Formation of *N*-Nitroso-*N*-methylurea in Various Samples of Smoked/Dried Fish, Fish Sauce, Seafoods, and Ethnic Fermented/Pickled Vegetables Following Incubation with Nitrite under Acidic Conditions

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In continuation of our previous studies on *N*-nitroso-*N*-methylurea (NMU) formation in cured meats following incubation with nitrite at gastric pH, we extended the investigation to other foods mentioned in the title of this paper. The main objective was to determine whether these foods have the potential to form NMU at pH's that can be found in the human stomach. This was done by nitrosating an aliquot (5 g for fish sauce, 10 g for the others) of each with 7.25 μM to 1.59 mM levels of sodium nitrite for 2 h at room temperature at pH 0.8−1.5 and measuring the amounts of NMU formed. Of the samples tested, fish sauce formed 2−712 ng of NMU, followed in decreasing order by herring (<0.3−688 ng); dried anchovy, shrimp, and other fishes (<0.3−134 ng); crab and lobster paté (<0.3−342 ng); sardines (6−59 ng); oysters and mussels (11−31 ng); dried squid (3−47 ng); kimchi (7−107 ng); and Japanese pickled radish (<0.3−72 ng). Incorporation of 200−2000 ppm of ascorbic acid in the fish sauce and other foods, prior to nitrosation, appreciably inhibited such NMU formation. Although previous researchers in China reported NMU formation in nitrosated samples of fish sauce, this is the first reported formation of NMU upon nitrosation of the other foods mentioned above, and the first reported inhibition of such formation by added ascorbic acid.

Keywords: *N-Nitroso-N-methylurea, NMU, nitrite, determination of NMU in foods, inhibition of NMU formation by ascorbic acid, fish and seafoods, fermented/pickled vegetables*

INTRODUCTION

Research over the past 25−30 years has focused much attention on the occurrence and formation of both volatile and nonvolatile nitrosamines in various foods and beverages and has generated extensive data in these areas (*1*, *2*). Similar data on *N*-nitrosamides (e.g., *N*-nitrosoureas, *N*-nitrosoguanidines), however, are almost nonexistent. Lack of sensitive and specific analytical methods for their determination in foods may have been one of the reasons for lack of progress in research in this area. Since most *N*-nitrosamides are unstable in aqueous medium (*3*, *4*), it is highly unlikely that these compounds will be found in foods in significant concentrations. However, traces of nitrosamides might be formed in the human stomach due to the reaction of nitrite with amides, both of which could be ingested through foods, with the former also originating from saliva (*5*, *6*). Salivary nitrite levels can reach very high levels (up to1.26 mM) after consumption of nitrate-rich meals containing leafy and root vegetables (*6*−*8*). Some researchers believe that in vivo nitrosation in the stomach may be a major source of human exposure to *N*-nitroso compounds, especially to nitrosamides, because the rate of formation of nitrosamides is high under acidic conditions similar to that existing in the human stomach (*9*−*15*). Since most nitrosamides are direct acting carcinogens, their formation in the stomach is of concern because upon spontaneous decomposition they form alkylating agents which can lead to cancer of the stomach or other organs (*16*).

Several studies have indicated a link between the high incidence of stomach cancer in the human population in Japan and China and consumption of dried fish, fish sauce, and salted pickled/fermented vegetables (*15*, *17*−*19*). It has also been suggested that, as a result of intragastric nitrosation, precursors present in such foods might be converted to *N*-nitroso-*N*-methylurea (NMU) or other nitrosamides which may be the causative agents responsible for the high incidence of stomach cancer in the population mentioned above. There are several published reports that indeed lend credence to this hypothesis. For example, deliberate nitrosation of many foods (e.g., fish, dried fish, fish sauce, various Japanese foodstuffs) formed strongly mutagenic compounds which required no metabolic activation, suggesting that they might be nitrosamides (*20*−*24*). Also, administration of such nitrosated food extracts to experimental animals over a prolonged period induced carcinoma of the glandular stomach (*17*). However, due to the unavailability of reliable analytical methods, levels of NMU or the presence of any specific nitrosamides in such nitrosated foods were not reported or determined. Furthermore, Weng et al. (*22*) concluded from their studies that mutagens formed after nitrosation of dried and salted fish were unlikely to be

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Table 1. Recoveries of Added NMU from Various Samples of Fish Sauce, Fish, Seafoods, and Fermented Vegetables[a]

food item used and division registry number	spiking levels of NMU, ppb	% recoveries of NMU
fish sauce 99-6	10	82
fish sauce 99-5	25	93
kippered herring 99-19	10	89
lobster paté 99-37	60	60
dried salted cod 99-46	60	67
herring fillet 99-40	60	104
smoked kipper fillet 99-38	10	92
herring in tomato sauce 99-39	25	76
herring in tomato sauce 99-39	25	74
smoked light tuna 99-42	25	81
smoked light tuna 99-42	10	78
soybean paste 99-78	10	83
kimchi	10	70
kimchi	5	100
average		81.8

[a] All samples were negative (<0.03 ppb) for NMU.

