HPLC '89

Poster Presentation

Identification of Single Residue Substitutions in Sequences by Microbore HPLC and Amino Acid Analysis

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ABSTRACT

Substitutions of amino acids (AA) in a sequence may result in altered biological activity. Such changes may be recognized by altered retention times on reversed phase (RP) HPLC. This approach requires absolute reproducibility in the optimized chromatographic separation. In addition, all aspects of sample handling protocols must be rigorously defined. Tryptic digests of cytochrome c from various species serve as the model for identifying single residue substitutions. Sample handling and separation protocols were optimized, and altered chromatographic mobilities were defined. Orientation within the complex chromatograms was simplified by photodiode detection. Peak identification was confirmed by AA analysis, and where appropriate, sequence determination. RP-HPLC followed by AA analysis is effective for determination of AA substitutions in proteins or synthetic peptides.

Introduction

Point mutations or single residue substitutions in the primary sequence of a protein or peptide may have deleterious effects on its biological activity. This type of alteration can occur during peptide synthesis. The altered peptide fragments must be separated from the desired product. An ideal method is by reversed phase (RP) HPLC. Strict control of chromatographic conditions such as gradient composition reproducibility, column re-equilibration time, and column temperature, enable generation of extremely reproducible, high resolution peptide maps. Using these maps, peptides differing by a single amino acid residue may be resolved.

Due to its well characterized primary structure, tryptic digests of cytochrome c from various species were chosen as models for the AA residue substitutions. Using photodiode array detection, landmark structural features, such as the covalently attached heme and single tryptophan (W) residue, would assist in peptide identification.

Materials and Methods

Tryptic Digestion. Cytochrome C (Sigma) from bovine, rabbit, chicken, and horse heart mitochondria (1mg/500µl) were suspended in 0.1M ammonium bicarbonate buffer, pH 8.0. Trypsin (0.1mg) was dissolved in 500µl buffer and added to the cytochrome C solutions. The digests were incubated for 24 h at 37°C. Following incubation, trypsin was de-activated by heating at 100°C for 5 min. The digests were separated into aliquots of 100 µl and frozen at -20°C until chromatographed. Prior to HPLC, digests were diluted 1:10 with eluent A.

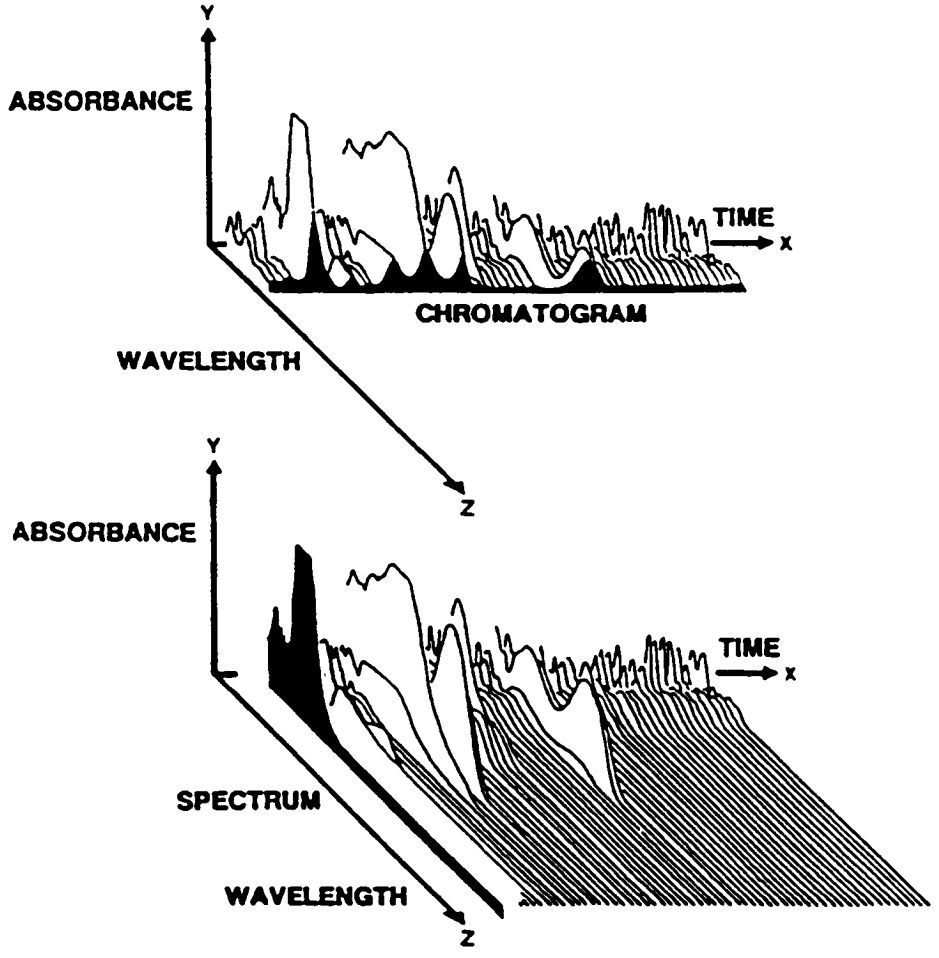
HPLC System. The tryptic digests were resolved by reversed phase HPLC at 35°C using a 2mm x 15cm Waters DeltaPak™ C18, 5µ, 300Å column on the Waters Peptide Analyzer. The Peptide Analyzer was equipped with Photodiode Array Detection (model 990+), and samples were analyzed in the wavelength range of 190-425 nm with 1.4 nm resolution. The gradient was optimized for the bovine tryptic digest in order to facillitate comparisons The eluents were: A=water/0.1%trifluoroacetic acid (TFA): B=acetonitrile/0.1%TFA in the following gradient:

Time (min)	Flow (ml/min)	%A	%B	Curve
0	0.25	95	5	*
3	0.25	90	10	6
13	0.25	87	13	6
67	0.25	60	40	6
77	0.25	40	60	6
87	0.25	40	60	6

Peptides were collected from RP-HPLC and amino acid composition determined using the Waters PicoTag™ protocol for hydrolysis samples.

