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**Applications of a
Chemiluminescent Nitrogen
HPLC Detector to the Analysis
of Proteins and Peptides
in Snake Venom**



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Introduction

About snake venom

- *Snake venom is a highly evolved saliva*
- *Dried venom composition is 80-90% proteins, peptides, and enzymes*
- *Components found in various snake venom*
 - *Acetylcholinesterase*
 - *Hyaluronidase*
 - *Adenosinetriphosphatase*
 - *Collagenase*
 - *L-amino acid oxidase*
 - *Proteinase*
 - *Phosphodiesterase*
 - *Many other components remain unidentified*
- *Protein composition determines nature and action of a snake's venom*

Introduction

Types and Actions of Venom

– Target Sites

- *Nervous system (neurotoxic)*
- *Circulatory system/surrounding tissue (hemotoxic/hemolytic)*

– Possible effects on target sites

- *Paralysis of heart or lungs*
- *Loss of blood pressure*
- *Initiates blood clotting*
- *Inhibits blood clotting*
- *Breakdown of proteins*
- *Severe pain*

Introduction

Applications of Snake Venom

– Snakes (whole venom)

- *Catching prey and beginning digestion*
- *Defense*

– Humans (venom components)

- *Anti-stroke medication*
- *Treating blood disorders*
- *Cancer research*
- *Antispasmodics*
- *Pain killers*

Introduction

Protein Analysis

- *HPLC*
 - *Reversed phase using 90Å and 300Å supports*
 - *Size exclusion*
- *Capillary electrophoresis*
- *Gel electrophoresis*

Standards

- *Identification*
- *Quantitation*
 - *High purity standard to calibrate detector response*
 - *Calibration standard for every target compound*

Introduction

Chemiluminescent Nitrogen Detector (CLND)

- *Proteins and amino acids contain nitrogen*
- *Direct response to total amount of nitrogen*
- *Proteins and amino acids contain nitrogen*
- *Quantitate with as few as one calibration standard*
- *Total or speciated detection modes*
- *Stand alone operation or as a part of a multiple detector system*

Introduction

Nitrogen Chemiluminescence Reaction



Equimolar Response for Various Amino Acids and Peptides Relative to Caffeine Response

Name	*RRF	Name	*RRF
Alanine	0.91	Gly-Gln	0.82
Arginine	1.05	Tyr-Gly-Gly	0.80
Glycine	1.02	Phe-Gly-Phe-Gly	1.07
Histidine	0.97	Leucine Enkephalin	1.05
Lysine	1.01	Thymopentin	0.96
Methionine	0.96	Bradykinin	1.06
Phenylalanine	1.01	Bombesin	1.19
Proline	0.99	Neurotensin	1.05
Serine	1.06		
Tryptophan	1.08		
Valine	0.99		

**RRF – relative response factor versus caffeine response*

Nitrogen Content of Amino Acids, Peptides, & Proteins

All 20 standard amino acids: 8 – 32 %N
All except 2 (Arg, His): 8 – 21 %N

Average: 14.7 %N
Median: 12.1 %N

	MW	%N
Bradykinin	1060	9.8
Leucine enkephalin	556	12.6
Bombesin	1620	20.8
Neurotensin	1672	17.6
Insulin aspart	5826	15.6
Ovalbumin	44000	13.4
Human serum albumin	68000	13.1
Bovine serum albumin	68000	14.4
Human IgG	150000	13.1
Bovine thyroglobulin	670000	12.9

