CHEMICAL METHODS FOR THE DETERMINATION OF THE FRESHNESS OF FISH[1] [2]

DARDJO SOMAATMADJA[3], JOHN J. POWERS, AND
DAN E. PRATT

Department of Food Technology,
University of Georgia, Athens, Georgia

(Received for publication July 15, 1960)

Various objective methods to estimate the degree of freshness of fish and fishery products were compared. The total volatile nitrogen (TVN) and ammonia-nitrogen of carp flesh reached values of 30-33 mg% when carp stored at 0-4°C. and 25-27°C. began to have a bad odor. For catfish stored at 0-4°C. and 25-27°C. these values reached 20-30 mg%. Trimethylamine was not found in fresh and spoiled carp and catfish. The determination of trimethylamine as an index of spoilage cannot be used for these fresh-water fish. The determinations of tyrosine and volatile reducing substances (VRS) cannot be used to estimate the degree of freshness of fish if the temperature at which the fish had been stored is not known. If these two methods were combined with TVN and ammonia-nitrogen one could determine the degree of freshness of fish and the temperature at which the fish were stored. The determination of tyrosine combined with TVN and ammonia-nitrogen can give further support as to whether fish are absolutely fresh. If the temperature under which fish have been stored is known each of the tests used in this study can be applied individually to determine the degree of freshness of fish.

Since fish are nutritious and are eaten by many peoples in the World, the freshness of fish intended for human consumption is important. Many objective methods have been proposed as aids in estimating the freshness of fish, but most of these methods have been applied to fish iced or otherwise chilled to retard spoilage. In areas of the World where refrigeration is not common, fish may be kept alive until sale, or, if they die, they may be held at ambient temperature until sale. In Indonesia, for example, fish are kept alive in bamboo vessels — made waterproof with coal tar — but sometimes dead fish may be left in the vessel to facilitate sale.

The objectives of the present study were to determine: (a) whether some of the methods used or suggested for refrigerated fish would apply to fish held at ambient temperatures, (b) whether such methods would be applicable to more than one species of fish, and (c) whether a combination of objective tests would yield a significantly higher correlation with sensory appraisal than use of any one test alone.

REVIEW OF LITERATURE

Numerous methods of estimating the degree of freshness of fish have been reported in the literature (*2, 4, 5, 6, 7, 9, 10, 12, 13, 19, 20 24*). Tarr (*27*) has reviewed many of the methods and biochemical changes known to occur in fish during decomposition. The temperature at which fish have been stored has been reported to influence the reliability of certain tests (*14, 15 23*). Since the components of fish vary with species and geographic area (*25*), the application of certain chemical methods is limited by these factors. Farber (*7*) stated that the determination of indole, volatile nitrogenous compounds, H_2S, and pH were of no significance for evaluating the early stages of spoilage of certain species of fish. Reay and Shewan (*21*) reported that the determinations of trimethylamine and dimethylamine are the best methods to determine the freshness of marine fish, but Castel (*3*) and Anderson and Fellers (*1*) were not able to detect the presence of trimethylamine in spoiled fresh-water fish.

EXPERIMENTAL PROCEDURES

Fish Samples.

Three different kinds of fresh-water fish were used in this study: carp, catfish, and bream. The carp were not less than 16 inches long with an average weight of one pound. The catfish were not less than 12 inches long with an average weight of 12 ounces. The bream were not classified according to size. All fresh-water fish were taken from a local fish pond and were kept alive in the laboratory until used. Two kinds of salt water fish were used, these were mullet and mackerel, and were obtained from a local fish market.

At the beginning of the experiment, some of the fresh-water fish were killed and eviscerated. The sample was divided into two batches; one was stored

[1]Data reported herein are from the M. S. thesis of Dardjo Somaatmadja, Univ. of Georgia, 1959.

[2]Contribution from College Experiment Station, Univ. of Georgia, Athens, Ga. Approved as Journal Paper No. 131.

[3]On leave from the Laboratory for Chemical Research, Indonesian Department of Industry, Bogor, Indonesia.

at 0-4°C. and the other at 25-27°C. in an unsealed jar. Some of the remaining fish were kept alive (*a*) until each of the test periods to provide fresh (control) fish for the sensory appraisal and chemical tests and (*b*) to provide fish for each of the different experimental storage periods.

