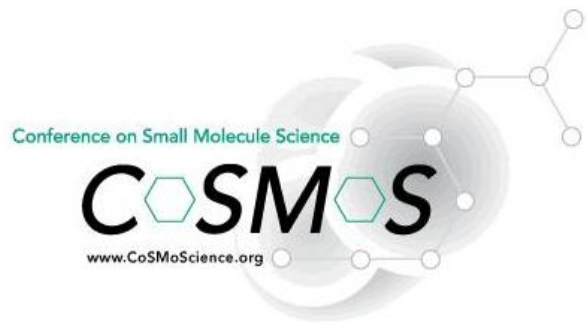


## Comparison of Chemiluminescent Nitrogen Detection, Evaporative Light Scattering, and NMR for High Throughput Quantification

Kenneth Lewis, Ph.D.  
OpAns, LLC  
RTP, NC



## Acknowledgements

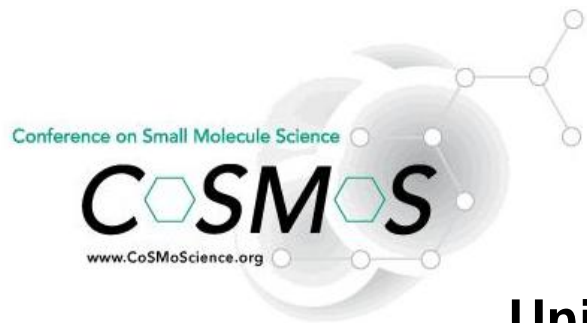
Coauthors on original presentation

- Kathryn Lawrence, Thomas Mitchell, Sandeep Kalelkar  
Previously at Eli Lilly & Co, Sphinx Laboratories, RTP, NC 27709
- Andrea Sefler  
GlaxoSmithKline, RTP, NC 27709

Jean-Francois Borny and Mark Homan of Antek Instruments.

Bill Fitch at Affymax

Dean Phelps at GlaxoSmithKline, RTP



## Why care about quantification without reference standards?

### Universal questions for compound analysis

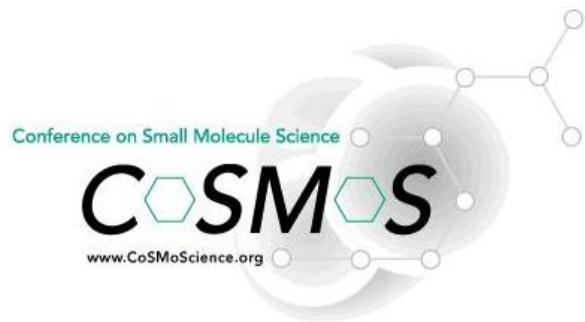
- Is the expected compound present?
- How pure is the sample?
- How much is there?

### Wide Applicability

- Chemistry Support
- Compound Management QA/QC
- ADME Applications
- Manufacturing QC

### Why do we need a solution?

- No generic solution currently available
- Reference Material is not available
- Not enough material to weigh
- Weighing is cumbersome
- Samples are impure



## Options for High Throughput Quantification

### NMR

#### Advantages

- Universal
- Very accurate

#### Drawbacks

- Requires assignment of the spectrum
- Not currently automated
- Best with pure samples
- Expensive instrumentation
- Poor sensitivity

### ELSD

#### Advantages

- Universal
- Inexpensive
- Easy to use
- Compatible with LC

#### Drawbacks

- Compound dependent response

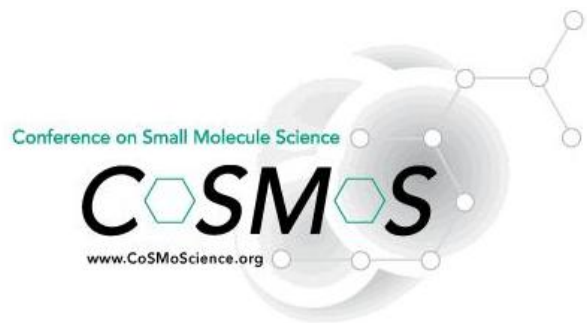
### CLND

#### Advantages

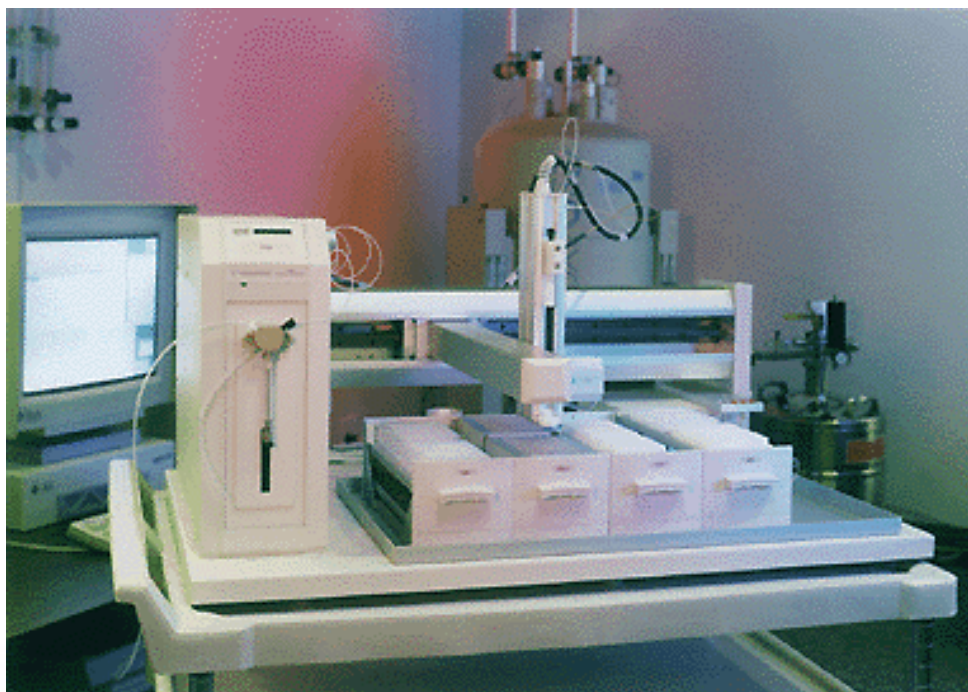
- Accurate without reference standards
- Linear response
- Compatible with LC

#### Drawbacks

- Compound must contain Nitrogen
- No acetonitrile
- Robustness?



## Direct Inject NMR

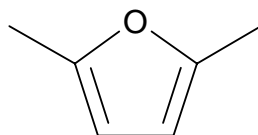


Varian (VAST)

Bruker (BEST)

## $^1\text{H}$ NMR Quantification

- Use 2,5-dimethylfuran as the internal standard

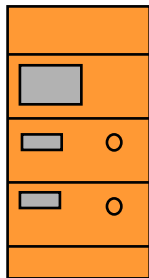


- Assign spectra and ratio integrated peak areas
- Dimethylfuran can be removed by vacuum concentration (bp 92 °C)
- Newer techniques reference internal probe signals.

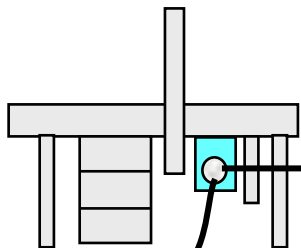
Samuel Gerritz and Andrea Seifler, *J. Comb. Chem.* **2000**, 2, 39-41

## Instrument Schematic

### HPLC Pump



### Autosampler



### Column Oven



**CLND**  
150 $\mu$ L/min

**ELSD**  
700 $\mu$ L/min

**MS**  
150 $\mu$ L/min

Column: 2.1 x 50 mm Luna C18

Mobile Phase: Water (0.1% Formic Acid) / Methanol (0.08% Formic Acid)

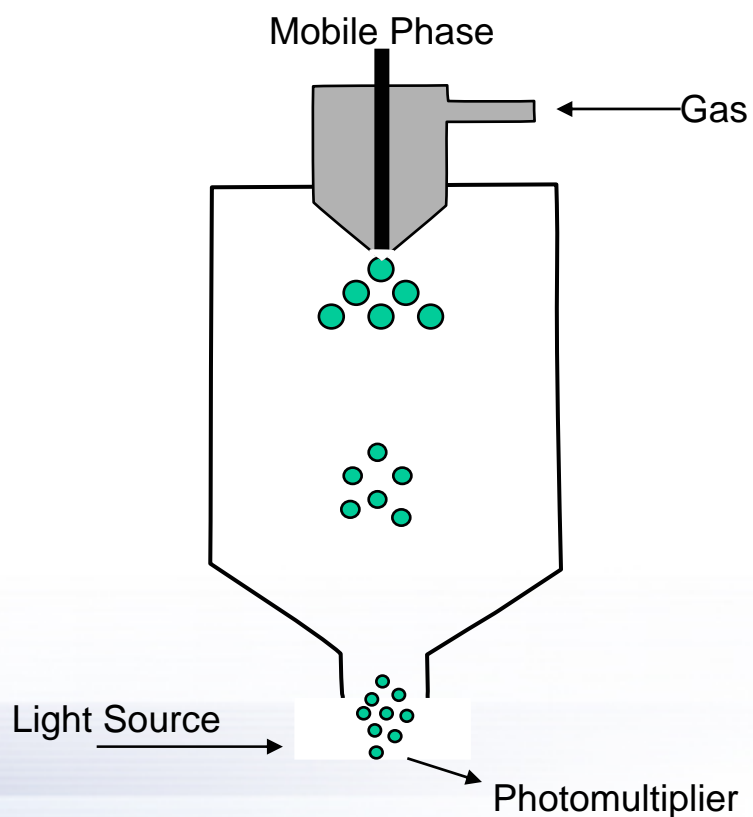
Gradient: 5 to 100% Methanol in 7.5 minutes, 30 second hold, 30 second recovery

