Methods to evaluate fish freshness in research and industry

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Current work in a European concerted action project 'Evaluation of Fish Freshness' (AIR3 CT94-2283) focuses on harmonizing research activities in the area of fish freshness evaluation in leading fish laboratories in Europe (see Box 1). The overall aim of the concerted action project is to validate methods for the assessment of fish freshness and to discuss the freshness criteria for fish commercialized within the European Union. The project's participants are working in subgroups studying sensory analysis, microbiology, volatile compounds, proteins, lipids, adenosine triphosphate and physical measurements with respect to fish freshness evaluation. In this article, the different subgroups have summarized changes that occur in fish and methods to evaluate fish freshness as a first step towards the definition of criteria for fish freshness.

Freshness makes a major contribution to the quality of fish or fishery products. For all kinds of products, freshness is essential for the quality of the final product. Figure 1 depicts the relationship between quality and freshness, focusing on the different characteristics of freshness as approached by the different subgroups of the project. Freshness can be explained to some extent by some objective sensory, (bio)chemical, microbial and physical parameters[1], and can therefore be defined as an objective attribute.

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Knowledge of the various descriptors of properties that are encountered in fish immediately after harvest or catch must be known as well as of the changes in properties that take place over time[2]. This information can be gained by performing controlled storage experiments that extend from the time of harvest until spoilage. Freshness, loss of freshness and spoilage can thus be monitored; once the dynamics and the rate of the various changes that occur have been measured, the next step is to try to develop a model. The future aim is to use a model to determine when a sample was harvested or predict the remaining shelf life of an unknown sample. To achieve this aim, it is useful to combine several measurements obtained by different methodologies and correlate the findings with sensory assessments, which are currently the most used method to evaluate fish freshness.

Sensory evaluation of fish freshness

Sensory evaluation is defined as the scientific discipline used to evoke, measure, analyse and interpret characteristics of food as perceived by the senses of sight, smell, taste, touch and hearing. Sensory tests can be divided into three groups: discriminative tests, which indicate whether there is a difference between samples; descriptive tests; and affective tests[3]. Discriminative and descriptive tests are objective analytical tests in which a trained panel is used. Affective tests are subjective consumer tests that are based on a measure of preference or acceptance. The choice of method depends on the purpose of the application of the sensory evaluation and whether it is used in product development, quality control, consumer studies or research. The most commonly used descriptive tests are structured scaling for quality assessment and profiling for a detailed description of one or more attributes.

Characteristic sensory changes occur in the appearance, odour, taste and texture of fish when they deteriorate[4]. In Europe, the most commonly used method for the quality assessment of raw fish in the inspection service and in the fishing industry is the European Union scheme[5]. This scheme does not take into account differences between species because only general parameters are used. Alternative scaling methods such as the quality index method (QIM) have been suggested[6,7], where the descriptions of the individual grades are precise, objective, independent and primary rather than a cluster of terms. The QIM is based on the significant sensory parameters for raw fish. The scores for all of the characteristics are then added to give an overall sensory score, the so-called quality index, which can also be used to predict storage life. In the fish industry, the grading of raw fillets also occurs. However, it is more common to cook fillets before carrying out sensory evaluation, and the Torry scheme[4] is the most commonly used scale for the freshness evaluation of cooked fish, both in the fish industry and in research laboratories throughout Europe.

In the fish industry, a few highly specialized trained assessors usually evaluate the freshness of fish[8]. Guidelines for the sensory evaluation of fish and shellfish in inspection

 0924-2244/97/$17.00
PII: S0924-2244(97)01049-2

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and regulation services[9] are currently under discussion by the Codex Committee on Fish and Fishery Products.

The future aim is to use trained panels to evaluate raw materials and products, as a part of the quality assurance programme performed by fish processing companies. The sensory evaluation of food has been described with the establishment of quality control programmes in mind[10]. In research laboratories, it is common to have trained panels with proven skills, complete sensory evaluation facilities and computerized data sampling. Some of the food research laboratories studying the quality of fish have received an accreditation for their sensory evaluation methods (e.g. VTT, Finland) based on the EN 45001 and the ISO/IEC Guide 25[11]. The ISO standards describe both the selection and training of panellists.

