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Evaluation of the Results of the QUASIMEME Lipid Intercomparison: the Bligh & Dyer Total Lipid Extraction Method

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The results of the QUASIMEME lipid intercomparison exercise were evaluated in relation to the Bligh & Dyer (1959) total lipid extraction method. Most of the participants provided detailed information on their methods and a comparison was made based on the following parameters: drying temperature; subsampling; sample intake; solvent composition of the extraction—and partition mixture; the use of a second extraction; mixing method; and the use of filtration. Only a small number of laboratories applied conditions which conformed strictly to the original method of Bligh & Dyer (1959). Although these conditions were originally specified for cod muscle tissue, they are applicable to mussel tissue as well. Some differences in the results could be attributed to deviations from the original method, but none of them were significant with the exception of subsampling. The latter resulted in significant differences between laboratories that used the same extraction method, caused by an inappropriate compensation for the amount of organic phase absorbed by the tissue (Smedes & Thomasen, 1996). Copyright © 1996 Elsevier Science Ltd

The importance of a reliable and reproducible lipid determination method has been recognised within the QUASIMEME project (Bailey & Wells, 1994). Laboratories involved in the project were, as a first step, invited to take part in a lipid intercomparison exercise. A mussel homogenate was distributed to the participating laboratories, together with a questionnaire to establish the detail of the methods used. The results of the exercise were discussed during a workshop in Dublin, held on 13–16 October 1994. One of the conclusions of the workshop was the recognition of the Bligh & Dyer (1959) total lipid extraction method (B&D) as the most reliable method currently available (Bailey & Wells, 1994). In the 30 years since the introduction of B&D, many adaptations have been made to the original method (de Boer, 1988). As B&D is an operationally defined method, such deviations from the original method can lead to variable results. Smedes & Thomasen (1996) recently evaluated the method and discussed the impact of changes on the analytical result by applying a theoretical extraction model. They found that the kinetics of the extraction are promoted by a multi-step approach (first dissolve then extract), and by a higher methanol content in the solvent mixture. This is another key parameter in determining the yield of the extraction. One of the main sources of reduced extraction efficiency was absorption of the organic phase (containing lipids) by the tissue.

The observed variability of the intercomparison exercise (CV on the total lipid determinations, 12.6%; Bailey & Wells, 1994) could be explained in part by modifications of the original method (B&D) by the participants. The variability obtained seems small when compared to those obtained in contaminant analysis, but much better results have already been obtained in food analysis. Hollman *et al.* (1993) obtained a relative standard deviation of 2% during a certification of milk powder and pork muscle. A much better overall CV than 12.6% should therefore be possible, especially considering the relative simplicity of the B&D method. It was suggested, therefore, that a thorough evaluation may indicate which of the changes made are responsible for the variation in data.

Experimental

The questionnaire circulated during the exercise did not provide adequate information on the methods used by the participants, and they were subsequently asked to supply a detailed description of their method. Of the 33 participants who attended the meeting, 25 responded. The different methods used by the participants were then compared to the method of Bligh & Dyer (1959) with a special emphasis on a number of parameters which were considered important for the efficiency of the method namely:

- Drying temperature
- Subsampling
- Sample intake
- Composition of the extraction and partition mixture
- The use of a second extraction step
- Mixing method
- Whether filtration was used
- Other conditions

Results and Discussion

General

An overview of the different methods that were used by the participants and the key parameters is given in Table 1. The lipid contents determined are shown in Fig. 1. Not all of the descriptions that were supplied contained sufficient detail to allow a proper comparison of methods. The participants were unable to use the same volumes as those given in B&D because of the limited amount of sample used in the exercise, but only a limited number of laboratories used a downscaled version of the original method, whilst still retaining the original solvent proportions (L10, L150, L230, L430, L440, L460, L500, L560, L650, L770a, L810, L840 and L00) and performing a re-extraction (L10, L150, L00; Fig. 1). The results in Table 1 of laboratories L790 and L810 immediately stand out. The latter laboratory reported the data on a freeze dried weight basis which explains the high result. The low result of L790 cannot be explained. The results of both laboratories were excluded from further evaluation. The variability between the laboratories using B&D, respecting the original solvent ratios, was 9%. The other laboratories deviated from the B&D method mainly by using a different partition mixture, sample intake and by using dichloromethane instead of chloroform.

