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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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the mixture homogenized for 3 min with 100 mL of methanol and 5 g of Celite (filtering aid) by using a Sorvall Omni-Mixer (Du Pont Instruments). The extract was carefully decanted and filtered through a Büchner filter funnel (using Whatman filter paper, No. 1) under mild suction. The residue in the mixer was rinsed with two 30-mL portions of methanol and filtered as above. About 20 mL of water was added to the above filtrate and the mixture extracted with 100 mL of *n*-hexane in a separatory funnel to remove any lipid and pigment that might be present in the malt. The hexane layer was discarded. The methanol–water layer was concentrated under vacuum (using a flash evaporator) to 2–5 mL (avoiding going to dryness) and the residue was quantitatively transferred (using a Pasteur pipet) into a graduated test tube. About 5–8 mL of water was used to rinse the evaporation flask, the rinse was added to the main extract in the test tube, and the final volume was made up to 15 mL. Exactly 2 mL of 3 N sulfuric acid was added to the extract and the mixture was poured into a 20 mL capacity Preptube or Clin Elut Extube.

After 5 min, the tube was washed (to remove pigments) with a 60 mL of mixture of *n*-hexane–dichloromethane (3:1) and the wash discarded. The tube was then eluted with 4 × 20 mL of ethyl acetate (waiting 2–3 min between each addition), the eluate was dried over anhydrous sodium sulfate (kept 30 min with occasional swirling) and filtered, and the filtrate was concentrated to ≃2 mL with a flash evaporator. The concentrated extract was quantitatively transferred (with 4 × 1 mL of ethyl acetate rinsing) into a test tube and the mixture concentrated to ≃1 mL in a gentle stream of nitrogen (avoiding going to dryness).

For ale and beer samples the preliminary extraction step with methanol was omitted. A 15–19-mL aliquot of the sample was mixed with 1 mL (for Preptubes) or 3 mL (if using Extubes) of 3 N sulfuric acid and 1 mL of 1% sulfamic acid, and the mixture was poured directly into the extraction tube. The washing step with 60 mL of hexane–dichloromethane (3:1) was also found to be unnecessary. The rest of the procedure was exactly as described above.

(*b*) *Esterification.* Two different methods of esterification were used. In the first method, the concentrated eluate from step a above was treated with excess diazomethane, and the reaction mixture allowed to stand in an ice bath for 30 min and then concentrated to 1.0 mL in a gentle stream of nitrogen (care must be taken to avoid dryness). In the second method, the eluate was concentrated to 0.1–0.2 mL (avoiding going to dryness) in a stream of nitrogen and mixed with 1.0 mL of BF_3–methanol reagent in the stoppered test tube, and the mixture was heated in a sand bath (60–70 °C) for 30 min in the dark. The sample was allowed to cool to room temperature and mixed with 4 mL of water. Exactly 1.0 mL of dichloromethane was added and the mixture was vigorously mixed for 2 min with a Vortex mixer. An aliquot (6 μL) of the dichloromethane layer, which contained the methyl esters, was used for the final GLC analysis as described below. Known amounts (1 μg each) of NSAR and NPRO standards (dissolved in ethyl acetate) were esterified similarly and used for quantitation. To avoid hydrolysis of the ester derivatives, the GLC analysis was carried out on the same day.

(*c*) *GLC–Thermal Energy Analyzer (GLC–TEA) Technique.* The basic principle and operation of the TEA detector have been well documented (Fine et al., 1975; Fine and Rounbehler, 1975). In the GLC mode it is highly accurate, sensitive, and selective for the determination of volatile *N*-nitrosamines or volatile derivatives of nonvolatile *N*-nitroso compounds. The GLC–TEA conditions were as follows: column, 9 ft × $^1/_8$ in. (o.d.) Ni tubing packed with 10% Carbowax 20 M on Chromosorb W, HMDS (60–80 mesh), *without* any added NaOH; temperatures, injection port 225 °C, transfer line 290 °C, GLC oven programmed from 160 to 200 °C at the rate of 10 °C/min, and TEA 450 °C; carrier gas (Ar) flow rate 30 mL/min; TEA vacuum chamber pressure ≃1 mm; TEA cold trap immersed in liquid N_2.

