Amines in Fish Muscle

I. Colorimetric Determination of Trimethylamine as the Picrate Salt

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ABSTRACT

A sensitive accurate colorimetric method for trimethylamine determination is presented, based on the extraction with toluene of an alkaline sample containing 0.002 to 0.02 mg. trimethylamine nitrogen, and the formation of the yellow coloured picrate by mixing with a picric acid reagent. The application of the method in fishery products and effects of interfering substances have been investigated.

Trimethylamine estimation is the most satisfactory measure so far developed of spoilage in sea fish where the concentration of trimethylamine oxide is sufficient to provide an adequate source of trimethylamine. The time-consuming nature of present procedures and the special apparatus required limit their general use for routine determinations in the plant, or even in the research laboratory (Beatty and Gibbons 1937, Boury and Schvinte 1935). No colorimetric method has heretofore been proposed.

Trimethylamine is produced in spoiling fish muscle by the bacterial reduction of the trimethylamine oxide present, and its rise parallels that of the bacterial population. The bacterial growth takes place on the surface until well on in the spoilage cycle (Wood, Sigurdsson and Dyer 1942), and since composite samples of whole fish fillet are usually used, the method must be capable of measuring the very small initial increase in trimethylamine in order to give useful information of the onset of spoilage. Ammonia, and small amounts of monomethylamine and dimethylamine are also produced along with the trimethylamine. Choline, betaine, and γ butyrobetaine, containing trimethylamine radicals, also occur in fish muscle (Kutscher and Ackerman 1933), and these compounds must not interfere in the determination.

An examination of the literature showed a number of reactions for amines which might possibly be used for the estimation of trimethylamine.

REACTIONS FOR AMINES

The chloranil test for amines (Sivadjian 1931) gives different colours with primary, secondary and tertiary amines, but its sensitivity was found to be insufficient for the amine concentration found in fish extracts.

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When potassium ferrocyanide is added to a solution of choline and cobaltinitrite a deep green colour is produced (Jacobs and Hoffman 1931). It was found that trimethylamine could be substituted for the choline and that the resulting colour was related to the amount present. However the optimum conditions for quantitative results were not found.

The Folin aminoacid reagent, sodium naphthoquinone-4-sulphonate (Folin 1922, E. G. Schmidt 1938, Frame, Russel and Wilhelmi 1943), also was found to react with trimethylamine, but since ammonia and formaldehyde interfered, the test was unsatisfactory.

The phenol reagent of Folin and Ciocalteu (1927) is reduced by trimethylamine as well as phenols. No previous mention has been made of this reaction with trimethylamine, and since the test is used for protein decomposition in fish muscle (Bradley and Bailey 1940, Wood Sigurdsson and Dyer 1942), the effect of trimethylamine must be taken into account. The interference is negligible at low trimethylamine concentrations, but in spoiled fish containing 50 mg. trimethylamine nitrogen per 100 g. tissue, the colour due to trimethylamine equals that given by the products of protein decomposition. The reaction with trimethylamine is only moderately sensitive, since it produces but one twenty-sixth as much colour as tyrosine at equimolar concentration. Since distillation would be necessary to separate the protein decomposition products, the test was not considered an improvement over existing methods.

Finally, the amine reagent of Richter (1938) and Richter, Lee and Hill (1941) was adapted for use. These authors determined amines in urine and blood by extracting an alkaline solution with petroleum ether or toluene, separating the amines from lecithin, etc., by shaking in acid solution, and reextracting the amines with the solvent from the acid extract after making alkaline with potassium carbonate. A two per cent solution of picric acid in chloroform was then added to an aliquot of centrifuged extract diluted with an equal volume of chloroform, the resulting yellow colour being compared with standards.

The original method was found to be unnecessarily complicated and gave inaccurate results. Thorough investigation of the various factors, concentration, reagents, etc., led to the development of a highly satisfactory method.

THE PICRATE METHOD

Reagents

Toluene. C.p. reagent, dried by shaking with anhydrous sodium sulphate. Interfering substances may be removed if necessary by shaking with N sulphuric acid followed by distillation and drying with sodium sulphate.

Picric acid. 0.02 per cent solution of picric acid (dry), c.p., in moisture-free toluene. A 2 per cent stock solution may be conveniently prepared and the dilute reagent made as required. The latter should be practically colourless.

Potassium carbonate. 100 g. potassium carbonate, c.p., dissolved in 100 g. water.

Formaldehyde. 10 per cent solution of formalin (40 per cent formaldehyde, commercial, shaken with magnesium carbonate and filtered) in water.

