A Rapid Method for Extraction of Total Lipids from Whey Protein Concentrates and Separation of Lipid Classes with Solid Phase Extraction

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ABSTRACT: A modified procedure for extraction of total lipids from whey protein concentrates was developed such that stable emulsion with extracting solvents was avoided and the solvent system remained monophasic. Nonlipid contaminants from the extract were removed using gel filtration instead of traditional aqueous washing to prevent any loss of polar lipids. The extraction of total lipids by the modified procedure was complete and comparable with a reference procedure. Traditional thin-layer chromatography is tedious and more qualitative than quantitative for lipid class separation. Total lipids were further separated into free fatty acids, phospholipids, cholesterol ester, triacylglycerol, cholesterol, diacylglycerol, and monoacylglycerol, using modified solid phase extraction procedure. Columns with 2 g amino propyl packing allowed separation of up to 80 mg of total lipids into lipid classes gravimetrically. The values for anhydrous milk fat for all lipid classes agreed with those in the literature. Separation of total lipids into lipid classes with solid phase extraction is easy, quantitative, and can also be performed on a preparative scale.

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KEY WORDS: Lipid class separation, solid phase extraction, total lipid extraction, whey protein concentrates.

Among the constituents of whey protein concentrate (WPC), total lipids have a significant effect on its functionality (1). In general, the higher the total lipid content, the poorer the functionality of WPC. Very little is known about the lipids of whey and WPC (2). Most data available relate to the total lipid content of WPC; however, de Wit *et al.* (3,4) and Theodet and Gandemer (2) have determined total phospholipids (PL), triacylglycerols (TG), free fatty acids (FA), and diacylglycerols (DG). More information about lipid classes and subclasses is vital in understanding their role in diminishing the functionality of WPC.

To study lipids of whey or WPC, it is necessary to achieve total extraction of these components without attendant chemical deterioration (5). Extraction of lipids from WPC is difficult due to: (i) low levels of total lipids, (ii) high protein content, which interferes during phase separation of solvents by producing a stable emulsion, and (iii) presence of stable lipid–protein complexes (2). Roese-Gottlieb and Mojonnier methods are used extensively for extraction and quantification of lipids in dairy products. These methods use base or acid in combination with heat to dissociate the lipid–protein complexes. However, heat in the presence of acid or base leads to oxidation and/or hydrolysis of PL and unsaturated FA. This is why it is essential that cold methods of extractions using binary solvent mixtures, such as chloroform–methanol (6), dichloromethane–methanol (7), or hexane–isopropanol (8), be used for total lipid extraction. In cold solvent extraction, alcohol helps in the dissociation of lipid–protein complexes and thus allows near complete extraction of lipids. Various authors have proposed modifications to these methods to adapt them to dairy products (9–12). Recently, Theodet and Gandemer (2) compared five different methods for quantitative extraction of lipids from WPC. They recommended the method of Clark *et al.* (10) as the most suitable method for quantitative and qualitative analysis of lipids from whey and WPC. However, they also noted that the method was time-consuming.

Most studies usually need qualitative and/or quantitative lipid class separation of total lipids for further studies. For qualitative lipid class separation, thin-layer chromatography (TLC) is the fastest and most convenient technique. However, it is tedious, time-consuming, and indirect for quantitative determination of lipid classes. Also, oxidative deterioration of polyunsaturated lipids during the TLC process and its sensitivity to sample load limit use of TLC as a sample preparation technique. Preparative high-performance liquid chromatography (HPLC) or column chromatography techniques are expensive, require large amounts of solvents, and recovery and purity of the samples are at risk, when the sample is a mixture of complex lipids, such as milk lipids. During recent years, solid phase extraction (SPE) has evolved as a useful technique for separation of lipid classes. Following the report of Kaluzny *et al.* (13), many scientists have reported use of SPE for lipid class separation (14–18). However, all the reported methods used very small quantity of the samples, and lipid classes were determined indirectly.

