AN IMPROVED METHOD TO ANALYZE TRIMETHYLAMINE IN FISH AND THE INTERFERENCE OF AMMONIA AND DIMETHYLAMINE

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ABSTRACT

The trimethylamine content of most marine fish, especially the gadoid species, is internationally accepted as an index of spoilage. However, ammonia, dimethylamine, and other amines also contribute to the trimethylamine value. Variations in the conditions of the three current methods used to analyze for trimethylamine content were studied in detail to determine the best condition to extract trimethylamine and to reduce the extraction of ammonia, dimethylamine, and other amines. Formaldehyde does not inhibit the interference from ammonia but the interference is negligible even in advanced spoilage. Formaldehyde inhibits the interference from dimethylamine to some extent if KOH is used as the base but increases the interference in the K_2CO_3 method. The extraction of di- and trimethylamine are highly dependent upon the base used and the temperature of extraction. A new method of extracting at −15° C using 45% KOH was developed that essentially eliminates interference from ammonia, dimethylamine, and other amines. To directly compare the methods, the trimethylamine content of a sample of spoiled walleye pollock, *Theragra chalcogramma*, flesh was determined by the three currently used methods and the cold method of extraction. All methods gave similar standard deviations but the K_2CO_3 method gave higher values than the KOH methods and the cold method gave the lowest value. Various levels of trimethylamine and dimethylamine simulating different qualities of fish and frozen storage times were added to samples of Pacific cod, *Gadus macrocephalus*, flesh. The cold method consistently extracts more accurate amounts of trimethylamine with less interference from dimethylamine than any of the other extraction methods.

The trimethylamine (TMA) content of most marine fish, especially the gadoid species, is accepted internationally as an index of spoilage. Dyer's 1959 method of analysis for TMA, except for the concentration of formaldehyde (FA), has been adopted by the Association of Official Analytical Chemists (Horwitz 1975). Trimethylamine is produced by the reduction of trimethylamine oxide by microorganisms (Poller and Linneweh 1926). Ammonia, dimethylamine (DMA), and other volatile bases are also formed when fish spoil and to some extent interfere with the measurement of TMA. In advanced spoilage, some of the higher aliphatic amines are formed by decarboxylation of amino acids and may cause interference (Dyer 1945).

A number of investigators studied the TMA method to improve the accuracy and reduce the effects of ammonia, DMA, and other amines. Dyer (1945) adapted the method of determining amines to fish and used 0.02% picric acid in dry toluene instead of chloroform (Richter 1938; Richter et al. 1941). Dyer and Mounsey (1945) used a trichloroacetic acid (TCA) extract of fresh cod instead of the unstable press juice or weighed samples. Hashimoto and Okaichi (1957) claimed that variation of temperature caused serious errors in the determination of TMA, and recommended a 30° C extraction with 25% KOH rather than 50% K_2CO_3 and room temperature. Tozawa et al. (1971) confirmed these findings and showed that 25% KOH reduced the interference caused by DMA and claimed the formation of a compound from FA and DMA which was not extracted in the presence of hydroxide ions. Murray and Gibson (1972) found that 45% KOH extracted more TMA and gave more linear and reproducible results than if extracted with 25% KOH or 50% K_2CO_3.

The three current methods of analysis for TMA employ 25% KOH, 45% KOH, or 50% K_2CO_3 to release TMA for extraction into the toluene layer and result in different absorbancies for the picrate color with DMA and TMA. In general, the use of K_2CO_3 results in a higher extraction of DMA and a lower extraction of TMA than if KOH were used. Ideally, the best method to measure TMA content should result in complete extraction of TMA and zero extraction of ammonia (NH_3), DMA, and other amines so that these components will not contribute to the TMA value. A new method was

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developed from information obtained from a detailed study of how temperature, type and concentration of base, and the presence (or not) of FA affect the extraction and subsequent absorbancies of the picrate color of NH_3, DMA, and TMA.

EXPERIMENTAL

Purification Procedures

Trimethylamine hydrochloride (TMA·HCl) and dimethylamine hydrochloride (DMA·HCl) were crystallized twice from hot 2-propanol and dried under high vacuum overnight. Reagent grade and previously used toluene was purified by shaking and partitioning with concentrated sulfuric acid in a separatory funnel followed by water, sodium hydroxide, and water; filtering through anhydrous sodium sulfate (NA_2SO_4); and distilling at 110° C. Reagent grade Formalin[2] (37% FA) was shaken with magnesium carbonate, filtered, and diluted 1:9 with water. Hexamethylenetetramine (HMTA) from Pfaltz and Bauer was crystallized twice from hot 2-propanol or from hot, dry toluene and dried overnight under high vacuum. N N N'N'-tetramethylmethanediamine (TMMD) from Pfaltz and Bauer was distilled using a column packed with glass helices. The first 25 ml fraction (72°-81° C) was discarded, the next 25 ml fraction (81° C) was used for analysis, and the final 25 ml distillate was discarded. Reagent grade ammonium chloride (NH_4Cl) was crystallized twice from hot water and dried under high vacuum overnight.

