Collaborative Study of Modified AOAC Method of Analysis for Nitrite in Meat and Meat Products

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A modified version of the AOAC method of analysis for nitrite in meat and meat products was tested collaboratively by 23 laboratories. Results were compared with those obtained by the official AOAC method. Recommended modifications include: (a) substitution of *N*-(1-naphthyl)ethylenediamine and sulfanilamide for Griess reagent, (b) separate addition and 1:10 dilution of the above reagents, (c) 20 min color development and absorbance read at 540 nm, (d) substitution of $NaNO_2$ for $AgNO_2$ and NaCl, (e) omission of mercuric chloride, (f) screening of filter paper for nitrite contamination, (g) more precise dilution of sample aliquot, and (h) standard curve linear up to 10 μg N/50 ml. Results were statistically treated by Youden's technique for comparing 2 methods, using a matched pair sample scheme. The random error for the modified method was significantly lower than the random error for the official method. A *t*-test showed no difference in bias between the 2 methods.

The official method of analysis for nitrite in meat products (1) was adopted by the AOAC in 1925 and has remained unchanged to the present time. In 1973, we published recommendations for simplifying and improving the official method (2). Subsequent findings have prompted us to recommend further modifications. The present report is a collaborative comparison of the official method (24.037–24.038) and a version of the official method which includes both the earlier and later modifications. The latter is called the "modified method" in this paper.

Collaborative Study

Frankfurter emulsion was used as the sample material for this study because the homogeneity of this product minimizes sampling error. Two frankfurter emulsions were chopped in a Koch Schnell cutter, Model 25. Both were standard formulations containing beef, pork, pork fat, sucrose, salt, spices, and sodium nitrite. The 2 emulsions were identical, except that Sample A was prepared with the legally permitted level of 156 ppm $NaNO_2$, and Sample B was prepared with twice that amount, that is, 312 ppm. This was done to ensure that there would be a difference in residual nitrite values in Samples A and B. Loss of nitrite in meat is a capricious process. It is quite possible to add widely different amounts of nitrite to 2 samples and, after processing, find the same amounts of residual nitrite in both.

Individual samples were packed in 2″ diameter by 3″ high cans which were then sealed under vacuum. The cans were heated in a circulating air oven set at 77°C until the contents reached an internal temperature of 72°C, which required 2.5 hr. They were cooled with cold tap water and frozen at −16°C. After 4 days, each participating laboratory was sent 1 can of Sample A and 1 can of Sample B. The frozen samples were packed in Styrofoam shipping containers with 20 lb of crushed Dry Ice, and shipped by air freight to each laboratory. Samples were shipped frozen to minimize loss of nitrite and to maintain the same temperature for all samples until they were analyzed. Most of the collaborators received their samples within 24 hr.

Experimental

The major problem in collaborative studies on methods for determining nitrite in meat is the wide variability in nitrite concentration from sample to sample. Loss of nitrite is primarily a function of time and temperature (3). Figure 1 shows the large initial loss of nitrite during pasteurization of a canned frankfurter emulsion prepared in our laboratory. After heating, half the cans were stored at 2°C and the remainder at −24°C. The rate of loss at −24°C was slightly less than

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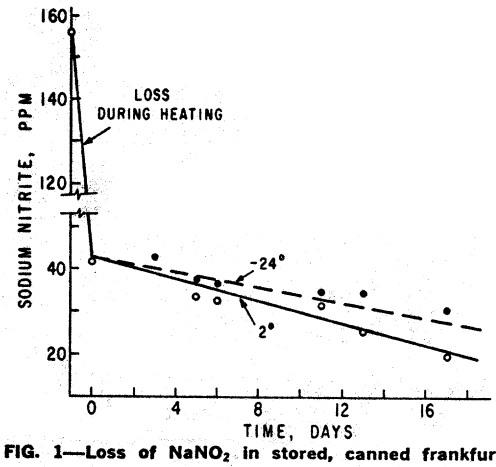

FIG. 1—Loss of $NaNO_2$ in stored, canned frankfurter emulsion.

at 2°C. In order to minimize nitrite variability from sample to sample, collaborators were asked to store the samples in Dry Ice and thaw them under cold tap water prior to performing the analyses, to run a single determination on 2 samples by each method for a total of 4 determinations, and to perform the analyses on a fixed date to further ensure that all samples contained the same level of nitrite.

