An Improved Fluorometric Method for the Determination of Ammonia and Volatile Amines in Meat Tissue by High-Performance Liquid Chromatography

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An improved method was developed for determining ammonia and volatile amines in meat tissue as fluorescent dansyl derivatives. Separations were carried out by reversed-phase high-performance liquid chromatography. The procedure minimizes formation of both dansyl acid and dimethylamine from dansyl chloride and decomposition of dansylated α-amino acids to dansylamide. Determination of ammonia by this method was more accurate and precise than that by the glutamate dehydrogenase assay.

Ammonia levels in meat tissue are presently being investigated in our laboratory as indicators of the microbial quality of meat. Some commonly used methods to determine ammonia and/or volatile amines in biological tissue are the glutamate dehydrogenase assay (Reichelt et al., 1964), ammonia-selective electrode (ASE) (Gerhart et al., 1979), Kjeldahl distillation (Brooks and Ammerman, 1978), and high-performance liquid chromatography (HPLC) (Jen-Kun Lin and Chen-Ching Lai, 1980). The enzyme assay is preferred since results are not affected by interfering volatile compounds potentially present in the tissue (Parris and Foglia, 1983). For example, ammonia in meat cannot be determined accurately by the ASE method since the electrode also responds to volatile amines (Parris and Foglia, 1983). The electrode's response to volatile amines is not quantitative. Ultraviolet detection of dabsyl chloride [4-(dimethylamino)azobenzene-4-sulfonyl chloride] derivatives by HPLC has been used to determine the ammonia and volatile amines in fish (Jen-Kun Lin and Chen-Ching Lai, 1980). However, significant side reactions have been reported for the more frequently used dansyl chloride [5-(dimethylamino)naphthalene-1-sulfonyl chloride] reactant, e.g., dansyldimethylamine (Seiler, 1970), dansyl acid (Dns-OH) (from the hydrolysis of dansyl chloride), and decomposition of dansyl α-amino acids to dansylamide (Neadle and Pollit, 1965). These side reactions can also occur in the dabsylation reaction.

This paper reports on an improved method for derivatizing ammonia and volatile amines in meat tissue as fluorescent dansyl derivatives and minimizes side reactions. Derivatized ammonia and volatile amines are partitioned by reversed-phase HPLC. Determination of ammonia using this method was found to be more accurate, precise, and applicable over a wider range of ammonia concentrations than the enzyme assay and permitted identification of volatile amines present in meat tissue that interfere with the ammonia-selective electrode (ASE) determination.

EXPERIMENTAL SECTION

Sample Source. Fish, shrimp, and preground beef samples were obtained from local supermarkets and usually analyzed on the same day they were obtained. If not, they were stored frozen at -30 °C.

Solvent Preparation. The water used in all the determinations was doubly deionized-distilled from a Mega-Pure System (Corning, Corning, NY). Acetonitrile used for the HPLC determination was distilled and stored under helium until used.

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Extraction. Ammonia and volatile amines were extracted from ground beef tissue with aqueous acetonitrile according to a previous procedure (Parris and Foglia, 1983). Aqueous acetonitrile was substituted for aqueous methanol since the latter reacts with dansyl chloride.

Acid Hydrolysis. Protein in fish and shrimp samples was hydrolyzed according to a previously reported procedure (Jen-Kun Lin and Chen-Ching Lai, 1980).

Enzyme. The ammonia present in the aqueous acetonitrile extract of meat samples and neutralized acid hydrolyzed fish samples was determined by a modified procedure based on the enzymatic (glutamate dehydrogenase) conversion of α-ketoglutarate to glutamate in the presence of plasma ammonia (Sigma Chemical Co., 1980). Acetonitrile present in the extract is diluted to 2.5% (v/v) with aqueous reagents and does not interfere with this assay.

Chromatography. Chromatographic separation were carried out on a Waters Associates (Milford, MA) HPLC system, which included the following components: Model 6000A solvent delivery system, Model U6K injector, 30 cm × 4 mm i.d. μBondapak C_{18} column, and Model 420 fluorescence detector. The mobile phase was 30 mM K_2HPO_4 (pH 7.20) (solvent A) and acetonitrile (solvent B). Isocratic conditions (60:40 buffer-acetonitrile) were used to determine ammonia in tissue, and a solvent gradient was required to determine ammonia and volatile amines. The flow rate was 1 mL/min. The filters used were 370 ± 110 nm excitation and 500 nm long pass emission.

