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Journal of Chromatography A, 985 (2003) 503–512

JOURNAL OF CHROMATOGRAPHY A

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Determination of nitrosamines in preserved sausages by solid-phase extraction–micellar electrokinetic chromatography

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Abstract

A new methodology for extraction, pre-concentration and analysis of nitrosamines in meat derived products was developed. The samples (canned sausages) were distilled in vacuum steam and the nitrosamines were pre-concentrated by solid-phase extraction with active carbon. Then, micellar electrokinetic chromatography was used for the separation and determination of the different nitrosamines contained in a real sample and gas chromatography with mass spectrometry detection was used as the confirmation technique. The method allowed the determination of nitrosamine compounds at trace levels with relative standard deviation ranging from 4.0 to 22%.
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Keywords: Meat; Food analysis; Nitrosamines

1. Introduction

Nitrite and nitrate are normally added to smoked meat products such as canned sausages, ham, and salamis to prevent the formation of toxins from *Clostridiun botulinum* [1]. Nitrite is also responsible for the development of the flavor and iron reaction with some meat pigments to produce the desirable red color, characteristic of these products [1,2]. An undesirable reaction between nitrite and amine or amino derivatives produces nitrosamines. These compounds are found in the environment and in various foods [3–13], and have been receiving considerable attention because of their high toxicity [1]. Approximately 80% of the nitrosamines that were tested in laboratory animals produced cancer. These compounds are amines with two organic groups (R) and one NO group bonded to a central nitrogen [1,2]. The alkyl nitrosamines are carcinogenic and mutagenic, being activated by oxidation and subsequent generation of carbonium ions, which can promote alkylation of DNA [2,4]. It has been calculated that the tolerable level of human exposure to the most volatile nitrosamines is in the range of 5–10 mg kg^{-1} body weight. In some countries the laws can be quite strict with only lower limits allowed. For this reason a minimum detection limit less than 10 μg l^{-1} (considering the final extraction solution) is normally accepted as an essential prerequisite for the determination of this class of

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0021-9673/02/$ – see front matter © 2002 Published by Elsevier Science B.V.
PII: S0021-9673(02)01825-3

compounds in foods and in environmental samples [2,8].

The analytical techniques that can detect μg l^{-1} levels usually involve gas or liquid chromatography after extraction from the original sample. These methods necessarily include time consuming clean-up procedures to remove any interferences, mainly because most chromatographic detectors are not specific for the functional group NNO [1–3].

Thermal energy analyzer (TEA) detection, the only detection method that is recognized as specific for nitrosamines, is based on the chemiluminescence generated by the decay of the NO_2 group when it is electronically excited [1,14]. This system is expensive and not available in most laboratories. This system requires the use of a spectroscopic technique for unequivocal detection of the chromatographic peaks [1]. The combination of gas chromatography and mass spectroscopy with ion monitoring using different columns has been used to the identification and quantitation of nitrosamines [1,15].

Capillary electrophoresis (CE) has emerged as an efficient and rapid separation technique in recent years and has been employed in many applications such as analysis of proteins, pharmaceutical compounds, cosmetics and environmental pollutants [2,16]. There are sufficient CE methods to separate a wide variety of analytes. However for some classes of compounds, such as nitrosamines, extra work is necessary to carry out separation using commercial equipment [17]. This technique represents an alternative to the analysis of aqueous samples, which is not possible by gas chromatography.

For the separation of hydrophilic, low molecular mass, neutral and polar compounds, such as nitrosamines, it is necessary to develop CE techniques to enhance their selectivity. The main reason is that these compounds do not interact strongly with commonly used surfactants such as sodium dodecyl sulfate (SDS) or other buffer modifiers such as cyclodextrins in electrokinetic chromatography [16–19].

Ng and others [18] developed the separation of nitrosamines by CE with γ-cyclodextrins and an open tubular column. Janin et al. (cited within Ref. 17) investigated the separation of a select group of nitrosamine compounds by CE using sub-ambient column temperature to improve the resolution of these compounds, resulting in the separation of their *sym* and *anti* conformers.

