Determination of total lipid using non-chlorinated solvents

The Analyst

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The restrictions on the use of chlorinated solvents under the Montreal Protocol makes it necessary to develop an alternative method to the Bligh and Dyer lipid extraction as currently applied to marine tissues. Several different solvent mixtures were systematically tested as a replacement for chloroform. The presence of a polar solvent is a prerequisite in order to obtain phase separation between the aqueous and organic phases, but too high a concentration of solvent in the aqueous phase prevents the more polar lipids from being extracted. A high content of water in the organic phase can result in co-extraction of non-lipids. Several combinations of solvents may be able to extract lipids, but for reasons of safety and toxicity, a propan-2-ol–cyclohexane–water (8 + 10 + 11 v/v/v) mixture has been proposed. The method is not sensitive to a wide range of sample-phase volume ratios provided that the solvent compositions remain constant. Application to plaice, mussel and herring samples showed results that were in agreement with the extraction following Bligh and Dyer using chloroform and methanol.

Introduction

The contaminant contents in marine organisms are often normalised to the lipid content as part of marine environmental studies and monitoring programmes. Although the question as to whether the total lipid or a specific lipid fraction should be used is still under debate[1–3] it remains necessary to use an adequate method to extract total lipids, regardless of the application. A method introduced by Folch *et al.*[4] and further developed by Bligh and Dyer[5] has been adopted as the standard method for the determination of total lipids in marine organisms. Methanol plus chloroform, chloroform and water are added to the tissue in a three-step extraction and, after phase separation, the lipids are determined in the chloroform phase.

Because of the adverse effect of chlorinated solvents on the environment, the use of chloroform should be strongly discouraged. Following the Montreal Protocol, EU regulations have been developed to control strictly the use of chlorinated solvents. To avoid a step change in lipid measurements, the development of any new method should give comparable results to the Bligh and Dyer method.

Soxhlet extraction of tissues can easily be performed with non-chlorinated solvents but the results are often lower than those given by Bligh and Dyer method and not considered as total lipids but as 'extractable' lipids. According to de Boer,[6] the fraction not extracted by the Soxhlet method was referred to as bound lipids. Furthermore, the yield of Soxhlet extraction is determined by the solvent composition and extraction time or, more precisely, the number of cycles. The results of Soxhlet extraction are very much operationally dependent and at the same time it is difficult to control the conditions. Campbell and Wells[7] reported that using increasingly polar solvents, the extractable amount increased correspondingly to become much higher than the result given by the Bligh and Dyer method. Therefore, Soxhlet extraction is not considered here as an alternative.

Randall *et al.*[8] demonstrated that different methods can lead to a three-fold difference in lipid contents. Also, the intake mass was of some influence on the result. In an intercomparison exercise to determine the comparability in 'extractable' lipid measurements, the results varied widely owing to differences in extraction methodology.[9] Laboratories in this exercise also applied the method of Bligh and Dyer, but in fact applied modifications of the method due to different interpretations. The exercise also revealed variable results which could be partly related to the adaptations made.[10] Since these deviations influenced the result obtained, it is of paramount importance to understand the Bligh and Dyer method before an alternative method can be developed. Previously, data obtained by Bligh and Dyer were evaluated according to a theoretical extraction model after measuring solvent compositions in the mixtures originally applied.[11] Despite the different solvent volumes used, solvent compositions were close to constant and the extraction yield was mainly determined by the fraction of the organic phase that could be recovered. This was subsequently confirmed by repeating the experiments of Bligh and Dyer.[12] Evaluation of the data was supported by the determination of the species in the extracted lipids by high performance liquid chromatography (HPLC). The extraction efficiencies were not related to phase ratios and all extracts showed similar lipid patterns and lipid contents. Only when the methanol content was varied over a wide range were there measurable differences in extraction yield. The yield of phospholipids was more sensitive to the methanol content than that of neutral lipids and free fatty acids.

This paper presents the results of the development of a new method for the extraction of 'total' lipids. Such a method should use readily available non-chlorinated solvents with low toxicity, non-olfactory, non-explosive properties and at low cost, be robust and easy to use and be applicable to a wide range of tissue types and lipid contents without the need to be modified.

Starting from the Bligh and Dyer method,[5] different solvents were tested. The importance of methanol in the Bligh and Dyer extraction already indicated that a polar solvent was a prerequisite to obtaining an efficient extraction yield. In previous work,[12] cod muscle was used to study the Bligh and Dyer method, but the present work was focused only on the lipid extraction from mussel (*Mytilus edulis*) tissue, since this is one of the most important species in international marine monitoring programmes. Depending on the availability, other tissues were also investigated.

This development work was undertaken at RIKZ in conjunction with the EU Project QUASH (Quality Assurance of Sampling and Sample Handling) which had the implementation

of lipid measurements in marine laboratories using non-chlorinated solvents as one of its objectives.

Experimental

Chemicals

Chloroform, methyl *tert*-butyl ether (MTBE) (Merck, Darmstadt, Germany) and hexane, methanol, cyclohexane, diisopropyl ether and propan-2-ol (Baker Chemicals, Deventer, The Netherlands) were all of analytical-reagent grade. Water was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). HPLC grade methanol and tetrahydrofuran (Baker Chemicals, Deventer, The Netherlands) were used.

