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A Quantitative Lipid Extraction Method for Residue Analysis of Fish Involving Nonhalogenated Solvents

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Numerous intercalibration exercises have indicated that the in Sweden frequently used, so called Jensen extraction method for total lipids and lipophilic pollutants gave satisfactory yields when applied to fatty aquatic organisms. However, a comparison with the classical Bligh and Dyer method and the forerunner, the Folch methods, revealed that in the case of very lean fish (fat content below 1%, e.g., cod), the lipid yields were about 25% too low for the Jensen method; consequently, residue levels quoted on a lipid weight basis were correspondingly too high. To rectify the unacceptably low fat recovery from lean marine organisms, the Jensen extraction method has been modified to give recoveries not significantly different from the Folch and Bligh and Dyer methods. In the modified version, acetone is replaced by 2-propanol and part of the hexane is replaced by diethyl ether. Comparison between the modified Jensen method and the Folch method for cod muscle gave the same recovery of total lipids but slightly lower than that obtained with the Bligh−Dyer method. A possible explanation for this small difference is discussed. It is anticipated that the reported increased yield for cod is due to the superior solubility of phospholipids in 2-propanol as compared to acetone. The possible use of correction factors for previously reported contaminant residual levels of lean and medium fat fish calculated on lipid basis in the future is suggested.

KEYWORDS: Phospholipids; total lipids; residue analysis; lean fish muscle; Bligh-Dyer; nonhalogenated; Jensen method

INTRODUCTION

In recent years, it has become increasingly accepted that it is more ecologically relevant to express the residue levels of lipophilic persistent pollutants on a fat weight basis rather than on the more traditional fresh weight basis in environmental monitoring programs. There are three major reasons for this gradual change in practice. One concerns food web monitoring studies where it is necessary to calculate on the basis of the lipids present in the sample in order to compare residues in different parts of the web. For example, the lean predatory cod shows lower levels of DDT than the herring on a fresh weight basis, whereas on lipid weight basis, the cod has higher levels than the herring, as would be expected. Second, the fat content in a sample, and consequently the amount of fat soluble substances to be analyzed, can differ between different subsamples of the same tissue and thus influence the residue level on a fresh weight basis. Third, to compare pollution levels in a single species from different parts of the world, it is necessary to use fat level-based data to compensate for differences in climate, seasons, and dietary qualities.

Lipid-based residue reports require correct determinations of both the total amount of lipophilic pollutants and the total lipids in the matrix. Today, the first demand is well-mastered by most environmental chemists and ecotoxicologists but the second is, however, more questionable. Numerous intercalibration exercises (*1*, *2*) have shown that very few extraction methods currently used in environmental research provide lipid recoveries under mild conditions that are comparable with those obtained using the so-called Bligh and Dyer (BD) extraction method (*3*) or its forerunner the Folch method (F) (*4*), which are commonly used in biochemical research. The BD and F methods utilize a mixture of water, MeOH, and chloroform ($CHCl_3$) as extraction medium, and the extractions are performed at room temperature. Since 1967, all residue levels in official monitoring programs run by the Swedish Museum of Natural History have been expressed on a fat weight basis. The environmental scientists considered it to be advantageous if the $CHCl_3$ solvent used in the BD or F methods was replaced with a nonhalogenated solvent since the organic pollutants of interest in those days all contained halogen. The so-called Jensen (J) extraction method (*5*) was therefore developed with the objective of providing the same total lipid yields as the F method but using only nonhalogenated solvents. The J method was developed and tested in parallel with the F method on herring samples.

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Numerous comparisons showed that the J method yielded lipid values identical with those of the F method if the fat content was above 3−5% (*5*) as in the case of herring muscle. The J method was accepted as a standard method by the Swedish Museum of Natural History in Sweden in 1969.

In the late 1990s, this museum, together with the Institute of Applied Environmental Research (ITM) at Stockholm University, participated in an intercalibration study arranged by the Quality Assurance of Information for Marine Environmental Monitoring in Europe (Quasimeme) (*6*). In this program, it was decided that the lipid content of a lean and a fat fish should be examined. It turned out that the J method gave the same recoveries as the BD method in the case of lipids from fat fish, e.g., herring, but from lean fish, unacceptably low levels of lipids (approximately 75%) were obtained, and consequently, residue levels were too high (*1*, *2*). It was concluded that the unsatisfactory recovery obtained with the J method was due to the low total lipid content in the lean cell material.

