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RAPPORT DES RECHERCHES COLLECTIVES

NO. XXX

INTERCALIBRATION EXERCISE ON THE QUALITATIVE AND QUANTITATIVE ANALYSIS OF FATTY ACIDS IN *ARTEMIA* AND MARINE SAMPLES USED IN MARICULTURE

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on behalf of the 'Working Group on the Mass Rearing of Juvenile Marine Fish' of the International Council for the Exploration of the Sea

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1. INTRODUCTION

A workshop held at the Second International Symposium on the Brine Shrimp *Artemia* noted that there was a large variation in the maximum amount of (n-3) highly unsaturated fatty acid (HUFA) enrichment reported by different groups of scientists (Simpson, 1987). It was not clear whether this variation was due to differences in enrichment technique or to differences in analytical methods and so an international inter-laboratory exercise was proposed to resolve the issue. The aim was to determine the variability associated with the preparation and analysis of fatty acids in two samples of *Artemia* supplied to the laboratories by the Artemia Reference Center (ARC). The results reported to the ARC indicated that intra-laboratory variability in determinations of total lipid content and fatty acid composition by the qualitative (i.e. expressed as area-percentages) method was generally low, with a mean coefficient of variation (CV) of around 5 to 7%. In contrast, intra-laboratory variability in determinations of fatty acid composition by the quantitative method (i.e. expressed as mg FA/g dry wt) was higher, with CVs greater than 10% in several cases. Inter-laboratory variability was generally much greater, with CVs of >20% for total lipid content, 8 to 27% and 12 to 49% for content of individual fatty acids determined by qualitative and quantitative methods respectively. Thus, the precision of data from any given laboratory may be adequate, but the accuracy may not be (Léger *et al.*, 1989).

Following this exercise, the ICES Working Group on Mass Rearing of Juvenile Marine Fish recommended that improvements in the analysis and reporting of fatty acids should be pursued (ICES, 1988). It suggested the preparation of a proposed methodology for the qualitative and quantitative analysis of fatty acids in *Artemia* and marine samples. This document was prepared by the Artemia Reference Center (Léger *et al.*, 1990) and submitted to the National Center for Mariculture (G. Kissil, IOLR, Eilat, Israel) and the Aquaculture Department of SINTEF (J. Rainuzzo, Trondheim, Norway) for verification and amendment of the proposed procedures. The resulting proposal, covering sample preparation, lipid extraction, esterification and recommendations for GC-analysis, was then submitted for final evaluation by J. Sargent (University of Stirling, Scotland, UK). The proposed methodology for (n-3) HUFA analysis resulting from these consultations was submitted to the ICES Mariculture Committee for adoption as a standard procedure.

The ICES Working Group on Mass Rearing of Juvenile Marine Fish then launched an inter-calibration exercise to verify the accuracy of this Standard Methodology when applied at different laboratories. In order to distinguish variability arising from chemical analysis from that attributable

to biological factors (e.g. preparation of live material), it was proposed that all participating laboratories used the same reference standard (GLC-standard GLC-68-B methylesters of the NU-CHECK-PREP Co) and two inter-calibration samples provided by the Artemia Reference Center. These were a formulated feed sample, subject only to variability from chemical analysis, and a sample of *Artemia* cysts, which, because they required hatching, separation of nauplii and estimation of naupliar dry weight, would in addition be subject to variation from biological sources. This report describes the results received from 11 laboratories which participated in this intercalibration exercise.

2. OUTLINE OF THE INTERCALIBRATION EXERCISE

The following materials were sent to each of 20 laboratories that expressed an interest in participating in the intercalibration exercise:

(a) Two vacuum-packed samples (5 and 10 g) of a dry formulated feed with a high (n-3) HUFA content.
(b) Three vacuum-sealed samples (each of 10 g) of *Artemia* cysts.
(c) Instructions for the participant (see Addendum II)
(d) The ICES Standard Methodology for (n-3) HUFA Analysis (see Addendum III)

3. DATA TREATMENT AND STATISTICAL ANALYSIS

From the data received from each participant, intra-laboratory means, standard deviations (sd), and coefficient of variations (CV) were calculated for lipid content and the content of six (*Artemia*) or seven (dry feed) selected major fatty acids. Since not all participants were able to separate 18:1n-7 and 18:1n-9, data for these two fatty acids were summed and subsequently treated as one, i.e. 18:1n-7/9. The data were summarised in the following way:

1. The intra-laboratory CVs for total lipid were used to calculate a mean intra-laboratory CV.
2. The mean lipid content for each laboratory was used to calculate an overall mean, sd and CV. This represented the inter-laboratory variability.
3. The CVs for the selected fatty acids from each laboratory were used to calculate a mean intra-laboratory CV. This represented a measure of the precision of each laboratory.

4. To evaluate the effect of introducing the Standard Methodology on the precision of the laboratories, the mean intra-laboratory CVs were averaged for all participants, the participants that followed the Standard Method and those that applied their own method.

5. For these three groups of laboratories, the mean content of a given fatty acid for each laboratory was used to calculate an inter-laboratory mean, sd and CV for that fatty acid.

6. To evaluate the effect of introducing the Standard Method on the inter-laboratory variability, the inter-laboratory CVs of all selected fatty acids were averaged for each group of laboratories (Tables 4, 5, 6, 8, 9).

Mean values for lipid and selected fatty acid content reported by the different laboratories were compared using one-way analysis of variance (ANOVA) and Tukey's multiple range test (Sokal and Rohlf, 1981). One laboratory (No. 11) was excluded from the statistical analysis since only one analysis was provided (Tables 4, 7, 10).

4. RESULTS AND DISCUSSION

4.1. Response to the excercise

A preliminary evaluation of the intercalibration exercise was presented to the meeting of the ICES Working Group on Mass Rearing of Juvenile Fish in Bergen, Norway (ICES, 1993). At that time, results had been received from only seven of the 20 laboratories to which samples had been sent, and only four of those had followed the prescribed ICES Standard Method for (n-3) HUFA analysis. Following a further appeal for submissions, the number of contributions increased to 11 of which five followed the Standard Method (Laboratory Nos. 1, 2, 3, 9, 10), five their in-house method (Laboratory Nos. 5, 6, 7, 8, 11), and one compared their in-house method with the Standard Method (Laboratory No. 4).

Not all laboratories provided all the data that were requested and very few followed the instructions with regard to replication of the analysis (Table 1). Laboratory Nos. 5 and 7 did not provide the fatty acid profiles on a quantitative (mg/g) basis for the dry feed and the *Artemia* sample, respectively. Total lipid content was reported by eight participants for each sample type. Overall, only 5 and 6 participants performed the fatty acid analysis according to the prescribed procedure for the dry feed and the *Artemia* sample respectively. In addition, data derived from in-house analytical methods were

provided for the dry feed and the *Artemia* sample by 6 and 5 participants, respectively. In this way, the present inter-calibration exercise allowed an evaluation of the effect of introducing a standard method on the precision (intra-laboratory variability) as well as on the accuracy (inter-laboratory variability) of the fatty acid analysis of a formulated dry feed and *Artemia* nauplii.

4.2. Analytical methods, instrumentation and operational parameters used by the participants

The information provided by the participants on the analytical methods used is given in Table 2. Two out of the six laboratories using the prescribed method reported some slight modifications of the procedure. The in-house methods of the participants were very different and included saponification followed by transmethylation (Laboratory Nos. 5, 7) and micromethods using direct transesterification (Laboratory Nos. 4, 6) or Bligh and Dyer (1959) extraction (Laboratory No. 8).

The following comments on the Standard Method were made by the participants:

- Transesterification in methanol-acetyl chloride at 24-29°C (Lepage and Roy, 1984) should be adopted to reduce the hazards of using toluene or benzene and having vials at 100°C.
- Phase separation filter papers to remove water from the extraction should be used instead of filtering over sodium sulphate.
- The internal standard should be added prior to methylation to correct for possible solvent losses.
- Internal standards consisting of saturated fatty acids may be preferred over unsaturated fatty acids because of their higher stability.
- Direct transesterification methods are less laborious and solvent consuming than the Standard Method which involves lipid extraction and esterification.
- The Standard Method requires excessively large samples (100 mg dry wt).

The participating laboratories differed with regard to choice of gas chromatograph, column, carrier gas, temperature programme, and injection system, whereas all laboratories that specified detector type used flame ionization detectors (Table 3). Of the laboratories following the Standard Method, only Laboratories 1, 2 and 3 used the recommended 20:2n-6 as internal standard. The in-house methods involved the use of an uneven saturated fatty acid as internal standard.

4.3. Total lipid analysis

About half of the laboratories that reported lipid content followed the Standard Method. The average

intra-laboratory variation (CV) in the determination of total lipid content was only 3.6% for the dry feed and 4.0% for the *Artemia* nauplii (Table 4). Inter-laboratory variation was somewhat higher being 5.2% and 8.7% for dry feed and nauplii respectively. Furthermore, significant differences were found between lipid content reported by the different laboratories (ANOVA; dry feed: $P<0.01$, *Artemia*: $P<0.001$). Although these differences between laboratories using the Standard Method were significant, the values for inter-laboratory variation were considerably lower than those generated in the inter-calibration exercise organised by Léger *et al.* (1989). These authors reported an inter-laboratory variation of 28.5% for decapsulated *Artemia* cysts and 22.8% for nauplii. This difference between the two studies may be explained by the use of a standard protocol for hatching and lipid extraction in the present inter-calibration exercise.

4.4. Fatty acid analysis

Results for the selected fatty acids for the dry feed are expressed both qualitatively as area percent values (Table 5) and quantitatively in terms of mg/g dry wt. (Table 6). The results of the statistical analyses are presented in Table 7. Equivalent data for *Artemia* nauplii are presented in Tables 8, 9, and 10. In addition, total fatty acid methyl esters (FAME, expressed in terms of mg/g dry wt.) recovered from the feed and *Artemia* are given in Tables 6 and 9, respectively.

Independent of sample type and variation level, qualitative data exhibited average coefficients of variation that were approximately half of those of the corresponding quantitative data (Tables 5, 6, 8, 9). For both the dry feed and the *Artemia* sample, average intra-laboratory variation for the selected fatty acids was below 6.3% and 11.0% for the qualitative and quantitative values respectively. The intra-laboratory variation, averaged for all participants, in the feed and *Artemia* respectively was as low as 3.3% and 2.7% for qualitative data, and 6.9% and 6.3% for quantitative data. In contrast, the inter-laboratory variation, based on the averaged data for all the fatty acids, in the feed and *Artemia* was 13.7% and 7.3% respectively for the qualitative data, and 24.5% and 11.5% respectively for the quantitative data.

