Lipid determination from monophasic solvent mixtures: influence of uneven distribution of lipids after filtration and centrifugation

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Abstract Reasons for the variation in erythrocyte lipid extraction were investigated. We found that there was an uneven distribution of lipids after filtration (up to 10% accumulated in the filtrates) or centrifugation (up to 10% in the top fraction) of several apparently monophasic extraction systems. The uneven distribution was more pronounced in the systems containing relatively more water, or, generally speaking, with more nonpolar solvents. Ill-defined procedures using either filtration or centrifugation, therefore, could be the cause for variations in lipid extraction. A way to prevent the uneven distribution has been developed and is discussed.—**Wang, W-Q., and A. Gustafson.** Lipid determination from monophasic solvent mixtures: influence of uneven distribution of lipids after filtration and centrifugation. *J. Lipid Res.* 1994. **35:** 2143–2150.

Supplementary key words lipids • cholesterol • phospholipids

The extraction of lipids from biological samples is frequently performed using alcohol–chloroform solvents. It has been reported (1) that the lipid extraction from erythrocytes is variable with solvents containing relatively more chloroform, such as methanol–chloroform 1:2 (v/v) by the method of Folch, Lees, and Sloane Stanley (2). This problem has been tested by using solvents containing more methanol (3–5) or isopropanol (1, 6), by using hemolyzed erythrocytes (1, 7) or cell ghosts (8), or by using exhaustive reextractions (3).

We found that a methanol–chloroform–water mixture is not always stable even when the mixture is apparently monophasic. Lipid distribution in such a monophasic solvent mixture may become uneven after filtration or centrifugation. This appeared to be one of the reasons for the variation in lipid extraction when the same sample was extracted with different methods. This suggestion was based on the observation that a slightly visible bilayer (although not a typical biphase) appeared at the top of a methanol–chloroform–water mixture after storage. Therefore, efforts were made in this study to verify an uneven distribution of lipids in a polar–nonpolar solvent mixture after filtration or centrifugation, and to show how such an uneven distribution was affected by the presence of water in the mixture.

MATERIALS

Analytical reagent grade chemicals and solvents were used. The following solvents and chemicals were obtained from E. Merck, D-6100 Darmstadt (Germany): chloroform, methanol, toluene, 1-butanol, acetic acid, acetone, petroleum ether, ether, and Silica gel 60 H. Isopropanol was purchased from Chemicon AB (Malmö, Sweden), and n-hexane was from Labscan Limited, Co. Dublin (Ireland). Cyclohexane was from Sigma Chemical Company (St. Louis, MO), and enzymatic kits for determination of total cholesterol were from Boehringer Mannheim GmbH Diagnostica. The following materials were used as references and obtained from Sigma Chemical Company: phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, lecithin, sphingomyelin, phosphatidic acid, lysophosphatidylcholine, lysophosphatidylethanolamine, cardiolipin, and 5(6)-cholesten-3β-ol.

METHODS AND RESULTS

Preparation of pooled lipid extract

Washed and packed erythrocytes (0.4 ml) were extracted with 4.5 ml methanol and 3 ml chloroform, with methanol added 30 min before chloroform (9). The extract supernatants were collected 30 min later, evaporated under nitrogen, redissolved in methanol–chloroform 1:1 and kept at −20°C before use.

Abbreviations: TLC, thin-layer chromatography.

