Comparison of three microquantity techniques for measuring total lipids in fish

Yuehan Lu, Stuart A. Ludsin, David L. Fanslow, and Steven A. Pothoven

Abstract: To measure lipids in juvenile and adult fishes, we refined three microquantity approaches (microgravimetric, microcolorimetric sulfophosphovanillan (SPV), and Iatroscan thin layer chromatography – flame ionization detection (TLC–FID)) that were originally developed to measure lipids in small aquatic invertebrates. We also evaluated their precision and comparability by quantifying the total lipid content of age-1+ walleye (*Sander vitreus*), yellow perch (*Perca flavescens*), and lake whitefish (*Coregonus clupeaformis*) collected in Lake Erie (US–Canada), Lake Michigan (USA), and Muskegon Lake (Michigan, USA). Our findings demonstrate that (*i*) microquantity approaches provide estimates of total lipids in juvenile and adult fishes similar to those of more traditional macroquantity approaches, (*ii*) the microcolorimetric SPV and microgravimetric approaches produce near identical estimates of total lipid content, and (*iii*) the Iatroscan TLC–FID approach underestimates total lipids relative to the other approaches for individuals with high lipid levels. Ultimately, our research makes available additional techniques for measuring total lipid content of fishes that are less expensive than traditional techniques, owing to a reduced need for large quantities of samples and solvents.

Résumé : Afin de doser les lipides dans les poissons juvéniles et adultes, nous raffinons trois méthodologies microquantitatives (microgravimétrie, microcolorimétrie à sulfophosphovanilline (SPV) et chromatographie sur couche mince Iatroscan couplée à un détecteur à ionisation de flamme (TLC–FID)) mises au point au départ pour mesurer les lipides dans de petits invertébrés aquatiques. Nous avons évalué leur précision et les avons comparées entre elles en dosant le contenu en lipides totaux de dorés (*Sander vitreus*), de perchaudes (*Perca flavescens*) et de grands corégones (*Coregonus clupeaformis*) d'âge 1+ récoltés aux lacs Érié (E.-U. – Canada), Michigan (É.-U.) et Muskegon (Michigan, É.-U.). Nos observations démontrent que (*i*) les méthodes microquantitatives fournissent des estimations des lipides totaux chez les poissons juvéniles et adultes semblables à celles des méthodologies macroquantitatives plus courantes, (*ii*) les méthodologies microcolorimétriques SPV et microgravimétriques donnent des estimations presque identiques du contenu lipidique total et (*iii*) la méthodologie TLC–FID Iatroscan sous-estime les lipides totaux par comparaison aux autres méthodes chez les individus qui ont un fort contenu lipidique. En fin de compte, notre recherche rend disponibles de nouvelles techniques pour mesurer le contenu lipidique total chez les poissons, qui sont moins coûteuses que les techniques habituelles, parce qu'elles requièrent de moins grandes quantités d'échantillons et de solvants.

[Traduit par la Rédaction]

Introduction

Lipids are an integral component of aquatic animals, and their estimation can provide insight into growth strategies, health and condition, and survival potential of aquatic invertebrates and vertebrates alike (e.g., Arts et al. 1993; Ludsin and DeVries 1997; Post and Parkinson 2001). In turn, numerous approaches for quantifying lipid content of aquatic biota have been developed. Older, more traditional lipid analyses (e.g., Folch et al. 1957; Bligh and Dyer 1959) require large quantities of both the sample and solvents (e.g., methanol, chloroform), which can be problematic when dealing with organisms with low lipid levels (e.g., pooling of individuals is required; see Miranda and Hubbard 1994) or a small budget. However, with improved technological and analytical capabilities, "microquantity" approaches that require only micrograms of sample and microlitres of solvents have been developed for estimating lipid content of aquatic organisms. In turn, sample processing costs have declined and the ability to assess variation in lipid content at the level of an individual has increased.

There are three microquantity techniques that have be-

Received 30 October 2007. Accepted 2 April 2008. Published on the NRC Research Press Web site at cjfas.nrc.ca on 30 September 2008.
J20243

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come commonly used in the aquatic sciences: (*i*) Van Handel (1985) developed a microquantity colorimetric sulfophosphovanillan (SPV) method to measure total lipids in mosquitoes; (*ii*) Gardner et al. (1985) developed a microgravimetric assay to measure total lipids in a variety of freshwater aquatic invertebrates (e.g., amphipods, oligochaetes); and (*iii*) Parrish (1986, 1987) introduced a microquantity thin-layer chromatographic approach, using an Iatroscan flame ionization detection system (Iatroscan TLC–FID; Mitsubishi Kagaku Iatron, Inc., Nishi-Goken-cho, Shinjuku-ku, Tokyo, Japan) to measure lipid classes (e.g., triglycerides, sterol esters, sterols, free fatty acids, phospholipids) and total lipids (by summing lipid classes), which has been adapted for use with zooplankton, benthic macroinvertebrates, larval fish, and juvenile fish (Fraser et al. 1985; Arts et al. 1993; Ludsin and DeVries 1997).

