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THE DETERMINATION OF NITRITE: A CRITICAL REVIEW*

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* Abbreviations: PNA = para-nitroaniline; SAN = sulfanilamide; PABA = para-aminobenzoic acid; SAA = sulfanilic acid; MAA = metanilic acid; PCA = para-chloroaniline; ANI = aniline; NED = N-(1-naphthyl) ethylenediamine; 1-NA = 1-naphthylamine.

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I. INTRODUCTION

The cause of both the joys and sorrows of nitrite determination lies in its extreme reactivity. The joy is that it is readily reacted with a wide variety of compounds to form distinctive and unique derivatives that are easily measured. The form in which nitrite is most commonly determined, a diazo dye, is absolutely specific for nitrite, for no other compound will form diazo pigments. The sorrow is that because nitrite is so reactive, sample clean-up can become a very complex problem of removing compounds that compete for nitrite with the very reagents used to determine it. The substrates in which nitrite is most commonly determined are cured meats, which are collections of nitrite-reactive substances. The aim of sample preparation procedures for cured meats is to rid the system of nitrite reactants without losing nitrite in the process. Put in this manner, it would appear that the only critical part of the procedure is sample preparation, not the determination step. Such is not the case. Nitrite does not react as the nitrite ion, but as the protonated form, nitrous acid. The first step of any reaction to make a nitrite derivative for measurement is the formation of a nitrosating species, usually an anhydride, from nitrous acid. The problem in this step is that nitrous acid undergoes a number of other reactions, including dismutation reactions that produce nonreactive or gaseous compounds. Even after the nitrosation reaction, the intermediate compounds produced are reactive. In the dye formation reaction, the first compound formed is a highly reactive diazonium ion which then couples with a second molecule to form a pigment. The diazonium ion is an electrophilic reagent, but so then are the original nitrosating species formed from nitrous acid. Anything to which the diazonium ion will couple will also react with nitrous acid. Nitrous acid will react with reductants, but the diazonium ion reacts faster.

In view of the many complexities involved, it is surprising that nitrite can be determined with any degree of precision or accuracy at all, and in a certain sense, it can not. Nitrite determination methods proliferate in the literature, sometimes with modifications so specific that it is difficult to decide *a priori* why they were made. Some techniques are relatively simple; some very complicated with many steps and many reagents. The intent of this review is to examine the causes of the problems involved in nitrite determination and evaluate the several methods of both sample preparation and nitrite determination with respect to their efficacy in solving the problems.

II. NITRITE CHEMISTRY

As previously noted, the chemistry of nitrite is really that of nitrous acid, but it has become customary to speak or write of "nitrite determination" for it is as the anion that the compound exists in nature. The pK_a of nitrous acid is 3.4, hence at pH values above 5 the anion comprises >98% of the total. Even so, all reactions that are observed in food products are due to the small amount of nitrous acid present. Because it is general usage, I shall use the terms "nitrite" and "nitrite determination" in the general sense, and refer specifically to nitrous acid when discussing reactions where the latter is the active species.

The determination of nitrite involves at least two and generally three or more reaction steps, but the first two steps for most determinations are the formation of a diazotizing species, followed by a diazotization reaction. Methods which determine nitrite as nitric oxide do not depend on a diazotization reaction, but the initial step still involves the formation of a diazotizing species. The reactions that will produce diazotizing species are summarized in Figure 1, as well as reactions that produce unreactive species.

The rate of disappearance of nitrous acid from the solutions is the sum of the rates of disappearance in each reaction, which in turn are equal to the rate constants times the concentrations of the reactants raised to the power of their molecularity in the reaction. The overall rate is that given by reaction 1.7. Where the reaction is reversible, a corresponding positive term must be added to the equation.

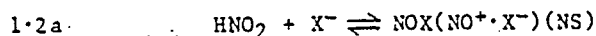
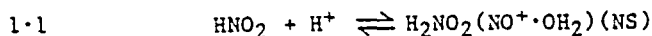
The reaction of nitrous acid is therefore not homogeneous and from Figure 1 we may deduce that the following factors govern what types of intermediates and/or products are formed and their concentrations:

1. The acidity of the solution
2. The presence of other anions
3. The concentration of nitrite
4. The temperature of the reaction solution

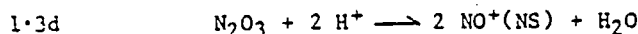
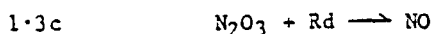
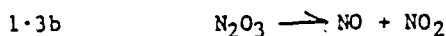
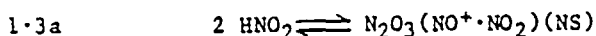
The first two factors are readily apparent. Factor 3 is the result of kinetics. The rate of a reaction is proportional to the concentration of each reactant raised to the power of the molecularity of the reactant. The most common reactions are numbers 1.1, 1.2a, 1.3a, and 1.4 representing three different orders of molecularity. Reactions 1.1, 1.2a, and 1.3a both produce nitrosating species (NS), but reaction 1.3a produces only half as much NS as do reactions 1.1 and 1.2a. Reaction 1.4 produces only nitric oxide, which is a nitrosating species only under certain conditions. Let us imagine a set of conditions where, with a given concentration of nitrous acid, the rate of product formation from the three reactions is 1:1:1. If the concentration of nitrous acid is increased tenfold the ratio of the initial rates becomes 1:100:1000. After a given period of time, the relative amounts of reactive and unreactive species will be quite different at the two concentrations, and the relative amounts of products formed will be quite different also. This kind of situation has been noted in practice for varying levels of nitrite. In nitrite determination at high nitrite levels, only yellow pigments are formed¹ and clearly the same set of reactions are not taking place as those that occur at lower levels of nitrite.

A similar situation prevails with regard to temperature. If the energies of activation are not the same, even for reactions of the same order, a change in temperature changes the relative amounts of nitrosating and unreactive species formed. In addition to the variations in product concentrations induced by the foregoing factors, the reactive species formed are not equivalent with respect to their nitrosating reactivity, the order being roughly $H_2NO_2^+ > NOX > N_2O_3$. N_2O_3 is not only a nitrosating species, but will decompose to yield nitric oxide (reaction 1.3b) which is a weaker nitrosating compound. Reductants, after having

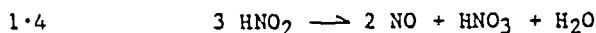
Monomolecular in nitrous acid



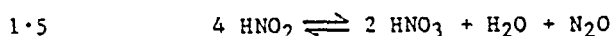
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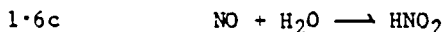
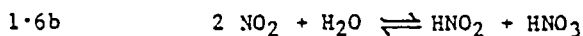
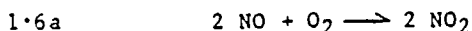
Termolecular



Quadrimeric



Reformation of nitrous acid



Rate of nitrite disappearance

$$1.7 \quad \frac{d\text{HNO}_2}{dt} = -k_1 [\text{HNO}_2] - k_2 [\text{HNO}_2] [\text{X}] - k_2 [\text{HNO}_2]^2 - k_3 [\text{HNO}_2]^3 - k_4 [\text{HNO}_2]^4$$

FIGURE 1. First step nitrous acid reactions. (NS), nitrosating species X^- , anions, Cl^- , Br^- , SCN^- , OAc^- , etc. Rd, reductants. Where a reaction is shown as reversible, the rate expression 1.7 should also contain the reverse rate term as a positive factor.

been nitrosated, can also produce nitric oxide (reactions 1.2b, 1.3c) and hence do not totally eliminate the nitrosating potential of the system.

The foregoing reactions have been fairly well characterized and further discussions of

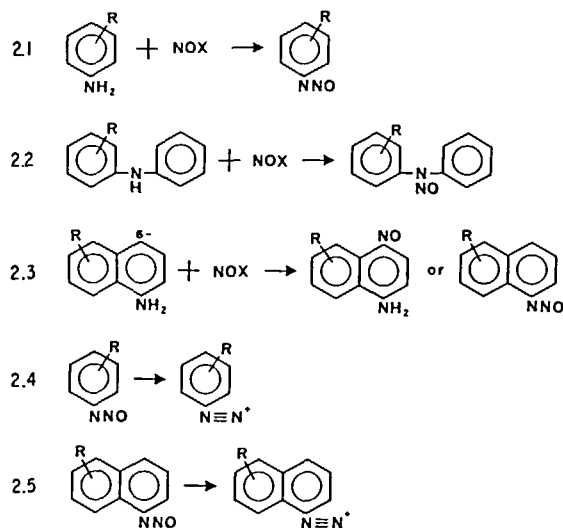


FIGURE 2. Second step nitrosation and diazonium salt formation.
 R: ring substituents, $-\text{SO}_3\text{H}$, $-\text{SO}_2\text{NH}_2$, $-\text{Cl}$, $-\text{COOH}$, $-\text{NO}_2$,
 etc. X: Cl, Br, NO_3 , .

them will be found elsewhere.²⁻⁴ The termolecular reaction 1.4 is one of the seven or eight established three-molecule reactions. Catalyzed reactions frequently have more molecules involved, but most of them are bound together or react sequentially, whereas reaction 1.4 represents a three-molecule collision in a Brownian system. The quadrimolecular reaction, 1.5, was given by Schwarz-Bergkamp⁵ to explain the formation of N_2O , but the reaction does not seem to be a likely one. Nitric oxide is readily oxidized to nitrogen dioxide which in turn hydrates and dismutates in water to form nitric and nitrous acids,⁶ reactions 1.6a and b. It is this cycle that explains why the reduction of nitrous acid results in the eventual conversion of nitrite to nitrate and probably accounts in part for the antioxidant effect of nitrite. However, Hardy et al.⁷ noted that nitrite would oxidize a large molar excess of ascorbate with but very little nitrite loss and proposed a one-electron oxidation of nitric oxide back to nitrous acid, reaction 1.6c.

