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THE ESTIMATION OF N-NITROSAMINES IN TROPICAL REGIONS BY REVERSED-PHASE PAPER AND THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A simple and convenient procedure based on reversed-phase paper and thin-layer chromatography is reported for the detection and estimation of N-nitrosamines in food products, particularly cured meat. The method can easily be applied in tropical regions when plenty of sunshine is available. It is particularly useful in food-testing laboratories where sophisticated analytical instruments such as the gas chromatograph, mass spectrometer, etc., are not available. By this procedure, it is possible to detect up to 10-μg and to estimate up to 75-μg (reversed phase) and 50-μg (thin layer) amounts of N-nitrosamines.

INTRODUCTION

N-Nitrosamines are formed during the curing of meat with nitrites and nitrates. Recently, there has been some concern that nitrosamines are highly toxic when ingested in certain concentrations[1–8]. Many widely used agricultural chemicals are derivatives of alkylureas and alkylcarbamic acids which react with nitrites under mild acid conditions to form dialkylnitrosamines or an N-nitroso derivative or a mixture of these compounds which are highly toxic[9,10]. The formation of N-nitrosamines by nitrosation of amines may take place in the human stomach as well as in stored food, and it has been shown that such compounds can be formed *in vitro* from secondary amines and nitrites when mixed together in human gastric juice[11,12]. Because of the possible formation of nitroso carcinogens, cured meat and meat products have to be thoroughly screened for the presence of such compounds. Since these toxicants occur only in microquantities, sensitive methods are required for their proper screening and estimation. Generally, gas chromatography (GC) and/or mass spectroscopic and polarographic techniques have been utilized.

Although reversed-phase paper chromatography (PC) has not been employed for the microseparation and estimation of nitrosamines, thin-layer chromatography (TLC) has been adopted by many workers[13–17]. Sen and co-workers[14–16] used TLC for the detection and semiquantitative determination of nitrosamines, while Eisenbrand[17] was able to estimate amounts of up to 80 μg of nitrosamines in foods by

TLC in conjunction with ultraviolet (UV) spectroscopy. The present paper describes a simple and convenient procedure for the estimation of N-nitrosamines which could be applied in remote areas where sophisticated and costly instruments are not available. It is possible to detect up to 10-μg and estimate up to 75-μg amounts of nitrosamines by PC and 50-μg amounts by TLC.

EXPERIMENTAL

Preparation of N-nitrosamines

N-Nitroso derivatives of dimethyl-, diethyl-, dipropyl- and dibutylamines and piperidine were prepared according to the standard procedure[18]. Each N-nitrosamine was distilled twice using an efficient column and only fractions having sharp boiling points were collected. The distillate was dissolved in dichloromethane, and interfering amines were removed by washing with glycine–HCl buffer (pH 2.1 $\pm$ 0.1). Further clean-up was achieved by means of chromatography on an alumina column (OH^-) using pentane as the stationary phase. The column was successively washed with the solvent mixtures dichloromethane–pentane (1:50, 1:10 and 1:5). The nitrosamines were eluted with dichloromethane, which was distilled off under reduced pressure and diffused light; they gave a single spot on two-dimensional TLC and were spectroscopically pure.

Adsorbents

The adsorbents silica gel G, alumina (H^-, OH^- and neutral; Type T) and magnesium silicate with 13% calcium sulphate were obtained from E. Merck, Darmstadt, G.F.R.

Solvents and technique

All the solvents were dried and freshly distilled. The ascending technique was employed for both PC and TLC, the temperature of irrigation being 26–28°.

Chromogenic reagent

Griess reagent: 1% sulphanilic acid in 30% acetic acid–0.1% α-naphthylamine in 30% acetic acid (1:1).

