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Method of Determination of Nitrosamines in Sausages by CO_2 Supercritical Fluid Extraction (SFE) and Micellar Electrokinetic Chromatography (MEKC)

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In this paper, the use of supercritical fluid extraction (SFE) and micellar electrokinetic capillary chromatography (MEKC) is proposed for the complete analysis of volatile nitrosamines in sausages. The extraction fluid used was CO_2 and variables such as density, temperature of thimbles, extraction time, modifier, fluid flow, and kind of traps were investigated. Several experiments were carried out to obtain the most favorable conditions for analysis of volatile nitrosamines in sausages. The recoveries ranged from 21 to 82% for the five nitrosamines studied. The optimal condition of extraction was 0.2 g of sample fortified with 10 mg/kg, using dynamic extraction during 20 min and with adsorbent Florisil in the trap. The solvent selected for the elution of the analytes was methanol.

KEYWORDS: Supercritical fluid extraction; micellar electrokinetic capillary chromatography (MEKC); nitrosamines; sausages

1. INTRODUCTION

The nitrosamines are found in the environment and in several foods (*1*−*6*) and are produced by nitrosation of secondary amines. Their formation is influenced by several factors including addition of nitrites or nitrates as preservatives (*7*−*9*). They have received considerable attention in function of their high toxicity. Approximately 80% of the known nitrosamines produced cancer in laboratory animals (*2*, *7*). These compounds are amines with two organic groups (R) and one NO group linked to the central nitrogen (*2*). The alkyl nitrosamines are carcinogenic and mutagenic, being activated by oxidation and subsequent generation of potential carbocations, which can promote the DNA alkylations (*10*−*15*).

Two isolation methods are currently used in the Food Safety Inspection Service Monitoring Program (FSIS) for analyzing nitrosamines in tissues; both have a disadvantage of the length of time required to perform the analyses and require, also, an extensive use of equipment and solvents (*16*).

The tendency to minimize the use of solvents and the exposure of the personnel of the laboratory to this family of compounds requires new extraction technologies (*7*, *15*, *17*).

One of these techniques is the supercritical fluid extraction (SFE). This extractive technique has had only limited application to nitrosamines, mainly those specific in tabacco products, where low extraction efficiencies were found (*18*−*20*). The SFE minimizes sample handling, provides fairly clean extracts, expedites sample preparation, and reduces the use of environmental toxic solvents (*16*−*23*).

The determination of nitrosamines has been usually carried out by gas chromatography (GC) using thermal energy analysis (TEA) (*21*, *22*). The nitrogen phosphorus detector (NPD) and the mass spectrometry (MS) have also been used (*23*).

Micellar electrokinetic capillary chromatography (MEKC) is a highly efficient, rapid, and flexible analytical separation technique which has become a popular separation technique over the past decade. Because of its high efficiency, it has been employed in many applications such as analysis of proteins, pharmaceuticals, and environmental pollutants (*24*−*26*). There is a sizable quantity of MEKC separation methods for a wide variety of analytes. In the hydrophilic separation of low molecular weight neutral and polar compounds as nitrosamines, it is necessary to develop the MEKC technique to enhance selectivity. The main reason is that these compounds do not interact strongly with commonly used surfactants (e.g., sodium dodecyl sulfate or other buffer modifiers as cyclodextrins in electrokinetic chromatography) (*24*−*30*).

The present paper shows the results for the rapid and efficient extraction of five volatile nitrosamines usually found in sausage samples, using SFE with carbon dioxide.

