

Supercritical Fluid Extraction of *N*-Nitrosamines in Hams Processed in Elastic Rubber Nettings

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A method for analysing *N*-nitrosamines in hams processed in elastic rubber nettings by supercritical fluid extraction (SFE) is described. The study was carried out with the prototype of a commercial extractor with a silica gel adsorption cartridge integrally attached to the variable restrictor. The SFE method was compared with a solid-phase extraction procedure currently used for ham analysis. Both methods used the same gas chromatographic-chemiluminescence detection conditions. No significant difference ($p < 0.05$) was found between results obtained with the 2 methods. Repeatability standard deviation of the SFE method was 1.7 ppb, with a coefficient of variation (CV) of 2.7%, compared with 2.2 ppb, with a CV of 3.5%, for solid-phase extraction. SFE permits minimal use of solvent and more rapid analysis of nitrosamines.

Supercritical fluid extraction (SFE) is rapidly becoming the method of choice for isolation of certain analytes from sample matrixes. Compared with standard extraction techniques, SFE offers substantial time savings because of

the unique properties of gases in their supercritical state (1). The polar and nonpolar characteristics of the supercritical fluid can be controlled by varying the pressure and temperature of the extraction system. This technique can be used both for sample extraction and analyte concentration. Carbon dioxide is the gas of choice because of its excellent physical properties in the supercritical state, low toxicity, and reasonable cost. The current emphasis on methods that use less solvent makes SFE an attractive alternative for the analysis of nitrosamines.

Since the discovery of carcinogenicity of *N*-nitrosodimethylamine (2), several hundred compounds containing the *N*-nitroso group have been found to be carcinogenic in a number of animal species. These compounds typically are isolated by distillation and/or solvent partition. However, only limited studies have been carried out on SFE of nitrosamines. Prokopczyk et al. (3) reported extraction efficiencies of 83 to 98% for the major nicotine-derived tobacco-specific nitrosamines in smokeless tobacco and snuff, with methanol-modified supercritical carbon dioxide. Recently, our group (4), using only carbon dioxide, obtained recoveries of 84 to 105% for 10 volatile nitrosamines, including aliphatic and alicyclic nitrosamines, in frankfurters fortified at 20 ppb. In these studies (3, 4), SFE was carried out with a self-assembled apparatus. In our study (4), a new design concept was necessary because of significant loss of nitrosamines at the restrictor-collector interface when these analytes were extracted with commercial SFE in-

struments. For this reason, a unique integral micrometering valve-collector assembly was developed to trap nitrosamines on the sorbent bed of commercial solid-phase extraction (SPE) cartridges. This assembly design was described previously for isolation of 3 nitrobenzamide antimicrobial drug residues in chicken liver tissue (5). In this paper, we report the development of an SFE method for determination of nitrosamines in boneless hams processed in elastic rubber nettings. The method uses the prototype SFE with the integral metering valve-collector assembly. The SFE method also was compared with an SPE technique currently used for analysis of nitrosamines in hams.

METHOD

Caution: *N*-Nitrosamines are potential carcinogens. Exercise care in handling these compounds.

Materials

(a) *Ham samples.*—Samples were obtained from local retail outlets or producers, or from the U.S. Department of Agriculture's Food Safety and Inspection Service (FSIS) and analyzed without further heating. The outer 1/4 in. of the ham was removed, ground through a 1/16 in. plate, and then thoroughly mixed. The comminuted sample was vacuum-packaged and stored at -20°C until analyzed.

(b) *Reagents.*—The sources and cleanup of Hydromatrix (Celite 566), Celite 545, anhydrous sodium sulfate, propyl gallate, silica gel, dichloromethane (DCM), pentane, and diethyl ether were described in detail elsewhere (4, 6, 7). Morpholine was doubly distilled before use and then checked for presence of *N*-nitrosomorpholine (NMOR) as a contaminant; none was found. Preparation of the SPE cartridge was described in detail elsewhere (4). Briefly, 1.0 g washed and sieved (70–150 mesh) silica gel was packed into an empty 6 mL SPE cartridge and then a frit was placed on top.

