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On the determination of zooplankton lipid content and the occurrence of gelatinous copepods

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Abstract. Wax esters extracted and purified from the copepod *Calanus pacificus* elicit a different response in quantitative thin-layer chromatography–flame ionization detection (TLC–FID) than simpler, saturated wax esters available from commercial sources and commonly used for instrument calibration. Failure to use native copepod wax esters in calibration procedures results in underestimates of 18–32% in wax ester content, depending on the mass of lipid loaded on chromarods. In contrast to wax esters, triacylglycerols from the copepod *Eucalanus californicus*, as well as phospholipids from *E. californicus* and *C. pacificus*, elicited no significant difference in detector response in comparisons with commercially available standard compounds. Extraction of copepod lipids in solutions of chloroform:methanol results in the release of non-lipid material. Therefore, indirect gravimetric measures of lipid content that rely on calculation of lipid mass by difference after ‘de-fating’ copepods greatly overestimate the true lipid content. Lipid-specific assays with appropriate calibration standards are essential for the accurate quantification of the lipid content of planktonic animals. The water content differs appreciably among co-occurring species of copepods, averaging 82–84% in *Rhinocalanus nasutus*, *C. pacificus* and *Metridia pacifica*, but 93% of body mass in *E. californicus*. The occurrence of gelatinous tissues in some planktonic copepod species underscores the need to take account of structural and life history diversity among taxa. Care must be taken in the selection of the independent variable used to scale zooplankton lipid content and other structural and metabolic relationships.

Introduction

Many life history traits of zooplankton are influenced by investments in depot lipids. For example, populations that experience seasonally interrupted production cycles commonly mitigate long intervals of food shortage by synthesizing lipid reserves, entering a state of dormancy, and reducing rates of metabolism (Lee, 1974; Ohman *et al.*, 1989; Arashkevich *et al.*, 1996). Subsequent development to the adult stage (Sargent and Henderson, 1986) and the onset of oögenesis (Hagen and Schnack-Schiel, 1996) can be affected by the presence of lipid stores that remain at the end of the dormant period. In cladocerans, maternal lipids invested in embryos affect the survivorship of neonates (Goulden and Place, 1993). In meroplankton studies, discussions of the life history traits of benthic invertebrate larvae have long centered on the trade-offs between investment of reserves (principally lipids) in lecithotrophic larvae and the release of planktotrophic larvae (e.g. Levin and Huggett, 1990). In other lipid studies, the analysis of spatial variations in individual lipid content has provided insights into spatial differences in nutritional status and the animals’ recent feeding history (Håkanson, 1987; Smith and Lane, 1991). Specific fatty acids and other lipid compounds have been used as biomarkers to infer dietary pathways (Graeve *et al.*, 1994; Ederington *et al.*, 1995). To address all such issues properly, accurate measures of lipid content are essential.

The introduction of quantitative thin-layer chromatography–flame ionization detection (TLC–FID; Ackman, 1981) made available a useful analytical approach for the analysis of total lipids and lipid class composition in plankton studies. The commercial instrument for TLC–FID, known as the Iatroscan, combines the sensitivity of TLC with the quantitative capability of FID. This method has the advantage that there is no need to fractionate or derivatize lipids prior to analysis. It is sensitive to ~0.2 µg of lipid and presents the capability to analyze lipids from as little as a single large zooplankton. Classes of storage lipids can be differentiated from structural lipids. Rapid analysis of 10 separate chromatographic units on silica-coated chromarods facilitates replication.

As with all analytical techniques, careful calibration of the TLC–FID with authentic standards is essential. It is well recognized that native lipids isolated and purified from the organisms being analyzed are the best standards for calibration purposes. Because lipids from natural marine organisms can be complex in structure, often with a high degree of unsaturation and elongate fatty acid chains (Sargent and Henderson, 1986; Kattner and Hagen, 1995), this principle might be expected to be of particular importance when quantifying marine lipids. However, the isolation and purification of native lipids is time consuming and requires substantial amounts of tissue, partially negating one of the advantages of TLC–FID. Furthermore, the polyunsaturated fatty acids typical of marine lipids are susceptible to enzymatic hydrolysis and peroxidation over time, making them undesirable as stable, standard reference compounds. This has led most investigators to use commercially available lipids for calibration purposes.

