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Comparison of Two Methods and Improvements for Colorimetric Determination of Nitrite in Cod Roe

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ABSTRACT

Attempts were made to develop a sensitive and reproducible method to determine nitrite in cod roe. Two diazotation-coupling reaction methods were considered; (*a*) the method defined by the Ministry of Health and Welfare of Japan (Method 1) and (*b*) the reference method of ISO (Method 2). Since the nitrite content in cod roe was much less than in meat products, Method 2 was modified to make it suitable for microanalysis at 1 ppm level as NO_2. Modifications included reducing volumes of color-development solutions and making changes in the color development process, thus making the color intensity four times as great as before. Carrying out corrections with both reagent and water blanks made the effect of the blank on measured values negligible. Recoveries of nitrite at 20- and 2-ppm levels were 94.7 and 88.1%, respectively, reproducibility being ± 7.9%, as the coefficient of variation. The obtained values by the modified method were, on the average, higher than those of the original method by 37.1%. Nitrite contents obtained by Method 1 were lower than those by the original Method 2. These low values might be attributed to loss of nitrite during extraction from the sample without pH adjustment, since the measured value showed a remarkable increase by addition of alkaline solution before extraction. Nitrite contents in imported cod roe were within the range 0.16-1.03 ppm expressed as NO_2.

Mentaiko is a kind of salted cod roe usually colored with new coccine or other red dye and students often eat it along with rice for their lunch. It is a product of Hakata and Kita-kyusyu districts of Japan and salted cod roe used as the raw material is imported from South Korea; the quantity of salted marine products that passed through the harbors of Shimonoseki and Moji during 1977 amounted to 1,296.3 tons. In Japan, use of nitrite (sodium nitrite) as a food additive for color fixative and for other purposes is limited. Concerning fish roe, it is used only with salmon roe (sujiko and ikura) with the residual level of 5 ppm as NO_2; its use on cod roe is strictly prohibited. It is well known, however, that cod roe contains small amount of nitrite as its own component.

The objectives of this work were to modify an existing nitrite method to use for determination of small amounts of naturally-occurring nitrite in cod roe, to show the validity of this method through recovery tests, to compare this method with the prescribed method now in use, and to demonstrate the usefulness of the method by use on cod roe samples.

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MATERIALS AND METHODS

Methods

It is prescribed in the Sanitary Inspection Guide (*1*) that the nitrite content in salmon and cod roe should be measured by use of both uranyl acetate and zinc acetate as precipitants. The former substance, however, is designated as one of the nuclear fuels and one must apply to the Director-General of the Science and Technics Agency for sanction every time he wishes to obtain it from a supplier. Harada (*2*) made it clear that mercuric chloride is an excellent precipitant applicable to roe, but use of this substance is not recommended because of its toxicity.

Accordingly, it was undertaken by us to apply the already established methods to determine nitrite in meat products for determination of the micro-quantity of nitrite in cod roe. Two methods are now available for this purpose; one is the analytical method for food additives in foodstuff authorized by the Food Chemistry Division of the Ministry of Health and Welfare of Japan (*3*), while the other is the international standard method established by ISO (International Standardization Organization) and adopted in our laboratory for inspection of imported meat products (*4*). Both methods are based on colorimetry of nitrite by the diazotation-coupling reaction though the former uses zinc sulfate-sodium hydroxide while the latter employs the Carrez reagent as the precipitant. The details of the procedure are as follows.

