

MEAT AND MEAT PRODUCTS

Interaction Between Sample Preparation Techniques and Three Methods of Nitrite Determination

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Meat samples containing nitrite and varying concentrations of ascorbate, cysteine, and sodium chloride were prepared for nitrite analysis. The methods used were the AOAC method of dilution and heating; the addition of mercuric chloride, charcoal, and Carrez reagents at 2 different pH values; and direct analysis of sample supernatants with no treatment (control). The effect of these initial conditions and preparation methods on measured nitrite was determined by using 3 different Griess reagent combinations and chemiluminescent and differential pulse polarographic techniques. Systematic variations were observed in samples treated with mercuric chloride, while the addition of Carrez reagents had little or no effect. Best results were obtained by the AOAC dilution/heating method under alkaline conditions, or by charcoal addition followed by chemiluminescent or colorimetric nitrite determination. Statistical analysis of the nitrite concentrations determined in the several samples showed that these 3 procedures were precise to about 5-6% CV, which was not significantly different from the CV value of 4% determined from replicate analyses.

Sample preparation methods for the determination of nitrite involve a number of processes and reagents designed to clarify the solutions for colorimetric analysis and to improve the yield of nitrite by either cleaving endogenous nitroso compounds (1, 2) or eliminating interferences (3, 4). Common clarifying agents are borate (borax) (1, 2, 5-8), mercuric chloride (1, 2, 6-9), Carrez reagents (1-3, 5-8, 10, 11), iron (Fe^{+3}) (12, 13), and/or aluminum (Al^{+3}) (14-17), all of which are commonly used to precipitate proteins. Mercuric chloride also has been claimed to cleave nitrosothiols, presumed form of the bound nitrite in meats (1, 2). Other procedures included alkalization (1-3, 12-17), dilution of the sample to various levels (1-3, 7, 10, 13, 14, 16-19), and heating (1, 2, 5, 7-11, 13-16, 18, 19).

Evaluations of these methods have always been carried out as entire procedures, without assessments of the effect of the individual steps

on either clarification or total nitrite, and seldom as direct comparison with others. Some of the methods involved as many as 4 different reagents and several processing steps, but it is not readily evident that they are all necessary. Furthermore, excessive handling of nitrite samples is not good procedure because nitrite is readily lost through reduction and/or oxidation reactions with endogenous compounds and with oxygen in the air during the preparation procedure. If nitrite is in fact bound to proteins, the use of protein precipitants could lead to a loss of nitrite, depending on the point at which they are used in the preparation procedure.

A systematic comparison of sample preparation methods for nitrite analysis therefore requires a direct comparison of the effect of each reagent or procedure on the amount of measurable nitrite in the sample. In an earlier study of nitrite in frankfurters (20), we used the criteria of maximal nitrite recovery and elimination of turbidity. In that study, the AOAC official first action method (18), which specifies diluting and heating the sample, gave the best results. Recently, we have shown that residual ascorbate has a differential effect on pigment production from different Griess colorimetric reagent combinations (21) and that this effect may be used to determine the effectiveness of sample preparation procedures (4). Using this criterion, it was found that the AOAC method was not as effective as mercuric chloride addition.

We have continued these studies to include other sample preparation methods and nitrite-measuring techniques to determine if there are more effective procedures and/or reagents, and to establish a criterion of accuracy by which to gauge their effectiveness.

Experimental

Reagents and Substrate

All reagents were reagent grade or highest purity obtainable. Darco G-60 activated carbon, tested to be free of nitrite, was used. The meat

substrate was a 1:2 slurry of pork skeletal muscle and water prepared by first blending an equal weight of pork and 6mM nitrite solution sparged with nitrogen. This slurry was then divided into 4 portions, and to each was added $\frac{1}{2}$ volume of water containing either 0, 9, or 36mM ascorbate or 60mM cysteine. Each portion, containing 2mM ascorbate or 20mM cysteine, was then divided again and each half was made 1M in sodium chloride by the addition of weighed portions of salt. The samples were then heated 1 h at 70°C to react the nitrite with the tissue components and the added reductants. The resulting curd was reblended under nitrogen. The pH of the final slurry was 5.53.

