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## Micromethod for lipids in aquatic invertebrates<sup>1</sup>

**Abstract**—Microtechniques with disposable, calibrated, capillary pipets were developed to measure amounts and classes of lipids in individual Lake Michigan benthic invertebrates. After lipids from an animal were partitioned into 100  $\mu$ l of extraction solvent and purified, measured portions were weighed with an electrobalance. Lipids in some samples were also characterized by thin layer chromatography with flame ionization detection (TLC-FID). The extraction method was calibrated gravimetrically with cod liver oil and evaluated with a dry, ground fish sample. Lipid content was proportional ( $r = 0.99$ ) to fish tissue dry weight over the range of 0.3–13 mg. TLC-FID measurements correlated well ( $r = 0.98$ ) with gravimetric analysis for portions of extracts of individual amphipods (*Pontoporeia hoyi*) and oligochaetes (*Stylodrilus heringianus*). TLC-FID analysis indicated that <5% of the measured gravimetric weights was due to the inclusion of nonlipid material in the extracts.

The metabolic role of lipids in cellular energy conversions and membrane transport (Prosser 1973) makes them important to energy and chemical fluxes in lake and marine systems. The relationship of lipids to energy storage and feeding conditions has been demonstrated by analyzing lipid components in marine invertebrates (Benson and Lee 1975; Lee 1975; Hakanson 1984). Visual observation of lipid droplets in daphnids (i.e. lipid index) has provided useful information concerning nutritional status, life stage, and reproductive state of freshwater cladocerans (Goulden and Hornig 1980; Tessier and Goulden 1982; Tessier et al. 1983; Holm and Shapiro 1984). Field measurements of lipid content and com-

position of freshwater invertebrates are needed to understand the biochemistry and physiology of these animals in nature and their role in energy transformations.

Detailed quantitative studies of lipids in freshwater invertebrates are lacking, probably because most methods for measuring lipids are not designed to conveniently analyze such small organisms. Gravimetric analysis, the preferred method for measuring total lipids, usually requires sample sizes of several milligrams (e.g. Green 1971) or the evaporation of several milliliters of extraction solvent from the lipid extracts (Herbes and Allen 1983). Evaporation of large volumes can cause high blanks for gravimetric lipid analysis if solvents contain impurities. Colorimetric methods can quantify total lipids from small samples (Holland and Gabbott 1971) but respond variably for different lipid materials and should be intercalibrated with gravimetric methods (Barnes and Blackstock 1973). For compositional studies, extracted lipids are traditionally fractionated into classes and then one or more classes are derivitized and analyzed by colorimetry or gas chromatography (Farkas 1970; Lee 1975; Cowgill et al. 1984; Goulden and Henry 1984). Thin layer chromatography on silica-coated quartz rods (Chromarods) has recently been combined with flame ionization detection (TLC-FID) to separate and quantify lipid classes in small samples (Ackman 1980). This technique has been applied to the measurement of wax esters, triglycerides, and phospholipids in marine copepods (Hakanson 1984) and to several lipid classes in *Artemia* (Sasaki and Capuzzo 1984). Like other gravimetric and chromatographic methods, TLC-FID requires the concentration of extracted lipids into a small volume

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of solvent for efficient sample application and analysis. Techniques are needed to extract and purify lipids from aquatic invertebrates in volumes of solvent that can be analyzed directly by gravimetry or chromatography.

We describe and evaluate a micromethod for lipids in aquatic invertebrates. Dried animals are extracted with 100  $\mu$ l of solvent and portions of the extract are transferred, centrifuged, measured, and purified with disposable capillary pipets. A clean, concentrated extract can be prepared from a dried animal in about 12 min.

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A microtissue-homogenizer (Potter Elvehjem style) was assembled by placing a Teflon and glass pestle into the chuck of a variable-speed rotary tool (Dremel model 3701) mounted vertically. Small (50- $\times$ 6-mm o.d.) disposable glass test tubes served as mortars for the homogenizer (Fig. 1a). For extraction, dried whole organisms or weighed tissue samples between 0.3 and 13 mg were placed in the tubes in ice water (Fig. 1a). The ice bath cooled the samples during extraction and prevented evaporative loss of solvent. We added 100  $\mu$ l of extraction solvent (chloroform : methanol, 2 : 1 vol/vol: Folch et al. 1957) and homogenized the cooled sample for 30 s at slow speed (ca. 5,000 rpm). After homogenization, the tube was slowly lowered to remove the pestle from the extraction mixture. The motor was turned off before the sample was completely removed. Between samples, we rinsed the pestle in chloroform in an 8-ml vial by running the motor briefly. The homogenate was drawn into a borosilicate glass microcapillary pipet (Pyrex disposable micro-sampling pipet, 100  $\mu$ l: Corning Cat. 7099S-100) to a height of not more than 65 mm, and the lower tip of the pipet was plugged with a moist inorganic clay preparation ("potter's clay" from an art supply store). The tube was shortened (by scoring and removing the upper end) to 75 mm to fit a hematocrit centrifuge and kept at 4°C until centrifugation. After a series of samples (up to 12 animals and three extraction