Despite development of an array of microquantity approaches for use with small aquatic organisms, not all techniques, including Van Handel's (1985) microcolorimetric SPV approach and Gardner et al.'s (1985) microgravimetric approach, have been adapted for use with comparatively large organisms such as juvenile and adult fish. Further, our understanding how these three microquantity techniques perform relative to one another is incomplete. Gardner et al. (1985), however, did find that their microgravimetric assay correlated strongly with Iatroscan TLC–FID measurements for individual amphipods and oligochaetes. Further, although Van Handel's (1985) microcolorimetric SPV approach has been preferred over Gardner et al.'s (1985) microgravimetric method for measuring total lipids of benthic macroinvertebrates, because it is more time- and labor-efficient (Landrum et al. 2002, 2004; Inouye and Lotufo 2006), a true comparison of the relative performance of these two microquantity methods has not yet been conducted for any organism.

Owing to differences in how lipids are quantified among gravimetric, colorimetric SPV, and Iatroscan TLC–FID techniques, a potential exists for method-specific biases. For example, gravimetric approaches have potential to be biased upwards because of inclusion of nonlipid materials (Hopkins et al. 1984). By contrast, colorimetric SPV approaches, which measure the absorbance of the red–purple complex produced from the reaction between double carbon bonds and the SPV reagent, may be biased downwards, as only compounds containing unsaturated carbon bonds are quantified (Barnes and Blackstock 1973). Finally, the Iatroscan TLC–FID approach, which sums individual lipid classes to generate an estimate of total lipids, has potential to underestimate lipids, owing to nonlinear responses of the TLC–FID detector (e.g., Parrish 1986, 1987). If reliable comparisons among studies employing these microquantity techniques to quantify total lipids are to be made, a critical evaluation of their relative performance is needed.

Herein, we conducted a laboratory study designed to determine how these microgravimetric, microcolorimetric SPV, and Iatroscan TLC–FID approaches compare with one another in terms of measuring total lipid content of three fishes. In so doing, we developed protocols for using Gardner et al.'s (1985) microgravimetric and Van Handel's (1985) microcolorimetric SPV approaches for use with juvenile and adult fishes. We also refined Parrish's (1986, 1987) protocol and provided an alternative to Ludsin and DeVries' (1997) protocol for using Chromarod Iatroscan TLC–FID to measure total lipids (by summing lipid classes) of juvenile and adult fishes. Ultimately, through development of these protocols and comparison of the relative performance of these techniques, we sought (*i*) to enhance the abilities of scientists to conduct inexpensive, yet reliable, analyses of lipid content in fishes, and (*ii*) to provide a means to compare findings from previous investigations that used any of these methods for measuring total lipids.

Materials and methods

Sample preparation

We analyzed the whole-body lipid content of age-1+ walleye (*Sander vitreus*), yellow perch (*Perca flavescens*), and lake whitefish (*Coregonus clupeaformis*), which are of economic and ecological importance in numerous freshwater systems throughout North America and represent a wide range of lipid levels in fish. Samples were collected from Lake Erie, Lake Michigan, or Muskegon Lake (a drowned-river mouth located at Muskegon, Michigan). We also analyzed lipid content of individual gonads (testes = 4, ovaries = 6) from a subset of 10 walleye, which originated in western Lake Erie but were being reared in the Michigan Department of Natural Resources' Saline Fish Hatchery (Saline, Michigan). All other fish were collected during May to September of 2005 or June to October of 2006 via bottom trawling (Table 1). Upon collection, fish were measured (to the nearest millimetre total length (TL)) and weighed (to the nearest gram). After fish reached a frozen consistency in the freezer (the grinder performs better on frozen material), they were immediately homogenized with a Waring Commercial blender (model HGB-300, Waring Corp., New Hartford, Connecticut, USA) with no additional liquid and stored in individually labeled glass jars at –80 °C until being freeze-dried. Freeze-dried samples were then reground with a Hamilton Beach hand blender (model 59775, Hamilton Beach Co., Glen Allen, Virginia, USA) and sifted through a 1.68 mm (no. 12) sieve. Although the average particle size was much smaller than 1.68 mm, this sieve was the smallest one that would allow the sample to readily pass through it (owing to clumping of lipid-rich particles), while still removing the few remnants of larger bone or scale. Samples were then stored at –80 °C until lipid analysis.

To verify that our microquantity methods were producing total lipid values on par with those of more traditional macroquantity methods, we first compared total lipid content of 19 randomly selected individuals (across all three species collected during 2006) using Folch et al.'s (1957) macroquantity method and Van Handel's (1985) microquantity colorimetric SPV method. We then compared total lipid values and precision of these estimates among the microgravimetric, microcolorimetric, and Iatroscan TLC–FID methods. All reagents used in this entire study were HPLC grade. We described the methods for all four techniques below.

Macroquantity method

Our macroquantity procedure followed that of Folch et al. (1957) with slight modifications. Each sample was run at least in triplicate, with solvent blanks also being analyzed.

Table 1. Summary statistics for fish samples analyzed in this study.

