Analytical Methods

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
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Cite this: *Anal. Methods*, 2022, **14**, 2665

Rapid measurement of total lipids in zooplankton using the sulfo-phospho-vanillin reaction†

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Zooplankton provide a vital source of nutrition to a variety of fish and marine predators. Measuring the total lipid content of zooplankton provides important information about diet quality available to predators, revealing details about trophic dynamics and ecosystem status. We analyze the performance of a microplate assay, utilizing the sulfo-phospho-vanillin (SPV) reaction, to quantify the total lipid content of various large crustacean zooplankton in a rapid and high throughput manner. Pilot experiments were performed by measuring the total lipid content of purchased freeze-dried zooplankton (*Calanus finmarchicus* and *Euphausia superba*) by both SPV and gravimetric analysis (low throughput and requires large sample size). The results of the SPV assay were not statistically different from gravimetric analysis for either species ($p > 0.05$). Further, an inter-laboratory comparison study was performed to measure the total lipid content (% of wet mass) of field-collected Arctic and North Pacific zooplankton (copepods ($n = 19$) and euphausiids ($n = 29$)) of various species utilizing multiple analysis methods. Results from thin layer chromatography with flame ionization detection (TLC-FID) demonstrated that lipid classes in zooplankton samples varied in composition of steryl/wax esters (3–95%), triacylglycerols (1–52%), free-fatty acids (0.4–25%), sterols (0–4%) and polar lipids (1–42%). Despite this variation in lipid class composition among samples, the results of the SPV assay agreed well with gravimetric analysis. The mean absolute and relative differences between SPV and gravimetric analysis for all zooplankton lipids in this study were 1.0% and 11.6%, respectively. The SPV assay is rapid (<2 hours), high throughput (25 samples processed in parallel), low cost (supplies <$ 0.67 per sample), precise (inter assay CV = 6.9%, intra assay CV = 6.0%), sensitive (limit of detection < 1.7 micrograms of lipid per analysis), and accurate when calibrated with appropriate standards.

Received 21st April 2022
Accepted 17th June 2022

DOI: 10.1039/d2ay00665k

rsc.li/methods

Introduction

Zooplankton are an essential component of aquatic food webs, creating a crucial link between primary producers and higher trophic level consumers.[1–3] Zooplankton provide a rich and abundant source of lipids, the densest form of molecular energy available in marine ecosystems.[4,5] On average, lipids yield greater than two-thirds more energy (per gram) than either carbohydrates or proteins, making them the ideal energy storage molecule for zooplankton.[6] The total lipid content of individual zooplankton is highly variable and influenced by factors both internal (species, sex, stage, reproductive status) and external (season, temperature, salinity, food availability, *etc.*).[5,7] Monitoring the total lipid content of large crustacean zooplankton (such as copepods and euphausiids) is a practice currently used by ecologists and fisheries scientists aiming to understand various ecosystem processes, such as the effect of climate change on trophic dynamics of commercially and ecologically important cold-water fish species.[8–11]

A variety of analytical methods currently exist for measuring lipids in zooplankton. These methods vary in the detail that they reveal about the sample, from simple estimates of total lipid mass to direct measurements of molecular composition and structure. Analytical methods should be carefully selected based on the needs and hypothesis of the user and the labor intensity required to generate appropriate granularity of data. Gravimetric methods, such as those reported by Folch and Bligh & Dyer, measure the total mass of lipid in a sample after extraction into an organic solvent.[12,13] This method is universal and considered the gold standard for total lipid measurements; however, it suffers from errors in selectivity (non-lipid contamination), difficulty measuring low-mass samples, and

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† Electronic supplementary information (ESI) available. See https://doi.org/10.1039/d2ay00665k

throughput. Similarly, a photographic method has been developed as a non-destructive approach to estimate the total lipid mass of calanoid copepods by measuring the area of the lipid-sac using a calibrated microscope and image analysis software, calculating an approximate volume of the lipid sac, and multiplying this value by an average lipid density of 0.9 g mL^{-1}. This method is proven both fast and accurate, but it is limited to the study of copepods with visible and undamaged lipid sacs. Both gravimetric and photographic analyses yield information on total lipid content, but they do not provide detailed information on lipid molecular composition.[14–16]