N-nitroso compounds because the addition of ascorbate during nitrosation did not decrease the mutagenicity.

The first reported evidence of NMU formation following nitrosation of common food ingredients, namely, creatinine and methylguanidine, came from studies by Mirvish et al. (*25*, *26*), Masuda et al. (*27*), and Kawabata et al. (*28*). More recently, Deng et al. (*29*) presented evidence for the formation of NMU in nitrosated Chinese fermented fish sauce "consumed traditionally in the highest risk area for stomach cancer in China". In Health Canada, we have also been working in this area and have developed a highly sensitive and specific analytical method for the determination of NMU and other nitrosamides in foods. Using this method, we have recently reported formation of traces of NMU in cured meats incubated under gastric pH with and without additional nitrite (*30*). The above-mentioned findings by Deng et al. (*29*) with fish sauce prompted us to repeat their study using a different analytical method as well as to confirm the identity of the formed NMU by gas chromatography−mass spectrometry (GC-MS) which was not carried out by the Chinese researchers at that time. In addition, we also wish to report some new findings on NMU formation upon nitrosation of various smoked and dried fish, seafoods, and some ethnic fermented/pickled vegetables that had not been investigated previously.

EXPERIMENTAL PROCEDURES

1. Apparatus. The details of the apparatus used in the study have already been described in detail (*30*). These included the following: (1) a high performance liquid chromatography (HPLC) system attached sequentially to a post-column chemical denitrosation device and a thermal energy analyzer (TEA) that was used for the preliminary determination of NMU; and (2) a gas chromatograph coupled to a high resolution mass spectrometer, which was used for confirmation as well as for determination of NMU at ultra trace levels. The mass spectrometer was operated at a resolution of 5000 (10% valley definition) in the selected ion monitoring (SIM) mode set to monitor a fragment at *m*/*z* 60.0324—the most prominent fragment ion in the mass spectrum of NMU, most likely corresponding to $CH_3N{=}NOH$ (*31*).

2. Chemicals and Reagents. NMU standard, L-ascorbic acid, sulfamic acid, and all other reagents and organic solvents were purchased from the same sources as reported previously (*30*). Also, the dilute NMU aqueous standards and that dissolved in CH_2Cl_2 used, respectively, for HPLC-TEA and GC-MS determinations were prepared exactly as in the above-mentioned study.

3. Samples. All samples were purchased locally in the Ottawa area, but many of these were imported from other countries as indicated in Tables 1-4. Except for the fish sauces, all other samples were homogenized using a blender (without any added water) and stored in screw cap glass jars at 4 °C until use. If not used within a week, the samples were stored at −20 °C. The fish sauces were stored in a refrigerator (4 °C) in the original bottles.

4. Incubation of Various Food Items with Nitrite. As mentioned earlier, the main purpose of these experiments was to determine NMU-forming potentials of different foods following nitrosation at gastric pH. To determine this, a suitable aliquot (5 g for fish sauce and 10 g for all others) of a well-homogenized sample was mixed with water (5−30 mL) and 1−2 mL of 0.145−36.23 mM nitrite solution (containing 0.01−5 mg of $NaNO_2$), and the pH of the mixture was adjusted (usually to 0.8−1.5) by the addition of 1 or 3 M HCl. The sample was then incubated in the dark for 2 h at room temperature. After 10−15 min of initial incubation, the pH of the sample was rechecked and, if necessary, was readjusted to the original level and the incubation was resumed. The sample was then processed as described below.

5. Determination of NMU in Incubation Mixtures of Nitrite and Various Food Items. Immediately after incubation, the sample was mixed with 100 mg each of ascorbic acid and sulfamic acid (pH 1−1.5) to destroy excess nitrite and prevent artifact formation. The mixture was saturated with NaCl and processed as described previously (*30*). Briefly, the steps consisted of (a) extraction of the mixture with ethyl acetate; (b) evaporation of the ethyl acetate extract to a small