Drying temperature

B&D suggested a drying temperature of 60°C which was also considered to be the minimum temperature at the QUASIMEME lipids workshop in Dublin (Bailey & Wells, 1994). Most laboratories used temperatures below 60°C, possibly to prevent evaporation of the lipids. Free fatty acids will slowly evaporate at higher temperatures, but other lipids, such as the triglycerides, can act as a keeper thereby minimizing the evaporation. Smedes (unpub. data) noted a weight reduction of only 1% when the drying temperature was increased to 100°C. Free fatty acids can also act as a keeper for water if lower temperatures are used.

Considering that one lab dried the sample at room temperature, and another 'until no more solvent was smelled', the results of the intercomparison exercise do not show the drying temperature to have a pronounced effect on the results. A minimum drying temperature of 60°C seems, therefore, to be advisable. Also, the shape of the drying container can be important. To allow for proper evaporation petri-dishes or aluminium cups should be used.

Subsampling

A number of laboratories used subsampling to determine the lipid content in the extract. This can be done in two ways, referred to as 'measured subsam-

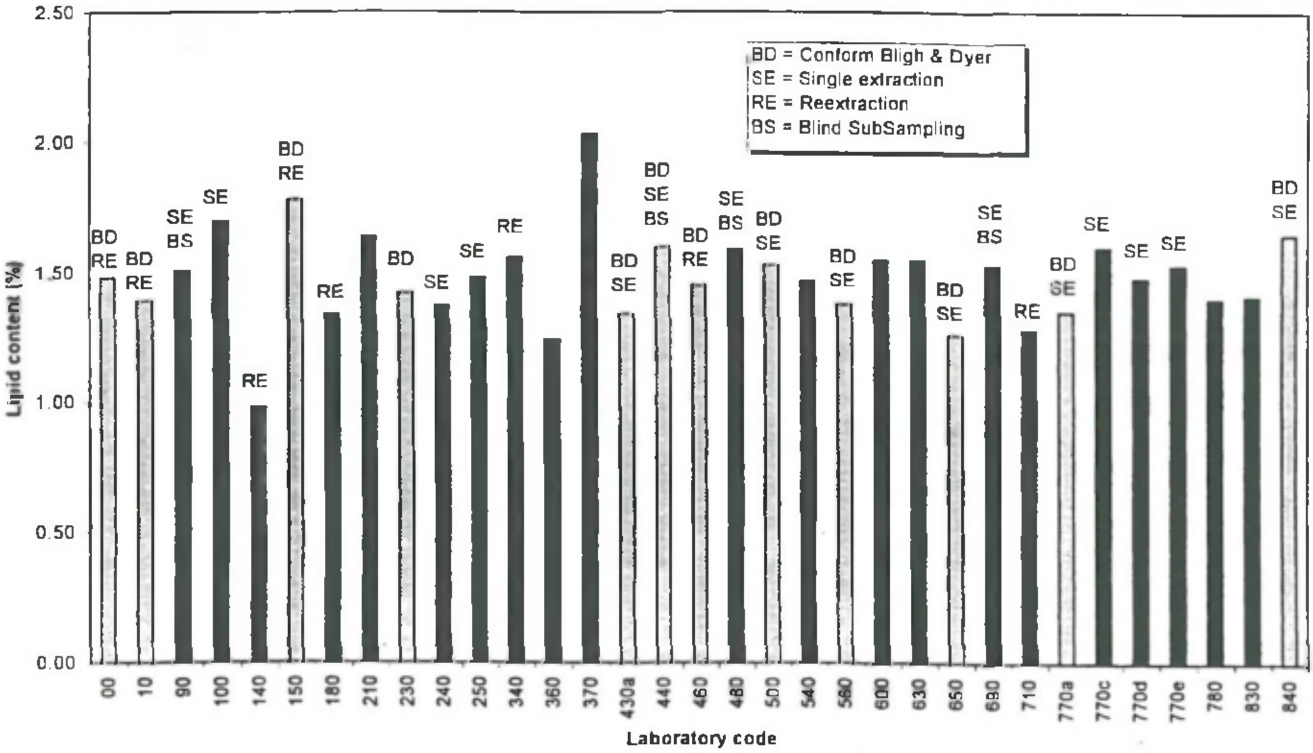

Fig. 1 Overview of the lipid contents obtained by the different participating labs with a number of key parameters: BD=partition mixture conforming to the original B&D method, SE=only a single extraction step was performed, RE=a second extraction was performed, BS=when blind subsampling (cf text) was used. The bars of the labs using the original partition mixture are in a lighter colour.

