Comparison of the Bligh and Dyer and Folch Methods for Total Lipid Determination in a Broad Range of Marine Tissue

Sara J. Iverson*, Shelley L.C. Lang, and Margaret H. Cooper

Department of Biology, Dalhousie University, Halifax, Nova Scotia B3H 4J1, Canada

ABSTRACT: For many studies, it is important to measure the total lipid content of biological samples accurately. The Bligh and Dyer method of extraction was developed as a rapid but effective method for determining total lipid content in fish muscle. However, it is also widely used in studies measuring total lipid content of whole fish and other tissues. Although some investigators may have used modified Bligh and Dyer procedures, rarely have modifications been specified nor has their effectiveness been quantitatively evaluated. Thus, we compared this method with that of the classic Folch extraction in determining total lipid content of fish samples ranging from 0.5 to 26.6% lipid. We performed both methods as originally specified, i.e., using the chloroform/methanol/water ratios of 1:2:0.8 and 2:2:1.8 (before and after dilution, respectively) for Bligh and Dyer and of 8:4:3 for Folch, and with the initial solvent/sample ratios of (3+1):1 (Bligh and Dyer) and 20:1 (Folch). We also compared these with several other solvent/sample ratios. In samples containing <2% lipid, the results of the two methods did not differ. However, for samples containing >2% lipid, the Bligh and Dyer method produced significantly lower estimates of lipid content, and this underestimation increased significantly with increasing lipid content of the sample. In the highest lipid samples, lipid content was underestimated by up to 50% using the Bligh and Dyer method. However, we found a highly significant linear relationship between the two methods, which will permit the correction of reported lipid levels in samples previously analyzed using an unmodified Bligh and Dyer extraction. In the future, modifications to procedures and solvent/sample ratios should be described.

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The total lipid content of biological samples is an important quantity used in many biochemical, physiological, and nutritional studies. Thus, reliable methods for the quantitative extraction of lipids from tissues are of critical importance. Natural lipids generally comprise mixtures of nonpolar components such as glycerides (primarily triacylglycerol) and cholesterol, as well as some free fatty acids and more polar lipids. Isolation, or extraction, of lipid from tissues is performed with the use of various organic solvents. In principle, the solvent or solvent mixture used must be adequately polar to remove lipids from their association with cell membranes and tissue constituents but also not so polar that the solvent does not readily dissolve all triacylglycerols and other nonpolar lipids (1). Folch *et al.* (2) were one of the first to recognize this and develop the chloroform/methanol/water phase system (the so-called "Folch" method), which, under various modifications, continues to be considered the classic and most reliable means for quantitatively extracting lipids. In the interest of economy, less exhaustive methods have been developed. By far the best known is the "Bligh and Dyer" method (3), which has become one of the most recommended methods for determining total lipid in biological tissues (4,5) and indeed has become the standard for lipid determination in many studies of marine fish (e.g., 1, 5–12) as well as for other types of samples such as milks (e.g., 13,14).

The primary advantage of the Bligh and Dyer method is a reduction in the solvent/sample ratio (1 part sample to 3 parts 1:2 chloroform/methanol followed by 1 or 2 parts chloroform) (1,3). In contrast, the Folch method employs a ratio of 1 part sample to 20 parts 2:1 chloroform/methanol, followed by several washings of the crude extract (2). Despite this solvent reduction, the Bligh and Dyer method is nevertheless thought to yield recovery of ≥95% of total lipids (1). Although the procedure was developed using cod muscle, it states (1,3) that it can be applied to any tissue containing (or modified to contain) 80% water. Hence, it has been used ubiquitously. Although the Bligh and Dyer method has undergone rigorous and favorable evaluations (e.g., 5,9,16), virtually all of these evaluations have been performed on samples containing less than 1.5% total lipid. Some studies report using a modified Bligh and Dyer method for lipid-rich samples; however, the modifications are often unspecified (e.g., 15), making the evaluation and comparison of results difficult. In other cases, investigators report the use of the Bligh and Dyer method even with samples having high lipid contents, but do not indicate that any modifications have been made. In the course of recent studies in our laboratory, we discovered that samples of a known high lipid content were greatly underestimated using the Bligh and Dyer method compared to the Folch method, although we did not detect any difference in the fatty acid composition under either method. Since much of the data published on the lipid contents of whole fish and other samples have been derived using the Bligh and Dyer method, we undertook a study to evaluate the relationship between these methods in their estimation of total lipid content.

