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High-throughput determination of total lipids from North Pacific marine fishes via the sulfo-phospho-vanillin microplate assay

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Abstract

Total lipid content is a valuable indicator of fish health, prey quality, survival potential, stock health, and ecosystem status. Here, we demonstrate an accurate method for measuring total lipids in fish tissues using the spectrophotometric sulfo-phospho-vanillin (SPV) assay, adapted to a 96-well plate format. Samples of dried homogenate were cross-analyzed via the SPV assay and standard gravimetric lipid analysis. Initial measurements of whole fish homogenates analyzed include Pacific herring (*Clupea pallasii*), Pacific cod (*Gadus macrocephalus*), walleye pollock (*G. chalcogrammus*), Pacific capelin (*Mallotus villosus*), Chinook (*Oncorhynchus tshawytscha*), and coho (*O. kisutch*) salmon. Samples of muscle tissue were analyzed from Chinook, pink (*O. gorbuscha*), sockeye (*O. nerka*), and chum (*O. keta*) salmon. All SPV measurements were calibrated using menhaden oil. The mean absolute and relative difference between gravimetric and SPV analysis was 0.5 and ~ 16.4%, respectively ($n = 121$). To improve the accuracy of SPV assay results, linear calibration models specific to taxa and tissue matrix type were developed, enabling calculation of *corrected* SPV assay values. The accuracy of using these calibration models was tested by analyzing additional fish samples ($n = 16$). The results of the *corrected* SPV assay were not statistically different ($p > 0.05$) from gravimetric analysis for any samples measured, and the mean absolute and relative difference between the two assays improved to 0.2% and 4.6%, respectively. The SPV assay provides a rapid (2 h), high-throughput (25 samples processed in triplicate), precise (interassay coefficient of variation = 5.6%), and accurate method for quantifying the total lipid content of homogenized fish tissue.

Lipids are a highly valuable source of energy in marine ecosystems, providing significantly higher caloric density than protein and carbohydrates (Parrish 2013). Fish accumulate lipids from their diet, utilizing them to fuel growth and storing them as reserves of energy for survival through periods of limited food availability or starvation (Tocher 2003; Lloret et al. 2013; Waters et al. 2022). Lipids are the first energy reserves to be catabolized during nonfeeding and reproductive stages (Adams 1999; Grant and Brown 1999; Lloret et al. 2013). Exhausted energy reserves weaken fish, increasing susceptibility to predation and disease, and decreasing growth rates and reproductive potential (Adams 1999; Brosset et al. 2016; Dutil et al. 2006; Marshall et al. 1999; Pangle et al. 2004; Sewall

et al. 2019). Therefore, measuring the total lipid content of fish is vital for understanding overall fish health and condition, as well as the availability of energy to predators (Neill et al. 2014; Lerner and Hunt 2023).

Deriving ecologically relevant data about fish stocks from measurements of individual specimens requires relatively large sample sizes. Traditional lipid analyses (gravimetric and chromatographic) are time consuming and low throughput, resulting in limited data availability on spatial and temporal trends. Furthermore, slow turnaround time between sample collection and finalized data hinders the ability of scientists to report data to ecosystem managers in a policy-relevant time frame. A rapid and high-throughput method of measuring total lipid content in fishes would enable efficient data reporting for use in annual ecosystem status reports and stock assessments.

The sulfo-phospho-vanillin (SPV) method of lipid quantitation has long been studied as a rapid alternative to traditional total lipid analyses. In brief, extracted lipids are subjected to heat and concentrated sulfuric acid followed by reaction with vanillin in the presence of phosphoric acid (Knight et al. 1972). This

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Additional Supporting Information may be found in the online version of this article.

reaction produces a pink chromophore that can be quantified using absorbance spectrophotometry, enabling the method to be processed in a microplate format using a plate reader and multichannel pipette for streamlined high-throughput processing (McMahon et al. 2013). The SPV method has been used successfully for quantifying the total lipid content of various biological sample types, such as zooplankton, mosquitoes, algae, several fishes, as well as human meibum and plasma (Frings et al. 1972; Knight et al. 1972; Barnes and Blackstock 1973; Van Handel 1985; Inouye and Lotufo 2006; Lu et al. 2008; Cheng et al. 2011; McMahon et al. 2013; Byreddy et al. 2016; Bailey et al. 2022; Pinger et al. 2022; Farinacci and Laurent 2023; Yang et al. 2024).

