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Analysis of volatile nitrosamines from a model system using SPME–DED at different temperatures and times of extraction

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Abstract

Solid-phase microextraction (SPME) coupled to a direct extraction device (DED) was evaluated as a method for extracting nitrosamine (NA) standard mix containing nine volatile nitrosamines from a solid food model system using several temperatures and times of extraction. The efficacy of extraction, the linearity of response and the sensitivity of this method for analysis of NA were determined at refrigeration (4 °C) and room (25 °C) temperature. Several extraction times (15, 30, 60,120 and 180 min) were also tested. Analysis was carried out with gas chromatography–mass spectrometry (GC–MS) in selected ion monitoring (SIM) mode. At 4 °C all NA were detected at all concentrations studied (ranged from 1 to 50 ng ml^{-1}) except for *N*-nitrosopyrrolidine (NPYR), *N*-nitrosomorpholine (NMOR) and *N*-nitrosodiphenilamine (NDPheA). Better results were obtained at 25 °C in terms of efficacy of extraction, linearity values (regression coefficients, R^2) and sensitivity values (limit of detection, LOD). Only 15 min of extraction time were enough to extract all NA from gelatines (10 ng ml^{-1}) at 25 °C. None of the NA reached the equilibrium, even when long times of extraction (20 and 24 h) were evaluated. SPME–DED appears to be a rapid and suitable technique for extracting NA from model food system at both refrigeration and room temperatures.
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Keywords: SPME–DED; Volatile nitrosamines; Gelatine; Temperature; Time

1. Introduction

NA are potentially carcinogenic, mutagenic and teratogenic compounds for animals and humans (Mirvish, 1975; Preussmann & Stewart, 1984; Scanlan, 2003). The potential NA sources for exogenous exposure in humans include tobacco (Wilp, Zwickenplug, & Richter, 2002), packaging materials (Fiddler, Pensabene, & Gates, 1997), pesticides (Rywotycki, 2003), soil (Mallik & Tesfai, 1981) and a variety of foods (Glória, Barbour, & Scanlan, 1997a; Scanlan, 2003; Vecchio, Hotchkiss, & Bisogni, 1986) and drinks (Havery, Hotchkiss, & Fazio, 1982; Lijinsky, 1999). NA are generated in foods by reaction between primary, secondary or tertiary amines and nitrosating agents (Tricker & Preussmann, 1991). Their formation is influenced by several factors including the addition of nitrites and/or nitrates as preservatives (Cassens, 1995, 1997).

The great concerns towards these compounds challenge researchers to develop satisfactory methods for the analysis of NA in foods. The extraction of NA in foods shows several difficulties. First, NA have been detected at very low levels and in a large variety of foods with different compositional characteristics. Additionally, some problems during isolation and detection of NA have been described such as the possibility of interferences with similar chemical compounds and the formation of NA while carrying out the method (Raoul, Gremaud, Biaudet, & Turesky, 1997). Therefore, it results necessary to develop sensitive, efficient and specific methods for NA analysis. In food systems, methods of analysis of volatile NA involve many preliminary steps such as sampling, extraction, clean-up and concentration. These procedures are time consuming and

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most of them require the use of toxic solvents. Solid phase microextraction (SPME) (Steffen & Pawliszyn, 1996) is a solvent free process that combines sampling, extraction, concentration and instrument introduction into a single step. Previous studies have demonstrated the feasibility of SPME for analysing tobacco specific nitrosamines (Mullet, Levsen, Borlak, Wu, & Pawliszyn, 2002) or *N*-nitrosodibutylamine and *N*-nitrosodimethylamine in smoked hams (Sen, Seaman, & Page, 1997). Recently, Andrade, Reyes, and Rath (2005) reported a method for the determination of volatile nitrosamines in sausages using headspace sampling by SPME. However, a direct extraction method cannot be performed in solid samples using SPME, because the SPME fibre is too weak to be pushed into the solid material. In previous papers (Andrés, Cava, & Ruiz, 2002; Ruiz, Ventanas, & Cava, 2001), we have described the use of a small direct extraction device (DED) which enables the introduction of the SPME fibre in the core of solid matrixes, allowing the analysis of volatile compounds from solid foods with little deterioration of the product, if any. In a previous work (Ventanas, 2003), we studied the feasibility of using SPME–DED followed by GC–MS for the analysis of NA extracted from gelatine matrices mimicking a food matrix. In such preliminary study, the aim was to develop a simple screening method for the assessment of NA in solid food systems. Moreover, we compared the efficiency of different types of SPME stationary phases for the extraction of these compounds and carboxen/polydimethylsiloxane (CAR/PDMS) 85 μm fibre was selected.

