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Analytical Studies on Isoxazoles. V.[1] Colorimetric Determination of Isouron and Its Isomer with *p*-Dimethylaminocinnamaldehyde

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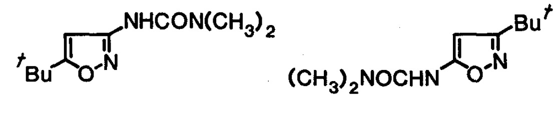
Isouron (**1**) and its isomer, 1-(3-*tert*-butylisoxazol-5-yl)-3,3-dimethylurea (**2**), were determined by a colorimetric method with *p*-dimethylaminocinnamaldehyde (DACA) as the reagent. Compounds **1** and **2** were hydrolyzed to give 3-amino-5-*tert*-butylisoxazole (**3**) and 5-amino-3-*tert*-butylisoxazole (**4**), respectively, which were transformed into colored substances by reaction with DACA. The former colored product was a Schiff base but the latter product had a different absorption maximum from the corresponding Schiff base. The latter color reaction resulted in a bathochromic shift as compared with Schiff base formation at the 5-position. Traces of **2** (more than 0.05%) in **1** could be estimated precisely together with the quantitation of **1** by making use of this bathochromic effect.

Keywords——isouron; 1-(3-*tert*-butylisoxazol-5-yl)-3,3-dimethylurea; herbicide; colorimetric assay method; *p*-dimethylaminocinnamaldehyde; hydrolysis of isouron; 3-amino-5-*tert*-butylisoxazole; 5-amino-3-*tert*-butylisoxazole; Schiff base

Since isouron (**1**) is being investigated as a potential herbicide, we previously developed a method to estimate the quality of the raw material.[2] The isomer of isouron, 1-(3-*tert*-butylisoxazol-5-yl)-3,3-dimethylurea (**2**), was inevitably formed in the synthetic process as a contaminant. Because **2** shows some herbicidal activity, it was improtant to determine its content in the raw material. In the previous papers,[1,2] we reported colorimetric and fluorometric methods for the assay of **1**, but the isomer **2** could not be determined by these methods, which were specific for **1**. We wished to determine **1** and **2** separately. Thus, **1** and **2** were derived to **3** and **4**, respectively, by hydrolysis. Compound **3** reacted with *p*-



1 **2**

Chart 1 ᵗBu = *tert*-butyl

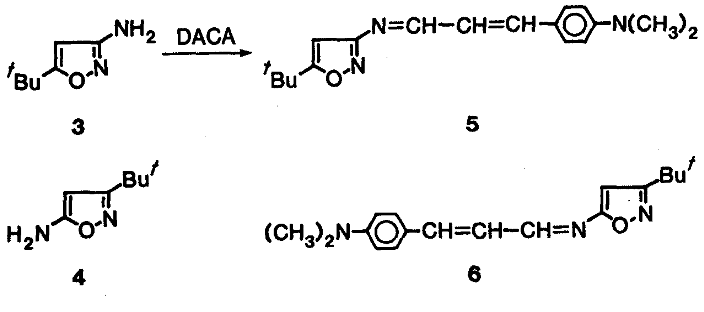


Chart 2

dimethylaminocinnamaldehyde (DACA) to form a Schiff base (**5**) but **4** was different from the Schiff base (**6**), and absorbed light at a longer wavelength than **6** did. A sensitive colorimetric method, by which both **1** and **2** could be assayed separately, was established by utilizing this bathochromic effect. Isouron could be determined by this method with essentially the same precision as in our previous two methods, and at the same time, trace amounts of **2** (more than 0.05%) could be assayed without affecting the precision, which was a great advantage. It was not necessary to cool the reaction mixture with ice, in contrast to the previous diazo coupling methods, and this is a further advantage of the present colorimetry.

It is known that, since **4** has an enamine structure, the carbon atom at the 4-position is active.[3] Thus, the colored substance derived from **4** may have been obtained by the reaction of DACA at C_4. The structure of the colored substance is now under investigation.

Experimental

Apparatus——A Hitachi model 624 recording spectrophotometer was used for absorbance measurements. An Iwaki KM shaker, type V-S, and a Sakuma centrifuge, model 90-4, were used. A thermomixer, model TM-100 (Thermonics Co., Ltd.), was used for mixing solutions.