Sample Preparation Techniques

Direct Analysis.—A portion of the slurry was centrifuged and measured volumes of the clear supernate were withdrawn with a syringe pipet for nitrite determination.

Mercuric chloride addition.—Samples were prepared by adding 0.2 mL saturated HgCl_2 to 2.0 mL slurry. After 30 min, the slurries were centrifuged and portions of the supernate were withdrawn for nitrite measurement. The precipitates were resuspended and the samples were heated $\frac{1}{2}$ h at 60°C, cooled, and centrifuged. The supernate was again analyzed for nitrite.

For the AOAC preparation, 1 mL slurry was measured by a positive displacement into a 100 mL volumetric flask, diluted to 80 mL, and heated 2 h at 80°C. The flasks were cooled, and the solutions were diluted to 100 mL and filtered through Whatman No. 2 paper which had been washed free of nitrite. The filtrates were analyzed for nitrite.

Carrez reagents addition.—Exactly 0.1 mL Carrez I (0.3M $\text{K}_4\text{Fe}(\text{CN})_6$) and 0.1 mL Carrez II (1.0M ZnSO_4) were added successively to 2.0 mL slurry. After $\frac{1}{2}$ h, the slurries were centrifuged and the supernates were analyzed for nitrite.

Charcoal treatment was performed by diluting 1 mL slurry to 10 mL and adding 0.1 g charcoal. The samples were shaken $\frac{1}{2}$ h and centrifuged, and the supernates were analyzed for nitrite.

Analysis under alkaline conditions.—The effect of alkaline conditions on the mercuric chloride, AOAC, and charcoal preparations was determined by adjusting portions of the slurry to between pH 10.0 and 10.3, which was the pH of most alkaline extractions. The various procedures were performed as before, maintaining the pH as necessary. Because the samples were not buffered, the pH was below 3.0 after addition of

the Griess reagents, but within the range of maximal pigment formation (22).

Saturated sodium borate (borax) and Carrez reagents.—Saturated $\text{Na}_2\text{B}_4\text{O}_7$, Carrez I ($\text{K}_4\text{Fe}(\text{CN})_6$), Carrez II (ZnSO_4), and the last 2 together were tested for their effect on the Griess reagents with and without 3mM ascorbate, by adding 0.1 mL of each reagent to 1 mL Griess reagent and 0.1 mL 2mM nitrite, and diluting the solution to 10 mL.

Nitrite Determinations

For Griess colorimetric analysis, a suitable portion of the clear supernate or filtrate was added to 1.0 mL Griess reagents in a 10 mL volumetric flask and diluted to 10 mL. The reagent combinations were sulfanilic acid-*N*-(1-naphthyl)ethylenediamine (SAA), sulfanilamide-1-naphthylamine¹ (SAN), and sulfanilamide-1,7-Cleve's acid (8 amino-2-naphthalene sulfonic acid) (1,7-C). The latter was used to test 1,7-Cleve's acid as a substitute for 1-naphthylamine which has been classified a Class I carcinogen (23). After $\frac{1}{2}$ to 1 h, absorbances were measured in a Cary 14 spectrophotometer.

Nitrite concentrations were also determined by differential pulse polarography (DPP) according to the technique of Chang et al. (24) and by a chemiluminescent technique developed in this laboratory (25). The former method was chosen because it is sensitive to residual ascorbate (24), and the latter because it uses large excesses of ascorbate to generate nitric oxide from nitrite for chemiluminescent detection (CLD) and would therefore be assumed to override the effect of residual ascorbate.

Nitrite content of the various sample preparations was determined by all 3 techniques.

Results

van Eck (26) found that borate slowed the Griess reaction, which, in view of the complex nature of the reaction, might be expected to result in different amounts of pigment formed from different Griess reagent combinations. Preliminary experiments confirmed van Eck's observation on rates, but did not show any reagent combination differences in the final nitrite concentrations. Therefore, we did not investigate the use of borate further, particularly since we had previously found that the reagent had no effect except to increase the pH of the solutions (20).

