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Erythrocyte Lipid Extraction in Alcohol–Chloroform Systems: A Comparative Study

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Lipid extraction efficiency is variable when different methods are used on the same tissue. After a systematic comparison on erythrocytes, we found that this variability was due to several factors, such as the addition of alcohol and chloroform as a mixture, the use of repeated extractions or partition procedures after the extraction. Most important, however, was the uneven distribution of solvents and lipids in an apparently monophasic extraction system. This formation reduces lipid extraction and causes accumulation of lipids in the extract supernatant, which leads to an overestimation of the extracted lipid content. We recommend a one-step 18.75 volume of methanol–chloroform (1.5:1 v/v) method for lipid extraction from erythrocytes, with methanol added before chloroform. This method combines simplicity in the extraction procedure, complete extraction of cholesterol and total phospholipids, and reliability in the quantitation of the extracted lipids.

The one-step methanol–chloroform (1:2 v/v) extraction of Folch,[1] and the repeated methanol–chloroform (2:1) of Bligh and Dyer[2] are the most frequently used methods of lipid extraction. When adapted for lipid extraction from erythrocytes, however, the extraction efficiency with these methods has been found to be quite variable.[3–7] The efficiency was improved by using specific methods developed for erythrocytes, involving for example methanol–chloroform (1:1),[3] or water–isopropyl alcohol–chloroform (1:11:7),[4] but these were either too time-consuming or gave a low phospholipid recovery.

In this study, lipid extraction efficiency from erythrocytes with different solvent systems is compared and reasons for the variation in the lipid extraction investigated. Our results indicated that lipid extraction and determination could be affected by several commonly used steps in the extraction. The most important finding was, however, the presence of an uneven distribution of lipids in the extraction system. It was possible to obtain complete lipid extraction from erythrocytes with a one-step extraction procedure, but attention had to be paid specifically to prevent the uneven distribution phenomenon.

Materials

Materials used in this study were the same as previously reported.[8,9]

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Methods and results

Cell preparation. Blood from fasting men was taken in vacuum containers with EDTA as anticoagulant. The blood was centrifuged at 1800*g* for 10 min. After removal of plasma, the cells were washed three times with 0.9% NaCl. Buffy coats were removed after each wash. Samples which underwent hemolysis during washing or which contained clots of extract residue during lipid extraction were excluded.

Recommended procedure for lipid extraction. To 0.4 ml of the prepared erythrocytes, 4.5 ml of methanol were added drop-by-drop with stirring on a mixer until the extract residue turned brownish-red. Thirty minutes later, 3 ml of chloroform was added in the same way. The extraction was complete 1 h later. Occasional stirring of the extraction system was unnecessary.

Lipid determination was affected by extracted pigment.[4,8] The whole extract supernatant was therefore transferred to a new tube, centrifuged at 30000*g* at 4°C for 30 min, and transferred again to another tube. Pigment absorbance was reduced from 0.5 per ml extract to less than 0.1 (at 400 nm), so the subtraction of the pigment absorbance in cholesterol analysis (absorbance measured at 500 nm) could be omitted.[8]

Aliquots of the extract supernatants were evaporated under nitrogen to dryness. The cholesterol content was analysed using an isopropyl alcohol–enzymatic kit method.[8] Total phospholipids were determined by

Table 1. Erythrocyte lipids extracted by different methods (mg/ml erythrocytes, mean±SD, $n=6$).[a]

Extraction method	Procedure	Cholesterol	Phospholipids
Folch[1]	M/C 1:2, 19 v	1.24(4)***	3.00(30)*
Reed[3]	M/C 1:1, 10 v, 4×	1.35(3)*	3.15(19)*
Portoukalian[14]	M/C 1:1, 20 v; then M/C 1:1, 10 v	1.23(2)***	3.18(10)**
Bligh and Dyer[2]	M/C 2:1, 3 v, mix C/W 1:1, 2 v	0.88(14)*** [0.01(1)]	3.02(15)** [0.11(3)]
Kates[13]	W 1 v, M/C 2:1, 7.5 v; then W/M/C 3:8:4, 9.5 v	0.97(4)*** [0.01(1)]	3.15(15)* [0.28(9)]
Svennerhom and Fredman[15]	W/M/C 2.22:8:4, 19.2 v; then W 2 v, M/C 2:1, 8 v	1.11(5)*** [0.08(4)]	3.24(5)*** [0.22(6)]
Rose and Oklander[4]	W/I/C 1:11:7, 19 v	1.41(4)**	3.25(7)**
Recommended	M/C 1.5:1, 18.75 v	1.37(3)	3.51(5)

*$p<0.05$, **$p<0.01$. ***$p<0.001$, compared to the values with the recommended method. [a] Values in square brackets [ ]: lipids analysed from the upper phase after partition.
Alcohol/chloroform: addition of alcohol before chloroform; alcohol/chloroform: addition of the solvents as a mixture. Abbreviations: M, methanol; I, isopropyl alcohol; C, chloroform; W, water; and v, volume.

