Automatic Colorimetric Determination of *N*-Nitroso Compounds

In this paper, a procedure is described for automatic analysis of any type of *N*-nitroso structure. The new method has been adapted from the classical approach of cleavage of the compound by uv irradiation followed by analysis of released nitrite by diazotization and coupling to form a dyestuff. Automation of the procedure has increased the sensitivity relative to background noise level and eliminated numerous sources of analytical error. When the irradiation used is in the region of 250 nm, the method is not entirely specific and problems arise when samples contain high concentrations of nitrate or *C*-nitroso structures. However, if the irradiation is in the region of 360 nm, the specificity is greatly increased with only a small sacrifice in yield, and the problem of interference by nitrate is eliminated. The analyzer is sufficiently sensitive for individual samples and chromatographic eluents, and has already proved extremely useful in studies of formation and decomposition mechanisms of several *N*-nitroso compounds.

The toxicity and carcinogenicity of most compounds with an *N*-nitroso structure are well established (Druckery *et al.*, 1967; Magee and Barnes, 1967); these compounds have recently been indicated as potential environmental health hazards. Thus it would be useful to have a method to screen large numbers of food and beverage samples at a reasonably low concentration level and in a convenient sample size.

The most specific, sensitive, and quantitive methods available are those which utilize gas chromatography (gc) and mass spectrometry after a suitable cleanup procedure (Howard *et al.*, 1970); the possibility also exists for highly specific gc detectors (Rhoades and Johnson, 1970). These methods, however, are tedious and are applicable only to volatile compounds.

Methods which may be more suitable for repetitive analysis, although they may be equally time-consuming, include the thin-layer chromatographic procedures modified from the initial work of Preussmann and coworkers (1964; modified by Sen *et al.*, 1969); the polarographic procedures (Heath and Jarvis, 1955; Walters *et al.*, 1970); and possible combinations. These methods are also useful for determination of nonvolatile compounds.

Since we found the polarographic procedures relatively nonspecific and the half-wave potential varied considerably according to the structure of the compound, we decided to modify the procedure introduced by Daiber and Preussmann (1964) which utilized the Griess Reagent to measure the amount of nitrite released from a compound after uv irradiation. This paper describes such a procedure which can be used either to process large numbers of individual samples or to monitor the eluate of a chromatographic column (Eisenbrand *et al.*, 1970). An automatic method for determining nitrate and nitrite in water and soil (Henriksen and Selmer-Olsen, 1970) and a comparison of 52 spectrophotometric methods for the determination of nitrite (Sawicki *et al.*, 1963) have been previously reported.

EXPERIMENTAL

Reagents and Chemicals. Dimethylnitrosamine (DMN) and diethylnitrosamine (DEN) were obtained from commercial sources (Eastman Organic Chemicals, Rochester, N.Y.). *N*-Nitrosopyrrolidine (NPy), *N*-nitrosoproline (NPr), and *N*-nitrososarcosine (NS) were synthesized from the parent amines and nitrite (White, 1955).

Sulfanilic acid reagent was prepared by adding 1 g to 50 ml of aqueous acetic acid (30% v/v), stirring for 1 min, and filtering. *N*-1-Naphthylethylenediamine dihydrochloride (0.2 g) was dissolved in 100 ml of 30% acetic acid.

All buffers were prepared from 0.1 *M* citric acid and 0.2 *M* K_2HPO_4. All other chemicals were reagent grade.

Automatic Analyzer. The flow diagram for the analyzer is shown in Figure 1. All equipment (except irradiator) and all tubing are of standard variety as supplied by the Technicon Co. (Tarrytown, N.Y.) and correspond to models listed under Autoanalyzer I. The uv irradiator consists of a black box fitted with two germicidal lamps (General Electric Co., Model G 15T8: short-wave; or Model F 15T8: long-wave). In the irradiator, the sample passes through a quartz coil (Syncor, Inc., Malden, Mass.) of the following dimensions: length, 6.5 ft; coil diameter, 2 in.; tubing inside diameter, 0.11 in.; tubing outside diameter, 0.19 in. The residence time in the irradiator was fixed at 30 min and the distance from the coil to the uv lamps was variable, although nominally 2 in. above the coil (energy density of 2.7 mW/cm^2).

