

Overview and appraisal of lipid extraction methods

	Principle of method	advantage	disadvantage	studies
Folch <i>et al.</i> (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 <i>et al.</i> 2000 Kondo <i>et al.</i> 2005 Rinchard <i>et al.</i> 2007 ; Nanton <i>et al.</i> 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)	Recommended by US-EPA (1996, 2003) for bioconcentration tests and field studies Schulz & Hayton 1994 Yakata <i>et al.</i> 2006 (OECD 305 test), Widenfalk <i>et al.</i> 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 protein - extract contains less nonlipids compared to chloroform-methanol extracts of Folch	- laborious - 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)

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 un toxic 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 -no filtration required	-laborious -the extraction of specific tissues, like liver, may lead to the formation of an emulsion which can be prevented by replacing the water by 1 M HClO₄ to denature the proteins. The addition of NaCl may also help.	Standard procedure for QUASIMEME Laboratory Performance Studies
Jensen method (Jensen <i>et al.</i> 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	- laborious - special glass apparatus required - suitability for small samples has to be checked - no interlaboratory study -further validation/calibration approaches required NB.: The original “Jensen method” (Jensen et al. 1972) uses a different solvent system leading to an underestimation of the lipid content of very lean fish	

Soxhlet method (AOAC, 1995)	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, extraction time, cycles) - conditions are difficult to control (continuous flow of solvents) - time consuming	Van Haelst <i>et al.</i> 1996: toluene/hexane Lang <i>et al.</i> 1997: acetone/petroleum Wu <i>et al.</i> 2001; Webster <i>et al.</i> 2007: MTBE Lu & Wang (2002) (OECD 305): cyclohexane/acetone/petroleum ether Zhao <i>et al.</i> 2005: methylene chloride/hexane Zhou <i>et al.</i> 2007/2008: hexane/acetone; Ferreira <i>et al.</i> 2008: n-hexane; Guo <i>et al.</i> 2008: acetone/dichloromethane Houde <i>et al.</i> 2008; Wu <i>et al.</i> 2008: no further details
Accelerated Solvent Extraction (ASE) (Richter <i>et al.</i> 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	- 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 <i>et al.</i> 2001 : dichloromethane/hexane Balmer <i>et al.</i> 2005: dichloromethane/cyclohexane Buckman <i>et al.</i> 2006 Law <i>et al.</i> 2006 Chu & Metcalf 2007: methanol Houde <i>et al.</i> 2008

ASE cont.	lipid is weighted. Essentially the same as Soxhlet but heating the solvent above its boiling point and keeping it liquid under pressure			
Supercritical Fluid Extraction (King 2002)	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 CO₂ needed	
Gardner <i>et al.</i> (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)	Microquantity colorimetric sulfophosphovanillin 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 H₂SO₄ - lower measurement precision compared to macroquantitative methods	Landrum <i>et al.</i> 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 by solvent extraction.	- requires sophisticated equipment - not widely available - not widely used as a method of determining lipid content	Darwish <i>et al.</i> 1989; Mathias <i>et al.</i> 1987; Cronin & McKenzie 1990
Parrish (1986, 1987)	Microquantity thin-layer chromatographic method with Iatroscan flame ionization detection system (Iatroscan TLC-FID) for lipid classes. Method works with solvent extracts (e.g. Gardner method).	- comparable results to macroquantitative methods with lower costs for solvents, reduced processing time, less chemical waste - no need to pool samples	- non linear response of the TLC-FID detector may lead to underestimation of total lipids - lower measurement precision compared to macroquantitative methods	

Parrish cont.	Total lipids by summarizing individual lipid classes. Method was established for zooplankton, benthic macroinvertebrates, larval and juvenile fish.		- 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 method or Schmid-Bondzynski-Ratzlaff method	Gravimetric quantification using acid hydrolysis followed by solvent extraction. Sample is heated in a water bath with hydrochloric acid. After cooling, lipid is extracted 3-4 times with diethyl ether and petroleum ether. Solvent is evaporated and lipid weighted.	- 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 and special equipment required - labour intensive	(James 1995)
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	- 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	(BSI - BS4401-4)

Weibull-Stoldt cont.	and Soxhlet extracted.		needed - special equipment required - results are very much operationally dependent (solvent composition, extraction time, cycles) - conditions are difficult to control - time consuming	
Caviezel method	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	(Pendl et al. 1998)
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	(Toussaint et al. 2002)

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 <i>et al.</i> 2005 Wolf & Pfannhauser 2007
--	--	---	---	--

References

AOAC (Association of Official Analytical Chemists): **Animal Feed**. Chapter 4 in Official Methods of Analysis. 16th edition. AOAC International, Arlington, VI, USA; 1995:30pp.

