Biomarkers of Human Exposure to Air Pollutants in Louisville

Environmental Directives and Biomarker Applications

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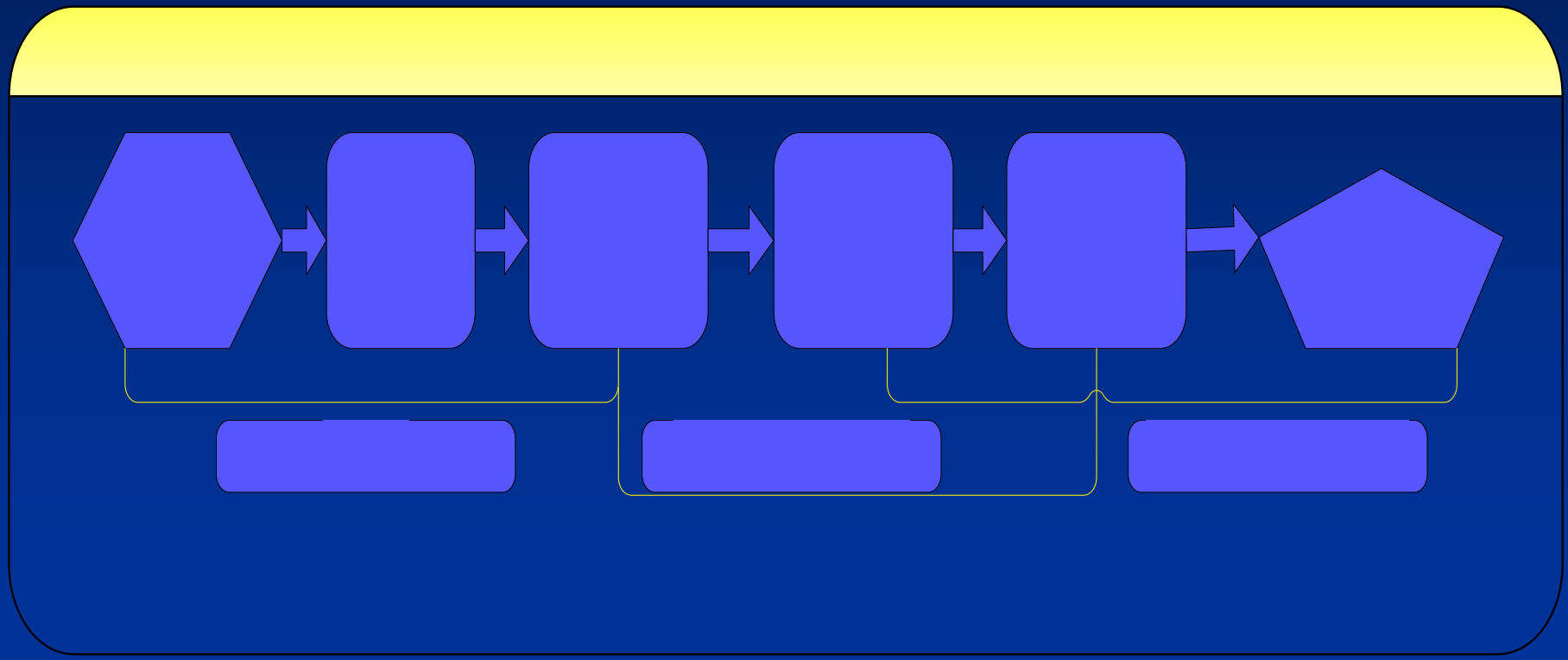
Biomarker Definition:

- Measurable internal indicator of change at molecular or cellular level to detect key event(s) linking specific exposure to health outcome

Bennett and Waters, Environ. Health Perspectives 108: 907-910 (2000)

Biomarkers in Environmentally-Induced Disease

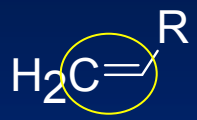
Biomarkers help define environmental exposure-disease relationships.



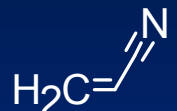
Exposure Assessment:

- Airborne chemical concentrations
  - Compendium Method TO-15 Determination Of Volatile Organic Compounds (VOCs) In Air Collected In Specially-Prepared Canisters And Analyzed By Gas Chromatography/Mass Spectrometry (GC/MS)
    - http://www.epa.gov/ttn/amtic/files/ambient/airtox/to-15r.pdf
  - Pro:
    - Methodology is well developed and sensitive
    - Can be used in regulatory action against pollution
  - Con:
    - Not internal biomarker, no measurement of internal dose
    - Non-integrating assessment (does not reflect cumulative effect)
    - No indication of metabolic activation or mechanism of action

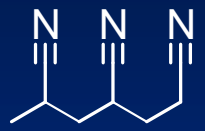
Vinyl Monomers Used in Polymer Production Are Present in Louisville Air

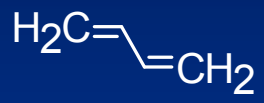


Vinyl group

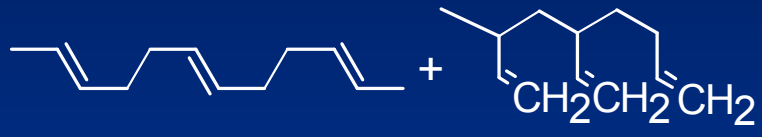


Acrylonitrile

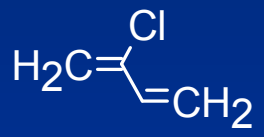




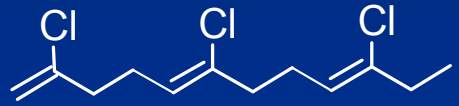
1,3-Butadiene

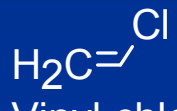


Homopolymers are shown. Heteropolymers also are extensively utilized.

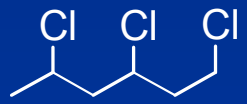


Chloroprene





Vinyl chloride



NNE View from Rubbertown
Kramers Lane & Campground Road

West Louisville Air Toxics Studies

- Monitoring of monomer and chemical concentrations in Louisville air has been conducted by the UofL Air Quality Lab since 1999.
  - Air concentration data available via web site:
    - http://www.kppc.org/EJP2/Air_Quality/Database/
  - West Louisville Air Toxics Study 1
    - April 2000 - April 2001
    - Risk assessment 2003 by Sciences International for the Louisville Metro Air Pollution Control District http://www.louisvilleky.gov/APCD/STAR/WLATS.htm
  - West Louisville Air Toxics Study 2
    - November 2001 – 2005
    - Risk assessment 2006 http://www.louisvilleky.gov/APCD/STAR/

Louisville Metro Area

Rubbertown and WLATS2 Air Monitoring Sites

Rubbertown Area

E->

Air Sampling Sites

(A) Firearms Training – 4210 Algonquin Pkwy.

Impact site for 3 polymer plants, 5 petrochemical facilities

(C) Ralph Ave./Campground Rd. – 4211 Campground Rd.

(2 monitors) - Impact site for 4 polymer facilities

(E) UofL Shelby Campus – 9001 Shelbyville Rd.

Urban control site to measure impact of urban activities

(F) Cane Run Elementary – 3951 Cane Run Rd.

Community exposure site for Hallmark and Algonquin neighborhoods

(I) Chickasaw Park –942 S. 47th St.

Community exposure site for Chickasaw neighborhood

(M) Farnsley Middle School – 3400 Lees Lane

Communities: Cane Run, Riverside Gardens, and Shively

Canister Air Sampler

Sample Station & Canister

- "TO-15 cans"
  - "summa canisters"
  - "silco- steel"
  - SS with fused silica liner & special SS valve
- 6 liter capacity
- *Very clean* – baked under vacuum

Air Quality Lab Tasks in Analysis of Volatile Organic Chemicals (VOC)

Mr. John Metaxas, Coordinator
Air Quality Lab

- Collect ambient air field samples
  - 6 Sites
  - Every 12 days
- Analyze for 78 VOC by gas chromatography/mass spectrometry (GC/MS)

  EPA Method TO -15
  MQL values ~ 0.1 ppbv
- Analyze, QC, store data, supply to web site/community/industry
- Clean/evacuate canisters for additional sampling