(*d*) *GLC–High Resolution Mass Spectrometric Confirmation (GLC–MS).* Prior to GLC–MS analysis the esterified extract was further purified as follows. About 9 mL of anhydrous *n*-hexane was mixed with 1 mL of esterified extract (prepared by the diazomethane method), and the mixture was passed through a 1 cm × 4 cm column of basic alumina deactivated with 3% water. The column was washed with 25 mL of anhydrous *n*-hexane and the washing discarded. About 50 mL of dichloromethane was passed through the column and the eluate carefully concentrated to ≃4 mL by evaporation in a Kuderna-Danish concentrator. The solution was then concentrated to 1.0 mL in a stream of nitrogen in the usual manner. A 5–10-μL aliquot of the cleaned up extract was analyzed by GLC–MS using the specific ion (for the molecular ion) monitoring technique as described previously (Sen et al., 1978). The MS (Varian Mat 311A) was operated at a resolution of 10000. The GLC column was the same as described above.

Table I. Percentage Recoveries of Added NPRO and NSAR from Malt and Beer

sample	spiking level, ppb, for each nitroso amino acid	% recoveries[a] NSAR	NPRO
malt[b]	40	81	88
malt	40	74	118
malt	40	81	74
malt	40	100	89
malt	10	82	79
	mean:	83.6	89.6
beer[b]	1.0	–[c]	100
beer	1.2	–	75
beer	1.2	–	100
beer	1.2	–	73
beer	1.2	–	88
beer	1.0	100	100
beer	50	97	99
	mean:	*d*	90.7

[a] The amount present, if any, in the sample was subtracted before calculating percent recoveries. [b] Each sample was of either a different brand or of a different lot number. [c] Not spiked with NSAR. [d] Insufficient data.

RESULTS AND DISCUSSION

The method described above for the analysis of NPRO and NSAR in malt and beer is very simple and rapid. As can be seen from the data in Table I, the percentage recoveries of added NSAR and NPRO to malt and beer are highly satisfactory. The minimum detection limit of the method is about 0.5–1 ppb depending on the sample size used for the analysis. The method has also been successfully applied to the analysis of other foods such as cured meats, fried bacon, fish, and skim milk powder (Sen et al., 1982).

In the early stages of the method development we used only Preptubes for extraction purposes. But later, when these tubes became commercially unavailable, we tried Clin Elut Extubes, which were also found to work extremely well with the exception that the sample extracts had to

Table II. Levels of NSAR and NPRO in Canadian Malts

sample no.	level, ppb NSAR	level, ppb NPRO
1	N[a]	23.2
2	N	5.6
3	N	21.0
4	N	13.6
5	N	27.1
6	N	21.8[b]
7	N	31.5[b]
8	N	37.0[b]
9	N	113.3[b]
10	<1	18.0[b]
11	<1	42.1[b]
	mean:	24.1[c]

[a] N = negative (<0.5 ppb). [b] Confirmed by GLC-MS. [c] Excluding no. 9.

Table III. Levels of NPRO in Beer and Ale

origin	no. of positives/ total	level, ppb[a] range	level, ppb[a] mean
Quebec, Canada	14/23	trace-6.0	1.3
Ontario, Canada	4/4	0.5-0.9	0.7
Scotland	2/2	3.1-5.0	4.0
Germany	1/1		1.8
Holland	1/1		1.5
Japan	1/1		3.5
Australia	1/1		3.8
France	1/1		2.7
Denmark	1/1		1.1
United States	1/1		1.8
Czechoslovakia	1/1		3.9
		overall mean:	1.7

[a] Uncorrected for percent recoveries.

be mixed with extra sulfuric acid (3 mL of 3 N sulfuric instead of 1 mL) for quantitative recovery of the nitroso amino acids. Probably, these compounds are adsorbed strongly on the hydrophilic matrix present in the Extubes that requires the addition of extra acid to release them in the free acidic form—the form easily extractable into ethyl acetate. The Preptubes, on the other hand, are packed with specially processed cotton gauze (similar to cheesecloth) that does not cause any such adsorption problems.

