Occurrence of Nitrate, Nitrite, Dimethylamine, and Dimethylnitrosamine in Some Fermented Nigerian Beverages

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A survey of the nitrate (NO_3^-), nitrite (NO_2^-), and dimethylamine (DMA) contents of some Nigerian fermented beverages (palm-wine, nono, burukutu, pito, and ogogoro), conducted in the Kwara and Benue States, has shown that these substances occur frequently in the drinks. The detection of a hazardous product, in one sample of palm-wine, presumed to be dimethylnitrosamine (DMN) and verified by mass spectrometry is also reported. NO_3^- (13.7–91.3 ppm), 0.86–2.9 ppm NO_2^-, 4.6–31.6 ppm DMA, and 0.6 ppb DMN were detected. Nitrate, nitrite, and dimethylamine were assayed colorimetrically or spectrophotometrically, and dimethylnitrosamine by thin-layer and gas–liquid chromatographic methods. Limits of detection were: 0.2 μg, 1 μg, and 2 μg/mL of NO_2^-–N, NO_3^-–N, and DMA–N, respectively. In the case of DMN, the limit of detection was 2 ng and recoveries of added compounds were good. The presence of nitrates, nitrites, and secondary amines in our foods and environment is discussed in relation to nitrosamine toxicology.

The first indication that the carcinogenic *N*-nitrosamines could be formed in our food was as a result of the detection and identification of dimethylnitrosamine in fish meal preserved with sodium nitrite (Ender et al., 1964). These workers subsequently showed that a chemical reaction between fish amine (dimethylamine) and sodium nitrite was responsible for the formation of dimethylnitrosamine in the fish meal.

It is now widely accepted that nitrate, nitrite, and secondary amines are precursors in the formation of nitrosamines and that the potential exists for the nitrosation reaction to occur in nature (Hawksworth, 1970; Hawksworth and Hill, 1971; Ayanaba et al., 1973; Ayanaba and Alexander, 1974). In view of the critical public health significance of the presence of carcinogenic nitrosamines in foods meant for human consumption, the International Agency for Research on Cancer (IARC) at a meeting, on nitrosamines, in Lyon, France (1970) has stressed the need for an evaluation of various beverages and foods for the presence of nitrosamine precursors. We have therefore investigated the occurrence of nitrates, nitrites, and dimethylamine in some Nigerian fermented beverages, namely, palm-wine, nono, burukutu, pito, and ogogoro. A possible consequent contamination of palm-wine by dimethylnitrosamine has also been investigated.

Palm-wine is essentially a dense suspension of bacteria and yeasts in fermenting palm-sap, and it is obtained by the tapping of palms (family Palmae). Two main species, *Elaeis guineensis* and *Raphia hookeri*, are tapped in Nigeria and tapping methods have been described by Bassir (1962) and Okafor (1972). Microorganisms contaminate the sap during tapping without conscious inoculation (Bassir, 1962). Nono is fresh cow milk that has been locally skimmed and allowed to ferment. Burukutu and pito (Ekundayo, 1969) are produced by the fermentation and cooking of cereals (guinea corn and millet), and ogogoro is gin locally distilled from stale palm-wine or sugar molasses. These beverages are usually hawked for sale and drunk frequently, in fairly large quantities, by many Nigerians.

MATERIALS AND METHODS

Test Beverages. *Sampling Plan.* The primary aim of the survey, conducted in the Kwara and Benue States of Nigeria, was to obtain data on the levels and distribution of nitrosamine precursors (nitrates, nitrites, and dimethylamine) in some of the popular local fermented beverages. No attempt, therefore, was made to follow a formal statistical sampling plan. However, sampling was carried out in such a way that, in most of the areas investigated, the various terrain and the different kinds of people were adequately covered. Twenty individual samples of each test beverage were collected randomly, in 1-L plastic containers, from a given location and preserved, prior to analysis, by the addition of a spatula-full (5 mg) of mercuric chloride ($HgCl_2$).