The following lipid standards (Sigma Chemicals, St. Louis, MO, USA) were used: cholesteryl palmitoleate (CHOLE), acyl-*sn*-glycero-3-phosphocholine (LPC), triolein as a representative of triglycerides (TG), oleic acid as a representative of free fatty acids (FFA), cholesterol (CHOL), 1,2-diacyl-*sn-glycero*-3-phosphoethanolamine (PE), 1,2-diacyl-*sn-glycero*-3-phosphocholine (PC), sphingomyelin (SM) and 1,2-diacyl-*sn-glycero*-3-phosphoserine (PS).

Samples

Approximately 1 kg of wet mussel tissue was obtained for the experiments from the RIKZ musselwatch programme. Fresh cod flesh was purchased from a fish market. Mackerel was used from the intercomparison exercises organised by QUASIMEME (Quality Assurance of Information for Marine Environmental Monitoring in Europe). Prior to extraction, samples were stored in a freezer at −20 °C. Plaice, mussel and herring samples, used for comparing the newly developed method with Soxhlet extraction and the Bligh and Dyer method, were provided by QUASH.

Bligh and Dyer extraction

The sample was weighed in a 100 ml glass jar followed by the addition of 20 ml of methanol and 10 ml of chloroform and mixing for 2 min with an Ultra Turrax mixer T25 (IKA Labortechnik, Staufen, Germany) with an 18 mm shaft. A further 10 ml of chloroform were added and mixed for 1 min. Water was then added to make a total of 18 ml including the water already in the sample and mixed for 1 min. The layers were separated by centrifugation for 10 min at 450 *g* in a thermostatic centrifuge (Sigma 3K12, Ostenrode Am Harz, Germany) at 20 °C. The lower layer was transferred to a pear-shaped flask with a Pasteur pipette. A 20 ml volume of 10% v/v methanol in chloroform was added to the residue in the glass jar and mixed for 2 min. After centrifugation, the lower phase was added to that from the first extraction. The solvent was evaporated to dryness and the extracted weight was recorded after drying for 1 h at 103 °C.

Extraction with alternative solvents

The same principle as in the Bligh and Dyer method was used for extraction with all solvents. The tissue was weighed in a 100 ml glass jar and one of the following polar solvents methanol (MeOH), ethanol (EtOH) or propan-2-ol (2PrOH) was added. The chloroform was replaced with an apolar solvent such as MTBE in hexane, diisopropyl ether (DIPE) or cyclohexane. Typically, 20 ml were added and the phases were mixed for 2 min. Accounting for the amount of water in the sample, water was added followed by mixing for 1 min. The phases were separated by centrifugation and the maximum volume of the upper layer was transferred to an evaporation flask and weighed. A weighed amount of the extract (*ca.* 0.5 g) was used to analyse the phase composition by gas chromatography. The solvent was evaporated to dryness and the extracted weight recorded after drying for 1 h at 103 °C. This residue was then dissolved in chloroform to make a solution of 50–100 mg ml^{-1} and subjected to HPLC to record the lipid pattern. Contrary to the objectives, chloroform was used here since its properties allow injection in reversed phase HPLC. Alternatives such as propan-2-ol or toluene were not investigated in the present study.

In a number of cases, as indicated in the Results section, the lipids remaining in the lower layer were measured. The lower layer was carefully isolated by means of a separation funnel, paying special attention to ensure that no residual organic phase was included. The isolated aqueous phase was extracted a second time using the same solvent mixture which gave the highest yield in the first extraction series.

Gas chromatographic analysis

The solvent compositions were determined using a Perkin-Elmer (Norwalk, CT, USA) Auto System gas chromatograph equipped with a thermal conductivity detector. The mixtures were injected (200 °C) on a PLOT fused silica Porapak-Q column (25 m × 0.53 mm id, 20 μm film thickness, Chrompack, Bergen op Zoom, The Netherlands) at an oven temperature of 170 °C. Helium was applied as a carrier gas at a column flow rate of 20 ml min^{-1} with a split flow of 300 ml min^{-1}. Chromatographic data were processed by computer utilising Turbochrom (Perkin-Elmer) software. Calibration was performed by injecting standard solutions and quantification was based on peak area.

HPLC analysis

The extracted lipids were separated on a Hewlett-Packard (Amstelveen, The Netherlands) Model 1050 HPLC system consisting of a solvent de-gasser, a quaternary pump and an automatic injector. The detector was an evaporative mass detector (PL-EMD 940, Polymer Laboratories, UK) used at a temperature of 55 °C and an air flow of 10 l min^{-1}. The eluent was sprayed with air in a heated tube in the detector where it evaporated. Non-evaporating compounds in the eluent remained in the air stream as small droplets and were detected by light scattering.

Lipid patterns were recorded by reversed phase HPLC using a Cartenoid column of 100 × 4.6 mm id (YMC-Pack C_{30}, S-03 μm, YMC Europe, Schermbeck, Germany). A 15 min linear aqueous tetrahydrofuran gradient running from 70 to 100% was used in combination with a programmed flow-rate from 0.7 to 1.2 ml min^{-1} also in 15 min. The gradient remained at 100% tetrahydrofuran for 10 min and then returned to the initial composition and flow rate in 5 min. Using an automatic injector, a fixed equilibrium time (5 min) between analyses was applied to obtain equal column activity and subsequently constant retention times. The intitial injection was discarded since it was required to equilibrate the system. Standards and extracts, in 100% chloroform, were injected to identify the lipid classes.