Data Analysis Formats in Photodiode Array Detection

The Waters model 990+ Photodiode Array (PDA) Detector collects and stores a three-dimensional (3-D) data set: time, absorbance, and wavelength. While such data is displayed as a 3-D plot, other data presentation formats are also used. A contour plot is a view from the top, showing time vs. wavelength with concentric circles of varying colors to indicate increasing absorbance. Another data format is the chromatogram plot showing absorbance as a function of time at a specific wavelength. Additionally, the absorbance spectrum at a given time of the data set may be obtained. The PDA computer has menu-driven software enabling the user to perform mathematical calculations such as addition, subtraction, and derivative calculation.



Comparison of Cytochrome C from Bovine, Rabbit, Chicken, and Horse

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                    10                  20
BOVINE    GDVEKGKKIFVQKCAQCHTVEKGGKH
RABBIT    GDVEKGKKIFVQKCAQCHTVEKGGKH
CHICKEN   GDIEKGKKIFVQKCSQCHTVEKGGKH
HORSE     GDVEKGKKIFVQKCAQCHTVEKGGKH

          30                  40                  50
BOVINE    KTGPNLHGLFGRKTGQAPGFSYTDAN
RABBIT    KTGPNLHGLFGRKTGQAVGFSYTDAN
CHICKEN   KTGPNLHGLFGRKTGQAEGFSYTDAN
HORSE     KTGPNLHGLFGRKTGQAPGFTYTDAN

                    60                  70
BOVINE    KNKGITWGEETLMEYLENPKKYIPGT
RABBIT    KNKGITWGEDTLMEYLENPKKYIPGT
CHICKEN   KNKGITWGEDTLMEYLENPKKYIPGT
HORSE     KNKGITWKEETLMEYLENPKKYIPGT

          80                  90                  100
BOVINE    KMIFAGIKKKGERADLIAYLKKATNE
RABBIT    KMIFAGIKKKDERADLIAYLKKATNE
CHICKEN   KMIFAGIKKKSERVDLIAYLKDATSK
HORSE     KMIFAGIKKKTEREDLIAYLKKATNE
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Figure 1. The primary structure of cytochrome c derived from bovine, rabbit chicken, and horse heart mitochondria. Sequence differences between species are indicated by **bold type.** Tryptic cleavage points, Arg and Lys, are displayed in outlined characters R and K, respectively.