Molecular-level detail of lipid composition is most often obtained using chromatographic methods to separate and identify individual components of the lipid extract. Both thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) have been used to successfully separate zooplankton lipids into their respective classes (*i.e.* wax ester, triacylglycerols, phospholipids, *etc.*) followed by quantitation commonly using a flame-ionization detector (FID), evaporative light scattering detector (ELSD), or densitometry.[17–22] Measuring individual lipid-class components, commonly utilizing the *Iatroscan* TLC-FID instrument, provides valuable information to scientists about how both external and internal factors affect the internal biochemistry and overall energetic condition of zooplankton (*e.g.* effect of ocean temperature on storage of wax esters).[7] Further, analysis of the total fatty acid/alcohol composition of lipids, commonly referred to as fatty acid methyl ester (FAME) analysis, provides information on feeding relationships and food web linkages.[23–25] Total FAMEs analysis utilizes gas chromatography (GC) and detection by either FID or mass spectrometry, making it a sensitive and highly quantitative method. The total lipid mass of a zooplankton extract can be accurately calculated from chromatography data by summing the masses of the separated components. Chromatographic methods are indispensable for their ability to provide highly quantitative molecular-level information about lipid extracts, but they are time consuming, have limited throughput, and require technical instrumentation and expertise.

The Nile Red fluorometric assay has been validated for use with zooplankton.[26] This microplate method utilizes a non-covalent interaction between Nile Red and lipid molecules to produce a fluorophore with unique excitation/emission spectra for different lipid classes, allowing semi-quantitative information about lipid class composition and total lipid content. The authors of this method show that the fluorescence spectra of different lipid classes vary significantly between different species of zooplankton. Therefore, in order for determinations of total lipid to be accurate, the assay must be calibrated using purified lipid extracts native to each individual species being analyzed. This can be prohibitive when analyzing the wide range of zooplankton species caught in a traditional sample collection, such as a plankton net.

The sulfo-phospho-vanillin (SPV) reaction is a popular method for determining total lipids in a variety of sample types: human serum, meibum, microalgae, mosquitos, fish, and more.[19,27–32] The reaction is performed in two steps: (1) treatment of lipids with concentrated sulfuric acid and heat to create carbocation chromogen intermediates, and (2) reaction of those intermediates with vanillin in the presence of phosphoric acid to generate a pink chromophore. The reaction mechanisms are complex and are reported in detail by McMahon *et al.*[28]

Herein, we analyze the performance of the SPV assay for measuring the total lipid content (% of wet weight) of various large crustacean zooplankton common to the Arctic and North Pacific oceans in an inter-laboratory comparison study. We also demonstrate accurate calibration of the assay using commercially available lipid extracts.

Methods

Preparation of SPV calibration standards

Three calibration standards containing zooplankton lipids were assessed by the SPV assay: (1) bulk extracted and purified wax ester-based copepod oil from late developmental stage *Calanus finmarchicus* produced using methods detailed in Olsen *et al.*,[33,34] (2) a commercially purchased nutritional supplement containing *Arctic Plankton Oil* (*C. finmarchicus*) sourced from Supersmart USA (Miami, FL), and (3) a commercially purchased triacylglycerol-rich *Fish Oil from Menhaden* (Menhaden Oil) sourced from Sigma (St. Louis, MO). The *Arctic Plankton Oil* supplement capsules were cut open with a scalpel and the oil was removed using a glass Pasteur pipette immediately before use.

To create calibration stock solutions, approximately 25.0 milligrams (mg) of each oil were measured into separate 50 milliliter (mL) volumetric flasks. The exact mass was recorded and the flasks were filled to the fiducial mark with a mixture of 2 : 1 (*v* : *v*) chloroform : methanol. A set of calibration standards were made from each stock *via* dilution with 2 : 1 chloroform : methanol. The standards were made to a total volume of 1.0 mL in glass test tubes and contained 30, 50, 100, 200, and 300 micrograms (μg) of lipid per mL.

The SPV assay was calibrated using *Menhaden Oil* for all analyses of euphausiids in this study, whereas the bulk-extracted and purified *C. finmarchicus* oil was used as a calibration standard for all copepods.

Preparation of freeze-dried zooplankton for SPV analysis

Freeze-dried *C. finmarchicus* and *Euphausia superba* were purchased from Bulk Reef Supply (Golden Valley, MN). Freeze-dried zooplankton were ground into a finely homogenized powder using a mortar and pestle. Then, 10–15 mg of homogenized zooplankton powder was placed into a pre-tared glass test tube, the mass was measured, followed by addition of 2.0 mL of 2 : 1 (*v* : *v*) chloroform : methanol. The vials were capped and sonicated for 30 minutes. Finally, a 1 : 10 dilution of each sample was prepared using 2 : 1 (*v*/*v*) chloroform : methanol and used for SPV analysis. In order to calculate intra-assay and inter-assay variation, three replicate samples of the *C. finmarchicus flakes* were weighed out and

analyzed within a single plate, and the experiment was repeated over three separate days.