Extraction Procedure for Fish Flesh

Procedures cited in the literature for the extraction of fish have used a specific amount of flesh plus water or TCA followed by shaking or blending and filtration. These methods assume a specific moisture content of the flesh, a total volume, and a complete extraction or uniform dispersion of TMA in the extract, e.g., 100 g flesh at 80% moisture plus 300 ml TCA solution would give a 1/95 aliquot for a 4 ml sample. To improve accuracy of the method, we used an exhaustive extraction-filtration procedure and dilution to a volume. Details of the procedure are given in the Recommended Procedures section.

[2]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

Methods of Analyses for TMA

Unless otherwise indicated, the three commonly used methods of analyses for TMA were modified slightly to fit our equipment and to reflect recent advances in the methods. The methods of Dyer (1945), Tozawa et al. (1971), and Murray and Gibson (1972) were used as follows: a 4 ml sample of a standard solution in 5% TCA or a 5% TCA extract of fish flesh was added to a 25 × 150 mm screw top test tube, followed by the addition of 10 ml toluene, 1 ml of 3.7% FA solution, and left to stand 5 min before the addition of 3 ml of base (25% KOH, 45% KOH, or 50% K_2CO_3). The tube was tightly sealed using a gasket of a double layer of 1 mil polyethylene film under the cap and shaken for 15 min at room temperature on a Burrell wrist action shaker modified by building-up the platform 20.3 cm with Styrofoam. After standing several minutes, about 7 ml of the toluene layer were removed and dried with 0.5 g anhydrous Na_2SO_4. After drying, 5 ml were added to 5 ml of 0.02% picric acid in dry toluene and the absorbance was determined at 415 nm on a Gilford modified Beckman D.U. spectrophotometer. A fourth method will be referred to as the "cold method" of extraction and uses 45% KOH (Murray and Gibson 1972) but the extraction is done at −15° C. The details of this method are given in the section on Recommended Procedures.

Standard Curves

Since four distinctly different methods were used to analyze for TMA content, complete blank determinations and standard curves were made for each method. The equations for the regression lines (standard curves) of absorbance on concentration of TMA (micrograms TMA-N/milliliter) for each of the methods were as follows:

25% KOH, room temperature	$Y = 0.071X - 0.007$	(1)
45% KOH, room temperature	$Y = 0.087X - 0.001$	(2)
50% K_2CO_3, room temperature	$Y = 0.067X - 0.012$	(3)
45% KOH, cold (−15° C)	$Y = 0.082X - 0.007$	(4)

where Y = absorbance and X = micrograms TMA-N/milliliter.

The trimethylamine values in milligrams TMA-N/100 g flesh were calculated from these equations and from the total volume (250 ml) of 5% TCA extract, weight of extracted fish flesh (75 g), 4 ml of sample extract per tube, and a dilution factor

(K), if present (e.g., for the cold method use Equation (4)):

$$\frac{(A + 0.007)(250\text{ ml})(100)(K)}{(0.082)(75\text{ g flesh})(1{,}000\ \mu\text{g/mg})} = \text{milligrams TMA-N/100 g flesh.} \quad (5)$$

RESULTS AND DISCUSSION

In the following sections, the influence of the presence of FA (or not), the type and concentration of base used, and temperature of extraction were determined separately on each component in the reaction and extraction mixture; i.e., 1) NH_3 and HMTA, 2) DMA and TMMD, and 3) TMA. The four methods were directly compared for precision by replicate analyses of a composite sample of minced fish flesh. Finally, accuracy (recovery of TMA and the interference of DMA) was determined for the four methods by direct addition of known quantities of TMA and DMA.

