Measurement of Formaldehyde in Fish Muscle Using TCA Extraction and the Nash Reagent

C. H. Castell and Barbara Smith

Fisheries Research Board of Canada
Halifax Laboratory, Halifax, N.S.

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The use of the Nash test, in conjunction with TCA extraction, for measuring formaldehyde in fish muscle was made more quantitative. This was done by means of a "recovery factor" which took into consideration the percent of formaldehyde added to the muscle extracted by the TCA solution. The average recovery from 15 different samples of cod muscle was 51.3% with an SD of 5.6. Because of differences between species in capacity to bind formaldehyde, it would appear that a different "recovery factor" may be required for each species of fish. As heating muscle increases its ability to bind formaldehyde, recovery factors developed for use with raw muscle are not applicable to the same muscle after it has been cooked. The percentage of added formaldehyde that was recovered varied with variations in the procedures used in preparing the muscle, making the extract, and carrying out the Nash test.

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Le test de Nash, couplé à l'extraction au TCA, pour le dosage du formaldéhyde du muscle de poisson a été rendu plus quantitatif. Nous nous sommes servis pour cela d'un "facteur de récupération" qui tient compte du pourcentage de formaldéhyde total dans le muscle soumis à l'extraction par la solution de TCA. La récupération moyenne observée sur 15 échantillons différents de muscle de morue est de 53.1%, avec écart type de 5.6. Par suite de différences dans leur capacité de fixation du formaldéhyde, chaque espèce de poisson semble exiger un "facteur de récupération" différent. Le chauffage du muscle augmente sa capacité de fixation du formaldéhyde. Pour cette raison, les facteurs de récupération établis pour le muscle cru ne s'appliquent pas au même muscle après cuisson.

Nous avons également étudié l'effet des variations de procédures dans la préparation du muscle, la fabrication de l'extrait et la conduite du test de Nash sur la proportion de formaldéhyde ajouté qui peut être récupérée.

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A procedure for the estimation of formaldehyde in biological materials was described by Nash (1953). It is based on the formation of diacetyldihydrolutidine from acetylacetone and formaldehyde in the presence of excess ammonium salt. He suggested (but did not support it with data) that the test could be carried out without the complete destruction of the biological material being examined. He went even further by stating "the conditions are mild enough to allow of its use with living material, and its sensitivity and degree of specificity are comparable to those of other reactions requiring more severe conditions." A careful reading of his paper shows that this proposed procedure is simply a colorimetric method for measuring free or loosely bound formaldehyde. Formaldehyde, however, reacts readily, and in some cases almost irreversibly, with proteins, amines, amino acids, and many other organic compounds. In biological materials much of this bound formaldehyde is not available to react with the Nash reagent; therefore, this test is not a satisfactory measure of the total amount of formaldehyde that is present in plant or animal tissues unless it is used in conjunction with some method of extraction that releases the more firmly bound formaldehyde. In most analytical procedures this is accomplished by the very methods that Nash suggested were not required, such as the complete destruction of the tissues by means of hot, concentrated sulphuric acid, as used by Eegriwe (1937). However, this drastic treatment poses a difficulty when applied to the tissues of many marine animals. Soudan (1962) has pointed out that strong acid treatment also liberates formaldehyde from trimethylamine oxide, which is a normal constituent of most sea fish. This oxide, therefore, has to be elim-

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inated or reduced before the test can be carried out.

Amano and Yamada (1964) attempted to adapt the Nash test to the measurement of formaldehyde in fishery products by a much gentler method of extraction. They first macerated the fish in a mortar and then extracted it with a 20% TCA solution, but they gave no data on the efficiency of the extraction. Somewhat later, Amano and Tozawa (1969) made the following significant comment: "Because of its highly reactive nature with proteins, or with many other components in fish tissue, a full determination of formaldehyde may not be feasible "(i.e. by means of the Nash test on a TCA extract).