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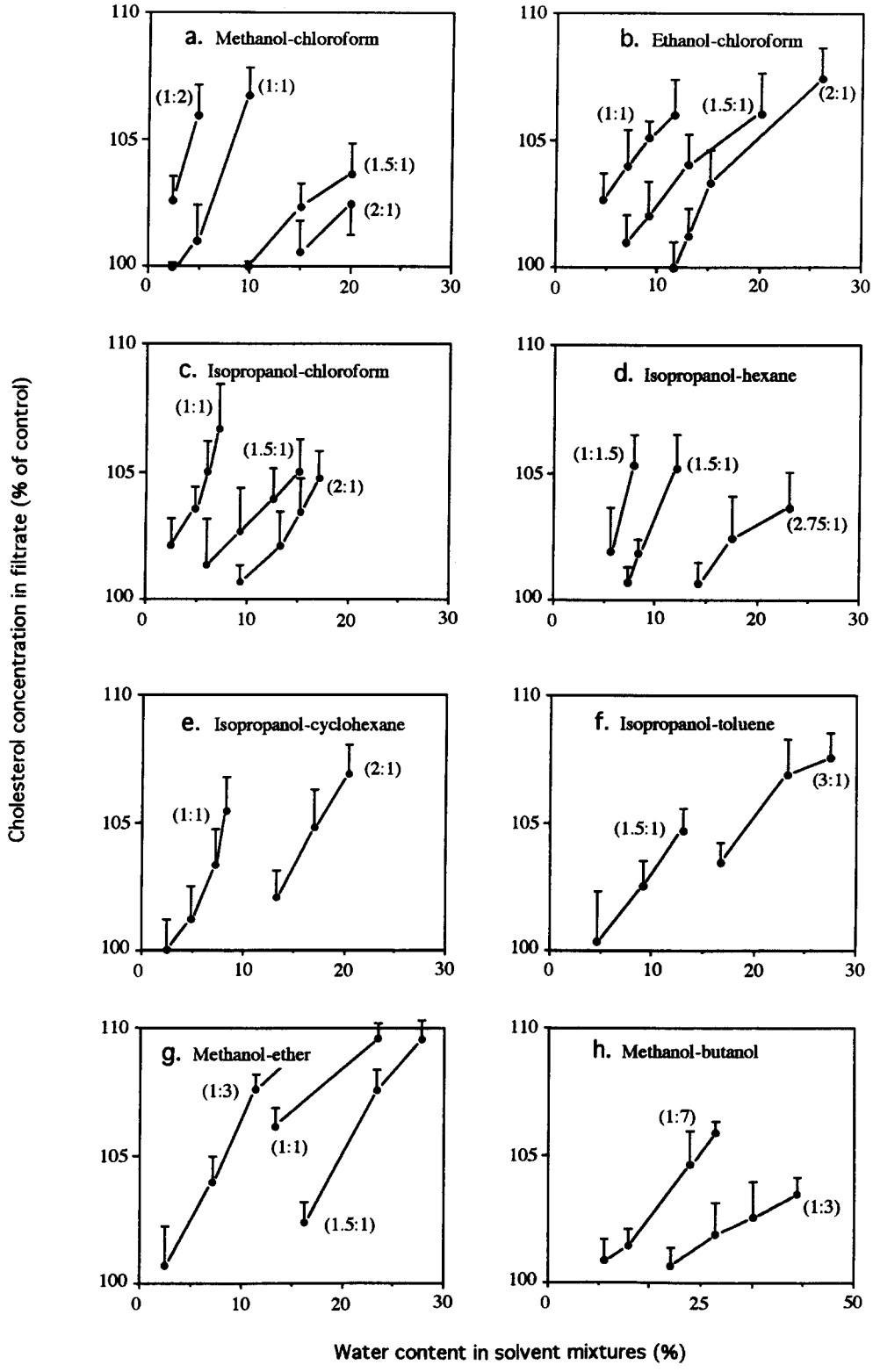

Fig. 1. Cholesterol accumulation in filtrates of monophasic polar–nonpolar solvent–water mixtures after filtration. Aliquots of prepared lipid extract were added to tubes and evaporated to dryness under nitrogen. Four ml of different polar–nonpolar mixtures were added, followed by distilled water. Apparent monophasic mixtures were filtrated through glass wool. Cholesterol concentration was determined from the filtrates. Results were compared to the cholesterol concentration in unfiltered control (n = 4 in each group).

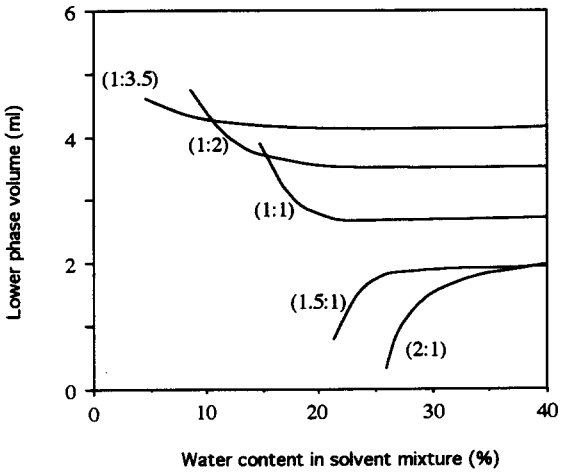

Fig. 2. Lower phase volume changes upon increasing water addition to biphasic methanol–chloroform mixtures. Distilled water was added to 4.5 ml methanol–chloroform 1:3.5, 1:2, 1:1, 1.5:1, and 2:1, until the water content reached 40%. Lower phase volumes were recorded after a biphase was formed.