The addition of Carrez I alone eliminated

¹ 1-Naphthylamine has been classified as a toxic and hazardous substance (23).

Table 1. Effects of NaCl, reductant, sample preparation methods, and Griess reagent on measured nitrite (mM NO₂) in pork samples heated 1 h at 70 °C ([NO₂] = initial 2.0mM)

NaCl (1) ^a	Reductant (2)	Reagent ^b (3)	Direct		HgCl ₂			AOAC		Carrez (11)	Charcoal		
			Day 1 (4)	Day 2 (5)	H ⁺ ^c (6)	Δ (7)	OH ^{-d} (8)	H ⁺ (9)	OH ⁻ (10)		H ⁺ (12)	OH ⁻ (13)	
0	0	SAA	1.36	1.35	1.27	1.09	1.25	1.63	1.59	1.38	1.36	1.45	
		1,7-C	1.31	1.33	1.16	1.05	1.53	1.58	1.54	1.29	1.43	1.54	
		SAN	1.41	1.40	1.24	1.09	1.41	1.62	1.57	1.37	1.47	1.53	
	3mM	SAA	1.53	1.47	1.18	0.89	1.09	1.66	1.54	1.47	1.46	1.26	
		Asc.	1,7-C	0.50	0.50	1.07	0.80	1.31	0.88	1.24	0.58	1.39	1.36
		SAN	0.86	0.84	1.14	0.79	1.25	1.12	1.38	0.87	1.38	1.36	
	12mM	SAA	0.78	0.76	1.02	0.36	0.90	1.01	1.35	0.78	1.06	1.04	
		Asc.	1,7-C	0.12	0.12	0.94	0.23	1.08	0.26	0.96	0.14	1.03	1.16
		SAN	0.28	0.28	0.98	0.25	0.98	0.48	1.10	0.27	1.02	1.16	
	20mM	SAA	0.86	0.91	1.10	0.61	1.02	1.00	1.35	0.83	1.13	1.17	
		Cys.	1,7-C	0.90	0.92	0.99	0.56	1.22	1.06	1.31	0.76	1.12	1.22
		SAN	1.14	1.01	1.07	0.58	1.12	1.13	1.35	0.82	1.13	1.32	
1M	0	SAA	1.39	1.47	1.68	1.63	1.21	1.47	1.68	1.25	1.21	1.43	
		1,7-C	1.24	1.16	1.45	1.50	1.45	1.40	1.45	1.22	1.19	1.37	
		SAN	1.34	1.25	1.50	1.51	1.43	1.44	1.46	1.24	1.16	1.45	
	3mM	SAA	0.97	1.00	1.03	0.79	0.68	1.01	1.11	0.88	0.86	0.81	
		Asc.	1,7-C	0.44	0.38	0.78	0.72	0.83	0.66	0.91	0.42	0.85	0.83
		SAN	0.58	0.65	0.87	0.73	0.83	0.76	0.91	0.59	0.82	0.83	
	12mM	SAA	0.56	0.51	0.83	0.35	0.50	0.67	0.69	0.49	0.67	0.51	
		Asc.	1,7-C	0.11	0.10	0.31	0.22	0.58	0.18	0.60	0.09	0.64	0.58
		SAN	0.22	0.19	0.45	0.19	0.58	0.33	0.64	0.18	0.60	0.59	
	20mM	SAA	0.93	1.00	1.01	0.59	0.89	0.95	1.12	0.82	0.91	0.90	
		Cys.	1,7-C	0.80	0.76	0.91	0.46	1.04	0.89	1.00	0.74	0.86	1.00
		SAN	0.86	0.84	0.92	0.48	0.97	0.91	1.01	0.77	0.85	0.96	

^a Column number.

^b SAA = reagent combination of sulfanilic acid and *N*-(1-naphthyl)ethylenediamine. 1,7-C = reagent combination of sulfanilamide and 1,7-Cleve's acid. SAN = reagent combination of sulfanilamide and 1-naphthylamine.