Standard trimethylamine solution. Stock solution: 0.682 g. trimethylamine hydrochloride (Eastman Kodak Co.) and 1 ml. hydrochloric acid, c.p., dissolved in 100 ml. water. As required, a dilute standard is prepared by diluting 1 ml. stock solution and 1 ml. hydrochloric acid to 100 ml. This contains 0.01 mg. trimethylamine nitrogen per ml. and 1 or 2 ml. may be used conveniently for the standard colour solution.

Procedure

An aliquot of a sample solution containing 0.002 to 0.02 mg. nitrogen as trimethylamine is made to 4 ml. in a test tube. One ml. formaldehyde, 10 ml. toluene, and 3 ml. potassium carbonate solution are added. The tube is stoppered with a cellophane-covered cork and is shaken vigorously approximately 40 times. Five ml. of the toluene layer is pipetted off into a small test tube and 0.3 to 0.4 g. granular anhydrous sodium sulphate is added. This is shaken a few times to dry the toluene, which is then poured off into a dry colorimeter tube containing 5 ml. of the 0.02 per cent picric acid reagent. After mixing, the yellow colour is read in a photoelectric colorimeter using a filter with maximum transmission at 4200 ångstrom units, the blank being made similarly except that water is used instead of trimethylamine or test solution. It has been found convenient to prepare two blanks, using the first to clean pipettes, corks, etc., from chromogenic substances, and using the second in the colorimeter.

A standard curve may be made up by using the standard trimethylamine solution but it has been found more convenient to use a K value with the Evelyn colorimeter ($K = \frac{2 - \log G}{c}$, where G is the galvanometer reading when the blank is set at 100, that is per cent light transmission; c is the concentration expressed as mg. nitrogen per tube). In routine use, it is only necessary to check standards with new reagents.

Development of Method

solvents and alkali

Richter et al. (1941) used a 1 to 1 solution of chloroform and either petroleum ether or toluene in the final colour solution. We have found that the colour is almost as intense (90 per cent) if toluene is used throughout. The recovery of solvents by fractional distillation is thus avoided.

Toluene, petroleum ether, benzene and xylene all gave good results as extracting solvents, and toluene was adopted as being the most suitable. The solvent is recovered by shaking with a small volume of N sulphuric acid to remove amines, decanting, distilling, and drying with anhydrous sodium sulphate. Various ratios of alkali, solution and solvent were tried. The ratio of toluene to solution should be between 1 and 2 to avoid emulsion formation. Using 5 ml. test solution and 10 ml. toluene, 3 ml. potassium carbonate solution was necessary to give constant recovery. The alkaline carbonate liberates the amine from its salts, so that it passes into the toluene phase on shaking, the salts being insoluble in the solvent. The tubes should be shaken vigorously 30 to 40 times. A cello-

phane-covered cork should be used. The first blank cleans this cork and the pipettes, etc., from chromogenic substances. The toluene extract contains appreciable suspended water which may be conveniently removed by shaking a few times with 0.3 to 0.4 g. granular anhydrous sodium sulphate, added from the point of a spatula, followed by decantation. More complete and convenient removal of water was thus obtained than by centrifugation.

PICRIC ACID CONCENTRATION

The blanks obtained with the 0.08 per cent picric acid reagent as used by Richter et al. were quite coloured. Therefore various concentrations between 0.008 and 0.4 per cent were tried. From 0.01 to 0.05 per cent was found most

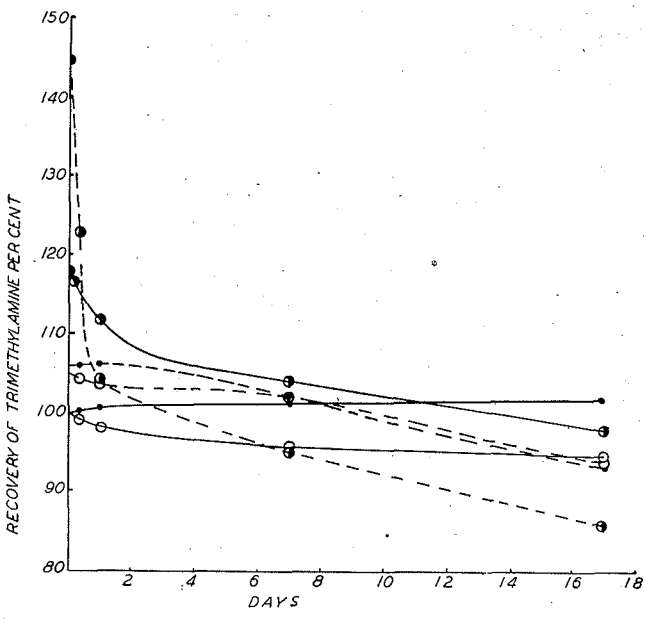

FIGURE 1 Effect of ammonia and formaldehyde on recovery of 0.01 mg. trimethylamine nitrogen. ——— Presence of formaldehyde, 1 ml 10 per cent solution. – – – No formaldehyde. ● 0.01 mg trimethylamine nitrogen; ○ 0.01 mg. trimethylamine nitrogen + 0.1 mg ammonia nitrogen; ◓ 0.01 mg trimethylamine nitrogen + 0.5 mg ammonia nitrogen.

