



Monitoring DNA Damaging Exposure Thresholds for a Foodborne Carcinogen Using LC-MS/MS and DNA Microarrays

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CoSMoS 2009 Boston, MA

Constant Assault



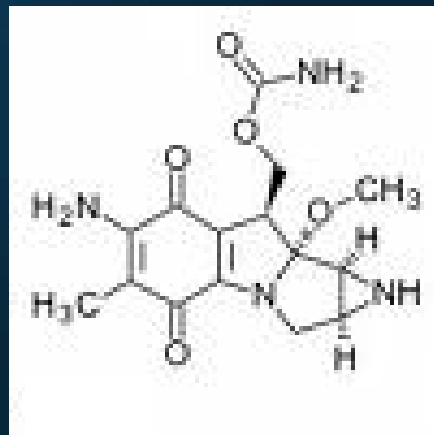
Heterocyclic aromatic amines



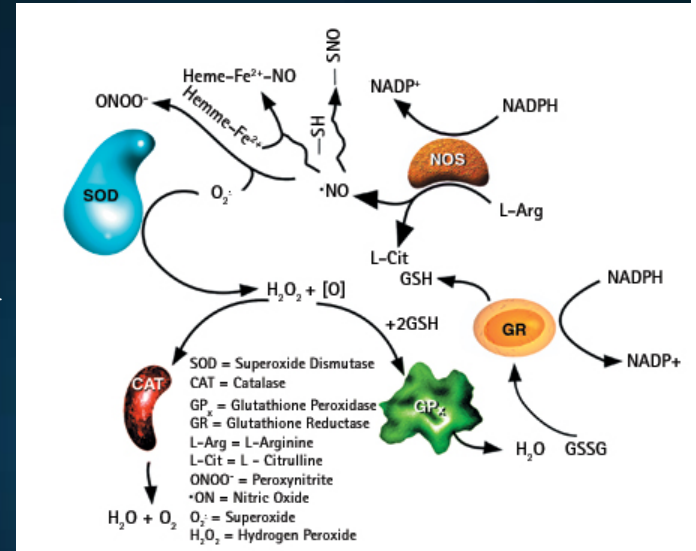
Cigarette smoke



UV B radiation



Pharmaceuticals

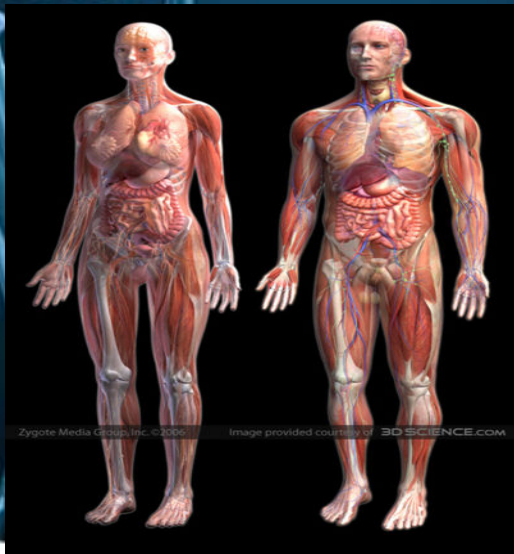


Oxidative damage



Polyaromatic hydrocarbons

DNA Adducts and You



Bladder - Archer, C., et al. *Cancer Letters* (Shannon, Ireland) (2000), 155(1), 55-60.

Intestine - Tsukamoto, T., et al. *Jap. Jour. of Cancer Res.* (1999), 90(7), 720-725.

Kidney - Yang, H., et al. *Environ. Mol. Mut.* (2002), 40(2), 116-121.

Liver - Soglia, J. et al., *Analytical Chemistry* (2001), 73(13), 2819-2827.

Mammary gland - Imaida, K., et al. *Jap. J. Cancer Res.* (1996), 87(11), 1116-20.

Pancreas - Hirose, M., et al. *Environ. Mol. Mut.* (2002), 39(2/3), 271-278.

Prostate - Shirai, T. et al. *Cancer Research* (1997), 57(2), 195-198.

Stomach/Colon - Pence, B., et al. *Food Res. Tech.* (1998), 207(6), 455-458.

Brain — Liu X., et al. *Analytical Chemistry* (2005), 77(18), 5982-5889.

Colon/Blood - Dingley, K., et al. *Cancer Epi. Biomar & Prev.* (1999), 8(6), 507-512.

Intestine - Klein, J., et al. *Carcinogenesis* (2001), 22(4), 619-626.

Liver - Baranczewski, P., et al. *Biomarkers* (2004), 9(3), 243-257.

Lung - Sinha, R., et al. *Cancer Res.* (2000), 60(14), 3753-3756.

Pancreas - Ricicki, E., et. al., *Chem. Res. Toxicology* (2005), 18(4), 692-9.

Prostate - Lawson, T., et al. *Cancer Let.* (Shannon, Ireland) (2002), 175(2), 141-6.



Project Objectives

- Develop sensitive and quantitative LC-MS methods for low-level DNA adducts for the foodborne carcinogen PhIP
- Evaluate the dose-response characteristics of PhIP using both MS and DNA microarrays
- Determine if there is a No Observable Transcriptional Effect Level or “NOTEL” for PhIP using DNA microarrays
- Determine if there is a correspondence between the NOTEL and No Observable DNA Adduct Level (NODAL)



No Observable Transcriptional Effect Level NOTEL

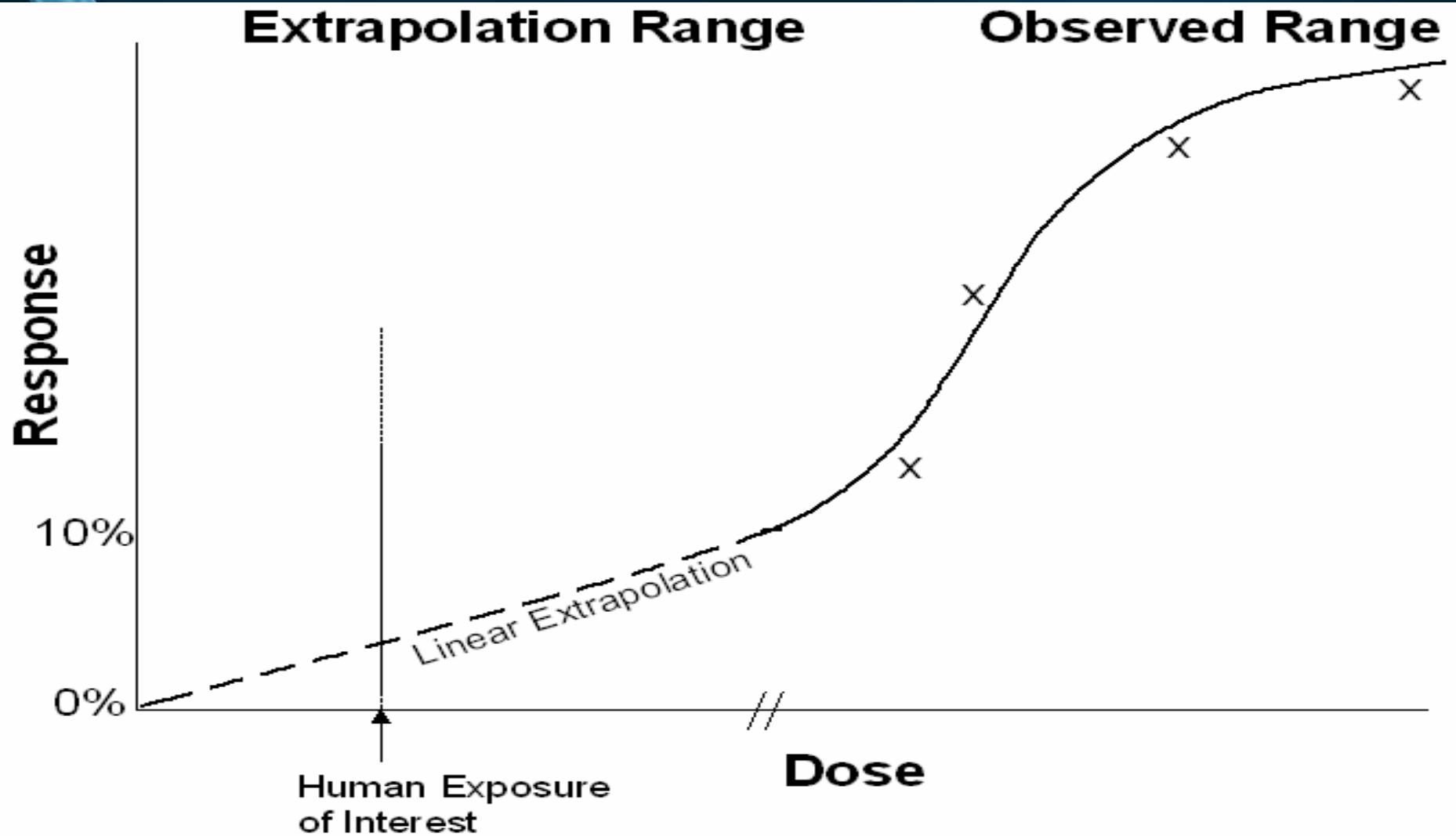
- The concentration of a compound at which no detectable changes in gene transcription are observed in exposed cells
- Extrapolation of dose-response effects from high concentration studies have shown nonlinear trends for some classes of compounds when actually tested at low concentrations



Why is a “NOAEL” Important ?