(c) *N-Nitrosodipropylamine (NDPA) internal standard solution.*—0.10 $\mu\text{g}/\text{mL}$ in DCM.

(d) *Gas chromatographic working standard solution.*—Each 0.10 $\mu\text{g}/\text{mL}$ in DCM: *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), NDPA, *N*-nitrosodibutylamine (NDBA), *N*-nitrosopiperidine (NPIP), *N*-nitrosopyrrolidine (NPYR), NMOR, and *N*-nitrosodibenzylamine (NDBzA). These nitrosamines were either purchased or synthesized from their corresponding amines and sodium nitrite according to a general procedure reported previously (8).

Apparatus

(a) *Supercritical fluid extractor.*—The extractor was a prototype of a commercial instrument developed jointly by our laboratory and Applied Separations (Allentown, PA) and now in commercial production. This instrument was configured for parallel extraction of 2 SFE vessels. Extraction vessels were connected to the system with hand-tightened, slip-free connectors (Keystone Scientific, Bellefonte, PA). The restrictors were micrometering valves (10 RMM2812, Autoclave Engineers, Inc., Erie, PA) encased in an aluminum block fitted with a cartridge heater and a thermocouple. The seat-retaining nuts of the

micrometering valves, which connect the valve to other devices, were replaced by a redesigned retaining nut fabricated locally. This redesigned nut, referred to as the integral seat retainer-column nut, enables a commercial 6 mL SPE cartridge to be attached directly to the micrometering valve without fittings or connecting tubing. Components of this prototype instrument are shown in Figure 1. A detailed description of the metering valve-SPE interface has been reported elsewhere (5).

(b) *Gas chromatograph-thermal energy analyzer (GC-TEA).*—The instruments and operating conditions used for separation and quantitation of nitrosamines were described elsewhere (7).

(c) *Other glassware and equipment.*—All other items needed for SFE or SPE have been described elsewhere (4, 6).

Sample Preparation (SFE)

Weigh 5.0 g comminuted ham sample into a 100 mL beaker. Add 250 mg propyl gallate. Using a 0.5 mL transfer pipette, spike the sample with either 0.5 mL NDPA internal standard solution or 0.5 mL GC working standard solution. Add 5.0 g Hydromatrix. Stir the mixture with a glass rod until uniform in appearance (ca 1 min). Seal one end of the high pressure extraction vessel (66015 SFE vessel, 24 mL volume or capacity, Keystone Scientific) and label it as "top." Transfer the dry, free-flowing mixture into the extraction vessel prepacked with a

