A GAS–LIQUID CHROMATOGRAPHIC PROCEDURE FOR THE DETECTION OF VOLATILE N-NITROSAMINES AT THE TEN PARTS PER BILLION LEVEL IN FOODSTUFFS AFTER CONVERSION TO THEIR CORRESPONDING NITRAMINES

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SUMMARY

A procedure is described which is capable of detecting volatile N-nitrosamines in foodstuffs at the 10-p.p.b.* level or better. The method is based on vacuum distillation, clean-up on an alumina column, oxidation with peroxytrifluoroacetic acid, further clean-up on a mixed magnesium oxide/alumina column and determination by electron capture gas–liquid chromatography.

The method has been evaluated for a range of N-nitrosamines added to a variety of foodstuffs.

INTRODUCTION

A wide range of analytical techniques have been proposed for the determination of N-nitrosamines in foodstuffs[1–5] but several recent papers[6–8] have indicated that many, if not all, of these earlier techniques suffer from a lack of sensitivity and/or specificity. The analytical requirements were considered by an IARC Panel in 1969[9] which concluded that, in order to provide adequate information, methods should be capable of determining individual nitrosamines down to concentrations of 1 to 10 parts in 10^9 (*i.e.* 1–10 p.p.b.). Several techniques have been reported[10–13] which are based on the use of gas chromatography–mass spectrometry (GC–MS) and give both the sensitivity and specificity required. However, the number of laboratories with GC–MS facilities is limited and a need exists for a purely GC method capable of screening at these levels.

In 1970 SEN[14] and ALTHORPE *et al.*[15] independently reported the outline of a technique based on the oxidation of N-nitrosamines to their corresponding nitramines with peroxytrifluoroacetic acid (PTFA)[16]. The nitramines so formed were determined by electron capture gas chromatography. The response of nitramines to the electron capture detector (ECD) compared to the response of the parent N-nitrosamines to the flame ionisation detector was greater by two to three orders of magnitude, depending on the compound studied.

This report covers the development of a method capable of screening for the

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* Throughout this article the American (10^9) billion is meant.

presence of a range of volatile N-nitrosamines in foodstuffs at a level of 10 p.p.b. or better.

EXPERIMENTAL

The efficiency of conversion of N-nitrosamines to their corresponding nitramines

Oxidation conditions are those reported by ALTHORPE *et al.*[15], given in full in the ANALYTICAL PROCEDURE.

Conversion efficiencies have been studied at levels of 10, 2.5 and 1 μg for a series of volatile N-nitrosamines, corresponding to levels of 40, 10 and 4 p.p.b. for a 250-g sample.

TABLE I

EFFICIENCY OF CONVERSION OF N-NITROSAMINES TO NITRAMINES USING PTFA

Nitrosamine	Conversion %			
	50 μg[a]	10 μg	2.5 μg	1 μg
Dimethylnitrosamine (DMN)	86	—	96	95
Methylethylnitrosamine (MEN)	85.5	—	—	—
Diethylnitrosamine (DEN)	84.5	—	86	92
Methylisopropylnitrosamine (Mi-Pn)	76	—	—	—
Diisopropylnitrosamine (Di-PN)	72	—	—	—
Di-*n*-propylnitrosamine (Dn-PN)	84	83	—	—
Diisobutylnitrosamine (Di-BN)	82.5	—	93	99
Di-*n*-butylnitrosamine (Dn-BN)	83	83	78	95
N-nitrosopiperidine (NN Pip)	25	nil	nil	nil
N-nitrosopyrrolidine (NN Pyrr)	85	87	84	95

[a] Values from ref. 15.

A series of pure nitramines was prepared[14] and their response to an ECD was determined. The efficiency of oxidation of each N-nitrosamine at the above level was then determined by comparing the peak heights of the nitramine formed with that of the appropriate nitramine standard.

Results are given in Table I, together with recoveries at 50-μg level previously reported by ALTHORPE *et al.*[15].

