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Research Article

Determination of Nitrosamines in Sausages by Solid Phase Extraction with Activated Carbon – GC/MS

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Abstract

This study shows a method for the detection and quantification of six volatile nitrosamines, dimethyl nitrosamine, diethylNitrosamine, N-nitrosopyrrolidine, N-nitrosopiperidine, N-nitrosomorpholine, N-nitrosoibuthylNitrosamine in meat products. The procedure based on: (a) Isolation of the compounds by vacuum steam distillation, (b) extraction in solid phase - SPE - from aqueous distillates using activated carbon and (c) analysis by gas chromatography-mass spectrometry. The recovery of the compounds from fortified samples (sausages and their preserved liquid) with 200 µg.Kg^{-1} ranged between 10.9 - 61.7% to solid sample and 20.9 - 80.4% to liquid sample. The direct application of this method to samples of canned sausages allowed the separation, identification and quantification of the nitrosamines at the µg.Kg-1 level with detection limit varying between 0.2 to 1.0 µg.Kg^{-1}.

Introduction

Nitrite and nitrate are normally added to meat products as canned sausages, ham and salam is aiming at preventing the formation of toxins from *Clostridiun Botulinum* [1,2]. Nitrite is also responsible for the development of the cured flavour and the desirable red coloration which are characteristic of these products [1-4]. The undesirable reaction between nitrite and amine or amino derivatives, like chloramines [5-7] can produce nitrosamines. The nitrosamines can be found in different environments, such as water [8-11], rubber products [12], cosmetics [13,1], fish [14], Beers [15-17] and meat products [18-22].

Nitrosamines have been receiving considerable attention as they are highly toxic [23]. Approximately 80% of the known nitrosamines have caused cancer in laboratory animals. These compounds are amines with specific functional group formed by a central nitrogen bonded to another nitrogen and an oxygen (N-NO) [1,3]. The alkyl nitrosamines are carcinogenic and mutagenic, being activated by oxidation and subsequent generation of carbocations, which can promote the alkylation of the DNA [1,23]. The levels of nitrosamines in food it has been calculated that the tolerable level of the most volatile nitrosamines for humans should range from 5 to 10 μg.Kg^{-1} of body weight. The minimum detection limit of 10 μg.kg^{-1} has been normally accepted for meat products [24,15].

The analysis done by the Thermal Energy Analyzer detector (TEA), it being recognized as specific for nitrosamines, it is based on the chemiluminescence generated by the decay of the NO_2 group when they are electronically excited [1,13,25]. These methods require long cleaning procedures to remove interferences, what increase the chance of analytical error [1,14]. This system also demands for the unequivocal detection of the chromatographic peaks, and the use of the mass spectroscopy [1,26,27].

The determination of the nitrosamines in foods it is very complex and may not be achieved directly without a pre-concentration step and/or sample clean up. Therefore, the combination of vacuum distillation with solid phase extraction - SPE [8,7] solid phase micro extraction-SPME [16,28] and liquid extraction [2,26] have been used in the process of the sample preparation. Activated carbon has been chosen for extraction and pre concentration of the analytes from aqueous extracts because it is cheap, versatile and easy to manipulate [21,29,30].

This study aims to develop the extraction, identification and quantification of nitrosamines in canned sausages using methodologies easy to implement, combined with activated carbon SPE and more sensitive and selective equipment such as GC / MS.

Materials and Methods

The analytical reagents were purchased from Fluka and Sigma. The standards as recommended by the EPA 8270 method were purchased from Sigma Aldrich and kept at 4°C in

the dark. The nitrosamines used in this work were the following: Dimethylnitrosamine (DMN), Diethylnitrosamine (DEN), N-nitrosopiperidine (NPIP), Dibuthylnitrosamine (DBN) and biphenyl (internal standard-IS), all beingn-Nitrosopyrrolidine (NPYR), N-Nitrosomorpholine (NMOR), supplied by Sigma.

The canned sausages used in this study were bought in local markets. After opening the cans, the sausages were separated from the liquid and both parts were analyzed in the same way.

A 1000 μg.mL^{-1} stock standard solution of each analyte was prepared in methanol and stored in the cooler without contact with the light. Working standard daily prepared by diluting the stock solution with ultrapure water (mili Q System millipore, bedfofd, MA). The granular activated carbon 8-20 mesh, untreated and granulated was purified by soxhlet extraction with Dichloromethane (DCM) for 24 hours and dried in an oven at 105°C.