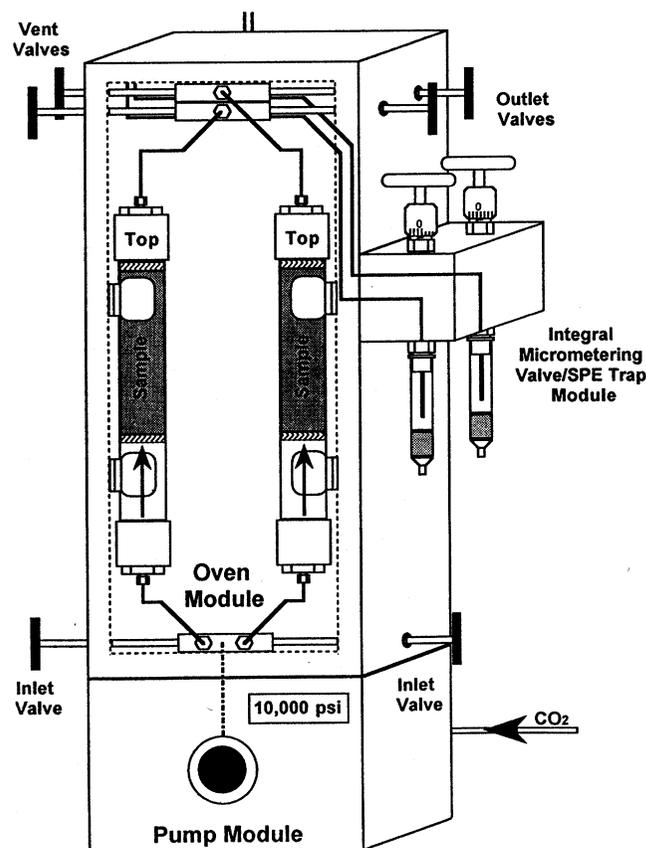


Figure 1. Schematic diagram of prototype SFE system.

plug of polypropylene wool (Aldrich Chemical Co., Milwaukee, WI). Tightly compress the mixture with a tamping rod that ensures uniform supercritical fluid flow. Finally, add a second plug of polypropylene wool to the extraction vessel and compress in place with the tamping rod. Tighten end fittings before the next step. The sample is now packed in the end labeled "top" (Figure 1).

SFE Procedure

Install the extraction vessels in the SFE as shown in Figure 1, with the ends labeled "top" connected to the upper fittings. Preheat the micrometering valves to 110°C. Close the oven shut-off and vent valves; open the inlet valves. Pressurize the SFE vessels with carbon dioxide to ca 9000 psi (612 bar); simultaneously set the oven temperature to 40°C and commence heating. Equilibrate the system by using a 10 min static holding period. When the system is equilibrated, adjust the pressure to a final setting of 10 000 psi (680 bar). During this period, pack empty SPE cartridges with silica gel and then attach the cartridges to the integral seat retainer-column nut of the micrometering valves (Figure 1). Attach the SPE cartridges with flexible tubing to a Floline SEF-51 flow meter-gas totalizer (Scott Specialty Gases, Plumsteadville, PA). The direction of fluid flow through the system is indicated by the arrow in Figure 1. After the 10 min heating period, open the outlet valves to direct flow to the micrometering valve module. Use these valves to adjust the flow of the expanded gas to 2.8 L/min through the SPE cartridges and maintain that rate throughout the experiment until 50 L are recorded on the gas totalizer. At that point, close the inlet and outlet valves and depressurize the SFE vessels by using the vent valves. During extraction, flow rates were kept between 2.7 and 2.9 L/min. Nitrosamine results indicated that this slight variation had no effect on analyte recoveries. Remove the extraction vessels from the oven module and attach Luer adapters to the upper slip-free connectors. Attach a filled syringe to each adapter and flush any trace residues of analyte-fat remaining in the discharge tube of the micrometering valves with 0.3 mL hexane. Remove the SPE cartridges containing the analyte-fat mixture from the seat retainer nut. Hold the cartridges below the seat retainer nuts and rinse the 1/16 in. stainless steel tubing of these assemblies with 0.1 mL hexane directly into the SPE cartridges to ensure quantitative recovery of nitrosamines.

Nitrosamine Recovery and Analysis (SFE)

Details of this procedure were described previously (4). Briefly, wash the SPE cartridge with two 4 mL portions of 25% DCM in pentane; discard the washes. Elute nitrosamines with two 4 mL portions of 30% ether in DCM. Concentrate to 1.0 mL and quantitate on the GC-TEA. The nitrosamine values of individual samples were corrected for recovery of NDPA internal standard. The minimum levels of reliable measurement were 0.5 ppb for NDMA and 1.0 ppb for the other nitrosamines.