The methylation procedure using diazomethane is similar to that reported by Pollock (1981) and Kawabata et al. (1974), but the cleanup procedures described in this paper are entirely different from those described by the above-mentioned workers. Moreover, the BF_3–methanol esterification method, as described here, is a new technique and has not been used by others for the analysis of nitroso amino acids in foods. We used diazomethane for esterification of NSAR and NPRO in the first phase of the study, but due to concern as to its toxic and carcinogenic properties it was later replaced with BF_3–methanol reagent, which is available commercially from several suppliers. The latter technique works extremely well, and gives results comparable to those obtained by the diazomethane technique (Figure 1). Moreover, diazomethane is too hazardous a chemical to be used for routine work; it can cause severe explosion if not handled properly. For these reasons, we recommend that its use should be avoided and it be replaced with BF_3–methanol or other suitable reagents for the esterification of nitrosoamino acids.

Tables II and III give levels of NPRO detected in some domestic Canadian malts and in 18 domestic and 10 imported samples of beer and ale, respectively. It should be noted that one of the malts (no. 9), which contained the highest levels of NPRO, came from an old stock that was dried by the direct drying method. The average of NPRO

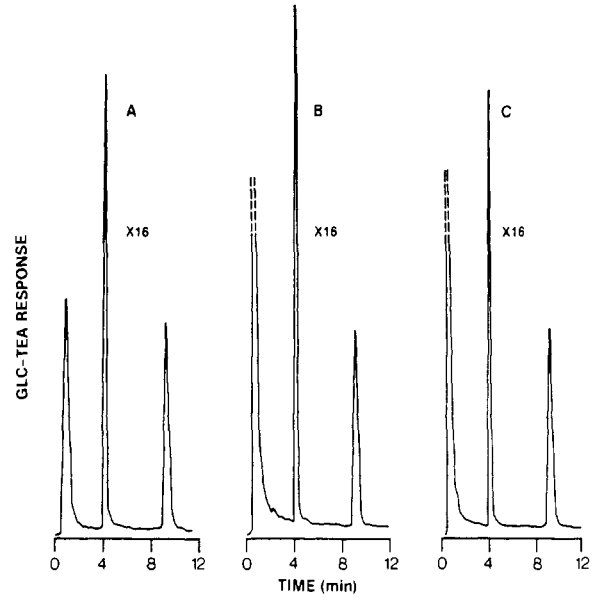

Figure 1. Chromatograms showing the comparison of two esterifying techniques. (A) 5.2 μL/1.0 mL NSAR (left) and NPRO (right) standards (1 μg/mL each) esterified by the BF_3–methanol method. (B) 5.2 μL/1.0 mL NSAR and NPRO standards esterified by diazomethane reagent. (C) A beer sample spiked with 1 μg each of NSAR and NPRO and taken through the entire method (BF_3–methanol reagent used for esterification); 5 μL/1.0 mL final solution injected. The percent recovery of the two compounds were 97.2% and 99.5%, respectively.

in the malt, excluding the value for sample 9, was found to be 24.1 ppb, and that for all 28 beers was 1.7 ppb. In six samples of malt (Table II) the identity of NPRO was confirmed by GLC–MS. There were indications of the presence of traces (<1 ppb) of NSAR in two samples of malt, but the levels were too low to be confirmed by GLC–MS. These data are comparable to those reported by Pollock (1981), who observed the presence of traces–20 ppb of NSAR and 8–286 ppb of NPRO in some 13 samples of malts produced by different drying procedures. The above researcher, however, did not investigate the possible presence of these compounds in beer and ale. Our results on the levels of NPRO in beer are much lower than that reported by Bogovski et al. (1982), who detected an average of 71 ppb of NPRO in some Russian beers. Perhaps, the drying method used to process the malts, from which the latter beers were made, and their high NPRO contents were responsible for such high values.

Thus far, we have shown that only traces of NPRO are present in malt and beer. Since NPRO is reported to be noncarcinogenic (Magee et al., 1976) these findings have little health hazard significance. However, the presence of NPRO can be taken as an indicator of the presence of other nonvolatile *N*-nitroso compounds for which analytical methodologies are not yet available. Therefore, studies should be continued along these lines in order to develop new methodologies that will be applicable to the analysis of a wide variety of nonvolatile *N*-nitroso compounds.