The recovery of nitrosamines at ultra trace level may require the development of an adequate pre-concentration method. Active carbon is suitable for this pre-concentration step due its low cost, versatility and easy application [20,21].

The aim of this work was to develop a methodology for the extraction, pre-concentration, separation and identification of nitrosamines in real samples using solid-phase extraction with active carbon and capillary electrophoresis with fused-silica capillary. The effects of buffer composition, instrumental variables, optimization of conditions and characterization of the solid-phase extraction (SPE) with active carbon were investigated. The developed methodology is applicable to the determination of these analytes in different samples such as aqueous synthetic samples and sausages.

The presence of these compounds was confirmed by GC–MS. This technique is used for unequivocal identification of nitrosamines in different matrices and integrates the procedure for analysis of volatile nitrosamines in nitrite-cured meat products utilized by the Food Safety Inspection Service (FSIS) of the US Department of Agriculture; the analytical technique gives us the required selectivity to determine nitrosamines.

2. Experimental

2.1. Reagents

All the reagents used were, at least, of analytical grade. Sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$), and sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$) were purchased from Fluka, γ cyclodextrin (CD) and sodium dodecyl sulfate (SDS), were purchased from Sigma. The standards were purchased from Sigma–Aldrich and were those recommended by the US Environmental Protection Agency (EPA) 8270 method: dimethylnitrosamine (DMN), diethylnitrosamine (DEN), *N*-nitrosopyrrolidine (NPYR), *N*-nitrosomorpholine (NMOR), *N*-nitrosopiperidine (NPIP) plus biphenyl, which was used as internal standard (I.S.). These compounds were maintained at 4 °C in the dark.

A 1000 mg ml^{-1} stock standard solution of each analyte was prepared in methanol and stored at 4 °C. Spiked solutions were prepared daily by diluting the stock solution with bi-distilled water.

The adsorbent used was an active carbon, with 100–400 mesh and surface area of 860 m^2 g^{-1}, purchased from Merck. The pore size distribution revealed a uniform distribution with a mean pore volume of ~0.945 ml g^{-1}. Active carbon was purified by Soxhlet extraction with dichloromethane, during 24 h, and dried in an oven at 105 °C for 8 h.

2.2. Equipment

2.2.1. CE

Experiments were carried out in a Beckman PACE 5500 CE instrument and a fused-silica capillary was used for the separation of the analytes. The system comprised a 0–30-kV voltage built-in power supply, a diode array detector and PACE software for system control data processing. The capillary (50 cm×75 mm I.D.) used was obtained from Beckman Instruments (Fullerton, CA, USA). The temperature was maintained at 20 °C by using a fluorocarbon-based cooling fluid.

2.2.2. GC–MS

A gas chromatograph with mass spectrometer Shimadzu QP 5050A was used for confirmation purposes. A 30 m×0.25 mm fused-silica capillary column HP-1 (0.25-μm film thickness) was used for the GC separation with the following oven temperature program: 35 °C (3-min hold) heating to 70 °C at 5 °C min^{-1} and heating to 200 °C at 15 °C min^{-1} (10-min hold). The interface temperature and injector temperature were maintained at 200 °C. A volume of 1.0 μl was injected in the split mode (1:20) and the detection was done mainly in the selected ion monitoring (SIM) mode (at m/z of 74, 100, 102, 114, 116, 154). In some applications, mainly for confirmation of identity, the full scan spectra were also obtained.

2.3. Sample preparation

The samples used in this work were canned sausages from Brazil (sample 1) and from Spain (sample 2), purchased from the local market.

Extraction was carried out by vacuum steam distillation in a rotary vacuum evaporator (Eyela NE-1). A 150-g sample was mixed with 100 ml of bi-distilled water and left to rest for 10 min. Then the flask containing the mixture was connected to the rotary evaporator and distillation took place. The maximum vacuum of a rotary oil pump was applied and the water bath was heated to 65 °C. The condenser was cooled with water (4 °C) and the distillate was received in a 500-ml flask in an ice bath. These conditions were maintained until no more distillate was produced, ~80 min. The distilled product was kept below 0 °C in the dark. This procedure was adapted from the method described by Telling et al. [15].