Species	Tissue	Collection lake	Collection period	TL (mm)	Mass (g)	Lipid (%)	CV (%)	*N*	Comparison
Walleye	Body	Hatchery (Erie)	April 2005	576±25	1758±321	27.0±5.2	8.7±4.5	10	SPV vs. MG
	Gonad	Hatchery (Erie)	April 2005	NA	200±225	24.7±12.8	8.8±4.2	10	SPV vs. MG
	Body	Muskegon, Michigan	June–October 2006	461±106	1103±784	33.2±8.6	8.9±6.3	26	SPV vs. TLC–FID
									SPV vs. Macro (N = 8)
Yellow perch	Body	Erie	May–September 2005	247±32	185±65	20.2±4.4	10.3±6.9	10	SPV vs. MG
	Body	Muskegon, Michigan	June–October 2006	266±33	235±91	22.1±5.6	8.9±6.5	18	SPV vs. TLC–FID
									SPV vs. Macro (N = 3)
Lake whitefish	Body	Michigan	June–October 2006	501±51	1130±298	28.6±8.8	7.2±5.2	41	SPV vs. TLC–FID
									SPV vs. Macro (N = 8)

Note: Walleye, *Sander vitreus*; yellow perch, *Perca flavescens*; lake whitefish, *Coregonus clupeaformis*. Samples were collected in Muskegon Lake, Lake Michigan, and Lake Erie during 2005 and 2006. The average total length (TL, ±1 standard deviation (SD)), wet mass (Mass, ±1 SD), percent lipids (Lipid, ±1 SD), and coefficient of variation (CV, ±1 SD) are reported, as are sample sizes (N). Percent lipids and CVs were determined from the microcolorimetric SPV method, as it was the only method used on all samples. We also indicate the comparative analyses conducted with each data set (SPV, microcolorimetric; TLC–FID, Iatroscan; MG, microgravimetric; Macro, macroquantity). NA, not applicable.

We extracted lipids from a 1 g subsample of homogenized freeze-dried tissue in a 20 mL chloroform–methanol mixture (2:1 by volume), using a 30 mL Wheaton tissue homogenizer with a Teflon pestle that was powered by a household drill (2500 r/min (1 r = 2πrad)). The homogenization was conducted in an ice bath to prevent evaporation. After the mixture was agitated thoroughly using a vortex, it was decanted into a 50 mL centrifuge tube and centrifuged at 2400 r/min for 15 min, which caused the solid and the liquid phases to separate. The liquid (lipid) phase was recovered into a graduated 50 mL centrifuge tube, using a pipette, to which 0.9% NaCl solution (0.2 volume of lipid phase) was added. The lipid–chloroform solution was then vortexed twice for ~5 s each time and centrifuged at 2400 r/min for 5 min to further purify the lipid phase.

After recording the volume of the lower chloroform phase (V_p), 5 mL (V_s) of it was transferred into preweighed vials using a graduated pipette. The vials were dried overnight at ~50 °C, while being purged continuously with nitrogen gas, until a constant mass was achieved. The percent dry mass of lipids (% lipid) was calculated as

$$(1) \qquad \%\ \text{lipid} = \frac{(W_m - W_b)V_p}{V_s M_f} \times 100$$

where W_m is the sample lipid mass (mg) and W_b is the mass of "lipids" from blanks (mg), V_p is the volume (mL) of the chloroform phase after NaCl purification and centrifuging, V_s is the volume of the lipid subsample (i.e., 5 mL in this method), and M_f is the dry mass of fish (mg).

Microquantity methods

Microcolorimetric SPV and Iatroscan TLC–FID lipid extraction and purification

The microquantity SPV and Iatroscan TLC–FID analyses were conducted on the same day, using the same lipid extracts. The procedure for extracting total lipids from our freeze-dried samples for these two microquantity approaches followed that of Gardner et al. (1985), with some modifications to improve the efficiency of time and labor. A 1 mg "control" extract was made from artificial plankton (Argent Chemical Laboratories, Redmond, Washington, USA), and blanks were taken through the entire procedure.

To extract lipids, a subsample of 1–3 mg (measured to nearest 0.001 mg) was measured into glass test tubes (50 mm × 6 mm), and 200 μL of the 2:1 chloroform–methanol extraction solvent was added to each sample. Samples were homogenized for 30 s at 5000 r/min using a variable-speed rotary tool (Dremel model 380-5) with a glass bead pestle. This homogenate was decanted into a 1.5 mL conical centrifuge tube. Subsequently, another 200 μL of the chloroform–methanol extraction solvent was added to each centrifuge tube and the mixture was immediately homogenized briefly (~5 s), on ice, to prevent evaporation.

To purify the lipid extract, 200 μL of a 0.9% NaCl solution was added to each sample, which then was mixed by vortexing. Centrifuge tubes were then placed in a centrifuge (model 5415, Eppendorf, Hamburg) for 15 s at 8000 r/min, or a relative centrifugal force of 5220*g*. At this force, the phase separation occurs almost immediately and increasing time did not improve separation in controlled trials. This

phase separation resulted in nonlipid compounds and methanol being partitioned into the upper aqueous phase and the lipid–chloroform extract being partitioned into the lower organic phase. From this organic lower phase, a microsampling pipette was used to remove 100 and 3 μL aliquots for microcolorimetric SPV analysis and Iatroscan TLC–FID analysis, respectively.