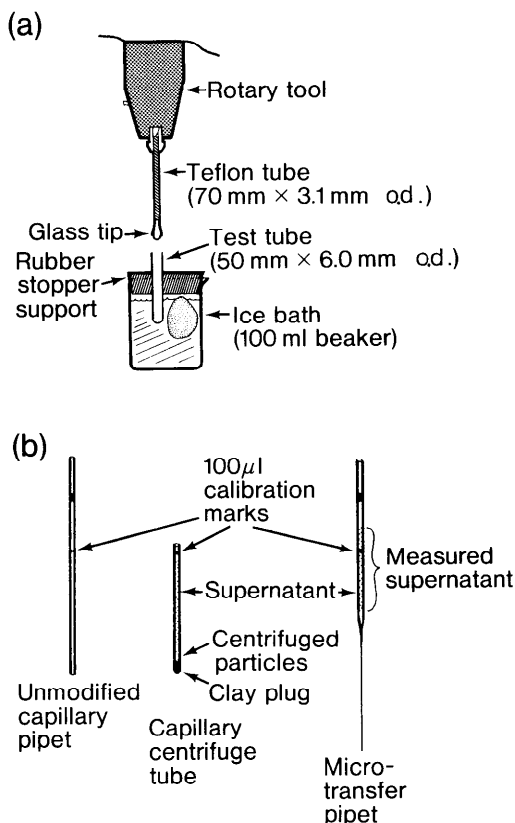


Fig. 1. Schematic diagram of apparatus for lipid extraction. a. Apparatus for homogenizing invertebrates in extraction solvent and for mixing extract with aqueous NaCl solution during purification. b. Unmodified and modified capillary pipets for centrifuging, transferring, and measuring lipid extracts after homogenization. Transfer pipets were made from capillary pipets with a flame.

blanks) was homogenized, we removed the particles from the extracts by spinning them for 1 min at 13,500  $\times$  g in the hematocrit centrifuge (IEC MB micro-hematocrit centrifuge). Most (about 80  $\mu$ l) of the supernatant from each was pulled off with a fresh transfer pipet made from a calibrated capillary pipet (Fig. 1b). We determined the volume of transferred solution by gently drawing the solution into the upper part of the uniform length of the transfer pipet and measuring its length in millimeters. Our calculations of volume were based on the ratio of fixed length to volume of the calibrated pipets. After volume measurement, the extract (usually about 60  $\mu$ l) was expelled into

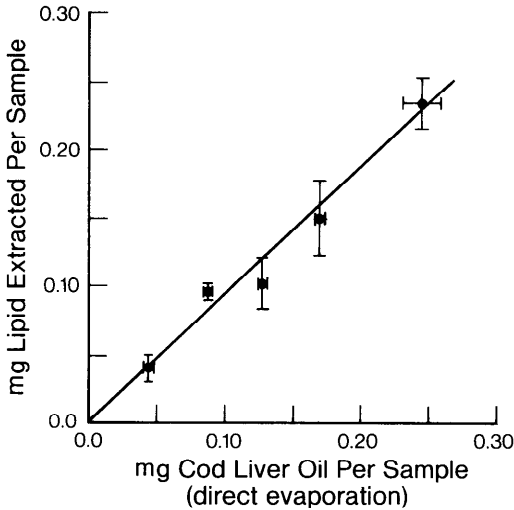


Fig. 2. Graph of cod liver oil recovery to determine calibration factor for solvent corrections. Error bars = SD ( $N = 4$ ). The calibration factor ( $C = 1.07$ ) was the inverse of the slope of the least-squares line ( $Y = -0.002 + 0.934X$ ;  $r = 0.995$ ) for the six points (mean values) after blanks were subtracted. Mean blank for direct evaporation =  $0.5$  (SD =  $6$ ;  $N = 4$ )  $\mu\text{g sample}^{-1}$ . Mean blank for extraction procedure =  $10$  (SD =  $7.5$ ;  $N = 25$ )  $\mu\text{g sample}^{-1}$ .