Reagent——Chloroform was purchased from E. Merck Co. (analytical grade, No. 2445) and DACA from Wako Pure Chemical Industries Ltd. (reagent grade). The amino isoxazoles (**3** and **4**) were supplied by our laboratory. The other chemicals were of reagent grade.

0.8% DACA Solution: Dissolve 0.8 g of DACA in $CHCl_3$ to make 100 ml.

2% DACA Solution: Dissolve 2 g of DACA in DMF to make 100 ml.

Methanolic HCl Solution: Dilute 6 ml of hydrochloric acid with MeOH to make 100 ml. Dilute 1 ml of the solution with MeOH to make 25 ml (*ca.* 0.03 N).

Test Solutions Containing Various Amounts of HCl: Dilute hydrochloric acid (1—10 ml) with MeOH to make 100 ml. Take 1 ml of the solution into a 25-ml volumetric flask and dilute to the mark with MeOH.

Preparation of Schiff Base 5——DACA (250 mg) and AcOH (15 ml) were added to an ethanolic solution (30 ml) of **3** (200 mg). The solution was stirred at room temperature for 1 h. After removal of the solvent under reduced pressure, the residue was recrystallized from EtOH to give 100 mg (24%) of **5** as yellow needles. mp 157—158 °C. IR (KBr): 1611, 1584 cm^{-1}. *Anal.* Calcd for $C_{18}H_{23}N_3O$: C, 72.69; H, 7.80; N, 14.13. Found: C, 72.57; H, 7.82; N, 13.99.

Preparation of Schiff Base 6——DACA (250 mg) and AcOH (15 ml) were added to an ethanolic solution (30 ml) of **4** (200 mg). The solution was stirred at room temperature for 1 h. After removal of the solvent under reduced pressure, the residue was recrystallized from EtOH to give 250 mg (59%) of **6** as yellow plates. mp 184—185 °C. IR (KBr): 1610, 1577 cm^{-1}. *Anal.* Calcd for $C_{18}H_{23}N_3O$: C, 72.69; H, 7.80; N, 14.13. Found: C, 72.75; H, 7.83; N, 14.08.

Assay Procedure——Assay of **1**: Accurately weigh about 50 mg of the sample into a 10-ml volumetric flask, dissolve it in and dilute to the mark with $CHCl_3$. Pipet 2 ml of the solution into a 12-ml centrifuge tube, add 2 ml of 1 N NaOH and shake with a shaker for 10 min. After centrifugation, pipet 0.5 ml of the $CHCl_3$ layer into a 20-ml volumetric flask. Use the aqueous layer for the assay of **2**. Dilute the $CHCl_3$ layer to the mark with $CHCl_3$. Transfer exactly 0.5 ml of the solution to a hydrolysis tube[2] and evaporate the solvent under reduced pressure. Add exactly 2 ml of 10% DMF-aqueous solution. Seal the tube and heat it at 145—155 °C for 1 h. After cooling the tube to room temperature, open it and transfer exactly 1 ml of the reaction solution to a 12-ml centrifuge tube. Add exactly 2.5 ml of $CHCl_3$ and shake with a shaker for 3 min. After centrifugation, discard the aqueous layer and transfer exactly 1 ml of the $CHCl_3$ layer to a 10-ml volumetric flask. Add 5 ml of $CHCl_3$, 1 ml of 0.8% DACA solution and 1 ml of methanolic HCl solution, mix, and dilute to the mark with $CHCl_3$. Separately, take 6 ml of $CHCl_3$ into a 10-ml volumetric flask. Add 1 ml of 0.8% DACA solution and 1 ml of methanolic HCl solution, and dilute to the mark with $CHCl_3$. Use the solution as the blank solution. After allowing each solution to stand for 15 min, read the absorbance at 533 nm (A_{533}).

Assay of **2**: Transfer exactly 1 ml of the aqueous layer to a hydrolysis tube.[2] Add 1 ml of 1 N HCl and 0.1 ml of 5% $NaHCO_3$ solution. Seal the tube and heat it at 145—155 °C for 20 min. After cooling the tube to room temperature, open it and transfer exactly 1 ml of the reaction solution to a 12-ml centrifuge tube. Add 1 ml of H_2O and 2 ml of CH_2Cl_2, shake with a shaker for 3 min, and centrifuge for 5 min. Discard the aqueous layer and transfer exactly 1 ml of the CH_2Cl_2 layer to a 12-ml centrifuge tube. Add 1.5 ml of CH_2Cl_2, 1 ml of 2% DACA solution and 25 μl of hydrochloric acid. Mix the solution with a thermomixer.