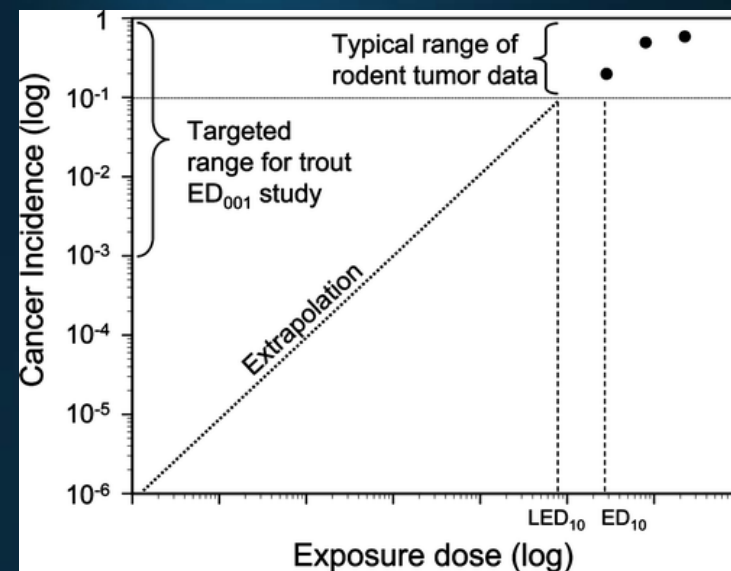
- Presently, risk assessors define “safe” human doses of toxicants and non-genotoxic carcinogens using the NOAEL (no observable adverse effect level, or threshold) or the lowest observed dose
- Default assumptions or safety factors are then used to set the “safe” human dose below the NOAEL:
 - 10X for cross species differences
 - 10X for genetic variation in sensitivity
 - 10X developmental stage differences
- NOAEL effectively assumes that there is no safe dose

Linear Low-dose Response Extrapolation for Genotoxic Carcinogens



Practical Implications of “Poor” Risk Assessment

- Feed 40,800 trout for 4 weeks with 0 - 225 ppm Dibenzo[*a*,*l*]pyrene
- Switch to regular feed for 9 months
- Examine for tumors
- Extrapolate how much carcinogen is required to induce 1 extra cancer case per million animals

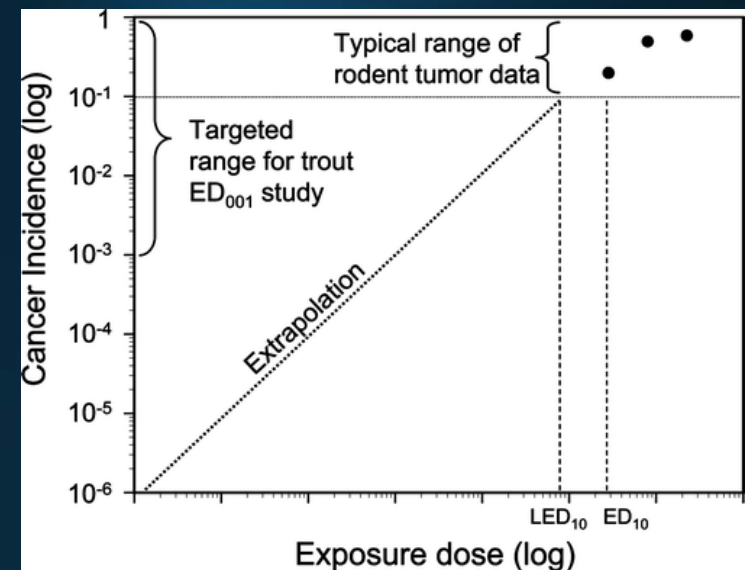
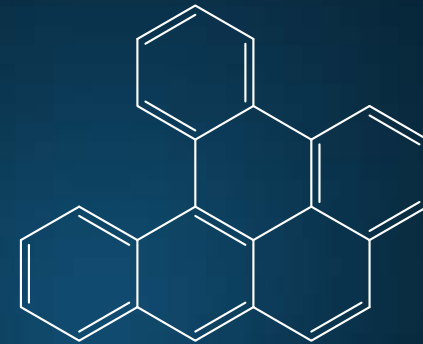


Practical Implications (cont.)

- Dosing range was 3 orders of magnitude lower than previous studies for DBP
- Dose-response was not linear
- Extrapolated carcinogen required to induce 1 extra cancer case per million animals:

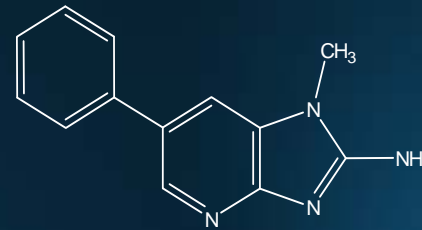
500 – 1500 fold higher concentration than the EPA linear extrapolation model

EPA's method underestimates the dose and overestimates the cancer risk!

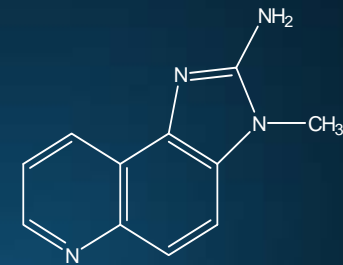


Heterocyclic Aromatic Amines

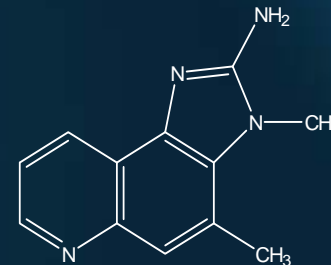
- Overcooked protein containing foods
- At least 20 identified in cooked meats
- ~ 1 to 100 ng/g meat*
- Carcinogenic in various animals and linked to breast and colorectal cancer
- PhIP is the most abundant



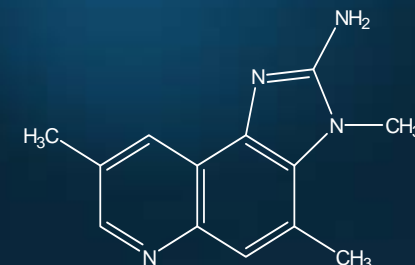
PhIP



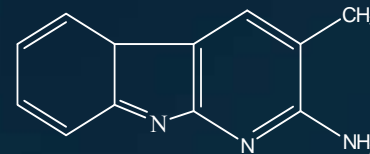
IQ



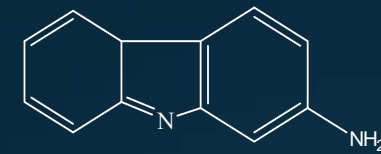
MeIQ



DiMeIQ



MeAαC



AαC

*IARC monograph, 1993, Vol. 56, Lyon, France, 211-228

Integrated Chip LC System

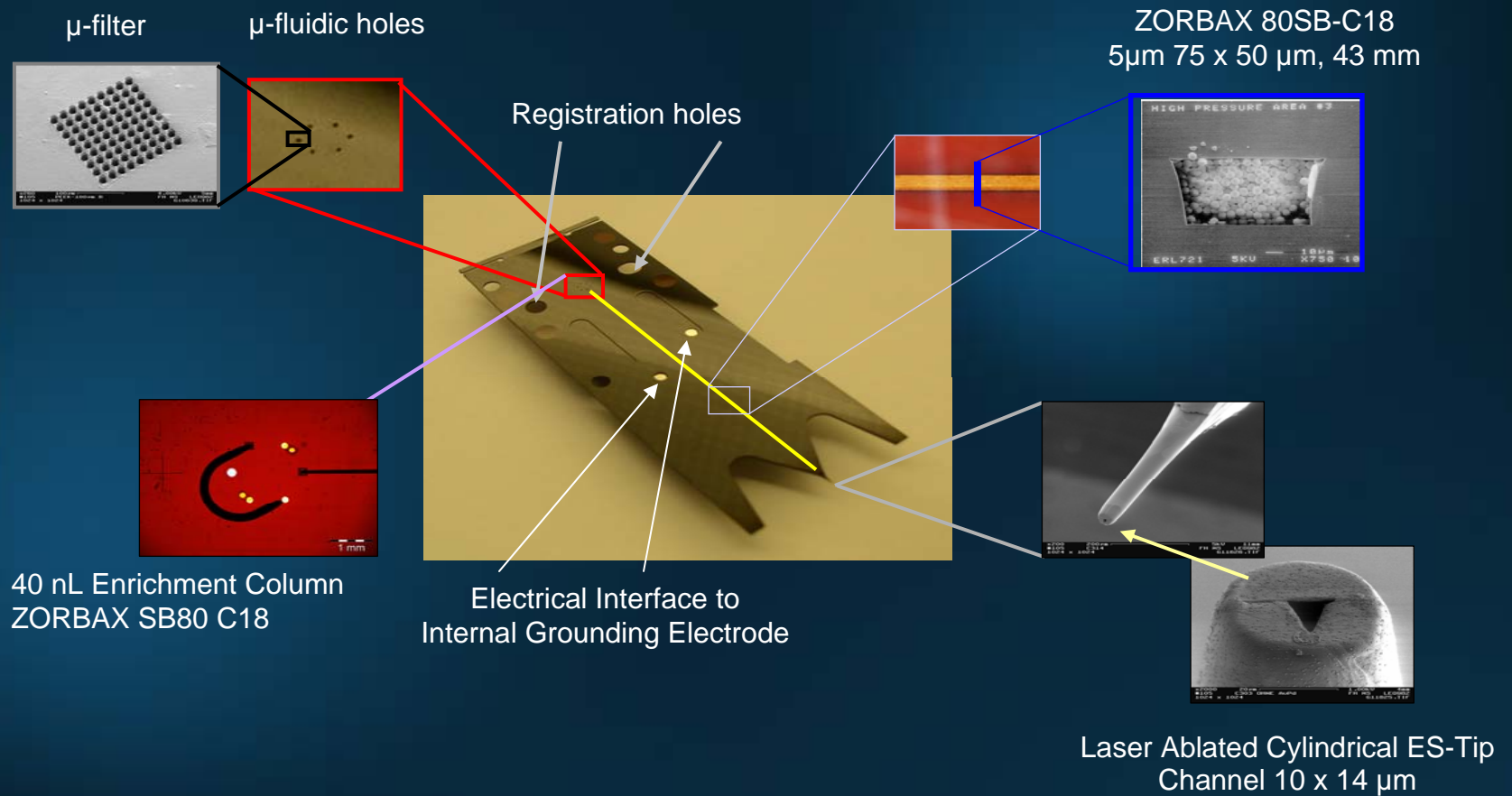


Photo Courtesy of Tom Trainor, Agilent Technologies, Inc.

