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Evaluation of Fish Tainting

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EVALUATION OF FISH TAINTING

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CONTENTS

A. SUMMARY AND CONCLUSIONS

1. A Test Guideline has been established for determining the potential of a chemical to taint seafood. This Guideline is based on the exposure of fish and the evaluation of the imparted taint by a triangular test.

2. The Test Guideline has been evaluated in a small ring test in which five laboratories tested four chemicals with three fish species.

3. Based on the limited results of the ring test, none of the following factors appear to have a systematic effect on the evaluation of the potential to cause taint :

   a) the performing laboratory;
   b) type of water used (sea- or freshwater);
   c) fish species;
   d) evaluation by flavour or by odour;
   e) method of preparation and presentation of samples for sensory evaluation.

4. One laboratory tested four chemicals following the proposed Test Guideline and the GESAMP guideline. Concordant results were obtained.

5. The proposed Test Guideline also contains a procedure for estimating the loss of taint from the test species during a depuration phase in clean water.

6. The hazard that a chemical will, after an accidental spill from a chemical tanker, taint seafood, depends not only on the intrinsic property of the chemical to cause tainting but also on the exposure of the seafood to the chemical. The probability that the tainted seafood will be marketed is determined by the likelihood of a spillage occurring, the intrinsic tainting property of the chemical, the exposure conditions and the likelihood of the tainted seafood to be caught or harvested. Calculations indicate that this probability is minimal under normal conditions.

B. INTRODUCTION

The value of recreationally and commercially important seafood might be reduced by the introduction of tainting compounds into the sea. Tainting compounds are taken here to be chemical substances which when taken up by aquatic organisms, result in a flavour or odour that is not typical of the species.

The IMO/FAO/UNESCO/WHO/IAEA/UNEP Joint Group of Experts on the Scientific Aspects of Marine Pollution (GESAMP) considered the possible tainting of sea food by chemical substances to be an important issue. When evaluating the hazards of harmful substances carried by ships, the potential of a chemical to cause tainting is one of the parameters used by GESAMP in establishing the hazard profile of the chemical. Under the MARPOL convention (the International Convention for the Prevention of Pollution from Ships, held in 1973) the hazard profile is used by IMO (International Maritime Organisation) to allocate the ship type in which the chemical should be transported by sea (GESAMP, 1982).

A number of authors have reviewed the literature dealing with off-flavours in fish caused by chemicals (Shumway, 1970; Shumway and Palensky, 1973; Stansby, 1978; Persson, 1981, 1984). In these reviews little attention has been paid to critical assessment of the test methods used. This is striking since taste evaluations are inherently subjective and the test method might be expected to strongly influence the test result. Although some standardisation has been attempted for specific purposes (ASTM, 1978) no generally accepted test method is available which defines the main components of taint assessment : the fish exposure conditions, the fish flesh preparations, the sensory evaluation by a panel and statistical treatment of the results.

Recognising this, GESAMP recently developed "draft guidelines for evaluating threshold values for fish tainting" (GESAMP, 1983) thereby stimulating efforts to develop generally acceptable standard test guidelines.

Having established guidelines for taint testing it is important to be able to evaluate the likelihood that a chemical with the potential to cause taint will in fact taint fish in the marine environment as a result of accidental spills or the discharge of tank washings.

In view of the requirement for a more detailed protocol for determining the potential for taint and the need to consider this potential in relation to the fate of chemicals in the sea a Task Force was set up. The Terms of Reference were :

1. To develop within the framework of the GESAMP guidelines (1983), a test guideline for determining the concentration of a chemical in water which taints the flesh of fish and, in addition, the subsequent effect of depuration.

2. To verify the test guideline experimentally and to modify it, as necessary, in the light of the results.

3. To consider in relation to the threshold tainting concentration the biological, physical and chemical factors which should be taken into account to assess the likelihood that a chemical will taint fish in practice.

C. BACKGROUND

Ships which transport chemicals in bulk by sea might introduce them into the marine environment as a result of accidents or permitted discharges (e.g. tank washings). When a chemical with the potential to cause the tainting of seafood is introduced into the marine environment the hazard it poses with regard to the tainting of seafood should be established. Factors which have to be taken into account when assessing this hazard are : the inherent flavour or odour of the chemical, the amount of chemical which might be introduced and the biological, physical and chemical factors which determine the fate of the chemical in the sea and subsequently in the marine organisms.

This report considers the establishment of a test guideline and the interpretation of the results from fish taint tests, taking into account the above mentioned factors.

According to the GESAMP guideline (1983) the laboratory experiment should represent an "acute case" i.e. provide information about the tainting of

commercially important seafood organisms which might occur following a short term exposure.

The probability of a tainted organism being caught is lower with tainting compounds which are rapidly metabolised to non-tainting substances or which are rapidly excreted. The guideline developed here for determination of taint also allows for an assessment of the importance of depuration.

D. ESTABLISHMENT OF A TEST GUIDELINE

1. Introduction

The aim of the guideline is to provide a procedure for the evaluation of the potential of a chemical to taint seafood. Whilst the importance is recognised of the concept of a taint threshold value, that is of a minimum concentration of a substance in water which results in tainted seafood, neither the definition of taint threshold value nor the principles by which it can be measured are established. ECETOC has therefore confined itself to establishing a procedure which allowing categorisation of chemicals according to the potential to cause taint.

An assessment of tainting must include a period of exposure of the test organism to a test substance followed by determination whether taint is present. In many situations the organisms exposed to tainting chemicals will have an opportunity to depurate before consumption; therefore consideration was given to a procedure to determine whether depuration occurs.

In outline, a procedure for evaluating the ability of a chemical to cause taint involves exposure of a test-organism to a chemical under defined conditions, followed by sensory evaluation to determine if the exposed organism is tainted. Depuration is then tested by transferring some exposed organisms to water free of the test substance and re-evaluating of the organism for taint (cf. Appendix 1).

2. Exposure of Fish

2.1. Information on test substance

The substance tested should be the grade which is transported in bulk at sea. A knowledge of the solubility, density, melting- and boiling-points, and the vapour pressure of the test substance at ambient atmospheric conditions is required. This information can be used to predict the behaviour of the test substance during exposure of the test organism and hence to determine the nature of the test system required, e.g. static aerated, semi-static with or without aeration, continuous flow or sealed.

Information on the hazard of the substance to man will also be required since certain health hazards may preclude its evaluation by the method of test described here.

2.2. Test organism

The GESAMP guideline for the assessment of tainting (GESAMP, 1983) refers to the "liability of substances to taint seafood". A wide variety of animals and plants can properly be considered as seafood; clearly it is not possible to test all seafoods.

The three principal groups of seafood organisms are fish, crustaceans and molluscs. It is clear that in various respects crustaceans and molluscs differ from fish, but the latter was selected as the most suitable group on which to base a guideline for assessment of taint because :

- they are the major source of seafood;
- they are readily available for testing; many species are capable of being reared in the laboratory and some are farmed on a commercial basis;
- they are maintained relatively easily under laboratory conditions;
- they are used extensively in aquatic toxicology. More laboratories have experience with fish than with molluscs or crustaceans.

The use of a marine fish species was considered to be desirable but no generally acceptable species could be found. The main reason for this was the limited availability of species of a suitable size and the lack of data on the acute toxicity of chemicals to marine fish in general.

Rainbow trout (Salmo gairdneri Rich.) was chosen as the preferred test organism. This fish trout is widely available, it has a moderate fat content and can be relatively easily kept in the laboratory. Being a euryhaline fish it can live in either fresh- or sea water and, if necessary, can be adapted from one medium to the other. In some situations it may be difficult to use rainbow trout as a test species and in such cases other species such as red sea bream (Pagrus maior) or carp (Cyprinus carpio) are considered suitable. Since the primary purpose of the test guideline is the determination of the taint level of chemicals transported in bulk by sea, sea water is the preferred medium, but the guideline can be used with freshwater.

Considerable evidence indicates that the fat concentration in fish may influence the accumulation of certain chemicals in the flesh. To ensure the comparability of data the fat content of the edible portion of the fish to be used for testing should be determined and lie in the range 3-10%. There are a number of suitable methods for determining the fat content (cf. Appendix 2).

The fish should not be fed immediately prior to or during the test since this removes the possibility of uptake of taint from test substance adsorbed onto the food and reduces the faecal contamination of the water. Knowledge of the previous diet of the fish is important in ensuring that no strong or atypical flavours result from the diet and mask the taint from the test chemical. If mortalities occur the test conditions should be reassessed.

2.3. Duration of exposure

Choice of the duration of exposure must inevitably be a compromise. In the environment some organisms might be exposed to high concentrations for short periods, others to low concentrations for long periods. It was considered that in general an exposure period of 24h, as recommended by GESAMP (1984), was acceptable.

