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A method for the determination of volatile *N*-nitrosamines in food by HS-SPME-GC-TEA

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Abstract

A method for the determination of volatile nitrosamines in sausages was developed using headspace sampling by solid-phase microextraction and gas chromatography with thermal energy analyzer detection (HS-SPME-GC-TEA). Two fused silica fibers, one coated with polydimethylsiloxane-divinylbenzene (PDMS-DVB) and another with poliacrylate (PA) were evaluated. A factorial fractional design was employed in order to evaluate the influence of the equilibrium time, ionic strength, extraction time and temperature for the extraction of *N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitrosopiperidine and *N*-nitrosopyrrolidine in sausages. The method was validated and applied for the determination of nitrosamines in sausages. The described method is simple, rapid, with adequate accuracy, selectivity, sensitivity and precision.

Keywords: Nitrosamines; Cured meat; HS-SPME-GC-TEA

1. Introduction

Nitrosamines are *N*-nitroso compounds formed in certain foods by the reaction of a nitrosating agent derived from either nitrite salts or nitrogen oxide with a substance having an amino group. They can be also formed endogenously in the human body, especially intragastrically. The majority of the nitrosamines tested have been carcinogenic in a variety of animal species. Furthermore, these compounds are toxic, mutagenic, teratogenic and act transplacentally (Craddock, 1990; Hotchkiss, 1987; Mirvish, 1975).

For analytical purposes the nitrosamines are divided into volatile and non volatile compounds. The volatile nitrosamines are a group of relatively nonpolar, low-molecular weight compounds which could be removed from the food matrix by distillation. A number of extraction and clean-up procedures for determining nitrosamines in food have been described, including solvent extraction on a dry celite column (Mitacek et al., 1999; Pensabene & Fiddler, 1988), low-temperature vacuum distillation (Glória, Barbour, & Scanlan, 1997a; Glória, Vale, Vargas, Barbour, & Scanlan, 1997b), supercritical fluid extraction (Fiddler & Pensabene, 1996; Reche, Garrigós, Marín, Cantó, & Jiménez, 2002), and solid-phase extraction (Raoul, Gremaud, Biaudet, & Turesky, 1997). These procedures are time consuming, labour intensive and also require the use of toxic solvents.

Because of US Environmental Protection Agency regulations (U.S. EPA, 1991), there is a strong incentive to reduce or replace the organic solvents, particularly those containing halogens, used in residue analysis. These regulations are designed to reduce the use of solvents that are potentially harmful to the environment and to reduce the costs of solvent disposal. Most current methods require selective separation of analyte from the

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sample matrix by multiple sample preparation treatments, including homogenization, distillation, solvent extraction-partition, concentration, and other cleanup steps. These procedures are time-consuming and labor intensive and may result in some analyte loss.

Solid-phase microextraction (SPME) is a relatively new procedure, first described by Pawliszyn and coworkers in 1990, that can be used to evaluate the volatile compounds present in the vapour and/or in the liquid phase of solid and liquid foods (Zhang & Pawliszyn, 1993). The advantages of the SPME method over other methods of extraction are numerous. SPME can be significantly faster and easier than solvent extraction methods, it is easily automated and it does not require the use of potentially toxic and expensive solvents (Arthur & Pawliszyn, 1990).

This paper describes the development of a simple method using headspace sampling by solid-phase microextraction gas chromatography with thermal energy analyzer detector (HS-SPME-GC-TEA) for the determination of *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosopiperidine (NPIP) and *N*-nitrosopyrrolidine (NPYR) in sausages. Because the SPME efficiency is influenced by several factors such as type of fiber coating, extraction time, ionic strength of the solution and temperature, a fractional factorial design and a central composite design were developed and employed to determine the optimal experimental conditions for the nitrosamine determination in the food sample matrix.

2. Materials and methods

2.1. Chemicals

All the reagents used were, at least, of analytical grade. The standards of *N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitrosopiperidine and *N*-nitrosopyrrolidine were purchased from Sigma–Aldrich and used without further purification.

