Simplification of Colorimetric Measurement of Secondary Amines by Nitrosation

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We studied the simplification of the usual colorimetric measurement of secondary amines, using nitrosation of secondary amines with KNO₂, degradation of KNO₂ with sulfamic acid, extraction of nitrosamines with CH₂Cl₂, and coloring of the nitrosamines. To skip the most time-consuming CH₂Cl₂ extraction step, the KNO₂ concentration for nitrosation was reduced from 60% to 40% in this modification to measure secondary amines more quickly and easily, but also to measure secondary amines yielding nitrosamines hardly extractable with CH₂Cl₂.

Secondary amines are nitrosated by nitrite in gastric juice to produce nitrosamines and some of the nitrosamines have strong mutagenic and oncogenic activities. Thus, measurement of secondary amines in foodstuffs is important, and Ito and Tanimura reported a method for colorimetric measurement of secondary amines. The method includes nitrosation of secondary amines by excess KNO₂, degradation of KNO₂ by sulfamic acid, CH₂Cl₂ extraction of nitrosamines, and coloring of nitrosamines with sulfanilamide and N'-1-naphthylthiyanediamine. However, we found that nitroso compounds of some secondary amine, such as catecholamines, were not extracted well with CH₂Cl₂. In this paper, we tried to omnit the CH₂Cl₂ extraction step and simplified the colorimetric measurement of secondary amines.

Materials and Methods

Materials. Sulfamic acid (SS), dimethyl nitrosamine (DMNA), proline, and 6-methylaminopurine (MAP) were dissolved in deionized water. Adrenaline (Fluka, AG.) and adrenaline (Fluka, AG.) were dissolved in 0.1N HCl and then neutralized with N NaOH before adjusting their concentrations. Other chemicals were obtained from Wako Pure Chemicals.

Measurement of secondary amines. Measurement of secondary amines basically followed the method of Ito and Tanimura shown in Fig. 1, with various modifications described in the text. For coloring of nitrosamines, 1g of sulfanilamide (SA) was dissolved in 100 ml of AcOH-ETO (3:7), and 180 mg of N'-1-naphthylthiyanediamine-HCl (NEDA) in 100 ml of AcOH:n-BuOH (3:7). A 7:1 mixture of n-BuOH and conc. HCl (n-BuOH:HCl) was used as the solvent of the coloring reaction. Absorbance and 552 nm of reaction mixture was measured using a Hitachi H200 spectrophotometer.

Results and Discussion

Optimization of nitrosation

As shown in Fig. 1, colorimetric measurement of secondary amines by Ito and Tanimura contains nitrosation

Mix sample sol. (1 ml), AcOH (2 ml), and 60% KNO₂ (2 ml)
Warm at 30°C for 30 min with stopper
Cool in ice bath and add 20% SS (8 ml)
Leave for 30 min at room temp. without stopper
Cool in ice bath, and add 30% NaOH (13 ml) and NaCl (2g)
Extract with CH₂Cl₂ (30 ml)
Add Na₂SO₄ (5 g) to CH₂Cl₂ extract
Filter with cotton
Add n-BuOH (5 ml), SA sol. (1 ml), NEDA sol. (1 ml), and n-BuOH:HCl (2:1) sol. (3 ml) to the filtrate (20 ml)
Heat at 55-60°C for 50 min
Heat at 100°C for 18 min
Make up to 10 ml with n-BuOH
Measure at 552 nm

Fig. 1. Schematic Diagram of Colorimetric Measurement of Secondary Amines by Ito and Tanimura.

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of secondary amines by excess KNO₂, degradation of residual KNO₂ by 20% SS, extraction of nitrosoamines with CH₂Cl₂, and coloring of the nitrosamines. Among these steps, the CH₂Cl₂ extraction step was most time-consuming and we found that some nitroso compounds could not be extracted well with CH₂Cl₂. Thus, we tried to omit the CH₂Cl₂ extraction step.

To skip the CH₂Cl₂ extraction step, complete degradation of KNO₂ is important. To measure the minimum amounts of KNO₂ for nitrosation, reaction mixtures containing 1 ml of 2 mM adrenalone, 0.5 ml of 2—1,000 mM KNO₂, and 0.5 ml of AcOH were incubated at 37°C for 10 min. After adding 2 ml of 20% SS, the reaction mixture was incubated at 37°C for 30 min. Then, 0.5 ml of the reaction mixture was mixed with 8 ml of n-BuOH: HCl (7:1), 1 ml of SA sol., and 1 ml of NEDA sol., and then heated at 100°C for 20 min. As shown in Fig. 2, nitrosation of adrenalone occurred at KNO₂ concentrations over 20 mM, increased rapidly between 40—400 mM, and then slowly over 400 mM.

Then the KNO₂ degrading ability of 20% SS was

examined at various KNO₂ concentrations. Reaction mixtures containing 1 ml of 1.5 mM DMNA (test) or H₂O (blank), 0.5 ml of AcOH, and 0.5 ml of 40—4,000 mM KNO₂ were mixed with 2 ml of 20% SS at 0°C and incubated at 37°C for 30 min. Then, 0.5 ml each of the reaction mixtures was tested for the coloring reaction. As shown in Fig. 3, blank values were negligible at KNO₂ concentrations below 400 mM, but increased rapidly thereafter. Test values with DMNA also increased with the increase in blank values, but net values (test-blank) were constant at KNO₂ concentrations between 40—4,000 mM. According to the above results, we used here 400 mM KNO₂ for nitrosation of secondary amines (final conc., 100 mM).

