Analysis of Nitrosamines in Aqueous and Biological Fluids Based on Measurement of Photochemically Liberated Nitrite

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Abstract ☐ A method is described for the analysis of nitrosamines in aqueous solution and in biological fluids (blood, plasma, and rat liver microsomal suspensions). The method is based on photochemical degradation of the nitrosamine in a controlled environment to yield the corresponding amine and nitrite ion, and the latter is subsequently used to form a chromophoric or fluorescent product. The analysis scheme is a modular three-component system consisting of a column to remove contaminating nitrite prior to photolysis, a photochemical reactor, and a chemical reactor. Additional modules are used to accommodate biological samples or large-volume (5–50 ml) aqueous samples. In this study, *N*-nitrosopyrrolidine, *N*-nitrosodimethylamine, and *N,N*-diethanolnitrosamine were utilized as substrates. Because of intersubstrate variability in the photochemical decomposition rate and overall nitrite yield, the structure (*i.e.*, photochemical behavior) of the particular nitrosamine in the sample must be known prior to analysis. With a colorimetric readout, the sensitivity for analysis of *N*-nitrosopyrrolidine was 800 ng/ml for a 5-ml sample and the measurement precision was ±6% in the biological fluids. Fluorometric analysis improved sensitivity to 4 ng/ml with a precision of ±10% in biological media.

Keyphrases ☐ Nitrosamines—analysis, chromophoric or fluorescent products of photochemical degradation, aqueous suspensions, biological fluids, rats, humans ☐ Carcinogens—nitrosamines, analysis, chromophoric or fluorescent products of photochemical degradation, aqueous suspensions, biological fluids, rats, humans ☐ Colorimetry—analysis, nitrosamines, aqueous suspensions, biological fluids ☐ Fluorometry—analysis, nitrosamines, aqueous suspensions, biological fluids

Nitrosamines are highly carcinogenic compounds (1). They are receiving considerable attention and concern because of their ubiquitous occurrence in the environment (2), food (3), cosmetics (4), and other matrixes. To evaluate the hazards of nitrosamines, adequate analytical methods are needed for their detection and quantitation. Such methods must be extremely sensitive, must give unequivocal and reproducible results, and must be applicable to a wide range of nitrosamines of varying physicochemical properties and in various matrixes.

Nitrosamines are monitored routinely by mass spectrometry (5) or thermal energy analysis (6, 7) after isolation by GLC or high-performance liquid chromatography (HPLC). In these cases, extensive cleanup has been required prior to chromatography. Nitrosamines also have been detected by polarography (8) or spectrophotometry after chemical derivatization (3, 9). These methods also have required extensive cleanup and enrichment and were not specific when applied to complex mixtures.

This report describes a method for nitrosamine analysis at nanograms per milliliter levels in various complex matrixes including water, human plasma, and liver homogenates. Analysis is based on the method described by Daiber and Preussmann (10), in which the nitrosamine is photochemically cleaved to yield an amine and nitrite ion; the nitrite is detected spectrophotometrically after diazotization of an amine and conversion of the resulting diazonium salt to an azo dye.

In this paper, emphasis is on cleanup and enrichment of samples containing nitrosamines to achieve selectivity and high sensitivity. *N*-Nitrosopyrrolidine, *N,N*-diethanolnitrosamine, and *N*-nitrosodimethylamine were used as model compounds.

EXPERIMENTAL

Reagents—*N*-Nitrosopyrrolidine[1], 2,6-diaminopyridine[1], *p*-chloroaniline[1], 1-decanol[2], *N*-(1-naphthyl)ethylenediamine dihydrochloride[2], and sulfanilamide[2] were used as received. *N*-Nitrosodimethylamine (11) and diethanolnitrosamine (1, 12) were synthesized by condensation of dimethylamine and diethanolamine with nitrous acid.