Table 2. Formation of NMU in Various Fish Sauce Following Incubation with Sodium Nitrite under Acidic Conditions[a]

fish sauce brand and country of origin	concentration of $NaNO_2$ in various final reaction mixtures	pH of incubation mixtures, respectively	amount of NMU formed (ng) per 5 g of fish sauce, respectively
A1, Thailand	nil; 1.59 mM	1−1.3; 1−1.3	negative;[b] 350
B1, Thailand	nil; 0.77; 0.39 mM	1; 1; 1	negative; 162; 57
	1.45 mM;[c] 1.45 mM	1−1.3; 1	295; 599
B2, Thailand	1.48 mM	0.8	123
C1, Canada	nil; 1.65 mM	1.2; 1.3	0.8; 84
D1, Thailand	nil; 0.83 mM; 0.83 mM[c]	1.3; 1.5; 1.2	0.8; 125; 67
E1, Thailand	nil; 7.25 μM;[d] 69.6 μM[d]	1; 1; 1	negative; 2.1; 12.4
	85.5 μM; 0.345 mM;[d] 1.59 mM	1.1; 1; 1	6.4; 17.1; 506
E2, Thailand	1.45 mM	0.9	379
F1, Thailand	1.59 mM; 0.40 mM	1; 1	206; 1.3
F2, Thailand	1.52 mM	0.9	244
G1, Thailand	1.52 mM	0.8	306

[a] Aliquots of fish sauce (5 g), water (10 mL), and $NaNO_2$ solution (0.05−2 mg dissolved in 1−2 mL of water) were mixed together, and the pH was adjusted with a dilute HCl solution (1−3.5 mL). [b] Less than 0.3 ng. All mixtures, except two, were incubated for 2 h in the dark at room temperature. [c] Two were incubated at 37 °C. Following incubation, the samples were processed as described under Experimental Procedures. [d] Used 5 mL of 10-fold diluted fish sauce.

Table 3. Formation of NMU in Incubation Mixtures of Various Fish and Seafoods and Sodium Nitrite Solutions under Acidic Conditions[a]

kind	country of origin	no. of positives/ total	[$NaNO_2$] in reaction mixture, mM	pH of incubation mixture	NMU formed, ng; mean (range)
herring (smoked fillet, canned in tomato or wine sauce, kippered)	Canada	5/7	2.03−2.9	1−1.1	130 (negative[b]−688)
	Germany	1/1[c]	0.65	1−1.1	53[c]
canned sardines (slightly smoked, in salt, or in hot tabasco sauce)	Canada, Italy	3/3	1.52−2.07	1−1.1	30.7 (6.3−59.2)
canned tuna	Canada, Philippines	0/2	2.17	1−1.1	negative
smoked oysters and mussels	Canada, Korea	3/3	2.11	1−1.1	20.6 (11.5−31.3)
shrimp (dried, salted fry, paste)	Hong Kong, Thailand, Canada, Philippines, Malaysia	3/5 (3/3 dried shrimp)	0.9−1.45	0.6−2	15.1 (negative-38.3); 25.2 (8−38.3) for the dried samples
anchovy (dried, salted, frozen)	japan, Vietnam, Italy, Thailand	2/4	1.48−2.07	0.6−2	33.6 (negative-68); one salted and one frozen anchovy were negative
miscellaneous salted/dried fish (cod, pollock)	Canada, China, Korea	2/4	0.69−1.93	0.6−2	26.4 (negative-101)
dried squid/cuttle fish	Taiwan, Hong Kong, China	3/3	1.03−2.23	1−1.1	32.1 (2.7−47)
pickled gouramy fish (cream style)	Thailand	1/1	2.07	1−1.1	134
lobster paté (some with cognac)	USA	3/3	2.07−2.23	1−1.1	51 (16−92.3)
		0/1	nil	1	negative
crab paté (devilled with vermouth)	USA	2/2	2.07−2.23	1−1.1	187 (32−342)
		0/1	nil	1	negative
crab meat	Thailand	0/1	2.23	1	negative

[a] Aliquots (10 g) of homogenized samples, water (10−30 mL), and 1−2 mL of $NaNO_2$ solution (5−60 mM) were mixed together, and the pH was adjusted with a 3 M HCl solution (1−11 mL). All mixtures were incubated for 2 h in the dark at room temperature. [b] Less than 0.3 ng. [c] The sample which formed 688 ng of NMU upon incubation with 2.23 mM of sodium nitrite in the incubation mixture (see row 1 above).

Table 4. NMU Formation in Nitrosated Samples of Various Ethnic Fermented/PickledVegetables[a]

kind	country of origin	no. of positives/ total	[$NaNO_2$] in reaction mixture, mM	pH of incubation mixture	NMU formed, ng; mean (range)
kimchi (a Korean dish)	Canada, Korea	4/4	1.16−3.19	0.6−2	39.1 (7.4−107)
		0/1	nil	1−1.1	negative[b]
pickled radish	Japan, Canada	3/4	1.88−4.2	1−1.5	21.7 (negative−72)
sacha sauce	Hong Kong	0/1	2.25	1−1.1	negative
fermented soybean chilli	Taiwan	1/1	4.06	1−1.1	13.4
black bean garlic sauce	Hong Kong	0/1	4.06	1−1.1	negative
fermented vegetable mixture	Thailand	0/1	2.03	1−1.1	negative
fermented soybean paste	Thailand	0/1	2.03	1−1.1	negative
shant pickles	China	0/1	1.88	1−1.1	negative