2.4. Exposure concentrations

An initital test, or limit test, should be carried out at a concentration of 10 $mg.l^{-1}$, or 1/10 of the 24h LC_{50}, or the limit of water solubility level whichever is the lowest. For most chemicals a concentration of 10

$mg.l^{-1}$ was considered to be the maximum concentration likely to be sustained over a period of 24h in the environment following an accidental or operational discharge from a ship (cf. Chapter F). The alternatives, of testing at 1/10 of the 24h LC_{50}, and the limit of water solubility concentration, are included to ensure that few if any of the exposed organisms will die during the test and that only concentrations below the water solubility are tested.

If taint is detected in the limit test further testing at at least two supplementary exposure concentrations (1.0 and 0.1 $mg.l^{-1}$) should be carried out. When taint is detected in the limit test the depuration should be used to assess the persistence of the taint.

Analysis of the test substance in the exposure medium at the beginning and at the end of exposure is necessary to check the stability of the concentrations throughout the test. The exposure concentration of the substance at the end of exposure should be within $\pm$ 50% of its initial concentration.

The use of co-solvents should be avoided if at all possible but they may be necessary when making up stock solutions of substances with low water solubility. If a co-solvent is used, evidence must be available to show that it is not capable of imparting taint to the test organism.

2.5. Test conditions

The preferred exposure medium is sea water and the fish should be acclimatized to it so that exposure to the test chemical does not occur whilst the fish is in a stressed condition. Acclimatization of Salmo gairdneri of the size required for testing can be achieved. The salinity of the sea-water should be 32-36 o/oo , encompassing the range typical for the open sea. Either natural or an artificial (reconstituted) sea-water is acceptable. In both cases care should be taken to ensure that the batch of water used does not itself possess any potential for taint. This should be checked by smelling the water and tasting some of the control fish before the experiment. Its detection at an earlier stage will avoid unnecessary expenditure and delays. In all tests a batch of control fish should be held under the same conditions as the exposed fish except that no test substance is present.

Continuous flow tests are considered preferable although semi-static or static tests are not excluded. Fish should not be stressed by overcrowding; the concentration of dissolved oxygen should remain above 60% of the air saturation value throughout the test. The water temperature should be controlled at 15 ± 2°C. Other fish species may need to be kept at a different temperature. The pH of the sea water is unlikely to vary significantly during the test but initial and final pH values should be recorded. It is advisable to check that concentrations of nitrite nitrogen in the water are less than 0.1 $mg.l^{-1}$ as concentrations in excess of this might cause mortality of Salmo gairdneri.

3. Uptake and persistence of taint

GESAMP (1982) stated that "where a substance causes taint it would be given a "T" rating even though it is known to have a relatively short half-life in the animal". Although GESAMP knew that substances can depurate from animals, they decided against the inclusion of a depuration phase in the assessment of taint. The purpose of a depuration phase is to allow an assessment of taint retention once exposure has ceased. Depuration might occur when free swimming organisms move out of the contaminated zone, when the contaminated water itself moves away from the zone the organisms inhabit, or when the compound itself rapidly decomposes. The mechanism of depuration can be passive physical loss, active metabolism or a combination of both.

GESAMP's decision not to include a depuration phase was made because they considered that "though absorption of chemicals is rapid, depuration is very slow" (GESAMP, 1984). There is, however, sufficient evidence in the literature to question the validity of this statement. Numerous experiments with different chemicals including a number known to impart taint to fish, have demonstrated a short "half-life" time for residues in fish tissues. It is, however, accepted that depuration will not always be rapid. The uptake and persistence of a chemical in the fish, and hence the persistence of taint, is dependent on :

- the concentration of the chemical accumulated during exposure;
- the rate of loss of the chemical due to metabolism;
- the rate of excretion of the chemical by active or passive means.

Experience has shown that the levels to which chemicals are accumulated under similar and constant conditions of exposure can differ greatly (Hamelink and Spacie, 1977). In addition, rates of depuration, once exposure has ceased, can vary from substance to substance (Binder et al.,1984).

3.1. Uptake

It is now widely accepted that non-ionized substances with a log-partition coefficient for n-octanol/water (log Pow) greater than three have the potential to bioaccumulate to a significant extent in aquatic organisms (OECD, 1984).

The organs in which the substance accumulates, e.g. adipose tissue, muscle or liver may differ according to the nature of the chemical. Conell (1978) demonstrated a highly significant correlation between the percentage of accumulated hydrocarbons in muscle tissue and the percentage of lipids in the muscle tissue.

3.2. Metabolism

Fish possess the ability to metabolize many xenobiotic compounds (Dewaide, 1971). Many fish species have a complement of enzyme systems (hydrolytic, reductive conjungative and mono-oxygenase systems) (Foureman and Bend, 1983) or show an activation of the enzyme systems capable of metabolizing xenobiotics (Elcombe et al.,1979).

3.3. Depuration

There are numerous examples of rapid depuration of chemicals from fish tissues. Veith et al.(1980) reported half life values of less than 24h for 16 out of 25 chemicals to which fish had been exposed for periods of 14-28 days. Persson (1984) reported the uptake and depuration behaviour of six chemicals and for three of these, purging times for the taint was 24h or less. Rapid depuration of naphthalene and methylnaphthalene was also recorded by Korn and Rice (1981). Branson et al. (1979) showed that fish tainting residues of diphenyl oxide were reduced by 50% each day. They also found a significant correlation between fish taint and the level of residues in the fish flesh. Ogata and Miyake (1978) found the half-life of benzene, toluene, m- or p-xylene and o-xylene to be 0.5, 1.4, 2.6 and 2.0 days respectively. However there are cases where the depuration times are

long, e.g. 2,5,2',5'-tetra-chlorobiphenyl has a half life of 1.76 years (Binder et al., 1984, Persson, 1984).

In light of the above evidence showing that relatively rapid depuration may be a common phenonenon it was thought desirable to include a depuration phase in the test procedures to be followed.

3.4. Comparison of Freshwater and Marine Fish

No evidence is available to suggest that different accumulation or depuration processes for chemicals occur in marine- and freshwater fish. On the contrary, investigations of toxicity and bioaccumulation with fish from both environments have given similar results (Niimi, 1983, Dawson et al., 1975/1977, Zaroogian et al., 1985). Further comparative data on the uptake and loss of tainting chemicals in fresh- and sea water fish are, however needed.

4. Sensory Evaluation of Taint

4.1. Preparation of fish for evaluation

The edible portion of fish gradually change in flavour on storage. If stored under refrigeration these changes are small over the first 48 h and will not affect evaluation of taint. Fish is prepared for cooking and subsequently cooked. This in itself imparts flavour or odour to the product. This potential problem can be overcome by the use of adequate controls and the arrangements of the triangular test. Suitable procedures for cooking include cooking in closed containers, in steam or boiling water, or by microwaves. The edible portion of the fish is then presented to the panel for sensory evaluation.

4.2. The choice of the triangular test for detecting taints

The experimental procedures described in these guidelines are intended to determine whether or not fish are tainted following exposure to chemicals in the water in which they are held. In this context taint is any flavour or odour foreign to the fish (ISO, 1983-a) and is not used in the more restricted meaning of an unpleasant flavour or odour.

Taint is a subjective experience and can only be evaluated by human sensory organs. A suitable procedure for evaluating whether or not a sample is tainted was considered to be the triangular test. In this

procedure an assessor is presented with 3 samples, 2 are identical (either test or control material) and the third is of the other treatment. The assessor is required to identify the single specimen from the pair. So long as the experimental procedure as laid down by ISO (1983-b) is followed, the test is free of bias. Appendix 3 gives more details why this technique was chosen.

4.3. Selection of assessors

On the basis of the principles described in Appendix 2, a panel of 15-20 people typical of the general population of consumers is recommended. The assessors should have some familiarity with the triangular test procedure and the normal flavour or odour of the species of fish used in the test. They should not be specially selected for acuity to the chemical under test.

There is a possibility that the assessment by the panels may differ consistently between and within laboratories and change as they become more experienced. These systematic differences are, however, likely to be small in comparison with other sources of variation arising from the experimental procedures.

4.4. Evaluation of results from a triangular test

The triangular test provides data which is amenable to statistical analysis. Detail instruction is given in ISO 4120 procedure (ISO, 1983-b). A discussion of the statistical principles involved is included in Appendix 2.

E. VALIDATION OF GUIDELINE

1. Participants in the Ring-test

Four West-European laboratories (3 industrial and 1 governmental) and one Japanese "contract" laboratory participated in the ring-test. Three laboratories performed both the exposure and the taint assessment phases. The other two laboratories only carried out the exposure of the fish to the chemicals and used specialised contract laboratories for the taint assessment.

Participating laboratories were : ICI (Brixham, UK), Hoechst (Frankfurt, FRG), Shell (Sittingbourne, UK), Torry (Aberdeen, UK), JETOC (Chemical Inspection and Testing Institute (Kurume, Japan)). The following laboratories participated in the assessment of taint : Food Preservation Research Association (Campden, UK) and Bundesforschungsanstalt für Ernährung (Karlsruhe, FRG).

