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DETECTION AND DETERMINATION OF N-NITROSAMINES BY THIN-LAYER CHROMATOGRAPHY USING FLUORESCAMINE*

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SUMMARY

A novel procedure is described for the detection and determination of N-nitrosamines (NAs) on thin-layer chromatographic plates. Ultraviolet irradiation of NAs on activated plates (silica gel or aluminum oxide) yields primary or secondary amines, which after spraying with fluorescamine reagent give fluorescent or non-fluorescent products, respectively. The majority of the 24 NAs examined afforded fluorophors. Visual detection limits of 10–15 ng (75 pmoles) were observed for most NAs, although the volatile dimethyl-, diethyl- and pyrrolidine-N-nitrosamines gave limits of 500, 40, and 40 ng, respectively. Spectrophotometric determinations of the relative fluorescence of 0.1–40 nmoles of NAs gave rise to nearly linear calibration curves when plotted on a log–log scale.

INTRODUCTION

Detection of N-nitrosamines (NAs) on thin-layer chromatographic (TLC) silica gel plates has been accomplished by cleavage with ultraviolet (UV) light or HBr to give the corresponding secondary amines and nitrous acid or its derivative. The secondary amine is then detected by reaction with ninhydrin[1,2] and nitrous acid with Preussmann reagent ($PdCl_2$–diphenylamine)[3,4], Griess reagent (sulfanilic acid–1-naphthylamine)[4], or NEDSA reagent (sulfanilic acid–N-1-naphthylenediamine)[5] to give coloured spots. About 1 μg of NA can be detected with these reagents. Sen *et al.*[6] reported that N-nitrosopyrrolidine affords a fluorescent spot after UV irradiation and reaction with ninhydrin, and that 50 ng produces a clearly visible spot. Reduction of NAs with lithium aluminum hydride yields asymmetric hydrazines, which upon treatment with appropriate aldehydes show coloured[7,8] or fluorescent spots[9].

In the course of investigating the reduction of NAs to hydrazines, it was observed that after UV irradiation on silica gel TLC plates and subsequent spraying with fluorescamine, the NAs afforded fluorescent spots and/or non-fluorescent dark blue spots when viewed under a UV lamp. This result was unexpected. Fluorescamine, 4-phenylspiro[furan-2(3H),1-phthalan]-3,3-dione, reacts to form fluorescent

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products only with compounds containing a primary amino group[10], and as described above, on TLC plates NAs have been reported[1,2] to produce secondary amines. Non-fluorescent products have been obtained[11,12] from the reaction of fluorescamine and secondary amines. A survey of the literature[13,14] revealed that photolysis of NAs in acidic solution generally yields secondary amines or their products, although primary amines have been observed as minor products in a few cases[15–17].

The present study reports the utility of fluorescamine as a reagent for the detection and determination of NAs on TLC plates.

EXPERIMENTAL

Materials

Diethyl-, dipropyl-, dibutyl-, diphenyl-, carbazole-, and piperidine-N-nitrosamines were purchased from Fisher Scientific (Pittsburgh, Pa., U.S.A.); dimethyl- and pyrrolidine-N-nitrosamines were purchased from Aldrich (Milwaukee, Wisc., U.S.A.); and all other NAs were synthesized from the corresponding secondary amines (obtained from either Fisher Scientific or Aldrich) and sodium nitrite (E. Merck, Elmsford, N.Y., U.S.A.) by standard methods[18]. All samples were checked for purity prior to use by gas chromatography and gave infrared (IR), nuclear magnetic (NMR) and mass spectra consistent with those reported in the literature. Standard solutions of NAs in methylene chloride were freshly prepared and stored in a refrigerator. Solutions of 0.1 mg/ml fluorescamine (Hoffmann-La Roche, Nutley, N.J., U.S.A.) in acetone were prepared daily and stored at room temperature in a stoppered flask. The primary amines used were analytical standards obtained from PolyScience, (Evanston, Ill., U.S.A.). All other reagents and solvents were of reagent grade and used as received from commercial sources. The pre-coated TLC sheets used in this study were: Macherey, Nagel & Co. Polygram Sil G 0.25-mm silica gel without gypsum or indicator (Brinkmann, Westbury, N.Y., U.S.A.); Eastman Chromatogram Sheet No. 13179 0.1-mm silica gel without fluorescent indicator (Fisher Scientific); and Baker-flex 1B-F aluminum oxide (J. T. Baker, Phillipsburgh, N.J., U.S.A.).

