

Critical Evaluation of the AOAC Method of Analysis for Nitrite in Meat

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Results of studies on the effects of 5 alternative procedures of sample preparation are presented. The standard deviations for the methods were 0.00505, 0.00142, 0.00307, 0.00265, and 0.00243, with the AOAC method giving the highest values for nitrite. The effects of some variables on the determination of the nitrite content of meat by the official method were also studied. These variables include the addition of mercuric chloride, duration of heating, rate of color reaction, and reagent preparation. The nitrite concentration as measured by the AOAC method varied with the initial dilution of meat sample. A number of compounds were added to the Griess reagent and nitrite solution to see whether they would affect the reaction. Reductants were found to interfere consistently. Suggestions for simplifying and improving the official method are made. These include omitting mercuric chloride, recrystallizing 1-naphthylamine before using, and allowing only 25 min for the completion of the color reaction, using a higher concentration of Griess reagent.

The method for analysis of nitrite in meat products was adopted by the AOAC in 1925 and has remained unchanged to the present (24.014-24.015). The procedure for extracting nitrite from the meat sample was developed by Kerr (1). The reagent used to determine the nitrite concentration was developed by Johann Peter Griess (2) in 1879, with later changes by Ilosvay (3) and Lunge (4). Briefly, the AOAC method calls for diluting 5 g comminuted meat sample to about 300 ml in a 500 ml volumetric flask. The diluted sample is heated 2 hr on a steam bath after which 5 ml saturated mercuric chloride is added. The flask contents are cooled, diluted to volume, and filtered. A suitable aliquot of the filtrate is added to 2 ml Griess reagent and diluted to 50 ml. Color is allowed to develop 1 hr after which the absorption is read in a spectrophotometer at 520 nm. The amount of nitrite present is determined by comparing the absorption with that of a standard nitrite curve.

We have critically examined the official method and are suggesting that several modifications be made. We have also measured the effects of altering some steps in the procedure. Recently, several new methods for nitrite in meat have been proposed, and we compared these for reliability with the official AOAC method.

Addition of Mercuric Chloride

The results of nitrite analyses on frankfurters, ham, and corned beef with and without the addition of mercuric chloride are shown in Table 1. For all but one pair of samples the amount of sodium nitrite measured was greater without the addition of mercuric chloride. Statistical analysis of the data showed the differences to be significant at the 95% level with an average difference of 6.4%. Two of the values, however, accounted for half of the sum of the differences; without them the average difference is 3.7%, which is approximately the standard deviation of the method.

Mercuric chloride is used primarily as a protein precipitant (5) but has also been found by Saville (6) to cleave nitrosothiols. The latter function has been suggested by Mirna (7) as being important in nitrite analysis. However, the prolonged cooking period of the AOAC method apparently serves both purposes and the addition of mercuric chloride does not increase the amount of nitrite measured. We have occasionally observed turbidity but this can be removed by centrifugation. We do not recommend filtration because this removes color from the solution, apparently because the azo compound precipitates on the filter paper. Since mercuric chloride is corrosive, extremely toxic, and a pollutant, we recommend discontinuing its use.

Preparation of 1-Naphthylamine Solution

The official method suggests that 0.1 g 1-naphthylamine be boiled in 20 ml of water until dissolved and then poured into 150 ml 15% acetic acid. However, commercial 1-naphthylamine contains a large percentage of decomposition prod-

Table 1. Sodium nitrite values (mM) with and without addition of mercuric chloride

Sample	With HgCl ₂	Without HgCl ₂	Diff.
Frankfurters	0.58	0.66	0.08
	0.64	0.68	0.04
	2.73	3.03	0.30
	2.82	3.28	0.46
	0.52	0.58	0.06
	0.52	0.57	0.05
	2.16	2.28	0.12
	2.28	2.30	0.02
	3.45	3.36	-0.09
	3.51	3.51	0
	3.23	3.77	0.54
	3.36	3.48	0.12
	2.29	2.45	0.16
	0.33	0.35	0.02
Corned beef	0.34	0.38	0.04
	0.56	0.65	0.09
Ham	0.55	0.60	0.05
	0.66	0.72	0.06
	0.65	0.70	0.05
	0.69	0.72	0.03
	0.69	0.73	0.04
Average	1.55	1.65	0.10
% Difference		6.4	

ucts which do not dissolve in boiling water or 15% acetic acid. It is more accurate to recrystallize the compound from hot water and weigh out the purified material. Recrystallization yields nearly colorless crystals which can be dissolved directly in the 15% acetic acid. If kept air-tight and refrigerated, recrystallized 1-naphthylamine will remain stable for several months.