Reaction of Ammonia

Sprung (1940) reported that FA reacts with NH_3 to form HMTA. Under vacuum distillation conditions at 30° C, the addition of FA to solutions of NH_4Cl and Na_2CO_3 rendered the NH_3 nonvolatile but in the absence of FA, NH_3 was volatile (Benoit and Norris 1942). Dyer (1945) claimed that the addition of 1 or 2 ml of 4% FA did not affect the recovery of TMA, and was sufficient to eliminate the interference of NH_3 up to 10-20 times over the usual concentration of trimethylamine nitrogen. Dyer, however, did not directly study the influence of FA on the development of the picric acid color in the presence of NH_3. Researchers studying the TMA method have followed the prior procedures and used FA to tie up NH_3 in the TMA test.

Standard solutions of NH_4Cl in 5% TCA were prepared and used in the three TMA methods. To simulate various stages of spoilage the concentration ranged from 6.6 to 66 μg N/ml; i.e., 2.2-22.0 mg N/100 g flesh. The absorbancies from the NH_4Cl solutions with and without FA (Table 1, columns 1-6) were low and of questionable significance except if 45% KOH was used. The absorbancies seemed to increase with concentration of NH_4Cl and were about the same whether FA were present or not if 50% K_2CO_3 and 25% KOH were used. Even at the higher concentrations, the absorbancies were low and would contribute little to the TMA value. When 45% KOH was used, the absorbancies were much higher than the absorbancies with the other bases and were not affected by FA except at the higher concentrations. Accordingly, FA and NH_3 did not react under these conditions and NH_3 could contribute to the TMA value. To determine if FA would react with NH_3 at elevated temperatures, the same standard solutions in 5% TCA were treated as before but were preheated in the presence of FA at 60° C for 30 min, cooled to room temperature, and 45% KOH added and extracted in the usual way. The absorbancies of the preheated samples were reduced significantly—see columns 5 and 7 in Table 1. The low absorbancies indicated that FA reacted with NH_4Cl when heated and there was no interference through 26.4 μg N/ml sample. The color development in the samples containing 33.0 μg N/ml and more could be caused by: 1) residual NH_3 through an incomplete reaction with FA, 2) NH_3 through a reverse reaction of the product which is assumed to be HMTA, by the law of mass action or chemical equilibrium, and 3) a partial extraction of HMTA by toluene and subsequent reaction with picric acid.

TABLE 1.—Ammonium chloride or hexamethylenetetramine (HMTA): the absorbancies of picrates in the trimethylamine test as affected by the addition of standard solutions of ammonium chloride or hexamethylenetetramine. Samples (columns 1-10) were extracted at room temperature for 15 min (except 9 and 10 which were extracted at −15° C for 60 s by hand), with formaldehyde (+) and without formaldehyde (0).

NH_4Cl or HMTA (μg N/ml)	NH_4Cl 50% K_2CO_3		NH_4Cl 25% KOH		NH_4Cl 45% KOH		NH_4Cl/HMTA 45% KOH		NH_4Cl 45% KOH	
	1 +	2 0	3 +	4 0	5 +	6 0	7[1] +	8 0	9 +	10 0
6.6	0.008	0.000	0.001	0.001	0.008	0.005	0.000	0.012	0.003	0.004
13.2	.012	.000	.001	.005	.024	.015	.000	.012	.013	.006
16.5	.012	.000	.001	.011	.029	.018	.000	.023	.016	.008
26.4	.016	.004	.010	.011	.027	.028	.000	.027	.017	.008
33.0	.004	.008	.000	.014	.042	.049	.023	.032	.019	.016
52.8	.012	.019	.010	.027	.066	.095	.019	.042	.022	.034
66.0	.020	.027	.011	.039	.072	.119	.044	.045	.024	.043

[1]After addition of FA, samples were heated at 60° C for 30 min to allow FA and NH_3 to react. Samples were then cooled, base added, and extracted at room temperature.

To determine if HMTA would react with picric acid, purified HMTA in dry toluene was added to the picric acid reagent. A strong color developed which indicated that HMTA reacted with picric acid. To determine if HMTA would be extracted under the standard conditions of the TMA test using 45% KOH, standard solutions of HMTA were prepared and used in the same manner as NH_4Cl solutions. The absorbancies for the HMTA samples (Table 1, column 8) were similar to the absorbancies of the samples represented by columns 5 and 7 of Table 1. Further, the absorbancies for HMTA samples were not 0 as would be expected from the literature and indicated that either HMTA was extracted by toluene and reacted with picric acid or a partial reverse reaction occurred and NH_3 was extracted and reacted with picric acid. Whether the picrate color was caused by NH_3 or by HMTA, FA did not eliminate the interference of NH_3 when 45% KOH was used. Formaldehyde gave some protection, however, if the reaction mixture was heated at 60° C for 30 min prior to the addition of 45% KOH.