It is noteworthy that the inter-laboratory variation for the dry feed analyses was considerably higher than that of the nauplii. This clearly demonstrated that the variation in the *Artemia* analyses was not simply the summation of analytical (i.e. comparable with the variation encountered in the analysis of the dry feed) and biological variation (e.g. hatching), but that other factors influenced the variability of the analyses. Heterogeneity among the distributed feed samples is not a likely cause

since great care was taken in the packaging of the samples to ensure homogeneity of the diet and this would have increased the intra-laboratory variation (most participants analysed the two samples that were provided). The more probable explanation is that the extraction of lipids and/or esterification of fatty acids is more critical in well-bound diets than in brine shrimp tissue. In this way, the higher variability in quantitative, as well as in qualitative data may be due to variable success in the extraction and/or methylation of the fatty acids from the extruded matrix of the diet. The higher variation in the total fatty acid content of the diet (26.6%) compared to that of the nauplii (14.4%) supports this contention.

The data generated by the Standard Method showed a slightly lower intra-laboratory and considerably lower inter-laboratory variation for the qualitative values than that produced by in-house methods (Table 11). However, the quantitative data showed the opposite trend, except for the inter-laboratory variation in the *Artemia* analyses. Again, the differences between inter-laboratory variability in quantitative data were more profound for the dry diet than for the nauplii.

Independent of the method applied by the laboratories, significant differences were observed between fatty acid profiles reported by different laboratories (Tables 7, 10). A possible factor that may have contributed to this is the lack of experience of some of the participants with either the method and/or the samples. In particular, the quantitative analysis of fatty acids may have benefitted from more experimentation with the method prior to acceptance of the results. The better accuracy of the qualitative analysis among laboratories using the Standard Method may be the result of the standardization of the extraction and esterification procedure.

The comparison of the Standard Method with the in-house procedure (i.e. a direct transesterification-extraction method) used by participant 4 not only deserved a special acknowledgement but, in addition, supported the need for a standardized method to prepare and analyze fatty acids. Although it should be pointed out that *actual* values are not known, the data generated by participant 4 may indicate that the direct method gives essentially the same results as the Standard Method but had the advantage of being more rapid. Closer examination of the data, however, shows this may not be the case. From Fig. 1 it is clear that the in-house method of participant 4 gave systematically higher values, both in qualitative and quantitative terms, for 16:0 and lower values for 20:5(n-3) and 22:6(n-3) than the Standard Method. In many cases, the latter differences were significant (ANOVA, Tables 7, 10). Although it is acknowledged that the Standard Method may be too lengthy and involved to be used as a routine procedure in the analysis of large numbers of samples, it could have

considerable value in intercalibrating the analytical procedures adopted by different laboratories.

Although the original goal of the present inter-laboratory exercise was to evaluate the accuracy of a standardised method for fatty acid analysis, it is interesting to note that the overall variability, both on the intra-laboratory as well as the inter-laboratory level, was significantly lower than that reported by Léger et al. (1989). These authors observed an average intra-laboratory CV in the fatty acid analyses of *Artemia* nauplii of 4.9% and 10.3% for qualitative and quantitative data respectively, whereas the equivalent values generated by the present exercise were 2.7% and 6.3%, respectively (Table 12). Similarly, the qualitative and quantitative values for average inter-laboratory variation of 18.1% and 24.5% respectively, reported by Léger *et al.* (1989) were higher than the equivalent values of 7.3% and 11.5% in this study. The extremely high variability observed by Léger *et al.* (1989) in the quantitative values of the decapsulated cysts may be due to variability of extraction success, as was suggested previously for the dry feed in the present study. Léger *et al.* (1989) aimed to assess the inter-laboratory variability of methodological and analytical procedures and intentionally did not provide specific instructions for *Artemia* hatching, sample preparation, and chromatographic analysis. The better accuracy obtained in the present exercise may be at least partially due to the stipulation of a standard procedure for hatching and preparation of the cyst sample in the instructions to the participants. Furthermore, the provision of a standard analytical method may have stimulated the participants, even those that did not follow it, to work more accurately. Finally, it is also possible that the intensive research on fatty acid requirements over the last decade has encouraged the improvement of and the experience in procedures for fatty acid analysis in analytical laboratories.

5. CONCLUSIONS

An international inter-calibration exercise was conducted to evaluate the accuracy of the ICES Standard Methodology for fatty acid analysis in a sample of *Artemia* and a formulated dry feed. Results were received from 11 of the 20 laboratories to which samples were sent. Five participants followed the Standard Method, five their own in-house method, and one laboratory compared their own in-house method with the Standard Method. Total lipid content was reported by eight participants.

The average intra-laboratory variation in the determination of total lipid content was only 3.6% (CV) for the dry feed and 4.0% for the *Artemia* nauplii. The inter-laboratory variation was somewhat

higher being 5.2% for the dry feed and 8.7% for nauplii. In addition, significant differences were found between lipid content reported by the different laboratories. Nevertheless, the inter-laboratory variation obtained in this study was considerably lower than that reported in a previous inter-calibration exercise (Léger *et al.*, 1989). It is suggested that this may have been because precise procedures were prescribed for both hatching the cysts and lipid extraction.

Intra- and inter-laboratory variability in the determination of fatty acid composition was on average twice as high for quantitative data as it was for qualitative data. The intra-laboratory variation, averaged for all the laboratories for the feed and *Artemia* respectively, was as low as 3.3% and 2.7% for qualitative data, and 6.9% and 6.3% for quantitative data. In comparison, the average inter-laboratory variation for the major fatty acids in the feed and *Artemia* respectively, was 13.7% and 7.3% for qualitative data, and 24.5% and 11.5% for quantitative data. The higher variability in the quantitative, as well as qualitative data, for the dry feed may have been due to a higher variability in the extraction and/or methylation of the fatty acids from the extruded matrix of the diet compared to brine shrimp tissue.

The laboratories using the Standard Method exhibited a somewhat lower intra-laboratory and inter-laboratory variation for the qualitative values than the laboratories applying their own in-house method. In contrast, the quantitative analyses revealed, particularly for the dry feed, a slightly higher variability for the laboratories following the Standard Method.

The overall variability in the present exercise, both on the intra-laboratory as well as inter-laboratory level, was significantly lower than that reported by Léger *et al.* (1989). The better accuracy obtained in the present exercise for the determination of fatty acid composition in *Artemia* nauplii is at least partially due to the stipulation of a standard procedure for hatching and analysis of the cyst sample in the instructions to the participants.

Although it is more elaborate and solvent consuming than many current methods for fatty acid analysis in routine use, the ICES Standard Method may be used to inter-calibrate the analytical procedures adopted by different laboratories to analyze fatty acids in *Artemia* and marine samples.

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The reference to proprietary products in this paper should not be construed as an official endorsement of these products, nor is any criticism implied of similar products which have not been mentioned.

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8. FIGURES AND TABLES

Fig. 1. Comparison of the content of the major fatty acids in *Artemia* nauplii and a dry diet obtained by Participant 4 using the in-house method and the ICES Standard Method. Bars represent differences between the ICES and the in-house method as a percentage of the value obtained with the Standard Method (A: area percent; B: mg/g data).

A: area%
B: mg/g dry wt.
ICES - OWN (as % of ICES)
ICES - OWN (as % of ICES)
15
10
5
0
-5
-10
-15
14:0
16:0
16:1
18:1
18:2
18:3
20:5
22:6
dry diet
Artemia

Table 1: Response to the intercalibration exercise on analysis of fatty acids in *Artemia* nauplii and dry feed: number of replicate analyses performed by each participant compared to the initial request.

Lab No.	Dry feed		*Artemia*	
	ICES method	Own method	ICES method	Own method
Requested	n=2		2 x 3 (n=1)†	
1	n=2		2 x 3 (n=1)‡	
2	n=2		2 x 1 (n=3)	
3	NA		2 x 3 (n=1)‡	
4*	A: n=6	B: n=6	A: 2 x 3 (n=1)	B: 2 x 3 (n=1)
5		n=2		NA
6		n=4		2 x 2 (n=5)
7		n=2		2 x 3 (n=1)
8		n=3		1 x 3 (n=1)
9	n=3		1 x 3 (n=1)	
10	n=2		2 x 3 (n=1)	
11		n=1		1 x 1 (n=1)
Total participants	5	6	6	5

†: a x b (n=c): a repetitions in time of b hatching incubations with c samples per incubation analyzed
‡: accidental loss of one replicate
*: Laboratory 4 used two procedures: A: ICES method, B: own method
NA: not available

Table 2: Methods for FAME extraction and preparation used by participants

Lab No.	Sample size (mg dry/wet sample)	Extraction/Saponification	Esterification	FAME extraction	Addition of internal standard	References cited
ICES method	150-200/1000	Extraction with $CHCl_3/CH_3OH$ (2:1)	toluene/CH_3OH (2:3) + $AcOCl/CH_3OH$ (5:100) @ 100°C for 1h	hexane	to prepared FAME as FAME	Folch et al. (1957), Ways and Hanahan (1964), Lepage and Roy (1984)
1	150-200/1000	ICES				
2	150-200/1000	ICES, except replacement of toluene by benzene and reduction of reagent volumes with 50%				
3	150-200/1000	ICES				
4A	150-200/1000	ICES				
4B	3-4/10-20	Direct transmethylation with 0.4ml toluene + 1.5ml $AcOCl/CH_3OH$ (5:50) @ 50°C for overnight		toluene	to freeze-dried sample as FFA	Christie (1981), Lepage and Roy (1984), Sukhija and Palmquist (1988), Christie (1989)
5	NS/1000	Saponification	14% BF_3 in CH_3OH	hexane	none	Shanta and Ackman (1990)
6	17-20/17-20	Direct transmethylation with 2N HCl in CH_3OH @ 100°C for 15h		hexane	to sample as FFA	NS
7	50-100/50-100	Saponification with KOH in CH_3OH for 30 min	BF_3 in CH_3OH	isooctane	to sample as FAME	NS
8	<40/NS	Extraction with $CHCl_3/CH_3OH$/water 2:2:1.8	14% BF_3 in CH_3OH	hexane	to freeze-dried sample as FFA or FAME	Bligh and Dyer (1959), Metcalfe et al. (1966)
9	150-200/1000	ICES				
10	130-190/1100	ICES, except transmethylation with 7% BF_3 in CH_3OH @ 100°C for 1h				Bligh and Dyer (1959), Morrison and Smith (1964)
11	NS	Folch modified with CH_2Cl_2 instead of $CHCl_3$, with 100 ppm BHT/Saponification 3 min at 90°C with HCl 6N, extracted with hexane	0.7M HCl in CH_3OH with vit C as antioxydant @ 90°C for 3 min	NS	NS	NS

NS: not specified by participant

Table 3: Instrumentation and operational parameters used by participants.