A Gas Chromatography (GC/MS) instrument with Mass Spectrometer Detector (Shimadzu QP5050A) was used for both confirmation and quantification purpose. A 30 m X 0.25 mm x 0.25 film thickness fused silica capillary column (sigma-aldrich) and HP-1 (0.25 μm film thickness) and a 30 m X 0.25 mm fused silica capillary column Carbowax 20M (0.25 μm film thickness) were used for the GC separation. The temperature of the oven was programmed as follows: 35°C (3min hold) with heating up to 70°C at 5°C min^{-1} and 6 heating up to 200°C at 15°C min^{-1} (10 min hold). The interface and injector temperature were of 200°C. The volume of 1.0μl was injected in split 1:20. The detection was done mainly in the Selected Ion Monitoring (SIM) mode (at m/z 74 u to DMN, m/z 102 u to DEN, m/z 116 u to NMOR, m/z 114 u to NPIP, m/z 100 u to NPYR, m/z 158 u to DBN, m/z 154 u to Biphenyl). In some applications, the full scan spectra were also obtained.

Calibration curve was constructed without any pre-concentration of the sample which ranged between 0.5 and 10 mg.L^{-1}. The Limit of Detection (LOD) was calculated by using 3-times the standard deviation of the linear coefficient divided by the slope, whereas Limit of Quantification (LOQ) was calculated by using 10-times its standard deviation of the linear coefficient divided by the slope. Eleven replicate analyses were performed on a synthetic sample 20.0 mg.L^{-1}, in order to evaluate the precision of the method for each of the compounds analyzed.

The extraction of the nitrosamines was carried out by vacuum steam distillation. This procedure was adapted from the method originally described by Telling [31] that was used modified later by Sanches [30]. It was weighed 150 g of the sample (solid or liquid) and mixed with 100 ml of double distilled water, 30 g of sodium chloride, 10 g of potassium carbonate and leaving macerate for 10 min. After this time the flask containing the mixture was connected to the rotary evaporator and the distillation took place. Maximum vacuum of a rotary oil pump was applied and the water bath heated to 65°C. The condenser was cooled with water (4°C) and the distilled substance was received in a flask of 500 ml in ice bath, the whole procedure lasted for approximately 60-90 min. The distilled product was kept at 0°C in the dark.

In the Pre concentration – SPE The aqueous distillate of each sample passed through the 1.0 g of granular activated carbon at 2.5 ml.min^{-1} in a column of 11.0cm x 1.1cm. The 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 DCM. The optimization of this methodology was presented in previous works [21].

Results and Discussion

The fractions were concentrated under stream nitrogen in ice bath. The volumes were adjusted to 1 mL and 2 mg.L^{-1} of biphenyl was added as an internal standard. All the determinations were made at least in triplicate. The levels of recovery of N-nitrosamines studied were obtained by extracting the sample fortified with 30 μg of, DMN, DEN, NPYR NPIP, NMOR, DBN, before the vacuum steam distillation. The extracts were analyzed by GC/MS using SIM mode and the percentage of nitrosamines recovered was calculated [14].

Quantitative determinations were done in the extracts obtained from both kinds of samples. The average recoveries and relative standard deviations (%RSD) of nitrosamines from solid sausages were 38.4%±23.3% DMN; 47.3%±16.4 DEN; 10.9%±34.8 NPIR; 15.2%±13.9 NMOR; 38.6%±12.5 NPIP e 61.7%±15.2 DBN, and from preserved liquid WERE 51.3% ±15.4 % DMN; 63.4%±12.8 DEN; 20.9%±20.3 NPIR; 40.7%±17.2 NMOR; 45.2%±21.0 NPIP e 80.4%±8.9 DBN. These results of recovery showed the difficulty in extracting the compounds of the solid matrix when the vacuum steam distillation method was used. When the sausages are grounded in the presence of water, it forms an aqueous dispersion that is constituted by non-polar and water immiscible compounds. The lipid-containing matrix influences the efficacy of the extraction and decreases the recovery of nitrosamines [20]. Low recovery values are common for both SPE and LLE. Nitrosamines are usually polar compounds, soluble in water with a low partition coefficient in Octanol/water and therefore, difficult to extract with organic solvents. The compounds are not adsorbed on non-polar surfaces [7]. One other factor that may contribute to the low levels of recuperation is the competition between nitrosamines and other compounds present in the distilled samples during the pre concentration process with activated carbon.