Reversed Phase HPLC-CLND Analysis

Column Conditions

Waters Symetry 300-C18

150mm x 2.1mm x 3.5 μ m

Gradient

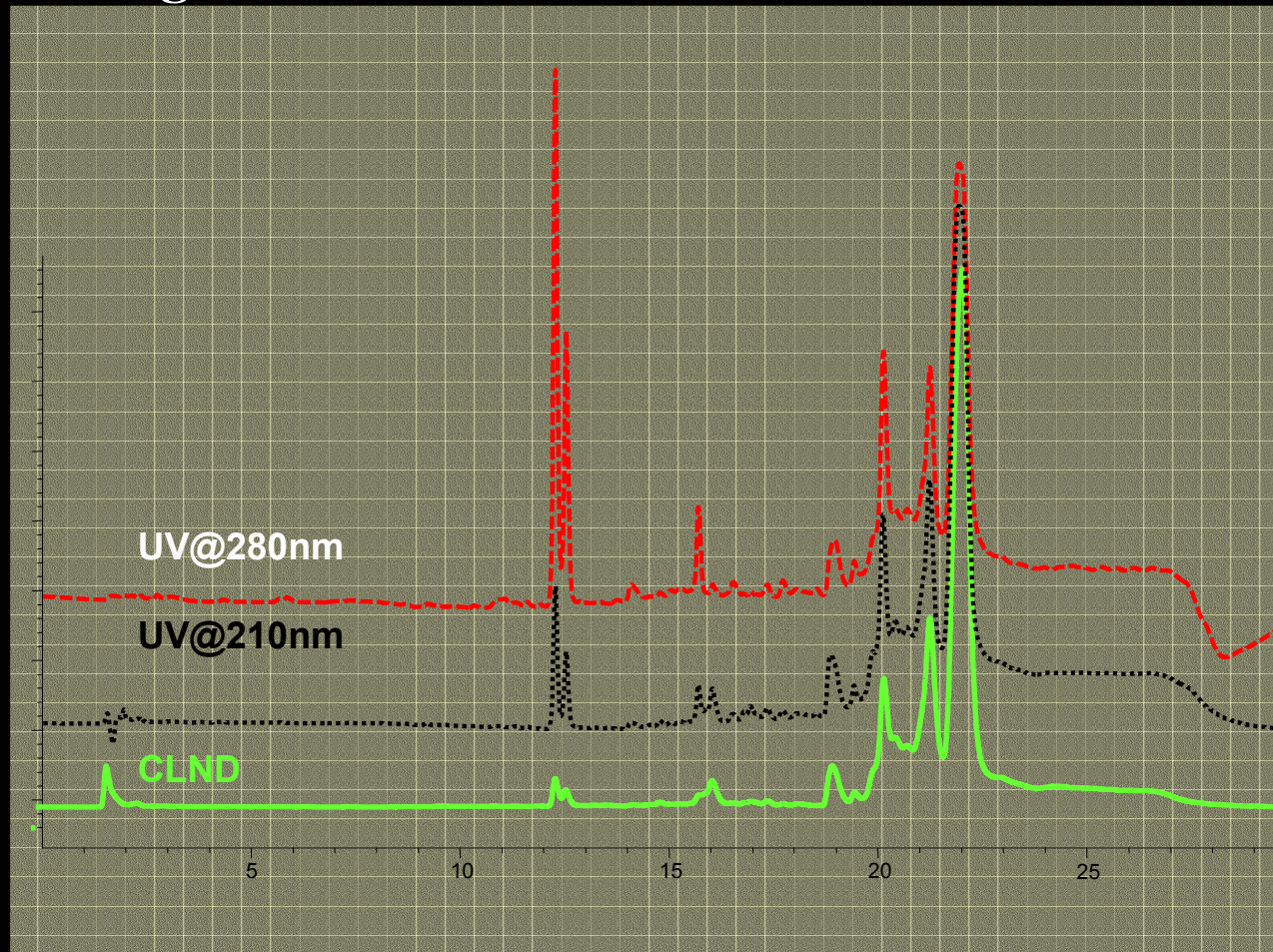
Water+0.1%TFA/Methanol

Flow rate

300 μ l/min

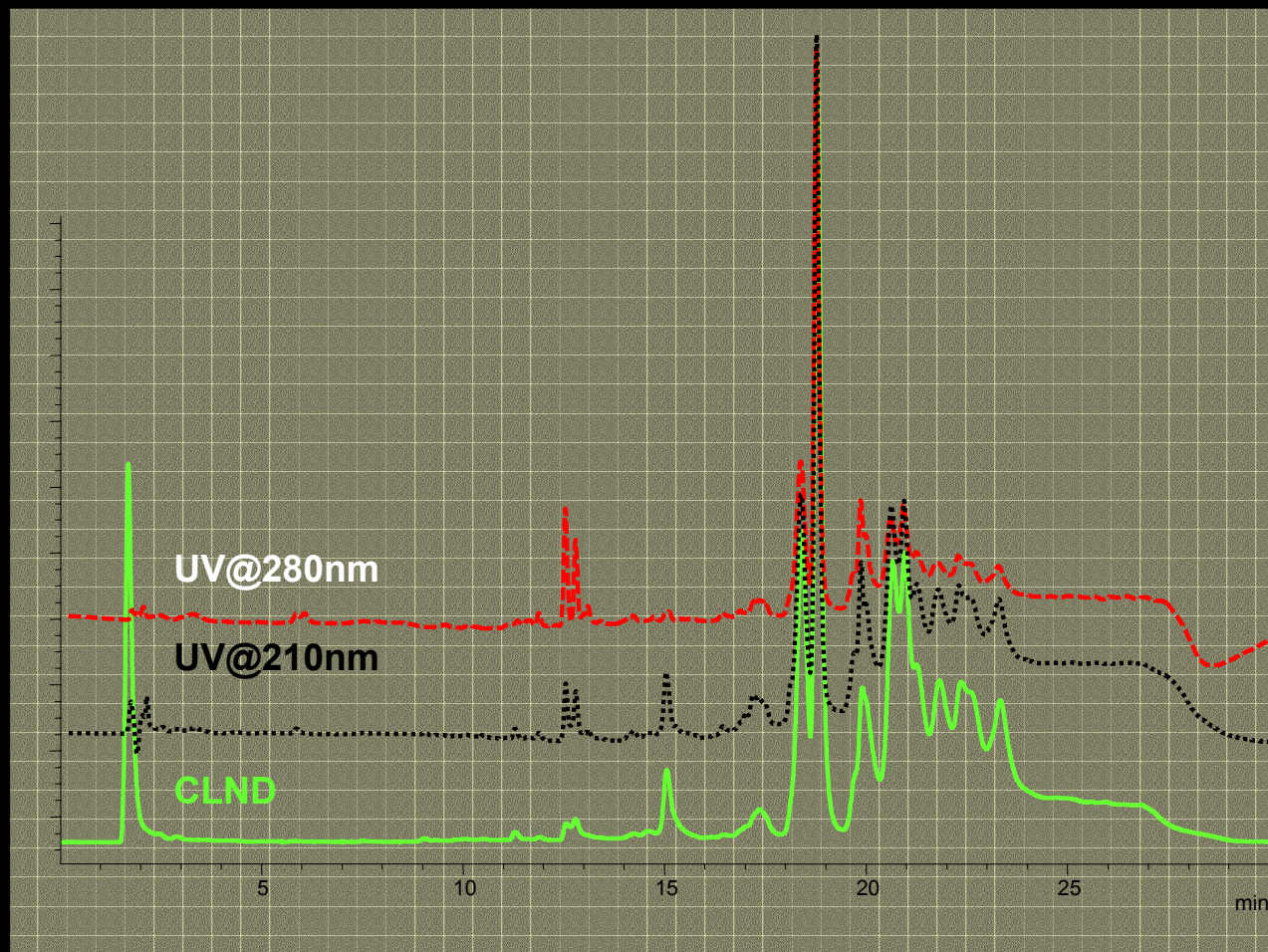
Western Diamondback Rattlesnake

27.1mg/10ml