2. Choice of Test Chemicals

The four test chemicals were butanol, pyridine, 1,2-dichlorobenzene and 2,4-dichlorophenol. In addition, two laboratories tested styrene. The principal properties of the test chemicals are summarised in Table 1. The test chemicals were chosen to encompass a range of octanol/water partition coefficients, solubilities in water and LC_{50}'s for fish. The chemicals also had a low acute toxicity to humans and no carcinogenic potential. Their taint classification according to GESAMP is given in Table 1.

3. Choice of Test Methods

Although, in the proposed guideline, preference was expressed for performing the tests in sea water, for practical reasons some laboratories conducted the tests with freshwater. Also one laboratory performed some tests according to the original GESAMP guideline (GESAMP, 1983). Four laboratories used rainbow trout and one used sea bream and common carp. For 1,2-dichlorobenzene and 2,4-dichlorophenol with the lowest LC_{50} values for fish the highest test concentration had to be reduced from 10 to respectively 5 and 4 $mg.l^{-1}$. The panel of one laboratory assessed tainting by odour only.

4. Test Results

The test results are summarised in Table 2. All concentrations given in Table 2 are nominal but measured concentrations were within the limits set in the test guideline.

5. Discussion of the Results

5.1. Comparison of results within laboratories. In general, for the chemicals which cause taint, there was a dose-response relationship. At low concentrations the number of assessors who correctly selected the odd sample is around 1/3 of the total, the proportion to be expected if all the panel members were guessing. This pattern is illustrated well in the results for pyridine, 1,2-dichlorobenzene and styrene. The results for a

particular chemical within a laboratory are what would be expected from the principles discussed in section D.4. and Appendix 2.

There is one anomaly in this set, the results of laboratory 1 for butanol, where a significant difference was found at the lowest concentration and not at higher concentrations, but even here the proportion of 9 correct out of 15 selections is just on the border of significance at the $p = 0.05$ level.

5.2. Comparison of results among laboratories. Systematic differences among laboratories could come from differences in exposure of the fish, in performance of the sensory analysis or in sensitivities of the assessors in the triangular test. The experimental design cannot distinguish between these factors and differences could be attributed to any of them, singly or in combination. It should be noted that the procedure used by laboratory 3 differed from that in the other laboratories in two important respects : they employed carp in freshwater and red sea bream in salt water whereas other laboratories used trout in both media, and they assessed the samples by odour whereas assessors in the other laboratories assessed them by taste and odour. Despite these differences the results from this laboratory are very similar to those from the other laboratories.

An inspection of the data in Table 2 does not reveal an obvious systematic difference between the results from the laboratories. Because the laboratories did not test all chemicals at the same concentrations, it is not possible to test the data by analysis of variance to determine laboratory effects. The best that can be done is to compare for a single concentration the results from the different laboratories (i.e. by rows in Table 2). If it is assumed that the results from each laboratory are a sample from a population of results then the best estimate of the proportion of correct selections for that population is obtained by pooling the results across laboratories. The pooled proportion can be used to calculate the expected number of correct selections for a laboratory that can be compared with the observed number in a Chi-squared test. It was found that the Chi-squared was not significant ($p=0.05$) and the original hypothesis that the data come from a single population therefore

cannot be rejected (no statistically-significant difference between the results of the laboratories).

5.3. Comparison between salt water and freshwater. There are 3 sets of results where a chemical was tested with fish in freshwater and in sea water. These 3 tests were carried out at 3 concentrations and included a depuration phase : laboratory 2 with pyridine and laboratory 3 with 1,2-dichlorophenol and styrene. There was a close correspondence between the results in the two media except for the data from the depuration test for styrene. The difference here, 5 out of 18 assessors detecting taint in sea water compared with 12 out of 18 in fresh water, is significantly different, $p = 0.01$, when tested by the comparison of proportions test. There is a single comparison by laboratory 1 of butanol at one concentration which gives different results - tainting in sea water but no tainting in freshwater.

Though there are only a limited amount of data available we may conclude that there are no striking or consistent differences in tainting results for freshwater and salt water experiments.

5.4. Tainting potential of individual chemicals. Only 1 out of 5 trials with butanol at a concentration of 10 $mg.l^{-1}$ produced a positive taint result and we may conclude that butanol does not taint fish even at the highest concentration tested (10 $mg.l^{-1}$). The result from the laboratory 1 trial at 0.1 $mg.l^{-1}$ shows significant tainting but in view of the results at higher concentrations this result must be considered anomalous.

The data for pyridine show a consistent pattern. It does not taint at the 1 $mg.l^{-1}$ or 0.1 $mg.l^{-1}$ levels. At 10 $mg.l^{-1}$, 4 out of 6 trials show a significant taint and it must be concluded that pyridine does taint at this concentration. Five out of six trials showed no tainting after depuration. It would seem that pyridine depurates quickly as defined by the procedure of this protocol.

Three out of 4 trials for 1,2-dichlorobenzene at 1.0 $mg.l^{-1}$ and 3 out of 4 trials at 0.5 $mg.l^{-1}$ indicated tainting of the fish. At 0.1 $mg.l^{-1}$ only 1 out of 4 trials showed tainting. It can be concluded that

1,2-dichlorobenzene taints at 0.5 $mg.l^{-1}$ but probably not at 0.1 $mg.l^{-1}$. Three of the depuration trials started from an exposure concentration of 0.5 $mg.l^{-1}$. None showed retention of taint. Three depuration trials started from an exposure of 1.0 $mg.l^{-1}$. One out of three trials showed retention of taint. These results suggest that the extent of depuration depends on the initial exposure concentration. Since some laboratories found mortalities at concentrations above 1 $mg.l^{-1}$ it can be concluded that depuration would occur rapidly after exposure to non-lethal concentrations.

2,4-Dichlorophenol taints at low concentrations; 4 out of 6 trials showed a significant taint at 0.1 $mg.l^{-1}$, the lowest concentration tested. The taint also appears to be retained. The results for this chemical indicate differences among the laboratories though these were not sufficient to be significantly different by the Chi-squared test. Two laboratories had very high proportions of correct selections at the 2 concentrations tested, 0.4 $mg.l^{-1}$ and 0.1 $mg.l^{-1}$; the other laboratories had low rates of detection and even where the results were significant, the numbers of correct selections were only at the minimum significance level. The laboratory 4 results were anomalous in that a tainting was not detected in fish exposed to 0.4 or 0.1 $mg.l^{-1}$ but was detected in one of the two depuration tests. As the pK_w value of this compound is very close to the pH at which the tests were conducted, it is possible that some differences in results between laboratories can be attributed to differences in degree of dissociation of the compound.

Two laboratories tested styrene. Laboratory 2 did not detect significant taint at 1.0 $mg.l^{-1}$ or 0.1 $mg.l^{-1}$ but anomalously detected taint in the depurated sample. Laboratory 3 detected taint in carp in freshwater at 5.0 $mg.l^{-1}$ and 1.0 $mg.l^{-1}$ and in red sea bream in salt water at 2.5 $mg.l^{-1}$ but not at 1.0 $mg.l^{-1}$. It would seem that styrene is on the borderline of tainting at 1.0 $mg.l^{-1}$. Depuration data are inconlusive.

The results obtained with the proposed detailed guideline are consistent with those obtained using the GESAMP framework guideline.

6. Necessity of Further Research

Although the results of the limited ring test have shown that the proposed guideline is satisfactory for the detection of chemicals which may taint fish, various gaps in our present knowledge exist. These may comprise the extent of the uptake and loss of chemicals by freshwater and seawater fish (with different lipid contents) and this for lipophylic, hydrophylic and ionic chemicals. A separate study could be devoted to determine the relationship in tainting potential between freshwater fish, sea-water pelagic and benthic fish and crustaceans and molluscs.

F. HAZARD ASSESSMENT FOR TAINTING OF SEAFOOD AND PROBABILITY OF CONSUMING TAINTED SEAFOOD

1. Hazard Assessment

In order to assess the likelihood that a chemical which causes tainting under defined experimental circumstances will cause tainting under real environmental conditions, the experimental and real environmental conditions must be compared. In this comparison the following factors have to be taken into account :

i) biota other than the test species might be exposed to the chemical;
ii) the nature and extent of exposure of seafood organisms to the chemical in the sea after a spill will not necessarily be represented by exposure in the laboratory test;
iii) the rate of depuration of tainting chemicals from exposed organisms.

1.1. The Biota

Since the criteria for selecting rainbow trout as the test species were practical in nature, the degree to which rainbow trout are representative in determining the potential of chemicals to cause tainting of seafood organisms might be considered limiting. Suitable species for testing might be found in marine pelagic or benthic fish species (e.g. cod, plaice), in pelagic or benthic molluscs (e.g.squid, mussel, oyster), pelagic and benthic crustaceans (e.g. shrimp, crab, lobster) and even seaweeds. However, tainting data from species other than fish, as well as comparisons between different fish species, especially those with

different fat contents, and between freshwater and sea water species, are scarce. Limited data from the small ring-test described in this report suggest that the differences between rainbow trout and carp, and between rainbow trout tested under freshwater and sea water conditions, are relatively small. More comparative research in this field is needed. For the time being, and mainly for practical reasons, rainbow trout appears to be a suitable species to determine the tainting potential of chemicals.