Microcolorimetric SPV analysis

Our microcolorimetric SPV analysis was conducted on all samples (both gonads and fish) collected during 2005 and 2006 (see Table 1), with all samples analyzed in triplicate at a minimum. Our methods followed those of Van Handel (1985).

Culture tubes (13 mm × 100 mm) containing 100 μL of lipid extract were heated at 100 °C for 15 min on a dry bath heater to remove the chloroform solvent. We made certain to complete evaporation of the chloroform as it has a positive interference with the phosphovanillin reagent. We added 0.25 mL of 96% H_2SO_4 to the bottom of the culture tube to make complete contact with the lipid residue, and samples were then vortexed and heated for 10 min at 100 °C on a dry bath heater. We added 5 mL of the colorimetric SPV reagent (consisting of 3 g vanillin, 0.5 L deionized water, 2 L concentrated phosphoric acid, stored in the dark) to each tube and thoroughly mixed tube contents by vortexing.

After incubating at room temperature for 30 min, the absorbance (A, nm; measured at 525 nm) was measured using a Turner spectrophotometer (model SP-870, Barnstead, Dubuque, Iowa). Solvent blanks were run through the entire procedure and used to "zero" the spectrophotometer. We used pure olive oil (10 mg·mL^{-1}) in the 2:1 chloroform–methanol solvent mixture as a stock standard (Knight et al. 1972; Ahlgren and Merino 1991). Working volumes of 0 (blank), 10, 20, 30, 40, and 50 μL of this standard solution were used to develop a standard curve for lipids (absorbance vs. mass (mg) of the standard), with the percent dry mass of lipids (% lipid) calculated as follows:

$$(2) \qquad \%\ \text{lipid} = \frac{AV_p}{V_s S_s M_f} \times 100$$

where V_p, V_s, and M_f are as described earlier (V_s = 100 μL in this method) and S_s is the slope of the regression line of the stock standard (nm/mg).

Iatroscan TLC–FID analysis

Individual lipid classes, which were summed into a total lipid estimate, were quantified on all fish collected during 2006 (see Table 1), with all samples run in triplicate. We followed Parrish's (1986) protocol, with slight modifications.

Iatroscan rods were spotted just below the origin, but well above the solvent level, with 3 μL of the same organic-phase extracts used for microcolorimetric SPV analysis. All developments were performed in 100 mL of solvent in 15 cm × 18 cm TLC development tanks. Developments were modified so as to allow for a single scan of the entire rod length (Ludsin and DeVries 1997). Specifically, we developed rods in a chloroform–methanol–water solution (5:4:1 by volume) until the leading edge of the solvent reached 1 cm above the origin. The rods were then developed in a hexane – diethyl ether – formic acid solution (99:1:0.05 by volume) for 52 min. Rods were then developed in a hexane – diethyl ether – formic acid solution (80:20:0.1 by volume) for 42 min. This scheme allowed for the separation of seven lipid peaks (i.e., hydrocarbons, sterol esters and waxy esters, triglycerides, free fatty acids, alcohols, sterols, and phospholipids) and allowed for maximum separation of the most abundant lipids (i.e., triglycerides, free fatty acids, and phospholipids). Prior to each development, rods were air-dried for 5 min and conditioned in a constant humidity chamber (~30%) that was saturated with aqueous $CaCl_2$.

Individual lipid classes were measured with an Iatroscan Mark IV (Iatron Laboratories, Tokyo, Japan) connected to a windows-based computer running Peak Simple Chromatography software (Shell USA, Fredericksburg, Virginia). The hydrogen flow rate was set at 180 mL·min^{-1} and scan rate was set at 4 mm·s^{-1}. Rods were analyzed in racks of 10 comprised of three samples in triplicate and an accompanying seven-lipid component standard consisting of hydrocarbons (squalene), sterol esters (cholestryl tridecanoate), triacylglycerols (tripalmitin), free fatty acids (palmitic acid), aliphatic alcohols (cetyl alcohol), alicyclic sterols (cholesterol), and phospholipids (phosphatidylcholine). To ensure cleanliness of rods, all rods were blank-scanned immediately prior to spotting with sample or standards. Subsequent to analysis, individual lipid classes were summed to estimate total lipids (W_m). The total lipid percents were calculated as follows:

$$(3) \qquad \%\ \text{lipid} = \frac{W_m V_p}{V_s M_f} \times 100$$

where W_m, M_f, V_p, and V_s (i.e., 3 μL in this method) are as described earlier.

Microgravimetric analysis

Because microgravimetric analysis is more time- and labor-intensive than microcolorimetric SPV analysis (Landrum et al. 2002, 2004; Inouye and Lotufo 2006), it was conducted only on samples collected during 2005. These samples included yellow perch, walleye, and walleye gonads (Table 1). Each sample was run in triplicate at a minimum. Our microgravimetric analysis followed Gardner et al.'s (1985) procedure with slight modifications. A 1 mg "control" extract was made from artificial plankton (Argent Chemical Laboratories), and blanks were taken through the entire procedure.