TABLE 1

Overview of the different lipid extraction methods used by the participants. Laboratories that used dichloromethane instead of chloroform are marked with a D next to the chloroform volume. Laboratories that used rotary evaporators are marked with a V next to the drying temperature. For unknown values and procedures the space is left blank.

	First step extraction Volumes in ml														Partition mixture Vol. in ml			
Lab code	Intake	Chloroform	Methanol	Water added	Water total	Mixing	Filtration	Subsampling	Second extraction	How	Back-extraction	Dry-ing CHCl3 phase?	Dry T in °C	Lipid content	$CHCl_3$	MeOH	H_2O	Remarks/comments
L00	12	30	30	17	27.3	Ultra Turrax 3 times 1 min	no	no	yes	Re-extraction with 25 ml $CHCl_3$	no		60	1.48	30	30	27	Ratio's and method matches exactly with B&D including re-extraction as described
L10	12	24	24	12	22.3	Ultra Turrax	yes	no	yes	Filter+residue with 25 ml $CHCl_3$ which is added to the first	no		60	1.39	49	24	22	Equal to B&D
L90	20	20	20	10	27.2	UltraTurrax	no	yes	no		no		40	1.51	20	20	27	Sample intake is extremely high and might result in extra water in the partition mixture
L100	20	20	40		17.2	Magnetic stirrer (30 min)	yes	no	no		After filtration 20 ml $CHCl_3$ and 20 ml H_2O was added		40	1.70	40	40	37	Actual extraction is performed at much higher methanol content than B&D. (Low drying temperature? particulate matter because of the long stirring?)
L140	8	15 D	30	5.1	12.0	Mechanical blending	yes	no	yes	Filter+residue with 15 ml CH_2Cl_2	To the mixture 15 ml water and 20g NaCl was added		103	0.98	30	30	27	Dichloromethane is used and extraction is performed at high methanol content. However that is does not explain the low result (compare Lab 100)
L150	26	52	52	26	47.5	Ultra Turrax	yes	no	yes	Filter+residue with 26+13 ml $CHCl_3$	no		45 V	1.78	91	52	48	Equal to B&D
L180	15	30 D	30	27	39.9	Shaking (10 min)	yes	no	yes	As the first extraction	no		30	1.34	30	30	40	Dichloromethane is used and extraction is performed by shaking. Drying until no solvent is smelled!
L230 B	10	40	40	25	36.6	Ultra Turrax	no	no	yes/no	The solid phase is washed with $CHCl_3$	no	Na_2SO_4	60 V	1.42	40	40	37	Centrifugation is used and the second extraction is actually a washing step, otherwise conform B&D
L240	25	50	50	0	21.5	Ultra Turrax	yes but no washing	no	no		no		105	1.37	50	50	22	Mixture is slightly deviating from B&D (less water) resulting in a high methanol content in the CHCl3 phase
L250	5	20 D	20	10	14.3	Magnetic stirrer (40 min)	yes + washing with 3 times 5 ml DCM	no	no		no		105	1.48	38	20	14	Dichloromethane is used
L340	1	20	10		0.9	Ultra Turrax	yes	no	yes	20 ml $CHCl_3$ +10 ml MeOH and subsequent washing	yes		105	1.55	60	30	33	Very low sample intake and extensive extraction (Folch & Stanley, 1957). Partition is not conform B&D
L430 A	5	10	10	5	3.3	Ultra Turrax	no	no	no ->	No extraction, only carefull washing to yield complete organic	no		100	1.34	10	10	9	B&D without re-extraction step
L430 B	5	10	10	5	8.3	Ultra Turrax	no	no	no ->	No extraction, only carefull washing to yield complete organ[illegible]	no		100	0.11	10	10	9	Result of application of a second extraction with 10 ml $CHCl_3$ as 430a
L440	10	20	20	10	18.6	Ultra Turrax	no	yes	no		no		?	1.60	20	20	19	Much care is taken to avoid evaporation. No volume correction is applied in subsampling
L460	5	10	10	5	9.3	Ultra Turrax	no	no	yes	The solid residue was washed two times with 2 ml $CHCl_3$ for 0.5 min on a vortex mixer	no	Na_2SO_4	60	1.45	10	10	8	B&D with gentle re-extraction with $CHCl_3$ using vortex mixing

TABLE 1 (continued)

