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Rapid semi-quantitative estimation of N-nitrosodibutylamine and N-nitrosodibenzylamine in smoked hams by solid-phase microextraction followed by gas chromatography–thermal energy analysis

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Abstract

A solid-phase microextraction (SPME) analytical method has been developed for the determination of N-nitrosodi-*n*-butylamine (NDBA) and N-nitrosodibenzylamine (NDBzA) in hams that is based on: (a) isolation of the compounds by steam distillation, (b) SPME from the distillate headspace using a polyacrylate coated silica fibre and (c) determination by gas chromatography–thermal energy analyzer technique or confirmation by gas chromatography–mass spectrometry. Recoveries of both NDBA and NDBzA from hams spiked at 5 to 160 μg/kg levels ranged between 41 to 112%. The overall method is fast, sensitive (detection limits, 1 to 3 μg/kg), precise (within 10%) and fairly accurate (average recoveries 86% and 70%, respectively). The results obtained by this technique for seven ham samples agreed fairly well with those obtained by an existing method (r^2=0.97). The new method is solventless, environmentally friendly and useful for rapid monitoring purposes. © 1997 Elsevier Science B.V.

Keywords: Food analysis; Extraction methods; Nitrosamines

1. Introduction

The newly developed solid-phase microextraction (SPME) technique, first reported by Belardi and Pawliszyn [1] in 1989, is increasingly being used for the gas chromatographic determination of a wide variety of volatile and semivolatile organic compounds in water or aqueous extracts of different substrates. Basically, it involves extraction of specific organic analytes directly from aqueous samples, or from the headspace of these samples in closed vials, onto a fused-silica fibre coated with a polymeric liquid phase, poly(dimethylsiloxane) or polyacrylate. After equilibration, the fibre containing the absorbed or adsorbed analyte(s) is removed and thermally desorbed in the hot injector of a gas chromatograph. The analytes are then analyzed by gas chromatography (GC) using an appropriate column and detector with or without cryofocusing. Further details of both the theoretical and practical aspects of the technique can be obtained from recent publications by Pawliszyn and coworkers [1–3] and others [4–6].

The technique is very simple, fast and does not employ any organic solvents either for sample preparation or cleanup. This makes it highly desir-

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able because, unlike other methods, it does not release environment-polluting organic solvents into the atmosphere. Thus far, the technique has been successfully applied to the determination of a wide variety of organic compounds in water [4], Hg^{2+} and CH_3Hg^+ in fish [5], and a large number of halogenated volatiles (e.g., vinyl chloride, 1,1-dichloroethane, CCl_4, chloro- and bromobenzenes) in flour, decaffeinated tea and coffee, spices, fruit juices and fruit drinks and milk [6]. The technique, however, has not been applied to the determination of N-nitrosamines (or nitrosamines) in foods or beverages that are potent carcinogens in laboratory animals [7]. It was thought, therefore, that the successful application of the technique to the determination of nitrosamines in the above items will eliminate the problems of widespread solvent use and lengthy and time-consuming sample preparation steps that are common with most published methods in this area [8–10].

In this paper, we wish to report such a method for the determination of N-nitrosodi-*n*-butylamine (NDBA) and N-nitrosodi-benzylamine (NDBzA) in smoked hams. These two nitrosamines are commonly detected in hams that have been packaged in elastic rubber nettings [11–14].

2. Experimental

2.1. Apparatus

The SPME device consisting of a manual holder and the appropriate fibres were obtained from Supelco (Sigma–Aldrich Canada, Mississauga, Canada). Headspace gas (HS) sampling by SPME was carried out in 30 ml crimp-top vials using 20 mm×2 mm laminated silicon–PTFE (0.2 mm) septa and aluminum seals (Supelco) as described previously [6]. The solutions in the HS vials were stirred continuously using magnetic stirrer (PTFE-coated bars); the speed of stirring was adjusted to give a vortex depth of about 1 cm. Vials containing the samples were heated in a surplus temperature controlled GC oven. Some structural alterations to the oven, however, had to be made in order to introduce the fibre holder through the top and to operate the magnetic stirring device from the bottom [15].

