1000 and 2000 ppm of nitrite chow diet increased with time, more than doubling over the 2-week storage period. For example, NDMA levels in the 1000 ppm of nitrite chow diet increased from 10 ppb at day 0 to 22 ppb on day 14 and respectively from 15 to 45 ppb for the 2000 ppm of nitrite chow diet. The former findings parallel values of 17 and 20 ppb for NDMA found (Wasserman, 1979) in a chow diet (NIH-007) containing 1000 ppm of added nitrite analyzed over several weeks by the FDA methanolic potassium hydroxide digestion procedure (Fazio et al., 1971). Prior to digestion, sulfamic acid was added to destroy the nitrite in the diet to prevent artifactual formation of nitrosamine. The low (3–4 ppb) levels of NDMA found in the chow diet with no added nitrite are within the range (<5 ppb) previously reported for such diets (Edwards et al., 1979).

Clearly, omission of the nitrosation inhibitors ammonium sulfamate and tocopherol resulted in artifactual nitrosamine formation during the analytical procedure, which demonstrates the need for careful control where the analysis of nitrosamines is of concern. This was evident in the chow diet where the artifactual nitrosamine formation was the greatest, implying that the possibility of in situ or in vivo nitrosation with chow diets is much greater than with either casein or agar diets.

The influence of these volatile nitrosamines in the cited nitrite feeding studies is unclear. Further, the effect of other nitrosation products potentially capable of being formed in situ or in vivo (e.g., nonvolatile *N*-nitrosoureas, *N*-nitrosamides, etc.) cannot presently be assessed until methods are developed that are capable of detecting and measuring these compounds as well.

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Registry No. NDMA, 62-75-9; NDEA, 55-18-5; NDPA, 621-64-7; NDBA, 924-16-3; NPIP, 100-75-4; NPYR, 930-55-2; NMOR, 59-89-2; nitrite, 14797-65-0; sodium nitrite, 7632-00-0.

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Nitrogen-Containing Heterocyclic Compounds Identified in the Volatile Flavor Constituents of Roasted Beef

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Volatile flavor compounds were isolated from 500 lb of roasted beef. The flavor isolate was subjected to extensive gas chromatographic fractionation, and the pure fractions obtained were identified by GC–mass spectrometry. A total of 44 nitrogen-containing heterocyclic compounds were identified. They included 15 thiazoles, 1 thiazoline, 6 oxazoles, 11 pyrazines, 6 pyrroles, 2 piperidines, and 3 pyridines.

Due to the great sensory and economic value of beef, an enormous amount of research has been conducted on beef flavor. Over 500 compounds have been mentioned in the literature as components identified in the volatiles of cooked beef (MacLeod and Seyyedain-Ardebili, 1981).

Heterocyclic compounds play an important role in roasted flavors and particularly in meat products (Ohloff and Flament, 1978). Some of them have interesting organoleptic properties and very low thresholds. The present paper reports on the identification of nitrogen-containing heterocyclic compounds in the volatile flavor of roasted beef.

EXPERIMENTAL SECTION

Preparation of Roasted Beef. A total of 25 lb of Longissimus beef muscle (cross rib roast), pieces averaging 3–4 lb, was pan-browned with no added fat. The browned

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Table I. Nitrogen-Containing Heterocyclic Compounds Identified in the Volatile Flavor of Roasted Beef