Lab No.	Gas chromatograph type	Integrator	Cappillary column specifications†	Carrier gas type, pressure, and flow rate	Temperature program	Injection system	Detection system	Internal standard
1	Carlo Erba Mega 5160 HRGC	Spectra Physics 4290	BPX70; SGE Australia 25m x 0.32mm x 0.21mm	H_2, 30 kPa, ± 2ml/min	110-150°C @ 10°C/min 150-168°C @ 3°C/min 168-178°C @ 0.5°C/min	On column	FID	20:2(n-6)
2	Carlo Erba GC6000 Vega	Shimadzu CR4A	PEG immobilised Superox4 10m x 0.32mm x 0.5mm	H_2, 30 kPa, 2.5ml/min	110-150°C @ 10°C/min 150-190°C @ 3°C/min 190-200°C @ 2°C/min 200°C for 13min	Automatic on 25cm precolumn with 10s cooling	FID 230°C	20:2(n-6)
3	Packard Model 436	Shimadzu CR3A	FFAP; Chrompack 50m x 0.22mm	H_2, 150 kPa, 1-2ml/min	50-180°C @ 39°C/min 180-225°C @ 3°C/min 225°C for 15min	On column	FID	20:2(n-6)
4	Perkin-Elmer 8500	built in	SP-2330; Supelco 30m x 0.25mm x 0.2mm	N_2, 10 psig	140-205°C @ 1°C/min	PTV injector 1min @ 45°C, 45-220°C @ 15°C/min	FID 250°C	19:0
5	Varian 3400	Spectra Physics 4270	DB Wax; J&W Scientific 30m x 0.25mm x 0.25mm	He 1.2 ml/min	180-240°C @ 4°C/min	Automatic Split/splitless @ 260°C	FID 250°C	none
6	HP 5890 7673A autosampler	(VG Multichrome data system)	DB23; J&W Scientific 30m x 0.32mm x 0.25mm	He 2.35 ml/min	145-216°C @ 2.8°C/min	Automatic Split/splitless @ 250°C 1min @ 60°C, fast to 145°C	FID 300°C	21:0
7	HP 5880A	NS	SIL88 50m x 0.25mm x 0.25mm	He 1 ml/min	80-220°C @ 5°C/min 220°C for 25min	Automatic Split @ 250°C	FID 300°C	17:0
8	Carlo Erba HRGC 5160	Shimadzu-Chromatopac C-R3A	Omegawax 250, Supelco	H_2	105-195°C @ 25°C/min 195°C for 3min 195-200°C @ 5°C/min 200°C for 38min	On column	FID	21:0
9	Carlo Erba 8560	Spectra Physics 4290	DB Wax; J&W Scientific 30m x 0.25mm	He	80-150°C @ 10°C/min 150-205°C @ 4°C/min 205-220°C @ 6°C/min 220°C for 10min	On column	FID	17:0
10	Varian 3400	Varian IBDH	Omegawax 320, Supelco 30m x 0.32mm	He, 3 ml/min, 50 psig	140-240°C @ 3.5°C/min 240°C for 12.15min	On column	FID 300°C	23:0
11	Packard 427	ENICA 21	CP-WAX-52 CB, Chrompack 25m x 0.32mm x 0.20mm	He, 0.7 bar	Isotherm oven 190°C	NS	FID 220°C	NS

NS: not specified by participant
†: column specifications are indicated as "Stationary phase; origin, length (m) x internal diameter (mm) x film thickness (mm)"

Table 4: Total lipid content (% of dry weight) of *Artemia* nauplii and dry feed. Data are expressed as mean ± standard deviation, with the coefficient of variation given in parentheses [mean ± sd (CV)]. Values in a column with different superscripts are significantly different (ANOVA, Tukey HSD, $P < 0.05$).

Lab No.	Dry feed	n	*Artemia*	n
1	16.63 ± 0.42 (2.6)^{ab}	2	18.13 ± 0.30 (1.7)^{abc}	2
2	15.78 ± 1.21 (7.7)^{ab}	2	16.20 ± 0.54 (3.3)^{a}	6
3	-	-	19.72 ± 0.51 (2.6)^{cd}	6
4	17.00 ± 0.36 (2.1)^{b}	6	21.09 ± 1.09 (5.2)^{d}	6
5	15.65 ± 0.06 (0.4)^{ab}	2	-	-
6	-	-	-	-
7	-	-	-	-
8	17.53 ± 0.34 (2.0)^{b}	3	19.46 ± 0.33 (1.7)^{cd}	3
9	15.25 ± 0.54 (3.5)^{a}	3	16.97 ± 1.06 (6.3)^{ab}	3
10	15.51 ± 1.02 (6.6)^{a}	4	18.46 ± 1.36 (7.4)^{bc}	6
11	15.45†	1	20.01†	1
Intralaboratory CV mean (min-max)	3.6 (0.4-7.7)		4.0 (1.7-7.4)	
Interlaboratory mean ± sd (CV)	16.1 ± 0.8 (5.2)	8	18.8 ± 1.6 (8.7)	8
ANOVA	$F_{6,15} = 5.95^{**}$		$F_{6,25} = 18.24^{***}$	

†: excluded from ANOVA (one replicate analysis only)

Table 5: Fatty acid content for seven major fatty acids in the dry feed (area percent basis). Data for each FAME are expressed as percentage of total FAMEs [mean ± sd (CV)]. Statistical analyses are given in Table 7.

Lab No.	14:0	16:0	16:1(n-7)	18:1(n-7/9)	18:2(n-6)	20:5(n-3)	22:6(n-3)	Intralaboratory CV mean (min-max)	
1	4.82 ± 0.20 (4.2)	13.93 ± 1.07 (7.6)	4.95 ± 0.52 (10.6)	13.12 ± 0.10 (0.8)	5.01 ± 0.23 (4.6)	11.85 ± 0.37 (3.1)	27.91 ± 1.03 (3.7)	5.0 (0.8-10.6)	
2	5.93 ± 0.06 (1.0)	14.12 ± 0.01 (0.1)	5.51 ± 0.08 (1.5)	12.83 ± 0.37 (2.9)	6.42 ± 0.07 (1.1)	12.90 ± 0.11 (0.8)	29.06 ± 0.47 (1.6)	1.3 (0.1-2.9)	
3	-	-	-	-	-	-	-	-	
4A	4.68 ± 0.09 (1.9)	13.45 ± 0.26 (1.9)	4.62 ± 0.05 (1.0)	11.70 ± 0.25 (2.2)	6.22 ± 0.15 (2.4)	11.92 ± 0.16 (1.4)	24.18 ± 0.76 (3.1)	2.0 (1.0-3.1)	
4B	4.55 ± 0.33 (7.2)	15.34 ± 1.00 (6.5)	4.35 ± 0.32 (7.5)	11.77 ± 0.65 (5.5)	6.71 ± 0.40 (5.9)	10.98 ± 0.55 (5.0)	21.55 ± 1.42 (6.6)	6.3 (5.0-7.5)	
5	5.64 ± 0.04 (0.8)	16.14 ± 0.88 (5.4)	5.83 ± 0.01 (0.1)	12.24 ± 0.15 (1.2)	6.53 ± 0.53 (8.1)	11.48 ± 0.45 (3.9)	21.79 ± 0.75 (3.4)	3.3 (0.1-8.1)	
6	5.13 ± 0.02 (0.4)	15.95 ± 0.07 (0.4)	4.90 ± 0.02 (0.3)	12.76 ± 0.04 (0.3)	7.45 ± 0.03 (0.5)	12.27 ± 0.02 (0.2)	26.01 ± 0.07 (0.3)	0.3 (0.2-0.5)	
7	4.80 ± 0.28 (5.9)	14.00 ± 0.85 (6.1)	4.30 ± 0.28 (6.6)	12.95 ± 0.64 (4.9)	9.60 ± 0.28 (2.9)	10.70 ± 0.28 (2.6)	21.95 ± 1.63 (7.4)	5.2 (2.6-7.4)	
8	5.04 ± 0.12 (2.4)	8.59 ± 0.17 (2.0)	6.75 ± 0.31 (4.6)	13.29 ± 0.26 (2.0)	4.46 ± 0.06 (1.2)	8.56 ± 0.19 (2.2)	21.28 ± 0.67 (3.2)	2.5 (1.2-4.6)	
9	4.76 ± 0.22 (4.5)	13.36 ± 0.10 (0.7)	5.10 ± 0.12 (2.4)	12.30 ± 0.11 (0.9)	5.23 ± 0.20 (3.8)	12.82 ± 0.17 (1.3)	29.31 ± 0.36 (1.2)	2.1 (0.7-4.5)	
10	2.61 ± 0.17 (6.5)	12.37 ± 0.84 (6.8)	5.21 ± 0.25 (4.8)	12.41 ± 0.69 (5.5)	6.29 ± 0.47 (7.4)	12.21 ± 0.01 (0.1)	28.53 ± 1.58 (5.5)	5.2 (0.1-7.4)	
11	4.40	13.00	5.10	11.80	6.30	12.40	27.50	-	
Interlaboratory mean ± sd (CV)								Interlaboratory CV mean (min-max)	mean of mean intralab CVs
A/ all laboratories									
	4.76 ± 0.85 (17.8)	13.66 ± 2.06 (15.1)	5.14 ± 0.70 (13.6)	12.47 ± 0.56 (4.5)	6.38 ± 1.36 (21.3)	11.64 ± 1.23 (10.6)	25.37 ± 3.28 (12.9)	13.7 (4.5-21.3)	3.3
B/ laboratories that applied ICES method									
	4.56 ± 1.20 (26.4)	13.44 ± 0.68 (5.1)	5.07 ± 0.33 (6.5)	12.47 ± 0.54 (4.4)	5.83 ± 0.66 (11.3)	12.34 ± 0.50 (4.0)	27.80 ± 2.09 (7.5)	9.3 (4.0-26.4)	3.1
C/ laboratories that applied own method									
	4.93 ± 0.45 (9.1)	13.84 ± 2.84 (20.5)	5.20 ± 0.94 (18.1)	12.47 ± 0.63 (5.1)	6.84 ± 1.68 (24.5)	11.07 ± 1.40 (12.7)	23.35 ± 2.69 (11.5)	14.5 (5.1-24.5)	3.5

Table 6: Fatty acid content for seven major fatty acids and total fatty acids in the dry feed (mg/g dry weight basis). Data are expressed as mg FAME per g dry weight of sample [mean ± sd (CV)]. Statistical analysis is given in Table 7.

