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Contributions to the Revision of TG OECD 305

Lipid measurement

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1 Introduction

Bioaccumulation of chemical substances can be caused by bioconcentration, mainly via respiratory membranes, or by biomagnification via dietary uptake. As partitioning between water or food and outer membranes of organisms represents the most important process of bioaccumulation, it is of particular interest when dealing with substances with certain physico-chemical properties, e.g. lipophilicity and persistence. In aquatic systems, sediments and soils, these substances tend to concentrate mainly in the lipid fraction of organisms and may lead to substantial physiological burdens. The accumulation of such residues in the food chain can reach levels toxic to predators and represents a risk for human health. Assessing the bioconcentration as well as the biomagnification potential is therefore an important issue for the environmental and human risk assessment of chemicals and one of the main features in environmental monitoring.

The TG OECD 305 focuses on the process of bioconcentration and aims at the determination of the bioconcentration factor (BCF), a proportionality constant relating the concentration of a chemical in an organism to its concentration in the ambient water at the steady state between uptake and depuration. Investigations in the aquatic environment have revealed a significant positive correlation between the accumulation of a chemical and the lipid content of organisms, and residue levels thus differ between individuals, species and size groups depending on their lipid contents (Baron 1990; LeBlanc 1995, Stow *et al* 1997). Therefore, variations in these data can be reduced, when BCF values are normalized by lipid content (U.S. EPA 2003, Jensen *et al.* 2003). Furthermore, this facilitates the extrapolation between species and sites.

However, it must be kept in mind that lipids are not a uniform compartment, and that different lipid classes, e.g. the relatively polar phospholipids common in cell membranes, the nonpolar triacylglycerols in storage lipids, the neutral steroids, glycolipids, triglycerids, and free fatty acids have quite different properties. Partitioning of lipophilic substances in the total lipid fraction is most probably related to these differences in polarity (Ewald and Larsson 1994, Randall *et al.* 1998).

Polarity also governs lipid solubility in water and organic solvents and thus the extraction of total lipids. Lipid fractions are usually characterized by different ratios of polar and non-polar lipids, mainly depending on the total lipid content of the animals/tissues. The use of appropriate extraction techniques based on solvent systems with the right polarity is thus essential (Randall *et al.* 1991, 1998). In addition, other factors, e.g. temperature, pH, light and oxygen, may influence lipid extractions (U.S. EPA 2003). In view of these considerations it is not surprising that up to now, many different extraction methods have been proposed but no standard method suitable for all tissues/species has been agreed upon.

Bioconcentration guidelines (e.g. OECD 305, U.S. EPA OPPTS 850.1730, Japanese Industrial Standards) emphasize the importance of lipid normalization, however, a clear guidance on lipid measurement in fish is missing. The results obtained with different extraction methods may differ quite considerably (De Boer 1988, Randall *et al*. 1991, 1998, Ewald *et al*. 1998, Smedes 1999, Iverson *et al*. 2001, Jensen *et al*. 2003, Zhuang *et al*. 2004, Inouye & Lofuto 2006, Lu *et al.* 2008) leading to over- or underestimations when normalizing BCF values to lipid contents. Therefore, the selection of appropriate extraction techniques using mixed systems of non-polar and polar solvents is required to ensure the complete extraction of total lipids from fish/tissue samples. In this context, the OECD 305 guideline recommends the use of chloroform/methanol for determination of fish lipid content. The microgravimetric assay for total lipids described by Gardner *et al.* (1985!, not 1995 as described in the OECD guideline) is given as a key reference.

As described before, the accurate determination of lipid content of fish samples is essential for lipid normalization of BCF values. To guarantee the comparability of BCFs obtained in different studies, the analytical procedures should be further standardized. Therefore, in the course of revising OECD guideline 305, lipid determination is one point that needs clarification.

The aim of the present study is therefore,

1) to give an overview over lipid extraction methods in bioaccumulation studies by reviewing current literature

2) to appraise each method with respect to strengths and weaknesses in terms of reproducibility, ease of conductance, and robustness taking into account its representation in literature

3) to compare the lipid content/BCF results obtained in different studies on the same substance. This would give information on whether different lipid measurement methods influence lipid normalized BCF results

4) to give a survey of lipid normalization percentages where they have been used.

