Journal of Biomolecular Techniques
17:308–326 © 2006 ABRF

ARTICLES

ABRF ESRG 2005 Study: Identification of Seven Modified Amino Acids by Edman Sequencing

D. Brune,[1] N.D. Denslow,[2] R. Kobayashi,[3] W.S. Lane,[4] J.W. Leone,[5] B.J. Madden,[6] J. M. Neveu,[4] and J. Pohl[7]

[1]*Arizona State University, Tempe, AZ;* [2]*University of Florida, Gainesville, FL;* [3]*UT MD Anderson Cancer Center, Houston, TX;* [4]*Harvard University, Cambridge, MA;* [5]*Pfizer, Inc., St. Louis, MO;* [6]*Mayo Clinic College of Medicine, Rochester, MN;* [7]*Emory University, Atlanta, GA*

Identification of modified amino acids can be a challenging part for Edman degradation sequence analysis, largely because they are not included among the commonly used phenylthiohydantion amino acid standards. Yet many can have unique retention times and can be assigned by an experienced researcher or through the use of a guide showing their typical chromatography characteristics. The Edman Sequencing Research Group (ESRG) 2005 study is a continuation of the 2004 study, in which the participating laboratories were provided a synthetic peptide and asked to identify the modified amino acids present in the sequence. The study sample provided an opportunity to sequence a peptide containing a variety of modified amino acids and note their retention times relative to the common amino acids. It also allowed the ESRG to compile the chromatographic properties and intensities from multiple instruments and tabulate an average elution position for these modified amino acids on commonly used instruments. Participating laboratories were given 2000 pmoles of a synthetic peptide, 18 amino acids long, containing the following modified amino acids: dimethyl- and trimethyl-lysine, 3-methyl-histidine, N-carbamyl-lysine, cystine, N-methyl-alanine, and isoaspartic acid. The modified amino acids were interspersed with standard amino acids to help in the assessment of initial and repetitive yields. In addition to filling in an assignment sheet, which included retention times and peak areas, participants were asked to provide specific details about the parameters used for the sequencing run. References for some of the modified amino acid elution characteristics were provided and the participants had the option of viewing a list of the modified amino acids present in the peptide at the ESRG Web site. The ABRF ESRG 2005 sample is the seventeenth in a series of studies designed to aid laboratories in evaluating their abilities to obtain and interpret amino acid sequence data.

Key Words: Edman sequencing, chemical sequencing, modified amino acid, phenylthiohydantoin (PTH) amino acid, retention time.

In recent years, more attention has been directed towards identifying amino acids in proteins that are post-translationally modified (PTM) because of their importance in protein structure and function. Some amino acid modifications, such as oxidation and carbamylation, can potentially occur during or as a result of the purification process. Edman sequencing and mass spectrometry are often the approaches taken to locate and identify modified amino acids in a protein or peptide, and success will often depend on the amount of protein as well as the sequence location and nature of the modification. While no one method is without its limitations, Edman sequencing offers the unique ability to determine the exact position of a modified amino acid within a peptide of lengths of up to 40 or more amino acids. The main drawbacks are that it requires pmol amounts of a relatively pure sample and relies on knowing where the phenylthiohydantoin (PTH) derivatives of the modified amino acids elute using the standard sequencing chromatography

Address correspondence and reprint requests to: Nancy D. Denslow, Department of Physiological Sciences, University of Florida, PO Box 110885, Gainesville, FL 32611 (phone: 352-392-4700 X 5563; fax: 352-392-4707; email: ndenslow@ufl.edu).

conditions. To address this issue, the Edman Sequencing Research Group (ESRG), a research group of the Association of Biomolecular Resource Facilities (ABRF), began compiling the elution characteristics for some of the common modified amino acids, starting with seven that were incorporated into the ESRG 2004 study sample,[1] including N,N-dimethyl arginine, N-ε-methyl lysine, phosphoserine, methionine sulfoxide, cysteine-S-proprionamide, N-ε-acetyl lysine, and 4-hydroxyproline.

The ESRG 2005 study sample, representing the seventeenth ESRG challenge,[1–16] builds on last year's study, with 6 additional modified amino acids being incorporated into an 18-amino-acid synthetic peptide and distributed for Edman sequencing analysis. The modified amino acids in this year's study are dimethyl lysine, trimethyl lysine, 3-methyl histidine, N-carbamyl-lysine, cystine, N-methyl-alanine, and isoaspartic acid. As in past studies, this allows participating facilities to evaluate their own performance compared to others, with the ultimate goal of validating procedures for the identification of PTMs, by Edman degradation. Again, participants were asked to return their results electronically, filling in a spreadsheet with the retention times and peak areas of the PTH-AA standards and the amino acids from the synthetic peptide. A list of the modified amino acids contained in the peptide and the original guide by Crankshaw and Grant[17] that first reported retention times for modified amino acids were placed on the ABRF Web site as a resource. Participants were challenged to identify as many modified amino acids as possible. The ESRG tabulated retention times and peak areas for both the known standards and amino acids in the peptide identified by the participating laboratories. They also calculated repetitive yields and other data related to the specific instruments used in the analyses.

MATERIALS AND METHODS

Fmoc derivatives of common amino acids were purchased from AnaSpec and Novabiochem. Fmoc derivatives of homocitrulline, 3-methyl-histidine, and trimethyllysine were generously donated by Dr. Mike Pennington, Bachem, and Fmoc derivatives of dimethyl-lysine and N-methyl-alanine were donated by Dr. Anita Hong, AnaSpec. Reagents used for Edman degradation sequencing by the research committee member laboratories were from Applied Biosystems, Inc. (ABI), except for those used in the Porton sequencer, which were as follows: R1, 5% PITC (ABI) diluted to 2% with heptane (Burdick & Jackson sequencing grade); R2, 2 mL diisopropylethylamine (Aldrich, double distilled) in 125 mL nanopure water; R3 and R4, 100% TFA and 25% TFA, respectively (JT Baker, high-performance liquid chromatography [HPLC] grade) in nanopure water; S1, 2.5–3.5 mg DTT (Research Organics) in 150 mL ethyl acetate (EMD Omnisolv) that had been treated with neutral alumina (100 g/L, Fluka type 507C) and filtered through a 0.2-μm nylon filter; and S2, 10% acetonitrile (Fisher Optima) in nanopure water.

The sequence of the synthetic 18-amino-acid peptide is shown in Figure 1. Structures of the modified amino acids are also illustrated. Similarly to the ESRG's 2004 study, we used repeats of tyrosine (positions 1, 10, 14) and alanine (positions 3, 8, 15) to help calculate initial and repetitive yields. We included an arginine residue at the C-terminus to impart a positive charge and minimize sample washout from the reaction cartridge during sequence analysis.

We used the following rationale for selecting the PTMs. Dimethyl-Lys, trimethyl-Lys and 3-methyl-His are all methylated amino acids, a common post-translational modification found in proteins such as histones, or actin.[18,19] The identification of these modifications is of functional importance. N-methyl-Ala is found at the N-terminus of many proteins[20] and thus may be encountered by most Edman sequencing groups. This modification can introduce a false preview of the following amino acid, which may lead to an incorrect assignment. N-carbamyl Lys (homocitrulline) was included because this modification occurs readily as a by-product of sample preparation when urea is used to dissolve proteins. The modification occurs spontaneously due to ammonium isocyanate present in the urea at pH > 7.[21] When this modification occurs, it prevents trypsin from cleaving at the lysine residue, resulting in a higher number of missed cleavages. Cystine was included to establish a PTH fingerprint for a disulfide-linked residue in a homodimer peptide. Mapping disulfide linkages provides structural and functional information about a protein, and we wanted to establish how this modification behaves under standard Edman degradation conditions on current protein sequencers. Lastly, we included iso-aspartic acid. This modification occurs via an aspartimide intermediate as a result of spontaneous asparagine deamidation in proteins, and can also occur during solid-phase peptide synthesis.[22] Hydrolysis of the aspartimide intermediate yields primarily isoaspartic acid, along with lower amounts of aspartic acid. Because of the position of the peptide bond, iso-aspartic acid is refractory to Edman degradation, and N-terminal sequencing terminates. Therefore, it was placed towards the end of the synthetic peptide.

Peptide Synthesis and Preparation

The ESRG 2005 peptide was synthesized on a Milligen-Biosearch 9050 peptide synthesizer using Fmoc chemistry,

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Tyr-[Me_2-Lys]-Ala-[3-Me-His]-Lys-His-[homoCit]-Ala-Cys-Tyr-[Me_3-Lys]-Gly-[N-Me-Ala]-Tyr-Ala-[isoAsp]-Val –Arg

S-S

Tyr-[Me_2-Lys]-Ala-[3-Me-His]-Lys-His-[homoCit]-Ala-Cys-Tyr-[Me_3-Lys]-Gly-[N-Me-Ala]-Tyr-Ala-[isoAsp]-Val –Arg

Structures of the modified amino acids:

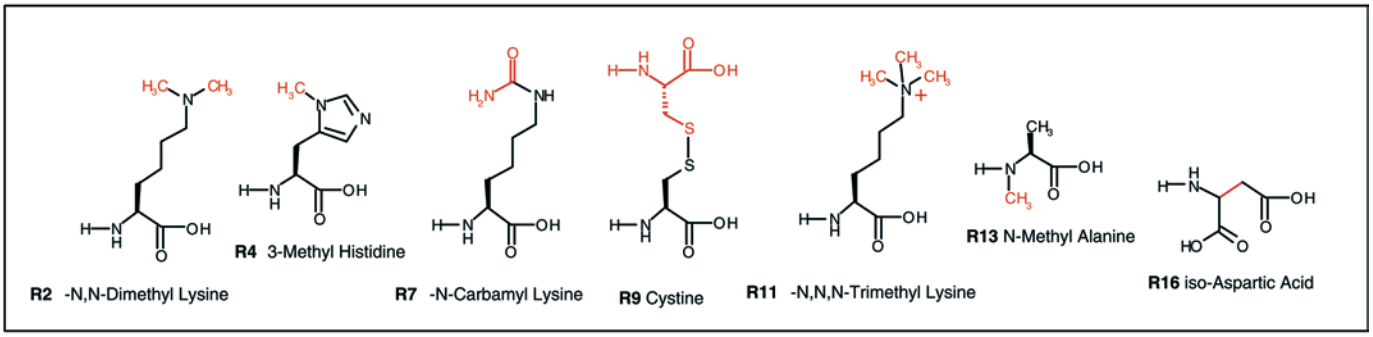

FIGURE 1

Description of the sample. A synthetic 18-amino-acid peptide was synthesized, containing seven modified amino acids. Cystine at position 9 linked the two chains together.

in the laboratory of one of the members of the ESRG. Synthesis was performed on Fmoc-Arg-PEG-PS resin (ABI) using a fourfold molar excess (0.8 mmol) of each Fmoc amino acid and the HATU coupling reagent, except in the cases of Fmoc-3-Me-His-OH and Fmoc-Lys(Me_3)-OH, which were available in smaller amounts. In these cases, PyAOP (0.7 mmol) was used as the coupling reagent. Three amino acids—Fmoc-Lys(Me_2)-OH, Fmoc-Lys(Me_3)-OH, and Fmoc-3-Me-His-OH—were injected manually during the synthesis; all others were dissolved and injected automatically. Coupling times for the methylated Lys residues were extended to 1 h, and coupling of Gly to N-Me-Ala was 45 min; all others were 30 min. The synthetic peptide was cleaved from the resin with 92.5% TFA with 2.5% each of triisopropylsilane, ethanedithiol, and water, precipitated by diethyl ether, and dried under vacuum.

The monomeric peptide was purified by reversed-phase HPLC on a Phenomenex C12 Proteo column (1 cm × 25 cm) using a gradient of 10–20% acetonitrile in water containing 10 mM TFA over 20 min. The disulfide-linked dimer was created by dissolving the peptide in 25 mM Na phosphate (pH 7.6) at a final concentration of 1.8 mM (determined from its 280-nm absorbance) followed immediately by addition of diamide to a final concentration of 0.9 mM.[23] After allowing the reaction to proceed for several minutes, the diamide concentration was increased to 1.3 mM, in order to drive the reaction to completion.

The dimeric peptide was then purifed by reversed-phase HPLC as described for the monomer, using a gradient of 12–22% acetonitrile in water containing 10 mM TFA over 20 min. The dimer product was confirmed by: (1) elution at a higher percentage of acetonitrile during reversed-phase HPLC, (2) the presence of a peak with twice the molecular mass of the monomer in MALDI-TOF mass spectrometry (monoisotopic $MH^+ = 4497.53 \pm 0.12$, average of two determinations) (Figure 2), and (3) earlier elution time than the monomer in size-exclusion chromatography on a Phenomenex SEC 2000 column (0.78 × 60 cm). The appropriate peptide peaks from all runs were pooled, aliquoted into PCR tubes for easy distribution, and stored at –20°C until shipped. The amount of the peptide dimer in each sample sent to study participants was determined to be 1000 pmol of the dimer (or twice that amount of the monomer, since the subunits were identical) from its absorbance at 280 nm. We calculated a molar extinction coefficient of 9065 for the peptide dimer, based on its composition of six Tyr residues and one disulfide bond. The calculation is based on molar extinction coefficients of 1490 and 125, for each Tyr and the disulfide bond, respectively.[24]

Sample Distribution

The ESRG announced the 2005 study by email to all ABRF members via the discussion board, as well as on the main ABRF page under "Open Research Studies" and on

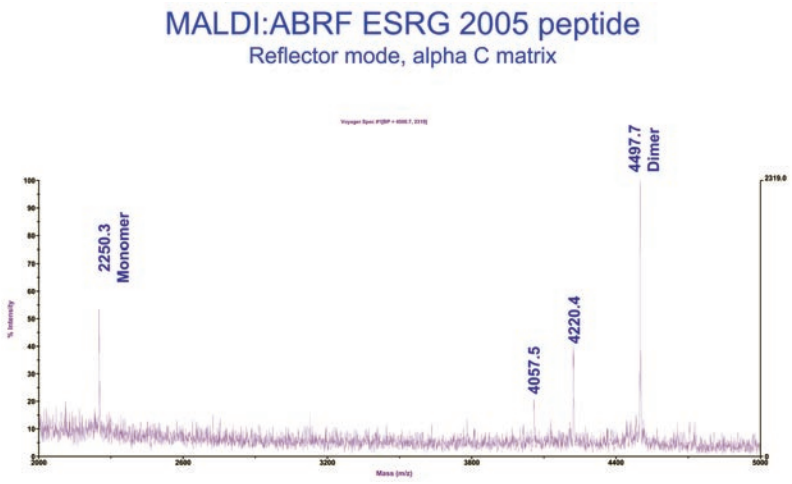

FIGURE 2

MALDI mass spectrometry results of ABRF ESRG synthetic peptide. The instrument was run in reflector mode using α-cyano-4-hydroxycinnamic acid matrix.

the ESRG page. A total of 49 requests for samples were received. Samples were sent out by regular mail to all who requested them.

Sample Analysis

ESRG members performed initial analyses to evaluate the quality of the peptide and data. The ESRG members analyzed the samples as they would normally for similar synthetic peptides, both by mass spectrometry and Edman sequencing. They obtained very comparable results, which imparted confidence in the design and synthesis of the peptide for the study. Participating laboratories were told that each sample contained approximately 1000 pmol of peptide that would be readily soluble in 50 µL to 100 µL of 30% acetonitrile containing 0.1% aqueous TFA. Using this knowledge of sample concentration, the laboratories were asked to load enough to obtain a good quality read for 18 cycles using their normal analytical conditions.

MALDI-TOF mass spectrometry was performed on the peptide dimer in two different laboratories using the Voyager DE STR and Voyager DE PRO mass spectrometers (ABI). Matrices used were α-cyano-4-hydroxycinnamic acid, sinapinic acid, and 4-hydroxybenzylidenemalononitrile, with similar results being obtained in all cases. Mass spectra were characterized by dominant peaks due to the singly charged peptide dimer (monoisotopic $m/z = 4497.53 \pm 0.12$) and the peptide monomer (monoisotopic $m/z = 2250.17 \pm 0.26$), with smaller peaks due to unidentified impurities with masses that were about 277.2 Da smaller than the monomer and dimer (Figure 2). In spite of the presence of two fixed positive charges per dimer due to the quaternary nitrogens of the trimethyl Lys residues, only a very weak peak corresponding to the doubly charged dimer (identified by 0.5-Da spacing between the isotopic peaks) was observed. This suggests that the dominant dimer peak contained a canceling negative charge, probably due to an unprotonated carboxyl group. The very low efficiency of the MALDI process in producing multiply charged ions has been noted previously, and is an important consideration in theoretical treatments of ion formation during this process.[25,26] The presence of a substantial monomer peak in the mass spectra of the peptide dimer may be due to disulfide reduction[27] or fragmentation of this bond during desorption and ionization.

Data Reporting

Each laboratory was asked to return data to the ESRG using an electronically transmitted Excel spreadsheet table that was sent to all participating laboratories. The table had entry slots for the retention times, pmol values, and peak areas for the standard amino acid phenylthiohydantoin derivatives (PTH-AAs) as well as for each of the 18 cycles of the synthetic peptide. As one of the modified PTH-AAs (cystine) results in more than one peak, the laboratories were asked to report the observed peaks in order of their elution from the column. Laboratories were asked to report known amino acids using the common three-letter amino acid code, to report "X" for unknown or modified amino acids, and to report "–" for no observed amino acid peaks for each cycle. They were also asked to indicate their confidence level in the call by placing parentheses around tentative calls.

In order to help participating laboratories identify the modified amino acids, the ESRG posted a list of the modified amino acids found in the peptide, but not their relative position in chromatograms. A reference of elution characteristics for commonly found modified amino acids, originally compiled by Crankshaw and Grant[17] in 1992, was also available on the ESRG Web site. Participants were asked to identify the unknown PTH-AAs using this

TABLE 1

Facility Number and Submitted Amino Acid Calls for Every Position in the Synthetic Peptide