To extract lipids, 100 μL of a 2:1 chloroform–methanol extraction solvent was added to a 0.3 to 1.3 mg subsample of freeze-dried tissue and homogenized 30 s at 5000 r/min. The homogenate was then drawn into a borosilicate glass microcapillary pipette to a height of no more than 65 mm, and the lower tip of the pipette was sealed with a moist inorganic clay preparation. The pipettes were shortened to fit into an IEC–MB microhematocrit centrifuge (International Equipment Company, Needham Heights, Massachusetts, USA), which was run for 1 min at 13 500 r/min to remove any nonlipid particles. The supernatant was then transferred into clean test tubes with a calibrated capillary pipette.

To generate a purified lipid extract of known quantity, we

first washed the crude lipid extract by adding 20% (by volume) of a 0.9% NaCl solution and next transferred it into a clean 100 µL capillary pipette. We then purified it by centrifuging for 1 min, discarding the aqueous, nonlipid layer. A known volume (V_s) of this subsample was transferred to a set of tared aluminum weighing tins, the solvent was evaporated on a heater (Themolyne Dri-Bath) set at 60 °C for 15 min, and the remaining lipids were then weighed. We calculated the percent dry mass of lipids (% lipid) as in eq. 1.

Statistical analysis

Because the microcolorimetric SPV analysis was the only technique conducted on all samples, all comparisons are made to this technique. Specifically, we compared the percent total lipid and coefficient of variation (CV = (sample standard deviation / sample mean) × 100) between (*i*) the microcolorimetric SPV and macroquantity techniques (n = 19 random samples from 2006), (*ii*) the microcolorimetric SPV and the microgravimetric methods (using 2005 data), and (*iii*) the microcolorimetric SPV and the Iatroscan TLC–FID methods (using 2006 data) (Table 1). For each comparison, we determined whether average total lipids for individuals significantly differed from a 1:1 line (i.e., slope = 1; intercept = 0) and also conducted an analysis of covariance (ANCOVA) to determine whether this relationship differed among species or sample groups: (*i*) walleye, yellow perch, and lake whitefish for microcolorimetric SPV vs. macroquantity methods; (*ii*) walleye, walleye gonads, and yellow perch for microcolorimetric SPV vs. microgravimetric methods; and (*iii*) walleye, lake whitefish, and yellow perch for the microcolorimetric SPV vs. Iatroscan TLC–FID methods (see Table 1). To compare measurement variability (i.e., CVs) between techniques (i.e., microcolorimetric SPV vs. macroquantity; microcolorimetric SPV vs. microgravimetric; microcolorimetric SPV vs. Iatroscan TLC–FID), we conducted paired t tests with all samples combined (all species and locations). We also conducted ANCOVAs for these comparisons with species and microquantity technique (per above) as independent factors.

After each regression analysis, we conducted a residual analysis to identify outliers (i.e., observations with standardized residuals > |3.5|; Faraway 2005). Only one outlier was identified (a 2006 walleye; standardized residual = –3.6) and removed from analyses. No data transformations were required as all data were normal (Kolmogorov–Smirnov normality tests; all $p > 0.05$) and variances were homogeneous (F tests; all $p > 0.05$). Our α level was set at 0.05 for all tests.

Results

Microcolorimetric SPV method vs. macroquantity method

The microcolorimetric SPV method provided estimates of total lipid content similar to those of Folch et al.'s (1957) macroquantity method. Specifically, we found a strong positive relationship between methods (R^2 = 0.82; F = 76.97, $p < 0.0001$), with the slope not different from one (95% confidence interval (CI) of slope = 0.98 to 1.60) and the intercept not different from zero (p = 0.59, 95% CI of intercept = –10.03 to 5.89). This relationship also did not vary among species (i.e., lake whitefish, walleye, yellow perch), nor did we find a species–method interaction (species effect, F = 0.13, p = 0.88; species–method interaction effect, F = 0.26, p = 0.77). By contrast, the average CV ± 1 standard deviation (SD) (3.2% ± 1.9%) of the macroquantity method was significantly lower than for the microcolorimetric SPV method (9.4% ± 7.4%) (paired t test, F = 20.36, p = 0.002). Thus, although the microcolorimetric SPV and macroquantity methods produced similar total lipid estimates, the precision in these estimates was better with the macroquantity approach than with the microcolorimetric SPV approach.

