Derivative Spectrophotometric Method for the Analysis of Tyrosine in Unhydrolyzed Protein, Food, and Feedstuff Samples

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A rapid and simple procedure for the analysis of tyrosine in proteinaceous materials has been developed. Sample is homogenized with sodium hydroxide solution, and the homogenate is centrifuged. Tyrosine is directly quantified in sample extract on the basis of the characteristic fourth derivative trough appearing at around 257.2 nm. Prior hydrolysis or additional cleanup of the extract is not required. The method can be directly applied to a large variety of samples including proteins, foods, and feedstuffs. When a number of pure proteins were analyzed by the method, the values of tyrosine residues found within proteins coincided quite well with sequence data of the literature.

INTRODUCTION

The chromatographic analysis of amino acids by ion-exchange, gas, and reversed-phase chromatography has been extensively investigated. Considerable advances have been made in increasing sensitivity, resolution, and reliability for measurement of amino acids in biological samples. However, for amino acid analysis of proteins, one major limiting parameter remains for both the precision and accuracy with which the amino acid composition can be determined and for the speed and cost with which those data can be obtained. This limiting parameter is the reliable preparation of the sample hydrolysate for the chromatographic method.

Hydrolysis of proteins is commonly carried out with 6 M hydrochloric acid at 110 °C for 18–24 h. The conditions used to hydrolyze proteins represent, in fact, a compromise aimed at yielding the best estimate of amino acid composition. Threonine and serine are susceptible to destruction during hydrolysis, while the peptide bonds in which valine and isoleucine are involved are difficult to hydrolyze. Cystine, cysteine, and methionine are also frequently destroyed to varying degrees depending on the sample matrix, while tyrosine may undergo progressive destruction by the presence of residual oxidizing agents, and some may be converted to chloro or bromo derivatives (White and Hart, 1992). Review papers (Roach and Gehrke, 1970; Davies and Thomas, 1973; Blackburn, 1978; Ambler, 1981) on the hydrochloric acid hydrolysis of proteins to amino acids discuss factors such as the concentration and purity of the acid, the hydrolysis time and temperature, the presence of carbohydrates in the matrix, the effects of aldehydes and metal impurities, and the kinetics of protein hydrolysis and amino acid destruction, and they describe various strategies for improving the yield of these refractory and labile amino acids. There is, therefore, increasing interest in developing alternative methods for the accurate determination of any of these labile amino acids in proteins, foods, and feedstuffs.

Alternative methods for the determination of tyrosine in pure proteins, without prior hydrolysis, have already been published (Servillo et al., 1982; Nozaki, 1990). They are both based on the prominent UV absorption of tyrosine residues within proteins, using second-derivative spectrophotometry to resolve the complex absorption spectra of the fully denatured samples into the contributions of all three aromatic amino acids. These methods have the potential of being an important addition to the chromatographic procedures for the determination of tyrosine in pure proteins, but the results drawn are frequently subject to variation according to the aromatic amino acid composition of the sample.

In this paper, a better approach to the measurement of tyrosine by derivative spectrophotometry is presented. Discrimination of tyrosine in intact proteins, foods, and feedstuffs is carried out at strongly alkaline pH by the fourth-derivative spectrophotometry. Quantification is performed on the basis of the intensity of the fourth-derivative trough at around 257.2 nm, a spectral region where the contribution of other aromatic amino acids to the ionized tyrosine is almost negligible.

MATERIALS AND METHODS

Instrumentation. A Shimadzu Model UV-160A double-beam spectrophotometer with 1-cm quartz absorption cells was used for all measurements. Fourth-derivative UV spectra were produced by electronic differentiation of the normal spectra obtained at a scanning speed of 480 nm/min. The derivative wavelength difference ($\Delta\lambda$), which depends on the measuring wavelength range and the setting of the key entry N, was 4.2 nm for a λ range $\leq$100 nm. In the case of fourth-order derivatization a setting of $N = 7$ was used.

A Retsch (Germany) laboratory mill equipped with a 0.5-mm screen, an Ultra-Turrax blender (Janke & Kunkel, GmbH, Germany), and a BHG Model 1100 (Germany) centrifuge were also used for sample treatment.

Reagents. Amino acid standards of *N*-acetyl-L-tryptophanamide, *N*-acetyl-L-tyrosinamide, *N*-acetyl-L-phenylalanine ethyl ester, and L-cystine were all purchased from Sigma Chemical Co. (St. Louis, MO).

