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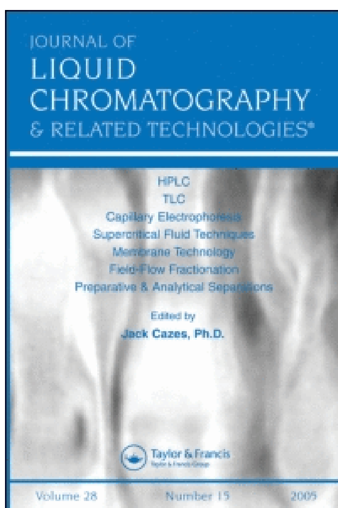

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IDENTIFICATION OF HEMORPHINS FROM BOVINE HEMOGLOBIN HYDROLYSATE: APPLICATION OF UV SECOND ORDER DERIVATIVE SPECTROSCOPY

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ABSTRACT

Aromatic amino acids have very informative second order derivative spectra. Whereas they exhibit overlapping maxima between 250 and 300nm in the zero order spectra, thin minima are obtained in their second order derivative spectra. This feature allowed to develop a method to identify aromatic amino acids, but also to calculate the ratio between these amino acids in peptides and proteins. This method has been used successfully for the detection of hemorphins in a peptic bovine hemoglobin hydrolysate. The constant ratios between aromatic amino acids are an important characteristic of lots of bioactive peptides; the advantage of this spectral method is to be non-destructive for the identification of these amino acids espacially for tryptophan.

INTRODUCTION

HPLC combined with sensitive and selective detection has become a widely used technique for both purification and identification of compounds [1, 2]. The advent of ultraviolet-visible (UV-VIS) diode-array detection system in HPLC has made it possible not only to perform on-the-fly real-time scanning but also to

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obtain their derivative spectra at the same time [3, 4]. The UV spectra of proteins and peptides are greatly dependent on their amino acids contents [5, 6]. Amino acids have very uninformative spectra except for the aromatics. Only phenylalanine, tyrosine and tryptophan exhibit absorption maxima between 250 and 300 nm. Nevertheless their UV-spectra are broad and they overlap. As a consequence, trying to use the unenhanced spectrum of a protein to determine the aromatic amino acids contents becomes very difficult. To estimate the relative ratio of these aromatic amino acids becomes almost impossible. The derivative spectroscopy, firstly developped by Savitzky and Goloy [7], offers the advantage of sharper spectral features when compared to conventional absorbance spectroscopy. For instance, a peak shoulder present in a zero order derivative spectrum can be transformed into a peak minimum when the second order derivative spectrum is obtained. Furthermore, overlapping bands can be transformed into resolved bands. Second order derivatives are usually performed instead of higher orders derivatives since they represent a compromise between selectivity (intensity of absorption minima and maxima increase with each derivative) and interference from false absorption (due to noise factors) [5]. So the derivative spectroscopy has become a revival as an analytical tool to identify and quantify aromatic amino acids in proteins and peptides [8, 9, 10].

We have previously reported isolation of two opioid peptides, LVV-hemorphin-7 and VV-hemorphin-7 from a very complex bovine hemoglobin peptic hydrolysate [11]. These peptides and those obtained either from bovine or human beta-chain of hemoglobin [12] contain important amounts of aromatic amino acids. The constant ratios between aromatic amino acids are an important characteristic of hemorphins. So these peptides provided us defined patterns to investigate second derivative spectra, and led us to develop a simple method for qualitative and quantitative determination of aromatic amino acids. This determination was efficient to improve the identification of hemorphins from a bovine hemoglobin peptic hydrolysate by UV-spectra comparison [13] and should be easily applied in peptide and protein hydrolysate research.

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EXPERIMENTAL

MATERIALS AND CHEMICALS

All common chemicals and solvents were of analytical grade from commercial sources. Tryptophan, tyrosine, phenylalanine, pepsin and rabbit lung

angiotensin converting enzyme (ACE) was purchased from Sigma Chemicals. Tuna myoglobin, cutinase and peptide GI were kindly provided by the colleagues of our laboratory. Hemorphin-7 was synthesized by C.Guillon, Laboratoire de Technologie Enzymatique, University of Compiègne, Compiègne, France

Bovine hemoglobin Hydrolysate, LVV-hemorphin-7 and VV-hemorphin-7

Bovine hemoglobin hydrolysate was obtained at pilot-plant scale by peptic proteolysis in an ultrafiltration reactor as previously described [14]. Active fraction FVII was prepared by gel permeation HPLC using TSK G2000 SWG column (19mm i.d. X 600 mm) and analyzed by reversed phase (RP) HPLC in order to obtain LVV-hemorphin-7 and VV-hemorphin-7 [11].