Cycle	1	2	3	4	5	6	7	8	9
Expected	Tyr	Me2Lys	Ala	3MeHis	Lys	His	HomoCit	Ala	Cystine
111	Tyr	Arg	Ala	Ala	Lys	His	X	Ala	X
112	Tyr	Arg	X	Ala	Lys	X	X	X	Cystine
113	Tyr	iso-Asp	Ala	3MeHis	Me2Lys	His	HomoCit	Ala	Tyr
114	Tyr	(Me-His)	Ala	X	Me2Lys	His	X	Ala	X
115*	Tyr	Me2Lys	Ala	3MeHis	Lys	His	HomoCit	Ala	Cystine
116	Tyr	OMe-Thr	Ala	N-Acetyl-Lys	Lys	His	CAM-Met	Ala	Cystine
117	Tyr	Me2Lys	Ala	3MeHis	Lys	His	HomoCit	Ala	Cys
118	Tyr	Arg	Ala	(Ala)	Lys[1]	His	CAE-Cys	Ala	X
119	Tyr	N-MeAla	Ala	HomoCit	Lys	His	X	Ala	(Arg)[2]
120	Tyr	(N-MeAla)	Ala	(3MeHis)	Lys	His	X	Ala	(Cystine)
121	Tyr	X	Ala	X	Lys	His	X	Ala	X
122	Tyr	X	Ala	X	Lys	His	(Cystine)	Ala	X
123	Tyr	Arg	Ala	X	Lys	His	CAE-Cys	Ala	pSer
124	Tyr	(Arg)	Ala	(3MeHis)	Lys	His	X	Ala	(Cystine)
125	Tyr	(X)	Ala	3MeHis	Lys	His	(X)	Ala	Me3Lys
126	Tyr	Arg	Ala	Me3Lys	(Me2Lys)	His	HomoCit	Ala	X
127	Tyr	Me2Lys	Hydroxy-Lys	N-Succinyl-Lys	Lanthionine	CAM-Met	Met sulfone	Hydroxy-Lys	pSer
128*	Tyr	Arg	Ala	(3MeHis)	(HomoCit)	His	(iso-Asp)	Ala	Cys
129	Tyr	(biotinyl-Lys)	Ala	3MeHis	Lys	His	(CAM-Cys)	Ala	(Cystine)
130	Tyr	(Me2Lys)	Ala	(3MeHis)	Lys	His	(HomoCit)	Ala	(Cys)
131	Tyr	(Me2Lys)	Ala	3MeHis	Lys	His	HomoCit	Ala	Cystine
132*	Tyr	Me2Lys	Ala	3MeHis	Lys	His	HomoCit	Ala	Cystine
133	Tyr	3MeHis	Ala	N-MeAla	Lys	His	isoAsp	Ala	(Ser)
134	Tyr	(Arg)	Ala	(3MeHis)	Lys	His	X	Ala	(MeLys)
135	Tyr	(N-MeAla)	Ala	3MeHis	Lys	(HomoCit)	(isoAsp)	Ala	Cys
136	Tyr	Me2Lys	Ala	3MeHis	Lys	His	HomoCit	Ala	Cystine
137	Tyr	(N-MeAla)	Ala	(3MeHis)	Lys	His	(HomoCit)	Ala	(Cystine)

[1] Co-elutes with Ser
[2] Cystine
[3] Order of residues 3,4,8, and 15: 8<15<3<4. Would be very easy to call all Ala.
* These facilities correctly assigned residues 17 and 18 as Val and Arg, respectively, by MS/MS

Table continued

reference or their own expertise and report their assignments at each cycle.

In addition to the peptide-sequencing challenge, an instrument and analytical conditions survey was conducted to determine how the participating laboratories conduct Edman degradation. Each laboratory was asked to provide information on their instruments, including HPLC gradient conditions, buffers and solvents, chemistry cycles, and other parameters that may have affected the results of the study.

RESULTS AND DISCUSSION

Consistent with the trend observed in the past few years, almost all of the instruments used for this year's study were from ABI. Members of the ESRG also used a Porton sequencer. Normalized retention times for the standards

TABLE 1 (continued)

Cycle	10	11	12	13	14	15	16	17	18
Expected	Tyr	Me3Lys	Gly	N-MeAla	Tyr	Ala	isoAsp	Val	Arg
111	Tyr	X	Gly	Arg	Tyr	Ala	X		
112	Tyr	X	Gly	X	Tyr	X[3]	isoAsp		
113	Tyr	3MeCys	Gly	Me3Lys	N-MeAla	Ala	Glu		
114	Cystine	(N-MeAla)	Gly	(Me3Lys)	X	Ala	X		
115*	Tyr	Me3Lys	Gly	N-MeAla	Tyr	Ala	isoAsp	Val*	Arg*
116	Tyr	OMeLys, OMeGlu	Gly	Abu, Canavnine MeArg	Tyr	Ala	Gla		
117	Tyr	Me3Lys	Gly	N-MeAla	Tyr	Ala	isoAsp		
118	Tyr	Arg	Gly	X	Tyr	Ala	X		
119	Tyr	X	Gly	Me3Lys	Tyr	Ala	X		
120	Tyr	(N-MeAla)	Gly	(Me3Lys)	Tyr	Ala	(isoAsp)		
121	Tyr	X	Gly	X	Tyr	Ala	X		
122	Tyr	Tyr	Gly	X	Tyr	Ala	Ala		
123	Tyr	(Me2- or Me3Lys)	Gly	Me2Arg	Tyr	Ala	Ala		
124	Tyr	X	Gly	Me3Lys	Tyr	Ala	X		
125	Me2Lys	Cystine	Gly	(X)	Me2Lys	Ala	X		
126	Tyr	X	Gly	Cys	N-MeAla	3MeHis	isoAsp		
127	Tyr	Hydroxy-Pro	Gly	Abu	Tyr	Hydroxy-Lys	pThr		
128*	Tyr	(N-MeAla)	Gly	(Me2Lys)	Tyr	Ala	(MeLys)	Val*	Arg*
129	Tyr	(Me2Lys)	Gly	(Me3Lys)	Tyr	Ala	(isoAsp)		
130	Tyr	(N-MeAla)	Gly	(Me3Lys)	Tyr	Ala	(isoAsp)		
131	Tyr	Me3Lys	Gly	N-MeAla	Tyr	Ala	(iso-Asp)		
132*	Tyr	Me3Lys	Gly	N-Me Ala	Tyr	Ala	isoAsp	Val*	Arg*
133	Tyr	HomoCit	Gly	Cystine	Tyr	Ala	Ala		
134	Tyr	X	Gly	X	Tyr	Ala	X		
135	Tyr	X	Gly	(Me2-or Me3Lys)	(Tyr)	Ala	Glu		
136	Tyr	Me3Lys	Gly	N-MeAla	Tyr	Ala	(iso-Asp)		
137	Tyr	(Me2Lys)	Gly	(Me3Lys)	Tyr	Ala	(iso-Asp)		

and modified amino acids are also reported for these instruments to aid the broader community.

Identification Accuracy

The results from the participating laboratories are shown in Table 1. Each entry shows the facility number and call for each position in the synthetic peptide. In general, the results were excellent for the standard amino acids, as would be expected, since each laboratory received a large amount of sample (Figure 3). The designation for assignment accuracy was the same as in previous studies, where PC stands for a positive (high confidence) correct determination, TC for a tentative correct determination, PW for a positive (high confidence) wrong determination, TW for a tentative wrong determination, and X indicating an unknown amino acid (– or X reported). Some participants designated all modified amino acids with an X, but included comments giving either a positive or tentative identification. In these cases, the modified residues named in the comments are given in Table 1. When participants used an X at a position where an unmodified (standard) amino acid occurred in the sequence, it was scored as an incorrect (PW) call. In this report we have grouped together all correct calls (PC and TC) and all

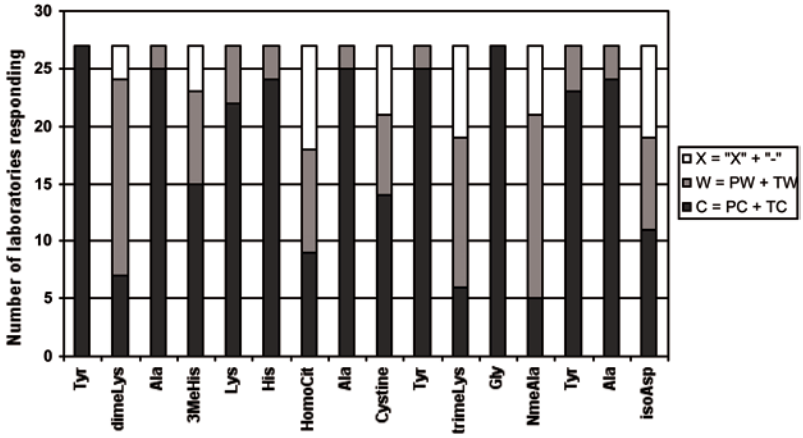

FIGURE 3

Accuracy of identification of each amino acid in the synthetic peptide. *C* is total correctly identified amino acids, including positive correct (PC) and tentative correct (TC) calls. *W* is total wrongly identified amino acids, including positive wrong (PW) and tentative wrong (TW). *X* is a position occupied by a modified amino acid for which there was no call.

incorrect calls (PW and TW). We expected that participating laboratories would have trouble with the modified amino acids, since these are not routinely encountered and only a few references of retention times have been published. One of the goals of the study was to provide a means to expand the experience for participating laboratories with these amino acids. In addition, we wanted to determine average retention times for each PTM to serve as a reference for future work.

Actual retention times for the modified amino acids for the Procise HT and cLC are shown in Figures 4 and 5, respectively. Four of the modified amino acids in the peptide were more problematic than the others, including dimethyl- and trimethyl-lysine, 3 methyl-histidine, and cystine. The PTH derivatives of these four amino acids exhibit different retention times depending on the conditions of the run, and can be difficult to identify because they sometimes elute close to the standard amino acids.

The first modified amino acid in the peptide, dimethyl-lysine, was at residue 2, and was among the three most incorrectly identified amino acids; the others were trimethyl-lysine (residue 11) and N-methyl alanine (residue 13). Both dimethyl- and trimethyl-lysine elute close to Arg on the Procise HT (Figure 4), and as expected they were misidentified as Arg or each other (Table 1). Both of these residues are broad peaks, similar to the shapes of His and Arg. On the Procise cLC, dimethyl-Lys elutes just before Arg, while trimethyl-Lys elutes just before Tyr (Figure 5), which caused some of the laboratories to misidentify trimethyl-Lys as Tyr (Table 1). Other incorrect calls for dimethyl-Lys included methyl-His, methyl-Ala, methyl-Thr, biotinylated-Lys, and iso-Asp. Other incorrect calls for trimethyl-Lys included methyl-Cys, methyl-Ala, methyl-Glu, cystine, hydroxy-Pro, and homocitruline.