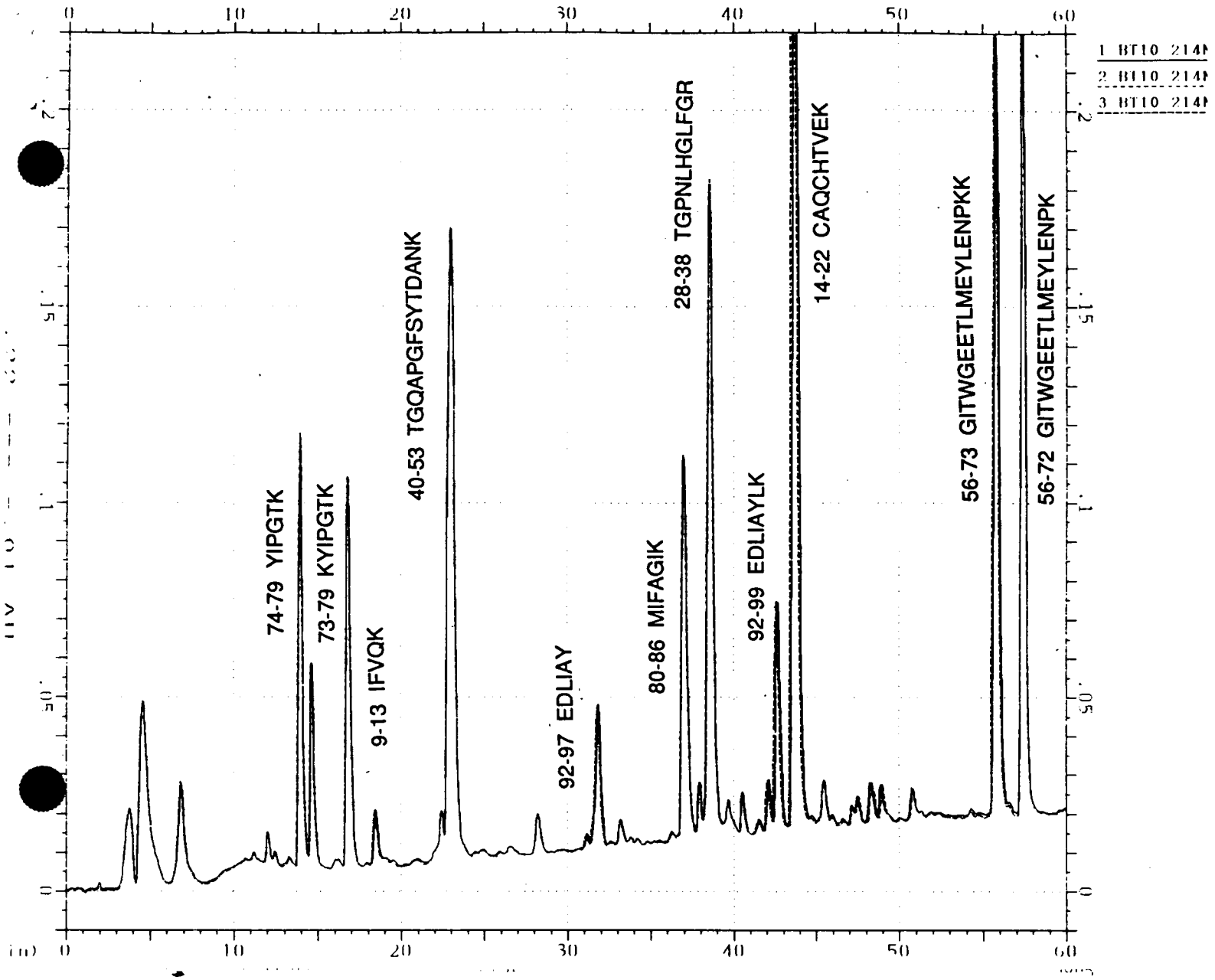

Figure 2. Peptide map of tryptic digest of bovine cytochrome c at 214 nm. Overlay of three separate injections of 1nmol showing excellent reproducibility of retention time and pattern of resolution. The sequence of each peptide fragment is displayed. The tryptic cleavage at Lys (K) residues 72 and 73 occurred with equal frequency producing two clearly resolved fragments differing by one Lys residue. It is interesting that a high-yield cleavage occurs at Tyr 97 (peptide 92-97 EDLIAY). Minor cleavages are often observed when the proteolytic enzymes are not purified immediately before use, even when the trypsin is TPCK-treated. However, the rate of cleavage at this particular tyrosyl residue is quite high. It is not obvious whether this is a consequence of the adjacent sequence or of the conformation. However, this was the only observed Tyr cleavage and is found in all four digests.

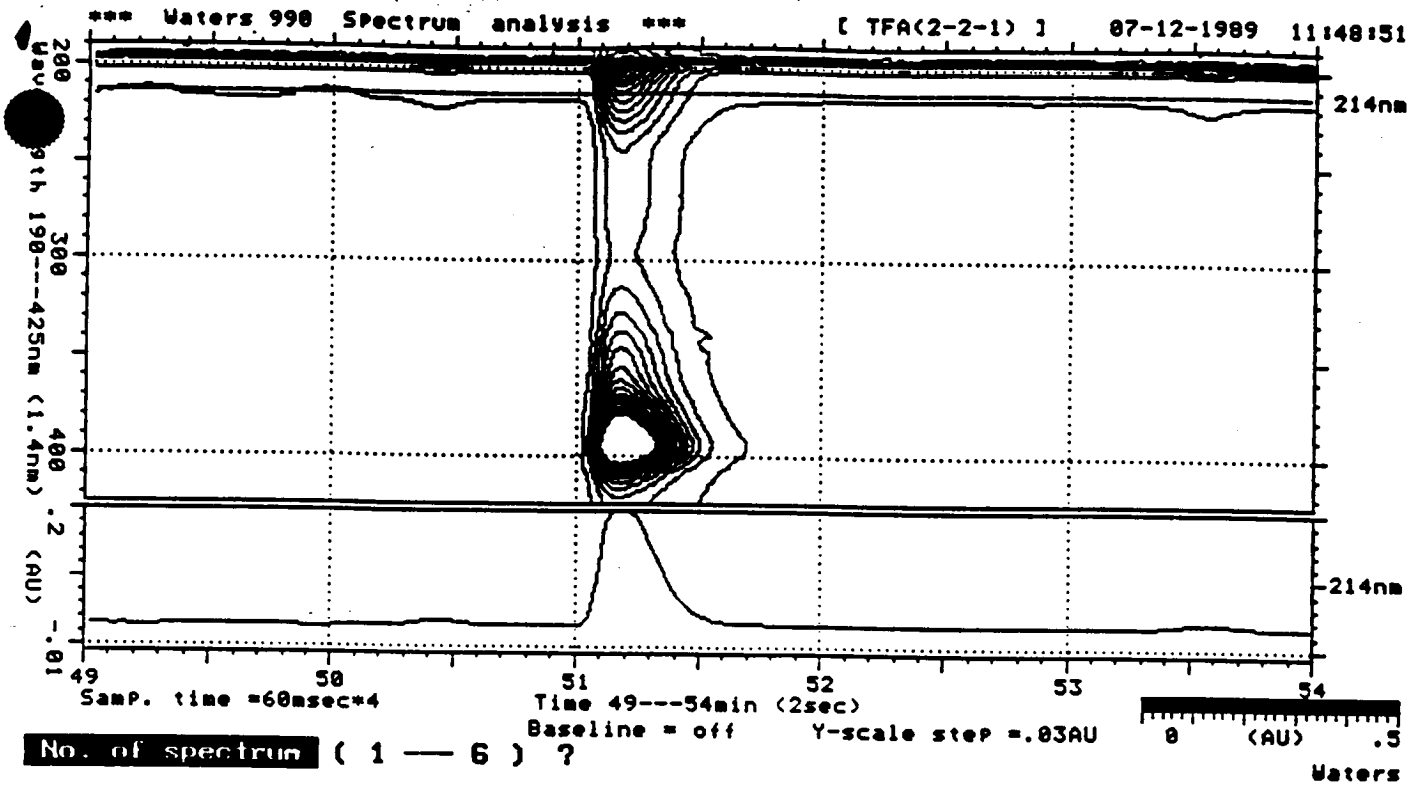

Figure 3. Contour plot of peptide 14-22 showing the heme group covalently attached to Cys residues 14 and 17. An initial step in the mapping procedure was the identification of this peptide by its distinctive spectra.