2. EXPERIMENTAL PROCEDURES

2.1. Reagents and Standards. Diatomaceous earth (acid-washed, approximately 95% SiO_2), sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$), sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$), and sodium dodecil sulfate (SDS) were purchased from Sigma Aldrich. The standards were also

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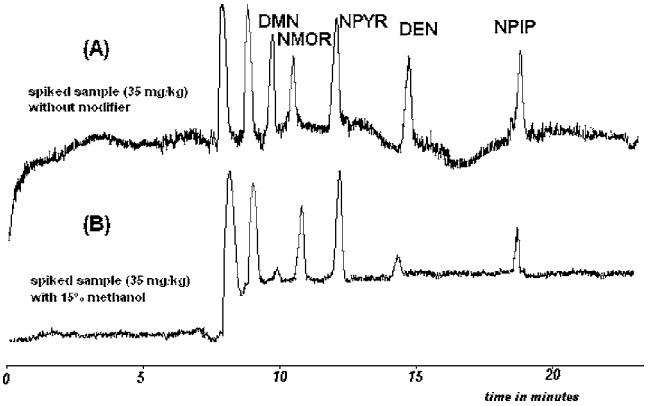

Figure 1. Comparative electropherograms for SFE extraction of spiked samples of sausages, using CO_2-SFE with and without modifier. Conditions are explained in the text.

Table 1. Effect of the Flow Rate of CO_2 in the Recovery of Nitrosamines from Sausage, Using SFE and MEKC (0.2 g of Sample Spiked with 10 mg/kg of Each Nitrosamine)

compounds	2 mL min^{-1}	RSD (%)[a]	3 mL min^{-1}	RSD (%)
DMN	2495.0	7.7	3539.0	8.2
NMOR	1456.0	11.9	1760.0	12.7
NPYR	2576.0	10.8	4677.0	11.9
DEN	2407.7	9.8	3580.7	9.4
NPIP	2829.0	8.5	4950.7	7.6

[a] RSD% = relative standard deviation for at least three repetitions for each sample.

Table 2. Effect of the Dynamic Extraction Time in the Recovery of Nitrosamines from Sausage, Using the Same Conditions as **Table 1**

	peak areas					
	10 min	RSD (%)[a]	15 min	RSD (%)	20 min	RSD (%)
DMN	2107.0	6.8	2209.0	7.9	2870.0	7.8
NMOR	1872.0	8.6	2560.0	9.1	2622.3	12.9
NPYR	2416.7	11.7	2871.0	12	4895.0	11.9
DEN	2831.0	13.1	3241.0	10.7	4095.3	9.8
NPIP	2982.7	11.9	3019.0	9.5	5054.6	8.3

[a] RSD% = relative standard deviation for at least three repetitions for each sample.

Table 3. Effect of the Extraction Temperature in the Recovery of Nitrosamines from Sausage, Using the Same Conditions as **Table 1**

	peak areas					
	40 °C	RSD (%)[a]	50 °C	RSD (%)	60 °C	RSD (%)
DMN	1369.0	5.4	1380.0	7.9	2960.6	7.4
NMOR	1180.0	12.7	2291.0	10.6	2629.0	6.9
NPIR	4599.0	11.6	4321.9	12.6	4817.0	8.4
DEN	3552.0	9.8	3327.0	9.9	3598.0	7.6
NPIP	5543.0	8.5	5499.0	5.9	5520.0	7.1

[a] RSD% = relative standard deviation for at least three repetitions for each sample.

purchased from Sigma Aldrich and were those recommended by the EPA 8270 method. They were maintained at 4 °C in the dark. The nitrosamines used in this work were dimethylnitrosamine (DMN), diethylnitrosamine (DEN), *N*-nitrosopyrrolidine (NPYR), *N*-nitrosomorpholine (NMOR), and *N*-nitrosopiperidine (NPIP). All the reagents and solvents used were, at least, of analytical grade, and were purchased form Merck.

A 1000 μg mL^{-1} stock standard solution of each analyte was prepared in methanol and was stored in the refrigerator. Working standard solutions were prepared daily by diluting the stock solution with methanol.

Table 4. Effect of the Kind of Trap in the Recovery of Nitrosamines from Sausage, Using the Same Conditions as **Table 1**

	peak areas					
	Florisil	RSD (%)[a]	diol	RSD (%)	CN	RSD (%)
DMN	2065.3	9.8	nd[b]		nd	
NMOR	4864.7	8.7	12502.7	7.5	5916.0	7.9
NPYR	19111.7	13.8	27028.3	12.7	2562.7	11.9
DEN	26527.7	8.6	23079.7	8.8	34144.0	8.1
NPIP	32395.3	8.4	31408.0	6.9	0	10.1

[a] RSD% = relative standard deviation for at least three repetitions for each sample. [b] nd = not detected.

