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CHROMATOGRAPHIC INVESTIGATIONS OF THE CONFIGURATIONAL AND GEOMETRICAL ISOMERISM OF ALLYLIC N-TERPENYL-N-HYDROXYETHYL-NITROSAMINES

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SUMMARY

A preparative adsorption column chromatographic method is reported for the separation of *cis* and *trans* geometrical isomers of two types of N-nitrosamines derived from allylic terpenyl ethanolamines (experimental fish toxicants). Column eluates were monitored by gas chromatography in which a Carbowax 20M stationary phase was used. Further separation of *E* and *Z* configurational isomers was achieved by reversed-phase and normal-phase high-performance liquid chromatography. In the reversed-phase high-performance liquid chromatography system (acetonitrile–water), the 6′,7′-acetylenic nitrosamines (NMOA) were efficiently resolved by using an argentous ($AgNO_3$) mobile phase, whereas the presence of sodium alkanesulfonate in the aqueous acetonitrile mobile phase favored the base-line resolution of the 6′,7′-olefinic nitrosamines (NDOA). For normal-phase separation on a silica column, addition of tetrahydrofuran to the mobile phase (methylene chloride-2-propanol) resulted in a varying degree of improvement in peak resolution (R) and column selectivity (α). Effects of temperature on the chromatographic behavior of the nitrosamine components are described. The high-performance liquid chromatographic separation method has proved to be applicable for the trace analysis of the title nitrosamines in organic tissues by way of thermal energy analysis.

INTRODUCTION

A number of N-nitrosamines with a wide range of structural variation are carcinogens, mutagens, and teratogens[1]. In an earlier article, Crosby and Sawyer gave a comprehensive account of the occurrence of several known N-nitrosamines in foodstuffs[2]. In 1980, we initiated a program with the objective of exploring the possibilities of nitrosamine formation in water and fish treated with chemicals normally used in fish culture or resource management[3]. Among the various chemicals examined, we have been most interested in GD-174, a disubstituted allytic terpenyl ethanolamine, 2-bis(3′,7′-dimethyl-2′,6′-octadienyl)aminoethanol (BDOA), because of its facile reaction with nitrite to give a four-component mixture of isomeric nitro-

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samines I, 2-nitroso-(3′,7′-dimethyl-2′,6′-octadienyl)aminoethanol (NDOA) as shown in Fig. 1. When BDOA was nitrosated under exhaustive conditions (high nitrite concentration, elevated temperature, and long reaction period), there was also obtained, in addition to the nitrosamine NDOA, a second product composed of four isomers of nitrosamine II, 2-nitroso-(3′-methyl-2′-octen-6′-ynyl)aminoethanol (NMOA) as shown in Fig. 2.

Both nitrosamines I and II were detected at trace concentrations (less than 10 ppb) in fish exposed to the precursor ethanolamine BDOA and nitrite[4]. Consequently, a quantitative analytical method was needed for sensitive and specific quantification of these nitrosamines in organic tissues, as well as for conformation studies. The conformation studies are relevant to the determination of energy barriers involved in the configurational (*E–Z*) isomerism of N-nitrosamines on the basis of accurate compositional data for isomers. In a previous publication[5], we demonstrated the existence of two pairs of geometrical (*cis–trans*) and configurational (*E–Z*) isomers of nitrosamine I as determined by a ^{13}C nuclear magnetic resonance (NMR) spectrometric method.

This paper describes a viable alternative method for the efficient separation and sensitive detection of nitrosamines I and II. The method entails a sequence of chromatographic techniques that include adsorption column chromatography (ACC), gas chromatography (GC), and normal- and reversed-phase high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Materials

All analytical reagent-grade chemicals were used as received from the manufacturer without further purification. Phosphoric acid (85%) and most inorganic and organic salts were obtained from Alpha Products (Danvers, MA, U.S.A.); sodium alkanesulfonate from Eastman-Kodak (Rochester, NY, U.S.A.); 2-propanol from Aldrich (Milwaukee, WI, U.S.A.); solvents (HPLC) and silica gel (ACC) from J. T. Baker (Philipsburg, NJ, U.S.A.); and other chromatographic solvents from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). The pure *trans* isomer and the *cis–trans* mixture of nitrosamine I were prepared from corresponding octadienyl chlorides and ethanolamine according to published procedures[5]. Preparation of the pure *trans* isomer and the *cis–trans* mixture of nitrosamine II followed the typical nitrosation method reported previously[5], except that a large excess of nitrite (amine:nitrite = 1:10) was used and the reaction period was extended to 4 h at 50°C. Pure *cis* isomers (and also *trans* isomers) of nitrosamines I and II were obtained from their isomeric mixtures by the ACC procedure that follows.

Adsorption column chromatography (ACC) and gas chromatography (GC) for the isolation of pure cis- (and trans-) N-nitrosamines I and II

The crude nitrosation product (2 g) that contained both the *cis* and *trans* isomers of nitrosamine I was chromatographed on a silica gel ("Baker analyzed" reagent, 40–140 mesh, No. 5-3404) column using about 90 g of packing material in a 50 cm × 2.5 cm I.D. glass column. The column was successively eluted with 200 ml each of hexane, hexane–diethyl ether (4:1, 2:1, 1:1, 1:2, 1:4), diethyl ether, diethyl

ether–methylene chloride (4:1, 2:1, 1:1), methylene chloride, and 400 ml methylene chloride–methanol (1:1). Fractions (20 ml) were collected at a rate of 1 drop/sec and each fraction was analyzed by GC on two parallel columns (5% Carbowax 20M and 3% OV-17, both on 100/120 Supelcoport in 200 cm × 2 mm I.D. glass coils). The isomeric nitrosamines were normally found in the hexane–ether eluates. The first component [fractions 33–36, 0.4 g (20% yield)] was identified as the *cis* isomer whose structure was confirmed by ^{13}C NMR. This isomer had a retention time of 19 min (5% Carbowax 20M, 220°C). Intermediate fractions (37–46) contained mixtures of isomers having different isomeric compositions. The pure *trans* component isolated from the column (fractions 47–58) amounted to 0.9 g (45% yield). A sample of this material eluted from GC (5% Carbowax 20M, 220°C) at 21 min and exhibited superimposable ^{13}C NMR spectral features as an authentic *trans* compound[5]. In a similar fashion, the crude nitrosation product containing the isomeric mixture of nitrosamine II was chromatographed to afford following pure components: *cis* isomer, fractions 37–38, 0.25 g (12.5% yield), GC retention time 27 min (Carbowax 20M, 220°C); *trans* isomer, fractions 65–72, 0.63 g (31.5% yield), GC retention time 29.2 min (Carbowax 20 M, 220°C). Spectral characteristics of the *trans* isomer are identical in every detail with those of a sample obtained from the *trans* nitrosamine I. The isomeric purity of each component isomer for both nitrosamines I and II averaged 99.3%.