Active carbon in powder (100–400 mesh) was utilized for pre-concentration of the aqueous extracts. The variables affecting the SPE, such as adsorbent mass (0.5–1.0 g), adsorption time (15–45 min) and aqueous sample volume (25–200 ml), were studied at different levels of concentration (0.05–0.8 mg l^{-1}). The recovery studies were performed in aqueous synthetic sample, spiked real samples and unspiked real samples.

A 200-ml aliquot of each sample (aqueous distillate of solid sample) was magnetically stirred with 1.0 g of active carbon for 45 min. Then the adsorbent was separated by filtration using a quantitative paper (Whatman, catalog No. 1442125, ashless, model 42).

Liberation of the compounds from the adsorbent was done by elution with 10 ml of acetone, 10 ml of dichloromethane and an additional volume of 10 ml of this last solvent.

The fractions were concentrated under N_2 stream in an ice bath. The volumes were adjusted to 1 ml and received 2 mg l^{-1} of biphenyl (as internal standard) for analysis by GC–MS and adjusted to 5 ml with bi-distilled water for analysis by capillary electrophoresis. All analyzes were done in triplicate at least.

2.4. Analysis of samples

2.4.1. Electrophoretic procedures

Prior to first use, each new capillary was subjected to a wash cycle, according to the following procedure: 1 M HCl solution was passed through the capillary (5 min), followed by 0.1 M NaOH (10 min)

and bi-distilled water (5 min). In order to maintain the capillary in good working condition, its surface was regenerated once a day by consecutive washing with water (5 min) followed by freshly prepared 0.1 *M* NaOH (5 min), water (5 min) and fresh buffer (50 m*M* phosphate–borate and 80 m*M* of SDS) during 20 min. The micellar electrokinetic chromatographic (MEKC) separation was performed in a fused-silica capillary at 20 °C. A voltage of 10 kV was used producing a mean current of 70 mA. Samples were injected using the electrokinetic injection mode at 10 kV for 10 s. Electropherograms were recorded at 236 nm. Separations were carried out from the anode to the cathode. The pH was corrected to 6.6 with 0.1 *M* phosphoric acid.

3. Results and discussion

3.1. Pre-concentration

The agitation time, mass and elution system were optimized to achieve the best efficiency of SPE with active carbon using synthetic sample. Table 1 shows the results in terms of recovery when the maximum values were used for each variable, such as 45 min of adsorption time, 1 g adsorbent, and 200 ml of sample volume to obtain the highest possible recovery.

The low recovery values (~40%) for the compounds NMOR, DMN and NPYR could be explained by the strong interaction with the aqueous matrix. These compounds show high polarity and can remain in the residual water after extraction. The study with the 0.4 mg l^{-1} concentration allowed detection of these compounds in the residual water. In the 0.8 mg l^{-1} concentration all the compounds were detected in the residual water, indicating saturation of the solid phase.

Another source of loss in the procedure can occur during the solvent change and volume reduction. This step is critical because some residues of acetone tend to interfere with the detection of NMOR and DMN in MEKC analysis.

The recovery values of the SPE procedure using solid spiked samples are low due to the difficulty in extraction from the solid matrix in the vacuum steam distillate and also because of the concurrence of nitrosamines and other compounds in the samples.

3.2. Analysis of real samples

3.2.1. Performance of the MEKC method

SDS (40–80 m*M*) was used to improve the separation of the compounds due to the fact that it is important for efficient separation in MEKC. It acts as a pseudo-stationary phase of a chromatographic separation. The use of 80 m*M* of SDS increased the capacity factor inducing a higher interaction of compounds with SDS and the resolution of the peaks was improved. For the development of this methodology a standard mixture of five standard compounds was used (Fig. 1). The optimized variables and the best results are shown in Table 2. Fig. 2 shows the electropherogram after optimization of the variables. As can be seen in Table 2, the addition of