The positions of dimethyl- and trimethyl-Lys change considerably depending on the concentration of premix used. On the cLC, for example, when premix is added at a concentration of 21 mL/L, it is easy to distinguish Tyr from both of the modified residues, dimethyl-Lys and trimethyl-Lys, but the two PTMs co-elute with each other at these conditions. But, at 14.5 mL/L, trimethyl-Lys is a shoulder on the Tyr peak and dimethyl-Lys is clearly resolved from both (Figure 6).

Only five respondents correctly identified N-methyl Ala, at position 13. It was the least correctly identified modified amino acid. It elutes as a major peak with a relative yield of about 70% in an uncrowded area of the chromatogram between Tyr and Pro. It was misidentified as Arg, dimethyl-Lys, canavanin, aminobutyric acid (Abu), methyl-Arg, and cystine.

3-Methyl-His was present at residue 4. Fifteen of the collaborating laboratories were able to identify it (Figure 3). It elutes as a broad peak close to Ala in both the Procise HT and cLC (Figures 5 and 6), and as expected, the most common misidentification was Ala. One facility (112) correctly noted the slight difference in retention times between 3-Me-His and Ala, but incorrectly identified residue 4 as Ala and designated Ala residues at positions 3, 8, and 15 as X. Other incorrect calls included N-Ac-Lys, homocitruline, trimethyl-Lys, and N-succinyl-Lys. The position of this amino acid also depends on the concentration of premix used (Figure 6). On the cLC, it can easily be distinguished from Ala when premix is used at a concentration of 21 mL/L, while it definitely co-elutes with Ala at a concentration of 14.5 mL/L. From these results, it appears that if modified amino acids are suspected or sought, two runs should be performed, using different concentrations of premix. Table 2 summarizes the effects on retention time that are observed on these three amino acids with changes in premix concentration.

The other relatively difficult modified amino acid to assign was cystine, which was incorporated into our

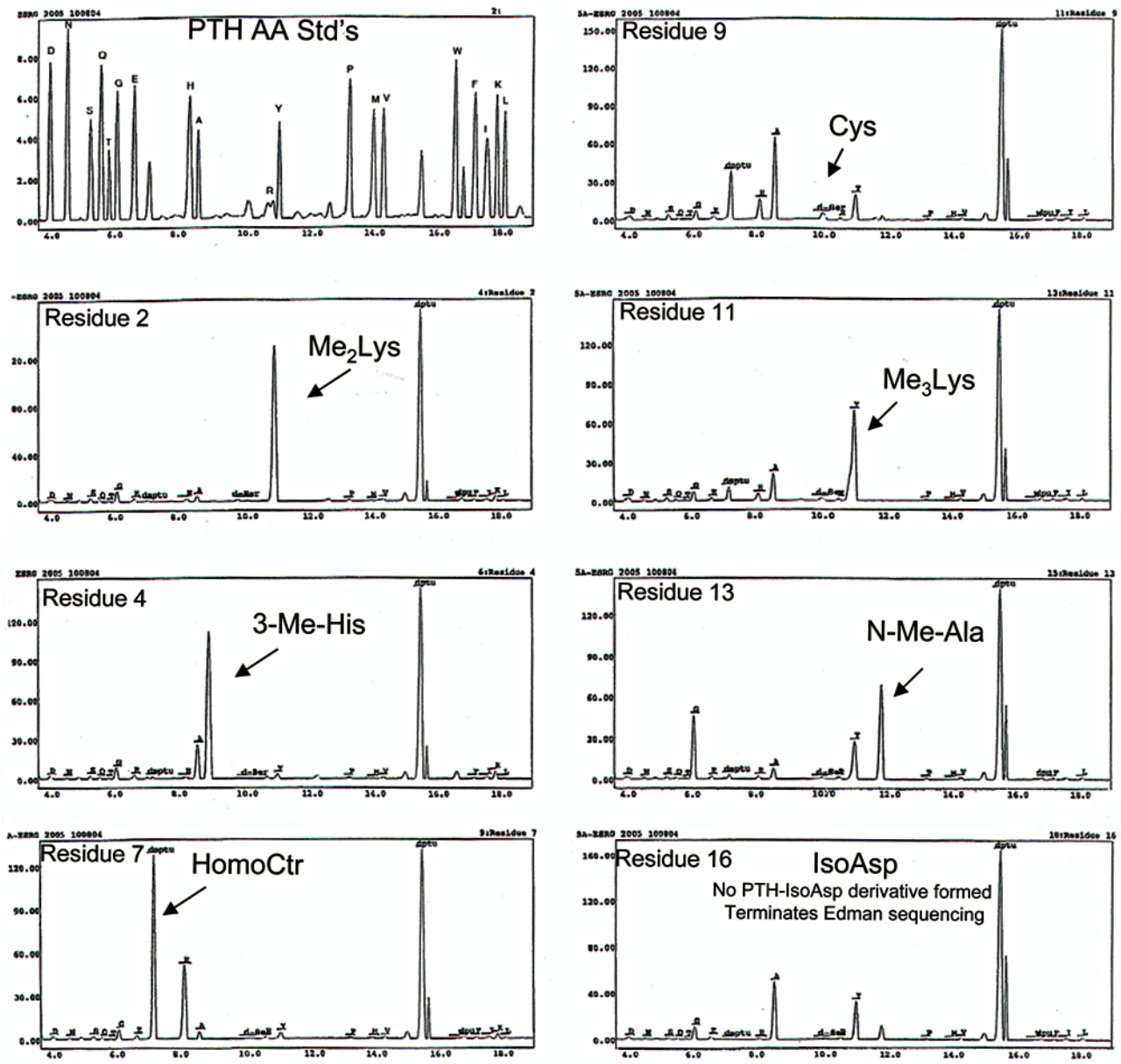

FIGURE 4

HT profiles. HPLC profiles for cycles for modified amino acids on the HT Procise instrument. Positions of the modified amino acids are indicated by arrows.

synthetic peptide at position 9. It was identified as cysteine or cystine, both of which were scored as correct, by fourteen of the participating laboratories. It was easily distinguishable from Tyr on both the HT and the cLC. It was misidentified as Arg, Ser, P-Ser, and methyl- and trimethyl-Lys. Cystine is highly unstable during Edman degradation. It is readily reduced to cysteine by DTT present in R4 and sometimes present in S2. It also is susceptible to beta elimination, desulfurization, and anhydroSer polymerization. AnhydroSer and its DTT adduct are also formed from cysteine. Identifying this difficult amino acid specifically as cystine requires prior knowledge of the behavior of its PTH derivative by Edman degradation. This identification is also facilitated by information derived from mass spectrometry.

How does one differentiate between cysteine and cystine based on Edman degradation alone? One ESRG member performed additional analyses in the presence and absence of DTT, to reduce the disulfide bond. There are at least two peaks that are present in the PTH chromatogram that derive from this amino acid. In the presence of DTT,

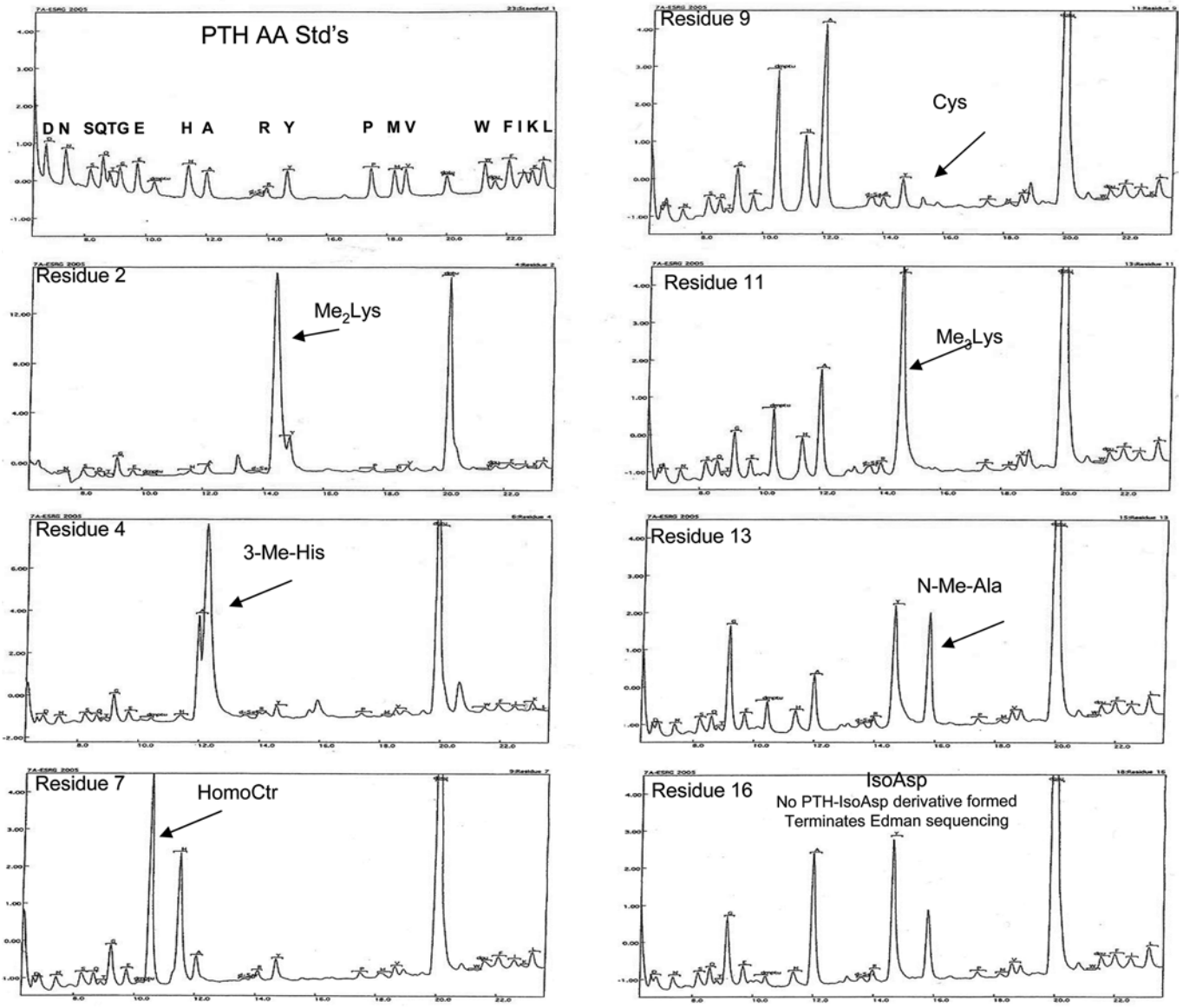

FIGURE 5

cLC profiles. HPLC profiles for cycles for modified amino acids on the cLC Procise instrument. Positions of the modified amino acids are indicated by *arrows*.