LVV-hemorphin-5, VV-hemorphin-5 and dipeptide Arg-Phe

According to LANTZ [15], 1 mg of either VV-hemorphin-7 or LVV-hemorphin-7 were dissolved in 1 ml 0.05 M Tris-HCl buffer pH 7.4 and incubated at 37 °C with angiotensin converting enzyme (ACE, 7.5 mU) for 7 h. The reaction mixture was resolved on a Nova-Pak C-18 column (3.9 mm i. d. X 150 mm). LVV-hemorphin-5, VV-hemorphin-5 and dipeptide Arg-Phe were identified by mass spectrometry.

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HPLC system

The liquid chromatographic system consisted of a Waters 600 automated gradient controller-pump module, a Waters Wisp 717 automatic sampling device and a Waters 996 photodiode array detector. Spectral and chromatographic data were stored on a NEC image 466 hard disc using a NEC image 466 computer. Millennium software was used to plot, acquire and treat chromatographic data.

METHODS

Mobile phase for Delta Pak C-18 column (19 mm i.d.X 300 mm)

The mobile phase was composed of 10 mM ammonium acetate buffer pH 6.0 as eluent A and acetonitrile as eluent B. The flow rate was 12 ml/min. Samples were dissolved in buffer A, filtered through 0.22 μm filters before injection. The gradient applied was 0-40% B in 80 min.

Mobile phase for Nova-Pak C-18 column (3.9 mm i.d. X 150mm)

The mobile phase comprised: 10 mM ammonium acetate buffer pH 6.0 as eluent A and acetonitrile as eluent B. A linear gradient (15-30% B in 15 minutes) was applied. The flow rate was 1.5 ml/min.

Mobile phase for TSK G2000 SWG column (7.6 mm i.d. X 600mm)

The elutions were performed with 5mM ammonium acetate buffer pH 6.0 . The flow rate was 0.9ml/min..

Procedure

Hemorphin-7, VV-hemorphin-7 and LVV-hemorphin-7 were chromatographed at room temperature on a Delta Pak C-18 and a Nova Pak C-18 columns respectively under conditions described above. Total hydrolysate was also injected on Delta Pak C-18 column under the same conditions. Tyrosine, phenylalanine, tryptophan and peptides were injected on the Nova Pak C-18 column and pepsin, tuna myoglobin and cutinase were loaded successively on the TSK G2000 SWG column. On-line instantaneous UV absorbance spectral scan was performed between 190 nm and 350 nm with a rate of one spectrum/second; spectral resolution was 1.2 nm. Chromatographic data were processed using Millennium software. Spectrum matching results (comparison between spectra of each peaks in the chromatographic profile with the library spectra of the hemorphins) [13] and second derivative spectra were obtained by Waters Millenium system.

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Amino acid analysis

Amino acids were analyzed using a Waters Picotag Work Station. Peptide hydrolysis was achieved with constant-boiling HCl containing 1% phenol, for 24h. at 109°C. Precolumn derivatization of amino acids with phenyl isothiocyanate and HPLC separation of derivatized amino acids on a Waters RP-Picotag column (150mm x 3.9mm i.d.) were performed according to Bidlingmeyer et al. [16]. The detection wavelength was 254 nm and the flow rate 1 ml/min.

Mass spectrometry analysis

Mass spectra, generated from Fast Atom Bombardment (FAB) mass spectrometry of the peptides, were recorded on a four sector "Concept II" tandem mass spectrometer (Kratos, Manchester, UK). Ions were produced in a standard

FAB source by bombarding the sample with xenon atoms having a kinetic energy of 8 KeV and the instrument was operated at an accelerating voltage of 8 KV. The peptide was dissolved in water (1 mg/ml) and 1ml of the solution was loaded on the stainless steel tip with thioglycerol as matrix. The mass range was scanned at 10 s/decade with a mass resolution of 3000. Caesium iodide was the standard for mass calibration.

RESULTS AND DISCUSSION

SPECTRAL ANALYSIS OF AROMATIC AMINO ACIDS AND PEPTIDES

Phe, Tyr, Trp and peptides listed in Table 1 were successively injected on RP-HPLC and their absorbance spectra were monitered with Waters 996 UV-Vis Photodiode array detector and processed to give second order derivative spectra. Figure 1 illustrates the second order spectra of each aromatic amino acid. The most characteristic feature of these second derivative spectra is that they exhibit a minimum at every maximum in the zero order spectra. As shown in table 2 the most prominent minima for Phe, Tyr, and Trp were 259nm, 283.5nm and 289.5nm respectively. These values were slightly different in regards to those of Palladino [8] and Zavitsanos [5], especially the secondary minimum for

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TABLE 1
Peptides, proteins and aromatic amino acids used for second order derivative spectral analyses.

