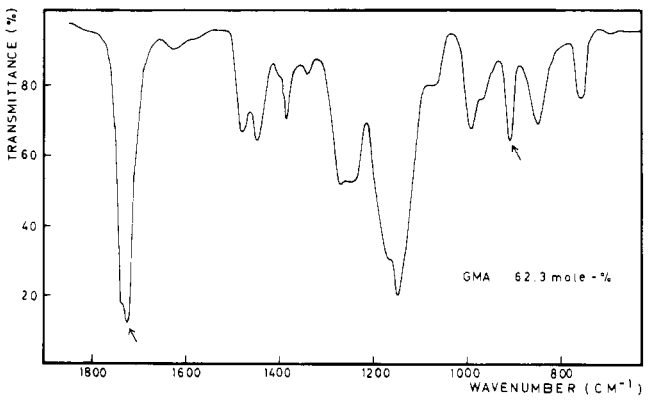

Figure 1. IR spectrum of an MMA–GMA copolymer containing 62.3 mol % GMA determined chemically

lymerization, using 2,2′-azo-bis(isobutyronitrile) (AIBN) as initiator at 60 °C in vacuum sealed ampoules. The copolymerization studies will be reported elsewhere (*8*).

Chemical Analysis. The content of oxirane oxygen and thereby the mole fraction of GMA in the MMA–GMA copolymers have been determined chemically by a hydrochlorination reaction (*8*).

Infrared Analysis. The IR analyses were made on dried potassium bromide pellets of 1.3-cm diameter containing 0.5 mg of copolymer sample in 200 mg of KBr. The pellets were prepared by pressing with about 10 tons under vacuum in a hydraulic press. The spectra were recorded on a Perkin-Elmer model 257 spectrophotometer, using medium scan rate and normal slit width.

RESULTS AND DISCUSSION

An infrared spectrum from 1800 and 625 cm^{-1} for one of the MMA–GMA copolymers is presented in Figure 1. The peaks at the wavenumbers 907 and 1717 cm^{-1} were selected as the most suitable ones for analysis of epoxy and carbonyl groups, respectively. Using the "base-line density" method, the values of the absorbances at 907 and 1717 cm^{-1} wavenumbers have been determined in triplicate. The average values of the absorbances, their ratio, and the GMA mole fraction determined chemically are presented in Table I.

Table I. Analytically Determined Mole Fraction of GMA in the MMA–GMA Copolymers and the Infrared Absorbances at 907 and 1717 cm^{-1}

Expt. No.	Mole fraction of GMA in the copolymer determined chemically	A(1717 cm^{-1})	A(907 cm^{-1})	Absorbance ratio $A(907)/A(1717)$
R50	0.218	1.366	0.128	0.093
R51	0.394	0.911	0.120	0.131
R52	0.584	0.629	0.115	0.182
R61	0.623	0.921	0.177	0.192
R53	0.706	0.955	0.204	0.213

The absorbance ratio at 907 cm^{-1} vs. 1717 cm^{-1} is linearly related to the GMA content in the copolymer and can be expressed by the following equation:

$$R = 0.25_0 X_G + 0.03_3$$

where R is the absorbance ratio at 907 and 1717 cm^{-1}, X_G is the mole fraction of GMA, containing the epoxy group in the copolymer. The term 0.033 is considered to be a correction factor arising from the very weak absorption due to poly(methyl methacrylate) at wavenumbers near 907 cm^{-1}.

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New Spectrophotometric Method for Simultaneous Determination of Tryptophan and Tyrosine

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Quantitative separation and determination of tryptophan and tyrosine in complex mixtures are very important, because normal growth is impossible in diets deficient in them. Two approaches are available for their determination simultaneously in mixtures. The first is based on the determination of each component in presence of the other one using a specific colorimetric reaction. The second is attained through the application of the more general colorimetric procedures of α-amino acids after chromatographic separation of each component (*1*). However, the first approach requires several samples and specific reactions, while the second is less accurate and time consuming.