a second clean test tube in the ice bath. We added a volume of NaCl solution (0.9% NaCl in water: Barnes and Blackstock 1973) 20% as large as the delivered extract volume with a  $50\text{-}\mu\text{l}$  Hamilton syringe to purify the extract (Folch et al. 1957) and mixed the contents of the tube for 10 s with the microhomogenizer. After the mixture was drawn into a new  $100\text{-}\mu\text{l}$  capillary pipet, we plugged the pipet with potter's clay, cut it to a  $75\text{-mm}$  length, and centrifuged it for 1 min to separate phases. The tube was scored (gently, to prevent possible weight errors from glass filings entering the extract) and broken about 3 mm below the liquid-liquid interface. The section containing the aqueous layer was discarded.

For gravimetric analysis, a portion of each purified extract was removed with a micro-transfer pipet, measured, and transferred to one of a set of small, tared weighing cups in a brass cup-holder (for efficient heat transfer). The weighing cups, formed by shaping  $2 \times 2\text{-cm}$  sheets of household aluminum foil over a  $7\text{-mm-o.d.}$  cylinder (e.g. the flat end of a pen), had a higher vol:wt

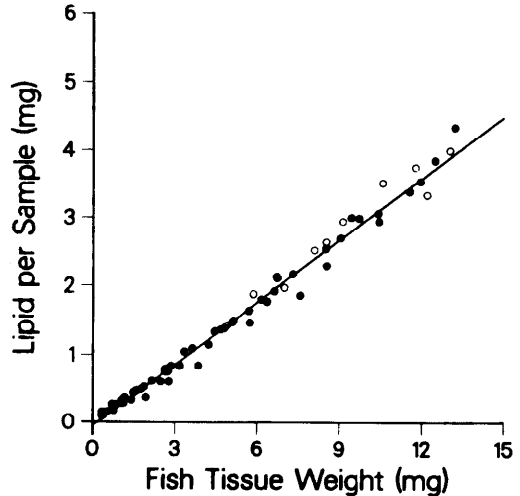


Fig. 3. Weight of lipid recovered per sample relative to weight of dry fish tissue extracted. ●—Sample extracted with  $100\ \mu\text{l}$  of extraction solvent; ○—with  $200\ \mu\text{l}$  of extraction solvent. The regression line shown ( $Y = -0.05 + 0.30X$ ;  $r = 0.994$ ;  $N = 55$ ) is for the former. The regression line for samples  $< 3\ \text{mg}$  is  $Y = 0.03 + 0.26X$  ( $r = 0.966$ ;  $N = 24$ ).

ratio than boats available from Cahn and could be used on the electrobalance at  $1\text{-}\mu\text{g}$  sensitivity ( $20\ \text{mg}$  scale). The remaining solvent was evaporated by placing the holder, with the cups, on a block heater (Thermolyne Dri-Bath) set at  $60^\circ\text{C}$  for 15 min, and the lipids were weighed on a Cahn electrobalance. We covered the heated block with a loosely capped bell jar to prevent particle contamination. A Petri-dish cover on the cup-holder prevented contamination when the cups were not in the drying oven.

We calculated the percentage of lipid on a dry weight basis as follows:

$$\% \text{ lipid} = [(WR/L) - B](CV/D)(100)$$

where  $W$  is the measured weight ( $\mu\text{g}$ ) of lipid in weighing cup;  $R$  the ratio of length (mm) to volume ( $\mu\text{l}$ ) for the capillary pipet used to deliver the extract to the cup;  $L$  the measured length (mm) of final extract delivered to the cup from the capillary tube;  $B$  the blank correction ( $\mu\text{g}$  lipids per initial  $100\ \mu\text{l}$  of blank extract = mean  $WR/L$  for blanks on a given day), for our measurements, blanks averaged  $10\ \mu\text{g}$  per extraction (SD =  $7$ );  $C$  the empirically determined volume calibration factor to account for methanol