Since NH_3 or HMTA was extracted by toluene at room temperature and reacted with picric acid, NH_3 was extracted at a low temperature to see if the interference could be reduced. The same standard solutions of NH_4Cl were treated as before but were extracted by the cold method. The data given in columns 9 and 10 of Table 1 showed that the absorbancies were at the same general level as the preheated samples and the presence of FA had little effect on absorbance. We conclude that 45% KOH was less effective in releasing NH_3 at −15° C than at room temperature (Table 1, compare columns 5 with 9, 6 with 10) or NH_3 was less extractable by toluene at −15° C. Although other researchers have used FA to eliminate the interference of NH_3, our data showed that FA does not tie up NH_3 under the usual conditions in the analysis for TMA. On practical grounds, the contribution of NH_3 to the TMA value would be quite low because the bases (except 45% KOH) released only a small amount of NH_3 or NH_3 was only slightly extracted by toluene. Even in advanced spoilage such as 22 mg NH_3-N/100 g flesh (66 μg N/ml), the contribution of NH_3 to the TMA value would be equivalent to 0.45 mg TMA-N/100 g flesh if extracted at room temperature with 45% KOH but only 0.10 mg TMA-N/100 g flesh if determined by the cold method.

TABLE 2.—Dimethylamine hydrochloride: the absorbancies of picrates in the trimethylamine test as affected by the three bases used and temperature of extraction. Samples were extracted for 60 s with vigorous hand shaking using 4 ml 15.9 μg DMA-N/ml, with formaldehyde (+) and without formaldehyde (0).

	25% KOH		45% KOH		50% K_2CO_3	
Temperature of extraction (°C)	1 +	2 0	3 +	4 0	5 +	6 0
−15	0.015	0.067	0.015	0.605	0.119	0.000
0	.016	.130	.031	.657	.200	.000
6	.024	.208	.053	.721	.251	.007
22	.062	.310	.157	.774	.327	.037
30	.079	.371	.361	.967	.453	.088

Reaction of DMA

Several researchers have found that DMA and TMA are not completely extracted by toluene under conditions used in the TMA test unless replicate extractions are made (Castell et al. 1974). Further, different bases resulted in different extractabilities of DMA. Accordingly, we determined the absorbancies of the picrate color using a standard solution of DMA (15.9 μg DMA-N/ml; i.e., 5.3 mg DMA-N/100 g flesh) under various conditions of the TMA test; temperature, base (K_2CO_3 and KOH), replicate extraction, and presence or absence of FA.

A standard solution of DMA·HCl in 5% TCA was extracted with and without FA by the usual methods but the temperature of extraction was varied (−15° C to +30° C) and the tubes were vigorously shaken by hand for 60 s. The extraction of DMA was strongly influenced by the base and by temperature (Table 2). In the absence of FA, the differences in reactivity of the bases in releasing DMA from the hydrochloride salt are shown in columns 2, 4, and 6 (Table 2). At −15° C, 45% KOH released about half of the DMA present, 25% KOH released 1/20th, and K_2CO_3 was unreactive and did not release DMA. The bases were more reactive at higher temperatures than at lower temperatures but had the same order of reactivity. The date also showed that, if released from the salt, DMA was extracted by toluene even at low temperatures. When FA was present however, a different order of release was evident (Table 2, columns 1, 3, and 5) and showed that FA reacted with DMA to give a product having different reactivity with the bases. The order of release (or extractability) for the product was different than for DMA, i.e., high absorbance with K_2CO_3, intermediate with 45% KOH, and low with 25% KOH. Considerable amounts of the product were extracted in the presence of FA at all temperatures in the carbonate system and would result in

a contribution by DMA of 1.69 mg N/100 g flesh to the TMA value at 22° C. In frozen flesh of gadoid fish, the DMA content might be high relative to TMA and would result in a substantial error in the TMA value unless determined at low temperatures with KOH where DMA would contribute 0.1 mg N or less.