Compound	Structure	Phe	Tyr	Trp
Phenylalanine		1		
Tyrosine			1	
Tryptophan				1
Dipeptide	Arg-Phe	1		
F2-8	Tyr-Gly-Ala-Glu-Ala-Leu		1	
P41	Ser-Ala-Ala-Asp-Lys-Gly-Asn-Val-Lys-Ala-Ala-Trp			1
VV-hemorphin-5	Val-Val-Tyr-Pro-Trp-Thr-Gln		1	1
LVV-hemorphin-5	Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln		1	1
Hemorphin-7	Tyr-Pro-Trp-Thr-Gln-Arg-Phe	1	1	1
VV-hemorphin-7	Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	1	1	1
LVV-hemorphin-7	Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	1	1	1
Peptide GI	Glu-Lys-Leu-Gly-Glu-Tyr-Gly-Phe-Gln	1	1	
Pepsine		14	16	5
Tuna myoglobin		6	2	1
Cutinase		8	5	1

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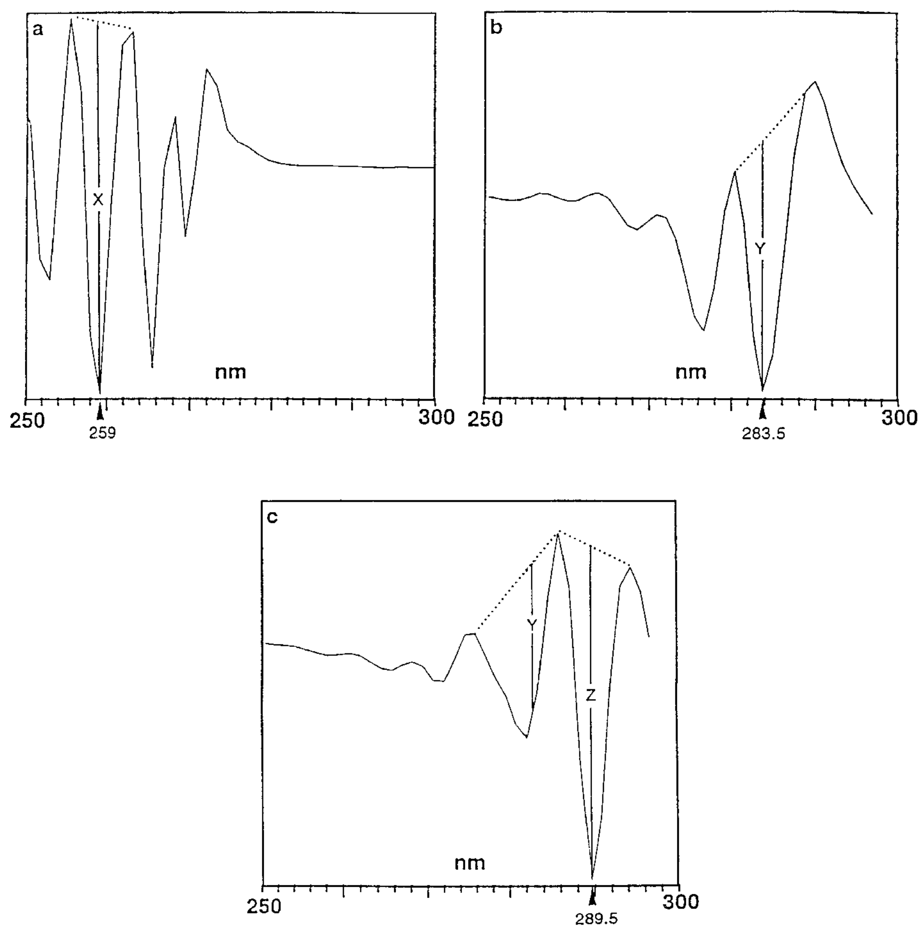

Figure 1. Second order derivative spectra of free aromatic amino acids. (a) phenylalanie, (b) tyrosine, (c) tryptophan. X, Y and Z: heights at the major minima.

TABLE 2
Major and side minima of aromatic amino acids second order derivative UV spectra.

Aromatic amino acids	Major minima	side minima
Phenylalanine	**259nm**	253nm, 264nm, 268nm
Tyrosine	**283.5nm**	276.5nm
Tryptophan	**289.5nm**	282nm

tryptophan (282nm instead of 278 nm). This may be due to the more precise spectral resolution of the 996 photodiode array detector. So the presence or absence of minima at 259, 283.5 and 289.5 nm allowed us to determine the presence or absence of Phe, Tyr and Trp. Figure 2 shows the second order derivative UV-spectra of the three known peptides Arg-Phe, FII 8, P41, containing Phe, Tyr and Trp respectively. The wavelengths of major minima were exactly the same as for free aromatic amino acids. This indicated that non-aromatic amino acids were not involved in the absorbance between 250nm and 300nm. This resulted in an easy identification of aromatic amino acids by their second order derivative spectra.

