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The Determination of Steam-volatile *N*-Nitrosamines in Foodstuffs by Formation of Electron-capturing Derivatives from Electrochemically Derived Amines*

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A method for the determination of six steam-volatile *N*-nitrosamines in a range of foodstuffs is described. *N*-Nitrosamines are isolated by steam distillation and are subjected to controlled-potential electrochemical reduction. The amines thus produced are converted into polyfluorinated amides, which are analysed by gas chromatography by means of electron-capture detection. Sensitivities of 1 μg per kilogram of original foodstuff are attained for all the *N*-nitrosamines studied. Low levels of some *N*-nitrosamines have been detected in several foodstuffs, some of which had not been treated with nitrate - nitrite preservatives.

THE carcinogenic properties of many of the *N*-nitrosamines are well documented.[1,2] Because of the widespread use of sodium nitrate and nitrite as preservatives, it has for some time been realised that there exists the possibility of the formation of *N*-nitrosamines in certain foodstuffs.[3] In recent years, much work has been directed towards the detection and determination of volatile *N*-nitrosamines with special regard to food analysis.

Among the methods developed for *N*-nitrosamine determination are thin-layer chromatography,[4] polarography,[5] spectrophotometry[6] and gas - liquid chromatography.[7] Many of these methods, including gas chromatography with non-specific detectors, have been hampered by their lack of sensitivity or specificity. Recently, gas-chromatographic methods that involve the use of specific detectors have been reported. These include the combination of detectors specific for nitrogen with the confirmation of results by use of a mass spectrometer.[8,9] Two reports of methods that involve the use of an electron-capture detector have also appeared. In one of these,[10] *N*-nitrosodimethylamine is oxidised to the electron-capturing nitramine, while in the other, due to Eisenbrand, G., and Preussmann, R. (private communication), the cleavage of *N*-nitrosamines with hydrogen bromide in acetic acid with subsequent reaction of the resultant amines with heptafluorobutanoyl chloride to yield volatile amides is used.

N-Nitrosamines are reduced electrochemically in alkaline solution to give high yields of the secondary amines.[11,12] This report describes the utilisation of such a reduction process in a method of determination that results ultimately in the preparation and gas-chromatographic determination of heptafluorobutanoyl derivatives of the electrolysis products of six *N*-nitrosamines: *N*-nitrosodimethylamine (DM), *N*-nitrosodiethylamine (DE), *N*-nitroso-di-n-propylamine (DP), *N*-nitrosodi-n-butylamine (DB), *N*-nitrosopiperidine (PIP) and *N*-nitrosopyrrolidine (PYR).

EXPERIMENTAL

APPARATUS—

Gas chromatographs—Pye series 104 gas chromatographs with 10 mCi nickel-63 electron-capture detectors were used. The detectors were operated at 500 μs pulse space and at a temperature of 250 °C. The columns were a 2·75 m × 2 mm i.d. stainless-steel column, packed with 15 per cent. *m/m* of FFAP (Carbowax 20M terminated with 2-nitroterephthalic acid) (Phase Separations Ltd.) on 80 to 100-mesh Chromosorb W, operated at a temperature of 60 °C; and a 5·8 m × 2 mm i.d. stainless-steel column, packed with 20 per cent. *m/m* of FFAP on 80 to 100-mesh Chromosorb W and operated at 110 °C. Nitrogen at a flow-rate of 50 ml min^{-1} was used as the carrier gas. Samples of 5-μl volume were injected.

Electrolysis cell—The cell (see Fig. 1) was a flat-bottomed vessel constructed from a 75 mm i.d. flat-flange joint. A tap was fitted in the centre of the base. Through the five

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sockets in the lid passed a stirrer, which reached the mercury cathode - electrolyte interface, the cathode connection, a gas bubbler, a saturated calomel electrode and an anode compartment. The anode compartment consisted of a tube closed at one end by a porous ceramic disc, 0·5 cm thick (Activion Ltd., Kinglassie, Fife). The anolyte was 0·1 M sodium hydroxide solution, and the anode was a platinum sheet (22 gauge, 5 × 5 cm).