NA compounds have been detected in refrigerated foods such as cheese (Glória et al., 1997b; Scanlan, 2003) and fish (Mitacek et al., 1999) and also in products stored at room temperature such as cured products (Holland, Wood, & Randall, 1981; Rywotycki, 2002; Vecchio et al., 1986). Thus, it is necessary to study the use of SPME–DED for the extraction of NA from solid systems both at refrigeration and at room temperatures.

SPME–DED is a headspace extraction method, but in this case, the headspace is created within the device (Ruiz et al., 2001). Headspace-SPME (HS-SPME) is an equilibrium method, and as consequence, it is strongly influenced by experimental conditions. The time to achieve the equilibrium determines the maximum amount of analyte that can be extracted by the fibres which controls the sensitivity of the method (Doong, Chang, & Sun, 2000). When extraction of analytes by SPME is performed once reached the equilibrium between the headspace and the SPME, the amount of extracted compound is only slightly affected by the extraction factors (Pawliszyn, 1997). The optimal extraction time should be the time needed to reach the equilibrium. However, for practical reason shorter extraction times are often needed. Therefore, it would be interesting to investigate whether the SPME–DED extraction of NA from solid gelatines can be carried out using short extraction times.

The aim of the present work was to evaluate the feasibility of SPME–DED–GC–MS for the analysis of volatile NA from solid matrix under different temperatures and extraction times.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals and reagents

NA standard was supplied by Sigma–Aldrich (EPA 8270 nitrosamines mix, Sigma–Aldrich, St. Louis, MO, USA). This solution contained nine analytes: *N*-nitrosodimethylamine (NDMA), *N*-nitrosomethylethylamine (NMEA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosopyrrolidine (NPYR), *N*-nitrosomorpholine (NMOR), *N*-nitrosodi-*n*-propylamine (NDPA), *N*-nitrosopiperidine (NPIP), *N*-nitrosodi-*n*-butylamine (NDBA) and *N*-nitrosodiphenylamine (NDPheA) (Table 1). Most of them have been reported in a variety of foods and drinks such as NDMA in beer, cheese or fish (Glória et al., 1997b; Scanlan, 2003), NPYR in cooked bacon (Fiddler & Pensabene, 1996) or NDBA in hams packaged in elastic rubber nettings (Fiddler et al., 1997).

Methanol was obtained from Scharlau Chemie (Barcelona, Spain) and was HPLC grade. Gelatine (300 bloom) was supplied Sigma–Aldrich (St. Louis, USA).

Table 1
Volatile NA included in the studied standard, together with the selected ion for detection in MS SIM mode, the retention time for each analyte and their molecular weight

Nitrosamines	*m/z*	Ret. time (min)	MW	log K_{ow}	Water solubility (mg l^{-1})	Vapour pressure (mm Hg)
N-nitrosodimethylamine (NDMA)	74	8.27	74	−0.57	1,000,000	2.7
N-nitrosomethylethylamine (NMEA)	88	12.02	88	0.04	300,000	2.09
N-nitrosodiethylamine (NDEA)	102	17.24	102	0.48	106,000	0.86
N-nitrosopyrrolidine (NPYR)	100	36.29	100	−0.19	1,000,000	0.06
N-nitrosomorpholine (NMOR)	116	36.40	116	−0.44	861,527.5	0.036
N-nitrosodi-*n*-propylamine (NDPA)	130	36.48	130	1.36	13,000	0.389
N-nitrosopiperidine (NPIP)	114	37.45	114	0.36	76,480	0.14
N-nitrosodi-*n*-butylamine (NDBA)	84	39.66	158	2.63	1270	0.0469
N-nitrosodiphenylamine (NDPheA)	169	43.04	198	3.13	35	0.0000697

Polarity expressed as log *K* octanol–water (log K_{ow}), water solubility in mg l^{-1} at 25 °C and vapour pressure at 25 °C for each NA from available literature are also included.