Flow: 1.0 mL/min

Instruments: Sedex 75 ELSD, Antek 8060 CLND, Micromass ZQ MS

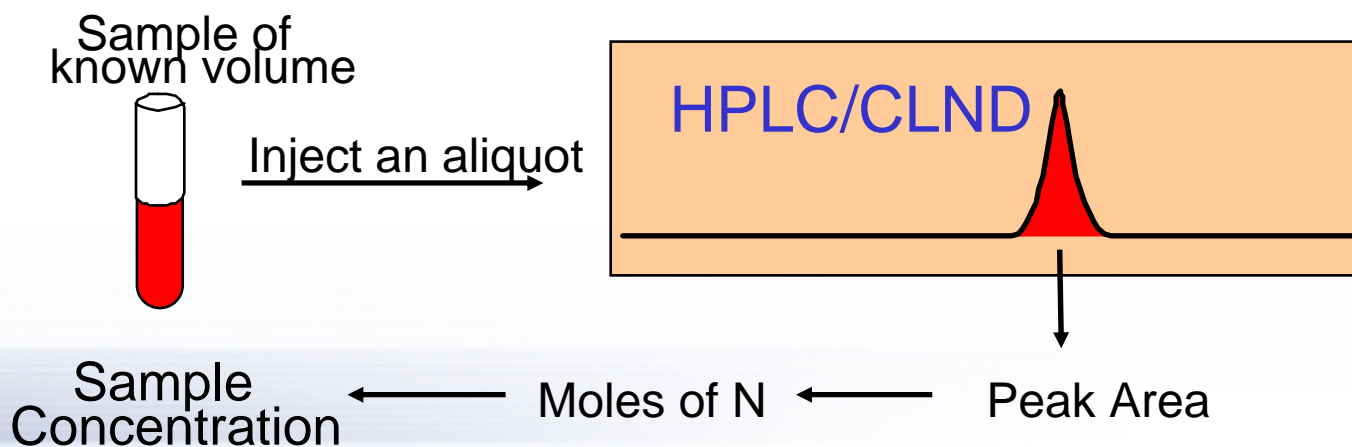
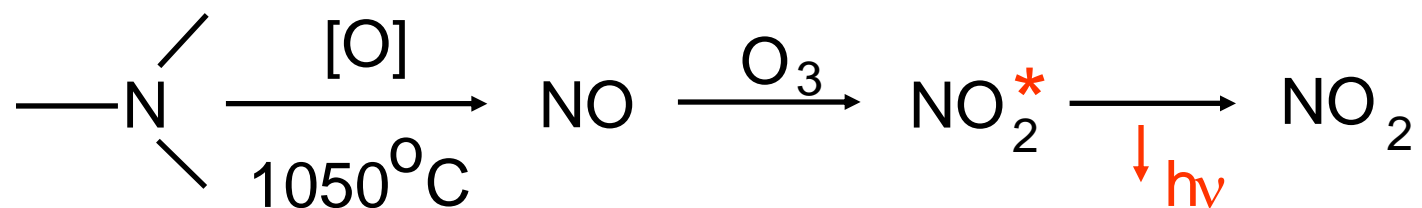
## Evaporative Light Scattering Detection

A destructive detector which detects the non-volatile residue content of a chromatographic peak.

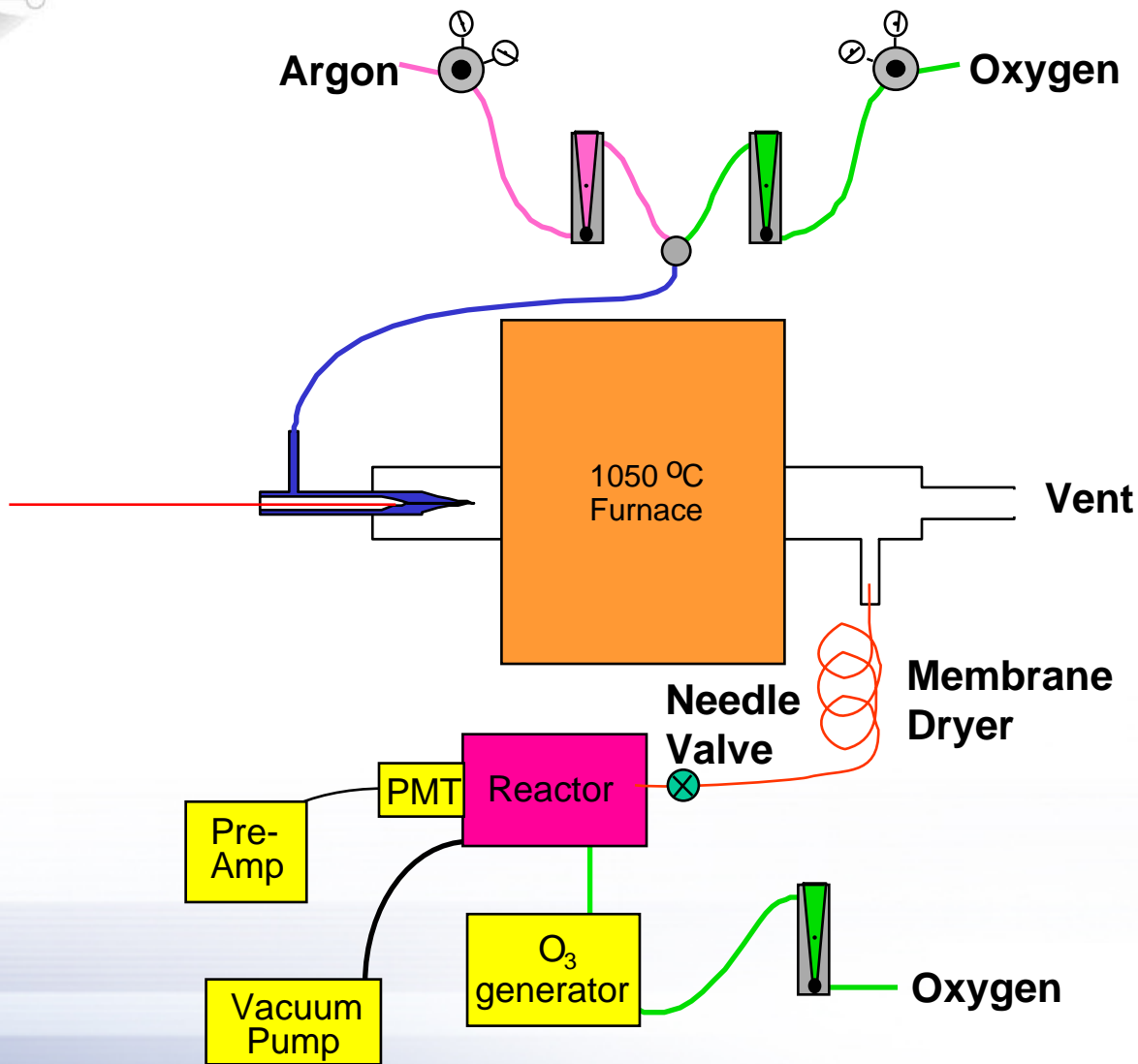


## Chemiluminescent Nitrogen Detector

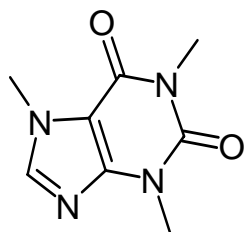
A destructive detector which detects the total nitrogen content of a chromatographic peak.



