

ABRF 2005 ESRG Study

Modified Amino Acids in Edman Sequencing

Members of the Committee

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Objectives of the Study

- Compile data on elution characteristics of modified PTH amino acids with currently used equipment
- Test the ability of participating laboratories to correctly identify modified amino acids

Description of the Sample

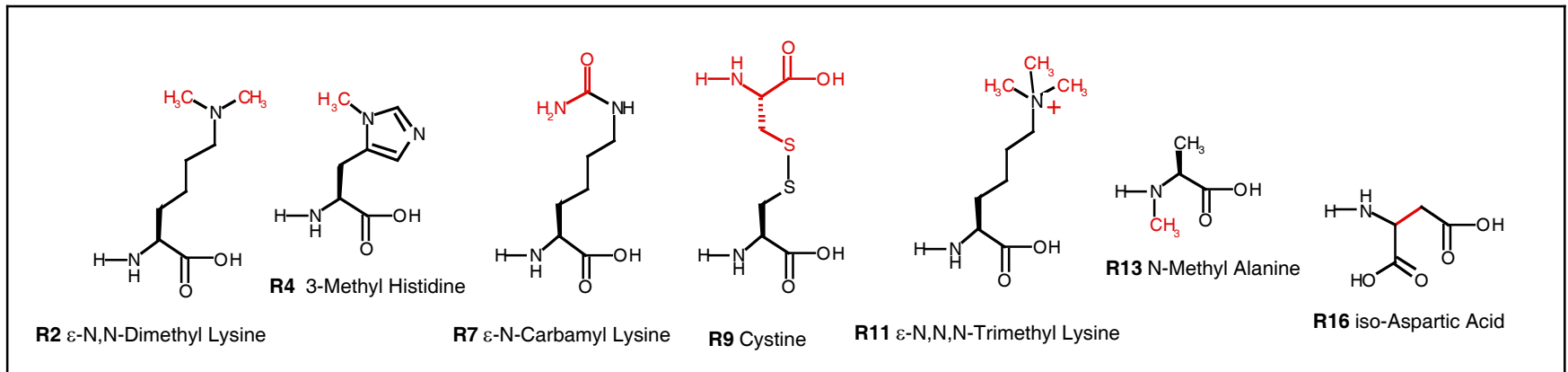
Synthetic, cysteine-9 disulfide-linked 18-mer peptide:

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Tyr-[Me₂-Lys]-Ala-[3-Me-His]-Lys-His-[homoCit]-Ala-Cys-Tyr-[Me₃-Lys]-Gly-[N-Me-Ala]-Tyr-Ala-[isoAsp]-Val-Arg



Tyr-[Me₂-Lys]-Ala-[3-Me-His]-Lys-His-[homoCit]-Ala-Cys-Tyr-[Me₃-Lys]-Gly-[N-Me-Ala]-Tyr-Ala-[isoAsp]-Val-Arg

Structures of the modified amino acids:



Sample Preparation

- **Solid-phase Fmoc peptide synthesis**
 - Fmoc-Arg-PEG-PS resin; 0.2 mmol scale
 - Fmoc-AA/HATU reagent for most AAs
 - PyAOP for Fmoc-3-Me-His and Fmoc Lys(Me₃)
- **Standard cleavage and RP-HPLC purification**
- **Cys oxidation to form a disulfide**
 - N,N,N',N',-tetramethylazodicarboxamide (“diamide”)
 - Meth. Enzymol. 143, 264-270, 1987

Requested Information

- **The amino acid sequence of the peptide**
- **Areas and retention times for peaks in each cycle**
- **Picomolar amounts, areas, and retention times for standards**
- **Information about sequencer, sample loading, HPLC equipment, gradient, solvents, flow rate, and column**
- **50 facilities requested the sample**
- **27 facilities returned sequencing data**

Sequencers Information

- 19 ABI 48X-HT, 7 ABI 49X-cLC, one 477A
- 23/27 used all ABI reagents
- 23 liquid phase, 4 gas phase
- 21 GFF, 6 PVDF or both (cycles matched type of support)
- Loaded 2-100% (40-2,000 pmol); mean 21.4% (430 pmol)
- All ABI Spheri-5 PTH columns (2.1mm or 0.8 mm I.D.)
- All ABI Solvent A (+ additives besides Premix)
- All ABI Solvent B or equivalent