Lab No.	14:0	16:0	16:1(n-7)	18:1(n-7/9)	18:2(n-6)	20:5(n-3)	22:6(n-3)	total FAME†	Intralaboratory CV mean (min-max)	
1	6.69 ± 0.16 (2.3)	19.31 ± 1.27 (6.6)	6.87 ± 0.89 (12.9)	18.20 ± 0.39 (2.1)	6.95 ± 0.47 (6.8)	16.43 ± 0.18 (1.1)	38.70 ± 0.99 (2.5)	138.75 ± 3.63 (2.6)	4.9 (1.1-12.9)	
2	5.16 ± 0.35 (6.7)	12.28 ± 0.95 (7.8)	4.79 ± 0.29 (6.1)	11.15 ± 0.53 (4.8)	5.58 ± 0.37 (6.6)	11.22 ± 0.95 (8.5)	25.28 ± 2.35 (9.3)	-	7.1 (4.8-9.3)	
3	-	-	-	-	-	-	-	-	-	
4A	5.86 ± 0.57 (9.7)	16.87 ± 1.90 (11.3)	5.79 ± 0.60 (10.3)	14.74 ± 1.57 (10.6)	7.81 ± 0.94 (12.1)	14.93 ± 1.48 (9.9)	30.34 ± 3.23 (10.6)	-	10.6 (9.7-12.1)	
4B	5.52 ± 0.13 (2.3)	19.20 ± 1.55 (8.1)	5.42 ± 0.24 (4.3)	14.71 ± 0.64 (4.3)	8.38 ± 0.33 (3.9)	13.68 ± 0.62 (4.5)	26.76 ± 1.86 (6.9)	-	4.9 (2.3-8.1)	
5	-	-	-	-	-	-	-	-	-	
6	5.33 ± 0.52 (9.8)	16.58 ± 1.59 (9.6)	5.09 ± 0.49 (9.6)	13.26 ± 1.24 (9.4)	7.73 ± 0.71 (9.2)	12.75 ± 1.22 (9.6)	27.02 ± 2.55 (9.4)	-	9.5 (9.2-9.8)	
7	5.66 ± 0.03 (0.5)	16.48 ± 0.11 (0.6)	5.09 ± 0.02 (0.4)	15.25 ± 0.30 (1.9)	11.37 ± 0.44 (3.9)	12.39 ± 1.62 (13.1)	26.02 ± 3.77 (14.5)	117.50 ± 7.78 (6.6)	5.0 (0.4-14.5)	
8	8.23 ± 0.13 (1.6)	14.02 ± 0.32 (2.3)	11.02 ± 0.61 (5.5)	21.68 ± 0.07 (0.3)	7.29 ± 0.24 (3.3)	13.98 ± 0.14 (1.0)	34.76 ± 1.88 (5.4)	163.24 ± 3.72 (2.3)	2.8 (0.3-5.5)	
9	4.28 ± 0.12 (2.9)	12.03 ± 0.73 (6.1)	4.59 ± 0.27 (5.9)	11.08 ± 0.81 (7.3)	4.71 ± 0.36 (7.7)	11.55 ± 0.69 (5.9)	26.41 ± 1.84 (7.0)	91.90 ± 7.54 (8.2)	6.1 (2.9-7.7)	
10	2.01 ± 0.28 (13.8)	9.48 ± 1.34 (14.2)	3.99 ± 0.48 (12.1)	9.51 ± 1.23 (12.9)	4.83 ± 0.71 (14.8)	9.34 ± 0.70 (7.5)	21.77 ± 0.42 (1.9)	76.48 ± 5.70 (7.5)	11.0 (1.9-14.8)	
11	5.20	15.30	6.00	13.90	7.40	14.60	32.40	117.79	-	
Interlaboratory mean ± sd (CV)									Interlaboratory CV mean (min-max)	mean of mean intralab CVs
A/ all laboratories										
	5.39 ± 1.59 (29.5)	15.15 ± 3.19 (21.1)	5.86 ± 1.98 (33.8)	14.35 ± 3.58 (25.0)	7.20 ± 1.94 (27.0)	13.09 ± 2.07 (15.8)	28.94 ± 5.07 (17.5)	117.61 ± 31.25 (26.6)	24.5 (15.8-33.8)	6.9
B/ laboratories that applied ICES method										
	4.80 ± 1.80 (37.4)	13.99 ± 3.99 (28.5)	5.20 ± 1.14 (21.8)	12.94 ± 3.51 (27.2)	5.97 ± 1.36 (22.7)	12.69 ± 2.90 (22.9)	28.50 ± 6.47 (22.7)	102.38 ± 32.43 (31.7)	26.9 (21.8-37.4)	8.0
C/ laboratories that applied own method										
	5.99 ± 1.27 (21.1)	16.31 ± 1.92 (11.7)	6.52 ± 2.54 (39.0)	15.76 ± 3.40 (21.5)	8.44 ± 1.70 (20.1)	13.48 ± 0.90 (6.7)	29.39 ± 3.93 (13.4)	132.84 ± 26.33 (19.8)	19.2 (6.7-39.0)	5.6

†: total FAME data are excluded from the calculation of mean intralaboratory and interlaboratory CVs

Table 7: Statistical analysis for the intralaboratory means given in Tables 5 and 6 on the fatty acid content for seven major fatty acids in the dry feed. Means in a column with different superscripts are significantly different (ANOVA, Tukey HSD, $P < 0.05$).

Lab No.	14:0	16:0	16:1(n-7)	18:1(n-7/9)	18:2(n-6)	20:5(n-3)	22:6(n-3)	total FAME
A: area percent								
1	4.82^{bc}	13.93^{bc}	4.95^{abc}	13.12^{b}	5.01^{a}	11.85^{c}	27.91^{de}	
2	5.93^{e}	14.12^{bcd}	5.51^{cd}	12.83^{ab}	6.42^{b}	12.90^{d}	29.06^{e}	
3	-	-	-	-	-	-	-	
4A	4.68^{b}	13.45^{b}	4.62^{ab}	11.70^{a}	6.22^{b}	11.92^{c}	24.18^{bc}	
4B	4.55^{b}	15.34^{cd}	4.35^{a}	11.77^{a}	6.71^{b}	10.98^{b}	21.55^{a}	
5	5.64^{de}	16.14^{d}	5.83^{d}	12.24^{ab}	6.53^{b}	11.48^{bc}	21.79^{ab}	
6	5.13^{cd}	15.95^{d}	4.90^{abc}	12.76^{ab}	7.45^{c}	12.27^{cd}	26.01^{cd}	
7	4.80^{bc}	14.00^{bcd}	4.30^{a}	12.95^{b}	9.60^{d}	10.70^{b}	21.95^{ab}	
8	5.04^{bcd}	8.59^{a}	6.75^{e}	13.29^{b}	4.46^{a}	8.56^{a}	21.28^{a}	
9	4.76^{bc}	13.36^{b}	5.10^{abcd}	12.30^{ab}	5.23^{a}	12.82^{d}	29.31^{e}	
10	2.61^{a}	12.37^{b}	5.21^{bcd}	12.41^{ab}	6.29^{b}	12.21^{cd}	28.53^{de}	
11‡	4.40	13.00	5.10	11.80	6.30	12.40	27.50	
ANOVA: $F_{9,23}$	41.33***	33.78***	27.43***	7.59***	71.52***	46.20***	34.20***	
B: mg/g dry weight								
1	6.69^{d}	19.31^{d}	6.87^{c}	18.20^{d}	6.95^{bc}	16.43^{d}	38.70^{d}	138.75^{c}
2	5.16^{bc}	12.28^{ab}	4.79^{ab}	11.15^{ab}	5.58^{ab}	11.22^{ab}	25.28^{ab}	-
3	-	-	-	-	-	-	-	-
4A	5.86^{cd}	16.87^{cd}	5.79^{bc}	14.74^{c}	7.81^{c}	14.93^{cd}	30.34^{bc}	-
4B	5.52^{c}	19.20^{d}	5.42^{b}	14.71^{c}	8.38^{c}	13.68^{bc}	26.76^{ab}	-
5	-	-	-	-	-	-	-	-
6	5.33^{c}	16.58^{cd}	5.09^{ab}	13.26^{bc}	7.73^{c}	12.75^{bc}	27.02^{ab}	-
7	5.66^{cd}	16.48^{bcd}	5.09^{ab}	15.25^{cd}	11.37^{d}	12.39^{abc}	26.02^{ab}	117.50^{b}
8	8.23^{e}	14.02^{bc}	11.02^{d}	21.68^{e}	7.29^{bc}	13.98^{bcd}	34.76^{cd}	163.24^{d}
9	4.28^{b}	12.03^{ab}	4.59^{ab}	11.08^{ab}	4.71^{a}	11.55^{ab}	26.41^{ab}	91.90^{a}
10	2.01^{a}	9.48^{a}	3.99^{a}	9.51^{a}	4.83^{a}	9.34^{a}	21.77^{a}	76.48^{a}
11‡	5.20	15.30	6.00	13.90	7.40	14.60	32.40	117.79
ANOVA: $F_{8,22}$†	55.07***	16.71***	50.37***	38.05***	27.34***	12.18***	13.44***	98.41***

†: except total FAME $F_{4,8}$

‡: excluded from ANOVA (one replicate analysis only)

Table 8: Fatty acid content for six major fatty acids in *Artemia* nauplii (area percent basis). Data for each FAME are expressed as percentage of total FAMEs [mean ± sd (CV)]. Statistical analyses are given in Table 10.