1.2. Environmental Exposure after a Chemical Spill from a Ship

Following a spill from a chemical tanker at sea, the exposure of marine organisms can be very different from the laboratory experimental situation and is strongly dependent not only on the physico-chemical properties of the spilled chemical but also on environmental parameters.

Since the tainting hazard is a function of the tainting potential of a chemical and the exposure conditions, it is justifiable to assess the experimentally determined tainting potential in relation to the exposure under realistic environmental conditions. To facilitate such an assessment, a proposal to rank chemicals on the basis of exposure concentrations predicted to occur under defined conditions at sea was presented by Poels and Wolff (1986).

Wolff and Poels (1986) used the following spill characteristics and environmental conditions to calculate exposure concentrations. The volume of the various tanks in a chemical tanker usually ranges from 60 m^3 to 2000 m^3 and were taken as extreme input data. Furthermore, it was assumed that spill could be very fast (instantaneous release) or extended (24 hours). The wind speed was taken to be 10 and 30 $m.s^{-1}$ because these values represent respectively average calm weather conditions and rough weather conditions. The water depth was taken to be 20 and 200 m, representing respectively depths of coastal water and of deeper seas where a thermal inversion layer occurs. The temperature of the water was taken as 10°C.

With all chemicals, the concentration is highest near the centre of the spill. The concentration of the chemical in water may vary significantly mainly depending on the water solubility. Concentrations in water exceeding 10 $mg.l^{-1}$ will only be found in a very restricted area near the

centre of the spill and this area will decrease with time. It was assumed for this reason that 10 mg/l is a realistic worst case value for the concentration of a spilled chemical in sea water and therefore this was chosen as a limit value for testing.

As indicated before, the approach taken by Wolff and Poels (1986) is conservative in that the values presented for concentrations and volumes are maximum values. In addition, transformation processes such as biodegradation and photodegradation are not taken into account, nor are adsorption phenomena which could especially be important for compounds which sink.

Although there are many groups of organic chemicals with similar physico-chemical properties, for practical purposes only three groups are considered in relation to environmental concentrations, contaminated surfaces, and volumes after a spill. These three groups are defined by the following environmentally relevant physico-chemical properties : water solubility, density and vapour pressure.

1.2.1. Compounds soluble in sea water. Water soluble compounds (e.g. pyridine, butanol, aniline) spilt from a chemical tanker will dissolve quickly and will not float or sink. The area as well as the volume of water with concentrations of up to a few hundreds of $mg.l^{-1}$ is restricted and limited to the centre of the spill. With time the concentration of the contaminants in water decreases while the volume of contaminated water increases. The volume of contaminated water one week after a spill of 60 or 2000 m^3 is of the same order of magnitude. The maximum volume of contaminated water directly after spill is about 0.001 km^3 and less than 0.01 km^3 after 7 days. This corresponds with maximum surface areas of respectively 0.05 and 0.5 km^2 (Wolff and Poels, 1986). Pelagic and benthic organisms will be exposed to these compounds after a spill, although benthic species are likely to be exposed to a lesser extent.

1.2.2. Poorly-soluble compounds with a density lower than sea water. Upon spillage, chemicals like benzene, xylene and styrene float on water, evaporate quickly. This, together with their limited water solubility, leads to less than 1% of the total spilt mass dissolving. It was shown by Wolff and Poels (1986) that compounds similar to benzene and xylene will

not reach a concentration of 1 $mg.l^{-1}$. The total contaminated volume directly after the spill does not exceed 0.001 km^3. The contaminated volume will be less than 0.1 km^3 after 7 days with a concentration around 0.01 mg/l. The surface of contaminated sea water immediately after the spill was calculated to be 0.01 - 0.13 km^2 (diameter 110-400 m) and 0.5 - 2 km^2 (diameter 800-1600m) after 7 days for xylene and benzene like compounds (Wolff and Poels, 1986). Only pelagic organisms are likely to be exposed to these compounds.

1.2.3. Poorly-soluble compounds with a density higher than sea water. These compounds (e.g. 1,2-dichlorobenzene and 2,4-dichlorophenol), after spillage, will sink to the bottom of the sea and spread over the seabed. The area initially covered is very small (the diameter varying from 12 up to 90 m, depending on the volume spilt and sea depth). This area may increase rapidly after the spill depending on many factors such as seabed topography and the current speed (Wolff and Poels, 1986).

Ultimately, the total volume of non-volatilised or degraded product spilt will dissolve in the sea water, the time period being primarily dependent on factors such as water solubility and current speed. The concentration of the chemical in water is highest directly below the spill. Concentrations of 0.03 and 1.2 $mg.l^{-1}$ were calculated for 1,2-dichlorobenzene and 2,4-dichlorophenol a short time after the spill. Depending on the water speed (0.5m/sec) a 10 resp. 100 fold dilution will take place within a 3 - 24 h period the contaminated volume of sea water being between less than 0.1 km^3 and 3 km^3 (Wolff and Poels, 1986). Compounds that sink to the bottom of the sea will adsorb on sediments. A slow release will lead to a presence of such compounds in sea water for longer periods although at very low concentrations. The hazard of these compounds is estimated to be higher than for compounds that float on sea water. For sessile organisms the hazard is higher than for free swimming (pelagic) organisms because they cannot avoid the contaminated area. The hazard for bottom dwellers will be intermediate between that for pelagic and sessile organisms.

1.3. Depuration of Tainting Chemicals from Seafood Organisms

As previously reported some chemicals are depurated rapidly from aquatic organisms. The depuration rate will be largely dependent on the properties of the chemical itself and the ability of the exposure species to metabolise it. The exposure concentration is important since higher concentrations will lead to a higher initial uptake in shorter exposure periods. In such cases the chance that the tainting threshold concentration will be surpassed is higher.

2. Probability of Consuming Tainted Seafood

The probability that tainted fish will be consumed depends on :

a) the probability of an accident with a chemical tanker leading to a spill of a chemical with tainting properties;
b) the hazard of the spilt chemical to cause tainting of fish;
c) the probability that tainted fish will be harvested.

The assessment of the probability of an accident is outside the scope of this report. The hazard that a chemical will cause taint in fish is discussed in the previous paragraphs. The probability of a tainted fish being harvested after a chemical spill depends on a numbers of factors, the most important being :

- the area and volume of the contaminated water or seabed;
- the volume of water and/or area of seabed swept by fishing operations;
- the duration of a haul and speed of the fishing boat (cf. Appendix 4).

Taking these factors into account two examples will be presented to illustrate the probability of harvesting tainted fish after a chemical spill.

Example 1 : In the case of a 2000 m^3 spill of a water soluble compound where a 0.3 km^2 area (diameter 400 m) is contaminated, pelagic trawling with a haul of 30 minutes and passing the contaminated area will only scoop up fish from less than 1% of the contaminated sea volume.

Example 2 : In the case of a 2000 m^3 spill of a relatively soluble compound

with a density higher than water (e.g. o-dichlorophenol) mainly benthic organisms are at risk. Assuming fish are caught with a bottom trawler, (duration of a haul is 4 h and speed 6 km/hr), and assuming that the trawler fishes over the full length of the plume of contaminated water, about 20% of the total volume fished during the haul will be from contaminated water. Where the direction of the trawler is at right angles to the plume only about 2% of the total volume fished is from contaminated water.

In both examples it is assumed that the chemical in the total volume of contaminated water is above the concentration causing taint. In fact the major part of the volume will have a concentration below that causing tainting.

The data used in these examples are taken from Appendix 3 and Wolff and Poels (1986) and suppose the absence of warning of a significant chemical spill from a bulk tanker, the absence of avoidance reactions of fish and random distribution of the fish in the contaminated and uncontaminated areas.

It is concluded from the above that the probability of consuming tainted fish after a chemical spill at sea is very limited.

G. RECOMMENDATIONS

1. Exposure of seafood organisms will be influenced by many factors including the quantity of the chemical spilt, the physico-chemical properties of the chemical and the state of the sea. Any hazard assessment should therefore take into account the intrinsic tainting potential of the chemical and the exposure conditions.

2. When regulatory bodies evaluate the hazard that chemicals will cause tainting of seafood organisms they should take account of their ability to depurate the chemical.

3. The models used to predict the environmental distribution of chemicals following an accidental spill from a ship at sea should be further validated.

4. The feasibility of a simple screening test for tainting potential based on direct additions of chemicals to fishflesh should be investigated.

5. Although acceptable test results were obtained in the ring test the proposed Test Guideline (cf. Appendix 1) should be further validated for more chemicals and by more laboratories.