2.1.1. Steam distillation apparatus

Basically, the apparatus employed a Garman 1 l steam distillation apparatus (Lurex, Vineland, NJ, USA) with some modifications as shown in Fig. 1. It consisted of a boiler (A) with sealed-in resistance wire for electric heating, a sample chamber (B) with a steam inlet tube, a pressure indicator (C) and a liquid sample introduction head. A side arm from the liquid injection head was attached via a 4-port valve (D) to the SPME vial which was cooled by circulating ice-cooled water. PTFE sleeves, rings or gaskets were used on all joints. The actual sequence of operations of the apparatus is described later in Section 2.5.

2.1.2. GC–thermal energy analysis (GC–TEA)

A Varian GC (Model 3400) interfaced with a TEA detector (Model 502, Thermedics Detection, Chelmsford, MA, USA) and a nitrogen converter (Model 610 R, Thermedics Detection), operating in the nitrosamine mode, were used for the determination of nitrosamines. The detector is highly sensitive and specific to nitric oxide which is formed from most N-nitroso compounds upon pyrolysis in the TEA furnace [16]. The GC operating conditions were as follows: 30 m×0.53 mm fused-silica capil-

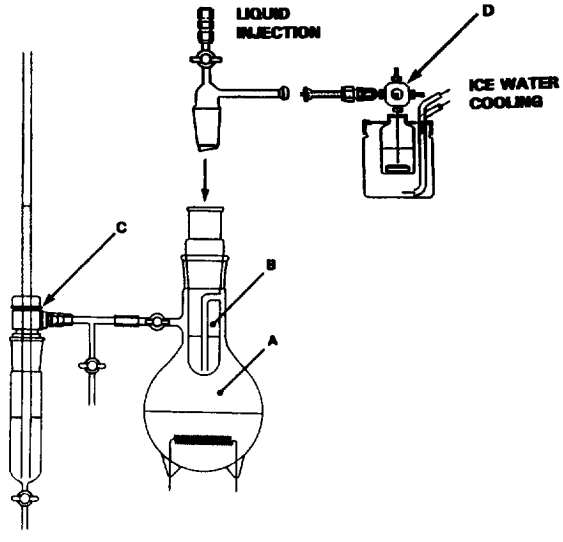

Fig. 1. Set-up for the steam distillation apparatus consisting of boiler (A), sample chamber (B), pressure indicator (C), liquid injection head, 4-port valve (D) with needle and a SPME vial for collection of distillate.

lary column coated with Supelcowax 10 (1 μm film thickness); injector temperature, 220°C; oven temperature was held at 40°C for 1 min (for cryofocusing), then heated to 160°C at 50°C/min and then to 220°C at 6°C/min. The TEA furnace and the GC–TEA interface temperatures were 800°C and 375°C, respectively. A glass injector port liner (0.53 megaliner direct, Chromatographic Specialties, Brockville, Canada) was inserted into the injector to guide the SPME fibre needle directly to the capillary column. The carrier gas (Ar) flow-rate was 8 ml/min.

2.1.3. GC–mass spectrometric (GC–MS) confirmation

A Hewlett-Packard gas chromatograph (Model 5890, series II) interfaced with a bench top mass selective detector (MSD, Hewlett-Packard, Model 5970B) was used for GC–MS confirmation purposes. A 30 m×0.25 mm fused-silica capillary column coated with DB-5 (0.25 μm film thickness) was used for the GC separation using the following oven temperature program: 40°C (2 min hold) with heating to 260°C at 10°C/min (4 min hold). The injector temperature was varied from 220 to 260°C. MSD was mainly in the selected ion monitoring (SIM) mode for the respective molecular ions (at m/z 158 for NDBA and at m/z 226 for NDBzA). In some applications, the full scan spectra were also obtained.

2.2. Chemicals and reagents

All chemicals used were of analytical grade and the organic solvents were glass distilled varieties. All nitrosamine standards, except N-nitrosodioctylamine (NDOA) and NDBzA, were purchased from Thermedics Detection. NDOA was synthesized by nitrosation of dioctylamine using a method similar to that reported by Lijinsky and Taylor [17]. Primary stock solutions of NDBA and NDBzA (90 to 156 μg/ml) were prepared in ethanol. Subsequent dilutions to appropriate concentrations were made in dichloromethane (DCM) which could be injected directly for GC analysis. Because DCM interfered with the SPME process (matrix effect), appropriate water standards were prepared by phase transfer from DCM to water. To avoid losses of the volatile NDBA during the phase transfer, a Kuderna–Danish (K–D) concentrator was used as described previously [18]. Whereas dilute standards of NDOA (insoluble in water) and NDPA, the two performance indicator internal standards (I.S.s) used in the study, were prepared in methanol and water, respectively. Appropriate amounts (1–4 ng of NDOA and 10–100 ng of NDPA) of each I.S. were added to ham extract just prior to steam distillation.