Comparison of the developed method with the Bligh and Dyer method

Plaice, mussel and herring tissue were extracted in triplicate using the following developed method. A sample mass containing ≤1 g of lipid and ≤8 g of water was weighed in a

100 ml glass jar, then 16 ml of 2PrOH and 20 ml of cyclohexane were added and mixed with an Ultra Turrax for 2 min. Taking in to account the water mass in the sample, water was added to obtain a total of 22 g and the mixture was mixed for a further 1 min. The phases were separated by centrifugation (450 *g*) and the organic phase was transferred into an evaporation flask with a glass pipette. For the second extraction, 20 ml of cyclohexane containing 13% w/w 2PrOH was added and mixed with an Ultra Turrax for 1 min. The organic phase was combined with the first extract and, after addition of boiling stones, the solvent was evaporated on a water-bath (85 °C). The residue was transferred quantitatively into a weighed, wide-mouthed aluminium cup or glass Petri dish by using a few millilitres of the cylohexane–2PrOH mixture or diethyl ether. This solvent was allowed to evaporate to dryness at the top surface of the water-bath (*ca.* 50 °C) and the cup was placed in an oven at at 103 °C for 1 h. From the mass of the residue and the intake mass, the lipid content was calculated.

The wet samples were also extracted with a chloroform–methanol mixture according to Bligh and Dyer as described above. Sub-portions of the samples were freeze-dried and subjected to hot Soxhlet extraction[13] for 24 h using acetone–hexane (1 + 3). Solvent evaporation and drying of the residue were performed as described above. Results from Soxhlet extraction were calculated back to wet weight.

Results

A. Methyl *tert*-butyl ether and methanol

The first alternative solvent tested was MTBE. A preliminary experiment showed that, compared with the Bligh and Dyer method, about 90% of the lipids could be extracted from cod flesh in a two-step extraction. However, during evaporation of the organic phase, precipitation occurred. Analysis of the phase compositions showed that about 10% water and 20% methanol were present in the organic phase. Evaporation of such an organic phase ended in a methanol–water mixture, due to an unfavourable azeotrope, causing the lipids to precipitate. Addition of MTBE followed by evaporation solved this problem, but such an extra step was not practically desirable.

To investigate the influence of the water content in the organic phase, the MTBE was gradually replaced with hexane, hence allowing less water in the organic phase. Fig. 1 shows the results for cod, mussel and mackerel in a series of extractions using different mixtures of hexane and MTBE (the right-hand scale applies to the lipid yield).

Extractions A-1–A-5 were carried out using 20 ml of solvent with the composition shown on the lower *x*-axis. In each case, 18 ml of water and 20 ml of methanol were present. The bars represent the yielded lipid contents of cod flesh, mussel and mackerel samples and the horizontal dotted line represents the lipid content using the Bligh and Dyer extraction procedure. Although the yields of the test mixtures were generally lower than those with the Bligh and Dyer method, it should be realised that only a single extraction was performed and not all the organic phase could be recovered. More organic phase was recovered for higher hexane contents as a result of a better phase separation. The extracted lipid yields in cod ranged from 3 to 6 mg g^{-1}, mussel from 12 to 18 mg g^{-1} and in mackerel from 180 to 320 mg g^{-1}, all based on wet weight. The solid and dotted curves in Fig. 1 represent the methanol and water content, respectively, in the organic phase.

B. Diisopropyl ether and methanol

Instead of controlling the water content in the organic phase by adding hexane to MTBE, DIPE was selected because of its lower solubility in water. The efficacy of DIPE for lipid extraction was tested in combination with various amounts of methanol. The stepwise addition of solvent and mixing were performed as described under Experimental. A 20 ml volume of DIPE was used in conjunction with different volumes of methanol and water. The results are plotted in Fig. 2. The four extractions, B-1–B-4, were performed with 10 g wet mussel tissue. The solid line shows the resulting methanol content in the organic phase and the dotted line refers to water. The

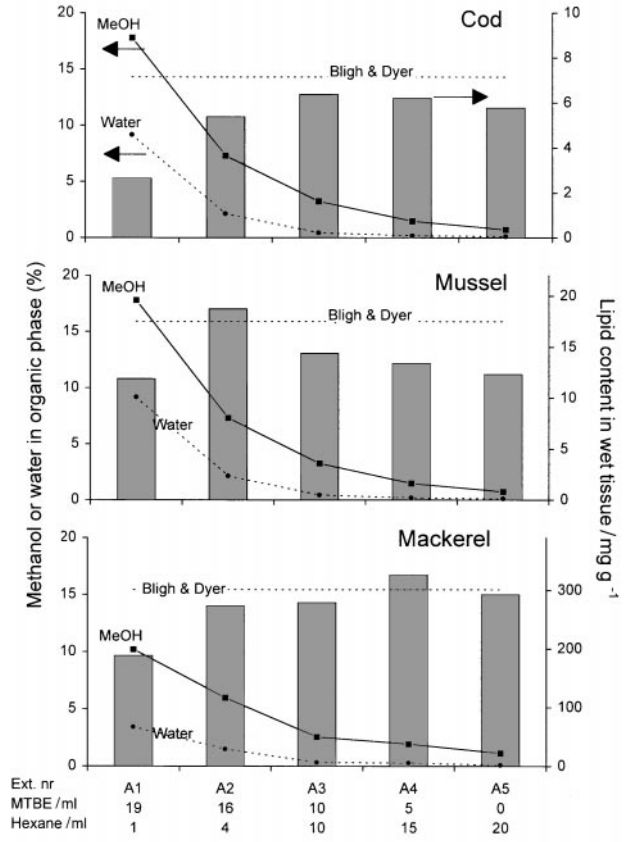

Fig. 1 Extraction of lipid from cod flesh, mussel and mackerel using amounts of MTBE and hexane as indicated below the *x*-axis. The amounts of MeOH and water were constant at 20 and 18 ml, respectively. The bars represent the lipid yield (mg g^{-1} wet tissue) and the horizontal dotted line shows the extracted lipid found obtained with the Bligh and Dyer method. All lipid contents refer to the right-hand *y*-axis. The curves indicate the MeOH and water contents and refer to the left-hand *y*-axis.