2.2. SPME fibers

An SPME holder (Supelco, Bellefonte, PA, USA) was used to perform the experiments. Fused silica fibres, coated with polydimethylsiloxane-divinylbenzene (PDMS-DVB, 65 μm) and polyacrylate (PA, 85 μm) coated fibers were evaluated to extract the volatile nitrosamines from the headspace. The fibers were conditioned prior use according to the instructions provided by the suppliers.

2.3. Sample preparation

The pork sausages were purchased from local markets (Campinas, SP, Brazil). The sausages samples were fried at 177 °C for 3 min each side and ground in a Waring blender with dry ice. For all extractions, samples (2.50 g) were placed in 24 mL vials sealed with Teflon–silicone septa.

2.4. Gas chromatography

For the nitrosamine determination an Agilent 6890 gas chromatograph interfaced with a thermal energy analyzer detector (TEA), Model 610 Thermo Orion, operating in the nitrosamine mode was employed. The chromatographic separations were carried out with a HP-INOWAX megabore column (30 m × 530 μm ID × 1 μm film thickness, from Agilent). The CG operating conditions were as follows: injector temperature, 200 °C; oven temperature was held at 100 °C for 3 min, then heated to 140 °C at 40 °C min^{-1} and then to 160 °C at 5 °C min^{-1}. The TEA furnace and the GC-TEA interface temperatures were 550 and 250 °C, respectively. The helium carrier gas flow-rate was 5 mL min^{-1}.

2.5. Standard stock solutions

Standard stock solutions of NDMA, NDEA, NPIR and NPYP were prepared by dilution of appropriate volume of the standards with methanol to a final concentration of 1000 μg L^{-1}. The working solutions (100 μg L^{-1}) were prepared daily by dilution of the standard stock solution with water purified in a Milli-Q system (Millipore).

2.6. Method optimization

2.6.1. HS-SPME – extraction of NDMA, NDEA, NPIR and NPYP in aqueous solution

Aqueous standard solutions (12 mL), containing 30 μg L^{-1} of NDMA, NDEA, NPIP and NPYR prepared in 36 % (m/v) NaCl, were poured into a 24 mL headspace vial. A small stirring bar was introduced and the HS vial was placed in a thermostated water bath. The solution was stirred magnetically and after 10 min the fiber was exposed in the vapor phase above the liquid sample. After the extraction time, the fiber is withdrawn into the needle, which was subsequently introduced into the injection port of the GC. The desorption procedure of the analyte from the fiber coating was performed at 200 °C for 8 min.

2.6.2. HS-SPME – extraction of NDMA, NDEA, NPIR and NPYP in fortified sausages

Samples about 2.5 g of previous ground and homogenized sausage were fortified with 360 ng of each nitrosamine. The fortified samples were added to the HS vial and enough water was added to make-up the volume to 12 mL. The HS-SPME extraction follows the same procedure as described for the aqueous solution.

The equilibrium time, temperature and extraction time were determined by an experimental design.

2.6.3. Design of experiments

A fractional factorial design (2^{4-1}) was carried out to distinguish the significant parameters affecting the SPME procedure. The results of this design were used to plan a subsequent higher order design 2^3 (central composite design), which was performed with the same procedure. All statistical calculations were developed with Statistic (Statsoft Inc., Tulsa – OK, EUA) v. 5.5.

2.7. Sample analyses by the standard addition method

Five portions (2.50 g) of the sausage samples (2.3) were placed in 24 mL vials sealed with Teflon–silicone septa and water was added to a volume of 12 mL. In the first sample a small stirring bar was introduced and the HS vial was placed in a thermostated water bath at 45 °C. The solution was stirred magnetically and after 10 min the PDMS-DVB (65 μm) fiber was exposed to the vapor phase above the sample. After an extraction time of 25 min, the fiber was withdrawn into the needle, which was subsequently introduced into the injection port of the GC. The desorption procedure of the analyte from the fiber coating was performed at 200 °C for 8 min and the chromatographic conditions employed are described above. A second sample, in a HS-vial, was spiked with 0.6 mL of a working standard solution containing 100 $\mu g\,L^{-1}$ of NDMA, NDEA, NPIR and NPYP and treated according to the procedure previous described for the sample. The other three vials were spiked with 2.4; 3.6; 4.8 and 7.2 mL, respectively. The entire procedure was carried out in duplicate.