Sample Pretreatment. Prior to analysis for nitrates and nitrites, aliquots of the test beverages were clarified as follows: activated animal charcoal was added to remove plant pigments and the slurry filtered through Whatman No. 30 filter paper. Each filtrate was centrifuged at 74600*g* for 15 min and 1 mL of the supernatant used for analysis; mercuric chloride added previously as a preservative served, also, as a good clarifying and deproteinizing agent.

One liter of a test beverage to be analyzed for dimethylamine or dimethylnitrosamine was steam distilled, in 3 N sodium hydroxide, to half-volume according to the method of Heath and Jarvis (1955), and 1 mL of the distillate (collected in a measuring cylinder immersed in an ice bath) was analyzed for dimethylamine. For the determination of dimethylnitrosamine in palm-wine, the distillate was treated as described in the appropriate section.

Preparation of Authentic Dimethylnitrosamine. Dimethylnitrosamine, used as a reference standard, was synthesized in our laboratory according to the method of Vogel (1956) and purified by continuous distillation until pure according to gas chromatography. It was characterized by infrared and ultraviolet spectrophotometry and mass spectrometry. The boiling point at atmospheric pressure was 152 °C (care must be taken in the handling of dimethylnitrosamine or substances suspected to contain nitrosamines because of their volatility, toxicity, and carcinogenicity).

Estimation of the Dimethylamine (DMA) Content of Test Beverages. The spectrophotometric method of Pribyl and Nedbalkova (1967) was used in the estimation of the dimethylamine content of the beverages. Optical density measurements of the yellow color produced were made at 445 nm using an SP600 Unicam spectrophotometer. Values of DMA were calculated from a standard

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Table I. Distribution of Dimethylamine Content of Beverages

Location	No. of samples	Dimethylamine, ppm ± SD				
		Palm-wine	Nono	Burukutu	Pito	Ogogoro
Ankpa[a]	20	7.17 ± 4.82	9.63 ± 7.06	14.98 ± 13.05	29.10 ± 15.09	24.61 ± 14.45
Dekina[a]	20	16.16 ± 4.60	12.09 ± 11.24	7.60 ± 4.28	15.94 ± 13.91	19.37 ± 14.02
Egbe	20	8.35 ± 4.17	5.78 ± 3.21	23.11 ± 19.69	8.45 ± 6.96	17.12 ± 9.31
Idah[a]	20	13.16 ± 8.56	9.63 ± 7.06	14.55 ± 7.70	11.45 ± 8.03	22.90 ± 19.15
Ilorin	20	14.02 ± 6.96	5.78 ± 3.32	11.98 ± 9.63	19.05 ± 15.84	16.26 ± 10.91
Jebba	20	14.02 ± 10.59	13.27 ± 2.25	12.52 ± 8.88	23.33 ± 18.51	31.57 ± 16.26
Kabba	20	4.06 ± 2.25	16.16 ± 14.12	9.95 ± 8.99	21.40 ± 16.05	10.27 ± 6.53
Kaima	20	23.86 ± 11.24	5.78 ± 4.17	15.09 ± 12.95	18.51 ± 16.48	14.12 ± 10.91
Lokoja	20	8.03 ± 5.24	10.38 ± 6.96	20.10 ± 17.55	22.79 ± 16.80	12.63 ± 9.84
Luma	20	12.09 ± 9.74	6.42 ± 4.39	29.96 ± 17.33	11.24 ± 9.10	9.84 ± 6.74
New Bussa	20	9.52 ± 7.70	6.53 ± 4.39	21.61 ± 14.34	30.50 ± 16.48	7.28 ± 4.92
Offa	20	8.67 ± 6.96	13.16 ± 10.27	14.23 ± 11.34	31.14 ± 14.98	11.34 ± 6.74
Okene	20	8.88 ± 6.21	27.29 ± 20.44	30.50 ± 16.37	14.66 ± 13.27	9.52 ± 8.35
Omu Aran	20	28.36 ± 12.20	9.10 ± 7.60	8.88 ± 6.96	20.01 ± 16.16	17.12 ± 15.19
Pategi/Lafiagi	20	5.03 ± 2.78	9.95 ± 6.31	11.13 ± 7.17	14.12 ± 10.38	15.09 ± 10.17

[a] Situated in Benue State.

curve of DMA. About 2 μg of DMA-N/mL could be detected with confidence. Beer's law was obeyed between 5–65 μg of DMA-N/mL. Each determination was done in duplicate.