LC-MS/MS Conditions

Mass Spectrometer:

- Agilent XCT Ultra Plus Ion Trap

Column:

- Agilent HPLC Chip
Zorbax SB80 C18 phase, 5 μm
4.3 cm x 75 μm w/40 nl enrichment col.
- Positive ESI voltage = 1.6kV
- Heated capillary = 325°C
- N₂ drying gas flow = 4.0 L/min

MS Parameters:

- MRM mode – scanning 310-510 Da
490 \pm 1 Da \rightarrow 374 Da PhIP-dG adduct
493 \pm 1 Da \rightarrow 377 Da d3-PhIP-dG (IS)
- Ultra scan mode, Ion Charge Control On
- 400 msec/scan 3 scans averaged
- He collision gas, 4.5mT pressure
- Collision voltage = 1.0 V

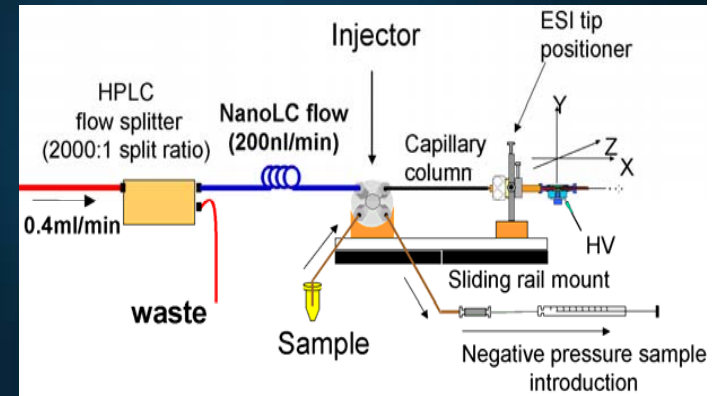
HPLC System:

- Loading pump (Agilent 1100 capillary pump)
4 $\mu\text{l}/\text{min}$
MP A = 3 mM Ammonium formate, pH 2.8*
MP B = 45:45:10 ACN:CH₃OH:3 mM
ammonium formate pH 2.8*
- Washing gradient:
Hold @ 5%B for 4 min, 5%B-90%B in 10
min, hold 4 min
- Nanopump (Agilent 1100/nanoflow)
600 nl/min
MP A = 3 mM Ammonium formate, pH 2.8
MP B = 45:45:10 ACN:CH₃OH:3 mM
ammonium formate pH 2.8
- Gradient:
Hold @ 30%B for 4 min, 30%B – 90%B in
14 min
- Agilent μ -wellplate Autosampler
96 wellplate /glass inserts

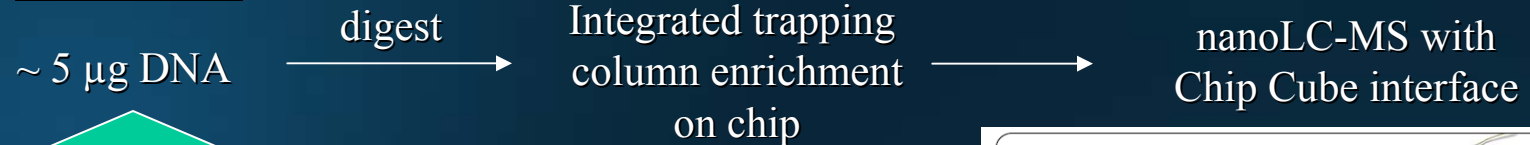
*Bianchi *et al*, J.Chrom B., 825 (2), 2005, 193-2000.