3. Results and discussion

The initial parameters included in the study of SPME for *N*-nitrosamines in sausages were the selection of the fiber coating, the ionic strength of the sample matrix, the equilibrium time, the sample temperature and the SPME fiber exposure time.

The choice of the fiber coating and the extraction mode depends on the properties of the analyte and the sample matrix. In spite of the volatile character of the nitrosamines under investigation and the complexity of the sample matrix, the HS-SPME extraction mode was chosen. The HS sampling mode is more appropriate for food matrices due to the fact that the fiber will not be in direct contact with the samples and, therefore, could have a longer lifetime. Two types of fiber coatings with different properties were evaluated: a strongly retentive PDMS-DVB fiber and a medium retentive PA fiber. The mixed mode coating fiber increases retention capacity due to the simultaneous effect of adsorption and distribution to the stationary phase (Kataoka, Lord, & Pawliszyn, 2000).

After the extraction time, the fiber was introduced into the injection port of the GC. The desorption procedure of the nitrosamines from the fiber coating was performed at 200 °C for 8 min and a typical chromatogram

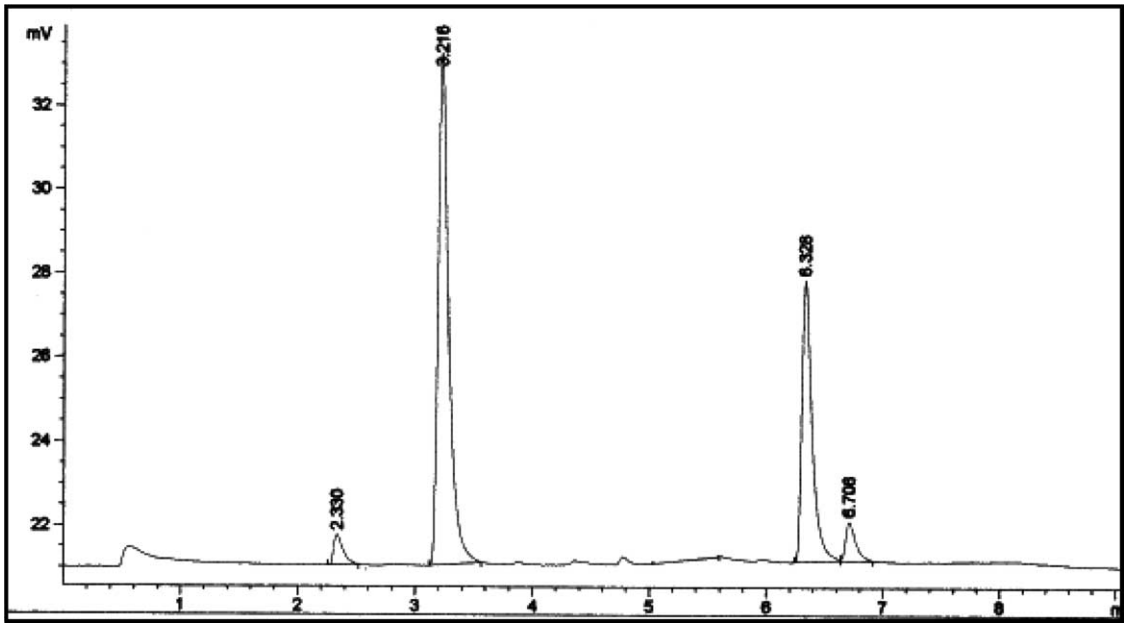

Fig. 1. Characteristic chromatogram (GC-TEA) for the separation of NDMA (t_r 2.33 min), NDEA (t_r 3.21 min), NPIP (t_r 6.33 min) and NPYR (t_r 6.71 min) at a concentration of 30 $\mu g\,mL^{-1}$. Chromatographic conditions: HP-INOWAX megabore column (30 m × 530 μm × 1 μm); Oven temperature: 100 °C (3 min), increased to 140 °C at a rate of 40 °C min^{-1}, increased to 160 °C at a rate of 5 °C/min; Injector temperature: 200 °C. The helium carrier gas flow-rate was 5 mL min^{-1}.

