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Ethyl Acetate/Ethyl Alcohol Mixtures as an Alternative to Folch Reagent for Extracting Animal Lipids

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The lipids of fresh egg yolk, boiled yolk, yolk powder, and raw animal tissues including pork loin, belly pork, and pork fat were extracted with the mixed solvents composed of ethyl acetate (EtOAc) and ethyl alcohol (EtOH) at 2:1 and 1:1 volume ratios, and the results were compared with those obtained with Folch reagent, that is, a mixture of chloroform and methyl alcohol (2:1, v/v). Extraction yields, lipid profiles, and fatty acid compositions were determined by weighing, TLC-FID, and GC, respectively. Data of the extracts obtained with the mixtures of EtOAc and EtOH were not significantly different from those obtained with Folch reagent, implying that the mixed solvent composed of EtOAc and EtOH (1:1 to 2:1, v/v) may replace Folch reagent, which is considered to be toxic and mutagenetic due to its component of $CHCl_3$, for lipid extraction.

KEYWORDS: Folch reagent; ethyl acetate; ethyl alcohol; egg yolk; animal tissues

INTRODUCTION

Lipids are defined as substances insoluble in water and salt solutions, but soluble in lipophilic solvents. Usually extracted with diethyl ether and petroleum ether, the extracts always contain hydrophobic substances other than lipids and therefore are called crude lipids. Because diethyl ether and petroleum ether are immiscible with water, the water content of foods and biological tissues affects the yield of lipid extraction. Folch et al. (*1*) successfully extracted lipids from animal tissues with the mixture of chloroform and methyl alcohol (2:1, v/v). Sheppard et al. (*2*) evaluated the effects of eight extraction methods on total fat and fatty acid composition of foods. The procedure included subjecting sausage to hydrolysis in a 4 N HCl solution at 60 °C for 30 min, heating in a 90 °C water bath for 30 min, and extracting with diethyl ether to get the most effective extraction. Obviously, this procedure was tedious and not applicable to various food samples. The mixed solvent developed by Folch et al. is widely used in lipid extraction currently, even though it contains the very harmful chloroform that was reported to decrease the activity of glutathione reductase and glutathione peroxidase in isolated rat hepatocytes, cause the death of human hepatocytes, and induce hepatoma in mice (*3*−*5*). Long-term use of Folch reagent is detrimental to human health. Therefore, it is necessary to develop a safe and effective method for lipid extraction. The objective of this work is to investigate the possibility of replacing the Folch reagent with nontoxic solvents such as ethyl acetate and ethyl alcohol for the sake of researchers' health.

MATERIALS AND METHODS

Materials. Packaged fresh eggs were purchased from a local supermarket. One yolk powder was prepared from boiled eggs. The boiled yolk was separated, pulverized, and air-dried at 50 ± 1 °C for 8−10 h. The other yolk powder and raw pork including loin eye, belly pork and fat tissue were obtained from the market. All solvents and reagents were of analytical grade.

Extraction of Lipids. Yolk and raw animal tissues were separately mixed with a solvent at a ratio of 1 to 10 (w/v). The solvents used included acetone, chloroform, acetonitrile, ethyl acetate, ether, *n*-hexane, ethyl alcohol, Folch reagent, and the mixtures of ethyl acetate and ethanol. After homogenization with a Polytron for 1 min, samples were settled and filtered. The extraction was done twice, and the two filtrates were combined together. The filtrate was evaporated under vacuum to remove solvent. Dried extracts were weighed to calculate the yields. All analyses were conducted in triplicate.

Analysis of Lipid Composition by TLC-FID. Extracts were dissolved in EtOAc/EtOH mixture (1:1, v/v) at 3% concentration and then applied to TLC rods (1.5 mm × 15 mm) (Chromarod-SIII, thin layer quartz rod; Iatron Laboratories, Inc., Tokyo, Japan). A mixed solvent of lower polarity, *n*-hexane/ether/formic acid (70:30:1, v/v), was utilized for the first development so that neutral lipids and cholesterol were separated and determined with a flame ionization detector (Iatronscan MK-5, TLC-FID analyzer) starting from the point ∼20% full length above the origin. Because the lipids retained in the origin were not burned, the TLC rod was then developed in chloroform/methanol/acetic acid/H_2O (75:45:1:1, v/v), and phospholipids were identified at this time.