Calibration curves were used to calculate the result for the real sample and the results were corrected by a factor based on the recovery obtained by the standard addition method. The curves showed linear correlation coefficients between 0,999 a 0.990 with an accuracy ranging from 0.8% (NPYIR) to 2.9% (DEN, NPIP). The detection limit for each nitrosamine was: 642 μg.l^{-1} DMN; 550 μg.l^{-1} DEN; 840 μg.l^{-1} NPIR; 500 μg.l^{-1} NMOR; 570 μg.l^{-1} NPIP and 165 μg.l^{-1} DBN and the quantitation limit was 1706 μg.l^{-1} DMN; 1880 μg.l^{-1} DEN; 1840 μg.l^{-1} NPIR; 1670 μg.l^{-1} NMOR; 1900 μg.l^{-1} NPIP e 540 μg.l^{-1} DBN, limits considered high when related to the requirement of the legislation that is 10 μg.Kg^{-1}.

Analysis of the Real Sample

The Figure 1 presents the order of elution of the standard from two columns, HP1 and Carbowax 20M. The change in the elution order is caused by the difference in the polarity of the stationary phase. The same comportment can be observed in Figure 2 in the analysis of the real sample.

The combination of SPE with GC/MS procedure improves the sensibility and the selectivity of the chromatographic analysis. The detection limit of all the procedures, extraction, pre concentration and the analysis by GC/MS was calculated considering the signal

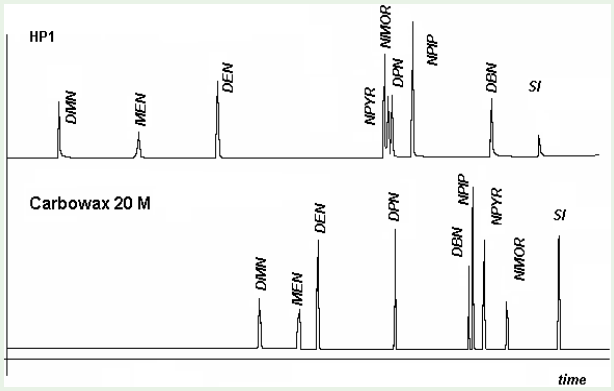

Figure 1: Chromatogram GC/MS to standard mix 10mg.L^{-1} in two chromatographic columns. DMN: Dimethylnitrosamine. DEN: diethylnitrosamine. NPIP: N-nitrosopiperidine, DBN: dibuthylnitrosamine. NPYR: n-nitrosopyrrolidine. NMOR: N-nitrosomorpholine.

of the blank (Ratio between the area from blank and the area of the internal standard), adding the standard deviation value multiplied by three, and LOQ adding ten times the value of standard deviation to the blank value.

Table 1 presents all the values ranging between 0.2 to 1.0 μg.Kg^{-1}, to LOD and 0.6 to 2.4 to LOQ, which are lower than the values determined by Ozel *et al* [18] and Andrade *et al* [20].

Analytical Applications

To demonstrate the applicability of the proposed method, we used it for the determination of nitrosamines in spiked and not spiked samples. The analysis with GC/MS of the eluates along with the study of retention time and mass spectrum, confirmed the presence of DMN, DEN, NPIP, NMOR, and NPIR. DMN, DEN and NPIR are normally originated from amino acids compounds. NPIR is a compound mostly found in products treated with nitrites that contain protein. Nitrite concentration and levels of hydroxyproline, proline and pyrolidine are involved in its formation [32], depending on the cooking temperature. The presence of these compounds is in agreement with the studies of Campillo [25].

NPIP originate from piperine, present in some pepper used as spices, but according to Drabik-Markiewicz [33] their formation is directly related to the presence of biogenic amines, such as cadaverine for exemplo, associated with high temperatures and high levels of nitrite in the manufacturing process.