	First step extraction Volumes in ml														Partition mixture Vol. in ml			
Lab code	Intake	Chloroform	Methanol	Water added	Water total	Mixing	Filtration	Subsampling	Second extraction	How	Back-extraction	Dry-ing CHCl3 phase?	Dry T in °C	Lipid content	$CHCl_3$	MeOH	H_2O	Remarks/comments
L480	2	13.3	6.7	5	6.7	Ultra Turrax 60s	yes without washing	yes	no		no		60	1.59	13	6.7	7	Mixture is deviating from B&D
L500	5	10	10	5	9.3	Whirle mixer	yes with 5 ml $CHCl_3$ for rinsing	no	no		no		60	1.53	15	10	9	Only deviation from B&D is washing the residue on the filter instead of re-extraction
L560	9	20	20	10	17.7	Ultra Turrax 30s	no	no	no		no		105	1.38	20	20	18	B&D without re-extraction step
L630	?	20	20		?	Ultrasonic/ blending	no	?	no		no		35	1.55				Insufficient information
L650	10	20	20	10	18.6	Hand shaking for 3 times 30s	no	no	no		no		35	1.26	20	20	19	B&D without re-extraction step. Probably the short shaking is not sufficient for complete extraction
L690	10	40	20	10	18.6	Shaking, 3 times for 7 minutes.	no	yes	no		no		35	1.53	40	20	19	Mixture is deviating from B&D
L710	10	10	20		8.6	Ultr Turrax	yes	no	yes	The residue was extracted with 10 ml $CHCl_3$	10 ml 0.9% NaCl was added after the extraction and filtration		45	1.28	10	20	9	The extraction mixture contains not enough $CHCl_3$ in the first step. In the second extraction much $CHCl_3$ stays in the tissue.
L770 A	10	20	20	10	18.6	Ultra Turrax	yes	no	no ->	Only washing of the residue with 15 ml $CHCl_3$	no		20	1.35	35	20	19	B&D with washing step instead of second extraction step. Drying temperature is low
L770 B	10	20	20	10	18.6	Ultra Turrax	yes	no	no ->		no		20	0.13	35	20	19	Result of application of a second extraction as 770A
L770 C	10	30	30		8.6	Ultra Turrax	yes	no	no ->		10 ml 0.9% NaCl was added after the extraction and filtration		20	1.60	53	30	19	Same as 770A but higher volume
L770 D	10	30	30		8.6	Ultra Turrax	yes	no	no ->		10 ml 0.9% NaCl was added after the extraction and filtration		20	1.48	53	30	19	Same as 770A but higher volume
L770 E	10	30	30		8.6	Ultra Turrax	yes	no	no ->		10 ml 0.9% NaCl was added after the extraction and filtration		20	1.53	53	30	19	Same as 770A but higher volume
L790	10	20	20	10	18.6	Ultra Turrax	no	yes	no		no		?	0.48	20	20	19	Refers to: de Boer, J., 1998
L810	25	50	50	25	45.0	Electric homogenisation	yes	yes	no		no		65	4.82	50	50	45	B&D without rextraction. Result was given at freeze dried weight and could not reliably be corrected
L840	5	25	25	20	24.3	Blending	yes	no	no		The $CHCl_3$ layer was washed with 20 ml of water		95	1.65	25	25	24	B&D without re-extraction step

pling' and 'blind subsampling'. In measured subsampling the recovered organic phase volume is measured and an aliquot is taken in which the lipid content is determined. The lipid content for the sample is then calculated for the total volume of the recovered organic phase (Bligh & Dyer, 1959). In blind subsampling, the lipid content is determined in a subsample of the organic phase and the total volume is considered to be equal to the added chloroform (de Boer, 1988; Randall *et al.*, 1991). Smedes & Thomasen (1996) demonstrated that absorption of the organic phase by the tissue prevents full recovery of the organic phase. As a consequence, this results in an incomplete isolation of the lipids although they were originally completely extracted to the organic phase. Measured subsampling does not correct for the amount of organic phase that is lost in this way, whereas blind subsampling accidentally compensates for this loss. In addition, Smedes & Thomasen (1996) calculated that the chloroform layer of mixture P in B&D, which Bligh & Dyer (1959) considered to be pure chloroform, actually contained 10.7% of methanol, and that the volume of the organic phase was 4% higher than the added chloroform in a procedural blank. The actual volume of the organic phase is thus higher, but losses through evaporation during the extraction will decrease the volume. Using the total volume of added chloroform to recalculate for subsampling tends to even out both effects although this is uncontrollable. Using blind subsampling will therefore result in higher, though probably more correct, lipid data. The latter is clearly illustrated by comparing the results of the laboratories L430 (1.34%) and L440 (1.60%). Both laboratories used an identical procedure but L430 measured the lipid content in the recovered organic phase, whereas L440 subsampled using the second method. Recalculating the result of L430 to the assumed volume of 20 ml leads to a lipid content of 1.60%, which is the same as determined by L440. Other laboratories (L90, L480, L690) used subsampling, but these data could not really be compared as the methods differed in more ways than just their use of subsampling (Table 1). Laboratory L90 applied a high sample intake which resulted in a slightly lower methanol content in the organic phase, and hence a lower yield (see later). The mixtures applied by laboratories L480 and L690 contained a higher amount of chloroform, but since the methanol:water ratio was not dramatically different from B&D, the methanol content of the organic phase was not expected to be different. The efficiency of extraction should, therefore, be comparable. The results (1.59% for L480 and 1.53% for L690) are indeed close to those of L440. Note also that L690 used shaking as the mixing method which might not be entirely efficient (see later).