Table 1
Recovery of the nitrosamines for different samples

Compound	Synthetic sample		Synthetic sample		Synthetic sample		Vacuum steam distillation	
	25 ml, 0.8 mg l^{-1}	RSD (%)	25 ml, 0.4 mg l^{-1}	RSD (%)	200 ml, 0.05 mg l^{-1}	RSD (%)	Solid sample, 1.0 mg kg^{-1} [a] 150 g	RSD (%)
DMN	61.5	6.9	44.7	7.9	68.3	5.2	–	
NMOR	44.2	6.9	39.0	10.9	37.6	13.8	42.3	13.8
NPYR	67.7	11.9	37.0	11.5	38.1	13.9	38.2	15.9
DEN	86.9	10.2	78.0	12.7	91.5	12.3	55.0	9.0
NPIP	102.9	7.9	74.8	12.2	86.6	5.3	45.9	7.5

RSD, relative standard deviation
[a] Amount of analytes added to the solid sample (spiked procedure).

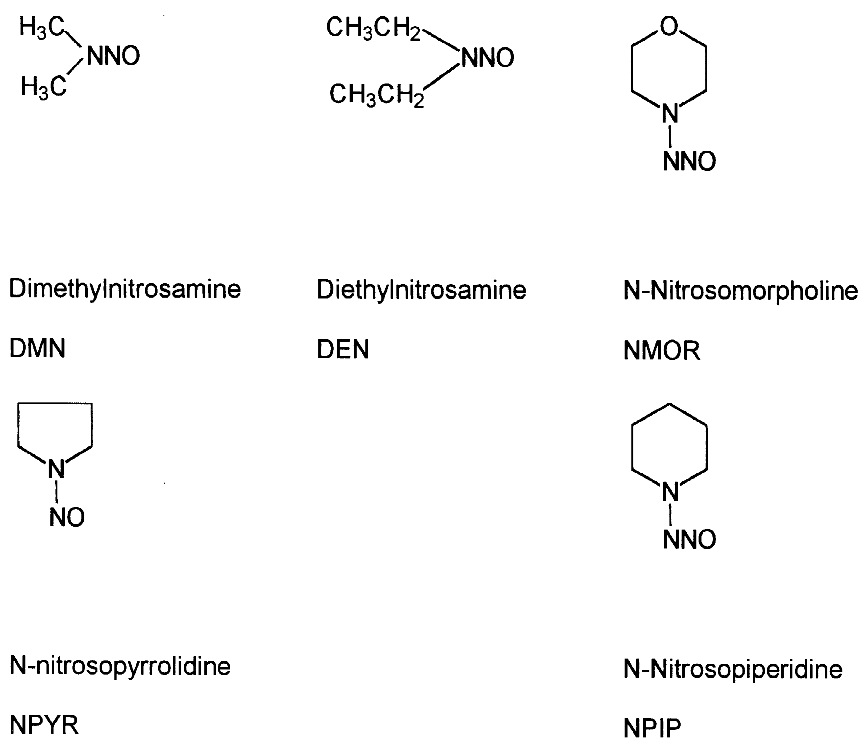

Fig. 1. Structure of the nitrosamines analyzed in this work.

γ-CD does not improve the separation and in the optimization method it was not added.

In order to check the performance of the electrophoretic method, a calibration curve was constructed without any pre-concentration of the sample in a concentration range from 0.50 to 10.00 mg l^{-1}. Each point of the calibration curve corresponds to the mean value obtained from at least three independent area measurements. The limit of detection (LOD) was calculated by using three times the standard deviation of the intercept divided by the slope, while the limit of quantitation (LOQ) was calculated by using ten times the standard deviation of the intercept divided by slope. The corresponding regression equation and other characteristic parameters for the determination of the nitrosamine compounds are shown in Table 3. A total of 11 replicate analysis were performed on synthetic sample at 2.0 mg l^{-1} in order to evaluate the precision of the method for each compound. The results are also summarized in Table 3. The LOD value was between 160 and 240 μg l^{-1} and LOQ value was between 520 and 820 μg l^{-1}.

