Oceanological and Hydrobiological Studies

International Journal of Oceanography and Hydrobiology

Volume 54, No. 3 September 2025
pages (160-170)

sciendo

ISSN 1730-413X
eISSN 1897-3191

Evaluation of three common methods of bulk lipid quantification in soft tissues of marine benthic invertebrates

by

Adam Sokołowski*, Kaja Czajkowska

DOI: **https://doi.org/10.26881/oahs-2025.1.16**
Category: **Original research paper**
Received: **April 08, 2025**
Accepted: **July 28, 2025**

University of Gdańsk, Faculty of Oceanography and Geography, Al. Piłsudskiego 46, 81-378 Gdynia, Poland

Abstract

Different analytical methods are used in biochemical laboratories to quantify lipid content in marine organisms, but the comparative utility of these protocols has not been assessed yet. This study evaluated three common methods of bulk lipid determination in the soft tissues of macrobenthic invertebrates in terms of their yield and labour intensity: the gravimetric method and two colourimetric methods of Marsh and Weinstein (1966) and Frings and Dunn (1970). Lipids were first extracted from three macrofaunal species, which were sampled in the coastal zone of the Gulf of Gdańsk (southern Baltic Sea): mussel *Mytilus trossulus*, shrimp *Crangon crangon* and polychaete *Hediste diversicolor*, using the Bligh and Dyer extraction technique (Bligh & Dyer, 1959). All tested methods proved accurate, precise and reproducible but differed in validation parameters and workload. The Marsh and Weinstein method provided an analytical procedure of the highest precision and recovery, the lowest limit of detection; however, it was laborious. The gravimetric method was the least labour-intensive but had the poorest validation parameters. The Frings and Dunn method produced more reliable results than the gravimetric method, but was the most time-consuming.

Key words: bulk lipid determination methods, efficiency, labour-effectiveness, marine benthic invertebrates

* Corresponding author: *adam.sokolowski@ug.edu.pl*

1. Introduction

Lipids (fats) are one of the main classes of biomolecules found in tissues of aquatic animals (Imbs et al., 2021; Koutsouveli et al., 2022; Lordan et al., 2017). The principal functions of lipids include energy storage, cell membrane construction, signalling, insulation and cell protection (Ridgway & McLeod, 2016). They form efficient storage depots, cellular barriers and extracellular and intracellular messenger molecules. Being largely composed of carbon and hydrogen, lipids are highly hydrophobic and can act as a solvent and absorption carrier for organic contaminants, providing efficient indication of pollutant bioaccumulation in marine ecosystems (Parrish, 2025). They are highly reduced non-polymeric chemical compounds and are, thus, essential fuels for oxidation (Parrish, 2013). Due to their high energy value (twice the specific energy, $kJ \cdot g^{-1}$, of proteins or carbohydrates), lipids are usually stored prior to periods of low food availability (e.g. in winter) and/or periods of enhanced energy expenditures, such as during reproduction or larval development, to serve as an important energy source. For example, Marshall et al. (2000) found a direct link between total egg production in cod and total lipid energy in the livers of mature females. Quantitative analyses of individually measured lipid classes or bulk lipid content in marine organisms have therefore become widely used in laboratory and field studies aiming at assessment of organisms' responses to changes of physic-chemical conditions, biochemical adaptations to different habitats, modifications of food alimentary value, in trophic ecology and bioaccumulation studies (Hines et al., 2007; Lee et al., 2006; Parrish, 2013; Peters et al., 2006; Prato et al., 2019). The content of lipid reserves along with energetic balance, metabolic rate and overall physiological performance have been shown to respond directly to alterations in the marine and coastal environments induced by human activity or resulting from natural processes (Acevedo-Whitehouse & Duffus, 2009). For example, significant differences in the content and composition of lipid compounds between two cryptic forms of the beadlet anemone (*Actinia equina*) allowed for defining two morphologically distinct subpopulations in the Black Sea: red and green forms that differed in adaptive capacity to water temperature (Stefanov et al., 1992). The morphological variation of the anemones was likely caused by different rates of metabolic pathways and physiological adaptation to various thermal conditions: the red form developed in warmer waters, and the green form inhabited cold, northern areas. Reinhardt and Van Vleet (1986) observed differences in the quantity of different lipid types (e.g. wax esters, triacylglycerols, and phospholipids) amongst fish and zooplankton living at different water depths, with species of higher bulk lipid content inhabiting water column below 230 m. A similar variation of bulk lipid content along a vertical profile was also reported by Friedrich and Hagen (1994) for the notothenioid fish from high-Antarctic waters (the Weddell Sea and the Lazarev Sea) with benthic species containing low lipid reserves (11.0%–11.9%) relative to pelagic species (40.0%–60.8%). Field studies on the copepod *Pseudocalanus acuspes* from the central Baltic Sea demonstrated apparent seasonal changes in the fatty acid profiles and lipid content in females. The highest wax ester content and individual total fatty acids and alcohols were measured in May, and the lowest in April and November, indicating that ciliates are the principal food source for copepods (Peters et al., 2006).

Although recent advances in chromatography and mass spectrometry have permitted the identification and quantitation of single lipid molecular species (lipidomic approach) and metabolic networking (Parrish, 2025), bulk lipid determination remains useful in ecophysiological and ecological studies. There are several different analytical methods for bulk lipid measurement in the soft tissues of marine organisms. The simplest and still broadly employed methods, which require basic analytical equipment and relatively little labour intensity, include the gravimetric method (Inouye & Lotufo, 2006) and the Marsh and Weinstein method (Marsh & Weinstein, 1966) after quantitative chloroform/methanol total lipid extraction following the protocol of Bligh and Dyer (1959). Advances in medical diagnostics and analytical technology allowed the development of the colourimetric method in 1970 (Frings & Dunn, 1970) and several methods based on thin-layer chromatography in the 80s and 90s (Gardner et al., 1985; Parrish, 1987). The gravimetric method (Lehtonen, 1996; Lehtonen & Andersin, 1998; Nielsen et al., 2005), the method described by Marsh and Weinstein (1966) and the colourimetric method of Barthel (1986) have been most commonly employed in biochemical studies of marine abiotic matrices (Dong-Young et al., 2022; Naeun et al., 2022; Pusceddu et al., 2009) and macrobenthic organisms (e.g. da Costa et al., 2013; da Silva-Castiglioni et al., 2010; Gora et al., 2018; Graham et al., 2024; Stelzer et al., 2021; Wołowicz et al., 2006) due to their relative ease of use in laboratory analytics. However, their utility for quantitative lipid determination has not been assessed yet, and evaluation of these methods for batch measurement of total lipid content in macrozoobenthic invertebrates provides an important and useful subject of comparative laboratory studies.