the main peak for cysteine (Cys_1) co-elutes with a peak that is normally present for Tyr. A second Cys_2 peak is small by comparison and is hard to distinguish from the baseline. In the absence of DTT, the second Cys_2 peak increases and is almost the same height as the Cys_1 peak (Figure 7). If one performs the run in the presence and absence of DTT, the relative changes in these two peaks will confirm the presence of cystine. In other words, if Cys_2:Cys_1 is less than 0.2 in the presence of DTT and about 1 in the absence of DTT, it points to a cystine at that position.

To confirm that this relative change in the two peaks is not peptide specific, a member of the ESRG used a different homodimer peptide, containing nine amino acids, with the cystine in position 3. This peptide did not contain tyrosine, and thus co-elution of Cys_1 with Tyr was not a problem (Figure 8). Again, in the presence of DTT the Cys_2 peak is relatively low compared to the Cys_1 peak, ratio less than 0.25. But in the absence of DTT, Cys_2:$Cys_1 \geq 1$. This experiment indicates that the Cys_1 peak can be identified as PTH-Cys, which is formed by reduction of PTH-cystine, which elutes as the Cys_2 peak. The earlier-eluting Cys_3 peak is due to the DTT adduct of anhydroSer (dehydroAla).

Homocitrulline (carbamyl Lys) was present at position 7. Only nine of the participating laboratories cor-

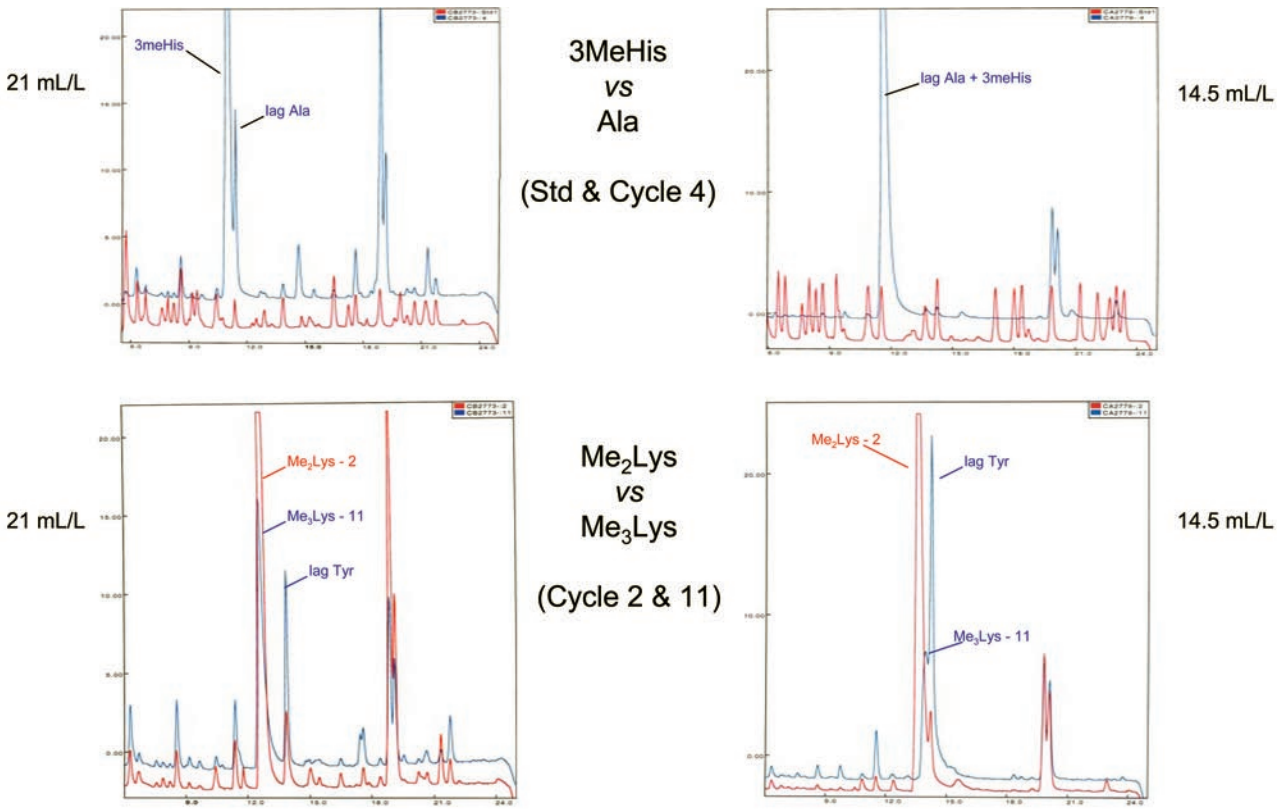

FIGURE 6

Effect of changing the premix buffer concentration from 21 mL/L to 14.5 mL/L on the elutions of PTH-3MeHis, PTH-Me_2Lys, and PTH-Me_3Lys. The Procise cLC was used for this analysis.

rectly identified this amino acid. It elutes in a clear area between Glu and His with a relative yield of about 80% on both the Procise HT and cLC, and should not have been difficult to assign. It was misidentified as carboxamidomethyl (CAM)-Met, Met(O_2) cystine, carboamidoethyl (CAE-Cys), Met sulfone, IsoAsp, and CAM-Cys.

IsoAsp was present at position 16 (Figures 4 and 5). This amino acid results in the termination of Edman chemistry sequencing. The presence of iso-Asp was inferred from a sudden drop in PTH signal and by a combination of Edman and MS/MS. Traces of Asp were found by some laboratories; this finding may be due to the beta-to-alpha conversion during SPPS. It was misidentified as Glu, Gla, Ala, pThr, and methyl-Lys. Three laboratories also correctly identified the two final amino acids, Val and Arg, by mass spectrometry.

Repetitive and Initial Yields

ESRG members calculated initial yield (IY) and repetitive yield (RY) for each of the participating laboratories. Peak areas of all PTH-AA standards and those of called amino

TABLE 2

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

	14.5 mL/L	21 mL/L	R.T. Effect	Base Type
3MeHis	Co-elutes with Ala	Separated from Ala	3MeHis	Tertiary
Me2Lys	Co-elutes with Arg	Co-elutes with Arg	On both	Tertiary
Me3Lys	Co-elutes with Tyr	Separated from Tyr Co-elutes with Me2Lys	Me2Lys Me3Lys	Quaternary

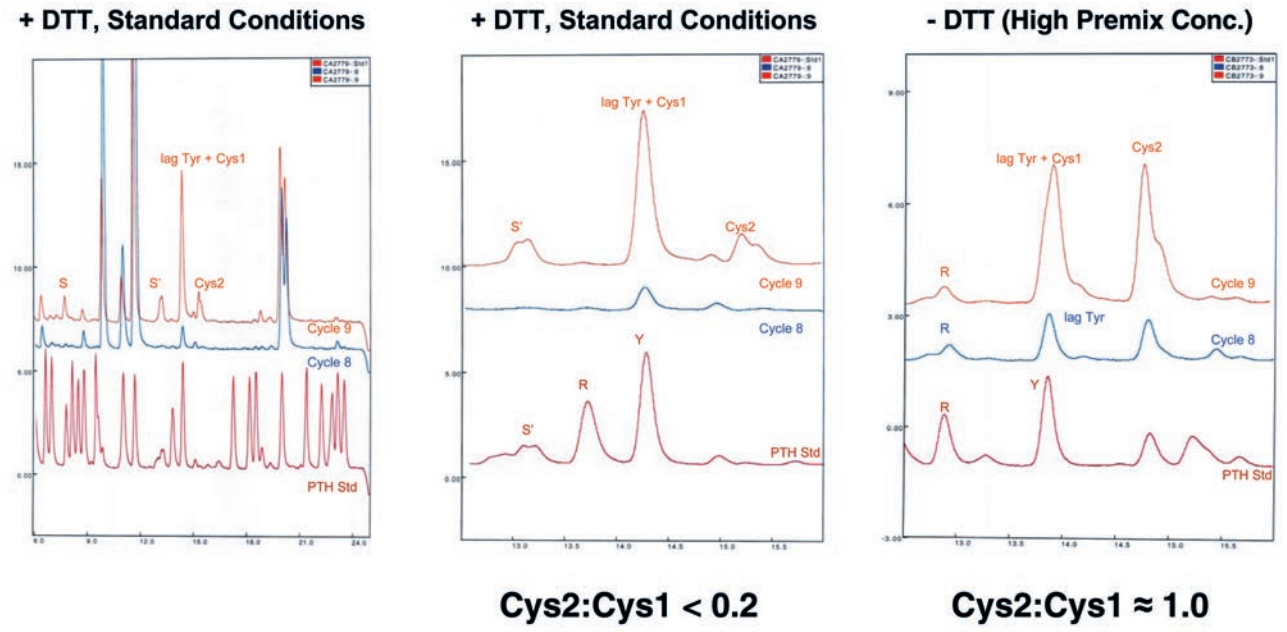

FIGURE 7

Effect of adding DTT to the R4 reagent (25% TFA) on the PTH-Cys elution profile in the test peptide.