Equipment

Gas chromatograms were obtained on a Varian Model 3700 gas chromatograph, operating at a detector (flame ionization) temperature of 350°C, an injector temperature of 250°C, an oven temperature of 220°C, and a carrier gas (nitrogen) flow-rate of 30 ml/min. The GC column specifications are described in the preceding section. Analytical HPLC was carried out on a Varian MicroPak MCH-10 (10 μm, 30 cm × 4 mm I.D.) C_{18} reversed-phase column, and on a Varian MicroPak Si-10 (10 μm, 30 × 4 mm I.D.) normal-phase silica column by using various mobile phases suitable for the specific separation of isomers (see Figs. 1–3 for appropriate mobile phase compositions). Preparative HPLC was performed under the same HPLC conditions, except that the column was 8 mm I.D. For all HPLC work, a Varian Model LC-5000 liquid chromatograph was used. This instrument was equipped with a column heater and interfaced with a variable-wavelength ultraviolet detector (Varian Varichrom) and a thermal energy analyzer (Thermo Electron Model 502) for respective isomerism studies and trace analyses of tissue samples[6]. The HPLC injection unit was composed of a Valco CV-6-UHPa-N60 injection valve and a 10-μl loop (100-μl loop for preparative separation). Analysis of tissue samples required a guard column (5 cm × 4 mm I.D.) packed with a Varian Vydac pellicular material (40 μm) matching the stationary phase in the analytical column. In a typical HPLC–thermal energy analysis, the instrument parameters were as follows: vacuum with oxygen, 1.8 Torr; output, 10 mV; and the following temperatures:furnace 475°C and trap −196°C. Natural-abundance ^{13}C NMR spectra of pure isomers were recorded on a Jeol FX 90-Q Fourier transform nuclear magnetic resonance spectrometer operating at 22.5 MHz.

Isomerization studies by HPLC

The *cis–trans* composition of a given nitrosamine mixture was determined either by GC (as described in the earlier part of this paper; note that the *E* and *Z* isomers are not separable by GC) or by HPLC. By using HPLC, the extent of *cis–trans* isomerization occurring concurrently with nitrosation was measured by direct injection of aliquot samples of the reaction solution onto the reversed-phase column. The respective mobile phase eluents for nitrosamine I and nitrosamine II were 65:35 water–[acetonitrile–water (9:1) + sodium octanesulfonate (0.01 *M*) + $HClO_4$ (0.01 *M*)] (65:35) and water–acetonitrile (70:30) containing $AgNO_3$ (0.1 *M*) at pH 3 (adjusted with phosphoric acid). When the nitrosation reaction was carried out at lower concentrations in the microgram range, it was necessary to work up the reaction mixture and isolate the nitrosamine products in methylene chloride prior to injection onto the normal-phase HPLC column for the analysis of isomers[7]. In this case, the mobile phase eluent used for both nitrosamines I and II was hexane–[methylene chloride–tetrahydrofuran (THF) (1:1)] containing 5% 2-propanol (85:15).

The HPLC systems described were utilized for the determination of the *E–Z* composition of a nitrosamine sample. Isolation of the pure *E* or *Z* isomer required the operation of preparative normal-phase HPLC at 0°C in a temperature-controlled chamber. Fractions of the column effluent that contained the pure isomer were combined and freeze-dried. The *E–Z* isomerization occurred at higher temperatures and the analytical HPLC data gave values of isomeric ratios of equilibrium mixtures at specific temperatures. In all analyses, the detector response was recorded on a Varian Model 9176 strip chart recorder and chromatographic peak parameters were obtained by a Varian Model CDS-111L data system.

Analysis of tissue samples

Fortified tissue samples (100 g each) containing 5 nCi of the ^{14}C-radiolabeled nitrosamine I (specific activity, 1.41 mCi/mmole)[7] were thoroughly extracted with diethyl ether–methanol–acetic acid (1:1:0.1) (2 × 200 ml). After removal of the solvents, the residual extract from each sample was chromatographed on a silica-gel column and eluted with a sequence of solvents in the same manner as described in the earlier ACC experiments. The radioactive fractions were combined and evaporated to *ca.* 0.5 ml; this material was then streaked onto a thin-layer chromatographic plate (Analtech Uniplate silica gel GHLF 0.05 × 20 × 20 cm, Analtech, Newark, DE, U.S.A.). The plate was developed with 2:1 methylene chloride–methanol and the radioactive band (R_F between 0.69 and 0.75) corresponding to that of reference nitrosamines I and II was scraped off. Extraction of this material first with 10 ml diethyl ether then with an equal portion of methanol followed by evaporation to dryness under a stream of nitrogen afforded an analytical sample. Aliquots (10 μl) of the sample in methylene chloride (total solution volume 0.2 ml) were quantified by normal phase HPLC–thermal energy analysis. Several enriched samples obtained from 500 to 1,000 g each of fortified tissues were analyzed by GC–chemical ionization mass spectrometry for the confirmation of analyte structures. The mass spectral procedure employed a Varian MAT 112 magnetic-sector, double focusing mass spectrometer equipped with a dual electron impact–chemical ionization source (isobutane was used as the reagent gas). The mass spectrometer was connected to a Varian 1440 gas chromatograph via a Varian MAT dual capillary-glass jet interface unit. Char-