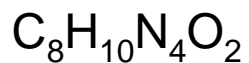
## Antek CLND Schematic



## Calibration Compounds

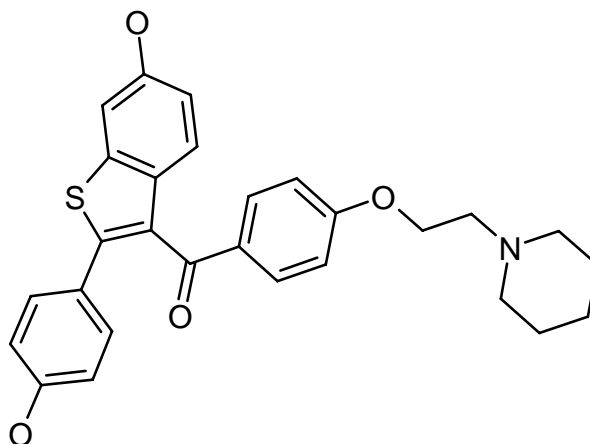


Caffeine

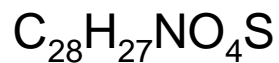


MW=194.2

Elutes at 24% MeOH

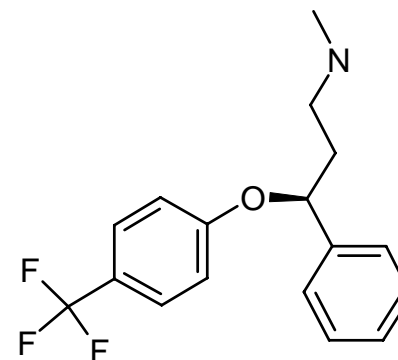


Raloxifene

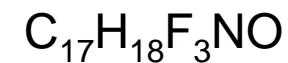


MW = 473.4

Elutes at 40% MeOH



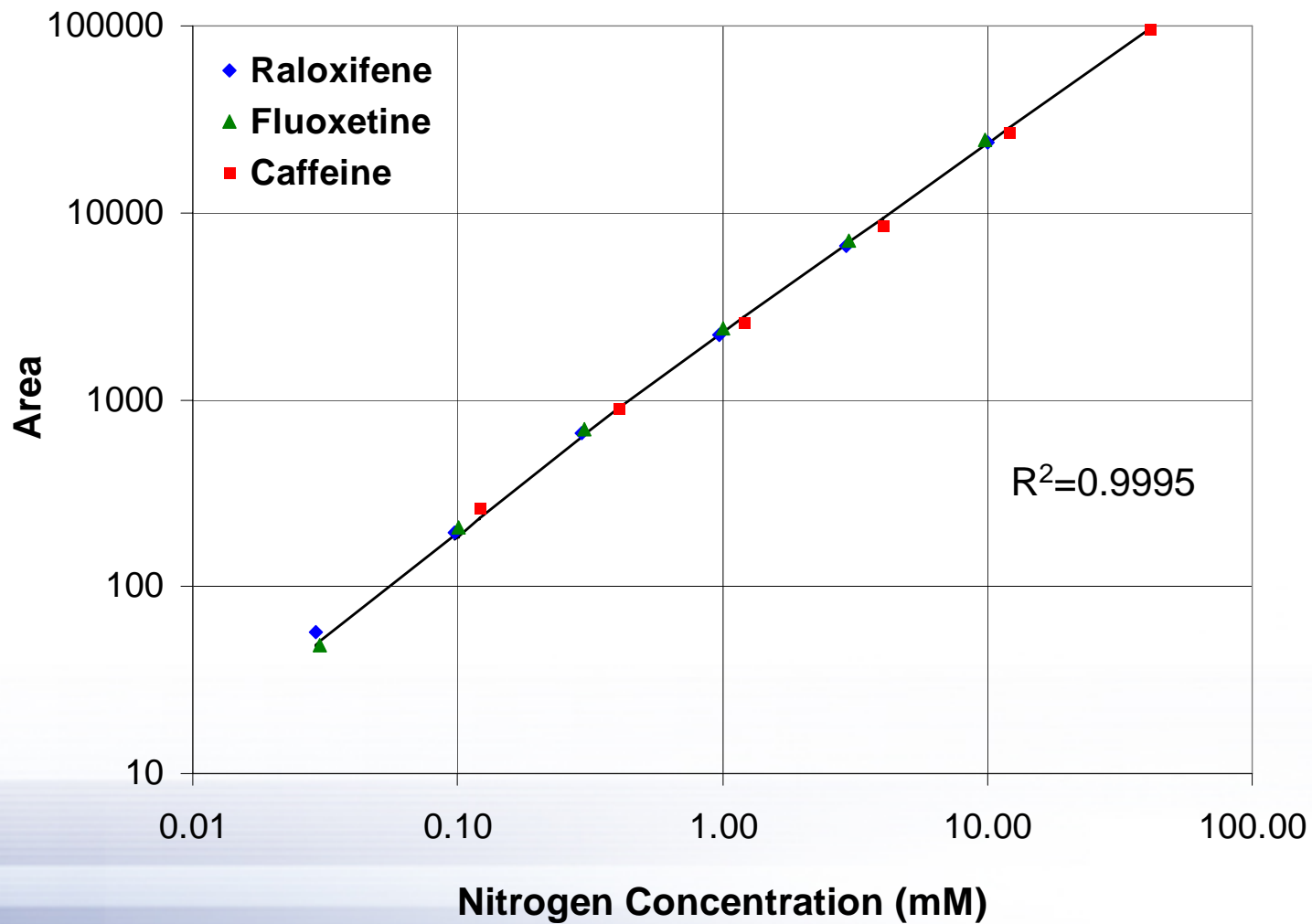
Fluoxetine



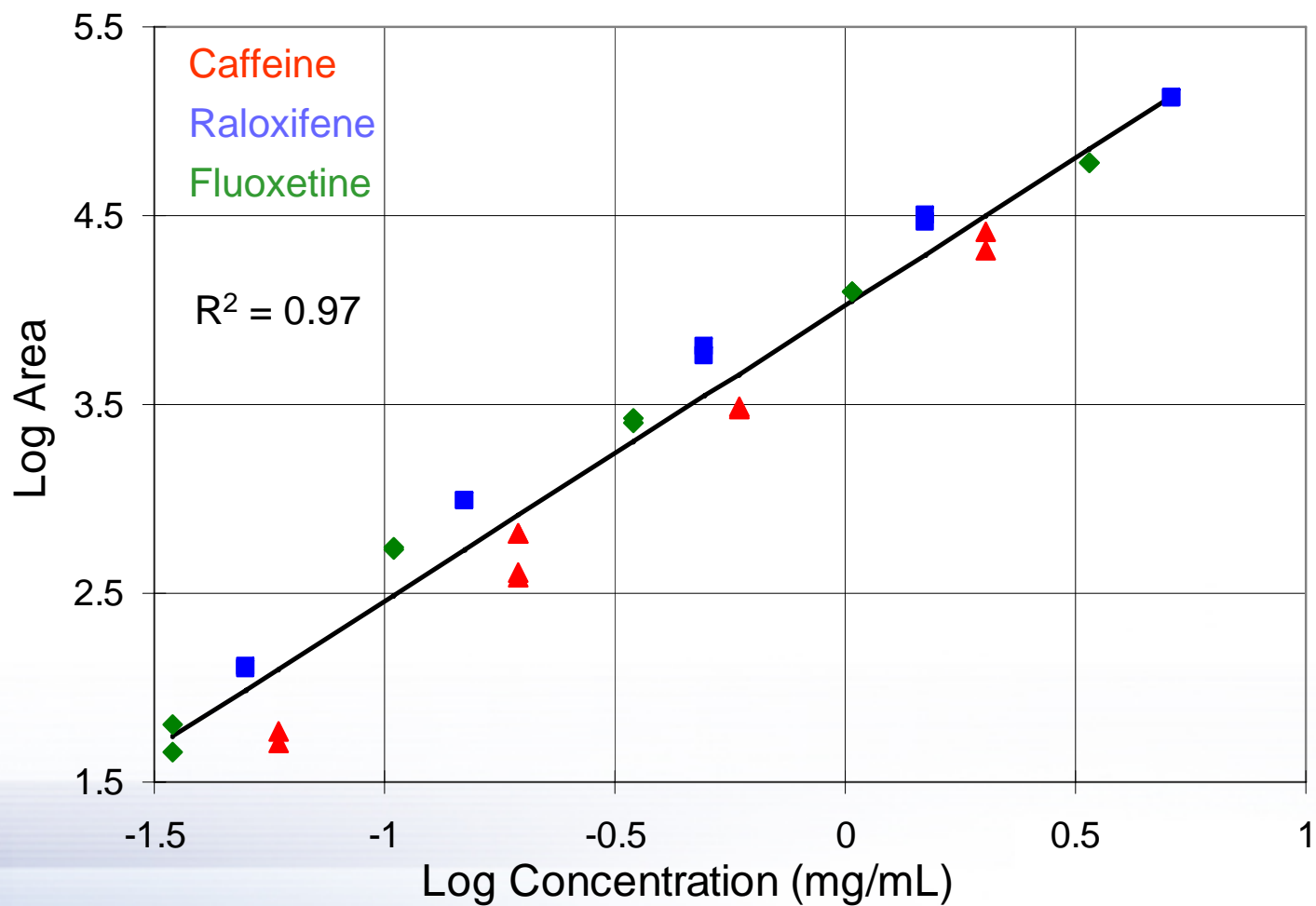
MW = 309.3

Elutes at 47% MeOH

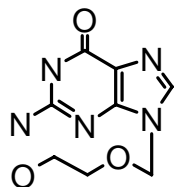
# CLND Calibration Curve



## ELSD Calibration Curve

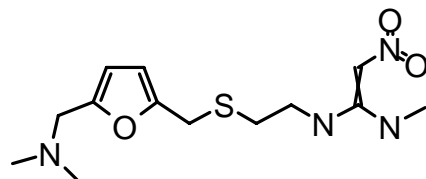


% Error - CLND ELSD CHN NMR



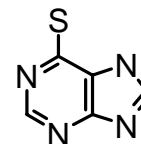
Acyclovir (Zovirax)