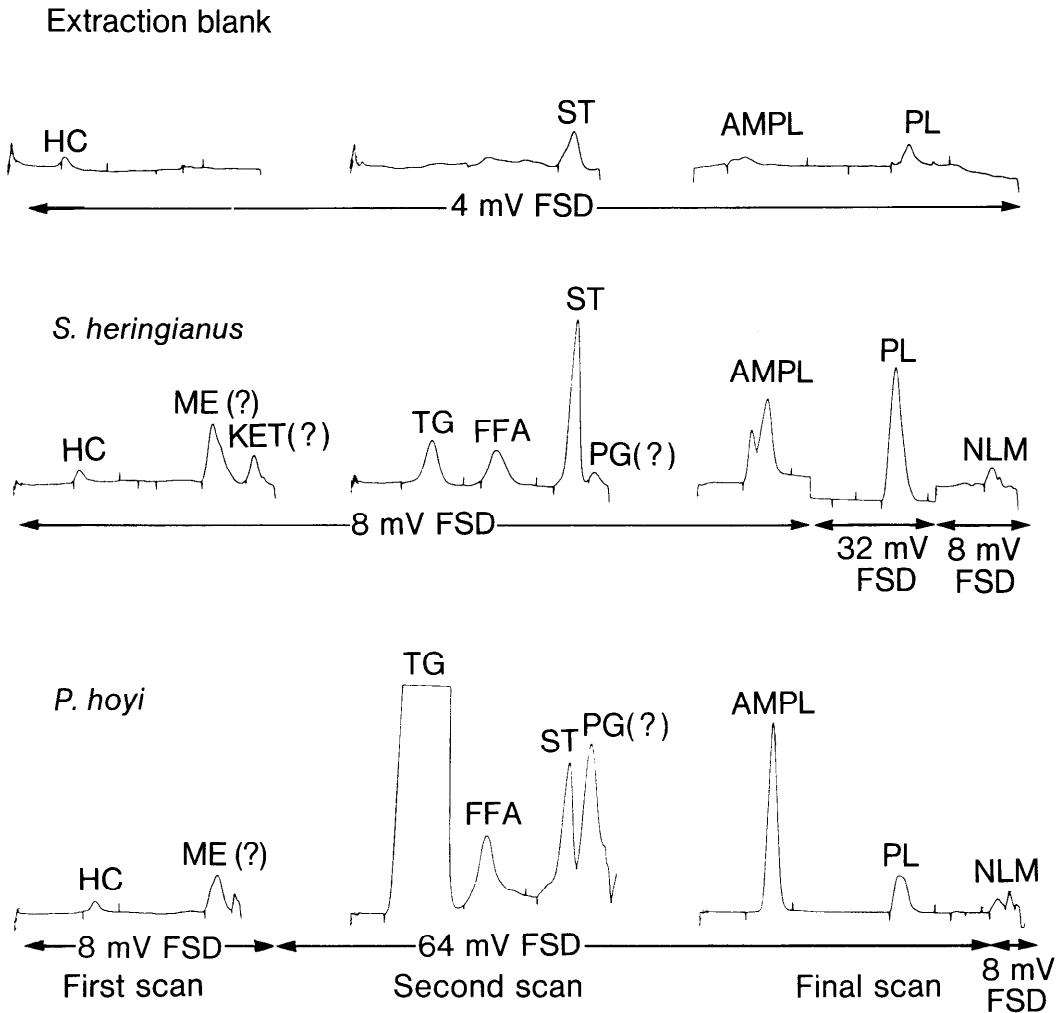


Fig. 4. Representative lipid chromatograms for an extraction blank and extracts of *Stylo-drilus heringianus* and *Pontoporeia hoyi*. Lipids were extracted from individual invertebrates as described in the text, and a portion (7–10  $\mu$ l) of the extract was spotted on a Chromarod and analyzed by TLC-FID. Each sample was developed in four different solvent systems and subjected to partial (not over the sample application point) scans with the flame ionization detector before the final complete scan. Developing direction was from right to left; scanning direction was from left to right. Integrator attenuations are given under each chromatogram. Lipid classes were quantified by comparing integrated areas with those of standard compounds. HC—Hydrocarbons; ME—methyl esters; KET—ketones; TG—triglycerides; FFA—free fatty acids; ST—sterols; PG—partial glycerides; AMPL—acetone-mobile polar lipids; PL—phospholipids; NLM—nonlipid material remaining at the point of application. Question marks indicate tentative identifications of some of the minor classes.

dissolved in the final chloroform extract and for slight solvent evaporation during centrifugation, for our experimental manipulations,  $C$  was calculated to be 1.07  $\mu$ l of final extract per 1  $\mu$ l pure chloroform in the extract (see below and Fig. 2);  $V$  the volume ( $\mu$ l) of chloroform in initial extraction solvent ( $V = 66.7 \mu$ l per 100  $\mu$ l of extraction

solvent); and  $D$  the dry weight ( $\mu$ g) of sample homogenized.