Typical Sample Preparation for LC-MS

Early 2000's

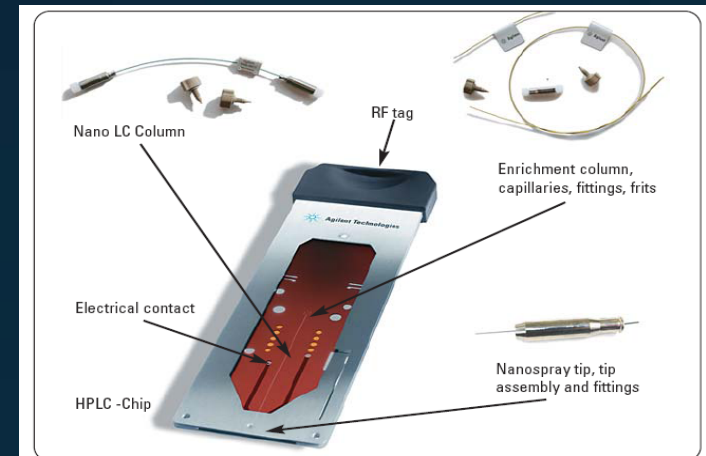


Late 2000's

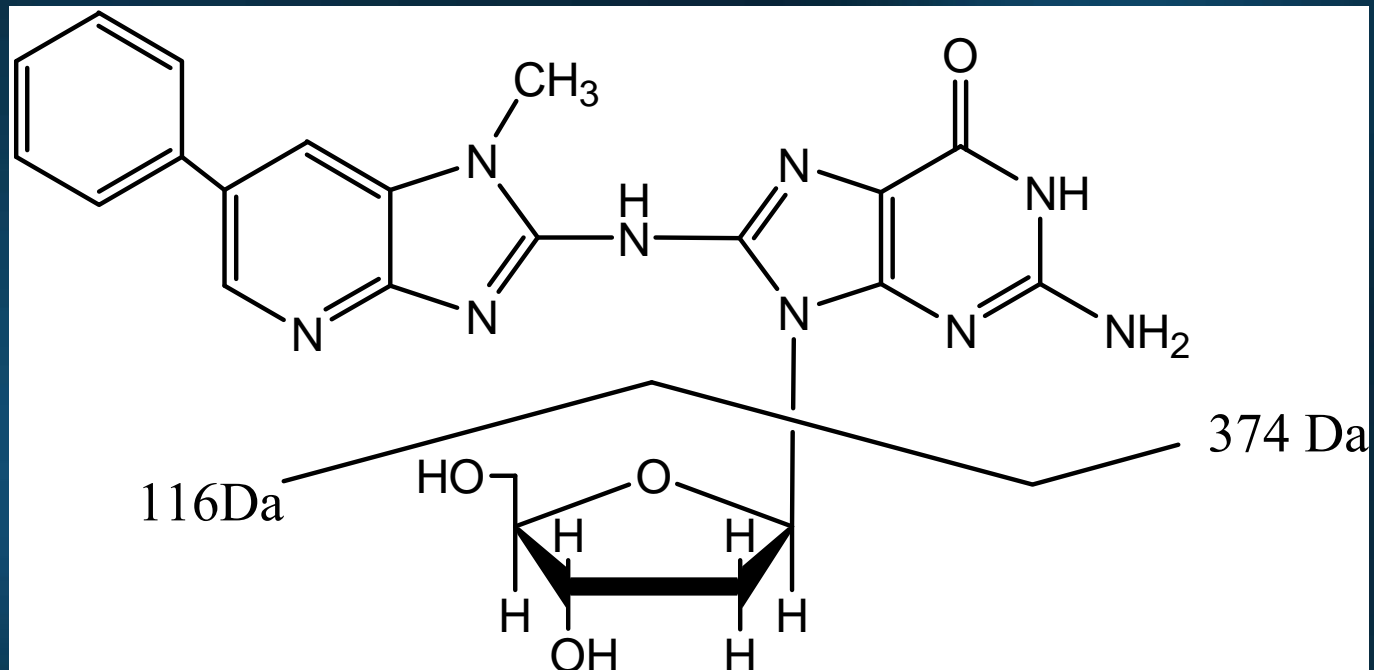


60-fold reduction
in DNA used

Elimination of SPE

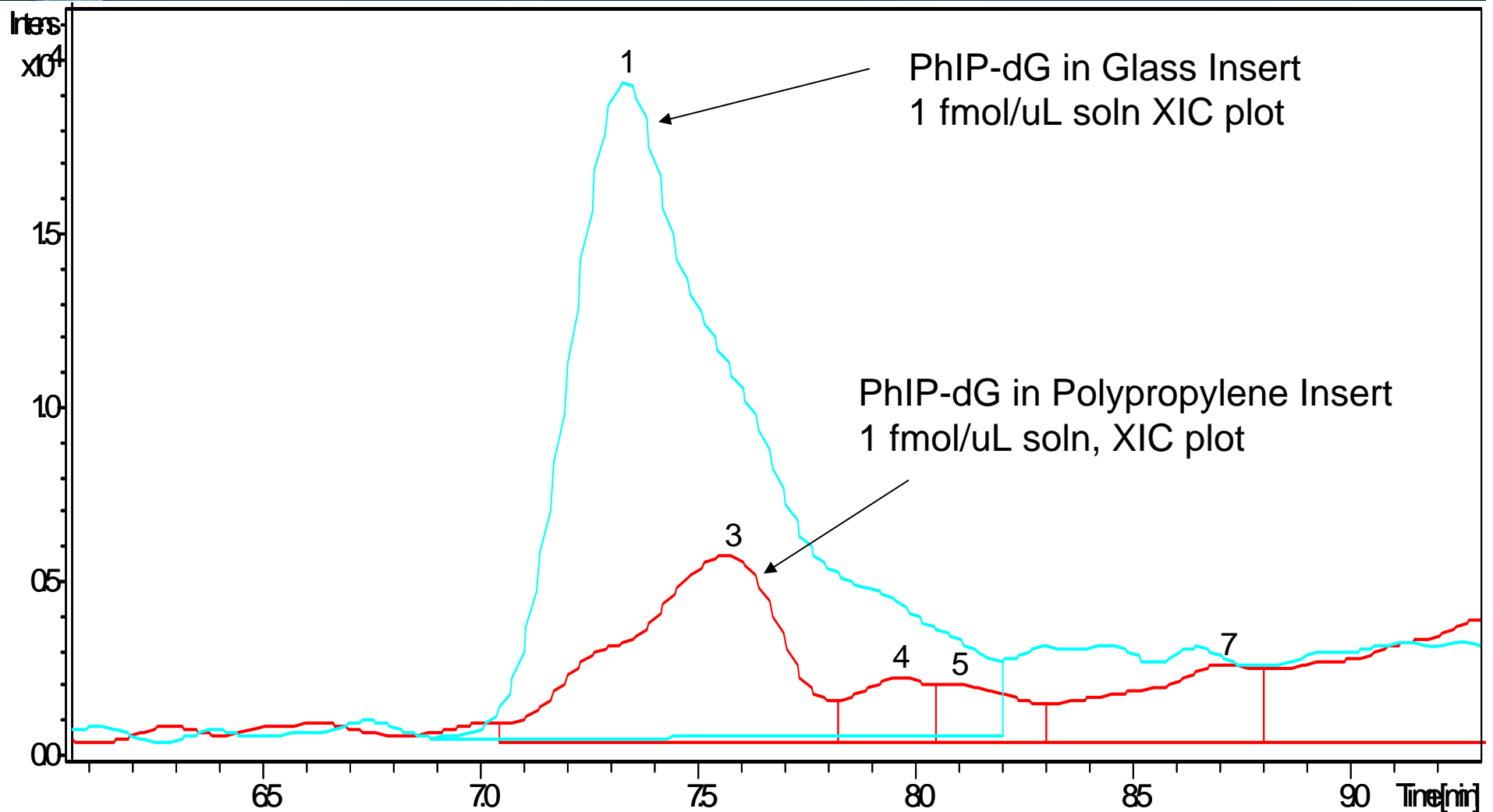


Problems in Method Development... or How to Make a Grown Man Cry



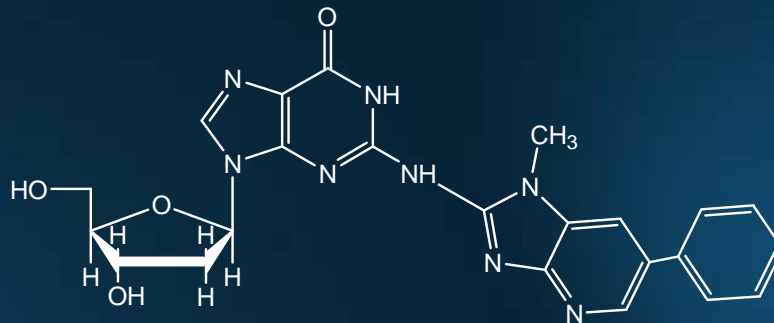
Problems in Method Development

Chapter 1 – Where did the PhIP-dG Go?

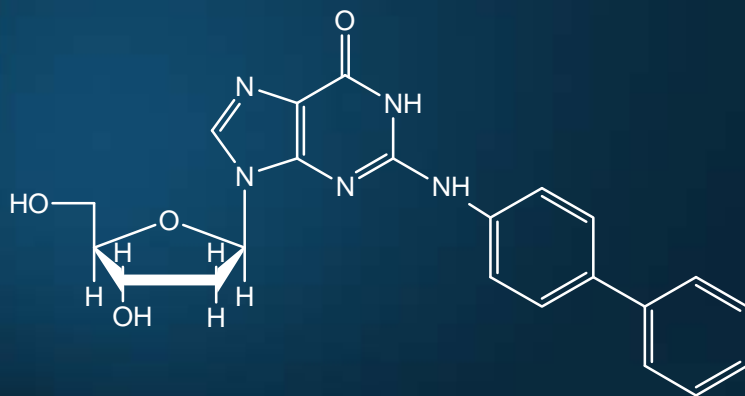


Problems in Method Development

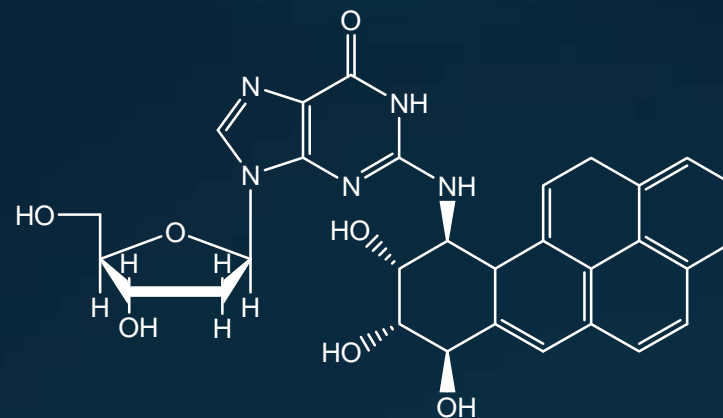
Chapter 1 – Where did the PhIP-dG Go?



PhIP-dG $\text{LogP} = 2.35$



ABP-dG $\text{LogP} = 3.03$

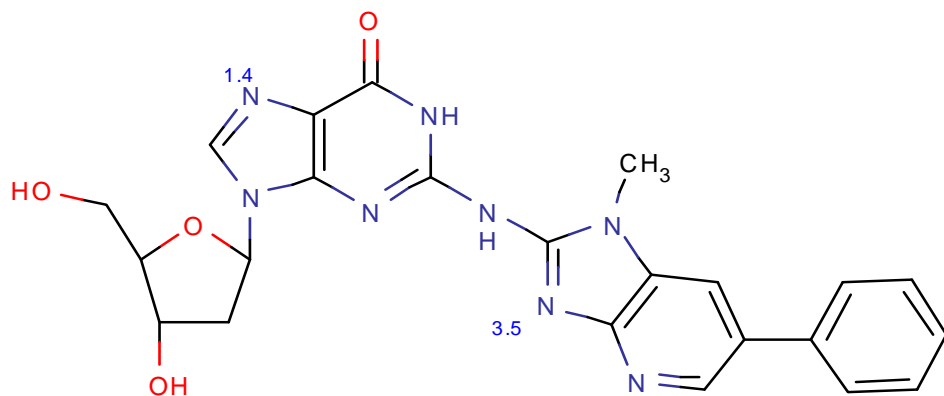


BPDE-dG $\text{LogP} = 4.42$

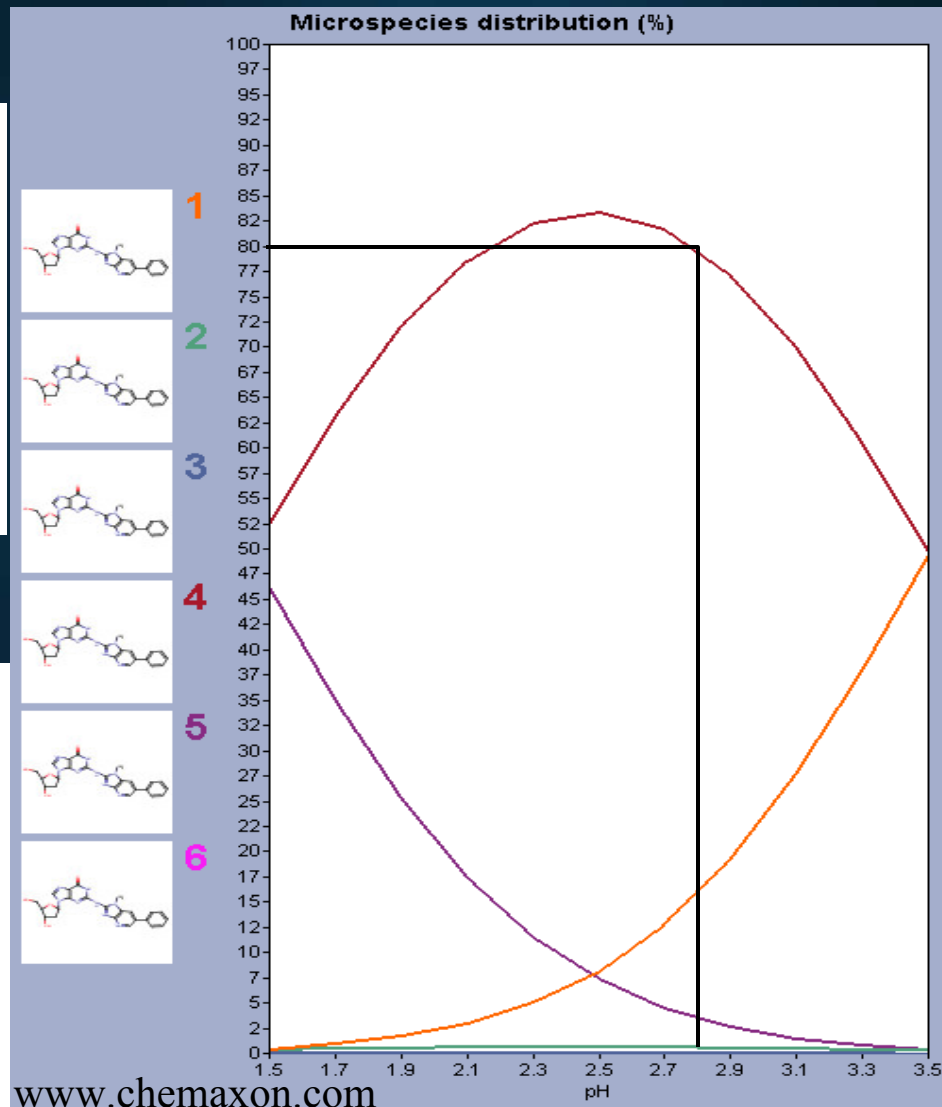
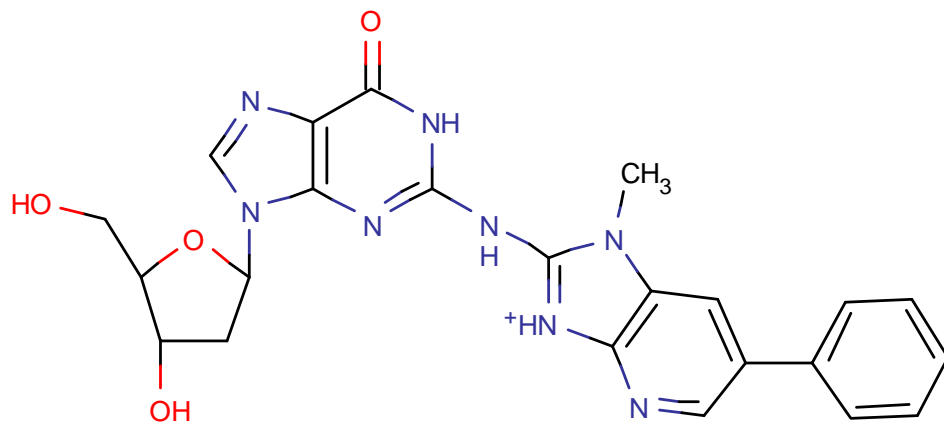
Problems in Method Development


Chapter 1 – Where did the PhIP-dG Go?