identification	mass spectrum reference
thiazoles and thiazolines	
2-methylthiazole	Vitzthum and Werkhoff (1974)
4-methylthiazole	Vitzthum and Werkhoff (1974)
2-propylthiazole[a]	Heller and Milne (1978)
2-methyl-4-ethylthiazole	Vitzthum and Werkhoff (1974)
trimethylthiazole	Vitzthum and Werkhoff (1974)
2-acetylthiazole	ten Noever de Brauw et al. (1980)
2-methyl-4-propyl-5-ethylthiazole[a]	Heller and Milne (1978)
2-methyl-4-ethyl-5-propylthiazole[a]	Heller and Milne (1978)
2-isopropyl-4,5-dimethylthiazole[a]	ten Noever de Brauw et al. (1980)
2-propyl-4-ethyl-5-methylthiazole[a]	Table II
2-butyl-4,5-dimethylthiazole[a]	ten Noever de Brauw et al. (1980)
2-butyl-4-methyl-5-ethylthiazole[a]	Table II
2-pentyl-5-methylthiazole[a]	Table II
2-pentyl-4,5-dimethylthiazole[a]	Table II
benzothiazole	Heller and Milne (1978)
2-acetyl-2-thiazoline	ten Noever de Brauw et al. (1980)
oxazoles	
4,5-dimethyloxazole[a]	Vitzthum and Werkhoff (1974)
trimethyloxazole	Vitzthum and Werkhoff (1974)
2-methyl-4-butyl-5-methyloxazole[a]	Table II
2,5-dimethyl-4-hexyloxazole[a]	Heller and Milne (1978)
2-propyl-4,5-dimethyloxazole[a]	ten Noever de Brauw et al. (1978)
benzoxazole[a]	Heller and Milne (1978)
pyrazines	
2-methylpyrazine	Maga and Sizer (1973)
2,3-dimethylpyrazine	Maga and Sizer (1973)
2,5-dimethylpyrazine	Maga and Sizer (1973)
2,6-dimethylpyrazine	Maga and Sizer (1973)
2-methyl-3-ethylpyrazine	Maga and Sizer (1973)
2-methyl-5-ethylpyrazine	Maga and Sizer (1973)
trimethylpyrazine	Maga and Sizer (1973)
2,5-dimethyl-3-ethylpyrazine	Maga and Sizer (1973)
2,3-dimethyl-6-ethylpyrazine	Maga and Sizer (1973)
tetramethylpyrazine	Maga and Sizer (1973)
2,5,6-trimethyl-3-ethylpyrazine	Maga and Sizer (1973)
pyrroles	
2-butylpyrrole[a]	MSDC
2-acetylpyrrole	Kinlin et al. (1972)
3-methyl-4-ethylpyrrole[a]	MSDC
N-methyl-2-formylpyrrole[a]	Kinlin et al. (1972)
indole	ten Noever de Brauw et al. (1980)
N-methylpyrrolidine[a]	Heller and Milne (1978)
piperidines	
piperidine[a]	Porter and Baldas (1971)
2-methylpiperidine[a]	Porter and Baldas (1971)
pyridines	
pyridine	Heller and Milne (1978)
2-methylpyridine	ten Noever de Brauw et al. (1980)
2-acetylpyridine	Heller and Milne (1978)

[a] Believed to be newly identified in heated beef.

meat was placed in a gas-heated oven at 166 °C and roasted to an internal temperature of 65 °C. The cooked meat was cubed into $^1/_2$-in. pieces.

Isolation and Concentration of Volatile Flavor Compounds. The volatile flavor compounds were isolated from 500 lb of roasted beef by using the apparatus described by Chang et al. (1977). The principle of the apparatus is the removal and subsequent condensation of the volatile compounds in the headspace of the roasted beef. Humidified nitrogen at a flow rate of 80 mL/min was used to remove the volatile compounds from roasted beef. A total of 25 lb of roasted beef was used for each isolation, which lasted 48 h at 50 °C. The headspace aroma was monitored during the isolation period. A total of 20 isolations were run. The total condensate collected in the traps cooled with dry ice and acetone was treated in a manner similar to that described by Herz and Chang (1966). The condensate was washed out and extracted with analytical-grade ethyl ether. The ethyl ether extract of the condensate was dried with anhydrous sodium sulfate and then concentrated to a volume of 50 mL with the use of a 32-plate Oldershaw column. It was finally concentrated to a volume of 5 mL with a spinning band still. The concentrate was evaluated for aroma and flavor by a panel of trained flavorists from General Foods Corp.

Fractionation of the Flavor Isolate. The initial preparative gas chromatography of the isolated volatile roasted beef flavor compounds was performed on a Beckman GC-55 gas chromatograph equipped with a flame ionization detector, fitted with a 2 mm i.d. × 3 m glass column, packed with 10% SP-1000 on 60–80-mesh Chromosorb W AW DMCS. The helium flow rate was 30 mL/min. The column temperature was held at 50 °C for 8 min and then increased by 2.5 °C/min to a holding temperature of 230 °C for 10 min. The preliminary chromatogram was separated into 21 fractions. Each fraction was accumulately collected in a "hairpin"-type trap, according to the method of Thompson et al. (1978).