Curing, cooking and dehydration of the mutton and the extraction of the N-nitrosamines

The mutton was cured and dehydrated according to the procedure of Bhatia *et al.*[19]. 1 kg of fresh, deboned and minced mutton was thoroughly mixed with 25 ml of an aqueous solution containing sodium citrate (2.5 g, 0.25% of the wet weight of the mutton) and sodium nitrite (0.10 g, 10 mg per 100 g of the wet weight of the mutton). After 1 h, 10 ml of 20% sodium carbonate and 10 ml of 1% sodium chloride were added, mixed and left for 30 min for complete curing. A portion of the cured mutton was cooked in a pressure cooker (15 lbs., 15 min). One portion of this cooked mutton was dehydrated by spreading it on perforated aluminium trays in an oven under hot air (60°, 2 h; ultimate moisture level, 5.5%). The N-nitrosamines were extracted from various mutton samples (wet weight, 1 kg) by the procedure of Sen *et al.*[14].

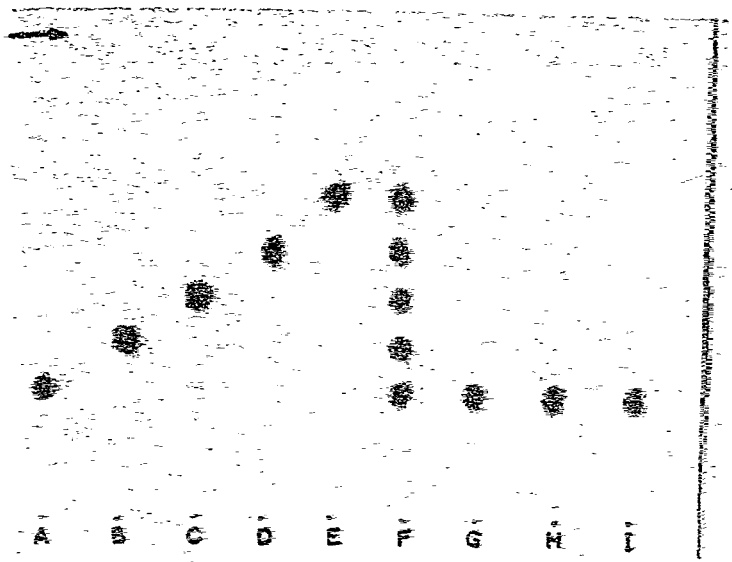

Fig. 1. Reversed-phase paper chromatogram of N-nitrosamines and samples of mutton. Paper, Whatman No. 1 impregnated with 5% decalin in light petroleum (b.p. 40–60°). Irrigation, *n*-butanol–pyridine–water (14:3:3) for 6 h. Colour reagent, 1% sulphanilic acid in 30% acetic acid–0.1% α-naphthylamine in 30% acetic acid (1:1). Samples: A = dimethyl-N-nitrosamine; B = diethyl-N-nitrosamine; C = N-nitrosopiperidine; D = N-nitrosodipropylamine; E = dibutyl-N-nitrosamine; F = a mixture of A–E; G = fresh cured mutton; H = cured cooked mutton; and I = cured, cooked and dehydrated mutton.

Paper chromatography

Estimation of the N-nitrosamines. Reversed-phase paper chromatography (PC) was employed. Whatman No. 1 paper (40 × 25 cm) was impregnated with a solution of 5% of decalin in light petroleum (b.p. 40–60°). Spots were made from 10 μl (10 μg/μl) of each nitrosamine solution in acetone and from the various mutton extracts. The paper was irrigated with *n*-butanol–pyridine–water (14:3:3) for 6 h. The entire procedure was carried out in the dark in order to prevent photoreduction of the N-nitrosamines. The chromatograms were dried, exposed to bright sunlight for 2 h and sprayed with the Griess reagent. The resulting reddish-purple spots were cut out, eluted with acetone, made up to a known volume and estimated colorimetrically at 520 nm. A typical chromatogram is shown in Fig. 1.

Thin-layer chromatography

Preparation of the plates. A homogeneous slurry of 30 g of the adsorbent in 100 ml of water–phosphate buffer (pH 5)–borax buffer (pH 9) was poured on to ten glass plates (20 × 30 cm), which were tilted from side to side in order to obtain a uniform coating. The plates were left overnight for drying, and activated at 110° for 1 h before use. Plates with and without thin-layer coatings were weighed and the average coatings (in mg/cm²) in each case were: kieselgel G, 7.2; alumina (H^+), 7.4; alumina (neutral), 7.1; alumina (OH^-), 7.0; and magnesium silicate with calcium sulphate, 8.3.