Separately, take 2.5 ml of CH_2Cl_2, and add 1 ml of 2% DACA solution and 25 μl of hydrochloric acid. Mix the solution with a thermomixer. Use the solution as the blank solution. After allowing each solution to stand for 100 min, read the absorbance at 608 nm (A_{608}).

Calibration Curve——Calibration curves were made according to the assay procedure. A linear relationship was obtained between the concentration of **1** or **2** and the absorbance. The ranges of linearity for **1** and **2** were 1.6—8 mg/ml and 6—30 μg/ml, respectively.

Examination of Conditions for Colorimetric Determination of 3——1) Solvent Effect: One ml of a $CHCl_3$ solution of **3** (*ca.* 7 μg/ml) was placed in a 10-ml volumetric flask, and 4% DACA solution (0.2 ml) was added. The mixture was evaporated under reduced pressure. Six ml of a solvent ($CHCl_3$, EtOH, 1,2-dichloroethane, benzene, MeOH, 3% aqueous MeOH, AcOEt or DMF) was added to the residue. The residue was dissolved well in the solvent with a thermomixer, then 0.1 ml of 0.3 N HCl (MeOH) was added. The solution was mixed and diluted to the mark with the same solvent. The absorbance at the maximum wavelength was measured at intervals of about 10 min using a solution obtained in the same manner but without **3** as the blank. The measurement was continued for 1 h.

2) Effect of HCl: One ml of a $CHCl_3$ solution of **3** (*ca.* 7 μg/ml) was pipetted into a 10-ml volumetric flask and 5 ml of $CHCl_3$ was added. Next, 1 ml of 0.8% DACA solution was added, followed by 1 ml of a methanolic HCl solution ranging in concentration from 0.0045 to 0.045 N. The solution was mixed and diluted to the mark with $CHCl_3$. The mixture was allowed to stand for 15 min, then A_{533} was measured. A solution obtained in the same manner but without **3** was used as the blank.

3) Effect of DACA: The same $CHCl_3$ solution of **3** (1 ml) as above was pipetted into a 10-ml volumetric flask, and 1 ml of methanolic HCl solution (*ca.* 0.03 N) and 1 ml of DACA solution ranging in concentration from 0.1 to 1% were added. After dilution of the mixture with $CHCl_3$ to 10 ml, A_{533} was measured.

Examination of Conditions for Colorimetric Determination of 4——1) Effect of HCl: One ml of a CH_2Cl_2 solution of **4** (*ca.* 3 μg/ml) was pipetted into a test tube and diluted with CH_2Cl_2 (1.5 ml), then 2% DACA solution (1 ml) was added, followed by a desired amount of hydrochloric acid (25, 50, 75 or 100 μl). The solution was mixed with a thermomixer. The absorbance (A_{608}) was measured at 10-min intervals using a solution obtained in the same manner but without **4** as the blank. The measurement was continued for 180 min.

2) Effect of DACA: The same CH_2Cl_2 solution of **4** was used, and 1 ml of DACA solution ranging in concentration from 0.5 to 5% was added. After the addition of hydrochloric acid (25 μl), A_{608} was measured in the same manner as described above. The measurement was continued for 2.5—3 h.

Measurement of Extraction Yield of 2——A $CHCl_3$ solution of **2** (*ca.* 35 μg/ml) was extracted with various concentrations of NaOH (0.01—2 N) according to the assay procedure. After centrifugation, each aqueous solution was neutralized with the same concentration of HCl solution as that of NaOH. After the addition of 5% $NaHCO_3$ (0.1 ml), the solutions were treated according to the assay procedure.

Measurement of Hydrolysis Yield of 2——Two ml of an aqueous solution of **2** (*ca.* 9 μg/ml) was placed in a hydrolysis tube, and heated at various temperatures. The resulting solutions were treated according to the assay procedure.

Measurement of Extraction Yield of 4——One ml of an aqueous solution of **4** (*ca.* 6 μg/ml) was placed in a 12-ml centrifuge tube, and the salt solution (1 ml) prepared by mixing 0.5 M NaCl (20 ml) and 5% $NaHCO_3$ (1 ml) was added. The solution was extracted with CH_2Cl_2 (2 ml). The shaking times were 3, 5 and 10 min. The resulting solutions were treated according to the assay procedure.

