

FOOD ADDITIVES

Determination of Volatile *N*-Nitrosamines in Frankfurters Containing Minced Fish and Surimi

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A method was developed to quantitatively measure volatile *N*-nitrosamines, particularly *N*-nitrosodimethylamine (NDMA), in cured minced fish or surimi-meat frankfurters. This method is free from artifact formation. First, 2 dry solid-phase extraction columns are prepared. Solvent is passed through the top column containing the fish-meat into a second column containing acid Celite. The eluate from the Celite column is then passed through a third column containing silica gel. Nitrosamines are eluted from the acid Celite column and then from the silica gel column into the same receiver. Recovery of the internal standard, *N*-nitrosoazetidine, added at the 10 ppb level, was 86.5%. In addition, a few samples of nitrite-treated salmon (lox) were also tested for *N*-nitrosamines. The results show that the method is applicable to samples containing nitrite-treated fish and fish-derived products.

Higher value-added uses for low-cost mechanically deboned fish flesh, particularly that derived from small, under- or non-utilized species or from larger fish after filleting, have been investigated for many years to more fully develop and support the U.S. domestic fishing industry (1). Proposals have been made to combine the high nutritional quality fish protein in the form of surimi or unwashed or washed minced fish with red meat or poultry to prepare a variety of processed or comminuted products (1, 2). These proposals include the use of fish in cured meat products such as frankfurters, bolognas, and salamis that comprise over 2 billion pounds of popular consumer products in the United States. Minced fish is mechanically separated (deboned) fish, whereas surimi is the Japanese term for a colorless, odorless, and tasteless salt-solubilized protein product obtained from minced fish that has gone through repeated washing steps.

In 1980, the National Marine Fisheries Service petitioned USDA's Food Safety and Inspection Service (FSIS) to amend the standard of identity for cooked sausage to permit the inclusion of up to 15% fish in frankfurters as an optional ingredient. One of the safety concerns raised by FSIS was the possible presence of nitrosamines, particularly *N*-nitrosodimethylamine (NDMA) in the case of cured (nitrite-treated) products, since fish contains a much higher concentration of dimethylamine and its precursors than does meat. Of particular concern is the enzymatic breakdown of trimethylamine oxide to formaldehyde and dimethylamine in commercially important gadoid fish (3), which includes cod, haddock, hake, pollock, and whiting. Part of the research data submitted to FSIS in support of the petition showed that higher levels of NDMA were found in combination fish-meat compared with all-meat (control) frankfurters (1). In addition, different NDMA values were obtained when various analytical methods were used, suggesting artifactual NDMA formation as a result of the method of analysis (1). Nitrosation inhibitors such as sodium ascorbate and α -tocopherol were not effective in eliminating artificial nitrosamine formation with the mineral oil distillation method (1).

Because of the latter occurrence and the questionable accuracy of the NDMA data previously obtained from this type of cured fish-meat product, we carried out investigations to develop a reliable method applicable to this type of substrate.

METHOD

Reagents

(a) *Celite 545*.—Not acid-washed (Fisher Scientific Co.). Test reagent blank before starting sample analysis, particularly if new bottle of Celite is used. If interfering chromatographic peaks are noted, prewash Celite twice with dichloromethane (DCM); then dry 4 h in 120°C vacuum oven before use.

(b) *Phosphoric acid*.—6N. Extract once with equal volume of DCM before use to remove potential impurities.

(c) *Sodium sulfate*.—Anhydrous, granular (Mallinckrodt No. 8024).

(d) *Dichloromethane (DCM), pentane, ethyl ether*.—Distilled in glass (Burdick and Jackson).

(e) *Silica gel*.—70–230 mesh (EM 7734), A. H. Thomas Co. (dried prior to use).

(f) *N-Nitrosoazetidine (NAZET) and N-nitrosohexamethylethylamine (NHMI) internal standard solution*.—0.10 μ g each/mL DCM.

(g) *GC working standard solution*.—NDMA, *N*-nitrosomethylethylamine (NMEA), *N*-nitrosodiethylamine (NDEA), NAZET, *N*-nitrosopiperidine (NPIP), *N*-nitrosopyrrolidine (NPYR), *N*-nitrosomorpholine (NMOR), and NHMI, 0.10 μ g each/mL DCM. All *N*-nitrosamines were synthesized from their corresponding amines and sodium nitrite according to general procedure published previously (4). *Note*: *N*-Nitrosamines are potential carcinogens. Exercise care in handling these materials.