Table 1 shows the differences in reagents and procedures between the official AOAC method and the modified method.

The compound α- or 1-naphthylamine has been listed as a carcinogen by the Occupational Safety and Health Administration and may be used only with elaborate precautions (4). The General Referee on Meat and Meat Products has recommended the substitution of *N*-(1-naphthyl)ethylenediamine and advised that no comparison of the 2 compounds be made. For this reason, *N*-(1-naphthyl)ethylenediamine and sulfanilamide were used as colorimetric reagents for both methods in the collaborative study. Sulfanilamide was substituted for sulfanilic acid because the former dissolves more readily in 15% acetic acid. The toxicity of *N*-(1-naphthyl)ethylenediamine is presently under investigation by the National Cancer Institute. Results of these studies are expected within the next year (S. Siegel (1975) National Cancer Institute).

N-(1-Naphthyl)ethylenediamine and sulfanilamide must be stored and added separately because, under acidic conditions, the 2 compounds compete for nitrite. Sulfanilamide is added first so that it forms the diazonium salt; after 5 min, *N*-(1-naphthyl)ethylenediamine is added to form the pink azo dye. Competition for nitrite was not a problem with 1-naphthylamine, because it reacts slowly with nitrite.

The 1:26 dilution of colorimetric reagents in the official method does not provide the 100-fold excess of reagents required for complete conversion of nitrite to chromophore and gives linear absorbance only up to 5 μg N/50 ml (2). A 1:10 dilution of colorimetric reagents provides complete conversion of nitrite and linear absorbance up to 10 μg N/50 ml, and allows faster formation of maximum color (20 min at 1:10, 60 min at 1:26 dilution).

Nitrite and the colorimetric reagents of the modified method form a compound with an absorption maximum at 540 nm, in comparison with the absorption maximum at 520 nm for the compound formed in the official AOAC method.

In the official method, nitrite standard solution is prepared by dissolving $AgNO_2$ and precipitating Ag with NaCl. The modified method substitutes $NaNO_2$ for what seems an unnecessary and archaic procedure, since reagent grade $NaNO_2$ is usually 99.9% pure.

Table 1. Differences between the official AOAC method for nitrite and the modified method under study

Item	Official AOAC method[a]	Modified method
Colorimetric reagents	α-naphthylamine and sulfanilic acid (Griess reagent); prepd sep. and stored mixed together	*N*-(1-naphthyl)ethylenediamine and sulfanilamide; prepd and stored sep.
Addn of colorimetric reagents	added together	added sep.
Diln of colorimetric reagents	1:26	1:10
Time for color development, min	60	20
Absorbance wavelength, nm	520	540
Nitrite std soln	dissolve 1.1 g $AgNO_2$; ppt with NaCl; contains 0.1 μg N/ml	dissolve 0.984 g $NaNO_2$; no ppt; contains 0.2 μg N/ml
Mercuric chloride soln, ml	5	none
Filter paper	—	check for nitrite contamination
Aliquot diln	aliquot dild to vol.; then reagent added	aliquot and reagents mixed and dild to vol.
Std curve	straight line to 5 μg N in final soln	straight line to 10 μg N in final soln

[a] Because of the suspected carcinogenicity of α-naphthylamine, *N*-(l-naphthyl)ethylenediamine and sulfanilamide were substituted for Griess reagent.