Measurement of Visible Absorption Spectra——The color reaction was performed with a $CHCl_3$ solution of **3** (*ca.* 7 μg/ml) according to the assay procedure. A CH_2Cl_2 solution of **4** (*ca.* 5 μg/ml) was treated according to the assay procedure. The color development of **5** was carried out with a $CHCl_3$ solution (*ca.* 18 μg/ml) under the same conditions as used for **3**. After the color developments, the visible absorption spectra were measured.

Degradation of **6** was measured spectrophotometrically by adding 0.1 ml of 0.001 N HCl (MeOH) to a CH_2Cl_2 solution (1 ml) of **6** (*ca.* 25 μg/ml) and diluting the mixture with CH_2Cl_2 to 10 ml. The absorption spectra were measured at regular time intervals.

Results and Discussion

Conditions for Colorimetric Determination of 3-Amino-5-*tert*-butylisoxazole (3)

Solvent Effect on Color Development——Isouron was hydrolyzed to give **3**. When methanolic HCl was added to a mixture of **3** and DACA, a red-colored Schiff base was formed. Since the color reaction was performed in the solvents except H_2O, the resulting **3** was transferred to an organic solvent. The most intense color was obtained in $CHCl_3$. The visible absorption spectrum in $CHCl_3$ is shown in Fig. 1 (No. 1), and is in accord with that of **5** (No. 2).

Figure 2 shows the absorbances at the maximum wavelengths of the colored solutions. The intensity of the absorbance was solvent-dependent. Water interfered with the coloration. The time-course of the absorbance showed that the color reaction went immediately to completion and the product **5** was generally stable. In benzene and AcOEt, the absorbance

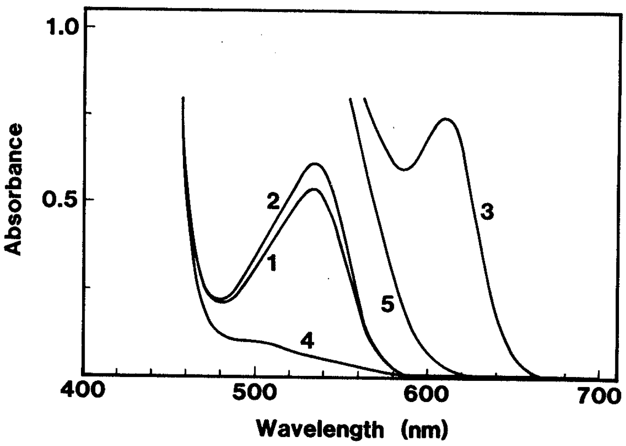


Fig. 1. Visible Absorption Spectra of the Colored Substances Obtained from **3** and **4**, and the Schiff Base (**5**) of **3**

1, reaction of **3** with DACA; 2, Schiff base (**5**); 3, reaction of **4** with DACA; 4, reagent blank (0.8% DACA); 5, reagent blank (2% DACA).