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This report presents a rapid method for extraction of total lipids from WPC and further preparative separation into lipid classes using modified SPE procedure.

MATERIALS AND METHODS

Samples for analysis. Dry whey powder (WP), acid WPC 34% protein, and cheese WPC 75% protein were obtained from Commonwealth Scientific and Industrial Research Organization, Australia, New Zealand Milk Products (Santa Rosa, CA), and New Zealand Dairy Research Institute (Palmerstown North, New Zealand), respectively. Anhydrous milk fat was purchased from Level Valley Dairy (West Bend, WI).

Reagents. Optima®-grade methanol, isopropanol (IPA), ethyl acetate, HPLC-grade chloroform, diethyl ether, methylene chloride, reagent-grade cupric sulfate, acetic acid, and phosphoric acid (85%) were obtained from Fisher Scientific Co. (Pittsburgh, PA). Reagent-grade glass distilled hexane was purchased from Burdick and Jackson (Muskegon, MI).

Extraction of total lipids. All the samples were removed from the freezer (–20°C) and allowed to equilibrate to room temperature. Then WPC 75%, WPC 34%, and WP were hydrated to a water to sample ratio (w/w) of 2:1, 2:1.5, and 2:2.5, and gently mixed for 15 min with magnetic stirrer. For extraction of total lipids 3, 4, and 5.4 g of hydrated solutions of WPC 75%, WPC 34%, and WP were used, respectively.

The procedure of Bligh and Dyer (19) was modified for total lipid extraction from dry whey products containing high protein contents. In this modified procedure, 35 mL of 1:1 (vol/vol) chloroform/methanol mixture as added to the above preweighed hydrated samples. Then the mixture was homogenized at 6000 rpm for 1 min in an Omni-mixer 17150 (Sorvall Inc., Newtown, CT). The contents were transferred to a 50-mL centrifuge tube and centrifuged at 2800 × *g* for 5 min, using a Mistral 3000i centrifuge (Curton Matheson Sci. Inc., Houston, TX). Supernatant was collected, and the pellet was reextracted with 35 mL of 2:1 (vol/vol) chloroform/methanol as above. Supernatant of the second extraction was collected and pooled with the previous supernatant. Solvents from the supernatants were evaporated using Buchi Rotavapor - R (Brinkmann Instruments, Westbury, NY) rotary vacuum evaporator at 35°C. The rotary vacuum evaporator was flushed with nitrogen before and at the end of evaporation to prevent the contact of atmospheric oxygen with lipids. Then the crude extract of lipids was vacuum desiccated for a minimum of 6 h to remove any remaining traces of moisture. Desiccator vacuum was broken with nitrogen. Nonlipid contaminants were removed using a gel filtration procedure (5). A glass column (20-mm diameter) with a Teflon stopcock and glass frit was packed with nonlipophilic Sephadex G-25 (Pharmacia LKB, Piscataway, NJ), hydrated in 1:1 (vol/vol) water/methanol mixture, to a height of 15 cm. Five cm of washed sea sand was added on the top to prevent disturbances in the packed beads during addition of sample or eluting solvents. The column was equilibrated with 19:1 (vol/vol) chloroform/methanol mixture saturated with water before applying the sample. Dry crude lipid extract was dissolved in less than 5 mL of 19:1 (vol/vol) chloroform/methanol mixture saturated with water. All lipids except nonlipid contaminants would be in solution. Then the contents were filtered through a sintered glass funnel (40–60 μm pore size), and the filtrate was loaded onto a gel filtration column. Total lipids, except gangliosides, were eluted with about 50 mL of 19:1 (vol/vol) chloroform/methanol mixture saturated with water (5) and collected in a preweighed vacuum evaporator flask. Nonlipid contaminants from the column were eluted by 1:1 (vol/vol) water/methanol mixture. The column was regenerated with 19:1 (vol/vol) chloroform/methanol mixture saturated with water before loading a new sample. The column could be used indefinitely. Solvents from the gel filtration eluent were evaporated using rotary vacuum evaporator at 35°C, and the total lipids were dried completely in a vacuum desiccator for a minimum of 4 h. Total lipids were determined gravimetrically.