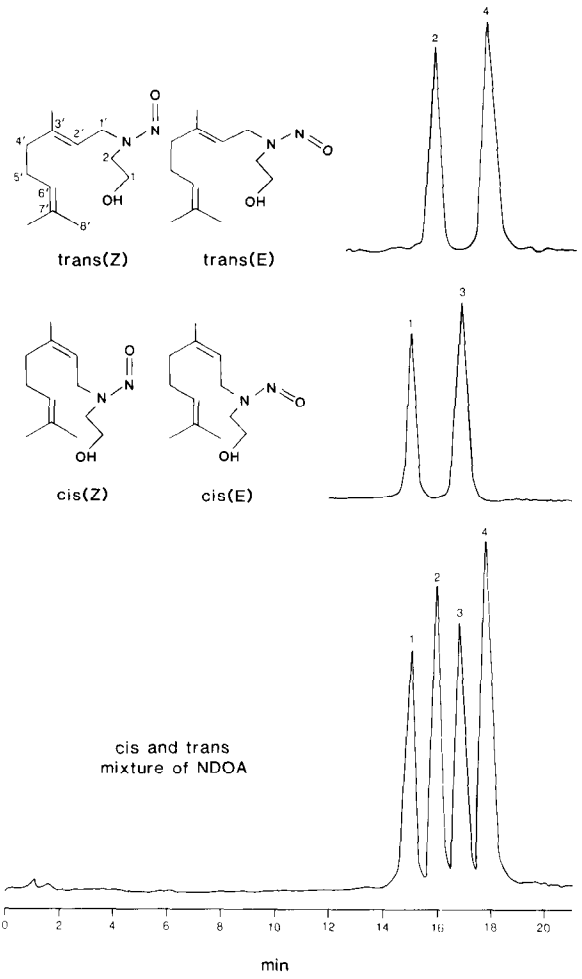

Fig. 1. Reversed-phase HPLC chromatograms and structures of nitrosamine I (NDOA). HPLC conditions: water–[acetonitrile–water (9:1) + sodium octanesulfonate + 0.01 *M* $HClO_4$] (65:35), 3 ml/min, isocratic elution. Peak identity, 1: *cis*(*Z*); 2: *trans*(*Z*); 3: *cis*(*E*); 4: *trans*(*E*).

acteristic mass spectral signals were *m/e* 227 ($M^+ + 1$), *m/e* 209 (M − OH), *m/e* 196 (M − NO), and *m/e* 137 ($M - C_2H_5N_2O_2$) for nitrosamine I; and *m/e* 211 ($M^+ + 1$), *m/e* 193 (M − OH), *m/e* 180 (M − NO), and *m/e* 121 ($M - C_2H_5N_2O_2$) for nitrosamine II.

RESULTS AND DISCUSSION

With reference to nitrosamine structures in Figs. 1 and 2, the *cis*-compounds in general seemed to have sufficiently higher mobility than the *trans*-counterparts in both ACC and GC to enable complete separation of the pure isomers by ACC. The average GC resolution (*R*) parameters are 1.75 and 1.63 for nitrosamine I and nitrosamine II, respectively. Because each geometric isomer consists of an *E* isomer and

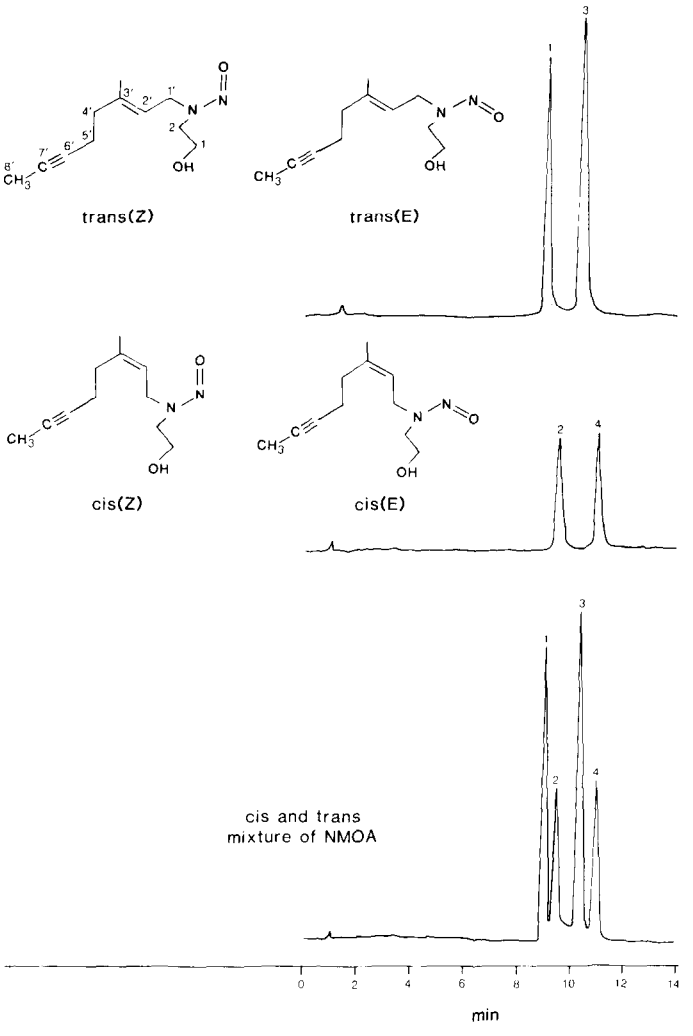

Fig. 2. Reversed-phase HPLC chromatograms and structures of nitrosamine II (NMOA). HPLC conditions: water–acetonitrile (70:30) containing 0.1 *M* $AgNO_3$ (pH 3), 2 ml/min, isocratic elution. Peak identity, 1: *trans(Z)*; 2: *cis(Z)*; 3: *trans(E)*; 4: *cis(E)*.

a *Z* isomer in a ratio that varies with temperature, it would be difficult to predict the chromatographic elution pattern on the basis of the isomeric structures, particularly in the absence of quantitative information on the polarity of contributing individual isomers. Comparative GC analyses of the two types of nitrosamines (I and II) revealed retention times in the order I < II. This ordering is representative for all of the ACC, GC, and normal-phase HPLC systems studied in that the 6′,7′-olefinic nitrosamines (I) elute before their 6′,7′-acetylenic analogues (II).