Calculated pKa's for PhIP-dG



Calculated Major Microspecies for PhIP-dG





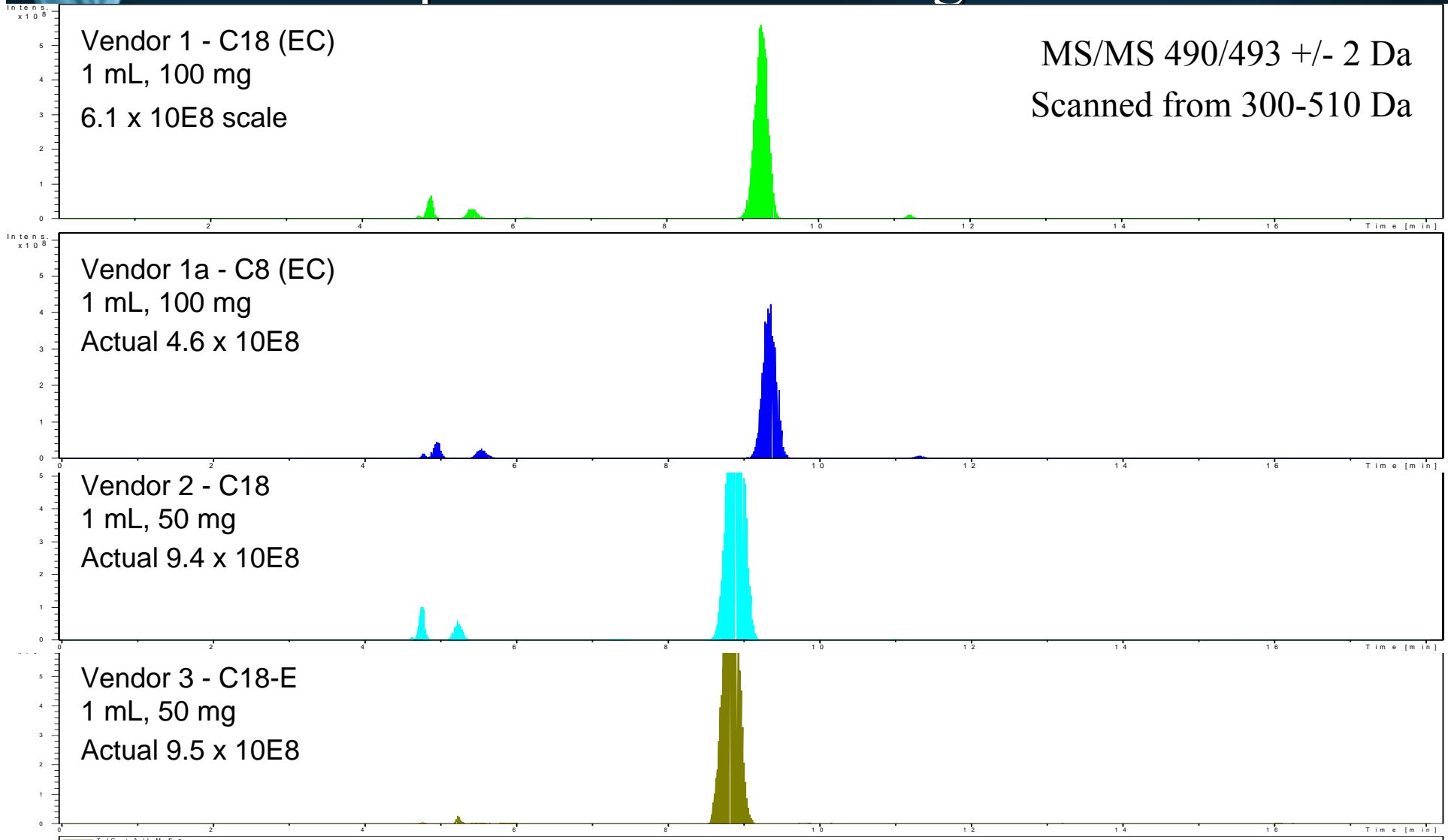
Problems in Method Development

Chapter 2 – The Case Against SPE

- Equilibrate cartridge w/ 3 x 1 mL 100% CH₃OH
- Equilibrate w/ 3x 1 mL 5% DMSO
- Load sample dissolved in 5% DMSO
- Rinse w/ 3 x 1 mL DI H₂O
- Rinse w/ 3 x 1 mL 10% CH₃OH w/ 0.1% HOAc
- Elute w/ 1 mL CH₃OH

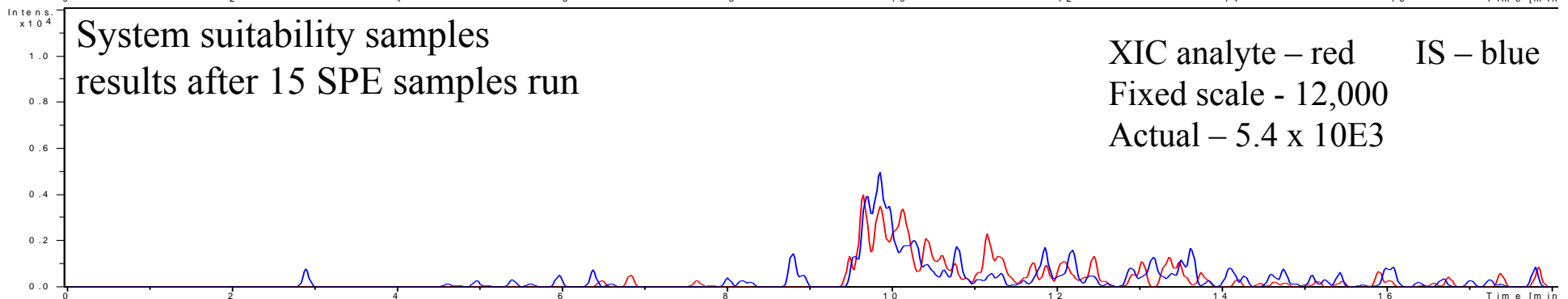
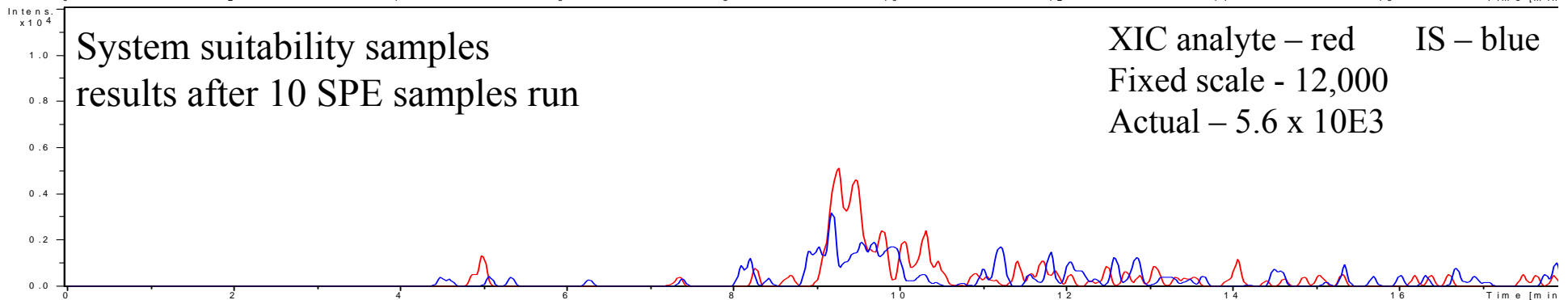
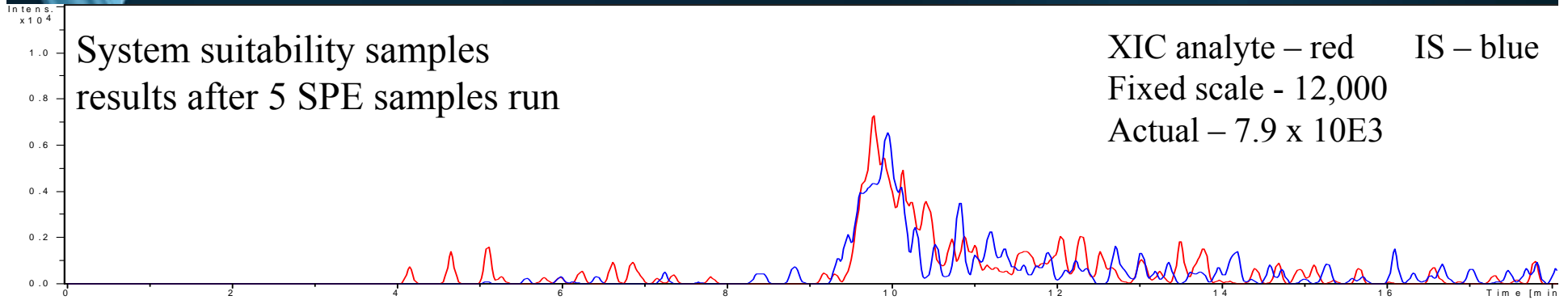
Problems in Method Development

Chapter 2 – The Case Against SPE



Problems in Method Development

Chapter 2 – The Case Against SPE



Problems in Method Development

Chapter 3 – Complete DNA Digestion?

Early 2000's ~ 300 μ g DNA

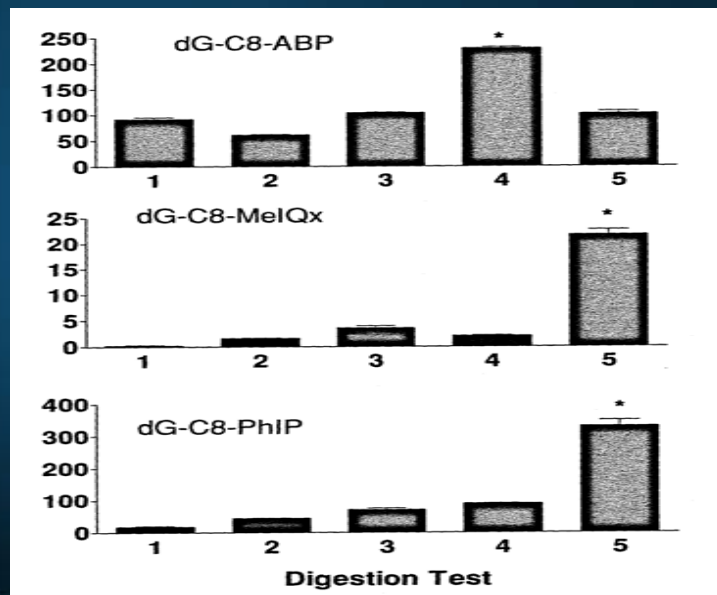
- DNase I (23 U) for 4 hr.
- Snake venom phosphodiesterase (1.1 U)
- Alkaline phosphatase (0.08 U)
- SVP and AP for 18 hrs.
- Does one size fit all adducts?