Balmer ME, Buser HR, Müller MD, Poiger T: **Occurrence of some organic UV filters in wastewater, in surface waters, and in fish from Swiss lakes**. *Environ Sc Technol* 2005, **39**:935-962.

Bligh EG, Dyer WJ: **A rapid method of total lipid extraction and purification**. *Can J Biochem Physiol* 1959, **37**:911-917.

BSI: **Determination of total fat content in meat and meat products**. BS4401-4:1970.

Buckmann AH, Wong CS, Chow EA, Brown SB, Solomon KR, Fisk AT: **Biotransformation of polychlorinated biphenyls (PCBs) and bioformation of hydroxylated PCBs in fish**. *Aquatic Toxicol* 2006, **78**(2):176-85.

Cartwright G, McManus BH, Leffler TP, Moser CR: **Rapid determination of moisture/solids and fat in dairy products by microwave and nuclear magnetic resonance analysis**. *J AOAC Internat* 2005, **88**(1):1-14.

Chu S, Metcalf CD: **Analysis of paroxetine, fluoxetine and norfluoxetine in fish tissues using pressurized liquid extraction, mixed mode solid phase extraction cleanup and liquid chromatography-tandem mass spectrometry**. *J Chromatogr A* 2007, **1163**(1-2):112-118.

Cronin DA, McKenzie K: **A rapid method for the determination of fat in foodstuffs by infrared spectrometry**. *Food Chemistry* 1990, **35**(1):39-49.

Darwish DS, van de Voort FR, Smith JP: **Proximate analysis of fish tissue by Mid-Infrared Transmission Spectroscopy**. *Can J Fish Aquatic Sci* 1989, **46**:644-649.

Ferreira M, Antunes P, Costa J, Amado J, Gil O, Pousão-Ferreira P, Vale C, Reis-Henriques MA: **Organochlorine bioaccumulation and biomarkers levels in culture and wild white seabream (*Diplodus sargus*)**. *Chemosphere* 2008, **73**(10):1669-1674.

Fisk AT, Stern GA, Hobson KA, Strachan WJ, Loewen MD, Norstrom RJ: **Persistent organic pollutants (POPs) in a small, herbivorous, arctic marine zooplankton (*Calanus hyperboreus*): Trends from April to July and the influence of lipids and trophic transfer.** *Mar Pollut Bull* 2001, **43(1-6)**:93-101.

Folch J, Lees M, Sloane-Stanley GH: **A simple method for the isolation and purification of total lipides from animal tissues.** *J Biol Chem* 1957, **226**:497-509.

Gardner WS, Frez WA, Cichocki EA, Parrish CC: **Micromethod for lipids in aquatic invertebrates.** *Limnol Oceanogr* 1985, **30(5)**:1099-1105.

Guo L, Qui Y, Zhang G, Zheng GJ, Lam PKS, Li X: **Levels and bioaccumulation of organochlorine pesticides (OCPs) and polybrominated diphenyl ethers (PBDEs) in fishes from the Pearl River estuary and Daya Bay, South China.** *Environ Pollut* 2008, **152(3)**:604-611.

Hara A, Radin N: **Lipid extraction of tissues with low-toxicity solvent.** *Anal Biochem* 1978, **90(1)**:420-426.

Harino H, Fukushima M, Kawai S: **Accumulation of butyltin and phenyltin compounds in various fish species.** *Arch Environ Contam Toxicol* 2000, **39(1)**:13-19.

Houde M, Muir DCG, Kidd KA, Guildford S, Drouillard K, Evans MS, Wang X, Whittle DM, Haffner D, Kling H: **Influence of lake characteristics on the biomagnification of persistent organic pollutants in lake trout food webs.** *Environ Toxicol Chem* 2008, **27(10)**:2169-2178.

