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Analytical Studies on Isoxazoles. I. A New Colorimetric Determination of 5-Substituted 3-Isoxazolecarboxylic Acids and Their Derivatives

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A new colorimetric method for the determination of 5-methyl-3-isoxazolecarboxylic acid (MIA) and 5-phenyl-3-isoxazolecarboxylic acid (PIA) was established. This method is based on the decomposition of 5-substituted 3-isoxazolecarboxylic acids to the corresponding β-ketonitriles, followed by condensation with *p*-dimethylaminobenzaldehyde (DABA). The 5-carboxylic isomers caused no interference in the determination of MIA and PIA.

Further applications of this method to perisoxal (II), N-[(dimethylamino)carbonyl]-5-methyl-3-isoxazolecarboxamide (III), and 3-(2-oxazolin-2-yl)-5-methylisoxazole (IV), by decomposition to the corresponding 5-substituted 3-isoxazolecarboxylic acids, followed by the color reaction with DABA, are described.

Keywords——colorimetric determination; 3-isoxazolecarboxylic acids; *p*-dimethylaminobenzaldehyde; Knoevenagel condensation reaction; α-benzoyl-*p*-dimethylaminocinnamonitrile; α-acetyl-*p*-dimethylaminocinnamonitrile

Various isoxazoles have been synthesized and some of them exhibit important pharmacological activities.

Although a number of investigations have been reported on chemical assay procedures for isoxazoles,[2] the reactivity of the isoxazole ring has not been effectively utilized for such assays.

Hirai and Nagai reported a color reaction for 5-phenyl-3-isoxazolecarboxylic acid (PIA).[3] This reaction is based on the decarboxylation of PIA, followed by Knoevenagel condensation with *p*-dimethylaminobenzaldehyde (DABA) to form α-benzoyl-*p*-dimethylaminocinnamonitrile (I), as shown in Chart 1.

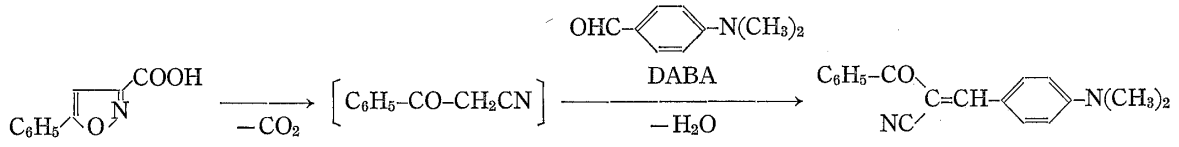
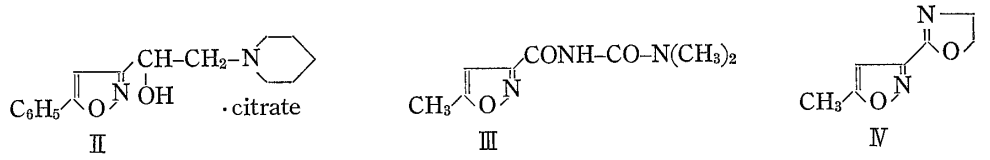


Chart 1

In the present paper, we describe the utilization of this color reaction for the determination of PIA and 5-methyl-3-isoxazolecarboxylic acid (MIA). Furthermore, this method is applied to assay some isoxazole derivatives, such as perisoxal (II),[4] N-[(dimethylamino)-carbonyl]-5-methyl-3-isoxazolecarboxamide (III),[5] and 3-(2-oxazolin-2-yl)-5-methylisoxazole

1) Location: *Sagisu, Fukushima-ku, Osaka, 553, Japan.*
2) C. Bergamini and G. Mattei, *Sperimentale, Sez. Chim. Biol.*, **6**, 13 (1955) [*C.A.*, **50**, 9941*h* (1956)]; J. Rieder and M. Roth, *Biochem. Pharmacol.*, **12**, 445 (1963); S. Pinzauti, V. Dal Piaz, and E. La Porta, *Farmaco, Ed. Prat.*, **29**, 136 (1974) [*C.A.*, **81**, 29576*a* (1974)]; R. Soliman and S.A. Belal, *Pharmazie*, **29**, 204 (1974).
3) E. Hirai and M. Nagai, *Shionogi Kenkyusho Nempo*, **22**, 1 (1972).
4) H. Kanō, I. Adachi, R. Kido, and K. Hirose, *J. Med. Chem.*, **10**, 411 (1967); H. Kanō and I. Adachi, *Shionogi Kenkyusho Nempo*, **18**, 56 (1968).
5) Shionogi and Co., Ltd., Japan. Patent 7321107 (1973) [*C.A.*, **79**, 105238*t* (1973)].