Analysis of Fatty Acid Composition by Gas Chromatography. Lipids were derivatized to generate methyl esters of fatty acids according to the method described by Alonso and Juarez (*6*) with a little modification. Briefly, 0.1 g of lipids was mixed with 3 mL of

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Table 1. Percentage Yields of Lipids Extracted with Different Solvents from Boiled Yolk and Yolk Powder[a]

solvent	boiled yolk	yolk powder
chloroform	33.84 ± 0.36a	49.02 ± 0.67a
EtOAc/EtOH (2:1)	33.59 ± 0.42a	49.26 ± 0.54a
diethyl ether	28.81 ± 0.23b	46.69 ± 0.42b
acetone	25.01 ± 0.35bc	40.38 ± 0.22c
ethyl acetate (EtOAc)	23.09 ± 0.12c	40.35 ± 0.36c
acetonitrile	12.51 ± 0.21d	11.47 ± 0.12d
n-hexane	8.70 ± 0.28e	46.20 ± 0.18b
Folch reagent	33.61 ± 0.32a	50.01 ± 0.52a

[a] Data are presented as mean ± SD. Values bearing different letters in the same column are significantly different in Duncan's multiple-range test.

diethyl ether and 1 mL of 20% methanolic tetramethylammonium hydroxide (TMAH) solution. After 10 min, water was added to stop the reaction, and methyl *n*-pentadecanoate was incorporated into the mixture as the internal standard. The organic layer was collected, dehydrated with anhydrous sodium sulfate, and subjected to GC analysis. A Hewlett-Packard 5890 gas chromatograph equipped with an HP 20M fused silica column (Carbowax 20M, 0.2 mm × 20 m) and a flame ionization detector (H_2 flow rate = 30 mL/min, air flow rate = 300 mL/min) was utilized. The column temperature was programmed at 180 °C initially, increased to 220 °C at 3 °C/min, and held for 15 min. Nitrogen was used as the carrier gas at a flow rate of 1 mL/min.

RESULTS AND DISCUSSION

Factors Affecting the Yield of Extraction. Boiled egg yolk and yolk powder were separately extracted with solvents of different polarity. As shown in **Table 1**, the yields of lipids extracted from boiled yolk and yolk powder were 8.70−33.84 and 11.47−50.01%, respectively.

The extraction yield of egg lipids was influenced by the water content of the egg products and the polarity of solvent. For instance, *n*-hexane, a lipophilic solvent, extracted only 8.70% lipids from boiled yolk but 46.20% lipids from yolk powder. Because water molecules interfered with the contact between *n*-hexane and lipids, the yield of lipids from boiled yolk with higher water content was significantly lower than that from yolk powder. On the other hand, solvents with low hydrophobicity such as acetonitrile extracted very few lipids regardless of the water content of the egg products.

Generally speaking, the performance of solvents with medium polarity was satisfactory no matter what the water content of the egg products. **Table 1** shows that the lipids extracted from boiled yolk with chloroform, Folch reagent, and ethyl acetate/ethyl alcohol (2:1, v/v) were weighed to 33.84, 33.61, and 33.59% of the moist samples, respectively. As for yolk powder without the interference of water, the yields obtained with chloroform, Folch reagent, and ethyl acetate/ethyl alcohol (2:1, v/v) increased to 49.02, 50.01, and 49.26%, respectively. The result that the 2:1 EtOAc/EtOH mixture extracted as much lipid as Folch reagent suggested it is an alternative choice for safer operation in lipid extraction.

Comparison of the Yields of Yolk Lipids Extracted with EtOAc/EtOH Mixture and Folch Reagent. When ethyl acetatate and ethyl alcohol were mixed at the same ratio as chloroform and methyl alcohol were mixed to prepare Folch reagent, yields of lipids extracted from fresh yolk and boiled yolk did not show significant difference. Although Folch reagent extracted 34.69% lipids from fresh yolk and 33.61% from boiled yolk, the lipid yields obtained with EtOAc/ EtOH (2:1, v/v) from fresh yolk and boiled yolk were 34.25 and 33.59%, respectively (**Table 2**). Similarly, the extraction yield from prepared yolk powder was increased to 65.07% for Folch reagent and increased to 63.84% for EtOAc/EtOH (2:1, v/v). This dramatic increase of lipid yield was due to the different water contents of the egg yolk samples. Because the water content of prepared yolk powder was lower than those of fresh yolk and boiled yolk, the solid content and lipid content were relatively higher in yolk powder. Assuming that the moist samples such as fresh yolk and boiled yolk contain 50% moisture, the lipid yields based on dry weight are corrected to about 68% for fresh yolk and 66% for boiled yolk, which are very close to the yields for the prepared yolk powder.

Table 2. Percentage Yields of Lipids Extracted from Different Types of Yolk with Folch Reagent and EtOAc/EtOH (2:1) Mixture[a]

yolk type	Folch reagent	EtOAc/EtOH (2:1)
fresh yolk	34.69 ± 0.93a	34.25 ± 0.78a
boiled yolk	33.61 ± 0.72a	33.59 ± 0.68a
prepared yolk powder	65.07 ± 0. 93a	63.84 ± 0.78a

[a] Data are presented as mean ± SD. Values bearing different letters in the same row are significantly in different Duncan's multiple-range test.