It is not sufficient to the complexity of the system, however, that the formation of the nitrosating species is a complicated proceeding, but the subsequent steps are equally so. Where nitrosation occurs, the rate of the reaction is not only a function of the nitrosating species, but also of the compound being nitrosated.⁸ These two-step reactions are shown in Figure 2, reactions 2.1 to 2.5. Aromatic amines (aniline and its substituted derivatives) are readily nitrosated (reaction 2.1), and under normal conditions undergo an internal rearrangement to form diazonium salts, reaction 2.4, as do *N*-nitrosanaphthylamines, reaction 2.5. Since the rate at which the internal rearrangement takes place is a function of the ring substituent, the overall rate for diazonium ion formation is a complex function of both the reactivity of the nitrosating species and the nitrosated compound in the nitrosation reaction, and the reactivity of the *N*-nitroso derivative in the rearrangement reaction.⁸

To form the pigment, the diazonium ion reacts with an electron-rich site on an aromatic nucleus (coupling reagent) to form a diazo dye. The coupling reagents themselves (usually substituted 1-naphthylamines) are attacked by nitrosating species (reaction 2.3). Both the nitrosating species and the diazonium salts are electrophilic reagents, hence anything with which the latter will react, the former will also. The electron-rich site of electrophilic attack on the naphthalene nucleus is the carbon in the position para to the ring substituent, the latter being typically an electron donor group of the positive induction type. If the latter is

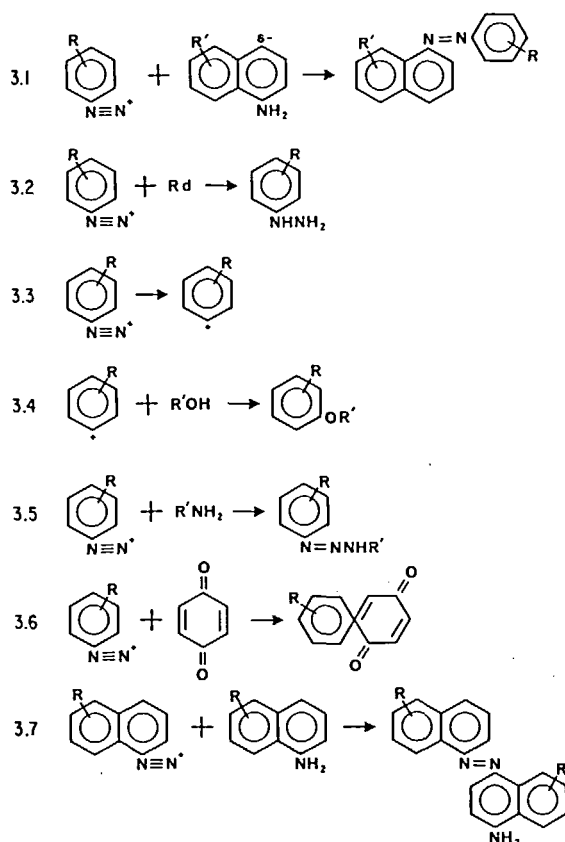


FIGURE 3. Fourth step diazotization and coupling reactions. R: ring substituents, -SO₃H, -SO₂NH₂, -Cl, -COOH, -NO₂, etc. X: Cl, Br, NO₂, OH₂⁺.

an especially strong electron donor, for example, the ethylene diamine group, the reactivity of the coupling reagent towards the nitrosating species approaches that of the aniline derivative, and loss of both NOX and coupling reagent reduces the amount of pigment produced.

The most common reason for the diazonium ion formation is the formation of a diazo dye but the coupling of a diazonium ion with a coupling reagent is also a complex reaction with a number of side reactions that do not lead to chromophore formation (Figure 3). Reaction 3.1 is the desired coupling reaction that produces the diazo dye, but coupling is not necessarily limited to chromogenic reagents.^{9,10} Amines, both aliphatic and aromatic, may couple with the diazonium ion (reaction 3.5) to produce diazoamino derivatives. With aromatic compounds, coupling may occur on the ring or with other groups on the ring such as the hydroxyl of phenolic compounds to produce colorless or different-colored compounds. Since the nitrosated species are aromatic compounds, the diazonium ion can react with an unnitrosated molecule of itself to form either colored or uncolored products. Other possible coupling reagents include conjugated aliphatic dienes, pyrroles, and indoles.

A second type of reaction may occur with diazonium ions in which the diazo group is lost. The simplest reaction is 3.3 and 3.4 in which nitrogen is lost and the highly reactive phenyl radical produced reacts with hydroxyl compounds to form the corresponding phenols or ethers. The diazo group may also be removed by reduction, in which the first step is the reduction of the diazo group to a hydrazine (reaction 3.2), a reaction readily accomplished by a number of relatively mild reductants. In addition, a number of substitution reactions

may take place such as the reaction with quinones (reaction 3.6), wherein the diazo group is replaced. It must be emphasized that these reactions may not necessarily take place in quantity in complex reaction mixtures, even if the necessary reactants are present, because of adverse conditions of pH or temperature, or the lack of catalyzing reagents. However, it may be categorically stated that if a reaction can take place it will to some extent, no matter to how small a degree. In a system where only very small quantities of pigments are being produced ($1.0\ \mu\text{M}$, 50 ppb), even very minor reactions can cause severe losses in pigment production.

These, then, are the major reactions which may take place both in formation and loss of pigment, but there are also a number of other minor reactions that take place among the reaction intermediates. These reactions, while kinetically or quantitatively insignificant, can in the sum cause appreciable losses of nitrite, both during sample preparation and nitrite determination. Indeed, in view of the complexity of the reaction system, one may well be astounded that the concentration of nitrite can be determined with any degree of precision or accuracy at all, and in fact, it is not. Collaborative studies have found an interlaboratory variation of 11 to 13%,^{11,12} which really is not too good since standard curves or replicates may be determined to $\pm 1\%$ or less. It may transpire that the higher figure is irreducible. For a method as old, tested, modified, and widely used as is the Griess colorimetric procedure, there still seems to be plenty of opportunities for analytical chemists to produce a new twist or combination of treatments to attempt to improve on the technique. Before examining some of these new techniques and/or procedures, let us briefly review the history of nitrite determination to see how analysts in the past have attempted to deal with these problems.

III. HISTORY

The structure of nitrite was well established by the beginning of the 19th century, but early methods of nitrite determination were not particularly sensitive since most of them depended on reduction of the nitrogen to N_2 , as by iodide or hydroxylamine, and measuring the gas evolved. In 1858 Johann Peter Griess (pronounced "grease") discovered the formation of diazo compounds from nitrous acid and aromatic amines¹³ and in 1862 he discovered diazo dyes.¹⁴ These two important discoveries led to the development of a very sensitive and rapid method for nitrite determination since dye concentrations as low as $1.0\ \mu\text{M}$ ($50\ \text{ppb NO}_2^-$) can be measured. Griess suggested the use of diazo dyes for nitrite determination in 1879¹⁵ and they appear not to have been in use before that date. The presence of nitrite as a naturally occurring salt in deposits and water was known earlier, but the ubiquity of the anion was suggested by Schonbein's 1862 discovery of nitrite in saliva and urine¹⁶ and Struve's 1870 demonstration of nitrogen oxides — equivalent to nitrite — in air.¹⁷ By the early 1880s Griess' colorimetric reagents were well established. In 1914 Bornand made a survey of existing nitrite determination methods and concluded that the use of Griess reagents was the preferred method.¹⁸

Shortly after its introduction, analytical chemists started studying this technique of nitrite determination. In 1889 Ilosvay found that the use of acetic acid gave better results than the hydrochloric or sulfuric acids in use up to that time,¹⁹ but he did not know why. He also reported one of the first observed interferences when he described a yellowing and reduced yield of pigments due to smoke. Because of the presence of oxides of nitrogen in air that would form the diazo dyes (nitrite equivalent nitrogen oxides), it was the practice to keep the reagents separate and, because of light-catalyzed oxidation of the reagents, in brown or opaque bottles. In 1889 Lunge²⁰ showed that the reagents could be premixed if the bottle was kept tightly stoppered to exclude both the nitrogen oxides and oxygen. He said the exclusion of light was not necessary, but subsequent experience seems to indicate it is a good idea to keep the mix in brown glass bottles. That excessively high concentrations of

reagents result in yellow pigments was observed by Ilosvay in 1890, but Lunge and Lwoff²¹ found that pigment formation was less than maximal if the reagent concentrations were less than 100 times the nitrite concentration. Conversely, Romijn²² found that if the concentration of nitrite was too high the pigment precipitated from the solution.

In 1916, Acel published a method for the determination of nitrite in meat²³ that is essentially the official AOAC method today, that is, dilution of the sample 100-fold, followed by heating for 1 or 2 hr. Indeed, this is the basic technique for almost all the official methods in use in Europe and America today. Four years later van Eck (1920) reported that chloride accelerates and borate slows the Griess reaction,²⁴ although he did not report whether or not either ion changed the final result. Kolthoff observed that neutral pH resulted in reduced pigment yields,²⁵ pointing out that the reaction is a rather delicately balanced acid reaction. Therefore by 1920 almost all the basic parameters of the reaction were established, with the one rather important omission of the identification of what is the major interference in the reaction — the effect of reductants. In 1957 Medina and Nicholas showed that reduced nicotinamide adenine dinucleotide (NADH), a reductant endogenous to bacteria plants and animals, reduced the amount of pigment formed.²⁶ Adriaanse and Robbers (1969) showed the same effect by ascorbate²⁷ and Lew (1977) showed that it was residual sulfite in beet sugar juices that caused reduced yields of the diazo dyes.²⁸ Rider defined the maximal pH range as being between 2.50 and 3.0,²⁹ which explains why acetic acid gives the best results since the pH of 15% glacial acetic acid is in this range. With these observations the groundwork was done for the cognizance and understanding of the problems which underlie nitrite determination. How to resolve the problems has been the subject of much research, and the process continues today. We shall now examine in detail the specific problems involved and the means that have been taken to resolve them.