A potentiostat [J. A. Radley (Laboratories) Ltd.] was used to control the cathode potential relative to that of the saturated calomel electrode.

MATERIALS—

Reagents were of analytical grade unless otherwise specified.
Cyclohexane, Spectrosol grade.
Heptafluorobutanoyl chloride (PCR Inc.), redistilled.
Sodium chloride.
Sodium carbonate.
Sodium hydroxide.
Sodium sulphate (anhydrous).
Sulphuric acid, 3 M.
Hydrochloric acid, 2 M.

N-*Heptafluorobutanoyl derivatives of amines*—Derivatives of diethylamine, di-n-propylamine, di-n-butylamine, piperidine and pyrrolidine were prepared from the amine and heptafluorobutanoic acid anhydride.[13]

NN-*Dimethylheptafluorobutanamide, boiling-point 44 °C at 18 mm of mercury pressure*—This compound was prepared by passing dry dimethylamine through a solution of heptafluorobutanoyl chloride in cyclohexane.

PROCEDURE—

Mince the sample and weigh 200 g into a 1-litre distillation flask together with 100 g of sodium chloride. Pass steam into the initially dry mixture until 250 ml of distillate have been collected. Add 30 g of sodium hydroxide to this distillate and distil the resulting solution until 150 ml have been collected. Then add 25 g of anhydrous sodium sulphate and 3 ml of 3 M sulphuric acid to the latter distillate, redistil, and divide the 100 ml of distillate into two equal portions.

Make one portion of the distillate alkaline with 5 ml of 0·01 M sodium hydroxide solution. De-gas the solution in the electrolysis cell for 1 minute by the passage of a stream of nitrogen and stirring. Electrolyse for 1 hour with stirring, at a cathode potential of −1·8 V *versus* a saturated calomel electrode. Acidify the reduced solution with 1 ml of 2 M hydrochloric acid and evaporate the mixture to dryness.

To the remainder of the distillate add 5 ml of 0·01 M sodium hydroxide solution and 1 ml of 2 M hydrochloric acid, and evaporate the solution to dryness.

Dissolve each dry residue in 2 ml of water and add 0·5 ml of 1 M sodium hydrogen carbonate solution and 1 ml of a 0·5 per cent. solution of heptafluorobutanoyl chloride in cyclohexane to each of the solutions. Shake the mixtures for 15 minutes, separate the cyclohexane layers and analyse them by gas chromatography.

Compare the differences in peak heights on the recorder traces given by the derivatives under consideration in the reduced and unreduced samples (diluted when necessary) directly with the peak heights from a 1 μg ml^{-1} standard solution of the six heptafluorobutanamides in cyclohexane.

RESULTS AND DISCUSSION

The response of the electron-capture detector was linear only with amounts of sample derivative below 7·5 ng. Above this level, the response varied in a manner peculiar to each detector, and the derivative solutions were diluted when necessary in order to maintain concentrations within the linear range. The lower limits of detection of the derivatives were measured and are reported in Table I.

NN-Di-n-butylheptafluorobutanamide had a retention time close to that of an impurity in the reagent at a column temperature of 60 °C and could be determined only at 110 °C, at which temperature the peaks due to *NN*-dimethyl- and *NN*-diethylheptafluorobutanamides were not resolved. Accordingly, gas-chromatographic analyses were carried out at both temperatures.