Sample Derivatization. Beef tissue extracts were optimally dansylated by adding 0.1 mL of extract (Parris and Foglia, 1983) to 0.05 mL of dansyl chloride (3.5 mg/mL acetone) followed by 0.09 mL of 0.1 M $KHCO_3$ adjusted to pH 8.0 with 6 N HCl. Three additional aliquots of dansyl chloride (0.05 mL) were added to the reaction at 15-min intervals, and the temperature was maintained at 25 °C. After 1 h the diluted reaction was quenched by acidifying to pH 2 with 2 drops of concentrated formic acid and then adjusted to 5 mL with the HPLC mobile-phase solvents. Derivatized extracts (25 μL) were injected onto the HPLC column.

Dabsylation of fish and shrimp samples was carried out by a procedure described by Lin et al. (Jen-Kun Lin and Chen-Ching Lai, 1980).

Each ammonia analysis included one blank, two standard ammonia solutions, and three tissue extracts. Ammonia levels in extract samples were based on peak height and were corrected for percent reaction of standard solution for each analysis.

RESULTS AND DISCUSSION

Comparison of glutamate dehydrogenase and dansyl derivatization methods for determination of ammonia in standard ammonium chloride solutions indicates that the

Table I. Ammonia Recovery from Beef Extract[a]

sample	ammonia added, μmol/g	enzyme total, μmol/g	enzyme found,[c] μmol/g	enzyme recovery, μmol/g	HPLC[b] total, μmol/g	HPLC[b] found,[c] μmol/g	HPLC[b] recovery, μmol/g
1	3.82	7.34	7.18 ± 0.25	98	7.50	7.50 ± 0.14	100
2	7.66	11.18	10.46 ± 0.25	94	11.34	11.68 ± 0.23	103
3	15.30	18.82	17.80 ± 0.64	95	18.98	19.22 ± 0.44	101

[a] The sample contained 3.52 and 3.68 μmol/g endogenous ammonia by enzyme and HPLC assays. [b] Samples were optimally derivatized as described under Experimental Section. [c] $n = 5$.

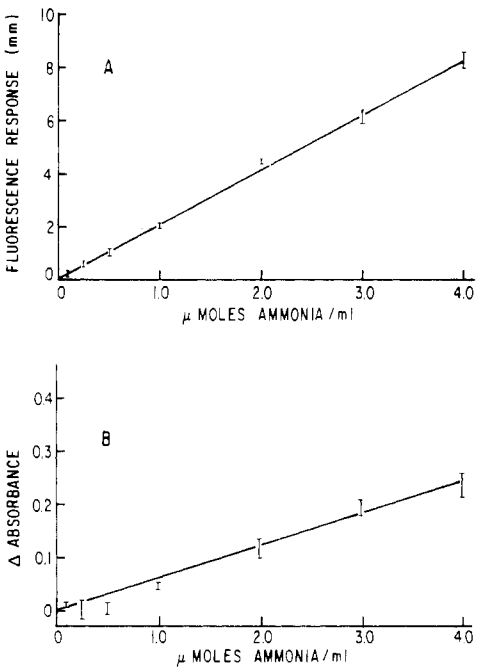

Figure 1. Comparison of HPLC and enzyme assays of ammonia standard solution ($n = 4$). (A) HPLC, fluorescence response (mm) = peak height (mm)/attenuation. (B) Enzyme.

enzyme assay is not as accurate or precise as the dansylation method (Figure 1). The coefficient of variation (CV) over the concentration range analyzed (0.01–0.40 μm/mL) was 6.3% for the HPLC and 16.5% for the enzyme assays. The variability of values for the HPLC assay is especially better at lower ammonia concentrations (Figure 1). In addition, the enzyme method has a limited reported concentration range of 0.02–1.18 μmol/mL (Sigma Chemical Co., 1980), whereas the lower detection limit by the HPLC assay is about 0.01 μmol/mL and the upper limit is determined by the solubility of ammonia in the aqueous acetonitrile extraction solvent. We found that 1.2% NH_4Cl (the principal form of ammonia in meat tissue) was soluble in (75:25) CH_2CN–H_2O at 25 °C, which is almost 1000 times greater than the concentration of ammonia in most meats (Parris and Foglia, 1983).