Safety Note. Since most *N*-nitroso compounds are carcinogenic and diazomethane is both carcinogenic and explosive, proper precautions should be taken while handling these chemicals.

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Registry No. *N*-Nitrosoproline, 7519-36-0; *N*-nitrososarcosine, 13256-22-9.

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Ergosterol, Ergosta-4,6,8(14),22-tetraen-3-one, Ergosterol Peroxide, and Chitin in Ergoty Barley, Rye, and Other Grasses

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Ergosterol, ergosta-4,6,8(14),22-tetraen-3-one (ETO), ergosterol peroxide (EP), and chitin contents in ergot sclerotia ranged from 204 to 827, 0.74 to 5.6, 41 to 152, and 8210 to 9020 μg/g, respectively, in barley, rye, and five grasses. Ergosterol and chitin contents of ergot sclerotia were high compared to those of ergot-free grain, and removal of ergot sclerotia from grain kernels leaves behind only background levels of ergosterol. Ergot contamination increased the ergosterol content significantly more than the chitin content of the grain. Maximum ergot contamination allowed by U.S. Grain Standards is 0.1% by weight in some grains (barley and oats) and 0.3% in others (rye and wheat). Only at or above the 0.3% allowed ergot level would increases in ergosterol contents become significant. Ergosterol, ETO, EP, and chitin are components of other fungi besides *Claviceps* spp.

Ergot sclerotia are formed in place of normal seeds during parasitic attack of cereals and grasses by *Claviceps* spp., mainly *Claviceps purpurea* (Bove', 1970; Lorenz, 1979). Because ergot sclerotia usually contain toxic alkaloids, their presence is highly undesirable in grain used for food or feed. Although ergot is noticeable in the heads of cereal grains and in harvested whole grain, its visibility is greatly reduced or completely lost after the grain is ground and processed into food or feed. Methods for detecting ergot in cereal grains or their products include various color tests, pigment determinations, microscopic examination for ergot fragments, tests for toxicity to experimental animals (not often used), and chemical determinations of ergot alkaloids (Lorenz, 1979). High-pressure liquid chromatography is particularly useful for detecting specific alkaloids, especially those that are physiologically active (Scott and Lawrence, 1980; Young, 1981).

Assays for ergosterol (Seitz et al., 1977, 1979) and chitin (Donald and Mirocha, 1977) have been proposed for measurement of fungal invasion in grains. Studies of those assays, however, did not include samples of ergoty grains or seeds. Therefore, we measured ergosterol and chitin content of ergoty and ergot-free grains and grass seeds to determine whether either assay would be useful for detecting ergot contamination and to what extent assays of ergosterol and chitin would be affected by the presence of ergoty grain.

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It has been know for a long time that ergosterol is a constituent of ergot (Tanret, 1889; Hart and Heyl, 1930), but quantitative information concerning its content in sclerotia or ergoty grains has been lacking. To the best of our knowledge little published information is available on the ergosta-4,6,8(14),22-tetraen-3-one (ETO) and ergosterol peroxide (EP) contents of cereals.

EXPERIMENTAL SECTION

Samples and Reference Compounds. Origins of samples of ergoty barley (*Hordeum vugare* L.), rye (*Secale cereale* L.), bromegrass (*Bromus sp.*), slender wheatgrass (*Agropyron sp.*), and an unidentified grass seed are given in Table I. Samples collected before 1978 were described by Pomeranz et al. (1975). Samples labeled "ergot free" in Table I represent grain or seed that remained after sclerotia were removed by hand.

Four commercial, ergot-free samples of barley (Table II) were obtained from the Federal Grain Inspection Service. The samples were selected to show a typical range in quality based on germination percentages and mold damage. Type of mold damage was not characterized.

Glucosamine and ergosterol were from Eastman Kodak Co. The latter was recrystallized twice from absolute ethanol. Authentic ETO and EP were prepared from ergosterol by the methods of Elks (1954) and White et al. (1973), respectively.

Assays. Ergosterol, ETO, and EP were extracted from ergot sclerotia, grains, and grass seeds as described previously (Seitz et al., 1979; Seitz and Paukstelis, 1977). Ergosterol and ETO were determined by a high-pressure liquid chromatography (HPLC) system consisting of a