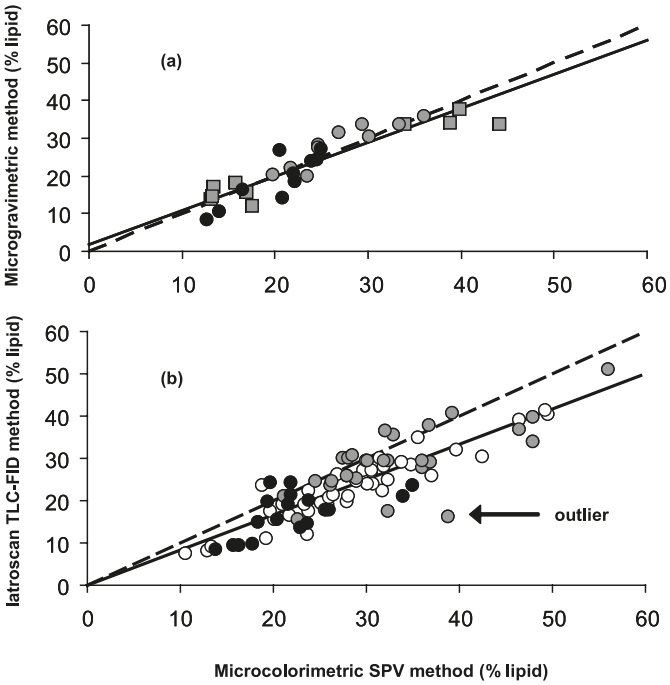

Fig. 1. Relationship between the microcolorimetric sulfophosphovanillan (SPV) and (*a*) microgravimetric method and (*b*) Iatroscan thin layer chromatography – flame ionization detection (TLC–FID) methods for estimating total lipid content (percent lipids) in fish tissue. Symbols: open circles, lake whitefish; solid circles, yellow perch; shaded circles, walleye; shaded squares, walleye gonad; solid line represents the regression line; broken line represents the y–x (1:1) line. The one outlier removed from this study is identified in *b*.

Microcolorimetric SPV method vs. microgravimetric method

Regression analysis indicated no difference in the ability of the microcolorimetric SPV and microgravimetric methods to measure total lipid content. The relationship between methods was positive and strong (R^2 = 0.88; F = 129.0, $p < 0.0001$; Fig. 1*a*), the slope was not different from one (95% CI of slope = 0.74 to 1.1), and the intercept did not differ from zero (p = 0.38; 95% CI of intercept = –2.3 to 5.9). Likewise, ANCOVA revealed no effect of sample type (i.e., walleye, walleye gonads, yellow perch) on this relationship (F = 2.0, p = 0.15) or a sample–method interaction (F = 2.4, p = 0.11).

Coefficients of variation ranged from 0.5% to 20.5% for

the microcolorimetric SPV method and from 1.4% to 25.0% for the microgravimetric method, with respective means (±1 SD) of 9.3% ± 5.2% and 11.5% ± 5.3%. In turn, no difference in measurement variability was evident between analytical techniques (paired t test, $t = 2.0$, $p > 0.5$), and ANCOVA revealed no effect of species or sample type ($F = 0.5$, $p = 0.62$) or species or sample – method interaction ($F = 0.3$, $p = 0.74$).

Microcolorimetric SPV method vs. Iatroscan TLC–FID method

We found a strong positive relationship between the microcolorimetric SPV and Iatroscan TLC–FID methods ($R^2 = 0.78$; $F = 292.0$, $p < 0.0001$) and also found that the intercept of the regression line did not differ from zero ($p = 0.68$; 95% CI of intercept = –3.6 to 2.4). However, the slope of the relationship was significantly less than one (slope = 0.86; 95% confidence intervals of slope = 0.76 to 0.96), indicating that the Iatroscan TLC–FID method underestimated total lipids relative to the microcolorimetric SPV method and that the magnitude of this underestimation increased with increasing lipid content (Fig. 1*b*). There was no effect of species (i.e., walleye, lake whitefish, yellow perch) on this relationship ($F = 1.3$, $p = 0.29$) or a species–method interaction ($F = 1.1$, $p = 0.34$).

No difference in average CVs was evident between techniques (paired t test, $t = 1.6$, $p = 0.11$; ANCOVA: species effect, $F = 1.2$, $p = 0.29$; method effect, $F = 1.3$, $p = 0.25$; species–method interaction, $F = 0.4$, $p = 0.69$). Coefficients of variation ranged from 0.6% to 26.7% for the microcolorimetric SPV method and from 0.3% to 24.6% for the Iatroscan TLC–FID method, with respective means (±1 SD) of 8.1% ± 5.8% and 7.8% ± 4.9%.

Discussion

Microcolorimetric SPV method vs. macroquantity method

Our comparison of Van Handel's (1985) microcolorimetric SPV method and Folch et al.'s (1957) macroquantity method, the latter of which has been widely accepted as a reliable technique with which to measure total lipid for fish (e.g., Rinchard et al. 2007; Nanton et al. 2007), indicates that microquantity and macroquantity approaches can estimate total lipid content of juvenile and adult fishes equally well, as no statistical differences between methods were found in our study. Although this finding may not hold for all microquantity methods (e.g., Iatroscan TLC–FID; see below) and does counter Inouye and Lotufo's (2006) finding that Van Handel's (1985) microcolorimetric SPV method consistently produced higher lipid estimates than their macroquantity method (i.e., Bligh and Dyer 1959), we are encouraged by the strong correspondence between results achieved with the two methods used in our study and recommend continued use of microquantity methods in most circumstances.