Lipid determination

Cholesterol was analyzed using an isopropanol enzymatic kit method (10). Total phospholipids were determined by Bartlett's modified method (11) after washing of dried extract by the Folch et al. (2) partition procedure (2 ml chloroform, 1 ml methanol, and 0.75 ml of 0.74% KCl). Phospholipids (μg) were determined as inorganic phosphorus (μg) × 25. Individual phospholipids were separated by thin-layer chromatography (12), and detected by a TLC-densitometer ("OneScanner"). Results are given as mean ± SD.

Uneven distribution of lipids after filtration

Cholesterol concentrations were higher in filtrates after filtration than found in control series without filtration (**Fig. 1a to 1h**). The extent of the cholesterol accumulation was related to the relative amount of water and nonpolar solvents, respectively, in the solvent mixtures.

The replacement of the lipid extract with a cholesterol standard reproduced these results, as tested in alcohol–chloroform 1:2 to 2:1 and in the isopropanol–hexane 1:1.5 mixture (13, 14) (data not shown). In these alcohol–chloroform mixtures, total phospholipids also accumulated in the filtrates. However, in isopropanol–hexane 1:1.5 after water addition (7%), total phospholipid concentration in the filtrate was lower and only 40% ($P < 0.001$) of that in the control.

Lipid accumulation in the filtrate was pronounced when glass wool was tightly packed. It was reproduced with filter paper, but not with a single glass bead. It was also seen at the addition of 0.9% NaCl or 4% Triton X-100, but less so with the Triton X-100 addition. Addition of acetic acid markedly prevented this lipid accumulation.

Trypan blue (5 μl in water), when added to the solvent mixtures as used in Fig. 1, appeared in the glass wool after filtration.

We also observed that in a biphasic methanol–chloroform–water mixture, filtration caused the volume of the lower phase to increase in methanol–chloroform 1:3.5, 1:2, and 1:1, but to decrease in methanol–chloroform 1.5:1 and 2:1, just as the lower phase volume changed when less water was added to unfiltered biphasic methanol–chloroform–water mixtures (**Fig. 2**).

TABLE 1. Influence of filtration and centrifugation on cholesterol concentration in extract supernatants of methanol–chloroform 1:1 extraction of erythrocytes

	Solvent Addition Method	
Supernatant Treatment	Methanol–Chloroform[a]	Methanol–Chloroform[b]
12.25 Volume extraction		
Intact supernatant[c]	0.099 ± 0.002	0.118 ± 0.002
Filtrated[d]	0.106 ± 0.002***	0.121 ± 0.002**
Centrifuged[e]	0.117 ± 0.002***	0.129 ± 0.002***
Centrifuged-transferred[f]	0.104 ± 0.002**	0.122 ± 0.002*
Transferred-centrifuged-transferred[g]	0.100 ± 0.002	0.119 ± 0.002
24.50 Volume extraction		
Intact supernatant[c]	0.052 ± 0.001	0.054 ± 0.001
Filtrated[d]	0.052 ± 0.001	0.054 ± 0.001
Centrifuged[e]	0.056 ± 0.001***	0.057 ± 0.001**
Centrifuged-transferred[f]	0.053 ± 0.001**	0.056 ± 0.001*
Transferred-centrifuged-transferred[g]	0.052 ± 0.001	0.053 ± 0.001

Erythrocytes were extracted with 12.25 or 24.5 volumes of methanol–chloroform 1:1, with methanol added together with chloroform (*a*) or 30 min before chloroform (*b*). Values are given as mg/ml extract supernatant, mean ± SD, n = 6. Cholesterol was determined from: (*c*) the intact supernatant (standing for 2 h at room temperature); (*d*) filtrate; (*e*) the top of the centrifuged extracts (1800 *g* for 10 min); (*f*) from the centrifuged, then transferred supernatants; or (*g*) the supernatant that was transferred to a second tube, centrifuged, and transferred again to another tube (transferred-centrifuged-transferred supernatant).