Estimation of the Nitrite Content of Test Beverages. The nitrite content of the test beverages was estimated by the colorimetric method of Montgomery and Dymock (1971). Optical density measurements of the resultant pink solutions were made at 550 nm using an SP600 Unicam spectrophotometer. Values of nitrite were calculated from a standard curve of nitrite and the limit of detection was 0.2 μg NO_2^--N/mL. One milliliter of a clarified test beverage was employed as a blank but the sulfanilic acid reagent was replaced by an equivalent amount of double-distilled water. Nitrite determination, per sample, was done in duplicate.

Estimation of the Nitrate Content of Test Beverages. A modification of the phenoldisulfonic acid method of Harper (1924) was used in the estimation of the nitrate content of the test beverages. Ten milliliters of a solution of nitrate-free silver sulfate (4 g/L) were added to about 50 mL of a clarified beverage sample to remove interfering chloride ions. Precipitated chloride was removed by filteration after the beverage had been stored away in a dark cupboard overnight.

One milliliter of the filtrate was mixed in a test tube with 0.2 g of magnesium oxide, to prevent loss of nitrate, and then gradually evaporated to dryness in a sand bath. Phenoldisulfonic acid "nitrate" reagent (0.5 mL) was added to the residue to form an intimate mixture, and the tube was placed in a boiling water bath for 10 min. After cooling, 9.5 mL of a diluting solution (220 mL of concentrated ammonium hydroxide and 5 mg of triammonium citrate in 1 L of double-distilled water) was added. The mixture was cooled, shaken vigorously, and allowed to stand for 15 min. The intensity of the resultant nitrophenoldisulfonic yellow color was measured in an Eel photoelectric colorimeter (violet filter). Values of nitrate were calculated from a standard curve of nitrate.

One milliliter of a clarified test beverage used as a blank, contained 0.5 mL concentrated sulfuric acid instead of 0.5 mL of the phenoldisulfonic acid reagent. Each determination was carried out in duplicate. Nitrite interference was prevented by the addition of a sulfamic acid paper [prepared according to the method of Montgomery and Dymock (1962)] into the clarified test sample, for 5 min, prior to the removal of chloride. One microgram of NO_3-N was detectable.

Analysis for Dimethylnitrosamine (DMN) in Palm-Wine. The steam distillate from palm-wine was extracted into dichloromethane (purified for use in gas chromatography), cleaned up and concentrated to 0.5 mL by the method of Sen and Dalpe (1972).

Thin-Layer Chromatography. The presence of dimethylnitrosamine was verified on thin-layer silica plates (0.5 mm) by the procedure of Preussmann et al. (1964). The plates were developed in a hexane–ether–dichloromethane solvent system (4:3:2).

Gas–Liquid Chromatography. A Beckman GC-45 gas chromatograph, equipped with a flame ionization detector, was used in the detection and quantification of dimethylnitrosamine. The steel column (1 m × 3 mm) was packed with Porapak Q (100–120 mesh), and operating temperatures were: 220, 190, and 240 °C, respectively, for the injector, column, and detector. The carrier gas was helium flowing at a rate of 30 mL/min. The limit of detection by this procedure was 2 ng of dimethylnitrosamine and the retention time of DMN was 128 s.

Mass Spectrometry. The fragmentation pattern of the palm-wine isolate, which had an identical R_f value on TLC plate with the authentic dimethylnitrosamine, was obtained using a Hitachi mass spectrometer (Model RMU-6E). Zones of this R_f value (0.38–0.40), from 20 plates, were scraped into a beaker and eluted with dichloromethane. Silica gel was removed by filtration through Whatman No. 30 filter paper. Dichloromethane was evaporated on a steam bath (56 ± 2 °C) and the residue analyzed for dimethylnitrosamine in the mass spectrometer.

Recoveries of Added Nitrogen Compounds. Prior to analysis of the test beverages, recoveries of added nitrate, nitrite, and dimethylnitrosamine were determined. No recovery test of dimethylamine was carried out.