2.3. Samples

The smoked hams were purchased locally in the Ottawa area. Each sample (whole ham) was cut into small pieces, homogenized thoroughly using a blender. If not analyzed within 2 to 3 days, the samples were stored in a closed glass container at −20°C.

2.4. Sample preparation

A 20-g aliquot of a sample (fresh or thawed) was mixed with 0.5 g of ascorbic acid or propyl gallate (to inhibit nitrosation) and 80 ml of water, and the mixture was homogenized for 5 min using a Polytron homogenizer. The suspension was made up to 100 ml with water, and a 10-ml aliquot was removed and diluted with water to 100 ml. 5-ml aliquots of either of the dilutions (well mixed before each use) were used for SPME experiments.

2.5. Micro steam distillation

The steam distillation set-up was assembled as shown in detail in Fig. 1, and the positions of the 4-port valve during various manipulations are shown separately in Fig. 2. The steam generator was turned on and the steam was allowed to exit and displace air in the liquid injector head, 4-port valve, needle and vent (Fig. 1). A sealed vial containing 6 g of NaCl, 1 ml of 30% acetic acid, 100 mg of ascorbic acid and a stirring bar, was fitted into the screw cap jar with clip and gasket (Fig. 1), the circulation of ice water was turned on, the septum on the vial was pre-pierced with a 19-gauge sideport needle, the 4-port valve (D) was set to position 2, and the jar and HS vial were raised to insert the needle. The 4-port valve was set to position 3 and the vial was evacuated for 1 min (water aspirator). A mixture of 5 ml meat

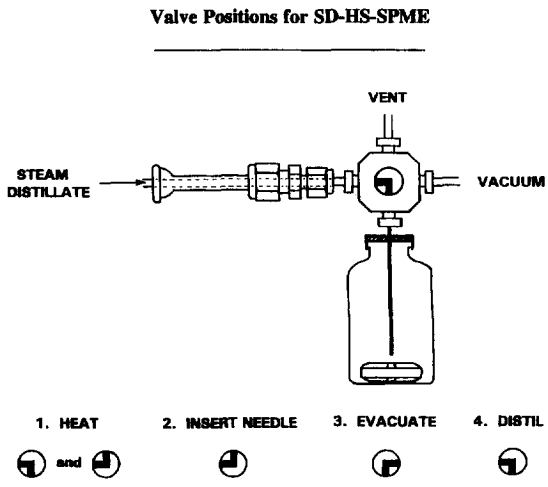

Fig. 2. Diagram showing the positions of the right-angle valve core and steam path before and during distillation.

suspension (equivalent to 0.1 or 1 g of meat), 0.5 ml of 30% acetic acid and the I.S. solution (NDOA or NDPA) was then injected into the sample chamber (Fig. 1) using a 10-ml glass BD syringe and a 12-cm long 19 gauge hypodermic needle (the two connected together with a luer lock), and the valve was turned to position 4 and the magnetic stirring started. The rheostat controlling the power supply to the heating element was adjusted so that the pressure indicator level was maintained almost at the top, thus ensuring a positive pressure inside the generator. When about 15 ml of distillate (6 to 7 min) was collected, the collector assembly was lowered, the ice water circulation stopped, and the vial was removed for HS-SPME analysis as described in Section 2.6.

2.6. HS-SPME

The cap on the vial was removed and about 2.5 g of solid KOH was added to the steam distillate. The vial was recapped with a new pre-pierced septum and seal, and the mixture was stirred magnetically at a low speed for about 10 min. The needle of the SPME fibre assembly was inserted through the septum hole, and the fibre extended to 1 cm above the liquid surface. The SPME equilibration (with constant stirring) inside the temperature controlled GC oven, retraction of the fibre following equilibration, and subsequent thermal desorption of the compounds in the GC injector and GC analysis were carried out as reported previously [6].