Method 1 (method of Ministry of Health and Welfare)

Reagents. 0.5 N NaOH, 12% $ZnSO_4 \cdot 7H_2O$ solution, 10% ammonium acetate buffer (dissolve 100 g of guaranteed ammonium acetate in 900 ml of water, adjust pH to 9.0 with 10% ammonia water, and make to 1,000 ml with water), 1% ammonium acetate buffer (dilute buffer) (dilute 10% ammonium acetate buffer to one tenth concentration and readjust the pH to 9.0 with 10% ammonia water), sulfanilamide solution [dissolve 0.5 g of guaranteed sulfanilamide in 100 ml of hydrochloric acid (1 + 1), stable for 4 weeks], naphtylethylenediamine solution (dissolve 0.12 g of N-(1-naphtyl)-ethylenediamine (guaranteed) in 100 ml of water, filter if necessary; keep in a refrigerater in a brown bottle; stable for 2 weeks), nitrite-nitrogen standard solution (weigh accurately 0.493 g of sodium nitrite previously dried for 24 h over sulfuric acid in a desiccator, dissolve in sterile water to make 1,000 ml; standard stock solution).

Take 10 ml of the standard stock solution, add water to make 100 ml, then take 2 ml from the 100 ml solution, add 10 ml of 10% sodium acetate solution and water to make 100 ml, and use thus prepared solution as the nitrite-nitrogen standard solution (prepare freshly before use). One ml of nitrite-nitrogen standard solution = 0.2 μg NO_2-N.

Preparation of sample solution. Weigh 10.0 g of sample cut in pieces, add adequate volume of water at about 80 C and homogenize. Pour the contents into a volumetric flask of 200 ml, wash the contents several times with warm water and add the washings to the same flask. Add 10 ml of 0.5 N NaOH, shake well, then add 10 ml of 12% $ZnSO_4 \cdot 7H_2O$ solution, shake well and then heat for 20 min in a water bath at 80 C, shaking occasionally. Cool to room temperature in cold water, add 20 ml of 10% ammonium acetate buffer, make to 200 ml with water, mix the contents well, filter through a dry filter paper for quantitative analysis (Toyo Filter Paper No.5C) into a ground stoppered conical

flask, discard the first 10 ml of filtrate and use the obtained clear filtrate as a sample solution. Carry out the procedure by use of 10 ml of water instead of sample to prepare a blank solution.

Determination. Place each 20 ml of sample solution and blank solution into separate volumetric flasks (25 ml) a, b, respectively; to each flask add 1 ml of sulfanilamide solution, mix, add 1 ml of naphtylethylenediamine solution, make to 25 ml with water, mix the contents well and let color develop. Place the same quantity of sample solution into another volumetric flask (25 ml) c, add 1 ml of dilute HCl (1 + 1) and water to make 25 ml, mix the contents well and measure the optical densities of contents of flasks a, b and c, after 20 min, at the wavelength of 540 nm using water as a reference. Aa: O.D. of flask a, Ab: O.D. of flask b, and Ac: O.D. of flask c.

Substract the sum of Ab and Ac from Aa, read the corresponding concentration from the calibration curve, and calculate the nitrite content in the sample (C_{NO_2}) by the following formula.

$$C_{NO_2}\ (\text{ppm}) = 3.28\ A$$

where A is the nitrite-nitrogen content in 20 ml of sample solution

Preparation of calibration curve. Place 0, 2, 5, 10, 15 and 20 ml of nitrite-nitrogen standard solutions into separate volumetric flasks of 25 ml, to each flask add dilute buffer to make to about 20 ml, add 1 ml of sulfanilamide solution, mix, add 1 ml of naphtylethylenediamine solution and water to make 25 ml, and mix the contents well. Place 20 ml of diluted buffer into another volumetric flask of 25 ml, then proceed the same as for the standard solution to prepare the blank solution. After 20 min, measure the optical density of each solution at the wavelength of 540 nm using this blank solution as a reference. Prepare a calibration curve.