Conversion efficiencies are fairly constant for up to 50 μg of individual N-nitrosamines. Conversion efficiencies for N-nitrosopiperidine were low and non-reproducible. When standard solutions of N-nitraminopiperidine in methylene chloride were exposed to the standard PTFA mixture the amount of nitramine detected decreased regularly with time, indicating that N-nitrosopiperidine is unstable in the presence of the oxidising agent.

We have also found that N-nitrosamines containing a phenyl substituent cannot be quantitatively oxidised by this technique.

Extraction and clean-up

Volatile N-nitrosamines can be extracted from foodstuffs by either steam or vacuum distillation. For the work here reported the vacuum distillation technique described by TELLING *et al.*[11] was used.

Recoveries of 2.5-μg quantities of a range of N-nitrosamines added to a range of meat and vegetable substrates and put through the distillation technique have been obtained using GC–MS as the determinative step[11,12]. Results are given in Table II.

These recoveries are similar to those reported by BRYCE AND TELLING[12] and it was found that recoveries of added nitrosamines are independent of the type of substrate used.

TABLE II

THE RECOVERY OF 2.5-μg QUANTITIES OF N-NITROSAMINES ADDED TO 250 g OF MEAT OR VEGETABLE SUBSTRATE THROUGH A VACUUM DISTILLATION TECHNIQUE

Nitrosamine	Recovery (%)		
	Max.	Mean	Min.
DMN	94	78	48
DEN	93	82	54
Di-BN	100	68	52
Dn-BN	65	54	47
NN Pyrr	61	41	25

The use of activated alumina for cleaning-up extracts containing N-nitrosamines has been described by several workers[9] but not at the levels required by the proposed technique.

The use of acid, neutral and alkaline Woelm alumina of activities 1–3 was examined and the elution profiles of N-nitrosamines and their corresponding nitramines with solvent systems based on n-hexane–diethyl ether and n-hexane–methylene chloride mixtures were studied.

Results may be summarised as follows:

(1) The order of elution of N-nitrosamines is always the same, viz. Di-BN, Dn-BN, Dn-PN, Di-PN, DEN, NN Pip, MEN, DMN and NN Pyrr.

(2) N-nitrosamines are eluted from acid aluminas at a much faster rate than from neutral and basic aluminas, which give similar elution rates.

(3) 1-ml samples in methylene chloride must be diluted with 4 ml of n-pentane or n-hexane before addition to the column to prevent solvent front carry-through and loss of column efficiency.

(4) Only N-Nitrosopyrrolidine can be isolated from other N-nitrosamines.

(5) Diethyl ether–n-hexane mixtures are better eluants than dichloromethane–n-hexane mixtures.

(6) Aluminas which are weaker than activity 3 show poor retention of N-nitrosamines, even when n-hexane is used as the eluting solvent.

(7) The elution pattern for nitramines is very similar to that of the corresponding N-nitrosamines.

(8) Relatively polar solvent mixtures are required to elute nitramines from high activity alumina columns (e.g. 25 % diethyl ether in n-pentane) whereas relatively non-polar eluting solvents (e.g. 1 % diethyl ether in n-pentane) will elute the majority of interfering distillate components.

(9) It was not possible to completely separate N-nitrosamines from co-extracted components using a single column of activated alumina.

(10) By obtaining partial clean-up on an alumina column, then oxidising the eluate with PTFA and again adding to an alumina column, it is possible to obtain a fraction containing all nitramines virtually free of interfering components.

(11) PTFA reagent contains electron capturing impurities and these are best removed from the oxidised mixture by incorporating magnesium oxide into the second column of activated alumina.