*To whom correspondence should be addressed. E-mail: siverson@is.dal.ca

MATERIALS AND METHODS

Fish and invertebrates were chosen to represent a wide range of lipid contents based on previous species estimates. A total of 36 individuals were used, which included pollock, herring, rock sole, rock fish, sculpin, octopus, and squid. Each whole animal was thoroughly ground and homogenous subsamples were taken for extraction. To increase the range of lipid contents evaluated, we also used weighed aliquots (n = 9) of a homogenous mixture of ground commercial fish (originally containing 2% fat) and commercial fish oil. Weighed quantities of oil were added to produce mixtures ranging from an estimated 21 to 26% lipid. Our primary interest was to evaluate the Bligh and Dyer method compared to the Folch method, but because of the high solvent volumes used in the Folch, we also evaluated the performance of a reduced-solvent Folch using a subset of these samples. Within each method, all samples were extracted and lipid contents were quantified in duplicate.

The Bligh and Dyer extraction was performed as originally outlined using the following ratios (1,3): Briefly, 100 g sample containing (or adjusted to contain) 80 g water (as determined by oven drying separate aliquots) is homogenized with 100 mL chloroform and 200 mL methanol (monophasic system). The solution is rehomogenized with 100 mL chloroform, following which 100 mL of either distilled water (3) or weak salt solution (e.g., 0.88% NaCl or KCl) (1,9) is added. After filtration is performed under suction, the final biphasic system is allowed to separate into two layers and the lower (chloroform) phase is collected. For quantitative lipid extraction (3), the tissue residue is then rehomogenized with 100 mL chloroform, filtered, and the filtrate added to the lower phase collected. Lipid content is then determined gravimetrically after evaporating a measured aliquot of the combined chloroform phase to dryness under nitrogen (see below). As Bligh and Dyer stated (3,16), the above volumes can be scaled down, as long as the critical ratios of chloroform, methanol, and water (1:2:0.8 and 2:2:1.8, before and after dilution, respectively) and of initial solvent to tissue [(3 + 1):1] are kept identical. Thus, we followed the above procedures but reduced the scale of all components (i.e., keeping all ratios the same) for use with a smaller sample amount (4 g sample in a 40 mL conical glass centrifuge tube), to allow both centrifugation of the final biphasic system and collection of the entire lower phase for evaporation and subsequent lipid estimation. Instead of applying manual pressure (3) to the small filter cake, we performed a second chloroform wash to improve removal of residual lipid during filtration.

The Folch extractions were performed as described, using the original extraction ratio of 20 parts 2:1 chloroform/methanol to 1 part tissue, which can be done on any scale that is technically feasible (2). A weak salt solution (e.g., 0.58–0.88% NaCl or KCl) is then added to achieve a final ratio of 8:4:3 chloroform/methanol/water after including the water contained in the tissue (1,2). We also compared the original ratio against a modified version using 30 parts 2:1 chloroform/methanol to 1 part tissue (1). After verifying that the 20:1 and 30:1 solvent/sample ratios produced similar results in our samples (n = 27, all <25% lipid; Fig. 1A), we analyzed the rest of the samples using only the 20:1 ratio as follows: 1.5 g tissue was homogenized with 30 mL 2:1 chloroform/methanol. Although Christie (1) reports improvement by first homogenizing with 10 mL methanol followed by 20 mL chloroform, we have tested both procedures without detecting differences (Iverson, S.J, Lang, S.L.C., and Cooper, M.H., unpublished results). The mixture was filtered and then washed several times with 2:1 chloroform/methanol, and 0.88% NaCl in water was added to the combined filtrate at a final ratio of 8:4:3 chloroform/methanol/water. Finally, we used a "reduced-solvent" Folch, where the ratios of solvent to sample were 7.5:1.0 (i.e., closer to that of the Bligh and Dyer method), but the chloroform/methanol/water ratio was kept the same (i.e., 8:4:3).