[a] Aliquots (10 g in most cases) of homogenized samples were mixed with 10−20 mL of water and $NaNO_2$ solution (2−5 mg dissolved in 2−5 mL of water), and the pH's of the mixtures were adjusted to the required level with the addition of 3 M HCl. The samples were incubated in the dark for 2 h and then processed as described under Experimental Procedures. [b] Less than 5 ng (by HPLC-TEA).

volume and transfer to water−methanol mixture (phase transfer); (c) removal of fats and lipids by partitioning between *n*-hexane and water−methanol; (d) re-extraction of the water−methanolic solution with CH_2Cl_2; (e) phase transfer again to a small volume of water; (f) cleanup on C_{18} and silica Sep-Pak cartridges; and finally (g) determination of NMU in the cleaned-up extract by HPLC−postcolumn chemical denitrosation−TEA (HPLC-TEA) and GC-MS techniques. Also, to facilitate partitioning of NMU in the organic phase, all aqueous solutions/extracts were saturated with NaCl before extraction with CH_2Cl_2. The details of each step and that of the two determinative techniques have been described previously (*30*). Since NMU is fairly unstable in aqueous solution, especially at pH >6−7, attempts were made to complete the analysis without interruption. If sample extracts had to be stored overnight, they were always kept in $CH_2Cl_2$and stored at −20 °C. Also, care was taken to avoid exposure of NMU standards and sample extracts to strong light and they were kept in an ice−water bath if prolonged interruptions were necessary during the analysis.

6. Effect of Ascorbic Acid on NMU Formation during Nitrosation of Various Foods with Nitrite. Previous studies by Mirvish et al. (*32*) and others as reviewed by Mirvish (*33*) have clearly demonstrated the effectiveness of ascorbic acid as a nitrosation inhibitor in both in vitro and in vivo systems. Hence, we wanted to determine whether incorporation of sufficient levels of ascorbic acid in various foods, before incubation with nitrite under acidic conditions, could appreciably inhibit NMU formation. In these experiments, an aliquot (5−10 g) of a food sample was first mixed with water and different levels of ascorbic acid. The mixture was allowed to equilibrate at room temperature for 10−15 min and was then nitrosated for 2 h at room temperature as described above in section 4. A control sample with no ascorbic acid was run simultaneously. The decrease in NMU formation in the ascorbic acid treated sample as compared to that in the control,

run under identical conditions, was taken as a measure of the extent of inhibition.

RESULTS AND DISCUSSION

Analytical Methodology. The analytical methodology used is essentially the same as that reported previously in a similar study with cured meats (*30*) and was thoroughly tested at that time. Further recovery studies with fish sauce and the other items were carried out to ensure that the method worked well and that the data generated were accurate and reliable. The average recovery of NMU added at the levels of 10−60 ppb to various samples was 82% (range 60−104%) (Table 1), which was thought to be acceptable, especially in view of the fact that NMU is a fairly unstable compound and the overall methodology is quite lengthy. If the procedure is strictly adhered to (*30*), there should not be any problems using the method. The minimum detection limits of the method by LC-TEA and GC-MS were about 0.5 ppb (5ng/10 g) and 0.03 ppb (0.3 ng/10 g), respectively. The GC-MS technique was used for the analysis of the majority of the samples. Only in selected cases, usually one or two in each category, were the extracts also analyzed by HPLC-TEA. Thus, the identity of NMU, at least in those samples, was confirmed by both techniques.

NMU Formation in Fish Sauce. Ten samples of fish sauce, consisting of seven different brands, were tested to determine their NMU-forming potential after nitrosation with 7.25 μM to 1.59 mM (in the final reaction mixture) levels of $NaNO_2$ (Table 2). In most cases, the samples were nitrosated for 2 h at room temperature at pH 0.8−1.5; only in two cases was the incubation carried out at 37 °C. In our previous studies on nitrosation of creatinine (*30*), it was observed that NMU is considerably less stable at 37 °C than at room temperature (20−22 °C). This was also observed to be true in this study (Table 2), where the higher temperature was chosen in two cases. In both cases, the amounts of NMU detected at 37 °C were approximately one-half of that detected at room temperature (295 ng vs 599 ng, and 67 ng vs 125 ng). Hence, the lower incubation temperature was chosen for incubation of all other samples.

