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An accelerated microdiffusion method for the determination of ammonia in cartilaginous fish

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NEXT to trimethylamine, ammonia is the most typical spoilage product in fish, molluscs and crustaceans. It is generally determined together with other volatile nitrogen bases, as total volatile basic nitrogen (TVB).

The objective quality determinations of cartilaginous fish such as dogfish and skate, however, raises special problems. These fish contain large quantities of urea (1.5 to 2 per cent.) present in all parts of the body (muscles, intestines, blood, slime, etc.)[1] By the action of a bacterial enzyme, the urease, urea is relatively quickly degraded to ammonia and carbon dioxide.

Apart from this decomposition, ammonia is also formed during spoilage by dissemination of amino acids, either free or split off from proteins, and allied compounds (e.g. creatine), by oxidation of amines and by decomposition of nucleic bases. Hence, the possibility should be considered that the determination of free ammonia gives a better estimation of the freshness than the TVB-test. Furthermore, this latter method is rather difficult to carry out owing to the hydrolysis of urea during analysis, which produces ammonia, interfering with the results.

Broad applications

For the direct determination of free ammonia, different volumetric and colorimetric methods are available. In biological tissues and fluids, however, many interfering substances are generally present, making the preliminary isolation of ammonia a necessity. This can be achieved by ion exchangers or distillation, but in most cases, preference is given to the microdiffusion technique.

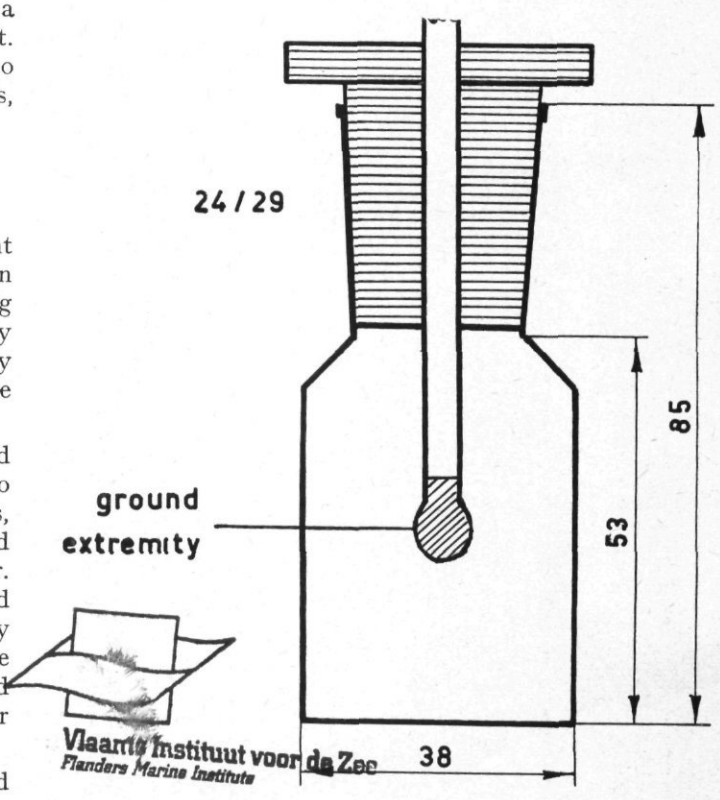
The microdiffusion method of Conway[2] has found broad applications, not only for determining ammonia, but also for a wide variety of other compounds (amines, acids, aldehydes, etc.). This method makes use of so-called Conway units with a concentric inner and outer chamber. Although relatively simple and very sensitive, this method has different disadvantages: the cell is comparatively expensive; the removal of the heavy grease used to make the cell airtight presents a cleaning problem; the method requires several hours; the liquid in the inner chamber must be pipetted out when applying colorimetry.

In order to avoid these difficulties, Seligson and Seligson[3] proposed an accelerated microdiffusion method for determining the free ammonia level in blood. They use penicillin bottles as diffusion cells; the rubber stopper is bored to fit a footed ground glass rod. The sample to be analysed is pipetted into the cell and made alkaline. The extremity of the rod is moistened with sulphuric acid. The cells are then rotated in horizontal position for a short period of time. The solution covers the inner wall completely, increasing the diffusion area considerably and keeping the depth of the layer at a minimal level. Moreover, the solution is constantly agitated.

All these factors considerably shorten the diffusion time. After diffusion, the ammonia is determined colorimetrically with Nessler reagent.

Vyncke and Merlevede[4] have adapted this method to

Fig. 1. Microdiffusion cell (dimensions in mm).



shell fish and demonstrated its usefulness for the objective quality assessment of these species. The method has now been improved and applied to elasmobranch fish.