Bartlett's (1959) modified method[10] after washing of the dried lipid extract by the Folch procedure.[1] Total phospholipids (mg) = inorganic phosphorus × 25. Individual phospholipids were separated by thin-layer chromatography.[9] In this one-step extraction, the content of extracted lipids was calculated taking into account the lipid concentration in the extract supernatant and the total solvent volume in the extraction system (including the solvent in the residue).

Cell ghosts were prepared according to Hanahan and Ekholm,[11] washed five to six times until they turned greyish-white, and mixed with 1 M NaOH for 48 to 72 h. Total protein content was determined by the Lowry method.[12]

Lipid extraction and analysis were performed at room temperature on the same day, otherwise the supernatant was kept at −20°C.

Comparison of lipid extraction efficiency for different methods. Lipid extraction efficiency from erythrocytes for several available methods and the recommended method was compared. Exact procedures were followed for these methods. The lipid content was determined from both upper and lower phases when a partition procedure was called for. Our results showed that not all methods were equally efficient in lipid extraction from erythrocytes (Table 1). The recommended method extracted more total phospholipids than all the others, but the water–isopropyl alcohol–chloroform (1:11:7) method of Rose and Oklander[4] extracted most cholesterol. The content of individual erythrocyte lipids extracted with the recommended method is listed in Table 2. The extent of the lipid extraction with the recommended method was tested by the following procedures: repeating the extraction four times, prolonging the extraction time to 48 h, or using 50 volumes of the solvent for the extraction.

Procedures influencing lipid extraction and determination. I. Solvent addition. We found that the addition of alcohol before chloroform to erythrocytes always led to greater extraction of lipids than when the two solvents were added as a mixture (Table 3).

II. Total solvent-to-sample ratio. Small-volume systems unexpectedly extracted more cholesterol in one-step extractions (Fig. 1). Small-volume systems also extracted more total phospholipids (data not shown).

Table 2. Erythrocyte lipids extracted by recommended method: 18.75 volumes of methanol/chloroform (1.5:1) solvent (mean±SD, $n=11$).

Lipids	Recovery
Cholesterol	1.37(5) mg/ml packed cells 202.5(92) μg/mg membrane protein
Total phospholipids	3.37(13) mg/ml packed cells 501.0(260) μg/mg membrane protein
Individual phospholipids (%)	
Phosphatidylserine	12.9(7)
Phosphatidylethanolamine[a]	27.6(5)
Phosphatidylinositol	1.9(2)
Phosphatidylcholine	30.0(4)
Sphingomyelin	24.7(7)
Lysophosphatidylcholine	1.5(1)
Phosphatidic acid	1.4(1)

[a] Includes lysophosphatidylethanolamine.

Table 3. Erythrocyte cholesterol extracted by addition of solvents separately or as a mixture (mg/ml erythrocytes, mean±SD, $n=3$).

Extraction solvent	Addition of alcohol and chloroform	
	As a mixture	Alcohol before chloroform
11.25 volumes of solvent extraction		
Methanol–chloroform 1:1	1.37(1)	1.49(4)*
Methanol–chloroform 1.5:1	1.26(1)	1.44(2)**
Methanol–chloroform 2:1	1.24(1)	1.42(1)**
Ethanol–chloroform 1.5:1	1.24(2)	1.41(1)**
Isopropyl alcohol–chloroform 1.5:1	1.13(1)	1.42(2)**
18.75 volumes of solvent extraction		
Methanol–chloroform 1:2	1.33(1)	1.45(2)*
Methanol–chloroform 1:1	1.34(1)	1.40(5)*
Methanol–chloroform 1.5:1	1.30(1)	1;38(1)*
Methanol–chloroform 2:1	1.32(1)	1.37(1)*
Ethanol–chloroform 1.5:1	1.31(1)	1.38(1)*
Isopropyl alcohol–chloroform 1.5:1	1.30(1)	1.36(1)*

*$p<0.05$. **$p<0.01$.