2.2. Methods

2.2.1. Preparation of NA standards

NA standard stock solution at 10 μg ml^{-1} was prepared by diluting 10 μl of the NA standard (2000 μg ml^{-1}) in 2 ml of methanol. NA standard working gelatines with different NA concentrations (1–50 ng ml^{-1}) were prepared by addition of the appropriate volume of the NA stock solution to 30 ml of gelatine solutions (20% in double distilled water) contained in 30 ml polypropylene tubes. Gelatine solutions were prepared by adding the appropriate amount of gelatine to boiling water and subsequent agitation. NA standard stock solution was added when the gelatine was at 60 °C. Working gelatines were immediately sealed with polyethylene caps, vortexed for 30 s and stored at 2–4 °C until analysis. Gelatines of each concentration were prepared in duplicate. Analysis of NA in gelatines by SPME–DED was carried out within the next 7 days of preparation.

2.2.2. SPME–DED extraction of NA

NA were directly extracted from gelatines by inserting the DED into the core of the gelatine and subsequent introduction of the SPME fibre into the DED following the procedure previously described by Ruiz et al. (2001) (Fig. 1). In order to control the temperature extraction (4 and 25 °C), tubes containing the gelatines were kept in a thermostatic water bath.

A SPME (Supelco, Co., Bellefonte, PA) fibre (10 mm length) coated with carboxen/poly(dimethylsiloxane) (85 μm thickness) couple to a small DED (Andrés et al., 2002; Ruiz et al., 2001) were used to extract the NA from gelatine model systems. Previous work showed that this coated fibre presented the best characteristics for a satisfactory extraction of NA from gelatine matrix (Ventanas, 2003).

Depending on the temperatures and times of extraction, two experiments were carried out:

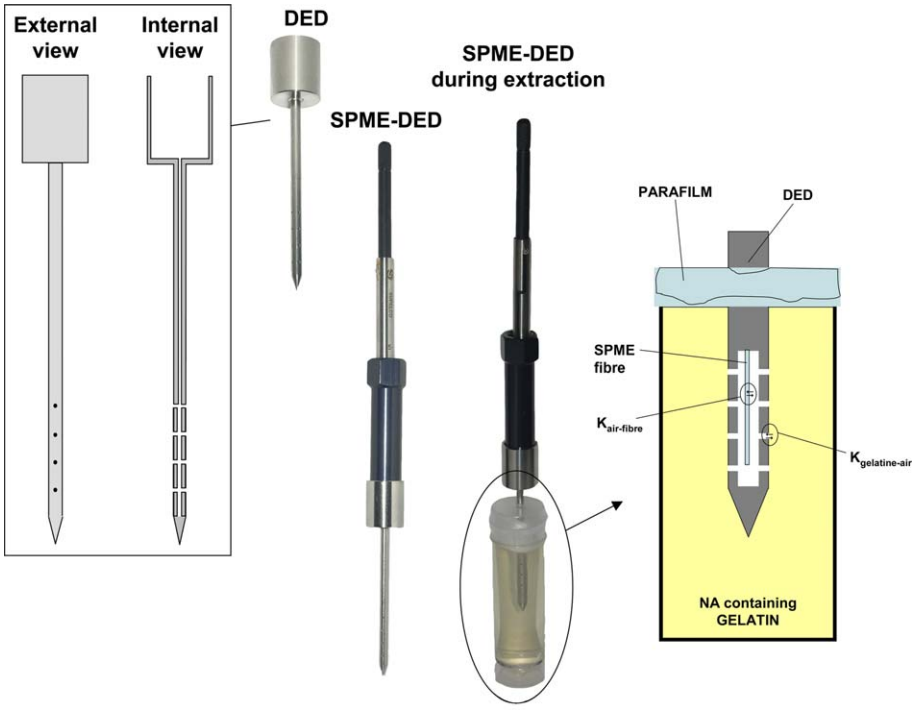

Fig. 1. Scheme of the use of SPME–DED in gelatines mimicking solid foods.

Table 2
Chromatographic areas (UA × 10^4) for the nine NA extracted from gelatines at different NA concentrations at 4 and 25 °C

	1 ng ml^{-1}		5 ng ml^{-1}		10 ng ml^{-1}		25 ng ml^{-1}		50 ng ml^{-1}	
	4 °C	25 °C	4 °C	25 °C	4 °C	25 °C	4 °C	25 °C	4 °C	25 °C
NDMA	0.12	1.55	0.26	4.37	0.69	13.92	2.57	24.70	2.70	47.17
NMEA	0.21	1.21	0.27	3.79	0.59	11.25	2.23	22.39	2.26	44.38
NDEA	0.16	1.42	0.26	5.09	0.87	14.74	3.14	32.54	3.67	61.65
NPYR	n.d.	0.10	n.d.	0.12	n.d.	0.42	0.03	0.43	0.40	2.62
NMOR	n.d.	0.28	n.d.	0.28	n.d.	0.40	0.02	0.51	0.03	2.24
NDPA	0.23	0.86	0.12	2.77	0.38	5.13	1.60	18.22	1.75	39.22
NPIP	0.04	0.53	0.36	1.17	0.13	3.80	0.53	6.20	0.45	21.19
NDBA	0.06	3.23	0.28	56.80	1.59	14.32	12.64	69.76	5.00	167.01
NDPheA	n.d.	3.13	0.17	3.44	0.42	5.44	0.53	3.69	0.84	23.09

n.d., not detected.