(IV),[6] which are convertible into the corresponding 5-substituted 3-isoxazolecarboxylic acids by oxidation or hydrolysis. Among them, II is a powerful analgesic and antiinflammatory agent, III possesses antidiabetic activity, and IV produces increased antibacterial activity in combination with sulfonamides or antibiotics.



II III IV

Chart 2

Experimental

Apparatus——Absorption spectra and intensities were measured with a Hitachi 356 dual-wavelength double-beam spectrophotometer. Infrared (IR) spectra were measured with a JASCO 403G spectrometer, and nuclear magnetic resonance (NMR) spectra were taken at 60 MHz with a Varian A-60 spectrometer. The chemical shifts are given in ppm from tetramethylsilane as an internal standard. The pH measurements were made using a Metrohm 436 potentiograph equipped with a Metrohm EA 121-X glass-calomel electrode.

Reagents——DMSO: Reagent-grade DMSO was purchased from Wako Pure Chemical Industries, Ltd. and used without further purification.

DABA: Reagent-grade DABA was obtained from Kanto Chemical Co., Inc. and purified by recrystallization from EtOH–H_2O (1: 1, v/v) mixture.

3.5% DABA Solution: DABA (3.5 g) was dissolved in 100 ml of DMSO.

5.0% DABA Solution A: DABA (5.0 g) was dissolved in 100 ml of DMSO–H_2O (9: 1, v/v) mixture.

5.0% DABA Solution B: DABA (5.0 g) was dissolved in 100 ml of DMSO.

7.5% DABA Solution: DABA (7.5 g) was dissolved in 100 ml of DMSO–H_2O (9: 1, v/v) mixture.

2.0% $K_2Cr_2O_7$ Solution: $K_2Cr_2O_7$ (2.0 g) was dissolved in 100 ml of 20% (v/v) H_2SO_4.

0.2 M Phthalate Buffer (pH 4.0).

0.4 M Phthalate Buffer (pH 4.0).

MIA and 3-Methyl-5-isoxazolecarboxylic Acid: These were prepared by the method of Sumimoto and Ishizuka[7]: MIA, mp 170—171°; 3-methyl-5-isoxazolecarboxylic acid, mp 208—209°.

PIA: This was prepared by the method of Quilico and Simonetta,[8] mp 160—161°.

3-Phenyl-5-isoxazolecarboxylic Acid: This was prepared by the method of Quilico and Speroni,[9] mp 179—180°.

Perisoxal (II),[4] mp 143—145°.

N-[(Dimethylamino)carbonyl]-5-methyl-3-isoxazolecarboxamide (III),[5] mp 84—85°.

3-(2-Oxazolin-2-yl)-5-methylisoxazole (IV),[6] mp 136.0—136.5°.

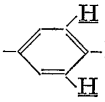
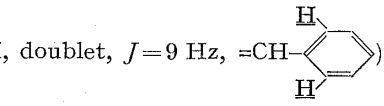
Analytical Procedure for MIA (or PIA)——Prepare a sample solution by dissolving 1.5—15.0 μg/ml of MIA or 2.5—25.0 μg/ml of PIA in DMSO–H_2O (1: 9, v/v) mixture. Pipette 1.0 ml of the solution into a 5 ml volumetric flask, and 1.0 ml of 5.0% DABA solution A (PIA: 1.0 ml of 7.5% DABA solution), and heat the mixture in a boiling water bath for 2 hr. After cooling to room temperature, dilute the reaction mixture with DMSO to the mark and measure the absorbance at 442 nm (PIA: 457 nm) against a reagent blank prepared similarly but without the sample.