In all the above extractions (both Bligh and Dyer and Folch), the final biphasic system was centrifuged, and the entire lower phase (along with washings) was collected into a

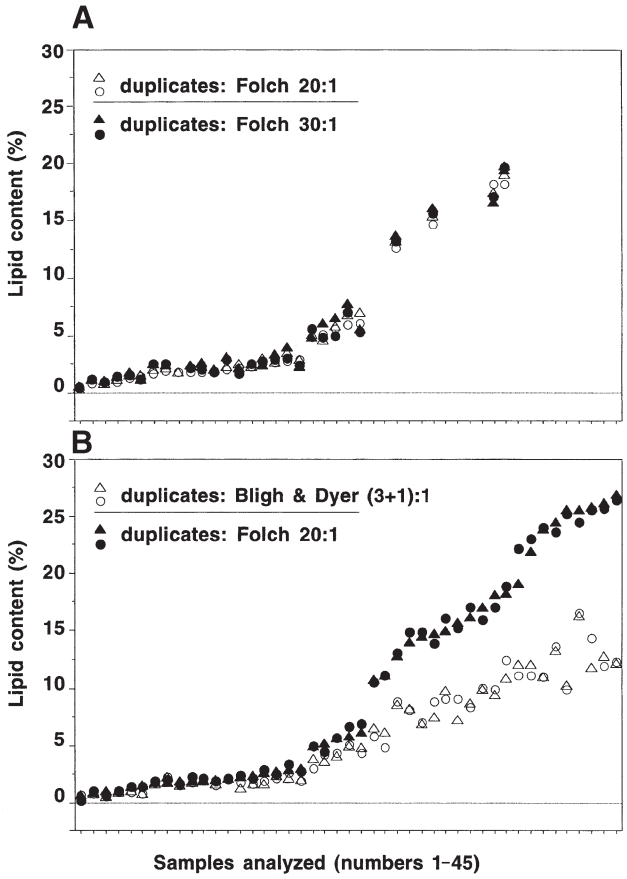

FIG. 1. Estimates of total lipid content determined in replicate aliquots: (A) samples (n = 27) extracted using both a 20:1 and a 30:1 solvent/sample ratio Folch and (B) all samples (n = 45) using the Bligh and Dyer method in comparison with the original Folch method. The last nine samples on the x-axis represent the homogenates of commercial fish and oil, which were produced to contain a range of 21–26% lipid. All samples were analyzed in duplicate in each of the extraction methods and are presented in approximate order of increasing lipid content.

preweighed glass tube and evaporated to dryness in an analytical high-speed nitrogen evaporator (24-position N-EVAP 112, Organomation Associates, Inc., Berlin, MA) fitted with stainless steel 14-in. × 19-gauge needles and equipped with a thermostatically controlled water bath maintained at 25–30°C. The nitrogen stream was continually moved so that it actively disturbed the evaporating surface of the sample until all detectable traces of solvent were gone. To remove all final traces of solvent and water, the sample tube was then wiped dry and placed in a sealed glass vacuum tube and flushed with nitrogen, and vacuum suction was applied for 5 min (BOC Edwards model RV3 vacuum pump; Crawley, West Sussex, United Kingdom). Lipid content was then determined gravimetrically. Since results of the Folch method using 20:1 or 30:1 solvent/sample ratio did not differ, we used the results from the 20:1 Folch method as the basis for comparison with and evaluation of the other extraction methods.

RESULTS

In general, duplicate analyses within each extraction method were very consistent, although more so for Folch extractions (n = 45, Fig. 1B). In samples containing <2% lipid (n = 11), results for the Bligh and Dyer method did not differ from those obtained by the Folch method (P = 0.150, paired t-test). However, for samples containing >2% lipid (n = 34), the Bligh and Dyer estimates of lipid content were significantly lower than those of Folch (P < 0.0001). In our nine samples of fish oil–supplemented homogenates, lipid content estimates (20.6–26.6%) using Folch extraction concurred with our estimated lipid contents (21–26%, as discussed in the Materials and Methods section); however, lipid content estimates using the Bligh and Dyer extraction were 50% lower (Fig. 1B). The next-highest lipid contents were found in herring samples (n = 12, 10.7–18.6% lipid by Folch), which were estimated to be about 45% lower (6.1–11.6% lipid) using the Bligh and Dyer method.

The underestimation of lipid content by the Bligh and Dyer method increased significantly with increasing lipid content (Fig. 2A). From 0% to approximately 2% lipid, results of the two methods agreed well. However, with increasing lipid content, the deviation from the one-to-one reference line increased. We were interested in describing the predictive relationship between the two methods to allow correction of previous lipid content analyses that we had performed using the Bligh and Dyer method. Using a log–log plot, we found a highly significant linear relationship between lipid content determined by the Folch method and that determined by the Bligh and Dyer method (Fig. 2B).

The results of the reduced-solvent Folch (7.5:1.0 solvent/sample ratio) were highly correlated with both the 20:1 and 30:1 Folch (r = 0.999, n = 34, and r = 0.987, n = 27, respectively); however, the reduced-solvent method tended to underestimate lipid content as lipid content increased. In samples containing ≤3% lipid (n = 19), there was no significant difference between the Folch extractions using the 20:1 vs.