Furthermore a calibration curve was constructed including the pre-concentration step in active carbon, with concentrations varying from 50 to 500 μg l^{-1}. In this case the calculated LOD was between 34 and 60 μg l^{-1} for 100 ml of sample and 22–35 μg l^{-1}

Table 2
Optimized variables and best results for the development of MEKC method using spiked solution

Variable	Studied	Optimized
SDS	40–80 m*M*	80 m*M*
γ-CD	0–20 m*M*	Without γ-CD
pH	4.0–6.6	6.6
Injection mode	Hydrodynamic electrokinetic	Electrokinetic
Injection time	5–30 s	10 s
Injection voltage	5–20 kV	10 kV
Separation voltage	5–10 kV	10 kV

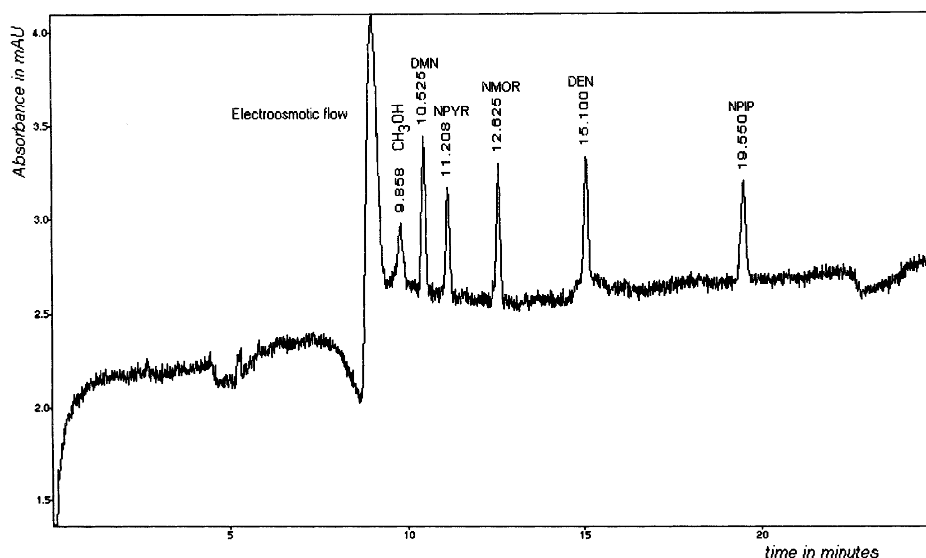

Fig. 2. Electropherogram of a nitrosamine standard mixture (2 mg l^{-1}). Analytical conditions: buffer: 50 m*M* phosphate–borate, pH 6.6, 80 m*M* SDS; injection: electrokinetic,10 kV, 10 s, voltage applied 10 kV.

for 200 ml of sample for all nitrosamines studied. The results are summarized in Table 4. The use of the pre-concentration procedure improved sensibility and detection limit.

3.3. GC–MS

This technique was applied to the Brazilian sausage sample to confirm the presence of the nitrosamines detected by MEKC.

A calibration curve was constructed without any pre-concentration of the sample in the range of 0.5–10 mg l^{-1} (Table 5). The LOD value was between 214 and 846 μg l^{-1} while the LOQ was between 900 and 2821 μg l^{-1}. These high LOD and LOQ values proved that the technique also needs

Table 3
Figures of merit of the electrophoretic method without pre-concentration of samples

Analyte	Equation	r	R^2	$S_{y/x}$	LOD	LOQ	RSD (%)
DMN	$a = -865.1 \pm 236.3$ $b = 4149.5 \pm 76.44$	0.993	99.4	672.6	170	570	4.5
NMOR	$a = -213.9 \pm 217.3$ $b = 2661.0 \pm 45.8$	0.995	99.6	695.6	240	820	5.0
NPYR	$a = 315.7 \pm 231.1$ $b = 3462.1 \pm 48.8$	0.995	99.6	739.7	200	670	4.7
DEN	$a = 195.3 \pm 149.4$ $b = 2871.0 \pm 31.51$	0.994	99.8	478.1	160	520	5.4
NPIP	$a = -684.9 \pm 237.4$ $b = 3332.2 \pm 50.1$	0.997	99.6	759.9	210	710	6.9

a, Intercept; *b*, slope; LOD, limit of detection; LOQ, limit of quantitation (LOD and LOQ are expressed in μg l^{-1}); *r*, correlation coefficient; R^2, curve fitting level (in %) obtained by analysis of variance (ANOVA) for validation of the model; RSD, relative standard deviation; $S_{x/y}$, standard deviation of residual.