Sample Preparation, Recovery, and Analysis (SPE)

The complete procedure for preparation, extraction, cleanup, and quantitation of ham samples by our SPE procedure

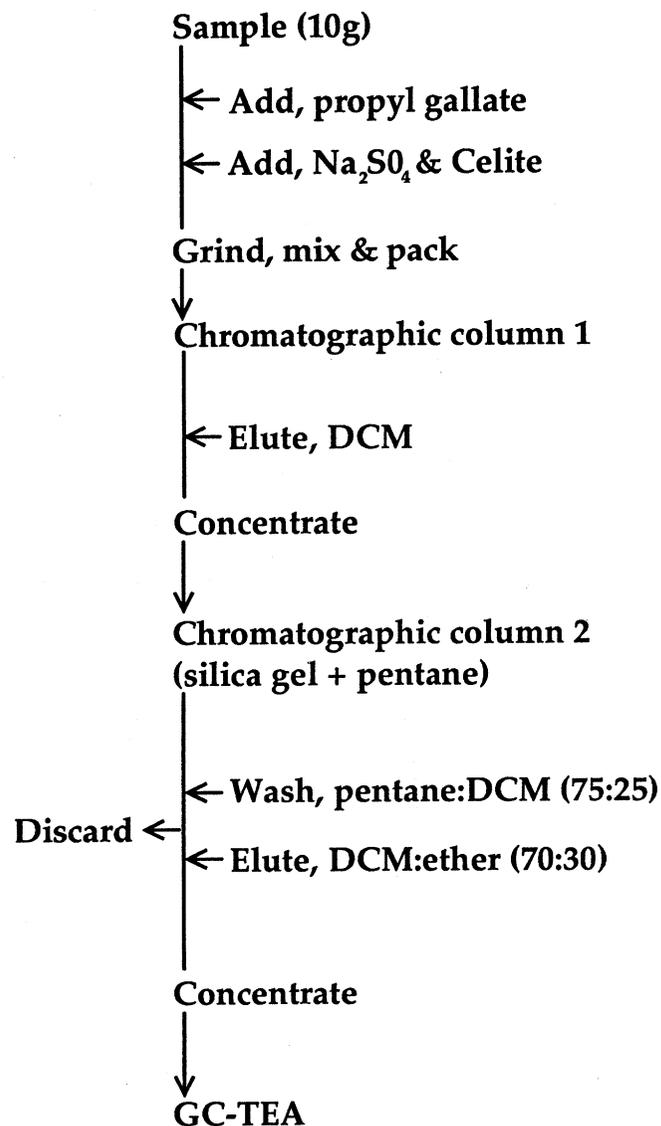


Figure 2. Flow diagram of SPE procedure.

cedure were described in detail elsewhere (6, 7). A flow diagram is shown in Figure 2. The minimum levels of reliable measurement were 0.2 ppb for NDMA and NDEA, and 1.0 ppb for the other nitrosamines.

Statistical Analysis

Data were analyzed by analysis of variance and means procedures of the Statistical Analysis System software distributed by SAS Institute, Inc. (9). These results were then interpreted according to the methods of Snedecor and Cochran (10).

Results and Discussion

An earlier SFE used for isolation of nitrosamines from frankfurters was assembled entirely in our laboratory (4). To obtain satisfactory recoveries, we fabricated an integral restrictor interface so that we could collect extracted analytes directly on sorbents in standard SPE cartridges (5) rather than from a cooled solvent, as with most laboratory-assem-

bled and commercially available SFE instruments. This interface adapter yielded excellent recoveries of nitrosamines and minimized post-SFE cleanup (4). We next attempted to modify a commercial multiport parallel SFE extractor, which used fixed restrictors vented into open refrigerated vials for analyte recovery, to a system where analytes are collected in SPE cartridges. We fabricated a retaining nut similar to that shown in Figure 3b (*see* reference 2) to retain the 6 mL SPE cartridges on the fixed restrictors of the commercial SFE. Although we were able to collect nitrosamines from samples with this modified system, we encountered several problems that we could not resolve. For example, the restrictors could not achieve the same flow rates that were used with the variable restrictors in the laboratory-assembled apparatus, partly because of the high fat content of the meat samples. Also, the extracted fat solidified in the SPE cartridges because of the inability of the restrictor heating block to keep the cartridges above freezing temperatures. As a result of the difficulties with the modified extractor, recoveries of nitrosamines were variable and low compared with those obtained with the laboratory-assembled apparatus. Experiments with this system were therefore discontinued. Instead, a Cooperative Research and Development Act (CRADA) agreement was signed with a commercial instrument manufacturer to build an SFE based on design concepts developed for the laboratory-assembled apparatus used in our previous studies (4, 5). The final prototype of this commercial SFE was used for all experiments described in this paper.