Microbial methods

The activity of microorganisms is the main factor limiting the shelf life of fresh fish. An estimation of the total viable counts (TVC) is used as an acceptability index in standards, guidelines and specifications. The objective of the collaborative work of this subgroup is to study the application of microbial methods for the evaluation of the freshness of fish from different countries and stored under different conditions of temperature (0–10°C) and atmosphere (air, vacuum or modified-atmosphere packaging). The remaining shelf life is used as a definition of fish freshness.

Newly caught fish contain a diverse microflora. TVC of 10^2–10^6 cfu/g are usual on whole fish and cut fillets. During chill storage, psychrotolerant microorganisms are selected; thus, differential counting of these microorganisms

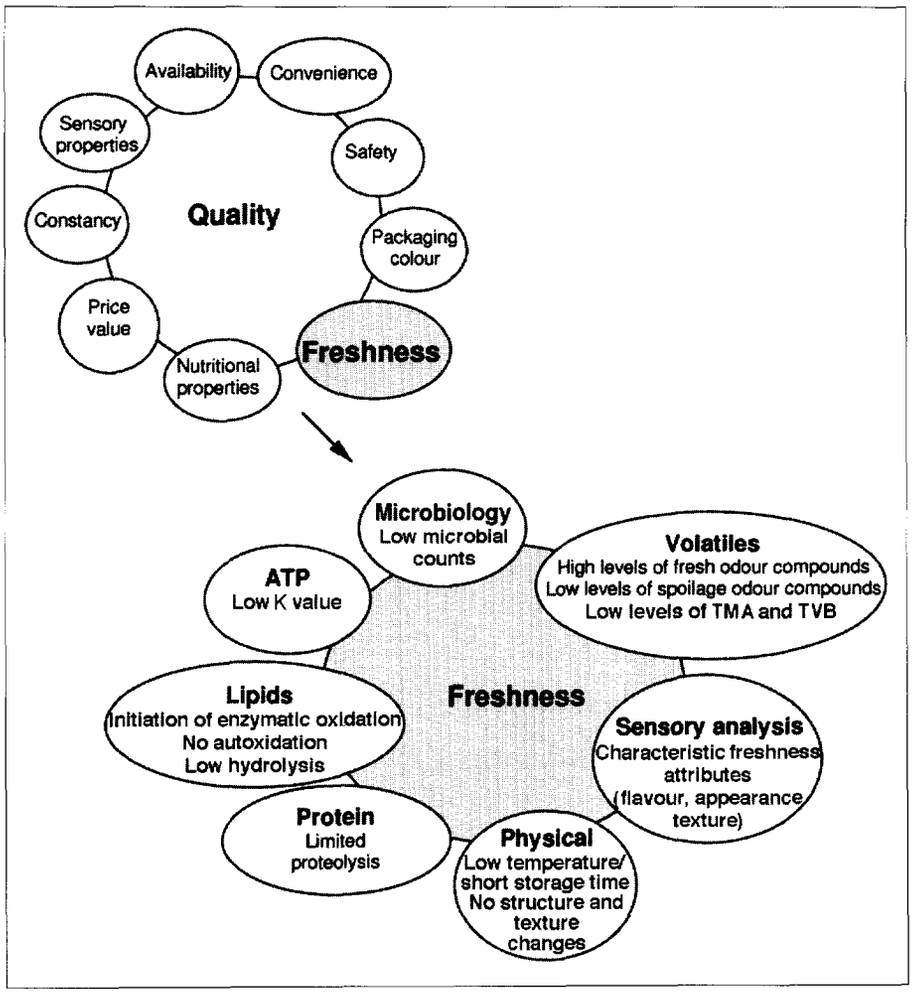

Fig. 1

Relationship between quality and freshness. Quality is a function of freshness; freshness is essential for quality but it is not *a priori* a quality factor. The upper 'quality' circle comprises the factors that contribute to quality, and the lower 'freshness' circle details the various approaches used to evaluate fish freshness. The K value is defined as the ratio of the sum of inosine and hypoxanthine concentrations to the total concentration of adenosine triphosphate (ATP) metabolites. TMA, trimethylamine; TVB, total volatile bases.