RESULTS AND DISCUSSION

The survey shows that dimethylamine (Table I), nitrates (Table II), and nitrites (Table III) usually occur in the fermented beverages assayed, except in ogogoro in which neither nitrate nor nitrite was detected.

Nitrates are normal constituents of plants and forage and are widely distributed in nature. Reports of the occurrence of secondary amines in nature include the identification of dimethylamine in cod, whitting, and herring (Shewan, 1938), unicellular green algae (Rolle et al., 1971); of dimethylamine, diethylamine, *N*-methyl-*n*-propylamine in higher plants, particularly tobacco (Irvine and Saxby, 1969; Bush, 1970); of dimethylamine, di-*n*-propylamine, morpholine, pyrrolidine, piperidine, methylethylamine, methyl-*n*-butylamine in beverages and meat or fish products (Singer and Lijinsky, 1976). In such

Table II. Distribution of Nitrate Content of Beverages

Location	No. of samples	Ppm nitrate ± SD				
		Palm-wine	Nono	Burukutu	Pito	Ogogoro
Ankpa[a]	20	36.33 ± 25.69	56.26 ± 26.58	26.58 ± 8.86	35.00 ± 16.02	Nd[b]
Dekina[a]	20	27.91 ± 14.18	34.11 ± 13.73	22.59 ± 8.86	47.4 ± 12.85	Nd
Egbe	20	23.04 ± 14.62	16.39 ± 8.42	60.25 ± 24.08	31.15 ± 8.86	Nd
Idah[a]	20	91.26 ± 45.94	20.82 ± 12.85	22.59 ± 10.19	13.73 ± 6.20	Nd
Ilorin	20	85.06 ± 47.84	46.96 ± 11.52	32.34 ± 13.29	50.50 ± 15.95	Nd
Jebba	20	20.38 ± 17.28	17.28 ± 11.08	54.93 ± 32.34	16.39 ± 9.75	Nd
Kabba	20	47.40 ± 37.21	31.90 ± 25.69	36.77 ± 30.57	39.87 ± 10.63	Nd
Kaima	20	81.07 ± 12.40	29.37 ± 11.08	32.78 ± 21.71	43.41 ± 16.39	Nd
Lokoja	20	73.54 ± 54.05	18.61 ± 8.86	41.64 ± 29.24	37.66 ± 15.06	Nd
Luma	20	18.16 ± 11.08	8.42 ± 3.99	49.17 ± 16.39	60.69 ± 31.45	Nd
New Bussa	20	18.61 ± 11.08	34.55 ± 20.82	41.20 ± 19.05	38.98 ± 17.28	Nd
Offa	20	59.81 ± 36.33	6.63 ± 7.97	24.37 ± 11.08	36.77 ± 19.94	Nd
Okene	20	28.35 ± 11.52	27.02 ± 17.28	27.47 ± 16.39	31.45 ± 13.29	Nd
Omu Aran	20	41.64 ± 17.28	16.83 ± 17.09	19.94 ± 8.86	37.21 ± 19.94	Nd
Pategi/Lafiagi	20	61.58 ± 25.69	34.55 ± 24.39	36.49 ± 10.63	30.12 ± 10.19	Nd

[a] Situated in Benue State. [b] Nd = no detection.