The purpose of this present work was to re-examine the procedure for measuring formaldehyde by means of TCA extraction and the Nash test. An attempt was made to estimate the proportion of the total formaldehyde present in fish muscle that can be recovered by TCA extraction. This was done by adding measured amounts of formaldehyde to samples of fish muscle, measuring the amounts recovered in the TCA extract, and then calculating the percentage of the added formaldehyde that was recovered. If this "recovery factor" is reasonably constant for muscle from a given species of fish, it would then be possible to convert the results of the Nash test on a TCA extract into values that more closely approximate the total amount of formaldehyde in the tissues. As the formaldehyde-binding capacity of muscle is primarily determined by its chemical composition, and as the composition of muscle varies slightly from one species to another, it is anticipated that a different "recovery factor" may be required for each species of fish. Most of the work described in this paper was confined to fresh cod muscle and to concentrations of formaldehyde not exceeding 0.01% or 100 ppm in the muscle. A study was also made of the effects of variations in the procedures used for preparing the muscle, making the extract, and carrying out the Nash test on the amount of formaldehyde that was recovered. In addition, some tests were carried out to determine the formaldehyde-binding effect of certain components of the muscle, as indicated by their ability to change the proportion of formaldehyde that was recovered when they were added to the muscle.

Materials and Methods

Most of the experimental work was carried out with muscle from Atlantic cod (*Gadus morhua*), but for the purpose of comparison tests were also made with muscle from Atlantic haddock (*Melanogrammus aeglefinus*), pollock (*Pollachius virens*), silver hake (*Merluccius bilinearis*), Atlantic wolffish (*Anarhichas lupus*), redfish (*Sebastus marinus*), winter flounder (*Pseudopleuronectes americanus*), Atlantic salmon (*Salmo salar*), and lobster (*Homarus americanus*). Most of the fish were obtained live from the station's aquaria and the tests were commenced before the fish had entered rigor mortis. A number of tests were also made with fillets obtained from commercial sources that were of good quality but which had passed through rigor.

The formaldehyde was analytical reagent grade. The basic procedures for the experimental work were as follows:

(1) Because of the difficulty of obtaining uniform distribution of formaldehyde when added to whole fillets, blended fillets were used. The blend was prepared by blending a whole fillet with an equal weight of water in a gallon size Waring blendor for approximately 45 sec at medium speed.

(2) The formaldehyde solutions were always prepared from the stock 40% solution immediately before being used. They were diluted in such a manner that the desired concentration was obtained when one part of formaldehyde solution was mixed with 2 parts of blend by weight. The final blend then contained 1 part of fish to 2 parts of water and 60 ppm of formaldehyde. All results are recorded as ppm (mg/1000 g) in the blend.

(3) After standing 30 min at room temperature, the blended muscle, containing the formaldehyde, was extracted by adding an equal weight of 10% TCA solution, stirred, and filtered. A series of comparative tests showed no difference in the amount of formaldehyde recovered from blends by using 10% TCA, 10% $HClO_4$, or plain water. In a typical test the recoveries were: 52.7% with TCA, 49.2% with $HClO_4$, and 52.0% with water. The disadvantage in using water was that it required extra centrifuging to remove the cloudiness that developed during the heating of the reaction tubes, which could not be eliminated by filtering. We also found no advantage in increasing the strength of the TCA solution from 10% to the 20% used by Amano and Yamada.

(4) Nash color reagent: A solution containing 2-M ammonium acetate, 0.05-M acetic acid, and 0.02-M acetylacetone was prepared by dissolving 150-g ammonium acetate, 3 ml of acetic acid, and 2 ml of acetylacetone (redistilled at 140 C) in water and adjusting the volume to 1000 ml. This solution was stored in a dark colored, airtight bottle at 0 C.

In reference to the stability of his color reagent, Nash (1953) stated that after 2 weeks at 20 C it "appeared to be unchanged." Our experience has shown it to be less stable than indicated. Even when stored close to 0 C the optical density values with known amounts of formaldehyde gradually decreased as the storage period increased. Cochin and Axelrod (1959) overcame this by preparing a slightly modified, double-strength Nash reagent (DSNR): 150-g ammonium acetate and 2 ml of acetylacetone in a volume made up to 500 ml with water. We found that when the DSNR was stored 6 months at 0 C it gave readings that were identical to those from freshly prepared reagents. Tests using both DSNR and freshly prepared Nash reagent gave similar results. Because of its better keeping quality, DSNR was used in most of the formaldehyde determinations recorded in this paper.