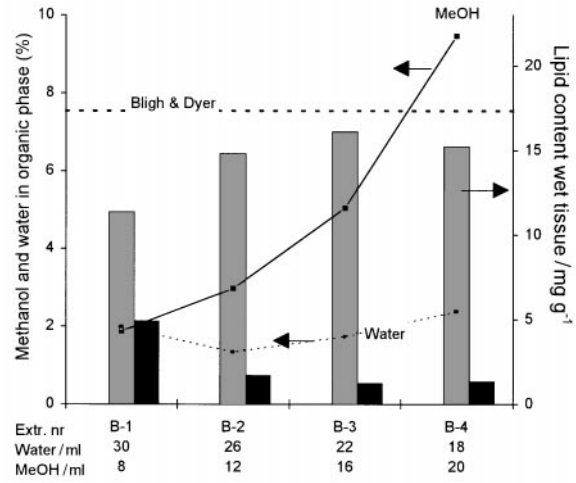

Fig. 2 Extraction of lipid from mussel using DIPE in the presence of various amounts of water and MeOH. The black bar represents the residual lipid extracted from the isolated aqueous tissue phase (right-hand *y*-axis). Other details as in Fig. 1.

extractable weight, determined in the isolated organic phase, is represented by a grey bar (right-hand scale) and the horizontal dotted line shows the result using the Bligh and Dyer method. HPLC analysis showed that lipid patterns were nearly indistinguishable from each other and the lipid yield was primarily determined by the recovery of the organic phase. In addition to a lipid determination in the organic phase, the aqueous and tissue phase was isolated after extraction and again extracted using the solvent composition that gave the highest yield in the first extraction (B-3). The black bars in Fig. 2 represent the weight extracted by this second extraction. HPLC of the lipid extracted from the aqueous phase confirmed incomplete extraction of B-1.

C. Cyclohexane and ethanol

The solvents cyclohexane and ethanol were of particular interest because of their low toxicity. Since encouraging results were obtained with hexane–methanol (A-5, see Fig. 1), it was felt that cyclohexane–ethanol should also prove applicable. Compared with hexane, cyclohexane is slightly more polar and ethanol dissolves better in hexane and cyclohexane than methanol. A wide range of water-to-ethanol ratios were used with a constant volume of cyclohexane. The procedures used were the same as before and the results are presented in Fig. 3. No phase separation could be obtained for C-1 and C-2 mixtures, even after prolonged centrifugation. The water content in the organic phase was negligible and the ethanol content also remained low. As a consequence, the ethanol content in the aqueous phase almost directly reflected the volume amounts. The extracted lipid yield clearly decreased with increase in ethanol, mainly caused by incomplete extraction of FFAs and phospholipids, as indicated by the HPLC lipid patterns of the second extract. It is most likely that for very high EtOH contents, as in C-6 (60%) and C-7 (75%), the solubility of FFAs and phospholipids in the aqueous phase became too high to allow complete extraction into the organic phase.

D. Cyclohexane and propan-2-ol

The use of propan-2-ol (2PrOH) was examined in parallel with the previous experiment. The results are given in Fig. 4. Compared with ethanol, 2PrOH showed a higher content in the cyclohexane phase while the water content in the organic phase remained low. The lipid yield of the first extraction (grey bars) increased as the percentage of 2PrOH increased. As a consequence, the residual lipid extracted from the tissue and aqueous phase mixture decreased (black bars). The HPLC traces of the latter extract confirmed that D-2 and D-3 contain the lowest amount of residual lipids (Fig. 5). An almost complete lipid pattern was still present in D-1 while the FFAs and the more polar lipids were not totally extracted from the tissue for D-4. The extraction D-3 demonstrated the best phase separation.

Because the differences were only marginal, the mixture was tested more thouroughly with a wider range of 2PrOH and with a subsequent second extraction. The composition of the organic phase was measured from the first extraction and the same composition was prepared and used for the second extraction. The two extracts were combined and after evaporation and drying, the weight of the residue was determined. These results together with the water and 2PrOH contents are plotted in Fig. 6. Compared with the single extraction, a lower percentage of 2PrOH does not result in a lower extracted weight of lipid using duplicate extraction. Alternatively, a large increase in extracted lipid was obtained when very large amounts of 2PrOH were

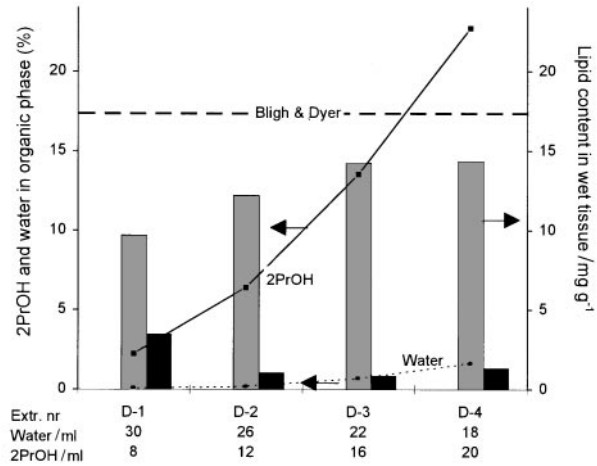

Fig. 4 Extraction of lipid from mussel with cyclohexane in the presence of various amounts of water and 2PrOH. Other details as in Fig. 1.