5) to review the literature for studies describing the distribution of contaminants over different organs and/or lipids, most notably storage *vs.* membrane lipids.

The overall aim is to select a preferred method which shall be included in the updated OECD 305 along with a sampling schedule of lipid measurement in fish.

2 Determination of fish lipid content

2.1 Literature review (Part 1) – overview and appraisal of lipid extraction methods

The review of methods for lipid extraction and an appraisal of each method are presented in **Annex 1**. The focus of this survey is on traditional extraction procedures using organic solvents (e.g. Folch *et al*. 1957, Bligh and Dyer 1959, Hara and Radin 1978, or the Soxhlet-method), however, also non-destructive instrumental methods based on near-infrared (NIR) spectroscopy or nuclear magnetic resonance (NMR) spectroscopy are presented. Each method currently employed for the determination of lipid content has its advantages and disadvantages and there is no procedure available which is suitable for all types of lipids. For choosing suitable methods for preparation of lipid extracts from fish tissues based on solvent extraction, four criteria must be considered: the toxicity of the solvents for humans (i.e. lab technicians) and the ease of disposal of the used solvents should be a prime

consideration. At the same time, the simplicity and efficiency of the method must be borne in mind. The choice of method used for the determination of lipid content will be dictated to a large extent by cost and by the urgency with which the results are required. The non-destructive instrumental methods are for instance exceptionally quick and involve very little sample preparation but expensive and sophisticated equipment is required which might not be widely available. In addition, their degree of accuracy is perhaps less than that of the destructive solvent extraction methods.

The amount of fish biomass sampled during bioaccumulation studies is usually limited due to the small size and low number of animals. Some procedures for solvent extraction might be principally suitable for extraction of lipids from fish tissues but relatively large sample volumes are required (e.g. Folch *et al*. 1957). In these cases a careful adjustment of the extraction procedure to reduced sample sizes is required. Several micro-gravimetric assays have been described (e.g. Gardner *et al*. 1985; Parrish 1986).

The relative merits of the different methods have to be considered when a preferred method for inclusion in the updated OECD 305 test guideline is selected.

2.2 Literature review (Part 2) – bioaccumulation studies incl. lipid determination

The vast majority of bioaccumulation studies available in the literature have been performed in the context of environmental monitoring or field studies. Depending on the aim of the respective studies, residue levels are either expressed on wet weight basis – e.g. when the focus is on consumer safety – or normalized to lipid weight.

Publications of bioconcentration studies according to OECD 305 (1996) or U.S. EPA OPPTS 850.1730 (1996) are rare:

Schettgen (2000) performed OECD 305 studies with Triclosan, and some pyrethroid pesticides. Total lipids were determined according to Hara & Radin (1978) using a two step extraction with hexane and isopropanol followed by the photometrical determination of the lipid-sulphosphovanillin complex after digestion with sulphuric acid (Merck 1974).

Fox *et al*. 1994 performed bioconcentration tests on PCBs according to OECD 305 E, but no details on the methods used for lipid extraction except the solvent combination (hexane/2-propanol) are presented.

Yakata *et al*. 2006 studied the influence of dispersants on the BCF of seven organic compounds in flow-through test systems according to OECD 305. Lipids were determined using the Bligh & Dyer method.
Other papers report on bioconcentration tests according to OECD 305 or U.S. EPA OPPTS 850.1730, without lipid determination being carried out (e.g., Min & Cha 2000, Seo *et al.* 2002, Satyanarayan & Ramakant 2004, Springer *et al*. 2008).
The outcome of the literature review with respect to guideline studies and lipid determination methods was thus not satisfactory due to a shortage of data. This is probably due to the fact that the majority of bioconcentration studies according to guidelines are performed in the context of regulatory risk assessment of chemicals. These studies have to be performed according to GLP (Good Laboratory Practice) and are thus of high quality. However, they are strictly confidential and not available to the public and could thus not be reviewed for this report.

The literature review was therefore amended by short interviews with laboratories that perform OECD 305 studies in the context of registration and notification.