This study aimed to evaluate three methods of quantitative bulk lipid determination in the soft tissues of marine benthic invertebrates of different taxonomic groups in terms of their efficiency and labour intensity: the gravimetric, Marsh and Weinstein (1966) and Frings and Dunn (1970) methods. Such evaluation can serve as a baseline for the selection of analytical methods for marine animals originating from wild populations and aquaculture, and for interlaboratory comparisons.

2. Materials and methods

2.1. Sample collection and treatment

Comparative analyses of bulk lipid content were carried out using three macrobenthic invertebrate species, representing various taxonomical classes and of different lipid compositions and contents in their soft tissues: the common ragworm *Hediste diversicolor* (O.F. Müller 1776) (Polychaeta), the mussel *Mytilus trossulus* (Gould 1850) (Bivalvia) and the brown shrimp *Crangon crangon* (Linnaeus 1758) (Malacostraca). The animals were sampled in the shallow (water depth down to 9 m) coastal zone of the Gulf of Gdańsk (southern Baltic Sea; Fig. 1). The ragworms were collected using a van Veen grab (catch area 0.1 m^2) at one site in the mouth of the Wisła Śmiała River (VIST) (φ 54°21'50.90"N, λ 18°47'00.50"E) at depth of 9 m in May 2017. The mussels were collected with a standard benthic dredge at one coastal site Mechelinki (MECH) (φ 54°36'26,40" N λ 18°31'35.40" E) in May 2018, and the shrimps were sampled using a landing net from the seashore at sandy beach adjacent to Gdynia harbour (GDY) (φ 54°29'54.73" N λ 18°33'43.73" E) in April 2018.

Special care was taken to select individuals of a limited length range i.e. *H. diversicolor* (42–46 mm), *M. trossulus* (28–41 mm) and *C. crangon* (26–39 mm) as size has been demonstrated to sometimes affect biochemical constituents and physiological condition of marine benthic invertebrates (Sukhotin et al., 2003). Immediately after sampling, live individuals were transported to the laboratory in containers filled with seawater collected *in situ* and frozen in separate vials at –80°C. Before freezing, the soft tissues of each bivalve were removed. Frozen organisms and soft tissue were lyophilised individually in the STERIS LYOVAC GT2 (Germany) freeze dryer at room temperature and 0.03 mbar pressure for 48 hr and then homogenised manually in a porcelain mortar. The homogenises of all individuals belonging to one species were then combined to create three aggregate samples (pools), each containing 30 individuals of polychaetes, 26 individuals of bivalves and 33 individuals of crustaceans.

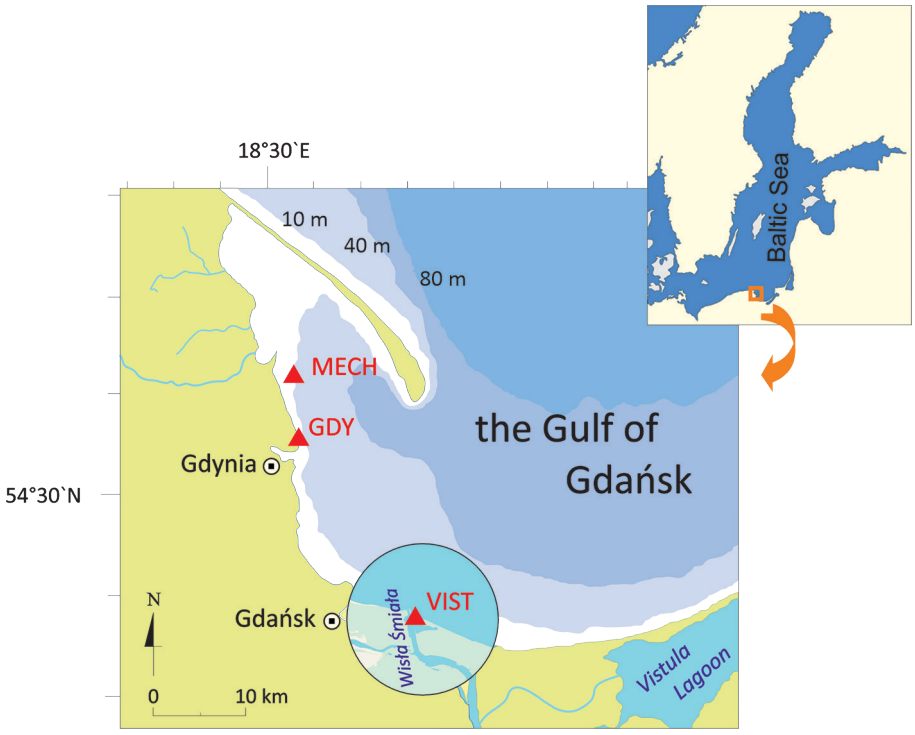

Figure 1

Location of sampling sites in the Gulf of Gdańsk.

2.2. Lipid extraction

Before the analysis, thick-walled SCHOTT DURAN (Germany) tubes were quenched several times in boiling and cold water (0°C). All test tubes and glass balls were washed with dishwashing liquid, rinsed with distilled water and dried at room temperature. To ensure that all residual lipids were removed, the tubes were rinsed with chloroform and dried in a laboratory air dryer at 45°C (Christian, 2009).