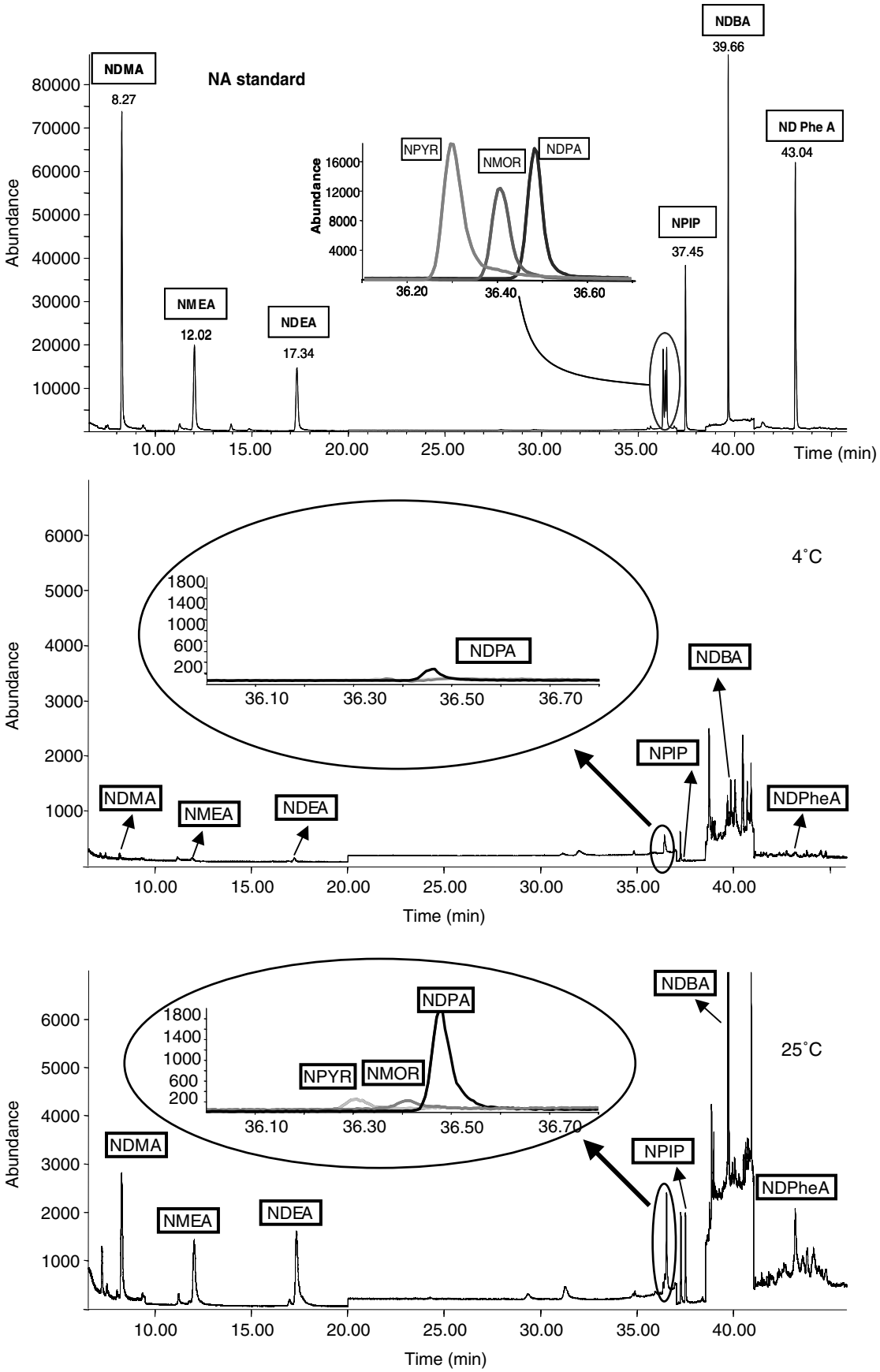

Fig. 2. SIM chromatograms of the NA standard directly injected in the GC–MS (NA standard) and of NA extracted using SPME–DED from gelatines at 10 ng ml^{-1} of concentration and at 4 and 25 °C of temperature. The inner graphics show the selected ions for the detection of NA eluted between 36 and 38 min.