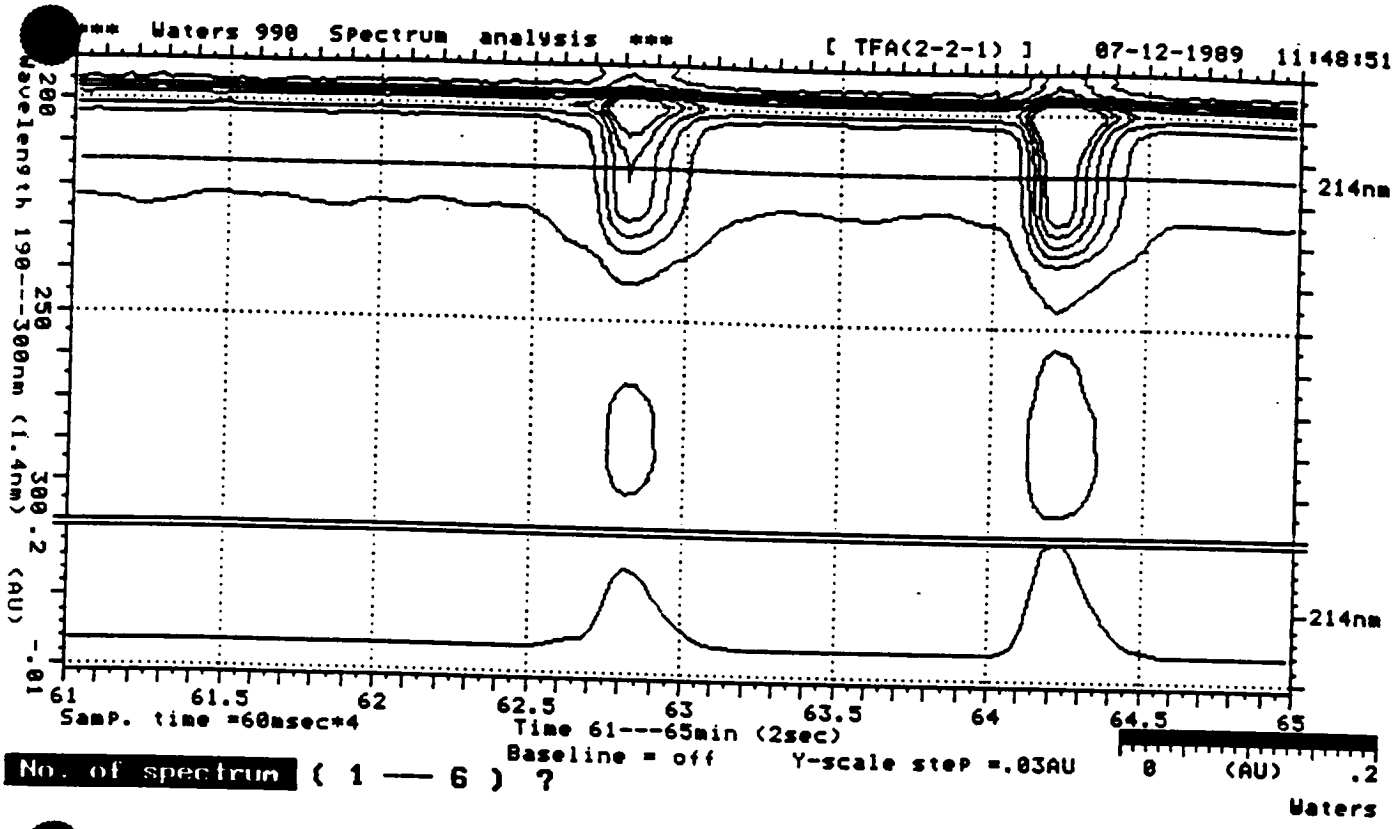

Figure 4. Contour plot of peptides 56-73 and 56-72 showing absorbance at 280 nm indicative of aromatic amino acids Trp and Tyr.