Preparation of freeze-dried zooplankton for gravimetric analysis

Gravimetric analysis of the purchased freeze-dried zooplankton was performed using a Dionex Accelerated Solvent Extractor (ASE) 350 (Thermo Scientific, Waltham, MA) employing a modified version of the *Folch method*.[13,35,36] Approximately 100–200 mg of freeze-dried zooplankton powder was mixed with 5 mL of hydromatrix (Agilent, Santa Clara, CA). The mixture was compressed into a 10 mL ASE cell containing a cellulose filter (ASE 350 cell filter, Thermo Scientific). The remaining cell volume was filled with sand (Sigma, St. Louis, MO). Total lipids were extracted using a 2 : 1 (*v*/*v*) chloroform–methanol solution. Lipid extracts were washed with 0.88% (*w*/*v*) potassium chloride and 1 : 1 (*v*/*v*) methanol–water before concentrating to ~1 mL with a Heidolph Laborota 4011 (Schwabach, Germany) rotary evaporator (40 °C, 500 mmHg). Lipid extracts were transferred to pre-weighed aluminum pans, dried overnight in a vacuum oven (VWR, Radnor, PA) at 700 mmHg and room temperature, and weighed (±0.00001 g). Prior to analysis, all glassware was baked at 400 °C for 4 hours to remove any organic contamination.

SPV analysis method

All chemical reagents were purchased from Fisher Scientific (Waltham, MA). An aliquot containing 100.0 μL of sample or standard was pipetted into each well of a glass 96-well plate (Product number: 3600500, Zinsser Analytic, Northridge, CA) in triplicate. Solvent was then evaporated by placing the glass 96-well plate on a hot plate (Reacti-Therm III, Thermo Scientific) set to 100 °C for 10 minutes. Then, 20.0 μL of concentrated sulfuric acid was added to each well and the samples were allowed to incubate at 100 °C for 10 minutes, after which the plate was allowed to cool to room temperature. After cooling, 280 μL of SPV reagent (6.8 mM vanillin, 2.6 M phosphoric acid) was added to each well, then the plate was incubated at room temperature with gentle shaking for 30 minutes. A Synergy H1 Hybrid Microplate Reader (BioTek, Winooski, VT) was used to measure the resulting absorbance at 490 nanometers (nm), as suggested by Van Handel.[29] All zooplankton samples in this study were analyzed at 490 nm. Samples containing low levels of lipid can be measured at 530 nm for increased sensitivity.[29] The absorbance of each well was measured both before and after the addition of the vanillin reagent in order to account for the background (matrix) absorbance of each sample. Lipid concentration was calculated by comparing the mean absorbance values of each sample to external standards of known concentration, and accounting for any dilutions. The total lipid (% of wet mass, or dry mass), was calculated by dividing the mass of lipid by the total mass of the sample prior to extraction. The limit of detection was calculated at both 490 nm and 530 nm, according to Thomsen *et al.*[37]

Sample collection

Arctic copepods and euphausiids were collected with plankton nets during surveys conducted in the Chukchi Sea in the spring and summer of 2017 to 2019, while *Euphausia pacifica* were collected using a ring net in the fall of 2019 from Puget Sound, WA, USA.[38,39] Following collection, zooplankton were frozen at −80 °C and were later shipped on dry ice to the Marine Lipid Ecology Laboratory at the Hatfield Marine Science Center (Oregon State University) in Newport, OR. Samples were processed within 12 months from the time of sampling. Briefly, samples were removed from frozen storage, identified on a Petri dish over crushed ice, blotted dry with a Kimwipe™, sorted into vials, weighed and stored at <−20 °C in 2 mL of chloroform under a blanket of nitrogen. Prior to storage, the total wet mass of zooplankton in each vial was measured on a microbalance and ranged from 10.0 to 303.9 mg (mean = 93 mg). Copepod species included: *Neocalanus flemingeri/plumchrus* (n = 10), *Neocalanus cristatus* (n = 4), and *Calanus marshallae/Calanus glacialis* (n = 5). Euphausiid species included *Euphausia pacifica* (n = 10), *Thysanoessa inermis* (n = 4), *Thysanoessa raschi* (n = 7), and mixed unidentified large Arctic euphausiids (n = 8). *N. flemingeri and N. plumchrus* were not identified separately during collection and are therefore combined. The same is true for samples containing *C. marshallae* and *C. glacialis*, as these species are difficult to differentiate using standard microscopy techniques.