Experiment 1. NA were extracted from gelatine for 60 min at room temperature (25 °C) and at refrigeration one (4 °C).

The linearity of the method was evaluated by increasing concentrations of NA over a range from 1 to 50 ng ml^{-1} since these levels of NA are similar to those usually detected in foodstuffs (Lijinsky, 1999; Scanlan, 2003). All extractions were in duplicate.

The LOD, defined as the concentration of the NA in gelatine that caused a peak with a signal-to-noise ratio greater than 10, was also calculated in order to test the sensitivity of the method at the studied temperatures.

Experiment 2. Different extraction times were tested: 15, 30, 60, 120 and 180 min at room temperature (25 °C) in gelatines with NA at 10 ng ml^{-1}. In addition, extractions at 20 and 24 h were also performed, in order to determine whether equilibrium time was reached for studied NA. All extractions were carried out in duplicate.

2.2.3. Gas chromatography–mass spectrometry

Once carried out the extraction, analytes were desorbed onto the GC column by inserting the SPME fibre in the injector port that was set at the appropriate temperature (280 °C for CAR/PDMS).

Analyses were performed using an Agilent 6890 series gas chromatograph (Agilent, Avondale, USA) coupled to a mass selective detector (Agilent 5973, Agilent, Avondale, USA). Analytes were separated using a 5% phenyl-methyl silicone (HP-5) bonded phase fused silica capillary column (Hewlett-Packard, 50 m × 0.32 mm i.d., film thickness 1.05 μm), operating at 6 psi of column head pressure, resulting in a flow of 1.3 ml min^{-1} at 40 °C. The SPME fibre were desorbed and maintained in the injection port at the appropriate temperature during the complete chromatographic run. The injection port was in splitless mode. The oven temperature program was 40 °C of initial temperature, raised to 80 °C at a rate of 5 °C min^{-1}, maintained at 80 °C for 27 min, and then raised to 250 °C at a rate of 6 °C min^{-1}, and held for 6 additional min. The transfer line to the MS was maintained at 280 °C. The mass spectra was obtained by electronic impact at 70 eV, a multiplier voltage of 1756V and collecting data at a rate of 1 scan s^{-1}. Detection of compounds was carried out in selected ion monitoring (SIM) mode (Table 1).

3. Results and discussion

3.1. Extraction of volatile NA at different temperatures

The efficiency of SPME–DED in the extraction of NA at different concentrations (from 1 to 50 ng ml^{-1}) at 4 and 25 °C is illustrated in Table 2. Fig. 2 shows the chromatograms obtained after extraction of NA at 10 ng ml^{-1} from solid gelatines at the two studied temperatures. Most NA were extracted at 4 and 25 °C at all studied concentrations. However, at 4 °C NPYR and NMOR were not extracted at 1, 5 and 10 ng ml^{-1} and NDpheA was not extracted at 1 ng ml^{-1}. At 25 °C, higher chromatographic areas of all NA at all concentrations were observed. Nevertheless, at refrigeration temperature SPME–DED successfully extracted most NA reported in foodstuffs, and therefore, it could be used as a screening method in products stored at such temperatures. The effect of temperature on the volatile extraction efficiency using HS-SPME can be attributed to different parameters (Ng, Teo, & Lakso, 1999). Heating provides of energy to the volatile analytes to overcome energy barriers, which bind them to the matrix, enhances the mass transfer process, increases vapour pressure of the volatiles and facilitates their release to the headspace (Boyd-Boland et al., 1994; Machiels & Istasse, 2003). Therefore, the extraction of NA is more efficient at 25 °C than at 4 °C. The second parameter is the ab/adsorption process on the SPME fibre coating (CAR/PDMS). This phenomenon is an exothermic process with the correlated decreased of the partition coefficients with higher extraction temperatures Machiels and Istasse (2003). Thus, there would be an optimum temperature whereby the extraction efficiency is maximised. Increasing the temperature beyond the optimum temperature will have a negative effect on the extraction process (Boyd-Boland et al., 1994). However, in the present study, the working temperatures (4 and 25 °C), were probably too low to evidence this latter phenomenon, since such a decrease in efficiency extraction has been reported at higher temperatures (70–80 °C) by others authors (Ng et al., 1999; Pawliszyn, 1997).