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TABLE 1

Properties of Test Chemicals used in Fish Tainting Experiments

Chemical	MP °C	BP °C	Density 20°/4°C	log Pow	Solubility in H_2O(20°C) $mg.l^{-1}$	Vapour pressure mmHg (25°C)	LC_{50}(fish) 24 h. $mg.l^{-1}$	LC_{50} used for setting the exposure levels in the tainting test	LD_{50}(oral rat) mg/kgbw/d	pK_w	Status Carcinog.	GESAMP Classif.
Butanol	-80	118	0.81	0.8	77,000	6.5	1940	100	790	NR**	NC***	O*
Pyridine	-42	115	0.98	0.6-1.0	451,000	20	100-1350	100	891	5.11-5.44	NC	T*
1,2-Dichlorobenzene	-18	180	1.30	3.4	100	1.6	14.3-105	50	500	NR	NC	Z*
2,4-Dichlorophenol	-43	217	1.38	3.3	4000	0.1	4	(40)	580	7.5	NC	T*
Styrene	-31	145	0.91	2.6	300	6.5	17-66	50	5000	NR	NC	T*

* Z : Bioaccumulated with attendant risk to aquatic organisms or human health, however with short retention in the order of one week or less.
T : Bioaccumulated, liable to produce tainting of seafood.
O : No evidence to support a rating.

** NR : Not relevant.

*** NC : Not carcinogenic (ACGIH, 1986-1987).

Table 2 : Fish Tainting Test Results obtained in 5 Laboratories according to Methods described in Text.

(In brackets ratio of number of correct decisions to number of tasters)

Laboratory	1		2		3 (G)*		3		4	5
Fish Type	Trout	Trout	Trout	Trout	Red sea bream	Carp	Red sea bream	Carp	Trout	Trout
Conc. mg.l^{-1}	SW *	FW *	SW	FW	SW (threshold)	FW	SW	FW	FW	SW
					n-BUTANOL					
10	T*(10/13)	N*(6/15)	N(8/16)						N(7/15)	N(4/18)
1		N(6/14)	N(2/16)							
0.1		T(9/15)	N(7/16)							
D*		N(8/16)	N(8/16)						N(3/15)	
					PYRIDINE					
10		N(2/16)	T(10/16)	T(11/16)				T(12/18)	N(6/15)	T(11/18)
						2.4				
1		N(6/16)	N(6/16)	N(8/16)				N(9/18)		N(5/18)
0.1		N(4/16)	N(6/16)	N(4/16)				N(5/18)		
D		N(6/16)	N(7/16)	T(9/16)				N(7/18)	N(6/15)	N(7/18)
					1,2-DICHLOROBENZENE					
5										lethality
1.0			T(13/16)					T(13/18)		T(14/18)
0.5		T(13/15)	T(10/16)					N(8/18)	T(10/16)	
0.1		T(9/16)	N(5/16)			0.12		N(8/18)		N(8/18)
D		N(8/18)	T(9/16)					N(8/18)	N(8/16)	N(9/18)
D (0.5)								N(4/18)		
Tween adj. (0.1)		N(7/16)								N(7/18)

* Abbreviations :

SW : sea water.

FW : freshwater

G : GESAMP Guideline

D : 24h depuration after exposure to the highest concentration, if otherwise indicated D(C)

T : tainting at 5% significance level

N : non-tainting

Table 2 (continued)

Laboratory	1		2		3 (G)*		3		4	5
Fish Type	Trout	Trout	Trout	Trout	Red sea bream	Carp	Red sea bream	Carp	Trout	Trout
Conc. $mg.l^{-1}$	SW *	FW *	SW	FW	SW (threshold)	FW	SW	FW	FW	SW
2,4-DICHLOROPHENOL										
4		T*(9/16)							N(6/17)	
0.4			T(9/16)				T(16/18)	T(16/18)	N(7/15)	T(17/18)
0.1		T(9/16)	N*(8/16)**				T(14/18)	T(15/18)	N(8/15)	T(16/18)
D*		T(10/16)	N(8/16)**		0.004	0.005	T(16/18)	T(16/18)	D(4):T(12/17) D(0.4):N(4/15)	N(8/18)
** = statistical taint level is (9/16)										
STYRENE										
5.0							T(12/18)			
2.5			lethality		1.2			T(11/18)		
1.0			N(8/16)			0.20	N(7/18)	T(11/18)		
0.1			N(5/16)				N(7/18)	N(7/18)		
D			T(10/16)				N(5/18)	T(12/18)		
D(1.0)							N(5/18)			

* Abbreviations :

SW : sea water.
FW : freshwater
G : GESAMP Guideline

D : 24h depuration after exposure to the highest concentration, if otherwise indicated D(C)
T : tainting at 5% significance level
N : non-tainting

APPENDICES

APPENDIX 1 : GUIDELINE FOR THE ASSESSMENT OF FISH TAINTING

1. INTRODUCTORY INFORMATION

° Prerequisites

- Water solubility
- Density
- Melting- and boiling point
- Vapour pressure
- 24 hr LC_{50} to the fish species
- Method of analysis for the quantification of the test substance in water
- Information necessary to evaluate the hazard to man during the conduct of the test

° Guidance information

- Composition of the test substance
- Octanol/water partition coefficient
- Chemical stability in water and light
- pK value of ionisable substances

° Qualifying statements

- Constant conditions should be maintained as far as possible throughout the test and, if necessary, semi-static or flow-through procedures should be used.

- It may not be possible to determine the tainting ability of chemicals of limited solubility under the test conditions.

° Standard documents

This guideline was based upon the GESAMP - Draft guidelines for evaluating threshold values for fish-tainting (1984).

2. METHOD

A. INTRODUCTION, PURPOSE, SCOPE, RELEVANCE, APPLICATION AND LIMITS OF TEST

The purpose of this test is the evaluation of tainting as a hazard of substances carried by ships and is related specifically to the column A "T"-rating in the hazard profiles as developed by GESAMP (1982).

It is proposed that the evaluation be carried out in three stages :

i. Limit test

ii. Definitive test to categorise the concentration of a chemical which taints fish

iii. Assessment of taint retention.

If no taint is detected at stage i (limit test) no further testing is required. A positive taint at stage i would require stages ii and iii to be carried out.

The test procedure consists of two parts which do not necessarily need to be undertaken within a single organisation : the exposure of fish and the assessment of taint.

This test procedure is considered to be non-applicable to chemicals which are considered to present an unacceptable hazard to man during testing.

° Definitions and units

Static test is a test in which no flow of test solution occurs and where the solutions remain unchanged throughout the duration of the test.

Semi-static test is a test without flow of solution, but with occasional batchwise renewal of the test solution after prolonged periods (e.g. 24h).

Flow-through test is a test in which water is renewed continuously in the test chambers, the test substance being transported with the water used to renew the test medium.

Taint is a taste or odour foreign to the species under test (ISO,1983-a).

Tainting concentration is the concentration to which fish are exposed and at which taint is detected.

Units - all concentrations of test substance in water are given in weight per volume (e.g. $mg.l^{-1}$).

° Reference substances

No reference substances are recommended for this test. Nevertheless, if a reference substance is used, the results should be given.

° Principle of the test method

Fish are exposed for a period of 24 hours to the test substance dissolved in water, at a range of specified concentrations. The exposed fish are harvested, cooked and a panel of assessors used to determine the presence or absence of a taint on the basis of a triangular test.

When a taint is detected at the limit test concentration a depuration step should be included. In this step exposed fish are held in uncontaminated water for 24h and subsequently assessed for taint.

° Conditions for the validity of the test

The dissolved oxygen concentration should be at least 60% of the air saturation value throughout the test. Aeration can be used provided it does not reduce the exposure level of the test substance to less than 50% of the initial concentration.

There should be evidence that the concentration of the substance being tested has been maintained at a level of at least 50% of the initial concentration.

B. DESCRIPTION OF THE TEST PROCEDURES

° Preparations

Equipment

i. For exposure. Normal equipment needed for exposure of fish to chemicals (OECD, 1981).

ii. For evaluation of taint. Facilities and equipment for the preparation and cooking of fish and for sensory testing by a panel of assessors.

Solutions of test substances

Stock solutions of the appropriate concentrations are best prepared by dissolving the appropriate amount of the test substance in the required volume of dilution water. Cosolvents should not be used except where unavoidable as they may impart unusual flavours in the test population. If adjuvants are used evidence must be supplied to ensure no background taint has been imparted.

Solutions at the specified test concentrations are usually prepared by dilution of a stock solution. Chemicals with a solubility below 10 $mg.l^{-1}$ are tested at their water solubility with due consideration to the test conditions, e.g. temperature, salinity, pH.

If ionisable substances with a pK value between 7 and 8 are to be evaluated the test should be performed with sea water or with freshwater adjusted to the pH of sea water (pH about 8)

T e s t f i s h

Selection of species

The test species should be a fin fish with a moderate fat content (3-10%). The recommended test species is the rainbow trout (Salmo gairdneri). Other species such as red sea bream (Pagrus maior) or carp (Cyprinus carpio) can be used.

The fish used in the test must come from the same source. They should be of similar size and have been fed a diet which does not impart any strong or unusual flavour or odour to the flesh of the fish. The test population must be sampled prior to testing for fat content (cf. this report, Appendix 2) and be checked for any strong or unusual flavour or odour.