Table 5. Optimization of SFE Variables

variables	range studied	optimum value
modifier/methanol (%)	0–15	0 (without modifier)
flow rate, mL min^{-1}	2–3	3
pressure, bar	129–360	360
extraction time, min	10–20	20
temperature thimble, °C	40–60	40
trap type	Florisil, cyano, diol	Florisil

The work with nitrosamines is extremely dangerous, a risk situation being any exhibition form. In this work, even the manipulation of the nitrosamines standard solutions as well as fortified samples was made in fume hoods, using gloves and protection masks. On the other hand, SFE is a closed system of extraction which represents an advantage in the handling of hazardous samples.

2.2. Supercritical Fluid Extraction (SFE). SFE was carried out in a Hewlett-Packard 7680T supercritical fluid extractor equipped with a Hewlett-Packard 1050 isocratic modifier pump with an automated variable restrictor. Various packed traps were used, such as diol, cyano, and Florisil (purchased from Supelco). Extractions were conducted in 7-mL thimbles. Each extraction was performed in triplicate and the extraction recoveries reported are the average of at least three extractions. Samples were submitted to dynamic extraction for 10, 15, and 20 min and to static extraction for 2 min. In the static way, the sample gets in touch with the fluid extractor and the thimble is closed, being that the sample is in contact with the same amount of supercritical fluid. After this period of time, the thimble is "opened" and the fluid extractor passes through the sample, under constant flow, dragging the analytes in the current until the trap where the analytes will be captured. The extraction was accomplished at a pressure from 129 to 360 bar using pure CO_2 or with methanol as modifier for the CO_2. Methanol was used to enhance the extraction power of CO_2 because of its polarity. The trap and nozzle temperatures were 65 °C and 70 °C, respectively, during the extraction and 25 °C during rinse. The rinse flow rate was 1.0 mL min^{-1}. Methanol (0−15%) was mixed online with CO_2. The extraction temperatures tested were 40, 50, and 60 °C. The flow rate was varied from 2 to 3 mL min^{-1} and the extracted analytes were collected in a solid sorbent trap of three packing materials: Florisil, ciano, and diol. After each extraction, the analytes were eluted from the trap at 20 °C with 2.0 mL of methanol.

2.3. Sample Preparation. Sample preparation was studied using three extraction procedures.

Procedure 1: SFE of Spiked Sausages. One-half gram of diatomaceous earth and 0.2 g of sample (ground fresh sausages) were put in the extraction thimble. To this thimble was added 70 μL of a working standard solution with 100 mg/L of DMN, DEN, NMOR, NPYR, and NPIP, corresponding to spiked sausage with 35 mg/kg of each nitrosamine. The same procedure was repeated by adding 70 μL of a working standard solution with 100 mg/L of the nitrosamines (spiked with 3.5 mg/kg). These procedures were used in the step of optimization of the main variables of the extraction procedure.

Procedure 2: SFE of Spiked Blanks (without Sausages). Seven-tenths of a gram of diatomaceous earth and 70 μL of standard solution at 100

Table 6. Recoveries (%) of Nitrosamines by SFE and MEKC

	diatomaceous earth spiked (0.7 g)				diatomaceous earth spiked + sample (0.5 g + 0.2 g)					
compounds	10 mg/kg [a,c]	RSD %[d]	10 mg/kg[b,c]	RSD %	10 mg/kg[c]	RSD %	1 mg/kg[c]	RSD %	0.4 mg/kg[c]	RSD %
DMN	103.5	5.8	70.9	8.9	60.5	9.7	29.8	13.7	20.9	12.9
NMOR	86.6	7.0	78.7	8.0	74.7	8.9	54.8	23.0	50.7	21.0
NPIR	82.4	7.6	63.5	7.6	53.9	8.0	61.9	11.7	65.5	12.6
DEN	84.3	3.8	77.9	5.1	70.3	6.1	87.9	12.0	81.6	9.8
NPIP	99.1	6.0	71.9	11.5	68.7	5.9	79.7	8.5	74.7	12.7

[a] Compounds extracted alone, determined by UV–vis. [b] Compounds extracted together, determined by MEKC. [c] Final concentration of the spiked analytes in the sample. [d] RSD% = relative standard deviation for at least three repetitions for each sample.

or 10 mg/L of each analyte separately (corresponding to 10 mg/kg or 1 mg/kg) were added to the extraction thimble. The extraction was carried out for each compound and the control was done by UV spectroscopy.