- ESRG members data collected on:
 - 4 ABI 48X-HT, 3 ABI 49X-cLC, one Porton

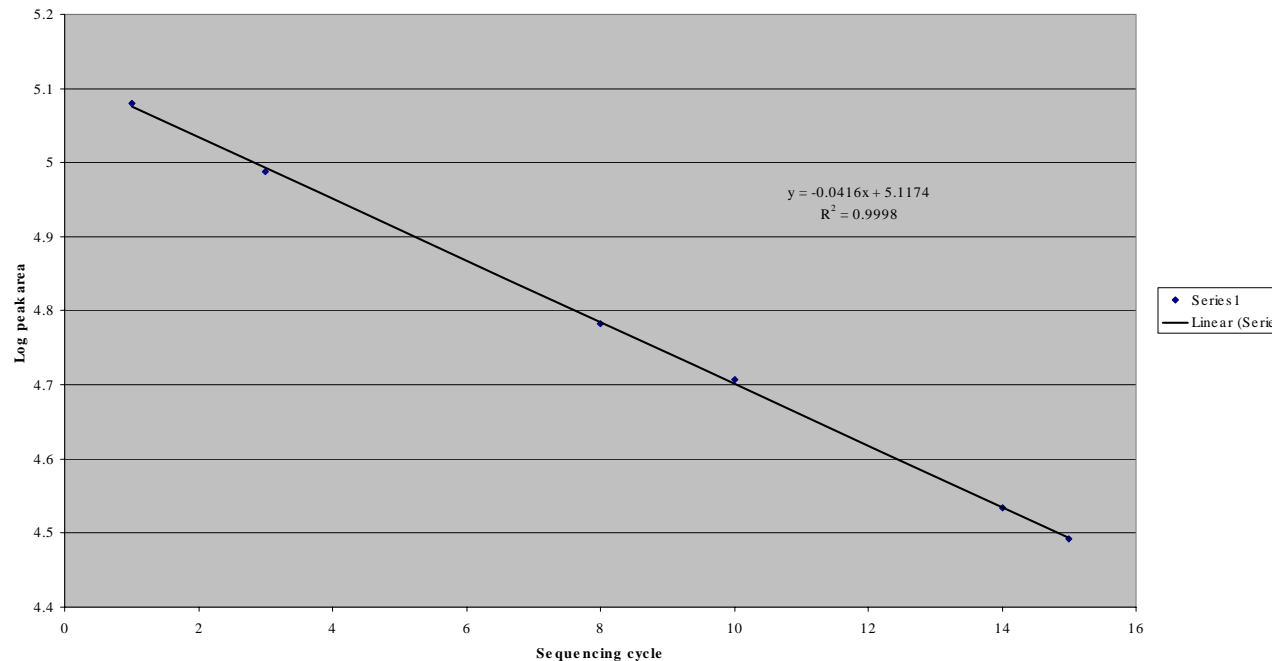
Initial & Repetitive Yields

1. Initial yields calculated from the equation:

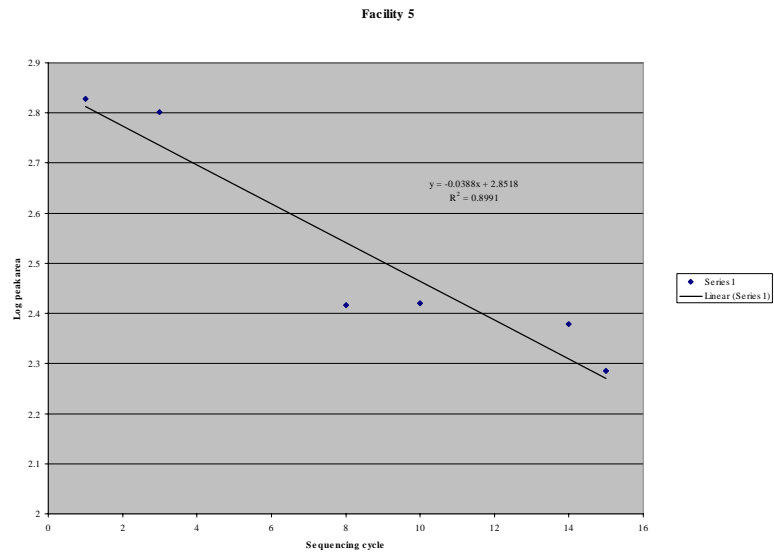
$$I.Y. = \frac{(\text{pmol of Tyr Std}) \times (\text{Area of Tyr 1 peak})}{2,000 \text{ pmol} \times (\% \text{ loaded}) \times (\text{Area of Tyr Std})}$$

2. Repetitive yields calculated from slope of trend line through plot of log A as a function of sequencing cycle, where values of A are peak areas of Tyr and Ala residues in the sample sequence. The slope of the trend line is the log of the R.Y.