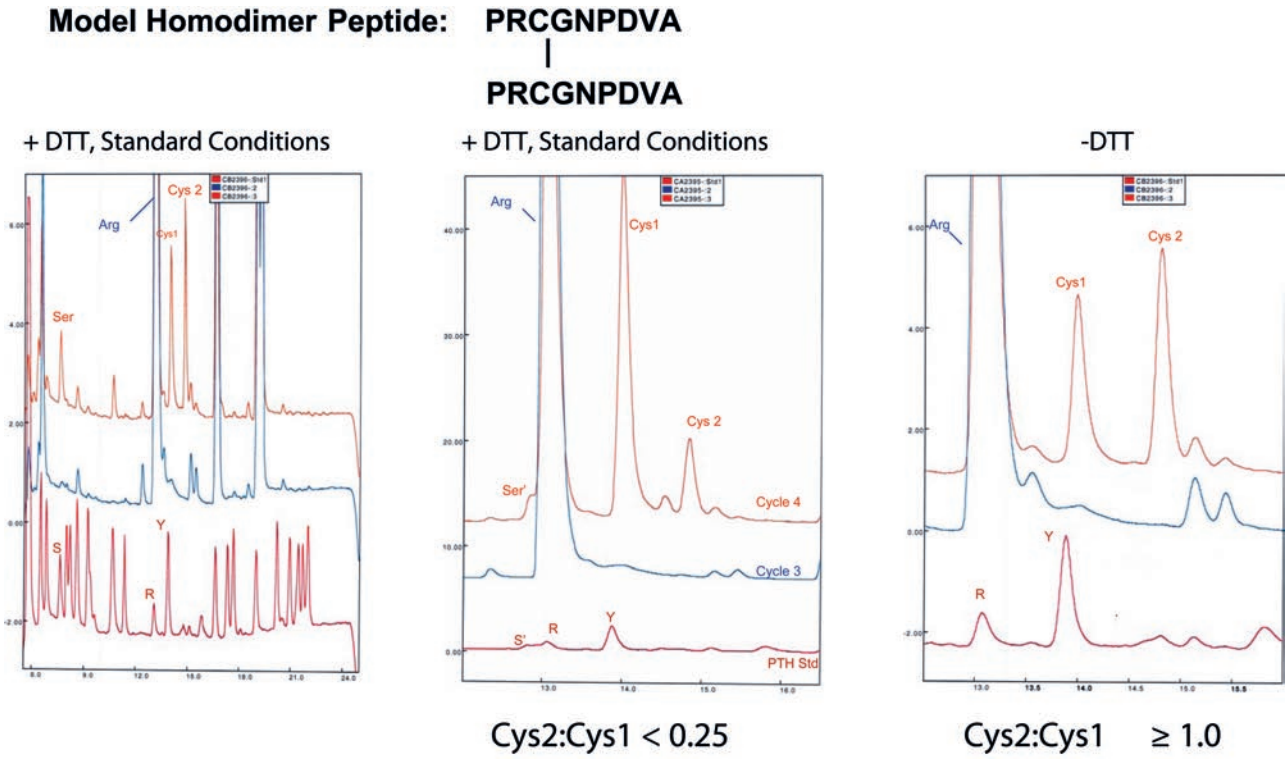

FIGURE 8

Effect of adding DTT to the R4 reagent on the PTH-Cys profile in the model homodimer peptide, PRCGNPDVA.

TABLE 3

Analysis of Data Submitted by Facilities

Facility Number	Instrument Type	% Loaded	Initial Yield	Repetitive Yield	PC	TC	PW	TW	X
111	494-HT	2.5%	126.4%	90.7%	9	0	3	0	4
112	494-HT	5.0%	48.9%	88.7%	7	0	6	0	3
113	494-HT	100.0%	29.8%	95.2%	9	0	7	0	0
114	477	25.0%	4.3%	90.4%	6	0	2	4	4
115	494-HT	10.0%		91.5%	16	0	0	0	0
116	494-cLC	10.0%	39.5%	91.7%	10	0	6	0	0
117	494-HT	10.0%	60.6%	95.1%	16	0	0	0	0
118	494-HT	50.0%	61.7%	91.4%	9	0	2	2	3
119	494-HT	15.0%	70.2%	92.8%	9	0	3	1	3
120	494-HT	30.0%	30.7%	96.7%	9	3		03	1
121	494-HT	20.0%	68.0%	91.7%	9	0	0	0	7
122	494-HT	20.0%	8.5%	95.2%	9	0	2	1	4
123	494-HT	10.0%	30.1%	89.9%	9	1	5	0	1
124	494-cLC	20.0%	2.0%	90.1%	9	2	1	1	3
125	494-cLC	10.0%	28.4%	90.6%	8	0	4	0	4
126	494-HT	100.0%	40.7%	88.0%	8	0	6	0	2
127	494-HT	30.0%	35.6%	91.4%	5	0	11	0	0
128	494-HT	15.0%	32.6%	90.9%	9	1	1	5	0
129	494-cLC	2.7%	33.8%	93.7%	10	2	0	4	0
130	494-HT	10.0%	53.1%	90.7%	9	5	0	2	0
131	494-HT	10.0%	31.6%	93.9%	14	2	0	0	0
132	494-cLC	2.0%	44.7%	93.2%	15	1	0	0	0
133	494-cLC	4.0%	15.9%	86.0%	9	0	6	1	0
134	494-HT	30.0%	62.5%	94.9%	9	1	0	2	4
135	494-HT	10.0%	46.2%	87.3%	9	1	1	4	1
136	494-cLC	4.0%	72.0%	91.4%	15	1	0	0	0
137	494-HT	50.0%	49.9%	93.5%	9	4	0	3	0

acids at each cycle were tabulated and applied in the following formula to obtain the IY:

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

"2000 pmol" was the total supplied sample for the study based on the two identical subunits, "% Loaded" was as reported by each facility, and the remaining values were as stated for each data set reported. The results for each facility are shown in Table 3.

Repetitive yields (RY) were calculated using the multiple Tyr and Ala residues designed specifically for this purpose in the synthetic peptide. Tyr was at positions one, ten and fourteen, while Ala was at positions three, eight and fifteen, giving six positions out of 18 from which to calculate the RY. Repetitive yields were calculated from the slope of the trend line of a plot of log A as a function of sequencing cycle, where values of A are peak areas of Tyr or Ala residues in the sample sequence. The slope of the trend line is the log of the RY, as shown in Figure 9A, which is an excellent result, with an R^2 of 0.9998. Some laboratories had slopes that showed intermediate goodness of fit (Figure 9B) and others had poor results (Figure 9C). Repetitive yields ranged from a low value of 86% to a high value of 96.7% and results were correlated to a certain extent with repetitive yield.

Retention Times of Standard and Modified PTH-AAs

As was done in last year's study,[1] we determined relative retention times for standard amino acids (Table 4) and the normal and modified amino acids of the synthetic peptide (Table 5). The absolute retention times reported by the facilities varied somewhat from each other due to column and gradient differences as well as other parameters influenced by the type of instrument used. The actual elution order for the amino acids on all the instruments was the same. We first separated the results by instrument type