was suggested as a measure of fish quality in early studies. More recently, the bacterium *Shewanella putrefaciens*, which produces hydrogen sulphide, was determined as the specific spoilage organism (SSO) of some chilled fresh fish. This microorganism can be enumerated in iron-containing agar, and correlation coefficients as high as −0.97 were achieved when comparing log numbers of *S. putrefaciens* with the remaining shelf life of aerobically stored fish, as determined by sensory evaluation. Owing to the selection of microorganisms in chilled fish, the correlation between SSO and freshness is usually higher than between TVC and freshness[12–15].

Most marine fish spoilage bacteria reduce trimethylamine oxide to trimethylamine (TMA), and this reduction is used as the basis for several rapid, automated conductance assays. The time required to detect changes in conductance showed good correlation with the freshness of various fish species, and correlation coefficients from −0.85 to −0.99 were reported both for aerobically stored and packed fresh fish[15,16].

Photobacterium phosphoreum was identified as the SSO in some modified-atmosphere packed (MAP) fish. This microorganism can be specifically detected using a conductance technique[17], and a good correlation was found between this SSO and the remaining shelf life of MAP cod fillets.

Clearly, microbial methods can provide useful measures of fish freshness; however, the most promising results have been achieved with relatively slow detection methods such as plate count and other growth techniques that involve a period of incubation. Therefore, the development of practical techniques for the concentration and separation of microorganisms from fresh fish will be important in improving the response time, sensitivity and specificity of both the classical and new rapid methods.

At the point of sensory rejection, the TVC of fish products are typically 10^7–10^8cfu/g. Nevertheless, standards, guidelines and specifications often use much lower TVC as indices of acceptability. In a recent European study by consumers, fish was assumed 'not to be in a good enough condition to be stored for long' when TVC were 10^6cfu/g (Ref. 18). Microbial criteria based on low TVC such as 10^6cfu/g are problematic to use because a correlation between TVC and the remaining shelf life is assumed but generally not known. Therefore, it has been suggested that microbial methods for the evaluation of fish freshness are developed together with mathematical models that express the effects of storage conditions such as temperature and atmosphere on the correlation between microbial numbers and remaining shelf life. Thus, rapid microbial methods could be useful not only at the time of analysis but also later on during storage of the fish.

Volatile compounds as indicators of freshness and spoilage

Odour is one of the most important parameters used to evaluate fish freshness. Measurements of characteristic volatile compounds can be used to monitor the freshness or spoilage stage of fish. The volatile compounds contributing to fish odour can be divided into three groups based on their origin, as illustrated in Fig. 2. Various compounds have been suggested as indicators of spoilage. Classical chemical methods for the analysis of total volatile bases and TMA (trimethylamine) have been used for the determination of fish freshness in the industry[2].

Headspace methods for the analysis of volatile compounds involve the collection and concentration of the volatiles for subsequent chromatographic separation to identify and quantify the separated compounds. Extremely volatile, low molecular weight compounds can be analysed by static headspace methods[19]. More efficient, dynamic headspace methods are necessary for collecting and concentrating less-volatile compounds[20] such as those contributing to 'fresh fish' and 'oxidized' odours. Higher-boiling compounds require even more efficient isolation methods such as solvent extraction with organic solvents or supercritical carbon dioxide[21]. Other approaches are simultaneous distillation and extraction in the gas phase[22] or high-vacuum distillation[23]. Once the volatiles have been trapped, they are transferred by thermal desorption or solvent extraction to a chromatograph for separation and identified by appropriate detectors.