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, compared with cholesterol concentration in intact supernatant of the same extraction.

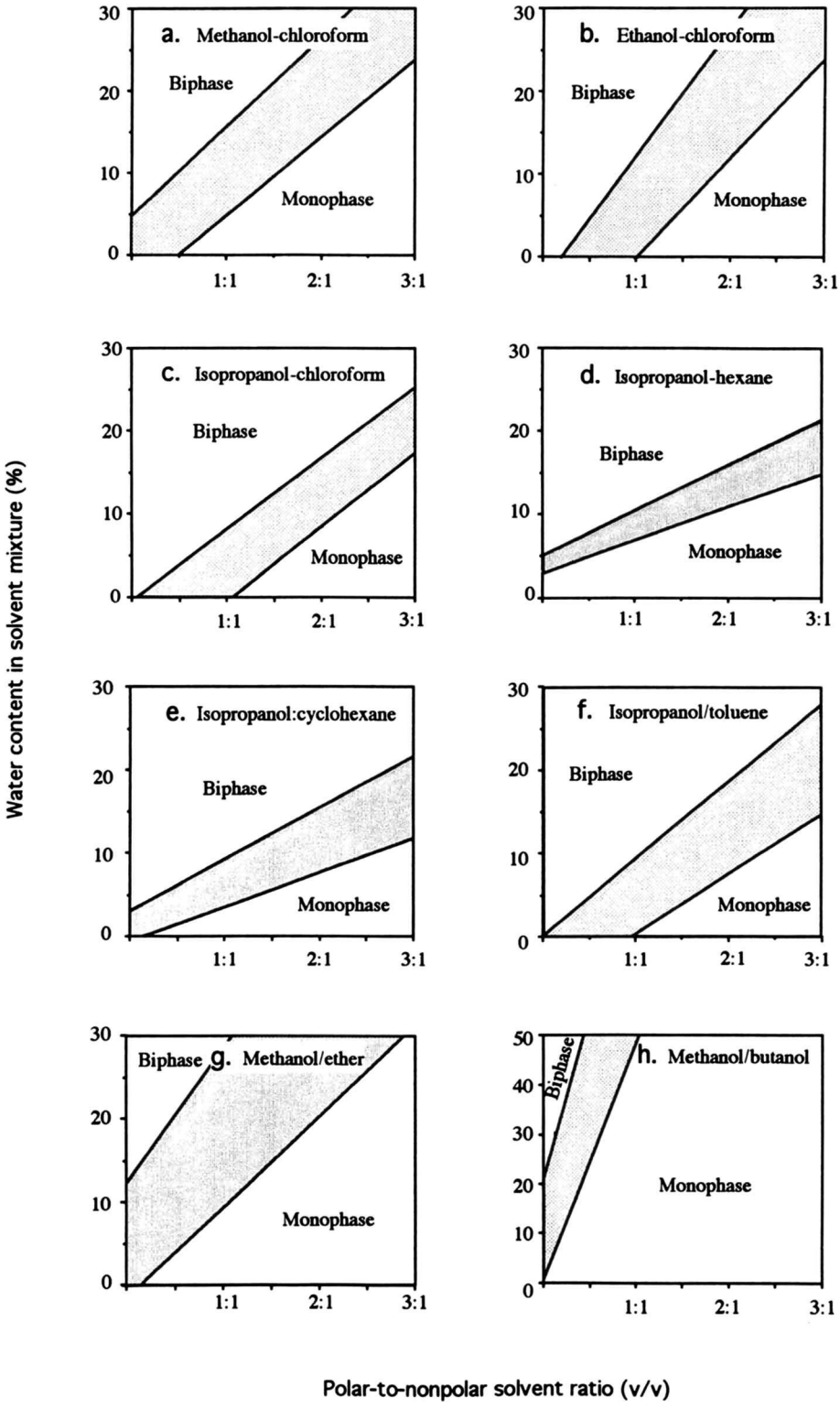

Fig. 3. Phase distribution in polar–nonpolar solvent–water mixtures. The stable monophase, unstable monophase (shaded squares), and biphase are distinguished by adding a minimum water content at which the cholesterol concentration showed an increase in the filtrate as tested in Fig. 1, and secondly at which the apparent biphase appeared.