During our earlier investigation on use of SFE to analyze cured meat products for volatile nitrosamines, our sample size was 2.5 g (4) because we loosely packed the sample-Hydratrix mixture, completely filling the extraction vessel. By changing to compressed packing without analyte loss, we increased sample size to 5.0 g, which resulted in increased sensitivity and without the need for more solvent to elute analytes from the SPE cartridges.

One problem initially encountered during development of the SFE method was artifactual nitrosamine formation (4). This occurred when the sample is heated (70°–80°C) in the presence of residual NaNO₂ prior to extraction with supercritical carbon dioxide. To eliminate this problem in analysis of frankfurters, we lowered the extraction temperature to 40°C and added propyl gallate, a nitrosamine inhibitor, to the samples. To determine whether nitrosamines could form artifactually in the ham samples, which have a lower fat content than frankfurters, morpholine, a rapidly nitrosated secondary amine precursor of NMOR, was added to several ham samples before SFE. No NMOR was detected in the SFE extract.

Recoveries of 7 volatile and 1 semivolatiles nitrosamine (NDBzA) added to nitrosamine-free ham at 10 ppb and analyzed by SFE are shown in Table 1. The mean recovery of NDBzA, the nitrosamine found in ham processed in elastic rubber netting, was 96.1 ± 4.5%. The mean recovery of all other nitrosamines by SFE ranged from 95.2 to 103.5%. This compares favorably with the range of recoveries (92.4 to 97.8%, excluding NDBzA) we reported for SFE of 10 volatile nitrosamines in frankfurters for-

Table 1. SFE recovery of nitrosamines from hams fortified at 10 ppb

N-Nitroso compound	Recovery, %			
	Range	Mean (n = 8)	SD	CV
NDMA	91.8–102.0	96.4	4.1	4.2
NDEA	98.4–106.1	102.6	2.5	2.4
NDPA	96.3–109.4	100.9	5.6	5.5
NDBA	90.0–102.4	95.2	4.8	5.0
NPPIP	96.4–109.1	103.5	4.5	4.4
NPYR	98.0–105.1	100.5	2.4	2.4
NMOR	97.3–107.5	102.8	3.7	3.6
NDBzA	88.4–101.2	96.1	4.5	4.6

tified at 20 ppb (4). Analysis for NDBzA in hams by SPE gave a mean recovery of 95.6 ± 4.5% (7).

The presence of nitrosamines in cured meat products processed in elastic rubber netting has been reported recently (6, 7, 11). Therefore, we chose this sample type for further investigation by SFE. Samples from the outer surface of 21 ham samples processed in elastic rubber netting were analyzed in duplicate for nitrosamines by both SFE and SPE. The outer ham surface has the maximum exposure to rubber in the netting and has the highest nitrosamine values. The SPE method was selected for comparison because it readily isolates volatile nitrosamines and NDBzA from the sample matrix. Raw data are compared in Table 2, and statistical results are given in Table 3. The samples analyzed contained nitrosamines in a wide range of concentrations, from none detected (ND) to 157 ppb. The internal standard for all extracted samples was NDPA. NDBzA was the only nitrosamine detected in the ham samples, reflecting the change in the formulation of the rubber netting, which previously yielded NDBA (6). As expected, highly significant differences ($p < 0.01$) were found among random samples. No significant difference ($p < 0.05$) in results was found for the 2 methods. The overall mean NDBzA from SFE-analyzed samples was 63.2 ppb, and the overall mean from the SPE-analyzed samples was 63.7 ppb. The repeatability standard deviation of the SFE method was 1.7 ppb, with a coefficient of variation (CV) of 2.7%, compared with 2.2 ppb and a CV of 3.5% for the SPE method. The overall mean recovery of NDPA from SFE-determined samples was 93.4%, and the overall mean from SPE samples was 85.0%. These results show that the SFE method is comparable with the SPE procedure for analysis of hams. GC-TEA chromatograms obtained from SFE-analyzed ham samples also appeared to be “cleaner” than those from the SPE-analyzed samples, even with the difference in sample size, 5 versus 10 g.