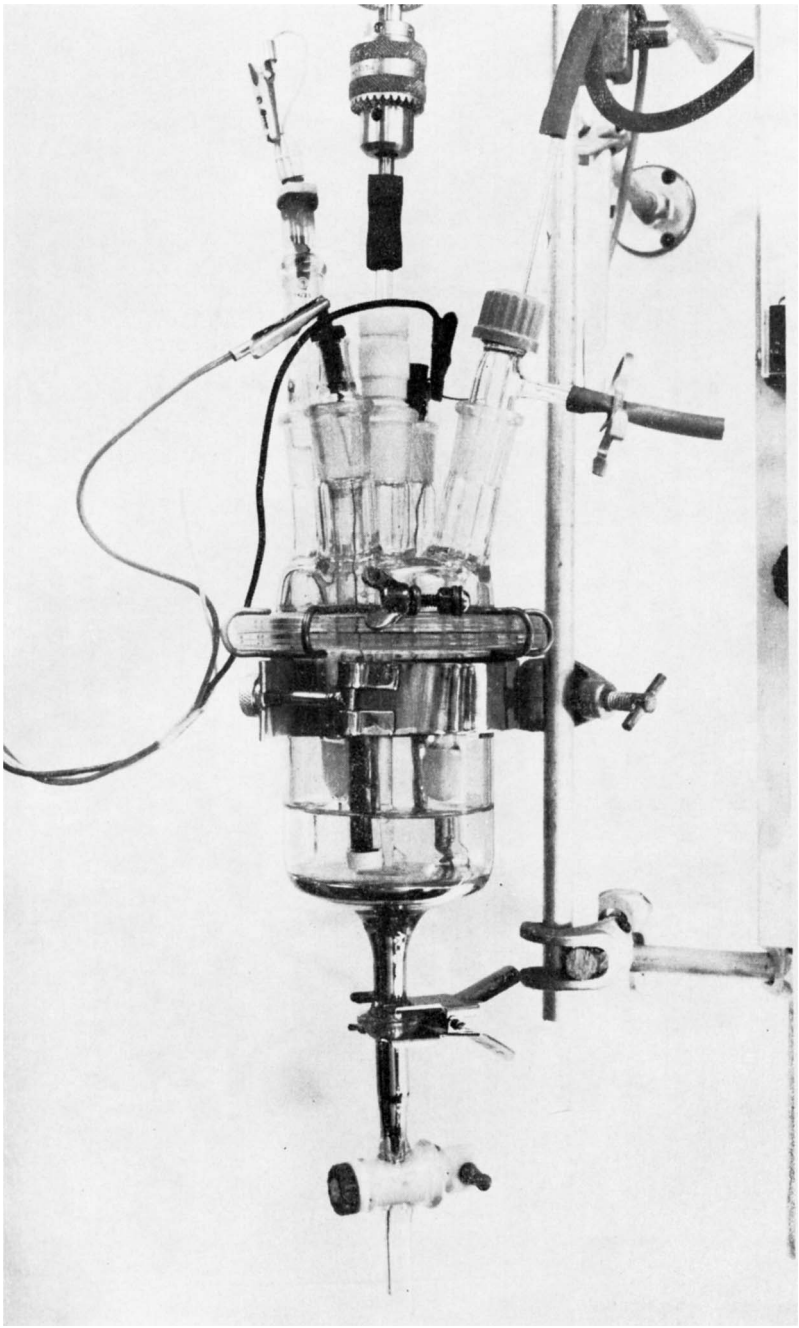

Fig. 1. Electrolysis cell

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TABLE I

LOWER LIMITS OF DETECTION OF HEPTAFLUOROBUTANAMIDES

Heptafluorobutanamide	Lower limits of detection/g, on— Column 1*	Column 2†
Dimethyl	$5{\cdot}3 \times 10^{-11}$	—
Diethyl	$8{\cdot}0 \times 10^{-11}$	—
Di-n-propyl	$1{\cdot}0 \times 10^{-10}$	$3{\cdot}5 \times 10^{-11}$
Di-n-butyl..	—	$5{\cdot}0 \times 10^{-11}$
Piperidyl	$5{\cdot}4 \times 10^{-10}$	$7{\cdot}8 \times 10^{-10}$
Pyrrolidyl	$6{\cdot}3 \times 10^{-10}$	$1{\cdot}0 \times 10^{-10}$

* Column of 15 per cent. *m/m* FFAP on Chromosorb W, 9 feet in length, temperature 60 °C.
† Column of 20 per cent. *m/m* FFAP on Chromosorb W, 19 feet in length, temperature 110° C.

The derivative preparation, a modification of the method of Eisenbrand and Preussmann (see above), resulted in quantitative yields of all six heptafluorobutanamides for periods of agitation greater than 5 minutes.