Spectral determinations

IR spectra were determined in carbon tetrachloride on a Beckman IR-20A spectrometer. NMR spectra were determined in carbon tetrachloride on a Varian T-60 spectrometer. Mass spectra were determined on a Finnigan 9500 gas chromatograph (1.8 m × 6.5 mm O.D. glass column containing 3% SE-30 ultraphase on high-performance Chromosorb W, 80–100 mesh) coupled to a 3100D quadrupole mass spectrometer and a 6000 computer controlled data aquisition system.

Gas chromatography

Purity of NA standards were determined on a Pye 104 gas chromatograph with a flame ionization detector and 1.5 m × 6.5 mm O.D. glass column containing 5% Carbowax 20M on high-performance Chromosorb W, 80–100 mesh.

Fluorescence spectrophotometer

Spectrophotometric determinations were made on a Hitachi Perkin-Elmer

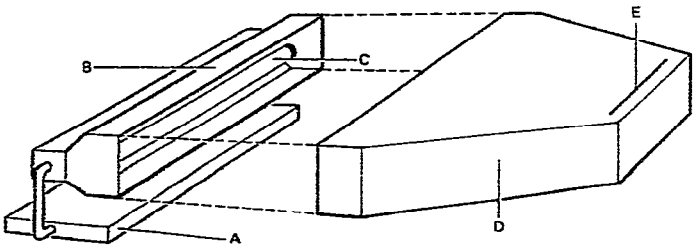

Fig. 1. Apparatus for UV irradiation of TLC plates (exploded view). A = stand; B = lamp shield; C = 18-in. UV germicidal lamp; D = cardboard enclosure lined with aluminum foil; E = 21-cm wide slit for lowering plates inside enclosure. Lamp C and slit E are 22 cm apart.

(Tokyo, Japan) Model MFP-2A fluorescence spectrophotometer equipped with a TLC accessory and strip chart recorder.

Ultraviolet light sources for irradiation

Several UV light sources were examined for their ability to cleave NAs photolytically on TLC plates. These included a Model SL 2537 9W short-wave mineral light (Ultra-Violet Products, San Gabriel, Calif., U.S.A.), a Model SL 3660 9W long-wave mineral light (Ultra-Violet Products), a 150-W Model 16200 mercury lamp (Hanovia, Newark, N.J., U.S.A.), and a 450-W Hanovia high-pressure mercury lamp. The best results, however, were obtained with a 15W General Electric 18-in. germicidal lamp to which the aluminum foil-lined cardboard enclosure shown in Fig. 1 had been attached.

Thin-layer chromatography

At first, the silica gel and aluminum oxide plates were stored in a dry atmosphere and used as received, but for reasons explained below, thereafter all plates were activated by heating at 110° for 1 h immediately to prior use. The plates were spotted with solutions of NA standards in methylene chloride, developed 8 cm in a suitable solvent mixture and visualized. Table I summarizes R_F values for the NAs in a variety of solvent mixtures on both silica gel and aluminum oxide plates.

The cleavage products from UV irradiation of NAs were visualized by a variety of methods. In the first[5], the developed plates were sprayed with 30% aqueous acetic acid, irradiated for 10 min, air-dried for 10 min, sprayed with a solution of 0.3% ninhydrin and 2% pyridine in ethanol, and heated at 80° for 10–15 min to give red-purple spots. In the second, the developed and irradiated plates were sprayed sequentially with fluorescamine solution and 10% triethanolamine[19] in methylene chloride and then viewed inside a dark chamber using a long-wave UV light source (Ultra-Violet Products). In the third[3], the developed plate was sprayed with a freshly prepared mixture of 1.5% diphenylamine in 95% ethanol–0.1% palladium(II) chloride in 0.2% aqueous sodium chloride (4:1) and irradiated for 10 min to give blue-violet spots. This last method was also modified, so that the developed plate was first irradiated and then sprayed, heated for 10 min at 80°, and viewed under long-wave UV light to give, in some instances, fluorescent spots.

Alternatively, the spotted plates were irradiated, developed in 95% ethanol–concentrated ammonium hydroxide (4:1), air-dried for 20 min, and then visualized.