(h) *Fish-meat samples*.—Unwashed minced fish-meat and surimi-meat frankfurters in which 15% of the meat was substituted were prepared by the National Marine Fisheries Service, Southeast Fisheries Center, Charleston Laboratory, following a typical industry procedure (1). In addition, 156 ppm sodium nitrite (NaNO_2) and 550 ppm sodium erythroate were added to the formulation prior to processing. The finished products were shipped to ERRC in Styrofoam insulated containers containing cold packs. Upon receipt, frankfurters were removed from their casings and refrigerated at 4°C until analyzed (1–3 days). Different brands of smoked salmon (lox) were purchased from local retail stores.

Apparatus

Usual laboratory equipment and the following items:

(a) *Mortar and pestle*.—Glass, 473 mL (16 oz; A. H. Thomas).

(b) *Chromatographic columns*.—(1) Glass, 350 \times 32 mm id with 60 \times 6 mm id drip tip, for step 1 of procedure. (2) Chromaflex, glass, 250 \times 14.5 mm id (Kontes No. K-420530), for step 2 of procedure, with 19/22 joint glassblown to top of column if possible.

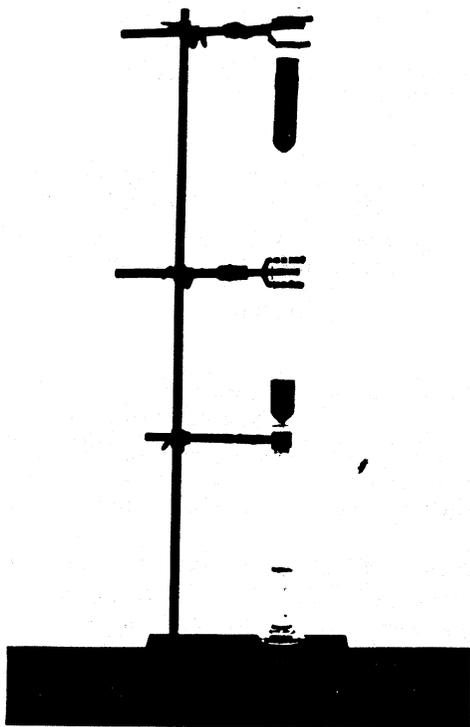


Figure 1. Elution of nitrosamines and amines from sample column to acid-Celite column.

(c) *Evaporative concentrator.*—Kuderna-Danish (KD), 250 mL and 125 mL; concentrator tube, 4 mL, graduated; Snyder (3-section) and micro-Snyder distilling columns (Kontes Glass Co.).

(d) *Tamping rod.*—Glass, 450 mm long with 12 mm diameter disk, prepared by local glassblower.

(e) *Gas chromatograph-thermal energy analyzer (GC-TEA).*—Complete details on the operating system have been published previously (5), except for the following change: column programmed from 120 to 220°C at 4°/min. Check chromatographic column to assure that individual nitrosamines in standard solution are sufficiently resolved for quantitation.

Procedure

(a) *Reagent control.*—Some brands of DCM are known to cause artifactual formation of nitrosamines or to contain TEA responsive peaks. Test the former by adding 100 mg “NMOR-free” morpholine to 200 mL DCM, let stand overnight at room temperature, and then concentrate to 0.5 mL prior to GC-TEA analysis. For the latter, concentrate 200 mL DCM to 0.5 mL and check for interfering TEA responsive peaks. In addition, reagent blank should also be analyzed.

(b) *Sample preparation.*—Grind uncooked fish-meat frankfurters twice through 1/8 in. plate prior to analysis. Grind all other products, as above, prior to analysis.

(c) *Sample analysis.*—Step 1: Isolation of NDMA, NPYR, and NMOR from sample matrix. Into 250 mL beaker, weigh 10.0 g Celite and add 10 mL 6N phosphoric acid, ca 3 mL at a time, while stirring with small glass rod until mixture is

fluffy and uniform in texture. Using powder funnel, pour all acid-Celite mixture into 350 × 32 mm chromatographic column containing glass wool plug at the bottom. Insert tamping rod through acid-Celite mixture and tamp to achieve height of ca 25 mm. Add 25.0 g anhydrous sodium sulfate to top of column.