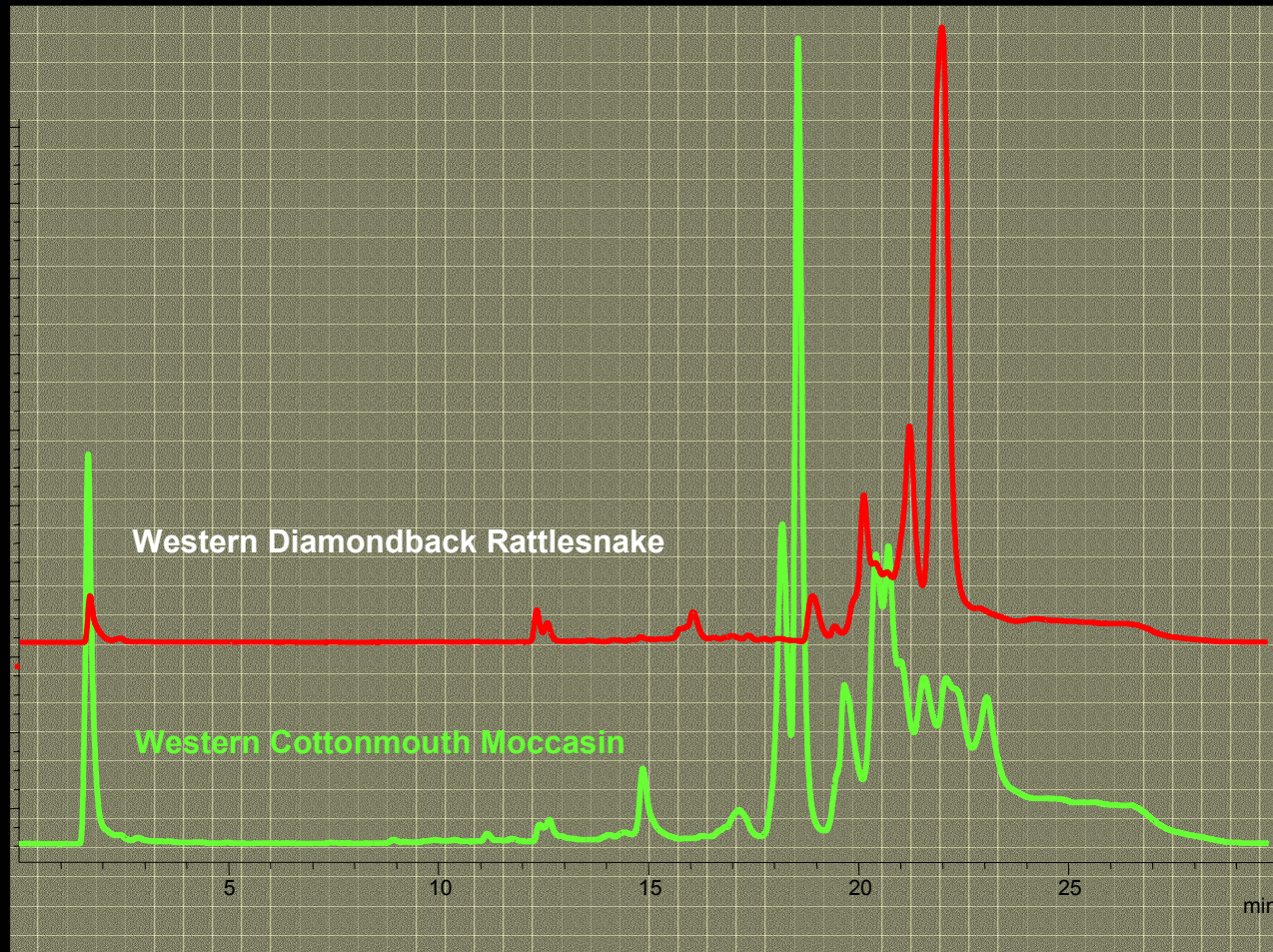


Western Cottonmouth Moccasin

25.0mg/10ml



Pit Viper Venom Comparison



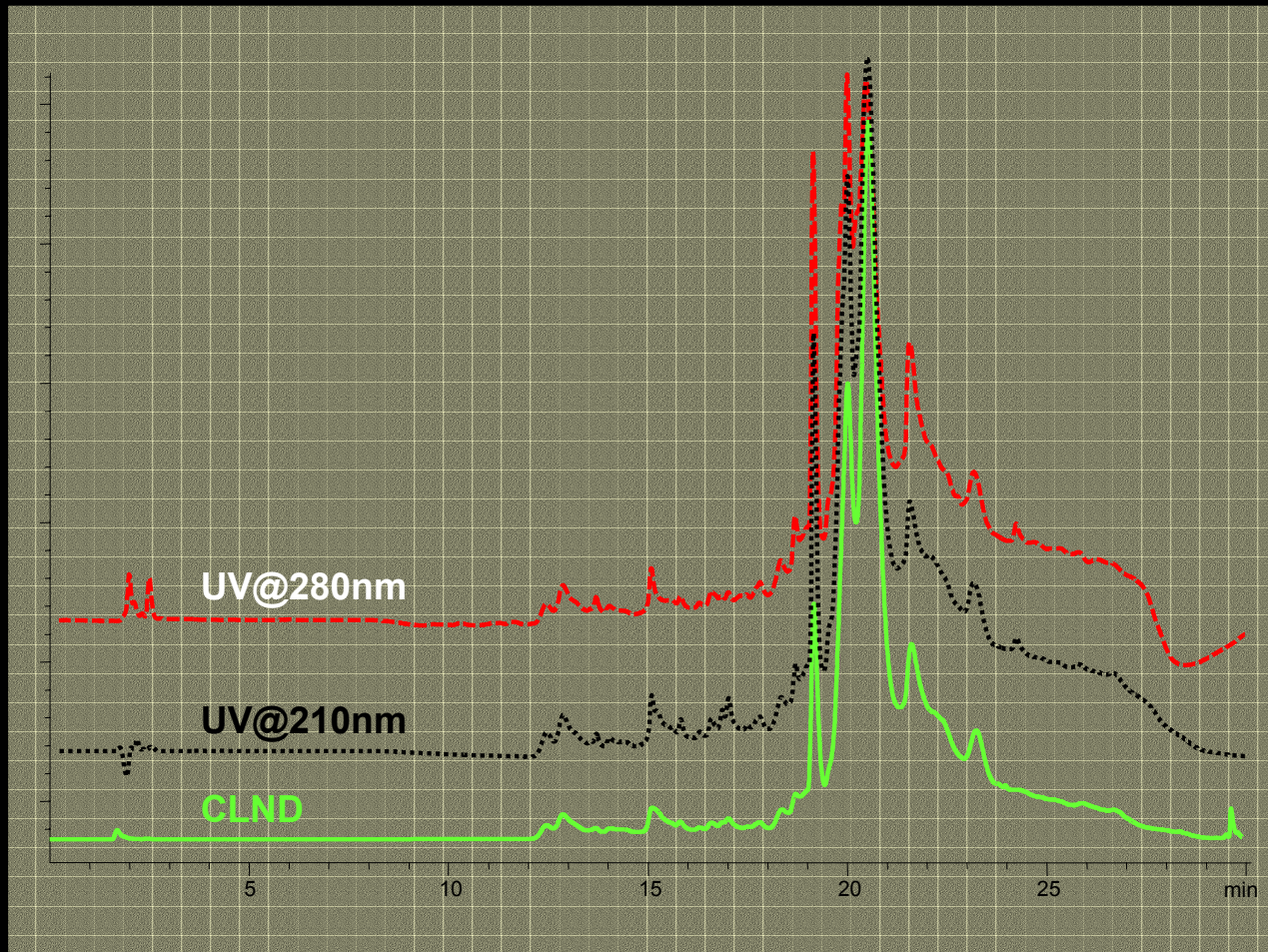
King Cobra

21.0mg/10ml

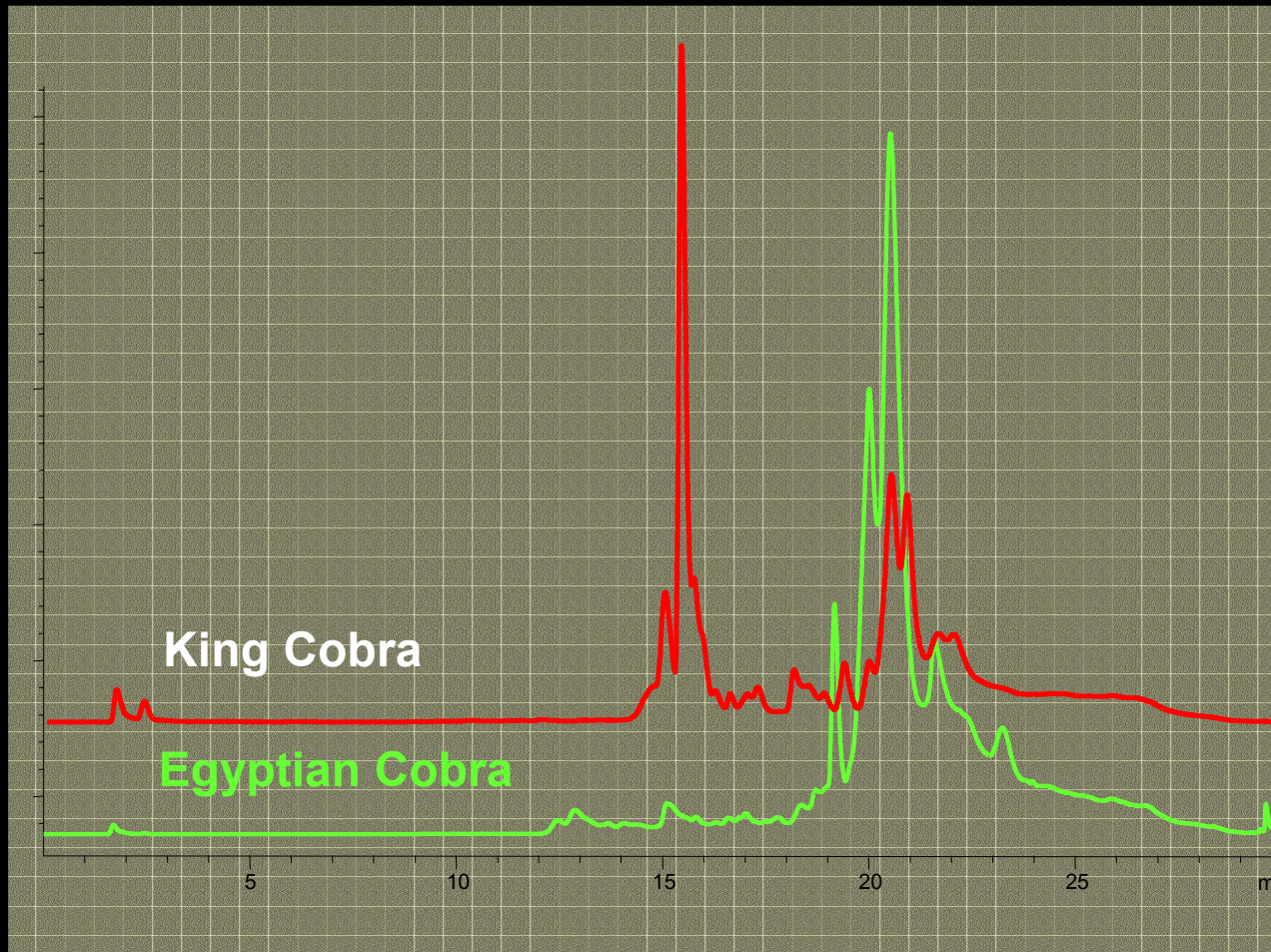


Egyptian Cobra

29.4mg/10ml



Cobra Venom Comparison