For HS-SPME of standards, appropriate amounts of the respective water standards, 6 g of NaCl, 1 ml of 30% acetic acid and 100 mg of ascorbic acid were added to the HS vial. Enough water was added to make up the volume to 15 ml, the mixture stirred magnetically at room temperature for 10 min, and 2.5 g of solid KOH were added. The vial was recapped and HS-SPME–GC–TEA analysis was carried out as above.

2.7. Determination of standard curves

Two sets of standard curves – one in a lower concentration range (5 to 40 ng of NDBA and 0.5 to 4 ng of NDBzA per vial) and the other in a higher range (15 to 1250 ng of NDBA and 9 to 720 ng of NDBzA per vial) – were constructed to determine the linearity of the response. Each was run in duplicate and the averages of the peak heights or peak areas were taken to construct the standard curves. This was, however, done only once to determine the range for linearity. For day-to-day analyses, the concentration of NDBA and NDBzA in ham distillates were calculated from their relative response to those of appropriate external standards analyzed on the same day.

3. Results and discussion

3.1. Applicability of the technique to nitrosamine determination

Initially, we attempted to develop a SPME method for the GC–TEA determination of all seven volatile nitrosamines (first seven in Table 1) commonly detected in cured meat products [8,9]. However, in the extraction time used, the SPME efficiency into a polyacrylate (PA) fibre from the water and HS was too low for most of these nitrosamines (Table 1). This suggested that the technique would not be sensitive enough for determination of the commonly found nitrosamines such as N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitrosopyrrolidine (NPYR), N-nitrosopiperidine (NPIP)

Table 1
Extraction efficiency of various nitrosamines from aqueous phase to PA coated fibre (via head space)[a]

Nitrosamine	% Extracted	
	Room temperature	80°C
NDMA	0.08	–[b]
NDEA	0.17	–
NDPA	2.04	2.04
NDBA	19.3	13.3
NPIP	0.07	–
NPYR	0.07	–
NMOR	0.02	–
NDBzA	1.9	34.8
NDOA	<1	26.2

[a] Equilibrated for 1 h at 80°C (salt saturated and made strongly alkaline).
[b] Not determined.

and N-nitrosomorpholine (NMOR) in foods. These relatively polar, water-soluble nitrosamines are readily steam distilled [8,13]. Thus, their poor HS-SPME must be attributed to an unfavourable partition into the fibre coating. Similar results were also obtained when a Carbowax–divinylbenzene (CW–DVB) coated fibre was used (data not shown). The relatively non-polar NDBzA and the longer-chain dialkyl nitrosamines, NDPA, NDBA and NDOA, were more efficiently extracted than those above. NDBzA and NDOA, however, required a HS-SPME temperature of 80°C to achieve acceptable extraction in 1 h. The more volatile NDPA and NDBA could be extracted effectively at room temperature as well as 80°C.

Having failed to achieve the initial objective, we decided to develop a SPME–GC–TEA technique for NDBA and NDBzA that appeared to be feasible from the extraction efficiency data presented in Table 1. This happened to be quite fortuitous because these two nitrosamines are frequently detected in hams packaged in elastic rubber nettings [11–14], and there appeared to be a need for a rapid and sensitive monitoring technique for their determination in such products.

3.2. Selection of fibre and optimization of conditions

In our hands, the fibres coated with either PA or CW–DVB polymers gave better yields of both NDBA and NDBzA than that obtained with polydimethylsiloxane (PDMS) coated silica fibre.

Recently, Shirey et al. [19] reported SPME–GC determination of NDMA and a few other nitrosamines in water using a similar (PDMS–DVB) fibre, but they did not apply the technique to the determination of these compounds in any food matrices. Although the performances of the PA and CW–DVM fibres appeared to be comparable, the former was chosen for it seemed to give slightly better results. Also, investigation on the effect of salt saturation and that of the addition of alkali (to 3 *M* KOH strength) suggested that both were required for achieving the maximum sensitivity. Hence, all solutions were made up to 3 *M* KOH strength and saturated with NaCl before beginning SPME equilibration.