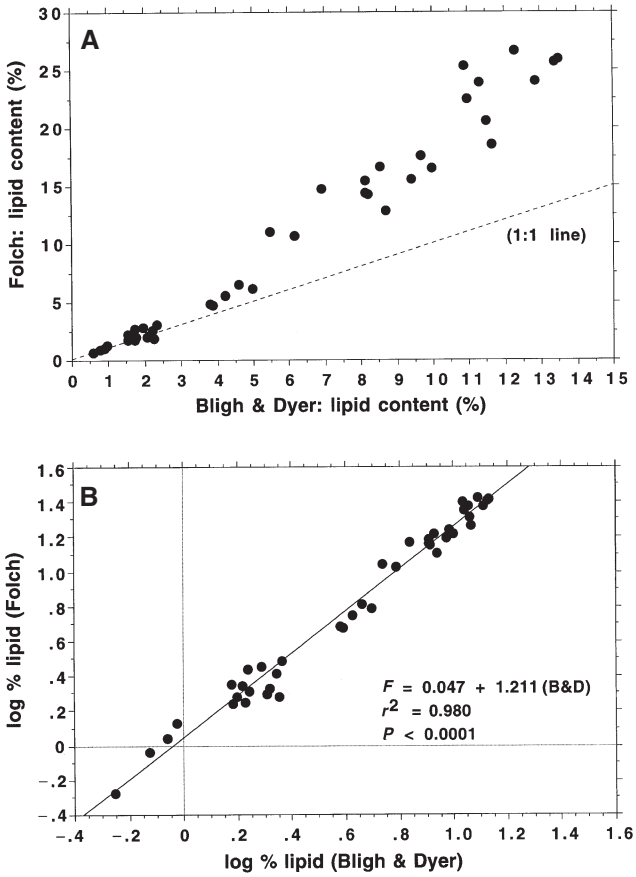

FIG. 2. (A) Correlation of the estimates of lipid content (duplicates averaged) in 45 samples using the Folch (20:1) vs. Bligh and Dyer methods (r = 0.9834, P < 0.0001); the dashed line represents the one-to-one reference line. (B) The log–log predictive relationship between estimates of lipid content using the Folch (F) vs. the Bligh and Dyer (B&D) method.

the 7.5:1 solvent/sample ratios (1.9 ± 0.16% vs. 1.9 ± 0.18% lipid, respectively; P = 0.9559, paired t-test), but in samples containing >3% lipid (n = 15), the reduced-solvent Folch significantly underestimated lipid content (10.7 ± 1.18% vs. 12.0 ± 1.30%, P < 0.0001). The lipid content estimates of these same 15 samples, using the Bligh and Dyer method, were even lower at 7.2 ± 0.65% lipid. In the highest-lipid natural fish sample tested (herring), lipid content was estimated as 18.6, 16.4, and 11.6% using the 20:1 Folch, the 7.5:1.0 Folch, and the Bligh and Dyer methods, respectively.

DISCUSSION

In the time since the Folch (2) and the Bligh and Dyer (3) methods for total lipid determination were published, there have undoubtedly been numerous modifications to both methods to improve the efficiency of lipid recovery from various tissues. However, in many publications where these methods have been used, modifications have been neither described nor validated. In other cases, investigators stated that lipids were quantified "according to" one or the other method, but they do not indicate whether any modifications were made,

implying that the methods were applied basically according to the original procedures, even though that may not have been the case. Given that many conclusions about tissue and whole-body lipid and energy values are based on published lipid contents, our purpose was to evaluate these two methods, as originally described, with the aim that investigators could evaluate previously published data and that appropriate modifications would be made and described in the future.

In numerous tests with samples containing <2% lipid, the Bligh and Dyer method has been shown to be very effective and reliable (4,5,9,16). Like other investigators (5), we found that lipid extraction using the Bligh and Dyer method produced estimates of total lipid content identical to those of Folch in samples containing <2% lipid. We also did not detect any differences in the subsequent fatty acid composition of duplicate samples extracted under either method, although this may require further investigation in very low fat samples that contain a higher phospholipid/neutral lipid ratio (e.g., alkali hydrolysis followed by methylation and fatty acid quantitation could also be used to examine any biases in total fatty acid recovery). However, in contrast to low-lipid samples, in all samples containing >2% lipid, the Bligh and Dyer method produced significantly lower estimates of lipid content, and this underestimation increased with increasing lipid content of the sample.