The same extraction method (Bligh & Dyer, 1959) was used for all lipid determination methods. A weight of 10 ± 0.1 mg of homogenised tissue (in 20 replicates for each species and each method, n = 20), 1 cm^3 of pure chloroform and 2 cm^3 of methanol (≥99.6%) were added to a thin-walled glass test tube, and the mixture was centrifuged for 10 min (3000 rpm, 10°C). The supernatant was then transferred into new thin-walled glass test tubes or thick-walled glass test tubes in the case of the Marsh and Weinstein method. The lipids remaining in the pellet were re-extracted using the same procedure, and the supernatant was collected again in the same test tube. Next, 4 cm^3 of distilled water was added to the supernatant derived from the double extraction. The two-phase mixture was separated by centrifugation for 10 min (3000 rpm, 10°C). The upper layer, which contained a mixture of methanol and distilled water, was removed carefully with an automatic pipette. Evaporation of the lower layer, which contained lipids dissolved in chloroform, was carried out in a laboratory air dryer at 45°C for 24 hr. After drying, white residue was visible at the bottom of the test tubes.

2.3. Lipid determination

2.3.1. Gravimetric method

Tubes with the extracted lipids were pre-weighted on a laboratory balance with a readability of 0.01 mg. Lipid content was determined using the following equation:

$$L = [(R - E)/M] \times 1000, \quad (1)$$

where R is the weight of the tube containing the residue, E is the weight of the empty tube before extraction and M is the weight of the homogenised dry tissue material from a single sample.

2.3.2. Marsh and Weinstein method

The original method developed by Marsh and Weinstein (1966) was modified to dissolve the entire residue at the bottom of the test tubes. The modification included the addition of 10 cm^3 of 95% sulphuric acid to the tubes with the residue instead of 2 cm^3 H_2SO_4 and heating at 200°C for 20 min instead of 15 min. After heating, 2 cm^3 of the solution was transferred to new thin-walled glass test tubes and the original procedure described by Marsh and Weinstein (1966) was then followed. In order to make a calibration curve, tripalmitate (SIGMA-ALDRICH® (Germany), ≥99%) was chosen from a list of standards as proposed in the original procedure. A stock standard solution (SSS) with a concentration of 750 µg · cm^{-3} was prepared by adding 15 mg of tripalmitate to 20 cm^3 of chloroform. Two series of standard solutions were prepared by diluting the tripalmitate stock solution with chloroform to obtain the following final concentrations: 0.0, 187.5, 375.0, 562.5 and 750.0 mg · cm^{-3}.

Based on the linear regression function between the concentrations of the standard solutions and their absorbance, which was measured at a wavelength of 360 nm, the lipid content in the samples was determined according to the following formula:

$$L = [Z/(5 \times M)] \times 1000, \quad (2)$$

where Z is the lipid content in the sample calculated using the linear regression function, and M is the tissue dry weight.

2.3.3. Frings and Dunn method

The procedure described by Frings and Dunn (1970) was modified because the original method was based on lipid determination in liquid serum, whilst powdered precipitate was used in this study. Serum-like liquid was made by dissolving the precipitate. The serum was prepared by adding 0.5 cm^3 of 99.8% ethanol to each tube containing the residue. 0.1 cm^3 of the solution was then transferred to new thin-walled glass tubes. Further steps were carried out according to the original procedure. To prepare a calibration curve, cholesterol was chosen from a list of standards proposed by Frings and Dunn (1970). A SSS with a concentration of 10 mg · cm^{-3} was prepared by adding 500 mg of cholesterol to 50 cm^3 of 99.8% ethanol. Two series of standard solutions were prepared by diluting the cholesterol stock solution with ethanol to obtain the following final concentrations: 0, 1, 2 and 4 mg · cm^{-3}.

Based on the linear regression function between the concentrations of the standard solutions and their absorbance, which was measured at a wavelength of 540 nm, the lipid content in the samples was determined according to the following formula:

$$L = [Z/(105 \times M)] \times 1000 \quad (3)$$

where Z is the lipid concentration in the measured sample, calculated using the linear regression function, and M is the tissue dry weight.

Data quality control was provided by a separate comparative study of 10 procedural blanks, which were analysed simultaneously according to the same procedures as the samples for each lipid determination method. In order to assess recovery (R), a method of standard addition of a known amount of analyte (1.9 ± 1.0 mg of tripalmitate) was used in five replicates for each method. The procedures of lipid extraction and lipid determination according to the analysed method were then followed.

2.4. Statistical and mathematical analysis

Routine quality control validation parameters and the time required for completing analysis (used here as a proxy of sample throughput) were determined for each method as factors that allowed evaluation of the lipid determination methods. In order to assess the efficiency of each method, precision (CD), limit of detection (LOD) and recovery (R) were calculated (Araujo, 2009) using the following formulas:

$$C_D = \frac{S_D}{\bar{L}} 100, \quad (4)$$

where S_D is the standard deviation (SD) of the average lipid content in a series of 20 replicates [%] and L is the arithmetic average lipid content in a series of 20 replicates [%],

$$LOD = \bar{b} + 3 \times S_{D\,blank}, \quad (5)$$

where $\bar{b}$ is the arithmetic average lipid content in a series of 10 blanks [%] and $S_{D\,blank}$ is the SD of the average lipid content in the blanks [%] and

$$R = \frac{(\overline{L_w} + C) - \overline{L_w}}{T} \times 100\%, \quad (6)$$

where C is a weight or concentration of tripalmitate [mg, mg · cm^{-3} and µg · cm^{-3} for the gravimetric, Marsh and Weinstein method and Frings and Dunn method, respectively], $\bar{L}_w$ is an average weight or concentration of lipids in a series of 20 replicates [mg, mg · cm^{-3}, µg · cm^{-3}] and T is the known weight or concentration of tripalmitate added to the tube [mg, mg · cm^{-3}, µg · cm^{-3}].

Labour intensity (workload) of each lipid determination method was calculated as the average time (rounded to 1 min) needed for performing the analysis of one series of samples ($n = 20$), preparation of two series of standard solutions and standard stock solutions and, in case of the Frings and Dunn method, also preparation of the phospho-vanillin solution.

The Shapiro–Wilk normality test was used to verify the distribution of data, and the Levene test was employed to check the homogeneity of variance. The significance of differences between variables for the main effects and interactions between them was estimated by analysis of variance (ANOVA), followed by Tukey's post-hoc test (α/n) when F was significant. The level of significance for all tests was $p < 0.05$. All statistical analyses were performed using Statistica 13.1 software (TIBCO Software Inc., San Ramon, USA).