To obtain standard curves, solutions of *N*-nitroso compounds were prepared in buffers or in water over the concentration range 1 to 100 $\times$ 10^{-6} mol/l. (1–100 μM). The solutions were then placed in sample cups for introduction into the analyzer. The sample volume was approximately 0.25 ml.

The uv irradiation process converts *N*-nitroso compounds to a variety of products, depending upon conditions (Chow, 1967), but nitrite is a major final reaction product under conditions of sufficiently high hydroxide ion concentration (Daiber and Preussmann, 1964). The irradiated stream is then mixed with sulfanilic acid and naphthylethylenediamine to form the coupled azo dye. The absorbance of the solution is then measured at the absorption maximum, 550 nm.

The analysis of nitrite and some other types of compounds (*e.g.*, alkyl nitrites) can be accomplished with the same system by simply bypassing the uv irradiator.

RESULTS

Standard Curves. Each of the *N*-nitroso compounds gave linear calibration curves of absorbance *vs.* concentration over the entire concentration range explored. The yields, however, were considerably less than 100% and were influenced by the pH of the sample solution.

The yield of each compound after short-wave irradiation in water and in pH 7 buffer is compared to that of nitrite in Table I. The results are expressed as absorptivities on a molar basis, and the yields can be calculated by comparison to the molar absorptivity of nitrite.

The complex pH behavior of nitrite and dimethylnitrosamine is given in Figures 2 and 3. The nitrite curves in Figure 2 are derived from irradiated and nonirradiated samples and indicate a constant pH-independent loss of nitrite due to

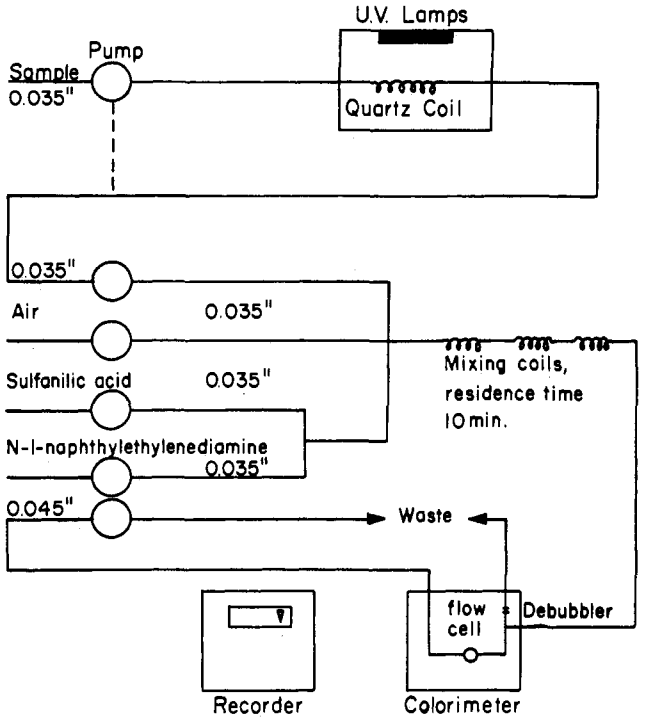

Figure 1. Flow diagram of apparatus for automatic analysis of *N*-nitroso compounds