Size Exclusion HPLC-CLND Analysis

Column Conditions

Phenomenex BIOSEP-S2000

300mm x 4.6mm

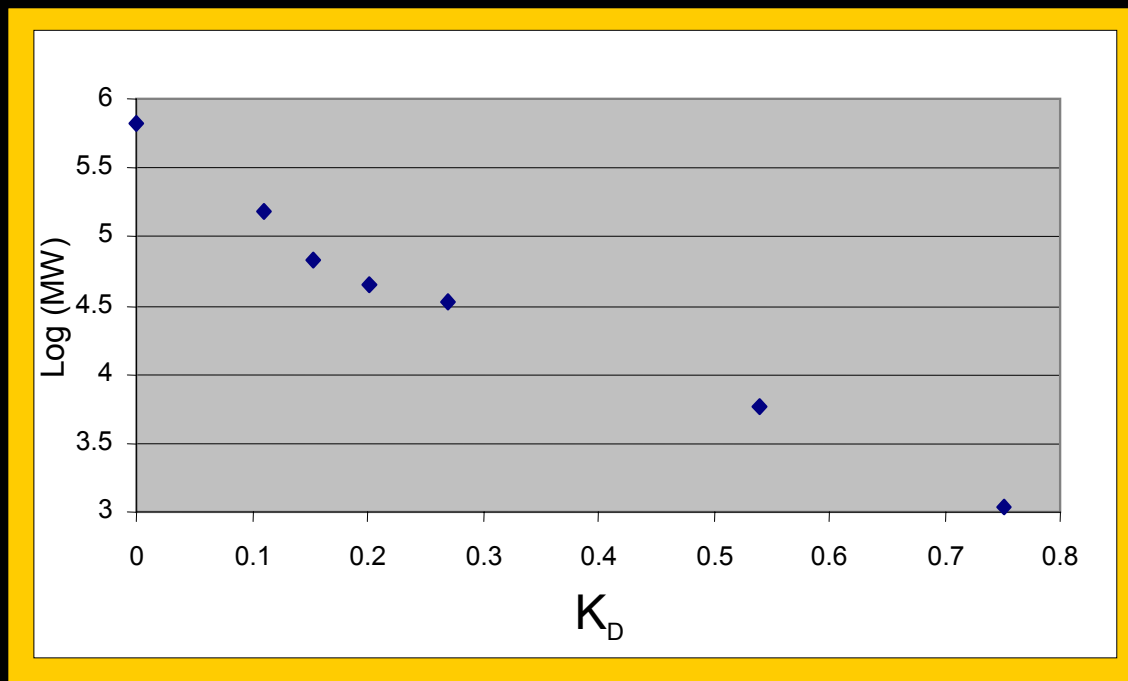
Elution

Water pH 6.6 25mM phosphate buffer

Flow rate

200 μ l/min

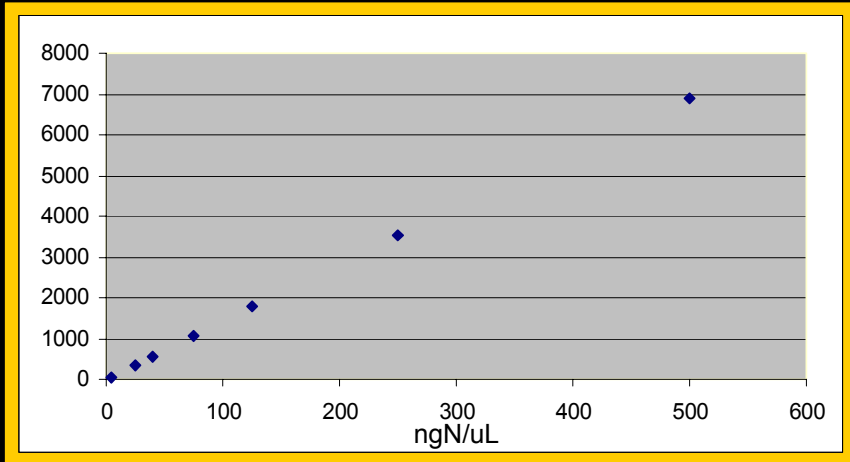
Size Exclusion LC-CLND Analysis



NAME	MW
Bradykinin	1080