Method 2 (reference method of ISO)

Reagents. Reagent I (dissolve 106 g of potassium ferrocyanide trihydrate [$K_4Fe(CN)_6 \cdot 3H_2O$] in water and dilute to 1,000 ml), Reagent II (dissolve 220 g of zinc acetate dihydrate [$Zn(CH_3COO)_2 \cdot 2H_2O$] and 30 ml of glacial acetic acid in water and dilute to 1,000 ml), borax solution, saturated (dissolve 50 g of sodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$) in 1,000 ml of tepid water and cool to room temperature), sodium nitrite standard solution (dissolve 0.150 g of sodium nitrite ($NaNO_2$) in water and dilute to 1,000 ml in a volumetric flask, pipette 10 ml of the solution into a 1,000-ml volumetric flask and dilute to the mark; 1 ml of this solution contains 1.0 μg of NO_2 per ml; the standard solution shall be made on the day of use), color development solution I (dissolve, by heating on a water bath, 2 g of sulfanilamide ($NH_2C_6H_4SO_2NH_2$) in 800 ml of cold water, filter, if necessary, and add 100 ml of HCl, while stirring, dilute to 1,000 ml with water), color development solution II (dissolve 0.1 g of N-1-naphtylethylene diamine dihydrochloride ($C_{10}H_7NHCH_2CH_2NH_2 \cdot 2HCl$) in water, dilute to 100 ml with water), and color development solution III (dilute 445 ml of hydrochloric acid to 1,000 ml with water.

Store the color development solutions in well-stoppered brown bottles. They shall be kept in a refrigerator for not longer than 1 week.

Procedure. Proceed from a representative sample of at least 200 g. Weigh, to the nearest 0.001 g, about 10 g of homogenized sample. Transfer the test portion quantitatively into a 300-ml conical flask, and add successively 5 ml of saturated borax solution and 100 ml of water at a temperature not below 70 C. Heat the flask for 15 min on a boiling water bath and shake repeatedly. Allow the flask and its contents to cool to room temperature and add successively 2 ml of reagent I and 2 ml of reagent II. Mix thoroughly after each addition. Transfer the contents to a 200-ml volumetric flask. Allow the flask to stand for 30 min at room temperature. Dilute to the mark with water. Mix the contents of the flask thoroughly and filter through a fluted filter paper.

Pipette a portion of the filtrate, but not more than 25 ml into a 100-ml volumetric flask and add water to obtain a volume of about 60 ml. Add 10 ml of color development solution I, followed by 6 ml of color development solution III; mix and leave the solution for 5 min at room temperature in the dark. Add 2 ml of color development solution II, mix and leave the solution for 3 min at room temperature in the dark. Dilute to the mark with water. Measure the absorbance of the solution in a 1-cm cell at a wavelength of 538 nm.

Pipette respectively into nine 100-ml volumetric flasks 0, 2, 4, 6, 8, 10, 12, 16 and 20 ml of sodium nitrite standard solution and add water to obtain a volume of about 60 ml. Proceed as described above, starting from "add 10 ml of color development solution I...". Draw the calibration curve by plotting the measured absorbances against the concentrations, in μg of NO_2 per 100 ml, of the solutions.

Calculate the nitrite content of the sample, expressed as milligrams of NO_2 per kg, using the formula

$$NO_2 = C \times \frac{200}{m \times V}$$

where

m is the mass, in grams, of the test portion;

V is the volume, in milliliters, of the portion of the filtrate taken for the determination; and

C is the concentration of nitrite (NO_2) in μg per 100 ml, read from the calibration curve.

The difference between the results of two determinations carried out simultaneously or in rapid succession, by the same analyst, shall not be greater than 10% of the assayed nitrite content. Report the result to the nearest 1 mg per kg of the product.

RESULTS AND DISCUSSION

Preparation of calibration curve in lower concentrations of nitrite

In this section, investigations were carried out by use of Method 2. Since the nitrite content of cod roe is, on the whole, much lower compared with that in meat products, the calibration curve has to be prepared at lower concentrations. Besides, it was undertaken to reduce the volume of color developing solution from 100 to 25 ml to increase the color intensity. The modified process of Method 2 is expressed in Fig. 1. The calibration curves obtained are as shown in Fig. 2. Good linearity was obtained by the modified procedure as well as the original procedure, and from the results it is evident that each 1 ml addition of color development solutions I, II and III was enough for determination of less than 5 μg of nitrite. We found it was not necessary to leave the solution for 5 min at room temperature in the dark after addition of color development solutions I and III.