The electrochemical reduction of *N*-nitrosamines to amines at a mercury cathode proceeds with a high yield at pH 12.[11] It was found to be possible to use 0·001 M sodium hydroxide solution as catholyte in order to minimise the amounts of solid residue resulting from the evaporation stage. Reduction of 15 and 60 minutes duration were carried out on solutions containing 0·25 μg of each of the six *N*-nitrosamines studied. The yields (after reduction and derivative preparation) are given in Table II. Reduction of solutions containing 1 and 5 μg of each *N*-nitrosamine gave results that fell well within the ranges quoted for the more dilute solutions. The short reduction time of 15 minutes resulted in lower and less reproducible yields; in consequence, the longer period was used in the analyses.

TABLE II

YIELDS OF HEPTAFLUOROBUTANAMIDES AFTER REDUCTION OF *N*-NITROSAMINES AND DERIVATIVE FORMATION

N-Nitrosamine	Amount/μg	Reduction time/minutes	Yield, per cent.
Dimethyl	0·25	60	103, 97, 95, 99, 99
	0·25	15	103, 98, 87, 97
	1·0	60	93
Diethyl	0·25	60	115, 106, 108, 114, 118
	0·25	15	105, 110, 88, 98
Dipropyl	0·25	60	82, 84, 79, 77, 82
	0·25	15	76, 71, 59, 56
	1·0	60	80
Dibutyl	0·25	60	72, 79, 72, 73, 77
	0·25	15	50, 49, 49, 48
	1·0	60	71
Piperidyl	0·25	60	96, 104, 100, 104
	0·25	15	104, 88, 81
	1·0	60	103
Pyrrolidyl	0·25	60	105, 105, 108, 115
	0·25	15	97, 92, 95
	1·0	60	106

Volatile *N*-nitrosamines have been isolated from food samples by steam distillation under various conditions with reported recoveries of 70 to 100 per cent.[5,8,14] The use of alkaline conditions often results in the decomposition of the sample, with attendant difficulties in distillation, while the use of acidic conditions might catalyse nitrosation reactions caused by the presence of sodium nitrite in the sample. A neutral steam distillation was therefore used to remove the volatile *N*-nitrosamines from the sample, and two further distillations, one from alkali and one from acid, were used to remove acidic and basic materials that co-distilled with the *N*-nitrosamines. Neutral, volatile materials were lost in the evaporation stage following the reduction. Adventitious amines (mostly dimethylamine and diethylamine from the air or glassware: the acidic distillation step was shown to be effective in retaining

TABLE III

YIELDS OF HEPTAFLUOROBUTANAMIDES PREPARED FROM AQUEOUS SOLUTIONS OF *N*-NITROSAMINES AND SPIKED FOODS

Amount of *N*-Nitrosamine/μg	Sample type	Yield of heptafluorobutanamides, per cent. Dimethyl	Diethyl	Dipropyl	Dibutyl	Piperidyl	Pyrrolidyl
0·25	Aqueous	93	113	78	53	90	55
		104	100	76	40	52	52
		70	84	52	44	70	52
		88	84	64	26	83	40
		90	92	70	—	88	66
		76	100	64	40	85	68
		92	108	76	52	90	64
		80	96	72	48	80	64
	Minced beef	76	76	64	40	80	*
		76	80	76	56	82	*
	Cheshire cheese	82	*	86	39	77	*
10·0	Aqueous	71	91	79	65	78	66
		71	83	78	55	86	69
0·1	Cod	*	*	69	35	89	*

* Unspiked sample contained the particular *N*-nitrosamine.

amines emanating from the sample) were monitored by analysis of the unreduced portion of the sample; the differences in peak heights obtained with the reduced and unreduced samples were taken as a measure of the *N*-nitrosamine content.

