International Council for the
Exploration of the Sea

C.M. 1992/F:4

REPORT OF THE WORKING GROUP ON MASS REARING OF JUVENILE MARINE FISH TO THE MARICULTURE COMMITTEE OF ICES

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1. MEMBERS

The present Working Group members are:

Belgium: P. Sorgeloos, C. Belpaire. **Canada:** J. Gagne, K. Waiwood. **Denmark:** M. Behrens, O. Bagge, I. Fjallstein, J. G. Støttrup. **France:** B. Chatain, J. Person. **Germany:** W. Nellen, G. Quantz. **Iceland:** B. Bjørnsson, G. Marteinsdottir. **Norway:** G. Adoff, Ø. Bergh, S. Bolla, D. Danielssen, A. Folkvord, K. Gravning, J. Gulbrandsen, K. Hamre, I. Holmefjord, I. Huse, E. Kjørsvik, T. Kleppe, I. Lein, A. Mangor-Jensen, G. v.d. Meeren, E. Moksness, K. Naas, Y. Olsen, I. Opstad, T. Pedersen, H. Reinertsen, G. Rosenlund, A. Skadsheim, S. Tilseth, V. Øiestad. **Poland:** W. Pelczarski, J. Wiktor. **Portugal:** J. Menezes, P. Pousao. **Spain:** J. R. Cejas, C. Fernandez-Pato, J. Iglesias, G. Minkoff. **Sweden:** H. Ackefors, J. Andersson, P.-O. Larsson, J. Pickova. **UK:** M. Gillespie, B.R. Howell, A. Munro.

2. TERMS OF REFERENCE

The following terms of reference were given by the Council in C. Res. 1991/2:42: The Working Group on Mass Rearing of Juvenile Fish (Chairman: Dr I. Huse, Norway) will work by correspondence in 1992 to:

a) develop and circulate a protocol for standardized monitoring of egg and larval quality;

b) plan an inter-laboratory investigation of egg and larval quality;

c) prepare a protocol for hygienic procedures in rearing systems;

d) prepare a protocol for standard nutrition research;

e) prepare a report on standard inert reference diets;

f) submit a written report on progress to the Mariculture Committee at the 1992 Statutory Meeting with the expectation of a meeting in 1993 in Bergen, Norway.

3. INTERIM ACTIVITY

All tasks spesified in the terms of reference are persued. Preparatory work has been and will be carried out in the interim period, and the tasks will be finalized at the Bergen W. G. meeting in 1993, and reported at the 1993 Council Meeting.

A standard procedure for quantitative (n-3)HUFA analyses initiated at the 1987 W.G. meeting has been further amended based on suggestions of Prof. J. Sargent (UK) and is now ready for application in an intercalibration study. Laboratory for Aquaculture and Artemia Reference Center (ARC) is willing to coordinate a study. The procedure is enclosed in the Appendix.

ARC has also selected two batches of Artemia cysts that can be used as reference starter feed for marine fish larvae to be used in nutrition studies. ARC also is willing to prepare a protocol for use of the selected batches. This is in accordance with item d) in the terms of reference.

The enrichment intercalibration exercise for (n-3)HUFA in Brachionus and Artemia is being prepared, and a protocol is worked out by Prof. P. Sorgeloos (Belgium) and Prof. J. Sargent (UK).

Different formulations of an extruded semi-purified diet that can be coated with lipid soluble components are beeing evaluated in order to prepare a reference diet for use in nutritional studies with marine fish.

4. RECOMMENDATIONS

The Working Group on Mass Rearing of Juvenile Marine Fish recommends that the group should continue its work and meet in Bergen on June 25-26, 1993 with BARI HOWELL (UK) as Chairman.

The following terms of reference are suggested based on C. Res. 1991/2:42.: The group should meet to establish:

a) a protocol for standardized monitoring of egg and larval quality;

b) an inter-laboratory investigation of egg and larval quality;

c) a protocol for hygiene procedures in rearing systems ;

d) a protocol for standard nutrition research;

e) a report on standard inert reference diets;

f) a progress report to the Mariculture Committee at the 1993 Statutory Meeting;

APPENDIX

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

Contents

IV.1. Intercalibration sample

A selected batch of a compound formulation of marine meals, containing a high (n-3) HUFA content, will be provided. FAME profile can be found in annex. The reference sample will be made available for analysis in 3 x 5g quantities.