Table III. Distribution of Nitrite Content of Beverages

Location	No. of samples	Ppm nitrite ± SD				
		Palm-wine	Nono	Burukutu	Pito	Ogogoro
Ankpa[a]	20	2.50 ± 0.52	1.84 ± 0.42	1.35 ± 0.76	1.28 ± 0.29	Nd
Dekina[a]	20	1.05 ± 0.21	Nd	2.38 ± 1.39	1.60 ± 0.89	Nd
Egbe	20	2.81 ± 0.81	2.07 ± 0.71	1.78 ± 0.80	1.79 ± 0.72	Nd
Idah[a]	20	1.29 ± 0.43	1.63 ± 0.62	2.67 ± 1.72	2.01 ± 1.15	Nd
Ilorin	20	2.26 ± 0.43	Nd	1.44 ± 0.95	1.34 ± 0.40	Nd
Jebba	20	2.07 ± 0.49	2.06 ± 0.51	Nd	2.63 ± 0.84	Nd
Kabba	20	Nd[b]	2.81 ± 0.79	0.98 ± 0.51	1.15 ± 0.35	Nd
Kaima	20	1.74 ± 0.40	1.47 ± 0.56	Nd	1.99 ± 0.89	Nd
Lokoja	20	2.65 ± 0.53	1.42 ± 0.36	Nd	Nd	Nd
Luma	20	Nd	1.78 ± 1.26	0.86 ± 0.46	1.33 ± 0.57	Nd
New Bussa	20	1.73 ± 0.75	2.90 ± 1.80	Nd	Nd	Nd
Offa	20	2.69 ± 0.63	2.46 ± 0.88	Nd	1.91 ± 0.83	Nd
Okene	20	1.55 ± 0.46	1.07 ± 0.73	Nd	Nd	Nd
Omu Aran	20	2.12 ± 0.98	0.99 ± 0.62	Nd	1.57 ± 0.48	Nd
Pategi/Lafiagi	20	1.89 ± 0.46	1.48 ± 0.87	Nd	1.78 ± 0.84	Nd

[a] Situated in Benue State. [b] Nd = no detection.

cases, they occur usually with primary and tertiary amines.

Methods of identification of secondary amines are not very well developed and those usually measured are dimethyl- and diethylamine. In this survey, the method used in the assay of dimethylamine did not give any coloration with primary, tertiary, or some secondary amines (morpholine and piperidine). Although positive coloration was obtained with secondary alkylamines like diethylamine and di-*n*-propylamine, under our conditions in which the peak absorption due to dimethylamine was 445 nm, there would be a preponderance of dimethylamine (bp 6.88 °C) in the distillate. Other secondary alkylamines have much higher boiling points and do not usually occur in biological material (Sidgwick, 1937).

Nitrite, however, rarely occurs abundantly in nature and usually arises as a result of microbial reduction of nitrate in plant or food during storage. Since secondary amines and nitrates occur frequently in plant foods and products, the nitrite content of these could, therefore, become a critical factor in the elaboration of nitrosamines in situ. The presence of nitrite in the test beverages could, therefore, be either as a result of microbial reduction of nitrate in the biological fluids, or due to its absorption from the soil in which it is generated by nitrifying and denitrifying bacteria; either from the oxidation of ammonium (Alexander, 1965; Stojanovic and Alexander, 1958), or reduction of nitrate (Broadbent and Clark, 1965). Eighty percent of all the beverages analyzed contained nitrate; sixty-eight percent of them contained nitrite; while all of them contained dimethylamine.

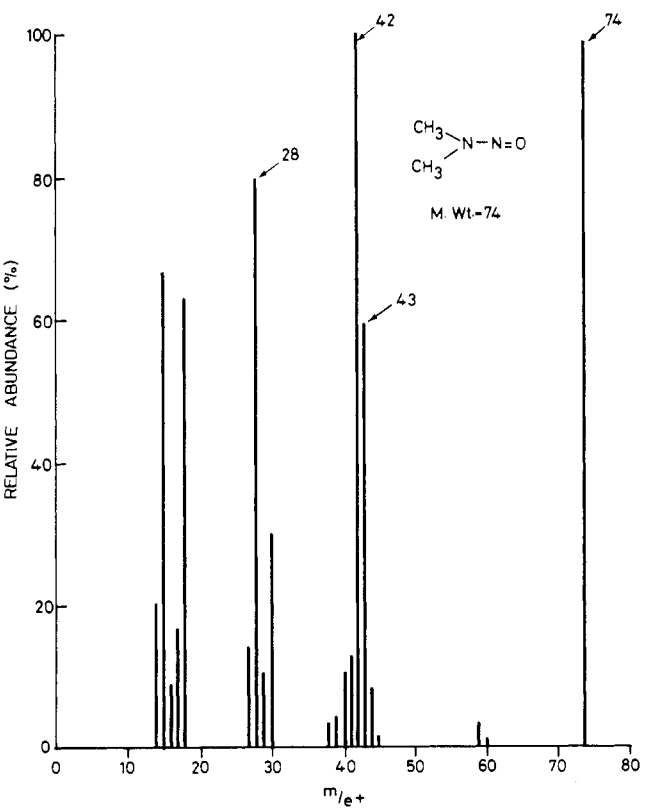

Figure 1. Mass spectrum of reference dimethylnitrosamine.