However, in instances when low within-sample measurement precision is critical, investigators may want to consider using macroquantity approaches, given that we found macroquantity measurement precision (CVs < 5%) to be superior to microquantity precision (CVs from 20% to 30%). These differences are likely due to a combination of nonhomogeneity in tissue subsamples (at the microscale) and the small subsample volumes of tissue analyzed in our microquantity approaches (i.e., 100 μL aliquots were analyzed for both the microcolorimetric SPV and microgravimetric methods, whereas 3 μL aliquots were analyzed for the Iatroscan TLC–FID method) versus our macroquantity approach (5 mL aliquots). We can only imagine that the differences between microquantity and macroquantity estimates of precision would be heightened had we not sieved samples for large pieces of bone and scales. Thus, investigators seeking high precision in their microquantity estimates of total lipids should (*i*) ensure that samples are finely homogenized, (*ii*) increase within-sample replication, or (*iii*) increase the volume of subsamples analyzed (which may defeat the purpose of using microquantity approaches).

Microcolorimetric SPV method vs. microgravimetric method

Previous investigations have shown a tendency for gravimetric approaches to overestimate lipid levels (Hopkins et al. 1984) and for colorimetric SPV approaches to underestimate them (Barnes and Blackstock 1973). However, we found no evidence to this effect in our study for these two microquantity approaches; we also did not find that the ability to estimate lipids between methods differed among species of varying lipid content or between tissue types (somatic vs. gonadal). Landrum et al. (2002) also demonstrated that the microcolorimetric SPV and microgravimetric methods produce comparable results for aquatic algae. Additionally, measurement precision did not differ between microquantity methods in this study, regardless of species or tissue type analyzed. Although we recommend use of the microcolorimetric SPV approach in future studies, as it has been shown to be more time- and labor-efficient than the microgravimetric approach (Kukkonen et al. 2004; Landrum et al. 2004; Inouye and Lotufo 2006), the lack of difference between these two microquantity approaches for a organisms of both low (algae) and high (somatic and gonadal fish tissue) total lipid content indicates that comparison of studies using these two methods can be conducted with little concern for potential bias.

Importantly, however, this latter conclusion also assumes that appropriate protocols are used to minimize the potential for bias. In this study, we provided three major modifications to extant protocols that we feel improved the accuracy of our total lipid measurements. First, because gravimetric methods measure all compounds in an extract (including potentially nonlipid materials), they have the tendency to overestimate lipid levels (Folch et al. 1957; Gardner et al. 1985). To minimize the potential for this bias, we included a saline (0.9% NaCl) wash to purify the extract (Barnes and Blackstock 1973; Gardner et al. 1985; our study). Although we did not conduct trials without this saline wash to quantify the amount of error that discarding this step would induce (but see Folch et al. 1957), the fact that our lipid estimates did not differ between our microgravimetric and microcolorimetric SPV approaches indicates that this step may be essential. Second, we used concentrated H_2SO_4 in the microcolorimetric SPV procedure to reduce the potential for our colorimetric method to underestimate lipid content,

which can be problematic because this approach only measures compounds that contain unsaturated bonds (Knight et al. 1972; Barnes and Blackstock 1973). The saturated lipids in our fish tissue, when heated with concentrated H_2SO_4, could most likely generate intermediate, unsaturated products (e.g., ketones) that could react with colorimetric SPV reagents (Ahlgren and Merino 1991). Third, we purified the lipid extract (by adding a saline rinse) in the microcolorimetric SPV procedure. Although previous studies have suggested that this purification step is unnecessary (Barnes and Blackstock 1973; Ahlgren and Merino 1991), preliminary protocol-development trials (n = 5 individuals measured in triplicate) demonstrated that the comparability of our microcolorimetric SPV and microgravimetric methods increased when a purification step was used for both approaches (D. Fanslow, unpublished data). Further, in contrast to Barnes and Blackstock (1973), who found nonlipid materials to have negligible impacts on colorimetric SPV measures, we found that taking pure glycogen and protein through the whole lipid extraction and microcolorimetric SPV quantification procedure resulted in measured "lipid" levels of 9.4% and 1.04% (g/g dry mass), respectively (D. Fanslow, unpublished data). Thus, nonlipid materials can bias microcolorimetric SPV estimates high, and total lipid estimates generated with protocols that lack a purification step should be treated with caution. A final reason for including a purification step in the microcolorimetric SPV technique is to remove residual fish tissues left behind in the homogenization and extraction process. The microcolorimetric SPV method initially developed by Van Handel (1985) was specifically designed for mosquitoes, the tissues of which are too small to interfere with the readings of absorbance values. For large organisms such as fish, however, we found that the solid traces turned brown after heating with concentrated sulfuric acid and absorbance values consequently became unstable.

Microcolorimetric SPV method vs. Iatroscan TLC–FID method

No differences in analytical precision (CVs) were found between the microcolorimetric SPV and Iatroscan TLF–FID techniques. We did, however, find that the Iatroscan TLC–FID approach yielded lower total lipid values than the microcolorimetric SPV method and that these differences increased with increasing lipid content. Although no comparisons were conducted between the microgravimetric and Iatroscan TLC–FID methods, we also expect that the Iatroscan TLC–FID method would underestimate lipids relative to the microgravimetric approach (especially at high lipid levels), given the similarity between the microgravimetric and microcolorimetric SPV approaches (per above).