At each test period, after evaluation of the odor, the fish were used for the tetrazolium tests. For this test the fish were not ground, but the test paper containing 2-P-iodophenyl-3-P-nitrophenyl-5-phenyl tetrazolium chloride (INTC) was placed in contact with the skin of the fish for five minutes after which the extracted red color was read at 550 mμ. Afterward, the entrails and bones were removed and the flesh blended in a Waring blendor. The blended flesh was used for the determination of volatile nitrogenous constituents, volatile reducing substances, tyrosine, and volatile acid number.

Determination of Nitrogen Constituents.

The methods reported by Winton and Winton (*28*) was used for total volatile nitrogen (TVN), ammonia nitrogen, and trimethylamine. In this study the suspension of the sample was not stored in the refrigerator before analysis as reported originally but was analysed immediately. Preliminary experiments showed that both procedures gave the same results. The method of Ottaway (*18*) was used for tyrosine.

Determination of Total Volatile Acid Number.

The method of the Association of Official Agricultural Chemists (*16*) was employed.

Determination of Reducing Substances.

The authors employed the method reported by Shewan and Liston (*22*) for tetrazolium reduction tests. Two different methods were used to determine the amount of reduction by volatile substances. The method reported by Lang, Farber, Yerman, and Beck (*11*) was slightly modified by using iodometric titration.

The second method was to measure the reducing substances volatilized by steam. In this method, a 5-g. ground sample was weighed into a 500-ml. distillation flask, followed by 200 ml. of water. The suspension was distilled for 30 minutes into an iodine flask containing 10 ml. of 0.05 *N* alkaline potassium permanganate solution. After cooling, 2 g. of KI were added followed by 15 ml. of 6 *N* sulfuric acid solution. The liberated iodine was titrated with 0.05 *N* sodium thiosulfate solution. The amount of reduction is reported as milliequivalent reduction per 100 gram of sample.

Organoleptic Evaluation.

For the odor evaluation of samples, scores ranged from 5 to 1. A score of 5 represented fresh fish and 1 represented decomposed fish. A score of 3 or below was considered to be organoleptically unacceptable.

RESULTS AND DISCUSSION

Both fresh carp and catfish contained volatile nitrogenous constituents. When carp were stored at 0-4°C. the TVN and ammonia nitrogen content reached approximately 32 mg% and 30 mg%, respectively, as carp started to possess an undesirable odor. The same values of TVN and ammonia nitrogen were found when carp were stored at 25-27°C. as may be seen in Table 1. When catfish were stored at 0-4°C. the

TABLE 1 — EFFECT OF STORAGE OF CARP AT 0-4°C AND 25-27°C ON ORGANOLEPTIC SCORES, TOTAL VOLATILE NITROGEN, (TVA), AMMONIA-NITROGEN, TRIMETHYLAMINE NITROGEN (TMA), VOLATILE REDUCING SUBSTANCES (VRS), AND TYROSINE

Days of storage	0		1		2		3	4	5	6
Temp. of storage, (°C)	0-4	25-27	0-4	25-27	0-4	25-27	0-4	0-4	0-4	0-4
Score	5	5	5	3	4	1	4	3	2	1
TVN, mg%	9.4-10.9	10.6-11.2	14.3-14.3	31.9-33.1	14.4-19.4	193.2-196.0	20.8-23.1	32.1-32.3	47.2-53.5	83.1-110.1
Ammonia-N mg%	8.1-11.7	7.6-10.7	12.1-12.8	32.4-32.9	14.0-15.1	193.5-183.6	18.1-24.6	29.0-31.4	39.9-44.8	73.8-101.8
TMA, mg%	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0-0
Micro-equiv. reduction/ 5 ml. fish juice	1.0-2.0	6.1-7.9	9.3-11.0	171.8-173.2	120.0-12.7	214.5-222.2	14.2-17.5	29.3-32.2	43.3-44.7	104.6-110.4
Tyrosine mg%	0-0	0-0	3.3	11.5-25.5	4.3	35.4-66.0	6.3	8.0	9.5	10.6

TABLE 2 — EFFECT OF STORAGE OF CATFISH AT 0-4°C AND 25-27°C ON ORGANOLEPTIC SCORES, TOTAL VOLATILE NITROGEN (TVN), AMMONIA-NITROGEN, TRIMETHYLAMINE NITROGEN (TMA), VOLATILE REDUCING SUBSTANCES (VRS), TYROSINE, TETRAZOLIUM TEST (INTC), AND TOTAL VOLATILE ACID NUMBER (TVA)