The goal of this manuscript is to evaluate the performance of the SPV assay for quantifying lipid content in various marine fishes with commercial and subsistence importance in the North Pacific Ocean. Further, we demonstrate a method for accurately calibrating the assay for samples for which species-specific commercial calibration standards are not available.

Materials and procedures

Sample collection

All samples analyzed in this study were opportunistically subsampled from long-term monitoring research investigations within multiple Alaska ecosystems. Whole Pacific herring (*Clupea pallasii*), walleye pollock (*Gadus chalcogrammus*), Pacific cod (*G. macrocephalus*), Pacific capelin (*Mallotus villosus*), pink salmon (*Oncorhynchus gorbuscha*), chum salmon (*O. keta*), and Chinook salmon (*O. tshawytscha*) were collected from ecosystem surveys conducted by the National Marine Fisheries Service in the northern and southern Bering Sea, the Gulf of Alaska, and Prince William Sound during 2021 and 2022. Sockeye (*O. nerka*), pink, and chum salmon were sampled from the North Pacific Ocean as part of the International Year of the Salmon survey in 2022. Additional pink, sockeye, and Chinook salmon were collected in 2022 as part of the Southeast Alaska Coastal Monitoring Project. Muscle tissue was sampled from adult Chinook salmon collected at Little Port Walter Biological Research Station on Baranof Island, Alaska, in 2021 and 2022. Juvenile Chinook and coho (*O. kisutch*) salmon were collected from the Yukon River in 2021 and 2022 as part of a juvenile salmon condition study. Whole fish and muscle tissue samples were frozen at −20°C and shipped to Auke Bay Laboratories (Juneau, Alaska) for processing and analysis.

Sample preparation

Prior to lipid analysis, whole fish and muscle tissue samples were homogenized and a subsample was dried in a LECO TGA801 thermogravimetric analyzer (LECO; St. Joseph, MI) set to 135°C. Samples were considered dry after reaching a constant mass, allowing for calculation of percent moisture. The dried fish homogenates were then ground to a fine powder using a mortar and pestle. Samples were stored in a desiccator at room temperature until use.

SPV analysis

The SPV assay was performed as previously reported with slight modifications (Pinger 2022). First, 10–30 mg of dry homogenized fish tissue was placed into a pre-tared glass test tube, the mass was recorded, and 2.0 mL of chloroform : methanol (2 : 1, v/v) solution was added. The test tubes were then capped and sonicated for 30 min. Next, a 1 : 10 dilution of each sample was made to a volume of 1.0 mL using chloroform : methanol (2 : 1, v/v) in a separate glass test tube. For each sample, 100 μL of the 1 : 10 dilution was added to a high purity, temperature resistant borosilicate glass 96-well plate (Zinnser Analytic) in triplicate wells (totaling 300 μL). Solvent was evaporated by placing the glass 96-well plate on a Reacti-Therm III hot plate (Thermo Scientific) set to 100°C for 10 min. Then, 20 μL of concentrated sulfuric acid (ACS reagent grade, 95–98%) was added to each well and the plate was incubated at 100°C for an additional 10 min and then cooled to room temperature. Once cooled, 280 μL of SPV reagent (6.8 mM vanillin, 2.6 M phosphoric acid) was added to each well and incubated at room temperature with gentle shaking on a plate shaker (Vortemp™-56, Labnet International) for 30 min. The lid of the plate shaker was closed to protect the developing chromophore from light exposure. Incubation time was optimized experimentally, as detailed in the Supporting Information document. Finally, absorbance at 490 nm was measured using a plate reader (BioTek Synergy H1 Hybrid). All samples in this study were analyzed at 490 nm; however, samples containing low levels of lipid may be measured at 530 nm for increased sensitivity (Van Handel 1985).