Since only 1 ml each of sulfanilamide and naphtylethylenediamine solutions are used in Method 1, it is impossible to reduce the final volume of the procedure as was done in Method 2.

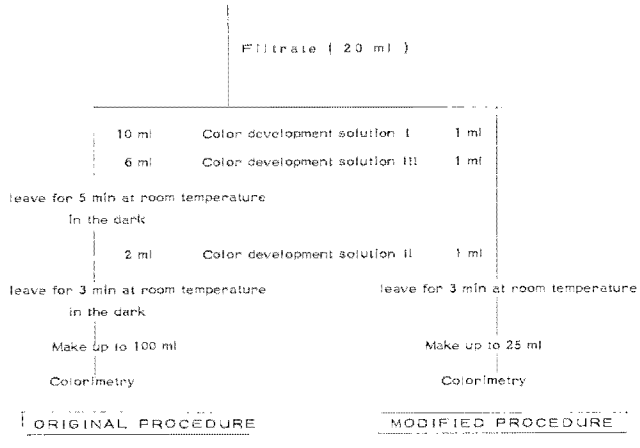

Figure 1. *Partial modification of Method 2.*

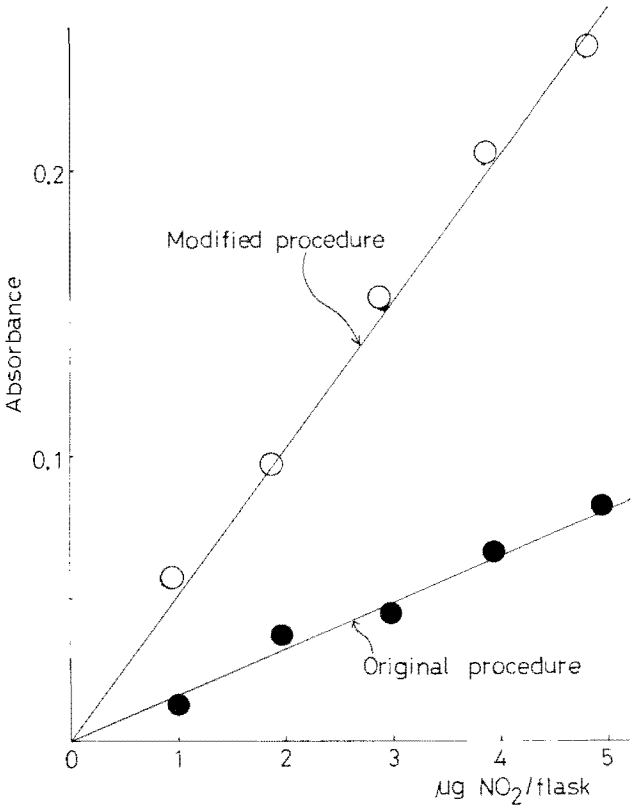

Figure 2. *Calibration curves of nitrites by Method 2.*

Problems with blank values

Three kinds of blank values were measured in the original and modified procedures of Method 2 to compare them to each other. *Absolute blank*: No nitrite-nitrogen standard solution was used in preparation of calibration curve. *Blank 1*: 10 ml of water was used instead of the sample. This value is used to know the degree of color development by reagents other than nitrite. *Blank 2*: The same quantity of water was used instead of color development solutions I and II. This value serves to show the degree of coloration of the sample.

If cod roe is naturally or artificially colored, the blank value 2 will not be negligible. Values of blanks 1 and 2 measured against the absolute blank are as shown in Table 1. Sometimes seemingly high values for nitrite in cod roe were obtained by use of the original procedure. Such a phenomenon, presumably, was partly due to the disturbance by blank values. Contrary to this, the effect of blank values was below 0.1 ppm as NO_2 in the modified procedure. Reproducibility of determined nitrite contents through the full process of the original and modified procedures of Method 2, expressed as coefficients of variation were 5.2 and 2.9%, respectively.