A number of *N*-nitrosamine solutions were analysed in order to determine the over-all yields and reproducibility of the method. These yields are reported in Table III, which also

TABLE IV

N-NITROSAMINES IN FOODSTUFFS

Sample	*N*-Nitrosamine content/μg kg^{-1} DM	DE	DP	DB	PIP	PYR
Cheddar cheese (1)	T	T	ND	ND	ND	ND
Cheddar cheese (2)	ND	ND	ND	ND	ND	ND
Cheshire cheese	T	1·5	ND	ND	ND	1·0
Norwegian goat's milk cheese	ND	ND	ND	ND	ND	ND
Corned beef	ND	ND	ND	ND	ND	2·0
Pork luncheon meat (1)	ND	ND	ND	T	ND	1·5
Pork luncheon meat (2)	ND	ND	ND	ND	ND	ND
Back bacon (uncooked)	ND	*	ND	ND	ND	1·5
Back bacon (fried)	ND	1·5	ND	ND	ND	3·0
Streaky bacon (uncooked)	ND	ND	ND	ND	ND	ND
Streaky bacon (fried)	T	ND	ND	ND	ND	3·5
Pig's liver (uncooked)	ND	1·5	ND	ND	ND	1·5
Pig's liver (fried)	ND	ND	ND	ND	ND	11·0
Haddock (uncooked)	ND	ND	ND	ND	ND	ND
Haddock (fried)	ND	ND	ND	ND	ND	ND
Fresh cod (uncooked) (1)	ND	ND	ND	ND	ND	2·1
Fresh cod (uncooked) (2)	T	ND	ND	ND	ND	1·3
Fresh cod (uncooked) (3)	ND	ND	ND	ND	ND	1·6
Fresh cod (fried)	ND	ND	ND	ND	ND	1·0
Stale cod (uncooked)	1·0	1·5	ND	ND	ND	6·0
Stale cod (fried)	1·0	1·5	ND	ND	ND	6·0
Smoked cod (1)	ND	ND	ND	ND	T	4·0
Smoked cod (2)	ND	ND	ND	ND	ND	ND
Tinned herrings	ND	ND	ND	ND	ND	2·5
Cooking fat (well used for frying bacon)	ND	ND	ND	ND	T	5·0

T = Trace amounts. ND = Not detected.

* Contamination by adventitious diethylamine (as shown in the chromatograms of the non-reduced sample) precluded measurement.

DM = *N*-nitrosodimethylamine; DE = *N*-nitrosodiethylamine; DP = *N*-nitrosodipropylamine; DB = *N*-nitrosodibutylamine; PIP = *N*-nitrosopiperidine; PYR = *N*-nitrosopyrrolidine.

gives yields of heptafluorobutanamides from meat, cheese and fish samples to which had been added a mixture of the six *N*-nitrosamines. In these last results no figures are given when prior analysis of the unspiked sample demonstrated the presence of a particular *N*-nitrosamine. From the values of the over-all yields, together with the limits of detection of the derivatives, it is calculated that an original concentration of 1 μg kg^{-1} of the *N*-nitrosamines in 200 g of sample can readily be determined by the proposed procedure.

Several food samples have been analysed in order to demonstrate the scope of the method, and the *N*-nitrosamine contents of these samples are summarised in Table IV. "Trace amounts" referred to in this table represent positive findings of less than 1 μg kg^{-1}, but greater than 0·5 μg kg^{-1}. Values below this lower limit are not reported. In only one sample, the fried pig's liver, was any one of the *N*-nitrosamines under investigation present to a level greater than 10 μg kg^{-1}. The other sample that contained levels of *N*-nitrosopyrrolidine greater than 5 μg kg^{-1} was a particularly strongly smelling sample of cod (although fresh samples contained significantly lower amounts of *N*-nitrosamines). It is of interest to note that neither of these samples had been cured with sodium nitrite. A few of the samples analysed have also been analysed by another method, involving gas chromatography and mass spectrometry, in this laboratory.[15] Preliminary results indicate reasonable agreement between results from the two methods.