When tyrosine and tryptophan coexisted in the same peptide, the primary minimum for tyrosine (283.5nm) and the second minimum for tryptophan (282nm) overlaped. As shown by Palladino[8] and Zavitsanos [5] this overlapping made the identification of tyrosine in the presence of tryptophan difficult. The amplitude of minima should be accurately defined in order to determine the presence of tyrosine and to calculate the ratio between aromatic amino acids. Figure 1 c illustrates how the amplitude of minima for Trp were evaluated (Y at 283.5nm, Z at 289.5 nm). For tryptophan (figure 1 C), the proportion between Z and Y was 1:0.45. In the presence of tyrosine, we must consider its contribution at 283.5 nm, so if we postulated that the amplitude at 283.5 nm was simply additional, the contribution of tyrosine at 283.5 nm minima was Y - 0.45 Z .

Figure 3 show the second derivative spectra of VV-hemorphin-5 (a), hemorphin-7 (b), VV-hemorphin-7 (c) and LVV-hemorphin-7 (d), and the relative values of their amplitude at 258.5nm (X), 283.5 nm (Y) and 289.5 nm (Z) are presented in table 3. When Phe, Tyr and Trp were present in a peptide with a molar ratio 1:1:1 (as for hemorphin-7, VV-hemorphin-7 and LVV-hemorphin-7), the relative values of their amplitude were 1:7.5:11. Considering the tryptophan contribution at 283.5 nm, the contribution of tyrosine at 283.5nm was calculated as

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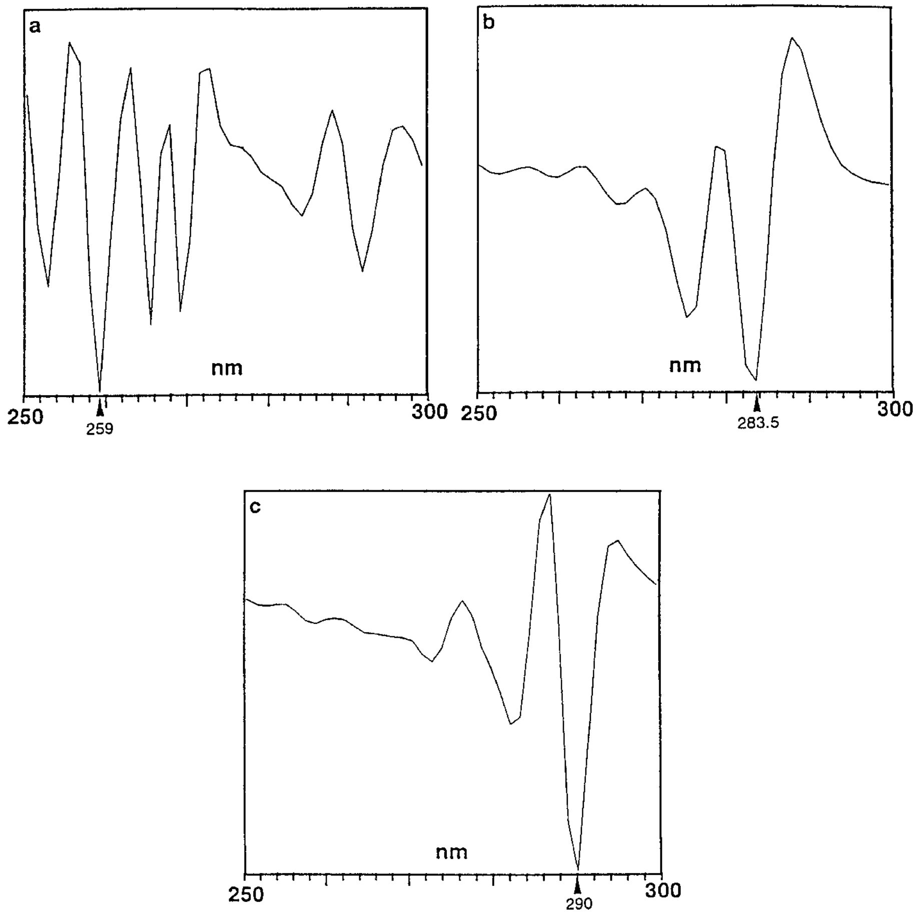

Figure 2. Characteristic minima in second order derivative UV-spectrum of small peptides indicating the presence of aromatic amino acids. (a) Arg-Phe, (b) FII 8, (c) P41. Peptide sequences: FII 8, Tyr-Gly-Ala-Glu-Ala-Leu; P41, Ser-Ala-Ala-Asp-Lys-Gly-Asn-Val-Lys-Ala-Ala-Trp.