Extraction for 1 h at four different temperatures (room temperature, 40°C, 80°C and 100°C) suggested that 80°C gave the best results for all four nitrosamines (NDPA, NDBA, NDOA and NDBzA). Using the above conditions (PA fibre, 1 h, 80°C), the relative standard deviations (R.S.D.s) of five replicate SPME analyses for NDPA, NDBA, NDOA and NDBzA standards (without ham) were found to be 2.2, 4.8, 8.9, and 6.5%, respectively.

3.3. Standard curve

For NDBA, the response was linear at least up to 0.625 μg/vial, and that for NDBzA it was linear up to 0.312 μg/vial. Above these amounts, the responses began to level off most likely due to saturation of the SPME fibre. Since a 0.1 g sample size was usually taken for the final SPME–GC–TEA determination, this would correspond to 6.25 ppm of NDBA and 3.12 ppm of NDBzA in ham. Any sample extract containing equal to or higher amounts of these nitrosamines should be further diluted before carrying out SPME.

3.4. Analysis of hams

It has been known from previous research [6] that nonpolar organic compounds such as food lipids can seriously interfere with the SPME of nonpolar analytes from aqueous solution. This was also found to be the case during determination of NDBA and

NDBzA in hams using SPME. When aqueous NDBA and NDBzA standards were added to dilute aqueous ham homogenates, only <20% (versus water) of the two compounds were extracted by HS-SPME. This suggested that some sort of prior cleanup would be needed to remove the fats and lipids that are present in ham. To overcome this problem, the steam distillation cleanup (simply called steam distillation) was adopted as described in Sections 2.1.1 and 2.5.

Distillation of ham homogenates followed by SPME–GC–TEA worked fairly well provided only a sample size equivalent to 0.1 g of ham was used. For some hams, a larger sample size (equivalent to 1 g of ham) could be used but that had to be predetermined by carrying out recovery studies with added NDBA and NDBzA, and comparing the results against direct HS-SPME of the same amounts of standards (in the absence of any ham extract). It should be emphasized that the term % recovery has been used throughout to describe such results. It is recommended, therefore, that all hams first be analyzed using a 0.1 g sample size at a TEA detector attenuator setting of 4. This should give a detection limit (×3 noise level) of about 3 μg/kg of NDBA and 1 μg/kg of NDBzA in hams that is considered adequate for monitoring purposes.

Despite these drawbacks, the overall method worked fairly well with most of the smoked hams analyzed thus far (Table 2). The recoveries of NDBA from various spiked hams ranged between 42 to 112% (average 86.6%), and that for NDBzA ranged between 41 to 107% (average 70.1%). These values are comparable to those obtained with nitrosamine water standards taken through the whole method (average 102%, range, 95 to 108% for NDBA and an average of 71.3%; range, 60 to 84% for NDBzA). The corresponding recoveries for NDOA varied more widely and were sometimes very high (average 103%; range, 42 to 163%). Whereas those for NDPA seemed to be more reproducible (average 91.1%; range, 58 to 124%), but the data (not shown in Table 2) did not correlate well with those of NDBA and NDBzA. In other words, neither of the I.S.s chosen were satisfactory. For these reasons, none of the results were corrected for I.S. recovery losses. Instead, they were corrected against recovery losses of NDBA and NDBzA as determined from the analysis of respective spiked samples. Further research will be necessary to search for a better I.S., that may eliminate the need to carry out recovery studies for each sample with added NDBA and NDBzA.

Table 2
Recoveries of NDBA and NDBzA from hams by this method

Ham	Spiking level (μg/kg)		Recovery (%)[a]	
	NDBA	NDBzA	NDBA	NDBzA
A	80	45	63	45
A	140	80	102	69
B	50	5	42	41
B	150	81	111	86
C	16	9	76	98
C	160	90	76	59
D	160	90	82	54
E	156	90	112	84
E	16	9	102	87
F	150	81	109	107
G	160	90	74	46
H	160	90	88	48
I	160	90	89	87
Average			86.6	70

[a] All recoveries were calculated against direct HS-SPME of standards.

The repeatability of the overall method was also highly satisfactory. For example, five replicate analyses of two hams containing incurred NDBzA gave values of 23.6±4.4 μg/kg and 51.3±7 μg/kg. Similarly, three replicate recovery studies with a ham, which had been found to be negative for nitrosamines, spiked with NDBA (160 μg/kg) and NDBzA (90 μg/kg), gave recovery values of 102%, 103%, 112% and 68.9%, 68.9% and 84.4%, respectively. Two representative chromatograms from some of the recovery studies are presented in Figs. 3 and 4.

