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Determination of N-nitrosodimethylamine in fish products using gas chromatography with nitrogen–phosphorus detection

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SUMMARY

A simple and rapid gas chromatographic method for the determination of N-nitrosodimethylamine (NDMA) in fish products is described. NDMA is extracted from a dried sample with methylene chloride, mixed with *n*-hexane and passed through a silica gel column. NDMA adsorbed on silica gel is eluted with methylene chloride–diethyl ether (7:3) and the eluate is passed through a Sep-Pak alumina A cartridge column, on which NDMA is adsorbed. NDMA is eluted from the cartridge with diethyl ether–methanol (2:1) and the solution is injected into a gas chromatograph with nitrogen–phosphorus detection. This method does not use solvent evaporation and concentration in the clean-up procedure, which eliminates the loss of volatile NDMA and artifactual formation of NDMA in the analytical procedure. The detection limit is 0.5–1 μg/kg and recoveries from salted pollack roe spiked at 40 and 4 μg/kg were 96.7% [relative standard deviation (R.S.D.) 3.6%] and 85.0% (R.S.D. 6.8%) respectively.

INTRODUCTION

There has been increasing concern over the occurrence of N-nitroso compounds in foods, because these compounds have been demonstrated to be carcinogenic in several species of animals and are therefore likely to be related to human cancer[1,2]. N-Nitrosodimethylamine (NDMA) causes liver damage in humans and rats[3] and is carcinogenic in rats[4]. Fish contains a high concentration of dimethylamine and its precursors[5,6], from which NDMA may be formed by reaction with various nitrosating agents (nitrous acid, nitrogen oxide and nitrous acidinium ion). Accordingly, fish and fish-derived foods must be routinely screened for NDMA content, especially in Japan where various types of fish products are used in the daily diet as protein sources.

A number of extraction and clean-up procedures for determining nitrosamines in foods have been described, including direct solvent extraction (followed by column chromatography)[7], solvent extraction on a dry Celite column[8,9], steam distilla-

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tion[10–12], low-temperature vacuum distillation[13] and high-temperature purge and trap methods[14–16]. These procedures are relatively cumbersome and time consuming, and include solvent evaporation and/or a high-temperature distillation step, which may cause losses[17] and artifactual formation of NDMA[8,18–20].

We have developed a simple and rapid extraction and clean-up method, in which a silica gel column and an alumina cartridge column clean-up are used successively. In this procedure, clean-up and concentration of the sample extract are carried out simultaneously, which avoids losses and artifactual formation of NDMA. Gas chromatography (GC) with a thermal energy analyser is generally the most accepted instrumental technique, because the latter is relatively specific for nitrosamines and is sufficiently sensitive enough. However, thermal energy analysers are expensive and are not commonly used in food quality control laboratories. We therefore used GC with nitrogen–phosphorus detection (NPD).

EXPERIMENTAL

Materials and chemicals

Pesticide-grade chemicals were used as received. *n*-Hexane, methanol, acetone and anhydrous sodium sulphate were obtained from Wako (Osaka, Japan). Alumina (neutral, activity 1; Woelm Pharma, Eschwege, F.R.G.) was used as received. Silica gel (No. 7734; Merck, Darmstadt, F.R.G.) was dried at 140°C for 3 h and stored in a desiccator. An alumina cartridge column (Sep-Pak alumina A) was obtained from Waters Assoc. (Milford, MA, U.S.A.). Methylene chloride and diethyl ether (pesticide grade; Wako) were chromatographed on an alumina column just before use. N-Nitrosodimetylamine (NDMA) was of gas chromatography standard grade (Wako) and used as received.

Standard solutions

NDMA stock standard solution was prepared by dissolving 10 mg of NDMA in methanol and diluting to 10 ml with methanol. Analytical working standard and spiking solutions were prepared by diluting the stock solution with acetone.

Caution: NDMA is a potent carcinogen which should be handled with extreme caution in a ventilated hood. Inhalation of NDMA vapour and contact of NDMA with skin and clothing must be avoided.

Instrumentation

The glass column for clean-up was 300 mm × 15 mm I.D. with a coarse fritted disc and a PTFE stopcock. The GC column was a DB-WAX fused-silica open-tubular column (15 m × 0.53 mm I.D.) (J&W Scientific, Folsom, CA, U.S.A.). A Model 5710A gas chromatograph equipped with a nitrogen–phosphorus detector (Model 18789A, Hewlett-Packard, Avondale, PA, U.S.A.) was used. A direct injection inlet conversion kit (Model 2-3797, Supelco, Bellefonte, PA, U.S.A.) and make-up gas detector adaptor kit (Model 2-3648, Supelco) were used to connect the DB-WAX column to the Model 5710A chromatograph, which was designed for use of only packed columns. The make-up gas line was connected to the second injection port and the flow was regulated with the flow regulator. The GC conditions were as follows: injection temperature, 200°C; column temperature, 60°C (isothermal); detector

temperature, 300°C; carrier gas (nitrogen) flow-rate, 5.5 ml/min; make-up gas (nitrogen) flow-rate, 31.5 ml/min; hydrogen flow-rate, 3 ml/min; and air flow-rate, 100 ml/min.