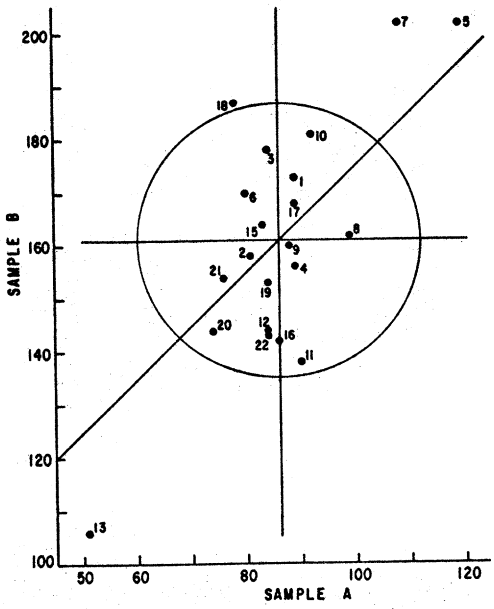

FIG. 2—Two-sample plot of $NaNO_2$ values (ppm) obtained by official AOAC method.

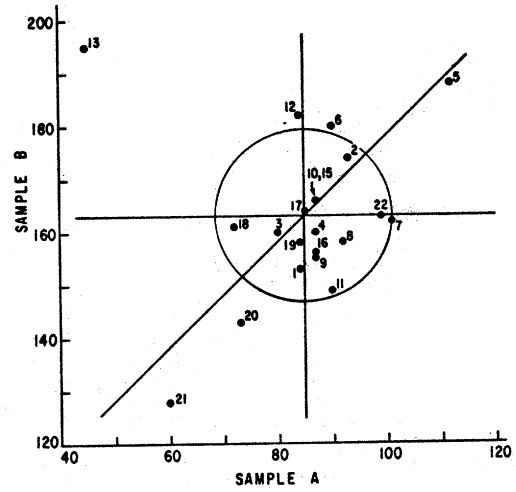

FIG. 3—Two-sample plot of $NaNO_2$ values (ppm) obtained by modified method.

In a previous publication (2), we showed that there was only a small difference in amount of nitrite found when pairs of meat samples were analyzed with and without the use of mercuric chloride. Since mercuric chloride is corrosive, extremely toxic, and a potential pollutant, we recommend discontinuing its use.

Another modification in the official method is based on the finding in our laboratory of samples of filter paper apparently contaminated with nitrite (5). Six of 28 boxes of filter paper examined contained sufficient nitrite to cause significant error in determining the nitrite content of meat. All filter paper should be tested for nitrite by analyzing 3 or 4 sheets of paper, at random, throughout the box. If any of the sheets is positive, none of the sheets in the box should be used.

In the official method, an aliquot of sample is diluted to 50 ml, and then 2 ml Griess reagent is added. Greater precision is achieved by diluting both aliquot and reagent to 50 ml.

Results and Discussion

Data were received from 22 of 23 collaborators who participated in the study. One collaborator did not report any data because the filter paper and glass wool available in his laboratory were contaminated with nitrite. The data from Collaborator 14 were rejected after a statistical outlier test was applied at the 1% level of significance (6).

Figures 2 and 3 show the pairs of data points for each method plotted according to Youden (7). The horizontal and vertical lines are drawn through the average values for Samples A and B. The radius of the circle drawn about the intersection of the 2 lines was obtained by multiplying S_r by 2.45. The circle would include 95% of the points if there were no systematic errors. The official method has 81% of the points within the circle, the modified method, 71%. Collaborator 5 submitted consistently high results for both sample materials and both methods, as indicated by the points lying far out but close to the 45° line. A point far out on this line suggests that this collaborator was working carefully, but was somehow consistently modifying the method. Collaborator 13 reported low results for both samples for the first method and one high and one low result for the second method. Based on these observations, the data from Collaborator 13 were rejected.

The plots of both methods show elliptical patterns of points which indicate that precision was good within one laboratory, but not so good between laboratories. This result confirms what has been commonly found by others. The smaller circle in Fig. 3 reflects the lower random standard deviation of the modified method.

