9.0
	500	441 \pm 15	3.0	-12.0
	700	736 \pm 19	3.0	5.0
Compound B	300	273 \pm 12	4.0	-9.0
	500	456 \pm 6	1.0	-9.0
	700	732 \pm 31	4.0	5.0
Compound C	300	277 \pm 9	3.0	-8.0
	500	448 \pm 8	2.0	-10.0
	700	742 \pm 18	2.0	6.0
Compound D	300	274 \pm 8	3.0	-9.0
	500	453 \pm 11	2.0	-9.0
	700	713 \pm 11	2.0	2.0

ELSD Intra-assay Precision and Accuracy for External QC std CP-122,817

Table 3. Intra-assay precision and accuracy for External Calibrant CP-122,817 (12/07/05) in DMSO

Analyte	Nominal conc. (ug/ml)	Calculated conc. (mean \pm SD, n=3) (ug/ml)	RSD (%)	Deviation (%)		
CP-122,817	500	544 \pm 15	3	9		

Representative ELSD/NMR results from LGG purified library

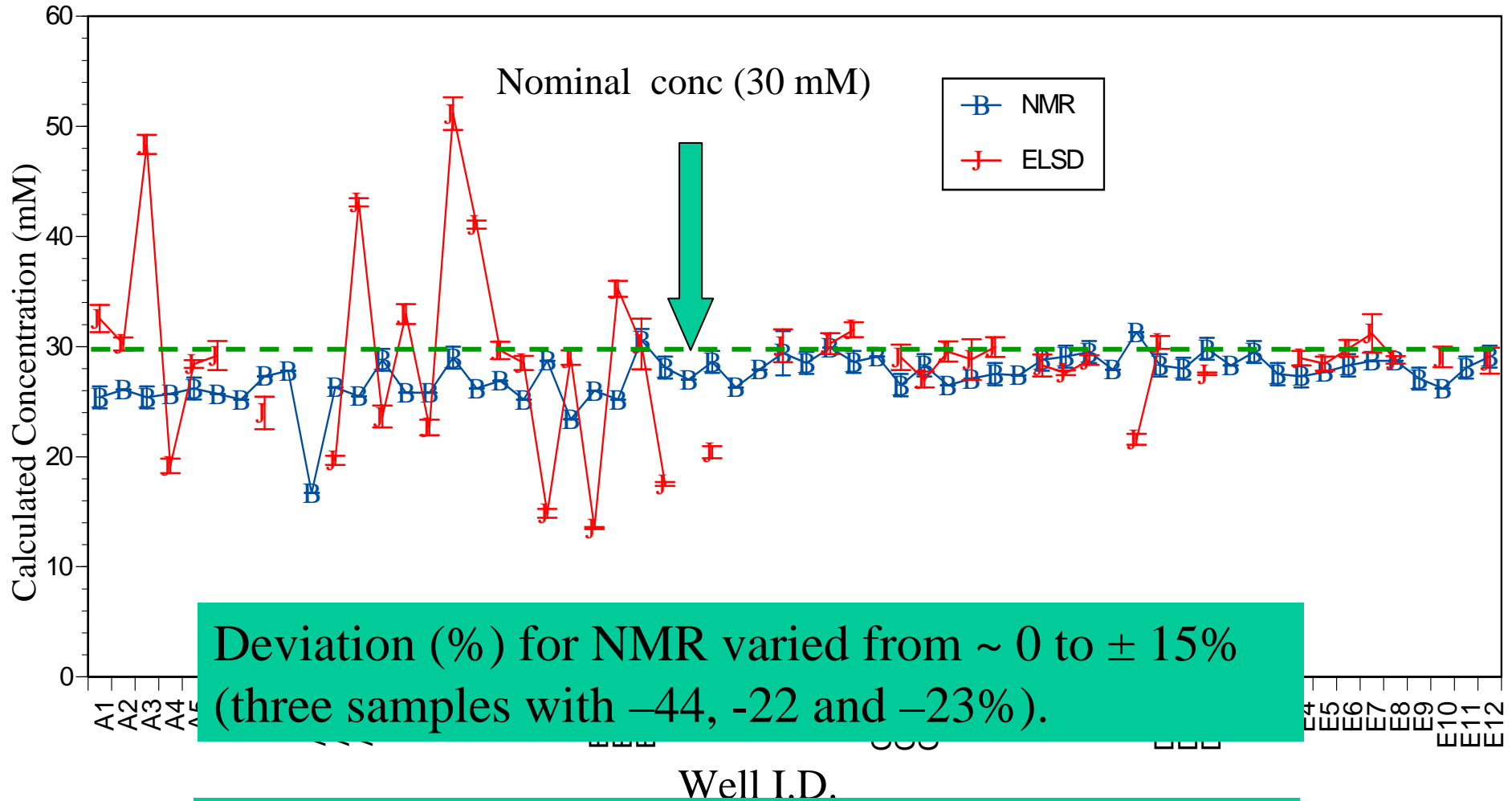