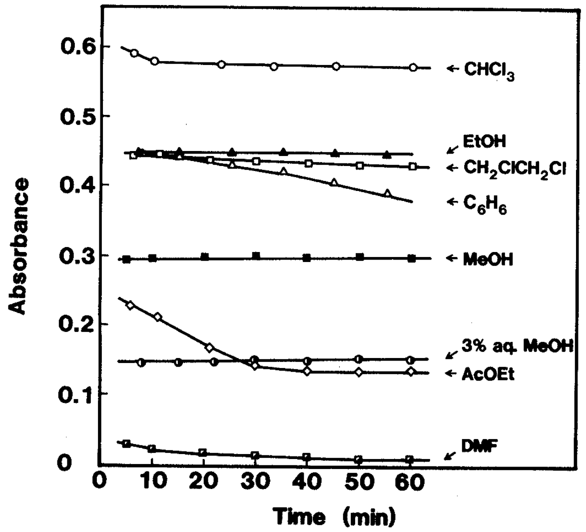


Fig. 2. Effect of Solvent on the Color Development of **3**

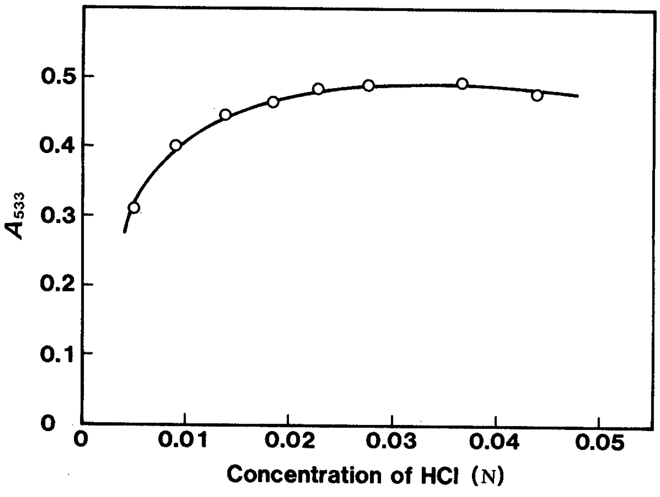


Fig. 3. Effect of Concentration of HCl on the Color Development of **3**

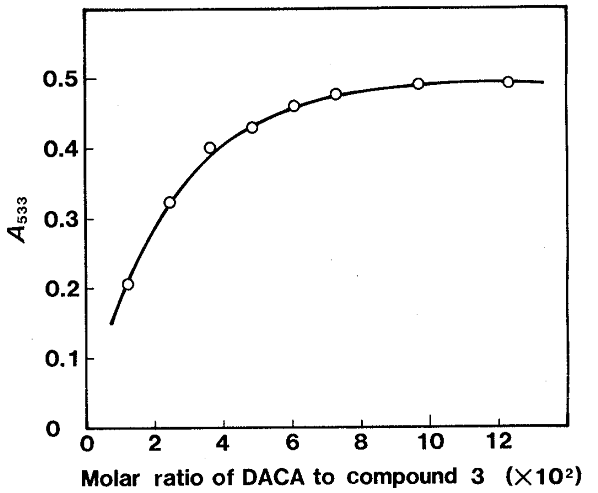


Fig. 4. Effect of Concentration of DACA on the Color Development of 3

decreased gradually with time.

Effect of HCl Concentration of Color Development——Methanolic HCl solution (1 ml) prepared in the concentration range of 0.0045 to 0.045 N was added to a $CHCl_3$ solution of **3** in the presence of DACA (0.8% solution). Figure 3 shows the effect of HCl concentration on the color development. The absorbance increased with concentration and became constant at 0.02—0.035 N.

Effect of DACA Concentration on Color Development——The effect of DACA concentration is shown in Fig. 4. Various concentrations of DACA from 0.1 to 1% were used for this color reaction. When DACA was present in a 700-fold molar excess over **3** (0.6%), a constant absorbance was obtained.

Conditions for Colorimetric Determination of 5-Amino-3-*tert*-butylisoxazole (4)

Compound **4** reacted with DACA in the presence of a strong acid to give a blue-colored substance. The most intense color was obtained in CH_2Cl_2. In many other solvents, the coloration was much weaker. The visible absorption spectrum of the colored substance in CH_2Cl_2 is shown in Fig. 1 (No. 3). The maximum wavelength was 608 nm.

Effect of Amount of HCl on Color Development——The color reaction of **4** with DACA was carried out using various amounts of hydrochloric acid (25—100 μl). The A_{608} values were measured at 10-min intervals after HCl addition for 3 h. As shown in Fig. 5, color development proceeded to the same intensity in each acid. Since the colored substance was unstable, it is necessary to find conditions where the degradation rate is slow. The maximum absorbance on addition of 25 μl was obtained after 100 min, and remained almost constant till 140 min. Although the absorbances after addtions of 50—100 μl reached the maximum values faster, the stability was less.

Effect of DACA Concentration on Color Development——The color reaction was carried out with various concentrations of DACA (0.5—5%). To ensure stability, 25 μl of hydrochloric acid was used. Figure 6 shows the absorbances measured at 10-min intervals after HCl addition for 2.5—3 h. The most stable absorbance was obtained with the 2% solution.

Conditions for the Total Assays of 1 and 2

Total Assay of 1——The raw material was dissolved in $CHCl_3$. To separate **2**, the $CHCl_3$ solution was extracted with 1 N NaOH. The $CHCl_3$ solution was then evaporated to dryness, and **1** was redissolved in 10% aqueous solution of DMF and hydrolyzed in the same manner as described in the previous paper.[2] After hydrolysis, the resulting **3** was extracted with $CHCl_3$. The extraction yield of **3** was found to be quantitative. The $CHCl_3$ layer was treated according to the assay procedure for **3**. The Schiff base formation proceeded quantitatively.