In GC measurements, after injection and initial elution of solvent peaks, it was occasionally necessary to re-zero the baseline.

Extraction and clean-up

A 10.0-g amount of finely comminuted sample and anhydrous sodium sulphate (in a sufficient amount to dry the sample, 1–8 g) were placed in a glass mortar (I.D. 8 cm, depth 6 cm) and mixed thoroughly with a pestle to dryness. The mixture of sample and sodium sulphate was quantitatively transferred into a 100-ml centrifuge tube. The mortar was washed with 40 ml of methylene chloride and the washings were added to the centrifuge tube. If the water content of the sample was below 20%, 10.0 g of the comminuted sample were directly weighed into a 100-ml centrifuge tube and 40 ml of methylene chloride were added. The centrifuge tube was shaken for 10 min and centrifuged at 1000 *g* for 10 min. The upper extract was filtered through a sodium sulphate bed on a glass filter with suction. The residue was mixed with 10 ml of methylene chloride, centrifuged, and the extract was filtered as above. The extracts were combined and *ca.* 100 ml of *n*-hexane (twice the volume of the extract) were added and mixed thoroughly.

A silica gel column was prepared by dry packing and tapping 5 g of silica gel into a chromatographic glass column and 3 g sodium sulphate were placed on top. The *n*-hexane added extract was passed through the silica gel column and the eluate was discarded (fraction 1). Then the column was eluted with 30 ml of methylene chloride–diethyl ether (7:3) and the eluate was collected (fraction 2). Fraction 2 was passed through the Sep-Pak alumina A cartridge column and the eluate was discarded. Then the cartridge column was eluted with 2 ml of diethyl ether–methanol (2:1) and the eluate was collected in a 10-ml graduated test-tube with a ground-glass stopper. The volume was measured and this solution was ready for injection into the gas chromatograph with NPD. This sample solution was stored in a refrigerator until GC analysis.

Gas chromatographic analysis

The GC parameters were adjusted as described under *Instrumentation*. A 2-μl volume of each standard solution (5–50 μg/kg) was injected into the gas chromatograph and a calibration graph was constructed by plotting the peak area against the amount of NDMA. A 2-μl aliquot of sample solution was injected and NDMA was identified from the retention times of the peaks. The peak area was compared with the calibration graph and the amount of NDMA in the sample solution was calculated. The concentration of NDMA in the sample was calculated by dividing the amount of NDMA by the amount of sample (10 g).

RESULTS AND DISCUSSION

In most previous work methylene chloride was used as the extraction solvent for NDMA, and we also chose it because of its good solubility for nitrosamines. Water-containing samples must be dried for efficient extraction, so such samples were

thoroughly mixed with anhydrous sodium sulphate in a mortar before extraction. For example, salted pollack roe contains *ca.* 60% water and hence must be dried, but most smoked foods can be directly extracted after comminution.

Methylene chloride and diethyl ether must be purified by alumina column chromatography just before use, because aged solvents occasionally gave lower recoveries in our experiments. Probably NDMA was decomposed by hydrochloric acid or peroxide produced in the solvents on standing. An unknown substance in methylene chloride that was reported to enhance artifactual NDMA formation[20] was also removed by this chromatography.

We investigated the clean-up of NDMA extracted from samples by the use of silica gel and alumina column chromatography. In our experiments using NDMA standard, NDMA was not eluted from the silica gel column with *n*-hexane–methylene chloride mixtures (3:1 to 1:1). Therefore, the methylene chloride extract (*ca.* 50 ml) was mixed with *n*-hexane (100 ml) and the solution was passed through a silica gel column (5 g). NDMA adsorbed on the silica gel was then eluted with 30 ml of methylene chloride–diethyl ether (7:3).

West and Day[21] used alumina column clean-up for the determination of volatile nitrosamines in crops and soils, and eluted NDMA from 4% deactivated alumina with 1-chlorobutane or benzene. We tried a Sep-Pak alumina A cartridge column and found that it has an unexpectedly strong adsorbing ability for NDMA, and benzene, methylene chloride, diethyl ether and ethyl acetate could not elute NDMA from this column. Methanol or methanol-containing eluents were necessary for elution of NDMA from Sep-Pak alumina A. Therefore, when methylene chloride–diethyl ether eluate from the silica gel column was passed through the Sep-Pak alumina A column, NDMA was completely adsorbed, and could subsequently be eluted with 2 ml of diethyl ether–methanol (2:1).

Hence, we were able to clean-up and concentrate the sample extract solution (*ca.* 50 ml) to *ca.* 2 ml by two successive column chromatographic steps on silica gel (5 g) and Sep-Pak alumina A without the need for solvent evaporation. With this procedure, we could avoid losses of low-boiling NDMA and artifactual formation of NDMA in the analytical procedure. Crosby[17] stated that solvent evaporation is the single most critical step in nitrosamine analysis, and the artifactual formation of nitrosamines in the analytical procedure has always been a problem in nitrosamine analyses, various nitrosation inhibitors being used[17,18–20].

