The United Nations University
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EVALUATION OF SUITABLE CHEMICAL METHODS FOR SEAFOOD PRODUCTS IN MOZAMBIQUE

Carlos Riquixo
Ministério de Agricultura e Pescas
Direcção Nacional de Pescas
Serviços Provinciais de Administração Pesqueira-Maputo
Tel. 258-1-428194/425678, Fax: 258-1-420335
Maputo- Mozambique

Supervisor: Sigurdur Einarsson,
Icelandic Fisheries Laboratories

ABSTRACT

Evaluation of suitable chemical methods for seafood products were investigated with respect to their accuracy and suitability for determination of additive in seafood products, freshness, nutritional value and scombroid poisoning in fish in Mozambique. Methods were tested using different fish species and deep shrimp at various freshness stages. The samples were from Iceland and were kept in insulated plastic boxes and in the most aseptic manner, some of them for subsequent microbiological, sensorial and chemical analyses. The methods selected were simple, rapid and inexpensive. Volhard titration and Mohr methods were used for salt determination and enzymatic method for sulphite determination. The enzymatic method for sulphite determination was based on the oxidation of sulphite ion to sulphate by oxygen in the presence of sulphite oxidise. Quantitative total volatile basic nitrogen (TVBN) and trimethylamine (TMA) methods were performed. For the TMA the procedure included a deproteinisation of primary and secondary amine using formaldehyde at alkaline pH and finally steam distillation of TMA. TVB and TMA were also performed using Flow Injection Gas Diffusion (FIGD) methodology. Due to unsteady baseline on the chart recorder the TMA procedure needed more time than TVB. The TVB could however be determined successfully. Good correlation was found between following methods: MgO, TCA-extract steam distillation with FIGD for TVB determination and FIGD with Picric acid for TMA determination. Kjeldahl and fluorometric methods were used for protein and histamine determination respectively. The assay of histamine consisted of histamine extraction with methanol, purified by cation-exchange resin and determination by photoflurometer. All analyses were performed with standardised solutions and reagents of purified grade. For each determination different chemical methods were used and the results were compared. It was concluded that the Volhard, Mohr, enzymatic, TCA-extract steam distillation, FIGD and MgO methods were suitable for and applicable to present Mozambican conditions.

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1 INTRODUCTION

Shrimp processing is an important sector in the Mozambican economy. Thus the demand for research and development work in this sector is expected to increase in Mozambique. Knowledge and experienced personnel in marine fish processing are key elements in this connection. A practical overview of different chemical methods to evaluate freshness quality of seafood products especially important. Therefore an evaluation of suitable chemical methods for the determination of additives in seafood, freshness, nutritional value and scombroid poisoning of fish in Mozambique was the subject of this study, which will be of relevance to the national program in the following ways:

1. Increase the confidence in the analytical results;
2. Increase the demand of research;
3. Provide guidelines for a quality assurance system in the chemical laboratory;
4. Provide information for chemists to rationalise laboratory operations;
5. Increase the quality of national fish production for national and international market.

Different chemical methods were investigated and compared with respect to their accuracy and suitability in Mozambique. Although a detailed organoleptic inspection is usually sufficient to determine the freshness of intact refrigerated or processed fish, chemical analyses are indispensable for the assessment of quality. Thus during this study the changes in the levels of TVBN and TMA during fish decomposition and relations between sensory and chemical analyses were investigated.

Most laboratories have determined the sulphite content with (modified) Monier-Williams method. Thus the accurate evaluation of enzymatic method for SO_2 determination was investigated for comparability with a modified Monier-Williams method. Investigations were also done for protein and histamine content. Since the histamine is neither volatile nor destroyed by cooking, a convenient method of detecting it in seafood samples is needed, particularly where decomposition is suspected. Therefore evaluation of histamine content resulting from different handling methods of the sample were investigated, which will be a basis for education on hygiene handling of raw material and proper process control of product. From these reasons, the purpose of this report is to provide important recent information on quality assurance in the laboratory concerning chemical aspects relating to the food industry. The report is organized and written to call attention to recognized practices and procedures. It is designed to provide useful guidelines for chemical analysis and provides information for chemists to rationalize laboratory operations. The report provides specific and sometimes general information on a particular phase of a chemical analysis that is considered essential for quality assurance in the chemical analyse and the phrase “chemical analysis” has been used in this report in the restricted sense of only those methods of analysis which use chemical processes.

I trust that this report will encourage laboratories to embark on or to improve quality assurance programs in the chemical laboratory and that the concepts, and chemical methods developed here will save many hours in assembling and implementing chemical analysis.

2 REVIEW MATERIAL

2.1 Quality assurance of reagents

It is of utmost importance in an analytical laboratory to be able to trust all reagents used for the sample preparation, as well as for other analytical preparation. A stringent quality assurance program for such reagents is necessary. This means that the quality of all inventoried and newly purchased chemicals must be assured. It also means that the integrity of the various solutions prepared and samples gathered for analytical purposes in the laboratory must be maintained.

a) ***Chemicals purchased***

Chemicals purchased and/or stored will probably have one of the following designations (Kenkel 1994):

- **Primary standard-** This is a specifically manufactured analytical reagent of exceptional purity for standardising solutions and preparing reference standards.

- **Reagent (ACS)** – Maximum limits of purity for most commonly used reagents mostly inorganic have been established by the committee on analytical reagents of the American Chemical Society (ACS).

- **CP "Chemically Pure**"- Grades of chemicals are offered by manufacturers. They meet or exceed USP or NF requirements, but are of a lower grade than "Reagent ACS" chemicals.

- **NF "National Formulary"** Chemicals labelled NF meet the requirements of the national formulary. Chemicals ordered with an NF label may not be useful for reagents. It will be necessary to check the national formulary in each case. This designation and the USP (US Pharmacopoeia) designation are now the same.

- **Practical-** These grade designation chemicals of sufficiently high quality to be suitable for use in some synthesis. Organic chemicals of practical grade may contain small amounts of intermediate isomers or homologues.

- **Purified-** This a grade of chemical where care has been exercised by the manufacturer to offer a product that is physically clean and of good quality but not meeting reagents ACS, Reagent, USP, or CP standards.

- **Reagent-**When the ACS has not developed specifications for a specific reagent, the manufacturers establishes its own standards, and the maximum limits of allowable impurities are shown on the labels of these reagents.

- **Technical-**This grade of chemical generally suitable for industrial use. Purity is not specified and is generally determined by on-site analysis.

- ***Spectro grade*** – This is a designation for organic solvents which have been prepared for use in ultraviolet or infrared spectroscopy also conform to Reagent or Reagent ACS standard.

- **HPLC-**This is a designation for organic solvents which have been specifically prepared for use as (High Pressure Liquid Chromatograph) HPLC.

b) The integrity of inventoried chemicals

The integrity of inventoried chemicals or chemicals that have been on the shelf for a period of time can be determined. This determination of integrity, or shelf-life, can plant, various reference sources, such as the Merck index, and/or various laboratory tests to determine purity.

c) *Samples and solutions gathered*

Samples and solutions gathered or prepared by laboratory personnel must also be properly labeled at the same time of sampling or preparation. It means that the label must include the name of the sample or the chemicals or chemicals dissolved and the date the sample was gathered or the solution prepared.

d) Notebook

The laboratory must maintain a notebook of all samplings and preparations, giving details of each sampling or preparation and the references which called for the particular chemicals or techniques used. This would allow tracking the reagent or sample in the event an error is suspected.

2.2 Sampling and sample preparation

The result of chemical analysis involves the manner in which the sample was brought into the laboratory, was obtained and handled and the manner in which the sample was prepared for analysis once it reached the laboratory, as well as what specific analysis was performed from beginning to end.

1. The sample is collected in a manner consistent with the goal of the analysis.
2. The sample, once collected, is handled in a manner so as to protect it in every way from contamination or alteration
3. The sample is brought into laboratory and prepared for the particular technique chosen.
4. The laboratory operation involved for the technique chosen is executed and the appropriate data obtained.
5. The data is worked up in such a way that the final desired result is determined

The first three steps of this scheme are dealt with in this report. These are steps which are common to all analysis, regardless of the technique chosen, and thus are considered here as group, separate from steps 4 and 5. These latter steps are what could be termed “the heart of the analysis” (Kenkel, 1994).

a) Obtaining the sample

Step one

The key word in any analysis is “**representative**”. A laboratory analysis sample must be representative of the whole so that the result of the chemical analysis represents the entire

system that it is intended to represent. If there is variation in the system, small samples must be taken from all suspected locations. If the results for the entire system are to be reported, these small samples are then mixed well to give the final sample to test. In some cases, analysis on the individual samples may be more appropriate.

b) Handling the sample

Step two

There are basically two considerations associated with how to get the sample from the sampling site to the laboratory without contamination or alteration.

1. Storage of the sample in a container which does not leach contaminants to such a degree that would be damaging to the integrity of the sample, particularly if trace amounts of the constituent are to be determined.
2. Preservation of the sample from problems which may be internal, e.g. temperature affects or bacterial affects.

For example, if trace amounts of metals are to be determined, one would not want to store the sample in glass container, since a glass can leach small amounts of metals.

c) Preparing the sample for analysis

Step three

Once steps one and two are completed the sample is in the laboratory ready for the analysis. Most of the time, however, it is still not in a state in which the chosen analytical technique, whatever it is, can be properly applied. The vast majority of all analytical procedures call for the sample to be in the dissolved state- a solution of the sample is what is required most of the time. Sometimes this will mean complete dissolution of the entire sample, while at other times it means only practical dissolution.

2.3 Quality assurance in the data and reported results

The most important aspect of the chemical analysis is to assure that the data and results that are reported are of the maximum possible quality. This means that the analyst must be able to recognize when the testing instrument is breaking down and when a human error is suspected. The analyst must be familiar with error analysis schemes that have been developed and able to use them to the point where confidence and quality are assured. Errors in the analytical laboratory are basically of two types:

- ***Determinate errors***
- ***Indeterminate errors***

a) Determinate errors

Determinate errors also called "***systematic"*** are avoidable blunders that were known to have occurred, or at least were determined later to have occurred, in the procedures. They arise from such avoidable sources as contamination, wrongly calibrated instruments, reagent impurities, instrumental malfunctions, poor sampling techniques, incomplete dissolution of the sample, errors in calculations etc. Sometimes correction factors can adjust for their occurrence; at other times the procedures must be repeated so as to avoid the error.

b) Indeterminate errors

Indeterminate errors, also called "***random***", are impossible to avoid. They are random errors, human errors which were not known to have occurred, or errors inherent in measurements. Such errors are known to occur, but can neither be accounted for directly nor avoided. (Example: problems inherent in the manner in which an instrument operates, errors inherent in reading a meniscus or a meter, errors, such as sample loss, that occur in sample and solution handling, etc).

The indeterminate errors are unavoidable and unknown; they affect the results both positively and negatively and are dealt with by statistics. A given result can be rejected as being too inaccurate based on statistical analysis indicating too great deviation from the established norm. The procedure to check for such errors involves running a series of identical testes on the same sample, using the same instrument or another piece of equipment, over and over. The results that agree within certain predetermined limits are averaged and the average is then considered the correct answer. Any results that fall outside these predetermined limits are ***rejected*** and are not used in the average. The rejectability parameters are the standard, which a given laboratory must determine and adopt for the particular situation.

2.4 Salt (NaCl)

Salt functions as a preservative through its effect on osmotic pressure and the destructive effect of the chloride ion itself on micro-organisms. Gram-negative bacteria are more sensitive to sodium chloride (concentrations of 8%) or less inhibit growth) than gram-positive bacteria, which sometimes grow in saturated solution (Egan *et al.* 1981). Salt can also retard enzymatic activity. The use of salt in high concentration is again limited to a relatively few foods, mainly fish and meat products, in which its presence is not objectionable.

Knowledge of the concentration of salt (sodium chloride) in foodstuffs is very often required. Normally it is sufficient to determine total chloride and express this in terms of sodium chloride. Minor components of foods can also contribute chloride ions but foods which require an analysis for salt are normally those in which salt is an added or significant ingredient.

Titrimetric methods (*Volhard and Mohr***)** are most commonly used. Salt must first be extracted from the food either by careful ashing at 500-550°C (alkali chlorides are relatively volatile at higher temperature) followed by dissolution of the ash, or by boiling of excess silver nitrate and back titration with ammonium thiocyanate (Egan *et al*. 1981) The latter method is generally more accurate and precise

$$Ag^+ + NO_3^- + Na^+ + Cl^- \longrightarrow AgCl + Na^+ + NO_3^-$$

$$Ag^+ + NO_3^- + NH_4^+ + SCN^- \longrightarrow AgSCN + NH_4^+ + NO_3^-$$

The first excess of NH_4SCN that precipitates in the solution reacts with the indicator $FeNH_4(SO_4)_2$ and turns the solution red.

2.5 Metabisulfite

Sulphur dioxide may be used in the form of gas, in solution as sulphurous acid, or as the sulphites of sodium, potassium or calcium, but for the purpose of the regulations the amount present is calculated as sulphur dioxide (SO_2). *Sulphurous acid inhibits the growth of mould, yeast's and aerobic bacteria and also prevents the browning of fruits and vegetables due to enzymatic reactions* (Egan *et al*. 1981). It assists in conserving vitamin C, but make vitamin B inactive by cleavage of the molecule. Sulphur dioxide reacts with the constituents of foods in both reversible and irreversible reactions. When added to food it undergoes association-dissociation reaction in the aqueous phase and equilibrium is set up between SO_2, H_2SO_3, HSO_3^- and SO_3^{2-}. The equilibrium depends both on the pH of the food and on other reactive species present in the food matrix such as free carbonyl groups in sugars or disulphide groups in proteins. The antimicrobial activity of sulphur dioxide is due to undissociated H_2SO_3 (Egan *et al*. 1981).

a) Free and bound sulphur dioxide

It is evident from the foregoing that sulphur dioxide when added to food enters into many reactions in which the reaction products may be stable or unstable. So far as the preservative regulations are concerned, the limit has been held to refer to that amount which is determined in the Monier-Williams method. This method will determine the total sulphur dioxide (free and reversibly combined) although as indicated above it is claimed that preservative power is related to the concentration of undissociated acid. Thus is apparent that the method required for analysis will depend on the problem to be answered. For these reason the literature contains a wide range of analytical methods and manipulation of chemical conditions will permit the determination of free and bound SO_2. Choice of method will also relate to the level to be determined, the physical state of the product and such special factors as the presence of other volatile sulphur compounds or other potential interfering substances in the food. The state of equilibrium in the food will be disturbed during the analysis if the process involves removal of sulphur dioxide, so that analysis designed to determine other than the total amount must be carried out under conditions which minimise the disturbing effects.

Losses of sulphur dioxide are accelerated by contact with air and elevation of temperature. It is preferable to submit unopened samples to the laboratory for analysis. Where this is not practical it is necessary to ensure that subsamples are of sufficient size to fill sample jars. The jars should be closed with an air-tight closure and should if possible be preserved in cool or frozen state (Stadtman e*t al*. 1946).

b) Estimation of total sulphur dioxide by indirect method

Direct methods for total sulphur dioxide, like those for free sulphur dioxide, are subject to interference from colour, other reactive compounds and recombination reactions. The extent of interference is dependent on the nature of the product and the level of sulphur dioxide.

