Multiresidue Recovery at PPB Levels of 10 Nitrosamines from Frankfurters by Supercritical Fluid Extraction

R.J. Maxwell*, J.W. Pensabene, and **W. Fiddler**
U.S. Department of Agriculture, ARS, Eastern Regional Research Center, 600 E. Mermaid Lane, Philadelphia, Pennsylvania 19118

Abstract

The design of a laboratory-assembled supercritical fluid extractor is described for the efficient recovery of volatile nitrosamines from a common-cured meat product, frankfurters. The principal feature of the apparatus was a newly designed restrictor–collector interface where a commercial solid-phase extraction cartridge was directly attached to the micrometering valve. This reduced the path length between the discharge tube and the 1 g silica gel sorbent bed. The elapsed time for each 2.5 g sample extraction with supercritical CO_2 was 17 min. The nitrosamines were separated and detected using a gas-chromatographic chemiluminescence (Thermal Energy Analyzer, Thermedics, Inc.; Woburn, MA) system. Recovery of 10 volatile aliphatic and alicyclic nitrosamines from frankfurters, fortified at the 20 ppb level, ranged from 84.3 to 104.8% with relative standard deviation of 2.34 to 6.13%.

Introduction

Some *N*-nitrosamines are carcinogenic compounds which may form at the ppb level in foodstuffs principally through the interaction of secondary amines with nitrite or nitrogen oxides or both (1). The levels of volatile nitrosamines in nitrite-cured meat products are monitored through USDA's Food Safety Inspection Service (FSIS) surveillance program. Two isolation methods currently are used in the FSIS Monitoring Program to analyze nitrosamines in tissues (2). The first method involves the vacuum distillation of the nitrosamines from the sample in a mixture of mineral oil and base. This is followed by dichloromethane extraction and then gas-chromatography–Thermal Energy Analyzer (GC–TEA) or chemiluminescence determination. Samples found to contain violative levels of nitrosamines then are reexamined with a more lengthy procedure. This includes low- temperature vacuum distillation from base, acidification of the aqueous distillate, extraction with dichloromethane (DCM), washing the DCM with acid and base, concentration, and a column chromatographic clean-up step before GC/MS (gas chromatography/mass spectrometry) confirmation. In addition to the obvious disadvantage of the length of time required to perform the analyses, both methods require the extensive use of equipment and solvents. By 1995, government-funded contracts and selected Federal laboratories, including those operated by FSIS, will be required by the Environmental Protection Agency to reduce their use of specified solvents by 50% (3). To comply with this requirement, new technologies need to be investigated. One of these is supercritical fluid extraction (SFE). This technique has had only limited application to the field of nitrosamines, mainly those specific to tobacco products where low extraction efficiencies were encountered (4). The use of SFE for the analysis of meat products poses a problem because of the coextraction of trace analytes with the large amount of lipid typically present in such samples.

We have recently assembled an SFE apparatus, which we first used to recover nitrobenzamide antimicrobial residues from liver tissue (5). The apparatus in its final configuration, designed to improve the extraction of these polar analytes, was applied to nitrosamines. In this paper, we describe the apparatus and method used for the rapid and efficient extraction of 10 volatile nitrosamines in fortified frankfurters using supercritical carbon dioxide.

Experimental

Apparatus

Supercritical fluid extractor. The SFE used in these studies was assembled from components obtained from various suppliers and from parts designed and fabricated in this laboratory. A schematic drawing of the apparatus is shown in Figure 1. The restrictor used in this system was a micrometering valve (10VRMM2812, Autoclave Engineers, Inc.; Erie, PA) encased in an aluminum block fitted with a cartridge heater and a thermocouple. The seat retainer nut of the micrometering valve, which connects the valve to other devices through lengths of 1/8 in tubing, was replaced by a retainer nut fabricated locally. This redesigned nut, referred to as the integral seat retainer–column nut, enabled commercial 6 mL SPE columns to be attached directly to the micrometering valve without the aid of fittings and connecting tubing. All of the components of the SFE system shown in Figure 1 have been described in detail elsewhere (5).

*Author to whom correspondence should be addressed.