Test 4 – 4 enzyme system

- DNase I (10 U) for 1 hr.
- Spleen phosphodiesterase (0.01 U) and SVP (0.01 U) for 6 hrs.
- Alkaline phosphatase (5 U) overnight

Test 5 – 6 enzyme system

- DNase I (10 U) for 1 hr.
- Micrococcal nuclease (5 U) and Nuclease P1 (5 U) and spleen phosphodiesterase (0.01 U) and snake venom phosphodiesterase (0.01 U) for 6 hr.
- Alkaline phosphatase (5 U) overnight



Problems in Method Development

Chapter 3 – Complete DNA Digestion?

Late 2000's ~ 5 μ g DNA

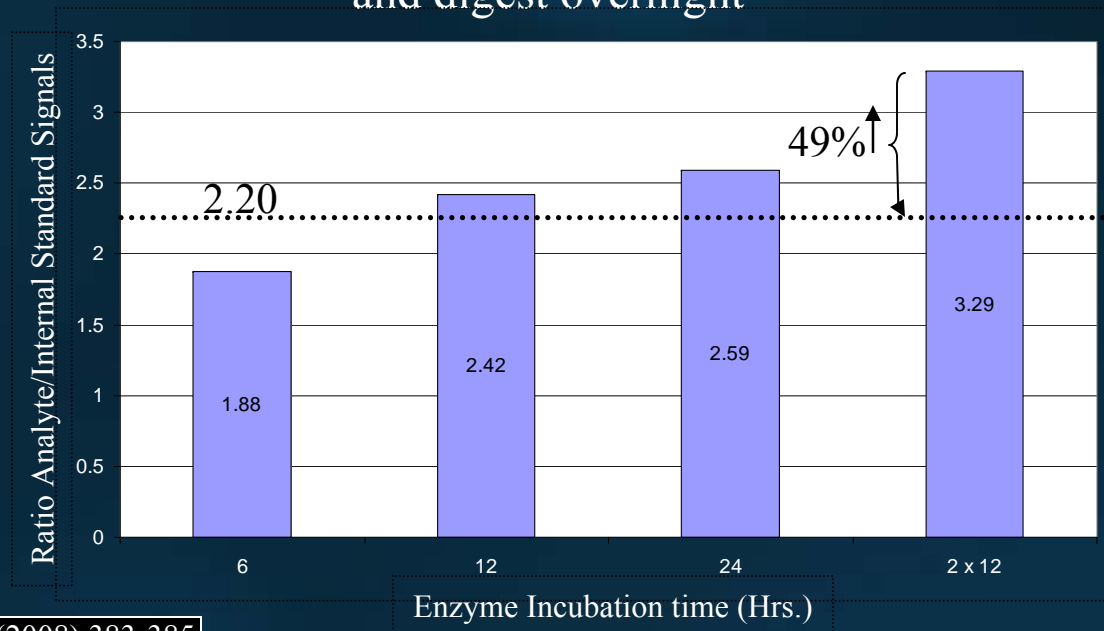
- Nuclease P1 (10 U) for 2 hr.
- Snake venom phosphodiesterase (0.010 U) for 2 hrs.
- Alkaline phosphatase (0.5 U) for 1 hr.

➤ Problems:

Extensive sample prep
Several pH changes
Right enzyme mix?

Current Digest Conditions*:

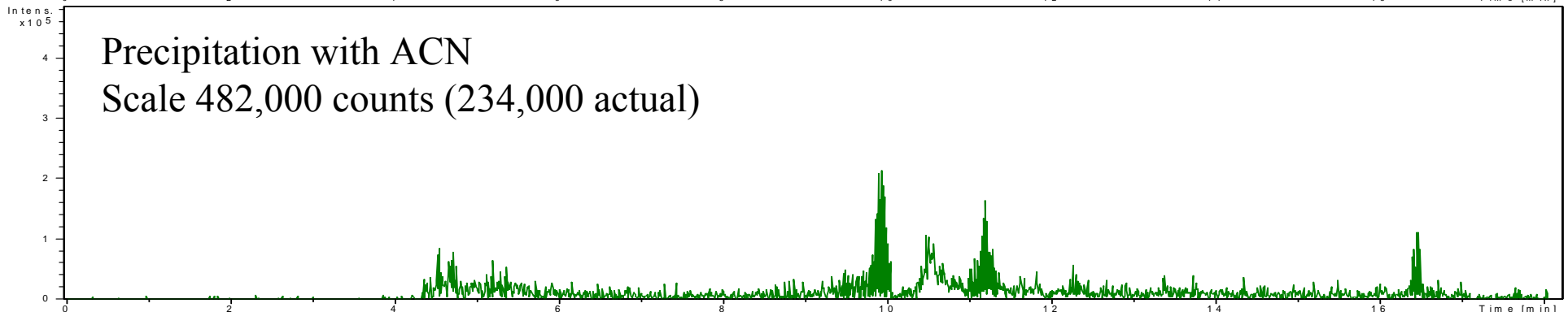
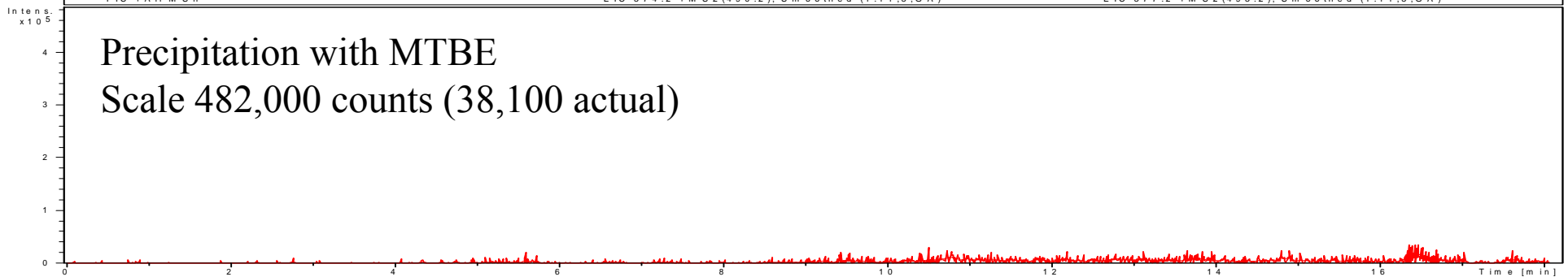
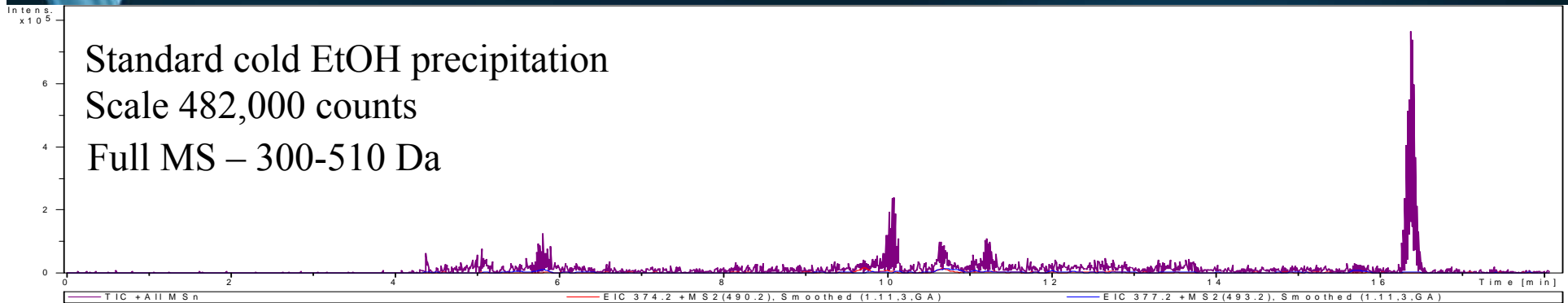
- Benzonase (1250 U) and SVP (1.5U) and AP (100U) in buffer at pH=7.9
- Incubate for 12 hrs. then add a second volume of enzyme mix and digest overnight



