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Determination of Phenylalanine, Tryptophan and Tyrosine in a Mixture of Amino Acids by Second Derivative Spectrophotometry

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The second derivative spectra of *N*-acetyl ethyl esters of tryptophan, tyrosine and phenylalanine were measured with various values of derivative wavelength difference, $\Delta\lambda$, at pH 7 and 13.

The second derivative spectra of tryptophan at pH 7 and pH 13 were very similar at any $\Delta\lambda$, as were those of phenylalanine. However, the derivative spectrum of tyrosine at pH 13 was quite different from that at pH 7; at pH 7 there were two peaks and troughs between 270 and 300 nm, whereas at pH 13, these spectral bands were not present but there were broad positive and negative spectral bands, due to acid dissociation of the phenolic -OH group.

A method for the quantitative determination of these amino acids was developed on the basis of the spectral properties of the three aromatic amino acids. This method should be suitable for determining the amounts of these three aromatic amino acid residues in proteins.

Keywords——derivative spectrophotometry; phenylalanine; tryptophan; tyrosine; determination of amino acid; satellite spectral band

The aromatic amino acids tyrosine, tryptophan and phenylalanine in proteins have characteristic absorption bands in the ultraviolet region. However, quantitative determination of these amino acid residues is very difficult owing to the spectral similarities of tyrosine and tryptophan at pH 7,[1] and to masking of the weak absorption bands of phenylalanine by the strong bands of tyrosine and tryptophan.[2]

Recently it was recognized that derivative spectrophotometry is very useful for characterizing an analyte band that is overlapped by other absorption bands with different half-widths, since the intensity of the absorption band with a small half-width increases more than that of a band with a large half-width on differentiation.[3] We developed a method for estimating the state and amount of phenylalanine residues in proteins by using second derivative spectrophotometry, thereby eliminating the effect of the strong absorption bands of tyrosine and tryptophan almost completely.[4,5] Balestrieri *et al.*[6] also reported that the number of phenylalanine residues can be determined directly from the second derivative spectra of denatured proteins.

However, it is difficult to separate completely the spectral bands of tryptophan and tyrosine from a mixture of all three chromophores.[6] Thus, it is necessary to develop methods for reducing the effect of interfering spectral bands and for analyzing the spectrum on the sloping base-line derived from the satellite components.[5] If such methods can be established, they could also be applicable for analysis of the spectral bands of various compounds besides aromatic amino acids. As a first step in analyzing the spectral bands of the three aromatic amino acid residues in proteins, a study on the characterization of the spectral properties of the chromophoric amino acids has been undertaken.

This paper deals with the second derivative spectral properties of tyrosine, tryptophan and phenylalanine under various conditions and reports a method for quantitative determination of the three aromatic amino acids by measurements of the overlapping spectral bands with

emphasis on the analysis of the spectral band on the satellite components.[5] In this study, *N*-acetyl ethyl esters of aromatic amino acids were used, since the spectral properties of these modified amino acids were essentially the same as those of the corresponding unmodified amino acids, and the modified amino acids are more suitable models for the amino acid residues in proteins.

Experimental

Materials——The *N*-acetyl ethyl esters of L-tryptophan, L-tyrosine and L-phenylalanine were purchased from E. Merck, A.G., Tokyo Kasei Kogyo Co., and Sigma Chemical Co., respectively. The concentrations of these amino acids were determined spectrophotometrically based on the following molar extinction coefficients: 1420 at 274.5 nm, 5550 at 279.9 nm and 197 at 257.4 nm for the N-acetylated esters of tyrosine,[7] tryptophan,[7] and phenylalanine,[8] respectively. Solutions of the amino acids were prepared just before experiments.

Measurement of the Derivative Spectra——Derivative spectra were recorded in a Shimadzu double-beam/difference/dual-wavelength recording spectrophotometer, model UV-300, with a derivative attachment, model DES-1. The output signals from the spectrophotometer were converted electrically into derivative signals. The recording conditions were as follows: slit width, 1 nm; scan speed, 37.5, 75 and 150 nm/min for $\Delta\lambda$=1, 2 and 4 nm, respectively; response time, 0.05 and 0.2 sec for the spectrophotometer and the derivative attachment, respectively.