Analytical Procedure for II——Prepare a sample solution by dissolving 5.0—50.0 μg/ml of II in 20% (v/v) H_2SO_4. Pipette 1.0 ml of the solution into a glass-stoppered test tube, add 1.0 ml of 2.0% $K_2Cr_2O_7$ solution, and heat the mixture in a boiling water bath for 1 hr. After cooling to room temperature, add 2.0 ml of 7 N NaOH to the reaction mixture and extract PIA three times with ethyl ether (1 ml each). Collect the ethereal solution in a 5 ml volumetric flask, evaporate to dryness at 40°, and dissolve the residue in 1.0 ml of DMSO–H_2O (1: 9, v/v) mixture. Assay this solution as described above for PIA. Measure the absorbance of the final solution at 457 nm against a blank prepared similarly but without the sample.

Analytical Procedure for III (or IV)——Prepare a sample solution by dissolving 2.0—20.0 μg/ml of III in 0.1 N HCl or 1.5—15.0 μg/ml of IV in 1.5 N HCl. Pipette 1.0 ml of the solution into a 5 ml volumetric flask and heat in a boiling water bath for 1 hr. After cooling to room temperature, add 0.1 ml of 1 N NaOH (IV: 0.5 ml of 3 N NaOH), 0.5 ml of 0.2 M phthalate buffer (IV: 0.5 ml of 0.4 M phthalate buffer), and 1.5 ml

6) Shionogi and Co., Ltd., Belg. Patent 670928 (1966) [*C.A.*, **65**, 7181*a* (1966)].
7) S. Sumimoto and I. Ishizuka, *Kogyo Kagaku Zasshi*, **64**, 1820 (1961).
8) A. Quilico and M. Simonetta, *Gazz. Chim. Ital.*, **77**, 586 (1947) [*C.A.*, **42**, 5904*b* (1948)].
9) A. Quilico and G. Speroni, *Gazz. Chim. Ital.*, **76**, 148 (1946) [*C.A.*, **41**, 961*g* (1947)].

of 5.0% DABA solution B (IV: 2.5 ml of 3.5% DABA solution) to the solution. Heat the mixture in a boiling water bath for 2 hr. After cooling to room temperature, dilute the reaction mixture with DMSO to the mark and measure the absorbance at 446 nm (IV: 447 nm) against a blank prepared similarly but without the sample.

Isolation of α-Acetyl-*p*-dimethylaminocinnamonitrile (V)——A mixture of MIA (0.2 g) and DABA (10 g) in 400 ml of DMSO–H_2O (1: 1, v/v) was heated in a boiling water bath for 2 hr. The reaction mixture was allowed to stand overnight at room temperature and the precipitate was collected by filtration. The crude product was isolated from the precipitate by preparative TLC using hexane–ethyl acetate (7: 3, v/v) as a developing solvent. Recrystallization from EtOH gave V (0.12 g), mp 108.0—108.5°. *Anal.* Calcd for $C_{13}H_{14}N_2O$: C, 72.87; H, 6.59; N, 13.07; O, 7.47. Found: C, 72.66; H, 6.43; N, 12.91; O, 7.52. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 2210 (CN), 1684 (C=O). NMR ($CDCl_3$) δ: 2.50 (3H, singlet, $COCH_3$), 3.12 (6H, singlet, $N\langle^{CH_3}_{CH_3}$), 6.70 (2H, doublet, $J=9$ Hz, ), 7.95 (2H, doublet, $J=9$ Hz, =CH– ), 8.00 (1H, singlet, =CH–).

Isolation of α-Benzoyl-*p*-dimethylaminocinnamonitrile (I)——α-Benzoyl-*p*-dimethylaminocinnamonitrile (I) was isolated in a manner similar to that described above for V and identified by comparison of the visible, IR, and NMR spectra with those of an authentic sample obtained by the method of Kauffmann,[10] mp 157—158°. *Anal.* Calcd for $C_{18}H_{16}N_2O$: C, 78.24; H, 5.84; N, 10.14; O, 5.78. Found: C, 78.14; H, 6.04; N, 10.06; O, 5.96.

Results and Discussion

Determination of MIA or PIA

The absorption spectra of the solutions obtained by the above assay procedure for MIA and PIA are shown in Fig. 1. The absorption maxima of the reaction mixture of MIA and that of PIA were at 442 nm and 457 nm, respectively. The developed colors were stable for at least 2 hr at room temperature for both MIA and PIA.