As can be seen from the results (Table 2), all the fish sauces formed detectable amounts of NMU (1.3−599 ng) after nitrosation. As expected, the amount of NMU formed generally increased with an increase in nitrite concentration in the incubation mixture. Also, there were variations in such formation both between as well as within the various brands of fish sauces tested. Most of the fish sauces by themselves (without nitrosation), however, were negative, but two contained traces of NMU. Detectable amounts of NMU were formed even when very low concentrations of $NaNO_2$ (e.g., sample E_1 with 85.5 μM $NaNO_2$ in the reaction mixture) were used or when a 10-fold diluted fish sauce was nitrosated with 7.25 μM to 345 μM levels of $NaNO_2$ (the latter formed 17.1 ng of NMU). This suggests that there is a good possibility of NMU formation in the stomach following ingestion of even small amounts of fish sauce and nitrite (through saliva or other foods). It would be very difficult, however, to prove this directly.

Our results confirm previously reported findings by Deng et al. (*29*) that nitrosation of fermented fish sauce can indeed form appreciable levels of NMU. These workers, however, used a much higher concentration of sodium nitrite (5 mM) in the final incubation mixture than that used in the present study (7.25 μM to 1.59 mM). The cleanup as well as the final analytical techniques used in this study are also different from those used by the Chinese researchers. Although both methods used HPLC for chromatography and TEA for final detection and quantitation, there are minor differences in the two methods. Our method is based on hydrogen-iodide-catalyzed postcolumn chemical denitrosation of NMU to nitric oxide, whereas the Chinese researchers used UV-induced photolysis to generate NO following preliminary HPLC separation. Both the methods are highly specific, and, therefore, complement each other. In addition, we have carried out additional cleanup of the extract and confirmed the identity of NMU formed by GC-MS. After our work had been completed, it came to our attention that Deng et al. (*34*, *35*) have also confirmed the identity of NMU, formed after nitrosation of fish sauce, by HPLC-electrospray ionization mass spectrometry. Therefore, in view of the positive tests in all these methods, it is highly likely that the compound was properly identified as NMU. More recently, the same group of Chinese researchers (*36*) have demonstrated NMU formation in stomach of minipigs and human volunteers given fish sauce and nitrite.

NMU Formation in Other Foods. The investigation was then extended to various fish and seafoods that are commonly consumed in Canada, USA, and many other countries. In total, 37 samples of such products (details presented in Table 3) were tested. The data (Table 3) suggest that most formed traces of NMU upon incubation with nitrite under the experimental conditions used. Although the number of samples tested in each category was relatively small, some of them formed appreciable levels of NMU (all results are based on a 10 g sample size). These included the herrings (up to 688 ng), sardines (up to 59.2 ng), smoked oysters and mussels (up to 31.3 ng), various dried fish (up to 134 ng), dried squid (up to 47 ng), and both lobster and crab paté (up to 342 ng). Although the NMU-forming potential of these items was not as high as that observed for the Chinese fermented fish sauce, these foods are consumed in larger quantities per meal than fish sauce, which is mainly used as a cooking adjunct and not consumed directly. Another interesting observation was that most of the fish sauces analyzed in this study (Table 2) were based on anchovy (a small fish of herring family) or anchovy extract. This is consistent with our data (Table 3) on anchovy because two out of four such fish tested formed 68 and 66.5 ng of NMU; the other two (one was frozen fish, the other was salted) did not form detectable NMU. Only one fish sauce contained capelin (small smelt-like fish) (C1, Table 2), which seemed to form the lowest amount of NMU when nitrosated under similar conditions. More samples of different kinds, however, should be tested before reaching any conclusion regarding the relationship between the type of fish used to prepare the sauce and the amount of NMU formed. Because of the unavailability of other types of fish sauces, we could not test other products. Moreover, the processing conditions, especially that used for fermentation, would also influence the formation of NMU precursors in the finished products. Figure 1 shows a typical example of GC-MS-SIM results obtained from the analysis of a nitrosated pollock.