The influence on cholesterol concentration by filtration was dependent on the condition of the residue in the whole extraction system. In the filtrate of methanol–chloroform 1:1 with 6.3% water extraction containing a well-dispersed residue, the cholesterol concentration was increased only by 2.5% as compared to 7.1% in the filtrate of the methanol–chloroform system containing a clotted residue (**Table 1**). When, on the other hand, the extraction was carried out in methanol–chloroform 1:1 with 3.2% water, the influence on the cholesterol concentration by filtration was not apparent (Table 1).

Prevention of the uneven distribution phenomenon due to filtration

The way to prevent the variation in cholesterol concentration in filtration is, therefore, to use a stable solvent mixture. Stable solvent systems appear to be those containing relatively more alcohol, but less water or less nonpolar solvent (Fig. 1). A more alcohol-rich mixture can accept more water before it becomes unstable. Data in Fig. 1 show that when the water content was limited within a certain range depending on the polar–nonpolar solvent used, the monophasic polar–nonpolar mixture became stable and the filtration procedure was satisfactory for quantitative estimation of the cholesterol concentration. For example, methanol–chloroform 1:1 containing up to 5% of water, or methanol–chloroform 2:1 containing up to 15% water were stable solvent systems for filtration (Fig. 1a). The stable and unstable monophasic solvent systems could be expressed in a different way as in **Fig. 3,** in relation to its minimum water content. It could be seen that the boundary separating a stable from an unstable monophasic solvent was parallel to that separating an unstable monophasic solvent from a biphasic solvent. A solvent mixture that forms an unstable monophase by water addition can thus be predicted.

Uneven distribution of lipids after centrifugation

Furthermore, storage and centrifugation markedly affected the distribution of cholesterol in a monophasic solvent mixture (**Fig. 4**). Cholesterol accumulated (2–3%) in the top fraction of a 15% water-containing methanol–chloroform 1.5:1 mixture when the solvent had been standing at room temperature for only 2 h (Fig. 4A). Centrifugation (1800 *g* for 10 min) of the mixture caused a more marked accumulation (10–13%) of cholesterol in the top (Fig. 4B). The centrifugation-caused accumulation of cholesterol was reduced (to 3–5%) after standing at room temperature for 24 h (Fig. 4C), but no further reduction was observed after another 24 h. It was interesting to note that storage of the solvents at −20°C preserved the lipid distribution (Fig. 4D and E). The uneven distribution phenomenon was enhanced by low temperature, high gravity, and longer centrifugation time.

