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

prepared by

Peter Coutteau and Patrick Sorgeloos
Laboratory of Aquaculture & Artemia Reference Center
University of Gent, Rozier 44, B-9000 Gent, Belgium

on behalf of the

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edited by

Bari Howell, Yngvar Olsen and José Iglesias

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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, JOLR, 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 intercalibration 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 methylster of the NU-CHECK-PREP Co) and two intercalibration 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 nauplius 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 Exercise

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 intercalibration 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 transesterification (Laboratory Nos. 5, 7) and micro methods 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-acetel chloride at 24-26°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 intercalibration 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 intercalibration exercise.

4.4 Results

Results are expressed as means ± standard deviation (Tables 5 and 6). The results are shown in Tables 8 and 9. The inter-laboratory variation for the percent recovery of the standards was approximately 2.7% for the fatty acids, 2.0% for the total lipids, 1.6% for the percent recovery of the lipid to fatty acid ratio. The individual laboratory results based on the Standard Methods and the qualitative and quantitative results are shown in Table 7.
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-bounded 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 benefited 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 Figure 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 standardized 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 intercalibration 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
quantitative 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.

6 ACKNOWLEDGEMENTS

We greatly acknowledge the participants for the time they
took to carry out their part in this exercise. This study was
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Research (FWO; Peter Couteau is a Senior Research
Assistant with the NFWO). The authors wish to thank
Geert Van de Wiele for his practical assistance in the
organization of this exercise.

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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Rearing of Juvenile Marine Fish to the Mariculture

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STP 1027
American
USA.

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extraction.

Metcalfe, K. Preparation of dry feed and fatty acid
extraction.

Morrison, J. Preparation of dry feed and fatty acid
extraction.

Shanthan, A. Preparation of dry feed and fatty acid
extraction.


Figure 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).

![Graph showing comparison of fatty acid content between Artemia nauplii and dry diet](image-url)
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.

<table>
<thead>
<tr>
<th>Lab No.</th>
<th>Dry feed</th>
<th>Artemia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ICES method</td>
<td>Own method</td>
</tr>
<tr>
<td>Requested</td>
<td>n=2</td>
<td>2 x 3 (n=1)†</td>
</tr>
<tr>
<td>1</td>
<td>n=2</td>
<td>2 x 3 (n=1)‡</td>
</tr>
<tr>
<td>2</td>
<td>n=2</td>
<td>2 x 1 (n=3)</td>
</tr>
<tr>
<td>3</td>
<td>NA</td>
<td>2 x 3 (n=1)‡</td>
</tr>
<tr>
<td>4*</td>
<td>A: n=6</td>
<td>B: n=6</td>
</tr>
<tr>
<td>5</td>
<td>n=2</td>
<td>NA</td>
</tr>
<tr>
<td>6</td>
<td>n=4</td>
<td>2 x 2 (n=5)</td>
</tr>
<tr>
<td>7</td>
<td>n=2</td>
<td>2 x 3 (n=1)</td>
</tr>
<tr>
<td>8</td>
<td>n=3</td>
<td>1 x 3 (n=1)</td>
</tr>
<tr>
<td>-9</td>
<td>n=3</td>
<td>1 x 3 (n=1)</td>
</tr>
<tr>
<td>10</td>
<td>n=2</td>
<td>2 x 3 (n=1)</td>
</tr>
<tr>
<td>11</td>
<td>n=1</td>
<td>1 x 1 (n=1)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Lab No.</th>
<th>Sample size (mg dry/wet sample)</th>
<th>Extraction/Saponification</th>
<th>Esterification</th>
<th>FAME extraction</th>
<th>Addition of internal standard</th>
<th>References cited</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICES</td>
<td>150-200/1000</td>
<td>Extraction with CHCl₃/CH₃OH (2:1)</td>
<td>toluene/CH₃OH (2.3) + AcOCl/CH₃OH (5:100) @ 100°C for 1h</td>
<td>hexane</td>
<td>to prepared FAME as FAME</td>
<td>Folch et al. (1957), Wys and Hanahan (1966), Lepage and Roy (1984)</td>
</tr>
<tr>
<td>2</td>
<td>150-200/1000</td>
<td>ICES, except replacement of toluene by benzene and reduction of reagent volumes with 50%</td>
<td>hexane</td>
<td>none</td>
<td>Shanta and Ackman (1990)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>150-200/1000</td>
<td>ICES</td>
<td>hexane</td>
<td>to sample as FFA</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>4A</td>
<td>150-200/1000</td>
<td>ICES</td>
<td>to sample as FAME</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4B</td>
<td>3-4/10-20</td>
<td>Direct transmethylation with 0.4ml toluene + 1.5ml AcOCl/CH₃OH (5:50) @ 50°C for overnight</td>
<td>toluene</td>
<td>to freeze-dried sample as FFA or FAME</td>
<td>Bligh and Dyer (1959), Metcalfe et al. (1966)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>NS/1000</td>
<td>Saponification</td>
<td>hexane</td>
<td>none</td>
<td>Shanta and Ackman (1990)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>17-20/17-20</td>
<td>Direct transmethylation with 2N HCl in CH₃OH @ 100°C for 15h</td>
<td>hexane</td>
<td>to sample as FFA</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>50-100/50-100</td>
<td>Saponification with KOH in CH₃OH for 30 min</td>
<td>hexane</td>
<td>to sample as FAME</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>&lt;40/NS</td>
<td>Extraction with CHCl₃/CH₃OH/water</td>
<td>14% BF₃ in CH₃OH</td>
<td>hexane</td>
<td>Bligh and Dyer (1959), Morrison and Smith (1984)</td>
<td></td>
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<tr>
<td>9</td>
<td>150-200/1000</td>
<td>ICES</td>
<td>14% BF₃ in CH₃OH</td>
<td>hexane</td>
<td>Bligh and Dyer (1959), Morrison and Smith (1984)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>130-190/1100</td>
<td>ICES, except transmethylation with 5% BF₃ in CH₃OH @ 100°C for 1h</td>
<td>hexane</td>
<td>to freeze-dried sample as FFA or FAME</td>
<td>Bligh and Dyer (1959), Morrison and Smith (1984)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>NS</td>
<td>Folch modified with CH₂Cl₂ instead of CHCl₃, with 100 ppm l-HCl, with 3 min at 90°C with HCl 6N, extracted with hexane</td>
<td>0.7M HCl in CH₃OH with vit C as antioxidant @ 90°C for 3 min</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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NS: not specified by participant