Similar studies with various ethnic fermented/pickled

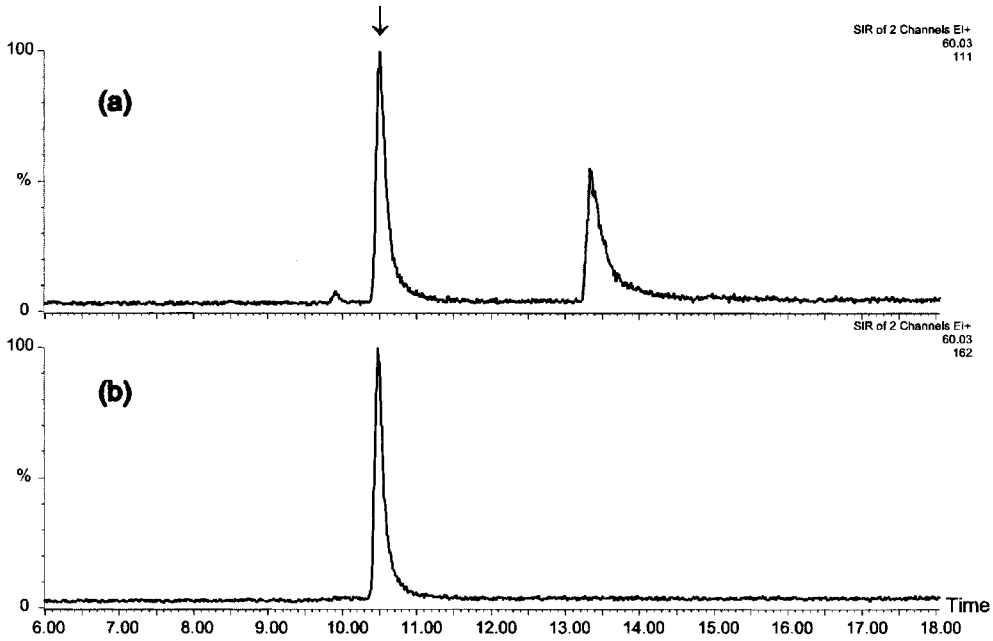

Figure 1. GC-MS-SIM results (*m*/*z* 60.0324; resolution 1 in 5000). (a) 4 μL of cleaned-up extract of a nitrosated dried pollock which formed a total of 61.2 ng of NMU/10 g of sample (for conditions, see text and Table 3). The arrow identifies the NMU peak. (b) 210 pg of NMU standard.

Table 5. Inhibition of NMU Formation in Fish Sauce and Other Foods by Ascorbic Acid Added Prior to Incubation with Sodium Nitrite under Acidic Conditions[a]

item	ascorbic acid added to the food		$[NaNO_2]$ in reaction mixture, mM	molar ratio of ascorbate to nitrite	NMU formed, ng	% inhibition of NMU formation
	mg	ppm				
fish sauce						
brand E1 (5 g)	nil	NA[b]	0.16	NA	5.3	NA
	5	1000	0.16	9.8	negative[c]	100
brand E1 (5 g)	nil	NA	0.41	NA	86	NA
	1	200	0.41	0.78	<5	94
brand B1 (5 g)	nil	NA	1.56	NA	712	NA
	2.5	500	1.56	0.49	542	19.3
brand B1 (5 g)	nil	NA	1.45	NA	599	NA
	5	1000	1.45	0.98	281	53
	10	2000	1.45	1.96	10	98
brand F1 (5 g)	nil	NA	0.14	NA	2	NA
	5	1,000	0.14	9.8	0.2	90
brand F1 (5 g)	nil	NA	1.59	NA	230	NA
	2.5	500	1.59	0.49	negative	100
kimchi						
brand J (10 g)	nil	NA	2.1	NA	38	NA
	2	200	2.1	0.39	24	37
	5	500	2.1	0.98	10	74
brand K (10 g)	nil	NA	0.58	NA	41	NA
	1	100	0.58	0.78	16	63
	2.5	250	0.58	1.96	10	75
pickled gouramy fish (10 g)	nil	NA	2.07	NA	134	NA
	2	200	2.07	0.39	39.4	70.6
	5	500	2.07	0.98	4.8	96.4
anchovy, salted (10 g)	nil	NA	2.1	NA	68	NA
	2	200	2.1	0.39	44	35
	5	500	2.1	0.98	<5	93

[a] Aliquots of homogenized samples were mixed with appropriate amounts of ascorbic acid and allowed to equilibrate for 5−10 min, and the samples were then mixed with sodium nitrite solutions. The pH's of the mixtures were adjusted to 1−1.1, and the samples were incubated in the dark for 2 h. The controls were processed similarly except no ascorbate was added to the samples. [b] NA = not applicable. [c] Less than 0.3 ng.

vegetables suggested that kimchi, a Korean dish, and pickled radish, which is popular in Japan and Korea, could also form traces of NMU (up to 107 ng/10 g for kimchi and 72 ng/10 g for pickled radish) upon nitrosation with nitrite under the conditions used (Table 4). Among the other products, only a fermented soybean chilli formed traces of NMU when nitrosated with a fairly high level of $NaNO_2$ (4.06 mM). Of the four kimchis, three were incubated with 1.16−2.17 mM and one with 3.19 mM levels of $NaNO_2$. Even with such high levels of nitrite, the last-mentioned sample formed only 7.4 ng of NMU, suggesting a low NMU-forming potential of this particular kimchi. Of the four pickled radish samples tested, one gave negative results even upon incubation with 1.93 mM levels of $NaNO_2$, one formed barely detectable levels (0.3 ng) of NMU by GC-MS, and