3. Results

3.1. Lipid content determined by different methods

Soft tissue lipid content varied significantly amongst the invertebrate species and three analytical methods for *M. trossulus* and *C. crangon* (ANOVA; Tables 1 and 2). The lipid content in the tissues of *H. diversicolor* did not differ statistically amongst analytical methods. Tukey's post-hoc test (α/n) detected differences in the lipid content of mussel tissues between the gravimetric and Frings and Dunn methods ($p = 0.036$) and between paired species for all methods ($p < 0.001$).

The highest lipid contents in the mussels (mean ± standard error [SE]; 14.85 ± 2.95%, $n = 20$) and polychaetes (10.81 ± 2.36%, $n = 20$) were assayed with the gravimetric method and the lowest using the Frings and Dunn method (13.27 ± 1.20%,

Table 1

Mean lipid content (%) in soft tissues of marine benthic invertebrates from the southern Baltic Sea determined by three analytical methods of quantitative bulk lipid determination following the Bligh and Dyer extraction.

Analytical method	Species	Mean	SD	SE	*n*
Gravimetric	*Mytilus trossulus*	14.85	2.95	0.66	20
	Crangon crangon	6.37	2.08	0.49	18
	Hediste diversicolor	10.81	2.36	0.53	20
Marsh and Weinstein	*Mytilus trossulus*	13.79	0.55	0.13	19
	Crangon crangon	7.41	0.34	0.08	18
	Hediste diversicolor	10.44	0.65	0.15	19
Frings and Dunn	*Mytilus trossulus*	13.27	1.20	0.27	20
	Crangon crangon	7.38	1.04	0.23	20
	Hediste diversicolor	10.64	1.08	0.24	20

SD, standard deviation; SE, standard error; *n*, number of replicates.

Table 2

Results of one-way ANOVA of differences in bulk lipid content amongst macrobenthic invertebrate species analysed by different analytical methods (a) and amongst three analytical methods for the same species (b).

a)			
Analytical method	***F***	***p***	**df**
Gravimetric	54.380	**<0.001**	2
Marsh and Weinstein	670.220	**<0.001**	2
Frings and Dunn	142.186	**<0.001**	2
b)			
Species	***F***	***p***	**df**
Mytilus trossulus	3.441	**0.039**	2
Crangon crangon	3.544	**0.036**	2
Hediste diversicolor	0.276	0.759	2

ANOVA, analysis of variance.
Significant values are in bold.

$n = 20$ and $10.64 \pm 1.08\%$, $n = 20$, for *M. trossulus* and *H. diversicolor*, respectively; Table 1). The lowest lipid content in crustacean tissues ($6.37 \pm 2.08\%$, $n = 18$) was determined by the gravimetric method. The intermediate lipid contents in the invertebrate tissues, except *C. crangon*, were measured using the Marsh and Weinstein method. The lowest values of SE were recorded for the Marsh and Weinstein method, intermediate values for the Frings and Dunn method and the highest using the gravimetric method (Table 1).

3.2. Quality control validation parameters

The largest CDs in routine replicated assays for all analysed species were recorded for the data obtained using the gravimetric method, the lower CDs were calculated using the Frings and Dunn method, and the highest precision was obtained for the Marsh and Weinstein method (Table 3). The results for lipid content in the tissues of *M. trossulus* were the most accurate when using all analytical methods. For the gravimetric and Frings and Dunn methods, the least accurate results were obtained for lipid content in the crustacean tissues, whereas for the Marsh and Weinstein method, in the nereid tissues.

The lowest LOD for all species was obtained using the Marsh and Weinstein method, and the highest, except for the nereid tissues, using the gravimetric method (Table 3). The Frings and Dunn method provided intermediate LOD; exceptions were the nereid tissues for which the LOD value was highest.

Recovery ranged from 89.1% to 102.5% with the highest value obtained by the Marsh and Weinstein method, except for *C. crangon* tissues, for which *R* was 95.2% (Table 3). The lowest efficiency was described for the gravimetric method, except for *C. crangon* tissues, for which *R* was 101.7%.

3.3. Labour intensity

An assay of 20 samples using the gravimetric method took only about 45 min, whereas analysis of a series of 20 replicates consumed 1 hr 15 min and 2 hr when using the Marsh and Weinstein and Frings and Dunn method, respectively (Table 4). In addition, the latter two procedures required prior preparation of SSSs, which ran for 20 min and 35 min, respectively. The preparation of the phospho-vanillin solution by the Frings and Dunn method took an additional 20 min. The average total time needed to complete the analysis using the gravimetric, Marsh and Weinstein and Frings and Dunn methods was estimated at 45 min, 95 min and 175 min, respectively.

Table 3

Precision (CD, %), limit of detection (LOD, %) and recovery (R, %) for analyses of bulk lipid content in marine benthic invertebrates using the gravimetric, Marsh and Weinstein and Frings and Dunn methods

Analytical method	Species	CD	LOD	R
Gravimetric	*Mytilus trossulus*	19.85	1.12	89.06
	Crangon crangon	32.68	0.98	101.67
	Hediste diversicolor	21.88	0.11	97.30
Marsha and Weinstein	*Mytilus trossulus*	4.30	0.01	102.50
	Crangon crangon	4.56	0.03	95.17
	Hediste diversicolor	6.21	0.01	100.79
Frings and Dunn	*Mytilus trossulus*	9.05	0.27	95.16
	Crangon crangon	14.05	0.29	94.85
	Hediste diversicolor	10.12	0.32	98.16

Table 4

Time consumption (min) of analyses of 20 samples (20S), preparation of SSS and OR using three analytical methods of quantitative bulk lipid determination

Analytical method	Time consumption			
	20S	SSS	OR	Total
Gravimetric	45	0	0	45
Marsha and Weinstein	75	20	0	95
Frings and Dunn	120	35	20	175

OR, other reagents; SSS, stock standard solution.