Lipid extraction from field-collected zooplankton

Within six months of sampling, frozen zooplankton collected from the field were homogenized in chloroform and methanol and total lipids were extracted according to Parrish using a modified Folch procedure.[13,17] Homogenized samples were sonicated and centrifuged four times in a biphasic system of chloroform : methanol : water 8 : 4 : 3 (*v*/*v*/*v*). A double pipet method was used to remove the bottom chloroform layer after each wash. Chloroform layers were pooled and dried under nitrogen to a final lipid extract volume of 0.5 to 1.5 mL.

Thin layer chromatography

Lipid class profiles and total lipids were determined using thin layer chromatography with flame ionization detection (TLC/FID) with a MARK VI Iatroscan (Iatron Laboratories, Tokyo, Japan) as described by Lu *et al.* and Copeman *et al.*[19,40] Extracts were spotted on duplicate silica-gel-coated Chromarods, and a three-stage development system was used to separate wax esters, triacylglycerols, free fatty acids, sterols and polar lipids. The polar lipid fraction is mostly comprised of phospholipids with minor amounts of other acetone mobile polar lipids. The first rod development was in a chloroform : methanol : water solution (5 : 4 : 1 by volume) until the leading edge of the solvent phase reached 1 cm above the spotting origin. The rods were then developed in hexane : diethyl ether : formic acid solution (99 : 1 : 0.05) for 28 or 38 min in Arctic and North Pacific zooplankton, respectively. Finally rods were then developed in a hexane : diethyl ether : formic acid solution

(80 : 20 : 0.1) for 38 min. After each solvent development, rods were dried (5 min) and conditioned (5 min) in a constant humidity chamber (~32%) that was saturated with aqueous $CaCl_2$. Following the last development, rods were scanned using Peak Simple software (ver. 4.89, SRI Inc.) and the signal detected in millivolts was quantified with calibration curves using the following standards from Sigma (St. Louis, MO, USA): palmitic acid (free fatty acids), cholesterol (sterols), L-alpha-phosphatidylcholine (polar lipids). Specialized standards were purified by column chromatography to be used for wax esters (*C. finmarchicus* oil) and triacylglycerols (*Boreogadus saida* liver oil) using methods from Ohman.[20]

Samples were run in duplicate and if the coefficient of variation for any major peaks was >15%, samples were run an additional time. Calibrated relationships between lipid class areas and standard lipid amounts (μg) had correlations with an $r^2 \geq 0.98$ for all classes. Lipid class values are reported as percent of total lipid mass (m/m).

Preparation of field-collected zooplankton for gravimetric analysis

Following lipid class analyses by Iatroscan, samples were gently evaporated to dryness under a stream of nitrogen and then were immediately diluted to 2 mL. Samples were then vortexed and sonicated on ice for 10 minutes before being split for gravimetric or SPV analyses. A volumetric pipet was used to transfer one quarter of the extract (0.5 mL) into new lipid clean vials for shipment to the AFSC's Auke Bay Laboratories (Juneau, Alaska, USA) for SPV total lipid analyses. Samples were shipped frozen overnight on dry ice. Finally, 200 μL of the original extract was used to perform gravimetric analysis on the lipid extract on the same day the sample was diluted and split for analysis by SPV. To prepare the field-collected sample extracts for SPV analysis, 75 μL of sample was diluted to 1 mL with 2 : 1 chloroform/methanol (*v*/*v*) in a glass test tube.

Gravimetric analysis of field-collected zooplankton samples was performed using a micropipette to transfer 200 μL of lipid extract in chloroform into a small (300 μL) tin foil weigh boat. Lipid extracts evaporated within 10 minutes under a gentle stream of nitrogen and the foil boats were reweighed on a microbalance (Sartorius R160P) to the nearest ±0.0001 mg. Samples were checked after an additional 10 minutes to ensure that the sample had dried to a consistent mass.