is shown in Fig. 1. The chromatogram and the calculated parameters plate count, tailing factor, resolution and repeatability, show that the GC-TEA system is adequate for the nitrosamine determination in sausages.

3.1. HS-SPME of NDMA, NDEA, NPIP and NPYR in aqueous solution

The performance of the PDMS-DVB and PA coating fibers were compared for the extraction of NDMA, NDEA, NPIP and NPYR in the vapor phase above an aqueous solution containing the nitrosamines. The equilibrium of the nitrosamines in the liquid–vapor phase, at 45 °C, was reached after 10 min. This value was experimentally determined and only after this time was the fiber inserted into the HS. The optimum experimental conditions were established by the evaluation of the following parameters: equilibrium time (t_{eq}), ionic strength (μ), temperature (T) and extraction time (t_{ex}) for both fibers. The optimum recoveries were obtained with the following experimental conditions: PDMS-DVB (t_{eq}: 10 min; μ: 36% (w/v) NaCl; T: 30 °C and t_{ex}: 30 min) and PA (t_{eq}: 10 min; μ: 36% (w/v) NaCl; T: 50 °C and t_{ex}: 20 min).

The PDMS-DVB-coated fiber showed better recoveries for the extraction of NDMA and NDEA in comparison with the PA-coated fiber, which presents higher efficiency for NPIP and NPYR (Fig. 2). The PDMS-DVB fiber has an intermediate polarity and is sensitive to amines (Alpendurada, 2000). The polar PA-coated fiber was more efficient for the extraction of the more polar compounds NPIP and NPYR. Due to the fact that NDMA and NDEA are more commonly found in sausages, and the PDMS-DVB-coated fiber gives higher recoveries for these compounds, this mixed coating fiber was employed for the studies with the sausages samples.

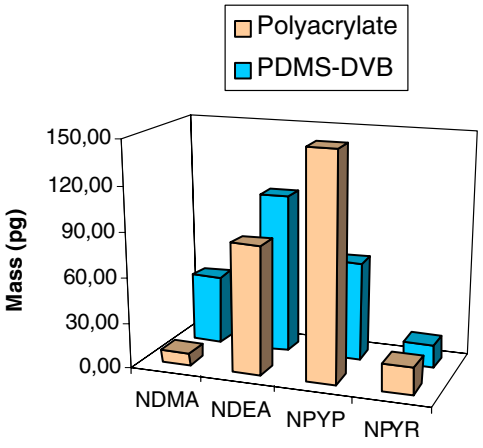

Fig. 2. Comparison of different fiber coating. Conditions: 30 μg mL^{-1} of NDMA, NDEA, NPIP and NPYR. PDMS-DVB (t_{eq}: 10 min; μ: 36% (w/v) NaCl; T: 30 °C and t_{ex}: 30 min) and PA (t_{eq}: 10 min; μ: 36% (w/v) NaCl; T: 50 °C and t_{ex}: 20 min).

3.2. HS-SPME of NDMA, NDEA, NPIP and NPYR in sausages

The sausage ground in the presence of water forms an aqueous dispersion, constituted of non-polar and water immiscible compounds. The lipid-containing matrix influences the extraction efficiency and decreases the recoveries of the *N*-nitrosamines by the PDMS-DVB coating fiber in HS sampling. In a preliminary study it was verified that the optimum experimental conditions established for the HS-SPME extraction of the nitrosamines from water were not the most adequate when the food sample matrix was present.

In order to evaluate the influence of the different factors affecting the HS-SPME process, a factional factorial design was used. Factors such as equilibrium time, presence of salt, extraction time and temperature were included in the design.