Reagents. All solutions should be prepared in ammonia-free water. Demineralized water meets this requirement. Normal distilled water should be purified by passing over an ion exchange column (e.g. Amberlite Monobed III or similar resin).

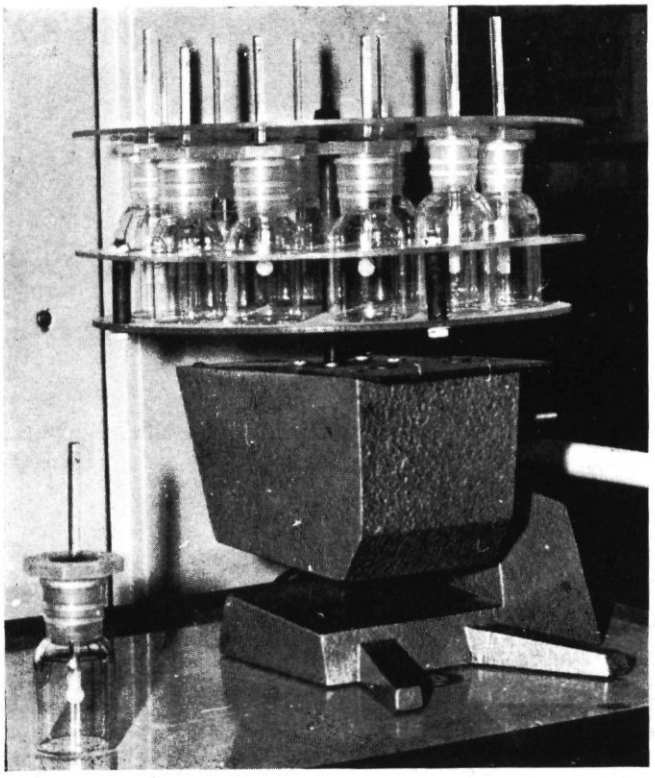
Nessler reagent. The following reagent was found to give maximum sensitivity, reliability and stability. Solution 1: dissolve 40 g red HgI_2 and 30 g potassium iodide in 100 ml water. Solution 2: dissolve 95 g sodium hydroxide in 1.000 ml water. Mix 1 and 2 and let stand for three days. Decant the clear supernatant into a brown flask and store in the dark. Dilute 1:25 before use.

Apparatus. Microdiffusion cells. The penicillin bottles proposed by Seligson and Seligson[3] were replaced after multiple modifications by flasks of 60 ml with conical sockets (24/29) fitted with polyethylene stoppers and footed ground glass rods (Fig. 1). The rod is fixed to the stopper with a household type clear adhesive, which also makes it airtight.

The 24/29 standard socket greatly facilitates the insertion and the removal of the rod without touching the wall. This opening is maximal when taking the small volume of the flask into consideration.

Microdiffusion rotator. The "Multi-Purpose Rotator" model 150 V (Scientific Industries, New York) was tried in the first experiments, but the device with clips for fixing the cells appeared not to be satisfactory. The rotation head was, therefore, replaced by a laboratory-made model consisting of three plexiglass discs of 27 cm diameter (Fig. 2). The first disc serves as a ground plate for the bottles. The second is bored to fit 12 bottles. Both are fixed to the spindle at a distance of 3.5 cm from each other. The third plate is loose and has 12 holes of 1 cm diameter fitting the outer extremities of the glass rods and rests on the polyethylene stoppers. By means of a butterfly-nut turning on the spindle, the upper disc is screwed tightly against the rubber stoppers, thus holding the bottles in position and perfectly sealing them at the same time. The rotator can be turned in both vertical and horizontal position, allowing easy handling of the bottles.

Fig. 2. Microdiffusion rotator with cells in vertical position.



Procedure. Ten g. of previously chopped fish is homogenized for two minutes with 400 ml of demineralized water; 1 ml of the supernatant is pipetted into a microdiffusion flask and 1 ml of a saturated potassium carbonate solution is added. The extremity of the footed glass rod is dipped into 1 N sulphuric acid and the excess shaken off. The stopper with rod is immediately replaced on the bottle.

All cells are now placed vertically into the rotator, turned in horizontal position and rotated for 30 min. at 50 rpm.

The rods, which now carry ammonium sulphate, are removed and dipped into 5 ml Nessler reagent 1:25. The absorbance of the yellow-orange solution is measured at 400 mμ after five minutes; the concentration of ammonia is read from a standard curve determined with solutions of 2.5, 5 and 7.5 μg NH_3-N.