Although NDBzA was the only nitrosamine detected in ham samples, the presence of NDBA and, to a lesser extent NPPIP, may still be possible. Their presence would be due to continued use of zinc dibutylthiocarbamate and dipentamethylene thiuram tetrasulfide as vulcanizing agents in rubber formulation. A spiked recovery study showed that these 2 nitrosamines

Table 2. Determination of N-nitrosodibenzylamine (NDBzA) in netted hams by SFE and SPE

Sample	SFE ^a		SPE ^a	
	NDPA, %	NDBzA, ppb ^b	NDPA, %	NDBzA, ppb ^b
A	89.9	110.5	89.7	111.3
B	93.9	9.0	66.6	9.1
C	96.2	6.1	71.8	7.0
D	97.3	9.2	77.9	9.7
E	97.5	132.0	69.8	132.4
F	94.5	146.9	94.9	139.6
G	93.4	ND ^c	88.8	ND
H	97.7	118.1	100.0	115.5
I	81.9	21.1	94.5	28.5
J	89.6	58.2	91.9	66.7
K	88.5	27.4	98.2	31.0
L	128.1	157.3	91.1	157.3
M	79.5	100.8	95.2	99.8
N	82.1	146.6	85.5	143.8
O	88.7	43.7	84.6	43.3
P	83.2	25.8	91.6	28.4
Q	99.3	69.7	79.5	69.3
R	107.9	6.6	75.4	4.9
S	88.4	24.1	73.2	26.9
T	86.7	69.0	78.8	68.1
U	97.9	45.1	85.8	44.5

^a Results are averages of duplicate determinations.

^b Data corrected for recovery of the NDPA internal standard.

^c ND, not determined.

could be isolated by SFE. However, to ensure that normally incurred NDBA and NPIP could be successfully extracted from ham by SFE, older ham samples previously found to contain these specific nitrosamines were analyzed. Again, the results showed no differences in NDBA and NPIP values between SFE and SPE methods.

Analysis for regulatory purposes requires use of standardized equipment and conditions to ensure good reproducibility of results. In this paper, we have reported a study conducted with a prototype commercial SFE instrument using an in-line nitrosamine collection system with an SPE cartridge, as shown in Figure 1. This SFE system avoided the use of a time consuming off-line transfer step and potential nitrosamine loss.

Conclusions

Although very little research has been done on the use of SFE to extract nitrosamines, this is a promising technique for extracting both volatile and semivolatile nitrosamines from complex food matrixes. SFE can extract various volatile nitrosamines, both aliphatic and alicyclic, and the semivolatile NDBzA from a low-fat cured meat product,

Table 3. Analysis of variance of SFE and SPE data

Source	Degrees of freedom	Sum of squares	Mean square	F value
Sample	20	222947.24	11147.36	2852.89
Method	1	4.79	4.79	1.22
Sample × method	20	224.59	11.23	2.87
Error	42	164.11	3.91	
Total	83	223340.73		

ham. The minimal use of solvent, 16 mL compared with approximately 500 mL for SPE, will help laboratories in meeting new Environmental Protection Agency guidelines for solvent reduction (12). SFE also will reduce analysis times: 20–24 samples per day can be analyzed by SFE, compared with 8–10 samples by SPE. This new method will also meet the needs of regulatory agencies and others who analyze foods for carcinogenic N-nitrosamines.

Acknowledgments

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