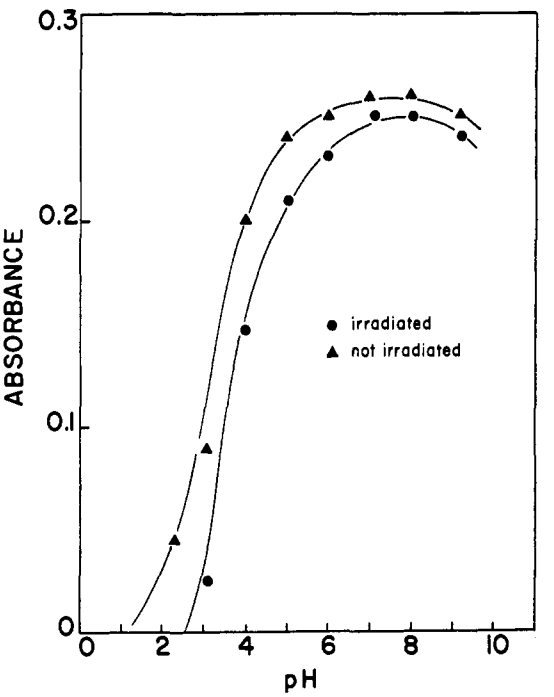

Figure 2. Influence of pH on analysis of nitrite

uv irradiation. The curve for DMN is typical of that of the other *N*-nitroso compounds, which all have linear portions in the pH range 5 to 8. For this reason, the Δ absorbance/Δ pH for each compound studied is given in Table II. Although increased sensitivity could be gained by using pH 8 buffers, a working condition of pH 7 was chosen to give leeway for possible pH variations in the samples.

Interfering Compounds. To determine whether salts of organic and inorganic acids, sugars, nucleoside bases, amino acids, and other compounds would interfere with the analysis, 0.1% solutions of the following compounds were tested: (salts and buffers) KH phthalate, Na acetate, KH_2PO_4, K_2HPO_4, K_2SO_4, Na tartrate, $NaNO_3$, NH_4 oxalate, NaCl, Na citrate, Na formate; (other biochemicals) glucose, fructose, proline, creatine, methionine, cysteine, histidine, tryptophan, *N*-acetylglucosamine, *N*-acetylarginine, arginine, sarcosine, and uracil. None of the compounds listed gave a color reaction with short-wave irradiation, or interfered with the reaction of *N*-nitroso compounds, with the exception of nitrate. Nitrate gives a color reaction only if the sample is introduced through the irradiator, equivalent to a 0.0167% yield of nitrite in distilled water and 0.157% in pH 7 buffer. Therefore, high concentrations of nitrates would be of significance, and the ubiquity of these salts would require special precautionary procedures.

Following the recommendation of Sander (1967), we converted the irradiator to long-wave uv (short-wave ≃250 nm; long-wave ≃360 nm) to eliminate nitrate interference. Under these conditions no detectable nitrite was formed from solu-

Table I. Molar Absorptivity Yields of *N*-Nitroso Compounds[a]

Compound	Absorptivity, l./mol × 10^3	
	Water	pH 7 Buffer
Dimethylnitrosamine	3.3	3.5
Diethylnitrosamine	2.3	3.2
N-Nitrosopyrrolidine	2.1	2.9
N-Nitrosoproline	2.0	3.1
Sodium nitrite (irradiated)	5.3	5.5

[a] Short-wave uv.

Table II. Influence of pH on Absorbance of *N*-Nitroso Compounds[a]

Compound	Δ A/Δ pH
Dimethylnitrosamine	0.035
Diethylnitrosamine	0.020
N-Nitrosopyrrolidine	0.045
N-Nitrosoproline	0.030

[a] Only over pH range 5 to 8.

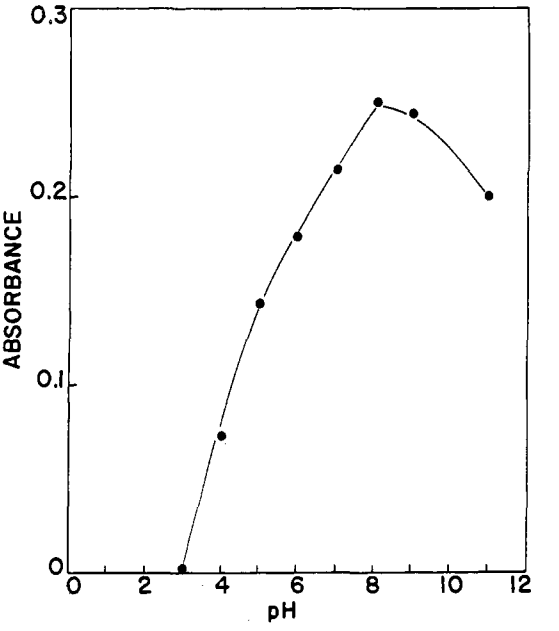

Figure 3. Influence of pH on analysis of dimethylnitrosamine

tions that contained 1000 ppm of nitrate; the molar absorptivity yield of DMN was reduced to 1.5×10^3. Nitrososarcosine also yielded 1.5×10^3.