^c Acidic samples, pH 5.53.

^d Alkaline samples, pH 10.

pigment production from all 3 reagent combinations regardless of the presence of ascorbate or cysteine. Ferrous ions interfere in Griess pigment formation (27) through reaction with the intermediate diazonium ion. This effect was reversed by adding Carrez II which precipitated Zn₂Fe(CN)₆, but we suspected that complete reversal of Carrez I inhibition by Carrez II would not occur in meats if sufficient zinc were removed from the system by protein precipitation. Since ferrocyanide is thus contraindicated, we would not have included it in further studies except that it is used in several published methods (3, 5, 8, 11).

The results of the Griess colorimetric analyses of the samples are given in Table 1. The data in columns 4 and 5 are replicates run on 2 different days. The pooled estimate of the standard deviation between replicates was 0.038 (\bar{X} = 0.848, CV = 4.4%). For assessing the results of this study, a given value or average will be considered outside the normal population if it is 4.56 σ (± 0.17) or more than the average (28). There was a 30% loss of nitrite in the samples with no added

ascorbate or cysteine (first row, Table 1) due to reaction with endogenous compounds, but they had no residual effect on the Griess reaction because all 3 reagents gave the same nitrite concentration. In the samples containing salt, SAA values are higher than are 1,7-C or SAN values due to enhancement of SAA-NED pigment production by chloride (29). With added ascorbate, the effects of enhancement of pigment production with SAA and decreased pigment formation with SAN are observed (4). The interference of ascorbate in pigment production is greater with 1,7-Cleve's acid and is due to the slower coupling rate of the acid with the sulfanilamide diazonium ion as compared with NED or 1-NA (21). Because the interference by ascorbate is due to reduction of the diazonium ion (21), slower coupling reagents produce less pigment in competition with a reductant. Cysteine caused a loss of nitrite, but did not affect the Griess reaction because the difference in measured nitrite values (0.074) is not significantly greater than the expected population variation (± 0.038).

Table 2. Effects of NaCl, reductant, and sample preparation methods on nitrite measured (mM NO₂) in cured meat slurries by Griess, CLD, and DPP methods

NaCl (1) ^a	Reductant (2)	Method ^b (3)	Preparation method					
			Direct (4)	AOAC (5)	AOAC(OH ⁻) (6)	Charcoal (7)	HgCl ₂ (8)	Carrez (9)
0	0	Griess	1.41	1.62	1.59	1.46	1.24	1.37
		CLD	1.52	1.52		1.51	1.07	1.16
		DPP	1.00	1.64		1.59	1.06	1.13
	3mM Asc.	Griess	0.86	1.12	1.38	1.34	1.14	0.87
		CLD	1.44	1.36		1.32	1.00	1.07
		DPP	0.63	1.00		1.30	0.94	0.56
	12mM Asc.	Griess	0.28	0.48	1.10	1.11	0.98	0.27
		CLD	1.02	1.03		1.19	0.73	0.66
		DPP	0.17	0.40		1.16	0.79	0.23
	20mM Cys.	Griess	1.14	1.13	1.17	1.16	1.07	0.82
		CLD	1.13	1.15		1.17	0.80	0.44
		DPP	0.58	1.13		1.01	0.64	0.57
1M	0	Griess	1.34	1.54	1.46	1.48	1.50	1.38
		CLD	1.48	1.59		1.45	1.28	1.13
		DPP	1.27	1.51		1.45	1.23	1.21
	3mM Asc.	Griess	0.58	0.51	0.91	0.93	0.87	0.56
		CLD	0.96	0.93		0.90	0.67	0.67
		DPP	0.52	0.16		0.87	0.64	0.52
	12mM Asc.	Griess	0.22	0.14	0.65	0.65	0.45	0.20
		CLD	0.60	0.65		0.64	0.22	0.35
		DPP	0.02	0.06		0.58	0.30	0.00
	20mM Cys.	Griess	0.86	1.09	1.01	1.00	0.92	0.69
		CLD	0.96	1.00		0.99	0.44	0.30
		DPP	0.20	0.87		0.87	0.46	0.19

^a Column number.