Insulin	5800
b-lactoglobulin	34000
Ovalbumin	44000
Human albumin	68000
IgG	150000
Thyroglobulin	670000

Nitrogen Calibration

Flow injection mode (no column) using caffeine standards



Ovalbumin (99%)

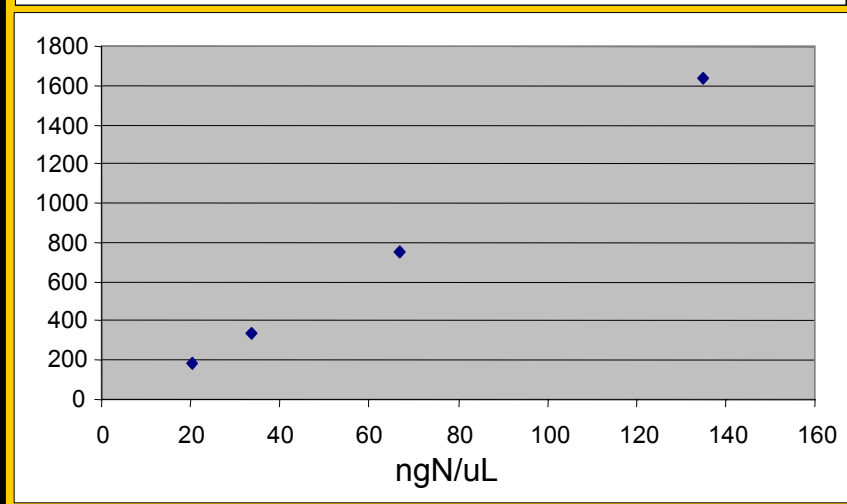
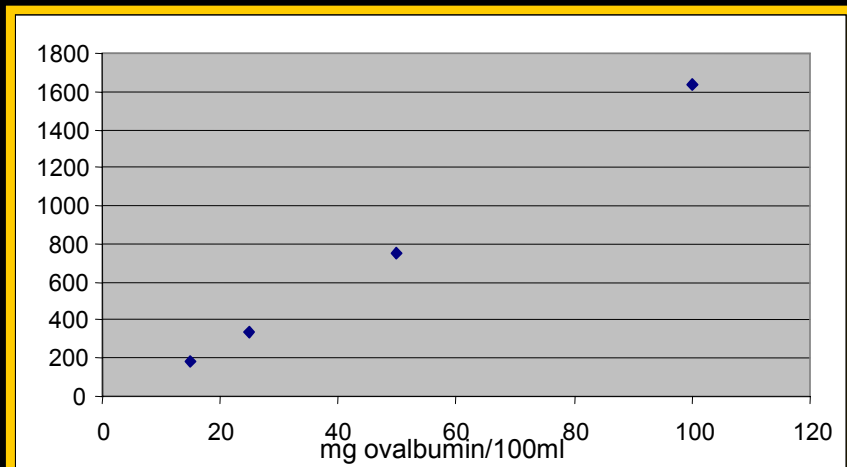
100.5 mg/100ml