Holding

Fish to be used in the test should be acclimatised to water of the quality to be used in the test for at least 7d before they are used.

Acclimatization: The rainbow trout is normally raised in freshwater but fish of above 100g can be acclimatized to sea water. Acclimatization to seawater is more readily achieved in springtime this being the natural smolting period for American steelhead trout (Salmo gairdneri). The physiological stress of acclimatization can be reduced by carrying out the transfer at, or below 10°C and in

several steps. Each step should last for at least 24 hours with small salinity increments during the initial phases,, e.g. 10, 25, 75, 100% sea water. At other than the optimum time of year it may be necessary to carry out the process over 2-3 weeks.If, during the acclimatization to sea water, fish show signs of darkening or loss of equilibrium, they should be kept at the same salinity and the food pre-soaked in freshwater prior to feeding until the condition of the fish returns to normal. After full acclimatization to the diluent and the test temperature, the fish should exhibit a normal feeding response for a minimum of 7 and preferably 14 days before they are used in a test (Prunet and Boeuf, 1985).

If freshwater is used as the diluent it should be of a quality suitable for the maintenance of fish for long periods without stress.

Fish should be fed daily during the acclimatization and holding periods, and continued until 24 hours prior to the start of a test.

The test population must be acclimatized to the test temperature at a rate not to exceed 2°C per 24 hours, and maintained at that

temperature for a minimum of 7 days before being used in a test.

Water: Natural sea water (32-36°/oo salinity) or freshwater from an uncontaminated source should be used. Reconstituted water may be used but should be well aerated before use.

Light : 12-16 hours photoperiod daily.

Temperature : For rainbow trout and sea water this should be 10°C or less during acclimatization to seawater and 15 ± 2°C for a minimum of 7 days before the test. For other species the appropriate temperatures should be used.

pH : The pH during acclimatization should be the same as in the definitive test within ± 0.5 pH unit.

Dissolved oxygen concentration : At least 60% of the air saturation value.

Prophylactic treatment : Prophylactic treatment should be avoided but if it has to be used there should be a period of at least 7 days between the end of the treatment and the start of the test.

Feeding : Daily, with a diet which does not impart a strong or atypical flavour or odour.

Mortality : There should be less than 5% mortality in the test population during the 7 days prior to the start of the study.

Performance of the test

The limit test should be carried out first. If no taint is detected no further testing is required; the detection of a taint requires the depuration phase of the limit test to be carried out and exposure to supplementary concentrations.

The concentration of the test substance must be determined in each test at the beginning and end of the exposure period.

The use of continuous flow test systems is recommended but static or semi-static procedures are acceptable provided that, during the exposure period, the concentration of test material does not drop below 50% of the initial value.

The test animals must be placed directly into the test solution. When using a continuous flow system, the apparatus must be run for a sufficient time for the test solutions to stabilise before the test animals are introduced.

i) Exposure

Duration : 24 hours

Tanks : Of suitable capacity in relation to the recommended loading.

Test concentration: For the limit test a single test concentration of 10 $mg.l^{-1}$, one-tenth of the 24h LC_{50}, or the maximum solubility in water whichever is the lowest.

The definitive test consists of 2 supplementary concentrations at 1 and 0.1 $mg.l^{-1}$ if these are not already included in the limit test.

A control group must be run under the same experimental conditions. If adjuvants are used evidence must be supplied to ensure no background taint has been imparted.

Dilution water : Natural sea water (salinity 32-36°/oo) or freshwater from an uncontaminated source should be used. Reconstituted sea water of similar salinity may be used and well aerated before use.

Light : 12-16 hours photoperiod daily.

Temperature : For rainbow trout this should be 15 ± 2°C. For other species the appropriate temperatures should be used.

pH : The pH during exposure should be between 6 and 8.5. If ionisable substances with a pK value between 7 and 8 are to be evaluated with freshwater, the pH should be adjusted to the pH of seawater (pH about 8).

Dissolved oxygen concentration : Not less than 60% of the air saturation value throughout the test.

Loading : For static and semi-static tests the loading must not exceed 1 g. of fish.l^{-1}. A lower loading or aeration must be used if the concentration of dissolved oxygen falls below 60% saturation during the test.

For flow-through tests the loading should not exceed 20 g of fish.l^{-1} of solution in the chamber. The flow rate should be at least 1 litre per 2 g of fish per day.

Numbers of animals : Sufficient fish should be exposed at each exposure concentration to give a minimum of 200 g of wet flesh (approximately 500 g of live fish) obtained from a minimum of 3 individual specimens. More fish flesh is required for the control. Less fish flesh is required when taint is assessed by odour only.

Mortality : It is unlikely that mortality of fish will occur in the exposed or control groups. If mortalities occur the test conditions should be reassessed.

Feeding : The test population must not be fed during the test period nor for 24 hours prior to the start of the test.

ii) Depuration

Purpose and performance of test :

The purpose of the depuration phase is to determine whether taint imparted under the conditions of the limit test is rapidly lost when the exposed fish are placed into diluent water.

It is recommended that the depuration phase be carried out as an extension of the limit test. This requires double the amount of fish to be exposed to the test substance during the limit test and also an additional amount of control fish.

Exposure :

Exposure of fish to the test substance must be carried out under the same conditions and at the same concentrations as for the limit test, ie. 24 hours exposure to 10 $mg.l^{-1}$, 1/10 24 hr. LC_{50} or the water solubility, whichever is the lowest. Sufficient fish should be exposed to give a minimum of 200 g of wet flesh from a minimum of 3 individuals. Less fish flesh is required when taint is assessed by odour only.

Depuration

After exposure the fish must be transferred to a clean test vessel containing diluent water. They must be held for a period of 24 hours under the same conditions as described for the limit test but

without the presence of any test substance.

iii) Evaluation of taint

Harvesting of the fish : Only live fish should be used for evaluation. At the end of the exposure period the fish should be harvested and stunned by a blow to the head. The fish should be gutted immediately, allowed to bleed and washed. If the fish are not to be cooked within 2 hours they should be packed in an aluminium or polymer foil and stored at 0° to 4°C in an odour free environment. The fish should be tested within 48 hours of harvesting.

Preparation of samples : All utensils and equipment used for preparing and holding samples should be free of taint. Where the same equipment is used successively to prepare samples, the lowest concentration should be processed first, then successively to the highest concentration, cleaning the equipment between each sample.

The fish may be washed briefly in potable water to remove blood, slime or ice. The head is removed and the belly cavity cleaned.

Cooking : The fish should be cooked in one of the following ways :

- Steaming method. The fish is placed in a casserole with a loose fitting lid or wrapped in aluminium foil. The casserole or wrapped fish is suspended over or in boiling water or steam for a period just sufficient to cook the fish. This period should be determined from prior experimentation.

- Boil-in-the-bag method. The fish should be put into a plastic bag intended for cooking foods in boiling water. The bag should be weighted by putting into it glass rods or weights and the bag should be closed loosely and clipped. The bag should be suspended in boiling water or steam for a period just sufficient to cook the fish. This period should be determined by prior experimentation.

- Microwave cooking. The fish should be cooked in a closed container and according to the optimum conditions as prescribed by the manufacturer.

Care should be taken that no taint is transferred between samples during the cooking process.

After cooking the flesh should be removed and freed from skin and bone. The flesh from all fish in each group should be pooled and mixed thoroughly. The test material should be dispensed into the lidded

containers in which it will be presented to the assessors (see Conduct of test) and kept at the temperature at which the taint or odour will be assessed.

Selection of assessors :

Fifteen to twenty assessors should be used. They should be familiar with the triangular test procedure and the normal flavour or odour of the species of fish used in the test. They should not be specially selected for acuity to the chemical under test.

Conduct of test:

The material at each test concentration is presented to the panel of assessors as a triangular test in comparison with the control (zero concentration). Detailed instructions for carrying out the triangular test are given in ISO 4120 (1983-b), or the equivalent national standard. The following is a summary of the procedure.

An assessor is presented with a set of 3 identical flavour and odour-free receptacles each containing at least 10 g of sample. The receptacles are coded with 3- or 4-digit random numbers. Two samples are identical and the third is different. The assessor is required to select the single sample. The assessor may assess the sample by odour or flavour or both. If the assessor can make the

selection on odour there is no need to proceed to tasting the samples.

The pair of identical samples can be from the test material or from the control, but across all the assessors there should be an equal (or near equal in the case of an odd number of assessors) presentation of the 2 possibilities. Further, within each of these 2 possibilities there are 3 ways the receptacles can be ordered when presented to the assessors giving 6 combinations in all. If the test and control materials are denoted as A and B the 6 combinations are AAB, ABA, BAA, BBA, BAB and ABB. As far as possible, considering the number of assessors, the combinations should be presented an equal number of times and distributed randomly among the assessors.

The assessment proceeds by presenting material from the test concentrations successively to assessors as triangular tests starting with the lowest concentration. At each concentration the proportion of assessors who correctly identify the odd sample is recorded. The ISO standard permits a "forced choice" or "no difference" option. A "forced choice" option is however recommended.