Total lipids of WPC 75% were also separately determined following the procedure of Clark *et al.* (10), the procedure of Hara and Radin (8), and three modifications of the procedure of Hara and Radin (8). The first modification consisted of increasing the polarity of the extracting solvent system, as suggested by Hara and Radin (8). This was achieved by changing the hexane to IPA ratio from 3:2 to 1:1. For the second and third modifications, acetonitrile or acetone, respectively, were added to the hexane/IPA mixture as a denaturing agent for WP. After preliminary trials, the proportion of solvents selected were hexane/IPA/acetonitrile, 3:5:1 and hexane/IPA/acetone, 1:3:1.

Lipid class separation using SPE. The procedure of Kaluzny *et al.* (13) was modified to separate total lipids into FA, PL, cholesterol esters (CE), TG, cholesterol (C), DG, and monoacylglycerols (MG). In this modified procedure, Mega Bond Elut (2 g) disposable aminopropyl SPE columns from Varian Sample Preparation Products (Harbor City, CA) were used, instead of Bond Elut (500 mg), and the optimum volume of each eluting solvent was determined (Table 1). During the elution of TG, the column was not piggy-backed as part of the C did not elute with TG (13). A schematic representation of the elution procedure is shown in Figure 1. A vacuum Manifold with twelve individual flow control valves and stainless-steel guide needles (Supelco, Bellefonte, PA) was used to elute the solvents through the SPE columns under 10–12 kPa vacuum. A center plate of the collection rack was fabricated to hold twelve 20 mm × 125 mm (18 mL) Pyrex screw cap tubes. The first aminopropyl Mega Bond Elut column was placed on the vacuum manifold and washed twice with 8 mL of hexane. Total lipids were dissolved in less than 2 mL of chloroform and applied to the column under vacuum. Chloroform was eluted leaving total lipids on the column. Then the column was sequentially eluted with 18 mL of each solvent A, B, and C, eluting neutral lipids (NL), FA, and PL, respectively, as shown in Figure 1. After separation, solvents from each SPE eluent were evaporated using rotary vacuum evaporator followed by vacuum desiccation until constant weight (usually 4 h). The NL fraction was dissolved in less than 1 mL of hexane and

TABLE 1
Solvents Required for Solid Phase Extraction with Mega Bond Elut[a] in Isolation and Purification of Lipid Classes

Name	Solvents	P'[b]	Amount (mL)[c]	Lipid class eluted[d]
A	2:1 (vol/vol) Chloroform/2-propanol	4.07	18	NL
B	2% (vol/vol) Acetic acid in diethyl ether	2.86	18	FA
C	Methanol	5.1	18	PL
D	Hexane	0.01	12	CE
E	1% (vol/vol) Diethyl ether, 10% (vol/vol) methylene chloride in hexane	0.437	36	TG
F	5% (vol/vol) Ethyl acetate in hexane	0.315	36	C
G	15% (vol/vol) Ethyl acetate in hexane	0.616	36	DG
H	2:1 (vol/vol) Chloroform/methanol	4.43	18	MG

[a]Varian Sample Preparation Products (Harbor City, CA).
[b]Solvent strengths (P') are from Reference 13.
[c]Solvent volumes represent about 80 or 60 mg of total lipids from whey protein concentrate or anhydrous milk fat, respectively.
[d]NL—Neutral lipids; FA—free fatty acids; PL—phospholipids; CE—cholesterol ester; TG—triacylglycerols; C—cholesterol; DG—diacylglycerols; MG—monoacylglycerols.