Column Preparation—XAD-2 beads[3] (20–50 mesh) were purified by 30-min successive washes with 5.25% sodium hypochlorite, double-distilled water (distilled from alkaline permanganate), 3 *M* HCl, water, and acetone. Fractured beads were removed from the acetone solution by decantation, and the remaining beads were isolated by filtration. Forty-five milliliters of the purified beads was slurried with acetone containing 3 ml of 1-decanol as described previously (13). The acetone was removed under reduced pressure on a rotary evaporator, and the beads (coated with decanol) were packed into a column made from a disposable pipet (14.6 cm × 7.6 mm o.d.).

Sample Purification—A sample of human plasma or rat liver homogenate (14) containing nitrosamine was subjected to ultrafiltration[4] at 1000×*g* for 30 min to deproteinize the mixture. The resulting ultrafiltrate was either passed directly through a column packed with anion-exchange resin (0.5 g AgI, 200–400 mesh, HO^-) or the pH of the ultrafiltrate was adjusted to 1.7 with 6 *N* H_2SO_4 and the mixture was passed through an XAD-2/decyl alcohol column (13).

The eluent was adjusted to pH 5–7, if necessary, and added to quartz vessels. These vessels were placed 16 cm from a 15-w, 46-cm germicidal lamp[5] (maximum output at 250 nm), and the mixtures were irradiated for 15 min. The freed nitrite could be detected colorimetrically or fluorometrically. Alternatively, the nitrite in the irradiated samples could first be concentrated on an XAD-2/decyl alcohol column (13), and the resulting decyl nitrite could be monitored colorimetrically or fluorometrically after its elution from the column with 1.2 ml of acetone.

Nitrite Detection—*Colorimetry (15)*—Sulfanilamide (0.15–0.50 ml of a 5% solution) was added to the acetone eluate (containing the decyl nitrite) or directly to the photolysis mixture (after acidification to pH 1.7) containing the liberated nitrous acid. The solution was mixed thoroughly and allowed to stand for 1 min. An equal volume of *N*-(1-naphthyl)ethylenediamine (0.25% solution) was added, and the absorbance of the final solution was measured[6] at 545 nm (a_m 4×10^{-5}) after 2 min was allowed for complete color development.

Fluorometry—Nitrite or decyl nitrite was monitored using a modification of the Dombrowski and Pratt procedure (16), in which either the photolysis mixture containing nitrous acid (after pH adjustment to 1.7) or the decyl nitrite (eluted from the XAD-2/decyl alcohol column with acetone) was used to diazotize *p*-chloroaniline (0.3 ml of a 0.06% solution in 0.1 *M* HCl). Samples in which the diazonium salt was formed from decyl nitrite were extracted with benzene (6 ml) to remove decyl alcohol.

All samples were reacted with ammonium sulfamate (0.3 ml of a 4%

[1] Aldrich Chemical Co., Milwaukee, Wis.
[2] Eastman Chemical Co., Rochester, N.Y.
[3] Rohm & Haas Co., Philadelphia, Pa.
[4] CF-50A conical filters, Amicon Corp.
[5] General Electric Corp.
[6] Cary model 118 spectrophotometer, Varian Associates, Palo Alto, Calif.

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Table I—Kinetics for Photochemical Nitrosamine Decomposition and Yield of Nitrite Formed [a]