Table 4
Figure of merit of electrophoretic method with pre-concentration active carbon powder using 200.0 ml of aqueous sample

Analyte	Equation	r	R^2	$S_{y/x}$	LOD	LOQ	RSD (%)
DMN	$a=1091.0\pm161.1$ $b=17.36\pm0.309$	0.998	99.6	412.8	27.8	92.6	4.0
NMOR	$a=342.0\pm124.4$ $b=10.4\pm0.239$	0.996	99.6	18.76	36.0	120.0	16.6
NPYR	$a=568.5\pm148.6$ $b=19.8\pm0.290$	0.998	99.7	389.9	22.5	75.1	17.4
DEN	$a=-1761.7\pm233.7$ $b=26.75\pm0.445$	0.998	99.6	598	26.1	87.3	22.7
NPIP	$a=-865.8\pm222.4$ $b=28.25\pm0.428$	0.998	99.7	570.0	23.6	78.74	20.7

a, Intercept; *b*, slope; LOD, limit of detection; LOQ, limit of quantitation (LOD and LOQ are expressed in $\mu g\ l^{-1}$); *r*, correlation coefficient; R^2, curve fitting level (in %) obtained by analysis of variance (ANOVA) for validation of the model; RSD, relative standard deviation; $S_{x/y}$, standard deviation of residual.

pre-concentration and has lower sensibility than MEKC: thus GC–MS analysis of the samples (only for confirmation purposes) was carried out using pre-concentration step.

3.4. Analytical applications

To demonstrate the applicability of the proposed method, spiked and unspiked real samples were used for the determination of nitrosamines. The recoveries obtained for the nitrosamines in different samples and the respective extraction procedure are summarized in Table 1. The methodology allowed extraction, pre-concentration and separation of the nitrosamines.

The study with synthetic aqueous samples was carried out by SPE–MEKC using three aqueous samples in distilled water. The concentration and the group of standards were varied . The recoveries obtained for nitrosamines, in the range of 68.8–105.0%, are summarized in Table 6.

Fig. 3 shows the electropherogram of the eluates of acetone from solid sample (A) unspiked and (B) spiked with the standards solution. In this figure it is possible to note the increasing area of some marked peaks, indicating the presence of these compounds in the original sample.

Table 5
Figure of merit of the GC–MS method without pre-concentration

Analyte	Equation	r	R^2	$S_{y/x}$	LOD	LOQ	RSD (%)
DMN	$a=-0.021\pm0.021$ $b=0.098\pm4.8\cdot10^{-3}$	0.994	98.8	0.054	214	900	1.9
NMOR	$a=-0.03\pm0.015.$ $b=0.09\pm3.0\cdot10^{-3}$	0.993	98.95	0.040	500	1667	1.0
NPYR	$a=-0.19\pm0.079$ $b=0.28\pm1.5\cdot10^{-3}$	0.981	99.0	0.204	846	2821	2.6
DEN	$a=-0.034\pm0.035$ $b=0.19\pm6.8\cdot10^{-3}$	0.993	98.9	0.090	552	1842	2.5
NPIP	$a=-0.17\pm0.04$ $b=0.21\pm7.8\cdot10^{-3}$	0.992	98.3	0.103	571	1900	2.9

a, Intercept; *b*, slope; LOD, limit of detection; LOQ, limit of quantitation (LOD and LOQ are expressed in $\mu g\ l^{-1}$); *r*, correlation coefficient; R^2, curve fitting level (in %) obtained by analysis of variance (ANOVA) for validation of the model; RSD, relative standard deviation; $S_{x/y}$, standard deviation of residual.