Sample intake

A high sample intake can, for the same volume of organic phase, result in a lower extraction efficiency (Smedes & Thomasen, 1996), due to increased absorption by the tissue, or the limited solubility of the lipids in the organic phase. However, in the case of the mussel tissue (a lean tissue) the effect is not expected to be dramatic. This is demonstrated by the results of laboratory L90 who used the highest sample-to-chloroform-ratio (1:1). This did not result in a very low result (1.51%) when compared with the other laboratories that applied blind subsampling. Considering the result of laboratory L340 (1.56%) which applied a sample to solvent ratio of 1:20 and eliminated absorption of organic phase through three sequential extractions, it seems unlikely that the sample to solvent ratio is an important parameter. Consequently, the solubility of the organic phase seems sufficient for mussel tissue, and adsorption of lipids to the tissue appears to be negligible.

Composition of the extraction/partition mixture

The composition of the extraction- and/or partition-mixture is very important if an optimal yield is to be obtained (Bligh & Dyer, 1959). Smedes & Thomasen (1996) have also shown that the methanol content is a key parameter. Since the optimum composition in B&D of the mixture was defined for cod muscle tissue, it could be questioned whether applying the same mixture in this exercise would result in adequate extraction. The mussel tissue that was used for the intercomparison contained a higher lipid content (about four times higher), and the composition of the mixture called 'lipids' was also different from cod muscle. The optimum methanol content could, therefore, be different. The results (given in Table 1) show that insufficient solubility in the organic phase is unlikely. Participants (L10, L150, L250, L340, L500, L690 and L770) with a lower methanol:chloroform ratio obtained results both comparable to others, and also higher. This was especially true for L150 that obtained a relatively high lipid value (1.78%), and used a relatively high chloroform to water ratio. By contrast, L340 (1.56%) used a partition mixture that finally had a methanol content of around 12%, which is higher than the P mixture of B&D. This could be regarded as the most exhaustive extraction. Further proof that the original B&D partition mixture results in a sufficient performance is found in the results of a second extraction by L430 and L770 (see later). Only 8–10% of additional lipids were recovered (B&D recovered 6%). One laboratory (L770) experimented with higher volumes of chloroform (up to factor of 1.5) and found a slight increase in lipid content. As they did not apply subsampling, a larger fraction of organic phase could be recovered, and this may explain the increase. Finally, three laboratories (L140, L180 and L250, marked with a D in Table 1) substituted dichloromethane for chloroform. Clearly, this will result in a somewhat different extraction system due to the change in solvent polarity, but the results for L180 (1.34%) and L250 (1.48%) certainly suggest an equal extraction potential.