3.3. Experimental designs

The low and high values for each parameter were selected according to preliminary experiments and codified to be −1 and +1. With the use of this transformation, every parameter is independent to the measured value. Table 1 lists the values of each factor and their corresponding codified value. The effect of the different variables affecting the SPME of *N*-nitrosamines was studied by a 2^{4-1} fractional factorial with two levels (−1 and +1) for the four factors previously indicated. This design requires eight experiments. With this experiment it was verified that the presence of salt is not significant when this matrix is employed, since the sample already contains salt. A complete factorial 2^3 with center point codified to be 0 was also realized. The result of this study indicated that the equilibrium between the liquid sample and the vapor phase was reached at 5 min. In spite of this, all the further experiments were carried out with a delay of 10 min before the introduction of the fiber into the HS-vial.

Since the salt effect and the equilibrium time between the liquid sample and the vapor phases are not significant, a central composite design was carried out for the other two factors – temperature and extraction time – in order

Table 1
Codification of factors

Levels	Ionic strength (%, w/m, NaCl)	Equilibrium time (min)	Temperature (°C)	Extraction time (min)
−1.41	–	–	24	4
−1	0	5	30	10
0	–	17.5	45	25
+1	36	30	60	40
+1.41	–	–	66	46

to refine the optimum SPME conditions for the extraction of *N*-nitrosamines. This design was constructed by the use of a full 2^2 factorial design with three central points and four axial points (located at an adequate distance from the center of the design). This distance was selected to be equal to −1.41 and +1.41 to assure the rotability condition of the central composite design.

Attending to the mathematical model assumed for this design, the predicted recoveries for each *N*-nitrosamine, resulting in response surfaces for each *N*-nitrosamine are shown in Figs. 3–6.

Headspace sampling was carried out using the PDMS-DVB fiber at different sampling temperatures for different extraction times. For alicyclic and cyclic nitrosamines, two different types of response surfaces were observed. Figs. 4 and 5 indicate that sampling at 45 °C for a period of time above 20 min produced higher recoveries of NDMA and NDEA from the sample

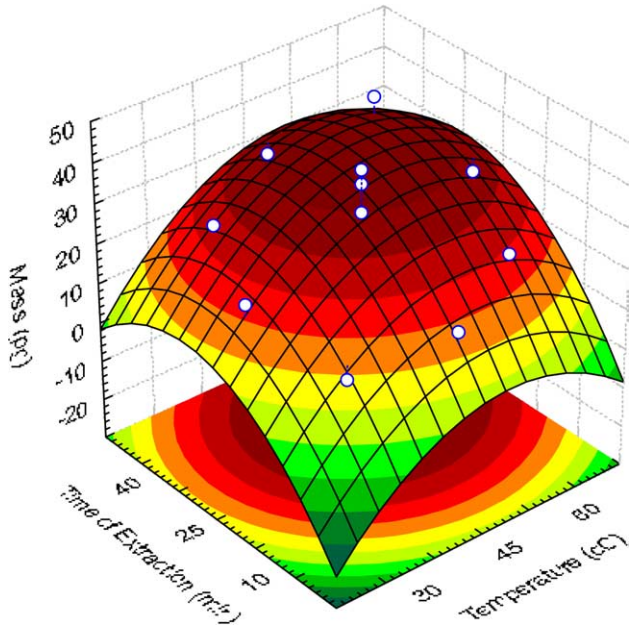

Fig. 3. Response surface for NDMA from the central composite design. Mass of NDMA vs. time of extraction and temperature.