Completeness of the aqueous acetonitrile extraction procedure was evaluated by recovery of ammonia in tissue to which known amounts of NH_4Cl standards have been added. The total ammonia concentration was about 2, 3, and 4 times that of the ground beef sample. Ammonia recovery from beef extracts was determined by enzyme and HPLC assays (Table I). Ammonia values determined by the HPLC method were slightly higher and had smaller variation than those obtained by the enzyme assay. Good agreement was obtained between aqueous acetonitrile extraction of ammonia from beef and alcohol extraction reported previously (Parris and Foglia, 1983).

Conventional dansylation reactions of amino acids and amine are carried out with an excess of dansyl chloride (Dns-Cl) under alkaline conditions (Neadle and Pollit, 1965; Seiler, 1970). Since ammonia is less reactive than amino acids or aliphatic, aromatic, and alicyclic amines toward Dns-Cl, it requires a large excess of reagent and elevated temperatures to obtain yields greater than 90% for standard ammonium chloride solutions. In tissue determination of ammonia as its dansyl derivative (Dns-NH_2) under these conditions, side reactions occur that can affect this determination. The amount of ammonia in beef tissue extract determined as Dns-NH_2 was about twice that determined by the enzyme assay for the same extract (13.02 vs. 7.10 μmol/g; Table II). These high values have been reported to result from the decomposition of dansylated α-amino acids and the subsequent formation of Dns-NH_2 (Neadle and Pollit, 1965). The yield of Dns-NH_2 varies with the amino acid present and with the reaction condition. Addition of a mixture of four α-amino acids, alanine, valine, leucine, and aminoisobutyric acid, to beef tissue extracts resulted in an 80% increase in Dns-NH_2 over that of the unspiked extracts. These results clearly indicate that the determination of ammonia in meat tissue cannot be determined accurately with standard dansylation techniques.

When more concentrated reaction conditions and incremental additions of Dns-Cl were used, so that the final Dns-Cl to NH_3 standard ratio was about 10:1, yields greater than 90% can be obtained at pH 8.0 and 25 °C (Table III). Ammonia levels found in the tissue extracts as Dns-NH_2 decreased with lower temperatures and approached the enzyme value at ambient temperatures. This demonstrates the dramatic effect of elevated temperatures on the decomposition of dansylated α-amino acids and the formation of Dns-NH_2 in tissue extracts. Also at elevated tem-

Table II. Dansylation of Ammonia in Tissue Extract[a]

sample	ammonia standard, %, reaction	ammonia in beef[b,c] tissue, μmol/g
1	95	11.80
2	90	12.60
3	94	12.38
4	95	15.32
5	92	13.00

[a] Reaction conditions: Dns-Cl–NH_3 standard (400:1); pH 10; 1 h; 37 °C; reaction volume = 3.9 mL. [b] Average value = 13.02 ± 0.07 μmol/g, CV = 10.5%. [c] Ammonia in beef tissue by enzyme assay = 7.10 μmol/g, CV = 3.1%.

Table III. Effect of Reaction Conditions on Dansylation of Ammonia[a]

temp, °C	no. of samples[b]	standard ammonia, % reaction	CV[c]	ammonia in beef tissue, μmol/g	CV
25	3	91	4	6.00	2
37	3	91	6	8.00	5
60	3	61	6	13.40	6

[a] Reaction conditions: Dns-Cl–NH_3 standard (10:1); pH 8.0; 1 h; reaction volume = 0.39 mL. [b] Run in triplicate. [c] Ammonia in beef tissue by enzyme assay = 5.60 μmol/g, CV = 3.5%.