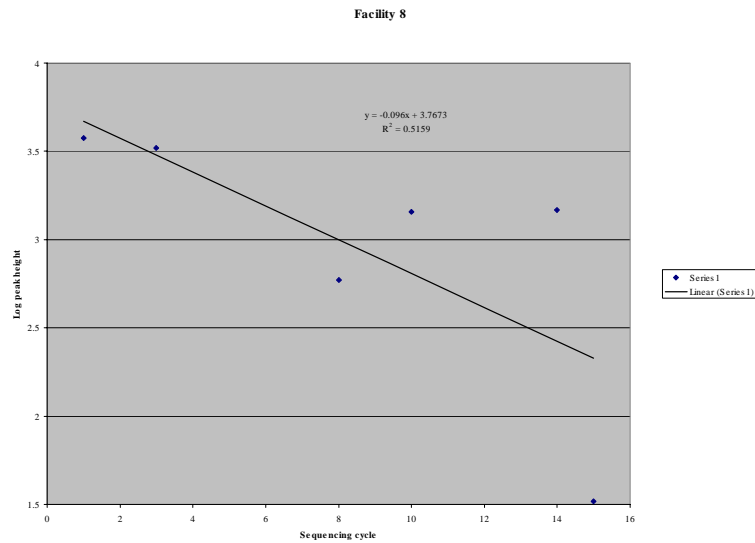
Facility 18



Repetitive Yield: Intermediate Result



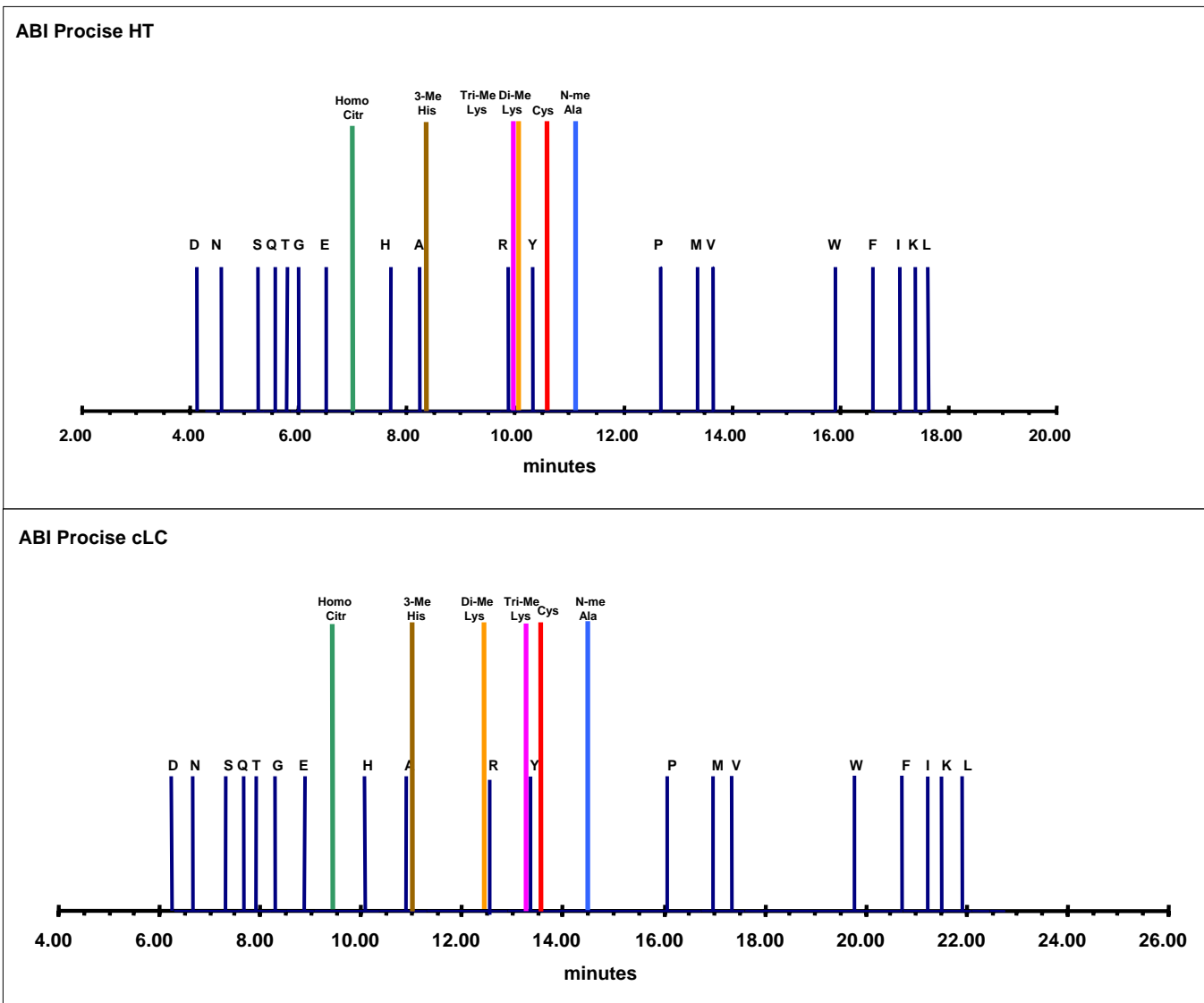
Repetitive Yield: Poor Result



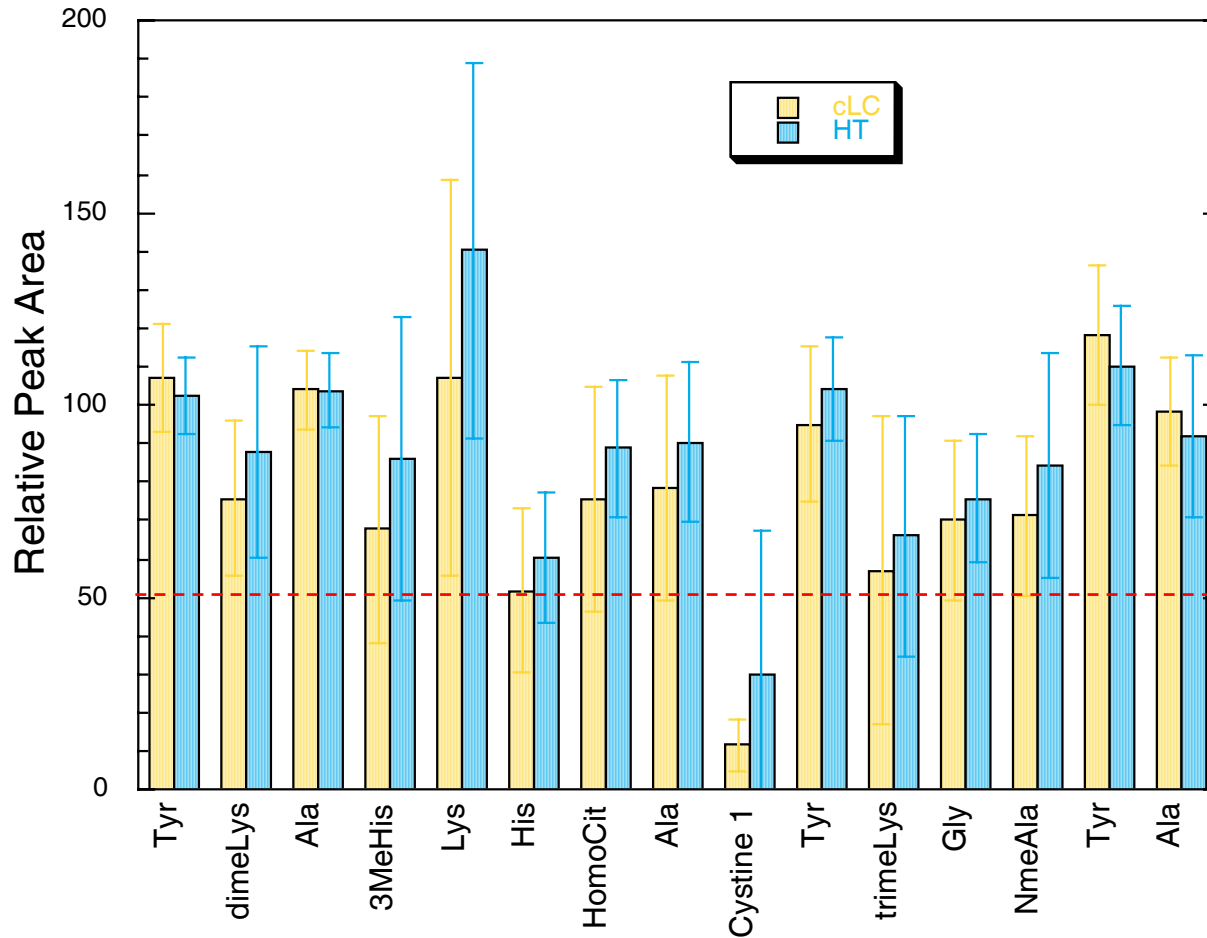
Normalized Retention Times for Amino Acids in Peptide

AA	494-HT Average SRTnA's	494-HT Av Full RT	494-HT Rel Peak Areas	n	494-cLC Average SRTnA's	494-cLC Av Full RT	494-cLC Rel Peak Areas	n	477	477 Full RT	Rel. Peak Area
Tyr	0.16	10.37	102.3%	23	0.16	13.37	107.2%	10	0.16	16.60	85.8%
dimeLys	0.13	9.91	88.0%	23	0.10	12.46	75.8%	10	0.12	15.60	58.0%
Ala	0.00	8.25	103.8%	23	0.02	11.18	104.1%	10	0.02	13.60	103.8%
3MeHis	0.01	8.41	85.9%	21	0.01	11.03	67.7%	10	0.02	13.50	71.6%
Lys	0.68	17.41	140.3%	22	0.68	21.47	107.0%	10	0.68	27.80	7.1%