Effect of DABA Concentration——The color development was significantly dependent on the concentration of DABA. As shown in Fig. 2, a constant absorbance was obtained

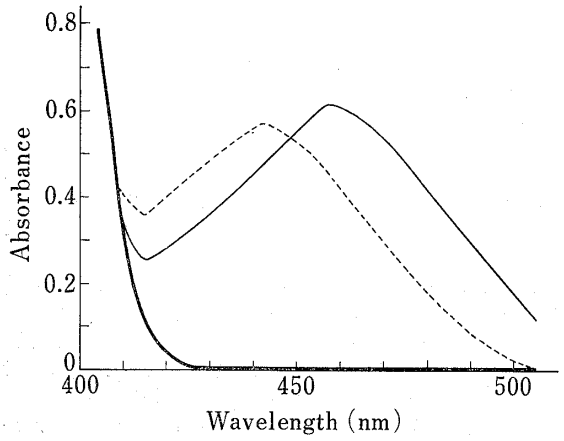


Fig. 1. Absorption Spectra of the Colored Solution and Blank Solution

····· : MIA (11.0 μg/ml),
—— : PIA (22.0 μg/ml),
▬▬ : reagent blank.

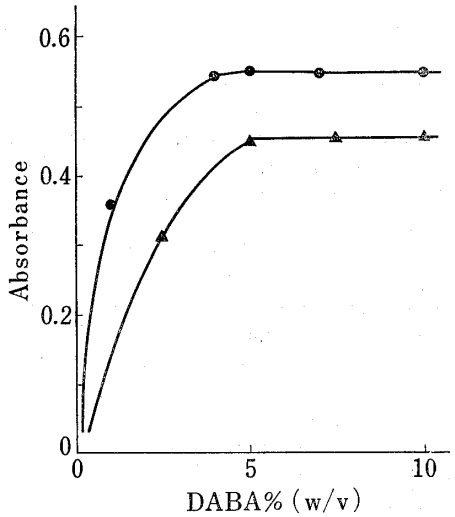


Fig. 2. Effect of DABA Concentration on Color Development

10.5 μg/ml of MIA and 17.0 μg/ml of PIA were treated according to the standard procedure but with various concentrations of DABA.
—●— : MIA (measured at 442 nm),
—▲— : PIA (measured at 457 nm).

10) H. Kauffmann, *Chem. Ber.*, **50**, 515 (1917).

in the concentration range of 4.0—10.0% DABA for MIA and 5.0—10.0% DABA for PIA. Such large amounts of DABA did not interfere with the determination of MIA and PIA, because DABA has no absorption at wavelengths longer than 430 nm, as shown in Fig. 1.

Effect of Solvent Composition——5-Substituted 3-isoxazolecarboxylic acids are decarboxylated on heating to give the corresponding β-ketonitriles. DMSO was useful as a reaction medium, permitting the use of relatively high temperature and being able to dissolve a large amount of DABA, while the color reaction was accelerated by the addition of H_2O. As shown in Fig. 3, a constant absorbance was obtained in the range of 50—65% H_2O in DMSO–H_2O mixture for MIA, and 40—60% H_2O for PIA.

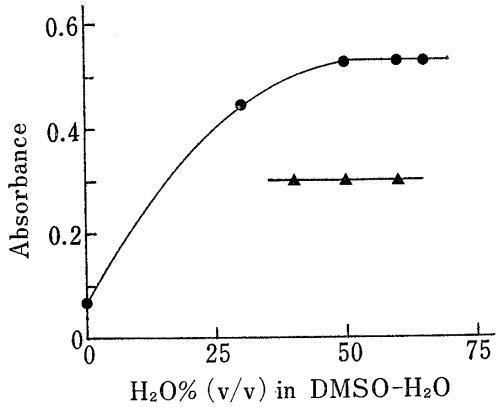


Fig. 3. Effect of H_2O% (v/v) in DMSO–H_2O on Color Development

10.0μg/ml of MIA and 11.0μg/ml of PIA were treated according to the standard procedure but with various levels of H_2O% (v/v) in DMSO-H_2O.
—●— : MIA (measured at 442 nm),
—▲— : PIA (measured at 457 nm).