We next studied the effects of the three bases and the presence or absence of FA on the total extractability of DMA. Similar extractions were performed by Castell et al. (1974) but these authors only considered the carbonate system with FA present. The same DMA solutions (15.9 μg DMA-N/ml) with or without FA were extracted as before but at room temperature for 15 min, i.e., 4 ml DMA solution, 10 ml toluene, 1 ml FA (or not), and 3 ml base. After removing about 7 ml toluene for drying and reacting with picric acid, the remainder of the toluene layer was carefully aspirated off, 10 ml toluene added, and reextracted. This process was repeated for a total of six extractions. In the absence of FA, DMA was released rapidly from the salt by 45% KOH, about half as fast by 25% KOH, and slowly by 50% K_2CO_3 (Table 3). If FA were present however, many extractions would be required to extract all of the DMA which, in agreement with Sprung (1940), showed that FA reacted with DMA to give TMMD. The data further showed that TMMD was relatively soluble in toluene but the extractabilities were different because each base had a different rate of reaction with TMMD, i.e., a rapid release of TMMD with K_2CO_3 and slow with 25% KOH. The possibility of each base having a different salting-out effect was eliminated when equal absorbancies were obtained if the same extractions were made with the addition of 0.5 g KCl (data not given).

The data also showed that in the carbonate method TMMD was released to the toluene phase more rapidly than DMA and explains the known interference of DMA in the presence of FA by the Dyer (1945) method. Formaldehyde might best be left out in the 50% K_2CO_3 method. The lower picrate color absorbancies in the KOH systems with FA present might also be explained by the law of mass action (equilibrium) as was done in the section on NH_3. In the equilibrium (FA+DMA ⇌TMMD) the concentrations of FA, DMA, and TMMD in the aqueous phase are dependent on the type and concentration of base. The products (FA and DMA) of the hydrolysis of TMMD would be formed at a rate dependent upon these same variables and DMA would be rapidly removed from the aqueous phase in the KOH systems because of the rapid extraction of DMA by toluene. Since the absorbancies in the KOH systems were relatively low in the presence of FA, the concentration of DMA from the hydrolysis of TMMD must have been low. In the carbonate system however, DMA from TMMD was slowly released from the aqueous phase into the toluene layer. Apparently a low concentration of DMA existed in the equilibrium formed in the carbonate system and favored the extraction of TMMD by toluene. It is likely that both TMMD and DMA were extracted by toluene at rates that depend on the base and temperature used.

To further study the extraction of TMMD, the same multiple extractions described for DMA were made using purified TMMD in 5% TCA but at a slightly lower concentration (15.0 μg TMMD-N/ml). The absorbancies of the TMMD-picrates (Table 4) were nearly the same as the absorbancies of the DMA-picrates (Table 3). The similarity of data between DMA and TMMD inferred again that FA and DMA react to give TMMD. The addition of FA forced the reaction toward TMMD where the type and concentration of base con-

TABLE 3.—Dimethylamine hydrochloride: the absorbancies of picrates in multiple extractions in the trimethylamine test as affected by the three bases used. Samples were extracted for 15 min at room temperature using 4 ml 15.9 μg DMA-N/ml, with formaldehyde (+) and without formaldehyde (0).

	25% KOH		45% KOH		50% K_2CO_3	
Extraction number	1 +	2 0	3 +	4 0	5 +	6 0
1	0.064	0.416	0.184	1.022	0.498	0.03
2	.042	.175	.160	.204	.273	.02
3	.049	.125	.149	.017	.195	.01
4	.040	.057	.114	.000	.114	.02
5	.049	.036	.117	.000	.074	.02
6	.049	.015	.092	.000	.051	.02

TABLE 4.—N N N'N'-tetramethylmethanediamine: the absorbancies of picrates in multiple extractions in the trimethylamine test as affected by the three bases used. Samples were extracted for 15 min at room temperature using 4 ml 15.0 μg TMMD-N/ml, with formaldehyde (+) and without formaldehyde (0).

	25% KOH		45% KOH		50% K_2CO_3	
Extraction number	1 +	2 0	3 +	4 0	5 +	6 0
1	0.051	0.308	0.193	0.882	0.417	0.041
2	.038	.198	.160	.180	.277	.041
3	.046	.123	.142	.028	.185	.044
4	.036	.067	.122	.002	.126	.048
5	.039	.031	.090	.000	.081	.028
6	.046	.022	.073	.000	.059	.025

trolled the degree of retention of TMMD in the aqueous phase or its release to the toluene phase. The use of 1 ml 3.7% FA and 4 ml 15.9 μg DMA-N/ml results in a large excess of FA, about 500 times over that required (1 FA to 2 DMA). Consequently, in the absence of added FA where only the stoichiometric amount of FA was present from TMMD, an equilibrium was established in the KOH systems that favored the formation of DMA and its rapid extraction by toluene. A different equilibrium was formed in the K_2CO_3 system that favored the release of TMMD and extraction by toluene.