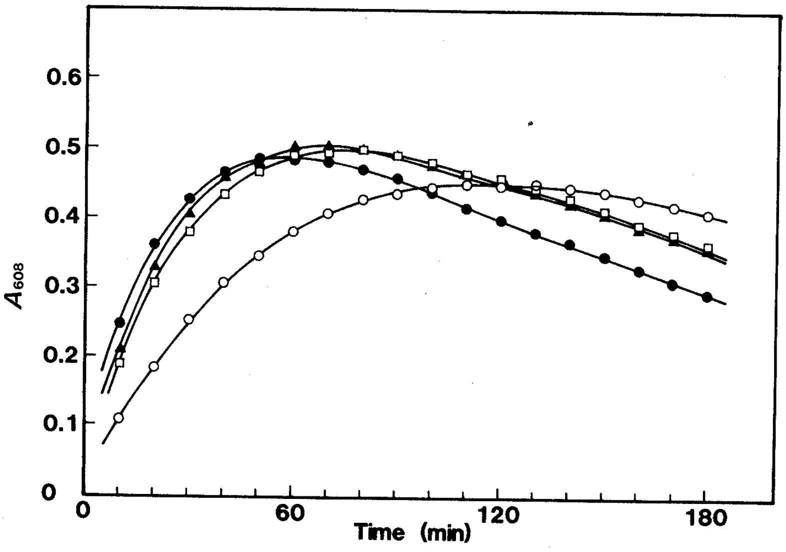


Fig. 5. Effect of Amount of HCl on the Color Development of **4**
○, 25 μl of HCl; □, 50 μl; ▲, 75 μl; ●, 100 μl.

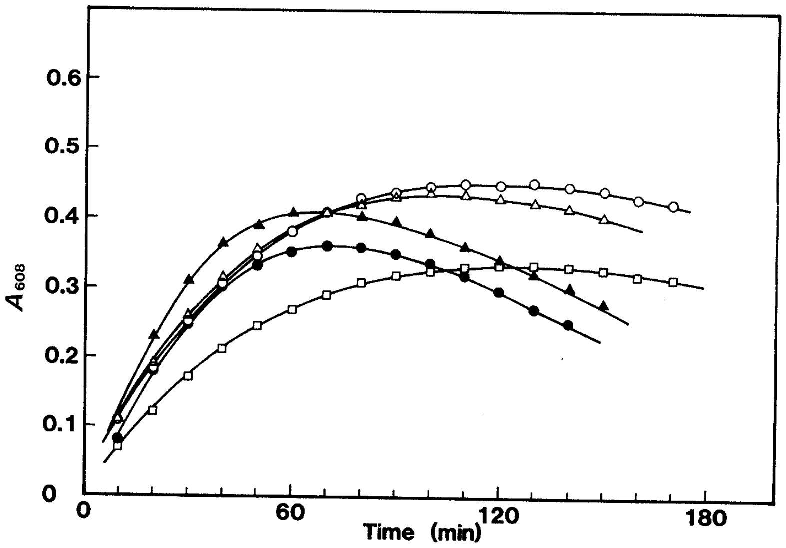


Fig. 6. Effect of Concentration of DACA on the Color Development of **4**
□, 0.5% DACA; △, 1%, ○, 2%; ▲, 3%; ●, 5%.

By this method, 98—100% of **1** was recovered as **5**.

Total Assay of 2——Since **2** was more acidic (pK_a: 10.83) than **1**, it was transferred from the $CHCl_3$ solution to the aqueous alkaline solution as described above. The extraction yield increased with increase of NaOH concentration, as shown in Fig. 7. About 85% of **2** was extracted with more than 1 N NaOH. The aqueous layer was taken, neutralized and hydrolyzed. Figure 8 shows the effect of temperature on the hydrolysis. Compound **4** was formed quantitatively at temperatures higher than 145 °C. After hydrolysis, **4** was extracted with CH_2Cl_2. The extraction yield was found to be 91—93%. The CH_2Cl_2 layer was treated according to the assay procedure for **4**. By this method, 75—80% of **2** was recovered as **4**, which was converted to the colored substance.