Lab No.	16:0	16:1(n-7)	18:1(n-7/9)	18:2(n-6)	18:3(n-3)	20:5(n-3)	Intralaboratory CV mean (min-max)	
1	11.26 ± 0.18 (1.6)	7.33 ± 0.23 (3.1)	23.51 ± 0.23 (1.0)	4.47 ± 0.06 (1.2)	17.90 ± 0.27 (1.5)	8.60 ± 0.25 (2.9)	1.9 (1.0-3.1)	
2	11.72 ± 0.26 (2.2)	9.03 ± 0.35 (3.9)	26.67 ± 3.56 (13.3)	4.48 ± 0.10 (2.3)	20.58 ± 0.40 (1.9)	9.84 ± 0.18 (1.8)	4.3 (1.8-13.3)	
3	10.71 ± 0.24 (2.2)	7.13 ± 0.15 (2.0)	24.48 ± 0.14 (0.6)	3.96 ± 0.05 (1.2)	17.58 ± 0.31 (1.8)	8.71 ± 0.09 (1.0)	1.5 (0.6-2.2)	
4A	10.96 ± 0.12 (1.1)	7.39 ± 0.10 (1.4)	24.89 ± 0.19 (0.7)	3.78 ± 0.04 (1.1)	17.40 ± 0.40 (2.3)	9.18 ± 0.68 (7.4)	2.3 (0.7-7.4)	
4B	12.02 ± 0.22 (1.9)	7.07 ± 0.22 (3.2)	23.06 ± 2.73 (11.8)	3.71 ± 0.06 (1.7)	17.11 ± 0.66 (3.9)	7.89 ± 0.36 (4.6)	4.5 (1.7-11.8)	
5	-	-	-	-	-	-	-	
6	12.50 ± 0.15 (1.2)	7.44 ± 0.12 (1.6)	26.12 ± 0.17 (0.6)	4.19 ± 0.03 (0.6)	19.68 ± 0.48 (2.4)	8.50 ± 0.07 (0.9)	1.2 (0.6-2.4)	
7	11.18 ± 0.08 (0.7)	7.57 ± 0.12 (1.6)	25.53 ± 0.23 (0.9)	4.10 ± 0.00 (0.0)	17.47 ± 0.27 (1.6)	8.40 ± 0.28 (3.3)	1.3 (0.0-3.3)	
8	9.50 ± 0.47 (4.9)	8.03 ± 0.27 (3.3)	24.62 ± 1.85 (7.5)	4.61 ± 0.22 (4.8)	16.88 ± 0.59 (3.5)	11.80 ± 0.97 (8.3)	5.4 (3.3-8.3)	
9	11.67 ± 0.21 (1.8)	7.52 ± 0.02 (0.3)	24.80 ± 0.29 (1.2)	3.99 ± 0.03 (0.8)	19.43 ± 0.16 (0.8)	9.36 ± 0.17 (1.8)	1.1 (0.3-1.8)	
10	11.11 ± 0.35 (3.1)	8.03 ± 0.38 (4.7)	26.18 ± 0.31 (1.2)	4.26 ± 0.19 (4.5)	19.01 ± 0.39 (2.0)	9.27 ± 0.22 (2.4)	3.0 (1.2-4.7)	
11	10.90	8.00	24.20	3.90	18.30	9.40	-	
Interlaboratory mean ± sd (CV)							Interlaboratory CV mean (min-max)	mean of mean intralab CVs
A/ all laboratories								
	11.23 ± 0.78 (7.0)	7.68 ± 0.56 (7.3)	24.91 ± 1.13 (4.5)	4.13 ± 0.30 (7.2)	18.30 ± 1.20 (6.6)	9.18 ± 1.03 (11.3)	7.3 (4.5-11.3)	2.7
B/ laboratories that applied ICES method								
	11.24 ± 0.40 (3.6)	7.74 ± 0.70 (9.1)	25.09 ± 1.16 (4.6)	4.15 ± 0.29 (7.0)	18.65 ± 1.24 (6.7)	9.16 ± 0.45 (5.0)	6.0 (3.6-9.1)	2.4
C/ laboratories that applied own method								
	11.22 ± 1.15 (10.3)	7.62 ± 0.40 (5.3)	24.71 ± 1.19 (4.8)	4.10 ± 0.34 (8.3)	17.89 ± 1.14 (6.4)	9.20 ± 1.55 (16.9)	8.7 (4.8-16.9)	3.1

Table 9: Fatty acid content for six major fatty acids and total fatty acids in *Artemia* nauplii (mg/g dry weight basis). Data are expressed as mg FAME per g dry weight of sample [mean ± sd (CV)]. Statistical analyses are given in Table 10.

Lab No.	16:0	16:1(n-7)	18:1(n-7/9)	18:2(n-6)	18:3(n-3)	20:5(n-3)	Total FA	Intralaboratory CV mean (min-max)	
1	14.49 ± 1.22 (8.41)	9.43 ± 0.93 (9.91)	30.25 ± 2.57 (8.49)	5.75 ± 0.46 (8.04)	23.03 ± 1.78 (7.73)	11.08 ± 1.09 (9.80)	128.66 ± 10.83 (8.42)	8.73 (7.73-9.91)	
2	12.02 ± 1.05 (8.75)	9.37 ± 0.92 (9.86)	28.63 ± 2.08 (7.27)	4.56 ± 0.39 (8.59)	20.99 ± 1.73 (8.24)	10.11 ± 0.67 (6.63)	-	8.22 (6.63-9.86)	
3	13.86 ± 0.99 (7.18)	9.21 ± 0.59 (6.41)	31.59 ± 1.76 (5.58)	5.11 ± 0.32 (6.16)	22.68 ± 0.98 (4.30)	11.24 ± 0.61 (5.43)	-	5.84 (4.30-7.18)	
4A	16.10 ± 0.29 (1.79)	10.85 ± 0.26 (2.38)	33.94 ± 5.42 (15.97)	5.56 ± 0.16 (2.85)	25.88 ± 0.66 (2.55)	13.49 ± 0.97 (7.22)	-	5.46 (1.79-15.97)	
4B	18.22 ± 0.88 (4.84)	10.71 ± 0.54 (5.09)	36.53 ± 1.92 (5.26)	5.62 ± 0.29 (5.23)	25.94 ± 1.68 (6.49)	11.72 ± 0.56 (4.75)	-	5.27 (4.75-6.49)	
5	-	-	-	-	-	-	-	-	
6	13.78 ± 0.35 (2.55)	8.20 ± 0.13 (1.64)	28.81 ± 0.78 (2.71)	4.61 ± 0.16 (3.51)	21.72 ± 1.19 (5.46)	9.37 ± 0.24 (2.54)	109.58 ± 2.77 (2.53)	3.07 (1.64-5.46)	
7	-	-	-	-	-	-	80.48 ± 0.92 (1.15)	-	
8	11.46 ± 1.20 (10.51)	9.68 ± 0.87 (8.95)	29.74 ± 3.80 (12.79)	5.55 ± 0.28 (5.00)	20.35 ± 1.67 (8.22)	14.19 ± 0.87 (6.16)	120.48 ± 6.70 (5.56)	8.60 (5.00-12.79)	
9	12.34 ± 0.54 (4.41)	7.94 ± 0.24 (2.96)	26.21 ± 0.82 (3.12)	4.22 ± 0.09 (2.24)	20.52 ± 0.40 (1.93)	9.89 ± 0.42 (4.22)	112.34 ± 2.88 (2.56)	3.15 (1.93-4.41)	
10	12.10 ± 1.16 (9.58)	8.74 ± 0.80 (9.21)	28.48 ± 2.20 (7.74)	4.62 ± 0.29 (6.32)	20.68 ± 1.64 (7.93)	10.10 ± 0.97 (9.64)	108.83 ± 8.82 (8.10)	8.40 (6.32-9.64)	
11	13.80	10.20	30.80	4.90	23.20	12.00	126.96	-	
Interlaboratory mean ± sd (CV)								Interlaboratory CV mean (min-max)	mean of mean intralab CVs
A/ all laboratories									
	13.82 ± 2.08 (15.1)	9.43 ± 0.98 (10.3)	30.50 ± 2.96 (9.7)	5.05 ± 0.54 (10.8)	22.50 ± 2.08 (9.2)	11.32 ± 1.58 (14.0)	†112.48 ± 16.23 (14.4)	11.5 (9.2-15.1)	6.3
B/ laboratories that applied ICES method									
	13.48 ± 1.64 (12.1)	9.26 ± 0.96 (10.4)	29.85 ± 2.70 (9.1)	4.97 ± 0.60 (12.2)	22.30 ± 2.05 (9.2)	10.99 ± 1.35 (12.3)	116.61 ± 10.58 (9.1)	10.9 (9.1-12.3)	6.6
C/ laboratories that applied own method									
	14.31 ± 2.82 (19.7)	9.70 ± 1.08 (11.2)	31.47 ± 3.47 (11.0)	5.17 ± 0.49 (9.5)	22.80 ± 2.40 (10.5)	11.82 ± 1.97 (16.7)	109.37 ± 20.55 (18.8)	13.1 (9.5-19.7)	5.7

†: total FAME data are excluded from the calculation of mean intralaboratory and interlaboratory CVs

Table 10: Statistical analyses for the intralaboratory means given in Tables 8 and 9 on the fatty acid content for six major fatty acids in *Artemia* nauplii. Means in a column with different superscripts are significantly different (ANOVA, Tukey HSD, $P < 0.05$).

Lab No.	16:0	16:1(n-7)	18:1(n-7/9)	18:2(n-6)	18:3(n-3)	20:5(n-3)	total FAME†
A: area percent							
1	11.26^{cde}	7.33^{ab}	23.51^{ab}	4.47^{f}	17.90^{a}	8.60^{abc}	
2	11.72^{ef}	9.03^{e}	26.67^{b}	4.45^{f}	20.58^{c}	9.84^{d}	
3	10.71^{b}	7.13^{ab}	24.48^{ab}	3.96^{bc}	17.58^{a}	8.71^{bc}	
4A	10.96^{bc}	7.39^{ab}	24.89^{ab}	3.78^{ab}	17.40^{a}	9.18^{cd}	
4B	12.02^{fg}	7.07^{a}	23.06^{a}	3.71^{a}	17.11^{a}	7.89^{a}	
5	-	-	-	-	-	-	
6	12.50^{ef}	7.44^{abc}	26.12^{ab}	4.19^{de}	19.68^{bc}	8.50^{abc}	
7	11.18^{bcd}	7.57^{bcd}	25.53^{ab}	4.10^{cde}	17.47^{a}	8.40^{ab}	
8	9.50^{a}	8.03^{cd}	24.62^{ab}	4.61^{f}	16.88^{a}	11.80^{e}	
9	11.67^{def}	7.52^{abcd}	24.80^{ab}	3.99^{bcd}	19.43^{b}	9.36^{cd}	
10	11.11^{bc}	8.03^{d}	26.18^{ab}	4.26^{e}	19.01^{b}	9.27^{cd}	
11‡	10.90	8.00	24.20	3.90	18.30	9.40	
ANOVA: $F_{9,40}$	45.11***	35.18***	2.77*	47.11***	45.95***	30.11***	
B: mg/g DW							
1	14.49^{cd}	9.43^{ab}	30.25^{ab}	5.75^{d}	23.03^{a}	11.08^{ab}	128.66^{c}
2	12.02^{ab}	9.37^{a}	28.63^{ab}	4.56^{ab}	20.99^{a}	10.11^{ab}	-
3	13.86^{bc}	9.21^{a}	31.59^{abc}	5.11^{bc}	22.68^{a}	11.24^{b}	-
4A	16.10^{d}	10.85^{c}	33.94^{bc}	5.56^{cd}	25.88^{b}	13.49^{c}	-
4B	18.22^{e}	10.71^{bc}	36.53^{c}	5.62^{cd}	25.94^{b}	11.72^{b}	-
5	-	-	-	-	-	-	-
6	13.78^{abc}	8.20^{a}	28.81^{ab}	4.61^{ab}	21.72^{a}	9.37^{a}	109.58^{b}
7	-	-	-	-	-	-	80.48^{a}
8	11.46^{a}	9.68^{abc}	29.74^{ab}	5.55^{cd}	20.35^{a}	14.19^{c}	120.48^{bc}
9	12.34^{abc}	7.94^{a}	26.21^{a}	4.22^{a}	20.52^{a}	9.89^{ab}	112.34^{b}
10	12.10^{ab}	8.74^{a}	28.48^{ab}	4.62^{ab}	20.68^{a}	10.10^{ab}	108.83^{b}
11‡	13.80	10.20	30.80	4.90	23.20	12.00	126.96
ANOVA: $F_{8,34}$†	29.56***	10.71***	6.09***	16.07***	12.30***	18.52***	30.57***

†: except total FAME $F_{5,21}$
‡: excluded from ANOVA (one replicate analysis only)

Table 11: Comparison of mean intralaboratory and interlaboratory coefficient of variation reported by participants that followed the ICES Standard Method and those that used their own method for the fatty acid analysis in dry feed and *Artemia* nauplii. Number of contributions on which the CV is based is given in parentheses [n].