Variable temperature (30–90°C) nitrosation of BDOA induced isomerization of the 2′,3′-allylic double bond and gave the nitrosamine products presented in Table I. The *cis–trans* ratios derived from GC measurements are in good agreement with

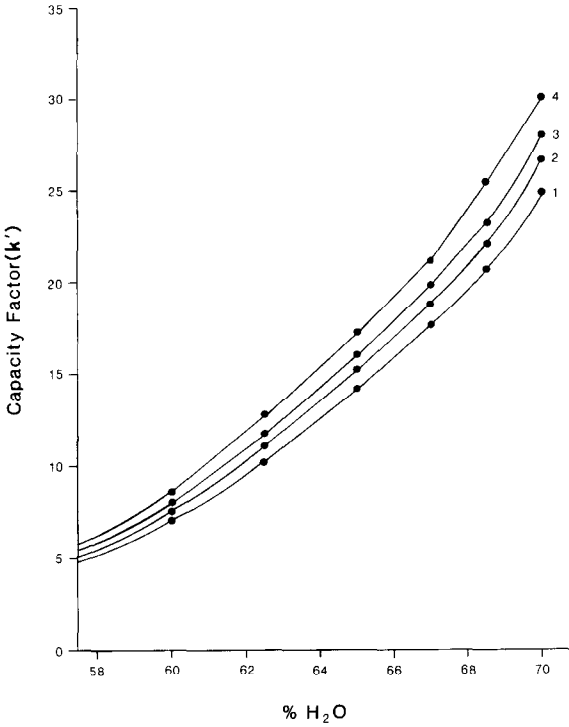

Fig. 3. Effect of water content in the mobile phase on the capacity factor of NDOA isomers. HPLC conditions: water–acetonitrile containing 0.1 *M* sodium octanesulfonate (pH 3 adjusted with H_3PO_4), 3 ml/min, isocratic elution. Component designations are as in Fig. 1.

those obtained by HPLC methods. The parity in *cis–trans* compositions between nitrosamine I and nitrosamine II in a reaction mixture is clearly evident. This implies that the transformation of nitrosamine I to II probably proceeds without isomerization.

At the outset of the HPLC work, numerous attempts were made to select suitable mobile phases for effecting component separation and to achieve optimization of HPLC conditions. Of the many solvent systems examined, we found that acetonitrile–water and methylene chloride–THF–2-propanol systems demonstrated the most satisfactory capability for use as eluents in respective reversed-phase and normal-phase HPLC. To ensure the unambiguous peak assignment for the four isomeric peaks [each geometric isomer (*cis* or *trans*) has two configurational isomers (*E* and *Z*) of a nitrosamine], a sample of the nitrosamine mixture was co-chromatographed with that of a pure isomer obtained by ACC and preparative HPLC. Such a peak-matching technique executed under different HPLC conditions should suffice to establish the peak identity of each component isomer. Typical HPLC chromatograms showing the separation of nitrosamine isomers in the reversed-phase mode are illustrated in Fig. 1 for nitrosamine I and in Fig. 2 for nitrosamine II. The separations of similar sets of isomeric compounds in the normal-phase mode are shown in Fig. 4 for comparison. Results of these studies with the two methods of component sep-

TABLE I

CIS-TRANS COMPOSITION OF MIXTURES OF NDOA AND OF NMOA FORMED FROM NITROSATION OF BDOA AT VARIOUS TEMPERATURES

Reactions were carried out in 70% aqueous dimethyl formamide solution buffered at pH 5 with phosphate salts. The initial amine (BDOA)/nitrite ratio was 1/15.

Temperature (°C)	*Reaction time (h)*	*Composition (cis–trans)**			
		NDOA		*NMOA*	
		GC S.D. × 10²	*HPLC S.D. × 10²*	*GC S.D. × 10²*	*HPLC S.D. × 10²*
30	24	0.13 ± 0.77	0.12 ± 0.85	0.13 ± 0.78	0.13 ± 0.82
40	24	0.23 ± 1.15	0.23 ± 1.43	0.22 ± 0.90	0.23 ± 1.33
50	12	0.34 ± 1.77	0.33 ± 2.11	0.32 ± 1.31	0.33 ± 1.49
60	12	0.40 ± 1.40	0.39 ± 1.51	0.38 ± 1.63	0.39 ± 2.03
70	6	0.43 ± 1.16	0.43 ± 1.12	0.42 ± 1.55	0.43 ± 1.68
80	4	0.47 ± 1.41	0.46 ± 1.86	0.46 ± 1.33	0.45 ± 1.62
90	3	0.49 ± 1.03	0.50 ± 1.90	0.48 ± 1.20	0.49 ± 1.47

* Composition measurements were based on the mean values of three replicate determinations. S.D. = standard deviation. Average coefficient of variation: NDOA, 3.90% (GC) and 4.76% (HPLC); NMOA, 3.86% (GC) and 4.61 (HPLC). The HPLC mobile phase was hexane–[THF–chloroform–2-propanol(1:1:0.1)] (87:13).

aration (reversed-phase partition and normal-phase liquid–solid adsorption) are discussed separately.

In reversed-phase HPLC, the presence of added salts to serve as modifiers and complexing agents in the mobile phases somewhat improved the peak resolution (R) and column selectivity (α) (Table II). To ascertain the possible interaction of sodium alkane sulfonate with the nitrosamine solute in the chromatographic process, the retention behavior of the nitroso-compounds was studied at several concentrations (5–20 mM) of sodium methane sulfonate in 30:70 acetonitrile–water buffered at pH 3 with phosphoric acid. From the retention data in Table II, it is obvious that the capacity factor (k') of a component isomer increases with increasing sulfonate concentration, indicating an increased degree of hydrophobic interaction between the mobile phase solutes and the octadecysilica bonded phase in the reversed-phase partitioning process. N-nitrosamines are known to be polar due to the dipolar electronic structure of the nitrosamine function with a positive charge on the amine nitrogen, which may interact with an ion-pairing reagent such as the alkaline salt of alkyl sulfonates to form a less polar complex in the above mentioned HPLC system.