The results presented in Miller and Morgan (1996) and in the present paper show that, in contrast to the assumptions of early investigators (including the present author), the response of the Iatroscan FID to wax esters isolated from the marine copepod genus *Calanus* can be substantially different than the response to other wax ester compounds that have commonly been used as calibration standards. The consequence is an underestimate of the true wax ester content of the copepods and a lesser underestimate of total lipid content, unless native copepod wax esters are used for calibration. The present paper shows that, unlike the result with wax esters, there is no evidence for different FID responses to triacylglycerols or phospholipids isolated from copepods when compared with pure compounds available commercially.

At times, it is not necessary or feasible to analyze the composition of different lipid classes by TLC–FID or other techniques and only the total lipid content of animals is of interest. Proper methods for the complete extraction and separation of lipids include tissue homogenization followed by the two-phase technique of Blich and Dyer (1959), the method of Folch *et al.* (1957) and perhaps related procedures (e.g. Gardner *et al.*, 1985). Extraction in chloroform:methanol without tissue homogenization will extract virtually all lipids from marine copepods, except for a small fraction (4–6%) of membrane-associated phospholipids (Ohman, 1988). This technique has the advantage that tissues are not disrupted and thus the copepod husks remaining after extraction in organic solvents are free of lipids, but otherwise appear relatively intact. It has been assumed (e.g. Håkanson, 1987; Ohman and Runge, 1994) that this ‘de-fatted’ copepod residue can be

added to the lipid mass to reconstruct the total dry mass of copepods accurately. In another variation of the de-fattening approach, various investigators (e.g. Smith and Lane, 1991; Miller, 1993) have sought to estimate total lipid as a fraction of dry mass by gravimetric measure of dry mass before and after removal of lipids by extraction with organic solvents. This approach assumes that all lipids are extracted and that only lipids are extracted. The present results show that extraction in chloroform:methanol removes more than the lipid material (cf. Hopkins *et al.*, 1984) and therefore the de-fattening approach results in substantial overestimates of total lipid content.

For the purposes of comparing lipid content among or within taxa, the lipid content of zooplankton is commonly scaled to total body mass. Most commonly, this is measured as dry tissue mass rather than wet mass, if techniques for analysis of constituents such as nitrogen (N), carbon (C) or protein are not available. Wet mass is analytically more difficult to measure than dry mass (Beers, 1976; Power *et al.*, 1991) and more prone to artifact. However, important evolutionary advantages may accrue to taxa that have a high water content (Alldredge and Madin, 1982) and indirect results suggest that even within the marine calanoid copepods, considerable differences may exist among genera in water content (Flint *et al.*, 1991). The scaling of lipid content and other organic constituents, as well as rate terms such as respiration, excretion and egestion, can differ markedly if the mass term in the denominator includes or excludes water (Childress and Nygaard, 1974; Flint *et al.*, 1991). Hence, we also evaluated the water content of four species of copepods from the California Current System.

Method

All copepods used in these experiments were collected in the upper 500 m of the California Current System with nets of mesh size 333 or 505 μm and frozen promptly at sea in liquid nitrogen after removal of excess water. They were thawed carefully in a shore-based laboratory, rinsed briefly in Milli-Q water, and analyzed for lipid content or mass. Previous results have shown that there is no significant difference between the dry mass or lipid content of fresh and frozen copepods when they are properly frozen and thawed (Ohman, 1996). Fresh analytical grade reagents were used for lipid extractions and analyses, and lipids were always maintained under nitrogen.

Lipid fractionation

To compare the response of latroscan TLC-FID to purified copepod lipids with that to commonly available standard compounds, lipids were extracted from 71–309 individuals of *Calanus pacificus* or *Eucalanus californicus* [either adult females or copepodid stage V (CV)]. Extraction was by homogenization and phase separation according to Bligh and Dyer (1959), except for *E. californicus* CV which were extracted by standing in chloroform:methanol (2:1, v/v). Individual lipid classes were then separated by silicic acid chromatography (after Nevenzal *et al.*, 1965; Kates, 1986) in a 6 ml column of silica gel (70–230 mesh)

pre-dried at 120°C. Copepod lipids were applied to the column in hexane and lipid classes eluted sequentially as shown in Table I. This solvent system successfully separated pure wax esters from *C.pacificus* and *E.californicus* (in fraction 2), but *E.californicus* triacylglycerols (TAGs) eluted in both fractions 3 (in a pure state) and 4 (co-eluted with sterols). Hence, the TAGs from fraction 4 were separated from sterols in a second column fractionation in which TAGs were eluted in 10% diethyl ether in hexane and sterols in 100% diethyl ether. Eluted lipids were dried in a stream of N₂ and weighed on an electrobalance. The purity of the separations was confirmed by TLC-FID and independently by plate TLC with colorimetric stains (Kates, 1986). Silicic acid columns were reconditioned by the application of 8 vols of methanol, followed sequentially by acetone, ether, and hexane. In this manner, wax esters and phospholipids were purified from *C.pacificus*, and TAGs and phospholipids from *E.californicus*.