his	-0.04	7.71	60.3%	23	-0.05	10.07	51.8%	10	-0.03	12.40	35.5%
HomoCit	-0.09	6.95	88.7%	22	-0.10	9.37	75.3%	10			
Ala	0.00	8.24	90.4%	23	0.00	10.87	78.3%	10	0.02	13.50	108.1%
Cystine 1	0.18	10.64	30.1%	20	0.17	13.55	11.6%	10			
Cystine 2	0.15	10.24	4.8%	8	0.15	13.17	7.0%	7			
Cystine 3	0.11	9.73	3.2%	7	0.10	12.48	2.4%	5			
Tyr	0.16	10.40	104.3%	23	0.16	13.37	94.9%	10	0.16	16.50	129.9%
trimeLys	0.12	9.89	65.9%	19	0.15	13.29	56.9%	10	0.19	17.10	41.8%
Gly	-0.16	6.03	75.6%	23	-0.17	8.23	70.0%	10	-0.14	10.10	130.1%
NmeAla	0.22	11.22	84.3%	22	0.23	14.47	71.3%	10		18.00	51.2%
Tyr	0.16	10.42	110.3%	23	0.16	13.36	118.1%	10	0.16	16.50	84.9%
Ala	0.00	8.25	91.9%	23	0.00	10.87	98.3%	10	0.01	13.30	93.9%

Time Lines for Elution of Standard and Modified Amino Acids on the Procise HT and Procise cLC