Weigh 10.0 ± 0.1 g sample into mortar, add 1.0 mL internal standard solution (equivalent to 10 ppb) with 1.0 mL transfer pipet, add 25.0 g anhydrous sodium sulfate, and mix with pestle ca 15 s; then add 20.0 g Celite and again mix with pestle 15–20 s until Celite is thoroughly mixed with sodium sulfate and sample. Grind entire mixture with moderate pressure an additional 1–1.5 min, and quantitatively transfer mixture into second chromatographic column (350 × 32 mm) containing glass wool plug; tamp with tamping rod to achieve a height of ca 75 mm. To top of column, add 25 g anhydrous sodium sulfate. Place second column containing sample mixture directly over first column containing acid-Celite such that solvent will flow through sample-containing column directly into acid-Celite column (Figure 1). Rinse mortar, pestle, and tamping rod with 15 mL pentane-DCM (95 + 5) and add rinse to top of column. Immediately add an additional 160 mL of the same solvent to column. Collect eluate that passes through both columns in 100 mL graduated cylinder. When solvent from top column stops dripping, remove and discard contents of top column. Change receivers to 250 mL K-D flask equipped with 4 mL concentrator tube (Figure 2). Save eluate collected in 100 mL graduate for further analysis (step 2). Add 125 mL DCM to bottom column containing acid-Celite mixture, and collect eluate. When column stops dripping, remove K-D flask and stopper and save it for collection of eluate from silica gel column described in step 2. *Note:* Procedure can be stopped at this point if NDMA, NPYR, and NMOR, contained in the K-D flask, are the only nitrosamines of interest.

Step 2: Isolation of NMEA, NDEA, NPIP, and NHMI from eluate in step 1. Add eluate in 100 mL graduated cylinder (containing NMEA, NDEA, NPIP, and NHMI) to silica gel column chromatography following a modification of procedure described previously (6): To Chromaflex column containing glass wool plug and 25 mL pentane, add 5.0 g silica gel and 5.0 g anhydrous sodium sulfate; then attach 125 mL K-D flask to top of column. Wash column with 25 mL pentane. Stop flow when liquid level is 2 mm above sodium sulfate. Quantitatively transfer contents of 100 mL graduated cylinder (from step 1) to silica gel column, with small volumes of pentane. Collect eluate in 500 mL Erlenmeyer flask (flow rate ca 70 drops/min). When liquid level in column reaches top of sodium sulfate layer, add 100 mL wash mixture (25% DCM in pentane) to 125 mL K-D flask. When liquid level in column again reaches sodium sulfate layer, attach 250 mL K-D flask containing eluate from acid-Celite column (step 1). Charge column with 125 mL 30% ether in DCM to elute nitrosamines (flow rate ca 70 drops/min). Attach Snyder column to K-D flask, and concentrate sample to 1.0 mL as described previously (5). Do not concentrate sample with stream of nitrogen.

(d) *Nitrosamine determination.*—Quantitatively measure volatile nitrosamines as described previously (5). Minimum detectable level of NDMA is 0.2 ppb.

(e) *Residual sodium nitrite.*—Determine residual sodium nitrite on 10.0 g uncooked sample by modified Griess-Saltzman procedure (7).

(f) *Statistical analysis.*—Statistical analyses were carried out according to the methods of Snedecor and Cochran as described previously (8).

Results and Discussion

Initially, we investigated a number of methods to determine their accuracy in quantitating NDMA in a cured combination fish-meat substrate without artifactual formation of nitrosamines. We eliminated the commonly used mineral oil distillation procedure because it has already been shown to form nitrosamines during analysis, especially when high levels of residual nitrite are present (5, 8). It was noted that extreme care should be exercised to obtain results with good repeatability, even when artifactual nitrosamine formation does not occur. For this reason, attention was focused on nondistillation methods that would minimize the reaction between any residual nitrite and nitrosatable amine as would occur during heating.

The ERRC dry column method (5), primarily designed to test for NPYR in fried bacon, was tested despite the fact it was not developed to handle food substrates containing extremely high levels of amines; it was found not to be useful. Therefore, in developing a new solid-phase extraction method, it was thought essential that the amines contained in the substrate be separated from the residual nitrite prior to analysis. In addition, the principal emphasis was placed on NDMA because this nitrosamine was of major concern in this type of product.