Calculated Nitrogen Concentration

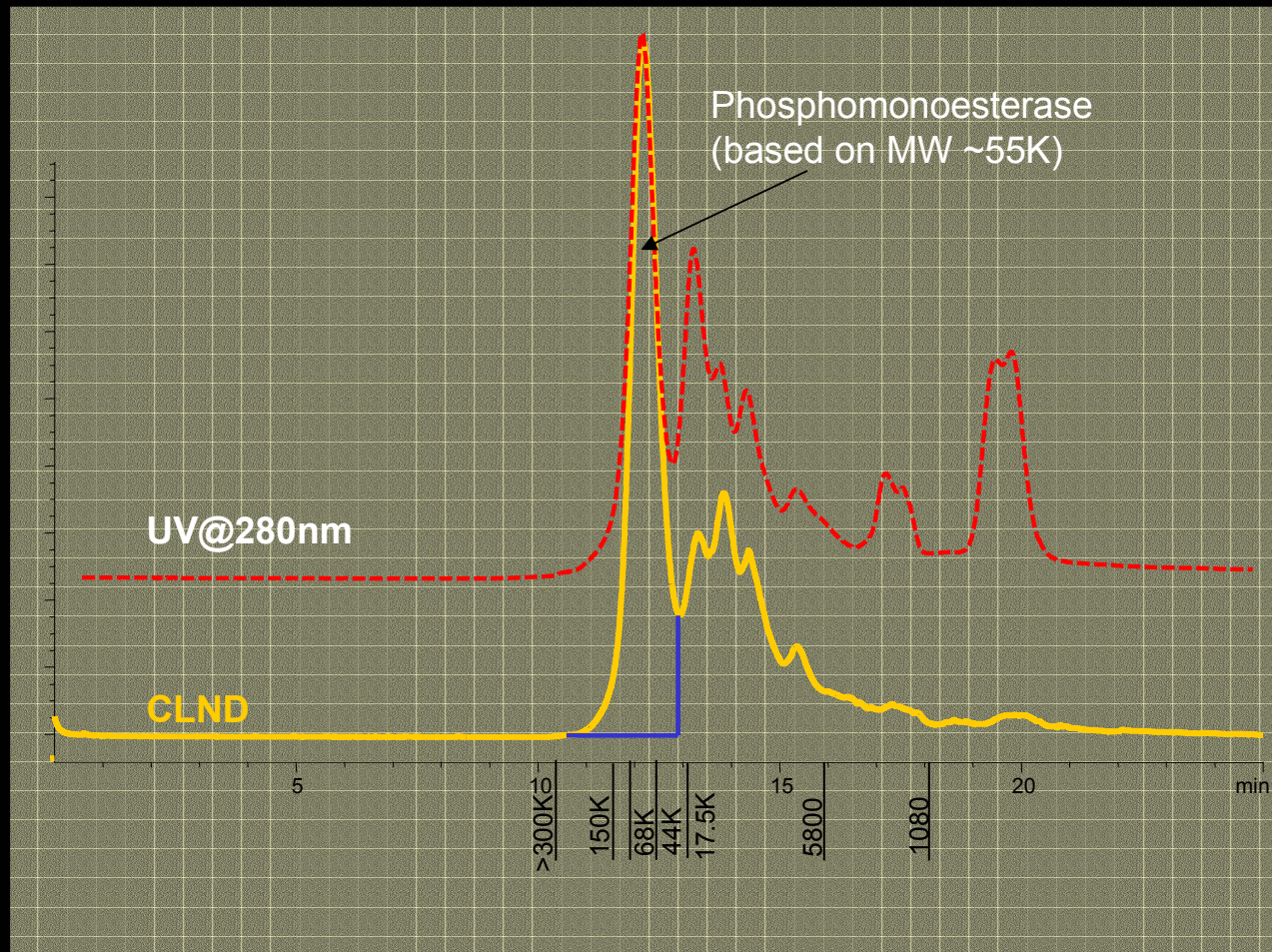
13.38 mgN/100ml

Nitrogen Calibration

Ovalbumin on SEC column



Western Diamondback Rattlesnake



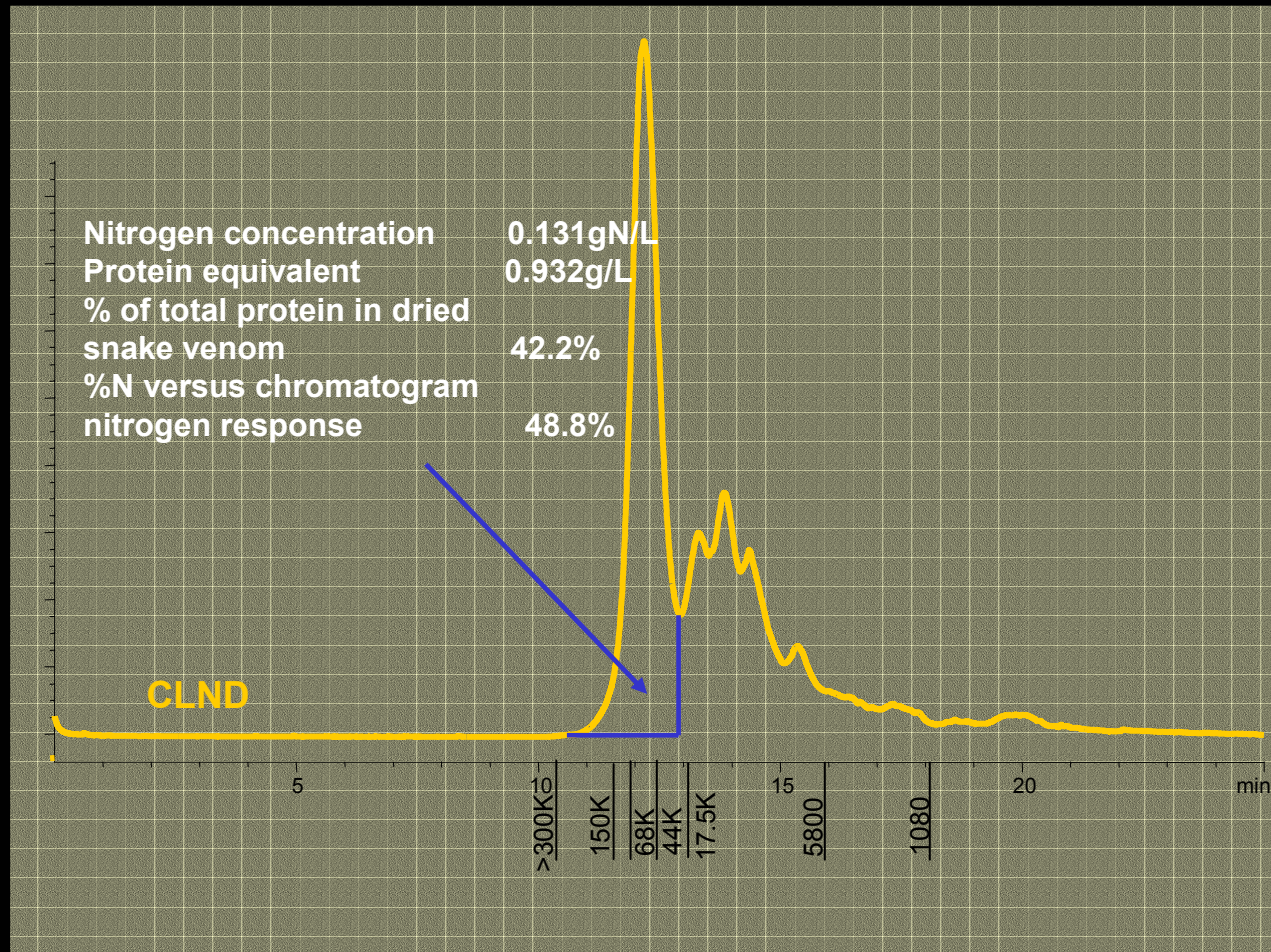
Total Nitrogen and Protein Content of Dried Venom Samples