(5) A 5-ml sample of the TCA extract was added to 10 ml of water and adjusted to *p*H 6.0 (with acetic acid

or NaOH solutions) and then made up to 25 ml with water. Five milliliters of this adjusted and diluted extract were added to 5 ml of the Nash color reagent followed by heating in a water bath for 5 min at 60 C and then cooling under running tap water. The optical density (OD) was read at 415 nm and the formaldehyde calculated by comparison of values obtained with a standard curve. During the course of the work three different spectrophotometers were used and compared: Unicam, Beckman DU, and Bausch and Lomb "Spectronic 20." They all gave satisfactory readings, but the "Spectronic 20" needed the use of a line voltage regulator to give steady readings.

Standard Curve and Recovery Factors

The curve obtained by plotting formaldehyde concentrations in aqueous solutions against OD readings, using the Nash color reagent (Fig. 1), shows a straight line relation up to a formaldehyde concentration of about 25 ppm (OD of about 0.7). Over a period of a year, using several lots of separately prepared Nash and DSNR solutions, 45 tests were made on standard solutions containing 20 ppm of formaldehyde. The average OD was 0.513 with an SD of 0.052. From these figures a factor was obtained for the conversion of OD values into formaldehyde concentrations (where the OD values were under 0.7): Each OD unit of 0.1 equals approximately 3.9 ppm of formaldehyde. The relation between the concentrations of added formaldehyde in the blend and the OD values that were obtained by TCA extraction and the Nash test (Fig. 1) was also linear for OD values up to 0.7. Calculations based on the values from which these curves were drawn show that for this particular sample of fish muscle approximately 44% of the added formaldehyde was recovered in the TCA extract.

Formaldehyde Recovery from Cod Muscle

The blend made from the two fillets from one cod fish, containing 60 ppm of formaldehyde, was divided into 10 portions and a formaldehyde determination was made on a TCA extract from each of the 10 portions. The average recovery was 55.6% and the SD was 0.51. Also, over a period of several months a similar concentration of formaldehyde was added to muscle from 15 different cod fish. In this case the average recovery was 51.3% and the SD 5.6. This indicates that, although there might be slight variations between muscles from individual fish, approximately 50% of the formaldehyde added to the cod muscle was recovered in the TCA extracts.

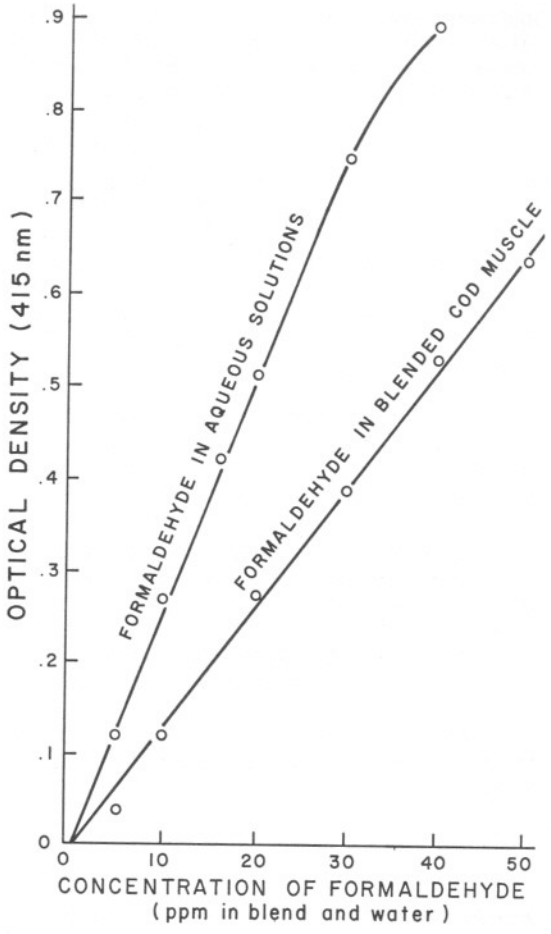

FIG. 1. Standard curve showing the relation between optical density and the concentration of formaldehyde in aqueous solutions, and the curve showing the relation for formaldehyde added to the fish muscle, obtained from the TCA extracts. Each plotted value is the average for five or more samples.