Sample Injection Process

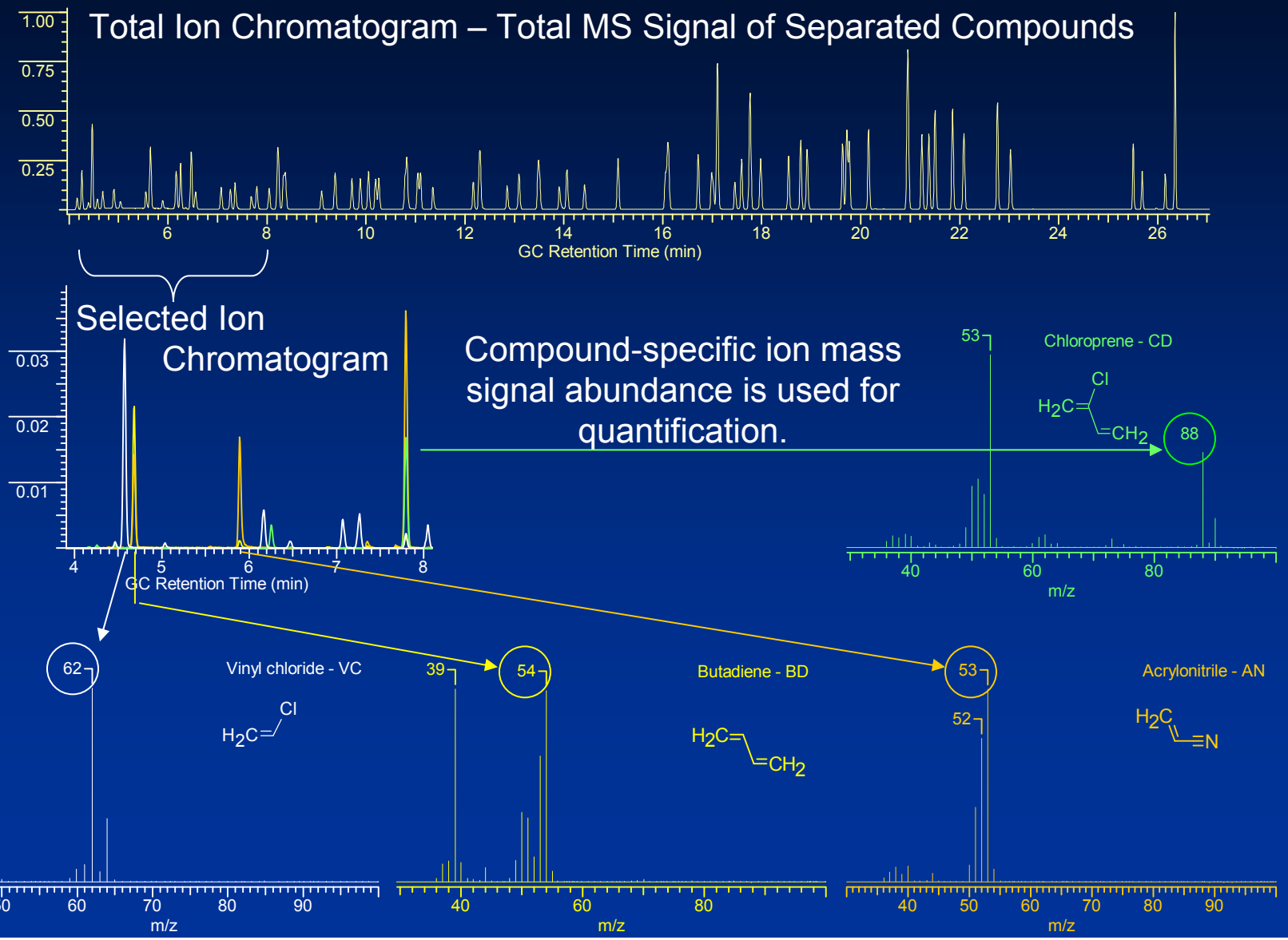
Automated Canister Sampling

- The Entech 7100 concentrator meters pump-out of each can into:
  - Module 1: cryo-traps VOCs, extracts H_20, N_2, O_2, CH_4 ->
  - Module 2: cryo-traps VOCs, extracts CO_2 ->
  - Module 3: -180˚C cryo-focuses VOCs for injection to gas chromatograph (GC)
- Injects concentrated sample into GC/MS for GC separation, mass spectral (MS) detection, and quantification.

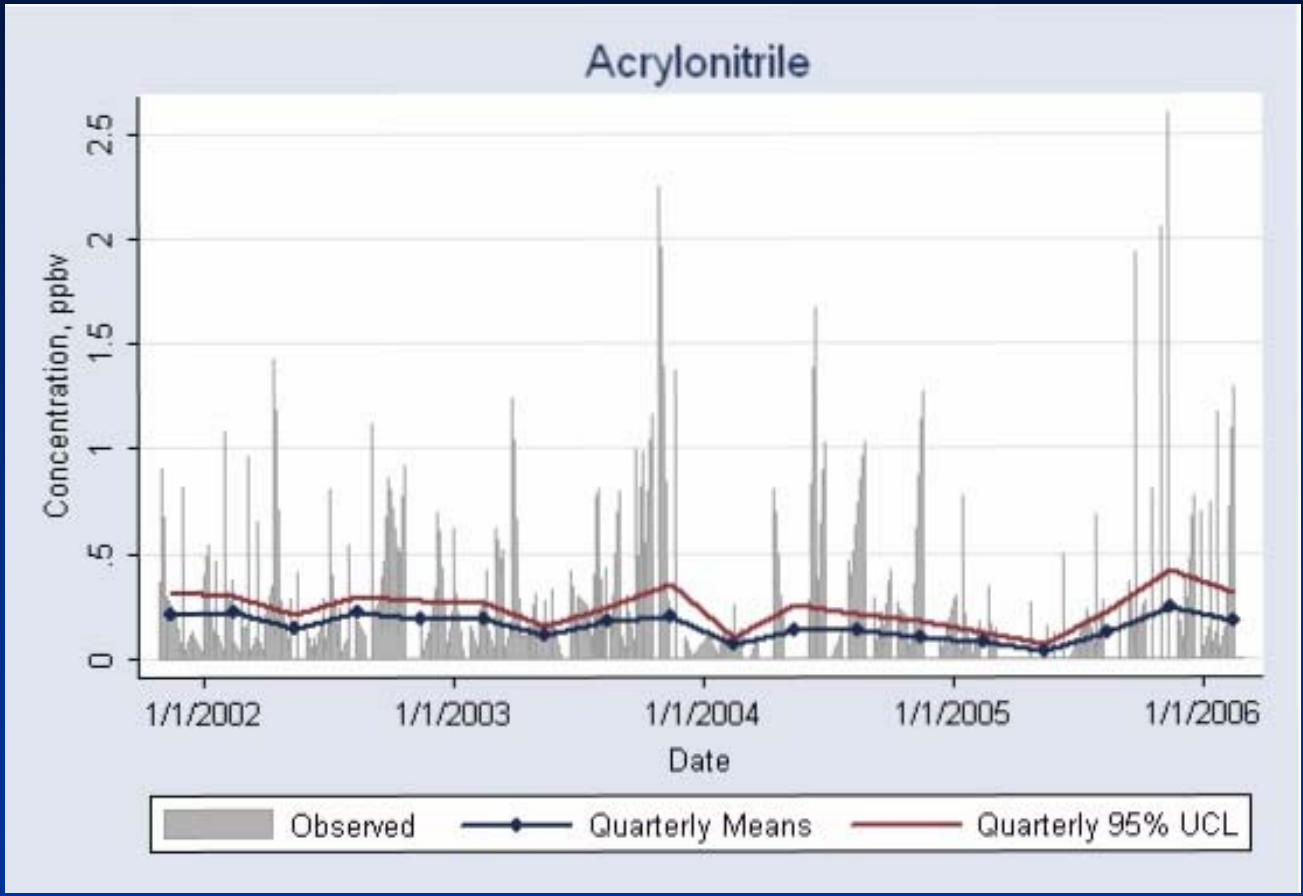
Overview of GC/MS Analysis

- Gas chromatography – Mixture separation
  - Separates organic chemicals by sequential He elution in a heated fused silica capillary column (60 m x 0.32 mm)
  - Mixture is injected into the column, and separated compounds elute with characteristic column retention time
- Mass spectrometry – Molecular fragmentation and mass analysis
  - Analysis of abundance vs. mass of ions (+ charged particles) from electron fragmentation of each compound's molecular structure
- GC/MS Analysis
  - Detection of each compound at a known retention time using compound-specific ions gives sensitive, selective means for quantification of volatile air pollutants.

GC/MS: Separation, Ion Atomic Mass Analysis



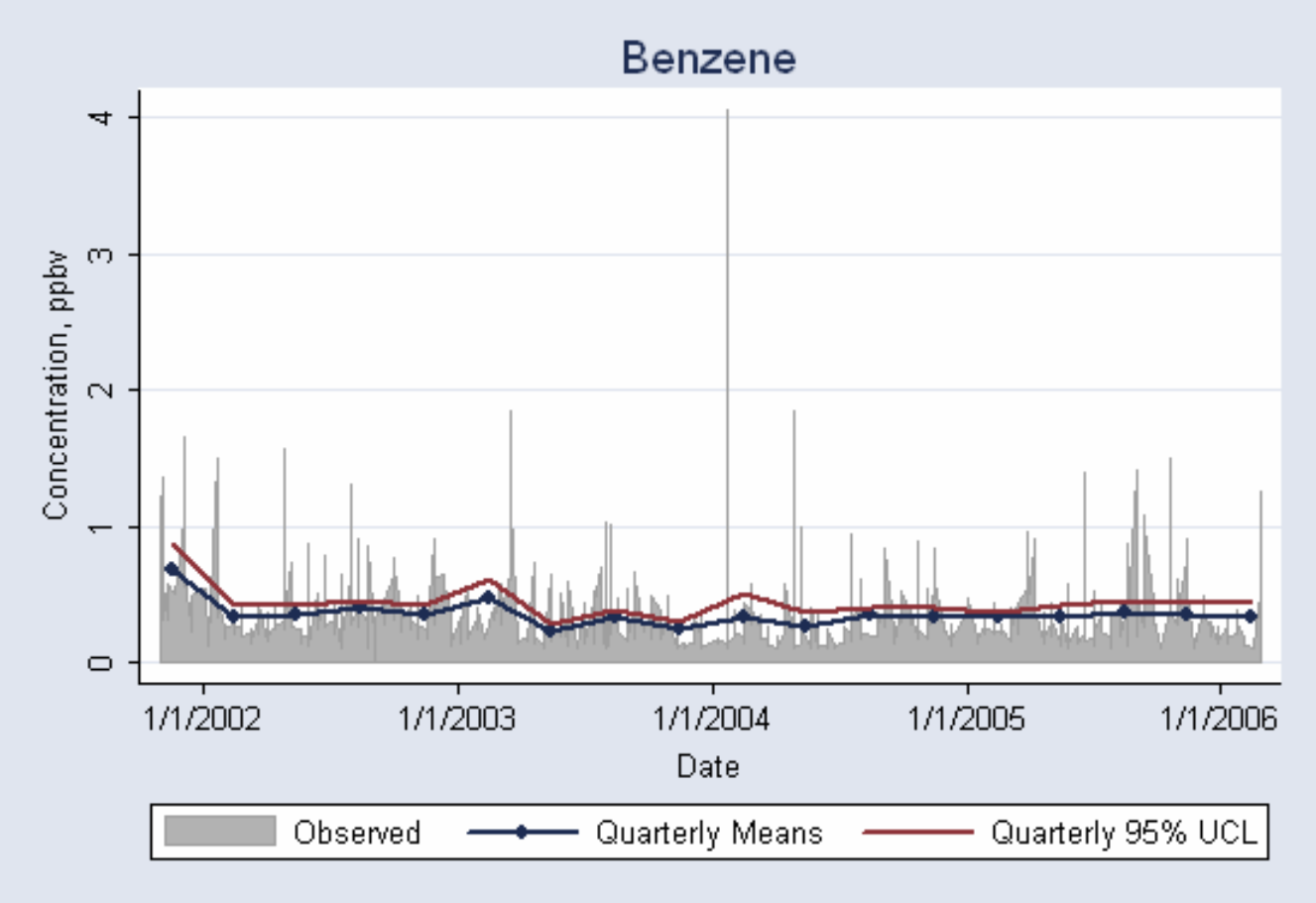
Acrylonitrile Concentrations
(all west Louisville sites)