In fish muscles, the lipids are present as constituents of the different cell membranes and intra- and extracellularly depot fat droplets (*7*). Generally, lipids in the membranes are composed mostly of polar phospholipids, typically about 70% of the membrane lipids; the remaining classes of lipids are neutral steroids (20%), glycolipids, and small amounts of triglycerides (*8*). It may be anticipated that the difference between lean and fat fish reflects the relative proportions between depot fat and membrane lipids in the organism.

The phospholipids are considerably more polar than the neutral fat and hence significantly less soluble in the J solvent mixtures. However, in fat fish, the phospholipid fraction is a lower proportion of the total lipids than that in lean fish. Thus, recovery of phospholipids has less influence on the total lipid recovery from fat fish than from lean fish.

The aim of the present project was to improve the original J method by investigating other nonchlorinated solvent mixtures that may result in quantitative lipid recoveries when applied to organisms with very low lipid contents. Furthermore, it was decided that the technique and equipment used in the original J method should be maintained.

In this work of investigations and comparisons, we have also included the method recently developed by Smedes (S) (*9*), using a technique similar to the BD method but involving a mixture of 2-propanol (IPR) and *c*-hexane as extracting medium. It deserves mentioning in this context that the frequently used Soxhlet continuous extraction technique commonly used for all kinds of matrixes, involving long time refluxing, seldom gives acceptable fat recovery and provides exposure of labile lipids and pollutants to both high temperature and oxygen. Often, this leads to erroneous results especially when applied to fish tissues (*1*, *2*, *10*). Continuous extraction techniques were not included in this investigation. However, in our opinion, a careful comparison between the liquid batch extractions and the continuous Soxhlet extraction on a defined lipid material from lean fish muscles is highly required.

MATERIALS AND METHODS

A fillet of cod from the North Atlantic, with an expected fat content of below 1%, was chosen as the test material. The fillet (500 g) was cut into 1−2 cm cubes, which were wrapped in six thin layers of aluminum foil and stored frozen. The fillet cubes, partly thawed in a refrigerator at +5 °C, were added in small portions to a Philips model HR 1392 kitchen food processor (blades top-inserted) and chopped together with solid carbon dioxide until frozen. The frozen material was transferred to a Dewar flask, which was covered with aluminum foil and placed in the freezer at −18 °C, to allow excess carbon dioxide to escape. The material prepared in this way forms a homogeneous fine-grained material, which can easily be portioned out even after several months in the freezer. Use of the traditional Waring Blender with fixed blades at the base of the vessel proved unsatisfactory.

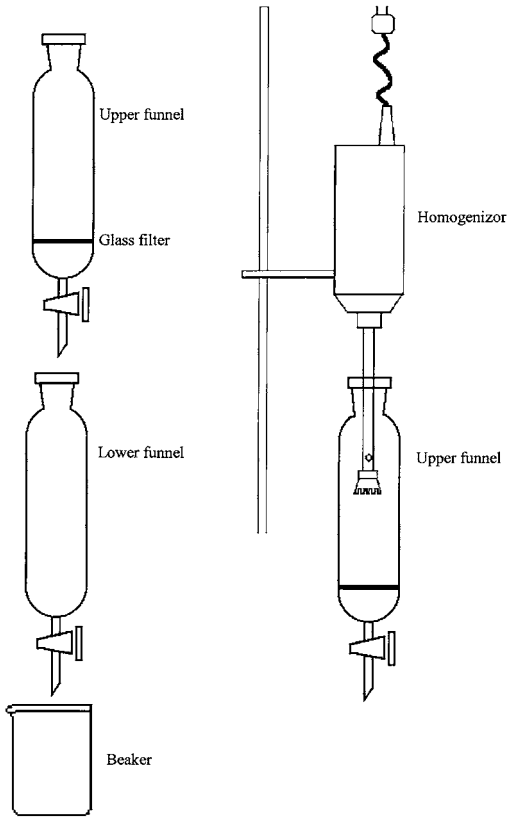

Figure 1. Glass apparatus used in the J methods.

Solvents. PA grade qualities of acetone (Ac), normal hexane (n-Hx), cyclohexane (c-Hx), methanol (MeOH), chloroform ($CHCl_3$), methyl-*tert*-butylether (MTBE), and 2-propanol (IPR) from Merck, Darmstadt, Germany, and diethyl ether (DEE) from Lab Scan, Dublin, Ireland, were used throughout the experiments.

Experimental Design. Six different extraction methods were compared, using 10 g of sample from the same cod fillet batch. Five replicates were run with each method, and the mean, standard deviation (SD), and coefficient of variation were determined.