TABLE I

THIN-LAYER CHROMATOGRAPHIC R_F VALUES AND VISUAL FLUORESCENCE DETECTION LIMITS OF N-NITROSAMINES ON SILICA GEL AND ALUMINUM OXIDE

Solvent systems A, B and C, hexane–ether–methylene chloride (4:3:2), (5:7:10), and (10:3:2), respectively; system D, ethyl acetate–hexane (4:1). Detection limit is the minimum amount of an N-nitrosamine (NA) that ultimately gives a detectable visual fluorescence. Range due to difference in visual acuity of the two observers. Determined on activated silica gel by spotting NA, developing, irradiating with UV light, spraying with fluorescamine reagent, and viewing under long-wave UV light. nf = Non-fluorescent products.

No.	*N-Nitrosamine*	*R_F value*				*Detection limit*	
		Silica gel			*Aluminum oxide*	*ng*	*pmoles*
		A	*B*	*C*	*D*		
1	Dimethyl	0.35	0.44	0.24	0.44	500*	7000
2	Diethyl	0.44	0.60	0.39	0.52	10–40**	100–400
3	Dipropyl	0.58	0.65	0.49	0.58	7–10	50– 75
4	Dibutyl	0.67	0.67	0.53	0.58	9–12	60– 90
5	Dipentyl	0.73	0.68	0.56	0.66	4– 6	20– 30
6	Dihexyl	0.76	0.76	0.57	0.68	9–12	40– 55
7	Diheptyl	0.77	0.78	0.58	0.69	9–12	35– 50
8	Dioctyl	0.79	0.79	0.59	0.70	8–11	30– 40
9	Diallyl	0.62	0.68	0.50	0.55	10–12	90–100
10	Di-iso-butyl	0.69	0.69	0.56	0.58	8–11	50– 75
11	Dicyclohexyl	0.66	0.76	0.52	0.59	12–14	55– 70
12	Dibenzyl	0.69	0.77	0.53	0.61	15–18	65– 80
13	Methyl, butyl	0.45	0.58	0.38	0.54	7–10	65– 85
14	Ethyl, butyl	0.55	0.62	0.47	0.55	8–11	60– 90
15	Methyl, phenyl	0.63	0.67	0.49	0.59	17–20	125–150
16	Propyl, phenyl	0.70	0.73	0.56	0.63	10–12	60– 75
17	Ethyl, benzyl	0.56	0.65	0.58	0.58	16–20	100–125
18	Phenyl, benzyl	0.70	0.75	0.55	0.60	7–10	35– 50
19	Pyrrolidinyl	0.41	0.50	0.36	0.51	20–40**	200–400
20	Piperidinyl	0.22	0.34	0.20	0.39	17–20	150–175
21	Diphenyl	0.71	0.75	0.57	0.63	nf	nf
22	Morpholinyl	0.21	0.33	0.23	0.39	nf	nf
23	N-Methyl piperazinyl	0.03	0.03	0.06	0.28	nf	nf
24	Carbazolyl	0.76	0.79	0.60	0.60	nf	nf

* If N-nitrosodimethylamine was not developed then detection limit was 10 ng (150 pmoles). Detection limits improved only slightly (*ca.* 25%) when non-volatile NAs were not developed.

** Range due to relatively high volatility of NA.

In some instances the spotted plates were sprayed with 30% aqueous acetic acid and air-dried for 20 min prior to irradiation, development, and visualization.

Visual detection limits

Limits of detectability for the visual fluorescence of NAs by this method were determined by spotting decreasing volumes of standard solutions of each compound on activated Eastman silica gel plates, developing, irradiating, spraying with fluorescamine reagent, and then viewing under UV light. In this context, "detection limit" is the minimum amount of an NA that ultimately gives a detectable visual fluorescence. Five observers viewed the resulting fluorescence for several NAs and all could

see about the same amounts (to within *ca.* 3 ng). The detection limits for the majority of NAs were determined by the two observers whose visual acuity was at the two extremes.

Fluorescence spectrophotometry

Fluorescence intensities of NAs developed and visualized with fluorescamine reagent as described above were measured on 10 × 20 cm activated Eastman silica gel plates, which had been spotted at 2-cm intervals with varying volumes of NA standard solutions. The excitation and emission wavelengths giving maximum response were determined for each NA and the plates then scanned. Peak areas of the recorder tracings were determined with an electronic integrator (Model AAG Integrator/Calculator; Technicon Chromatography, Ardsley, N.Y., U.S.A.) and the relative fluorescence values normalized by assigning to 1.0 nmole of each NA a value of 10. Unless noted otherwise, measurements were made in triplicate.