TABLE III

RECOVERIES OF N-NITROSAMINES AND THEIR CORRESPONDING NITRAMINES THROUGH THE COLUMN CLEAN-UP STEPS

Nitrosamine	Recovery (%)	
	1st Column (Nitrosamine)	2nd Column (Nitramines)
DMN	78	77
DEN	94	95
Di-BN	97	95
Dn-BN	98	92
NN Pyrr	95	92

Working from these observations, a technique has been developed based on initial clean-up on an alumina column, oxidation, and final clean-up on a mixed alumina/magnesium oxide column. Recoveries through the clean-up stages for 2.5 μg of individual N-nitrosamines are given in Table III.

Losses through the two clean-up stages are small for compounds other than DMN and its corresponding nitramine. The major source of loss of DMN is by evaporation, rather than during either of the elution steps.

Gas–liquid chromatographic determination

Various stationary phases and operating parameters have been examined. PEG 20M was found to be the most useful stationary phase and an operating temperature of 140–150° gave satisfactory elution times.

Using a Pye [63]Ni detector at 1 × 500 attenuation under the conditions given in the METHODS section the response data given in Table IV were obtained.

TABLE IV

RESPONSE DATA FOR NITRAMINES USING A PYE [63]Ni DETECTOR

Corresponding nitramine	Detection limit (ng)	Corresponding approximate level in a 250-g sample (p.p.b.)
DMN	0.025	0.2
DEN	0.03	0.2
Di-BN	0.06	0.4
Dn-BN	0.11	0.7
NN Pyrr	0.11	0.7

Equivalent to a peak height of 5 % f.s.d. on attenuation setting 1 × 500 (full scale is 2 × 10^{-9} A).

As shown in Tables I and II, the recoveries of N-nitrosamines through the distillation and oxidation stages are the principal factors governing the sensitivity of the method rather than the response of the ECD.

Because of the characteristics of the ECD it is essential to determine the linear response range for each nitramine. This response was found to deviate from linearity above a level of 1.2–1.5 ng injected, depending upon the particular nitramine.

For the experimental conditions described in the following method a 1.2-μl injection aliquot obtained from an original sample containing 10 p.p.b. of N-nitrosamine will contain ca. 1.1 ng of nitramine corresponding to DMN, DEN and Di-BN and hence will be at the top of the linear dynamic range of the detector. For Dn-BN and NN Pyrr, 1.2 μl of the final 2-ml solution will contain 0.6–0.7 ng of the corresponding nitramines and hence an injection volume of 2.4 μl could be used in the examination of these compounds.

ANALYTICAL PROCEDURE

Application

Products containing volatile N-nitrosamines other than N-nitrosopiperidine and those with a phenyl substituent.

Safety note

Some N-nitrosamines are known to be carcinogenic compounds and all experimental work should be done in a well ventilated area; safety gloves should be worn whenever N-nitrosamines are being handled.

Apparatus and reagents

Vacuum distillation apparatus; gas chromatograph fitted with an ECD; 10-ml graduated tubes; Kuderna–Danish evaporators; chromatographic columns 30 × 1 cm I.D.; dichloromethane, redistilled; *n*-pentane, distilled from sodium hydroxide; diethyl ether, AnalaR grade; sodium chloride, AnalaR grade; potassium carbonate, AnalaR grade.

Activated alumina, neutral Woelm, Brockmann Grade 3.

Deactivate aluminium oxide, neutral, activity 1 by the addition of 6 ml of water to each 94 g of alumina contained in a suitable flask. Stopper the flask tightly and shake until no visible lumps remain and the exothermic reaction has ceased. Equilibrate for 8 h in the closed flask before use.

Magnesium oxide, dried at 105° for 2–3 h; trifluoroacetic hydroxide, redistilled; hydrogen peroxide 85–90 % w/w (obtainable from Laport Industries Limited, Warrington, Lancs.); sodium sulphate, anhydrous; calcium carbonate, AnalaR grade.

Peroxytrifluoroacetic acid (PTFA).

Place 4–5 ml of redistilled dichloromethane in a 10-ml volumetric flask and carefully add 0.4 ml of 85–90 % w/w hydrogen peroxide by pipette. Slowly add by pipette 2.5 ml of redistilled trifluoroacetic anhydride, swirl gently and allow to cool in an ice-bath for 5 min. Allow the contents of the flask to attain room temperature, then dilute to volume with redistilled dichloromethane.