Extraction of TMA

The extraction of TMA under various conditions of base, temperature, and FA was examined. A standard solution of TMA·HCl in 5% TCA was prepared (15.9 μg TMA-N/ml, i.e., 5.3 mg TMA-N/100 g). This concentration was chosen as it is near the point of unacceptable quality for fish. In the carbonate method (Table 5), the extraction of TMA was highly dependent upon temperature and would result in a lack of precision unless the temperature was controlled as suggested by Hashimoto and Okaichi (1957). Absorbancies were not as dependent upon temperature in the 25% KOH method as with K_2CO_3 and were nearly independent of temperature with 45% KOH. The slightly lower absorbancies with FA present than if not present might be caused by an impurity of DMA or an interference from FA even though FA would not be expected to react with a tertiary amine. As stated in the section on DMA, FA might best be left out in the carbonate method, i.e., only 10% less TMA was extracted than was extracted in the 45% KOH method.

To determine the conditions for maximum extractions of TMA, the same multiple extractions were done as with DMA. In the 45% KOH test (Table 6), 97% of the TMA was removed in the first extraction and the remainder was removed in the second extraction. The first, second, and third extractions removed 80, 17, and 3% with 25% KOH and removed 72, 21, and 6% with 50% K_2CO_3. Standard curves are assumed to compensate for constant experimental errors such as slightly less than 100% extraction of TMA, but the reliability of the data would be questionable with the low recoveries reported here for 25% KOH and 50% K_2CO_3. Neither do standard curves compensate for variable errors such as the observed strong dependence on temperature of the extraction of TMA in the 25% KOH and 50% K_2CO_3 methods (Table 5).

TABLE 5.—Trimethylamine hydrochloride: the absorbancies of picrates in the trimethylamine test as affected by the three bases used and temperature of extraction. Samples were extracted for 60 s with vigorous hand shaking using 4 ml of 15.9 μg TMA-N/ml, with formaldehyde (+) and without formaldehyde (0).

Temperature of extraction (°C)	25% KOH		45% KOH		50% K_2CO_3	
	1 +	2 0	3 +	4 0	5 +	6 0
−17	0.685	0.854	1.312	1.402	0.286	0.682
0	.921	1.099	1.366	1.412	.630	1.027
6	1.050	1.138	1.378	1.391	.722	1.095
21	1.136	1.235	1.373	1.350	1.013	1.218
30	1.198	1.269	1.408	1.420	1.150	1.303

TABLE 6.—Trimethylamine hydrochloride: the absorbancies of picrates in multiple extractions in the trimethylamine test as affected by the three bases used. Samples were extracted for 15 min at room temperature using 4 ml 15.9 μg TMA-N/ml, with formaldehyde (+) and without formaldehyde (0).

Extraction number	25% KOH		45% KOH		50% K_2CO_3	
	1 +	2 0	3 +	4 0	5 +	6 0
1	1.113	1.223	1.384	1.403	0.987	1.245
2	.233	.159	.050	.033	.282	.130
3	.044	.020	.000	.001	.079	.009
4	.003	.005	.000	.000	.016	.003

Comparative Analyses Using Fish Flesh

Walleye pollock, *Theragra chalcogramma*, were held in slush-ice for 9 d and filleted. Twelve separate TCA extractions were made on a composite sample of the ground flesh. Each extract was analyzed in duplicate by each of the three TMA methods and the cold method. Portions of the extracts were neutralized and analyzed for DMA by Dowden's 1938 method, modified slightly by increasing the time of extraction to 15 min on the modified mechanical shaker.

All methods (Table 7) resulted in similar standard deviations but the TMA values were higher in the K_2CO_3 method than in the KOH methods and the cold method of extraction gave the lowest value. The absorbancy data at 22° C of Table 2 can be used to approximate the degree of contribution of DMA to the TMA values in Table 7. The flesh contained 2.25 mg DMA-N/100 g (6.75 μg/ml) and would contribute different amounts to the TMA value according to the method of analysis employed. The data of Table 2 for K_2CO_3 (0.327 *A* at 22° C using 15.9 μg DMA-N/ml) are equivalent

TABLE 7.—Trimethylamine content in mg TMA-N/100 g flesh from 9-d-old walleye pollock using four methods of analysis.