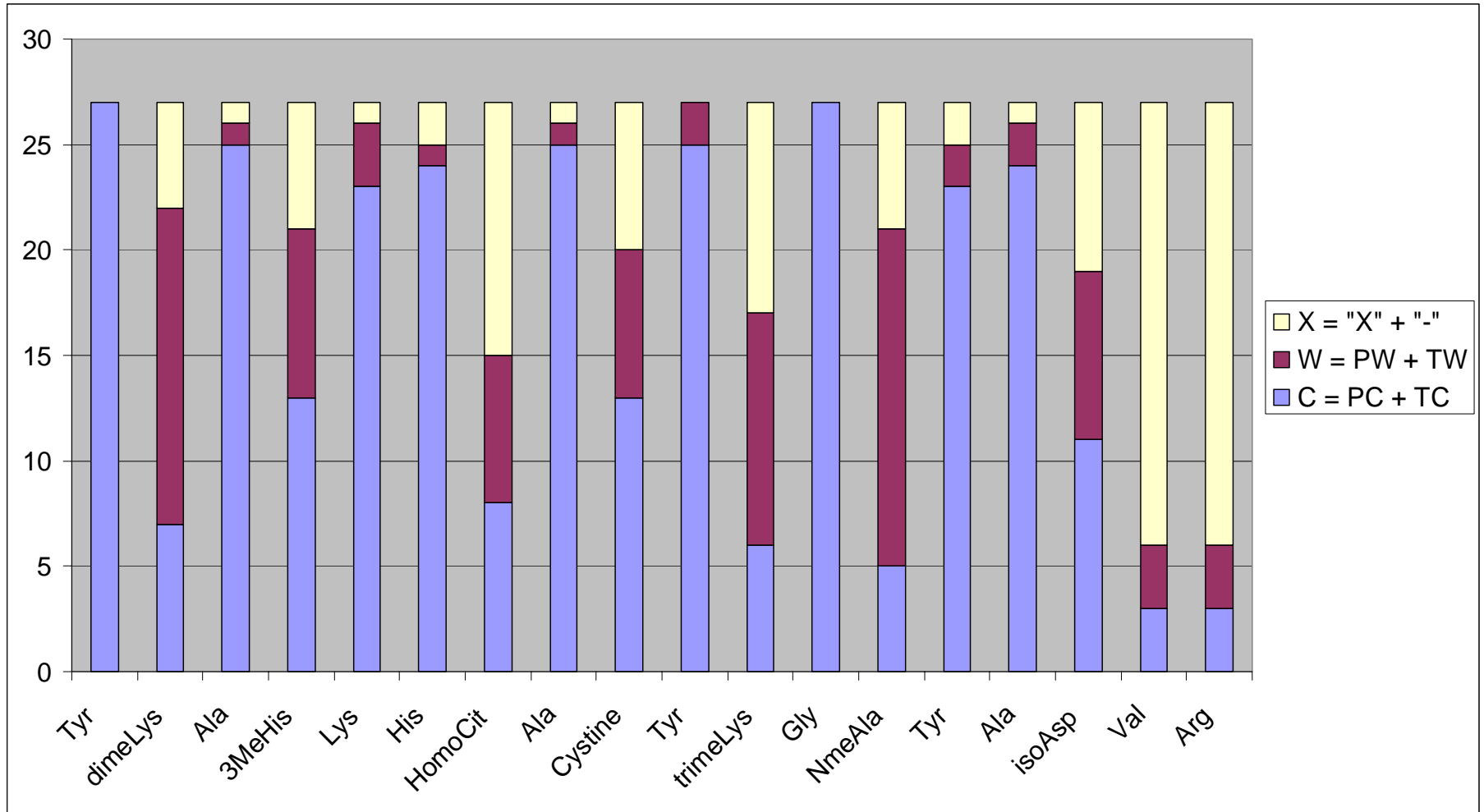
Average PTH Yields



ESRG2005 Sequence Assignment

- Provide positive calls (PC) for the primary amino acid for each position in the peptide
- Place tentative calls (TC) in parentheses
- Use "X" to denote unidentified peaks, and "-" when no peak observed
- Provide additional information as necessary in the Comments Section
- ESRG evaluation of accuracy of identifications:
 - PC = high confidence correct
 - TC = tentative correct
 - PW = high confidence wrong
 - TW = tentative wrong
 - X = "X" or "-" reported

Accuracy of Identification



Results / Discussion

- **IsoAsp (11/27)**

- Expected to block Edman degradation
- Presence of isoAsp-16 was inferred:
 - Drop of PTH signal in cycle 16
 - Combination of Edman and MS/MS to ID (several labs)
- Traces of Asp found in some labs
 - Beta-to-alpha conversion during SPPS
- Incorrectly identified: (8/27)
 - 3x Ala, 2x Glu, 1x Gla, 1x pThr, methylLys
 - PTH LC profiles unavailable to ESRG to allow detailed evaluation
 - Relative yields not calculated

Results / Discussion

- **HomoCitruline (8/27)**
 - Result of acylation with ammonium isocyanate
 - Major peak eluting between Glu / His
 - Incorrectly identified (7/27):
 - Cam-Met, Met(O₂), Cys, Asp
 - Relative yield: ~80%

Results / Discussion

- **N-methyl-Alanine (5/27)**
 - Major peak eluting between Tyr / Pro
 - Incorrectly identified: (16/27)
 - Arg, dimethyl-Lys, trimethyl-Lys, Abu, Canavanin
 - Relative yield: ~70%

