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Note

High-performance liquid chromatographic determination of 4-nitroso- and 4-nitrophenols in the presence of phenol and alkylphenols

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The carcinogenic properties of N-nitrosamines in laboratory animals[1,2] and the formation of these compounds in foods has created interest in the effect of various compounds on the formation of N-nitrosamines. Phenols are of interest because they may either inhibit[3,4] or accelerate[5–9] the formation of N-nitrosamines, depending primarily on the reaction conditions. Phenols are present in hardwood smoke used in the smoking of cured meat and fish products and could be involved in catalysing the nitrosamine reaction.

The smoking of bacon with hardwood smoke resulted in the incorporation of phenols in the meat matrix, which consisted primarily of phenol itself, cresols (methylphenols), 4-substituted guaiacols (2-methoxyphenols) and syringols (2,6-dimethoxyphenols), with approximately half of the phenols substituted in the *para*-position[10]. Heavily smoked bacon has been shown to contain up to 280 ppm of phenols[10]. The reaction of the nitrite in the curing mixture used for the preparation of bacon with the phenols from the smoke resulted in the formation of a variety of *ortho*-substituted nitro- and nitrosophenols[11]. Nitrosation of a liquid smoke preparation resulted in a larger variety of nitrophenols, a few substituted in the *para*-position and the remainder in the *ortho*-position[12]. In this extract no nitrosophenols could be identified, but the nitrophenols were believed to be derived from the corresponding nitroso derivative, which can undergo facile oxidation during the reaction period[13]. 2-Nitrosophenols are amenable to gas chromatographic separation owing to intramolecular hydrogen bonding. 4-Nitrosophenols do not exhibit this type of bonding, thereby decreasing their volatility; they have long retention times with some on-column decomposition[14], resulting in poor separations and low sensitivity. The separation of some nitrosophenols by paper chromatography has been reported[15,16].

In Poland there is interest in the use and composition of hardwood smoke, and therefore studies of its major phenolic components and the chemistry of the nitrosation of the phenols are of interest. There is a need, however, for a more sensitive method for the determination of both *o*- and *p*-nitrosophenols (at parts per 10^9 levels) in the presence of nitrophenols and phenols. An improved high-performance liquid chromatographic (HPLC) method is reported in this paper.

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EXPERIMENTAL

Reagents

The following chemicals were used: methanol (distilled in glass) from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.), anhydrous diethyl ether from J. T. Baker (Phillipsburg, NJ, U.S.A.), 4-nitrosophenol, 4-nitroso-2,6-dimethylphenol and 4-nitro-2,6-dimethylphenol from Aldrich (Milwaukee, WI, U.S.A.), 4-nitroso-2-methylphenol from Pfaltz and Bauer (Stamford, CT, U.S.A.), 4-nitrophenol from Chem Service (Media, PA, U.S.A.) and phenol, 2-methylphenol and 2,6-dimethylphenol from PolyScience Corp. (Niles, IL, U.S.A.). Diethyl ether was carefully redistilled in glass; all other reagents were used as received without further purification.

Preparation and conditioning of resin columns

Amberlite XAD-4 resin from Rohm and Haas (Philadelphia, PA, U.S.A.) was ground and sieved to give 60–80, 80–100, 100–150 and 150–200 mesh fractions. Resin of each mesh size was washed with methanol to remove dust, sieved again, then a 0.5- or 1-g amount was suspended in a 20 cm × 10 mm I.D. glass column containing 20 ml of methanol to wet the resin; the resin then settled to the bottom of the column. After washing with additional methanol and distilled water, the column was ready for use.

Adsorption and elution of sample on resin

Aqueous phenolic solutions were passed through the column at a flow-rate of 1–3 ml/min depending on the particle size of the resin. The column was washed with two 1-ml volumes of distilled water and the phenolic compounds were eluted with 2.5 ml of diethyl ether. The eluate was concentrated in a water-bath at 70°C with a micro Snyder column and diluted to 1 ml with water–methanol (65:35); 20 μl were injected into the HPLC system for analysis.