Underestimation by the Iatroscan TLC–FID method may result from multiple factors. First, although the Mark IV model drastically improved linearity of the FID detector response than the earlier version, some lipids (e.g., cholesterol, monacylglycerol, glycolipids) still generate a curvilinear relationship with sample loads (Parrish 1986; Parrish et al. 1996; Indrasena et al. 2005). Further, this nonlinearity can be exacerbated when microquantities of lipid material (such as used in this study) are used (Kaitaranta and Nicolaides 1981; Ohman 1997; Indrasena et al. 2005), resulting in a weaker FID response that can lead to underestimation of lipids. Second, decomposition of lipid components during sample analysis could affect the accurate measurement of individual classes of lipid on TLC–FID but might not measurably bias the microcolorimetric SPV method. For example, the degradation of some monoacylglycerol polar lipids (e.g., P-choline, P-ethanolamine), which is signified by an increase in free fatty acids, was observed in Iatroscan TLC–FID analysis of both fish and *Artemia* (brine shrimp) lipids (Bligh and Scott 1966; Braddock and Dugan 1972; Sasaki and Capuzzo 1984). Moreover, although methanol is essential for lipid extraction, it also is considered an undesirable solvent because it can react with samples after extraction. Esterification of carboxylic groups with methanol has been considered a serious problem in extracts of airborne particulate (Clement et al. 1982). The presence of methyl esters in the lipid extracts of benthic invertebrates was attributed to the reaction of lipid components with the methanol during sample analysis (Gardner et al. 1985).

Previous studies with aquatic invertebrates have drawn similar conclusions as our own, finding that the Iatroscan TLC–FID methods tend to underestimate total lipids (Fraser et al. 1985; Parrish 1986, 1987). For example, Sasaki and Capuzzo (1984) found that the slope of the relationship between total lipids measured with a macro-Iatroscan TLC–FID approach and macrogravimetric approach was 0.88 for *Artemia* extracts, which was quite similar to that found in our study (0.86). Unfortunately, additional details about Sasaki and Capuzzo's (1984) relationship were not provided. Similarly, Gardner et al. (1985) found that micro-Iatroscan TLC–FID measurements of total lipids were generally lower than microgravimetric measurements of oligochaetes. Given that underestimation of total lipids by Iatroscan TLC–FID approaches appears general to both aquatic invertebrates and fishes, we recommend that this approach only be used to estimate lipids of individual classes, cautioning against the common practice of summing up these results to measure total lipids. Additionally, owing to the potential for Iatroscan TLC–FID to underestimate total lipids in "fatty" fishes, care also should be taken when using lipid values from previous studies that used Iatroscan TLC–FID to quantify total lipids (e.g., Scott et al. 2002; Zhou et al. 1995). Given the range of total lipid values spanned in this study, a comparison of total lipid levels in other studies with those in ours here should provide a good means to determine whether previous Iatroscan TLC–FID studies may indeed be biased low.

In summary, based on our comparison of our primary microquantity approach (microcolorimetric SPV) and a more traditional macroquantity approach, we feel that microquantity approaches offer clear advantages over macroquantity approaches when it comes to estimating total lipids of aquatic organisms, including fish. Most importantly, microquantity approaches can provide results comparable to those of macroquantity approaches with reduced processing time, solvent costs, and chemical waste. In addition, a reduced need for large tissue subsample volumes will in most cases eliminate the need to pool samples (per Ludsin and DeVries 1997), which has shown to be necessary when using macroquantity approaches (e.g., Miranda and Hubbard 1994). Even so, macroquantity approaches still

may prove advantageous over microquantity approaches in some cases. In particular, we recommend the use of macroquantity approaches in situations in which high measurement precision, not just a reasonably accurate estimate of lipid levels, is required. In addition, although our microgravimetric and microcolorimetric SPV approaches appear suitable for organisms with both high and low lipids levels, our results suggest that the Iatroscan TLC–FID approach might only be suitable for quantifying total lipids in organisms with lower lipid levels than species such as walleye and lake whitefish.

Acknowledgments

We thank Joann Cavaletto for teaching us the Iatroscan TLC–FID method, as well as Pete Landrum for assistance with the microcolorimetric SPV method. We thank the Laurentian boat crew for their assistance with fish collections, as well as Tomas Höök, Marco Costantini, and James Roberts for their assistance with the 2005 field collections, conducted as part of the International Field Years on Lake Erie (IFYLE) Program. Much appreciation goes to Michigan Department of Natural Resources for providing access to walleye at their Saline Fish Hatchery, including Jim Breck who helped process those fish. This work was funded by NOAA's Great Lakes Environmental Research Laboratory (GLERL), as well as a 2006 NOAA GLERL / Cooperative Institute for Limnology and Ecosystems Research (CILER) Summer Fellowship to Yuehan Lu. This publication represents NOAA GLERL contribution No. 1469.

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