Results / Discussion

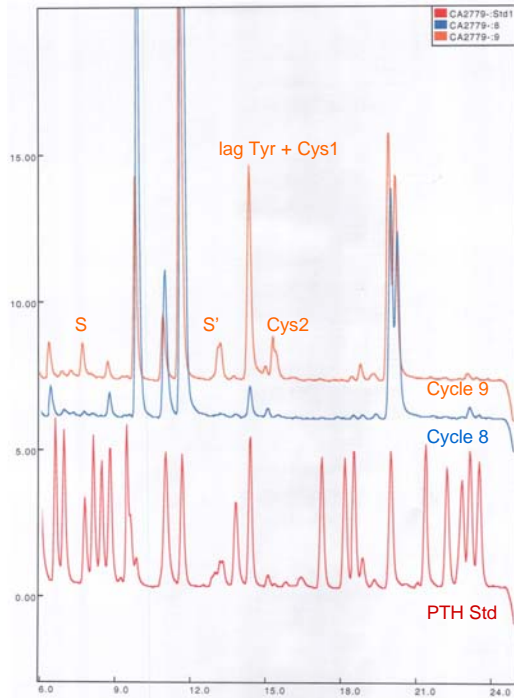
- **Cystine (13/27)**

- Highly unstable to Edman chemistry:
 - Reduction to CysSH by DTT in R4 (and S2)
 - Beta-elimination to anhydroSer (+ polymerization)
 - anhydroSer-DTT (S') adduct (possible co-elution w/Arg); Ser
- Close elution with Tyr (esp. on cLCs)
 - Relatively low yields: 10%-20%
 - Minor peak eluting after Tyr
- Cystine was assigned based on:
 - Prior knowledge of behavior PTH-Cysteine/Cystine
 - Combination of Edman and/or MS evidence before/after reduction&alkylation
- Incorrectly identified (7/27) as:
 - Tyr, Arg, Ser, pSer, methyl-Lys

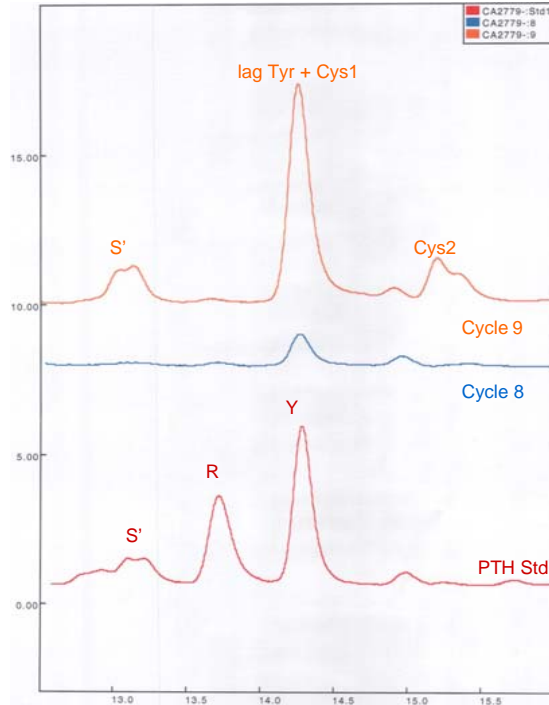
PTH Profile for Cystine Residue in Cycle 9 of

Effect of DTT in 25% TFA (R4) During Conversion

+ DTT, Standard Conditions

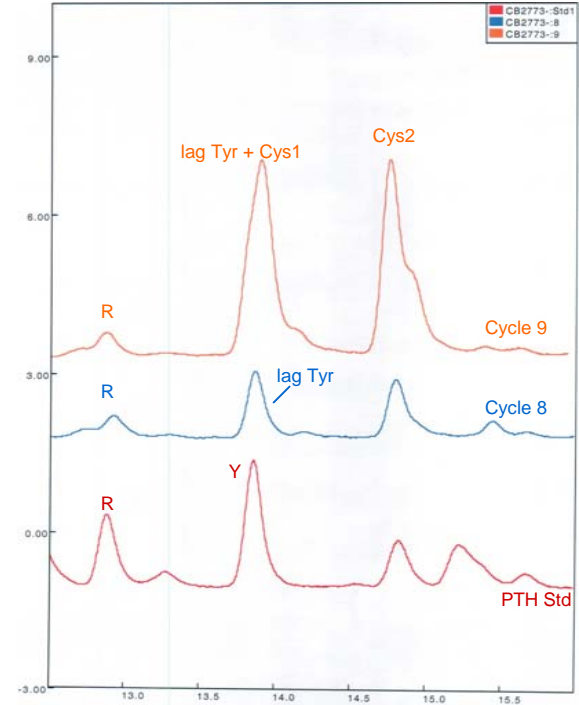


+ DTT, Standard Conditions



Cys2:Cys1 < 0.2

- DTT (High Premix Conc.)

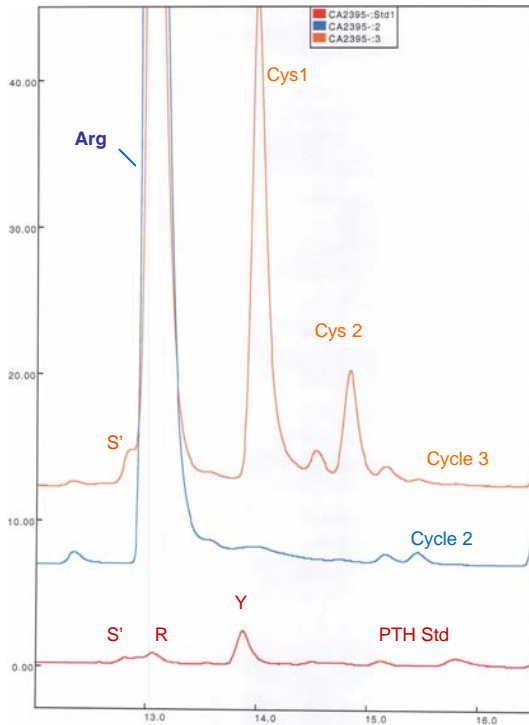


Cys2:Cys1 ≈ 1.0

Effect of (+/-) DTT on PTH Profile of Cystine Residue: Cycle 3, Model Homodimer Peptide