Each of the 21 fractions was subjected to a second fractionation using a 2 mm i.d. × 3 m glass column packed with 10% OV-17 on 60–80-mesh Chromosorb W AW DMCS.

Table II. Mass Spectral Data of New Thiazoles and Oxazoles Identified in Roasted Beef Flavor by Comparison with Synthetic Authentic Compounds

compound	aroma	characteristic MS data,[a] m/z (rel intensity)
2-propyl-4-ethyl-5-methylthiazole ($C_9H_{15}NS$)	rubbery, minty, spicy	27 (64), 28 (50), 39 (54), 41 (67), 53 (16), 59 (73), 65 (12), 67 (27), 77 (4), 85 (72), 99 (16), 100 (23), 112 (3), 113 (5), 126 (9), 127 (5), 140 (27), 141 (100), 154 (29), 155 (4), 168 (11), 169 (16); M = 169
2-butyl-4-methyl-5-ethylthiazole ($C_{10}H_{17}NS$)	green, pleasant vegetable green	27 (23), 28 (20), 41 (24), 45 (36), 53 (6), 55 (6), 67 (10), 71 (43), 84 (6), 85 (8), 99 (5), 100 (3), 112 (14), 113 (9), 125 (4), 126 (2), 141 (100), 142 (11), 154 (23), 155 (41), 168 (14), 169 (2), 182 (6), 183 (5); M = 183
2-pentyl-5-methylthiazole ($C_9H_{15}NS$)	vegetable, leek-like, sulfury	27 (25), 29 (21), 39 (22), 45 (28), 54 (3), 55 (4), 71 (27), 72 (26), 85 (2), 99 (1), 100 (2), 112 (9), 113 (100), 126 (25), 127 (7), 139 (3), 140 (9), 152 (4), 153 (1), 168 (1), 169 (3); M = 169
2-pentyl-4,5-dimethylthiazole ($C_{10}H_{17}NS$)	rubbery, sulfury	27 (13), 29 (8), 41 (13), 45 (12), 53 (10), 59 (11), 68 (2), 71 (19), 85 (9), 86 (17), 94 (1), 112 (1), 113 (1), 126 (16), 127 (100), 140 (29), 141 (9), 154 (11), 155 (2), 168 (1), 182 (7), 183 (7); M = 183
2-methyl-4-butyl-5-methyloxazole ($C_9H_{15}NO$)	green, fatty, vegetable	27 (11), 29 (4), 42 (33), 43 (78), 53 (3), 55 (9), 69 (39), 70 (13), 82 (2), 83 (2), 96 (3), 97 (2), 110 (77), 111 (100), 124 (6), 125 (3), 138 (5), 153 (21), 154 (3); M = 153

[a] The two most intense ions every 14 mass units above 20 are listed.

Mass Spectrometry of the Gas Chromatographic Fractions. Mass spectrometry was performed on a Du Pont 21-490 mass spectrometer with a jet separator interfaced to a Varian Moduline gas chromatograph with an FID detector and a 2 mm i.d. × 3 m glass column packed with 10% OV-101 on 60–80-mesh Chromosorb W AW DMCS. The flow rate was 30 mL/min. The column temperature was held at 40 °C for 5 min and then increased by 5 °C/min to a holding temperature of 240 °C for 10 min. Spectra were taken at 70 eV.

Materials. Alkylthiazoles were synthesized according to the method of Kurkjy and Brown (1952). 2-Methyl-4-ethyl-5-propyloxazole was synthesized according to the method of Theilig (1953).

RESULTS AND DISCUSSION

The volatile flavor constituents were isolated from 500 lb of roasted beef held at 50 °C for 48 h in a headspace flushing apparatus. The aroma and flavor of the concentrate was found to possess the characteristics of a roasted beef flavor. A total of 44 nitrogen-containing heterocyclic compounds were identified. They consisted of 15 thiazoles, 1 thiazoline, 6 oxazoles, 11 pyrazines, 6 pyrroles, 2 piperidines, and 3 pyridines (Table I).