The lipid content of the sample extract was calculated by comparing sample absorbance values to a calibration curve of external standards of known concentration. The standards were prepared from commercially purchased menhaden oil (Sigma Aldrich). Briefly, 25 mg of menhaden oil was diluted to 50 mL in a volumetric flask with chloroform : methanol (2 : 1, v/v) and mixed well to create a stock solution. Standards were made by pipetting the stock solution into glass test tubes and diluting to 1.0 mL with chloroform : methanol (2 : 1, v/v) to contain 0, 30, 50, 100, 200, and 300 (μg) of lipid per mL.

The final result, reported as % lipid, was calculated by dividing the measured lipid content by the total mass of the sample weighed prior to extraction and accounting for the % moisture content in the original sample as follows:

$$\text{Sample Wet Mass} = \frac{\text{Sample Dry Mass}}{1 - \left(\frac{\% \text{ moisture}}{100}\right)}$$

$$\% \text{ Lipid} = \left(\frac{\text{Lipid Mass}}{\text{Sample Wet Mass}}\right) \times 100\%$$

All % lipid measurements reported in this paper are calculated with respect to wet mass.

Gravimetric lipid analysis

Gravimetric lipid analyses were conducted following a modified version of the method described by Folch (Folch et al. 1956; Iverson et al. 2001) and briefly summarized here. First, approximately 100 mg of dry homogenate was measured into a glass centrifuge vial (Reacti-Vial—13223, Thermo Scientific) and the mass of the homogenate was recorded to the nearest 0.00001 g. Then, approximately 3 mL of chloroform : methanol (2 : 1, v/v) was added followed by 750 μL of 0.1 M KCl. The samples were vortexed briefly and sonicated for 10 min. Following sonication, the samples underwent centrifugation at 3000 RCF for 5 min at 4°C. The bottom phase, which contained roughly 2 mL of chloroform and the extracted lipids, was transferred to a new vial. An additional 2 mL of chloroform was added to the vial and the centrifugation and transfer steps were repeated twice more, resulting in three total washes. The vials containing the extracted lipids and chloroform were centrifuged at 3000 RCF for 10 min at 4°C. Subsequently, the supernatant was decanted into pre-weighed aluminum weigh boats and left to evaporate overnight in a fume hood. The residual mass in the weigh boat was then measured to determine the total lipid mass.

Quality assurance

The SRM 1946 standard reference material of Lake Superior Lake trout (*Salvelinus namaycush*) homogenate (hereafter SRM 1946) was purchased from the National Institute of Standards and Technology (Choquette 2016). The SRM 1946 material has a certified reference value for total lipid and moisture content. The material was used to verify the accuracy of the gravimetric analysis method. The SRM 1946 homogenate was lyophilized prior to analysis.

A stock of dry walleye pollock homogenate was created and used as an in-house quality control standard to run routinely alongside samples on each SPV analysis plate. Additionally, two quality control standards were run with each analysis. Quality control standards were handled identically to unknown samples. Quality control standard values were recorded for individual analysis runs ($n = 20$) to calculate an interassay coefficient of variation (CV).

Data analysis

An initial batch of samples ($n = 121$) were analyzed using both gravimetric and SPV methods. Samples were grouped taxonomically (i.e., by genus) and by tissue matrix type (e.g., *gadids—whole body*, *salmon—muscle*, etc.). The results of the two assays were compared by linear regression plots (Fig. 1a–e). The equation of the line-of-best-fit was used to generate linear calibration models between the two assays for each group. A separate test batch of samples ($n = 16$) were analyzed by both SPV and gravimetric analyses. For robustness, the test batch consisted of whole fish and muscle tissue sourced from either different marine ecosystems, different years, or different species as those measured in the initial

batch. Results from the SPV assay of the test batch were input into the linear calibration models (reported in Table 1) to calculate a *corrected SPV % lipid* value, reported in Table 2.