loaded on second aminopropyl column previously washed twice with 8 mL of hexane. Then the column was sequentially eluted with 12, 36, 36, 36, and 18 mL of solvents D, E, F, G, and H eluting (see Table 1) CE, TG, C, DG, and MG, respectively. Again, eluting solvents were removed by rotary vacuum evaporator from each fraction, followed by vacuum desiccation. Each class of lipid was then determined gravimetrically and expressed as a percentage of the total lipids. Samples of all the lipid classes were dissolved in chloroform and transferred into amber glass screw cap vials. Chloroform was evaporated under gentle flow of dry nitrogen. Then the vials were immediately capped with Teflon-lined caps and stored at −20°C until further analysis.

TLC. Routine visual checks of the purity of lipid classes separated by SPE were performed using silica gel EM 60 HPTLC plates (EM Separations, Gibbstown, NJ). Samples were applied using a capillary (~2-mm spot diameter). About 0.5 μg of each lipid sample was spotted against known standards, and in some cases samples were overloaded (>2 μg) to assess any low contamination of other lipid class. Plates were developed vertically in a solvent system of hexane/diethyl ether/acetic acid (70:30:1, vol/vol/vol). Lipids on the plate were charred with 10% cupric sulfate in 8% phosphoric acid for visualization (20) as follows: the plates were dipped in the visualization reagent for 3 s, air-dried for 10 min, and heated in a gas chromatograph oven programmed from 30 to 180°C at 8°C/min with a final hold of 8 min.

Statistical analysis. Extraction of total lipids and separation of the lipid classes were performed in triplicate for each sample. The data were analyzed with Minitab for Windows program, release 10 (Minitab Inc., State College, PA).

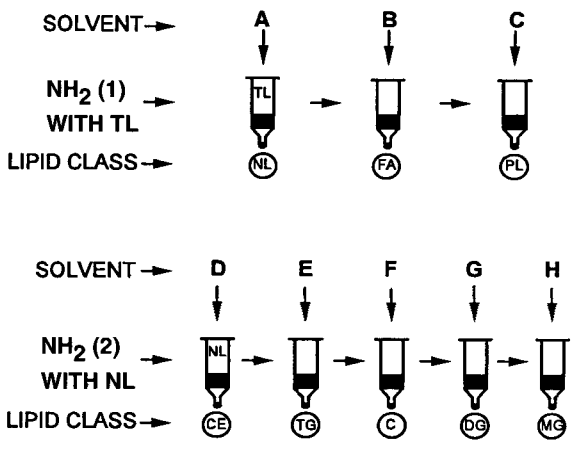

FIG. 1. Diagramatic representation for separation of lipid classes using solid phase extraction; NL, neutral lipids; FA, free fatty acids; PL, phospholipids; CE, cholesterol ester; TG, triacylglycerols; C, cholesterol; DG, diacylglycerols; MG, monoacylglycerols.

RESULTS AND DISCUSSION

Extraction of total lipids. All samples were hydrated before extraction, as quantitative extraction of total lipids from dry powders would be incomplete (21). However, the water content of the hydrated sample for total lipid extraction is very critical and should not turn the monophasic ternary system of chloroform/methanol/water biphasic during extraction (19). If water content of the hydrated sample for extraction exceeds 6 mL for 35 mL of 1:1 chloroform/methanol, the ternary system would turn biphasic. Phase separation before extraction could be prevented by either increasing the amount of chloroform/methanol mixture (i.e., 45 instead of 35 mL) or increasing the proportion of methanol (i.e., use 1:2 instead of 1:1 chloroform/methanol mixture) in the extracting solvents. Total crude and purified lipids extracted from WPC 75% using different solvent systems are presented in Table 2. Recently, Theodet and Gandemer (2) studied five different methods of extraction of total lipids from whey and WPC and suggested the method of Clark *et al.* (10) as the best method for extraction of total lipids. The method of Clark *et al.* (10), a