Results and Discussion

Spectral Properties of Aromatic Amino Acids

Fig. 1 shows the second derivative spectra of *N*-acetyl ethyl ester of phenylalanine (Ac–Phe–OEt, curves *a* and *d*), tryptophan (Ac–Trp–OEt, curves *b* and *e*) and tyrosine (Ac–Tyr–OEt, curves *c* and *f*) in 0.1 M phosphate buffer, pH 7 (solid lines), and 0.1 M KOH, pH 13 (broken lines), measured at the derivative wavelength difference, $\Delta\lambda$=1 nm. As can be seen in Fig. 1, the spectra of Ac–Phe–OEt at the two pH values are very similar, as are those of Ac–Trp–OEt, but the spectrum of Ac–Tyr–OEt depends markedly on the pH of the medium, owing to acid dissociation of its phenolic –OH group.

As reported previously,[4] the second derivative spectrum of Ac–Phe–OEt at pH 7 (curve *a*) has seven peaks and troughs between 240 and 270 nm. On changing from pH 7 to pH 13, the positions of the peaks and troughs of the spectrum shifted slightly (less than 1 nm) to longer wavelength with an increase in intensity; at pH 13, the peaks are at 243, 248, 254, 258, 261, 265 and 269 nm, and the troughs are at 240, 245, 250, 256, 259, 263 and 267 nm.

The spectrum of Ac–Trp–OEt shows a slight red-shift and a slight decrease in intensity on increasing the pH; at pH 13, the λ_{max} values are 275, 278, 285 and 292 nm, and the λ_{trough} values are 271, 277, 281 and 288 nm. The absorbance due to Ac–Trp–OEt between 245 and 269 nm is very small.

Unlike the derivative spectra of phenylalanine and tryptophan, that of tyrosine is pH-dependent. At pH 7 there are 2 peaks and troughs between 270 and 300 nm; the λ_{max} values are 278 and 287 nm, and the λ_{trough} values are 275 and 282 nm. The rather broad band at about 235 nm is due to the effect of satellite components.[5] On acid dissociation of the phenolic –OH of tyrosine, the sharp peaks and troughs disappear, with the appearance of a broad band with a maximum at about 310 nm and a trough with a flat bottom between 286 and 300 nm (curve *f*). The spectral band due to the satellite components moved to a higher wavelength of about 256 nm, owing to a red-shift of the normal absorption spectrum on acid dissociation. Fig. 1 shows that among the aromatic amino acids, only tryptophan has characteristic derivative spectral bands at above 275 nm in alkaline pH, where the phenolic –OH of tyrosine is completely dissociated.

Next, to see how the second derivative spectra of aromatic amino acids change with the derivative wavelength difference, $\Delta\lambda$, we measured the derivative spectra of Ac–Phe–OEt,

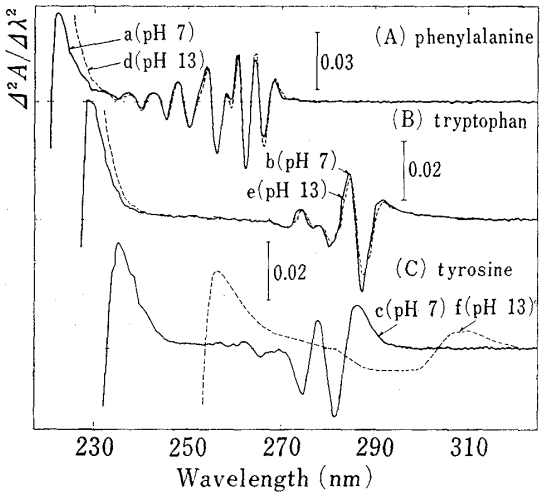

Fig. 1. Second Derivative Spectra of *N*-Acetyl Ethyl Esters of Phenylalanine (curves a and d), Tryptophan (curves b and e) and Tyrosine (curves c and f) measured with $\Delta\lambda=1$ nm

Concentrations: phenylalanine, 12.4×10^{-4} M; tryptophan, 1.20×10^{-4} M; tyrosine, 6.06×10^{-4} M. Spectra were recorded at pH 7 (curves a, b and c) and pH 13 (curves d, e and f).