Comparative lipid determination techniques

The total lipid content of adult female *E.californicus* and CV *C.pacificus* was determined by three methods: (i) removal of chloroform–methanol-soluble material from dry copepod tissues followed by gravimetric measure of the residue; (ii) removal of chloroform–methanol-soluble material from wet copepod tissues and gravimetric measure of the residue; (iii) removal of chloroform–methanol-soluble material from wet copepod tissues and TLC–FID analysis of the extract. First, 13–18 lots of three animals each were dipped in Milli-Q water and placed on dry, tared aluminum boats. These were dried for 24 h at 55°C for measurement of total dry mass on a Cahn 29 electrobalance. Lipid method (i), dry tissue extraction: 2:1 chloroform:methanol (v:v) was added to each boat containing dried copepods, allowed to extract lipids, then the solvent pipetted out. This was repeated with fresh solvent 10–12 times over 4 h. The lipid-free copepods were then dried overnight at 55°C and re-weighed to obtain ‘de-fatted’ copepod dry mass. Total lipid was taken as the difference between total dry mass and de-fatted dry mass. Lipid method (ii), wet tissue extraction: copepods from the same field sample used for total dry mass were dipped in Milli-Q water and added in lots of 6–8 individuals each to vials containing 2:1

Table I. Solvent systems used to separate copepod lipid classes by silicic acid column chromatography. Column volumes are expressed as multiples of the packed column bed volume

Fraction	Lipid class	Solvent	Column volumes
1	Hydrocarbons	100% hexane	3
2	Wax esters	1% diethyl ether in hexane	6
3	Triacylglycerols	5% diethyl ether in hexane	5
4*	Polar lipids	100% diethyl ether	5
5	Phospholipids	100% methanol	5

*Fraction 4 from *E.californicus* included some TAGs. In a second fractionation, TAGs eluted in a pure state in 10% diethyl ether in hexane, followed by elution of cholesterol and free fatty acids in 100% diethyl ether.

chloroform:methanol ($N = 10$ extractions). Lipids were extracted for 48 h under N_2 at 4°C (Ohman, 1988), the copepods removed from the solvent to tared dry boats, dried at 55°C, and weighed. Total lipid was taken as the difference between total dry mass and de-fatted copepod mass. Lipid method (iii), TLC-FID: the lipid extracts from method (ii) were dried under N_2 , resuspended in pure chloroform, and analyzed by TLC-FID. TLC-FID analysis was carried out in triplicate on SIII chromarods using an Iatroscan Mark V. Lipids were spotted in pure chloroform using a Hamilton microliter syringe. Chromarods were developed in hexane:diethyl ether:formic acid (82:18:0.1) for 33 min, dried, then scanned twice at 30 s scan⁻¹ with air flow at 2000 ml min⁻¹ and H_2 flow at 160 ml min⁻¹. Lipid standards used were purified wax esters isolated from *C.pacificus* CV (>99% pure), and palmitic acid palmityl ester (16:0, 16:0; C atoms:number of double bonds for wax ester fatty acid, wax ester fatty alcohol), palmitic acid stearyl ester (16:0, 18:0), palmitoleic acid stearyl ester (16:1, 18:0), tripalmitin, myristic acid, cholesterol and L- α -phosphatidylcholine (type V-EA), from Sigma Biochemical Co. The purity of lipid standards was always checked prior to analysis. Total lipid was taken as the sum of wax esters, TAGs, sterols, free fatty acids and phospholipids.

Power function calibration relationships were fitted to wax ester data using non-linear regression employing the Simplex method and comparisons between fitted curves made using the 95% confidence limit (CL) estimated from the asymptotic standard error (Systat). Linear calibration relationships were fitted to TAG and phospholipid data, and the regressions compared by analysis of covariance.