^b CLD = chemiluminescent detection. DPP = differential pulse polarography.

Mercuric Chloride

While the addition of mercuric chloride was largely effective in eliminating the ascorbate effect as judged by the comparable nitrite concentrations measured by the 3 colorimetric reagents, a detailed examination of the data in Tables 1 and 2 raises a question as to the efficacy of the use of HgCl₂. Table 1 shows that the amount of measured nitrite for the 3 Griess reagents is in the order SAA > SAN > 1,7-C in the acid-Hg samples (column 6), and 1,7-C > SAN > SAA for the alkaline Hg samples (column 8). This order was common to all samples and is significant at the *P* = 0.001 level. The order in the acidic (H⁺) samples is the same as that for residual ascorbate, suggesting that mercuric ion is not totally effective in removing residual ascorbate.

The samples were heated and made alkaline, to determine if the observations of Olsman and van Leeuwen (1) on the release of protein-bound nitrite were applicable to whole meat systems. They found that adding HgCl₂ before heating and keeping the pH between 5 and 6 resulted in higher yields of nitrite. Our results show that heating whole meat samples at the lower pH results in overall nitrite loss, while making the

samples alkaline results only in reversing the order of pigment yields with the 3 reagent combinations. Although the release of protein-bound nitrite may be a factor in nitrite analysis of cured meats, it is evident that there are other factors involved that are of greater importance. Mercuric ion interfered in the CLD and DPP measurements (Table 2, column 8). The measured nitrite values for these 2 methods were consistently, significantly, and appreciably lower than were the Griess values. The most consistent argument for the lowering is a mercuric ion interference in the nitrosation reaction which is the first step in the sequences involved in all 3 methods of measurement.

Carrez Reagents

The addition of Carrez reagents did not result in any change over direct measurement in the amounts of nitrite measured by the Griess reagents (Table 1) or by DPP (Table 2). They also interfered with the CLD measurement of nitrite in that the Carrez-CLD values were about 2/3 of the direct measurement values (compare columns 4 and 9, Table 2) with the no added reductant and ascorbate samples, and about one

half with the cysteine samples. To the extent that neither the CLD nor the DPP method requires solution clarification, the finding is superfluous except that it does indicate an interference in nitrosation reactions that probably also takes place in the Griess reaction.

AOAC Method

Use of the AOAC method resulted in an increase in measured nitrite in the meat samples with no reductant but was only partially effective in eliminating ascorbate interference because the residual reductant effect on the different Griess reagents was still observed. Making the samples alkaline almost completely eliminated the residual ascorbate effect (Table 1, column 10) and gave high nitrite values (Table 1, column 10; Table 2, column 6). A pH of 8 has been effective in elimination of ascorbate interference (30, 31) and it is apparent that pH values of 6 or above in the dilute AOAC solutions during heating are effective (32). The effect is attributed to the oxidation of ascorbate at a pH at which nitrite is essentially unreactive (30).

Charcoal Preparation

Of the preparation techniques tested, charcoal gave the most uniform results, both for the 3 Griess reagent combinations (Table 1, columns 12 and 13) and for the 3 different measurement methods (Table 2, column 7). The measured nitrite values for any given reductant level were the same and the pooled σ value for both the H^+ and OH^- charcoal data in Table 1 was 0.0734, indicating no residual ascorbate or cysteine effect. The values so obtained were also the highest for any preparation method tested (Tables 1 and 2).

Chemiluminescent and Polarographic Detection

The nitrite concentrations measured by CLD were the highest and most uniform of any of the measurement methods for the direct, AOAC, and charcoal-treated samples. As previously noted, the addition of $HgCl_2$ or Carrez reagents interfered in the CLD determination. In contrast, the DPP measurements gave low values with added reductants, with an even greater interference by ascorbate in the DPP measurement than was observed in the Griess measurement. Furthermore, there was an interference by sulfhydryl groups in the DPP measurements (direct reading), which was removed by using either the AOAC method or charcoal addition. In view of

the sensitivity to interference, the slowness of the procedure, and the inferior results, differential pulse polarography does not recommend itself as a nitrite measurement method.