Table II also lists the k' values of the nitroso-compounds obtained from argentation of the mobile phase [acetonitrile–water (30:70), adjusted to pH 3 with phosphoric acid] with various concentrations of silver nitrate (5–500 mM). As the concentration of the latter reagent increased, each of the nitrosamine isomers tended to be retained less (smaller k' value) by the octadecylsilica column. This result, coupled with the observation of improved resolution (higher R value; Table II) upon argentation, is suggestive of the effectiveness of the complexation of the silver ion with the olefinic bond to yield an analyte species of reduced hydrophobicity. At this point, it must be stated that, among all of the reversed-phase HPLC techniques investigated in the present study, the argentation method described here has been the unique

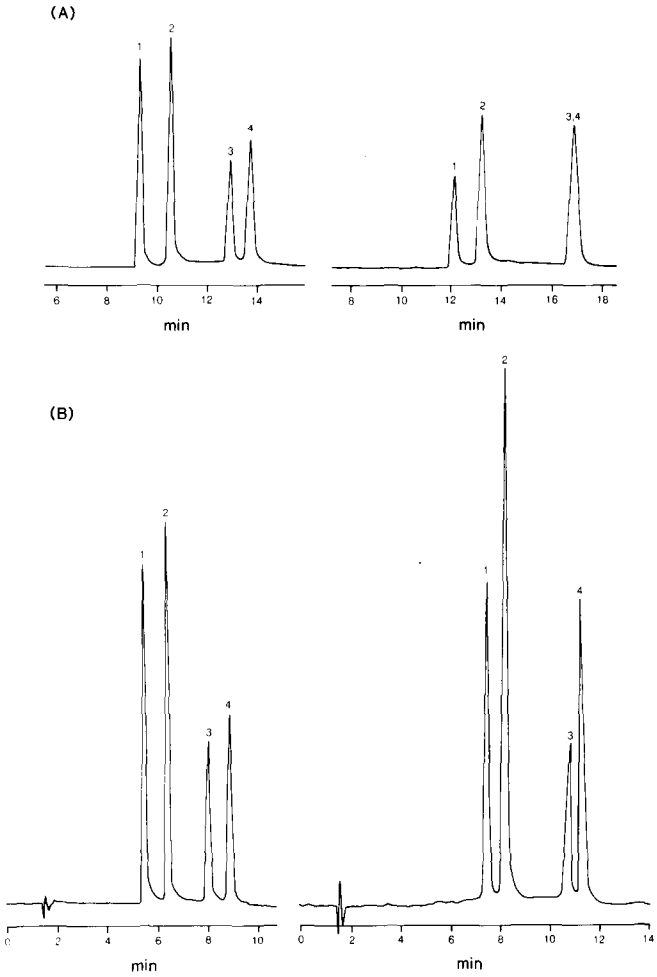

Fig. 4. Normal phase HPLC chromatograms of NDOA and of NMOA, HPLC conditions: (A) hexane–[methylene chloride–2-propanol (93:7)](85:15), 2 ml/min, isocratic elution, and (B) hexane–[THF–methylene chloride–2-propanol (1:1:0.1)] (85:15), 2 ml/min, isocratic elution. Peak identity for both NDOA (left) and NMOA (right), 1: *cis* (*E*); 2: *trans*(*E*); 3: *cis*(*Z*); 4: *trans*(*Z*).

method of choice for achieving base-line resolution of the four isomers of nitrosamine II.

Comparisons of pertinent chromatographic characteristics given in Table II for nitrosamines I and II show vast differences in chromatographic behavior between the two types (I and II) of nitrosamines studied under identical reversed-phase HPLC conditions, in spite of the minor dissimilarity in their structures. Components of nitrosamine I required a much longer time (nearly five times as long) to elute through the reversed-phase column than did those of nitrosamine II. Within a mobile phase system where either sodium methane sulfonate or silver nitrate was present, the reversed-phase HPLC data indicated an apparent reversal of the elution order between the corresponding isomeric pairs of the two nitrosamines (I and II). This is better

TABLE II

REVERSED-PHASE (ODS) HPLC OF NITROSAMINES I (NDOA) AND II (NMOA) AT 25°C

The mobile phase consisted of acetonitrile–water (30:70) and its pH was adjusted to 3.00 with phosphoric acid.

Concentration of added salt (M)	*Chromatographic characteristics**													
	NDOA component peak							*NMOA component peak*						
	1		*2*		*3*		*4*	*1*		*2*		*3*		*4*
	k'	*R* (α)	*k'*	*R* (α)	*k'*	*R* (α)	*k'*	*k'*	*R* (α)	*k'*	*R* (α)	*k'*	*R* (α)	*k'*
Sodium methane sulfonate														
0.005	42.0	1.20 (1.04)	43.8	4.27 (1.15)	50.2	0.87 (1.02)	51.0	8.00	0.00 (1.00)	8.00	2.14 (1.14)	9.10	0.80 (1.04)	9.50
0.010	44.2	1.47 (1.05)	46.4	4.47 (1.14)	53.1	0.93 (1.03)	54.5	8.28	(0.14 (1.01)	8.35	2.36 (1.14)	9.53	0.94 (1.05)	10.0
0.015	46.7	1.48 (1.08)	48.9	4.73 (1.15)	56.0	0.96 (1.02)	57.3	8.64	0.34 (1.02)	8.81	2.42 (1.15)	10.1	0.96 (1.05)	10.6
0.020	49.0	1.50 (1.08)	53.2	5.13 (1.17)	62.7	1.00 (1.05)	65.8	9.93	0.40 (1.03)	9.20	2.60 (1.15)	10.6	1.20 (1.06)	11.2
Silver nitrate														
0.010	60.3	1.73 (1.04)	62.9	5.26 (1.14)	72.1	1.31 (1.03)	74.2	9.20	0.86 (1.03)	0.94	1.48 (1.09)	10.2	1.33 (1.04)	10.6
0.050	52.0	1.80 (1.05)	54.7	5.30 (1.14)	62.2	2.07 (1.05)	65.3	7.89	0.98 (1.02)	8.08	2.40 (1.10)	8.80	1.47 (1.05)	9.24
0.100	46.4	1.87 (1.06)	49.2	5.33 (1.15)	56.6	2.10 (1.05)	59.5	6.71	1.43 (1.04)	7.00	2.95 (1.11)	7.79	2.38 (1.06)	8.29
0.200	38.6	1.93 (1.08)	41.5	5.36 (1.16)	48.2	2.30 (1.06)	51.1	6.57	1.56 (1.05)	6.87	3.11 (1.11)	7.65	2.41 (1.07)	8.22
0.300	36.3	2.05 (1.08)	39.2	5.51 (1.17)	46.5	2.44 (1.08)	50.2	6.21	1.58 (1.06)	6.60	2.99 (1.12)	7.38	2.44 (1.09)	8.07

* All resolution (*R*) and selectivity (α) values were determined based on the peak characteristics of adjacent components. For component peak identification, see Figs 1 and 2.

represented by the order of increasing retention time as follows: for nitrosamine I, *cis*(*Z*) < *trans*(*Z*) < *cis*(*E*) < *trans*(*E*); for nitrosamine II, *trans*(*Z*) < *cis*(*Z*) < *trans*(*E*) < *cis*(*E*). Although we found it difficult to correlate the isomeric structural parameter with the component retentivity, the *E* isomers in the two series of nitrosamine components illustrated above were generally more retentive on the reversed-phase column than the *Z* isomers. The least retained components were *cis*(*Z*) for nitrosamine I and *trans*(*Z*) for nitrosamine II. In the reversed-phase HPLC of these nitrosamines, the water content in the mobile phase had a marked effect on the retention behavior of a component isomer. Fig. 3 shows, as an example, how a small variation in water content (58–70%) gave rise to a large change in the capacity factor, k', (5–30) of the components in nitrosamine I.