James CS: **Analytical Chemistry of Food.** Blackie Academic and Professional, London, UK, 1995. pp.91-105.

Jensen S, Johnels AG, Olsson M, Otterlind G: **DDT and PCB in herring and cod from the Baltic, the Kattegat and the Skagerrak.** *Ambio Spec Rep* 1972, Rep. No 1 (1) pp.71-85.

Jensen S, Häggberg L, Jörundsdóttir H, Odham G: **A quantitative lipid extraction method for residue analysis of fish involving nonhalogenated solvents.** *J Agric Food Chem* 2003, **51**:5607-5611.

King JW: **Supercritical Fluid Technology for Lipid Extraction, Fractionation and Reactions**. In *Lipid Biotechnology*. Edited by Kuo TM, Gardner H., Marcel Dekker Inc., New York, USA 2002, pp 663-687.

Kondo T, Yamamoto H, Tatarazako N, Kawabe K, Koshio M, Hirai N, Morita M: **Bioconcentration factor of relatively low concentrations of chlorophenols in Japanese medaka**. *Chemosphere* 2005, **61(9)**:1299-1304.

Landrum PF, Gedeon ML, Burton GA, Greenberg MS, Rowland CD: **Biological response of *Lumbricus variegatus* exposed to fluoranthene-spiked sediment**. *Arch Environ Contam Toxicol* 2002, **42(3)**:292-302.

Lang PZ, Wang Y, Chen DB, Wang N, Zhao XM, Ding YZ: **Bioconcentration, elimination and metabolism of 2,4-dinitrotoluene in carps (*Cyprinus carpio* L.)**. *Chemosphere* 1997, **35(8)**:1799-1815.

Law K, Palace VP, Halldorson T, Danell R, Wautier K, Evans B, Alaei M, Marvin C, Tomy GT: **Dietary accumulation of hexabromocyclododecane diastereoisomers in juvenile rainbow trout (*Oncorhynchus mykiss*) I: Bioaccumulation parameters and evidence of bioisomerization**. *Environ Toxicol Chem* 2006, **25(7)**:1757-1761.

Lu Y, Wang Z: **Bioconcentration of trace organochlorine pesticides by the rainbow trout**. *J Environ Sci Health A* 2002, **37(4)**:529-539.

Mathias JA, Williams PC, Sobering, DC: **The determination of lipid and protein in freshwater fish using near-infrared reflectance spectroscopy**. *Aquaculture* 1987, **61**:303-311.

McLean B, Drake P: **Review of methods for the determination of fat and oil in foodstuffs**. Review No. 37. Campden & Chorleywood Food Research Association Group (CCFRA) 2002, pp 52.

Merck E: **Gesamtlipide. I. Photometrische Bestimmung**. In *Klinisches Labor*. Edited by Merck E. Darmstadt, Germany, 12. Aufl., 1974:247-249.

Nanton DA, Vegusdal A, Rørå AMB, Ruyter B, Baeverfjord G, Torstensen BE: **Muscle lipid storage pattern, composition, and adipocyte distribution in different parts of Atlantic salmon (*Salmo salar*) fed fish oil and vegetable oil.** *Aquaculture* 2007, **265(1-4)**:230-243.

OECD 305: **OECD Guidelines for testing chemicals.** Proposal for updating guideline 305. Bioconcentration: Flow-through fish test. OECD; 1996.

Parrish CC: **Dissolved and particulate lipid classes in the aquatic environment.** *PhD thesis.* Dalhousie University, Halifax, Nova Scotia, Department of Oceanography; 1986.

Parrish CC: **Separation of aquatic lipid classes by Chromarod thin-layer chromatography with measurement by latroscan flame ionization detection.** *Can J Fish Aquat Sci* 1987, **44**:722-731.

Pendl R, Bauer M, Caviezel R, Schulthess P: **Determination of total fat in foods and feeds by the Caviezel Method, based on a gas chromatographic technique.** *J AOAC Int* 1998, **81(4)**:907-917.

Quasimeme [www.quasimeme.org]

Richter BE, Jones BA, Ezzell JL, Porter NL, Avdalovic N, Pohl C: **Accelerated solvent extraction: a technique for sample preparation.** *Anal Chem* 1996, **68(6)**:1033-1039.