Indirect methods are dependent on the removal of sulphur dioxide by distillation from mineral acid; the choice is usually limited to phosphoric or hydrochloric acid. The gas

evolved may be displaced by a stream of inert gas and collected in absorbing solution. Hydrochloric acid gives a rapid liberation of sulphur dioxide from foods but problems may be encountered with the release of other volatile sulphur compounds which react with iodine and foods containing material such as mustard and onion will give high results. Interference may be prevented by addition of copper acetate prior to the acidification with hydrochloric acid (Egan *et al*. 1981*)*. When phosphoric acid is used, the liberation of sulphur dioxide is slower than with hydrochloric acid, especially in the presence of high amount of sugars.

c) Sulphite contents

Experience shows that the use of sulphite can be said abusive since residuals in edible parts of raw shrimp are greater than 100 mg total SO_2 /100g. Much higher concentration is found in edible parts. Boiling caused a mean reduction of 53.5 +/- 8.5% free sulphite and 33.0 +/- 4.5% total sulphite in edible parts. Boiling the shrimp not cause modifications in the distribution of sulphites between edible and inedible parts, although it did affect the ratio of free and bound sulphite in the inedible parts. The presence of elevated amounts of sulphite in the boiling water was noted (FSTA current 1990-1998/09).

d) Methods

The method required for analysis will depend on the problem to be answered. For this reason the literature contains a wide range of analytical methods and manipulation of chemical conditions permitting the determination of free and bound SO_2. Choice of method will also relate to the level to be determined, the physical state of the product and such special factors as the presence of other volatile sulphur compounds or other potential interfering substances in the food.

A method for determination of total sulphite in shrimp, potatoes, dried pineapple, and a white wine by Flow Injection Analysis (FIA) was collaboratively studied by 8 laboratories. Recoveries of sulphite added to samples averaged 80%. Comparison of FIA with the Monier Williams method indicated comparable results by the 2 methods (FSTA current 1990-1998/09). The enzymatic method is recommended because it is rapid, sensitive, straightforward, and free from interference. The Monier Williams is also recommended since accurate results can be obtained by limiting distillation to 60 min (FSTA, current 1990-1998/09) and it is also time saving and utilises basic laboratory equipment.

2.6 Total volatile bases Nitrogen (TVBN) and Trimethylamine (TMA)

a) Total volatile bases nitrogen (TVB-N)

Total **V**olatile **B**ases **N**itrogen (TVB-N) is one of the most widely used methods today to estimate the degree of decomposition of fish. It includes the measurement of trimethylamine (produced by spoilage bacteria), *dimethylamine* (produced by autolytic enzymes during frozen storage), *ammonia* (produced by the deamination of amino-acids and nucleotide catabolites) and other volatile nitrogenous compounds associated with seafood spoilage (Malle and Poumeyrol, 1989). Although TVB analysis is relatively simple to perform it generally reflects only later stages of advanced spoilage and is

generally considered unreliable for the measurement of spoilage during the first ten days of chilled storage of several species. (Malle and Poumeyrol, 1989). It is particularly useful for the measurement of quality in cephalopods such as squid, industrial fish for meal and silage and crustaceans. However, it should be kept in mind that values do not reflect the mode of spoilage (bacterial or autolytic), and results depend to a great extent on the method of analysis (Malle and Poumeyrol, 1989).

The level of TVBN for white fish is generally considered to be fresh if the TVB is less 20 mg N/100 g sample. If the TVB reaches 30 mg N/100 g most authorities consider the fish to be stale, whilst at level of 40 mg N/100 g the fish is regarded as unfit for consumption. (Egan *et al.* 1981)
The Codex Alimentarius Committee proposed in 1968 the TVB assay by steam distillation (Egan *et al.* 1981).

b) Trimethylamine (TMA)
The total volatile bases developed during the storage of unfrozen fish consist primarily of ammonia and trimethylamine (TMA). The suitability of using TMA content itself as chemical method of evaluating freshness quality of seafood has been investigated extensively. Depending on the species (ground fish, pelagic species, and shellfish), it has been observed to be a useful measure of freshness quality (particularly flavour and odour aspects) of a variety of seafood, but this usefulness depends upon the time of the year and/or the location of catching, stage of spoilage, type of processing, and/or storage, and method of analysis (Malle and Poumeyrol, 1989).

Trimethylamine provides an accurate indication of bacterial spoilage in some species (Malle and Poumeyrol, 1989). It is a pungent volatile amine often associated with the typical (fish odour of spoiling seafood. Its presence in spoiling fish is due to the bacterial reduction of trimethylamine oxide (TMAO) which is naturally present in the living tissue of many marine fish species. Although TMA is believed to be generated by the action of spoilage bacteria, the correlation with bacterial numbers is often not very good. This phenomenon is now thought to be due to the presence of small numbers of "specific spoilage" bacteria which do not always represent large amounts of spoilage-related compounds such as TMA.

One of these specific spoilage organisms, *Photobacterium phosphoreum*, generates approximately 10-100 folds the amount of TMA than that produced from the more commonly-known specific spoiler, *Shewanella putrefaciens* (Dalgaard, 1995) TMA is useful as rapid means of objectively measuring the eating quality of many marine demersal fish. The chief advantages of TMA analysis over the enumeration of bacterial numbers are that TMA determinations can be performed far more quickly and often reflect more accurately the degree of spoilage than do bacterial counts (Malle and Poumeyrol, 1989).

The chief disadvantages of TMA analyses are that they do not reflect the earlier stages of spoilage and are only reliable for certain species. The level of TMA found in fresh fish rejected by sensory panels varies between fish species, but is typically around 10-15 mg/

TMA-N/100 g in aerobically stored fish and at level of 30 mg TMA-N /100 g in packed cod (Malle and Poumeyrol, 1989).

2.7 Relation between sensory and chemical analyses

One of the most important aspects of fish and fish products is freshness. Due to consumer preferences there is a strong tendency to select very fresh fish. Sensory evaluation is the most important method for freshness and quality evaluation in the fish sector and fish inspection services. Sensory evaluation is the scientific discipline used to evoke, measure analyse and interpret reactions to characteristics of food perceived through the senses of sight, smell, taste, touch and hearing. The first sensory changes during storage of fish are those that concern appearance and texture. Odour is one of the most important aspects of sensory analysis to evaluate fish freshness and odour changes during storage of fish**.**

Sensory methods are fast, simple, sensitive and objective, but the method relies on human judgement and proper training of panels. Sometimes sensory tests are also perceived to be inherently subjective (Krzymien and Elias, 1990). A detailed sensory inspection is usually sufficient to determine the freshness of iced fish, frozen, and processed fish, but in the case of dispute, laboratory examinations are indispensable and rapidly provide objective data for the assessment of quality.

Fish decomposition is mainly due to bacterial growth resulting in the production of various volatile substances. Some of theses substances are not normally found in live muscle tissue, while others, which are already present in the muscle, increase logarithmically in parallel with microbial growth (Malle and Poumeyrol, 1989). The assay of some of these substances usually provides useful data for the evaluation of fish freshness or quality. The determination of total volatile bases nitrogen (TVBN) first proposed by Boury in 1935, is widely used today to estimate the degree of decomposition of fish.

Chemical tests usually measure the amounts of breakdown product derived from enzymatic, bacterial or oxidative activity and have trimethylamine, histamine; ammonia and several other breaksdown products can be chemically recorded to evaluate the fish quality. Chemical methods, although precise and objective, require laboratory equipment and must be performed by technically qualified personnel (Krzymien and Elias, 1990).

2.8 Protein

The seas offer a wealth of nutritional resources. They are already a principal source of protein. Good quality proteins are not universally available in sufficient quantities in the form of agricultural products. In view of this, it is not surprising that, in their quest for food scientists and industrialists all over the world have again focused their attention upon the seas as a most potential source of nutrients. The most important function of dietary protein is to supply amino acids either directly or indirectly. These amino acids are then used by an organism to synthesise its own proteins. The well-known fact that

proteins differ in their nutritive values, is due to the variability of amino acid composition.

The complex chemical composition of proteins makes them quite difficult to characterise by simple chemical or physical procedures. However, their component amino acids may be conveniently detected by various specific chemical tests. The standard method for determining nitrogen in inorganic compounds is the Kjeldahl method (Persson, 1995).

a) Kjeldahl method

Until comparatively recent times, the protein content of foods could be estimated from the organic nitrogen by Kjeldahl procedure, Nowadays there are several alternative chemical and physical methods available, some of which have been automated or semi-automated. Although it has been subject to modification over the years, the basic Kjeldahl procedure still maintains its position as the most reliable technique for the determination of organic nitrogen. In consequence it is included in official and statutory methods and approved by international organisations. Furthermore, the results obtained by Kjeldahl are used to calibrate physical and automatic methods (Pearson, 1995).

The Kjeldahl method is based on the wet combustion of the sample by heating with concentrated sulphuric acid in the presence of metallic and other catalysts to reduce of organic nitrogen in the sample to ammonia, which is retained in solution as ammonium sulphate. The digest, having been made alkaline, is distilled or steam distilled to release the ammonia which is trapped and titrated. Mercury, as mercuric oxide is generally agreed to be the most affective catalyst, with selenium almost as effective, but both have toxic hazard and waste disposal problems. Moreover, mercury forms ammonia complexes in the digest requiring the addition of sodium thiosulphate to break the complex and to release the ammonia. The use of a mixture of copper (II) sulphate and titanium dioxide is recommended (Williams 1978). However, Wall and Gehrke (1975) considered this mixture to be at least as effective.

Reduction in the digestion time has also been achieved by addition of potassium or sodium sulphate which raises the digestion temperature. Metal catalysts are conveniently available in tablet form compounded in a potassium sulphate base. The addition of hydrogen peroxide significantly accerates digestion and decrease foaming (Persson, 1995). Traditionally, the ammonia liberated from the digest having been made alkaline is distilled into a standard quantity of dilute acid which is finally titrated with standard alkali to give the organic nitrogen content of the sample. More popular nowadays is to distil into 4 per cent boric acid solution and to titrate the ammonia directly with standard sulphuric acid.

b) Limitation of Kjeldahl digestion

The Kjeldahl method was originally designed for the determination of proteins. Modifications to the original method have increased versatility so that many different sample types can be determined. However, there are still many compounds that are classified as refractory compounds since their nitrogen can not be easy recovered. Refractory compounds are those containing the nitrogen in an oxidised form or as heterocyclic compound For example of such substances are nitrate, nitrite, alkaloids,

pyridine, quinoline derivates, triazoles etc. these substance need to be pre-treated before the Kjeldahl digestion. For example, by adding reducing agents, nitrate can be included in the analysis (Persson, 1995).

c) Sample preparation

*S*ample preparation for Kjeldahl analysis should be carefully performed to avoid errors in the final result. This procedure must involve one or more treatments to homogenise the sample, i. e. the particle size of the sample must be reduced to a size <1 mm. Homogeneity of the method offers the possibility to reproducibility of the method and also offers the possibility to reduce the sample size without sacrificing the quality of the final results. Usually the speed of the digestion will be improved when small particle sizes are used. A good sample blender is necessary for the sample preparation.

The *Cyclotec* mill is generally the most suitable choice for dry products such as grains and feed samples. The *Cemotec* mill offers the possibility of accurate moisture tests on the ground sample, whereas the *Knifetec* mill is suitable for samples with a high moisture and / or fat content.

d) Controller

Most digestions can be done using a constant temperature of the block digestor throughout the whole procedure. Usually 420 °C is used (Persson 1995). However for samples with foam or when large sample volumes are used, a temperature controller can be used to gradually raise the temperature of the block. By slowly increasing the temperature of the block, foaming is decreased by the slower speed of digestion. Larger volumes of liquid can easily be evaporated using this procedure. The exhaust is operated at high flow rate during this evaporation process.

e) Quality control in Kjeldahl procedures

In order to verify the accuracy of the digestion process it is important to have access to a detection procedure that performs with an acceptable recovery. For distillation/titration systems usually recoveries better than 99.5% should be achieved. If the detection system performs with the proper recovery it can be used to control the digestion procedure.

The distillation principle is to convert ammonium (NH_4+) into ammonia (NH_3) by adding alkali (NaOH) and steam distil into a receiver flask containing boric acid with mixed indicators. Titration with standard acid solution using colour sensing titrimetric end-point detection is officially approved for the final determination (Persson 1995). Since all nitrogen in the samples after digestion form ammonium sulphate it can be used as standard to check the recovery of the distilling unit.

2.9 Histamine

Tuna, bonito, mackerel etc. are most frequently incriminated in scombroid poisoning. They have a high concentration of free histidine in their tissues. Bacteria producing histidine decarboxilase convert histidine to histamine and possibly other biogenic amines. *Proteus morganii* appears to be the most active species in producing the scombrotoxin but members of the genera Salmonella, Shigella, Clostridium, and Escherchia are also able to do so (Clark, 1978).

Histamine and perhaps other heat-stable biogenic amines appear to be the responsible agents. The histamine content of toxic fish is usually insufficient, by itself, to cause scombroid poisoning. Usually the fish does not become toxic unless it held for many hours to a few days at room temperature (Clark, 1978). The normal psychrophilic and psychrotolerant micro-organisms do not produce scombrotoxin when growing on refrigerated fish. Toxic canned tuna undoubtedly has become toxic prior to canning, probably as result of inadequate refrigeration on the fishing vessels (Clark, 1978).

The chemical assay of histamine AOAC, 1975 does not measure the other related toxic amines. However, a biological test, namely contraction of an isolated guinea pig intestine, apparently measures the entire scombrotoxin complex (Clark, 1978). In the, current method, pure histamine is the standard used to stablish a response curve, so that scombrotoxin can be measured in *quantitative terms as mg histamine per 100 grams of food* (Clark 1978**).** Since histamine is neither volatile nor destroyed by cooking, a convenient method of detecting in seafood samples is needed, particularly where decomposition is suspected.

A variety of alternative assays exist for histamine in fish products. Most involve chromatography of histamine derivatives using costly instrumentation such as High Pressure Liquid Chromatograph (HPLC) or Gas Chromatograph (GC). The method of the Association of Official Analytical Chemists (AOAC 1990) involves extraction of histamine from sample (tuna) with hot methanol, ion exchange chromatography, derivatization by o-phthaldehyd and fluorometric quantitation (AOAC 1990). This method, while sensitive, yields a colourless product and requires a fluorometer for product detection (Ohashi *et al*. 1994). This fluorometric method using o-phthaldehyd to yield a fluorophore is the most widely used for histamine determination in fish because of its sensitivity (Ohashi *et al*. 1994).

Spoiled scombroid fish containing about 100 mg or more of histamine per 100 g fish muscle is generally toxic to human, but in one recent outbreak, strongly suspected to be scombroid poisoning, no histamine was detected in uneaten remnants of incriminated food (CDC, 1973e) 5-10 mg could be a sign of histamine poisoning; 50-100 mg will always give histamine poisoning and 10 mg is maximal in food (Arnold and Brown, 1978).

3 MATERIAL AND METHODS

Samples of different fish species and deep shrimp at various freshness stages were analysed using different chemical methods. Equipment and chemical reagents were from Icelandic Fisheries laboratory (IFL).

3.1 Comparison of salt determination methods

Different chemical salt determination methods were used and compared to find the best one. The Volhard and the Mohr methods were done based on the precipitation reactions.

These involve principally reactions utilising the highly insoluble nature of silver compounds.