Effect of time of irradiation

To determine the effect of time of UV irradiation on the yield of fluorophor from fluorescamine-treated NAs, 1.0 nmole N-nitrosodibenzylamine was spotted several times at 2-cm intervals on activated Eastman silica gel TLC plates. The plates were developed in hexane–ether–methylene chloride (5:7:10), each spot was irradiated for varying lengths of time, the plates sprayed with fluorescamine reagent, and the relative fluorescence intensities of each spot determined spectrophotometrically.

Safety precautions

Many NAs are known to be potent carcinogens. Thus, safety precautions to prevent skin contact and inhalation must be exercised at all times.

RESULTS AND DISCUSSION

In the initial stages of this work, irradiation of NAs on pre-coated silica gel TLC plates (used as received) followed by spraying with fluorescamine reagent afforded a pale blue fluorescent "doughnut" with a dark blue "hole", all on a faint fluorescent background (Fig. 2b). Since fluorescamine yields fluorescent products only with primary amines[10] and on TLC plates NAs reportedly[1,2] give secondary amines upon UV irradiation, this result was unexpected.

To shed light on the nature of the doughnut and the hole, N-nitrosodipentylamine and its corresponding primary and secondary amines were spotted, irradiated and then developed. Figs. 3a and 3b indicate that both the primary (major product) and secondary amines were formed. Thus, the fluorescent doughnut in Fig. 2b arises from the primary amine and the dark hole from the secondary amine. The chemistry of NA photolysis on TLC plates will be the subject of a separate publication. By analogy with UV photolysis of NAs in solution, which primarily gave secondary amines, earlier workers[1,2] may have assumed from a positive reaction with ninhydrin (*e.g.*, Fig. 2a) that only the secondary amine had formed on their TLC plates. Ninhydrin, of course, reacts with both primary and secondary amines (Figs. 3a and 4a). It was then thought that the presence of acetic acid as used by others prior to irradiation might have influenced the outcome; Figs. 4a and 4b show no apparent dif-

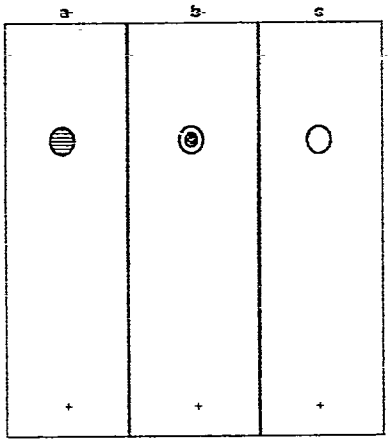

Fig. 2. Thin-layer chromatogram (silica gel) of N-nitrosodipentylamine developed with hexane–ether–methylene chloride (5:7:10) and irradiated with UV light. (a) Plate was sprayed with aqueous acetic acid prior to irradiation, then sprayed with ninhydrin reagent and heated to give purple spot. (b) Plate sprayed with fluorescamine reagent and viewed under long-wave UV light to give pale blue fluorescent "doughnut" and a dark hole", all on a faintly fluorescent background. (c) Same as (b) except activated plate was used. Spots: ⊖ = purple spot; ⊗ = dark blue spot under UV light; ○ = fluorescent spot under UV light.

ference in the resulting products when compared with the absence of acid (Figs. 3a and 3b).

The course of this photolysis appears to be affected by the water content of the silica gel layers; only the primary amine was observed when the plate was activated by heating at 110° for 1 h (Figs. 2c, 3c and 4c). The course also appears to be affected by the nature of the surface; although primary amines form on silica gel and aluminum oxide plates, they were not observed on a variety of paper surfaces.