We have several reasons to believe that the total lipid contents of all samples were accurately determined using the Folch extraction method. First, as stated above, in low-lipid samples both the Folch and Bligh and Dyer results were identical. Second, the estimates of percent lipid in the high-lipid fish oil–supplemented homogenates, using the basic Folch extraction, agreed with our calculated lipid contents; furthermore, an increased (30:1) solvent/sample ratio Folch produced the same values. Finally, these homogenates were also analyzed for protein content (by macro-Kjeldahl), as well as dry matter (Cooper, M.H., unpublished data). The amount of dry matter not accounted for by protein and lipid in these samples was reasonably consistent with expectation at 2–4% using the lipid values obtained by Folch extractions, but was quite high (14–20%) using the lipid values obtained by the Bligh and Dyer extractions.

Bligh and Dyer (3) developed their method using fish fillets (i.e., muscle) that generally contained low levels of lipid and a high proportion of phospholipid. In whole animals and in tissue, an increase in total lipid content is due predominantly to increases in triacylglycerol. Indeed, subsets of our isolated lipid subjected to thin-layer chromatography (17) showed that the primary component in the extract was triacylglycerol (especially as lipid content increases), followed by minor amounts of more polar lipid classes. Although Bligh and Dyer (3) stated that their method could readily be applied to other biological tissues, they, as well as others, acknowledged that lipid-rich samples may require modifications. For instance, Christie (1) suggested that very lipid-rich tissues such as adipose tissue and oil seeds should be extracted first with a nonpolar solvent such as diethyl-ether or chloroform, after which the remaining lipid could be recovered effectively using Bligh and Dyer methods. However, this appears to have often gone unrecognized. The total yield of lipids may be more reduced than most investigators have suspected, especially given the widespread use of apparently unmodified Bligh and Dyer extractions for whole fish and other tissues. Even in samples containing 2–10% lipid (which is common for many marine fish and invertebrates), underestimation will still be a significant problem (e.g., Fig. 1), and this has likely been neglected.

The reduced efficiency of the Bligh and Dyer method with increasing tissue lipid contents might be explained from several standpoints. One cause of reduced lipid yield at high lipid concentrations could be the limited solubility of the predominantly nonpolar lipids, such as triacylglycerols, in the seemingly relatively polar solvent solution (1:2 vol/vol chloroform/methanol) employed in the Bligh and Dyer method, which was designed chiefly to extract phospholipid efficiently. However, although the initial solvent ratios are different in the Bligh and Dyer vs. the Folch methods, they do not result in measurably different contents of methanol in the final organic (chloroform) phase (e.g., 16). Hence, this is not likely to be a significant factor. Smedes and Thomasen (16) found that the absorption of the organic phase by the tissue was one of the main causes of incomplete lipid yield. Relatively constant amounts of the organic phase are absorbed by the tissue such that using greater volumes of organic-phase solvents reduces the fraction of the organic phase that is lost in this manner (16). When tissues with increasing lipid contents are extracted (using the same volumes of solvents), the lipid concentration in the organic phase should also increase, assuming that limits of solubility are not reached. This would result in increased loss of lipid in the fraction of organic phase absorbed by the tissue, causing a reduction in final lipid yield. Thus, in addition to maintaining critical solvent and water ratios, perhaps the most important consideration is simply the ratio of solvent to dry-weight sample (and expected fat content), as even with the Folch method, a reduced ratio produced significant underestimates of lipid content.

Our results do indicate that all methods used to estimate lipid contents were highly correlated. Fortunately, there is a highly predictable relationship between the Bligh and Dyer and Folch methods (Fig. 2B), potentially allowing correction of reported values from previous analyses that used an unmodified Bligh and Dyer extraction. It may also be the case that investigators have used a modified Bligh and Dyer extraction employing an increased solvent/sample ratio that produced reliable results and have simply not stated this. It will be important in the future that investigators specify modifications to any of these procedures, especially the precise solvent/sample ratio used. For instance, although an increase in the solvent/sample ratio (i.e., to 30:1) from the original Folch did not appear to alter the estimated lipid content significantly (Fig. 1A), we would not recommend making this assumption for tissues containing greater than 25% lipid (i.e. adipose tissue, milks of many species) unless verified. In such samples,

a further increase in the solvent/sample ratio and/or further multiple extractions may be necessary for quantitative lipid evaluation (e.g., 1), as we have found for marine mammal milks (Iverson, S.J., personal communication).

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