To determine the calibration factor,  $C$ , we added different volumes (0, 5, 10, 15, 20, and 30  $\mu$ l) of 1% cod liver oil in chloroform to both mortar test tubes and weighing cups and removed the chloroform in the drying oven. The samples in the mortars were ex-

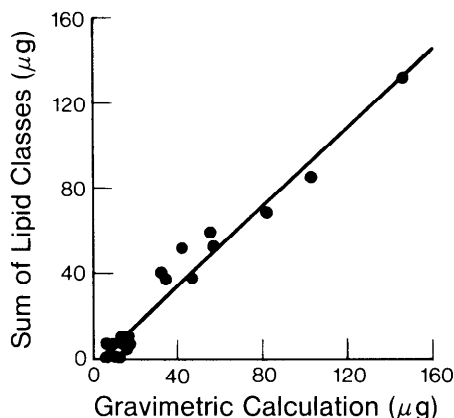


Fig. 5. Comparison of total lipids, in invertebrate samples, determined by calculated gravimetric weight vs. the sum of the individual FID quantified lipid classes in corresponding samples.  $Y = -2.65 + 0.93X$  ( $r = 0.981$ ;  $N = 19$ ).

tracted and weighed gravimetrically, exactly as described above for tissue samples, and the mean weights calculated from the extracts were compared to the mean weights of the samples added directly to the cups after blanks were subtracted from each. The calibration factor, the inverse of the slope of the regression line, was 1.07.

We examined the possible dependence of extraction efficiency on tissue sample size by gravimetrically analyzing different amounts (between 0.3 and 13 mg) of a dried, ground, sieved fish sample. Our results (Fig. 3) indicated that the procedure's extraction efficiency was generally linear with samples between 0.3 and 13 mg dry wt. The amount of lipid recovered was directly proportional to the amount of tissue analyzed ( $r = 0.994$  for samples ranging in size from 0.3 to 13 mg;  $r = 0.966$  for those ranging from 0.3 to 3 mg).

To apply the technique to the analysis of lipids in invertebrates, we collected and analyzed *Pontoporeia hoyi* and *Stylodrilus heringianus* from Lake Michigan. The amphipod *P. hoyi* (adult size: 0.7–4 mg ash-free dry wt) constitutes more than half of the macrobenthic biomass in the upper Great Lakes (Cook 1975; T. Nalepa pers. comm.) and, as a major food for small fish (Wells 1980), is an intermediate between settling detritus and the upper food web in

the lakes. Oligochaetes, including *S. heringianus* (adult size: 0.4–0.7 mg ash-free dry wt), are the second most abundant macroinvertebrate group in the upper Great Lakes and represent >30% of the macrobenthic biomass. The samples were collected by Ponar grab (three per site) from two sites (water depths of 24 and 45 m) offshore from Grand Haven, Michigan, in August 1984. The intact Ponar samples from each site were combined, taken on ice to the laboratory, and stored overnight at 4°C until the invertebrates were removed. Individual animals were removed from the sediments, rinsed with lake water, placed in tared test tubes, and dried at room temperature for 22 h at reduced pressure under a nitrogen atmosphere in a desiccator containing silica gel. The dried organisms were weighed in the mortar tubes and extracted. We analyzed separate portions of extract for lipids gravimetrically and by TLC-FID.

For TLC-FID analysis, about 10 µl of purified extract was drawn into a transfer pipet, made from a 50-µl capillary pipet, under an atmosphere of nitrogen. The volume was measured, and the tube was flame-sealed for storage until TLC-FID analysis. Except for 1–2 days during an unexpected shipping delay, the samples were kept at <–20°C until analysis within 5–8 days. A second portion of extract was analyzed gravimetrically to estimate total lipids in the TLC-FID sample. At the time of TLC-FID analysis, we broke off the sealed fine tips of each sample with forceps and spotted the sample onto the silica-layered rod (Chromarod) directly from the capillary tube. Lipid classes were separated and quantified in a stepwise sequence by partial scanning between developments with modifications of previously described techniques (Delmas et al. 1984; Parrish and Ackman 1985).