Table 6
Analysis of aqueous synthetic samples by SPE–MEKC (concentration in mg l^{-1})

Sample	Analyte	Added (mg l^{-1})	Found (mg l^{-1})	Error (%)	Recovery (%)
1	DMN	0.300	0.237	21	79.0
	NMOR	0.050	0.044	12	88.0
	NPYR	0.300	0.277	7.7	92.3
	DEN	1.000	0.988	1.2	98.8
	NPIP	0.800	0.734	9.0	91.0
2	NMOR	0.700	0.617	11.9	88.1
	NPYR	0.900	0.882	3.0	97.0
	DEN	0.200	0.168	6.0	84.0
	NPIP	0.400	0.333	6.8	83.2
3	DMN	0.300	0.200	31.4	68.6
	NMOR	0.500	0.343	31.4	68.6
	NPYR	0.800	0.570	28.8	71.2
	DEN	0.600	0.633	5.0	105
	NPIP	0.300	0.252	16.0	84.0

Fig. 4 shows the electropherogram of the eluate of acetone from solid sample (B) previously spiked with the standard solution at a concentration of 1 ppm before extraction, (C) subsequently spiked with the standards after extraction, and (A) the standard solution at 10 mg l^{-1}, for comparison. Study of the figures shows the ability of steam vacuum distillation to extracted these compounds from real samples and to separate them by MEKC.

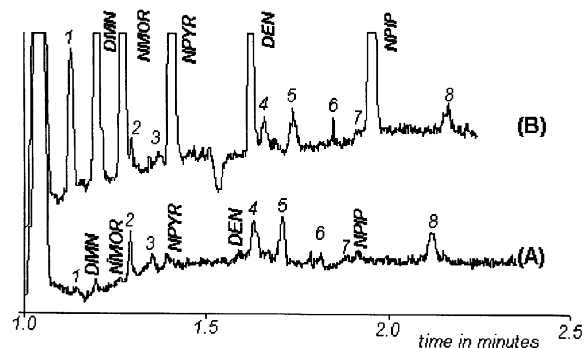

Fig. 3. Electropherogram of acetone eluates of the nitrosamine extracted from 150 g of the sausages unspiked (A) and spiked (B). Analytical conditions: buffer: 50 m*M* phosphate–borate, pH 6.6, 80 m*M* SDS; injection: electrokinetic,10 kV, 10 s, voltage applied 10 kV.

The results suggest the presence of NPIP, NPYR, NMOR and DEN in the samples studied.

Fig. 5 shows the SIH chromatogram of the acetone eluate obtained by SPE after vacuum steam distillation of the sample of sausages. This figure shows, through the study of retention time and mass spectra, the presence of the compounds NPIP, NMOR and NPYR.

The identification of these compounds by the methodology proposed here is very important because it indicates a high concentration in foodstuffs, representing a risk to human health.

4. Conclusions

The optimization of a methodology using SPE with active carbon and vacuum steam distillation to separate nitrosamines from sample of sausage and aqueous samples was achieved. The developed technique is simple and highly suitable for extraction, pre-concentration, separation and determination of nitrosamines at low levels in foodstuffs.

The combination of this extraction procedure with MEKC and GC–MS shows that it is very suitable for the study of nitrosamines in the proposed samples. The method has a short analysis time, high efficiency, suitable sensitivity and is simple to use.