On the other hand, few specific reactions are known for tryptophan and tyrosine. The color intensities which were developed by many of these reactions did not provide a suitable quantitative measure of these acids in mixtures with other amino acids (*2*). However, colorimetric determination of tryptophan in amino acid mixtures without prior separation may be achieved by virtue of its ability to undergo facile specific reaction with xanthydrol in concentrated hydrochloric acid (*3, 4*), or by reaction with *p*-dimethylaminoazobenzaldehyde (*5–7*). The only specific reaction recorded, so far, for tyrosine which is based on a reaction with 1-nitroso-2-naphthol (*8*) gives low recoveries

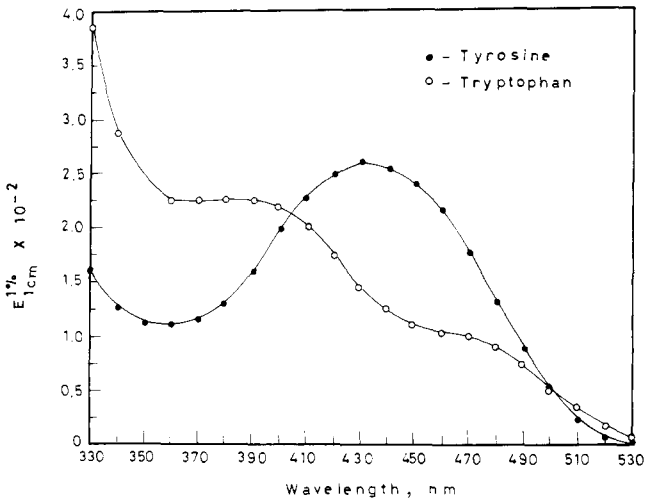


Figure 1. Absorption spectra of the nitration products at 50 °C of tryptophan and tyrosine in sodium hydroxide solution

and tryptophan; cysteine as well as a number of metallic ions interfere (*9*).

The present work was planned as a systematic study to develop a new spectrophotometric method for simultaneous determination of tryptophan and tyrosine in mixtures without prior separation. Considering the simplicity, procedural time, color stability, adherance to Beer's law, sensitivity, and accuracy, a single nitration reaction followed by alkali treatment is described for both amino acids.

EXPERIMENTAL

Apparatus. Absorbance measurements were recorded with a Unicam SP 8000 spectrophotometer. Matched quartz cells of 1.00-cm path length were used.

Reagents. All the reagents were of analytical grade unless otherwise specified: 16*M* nitric acid, 5*M* sodium hydroxide solution, and ethyl alcohol. The α-amino acids used were chromatographic grade (B.D.H.). Stock solutions of tryptophan and tyrosine were freshly prepared by dissolving 150 mg of each in about 150 ml of methyl alcohol, and 2 ml of 10*M* hydrochloric acid was added dropwise till complete dissolution. The solutions were then completed to 1 liter with ethyl alcohol.

Procedure. Construct standard calibration curves for pure tryptophan and tyrosine as follows: Deliver 0.10, 0.20, 0.40, 0.50, 0.70, 0.90, 1.00, and 1.20 ml aliquots of the above stock solutions into 20-ml graduated tubes. Evaporate on a boiling water-bath till complete dryness. Add 1.00 ml of 16*M* nitric acid and place the tubes for 10–15 minutes in a water-bath adjusted at 50 °C. Cool at room temperature, add 4.0 ml of 5*M* sodium hydroxide solution portionwise, and complete to 10 ml with ethyl alcohol. Mix the contents and measure the absorbances at 360 and 430 nm in the case of tryptophan and tyrosine, respectively, using 1.00-cm cuvettes. A blank experiment is prepared under identical conditions and is measured against the experiment. Draw a graph connecting the relation between the absorbance and concentration (μg/ml) for each amino acid. For samples of unknown concentration, follow the same procedure and compare with the respective calibration graph.

For simultaneous determination of tryptophan and tyrosine, follow the above procedure and calculate the concentration of each component using the following equations:

$$\text{Tryptophan } (\mu g/ml) = \frac{260\,A_{\lambda_1} - 110\,A_{\lambda_2}}{225 \times 260 - 110 \times 150} = 0.61905\,A_{\lambda_1} - 0.2619\,A_{\lambda_2} \quad (1)$$

$$\text{Tyrosine } (\mu g/ml) = \frac{225\,A_{\lambda_2} - 150\,A_{\lambda_1}}{225 \times 260 - 110 \times 150} = 0.5357\,A_{\lambda_2} - 0.35714\,A_{\lambda_1} \quad (2)$$

where, A_{λ_1} and A_{λ_2} are the absorbance readings at 360 and 430 nm, respectively.