Discussion

Nitrite Measurement

Because nitrite reacts with the substrate to which it is added, particularly meat products, there can be no absolute value by which to judge the effectiveness of any preparation procedure or nitrite measurement method. Nevertheless, it is only in a system where nitrite has had an opportunity to react that the effectiveness of any procedure can be assessed. Under these circumstances, effectiveness may be gauged by a consensus of those techniques, which by practical experience, on theoretical grounds, or both, give the highest and most consistent yields. Applying these criteria to the present study, the relevant sample preparation and/or nitrite measurement methods are charcoal addition, chemiluminescent detection, and, to a lesser degree, the AOAC method under alkaline conditions. The mean values for the charcoal-treated samples of Table 1 and the CLD, charcoal, and AOAC alkaline method data of Table 2 are listed in Table 3. It is evident that the 2 sets of data are equivalent. The data for 1M NaCl/O reductant do differ significantly, but on examination of the data in Table 1, there appears to be

Table 3. Mean values for nitrite measured (mM NO_2^-) by different sample preparation procedures and nitrite measurement methods

NaCl	Reduc-tant	Charcoal/Griess Table 1 $n = 6$	CLD, ^a Charcoal, AOAC (OH^-), Table 2 $n = 6$
0	0	1.46 ± 0.067	1.52 ± 0.046
0	3mM Asc.	1.37 ± 0.065	1.35 ± 0.054
0	12mM Asc.	1.08 ± 0.065	1.08 ± 0.10
0	20mM Cys.	1.18 ± 0.077	1.12 ± 0.065
1M	0	1.30 ± 0.13	1.47 ± 0.017
1M	3mM Asc.	0.83 ± 0.018	0.92 ± 0.038
1M	12mM Asc.	0.60 ± 0.055	0.62 ± 0.033
1M	20mM Cys.	0.91 ± 0.058	0.96 ± 0.059
Spooled CV, %		0.073 6.7	0.059 5.2

^a Excluding the $HgCl_2$ and Carrez values.

a systematic error in the acidic charcoal samples. The pooled standard deviations in both sets are less than twice the expected value from replicate analysis (0.038) and we conclude that both sets of data are for the same population, and that the criterion of uniformity is valid. The criterion of maximal yield is also met with respect to ascorbate for the charcoal and AOAC alkaline methods, since they gave the same measured nitrite as did the CLD method. Because the latter generates NO with excess ascorbate, which is a more powerful reductant than sulfhydryl groups, the criterion also applies to the cysteine interference. The results indicate, therefore, that uniform and maximal yield of measured nitrite in meat systems may be obtained colorimetrically with the use of either charcoal or the AOAC alkaline procedure, or by chemiluminescent detection of nitric oxide produced from nitrite by reduction with ascorbic acid. We found that the use of mercuric ion or Carrez reagents is not advisable, either as a preparation procedure or as a step therein.

Removal of Turbidities

All of the referenced methods (1-3, 5-18) filter the samples to remove precipitates generated by the added reagents or procedures. In this study, we used centrifugation to remove precipitates. On occasion, especially in samples containing mercuric chloride, we found that turbidities that were not removable by filtration were readily cleared by centrifugation. As a result, when we tested for turbidity in the samples, we found that it was a negligible factor.

We recommend that the use of charcoal and the AOAC alkaline procedures as sample preparation methods be investigated in a collaborative study, with the objective of replacing the present AOAC method. Such a study should, if possible, include an evaluation of the effectiveness of centrifugation and filtration in removing turbidities from samples before colorimetric analysis. While the use of chemiluminescent determination of nitrite does not appear to be a suitable standard method now because of the time and equipment involved, it may be used as a reference method by which to judge standard methods. Further studies on reagent additions and/or preparation techniques should be strictly confined to the demonstration, by direct comparisons, of the effectiveness, utility, and necessity for the reagent or technique.

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