Recovery of nitrite

Tests were done with Method 2. Two ml of stock nitrite solution (0.1 mg NO_2/ml) or 2 ml of nitrite standard solution was used and both the original and modified procedures were followed. Next the same quantity of nitrite was added to 10 g of cod roe (corresponding to 20 and 2 ppm, respectively) and the spiked samples were subjected to the two analyses. Results are summarized in Table 2. Recoveries were satisfactory for both original and improved procedures either with or without cod roe. At the added level of 2 ppm, however, recovery of the original procedure was low compared with that of the modified procedure, the obtained values being scattered. It was proved that the modified Method 2 was particularly adequate for determination of a micro-quantity of nitrite in cod roe.

TABLE 2. *Recovery of added nitrite by Method 2.*

	Without cod roe		With cod roe	
Added level (ppm No_2) Method 2	20	2	20	2
Recovery (%)				
Original method	99.6	77.5	93.8	73.5
Modified method	98.9	91.5	94.7	88.1

Inspection on imported cod roe

The original and modified Method 2 were applied to 12 samples of imported cod roe; results are in Table 3. Seemingly high values were obtained by use of the modified procedure. No reliable data have been published up to now in Japan on a survey of the natural nitrite content in cod roe.

Comparison of Method 2 versus Method 1

Since Method 1 is presently established as the analytical method for nitrite by the Ministry of Health and Welfare of Japan, a comparison of Method 2 with Method 1 has to be done. Preliminary examination of several samples of cod roe revealed that the values obtained by Method 1 were, on the average, 48.4% lower than those of the original Method 2. Furthermore, recovery of nitrite added at the level of 5 ppm of NO_2 by Method 1 was as low as 58.0%. Since it is agreed that in

TABLE 1. *Comparison of blank values in the original and modified Method 2.*

	Original procedure		Modified procedure	
	O.D. at 538 nm	Equivalent to NO_2 ppm	O.D. at 538 nm	Equivalent to NO_2 ppm
Blank 1[a]	0.004	0.33	0.003	0.06
Blank 2				
Sample No. 1	0.001	0.08	0.001	0.02
Sample No. 2	0.000	0.00	0.003	0.06
Sample No. 3	0.002	0.16	0.005	0.10
Sample No. 4	0.005	0.40	0.005	0.10
Sample No. 5	0.003	0.24	0.002	0.04

[a]Mean value of five trials.

TABLE 3. *Comparison of measured values of nitrite contents in imported cod roe by Method 2.*

Sample	Nitrite content (NO_2 ppm) (1) Original procedure	(2) Modified procedure
No.1	0.64	0.73
" 2	0.09	0.16
" 3	0.18	0.37
" 4	0.18	0.26
" 5	0.24	0.39
" 6	0.30	0.37
" 7	0.67	1.03
" 8	0.64	0.69
" 9	0.43	0.61
" 10	0.40	0.80
" 11	0.60	0.60
" 12	0.50	0.50
Mean value	0.41	0.54
[(b)/(a)] × 100	100	131.7

Method 1 the final volume of color developing solution, that started from 20 ml of filtrate, is to be made to 25 ml, it is impossible to reduce any more the final volume of color developing solution. In Method 2, it is described that sample is to be heated at not below 70 C after the pH of the solution has been made alkaline by addition of saturated, borax solution. The test sample is homogenized in Method 1 with hot water at about 80 C before addition of the alkaline solution (0.5 N NaOH), and it is presumed that homogenizing at a slightly acidic pH might lead to a partial decomposition of nitrite. Accordingly, it was undertaken to modify the preparation process of the sample solution in Method 1 to that shown in Fig. 3. In the modified procedure, the sample is heated after addition of the alkaline solution. Results are as shown in Table 4. It became clear that the values for nitrite by Method 1 were doubled by this modification, indicating that a considerable amount of nitrite was lost in the original procedure during heating at 80 C. The obtained values, however, were a little lower than those of the modified Method 2.