Results and discussion

Study of appropriate calibration standards

Initial experiments were performed in order to identify appropriate calibration standards for the SPV assay when analyzing copepods and euphausiids. Menhaden Oil was chosen as a calibration standard for euphausiids because it provides a representative mixture of marine lipid classes and fatty acids, and it is a recommended reference material for marine oils by the *American Oil Chemists' Society (AOCS) Official Method Ce li-07*. The response of the SPV assay for Menhaden Oil was compared to two different sources of *C. finmarchicus* oil in Fig. 1. *C. finmarchicus* supplement capsules were explored as a commercially available source of pure copepod oil. The capsules are reported to each contain 500 mg of *C. finmarchicus* oil and can easily be sliced open using a knife or scalpel, revealing a liquid oil-filled cavity. Calibration curves for each oil are graphed in Fig. 1, and the slopes of the best-fit-line for each curve were compared using the Student *t*-test at the 95% confidence level. The SPV response of oil derived from a bulk extraction of *C. finmarchicus* ($y = 0.035(\pm0.002)x + 0.10(\pm0.01)$, $r^2 = 0.99$) was not statistically different from purchased supplement capsules ($y = 0.033(\pm0.002)x + 0.10(\pm0.01)$, $r^2 = 0.99$) ($n = 3$, $p > 0.05$). However, the response of the Menhaden Oil ($y = 0.0238(\pm0.0004)x + 0.09(\pm0.01)$, $r^2 = 0.99$) was approximately 30% lower than the oils from *C. finmarchicus* ($n = 3$, $p < 0.05$). This could be explained by a variety of differences in the molecular composition of lipids from copepods in comparison to other marine species, such as high proportions of polyunsaturated fatty acids in wax ester moieties and relatively low proportions of polar phospholipids. Differences in molecular structure are known to cause slight variations in the SPV response (*e.g.* degree of saturation, molecular mass, and steric hindrance).[28,41] For purposes of accuracy, all copepod samples in this study were calibrated using the bulk-extracted and purified wax ester-based copepod oil, whereas all euphausiids were calibrated with Menhaden Oil. The limit of detection of the SPV assay was determined to be approximately 1.7 (±0.2) μg of lipid per analysis when measured at 490 nm, and 1.0 (±0.1) μg of lipid per analysis when measured at 530 nm.

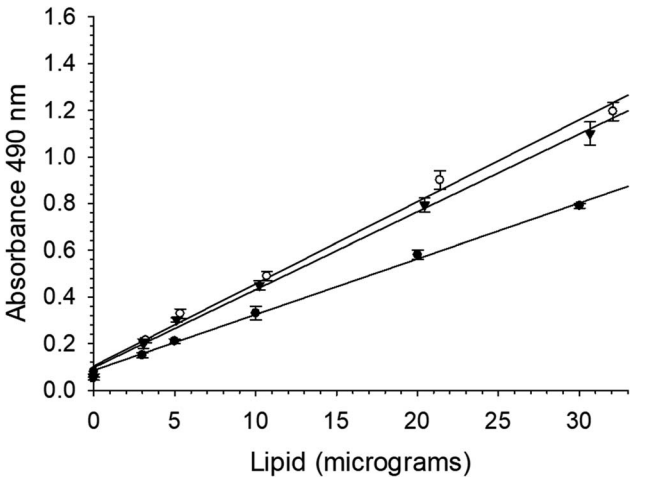

Fig. 1 External standard calibration curves. Oil from Menhaden (filled circles, $y = 0.024x + 0.09$, $r^2 = 0.99$), bulk extracted *Calanus finmarchicus* oil (open circles, $y = 0.035x + 0.10$, $r^2 = 0.99$), oil from *C. finmarchicus* supplement capsules (filled triangles, $y = 0.033x + 0.10$, $r^2 = 0.99$). $n = 3$, error = s.d.

Total lipids of freeze-dried zooplankton

Freeze-dried *C. finmarchicus* and *E. superba* were purchased for use as standard reference materials, as the total lipid content (% of dry mass) of each was reported by the manufacturer as 26% and 16%, respectively. Therefore, the following

experiments were performed in order to determine if the aforementioned standards (Fig. 1) could be used to calibrate the SPV assay for accurate determination of the lipid content of these reference materials. First, the lipid composition reported by the manufacturer of these materials was confirmed *via* ASE-gravimetric analysis to be 26.0 ± 2.7% ($n = 9$) and 12.8 ± 1.8% ($n = 8$), respectively. The SPV assay was performed on the same materials, using the Menhaden Oil standard curve (Fig. 1) for calibration of the *E. superba* flakes, and the bulk extracted *C. finmarchicus* oil standard curve for calibration of the *C. finmarchicus* flakes. According to the Student *t*-test, the results of the SPV assay (26.5 ± 2.8% ($n = 9$) and 14.5 ± 0.5% ($n = 4$)) were not statistically different from gravimetric analysis for either sample ($p > 0.05$) at the 95% confidence level, as observed in Fig. 2. Further, the inter-assay and intra-assay coefficients of variation (CV) were measured as 6.9% and 6.0%, respectively.