While the occurrence of NPYR, presumably from putrescine, does not appear to be widespread, there have been a few reports of its occurrence in fish-related foods (9, 10). Morpholine is sometimes used as an anti-corrosive agent in steam boilers. If morpholine-containing steam is used during surimi processing, NMOR could be formed in a surimi-meat frankfurter product similar to that found in soy protein concentrate by Fazio and Havery (11), or in steam used during smokehouse processing by Gray et al. (12). We subsequently developed a method that is applicable for the determination of NDMA, NPYR, and NMOR in cured fish-meat frankfurters. We also demonstrated that NAZET can serve as an internal standard for the analysis of NDMA with this procedure because of the high correlation between the 2 nitrosamines ($P < 0.01$, $N = 11$, correlation coefficient = 0.7621).

We have since added a second step to the procedure, based on the method developed by White et al. (6), to quantitate NDEA, which can be occasionally found in fish (13), and NPIP, which is commonly associated with piperine from pepper (14), and with cadaverine from the bacterial/enzymatic decarboxylation of lysine (15). This may be especially important since fish protein has been reported to have a higher lysine content than beef, pork, lamb, and poultry (16). It should be noted, however, that initial investigations (1) and our studies, to date, have indicated that NDMA is the only volatile nitrosamine detected in the fish-meat samples.

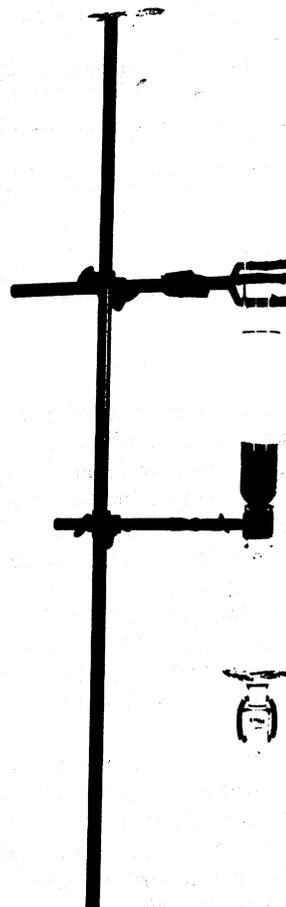


Figure 2. Elution of nitrosamines from acid-Celite column.

A comparison was made between our new solid-phase extraction technique for combination fish-meat samples and the ERRC dry column method which combines the sample matrix and the acid-Celite into the same column. In addition, we also included a variation of the dry column method used to determine NDMA in beer or malt which incorporates sulfamic acid into the sample matrix to destroy residual nitrite prior to analysis (17). Each sample was spiked with 50 ppm morpholine, a rapidly nitrosated amine, prior to analysis to assess the degree of artifactual formation by subsequent measurement of the NMOR content. The results (Table 1) show that the ERRC dry column method formed small amounts of NMOR in some of the samples. Significantly higher levels were detected in the same samples when sulfamic acid was added to destroy residual nitrite. Perhaps the acidic conditions caused by the use of sulfamic acid favored

Table 1. *N*-Nitrosodimethylamine in meat frankfurters containing 15% minced fish or surimi (method comparison)*

Sample	Residual NaNO ₂ , ppm	New method, ppb		ERRC DC method, ppb		DC-sulfamic acid method, ppb	
		NDMA	NMOR	NDMA	NMOR	NDMA	NMOR
Menhaden	23	ND	ND	ND	1.0	ND	21.0
Cod	14	3.3	ND	3.2	Tr	3.0	16.0
Cod	5	ND	ND	ND	ND	ND	ND
Mullet	18	0.3	ND	0.5	Tr	ND	ND
Pollock	65	1.2	ND	2.4	5.9	1.7	8.6
Red hake	84	6.9	ND	7.7	1.6	20.5	4.3
Cod	68	1.5	ND	1.6	3.8	2.5	8.5
Whiting	31	1.3	ND	0.9	1.5	1.2	3.1
Pollock surimi	70	ND	ND	ND	2.0	ND	2.6
Menhaden surimi	28	ND	ND	ND	ND	ND	ND

Table 2. Statistical analysis on repeatability of solid-phase extraction method

Sources of variation	NDMA, corrected				Recovery of NAZET, %		
	Degrees of freedom	Sum of squares	Mean square	F-value	Sum of squares	Mean square	F-value
NDMA	7	3826.27	546.61	2348.99**	909.45	129.92	3.20
Error	8	1.86	0.23		324.91	40.61	
Total	15	3828.13	—		1234.36	—	
Repeatability*			0.48 ppb			6.37%	