Water content analysis

Extreme care is required to obtain reliable measures of the wet mass of individual copepods. Pooled groups of copepods cannot be used because free water remains trapped between animals. Measurements were made in a humidity-regulated laboratory with a Cahn 29 electrobalance containing fresh desiccant and ²¹⁰Po α -emitter. Only animals with intact appendages and body turgor were used. This was especially important with *E.californicus* because individuals with even slight perforations in the exoskeleton lose fluids and become flaccid when removed from water. Animals were briefly dipped in Milli-Q water, blotted lightly against a tissue to remove water trapped under the antennules, mouthparts and swimming legs, then transferred to a tared aluminum boat using dry microforceps. The animal plus boat mass was recorded after 30 s, to permit the electrobalance to stabilize, then at subsequent 30 s intervals to measure the time course of water loss. The initial copepod wet mass was determined from the y-intercept of a linear regression of mass as a function of time. Boats were then dried at 55°C and reweighed for dry mass. Water content was determined as the difference between wet and dry mass. There was no known difference in the spawning state of the four species of females analyzed. Sample sizes were 20–33 individuals weighed per species.

Results

TLC-FID response factors

The response of the Iatroscan FID did not differ among three saturated or mono-unsaturated wax esters: palmitic acid palmityl ester, palmitic acid stearyl ester and palmitoleic acid stearyl ester. The slopes and intercepts for power functions fitted to these compounds were not different ($P > 0.05$), so these data were then pooled. The FID response to these wax esters differed significantly from the response to pure wax esters from *C.pacificus* CV (Figure 1A). The intercept was higher ($P < 0.05$) for the commercial wax esters and the exponent was slightly (but not

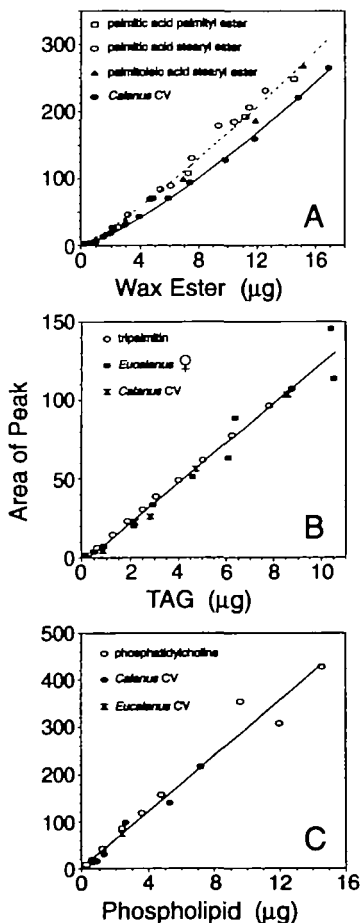


Fig. 1. Response of Iatroscan TLC-FID to purified lipids extracted from calanoid copepods (filled symbols) and to commercially available reference compounds (open symbols). (A) Wax esters (WE). *Calanus pacificus* CV: area = $7.114 \times WE^{1.274}$, $N = 13$, $r^2 = 0.99$. Commercial wax esters: area = $11.735 \times WE^{1.156}$, $N = 20$, $r^2 = 0.98$. (B) Triacylglycerols (TAG). Combined regression: area = $-3.546 + 12.667 \times TAG$, $N = 25$, $r^2 = 0.98$. (C) Phospholipids (PL). Combined regression: area = $3.679 + 29.385 \times PL$, $N = 16$, $r^2 = 0.97$.

significantly) different ($P > 0.05$). These analyses were repeated on a separate set of chromarods with the same diminution in FID response when *Calanus* wax esters were applied.

The error in wax ester determination varies in a non-linear manner with the mass of lipid loaded on chromarods, because the calibration curves for wax esters do not have identical exponents. Figure 2 illustrates that the error in the absolute mass of wax ester varies from $\sim 0.16 \mu\text{g}$ wax ester for $0.5 \mu\text{g}$ applied to chromarods to $2.2 \mu\text{g}$ for $12 \mu\text{g}$ wax ester applied. This corresponds to a percentage error declining from 32% at the lower loadings to 18% at the higher loadings. In all cases, the true wax ester content is underestimated when using commercial wax esters of the kind used here for calibration purposes.

Sufficient triacylglycerols were extracted and purified for calibration purposes from *E.californicus*. [*Eucalanus californicus* stores mainly TAGs, while *C.pacificus* stores primarily wax esters (Ohman, 1988).] Separate column fractionations were performed for lipids from CV and adult female *E.californicus*. Comparison of FID response to a saturated TAG standard (tripalmitin) and copepod TAGs showed no difference (Figure 1B, similar slopes and intercepts by ANCOVA, $P > 0.30$). This same result was found when the analyses were repeated on an independent set of chromarods.

The FID response to purified copepod phospholipids was no different than that to pure phosphatidylcholine (Figure 1C, $P > 0.30$, ANCOVA). These analyses were also repeated independently with the same results.