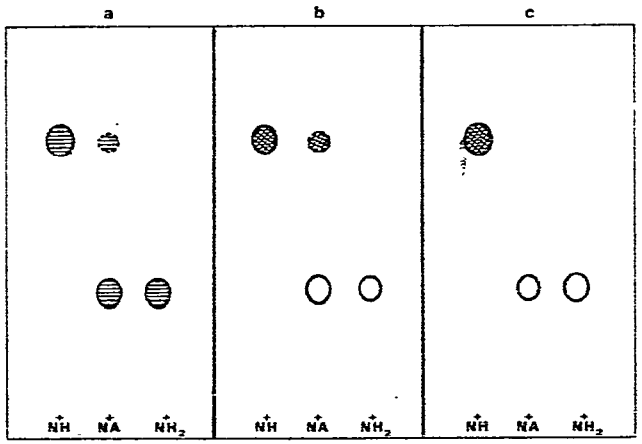

Fig. 3. Thin-layer chromatogram (silica gel) of dipentylamine (NH), N-nitrosodipentylamine (NA) and pentylamine (NH_2). Plate was irradiated with UV light, developed with ethanol–ammonium hydroxide (4:1), sprayed with (a) ninhydrin reagent and heated, and (b) fluorescamine reagent; (c) same as (b) except that activated plate was used. Spots: see legend of Fig. 2.

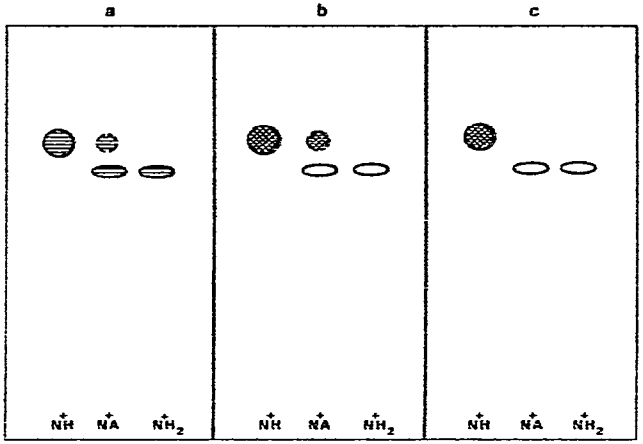

Fig. 4. Thin-layer chromatogram (silica gel) of dipentylamine (NH), N-nitrosodipentylamine (NA), and pentylamine (NH_2). Plate was sprayed with aqueous acetic acid, irradiated with UV light, developed with ethanol–ammonium hydroxide (4:1), sprayed with (a) ninhydrin reagent and heated, and (b) fluorescamine reagent; (c) same as (b) except that activated plate was used. Spots: see legend of Fig. 2.

Visual detection limits

The limits of detection for the visual fluorescence detection of irradiated NAs with fluorescamine were determined and are summarized in Table I. Detection limits of 10–15 ng (about 75 pmoles) were observed for most NAs, but the limits were higher (poorer) for the more volatile diethyl-, pyrrolidine-, and especially the dimethyl compounds. The high limit (500 ng) for N-nitrosodimethylamine is probably due to evaporative losses of the parent NA and/or the resulting primary amine (the spots were much more diffuse than those from any other NA). As little as 6 ng (90 pmoles) of the non-volatile methylamine hydrochloride could be detected, and if the NA is visualized without development the detection limit is about 10 ng (150 pmoles).

It is of interest that the heterocyclic pyrrolidine and piperidine NAs afforded primary amines. N-Nitrosomorpholine also afforded some primary amine, however the resulting fluorophor was masked by other non-fluorescent products. The diphenyl-, carbazolyl-, and N-methylpiperazinyl-NAs yielded only non-fluorescent products.

Calibration curves

Quantitative analyses of various primary amines on TLC plates using fluorescamine have been reported[20–23]. Initially, activated Macherey, Nagel & Co. silica gel plates were used for the spectrophotometric quantitation of fluorescence derived from the NAs. However, their use was abandoned because of the large (20–30%) relative standard deviations (R.S.D.'s) and a weak fluorescent background. After switching to activated Eastman silica gel plates, R.S.D.'s of 5–10% and 10–20% for the same and different plates, respectively, were obtained. Kirchner[24] reported R.S.D.'s of 4–6% on the same plate and of 9–12% on different plates with fluorescence measurements. A variation of about $\pm 3.5\%$ in R_F values was noted with the Eastman plates but this did not interfere with the fluorescence measurements.

Spectrophotometric measurements[25–27] on various fluorophors derived from

fluorescamine have revealed weak and strong excitation bands at about 275 and 390 nm, respectively, and an emission band at 475–495 nm. Excitation and emission wavelengths giving the maximum response on the spectrophotometer were determined for the fluorophors derived from each NA. Those compounds containing a phenyl group and producing a fluorophor showed excitation-emission maxima at 398 and 484 nm, respectively. The remaining NAs afforded fluorophors having an excitation band with two maxima at 382 and 396 nm and an emission band with a maximum at 460–465 nm. In these latter cases, the greater of the two maxima at 382 or 396 nm was chosen for excitation.