IV. SAMPLE PREPARATION

The specific goals in sample preparation for nitrite determination are

1. To extract nitrite into solution in a form in which it may be determined
2. Remove substances that interfere in the nitrite determination
3. Remove turbidities that interfere in colorimetric determination

A. Nitrite Extraction

The first step in any sample preparation is to homogenize and extract nitrite from the sample. In the AOAC procedure this is accomplished by taking a large enough portion to be sure it is representative of the whole product and grinding and mixing several times to ensure homogeneity. The first step is obvious, but sometimes the objective is not met. A number of years ago the American Meat Institute Foundation ran a set of collaborative check samples of a number of different types of products. A Youden's plot of the various laboratories' results for one set of samples is shown in Figure 4. The bologna values are rather widely spread but are homogeneously distributed about the mean as might be expected for a well mixed, comminuted product. However, the ham values are grouped in three, possibly four, distinct groups, clearly showing that different parts of the original ham had different levels of nitrite. Does this mean that the only way to get a homogeneous ham sample is to grind the whole ham? Let us hope not. Another solution to the homogeneity problem is to blend the sample. Perryman used a paddle homogenizer to mix and extract samples and reported that the homogenization gave equivalent results to the heating procedure of the AOAC.³⁰ Blenders are readily available, and blending is an easier way of preparing a homogeneous sample. It is simpler to blend a relatively large sample with water and take a measured portion than it is to thoroughly mix a ground sample and remove a measured portion for subsequent nitrite determination. The blending step includes mincing, homog-

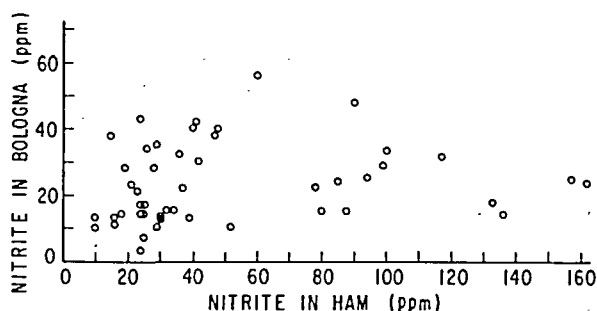


FIGURE 4. Youden plot of the nitrite content of ham and bologna samples as determined by 47 laboratories. From an American Meat Institute Foundation collaborative study.

enization, and extraction in one step, and we have found that precise portions of the resulting slurry are readily dispensed using a positive displacement pipet or syringe with a relatively large orifice.

The extraction process is usually accomplished with an excess of water, with ratios ranging from a 5:1 water to sample³¹ ratio, up to a 60:1 water to sample ratio in the AOAC procedure³² with other methods ranging between these values.^{30,33-50} The rather large excess of water tends to dilute what is usually an already low concentration of nitrite, but may be critical to obtaining maximal and uniform results, as it has been shown that the more dilute the sample during the heating process of the AOAC procedure, the higher the measured nitrite value.⁵¹ Whether this dilution effect also occurs in other methods of sample preparation is not known.

After the initial sample dispersion into water, the various methods of sample preparation fan out into a wide variety of treatments. It would not be feasible to recount the many different sequences of treatments and additions that are in use or have been proposed, nor would it be particularly useful. Therefore individual procedures or chemical additions will be examined in terms of what they are known or presumed to do to the sample.

B. Removing Interferences: Heating

Most methods heat the sample for periods of 15 min to 2 hr, with the addition of various chemicals either before or after the heating. In general if chemical addition serves the function of reacting with and removing unwanted components, it would be expected that the addition should precede the heating, but this may not always be true. It appears in fact to be more common to add chemicals after heating than before. The heating period is presumed to serve three purposes: to remove interfering substances, to extract or solubilize nitrite, and to precipitate proteins or other substances. The length of time it takes to accomplish the desired effect seems to be quite variable. The official U.S. (AOAC), Scandinavian (NM-KL), and other methods call for heating for 2 hr.^{32,36,45,49} Other heating times include 15 min,^{34,38,40,42} 30 min,^{31,32} and 45 min.³⁵ Some methods heat the sample to 80°C or boiling, then cool.^{37,39} Some methods merely leave the extracts at room temperature or warm them to approximately 50°C, hold for 10 to 20 min, then cool.^{30,43,44,46-48} In a study of the AOAC procedure it was reported that at least 1.5 hr was necessary for maximum nitrite yield, and therefore, 2 hr should be used as a safety factor.⁵¹ Quite probably the length of heating time required to obtain maximal yield is dependent on the type of chemicals added prior to heating or the pH of the solution, but the matter has not received any systematic investigation, and it is not known which of the three functions of heating mentioned previously is time-limiting. Since almost all preparation methods call for heating for some specified period it may be assumed heating is a requisite for maximal yield of nitrite, but this assumption may not be

correct. Clearly, it is to the advantage of the method to keep heating periods as short as possible, both to save time and to minimize nitrite oxidation to nitrate. The primary function of the heating is to extract and/or solubilize nitrite, but it may not be necessary to the process. Rougié et al.⁵² found that successive extractions, up to six, continued to yield more nitrite in decreasing increments. This phenomenon suggests an equilibrium, as does Nicholas and Fox's⁵¹ observation that the larger the volume of the extraction solution the greater the amount of measured nitrite. An equilibrium process suggested the possibility of improved nitrite yield by extracting with a salt solution to stamp out charge effects, but when tested, the amount of nitrite recovered from cured meat samples was the same with or without salt.

Nitrite in foods may either be bound, complexed (in meat), or reacted (BCR nitrite), as well as free in solution. Nitrite in meat may form a complex with heme pigments, either as the ionic nitrite complex or the coordinate-covalent nitric oxide complex. This form is readily dissociable and determined as nitrite. Reacted nitrite is usually presumed to be in the form of nitrosothiols (also erroneously called thionitrites). In this form, since the release of nitrite from the reacted form is a time process, increased yields of nitrite would be expected just from allowing time for the extraction. The heating and multiple extraction effect of increasing nitrite yields is most consistent with bound nitrite, either through general ionic bonds to the substrates or to a specific ion-binding site. It would be of some interest to identify just which of these forces is involved in BCR nitrite, and could be of very practical significance if it would lead to shortened extraction procedures.

C. Chemical Addition

The addition of salts is done with the primary intent of coagulating proteins or other compounds, principally to clarify solutions for colorimetric analyses. To this end the salts are protein precipitants, either cations, mercury, iron, aluminum, lead, zinc, or copper, or anions, ferricyanide, phosphotungstate, borate, or trichloroacetic acid.⁵³ As in the case of heating, it is not possible to say with any certainty which of these anions or cations is most effective since only limited comparisons have been made, generally in specific procedures on one substrate. Again, it is possible that no one precipitant will work equally well with all proteins, although a number of procedures have been proposed for multiple substrates or commodities.^{27,47,48}

Solutions of potassium ferrocyanide in the range 0.25 to 0.40 *M* and zinc sulfate or acetate in the range 0.75 to 1.8 *M* are known as Carrez I and Carrez II (or Reagent I and Reagent II), respectively, and are usually used together. Why the combination is used is not quite clear, but presumably it is to precipitate different compounds, that is, those that precipitate with zinc and those that precipitate with ferrocyanide. However, there does not seem to be any *a priori* justification for adding both, especially since it has been shown that ferrocyanide interferes in the Griess reaction,⁵⁴ probably because of the ferrous ion.^{55,56} While the interference was reversed by the addition of zinc to precipitate zinc ferrocyanide,⁵⁴ there is a conceivable danger in the use of the two reagents. If, during sample preparation, most of the zinc were to be removed by precipitation with endogenous compounds, incomplete removal of ferrocyanide would result with a concomitant decrease in pigment formation. Zinc alone appears to be sufficient to the purpose of removing turbidities,⁵⁴ in which case ferrocyanide is neither necessary nor desirable.

Mercuric ion is a commonly added precipitant used to clarify solutions for colorimetric use^{30,34,46} and has been proposed as an extraction agent for nitrite. Mercuric ion cleaves nitrosothiols,⁵⁷ one of the forms in which nitrite is believed to be bound in meats, and experiments have been carried out to determine the amounts of "bound" nitrite by measuring nitrite released by mercuric chloride addition to partially purified meat extracts.^{46,58} In Mirna's original study of the acetone-washed precipitate of cured meats,⁴⁶ the mercury was added after a heating period. Olsman and van Leeuwen⁵⁸ claimed adding the mercuric chloride

before heating improved the yield from the acetone precipitate, possibly by increasing the rate of the mercuric reaction. Some questions may be raised as to the interpretation of the results. Saville,⁵⁷ who first reported the cleavage, followed the reaction in acid solution under conditions where the nitrosothiol forms and is otherwise stable. He did not study the reaction at neutral pH or the mildly acid (pH 5+) conditions of meat and it has not been established that mercuric ions do in fact cleave nitrosothiols at neutral pH. Such cleavage may not be necessary. Dennis et al.⁵⁹ made the red nitrosocysteine at acid pH then neutralized solutions to varying pH values. They found that the color faded rapidly at pH 5.5, being about 80% decomposed in 30 min. Since nitrite is reduced to nitric oxide by cysteine at meat pH values (5 to 7), it is assumed that nitrosothiols are formed, but the reduction is very slow and the mechanism has not been established. A slow rate of formation coupled with a fairly rapid rate of decomposition means that the concentration of nitrosothiols in cured meats is probably very low. If so, the release of nitrite from proteins precipitated from meat extracts is not a specific result of the cleavage of nitrosothiols.