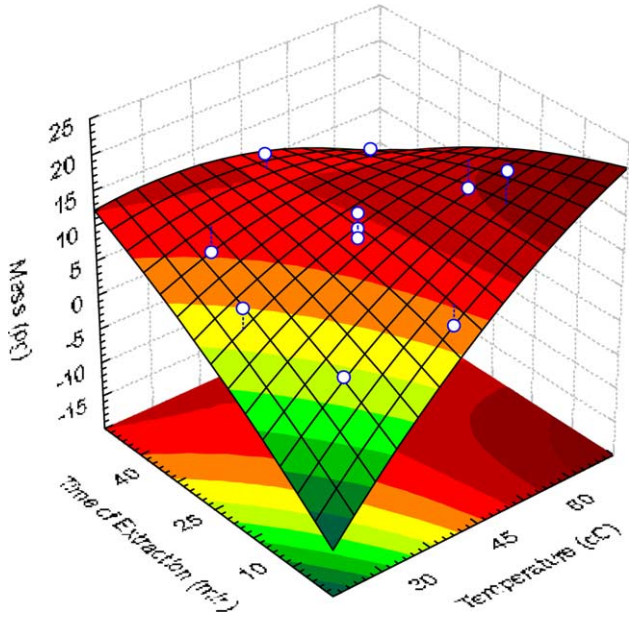

Fig. 5. Response surface for NPIP from the central composite design. Mass of NDMA vs. time of extraction and temperature.

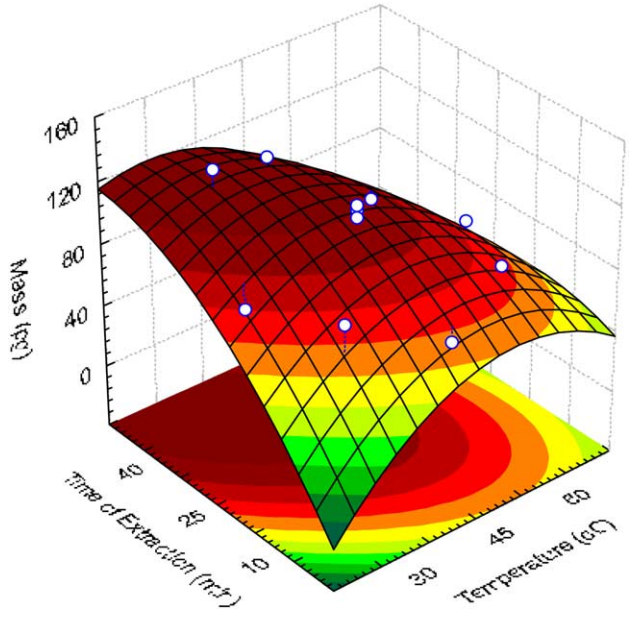

Fig. 4. Response surface for NDEA from the central composite design. Mass of NDMA vs. time of extraction and temperature.

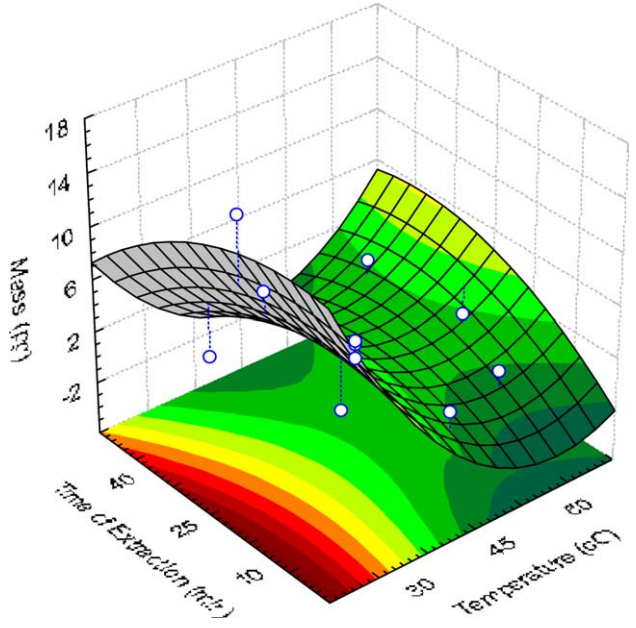

Fig. 6. Response surface for NPIR from the central composite design. Mass of NDMA vs. time of extraction and temperature.