Agreement between the *corrected SPV* and gravimetric methods was assessed using the Student's *t*-test. Additionally, the Bland–Altman analysis method was used as a graphical approach to evaluate the comparability between the SPV assays (uncorrected and corrected) and the gravimetric analysis for the samples in the test batch (Fig. 2). The Bland–Altman analysis is recommended as a straightforward way of evaluating bias and agreement intervals (95% of differences) between two analytical methods (Altman and Bland 1983; Giavarina 2015). SigmaPlot 14.0 software, the R programming language (R Core Team 2023), and ggplot2 (Wickham 2016) were used for data visualization and analysis.

ASSESSMENT

The comparison between SPV and gravimetric methods demonstrated considerable precision for all species and tissue types analyzed. Linear regression models for each sample type resulted in a correlation coefficient near unity ($r^2 = 0.97$–0.99; Fig. 1; Table 1). The accuracy of the SPV assay, as compared to standard gravimetric analysis, varied among groups. This variation is evident in the slope of the best-fit line for each group's linear regression model. SPV assay measurements of whole Pacific herring (Fig. 1a) lipids showed the closest relative agreement with gravimetric measurements; the slope was near unity and the mean relative difference between the two assays was 9.5% (mean absolute difference: 0.5%, $n = 12$). This close agreement is likely due to the use of menhaden oil as an in-plate calibration standard. Pacific herring and menhaden are both Clupeiformes and are likely to have similar lipid molecular composition. The SPV assay method exhibited fair accuracy for whole gadid (i.e., Pacific cod and walleye pollock; Fig. 1b) lipid content measurement; the mean relative difference from gravimetric analysis was 11.7% (mean absolute difference: 0.2%, $n = 25$). Analysis of whole Pacific capelin (Fig. 1c), and whole Chinook and coho salmon (Fig. 1d) lipid content using the SPV assay was also fairly accurate compared to gravimetric analysis. The mean relative difference for Pacific capelin was 16.6% (mean absolute difference: 0.7%, $n = 8$), and for Chinook and coho salmon it was 14.6% (mean absolute difference: 0.3%, $n = 30$). The SPV assay tended to overestimate the lipid content of salmon muscle tissue (Fig. 1e), especially in fattier tissue samples (> 2.5% lipid). The mean relative difference from gravimetric analysis for salmon muscle tissue was 22.0% (mean absolute difference: 0.8%, $n = 47$). For all samples analyzed, the mean absolute and relative % difference was 0.5% and 16.4%, respectively ($n = 121$), when directly comparing results from the SPV assay to standard gravimetric analysis.

