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Note

Method for high-performance liquid chromatographic measurement of N-nitrosamines in food and beverages

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Trace analysis of N-nitrosamines by high-performance liquid chromatographic (HPLC) methods has hitherto been limited to non-polar N-nitrosamines[1], polar nitrosamines such as N-nitrosodiethanolamine[2] and only those ionisable N-nitrosamines (*e.g.* N-nitrosoamino acids) where the ionisation can be suppressed by the solvent[3]. Routine analysis of even these compounds is further hampered by problems associated with the cold traps[4] normally used with the thermal energy analyser (TEA) which has been used as detector. The limitation on HPLC analysis of non-volatile N-nitrosamines is due to (i) the loss of performance of the catalytic pyrolysis tube if inorganic buffers or ion-pairing agents are used and (ii) the seriously adverse effect on base-line stability of the detector when solvents containing more than trace amounts of water are used. Recent work[5] provides at least a partial solution to these problems by the use of reversed-phase microbore HPLC using an organic ion-pairing agent and a post-column acetone make-up solvent prior to a TEA detector. The present work involves a very simple clean-up procedure which allows routine determination of polar N-nitrosamines in cured meats and beverages. The method involves a "Preptube" extraction stage and avoids the need for liquid–liquid extractions and centrifugation of the resultant emulsions.

EXPERIMENTAL

Materials

"Preptubes" (Thermo-Electron Corp., Waltham, MA, U.S.A.) for non-volatile N-nitrosamine analysis were conditioned with either 20 g 2 *N* sulphuric acid containing 1 g ammonium sulphamate and 3.5 g sodium chloride or 20 g 1.0 *M*, pH 7.0, phosphate buffer containing 0.2 g sodium azide and 3.5 g sodium chloride (for acidic or neutral extracts, respectively) and then eluted sequentially with 100 ml acetone and 100 ml ethyl acetate prior to use.

Reagent blanks were regularly analysed for the volatile and non-volatile N-nitrosamine analyses and found to be negligible in all cases. All solvents were glass distilled before use.

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Methods

Volatile N-nitrosamines in beer were determined by a gas chromatographic (GC) method in which sodium azide (0.5 g) and internal standard (N-nitrosodipropylamine) were added to beer (45 g) which was then adsorbed on three Preptubes prewetted with dichloromethane and eluted with 6 × 8 ml of this solvent. Concentration to 1.0 ml in a Kuderna–Danish evaporator at 55°C was followed by GC analysis with 20% Carbowax 20M terephthalic acid (TPA) + 2% KOH on Chromosorb W AW at 190°C using a TEA detector. Recoveries were 85–100%.

Extracts of non-volatile N-nitrosamines from cured meats (20 g) were prepared by homogenising for 3 minutes at 45°C with 20 g Celite and 90 ml of *either* 2 *N* sulphuric acid containing 5 g ammonium sulphamate (for acidic extracts) *or* 1 *M* phosphate buffer (pH 7.0) containing 1 g sodium azide (for neutral extracts). After cooling to 5°C, the mixture was filtered and 50 g of the filtrate saturated with sodium chloride. The filtrate (3 × 15 g) was absorbed onto three Preptubes and each Preptube eluted with 100 ml ethyl acetate. The eluates were combined and concentrated to dryness on a rotary evaporator at 35°C. The residue was suspended in 2 × 50 ml acetone and concentrated to dryness at 35°C to remove traces of water. The residue was suspended in 5 ml acetone, filtered, the filtrate blown down to dryness with nitrogen and redissolved in 0.5 ml acetone.

Extracts of non-volatile N-nitrosamines from beer (50 g) were prepared by adding 10 g of sodium chloride and *either* 5 ml 10 *N* H_2SO_4 containing 2.5 g ammonium sulphamate *or* 0.5 g sodium azide and sufficient 2 *N* sodium hydroxide (ca. 2 ml) to adjust the pH to 7.0. The sample (3 × 15 g) was absorbed onto three Preptubes and treated as described for cured meats.

Acidic extracts (20 μl aliquots) were analysed with a Spherisorb 5 silica column (25 cm × 5 mm) pre-conditioned with 1 l of 10% acetic acid in acetone. The mobile phase was linearly programmed from 98:1:1 to 89:10:1 hexane–ethanol–acetic acid over 20 min at 2 ml/min. N-Nitrosopipecolic acid was used as internal standard and eluted after 11.8 min.