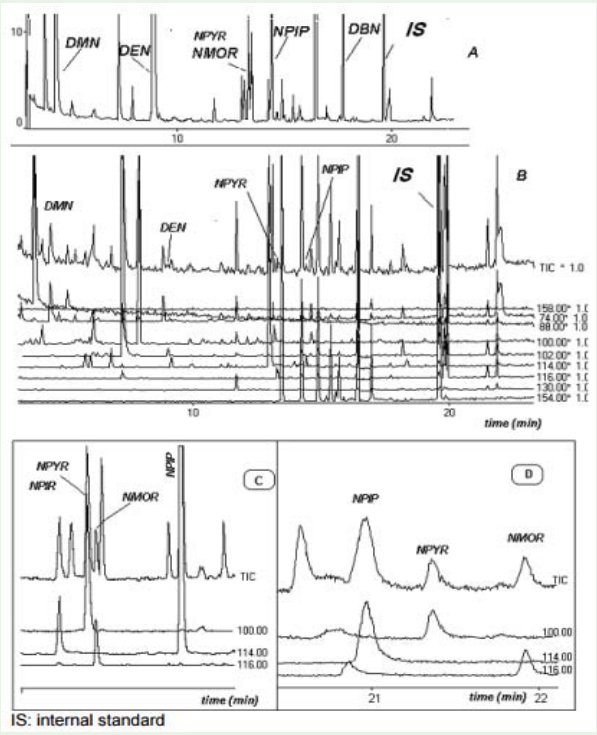

Figure 2: Chromatogram GC/MS (A) Eluate acetone spiked sausage sample (B) Eluate acetone sausage sample (without spiked) (c) Zoom of the chromatogram to eluate acetone spiked sausage sample- column HP1. (d) Zoom of the chromatogram to eluate acetone without spiked sausage sample- column Carbowax 20M. DMN: Dimethylnitrosamine. DEN: diethylnitrosamine. NPIP: N-nitrosopiperidine, DBN: dibuthylnitrosamine. NPYR: n-nitrosopyrrolidine. NMOR: N-nitrosomorpholine.

N-Nitrosomorpholine (NMOR) has been reported to occur sporadically in foods [34] and these authors have speculated that the use of morpholine as a corrosion inhibitor in boiler feed waters has led to the occurrence of NMOR in food. Figure 1 shows a chromatogram (A) of the eluates acetone, from the spiked solid sample. The figure also shows a chromatogram (B) of the eluates of acetone, from not spiked solid sample. This image shows the increase in the area to some peak indicated with standard name. The level of the compounds in the preserved liquid of the sausages was higher than the one of the solid sample.

Table 1: Levels of nitrosamines sample of sausages and preserved liquid by GC/MS.

Analyte	Preserved liquid			Sausages			LOD[b]	LOQ[c]
	*m*g.Kg^{-1} ± %RSD	mol.Kg^{-1}	*m*g.Kg^{-1} In DMN	*m*g.Kg^{-1} ± %RSD	*m*g.mol Kg^{-1}	*m*g.Kg^{-1} In DMN	*m*g.Kg^{-1}	*m*g.Kg^{-1}
DMN	1.6±5.0	0.02	1.6	3.9±9.5	0.05	3.9	0.4	1.5
DEN	<LOD	nc[a]	nc	2.0±6.0	0.02	1.5	0.2	0.9
NPIR	81.1±2.1	0.81	59.0	9.8±12.0	0.06	4.6	1.0	2.4
NMOR	7.0±2.8	0. 06	4,4	1.1±15.9	0.01	0.7	0.3	1.1
NPIP	32.6±2.2	0.29	21.2	5.2±11.1	0.05	3.4	0.3	0.8
DBN	<LOD	nc	nc	<LOD	Nc	nc	0.2	0.6
total	126.8	1.18	86.2	22.01	0.20	14.0		

[a]not calculed, values <LOD; [b]Limit of detection; [c]Limit of Quantification.

The main compound is NPIR, for both solid and liquid samples, being the levels higher than the ones permitted by the legislation for liquid samples. It indicates the great toxicity of the preserved liquid of canned sausages. Figure 2 shows a GC/MS chromatogram in SIM mode of the eluate of acetone from the preserved liquid of the sausages.

Considering that each of the Nitrosamine studied shows one NNO functional group and that the molar ratio of each compound like DMN is 1:1, we converted all the analytes using their molecular masses for DMN. It is observed that the preserved liquid spans more than 6 times the recommended limit and sausage also shows values above the recommended.

Conclusion

The optimization of a methodology to determinate nitrosamines from sausages samples and aqueous sample was investigated. The method developed was simple and suitable for the extraction (by vacuum steam distillation), pre concentration (Through the SPE columns with granular activated carbon), and determination of nitrosamines (by GC/MS) in real samples. The method has limits of detection and quantification lower than10 $\mu g.Kg^{-1}$, using simple and inexpensive equipment and reagents for the preparation of the sample. The use of GC/MS in SIM mode allowed the clean-up (electronic) of the extracts, ensuring adequate levels of selectivity and sensitivity to the nitrosamine concentrations found in samples of meat products. The samples presented concentration levels above the limits recommended by the control agencies, representing a risk to human health.

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