Data and Reporting

Treatment of results

If it is observed that the stability or homogeneity of the test solutions cannot be maintained, care should be taken in the interpretation of the results and note made that these may not be reproducible.

The method used to process the results of the triangular tests is the International Standard ISO 4120 (1983-b). The total number of correct assessments for each test concentration is determined.

Probability tables are used to interpret the result of the triangular test (cf. Table), and normally the significance level of 5% is accepted, e.g. if a total of 15 assessors were to make 9 correct selections of the odd samples, this would indicate a difference in taste between the two samples at the 5% confidence level.

TABLE 1

Minimum numbers of correct replies to establish a difference at various significance levels for the triangular test

Number of replies	Minimum Number of Correct Replies for a significance level of		
	5 %	1 %	0,1 %
15	9	10	12
16	9	11	12
17	10	11	13
18	10	12	13
19	11	12	14
20	11	13	14

Test report

The test report should include the following information :

Test substance: chemical identification data

Test organisms: scientific name, size, supplier, fat content, acclimatization, any pretreatment, etc.

Test conditions:
- test procedure used (e.g. static, flow-through, aeration, fish loading, etc.)
- diluent water quality characteristics (treatment, source, pH, salinity, any other information available)
- test solution characteristics (dissolved oxygen concentration, pH values, temperature)
- method of preparation of stock and test solutions
- information on the maintenance of the concentration of the test substance in the test solutions
- number and mortality of fish at each test concentration

Evaluation of taint :
- method of preparation of samples for triangular test
- conduct of the test (number of assessors, method of presenting the samples, any other relevant details)
- method of assessment : i.e. odour and/or flavour

Results :
- results of the triangular tests

- lowest concentration causing taint and when taint was observed at the limit test, the effect of depuration
- incidents in the course of the test which might have influenced the results
- any deviation from the Test Guidelines

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APPENDIX 2 : DETERMINATION OF LIPID CONTENT

Lipid content can be determined by any of the following methods.

1. Ether Extractable Lipid

Principle

The method is based on the continuous extraction of lipid by ether from a chemically dried sample.

Apparatus

1. Analytical balance with at least 0.5 mg accuracy
2. Soxhlet extraction apparatus or Goldfisch apparatus
3. 50 ml round or flat bottomed flask
4. Controllable heater
5. Rotary evaporator
6. Vacuum drying cabinet
7. Vacuum rotary oil pump
8. Desiccator

Reagents

1. Dry diethyl ether, practically free of peroxides and ethanol, spec. weight 0.720, BP 34.5°C
2. Sodium sulphate AR, dry

Procedure

The sample (10g) is accurately weighed and thoroughly mixed with an equal weight of sodium sulphate. The mixture is transferred to a fat-free extraction thimble which is closed with fat-free cotton wool. Extraction with ether is carried out in either a Soxhlet or Goldfisch apparatus for 6 hours with the temperature so regulated that syphoning occurs about 15 times per hour.

The ether extract can be treated in either of two ways. In the first it is collected in a dried, tared flask, the ether is evaporated in a rotary evaporator and the flask and residue dried in a vacuum cabinet for 30 min. at 100°C. Afterwards flask and residue are cooled to room temperature (30-60 min.) in a desiccator and weighed. In the second, the ether extract is collected in an untared flask, the ether evaporated in a rotary evaporator

and the residue quantitatively transferred with ether to a tared 50 ml flask. The ether is again evaporated in a rotary evaporator and the residue dried to constant weight by evacuating (repeatedly if necessary) the flask with a rotary oil pump at room temperature for 15 min.

Calculation

$$\% \text{ 'crude' fat} = \frac{\text{gain in weight of flask in g} \times 100}{\text{weight of sample in g}}$$

The results should be quoted in terms of ether-extractable lipid.

2. Modified Bligh & Dyer Method

Principle

The method is based on that of Bligh & Dyer (1959) modified by Hanson & Olley (1963).

Apparatus

1. Centrifuge capable of spinning 200 ml cup at 2000 g
2. Ultra Turrax homogenizer (TP 18/2N; Janke & Kunkel, Staufen ir Br, West Germany)
3. Fast analytical balance with at least 0.5 mg accuracy
4. 50 ml round bottomed flask
5. Rotary evaporator
6. Vacuum drying cabinet
7. Vacuum rotary oil pump
8. Desiccator

Reagents

1. Chloroform
2. Methanol

Procedure

About 10g of sample is accurately weighed into a 250 ml centrifuge cup and 8 ml of water is added. Add 20 ml chloroform measured accurately and 40 ml methanol and homogenize for 1 min. The homogenization is carried out with the aid of an Ultra Turrax homogenizer flask, the stem of which is fitted with a solvent resistant stopper which forms an airtight fit with the centrifuge cup. A further 20 ml chloroform is pipetted into the cup and the

mixture homogenized for 30 sec. Add 30 ml of water and homogenize for 30 sec..

The cup is centrifuged at 2000 rpm for 20 min and 20 ml of the chloroform layer is pipetted out after removal of the aqueous layer. Or the homogenate can be vacuum filtered through filter paper on a Buchner funnel and the chloroform layer allowed to separate in a separating funnel. Small amounts of water in the chloroform aliquot can be removed by running it through dry filter paper followed by washing the paper with fresh chloroform.

The lipid in the 20 ml chloroform is determined after evaporation of the chloroform (plus washings) in a dried tared flask according to the previous procedure.

Calculation

It is assumed that the final volume of the chloroform layer is 40 ml, ie. there have been no losses by evaporation.

$$\% \text{ lipid} = \frac{\text{gain in weight of flask in g} \times 200}{\text{weight of sample in g}}$$

3. Fosslet method

The lipid content is measured according to the apparatus manufacturers' handbook (A/S N Foss Electric, Denmark).

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APPENDIX 3 : EVALUATION OF TAINT

1. The Choice of the Triangular Test for Detecting Taints

Consider the situation where small amounts of a chemical are successively added to fish meat with a sample being tasted after each addition. At first, an assessor cannot detect any difference in flavour between the test sample and the untreated control but as the concentration of added substance is increased he, or she, can detect a difference though perhaps not being able to recognise the difference as characteristic of the chemical. The concentration at which a change is detected can be considered a threshold - below it no effect is noticed, above an effect is noticed - and is referred to as the detection threshold. As the concentration of added chemical is increased a value is reached when the assessor can recognise the new flavour as being due to the chemical. This is the recognition threshold. A taint has already been defined as any change in flavour from the intrinsic flavour of the fish so any concentration of chemical above the detection threshold will cause the fish to be tainted.

The concept of a threshold as being a sharp and invariant boundary between no effect and effect is a theoretical construct; in practice the threshold is a more diffuse boundary. If an assessor is repeatedly given samples with added chemical at concentrations in the region of the threshold he, or she, will sometimes detect a change in flavour at a particular concentration and sometimes not. At each concentration the observations from such a trial can be expressed as a probability of detection, the proportion of times the flavour is detected, and the data plotted as probability distribution curve. The curve has a sigmoid shape when probability is plotted against logarithm of concentration and the assessors's threshold is the concentration at the inflection point, the value when the probability of detection is 0.5.

The situation is similar if detection is replicated over the assessors rather than over occasions with a single assessor; the proportion of assessors who detect a difference increases as the concentration of the added chemical increases and the probability distribution when plotted against logarithm of concentration is a sigmoid-shaped curve. If thresholds are determined for a large number of assessors the frequency distribution curve of threshold against logarithm of concentration is symmetric and has the shape of the normal distribution curve. (The distribution is symmetric and normal in shape

for most favouring substances; for some the distribution is skewed, or is even bimodal, indicating some proportion of the population is unusually insensitive or sensitive to the flavour). The range of thresholds for a particular flavouring compound among individuals is quite wide; typically the ratio of concentrations at the limits enclosing 95% of a population is around 100:1.

This relationship between detection and concentration is typical of dose - effect relationships in general and the concept of the threshold being the point when there is a probability of the effect being observed of 0.5 is analogous to the LC_{50} concept in toxicology.

A variety of sensory procedures have been used in reported investigations of taints in fish and shellfish but they can be classified into 2 broad groups : those in which assessors detect a taint and rate its intensity in order to derive a measure of a tainting threshold, and those in which assessors detect a taint or change in flavour or odour, usually in comparison with a reference, in order to determine if a taint is present or not.

The first approach is exemplified by the work of Shumway and Palensky (1973) who exposed fish to a range of concentrations of potential tainting chemicals and asked the assessors to rate the intensity of taint in samples after cooking. The scores after correcting for a 'blank' were extrapolated to zero intensity in order to determine the threshold level. This procedure requires that the assessors recognise the taint in order to rate its intensity and that there are sufficient test concentrations above the threshold level to give a reliable extrapolation. The guidelines described in this report does not require that a threshold be determined and only a small range of concentrations - and not necessarily encompassing the threshold concentration - are required to be tested. Hence a sensory procedure based on measuring intensity is not required and possibly could not be applied.