IV.2. Total lipid extraction procedure

IV.2.a. Modified procedure of Ways and Hanahan

Note 1: Amount of sample to be analyzed 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 our standard for lipid extractions and subsequent fatty acid analyses. The total lipid content is important for determining the quantity of internal standard to be added.

Examples

Product	weight of sample in 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
Artificial Diet	± 0.2	± 98	± 15	± 30

Note 2: Because of safety and health reasons, benzene was replaced by toluene. 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.

Modified procedure of Ways and Hanahan (1964)

The lipids are extracted with a binary solvent mixture ($2CHCl_3$: $1CH_3OH$). 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) or wet sample (1 g) is transferred into a centrifuge tube (50 ml) and thoroughly homogenised in 30 ml solvent mixture ($2CHCl_3$: $1CH_3OH$) 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 18 ml KCl 0.1 M or 0.745 %.

Another two reextractions are done by adding the same amount of solvent to the sediment each time. The final proportions $CHCl_3$, CH_3OH, H_2O are 10:5:3 v/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) 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 reextracted 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 lipids are transferred with 5 ml methanol/toluene solution (3:2 v/v) in a pyrex centrifuge glass-tube with a teflon lined cap. Storage in a freezer (-18°C) is recommended.

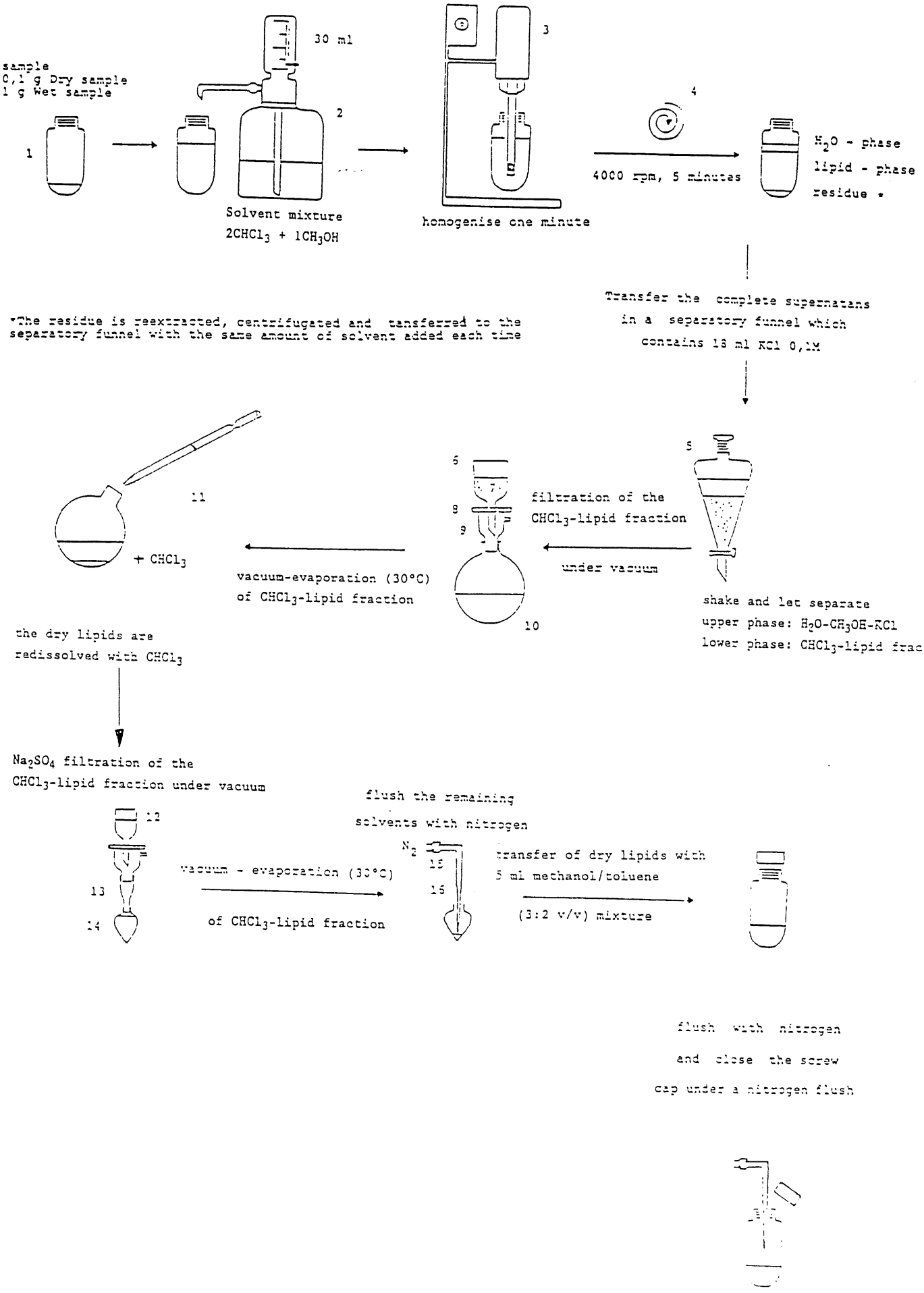
Remark:

Another quantification method for determining total lipids could be the use of a Reagent kit, Mercko test 3321, according to Zollner-Pirsh. The lipid concentration is compared with standards and determined photometrically. The first trials were positive but, further optimization appears necessary.

IV.2.c. List of chemical products

Product	Chemical formula	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

Fluxogram A: the total lipid extraction according to the method from Ways and Hanahan (1964)



IV.3. Esterification procedure

IV.3.a. Modified procedure of Lepage and Roy (1984)

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

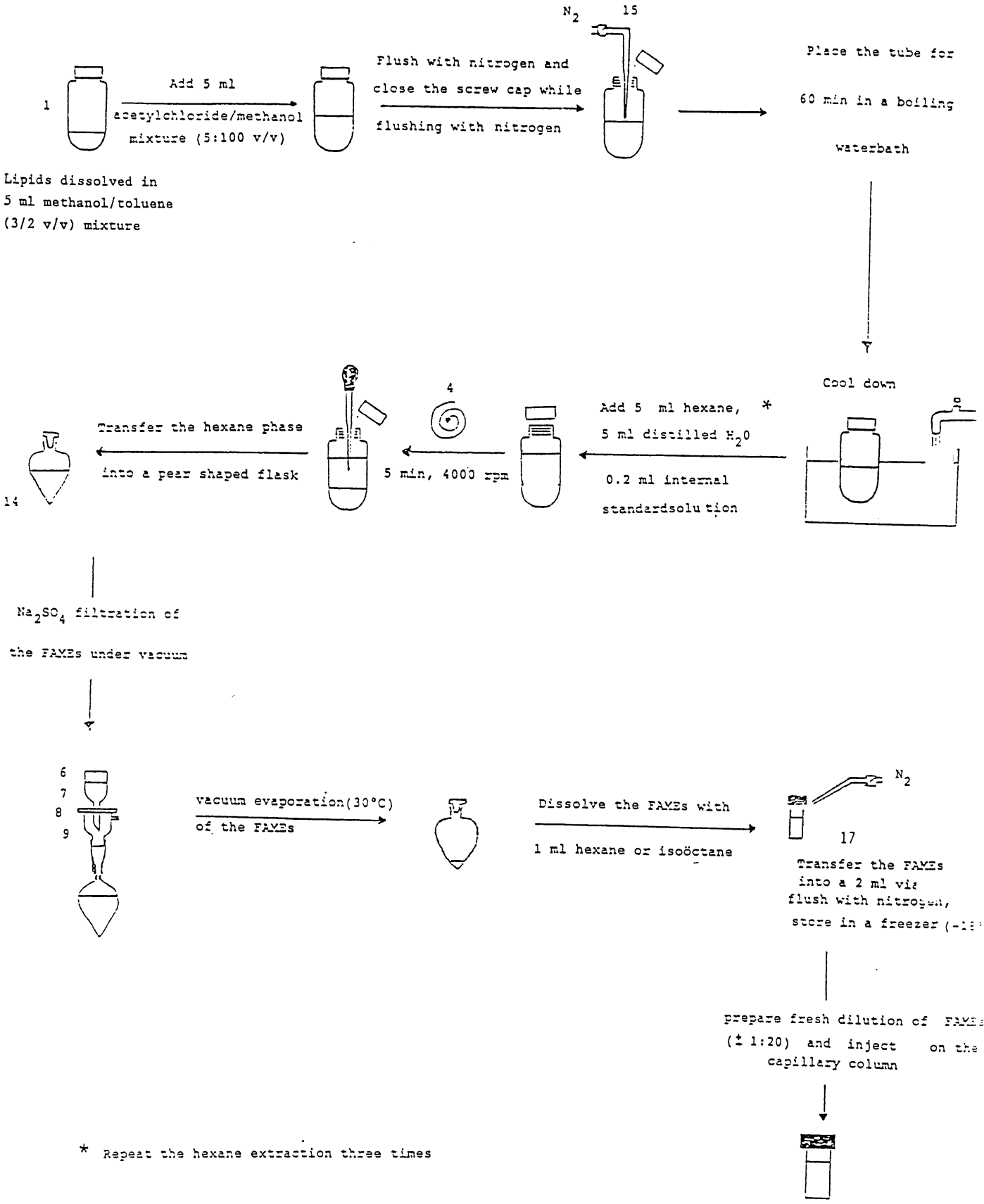
Description of the method, step by step

- 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) (see note 2).
- 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 centrifuge glass tube.
- Add 5 ml hexane and 5 ml distilled H_2O.
- Add 0.2 ml internal standard solution (see note 3; concentration and preparation in text).
- 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 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 (vacuumevaporator). Temperature 30°C.
- Flush with nitrogen to remove remaining solvents.
- Dissolve the FAMEs with 1 ml hexane or isooctane 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 (-18°C).

IV.3.c. List of chemical products

Product	Chemical formula	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

Fluxogram B: direct esterification according to the method modified from Lepage and Roy (1984)