Gravimetric analysis was performed on a quality control sample of fish muscle tissue with a certified lipid content
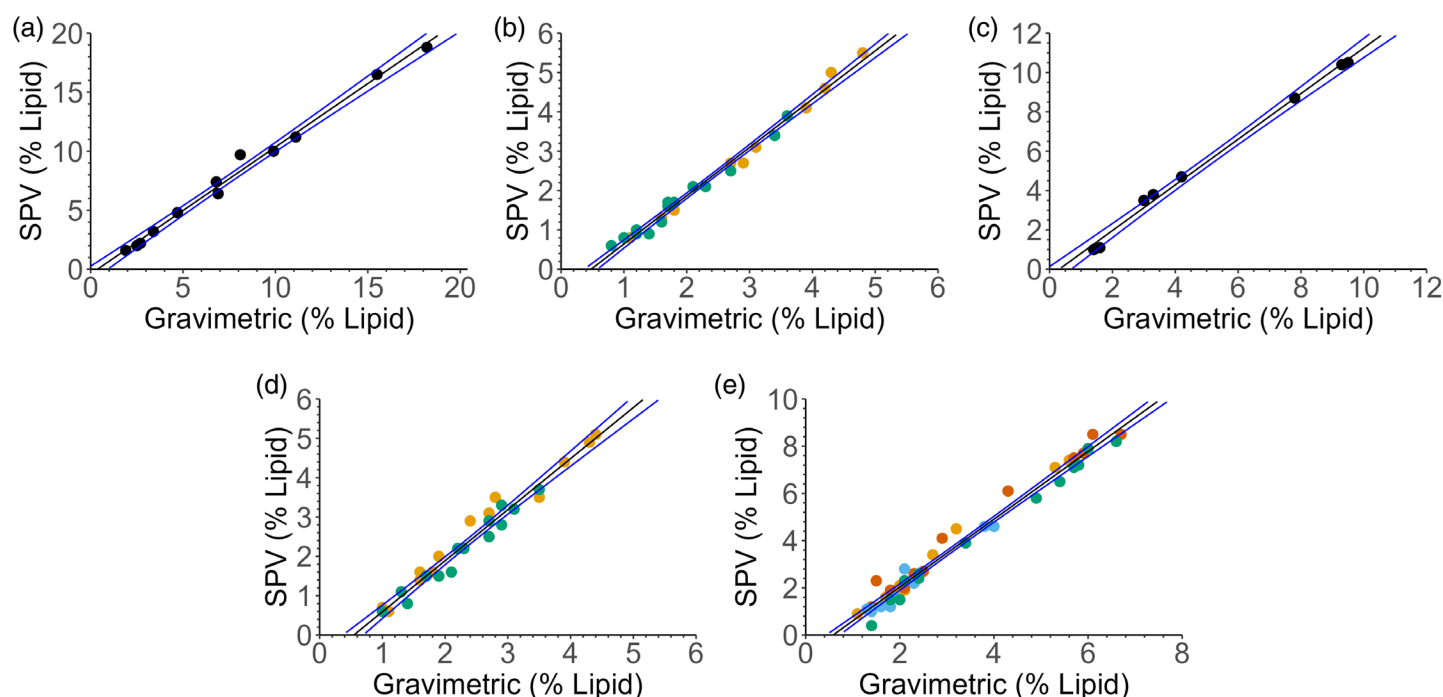
Fig. 1. Method comparisons between SPV assay and gravimetric analysis for marine fishes from the North Pacific. Blue lines represent 95% confidence intervals for: (**a**) Pacific herring (whole); (**b**) mixed gadids (whole, Pacific cod = green, walleye pollock = yellow); (**c**) Pacific capelin (whole); (**d**) mixed salmon (whole, Chinook = green, coho = yellow); (**e**) mixed salmon (muscle, Chinook = green, chum = yellow, sockeye = orange, pink = blue). See Table 1 for species and counts for each group.

(Choquette 2016). The gravimetrically measured total lipid content ($10.2\% \pm 0.2\%$, $n = 3$) agreed with the certified reference value ($10.17\% \pm 0.48\%$), thus validating the gravimetric methods used here. Further, the precision of the SPV assay was validated by replicate measurement of an in-house standard reference material across separate analysis runs. The interassay CV was 5.6% ($n = 20$), confirming the precision of the SPV assay.

To improve the accuracy of the SPV assay we used the equation of the best-fit line for each group (Table 1) as a calibration

model. We tested the accuracy of these calibration models on an additional group of samples, hereafter referred to as the test batch. The initial SPV assay measurements of these test batch samples are reported in Table 2, as well as the corrected SPV value calculated using the calibration model. The results of the corrected SPV assay were not statistically different from gravimetric analysis for the samples measured here (Student's *t*-test, $p > 0.05$).

The Bland–Altman analyses (Fig. 2) showed relatively good agreement between the uncorrected SPV assay and the

Table 1. Equations of the best-fit line from method comparisons between SPV assay and gravimetric analysis for marine fishes from the North Pacific. Samples were either whole fish or muscle only samples. Species in each mixed group and number of samples is shown.

Sample type	Species (n)	Equation of best-fit line	Correlation coefficient r^2
Pacific herring—whole	Pacific herring (12)	$y = 1.07\,(\pm 0.03)x - 0.4\,(\pm 0.3)$	0.99
Mixed gadids—whole	Pacific cod (14)	$y = 1.23\,(\pm 0.03)x - 0.59\,(\pm 0.07)$	0.99
	Walleye pollock (11)		
Pacific capelin—whole	Pacific capelin (8)	$y = 1.16\,(\pm 0.03)x - 0.3\,(\pm 0.2)$	0.99
Mixed salmon—whole	Chinook (15)	$y = 1.30\,(\pm 0.05)x - 0.7\,(\pm 0.1)$	0.97
	Coho (15)		
Mixed salmon—muscle	Chinook (13)	$y = 1.44\,(\pm 0.03)x - 0.8\,(\pm 0.1)$	0.98
	Chum (11)		
	Sockeye (12)		
	Pink (10)		

Table 2. Results comparing each analysis method on samples in the test batch.