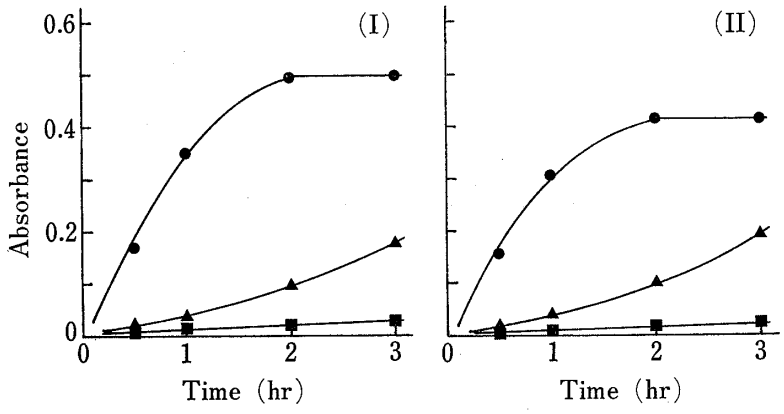


Fig. 4. Effects of Reaction Temperature and Time on Color Development

9.5μg/ml of MIA and 15.0 μg/ml of PIA were treated according to the standard procedure except that various reaction times were used at the following temperatures.
—●— : 98°, —▲— : 80°, —■— : 60°.
(I) : MIA (measured at 442 nm), (II) : PIA (measured at 457 nm).

Effects of Reaction Temperature and Time——MIA and PIA both gave a constant absorbance on heating at 98° for 2—3 hr as shown in Fig. 4. The rate of the color development became markedly slower with a decrease in temperature. The colors scarcely developed on heating at 60°.

Calibration Curve——As shown in Fig. 5, linear relationships were obtained between color intensity and concentration in the range of 1.5—15.0 μg/ml for MIA and 2.5—25.0 μg/ml for PIA.

Accuracy and Precision——The precision of the assay procedure was examined on replicate runs for ten sample solutions containing 5.46 μg/ml of MIA or 9.31 μg/ml of PIA. The data presented in Table I indicate that the proposed method is accurate and precise.

Interference by the 5-Carboxylic Isomer——3-Isoxazolecarboxylic acids are presumed to contain the corresponding 5-isoxazolecarboxylic acid isomer as a by-product of the synthetic process. Regression analysis was performed to examine interference by the isomer. As shown in Tables II and III, the isomer caused no interference in the assay of MIA and PIA.

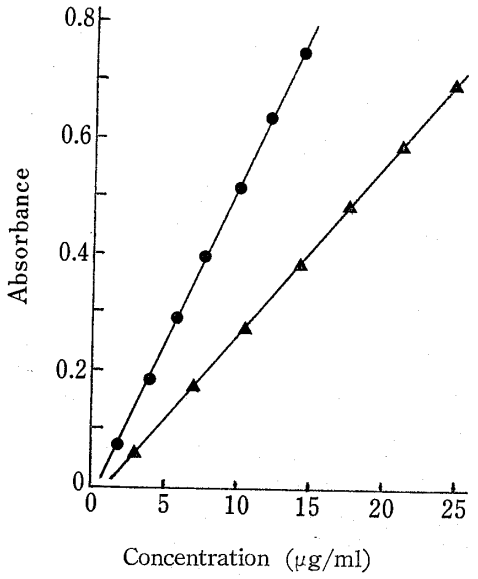


Fig. 5. Calibration Curves for MIA and PIA

—●— : MIA (measured at 442 nm),
—▲— : PIA (measured at 457 nm).

Table I. Reproducibility of Color Development of Replicate Samples of MIA and PIA

Added (μg/ml)		MIA 5.46	PIA 9.31
Found (μg/ml)	1	5.55	9.28
	2	5.59	9.55
	3	5.61	9.45
	4	5.30	9.82
	5	5.46	9.35
	6	5.48	9.42
	7	5.51	8.91
	8	5.51	9.32
	9	5.61	9.48
	10	5.51	9.69
Average		5.51	9.43
Standard deviation		0.09	0.25
Coefficient of variation		1.7%	2.6%

Determination of II

II was oxidized with $K_2Cr_2O_7$ in an acidic medium to yield PIA, followed by reaction with DABA. The absorption spectrum of the solution obtained by the above assay procedure for II was identical with that of the final reaction mixture of PIA and DABA described above.