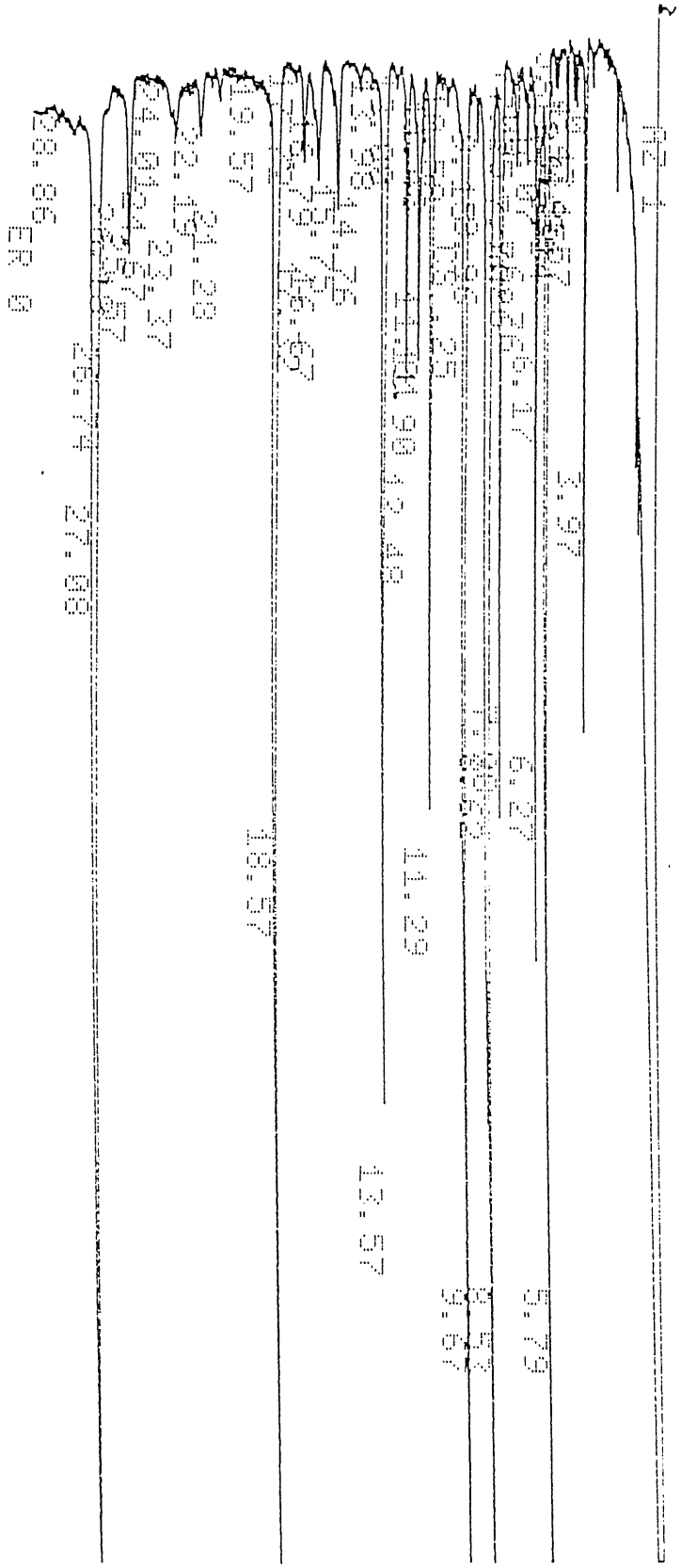


* Repeat the hexane extraction three times

IV.3.d. Legend to fluxogram A + B

1. Centrifuge glass-tube with a teflon lined screw cap
2. Dispenser
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
8. Rubber device for vacuum sealing
9. Adaptor unit
10. 100 ml flask (vacuumproof)
11. pipette
12. Filter funnel, diam. 55 mm, coarse n°3
13. Adaptor unit
14. Pear shaped flask 50 ml (vacuumproof)
15. Nitrogen
16. Pasteur pipet
17. 2 ml amber vial with a teflon lined screw cap

Compound formulation of marine meals
sample chromatogram of FAME-composition



Compound formulation of marine meals
FAME-composition

Component	Area %	mg/g DW
14:0	2.3	1.8
14:1	0.2	0.2
15:0	0.4	0.4
15:1	0.2	0.2
14:2	-	-
16:0	12.8	10.1
16:1	3.0	2.3
17:0	0.3	0.2
16:2	-	-
16:3	0.5	0.4
17:1	-	-
18:0	3.5	2.7
18:1	18.1	14.3
19:0	0.1	0.1
18:2	12.9	10.2
19:4	0.1	0.1
20:0	-	-
18:3(n-6)	3.9	3.1
20:1	2.1	1.7
18:3(n-3)	1.5	1.2
18:4	-	-
I.S.		
20:3(n-6)	-	-
20:3(n-3)	-	-
20:4(n-6)	1.5	1.2
20:4(n-3)	1.0	0.8
22:1	-	-
21:5	0.8	0.6
20:5(n-3)	12.0	9.5
24:0	-	-
24:1	-	-
22:3(n-3)	0.4	0.3
22:4(n-6)	0.2	0.2
22:4(n-3)	-	-
22:5(n-6)	-	-
22:5(n-3)	1.6	1.2
22:6(n-3)	18.8	14.8
Σ HUFA (n-3) ≥ 20:3(n-3)	33.8	26.6

IV.4. Internal standard (method) and preparation of sample for GC-analysis

IV.4.a. Criteria for selecting a suitable internal standard

- 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 IV.5.).

IV.4.b. 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 -18°C in a freezer.