Effect of 2 on the Determination of 1——A constant amount of **2** (50 mg) was added to a $CHCl_3$ solution of **1** in the concentration range 2 to 8 mg/ml (10 ml). Compound **1** in each sample solution was assayed using a calibration curve made by the use of standard solutions

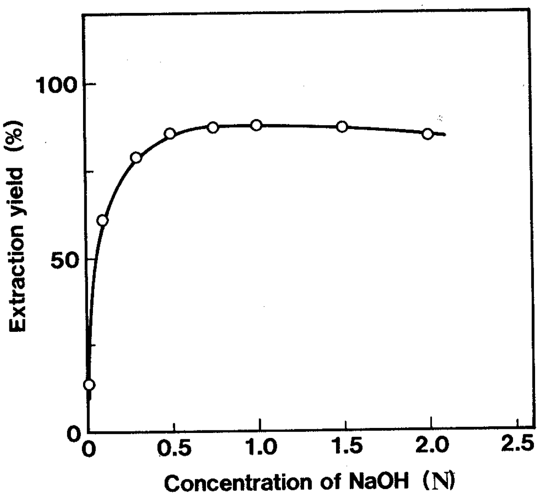


Fig. 7. Effect of Concentration of NaOH on the Extraction of **2**

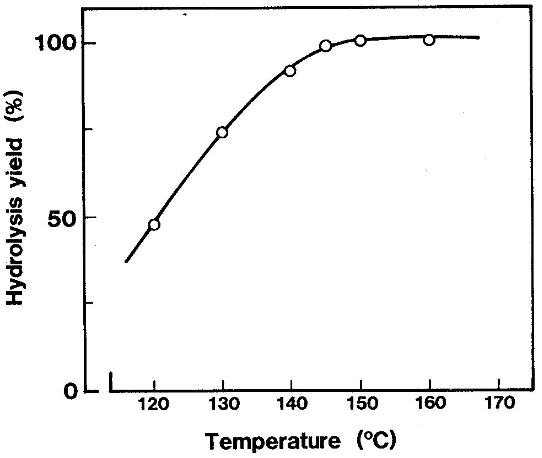


Fig. 8. Effect of Temperature on the Hydrolysis of **2**

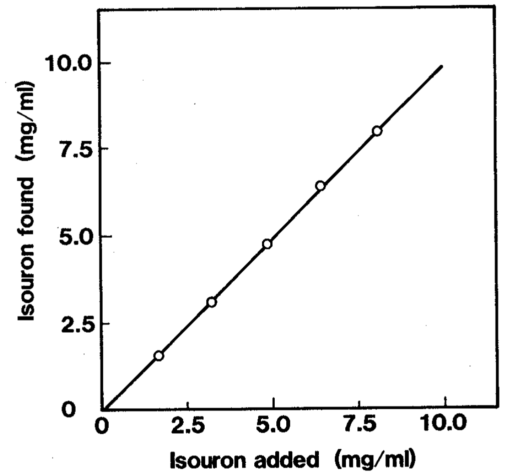


Fig. 9. Recovery of Isouron (**1**) from a Mixture Containing the Isomer (**2**) (5 mg/ml)

Regression equation: $y=0.9953x-0.048$, $s=0.049$. The plot was assumed to pass through the origin.

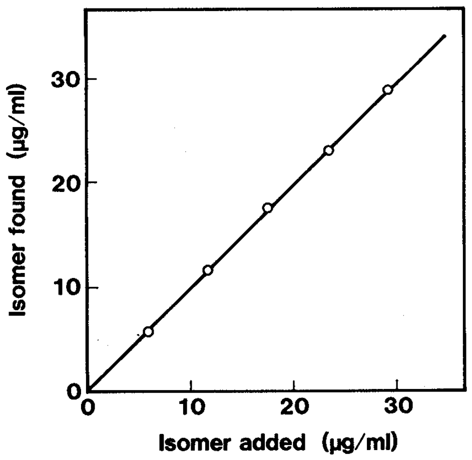


Fig. 10. Recovery of the Isomer (**2**) from a Mixture Containing Isouron (**1**) (3.6 mg/ml)

Regression equation: $y=0.9810x+0.153$, $s=0.128$. The plot was assumed to pass through the origin.

of **1**. As shown in Fig. 9, no interference of **2** was observed in the assay. The result shows that this method is applicable even to samples which contain more than 50% **2**.