Volhard method for silver

$Ag^+ + SCN^- \longrightarrow$ **AgSCN**

Mohr method for chloride and also the "chloride method" for silver

$Ag^+ + Cl^- \longrightarrow$ **AgCl**

The chloride titration method was based on the quantab unit's scale, which can be ready through the peak of the yellow/white colour. Each quantab unit means certain salt concentration, which can be seen in Appendix 4 (scale in quantab units).

a) Preparation of standard solution of silver nitrate

It was dissolved slightly more than theoretical wt of Ag NO_3 (equivalent wt, 169.87 g) in halogen-free H_2O and diluted. To volume. Thoroughly clean glassware, avoiding contact with dust. The solution was kept in amber g-s bottles away from light.

1- Titration's reagent

Potassium chloride-recrystallis KCl three times from H_2O, dried at 110°C, and then heated at ca 500 °C const wt Equiv. wt KCl=74.555.

-Potassium chromate solution *(as indicator)*-5 % solution of K_2CrO_4 in H_2O

-Saturated ferric ammonium sulphate $FeNH_4 (SO_4)_2$.

2- Standardisation

It was accurately weighed KCl enough to yield titration of ca 40 ml (ca 0.3000 g for 0,1 N solution) and transferred to 250 ml glass Erlenmeyer flash with 40 ml distilled water. Added 1 ml K_2CrO_4 5 % solution and titr. With $AgNO_3$ solution until first perceptible pale red-brown appears.

The amount of $AgNO_3$ solution (in ml) required to produce and print colour was subtracted from the titration volume. Then the normality of $AgNO_3$ was determined.

$$\text{Normality} = \frac{\text{g KCl} \times 1000}{\text{Net Vol } AgNO_3 \times 74.555}$$

3- Calculation

- Mass of KCl I: 0.3002
  II: 0.3002
- Titration volume of $AgNO_3$ I: 40.25 ml
  II: 40.25 ml
- Normality of $AgNO_3$

$$\text{Normality} = \frac{\text{g KCl} \times 1000}{\text{Vol } AgNO_3 \times 74.555}$$

$$N = \frac{0.3002\ g \times 1000}{40.25\ ml \times 74.555} = 0.100036\ N$$

Mass I = mass II and Vol I = Vol II means NmI = NmII = 0.100036 = 0.1000 N

b) Preparation of standard solution of ammonium thiocyanate

Ca 0.1 N solution was prepared from reagent that shows no Cl, using 461.3g NH_4SCN with 100% purity for 6 litre volume.

1-Titration's reagents

-$AgNO_3$ 0.1 N

-$FeNH_4(SO_4)_2$ *(as indicator)*

2-Standardisation

1 ml of $FeNH_4(SO_4)_2$ was added to 10 ml 0.1 N $AgNO_3$ from 4.1.1.2 a) and titrated with NH_4SCN.

3-Calculation

MM NH_4SCN = 76.12 G

Purity = 99%

Volume needed = 6 litre

Concentration needed = 0.1000

- Purity mass of NH_4SCN necessary for 6 litter 0.,1 N

76, 12 g /mol X 6 = 456.72

456.72 – 99 %

X - 100 %

X = 461.3 g (mass necessary for 6 litre 1 N NH_4SCN

For 6 litres 0.1 N is needed 46.13 g of NH_4SCN

Because it was very good to have slightly more than theoretical wt, it had to be weight 46.3 g of NH_4SCN

- Accurate concentration of NH_4SCN **I**

-Volume of $AgNO_3$ = 10 ml

-Accurate concentration of $AgNO_3$ = 0.1000 N

-Titration's volume of NH_4SCN = 9.9 ml

-Accurate concentration =?

$C_1 . V_1 = C_2 . V_2$

0.1000 x 10 ml *($AgNO_3$)* = C_2 x 9.9 ml (NH_4SCN)

C_2 (NH_4SCN) = 0.1010

- To have concentration of 0.1000 N NH_4SCN it was necessary to add some ml of water. *Volume of water to add:*

C_1 x V_1 (NH_4SCN) = C_2 x V_2 (NH_4SCH)

0.1010 x 5975 ml (NH_4SCN) = 0.1000 x V_2 (*5975 ml because from 6 litre NH_4SCN. 25 ml was used for titration)*

V_2 = 6034.75
Volume to add = $V_2 - V_1$ x 93 % = 6034.75 –5975 = 59.56 ml x 0.93 = 55.56 ml
55 ml. were needed.

- Accurate concentration of NH_4SCN **II**

-Volume of $AgNO_3$ = 10 ml
-Accurate concentration of $AgNO_3$ = 0.1000 N
-Titration's volume of NH_4SCN = 10 ml
-Accurate concentration =?
$C_1 \cdot V_1 = C_2 \cdot V_2$
0.1000 x 10 ml *($AgNO_3$)* = C_2 x 10 ml (NH_4SCN)
C_2 (NH_4SCN) = 0.1000

3.1.1 Volhard method

The Volhard method was done in two variations:

- Shaking the sample
- Boiling the sample

Both variations were used.

1-Shaking the sample

a) Reagents
-0.1 N $AgNO_3$ std solution
-0.1 N NH_4SCN std solution
-$FeNH_4 (SO_4)_2$ (indicator)

b) Equipment
-Erlenmeyer flasks
-Burette (graduation 0.1ml)
-Shaker machine

c) Procedure
200 ml distilled H_2O added to 5.0 g sample and shaked for 45 minutes. 20 ml from shaked solution was added to 5 ml 0.1 N $AgNO_3$ std. soln. And 1 ml $FeNH_4(SO_4)_2$ in a 100 ml Erlenmeyer flask. Titrated with 0.1 N NH_4SCN std. solution until the solution becomes permanent light brown. Then ml 0.1 N NH_4SCN std. solution used was subtracted from ml 0.1 N $AgNO_3$ std. solution added and difference determined as NaCl. The concentration of NaCl% was determined using calculation *1* and the results tabulated in Table 1.

1 ml 0.1 M $AgNO_3$ = 0.005844 g NaCl

Calculation 1

$$\text{NaCl \%} = \frac{(\text{ml } AgNO_3 - \text{ml } NH_4SCN) \times 0.1 \text{ N } AgNO_3 \times 58.45 \text{ g /mol NaCl} \times 200 \times 100}{\text{Sample (g)} \times 1000 \text{ ml/L} \times 20}$$

2-Boiling the sample

a) Reagents

-0.1 N $AgNO_3$ std. solution
-0.1 N NH_4SCN std. solution
-$FENH_4(SO_4)_2$ (indicator)
-HNO_3 68%

b) Equipment

-Erlenmeyer flasks
-Burette (graduation 0.1ml)
-Hot plate
-Boiling flat flask

c) Procedure

Into a boiling flat flask 0,1 N $AgNO_3$ std soln, 1 g sample, 25 ml H_2O and 20 ml HNO_3 68% (concentrate) were added. First it was gently boiled on hot plate (it can be done on sand bath) until all solids except AgCl dissolve (usually 15 min). Secondly it was cooled, 50 ml H_2O and 1 ml indicator $FeNH_4(SO_4)_2$ added and titrated with 0.1 N NH_4SCN std .soln. until the solution. becomes permanently light brown. Finally ml 0.1 N NH_4SCN std. soln. used was subtracted from ml 0.1 N $AgNO_3$ std. soln. added and difference determined as NaCl. Concentration of Sodium chloride (NaCl%) was determined using *calculation 2.*

Calculation 2

$$\text{NaCl \%} = \frac{(\text{ml } AgNO_3 - \text{ml } NH_4SCN) \times 0.1 \text{ N } AgNO_3 \times 58.45 \text{ g NaCl /mol} \times 100}{\text{Sample (g)} \times 1000 \text{ ml/L}}$$

The accuracy of the Volhard method was checked by following procedure***:***

-Checking the reagents

Into 100 ml Erlenmeyer flask was added 1 ml of $FeNH_4(SO_4)_2$ –indicator to 10 ml 0.1 N $AgNO_3$ std. solution titrated with 0.1 N NH_4SCN std. soln. The volume of the NH_4SCN 0.1 N had to be equal with the volume of the 0.1 N $AgNO_3$ std. solution.

-*Checking the Volhard method*

1%, 3% and 5% NaCl 99.5% pure was put into 3 flat solution flasks. Three new 100 ml solutions using 1 ml from the first solutions (1%, 3% and 5%) respectively were prepared and added 5 ml 0.1 N $AgNO_3$ std. soln., 1 ml $FeNH_4(SO_4)_2$ to 20 ml from each new solution and titrated with 0.1 N NH_4SCN std. soln. Finally the ml 0.1 N NH_4SCN std. soln. used were subtracted from ml 0.1 N $AgNO_3$ std. soln. added and difference determined as NaCl. NaCl % was determined using *calculation 3* and the results are shown in Table 3.

Calculation 3

$$\text{NaCl \%} = \frac{(\text{ml } AgNO_3 - \text{ml } NH_4SCN) \times 0.1 \text{ N } AgNO_3 \times 58.45 \text{ g NaCl /mol} \times 100 \times 100}{1000 \text{ ml/L} \times 20 \text{ ml}}$$

3.1.2 Mohr method

1%, 3% and 5% NaCl 99.5% pure was put into 3 flat solution flasks respectively. Three new 100 ml solutions were prepared using 1 ml from the first solutions (1%, 3% and 5%). Add 1 ml K_2CrO_4 5% to 20 ml from each new solution and titrated with 0.1 N $AgNO_3$ std. soln. NaCl% was determined using *calculation 4* and the results shown in Table 4.

1 ml 0.1 M $AgNO_3$ = 0.005 844 g NaCl

Calculation 4

$$\text{NaCl \%} = \frac{\text{ml } AgNO_3 \times 0.1 \text{ N } AgNO_3 \times 58.45 \text{ g /mol} AgNO_3 \times 100 \times 100}{1000 \text{ ml/L} \times 20 \text{ ml}}$$

3.1.3 Chloride titrator method

a) Preparation of the sample

From 1% solution of 1% NaCl 99.5% purity, was new 5 solutions prepared with following concentrations: 50 ml of NaCl 100, 25 ml/100, 10 ml/ 100, 5 ml/100 and 1 ml /100 ml. The concentration of NaCl % was determined by chloride titrator.

b) Procedure

First a titrator was removed from the bottle and replaced immediately. The lower end of the titrator was then lowered into solution. (Do not allow solution to reach yellow completion string at top of titrator). Then the solution was allowed to saturate the titrator and turn the yellow completion string dark blue. Note where the tip of the yellow /white peak on reacted titrator falls on the numbered scale in Quantab units. Finally using the quantab table (Appendix 4) the salt concentration was determined. Filtration of the sample solution had to be made to prevent obstruction of the titrator. The results are shown in Table 5.

3.2 Evaluation of enzymatic method for SO_2 determination

The determination of sulphur dioxide was done taking two samples (*sodium sulphite and sodium metabisulfite*) with known concentration were analysed using an enzymatic method. The experimental and expected results were compared and the accuracy of the method assessed. The method is based to the fact that sulphite (sulphurous acid) is oxidised by sulphite oxidase (SO_2-OD) to sulphate in the presence of oxygen.

$$SO_2^{--} + O_2 + H_2O \xrightarrow{SO_2\text{-OD}} SO_4^{++} + H_2O_2 \quad (1)$$

The hydrogen peroxide formed in this reaction is reduced by the enzyme NADH-peroxidase (NADH-POD) in the presence of reduced nicotinamide-adenine dinucleotide (NADH).

$$H_2O_2 + NADH + H^+ \xrightarrow{\text{NADH-POD}} 2H_2O + NAD^+ \quad (2)$$

The amount of NADH oxidised in reaction 2 is equivalent to the amount of sulphite or to aldehyde chemically-bound sulphite. NADH is determined by means of its light absorbance at 334, 340 or 365 nm.

a) Reagents

1. Bottle 1 with approx. 30 ml solution, consisting of triethanolamine buffer, pH approx. 8.0; stabilisers.
2. Bottle 2 with approx. 30 tablets, each tablet contains NADH, approx.0.4 mg; stabilisers.
3. Bottle 3 with approx. 0.3 ml suspension, consisting of NADH-POD, approx. 3U; stabilisers.
4. Bottle 4 with approx. 1.6 ml suspension, consisting of: SO_2-OD, stabilisers.

b) Preparation of solutions

1. The content of bottle 1 was used undiluted
2. For each assay was one tablet of bottle 2 dissolved with 1 ml solution of bottle 1 in centrifuge reagent tube (blank and samples) depending on number of determinations. Forceps were used to take the tablets out of the bottle 2. This results in reaction mixture 2.
3. The contents of bottle 3 and 4 were applied undiluted.

c) Stability of reagents

- Solution 1, the content of the bottle 2, 3 and 4 were stable at +4 °C (seen on the package label)
- Solution 1 and mixture 2 were brought to 20-25°c before use.
- Reaction mixture 2 is stable for one week at +4°C

Only freshly distilled water was used for the assay or treat demineralised water with activated charcoal (1g/100 ml): charcoal was mixed into water while stirring and filtered after 3 min.

d) Procedure

4 cuvettes were taken containing the blank, the sample of sodium sulphite and sample of metabisulfite and performed as indicated by the table below:

Pipette into cuvettes	Blank	Sample
Reaction mixture 2	1.000 ml	1.000 ml
Sample solution	-	0.100 ml
Redistilled water	2.000 ml	1.900 ml
Suspension 3	0.010 ml	0.010 ml
The mixture was mixed and the absorbances of the solutions read after 5 min (A1) using spectrophotometer with wavelenght of 340 nm . Start the reaction by addition of :		
Suspension 4	0.050 ml	0.050 ml
Mixture was wait for the completion of the reaction 30 min. The absorbance of the solution (A2) read. If the reaction has not stopped after 30 min, continue to read the absorbance at 5 min intervals until the absorbance decreases constantly over 5 min.		

Both difference of the blank and sample was determined (A_1-A_2). The absorbance difference of the blank from the absorbance difference of the sample

$\Delta A = (A_1\text{-}A_2)$ sample $-(A_1\text{-}A_2)$blank

The measured absorbance differences should, as a rule, be at least 0.100 absorbance units to achieve sufficiently accurate results. Occasionally negative value with (A1-A2)blank is obtained. This value is then to be added to (A_1-A_2) sample according to the calculation formula. If the absorbance difference of the sample (ΔAsample) is higher than 1,000 (measured at 340 nm, or Hg 334 nm, respectively) or 0.500 (measured at Hg 365), the concentration of sulphite in the sample solution is too high. The sample is to be diluted according to the dilution table in that case.

SO_2 g/g was determined using *calculation 5* and the results are shown in Table 6.

Calculation 5

$$C = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A \text{ (g/L)}$$

V= final volume (ml)
v = sample volume (ml)
MW = Molecular weight of the substance to be assayed (g/mol)
d = light path (cm)
ε = Extinction coefficient of NADH at
340 nm = 6.3 (l x $mmol^{-1}$ x cm^{-1})
Hg 365 nm = 3.4 (l x $mmol^{-1}$ x cm^{-1})
Hg 334 nm = 6.18 (l x $mmol^{-1}$ x cm^{-1})
for sulphite (SO_2)

Calculation 5

$$C = \frac{3.060 \times 64.06}{\varepsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A = \frac{1.960}{\varepsilon} \times \Delta A \text{ (gSO2/l sample solution)}$$

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor F.