All microdiffusion analyses should be performed in strictly uniform circumstances. Volume of cells, diffusion area and rotation speed can easily be kept constant and are therefore of less importance in practice. Marked variations in room temperature on the other hand should be avoided because rate of diffusion is affected by temperature.[2]

The experimental conditions of the method were fixed in previous experiments.[4] The rotation time, however, was increased to 30 minutes since this time need not be very strictly adhered to. Moreover, small fluctuations in rotation speed are of no importance in this case.[3]

Interfering substances. With ammonia, different other compounds also produce colours with Nessler reagent. In fish, trimethylamine and (in teleost fish) dimethylamine are especially concerned; they are also liberated by alkalies, they diffuse and are bound as sulphate on the rod. Analyses carried out with 10 μg N of both amines, corresponding to 40 mg N per cent. fish, gave no absorbance, indicating that trimethylamine and dimethylamine do not interfere with the determination of ammonia.

The possible hydrolysis of urea was also investigated. Experiments with 500 μg of urea produced no ammonia, which is probably due to the short diffusion (and alkalinisation) time. The method thus allows the determination of ammonia in fish containing high amounts of urea. This should be emphasized since other methods (e.g. distillation) do not always meet this requirement.

Some precautions should be taken before carrying out

free ammonia determinations on dogfish (*Squalus acanthias L*). In the slime and the blood on the skin of the fish, ammonia is easily formed, especially when the fish has been exposed to higher temperatures for some time, such as on deck before stowage in the hold or in the auction hall. In this case, ammonia can easily be detected by smell, but this does not always indicate the fish to be of definitely inferior quality. In the muscles themselves, it is very possible that little or no free ammonia had yet been formed. Before sampling, all fish must thus be thoroughly washed with ice cold water.

In Belgium, as in several other countries, dogfish is usually beheaded and skinned shortly after auction to be sold as "sea-eel". During this operation, much blood is released at the surface of the skinned fish (the red colour being an index of freshness) and ammonia can be formed easily. This skinned fish should also be washed carefully before sampling.

An average spoilage curve for dogfish is shown in Fig. 3. The fish were gutted and stored in ice at 1 deg. C immediately after capture. They were caught in the southern part of the North Sea during the months of October to January. Each dot on the figure represents an average of ca. 30 analyses. The range of the observations is also indicated.

It can be noted that ammonia increases very slowly during the first eight days of storage but rather quickly after that period, to reach values of 75 mg N per cent. after 14 days. The limit of edibility can be set tentatively at about 60 mg N per cent.

Ammonia content correlated fairly well with organoleptic judgment which indicates that the method could be useful for the objective quality assessment of elasmobranch fish. Further experiments, however, are necessary to draw a definite conclusion in this matter.

Finally, it should be emphasized that the method was shown to be very valuable for comparative experiments. It was already used with good results for the study of the influence of different temperatures on the shelf life of dogfish.[5]

Summary

The microdiffusion method described makes use of small diffusion cells with conical polyethylene stoppers fitted with a glass rod and ground extremity, which is moistened with sulphuric acid. A fish extract is pipetted into the flask and made alkaline with potassium carbonate. By rotating the cells in a special microdiffusion rotator, diffusion time is shortened to 30 minutes. Ammonia is determined with Nessler reagent.

There is no interference from urea, dimethylamine, or trimethylamine. The method is very simple and can be used for quality assessment of elasmobranch fish. An average spoilage curve for dogfish (*Squalus acanthias L.*) is given.

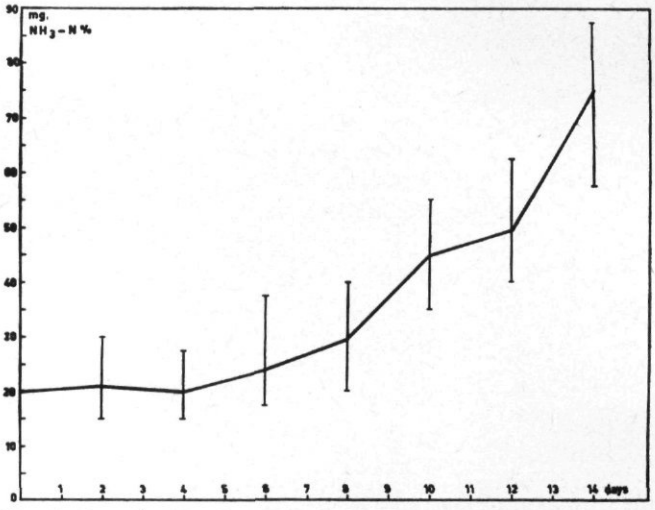


Fig. 3. Average spoilage curve for dogfish ("Squalus acanthias L.") kept at 1 deg. C in ice.

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