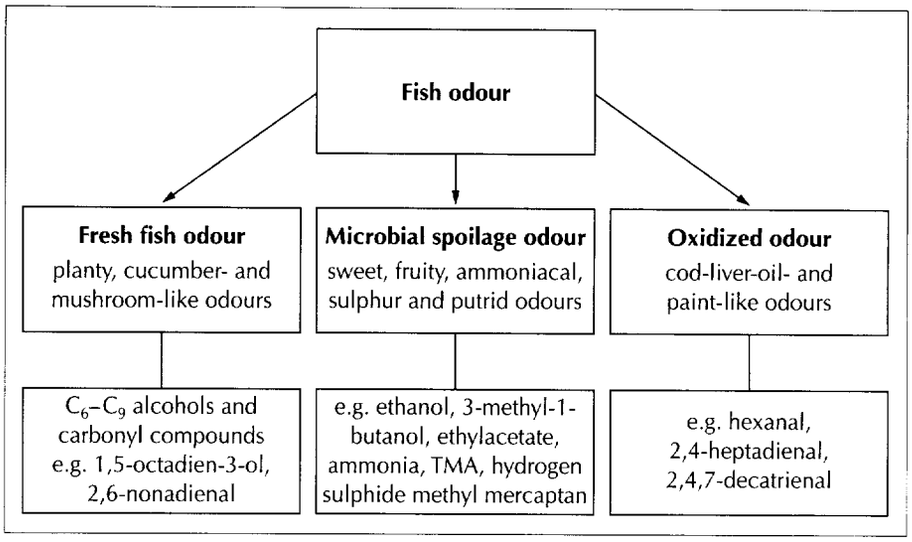

Fig. 2
Categorization of fish odours and the volatile compounds that contribute to the characteristic odour of fresh, spoiled and oxidized fish. TMA, trimethylamine.

Although instruments with a high degree of automation are available for the trapping and chromatography steps, the complexity, cost, and lengthiness of volatile analysis methods make them suitable only for specialized research and analytical laboratories.

The rapid assessment of volatile compounds in food using arrays of gas sensors, so-called electronic noses, is of increasing interest[24], and an instrument with electrochemical gas sensors has been developed for the rapid detection of fish freshness[25]. A validation of an electronic nose designed to detect spoilage in minced beef showed that its reproducibility, repeatability and discriminative power need to be improved[26]. However, assessment of the quality of salmon (*Salmo salar*) and whiting (*Merlangius merlangus*) using an electronic nose has shown that samples can be classified into three sensory categories, namely good, acceptable and not acceptable (J. Luten and P. Scheerman, pers. commun.; M. Etienne and J. Fleurence, unpublished).

For the future development of rapid gas sensor techniques for fish freshness application, it will be necessary to define standard methods based on gas sensors and to validate their usage for detecting the characteristic volatile compounds that are indicative of the freshness stage of fish.

The effects of postmortem storage of fish on proteins

After water, which accounts for ~80% (w/w), proteins are the major constituents (15–20%) of fish flesh. The proteins found in muscle can be broadly categorized as: water-soluble or sarcoplasmic proteins; contractile proteins (e.g. myosin and actin), which are extractable in solutions of relatively high ionic strength, and proteins that are insoluble in solutions of either high or low ionic strength (collagens)[27].

On postmortem storage, the sarcoplasmic proteins undergo no change in either composition or enzymatic activity. However, in the case of the myofibrillar proteins, the cytoskeletal proteins (α-actinin and α-connectin) undergo proteolysis. The Z-disc region of the myofibril appears to be destroyed by the action of proteases, releasing α-actinin. It has also been shown that α-connectin, an extremely high molecular weight protein that runs longitudinally along the myofibril, is broken down to β-connectin by proteolysis[28]. However, changes have not been reported in the major contractile proteins myosin and actin. The collagen proteins are relatively minor components of muscle and, despite conflicting evidence, it is generally agreed that some degree of breakdown by collagenases takes place[29].