Jensen (J) Method. Because it is important to keep the water content of the final organic extract to a minimum, the practical handling of the method is described in some detail.

The glass apparatus used in all three compared J modifications consisted of two cylindrical 100 mL separatory funnels (h, 150 mm; i.d., 30 mm), one placed above the other (**Figure 1**). The upper funnel was equipped with a glass filter at the bottom. (For further details, see refs *1*, *2*, *11*.) The common procedure for all three J methods was as follows: A 10 g sample was transferred to the upper funnel and homogenized (Ultra Turrax, Tarmno Gmbh, Staufen, Germany) for 1 min with solvent mixture A (see **Table 1**) and transferred to the lower funnel through the filter by exerting gentle pressure from compressed nitrogen. In the standard procedure, the lower funnel contained 50 mL of 0.1 M phosphoric acid in an aqueous 0.9% sodium chloride solution, but the phosphoric acid was omitted where the aim was to compare the results with the BD method. The sample remaining in the top funnel was then homogenized for 1 min with solvent mixture B and transferred to the lower funnel as described above. Finally, the sample was shaken or stirred with a glass rod with solvent mixture C and also transferred to the lower funnel. During homogenization, small particles of tissue may be collected in the liquid present between the filter plate and the tap. To avoid this material to pass into the lower funnel, a small portion of the extract was first tapped off into a test tube without nitrogen pressure until a clear phase appeared and this was passed back to the top funnel. To avoid formation of an emulsion, the lower funnel was

Table 1. Solvents Used in the J Methods (10 g of Fillet)

	solvent mixtures		
method	A	B	C
J, original	Ac (25 mL) n-Hx (10 mL)	n-Hx/DEE 9:1 (25 mL)	n-Hx/DEE 9:1 (25 mL)
J, mod. I	Ac (25 mL) DEE (10 mL)	n-Hx/DEE 9:1 (25 mL) IPR (10 mL)	n-Hx/DEE 9:1 (25 mL)
J, mod. II	IPR (25 mL) DEE (10 mL)	n-Hx/DEE 9:1 (25 mL) IPR (10 mL)	n-Hx/DEE 9:1 (25 mL)

not shaken but just sealed and turned upside down 30 times. After phase separation, the lower aqueous phase was transferred to a 100 mL beaker. To avoid water in the organic extract, it was necessary to effectively rotate the lower funnel and transfer any additional water into the beaker. The organic phase was decanted into a preweighed beaker. Finally, the aqueous phase was returned to the lower funnel and reextracted with 15 mL of hexane:DEE (9:1 v/v) as above. The combined organic phases in the beaker were evaporated in a cupboard overnight at room temperature and reweighed until constant weight.

Bligh and Dyer (BD) Method (*3*). A 10 g sample was weighed into a 100 mL centrifuge tube and homogenized for 2 min with 30 mL of a MeOH:$CHCl_3$ mixture (2:1 v/v) followed by 10 mL of $CHCl_3$ and mixing for 1 min. Water was then added to make a total of 18 mL including the water present in the sample and mixed for 1 min. The sample was centrifuged for 10 min at 450*g*, and the lower organic phase was transferred with a pipet to a preweighed 100 mL glass beaker. This procedure was repeated for the residue after addition of 20 mL of a $CHCl_3$:MeOH mixture (9:1 v/v). The beaker was finally placed in the cupboard overnight as described for the J method.

Smedes (S) Method (*9*). A 10 g sample in a 100 mL glass centrifuge tube was homogenized with 16 mL of IPR and 20 mL of c-Hx for 2 min. Water was then added to give a total water volume of 22 mL. The water content of the sample was approximately 8 mL; hence, 14 mL of water was added. The phases were separated by centrifugation for 10 min at 450*g*. The organic phase was pipetted to a 100 mL preweighed beaker. The procedure was repeated after addition of 20 mL of c-Hx:IPR (87:13 v/v). Finally, the beaker was placed in the cupboard overnight as above.

Modified Folch (F) Method (*12*). A 10 g sample was homogenized with 50 mL of MeOH for 1 min in a blender. A 100 mL amount of $CHCl_3$ was added, and the homogenization was repeated for 2 min. The mixture was transferred to a 300 mL cylindrical separatory funnel with a sintered glass filter at the bottom, and the extract was pressed to a second separatory funnel with nitrogen gas. The residue in the upper funnel was reextracted by shaking for 3 min with 150 mL of a 2:1 v/v mixture of $CHCl_3$:MeOH followed by filtration as above. The combined extracts were washed by shaking thoroughly with 75 mL of 0.9% potassium chloride in water, and the phases were allowed to settle. The organic lower phase was transferred to another separatory funnel and washed twice with 50 mL portions of a 1:1 v/v mixture of MeOH: 0.9% potassium chloride in water. The organic lower phase was transferred to a 200 mL beaker, and the volume was reduced to about 50 mL in a water bath (60 °C) under a stream of nitrogen. Finally, it was transferred to a preweighed 100 mL glass beaker, and the solvents were allowed to evaporate overnight.