The addition of mercuric chloride to whole cured meat extracts has led to variable results and is subject to systematic interferences. On the positive side, mercuric ion eliminates ascorbate interference in the Griess reaction by precipitating the ascorbate as the insoluble mercuric salt. However, the effect of mercury in cured meats as reported in the literature is variable. Mercuric ion increased measured nitrite in cold water extracts,^{55,60} but after heating it either slightly increased⁶⁰ or decreased⁵⁴ the measured values. The major increases in measured nitrite in both of these experiments were due to the heating, compared with which the mercuric ion variation was minor. In addition, mercuric ion (possibly the associated chloride?) has been shown to have some specific interactions with methods of nitrite determination. It interferes in chemiluminescence and polarographic determination of nitrite,⁵⁴ and introduces systematic variations in the amount of pigment formed from different Griess reagent combinations, depending on the pH of the extracting solutions.⁶¹ The anion also introduces a systematic variation in colorimetric determinations. Chloride increases the amount of pigment formed from a given concentration of nitrite if sulfanilic acid is used as the nitrosated species,⁶² an effect that does not occur if sulfanilamide is used.⁶¹ The net effect is a higher measured nitrite with sulfanilic acid than with sulfanilamide. This observation leads to the caution that with certain Griess reagent combinations it may be necessary to use an ionic strength adjusting solution to obtain consistent results in samples with high initial chloride concentration or with added salts containing chloride. In view of the inconsistent results, the relatively low increase in measured pigment, the systematic interference in the methods of nitrite determination, and, as Bousset observed,⁶⁰ its toxicity, mercuric chloride does not appear to be a precipitant or clarifying agent of choice, and its use probably should be ended.

Insofar as the rest of the cations and anions are concerned (iron, aluminum, lead, copper, phosphotungstate, borate, and trichloroacetic acid), there is not enough evidence in the literature to definitively evaluate their effectiveness in sample preparation since it is not a common practice to compare samples before and after (with and without) addition of specific reagents. Henrioul⁶³ used lead, tungstate trichloroacetic acid, mercury, and aluminum and found not one of them satisfactory. In another study, we compared samples with and without borate, iron, and aluminum⁶⁴ and found none of them to have much of any effect, especially in heated samples. From personal experience the author has found that zinc alone (Carrez II) clarifies solutions quite satisfactorily and does not interfere in nitrite determination, and therefore is his reagent of choice. It must be noted that zinc ascorbate is soluble; therefore zinc does not remove ascorbate interference which must be eliminated by other procedures.

One interesting question remains: what is being precipitated by these ions? The generic function of all of the aforementioned ions is protein precipitation, but since most meat products are processed to temperatures sufficient to denature the sarcoplasmic proteins, the

soluble protein content of extracts is very low or nil. Tyrosine and tryptophane are constituents of all muscle proteins, yet cooked, cured meat extracts do not have the 260 nm absorption bands of these two amino acids, i.e., no proteins.⁶⁴ There are of course other tissue components in cured meat extracts that are precipitable by heavy metal ions (for example, lipids), but with the exception of mercuric ascorbate, no specific compounds have yet been identified.

D. Alkalization

Common practices are the addition of ammonium chloride buffer, pH 9.6,^{27,33,35,37,39,43,44,47} sodium hydroxide,^{42,48,65} sodium carbonate,⁴⁵ or sodium borate^{34,38,40,46} to make the extracts alkaline. Since nitrite reacts primarily as nitrous acid, raising the pH shifts the ionization equilibrium to the unreactive nitrite ion. Under these conditions, processes such as nitroso-compound decomposition, nitrite extraction, and elimination of interfering substances may be carried out without further loss of nitrite through reaction processes. The oxidation of ascorbate, for example, proceeds apace at higher pH values, thus eliminating one interference in the Griess reaction (see below). Fiddler⁶⁶ had proposed that meat extracts be raised to pH 6 before the AOAC heating procedure, and Sen and Donaldson⁴⁷ demonstrated that alkalization to pH 8 improved measured yields from acid (fermented) meat products. In a study of the effects of various preparation procedures on the elimination of ascorbate interference, we found that alkalization improved nitrite yields⁵⁴ regardless of the initial pH of the sample, and almost totally eliminated the ascorbate effect at the levels at which ascorbate is normally added to meat. The only problem is that in alkali, proteins and/or other compounds tend to form colloidal suspensions that interfere in colorimetric analysis. The heavy metal anions are added to eliminate these colloids. Which of these is the most effective in eliminating alkaline turbidities is not known since they have not been compared under similar circumstances, but the most commonly used is zinc (Carrez II). Both mercury and zinc have been added,⁴⁶ but whether or not the dual addition has any more effect than the lone addition of zinc, or mercury for that matter, is not known. Another solution to the problem was suggested by Grau and Mirna.⁴⁰ They used borate, which is a mild alkalizing agent and does not take the pH above 6.5 to 6.7. They stated that above this pH, precipitation was incomplete and the solutions could not be clarified. It would appear that there is a relatively narrow range of pH where nitrite is lost at the lower end and unremovable turbidities develop at the upper.

Another consideration with alkalization is that the pH must be reduced subsequently to 3 or below for nitrite determination²⁹ since the nitrosation reactions of nitrite determination require the acid form. Technically, alkalization with a buffer could lead to higher pH values in the nitrite determination solutions (NDS) when larger volumes of low nitrite concentration samples are taken. Since higher pH values in the NDS led to reduced pigment formation, this would mean a lessened sensitivity at lower nitrite concentrations. One method adds buffer to the standard as well as the samples, then reacidifies both⁴⁷ to ensure that both have the same pH in the NDS, although the success of the procedure was not specified. It would seem more prudent merely to add alkali to about pH 8, for then the 15% glacial acetic acid of the colorimetric reagents will bring the pH of the NDS within the right range.

E. Charcoal Addition

As will be discussed in detail later, ascorbate interferes in the Griess reaction. The first direct addressing of the elimination of ascorbate was Adriaanse and Robbers'²⁷ addition of charcoal to the extracts to adsorb the ascorbate. Fudge and Truman³⁸ heated the charcoal-treated extracts, but did not specify if the heating procedure improved the yield over not heating. The addition of charcoal would not be expected to release bound nitrite, although the heating might have done so. Charcoal treatment was found to be an effective technique in terms of maximum measured nitrite values and elimination of the ascorbate interference,

but at low nitrite concentrations it gave lower yields than did making the samples alkaline and heating at 80°C for 2 hr.⁶⁷ While on the subject of reductants, it may be noted that the elimination of sulfite (which also interferes in color formation) from beet sugar juices was accomplished by the addition of formaldehyde.²⁸

F. Clarification

The final step before colorimetric determination of nitrite is the removal of the precipitated materials and/or turbidities, most commonly performed by filtration. Since many commercial filter papers contain nitrite, it is necessary either to choose nitrite-free papers¹² or to discard the first 20 ml of filtrate. We found that 300 to 400 ml of water was required to completely eliminate color development and washings should be tested to see if the last of the nitrite has been removed.⁶¹ Filtration is not always successful, especially if it is desired to prepare samples in as short a time as possible. Very fine turbidities develop, especially in alkaline solutions, which even the addition of heavy metal ions does not always precipitate. In fact, we found mercury sometimes exacerbates the problem.⁵¹ These precipitates are often gelatinous in nature and tend to clog filter papers fine enough to remove them. We have found that centrifugation is superior to filtration in removing these finely dispersed precipitates, and that zinc (Carrez II) coagulates the colloidal material quite satisfactorily.

G. Separation of Nitrite from Substrate

One may either remove the interfering compounds from nitrite, as has been discussed, or remove the nitrite from the interfering compounds. From a theoretical standpoint, the latter procedure is the better way of preparing samples since the nitrite ion is obtained in a more or less pure state. Nitrite is not extractable from water, but may be isolated by chromatography on ion exchange resins. Small et al.⁶⁸ developed an ion exchange technique where nitrite in strong alkaline solution with other anions is chromatographed on a weak anion exchange resin. As the solution with the separated ions leaves the separating column it is run through a stripper column, a strong cation exchange resin in the H⁺ form which removes the buffer cation, generally sodium, thereby neutralizing the solutions. The anion concentration is then determined by conductivity, but any determination procedure could be used. Other methods of nitrite isolation by chromatography include other ion exchange,^{69,70} high pressure liquid,⁷¹⁻⁷⁴ thin layer,^{75,76} and paper⁷⁷ chromatography. Removal of nitrite from solutions containing nitrate also was accomplished by making the solutions strongly acid, steam distilling the solutions, and trapping the volatile nitrogen oxides from nitrous acid⁷⁸ in alkali. The possible effects of interfering substances were not investigated. The volatilization of nitrogen oxides has been used for chemiluminescent⁷⁹⁻⁸² or pneumatoamperometric⁸³ determination of nitric oxide by either acidification^{79,80} or nitrous acid reduction by reductants.⁸¹⁻⁸³ Reduction probably produces a more homogeneous nitric oxide since acidification does result in low amounts of other nitrogen oxides being produced.

Whether any sample preparation method is of utility is a question of the amount of sample preparation, the time required for the determination, and the sensitivity, all functions of the substrate being analyzed. I would speculate that in meat which contains a wide variety of soluble compounds, sample preparation for chromatography may be as difficult a problem as preparing samples for colorimetric analysis. Volatilization of nitric oxide, especially by reduction, requires little sample preparation, but sampling is slow. To the author's knowledge, no one has ever examined by experimentation the question of whether it is preferable to isolate nitrite from its contaminants, or eliminate the interferences from nitrite solutions.

H. Summary of Preparation Methods

In summarizing preparation methods, the author feels that it is highly significant that almost all official methods, including those of the International Standards Organization,³⁴

The Nordisk Komité for Levnedsmidler,⁴⁵ The European Economic Community,³³ Japan,⁸⁴ Canada, and the U.S. (Association of Official Analytical Chemists),³² use some variation of the dilution and heating method originally proposed by Acel.²³ The alkalization of samples before heating is common, as is the addition of precipitating reagents, usually zinc. As a result of a recent study wherein we obtained maximal yields and most consistent results following these practices,⁶⁷ the author is strongly of the opinion that dilution/alkalization/heating/zinc is the preferred method of preparation of samples for nitrite determination. In view of the closeness of the various official techniques, it should not be at all difficult to establish a worldwide standard for sample preparation.

V. NITRITE DETERMINATION

A. Fundamental Considerations

As noted previously, the determination of nitrite is a procedure fraught with a number of difficulties related primarily to the reactivity of nitrous acid. Obviously, the more reaction steps involved, the greater the chance of loss of nitrite or reaction intermediates due to side reactions, with less of the measured product being formed. Therefore, for discussion and evaluation I shall consider nitrite determination procedures according to the number of reaction steps or the kind of reaction involved. Accordingly, methods of nitrite determination have been grouped as follows: no reaction; nitrosation and decomposition (two-step) reactions; reduction and complex formation (three-step) reaction; coupling (four-step) reactions, oxidation to nitrate, catalysis, enzyme reduction, and ring formation.