III. Uneven distribution phenomenon. (1) Extra alcohol addition. When extra methanol was added to the 'extraction-finished' methanol–chloroform (1:1 and 2:1) systems, the initially higher recovery values (than those obtained with the recommended method) were reduced below the values with the recommended method (Fig. 2). Similar results were obtained when extra methanol was added to methanol–chloroform (1:2 and 1.5:1).

(2) Exogenous cholesterol application. Addition of exogenous cholesterol revealed that the cholesterol standard accumulated in the supernatant of small-volume extraction systems (Fig. 3). This experiment was performed by determining the cholesterol concentration (*a*) in the supernatant of the extraction system (see Fig. 1), and in the supernatant of a parallel sample extraction with the addition of a known cholesterol standard (*b*). Cholesterol concentration (*c*) was also determined in a control series, prepared by adding the same amount of cholesterol

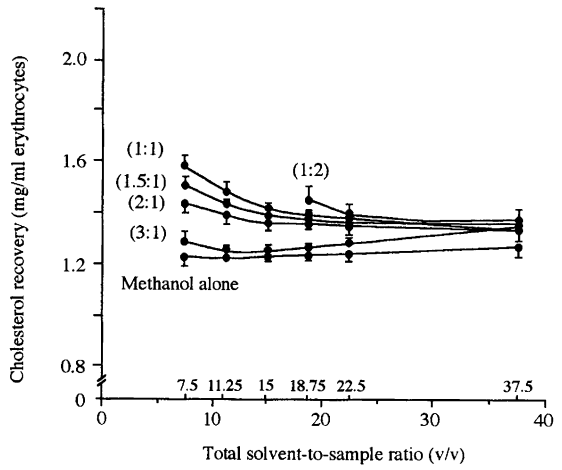

Fig. 1. Erythrocyte cholesterol extracted by one-step extraction with methanol–chloroform solvents. Aliquots of washed and packed erythrocytes were extracted with 7.5 to 37.5 volumes of methanol–chloroform (1:2), (1:1), (1.5:1), (2:1), (3:1) or methanol alone. Methanol was added before chloroform. Cholesterol was determined from the intact supernatant (without filtration or centrifugation) of the apparently monophasic extraction system.

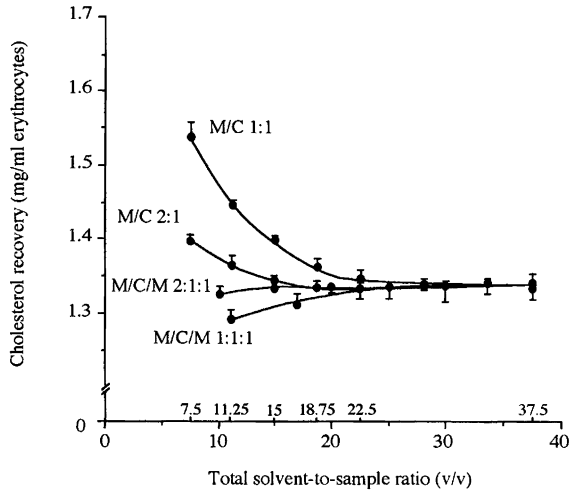

Fig. 2. Addition of extra methanol to 'extraction-finished' methanol–chloroform systems. Erythrocytes were extracted first with 7.5 to 37.5 volumes of methanol–chloroform (M–C) (1:1) or (2:1) (with methanol added before chloroform). One hour later extra methanol was added to the extractions, changing the initial methanol–chloroform 1:1 and 2:1 proportions to methanol–chloroform–methanol (1:1:1) and (2:1:1), respectively. After 1 h the extracted cholesterol content was determined. Extractions without extra methanol addition were used as a reference.