Stock solutions of *N*-acetyl-L-tyrosinamide were prepared by weighing ca. 25 mg of the amino acid and dissolving in and diluting to 25 mL with 0.1 M sodium hydroxide solution. Aliquots of these stock solutions were further diluted to give working solutions in the range 3.4–27.4 μg of tyrosine/mL or 18.8–150.9 μM. Stock solutions were prepared fresh weekly and kept in a refrigerator when not in use.

Protein Samples. Gluten (wheat) and gliadin (wheat) were obtained from BDH Ltd. (Poole, England). Casein (cow milk),

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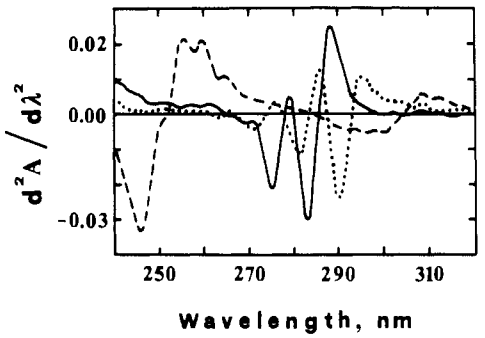

Figure 1. Second-derivative UV spectra of 60.9 and 24.4 μM *N*-acetyl-L-tyrosinamide at pH 6.7 (—) and 13 (- - -), respectively, and of 12.2 μM *N*-acetyl-L-tryptophanamide (···) at both neutral and strongly alkaline pH.

lysozyme (chicken egg white), ovalbumin (chicken egg), albumin bovine, and histone (calf thymus) were all purchased from Sigma. Cytochrome *c* (horse heart) was obtained from Boehringer Mannheim (Germany).

Pure protein concentrations were determined spectrophotometrically using the following molar extinction coefficients: 4.6×10^4 at 278 nm for albumin bovine (Ichikawa and Terada, 1981), 29.5×10^3 at 550 nm for cytochrome *c* (Servillo et al., 1982), 37.8×10^3 at 280 nm for lysozyme (Servillo et al., 1982), and 3.3×10^3 at 280 nm for ovalbumin (Dayhoff, 1972). The nitrogen content of other proteins was determined according to Method 960.52 (AOAC, 1990).

Food and Feedstuff Samples. Corn, barley, wheat, skimmed milk powder, cow milk, soybean meal, isolated soy protein, minced beef, yogurt, feta cheese, and fish meal were all purchased from local markets. Nitrogen contents were determined on each sample by AOAC (1990) methods (Methods 920.87, 920.105, 928.08, and 954.01).

Analytical Procedure. A quantity of 15–20 mg of protein sample, or an equivalent amount of finely ground (0.5-mm screen) food/feedstuff sample, was weighed into a 50-mL tube. A volume (25 mL) of 0.1 M sodium hydroxide solution was added, and the content of the tube was blended for 1 min at high speed. The homogenate was centrifuged for 3 min at 2000*g* to precipitate any suspended material, and an aliquot of the supernate was submitted to UV spectrophotometry and then to fourth-derivative processing. In cases when the recorded absorbance value at around 257.2 nm was higher than 1, sample extracts were appropriately diluted.

Determination. Normal and fourth-derivative UV spectra of both sample extract and series of working solutions were obtained according to described conditions. The calibration curve was constructed by plotting values of trough depth at 257.2 nm, as they were printed on the instrumental chart in arbitrary units, vs concentration of each of the working solutions. The concentration of tyrosine in sample extracts was calculated using slope and intercept data of the computed least-squares fit of the calibration curve. The percentage of tyrosine in proteinaceous samples or the number of tyrosine residues in protein molecules was determined using appropriate dilution factors.

RESULTS AND DISCUSSION

The UV spectrophotometric determination of tyrosine in pure proteins by existing second-derivative methodology (Servillo et al., 1982; Nozaki, 1990) is seriously affected by the presence of tryptophan. Figure 1 shows the large spectral overlap between these two amino acids, at pH 6.7, above 275 nm. Tryptophan tends to increase the intensity of the tyrosine trough centered at 283 nm and to reduce, concurrently, the intensity of the tyrosine peak at 289 nm. As these second-derivative bands are those employed as reference points for tyrosine quantification, the accuracy of the available second-derivative spectrophotometric methods depends largely, in fact, on the tryptophan/tyrosine ratio of the analyzed sample.

