Rapid Determination of Volatile N-Nitrosamines in Nonfat Dry Milk

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ABSTRACT
A rapid gas chromatographic-thermal energy analysis method was developed for determination of volatile nitrosamines in nonfat dry milk. The average recovery of nitrosodimethylamine added to nonfat dry milk at 3 ppb was 96%. In a nationwide survey of nonfat dry milk, nitrosodimethylamine was found in 48 of 57 samples from .1 to 3.7 ppb, and at an average .6 ppb. Apparent nitrosopyrrolidine and nitrosopiperidine averaged approximately .1 ppb, the detection limit of the method.

INTRODUCTION
In 1978 nitrosodimethylamine (NDMA), a potent animal carcinogen, was identified in West German-produced beers (12). Investigations by other researchers corroborated these findings in beers manufactured throughout the world (2, 3, 4, 9, 11, 13). Malt was the source of the nitrosamine. Further studies have suggested that the nitrosamine may have been formed during the direct drying of the malt when nitrogen oxides from the combustion of the fuel reacted with naturally occurring amines in the malt (3, 9).

Since nitrosamine formation has been linked to the direct drying process, other similarly dried foods also may contain nitrosamines. In the United States, for example, 907 billion kg of nonfat dry milk (NDM), the majority of which is dried by direct flame, are produced annually for use in other foods and for reconstitution for drinking. Reineccius and Coulter (7) investigated NDM that was dried in both direct flame and indirectly heated dryers but found no nitrosamines by a method with a 10-ppb detection limit. More recently, Lakritz and Pensabene (5), in a survey of milk and dairy products, found NDMA in 9 of 10 NDM samples at an average 1.7 ppb. Sen et al. (10) identified NDMA in NDM by a gas chromatograph coupled to a thermal energy analyzer. Libbey et al. (6) found NDMA in NDM at an average 1.9 ppb and confirmed their results by mass spectrometry (MS). These findings prompted the Food and Drug Administration to conduct a national survey of NDM for volatile nitrosamines. This paper describes a rapid method for the analysis of NDM for volatile nitrosamines and reports the results of a survey of United States NDM producers.

MATERIALS AND METHODS
A simple column chromatographic elution of the nitrosamine from an NDM-Celite-water mixture was followed by concentration and gas chromatographic/thermal energy analysis (GC/TEA).

Reagents
Reagents include Celite 545 (Fisher; fired overnight at 700°C in a muffle furnace before use), ammonium sulfamate (ACS, Fisher), sulfuric acid (reagent, Baker), anhydrous sodium sulfate (granular, ACS, Mallinckrodt), dichloromethane (Burdick and Jackson), distilled water (double distilled), glass wool (Pyrex), carborundum grains, and nitrogen (ultra-high purity). An ammonium sulfamate-sulfuric acid solution was prepared by dissolving 1.0 g of ammonium sulfamate in 100 ml of 1 N sulfuric acid. Reagent blanks were analyzed to ensure the absence of interfering peaks.

A standard mixture of 14 volatile nitrosamines, including nitrosodimethylamine, nitrosopyrrolidine, and nitrosopiperidine (Aldrich or Eastman), was prepared by weighing 100 mg of each compound into separate flasks followed by serial dilution with dichloromethane to obtain a concentration of 100 μg/ml. An aliquot of each diluted nitrosamine was mixed together to produce a standard sample.

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containing .5 μg/ml of each of the 14 volatile nitrosamines.

Sample Analysis

In a 600 ml beaker, 25.0 g of NDM and 40 g of Celite were mixed. A 25-ml aliquot of the ammonium sulfamate-sulfuric acid solution was added and mixed until homogeneous. The mixture appeared fluffy in consistency. A plug of glass wool was placed in the chromatographic column (32 mm i.d. by 400 mm) and covered with 20 g of anhydrous sodium sulfate. The tamping rod (19 mm diameter disc) and powder funnel (145 mm) were placed in the column with the end of the tamping rod extending through the funnel opening. The NDM-Celite mixture was added to the column and tamped firmly in small increments to a total bed depth of 16 to 20 cm. The beaker was rinsed with 125 ml of dichloromethane and poured onto the column. The eluate was collected in a 250-ml Kuderna-Danish evaporative concentrator fitted with a 4-ml concentrator tube (Kontes). If necessary, the column flow was adjusted to a rate of 1 to 2 ml/min. When the column had stopped dripping, carborundum grains were added, a three-chamber (Kontes) Snyder column was attached to the flask, and the eluate was concentrated in a 60°C water bath to about 4 ml. When the Snyder column had drained completely, the concentrator tube was removed and the extract further concentrated to 1.0 ml under a gentle stream of nitrogen.

Gas Chromatography/Thermal Energy Analyzer Conditions

The nitrosamines were separated on a 2.7 m by 4 mm i.d. glass column packed with 10% Carbowax 1540 (Analabs) plus 5% KOH (ACS, Fisher) on 100 to 120 mesh Chromosorb WHP (Supelco) with either a Hewlett-Packard Model 5710A or a Fisher Series 4400 gas chromatograph. The column was programmed from 100 to 180°C at 4°C/min for each analysis. The carrier gas was argon at a flow rate of 40 ml/min. The injection port was maintained at 200°C. The gas chromatograph was coupled to a Model 5021 thermal energy analyzer (Thermo Electron Corp., Waltham, MA), which was operated at a vacuum of approximately 1.1 mm of mercury, furnace at 450°C, and in the x 2 to x 8 attenuation ranges, with a liquid nitrogen trap. Chromatograms were obtained with either a Tracer Model MT 21 or a Fisher Series 100 recorder operated at 1.3 cm/min.