Table II. Determination of MIA in the Presence of Its 5-Carboxylic Isomer

No.	Added (μg)		Found (μg)
	Isomer	MIA (x)	MIA (y)
1	0.32	1.80	1.83
2	0.32	3.60	3.15
3	0.32	5.40	5.44
4	0.32	7.20	7.23
5	0.32	9.00	9.05
6	0.32	10.79	10.57
7	0.32	12.59	12.42

Regression equation: $y=0.9974x-0.079$, $s=0.21$.

Table III. Determination of PIA in the Presence of Its 5-Carboxylic Isomer

No.	Added (μg)		Found (μg)
	Isomer	PIA (x)	PIA(y)
1	12.63	3.56	3.64
2	12.63	7.13	6.87
3	12.63	10.69	10.70
4	12.63	14.25	14.00
5	7.90	17.82	17.56
6	6.32	21.38	21.25
7	3.16	24.94	25.67

Regression equation: $y=1.0195x-0.289$, $s=0.35$.

Effect of $K_2Cr_2O_7$ Concentration——On heating in a boiling water bath, II was easily oxidized by $K_2Cr_2O_7$. A constant absorbance was obtained in the concentration range of 0.5—5.0% (w/v) $K_2Cr_2O_7$.

Effect of Oxidation Time——A constant absorbance was obtained in the time range of 0.5—3.0 hr when II was heated in a boiling water bath.

Effect of NaOH Concentration——The reaction mixture of II should be partly neutralized by NaOH to extract PIA efficiently. It was found to be most effective to use 2.0 ml of 7N NaOH.

Calibration Curve——A linear relationship was obtained between color intensity and concentration in the range of 5.0—50.0 μg/ml.

Precision——The precision of the assay procedure was examined on replicate runs for ten sample solutions containing 21.4 μg/ml of II. Each solution was assayed by the proposed procedure. The coefficient of variation for the ten replicate sample solutions was 3.4%.

Determination of III or IV

III and IV were each hydrolyzed with hydrochloric acid to afford MIA, followed by reaction with DABA.

Effect of Hydrochloric Acid Concentration——On heating in a boiling water bath, III and IV were easily hydrolyzed by hydrochloric acid. As shown in Fig. 6, a constant absorbance was obtained in the concentration range of 0.08—1.00 N HCl for III and that of 1.2—2.0 N HCl for IV.

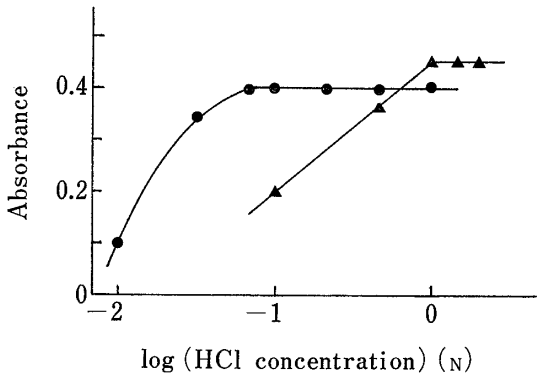


Fig. 6. Effect of HCl Concentration on Hydrolysis of III or IV

10.3μg/ml of III and 10.0μg/ml of IV were treated according to the standard procedure but with various concentrations of HCl.
—●— : III (measured at 446 nm),
—▲— : IV (measured at 447 nm).

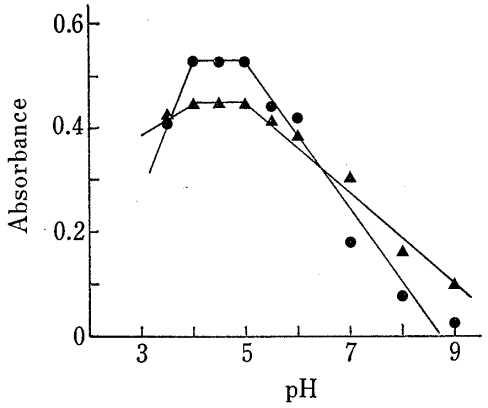


Fig. 7. Effect of pH after the Hydrolysis of III or IV on Color Development

12.7 μg/ml of III and 10.0 μg/ml of IV were treated according to the standard procedure but with various pH values after hydrolysis.
—●— : III (measured at 446 nm),
—▲— : IV (measured at 447 nm).