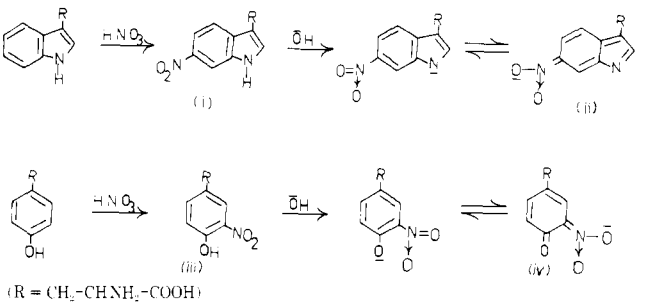
RESULTS AND DISCUSSION

Characteristics of the Spectra. Alcoholic solutions of tryptophan and tyrosine show absorption maxima at 215 and 205 nm, respectively, with a broad absorption band at 240–280 nm. No absorption above 300 nm is indicated by any of these acids. Treatment of tryptophan and tyrosine with 16*M* nitric acid at 50 °C, introduces a strong chromophoric nitro group in the aromatic moiety of the molecules. The alkaline solution of these nitration products exhibits a maximum absorption (broad band) at 360–400 nm with $E^{1\%}_{1cm}$ of 225 in case of tryptophan and a maximum absorption (sharp peak) at 430 nm with $E^{1\%}_{1cm}$ of 260 in case of tyrosine (Figure 1).

Nature of the Reaction. The nitration product of tryptophan at 50 °C was isolated, crystallized out from methyl alcohol, and identified. The infrared spectrum shows that 6-nitrotryptophan (*i*) is a possible reaction product. The finding of other workers, that tryptophan (*10*) and 3-substituted indole (*11*) undergo nitration reaction in position 6, is in agreement with the present results.

The absorption spectrum of the nitration product of tyrosine in sodium hydroxide solution (λ_{max} 430 nm, $E^{1\%}_{1cm}$ 260) was compared with the spectra of *o*-, *m*-, and *p*-nitrophenols in sodium hydroxide. *o*-Nitrophenol displayed the same absorption spectrum. It is possible, therefore, that the phenolic group by virtue of its +M effect activates the ortho position leading to the formation of 2-nitrotyrosine (iii).

The formation of colored species (ii) and (iv) by the action of alkalies on these nitro derivatives is ascribed to their ability to tautomerize to yield salts of quinoidal nitronic acid (*12*). These reactions may be represented as follows:



Effect of Reaction Temperature. The effect of temperature on the reaction of tryptophan and tyrosine was studied by carrying out the nitration for 10 minutes at temperatures ranging from 0 °C up to 100 °C. With tryptophan, constant absorbance values were obtained when the nitration was carried out at 20 °C up to 70 °C, above which the absorbance was slightly increased. In the case of tyrosine, maximum and constant absorbance value was noticed by nitration above 20 °C up to 100 °C (Figure 2).

Effect of Reaction Time. Tryptophan and tyrosine were subjected to nitration reactions at 50 °C for 5, 10, 20, and 30 minutes. After that, sodium hydroxide solution and alcohol were immediately added and the absorbance was measured. No effect due to the change in time, within the range of 5 to 20 minutes, was noticed on the absorbance of the reaction products of both amino acids (Figure 3).

It is recommended, therefore, to carry out the nitration at 50 °C for 10–15 minutes. Under these conditions, unavoidable experimental increase or decrease in the nitration temperature within ±10 °C or nitration time to ±5 minutes does not affect the absorbance of the reaction products.

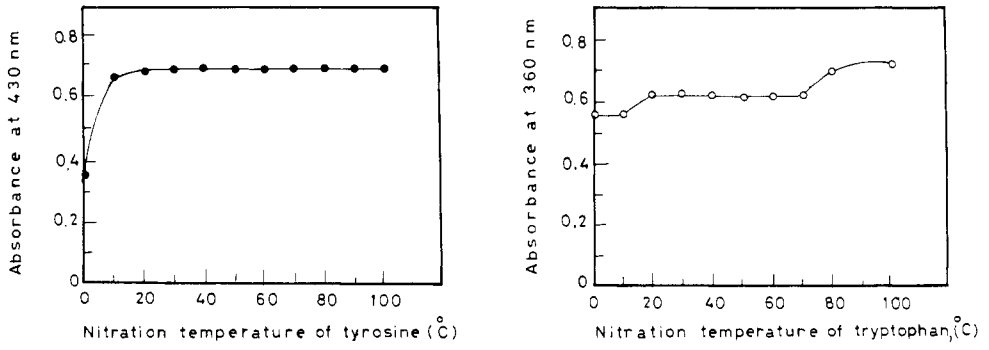


Figure 2. Effect of the nitration temperature of tyrosine and tryptophan (27 μg/ml) for 15 minutes on the absorbance of the nitration products