Measurement of Formaldehyde Formed in Galoid Fillets During Storage

In dealing with commercial fish one would rarely be required to measure the amount of formaldehyde that has been purposely added to the muscle. Of much more interest is the formaldehyde that is formed in situ as the muscle undergoes certain types of deterioration or processing. An example of this is the formaldehyde that is formed in the muscle of gadoids when the fillets are frozen and stored at temperatures above –25 C (Tokunaga 1964, 1965; Castell 1971). In this case the formaldehyde has its origin in the trimethylamine oxide (TMAO) of the muscle (Amano and Yamada 1964a, 1964b; Yamada and Amano 1965), which decomposes through enzyme action to form equimolar amounts of DMA and formaldehyde. During storage the DMA accumulates, but at least part of the formaldehyde is firmly bound by the tissues and cannot be recovered by TCA extraction. With this in mind, DMA and formaldehyde (using the copper dithio-carbonate procedure of Dyer and Mounsey 1945) determinations were carried out on some old frozen fillets that had been stored under poor storage conditions. The purpose was to compare the amounts of DMA and formaldehyde that had formed during storage in terms of millimoles per 100 g of muscle. To begin with,

the formaldehyde was measured in the usual manner by TCA extraction and the Nash color reagent, but without the use of a "recovery factor." The average values from four separate fillets obtained in this way were as follows:

	DMA	HCHO
mmole/100 g	0.82	0.44
molar ratio	1	0.536

This would suggest that during storage only half a mole of formaldehyde was formed for each mole of DMA. However, if the average "recovery factor" for cod muscle (Table 1) is taken into consideration, quite a different set of values is obtained:

	DMA	HCHO
mmole	0.82	$\frac{100}{51.5} \times 0.44 = 0.85$
molar ratio	1.00	1.04

indicating that very close to equal molar amounts of DMA and formaldehyde were produced in the stored frozen fish.

Factors Affecting the Recovery of Formaldehyde

This section indicates how some factors affect the amount of formaldehyde that can be recovered from cod muscle.

Proportions of Muscle and Water in the Blends

When the proportions of muscle and water in the blends were changed, there was a corresponding change in the amounts of added formaldehyde that were recovered. As the proportion of muscle increased the amount of recoverable formaldehyde decreased (Fig. 2), indicating that the unrecovered fraction was bound by the tissues.

Binding of Formaldehyde by the Soluble and Insoluble Components of the Muscle

Both the solid and the liquid fractions of the muscle were able to bind the formaldehyde, but the greatest "loss" occurred with the insoluble materials, as shown by the following experiment: A 1:1b lend of cod muscle was centrifuged at 41,300 *g*. The supernatant liquid was drained off by inverting the tubes and letting these stand for 10 min. This gave, by weight, 31% very compact solid material and 69% clear, liquid solubles. The liquid was then divided into two portions, one of which was treated with TCA and filtered to remove soluble proteins and other materials precipitated by the TCA. Thirty-one grams of the solids were made up to 100 g