*Adapted from Quiulivan and Gregory, Anal. Biochem, 373 (2008) 383-385

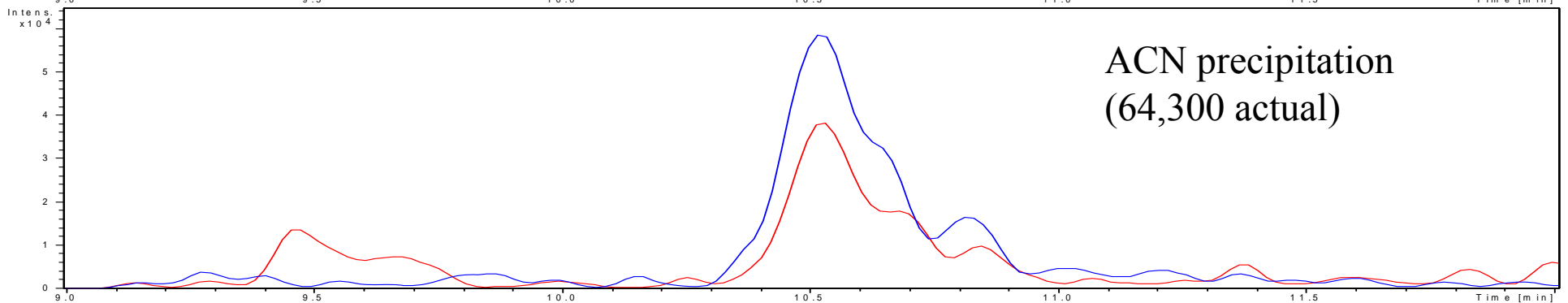
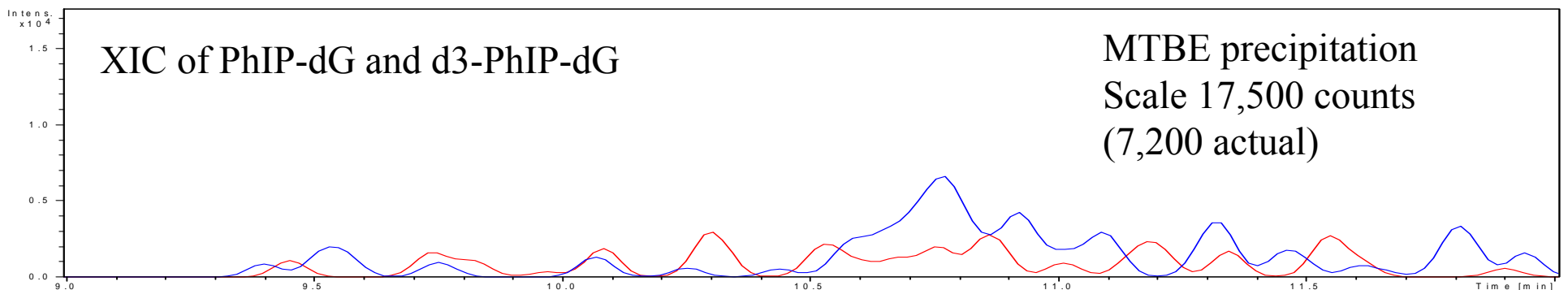
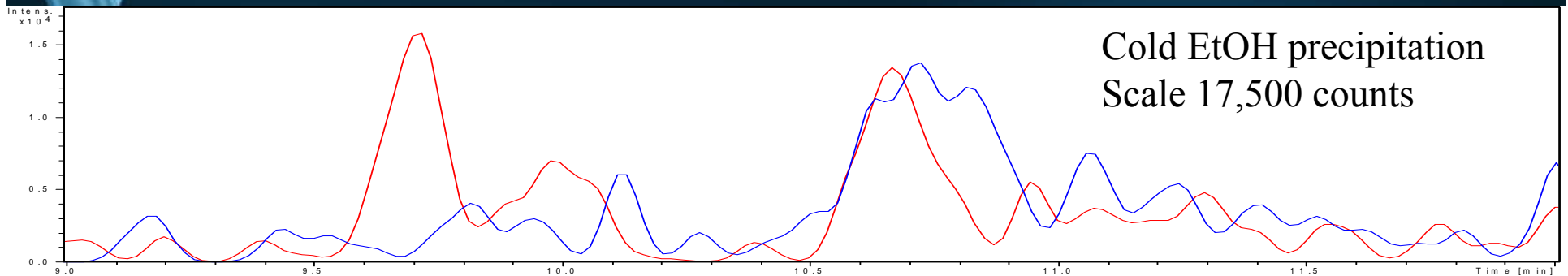
Problems in Method Development

Chapter 4 – Protein ppt after DNA Digestion



Problems in Method Development

Chapter 4 – Protein ppt after DNA Digestion



DNA Sample Preparation for LC-MS

Culture human
BEAS-2B cells
(lung epithelial cells)

Dose with N-OH-PhIP
Incubate for 24 hrs.
→
Dosing levels from
 1.0×10^{-6} to 1.0×10^{-10} M

Isolate mRNA and DNA from cells
using Qiagen All-Prep kit

Determine quantity of DNA with
Invitrogen Qubit system

0.2 – 10 μ g DNA isolated

5 μ g DNA used for analysis

Add 50 μ L enzyme digest buffer*

20 mM Tris-HCl pH=7.9
100 mM NaCl
20 mM MgCl₂
1250 Benzonase
1.5 U Snake venom phosphodiesterase
100 U Alkaline phosphatase

→
Incubate @ 37°C, 12 hrs.
Add 50 μ L enzyme digest buffer and
incubate overnight

Precipitate enzymes with
ACN, transfer and
SpeedVac to dryness

←
5 μ l injection LC-MS/MS mode
(1.25 μ g total DNA used for analysis)