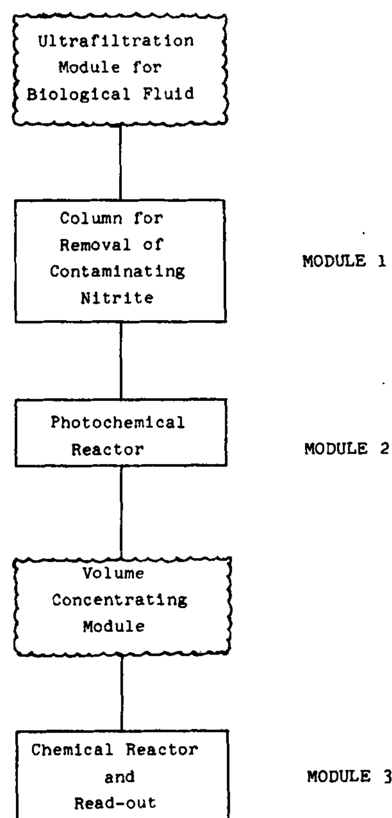
Compound	Fluid	Rate Constant ± SD^b, min^{-1}	Yield of Nitrite[c] ± SD^b, %
N-Nitrosopyrrolidine	Distilled water	0.291 ± 0.029	45.3 ± 5.57
	0.1 *M* H_2SO_4	0.513 ± 0.149	9.7 ± 2.20
	0.1 *M* NaOH	0.275 ± 0.007	51.1 ± 5.23
	Buffer pH 3[d]	0.443 ± 0.081	32.7 ± 3.93
	Buffer pH 5[d]	0.360 ± 0.014	34.8 ± 3.87
	Buffer pH 7[e]	0.274 ± 0.073	40.8 ± 2.86
	Buffer pH 9[e]	0.266 ± 0.084	34.3 ± 0.85
	Plasma[f]	0.18 ± 0.084	33.3 ± 8.7
	Liver microsomes[g]	0.24 ± 0.056	28.5 ± 3.1
N-Nitrosodimethylamine	Distilled water	0.154 ± 0.017	59.8 ± 0.83
	Buffer pH 7[e]	0.382 ± 0.014	56.3 ± 3.12
	Plasma[f]	0.140 ± 0.014	31.9 ± 2.53
	Liver microsomes[g]	0.067 ± 0.008	30.3 ± 2.63
N,N-Diethanolnitrosamine	Distilled water	0.411 ± 0.033	41.6 ± 1.40
	Buffer pH 7[e]	0.529 ± 0.017	41.9 ± 6.22
	Plasma[f]	0.140 ± 0.014	35.4 ± 0.30
	Liver microsomes[g]	0.134 ± 0.040	31.2 ± 0.30

[a] Reaction carried out as described under *Experimental*. [b] Values represent the mean ± *SD* for three determinations. [c] Yield was determined spectrophotometrically at 545 nm and arose from the azo compound produced by successive reaction of nitrite with sulfanilamide and *N*-(1-naphthyl)ethylenediamine. [d] Acetate buffer (0.5 *M*). [e] Britton–Robinson buffer. [f] Obtained from the Kansas City Blood Bank and used as received. [g] Prepared from rat liver as previously described (14).

solution) followed by 2,6-diaminopyridine (0.05% solution prepared in 1 *M* acetate buffer, pH 5). The reaction was continued for 15 min, and the resulting azo dye was extracted with benzene (6 ml) and washed with water (6 ml). The benzene layer was evaporated to dryness under a nitrogen stream. The yellow residue was redissolved in water (2 ml), ammoniacal cupric sulfate (0.4 ml of a 0.725 *M* aqueous solution) was added, and the solution was heated in boiling water for 30 min. The mixture was acidified with 0.4 ml of 6 *M* HCl to yield a triazole, which was monitored fluorometrically[7] at 420 nm ($\lambda_{excitation}$ = 360 nm).

RESULTS AND DISCUSSION

Analysis of nitrosamines in water and biological fluids (human plasma and rat liver microsomal suspensions) will be described as a modular system (Scheme I) requiring at least three components: (*a*) a column packed with either anion-exchange resin or XAD-2 beads coated with decyl alcohol to remove any nitrite contaminants, (*b*) a photochemical reactor to convert nitrosamines to the corresponding amine and nitrite ion, and (*c*) a chemical reactor to transform the liberated nitrite into a readily monitored product. In this system, the nitrite was used to diazotize a primary aromatic amine and the diazonium salt was coupled with a reactive arene to yield a product that could be measured colorimetrically or fluorometrically, depending on the amine-coupling agent pair chosen.