No statistically significant change

10^{-6} risk level =.007 ppbv

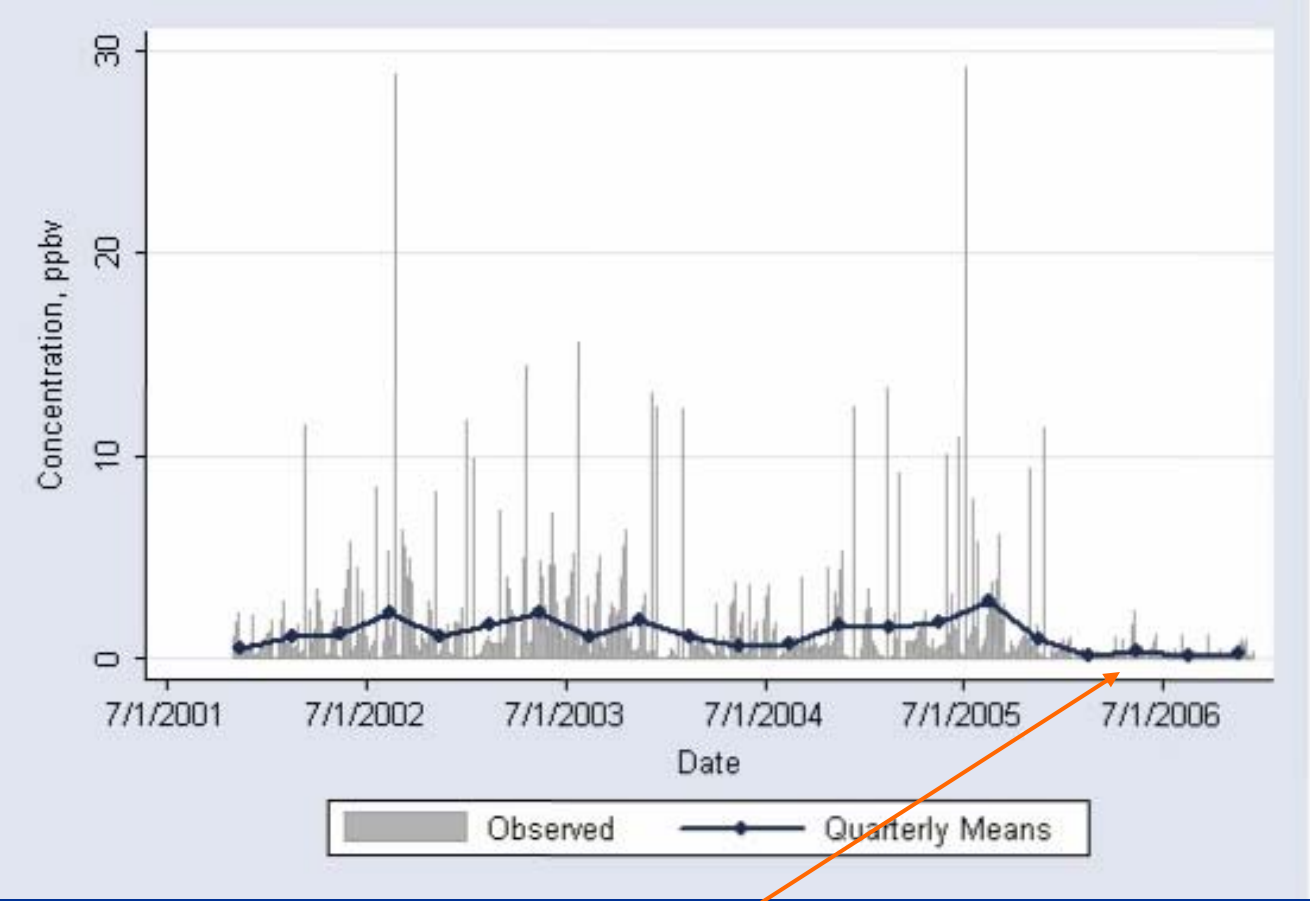
Benzene Concentrations
(all west Louisville sites)



No statistically significant change

10^{-6} risk level =.04 ppbv

1,3-Butadiene Concentrations
(all west Louisville sites)



85% drop in 2006 from 2001-2005

10^{-6} risk level = 0.014 ppbv

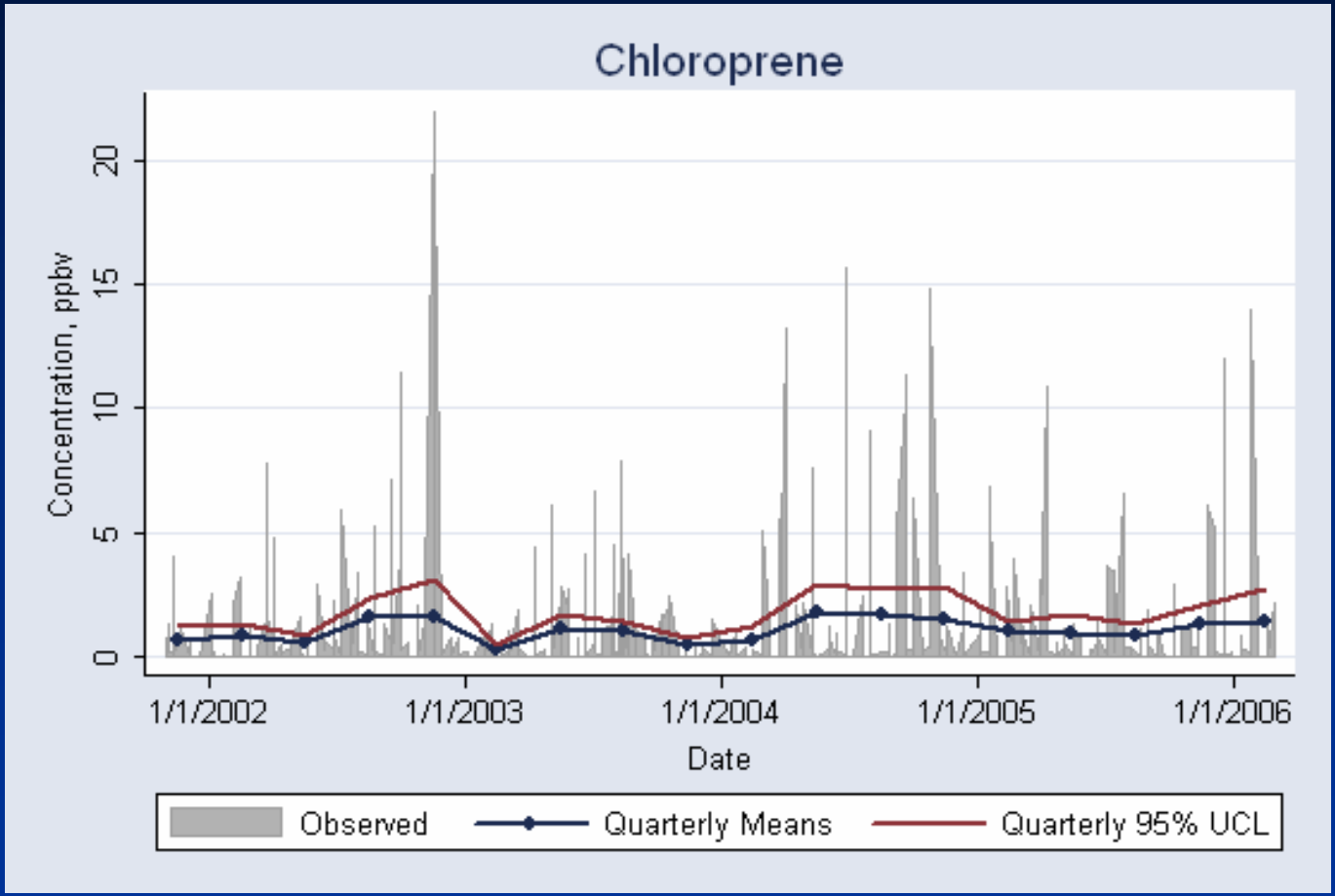
Analysis: Single Plant Impact on 1,3-Butadiene Air Concentrations

- American Synthetic Rubber - (ASR, Michelin Tire Co.)
  - Major BD user for production of butadiene-styrene rubber for tires.
    - Pre-2005 ASR used flare to burn BD emissions (~98% efficient)
    - In 2003 Reported 83% of Jefferson Co. butadiene releases (118,448 lbs)
      - US EPA Toxics Release Inventory (http://www.epa.gov/triexplorer/)
  - Participated in BD air study: plant operation vs. shutdown (Below)
    - BD in air was compared during operation and shutdown (Barnett, 2004)

	Ralph Ave. Site (C) (ppb)			Cane Run Elem. Site (F) (ppb)			Fire Arms Train. Site (A) (ppb)		
	Op.	Shut	Dec.	Op.	Shut	Dec.	Op.	Shut	Dec.
Mean	2.51	0.97	61%	0.72	.23	68%	1.11	.47	58%
Median	0.78	0.76	3%	0.32	0.17	47%	0.47	.093	17%
95% UCL	3.43	1.88	45%	0.98	.044	55%	1.50	0.77	49%

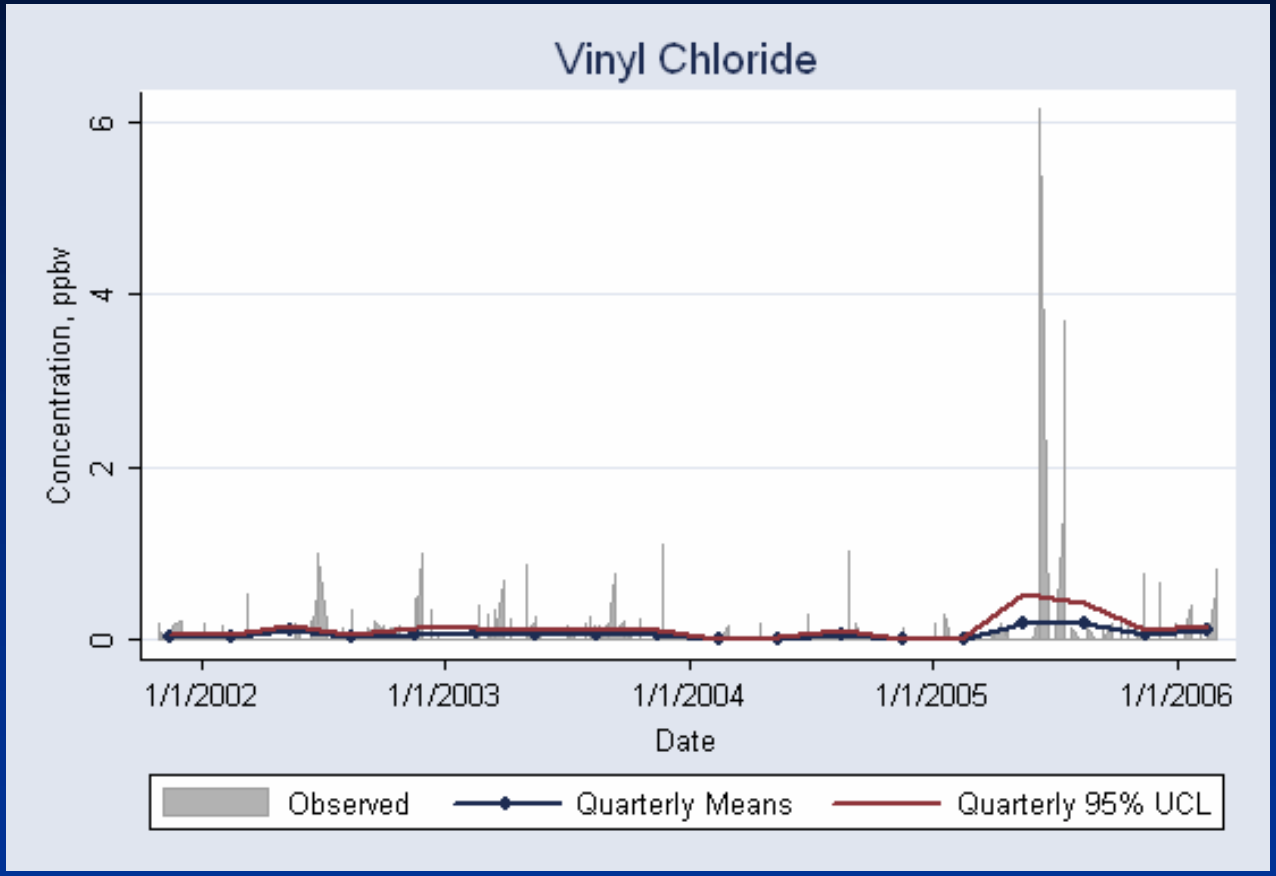
  - Late 2005 ASR installed thermal oxidizer technology (99.99+% efficiency)
    - 62% decrease in BD TRI point source emissions
    - 92% decrease in BD TRI fugitive emissions