suitable, low recovery being obtained with concentrations greater than 0.1 per cent. The lower the concentration, the less is the error introduced by the blank due to the colour of the picric acid. A 0.02 per cent solution proved satisfactory. About 0.01 to 0.02 mg. of trimethylamine nitrogen per tube is usually present and the excess of picric acid is thus three to six times over the amount required to form the trimethylamine picrate salt, assuming maximum extraction of trimethylamine by the solvent.

AMMONIA AND FORMALDEHYDE

Ammonia gave cloudy solutions which could not be read in the colorimeter. The addition of 1 or 2 ml. of the 10 per cent formaldehyde reagent did not affect the recovery of trimethylamine, and was sufficient to eliminate the interference

of ammonia up to 0.2 mg. nitrogen, an excess of 10 to 20 times over the usual concentration of trimethylamine nitrogen. The formaldehyde itself gave a slight colour but the effect was eliminated when it was added to the blank tube. The effect of ammonia and of formaldehyde on the recovery of 0.01 mg. trimethylamine nitrogen up to 18 days is shown in figure 1.

Stability

The formaldehyde also increased the stability of the colour solution. If the tubes are stoppered to prevent evaporation, the colour is quite stable. Only a very small change in colour intensity occurred up to one month, particularly if the solution contained no ammonia. Thus colour standards for visual comparison may be kept for considerable periods, an important point in plant routine work.

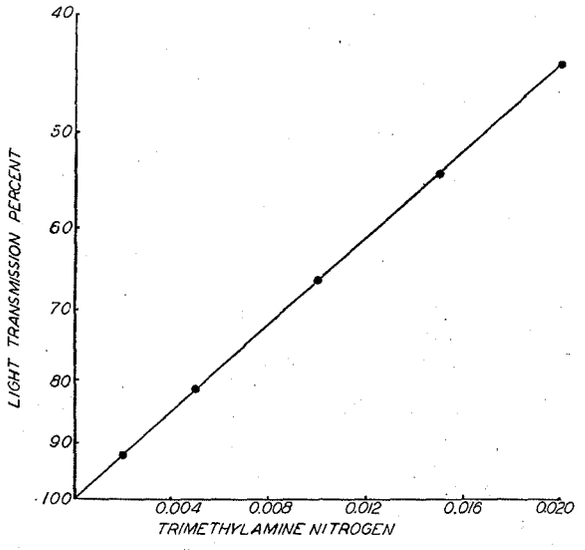

Figure 2. Standard curve for trimethylamine picrate. Per cent light transmission, logarithmic scale, plotted against mg. trimethylamine nitrogen per tube.

Standard Curves

A standard curve obtained by using various amounts of the standard trimethylamine solution is shown in figure 2. K values from a concentration of 0.002 to 0.020 mg. nitrogen per tube average 18.0 ($\pm$ 0.3), and Beer's law is followed over this range. Results from 35 points obtained on standard solutions over a period of three months under routine conditions gave similar results, with a slightly greater standard deviation, $K = 18.0$ ($\pm$ 0.9). Thus the reproducibility of the method is quite satisfactory.

Interferences

A solution containing 1 mg. nitrogen per tube of each of a number of the following amino acids and other substances were found to give no interference; glycine, asparagine, cysteine, cystine, tryptophane, histidine, tyrosine, gluta-

thione, creatine, creatinine, allantoin, urea, hydroxylamine, guanine, betaine, choline and trimethylamine oxide. Many of these are present in fish muscle and give picrate salts, but do not interfere because of insolubility in the toluene extractant. γ butyrobetaine, also present in fish (Kutscher and Ackerman 1933), was not tested but it probably behaves like betaine.

Lecithin gives a cloudy yellow colour. This probably explains the cloudy solutions obtained with extracts of fatty fish. No interference from this source occurred with cod or similar fish, although Kaucher et al. (1943) finds about three per cent lecithin in dried cod muscle. This interference may be eliminated when necessary, by a preliminary extraction with solvent from acid solution, under which condition trimethylamine is not extracted.

The primary, secondary and tertiary methylamines and ethylamines all react as shown in table I. With the exception of indole, the hydrochloride salts twice recrystallized from alcohol were tested. Only methylamine and dimethylamine

TABLE I. Colour values of various amines.