4. Discussion

4.1. Bulk lipid content in benthic invertebrates

Bulk lipid content in soft tissues of marine macrobenthic fauna varies with environmental conditions (e.g. temperature, salinity, food quantity and its nutritional value) and biological factors (e.g. species, physiological state, age and sex) and increases before reproduction and during periods of low food availability (Lehtonen, 2004; Wiklund et al., 2009). For example, lipid content in tissues of the brown shrimp *C. crangon* collected along the coast of Great Britain between late April and early May (i.e. before the reproductive period) ranged from 18% to 20% of dry weight (Moore, 1976). According to Szaniawska (1983), the lipid level in tissues of the same species from the Gulf of Gdańsk (southern Baltic Sea) fell in 2.6%–13.7% depending on the season whilst Mika et al. (2012) showed that in spring total lipid content of the shrimp muscle tissue from the Baltic Sea was only 3.4%. The results obtained in this study (6.4%–7.4%) are within the range of values presented by Szaniawska (1983) but differ from the data of Mika et al. (2012), which results likely from different sample types analysed i.e. muscle tissue versus whole organism.

In the shallow coastal waters of the southern Baltic, lipid content in soft tissue of the mussel *M. trossulus* varies seasonally, increasing from March to April, when it reaches its maximum level (24%), remaining high even until June (Szaniawska, 1983; Wołowicz et al., 2006). The accumulation of lipid reserves in the bivalves in this period is induced by elevated food consumption after spring algal blooms to recover after winter starvation and to prepare for reproduction. Lipid content measured in this study is fairly low (13.3%–14.9%), which probably stems from the earlier reproduction of the bivalves in the year of sampling. The release of lipid-rich gametes into the external environment induces a substantial reduction in total lipid content in the mussels. According to Martínez-Pita et al. (2012), during the pre-reproduction period, females of *Mytilus galloprovincialis* may contain up to twice as much fat as males. However, its reproductive effort might be so high that it can lead to a decrease in the physiological state and, in the extreme case, to the death of an organism (Wołowicz et al., 2006). Another reason for such a low lipid content in the mussels assayed in this study is the salinity variations in the overlying bottom zone in the shallow area where the mussels were collected (Sokołowski et al., 2017). In a laboratory experiment described by Lasota et al. (2018), variations in salinity resulted in a significant decrease in bulk lipid content in the soft-shell clam *Mya arenaria* and the Baltic clam *Macoma balthica* from the southern Baltic Sea. Salinity-induced osmotic shock can cause cell breakdown and lipid peroxidation (Abele & Puntarulo, 2004) or a change in the lipid-protein ratio (Sokołowski et al., 2003).

Empirical data on the lipid content in tissues of *H. diversicolor* from the Baltic Sea are scarce. The nereids from the coast of Great Britain have been reported to store 31.5% and 14% lipid reserves in winter and summer, respectively (García-Alonso et al., 2008). According to Wang et al. (2019), the bulk lipid content of *H. diversicolor* from the Norwegian coast ranged from 13% to 16% depending on its diet: similar lipid levels were noted in individuals with a diet based on microalgae and fish bits whilst lower values were observed for individuals fed with waste from juvenile salmonids and a mixture of fish waste and microalgae. Anglade et al. (2023) reported that the bulk lipid content was between 12.3% and 19.6% of the polychaetes reared on different types of aquaculture sludge in the Trondheimsfjord in Buvika, Norway. In this study, the lipid content was lower than that in the nereids from the Norwegian coast and ranged from 10.4% to 10.8%.

4.2. Comparison of validation parameters

The quality of the results of biochemical analyses is affected by several factors e.g. diligence of sampling, sample pretreatment and laboratory procedures, analytical equipment and quality of reagents. In order to manage the quality of the analytical method, control analysis and analysis of procedural blank samples are routinely performed (Skoog et al., 2013). In this study, standard validation parameters were calculated for each method, including precision, LOD and recovery. Tukey's post-hoc tests showed no significant differences in the bulk lipid content of a given species between paired analytical methods; the exception was *M. trossulus,* which demonstrated a significant difference between the results obtained using the gravimetric and Frings and Dunn methods. The observed differences were likely caused by a random experimental error resulting from e.g. accidental sample contamination, loss of a part of the weighed sample, instability of analytical equipment or analyst fatigue (Taylor, 2022). An increasing number of replicates usually offers a compromise leading to a reduction in random error but not to its total elimination. The lack of significant differences between the methods tested indicates that each method can be used successfully in analyses of both low-lipid and lipid-rich tissues of marine macrobenthic invertebrates. The calculated SD and R of each method fall in most cases in a satisfactory range of 0%–8% and 90%–100%, respectively

(Taverniers et al., 2004). All methods used in this study proved to provide accurate and highly reproducible quantitative measures of total lipid content in the whole soft body of benthic invertebrates.

The Marsh and Weinstein method offers the most accurate analytical protocol for lipid quantification (the highest precision, low LOD, SD and SE) regardless of the analysed animal tissue. The obtained recovery (mean for three invertebrate species, 99.5%) was similar to that reported by Reisenbichler and Bailey (1991) (95%–100% depending on the analysed standard), who indicated that the possible difference in *R* amongst different standards might have been caused by the modification of the lipid extraction method. When considering all validation parameters, the best overall performance of the analyses was documented for the Marsh and Weinstein method, moderate for the Frings and Dunn method and worst for the gravimetric method. In addition, the recovery of the Frings and Dunn method fell into a wider range than that obtained using the Marsh and Weinstein method, but was narrower than that calculated for the gravimetric method. The exception was lipid analyses of crustacean tissues for which *R* varied from 94.9% to 101.7%. In the original study of Frings and Dunn (1970), precision in repeated assays of 60 randomly selected serum samples was 3.5% and Jacobs and Henry (1962) reported a CD of 9.6% for the gravimetric method. In terms of validation parameters, the Marsh and Weinstein method can be, therefore, recommended for quantitative total lipid determination in macrobenthic invertebrates of various fat content.

4.3. Comparison of time consumption

Besides high precision and accuracy, consideration should also be given to another important design criterion of the quantitative lipid determination method: workload. Estimates of the workload required to complete laboratory analysis can vary widely with analyst experience and practical skills, ease of operation of analytical equipment, organisation of laboratory workspace and complexity of the analytical procedure. According to Harvey (2000), depending on the technical advancement of the analytical equipment, the time spent on staff training should also be considered working time. Several steps in analytical procedures have, however, fixed times (e.g. centrifuging, drying, etc.), which do not vary with analyst or laboratory conditions. In the case of a single run of many series of samples, the most optimal in terms of time consumption are continuous and/or automated methods (Webster et al., 2005). However, these methods were not used in this study due to the small number of samples (n = 18–20).