### Table 3  
Instrumentation and operational parameters used by participants

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<tr>
<th>Lab</th>
<th>Gas chromatograph type</th>
<th>Injector</th>
<th>Capillary column</th>
<th>Carrier gas type</th>
<th>Temperature program</th>
<th>Injection system</th>
<th>Detector</th>
<th>Internal</th>
<th>External</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>Lab No.</td>
<td>Gas chromatography type</td>
<td>Integrator</td>
<td>Capillary column specifications</td>
<td>Carrier gas type, pressure, and flow rate</td>
<td>Temperature program</td>
<td>Injection system</td>
<td>Detection system</td>
<td>Internal standard</td>
<td></td>
</tr>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>Carlo Erba Mega 5160 HRGC</td>
<td>Spectra Physics 4290</td>
<td>BPX70; SGE Australia 25m x 0.32mm x 0.21mm</td>
<td>He, 30 kPa, 2.0ml/min</td>
<td>110-150°C; @ 10°C/min, 150-168°C; @ 3°C/min, 168-178°C; @ 0.5°C/min</td>
<td>On column</td>
<td>FID</td>
<td>20.2(±6)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Carlo Erba GC6000 Vega</td>
<td>Shimadzu CR4A</td>
<td>PEG immobilised Superoxel 10m x 0.32mm x 0.5mm</td>
<td>He, 30 kPa, 2.5ml/min</td>
<td>110-150°C; @ 10°C/min, 150-190°C; @ 3°C/min, 190-200°C; @ 2°C/min, 200°C for 13min</td>
<td>Automatic on 25cm precolumn with 10b cooling</td>
<td>FID 230°C</td>
<td>20.2(±6)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Packard Model 436</td>
<td>Shimadzu CR3A</td>
<td>FFAP; Chrompack 50m x 0.22mm</td>
<td>He, 150 kPa, 1-2ml/min</td>
<td>50-180°C; @ 3°C/min, 180-225°C; @ 3°C/min, 225°C for 15min</td>
<td>On column</td>
<td>FID</td>
<td>20.2(±6)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Perkin-Elmer 8500</td>
<td>built in</td>
<td>SP-2330, Supelco 30m x 0.25mm x 0.22mm</td>
<td>N2, 10 psig</td>
<td>140-205°C; @ 1°C/min</td>
<td>PTV injector</td>
<td>FID 250°C</td>
<td>19.0</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Varian 3400</td>
<td>Spectra Physics 4270</td>
<td>DB Wax; J&amp;W Scientific 30m x 0.25mm x 0.25mm</td>
<td>He</td>
<td>1.2 ml/min</td>
<td>Automatic Split/splitless @ 260°C</td>
<td>FID 250°C</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>HP 5890 7673A autosampler</td>
<td>(VG Multichrom data system)</td>
<td>DB23; J&amp;W Scientific</td>
<td>He</td>
<td>2.35 ml/min</td>
<td>Automatic Split/splitless @ 250°C, 1min @ 60°C, fast to 145°C</td>
<td>FID 300°C</td>
<td>21.0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>HP 5880A</td>
<td>NS</td>
<td>SIL88 50m x 0.25mm x 0.25mm</td>
<td>He</td>
<td>1 ml/min</td>
<td>80-220°C; @ 5°C/min, 220°C for 25min</td>
<td>Automatic Split @ 250°C</td>
<td>FID 300°C</td>
<td>17.0</td>
</tr>
<tr>
<td>8</td>
<td>Carlo Erba HRGC 5160</td>
<td>Shimadzu-Chromatopac C-R3A</td>
<td>Omegawax 250, Supelco</td>
<td>He</td>
<td>105-195°C; @ 25°C/min, 195°C for 3min, 195-200°C; @ 5°C/min, 200°C for 38min</td>
<td>On column</td>
<td>FID</td>
<td>21.0</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Carlo Erba 8560</td>
<td>Spectra Physics 4290</td>
<td>DB Wax; J&amp;W Scientific 30m x 0.25mm</td>
<td>He</td>
<td>80-150°C; @ 10°C/min, 150-205°C; @ 4°C/min, 205-220°C; @ 2°C/min, 220°C for 10min</td>
<td>On column</td>
<td>FID</td>
<td>17.0</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Varian 3400</td>
<td>Varian BDH</td>
<td>Omegawax 320, Supelco 30m x 0.22mm</td>
<td>He, 3 ml/min, 50 psig</td>
<td>140-240°C; @ 3.5°C/min, 240°C for 12.15min</td>
<td>On column</td>
<td>FID 300°C</td>
<td>23.0</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Packard 427</td>
<td>ENICA 21</td>
<td>CP-WAX-52 CB, Chrompack 25m x 0.32mm x 0.20mm</td>
<td>He, 0.7 bar</td>
<td>Isotherm oven 190°C</td>
<td>NS</td>
<td>FID 220°C</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

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).

<table>
<thead>
<tr>
<th>Lab No.</th>
<th>Dry feed</th>
<th>n</th>
<th>Artemia</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.63 ± 0.42 (2.6)\textsuperscript{a}</td>
<td>2</td>
<td>18.13 ± 0.30 (1.7)\textsuperscript{ab}</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>15.78 ± 1.21 (7.7)\textsuperscript{a}</td>
<td>2</td>
<td>16.20 ± 0.54 (3.3)\textsuperscript{b}</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>19.72 ± 0.51 (2.6)\textsuperscript{ad}</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>17.00 ± 0.36 (2.1)\textsuperscript{b}</td>
<td>6</td>
<td>21.09 ± 1.09 (5.2)\textsuperscript{b}</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>15.65 ± 0.06 (0.4)\textsuperscript{b}</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>17.53 ± 0.34 (2.0)\textsuperscript{b}</td>
<td>3</td>
<td>19.46 ± 0.33 (1.7)\textsuperscript{ad}</td>
<td>3</td>
</tr>
<tr>
<td>9</td>
<td>15.25 ± 0.54 (3.5)\textsuperscript{b}</td>
<td>3</td>
<td>16.97 ± 1.06 (6.3)\textsuperscript{b}</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>15.51 ± 1.02 (6.6)\textsuperscript{b}</td>
<td>4</td>
<td>18.46 ± 1.36 (7.4)\textsuperscript{b}</td>
<td>6</td>
</tr>
<tr>
<td>11</td>
<td>15.45\textsuperscript{b}</td>
<td>1</td>
<td>20.01\textsuperscript{b}</td>
<td>1</td>
</tr>
</tbody>
</table>

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  \( F_{8,15} = 5.95^{**} \)  \( F_{6,23} = 18.24^{***} \)

\( \dagger \): 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.