SPV analysis of field-collected zooplankton

The results of the SPV assay were both precise and accurate in measuring the total lipid content of field-collected Arctic copepods in comparison to gravimetric analysis. The results of the method comparison are displayed in Fig. 3A, which show a correlation coefficient and slope of the best-fit-line both near unity ($y = 0.91(\pm0.02)x + 1.36(\pm0.36)$, $r^2 = 0.99$, $n = 19$). The samples provided a wide range of total lipid content from 2 to 52%, and encompassed several different species of calanoid copepods, including *N. flemingeri*/*N. plumchrus* ($n = 10$), *N. cristatus* ($n = 4$), and *C. marshallae*/*C. glacialis* ($n = 5$). Iatroscan results (Table S1†) reveal that copepod lipids analyzed here were primarily composed of wax esters (range = 55.0–94.5% of lipid mass, mean = 86.6%), and contained low levels of both triacylglycerols (range = 0.6–9.1% of lipid mass, mean = 3.7%) and polar lipids (range = 1.1–42.1% of lipid mass, mean = 7.3%). These results suggest that the SPV assay can be utilized to accurately measure lipids in a variety of copepod species when calibrated using oil derived from copepods.

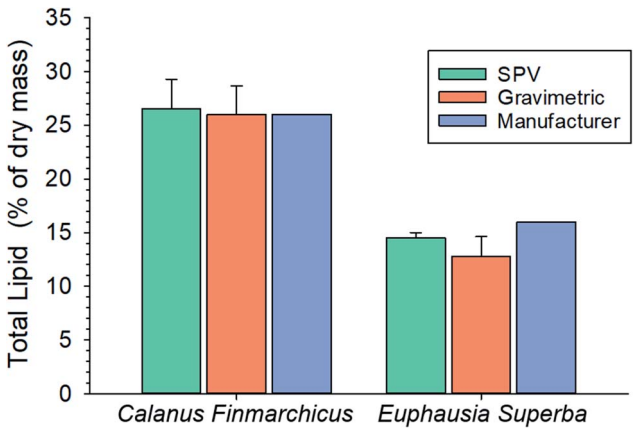

Fig. 2 Total lipid (% of dry mass) of purchased freeze-dried zooplankton measured by SPV and ASE-gravimetric analysis and compared to the reference value provided by the manufacturer.