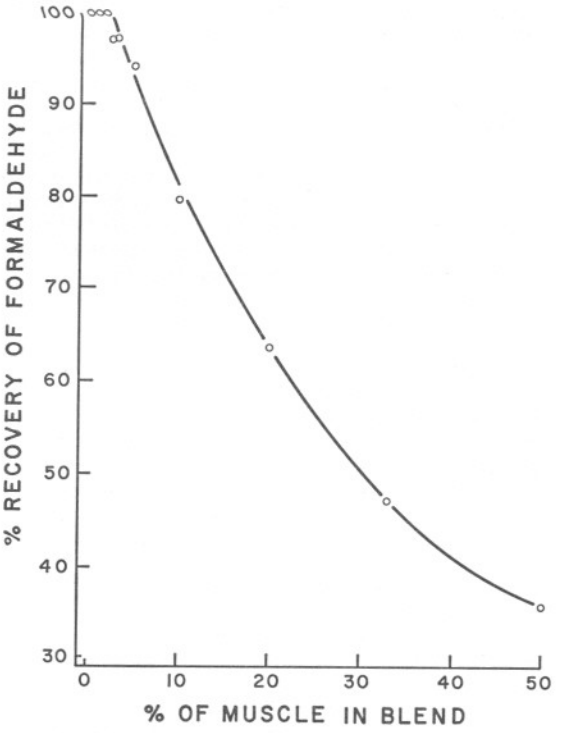

FIG. 2. Effect of the proportions of water and muscle in the blend on the recovery of added formaldehyde. The formaldehyde was added to give a concentration of 60 ppm in each of the blends.

by adding water and dispersed by vigorous stirring. Sixty-nine grams of each of the liquid fractions were also made up to 100 g by the addition of water. To each lot formaldehyde was added to give a concentration of 60 ppm and, after standing for 30 min, they were tested as usual:

Fraction	% Recovery of HCHO
Insolubles	49.2
Solubles	66.3
TCA-treated solubles	69.7

It is noteworthy that removal of the soluble proteins from the liquid fraction by TCA treatment made relatively little difference in its capacity to bind formaldehyde.

Blending Time

Insufficient blending, where the tissues were not completely disintegrated, resulted in a greater recovery of the added formaldehyde; but once the blend had reached a smooth creamy texture more blending

had no effect. With most fish muscle samples this creamy stage was reached after 1–1.5 min at medium speed in a Waring blendor.

Time Between Adding Formaldehyde and Testing

In well blended muscle the "loss" of added formaldehyde occurred rapidly. With samples left for 3–120 min between the addition of formaldehyde and extraction with TCA solution, the percentage recoveries showed no significant differences:

Time (min)	3	5	25	60	120
Recovery (%)	49.7	50.8	51.4	54.1	50.8

Heating the Reaction Tubes

Using DSNR for determining the recoverable amounts of formaldehyde added to blended cod muscle, we found that heating the reaction tubes beyond 5 min at 60 C resulted in decreasing optical density readings (Fig. 3). This agrees with the results obtained by Nash using the ordinary color reagent, in which he found the reaction time for 99% completion of the color change to be 40 min at 37 C or 5 min at 58 C.

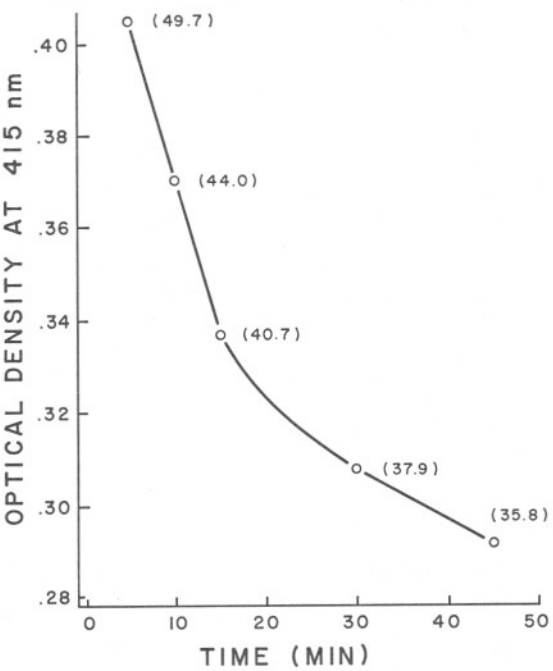

Fig. 3. Effect of holding time at 60 C on color development in reaction tubes containing the double strength Nash reagent and TCA extracts from blended cod muscle to which formaldehyde had been added to give a concentration of 60 ppm. The figures in brackets show the percentage recoveries of formaldehyde.