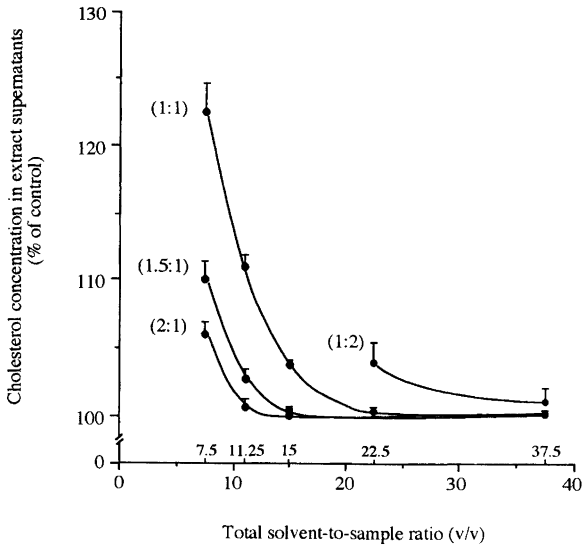

Fig. 3. Accumulation of exogenous cholesterol standard in the supernatants of methanol–chloroform extraction systems. Erythrocytes were extracted first with 7.5 to 37.5 volumes of methanol–chloroform (1:2), (1:1), (1.5:1) or (2:1), with methanol added before chloroform. One hour later, 25 µl cholesterol standard (13.3 µg in chloroform) were added and mixed. Parallel extractions were also performed but with 25 µl chloroform addition. A control series was prepared by adding the same amount of cholesterol standard to methanol–chloroform (1:1) without the erythrocyte sample but with the same volumes including the water content from the erythrocytes.

Accumulation of cholesterol in supernatant =

$$\frac{\text{Cholesterol concentration (after addition of cholesterol standard minus that before the addition)}}{\text{Cholesterol concentration in control series}} \times 100\%$$

standard to methanol–chloroform (1:1) without the erythrocytes but with the corresponding water content. Presumably, the net increase in the cholesterol concentration in the supernatant of the extraction after the addition of the cholesterol standard ($b - a$) should be the same as the cholesterol concentration (c) in control series, e.g., $(b - a)/c = 1$. However, we repeatedly observed that the net increase in cholesterol concentration after the addition of cholesterol standard to the extraction systems exceeded that in the control, e.g., $(b - a)/c > 1$. This was most marked in the small-volume extractions (Fig. 3). When corrected for the percentage increase in the concentration of the cholesterol standard (Fig. 3), the 'true' concentration of the extracted cholesterol in the supernatant was reduced to below that obtained with the recommended method.

The principle of using the cholesterol standard in monitoring the uneven distribution of the extracted cholesterol was used throughout the study.

(3) 0.9% NaCl or water addition. The cholesterol was also extracted from erythrocytes with methanol–chloroform

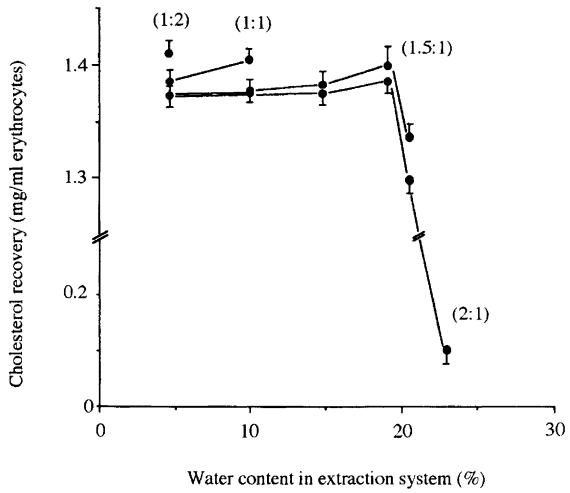

Fig. 4. Addition of 0.9% NaCl to erythrocytes before cholesterol extraction with methanol–chloroform systems. Erythrocytes were first mixed with 0.9% NaCl, then extracted with 18.75 volumes of methanol–chloroform (1:2), (1:1), (1.5:1) or (2:1), with the alcohol added before chloroform making the water content in the whole extraction systems up to 30% or until a biphase appeared. Cholesterol was determined 1 h later from monophasic supernatants, exclusively.

(1:2 to 2:1) with increasing addition of 0.9% NaCl before extraction until a biphase appeared (Fig. 4). Cholesterol recovery was increased in the monophasic methanol–chloroform (1:2 and 1:1) extraction systems with the 0.9% NaCl addition. The addition of 0.9% NaCl (more than 16.7%) in methanol–chloroform (1.5:1) and (2:1), however, markedly decreased the cholesterol recovery. The same phenomenon was observed with the addition of water.

Application of the cholesterol standard in these systems also showed that the net increase in the cholesterol concentration in the extract supernatant exceeded that expected and that this was related to the volumes of the saline or water addition (data not shown).