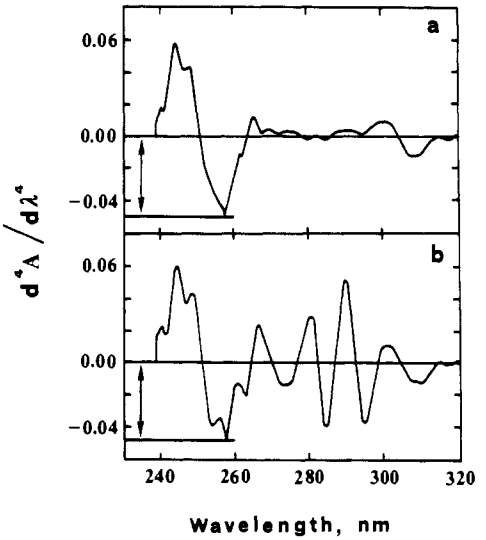

Figure 2. Fourth-derivative UV spectra of 54.3 μM *N*-acetyl-L-tyrosinamide (a) and of a mixture (b) containing 54.3 μM *N*-acetyl-L-tyrosinamide, 218 μM *N*-acetyl-L-phenylalanine ethyl ester, 288 μM L-cystine, and 20 μM *N*-acetyl-L-tryptophanamide in 0.1 M sodium hydroxide solution.

To eliminate tryptophan interference, other workers (Balestrieri et al., 1980) suggested an alternative analytical scheme for measuring tyrosine in mixtures containing all three aromatic amino acids. Their method benefited from the large difference between the UV absorption spectra of the ionized and nonionized forms of *N*-acetyl-L-tyrosinamide. Figure 1 illustrates second-derivative spectra of *N*-acetyl-L-tyrosinamide and *N*-acetyl-L-tryptophanamide recorded at both neutral and strongly alkaline pH. It becomes clear that the *N*-acetyl-L-tryptophanamide spectrum does not change with pH, while that of *N*-acetyl-L-tyrosinamide is dramatically changed. The finely detailed derivative pattern of *N*-acetyl-L-tyrosinamide at neutral pH between 275 and 295 nm is essentially lost with alkalinization at pH 13, and new characteristic derivative peaks appear at 255, 260, and 309 nm. On the basis of the peak centered at 260 nm, Balestrieri et al. (1980) were able to isolate tryptophan contribution to tyrosine determination, but new interferences arising from both phenylalanine and ionized cysteine concurrently appeared.

In an attempt to improve the above-mentioned analytical scheme, our preliminary experiments were directed toward examining whether the second-derivative peak of tyrosine at 309 nm might also be useful for tyrosine quantification at alkaline conditions. At 309 nm, phenylalanine and ionized cysteine were not expected to interfere with the analysis because their second-derivative spectra are essentially flat above 275 nm. However, considerable interference from tryptophan reappeared when the tryptophan/tyrosine ratio of the amino acid mixture was as low as 1/3.

Since second-derivative spectrophotometry could not help in further improving band discrimination, the resolving power of fourth-derivative spectrophotometry was alternatively investigated. Figure 2 illustrates fourth-derivative spectra of a 54.3 μM *N*-acetyl-L-tyrosinamide solution and of a mixture containing, in excess of *N*-acetyl-L-tyrosinamide, 218 μM *N*-acetyl-L-phenylalanine ethyl ester, 288 μM L-cystine, and 20 μM *N*-acetyl-L-tryptophanamide in 0.1 M sodium hydroxide solution. It is readily seen that great transformation of the derivative spectrum of tyrosine occurs due to the additional differentiation process. A distinct fourth-derivative trough appears at

Table I. Effect of the Presence of L-Cystine, *N*-Acetyl-L-tryptophanamide, and Various Amounts of *N*-Acetyl-L-phenylalanine Ethyl Ester on the Fourth-Derivative Spectrophotometric Determination of *N*-Acetyl-L-tyrosinamide at pH 13

composition of amino acid mixtures, μM				
N-acetyl-L-tyrosinamide	L-cystine	*N*-acetyl-L-phenylalanine ethyl ester	*N*-acetyl-L-tryptophanamide	found[a] concn of *N*-acetyl-L-tyrosinamide, μM ± SD
0	0	0	273.5	0.0 ± 0.0
0	288	0	0	0.0 ± 0.0
54.3	0	0	0	54.3 ± 0.0
54.3	288	0	58.1	54.3 ± 0.0
0	0	54.5	0	0.0 ± 0.0
54.3	288	54.5	58.1	54.3 ± 0.5
54.3	288	109	58.1	53.2 ± 0.8
54.3	288	163.5	58.1	52.1 ± 0.5
54.3	288	218	58.1	51.0 ± 0.5

[a] Average of five determinations.