If the guidelines are used to measure threshold concentration by evaluating a wider range of concentrations then it should be noted that there are some fundamental problems associated with the approach just described. The assessors know the general nature of the test and may imagine they perceive a taint even when the causative chemical is not present or is below the threshold level of detection. This is seen when control samples, that is

material not exposed to the chemical, are present, unidentified, among test samples. Some assessors will give non-zero scores for intensity and the panel mean score is then non-zero. Similarly test samples at low concentrations of chemical below the threshold level will have positive mean scores. These false positives represent the 'blank' referred to in the previous paragraph. Another problem is that the assessor has to recognise the taint in order to rate its intensity. The flavour or odour of fish is intrinsically variable and it is difficult to recognise a taint at low levels of intensity against this natural variation. The assessors must therefore be experienced in the natural range of flavour and odour of the test species and of the odour or flavour of the test chemical.

Test procedures which require only that assessors detect a taint, or change in flavour or odour, are appropriate to the protocol described here and resolve some of the problems just described. These procedures require an assessor to taste or smell the sample and report if a taint is present or not usually in comparison with a control sample. There are several ways the test can be performed.

The simplest, and the most economical of material, is to present only a test sample and the assessor is required to state if it is tainted. This approach has the problems described earlier; the assessor knowing it is a test sample has an expectation that it is tainted and is biased to give a positive answer, and, the assessor must be familiar with the sensory properties of the test species and of the sensory nature of the taint in order to detect the taint. This experimental approach then is flawed and is not advised for the guidelines recommended in this report.

The problem of the variation in the sensory properties of the test species is overcome by comparing the test sample with a control and any difference between them is an indication of the presence of taint. This pair comparison procedure can still be biased if the control and test are identified as such and the assessor is asked to state if the test is tainted. There is an expectation that the test sample would be tainted with a consequent bias to answer positively if there is some doubt. This bias can be overcome if the nature of the samples in the pair are not revealed and the assessor is required to select the one that is tainted. This procedure does not get over the problem of recognition since the assessor, perceiving a difference

between the samples, has to decide which one has the taint - it is not sufficient just to record there is a difference because there is an expectation bias that there should be a difference. The assessor therefore has to be familiar with the natural sensory properties of the species and of the flavour or odour of the test chemical.

A procedure which is free of expectation bias and does not require any, or at best only little, familiarity with the sensory properties of either the test species or the chemical is the triangle test. In this procedure the assessor is presented with 3 samples, 2 are identical, and can be either test or control material, and the third is of the other treatment. The assessor is required to identify the single specimen from the pair. So long as the experimental procedure as laid down in standard methods is followed the test is free of bias and, since the assessor is not required to state anything about the nature of the difference between the pair and the single nor which, the pair or the single, is tainted there is no element of recognition involved. Hence the triangular test does not have the problems associated with procedures involving 1 or 2 samples. Though it requires more of both test and control material that the others this disadvantage is outweighed by the advantages of a sounder experimental approach and is recommended for inclusion in these guidelines. A standard for the experiment procedure has been issued (ISO 4120, 1983).

2. Selection of Assessors

Assessors differ in their abilities to detect low intensities of flavour of odour and some thought has to be given to the selection of members of the sensory panel. If the persons selected for inclusion in the sensory panel are very sensitive then taint will be detected at a lower exposure level than a panel of insensitive persons will. It is usual for some purposes in the sensory testing of foods to deliberately select individuals who are sensitive to the odours and flavours of interest. For example in product development the experimenter may wish to determine the effect of additives on flavour or odour and would choose as assessors those persons who are known to respond to those additives. In quality control of food products a taste panel for off flavours would be selected from those persons who had been tested and shown to be sensitive to the off flavour.

In the context of these guidelines it is not necessary to determine the lowest possible concentration that can be detected and to use sensitive assessors; the IMO Working Group has referred to tainting as a hazard in relation to the acceptability of fish to consumers generally. It is reasonable therefore to use as assessors in evaluating tainting, people who are typical of the general population of consumers in respect of their abilities to detect changes in flavour.

It is not possible to select a panel which is truly representative of this population in some sampling sense because it is not known how sensitivity to the flavour of chemicals that are likely to be tested by the guidelines is distributed among individuals. The best than can be achieved in practice is to use a reasonably large panel, in the region of 15-20 people, on the assumption that it will contain a typical spread of sensitivities.

There is a possibility that panels from different laboratories and, within laboratories, from time to time, will be consistently different from each other, that is will be biased, but these systematic errors are likely to be small and would not be a significant source of error in comparison with other sources of variance arising from the experimental procedures included in the guidelines.

3. Evaluation of Results from a Triangular Test

The principle of the triangular test is that the assessor has to distinguish a single sample from a pair of identical samples when they are presented together as a set of 3. If the concentration of flavouring substance, either in the single sample or in the pair, is below the threshold level for all assessors in the panel the assessors have to guess which is the odd sample. In this case 1/3 of the assessors should correctly select the odd sample. This fraction, one third, of correct selections is the limiting value as the number of assessors approaches infinity; with practical sized panels the number of assessors correctly selecting the odd sample will be around 1/3. As the concentration of flavouring compound increases it will eventually exceed the threshold of some assessors and so a higher proportion of the panel will correctly select the odd sample. The proportion of correct selections increases as the concentration increases until ultimately all the assessors correctly select the odd sample. It is then necessary to decide on what

number of correct selections above the chance level of 1/3 of the total gives confidence that the panel is truly detecting a difference.

The probability that a particular number of correct selections would be obtained by chance in the triangular test can be calculated from the terms of the expansion of the binomial expression $(p + q)^n$ where $p = 1/3$, $q = 1 - 1/3 = 2/3$ and n is the number of assessors in the panel. The probability of C or less correct selections is given by summing the first C terms of the expansion. The minimum numbers of correct selection for selected levels of confidence are tabulated for a range of panel sizes in manuals of sensory testing and a typical example is reproduced in the Table of the proposed Test Guideline (Appendix 1).

It can be seen from this table that as the panel size increases the proportion of correct selections needed for a significant effect decreases. This is in accordance with the usual principle that the larger the sample size the greater the confidence that the measured value presents the true value. In the case of the triangular test the true value is a proportion of 0.333 if there is no detectable difference; any higher value suggests a difference. For a given panel size the observed proportion has to exceed this value sufficiently for the experimenter to be confident that there is a detectable difference between the test sample and the reference. In the case of a panel of 15 assessors the minimum number of correct selections has to be 9 for a significant difference at the 95% confidence level, that is 60% of the total compared with 33.3% if there is no difference and all panel members are guessing. If a panel size of 30 assessors is used the minimum number of correct selections is 15 (this value is not included in the Table of Appendix 1), 50% of the total. Another way of expressing this property is that for a particular distribution of sensitivities among a population of assessors, a large panel selected from this population will detect a smaller difference between test and reference than will a smaller panel, that will detect taint at a lower concentration of contaminating chemical.

Hence it is important in evaluating taint by this protocol that the panel size should be close to the recommended 15 assessors if results from different laboratories are to be compared.

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APPENDIX 4 : THE VOLUME OF SEA AND AREA OF SEA BED SWEPT BY FISHING OPERATIONS

1. Bottom Trawling

A bottom trawl consists of a mesh bag dragged along the sea floor. The mouth of the bag is held open in the vertical axis by means of a weighted bottom rope and a buoyant head rope. The sides of the net are attached by ropes to vanes which hold the mouth open in the horizontal dimension as the net is towed through the water.

In a typical North Sea trawl the opening of the net when it is being towed is 4.5 m high and 19 m wide. The net opening is approximately rectangular in shape with a bowed upper line but for the purposes of calculating the volume swept by the net it will be sufficient to consider it as a rectangle. The cross sectional area is then 85.5 m^2.

The vessel will tow the net at 3-4 knots (5.5 - 7.5 $km.h^{-1}$) for 2-4 hours. The distance covered is therefore from 11 to 30 km and the volume swept by the net will be 9.4×10^5 - 2.6×10^6 m^3. The area of sea bed swept by the net will be 2.1×10^5 - 5.7×10^5 m^2.

2. Pelagic Trawling

Pelagic trawling uses a net similar in size to one used for bottom trawling but the gear is designed so that the net operates in mid-water and can be manoeuvered to some extent. It is intended for catching shoaling fish such as herring and mackerel. The shoal is located and the net is shot and manoeuvered to scoop up the fish. In this case a haul will last for about 30 minutes and the volume swept by the net will be 2.8×10^5 m^3.

3. Bottom Seine Netting

In seine netting the net, similar in size and shape to a bottom trawl net, remains more or less stationary on the sea bed. The vessel pays out long ropes in a triangular pattern with the vessel ultimately at the apex of 2 long sides and the net in the centre of the short side. The ropes are hauled in thus closing the triangle towards the net. This action herds the fish into the net which is then hauled in. It can be considered that the fish enclosed within the original triangle are caught.

Typically the triangle is about 500 m along the short side and 1500 m along the long side. The area of sea bed swept by this type of fishing operation is about 3.8×10^5 m².

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