Chromatographic results showed that the lipid extracts were relatively free of nonlipid material (Fig. 4). Blanks for most lipid classes were either low or undetectable. "Nonlipid material" (FID-responsive substances remaining at the point of application on the Chromarod) constituted <5% of total measured lipid weights for inver-

tebrate samples. Similar lipid purity in extracts analyzed by TLC-FID was achieved by Sasaki and Capuzzo (1984), who also washed their extracts with NaCl solution. If extracts are not washed, as is common in invertebrate analysis, gravimetric results may be biased upward (Barnes and Blackstock 1973) and high levels of nonlipid material may be observed in TLC-FID analyses. This is not a problem if polar lipids are separated from nonlipid material, as in Fig. 4, but could bias results for simpler separations in which polar lipids, including phospholipids, remain at the point of application. When concentrations of individual identified groups were quantified, summed, and compared to gravimetric total lipid values on the same samples, results from the two approaches were closely related (Fig. 5). However, for oligochaete samples, having levels of  $<20 \mu\text{g}$  total lipids per TLC-FID injection, TLC-FID measurements were generally lower than gravimetric results. A comparison of TLC-FID to gravimetric results on *Artemia* extracts gave a ratio of 0.88 (Sasaki and Capuzzo 1984).

Some decomposition and reaction of lipid components with the methanol was suggested by the presence of free fatty acids and methyl esters in some of the extracts (Fig. 4). Although partial lipolysis would not measurably affect gravimetric results, it could affect precise measurements of individual classes of lipids (e.g. phospholipids) in the extracts. Further work is needed to determine how much of this lipid decomposition occurred during drying and extraction and how much during storage of the extract before analysis. There may have been some decomposition during the shipping delay or during the freezing process, but decomposition of lipids should be minimal during storage of lipids in chloroform:methanol under nitrogen at  $-20^\circ\text{C}$  (Sasaki and Capuzzo 1984).

A  $2 \times 2$  fixed-factor ANOVA of gravimetric results from the field samples indicated that the method could distinguish significant ( $P < 0.01$ ) site and species differences in total lipid levels (Table 1). *Pontoporeia hoyi* consistently had higher

Table 1. Statistical comparison of total lipids, expressed as % AFDW (ash-free dry wt), in *Pontoporeia hoyi* and *Stylodrilus heringianus* sampled from two sites in Lake Michigan, offshore from Grand Haven, Michigan, in August 1984. Cell variances were not significantly different ( $P > 0.05$ ).

Site	Depth sampled (m)	<i>P. hoyi</i>		<i>S. heringianus</i>	
		Mean (SE)	N	Mean (SE)	N
A	24	43(2)	6	11(2)	6
B	45	33(1)	4	14(1)	5

ANOVA table ( $2 \times 2$  fixed factor)

Source of variation	df	F	P
Site	1	4.7	0.05
Species	1	200	0.0001
Site $\times$ species	1	14.4	0.002

levels of lipid than *S. heringianus*. In contrast to *S. heringianus*, *P. hoyi* had most ( $>60\%$ ) of its lipid as triglycerides (e.g. Fig. 4). *Pontoporeia hoyi* also had higher levels of total lipid at the 24- than at the 45-m-depth site, whereas total lipid levels in *S. heringianus* were not significantly different at the two sites.

Although the micromethod described above requires that invertebrate samples be dried before analysis, it has several advantages for recovering and purifying lipids from small samples. Glassware washing and potential cross-sample contamination is prevented by using disposable glass tubes and weighing cups. Concentration of solvent impurities is minimized by using small solvent volumes. The hematocrit centrifuge rapidly removes particles from solvents (Gardner et al. 1980) and separates phases during extract purification. Because all calibrated pipets from a given batch have the same length and diameter, the volume of transferred extract is easily calculated by drawing it up into the uniform section of the transfer pipet and measuring its length with a ruler. The direct measurement of transfer volumes in the capillary tubes eliminates the need to use extra solvent to quantitatively transfer the complete extracts through the cleanup steps, but still permits direct calculation of total lipid concentrations in the samples.

Wayne S. Gardner  
William A. Frez  
Elizabeth A. Cichocki

National Oceanic and Atmospheric  
Administration  
Great Lakes Environmental  
Research Laboratory  
2300 Washtenaw Avenue  
Ann Arbor, Michigan 48104

Christopher C. Parrish

Department of Oceanography  
Dalhousie University  
Halifax, Nova Scotia B3H 4J1

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