It can be concluded that, in the main, the proteins of muscle are largely unaffected during postmortem storage, and that the softening of muscle is due not to the breakdown of myofibrils but to proteolytic digestion of minor cell components that link the major structural units together[30,31]. These changes can be observed by light and electron microscopy and can be measured to some extent by texturometers. There are, however, difficulties associated with the use of texturometers on whole muscle because of the inherent nature of muscle tissues. Changes in the size of proteins can be determined by electrophoretic and chromatographic techniques, but these techniques are not suitable for industrial use. Similarly, the isolation of proteins involves lengthy extraction and fractionation procedures, which are only suitable for a research laboratory. However, once isolated, collagens, for example, can be characterized using relatively simple techniques to determine their solubility properties and thermal denaturation temperatures.

To date, there are no rapid methods for determining changes in muscle proteins during postmortem storage; furthermore, until it is possible to detect the release of proteolytic enzymes in muscle tissue, there is little prospect of the emergence of methods that are suitable

for industry. However, instrumental methods of measuring texture show more promise (see 'Physical measurements', below).

Measurements of lipid oxidation in fish

The highly unsaturated lipids of fish easily become oxidized, resulting in alterations in smell, taste, texture, colour and nutritional value. Oxidation starts immediately after catch, but becomes particularly important for shelf life only at temperatures <0°C[32], when oxidation rather than microbial activity becomes the major spoilage factor.

The initiation of lipid oxidation arises from various early postmortem changes in fish tissues. These changes, which disturb the natural balance between antioxidants and pro-oxidants, include the accumulation of active oxygen species, the activation of haemoproteins, an increase in free iron and the consumption of antioxidants[33].

Once initiated, the extent of lipid oxidation can be followed using either the reactants or the products. Measurements of oxygen consumption can be monitored with an oxygen electrode[34], whereas the loss of fatty acids and antioxidants can be measured using gas chromatography (GC) and high-performance liquid chromatography (HPLC)[35]. The peroxide value is the most common measure of lipid hydroperoxides, also called primary lipid oxidation products. Other methods are HPLC in combination with, for example, chemiluminescence detection[36] or, if conjugated double bonds are present, simple spectrophotometry[37]. The primary products easily break down into secondary products, such as aldehydes and ketones. The volatile nature of these compounds makes them suitable for both GC and sensory analysis. Aldehydes can also be measured using several colorimetric methods, such as the method that determines the anisidin value, or the widely used thiobarbituric-acid-reactive substances (TBARS) test[37]. Tertiary products, arising from interactions between oxidizing lipids and nitrogen-containing compounds, can be followed using fluorescence spectroscopy or, in later stages, by visual assessment or colorimetry[38].

All of these techniques are used in research, but only a few of them are routinely applied in the fish industry, because they are time-consuming and require expensive laboratory equipment and trained personnel. To monitor the progression of lipid oxidation, it is important to use more than one method, especially when comparing different types of fish products. Otherwise, the instability of the various oxidation products makes the results difficult to interpret and extremely misleading.

ATP as a freshness indicator in fish

Following death, ATP is rapidly degraded to inosine monophosphate (IMP) by endogenous enzymes (autolysis). The further degradation of IMP to inosine and hypoxanthine is much slower, and is catalysed mainly by endogenous IMP phosphohydrolase and inosine ribohydrolase, with a contribution from bacterial enzymes as storage time increases. The degradation of ATP was found to parallel the perceived loss of freshness of fish as determined by trained analysts[39].

ATP as a chemical indicator of freshness

A chemical index of fish freshness is appealing because it is quantifiable, objective and lends itself to automation. ATP alone cannot be used because it is so rapidly converted to IMP. Concentrations of its intermediate degradation products rise and fall, making them unreliable indexes of freshness. As a result, attention has focused on inosine and hypoxanthine, the terminal catabolites of ATP. Inosine accumulates in some species of fish whereas hypoxanthine accumulates in others as terminal catabolites[39].