RESULTS AND DISCUSSION

Table 2 shows the mean of five fat determinations for each method of the same homogenate using six different methods. The SDs are acceptably low, which indicates that the reported method for preparation of a homogenate sample of up to 500 g of fish fillet with solid carbon dioxide is effective. The crushed frozen homogenate stored in a Dewar flask at −18 °C appeared intact over a period of 6 months. No sign of water sublimation was observed; that is, no ice crystals were seen on the inside of the container probably due to the evacuated double glass wall of the Dewar flask.

Table 2. Extractable Fat in 10 g of Cod Fillet Expressed in Percent Using Six Different Extraction Methods[a]

method	lipids (%)	SD	variation coefficent	yield rel BD 100%	yield rel F 100%
J, original	0.623	0.031	5.0	75.7	78.5
J, mod. I	0.676	0.018	2.7	82.1	85.1
J, mod. II	0.801	0.013	1.6	97.3	100.9
S	0.694	0.029	4.1	84.3	87.4
BD	0.823	0.017	2.1	100.0	103.7
F mod.	0.794	0.015	1.9	96.5	100.0

[a] All determinations were performed in five replicates except for F, which was four replicates.

The BD method is generally accepted to extract lipids quantitatively and was thus primarily set to 100% recovery. As seen from **Table 2**, the original J method gives by far the lowest fat recovery values (75.6%) for this lean tissue, even though the method was developed to give lipid yields identical with those of the official F method for fat fish. The levels of lipid soluble accumulating substances, such as PCB and DDT, were also extracted quantitatively with the original J method on lean fish (*1*, *2*). Consequently, the pollutant residues previously reported on a lipid weight basis can be 25% too high where the original J method has been used for tissues with less than about 1% of fat. This fact must be taken into account when comparing residual levels between the different species and the decline of levels over time.

As mentioned, a very likely explanation for the low lipid recovery is that in lean fish such as cod and pike, most of the lipids are present in the cell membranes. Here, the phospholipids dominate and can make up for more than 50% of the total lipids (*13*). For herring, having up to 5% fat, the phospholipids account for a minor amount of the total lipids. Very likely, Ac as used in the J method is not effective enough to extract the phospholipids, which leads to poor lipid recovery from lean matrixes. The aim of the present work was to improve the J method, still avoiding chlorinated solvents and applying the same technique as before to achieve the same lipid extraction efficiency as the BD method. The fundamental idea behind the original J method is that the first step in the extraction acts as a dehydrating step on the matrix thus making the fat accessible for the extraction. In the original method, the dehydration was carried out with Ac, which is known to be a poor solvent for phospholipids (*14*), it has been substituted for IPR in the modified method (J modification II).

In the original J method, the first extraction solvent Ac also contains 10% (v/v) of *n*-hexane. By replacing the *n*-hexane with DEE, as in J modification I, the yield of lipids from cod increased from 75.6 to 82.0% as compared to the BD method (100%). An attempt to replace DEE with MTBE resulted in a strong emulsion. When Ac was replaced with IPR (modification II) the lipid recovery increased from 82.0. to 97.2% of the BD recovery. In contrast to the original J method, the modification

Table 3. Extractable Lipids from Different Aqueous Organisms Expressed in Percent Using the Original J Method and the Modification II, Respectively[a]

			original J			modified J			k = ratio
		n	fat (%)	SD	CV	fat (%)	SD	CV	of fat %
cod[b]	spring	5	0.623	0.031	5.0	0.801	0.013	1.6	0.78
pike[c]	autumn	5	0.561	0.023	4.1	0.753	0.036	4.8	0.75
flounder[d]	spring	10	1.545	0.039	2.5	1.670	0.032	1.9	0.93
herring[e]	autumn	5	5.474	0.225	4.1	5.892	0.096	1.6	0.93

[a] n, number of analysis. [b] Same homogenate as in **Table 2**. [c] Homogenate of pike from the Lake Bolmen in Sweden. [d] Homogenate of flounder from the Western-Scheldt. [e] Homogenate of herring from the Archipelago of Stockholm.