Table II. Raw Date and Regression Equations of Calibration Curves for Tyrosine Determination by Fourth-Derivative Spectrophotometry at Wavelength Difference (Δλ) of 4.2 or 5.4 nm

tyrosine concn in standard solution		trough depth, arbitrary units	
μg/mL[a]	μM[b]	Δλ = 4.2 nm	Δλ = 5.4 nm
3.4	18.9	0.017	0.036
6.8	37.7	0.034	0.073
10.3	56.6	0.052	0.110
13.7	75.5	0.068	0.143
20.5	113.2	0.101	0.213
27.3	150.9	0.135	0.280

[a] Regression equation (Δλ = 4.2 nm): $Y = (6.6 \times 10^{-4}) + (4.91 \times 10^{-3})X$ (correlation coefficient, $r = 0.99993$). Regression equation (Δλ = 5.4 nm): $Y = (3.4 \times 10^{-3}) + (10.17 \times 10^{-3})X$ correlation coefficient, $r = 0.99983$). [b] Regression equation (Δλ = 4.2 nm): $Y = (6.6 \times 10^{-4}) + (8.90 \times 10^{-4})X$ (correlation coefficient, $r = 0.99993$). Regression equation (Δλ = 5.4 nm): $Y = (3.4 \times 10^{-3}) + (1.84 \times 10^{-3})X$ (correlation coefficient, $r = 0.99983$).

257.2 nm, the depth (Figure 2a) of which can be directly related to tyrosine concentration. Table I shows that the presence of both *N*-acetyl-L-tryptophanamide and L-cystine does not exert any influence on the depth of the tyrosine trough. *N*-Acetyl-L-phenylalanine ethyl ester, on the other hand, exerts some influence on the tyrosine trough, but this is almost negligible when the molar ratio of these amino acids is up to 1/1. At higher ratios this interference is progressively increased, leading to a 6.1% underestimation of tyrosine when the ratio becomes as high as 4/1. As compensation for interfering *N*-acetyl-L-phenylalanine ethyl ester could be readily made by a standard addition method, the fourth-derivative trough at 257.2 nm was considered to be a good reference point for measuring tyrosine concentration.

To further optimize the quantification, the influence of the derivative wavelength difference (Δλ) setting of the instrument on the intensity of the tyrosine trough at 257.2 nm was also examined. Although this intensity became greater with increasing Δλ setting, the regression equations (Table II) between the concentrations of the working solutions and trough depths at two selected Δλ settings pointed out that such an increase exerts some influence on the linearity of the calibration curve. Considering that the slight decline in linearity observed with the 5.4-nm setting was possibly due to deterioration of spectral resolution, the derivative wavelength difference setting at 4.2 nm was finally selected.

The accuracy and precision study of the method, performed with a number of pure proteins of which sequence data have been established (Dayhoff, 1972), is summarized in Table III. Excellent recovery was found, and relative standard deviation values indicate that sodium

Table III. Precision and Accuracy Data for the Determination of Tyrosine Residues in Proteins by Fourth-Derivative Spectrophotometry

protein	number of tyrosine residues		recovery, %	RSD, %
	determined[a]	literature[b]		
albumin bovine	18.70 ± 1.06 (19.66)[c]	20	93.5 (98.3)	5.4
cytochrome *c*	3.90 ± 0.05	4	97.5	1.3
histone	4.04 ± 0.06	4	101.0	1.5
lysozyme	2.95 ± 0.08	3	98.3	2.7
ovalbumin	10.10 ± 0.05	10	101.0	0.5

[a] Mean of triplicate analysis ± SD. [b] Literature values are those of Dayhoff (1972). [c] Value in parentheses is by standard addition method.

hydroxide assists in the liberation of any masked tyrosine residues in the protein molecules, a finding that lends support to previous studies (Bencze and Schmid, 1957). Nevertheless, a minor discrepancy between the present and literature data appears for bovine albumin, but this could be readily arranged by applying a standard addition method. The data of Table III may also suggest that substances which produce a constant background absorption or a gradual background variation can be easily normalized by the fourth-derivative function which takes zero value. This makes the developed method particularly useful for analysis in the presence of turbidity or when the background absorbance is high, such as in foods and feedstuffs.

The results from application of the method to a number of protein, food, and feedstuff samples selected to include a range of protein contents and a variety of protein sources are summarized in Table IV. Although comparison of the present data with those reported in the literature for one sample of a given food/feedstuff can be approximate only, Table IV shows that most of the drawn data are close to those reported by other workers. Regarding corn, there is a discrepancy between the value reported by Elkin and Griffith (1985) and the present value, but this might be due to difference in protein quality of the compared samples or, even, to the different quantification systems applied. Elkin and Griffith (1985), studying the amino acid analysis of corn, have already pointed out that tyrosine and phenylalanine values can be significantly lower by reversed-phase compared with ion-exchange chromatography.