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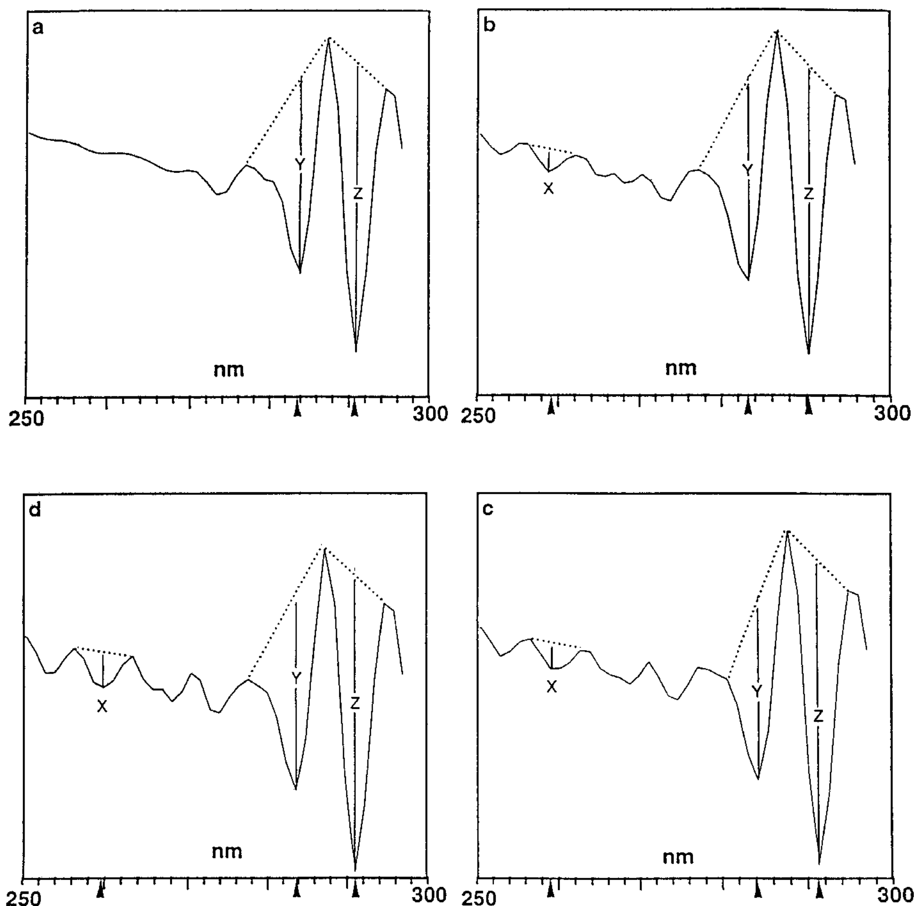

Figure 3. Characteristic minima in second order derivative spectra of some hemorphins indicating the content of aromatic amino acids. (a) VV-hemorphin-5, (b) Hemorphin-7, (c) VV-hemorphin-7, (d) LVV-hemorphin-7. X, Y and Z: as in figure 1.

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TABLE 3
Second order derivative spectra of some hemorphins: relative amplitudes at 259nm, 283.5nm and 289.5nm.

Hemorphins	Amplitude at 259 nm (Phe)	Amplitude at 283.5nm (Tyr+Trp)	Amplitude at 289.5 nm (Trp)
VV-hemorphin-5		7.5	11
Hemorphin-7	1	7.5	11
VV-hemorphin-7	1	7.5	11
LVV-hemorphin-7	1	7.5	11

7.5 - 0.45 x 11 = 2.55. From here we can deduce the relation between the ratios of the three aromatic amino acids, determined by their amplitude, as follows:

$$\text{Phe} : \text{Tyr} : \text{Trp} = X/1 : (Y - 0.45Z) / 2.55 : Z/11, \quad (1)$$

When Z = 0, tryptophan was absent, Phe : Tyr = X : Y/2.55 (2)
When Y = 0.45Z, tyrosine was absent Phe : Trp = X : Z/11 (3)
When X = 0, phenylalanine was absent Tyr : Trp = (Y - 0.45Z)/2.55 : Z/11 (4)

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VALIDATION OF THESE METHODS OF CALCULATIONS WITH SOME PEPTIDES OF KNOWN COMPOSITION

These rules were then checked with some known peptides (figure 4). In figure 4a the secondary derivative spectrum of peptide GI is presented. A zero amplitude at 289.5 nm (Z=0) indicated the absence of tryptophan. On the contrary, the presence of phenylalanine and tyrosine were demonstrated by minima at 258.5nm and 283.5 nm. The proportion between the two amplitudes was 1:2.45. So, according to equation (2) the ratio between Phe and Tyr in this peptide was 1:1. In the same way, the presence of Tyrosine and tryptophan in LVV-hemorphin-5 was verified by its second derivative spectrum (Figure 4b). According to equation (4) the ratio between tyrosine and tryptophan was also calculated as 1:1.