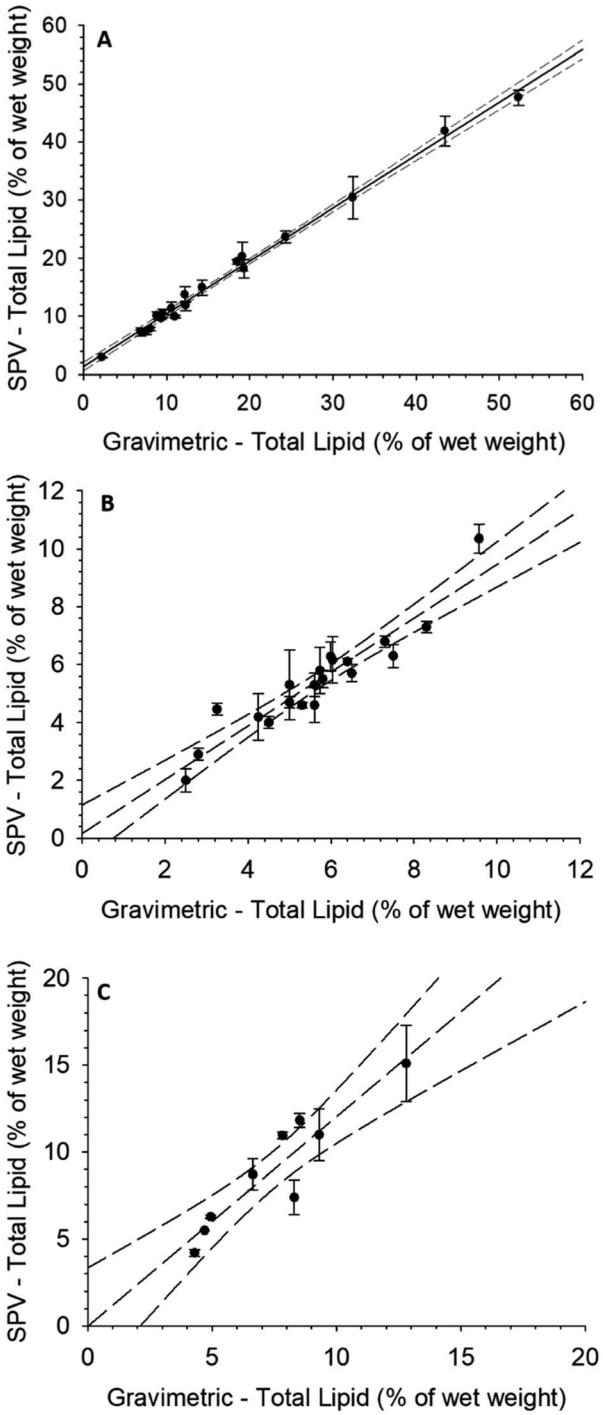

Fig. 3 Inter-laboratory comparison of gravimetric and SPV analyses of field-collected zooplankton. Dashed lines represent 95% confidence intervals. (A) Arctic copepods, various species, $n = 19$. ($y = 0.91(\pm0.02)x + 1.36(\pm0.36)$, $r^2 = 0.99$). (B) Triacylglycerol-based euphausiids, $n = 20$. ($y = 0.93(\pm0.08)x + 0.17(\pm0.47)$, $r^2 = 0.88$). (C) Wax-based euphausiids, $n = 9$. ($y = 1.2(\pm0.2)x - 0.02(\pm1.4)$, $r^2 = 0.86$).

Further studies analyzed the performance of the SPV assay when measuring various species of euphausiids ($n = 29$) collected from both the Arctic and North Pacific. The data in

Fig. 3B feature euphausiids containing triacylglycerol as the primary storage lipid (range = 13.1–52% of lipid mass, mean = 39.5%) while the data in Fig. 3C feature euphausiids containing wax ester as the primary storage lipid (range = 19.7–42.1% of lipid mass, mean = 35.9%). Importantly, both groups (Fig. 3B and C) were calibrated using Menhaden Oil, and the SPV assay performed well as displayed by the characteristics of the best-fit-line in each graph (triacylglycerol-based euphausiids: ($y = 0.93(\pm0.08)x + 0.17(\pm0.47)$, $r^2 = 0.88$), wax-based euphausiids: ($y = 1.2(\pm0.2)x - 0.02(\pm1.4)$, $r^2 = 0.86$)). These data suggest that Menhaden Oil standards can be used to calibrate the SPV assay for various species of euphausiids, regardless of their primary lipid class.

The compiled results from all zooplankton used in this inter-laboratory comparison study are shown in Fig. 4A and B. Fig. 4A shows good overall agreement between gravimetric and SPV analyses ($y = 0.93(\pm0.02)x + 0.83(\pm0.25)$, $r^2 = 0.98$). The mean absolute and relative difference between SPV and gravimetric analysis for all zooplankton lipids were 1.0% and 11.6%, respectively. The SPV assay was also compared to the Iatroscan method for measuring total lipids, as shown in Fig. 4B. The Iatroscan tended to underestimate the total lipid content in comparison to both SPV and gravimetric analyses (mean relative underestimation of 12% and 10%, respectively); however, this has been previously acknowledged in the literature and can be systematically accounted for.[17,19,42] Table S1 in the ESI† document contains the total lipid content of each sample as measured by SPV, gravimetric, and TLC-FID, as well as the lipid class composition.