3.3 Evaluation of TVB and TMA methods monitoring the changes of fish fillets of herring and shrimp kept at 0°C and 5°C

Samples of haddock were used for comparison of TVB and TMA levels in the same stage of decomposition. Samples of herring and shrimp stored at 0 and 5°C for up 13 days for herring and 10 days for shrimp were also used. Different chemical methods were used for evaluation of the changes in the levels of TVB and TMA during decomposition as well evaluation of suitable methods for freshness determination. Methods used for the TVB and TMA determination were following:

1- TVB-N- Determination of Total Volatile Bases Nitrogen (TVB-N) by steam distillation of Trichloroacetic Acid (TCA) extract (modified method of Malle and TAO 1987)

2- TVB-N –Determination of TVB-N with the Struer automatic distillation – using MgO (N. Antonacopulus, 1968)

3-TVB using FIGD-Flow Injection Gas Diffusion (IFL-Icelandic Fisheries Laboratory-1998)*

4-TMA- Determination of TMA by steam distillation of Trichloroacetic Acid (TCA) extract (Malle and Poumeyrol, 1989)

5-FIGD-Flow Injection Gas Diffusion (IFL-Icelandic Fisheries Laboratory-1998)*

6-PAC-Picric acid method- Trimethylamine nitrogen in seafood (AOAC 1990)

* For the FIGD-method is being researched by Icelandic Fisheries Laboratory (IFL) Iceland; University of Hull International Institute (HIFI) U.K.; Technological Educational Institution (TEI) of Athens, Greece; Institute Portuguese da Investigação das Pescas e do Mar (IPIMAR), Lisboa, Portugal; and Departamento de Nutrició I Bromatologia, Facultat de Farmacia, Universitat de Barcelona (UBNB-Barcelona), Barcelona, Spanin.

3.3.1 Comparison of TVB and TMA levels of Fish in the same stage of decomposition

Using FIGD method three different samples of haddock stored in the refrigerator for one week were used for TVB and TMA determination. Comparison was done between the TVB and TMA levels and the results are shown in Tables 7 and 8.

a) Apparatus and reagents

1- Apparatus

-Ismatec (Reglo-Digital MS-4/8-100) peristaltic pump with variable flow rate (1-100 rpm) 4 channels and 8 rollers
-Detector
-Chart recorder
-Gas permeable membrane
-Reodyne 5020 low pressure injection valve

2- Reagents

-NaOH (0.1 N)
-Formaldehyde 20% (v/v)
-Bromothymol blue water soluble (BTB) 0.3 g/L
-Trichloracetic acid (TCA) 7.5%
Trimethylamine hydrochloride, stock solution (0.05 M) in TCA 7.5%

b) Preparation of ammonium chloride and TMA-HCl as standard solutions

The standard solutions were prepared by weighing 23, 9 mg TMA-HCl into 500 ml of trichloracetic acid (TCA) to give 0,5 mM TMA-HCl stock solution. 50 ml from the solution were loaded into the distillation tube followed by 30 ml of 10% NaOH. Steam entrainment was performed using Struer automatic distillation. A beaker containing 15 ml of 0.27% boric acid and 2 drops Mishindicator (Methyl red and bromocresol green) was placed under the condenser for the titration of TMA. Complete neutralisation was obtained when the colour turned pink on the addition of a further drop of sulphuric acid 0.03 N (when the colour was too green). Distillation was started and steam entrainment continued for 12 min. The concentration of TMA in N was determined from the titration volume of 0.03 N sulphuric acids (ml) in the following calculation:

$C_1 \times V_1 = C_2 \times V_2$

Where
C_1 Concentration of sulphuric acid = 0.03 N
V_1 Volume of sulphuric acid = 13.5
C_2 Concentration of TMA-HCl = ?
V_2 volume of TMA-HCl = 50 ml

$C_1 \times V_1 = C_2 \times V_2$
$C_2 = C_1 \times V_1 / V_2 = 0.03 \times 13,5 / 50 = 0.0008$ N

The procedure of ammonium chloride as standard solution was performed as followed for TMA-HCl by weighing 14.6 g NH_4Cl.

1- Peak height
From the obtained standard solutions new solutions were prepared with following concentrations: 50 μ M/L, 100 μ M/L 150 μM/L 200 μM/L and peak height was determined .

c) TVB and TMA determination of haddock by FIGD method
Fish extracts for TMA and TVBN determination were prepared by blending 100 g samples of macerated flesh with 200 ml of 7.5 % trichloracetic acid (TCA, Merck 80%) for 2 min. in Waring blender at high speed and filtered to produce a clear solution. H2 from the clear solution. New dilutions were prepared (50x, 20x and 10x). Some ml of the diluted solution were introduced through peristaltic pump of 0.8 mm using in 3 others teflon tubing of 0.6 mm 1 N NaOH, 20% (v/v) formaldehyde and 0.3 g/l bromothymol blue(BTB) Merck 3026. The peaks were made by detector and into Chart recorder recorded at speed of 1 mm /min. The TMA (mg) was determined from the peak of the solution through ***calculation 6*** and results are shown in tables 7 and 8.

Calculation 6

$$\text{TMA (mg)} = \frac{\text{(Peak of the sample – peak of the blank) (dilution of the sample)x 14 g/mol x 100}}{\text{Peak height x 1000 x 357.14}}$$

357.14 means
50 g of the fish with a 80% moisture content in 100 ml TCA
The peak height is from standard solution of Ammonium chloride for TVB and Trimethylamine-hidrochloride for TMA .

3.3.2 TVB assay of herring and shrimp by TCA-extract steam distillation method

200 ml of 7.5% aqueous TCA solution was added to 100g of fish muscle and shrimp; after homogenisation, the mixture was filtered using Whatman filter paper. 25 ml of filtrate were loaded into the distillation tube followed by 6 ml of 10% NaOH. Steam entrainment was performed using a Struer automatic distillation.

A beaker containing 15 ml of 4% boric acid and 0.04 ml of methylred and bromocresol green indicator was placed under the condenser for the titration of TVBN. Distillation was started and steam entrainment continued until a final volume of 100 ml obtained in the beaker. The results of TVB was obtained with *calculation 7* and the results are shown in tables 9 and 10.

Calculation 7

$$\text{TVB mg- N/100g} = \frac{\text{(ml) (0.055 mol/l) (14 N g /mol) 100}}{8.333}$$

3.3.3 TVB assay of herring and shrimp by FIGD method

The procedure for TVB assay of herring and shrimp as well the calculations were performed as in 3.3.1 c) and *calculation 6.* The results are shown in tables 9 and 10.

3.3.4 TVB-N assay of shrimp by the Struer automatic distillation unit
(Antonacopulus, N. 1968) – MgO method

Freshness evaluation of whole shrimp was performed, using the TCA-extract for TVB and TMA assay; Picric acid method for TMA; FIGD and MgO for TVB assay methods. The procedure of TCA-extract and FIGD methods were performed as for herring. (point 3.3.2 and 3.3.3) The MgO was based on the method of Antonacopulus, N. 1968 using Struer automatic distillation.

a) Reagents:
0.05 N H2O4
0.027 % boric acid
Mischindikator 5, pH 4.4-5.8 from Merck Art. 6130
MgO

b) Procedure*:*
10.0 g sample was placed in a distilling flask, 3 g MgO and 100 ml H_2O added. TVB-N was distilled at rate of 10 ml/min for 12 minutes in Struer automatic distillation unit, which has been pre-heated. The distillate was collected in 100 ml of 0.27 boric acid containing 8 drops of mishindicator and titrated with 0.050 N H_2SO_4 simultaneously during the distillation to the equivalence point (a light grey colour). The mg of TVB-N is determined using calculation 8 and the results are shown in Table 10.

Calculation 8
mg –N/100g = volume of titration X Normal of acid X 14 X 10

3.3.5 TMA assay of herring by TCA-extract steam distillation

To assay TMA using TCA-extract by steam distillation, the processed as for TVB (3.3.2) The only difference was the addition of 20 ml 35% of formaldehyde to the distillation tube to block the primary and secondary amines.

- The boric acid solution turned green when alkalinised by the distilled TVBN which was titrated with aqueous 0,03 N sulphuric acid solution using 0,01 ml graduated microburette.
- It had to be added 1 drop of 0,03 N H_2SO_4 to confirm that it had reached the equivalence point. (adding 1 drop of 0,03 N H_2SO_4 the colour changes from grey to red)
- The quantity of TMA in mg was determined from the volume of sulphuric acid (ml)
- The results of TMA was obtained as followed in the *calculation 7* and the results were in the table 10 and 11 established.

3.3.6 TMA assay of herring by FIGD method

The procedure for TMA assay was taken as TVB- procedure occurred (point 3.3.3) The only difference between the TVB determination by -FIGD method and TMA determination by FIGD was in the calculation, where the peak height must be of TMA standard solution (TMA-HCl) while the peak height of TVB was Ammonium chloride. Thus the TMA (mg-N/100g) and TVB (mg-N/100g) are determined from the peaks of the solutions as followed in the *Calculation 6.* The results were shown in Table 11.

3.3.7 TMA assay of herring and shrimp by picric acid method

a) Reagents

1-Trichloracetic acid soln. –7.5% aq. Soln

2-Toluene-Dried over anhyd. Na_2SO_4. To remove interference, was shacked 500 ml toluene with 100 ml 1 N H_2SO_4, distil, and dry with anhyd. Na_2SO_2

3-Picric acid solns- 1 (stock soln) –dissolve 2 picric acid in 100 ml H_2O-free toluene

-2 (working soln)-Dil 1 ml stock soln to 100 ml with H_2O-free toluene

4-Potassium carbonate cleaned

5-Formaldehyde-20%.

6-Indicator soln-1 methyl red-methylene blue- It was mixed 2 parts 0.2% alc. Me red soln with 1 part 0.2% alc. Methylene blue soln; or 2 methyl red-bromocresol green soln-Mix 1 part 0.2% alc. Me red soln. with 5 parts 0.2 alc. Bromocresol green soln.

7-Trimethylamine (TMA) standard solns-1 (stock soln- Add 0.682 g $(CH_3)_3N.HCl$ to 1 ml HCl (1 +3) and dil. to 100 ml with H_2O. Check basic N content of 5 ml aliquots by adding 6 ml 10% NaOH son, distg into 10 4% boric acid in micro-Kjeldahl distn app., . and titrg with 0.1 N H_2SO_4, using indicator, (f), This soln is stable. *2-Working soln*-0.01 mg TMA-N/ml. It was added 1 ml stock soln to 1 ml HCl (1 +3) and dil. to 100 ml with H_2O.

b) Apparatus

Spectophotometer

c) Preparation of sample

It was weight 100 m g minced, well mixed sample and added 200 ml 7.5% Trichlor-acetic acid and blended 1 min and the blended soln filtrated to receive clear solution.

d) Procedure

Into reagent glass was added 1 ml sample, 3 ml dist. H_2O, 1 ml 20% formaldehyde, 3 ml 45% KOH and 10 ml clean toluen. The reaction glass was shacked 10 times strongly. 7-8 ml of over side liquid containing toluene and TMA- extract was decanted using pump pipette and introduced to other glass containing 0.3g of dry Na_2SO_4 . The mixture was shacked in the shaker machine. 4 ml of the solution was put into new reagent glass containing 4 ml of picric acid 0.2%. The colour turned yellow and more yellow when the TMA content increased. The TMA mg-N/100g was determined using following calculation 8 and the results were shown in Table 12.

Calculation 8

1) TMA mg-N /ml standard solution = Concentration of standard x volume of standard
Absorbance of standard (AB)

2) TMA mg-N/ 100 g sample =(Absorbance of the sample – Absorb of blank) x 3 x TMA mg-N /100 standard x dilution of sample

3.3.8 Relation between sensory and chemical analyses

10 samples of cod stored at 0°C for up to 15 days were investigated. The TVB and TMA were determined in each sample and the results were given in the table 15.

a) Samples
The Cod was caught in North Atlantic Ocean in Iceland. The samples were brought to the Marine Research Institute in insulated plastic boxes and in the most aseptic manner for subsequent chemical, microbiological and sensory analyses.

b) Sensory examination
The sensory evaluation was conducted in accordance with EEC freshness rating system and the attributes were included: rancid odors, general appearance of the mucus, skin, eyes, gills, odour of the gills and condition of the flesh. For cooked fish, fillets were steamed at 80°C for five minutes and the eating quality was assessed using the necessary scoring scale (10-1).

c) Chemical analyses
The freshness of the fish samples were evaluated by measuring the levels of TVBN using TCA-extract , MgO and FIGD methods and TMA using TCA-extract method. The procedure all these methods were performed as in the point 3.3.2, 3.3.3 and 3.3.5 and the results of sensory and chemical analyses were in the table 14 ordered.

3.4 Evaluation of protein content and digestions time

Samples of capelin, cod, acetanilide+sucrose and sucrose were taken to determine the protein content and digestions time using Kjeldahl method.

a) Verifying the distillation system
It was weight 0.15 g ammonium sulphate (*(NH4)2SO4 99.5% purity Mol weight = 132.14 g/mol; % Nitrogen in ammonium sulphate 99.5 % = 21.09%)* into distillation tube, then added 100 ml distilled water and 50 ml 40% NaOH and distillation was performed. The recovery was determined with following *calculation 9.*

Calculation 9
% Nitrogen = (ml –blank) N x 1.401)
g sample
N = Normality of titrant to 4 places of decimal

$$\% \text{ Recovery} = \frac{\text{Actual \% Nitrogen}}{21.09} \times 100$$

b) procedure

The block digestor was pre-heated 40 min before it was required to 370-420°C. The weighed homogenised sample was transferred on a filter paper to a digestion tube, taking an amount calculated to provide a titration of about 25 ml of 0.05 M sulphuric acid, or a maximum weight of 2 g. 2 catalysts tablet were added (5 g $K2S2O4$, =,15 g $CuSO4$, $5H2O$, 0.15 f $TIO2$-Kjeltab CT tablets, Thompson and Capper Ltd, Liverpool). Then 20 ml digestion acid (95% concentrated sulphuric acid,) was added and mixed. Left for a few minutes. The tube in the heated digestor was placed and attached the venting tube which removes the acid vapours. Allowing digestion to proceed for three hours. The tube from the block digestor were removed and allowed to cool for 60 min in the metal rack. 75 ml distilled water were added and mixed. 50 ml boric acid solution 4% ((0,125 g methyl red and 0.082g methylene blue per 100 ml ethanol) in titration receiver flask were placed and placed on the start button to effect a metered addition of sodium hydroxide solution and to initiate the steam distillation. The distillate was titrated with 0.05 M sulphuric acid solution to grey end point. Carry out a blank determination. The protein content were determined in the below *calculation 10* and the results were shown in Table 15.