In the literature, the extent of ATP degradation is expressed as the K value, which is defined as the ratio of the sum of inosine and hypoxanthine concentrations to the total concentration of ATP metabolites. A fresh fish will have a low K value. There is abundant evidence in the literature to suggest that the K value is a reliable indicator of freshness that is applicable for frozen fish, smoked fish and fish stored under modified atmospheres[39,40].

A shortcoming of the K value as a freshness index is its dependence on a variety of variables[41]. It varies between species owing to differences in rates of ATP degradation. It also varies with postmortem time and temperature storage conditions, handling conditions[42] and method of kill[43]. Thus, a profile of K value versus time must be established for each species and its specific handling and storage conditions before K-value measurements can be used to evaluate freshness.

K-value measurement

Following acid extraction and neutralization, metabolites are separated by ion-exchange chromatography or HPLC and quantified by their absorbance. Although other methods have used enzymatic assays and biosensors[39], it is generally agreed that the HPLC method is the most reliable.

The use of ATP metabolites as freshness indexes is a research technique that is not widely used in industry owing to the time and expense involved in the measurements. The future development of this approach requires cheap, reliable and rapid methods for ATP catabolite measurement.

Physical measurements

Physical changes in fish that result in the decline of freshness are mainly related to structure and colour. Texture measurements can be used to determine structural changes. The instruments used are texturometers fitted with a wide variety of accessories for the different types of analyses. The texture of whole fish muscle is difficult to measure because it lacks a uniform structure, making the preparation of samples of standard size difficult. This has led to a variety of sample preparation procedures and hence variable results and applications for the different methods. Comparisons of texture measurements of fish with sensory analysis have shown good correlation in some cases[44,45].

Another means of assessing the structure of fresh fish is microstructural characterization of the fish muscle. Different techniques are available: macroscopy [low

(2–10 times) magnification], light microscopy, confocal laser scanning microscopy, scanning electron microscopy and transmission electron microscopy. Structural changes to fish collagen during the postmortem period and their effects on further processing have been described by Bremner[46]. Weakening of the pericellular connective tissue has been shown to be one of the reasons for postmortem tenderization of fish muscle[47]. Postmortem changes to the microstructure of cod and salmon also affect the liquid-holding capacity[48].

Changes in fish freshness can also be determined by measuring the electrical properties of the fish muscle. Three different instruments are available to measure the change in electrical properties: the Torrymeter (Distell Industries Ltd, Fauldhouse, West Lothian, UK), the Fishtester VI (FT) (Intellectron International Electronics, Hamburg, Germany) and the RT-Freshness Grader (RT Rafagnataekni, Reykjavik, Iceland), which all show good correlation with sensory scores of fish freshness, when used within their applicable range of operation[49,50–52]. These meters cannot be used with thawed fish or fish that have been stored in chilled seawater, and their use for fillets is limited to a few days; water-ice with a high salt content and mechanically damaged fish cause erroneous results. The advantage of electrical testers is their immediate response and their suitability for field use and for use by personnel without previous experience.

Changes in fresh fish can be related to changes in colour measurements. Instrumental colour measurements are becoming important in quality control in the food industry. Advanced technologies have simplified the performance of these methods[53]. Recently, the effects of icing and storage temperatures on salmon quality attributes, and the postmortem changes in colour of carp and trout were studied[54,55]. However, there is a need for the standardization of instrumental colour measurements.

During the past few years, spectroscopic methods have gained importance in the evaluation of food quality parameters. The advantages of spectroscopic methods are their ability to provide rapid analysis and simultaneous evaluation of several parameters, and their potential for on-line or at-line use. These techniques have also been introduced into seafood analysis. Fluorescence spectra can be used to indicate whether a fish has been frozen, and the intensity of the fluorescence from fish muscle decreases with storage time on ice[56]. Nagashima *et al.*[57] reported the use of absorbance spectroscopy in the UV-visible range to determine the freshness of yellowfin tuna. Although spectroscopic methods have so far not proven sufficient to characterize fully the properties of fresh fish, developments in instrumentation and the techniques used to evaluate spectral data are likely to facilitate the collection of more information on the characteristics of fish. An example of this is the preliminary research performed at Fiskeriforskning in Norway, which indicates that the application of near-infrared spectroscopy may reveal information on the storage time of fresh fish.