Trimethylamine and Dimethylamine

In defining some of the limitations of his colorimetric procedure Nash (1953) pointed out that certain amines can compete with the ammonia in the reagent, causing "an apparent loss of determined formaldehyde." As both trimethylamine (TMA) and dimethylamine (DMA) are frequently found in marine fishery products, it is important to know whether either one of these amines interferes with the recovery of formaldehyde from fish muscle. To answer this, cod muscle blends were prepared from freshly killed fish, under conditions where neither TMA or DMA has a chance to develop. TMA–HCl and DMA–HCl were each added to give a range of amine values between 6 and 66 mg of amine nitrogen per 100 g of muscle. These concentrations extend beyond the limits normally encountered in badly deteriorated commercial fish. The blends were tested 30 min after adding the amines and the results showed that in the concentrations used they had little or no effect on the recovery of the formaldehyde:

Concn of amine in blend (mg amine N/100 g)	0	6	12	20	27	33	66
Recovery of HCHO(%)							
With TMA	46	52	47	46	44	46	44
With DMA	46	47	48	49	47	49	41

Other tests in which the *p*H of the blend was adjusted to an alkaline range of *p*H 7.0–9.0 (in order to free the amine from the hydrochloride) gave similar negative results.

Proteins and Amino Acids

The addition of various proteins (casein, gliadin, gluten, zein, and peptone) to give a concentration of 1% in the blends added little or nothing to the normal formaldehyde-binding activity of the muscle itself, as indicated by the amounts recovered in the TCA extract. If much larger concentrations were added, or if the muscle was heated before extracting it with the TCA, additional binding of the formaldehyde became evident. The same remarkable result was obtained with most of the free amino acids that were added to the muscle. One decided exception was with cysteine, and to a somewhat lesser extent with the closely related peptide, glutathione. The effects of cysteine on the recovery of formaldehyde, both from aqueous solutions and from blended cod muscle, are shown in Fig. 4. In the case of the blends, the muscle itself reduced the recovery by approx-

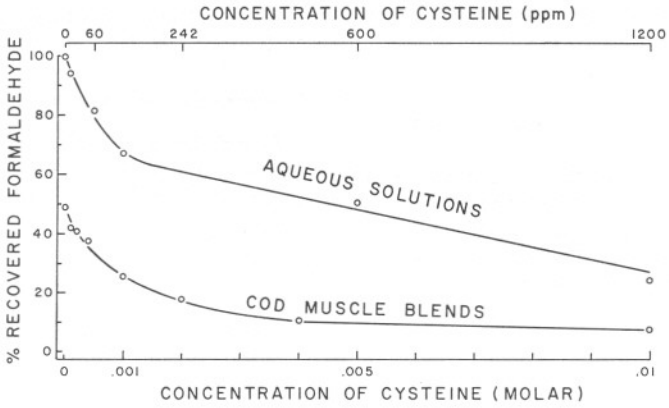

FIG. 4. Effect of cysteine on the recovery of formaldehyde from aqueous solutions and from blended cod muscle.

imately 50%, but the addition of relatively small amounts of cysteine further reduced the amounts recovered. The addition of 60 ppm (0.006%) cysteine was more effective in reducing the recovery of formaldehyde from the blend than the addition of 5% of any of the proteins that were tested.

The results with cysteine were not unexpected. It is known that it combines with formaldehyde to form thiazolidine-4-carboxylic acid, which is a remarkably stable compound towards both acid and alkali (Ratner and Clarke 1937). In addition to reacting with formaldehyde, cysteine also reacts directly with the Nash color reagent, seriously interfering with the results of the test. Fortunately, this can be prevented by precipitating the cysteine in the TCA extract with a weak solution of $CuSO_4$ and filtering immediately before the Nash reagent is added.

HEATING THE MUSCLE

Preheating the muscle increased its capacity to bind formaldehyde and therefore decreased the percentage of added formaldehyde that could be recovered. For example: four fillets were cut from two fish. One fillet from each fish was autoclaved for 15 min at 15 lb pressure, while the two corresponding fillets were left unheated. These were each blended separately and formaldehyde was added and extracted in the usual manner. The recoveries were 47.6 and 49.8% from the unheated muscle and 21.6 and 24.0% from the corresponding heated fillets. Heating the blends after the formaldehyde had been added resulted in even lower recoveries than when heat was applied to the whole fillet before it was blended and the formaldehyde added. For example: fillets were blended in the normal manner and the blends were mixed and distributed in heat-stable plastic bags. One was left unheated; a second was heated by immersing it for 10 min in boiling water before adding formaldehyde; the third had formaldehyde added before heating. The recoveries were as follows: Unheated, 56.8%; heated prior to adding formaldehyde, 33.1%; heated after adding formaldehyde, 23.7%.