Comparative lipid determination

The total dry mass of *E.californicus* females averaged $347 \pm 32 \mu\text{g}$ (Table II). Total lipids of *E.californicus* females averaged $178 \mu\text{g}$ copepod⁻¹ (51.3% of dry mass) when calculated from the difference between total dry mass and the dry mass of tissues de-fatted when dry [method (i)], $227 \mu\text{g}$ copepod⁻¹ (65.4% of dry mass) when calculated from the difference between total dry mass and dry mass of tissues de-fatted when wet [method (ii)], and $21 \mu\text{g}$ copepod⁻¹ (6.1% of dry mass)

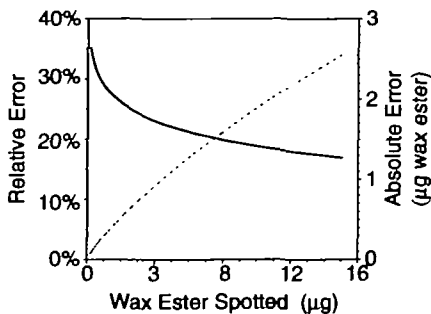


Fig. 2. Error in assessment of the wax ester content of *C.pacificus* by calibration of TLC-FID with simple saturated wax ester standards rather than native wax esters extracted from the copepods. Dashed line, absolute error (as μg wax ester); solid line, percent error. Error varies with the mass of wax ester spotted onto chromarods (see Figure 1A).

Table II. Comparison of copepod total lipid content from three different techniques: (i) extraction of dry tissues with 2:1 chloroform:methanol followed by weighing the non-lipid residue; (ii) extraction of wet tissues with 2:1 chloroform:methanol followed by weighing the non-lipid residue; (iii) extraction of wet tissues with 2:1 chloroform:methanol followed by measurement of extracted lipids by TLC-FID. Entries are $\bar{x} \pm 95\%$ CL (*N*)

	Total dry mass (μg)	(i) Dry tissue extraction			(ii) Wet tissue extraction			(iii) TLC-FID lipids	
		'De-fatted' copepod mass (μg)	Lipids by difference		'De-fatted' copepod mass (μg)	Lipids by difference		Lipid mass (μg)	Lipid/DM (%)
			Lipid mass (μg)	Lipid/DM (%)		Lipid mass (μg)	Lipid/DM (%)		
<i>Eucalanus californicus</i> ♀	347 \pm 32 (15)	169 \pm 23 (15)	178 \pm 26 (15)	51.3	120 \pm 10 (10)	227 \pm 24 (10)	65.4	21.2 \pm 5.2 (10)	6.1
<i>Calanus pacificus</i> CV	220 \pm 12 (13)	91 \pm 7 (13)	129 \pm 8 (13)	58.7	–	–	–	–	–
<i>Calanus pacificus</i> CV	200 \pm 11 (18)	–	–	–	67 \pm 6 (10)	133 \pm 9 (10)	66.5	79.8 \pm 10.2 (10)	39.9

when directly analyzed by Iatroscan TLC–FID [method (iii), Table II]. These *E.californicus* lipids were 6.0% wax esters, 31.9% TAGs, 3.2% free fatty acids, 3.8% sterols and 55.2% phospholipids.

The total dry mass of the *C.pacificus* CV used for method (i) was $220 \pm 12 \mu\text{g}$ and that of a second group used for methods (ii) and (iii) was $200 \pm 11 \mu\text{g}$ (Table II). Total lipids of *C.pacificus* CV averaged $129 \mu\text{g copepod}^{-1}$ (58.7% of dry mass) by method (i), $133 \mu\text{g copepod}^{-1}$ (66.5% of dry mass) by method (ii), and $79.8 \mu\text{g copepod}^{-1}$ (39.9% of dry mass) when analyzed by TLC–FID [method (iii)]. These *C.pacificus* lipids were 82.9% wax esters, 3.0% TAGs, 0.6% free fatty acids, 0.8% sterols and 12.7% phospholipids. To test for any bias in TLC–FID measurements of total lipids, aliquots of these same lipids were pipetted onto aluminum boats in pure chloroform, dried, and weighed. There was no difference between the total lipid mass determined by TLC–FID (calibrated with *Calanus* wax esters) and the total lipid mass when weighed on an electrobalance ($P > 0.40$, paired *t*-test, $N = 13$; mean difference = 6.9%), confirming the accuracy of the TLC–FID measurements. Because non-lipid materials are insoluble in pure chloroform, the solutions compared at this step by weighing and TLC–FID analysis would contain only lipids.