0 -27 1 3



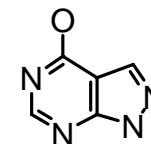
Ranitidine (Zantac)

-1 53 2 2



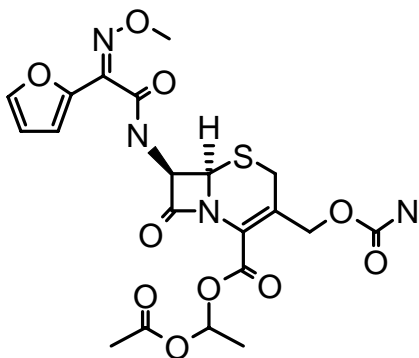
Mercaptopurine (Purinethol)

-2 -65 0 3



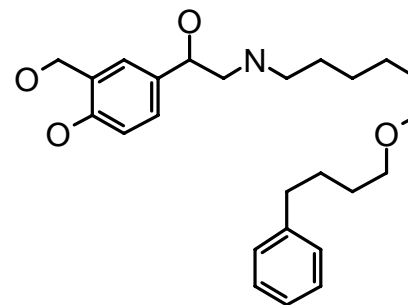
Allopurinol (Zyloprim)

-2 -30 0



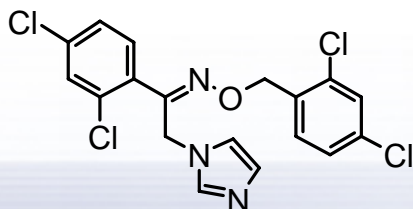
Cefuroxime axetil (Ceftin)

1 -42 1



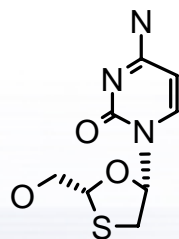
Salmeterol (Serevent)

-1 30 3 3



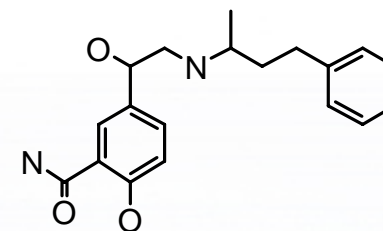
Oxiconazole (Oxistat)

2 24 -1 3



Lamivudine (Epivir)

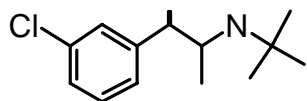
0 42 2



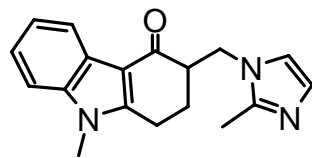
Labetalol (Trandate)

-2 13 2 1

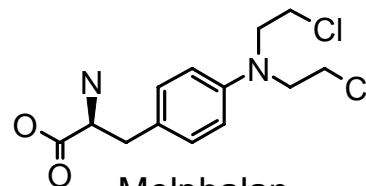
% Error - CLND ELSD CHN NMR



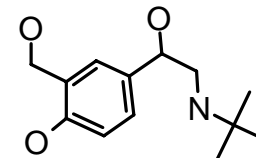
Bupropion  
(Wellbutrin)  
3 N/D 1 2



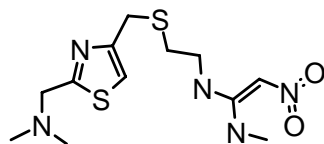
Ondansetron  
(Zofran)  
-1 -28 -1 3



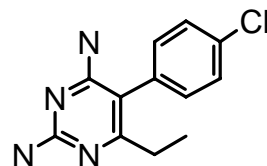
Melphalan  
(Alkeran)  
1 -6 4 9



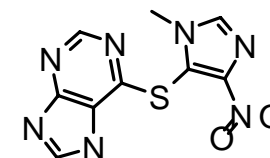
Salbutamol  
(Ventolin)  
0 46 3



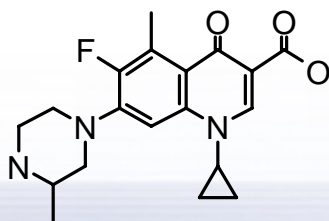
Nizatidine  
(Axid)  
2 9 1



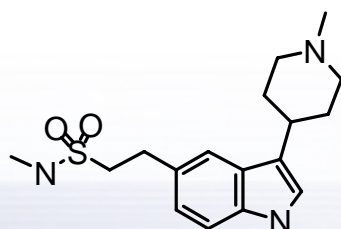
Pyrimethamine  
(Daraprim)  
-1 -34 1 -2