Table 3. Comparison of the Yields of Lipids Extracted from Fresh Yolk with EtOAc/EtOH Mixtures and Folch Reagent

solvent	yield[a] (%)	solvent	yield[a] (%)
ethyl acetate (EtOAc)	25.41 ± 0.95e	EtOAc/EtOH (2:1)	34.25 ± 0.78a
ethyl alcohol (EtOH)	14.93 ± 1.03g	EtOAc/EtOH (3:1)	33.06 ± 1.09a
EtOAc/EtOH (1:1)	34.69 ± 0.94a	EtOAc/EtOH (4:1)	33.92 ± 0.09a
EtOAc/EtOH (1:2)	30.21 ± 1.01c	EtOAc/EtOH (5:1)	34.64 ± 0.93a
EtOAc/EtOH (1:3)	25.16 ± 0.99e	EtOAc/EtOH (6:1)	32.72 ± 1.01b
EtOAc/EtOH (1:4)	21.66 ± 1.31f	EtOAc/EtOH (7:1)	29.65 ± 0.83d
Folch reagent	34.69 ± 0.93a	EtOAc/EtOH (8:1)	27.73 ± 1.32d
		EtOAc/EtOH (9:1)	26.12 ± 1.01de

[a] Data are presented as mean ± SD. Values bearing different letters are significantly different in Duncan's multiple-range test.

Comparison of the Yields Extracted from Fresh Yolk with EtOAc/EtOH Mixtures and Folch Reagent. With a single solvent, the extraction yields from fresh yolk were 25.41 and 14.93% when ethyl acetate and ethyl alcohol were respectively used, significantly lower than the yield obtained with Folch reagent (**Table 3**). Interestingly, the mixtures of ethyl acetate and ethyl alcohol at ratios of 1:1, 2:1, 3:1, 4:1, and 5:1 were as effective as Folch reagent, and the corresponding extraction yields were 34.69, 34.25, 33.06, 33.92, and 34.64%. However, with the increase of either ethyl alcohol or ethyl acetate, the yield was inversely decreased. For instance, the yield obtained with 6:1 EtOAc/EtOH was 32.72% and significantly lower than that with the 5:1 mixture by 5.5%. As the ratio of EtOAc/EtOH mixture was further increased to 7:1, the yield was significantly decreased by 14.4%. Similarly, the yield obtained with 1:2 EtOAc/EtOH was significantly lower than that with the 5:1 mixture by 12.78%. When the ratio of the EtOAc/EtOH mixture was 1:4, the yield was even lower by 37.47%. Therefore, the 2:1 EtOAc/EtOH mixture may be used in place of Folch reagent to extract lipids from fresh yolk.

Comparison of the Yields Extracted from Yolk Powder with EtOAc/EtOH Mixtures and Folch Reagent. Table 4 shows that when a single solvent such as ethyl acetate or ethyl alcohol was used for extraction, the extraction yields of yolk powder were significantly lower than that with Folch reagent. However, more lipid was extracted by mixtures of these two solvents. The yields obtained with 1:1, 2:1, and 3:1 EtOAc/EtOH mixtures were close to that obtained with Folch reagent, but with the increase of either ethyl acetate or ethyl alcohol, the yield was inversely decreased. The yields obtained with 1:5 and 5:1 EtOAc/EtOH mixtures were significantly lower than

Table 4. Comparison of the Yields of Lipids Extracted from Yolk Powder with EtOAc/EtOH Mixtures and Folch Reagent

solvent	yield[a] (%)	solvent	yield[a] (%)
ethyl acetate (EtOAc)	52.53 ± 0.97d	ethyl alcohol (EtOH)	26.07 ± 1.23d
EtOAc/EtOH (1:1)	64.97 ± 0.96a	EtOAc/EtOH (2:1)	63.84 ± 0.78ab
EtOAc/EtOH (1:2)	62.40 ± 1.21b	EtOAc/EtOH (3:1)	63.99 ± 1.39ab
EtOAc/EtOH (1:3)	60.40 ± 0.99bc	EtOAc/EtOH (4:1)	62.78 ± 1.09b
EtOAc/EtOH (1:4)	56.06 ± 1.61c	EtOAc/EtOH (5:1)	56.04 ± 0.39c
EtOAc/EtOH (1:5)	56.55 ± 2.31c	Folch reagent	65.07 ± 0.93a

[a] Data are presented as mean ± SD. Values bearing different letters are significantly different in Duncan's multiple-range test.

Table 5. Comparison of the Yields of Lipids Extracted from Animal Tissues with EtOAc/EtOH (2:1) Mixture and Folch Reagent[a]

	EtOAc/EtOH (2:1)	Folch reagent
loin eye	4.58 ± 0.47a	5.01 ± 0.51a
belly pork	34.57 ± 0.82b	35.03 ± 1.23b
fat tissue	91.27 ± 0.58c	91.46 ± 1.03c

[a] Data are presented as mean ± SD. Values bearing different letters in the same row are significantly different in Duncan's multiple-range test.