Method finally established

Taking into account reproducibility and other factors, we finally decided to choose the modified Method 2. The finally adopted method is as follows:

As for the reagents and solutions used, refer to Method 2. Proceed as described in Method 2 to obtain clear filtrate.

Pipette 20 ml of the filtrate into a 25-ml volumetric flask, add each 1 ml of color development solutions I, III and II in turn, mixing the contents well after each addition. Leave the solution for 3 min at room temperature and then dilute to the mark with water. Measure the absorbance (A) of the solution in a 1-cm cell at a wavelength of 538 nm against a reagent blank prepared in the same manner starting from 10 ml of water. Prepare sample blank from 10 g of the same sample without addition of color development solutions and measure the color intensity at the wavelength of 538 nm

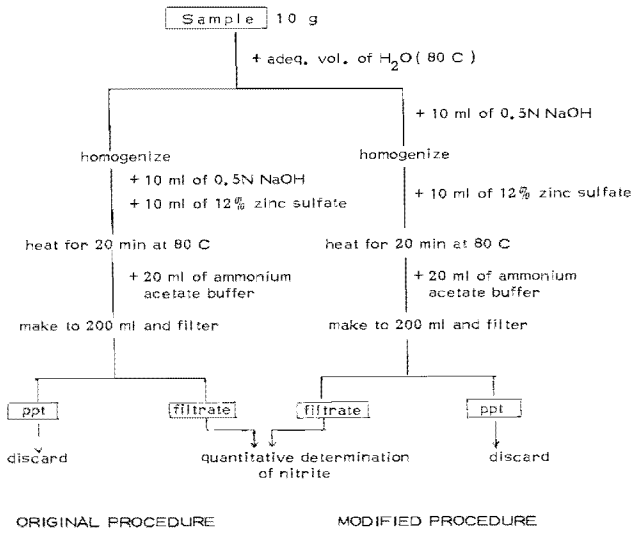

Figure 3. *Partial modification of Method 1.*

TABLE 4. *Comparison of original and modified procedures of Method 1 and Method 2.*

Sample	Nitrite content (NO_2 ppm) Method 1 Original procedure	Method 1 Modified procedure	Method 2 Original procedure	Method 2 Modified procedure
No. 1	0.27	0.33	0.46	0.47
" 2	0.45	0.99	0.67	1.03
" 3	0.24	0.59	0.43	0.61
Index	100	193.8	162.5	219.8

against a water blank prepared starting with 10 ml of water in the same manner without addition of color development solutions (B). Subtract B from A.

Pipette, respectively, into nine 25-ml volumetric flasks 0, 2, 4, 6, 8, 10, 12, 16 and 20 ml of nitrite standard solution and add water to obtain a volume of about 20 ml. Proceed as described in the measurement of absorbance (A) starting from "add each 1 ml of color development solution I, II and III...", and read the absorbance of each flask against the first solution into which no nitrite was pipetted. Draw the calibration curve by plotting the measured absorbances against the concentrations, in μg of NO_2 per 25 ml of the solution. Calculate the nitrite content of the sample by use of the calibration curve.

REFERENCES

1. Food Chemistry Division of Ministry of Health and Welfare. 1977. Analytical methods for food additives in foodstuff. Food Sanitation Res. 27:37-50.
2. Harada, M. 1971. Determination of nitrite in cod roes. Food Sanitation Res. 21:1203-1212.
3. Iwaida, M., Y. Kaneda, and M. Nishikawa. 1976. Determination of nitrite in imported meat and meat products. Bull. Nat. Inst. Hyg. Sci. 94:122-124.
4. Supervised by Ministry of Health and Welfare. 1971. Sanitary Inspection Guide Vol. 1. Japan Food Hygiene Association p. 488.