Extract number	Room temperature extraction			−15° C extraction
	25% KOH	45% KOH	50% K_2CO_3	45% KOH
1	9.52	9.84	10.42	9.18
2	9.73	9.76	10.27	9.14
3	9.55	9.78	10.18	9.08
4	9.66	10.01	10.28	8.84
5	9.53	9.92	10.34	8.84
6	9.69	10.07	10.25	9.10
7	9.51	9.93	10.39	9.28
8	9.56	9.78	10.28	8.97
9	9.49	9.82	10.22	9.18
10	9.65	9.79	10.21	8.91
11	9.65	9.75	10.22	9.11
12	9.55	9.82	10.39	9.11
Mean	9.59	9.85	10.29	9.06
SD	.08	.10	.08	.14

to 0.139 A for 6.75 μg DMA-N/ml by a simple ratio, i.e., $0.327A/(15.9\ \mu g$ DMA-N/ml):$X/(6.75\ \mu g$ DMA-N/ml). An equivalent TMA value was calculated to be 0.75 mg TMA-N/100 g flesh from Equations (3) and (5). If corrected for DMA, the TMA value from Table 7 (K_2CO_3) would be 10.29 − 0.75 = 9.54 mg TMA-N/100 g flesh. Similar calculations for the 25 and 45% KOH methods gave corrected values of 9.45 and 9.59 mg TMA-N/100 g flesh. The small contribution of DMA at −15°C (0.015 A) would be 0.05 mg TMA-N/100 g flesh and give a corrected value of 9.01 for the cold method. The TMA values obtained by the three methods of analysis were in good agreement if corrected for DMA. The cold method of extraction gave slightly lower and more accurate values than the other methods. Cold extraction reduced the release and extractability of numerous other interfering substances discussed by Dyer (1945).

Extraction of Fish Flesh with Added TMA and DMA

To determine the recovery of TMA and the interference of DMA, varying amounts of both were added to blended flesh of Pacific cod, *Gadus macrocephalus*, extracted with TCA in the usual way and analyzed for TMA content by four methods. The sample of flesh contained 3.25 mg DMA-N/100 g by Dowden's method (1938). The amount of amine added, the resulting TMA value, and the percentage of the theoretical value (recovery) by each method of analysis are given in Table 8. The TMA values of cod flesh with added TMA (3, 6, 9, and 12 mg) resulted in similar recoveries of TMA by all methods. If 5, 15, 30, and 50 mg DMA were added to the blended flesh, however, the TMA values were unacceptably high by the 50% K_2CO_3, 25% KOH, and 45% KOH methods. The cold method gave acceptable values although the addition of 50 mg DMA-N increased the TMA value from 1.59 to 2.12, i.e., 133% of theory. If the same quantities of DMA were added plus a small amount of TMA (3 mg), only the cold method gave acceptable TMA values. The other methods were strongly influenced by the presence of DMA. However, if larger amounts of TMA were added (12 mg), along with DMA, the influence of DMA was reduced considerably and the 25% KOH and cold methods gave acceptable results.

All methods gave about equal recovery of added TMA provided the DMA content was low. Trimethylamine values by the three published methods were strongly influenced by the relative

TABLE 8.—Trimethylamine values in mg TMA-N/100 g flesh of Pacific cod as affected by different methods of analysis when varying amounts of the TMA·HCl and DMA·HCl salts were added to the flesh before extracting with TCA.

Levels of TMA and DMA added	25% KOH		45% KOH		50% K_2CO_3		Cold method	
	TMA-N (mg)	Recovery (%)	TMA-N (mg)	Recovery (%)	TMA-N (mg)	Recovery (%)	TMA-N (mg)	Recovery (%)
Sample, as is	1.65		1.94		2.36		1.59	
3 mg TMA	4.81	103	5.17	105	4.89	91	4.81	105
6 mg TMA	7.34	96	8.28	104	7.39	88	8.12	107
9 mg TMA	11.62	109	10.71	98	11.39	100	11.32	107
12 mg TMA	13.94	102	15.23	109	17.04	119	14.90	110
5 mg DMA	2.00	121	2.83	146	3.79	161	1.72	108
15 mg DMA	2.47	150	4.24	219	6.32	268	1.77	111
30 mg DMA	3.18	193	5.89	304	10.16	431	1.89	119
50 mg DMA	3.83	232	8.09	417	14.46	613	2.12	133
3 mg TMA+5 mg DMA	5.21	112	6.14	124	6.84	128	4.60	100
3 mg TMA+15 mg DMA	5.70	123	7.59	154	9.25	173	4.81	105
3 mg TMA+30 mg DMA	6.35	137	9.74	197	11.87	221	4.97	108
3 mg TMA+50 mg DMA	7.78	167	13.10	265	16.35	305	4.98	108
12 mg TMA+5 mg DMA	14.48	106	16.42	118	19.39	135	15.24	112
12 mg TMA+15 mg DMA	14.88	109	16.71	120	19.83	138	15.34	113
12 mg TMA+30 mg DMA	14.93	109	17.90	128	21.17	147	15.16	112
12 mg TMA+50 mg DMA	15.79	116	19.72	141	24.27	169	15.19	112

amounts of TMA and DMA in the sample. Only the cold method gave TMA values that were nearly independent of the DMA content of all levels of DMA and TMA. If methods other than the cold method are used to analyze for TMA, the history of the fish and sample storage should be known or the DMA content should be determined separately.