Table 2
Validation parameters

Validation parameter	NDMA	NDEA	NPIP	NPYR
Linear range (ng)	60 a 720	60 a 720	60 a 720	60 a 720
Linearity[a]	0.9935	0.9870	0.9955	0.9944
Sensitivity (u.a. ng^{-1})	1.60×10^{-3}	8.21×10^{-3}	10.7×10^{-3}	2.9×10^{-3}
Repeatability (% RSD, $n = 6$) 360 ng	5	5	6	12
Recovery (%)[b]	105	110	105	107
Limit of detection ($\mu g\ Kg^{-1}$)	3	3	3	3
Limit of quantification ($\mu g\ kg^{-1}$)[c]	10	10	10	10

[a] Linearity is expressed by the correlation coefficient of the calibration graph.
[b] The recovery was calculated through the standard addition method.
[c] Sausage sample weight = 2.500 g.

matrix. However, NPIP and NPYR presented a different behavior; the optimum extraction time was greater than 10 min for both nitrosamines with than temperatures above 50 °C or lower than 30 °C, for NPIP and NPYR, resulting in higher recoveries, respectively (Figs. 5 and 6). Considering that in sausages the main nitrosamines formed are NDMA and NDEA, the optimum conditions for the HS-SPME extraction are: (t_{eq}: 10 min; μ: not adjusted; T: 45 °C and t_{ex}: 25 min).

The extraction efficiency for the 65 μm PDMS-DVB coating fiber, calculated in relation to the aqueous solution, was 0.008%, 0.32%, 0.13% and 0.01% for NDMA, NDEA, NPIP and NPYR, respectively.

3.4. Validation

With the established experimental conditions for the HS-SPME extraction, the method was *in-house* validated for the nitrosamines determination in sausages using the following performance criteria: linearity and range, sensitivity, selectivity, precision (repeatability), limit of detection (LOD), limit of quantitation (LOQ) and accuracy. The results are presented in Table 2. The linearity, range and sensitivity were obtained from a graph using the standard addition method with four concentration levels, with duplicate analysis.

The approach employed to evaluate the accuracy of the method was based on the recovery of known amounts of each nitrosamine spiked into sausage samples in four levels, using duplicate analyses. The amount of the standard analyte added was between two and five times the estimated amount of the analyte in the sample.

The LOD and LOQ were determined at a signal-to-noise ratio of 3 and 10, respectively, measured at the approximate retention time of the corresponding analyte peak. These values were validated by the analyses of fortified samples of sausages at this level.

3.5. Sample analyses

Due the fact that (i) the sausages samples are complex food matrices that contain water, carbohydrates, proteinaceous material and fat, among other constituents, (ii) samples from different producers are different in composition, and (iii) the extraction efficiency of HS-SPME is strongly influenced by the matrices, the determination of the volatile nitrosamines in sausage samples was carried out by the standard addition method. In both samples only NDMA was identified. The results obtained for NDMA ($P < 0.05$) were $43.5 \pm 6.5\ \mu g\ kg^{-1}$ ($n = 3$) and $15.0 \pm 2.2\ \mu g\ kg^{-1}$ ($n = 3$).

It must be mentioned that an enforcement level of 10 $\mu g\ kg^{-1}$ (ppb) has been established for the presence of nitrosamines in pumped bacon. Also, in order to determine that no confirmable levels of nitrosamines are present in a sample tested, the testing must be performed by methodology and procedures that would detect the presence of any nitrosamines at 10 ppb (USDA, 2004). In addition, an action level of 10 $\mu g\ kg^{-1}$ is currently being observed for NDMA in meat products (Canada, 2004). Therefore, the level of NDMA determined in the sausages analyzed (greater than 10 $\mu g\ kg^{-1}$) is considered unacceptable contamination and the product is susceptible to having legal action taken against it.

4. Conclusions

Solid-phase microextraction with headspace sampling has demonstrated to be an advantageous extraction technique for *N*-nitrosamine determination by GC-TEA in cured meat. The method is simple, permits fast analysis, without the use of solvents, little manipulation, is free from matrix interferences and presents adequate sensitivity to attend the recommendations of current food legislation.