An 8-μl aliquot of the final sample extract was injected into the GC/TEA system. Nitrosamines were identified by comparison of retention times with those of reference standards. Peak heights were used to calculate nitrosamine concentrations.

RESULTS AND DISCUSSION

To determine the repeatability of the method, fifteen 25 g portions of the same NDM were analyzed by the column elution method for volatile nitrosamines. The NDMA was at an average of 4.29 ppb with a standard deviation of .7 and a coefficient of variation of 16%. The method had a detection limit of .1 ppb, calculated by using a ratio of signal to noise of 5:1.

The efficiency of the method was determined by spiking 11 NDM samples with NDMA at 3 ppb. Recoveries of NDMA were 95, 90, 94, 92, 93, 102, 94, 102, 108, 92, and 95% (range 90 to 108, average 96).

Artifact Study

To determine if nitrosamines could be formed as a result of the analytical method, an NDM sample was spiked at 10 ppm with both sodium nitrite and morpholine. Analysis of the sample showed neither enhancement of NDMA previously in the NDM, nor the presence of nitrosomorpholine, verifying that the method did not produce artifactual nitrosamines.

The NDM samples, collected by government inspectors from plants in their respective districts, were analyzed for volatile nitrosamines. The results of the survey of 57 samples are shown in Table 1. The NDMA was in 84% of NDM samples and averaged .6 ppb. Ten samples contained greater than 1 ppb NDMA, whereas 9 samples had no detectable NDMA. Quantities of NDMA in this survey are similar to those observed by other researchers (5, 6). Typical chromatograms of an NDM sample and a nitrosamine standard are in Figure 1.

Two other volatile nitrosamines, nitrosopiperidine and nitrosopyrrolidine, were in 35% and 46% of the samples, respectively, but
TABLE 1. Volatile nitrosamines in nonfat dry milk.

<table>
<thead>
<tr>
<th>Nitrosamine</th>
<th>No. samples</th>
<th>ppb Found</th>
<th>Range (ppb)</th>
<th>Avg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrosodimethylamine</td>
<td>9</td>
<td>ND</td>
<td>ND—3.7b</td>
<td>.6</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>&lt;1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>&gt;1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrosopyrrolidine</td>
<td>31</td>
<td>ND</td>
<td>ND—.8</td>
<td>.1</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>&lt;1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>&gt;1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrosopiperidine</td>
<td>37</td>
<td>ND</td>
<td>ND—.5</td>
<td>&lt;.1</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>&lt;1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>&gt;1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aNone detected.  
bConfirmed by gas chromatography/mass spectrometry.

Averaged at the detection limit of the method as calculated by averaging all measurements, assigning zero to those below .1 ppb.

Nitrosamines in NDM are generally lower than those in other foods, especially as NDM is not consumed directly but is mixed with other foods or water. By comparison, NDMA is in beer and malt at averages of 1 ppb and 3 to 13 ppb, respectively (3), and nitrosopyrrolidine is in fried bacon at 10 to 20 ppb according to the most recent data (private communication). A review of nitrosamines in foods has been published (8).

The gas chromatograph/thermal energy analyzer used for nitrosamine detection and quantitation is a highly sensitive and relatively selective detector for nitrosamines. However, compounds other than nitrosamines give a positive GC/TEA response (1). Because nitrosamines encountered in these NDM samples were so low, confirmation by gas chromatography/mass spectrometry (GC/MS) was attempted only on the sample containing the greatest quantity (3.7 ppb) of NDMA. By scaling up the analysis (300 g) and washing the dichloromethane extracts with 25 ml of 6 N hydrochloric acid, 25 ml of 5 N sodium hydroxide, and 25 ml of 20% sodium bisulfite solution, NDMA was identified positively by GC/MS. Recently Libbey et al. (6), in a survey of dried dairy products, positively confirmed NDMA by GC/MS in NDM by scaling up the analysis and using a multiple injection and trapping technique. Further evidence for NDMA in NDM was provided by Lakritz and Pensabene (5), who subjected NDM extracts to ultraviolet (UV) photolysis, successfully destroying the UV-sensitive nitrosamine.

The mechanism of nitrosamine formation in direct flame dried foods is uncertain at this time. Volatile nitrosamines in malt and NDM dried by a direct flame suggest that perhaps a
nitrosating species generated by combustion of the fuel reacts with amines occurring naturally in the food being dried, forming nitrosamines. There is strong evidence to support this hypothesis. In malt, introduction of sulfur dioxide into the hot flame gases during drying significantly reduces the quantity of nitrosamine formed in the malt (3). Also, malt dried by an indirect heat source contains little or no nitrosamines (unpublished data). Recently however, Lakritz and Pensabene (5) found NDMA in 11 of 21 samples of pasteurized fluid milk at an average .1 ppb, and in 9 of 10 NDM samples at an average 1.7 ppb. When NDM is reconstituted at a ratio of 1:10, NDMA approximates concentrations in pasteurized milk. Lakritz and Pensabene (5) concluded that if nitrosation is occurring during the drying process, it is proceeding at a relatively low rate. In the same study, no nitrosamines were in raw milk, suggesting that nitrosation is during pasteurization. Further research is needed to determine the source and cause of nitrosamine formation in dairy products.

REFERENCES