Chloroprene Concentrations
(all west Louisville sites)



No statistically significant change

Vinyl Chloride Concentrations
(all west Louisville sites)



Statistical increase over observation period + 29%; 10^{-6} risk level = 0.09 ppbv

Air Monitoring Studies: Summary of Benefits

- Air monitoring using established methodology gives specific, reproducible estimates of VOC concentrations.
- Air monitoring studies provide:
  - Objective data for risk assessment, planning, and decision making
  - Indication of trends in emissions
  - Record of episodic releases due to malfunctions
  - Understanding of wide dispersion of airborne pollutants

Biomarkers of Internal Dose:

Can biomarkers assess internal exposure or examine mechanisms of vinyl monomer toxicity?

Vinyl Monomer Toxicity

- Significance: vinyl monomers are not toxic per se.
- They are converted to reactive epoxides in cells by membrane-bound Cytochrome P450 enzymes.
  - Example: oxidation of 1,3-butadiene:

1,3-Butadiene BD epoxide BD diepoxide

    - CYP2E1 has selectivity for small molecules such as vinyl monomers.
- Vinyl epoxide metabolites are reactive electrophiles.
  - Can react with key cell structures (e.g. DNA, proteins).
  - Are detoxified by cellular antioxidants (glutathione) and enzymes (glutathione S-transferase & epoxide hydrolase).

Genotoxic Events Leading to Vinyl Chloride Carcinogenesis

1. Oxidation of VC by CYP2E1 to chloroethylene oxide (VCO, vinyl chloride epoxide)
2. VCO reaction with DNA to form cyclic etheno-DNA adduct(s)
3. Lack of repair of DNA adducts in key genes
4. Miscoding transmutation of DNA sequences (G $\rightarrow$ A,
5. A $\rightarrow$ T transitions)
6. Mutations of key genes in susceptible cells:
    a. Proto-oncogenes (*ras*-oncogenes) – turn on cell replication
    b. Tumor suppressor genes (*p*53 gene) – stop cell cycle replication, enable apoptosis (programmed cell death)
7. Clonal expansion of malignant cells

Biomarkers of Tissue Dose and Mechanism of Action

- Surrogate tissue sampling and analysis
  - DNA adducts for genotoxic compounds
    - Tissue and cell type must be selected
    - DNA repair can confound
  - Persistent protein adducts can give integrated measure of exposure over time
    - Albumen adducts (days to weeks)
    - Hemoglobin adducts (weeks to months)

Hemoglobin (Hb)

4 Protein Chains:

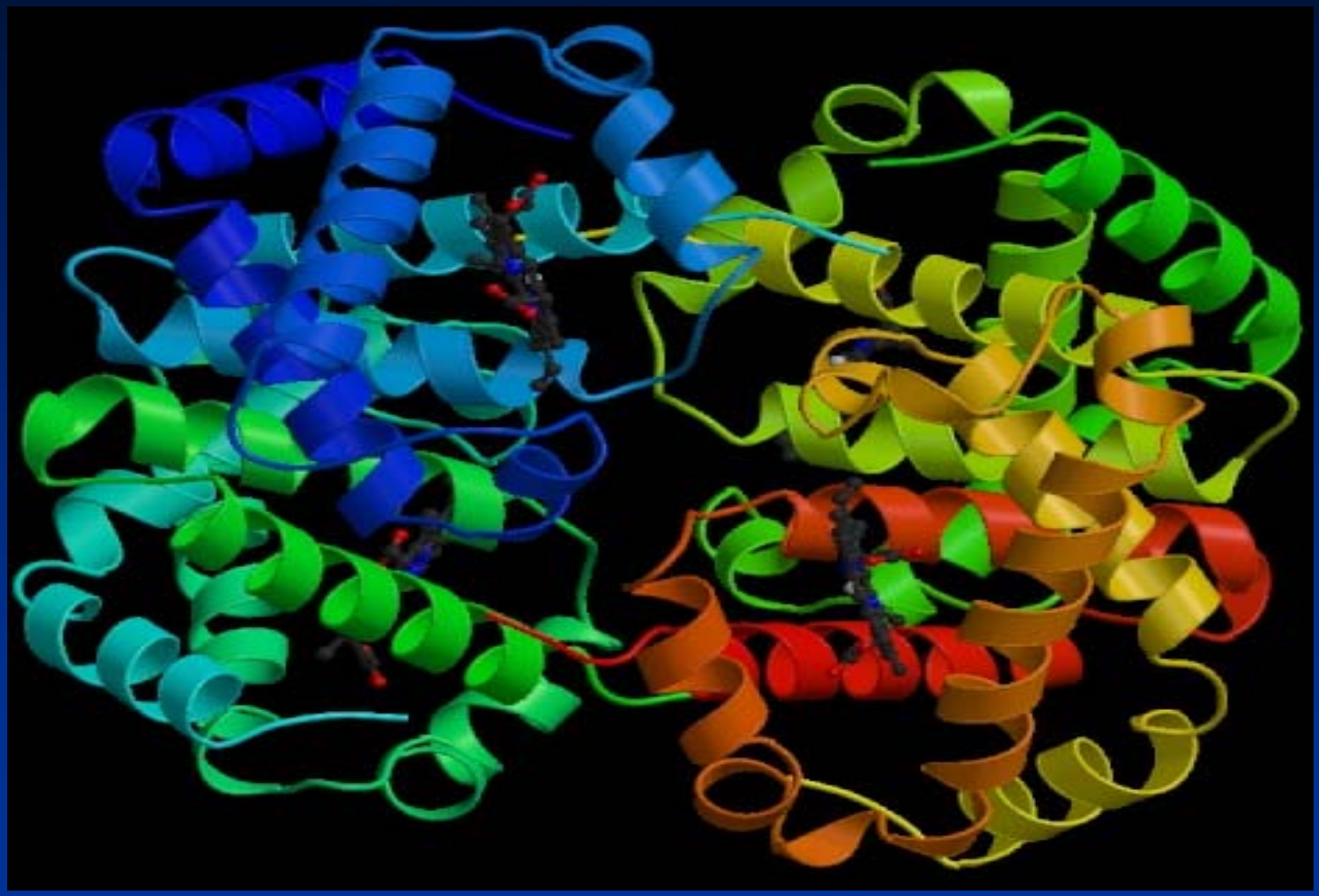
2 α Chains
141 AA

MW=15100 daltons

2 β Chains
146 AA

MW=15851 daltons

N-terminal amino acids are valine

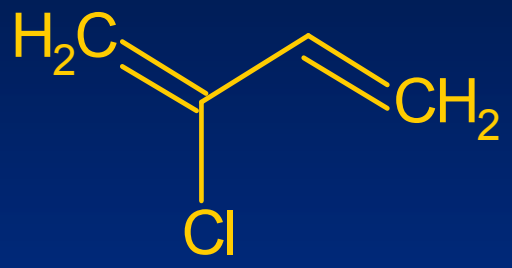


Hemoglobin Adducts as Biomarkers Hb:

- Abundant accessible protein for biomarker
- Monitors recent past exposure over red blood cell lifetime:
  - 120 days in human, 60 days in rat, 40 days in mouse
- Exposure biomarker related to mechanism
  - Most cancer-causing chemicals are reactive electrophiles.
- Traps electrophilic chemicals and reactive metabolites at nucleophilic sites:
  - N-terminal valine on α and β chains, cysteine, other sites
- Surrogate monitor for mutagenic DNA adducts

Chloroprene

- CAS 126-99-8
- M.W. = 88.54, B.P. = 59.4ºC
- Monomer for production of:
  - Polychloroprene, Neoprene
- Multi-organ rodent carcinogen
  - (Melnick et al, 1999)
- *"Reasonably anticipated to be a human carcinogen"*
  - (NTP, 1998)
- Present in Jefferson Co. KY air at ppb conc. from fugitive emissions
  - http://www.kppc.org/EJP2/Air_Quality/Database/



2-chlorobuta-1,3-diene

Chloroprene Bioactivation

2-chlorobuta-1,3-diene

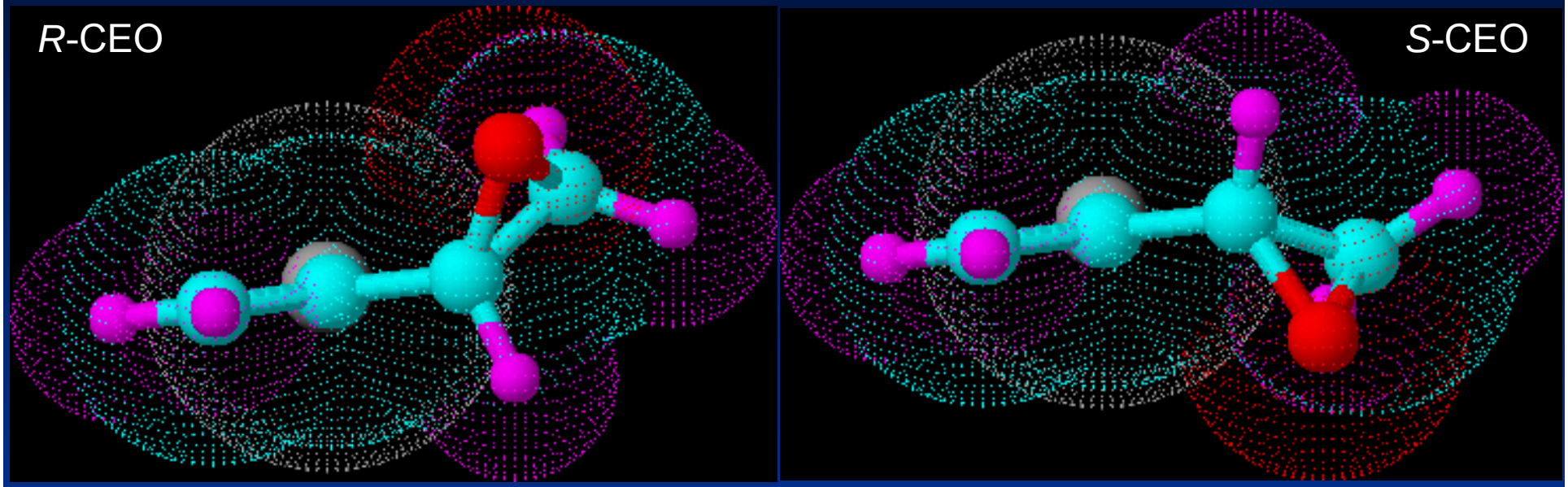
Chloroprene

(1-chloroethenyl)oxirane

CEO

- Cytochrome P450 oxidative enzymes (CYP2E1) metabolize chloroprene to a reactive electrophilic epoxide, (1-chloroethenyl)oxirane (CEO).
- CEO exists as a pair of enantiomers, due to the asymmetric 2-carbon of the oxirane ring.