**Courses of the nitrosation reaction**

Reaction mixtures containing 10 ml of secondary amine solutions (2 mM), 5 ml of 400 mM KNO₂, and 5 ml of AcOH were incubated at 37°C and 0.5 ml of the reaction mixture was sampled periodically for the coloring reaction (100°C, 20 min). As shown in Fig. 4, adrenalone gave a maximum A₅₅₂ value within 10 min and then the value decreased in the reactions longer than 30 min. In the case of proline, A₅₅₂ gave a maximum value at 15 min and then the value decreased. In the case of MAP, the value reached a maximum at 20 min and the value was unchanged up to 60 min of reaction. According to these results, nitrosation continued for 15 min at 37°C thereafter. Efficiency of nitrosation varied with secondary amines under these nitrosation conditions.

**Optimization of coloring reaction**

To establish the optimum concentrations of coloring reagents, SA and NEDA solutions were diluted with each

![Fig. 2. Effects of KNO₂ Concentration on Nitrosation of Adrenalone.](image)

![Fig. 3. Degradation of KNO₂ by Sulfamic Acid. Blank, (□); test, (■).](image)

![Fig. 4. Courses of Nitrosation of Secondary amines. Adrenalone, (○); proline, (●); MAP, (□).](image)

![Fig. 5. Dose Response Effect of Coloring Reagents on Coloring of DMNA. Various concentrations of coloring reagents were used for coloring of 0.4 mM DMNA.](image)

![Fig. 6. Courses of Coloring of Nitrosated Catecholamines. Adrenoline, (○); adrenalone, (●).](image)
Mix sample sol. (1 ml), AcOH (0.5 ml), 400 mM KNO$_2$ (0.5 ml)
↓
Warm at 37°C for 15 min
↓
Cool in ice bath and add 20% SS (2 ml)
↓
Warm at 37°C for 30 min
↓
Take 0.5 ml of the reactant, and add n-BuOH:HCl (7:1) sol. (8 ml), SA sol. (1 ml), and NEDA sol.(1 ml)
↓
Heat at 100°C for 20 min
↓
Make up to 10 ml with n-BuOH
↓
Measure at 552 nm

Fig. 7. Schematic Diagram of Modified Colorimetric Determination of Secondary Amines.

![Calibration Curves of Secondary Amines](image)

Fig. 8. Calibration Curves of Secondary Amines.
Adrenaline, (○); adrenaline, (●); proline, (□).

solvent and used for the coloring of DMNA. The concentrations of the two coloring reagents were changed simultaneously. As shown in Fig. 5, coloring was increased with the increase in concentration of coloring reagents and gave a maximum coloring at concentrations over 2 times the diluted solution. Thus, the concentrations of coloring reagents followed the original directions of Ito and Tanimura.

To examine stabilities of coloring reagents, an 1:1 mixture of SA and NEDA solutions was incubated for up to 4 hr at 37°C and used for coloring of DMNA, but there was no difference in coloring of DMNA during the 4-hr incubations at 37°C (data not shown). After the coloring reaction, the reaction mixture was left at room temperature for up to 18 hr to examine the stability of coloring, but there was no change in $A_{552}$ during the 18-hr incubation (data not shown).

Course of coloring

Adrenaline and adrenaline were nitrosated as described above, and then reacted with SA and NEDA at 100°C for various periods. The two nitrosated compounds gave maximum values within 10 min and no decrease of coloring was observed within 50 min (Fig. 6). Thus, the reaction time for coloring was decided to be 20 min at 100°C.

According to the above results, we modified the colorimetric measurement of secondary amines, as shown in Fig. 7. Figure 8 shows calibration curves of adrenaline, adrenaline, and proline obtained using the modified methods. Adrenaline and proline gave similar calibration curves at 0—2 mM, but adrenaline gave about 30% higher values than the others at each points. We showed previously that adrenaline gave mono- and di-nitroso compounds by its nitrosation. The higher $A_{552}$ value of adrenaline may be due to the production of a di-nitroso compound.

In the modified method, we increased the temperatures of nitrosation and KNO$_2$ degradation from 30°C to 37°C. Irrespective of reduction of the KNO$_2$ concentration from 60% to 400 mM, a 15-min reaction was enough for nitrosation of secondary amines. By the reduction of KNO$_2$ concentration, we could omit time-consuming CH$_2$Cl$_2$ extraction, cotton filtration, and CH$_2$Cl$_2$ removal steps. In total, the method became simpler and less time-consuming than the method of Ito and Tanimura. In addition to that, the method has wider applicability, since it can be used for the measurement of nitroso compounds hardly extractable with CH$_2$Cl$_2$.

The omission of CH$_2$Cl$_2$ extraction may increase the blank value in the secondary amine measurement, when the sample solution is colored. The increase of coloring can be easily compensated for by deduction of blank values from test values. In the presence of nitrite, phenolic compounds are also nitrosated and modify N-nitrosation of secondary amines. The C-nitroso phenols can be colored by the coloring reaction used here (unpublished result). This may lead to higher secondary amine contents obtained by our simplified method than those by the method of Ito and Tanimura. Correctly, our method is a simple measurement of nitrosatable food components and we are studying the mutagenecity of C-nitroso compounds as well as N-nitroso compounds in foodstuffs.

References