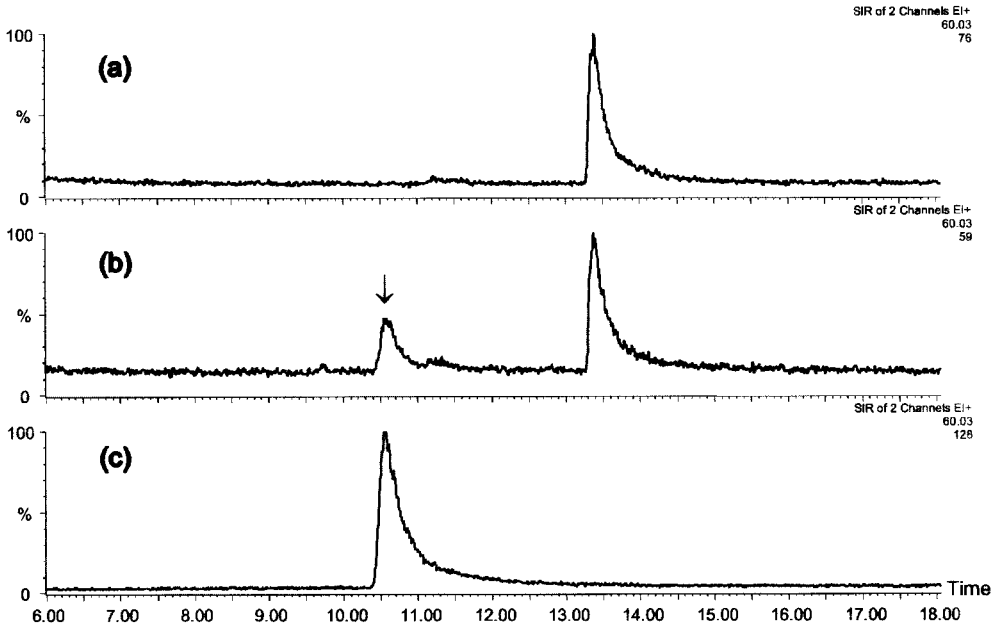

Figure 2. GC-MS-SIM results showing the inhibitory effect of added ascorbic acid on NMU formation. (a) 4 μL of cleaned-up extract of a nitrosated fish sauce (see row 2, Table 5) that had been premixed with 1000 ppm of ascorbic acid (5 mg/5 g) before nitrosation (the inhibition was nearly 100%). (b) 4 μL of cleaned-up extract of the same nitrosated fish sauce without any added ascorbic acid that formed a total of 5.3 ng of NMU (5 g sample nitrosated for 2 h with 0.156 mM levels of $NaNO_2$ at room temperature at pH 1). (c) 200 pg of NMU standard.

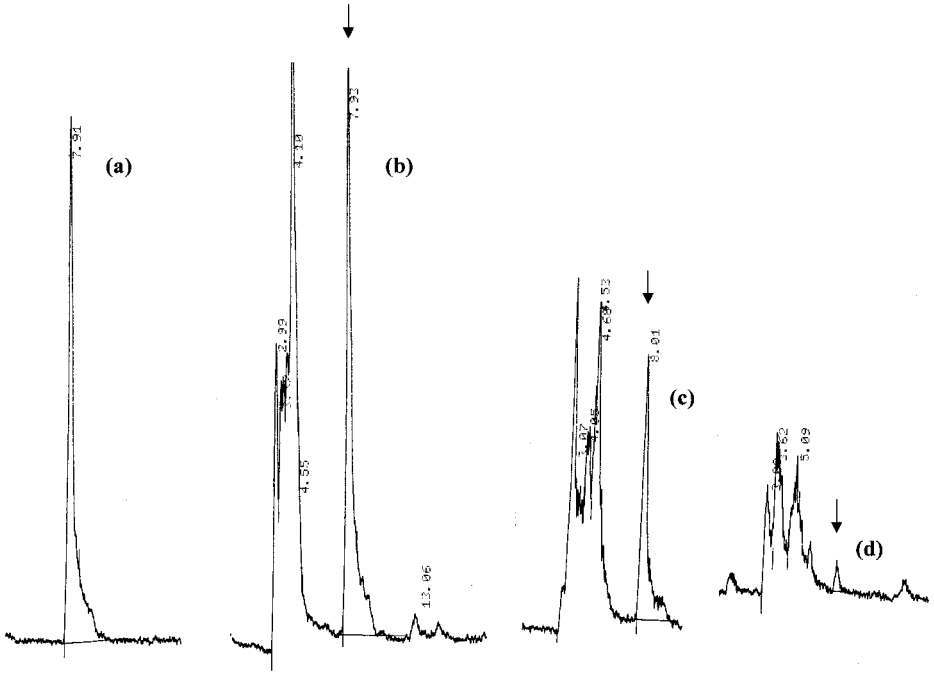

Figure 3. HPLC-TEA results demonstrating the inhibitory effect of added ascorbic acid on NMU formation following nitrosation of a pickled gouramy fish. (a) 6.24 ng of NMU standard. (b) 50 μL of cleaned-up extract of the nitrosated fish which formed a total of 134 ng of NMU (10 g sample nitrosated for 2 h with 143 ppm levels of $NaNO_2$ at room temperature at pH 1).(c) As above, except the fish was premixed with 200 ppm of ascorbic acid (2 mg/10 g) prior to nitrosation (70.6% inhibition). (d) As above, except the fish was premixed with 500 ppm of ascorbic acid before nitrosation (96.4% inhibition). The arrows indicate MMU peaks in the sample chromatograms.

the remaining two formed 14.6 and 72 ng of NMU upon incubation with 2.17 and 4.2 mM levels of $NaNO_2$, respectively. In total, 14 such samples, including six of miscellaneous sauces and pickles, were tested. To obtain a wider database, however, a much broader survey of such products would be needed.