When lipids were removed in pure chloroform from the extraction vials used for method (ii), a whitish residue remained in the vials. The amount of residue was greater in the vials from *E.californicus* than from *C.pacificus*. This residue of non-lipid material had been released from copepod tissues in the more polar chloroform:methanol solution, but was not soluble in pure chloroform. The mass of this non-lipid material was not determined, but it likely accounts for the major discrepancy between the indirect calculation of lipid content by difference between total and ‘de-fatted’ dry mass and the direct measures of lipid mass.

Water content

Following a first measure of copepod wet mass 30 s after transfer to the weighing chamber, the mass declined in a highly linear manner with a constant slope of -0.8 to $-1.1 \mu\text{g s}^{-1}$. The initial wet mass was therefore obtained by fitting a linear regression to successive measurements and solving for the *y*-intercept. Wet mass for individual adult females ranged from $4033 \pm 246 \mu\text{g}$ ($\bar{x} \pm 95\%$, $N = 33$) for *E.californicus* to $375 \pm 23 \mu\text{g}$ ($N = 25$) for *Metridia pacifica* (Figure 3A). From these and dry mass determinations on the individuals (Figure 3B), the percent of total wet mass as water was obtained (Figure 3C). Adult females of three species (*Rhincalanus nasutus*, *C.pacificus* and *M.pacifica*) had water contents in the range of 82.3–84.3%, all of which differed significantly ($P < 0.001$, ANOVA with Tukey HSD test) from the mean for *E.californicus* (92.9% of wet mass as water).

Discussion

The results presented here highlight several sources of error that introduce variability and bias into measures of the lipid content of marine copepods. For Iatroscan TLC–FID analyses, failure to calibrate the instrument with native wax

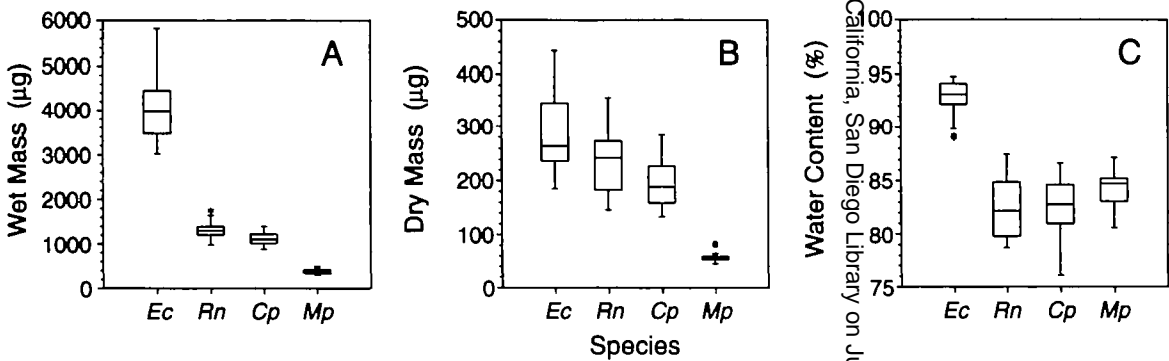


Fig. 3. Box and whisker plots (Tukey, 1977) of the mass and water content of adult females of four species of calanoid copepods. *Ec*, *Eucalanus californicus*; *Rn*, *Rhincalanus nasutus*; *Cp*, *Calanus pacificus*; *Mp*, *Metridia pacifica*. (A) Wet mass; (B) dry mass; (C) water content. Horizontal line, median; boxes, 25th and 75th percentiles; vertical lines, upper and lower fences ($= 1.5 \times (\text{H-spread})$, where H-spread = $1.75 \times (\text{75th} - 25\text{th tile})$). *Outlier.

esters can lead to appreciable underestimates of the true wax ester content. For measures of total lipid obtained by de-fatting animals in organic solvents and weighing the residue, substantial overestimates of the true lipid content occur, and because copepod taxa differ in organic and water content, the selection of dry or wet mass as the independent variable in scaling the lipid content per unit body mass can appreciably change comparisons among species. Beyond their significance for the accuracy of analytical measurements, such factors can lead to bias in the interpretation of the ecological causes for lipid variations (see the Introduction).