Species—sample ID	Tissue type	Catch origin and year	SPV % Lipid, $n = 3$–5 ($\pm$ SD)	*Corrected* SPV % Lipid, $n = 3$–5 ($\pm$ SD)	Gravimetric % Lipid, $n = 2$ ($\pm$ SD)	*p*-value Corrected SPV vs. gravimetric
Pacific herring—1	Whole	PWS—2022	7.6 $\pm$ 1.0	7.4 $\pm$ 0.9	6.98 $\pm$ 0.09	0.492
Chinook salmon—1	Muscle	LPW—2022	4.0 $\pm$ 0.2	3.4 $\pm$ 0.3	2.99 $\pm$ 0.11	0.052
Chinook salmon—2	Muscle	LPW—2022	4.9 $\pm$ 0.7	4.0 $\pm$ 0.5	4.01 $\pm$ 0.07	0.823
Chinook salmon—3	Muscle	LPW—2022	4.4 $\pm$ 0.2	3.6 $\pm$ 0.1	3.57 $\pm$ 0.02	0.561
Pink salmon—1	Whole	SEAK—2022	1.54 $\pm$ 0.02	1.73 $\pm$ 0.01	1.82 $\pm$ 0.03	0.141
Sockeye salmon—1	Whole	SEAK—2022	2.7 $\pm$ 0.1	2.7 $\pm$ 0.1	2.81 $\pm$ 0.00$_4$	0.125
Chinook salmon—1	Whole	SEAK—2022	1.0 $\pm$ 0.2	1.3 $\pm$ 0.1	1.39 $\pm$ 0.03	0.429
Pacific capelin—1	Whole	NBS—2021	6.9 $\pm$ 0.2	6.3 $\pm$ 0.2	6.03 $\pm$ 0.04	0.174
Pacific capelin—2	Whole	NBS—2021	8.4 $\pm$ 0.3	7.5 $\pm$ 0.2	7.72 $\pm$ 0.05	0.313
Pink salmon—2	Whole	NBS—2022	2.4 $\pm$ 0.3	2.4 $\pm$ 0.3	2.51 $\pm$ 0.03	0.765
Chum salmon—1	Whole	NBS—2022	5.3 $\pm$ 0.3	4.7 $\pm$ 0.2	4.72 $\pm$ 0.13	0.753
Pacific cod—1	Whole	SBS—2022	1.8 $\pm$ 0.2	1.9 $\pm$ 0.2	2.26 $\pm$ 0.30	0.343
Walleye pollock—1	Whole	SBS—2022	5.2 $\pm$ 0.2	4.7 $\pm$ 0.2	4.51 $\pm$ 0.07	0.137
Walleye pollock—2	Whole	SBS—2022	4.2 $\pm$ 0.3	3.8 $\pm$ 0.2	3.82 $\pm$ 0.31	0.931
Chinook salmon—2	Whole	NBS—2022	2.9 $\pm$ 0.3	2.8 $\pm$ 0.3	2.88 $\pm$ 0.02	0.644
Chum salmon—2	Whole	SBS—2022	2.8 $\pm$ 0.1	2.7 $\pm$ 0.1	2.76 $\pm$ 0.11	0.607

LPW, Little Port Walter; NBS, Northern Bering Sea; PWS, Prince William Sound; SBS, Southern Bering Sea; SEAK, Southeast Alaska.

gravimetric analysis, a moderate positive trend of differences proportional to the magnitude of the lipid content, and limits of agreement between −0.7% and 1.4%. The corrected SPV assay showed close agreement with gravimetric analysis, improved limits of agreement between −0.5% and 0.5%, and no trend of differences with increasing lipid content. Therefore, the two methods agree exceptionally, and the use of tissue-specific and taxa-specific calibration models further improves this agreement.