ELSD and NMR Precision and accuracy for test sample (12/07/05) in DMSO (Castrodad NAR 101375-162 and 101375-195)								
Well I.D.	Nominal conc. (mM)	Calculated conc. (mean \pm SD, n = 3) (mM)		RSD (%)		Deviation (%)		
		NMR	ELSD	NMR	ELSD	NMR	ELSD	
A1	30	25.4 \pm 1	32.5 \pm 1	4	4	-15	8	
A2							1	
A3							61	
A4	30	25.7 \pm 0	19.2 \pm 1	0	3	-15	-36	
A5	30	26.2 \pm 1	28.4 \pm 0	4	1	-13	-5	
A6							-3	
A7							n/a	
A8							-20	
A9							n/a	
A10	30	16.7 \pm 0	<LLOQ	0	n/a	-44	n/a	
A11	30	26.3 \pm 0	19.7 \pm 0	0	2	-12	-34	
A12							44	
B1							-21	
B2							10	
B3							-24	
B4	30	29 \pm 1	51.2 \pm 1	3	3	-3	71	
B5	30	26.2 \pm 0	41.1 \pm 0	0	1	-13	37	
B6	30	26.9 \pm 0	29.7 \pm 1	0	3	-10	-1	
B7	30	25.2 \pm 0	28.5 \pm 1	0	2	-16	-5	
B8	30	28.7 \pm 0	14.9 \pm 0	0	3	-4	-50	