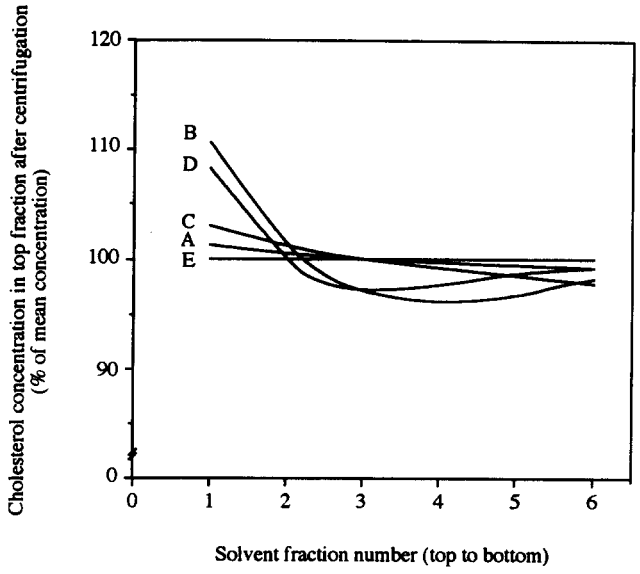

Fig. 4. Cholesterol accumulation in the top fractions of methanol–chloroform–water mixtures after standing or centrifugation. Aliquots of prepared lipid extract were added to tubes. After evaporation, 6.5 ml methanol–chloroform 1.5:1 and then 1.15 ml distilled water were added and mixed well. In A, the mixture was left at room temperature for 2 h; B, the mixture was centrifuged at 1800 *g* for 10 min, and cholesterol concentration was determined within 1 h; C, after the centrifugation, the mixture was left to stand at room temperature for 24 h; D, after the centrifugation, the mixture was left to stand at −20°C for 24 h; and in E, the mixture was not centrifuged but kept at −20°C for 24 h. Six fractions of solvents (1 ml each from top to bottom) were then transferred to other tubes, respectively. Cholesterol concentration was determined from each fraction and the mean value (n = 3 in each treatment) was compared to that from the uncentrifuged mixture (n = 6).

The uneven distribution phenomenon could be reproduced in other solvent mixtures, and became more pronounced with relatively more water in these solvents (**Fig. 5**). Generally speaking, a stable solvent system after centrifugation was then characterized by a lower content of water. However, in most solvent systems, the uneven distribution of lipids occurred even when the water content was zero, except in methanol–ether (Fig. 5g), in which 3 and 13% water, respectively, had to be added. That the stability of the solvent mixtures was related to the alcohol content was observed in isopropanol–chloroform (Fig. 5c), methanol–ether (Fig. 5g), and in isopropanol–cyclohexane (Fig. 5e) systems. For example, for a given water content (6%), centrifugation resulted in a 5% accumulation of cholesterol in the top fraction of an isopropanol–chloroform 1:1 mixture, while under the same conditions, it was negligible in an isopropanol–chloroform 2:1 mixture (Fig. 5c). In other systems (Fig. 5a to 5d, 5f, 5h), more alcohol made the systems unstable and produced uneven distribution of lipids after centrifugation. For methanol–chloroform mixtures, a given water content of 9% resulted in a 2–3% accumulation of cholesterol in the top fraction of a methanol–chloroform