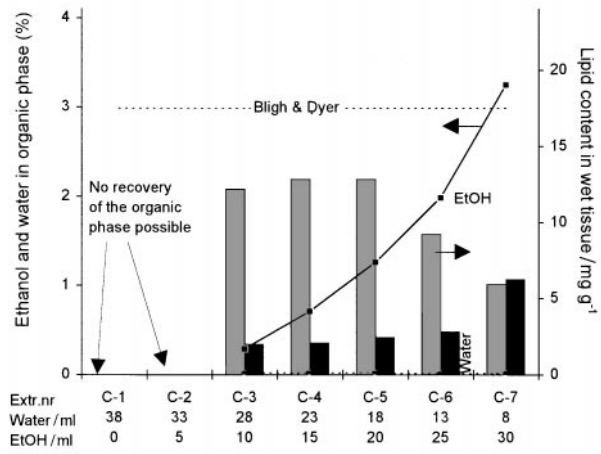

Fig. 3 Extraction of lipid from mussel with cyclohexane in the presence of various amounts of water and EtOH. Other details as in Fig. 1.

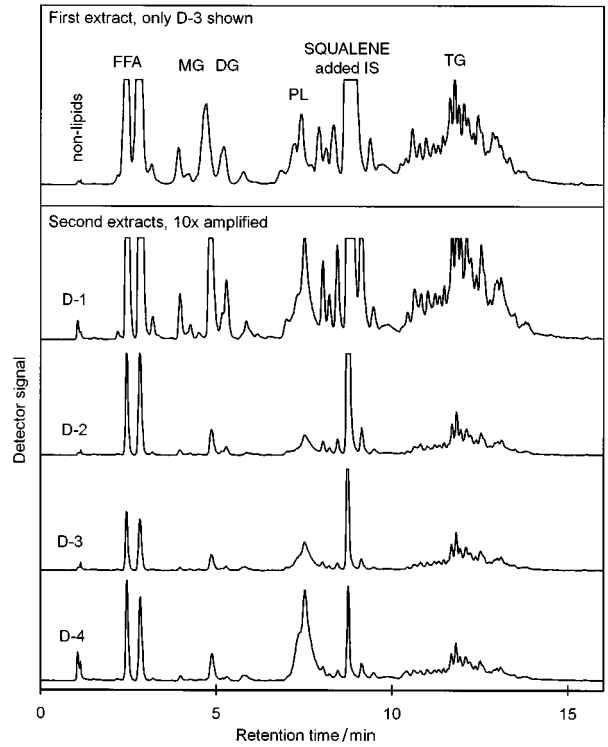

Fig. 5 HPLC of the lipid remaining in the aqueous tissue phase. Lipid classes are indicated in the upper chromatogram. For abbreviations, see Experimental.

used. However, when dissolving the material for HPLC analysis, a small amount of D-12 and about half of D-13 did not dissolve, which clearly indicated that non-lipids were extracted. This non-dissolvable fraction is reflected by a break point in the right-most bar in Fig. 6. Co-extracted non-lipids are usually more polar than lipids and are not retained on the HPLC column during separation. In HPLC of D-5–D-8, the peak heights of the unretained compounds were small and constant. These compounds increased slightly in concentration ongoing from D-9–D-11, but substantially for the extracts D-12 and D-13. Apart from this unretained peak, the lipid patterns for all extracts in this series were indistinguishable from each other and it was therefore concluded that the D-5–D-8 mixtures extracted only lipids. The percentages of 2PrOH for D-6–D-8 covered the same range as D-2 and D-3 in the previous experiment, with D-3 being the most practical in use with a view to phase separation. The extraction using the composition of D-3 was further investigated.

E. Variation of organic phase

This experiment was performed with different weights of wet mussel tissue. 2PrOH was added to each individual sample to provide an aqueous phase containing 30% w/w of 2PrOH, which was the equilibrium of the solvent ratios used in D-3 and D-8. The corresponding organic phase composition of 13.5% of 2PrOH in cyclohexane was prepared separately and added in weighed quantities to the tissue to obtain a range of organic phase-to-sample ratios. Because the phases were prepared with equilibrium composition, the organic phase volumes added also resulted in the final volumes. To maintain this volume evaporation was prevented. Recording the weights before and after extraction revealed that the losses did not exceed 2% of the added organic phase weight.

Following extraction and centrifugation, a small sub-sample of the organic phase was taken to confirm the composition by GC analysis. The weight of the remaining extract was recorded and the lipid content determined. The results are given in Table 1. The selected intake mass and organic phase volume resulted in a variation in the organic phase-to-sample ratio by a factor of 25. The measured percentages of 2PrOH in the organic phase were very close to the nominal value of 13.5%. As expected, the lipid content in the organic phase decreased when the organic phase volume increased. The lipid content in the sample resulting from these measurements is given in the last column of Table 1 and is fairly constant. It further shows no relation to the organic phase-to-sample ratio.