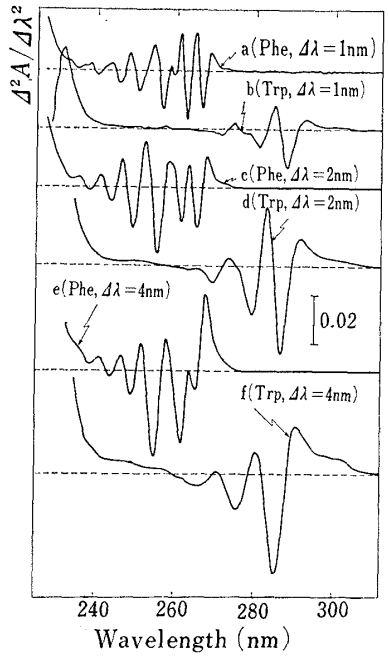

Fig. 2. Variation of the Second Derivative Spectra of *N*-Acetyl Ethyl Esters of Phenylalanine (curves a, c and e) and Tryptophan (curves b, d and f) with the Derivative Wavelength Difference, $\Delta\lambda$, measured at pH 13

Curves a and b, with $\Delta\lambda=1$ nm; curves c and d, with $\Delta\lambda=2$ nm; curves e and f, with $\Delta\lambda=4$ nm.
Concentrations of phenylalanine: 8.10×10^{-4} M (curve a) and 4.86×10^{-4} M (curves c and e).
Concentrations of tryptophan: 9.07×10^{-5} M (curve b), 6.71×10^{-5} M (curve d) and 3.35×10^{-5} M (curve f).

Ac–Trp–OEt and Ac–Tyr–OEt at various values of $\Delta\lambda$ at pH 13. Fig. 2 shows the second derivative spectra of phenylalanine and tryptophan with $\Delta\lambda=1$, 2 and 4 nm. With $\Delta\lambda=2$ nm the spectrum of phenylalanine shows maxima at 241, 247, 252, 259, 264 and 268 nm and minima at 244, 249, 255, 262 and 266 nm between 240 and 270 nm (curve *c*). The resolution of these spectral bands is not better than with $\Delta\lambda=1$ nm, but the intensities of the bands are about twice as great. In the spectrum with $\Delta\lambda=4$ nm the positions of the peaks and troughs, and the intensities of the spectral bands are very similar to those in the spectrum with $\Delta\lambda=2$ nm. The second derivative spectra of tryptophan (curves *b*, *d* and *f*) are very similar in all three cases, except that the optical intensities with $\Delta\lambda=2$ nm and $\Delta\lambda=4$ nm are 3 and 7 times that with $\Delta\lambda=1$ nm, respectively. The absorbances due to the satellite components in the region below about 260 nm increased as $\Delta\lambda$ increased, and with $\Delta\lambda=4$ nm (curve *f*) the absorbance at 245 nm was about 20 times that with $\Delta\lambda=1$ nm (curve *b*).

Fig. 3 shows the $\Delta\lambda$-dependence of the second derivative spectrum of the anionic form of tyrosine measured at pH 13. The trough between 286 and 300 nm seen with $\Delta\lambda=1$ nm became deeper as $\Delta\lambda$ was increased. Like the trough, the positive band at 310 nm became sharper with increase in $\Delta\lambda$ and showed a blue shift to 306 nm with $\Delta\lambda=4$ nm; the absorbance at 306 nm with $\Delta\lambda=4$ nm was 16 times more intense than the maximum with $\Delta\lambda=1$ nm. The intensity of the absorbance below 280 nm due to the satellite components also became greater as $\Delta\lambda$ increased.