Procedure 3. One-half gram of diatomaceous earth was added to 0.2 g of sample in five thimbles (total mass of sausages = 1 g) and each thimble was spiked with 28 μL of a 10 mg/L standard solution. The samples were preconcentrated in the trap by five successive extractions and only one elution time. This procedure was repeated in the same conditions with nonspiked sausages.

2.4. Analysis by Micellar Electrokinetic Capillary Chromatography (MEKC). Experiments were carried out with a Beckman P/ACE 5500 CE instrument for the separation and quantification of the analytes. The system comprised a 0−30 kV voltage built-in power supply, a diode array detector (DAD), and PACE software for system control data processing. The fused silica capillary used (50 cm × 75 μm I.D) was obtained from Beckman Instruments (Fullerton, CA). The temperature was controlled by using a fluorocarbon-based cooling fluid.

The methanol extracts were concentrated in an ice bath under N_2 flow and after this they were dissolved in ultrapure water (2 mL). Prior to first use, each new capillary was subjected to a standard wash cycle according to the following procedure: washing with HCl 1 mol/L (5 min) followed by NaOH 0.1 mol/L (10 min) and ultrapure water (5 min).

To maintain the capillary under good working condition, its surface was regenerated once a day by consecutive washing with water (5 min) followed by freshly prepared 0.1 mol/L NaOH (5 min), ultrapure water (5 min), and fresh buffer (50 mM phosphate/borate, pH 6.6, 80 mM sodium dodecyl sulfate (SDS)).

MEKC separation was performed at 20 °C. The carrier electrolyte was 50 mmol/L phosphate−borate. A voltage of 10 kV was used, which produced a mean current of 70 μA. Samples were injected by using the electrokinetic injection mode at 10 kV for 10 s. In this injection method, the introduction of the sample in the capillary is made through the application of a potential difference in a period of time, being able to optimize these two items for each case. This way, the analites will migrate inside of the capillary with higher selectivity. Eletropherograms were recorded at 236 nm. Separations were carried out from the anode to the cathode. The pH 6.6 was corrected with phosphoric acid 0.1 mol/L. The effects of buffer composition and instrumental variables were investigated and optimized in previous works (*31*, *32*).

3. RESULTS AND DISCUSSION

3.1. Supercritical Fluid Extraction. The influence of the amount of methanol added to CO_2 on the recovery of SFE extraction was tested by using procedure 1 with a spiking of 100 mg/L of each nitrosamine (DMN, DEN, NMOR, NPYR, and NPIP). The results are shown in **Figure 1**.

There was different extraction behavior of the nitrosamines according to their polarity: more polar compounds, such as NMOR and NPIR, presented increased recovery with increasing the amount of methanol while the other compounds showed smaller recovery. This behavior is probably caused by a decrease in the trapping efficiency because of the presence of methanol in the extraction fluid. This fact is more evident at amounts higher than 10%, and there was a decrease in the area for the all compounds. So, the performance of extraction without modifier was selected to further experiments.

The influence of the dynamic extraction time and flow rate was studied by using procedure 1 with a spiking of 10 mg/L of DMN, DEN, NMOR, NPYR, and NPIP.

Two flow rates (2 and 3 mL min^{-1}, **Table 1**) and three extraction times (10, 15, and 20 min, **Table 2**) were studied. All the experiments were made at least in triplicate. A flow rate of 3 mL min^{-1} gave the highest recovery in all cases. The study of dynamic extraction time indicated a light increase in the recovery of the analytes with increasing the time to 20 min with very good results. The best group of the conditions that result in minimal time extraction and maximum recovery was 2 min for static and 20 min for dynamic extraction time.