Neutral extracts (20 μl aliquots) were analysed with a Spherisorb 5 silica column (25 cm × 5 mm) pre-conditioned with 1 l of 2% acetic acid in acetone. The mobile phase was linearly programmed from 2:98 to 98:2 acetone–hexane over 30 min at 2 ml/min.

The HPLC analyses were performed using a TEA Model 502 chemiluminescence detector. The TEA pyrolyser was operated at 550°C and two sets of cold traps (isopropanol–solid carbon dioxide and liquid nitrogen) were used to trap the vapourised mobile phase.

Total N-nitrosamine levels were measured using a procedure[6] in which beer (1 g) was added to refluxing ethyl acetate (50 ml) containing α-tocopherol (0.5 g) and treated sequentially with 2 ml acetic acid and then 2 ml 15% HBr in acetic acid. The reaction mixture was connected in series via two 6 *N* KOH traps, a solid carbon dioxide–isopropanol trap, a needle valve and a liquid nitrogen trap to the reaction chamber of the TEA operating at 7 mmHg.

RESULTS AND DISCUSSION

Using the Preptube extraction methods described in the Experimental section

TABLE I

N-NITROSAMINE CONTENT OF BEER

Assuming stoichiometric release of NO from all N-nitrosamines; for NDMA 0.014 μmol/kg is equivalent to 1.0 μg/kg.

Sample	*Concentration (μmol/kg)*			
	N-Nitroso-dimethylamine	*N-Nitroso-proline*	*Major neutral N-nitrosamine*	*Total N-nitrosamine*
1	0.014	0.162	0.25	17.4
2	0.012	0.052	0.17	6.0
3	0.014	0.040	0.01	1.1
4	0.012	0.012	0.01	0.2

the recovery of N-nitrosoproline added to cured meat at 50 μg/kg was 76% (S.D. = 9.3; $n = 6$), detection limit 5 μg/kg; the recovery of N-nitrosoproline added to beer at 20 μg/kg was 91% (S.D. = 14.4, $n = 12$), detection limit 1 μg/kg. These data compare favourably with other analytical methods for N-nitrosoproline[7–9] and the use of Preptubes facilitates the extraction stage by eliminating liquid–liquid extractions and centrifugation of resultant emulsions. Using the procedures described above measurements were made of N-nitrosodimethylamine (NDMA), acidic and neutral non-volatile N-nitrosamines and total N-nitrosamines in beer. Results from four samples are given in Table I. N-Nitrosoproline eluted in the acidic extract as twin peaks corresponding to the two conformational isomers and was the major component; no other N-nitrosoamino acids were detected. The chromatograms from the neutral extract of sample 1 showed a peak which was much greater than those found in the other samples. All results are expressed as μmol/kg of NO assuming that each of the N-nitrosamines present gives a stoichiometric yield of NO in the catalytic pyrolyser of the TEA. All samples were light beers; the NDMA levels correspond to 0.9–1.1 μg/kg and are at the upper end of the range encountered in beer on sale in Great Britain early in 1981 (levels were typically around 0.4 μg/kg). It is notable that levels of total N-nitrosamines are much greater than can be accounted for in terms of the chromatographic peaks obtained from the extracts. The amount of material unaccounted for can be relatively large. The microbore HPLC method[5] will provide information about ionic N-nitrosamines which would not be extracted by the procedures reported here. It is also notable that whereas the amounts of total N-nitrosamine and of the major neutral N-nitrosamine are highest in sample 1 and decrease in samples 2 and 3 to the lowest in sample 4 there is no simple correlation between these figures and those for NDMA and N-nitrosoproline.

The HPLC–TEA chromatogram of the acidic fraction of sample 2 is shown in Fig. 1; with the exception of N-nitrosoproline no N-nitrosoamino acids were detected in any of the samples. The HPLC–TEA chromatogram of the neutral non-volatile fraction of sample 1 is shown in Fig. 2. Treatment of this neutral fraction with acetic acid and also with HBr–acetic acid[10] showed all the peaks to be unaffected by acetic acid but destroyed by the HBr–acetic acid reagent, thus indicating the presence of the N-nitroso functional group. Further studies are being conducted to establish the identities of these unknown compounds.