Calibration curves for a variety of different types of NAs were drawn and the individual data points for each are shown in Fig. 5. The relative fluorescence of the fluorophor from 1.0 nmole of each NA was arbitrarily given a value of 10 and since the amounts studied extended beyond two decades (from about 0.1 to 40 nmoles) the data were plotted on a log–log scale. Although the absolute fluorescence values for a given amount of the various NAs are not the same, it is readily apparent from Fig. 5 that the relative slopes of the various curves are not significantly different. The deviation from linearity at the higher levels may be due to quenching (reported by others for both TLC[21] and fluorometric[25] determinations with fluorescamine), generation of spots larger than the slit width of the spectrophotometer, and for the phenyl NAs formation of some yellow non-fluorescent material that partially masked the fluorescence.

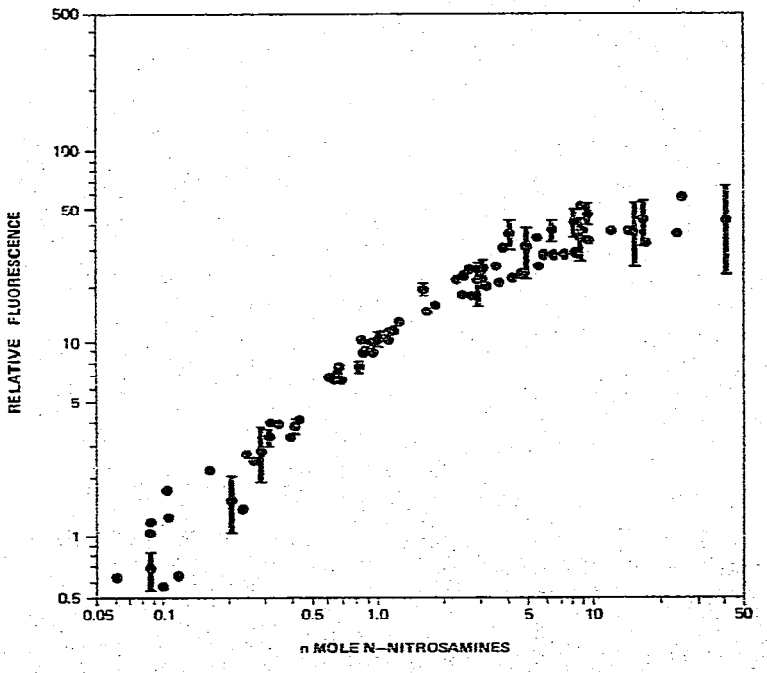

Fig. 5. Calibration curves for UV irradiation and fluorescamine treated N-nitrosamines (NAs) on Eastman silica gel TLC plates. Fluorescence measured for seven different amounts of each NA and values normalized so that 1.0 nmole gave a relative fluorescence of 10. Error bars are standard deviations calculated from measurements made in triplicate; all other data points are for single measurements. NAs studied (see Table I): 3, 6, 7, 8, 12, 15, 16, 17 and 18.

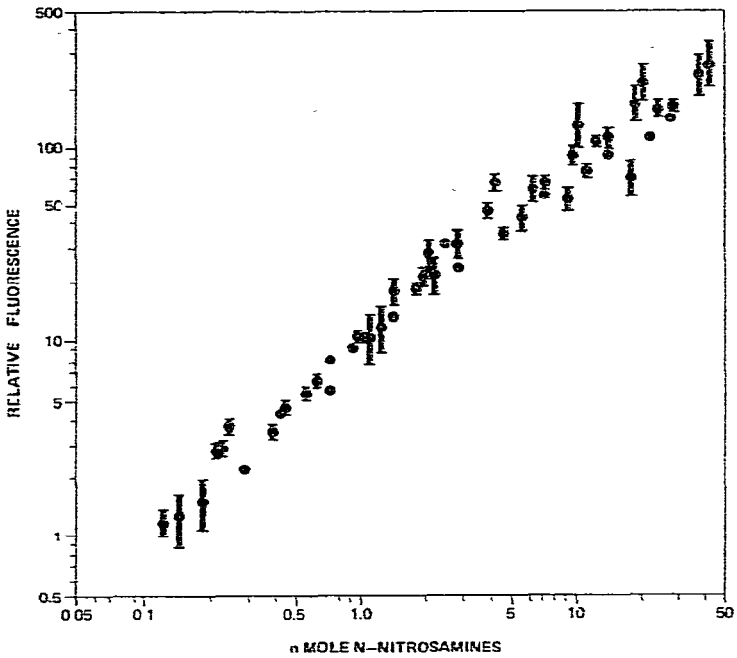

Fig. 6. Calibration curves for UV irradiated and fluorescamine treated N-nitrosamines (NAs) on Eastman silica gel TLC plates. NAs studied (see Table I): 9, 10, 11, 12, 13, 14, 19 and 20. Error bars: see legend of Fig. 5.