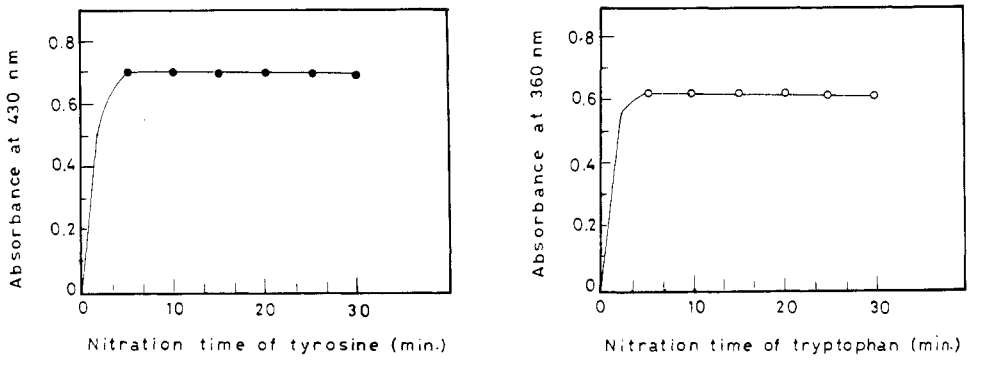


Figure 3. Effect of the reaction time of tyrosine and tryptophan (27 μg/ml) at 50 °C on the absorbance of the nitration products

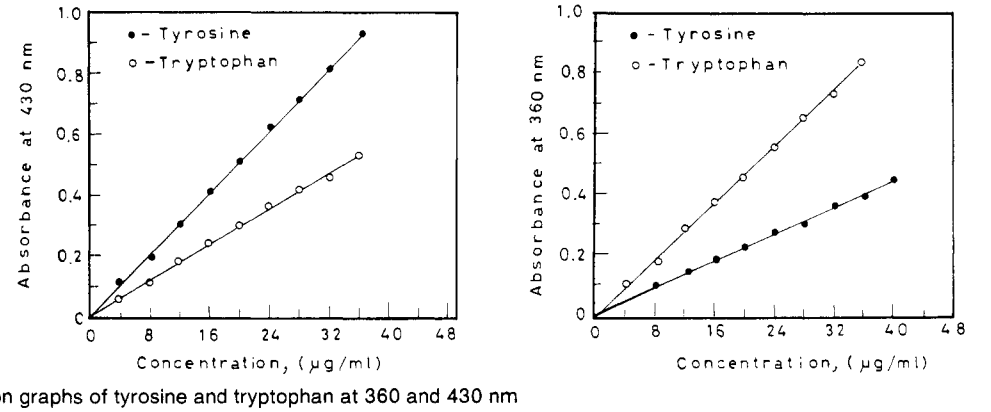


Figure 4. Calibration graphs of tyrosine and tryptophan at 360 and 430 nm

Effect of Alkali Concentration. Since 1 ml of 16*M* nitric acid is used in the nitration process, 0.7 gram of sodium hydroxide is needed to render the reaction mixture alkaline. An aliquote of 4 ml of 5*M* sodium hydroxide solution is quite sufficient. Higher concentrations of alkali (up to 1.2 gram) do not affect the absorbance values.

Effect of Solvent. Nitration reaction in the presence of organic solvents, specially alcohols, is not recommended. If the sample is already dissolved in an organic solvent, prior dryness before nitration must be done. On the other hand, addition of water-miscible organic solvents to the nitration products after alkalization appears to be necessary for full development of the color. Different organic solvents (e.g., ethyl alcohol, acetone, dimethylformamide, and dioxan) were used. No precipitation and maximum absorption were noticed in 50% ethyl alcohol solution.

Calibration Curves and Beer's Law. Beer's law is obeyed at 360 and 430 nm for tryptophan and tyrosine, respectively, in the concentration range of 2–40 μg/ml. Moreover, Beer's law is obeyed by each amino acid at these two wavelengths (Figure 4), and the color is stable for at least 20 hours.

The results obtained for the analysis of either tryptophan or tyrosine (Table I), show that the method possesses great advantages on the score of ease and accuracy since an average recovery of 99% is obtained. A standard deviation of ±0.5% was calculated from a series of 20 measurements for replicate analysis of 15 μg/ml of both α-amino acids.