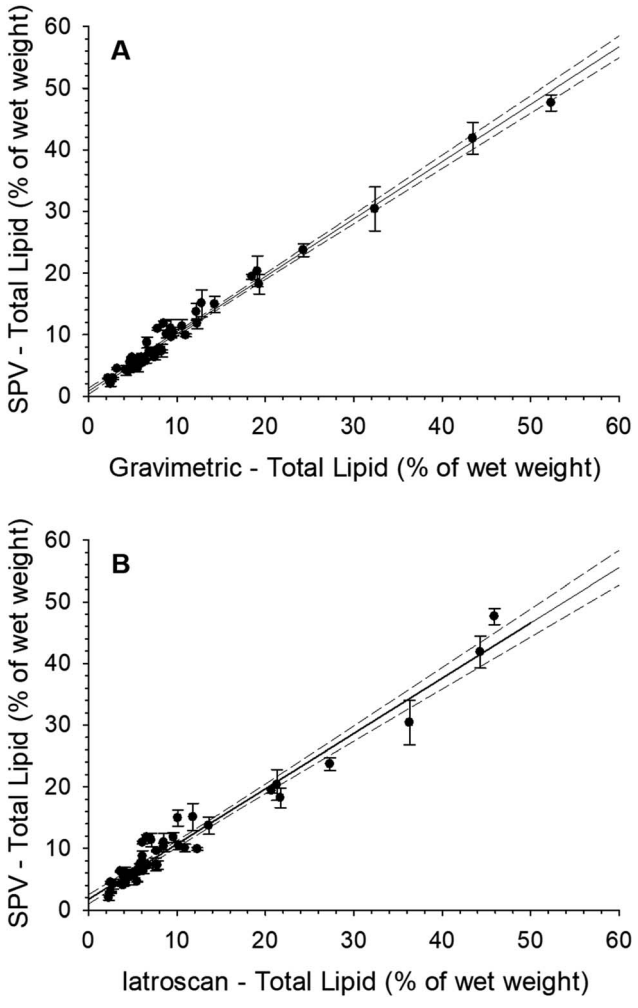

Fig. 4 Inter-laboratory comparison of Iatroscan, gravimetric, and SPV analyses. Dashed lines represent 95% confidence intervals. $n = 48$. (A) All zooplankton, SPV *vs.* gravimetric ($y = 0.93(\pm0.02)x + 0.83(\pm0.25)$, $r^2 = 0.98$). (B) All zooplankton, SPV *vs.* Iatroscan ($y = 0.90(\pm0.03)x + 1.74(\pm0.38)$, $r^2 = 0.96$).

Conclusion

Overall, the results of the SPV assay agree well with both gravimetric analysis and TLC-FID for measuring total lipid content in large crustacean zooplankton. In comparison to other methods, the SPV assay is quick and straightforward, and agrees well with more laborious traditional methods. The results of the SPV method agreed better with gravimetric analysis when the calibration standard more closely resembled the sample (*i.e.* calibrating with an arctic copepod oil standard when measuring samples of arctic copepods, or using a triacylglycerol-based oil when measuring triacylglycerol-based euphausiids). Authors of the *Nile Red* method report the necessity of a species-specific calibration curve.[26] It is impractical to obtain a stock of calibration oil (of appreciable quantity) for every different zooplankton species. However, there is room for improvement, and the accuracy of the SPV assay may possibly be improved by using species-specific calibration standards from native lipids. A method which utilizes universal calibration solutions, as reported here, improves the approachability of the method. The SPV method may also be accurate for measuring lipids in other zooplankton taxa not analyzed here (*e.g.* jellyfish, amphipods, chaetognaths, *etc.*), but this would need to be experimentally validated.

The lipid composition of zooplankton is diverse and contains varying levels of saturated, monounsaturated, and polyunsaturated lipids. Initial studies published on the mechanism of the SPV reaction reported that a measurable response necessitated a pre-existing carbon–carbon double-bond (C=C) or hydroxyl group.[41] However, more recent studies revealed that fully saturated wax esters (containing no C=C or hydroxyl) yield a strong SPV response, comparable to unsaturated wax esters.[28] This is due to the measurable formation of a C=C double bond intermediate product in the reaction of aliphatic esters with hot sulfuric acid in the first step of the reaction, which can then form the carbonium ion needed to react with vanillin in the second step of the reaction to form a stable chromophore. Further, it is unlikely for multiple chromophores to be present on a single lipid molecule due to steric hindrance. Therefore, polyunsaturated lipids yield a similar response to their monounsaturated and saturated counterparts. This low specificity of the SPV reaction between lipids is important, as it leads to accurate measurements of complex lipid mixtures observed in zooplankton. Additionally, the method is highly specific for

lipids and does not react with other biomolecules, such as proteins or carbohydrates.[28,30]

With the use of a multi-channel pipette, up to twenty-five samples, five calibration standards, and two reference material standards for quality assurance can be analyzed (in triplicate) in under two hours on a single 96-well plate. The short time from start to finish of a single batch of samples allows for multiple batches to be analyzed in a single day. The ability to monitor the lipid content of our oceans' zooplankton is of critical importance to ecosystem and fishery managers. The rapid and high-throughput nature of this method enables quick turnaround time between high-volume sample collections and data reporting. This information may be utilized in ecosystem status reports and models, empowering managers with knowledge of zooplankton quality as forage for fish, birds, marine mammals, and other marine organisms in a timely manner.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

We are thankful to research scientists that collected zooplankton as part of larger field ecological or laboratory experiments. These included Drs Lisa Eisner, David Kimmel, Shallin Busch, and Russell Hopcroft. Ecological studies on Arctic zooplankton were funded by the North Pacific Research Board, specifically as part of the Arctic Integrated Ecosystem Study (NPRB publication: *ArcticIERP-45*). *Euphausia pacifica* were obtained from ocean acidification laboratory experiments funded as part of the NOAA Ocean Acidification Program. We thank Jami Ivory, Kelia Axler, Kylie Welch, and Jessica Andrade for help with processing zooplankton samples in the Marine Lipid Ecology Laboratory at the Hatfield Marine Sciences Center. We also thank Drs C. Langdon and R. Olsen for access to Bulk extracted and purified wax ester-based copepod oil. The scientific results and conclusions, as well as any views or opinions expressed herein, are those of the authors and do not necessarily reflect those of NOAA or the U.S. Department of Commerce. Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

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