TLC-FID response

The Iatroscan FID response is not measurably different to TAGs extracted from *E.californicus* and to tripalmitin, a rather simple compound comprised of three C16:0 fatty acids. Although the fatty acid composition of *E.californicus* TAGs is unknown, TAGs of *C.pacificus* include unsaturated and longer chain fatty acids (Lee *et al.*, 1971). Consistent with the present results, Fraser *et al.* (1985) found that the degree of unsaturation of TAGs (from the fish oil Marinol) did not affect the Iatroscan FID response. Care is nevertheless warranted in the selection of TAG standards because a 1.7-fold difference in FID response has been observed between other TAG compounds (i.e. pure triolein and Marinol; Fraser and Taggart, 1988).

The equivalent FID response to pure phosphatidylcholine (PC) and phospholipids extracted from copepods is consistent with the known composition of *C.pacificus* phospholipids; those of *E.californicus* have not been reported. Patton *et al.* (1972) found that PC was the dominant phospholipid in '*Calanus helgolandicus*' (= *Calanus pacificus* Brodsky) and together with phosphatidylethanolamine (PE) comprised 61% of total phospholipids. The Iatroscan FID response is quite similar to PC and PE (Hazel, 1985), although it differs for other phospholipid compounds. Hence, for animals whose native phospholipids are dominated by PC and PE, either should be a suitable calibration standard.

In contrast to phospholipids and TAGs, simple wax esters elicit a significantly greater FID response than an equal mass of wax esters extracted from *C.pacificus* CV and are not appropriate for calibration purposes. Wax esters from members of the copepod family Calanidae typically include appreciable longer chain, mono- and polyunsaturated fatty acids and fatty alcohols (e.g. Lee *et al.*, 1972; Sargent and Henderson, 1986; Kattner *et al.*, 1989). It is, therefore, surprising that the present study found a lower, rather than a higher, response to these native wax esters because C as a proportion of wax ester mass increases with both longer fatty acid chain length and more double bonds. In the TLC-FID system, lipids are pyrolyzed in a hydrogen burner, then the stream of ionized C atoms is collected by the FID (Ackman, 1981). In principle, the higher the proportion of ionizable C present in a lipid, the larger the expected detector response per unit lipid mass. Since the copepod wax esters have a higher proportion of ionizable C than the commercial standards, one would expect a higher detector response.

Evidently, some structural characteristics of the copepod wax esters cause them to be either less efficiently combusted by the hydrogen burner, less volatile, or less efficiently detected by the FID than the commercially available, shorter chain standards. All of the commercial wax ester standards used here were synthesized from fatty acid and fatty alcohol precursors of plant origin (Sigma Biochemical Co., personal communication). Analysis by NMR or other techniques is necessary to resolve the structural basis of these different responses.

Miller and Morgan (1996) recently reported a lower TLC–FID response to wax esters extracted from *Calanus finmarchicus*. The present results are in agreement with their findings and suggest that this difference is likely to hold for wax esters from a variety of copepod species and perhaps other marine crustaceans.

It should be noted that despite the obvious importance of correctly calibrating the Iatroscan FID detector for quantitative measures of lipid content and composition, qualitative patterns such as the spatial match of copepod TAGs and wax esters with mesoscale variations in food supply (Håkanson, 1987), or the seasonal accumulation of lipid reserves in high-latitude zooplankton populations (Schnack-Schiel *et al.*, 1991), will not be affected in a qualitative sense by such corrections.

Inspection of 280 recent Iatroscan analyses of four species of calanoid copepods (*E. californicus*, *C. pacificus*, *R. nasutus*, *M. pacifica*) in this laboratory showed that the average mass of wax ester spotted was 0.8 μg , which would have resulted, on average, in a 30% underestimate of the true wax ester content if commercial wax esters had been used for calibration (see Figure 2). The total lipid content is less biased than the wax ester content, since wax esters as a function of total lipids vary appreciably among copepod taxa (e.g. Ohman, 1988; Hagen *et al.*, 1993). For these same four copepod species, the total lipid mass would have been underestimated by an average of 10.1%.

Application of the calibration relationship for *C. pacificus* CV wax esters to adult females of this species and to CVs or females of three other species resulted in total lipid values consistent with those measured directly by weighing. Therefore, from these results, there was no evidence that wax esters must be extracted separately from every developmental stage and species analyzed, although this assumption must be checked in every field situation. The fatty acid and fatty alcohol content of some species' lipids change in time and space (e.g. Kattner *et al.*, 1989), partly in response to changing dietary substrates, although this is not always the case (e.g. Ohman *et al.*, 1989). More data are needed concerning the variability in TLC–FID responses to wax esters arising from different species and different field conditions. However, because the Iatroscan TLC–FID response varies with many factors (Ackman, 1981; Parrish and Ackman, 1985; Ackman *et al.*, 1990), there are limits to the analytical accuracy and precision that can be obtained with this method.