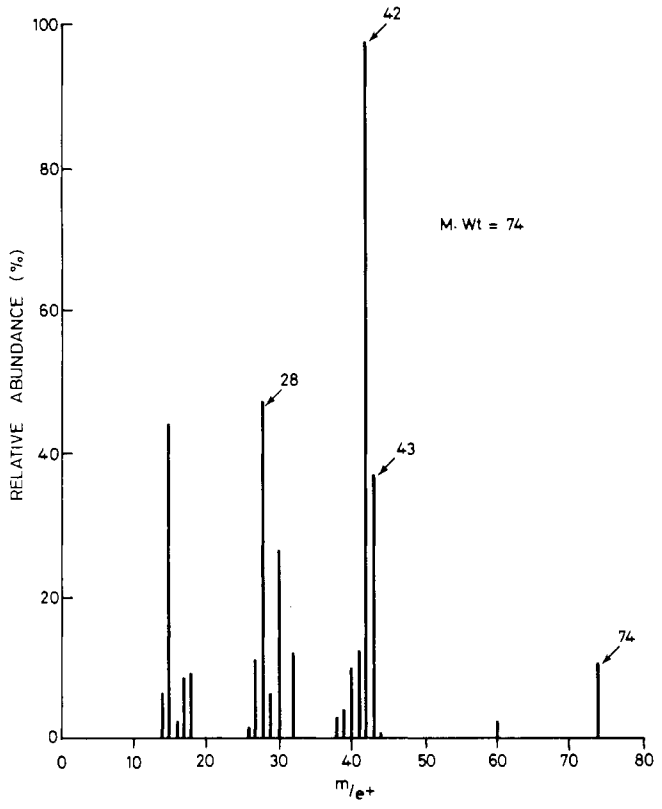

Figure 2. Mass spectrum of the unknown product, in the concentrate of palm-wine extract, corresponding to the R_f value of 0.38–0.40 on a thin-layer silica plate.

The mass spectra of the authentic reference dimethylnitrosamine and the product in an isolate of a sample of palm-wine, presumed to be dimethylnitrosamine, are shown in Figures 1 and 2, respectively. Although their fragmentation patterns differ, notably in the relative abundance of the parent ion m/e 74 and the ion m/e 43, the thin-layer chromatographic value (0.38–0.40) and gas chromatographic retention time value (128 s) were in agreement between the authentic DMN and the isolate. In addition, such results are frequently obtained when the amounts of compound introduced into the mass spectrometer are quite different for the known and unknown compound. Fazio et al. (1971) and Pensabene et al. (1972) have each reported mass spectra of authentic dimethylnitrosamine which were different from each other, notably in the relative abundance of the parent ion m/e 74 and ions of m/e 42 and 43. Dimethylnitrosamine (0.6 ppb) was detected in one sample of palm-wine. At present, work is in progress for the determination of nitrosamines in the other beverages. Recoveries of dimethylnitrosamine (added at the 1 ppb level), nitrate, and nitrite in palm-wine were 90 ± 5, 99.4 ± 0.57, and 98.3 ± 0.70%, respectively.

N-Nitrosamines are toxic (Barnes and Magee, 1954) and carcinogenic (Magee and Barnes, 1956). Therefore, the dangers which are inherent in the formation of nitrosamines in the human body from ingested precursors cannot be overemphasized, particularly in the light of the findings of Sander (1967) that the nitrosation reaction can occur in vitro in the gastric juice of various animal species, including man, and also in vitro at near neutral pH levels in the presence of enteric bacteria (Sander, 1968). Although small amounts of nitrite were detected in the test beverages, and so are unlikely to give rise to any appreciable amount of nitrosamine, their presence cannot entirely be ignored because no amount of a nitrosamine in food should be regarded as safe. In fact, the presence of a product in palm-wine, presumed to be dimethylnitrosamine, underlines the serious health hazard posed to public health by the presence of nitrates, nitrites, and secondary amines in food meant for human consumption.

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