Dilution of Griess Reagent

In the AOAC official method the final dilution of Griess reagent is 1:25, which, versus nitrite, gives linear optical absorption up to 5 $\mu\text{g N}/50\text{ ml}$ (7.14 μM) at which concentration the concentrations of Griess reagents are 326 μM sulfanilic acid (40 \times) and 87.2 μM 1-naphthylamine (12 \times). Lunge and Lwoff (8), however, reported that at least a 100 \times concentration of Griess reagent was required for complete conversion of nitrite to chromophore. We have found that if a 1:10 dilution of Griess reagent is used, linear optical absorption versus nitrite concentration is obtained up to 16 μM nitrite (equivalent to 10 $\mu\text{g N}/50\text{ ml}$), as shown in Fig. 1. Above 16 μM nitrite the limiting factor is the development of turbidity. Our experience is that above 7–8 μM nitrite (5 $\mu\text{g N}/50\text{ ml}$), at a 1:25 dilution, there is incomplete color conversion, but as shown in Fig. 1, even below this concentration the reaction has not

gone to completion. The reaction rate at the 1:25 dilution is much slower than at the 1:10 dilution. In a timed experiment it took 80 min for the reaction to reach completion at the former dilution as compared to 25 min at the latter. Since the pink chromophore fades with time the faster reaction time is preferred.

Dilution of the Meat Sample

The initial dilution of the meat sample in the AOAC method is 1:100. To investigate the effect of degree of dilution on sodium nitrite measured, we varied the initial dilution from 1:2 to 1:1000, using commercial frankfurters. The procedure was essentially that described in the official method except that the samples diluted 1:2 through 1:20 were heated in test tubes for 2 hr in an 80°C water bath. The other samples were heated 2 hr at 80°C on a steam bath. The results are shown in Fig. 2 in which the amount of sodium nitrite found is measured against the dilution. There is apparently a curvilinear relationship between the 2 variables. A possible explanation of obtaining a curve instead of a straight, horizontal line is that an increasing ratio of water to meat results in an increasing ratio of total dissolved oxygen in the water to endogenous reductants in the meat; this results in more extensive oxidation of compounds that would later interfere in the Griess-nitrite reaction. The dilution suggested in the official method occurs in a region of the curve where the slope is greatest. Because of this, it is important to follow closely the dilution recommended by the official method since a slight deviation could significantly affect the amount of sodium nitrite measured.

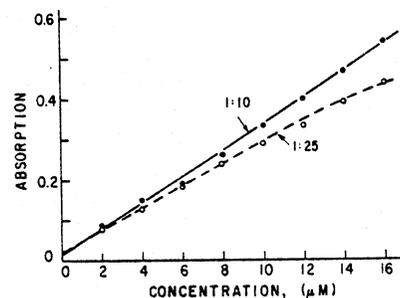


FIG. 1—Absorption at 525 nm vs. concentration of NaNO₂ at 2 dilution levels of Griess reagent, 1:10 and 1:25. Absorption was recorded at maximum color development for both dilution levels of Griess reagent.

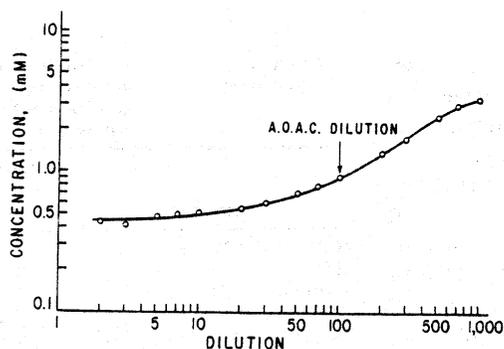


FIG. 2—Measured amount of NaNO_2 vs. dilution of meat sample.

Heating Time

The official method requires heating the diluted sample 2 hr on a steam bath. To determine the effect of length of heating time on the amount of sodium nitrite measured, we cooked samples 0.5, 1.0, 1.5, 2.0, 3.0, and 4.0 hr. The amount of sodium nitrite measured increased 16% over the period from 0.5 to 2.0 hr. Beyond 2 hr of cooking there was no real difference in measured nitrite. We do not recommend that any change be made in the required cooking time.

Effect of Added Compounds on Griess-Nitrite Reaction

There is mention in the literature of compounds that interfere in the Griess reaction (9). A number of compounds were added to Griess-nitrite solution to see whether they would affect the reaction. The compounds were added in amounts equivalent to the nitrite concentration. They were first mixed with the Griess reagent, and then the sodium nitrite was added. The compounds used were ammonium sulfate, sodium sulfide, sodium nitrate, sodium citrate, sodium thiocyanate, potassium chloride, magnesium chloride, calcium chloride, potassium permanganate, mercuric chloride, borax, sodium dithionite, urea, niacinamide, lysine, ascorbic acid, cysteine, and reduced and oxidized nicotinamide adenine dinucleotide (NADH and NAD). Ascorbic acid, cysteine, sodium dithionite, and NADH resulted in formation of less azo dye than would have been expected from the amount of sodium nitrite present. The remaining compounds showed no effect. All the compounds which interfered in the reaction are reductants. Since cured meats and meat products contain natural reductants in varying amounts and sodium erythorbate or sodium

ascorbate is added in processing, their effect on nitrite analysis must also be variable. Not until Adriaanse and Robbers (10) proposed adsorbing ascorbic acid on charcoal had the problem of reductants interfering with accurate nitrite analysis been directly investigated. We compared nitrite analyses of frankfurters after treatment of extracts with and without charcoal, according to Adriaanse and Robbers, and found no essential difference in nitrite concentrations. In terms of removing residual reductants, the AOAC method, consisting of heating 2 hr on a steam bath, is still the best available.