	Mean intralaboratory CV†		Mean interlaboratory CV‡	
	area%	mg/g DW	area%	mg/g DW
Participants following ICES Standard Method				
Dry feed	3.1 [5]	8.0 [5]	9.3 [7]	26.9 [7]
Artemia nauplii	2.4 [6]	6.6 [6]	6.0 [6]	10.9 [6]
Participants following own method				
Dry feed	3.5 [5]	5.6 [4]	14.5 [7]	19.2 [7]
Artemia nauplii	3.1 [4]	5.7 [3]	8.7 [6]	13.1 [6]

†: intralaboratory means of the CVs of each fatty acid, averaged for n laboratories
‡: interlaboratory CV for each fatty acid, averaged for n fatty acids

Table 12: Comparison of mean intralaboratory and interlaboratory coefficient of variation observed in the present intercalibration exercise and the one organized by Léger et al. (1989) on fatty acid analysis. Number of contributions on which the CV is based is given in parentheses [n].

	Mean intralaboratory CV†		Mean interlaboratory CV‡	
	area%	mg/g DW	area%	mg/g DW
Present intercalibration exercise				
Dry feed	3.3 [10]	6.9 [9]	13.7 [7]	24.5 [7]
Artemia nauplii	2.7 [10]	6.3 [9]	7.3 [6]	11.5 [6]
Léger et al. (1989)				
decapsulated *Artemia* cysts	4.6 [9]	7.9 [6]	16.8 [6]	42.0 [6]
Artemia nauplii	4.9 [9]	10.3 [6]	18.1 [6]	24.5 [6]

†: intralaboratory means of the CVs of each fatty acid, averaged for n laboratories
‡: interlaboratory CV for each fatty acid, averaged for n fatty acids

9. ADDENDUM I:
LIST OF PARTICIPANTS

Participants are ordered by country independent from their reference number in the report.

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10. ADDENDUM II:
INSTRUCTIONS PROVIDED TO THE PARTICIPANTS IN THE INTER-CALIBRATION EXERCISE

Samples to be analysed:

1. Dry formulated feed, high in (n-3) HUFA. Two vacuum-packed samples are provided: one 10-g and one 5-g sample. Store in refrigerated conditions. Use immediately upon opening. Perform two complete analyses (repeated in time). Evenso, take at least three samples of approximately 200 mg each and run dry weight analyses (oven-drying, 60°C, 24 hrs).

2. Freshly-hatched *Artemia* nauplii. Three 10-g vacuum sealed cyst samples are provided. Hatching needs to be performed under standard conditions:

a. The nauplii hatched from 3 g cysts are to be used for one HUFA-analysis. Hatching should be performed under standard conditions: 35 ppt (natural or artificial) seawater, constant temperature of 28°C, continuous illumination of min. 1000 lux, funnel-shaped hatching device with aeration from the bottom of the cone, 3g cysts in 1 l.

b. After 24 h incubation, remove aeration and let stand for 5 to 10 minutes. A separation will occur between the empty cyst shells, floating at the surface, and the freshly-hatched nauplii, sinking to the bottom.

c. Siphon the nauplii from the bottom over a sieve of 200-300 mm. Rinse with tapwater to remove all salts. Dip-dry bottom of sieve with paper towel to wet-dry the naupliar biomass. Transfer at least 3 g wet-dry naupliar biomass in a dark glass vial. Flush with nitrogen and store until analysis at -30°C in a freezer. Take (representative) sample for lipid analysis and also run dry weight analysis.

Perform three hatching incubations at a time (to verify variability) and repeat once in time. Correct water content in the analytical data for the naupliar biomass and express results in mg/g DW.

Expected data:

hatching run 1: 3 separate analyses (DW+GC)
hatching run 2: 3 separate analyses

Add copy of chromatograms and report results in area percent and mg/g DW following the examples given in Annexe 2.1 and 2.2. to the ICES-Standard Methodology.

Provide the following details of gas chromatograph used:

- Gas chromatograph type
- Cappillairy column specifications
- Carrier gas type, pressure and flow rate
- Temperature program
- Injection system
- Detection system
- Internal standard used for quantitation

11. ADDENDUM III:
ICES-STANDARD METHODOLOGY FOR (N-3) HUFA ANALYSIS

1. Total lipid extraction procedure

Modified procedure of Ways and Hanahan (1964)
Schematic outline of procedure is given in fluxogram A.

The lipids are extracted according to Folch et al. (1957) with a binary solvent mixture (2 $CHCl_3$: 1 CH_3OH).
A 0.1 M or 0.745 % KCl solution is added to separate the accompanied non lipid substances.

For this, the dry sample (100 mg; see Note 1.1.) or wet sample (1 g; see Note 1.1.; accurate dry weight analysis of identical sample is required!) is transferred into a centrifuge tube (50 ml) and thoroughly homogenised in 30 ml solvent mixture (2 $CHCl_3$: 1 CH_3OH) for 1 minute using a Kinematica Polytron PT 10 S (4000 rpm) or analogous equipment. The residue is separated by centrifugation at 4000 rpm for 5 minutes and the supernatant is transferred into a separatory funnel containing 40 ml KCl 0.1 M or 0.745 %.

Another two re-extractions are done by adding the same amount of solvent to the sediment each time. The final proportions $CHCl_3$, CH_3OH, H_2O are 10:5:3v/v. Shake the funnel for approximately 1-2 min. After two more minutes a separation will occur:

- phase above : H_2O-CH_3OH-KCl
- phase below : $CHCl_3$ - lipids

The $CHCl_3$-lipid fraction is then filtered through a water free Na_2SO_4-filter (see Note 1.2.) into a vacuum proof flask, first without vacuum and afterwards with vacuum.
This filter is then rinsed with $CHCl_3$. The water-methanol fraction is re-extracted with $CHCl_3$. The $CHCl_3$-lipid fraction is filtered through a waterfree Na_2SO_4-filter. This filter is then rinsed again with $CHCl_3$.

The combined $CHCl_3$-fractions are evaporated till nearly dry using a Büchi rotavapor (vacuum-evaporator). Temperature 30°C.

The lipids are then dissolved in $CHCl_3$ and rinsed over a waterfree Na_2SO_4-filter in a pear-shaped flask (50 ml) of a known weight.

The extract is evaporated till near dryness and the remaining solvents are flushed out with nitrogen (± 10 min). Optional: the amount of the total lipids is determined gravimetrically and expressed in percent. The amount of lipids is also necessary to calculate the dilution factor for GC-injection.

The lipids are transferred with 5 times 1 ml of methanol/toluene solution (3:2 v/v) (see Note 1.3.) in a pyrex centrifuge glass-tube with a teflon lined cap. The tube is then flushed with nitrogen. Storage in a freezer (-30°C) is recommended.

Note 1.1.: Amount of sample to be analysed for total lipid extraction.

In order to obtain accurate and reproducible results, the amount of sample is calculated taking into account, the total lipid content. About thirty mg of fat is proposed for lipid extractions and subsequent fatty acid analyses. The total lipid (TL) content is important for determining the quantity of internal standard to be added. See examples in following table.

Product	weight of sample (g)	approx. dry weight (%)	approx. total lipid content (%)	absolute TL content (mg)
Artemia Instar I	± 1.0	± 12	± 15-20	± 25
24-h enriched *Artemia*	± 1.0	± 9	± 25-30	± 30
Emulsion/oil	± 0.040	± 85-100	± 85-100	± 30-40
Formulated feed	± 0.2	± 98	± 15	± 30

Note 1.2.: Preparation of a waterfree Na_2SO_4-filter
- Take the filter-funnel of Æ 70 mm or 55 mm (porosity n° 3)
- Put Na_2SO_4 in the filter
- Wash the Na_2SO_4 with $CHCl_3$, under vacuum. Washing of the Na_2SO_4-filter is not necessary if anhydrous Na_2SO_4 of pro analysis quality is used.

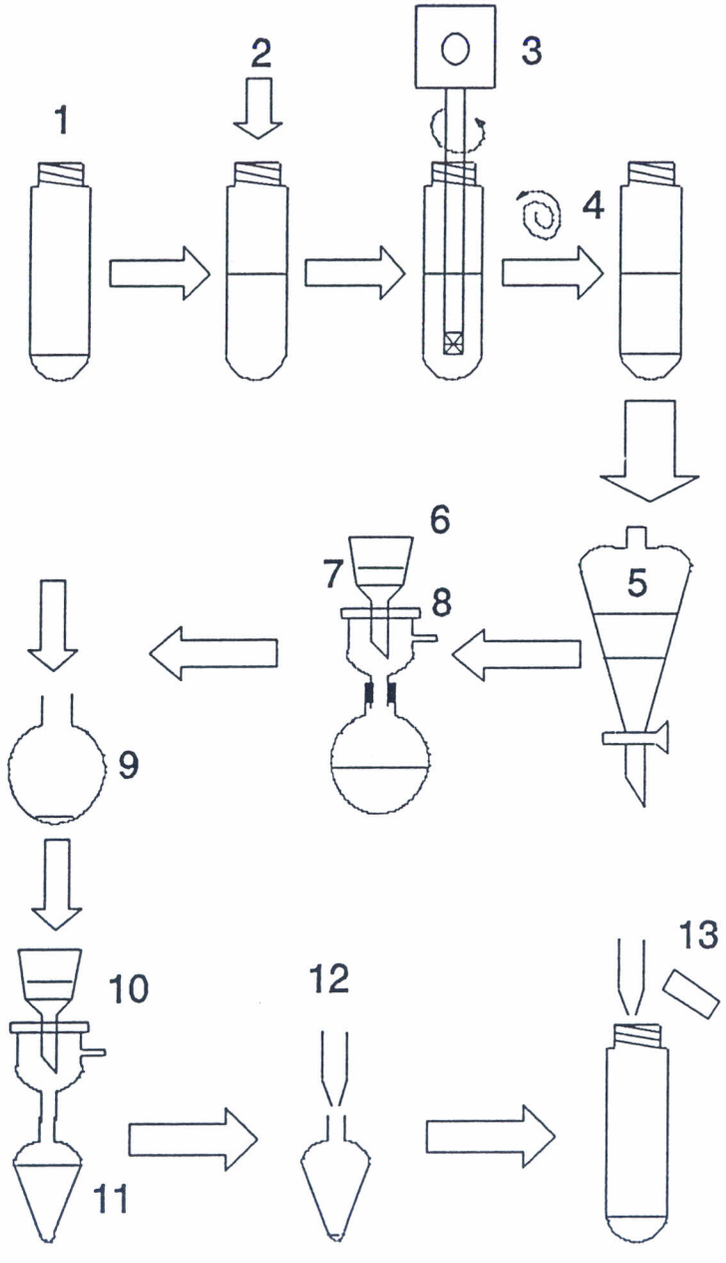
Note 1.3.:
Because of safety and health reasons, toluene is proposed instead of benzene (former procedure). The toxicity of toluene is much lower compared to benzene. The substitution can be done without modifications to the extraction or esterification procedures. However, it might be necessary to prolong nitrogen flushing time (due to the higher boiling point of toluene compared to benzene) at the end of the esterification procedure to remove all remaining solvents.