Spotting, irrigation and estimation of the N-nitrosamines. Spots were made from 10 μl of the acetone solutions of each nitrosamine (5 μg/μl) and from the various

TABLE I

R_F VALUES OF N-NITROSAMINES* FROM TLC ON VARIOUS ADSORBENTS

Irrigating solvent	*Alumina*						
	H^+					*Neutral*	
	a	*b*	*c*	*d*	*e*	*a*	*b*
Cyclohexane–chloroform (1:3)	0.86	0.88	0.90	0.92	0.95	0.88	0.9
Light petroleum–cyclohexane (19:1)	0.40	0.42	0.46	0.49	0.51	0.72	0.7
Light petroleum–xylene (4:1)	0.18	0.22	0.25	0.28	0.32	0.35	0.3
Light petroleum–dichloromethane (4:1)	0.52	0.54	0.55	0.58	0.62	0.39	0.4
Chloroform–xylene (19:1)	0.23	0.25	0.26	0.27	0.29	0.40	0.4
Toluene–dichloromethane (9:1)	0.55	0.59	0.61	0.63	0.66	0.52	0.5
Chloroform–dichloromethane (9:1)	0.82	0.85	0.85	0.88	0.89	0.91	0.93
Toluene–cyclohexane–dichloromethane (6:3:1)	0.32	0.36	0.38	0.46	0.49	0.68	0.70
Hexane–diethyl ether–dichloromethane (4:3:2)	0.15	0.18	0.19	0.21	0.23	0.21	0.24
	Silica gel G						
	H^+					*Neutral*	
	a	*b*	*c*	*d*	*e*	*a*	*b*
Cyclohexane–chloroform (1:3)	0.25	0.28	0.30	0.33	0.35	0.21	0.27
Light petroleum–cyclohexane (19:1)	0.86	0.88	0.89	0.95	0.97	0.25	0.32
Light petroleum–xylene (4:1)	0.12	0.14	0.16	0.17	0.19	0.22	0.24
Light petroleum–dichloromethane (4:1)	0.23	0.25	0.27	0.30	0.33	0.23	0.29
Chloroform–xylene (19:1)	0.37	0.40	0.41	0.45	0.48	0.21	0.25
Toluene–dichloromethane (9:1)	0.13	0.16	0.17	0.19	0.21	0.29	0.35
Chloroform–dichloromethane (9:1)	0.21	0.25	0.24	0.28	0.30	0.66	0.69
Toluene–cyclohexane–dichloromethane (6:3:1)	0.20	0.23	0.25	0.26	0.29	0.20	0.25
Hexane–diethyl ether–dichloromethane (4:3:2)	0.22	0.25	0.24	0.30	0.33	0.36	0.60

* a = Dimethyl-N-nitrosamine, b = diethyl-N-nitrosamine, c = N-nitrosopiperidine, d = dipropyl-N nitrosamine, and e = dibutyl-N-nitrosamine.

mutton extracts. Before irrigation, the plates were equilibrated for 30 min with the appropriate solvent system. When the latter contained diethyl ether, the irrigation temperature was less than 5°. The plates were irrigated in the dark, dried in air, exposed to bright sunlight for 2 h and sprayed with the Griess reagent. The resulting reddish-purple spots were scraped off with a microspatula, eluted with acetone, made up to a known volume and their intensities recorded at 520 nm. Extracts of equal areas adjacent to the spots were used as blanks. R_F values for the different solvent systems are given in Table I, and those of dimethyl-N-nitrosamine present in different mutton samples are shown in Table II.