Effect of Ascorbic Acid on NMU Formation during Nitrosation of Various Foods with Nitrite. The results on the inhibition of NMU formation by added ascorbic acid in a few selected samples of such foods are presented in Table 5. The extent of inhibition varied widely depending on both the type of sample used and the molar ratios of ascorbic acid to sodium nitrite. As expected, a molar ratio of >1 was needed to achieve appreciable (75−100%) inhibition of NMU formation. In a few cases, however, >70% inhibition of NMU formation was observed even with molar ratios of <1 (e.g., data in rows 4, 13, 16, 21, 22 and 25). These results

suggest that a 2:1 molar ratio of ascorbic acid to nitrite in the reaction mixture would most likely be required to minimize NMU formation. Figures 2 and 3 show the effect of ascorbic acid on NMU formation after nitrosation of a fish sauce and a pickled gouramy fish, respectively.

It is recognized that the in vitro nitrosation conditions used in these experiments do not reflect the true gastric conditions of the human stomach. First, the concentrations of nitrite used in some of the experiments were well in excess (e.g., in Tables 2-4, the high range of nitrite levels used was between 0.4 mM- 4.2 mM) of that found in vivo under normal or even unusual conditions (see below, next paragraph). Second, since only HCl was added to the incubation mixtures, the reaction media did not reflect the complex nature of gastric juice which, besides HCl, contains a wide variety of both organic and inorganic ingredients. For example, gastric fluid (and saliva) normally contains thiocyanate (*14*), which is catalytic for nitrosation. Therefore, the results reported should be interpreted with caution. But it should be mentioned that high levels of nitrite were not used in all cases. For example, in some of the experiments described in Table 2, very low levels (7.25−85.5 μM) of nitrite were used that are comparable to normal (4.3 μM and 0.11 mM) levels of nitrite found in the human stomach. Moreover, in the presence of thiocyanate ions, which were not included in the reaction media, the extent of NMU formation would be expected to be higher not lower. Therefore, there is a good possibility that traces of NMU might be formed in the stomach following ingestion of some of these foods.

Nitrite concentrations in human gastric contents can vary widely depending on many factors such as individual variations, disease conditions (atrophic gastritis, pernicious anaemia, gastric cancer), and meals consumed (e.g., nitrate-rich or not, presence or absence of nitrite scavenger). Under normal situations, the concentration of nitrite in the human gastric juice is very low and usually ranges between 0.3 and 7.6 ppm (4.3 μM and 0.11 mM) (*37*, *38*). But these values can rise to 9−32 ppm (0.13−0.46 mM) following consumption of nitrate-rich vegetables or salad (*38*) or in people with gastric pathology (*37*). Although incorporation of 1000−2000 ppm of ascorbic acid in fish sauce and the other foods effectively inhibited NMU formation under the in vitro conditions tested, it is doubtful these levels of addition would be adequate in inhibiting the same in vivo in the stomach because, upon ingestion, the ascorbate will be diluted considerably. In people with chronic atrophic gastritis or other diseases, which tend to elevate gastric nitrite levels, an additional dose of ascorbic acid perhaps as an oral supplement just before consuming these foods, would be required to inhibit such NMU formation. The addition of ascorbic acid to the food, however, would prevent formation of NMU or other *N*-nitroso compounds during storage of the food.

It appears from the results of this study and from that reported previously by Deng et al. (*29*), that consumption of fish sauce, other fish and seafoods, and some fermented pickled vegetables may lead to formation of traces of NMU in the human stomach if adequate levels of nitrite are also ingested (through saliva or foods) at the same time. It is not, however, clear whether this actually happens under normal situations. Further research is needed to establish this point. Additional research is also needed to determine the precursor amides in such foods that form NMU upon nitrosation. Previous research by Mirvish et al. (*25*, *26*) and Masuda et al. (*27*) have shown that creatinine, methylurea, and methylguanidine can all be nitrosated to form NMU, but very little data are available on the levels of these compounds in such foods.

SAFETY PRECAUTION

Since NMU is a potent animal carcinogen, extreme care must be taken while handling the chemical or working with dilute standards.

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