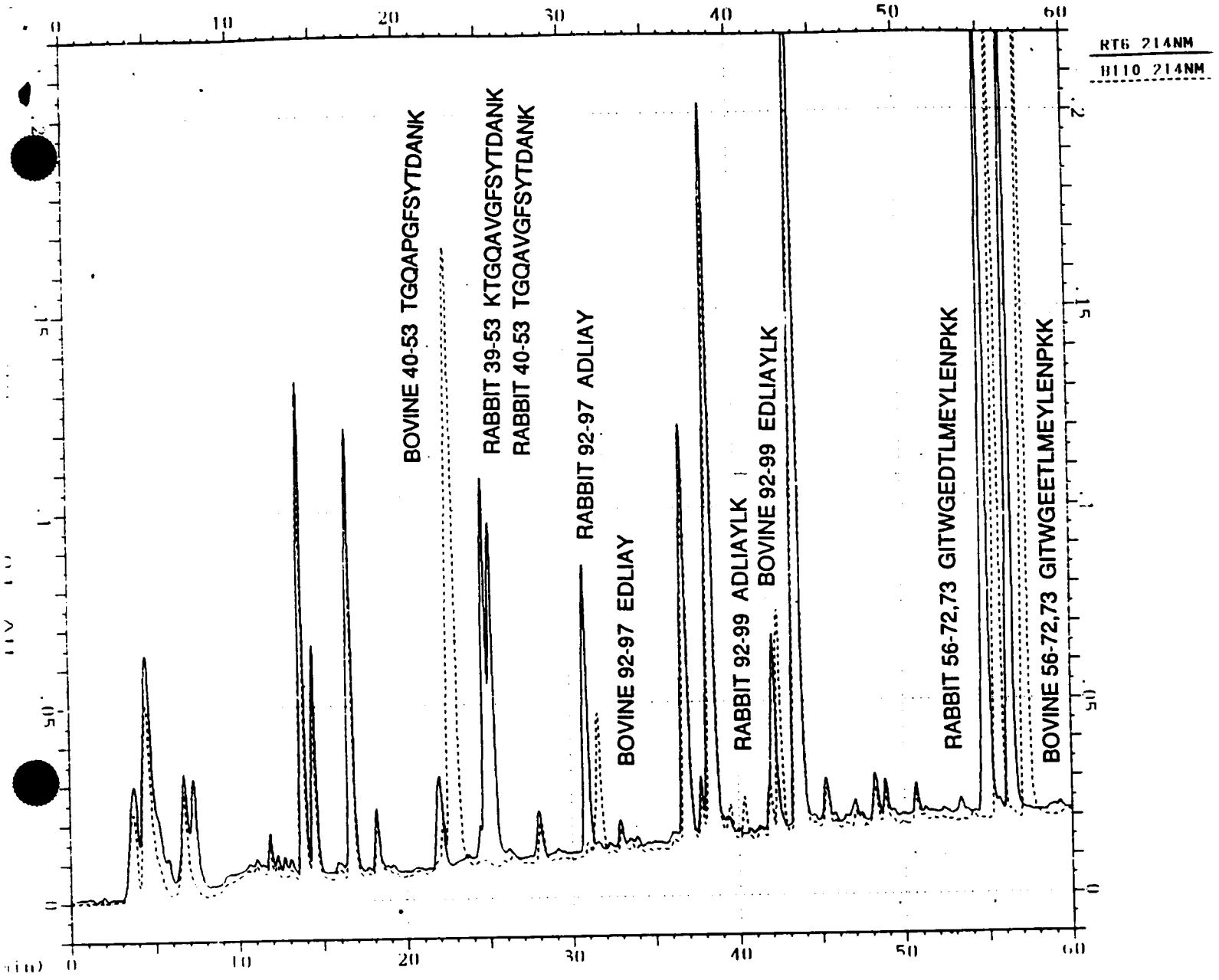

Figure 5. Overlay plot of the tryptic digests of cytochrome c from bovine (dashed line) and rabbit (solid line). Residue 44 is Pro (P) in bovine and Val (V) in rabbit as reflected in increased retention of the rabbit peptides 39-53 and 40-53 when compared to bovine peptide 40-53. Substitution of Ala (A) in rabbit for a Glu (E) in bovine at residue 92 altered the retention of two sets of peptides: 92-97 and 92-99 in the rabbit are less retained than the corresponding bovine peptides. In bovine, residue 62 is Glu (E), and the rabbit, Asp (D). Peptides 56-72 and 56-73 of rabbit that elute prior to the corresponding bovine peptides, consistent with a substitution of Asp for Glu at residue 62.

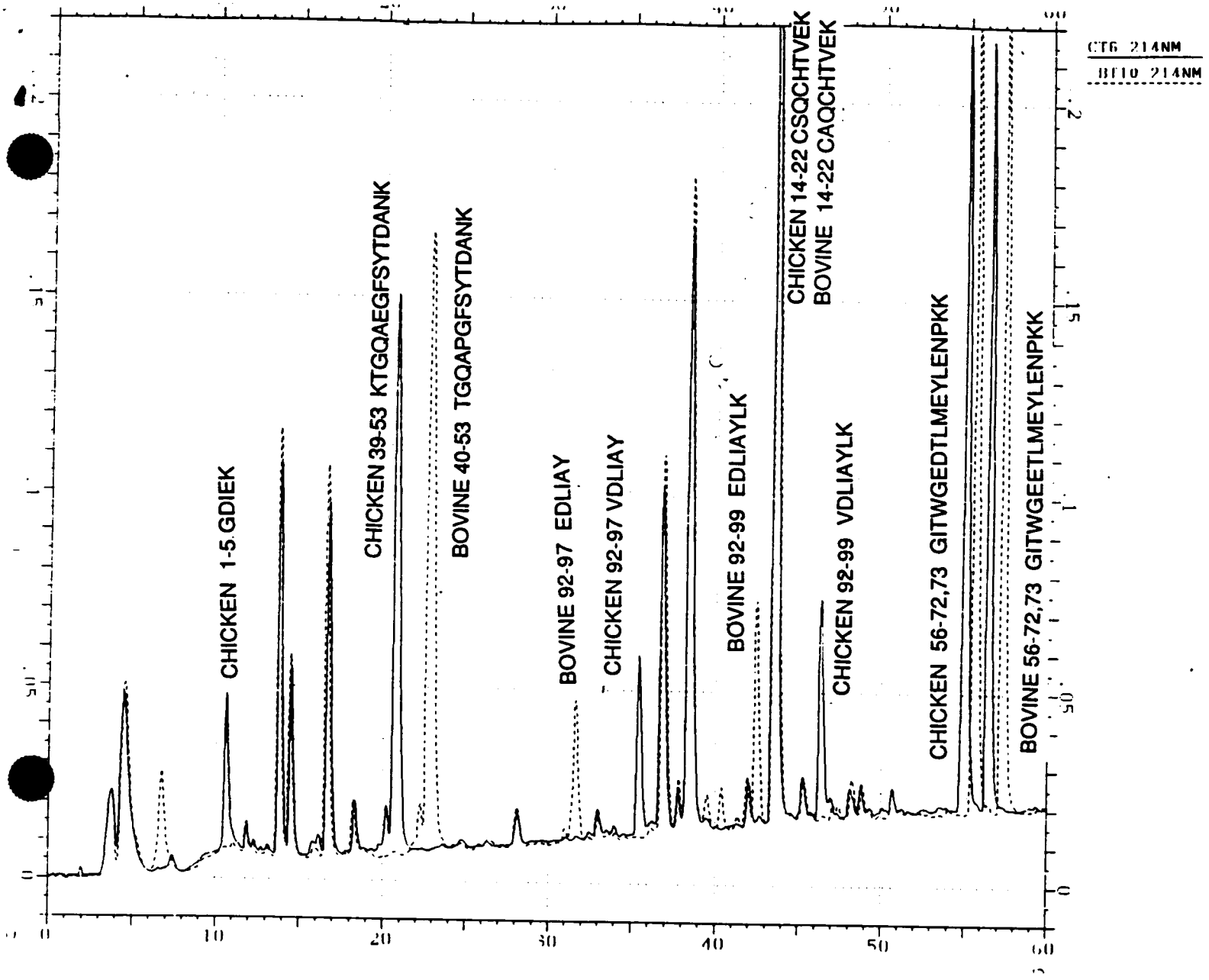

Figure 6. Overlay plot of the tryptic digests of cytochrome c from bovine (dashed line) and chicken (solid line). Residue 44 is Pro (P) in bovine and Glu (E) in chicken, resulting in decreased retention of peptide 40-53. Substitution at residue 92 of a Val (V) for a Glu (E) in the chicken resulted in greater retention of two peptides: 92-97 and 92-99. In bovine, residue 62 is Glu (E) while the chicken, like the rabbit, has Asp (D). This alteration is reflected in peptides 56-72 and 56-73 where the Asp-substituted peptides of the chicken elute prior to the bovine peptides.