Simultaneous Determination of Tryptophan and Tyrosine. The use of the following equations permits the analysis of both tryptophan and tyrosine in mixtures: Tryptophan concentration = $E_2\lambda_2A\lambda_1 - E_2\lambda_1A\lambda_2/E_1\lambda_1E_2\lambda_2 - E_2\lambda_1E_1\lambda_2$. Tyrosine concentration = $E_1\lambda_1A\lambda_2 - E_2\lambda_1A\lambda_1/E_1\lambda_1E_2\lambda_2 - E_2\lambda_1E_1\lambda_2$. E_1 and E_2 were calculated from the absorbance of 1% solution in a 1.00-cm cell (i.e., $E^{1\%}_{1cm}$ values). These values were found to be as follows:

Table I. Spectrophotometric Determination of Tryptophan and Tyrosine

Sample	μg/ml Taken	μg/ml Found	Recovery, %
Tryptophan	6.0	5.9	98.3
	9.0	8.9	98.8
	12.0	12.1	100.8
	18.0	18.2	101.1
	25.0	24.8	99.2
Tyrosine	6.3	6.2	98.4
	9.7	9.8	101.0
	12.6	12.5	99.2
	18.9	18.7	98.9
	25.2	25.0	99.2

Table II. Simultaneous Spectrophotometric Determination of Tryptophan and Tyrosine in Binary Mixtures

Tryptophan, μg/ml Taken	Found	Recovery %	Tyrosine, μg/ml Taken	Found	Recovery %
6.0	5.9	98.3	12.6	12.4	98.4
6.0	5.9	98.3	18.8	18.9	100.5
12.0	12.1	100.8	6.3	6.2	98.4
18.0	18.1	100.5	6.3	6.1	96.8
12.0	11.9	99.1	9.5	9.3	97.9
9.0	9.0	100.0	12.6	12.5	99.2
15.0	15.3	102.0	9.5	9.3	97.9
9.0	8.8	97.8	15.5	15.7	101.3
12.0	12.2	101.7	12.6	12.3	97.6
6.0	6.1	101.6	6.3	6.3	100.0

Tryptophan [$E_1\lambda_1$(i.e., $E^{1\%}_{1cm}$ at 360 nm) =

225, $E_1\lambda_2$ (i.e., $E^{1\%}_{1cm}$ at 430 nm) = 150] (3)

Tyrosine [$E_2\lambda_2$ (i.e., $E^{1\%}_{1cm}$ at 430 nm) =

260, $E_2\lambda_1$ (i.e., $E^{1\%}_{1cm}$ at 360 nm) = 110] (4)

To test the above equations, several known mixtures of tryptophan and tyrosine in varying proportions ranging from 1:3 to 3:1 were made up and analyzed. Table II, shows that the results are fairly accurate. An average recovery of 99% and a standard deviation of ±0.6% are obtained for both components.

Interferences. No interferences are caused by glycine, phenyl alanine, alanine, asparagine, aspartic acid, glutamic acid, proline, lysine monohydrochloride, valine, cystine, norleucine, glutathione, and threonine. Addition of these amino acids up to 1000-fold molar excess over tryptophan or tyrosine do not affect the sensitivity of the method. However, milligram amounts (above 2 mg) of histidine, cysteine, arginine monohydrate, and methionine slightly interfere.

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Spectrophotometric Determination of Dipyrone with Neotetrazolium Chloride

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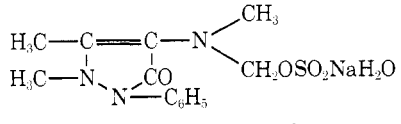
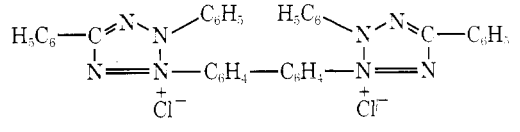
A previous paper describes the use of triphenyltetrazolium chloride (TTC) in the chromatographic investigation of sodium [*N*-(1,5-dimethyl-3-oxo-2-phenylpyrazolin-4-yl)-*N*-methylamino]methanesulfonate monohydrate (Dipyrone) (*1*).



The results obtained suggest that the tetrazolium salts emerge as prospective analytical reagents in Dipyrone determination.

Further investigations elucidate the problem to a still greater extent.

Neotetrazolium chloride [2,2′,5,5′-tetraphenyl-3,3′-(4,4′-biphenylene)-ditetrazolium chloride] represents a ditetrazolium salt, which similarly to TTC reacts with Dipyrone in alkaline medium.



A yellow colored compound, soluble in ethanol–hydrochloric acid has been obtained as a result of the aforesaid reac-