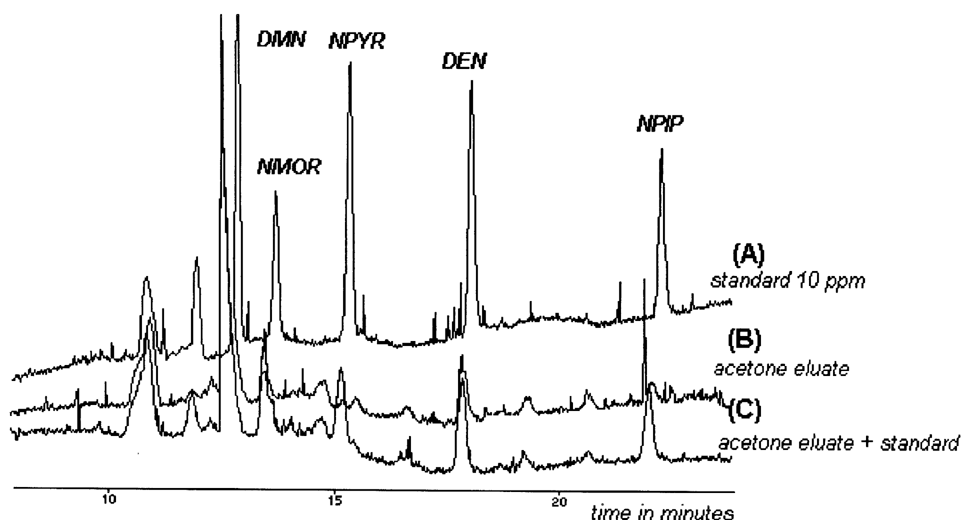

Fig. 4. Electropherogram of (A) a nitrosamine standard solution at 10 mg l^{-1}, (B) the acetone eluates of the extracted nitrosamine from sausages previously spiked, and (C) the acetone eluates of the extracted nitrosamine from sausages subsequently spiked. Analytical conditions: buffer: 50 m*M* phosphate–borate, pH 6.6, 80 m*M* SDS; injection: electrokinetic,10 kV, 10 s, voltage applied 10 kV.

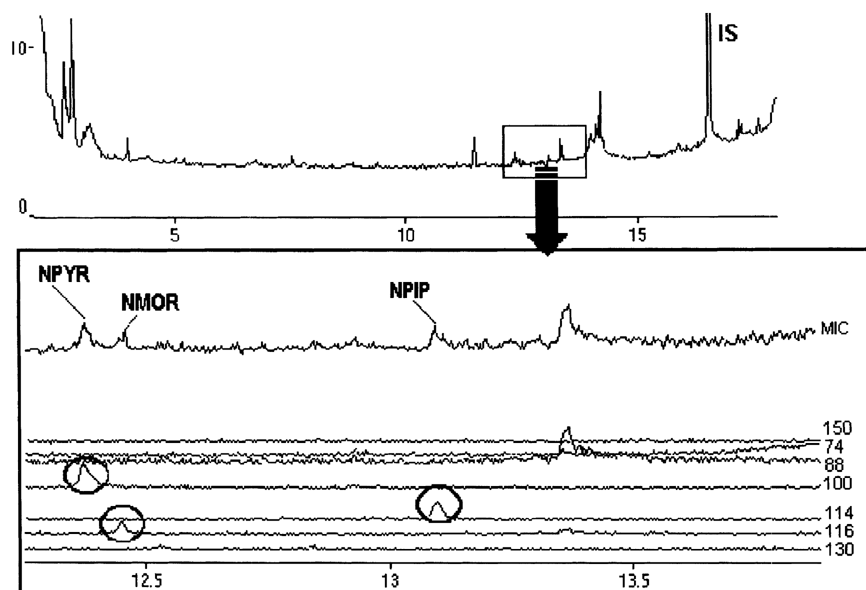

Fig. 5. SIM chromatogram of the acetone eluate from the sausages. Chromatographic conditions: column HP-1 (30 m×0.25 mm, 0.25 μm), temperature program: 35 °C (3-min hold) heating to 70 °C at 5 °C min^{-1} and heating to 200 °C at 15 °C min^{-1} (10-min hold), interface temperature and injector temperature at 200 °C, split 1:20.

Acknowledgements

Financial support provided by DGICyT (Project No. PB95-0977) is gratefully acknowledged. One of the authors (P.J.S.F.) thanks the University of Cordoba Department of Analytical Chemistry and Ecology, the Group of the Investigation in Viticulture and Enology of the Sciences Faculty and CAPES (Brazil) for a scholarship.

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