2.3 Survey (Part 1) – Lipid extraction procedures currently used in bioaccumulation studies carried out by governmental, academic and industrial labs

Eight international laboratories involved in bioaccumulation studies were asked for information about their SOP's used for lipid extraction from fish tissues. Our survey showed that despite of the broad range of methods available for lipid extraction, only a small group of techniques is routinely applied in bioaccumulation studies. Five of the eight laboratories interviewed stated that they commonly use solvent extraction methods based on chloroform/methanol according to Bligh and Dyer (1959) or Randall *et al.* (1991) which is a modification of the procedure originally described by Folch *et al*. (1956). Two laboratories commonly use the Smedes (1999) method which is based on non-chlorinated solvents. Only one lab mentioned to use a non-destructive instrumental method based on near-infrared spectroscopy (NIR). The results show that most labs follow the current guidelines of OECD 305 where the use of chloroform/methanol extraction techniques is recommended for

analysis of fish samples. The Smedes-method (Smedes 1999) was developed for the determination of total lipid in fish and extensively compared with the Bligh and Dyer method for different fish and shellfish samples. The results were in agreement with the extraction following Bligh and Dyer using chlorinated solvents and the method was thus recommended by QUASIMEME (www.quasimeme.org) as a "low-toxic" method for determination of total lipid in fish. It can be assumed that the results of all seven labs which use solvent extraction procedures for analysis of fish samples are comparable. Also the values obtained by NIR are most likely in agreement with those obtained by solvent extraction, as described by Darwish *et al.* 1989 and Mathias *et al.* 1987.

In conclusion, the results of our survey indicate that analytical methods commonly applied by governmental, academic and industrial labs for the determination of lipid content in fish sampled from bioaccumulation studies are of high quality. The estimation of possible impacts of unsuitable extraction techniques on lipid normalized BCF results might therefore be a rather theoretical issue.

2.4 Literature review (Part 3) – Do different lipid extraction procedures influence lipid normalized BCF results?

Principally, extraction differences may lead to substantial differences in comparing the lipid-normalized BCFs across studies and among species of varying lipid composition (Randall *et al*.1991). However, a critical investigation of different methods for lipid determination with respect to their impact on lipid normalization is missing. Due to the limited amount of published results from bioaccumulation studies which were carried out according to OECD 305, the specific comparison of normalized BCFs estimated for single contaminants is difficult. Geyer *et al*. 1985 described the relationship between the lipid content of fish and their bioconcentration potential of 1,2,4-Trichlorobenzene. This study presents a valuable collection of references of older bioaccumulation studies. For instance, in a study on rainbow trout (*Oncorhynchus mykiss*), presented by Galassi and Calamari (1983), a lipid content of 3.2% was estimated for the newly hatched animals leading to a wet-weight based BCF_W of 349 (based on lipid weight the respective BCF_L was 10906). In a further study on the same species but with bigger animals a lipid content of 8.3% was determined leading to a BCF_W of

1300 (BCF_L = 15660) (Oliver and Niimi, 1983). Lipid contents in both studies were determined by Soxhlet extraction. However, different solvent systems (n-hexane *vs.* acetone/hexane) and different boiling periods (8hr *vs*. 24h) were applied. It can be assumed that in the first study, due to the neutral solvent (n-hexane) only the neutral lipids were removed leading to a lower lipid content and thus a lower BCF_L value. In contrast, the lipid content presented in the second study seems to be exceptionally high for animals of the given size.

3 Literature review (Part 4) – Differences in residue distribution over different organs and/or lipids

Bioconcentration factors on a wet weight basis (BCF_W) increase with increasing lipid content. The generally held view is that neutral storage and membrane lipids are the most important classes for the bioaccumulation of nonpolar and polar residues, respectively. Therefore, apart from the lipid content, also the lipid composition might have an effect on the bioaccumulation potential of an animal. However, with respect to differences in residue distribution over different lipid types, only little data are available. Chefurka and Gnidec (1987) found that DDT binds to the relatively polar membrane-lipids. Chlorobiphenyls were detected in both, the membrane-bound and the unbound lipid fraction in fish (de Boer 1988). For extractable PCBs, Randall *et al*. (1998) reported about one third to be associated with membrane-bound lipids whereas two thirds were found in the neutral lipid fraction (Randall *et al.* 1998). Roche *et al.* (2000) found infrequent positive correlations between lipid contents in tissues and contaminant levels: in eel (*Anguilla anguilla*) muscular γHCH correlated with neutral lipids. In crucian carp (*Carassius carassius*) muscular γHCH correlated with total lipids and hepatic ΣPCB with phospholipids. In catfish liver (*Ictalurus nebulosus)* a positive correlation was detected between γHCH and total as well as neutral lipids. Generally, the evidence for the role of lipid composition on differences in bioaccumulation potential is missing and the normalization of BCF values should be better related to total lipid content than to any one fraction.