The results of labour intensity estimates showed that bulk lipid content determination using the gravimetric method is the most time-effective. Two other methods were more complex and included several additional phases such as dosing reagents, sample incubation and optical density measurements, which all extended the total time required to complete the analysis. Larger quantities of the SSSs (and the phospho-vanillin solution in the case of the Frings and Dunn method) can be, however, used in subsequent analyses to reduce the average time for one series of samples. González et al. (2004) indicated that maintaining a balance between time consumption and reliability of the obtained results can be done by selecting the appropriate number of quality control analyses and choosing less or more accurate analytical methods. The gravimetric method is a fast alternative to colourimetric methods but has the lowest precision and the highest LOD values. The choice of analytical method requires, therefore, careful consideration of different criteria.

5. Conclusions

Although all methods of bulk lipid quantification in the whole body of marine benthic invertebrates proved accurate and highly reproducible, comparative studies unravelled important differences in yield and efficiency. The gravimetric method appeared most advantageous in terms of labour intensity but was the worst-performing when considering the validation parameters. The Marsh and Weinstein method provided an analytical procedure with the highest precision and recovery, and the lowest LOD. It was more time-consuming than the gravimetric method but less labour-intensive than the Frings and Dunn method. The Frings and Dunn method produced more reliable results than the gravimetric method, but completing the analysis required the most time.

References

Abele, D., & Puntarulo, S. (2004). Formation of reactive species and induction of antioxidant defence systems in polar and temperate marine invertebrates and fish. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 138*(4), 405–415. https://doi.org/10.1016/j.cbpb.2004.05.013

Acevedo-Whitehouse, K., & Duffus, A. L. J. (2009). Effects of environmental change on wildlife health. *Philosophical Transactions of the Royal Society B: Biological Sciences, 364*(1534), 3429–3438. https://doi.org/10.1098/rstb.2009.0128

Anglade, I., Dahl, T. H., Kristensen, B. S. B., Hagemann, A., Malzahn, A. M., & Reitan, K. I. (2023). Biochemical composition of *Hediste diversicolor* (OF Müller, 1776) (Annelida: Nereidae) reared on different types of aquaculture sludge. *Frontiers in Marine Science, 10*, 1197052. https://doi.org/10.3389/fmars.2023.1197052

Araujo, P. (2009). Key aspects of analytical method validation and linearity evaluation. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 877*(23), 2224–2234. https://doi.org/10.1016/j.jchromb.2008.09.030

Barthel, D. (1986). On the ecophysiology of the sponge *Halichondria panicea* in Kiel Bight. I. Substrate specificity, growth and reproduction. *Marine Ecology Progress Series, 32*(1/2), 291–298. https://doi.org/10.3354/meps032291

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology, 37*(8), 911–917. https://doi.org/10.1139/o59-099

Christian, G. D. (2009). Herramientas y operaciones básicas de la química analítica. In G. D. Christian (Ed.), *Química Analítica* (pp. 20–64). McGraw-Hill Education. (in Spanish).

da Costa, F., Nóvoa, S., Ojea, J., & Martínez-Patiño, D. (2013). Biochemical and fatty acid dynamics during larval development in the razor clam *Ensis arcuatus* (Bivalvia: Pharidae). *Aquaculture Research, 44*(12), 1926–1939. https://doi.org/10.1111/j.1365-2109.2012.03197.x

da Silva-Castiglioni, D., Oliveira, G. T., & Buckup, L. (2010). Metabolic responses of *Parastacus defossus* and *Parastacus brasiliensis* (Crustacea, Decapoda, Parastacidae) to hypoxia. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 156*(4), 436–444. https://doi.org/10.1016/j.cbpa.2010.03.025

Dong-Young, L., Jung Hyun, K., Tae Hee, P., Hee Yoon, K., Dongyoung, K., Sungmin, H., & Hyun Je, P. (2022). Seasonal variations in biochemical and stable isotope compositions of particulate organic matter in two contrasting temperate coastal lagoons of Korea. *Frontiers in Marine Science, 9*, 953648. https://doi.org/10.3389/fmars.2022.953648

Friedrich, C., & Hagen, W. (1994). Lipid contents of five species of notothenioid fish from high-Antarctic waters and ecological implications. *Polar Biology, 14*, 359–369. https://doi.org/10.1007/BF00240256

Frings, C. S., & Dunn, R. T. (1970). A colorimetric method for determination of total serum lipids based on the sulfo-phospho-vanillin reaction. *American Journal of Clinical Pathology, 53*(1), 89–91. https://doi.org/10.1093/ajcp/53.1.89

García-Alonso, J., Müller, C. T., & Hardege, J. D. (2008). Influence of food regimes and seasonality on fatty acid composition in the ragworm. *Aquatic Biology, 4*(1), 7–13. https://doi.org/10.3354/ab00090

Gardner, W. S., Frez, W. A., Cichocki, E. A., & Parrish, C. C. (1985). Micromethod for lipids in aquatic invertebrates. *Limnology and Oceanography, 30*(5), 1099–1105. https://doi.org/10.4319/lo.1985.30.5.1099

González, F. J. E., Hernández Torres, M. E., Frenich, A. G., Martínez Vidal, J. L., & Campaña, A. M. G. (2004). Internal quality-control and laboratory-management tools for enhancing the stability of results in pesticide multi-residue analytical methods. *Trends in Analytical Chemistry, 23*(5), 361–369. https://doi.org/10.1016/S0165-9936(04)00520-5

Gora, A., Jayasankar, V., Rehman, S., Kizhakudan, J. K., Laxmilatha, P., & Vijayagopal, P. (2018). Biochemical responses of juvenile rock spiny lobster *Panulirus homarus* under different feeding regimes. *Journal of Applied Animal Research, 46*(1), 1462–1468. https://doi.org/10.1080/09712119.2018.1533475

Graham, Z. A., de Jesus Florentino, J., Smithers, S. P., Menezes, J. C. T., de Carvalho, J. E., & Palaoro, A. V. (2024). Claw coloration in the fiddler crab *Leptuca uruguayensis* has no correlation with male quality. *Current Zoology, 71*(1), 109–123. https://doi.org/10.1093/cz/zoae035

Harvey, D. (2000). The language of analytical chemistry. In D. Harvey (Ed.), *Modern analytical chemistry* (pp. 35–52). McGraw-Hill Higher Education.