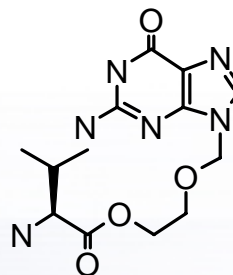
Azathioprine  
(Imuran)  
2 -31 -2 1



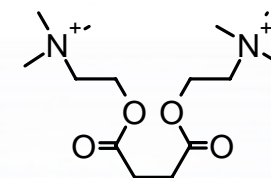
Grepafloxacin  
(Raxar)  
-2 -3 1 8



Naratriptan  
(Amerge)  
1 -3 2 -1

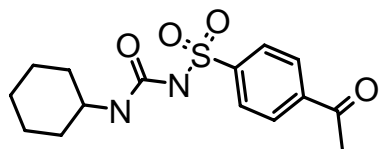


Valacyclovir  
(Valtrex)  
1 -35 2 2

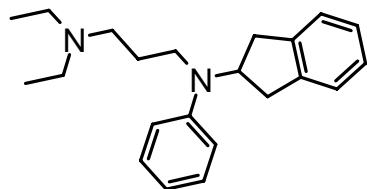


Succinylcholine  
(Anectine)  
0 -11 -8

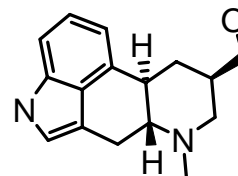
# % Error - CLND ELSD CHN



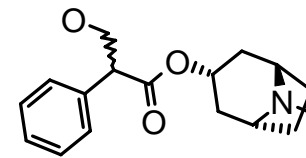
Acetohexamide  
(Dymelor)  
-3 -9 2



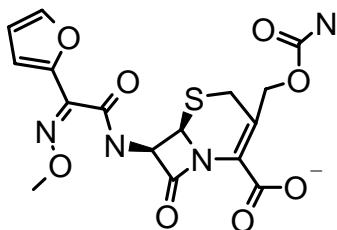
Aprindine  
2 5 3



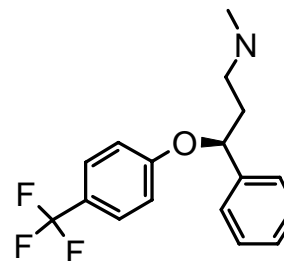
Dihydroelymoclavine  
-3 -4 -2



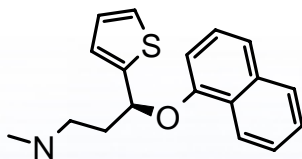
Atropine  
0 -27 12



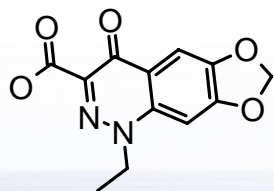
Cefuroxime  
(Kefurox)  
-6 -31 4



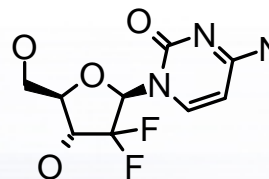
Fluoxetine  
(Prozac)  
1 3 -1



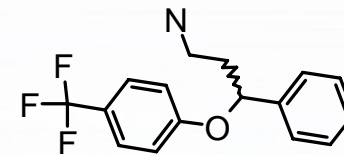
Duloxetine  
1 20 0



Cinoxacin  
(Cinobac)  
-7 -31 -2



Gemcitabine  
(Gemzar)  
0 -17 0

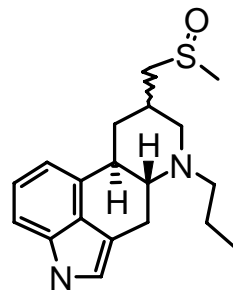


Norfluoxetine  
-10 -4 -1

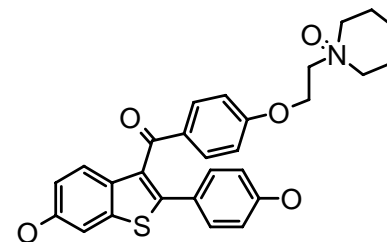
% Error - CLND ELSD CHN



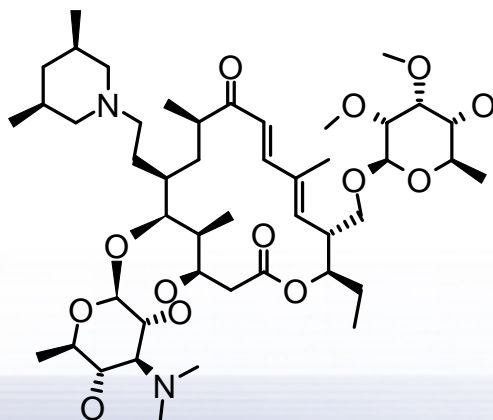
Pergolide mesylate  
(Permax)  
-2 16 2



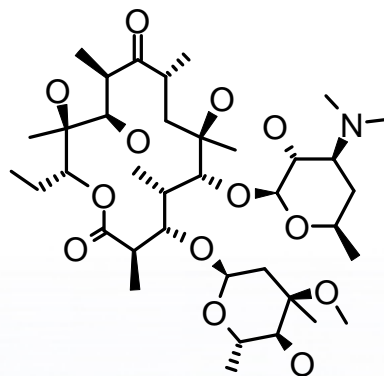
Pergolide sulfoxide  
-1 -8 1



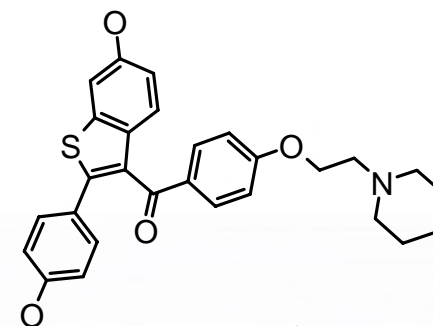
Raloxifene N-Oxide  
1 16



Tilmicosin  
(Micotil)  
-5 -14 -4

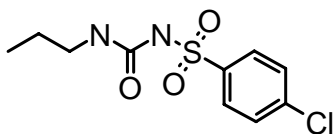


Erythromycin  
(Ilotycin)  
-8 -19 -6

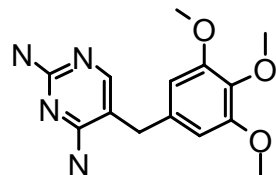


Raloxifene  
(Evista)  
-7 32 0

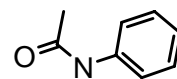
# % Error - CLND ELSD CHN



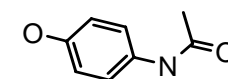
Chlorpropamide  
0 4 1



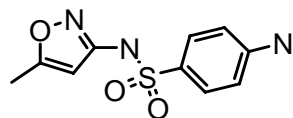
Trimethoprim  
-3 -30 0



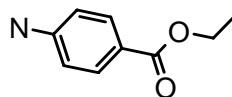
Acetanilide  
3 -85 1



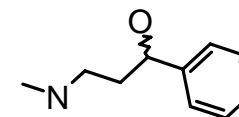
Acetaminophen  
2 -27 1



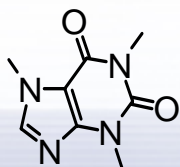
Sulfamethoxazole  
2 -3 0



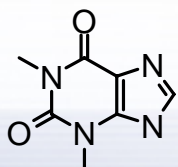
Benzocaine  
7 -76



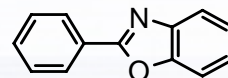
Phenylmethlamino propanol  
-2 28 -2



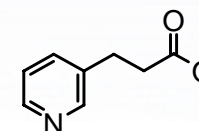
Caffeine  
-2 -37 1



Theophylline  
-4 -59 0



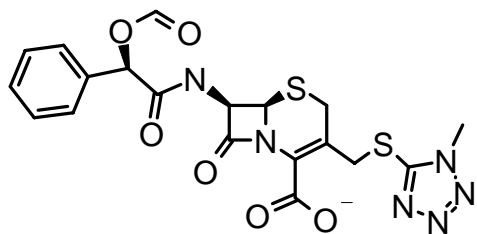
2-Phenylbenzoxazole  
-2 -98 0



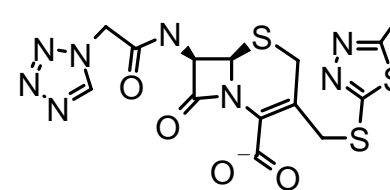
3-Pyridinepropionic acid  
-1 14 3

# Problem Structures for CLND

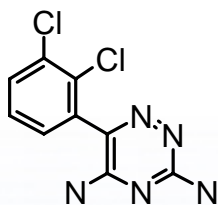
% Error - CLND **ELSD** CHN



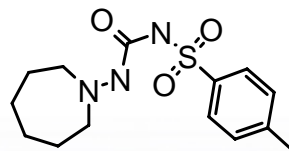
Cefamandole  
(Mandol)  
**-37 -36 0**



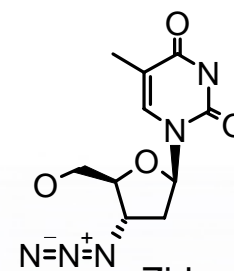
Cefazolin  
(Kefzol)  
**-39 -42 11**



Lamotrigine  
(Lamictal)  
**-19**



Tolazamide  
**-30 -16 1**



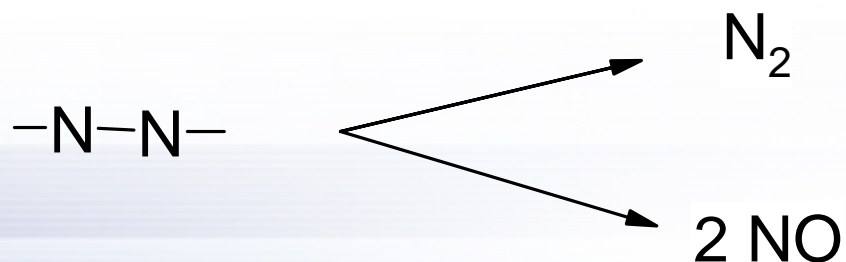
Zidovudine  
(Retrovir)  
**-42 -61**

## Response Rules

1. Azo groups are NOT detected

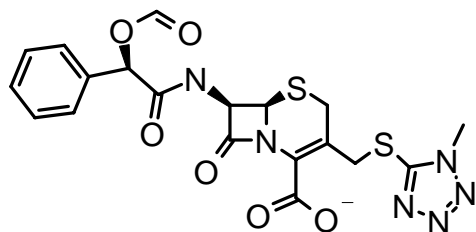


2. N-N bonds produce ~50% response



## Problem Structures for CLND

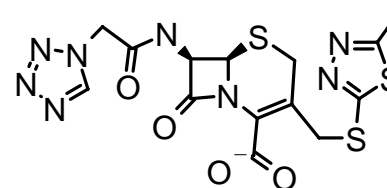
# N and % CLND Error Before Rule  
 # N and CLND Error After Rule



Cefamandole  
 (Mandol)

**6 -37%**

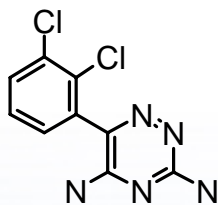
**4 -5%**



Cefazolin  
 (Kefzol)

**8 -39%**

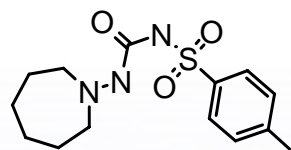
**5 3%**



Lamotrigine  
 (Lamictal)

**5 -19%**

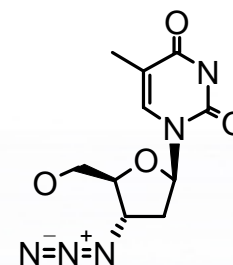
**4 1%**



Tolazamide

**3 -30%**

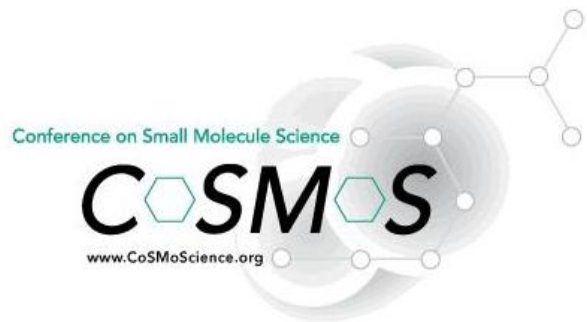
**2 5%**



Zidovudine  
 (Retrovir)