Discussion

The SPV method is well established as a rapid method to measure lipid content in various biological matrices, and many reports have demonstrated high accuracy when properly calibrated. However, proper calibration often requires using an oil standard with approximately similar molecular composition to that of the samples being analyzed, which can be difficult when analyzing an array of species and tissue types. When a species-specific standard is not available, a
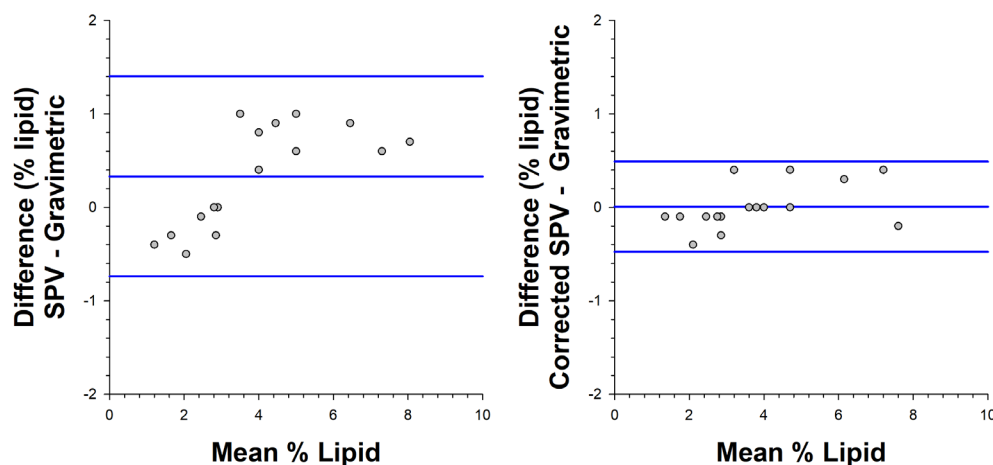

Fig. 2. Bland–Altman analysis method comparison results between SPV assay and gravimetric analysis for samples in the *test batch*. Blue lines represent limits of agreement (95% confidence).

standard derived from a closely related taxa as the samples being analyzed is typically used (Cheng et al. 2011; McMahon et al. 2013; Byreddy et al. 2016; Pinger et al. 2022). The menhaden oil used here as an in-plate calibration standard is commercially available and recommended as a reference material for marine oils. The results of the SPV method here show high precision and moderately good agreement with standard gravimetric analysis; however, systematic differences between the two assays were evident and dependent on the taxa and tissue matrix of the samples. In lieu of commercially available fish oil standards specific to each sample of interest, we developed linear calibration models to correct SPV assay determined lipid content. This method was successful in accurately determining the lipid content of a variety of samples.

Developing and using linear calibration models to compare SPV assay results directly to those obtained from gravimetric analysis may not be necessary for all applications. The decision of whether to employ such calibration methods depends on the application and needs of the user. The SPV assay enables precise monitoring of total lipid content within a specific taxa and tissue type, with or without specific calibration. However, the calibration method described here may be desired when comparing SPV results to previously published literature values.

The SPV assay offers exceptional improvement on sample throughput when compared to traditional gravimetric analysis. One technician can reasonably run three microplate SPV assays per 8-h period. Each assay consists of 23 unknown samples, 5 calibration standards, 1 blank, 1 biological replicate sample for a quality control precision check, and 2 standard reference material samples for an accuracy check, all measured in triplicate. This allows for up to 69 samples to be analyzed per day, per technician, with finalized data available daily. Comparatively, we find that 15 samples analyzed in singlicate is the maximum reasonable number to process daily when performing gravimetric analysis. The use of specialized extraction instrumentation (i.e., an automated accelerated solvent extractor) allows for up to 20 samples to be extracted daily, but this method requires a second day of processing to perform saline washes and bulk evaporation of solvent on each individual sample. Summarily, we find that the SPV assay increases our throughput by approximately threefold to sevenfold. Not only does the efficiency of the accurate processing of samples increase, scientists are able to provide information to managers and stakeholders in a more timely manner, therefore improving management of these commercially and ecologically important marine resources.

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Conflict of Interest

None declared.