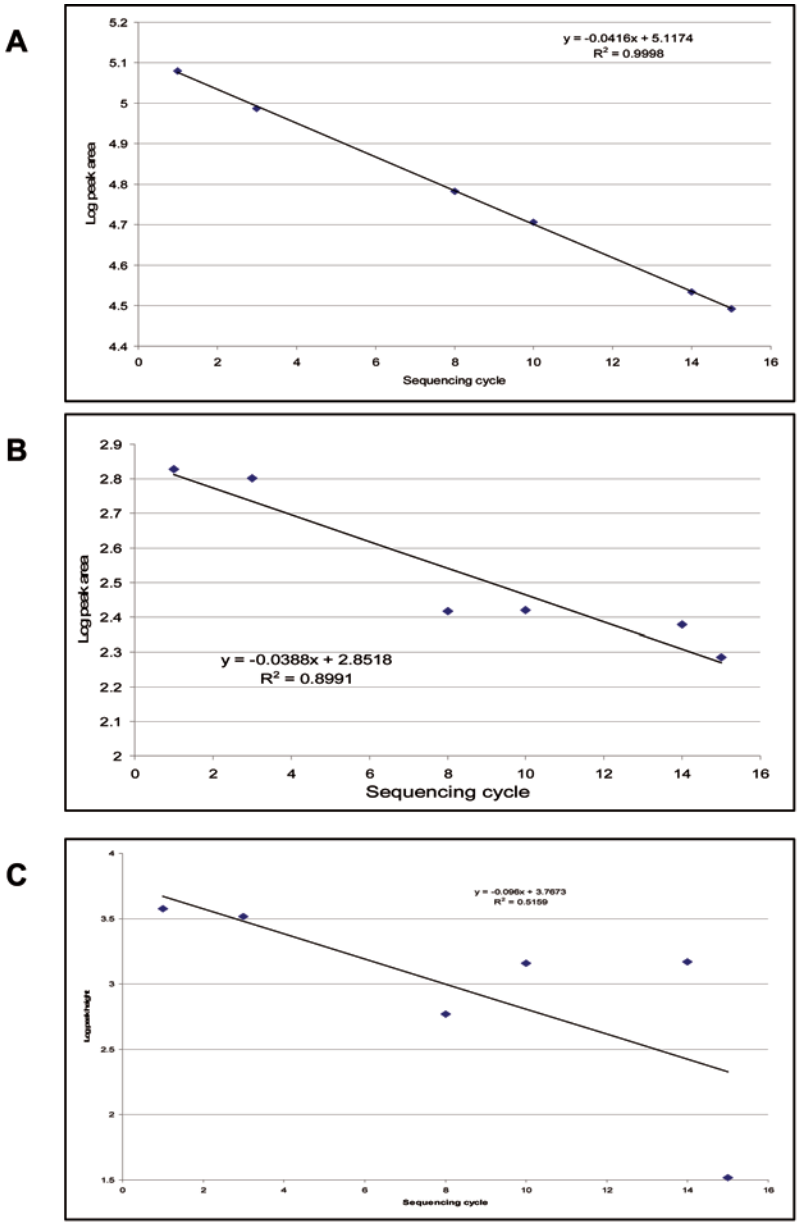

FIGURE 9

Calculation of repetitive yield from the slope of the trend line through a plot of log of the peak areas for Tyr and Ala residues as a function of sequencing cycle. (**A**) Repetitive yield of excellent quality, (**B**) repetitive yield of intermediate quality; and (**C**) repetitive yield of poor quality.

and then normalized the retention time data within each set to the time for Ala, using the following formula:

$$RTnA = [RTx - RTA] / [RTD - RTL]$$

where: RTnA is the retention time of any amino acid normalized to Ala; RTx is the raw retention time of amino acid x; RTA is the raw retention time of Ala; RTD is the raw retention time of Asp, the first amino acid to elute; RTL is the raw retention time of Leu, the last amino acid to elute.

This formula divides the retention time difference between amino acid x and Ala by the total length of the chromatographic run from Asp to Leu. The results for standard amino acids for both the Procise HT and the cLC shown in Table 4 give RTnAs of around –0.3 min for Asp, RTnAs of 0 min for Ala, and RTnAs of around 0.7 min for Leu. We calculated standard deviations for all of the normalized values. For the HT, the greatest standard deviation (0.03) was observed at the beginning and end of the chromatographic separations for Asp and Lys and Leu. On the cLC, calculated standard deviations did not exceed 0.01. These standard deviations are much tighter than was seen in last year's study, which showed standard deviations as high at 0.06.[1] The normalized values were then converted back to real scaled retention times (scaled RT), based on the average run time for each instrument (Figure 10).

$$Scaled\ RT = RTnA \times (AvRTL - AvRTD) + AvRTA$$

TABLE 4

Relative Retention Times of Standard Amino Acids Normalized to Ala and Scaled to the Average Run Time

	494-Ht Instruments (n = 19)			494-cLC Instruments (n = 8)			
	494-HT Av RTnAs	494-HT Std Dev of RTnAs	494-HT Av Full RT	494-cLC Av RTnAs	494-cLC Std Dev of RTnAs	494-cLC Av Full RT	
D	−0.30	0.03	4.20	−0.30	0.01	6.18	D
N	−0.27	0.02	4.62	−0.27	0.01	6.60	N
S	−0.22	0.01	5.25	−0.23	0.01	7.35	S
Q	−0.20	0.01	5.52	−0.20	0.01	7.69	Q
T	−0.18	0.01	5.75	−0.19	0.01	7.92	T
G	−0.17	0.01	6.00	−0.17	0.00	8.24	G
E	−0.13	0.01	6.48	−0.13	0.00	8.79	E
H	−0.04	0.02	7.69	−0.05	0.01	10.12	H
A	0.00	0.00	8.22	0.00	0.00	10.89	A
R	0.12	0.02	9.78	0.11	0.01	12.54	R
Y	0.16	0.01	10.36	0.16	0.01	13.38	Y
P	0.33	0.00	12.62	0.33	0.00	16.06	P
M	0.38	0.01	13.34	0.38	0.01	16.87	M
V	0.41	0.00	13.66	0.41	0.00	17.24	V
W	0.55	0.01	15.56	0.57	0.01	19.87	W
F	0.63	0.02	16.65	0.63	0.01	20.70	F
I	0.66	0.02	17.09	0.66	0.01	21.20	I
K	0.65	0.03	16.90	0.68	0.01	21.53	K
L	0.70	0.03	17.62	0.70	0.01	21.83	L

AvRTL, AvRTD, and AvRTA are the average retention times for Leu, Asp, and Ala, respectively, for each instrument type.

The retention times for the amino acids (normal and modified) from the synthetic peptide were similarly normalized based on Ala (Table 5). There was a wider range of values for the called amino acids compared to the standards, with standard deviations ranging from 0.01 up to 0.13 for the HT and up to 0.19 for the cLC. High variability was observed for cystine peaks, which proved to be quite inconsistent depending on the conditions of the run. Also, there was variability in the positions of dimethyl-Lys, trimethyl-Lys, and 3-methyl-His. As for the standards, we scaled the retention times back to actual minutes based on the average length of run. This was done to allow a more normal description of the retention times of the modified amino acids.

As in last year's study, we determined relative yields of each of the amino acids (normal and modified) based on the repetitive yield calculation and theoretical yield of each amino acid. This theoretical yield was determined from the TREND function in Excel used to calculate the repetitive yields. These values were plotted in Figure 11. The intention in determining these values was to show how the modified amino acid peak areas compared to those of the unmodified amino acids. Relative yields varied between 1% (Cys_3 peak) and 140% (Lys) for the instrument types represented by more than a single example, showing the difficulty in seeing all of the peaks for some of the modified amino acids.

Instrument Survey

The instrument and methods survey is found in Tables 6 and 7. In brief, there were 19 ABI Procise 49x-HT systems (average age of 7 y), 7 ABI Procise 49x-cLC systems (average age 6.7 y), 1 ABI 477A (10 y), and one Porton 2090e contributing to this year's study. Nearly all of these instruments were used with standard manufacturer's reagents and solvents, columns, and chemistry cycles, although a few laboratories prepared some of the reagents themselves. Common additives included TCEP or DTT in R4, and one laboratory included DTT in S3. Six laboratories included DTT in their S2 reagent, while 21 did not.

TABLE 5

Relative Retention Times of Modified Amino Acids Normalized to Ala and Scaled to the Average Run Time for Each Group of Instruments

		ABI 494-HT					
Cycle	Amino Acid	Average SRTnAs	Std Dev of SRT-nAs	Av Full RT	Rel Pk Area	Rel. Area Std Dev	n
1	Tyr	0.16	0.01	10.37	102.3%	9.8%	23
2	DimeLys	0.11	0.04	9.75	88.0%	27.5%	23
3	Ala	0.00	0.00	8.24	103.8%	9.6%	23
4	3MeHis	0.01	0.07	8.35	85.9%	36.7%	21
5	Lys	0.61	0.03	16.46	140.3%	48.8%	22
6	His	−0.04	0.02	7.69	60.3%	16.9%	23
7	HomoCit	−0.09	0.01	7.01	88.7%	17.9%	22
8	Ala	0.00	0.01	8.24	90.4%	20.6%	23
9	Cystine 1	0.17	0.12	10.49	30.1%	37.3%	20
9	Cystine 2	0.09	0.15	9.48	4.8%	2.0%	8
9	Cystine 3	0.05	0.14	8.92	3.2%	1.5%	7
10	Tyr	0.15	0.01	10.27	104.3%	13.5%	23
11	TrimeLys	0.11	0.06	9.63	65.9%	31.2%	19
12	Gly	−0.15	0.01	6.15	75.6%	16.6%	23
13	NmeAla	0.21	0.02	11.03	84.3%	29.5%	22
14	Tyr	0.16	0.02	10.30	110.3%	15.5%	23
15	Ala	0.00	0.01	8.24	91.9%	20.8%	23
16	IsoAsp						

Table continued

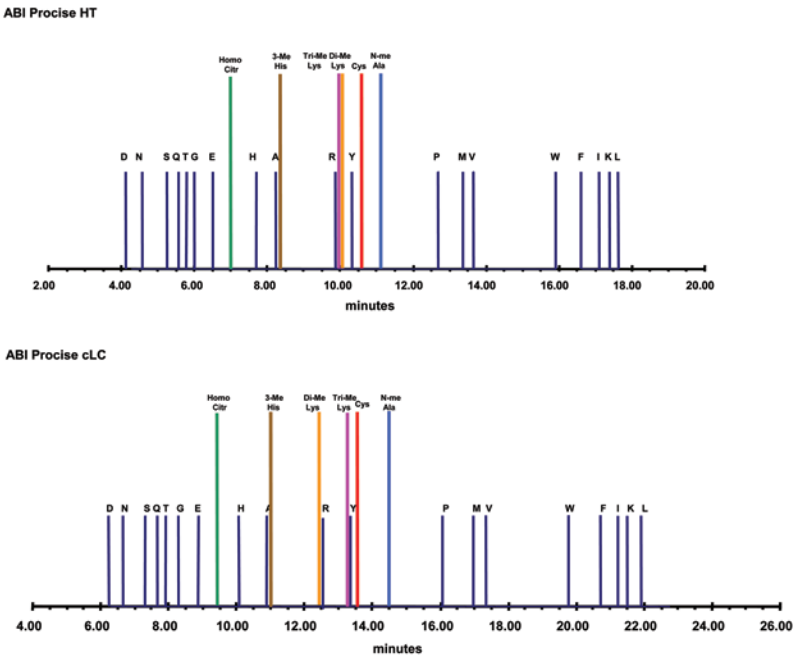

FIGURE 10

Normalized time lines for elution of standard and modified amino acids on the Procise HT and the Procise cLC.