Apparatus

The HPLC system consisted of an Altex (Palo Alto, CA, U.S.A.) dual-pump Model 100 instrument controlled by an Altex Model 420 processor and connected to a Waters Assoc. (Milford, MA, U.S.A.) Model 450 variable-wavelength detector.

Operating conditions

An Altex Ultrasphere ODS (5 μm) column (25 cm × 4.6 mm I.D.) was eluted isocratically with water–methanol–acetic acid (50:50:0.12) as the mobile phase at a flow-rate programmed from 0.5 ml/min at 0.1 ml/min, or by gradient elution with water–methanol–acetic acid (solution A, 65:35:0.15; solution B, 20:80:0.1) as the mobile phase at a flow-rate programmed from 0.5 to 1.5 ml/min at 0.05 ml/min.

RESULTS AND DISCUSSION

The determination of low levels of nitroso- and nitrophenols in the presence of phenols in aqueous solutions requires isolation followed by concentration and analysis. Amberlite XAD-4 is recommended by the manufacturers for the isolation of

phenols from aqueous solutions, so this resin was tested for its ability to concentrate the phenolic compounds from aqueous solutions. The recovery of the phenolic compounds varying from an initial concentration of 3.48 μg of 4-nitrosophenol to 33.36 μg of 2,6-dimethylphenol in 10 ml of solution are summarized in Table I. The particle size of the Amberlite XAD-4 resin affected the recovery of the phenolic compounds.

TABLE I

EFFECT OF PARTICLE SIZE OF AMBERLITE XAD-4 RESIN ON THE RECOVERY OF PHENOLS

Compound	*Amount added (μg)**	*Recovery (%)***			
		60–80 mesh	*80–100 mesh*	*100–150 mesh*	*150–200 mesh*
4-Nitrosophenol	3.48	61	50	43	22
4-Nitroso-2-methylphenol	6.44	87	86	77	47
4-Nitroso-2,6-dimethylphenol	11.4	91	90	85	53
4-Nitrophenol	10.34	97	97	96	83
4-Nitro-2,6-dimethylphenol	11.16	81	89	77	81
Phenol	18.66	95	99	94	63
2-Methylphenol	25.84	98	87	86	63
2,6-Dimethylphenol	33.36	85	84	78	53

* In 10 ml of aqueous solution.

** Average of three determinations.

Greater recoveries were obtained with the resins of larger particle size (60–80 and 80–100 mesh) than with those of smaller particle size (100–150 and 150–200 mesh). With the 60–80 mesh resin, 4-nitrosophenol gave the lowest recovery among the eight phenolic compounds tested (61 %), the recoveries for the others being in the range 81–98 %. The recoveries and standard deviations for two concentrations of eight phenolic compounds from aqueous solution on 60–80 Amberlite XAD-4 resin are given in Table II. The recovery of 4-nitrosophenol (60 %) was again the lowest, those of the other phenolic compounds varied from 82.7 to 108.8 %. With 0.5–1 g of resin the phenolic compounds can be eluted with 3 ml or less of diethyl ether. It is therefore possible to concentrate low levels of nitroso- and nitrophenols from fairly large volumes of aqueous solutions by this method.

The HPLC separation of the phenolic compounds with an Ultrasphere ODS (5 μm) column with a water–methanol gradient as the mobile phase was found to be satisfactory. The addition of acetic acid (0.1–0.5 %) to the mobile phase improved the symmetry of the peaks, especially those of nitrophenols. Initially a fixed-wavelength detector (254 nm) was used. However, the absorption maxima for phenol and the cresols have been reported to be 270 or 275 nm[17], whereas that for the 4-nitrosophenols was determined to be 305 nm. A variable-wavelength detector was therefore utilized. The absorption of phenols was slightly lower at 280 nm than 270 nm, but decreased sharply when the wavelength was increased further. The response of 4-nitrosophenols decreased gradually as the wavelength was decreased from 305 nm. As a single wavelength was needed for the detection of all of the phenolic compounds,

TABLE II

RECOVERIES OF AQUEOUS SOLUTIONS OF PHENOLS ON AMBERLITE XAD-4 RESIN

0.5 g of 60–80 mesh resin.