B. Direct Measurement Without Reaction

Considering the inhomogeneity of nitrous acid reactions, there is an advantage to determining nitrite by some physical characteristic of the molecule. Nitrite may be determined conductimetrically,⁶⁸ electrocatalytically,^{85,86} or spectrally, either by its optical absorption⁷¹⁻⁷⁴ or lack thereof.⁷⁰ Nitrite has absorption bands in the ultraviolet at approximately 340 and 210 nm. The former has a millimolar absorption of about 0.01 AU and is not useful for nitrite determination, but the latter has a millimolar absorption of 3.8×10^3 ,^{3,71} which may be used to determine nitrite down to parts per billion. Small and Miller⁷⁰ have developed an interesting technique wherein they equilibrate an ion exchange column with an ion that has a strong optical absorption in the ultraviolet. Eluted ions with no or less absorption show up as troughs or negative peaks in the basal absorption. The infrared absorption bands of nitrite and nitrate have been used to quantitate these compounds, but the sensitivity is only 0.02 M.⁸⁷ The principal disadvantage of these techniques is that they require purified nitrite. It is usually prepared by chromatography, which in biological substrates is not necessarily an easy task. In meat, for example, the principal contaminants are complex biological compounds that tend to precipitate on columns, eventually plugging them. Preparing samples for column chromatography generally requires the same amount of sample preparation as do optical absorption methods.

C. Nitrosation and Decomposition

The simplest techniques of nitrite determination through reaction involve first the formation of a nitrosating species from nitrous acid, followed by either a nitrosation reaction or a decomposition to produce nitric oxide. Both second steps will go simultaneously, of course. While under a given set of environmental conditions and over a narrow range of nitrous acid concentrations a constant proportion of nitrosated species to nitric oxide may be produced, though there is no assurance there will be. The course taken is controlled by the conditions of the system and the reactants available, as has been noted. Decomposition to nitric oxide is a relatively slow reaction compared to nitrosation, is recyclable to reform

nitrous acid (reactions 1.6a, b, and c), and is acid dependent being favored at high acidities. In contrast, since nitrosation takes place between a nitrous acid derivative and an unprotonated nitrosatable compound, nitrosation is favored over nitric oxide production in mildly acid conditions, with compounds having a pKa about that of nitrous acid, 3.4, or less. With such compounds in large excess over nitrite, the reaction goes nearly to completion.

Some of the reactants (and techniques) proposed for the determination of the products of the nitrosation reaction are diphenylamine⁸⁸ (differential pulse polarography); 2,4,6-triaminopyrimidine,⁸⁹ mepazine,⁹⁰ 2-mercaptoethanol,⁹¹ 2,6-xyleneol,⁹² chromotropic acid,⁹³ 2,7-diaminofluorene⁹⁴ (colorimetric); thiourea,⁹⁵ resorcinol⁹⁶ (colored complex formation); diaminonaphthalene^{97,98} (colorimetric and fluorometric); sulfanilic acid,³⁵ *p*-aminobenzoic acid⁹⁹ (fluorescence reduction); 5-aminofluorescein⁵⁵ (fluorescence enhancement); 1-hydrazinophthalazine,¹⁰⁰ and *p*-bromoaniline¹⁰¹ (volatile, gas liquid chromatography).

Decomposition reactions mainly produce nitric oxide (reactions 1.3b and 1.4) but in the process also produce other compounds, nitrogen dioxide, and nitric acid. In strong acid solutions some nitrous oxide is formed (reaction 1.5), but the quantities are negligible. In the absence of a nitrosatable compound, the reaction will go towards nitric oxide, nitrogen dioxide, and nitrous oxide since they, as gases, will act as sinks. In the gaseous state, nitric oxide has been determined by chemiluminescence⁷⁹ and ion-specific gas electrodes.⁴⁹ While these two methods measure only nitric oxide, other methods measure all three gases by optical absorption⁸⁰ or nitric oxide and nitrogen dioxide by dissolving the gases in alkali and determining the resulting nitrite colorimetrically.⁹³ For any of these methods it is imperative that the relative amounts of the three gases produced remain constant over the range of nitrite concentrations being determined. In addition, all of these except the ion-specific electrode require the dissolution of the nitric oxide either by steam distillation⁷⁸ or purging with a carrier gas.^{79,82} The ion-specific electrode relies on normal dissolution and diffusion through a hydrophobic membrane, which means the electrode is competing with dissolution from the solution surface into the atmosphere. All of these methods are therefore dependent not only on the varieties of the reactions that produce nitric oxide, but also upon the solubility and rate of diffusion of the gases. Obtaining precise and accurate determinations is therefore dependent on very good, painstaking technique.

The driving force for completion of either nitrosation or decomposition is that either product acts as a sink. Clearly, if there is any other irreversible reaction taking place, less than maximal formation of measured product from either reaction is formed. Nitric oxide cannot be determined accurately at all if there are any nitrosatable species present. Again, sample cleanup is almost always involved to remove extraneous nitrosatable species, mainly reductants, ascorbate, reduced coenzymes, sulfite, etc.

D. Three-Step Reactions

One way to reduce the vagaries of decomposition reactions is to drive nitric oxide formation with a reductant. The reaction steps are formation of the nitrosating species, nitrosation of the reductant, and an internal electron transfer to form nitric oxide. Reductants are the most nucleophilic of reagents and react much faster than other nitrosatable compounds, hence, from sheer rate of reaction, produce mainly nitric oxide from nitrite. Reductants used for this process are hydroquinone,⁸³ iodide,⁸¹ and ascorbic acid.⁸² Hydroquinone was used to produce nitric oxide for pneumatoamperometry⁸³ while the last two were used in chemiluminescent detection methods. Since nitric oxide formation by nitrous acid reduction will go under mild acid conditions, where the higher order reactions producing other nitrogen oxide gases as well as nitrosation reactions are not favored, reduction reactions would appear *a priori* to be preferred over acid dismutation to form nitric oxide. Under any circumstances, the formation must be carried out anaerobically to prevent nitric oxide oxidation.

There are other three-step reactions in which the nitrosation reaction product is complexed

with metal ions to form colored complexes. Nitrous acid and thiourea will form thiocyanate ion, which is determined as the red complex with iron.⁹⁵ Nitrous acid and resorcinol form a nitroso-derivate that produces a yellow complex with zirconium ion.⁹⁶ Both methods are rapid and specific, the only expected interferences being those for a two-step reaction, since the complex formation is quite specific.

E. Coupling Reactions: Four-Step

The coupling reaction group is the largest, for it includes the formation of diazo dyes, the principal technique by which nitrite is determined today. The first two steps are the same as before, with a third step internal rearrangement of an aromatic nitrosamine to form a diazonium ion, followed by a fourth step coupling reaction. The rearrangement is an internal reaction which is determined solely by the characteristics of the aniline derivative and, although the reaction actually involves several steps itself, from a practical standpoint it may be looked upon as a single step. Furthermore, because the intermediate nitrosamine has no particular distinguishing characteristics, the relative rates of nitrosation and rearrangement are not known for any of the aniline derivatives used for nitrite determination. The formation of the diazonium ion therefore is known kinetically only as a bimolecular reaction controlled by the nitrosating species and aniline derivative concentrations. From the standpoint of analyzing the critical parameters in dye formation, this is probably all the information necessary.⁸

The coupling reaction is a function of diazonium ion and coupling reagent concentrations and reactivity, but as the reader may again expect, the reaction is not simple. The author has made a study of the kinetics and mechanism of the Griess reaction and has characterized a number of the possible variations that may occur.⁸ To summarize briefly, the amount and stability of pigment formed was a function of the kind and relative amounts of reagents used. High concentrations of highly reactive coupling reagents resulted in less pigment formed due to reaction with the nitrosating species: low concentrations of coupling reagents resulted in less pigment formed due to unfavorable mass action conditions. Stability of the pigment formed decreased with increasing concentrations of nitrosated species and increased with increasing concentrations of coupling reagent. Some pigments faded to colorless compounds, some changed to compounds with different absorption spectra. Fading, which usually began before full pigment formation, was primarily an oxidative process, but the reaction and the addition of reductants was only partly effective against fading. There were several other phenomena described, but without going into further detail, the summary given in the paper⁸ of the critical factors involved in color production from aniline and naphthylamine derivatives is repeated here:

1. Kind and concentration of reagents used, including position of ring substituents
2. Specific combinations and relative concentrations of nitrosatable species and coupling reagents
3. Reaction of nitrite with ring substituents other than the amino group
4. Reaction of nitrite with the coupling reagent
5. Formation of more than one pigment
6. Oxidation of the diazonium ion intermediate
7. Oxidation of the pigment
8. Oxides of nitrogen in the air
9. Reduction of the diazonium ion by residual reductants
10. Formation of semistable nitroso-reductant intermediates
11. Pre-reaction of NS and nitrite

These factors, in addition to pH and temperature, are important in some degree to all reagents

studied and, it may be assumed, to any other compounds that have been or may be proposed for the purpose of determining nitrite. I know of no papers on nitrite determination where new or different reagents have been proposed in which more than one of these factors has been investigated. Since the operation of many of these factors results in significantly different total amounts of pigment formed, no new reagents or combinations thereof should be recommended without an assessment of the effects of these factors.

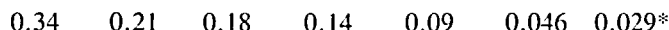
The number of reagent combinations that have been tried is legion. Sawicki et al.¹⁰² listed 52 different combinations reported in the literature, but not the methods of sample preparation. Since there is an interaction between sample preparation and nitrite determination methods,⁶¹ it is not possible to evaluate the combination with respect to each other. However, some general conclusions may be made by examining the characteristics of the two reagents involved, the nitrosated species (diazonium ion) and the coupling reagent.