Figure 4c presents the second derivative spectrum of a peptide selected at random in a total bovine hemoglobin hydrolysate chromatographed on a Delta Pak C-18 column (figure 6, peak 33). The absence of tyrosine was demonstrated by Y-0.45Z = 0 and the presence of phenylalanine and tryptophan was evidenced by the minima at 258.5nm and 289.5nm. The proportion between amplitudes at 258.5nm and 289.5nm was 3.2:12. So according to equation (3), the ratio of phenylalanine and tryptophan in this peptide was 3:1. When the known amino acid

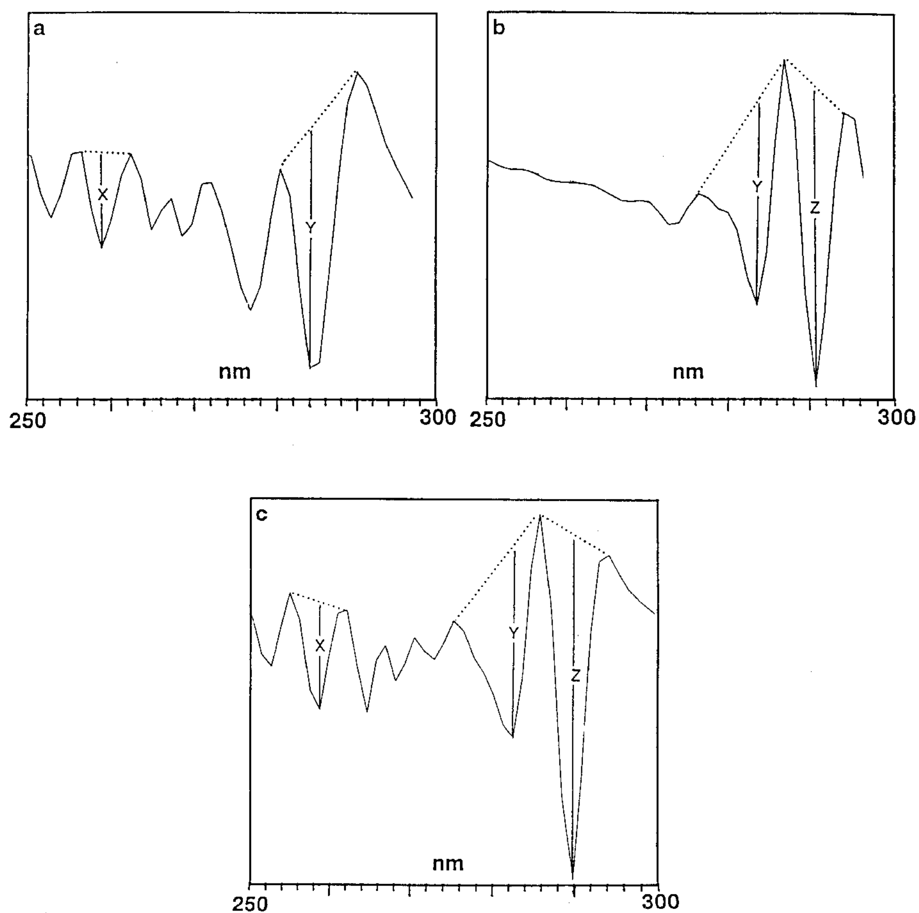

Figure 4. Second order derivative spectra of test peptides containing aromatic amino acids. The Tyr/Trp ratio is known from free amino acids and is used here to analyse spectral results. (a) peptide GI, (b) LVV-hemorphin-5, (c) peptide from bovin hemoglobin peptic hydrolysate -peak 33, see figure 6.

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TABLE 4
Analysis of aromatic amino acid content of some proteins by second order derivative spectra: comparison of real and calculated content.

Proteins	Calculated content			Real content		
	Phe	Tyr	Trp	Phe	Tyr	Trp
Porcine pepsine	18	16	5	14	16	5
Tuna myoglobin	5	2	2	6	2	1
Cutinase	4	4	1	8	5	1

sequences of alpha and beta bovine globins were examined for regions containing these amino acids, it was clear that the composition found was only compatible with peptide including residues 36 to 44 of the bovine hemoglobin beta chain. The N-terminal residue of this peptide may be proline or tryptophan because of the presence of tryptophan and absence of tyrosine. So the non-destructive identification of tryptophan and the informations resulting from secondary derivative UV spectra are of great interest in peptide maps.