<table>
<thead>
<tr>
<th>Lab No.</th>
<th>14:0</th>
<th>16:0</th>
<th>16:1(n-7)</th>
<th>18:1(n-7:9)</th>
<th>18:2(n-6)</th>
<th>20:5(n-3)</th>
<th>22:6(n-3)</th>
<th>Intralaboratory CV mean (min-max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.82 ± 0.20 (4.2)</td>
<td>13.91 ± 1.07 (7.6)</td>
<td>4.95 ± 0.52 (10.6)</td>
<td>13.12 ± 0.10 (0.8)</td>
<td>5.01 ± 0.22 (4.6)</td>
<td>11.85 ± 0.37 (3.1)</td>
<td>27.91 ± 1.03 (3.7)</td>
<td>5.0 (0.8-10.6)</td>
</tr>
<tr>
<td>2</td>
<td>5.95 ± 0.06 (1.8)</td>
<td>14.12 ± 0.01 (0.1)</td>
<td>5.51 ± 0.08 (1.5)</td>
<td>12.83 ± 0.37 (2.9)</td>
<td>6.42 ± 0.07 (1.1)</td>
<td>12.90 ± 0.11 (0.8)</td>
<td>29.06 ± 0.47 (1.6)</td>
<td>1.3 (0.1-2.9)</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4A</td>
<td>4.68 ± 0.09 (1.9)</td>
<td>13.45 ± 0.26 (1.9)</td>
<td>4.62 ± 0.05 (1.0)</td>
<td>11.70 ± 0.25 (2.3)</td>
<td>6.22 ± 0.15 (2.4)</td>
<td>11.92 ± 0.16 (1.8)</td>
<td>24.18 ± 0.76 (3.1)</td>
<td>2.0 (0.3-3.1)</td>
</tr>
<tr>
<td>4B</td>
<td>4.55 ± 0.33 (7.2)</td>
<td>15.34 ± 1.00 (0.5)</td>
<td>4.35 ± 0.32 (7.5)</td>
<td>11.77 ± 0.65 (5.5)</td>
<td>6.71 ± 0.40 (5.9)</td>
<td>10.98 ± 0.55 (5.0)</td>
<td>21.55 ± 1.42 (6.6)</td>
<td>6.3 (5.0-7.5)</td>
</tr>
<tr>
<td>5</td>
<td>5.64 ± 0.04 (0.8)</td>
<td>16.14 ± 0.88 (5.4)</td>
<td>5.83 ± 0.01 (0.1)</td>
<td>12.24 ± 0.13 (1.2)</td>
<td>6.53 ± 0.53 (8.1)</td>
<td>11.48 ± 0.45 (3.9)</td>
<td>21.79 ± 0.75 (3.4)</td>
<td>3.3 (1.8-5.1)</td>
</tr>
<tr>
<td>6</td>
<td>5.13 ± 0.02 (0.4)</td>
<td>15.95 ± 0.07 (0.6)</td>
<td>4.90 ± 0.02 (0.3)</td>
<td>12.76 ± 0.04 (0.3)</td>
<td>7.45 ± 0.03 (0.5)</td>
<td>12.27 ± 0.02 (0.2)</td>
<td>26.01 ± 0.07 (0.3)</td>
<td>0.3 (0.2-0.5)</td>
</tr>
<tr>
<td>7</td>
<td>4.80 ± 0.28 (5.9)</td>
<td>14.00 ± 0.85 (6.1)</td>
<td>4.30 ± 0.28 (6.6)</td>
<td>12.95 ± 0.64 (4.9)</td>
<td>9.60 ± 0.28 (2.9)</td>
<td>10.70 ± 0.28 (2.6)</td>
<td>21.95 ± 1.63 (7.4)</td>
<td>5.2 (2.6-7.4)</td>
</tr>
<tr>
<td>8</td>
<td>5.04 ± 0.12 (2.4)</td>
<td>8.59 ± 0.17 (2.0)</td>
<td>6.75 ± 0.31 (4.6)</td>
<td>13.29 ± 0.26 (2.0)</td>
<td>4.46 ± 0.06 (1.2)</td>
<td>8.56 ± 0.19 (2.2)</td>
<td>21.28 ± 0.67 (2.3)</td>
<td>2.5 (1.2-4.6)</td>
</tr>
<tr>
<td>9</td>
<td>4.76 ± 0.22 (4.5)</td>
<td>13.36 ± 0.10 (0.7)</td>
<td>5.10 ± 0.12 (2.4)</td>
<td>12.30 ± 0.11 (0.9)</td>
<td>5.23 ± 0.20 (3.8)</td>
<td>12.82 ± 0.17 (1.3)</td>
<td>29.31 ± 0.36 (1.2)</td>
<td>2.1 (0.7-4.5)</td>
</tr>
<tr>
<td>10</td>
<td>2.61 ± 0.17 (6.5)</td>
<td>12.37 ± 0.84 (6.5)</td>
<td>5.21 ± 0.25 (4.8)</td>
<td>12.41 ± 0.69 (5.8)</td>
<td>6.29 ± 0.47 (7.4)</td>
<td>12.21 ± 0.01 (0.1)</td>
<td>28.53 ± 1.58 (5.5)</td>
<td>5.2 (1.0-7.4)</td>
</tr>
<tr>
<td>11</td>
<td>4.40 ± 1.00 (2.0)</td>
<td>13.00 ± 0.00 (1.8)</td>
<td>5.10 ± 0.00 (1.1)</td>
<td>11.80 ± 0.00 (1.8)</td>
<td>6.30 ± 0.00 (1.1)</td>
<td>12.40 ± 0.00 (1.1)</td>
<td>27.50 ± 0.00 (1.1)</td>
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</tr>
</tbody>
</table>
## 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.