F. The Nitrosated Species

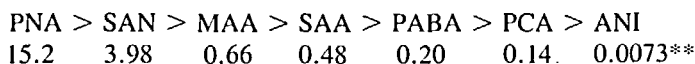
Since the first part of the reaction sequence is the formation of the diazonium ion of the aniline derivative, the latter should react swiftly with the nitrosating compound to preclude nitrosation of other nitrite-reactive compounds in the system. It would be expected that the order in which the derivatives nitrosate is the order of the ability of the substituent to shift electrons to the amino group through induction or hyperconjugation, thereby providing an electron-rich site for the nitrosating reaction. The rate of diazonium ion formation if ordered according to the Hammett σ -values would be



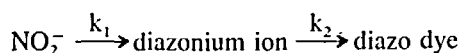
whereas it is



that is, both of the para-SO₂R anilines are switched with their respectively higher σ^- predecessors. This is probably due to a faster internal rearrangement to form the diazonium ion, for the -SO₂R group is more electronegative than the -COOH, -NO₂, or -Cl group. MAA was somewhat more reactive than its reported Hammett constant would indicate, from which it has been concluded that the effect is due to negative inductance since this is the only effect of -SO₂R groups in the meta position. Once formed, the diazonium ions coupled to form diazo dyes at rates proportional to the normal σ values expected for the various substituents in the order:



This is not however, the order of rates of pigment formation. For the reaction:



* The figures below the reagent are the first order rate constants (k_1) at 20°C.

** The figures below the reagent are the first order rate constants (k_2) for the coupling of the diazonium ions at 20°C.

each step may be treated as a first order reaction as a first approximation since nitrite is limiting in the first step and the diazonium ion in the second. The amount of diazo dye at time t is

$$[\text{diazo dye}]_t = [\text{NO}_2^-]_0 \left(1 + \frac{k_2 e^{-k_1 t} - k_1 e^{-k_2 t}}{k_1 - k_2} \right)$$

If the various aniline derivatives are arranged in terms of increasing time for 99% conversion to pigment, the following sequence is obtained, with 1-NA as the coupling reagent.

Reagent	SAN	>	PNA	>	SAA	>	PABA	>	MAA	>	PCA	>	ANI
minutes	14		20		28		41		53		110		660

This sequence also holds if NED or 1,6-Cleve's acid is used as the coupling reagent, with approximately the same relative times for all the listed reagents with NED. With SAN and PNA, 1,6-Cleve's acid produces pigment at about the same rate, but with the rest of the reagents the time for 99% conversion is in the hundreds of minutes. From the standpoint of rate of pigment formation, therefore, *p*-nitroaniline or sulfanilamide are the reagents of choice.

This sequence is only a relative measure of how fast the pigment is formed, and has almost no relationship with interference in the reaction. The latter is a function of the relative reactivity of the color-forming and interfering reactions. For example, while 1-nitrophenyldiazonium ion reacts very rapidly with coupling reagents, it also reacts rapidly with ascorbate. Interference by ascorbate did not vary greatly for various reagent combinations, and was not related to the rate of coupling.⁸

G. The Coupling Reagent

The other reactant of the coupling is usually 1-naphthylamine or a derivative thereof. Since the diazonium ion is an electrophilic reagent it couples with an electron-rich site on the naphthyl nucleus. The most effective substituents are those that shift electrons on the aromatic ring through positive resonance and inductive effects. Positive resonance effects are exerted only in the para or ortho positions; hence an amine group in the 1 position on naphthylene activates the 4 position. Meta substituents have almost no effect: positive resonance substituents in the meta (2) position increase the reactivity of naphthalene very little, and negative inductance groups in the same position reduce the reactivity of 1-naphthylamine very little. Substituents on the second ring of 1-naphthylamine lower the reactivity of the 4 position, although, as might be expected, the effect is least when the substituent is in the 6 or 7 position. However, in terms of positive electron shifts, there can be too much of a good thing. When the positive resonance effect of the 1-position substituent is increased, as when an ethyleneamine group is added to the amino group (*N*-(1-naphthyl)ethylenediamine), the 4 position becomes so electron-rich (nucleophilic) that its reactivity towards the initial nitrosating species approaches or becomes equal to that of the aniline derivative (nitrosated species) and reduced pigment formation occurs partly through diversion of the nitrosating species from the aniline and partly to inactivation of the naphthyl derivative. The effect is not total or permanent since the nitrosation of the coupling reagent is partially reversible, but does cause appreciable reduction in the amount of pigment formed from a given amount of nitrite. With these relationships and reactivities in mind, let us examine some specific reagent combinations.

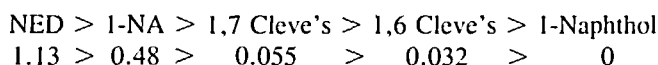
H. Reagent Combinations — Sulfanilamide

Let us start with the most common combination in use today, SAN and NED. This

combination is used in the official methods of the Scandinavian countries,⁴⁵ the ISO,³⁴ the EEC,³³ the U.S.S.R.,¹⁰³ Japan,⁸⁴ Sri Lanka,¹⁰⁴ and the U.S.³² and is the reagent pair in a number of (proposed) methods.^{28,31,36,38,41,105-112} As the most reactive of the substituted anilines and substituted 1-naphthylamines, the combination represents the theoretically best combination available. NED has, however, an unfavorable ratio of nitrosation to coupling rates. When pigment formation was measured in nitrite solutions where SAN and NED were added either simultaneously or separately with a 5-min reaction period between additions, the former produced only 66% as much pigment as did the latter.⁸ After simultaneous addition an appreciable amount of nitrite was lost by reaction with NED since both SAN and NED nitrosate at the same rate ($k_{1st} = 0.18 \text{ min}^{-1}$ for both). NED was present in lower concentrations than was SAN (0.2 mM and 1.0 mM, respectively), and there was some reversal of the nitrous acid/NED reaction; otherwise yields would have been lower. Pre-reaction with SAN eliminated the NED nitrosation by removing the nitrite through formation of the *p*-sulfonamidophenyldiazonium ion. In terms of coupling reagents, 1-NA was found superior to NED since simultaneous addition produced 94% as much pigment as did sequential addition, principally because the nitrosation rate of 1-NA is much lower than that of NED ($k_{1st} = 0.045$ and 0.18 min^{-1} , respectively). From this standpoint, 1-NA would appear to be the preferred coupling reagent, but has been used only in the penultimate AOAC procedure.¹¹¹ Its use has been discouraged, although not proscribed or forbidden, because the Occupational Safety and Health Administration has classified it as a Class I toxic chemical because of its reputed carcinogenicity.¹¹³

I. Sulfanilic Acid

An equally popular nitrosated compound is sulfanilic acid (SAA), but it is used with a wider variety of coupling reagents: NED (the combination also known as Griess-Saltzman),^{31,39,47,62,108,114-115} 1-NA (the combination also known as Griess-Ilosvay-Lunge reagent),^{1,29,40,50,77,116-121} 1-naphthol,³⁷ 1,7 Cleve's acid (8-amino-2-naphthalenesulfonic acid),²⁷ 1,6-Cleve's acid (5-amino-2-naphthalenesulfonic acid),¹²² phenol,⁶⁵ and *m*-aminophenol.¹²³ SAA forms a diazonium salt at little better than half the rate of SAN, but couples with rates varying from one third to one tenth that of SAN. Five of the above coupling reagents were reacted with the (4-sulfonic acid)-phenyldiazonium ion.⁸ The reaction rate order was



for which the time to 99% conversion was

$$4.1 < 9.6 < 83.7 < 144 < \infty \text{ min}$$

In practical terms, only NED and 1-NA have reasonable reaction rates, but NED produced even less pigment with SAA than it did with SAN (38% compared with 66%). Again, 1-NA was better since it produced 81% of the maximum possible pigment with SAA. SAA has other disadvantages that make it less suitable for colorimetric determination of nitrite as compared with SAN. Increasing sodium chloride concentrations cause increasing amounts of pigment to be formed from SAA/NED for given concentrations of nitrite,⁶² requiring control of the salt concentrations in color reaction solutions. In comparison, SAN is insensitive to salt.⁶¹ Residual ascorbate has an unusual effect on the SAA/NED reagent combination in that up to approximately 30 μM ascorbate in the color reaction solutions increases the amount of pigment formed.⁶¹ Above this concentration, increasing ascorbate causes decreasing pigment production. Of other nitrosated species tried,⁸ only metanilic acid showed the effect of increasing yields; with all other reagents residual ascorbate at any concentration

caused reduced pigment yields. The difference in the effect of ascorbate on SAA and SAN has led to an apparent disagreement in the literature. At comparable levels of ascorbate, Sen and McPherson found a 1.5% decrease in nitrite,¹²⁴ while Adriaanse and Robbers found a 49% decrease.²⁷ As expected, the former used SAA and the latter SAN. The true value probably lay in between. Since neither too little nor too much pigment formed is acceptable from an analytical standpoint, the only answer is the complete removal of ascorbate, since no reagent combination can be proof against either effect. This is especially true for slower coupling reagents such as the 1,6 and 1,7 Cleve's acids.

Because the rate of diazonium ion formation of SAA is comparable to the nitrosation rates of the faster coupling reagents, another phenomenon is observed. In some recent studies of nitrite in cured meat products it was observed that the variation in pigment production from SAA/NED was much greater than that from the AOAC combination of SAN/NED or SAN/1-NA.⁶⁷ The cause lies in the kinetics of the reactions. Where diazonium ion formation goes much faster than coupling reagent nitrosation, minor variations in the rate of the faster reaction will not have much effect on the amount of pigment formed. If the two rates are close or equal, minor variations in either rate will cause relatively large variations in the amount of pigment formed. Such reaction variations can come about as a result of changes in temperature, concentration due to mixing, pH, salt, residual reductants, etc. Unless such factors are carefully controlled, variations in the pigment formed are to be expected. From this standpoint alone, SAA, as well as any nitrosated species reacting even slower, is not the reagent of choice. Since the problem is a slow rate of diazonium ion formation, the obvious answer is one of the faster reacting aniline derivatives.