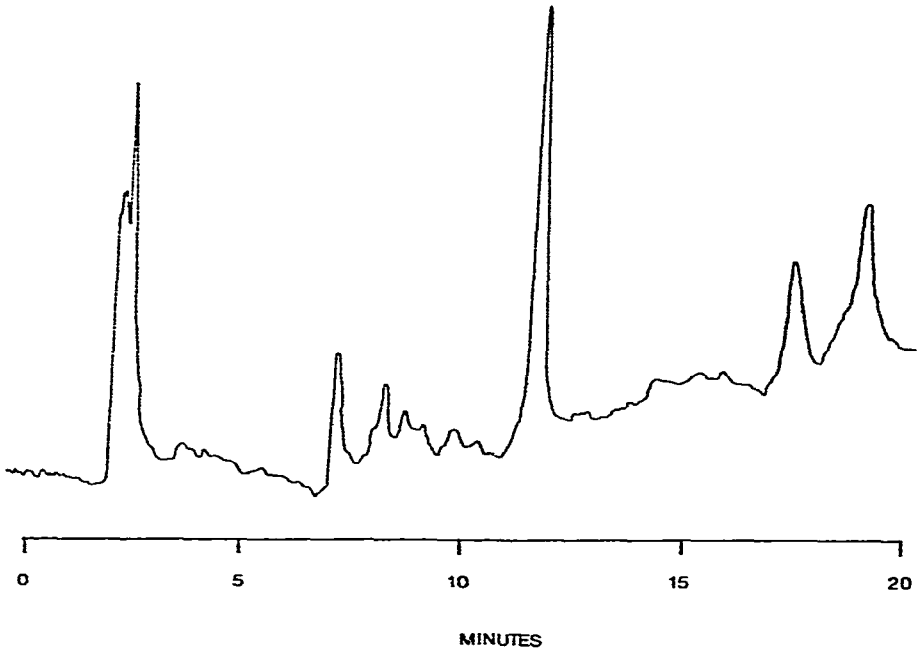

Fig. 1. HPLC–TEA Chromatogram of acidic beer extract with N-nitrosopipecolic acid internal standard (elution time 11.8 min). TEA attenuation × 16.

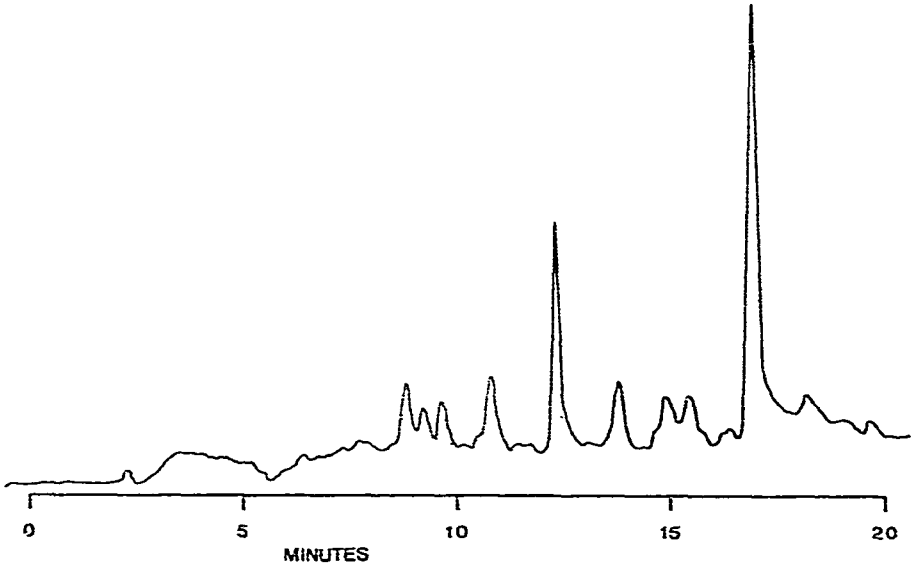

Fig. 2. HPLC–TEA Chromatogram of neutral beer extract. TEA attenuation × 64 for main peak, otherwise × 16.

It is known that malt is the only significant source of NDMA in beer but it remains to be established whether or not the same relationships hold with respect to the other nitrosamines. If malt is the source then the reasons for lack of correlation between the abundances of the various classes of nitrosamine in different beers will have to be sought in terms of differences in malting practice and in the various palliative measures applied in ensuring low levels of NDMA. The method described here is capable of being used on a routine basis for investigations of this sort or for quality control purposes.

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