↓
Reconstitute in 20 μ l DI H₂O

*Adapted from Quiulivan and Gregory, Anal. Biochem, 373 (2008) 383-385

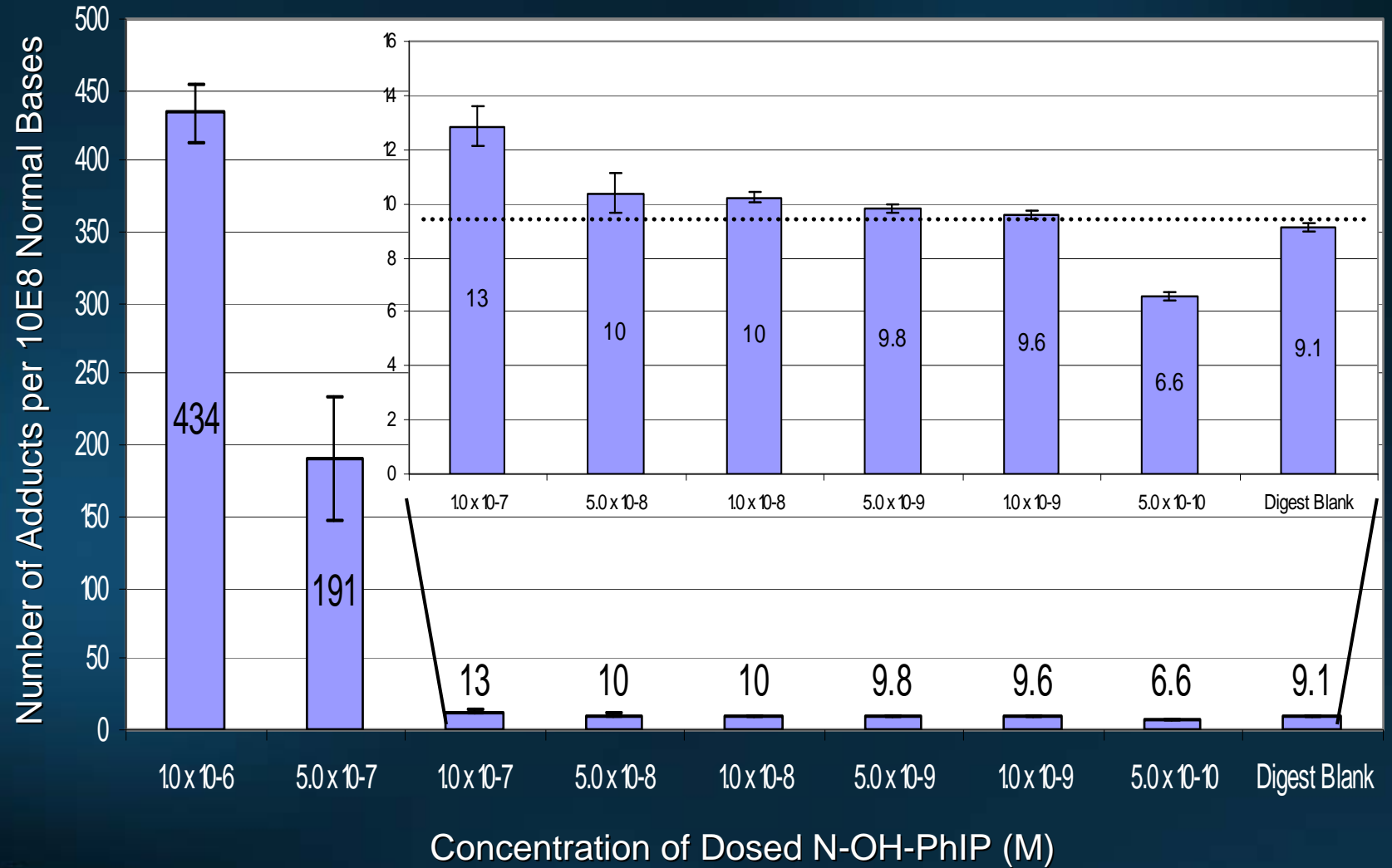
DNA Adduct Quantitation

$$y = 0.2571x - 0.076$$

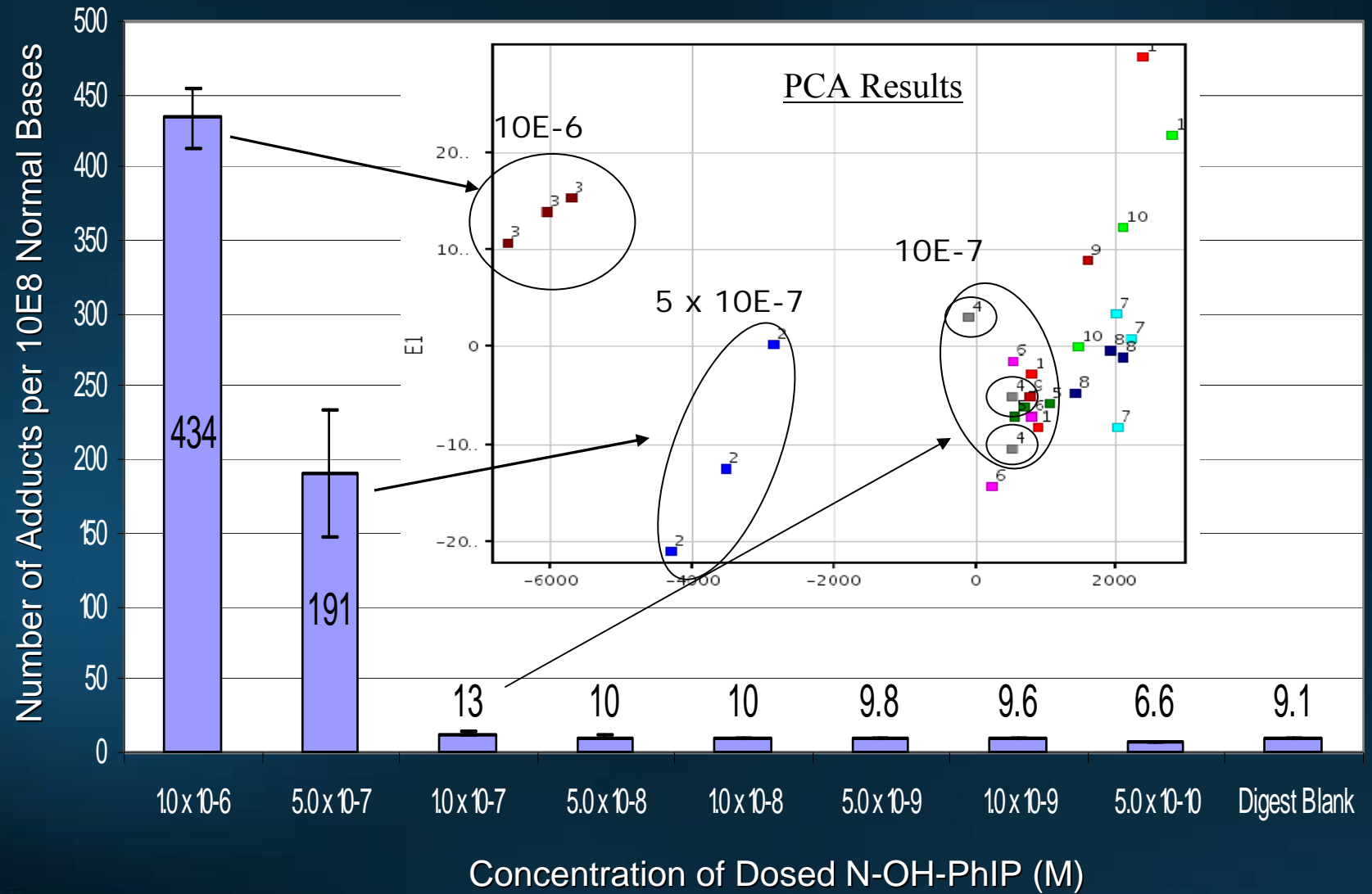
$$R^2 = 0.994$$

0.5 – 50 pg/ μ L

LOD = 0.13 pg/ μ L

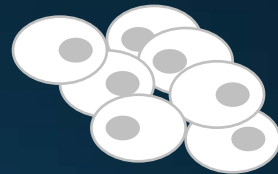


DNA Adducts and Microarray Data



DNA Microarray Analysis Procedure

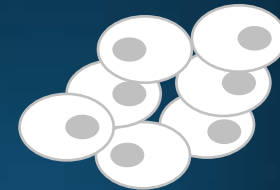
Control Cell Population
(DMSO)



Extract mRNA and DNA

Convert RNA to cRNA

Exposed Cell Population
N-OH-PhIP in (DMSO)



Extract mRNA and DNA

Convert RNA to cRNA

Hybridize in duplicate to
Affymetrix U133 Human GeneChip array
(~38,000 probes sets)

Scan

Data extraction
normalization
and analysis

Comparison of gene
expression profiles
at different doses

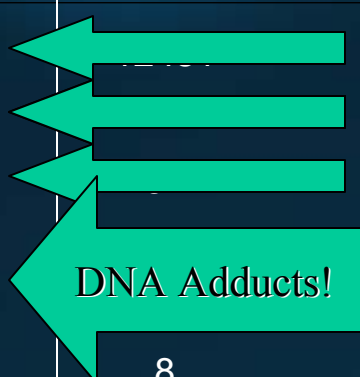
Cell exposures were for 24 h, beginning at a dose 10x below the dose that gave 50 % cell death



Bayesian Robust Inference for Differential Gene Expression (BRIDGE)

➤ Detects differentially expressed genes under multiple experiments

Dose (M)	# of genes with posterior probabilities > 0.8	# of genes with posterior probabilities > 0.7	# of genes with posterior probabilities > 0.6	# of genes with posterior probabilities > 0.5	# of genes with posterior probabilities > 0.1	Dose (M)
10^{-6}	4144	5099	5948	6857		10^{-6}
5×10^{-7}	968	1214	1473	1757		5×10^{-7}
10^{-7}	1	1	2	2		10^{-7}
5×10^{-8}	0	0	0	0		5×10^{-8}
10^{-8}	0	0	0	0		10^{-8}
5×10^{-9}	0	0	0	0	8	5×10^{-9}
10^{-9}	0	0	0	0	5	10^{-9}
5×10^{-10}	0	0	0	0	3	5×10^{-10}

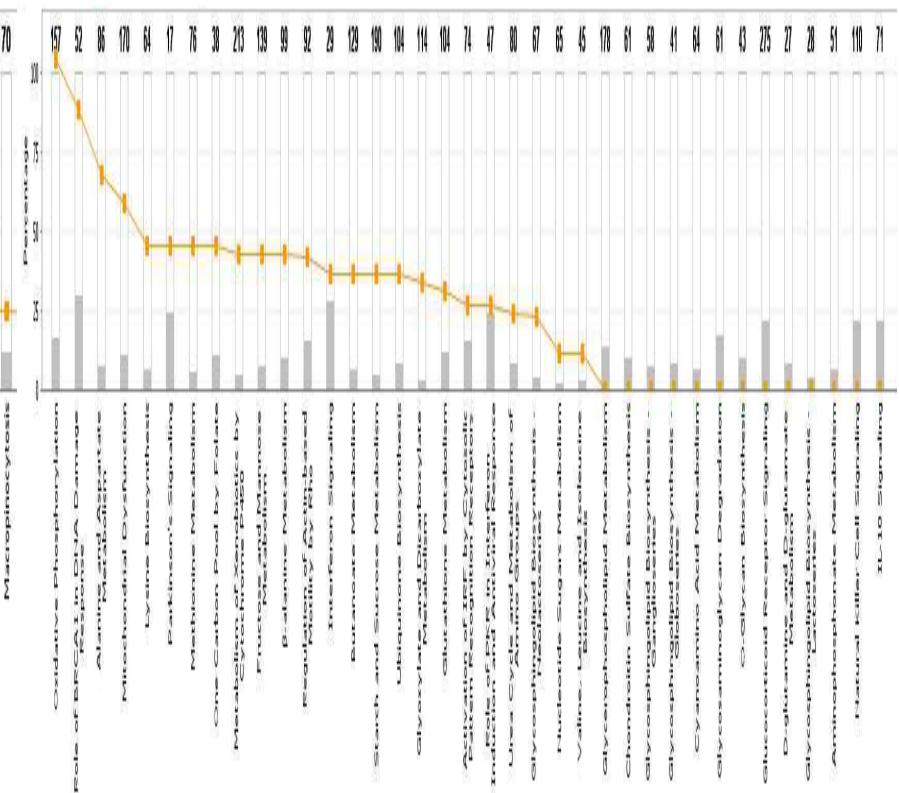
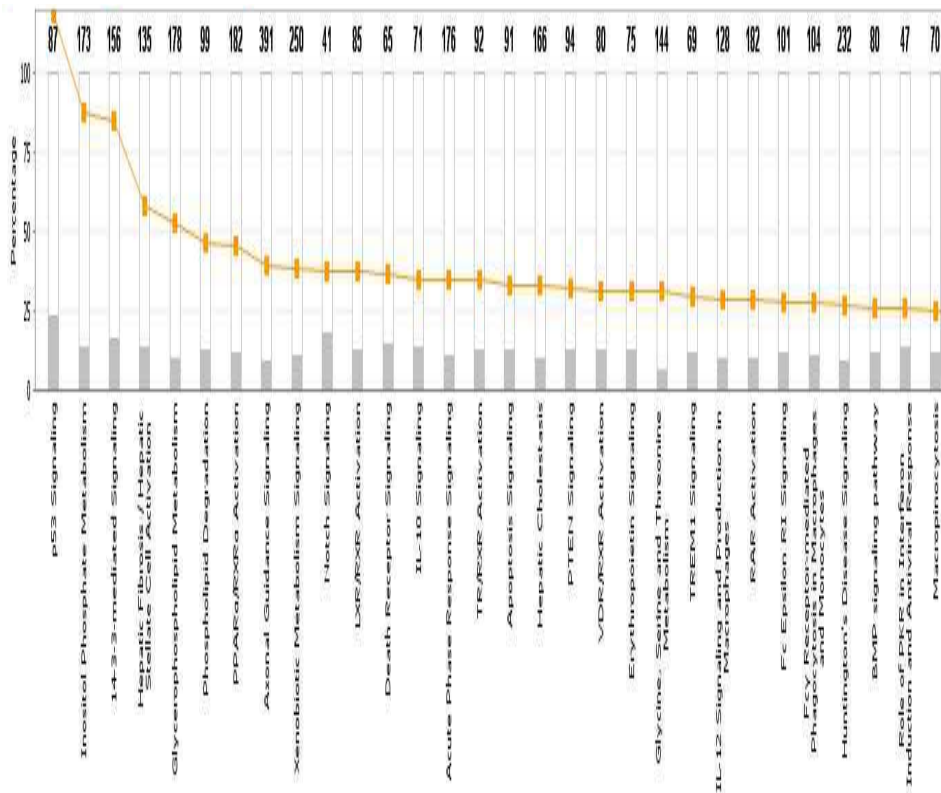


Comparative Ingenuity Pathway Analysis

➤ Different dosing concentrations yield different gene expression profiles

Top Six Canonical Pathways @ 1.0×10^{-6} M

Top Six Canonical Pathways @ 5.0×10^{-7} M





Implications for Risk Assessment?

- Risk assessors set human exposure limits for toxicants and non-genotoxic carcinogens using the NOAEL (no observable adverse effect level, or threshold) or the lowest observed effect level (LOEL)
- NOTEL/ NODAL could be used in place of NOAEL/ LOEL in point of departure (POD) modeling
- Incorporating NOTEL and/or NODAL values into the dose response curves for genotoxic compounds and carcinogens may further inform risk assessment by :
 - Providing threshold values that are based on mechanistically linked experimental data
 - Facilitating the selection of the human dose that is associated with to an acceptable increase in risk



Conclusions

- Integration of LC-MS/MS and DNA microarrays provide a sensitive means for detecting low-level DNA damage
- Complete DNA digestion can significantly lower the limit of detection for DNA adducts
- Gene expression is dramatically impacted by small changes in dosing concentration
- NOTEL and NODAL are closely correlated in this system
- The NODAL level may be an order of magnitude lower than the NOTEL which may have implications for determining exposure thresholds
- Large-scale gene expression screening may provide insights into the complicated biochemical mechanisms of carcinogen exposure



Acknowledgements

This study was supported by Public Health Services grant numbers:

\$\$ RO1 CA112231 (Paul Vouros, P.I., H. Zarbl, Co-P.I.)

\$\$ U19 ES011387 (H. Zarbl P.I.)

\$\$ P30 ES005022 (H. Zarbl, P.I.)

We also acknowledge the NCI Chemical Repository for providing pure N-OH-PhIP for this study.