Standard nitramine solutions.

(a) Prepare a solution containing 7.2 μg/ml of dimethylnitramine, 7.0 μg/ml of

diethylnitramine, 7.2 μg/ml of diisobutylnitramine, 4.4 μg/ml of di-*n*-butylnitramine, 3.4 μg/ml of N-nitraminopyrrolidine in dichloromethane.

(b) Dilute 0.25 ml of stock solution to 2.0 ml with dichloromethane.

METHODS

Vacuum distillation

Weigh 250 g of minced sample into the container of a macerator, add 50 g of sodium chloride, 10 g of potassium carbonate and 250 ml of water and macerate the mixture for 10 min. Transfer the resultant suspension to a 2-l round bottomed flask, washing out the macerator with a minimum of water. Immerse the flask in a water-bath at < 10° and connect it as part of a vacuum still, passing ice-water through the condenser and cooling the 500-ml receiver in an ice-bath.

Apply the maximum vacuum of a rotary oil pump and heat the water-bath to 65°, ensuring that the whole surface of the distillation flask is kept at this temperature. Maintain these conditions until no more distillate is produced (3–5 h). Release the vacuum and wash the condenser and adaptors with a minimum of water, adding the washings to the distillate. Note the volume of distillate (not less than 250 ml). Extract the distillate and washings with two 250-ml portions of dichloromethane, running the dichloromethane extracts into a Kuderna–Danish evaporator.

Immerse the lower part of the evaporator in a water-bath held at 50° and evaporate the dichloromethane, making sure that the level of solution in the evaporator is always above the water level in the bath.

When the volume of the extract is approximately 5 ml, remove the bulb from the collection tube, fit a Quickfit cone to the tube, and continue evaporating until the volume is reduced to 1 ml.

Initial clean-up

Weigh out 4 g of neutral alumina of Brockmann activity 3 and transfer to a chromatography column half filled with *n*-pentane, gently tapping the column between additions to assist settling. Add anhydrous sodium sulphate to form approximately a 1-cm layer on top of the alumina and run the level of the pentane just into the top of the sodium sulphate layer. Dilute the 1-ml concentrated dichloromethane solution with 4 ml of pentane and transfer the mixture to the top of the column. Place a 50-ml measuring cylinder under the column and run the solvent layer just into the top of the salt layer. Add 2 ml of *n*-pentane to the tube, transfer to the column and again run the level into the salt layer. Fill the column with a 1 % v/v solution of dichloromethane in *n*-pentane and elute until a total of 50 ml of eluate have been collected in the cylinder. Discard the eluate. Place a second 50-ml measuring cylinder under the column, fill the column with a 50 % v/v solution of dichloromethane in pentane and elute until a total of 50 ml of eluate has been collected. Transfer the eluate to a Kuderna–Danish evaporator and concentrate to approximately 5 ml on a water-bath at 50°. Disconnect the bulb, replace with a Quickfit cone to the tube and evaporate to 1 ml.

Oxidation

To the 1 ml of concentrated eluate add 0.2 ml of PTFA reagent, stopper the

tube, mix by swirling and allow the mixture to stand for at least 3 ½ h. Add 4 drops of distilled water, shake gently and allow to stand for 1 min. Add excess of calcium carbonate (*ca.* 0.25 g) and, when effervescence ceases, add 0.25 g of powdered anhydrous sodium sulphate. Shake gently, add 4.0 ml of *n*-pentane and mix.