RESULTS AND DISCUSSION

The intensity of the spots obtained with the Griess reagent and the corresponding transmission at 520 nm were optimised when the paper and thin-layer chromatograms were exposed to sunlight for 2 h. There was a linear relationship between the percentage transmission and the concentrations of the nitrosamines, and the results were reproducible. This eliminates the necessity of irradiating with a high-intensity UV source, since bright sunlight is plentiful in tropical countries.

			OH⁻				
c	*d*	*e*	*a*	*b*	*c*	*d*	*e*
0.9(	0.92	0.95	0.30	0.32	0.35	0.40	0.42
0.8(	0.84	0.87	0.25	0.28	0.30	0.36	0.42
0.3(	0.42	0.43	0.12	0.12	0.13	0.14	0.14
0.4(	0.46	0.48	0.10	0.15	0.18	0.20	0.22
0.4(	0.46	0.47	0.01	0.10	0.12	0.15	0.16
0.56	0.60	0.65	0.15	0.17	0.18	0.20	0.22
0.9(	0.96	0.98	0.32	0.34	0.35	0.39	0.41
0.72	0.76	0.80	0.28	0.32	0.34	0.37	0.40
0.24	0.28	0.30	0.12	0.15	0.16	0.19	0.22

								Magnesium silicate				
			OH⁻									
c	*d*	*e*	*a*	*b*	*c*	*d*	*e*	*a*	*b*	*c*	*d*	*e*
0.29	0.31	0.34	0.18	0.21	0.22	0.32	0.34	0.46	0.50	0.52	0.55	0.58
0.34	0.38	0.41	0.16	0.19	0.18	0.23	0.25	0.62	0.72	0.75	0.84	0.94
0.25	0.29	0.33	0.13	0.15	0.15	0.17	0.18	0.15	0.17	0.19	0.21	0.23
0.31	0.51	0.61	0.14	0.16	0.16	0.18	0.20	0.20	0.27	0.29	0.35	0.40
0.26	0.29	0.31	0.11	0.13	0.13	0.16	0.18	0.56	0.58	0.62	0.69	0.70
0.38	0.41	0.46	0.11	0.17	0.18	0.20	0.22	0.48	0.68	0.70	0.78	0.86
0.71	0.74	0.78	0.21	0.22	0.23	0.25	0.29	0.45	0.49	0.50	0.54	0.58
0.26	0.28	0.30	0.11	0.13	0.14	0.17	0.19	0.32	0.36	0.38	0.46	0.50
0.62	0.80	0.90	0.11	0.15	0.14	0.17	0.18	0.46	0.50	0.51	0.54	0.57

It was possible to detect up to 10-μg amounts of nitrosamines on both PC and TLC using a UV lamp (254 nm filter) and to estimate up to 75 μg (by PC) and 50-μg amounts (by TLC). Use of the Griess reagent did not produce coloured spots when the amount of nitrosamine was less than 25 μg. In all the cured mutton samples only dimethyl-N-nitrosamine was found. This was confirmed by two-dimensional TLC where an authentic sample of dimethyl-N-nitrosamine and that from mutton gave superimposable spots. The samples were also spectroscopically identical. No coloured spots were obtained for samples of cured dehydrated mutton, but when viewed under a UV lamp the amount of dimethyl-N-nitrosamine present was *ca.* 10 μg when compared with the standard. It is interesting to note that the amount of dimethyl-N-nitrosamine in cured mutton decreased slightly on cooking, and decreased largely on its subsequent dehydration.

PC on untreated paper resulted in tailing of the compounds when different solvent systems were employed. Papers treated with dimethyl sulphoxide (50% in toluene), groundnut oil (1% in acetone) and silicone oil (2% in acetone) gave poor resolutions with the solvent systems *n*-butanol–acetic acid–water (3:1:1); ethyl acetate–acetic acid–methanol–water (31:7:7:5), ethyl acetate–pyridine–tetrahydrofuran–water (58:20:11:11), methanol–chloroform–acetone–25% ammonia (42:17:

TABLE II

DETERMINATION OF THE AMOUNT (*µg*/kg) OF DIMETHYL-N-NITROSAMINE PRESENT IN MUTTON

Mutton sample	*Batch no.*	*PC*	*TLC*			
		Butanol–pyridine–water (14:3:3)	*Magnesium silicate*		*Alumina (neutral)*	
			*a**	*b***	*a**	*b***
Minced, cured, raw mutton	I	92	90	88	92	90
	II	110	102	100	102	104
	III	85	88	86	88	90
Minced, cured, cooked mutton	I	88	86	86	88	88
	II	105	106	100	100	100
	III	82	84	82	84	86
Minced, cured, cooked and dehydrated mutton	I	*ca.* 10	*ca.* 10	*ca.* 10	*ca.* 10	*ca.* 10
	II	*ca.* 10	*ca.* 10	*ca.* 10	*ca.* 10	*ca.* 10
	III	*ca.* 10	*ca.* 10	*ca.* 10	*ca.* 10	*ca.* 10

* a: solvent, light petroleum–cyclohexane (19:1).
** b: solvent, toluene–dichloromethane (9:1).

24:17) and *n*-butanol–pyridine–water (14:3:3). The best resolutions were obtained with the latter system when paper treated with decalin [5% in light petroleum (b.p. 40–60°)] was employed.

Magnesium silicate was found to be the best adsorbent for TLC resolutions of nitrosamines; the spots were particularly distinct when light petroleum–cyclohexane (19:1) and toluene–dichloromethane (9:1) were employed. In general, the mobility of the nitrosamines on the different types of alumina was in the order neutral > H^+ > OH^-. Silica gel G (OH^-) resulted in the smallest migrations of the compounds when compared with the other two types of adsorbent. Halogenated solvents had more affinity for the nitrosamines when compared to the other solvents.

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REFERENCES

1 J. M. Barnes and P. N. Magee, *Brit. J. Ind. Med.*, 11 (1954) 167.
2 P. N. Magee and J. M. Barnes, *Brit. J. Cancer*, 10 (1956) 11.
3 H. Druckery, R. Preussmann, S. Ivanokovic and D. Schmahl, *Z. Krebsforsch.*, 69 (1967) 103.
4 P. N. Magee and J. M. Barnes, *Advan. Cancer Res.*, 10 (1967) 163.
5 D. Schmahl and H. Osswald, *Experientia*, 23 (1967) 497.
6 W. Lijinsky and S. Epstein, *Nature (London)*, 225 (1970) 21.
7 P. N. Magee, *Food Cosmet. Toxicol.*, 9 (1971) 207.
8 P. Issenberg, *Abst. Amer. Chem. Soc.*, 163 (1972) AGFD 21.
9 R. K. Efespuru and W. Lijinsky, *Food Cosmet. Toxicol.*, 11 (1973) 807.
10 G. Eisenbrand, O. Ungerer and R. Preussmann, *Food Cosmet. Toxicol.*, 12 (1974) 229.
11 J. Sander, *Arch. Hyg. Bakt.*, 151 (1967) 22.

12 N. P. Sen, D. C. Smith and L. Schwinghamer, *Food Cosmet. Toxicol.*, 7 (1969) 301.
13 R. Preussmenn, G. Neurath, L. G. Wulf, D. Daiber and H. Hengy, *Z. Anal. Chem.*, 202 (1964) 187.
14 N. P. Sen, C. S. Dorothy, L. Schwinghamer and J. J. Marleau, *J. Ass. Offic. Anal. Chem.*, 52 (1969) 47.
15 N. P. Sen and C. Dalpe, *Analyst (London)*, 97 (1972) 216.
16 N. P. Sen, D. C. Smith, L. Schwinghamer and B. Howsam, *Can. Inst. Food Technol.*, 3 (1970) 66.
17 G. J. Eisenbrand, *J. Chromatogr.*, 51 (1970) 503.
18 J. W. Pensabene, W. Fiddler, C. J. Dooley, R. C. Doerr and A. E. Wassermann, *J. Agr. Food Chem.*, 20 (1972) 274.
19 B. S. Bhatia, V. K. Mathur and S. A. Das, personal communication, 1974.