Rinchard J, Czesny S, Dabrowski K: **Influence of lipid class and fatty acid deficiency on survival, growth, and fatty acid composition in rainbow trout juveniles.** *Aquaculture* 2007, **264(1-4)**:363-371.

Schettgen C: **Bioakkumulation von Triclosan bei verschiedenen pH-Werten des Wassers und der Pyrethroide Cyfluthrin, Cypermethrin, Deltamethrin und Permethrin.** *PhD thesis.* Universität Oldenburg; 2000.

Schultz IR, Hayton WL: **Body size and the toxicokinetics of Trifluralin in rainbow trout.** *Toxicol Appl Pharmacol* 1994, **129(1)**:138-145.

Smedes F: **Determination of total lipid using non-chlorinated solvents.** *Analyst* 1999, **124**:1711-1718.

Toussaint CA, Medale F, Davenel A, Fauconneau B, Haffray P, Akoka S: **Determination of the lipid content in fish muscle by a self-calibrated NMR relaxometry method: comparison with classical chemical extraction methods.** Journal of the Science of Food and Agriculture 2002, **82(2)**:173-178.

U.S. Environmental Protection Agency EPA: **Methodology for deriving ambient water quality criteria for the protection of human health.** Technical Support document Vol. 2: Development of national bioaccumulation factors. EPA-822-R-03-030. US EPA; 2003.

U.S. Environmental Protection Agency EPA: **Ecological effects test guidelines OPPTS 850.1730 Fish BCF.** Washington DC; 1996.

Van Haelst AG, Zhao Q, Van der Wielen FWM, Grovers HAJ, De Voogt P: **Determination of bioconcentration factors of eight tetrachlorobenzyltoluenes in zebra mussel *Dreissena polymorpha*.** *Ecotox Environ Saf* 1996, **34(1)**:35-142.

Van Handel E: **Rapid determination of total lipids in mosquitos.** *J Am Mosq Control Assoc* 1985, **1**:302-304.

Webster L, Russell M, Phillips L, McIntosh A, Walsham P, Packer G, Dalgarno E, McKenzie M, Moffat C: **Measurement of organic contaminants and biological effects in Scottish waters between 1999 and 2005.** *J Environ Monit* 2007, **9(6)**:616-629.

Widenfalk A, Bertilsson S, Sundh I, Goedkoop W: **Effects of pesticides on community composition and activity of sediment microbes – response at various levels of microbial community organization.** *Environ Pollut* 2008, **152(3)**:576-584.

Wolf A, Pfannhauser W: **A quick combined microwave/NMR-Method for routine analysis of fat and water content in meat products.** Poster at Food Chem XIV, Paris 2007.

[http://www.cem.de/images/produkte/fettbestimmung/Food_Chem_Paris_XIV_2007.pdf]

Wu JP, Luo XJ, Zhang Y, Luo Y, Chen SJ, Mai BX, Yang ZY: **Bioaccumulation of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls ((PCBs) in wild aquatic species from an electronic waste (e-waste) recycling site in South China.** *Environ Int* 2008, **34**:1109-1113.

Wu WZ, Schramm KW, Xu Y, Kettrup A: **Accumulation and partitioning of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/F) in the muscle and liver of fish.** *Chemosphere* 2001, **43(4-7)**:633-641.

Yakata N, Sudo Y, Tadokoro H: **Influence of dispersants on bioconcentration factors of seven organic compounds with different lipophilicities and structures.** *Chemosphere* 2006, **64(11)**:1885-1891.

Zhao X, Zheng M, Liang L, Zhang Q, Wang Y, Jiang G: **Assessment of PCBs and PCDD/Fs along the Chinese Bohai Sea coastline using molluscs as bioindicators.** *Arch Environ Contam Toxicol* 2005, **49(2)**:178-185.

Zhou R, Zhu L, Kong Q: **Persistent chlorinated pesticides in fish species from Qiantang River in East China.** *Chemosphere* 2007, **68(5)**:838-847.

Zhou R, Zhu L, Chen Y, Kong Q: **Concentrations and characteristics of organochlorine pesticides in aquatic biota from Qiantang River in China.** *Environ Pollut* 2008, **151(1)**:190-199.