Hines, A., Yeung, W. H., Craft, J., Brown, M., Kennedy, J., Bignell, J., Stentiford, G. D., & Viant, M. R. (2007). Comparison of histological, genetic, metabolomics, and lipid-based methods for sex determination in marine mussels. *Analytical Biochemistry, 369*(2), 175–186. https://doi.org/10.1016/j.ab.2007.06.008

Imbs, A. B., Ermolenko, E. V., Grigorchuk, V. P., Sikorskaya, T. V., & Velansky, P. V. (2021). Current progress in lipidomics of marine invertebrates. *Marine Drugs, 19*(12), 660. https://doi.org/10.3390/md19120660

Inouye, L. S., & Lotufo, G. R. (2006). Comparison of macro-gravimetric and micro-colorimetric lipid determination methods. *Talanta, 70*(3), 584–587. https://doi.org/10.1016/j.talanta.2006.01.024

Jacobs, S. L., & Henry, R. J. (1962). Studies on the gravimetric determination of serum lipids. *Clinica Chimica Acta, 7*(2), 270–276. https://doi.org/10.1016/0009-8981(62)90020-7

Koutsouveli, V., Balgoma, D., Checa, A., Hedeland, M., Riesgo, A., & Cárdenas, P. (2022). Oogenesis and lipid metabolism in the deep-sea sponge *Phakellia ventilabrum* (Linnaeus, 1767). *Scientific Reports, 12*(1), 6317. https://doi.org/10.1038/s41598-022-10058-6

Lasota, R., Sokołowski, A., Smolarz, K., Sromek, L., & Dublinowska, M. (2018). Multimarker response to salinity stress in two estuarine bivalves of different genetic

diversity: *Mya arenaria* and *Limecola balthica* from the Gulf of Gdańsk (southern Baltic Sea). *Invertebrate Biology, 137*(3), 250–263. https://doi.org/10.1111/ivb.12224

Lee, R. F., Hagen, W., & Kattner, G. (2006). Lipid storage in marine zooplankton. *Marine Ecology Progress Series, 307*, 273–306. https://doi.org/10.3354/meps307273

Lehtonen, K. K. (1996). Ecophysiology of the benthic amphipod *Monoporeia affinis* in an open-sea area of the northern Baltic Sea: Seasonal variations in body composition, with bioenergetic considerations. *Marine Ecology Progress Series, 143*, 87–98. https://doi.org/10.3354/meps143087

Lehtonen, K. K. (2004). Seasonal variations in the physiological condition of the benthic amphipods *Monoporeia affinis* and *Pontoporeia femorata* in the Gulf of Riga (Baltic Sea). *Aquatic Ecology, 38*(3), 441–456. https://doi.org/10.1023/B:AECO.0000035165.97619.78

Lehtonen, K. K., & Andersin, A. B. (1998). Population dynamics, response to sedimentation and role in benthic metabolism of the amphipod *Monoporeia affinis* in an open-sea area of the northern Baltic Sea. *Marine Ecology Progress Series, 168*, 71–85. https://doi.org/10.3354/meps168071

Lordan, R., Tsoupras, A., & Zabetakis, I. (2017). Phospholipids of animal and marine origin: Structure, function, and anti-inflammatory properties. *Molecules, 22*(11), 1964. https://doi.org/10.3390/molecules22111964

Marshall, C. T., Yaragina, N. A., Adlandsvik, B., & Dolgov, A. V. (2000). Reconstructing the stock-recruit relationship for Northeast Arctic cod using a bioenergetic index of reproductive potential. *Canadian Journal of Fisheries and Aquatic Sciences, 57* (12), 2433–2442. https://doi.org/10.1139/f00-222

Marsh, J. B., & Weinstein, D. B. (1966). Simple charring method for determination of lipids. *Journal of Lipid Research, 7*(4), 574–576. https://doi.org/10.1016/s0022-2275(20)39274-9

Martínez-Pita, I., Sánchez-Lazo, C., Ruíz-Jarabo, I., Herrera, M., & Mancera, J. M. (2012). Biochemical composition, lipid classes, fatty acids and sexual hormones in the mussel *Mytilus galloprovincialis* from cultivated populations in south Spain. *Aquaculture, 35*(8–359), 274–283. https://doi.org/10.1016/j.aquaculture.2012.06.003

Mika, A., Golbiowski, M., Skorkowski, E. F., & Stepnowski, P. (2012). Composition of fatty acids and sterols composition in brown shrimp *Crangon crangon* and herring *Clupea harengus* membras from the Baltic Sea. *Oceanological and Hydrobiological Studies, 41*(2), 57–64. https://doi.org/10.2478/s13545-012-0017-z

Moore, J. W. (1976). The proximate and fatty acid composition of some estuarine crustaceans. *Estuarine and Coastal Marine Science, 4*(2), 215–224. https://doi.org/10.1016/0302-3524(76)90043-8

Naeun, J., Seok-Hyun, Y., HuiTae, J., Hyo Keun, J., Yejin, K., Sanghoon, O., Jaesoon, K., Kwanwoo, K., Jae Joong, K., & Sang Heon, L. (2022). Seasonal variations in biochemical (biomolecular and amino acid) compositions and protein quality of particulate organic matter in the Southwestern East/Japan Sea. *Frontiers in Marine Science, 9*, 979137. https://doi.org/10.3389/fmars.2022.979137

Nielsen, D., Hyldig, G., Nielsen, J., & Nielsen, H. H. (2005). Lipid content in herring (*Clupea harengus* L.) - Influence of biological factors and comparison of different methods of analyses: Solvent extraction, Fatmeter, NIR and NMR. *LWT - Food Science and Technology, 38*(5), 537–548. https://doi.org/10.1016/j.lwt.2004.07.010