Final clean-up

Weigh out 4 g of neutral alumina of Brockmann activity 3 and transfer to a chromatographic column half filled with *n*-pentane, gently tapping the column between additions to assist settling. Weigh out 2 g of magnesium oxide and add to the top of the column, gently tapping the column between additions. Add anhydrous sodium sulphate to form approximately a 1-cm layer on top of the magnesium oxide and run the level of the pentane just into the top of the sodium sulphate layer. Transfer the diluted oxidised mixture to the top of the column, place a 25-ml measuring cylinder under the column and run the solvent level just into the salt layer. Wash the tube with 2 ml of *n*-pentane, transfer to the top of the column and again run the level just into the salt layer. Elute the column with a 1 % v/v solution of diethyl ether in *n*-pentane and collect a total volume of 25 ml in the cylinder. Discard this eluate. Place a 50-ml measuring cylinder under the column and fill the column with a 25 % v/v solution of diethyl ether in *n*-pentane. Collect exactly 35 ml of eluate, transfer to a Kuderna–Danish evaporator and concentrate to a volume of 2.0 ml. Stopper the tube and reserve for GC analysis.

Determination

Inject 1.2 μl of sample into a suitable gas chromatograph run under the conditions described below and obtain a chromatogram. Inject 1.2 μl of a standard nitra-
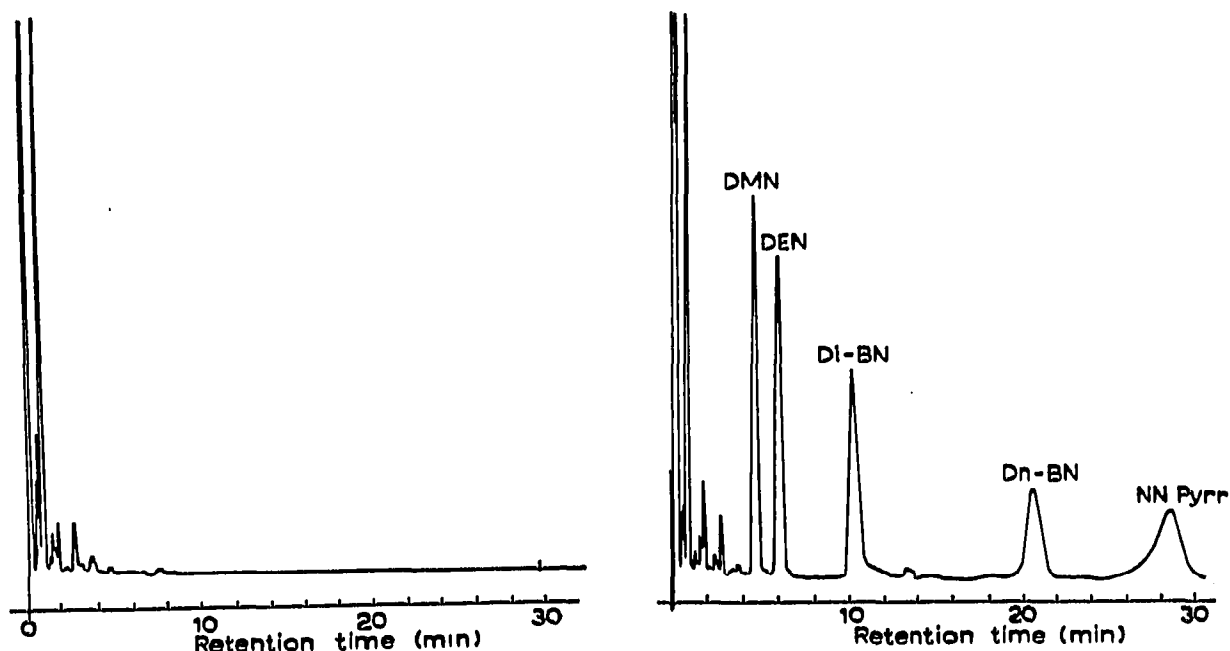
Fig. 1. Chromatogram of spinach taken through the process.

Fig. 2. Chromatogram of processed meat to which five nitrosamines have been added at 10-p.p.b. level and taken through the process.

mine mixture containing nitramines at a level which makes allowance for losses through the distillation and oxidation stages of the method. Compare the chromatograms for sample and standard.