List of chemical products

Product	Chemical formula	Purity specification
Chloroform	$CHCl_3$	for liquid chromatography
Methanol	CH_3OH	for liquid chromatography
Potassium chloride	KCl	extra pure
Sodium sulphate	Na_2SO_4	anhydrous, pro analysis
Toluene	C_7H_8	for liquid chromatography
Nitrogen gas	N_2	very pure

Remark : All recipients used in the procedure should be made out of glass. The use of plastic ware should be avoided. Contaminants might be extracted from the plastic and distort peak quantitation.

Fluxogram A



Legend to numbers of fluxogram A

1. Centrifuge glass-tube with a teflon-lined screw cap, containing dry or wet sample
2. Add 30 ml of solvent mixture($2CHCl_3$:CH_3OH) to sample
3. Homogenizer Kinematic Polytron PT 10 S (4000 rpm)
4. Centrifuge
5. Separatory funnel (100 ml)
6. Filter funnel, diam. 70 mm, coarse n°3
7. Na_2SO_4 anhydrous pro analysis
8. Rubber device for vacuum sealing and adaptor unit
9. 500 ml flask (vacuumproof)
10. Filter funnel, diam. 55 mm, coarse n°3 and adaptor unit
11. Pear-shaped flask
12. Flush remaining solvents with nitrogen
13. Transfer to centrifuge glass tube, flush with nitrogen, close and transfer to freezer at -30°C.

2. Esterification procedure

Modified procedure of Lepage and Roy (1984)
Schematic outline of procedure is given in fluxogram B.

According to Lepage & Roy (1984) and Christie (1981) most total lipids can be esterified directly. Therefore the saponification step is superfluous. Christie demonstrated that a good esterification can be achieved with an acetylchloride/methanol mixture (5:100 v/v).

Step by step description of the method

- Dissolve the dry lipids with 5 ml methanol/toluene solution (3:2 v/v) in a pyrex centrifuge glass-tube with a teflon lined screw cap.
- Add 5 ml of a freshly prepared acetylchloride/methanol mixture (5:100 v/v). When preparing the mixture, add acetyl chloride slowly to cooled methanol (to prevent splashing).
- Flush the tube with nitrogen, close off well and shake.
- Place the tube for 60 minutes in a boiling water bath (100°C) and shake regularly.
- Cool down the glass tube.
- Add 5 ml hexane and 5 ml distilled H_2O.
- Add 0.2 ml internal standard solution (see Note 2.1.).
- Centrifugate the glass-tube during 5 minutes (4000 rpm).
- Transfer the hexane phase into a pear shaped flask.
- Repeat the hexane extraction three times.
- Filtrate the hexane phase through a waterfree Na_2SO_4-filter (see Note 1.2.) into a vacuum proof pearshaped flask (elimination of possible H_2O contamination).
- The hexane phase is then evaporated till near dryness using a Büchi rotavapor (vacuum/evaporator). Temperature 30°C.
- Flush with nitrogen to remove remaining solvents.
- Dissolve the FAMEs with 1 ml hexane or iso-octane and transfer to a 2 ml amber vial with a screw cap and a teflon-faced silicone septaliner.
- Flush with nitrogen and store in a freezer (-30°C).

List of chemical products

Product	Chemical formula	Purity specification
Acetyl chloride	C_2H_3ClO	pro-analysis
Methanol	CH_3OH	for liquid chromatography
Hexane	CH_3-$(CH_2)_4$-CH_3	for liquid chromatography
Internal standard	cis 11, 14 eicosadiënoate	Nu-Chek-Prep U-68-M
Sodium sulphate	Na_2SO_4	anhydrous, pro analysis
Isooctane	CH_3-CH_3CH-$(CH_2)_4$-CH_3 (C 2,2,4-trimethylperitane)	for liquid chromatography

Note 2.1.: Internal standard and preparation of sample for GC-analysis

2.1.1. Criteria for selecting a suitable internal standard (I.S.)

- The I.S. should not be present in the extracted sample.
- The retention time of the I.S. should be in between the retention time of the first and last important peak.
- The I.S. should be chosen in function of the column. Care should be taken that no overlap occurs with other FAME-peaks present in the sample.
- e.g. 20:2n-6 or 19:0 fulfill these conditions for the given column (see under 3).

2.1.2. Preparation of the internal standard

The I.S., methyl 11, 14 eicosadiënoate (20:2n-6) is dissolved in iso-octane (b.p. 99°C) in a final, accurate concentration of 5 mg per ml. The I.S. solution should be flushed with nitrogen and stored at -30°C in a freezer.

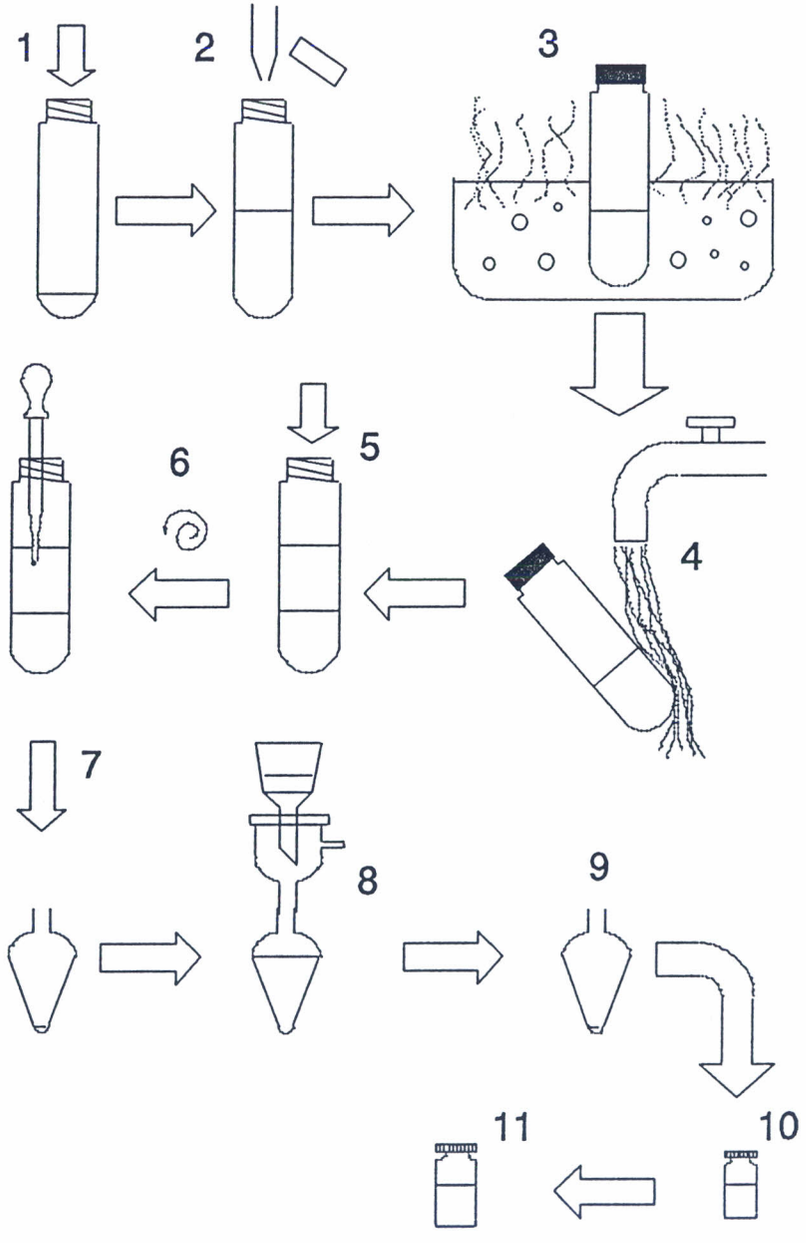
2.1.3. Preparation of the sample for G.C.-analysis

0.2 ml of the I.S.-solution is introduced in the esterified total lipid sample. Before introduction of the I.S., which is kept in the freezer at -30°C, room temperature equilibration is imperative.
The relation of the I.S. with the rest of the FAMEs is approximately 3-5%.
To obtain this ratio, 1 mg is added to approx. 20-30 mg esterified total lipids.
The required concentration of the FAMEs for capillary column injection is ± 2 mg/ml. For this we dilute the concentrated FAMEs solution prior to injection (dilution factor is ± 1:15). For the on-column injection on a capillary column we inject 0.2 ml of this dilution, which is also prepared in iso-octane. This means we inject ± 0.4 mg directly into the capillary column.

2.1.4. Data expression and treatment

The data for each FAME are expressed as percentage of total FAMEs (relative values) and as mg per g dry weight of tissue (absolute values) (see example of chromatogram and of data reporting in annex 2).

Fluxogram B



Legend to numbers of fluxogram B

1. Add 5 ml acetylchloride/methanol mixture (5:100 v/v) into the centrifuge glass tube
2. Flush with nitrogen and close screw cap
3. Place for 60 minutes in boiling waterbath
4. Cool down the tube
5. Add 5 ml hexane, 5 ml distilled water and exactly 0.2 ml Internal standard solution
6. Centrifuge for 5 minutes at 4000 rpm
7. Transfer hexane phase into pear-shaped flask
8. Filtrate hexane phase through waterfree Na_2SO_4 filter
9. Vacuum evaporate the FAMEs, flush with nitrogen
10. Dissolve FAMEs with 1 ml of hexane or iso-octane and transfer into 2 ml vial, flush with nitrogen, store in freezer.
11. Prepare fresh dilution of FAMEs to ± 2mg/ml and inject on the capillary column

3. Example of gaschromatographic conditions currently in use at the Artemia Reference Center.

Fatty acid methylesters are injected on a capillary column (25m fused silica, i.d. : 0.32 mm, liquid phase: BPX70 (very polar) SGE Australia, film thickness: 0.21 mm) installed in a Carlo Erba Mega 5160 HRGC gas chromatograph.