Days of storage	0		1		2		3	4	5	6
Temp. of storage, (°C))	0-4	25-27	0-4	25-27	0-4	25-27	0-4	0-4	0-4	0-4
Score	5	5	5	3	5	1	5	4	3	2
TVN, mg%	8.1-9.4	7.2-9.3	9.9-13.4	29.1-30.1	10.2-19.4	102.3-107.4	11.9-17.0	14.6-15.6	22.1-26.1	39.3-43.1
Amm.-N, mg%	9.2-9.0	6.3-8.6	8.1-12.7	26.2.-28.2	9.3-18.0	98.5-102.5	12.9-16.8	18.1-15.1	19.8-20.4	24.1-37.3
TMA, mg%	0-0		0-0		0-0		0-0	0-0	0-0	0-0
Micro-equiv. reduction/ 5 ml. fish juice	0.0-2.0	3.9-7.8	0.9-2.3	119.0-134.0	0.1-1.1	236.0-263.0	0.0-1.2	9.5-26.0	61.7-81.8	83.3-119.8
Tyrosine, mg%		0.0-1.5		8.2-15.7		52.6-56.5				
INTC, O. D.		0.002-0.021		0.205-0.255		O.235-0.390				
TVA		23.9-26.6		57.4-62.2		105.0-107.5				

TVN and ammonia nitrogen reached 22-26 mg% as the fish gave off a putrid odor. Approximately the same values were found when catfish were stored at 25-27°C. as may be seen in Table 2.

These results agreed with those of Tagawa, Yoshimo, and Miyauchi (*26*). They found in four species of fish that the ammonia nitrogen exceeded 30 mg% when the fish began to possess a putrid odor.

Trimethylamine values remained nil when both carp and catfish became completely unaccepatble. Castel (*3*) reported that fresh-water fish did not contain trimethylamine oxide which compound was reduced into trimethylamine during spoilage. Anderson and Fellers (*1*) reported that the presence of trimethylamine was hardly detectable in spoiled fresh-water fish although they were able to observe the presence of trimethylamine oxide. In this study ammonia nitrogen accounted for nearly all of the volatile nitrogenous constituents. The correlation coefficient between the two constituents was 0.994.

The volatile reducing substances (VRS) of fresh carp ranges from 1 to 18 units of reduction. When carp were stored at 0-4°C. the VRS values reached 29 to 32 units as the fish gave off a definite off-odor. The VRS values increased rapidly when the carp were stored at 25-27°C. and they reached 172 to 173 units when the fish were assigned the score of 3, as may be seen in Table 1. The data in Table 2 indicate that the VRS of catfish stored at 0-4°C. did not increase until the fourth day of storage, and the values reached 10-26 units as the fish started to possess an undesirable odor. When the fish were stored at 25-27°C. the VRS values increased at the same rate as those of carp stored at the same temperatures, and they reached 119 to 134 units when odor became bad. In this study ammonia nitrogen accounted for nearly all the VRS in carp and in catfish. The correlation coefficient between the two was 0.967.

These results agreed with those of Farber (*7*) and Farber and Peter (*8*) who reported that fresh fish contained small amounts of VRS, and the values in decomposed fish varied with the kind of fish. The results, however, contradict the findings of Wittfogel and Bighardt (*29*) who reported that fresh fish was devoid of VRS.

The volatile reducing substances obtained by distillation of mullet muscles reported as milliequivalent reduction per 100 g. of flesh is shown in Table 5. The values increased as the fish became putrid. Using this method, not only were the volatile substances produced during spoilage volatilized, but also substances which were liberated by boiling fresh tissues.

No tyrosine could be detected in freshly killed carp. When carp were stored at 0-4°C. the values reached 8 mg% as the fish became putrid. When the carp were stored at 25-27°C. the tyrosine content reached 11.5 to 25.5 mg% when the fish were assigned the score of 3, as may be seen in Table 1. Extremely low tyrosine content was found in freshly killed catfish and bream as may be seen in Tables 2 and 5, respectively. It seems that the determination of tyrosine was very useful to estimate whether the fish was absolutely fresh. The amount of tyrosine in fish which spoiled at 0-4°C. was lower than that of fish

which spoiled at 25-27°C. The amount of tyrosine found in carp and catfish stored at 25-27°C. varied with the individual fish ($P < 0.01$).

Bradley and Bailey (*2*) reported that determination of tyrosine was not of any value in determining the degree of freshness of fish which were stored at 0°C. Luijpen (*14*) stated that the formation of tyrosine in fish muscles was inhibited by low temperature and by the presence of salt. He also reported that the determination of tyrosine was inadequate for the objective evaluation of the freshness of cod fillets which had been held at temperatures between -1.25 and 4°C. (*15*).