Scheme I—Modular nitrosamine analysis system (〰, optional modules)

If nitrosamines are to be analyzed in biological fluid (human plasma rat liver microsomal suspensions), an ultrafiltration module must be introduced into the modular system (Scheme I). Biological samples were initially deproteinized by centrifugal ultrafiltration and then processed through Modules 1–3. For nitrosamine analysis in large-volume (>5 ml) aqueous samples, a second column packed with XAD-2 beads coated with decyl alcohol was introduced after the photolysis step to concentrate the liberated nitrite by extractive alkylation (13) prior to diazotization. Where sample size was restricted (<5 ml), sample concentration and cleanup were carried out primarily after diazotization.

Sample Purification—Samples of biological fluid containing *N*-nitrosopyrrolidine, *N*-nitrosodimethylamine, or *N,N*-diethanolnitrosamine (1–2000 ng/ml) were deproteinized by centrifugal ultrafiltration through membranes that excluded material of molecular weight ≥50,000. This procedure removed ≥98% of the protein, *i.e.*, material responsive to the biuret or Lowry assays (17). When aqueous buffer samples containing these nitrosamines (10–10,000 ng/ml) were carried through analysis before and after ultrafiltration, no statistical difference in nitrosamine levels was observed, indicating that the nitrosamine was not retained by the filter. Deproteinized samples were carried through the analysis scheme as described for aqueous samples.

Because the final analysis of nitrosamines in all matrixes was based on measuring liberated nitrite, it was necessary to ensure that any residual, contaminating nitrite was removed prior to photolysis. This elimination could be accomplished by passing the ultrafiltrate or aqueous sample through a column packed with an anion-exchange resin or through a column containing XAD-2 beads coated with decyl alcohol. Failure to deproteinize biological samples prior to applying samples to these columns resulted in erratic and incomplete (<35%) nitrite removal.

The anion-exchange resin quantitatively removed added nitrite from the solution (as well as other anions such as nitrate); however, some nitrosamine was also retained by the resin (Fig. 1). Total nitrite retention by the column was observed, even at the 5-μg/ml concentration when nitrite was present in a 500-fold *M* excess with respect to nitrosamine. The amount of nitrosamine held by the resin was a function of the amounts of nitrosamine and resin present. The amount of resin was optimized so that nitrite was quantitatively removed, but the loss of nitrosamine was minimized. With the assumption of nitrosamine concentrations of 1–1000 ng/ml and nitrite levels of 5 μg/ml, 0.5 g (wet weight) of anion exchanger gave optimum response: quantitative removal of nitrite and 25 ± 4% loss of nitrosamine.

When nitrite decontamination was carried out by passing the sample through a column packed with XAD-2 beads coated with decyl alcohol, the nitrite removed from the solution was inversely proportional to the

[7] Model MPF-2A spectrofluorometer, Perkin-Elmer Corp., Norwich, Conn.

Table II—Colorimetric Assay [a] for N-Nitrosopyrrolidine

Fluid	Standard Curve [b] Slope	Intercept	Regression Coefficient	Sensitivity, ng/ml	Precision [c], %
		Method I			
Water	0.057	0.048	0.998	800	5.7
Plasma [d]	0.065	0.023	0.997	800	9.8
		Method II			
Water (10-ml sample)	0.049	0.065	0.997	200	6.6
Water (100-ml sample) [e]	0.017	0.124	0.993	15	4.3

[a] As described under *Experimental*. [b] Standard curve was prepared from five 5-ml samples of N-nitrosopyrrolidine dissolved in the indicated fluid in the concentration range of 0.75–20 μg/ml and carried through the described assay. Each sample was assayed in triplicate. [c] Values represent the precision at the minimum sensitivity and represent the average for four determinations. [d] Obtained from the Kansas City Blood Bank and used as received. [e] Standard curve was prepared from five 5-ml samples of N-nitrosopyrrolidine dissolved in the indicated fluid in the concentration range of 1–50 ng/ml and carried through the described assay. Each sample was assayed in triplicate.