Compound	*Amount added (μg)**	*Recovery (%)*	
		*Mean***	*Standard deviation*
4-Nitrosophenol	3.48	60.2	3.75
	0.35	32.0	1.42
4-Nitroso-2-methylphenol	6.44	96.3	3.13
	0.64	82.7	4.06
4-Nitroso-2,6-dimethylphenol	11.4	100.6	5.29
	1.14	87.8	7.10
4-Nitrophenol	10.34	105.7	4.66
	1.03	97.4	6.85
4-Nitro-2,6-dimethylphenol	11.16	102.5	4.84
	1.12	84.6	8.87
Phenol	18.66	102.2	4.75
	1.87	94.6	4.65
2-Methylphenol	25.84	103.0	4.83
	2.58	97.2	6.25
2,6-Dimethylphenol	33.36	108.8	4.27
	3.34	91.5	4.09

* In 10 ml of aqueous solution.

** Mean of five determinations.

the wavelength selected had to minimize the loss of response from both types of phenolic compounds. A wavelength of 280 nm filled this requirement.

The response of these phenolic compounds at 280 and 305 nm relative to their response at 254 nm are given in Table III. The phenols gave no response at 305 nm,

TABLE III

RELATIVE RESPONSES AND DETECTION LIMITS OF PHENOLS AT WAVELENGTHS OF 280 AND 305 nm

Mobile phase: increase concentration of B in A (water–methanol–acetic acid; A = 65:35:0.15, B = 20:80:0.1) from 0 to 50% in 15 min, then from 50 to 100% in 7.5 min; flow-rate programmed from 0.5 to 1.5 ml/min at 0.05 ml/min.

Compound	*Relative response**		*Detection limit (ng)*	
	280 nm	*305 nm*	*280 nm*	*305 nm*
4-Nitrosophenol	5.31	9.22	1.29	0.77
4-Nitroso-2-methylphenol	5.00	10.4	2.02	1.02
4-Nitroso-2,6-dimethylphenol	4.94	11.9	3.78	1.66
4-Nitrophenol	2.81	6.22	3.74	1.79
4-Nitro-2,6-dimethylphenol	1.17	3.13	7.39	2.88
Phenol	3.26	NR**	7.83	NR
2-Methylphenol	4.45	NR	10.3	NR
2,6-Dimethylphenol	3.82	NR	14.0	NR

* Response relative to that at 254 nm.

** NR = No response.

making it possible to detect trace levels of nitroso- and nitrophenols in the presence of high concentrations of phenols at this wavelength. The detection limit, which was 5% at 0.01 a.u.f.s., varied from 7.83 to 14.0 ng at 280 nm for the phenols and from 0.77 to 2.66 ng at 305 nm for the nitroso- and nitrophenols. When the mobile phase was water–methanol–acetic acid (50:50:0.12) with the flow-rate programmed from 0.5 to 1.5 ml/min, the detection limit was reduced to 0.45–2.33 ng for the nitroso- and nitrophenols at a wavelength of 305 nm.

An HPLC trace of the phenolic compounds at a wavelength of 280 nm is shown in Fig. 1. The amounts of the compounds in the injected sample varied from 70 ng for 4-nitrosophenol to 667 ng for 2,6-dimethylphenol. This took into account the variation in response at this wavelength so that all the compounds could be observed with approximately the same peak heights.