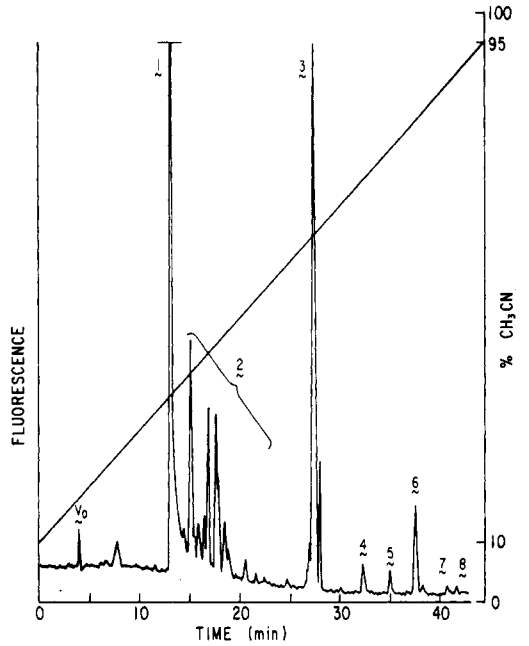

Figure 2. Gradient elution of dansyl components in beef extract. Column: μBondapak C_{18}. Solvents: A = 30 mM K_2HPO_4, pH 7.20; B = CH_3CN. Gradient 10%–95% B, curve no. 6 (linear), 1 h. Flow rate: 1 mL/min. Sample size: 25 μL. Detector gain: 32×. Peaks: 1, DNS-OH; 2, DNS-amino acids; 3, DNS-NH_2; 4, DNS-methylamine; 5, DNS-ethylamine; 6, DNS-dimethylamine; 7, DNS-butylamine; 8, DNS-diethylamine.

peratures, reaction yields are not as great as indicated for ammonia standard solutions at 60 °C in Table III. This is probably the result of Dns-Cl hydrolysis.

Ammonia and volatile amines have been determined for fish as their dabsyl chloride (Dbs-Cl) (Jen-Kun Lin and Chen-Ching Lai, 1980). In this work, quantitation was based on ammonia recovery from shrimp and fish extracts supplemented with a known amount of ammonia. Comparison of the ammonia values from shrimp determined by the dabsyl and enzyme methods for the same extract indicated that the dabsyl assay values for ammonia were over 3 times greater (17.47 vs. 4.92 μmol/g, n = 5). The elevated ammonia values probably result from the excess Na_2CO_3 and dabsyl chloride required in this method. Addition of the same four α-amino acids to the shrimp sample resulted in an 88% increase in Dbs-NH_2, so the decomposition of dabsylated α-amino acids was comparable to the amount of Dns-NH_2 formed in the previous experiment. Therefore, both dabsyl and dansyl α-amino acids can decompose to the corresponding amide derivative.

In addition to ammonia and α-amino acids, other amines can be identified as dansyl derivatives. From the chromatogram of beef extract (Figure 2), the dansylated volatile amines elute after Dns-NH_2. Five volatile amines in beef have been identified based on their retention times compared with standards but were not quantified since dimethylamine found in the extract can also result from the dansylation and dabsylation reactions and is related to reaction conditions (Table IV). Dabsylation and dansylation of ethylamine standard resulted in the formation of fluorescent dimethylamine, which is directly related to the excess amount of dansyl or dabsyl chloride present during the reaction. Less dimethylamine was also formed when the dansylation reaction was carried out at a lower pH. Dimethylamine probably results from the decomposition of dabsyl or dansyl chloride since it was present in their blanks but not detected in reactions using 1-naphthalenesulfonyl chloride (Ns-Cl) as the derivatizing agent under the same conditions. Dimethylamine is not formed in constant amounts with either dabsyl or dansyl reagents and should not be quantified by using this method. However, much less dimethylamine is formed at a lower pH and a smaller excess of Dns-Cl.

Table IV. Formation of Dimethylamine from Dabsyl and Dansylation Reactions

reaction[a]	ethylamine standard, mmol/L	dimethylamine derivative/ ethylamine derivative,[d] %
dabsyl[b]	0.5	9.8
	1.0	4.7
	1.5	2.8
dansyl[b]	0.5	7.9
	1.0	4.3
	1.5	2.8
dansyl[c]	0.5	2.1
	1.0	1.0
	1.5	0.7

[a] Dns- and Dbs-Cl = 5 mmol/L. [b] Na_2CO_3 buffer, pH 10.0 [c] $NaHCO_3$ buffer, pH 8.0. [d] The amount of dimethylamine derivative formed over the amount of ethylamine derivative formed times 100.

In conclusion, determination of ammonia as Dns-NH_2 was shown to be more accurate and precise than that by the enzyme assay. The method developed for the determination of ammonia and identification of volatile amines in meat tissue as fluorescent dansyl derivatives minimized the formation of Dns-OH, Dns-$N(CH_3)_2$, and Dns-NH_2 resulting from the hydrolysis of Dns-Cl, decomposition of Dns-Cl, and decomposition of α-amino acids, respectively. These same UV-absorbing derivatives were also formed for the less reactive dabsyl chloride method in fish and shrimp tissue.

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Registry No. Ammonia, 7664-41-7; methylamine, 74-89-5; ethylamine, 75-04-7; dimethylamine, 124-40-3; butylamine, 109-73-9; diethylamine, 109-89-7.

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