Calculation 10

$$\% = \frac{(0.1062 \text{ mol/L}) (14 \text{ g/mol}) (100) (\text{ml titrated}) - (\text{ml blank}) \times 6.25}{(\text{weight of the sample}) \times 1000 \text{ ml/L}}$$

3.5 Evaluation of histamine content by different fish handling methods

Histamine was determined in samples of salted herring and smoked salmon. 1 sample of herring was stored at 0°C for one day, the second was stored at 0°C for one day and left at room temperature during the night, the third was stored in the refrigerator during two weeks and the sample 4 was smoked salmon from Icelandic producer was rejected by Italian buyer due to too high histamine level. All plastic and glass containers were rinsed with HCl (1+3) and water before use.

a) Apparatus

-Photofluorometer
-Chromatographic tube
-Repipets –1 and 5 ml
-Burette

b) Reagents

1- ***Ion exchange resin-*** The –OH form was converted by adding ca 15 ml 2 N NaOH/g resin to beaker. The mixture was swirled and left stand < 30 min. Then the liquid was decanted and repeated with addnl base. Thoroly wash resin with H_2O, it was slurred into fluted paper (S&S N° 588), and washed again with H_2O. The resin was prepared fresh weekly and stored under H_2O. A glass wool plug in base of tube was placed and slurred in enough resin to form 8 cm bed. The resin was never regenerated in packed column,

rather, used batch regeneration in beaker when necessary. The column was washed with ca 10 ml H_2O before applying each ext.
2- *3.57 N Phosphoric acid-it* was diluted 121.8 ml 85% H_3PO_4 to 1 litter. 3- *0.1 % O-phthalicdicaboxaldehyd (OPT) soln*- It was dissolved 100 mg OPT in 100 ml distd-inglass MeOH. The solution was stored in the refrigerator and fresh prepared weekly.

c) Histamine Standard solutions
4-1-Stock solution- It was accurately weight ca 169.1 mg histamine.2HCl into 100 ml volumetric flask, and dissolved to vol with 0.1 N HCl fresh prepared.
4-2- 10 μg/ml intermediate solution it was pipette 1 ml sock solution (4-1) into 100 ml volumetric flask, and diluted to Vol with 0.1 N fresh HCl.
4-3-0.5 ; 1.0 and 1.5 μg/5 ml workings solution - It was pipetted 1, 2 and 3 ml intermediate Lon (4-2) into 100 ml volumetric flasks, and diluted each to vol with 0.1 N fresh HCl.

d) Preparation of the sample
1- 10 g of the sample was added 50 ml MeOH into Becher glass and mixed during 15 min with magnetic mixer then it was introduced into 100 ml volumetric flask, washing the Becher glass with a little water.
2-into second Becher glass was added 10 g sample, 1 ml of std. working solution 0.5 μg/5 ml and 50 ml MeOH, mixed also 15 min in the magnetic mixer and introduced into 100 ml volumetric flask too, washing the Becher glass with a little water.
3- Both sample (c-1 and c-2) was taken into water bath for 1 hour. Then filtration was performed to get clear filtrate. To fill 100 ml volumetric flask, the filtrate was added with a little dest. Water.
4- 1 ml of filtrate was eluted in Ion-exchange resin passing dest. Water until fill up 50 ml volumetric flask.

e) Preparation of standard curve
Into 50 ml 3 Erlenmeyer glasses was pipette 5 ml aliquots of each working std solution (0.5. 1.0 and 1.5 μg/5 ml) and 2 Erlenmeyer glasses was pipette 5 ml of the samples and added with 10 ml 0.1N HCl to each flask and mixed. 3 ml 1 N NaOH was added and mixed within 5 min, then 1 ml OPT solution was added and mixed immediately. After exactly 4 min 3 ml 3.57 N H3PO4 was added and mixed immediately too. It was important to mix after each addition and least once during OPT reaction in shake machine.

The blank was prepared by substituting 5 ml 0.1 N HCl for histamine. Within 90 minute, was recorded to the Photofluorometer Intensity (I) of working standard soln standardising with dest. Water and using excitation wavelength of 350 nm and emission wavelength of 444 nm. mg histamine/100 g fish was determined with *calculation* 11 and the results are shown in Table 17.

Calculation 11
mg histamine /100g fish = 10 x F/m x Is

Where F= dilution factor = (ml eluate + ml 0.1 N HCl /ml eluate) = 1 for undiluted eluate.

$$m = \frac{(Ia/1.5) + (Ib/1) + (Ic/0.5)}{3}$$

Ia, Ib and Ic are fluorescence from sample 1,5; 1.0 and 0.5 μg histamine standards, respectively.
Is = fluorescence from the sample

4 RESULTS

4.1 Comparison of salt determination methods

a) Comparison between shaking and boiling methods by Volhard titration method
By Comparing the both results of shaking (Table 1) and boiling (Table 2) methods by Volhard titration method it was observed that the results of both methods were similar although the results of shaking method were slightly higher than the results of boiling method. The differences are shown in Figure 1.

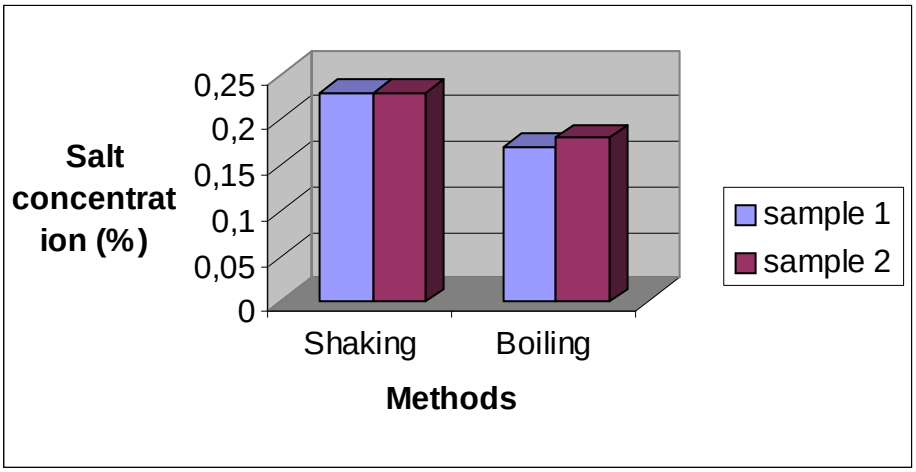

Figure 1. The difference between the shaking and boiling methods to determine sodium chloride by the volhard titration method in sample of light salted fillets haddock.

Table 1. Results of salt determination using shaking method by the Volhard titration method in samples of light salted fillets of haddock.

Sample	Wt mass (g)	Added vol. $AgNO_3$	Titration vol. NH_4SCN	Vol. NaCl (Difference)	% NaCl
I	AI = 5.07	5 ml	4.80	0.2 ml	0.23

	AII = 5.04	5 ml	4.80	0.2 ml	0.23
II	BI = 5.02	5 ml	4.80	0.2 ml	0.23
	BII = 5.03	5 ml	4.80	0.2 ml	0.23

Table 2. Results of salt determination using boiling method by the Volhard titration method in samples of light salted fillets of haddock.

Sample	Wt mass (g)	Added vol. $AgNO_3$	Titrration vol. NH_4SCN	Vol. NaCl (Difference)	% NaCl
I	1.03	5 ml	4.70 ml	0.3 ml	0.17
II	1.02	5 ml	4.65 ml	0.35 ml	0.18

b) Accurate evaluation of the Volhard titration method

By comparing the results of salt concentration by Volhard method with known salt concentration of salted water solution (Table 3), it was observed that the method was accurate.

Table 3. Salt determination for accurate evaluation of Volhard method using samples of salted water solutions with known salt concentration.

Sample - %	Wt mass (g)	Added vol. $AgNO_3$	Titration vol. NH_4SCN	Vol. NaCl (Difference)	% NaCl
I- 1%	1.01	5 ml	4.65 ml	0.35 ml	1.02
II-3%	3.02	5 ml	4.0 ml	1 ml	2.92
III-5%	5.03	5 ml	3.3 ml	1.7 ml	4.96

c) Comparison between Volhard titration method and Mohr method

The results of both methods Volhard and Mohr using the same sample were compared. The results were similar although the Mohr results were slightly higher than of Volhard. The results are shown in Table 4 and Figure 2.

Table 4. Differences between Volhard and Mohr methods using the same samples of salted water solutions.

SAMPLE-%	RESULTS (%)	
	Volhard method	Mohr method
I-1 %	1.02	1.17
II-3%	2.92	3.21
III-5 %	4.97	5.41

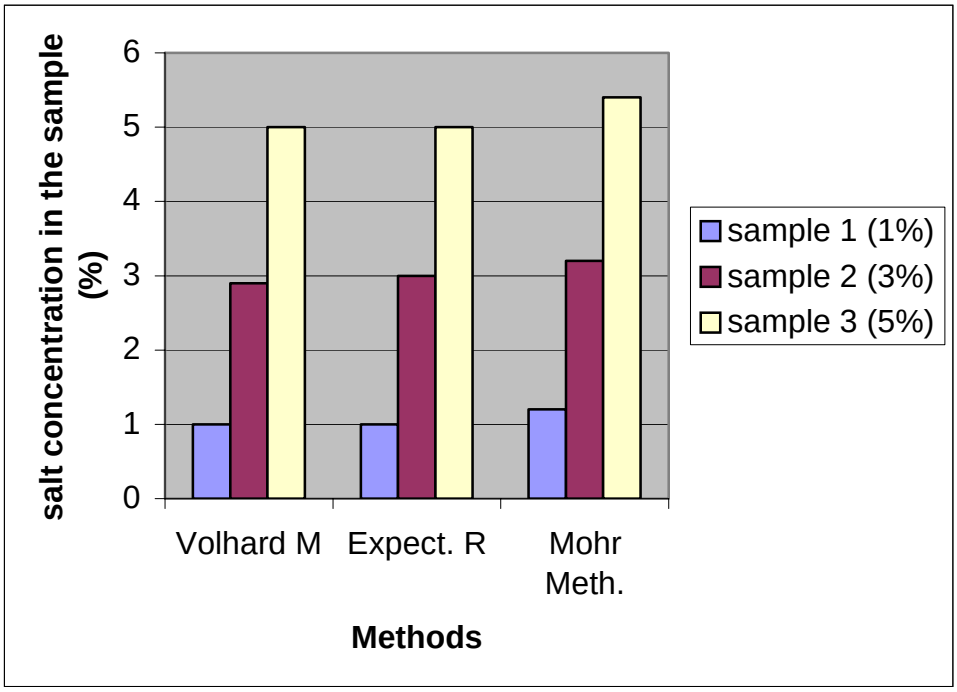

Figure 2. Results from the Volhard and the Mohr methods against expected values in salted water solutions.

d) Accurate evaluation of chloride titrator method

Comparison was also done between the results of chloride titrator and expected results (Table 5). From this method was observed that it was accurate method (Figure 3) but it was limited for certain range of salt concentration (0.05 to 1 %)

Table 5. Results of salt determination using chloride titrator method in samples of salted water solutions.

Sample	Dilution NaCl /100 ml	Quantab	% NaCl	Expected % NaCl
I	5.03 g/100	-	-	5.0
II	3.02 g/100	-	-	3.0
III	1.02 g/100	7.8	1.052	1.0
IV	50 ml/100	6.2	0.562	0.50
V	25 ml/100	4.4	0.264	0.25
VI	10ml/100	2.6	0.112	0.1
VII	5 ml/100	1.8	0.065	0.05
VIII	1ml / 100	-	-	0.001

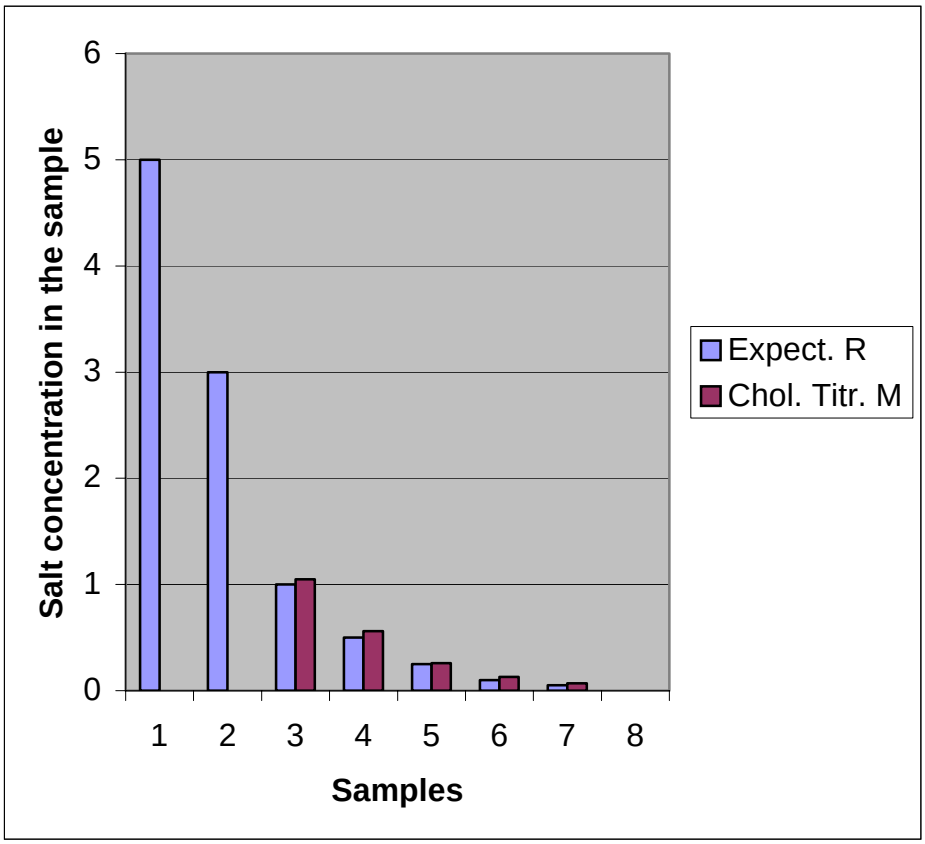

Figure 3. Results of chloride titrator method and expected values in salted water solutions.

4.2 Results of evaluation of enzymatic method

From the procedure of the enzymatic method and obtained results, it was observed that the enzymatic method is rapid, straightforward, accurate and free from interference.
The results of SO_2 evaluation using the enzymatic method are shown in Table 6.

Table 6. Expected and experimental results of SO_2 using enzymatic method.

Results of SO_2 using enzymatic method								
Samples	Wavelength		A1	A2	ΔA	ΔE	SO_2 g /L	
	Nm	ε					Exper. Result	Expec Result
Blank 1	340	6.3	1.980	1.932	0.048	0.1	3.1	3 g
Sodium sulphite	340	6.3	1.374	1.226	0.148			
Blank 2	340	6.3	1.050	1.002	0.048	0.1	3.1	3
Metabisulfite	340	6.3	1.122	0.974	0.148			

4.3 Results of evaluation of TVB and TMA methods monitoring the changes of fish fillets of herring and shrimp kept at 0°C and 5°C

4.3.1 Comparison of TVBN and TMA levels in the same stage of fish decomposition

By comparing the results of TVB levels (Table 7) and TMA levels (Table 8) of haddock it was observed that the TVB results were higher than the TMA results in all three samples of haddock (Figure 4). This phenomena was also observed in all other samples of herring, shrimp and cod (Table 9/11, 10/12 and 14).

Table 7. TVB results of three samples of haddock using FIGD methods.

Standard solution	Dilution	Peaks/sample (mm)	Average (mm)	TVB mg-N/100g
Haddock 1	50x	229	228	34.38
		227		
Haddock 2	50x	230	231	34.83
		232		
Haddock 3	50x	211	211	31.81
		211		

Table 8. TMA results of three samples of haddock using the FIGD method.