Table 2 shows the results from 22 laboratories for analyses of 2 samples by 2 methods. Means, standard deviations, and F values are shown. Computations were based on Youden's technique

Table 2. Collaborative results for determination of $NaNO_2$ (ppm) in matched pair samples by the official AOAC and modified methods

	Official method				Modified method			
Coll.	Sample A	Sample B	Diff.	Total	Sample A	Sample B	Diff.	Total
1	89	173	84	262	84	153	69	237
2	81	158	77	239	93	174	81	267
3	84	178	94	262	80	160	80	240
4	89	156	67	245	87	160	73	247
5	119	202	83	321	112	188	76	300
6	80	170	90	250	90	180	90	270
7	108	202	94	310	101	162	61	263
8	99	162	63	261	92	158	66	250
9	88	160	72	248	87	155	68	242
10	92	181	89	273	87	166	79	253
11	90	138	48	228	90	149	59	239
12	84	144	60	228	84	182	98	266
13	(51)	(106)	(55)	(157)	(45)	(195)	(150)	(240)
14	(40)	(64)	(24)	(104)	(44)	(74)	(30)	(118)
15	83	164	81	247	87	166	79	253
16	86	142	56	228	87	156	69	243
17	89	168	79	257	85	164	79	249
18	78	187	109	265	72	161	89	233
19	84	153	69	237	84	158	74	242
20	74	144	70	218	73	143	70	216
21	76	154	78	230	60	128	68	188
22	84	143	59	227	99	163	64	262
Mean	84.00	156.77	72.77	240.77	82.86	158.86	76.00	241.73
S_d	16.14	30.07		31.50	16.20	23.99		24.72
S_r			13.14				15.07	
S_b			20.24				13.86	
Mean excluding Coll. 14	86.10	161.19	75.10	247.29	84.71	162.90	78.19	247.62
S_d	13.12	22.33		23.49	14.01	15.08		15.51
S_r			10.91				13.53	
S_b			14.72				5.36	
Mean excluding Colls. 13 & 14	87.85	163.95	76.10	251.80	86.70	161.30	74.60	248.00
S_d	10.64	18.89		18.86	10.94	13.50		15.86
S_r			10.68				7.09	
S_b			10.99				10.03	

F for S_d^2 ratio = 1.41
F for S_r^2 ratio = 2.27
F for S_b^2 ratio = 1.20
$F_{0.05}$, 19 degrees of freedom = 2.17.

for comparing 2 methods (8). In order to use this technique, by which precision can be determined without duplicate analyses, it was assumed that both Samples A and B would have the same random and systematic errors at a given laboratory. Previous experience with nitrite analyses in our laboratory supports this assumption.

S_d is the overall standard deviation, S_r is the random or within-laboratory contribution by all laboratories for a given method, and S_b measures the between-laboratory error. Rejection of results from Collaborator 14 reduced all the standard deviations but made S_b smaller than S_r for the modified method. Between-laboratory error is almost always larger than within-laboratory error. Rejection of results from both Collaborators 13 and 14 decreased S_r and increased S_b for the modified method so that S_b was then larger than S_r, as is normally found.

In order to compare the final standard deviations of the 2 methods, F values were calculated from the ratios of the squares of S_r, S_d, and S_b. The calculated F values were then compared with the tabular F values. The S_r value for the modified method was significantly lower than S_r

for the official method. The reproducibility standard deviations (σ_x) were 15.33 for the official method and 12.29 for the modified method. To test for the difference in the systematic errors of the 2 methods, a t-test was calculated and compared with a tabular t value. The calculated t value was 0.75; the tabular value for 19 degrees of freedom at the 95% level was 2.09. There was no indication of a difference in bias between the 2 methods.

Collaborators' Comments

Collaborators 8 and 12 reported difficulty in reproducing the standard curve for the official method.

Collaborators 4, 7, 10, 16, and 21 had no $AgNO_2$, so used $NaNO_2$ to make the nitrite standard solutions for both methods. Collaborator 22 used an old sample of $AgNO_2$.

Collaborator 5 received the samples late and performed the analyses 1 day after the recommended date.

Collaborator 15 used 10 g samples of meat in 1 L flasks.

Collaborator 9 performed the official method both with Griess reagent and with the recommended substitutes. He also performed the analyses on the required date and 4 days later. There were no significant differences either between methods or between days. Collaborator 4 used Griess reagent instead of the recommended substitutes.

Collaborator 18 reported using a Spectronic 20 spectrophotometer, which has minimal accuracy at the low end of the scale used in the official method.

Recommendation

Based on the results obtained from this study, it is recommended that method **24.037–24.038** be modified according to the suggestions in this report.

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