Table 4. Extractable Lipids from Flounder Homogenates from Four Different Locations from the Helsingborg Harbor in the South of Sweden and the Danish Sounds, Caught the Same Day in the Autumn of 2001[a]

	original J fat (%)	modified J fat (%)	k = ratio of fat %
homogenate 1	0.824	1.117	0.74
homogenate 2	0.855	1.011	0.85
homogenate 3	1.201	1.212	0.99
homogenate 4	1.887	2.008	0.94

[a] A total of 3–21 individuals were homogenized to represent each location.

II involving the solvents IPR, DEE, and water (from the matrix) constitutes a one phase ternary solvent system. In this respect, the modified J method operates according to the same extraction principle as that of the BD method.

We were not able to obtain acceptable recoveries with the S method (*9*) (84.2 ± 2.6%). Smedes reports a fat recovery of 92% relative to BD for plaice with a fat content level of 1.4%. The poor recovery experienced by us using the S method may be due to the very low fat content of the cod used in our experiment (0.8%). To study if a fat range of 1−2% is especially critical for the truthfulness of the original J method, we studied flounder with a fat percent in this interval (see **Table 3**). The fat recovery has improved with the original J method to give a fat recovery of 93% (see **Table 3**) as compared to the modified method. The same picture is indicated in **Table 4** with flounder homogenates from four nearby localities in the Helsingborg harbor and the Danish sound.

Both the BD and the S methods rely on well-balanced proportions of the extraction solvent mixture and water. The lipids and the lipid soluble contaminants such as PCB and DDT will partition to either the lower $CHCl_3$ or the upper c-Hx layers, respectively. As seen from **Table 2**, the BD method gave higher lipid residual levels than the F method despite the fact that the F method uses five times more of the same solvent mixture than the BD method, but in our experience, the BD method often suffers from the heavy formation of emulsions. We also found that both the BD and the S methods created difficulties to separate the phases.

The contamination with water in the final extract may be a general problem in the BD and S methods since it is indicated that it is necessary to heat the organic extract to 103 °C before the fat determination, a temperature that in the presence of oxygen will destroy many polyunsaturated fish lipids. Following the detailed description of the J method, it is possible to avoid water in the final extract and thus to be able to evaporate the solvent at room temperature.

To check whether the variances among groups differed, Bartlett's test for homogeneity of variances was used. Although unduly sensitive to departures from normality (*15*), the test revealed no statistically significant differences regarding the variances. The number of samples is however low, and consequently, the power of such a test is also low.

When the arithmetic means of fat concentrations from the BD, S, F, and J method (modification II), respectively, were compared, significant differences were found (analysis of variance (ANOVA), $p < 0.001$, and Kruskal-Wallace, $p < 0.002$). When post-hoc tests (LSD, least significant difference) and Scheffé (*15*), for detailed comparisons of means were applied, the S method showed significantly lower mean fat values than the other three methods. With the LSD method also, the F and BD method turned out to be significantly different, $p < 0.05$, whereas Scheffé's more conservative test for unplanned comparisons gave a nonsignificant result between these two methods.

Assuming that the recoveries of the lipophilic environmental contaminants are quantitative using any of the investigated batch liquid methods (*6*), it may become appropriate to use a correction factor to adjust for the differences between old and recent reports on lipid basis. Using the data in **Table 2** for cod, this correction multiplier should be 0.78 for lipophilic contaminant residues reported on a lipid weight basis.

Table 3 shows the result from the analyses of some further fish species with different levels of lipid concentrations using the traditional and modified J methods. Pike from lake Bolmen (southern Sweden) sampled during spring showed figures very close to those of the cod (**Table 2**) suggesting the use of a correction factor (k) for the original J method. The k value for herring (Baltic Sea) was close to the expected (1.0), whereas lower k values were calculated for flounder.

Apart from extracting quantitatively the phospholipids from lean fish, the modified J method merits from its simplicity enabling the handling of a large number of samples. Fifteen lipid determinations can easily be performed per day by a skilled person.

The different polarities between the membrane and the storage lipid regions in the muscle cell raise points of considerable interest with respect to the localization of the hydrophobic pollutants in the intact cells. On the basis of solubility aspects only, one would expect a higher ratio of pollutants in the storage lipids than in the membranes. However, little is known about the behavior of the pollutants on a subcellular level, presumably due to experimental difficulties. The presence of hydrophobic pollutants in the membranes and their possible concentration in this matrix would imply physiological effects on the membrane functions. Interesting future work should therefore include determinations of pollutants in plasma membranes and cellular organelles.

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