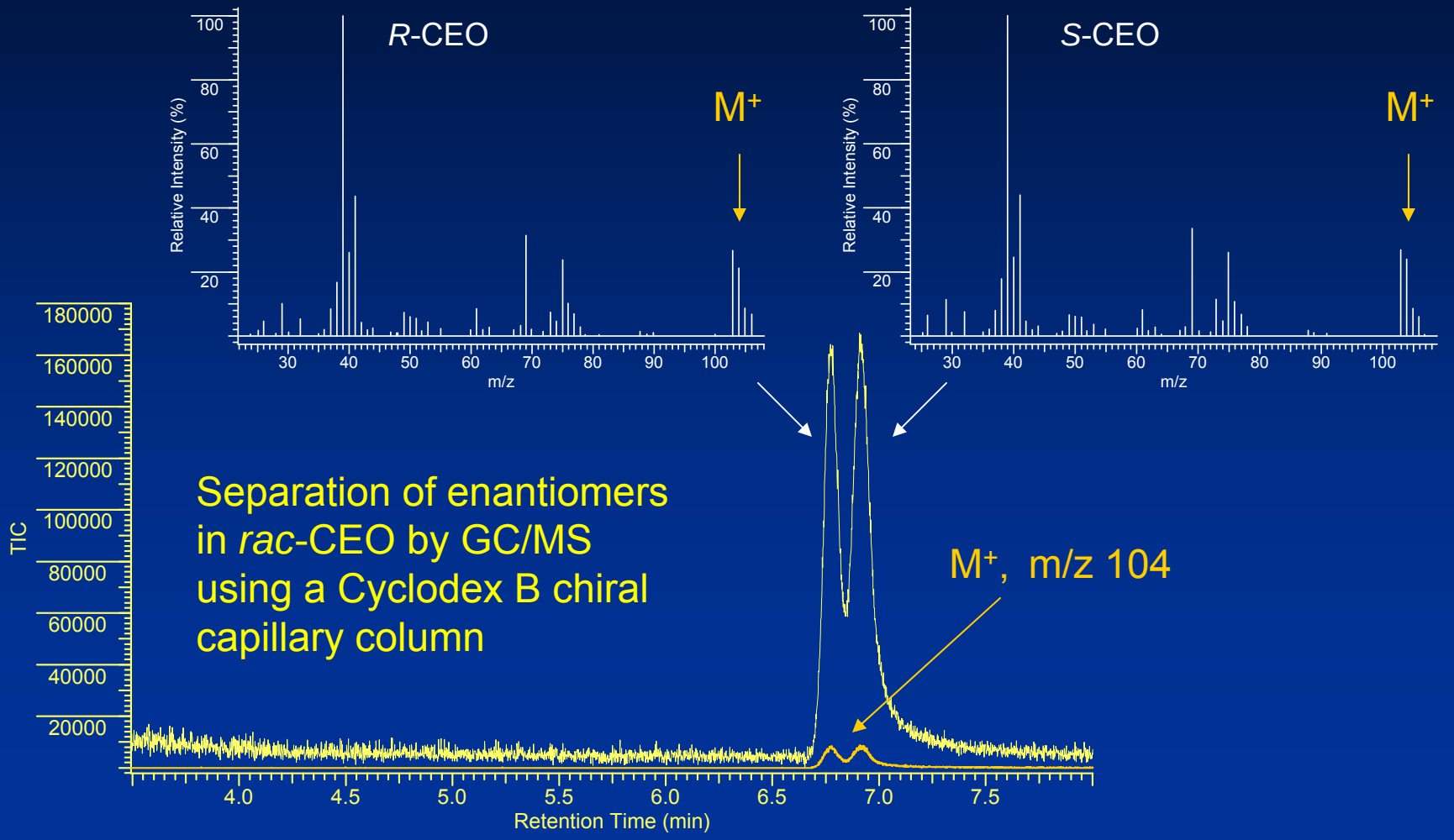
3-D Configuration of (1-chloroethenyl)oxirane



R- and *S*-CEO are enantiomers, non-superimposable mirror image isomers. *R, S* isomers are optically active and rotate plane-polarized light in opposite directions.

In a racemic mixture both are present in equal proportion.

Mass Spectra of CEO Enantiomers

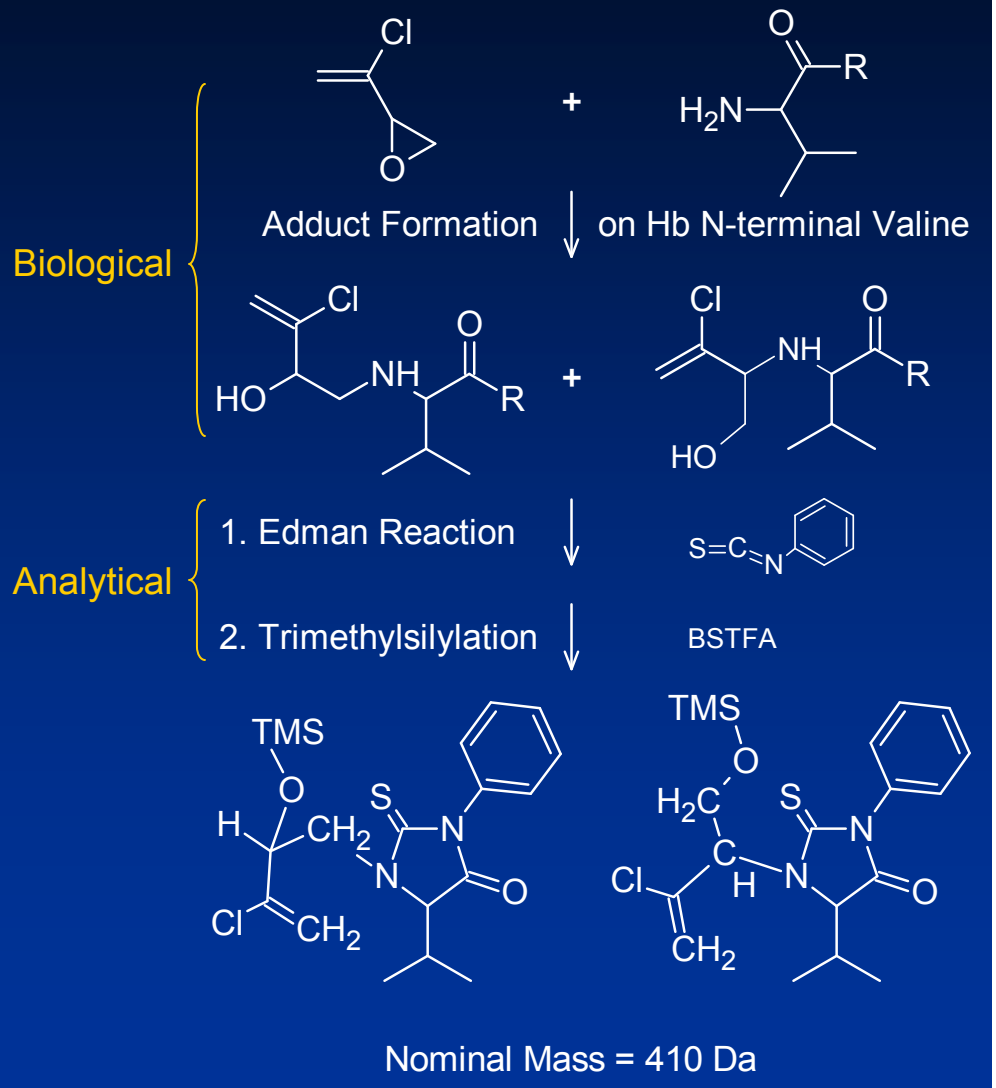


Chloroprene Biomarker Strategies

- Use Hb N-terminal valine adducts as biomarkers of CEO electrophilic reactions
  - Hurst, H.E. and Ali, M.Y. Analyses of (1-chloroethenyl)oxirane headspace and hemoglobin *N*-valine adducts in erythrocytes indicate selective detoxification of (1-chloroethenyl)oxirane enantiomers. Chem.-Biol. Interact. **166:** 332-340, 2007.
- Analytical approach:
  - Synthesize and purify standards: CEO-adducts to Valine (CEO-Val), Valine-Tyrosine-Valine (CEO-VYV), and CEO-$^{13}C_5$ –Val.
  - Isolate globin from blood exposed to CEO
  - Use: Edman reaction for Hb N-terminal valine cleavage &
    TMS derivatization for GC separation
  - Use mass spectrometry for detection, measurement