Some chromatographic profiles of the isomeric nitrosamines obtained from normal-phase HPLC on a silica column are displayed in Fig. 4 to demonstrate the effect of mobile phase solvents on the isomer separation. Elution of the various isomers followed the same pattern as that observed in ACC and GC: *cis* < *trans*; nitrosamine I < nitrosamine II (in the order of increasing adsorption on the silica stationary phase). Between the two nitrosamine (I and II), there was a consistency in the elution sequence: the *cis* (E)-isomer emerged first from the column effluent, followed successively by *trans*(*E*)-, *cis*(*Z*)-, and *trans*(*Z*)-isomers. Examination of the left two chromatograms in Fig. 4 reveals, in each case, the four well-resolved peaks of nitrosamine I, although the chromatograms were obtained individually with two different mobile phases: (1) mobile phase A (Fig. 4A), hexane–[methylene chloride–2-propanol (93:7)] (85:15) and (2) mobile phase B (Fig. 4B), hexane–[methylene chloride–THF–2-propanol (1:1:0.1)] (85:15). As in the separation of nitrosamine I, the isomeric components of nitrosamine II were efficiently separated by using mobile phase B as shown in Fig. 4B (right). However, we experienced difficulty in resolving component peaks 3 and 4 depicted in Fig. 4A (right) when the column was eluted with the mobile phase A eluent. Presumably this was a reflection of the favorable effect of THF in mobile phase B, increasing the column selectivity for the isomers of nitrosamine II. The higher differentiability generally observed for nitrosamine I relative to nitrosamine II may be best explained by recognizing that the presence of the non-linear carbon chain along the carbons at 5′, 6′, 7′ and 8′ positions with an additional methyl group at the 7′ carbon in nitrosamine I (note the linear arrangement of the corresponding carbons without a 7′-methyl group in nitrosamine II) will impart comparatively more effective interactions with the silica stationary phase.

Table III is a compilation of the resolution data together with the k' values determined under various mobile phase conditions. The experimental information in this table supports the concept that the capacity factor, k', decreased with increasing solvent strength as a result of competition for adsorption sites between the analyte solutes and solvents, and that resolution, R, increased with the decreasing solvent strength within the same solvent system. In a few instances where ternaries of THF–methylene chloride–[2-propanol (10%)] were used as mobile phases, an increase in the THF content in the mobile phase caused a slight increase in the R value. Also included in Table III are the α values to show the significant dependence of these selectivity parameters on polar constituents of the mobile phase employed. The representative curves in Fig. 5 illustrate the capacity factor k' of nitrosamine II as a function of the percent composition of hexane in a mobile phase composed of hexane

TABLE III

NORMAL-PHASE HPLC (Si) OF NITROSAMINES I (NDOA) AND II (NMOA) AT 25°C

*Mobile phase**	*Chromatographic characteristics***													
	NDOA component peak							*NMOA component peak*						
	1		*2*		*3*		*4*	*1*		*2*		*3*		*4*
	k'	*R* (α)	*k'*	*R* (α)	*k'*	*R* (α)	*k'*	*k'*	*R* (α)	*k'*	*R* (α)	*k'*	*R* (α)	*k'*
A	5.57	2.00 (1.18)	6.57	7.50 (1.25)	8.21	2.53 (1.07)	8.79	7.20	2.25 (1.08)	7.81	9.75 (1.33)	10.4	0.00 (1.00)	10.4
B	4.43	3.15 (1.26)	5.57	3.75 (1.24)	6.93	2.75 (1.14)	7.93	6.86	2.38 (1.13)	7.72	7.88 (1.37)	10.6	1.38 (1.05)	11.1
C	2.68	1.74 (1.23)	3.29	2.03 (1.26)	4.15	1.51 (1.15)	4.77	4.00	1.53 (1.13)	4.53	4.23 (1.37)	6.21	0.74 (1.09)	6.74
D	2.50	1.80 (1.26)	3.15	2.15 (1.27)	4.00	1.58 (1.16)	4.64	3.85	1.56 (1.17)	4.50	4.35 (1.34)	6.01	0.78 (1.08)	6.50
E	2.34	1.83 (1.26)	2.95	2.35 (1.28)	3.78	1.78 (1.17)	4.43	3.73	1.68 (1.19)	4.43	4.73 (1.33)	5.89	0.81 (1.07)	6.29
F	5.14	3.95 (1.28)	6.58	5.70 (1.31)	8.65	3.75 (1.16)	10.0	8.29	2.95 (1.13)	9.36	10.5 (1.40)	13.2	1.78 (1.05)	13.8
G	3.21	2.38 (1.27)	4.07	3.35 (1.30)	5.28	1.98 (1.14)	6.00	5.00	1.79 (1.13)	5.64	6.53 (1.42)	8.00	0.80 (1.04)	8.28
H	2.78	1.98 (1.26)	3.58	2.75 (1.29)	4.50	1.96 (1.16)	5.21	4.21	1.62 (1.13)	4.79	5.33 (1.40)	6.71	0.69 (1.04)	7.00
I	2.45	1.60 (1.23)	3.01	2.55 (1.30)	3.92	1.78 (1.16)	4.53	3.69	1.40 (1.13)	4.18	4.73 (1.40)	5.84	0.58 (1.03)	6.04
J	2.57	2.52 (1.14)	2.93	4.05 (1.19)	3.50	2.03 (1.08)	3.79	3.29	3.00 (1.13)	3.71	5.21 (1.25)	4.64	0.00 (1.00)	4.64

* Each mobile phase in the A–I series was composed of 85% hexane and 15% other solvents corresponding to the following combinations: A, CH_2Cl_2–2-propanol (ISP) (93:7); B, THF–CH_2Cl_2–ISP (1:1:0.1); C, THF–CH_2Cl_2–ISP (1:1:0.2); D, THF–CH_2Cl_2–ISP (2:1:0.3); E, THF–CH_2Cl_2–ISP (3:1:0.4); F, THF–$CHCl_3$–ISP (1:1:0.1); G, THF–$CHCl_3$–ISP (1:1:0.2); H, THF–$CHCl_3$–ISP (1:1:0.3); I, THF–$CHCl_3$–ISP (1:1:0.4). Mobile phase J was composed of 75% hexane and 25% CH_2Cl_2–CH_3OH (95:5).