Table III—Fluorometric Assay [a] for N-Nitrosopyrrolidine (Method I)

Fluid	Standard Curve [b] Slope	Intercept	Regression Coefficient	Sensitivity, ng/ml	Precision [c], %
Water	4.26	7.4	0.99	4	9.9
Plasma [d]	0.21	25.0	0.99	40	9.6
Liver microsomes [e]	0.20	8.0	1.00	80	12.0

[a] As described under *Experimental*. [b] Standard curve was prepared from five 5-ml samples of N-nitrosopyrrolidine dissolved in the indicated fluid in the concentration range of 1–250 ng/ml and carried through the described assay. Each sample was assayed in triplicate. [c] Values represent the precision at the minimum sensitivity and are the average of four determinations. [d] Obtained from the Kansas City Blood Bank and used as received. [e] Prepared from rat liver as previously described (14).

flow rate through the column, as previously discussed (13). At a flow rate of 44 ml/hr, solutions containing N-nitrosopyrrolidine (1–20 μg/ml) were cleared of 70 ± 2% of added nitrite (4.6 μg/ml) (Fig. 1), with no detectable loss of nitrosamine from solution. Thus, the anion exchanger is a more efficient medium for removing contaminating nitrite but suffers from the disadvantage of retaining some nitrosamine. The XAD-2 column does not retain any nitrosamine but, unfortunately, incompletely removes nitrite contaminants.

Varying combinations of ion-exchange resin and XAD-2 beads coated with decyl alcohol did not improve nitrosamine recovery and concomitant nitrite removal. The major difficulty was the dependency of on-column nitrosation on an acidic (pH ≤3) environment and ion-exchange removal of nitrite optimally requiring a medium of pH ≥ 5.

In all cases, ion-exchange and XAD-2 columns were not reused, so problems with residuals were not encountered.

Nitrite Liberation from Nitrosamine—Initial studies were carried out to determine the feasibility of cleaving nitrosamines to amines and nitrous acid with acid (18). However, when N-nitrosopyrrolidine and N-nitrosodimethylamine were heated in a sealed vial with acid (hydrochloric, hydrobromic, or sulfuric), inconsistent (±20%) and low (≤40%) yields of nitrous acid were obtained (monitored colorimetrically). In separate experiments under acid hydrolysis conditions, nitrite was consumed as a function of time, perhaps through oxidation by a contaminant in the acid.

A more satisfactory method for liberating nitrite from nitrosamines involved photolysis (10). The formation rate and yield of nitrite were functions of the particular substrate being analyzed, light intensity, wavelength, pH, and photolyzed sample matrix. As shown in Table I, the nitrite formation rate was faster at low pH than at neutral or high pH; however, the nitrite recovery was poorer from acidic solutions and improved at higher pH values. Conditions were chosen that produced the maximum nitrite yield, *i.e.*, pH 6–7. The nitrite liberation rate was sufficiently rapid over the pH 1–13 range to suggest that the reaction speed could be sacrificed in favor of maximum conversion to nitrite.

The nitrite yield from photolysis of aqueous nitrosamine solutions varied among the three model compounds studied; however, the reproducibility of the nitrite yield from sample to sample for a given nitrosamine was high (±3%). This variability in nitrite yield, however, precluded nitrosamine analysis of samples containing more than one nitrosamine or nitrosamines whose response to photolysis was unknown. The nitrite yield from biological fluid samples (blood plasma or liver microsomal homogenates) containing nitrosamine did not show as much variability among the three model substrates as did the water samples. An average of 31.9 ± 2.6% nitrite was obtained from photolysis of plasma and liver microsomal suspensions containing dimethyl-, diethanol-, or pyrrolidinenitrosamine. The nitrite yield from nitrosamines present in ultrafiltrates from biological samples was ~50–80% of that obtained in buffers of similar pH to the ultrafiltrate. The nitrite production was also somewhat slower in ultrafiltrate than in buffer. This finding may have been due to extraneous material in the ultrafiltrate lowering the photon flux available for reaction with the nitrosamine. The lamp output and the distance between the lamp and sample cell were chosen to give maximum nitrite recovery. A lamp with maximum intensity at 250 nm placed 16 cm from the quartz reaction vessel gave the optimum rate and nitrite yield.