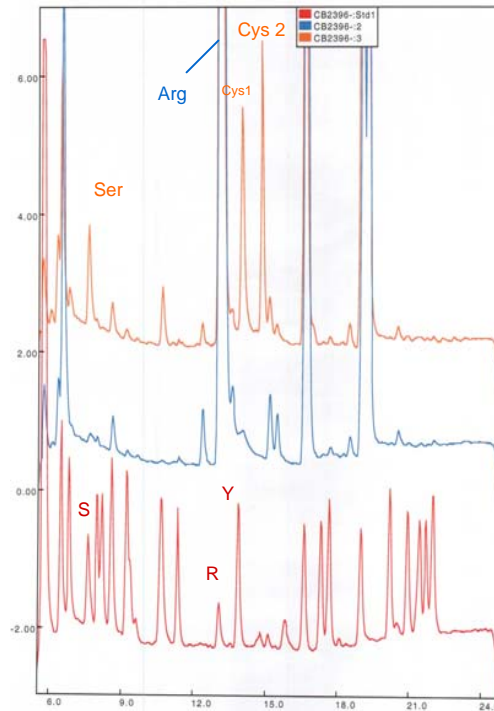


+ DTT, Standard Conditions

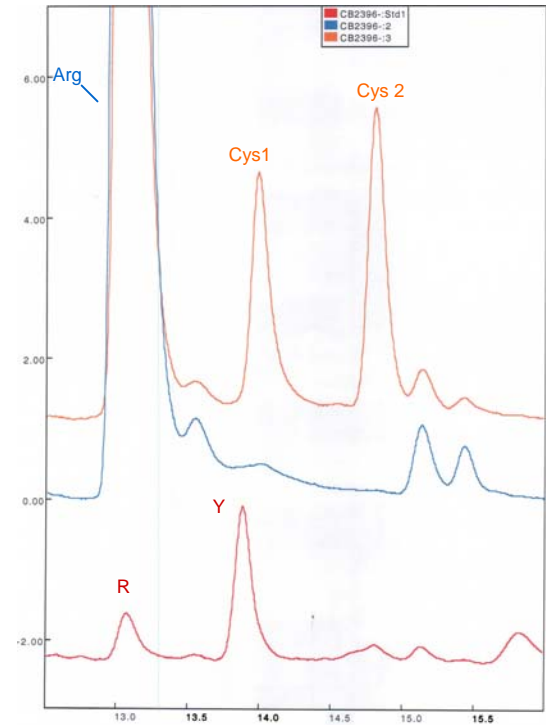


Cys2:Cys1 < 0.25

- DTT



- DTT



Cys2:Cys1 ≥ 1.0

Results / Discussion

- **3-Methyl-His (13/27)**
 - Difficult to assign: $W=8$
 - Partial co-elution with Ala (PW=3) on HTs and cLCs
 - Overlap with lag of Ala-3
 - Other PWs: Acetyl-Lys and Succinyl-Lys
 - Relative yield: ~70%

Results / Discussion

- **N- ϵ -dimethyl Lysine (7/27)**
 - Most incorrectly assigned (15/27)
 - Partial co-elution with Arg (PW=7) on HTs and cLCs
 - Other PWs: 3-Me-His, N-Me-Thr
 - Relative yield: 70%
 - Broader peak (similar to His and Arg)

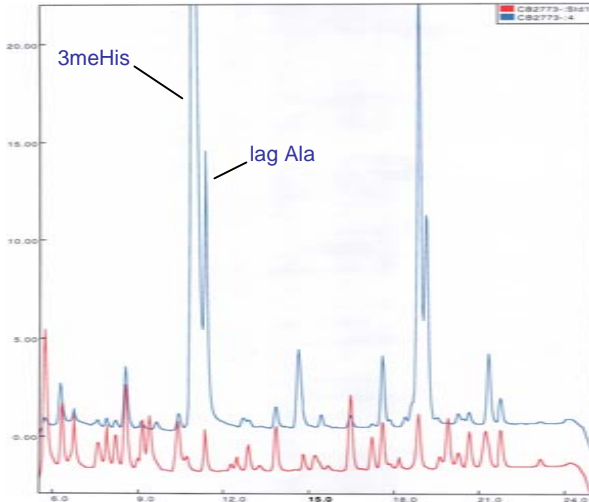
Results / Discussion

- **N- ϵ -trimethyl Lysine (6/27)**
 - 2nd most difficult to assign (W=11/27)
 - Partial co-elution with Arg and dimethylLys on HTs
 - Partial co-elution with Tyr on cLCs
 - Additional difficulty: lag of Tyr-10
 - Other PWs: Hyp, N- ϵ -Methyl-Lys, N-Me-Ala
 - Relative yield: ~60%
 - Broader peak (similar to His and Arg)