Second extraction

A second extraction with chloroform (as proposed by B&D) yielded, in their case, 6% additional weight of lipid. If the first extraction has completely extracted the lipids, then the second extraction recovers only the residual chloroform adsorbed to the tissue (see also subsampling). Two laboratories (L430 and L770) reported a separate result for a second extraction. After their single step extraction, the sample was again subjected to the same procedure. Compared to the first extraction, 8–10% additional lipid was obtained. Considering that about 20% of the organic phase is absorbed to the tissue (see subsampling), it can be concluded that the non-recovered chloroform phase seems not to contain the same lipid content as the primary recovered chloroform phase. This is further supported by comparing the lipid pattern of the first and second extraction by HPLC analysis. Although all lipids that are present in the first extract are also present in the second extract, the more polar lipids are dominant (Smedes & Thomasen, 1994). The procedure for the second extraction as proposed by B&D (step 5) is not expected to be very effective for polar lipids, and so it is not clear whether it will yield the same amount of lipid as a subsequent primary extraction. When comparing the results of L430A+B (1.47%) and L770A+B (1.48%) with the results of L00 (1.48%) and L460 (1.45%), it seems that either method can give similar results. Three subsequent extractions may yield a slightly higher result (L340, 1.56%). Smedes (unpub. data) extracted the methanol-water phase (after separation from the tissue) twice with chloroform, but gained only 1% of the total extracted weight. Further HPLC analyses of the extract showed that only non lipids were present. It can be concluded, therefore, that lipids extracted the second time will originate only from the tissue and that the aqueous phase can be discarded after the first extraction.

Mixing

Most participants applied Ultra Turrax for mixing. Prolonged stirring will yield similar results (L100 and L250). Shaking was applied by three participants (L180, L650 and L690) but resulted in a somewhat lower lipid content, particularly for L650. Laboratory L690 used extensive shaking, (3 × 7 min), which yielded a high lipid concentration. The latter result could also be explained as a result of an overestimation, as blind subsampling was used to determine the final lipid content (see earlier) and considerable evaporation of solvent may have occurred as a result of the long shaking time. Ultrasonic agitation by a sonoprobe (not an ultrasonic bath, which imparts lower energy) also accomplishes an effective extraction. If continued for a long time it may result in the formation of very fine particles of tissue which could enter the organic phase and contribute to the extractable weight, particularly if centrifugation is used. In the absence of cooling, extended ultrasonication can also denature the tissue. This denatured tissue can form a homogenous mixture with the chloroform layer, so it can no longer be separated by centrifugation.

Filtration

More than half of the participants used filtration, on glass fibre as well as paper filters, to remove the remaining tissue. As filtration is a rather laborious process some participants chose centrifugation as an alternative. When filtration is applied, the remaining tissue should be washed to recover the residual organic phase. This results for a single extraction step, in solvent ratios that are different from the original partition mixture (see Table 1). Note that in the B&D method washing is only applied in the re-extraction, and chloroform is not added to the partition mixture. From the present results, it can be concluded that as long as a given technique does not interfere with the partition mixture, it can still be regarded as valid.

Other conditions

Two laboratories (L230 and L460) filtered the organic extract over Na_2SO_4. This step removes water and particulate matter from the extract. Despite using only a weak second extraction (washing of the residue) their results (1.42 and 1.45%, respectively) demonstrate that filtration over Na_2SO_4 does not lower the yield. It is, therefore, expected to be a valuable contribution to the robustness of the extraction method, especially when centrifugation is applied.

Conclusions

Although some differences between the results could be attributed to the methods, most proved insignificant. Blind subsampling and re-extraction resulted in somewhat higher results compared to the original single step method of Bligh & Dyer (1959), as the original method does not recover all the lipids present.

Randall *et al.* (1991) demonstrated a very small variation between three laboratories, all applying the same method including blind subsampling. It is, therefore, highly likely that had laboratories used equal B&D methods in the QUASIMEME intercomparison exercise, the variability among the results would have been smaller. However, better comparability of results does not guarantee that the true lipid content has been determined.

Both subsampling and re-extraction yielded higher lipid contents, the former being partly due to the evaporation of the solvent during extraction. The true lipid content could, therefore, be stated to be in the range between both sets of results. The true lipid content is likely to be about 1.5%. However, although blind subsampling performed well in this exercise, it cannot be recommended for regular use. Evaporation, an uncertain phase volume, and variations in the lipid pattern in the recovered and absorbed organic phase make it difficult to control effectively. A B&D method with a subsequent second extraction is therefore recommended. For a complete yield, the sample intake should be chosen in such a way that at least 90% of the organic phase can be recovered with each extraction.

Even though the extraction described by Bligh & Dyer (1959) proved to be suitable for the mussel homogenate, this does not imply that this will be the case for all biological tissues. As B&D remains an operationally defined method, any deviation from the defined procedure can yield a different lipid result. Further work should, therefore, not only focus on the comparability of the method, but also on its validity for normalization of contaminant data.

Finally, the authors wish to thank all the participants of the exercise and the people of the QUASIMEME office for their co-operation.

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