As in other published methods [11–14,20], the possibility of artifactual formation in this method was prevented or minimized by incorporating ascorbic acid, propyl gallate and acetic acid (ascorbic acid destroys nitrite faster at an acidic pH) in the sample or sample extract during analysis. No evidence of artifactual formation was observed when a ham, which was negative for NDBzA, was analyzed by

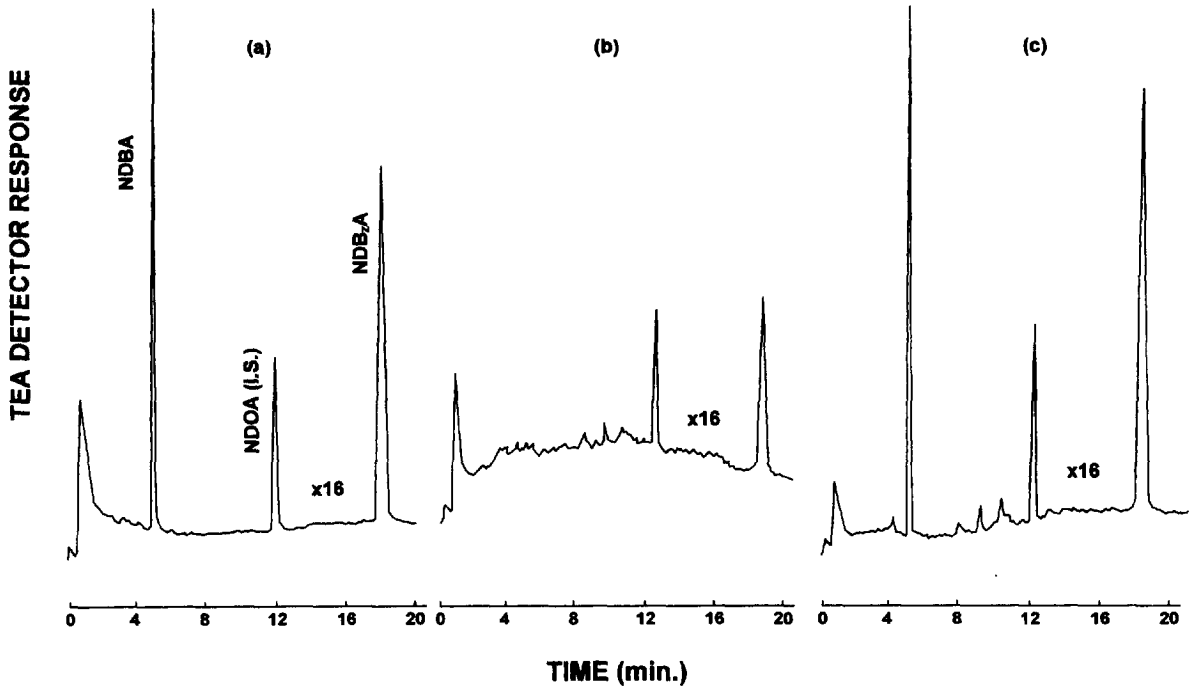

Fig. 3. Chromatograms of SPME analyses: (a) NDBA (14 ng), NDOA (I.S.), 4 ng, and NDBzA (8.1 ng) standards; (b) an unspiked ham extract (equivalent to 0.1 g of ham) taken through all the steps and showing the presence of 39.8 μg/kg of NDBzA (82% recovery for I.S.) and (c) same ham spiked with 140 μg/kg of NDBA (102% recovery) and 81 μg/kg of NDBzA (69% recovery) (112% recovery of I.S.).

this method following the addition of dibenzylamine (1 mg). Therefore, it is highly unlikely that the method is susceptible to artifactual formation.