Elution Profiles of 3MeHis, Me₂Lys and Me₃Lys on cLC

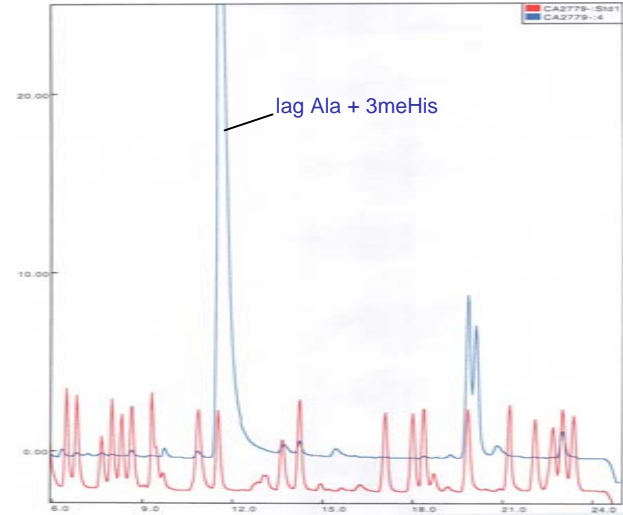
Effect of Premix Concentration: 21 ml/L vs. 14.5 ml/L

21 ml/L

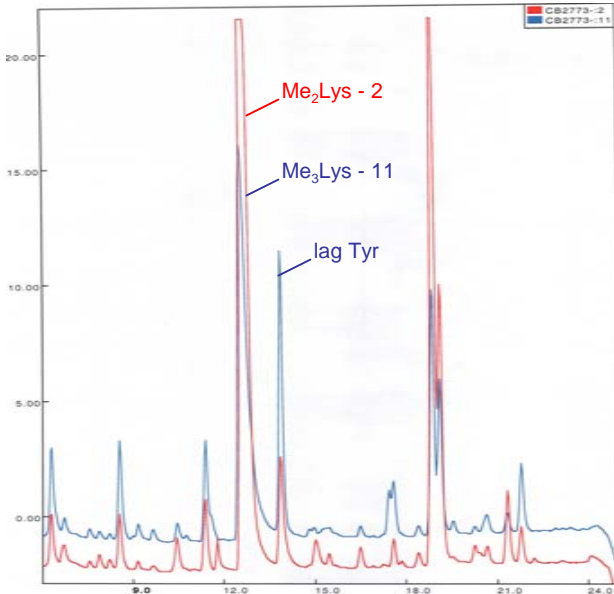


3MeHis
vs
Ala
(Std & Cycle 4)

14.5 ml/L

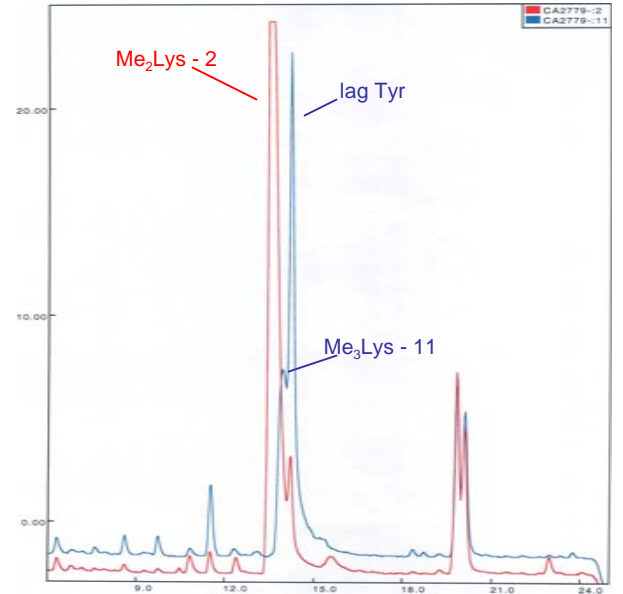


21 ml/L



Me₂Lys
vs
Me₃Lys
(Cycle 2 & 11)

14.5 ml/L



Effect of Increasing Premix Concentration on Elution Behavior of (+) Charged N-Methyl PTH-AAs

	<u>14.5 ml/L</u>	→ <u>21 ml/L</u>	<u>R.T. Effect</u>	<u>Base Type</u>
<u>3MeHis</u>	co-elutes Ala	separated Ala	← 3MeHis	tertiary
<u>Me₂Lys</u>	co-elutes Arg	co-elutes Arg	← on both	tertiary
<u>Me₃Lys</u>	co-elutes Tyr	separated Tyr co-elutes Me ₂ Lys	← ← Me ₃ Lys	quaternary

- The effect Premix is similar to effects on His and Arg
- The effect on Me₃Lys is more pronounced compared to 3MeHis, Me₂Lys and Arg
- PTH conditions may need to be optimized for modified AAs

“Sub-Optimal” Sequence of ESRG2005

- Placing closely eluting amino acid residues in juxtaposed positions in the sequence made correct assignment of modified amino acids more challenging:
 - Ala-[3MeHis]
 - [Cystine]-Tyr
 - Tyr-[Me₃Lys]

Conclusions

- **Relative retention times of the modified amino acids between similar instruments were very consistent.**
- **Sequencing and elution properties of the modified amino acids on the ABI Procise HT and cLC have been well characterized, along with a single example from an ABI 477 and a Porton sequencer.**
- **Assignment of the positively charged AAs proved to be challenging due to their co-elution with Ala, Arg, and Tyr.**

Acknowledgements

Thanks to all the participating laboratories for taking the time to analyze the sample and sending in their results. Without their participation, this effort would not have been successful.

Thanks to Dr. Anita Hong, Anaspec, Inc. for donating Fmoc-N-Me-Ala and Fmoc-Me₂Lys, and to Dr. Michael Pennington, Bachem Bioscience, Inc. for donating Fmoc-Me₃Lys, Fmoc-homoCit, and Fmoc-3-Me-His. Thanks also to Melinda Miller for removing identifiers from responding laboratories.