<table>
<thead>
<tr>
<th>Lab No</th>
<th>14:0</th>
<th>16:0</th>
<th>16:1(n-7)</th>
<th>18:0(n-9)</th>
<th>18:2(n-6)</th>
<th>20:5(n-3)</th>
<th>22:6(n-3)</th>
<th>Total FAME</th>
<th>Intralaboratory CV mean (min-max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.69 ± 0.16</td>
<td>19.31 ± 1.27</td>
<td>6.87 ± 0.89</td>
<td>18.20 ± 0.39</td>
<td>6.95 ± 0.47</td>
<td>16.43 ± 0.18</td>
<td>38.70 ± 0.99</td>
<td>138.75 ± 3.63</td>
<td>4.9 (1.1-12.9)</td>
</tr>
<tr>
<td>2</td>
<td>5.16 ± 0.35</td>
<td>12.28 ± 0.95</td>
<td>4.79 ± 0.29</td>
<td>11.15 ± 0.53</td>
<td>5.58 ± 0.37</td>
<td>11.22 ± 0.95</td>
<td>25.28 ± 2.35</td>
<td>-</td>
<td>7.1 (4.8-9.3)</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4A</td>
<td>5.86 ± 0.57</td>
<td>16.87 ± 1.90</td>
<td>5.79 ± 0.60</td>
<td>14.74 ± 1.57</td>
<td>7.81 ± 0.94</td>
<td>14.93 ± 1.48</td>
<td>30.34 ± 3.23</td>
<td>-</td>
<td>10.6 (9.7-12.1)</td>
</tr>
<tr>
<td>4B</td>
<td>5.52 ± 0.13</td>
<td>19.20 ± 1.55</td>
<td>5.42 ± 0.24</td>
<td>14.71 ± 0.64</td>
<td>8.38 ± 0.33</td>
<td>13.68 ± 0.62</td>
<td>26.76 ± 1.86</td>
<td>-</td>
<td>4.9 (2.8-3.1)</td>
</tr>
<tr>
<td>5</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>6</td>
<td>5.33 ± 0.52</td>
<td>16.58 ± 1.59</td>
<td>5.09 ± 0.49</td>
<td>13.26 ± 1.24</td>
<td>7.73 ± 0.71</td>
<td>12.75 ± 1.22</td>
<td>27.02 ± 2.55</td>
<td>-</td>
<td>9.5 (9.2-9.8)</td>
</tr>
<tr>
<td>7</td>
<td>5.66 ± 0.03</td>
<td>14.88 ± 0.11</td>
<td>5.09 ± 0.02</td>
<td>15.25 ± 0.30</td>
<td>11.37 ± 0.44</td>
<td>12.39 ± 1.62</td>
<td>26.02 ± 3.77</td>
<td>117.50 ± 7.78</td>
<td>0.0 (4.4-14.5)</td>
</tr>
<tr>
<td>8</td>
<td>8.23 ± 0.13</td>
<td>14.02 ± 0.32</td>
<td>11.02 ± 0.61</td>
<td>21.68 ± 0.07</td>
<td>7.29 ± 0.24</td>
<td>13.98 ± 0.14</td>
<td>34.76 ± 1.88</td>
<td>163.24 ± 3.72</td>
<td>2.8 (0.3-5.5)</td>
</tr>
<tr>
<td>9</td>
<td>4.28 ± 0.12</td>
<td>12.03 ± 0.73</td>
<td>4.59 ± 0.27</td>
<td>11.08 ± 0.81</td>
<td>4.71 ± 0.36</td>
<td>11.55 ± 0.69</td>
<td>26.41 ± 1.84</td>
<td>91.90 ± 7.54</td>
<td>6.1 (2.9-7.7)</td>
</tr>
<tr>
<td>10</td>
<td>2.01 ± 0.28</td>
<td>9.48 ± 1.34</td>
<td>3.99 ± 0.48</td>
<td>9.51 ± 1.23</td>
<td>4.83 ± 0.71</td>
<td>9.34 ± 0.70</td>
<td>21.77 ± 0.42</td>
<td>76.48 ± 5.70</td>
<td>11.0 (1.1-14.8)</td>
</tr>
<tr>
<td>11</td>
<td>5.20</td>
<td>15.30</td>
<td>6.00</td>
<td>13.90</td>
<td>7.40</td>
<td>14.60</td>
<td>32.40</td>
<td>117.79</td>
<td>-</td>
</tr>
</tbody>
</table>

### Interlaboratory mean ± sd (CV)

<table>
<thead>
<tr>
<th>A/ All laboratories</th>
<th>5.39 ± 1.59</th>
<th>15.15 ± 3.19</th>
<th>5.86 ± 1.98</th>
<th>14.35 ± 3.58</th>
<th>7.20 ± 1.94</th>
<th>13.09 ± 2.07</th>
<th>28.94 ± 5.07</th>
<th>117.61 ± 31.25</th>
<th>6.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>B/ Laboratories that applied ICES method</td>
<td>4.80 ± 1.80</td>
<td>13.99 ± 3.99</td>
<td>5.20 ± 1.14</td>
<td>12.94 ± 3.51</td>
<td>5.97 ± 1.36</td>
<td>12.69 ± 2.90</td>
<td>28.50 ± 6.47</td>
<td>102.38 ± 32.43</td>
<td>8.0</td>
</tr>
<tr>
<td>C/ Laboratories that applied own method</td>
<td>5.09 ± 1.27</td>
<td>16.31 ± 1.92</td>
<td>6.52 ± 2.54</td>
<td>15.76 ± 3.40</td>
<td>8.44 ± 1.70</td>
<td>13.48 ± 0.90</td>
<td>29.39 ± 3.93</td>
<td>132.84 ± 26.33</td>
<td>5.6</td>
</tr>
</tbody>
</table>

Notes:
- Total FAME data are excluded from the calculation of mean intralaboratory and interlaboratory CVs.
<table>
<thead>
<tr>
<th>Lab No.</th>
<th>14:0 (n=7)</th>
<th>16:0 (n=7)</th>
<th>16:1 (n=7/9)</th>
<th>18:1 (n=6)</th>
<th>18:2 (n=3)</th>
<th>20:5</th>
<th>22:6 (n=3)</th>
<th>Total FAME</th>
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</tr>
<tr>
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<td>13.93&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>4.95&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>13.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.85&lt;sup&gt;c&lt;/sup&gt;</td>
<td>27.91&lt;sup&gt;bc&lt;/sup&gt;</td>
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<td>14.12&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>5.51&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>12.83&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>6.42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.90&lt;sup&gt;c&lt;/sup&gt;</td>
<td>29.06&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>11.70&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.22&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>4.35&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.77&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.71&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>12.24&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.48&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21.79&lt;sup&gt;bc&lt;/sup&gt;</td>
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<td>15.95&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.90&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>12.76&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.27&lt;sup&gt;c&lt;/sup&gt;</td>
<td>26.01&lt;sup&gt;cd&lt;/sup&gt;</td>
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<td>4.46&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.56&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21.28&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
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<td>4.76&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>13.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.10&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>12.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.23&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>5.21&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>12.41&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.29&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>6.30</td>
<td>12.40</td>
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ANOVA: *F<sub>9.23</sub> = 41.33<sup>***</sup> 33.78<sup>***</sup> 27.43<sup>***</sup> 7.59<sup>***</sup> 71.52<sup>***</sup> 46.20<sup>***</sup> 34.20<sup>***</sup> 100<sup>***</sup>