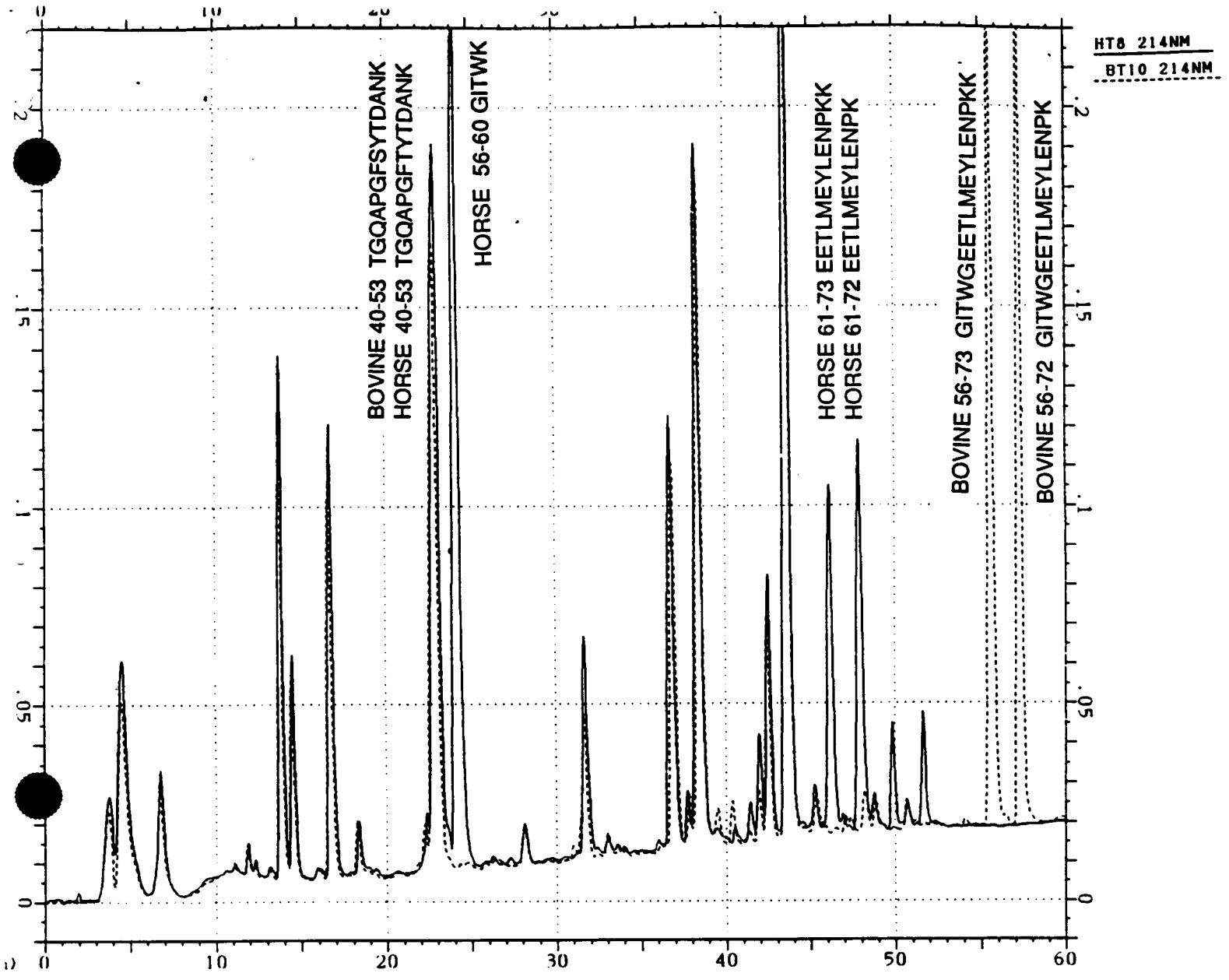

Figure 7. Overlay plot of the tryptic digests of cytochrome c from bovine (dashed line) and horse (solid line). There is a substitution at residue 47 of a Ser (S) in bovine for a Thr (T) in horse. However, these peptides co-elute in the present gradient. An additional substitution at residue 60 of a Gly (G) residue in bovine for a Lys (K) in horse results in an altered cleavage pattern. The bovine digest has two large hydrophobic peptides 56-73 and 56-72 that both contain 1 Trp (W) and 1 Tyr (Y). This sequence in the horse results in 3 peptides: 56-60, 61-73, and 61-72. Horse peptide 56-60 can be identified by the Trp (W) residue and peptides 61-73 and 61-72 should have spectral characteristics of Tyr (Y), as discussed below.