Thiazoles and oxazoles are well recognized as extremely potent odorants (Maga, 1975, 1978). Thiazoles have lower odor threshold values than the oxazoles and are therefore likely to be more important contributors to roasted beef flavor. Four thiazoles and one oxazole identified in this study were new to the flavor of food. They were synthesized and their mass spectral data are listed in Table II. Among the six oxazoles identified in this study, only 2,4,5-trimethyloxazole has been reported previously in the volatile flavor of cooked beef (Chang et al., 1968; Chang and Peterson, 1977). The mechanisms for the formation of 2,4,5-trimethyloxazole and 4,5-dimethyloxazole have been proposed (Rizzi, 1969; Ho and Hartman, 1982).

The *N*-methylpyrrolidine identified was evaluated to have an amine-like odor. Pyrrolidines have been identified in numerous foodstuffs (Ohloff and Flament, 1978) but have not been reported in beef flavor.

Two piperidines identified were piperidine and 2-methylpiperidine. These compounds were evaluated to possess a dry, nutty aroma. Piperidines may represent a new class of compounds in cooked beef.

Other nitrogen-containing heterocyclic compounds identified in roasted beef flavor included pyrazines and pyridines. The importance of these compounds to the flavor of meat has been reviewed (Ohloff and Flament, 1978).

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Registry No. 2-Methylthiazole, 3581-87-1; 4-methylthiazole, 693-95-8; 2-propylthiazole, 17626-75-4; 2-methyl-4-ethylthiazole, 32272-48-3; trimethylthiazole, 13623-11-5; 2-acetylthiazole, 24295-03-2; 2-methyl-4-propyl-5-ethylthiazole, 4276-67-9; 2-methyl-4-ethyl-5-propylthiazole, 41981-75-3; 2-isopropyl-4,5-dimethylthiazole, 53498-30-9; 2-propyl-4-ethyl-5-methylthiazole, 86290-19-9; 2-butyl-4,5-dimethylthiazole, 76572-48-0; 2-butyl-4-methyl-5-ethylthiazole, 86290-20-2; 2-pentyl-5-methylthiazole, 86290-21-3; 2-pentyl-4,5-dimethylthiazole, 86290-22-4; benzothiazole, 95-16-9; 2-acetyl-2-thiazoline, 29926-41-8; 4,5-dimethyloxazole, 20662-83-3; trimethyloxazole, 20662-84-4; 2-methyl-4-butyl-5-methyloxazole, 86290-23-5; 2,5-dimethyl-4-hexyloxazole, 20662-86-6; 2-propyl-4,5-dimethyloxazole, 53833-32-2; benzoxazole, 273-53-0; 2-methylpyrazine, 109-08-0; 2,3-dimethylpyrazine, 5910-89-4; 2,5-dimethylpyrazine, 123-32-0; 2,6-dimethylpyrazine, 108-50-9; 2-methyl-3-ethylpyrazine, 15707-23-0; 2-methyl-5-ethylpyrazine, 13360-64-0; trimethylpyrazine, 14667-55-1; 2,5-dimethyl-3-ethylpyrazine, 13360-65-1; 2,3-dimethyl-6-ethylpyrazine, 15707-34-3; tetramethylpyrazine, 1124-11-4; 2,5,6-trimethyl-3-ethylpyrazine, 17398-16-2; 2-butylpyrrole, 1551-10-6; 2-acetylpyrrole, 1072-83-9; 3-methyl-4-ethylpyrrole, 488-92-6; *N*-methyl-2-formylpyrrole, 1192-58-1; indole, 120-72-9; *N*-methylpyrrolidine, 120-94-5; piperidine, 110-89-4; 2-methylpiperidine, 109-05-7; pyridine, 110-86-1; 2-methylpyridine, 109-06-8; 2-acetylpyridine, 1122-62-9.