** For *R* and α determinations, see footnote to Table II. For component peak identification, see Fig. 4.

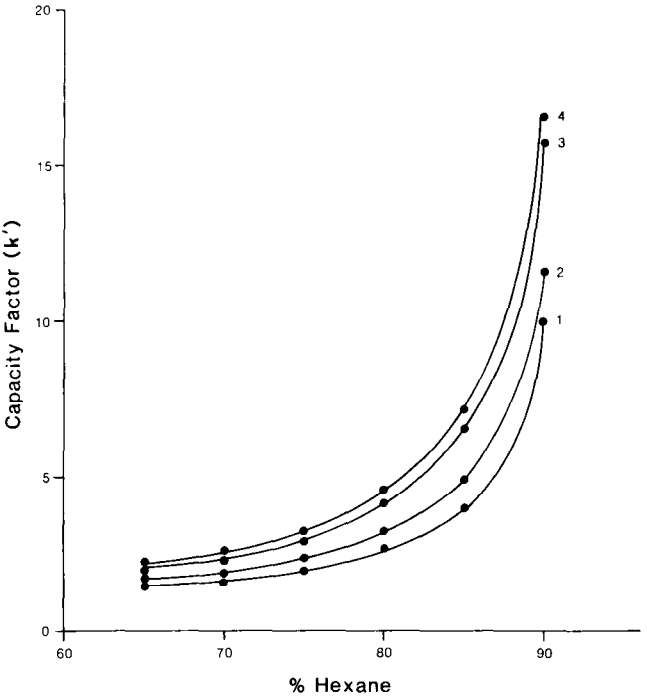

Fig. 5. Effect of hexane content in the mobile phase on the capacity factor of NMOA isomers. HPLC conditions are as in Fig. 4B. Component designations are as in Fig. 4.

and THF–methylene chloride–2-propanol (1:1:0.1). Evidently, a gradual change in the percent content of hexane in the mobile phase from 65% to 90% produced a large change in resolution parameters, especially between the sets of E and Z configurational isomers. The effect was most pronounced at the upper extreme of the hexane content (90% in this case), as evidenced by the marked increase in $R^{2,3}$ values: from 1.31 (65% hexane) to 10.7 (90% hexane) for peaks 2 and 3 (Fig. 5). When the mobile phase contained a high proportion of nonpolar solvents, such as hexane, the adsorption process seemed to be dictated by the polarity differences between the *E* and *Z* isomers.

An important application of the HPLC separation technique was to determine the energy barrier for the interconversion of *E* and *Z*-nitrosamines by measuring the first order rate of equilibration (starting from either pure *E* or pure *Z* compounds). The calculated free energy of activation had average values of 25.8 kcal/mole for nitrosamine I and 22.1 kcal/mole for nitrosamine II. The requisite of *E–Z* compositional data at different temperatures for the above computation of activation parameters led us to study the influence of temperature on retention behavior and other chromatographic properties of the nitrosamine isomers (Table IV). The *E–Z* isomeric ratios obtained from normal- and reversed-phase HPLC were clearly disparate. Isomer ratios reported in Table IV are reflective of the equilibrium constants at given temperatures. The predominant occurrence of the *E* rotamer, over the *Z* rotamer in relatively less polar solvents (normal phase eluents) may be attributed to

TABLE IV

EFFECT OF TEMPERATURE ON THE CHROMATOGRAPHIC BEHAVIOR OF NITROSAMINE ISOMERS

Mobile phase solvent compositions were as follows: reversed-phase HPLC, water–[acetonitrile–water (9:1) containing 0.01 *M* $HClO_4$ + 0.01 *M* $NaC_8H_{17}SO_3$] (65:35) for NDOA: water–acetonitrile (70:30) containing 0.1 *M* $AgNO_3$ at pH 3 for NMOA. Normal-phase HPLC, hexane–[THF–$CHCl_3$–ISP (1:1:0.1)] (85:15). C.V. = coefficient of variation.

Temperature (°C)	*Nitrosamine component**							*E–Z isomeric ratio*			
	1		*2*		*3*		*4*	*cis*	*(C.V.)*	*trans*	*(C.V.)*
	k'	*R*	*k'*	*R*	*k'*	*R*	*k'*				
Reversed-phase											
0.01 *M* $HClO_4$ + 0.01 *M* $NaC_8H_{17}SO_3$		*NDOA*									
25	13.80	1.22	15.0	1.21	15.8	1.29	16.9	1.11	(3.1)	1.15	(2.9)
35	12.3	1.18	13.3	0.99	14.0	1.25	14.9	1.13	(3.4)	1.16	(2.6)
45	11.0	1.04	11.8	0.96	12.2	1.09	12.8	1.15	(3.5)	1.16	(2.8)
55	10.1	0.82	10.6	0.81	10.9	0.87	11.4	1.18	(5.0)	1.20	(3.8)
65	8.85	0.59	9.20	0.69	9.38	0.74	9.67	1.22	(6.6)	1.25	(5.3)
0.1 *M* $AgNO_3$		*NMOA*									
25	6.71	1.43	7.00	2.95	7.79	2.38	8.29	1.03	(2.7)	1.05	(2.3)
35	6.36	1.09	6.57	2.75	7.35	2.11	7.72	1.05	(2.9)	1.11	(2.7)
45	5.93	0.78	6.21	2.00	6.86	1.28	7.15	1.09	(3.3)	1.13	(3.0)
55	5.57	0.63	5.71	1.77	6.28	0.99	6.57	1.11	(4.7)	1.24	(4.1)
65	5.50	0.40	5.67	1.58	6.18	0.82	6.36	1.14	(4.9)	1.28	(5.5)
Normal-phase											
THF–$CHCl_3$–ISP (1:1:0.1)		*NDOA*									
25	5.14	3.95	6.58	5.70	8.65	3.75	10.0	2.19	(3.2)	2.10	(2.5)
35	5.09	3.73	6.52	5.33	8.48	3.71	9.75	2.21	(2.8)	2.11	(2.8)
45	5.00	3.53	6.30	4.73	8.06	3.63	9.11	2.23	(3.5)	2.13	(3.7)
50	4.89	3.18	6.06	4.13	7.76	3.55	8.77	2.25	(6.3)	2.17	(5.8)
		NMOA									
25	8.29	2.95	9.36	10.5	13.2	1.78	13.8	2.12	(2.9)	1.84	(2.7)
35	8.13	2.35	9.11	10.0	12.7	1.54	13.3	2.12	(3.8)	1.85	(3.5)
45	7.89	2.23	8.76	9.48	12.0	1.35	12.5	2.14	(4.3)	1.88	(4.6)
50	7.34	2.16	8.15	8.77	11.0	0.95	11.3	2.19	(6.5)	1.93	(5.4)