Application of the Analyzer to the Analysis of Nitrite and *N*-Nitroso Compounds. CASE 1: *N*-NITROSO COMPOUNDS, NO INTERFERENCE BY NITRITE. The sample pH should be known and be in the pH range 5 to 8. The sample is introduced through the irradiator section and its concentration determined with the appropriate calibration curves.

CASE 2: NITRITE, NO *N*-NITROSO COMPOUNDS. The pH of the sample is measured and controlled as above and the sample is introduced after the irradiator.

CASE 3: *N*-NITROSO COMPOUNDS AND NITRITE. The sample is analyzed with and without irradiation, and the sample pH is controlled as in Case 1. The absorbance recorded for the irradiated sample is the sum of that yielded from the *N*-nitroso compound and that from the nitrite. Since some nitrite is lost in the irradiation process, the actual nitrite contribution to the absorbance of the irradiated sample must be corrected for this loss (Figure 2) calculated from the absorbance of the unirradiated sample. Appropriate pH corrections can be made, if necessary, and the concentration of *N*-nitroso compound can be calculated from the corrected absorbance.

DISCUSSION

Several significant improvements in the colorimetric procedure for *N*-nitroso compounds are provided by conversion to automatic analysis. No corrections are necessary for evaporative weight losses in the sample. Problems of unevenness of the irradiation field are eliminated. Since the samples are irradiated in the absence of air, background absorbance is reduced almost to zero (in the presence of air, nitrogen oxides are formed which leads to introduction of exogenous nitrite into the irradiated sample). Irradiation time is controlled, so that yields and background noise are constant.

In addition, problems of variable sample pH and of possible interference by nitrite in the sample had not been previously considered. The pH behavior is readily interpretable from the chemistry of the process. In the irradiation process, the *N*-nitroso compound is usually postulated to yield a short-lived species, NO or NO^+. Under acidic conditions, this species is thought to be recaptured by the elimination product of the parent compound (Chow, 1967), but under conditions of lower acidity, there will be competition from hydroxide ions to yield HONO. Since the pK for that acid is approximately 3.4, at pH's above 4, the acid will exist almost entirely as NO_2^-, effectively driving the reaction to form nitrite.

The final pH of the mixture of sample and color reagents also determines the rate of color formation for a fixed nitrite concentration. Although the diazotization reaction of sulfanilic acid proceeds at low pH, the rate of the coupling reaction (with the aromatic amine) increases with increasing pH, until the concentration of free amine is a maximum (Fieser and Fieser, 1956). Thus the yield for a fixed reaction time would increase over the pH range 2 to 6. Similar arguments can be used to explain the decreased yields at sample pH's above 9, due to effects on the rate of the diazotization reaction.

Certain problems of interference have not been solved, and may require chromatographic separation or cleanup. Walters *et al.* (1970) have demonstrated interference qualitatively by a variety of compounds containing the NO group (nitramines, *C*-nitro compounds, alkyl nitrites, and nitrates). Interference by alkyl nitrites can be corrected by procedures similar to those used for nitrite salts. If the other types of compounds prove to be fairly ubiquitous, one might resort again either to chromatographic procedures or to the use of long-wave uv irradiation, which we have confirmed eliminates the problem of interference by nitrate. Sander (1967) has also indicated that long-wave uv is relatively specific for compounds having the N–NO structure, and is therefore insensitive to C–NO compounds.

The present method provides a linear response to concentrations of *N*-nitroso compounds of less than 1 ppm. This is also the approximate sample concentration required for gas chromatographic analysis with a flame ionization detector (Issenberg, 1971), although the total volume of sample required for gc may be five times less. The selective gc detector of Rhoades and Johnson (1970) would provide even more sensitivity at a lower background noise level. Although a range-expander on the recorder could increase the sensitivity of our analyzer, in practice it is the signal/noise ratio that establishes the actual detection limit. On this basis, it appears that combining the present method with an appropriate liquid chromatographic separation would allow analyses of nonvolatile *N*-nitroso compounds at concentration levels similar to those achievable for volatile compounds with gc and an ionization detector. At the same time, the method will be immediately useful for studies of the kinetics of *N*-nitroso compound formation and decomposition.

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