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<tr>
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<th>2</th>
<th>3</th>
<th>4A</th>
<th>4B</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
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<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.69&lt;sup&gt;d&lt;/sup&gt;</td>
<td>12.28&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.09&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>13.26&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>7.73&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12.75&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.43&lt;sup&gt;d&lt;/sup&gt;</td>
<td>14.72&lt;sup&gt;d&lt;/sup&gt;</td>
<td>14.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.02&lt;sup&gt;d&lt;/sup&gt;</td>
<td>21.68&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.98&lt;sup&gt;c&lt;/sup&gt;</td>
<td>26.04&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

ANOVA: *F<sub>9.22</sub> = 55.07<sup>***</sup> 16.71<sup>***</sup> 50.37<sup>***</sup> 38.05<sup>***</sup> 27.34<sup>***</sup> 12.18<sup>***</sup> 13.44<sup>***</sup> 98.41<sup>***</sup> 100<sup>***</sup>

<sup>†</sup>: except total FAME *F<sub>14</sub>

<sup>‡</sup>: 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.

<table>
<thead>
<tr>
<th>Lab No.</th>
<th>16:0</th>
<th>16:1(n-7)</th>
<th>18:1(n-9)/9</th>
<th>18:2(n-6)</th>
<th>18:3(n-3)</th>
<th>20:5(n-3)</th>
<th>Intralaboratory CV mean (min-max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.26 ± 0.18 (1.6)</td>
<td>7.33 ± 0.23 (3.1)</td>
<td>23.51 ± 0.23 (1.0)</td>
<td>4.47 ± 0.06 (1.2)</td>
<td>17.90 ± 0.27 (1.5)</td>
<td>8.60 ± 0.25 (2.9)</td>
<td>1.9 (1.0-3.1)</td>
</tr>
<tr>
<td>2</td>
<td>11.72 ± 0.26 (2.2)</td>
<td>9.03 ± 0.35 (3.9)</td>
<td>26.67 ± 3.56 (13.3)</td>
<td>4.48 ± 0.10 (2.3)</td>
<td>20.58 ± 0.40 (1.9)</td>
<td>9.84 ± 0.18 (1.8)</td>
<td>4.3 (1.8-13.3)</td>
</tr>
<tr>
<td>3</td>
<td>10.71 ± 0.24 (2.2)</td>
<td>7.13 ± 0.15 (2.0)</td>
<td>24.48 ± 0.14 (0.6)</td>
<td>3.96 ± 0.05 (1.2)</td>
<td>17.58 ± 0.31 (1.8)</td>
<td>8.71 ± 0.09 (1.0)</td>
<td>1.5 (0.6-2.2)</td>
</tr>
<tr>
<td>4A</td>
<td>10.96 ± 0.12 (1.1)</td>
<td>7.39 ± 0.10 (1.4)</td>
<td>24.89 ± 0.19 (0.7)</td>
<td>3.78 ± 0.04 (1.1)</td>
<td>17.40 ± 0.40 (2.3)</td>
<td>9.18 ± 0.68 (7.4)</td>
<td>2.3 (0.7-7.4)</td>
</tr>
<tr>
<td>4B</td>
<td>12.02 ± 0.22 (1.9)</td>
<td>7.07 ± 0.22 (3.2)</td>
<td>23.06 ± 2.73 (11.8)</td>
<td>3.71 ± 0.06 (1.7)</td>
<td>17.11 ± 0.66 (3.9)</td>
<td>7.89 ± 0.36 (4.6)</td>
<td>4.5 (1.7-11.8)</td>
</tr>
<tr>
<td>5</td>
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<tr>
<td>6</td>
<td>12.50 ± 0.15 (1.2)</td>
<td>7.44 ± 0.12 (1.6)</td>
<td>26.12 ± 0.17 (0.6)</td>
<td>4.19 ± 0.03 (0.6)</td>
<td>19.68 ± 0.48 (2.4)</td>
<td>8.50 ± 0.07 (0.9)</td>
<td>1.2 (0.6-2.4)</td>
</tr>
<tr>
<td>7</td>
<td>11.18 ± 0.08 (0.7)</td>
<td>7.57 ± 0.12 (1.6)</td>
<td>25.53 ± 0.23 (0.9)</td>
<td>4.10 ± 0.00 (0.0)</td>
<td>17.47 ± 0.27 (1.6)</td>
<td>8.40 ± 0.28 (3.3)</td>
<td>1.3 (0.3-3.3)</td>
</tr>
<tr>
<td>8</td>
<td>9.50 ± 0.47 (4.9)</td>
<td>8.03 ± 0.27 (3.3)</td>
<td>24.62 ± 1.85 (7.5)</td>
<td>4.61 ± 0.22 (4.8)</td>
<td>16.88 ± 0.59 (3.5)</td>
<td>11.80 ± 0.97 (8.3)</td>
<td>5.4 (3.3-8.3)</td>
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<td>9</td>
<td>11.67 ± 0.21 (1.8)</td>
<td>7.52 ± 0.02 (0.3)</td>
<td>24.80 ± 0.29 (1.2)</td>
<td>3.99 ± 0.03 (0.8)</td>
<td>19.43 ± 0.16 (0.8)</td>
<td>9.36 ± 0.17 (1.8)</td>
<td>1.1 (0.3-1.8)</td>
</tr>
<tr>
<td>10</td>
<td>11.11 ± 0.35 (3.1)</td>
<td>8.03 ± 0.38 (4.7)</td>
<td>26.18 ± 0.31 (1.2)</td>
<td>4.26 ± 0.19 (4.5)</td>
<td>19.01 ± 0.39 (2.0)</td>
<td>9.27 ± 0.22 (2.4)</td>
<td>3.0 (1.2-4.7)</td>
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<tr>
<td>11</td>
<td>10.90</td>
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<td>24.20</td>
<td>3.90</td>
<td>18.30</td>
<td>9.40</td>
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</tbody>
</table>

Interlaboratory mean ± sd (CV)

| 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) |
| 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) |
| 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) |

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.
### 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.