J. *p*-Nitroaniline (PNA)

p-Nitrophenyldiazonium ion is the fastest coupling intermediate, and PNA has been proposed for nitrite determination in combination with 1-naphthol¹²⁵ and azulene.¹²⁶ The latter combination was chosen for rapid color development. However, as discussed previously, coupling rates are not the only rate factors involved since the rate of diazonium ion formation, which is generally slower, must be taken into account. Thus, there is not too much choice between PNA and SAN or SAA in terms of rapidity, but PNA does have one disadvantage in that not all of its dye derivatives are stable. The azulene product was stable for one hour¹²⁶ and the 1-NA and NED derivatives were stable for several hours.⁸ However, both 1,6 and 1,7 Cleve's acid, which are commonly used and have been proposed as 1-NA substitutes, were unstable. The 1,6 Cleve's acid derivative faded slowly over a period of hours while the 1,7 Cleve's acid pigment began appreciably fading as soon as it had been formed. It is, of course, inconvenient to have to time reactions in order to record maximal pigment formation, but of greater importance is that catalysis or inhibition of either color formation or destruction will change both the time and amount of maximal pigment formation.

K. Other Reagent Combinations

Other reagent combinations have been proposed for nitrite determination, including aniline/1-naphthol,¹²⁷ 4-aminoacetophenone/*N*-phenyl-1-naphthylamine,¹²⁸ and *p*-aminobenzoic acid/1-naphthol,¹²⁹ but the effectiveness of these compounds under a variety of conditions has not been tested. A proliferation of reagents all to do the same thing is not necessarily productive unless it is shown that the proposed combination results in an improvement over existing reagents. There are hundreds each of both nitrosatable organic amines and coupling reagents known to the dyestuff industry, leading to hundreds of thousands of possible diazo pigments. To assess all possible combinations would be a monumental task and which might not be fruitful anyway. Sawicki et al.¹⁰² reported a number of reagent combinations that have significantly higher absorption coefficients than do the combinations discussed previously (ϵ mM \approx 80 to 90 AU compared with 20 to 50 AU), but a twofold increase in

sensitivity may not necessarily justify the testing necessary to bring a reagent combination to a level of utility.

L. Pigment Formation from One Reagent

The formation of a diazo dye using only one reagent was used by Kamm et al. to determine nitrite by the nitrosation of 1-naphthylamine in a large excess of the reagent.⁴³ The diazonium salt formed coupled with free 1-naphthylamine to form a purple pigment which they extracted into chloroform, further concentrating the pigment. This is still a four-step reaction with the same problems of nitrite and coupling reagent losses due to nitrosation of the 4 position of the 1-naphthylamine.

M. Other Griess Pigment Measurements

There are other ways in which pigment formation may be used for nitrite determination other than measuring the optical absorption of the pigment. A particularly useful technique was developed by Bodine and Janzen¹⁰⁵ in which the pigment was formed, then titrated by oxidizing it to a colorless form. As the authors point out, such a technique could be useful in the field where a spectrophotometer might not be available. Whether or not the titration step has any interferences was not specified. Furuya et al.¹¹⁶ formed the pigment from sulfanilic acid and 1-naphthylamine and measured the resonance Raman absorption, which technique requires a colored compound. The instrumentation is rather expensive compared to a spectrophotometer, but the sensitivity was an order of magnitude greater than that of optical absorption.

N. Noncoupling Reactions of Diazonium Ions

Diazonium ions are relatively unstable and may be reacted to yield nitrogen or identifiable deaminated compounds. This property has been used to determine nitrite, measuring evolved nitrogen from pyrolysis by gas chromatography,¹³⁰ or the benzene derivative formed by reductive cleavage of the diazonium ion by hypophosphorous acid.¹³¹ The success of these techniques is dependent on the ease of diazonium ion formation, the stability of the ion, and the specificity of the pyrolysis or hydrolysis. The full extent of the effect of these factors is not known, since neither the sensitivity of nor interferences in the reaction were investigated.

O. Ring Formation

The nitrosation of hydrazines¹⁰⁰ or vicinal diamine aromatics¹³² leads to the formation of polyazo ring compounds. These compounds are organic solvent soluble and volatile, either as formed¹⁰⁰ or after forming the trimethylsilyl derivative,¹³² and may be determined by gas chromatography. Since there is only one reagent to react with nitrite, the rates of nitrosation and cyclization are not highly critical and the techniques are fairly clean and specific. Both, however, are quite lengthy, requiring extensive purification and solvent extraction.

P. Oxidation

Nitrite and nitrate are readily converted one to the other, and perhaps the best method of nitrate determination is to reduce it to nitrite and react the nitrite. Conversely, methods have been developed wherein nitrite is converted to nitrate and the nitrate determined by nitration of benzene or a substituted phenol and extracting the nitro-derivative into an organic solvent.^{48,133} The question arising is whether or not the reactions of nitric acid are any more specific than those of nitrous acid. Certainly to the extent that the most common nitrous acid determination technique, dye formation, is highly specific for nitrous acid, determining nitrite would appear to be advantageous. Nitric acid reactions are as heterogeneous as those of nitrous acid and it is probably better to bear nitrous acid ills than to fly to nitric acid maladies. One technique that technically is better with nitrate than with nitrite is ion-specific

electrode determination.¹³⁴ The problems associated with the gas electrode whereby nitrite is determined have already been discussed. In contrast, the nitrate electrode should not have most of these problems and should be more precise. The details of this technique have not been investigated as far as the author knows.

Q. Catalytic Methods

Sawicki et al.¹³⁵ investigated pigment formation from various azines, hydrazones, and diamines catalyzed by nitrite. The process is the oxidative formation of radicals, which then couple to form dyes. Since in the process nitrite is reduced to nitric oxide which then cycles back to nitrite, it may form quantities of pigment equivalent to absorptivities of 200 to 1200 $\text{mM}^{-1} \text{cm}^{-1}$. The problem with the technique is that the amount of pigment formed is dependent on time, temperature, reagent concentrations, and rate constants. To eliminate the time effect, the pigment absorption is determined at a fixed time after mixing reagents with nitrite since the concentration of pigment is constantly changing with time. But the amount of pigment formed at any given time is dependent on the rate of formation, hence, temperature, requiring temperature control. Since the effect of nitrite is catalysis, the proper measurement is not the amount of pigment formed in a given time, but rather the increase in the reaction rate constant with increasing nitrite concentration. Obviously, if anything else catalyzes or inhibits the system, wrong nitrite values will result. Because of the catalytic effect of nitrite, varying amounts of nitrite loss during the reaction, and relative concentration effects, the nominal absorptivity varies with the nitrite concentration, which leads to the odd result that the sensitivity decreases as the nitrite concentration decreases. Since the net gain in sensitivity at low levels of nitrite is only \sim twofold at best, and in view of the careful control that must be exercised, the technique does not seem to be particularly useful, especially for routine analyses.

R. Enzyme Reduction

Kiang et al. used a nitrite reductase from spinach leaves to reduce nitrite to ammonia and measured the latter with an air gap electrode.¹³⁶ The method was rather lengthy and not too sensitive (5 ppm). Since enzyme catalysis is substrate specific, the technique might be of utility in a mixture where nitrite could not be separated from interfering compounds, although in a system containing ammonia or substrates convertible to ammonia the technique could not be used.

S. 5-Step Formation of a Fluorescence Compound

Dombrowski and Pratt developed a fluorometric method for nitrite determination¹³⁷ involving diazotization and coupling, followed by reaction with ammoniacal cupric sulfate to form a fluorescent triazole. The procedure is time consuming and still requires sample clean-up, but is of an order of magnitude more sensitive than colorimetric analysis, an advantage of fluorescence.

T. Concentrating the Diazo Dye

Theoretically, the determination of nitrite no matter how dilute is only limited by the volume of solution available if the nitroso (diazo, etc.) product used for measurement is extractable into an organic solvent or can be adsorbed on an insoluble substrate. Practically, there has to be a limit, but what it is has not been definitely established. Kamm et al. extracted 4-(1-naphthylazo)-1-naphthylamine into chloroform, affecting a fourfold increase in concentration in so doing.⁴³ They detected 3 ppb nitrite (from nitrate), but when they increased the ratio of sample to extracting solution (thereby effectively lowering the detectable nitrite concentration) they ran into interference originating from the sample. Wada and Hattori¹¹⁵ investigated the lower limit of nitrite determination in sea water by forming the

SAA/NED pigment and adsorbing it on Dowex® 1-X8. Eluting the dye with 60% acetic acid yielded a concentrated solution of the pigment. However, sea water does not contain many interfering substances and whether minute concentrations of pigment could be formed in substrates with residual reductants is questionable, especially in view of Kamm et al.'s results.

U. Summary of Nitrite Determination Techniques

I have often suspected that if an analytical chemist approached an organic chemist for a method of nitrite determination, about the last reaction the organic chemist would suggest would be the Griess reaction with its four-step dye formation. Yet it has survived, and, within its limitations, remains the standard. Other methods are more sensitive, but none are faster. Mild acid conditions and room temperature reactions keep the determination clean and simple. I have run experiments, prepared samples, and determined nitrite in up to 60 to 70 samplings in 1 day. On routine analyses, two of us have run up to 168 colorimetric nitrite determinations after lunch. The process has been automated,^{105,120,139} but the machine determines nitrite in samples sequentially and is thereby slowed whereas one operator by hand can be 40, 50, or more samples developing color simultaneously. Many other methods promise cleaner results and maybe even faster determinations, i.e., ion exchange high pressure liquid chromatography, but these techniques have not been tested under fire. Until they have, colorimetric determination by the Griess reaction, like the infantry, remains the "queen of battles."