**5 -42%**

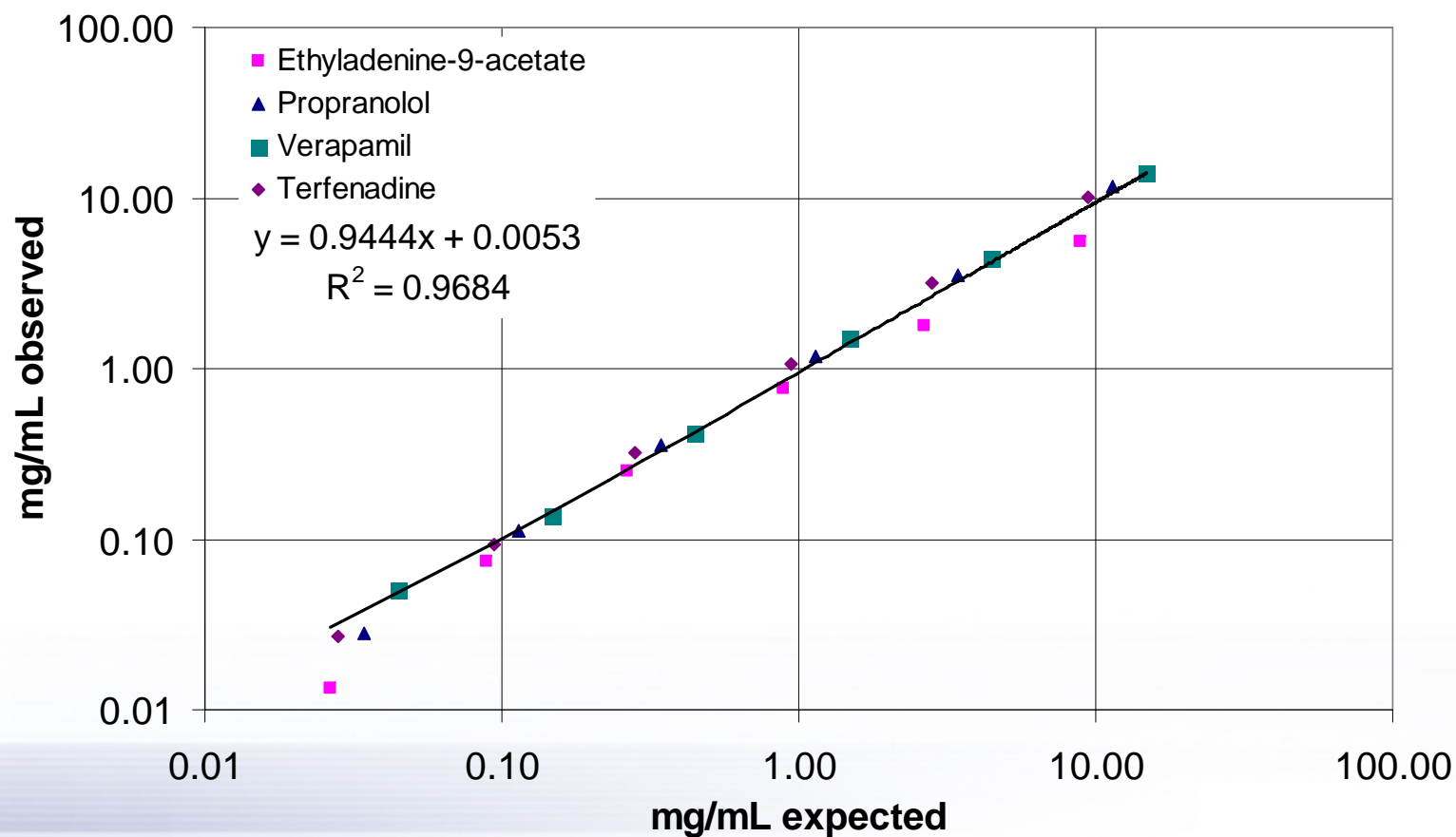
**3 -3%**

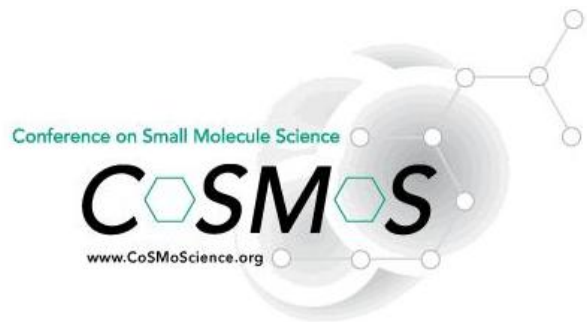


## What about the Corona Detector?

- ESA licensed technology from TSI (Stan Kaufman).
- Similar to ELSD except with detection based on charge/current instead of light scattering.
- Same limitations (volatility, etc) as ELSD
- Lower detection limit than ELSD.
  
- Significantly affected by solvent composition
- Signal must be squared, not cubed.

# Signal Squared, Gradient Adjusted CAD Quantification Accuracy





## Conclusions

- No method is perfect all of the time.
- NMR requires pure samples and internal standards for accurate quantification.
- CLND and ELSD rely on good chromatography.
- CLND is the best technique when little sample is available and it is of questionable quality.
- ELSD is easy and robust for ballpark quantification.

A bright sun is positioned in the upper right quadrant of the image, casting a wide, soft glow across the sky. The sun's rays are visible as a faint, circular pattern of light. The sky is a clear, light blue color. The word "Applications" is centered in the middle of the image in a bold, green, sans-serif font.

# Applications

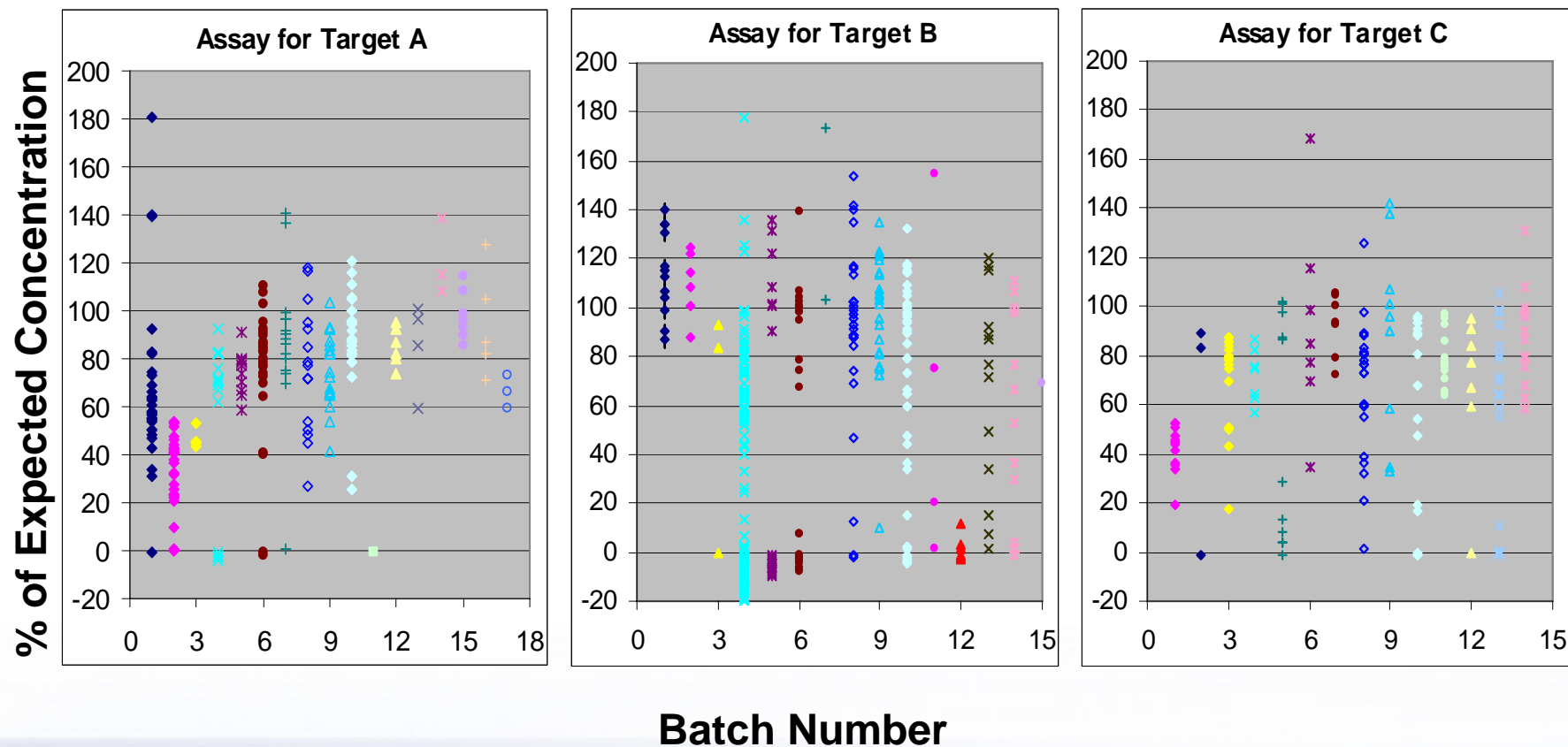
# The LC/DAD/CLND/ELSD/MS System @ RsDqv