Compound	*K* value	Colour as per cent of colour given by an equivalent concentration of trimethylamine
Methylamine	2.00	11
Dimethylamine	3.78	21
Trimethylamine	18.0	100
Ethylamine	9.7	54
Diethylamine	18.9	105
Triethylamine	25.2	140
N-Propylamine	12.8	71
Histamine	5.4	30
Indole	0.07	0.4

are likely to be found in extracts from fish muscle so far as is known (Shewan 1939, Beatty and Collins 1940), and these only in small amounts, so that interference from this source is negligible. The trialkylamines give more colour than the di- or mono-derivatives, and the colour increases with length in the carbon chain, at least up to propylamine. Most of the higher aliphatic monoamines and various other amines have not been tested. These are not present in fresh tissue, but some are produced in advanced stages of spoilage by bacterial decarboxylation of aminoacids (C. L. A. Schmidt 1938, p. 236, Gale 1940, Richter et al. 1941, Gale and Epps 1944); for instance, histamine, indole, phenylethylamine, tyramine, cadaverine, and putrescine.

It was found that these nonvolatile amines were not produced in fish until spoilage was well advanced. Dyer and Mounsey (1945) demonstrated a difference between the colour values on the extract and on the distillate from the extract only when the trimethylamine values had reached 60 to 75 mg. nitrogen per 100 g. tissue. Fish are becoming unpalatable at levels of 10 to 15. Histamine gave 30

per cent of the colour of trimethylamine, and indole only 0.4 per cent. Richter et al. (1941) found that with his procedure, tyramine and putrescine did not give colours, while phenylethylamine yielded a colour about equal to trimethylamine. The presence of protein did not appreciably affect the recovery of trimethylamine in muscle extracts.

Comparison with Distillation Methods

The distillation methods of Beatty and Gibbons (1937), both the semimacrodistillation under reduced pressure and their modification of the Conway technique, were compared with the picrate procedure. Representative results obtained on fish extracts are given in table II.

Table II. Comparison of colorimetric and distillation methods.

Extract	Trimethylamine (mg. nitrogen per 100 g. muscle)				
	Colorimetric		Conway		Distillation (semi-macro)
Cod	0.20	$(\pm 0.04)_6$	0.22	$(\pm 0.05)_6$	
	0.84	$(\pm 0.1)_3$	0.80	$(\pm 0.1)_3$	
	2.7	$(\pm 0.2)_3$	2.7	$(\pm 0.1)_3$	
	11.2	$(\pm 0.3)_3$	10.8	$(\pm 1)_2$	
	33.5	$(\pm 0.5)_3$	34.3	$(\pm 0.5)_3$	
	90				73
	109	$(\pm 3)_3$	94	$(\pm 1)_3$	
Haddock	2.1	$(\pm 0.3)_3$	2.1	$(\pm 0.2)_3$	
	7.9	$(\pm 0.3)_3$	7.9	$(\pm 0.1)_3$	
	39.0	$(\pm 1)_2$	40.2	$(\pm 0.5)_2$	
	81				71
Smoked cod	11.0		9.8		11.1
	34.0		32.0		33.0
	34.3		33.5		
	93				93
Chicken haddie pickle	58.4		59.0		60.1
	28.0		30.0		27.0
Salt cod	76				78
Mackerel	4.5		2.4		2.5
	3.2		1.8		1.9

The results show very good agreement between all three methods. Since the colorimetric method is much shorter and is adaptable to the analysis of large numbers of routine tests, it is the most satisfactory for general use.

The method has been satisfactorily used on more than 8,000 estimations of trimethylamine on trichloracetic acid and formaldehyde extracts of fish and directly on the pickle from canned fish over a period of a year, and is now in use

in several laboratories. A simplified modification for use with visual comparator tubes, as given by Dyer (1943), is also in use in one plant laboratory. It should be noted, however, that a 0.02 per cent solution of picric acid in toluene is now used instead of a chloroform solution.

SUMMARY

A sensitive colorimetric method for estimating the spoilage of fish by trimethylamine determination has been developed. A sample containing 0.002 to 0.02 mg. nitrogen as trimethylamine is made alkaline with potassium carbonate in the presence of formaldehyde, extracted with toluene, and the dried extract mixed with a toluene solution of picric acid. The yellow colour of trimethylamine picrate is measured in the colorimeter. The colour is stable and obeys Beer's law over the range given.

Ammonia, aminoacids, choline, betaine, urea and guanine do not interfere. Methylamines and ethylamines give some colour. Lecithin interferes but this interference can be eliminated. The higher amines produced during protein putrefaction also give the test.

The method agrees with distillation methods on fish products and has been found satisfactory.

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