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APPLICATION OF THESE METHODS TO SOME PROTEINS OF KNOWN COMPOSITION

Porcine pepsin, tuna myoglobin and cutinase were chromatographed by gel permeation HPLC under conditions described in experimental section and their second order derivative spectra were obtained (figure 5). The presence of the three aromatic amino acids in these proteins was clearly evidenced by the minima at 259, 284 and 290nm. The ratios between the aromatic amino acids were calculated (Table 4). It indicated that the presence of aromatic amino acids was identified qualitatively but the correct proportion was not obtained. This might be the result of the three-dimentional structure of native proteins. In fact, when the polypeptidic chain folds into a secondary or tertiary structure, aromatic amino acids can be buried within the molecule. Thus, these hidden aromatic amino acids are not involved in the protein absorbance. This observation shows that there is some limitation in the use of second derivative spectra for an accurate quantitative determination of native protein aromatic amino acid content. Further investigation should be undertaken with denatured proteins. Nevertheless, this method is of great interest for the analysis of oligopeptides.

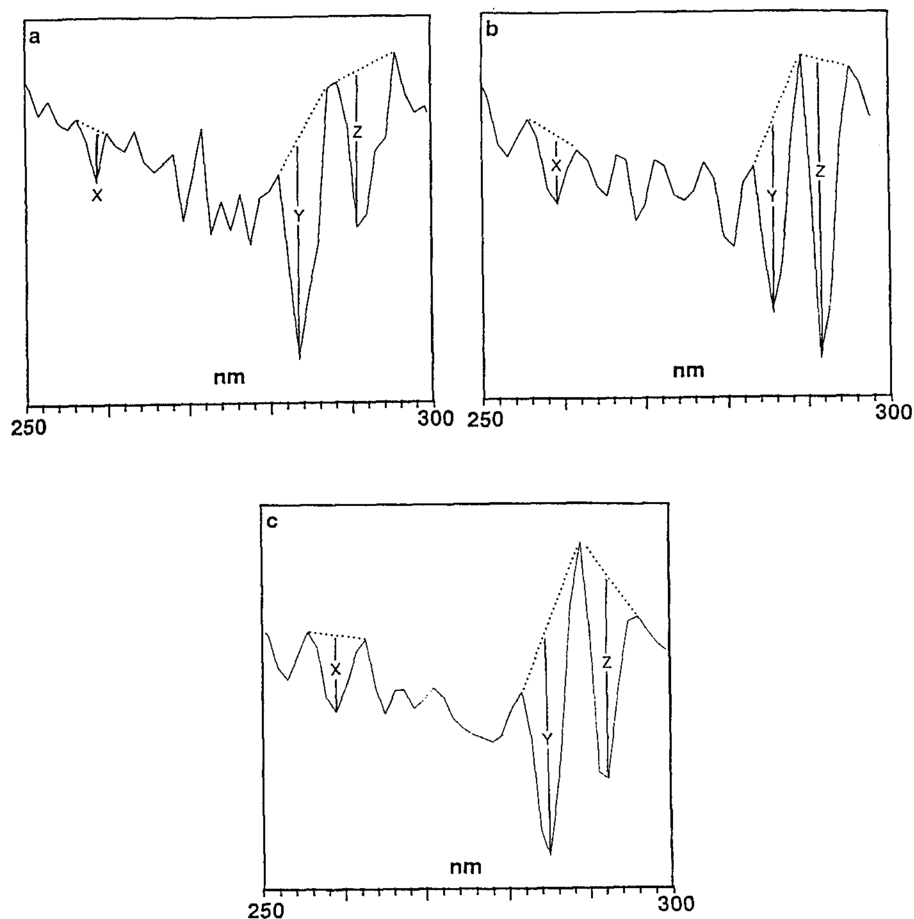

Figure 5. Second order derivative spectra of some proteins. (a) Porcine pepsin, (b) Tuna myoglobin, (c) cutinase. Estimation of their aromatic amino acid contents is carried out using the methods presented in the results and discussion section.

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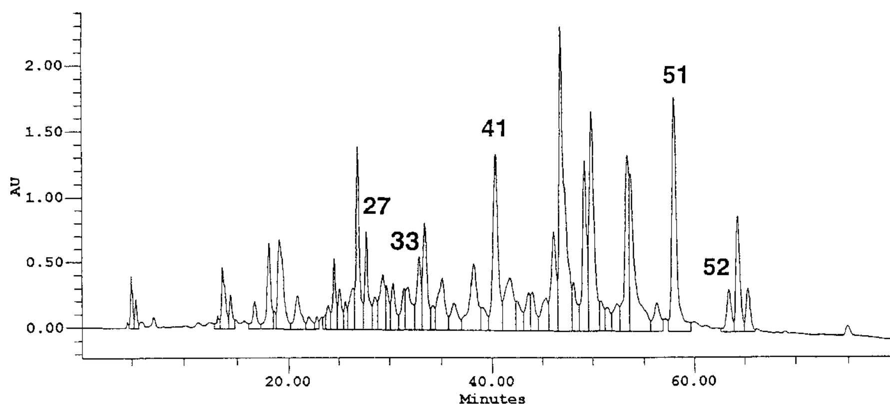

Figure 6. Reverse phase HPLC of total peptic bovin hemoglobine hydrolysate on a Delta pak C18 column. Peptides were eluted with a linear gradient of acetonitrile (eluent B) from 0% to 40% in 80min. (solvent A: 10mM ammonium acetate, pH6.0).