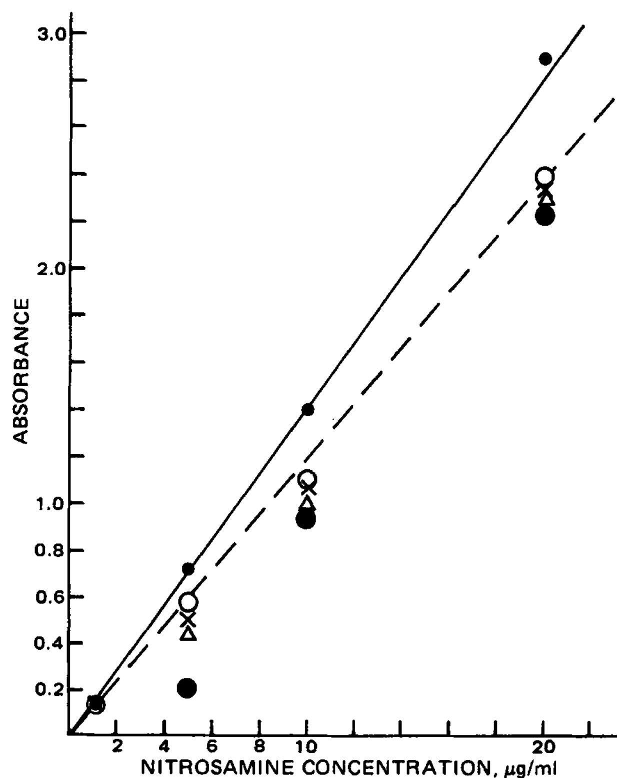
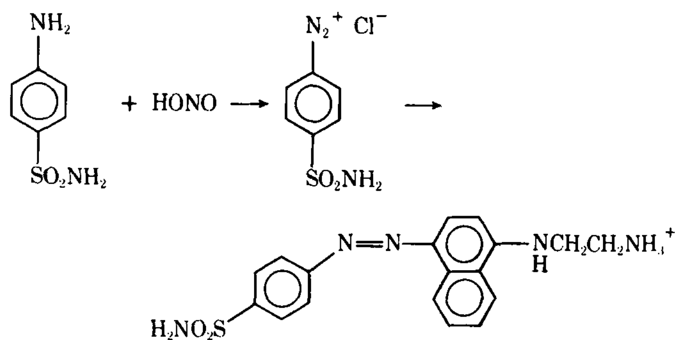


Figure 1—*Loss of nitrosamine to the anion-exchange column during removal of background nitrite prior to photolysis. Loss was determined as a function of total amount of exchange resin. Key:* ●, *no resin;* ○, *0.25 g of wet weight;* ×, *0.5 g of wet weight;* △, *1 g of wet weight; and* ●, *2 g of wet weight.*