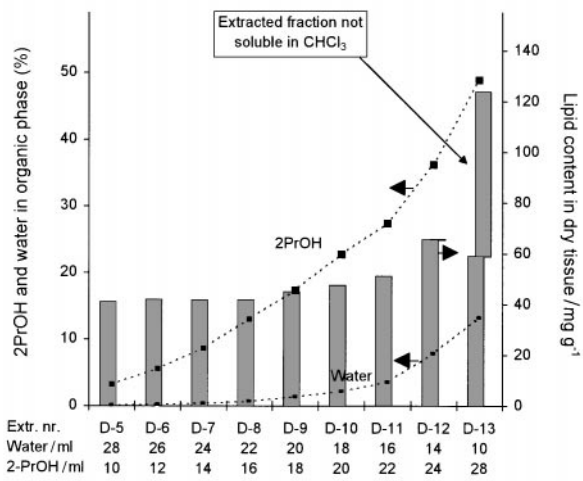

Fig. 6 As Fig. 4 except that a duplicate extraction was performed on dried mussel tissue. The ratio between water and 2PrOH was varied over a the much larger range. For D-13, the extracted weight above the break point in the bar could not be dissolved in chloroform.

F. Variation of aqueous phase

The amount of aqueous phase was varied with the amount of organic phase remaining constant. For this experiment different masses of pre-mixed aqueous phase were added to 2 g of dried mussel tissue (Table 2). Next, a weighed amount of about 20 g of organic phase (13.5% w/w of 2PrOH in cyclohexane) was added and the extraction was performed. After extraction and centrifugation, the percentage of 2PrOH was determined in the aqueous phase. All results were close to the nominal value of 30%, with a small deviation for F-1. The lipid contents were determined in an aliquot of the organic phase and recalculated for the total organic phase amount added. Only the sample with the largest aqueous phase (F-6) showed a slight decreased lipid yield, the other lipid contents being relatively constant and not showing a dependence on the size of the aqueous phase.

G. Comparison of methods

Three tissue types, plaice, mussel and herring, were extracted in triplicate using the Bligh and Dyer and Soxhlet methods and the optimised method as described under Experimental (Table 3). Clearly, lower results were found with the Soxhlet extraction for plaice (−30%) and mussel (−20%), whereas for herring the results were in the same range for all methods. When compared with the Bligh and Dyer method, the results of the method

Table 1 Variation of the organic phase to sample ratio

	Extraction number					
Parameter	E-1	E-2	E-3	E-4	E-5	E-6
Intake mass/g	21.1	10.5	10.7	10.4	5.4	2.6
Organic phase mass/g	8.0	7.4	15.8	31.1	30.6	30.8
Organic phase-to-sample ratio (w/w)	0.4	0.7	1.5	3.0	5.7	11.8
2-PrOH content (% w/w)	15.5	13.7	13.7	13.8	13.9	14.2
Lipid content in organic phase/mg g^{-1}	53.6	30.3	14.5	6.7	3.5	1.7
Lipid content in tissue/mg g^{-1}	20.5	21.4	21.5	20.1	19.7	19.5

Table 2 Variation of the aqueous phase to sample ratio

	Extraction number					
Parameter	F-1	F-2	F-3	F-4	F-5	F-6
Aqueous phase/g	7.5	15.3	22.8	30.6	30.4	60.5
Aqueous-to-organic phase ratio (w/w)	0.4	0.8	1.1	1.5	1.5	3.0
2-PrOH content in aqueous. phase (% w/w)	26	30	30	29	30	29
Lipid content in mussel/mg g^{-1}	37	35	34	37	36	31

Table 3 Lipid contents on a wet weight basis from different extraction procedures using three tissue types [Sets with different letters were significantly different ($P < 0.01$)]

	Concentration/mg g^{-1}		
Sample	Soxhlet method	Bligh and Dyer method	This work
Plaice (muscle)	9.5[a]	13.9[b]	12.8[c]
	9.3	13.7	12.6
	9.7	13.7	12.5
Mussel	20.0[a]	25.6[b]	24.5[c]
	20.6	25.6	24.5
	20.3	25.5	24.6
Herring (muscle)	110.7[a]	No result[b]	109.8[a]
	109.9	104.0	109.9
	109.4	102.6	108.8

developed and reported in this paper showed values which were 6% higher for herring and 8 and 4% lower for plaice and mussel, respectively.

Discussion

A method for the determination of lipids by extraction is, by its nature, operationally defined. The resultant measurement is in fact the residual mass after evaporation of the solvent of an extract and obtained under clearly defined extraction conditions. Because of the operationally defined character, it is essential for new and existing methods for lipid determination that (i) the critical parameters in the measurement are clearly evaluated, (ii) the robustness of a method is known and (iii) that any intra- or interlaboratory measurements clearly follow well defined procedures. The need to have such a method to measure lipid as a co-factor within national and international marine monitoring programmes is paramount in the interpretation and assessment of environmental data.