RSD (%) for NMR and ELSD was within 10%

Deviation (%) for NMR varied from \sim 0 to \pm 15% (three samples with -44, -22 and -23%).

Deviation (%) for ELSD varied from \sim 0 to \pm 40% (three samples with -55, 61 and 71%).

ELSD/NMR results from LGG purified library



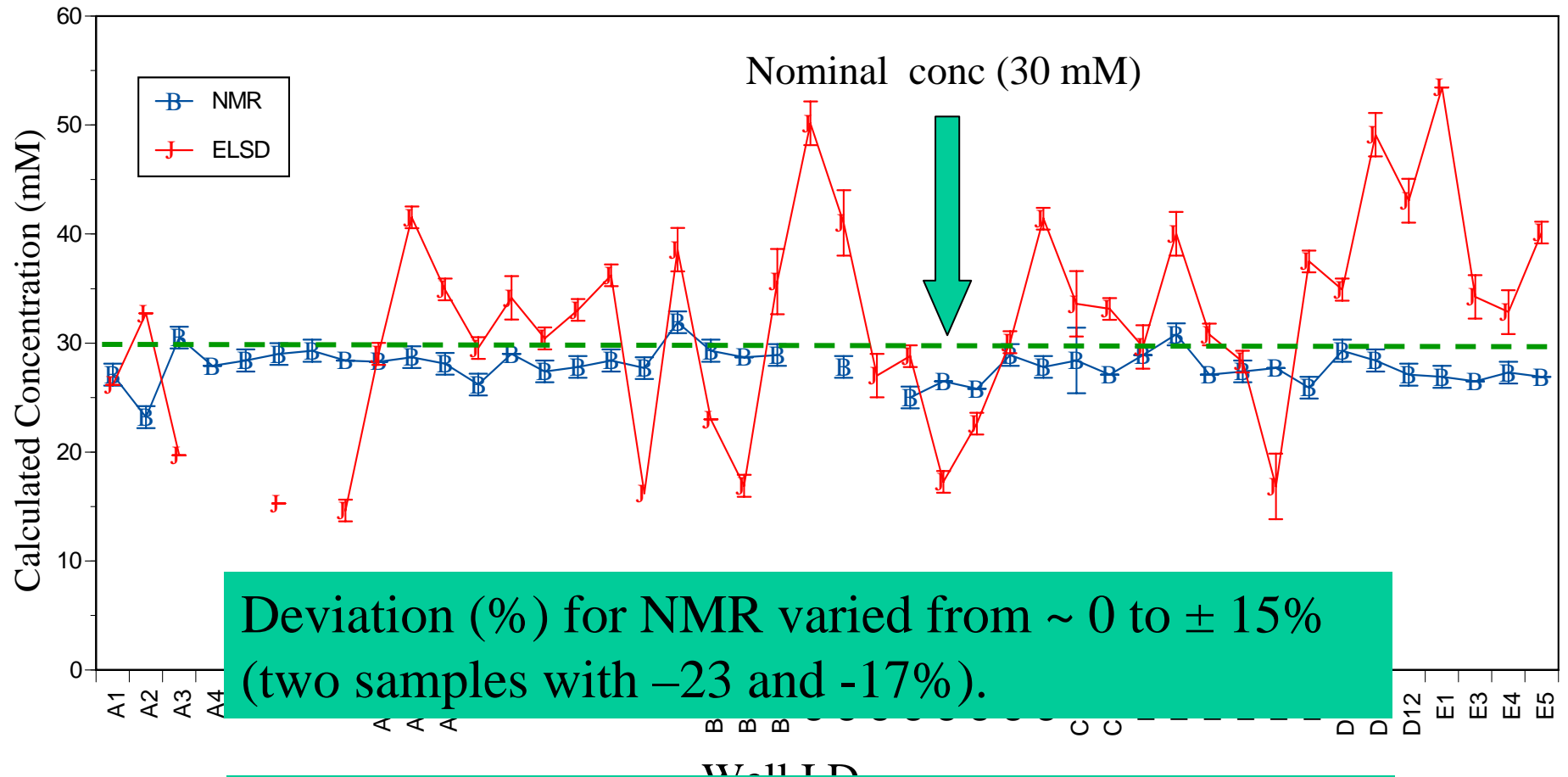
Deviation (%) for ELSD varied from ~ 0 to ± 40% (three samples with -55, 61 and 71%).

ELSD and NMR bars indicate the SD) for three replicates of each test sample. Gaps represent missing data points.