Time–temperature indicators (TTIs) are devices or materials that can be attached to, or incorporated into, foods to indicate the time–temperature history of the food. The time–temperature history is recorded by using some biological, physical or chemical process that depends on time and temperature. The temperature record can be used to develop appropriate models of the effects of bacterial or enzymatic spoilage on shelf life[58,59]. It is likely that TTIs will gradually be introduced into the wholesale and retail food chain, starting with temperature-sensitive, high-value foods such as fish.

Although there are many different physical measurements that provide information on parameters related to fish freshness, as discussed above, none of these methods is able to determine unambiguously whether a fish is fresh or not.

Box 2. Conclusions and future aims for fish freshness evaluation

- **Sensory evaluation** is currently the most important method for freshness evaluation in the fish sector. The trend is to standardize sensory evaluation by improving the methodologies and training of panels to make sensory evaluation an objective measurement.
- The development of **microbial methods** for evaluation of fish freshness together with mathematical models expressing the effects of storage conditions (temperature and atmosphere) on the growth of relevant spoilage microorganisms has been suggested.
- The rapid assessment of **volatile compounds** in fish using gas sensors to determine freshness is of increasing interest.
- To date, there are no rapid methods to determine changes in muscle **proteins** during postmortem storage.
- Most of the techniques that have been described to monitor **lipid** oxidation are suitable only for research purposes, and few are routinely applied in the fish industry. To monitor the progression of lipid oxidation, it is important to use more than one method, especially when comparing various types of fish products.
- The use of **ATP** (adenosine triphosphate) metabolites as freshness indexes is a research technique that is not widely used in industry. The future trend is the development of rapid techniques for the measurement of ATP metabolites.
- The following **physical measurements** provide information on parameters that are related to fish freshness. However, none of these methods is sufficient to determine unambiguously whether a fish is fresh.
  - It is likely that **time–temperature indicators** will gradually be introduced into the wholesale and retail food chain, starting with temperature-sensitive, high-value foods such as fish.
  - **Texture measurements** of fish have been compared with sensory analysis, and some studies have shown good correlation.
  - Changes in the **microstructure** of fish muscle have been related to the postmortem tenderization and liquid-holding capacity of fish muscle.
  - Changes in the freshness of fish can be monitored by measuring the **electrical properties** of fish muscle. The advantage of electrical testers is their immediate response and their suitability for field use and for use by personnel without previous experience.
  - Changes in **colour measurements** can be related to changes in fresh fish.
  - **Spectroscopic methods** have recently gained importance in the evaluation of food quality parameters.

Future aims for fish freshness evaluation

The overall conclusions and future trends for the methods of fish freshness evaluation that have been reviewed in this article are summarized in Box 2. The

future aim is to combine different standard methods that use rapid measurement techniques with a mathematical model to predict the freshness, number of days postharvest or remaining shelf life of an unknown fish sample. Such a model could complement sensory analysis for fish freshness evaluation in the near future. However, more research is needed; this should include controlled storage experiments of different species of fish, to obtain valid parameters for use in mathematical models.

Contributing coauthors

This review article is a collaborative work of the participants of the concerted action project 'Evaluation of Fish Freshness' (AIR3 CT94-2283), funded by the AIR programme of the European Union. Special thanks are due to the following, who have contributed to the preparation of this manuscript: J. Luten, M. Etienne, K. Randell, M. Proctor, P. Nesvadba, R. Schubring, K. Heia, J. Borderias, K. Nilsson and B. Jørgensen.

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