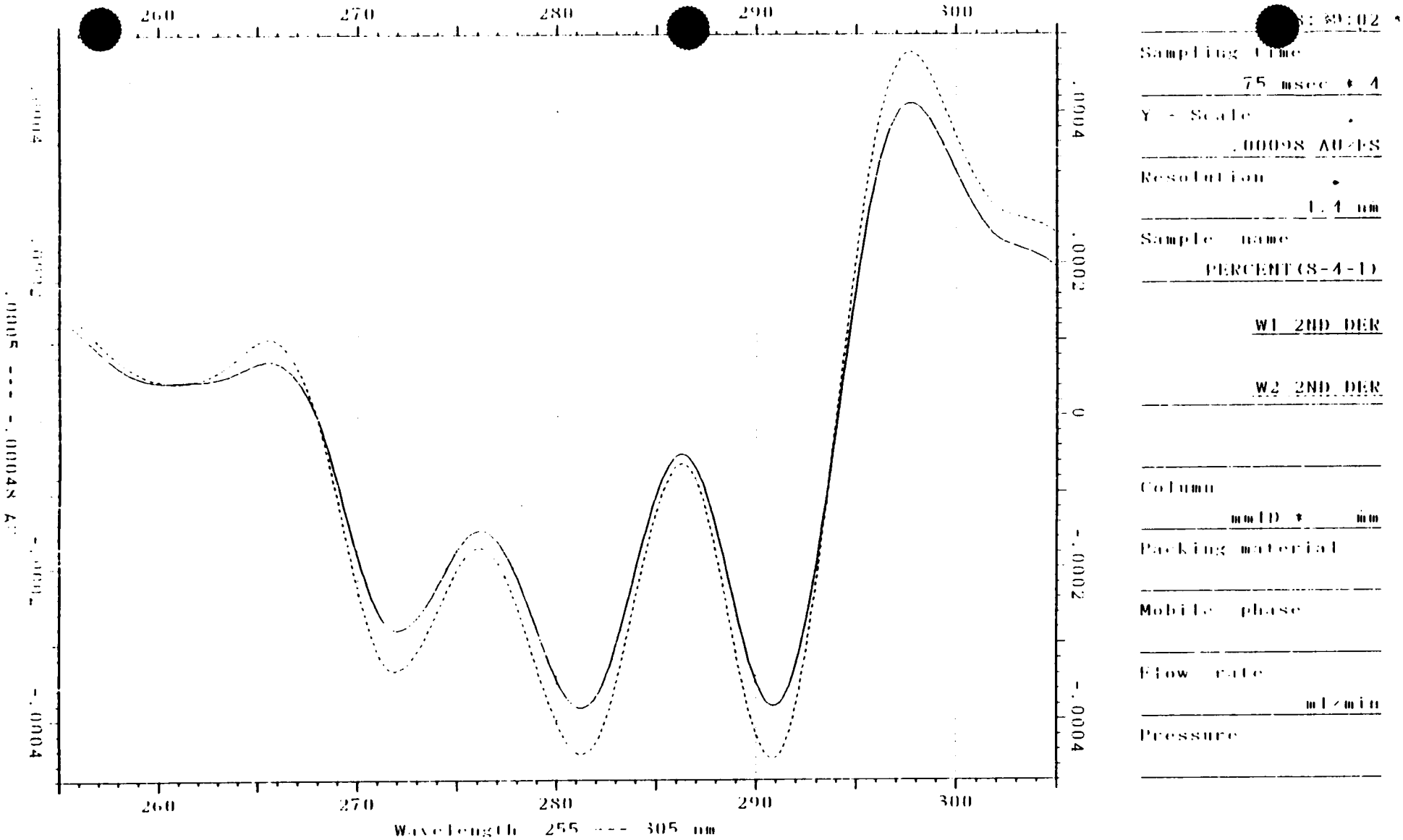

Figure 8. Second derivatives of the spectra from bovine peptides 56-73 (solid line) and 56-72 (dashed line) with the absorbance minima at 281 nm and 291 nm and maximum at 297 nm, confirming the presence of Trp in both peptides. The relative positions of these minima suggest that the peptides contain both Trp and Tyr. Compare these curves to those shown in figures 9 and 10 where Tyr 67 and Trp 59 have been separated by cleavage at Lys 60.