IV.4.c. Preparation of the sample of 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 -18°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 µl of this dilution, which is prepared in size.
This means we inject ± 0.4 µg directly into the capillary column.

IV.4.d. Data expression and treatment

The data for each FAME are expressed as percentage of total FAMEs (relative values) and as mg per g dry wight of tissue (absolute values).

IV.5. Example of Gaschromatographic conditions

The following conditions are currently used at the Laboratory for Aquaculture and Artemia Reference Center.

Fatty acid methylesters are injected on a capillary column (25m fused silica, i.d. : 0.32 mm, liquid phase : CP-Sil-88 Chrompack Europe, film thickness: 0.21 µm) 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: 105°C to 150°C at 7.5°C/min and 150°C to 180°C at 1°C/min. Peak identification and quantification is done with a calibrated plotter integrator (Spectra Physics SP 4290) and reference standards.
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.

IV.6. Notes + practical information

1. Preparation of a waterfree Na_2SO_4-filter
   - Take the filter-funnel of ⌀ 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.

2. Preparation of acetyl chloride/methanol mixture
   - Add acetyl chloride slowly to cooled methanol (to prevent splashing)

Remark :

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

IV.7. Evaluation of results

The intercalibration results are to be sent to:

Laboratory for Aquaculture and Artemia Reference Center
University of Ghent
Rozier 44
9000 Ghent, BELGIUM