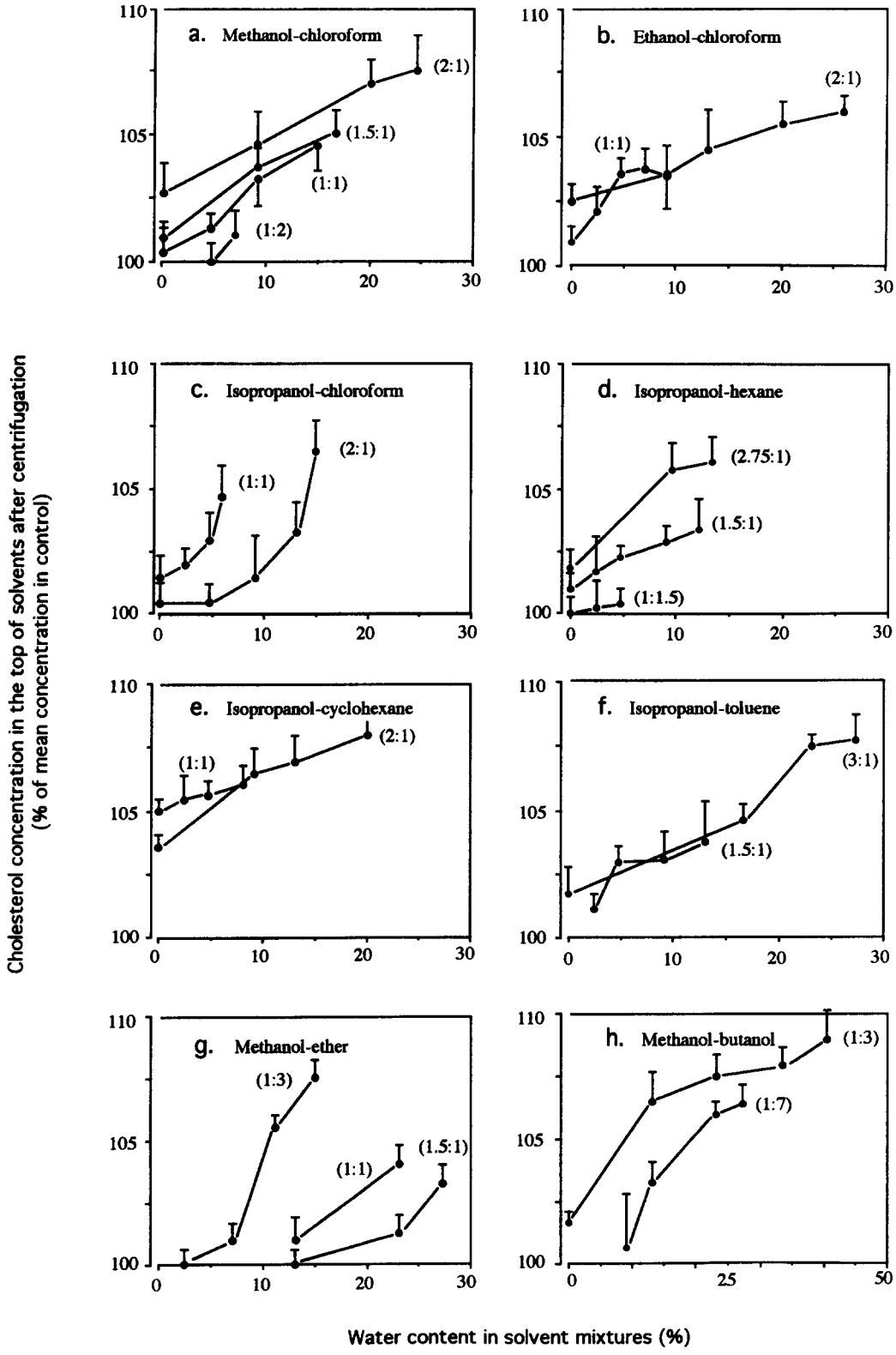

Fig. 5. Cholesterol accumulation in the top of polar–nonpolar solvent–water mixtures after centrifugation. Different lipid-containing solvent mixtures (as in Fig. 1) were centrifuged at 1800 *g* at room temperature for 10 min. Cholesterol concentration was determined from the top of the centrifuged mixture (no transferring). Results were compared with those in uncentrifuged controls (n = 4 in each group).

1:1 mixture, and 4–5% in methanol–chloroform 2:1 after centrifugation (Fig. 5a). In most solvent mixtures, however, the maximum accumulation of cholesterol reached up to 5 to 10% of mean cholesterol concentration.

Addition of a cholesterol standard confirmed the uneven distribution pattern after centrifugation in methanol–butanol 1:3 (**Fig. 6a**), as well as in isopropanol–hexane 2.75:1, isopropanol–cyclohexane 3:1, isopropanol–toluene 3:1, methanol–ether 1:3, and methanol–butanol 1:3 (data not shown), but not in methanol–chloroform 2:1 (Fig. 6b).

Total phospholipid concentration also increased after centrifugation as tested in methanol–chloroform 1:2 to 2:1 mixtures. Relatively more phosphatidylcholine and sphingomyelin accumulated in the top fractions, while other phospholipids appeared in the middle and bottom fractions.

Although the maximum accumulation of cholesterol (and total phospholipids) in the top of these solvent mixtures after centrifugation was only 5 to 10% (Fig. 5), the significance of this accumulation cannot be neglected. The uneven distribution of cholesterol between the top (fraction 1) and the middle (fractions 3–4) was clearly more than 10% (see Fig. 4B and D).