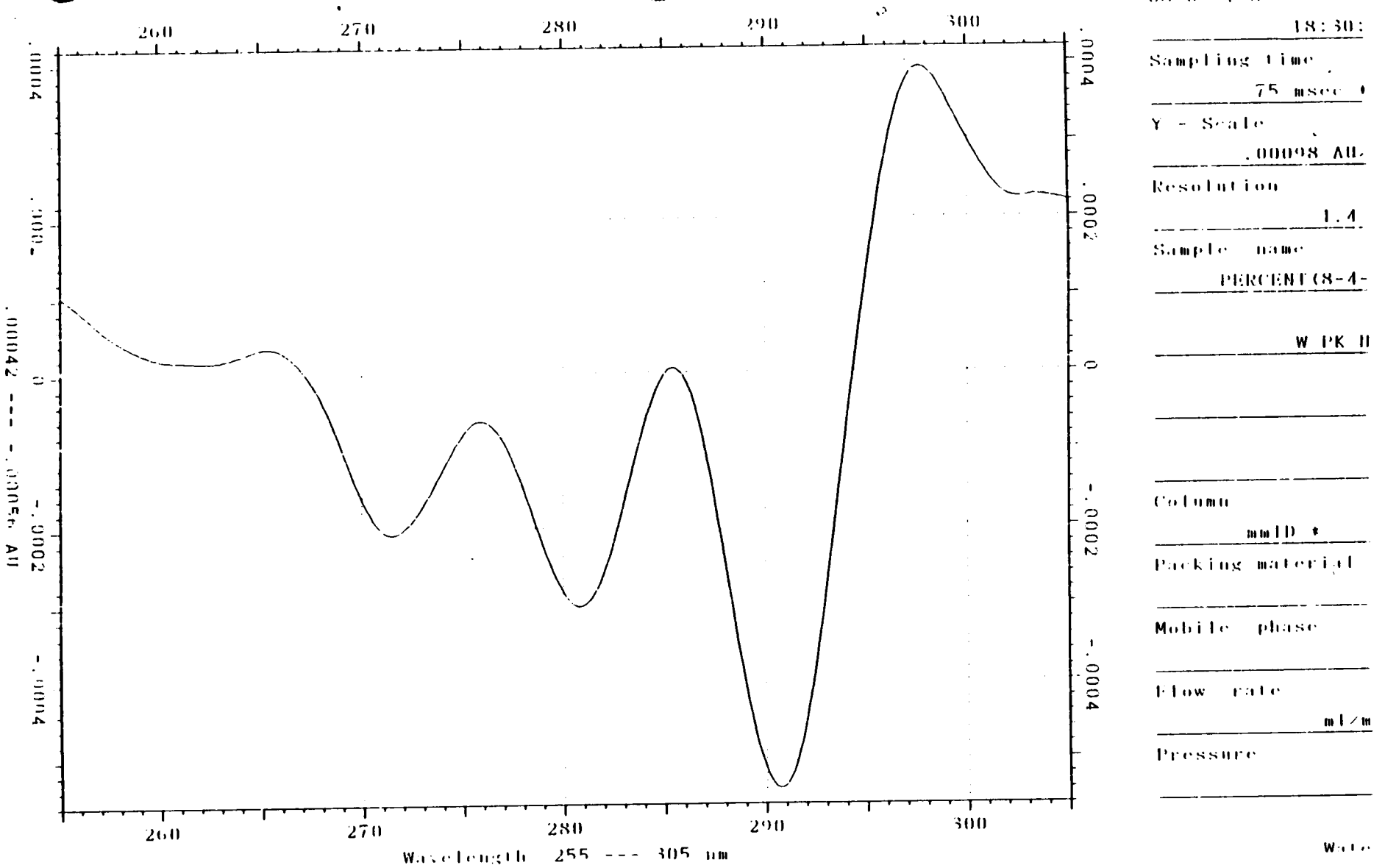

Figure 9. Second derivative of the spectrum for horse peptide 56-60 showing minima at 281 nm and 291 nm and maximum at 285 nm as expected for Trp (W).

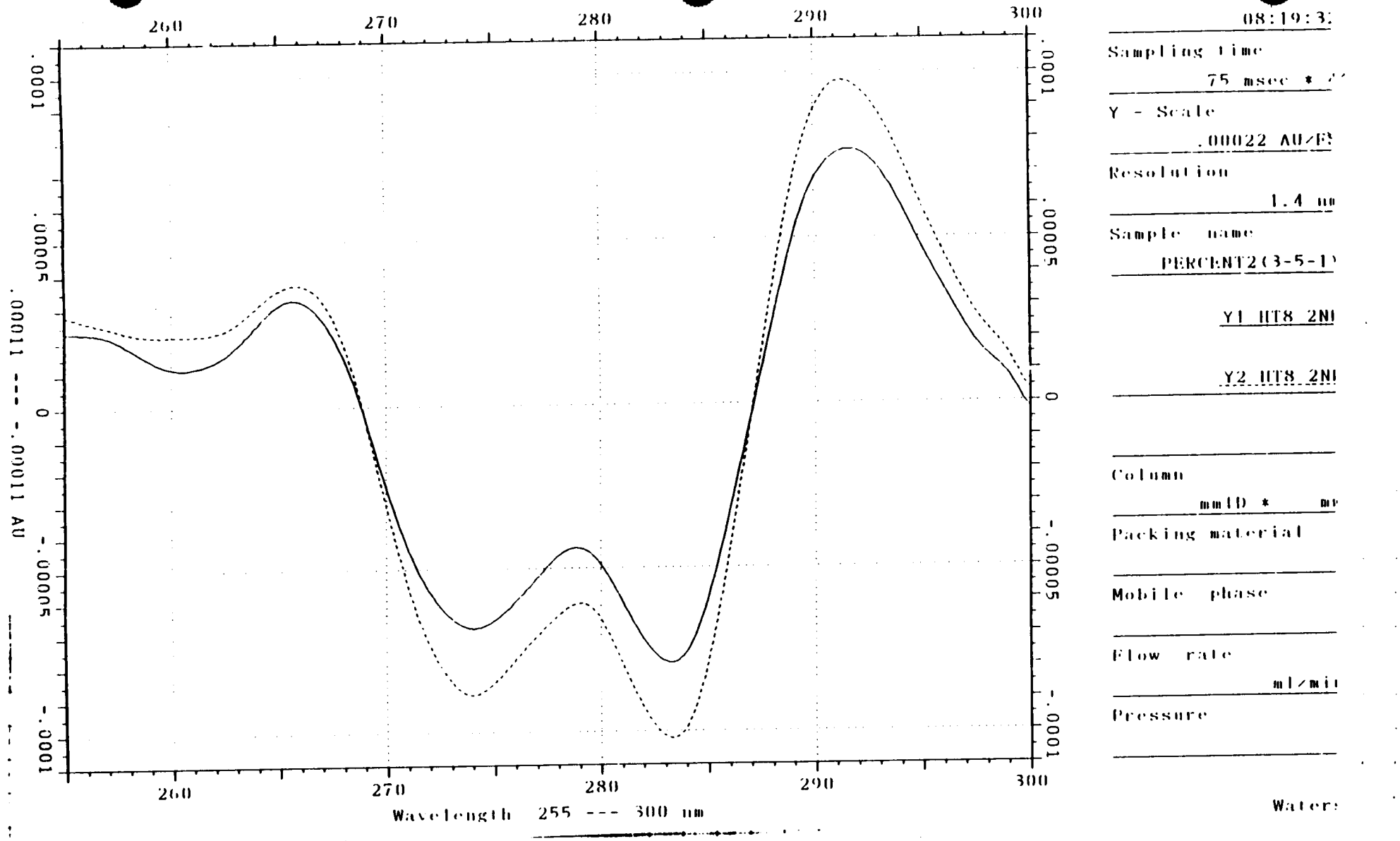

Figure 10. Second derivative of the spectra for horse peptides 61-73 (solid line) and 61-72 (dashed line). The minimum at 284 nm and maximum at 291 nm confirm the presence of Tyr (Y) in both peptides.

Conclusions

1. Optimized reverse phase chromatography using a reproducible HPLC system produces a characteristic peptide map for a protein digested under controlled conditions.
2. Single residue substitutions alter the chromatographic retention of the corresponding peptide. However, not all such substitutions can be resolved under the same conditions.
3. Single substitutions can affect the pattern of digestion for a protein by introduction or elimination of cleavage points and/or alteration of hydrolysis rates leading to complex changes in elution profiles.
4. Amino acid analysis by the Pico-Tag™ method is an effective tool for confirming the identity of peptides.
5. Additional information can be extracted from a peptide map by using photodiode array detection, particularly for identification of peptides containing aromatic amino acids or other chromophores.
6. Spectral data can provide more detailed information by the calculation of second derivatives. This procedure is a simple, menu-driven function of the Waters 990+ photodiode array detector. This approach readily distinguishes among peptides containing Trp, Tyr, or both aromatic amino acid residues.