Operating conditions are as follows: on column injection, carrier gas: hydrogen (30 kPa), flow rate : ± 2 ml/min, FID detection oven temperature program: 110°C to 150°C at 10°C/min, 150°C to 168°C at 3°C/min and 168°C to 178°C at 0.5°C/min.

Peak identification and quantification is done with a calibrated plotter integrator (Spectra Physics SP 4290) and reference standards for the most common fatty acid methyl esters.
These include 14:0, 14:1(n-5), 16:0, 16:1(n-7), 18:0, 18:1(n-9), 18:1(n-7), 18:2(n-6), 18:3(n-6), 18:3(n-3), 20:0, 20:1(n-9), 20:2(n-6), 20:3(n-6), 20:4(n-6), 20:3(n-3), 22:0, 22:1(n-9), 22:3(n-3), 22:4(n-6), 24:0, 24:1(n-9), 20:5(n-3) and 22:6(n-3).

Other specific columns available in the market in which FAME's and non- saponifiable material do not co-elute can also be used. Gas chromatographic conditions can differ depending on the column being used.

4. Use of calibration sample for gas chromatograph

Proper operation of the gas chromatograph can be verified by the use of a reference standard suitable for marine HUFA analyses. For this we have selected the GLC-standard GLC-68-B (methylesters) of the NU-CHECK-PREP company, P.O. Box 295, Elysian, MN 56028, USA, fax +1-507-267-4790. European distributor: Bast of Copenhagen, 44 Ingerslevsgade DK-1705, Copenhagen V, Denmark, fax +45-3131-9364) Costs are US$ 35.00 per 100 mg (+US$ 13.00 airmail shipment). Composition and sample chromatogram are added in annex 1. The standard is to be diluted with 5 ml of isooctane, to a concentration of 20 mg/ml. The solution is stored in a dark vial, flushed with nitrogen and kept in the freezer at -30°C until use. Before injection, the standard solution has to be diluted 40 times with isooctane, to a final concentration of 0.5 mg/ml. Of this solution, 0.2 ml is injected into the gas chromatograph.

Literature cited

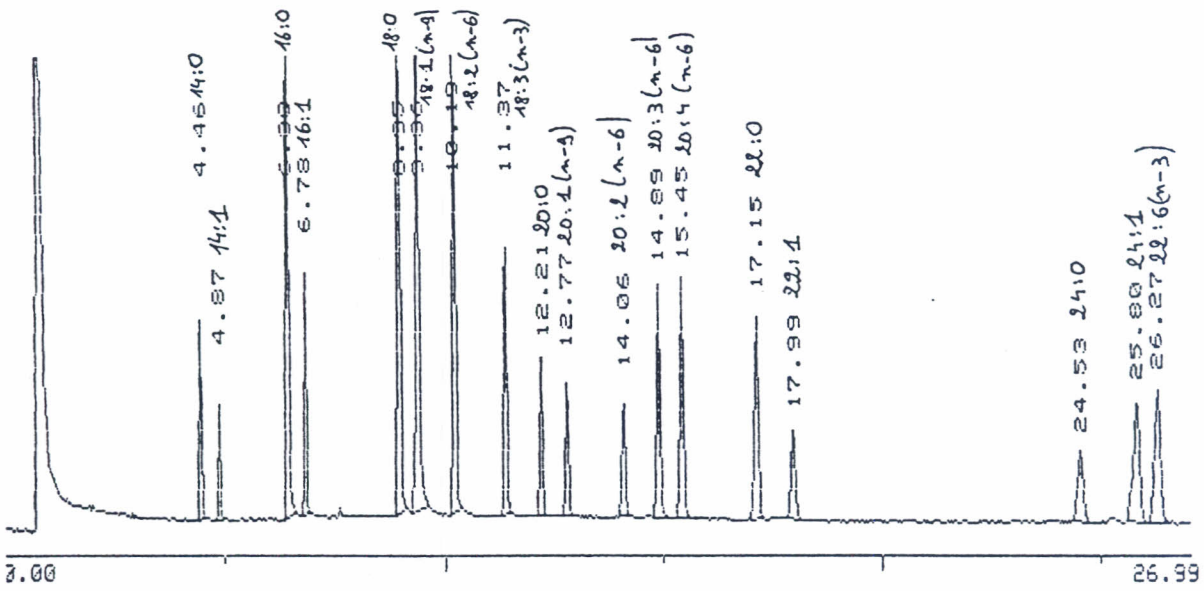
Christie, W.W., - 1981. Lipid analysis. Pergamon Press, Oxford UK, 2nd edition

Folch, J., Lees, M., and Sloane-Stanley, G.H., - 1957. J. Biological Chemistry 225:497-509.

Lepage, G. and Roy, C.C., - 1984. J. Lipid Research 25; 1391-1396

Ways, P. and Hanahan, D.J., - 1964. J. Lipid Research 5: 318.

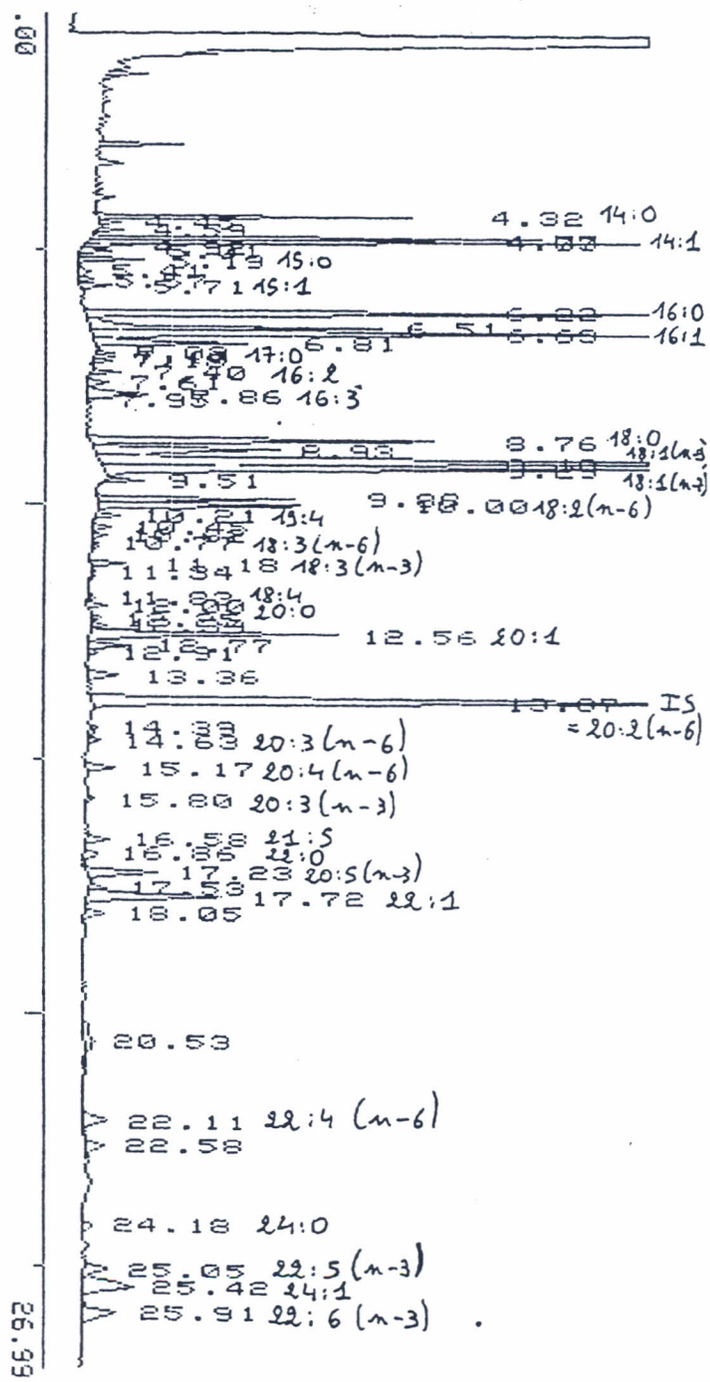
Chromatogram and composition of GLC-standard GLC-68-B (methylesters) of the NU CHECK PREP company.



Peak	% known	% found
14:0	3.0	2.9
14:1	1.0	1.0
16:0	10.0	10.4
16:1	2.0	2.0
18:0	15.0	14.9
18:1(n-9)	25.0	25.8
18:2(n-6)	10.0	10.3
18:3(n-3)	4.0	3.9
20:0	2.0	1.9
20:1(n-9)	2.0	1.9
20:2(n-6)	2.0	2.0
20:3(n-6)	4.0	3.9
20:4(n-6)	4.0	3.9
22:0	4.0	3.9
22:1(n-9)	2.0	1.8
24:0	2.0	2.0
24:1(n-9)	4.0	3.6
22:6(n-3)	4.0	3.9

Annex 2.1.

Example of fatty acid analysis of *Brachionus plicatilis*: chromatogram



Annex 2.2.

Example of fatty acid analysis of *Brachionus plicatilis*: data reporting. Analyses were performed on 1.6918 g live-weight *Brachionus* with a dry weight content of 6.52%. Analytical data were corrected for expression of results on a dry weight basis.

Fatty acid	Area %	mg/g DW
14:0	2.0	1.2
14:1	3.1	1.9
15:0	0.5	0.3
15:1	0.3	0.2
16:0	7.2	4.4
16:1	15.3	9.3
17:0	0.1	0.1
16:2	0.3	0.2
16:3	1.1	0.7
18:0	4.3	2.6
18:1(n-9)	25.6	15.6
18:1(n-7)	6.9	4.2
18:2(n-6)	2.5	1.5
19:4	0.2	0.1
18:3(n-6)	0.2	0.1
18:3(n-3)	0.5	0.3
20:0	0.2	0.1
20:1(n-9)	2.8	1.7
20:3(n-6)	0.1	0.1
20:4(n-6)	0.4	0.2
21:5	0.3	0.2
22:0	0.2	0.1
20:5(n-3)	1.1	0.7
22:1	2.7	1.7
22:4(n-6)	0.6	0.4
24:0	0.2	0.1
22:5(n-3)	0.5	0.3
24:1(n-9)	1.6	1.0
22:6(n-3)	0.8	0.5
Sum (n-3) HUFA $\geq$ 20:3(n-3)	2.4	1.5