TABLE 5 (continued)

		ABI 494-cLC						ABI 477 (n = 1)			Porton (n = 1)		
Cycle	Amino Acid	Average SRTnAs	Std Dev of SRT-nAs	Av Full RT	Rel Pk Area	Rel. Area Std Dev	n	SRTnA	477 Full RT	Rel Pk Area	SRTnA	Full RT	Rel Pk Area
1	Tyr	0.18	0.01	13.69	107.2%	13.9%	10	0.16	16.60	85.8%	0.14	13.53	77.1%
2	DimeLys	0.13	0.04	12.93	75.8%	20.3%	10	0.12	15.60	58.0%	0.22	14.70	116.4%
3	Ala	0.00	0.00	10.86	104.1%	10.3%	10	0.02	13.60	103.8%	0.00	11.18	108.0%
4	3MeHis	0.03	0.05	11.33	67.7%	29.5%	10	0.02	13.50	71.6%	0.10	12.77	129.6%
5	Lys	0.77	0.01	22.86	107.0%	51.4%	10	0.68	27.80	7.1%	0.67	21.93	143.8%
6	His	−0.05	0.01	10.13	51.8%	21.3%	10	−0.03	12.40	35.5%	−0.05	10.50	178.1%
7	HomoCit	−0.11	0.00	9.21	75.3%	29.3%	10	—	—	—	−0.12	9.27	158.9%
8	Ala	0.00	0.00	10.87	78.3%	29.2%	10	0.02	13.50	108.1%	0.00	11.22	135.8%
9	Cystine 1	0.18	0.15	13.66	11.6%	6.8%	10	—	—	—	0.06	12.25	16.9%
9	Cystine 2	0.20	0.05	13.95	7.0%	5.5%	7	—	—	—	0.17	13.88	22.1%
9	Cystine 3	0.09	0.22	12.24	2.4%	0.8%	5	—	—	—	—	—	—
10	Tyr	0.18	0.01	13.69	94.9%	20.2%	10	0.16	16.50	129.9%	0.15	13.60	115.1%
11	TrimeLys	0.21	0.05	14.16	56.9%	40.1%	10	0.19	17.10	41.8%	0.31	16.20	115.2%
12	Gly	−0.19	0.01	7.89	70.0%	20.6%	10	−0.14	10.10	130.1%	−0.17	8.58	99.7%
13	NmeAla	0.26	0.01	14.99	71.3%	20.7%	10		18.00	51.2%	0.25	15.30	109.9%
14	Tyr	0.18	0.01	13.68	118.1%	18.0%	10	0.16	16.50	84.9%	0.15	13.63	87.7%
15	Ala	0.00	0.00	10.87	98.3%	13.8%	10	0.01	13.30	93.9%	0.00	11.25	87.5%
16	IsoAsp	—	—	—	—	—	—	—	—	—	—	—	—

For the HPLC separations, most of the contributing laboratories used the ABI/Brownlee Spheri-5 PTH columns for either the HT (2.1 mm × 220 mm) or cLC (0.8 mm × 250 mm). Column ages varied from 20 to 2000 cycles. All users of ABI sequencers used ABI solvent A, but there were a number of different additives besides premix, which are listed in Table 7. Solvent B was isopropanol/acetonitrile.

Judging from the results of this study, most of the laboratories appear to do a good job with their instrument upkeep and analytical approach. The lack of competing manufacturers means that little effort is devoted

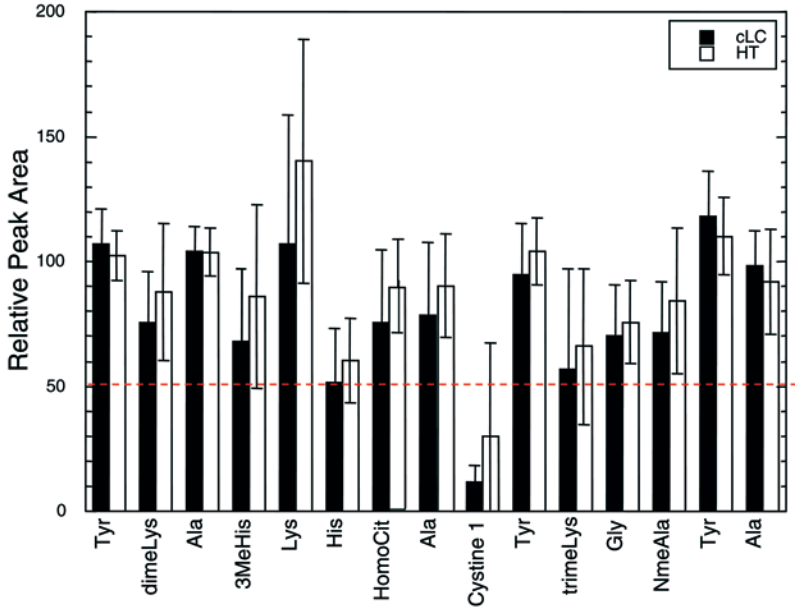

FIGURE 11

Average PTH yields for each amino acid in the synthetic peptide.

TABLE 6

Sequencer Information	
Manufacturer and Model	19 ABI 49X-HT (avg age of 7 y)
	7 ABI 49X-cLC (avg age of 6.7 y)
	1 ABI 477A (10 y old)
Reagents	23 used all instrument manufacturer reagents
	1 used some mfg. R1, R2C, R4, R5, & Premix
	1 said almost all mfg. reagents
	1 said partially
	1 said S4 home made
TFA Cleavage	24 liquid and 2 gas phase and 1 both
Sample Support	21 GFF, 4 PVDF,
	1 said both GFF and PVDF (probably answered in general)
Chemistry Cycle	All ABI users seemed to use the cycles that matched the support (i.e., GFF or PVDF)
DTT in S2	6 yes and 21 no
Other Additives	1 TCEP to R4A, 1 DTT in R4A & 1 DTT in S3
Sample Solvent	18 used 0.1% TFA/30% acetonitrile
	2 used 0.1% TFA/20% acetonitrile
	2 used 0.3% TFA/ 30% acetonitrile
	1 used 0.05% TFA/70% acetonitrile
	4 users were unclear
% Loaded	Ranged from 2% to 100% Mean 5.33%

TABLE 7

HPLC Information	
Column Manufacturer,	All 477A and all 49X-HT used ABI/Brownlee Spheri-5
Type, Size	PTH column 2.1 x 220 (or 150) mm
	6 cLC used ABI/Brownlee Spheri-5 PTH 0.8 x 250 mm
	1 cLC used ABI/Brownlee Spheri-5 PTH 0.8 x 150 mm
Column Age	Ranged from 20 to 2000 cycles
	Mean 611 cycles
Avg. Column Life	ABI 0.8 x 250 mm: 300–1,400 (mean 855)
	ABI 2.1 x 220 mm: 400–3,000 (mean 1405)
Solvent A	All used ABI Solvent A
Additives to Solvent A	Acetic acid/Na Acetate/ 0.2% 1-hexane sulfonic acid
besides Premix	• Na_2HPO_4/TFA
	Na phosphate/acetone
	• Acetone only
	• TFA only
	• TFA/acetone
	• Trp/TFA/KH_2PO_4
	• TFA/KPO_4/acetone
	• KH_2PO_4/acetone
	• Acetone/0.1M phosphate,pH5/1M NaOH
Solvent B	All use ABI Solvent B2 (isopropanol/acetonitrile) or equivalent (some users are not clear whether they use ABI B2 solvent).

to improving the performance of current instruments or designing newer, more powerful instruments. Also, there are few supply alternatives.

CONCLUSIONS

The seven modified amino acids examined in this study (dimethyl- and trimethyl-lysine, 3-methyl-histidine, N-carbamyl-lysine, cystine, N-methyl alanine, and iso-aspartic acid) add to the list of seven (N,N-dimethyl-arginine, N-ε-methyl lysine, S-β-propionamidocysteine, N-ε-acetyl lysine, 4-hydroxyproline, phosphoserine, methionine sulfoxide) analyzed last year, for a total of 14 modified amino acids. This study suggests that Edman degradation can do a good job identifying these residues, even though some are difficult to analyze. The most problematic modified amino acids in this year's study were the dimethyl- and trimethyl-lysine, 3-methyl-histidine, and cystine. The first three of these can be better analyzed in two runs by varying the concentration of premix from 14.5 mL/L to 21 mL/L. Cystine can be better identified by two runs in the presence and absence of DTT.

By normalizing to the retention time of Ala, we were able to determine elution characteristics for the seven modified amino acids, which should provide a means to make confident assignments of these amino acids in the future. The relative retention times for the modified PTH-AAs have been posted on the ABRF website at www.abrf/esrg.org.

ACKNOWLEDGMENTS

We thank Mike Pennington at Bachem for donating Fmoc derivatives of homocitrulline, 3-methyl histidine, and trimethyl-lysine, and Anita Hong at AnaSpec for donating Fmoc derivatives of dimethyl-lysine and N-methyl alanine. We thank Melinda Miller for graciously removing identifiers from the contributing laboratories. And finally, we especially thank all the participating laboratories for taking their time and effort to analyze the sample and sending us their results. Without their participation, this effort would not have been successful.

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