Lipid concentrations in different organs – namely filet and liver – can vary leading to differences in residue distribution. For instance, Wu *et al.* (2001) reported that PCDD/Fs accumulate mainly in liver and that muscle concentrations correlate with liver concentrations. Variability in muscle concentrations between fish species decreased when concentrations were normalized to lipid content. Several studies describe the residue distribution over different organs (e.g. Gunkel and Streit 1980; Roche *et al.* 2000; Jabber *et al*. 2001; Storelli and Marcotrigiano 2001; Satyanarayan & Ramakant 2004; Pääkkönen *et al.* 2005; Zhou *et al.* 2007; Blanes *et al.* 2008; Serrano *et al*. 2008). However, bioconcentration studies according to OECD 305 are performed to obtain BCF values which – *per definitionem* - refer to the whole fish and differences in residue distribution over different organs are thus not essential.

4 Lipid normalization

4.1 Literature review (Part 5) – Default values (whole body lipid content) for BCF normalization

Fish lipid content varies according to species, age, season and location and it can range from around 0.5% to 20% w/w or more in the wild (e.g. Geyer *et al*. 1994). BCF values on a wet weight basis (BCF_W) increase with increasing lipid contents. Normalization of BCF values to lipid content is one way to reduce variability when comparing measured BCF values for instance for different species or animals of different life stages. Lipid contents are commonly used to calculate BCF values on a per cent lipid basis (BCF_L) and can be further used to calculate a normalized whole body BCF assuming a fixed whole body lipid content. A default value of 5% is most commonly used as this represents the average lipid content of the small fish used in OECD 305 including rainbow trout (*Oncorhynchus mykiss*) Bluegill sunfish (*Lepomis macrochirus*), zebrafish (*Danio rerio*) fathead minnow (*Pimephales promelas*) and common carp (*Cyprinus carpio*) (Pedersen *et al.* 1995; Tolls *et al.* 2000 cited in the REACH TGD (R.7.10.4). No percent normalization was performed in the published OECD 305 bioaccumulation studies (Schettgen 2002, Yakata *et al*. 2006).

4.2 Survey (Part 2) – Default values (whole body lipid content) for BCF normalization currently used in bioaccumulation studies carried out by governmental, academic and industrial labs

Fish lipid contents should be always measured and reported together with the calculated BCF values. The interviews revealed that fish lipid contents are usually measured and reported but that BCFs are not necessarily further normalized on a lipid basis. Only one lab mentioned that BCF values are normalized to a default value of 6% which represents the average lipid content of bluegill sunfish (*Lepomis macrochirus*) used in their bioaccumulation studies. A common default value (e.g. 5%, as described above) should be defined in the revised OECD guideline to give a clear basis for the comparison of BCF values across studies and among species.

5 General conclusions and recommendations

5.1 Suggestion of a preferred method for inclusion in the updated OECD 305 test guideline

As stated above, the selection of a suitable lipid determination method should consider the methods reproducibility, robustness and ease of use. Furthermore, it should not be too expensive and – ideally – work without toxic solvents. Another crucial point is the applicability to small quantities of samples. Many of the methods listed above were established for samples of 1 – 10 g and have to be modified when working with sample sizes of < 1g. The chloroform/methanol extraction technique recommended as standard method is well established and accepted. After the Soxhlet-method, which is considered unsuitable for lipid extraction of fish tissues because of its dependency on conditions which are difficult to control (i.e. no precise determination of extraction cycles due to continuous flow), the Bligh and Dyer method is probably the second most common lipid extraction procedure reported in the scientific literature. Due to the simultaneous use of the non-polar chloroform and the polar methanol as solvents, this technique is characterized by high extraction efficiency. The lipid obtained can be subjected to further analysis if required. However, the major drawback of this method is the use of highly toxic solvents. We therefore suggest that as a replacement for the Bligh and Dyer procedure the Smedes-method (Smedes 1999) should be recommended as an alternative technique. This method is characterized by a comparable

efficiency of extraction, high accuracy, the use of less toxic organic solvents and ease of performance. Cyclohexane has a lower density than chloroform and therefore separates at the top of the extraction mixture. Particulate matter which is centrifuged to the bottom of the jar is automatically removed from organic phase. Therefore no filtration is necessary to remove it as with Bligh and Dyer where tissue residues form on top of the lower chloroform phase.