Parrish, C. C. (1987). Separation of aquatic lipid classes by Chromarod thin-layer chromatography with measurement by Iatroscan flame ionization detection. *Canadian Journal of Fisheries and Aquatic Sciences, 44*(4), 722–731. https://doi.org/10.1139/f87-087

Parrish, C. C. (2013). Lipids in marine ecosystems. *International Scholarly Research Notices, 604045*, 16. https://doi.org/10.5402/2013/604045

Parrish, C. C. (2025). Production, transport, fate and effects of lipids in the marine environment. *Marine Drugs, 23*(2), 52. https://doi.org/10.3390/md23020052

Peters, J., Renz, J., Van Beusekom, J., Boersma, M., & Hagen, W. (2006). Trophodynamics and seasonal cycle of the copepod *Pseudocalanus acuspes* in the Central Baltic Sea (Bornholm Basin): Evidence from lipid composition. *Marine Biology, 149*(6), 1417–1429. https://doi.org/10.1007/s00227-006-0290-8

Prato, E., Biandolino, F., Parlapiano, I., Giandomenico, S., Denti, G., Calò, M., Spada, L., & Di Leo, A. (2019). Proximate, fatty acids and metals in edible marine bivalves from Italian market: Beneficial and risk for consumers health. *Science of the Total Environment, 648*(6), 153–163. https://doi.org/10.1016/j.scitotenv.2018.07.382

Pusceddu, A., Dell'Anno, A., Fabiano, M., & Danovaro, R. (2009). Quantity and bioavailability of sediment organic matter as signatures of benthic trophic status. *Marine Ecology Progress Series, 5*, 41–52. https://doi.org/10.3354/meps07735

Reinhardt, S. B., & Van Vleet, E. S. (1986). Lipid composition of twenty-two species of Antarctic midwater zooplankton and fish. *Marine Biology, 91*, 149–159. https://doi.org/10.1007/BF00569431

Reisenbichler, K. R., & Bailey, T. G. (1991). Microextraction of total lipid from mesopelagic animals. *Deep Sea Research Part A. Oceanographic Research Papers, 38*(10), 1331–1339. https://doi.org/10.1016/0198-0149(91)90030-J

Ridgway, N., & McLeod, R. (2016). *Biochemistry of lipids, lipoproteins and membranes* (6th ed.). Elsevier.

Skoog, D. A., West, D. M., Holler, F. J., & Crouch, S. R. (2013). Random errors in chemical analysis. In D. A. Skoog, D. M. West, F. J. Holler, & S. R. Crouch (Eds.), *Fundamental of analytical chemistry* (pp. 93–122). Cengage Learning.

Sokołowski, A., Wołowicz, M., & Hummel, H. (2003). Free amino acids in the clam *Macoma balthica* L. (Bivalvia, Mollusca) from brackish waters of the southern Baltic Sea. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative*

Physiology, 134(3), 579–592. https://doi.org/10.1016/S1095-6433(02)00360-4

Sokołowski, A., Ziółkowska, M., Balazy, P., Plichta, I., Kukliński, P., & Mudrak-Cegiołka, S. (2017). Recruitment pattern of benthic fauna on artificial substrates in brackish low-diversity system (the Baltic Sea). *Hydrobiologia, 784*(1), 125–141. https://doi.org/10.1007/s10750-016-2862-z

Stefanov, K. L., Christie, W. W., Brechany, E. Y., Popov, S. S., & Andreev, S. N. (1992). Lipid composition of the red and green forms of *Actina equinia* from the black sea. *Comparative Biochemistry and Physiology. Part B, Biochemistry, 103*(3), 687–690. https://doi.org/10.1016/0305-0491(92)90391-4

Stelzer, P. S., Mazzuco, A. C. A., Gomes, L. E., Martins, J., Netto, S., & Bernardino, A. F. (2021). Taxonomic and functional diversity of benthic macrofauna associated with rhodolith beds in SE Brazil. *PeerJ, 9*, e11903. https://doi.org/10.7717/peerj.11903

Sukhotin, A. A., Lajus, D. L., & Lesin, P. A. (2003). Influence of age and size on pumping activity and stress resistance in the marine bivalve *Mytilus edulis* L. *The Journal of Experimental Marine Biology and Ecology, 284*(1-2), 129–144. https://doi.org/10.1016/S0022-0981(02)00497-5

Szaniawska, A. (1983). Seasonal changes in energy content of *Crangon crangon* L. (Crustacea, Decapoda). *Polish Archives of Hydrobiology, 30*(1), 45–56.

Taverniers, I., De Loose, M., & Van Bockstaele, E. (2004). Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance. *Trends in Analytical Chemistry, 23*(8), 535–552. https://doi.org/10.1016/j.trac.2004.04.001

Taylor, J. (2022). An introduction to error analysis: The study of uncertainties in physical measurements (3rd. ed.). University Science Books.

Wang, H., Seekamp, I., Malzahn, A., Hagemann, A., Carvajal, A. K., Slizyte, R., Standal, I. B., Handå, A., & Reitan, K. I. (2019). Growth and nutritional composition of the polychaete *Hediste diversicolor* (OF Müller, 1776) cultivated on waste from land-based salmon SMOLT aquaculture. *Aquaculture, 502*(15), 232–241. https://doi.org/10.1016/j.aquaculture.2018.12.047

Webster, G. K., Kotts, L., & Maloney, T. D. (2005). Considerations when implementing automated methods into GXP laboratories. *Journal of Laboratory Automation, 10*(3), 182–191. https://doi.org/10.1016/j.jala.2005.03.003

Wiklund, A. K. E., Dahlgren, K., Sundelin, B., & Andersson, A. (2009). Effects of warming and shifts of pelagic food web structure on benthic productivity in a coastal marine system. *Marine Ecology Progress Series, 396*, 13–25. https://doi.org/10.3354/meps08290

Wołowicz, M., Sokołowski, A., Bawazir, A. S., & Lasota, R. (2006). Effect of eutrophication on the distribution and ecophysiology of the mussel *Mytilus trossulus* (Bivalvia) in southern Baltic Sea (the Gulf of Gdańsk). *Limnology and Oceanography, 51*(1, part 2), 580–590. https://doi.org/10.4319/lo.2006.51.1_part_2.0580