These results raise a concern common to all analytical methods, not TLC–FID alone. Proper selection of standard compounds and demonstration that they elicit responses identical to that of native lipids are necessary for all methods for lipid determination, including gas–liquid chromatography, HPLC, spectroscopic techniques, colorimetric reactions, quantitative densitometry, and others.

Total lipid

The large disparity in measures of total lipid content when assessed by de-fatting copepods and by direct measurement using methods specific for lipid compounds confirms that extraction in chloroform:methanol releases considerably more organic matter than lipids alone. These solvents are known also to liberate such alcohol- and water-soluble constituents as carbohydrates, amino acids, proteins and salts (Hopkins *et al.*, 1984; Kates, 1986), especially when combined with tissue water. The release of non-lipid material is especially pronounced in *E.californicus*, where the lipid content was thereby overestimated by a factor of 8–10 \times . This effect likely accounts for the high lipid content (mean of 46%, range 29–58% of dry mass) reported for *E.californicus* in Smith and Lane (1991), who estimated lipid content by de-fatting, comparable to the overestimate of 51% of dry mass measured by the de-fatting method (ii) in the present study. In contrast, the true lipid content of this species was found to be 6% in the present study and 11% in the study of Ohman (1988). Use of the de-fatting technique by Lee (1974) probably accounts for the unusually high lipid values (to 77% of dry mass) he reported for *Calanus hyperboreus*, which from the present results appear to be inaccurate. The reliability of the lipid measures by de-fatting with other organic solvents such as methylene chloride (e.g. Miller, 1993) remains uncertain until verified.

Water content

Body mass is commonly used as an independent variable in understanding allometric relationships of respiration, excretion, growth and fecundity, as well as lipid content of zooplankton. Implicit in many such comparisons is the assumption that the relationship of body dry mass to wet mass, protein, C or other indices is constant among taxa. The present results show that even within the calanoid copepods, there are appreciable differences among taxa in the water content of tissues. While three species (*C.pacificus*, *R.nasutus* and *M.pacifica*) all had 82–84% water content, which overlaps the modal value for many different copepod taxa reported by Båmstedt (1986), *E.californicus* stands out with 93% of its tissues in the form of water. If one assumed that *E.californicus* had the average water content of copepods, their dry mass would be overestimated by a factor of 2.4. Any other derived measure (C, protein, etc.) would be similarly overestimated and mass-specific rates consequently underestimated.

Flint *et al.* (1991) showed that the eucalanids *Eucalanus inermis* and '*E.elongatus* f. *hyalinus*' have unusual organic composition, with lower lipid, protein, phosphorus and organic carbon content per unit wet body mass than members of the genus *Calanus*. They termed these distinctive species 'jelly-bodied' copepods, but pointed out that not all members of the Eucalanidae share such characteristics. In their study, wet body mass was estimated from published length–mass regressions rather than measured directly. Here we found by direct measurement that the water content of *E.californicus* is substantially higher than that of three other species with which it regularly co-occurs. With a water content of 93%, *E.californicus* is quite close to the common definition of gelatinous zooplankton

as organisms containing $\geq 95\%$ water (Alldredge and Madin, 1982). The high water content of these animals confers some of the ecological benefits of larger body size (e.g. Kjørboe and Sabatini, 1995) while requiring little investment in organic tissues.

Summary and recommendations

- (i) The response factor of the Iatroscan FID detector to wax esters extracted from copepods is significantly lower than the response to some commercially available saturated, shorter chain wax esters. The TLC–FID should be calibrated with native wax esters extracted from the zooplankton species of interest and lipid standards reported carefully in publications. This recommendation applies equally to other analytical methods for lipids until response factors are proven equivalent.
- (ii) Total lipid content should not be estimated by de-fatting zooplankton with organic solvents, as non-lipid materials are also liberated.
- (iii) If lipids are measured gravimetrically, they must first be purified to remove non-lipid residues (cf. Folch *et al.*, 1957; Bligh and Dyer, 1959; Hopkins *et al.*, 1984).
- (iv) Calanoid copepods differ appreciably in water content, with remarkably watery tissues in *E.californicus*. Care must be taken in converting between different measures of biomass and in the choice of independent variables for scaling lipid content, metabolic rates, growth rates, and fecundity relationships among taxa.

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