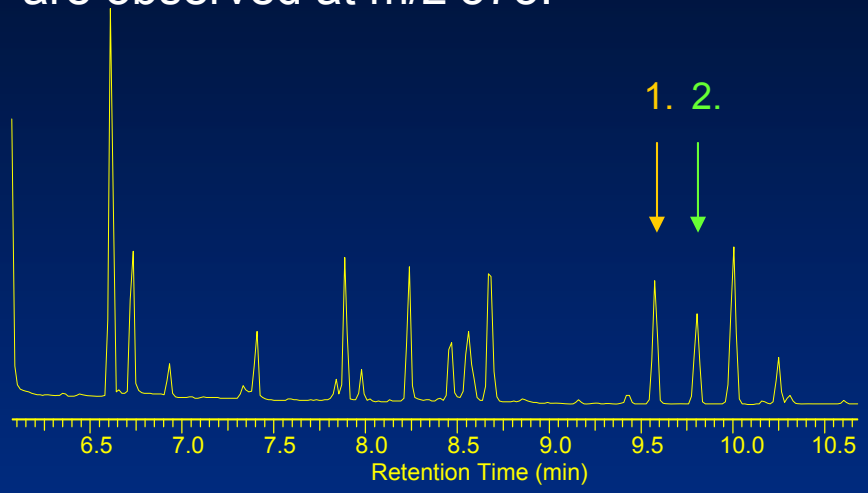
Biomarker Reactions

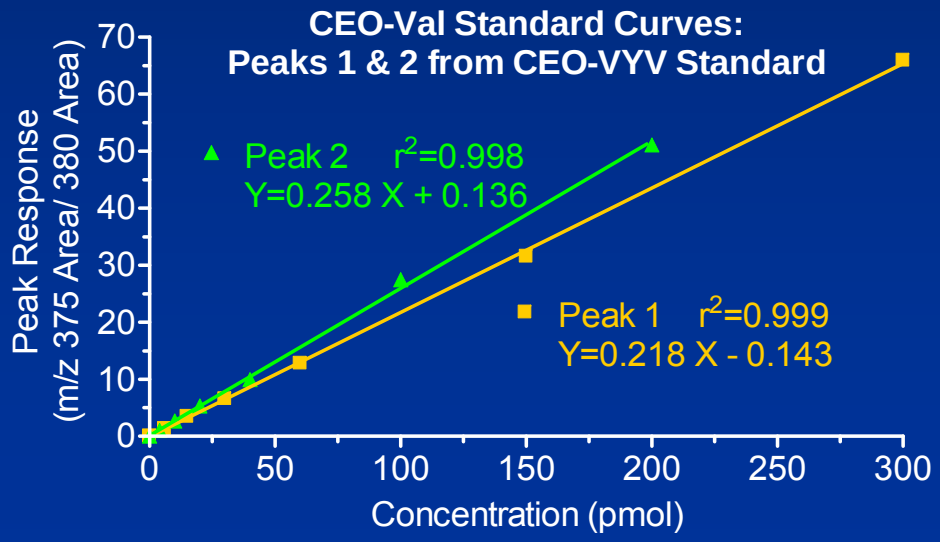
1. Edman degradation
   - Cleaves N-terminal adduct-valine
   - Produces stable cyclic derivative
2. Trimethylsilylation of hydroxyl group for volatility
3. Detect and measure by GC/MS using selected ion monitoring (SIM)



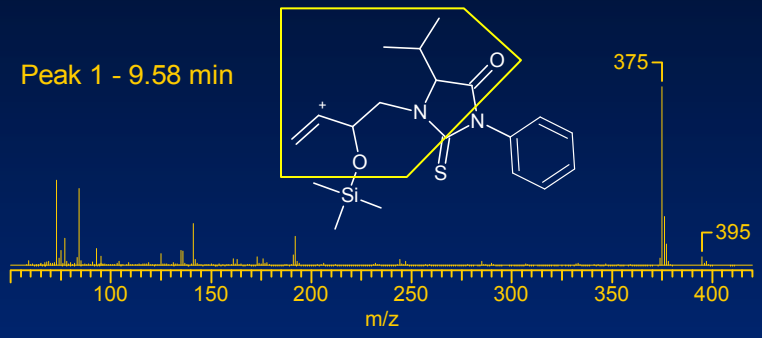
GC/MS of TMS, PITC Derivatives of CEO-Valine Adducts

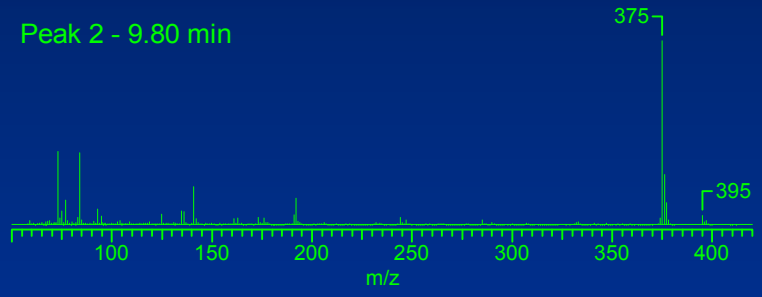
Two adduct-related GC/MS SIM peaks are observed at m/z 375.

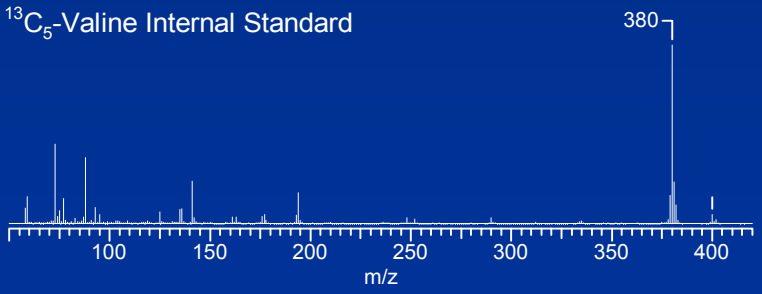




Mass spectra of derivatives



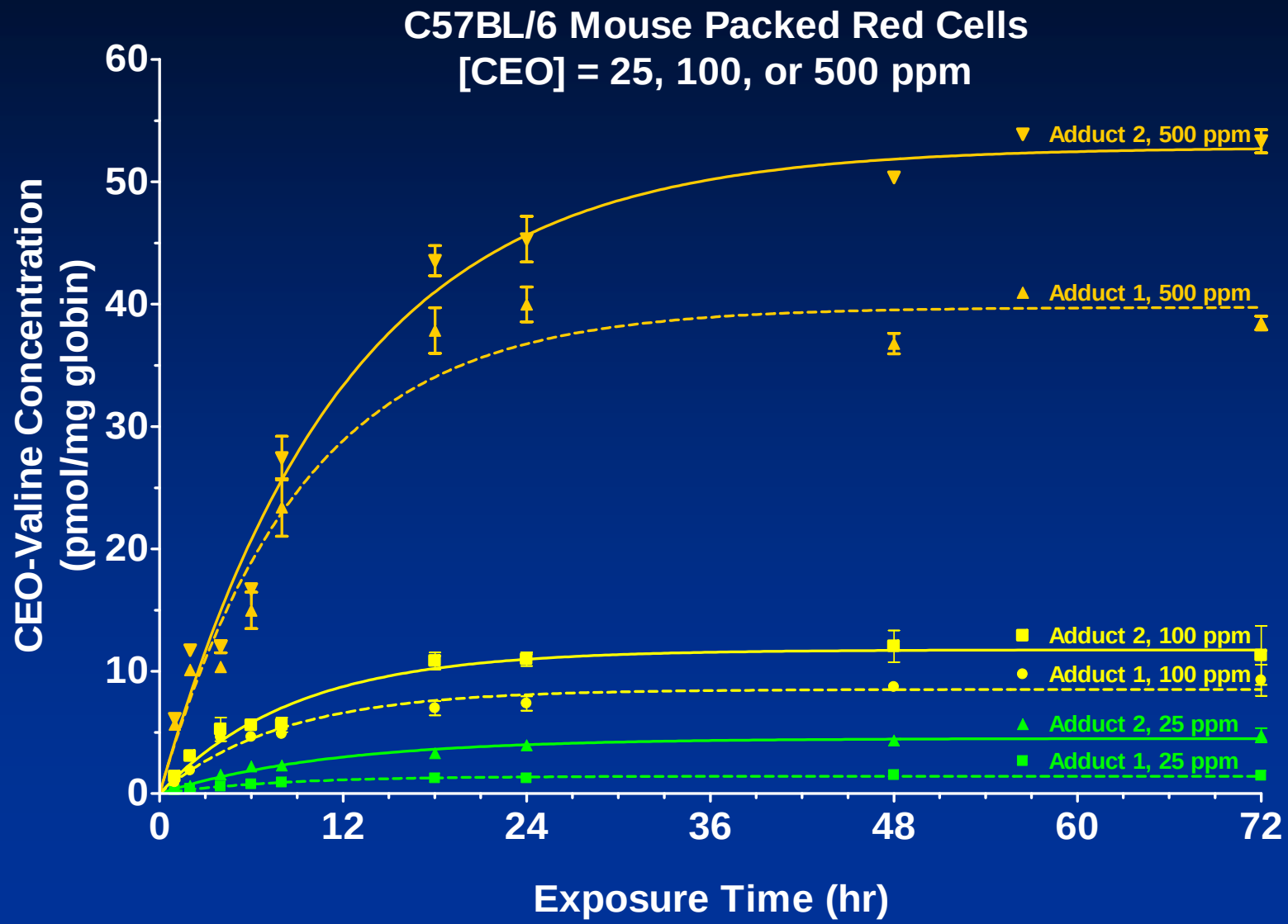




Hb Adducts from Mouse Globin CEO Exposure In Vitro

- Erythrocytes from C57Bl/6 mice
  - 125 µL packed red cells diluted to 2.0 mL in phosphate-saline at pH 7.4
    - [Hb] = 0.5 mmolar; (1.1 µmoles or 71 mg / vial)
    - Gentle shaking at 37ºC
- CEO
  - Introduced as vapor into septum vial
    - 500 ppm high level 49 µg (470 nmoles) / vial
    - 100 ppm medium level 9.8 µg (94 nmoles) / vial
    - 25 ppm Low level 2.4 µg (23 nmoles) / vial
  - CEO partitioned into liquid phase of red cell suspension
    - CEO blood / air partition coefficient = 122 [a]
      - [a] Himmelstein (2004) personal communication
- Sampling
  - Hb-CEO vials analyzed at exposure times up to 72 hr
- Analysis
  - GC/MS: + EI SIM m/z 375
  - Kinetics: Nonlinear fit of first order adduct formation data

Kinetics of CEO-Valine Adduct Formation In Vitro



[CEO-Valine Hb] vs. Time
Best Fit: Exponential Association Kinetics

$$[Adduct]_t = [Adduct]_{Max} \cdot (1 - e^{-k \cdot t})$$

PPM	500	500	100	100	25	25
Peak	1	2	1	2	1	2
$[Adduct]_{Max}$ (pmol/mg globin)	40	53	8.5	12	1.4	4.5
k (hr^{-1})	0.11	0.083	0.12	0.11	0.13	0.090
$t_{1/2}$ (hr)	6.4	8.3	5.6	6.1	5.4	7.7
Fit (r^2)	0.948	0.977	0.910	0.900	0.948	0.961

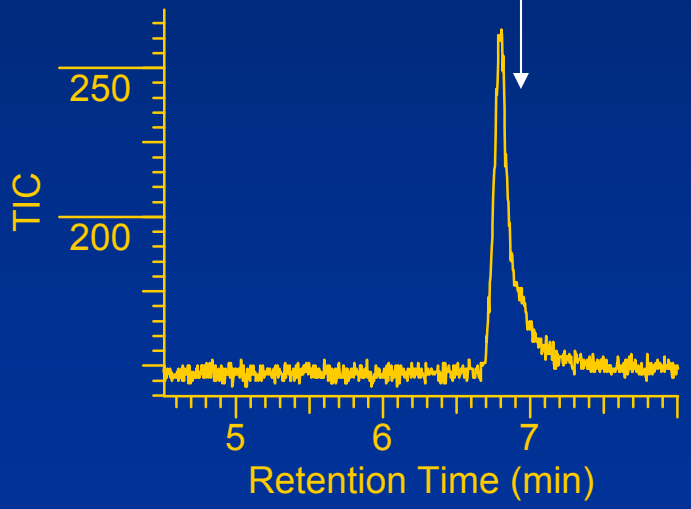
Enantiomeric Selectivity in CEO Detoxication

Erythrocyte incubation biomarker studies provide insight.