Quantitative Determination of Aromatic Amino Acids

From the results in Fig. 1, it is concluded that at pH 13, the optical properties of phenylalanine do not have any effect, and those of tyrosine have very little effect, on the spectrum of tryptophan between 286 and 300 nm when the spectrum is measured with $\Delta\lambda$=1 nm. Thus, the concentration of tryptophan, C_{Trp}, in a mixture of aromatic amino acids can be determined without influence by the spectral bands of phenylalanine and tyrosine from the difference of the second derivative absorption, $\Delta(\Delta^2A/\Delta\lambda^2)$, between the peak and the trough of the analyte bands of tryptophan in the region of 286 and 300 nm at pH 13 with $\Delta\lambda$=1 nm. The concentration can be calculated from Eq. (1), where l is the optical path length (=1 cm), and $\Delta(\Delta^2\varepsilon/\Delta\lambda^2)_{Trp}$ is the molar derivative extinction coefficient of tryptophan.

$$C_{Trp} = \frac{\Delta(\Delta^2A/\Delta\lambda^2)}{\Delta(\Delta^2\varepsilon/\Delta\lambda^2)_{Trp} \cdot l} \qquad (1)$$

For the determination of C_{Trp}, the absorption difference between 288 and 292 nm is the most useful, since in this wavelength region the absorbance of tyrosine always has almost the same value, as shown in Fig. 1, and thus the effect of tyrosine on the spectrum of tryptophan can be cancelled out almost completely by using the difference of the derivative absorbance for this wavelength pair. The value of $\Delta(\Delta^2\varepsilon/\Delta\lambda^2)_{Trp}$ for this wavelength pair was determined as 211 cm^{-1} nm^{-2} M^{-1} (ref. 9, and *cf.* Table I).

For determination of the concentration of tyrosine C_{Tyr} in a mixture of aromatic amino acids from the derivative spectrum at pH 13, the absorbance from the base-line at about 310 nm can be used. At this wavelength, phenylalanine has no effect and the absorbance due to tryptophan is rather small.

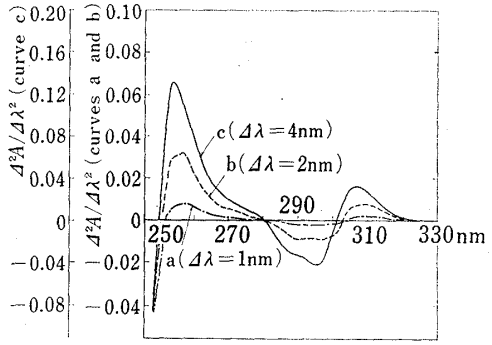

Fig. 3. Variation of the Second Derivative Spectrum of the *N*-Acetyl Ethyl Ester of Tyrosine with the Derivative Wavelength Difference, $\Delta\lambda$, measured at pH 13

Curve a, with $\Delta\lambda$=1 nm; curve b, with $\Delta\lambda$=2 nm; curve c with $\Delta\lambda$=4 nm.
Concentration of tyrosine: 2.02×10^{-4} M.

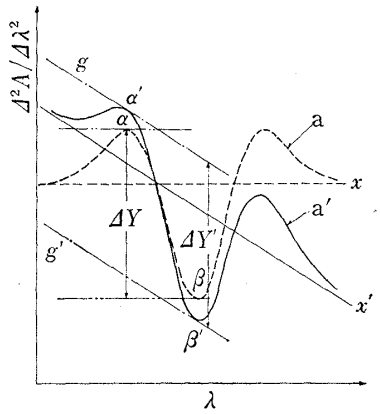

Fig. 4. Schematic Representation of the Second Derivative Absorption Spectrum based on the Gaussian Function

To amplify the derivative absorbance of tyrosine, which is small owing to the acid dissociation of phenolic –OH relative to that of tryptophan, the derivative absorbance with $\Delta\lambda$=4 nm is measured at 306 nm (the position of the maximum), since the absorption at this wavelength is 16 times the intensity at the maximum observed with $\Delta\lambda$=1 nm (*cf.* Fig. 3). At 306 nm the molar second derivative extinction coefficient of tryptophan with $\Delta\lambda$=4 nm was found to be 67.4 cm^{-1} nm^{-2} M^{-1}. Thus, the concentration of tyrosine can be determined according to Eq. (2) from the value of the second derivative absorbance at 306 nm of a solution containing aromatic amino acids and the concentration of tryptophan, C_{Trp}, which has been determined by Eq. (1). The value of $(\Delta^2\varepsilon/\Delta\lambda^2)_{Tyr}$ at 306 nm was determined as 159 cm^{-1} nm^{-2} M^{-1} (*cf.* Table I).