Gas chromatograph-Thermal Energy Analyzer. This system consists of a gas chromatograph (Model GC-14A, Shimadzu; Columbia, MD) equipped with AOC-14 auto-injector or equivalent, interfaced to a Thermal Energy Analyzer Model 502A (Thermedics, Inc.; Woburn, MA). Operating conditions are 2.7-m x 2.6-mm glass column packed with 15% Carbowax 20M-TPA on 60-80 mesh Gas Chrom P, He carrier gas 35 mL/min, injector 180 °C, TEA furnace 475 °C, TEA vacuum 0.4 mm, liquid nitrogen cold trap, and a column programmed from 120 to 220 °C at 4 °C/min (6).

Materials

Hydromatrix (Celite 566) was obtained from Varian-Sample Preparation Products Co. (Part No. 0019-8003; Harbor City, CA). Propyl gallate was a product of the Aldrich Chemical Co. The silica gel (7734) used in the SPE columns was obtained from E. Merck (Cherry Hill, NJ). The 70-230 mesh (grade 60) material was washed twice with dichloromethane (DCM), filtered and dried 4 h in a vacuum oven set at 60 °C. It was sieved to a particle range of 70-150 mesh before use. The sieved silica gel was packed into empty 6 mL SPE columns and frits provided by Applied Separations, Inc. (Allentown, PA). DCM, ethyl ether, and pentane (all liquid-chromatographic grade) were from Burdick and Jackson (Muskegon, MI). The internal standard solution was 0.10 g/mL of *N*-nitrosodipropylamine (NDPA) in DCM. The standard nitrosamine solution used to fortify frankfurters was prepared from the following components whose chemical structures are shown in Figure 2: *N*-nitrosodimethylamine (NDMA), *N*-nitrosomethylethylamine (NMEA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosodipropylamine (NDPA), *N*-nitrosodibutylamine (NDBA), *N*-nitrosoazetidine (NAZET), *N*-nitrosopyrrolidine (NPYR), *N*-nitrosopiperidine (NPIP), *N*-nitrosohexamethyleneimine (NHMI), and *N*-nitrosomorpholine (NMOR), each 0.10 g/mL in DCM. Alaska Pollock-fish meat frankfurters were prepared by the National Marine Fisheries Service, Southeast Fisheries Center, Charleston Laboratory. These frankfurters, which contained from 18 to 30% fat, were made from a mixture of 50% Alaska Pollock and 50% beef-pork by a typical industry processing and hot smoking procedure (7). Sodium nitrite (156 ppm) and sodium erythorbate (550 ppm) were among the additives used in the processing of these frankfurters. All-meat frankfurters containing 30% fat were also prepared.

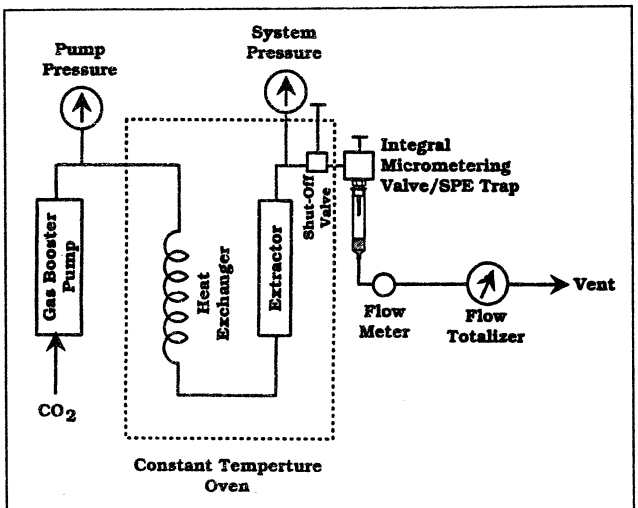

Figure 1. Schematic diagram of SFE fluid system showing details of the integral micrometering valve SPE column holder assembly (5).

