## Overview and appraisal of lipid extraction methods

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<th>Principle of method</th>
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| 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) | 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 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) |
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| 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 nontoxic 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 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 required  
- 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  
<p>| 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 |</p>
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<td>Soxhlet method (AOAC, 1995)</td>
<td>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.</td>
<td>- simple&lt;br&gt;- not very labour intensive&lt;br&gt;- can be operated with non chlorinated solvents&lt;br&gt;- lipids can be used for further determinations</td>
<td>- results lower than those of Bligh &amp; Dyer method&lt;br&gt;- extractable lipids are determined, not total lipids&lt;br&gt;- large amounts of solvents needed&lt;br&gt;- special equipment required&lt;br&gt;- possibly adverse effects on labile lipids and test substance by high temperatures and oxygen&lt;br&gt;- results are very much operationally dependent (solvent composition, extraction time, cycles)&lt;br&gt;- conditions are difficult to control (continous flow of solvents)&lt;br&gt;- time consuming</td>
<td>Van Haelst et al. 1996: toluene/hexane&lt;br&gt;Lang et al. 1997: acetone/petroleum&lt;br&gt;Wu et al. 2001; Webster et al. 2007: MTBE&lt;br&gt;Lu &amp; Wang (2002) (OECD 305): cyclohexane/acetone/petroleum ether&lt;br&gt;Zhao et al. 2005: methylene chloride/hexane&lt;br&gt;Zhou et al. 2007/2008: hexane/acetone; Ferreira et al. 2008: n-hexane; Guo et al. 2008: acetone/dichloromethane&lt;br&gt;Houde et al. 2008; Wu et al. 2008: no further details</td>
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<td>Accelerated Solvent Extraction (ASE) (Richter et al. 1996)</td>
<td>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</td>
<td>- not very labour intensive&lt;br&gt;- lipids suitable for further analysis&lt;br&gt;- techniques take out environmental contaminants (e.g. PCBs, dioxins, pesticides)</td>
<td>- expensive&lt;br&gt;- not all lipids extracted.&lt;br&gt;Various mixtures of solvents, temperatures and pressures needed for specific samples to ensure that all free fat is extracted.&lt;br&gt;- drying of samples required (or low in moisture). Blending with a suitable matrix may be useful.&lt;br&gt;- complex equipment</td>
<td>Fisk et al. 2001 : dichloromethane/hexane&lt;br&gt;Balmer et al. 2005: dichloromethane/cyclohexane&lt;br&gt;Buckman et al. 2006&lt;br&gt;Law et al. 2006&lt;br&gt;Chu &amp; Metcalf 2007: methanol&lt;br&gt;Houde et al. 2008</td>
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<td>ASE cont.</td>
<td>lipid is weighted. Essentially the same as Soxhlet but heating the solvent above its boiling point and keeping it liquid under pressure</td>
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| 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, CO₂ 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 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 |
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<td>Van Handel (1985)</td>
<td>Microquantity colorimetric sulfophosphovanillan method (SPV) for total lipids. Measurement of absorbance of red-purple complex produced from reaction between SPV-reagent and carbon double bonds</td>
<td>Comparable results to macroquantitative methods with lower costs for solvents, reduced processing time, less chemical waste - No need to pool samples</td>
<td>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</td>
<td>Landrum et al. 2002</td>
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<td>Near infrared spectroscopy (NIR)</td>
<td>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.</td>
<td>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.</td>
<td>Requires sophisticated equipment - not widely available - not widely used as a method of determining lipid content</td>
<td>Darwish et al. 1989; Mathias et al. 1987; Cronin &amp; McKenzie 1990</td>
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<td>Parrish (1986, 1987)</td>
<td>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).</td>
<td>Comparable results to macroquantitative methods with lower costs for solvents, reduced processing time, less chemical waste - no need to pool samples</td>
<td>Non-linear response of the TLC-FID detector may lead to underestimation of total lipids - lower measurement precision compared to macroquantitative methods</td>
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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

<p>| Additional methods recommended by CCFRA for fat analysis in meat and fish (taken from McLean &amp; 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) |</p>
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<thead>
<tr>
<th>Method</th>
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<th>Pros</th>
<th>Cons</th>
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| 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                                                                 | (Pendl et al. 1998)                                                                                           |
| 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 et al. 2005  
Wolf & Pfannhauser 2007 |
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BSI: Determination of total fat content in meat and meat products. BS4401-4:1970.


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