FREEZING

Freezing fresh cod fillets at –40 C for 2 hr and then defrosting and adding formaldehyde did not change the amount of formaldehyde recovered, as compared with similar fillets that had not been frozen. Determining the combined effects of freezing and frozen storage was a much more difficult problem because of the many variables that are involved. However, as a result of many tests, including some with fish that had been stored under adverse conditions, we concluded that the changes occurring in the muscle during frozen storage had relatively little or no effect on its formaldehyde-binding capacity, as indicated by the recovery from TCA extracts.

INTRA AND INTER SPECIES DIFFERENCES

Because of the inherent differences in the chemical composition of different species of fish and shellfish, it is possible that there may be corresponding differences in the formaldehyde-binding activity of muscle from different species. As a preliminary check on this problem determinations were made on the percent recovery of formaldehyde added to blended muscle from 10 species of commercial fish and shellfish (Table 1). Although these limited data are insufficient for a satisfactory statistical analysis, they

TABLE 1. Percentage recoveries of formaldehyde[a] that had been added to blends prepared from the muscle of various species of fish and shellfish using the Nash test and 10% TCA as the extractant.

Species	No. of fish	% Recovery	
		Range	Avg
Scallops	7	66.7–71.7	69.1
Lobster	4	59.1–64.1	61.7
Atlantic salmon	1	–	66.9
Winter flounder	2	53.4–54.8	54.1
Cod	15	40.3–58.6	51.5[b]
Haddock	5	44.1–54.7	48.4
Hake	2	45.1–51.3	48.2
Pollock	3	39.7–49.4	43.7
Wolffish	5	33.1–44.2	40.2
Redfish	1	–	39.9

[a]Formaldehyde added to give a concentration of 60 ppm in the blend.
[b]Standard deviation from the mean: 5.6.

do suggest that a species difference may exist. The range of formaldehyde recoveries from the scallops and lobsters was considerably higher than those from the fish. Among the species of fish, the gadoids, as a group, had recoveries that were closer to each other than to those of the shellfish or the nongadoid species of fish. However interesting these results appear to be, they need the confirmation that can only be obtained by a much more extensive set of measurements. It is also very probable that, with some species, the recovery factors may differ with some of the chemical changes that occur in the muscle during the annual cycles of feeding and spawning.

Discussion

This work started out with the object of determining the value of TCA extraction in conjunction with the Nash color reagent (Amano and Yamada 1964) as a measure of small amounts of formaldehyde in fish muscle. The work is far from being completed because so many factors are involved. There are the problems resulting from different species of fish, variations in the composition of muscle resulting from the cycles of spawning and feeding, and the effects of various ways of storing and processing the muscle.

The results do show, however, that the test as it has been used in the past is far from quantitative. However, by determining the percent of formaldehyde added to the muscle that can be recovered by extraction with TCA, a value much closer to the total amount of formaldehyde present can be obtained. It was shown that for added formaldehyde the recovery from cod muscle is approximately 50%. The very limited number of tests with muscle from other species suggests that some at least may differ from cod in their formaldehyde-binding activity, as indicated by extraction with TCA.

Knowing the extreme reactivity of formaldehyde with many compounds it was a little surprising to find that the addition to the muscle of 1% of several different proteins made relatively little change in the recovery of added formaldehyde. But it must be remembered that these small amounts of protein were added to a very complex system already containing proteins and many other compounds with which formaldehyde will combine. In contrast to this, the extremely effective binding of formaldehyde when trace amounts of cysteine were added to the muscle was equally interesting and suggests the need for the examination of other sulphydral compounds in this connection.

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