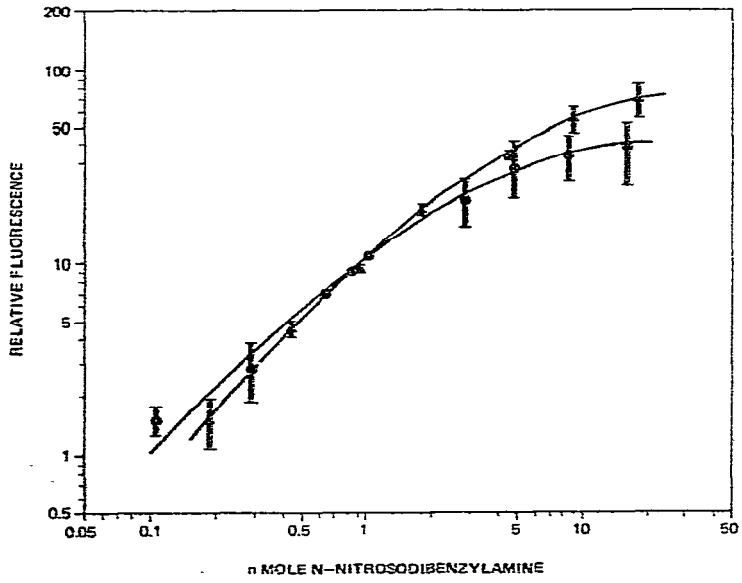

Fig. 7. Calibration curves for UV irradiated and fluorescamine treated N-nitrosodibenzylamine on two different lots of Eastman silica gel TLC plates. ●, Lot for NAs in Fig. 5; ▲, lot for NAs in Fig. 6. Error bars are standard deviations calculated from measurements made in triplicate.

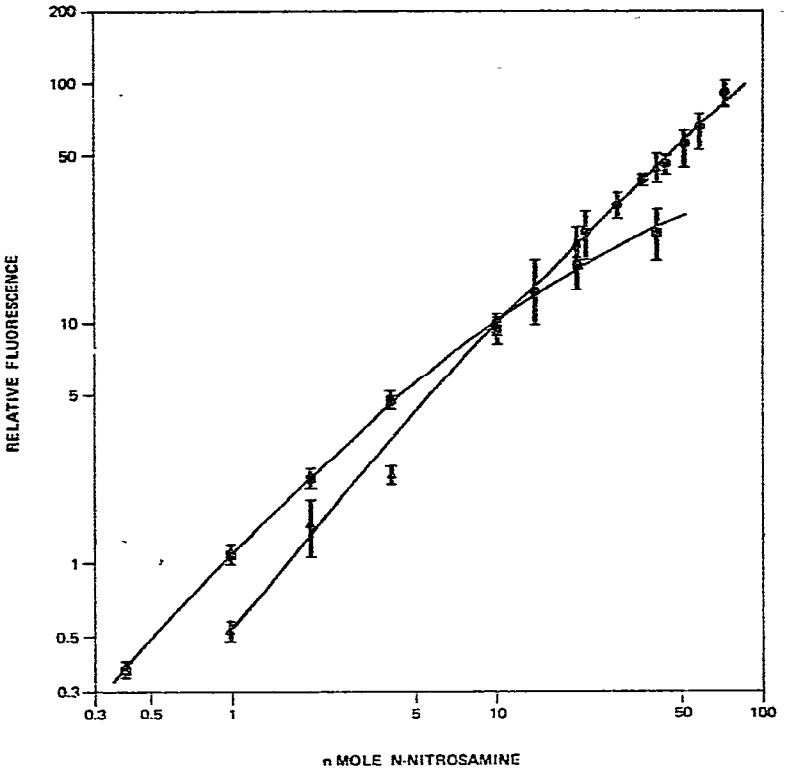

Fig. 8. Calibration curves for UV irradiated and fluorescamine treated N-nitrosodimethylamine (●), N-nitrosodiethylamine (▲), and N-nitrosopyrrolidine (■). Fluorescence values normalized so that 10 nmoles gave a relative fluorescence of 10. Error bars are standard deviations calculated from measurements made in triplicate.