- 1100 Binary HPLC stack
- 1956 SL single quad MS
- MultiMode ionization source
- MSD nebulizer is mounted directly underneath DAD cell
- ELSD and CLND inlets close to MSD nebulizer
- Recently replaced 1100 HPLC with 1200SL RR Stack



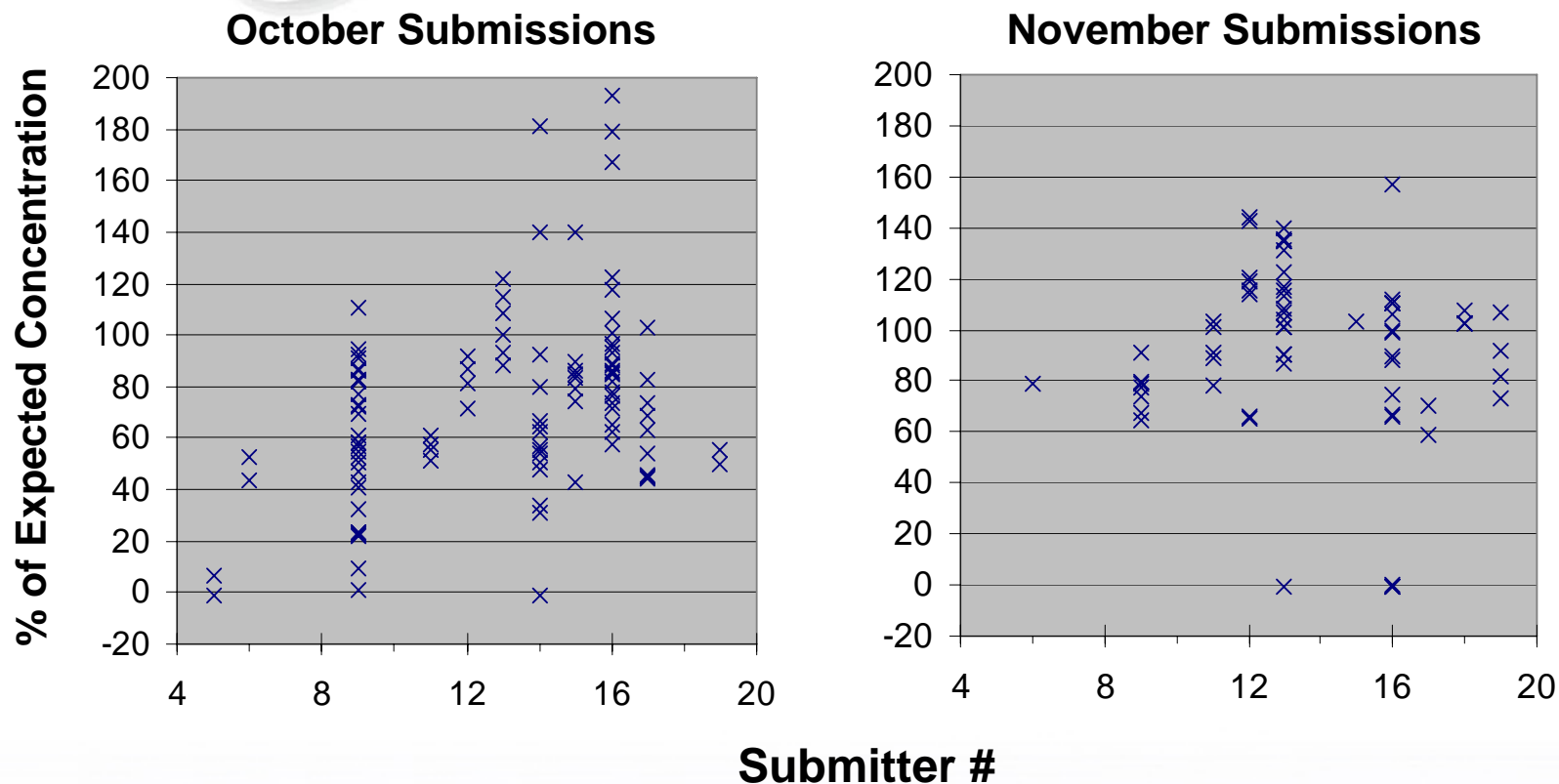
# Example QC Results

## Lead Validation Samples



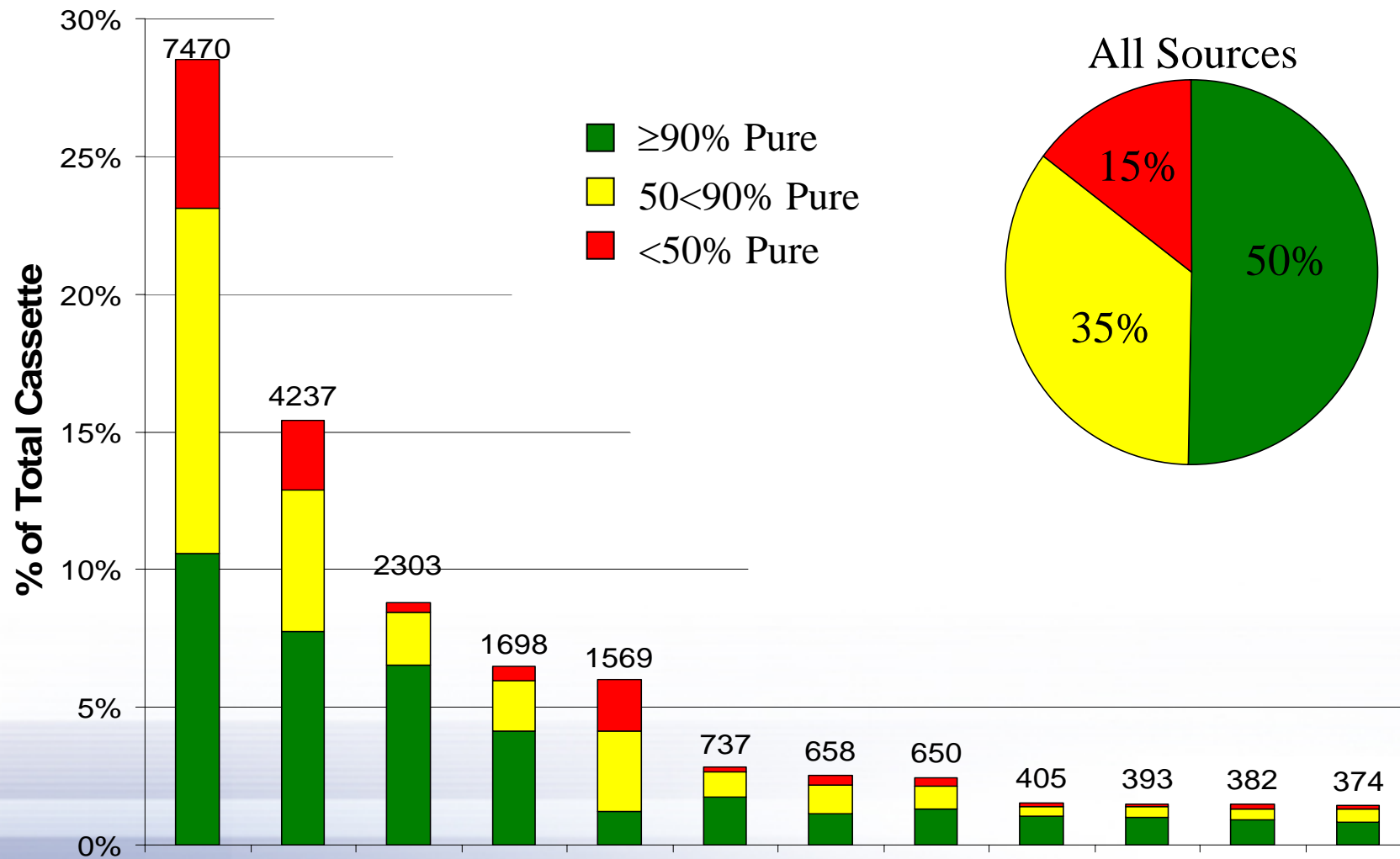
- Samples were synthesized specifically for each SAR project.
- Samples were weighed by the chemist and dissolved by hand.