Effect of 1 on the Determination of 2——A constant amount of **1** (36 mg) was added to a $CHCl_3$ solution of **2** in the concentration range of 6 to 30 μg/ml (10 ml). These samples correspond to raw materials containing **2** as an impurity in ratios of 0.2—0.8%. Compound **2** in each sample was assayed using the calibration curve made by the use of standard solutions of **2**. As shown in Fig. 10, no interference by **1** was observed in the assay.

Precision and Quantitation Limit of 2——According to the standard method, **1** and **2** were determined separately with coefficients of variation of 1.49 and 1.66% ($n=10$ each), respectively. The quantitation limit of **2** was 0.05% in the raw material.

Contents of 1 and 2 in the Raw Material——Both **1** and **2** were determined, and the results are summarized in Table I. The raw material (**1**) contained 0.06—0.38% **2**. The high value of **1** observed in Lot 38-16 seemed to be due to the presence of bis[5-*tert*-butylisoxazol-3-yl]urea (**7**), which was detected by thin layer chromatography. This compound was hydrolyzed to

Table I. Analytical Results for Isouron and Its Isomer in Raw Material Samples

Lot No.	Content (%)	
	Isouron	Isomer
38-16	103.6	<0.05[a]
38-18	99.4	<0.05
1669-21	96.5	0.21
1669-31	98.3	0.38
1669-41	100.4	0.35
1778	98.6	<0.05
76609	101.8	0.06

a) Less than 0.05%.

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Chart 3

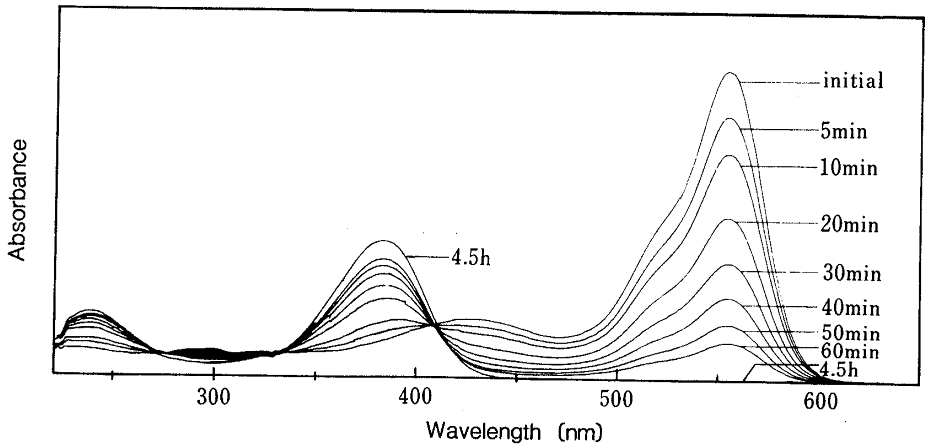

Fig. 11. Visible Absorption Spectra of the Schiff Base (**6**) in the Presence of HCl

2 mol of **3**.

Characteristics of the Assay Method——The raw material could be well evaluated by the present method, which can separately determine both isouron **1** and an active impurity **2**. Our previous methods have the defect that, because the diazonium salt of the isoxazole (**3**) is unstable, unlike that of aniline, the procedures should be carried out rapidly under ice-cooling. Since the Schiff base **5** is stable, such precautions were not necessary in the present method.

Stability of the Schiff Base 6——The Schiff base **6** is very unstable in the presence of acid as compared with **5**. The compound decomposed on addition of a small amount of HCl. Figure 11 shows the visible absorption spectra in CH_2Cl_2 observed after the addition of 0.001 N HCl. The color disappeared after 4.5 h. The absorbance at 385 nm was in accord with that of DACA. The color reaction of **4** in the assay procedure was performed in a more concentrated HCl solution (*ca.* 0.08 N) in the presence of excess DACA. In such an acid solution, the color of **6** disappeared instantaneously. This shows that the absorbance at 608 nm is not due to **6** but to a different colored substance. An assay method based on Schiff base formation could not be established even by using suitably diluted acid, but was possible by using the hydrogen sulfate of DACA as the reagent in the absence of acid.[4] This method, however, is not suitable for the determinations of both **1** and **2**. An investigation is planned to determine the structure of the new type of colored substance.

Acknowledgement The authors are grateful to Drs. S. Sumimoto and I. Ishizuka for providing samples.

References and Notes

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4) The salt was prepared by adding an equimolar amount of H_2SO_4 to an ethereal solution of DACA. mp 154—158 °C.