* The *R* determinations are the same as in Table II. For component peak identification, see Figs. 1, 2 and 4.

intramolecular hydrogen bonding[8] involving the *E* rotamer in which the hydroxyethyl group is *cis* to the nitroso oxygen. In accordance with this consideration, the equilibrium compositions of *E–Z* rotamers in more polar solvents, including the reversed-phase eluents, were altered to some extent in favor of the *Z* rotamers as evident from the results in Table IV, though the *E* rotamers remained in slight excess in all of the equilibrium mixtures under analysis. The examples in Table IV reveal a rather small variation in the *E–Z* composition with temperature variations but a notable temperature dependence for *k'* and *R*. Parallel to the effect of polar solvents on the chromatographic parameters, the elution of nitrosamine components were facilitated (lowering the *k'* value) by an increase in temperature, whereas elevation of temperature adversely affected the efficiency of peak resolution (lowering the *R* value). As

TABLE V

NORMAL-PHASE HPLC–THERMAL ENERGY ANALYSIS OF FORTIFIED FISH TISSUE SAMPLES

The mobile phase was composed of hexane–[THF–chloroform–2-propanol (1:1:0.1)] (87:13) and the flow-rate was maintained at 2 ml/min under isocratic elution.

Sample	*Nitrosamine level (ng/g)**															
	NDOA component peak								*NMOA component peak*							
	1		*2*		*3*		*4*		1		*2*		*3*		*4*	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*
1	0.311	0.255	0.357	0.305	0.142	0.126	0.170	0.143	0.025	0.021	0.026	0.022	0.012	0.010	0.014	0.011
2	1.205	1.038	1.182	0.997	0.550	0.487	0.563	0.476	0.114	0.920	0.116	0.092	0.054	0.045	0.063	0.052
3	1.866	1.513	1.825	1.639	0.852	0.796	0.869	0.703	0.176	0.138	0.162	0.129	0.083	0.070	0.088	0.073
4	2.262	1.886	2.312	2.060	1.033	0.911	1.101	0.880	0.208	0.174	0.223	0.185	0.113	0.089	0.121	0.098
5	2.751	2.397	2.707	2.434	1.256	1.169	1.289	1.130	0.267	0.227	0.254	0.209	0.126	0.101	0.138	0.111
6	3.221	2.801	3.173	2.676	1.471	1.232	1.511	1.363	0.314	0.269	0.282	0.218	0.148	0.120	0.153	0.123
7	3.778	3.348	3.912	3.474	1.725	1.463	1.863	1.605	0.382	0.336	0.347	0.294	0.180	0.159	0.189	0.150
8	4.529	4.005	4.652	4.010	2.068	1.738	2.215	1.904	0.445	0.483	0.423	0.361	0.210	0.178	0.230	0.193
Average recovery (%)	85.44		87.13		88.06		84.93		83.54		82.11		82.81		80.89	

* A = amounts of the nitrosamine added; B = amounts of the nitrosamine found. Nitrosamine level values are based on the mean values of three determinations. Coefficient of variation for all the analyses ranged 5.82–8.75%. For component peak identification, see Fig. 4B.

to the question on the generality of component differentiation among the isomers, the magnitude of ^{13}C NMR signal separations for the *cis–trans* pairs ($\Delta\delta$, 7.40–8.11 ppm) and for the *E–Z* pairs ($\Delta\delta$, 1.10–9.32 ppm) has been recently demonstrated[5]. For normal- and reversed-phase HPLC, the separation of the *E–Z* configurational isomers was always greater than that of the *cis–trans* geometric isomers (Figs. 1, 2 and 4).

In the course of applying the chromatographic methodology developed in the present work to the analysis of the title nonvolatile nitrosamines in organic tissues, we found that the combined use of HPLC–thermal energy analysis allowed sensitive detection of the nitrosamine components with desired selectivity and specificity. The utility of this HPLC–thermal energy analysis procedure was demonstrated in the quantitative determination of fish tissue samples for trace residues (< 10 ppb) of the nitrosamines shown in Table V. In these analyses, tissue samples fortified with known amounts of the nitrosamine analytes were assayed (see Experimental section for the cleanup procedure) to establish the validity of the analytical method. Calibration data were furnished by plotting the detector response against the concentration of standard solutions including that of an internal standard, N,N-bis (3′,7′-dimethyl-2′,6′-octadienyl) nitrosamine. These calibration curves for all four of the component isomers of each type of nitrosamine showed a linear response over the entire dynamic range from the detection limit (0.1 ng/g) to 10^3 ppb (slope, 1.0, correlation coefficient, 0.997–0.999). As indicated in Table V, the average recovery and coefficient of variation for the eight-sample analyses ranged from 80.89 to 88.06% and 5.82 to 8.75%, respectively.

We have assessed the relative merits of HPLC–UV and HPLC–thermal energy analysis methods for tissue analysis and concluded that the HPLC–UV method experiences considerably higher sample matrix effects. The sensitivity of the HPLC–UV method (detection limit, 0.5 μg/g) was too low for trace residue analysis. The nitrosamine-specific characteristics of thermal energy detection are expected to provide a unique trace analytical technique (Table V) with practical importance, in view of the reported detection of trace levels of nitrosamine residues (less than 10 ppb) in chemically treated fish[4].

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