## The Effect of QC on Quantity



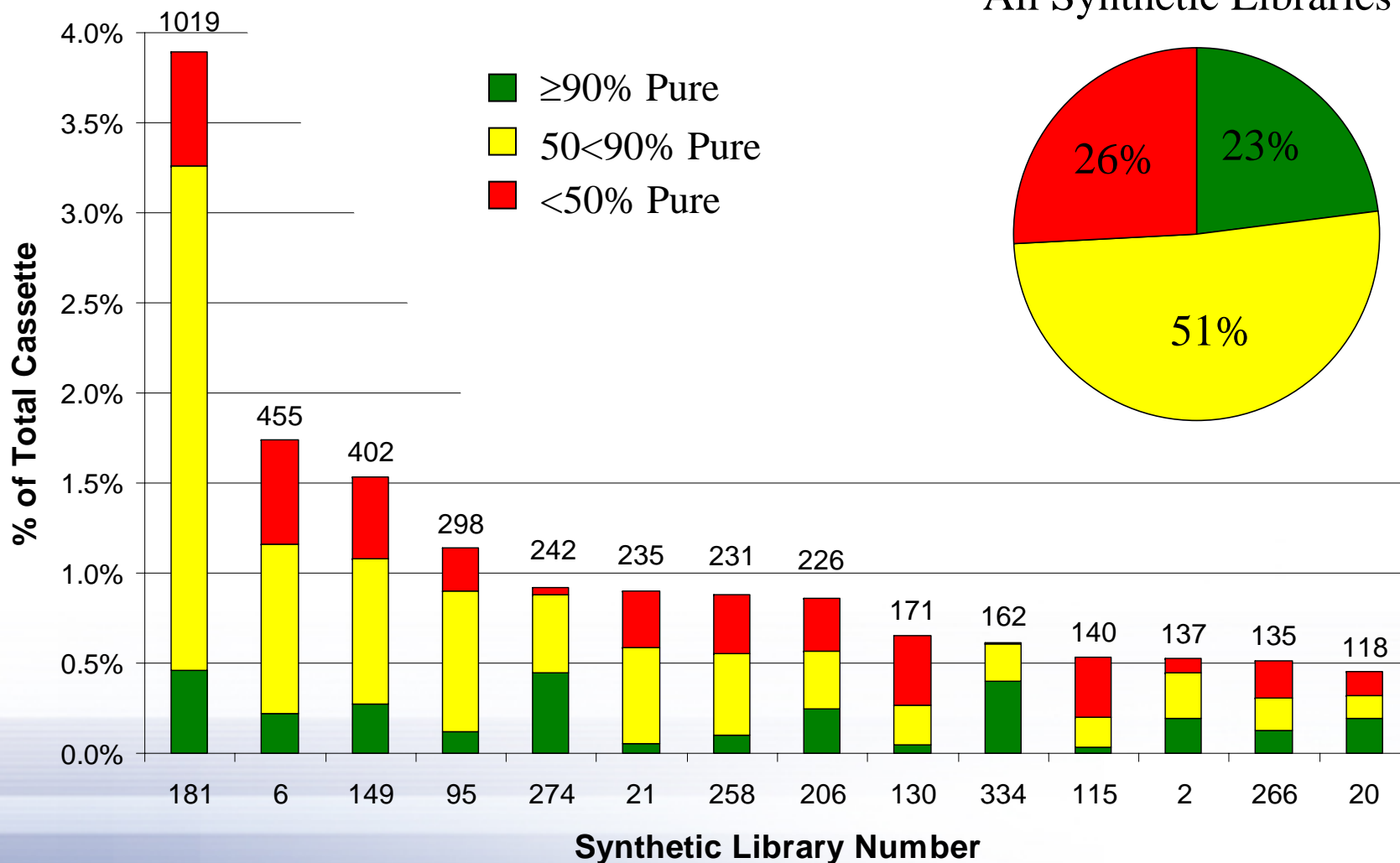
- All samples were for SAR projects.
- Samples were weighed by the chemist and dissolved by hand.
- Only process change was the implementation of QC in October.

## Compound Quality by Source

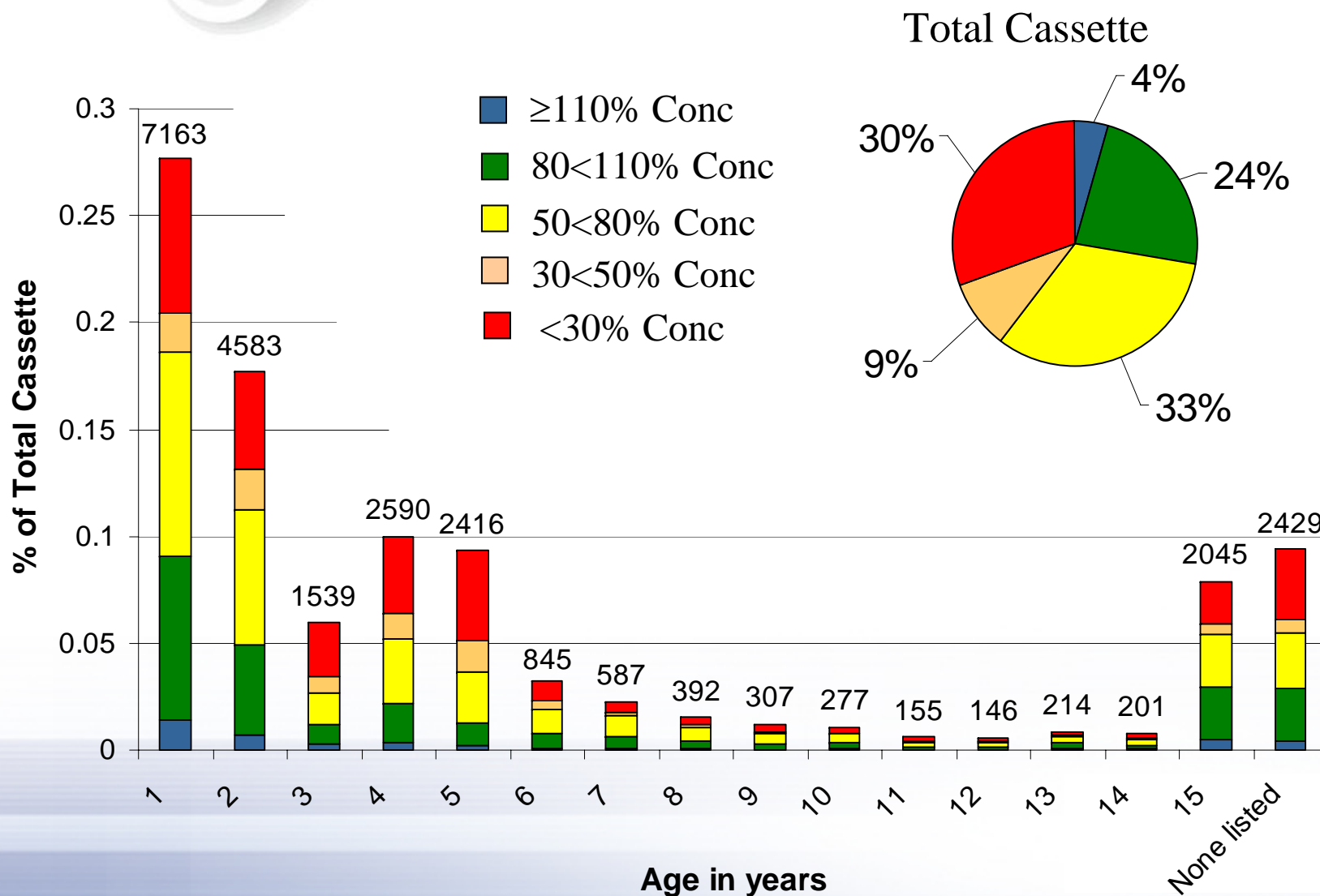


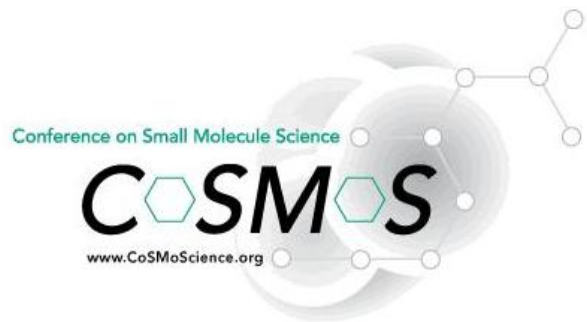
# Compound Quality by Synthetic Library

All Synthetic Libraries



## Quantity for Samples >90% Pure





## Comparison of Chemiluminescent Nitrogen Detection, Evaporative Light Scattering, and NMR for High Throughput Quantification

Kenneth Lewis, Ph.D.  
OpAns, LLC  
RTP, NC