Representative ELSD/NMR results from Std set study

ELSD and NMR Precision and accuracy for std set study (12/07/05) in DMSO									
Calculations relative to 2 stds Sulindac and Acetanilide									
Well I.D.	Analyte	Nominal conc. (mM)	Calculated conc. (mean \pm SD, n = 3) (mM)		RSD (%)		Deviation (%)		
			NMR	ELSD	NMR	ELSD	NMR	ELSD	
A1	Methimazole	30	27.1 \pm 1	26 \pm 0	4	1	-10	-13	
RSD (%) for NMR and ELSD was within 10% (one sample 17%)									
A4	Menadione	30	27.9 \pm 0	#	0	n/a	-7	n/a	
A5	Acetylsalicylic Acid	30	28.4 \pm 1	#	4	n/a	-5	n/a	
A6	Caffeine	30	29 \pm 1	15 \pm 0	3	1	-3	-49	
A8	Ibuprofen							n/a	
A9	Nicotinic acid							-51	
A10	Acyclovir							-3	
A11	Carbamazepine							39	
A12	Zileuton	30	28.1 \pm 1	35 \pm 1	4	2	-6	16	
B1	Oxcarbazepine	30	26.2 \pm 1	30 \pm 1	4	4	-13	-1	
B2	Ketoprofen							14	
B3	Lamotrigine							1	
B4	Estradiol							10	
B5	Letrozole							21	
Deviation (%) for NMR varied from ~ 0 to \pm 15% (two samples with -23 and -17%).									
B6	Zolmitriptan	30	27.7 \pm 1	16 \pm 1	4	8	-8	-46	
B7	Disulfiram	30	31.9 \pm 1	39 \pm 2	3	4	6	29	
B10	Fluconazole	30	29.3 \pm 1	23 \pm 0	3	0	-2	-23	
B11	Quinidine	30	28.7 \pm 0	17 \pm 1	0	8	-4	-44	
B12	Clozapine	30	28.9 \pm 1	36 \pm 3	3	7	-4	19	
Deviation (%) for ELSD varied from ~ 0 to \pm 45% (two samples with -51 and 67%).									