Due to the specific extraction procedures required for the analysis of many test substances, the use of techniques based on NIR or NMR spectroscopy (e.g. CEM Smart trac ™) has high potential for the lipid determination in fish sampled from bioaccumulation studies. However, the lack of intercalibration studies and the limited availability and high cost of equipment are currently the major bottleneck for the use of these techniques.

5.2 Suggestion of a standardized sampling schedule for lipid measurement

The OECD 305 guideline states that, if possible, lipid determination and residue analysis should be performed using the same sample. However, analysis of test substances often requires specific extraction procedures which might be in contradiction to the guidelines for gravimetric lipid determination by solvent extraction. In this case (until suitable non-destructive instrumental methods are available) it is recommended to determine the fish lipid content by solvent extraction on independent fish sampled at the start and at the end of the experiment. The amount of fish per tank should be adjusted accordingly. The analysis of fish from the test and control group at the end of the experiment is important to confirm the equal lipid content of test and control animals. As described in the OECD 305 guideline the lipid content of the fish (as mg/kg wet weight) at the end of the experiment should not differ from that at the start by more ± 25%.

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7 Annex 1 Methods for lipid determination

	Principle of method	advantage	disadvantage	studies
Folch et al. (1957)	Gravimetric quantification using 1-step solvent extraction with mixture of water and chloroform:methanol (2:1) followed by a wash with 0.9% KCl	- standard method - well established to determine total lipids	- adverse effects of chloroform on the environment (EU regulation controlling chlorinated solvents) - laborious (filtration etc)	Harino *et al.* 2000 Kondo *et al*. 2005 Rinchard *et al.* 2007 ; Nanton *et al.* 2007: Modified Folch-Method including butylated hydroxyltoluene as antioxidant
Bligh and Dyer (1959)	Gravimetric quantification using 3-step solvent extraction: (1) methanol + chloroform (2) chloroform and (3) water are added to the tissue. After phase separation total lipids are determined in the chloroform phase by gravimetric analysis following evaporation of the solvent	- simple - standard method, well established - determines total lipids - Samples can be analysed directly with no pre-drying necessary - lipids can be used for further determinations	- adverse effects of chloroform on the environment (EU regulation controlling chlorinated solvents) - laborious (filtration etc) - Special equipment required	Recommended by US-EPA (1996, 2003) for bioconcentration tests and field studies Schulz & Hayton 1994 Yakata *et al*. 2006 (OECD 305 test), Widenfalk *et al*. 2008
Hara & Radin (1978)	Gravimetric quantification using 1-step solvent extraction with hexane/isopropanol (3:2) followed by a wash with aqueous sodium sulphate	- Solvents less toxic and cheaper than chloroform and methanol - No interference in processing by proteolipid	No extraction of gangliosides, a minor fraction of total lipids	Recommended by US-EPA for field studies Schettgen (2000): extraction method in OECD 305 studies. Lipid determination was done photometrically (Merck 1974)

Hara & Radin cont.		protein - extract contains less nonlipids compared to chloroform-methanol extracts of Folch		
Smedes-Method (Smedes 1999)	Gravimetric quantification using 3-step solvent extraction with isopropanol and cyclohexane. Same principles as Bligh & Dyer	- No chlorinated solvents required - Relatively untoxic solvents - robust enough for routine use - No step-change in international monitoring data which have so far used Bligh & Dyer as standard method - more practicable than Bligh & Dyer because of lower density of cyclohexane	- further testing necessary with different tissues - laboratory performance test necessary Jensen *et al*. (2003): - difficulties in separating phases - extraction of lean fish critical	Recommended by QUASIMEME