Enantiomer Specific Loss of CEO Incubated with Red Cells

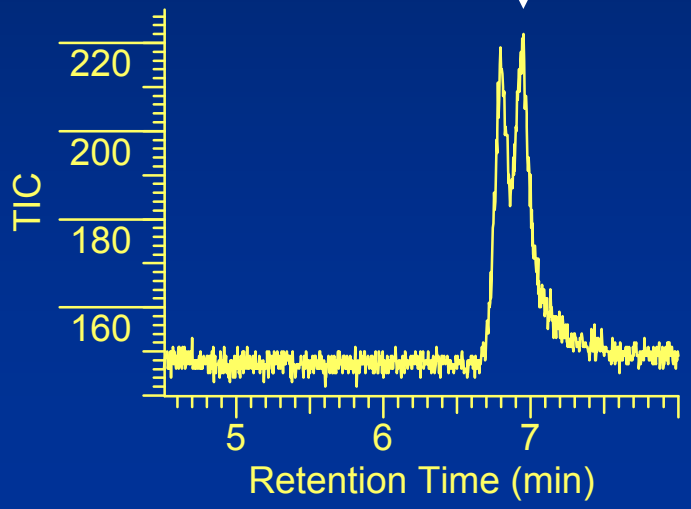
CEO in headspace over fresh RBC in PBS buffer

Peak 2 *S*-enantiomer ?



CEO in headspace over old hemolyzed RBC in PBS buffer

Peak 2 *S*-enantiomer



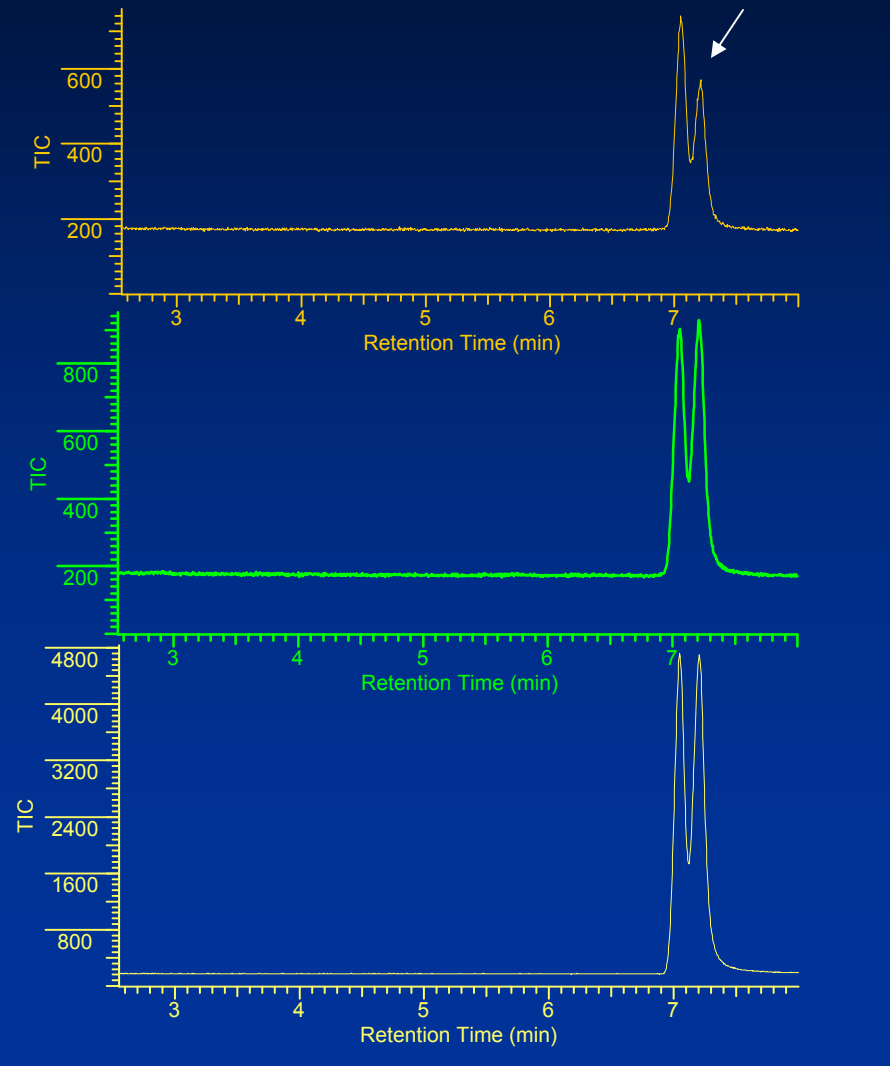
Selective Enantiomer Disappearance Is a Biological Process

CEO Incubation with:

Mouse Red Blood Cells

Phosphate-Saline Buffer

Empty Vial



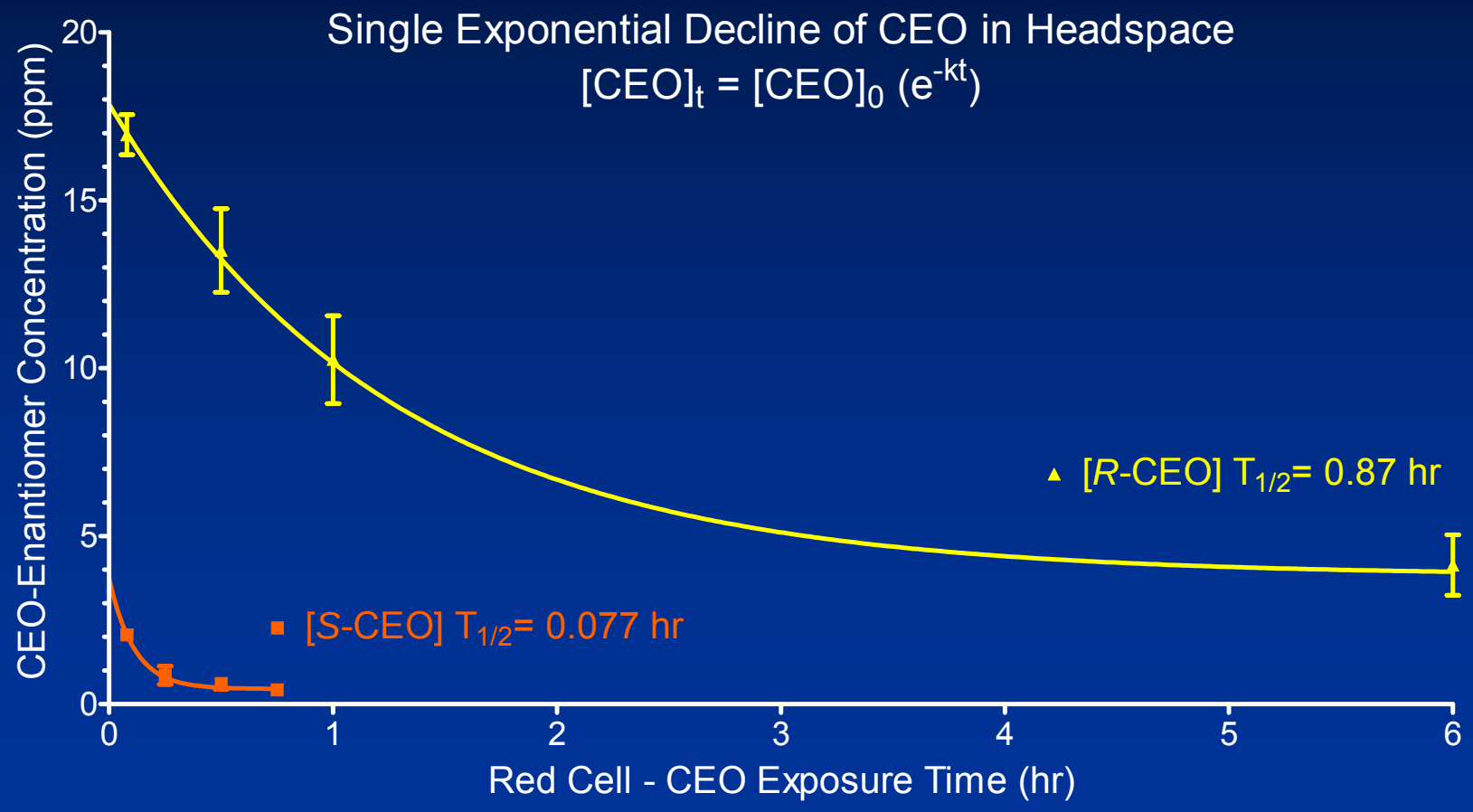
Possibilities for Stereospecific Destruction of CEO in Red Blood Cells?

- Chemical reactivity of CEO enantiomers should be identical.
- Enzymes convey stereo specificity due to differences in fit of *R*-, or *S*-CEO into active site.
- Likely candidates are:
  - Epoxide hydrolase
  - Glutathione *S*-transferase

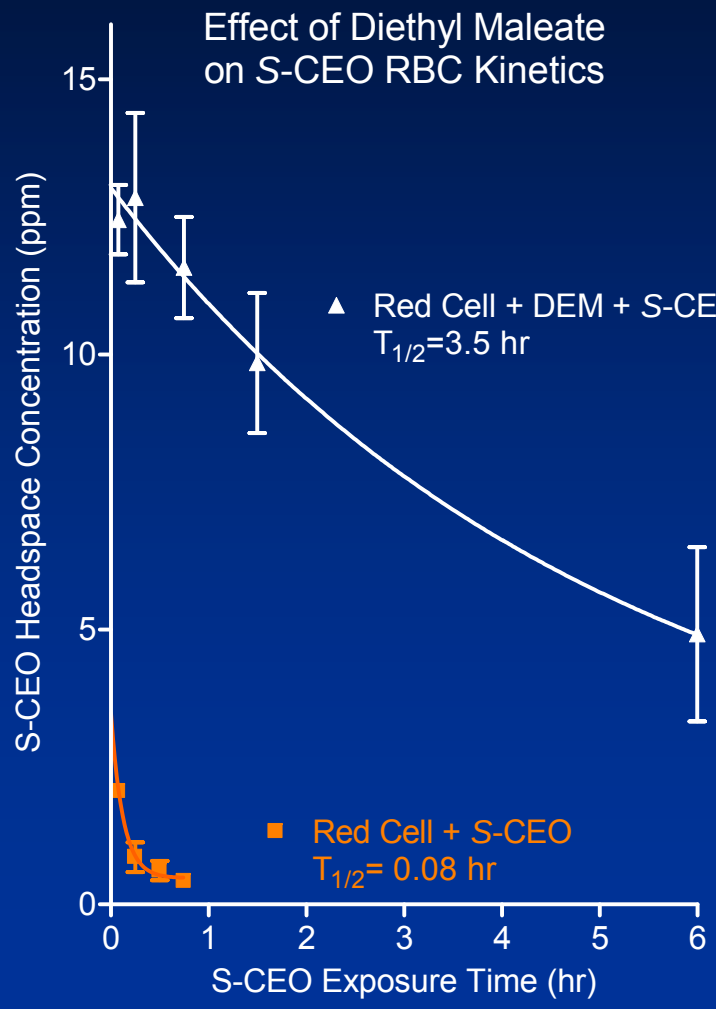
Biomarker Probe of Detoxication Mechanism