Table 3
Regression coefficient (R^2), linear regression equation and limits of detection (LOD) for NA extracted from solid gelatines by SPME–DED at 4 and 25 °C

	4 °C			25 °C		
	R^2	Equation	LOD (ng ml^{-1})	R^2	Equation	LOD (ng ml^{-1})
NDMA	0.8312	$y = 578.17x + 2145.5$	1.229	0.9896	$y = 9213.5x + 15{,}735$	0.097
NMEA	0.8085	$y = 470.65x + 2563.8$	0.312	0.9959	$y = 8781.3x + 6214.4$	0.028
NDEA	0.8825	$y = 783.03x + 1943.3$	0.227	0.9968	$y = 12{,}348x + 6165.4$	0.011
NPYR	0.8944	$y = 9.5016x - 47.902$	14.814	0.8751	$y = 498.74x - 1677.8$	0.206
NMOR	0.8954	$y = 6.7284x - 34.082$	24.997	0.8743	$y = 394.82x + 221.41$	0.357
NDPA	0.8508	$y = 385.52x + 721.59$	0.345	0.9953	$y = 8012.1x - 13417$	0.075
NPIP	0.6884	$y = 98.098x + 578.55$	3.308	0.9519	$y = 4141.4x - 9589.9$	0.080
NDBA	0.2923	$y = 1425.1x + 13237$	0.763	0.8803	$y = 30{,}490x + 67{,}337$	0.042
NDPheA	0.9006	$y = 154.56x + 1093.9$	3.140	0.7998	$y = 3859x + 7335.9$	0.149

The linearity of the SPME–DED method for analysing NA from solid gelatines at 4 and 25 °C was assessed by plotting calibration curves of obtained chromatographic areas vs. the concentration of the nitrosamines in the gelatines with concentrations ranging from 1 to 50 ng ml^{-1} (Table 3). Most NA showed a linear response both at 4 and at 25 °C, with regression coefficients (R^2) above 0.80 and 0.95 respectively. At 25 °C values of R^2 ranged from 0.7998 (NDPheA) to 0.9968 (NDEA) while at 4 °C ranged from 0.2923 (NDBA) to 0.9006 (NDPheA). Except for NDPheA, NMOR and NPYR, all R^2 obtained were lower at 4 °C than at 25 °C, probably due to the poorer