The total volatile acids number (TVA) of mackerel stored at 25-27°C. is shown in Table 4. The values of TVA of fresh mackerel ranged from 13 to 29, and increased as the fish became putrid. The TVA numbers of fresh and spoiled fish varied with the individual fish due to the fact that hydrolysis depends on the type of spoilage organisms predominant on fish during the spoilage process, confirming results reported by Farber (*7*).

Positive INTC tests were observed in freshly killed catfish and bream, as may be seen in Tables 2 and 5. Slight differences in the intensity of the red color of formazan were obtained from the skin of the fish next to the surface of the container and that from the surface of the skin exposed to the air. The authors felt that high INTC values were more closely related to aerobic growth than to spoilage produced by all types of organisms. Moorjani, Iyenjar, Bhatia, and Subrahmanjan (*17*), who used a different tetrazolium salt as indicator, stated that their method was especially useful for estimating the freshness of fish which was heavily contaminated with bacteria.

The determination of TVN and ammonia-nitrogen yielded values within the range of 30 to 33 mg% irrespective of whether the fish were stored at 0-4°C. or at 25-27°C. when the fish became putrid. Catfish acted similarly at both temperatures except that the values were lower (20-30 mg%). Without knowing the temperature at which fish had been stored, the determinations of tyrosine and VRS could not be applied to estimate the degree of freshness of fish. The determination of tyrosine could be used to estimate whether fish were absolutely fresh.

By applying the TVN or ammonia determination combined with VRS determination, one could determine with reasonable certainty whether or not carp or catfish was spoiled and the temperature at which the fish had been stored. By combining the two methods with tyrosine determination further support could be obtained on whether the fish was absolutely fresh. If the temperature at which the fish were stored was known each test (TVN, ammonia-nitrogen, VRS, and tyrosine determinations) could be applied to estimate the freshness of fish.

TABLE 3 — EFFECT OF STORAGE OF MULLET AT 25°-27°C ON ORGANOLEPTIC SCORES AND STEAM VOLATILE REDUCING SUBSTANCES (VRS)

Days of storage	Score	VRS[a]
0	5	2.1-4.4
1	3	6.2-8.2
2	1	8.7-12.3

[a]Results reported as milliequivalent reduction per 100 gram of flesh.

TABLE 4 — EFFECT OF STORAGE OF MACKEREL AT 25-27°C ON ORGANOLEPTIC SCORES AND TOTAL VOLATILE ACID NUMBER (TVA)

Days of storage	Score	TVA[a]
0	5	12.6-28.8
1	3	39.0-82.4
2	1	128.0-209.0

[a]Results reported as ml. of 0.01 NaOH per 100 g. fish flesh

TABLE 5 — EFFECT OF STORAGE OF BREAM AT 25-27°C ON ORGANOLEPTIC SCORES, TETRAZOLIUM REDUCTION TEST, (INTC), TYROSINE, AND VOLATILE REDUCING SUBSTANCES (VRS)

Days of storage	Score	INTC O. D.	Tyrosine mg%	VRS μ eq./5 ml. juice
0	5	0.011-0.017	1.9-2.7	5.8-8.7
1	2	0.130-0.245	39.2-41.1	113.0-132.0

REFERENCES

1. Anderson, D. W., Jr. and Fellers, C. R. The occurrence of trimethylamine and trimethylamine oxide in fresh-water fish. Food research, **17**: 472-474. 1952.

2. Bradley, H. C. and Bailey, B. E. Estimation of decomposition of fish muscle. Food Research, **5**: 487-493. 1949.

3. Castel, C. H. The several characteristics of trimethylamine and its effect on sea fish spoilage. Food in Canada, **9** (4): 44-45. 1949.

4. Connel, J. J. Studies of the protein of fish skeletal muscle 1. Electrophoretic analysis of codling extract of low ionic strength. J. Biochem., **54**: 119-126. 1953.

5. Connel, J. J. Studies of the protein of fish skeletal muscle IV. Ultracentrifugal analysis of codling extract. J. Biochem., **69**: 5-12. 1958.

6. Dyer, W. J., Sigurdson, G. J., and Wood, A. J. A rapid test for determination of spoilage in sea fish. Food Research, **9**: 183-187. 1944.

7. Farber, L. A comparison of various methods for de-

termination of spoilage in fish. Food Technol., **6**: 319-324. 1952.