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Determination of *N*-Nitrosoproline and *N*-Nitrososarcosine in Malt and Beer

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A rapid and sensitive method is described for the determination of *N*-nitrosoproline and *N*-nitrososarcosine in malt and beer. It consists of extraction of the sample with methanol, cleanup on commercially available extraction tubes, and preparation of the methyl ester derivatives by treatment with diazomethane or BF_3–methanol. The final determination is carried out by gas–liquid chromatography using a thermal energy analyzer detector. The average percentage recoveries of both the compounds added to malt and beer were highly satisfactory (84–90%). The average levels of *N*-nitrosoproline detected in 11 malt (both old and recent) and 28 beer samples (mostly recent) were found to be 24.1 ppb (range 5.6–113.3) and 1.7 ppb (range trace–6.0), respectively. Only two samples of malt contained traces (<1 ppb) of *N*-nitrososarcosine. In view of the noncarcinogenicity of *N*-nitrosoproline in experimental animals, these findings are unlikely to pose any hazard to human health.

Studies carried out during the past few years have paid considerable attention to the analysis of beer and ale for the presence of volatile nitrosamines, mainly *N*-nitrosodimethylamine (NDMA). It is now well established that most beer and ale contain traces of NDMA, which is a potent carcinogen, and that NDMA in these beverages originates from malt produced by the so-called "direct drying" technique in which hot flue gas containing nitrogen oxides is passed directly over the malt during the drying process. The details of the findings have been published by Spiegelhalder et al. (1980) and others (Fazio et al., 1980; Goff and Fine, 1979; Hotchkiss et al., 1980; Sen et al., 1980). Similar data on the contents of nonvolatile *N*-nitroso compounds of beer and ale are, however, lacking mainly because of lack of adequate methodologies for these compounds. An understanding of the total *N*-nitrosamine contents of the products is desirable in order to make a full assessment of the health hazard arising from the consumption of these beverages.

Since there are many types of nonvolatile *N*-nitroso compounds that could be present in beer and ale, no single method is likely to be adequate for their analyses. Therefore, our initial study was concentrated on the development of methodologies for *N*-nitrosoamino acids such as *N*-nitrososarcosine (NSAR) and *N*-nitrosoproline (NPRO), both of which have already been reported to occur in raw bacon and other cured meat products (Eisenbrand et al., 1978; Pensabene et al., 1979; Bogovski et al., 1982; Sen et al., 1978, 1982). Preliminary reports of the occurrence of NPRO in malt and beer have also been published (Bogovski et al., 1982; Pollock, 1981; Sen et al., 1982). This paper reports the development of a rapid method for their determination in malt and beer and presents some data on the levels of NPRO in these products.

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EXPERIMENTAL SECTION

Materials. All reagents used were of analytical grade and the solvents were of glass-distilled varieties obtained from commercial suppliers. NPRO and NSAR standards were gift from Drs. W. Lijinsky and C. L. Walters, respectively. The Preptubes (20 mL) and the Clin Elut Extubes (20 mL) were purchased from Thermo Electron Corp., Waltham, MA, and Analytichem International, Harbor City, CA, respectively. BF_3–methanol reagent was obtained from Applied Science, Milton Roy Industries, Ltd., Rexdale, Ontario, Canada. Diazomethane was prepared in situ from Diazald (Aldrich Chemicals, Milwaukee, WI) and collected by bubbling (swept by N_2) through ice-cold ether containing 5% methanol according to the method of Schlenk (1960). The solution was either immediately used or stored over dry ice in an insulated box (placed in fume hood) until used. Celite 545 was obtained from Fisher Scientific Co., Montreal, Quebec, Canada, and heated overnight at 600 °C before use.

The malt samples were collected from various plants in Canada through the courtesy of Field Operations Directorate, Health Protection Branch. A 100–150-g aliquot was finely ground in a blender and stored in a tightly sealed mason jar until analyzed. Both the domestic and imported beers and ales were purchased locally in the Ottawa–Hull area.

Procedure. (*a*) *Extraction and Cleanup.* A 15–20-g aliquot of the ground malt was mixed with 10 mL of 1 N sulfuric acid containing 1% dissolved sulfamic acid (to prevent artifactual formation of *N*-nitroso compounds) and

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