The 'standard' method for lipid extraction has been the Bligh and Dyer total lipid determination, which originally was not very strictly defined. For example, the choice of performing a second extraction was left to the analyst. In addition, the practical drawbacks such as the large volumes and filtration step were sufficient reason for the analyst to develop an adapted version.[6,14,15] This created much of the basis of the variability of results between laboratories.[10] Nevertheless, Bligh and Dyer originally selected an appropriate combination of solvents for the lipid extraction. The strength of the solvent combination lies in the properties of chloroform, which dissolves a proportion of methanol without the co-extraction of water.[11] Clearly, this is not due to the properties of the chloroform alone, because an extraction of cod tissue without methanol results in an unbreakable emulsion.[12] The chloroform is entirely sorbed by the tissue, which can only be prevented by the addition of the correct amount of a polar solvent such as methanol. By increasing the addition of the polar solvent, both phases inevitably dissolve each other until they mix. The first solvent to replace chloroform was selected on the basis that a polar solvent is a prerequisite and that the solvent which is not miscible with water has a high solubility for lipids.

Quaternary system

MTBE dissolves lipids easily and has a low boiling-point, which is an advantage in the evaporation stage. Although the preliminary extractions of cod flesh appeared promising, the large amount of co-extracted water caused a practical problem in the evaporation (see Section A). Furthermore, it seemed unlikely that large amounts of apolar triglycerides which occur in most lipid-rich tissues would dissolve in an organic phase containing 10% water. This was endorsed by the extraction (A-1) of the mackerel sample. This lipid-rich tissue contained mainly triglycerides, which resulted in a lipid concentration of about 30 mg ml^{-1} in the organic phase. The triglycerides from the mackerel sample only allowed a small percentage of water in the organic phase, resulting in lower water (3×) and methanol (2×) contents compared with the cod and mussel extractions (Fig. 1). A third phase was also observed between the organic and aqueous phases in the mackerel extraction.

The extracts were analysed by HPLC and revealed comparable lipid patterns with the exception of A-1, the extraction with the highest water content. This similarity indicates that the amount of organic phase recovered may have been more important for the yield than the exact composition. A distinct separation between phases is therefore a serious criterion for a lipid extraction method. Comparing the lipid yield of this single-step extraction with the results obtained by the Bligh and Dyer method, it is likely that a second extraction may have resulted in an acceptable extraction efficiency. Nevertheless, the solvent combination was not studied any further since the complex nature of the quaternary system makes optimisation and robust operation considerably more difficult without any significant improvement in method performance.

Aqueous phase

The evaluation of the quaternary system identified that both the yield of the organic phase and the partitioning of the different lipids between the aqueous tissue phase and the organic phase were key factors in the optimisation of the method. The yield of the organic phase was primarily determined by an adequate phase separation. If $>80\%$ of the organic phase was recovered, then a second extraction under the same conditions would complete the extraction. This was only true when the extraction efficiency, in terms of partition, was already complete, *i.e.*, all lipids were transferred to the organic phase during a single extraction. To study whether the partition hindered a complete extraction, it was necessary to measure the residual lipids in the aqueous tissue phase after a single extraction. The optimum extraction efficiency was found more accurately by determining the minimum residual lipid in the aqueous phase than determining the fraction which had already been extracted. Also, the variation of the lipid patterns in the organic phase was so small as to provide little information on the extraction efficiencies of the different lipid species. Therefore, in experiments B, C and D, the aqueous phase, including the tissue, was isolated and extracted again after the the first organic extraction. The difference between this and the *normal* second extraction was that care was taken not to include any of the remaining organic phase layer because the lipids present in that layer would dominate the relatively small amount dissolved in the aqueous phase or sorbed by the tissue. Furthermore, for the second extraction the conditions, in terms of phase compositions, were selected that gave the highest yields in the first extraction.

Evaluating the results of the second extraction of the aqueous tissue phase in experiments B, C and D showed that there was no strict optimum solvent ratio for the different solvent pairs. There was always a range of solvent ratios with minimal lipid content remaining in the aqueous tissue phase which surely favoured the robustness. Only for the extractions using EtOH and cyclohexane was the range of applicable solvent ratios rather narrow. A decrease in extraction efficiency occurred with more EtOH (Fig. 3) and for the lower ethanol contents no organic phase could be recovered as a result of the emulsions that formed. Therefore, cyclohexane–EtOH is not an ideal solvent pair for lipid extraction.

Selection of the solvent pair

Compared with EtOH–cyclohexane, the contents of MeOH in the DiPE were much higher for the same content of the polar solvent (methanol) in the aqueous phase. Likewise, 2PrOH dissolved better in the organic phase and resulted in a similar effect. Indeed, the results of these solvent combinations in Figs. 2 and 4 showed a striking similarity. The slightly lower lipid contents in the aqueous tissue phase and the corresponding lower response found by HPLC for the 2PrOH–cyclohexane mixtures were not a sufficient reason for a preference for this solvent pair. Given the similar performance, ethers were not the preferred choice on grounds of safety, smell and toxicity. Therefore, 2PrOH and cyclohexane were selected as the most favourable combination for lipid extraction.

Optimal composition

Extractions were made using a wide range of 2PrOH-to-water ratios to investigate whether a decrease in extraction yield would occur with a much higher concentration of 2PrOH. This procedure used two sequential extractions (see Section D). To obtain the maximum recovery, two extractions were essential, since it was not practically possible to recover the organic phase completely from the first extraction. However, the results showed an increase in extractable weight for the higher 2PrOH contents instead of a decrease as found for EtOH (Fig. 6). The fact that the extract did not entirely dissolve in chloroform indicated that it was unlikely to be lipids. In addition, the HPLC pattern showed a non-lipid fraction proportional to the increasing extractable weight. Apparently, at very high 2PrOH contents, the organic phase became relatively more attractive for non-lipids. This extraction of non-lipids only took effect when the water content in the organic phase became significant (Fig. 6). Where the water content was low, equal results were obtained over a wide range of 2PrOH contents. Based on the observation that the phase separation improved with higher 2PrOH concentration and the requirement that non-lipids should not be extracted, water–2PrOH–cyclohexane (22 : 16 : 20) was selected as the solvent (*i.e.* D-3 and D-8) for further investigation.