Jensen -Method (Jensen *et al.* 2003)	Gravimetric quantification using 3-step solvent extraction: - 2-propanol (IPR) & diethyl ether (DEE) - n-hexane/DEE and IPR - n-hexane/DEE	- No halogenated solvents - gentle method without heating - easy to handle - gives B&D comparable results for fat and lean fish	- Suitability for small samples has to be checked	
Soxhlet-Method	Gravimetric quantification using solid-liquid extraction in a Soxhlet Apparatus. Constant flow of organic solvent over material. Solvent is boiled, condenses and passes the tissues several times thereby extracting the lipids. After a suitable time the process is stopped, solvent evaporated and fat weighted	- simple - not very labour intensive - can be operated with non chlorinated solvents - lipids can be used for further determinations	- results lower than those of Bligh & Dyer-Method - extractable lipids are determined, not total lipids - large amounts of solvents needed - special equipment required - possibly adverse effects on labile lipids and test substance by high temperatures and oxygen - results are very much operationally dependent (solvent composition,	Van Haelst *et al.*1996: toluene/hexane Lang et al. 1997: acetone/petroleum Wu *et al.* 2001; Webster *et al*. 2007: MTBE Lu & Wang (2002) (OECD 305): cyclohexane/acetone/petroleum ether Zhao *et al*. 2005: methylene chloride/hexane Zhou *et al.* 2007/2008: hexane/acetone; Ferreira *et al.* 2008: n-hexane; Guo *et al*. 2008: acetone/dichloromethane Houde *et al*. 2008; Wu *et al*. 2008: no further details

Soxhlet-Method cont.			extraction time, cycles) - conditions are difficult to control (continous flow of solvents) - time consuming	
Accelerated Solvent Extraction (ASE) (Richter *et al.* 1996)	Gravimetric quantification using accelerated solvent extraction apparatus Solvent is pumped into sealed tube with sample and support material at elevated temperature and pressure. After a suitable time the solvent is pumped out, collected, and tube filling and emptying is repeated a number of times. After solvent evaporation the lipid is weighted. Essentially the same as Soxhlet but heating the solvent above its boiling point and keeping it liquid under pressure	- not very labour intensive - lipids suitable for further analysis - techniques take out environmental contaminants (e.g. PCBs, dioxins, pesticides)	- Expensive - Not all lipids extracted. Various mixtures of solvents, temperatures and pressures needed for specific samples to ensure that all free fat is extracted. - drying of samples required (or low in moisture). Blending with a suitable matrix may be useful - complex equipment	Fisk *et al.* 2001 : dichloromethane/hexane Balmer *et al.* 2005: dichloromethane/cyclohexane Buckman *et al* 2006 Law *et al.* 2006 Chu & Metcalf 2007: methanol Houde *et al.* 2008

Supercritical Fluid Extraction	Gravimetric quantification using supercritical fluid extraction. Sample is extracted with liquid carbon dioxide* which serves as solvent. After extraction it is allowed to evaporate and the remaining lipids are weighed. * under normal pressure, CO_2 is either gaseous or solid. Under pressure it is taken past its supercritical point and all three states can exist.	- Rapid - No organic solvent or acid needed - Lipids can be used for further analysis.	- Very expensive equipment - complex equipment - Supply of CO_2 needed	
Gardner *et al.* (1985)	Microgravimetric assay for total lipids using solvent extraction with chloroform-methanol followed by a wash with NaCl. (established for freshwater invertebrates).	- Comparable results to macroquantitative methods with lower costs for solvents, reduced processing time, less chemical waste - No need to pool samples	- Inclusion of non-lipid materials (upward bias) - Laborious and time intensive compared to SPV-method (see next entry) - lower measurement precision compared to macroquantitative methods	