$$C_{Tyr} = ((\Delta^2A/\Delta\lambda^2) - 67.4 \cdot l \cdot C_{Trp})/(\Delta^2\varepsilon/\Delta\lambda^2)_{Tyr} \cdot l \quad (2)$$

It is rather complicated to determine the amount of phenylalanine from the second derivative spectrum at pH 13, since the effect of satellite components due to tryptophan and tyrosine cannot be ignored between 240 and 270 nm, where characteristic absorption bands of phenylalanine are present. Thus, a method for quantitative analysis of spectral bands superimposed on the absorbance due to satellite components had to be developed.

Generally the absorption due to satellite components can be regarded as a straight line with a certain slope over a certain wavelength region. This is shown schematically as line x' in Fig. 4, which also shows the second derivative spectrum with a peak α and trough β (curve a) on the normal base-line x based on a Gaussian function (ΔY is the derivative absorbance difference between α and $\beta(=\Delta(\Delta^2A/\Delta\lambda^2))$). The derivative spectrum can be regarded as being represented by a Gaussian function over a limited wavelength region. The sum of spectrum a and the spectrum due to the satellite components (line x'), gives curve a' with a peak α' and a trough β', the positions of α' and β' being different from those of α and β. If the slope of the line x' is known, $\Delta Y'$ in curve a' can be determined from the perpendicular distance between lines g and g', which are drawn as tangents to peak α' and trough β', respectively, with the same slope s as line x'. The value of $\Delta Y'$ obtained is equal to that of ΔY. This is proved by the following treatment.

To determine the true perpendicular distance between the peak and trough in the derivative spectrum on the sloping linear base-line, a quadratic curve (Eqs. (3) or (4)) is used instead of a Gaussian one as a model of the second derivative absorption spectrum.

$$y_1 = px^2 + qx + r \quad (3)$$

$$y_2 = -px^2 - qx - r \quad (4)$$

where p, q and r are constants with positive signs.

The linear base-line (line x' in Fig. 5) with a slope s is shown in Eq. (5), where u is a constant.

$$y_3 = sx + u \quad (5)$$

When the slope s and the constant u are equal to zero, Eqs. (3) and (4) represent, respectively, spectra a and b in Fig. 5.

The perpendicular distance between the trough of spectrum a and the peak of spectrum b ($=\Delta Y$) can be calculated as $(-q^2+4pr)/2p$ from Eqs. (3) and (4).

In the case of $s>0$ and $u>0$, spectra a and b on the base-line x' (curves a' and b', respectively) are shown in Eqs. (6) and (7) based on Eqs. (3)—(5).

For spectrum a':

$$y_4 = px^2 + (q+s)x + (r+u) \quad (6)$$

Similarly for spectrum b':

$$y_5 = -px^2 - (q-s)x - (r-u) \quad (7)$$

The tangents with slope s to spectra a' and b' are given by Eqs. (8) and (9), respectively.

$$y_6 = sx + [-q^2+4p(r+u)]/4p \quad (8)$$

$$y_7 = sx + [q^2-4p(r-u)]/4p \quad (9)$$

The perpendicular distance between the two tangents, $\Delta Y'$, is obtained as $(-q^2+4pr)/2p$. This value is the same as that obtained as the perpendicular distance between spectra a and b, ΔY.

In the region between 260 and 267 nm, the derivative spectra of tryptophan and tyrosine at pH 13 with $\Delta\lambda=1$ nm were found to be almost linear with slopes of $0.696 \cdot l \cdot C_{Trp}$, and $2.25 \cdot l \cdot C_{Tyr}$ nm^{-3}, respectively. Thus, the spectral bands due to satellite components in the wavelength region where the analyte bands of phenylalanine are present can be regarded as a straight line of slope s (per nm), as shown in Eq. (10).

$$s = (0.696 \cdot C_{Trp} + 2.25 \cdot C_{Tyr}) \cdot l \quad (10)$$

The concentration of phenylalanine, C_{Phe}, in the presence of tryptophan and tyrosine can be determined by means of Eq. (11) from the second derivative spectrum at pH 13 with $\Delta\lambda$=1 nm by using the value for the perpendicular distance $\Delta Y'$ between the tangent lines g and g' to the peak at about 265 nm and the trough at about 267 nm, respectively, as shown in Fig. 6.

$$C_{Phe} = \frac{\Delta Y'}{\Delta(\Delta^2\varepsilon/\Delta\lambda^2)_{Phe} \cdot l} \qquad (11)$$

The value of $\Delta(\Delta^2\varepsilon/\Delta\lambda^2)_{Phe}$ at this wavelength pair in the absence of tryptophan and tyrosine was determined as 37.2 $cm^{-1}\,nm^{-2}\,M^{-1}$.