<table>
<thead>
<tr>
<th>Lab No.</th>
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<th>16:1 (n-7)</th>
<th>18:1 (n-9)</th>
<th>18:2 (n-6)</th>
<th>18:3 (n-3)</th>
<th>20:5 (n-3)</th>
<th>Total FA</th>
<th>Intra-laboratory CV mean (min-max)</th>
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<tbody>
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<td>14.49 ± 1.22 (8.41)</td>
<td>9.43 ± 0.91 (9.91)</td>
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<td>5.75 ± 0.46 (8.04)</td>
<td>23.03 ± 1.78 (7.73)</td>
<td>11.08 ± 1.09 (9.80)</td>
<td>128.66 ± 10.83 (8.42)</td>
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<td>12.02 ± 1.05 (8.75)</td>
<td>9.37 ± 0.92 (9.86)</td>
<td>28.63 ± 2.08 (7.27)</td>
<td>4.56 ± 0.39 (8.59)</td>
<td>20.99 ± 1.73 (8.24)</td>
<td>10.11 ± 0.67 (6.63)</td>
<td>-</td>
<td>8.22 (6.6-9.86)</td>
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<tr>
<td>3</td>
<td>13.86 ± 0.99 (7.18)</td>
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<td>31.59 ± 1.76 (5.58)</td>
<td>5.11 ± 0.32 (6.16)</td>
<td>22.68 ± 0.98 (4.30)</td>
<td>11.24 ± 0.61 (5.43)</td>
<td>-</td>
<td>5.84 (4.3-7.18)</td>
</tr>
<tr>
<td>4A</td>
<td>16.10 ± 0.29 (1.70)</td>
<td>10.85 ± 0.26 (2.38)</td>
<td>33.94 ± 5.42 (15.97)</td>
<td>5.56 ± 0.16 (2.85)</td>
<td>25.88 ± 0.66 (2.55)</td>
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<td>5.46 (1.79-15.97)</td>
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<td>18.22 ± 0.88 (4.84)</td>
<td>10.71 ± 0.54 (5.09)</td>
<td>36.53 ± 1.92 (5.26)</td>
<td>5.62 ± 0.29 (5.23)</td>
<td>25.94 ± 1.68 (6.49)</td>
<td>11.72 ± 0.56 (4.75)</td>
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<tr>
<td>6</td>
<td>13.78 ± 0.35 (2.55)</td>
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</tr>
<tr>
<td>8</td>
<td>11.46 ± 1.20 (10.15)</td>
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<td>5.55 ± 0.28 (5.00)</td>
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<td>4.62 ± 0.29 (6.32)</td>
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</table>

**Inter-laboratory mean ± sd (CV)**

**Mean (min-max)**

**Intra-laboratory CV mean (min-max)**

| 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) |
| 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) |
| 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) |

* total FAME data are excluded from the calculation of mean intra-laboratory and inter-laboratory 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).

<table>
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<th>Lab No.</th>
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<th>18:1(n-7/9)</th>
<th>18:2(n-6)</th>
<th>18:3(n-3)</th>
<th>20:5(n-3)</th>
<th>total FAME $^d$</th>
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<tr>
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<td>7.33$^b$</td>
<td>23.51$^{ab}$</td>
<td>4.47$^f$</td>
<td>17.90$^a$</td>
<td>8.60$^{abc}$</td>
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</tr>
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<td>2</td>
<td>11.72$^{ef}$</td>
<td>9.03$^c$</td>
<td>26.67$^{ab}$</td>
<td>4.45$^f$</td>
<td>20.58$^c$</td>
<td>9.84$^d$</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10.71$^{h}$</td>
<td>7.13$^b$</td>
<td>24.48$^{ab}$</td>
<td>3.96$^{bc}$</td>
<td>17.58$^a$</td>
<td>8.71$^{bc}$</td>
<td></td>
</tr>
<tr>
<td>4A</td>
<td>10.96$^{bc}$</td>
<td>7.39$^{ab}$</td>
<td>24.89$^{ab}$</td>
<td>3.78$^{ab}$</td>
<td>17.40$^a$</td>
<td>9.18$^{cd}$</td>
<td></td>
</tr>
<tr>
<td>4B</td>
<td>12.02$^{h}$</td>
<td>7.07$^a$</td>
<td>23.06$^{a}$</td>
<td>3.71$^a$</td>
<td>17.11$^a$</td>
<td>7.89$^a$</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>12.50$^{ef}$</td>
<td>7.44$^{bc}$</td>
<td>26.12$^{ab}$</td>
<td>4.19$^{bc}$</td>
<td>19.68$^{bc}$</td>
<td>8.50$^{bc}$</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>11.18$^{bd}$</td>
<td>7.57$^{bd}$</td>
<td>25.53$^{ab}$</td>
<td>4.10$^{bc}$</td>
<td>17.47$^{a}$</td>
<td>8.40$^{ab}$</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>9.50$^a$</td>
<td>8.03$^{cd}$</td>
<td>24.62$^{ab}$</td>
<td>4.61$^f$</td>
<td>16.88$^{a}$</td>
<td>11.80$^a$</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>11.67$^{ef}$</td>
<td>7.52$^{bd}$</td>
<td>24.18$^{ab}$</td>
<td>3.99$^{bd}$</td>
<td>19.43$^b$</td>
<td>9.36$^{cd}$</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>11.11$^{bc}$</td>
<td>8.03$^{d}$</td>
<td>26.18$^{ab}$</td>
<td>4.26$^f$</td>
<td>19.01$^b$</td>
<td>9.27$^{cd}$</td>
<td></td>
</tr>
<tr>
<td>11$^f$</td>
<td>10.90</td>
<td>8.00</td>
<td>24.20</td>
<td>3.90</td>
<td>18.30$^{a}$</td>
<td>9.40</td>
<td></td>
</tr>
</tbody>
</table>