(4) Uneven distribution in solvent containing different alcohols. To test whether the lipid extraction would be affected by different alcohols and whether the uneven distribution of cholesterol also occurred in other alcohol extractions, erythrocytes were extracted with 7.5 to 37.5 volumes of methanol–chloroform (1.5:1), ethanol–chloroform (1.5:1), or with isopropyl alcohol–chloroform (1.5:1). The addition of the cholesterol standard in these extractions showed that the net increase in the cholesterol concentration in these extract supernatants of small-volume extraction systems also exceeded that expected (Fig. 5). The 'accumulation' of the exogenous cholesterol in the supernatants disappeared when the total solvent-to-sample ratio reached 15:1, 29:1 and 37.5:1, respectively, for methanol–chloroform (1.5:1), ethanol–chloroform (1.5:1) and isopropyl alcohol–chloroform (1.5:1) (Fig. 5).

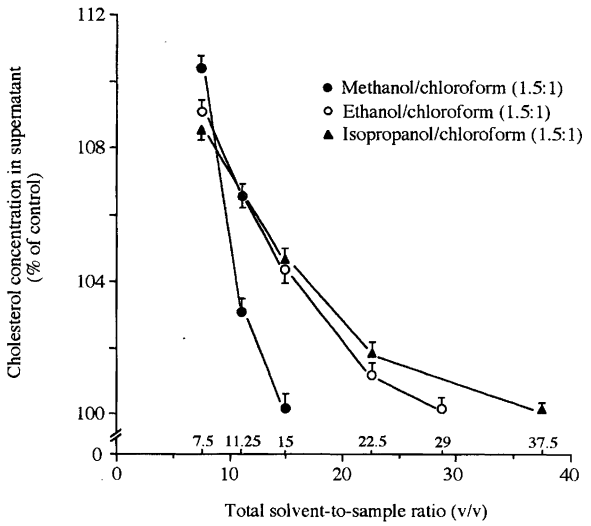

Fig. 5. Accumulation of exogenous cholesterol in the supernatants of different alcohol–chloroform extraction systems. Erythrocytes were extracted with 7.5 to 37.5 volumes of methanol–chloroform (1.5:1), ethanol–chloroform (1.5:1), or isopropyl alcohol–chloroform (1.5:1), with the alcohol added before chloroform. One hour later, cholesterol standard was added and mixed well. Parallel extractions without the addition of the exogenous cholesterol were performed. The net increase in the exogenous cholesterol in the extract supernatant was calculated as described in Fig. 3.

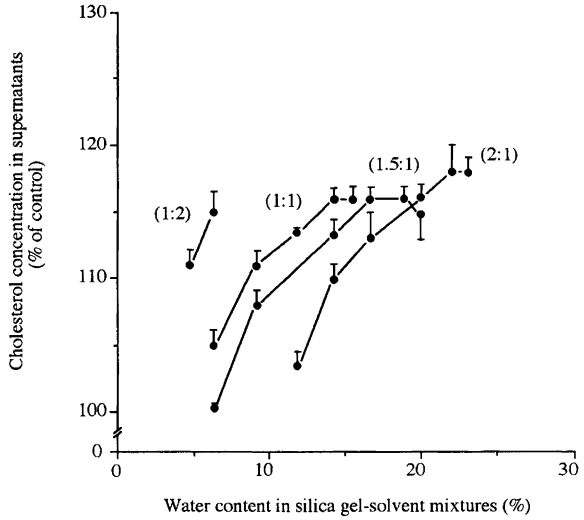

Fig. 6. Accumulation of extracted cholesterol in the supernatants of silica gel-containing methanol–chloroform–water mixtures. Four ml of methanol–chloroform (1:2), (1:1), (1.5:1) or (2:1), were added to tubes containing dried erythrocyte extracts (containing 6.35 μg cholesterol) and 0.34 g of silica gel H. Distilled water was added, so that the relative water content in the systems was in the range 1–25%. Identical mixtures without silica gel were used as a reference (100%).

We observed that the water–isopropyl alcohol–chloroform (1:11:7) extraction by Rose and Oklander gave 2.4% ($p<0.01$) higher cholesterol recovery than the recommended method (see Table 1). Application of the cholesterol standard test, however, showed that the concentration of the exogenous cholesterol in the extract supernatant was 7.1% ($p<0.001$) higher than expected. Corrected for the 7.1% higher concentration of extracted cholesterol, the true value of cholesterol recovery with the water–isopropyl alcohol–chloroform (1:11:7) extraction is only 1.30, and this is less than the 1.37 (mg/ml cells) obtained with the recommended method.