Standard solution	Dilution	Peaks/sample (mm)	Average (mm)	TMA (mg)
Haddock 1	50x	55	55.67	18.2
		56		

		56		
Haddock 2	50x	58	57.3	18.7
		57		
		57		
Haddock 3	50x	37	37	12.0
		37		
		37		
		50		

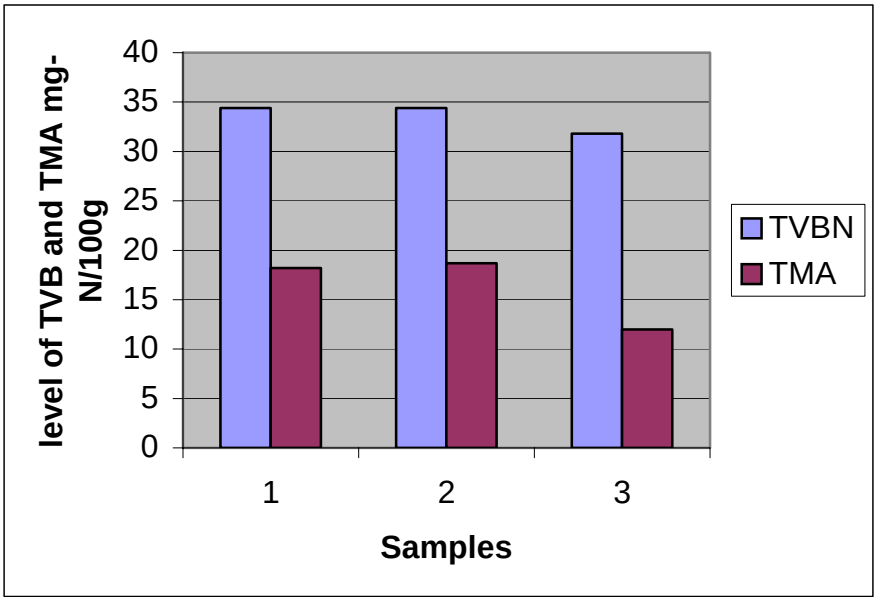

Figure 4. Differences between TVB and TMA levels in 3 samples of haddock kept at 0°C in the same period of time.

4.3.2 Results comparison of TVB methods

a) Comparison of TCA and FIGD methods

By comparing the results of TCA –extract and FIGD methods used for freshness evaluation of herring kept at 0°C and 5°C, it was observed that the TCA-extract method presented relatively higher results than the FIGD method at 0°C as well at 5°C (Table 9). The difference can be seen in Figure 5 for storage at 0°C and Figure 6 for storage at 5°C for up 13 and 11 days respectively.

Table 9. Results of TVB levels of herring using TCA-extract and FIGD methods.

Sample				**I**						**II**				
Storage temperature				**0 °C**						**5°C**				
Days of storage				2nd	4th	7th	9th	11th	13th	2nd	4th	7th	9th	11th
	R E	**TCA-** Extract	**Tit (ml)**	2.2	3.9	8	10	13.5	17.7	2.2	8.3	19.1	23.9	26.7

			Mg-N/100g	10.41	18.4	37.6	47.0	63.5	83.2	10.4	39	89.8	112	126
T		**FIGD-** Method	**Peak** (mm)	150	85	162	89	190	205	150	105	207	82	200
V			Mg-N/100g	9.1	11.7	16.8	37.9	54.3	61.8	9.1	14.4	53.4	69.9	115
B	*Peak height of TVB std. solution*			1.3	0.57	0.38	0.23	0.34	0.65	1.3	0.57	0.38	0.23	0.34
	Dilution of the sample			20x	10x	10	25x	25x	50x	20x	20x	25x	50x	50x

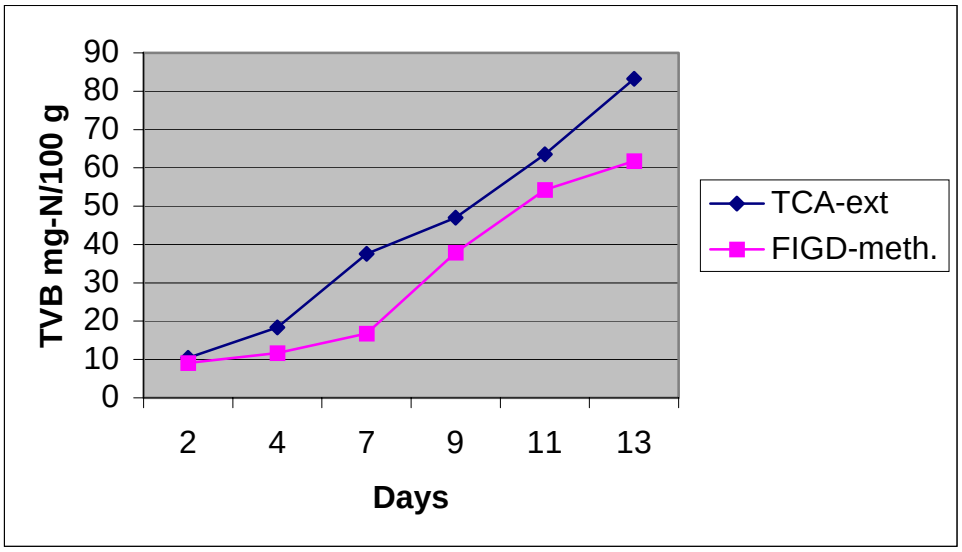

Figure 5. TVB analytical methods (TCA-extract , FIGD) used to determine freshness of herring that was stored at 0°C for up to 13 days.

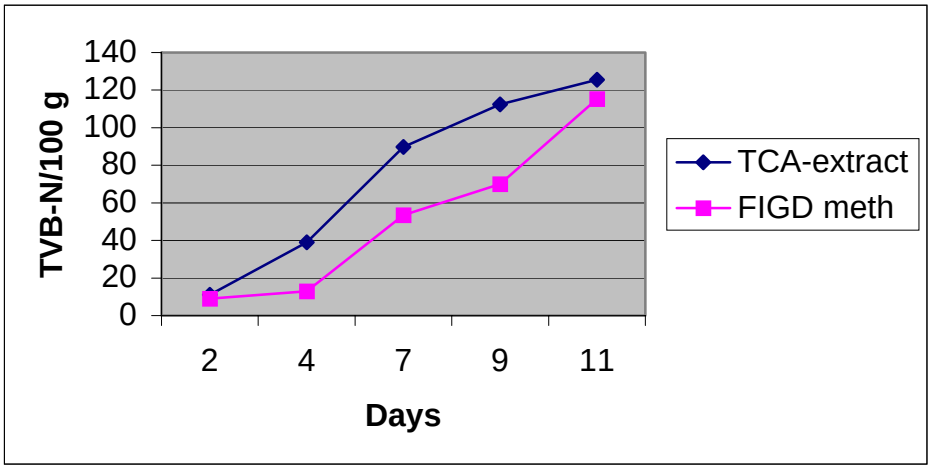

Figure 6. TVB analytical methods (TCA-extract and FIGD) used to determine freshness of herring that was stored at 5°C for up to 11 days.

b) TVB levels of shrimp using TCA-extract, FIGD and MgO methods

By comparing the results the TCA-extract with MgO methods (Table 10) by monitoring the changes of shrimp kept at 0°C and 5°C for up to 11 days, it was observed that the all three methods were similar although some deviations were observed (Figures 7 and 8).

Table 10. TVB and TMA results of shrimp kept at 0°C and 5 °C during 10 days using TCA-extract, FIGD and MgO methods.

Sample				I					II			
Storage temperature °C				0					5			
Days of storage				2nd	4th	6th	8th	10th	2nd	4th	6th	8th
RESULTS	TVB	TCA Extract	Titration (ml)	4.6	7.6	16	19.6	24	4.6	16.1	48.6	65.8
			mg N/100g	21.6	35.7	66.5	81.4	99.7	21.6	75.7	202	273
		MgO-Method	Titration (ml)	-	9	-	22.8	26	-	18.9	49	78.5
			Mg N/100g	22.5	37.8	68.2	84.3	96.5	22.5	79.4	205	290
		FIGD Method	Peak (mm)	161	124	46	57	174	161	96	101	167
			Mg N/100g	24.7	37.4	70.4	86	105	24.7	57.3	153	252
	TMA	TCA-extract	Titration (ml)	-	4	6.2	13.4	14.6	-	8.5	17.4	20.2
			mg N/100g	0.5	18.8	25.8	55.6	60.7	0.5	40	72.3	83.9
	Peak height of std solution			0.26	0.65	0.26	0.26	0.65	0.26	0.65	0.26	0.26
	Dilution of the sample			10x	50x	100x	100x	100x	10x	100x	100x	100x

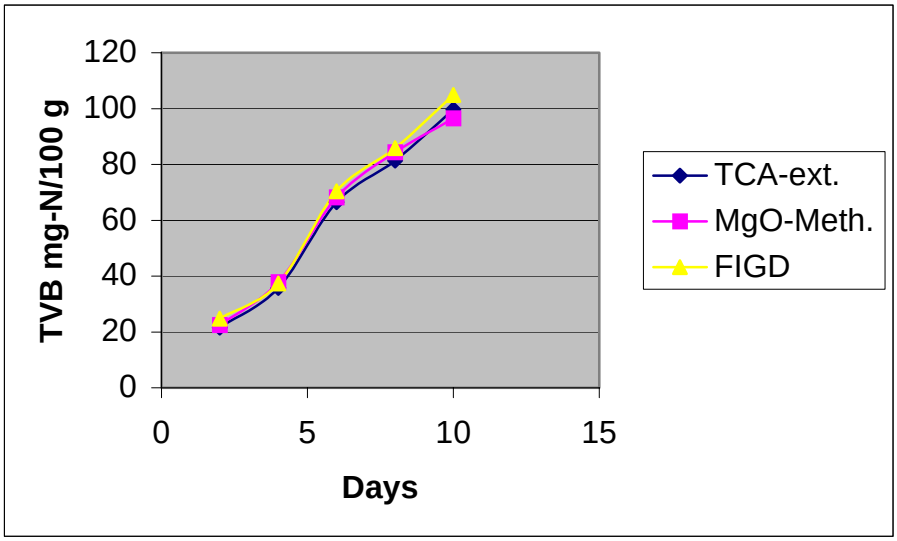

Figure 7. TVB analytical methods (TCA-extract, MgO and FIGD) used to determine freshness of shrimp that was stored at 0°C for up to 11 days.

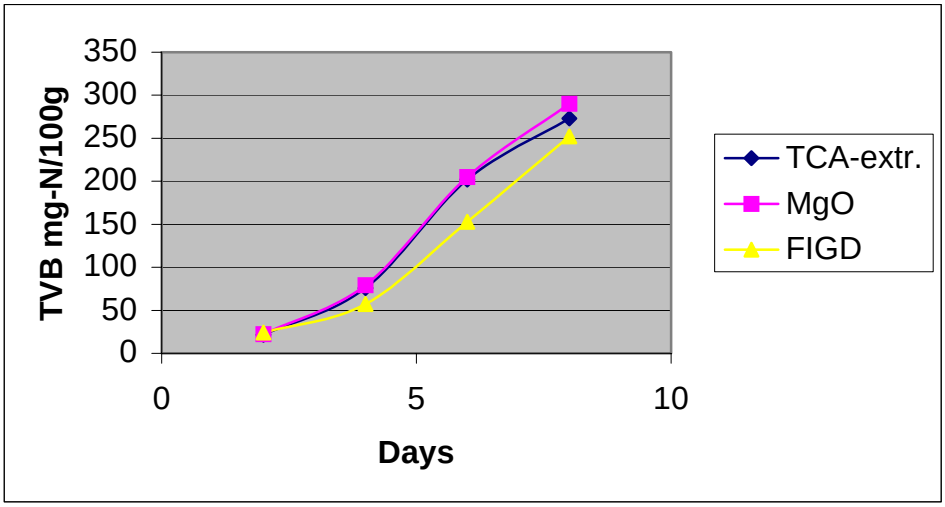

Figure 8. TVB analytical methods (TCA-extract, MgO and FIGD) used to determine freshness of shrimp that was stored at 0°C for up to 8 days.

4.3.3 Results comparison of TMA methods

By comparing the results of TCA-extract and FIGD methods (Table 11) and Picric acid method (Table 12 and 13) by monitoring the changes of herring and shrimp kept at 0°C and 5°C for up to 13 and 11 days it was observed that the Picric acid method is more similar to the FIGD than it was to the TAC –extract method. The correlation of results of FIGD and Picric acid method at 0°C and 5°C can be seen in Figures 8 and 9.

Table 11. TMA-results of herring kept at 0°C and 5°C for up to 13 days using TCA-extract and FIGD methods.

Sample				**I**						**II**				
Storage temperature				**0°C**						**5°C**				
Days of storage				2nd	4th	7th	9th	11th	13th	2nd	4th	7th	9th	11th
TMA	**RESULTS**	**TCA-**Extract Method	**Titration (ml)**	-	1.8	3.6	6.9	10.2	12.5	-	4.5	11.2	14	18
			Mg-N/100g	0.5	8.5	16.9	32.5	57.8	63.1	0.5	23.1	57.4	71.7	92.2
		FIGD-Method	**Peak**	150	18	88	116	188	128	150	55	132	126	162
			Mg-N/100g	0.98	2.21	10.5	35.5	51.2	69.7	1	16.8	38.0	72.6	88.2
	Peak height of TMA std. solution			0.6	0.32	0.34	0.34	0.36	0.36	0.60	0.32	0.34	0.34	0.36
	Dilution of the sample			Not Dilution	10x	10x	25x	25x	50x	Not Dilution	25x	25	50x	50x

Table 12. TMA results of herring and shrimp kept at 0°C and 5°C using picric acid method.

Order	Sample	Days	Storage T. °C	Dilution	Absorbance	TMA mg N/100 g
1	Blank				000	000
2	Standard 3				371	6.94
3	Standard 4				515	6.66
4	Herring	2	0		0.065	1.3
5		4	0		0.202	4.12
6		7	0		0.449	9.16
7		9	0	10x	0.159	32.43
8		11	0	10x	0.239	48.76
9		13	0	10x	0.274	55.90
10		4	5		0.383	7.75
11		7	5	10x	0.201	41.00
12		9	5	10x	0.388	79.15
13		11	5	10x	0.486	99.14
	Shrimp	2	0		0.025	0.51
5		4	0	10x	0.046	9.38
6		6	0	10x	0.094	19.18
7		8	0	10x	141	28.76
8		4	5	10x	0.097	19.79
9		6	5	10x	0.343	69.97
10		8	5	10x	0.423	86.3

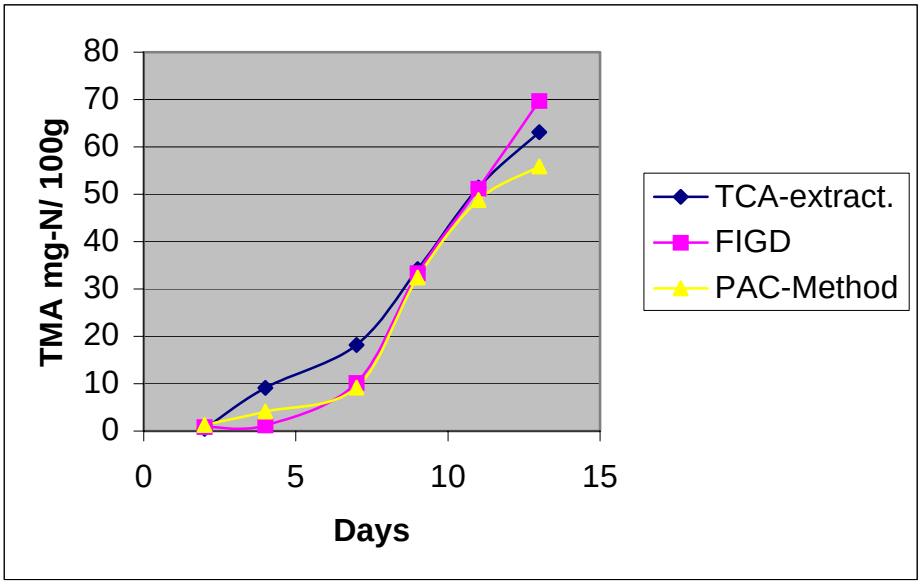

Figure 9. Comparison of TMA results of herring stored at 0°C for up to 13 days by using TCA-extract, FIGD and Picric acid methods.