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APPLICATION OF THIS METHOD TO THE DETECTION OF HEMORPHINS IN A TOTAL BOVIN HEMOGLOBIN HYDROLYSATE

The total bovine hemoglobin hydrolysate chromatographic profile is shown in figure 6. UV-spectra comparison performed as previously described [13] originally revealed four peaks (P27, P41, P51 and P52) as hemorphins. Analysis of their second order derivative spectra was carried out to evaluate their aromatic amino acid content. Figure 7 exhibited the second derivative spectra of peak 27 (a), 41 (b), 51 (c) and 52 (d). Figure 7a revealed the presence of tryptophan (minima at 289.5 nm) and the absence of phenylalanine and tyrosine ($X = 0$ and $Y - 0.45Z = 0$). The same results were obtained from figure 7b. This indicated that tryptophan was the only aromatic amino acid present in the peptides P27 and P41. Regarding the structure of hemorphins, these peptides could not be classified in the category of hemorphins. On the contrary, figure 7c and 7d demonstrated the presence of Phe, Tyr and Trp in peaks 51 and 52 with the ratio 1:1:1 which is representative of hemorphins. These results were verified by amino acid analysis.

Therefore, the successive application of UV-spectra comparison [13] and second order derivative spectra could be an accurate procedure to determine quickly and unambiguously the presence of hemorphins in complexe enzymatic hydrolysate.

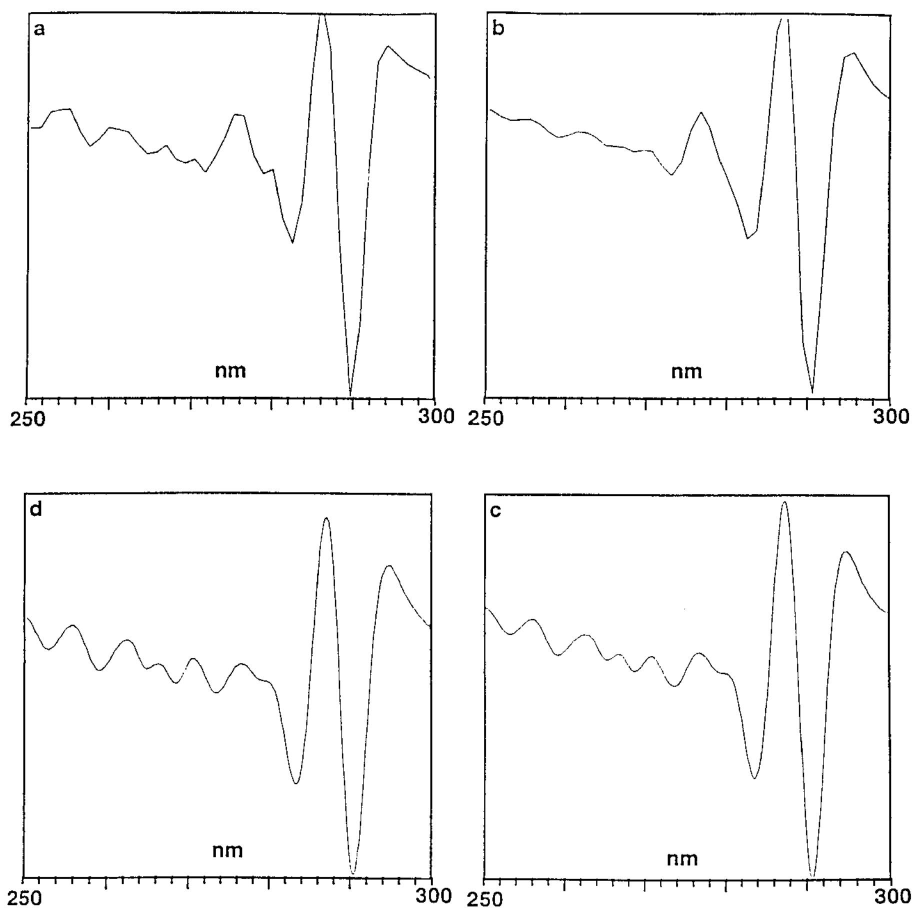

Figure 7. Second order derivative spectra of P27 (a), P41 (b), P51 (c) and P52 (d) from the total peptic bovin hemoglobine hydrolysate chromatographed on a Delta pak C18 column, selected as hemorphins by UV-spectra comparison.

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