(5) Uneven distribution in solvents containing silica gel or cell ghosts. The uneven distribution phenomenon was even more pronounced in solvent mixtures containing silica gel, assumed to bind water and to transfer lipids to the solvent supernatant (Fig. 6). On the other hand, when the extractions were performed in the presence of erythrocyte ghosts, the uneven distribution was prevented to a large extent (data not shown).

IV. Repeated extractions. Theoretically, with lipid-containing solvents left in the residue, the recovery of the extracted lipids in repeated extractions cannot be complete. Using the cholesterol content obtained in the one-step procedure using 18.75 volumes of methanol–chloroform (1.5:1) extraction as a reference (100%), extraction with methanol–chloroform (1.5:1; 18.75 volumes followed by 10 volumes) recovered 95.4 ± 0.6% ($p<0.001$) of cholesterol. When erythrocytes were extracted with first 11.25 volumes, then 10 more volumes of methanol–chloroform (1:1), the recovery of cholesterol was only 92.6 ± 0.9% ($p<0.001$). Exhaustively repeated extractions (more than three times) increased the total cholesterol recovery, but extracted more pigment.

Discussion

Our results show that lipid extraction and quantification from erythrocytes was affected by addition of polar–nonpolar solvents as a mixture (Table 3), by water addition (Fig. 4), by partition (Table 1) and repeated extractions. Most important, however, was the presence of an uneven distribution of lipids in the extraction system, reflecting an uneven distribution of extraction solvents (Figs. 1–5). This phenomenon has not often been recognised. It might explain why lipid extraction efficiency is so variable when different methods are used to extract erythrocytes (Table 1). Complete lipid extraction was, however, possible in one step by the recommended method, in which the uneven distribution could be avoided.

The addition of alcohol before chloroform disperses the residue, whereas the addition of the two solvents as a mixture causes a clot.[3] For a good dispersion of the residue, more than nine volumes of methanol were necessary to be added before the chloroform. Lipid extraction was reduced in systems containing clotted residues since complete contact of the solvents with the residue particles appeared to be prevented. Good dispersion of

the residue makes a complete lipid extraction possible, but care must be taken to avoid the uneven distribution phenomenon.

The extent of the uneven distribution, on the one hand, was enhanced by a relatively higher chloroform, lower methanol and/or higher water content in the extraction systems (Figs. 1–6), or was inversely related to the polarity of the alcohol (Fig. 5). On the other hand, the uneven distribution of lipids required the presence of well-dispersed residue-like particles in the system, and this was further evidenced by the addition of finely dispersed silica gel (Fig. 6). The uneven distribution was eliminated by using cell ghosts.

In a solvent system containing a well-dispersed residue, it is likely that water tends to combine with the particles. The extract supernatant becomes chloroform-rich, while the solvent in the residue becomes water-rich. The extra methanol added to the solvent eliminates the uneven distribution so that the lipid accumulation in the solvent supernatant is reduced. As in the formation of a biphase in the methanol–chloroform–water systems, methanol is a stabilising factor, while chloroform and water are non-stabilising. This is also true in these apparently monophasic solvent systems.

The accumulation of cholesterol standard in the supernatant supports the uneven distribution hypothesis. This accumulation of cholesterol in the supernatant (a higher than expected net increase in the concentration in the supernatant) is believed to be due to failure of the cholesterol standard to become distributed in the water-rich portion in the residue, thereby accumulating in the supernatant. The accumulation of the extracted lipids in the supernatant leads to an overestimation of the lipid content. On the other hand, reduced lipid extraction was evidenced after the addition of extra methanol, even after correction for the percentage increase in the exogenous cholesterol concentration. The binding of water to the extract residues might prevent sufficient contact of the chloroform-rich portion with the residue for complete extraction. In most instances, however, the effect of lipid accumulation in the supernatant caused by the uneven distribution of solvents overcomes this reduction in lipid extraction.

For a long time, the water content has been believed to be an important factor in enhanced lipid extraction. Our data contradict this, as do the findings by others.[7] The danger of water addition cannot be overemphasised since the water content is different in various tissues and even in different samples of erythrocytes it may vary *in vivo* and *in vitro*. According to our present data, the variation in water content between samples causes a large variation in lipid extraction when solvents containing relatively more chloroform than methanol are being used.

We predict, furthermore, that the dispersion capacity of different tissues may be variable upon addition of the same extraction solvent, so that the extent of the uneven distribution phenomenon might vary even more in other extraction systems.

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