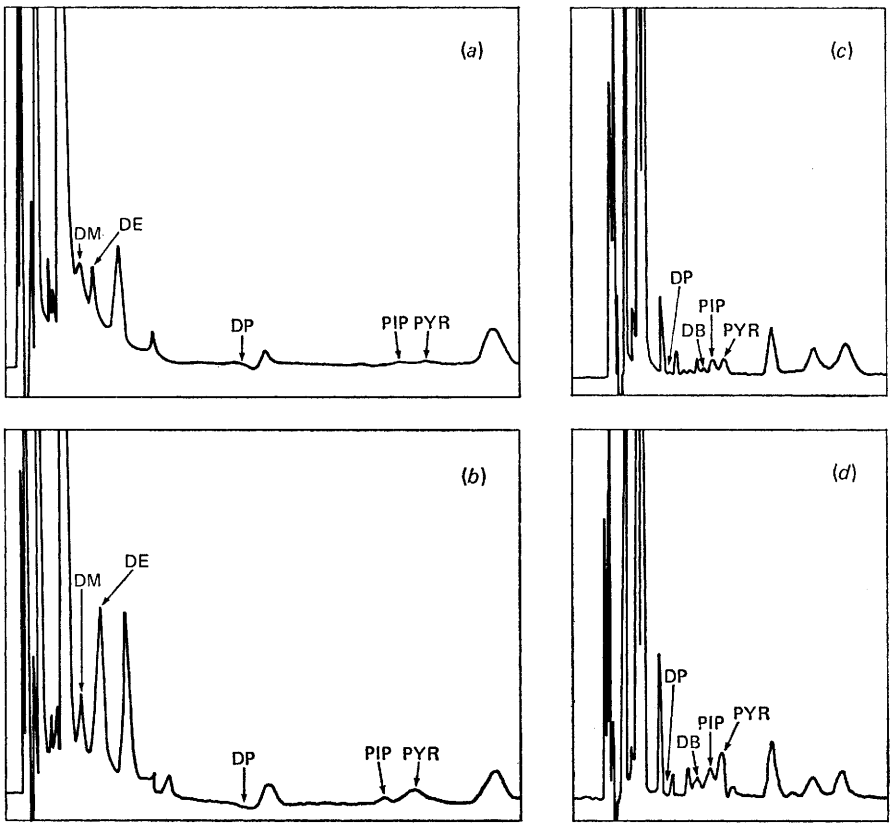

Fig. 2. Chromatograms of heptafluorobutanamides from analysis of Cheshire cheese: (*a*), unreduced sample; (*b*), reduced sample (60 °C, 15 per cent. FFAP on Chromosorb W, 9-foot column; (*c*) unreduced sample; (*d*), reduced sample (110 °C, 20 per cent. FFAP on Chromosorb W, 19-foot column). For abbreviations, see text

None of the foodstuffs analysed gave rise to interfering peaks in the chromatograms, although with some samples a number of components that had retention times longer than those of the amine derivatives were seen. A typical pair of chromatograms (reduced and unreduced portions) from the analysis of Cheshire cheese are shown in Fig. 2.

The present method can be extended to the determination of other steam-volatile *N*-nitrosamines. In Table V relative retention times for a number of heptafluorobutanamides, including the six under detailed investigation, are listed, although during the analyses reported in Table IV no evidence for the presence of the *N*-nitrosamine precursors of the other heptafluorobutanamides was found. The extension of this method to the analysis of the less volatile and non-volatile *N*-nitrosamines is currently being investigated.

TABLE V

RELATIVE RETENTION TIMES FOR HEPTAFLUOROBUTANAMIDES

Heptafluorobutanamide	Relative retention time	
	Column 1*	Column 2†
Dimethyl	0·33	—
Methylethyl	0·40	—
Diethyl	0·41	—
Methylisopropyl	0·45	—
Methyl-n-propyl	0·56	—
Ethyl-n-propyl	0·64	—
Di-n-propyl	1·00	1·00
Diallyl	1·14	—
Methyl-n-butyl	1·17	—
Ethyl-n-butyl	1·31	—
Methylisopentyl	1·56	—
Piperidyl	2·51	2·45
Pyrrolidyl	2·58	2·62
Di-n-butyl	3·32	2·19

* Column of 15 per cent. *m/m* FFAP on Chromosorb W, 9 feet in length, temperature 60 °C.
† Column of 20 per cent. *m/m* FFAP on Chromosorb W, 19 feet in length, temperature 110 °C.

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