** $P < 0.01$.

* Repeatability = $\sqrt{MS_{error}}$.

the nitrosation of morpholine over destruction of nitrite. The separation of the nitrosamines and amines from the residual nitrite in our new method, prior to exposure to the acid-Celite, prevented artifactual nitrosamine formation. This was supported by the lack of detectable NMOR in any of the 10 samples tested. All of the minced fish franks yielded less than 10 ppb NDMA, with no detectable levels in the 2 surimi-based franks. Of interest, the highest NDMA value in the minced fish products tested occurred in red hake, the same species initially found to have high values by the mineral oil distillation method, albeit with possible artifact formation (1).

Eight fish-meat frankfurter samples were analyzed in duplicate to determine the within-laboratory repeatability of the new solid-phase extraction method. Determinations of NDMA ranged from 0.66 to 50.33 ppb (corrected). The recovery of the NAZET internal standard (10 ppb) ranged from 64.4 to 101.3% with a mean of 86.5%. The analysis of variance (Table 2) showed that the variable within-laboratory repeatability standard deviation was 0.48 ppb for NDMA, and 6.37% for recovery of the NAZET standard.

A ruggedness test of the method for the determination of 1.0 and 10.0 ppb NDMA, conducted by varying the grinding, packing, and solvent elutions steps specified in the experimental section, showed that only packing the top column too tightly (60–65 mm) may cause NDMA values to vary significantly. This particular step has been shown to be a possible source of error in all of the procedures that utilize this solid-phase extraction method (5, 18).

The recoveries of 8 volatile *N*-nitrosamines added to nitrosamine-free fish-meat frankfurters at the 10 ppb level were as follows: NDMA, $97.1 \pm 4.5\%$; NMEA, $80.8 \pm 9.6\%$; NDEA, $69.6 \pm 7.4\%$; NPIP, $95.3 \pm 9.2\%$; NPYR, $92.5 \pm 3.3\%$; NMOR, $94.1 \pm 4.0\%$; NHMI, $85.3 \pm 7.4\%$ and NAZET, $92.5 \pm 2.3\%$. These results show that our method can be applied to a wide variety of volatile nitrosamines that may be found in cured meats and other types of food products. In addition, the method is applicable for rapidly screening the fish-meat frankfurters for NDMA by using only step 1 of the procedure. This shortened procedure can be useful if it can be demonstrated that NDMA is the only volatile *N*-nitrosamine of interest in this type of product.

Finally, to determine if this method is applicable to other fish-based substrates, particularly a nitrite-treated fish product such as smoked salmon (lox), 8 nitrosamines were added at the 10 ppb level to 6 different smoked salmon samples that initially had no detectable nitrosamines. While salmon does not contain the large amount of trimethylamine oxide that is present in members of the gadoid family, it does contain significantly more than many freshwater fish (19). Since salmon is consumed fresh or is cured with nitrite and subjected to smokehouse processing, the possibility exists that NDMA may be present (20). The analysis of the smoked salmon samples included both the solid phase extraction

columns (step 1) and the silica gel column (step 2). Average recoveries of the nitrosamines were as follows: NDMA, $77.2 \pm 6.7\%$; NMEA, $56.0 \pm 3.5\%$; NDEA, $49.2 \pm 2.6\%$; NPIP, $84.7 \pm 2.3\%$; NPYR, $95.2 \pm 3.6\%$; NMOR, $93.6 \pm 3.6\%$; NHMI, $87.9 \pm 2.5\%$; NAZET, $86.3 \pm 2.7\%$. Inexplicably, the recoveries of NMEA and NDEA were not as high in the smoked salmon samples as in the minced fish-meat franks; however, the nitrosamines of primary interest will be detected with this procedure. The apparent NDMA detected by GC-TEA in both the smoked salmon and the minced fish frankfurters was confirmed by the UV-photolysis-GC-TEA procedure described by Doerr and Fiddler (21).

In conclusion, we are reporting a method for the determination of volatile nitrosamines in meat frankfurters containing minced fish or surimi which is not susceptible to artifactual nitrosamine formation. This method can be used to rapidly determine NDMA in this type of product or to screen for other volatile nitrosamines by including the silica gel column step in the procedure. This method is also applicable to nitrite-treated fish products that may contain potentially high levels of nitrosatable amines (unpublished data).

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