On the other hand, factorial designs can be considered as an effective approach to study the influence of the parameters affecting SPME, and they permit the acquisition of more robust results with a reduced number of experiments when compared to the classical one at a time approach.

It is recommended that action levels or tolerances be established in the Brazilian food laws and that a study of

the presence of nitrosamines in sausages be conducted in order to avoid the presence of these deleterious substances.

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References

Alpendurada, M. F. (2000). Solid-phase microextraction: a promising technique for sample preparation in environmental analysis. *Journal of Chromatography A, 889*, 3–14.

Arthur, C. L., & Pawliszyn, J. (1990). Solid-phase microextraction with thermal-desorption using fused-silica optical fibers. *Analytical Chemistry, 62*, 2145–2148.

Canada (2004). Canadian Food Inspection Agency. Animal products. Food of animal origin. Meat hygiene manual of procedures. <http://www.inspection.gc.ca/english/anima/meavia/mmopmmhv/chap5/5.4-7e.shtml> (02/02/2004).

Craddock, V. M. (1990). Nitrosamines, food and cancer: assessment in Lyon. Meeting report. *Food and Chemical and Toxicology, 28*, 63–65.

Fiddler, W., & Pensabene, J. W. (1996). Supercritical fluid extraction of volatile *N*-nitrosamines in fried bacon and its drippings method comparison. *Journal of the Association Official Analytical Chemists International, 79*, 895–901.

Glória, M. B. A., Barbour, J. F., & Scanlan, R. A. (1997a). Volatile nitrosamines in fried bacon. *Journal of Agriculture and Food Chemistry, 45*, 1816–1818.

Glória, M. B. A., Vale, S. R., Vargas, O. L., Barbour, J. F., & Scanlan, R. A. (1997b). Influence of nitrate levels added to cheesemilk on nitrate, nitrite, and volatile nitrosamine contents in Gruyere cheese. *Journal of Agriculture and Food Chemistry, 45*, 3577–3579.

Hotchkiss, J. H. (1987). A review of current literature on *N*-nitroso compounds in foods. *Advances in Food Research, 31*, 54–115.

Kataoka, H., Lord, H. L., & Pawliszyn, J. (2000). Applications of solid-phase microextraction in food analysis. *Journal of Chromatography A, 880*, 35–62.

Mirvish, S. S. (1975). Formation of nitroso compounds: chemistry, kinetics, and in vivo occurrence. *Toxicology and Applied Pharmacology, 31*, 325–351.

Mitacek, E. J., Brunnemann, K. D., Suttajit, M., Martin, N., Limsila, T., Ohshima, H., & Caplan, L. S. (1999). Exposure to *N*-nitroso compounds in a population of high liver cancer regions in Thailand: volatile nitrosamine (VNA) levels in Thai food. *Food and Chemical Toxicology, 37*, 297–305.

Pensabene, J. W., & Fiddler, W. (1988). Determination of volatile *N*-nitrosamines in Frankfurters containing minced fish and surimi. *Journal of the Association Official Analytical Chemists, 71*, 839–843.

Raoul, S., Gremaud, E., Biaudet, H., & Turesky, R. J. (1997). Rapid solid-phase extraction method for the detection of volatile nitrosamines in food. *Journal of Agricultural and Food Chemistry, 45*, 4706–4713.

Reche, F., Garrigós, M. C., Marín, M. L., Cantó, A., & Jiménez, A. (2002). Optimization of parameters for the supercritical fluid extraction in the determination of nitrosamines in rubbers. *Journal of Chromatography A, 963*, 419–426.

USDA. (2004). US Code of Federal Regulations. Food Safety and Inspection Service, USDA § 424.22. Certain other permitted uses. 9 CFR Ch. III (1-1-03 Edition). Available from: <http://www.usda.gov> (02/02/2004).

U.S. EPA Pollution Prevention Strategy. (1991). *Federal Register, 56*: 7849.

Zhang, Z., & Pawliszyn, J. (1993). Headspace solid phase microextraction. *Analytical Chemistry, 65*, 1843–1852.