8. Farber, L. and Peter, A. L. A review of the value of volatile reducing substances for the chemical assessment of the freshness of fish and fishery product. Food Technol., **12**: 677-680. 1958.

9. Hillig F., Shelton, L. R., JrJ. and Loughrey, J. H. Chemical indices of decomposition in haddock. J. Assoc. Offic. Agr. Chemists, **42**: 702-708. 1959.

10. Kurtzman, C. H., Snyder, D. G., and Nilson, H. W. Cystine and total sulfhydryl content of unspoiled and spoiled shrimp. Food Research, **25**: 237-244. 1960.

11. Lang, O. W., Farber, L., Yerman, F., and Beck, C. Determination of spoilage protein foodstuffs with particular reference to fish. Ind. Eng. Chem. (Anal. Ed.), **16**: 490-495. 1944.

12. Lartigue, D., Novak, A. F., and Fiegar, E. A. An evaluation of the indole and trimethylamine tests for oyster quality. Food Technol., **14**: 109-112. 1960.

13. Love, R. M. Post mortem changes in the lenses of fish eyes. Assessment of storage time and fish quality. J. Sci. Food and Agri., **5**: 566-572. 1954.

14. Luijpen, A. F. M. G. Changes in Dutch salted matjesharing during storage and spoilage. Proceedings, Symposium on Cured and Frozen Fish Technology. Swedich Institute for Food Preservation Research. Goteborg, Sweden, November, 1953, pp. 95-102.

15. Luijpen, A. F. M. G. Objective test for fish stored under condition other than normal chilling in ice. J. Sci. Food and Agr., **9**: 410-417. 1958.

16. *Methods of Analysis of the Associatino of Official Agricultural Chemists*. 8th ed. 1955. Washington, D. C.

17. Moorjani, M. N., Iyenjar, J. R., Bhatia, D. S., and Subrahmanjan, V. Use of 2, 3, 5-triphenyl tetrazoliumchloride (TPTZ) for assessing the quality of fish. Food Science (Mysore, India), **6**: 275-276. 1957, *C. F.* Chem. Abstr., **52**: 14020, i. 1958.

18. Ottaway, J. H. A colorimetric method for the estimation of tyrosine. J. Biochem., **68**: 239-244. 1958.

19. Proctor, B. E., Nickerson, J. T. R., Fazzina, T. L., Ronsivalli, L., Smith, R. K. and Stern, J. Rapid determination of the quality of whole, eviscerated haddock. Food Technol., **13**: 224-228. 1959.

20. Ranke, E. and Bramstedt, F. Paper-electrophoretic studies on fish muscle protein. Arch. Fishereiwiss, **5**: 43-63. 1954. *C. f.* Chem. Abstr., **52**: 8410, b. 1958.

21. Reay, G. A. and Shewan, J. M. The spoilage in fish and its preservation by chilling. Adv. Food Research, **11**: 343-398. 1944.

22. Shewan, J. M. and Liston, J. The use of tetrazolium salt for assessing the quality of iced white-fish. J. Sci. Food and Agr., **8**: 222-226. 1957.

23. Sigurdsson, G. J. Comparison of chemical tests of the quality of fish, Ind. Eng. Chem. (Anal. ed.), **19**: 892-902. 1947.

24. Stansby, M. E. and Lemon, J. M. Electrometric study of fish protein. Ind. Eng. Chem. (Anal. Ed.), **5**: 208-211. 1933.

25. Stansby, M. E. Composition of certain species of fresh water fish. Food Research, **19**: 231-234. 1959.

26. Tagawa, S., Yoshino, N., and Miyauchi, K. Electrophoretic studies of the fish meat putrifaction process. I. Determination of ammonia nitrogen. J. Shimonoseki College Fisheries, **4**: 21-24. 1955. *C. f.* Chem. Abstr., **52**: 8408, e. 1958.

27. Tarr. H. L. A. Biochemistry of fish. Annual Review of Biochemistry, **27**: 223-247. 1958.

28. Winton, A. L. and Winton, K. B. *The analyses of Foods*. 2nd. ed. pp. 840-841. 1947. John Wiley & Sons, Inc., New York.

29. Wittfogel, H. and Gibhardt, R. The determination of volatile reducing substances as an aid in the objective estimation of the freshness of sea fish. Arch. Lebenmittel Hyg., **8**: 241244. 1957. *C. f.* Chem. Abstr., **52**: 8410, i. 1958.