VI. SUMMARY

A critical evaluation of the foregoing nitrite determination procedures is very difficult. Many of them are *de novo* techniques and only a very few of them have been compared with any other nitrite determination procedures. Seldom are the critical parameters such as sensitivity, reproducibility, precision, repeatability, specificity, accuracy, speed, expense, ease, or ruggedness given consideration, and never have all the parameters been considered at one time. Most of them are methods of nitrite determination only and are not associated with any specific substrate or method of sample preparation, requiring potential users to evaluate the procedure in their own substrates. This would have to be done in any case if the substrate used for the initial evaluation was different. From an operational standpoint, for whatever it is worth, I personally detected no obvious flaws in the procedures as described, and whether or not any of them are used may be dictated less by the intrinsic merit of the procedure than by the ownership of the equipment needed to perform the determination. After all, with the exception of measurements of a physical property, all methods involve some aspect of nitrous acid reactivity. Thus, all reaction methods, regardless of their sophistication, are at the mercy of the heterogeneous nature of nitrous acid reactions.

Of all the critical parameters just noted, there is one factor that can justify deviations such as complicated preparation or determination procedures, expensive equipment, high variability in precision or accuracy, etc., and that factor is high sensitivity. Clearly, if the situation calls for measuring 1 to 2 ppb nitrite, colorimetric determination (50 ppb sensitivity) is of no use. Table 1 lists the sensitivities of most of the techniques in use or proposed for use. The sensitivity limits shown are for the solutions of measurement, and translate to larger values in the original solutions if diluted. The limits are those claimed by the authors or if not given, calculated from the data using the criteria given by Ingle.¹⁴⁰ For gas chromatography, the sensitivity limits are calculated for a presumed 0.1 mL volume of injected sample. The relative merits of the methods listed can be discussed only with the *caveat* that all factors other than sensitivity are considered equal. Methods less sensitive than or equal to colorimetric methods might be of value if they provide quick and reasonably accurate determi-

Table 1
DETECTION LIMITS OF VARIOUS
METHODS OF NITRITE
DETERMINATION

Methods less sensitive than colorimetric ppb^a

Paper chromatography	10 ^a
Enzyme (NH ₃ electrode)	5000
Complex formation	2000
Catalytic voltammetry	500
Optical absorption of NOX gases	200
Pneumatoamperometry	200

Methods equivalent to colorimetric

Electrode	50
Titration	50
Fluorescence quenching	50
Direct optical absorption	20
Catalysis	20

Methods better than colorimetric

Conductimetric	10
Indirect photometric chromatography	10
Electron capture	5
Chemiluminescence	5
Resonance raman	5
Solvent extraction	5
Fluorescence	2
GLC (derivatization)	1
Polarography	0.3
Dye absorption	0.001

In solution being measured. If sample requires dilution,
detection limit will be higher in sample.

nations, are particularly specific, or have special applications. In the latter category we have mentioned titration of a diazo dye as being useful for field studies. Of the less sensitive methods, enzyme reduction would be of special value in that enzymes are highly specific in their choice of substrates. The other methods require both some sample preparation procedure and sophisticated equipment and, as developed, do not appear to have any particular advantage over colorimetric determination. Of the methods more sensitive to nitrite, some have more than sensitivity in their favor. Chemiluminescent determination of nitric oxide formed by nitrite reduction^{81,82} would be useful in substrates with residual ascorbate such as cured meats, fruits, and some vegetables. The indirect photometric chromatographic technique, wherein an optically absorbing buffer is displaced by nonabsorbing ions, at once separates and determines nitrite.⁷⁰

VII. SUBSTRATES AND COMMODITIES

Nitrite has been determined in a wide variety of substrates and commodities. The most common of course is meat.^{11,12,22,23,27,30,32-35,37-40,43,45-48,51,52,54,58,60,61,64,65,67,78,82,84,88,122,124,133,141} Other substrates and commodities include water;^{24,63,75,96,101,107,115,116,124,128,131} vegetables;^{27,47,50,142} plant tissues,¹⁴³ including tea;¹⁴⁴ milk,^{44,100,145} including whey;¹⁴⁶ cheese;^{47,48} fish;^{42,48,49} cosmetics;^{110,123} dyes;⁶⁹ cutting fluids;¹⁴⁷ gun powder;¹¹⁹ soil;²⁷ saliva;¹⁴ air;^{108,113}

egg white, soil, and vegetable oils;³⁶ beet sugar juices;²⁸ blood;¹²⁷ and curing pickle.⁴⁰ A critical comparison of them again is not possible because only seldom have two or more of them been studied with respect to a given method of either sample preparation of nitrite determination. However, as suggested by previous discussions, the methods of determination almost all involve several steps of nitrite reaction, with interferences arising from compounds in the substrate or commodity. During the following discussion, it should be kept in mind that with very few exceptions nitrite does not occur in large quantities in foods or most other substrates. The exceptions are cured meats and fish, or vegetables in which casual contamination by nitrate-reducing bacteria has occurred. Thus, in many of the substrates or commodities listed above, the effect of the substrate on nitrite determination was studied in spiked samples, since the substrate itself had very little or no endogenous nitrite. In fact, nitrite does not commonly occur in nature, since nitrite is either oxidized to the more chemically stable nitrate, or reduced by bacteria and other microorganisms to ammonia.

Two of the most extensively studied materials, the commodity cured meats, and the naturally occurring substrate, water, represent opposite ends of the nitrite determination spectrum. Nitrite occurs in readily determinable quantities in cured meats (and fish) but the products are loaded with interfering materials, principally reductants, either endogeneous reductones or added ascorbate (erythorbate). In contrast, water has very low levels of nitrite, but almost no interferences. The solutions to these problems have been discussed previously. Fruits and vegetables normally contain very little or no endogenous nitrite, but do contain nitrate and ascorbate. Bacterial conversion of nitrate to nitrite usually produces only very little nitrite in addition, so that the problem becomes one of determining low nitrite concentrations in the presence of the major interfering compound, ascorbic acid. The extent to which residual ascorbate can interfere was observed by Kamm et al.⁴³ who added varying amounts of baby foods to a 5- μ g nitrite-nitrogen spike in a fixed volume of extract. With 2 g of strained prunes or applesauce they obtained almost total recovery of the spike, but with 50 g the yield was reduced to 3 and 12%, respectively. Fifty grams of carrots, liver, or chocolate custard resulted in ca 60% recovery. Adriaanse and Robbers²⁷ took special care to remove ascorbate by charcoal addition and found trace quantities of nitrite in kale, french beans, and spinach with satisfactory recovery of added nitrite spikes. Milk apparently has no endogenous nitrite,¹⁰⁰ and processed dairy products have only very low concentrations,^{44,48,146} most likely taken in from nitrogen oxides in the air during processing. Milk, and most dairy products, have very low ascorbate concentrations (approximately 10 ppm) and there are no significant interferences in nitrite determination, as attested to by the good recoveries of spikes from milk samples.^{44,100,145} Many other products containing low nitrite concentrations present no problems of nitrite determination. Cosmetics, oils, soil, urine, feeds, and gun powder are easily extracted with water or analyzed for nitrite directly, and spike recovery data indicate there are little or no interfering substances in these materials. With no interferences, the specificity of nitrite in the formation of diazo dyes makes colorimetric analysis particularly attractive.

Nitrite determination in beet sugar juices presented a unique problem,²⁸ the solution of which might apply to other commodities. Sulfite is added to sugar beet extracts as an antibacterial preservative but, being a reductant, interferes in nitrite determination. The addition of formaldehyde to form the sulfite addition product eliminated the sulfite interference, but formaldehyde concentrations higher than 0.3% in the reacting solutions interfered in color formation.

A. Summary of Substrate/Commodity Nitrite Determination

For the most part, nitrite determinations in various substrates and commodities have been treated as separate entities, although some analysts have tested their procedures on a number of materials. It does not appear likely, or even necessary, that a single method for everything

will be developed. It would not be rational to boil sea water for 2 hr or put milk through a procedure to remove ascorbate. In the author's opinion, it would be better to analyze substrates or commodities for known interferences, then tailor the sample preparation procedure to the material, rather than to try to develop a unimethod for all. Kamm's technique of varying sample size in a constant spike/volume would be good for detecting general interferences.⁴³ Our technique of differential color development^{54,61} is a good specific for ascorbate interference, and other similar techniques or criteria may be developed to aid in the task of finding the best and most appropriate methods of nitrite determination in given substrates or commodities.

VIII. CONCLUSION

In conclusion, I should like to reiterate and expand on some comments made earlier. As has been pointed out,¹⁴⁸ bringing an analytical or determination method into practice is an expensive process, which is true of research in general. In the recommendations to analytical chemists contemplating collaborative studies,¹⁴⁸ the author poses some hard questions. Under "Purpose" the questions are asked "Is the method needed?" and "by whom?" Unfortunately, all too frequently this author has read through some methods and ended up asking himself why the work was done at all. In one paper, the authors deprecate previous methods as being time consuming, and then proceed to give a method which requires, with dilutions, additions, centrifugations, transfers, etc., at least 2.5 hr of manipulations per sample. At least in the AOAC procedure (and others) during the heating and color development periods, the analyst has time to do other work, drink coffee, read the newspaper, etc. Proposed methods should be examined critically with respect to existent methods and only presented if they have clear-cut advantages, not just because they are different.

Another shortcoming, and the author has been guilty of this on occasion, is not using standard procedures in presenting material, including definitions, statistical analysis, comparisons, etc. For example, the table of sensitivities has some dubious values listed simply because the data were not given or were vague. In more than one instance, two different sensitivity values were given in a paper, and it was not apparent which value was applicable. While styles vary from journal to journal, there are standard definitions, terminology, procedures, and statistical methods available.¹⁴⁸⁻¹⁵² Authors should use them; reviewers should insist on their use.

A. Suggested Reading

The discoverer of the most universal colorimetric reaction used today for nitrite determination was Johann Peter Griess, who is also originator of the dyestuff industry. There were of course many dyestuffs before his time, but the discovery of the diazo dyes laid the foundation of the synthetic dye industry. I recommend Cliffe's biography¹⁵³ of Griess to the reader. Usher and Telling¹⁵⁴ have covered some aspects of nitrite determination I have only hinted at or touched lightly upon. I have more or less tried not to repeat their conclusions, but rather to update and augment their review.

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