Effect of Hydrolysis Time——A constant absorbance was obtained at hydrolysis times of 1.0—1.5 hr for both III and IV on heating in a boiling water bath.

Effect of pH after Hydrolysis——Because of poor extraction of MIA, the reaction mixture after the hydrolysis of III or IV was partly neutralized for direct reaction with DABA. As shown in Fig. 7, a constant absorbance was obtained in the range of pH 4.0—5.0 for both III and IV. Therefore, in order to adjust the pH in the reaction mixture after hydrolysis to 4.5, 0.1 ml of 1 N NaOH and 0.5 ml of 0.2 M phthalate buffer were added in the case of III, and 0.5 ml of 3 N NaOH and 0.5 ml of 0.4 M phthalate buffer in the case of IV.

Effect of DABA Concentration——The effect of DABA concentration on the color development after the hydrolysis of III or IV was investigated. A constant absorbance was obtained in the concentration range of 4.0—8.0% DABA for III and 3.0—4.0% DABA for IV.

Table IV. Determination of MIA in the Presence of 1,1-Dimethylurea

No.	Added (μg)		Found (μg)
	1,1-Dimethylurea	MIA (x)	MIA (y)
1	5.13	1.76	1.75
2	5.13	3.53	3.49
3	5.13	5.29	5.22
4	5.13	7.05	7.26
5	5.13	8.81	8.74
6	5.13	10.58	10.62
7	5.13	12.34	12.20

Regression equation: $y=0.9953x-0.022$, $s=0.12$.

Table V. Determination of MIA in the Presence of 2-Aminoethanol

No.	Added (μg)		Found (μg)
	2-Aminoethanol	MIA (x)	MIA (y)
1	3.44	1.61	1.46
2	3.44	3.22	3.19
3	3.44	4.83	4.71
4	3.44	6.44	6.60
5	3.44	8.05	8.05
6	3.44	9.66	9.73
7	3.44	11.27	11.19

Regression equation: $y=1.0118x-0.097$, $s=0.11$.

Calibration Curve——Linear relationships were obtained between color intensity and concentration in the range of 2.0—20.0 μg/ml for III and 1.5—15.0 μg/ml for IV.

Precision——The precision of the assay procedure was examined on replicate runs for ten sample solutions containing 10.5 μg/ml of III or 7.27 μg/ml of IV. Each solution was assayed by the proposed procedure. The coefficient of variation for the ten replicate sample solutions was 2.4% for III and 1.8% for IV.

Effect of Hydrolysis Product Other than MIA——MIA and 1,1-dimethylurea or MIA and 2-aminoethanol are produced by the hydrolysis of III or IV, respectively. Thus, the influence of 1,1-dimethylurea or 2-aminoethanol on the color development of III or IV was examined. Regression analysis in the presence of 1,1-dimethylurea or 2-aminoethanol showed reasonable agreement between the added and the found amounts of MIA, as shown in Tables IV and V. It is concluded that the coloration of III and IV is based on the reaction of MIA with DABA.

Because this color reaction was in equilibrium with the hydrolysis of the colored substance, a large excess of reagent was required; however, this did not interfere with the determination of 5-substituted 3-isoxazolecarboxylic acids and their derivatives.

In conclusion, the procedure described here was specific for the isoxazole ring with reasonable accuracy and precision. The proposed method may be applicable to 5-substituted isoxazoles with unsubstituted 3- and 4-positions which are convertible into the corresponding β-ketonitriles on alkaline treatment.[11] Studies on the mechanism of the hydrolysis of the colored substance are in progress in connection with the mechanism of the color reaction.

11) A. Quilico, "The Chemistry of Heterocyclic Compounds: Five- and Six-Membered Compounds with Nitrogen and Oxygen," Vol. 17, ed. by R.H. Wiley, John Wiley and Sons, Inc., New York, 1962, pp. 44—45.