Chromatographic conditions. Column: 5 ft. 10 % PEG 20*M* on acid washed HMDS treated Celite; temperature: isothermal at 150°; carrier gas: argon; inlet pressure 28 p.s.i.; flow-rate: *ca.* 36 ml/min; attenuation: 1 × 1000 for 8 min, then 1 × 500.

RESULTS AND DISCUSSION

A wide range of vegetables and meats have been examined by this method. Final chromatograms were generally very clean, with the exception of spiced meat products where a few non-nitramine peaks were observed.
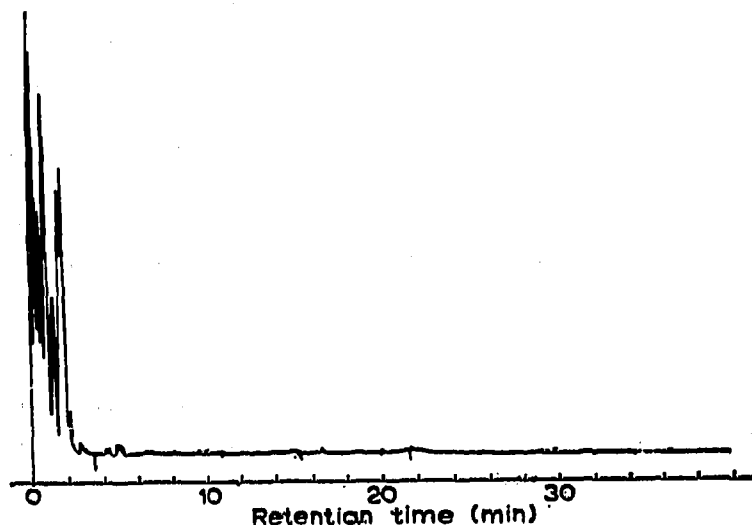

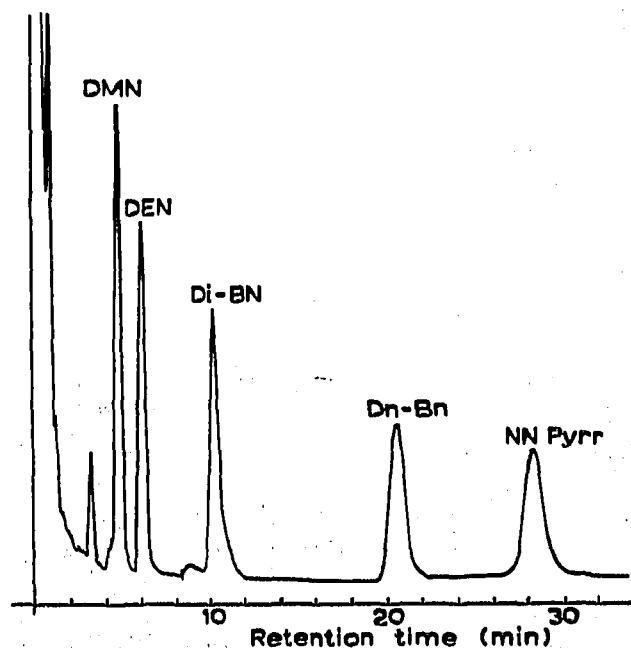Fig. 3. Chromatogram of processed meat taken through the process.

Fig. 4. Chromatogram of standard nitramine mixture.

Typical chromatograms obtained from vegetable and meat product substrates with and without added N-nitrosamines are given in Figs. 1–3 together with the chromatogram of the standard nitramine mixture (Fig. 4). Attenuation 1 × 1000 for 8 min, then 1 × 500.

As already discussed, the factors governing the accuracy of this technique are the distillation and oxidation stages. Recoveries through the latter are fairly constant and hence the variability of recoveries through the distillation stage are the most significant factor.

If some alternative, more reproducible separation technique could be used in conjunction with the oxidation and clean-up stages described above, it would be possible to use the technique on a quantitative basis.

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