Finally, a few selected samples of smoked hams packaged in elastic rubber nettings that had previously been analyzed using a conventional solvent extraction and alumina cleanup procedure [12], were reanalyzed by this newly developed SPME method. The final determinative step in both the methods were, however, the same, i.e., GC–TEA detection. The two sets of results (Table 3) agreed well (r^2= 0.97). The older method is, however, lengthy and time-consuming and employs large amounts of organic solvents for sample preparation. It takes a minimum of a full day to complete an analysis. On the other hand, the SPME method is very fast. A complete analysis can be carried out within 2 to 2.5 h. The older method [12], however, gives higher recoveries (average>90%) and is less susceptible to matrix effects. Therefore, the two methods complement each other. The SPME method is better suited for rapid monitoring purposes (semi-quantitative estimation), whereas the older method should be used when a higher accuracy is desired.

3.5. GC–MS confirmation

If a ham sample is found to be positive for either NDBA or NDBzA on initial determination by SPME–GC–TEA, the results can be confirmed by SPME–GC–MS in the same manner without any additional cleanup. To demonstrate this, two ham samples (F and I in Table 3) were reanalyzed by SPME–GC–MS using the SIM mode, and the presence of NDBzA in both cases were confirmed. Additionally, the identity of NDBzA in the second sample was confirmed by full scan MS (Fig. 5). Since none of the samples analyzed in this study

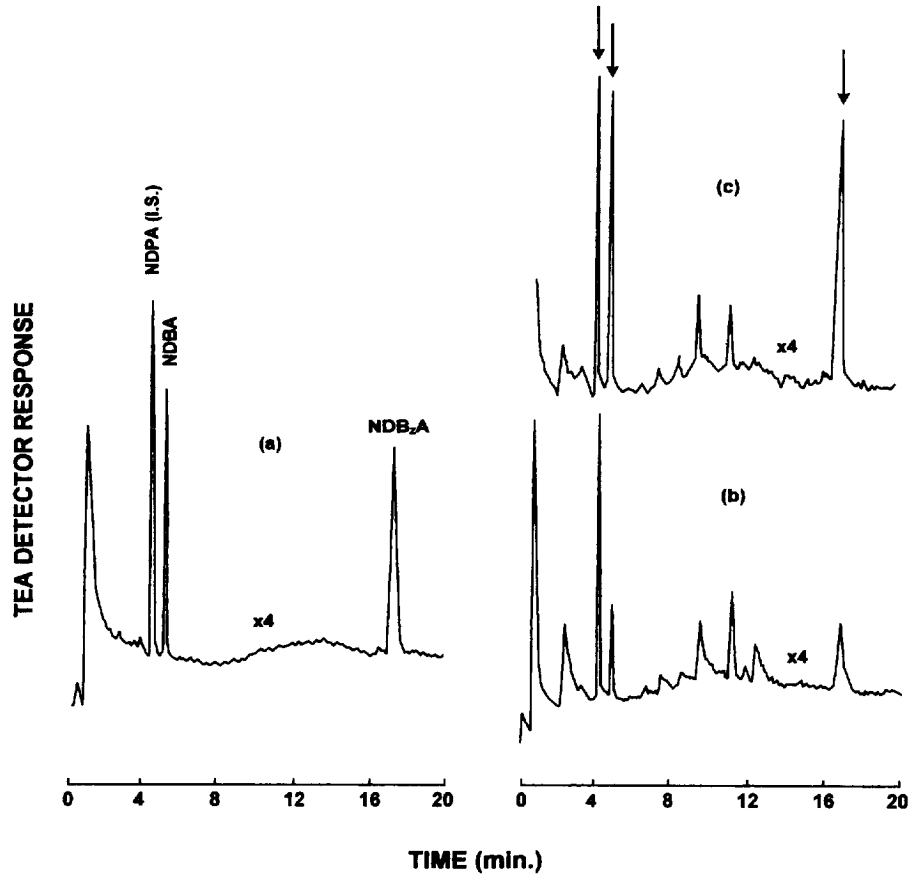

Fig. 4. Chromatograms of SPME analyses of hams at lower levels: (a) NDPA (I.S.), 10 ng, NDBA (1.6 ng) and NDBzA (0.9 ng) standards; (b) a ham extract (equivalent to 0.1 g of ham) showing the presence of 5.6 μg/kg of NDBA and 2.6 μg/kg of NDBzA (I.S. recovery 79%) and (c) same ham spiked with 16 μg/kg of NDBA (76% recovery) and 9 μg/kg of NDBzA (98% recovery) (88% recovery of I.S.).