Sample preparation

A 2.5-g comminuted frankfurter sample was weighed in a 100 mL beaker. Propyl gallate (250 mg) was added followed by 0.5 mL of the NDPA internal standard solution or the 10 nitrosamine standard solution. Then Hydromatrix (5.0 g) was weighed into the beaker. This mixture was stirred with a glass rod until uniform in appearance (about 1 min). The dry, free-flowing mixture then was transferred into a high pressure extraction vessel (Couplings 20F12463, nipple CNLX1208; Autoclave Engineers, Inc.). The volume of this vessel was approximately 26 mL.

SFE Procedure

The extraction vessel was installed in the SFE as shown in Figure 1, and the Autoclave micrometering valve was preheated to 110 °C. The oven shut-off valve was closed, and the fluid system was pressurized with carbon dioxide to 10,000 psi (680 bar) using a gas booster pump (Model AGT-62/152C, Haskel Engineering Co.; Burbank, CA); simultaneously, the oven was set to a temperature of 40 °C and heating commenced. To equilibrate the fluid system, a 2 min static holding period was used. During this time a 6 mL solid-phase extraction (SPE) column (Applied Separations) containing 1.0 g sieved silica gel was attached to the integral seat retainer nut of the micrometering valve (Figure 1). The SPE column was attached by tubing to a flow meter (Model 110, McMillian Co.; Georgetown, TX) and a gas totalizer (Model DTM-115, American Meter Co.; Philadelphia, PA).

At the end of the 2-min heating period, the shut-off valve was opened and flow was directed through the discharge tube of the micrometering valve to the SPE column sorbent bed. The flow of the expanded gas was adjusted to 3.0 L/min and maintained at that rate throughout the experiment until 50 L was recorded on the gas totalizer. At that point, the shut-off valve was closed, the SPE column containing the nitrosamine–fat mixture was removed from the seat retainer nut and the fluid system was depressurized. Trace residues of fat remaining on the discharge tube of the metering valve were washed into the SPE column with 0.25 mL of hexane using a syringe with a stainless steel needle.

Nitrosamine recovery and analysis

The SPE column sorbent bed containing the nitrosamine–fat matrix first was washed with 2- × 4-mL portions of 25% DCM

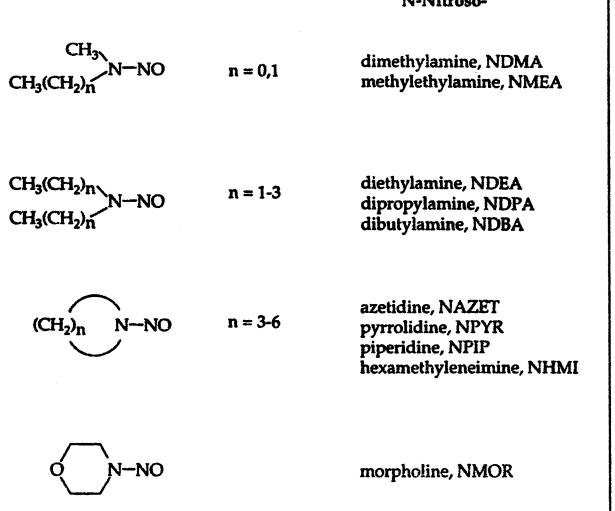

Figure 2. Chemical structures of 10 nitrosamines added as a mixture to fortified frankfurters.

in pentane. These washings, containing the recovered fat, were discarded. The nitrosamines then were eluted from the column by passing 2 × 4 mL of a mixture of 30% ethyl ether in DCM through the sorbent bed. These washings were collected in a 10-mL concentrator tube, which then was attached to a micro Snyder column (Kontes Glass Co.; Vineland, NJ) and the washings were concentrated at 70 °C to a 1.0 mL volume. To determine the nitrosamines, the solution was injected into a gas chromatograph interfaced to a nitrosamine specific chemiluminescent detector (GC-TEA), according to a procedure described previously (6). The limit of detection for the 10 nitrosamines ranged from 1.0 ppb for NDMA to the highest of 3.0 ppb for NDBA.

Results and Discussion

The SFE apparatus shown in Figure 1 was designed specifically to address the problems associated with the recoveries of trace analytes from biological matrices. Most commercial instruments are configured so that extracted solutes are collected in flasks or vials. They also may be trapped by bubbling the CO_2 gas from the discharge tube directly into a vial containing cooled solvent. While these approaches to solute trapping, for example, may be satisfactory for polyaromatic hydrocarbons, pesticides, or other analytes extracted from soils, water, or sediment, they pose difficulties for the recovery of polar analytes from meat tissue.