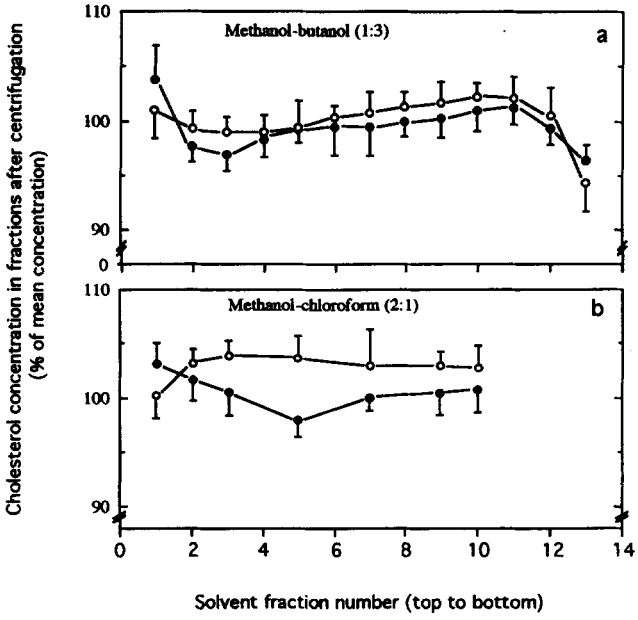

Fig. 6. Distribution of cholesterol standard (○) and extracted cholesterol (●) in fractions of two solvent mixtures after centrifugation. To tubes containing dried lipid extract, 4 ml methanol–chloroform 2:1 or methanol–butanol 1:3 were added, followed by the addition of 1.25 ml or 2.7 ml of distilled water, respectively. The mixtures were centrifuged at 1800 *g* for 10 min. Fractions (0.5 ml each) were taken from the top to the bottom. In another group, cholesterol standard was used instead of the extracted cholesterol. Cholesterol concentrations in these fractions were compared with those in uncentrifuged controls (n = 4 in each group).

Prevention of the uneven distribution phenomenon due to centrifugation

To prevent the uneven distribution phenomenon, we suggest that any centrifuged supernatant be transferred to another tube for lipid quantification. This procedure works with pure solvent mixtures and solvent mixtures that contain little sediment. However, in most lipid extractions, there is a large volume of extract residue in the extract mixture.

In one-step extractions (1, 2), the lipid concentration may differ in the supernatant and in the solvent left in the bottom residue after centrifugation. For a quantitative estimation of lipid concentration in the solvent in this case, we recommend that the entire supernatant be transferred to a tube for centrifugation, and then transfer the centrifuged supernatant to another tube to eliminate the uneven distribution of lipids produced after centrifugation (the transferred–centrifuged–transferred procedure) (Table 1).

DISCUSSION

Our data show that lipid distribution was uneven after filtration or centrifugation in apparently monophasic polar–nonpolar solvent mixtures with or without water. This phenomenon, as far as we know, has not been well recognized before.

The higher than expected lipid concentration in filtrate after filtration, can be explained by a water-rich solvent portion remaining in the filtering medium (glass wool or filter paper). This possibility was supported by the accumulation of cholesterol in the filtrate (Fig. 1), and also by the volume change in the lower phase in a biphasic methanol–chloroform solvent mixtures (Fig. 2), presumably through a physical trapping of a part of the water-rich portion by the glass wool through a capillary absorption phenomenon. The uneven distribution phenomenon was repeated with the use of filter paper, but not with a single glass bead. The relatively small accumulation of cholesterol in the filtrate of the 12.25 volumes of methanol–chloroform 1:1 (Table 1) was most likely due to a competitive trapping of a portion of water in the well-dispersed residue. This appears to make the extract supernatant relatively more "stable" as compared to an extract containing a clotted residue.

The uneven distribution of lipids after centrifugation was shown to be due to a layering of lipids within the solvent. It might be a density-related mechanism (Fig. 4 to 6), as the solvents used are less dense than water, and thereby the upper solvent-rich fraction contained more lipids. This hypothesis may explain the accumulation of cholesterol in the top fraction of most solvent mixtures tested, but is not applicable to the accumulation of cholesterol in the top fraction of methanol–chloroform–water (Fig. 5a). In the latter, chloroform is heavy

and tends to sink to the bottom. Cholesterol should accumulate in the bottom chloroform-rich fraction rather than float at the top. A possible explanation might be that, in this system, cholesterol forms a micellar complex, becomes hydrophilic, and thereby causes the accumulation in the upper water-rich portion. The formation of the micellar complex may be facilitated by the presence of phospholipids, as the use of cholesterol standard without phospholipids did not result in the accumulation phenomenon (Fig. 6b).

Generally speaking, a solvent mixture that is unstable during filtration may be more stable during centrifugation, and vice versa (Figs. 1 and 5). In the literature, the composition of polar solvents, nonpolar solvents, and water vary largely among different extraction methods and whole extraction systems. The filtration or centrifugation procedure used in a recommended method may not always be identically applied. These are all possible reasons for the observed variation in lipid extraction when different methods are used on the same tissue, or when the same method is used on different tissues with different or varying water content.

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