Representative fourth-derivative spectra of the analyzed samples are illustrated in Figure 3. They all show the same general features of the amino acid mixture presented in Figure 2b. From a comparison of these features with the spectrum of the standard *N*-acetyl-L-tyrosinamide (Figure 2a), it becomes evident that the latter matches nearly perfectly those of tyrosine residues within proteins.

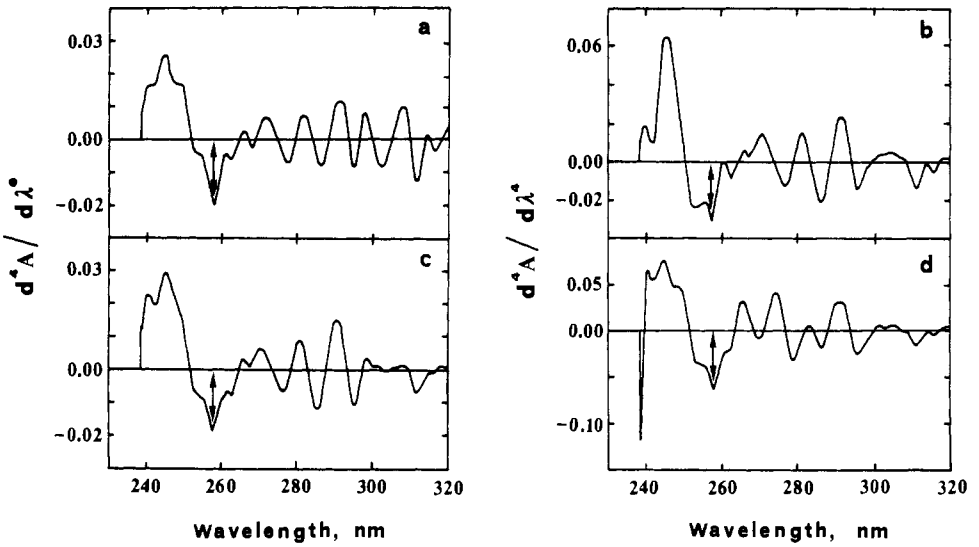

Figure 3. Representative fourth-derivative UV spectra of sample extracts: (a) milk; (b) barley; (c) yogurt; (d) fish meal.

Table IV. Tyrosine Content of Proteins, Foods, and Feedstuffs As Determined by Fourth-Derivative Spectrophotometry

type of sample	tyrosine,[a] g/100 g of protein		literature data
casein	6.06	5.2–5.4	Knipfel et al., 1971
		6.04	Sarwar et al., 1983
		5.94	FAO, 1970
		6.3	Ashworth et al., 1966
gluten	3.16		
gliadin	2.46		
minced beef	3.56	3.14	Sarwar et al., 1983
		3.6	FAO, 1970
isolated soy protein	3.02		
barley	4.50		
corn	3.04	4.2–4.4	Elkin and Griffith, 1985
wheat	3.02	3.5–3.7	Kan and Shipe, 1981
		3.4	Gehrke et al., 1985
		2.9	Sarwar et al., 1983
		3.0	FAO, 1970
cow milk	5.83		
skimmed milk powder	6.56		
yogurt	5.55		
feta cheese	5.11		
soybean mean	3.38	3.3–3.5	Knipfel et al., 1971
		3.75	Cavins et al., 1972
		3.6–3.8	Gehrke et al., 1985
		3.4–4	Elkin and Griffith, 1985
		4	Sarwar et al., 1983
		3.14	FAO, 1970
fish meal	3.17	3.2–3.3	Knipfel et al., 1971
		3.5–3.7	Gehrke et al., 1985

[a] Values given are average of duplicate analyses.

There is a slight red shift of the tyrosine trough into proteins, but this is, in all cases, less than 0.4 nm and has no influence on tyrosine determination. It can be considered, therefore, that *N*-acetyl-L-tyrosinamide in 0.1 M sodium hydroxide provides an excellent model compound for determining tyrosine residues.

On the basis of the above, it can be concluded that the developed fourth-derivative spectrophotometric method might be useful as a low-cost and fast alternative to existing chromatographic methods for accurate and precise determination of tyrosine in proteinaceous materials. A laboratory can have tyrosine analysis capability inherent in its spectrophotometric system without any compromise in instrument versatility. These advantages make the developed method particularly useful in cases where dedicated instrumentation and/or experienced staff cannot be found.

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