Biological matrices such as animal tissues contain lipids (fat) at various levels depending on tissue type. Tissue lipids generally are more soluble in supercritical CO_2 than the desired trace analyte (8,9). Therefore, SFE methods derived for the recovery of analytes such as nitrosamines from tissue would result in the co-extraction of varying amounts of fat.

In designing a collection device for trapping solutes from tissue matrices, two problems must be addressed: first, the need to quantitatively collect the analyte–fat mixture; and second, the need to separate the analyte from the fat after extraction for subsequent chromatographic analysis. We have addressed both of these difficulties by designing a restrictor–collector interface for the SFE (Figure 1), which allows for the collection of solutes directly on the frit above the sorbent bed of commercial solid-phase extraction (SPE) columns (5). This interface shortens the path length between the restrictor (micrometering valve) and the SPE column.

In this study, this same system and earlier metering valve–collector designs were tested in the process of developing a method for the recovery of 10 volatile aliphatic and alicyclic nitrosamines (Figure 2) from fortified frankfurters. The frankfurters used initially were a blend of 50% unwashed Alaska pollock mince and 50% of a mixture of beef and pork. Frankfurters having this composition were chosen for this investigation because, unlike all-meat frankfurters, the addition of fish to the formulation raises the possibility of artifactual nitrosamine formation occurring during the extraction process. This worse case scenario would result from the simultaneous presence of nitrite used to make the product and the amines present in the fish. It was thought that this product would test the SFE system more adequately than other cured-meat products having less potential for artifact formation.

In early experiments, the metering valve was heated with heating tape or heat guns or both and the sorbent (silica gel) used to trap the solutes was packed in a stainless steel tube fitted into an aluminum housing and attached to the valve through a length of tubing. Although the nitrosamines in the fat matrix were trapped on the sorbent bed, the uneven heating of the micrometering valve resulted in CO_2 gas being vented from the discharge tube at low temperatures (<10 °C). This in turn caused the water vapor from the tissue sample to condense and fat to solidify on the sorbent in the trap, which occasionally blocked the gas flow. The water condensation affected the activity of the silica gel sorbent bed and resulted in inconsistent nitrosamine recoveries. The fat–water condensation and gas flow problems were rectified first by encasing the micrometering valve in a temperature controlled aluminum housing. By setting the temperature of the encased metering valve at 110 °C, the CO_2 exiting the discharge tube could be maintained at 60 °C throughout the course of the experiment thereby eliminating fat solidification and water condensation on the sorbent bed. Additionally, an interface was designed that allowed commercial SPE (6 mL) columns packed in-house rather than laboratory assembled tubes to be attached directly to the micrometering valve (5). This innovation shortened the length of the discharge tube and also resulted in a reduction of the requirement of 6 g of silica gel in the laboratory prepared tube. Instead a commercial SPE column could be substituted containing only 1.0 g of the sieved sorbent because of the improved packing of the sorbent in the standard commercial column.

Table I. SFE Recovery of 10 Nitrosamines from Fortified* All-Meat Frankfurters

N-Nitroso† Compound	Range (%)	Mean‡ (%)	RSD (%)	CV (%)
NDMA	94.0-100.9	97.50	2.85	2.92
NMEA	87.0-92.6	89.45	2.34	2.62
NDEA	84.3-92.5	88.35	2.83	3.20
NDPA	84.8-98.6	92.37	6.13	6.34
NAZET	94.3-104.8	100.57	3.55	3.53
NDBA	92.2-101.8	95.22	3.54	3.72
NPIP	91.1-101.8	97.80	5.89	6.02
NPYR	91.0-103.2	95.17	4.41	4.63
NMOR	97.1-100.9	100.86	3.87	3.84
NHMI	91.5-102.0	97.62	4.06	4.16

*Fortification level - 20 ppb/nitrosamine.
†Figure 2.
‡Average of 6 determinations.