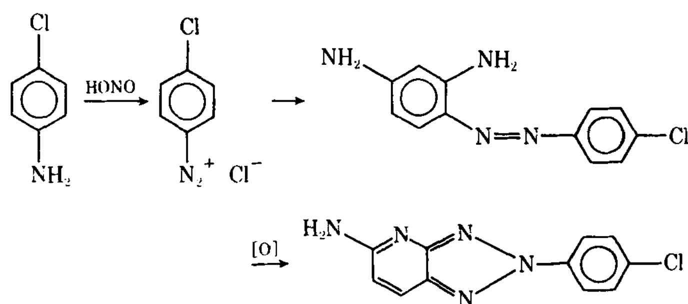
Scheme II

Detection of Liberated Nitrite—*Method I*—When the available sample size was limited (≤5 ml, *e.g.*, biological samples), liberated nitrite was reacted immediately with an aromatic amine under acid conditions to produce a diazonium salt, which was then coupled with an arene to yield either a chromophoric or a fluorescent product (Scheme II). The colorimetric assay was based on diazotization of sulfanilamide and coupling with *N*-(1-naphthyl)ethylenediamine. The reaction proceeded rapidly and could be carried out with minimum sample manipulation. The sensitivity attained using this method was ~800 ng of nitrosamine/ml of solution (Table II).

Sensitivity could be improved greatly by fluorometric monitoring of liberated nitrite (Scheme III). In this case, the nitrite was reacted with *p*-chloroaniline and the resulting diazonium salt was coupled with 2,6-diaminopyridine. This resulting azo compound was extracted into benzene, the benzene was removed by evaporation, and the residue was redissolved in water. The azo dye was oxidized to a fluorescent thiazole with ammoniacal cupric sulfate. Conversion to this fluorescent species permitted detection of nitrosamines at levels of 4 ng/ml in aqueous solution, 40 ng/ml in plasma, and 80 ng/ml in liver microsomes. In biological fluid, a precision of ±10% was readily attainable. The limitation preventing nitrosamine detection at lower levels by the fluorescence method was the appreciable background fluorescence (note the large intercept observed in standard curves constructed for fluorescence analysis of nitrosamine samples of varying concentration; Table III).

For both fluorescence and colorimetric analysis, quantitation was based on comparing detector response with a standard curve constructed for each nitrosamine in the matrix. The curve was prepared by carrying samples containing known amounts of the nitrosamine through the entire assay. The disadvantages of the fluorescence procedure were: (*a*) the extensive sample preparation required for fluorescence analysis and (*b*) the background fluorescence inherent in the method at low nanogram per milliliter concentrations.

Method II—For samples where sample volume was not limited (*e.g.*, water samples, pharmaceutical preparations, and food), nitrite, liberated by photolysis of nitrosamines, was first concentrated on a column packed with XAD-2 beads coated with decyl alcohol before being processed through the chemical reactor module (Scheme I). It was demonstrated previously (13) that when an aqueous solution of nitrous acid is passed through such columns, the nitrous acid is extracted into the decyl alcohol phase as decyl nitrite, formed on-column. An optimum flow rate of 44 ml/hr was reached at which 70 ± 2% of the nitrite present in a photolyzed sample (5–50-ml volumes) was retained on the column. With sample volumes >75 ml, however, the percent of nitrite retained by the column decreased in a nonlinear fashion, as discussed previously (13). Therefore, sample size was maintained below 100 ml.



Scheme III

The nitrite concentrated on-column in the form of decyl nitrite was eluted from the resin with 1.2 ml of acetone passed through the bed. The decyl nitrite present in the acetone eluate was then used to diazotize a primary aryl amine, as described for nitrous acid. Thus, a 50-ml aqueous sample can be concentrated to <2 ml, providing a 15-fold increase in sensitivity over that obtained when the XAD-2 decyl alcohol column module is not used. Some nitrite loss did take place with this column since only 70% of the nitrite passed through it was retained on-column as decyl nitrite, thus accounting for the observation that a decrease in sample volume by a factor of 25 only increased sensitivity by a factor of 15 (Tables II and III).

In conclusion, an analytical method has been described for the analysis of nitrosamines in aqueous solutions and biological fluids. No highly sophisticated instrumentation is required, and the methodology is adaptable to automation. The procedure is described as a modular system requiring a minimum of three components, but it can accommodate biological samples or large-volume aqueous samples by the introduction of additional modules. Sensitivity is adequate for many quality control applications.

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