Fig. 1 shows gas chromatograms obtained with NPD of standard NDMA and extracts from salted pollack roe. There were no interfering peaks on the sample extract chromatograms, demonstrating that the sample extracts were effectively cleaned up by the two successive column chromatographic steps.

The detector response varied with the experimental conditions such as hydrogen and air flow-rates, activity of alkali source, collector voltage and detector temperature. Amounts of 3–5 pg of NDMA could be detected using the optimum conditions in our equipment, and ordinarily 10–20 pg of NDMA could be detected. Hence, the eluate from the Sep-Pak alumina A column (*ca.* 2 ml) can be injected directly into the gas chromatograph with a nitrogen–phosphorus detector. The detection limit in the sample is 0.5–1 μg/kg, and 0.3 μg/kg using the optimum conditions.

The recoveries of NDMA added to NDMA-free salted pollack roe at 40 and 4 μg/kg are given in Table I. These recoveries are higher than those obtained by a steam

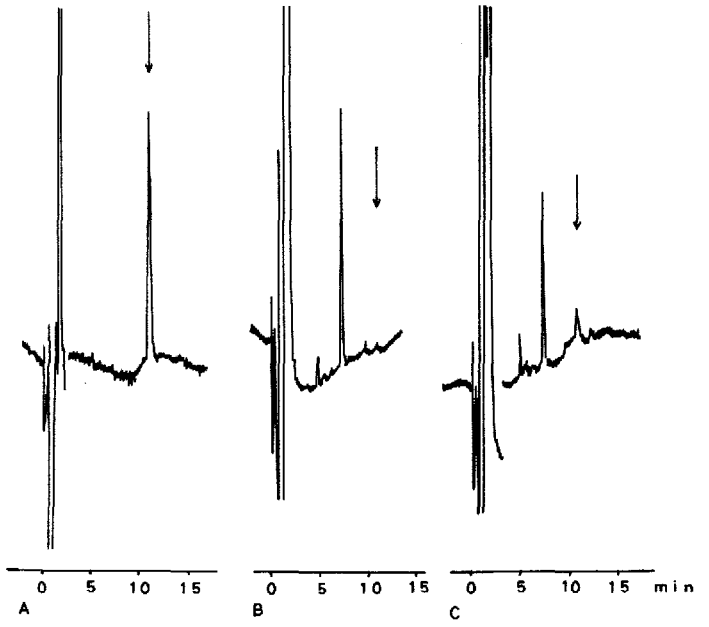

Fig. 1. Gas chromatograms. (A) Standard NDMA (0.7 ng); (B) extract from salted pollack roe (blank); (C) extract from salted pollack roe containing 0.7 μg/kg of NDMA. Arrows indicate retention time of NDMA.

distillation method (67–100%)[12] and a low-temperature vacuum distillation method (70–90%)[13], and compare well with those obtained with a Celite column (85–95%)[8] and a high-temperature purge and trap method (92%)[15]. In these reported methods, great care must be exercized to avoid unacceptable losses of volatile NDMA. The present results show that this procedure is applicable for rapidly screening fish products for NDMA in food quality control laboratories. Our method enables 10–12 samples to be extracted and cleaned up in a day, compared with six samples in a day using reported methods such as a Celite dry column[8,9] or steam distillation[10–12].

Several fish products obtained in local retail stores were analysed by this method and the results are given in Table II. These results are in the same range as reported

TABLE I

RECOVERY OF N-NITROSODIMETHYLAMINE ADDED TO SALTED POLLACK ROE

NDMA added (μg/kg)	*Recovery (%)*	*Average (%)*	*Relative standard deviation (%)*
40	96.8, 92.6, 96.5, 94.8, 103.0	96.7	3.6
4	74.7, 88.4, 88.4, 81.8, 91.7	85.0	6.8

TABLE II

N-NITROSODIMETHYLAMINE IN FISH PRODUCTS

Sample	*No. of samples analysed*	*No. of samples meeting NDMA level (μg/kg)*			
		ND[a]	*1–5*	*5–10*	*>10*
Salted pollack roe	47	43	4		
Smoked cuttlefish	11	2	3	3	3
Dried sardine	8	7		1	
Dried fish	6	6			

[a] Not detected (< 1 μg/kg).

earlier[22]. Commercial salted pollack roe is manufactured by dipping raw pollack roe in salt solution containing sodium nitrite and ascorbic acid. The low content of NDMA in salted pollack roe in Table II, in spite of the high concentration of dimethylamine in the raw pollack roe (150–200 μg/kg)[5,6], indicates that the formation of NDMA was suppressed by the ascorbic acid present.

This method may also be applicable to the determination of other nitrosamines in foods and this application is now being investigated.

In conclusion, this method for the determination of NDMA in fish products is simple, rapid and accurate and has a low susceptibility to artifactual NDMA formation. NPD is now widely used in food quality control laboratories and consequently this method is suitable for routine screening of NDMA in fish products.

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