Key to Abbreviations

SFE	Supercritical Fluid Extraction
FSIS	USDA's Food Safety Inspection Service
GC-TEA	Gas Chromatographic-Thermal Energy Analyzer
DCM	Dichloromethane
NDMA	*N*-nitrosodimethylamine
NMEA	*N*-nitrosomethylethylamine
NDEA	*N*-nitrosodiethylamine
NDPA	*N*-nitrosodipropylamine
NDBA	*N*-nitrosodibutylamine
NAZET	*N*-nitrosoazetidine
NPYR	*N*-nitrosopyrrolidine
NPIP	*N*-nitrosopiperidine
NHMI	*N*-nitrosohexamethyleneimine
NMOR	*N*-nitrosomorpholine

In the initial SFE experiments undertaken to optimize nitrosamine recoveries, the following parameters were used: the oven temperature was set at 80 °C, 200 L of CO_2 (expanded gas) was recorded, a flow rate of 4 L/min was maintained, the pressure was set at 680 bar, and 6.0 g of silica gel (70-230, grade 60, mesh-commercial product) was used in the sorbent trap.

Two problems were noted using these experimental conditions: (1) the commercial grade (70-230 mesh) silica gel initially caused a backflow problem because of compressed packing that was corrected by sieving the silica gel to 70-150 mesh and (2) the formation of artifactual nitrosamines. The latter observation was confirmed by comparing the results of the SFE recovery procedure with those obtained using a nondistillation, column chromatographic method for nitrosamines from cured meats developed by Pensabene and others (6,10,11). The nitrosamine values obtained for the fish-meat frankfurters by the above SFE conditions were six times higher than those found by the other method. These results suggested that substantial artifactual nitrosamine formation occurred.

The SFE operating parameters then were modified by adjusting flow rates and the temperatures of the heated zones until the lowest level of artifacts were obtained. The final conditions were oven temperature 40 °C, micrometering valve temperature 110 °C, pressure 680 bar, flow rate (CO_2-expanded gas) 3 L/min, and a total expanded gas volume of 50 L. A 2 min static period was incorporated into the procedure to equilibrate the fluid system before extraction. The total time required to complete an SFE extraction then became 17 min/sample.

These optimized conditions (*vide supra*), however, did not entirely eliminate artifactual nitrosamine formation. Approximately 17 ppb NDMA was still formed at 40 °C as compared to 7.5 ppb when the sample was analyzed by the column chromatographic method. To eliminate the formation of these artifacts, propyl gallate, a known nitrosation inhibitor, was added to the fish-meat frankfurter samples before extraction by SFE. The addition of propyl gallate to the samples resulted in values comparable with those levels obtained with the column chromatographic method.

The low temperature SFE (40 °C) and sample preparation conditions established for the fish-meat product then were used to extract multiple samples of all-meat frankfurters fortified with ten volatile nitrosamines (Table 1). Each of the nitrosamines was added as part of a mixture to the samples at a level of 20 ppb. Recovery of the nitrosamines from the SPE columns after SFE (Experimental–Nitrosamine recovery and analysis) used procedures similar to those developed by Pensabene and others to isolate these compounds using their column chromatographic method (6). The GC-TEA conditions were identical for the extracts obtained by both methods.

The mean recovery for each nitrosamine was an average of six determinations. The means ranged from 88.4 to 100.9. The range of values for each nitrosamine was narrow. Only two of the nitrosamines had average means below 90% (NDEA, 88.4% and NMEA, 89.5%). The next lowest mean was for NDPA at 92.4%, while the range for the remaining seven nitrosamines was focused between 95.2 and 100.9%. For instance, NMEA was 87.0-92.6% while that of NMOR was 97.1-100.9%. The RSDs for the 10 compounds ranged from 2.34 to 6.13%.

Conclusion

The results suggest that supercritical extraction combined with analyte trapping on SPE columns represents a promising approach to the difficult problem of isolating nitrosamines. This method yields extracts containing high recoveries of volatile nitrosamines with reduced sample preparation time, while only using small amounts of solvent in the analytical scheme.

Acknowledgment

Reference to a brand or firm does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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