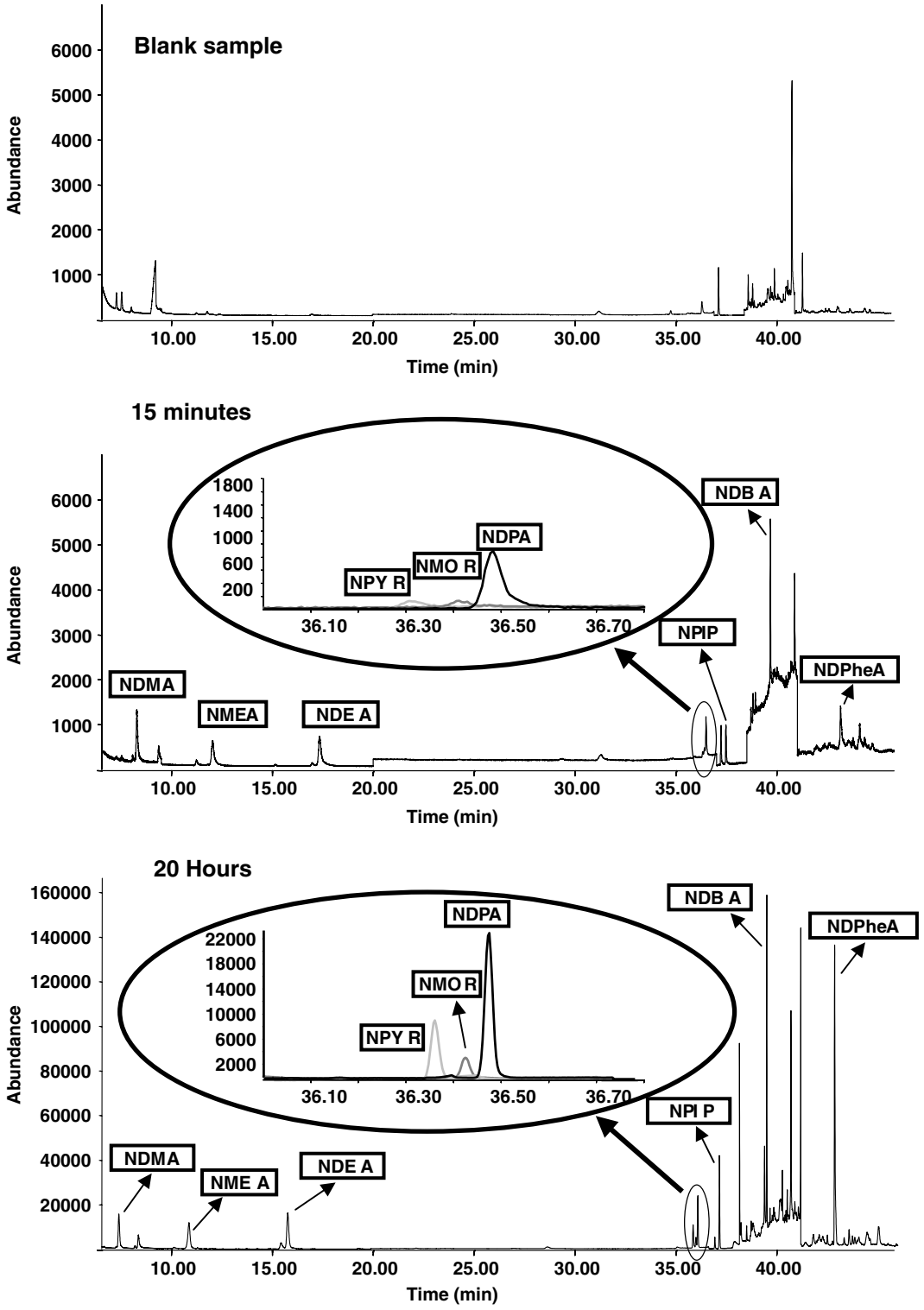

Fig. 3. SIM chromatograms of a blank sample (gelatine without NA extracted at 25 °C) and of NA extracted using SPME–DED from gelatines at 10 ng ml^{-1} of concentration and for 15 min and 20 h. The inner graphics show the selected ions for the detection of NA eluted between 36 and 38 min.

reproducibility at 4 °C. In addition, some authors have reported that the fibres containing carboxen, such as CAR/PDMS, show worse reproducibility results than other types (Elmore, Mottram, & Hierro, 2000; Pérez-Trujillo, Frías, Conde, & Rodríguez Delgado, 2002).

The LOD, defined as the concentration of the analyte in gelatines which caused a peak with a signal-to-noise ratio greater than 10, were calculated at 25 and 4 °C and are presented in Table 3. LOD was from 0.011 ng ml^{-1} (NDEA) to 0.357 ng ml^{-1} (NMOR) and from 0.227 ng ml^{-1} (NDPhe) to 24.997 ng ml^{-1} (NMOR) at 25 and at 4 °C respectively. All NA showed lower LOD at 25 °C than at 4 °C, notably for NPYR, NMOR, NPIP and NDpheA. The increase in efficiency with temperature explains the best sensitivity of the method at 25 °C. These results are in accordance with other authors who have reported better LOD values with increasing temperatures for different analytes such as butyltin compounds in wines (Azenhan & Vasconcelos, 2002) and trihalomethanes in drinking water (Cho, Kong, & Oh, 2003). Nevertheless, at 4 °C the LOD of NA extracted with SPME–DED from gelatines model, were low enough for detecting most of these compounds at the levels commonly reported in solid foodstuffs stored at refrigeration temperatures. Only NPYR showed a LOD (14.8 ng ml^{-1}) higher than the limit established by the Food and Drug Administration (FDA, 1978, 1980) for most NA, which is 5 ng ml^{-1} (ppb) in beer and bacon.

3.2. Extraction of volatiles NA at different times

The chromatograms obtained after 15 min and 20 h of extraction are presented in Fig. 3. Fig. 4 illustrates the extraction time profile for NA extracted from gelatines at 10 ng ml^{-1} at 15, 30, 60, 120, 180, 1200 (20 h) and 1440 min (24 h).

Only 15 min of extraction were enough to satisfactorily extract all the NA presented in the gelatines. Thus, the extraction of NA from a solid system by SPME–DED at the concentrations commonly reported in foods, at least at 25 °C, could be carried out rapidly (15 min). Most current analytical methods for NA extraction from foods need considerable longer extraction time.