RECOMMENDED PROCEDURES

Extraction Procedure for Fish Flesh

Blend a thoroughly mixed composite sample of fish flesh (75 g) with 90 ml of 8.2% (weight/volume) TCA for 5 min at high speed in a Vertis blender. Pour contents of blender jar into a 150 ml medium porosity sintered glass funnel and filter under vacuum. To prevent foaming and plugging of filter, clamp off the suction line after filtering starts and briefly open when required. Reextract residue with 70 ml of 5% TCA for 2 min and filter into the same filter flask and rinse with 5% TCA from a wash bottle. Quantitatively transfer the combined filtrates and washings to a 250 ml volumetric flask and dilute to the mark with 5% TCA. The extract (4 ml) is used in the TMA analysis without dilution but, if required, 4 ml of a diluted extract is used rather than smaller volumes of extract.

Cold Method of Analysis for TMA

Add 4 ml samples of standard solutions of TMA·HCl in 5% TCA or 5% TCA extracts, 10 ml toluene, and 1 ml of 3.7% FA to 25 × 150 mm screw top test tubes. Allow to stand for 5 min then place tubes in an ice-water bath in an effort to avoid the possible yellow color caused by the addition of concentrated KOH (Castell et al. 1974). When completely chilled, add 3 ml 45% KOH and tightly seal tubes, invert twice, and place in a mixture of salt and precooled saturated brine-ice at −15° C. Use a pump or stirring motor to maintain constant temperature by circulating the brine through the salt, brine-ice mixture. After 2 min, remove the test tubes and shake vigorously by hand for 15 s and replace in the cold bath for 2 min. Repeat this procedure three times for a total of 60 s of vigorous hand shaking. After settling (almost immediately), transfer about 7 ml of the toluene layer to clean dry 18 × 150 mm test tubes and dry with about 0.5 g anhydrous Na_2SO_4 by swirling (Vortex Mixer). After drying, remove 5 ml and add to 5 ml of 0.02% picric acid in dry toluene. Determine the absorbance at 415 nm using 1 cm standard silica cells and a Gilford modified Beckman D.U. spectrophotometer. Determine the blank in the same manner but use 4 ml TCA. Calculate the TMA content in mg TMA-N/100 g from the absorbance and Equations (4) and (5).

SUMMARY

Although NH_3, DMA, and other amines contribute to the TMA value, the TMA content of some marine fish, especially gadoid species, is accepted internationally as an index of spoilage. Variations in the conditions of the three methods used to analyze for TMA were studied to determine the best condition to extract TMA and to reduce the extraction of HN_3, DMA, and other amines. We found that NH_3 was not tied up by FA as suggested in the literature but has little affect on the TMA value of fish even in advanced spoilage. The amount of DMA extracted was strongly dependent on the temperature of extraction, the base used, and the presence or absence of FA. Formaldehyde and DMA reacted to form a compound that was rapidly extracted by 50% K_2CO_3 and very slowly by 25% KOH. If DMA and TMMD were extracted, the absorbancies were nearly the same which infers that the compound formed from FA and DMA was TMMD. The amount of TMA extracted was strongly dependent on the temperature of extraction when 25% KOH or 50% K_2CO_3 was used as the base but nearly independent when 45% KOH was used. A cold method of extraction (45% KOH and −15° C) was developed that essentially eliminated the contribution of DMA to the TMA value. Trimethylamine was determined in spoiled fish flesh by the cold method and the three other methods. Standard deviations were similar for all four methods. The K_2CO_3 method gave the highest value and the cold method gave the lowest value. If varying amounts of TMA and DMA were added to Pacific cod flesh and analyzed by the three published TMA methods, the recovery of TMA and interference from DMA was strongly influenced by the relative amounts of TMA and DMA present. Relative to the other methods, the cold method gave TMA values that were independent of the presence of DMA or the relative amounts of DMA and TMA. We recommend that the cold method be used because it extracts most of the TMA (97%), gives good recovery of added

TMA, is nearly independent of DMA content, and is not affected by other amines or NH_3.

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