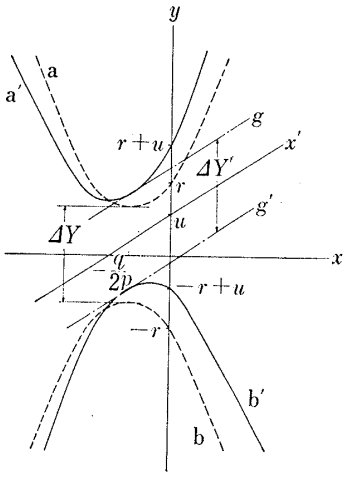

Fig. 5. Quadratic Curves as Models of the Second Derivative Spectrum

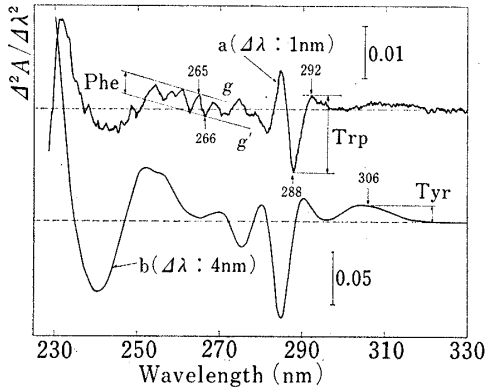

Fig. 6. Second Derivative Spectra of a Solution Containing Phenylalanine, Tryptophan and Tyrosine at pH 13.

Curve a, with $\Delta\lambda$=1 nm; curve b, with $\Delta\lambda$=4 nm.

The concentrations of *N*-acetyl ethyl esters of phenylalanine, tryptophan and tyrosine were 1.04×10^{-4} M, 7.06×10^{-5} M and 7.57×10^{-5} M, respectively.

TABLE I. Molar Second Derivative Extinction Coefficients ($\Delta(\Delta^2\varepsilon/\Delta\lambda^2)$) of *N*-Acetyl Ethyl Esters of Tryptophan, Tyrosine and Phenylalanine at Various Concentrations at pH 13

Experiment	Concentration (10^{-4} M)			$\Delta(\Delta^2\varepsilon/\Delta\lambda^2)$ ($cm^{-1}\,nm^{-2}\,M^{-1}$)[a]			Remarks
	Trp	Tyr	Phe	Trp	Tyr	Phe	
a	1.37	0	0	211	—	—	$\Delta\lambda$=1 nm
b	3.84	0	0	210	—	—	$\Delta\lambda$=1 nm
c	0	0.672	0	—	158	—	$\Delta\lambda$=4 nm
d	0	2.69	0	—	159	—	$\Delta\lambda$=4 nm
e	0	0	8.10	—	—	37.2	$\Delta\lambda$=1 nm
f	0	0	32.4	—	—	37.1	$\Delta\lambda$=1 nm
g	0.485	5.87	0	200	—	—	$\Delta\lambda$=1 nm, Tyr/Trp=12.1
h	1.77	0.921	0	—	159	—	$\Delta\lambda$=4 nm, Trp/Tyr=1.9
i	0	5.93	1.89	—	—	37.1	$\Delta\lambda$=1 nm, Tyr/Phe=3.1
j	1.98	0	1.89	—	—	37.1	$\Delta\lambda$=1 nm, Trp/Phe=1.1
k	1.98	5.93	1.89	209	—	37.2	$\Delta\lambda$=1 nm, Tyr/Trp=3.0 Tyr/Phe=3.1
l	0.706	0.757	1.04	212	159	37.2	$\Delta\lambda$=1 nm, Tyr/Trp=1.1 $\Delta\lambda$=4 nm
m	1.20	6.06	1.13	210	161	37.5	$\Delta\lambda$=1 nm, Tyr/Phe=5.4 $\Delta\lambda$=4 nm
n	1.20	0.253	0.517	212	162	37.3	$\Delta\lambda$=1 nm, Trp/Phe=2.3 $\Delta\lambda$=4 nm, Trp/Tyr=4.7