Griess Investigation

Griess reagent is a mixture of dilute sulfanilic acid and 1-naphthylamine separately dissolved in 15% acetic acid. In 1889, Lunge (4) suggested that the 2 compounds could be mixed together and the nitrite added to the mixture. In the nitrite method recently adopted by the European Economic Community (EEC) (P. L. Schuller, 1972), color-forming reagents are sulfanilamide and *N*-1-naphthylethylenediamine; the nitrite is added first to the sulfanilamide, and then the *N*-1-naphthylethylenediamine is added. We investigated to determine whether adding nitrite to the Griess components separately or mixed together yields different results. The mechanism of the Griess reaction is the nitrosation of sulfanilic acid to form the diazonium salt of sulfanilic acid, which then couples with 1-naphthylamine to form a brilliant pink azo compound. Figure 3 shows the 2 possible first-step reactions: (1) the nitrosation of sulfanilic acid, and (2) the nitrosation of 1-naphthylamine. The next step is a coupling of the diazonium ion with 1-naphthylamine to form either 1-sulfanylazophthylamine or 1-naphthylazonaphthylamine. The first is a pink dye with an absorption maximum at 525 nm; the second is insoluble in water. Since either sulfanilic acid or 1-naphthylamine may react with nitrite

NITROSATION OF SULFANILIC ACID AND 1-NAPHTHYLAMINE

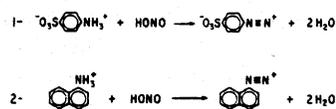


FIG. 3—Nitrosation of sulfanilic acid and 1-naphthylamine.

in the mixture, the relative amounts of intermediates, and therefore end products, depend on the relative reaction rates and concentration. If reaction (2) were of consequence with respect to (1), the amount of pink color would be reduced, that is, reaction (2) would interfere with normal color development. Such interference would be reduced by pre-incubation of sulfanilic acid with nitrite followed by addition of 1-naphthylamine. Nitrite was added to sulfanilic acid and the mixture was allowed to stand 20 min before 1-naphthylamine was added. The resulting amount of azo dye was the same as that formed when nitrite was added to a mixture of sulfanilic acid and 1-naphthylamine. It is clear that the rate of reaction (1) is faster than reaction (2) under conditions of Griess reagent analysis.

The use of more concentrated acetic acid and substitution of sulfuric and hydrochloric acids for acetic acid were investigated. Nitrite solution and Griess reagent were also reacted at 0°C instead of the usual room temperature. None of these altered conditions affected the amount of azo compound formed.

Comparison of Methods for Nitrite in Meat

A study was made of several recently proposed procedures for sample preparation for nitrite analysis. The procedures compared include the method adopted by the EEC and methods proposed by Kamm *et al.* (11), Mirna (7), and Adriaanse and Robbers (10). The official method was used as the standard for comparison. We were interested only in comparing the procedures for sample preparation from the above methods. We used Griess reagent for the color reaction for all the methods except that of Kamm *et al.* We considered their use of 1-naphthylamine alone to be an essential part of the method and did not substitute Griess reagent. Six replicate samples of ham and frankfurter were used for each method. The standard deviations for the methods and a typical set of sodium nitrite values are shown in Table 2. Although the standard deviations are comparable, the AOAC method always gave the highest sodium nitrite values. If, as our studies and the results of Adriaanse and Robbers indicate, the interference with the Griess reaction is due to residual reductants in meat, it may be con-

Table 2. Comparison of sodium nitrite values in frankfurters as measured by several methods for sample preparation with their standard deviations

Method	NaNO ₂ Values, mM	Std dev.
AOAC 24.014-24.015	0.28	0.00505
EEC	0.20	0.00142
Kamm <i>et al.</i> (11)	0.11	0.00307
Mirna (7)	0.19	0.00265
Adriaanse and Robbers (10)	0.20	0.00243

cluded that the long digestion period at higher temperatures of the AOAC method provides more complete oxidation. Since none of the procedures of the other methods tested, with the exception of the Adriaanse and Robbers method, seem to be addressed to the removal of reductants, in view of our comparison study, we find nothing to recommend any of the other methods over the AOAC method.

In summary we suggest (1) that the addition of mercuric chloride be discontinued, (2) that 1-naphthylamine be recrystallized before weighing, (3) that the Griess reagent be diluted 1:10 in use, and (4) at the 1:10 dilution level, that 25 min instead of 1 hr be allowed for color development.

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