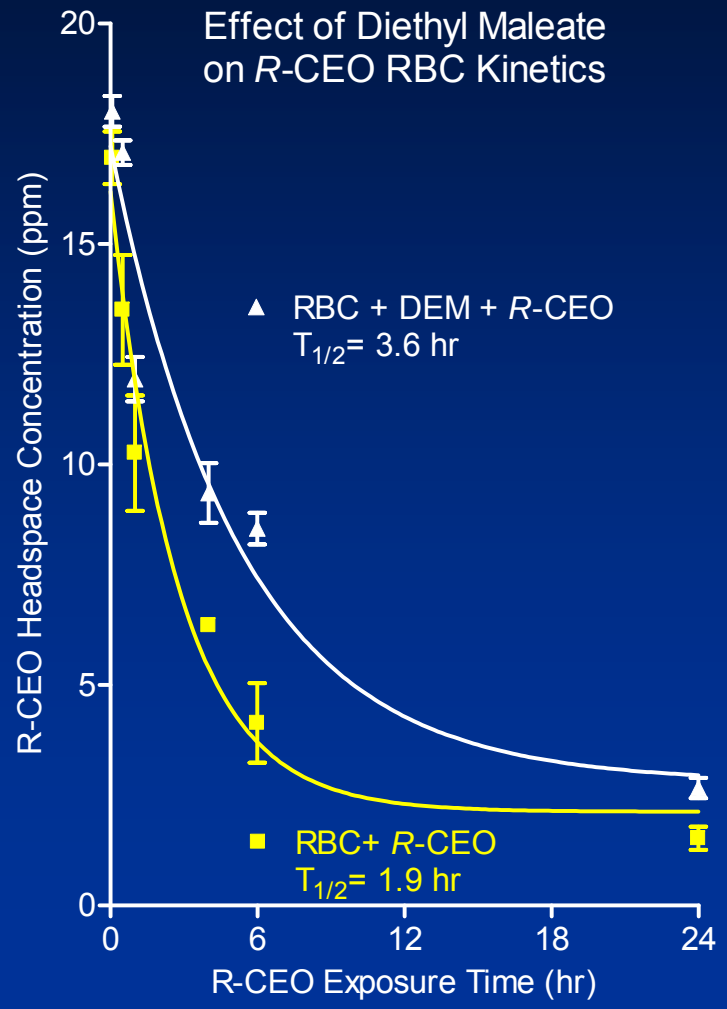
- Hypothesis:
  - GST mediated glutathione (GSH) conjugation preferentially detoxifies Peak 2 CEO enantiomer in fresh cells.
- Examine RBC destruction of CEO
  - Incubate RBC with chemicals that deplete RBC glutathione prior to CEO exposure
- Examine N-terminal valine adduct formation to assess impact

Kinetics of CEO Enantiomers Incubated with Red Blood Cells



RBC Pre-Incubation with Diethyl Maleate Markedly Reduces *S*-CEO Loss Rate In Vitro

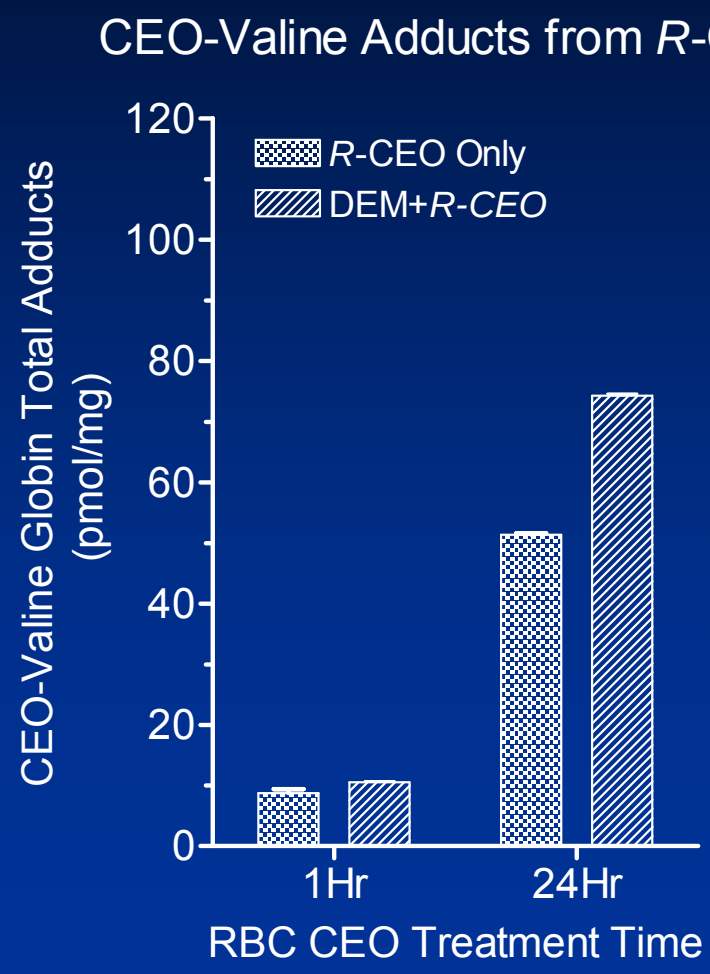


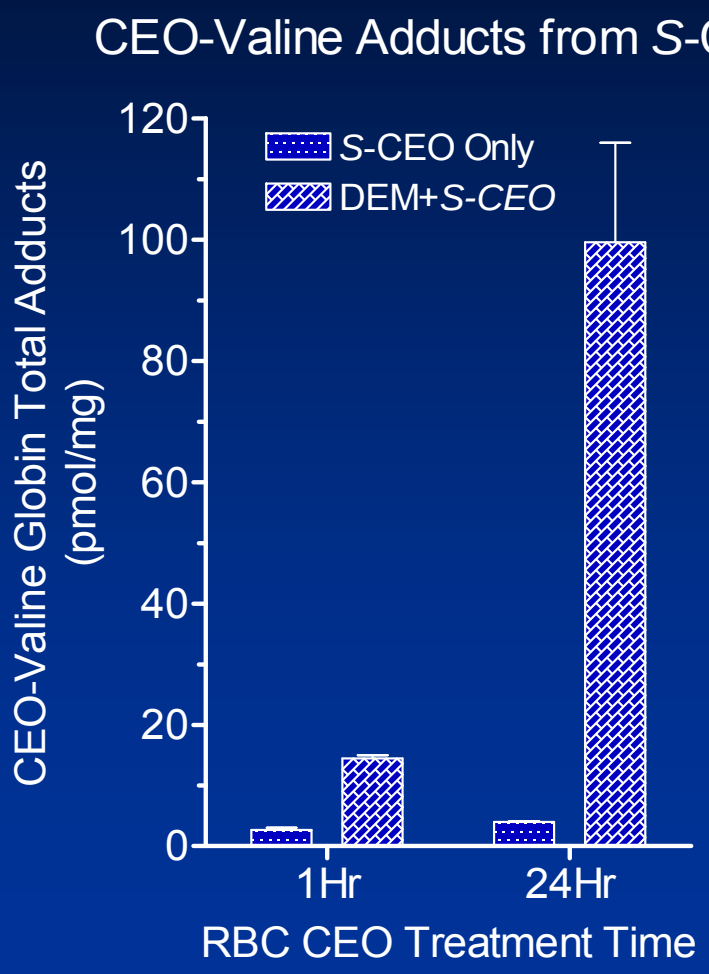


CEO Headspace Study Conclusions

- CEO exists as *R*- and *S*-enantiomers.
  - *R*- and *S*-CEO can be separated using a chiral Cyclodex-B GC column.
  - *R*-CEO elutes before *S*-CEO, allowing separate enantiomer quantification.
  - Mass spectra of the enantiomers are identical.
- With untreated C57BL/6 mouse red blood cells in vitro, CEO enantiomer half lives are:
  - *S*-CEO $T_{1/2}$= 0.08 hr; *R*-CEO $T_{1/2}$ = 1.9 hr.
  - *S*-CEO is eliminated much more rapidly than *R*-CEO by the red cell.
- If RBC are pretreated with diethyl maleate (glutathione depletor):
  - *S*-CEO $T_{1/2}$= 3.5 hr; *R*-CEO $T_{1/2}$= 3.6 hr
  - Resultant *S*-CEO levels are much higher, *R*-CEO levels are slightly higher, than when incubated with untreated RBC
  - This is a pronounced inhibition of *S*-CEO destruction.
- Glutathione S-transferase mediated conjugation with glutathione (GSH) is a likely mechanism for the more rapid elimination of *S*-CEO.

CEO-Valine Hb Adduct Formation Correlates with Enantiomer Loss Differences





Conclusions from Hb Adduct Studies

- CEO reacts in vitro with valine in RBC Hb giving two adduct isomers measurable by GC/MS after Edman derivatization and trimethylsilylation.
  - Isomers can be separated by GC, but mass spectra are identical.
  - Adducts likely are regioisomers from differential epoxide ring opening.
- CEO-valine adduct levels increase as CEO exposure levels increase.
  - In vitro formation half-life: ~ 6.5 hr.
- If RBC are pretreated with DEM, CEO-valine adduct levels increase; adducts from *S*-CEO markedly increase.
- *S*-CEO is more rapidly detoxified by GSH-dependent mechanism(s).
- *R*-CEO is more persistent, thereby enabling more adduct formation.
- Biomarker studies predict greater toxicity of the CEO *R*-enantiomer.

Air Concentrations vs. Hb Adduct Biomarker Measurements

- Air concentration measurements of about 80 volatile organic compounds can be accomplished using reliable, EPA-approved TO-15 methodology that is sensitive to sub-ppb pollutant concentrations.
- Hemoglobin adduct biomarkers enable examination of chemical mechanisms of toxicity, and offer promise to measure impact of reactive metabolites.
- Hemoglobin adduct measurement sensitivity?
  - May be adequate to detect adducts with industrial exposure to vinyl monomers at ppm concentrations.
  - Insufficient to examine possible formation of Hb adducts from vinyl monomers at ppb environmental concentrations.

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