Protein conversion factor 14%N

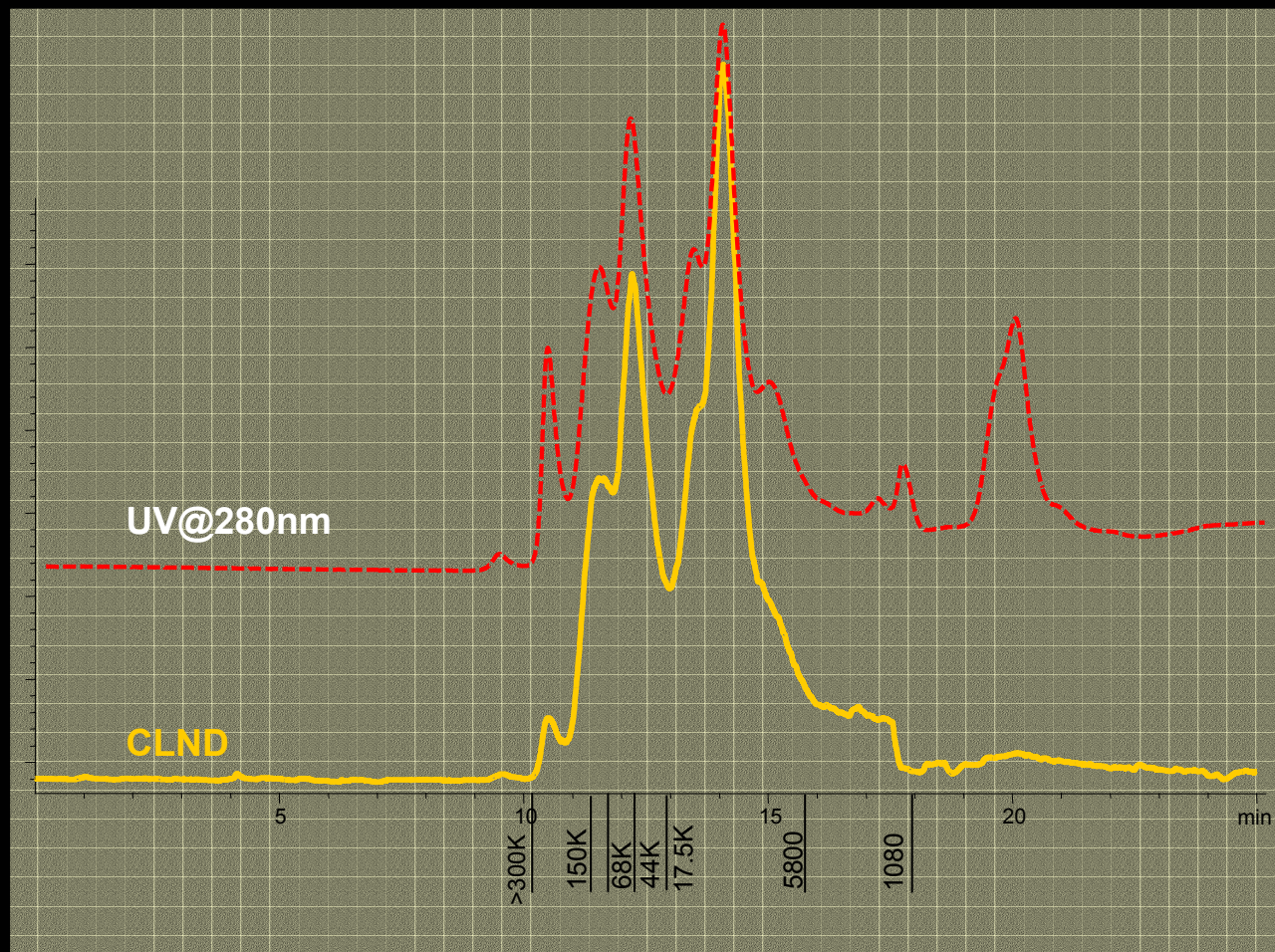
- **Western diamondback rattlesnake venom**
 - *12.4% total N*
 - *88.3% protein by weight*
 - *2.21g/L total protein in original 25mg/10ml sample*

Western Diamondback Rattlesnake

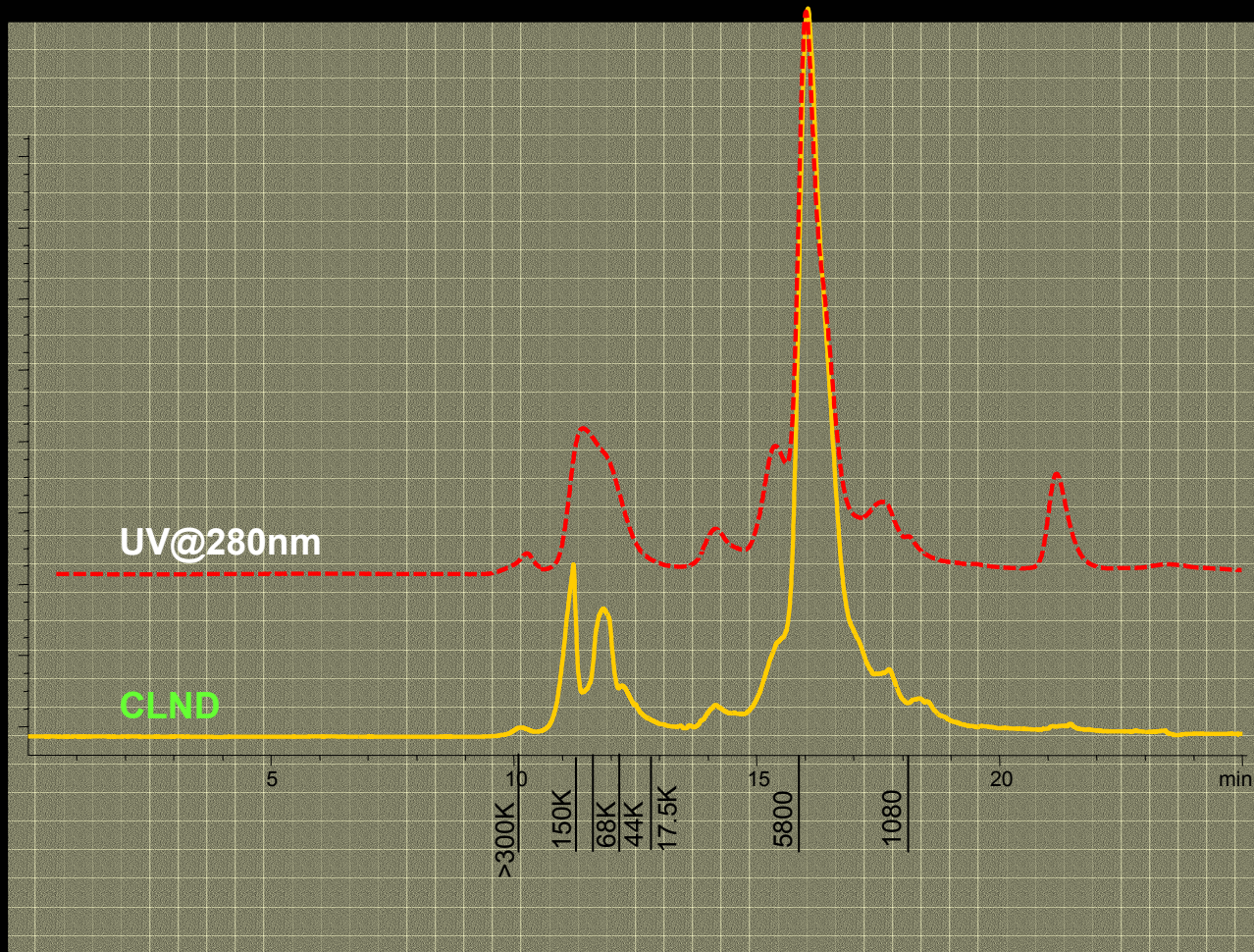
Semi-quantitation of protein content in dried venom