Table 6. Lipid Profiles of the Extracts Obtained from Fresh Yolk with Various Solvents

solvent	triglycerides (%)	cholesterol (%)	phospho-lipids (%)	other lipids (%)
chloroform	68.36	3.63	23.45	4.56
diethyl ether	78.83	3.56	12.40	5.21
n-hexane	78.68	3.07	13.11	5.14
ethyl acetate	80.21	3.04	14.11	2.64
EtOAc/EtOH (2:1)	69.98	2.13	19.40	8.49
acetonitrile	53.07	13.64	28.82	4.46
acetone	86.88	2.96	3.19	6.96
ethyl alcohol	7.21	12.92	71.16	8.71
Folch reagent	76.32	3.49	13.17	7.01

that obtained with the 2:1 mixture. The extraction yields were less by 11.42 and 12.22%, respectively.

Comparison of the Yields Extracted from Animal tissues with 2:1 EtOAc/EtOH Mixture and Folch Reagent. When an EtOAc/EtOH (2:1) mixture was used to extract lipids from loin eye, belly pork, and pork fat, the yields were close to that obtained with Folch reagent (**Table 5**). This implies that the 2:1 EtOAc/EtOH mixture can be used as an alternative to Folch reagent.

Lipid Profiles of the Extracts Obtained from Yolk with Various Solvents. Table 6 shows the lipid profiles obtained with solvents of different polarities. The more polar solvents such as ethyl alcohol, acetonitrile, and chloroform tend to extract the phospholipids more easily. Their lipid extracts contain about 71, 29, and 23% of phospholipids, respectively, and contain 7, 53, and 70% of triglycerides in that order. On the contrary, the solvent of lower polarity extracts fewer phospholipids but more triglycerides from yolk powder. As shown in **Table 7**, when *n*-hexane was used, the extract contained 4.84% phospholipids and 81.01% triglycerides. **Tables 6** and **7** indicate that the lipid profiles of extracts obtained with the 2:1 EtOAc/EtOH mixture were similar to that obtained with Folch reagent.

Effects of Solvent on Fatty Acid Compositions of the Extracts from Yolk. Table 8 shows that the fatty acid compositions of the extracts from boiled yolk were not significantly influenced by the species of solvent. Oleic acid (18:1), palmitic acid (16:0), and linoleic acid (18:2) were generally the three most abundant compositions in order of content.

Table 7. Lipid Profiles of the Extracts Obtained from Yolk Powder with Various Solvents

solvent	triglycerides (%)	cholesterol (%)	phospho-lipids (%)	other lipids (%)
chloroform	72.68	5.13	17.11	5.08
diethyl ether	79.02	3.52	13.15	4.31
n-hexane	81.01	5.84	4.84	8.31
ethyl acetate	83.63	3.82	8.72	3.83
EtOAc/EtOH (2:1)	75.08	5.82	12.34	6.76
acetonitrile	46.65	27.99	20.78	4.58
acetone	87.08	4.89	1.66	6.37
ethyl alcohol	7.72	12.85	70.76	8.66
Folch reagent	74.06	3.49	17.67	4.78

Table 8. Effects of Solvent on Fatty Acid Compositions of the Extracts Obtained from Boiled Yolk[a]

fatty acid	chloroform ($CHCl_3$)	acetonitrile (CH_3CN)	CH_3CN/ EtOH (2:1)	EtOAc/ EtOH (1:1)	Folch reagent
14:0	0.43	0.46	0.35	0.53	0.35
16:0	25.05	27.95	25.75	26.36	28.12
16:1	4.12	5.23	3.86	4.63	4.01
18:0	7.94	7.83	9.57	7.64	8.15
18:1	42.58	31.94	34.34	41.55	37.15
18:2	14.34	17.42	15.29	13.77	14.78
18:3	0.47	0.85	0.67	0.43	1.06
20:4	1.12	1.38	2.44	1.12	0.95
22:6	1.89	3.98	4.62	1.66	1.15
others	2.06	2.96	3.11	2.30	4.28

[a] Data are shown in percentage.

In conclusion, we found that when an EtOAc/EtOH mixture of either 1:1 or 2:1 ratio was used to extract lipids from fresh yolk, boiled yolk, yolk powder, and animal tissues, the extraction yields, lipid profiles, and fatty acid compositions were similar to those obtained with Folch reagent. Considering the toxicity and carcinogenicity of the Folch reagent, it should be replaced by the 1:1 or 2:1 EtOAc/EtOH mixture for the sake of the researcher's health.

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