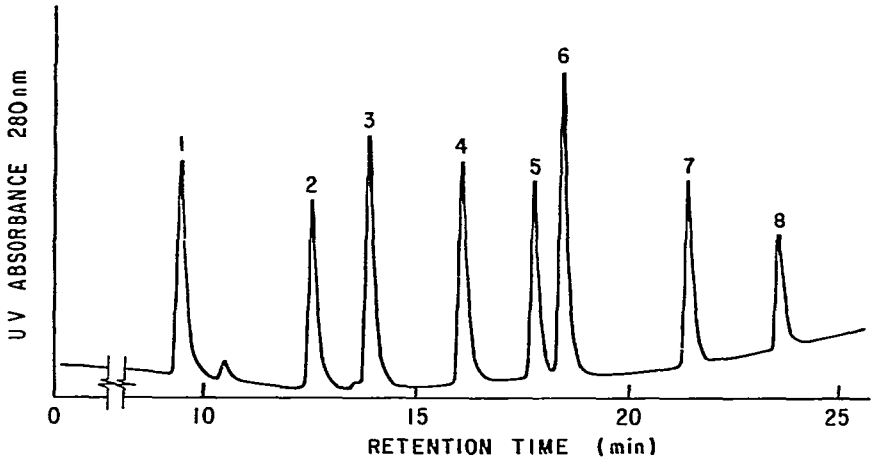

Fig. 1. Separation of (1) 4-nitrosophenol, (2) phenol, (3) 4-nitroso-2-methylphenol, (4) 4-nitrophenol, (5) 2-methylphenol, (6) 4-nitroso-2,6-dimethylphenol, (7) 2,6-dimethylphenol and (8) 4-nitro-2,6-dimethylphenol on a 25 cm × 4.6 mm I.D. Ultrasphere ODS (5 μm) column. Mobile phase: water–methanol–acetic acid (65:35:0.15 for A and 20:80:0.1 for B); from 0 to 50% B in 15 min, then from 50 to 100% B in 7.5 min, at a flow-rate programmed from 0.5 to 1.5 ml/min at 0.05 ml/min.

With this method it should be possible to concentrate relatively large volumes of aqueous extracts of phenolic compounds on Amberlite XAD-4 followed by elution with small volumes of diethyl ether. Then the phenolic compounds can be determined at a wavelength of 280 nm, followed, for greater sensitivity, by the nitroso- and nitrophenols at a wavelength of 305 nm. Analysis of smoke components and products of reactions of nitrite and phenols for nitroso- and nitrophenols in the presence of phenols will be investigated using this method.

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REFERENCES

1 P. N. Magee and J. M. Barnes, *Advan. Cancer Res.*, 10 (1967) 163.
2 H. Druckrey, R. Preussman, S. Ivankovic and D. Schmail, *Z. Krebsforsch.*, 69 (1967) 103.
3 B. C. Challis and A. J. Lawson, *J. Chem. Soc.*, (1970) 770.
4 B. C. Challis, *Nature (London)*, 244 (1973) 466.
5 B. C. Challis and C. D. Bartlett, *Nature (London)*, 254 (1975) 532.
6 E. A. Walker, B. Pignatelli and M. Castegnaro, *Nature (London)*, 258 (1975) 176.
7 E. A. Walker, B. Pignatelli and M. Castegnaro, *J. Agr. Food Chem.*, 27 (1979) 389.
8 R. Davies, M. J. Dennis, R. C. Massey and D. J. McWeeny, in E. A. Walker, M. Castegnaro, L. Griciute and R. E. Lyle (Editors), *Environmental Aspects of N-Nitroso Compounds*, IARC Scientific Publication No. 19, International Agency for Research on Cancer, Lyon, 1978, p. 183.
9 T. Kawabata, M. Ohshima, J. Vibu, M. Nakamura, M. Matsui and M. Hamano, *Proceedings of N-nitrosamines Meeting, Budapest, October, 1979*, in press.
10 A. O. Lustre and P. J. Issenberg, *J. Agr. Food Chem.*, 18 (1970) 1056.
11 M. E. Knowles, J. Gilbert and D. J. McWeeny, *Nature (London)*, 249 (1974) 672.
12 M. E. Knowles, J. Gilbert and D. J. McWeeny, *J. Sci. Food Agr.*, 26 (1975) 267.
13 B. C. Challis and R. J. Higgins, *J. Chem. Soc. Perkin Trans. II*, (1972) 2365.
14 M. E. Knowles, J. Gilbert and D. J. McWeeny, *Biomed. Mass Spectrom.*, 1 (1974) 286.
15 J. Green and S. Marcinkiewicz, *J. Chromatogr.*, 10 (1963) 354.
16 J. Gasparič, *J. Chromatogr.*, 13 (1964) 459.
17 B. K. Afghan, P. E. Belliveau, R. H. Larose and J. F. Ryan, *Anal. Chim. Acta*, 71 (1974) 355.