Results suggest that the longer the extraction time, the higher the chromatographic areas obtained for all studied NA. The area of extracted NA still increased after 24 h of extraction. Therefore, it seems that the equilibrium was not reached for any of the studied NA after this extraction time. Since SPME is an equilibrium extraction method, the equilibrium time determines the maximum amount of analyte that can be extracted by the SPME fibre (Steffen & Pawliszyn, 1996). In our case, even with a long time of extraction, the equilibrium was not reached. NA compounds can be established chemical interactions with the polar group of protein of gelatine. Additionally, in solid matrixes, NA can be retained physically inside the cells of the gel which constitutes the solid gelatine matrix (Steffen & Pawliszyn, 1996). These phenomena might hinder the transfer of NA through the complex matrix and thus the equilibrium process. Other authors, using HS-SPME, have reported shorter times to reach the equilibrium but in less complex system (Zambonin, Cilenti, & Palmisano, 2002). Moreover, some authors have observed that CAR/PDMS fibres need long times of exposition in order to reach the equilibrium (Gianelli, Flores, & Toldrá, 2002; Machiels & Istasse, 2003).

Although it is preferable to use the equilibrium time as the extraction time because of the optimal extraction

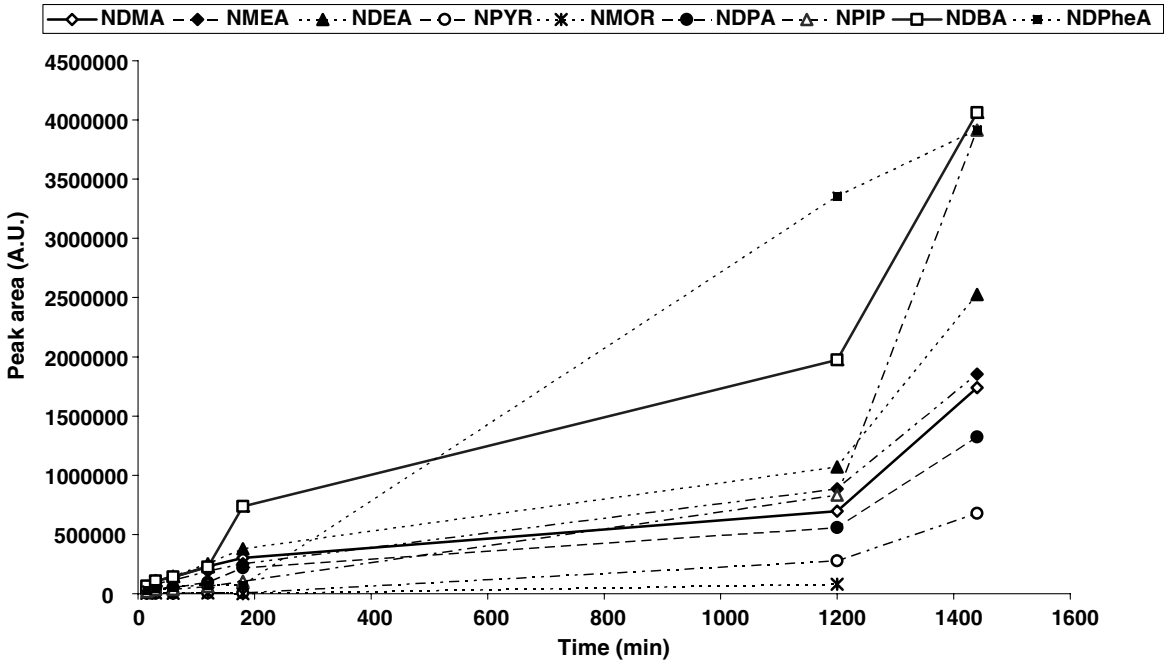

Fig. 4. Chromatographic areas (AU × 10^4) for the nine NA extracted from gelatines (10 ng ml^{-1}) using SPME–DED at different times of extraction.

efficiency and precision, practical limitations can be considered (Ng et al., 1999). In fact, our objective was to study whether it was possible to detect NA from a solid matrix using an extraction time as short as possible, in order to evaluate the potential application of SPME–DED–GC–MS as a screening analytical technique in real situations.

4. Conclusions

SPME–DED–GC–MS appears as a suitable technique to carry out a preliminary and qualitative analysis of volatiles NA commonly detected in solid foodstuff even at refrigeration temperatures and with short times of extraction. Nevertheless, the extraction of NA at room temperature is preferable if the temperature is not a limiting factor.

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