ANOVA: $F_{9,49}$ 45.11*** 35.18*** 2.77* 47.11*** 45.95*** 30.11***

B: mg/g DW

| 1 | 14.49$^{d}$ | 9.43$^{ab}$ | 30.25$^{ab}$ | 5.75$^d$ | 23.03$^a$ | 11.08$^{ab}$ | 128.66$^i$ |
| 2 | 12.02$^{d}$ | 9.37$^c$ | 28.63$^{ab}$ | 4.56$^{ab}$ | 20.99$^a$ | 10.11$^{ab}$ | - |
| 3 | 13.88$^{bc}$ | 9.21$^a$ | 31.59$^{bc}$ | 5.11$^{bc}$ | 22.68$^{a}$ | 11.24$^{b}$ | - |
| 4A | 16.10$^{d}$ | 10.85$^a$ | 33.94$^{bc}$ | 5.56$^{a}$ | 25.88$^{b}$ | 13.49$^c$ | - |
| 4B | 18.22$^{d}$ | 10.71$^{bc}$ | 36.53$^{c}$ | 5.62$^{a}$ | 25.94$^{b}$ | 11.72$^{b}$ | - |
| 5 | - | - | - | - | - | - |
| 6 | 13.78$^{bc}$ | 8.20$^{d}$ | 28.81$^{ab}$ | 4.61$^{ab}$ | 21.72$^{a}$ | 9.37$^{a}$ | 109.58$^b$ |
| 7 | - | - | - | - | - | - | 80.48$^a$ |
| 8 | 11.46$^d$ | 9.68$^{bc}$ | 29.74$^{a}$ | 5.55$^{ab}$ | 20.35$^{a}$ | 14.19$^c$ | 120.48$^{bc}$ |
| 9 | 12.34$^{bc}$ | 7.94$^a$ | 26.21$^{a}$ | 4.22$^a$ | 20.52$^{a}$ | 9.89$^{ab}$ | 112.34$^{b}$ |
| 10 | 12.10$^{bc}$ | 8.74$^a$ | 28.48$^{ab}$ | 4.62$^{ab}$ | 20.68$^{a}$ | 10.10$^{ab}$ | 108.83$^b$ |
| 11$^f$ | 13.80 | 10.20 | 30.80 | 4.90 | 23.20 | 12.00 | 126.96 |

ANOVA: $F_{9,49}$ 29.56*** 10.71*** 6.09*** 16.07*** 12.30*** 18.52*** 30.57***

$^f$: except total FAME $F_{3,49}$
$^f$: 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].

<table>
<thead>
<tr>
<th></th>
<th>Mean intralaboratory CV¹</th>
<th>Mean interlaboratory CV²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>area% mg/g DW</td>
<td>area% mg/g DW</td>
</tr>
<tr>
<td><strong>Participants following ICES Standard Method</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Participants following own method</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry feed</td>
<td>3.5 [5]</td>
<td>5.6 [4]</td>
</tr>
<tr>
<td>Artemia nauplii</td>
<td>3.1 [4]</td>
<td>5.7 [3]</td>
</tr>
</tbody>
</table>

¹: intralaboratory means of the CVs of each fatty acid, averaged for n laboratories

²: 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].

<table>
<thead>
<tr>
<th></th>
<th>Mean intralaboratory CV¹</th>
<th></th>
<th>Mean interlaboratory CV²</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>area% mg/g DW</td>
<td>area% mg/g DW</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present intercalibration exercise</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Léger et al. (1989)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

LIST OF PARTICIPANTS

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

Peter Couteau & Geert Van de Wiele
Laboratory of Aquaculture
& Artemia Reference Center
University of Gent
Rozier 44, B-9000 Gent
BELGIUM

John D. Castell & Linda Boston
Department of Fisheries & Oceans
Halifax Laboratory
P.O. Box 550, 1707 Lower Water St. Halifax, Novia Scotia
CANADA B3J 2S7

Geneviève Corraze
INRA - Station d'Hydrobiologie
Laboratoire de Nutrition des Poissons
B.P. 3
64510 St Pée-sur-Nivelle
FRANCE

Jean Robin & Stephan Caeva
IFREMER
B.P. 70
29280 Plouzané
FRANCE

A.N. Sagredos
NATEC
Bebningstrasse 154
D-2000 Hamburg 50
GERMANY

Johan Verreth & Peter Tijssen
Dept. of Fish Culture & Fisheries
Wageningen Agricultural University
P.O. Box 338
6700 AH Wageningen
NETHERLANDS

Arild Folkvord (1), Inger Haaland (2)
& Otto Grahl-Nielsen (2)
(1) Dept. of Fisheries and Marine Biology
(2) Dept. of Chemistry
University of Bergen
N-5020 Bergen
NORWAY

José Rainuzzo & Yngvar Olsen
SINTEF Center of Aquaculture
N-7034 Trondheim
NORWAY

Isabel Medina & J.L. Garrido
Instituto de Investigaciones Marinas
Av. Eduardo Cabello, 6
E-36208 Vigo
SPAIN

Lesley McEvoy
University of Stirling
Department of Biological and Molecular Sciences
Stirling FK9 4LA
Scotland
UNITED KINGDOM

A.R. Child
MAFF
Fisheries Laboratory
Conwy
Gwynedd LL32 8UB
UNITED KINGDOM
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, 3 g 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 -50°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 Annex 2.1 and 2.2. to the ICES-Standard Methodology.

Provide the following details of gas chromatograph used:

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

ICES-Standard Schematic

The lipid class analysis is performed in triplicates.

For the qualitative analysis, the extract is screened against a fatty acid mixture, identified with a database.

The results are reported as % of the total lipid.

Another analysis is performed in triplicates. This procedure is repeated before separating the lipid classes.

The C16:0 results are compared with the C16:0 first was.

This fatty acid is filtered out of the mixture.

The C18:2 results are expressed in mg/g FW.

The lipids are identified with a database known as the FAME Base.

The complete list of fatty acids is provided.

Optionally, it is recommended to expand the list of fatty acids in the glassware.
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 CHCl₃ : 1 CH₂OH).
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 CHCl₃ : 1 CH₂OH) 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 CHCl₃, CH₂OH, H₂O are 10:5:3 v/v. Shake the funnel for approximately 1-2 min. After two more minutes a separation will occur:

- phase above : H₂O-CH₂OH-KCl
- phase below : CHCl₃ - lipids

The CHCl₃-lipid fraction is then filtered through a water free Na₂SO₄-filter (see Note 1.2.) into a vacuum proof flask, first without vacuum and afterwards with vacuum.

This filter is then rinsed with CHCl₃. The water-methanol fraction is re-extracted with CHCl₃. The CHCl₃-lipid fraction is filtered through a waterfree Na₂SO₄-filter. This filter is then rinsed again with CHCl₃.

The combined CHCl₃-fractions are evaporated till nearly dry using a Büchi rotavapor (vacuum-evaporator). Temperature 30°C.