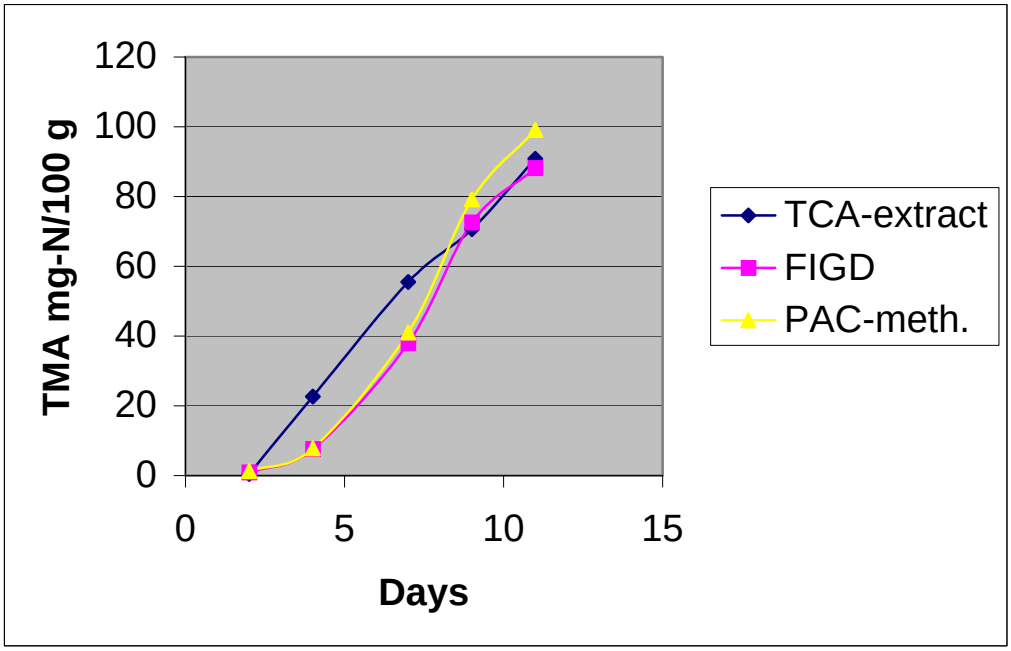

Figure 10. Comparison of TMA results of herring stored at 5°C for up to 13 days by using TCA-extract, FIGD and Picric acid methods.

4.3.4 Results of freshness comparison of herring kept at 0°C and 5°C

The freshness of herring kept at 0°C and 5°C using the FIGD as TVB and TMA freshness analytical methods were compared and it was observed that the TVB and TMA levels at 0°C were lower than at 5°C (Figures 10 and 11).

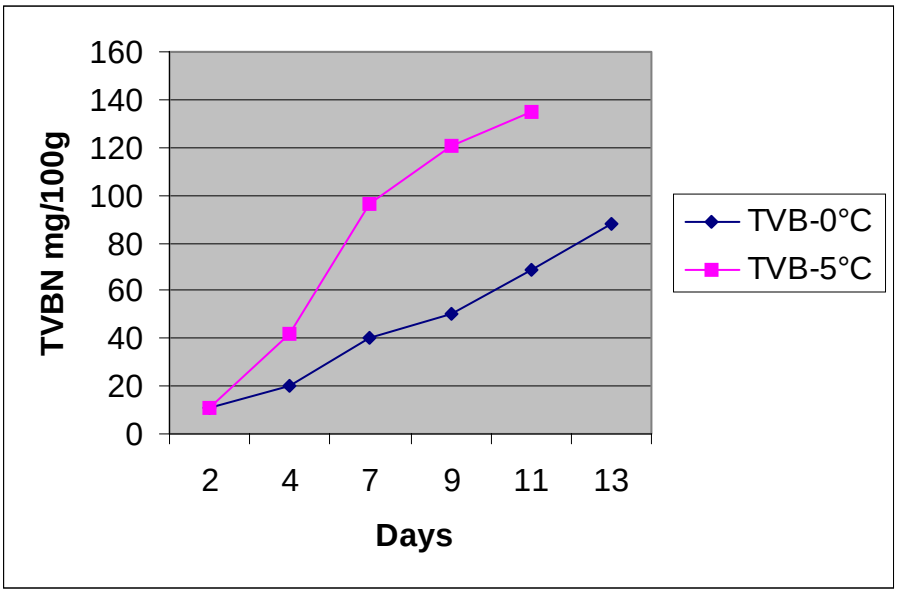

Figure 11. The difference of TVB levels of herring kept at 0°C and 5°C for up to 13 days using FIGD as TVB- freshness analytical method.

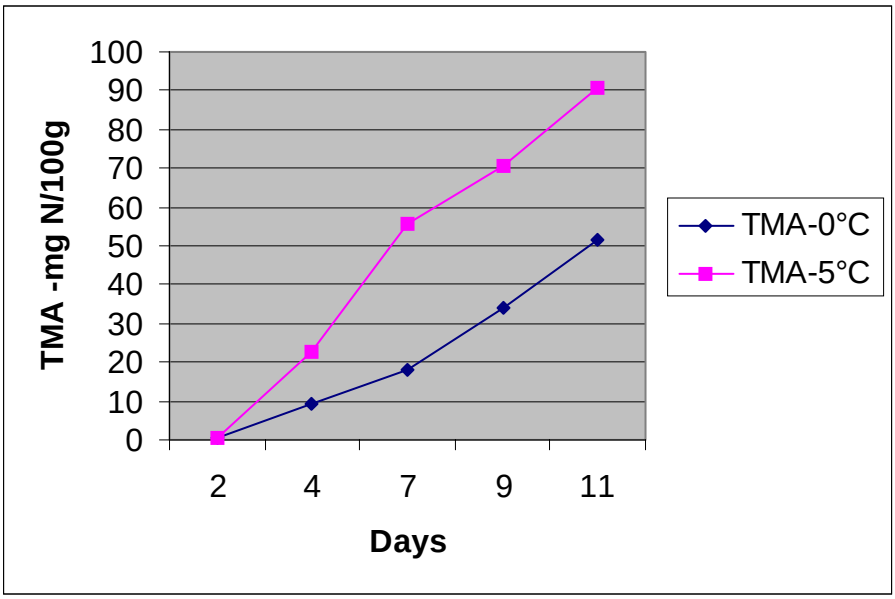

Figure 12. The difference of TMA values of herring stored at 0°C and 5°C for up to 13 days using the FIGD as TMA analytical method.

4.3.5 Freshness comparison of herring and shrimp

By comparing the TVB results of the herring and shrimp after 10 days in the same storage conditions (0°C and 5°C) , it was observed that the shrimp were more perishable than the herring at 0°C as well at 5°C. The results of both comparisons are seen in Table 13 and Figure 13.

Table 13. Perishable comparison of Herring and shrimp both kept at 0°C and 5°C.

TVB Sample	Method	Storage	Temperature	Days					
				2nd	4th	7th	9th	11th	13th
Herring	TCA-extract	13 days	0°C	11	19.7	40.4	50.5	68.6	88.3
Shrimp	TCA-Extract	10 days	0°C	23.2	38.3	71	87.9	107	-
Herring	TCA-extract	11 days	5°c	11	41.9	96.4	120.6	134.7	-
Shrimp	TCA-extract	8 days	5°C	23.2	81.2	215.8	292	-	-

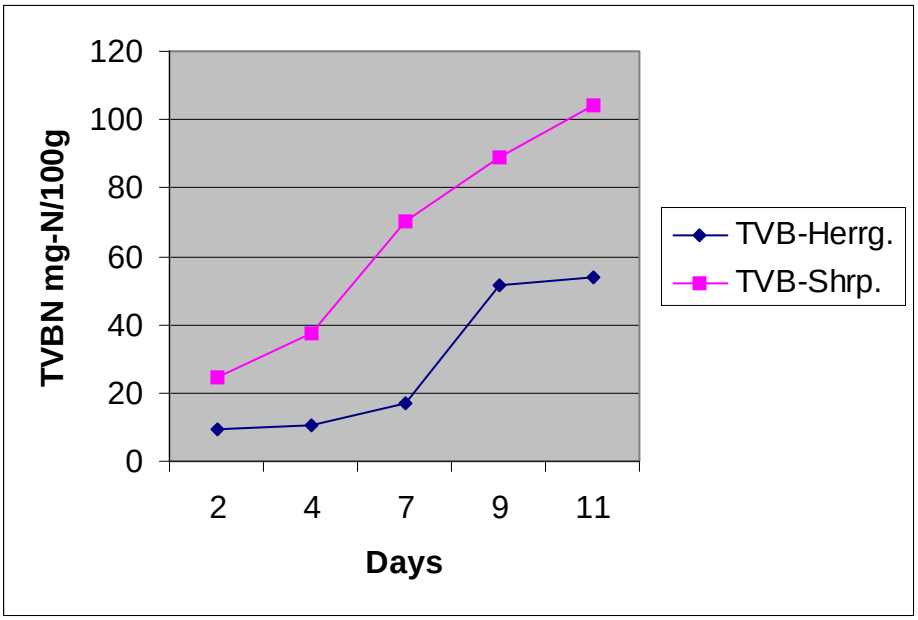

Figure 13. The difference of TVB levels between herring and shrimp kept at 0°C for up 10 days.

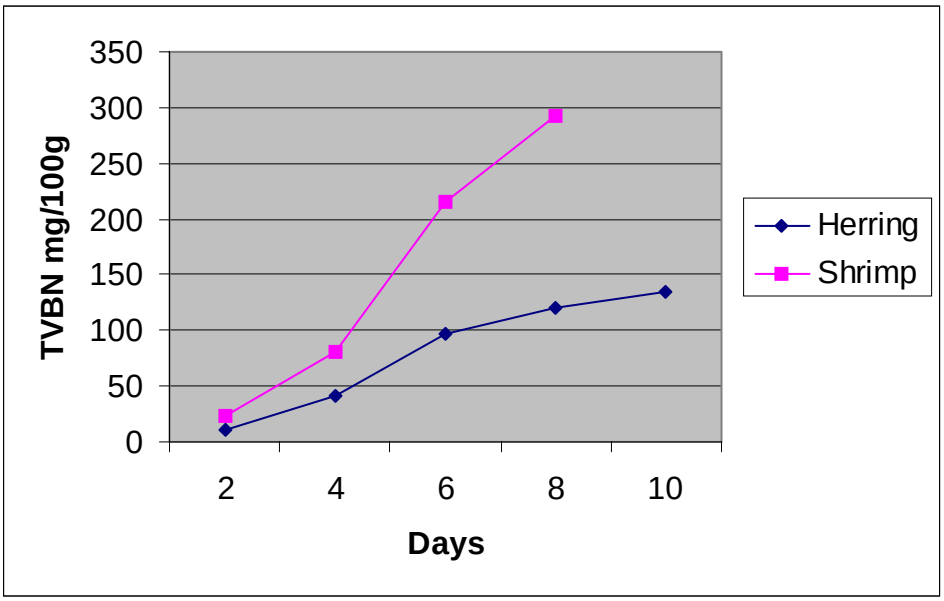

Figure 14. The difference of TVB levels between herring and shrimp kept at 5°C for up 10 days.

4.3.6 Results of relation between sensory and chemical analyses

Ten samples of cod stored at 0°C for 15 days were tested. The TMA and TVB were determined in each sample and the results are given in Table 14. The decomposition index was plotted on the x-axis and the amine levels on the y-axis. P is defined as the percentage TMA in the TVB (P= TMA/TVBN x %). The P values are showing in Figure 17. By comparing the changes in three criteria (TVBN, TMA, P) the shape of the slather diagram of TVB (Figure 15) show that the increase in TVBN is not very significant at start of decomposition. On the other hand the slather diagram of TMA (Figure 16) and P (Figure 17) did not give good information.

Table 14. Sensory and chemical results of cod stored at 0°C during 15 days.

5 Days	TVB	TMA	P (ratio)	QIM
2	14,4	0,5	3,5	3
5	16,9	0,5	3,0	5,2
9	19,7	0,5	2,5	9,8
12	21,7	2,2	10	12,9
15	26,7	6,5	24,3	15,7

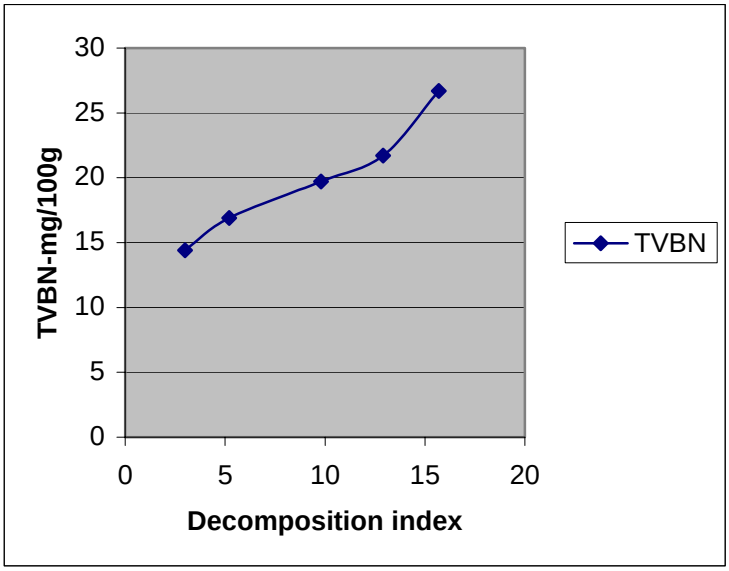

Figure 15. Correlation between the decomposition index and TVBN levels for cod stored at 0°C.

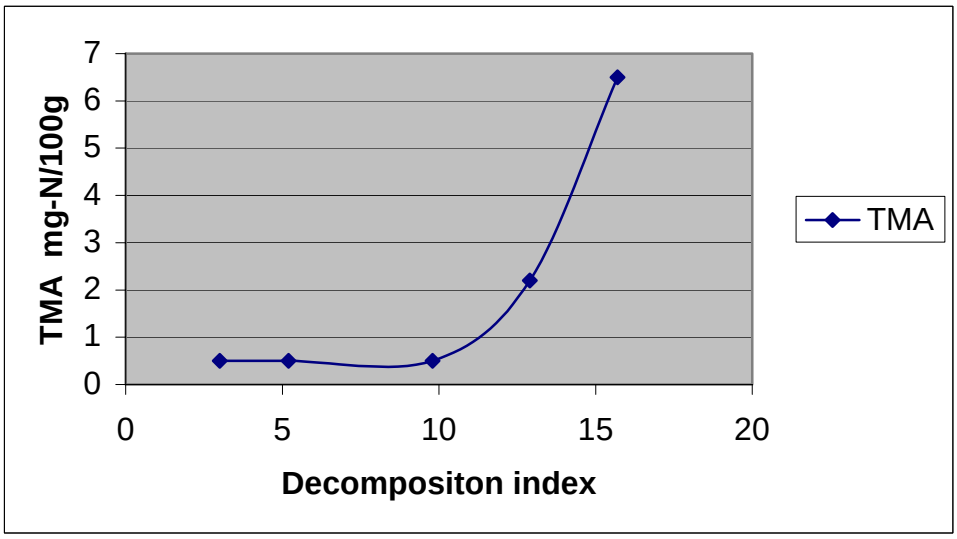

Figure 16. Correlation between the decomposition index and TMA levels for cod stored at 0°C.