The effect of extraction pressure on the recovery was studied at a constant temperature (40 °C). The investigation of extraction was carried out by using procedure 1, with spiking of 10 mg/L. The studied pressures of 129−360 bar at the temperature of 40 °C, resulting in densities of supercritical CO_2 of 0.74−0.94 g cm^{-3}, did not show significant differences, so, we opted for using 360 bar. At this pressure, the extraction of lipids was hindered, reducing the competition between nitrosamines and these interferent compounds, and so the extracts obtained are clean.

The influence of temperature on the efficiency of extraction was studied at constant pressure (200 bar) using procedure 1 with spiking of 10 mg/L. Three temperatures were tested: 40, 50, and 60 °C. Although the general behavior showed an increase in power solvency (capacity of the supercritical fluid to extract the analytes) with an increase of the temperature, this power solvency increased more with lipids than analytes. The temperature of 40 °C was selected (**Table 3**). This temperature was according to one work that related it to a decrease in the synthesis of nitrosamines in situ (*10*).

The trapping/collection efficiency of three types of trap (Florisil, cyano, diol) was evaluated by comparing the peak areas obtained for five nitrosamines studied in the extracts. The extractions were carried out with CO_2 at 360 bar, 40 °C (0.94 g mL^{-1} density), and 3 mL min^{-1} flow rate for 20 min of dynamic extraction after 2 min static extraction. The experiments were done in samples spiked with 70 μL of working standard solution at 100 mg/L (DMN, DEN, NMOR, NPYR, and NPIP). **Table 4** presents the results of the recovery using different traps where it is possible to observe that Florisil trap showed the best result for all extractions.

The effect of experimental variables was evaluated to develop a quick and quantitative SFE method. The variables, limits studied, and optimum values found are listed in **Table 5**.

3.2. Sample Application. After optimization of all the variables, the recovery studies were made by using 1 g of sample (spiked with 0.4, 1.0, and 10.0 mg/kg of the standards) divided

in five thimbles (0.2 g in each one), giving a total of five consecutive extractions and just one elution (procedure 3). The results are shown in **Table 6**. In parallel, it was applied to procedure 2 (blank analysis) for comparative purposes. This blank analysis was done in two ways: each compound extracted alone (first column in **Table 6**) and all the nitrosamines extracted together (third column in **Table 6**).

The recoveries from spiked sausages varied from 20.9% to 81.6%, and the low recovery values can be explained not only by the competition with the lipids but also by the losses during the evaporation and change of solvent. This can be observed by comparing columns 1 and 3 of **Table 6**. In column 1, the process was controlled by UV−vis using the sample directly from SFE without changing the solvent. In column 3, the control was made by MEKC changing methanol by water, presenting lower values. When observing the other columns where there was added sample (0.2 g), the values are reduced because of the competition with other compounds.

3.3. Conclusions. Optimization of the extraction method was obtained and the results demonstrated that this technique is a good extraction method for volatile nitrosamines with fast analysis and reduction in the solvent waste and in the analysis time. The recoveries obtained (**Table 6**) are higher than those acceptable by EPA methods for nitrosamines (*33*). This methodology represents an advantage in application of SFE commercial equipment. The use of the MEKC allowed the separation and determination of compounds being considered to be a good analytical tool to perform the extractive experiments. The restrictions are the poor selectivity in detection of real samples and the small amount of sample on the extractor (thimble). The results showed that supercritical fluid extraction presents a promising approach to the analysis of the nitrosamine isolation.

Although the nitrosamine levels found in food samples are in the range of μg/kg, this work was developed in the range of mg/kg in function of instrumental limitations (extraction equipment and analysis method). The amount of sample used in each thimble should not overcome 0.2 g for this equipment, and therefore the amount of analyte obtained, starting from a real sample, would be smaller and would disable the detection and consequently the study of the behavior of this analyte related to the supercritical fluid extraction (*32*).

By using extractors that allow work with a larger amount of samples and by combining other preconcentration techniques, the SFE method can be applied to real samples.

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