Robustness

Although lipid determination would always be an operationally defined method, it can still be a reliable measurement. The above results showed that limited variations in the solvent composition did not have a measurable effect on the result. Furthermore, for a lipid extraction method, it is important that variables such as phase volumes and sample mass do not bias the result. If the efficiency of the extraction is a competition between solubility in the organic phase and sorption of lipids by the tissue, then an increasing tissue mass would decrease the result. However, using the phase composition that resulted from the selected solvent ratios, equal lipid contents were measured when the organic phase volume was varied from 0.4 to 12 ml per gram of sample (see Table 1). Consequently, sorption of lipids by the tissue was insignificant under the given conditions. With a normal organic phase volume of 20 ml, the sample intake mass did not influence the measured lipid content over the the range 2–50 g wet mussel tissue.

Solubility of lipids in the aqueous phase would be a significant factor if the volume of that phase would influence the result. This was tested by varying the mass of the aqueous phase from 0.4 to 3 times that of the organic phase with all other conditions constant (see Section F). Only the extraction with the largest aqueous phase showed a slight decrease in the lipid content, indicating that the mass of the aqueous phase should be limited to about 1.5 times that of the organic phase.

Practical considerations

The investigations reported here resulted in an optimised method for the extraction of lipid from marine tissues. The same principles were followed as for a Bligh and Dyer extraction but with different solvents. One key advantage of cyclohexane over chloroform was its lower density, which consequently separated on top of the extraction mixture. The tissue residue which previously formed at the top of the chloroform layer now did not interfere with the isolation of the organic phase. In addition, particulate matter which is centrifuged to the bottom of the extraction jar is automatically removed from the organic phase using cyclohexane instead of being driven to the organic phase when chloroform is used. Consequently, the laborious filtration step used by Bligh and Dyer was unnecessary.

The work reported here has shown that a wide range of phase volumes and sample intake masses did not influence the measured lipid content. This was for a mussel tissue, which is not a particularly lipid-rich tissue. However, higher levels of lipids in marine biota are always present as triglycerides, which readily dissolve the organic phase. Nevertheless, high lipid concentrations in the organic phase must be avoided as the nature of the organic phase may be affected. A lipid content in the organic phase of 50 mg g^{-1} did not affect the solvent composition (Table 1). For an organic phase of approximately 20 g this corresponds to 1 g of lipid, which is set as the maximum for the selected method and is sufficient for all lipid determinations. The limit on the amount of lipid to be extracted (1 g) can be controlled by adjusting the sample amount.

Method comparison

From Table 3, it is clear that Soxhlet extraction extracted all lipids only from the lipid-rich herring sample. For plaice and mussel, Soxhlet extraction gave an unacceptably low yield. This confirms that Soxhlet extraction is not a serious candidate for the extraction of total lipids.[6,9]

The differences in extraction yield between the developed method and the Bligh and Dyer method were much smaller with the discussed method both lower for plaice and mussel and higher for herring. The average difference was only 2%. Although the differences were relatively small, they were found to be statisically significant ($P \leq 0.01$). However, it should be realised that two operationally defined methods will give, almost by definition, different results and always lead to a discussion of which one gives the true value. For these data, it was only due to the extremely good precision that a bias could be confirmed statistically. The main criterion for adopting a new method as a standard operating procedure was that no step change in international monitoring data would occur. The acceptance of a small bias should therefore be considered with respect to the variability of those international data. In an interlaboratory study,[9,10] where all laboratories used the Bligh and Dyer method to determine the lipid in a mussel tissue, a robust relative standard deviation of 13% was found. In that respect, the results of both methods applied here were in agreement. Furthermore, the adapted version of the Bligh and Dyer method used here was the result of extensive research[12] and would probably have produced results in the upper range of those obtained by the different internationally applied adaptions of the Bligh and Dyer method.

The discussion above reveals (1) that comparison of the two methods should be performed for a wider range of tissue types and (2) further testing of the method should involve more laboratories. Only in a laboratory performance test in which laboratories apply their regular methods in comparison with the new developed method can the significance of a possible step change in marine monitoring programmes be estimated. Both points were endorsed by the QUASH project and executed accordingly. The results will be published elsewhere.

Conclusions

Of the non-chlorinated solvents tested, the solvent pairs DiPE–MeOH and cyclohexane–2PrOH were the best combinations for lipid extraction from marine tissues. Both solvent mixtures showed good phase separation and sufficient extraction properties over a reasonable range of compositions. Both solvent pairs have low toxicity and are cost effective.

The selection of cyclohexane–2PrOH as an alternative for chloroform–MeOH was based on the adverse properties of DiPE, *i.e.*, explosive nature and irritating smell.

Similarly to the Bligh and Dyer method, the method developed and reported here is still operationally defined.

With respect to phase composition and phase volumes, the developed method proved sufficiently robust for routine use.

The results were sufficiently promising for the QUASH project to test the method further for a wide range of tissues and to be included in a laboratory performance test.

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