ELSD/NMR results from Std set study

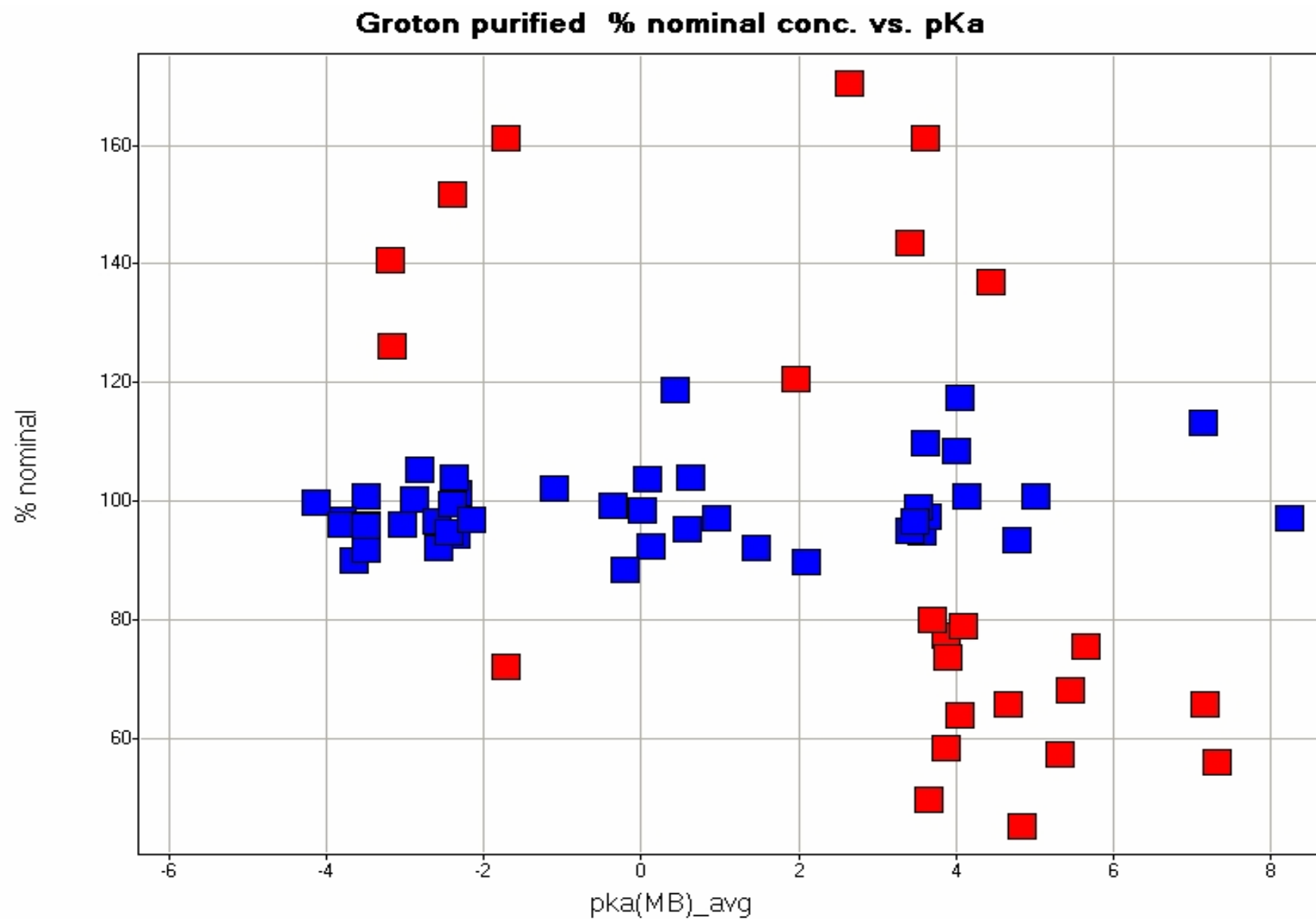


Deviation (%) for NMR varied from ~ 0 to $\pm 15\%$ (two samples with -23 and -17%).

Deviation (%) for ELSD varied from ~ 0 to $\pm 45\%$ (two samples with -51 and 67%).

ELSD and NMR results are shown. The green arrow represents the mean value (error bars indicate the SD) for three replicates of each test sample. Gaps represent missing data points. NMR calculations are relative to 2 stds sulindac and acetanilide (slight low shift of NMR calculated values may be attributed to balance)

% nominal concentration vs calculated pKa of purified compounds



Correlation Coefficient table for the physicochemical properties (Groton standard compound set)

	MP	BP	VP	MW	pKa	LogD
MP	1.0	0.93	- 0.85	0.83	0.33	- 0.13
BP	0.93	1.0	- 0.97	0.92	0.28	- 0.11
VP						0.11
MW	0.83	0.92	- 0.91	1.0	0.38	0.05
pKa						
LogD	- 0.13	- 0.11	0.11	0.05	- 0.50	1.0

BP, MP, VP and MW are highly intercorrelated thus providing redundant information for the analyses.

LogD and pKa , however, though moderately correlated with each other, were not related to any of the other four measurements

Conclusions

- Both HPLC/ELSD and NMR methods have been applied successfully for the quantitative determination of purified libraries as well as std set samples and results were compared
- Linear dynamic range and quantification using a “nearest calibrant” approach are essential to minimize errors in quantitative determination
- ELSD intra-assay precision and accuracy for the QC and external std were within $\pm 12\%$ and thus added confidence to the robustness of the assay.
- For both purified library and std set samples, the RSD (%) for NMR and ELSD was within 10% (one sample 17%)
- For both purified library and std set samples, the deviation (%) for NMR varied from ~ 0 to $\pm 15\%$ (three samples with -44 , -22 and -23%).

Conclusions (cont'd)

- For both purified library and std set sample, the deviation (%) for ELSD varied from ~ 0 to $\pm 40\%$ (three samples with 61, 67 and 71%)
- Graphical and statistical analyses showed that none of the 6 physicochemical measurements in either isolation or in combination was a useful predictor of the accuracy (± 20) to be expected from an ELSD concentration measurement.
- Statistical analysis also showed that BP, MP, VP and MW were highly intercorrelated thus providing redundant information for the analyses.
- LogD and pKa, however, though moderately correlated with each other, were not related to any of the other four measurements.