Van Handel (1985)	Microquanitity colorimetric sulfophosphovanillan method (SPV) for total lipids Measurement of absorbance of red-purple complex produced from reaction between SPV-reagent and carbon double bonds	- Comparable results to macroquantitative methods with lower costs for solvents, reduced processing time, less chemical waste - No need to pool samples	- measures detects only compounds with unsaturated carbon bonds (saturated fatty acids are not detected) therefore Lu et al (2008) modified the protocol adding H_2SO_4 - lower measurement precision compared to macroquantitative methods	Landrum *et al*. 2002
Near infrared spectroscopy (NIR)	Measurement depends on the absorption of infrared energy at specific wavelength by functional groups such as the carbonyl group in the ester linkage of lipids.	-Non-destructive method. -Sample can be used for other measurements e.g. contaminant analysis -Can be used on whole fish without removing skin and scales. -Time -Accuracy. The values obtained by NIR agree well with those obtained	-Requires sophisticated equipment. -Not widely available. -Not widely used as a method of determining lipid content	Darwish *et al.* 1989; Mathias *et al.* 1987

		by solvent extraction.		
Parrish (1986/1987)	Microquantity thin-layer chromatographic method with Iatroscan flame ionization detection system (Iatroscan TLC-FID) for lipid classes. Methods works with solvent extracts (e.g. Gardner – Method) Total lipids by summarizing individual lipid classes. Method was established for zooplankton, benthic macroinvertebrates, larval and juvenile fish.	- Comparable results to macroquantitative methods with lower costs for solvents, reduced processing time, less chemical waste - No need to pool samples	- Nonlinear response of the TLC-FID detector may lead to underestimation of total lipids - lower measurement precision compared to macroquantitative methods - suitable only for organisms with low fat content	
Additional methods recommended by CCFRA for fat analysis in meat and fish (taken from McLean & Drake 2002)				
Werner Schmid	Gravimetric quantification using acid hydrolysis followed by solvent extraction Sample is heated in a water bath with hydrochloric acid.	- Extracts all lipids - Cheap - Samples can be analysed without pre drying	- Triglycerides can be degraded by acid hydrolysis, therefore lipids can not be used for other determinations (e.g. fatty	

Werner Schmid cont.	After cooling, lipid is extracted 3-4 times with diethyl ether and petroleum ether. Solvent is evaporated and lipid weighted.		acid profiles) - Relatively labour intensive - Large amounts of solvents and special equipment required - labour intensive	
Weibull-Stoldt (= Weibull-Berntrop Method)	Gravimetric quantification using acid hydrolysis followed by Soxhlet extraction. Sample is mixed with hydrochloric acid and boiled for 30 min. Extract is cooled, filtered and filter washed until free of acid. Residue is dried and Soxhlet extracted.	- Extracts all lipids - Cheap - Samples can be analysed without pre drying	- Triglycerides can be degraded by acid hydrolysis, therefore lipids can not be used for other determinations (e.g. fatty acid profiles) - Relatively labour intensive - Large amounts of solvents needed - Special equipment required - results are very much operationally dependent (solvent composition, extraction time, cycles) - conditions are difficult to control	

			- time consuming	
Caviezel	Sample is extracted and fat saponified. Analysis is performed by GC-measuring of the whole fatty acids present. This is used to calculate the lipid content	- Other analysis can be performed at the same time - If equipment exists, it is not very cost intensive.	- Equipment costs are high - Results are not comparable with other methods .Therefore validation is required	
Nuclear Magnetic Resonance (NMR)	Quantification using the measurement of a generated signal from the fat molecules. Sample is dried, then inserted into the NMR and the signal applied. The signal created by the stimulated protons are measured and used to quantify the fat within the sample.	- Rapid if combined with fast moisture methods (microwave) - No organic solvents or acids needed - Does not rely on the removal of fat from the sample - Fat can be extracted and used for further analysis - Relatively simple	- Expensive - Sample has to be dried - Relatively new technique, needs further testing and validation - Other proton-containing substances may interfere	

NMR-Method: CEM Smart trac™	Sample is dried in a microwave and total fat (free and bound) is determined by NMR (Nuclear magnetic resonance	- fully automated - no solvents needed - easy to handle - fast - good reproducibility - applicable to all probes - same material can be used for determination of fat and test substance - works with sample sizes of 50-100 mg - no calibration required - used in foodstuff analyses - accepted as official AOAC method	- applicability has to be checked - can tissue percent solids be determined precisely to allow conversion of lipid concentration (per wet weight) to dry weight basis? - cost of system CEM smart trac™ (60.000 Euro) - commercial product	Cartwright et al. 2005 Wolf & Pfannhauser 2007