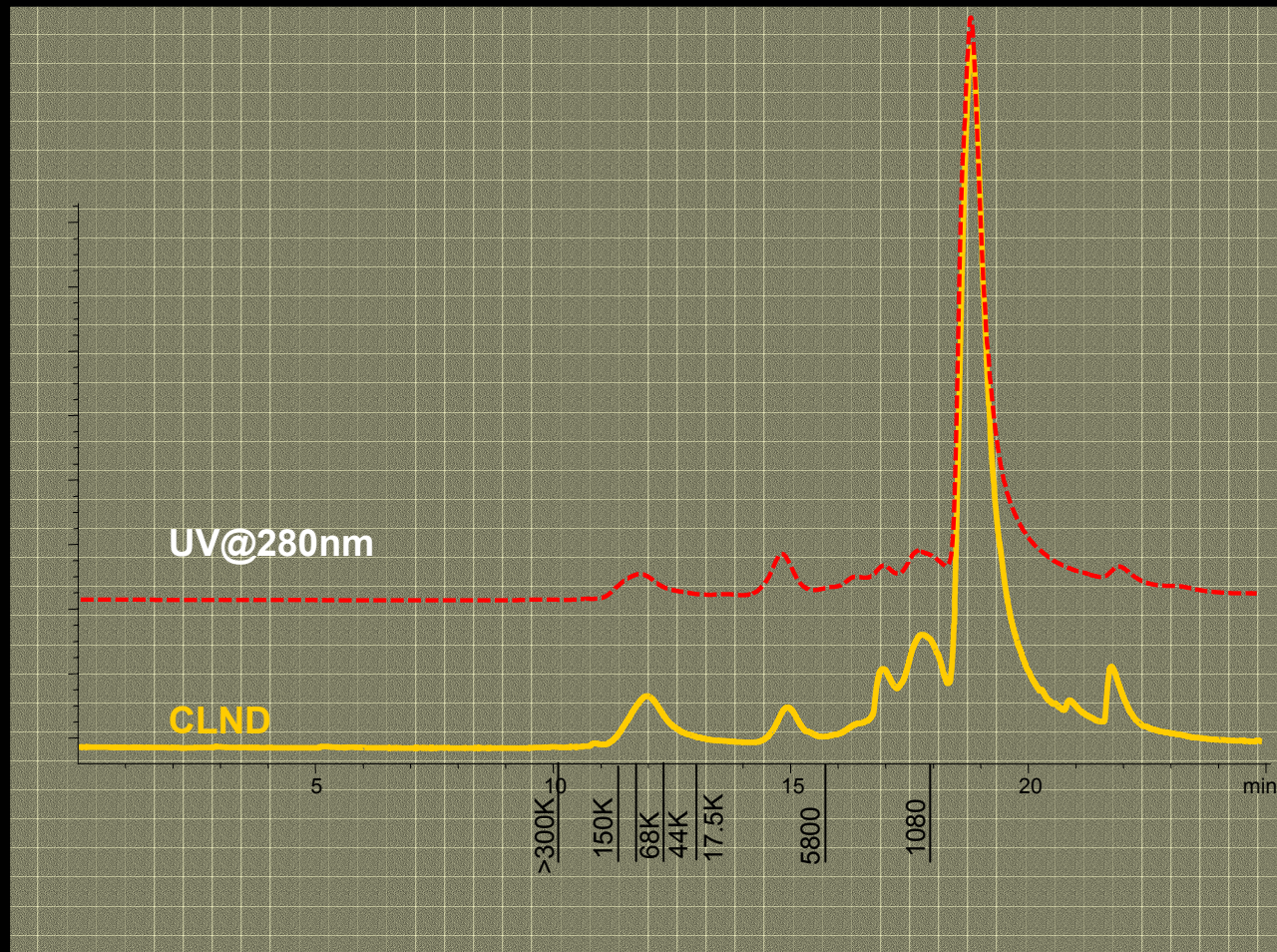
Cottonmouth Moccasin



King Cobra



Egyptian Cobra



Total Nitrogen & Protein Content of Dried Venom Samples

Protein conversion factor 14%N

- ***Western diamondback rattlesnake***
12.4% total N, 88.3% protein
- ***Cottonmouth moccasin***
11.9% total N, 85.0% protein
- ***King cobra***
12.7% total N, 90.5% protein
- ***Egyptian cobra***
10.8% total N, 76.8% protein

Conclusions

- **CLND provides:**
 - *Equimolar detector response to nitrogen in proteins*
 - *Rapid quantitation of nitrogen containing compounds*
 - *Total or speciated nitrogen results*

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