The lipids are then dissolved in CHCl₃ and rinsed over a waterfree Na₂SO₄-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.

<table>
<thead>
<tr>
<th>Product</th>
<th>weight of sample (g)</th>
<th>approx. dry weight (%)</th>
<th>approx. total lipid content (%)</th>
<th>absolute TL content (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artemia Instar 1</td>
<td>± 1.0</td>
<td>± 12</td>
<td>± 15-20</td>
<td>± 25</td>
</tr>
<tr>
<td>24-h enriched Artemia</td>
<td>± 1.0</td>
<td>± 9</td>
<td>± 25-30</td>
<td>± 30</td>
</tr>
<tr>
<td>Emulsion/oil</td>
<td>± 0.040</td>
<td>± 85-100</td>
<td>± 85-100</td>
<td>± 30-40</td>
</tr>
<tr>
<td>Formulated feed</td>
<td>± 0.2</td>
<td>± 98</td>
<td>± 15</td>
<td>± 30</td>
</tr>
</tbody>
</table>

Note 1.2: Preparation of a waterfree Na₂SO₄-filter

- Take the filter-funnel of Ø 70 mm or 55 mm (porosity n° 3)
- Put Na₂SO₄ in the filter
- Wash the Na₂SO₄ with CHCl₃ under vacuum. Washing of the Na₂SO₄-filter is not necessary if anhydrous Na₂SO₄ 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

<table>
<thead>
<tr>
<th>Product</th>
<th>Chemical formula</th>
<th>Purity specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>CHCl₃</td>
<td>for liquid chromatography</td>
</tr>
<tr>
<td>Methanol</td>
<td>CH₃OH</td>
<td>for liquid chromatography</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>KCl</td>
<td>extra pure</td>
</tr>
<tr>
<td>Sodium sulphate</td>
<td>Na₂SO₄</td>
<td>anhydrous, pro analysis</td>
</tr>
<tr>
<td>Toluene</td>
<td>C₇H₈</td>
<td>for liquid chromatography</td>
</tr>
<tr>
<td>Nitrogen gas</td>
<td>N₂</td>
<td>very pure</td>
</tr>
</tbody>
</table>

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.
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(2CHCl₃:CH₃OH) 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. Na₂SO₄ 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 figure 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 acetyl chloride/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 acetyl chloride/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 H₂O.
- Add 0.2 ml internal standard solution (see Note 2.1).
- Centrifuge 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 Na₂SO₄-filter (see Note 1.2.) into a vacuum proof pearsheaped flask (elimination of possible H₂O 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

<table>
<thead>
<tr>
<th>Product</th>
<th>Chemical formula</th>
<th>Purity specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl chloride</td>
<td>C₅H₅ClO</td>
<td>pro-analysis</td>
</tr>
<tr>
<td>Methanol</td>
<td>CH₃OH</td>
<td>for liquid chromatography</td>
</tr>
<tr>
<td>Hexane</td>
<td>CH₃(CH₂)ₓCH₃</td>
<td>for liquid chromatography</td>
</tr>
<tr>
<td>Internal standard</td>
<td>cis 11, 14 eicosadienoate</td>
<td>Nu-Chek-Prep U-68-M</td>
</tr>
<tr>
<td>Sodium sulphate</td>
<td>Na₂SO₄</td>
<td>anhydrous, pro analysis</td>
</tr>
<tr>
<td>Isooctane</td>
<td>CH₃(CH₂)ₙCH₃</td>
<td>for liquid chromatography</td>
</tr>
<tr>
<td></td>
<td>(C 2,2,4-trimethylperitane)</td>
<td></td>
</tr>
</tbody>
</table>

Note 2.1:
- The final volume of methanol/toluene should be 10 ml.
- The ratio of acetyl chloride to methanol (5:100) must be precise.
- The internal standard should be known exactly.
- e.g. 2,2,4-trimethylpentane (see Table 2).

2.1.2. Post esterification

The 1 Saponification is performed after esterification and the concentration of the fatty acid methyl esters is measured.

2.1.3. Preparation of the internal standard

0.2 ml of a 1 mg/ml solution is prepared and kept in the freezer (see Note 2). The relative standard deviation is calculated and the concentration of the internal standard is determined.

To obtain a 1 Saponification, the concentration of the fatty acid methyl esters must be known. The concentration of the fatty acid methyl esters is determined using capillary GC. A peak of 100% on a capillary column is equivalent to 1 mg direct injection.

2.1.4. Determination of fatty acid composition

The data is interpreted to determine the composition of tissue fatty acids.
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 eicosadienoate (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).
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 water bath
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 Na₂SO₄ 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 gas chromatographic conditions currently in use at the Artemia Reference Center.

Fatty acid methyl esters 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), 18:2(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), 20:4(n-3), 22:0, 22:1(n-9), 22:2(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, Elysonian, 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


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

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<th>Peak</th>
<th>% known</th>
<th>% found</th>
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</tr>
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</tr>
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<td>10.0</td>
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<td>14.9</td>
</tr>
<tr>
<td>18:1(n-9)</td>
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<td>25.8</td>
</tr>
<tr>
<td>18:2(n-6)</td>
<td>10.0</td>
<td>10.3</td>
</tr>
<tr>
<td>18:3(n-3)</td>
<td>4.0</td>
<td>3.9</td>
</tr>
<tr>
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<td>1.9</td>
</tr>
<tr>
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</tr>
<tr>
<td>20:2(n-6)</td>
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<td>2.0</td>
</tr>
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<tr>
<td>20:4(n-6)</td>
<td>4.0</td>
<td>3.9</td>
</tr>
<tr>
<td>22:0</td>
<td>4.0</td>
<td>3.9</td>
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<tr>
<td>22:1(n-9)</td>
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</tr>
<tr>
<td>24:0</td>
<td>2.0</td>
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<td>3.6</td>
</tr>
<tr>
<td>22:6(n-3)</td>
<td>4.0</td>
<td>3.9</td>
</tr>
</tbody>
</table>
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.

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<th>mg/g DW</th>
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<td>1.9</td>
</tr>
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<td>0.3</td>
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</tr>
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<td>0.1</td>
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</tr>
<tr>
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<td>0.2</td>
</tr>
<tr>
<td>21:5</td>
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<td>0.2</td>
</tr>
<tr>
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<td>0.1</td>
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<td>24:0</td>
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**Sum (n-3) HUFA**

| 20:3(n-3) | 2.4 | 1.5 |