Table 3
Comparison of results on NDBzA levels in hams as obtained by this method with that obtained by an older conventional method[a]

Hams	NDBzA level (μg/kg) detected	
	This method	Conventional method
A	40.6	34
F	22.5	25
G	99	107
H	31.9	41
I	316	375
J	81	90
K	230	208

[a] As described by Sen et al. [12].

contained any detectable levels of NDBA, the applicability of SPME–MS for its confirmation was demonstrated by analyzing a sample spiked with 156 μg/kg levels of NDBA. It should be emphasized that in the event, unknown fragment ions are present in a sample (ham) mass spectrum, additional analyses such as SIM under high-resolution or reanalysis after further cleanup of the sample extract will be required for unequivocal confirmation.

4. Conclusions

The concentration of both NDBA and NDBzA in hams could be easily analyzed by SPME–GC–TEA

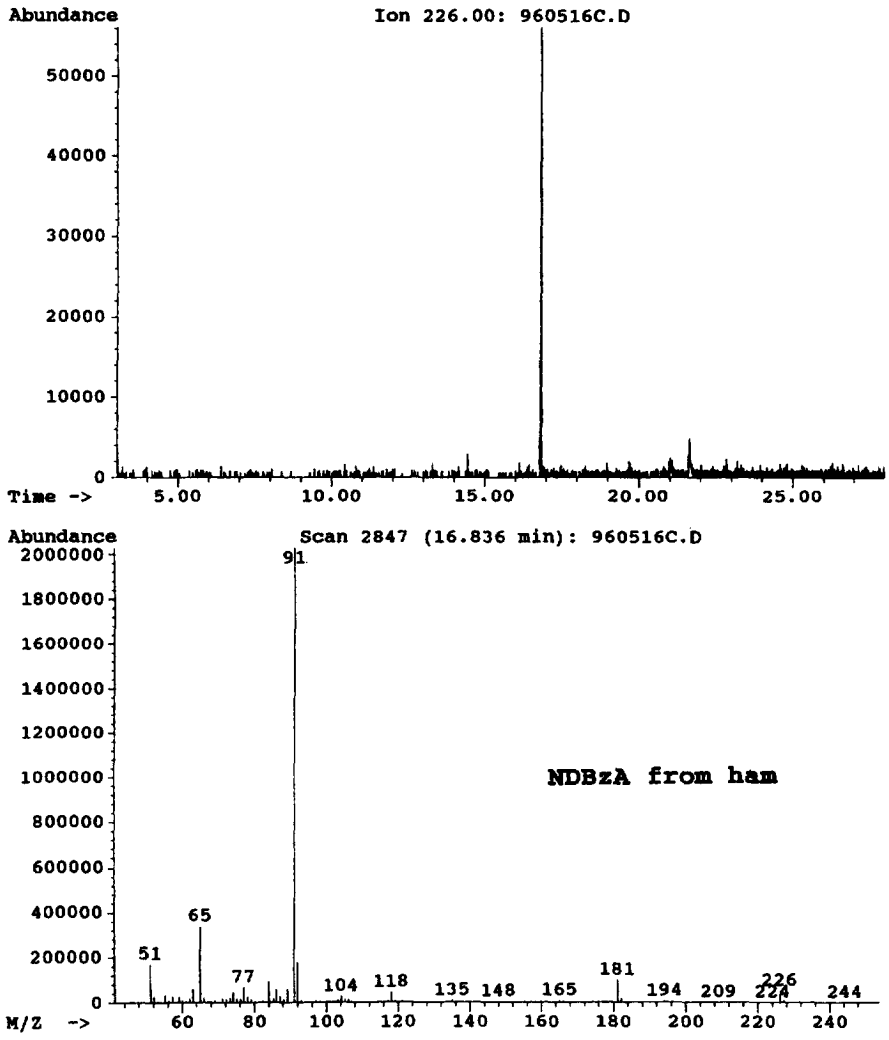

Fig. 5. SPME–GC–MS tracings of NDBzA (316 μg/kg) in a ham both in the selected ion monitoring (top) mode (at m/z 226) and the scan mode (bottom), showing the full spectrum.

using a PA coated fibre. The method is highly sensitive and fast, and should be useful for rapid semi-quantitative estimation of the levels of these two nitrosamines in hams packaged in elastic rubber nettings.

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