A change in the calibration curves was noted when a second lot of Eastman silica gel plates was used. Fig. 6 shows that the slopes of the various curves are, as in Fig. 5, not significantly different, but they are more linear over the range studied. Except for N-nitrosodibenzylamine, which was used in both figures, different NAs are represented in the two sets of data. However, since the NAs in both sets are similar, it is assumed that the variance in slope was due to the different lots of TLC plates used. The calibration curves for N-nitrosodibenzylamine from the two lots of plates are shown in Fig. 7. Other differences, such as slightly lower R.S.D.'s, more compact spots, and more uniform R_F values were also observed with the second lot.

Individual calibration curves for the three NAs (dimethyl-, diethyl-, and pyrrolidine) that have been observed in some foods are shown in Fig. 8. Because of their higher limits of detection, the relative fluorescence values have been normalized at 10 nmoles.

Effect of UV irradiation time on fluorophor formation

Fig. 9 shows that UV cleavage of N-nitrosodibenzylamine on silica gel to a

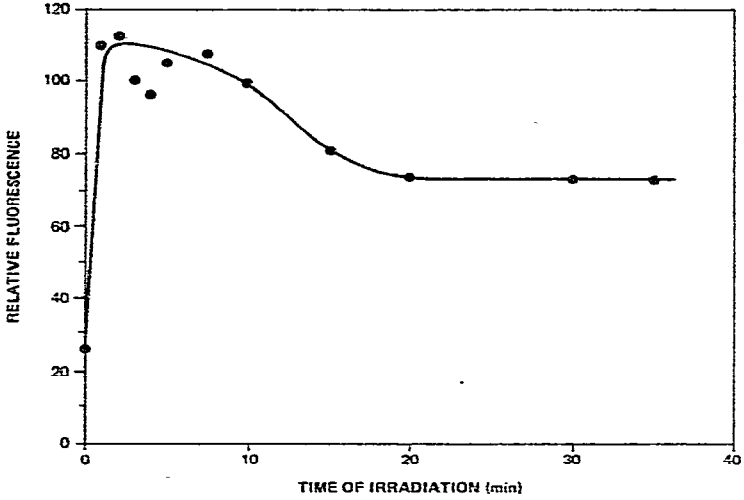

Fig. 9. Effect of UV irradiation time on fluorophor formation from 1.0 nmole N-nitrosodibenzylamine.

primary amine is essentially complete after only a few minutes and the yield of fluorophor from fluorescamine thereafter slowly decreases to a constant level.

Other fluorophors

Fluorophor formation has been reported for UV irradiated and ninhydrin treated N-nitrosopyrrolidine[7] and for NAs that can be reduced to an asymmetric hydrazine and then coupled with 9-anthraldehyde or 9-phenanthraldehyde[9]. While working with the Preussmann reagent ($PdCl_2$–diphenylamine)[3,4] fluorophor formation was observed on silica gel for a number of NA's when UV irradiation was followed by spraying with the reagent and then heating at 80°. The results are summarized in Table II. The resulting fluorophors were masked to varying degrees by non-fluorescent products. It is interesting to note that N-nitrosodiphenylamine and N-nitroso-N-methylpiperazine form fluorophors with this reagent, whereas they do not with fluorescamine. The limits of detection using the modified Preussmann method were not determined, however they are noticeably higher than those for the fluorescamine method.

TABLE II

FORMATION OF FLUOROPHORS FROM N-NITROSAMINES AND PALLADIUM CHLORIDE–DIPHENYLAMINE REAGENT

Fluorophor formation was determined by spotting N-nitrosamines on silica gel TLC plates, developing, irradiating with UV light, spraying with $PdCl_2$–diphenylamine reagent, heating for 10 min at 80°, and viewing under long-wave UV light. For N-nitrosamine number see Table I.

Fluorophor formation	*N-Nitrosamine number*
Strong	4, 5, 7, 8, 13, 14, 21
Weak	2, 3, 6, 10, 20, 23
None	9, 11, 12, 15, 16, 17, 18, 22, 24

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