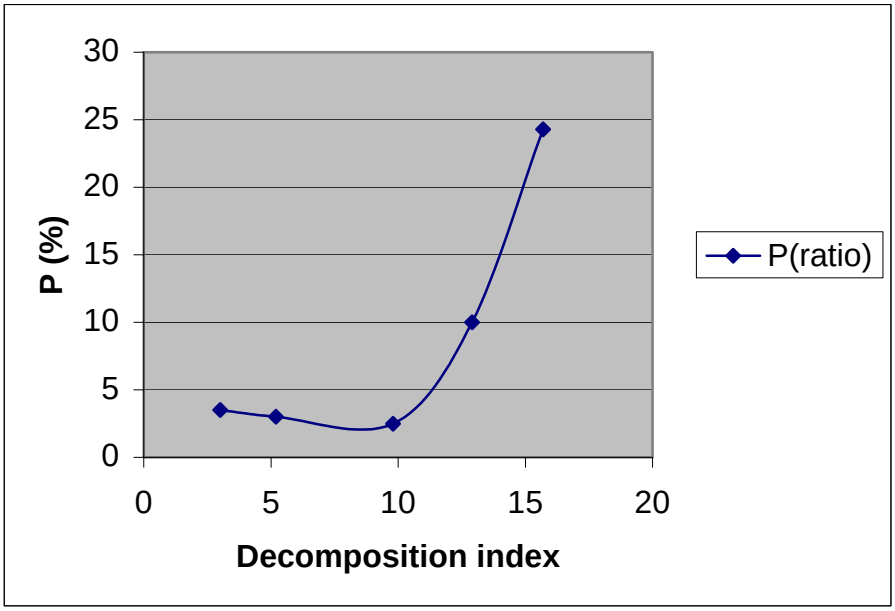

Figure 17. Correlation between decomposition index and P ratio (TMA/TVB x100) for cod stored at 0°C.

4.4 Results of evaluation of protein content and digestion time

By comparing the results of the four samples (cod, capelin, Acetanilide + sucrose and Sucrose) it was observed that the capelin had more protein content than all other samples. By comparing protein digestion at 3 hours, 2, 1, and half hour, were observed that 3 hours was the best time and for half hour any clear solution was found (Table 17).

Table 15. Results of protein content in the samples of Cod, capelin, Sucrose+ Acetanilide and Sucrose.

Time (hrs.)	S AM PLE S											
	COD			CAPELIN			ACETANILIDE + SUCROSE			SUCROSE		
	Wgt (g)	Vol (ml)	***Prot (%)***	Wgt (g)	Vol. (ml)	***Prot (%)***	Wgt (g)	Vol. (ml)	***Prot (%)***	Wgt (g)	Vol. (ml)	***Prot (%)***
3	2.6	60.4	***21.5***	0.51	38	***69.2***	1	35.1	***32.6***	0.51	0.4	***0.7***
2	2.1	39.8	***17.6***	0.51	38	***69.2***	1	35	***32.6***	0.51	0.4	***0.7***
1	2.1	38.3	***16.9***	0.51	37,7	***68.7***	1	32.9	***30.6***	0.50	0.2	***0.35***
0.5	2.1	-	-	0.51	-	-	1	-	-	0.50	-	-

4.5 Results of evaluation of histamine content by different handling methods

By using the photofluorometric method was observed that the level of histamine increase from 1st to 3rd sample (table 16). Using HPLC and Photofluorometric methods was found similar results (*lower than the limit)* of the sample which was rejected from Italian buyer.

Table 16. Results of histamine from four different samples.

Sample	Fluorescence Is	F	m	Histamine Mg /100g fish
1	50.1	1	89.4	5.6
2	54.6	1	64.7	8.4
3	69.5	1	64.7	10.7
4*	26.7	1	0.667	0.24 = 2.4 ppm 2 ppm (HPCL)

4* = Two different analytical methods were used (Photofluorometric and HPCL methods)

5 DISCUSSION OF RESULTS AND CONCLUSIONS

5.1 Comparison of salt determination methods

Salted haddock and salt (99.5% pure) were used as samples for salt determination using the Volhard titration method, Mohr and chloride titrator methods. The comparison of results is in Table 4 and Figure 2. Comparison of the chloride titration method and expected results is in Table 5 and Figure 3. From all used salt determination analytical methods, it was concluded that the Volhard method was the best against the other methods. It is an easy, simple, accurate and inexpensive method. The Mohr method gave relatively high results and the chloride was a very limited method since it only useful for a certain range of salt concentration (0.05-1%).

5.2 Evaluation of enzymatic method for SO_2 determination

The method is based to the fact that sulphite (sulphurous acid) is oxidised by sulphite oxidase (SO_2-OD) to sulphate in the presence of oxygen

$$SO_2^{--} + O_2 + H_2O \xrightarrow{SO_2\text{-OD}} SO_4^{++} + H_2O_2 \quad (1)$$

The hydrogen peroxide formed in this reaction is reduced by the enzyme NADH-peroxidase (NADH-POD) in the presence of reduced nicotinamide-adenine dinucleotide (NADH).

$$H_2O_2 + NADH + H^+ \xrightarrow{\text{NADH-POD}} 2H_2O + NAD^+ \quad (2)$$

The amount of NADH oxidised in reaction 2 is equivalent to the amount of sulphite or to aldehyde chemically-bound sulphite. NADH is determined by meas of its light absorbance at 334.340 or 365 nm.

The determination of sulphur dioxide was done by analysing two samples (*sodium sulphite and sodium metabisulfite*) with known concentration using the **enzymatic method**. The experimental and expected results were compared and the accuracy of the method evaluated (Table 6) From the procedure of the method and obtained results, it was concluded that the enzymatic method is rapid, straightforward, accurate and free from interference.

5.3 Evaluation of TVB and TMA methods monitoring the changes of fish Fillets of herring and shrimp kept at 0°C and 5°C

a) Comparison of TVB and TMA levels

TVB-N is one of the most widely used methods today to estimate the degree of decomposition of the fish and TMA provides an accurate indication of bacterial spoilage in some species. Both the Total Volatile Basic Nitrogen (TVBN) and Trimethylamine (TMA) were determined in three samples of haddock kept at 0°C for four days. The comparison of these two parameters showed that TVBN levels are higher than TMA levels at the same stage of decomposition (Figure 4) although the TMA increases more rapidly than TVB when the product is stored in ice. This confirms the known fact that the TVB is general term which includes the measurement of TMA (produced by spoilage bacteria), DMA (produced by autolytic enzymes during frozen storage, ammonia produced by deamination of aminoacids and nucleotides and other compounds associated with seafood spoilage. While the presence of TMA in spoiling fish is due to the bacterial reduction of TMA-Oxide.

b) Comparison of TVB methods

The TCA-extract, MgO and FIGD methods for TVBN assay were used. Results from the extracted samples of herring and shrimp kept at 0°C and 5°C for 13 days using FIGD and TCA –extract steam distillation and MgO were shown in Tables 9, 10 and Figures 5, 6, 7 and 8. These results lead to the conclusion that all three methods are similar. The slight difference observed between TCA-extract and MgO methods by TVB assay of herring, is thought to be caused by different chemical composition of herring and shrimp. The MgO method showed to be more similar to the TCA-extract method (Figure 8). Thus by TCA- extract steam distillation volatile basic nitrogen and other volatile and non volatile compounds are distilled (Figures 5 and 6) while by FIGD method only volatile basic nitrogen are distillated (Figure 7). This fact was confirmed with results of shrimp kept at 5°C where the results of FIGD were lower than of the TCA-extract when the shrimp was too spoiled (Figure 8). The difference of chemical composition between the herring and shrimp was confirmed when both samples were spoiled. The shrimp smelt strongly of ammonia while the sample of herring smelt of other chemical compounds.

c) Comparison of TMA methods

TMA results from determination of the extracted samples of herring and shrimp kept at 0°C and 5°C using FIGD, steam distillation and Picric acid methods are shown in Figures 9 and 10. It was concluded that all three methods were similar although the Picric acid method was more similar to the FIGD than to the TCA. The relatively high results of TCA-extract steam distillation is thought to be caused by not a total neutralisation of

primary and secondary amine by Formaldehyde. The results for TMA measured by FIGD method were sometimes not easy because an unsteady baseline on the chart recorder was observed. Thus the low standards for TMA could therefore not always be measured because of the fluctuations when only reagents passed by diodes.

d) Comparison of herring at 0°C and 5°C

By comparing the TVB-N and TMA levels of the all samples of shrimp and all samples of herring both kept at 0°C and 5°C (Figures 11 and 12) it was confirmed the known fact that 0°C increase the shelf-life of sea productability than 5°C.

e) Comparison of perishability of herring and shrimp

The TVB levels of herring and shrimp kept at 0°C (Figures 13) and 5°C (Figure 14) shows that the amine levels are different in the two species at a given stage of decomposition. This confirm the well known fact that the variability of the TVB and TMA content is due the different species related factors such as the microbiological flora, the condition of capture and storage. Figures 13 and 14 show how shrimp is more perishable than herring under the same time and storage conditions. (10 days under 0°C and 5°C).

5.4 Relation between sensory and chemical analyses

Ten samples of cod stored at 0°C for 15 days were tested. The TMA and TVB were determined in each sample and the results were given in Table 14. The decomposition index is plotted on the x-axis and the amine levels on the y-axis. P was defined as the percentage TMA in the TVB (P= TMA/TVBN x %). The P values were showing in Figure 17. By comparing the changes in three criteria (TVBN, TMA, P) the shape of the slather diagram of TVB (Figure15) show that the increase in TVBN is not very significant at start of decomposition. On the other hand the slather diagram of TMA (Figure 16) and P (Figure 17) did not give good information. This fact is thought to be caused by low Total Plate Count (TPC) of bacteria which were observed in all stored samples.

5.5 Evaluation of protein content and digestion time

Three samples (cod, capelin, sucrose+acetanilide and sucrose) were analysed in different times (3 hours, 2 hours, 1 hour and 30 min). By comparing the results of these four samples it was concluded that the capelin had higher protein content than all other samples. By comparing protein digestion at 3 hours, 2 ,1, and half hour, were concluded that 3 hours were the best time and for half hour any clear solution was found (Table 15).

5.6 Evaluation of histamine content by different fish handling method

Histamine was determined in samples of salted herring and smoked salmon. One sample of herring was stored at 0°C for one day, the second was stored at 0°C for one day and left at room temperature during the night, the third was stored in the refrigerator for two weeks and the fourth sample (smoked salmon) from an Icelandic producer was rejected by an Italian buyer due to too high histamine level.

Different levels of histamine were observed (Table 16). The increase of histamine levels found in samples 2 and 3 confirm the well known fact that usually fish does not become toxic unless held for many hours to a few days at room temperature, or indicate poor handling and/or processing of these products. Education on hygiene handling of raw material and proper process control are recommended for a local health authorities and producers. Using HPLC and Photofluorometric method was found similar results of the sample which was rejected from Italian, for this case, it was concluded that the histamine content in the analysed sample is lower than allowed limit.

6 CONCLUSIONS

From this study were concluded:

1. The Volhard and Mohr methods for salt determination, enzymatic method for metabisulfite, TCA-extract and FIGD for TVB and TMA, MgO for TVB determination were suitable and applicable methods for today Mozambican conditions.

2. Full understanding of the chemistry involved in the methodology, from extraction to the end determinations and full appreciation of methods are extremely necessary.

3. Education on hygiene handling of raw material and proper process control (storage conditions) of product can decrease the histamine level in the product and increase the shelf-life of the product.

4. Shrimp is one of the most perishable products, thus recommendations on Good Manufacture Practice (GMP) to the producer are extremely important.

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APPENDIX 1: Reference of selected chemical methods

Analyse	Methods used	Reference of the method
Salt Determination	Volhard method	Egan, H.; Kirk S.R. and Sawyer, R; Pearson´s chemical analyses of foods; 8th Edition; 1981, London-UK
	Mohr method	Kenkel, J; Analytical chemistry for Technicians 2nd Edition, 1994- Florida USA
	Chloride titrator	Environmental Test System, INC; Elkhart, 46514, USA.
Metabisulphite	Monier Williams	Egan, H.; Kirk S.R. and Sawyer, R; Pearson´s chemical analyses of foods; 8th Edition; 1981, London-UK
	Enzymatic	Maija-Lisa A. *et al.* Enzymatic Determination of sulfide in foods: NMKL, Interlaboratory study; journal of AOAC International Vol (76) No 1: 53-58; 1993
TVBN	TCA-extract	Malle, P. and Tao, S.H., rapid quantitative determination of TMA using Steam distillation; Journal of Food Protection; Vol (9):756-760; September 1987
	FIGD	Einarsson, S. The evaluation of a simple, cheap, rapid method of non-protein nitrogen determination in fish products through the processing 1998-Reykjavik-Iceland
	MgO	Antonacopuls, N. Handbuch der Lebensmittel chemie, Bd. III/1 Ed. Pp: 1493-1494 Acker I. Spring Verlag, Berlin Germany.
TMA	TCA	Malle, P. and Tao, S.H., rapid quantitative determination of TMA using Steam distillation; Journal of Food Protection; Vol (9):756-760; September 1987
	FIGD	Einarsson, S. The evaluation of a simple, cheap, rapid method of non-protein nitrogen determination in fish products through the processing ;Interlaboratory study; 1998-Reykjavik-Iceland
	Picric Acid	Helrich, K. Official Methods of Analysis 15 Edition AOAC ; 1990-Virginia-USA
Protein	Kjeldahl	Persson, J. A. handbook of Kjeldahl Digestion; 1995- Höganas- Sweden
Histamine	Photofluorometric	Helrich, K. Official Methods of Analysis 15 Edition AOAC ; 1990-Virginia-USA

APPENDIX 2: Table in quantab units

Quantab	NaCl %	ppm (mk /L)
1.4	0.044	265
1.6	0.054	330
1.8	0.065	394
2.0	0.076	459
2.2	0.086	524
2.4	0.097	589
2.6	0.112	678
2.8	0.128	776
3.0	0.144s	874
3.2	0.160	972
3.4	0.176	1070
3.6	0.193	1168
3.8	0.209	1268
4.0	0.225	1365
4.2	0.241	1463
4.4	0.264	1600
4.6	0.294	1786
4.8	0.325	1971
5.0	0.356	2137
5.2	0.366	2343
5.4	0.417	2538
5.6	0.447	2714
5.8	0.478	2899
6.0	0.514	3116
6.2	0.562	3416
6.4	0.611	3706
6.6	0.660	4001
6.8	0.708	4296
7.0	0.760	4612
7.2	0.833	5054
7.4	0.906	5496
7.6	0.979	5938
7.8	1.052	6380