a) The value for tryptophan was calculated from the difference between $(\Delta^2\varepsilon/\Delta\lambda^2)$ at 288 and at 292 nm. The value for tyrosine was determined by means of Eq. (2), as the value of $(\Delta^2\varepsilon/\Delta\lambda^2)$ from the base-line at 306 nm. The value for phenylalanine was calculated from the difference between $(\Delta^2\varepsilon/\Delta\lambda^2)$ of the peak at about 265 nm and of the trough at about 267 nm, according to Eq. (11).

To examine the availability of this procedure for quantitative determination of aromatic amino acids, the second derivative spectra of solutions of various amounts of the three aromatic amino acids were recorded at pH 13 with $\Delta\lambda$=1 nm and 4 nm. The values of the molar derivative extinction coefficients at the analyte bands of each of the three amino acids are listed in Table I. As a typical example of these derivative spectra, the spectra due to 1.04×10^{-4} M Ac–Phe–OEt, 7.06×10^{-5} M Ac–Trp–OEt and 7.57×10^{-5} M Ac–Tyr–OEt with $\Delta\lambda$=1 nm (*a*) and $\Delta\lambda$=4 nm (*b*) are shown in Fig, 6. Above 270 nm the two spectra are very similar, but between 240 and 270 nm, the characteristic spectral bands due to phenylalanine are not seen in the spectrum with $\Delta\lambda$=4 nm, because the derivative wavelength difference, $\Delta\lambda$, is too large in comparison with the half-width of the spectral bands of phenylalanine. The positions of the positive and negative peaks due to these aromatic amino acids are slightly different (about 1 nm) from those observed in the spectra of the three amino acids singly (*cf.* Fig. 1).

Table I shows that when the ratio of the concentration of tyrosine to that of tryptophan (Tyr/Trp) is small, as in experiments k and l (Tyr/Trp=3 and 1, respectively), the values of $\Delta(\Delta^2\varepsilon/\Delta\lambda^2)$ between 288 and 292 nm due to tryptophan can be determined very accurately. However, when Tyr/Trp is high, as in experiment g (Tyr/Trp=12), the molar derivative extinction coefficient of tryptophan is slightly influenced by tyrosine, being about 5% less than in the absence of tyrosine and phenylalanine.

For the determination of tyrosine, the effect of tryptophan should be taken into account as shown in Eq. (2). The results in Table I clearly show that even in the presence of a large amount of tryptophan, the value of $(\Delta^2\varepsilon/\Delta\lambda^2)$ of tyrosine at about 306 nm is the same as that determined with tyrosine alone, as typically seen in experiments h and n.

Our previous work[5] showed that the amount of phenylalanine in a mixture of aromatic amino acids and in proteins can be determined very easily from the derivative absorbance difference between 265 and 267 nm, without any effect of satellite components due to tryptophan and tyrosine. Though the absorbance difference at this wavelength pair at pH 13 is affected mainly by rather large satellite bands of dissociated tyrosine, it would be very convenient to determine the amount of phenylalanine from the derivative spectrum at pH 13 simultaneously during the course of the determination of other aromatic amino acids. The results in Table I clearly indicate that even in the presence of a large amount of tyrosine, as in experiments i, k and m, the value of $\Delta(\Delta^2\varepsilon/\Delta\lambda^2)_{\mathrm{Phe}}$ between the peak at about 265 nm and the trough at about 267 nm can be determined by means of Eq. (11) without any effect of satellite components.

Since the relative amounts of the three amino acids listed in Table I cover those of a wide variety of proteins,[10] it should be possible to determine the amounts of aromatic amino acid residues in various proteins directly from the second derivative spectra of the proteins by a treatment similar to that described in this paper.

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