TABLE 2
Total Crude and Purified Lipids of 75% Whey Protein Concentrate, Using Different Extraction Methods

Method of lipid extraction	Total crude extract (% w/w)	Total lipids (% w/w)	Total time (h)
Clark *et al.* (Ref. 10) [modified Folch *et al.* (Ref. 6) method]	—	7.68 ± 0.31^a	50–60
Hara and Radin (Ref. 8) (hexane/IPA, 3:2)	7.05 ± 0.23^a	5.95 ± 0.09^c	
Hara and Radin (Ref. 8) modified (hexane/IPA, 1:1)	7.11 ± 0.26^a	5.83 ± 0.18^c	
Hara and Radin (Ref. 8) modified (hexane/IPA/ACN, 3:6:1)	7.70 ± 0.17^b	6.50 ± 0.11^b	
Hara and Radin (Ref. 8) modified (hexane/IPA/acetone, 1:3:1)	7.74 ± 0.20^b	6.52 ± 0.15^b	
Present method [modified Bligh and Dyer (Ref. 19)]	14.12 ± 0.28^c	7.87 ± 0.25^a	12–14

[a–c]Reported values are means ± standard deviations. Values within a column followed by a different letter are significantly different ($P < 0.05$).

modified procedure of Folch *et al.* (6), was used as a reference method for this study. The solvent system of Hara and Radin (8), which uses hexane and IPA, is less toxic than the conventional solvent system of chloroform/methanol, and also extracts very low levels of nonlipid contaminants. These two specific advantages encouraged us to evaluate this solvent system as such and with three modifications as described in the Materials and Methods section. The solvent system of Hara and Radin (8) extracted the lowest total lipids from WPC 75% protein among the different solvent systems studied. Even increasing the polarity of the extracting solvents by changing the ratio of hexane/IPA from 3:2 to 1:1 did not improve extraction of total lipids. This may be due to inability of hexane and IPA to break the tertiary structure of the whey proteins. It is well established that denaturation or loss of tertiary structure of protein in the sample is essential for breaking the lipid protein interactions and thus complete extraction of total lipids (22). For the next two modifications, a mixture of hexane and IPA was added with denaturing solvent like acetonitrile or acetone. Addition of either acetonitrile or acetone to the extracting solvents hexane and IPA significantly increased the extraction of total lipids as compared to the method of Hara and Radin (8). However, when compared to the method of Clark *et al.* (10), the total lipids were still significantly lower. This indicated that solvent systems of Hara and Radin (8), with and without modifications, though less toxic, were unable to extract total lipids from high protein products.

Table 3 shows the total lipids content of different dry whey products, containing varying amounts of protein, using the modified procedure presented here. The present method extracted total lipids from all the three products studied, and is applicable to high protein products with low moisture content.

Lipid class separation using SPE. Purity check for each lipid class separated was performed using TLC. Repeated TLC indicated that contamination with other lipid classes was nondetectable by TLC. Also, because of higher quantity of the SPE packing material (2 g) and low cholesterol content, piggybacking of the column during TG elution was not necessary. Separation of CE was the most critical step. Because TG from milk fat contains fatty acids of varied polarity, either high humidity (>50% RH) or longer exposure of SPE column and/or hexane to atmosphere before use or improper preparation of SPE column can lead to partial elution of TG into CE fraction. Hence, it is highly recommended that SPE columns are opened just before use, hexane is kept practically moisture-free, extraction is carried out in low-humidity environment, and the SPE column is well prepared by flushing it with the required quantity of hexane before loading the sample.

Table 4 shows the percent of lipid classes, determined using modified SPE procedure, for anhydrous milk fat, WPC 75%, WPC 34%, and whey powder. Lipid class values for anhydrous milk fat from literature (23) using TLC are shown for comparison. The values for anhydrous milk fat for all lipid

TABLE 3
Total Lipids of Dry Whey and Whey Protein Concentrates with Varied Protein Content Using Modified Lipid Extraction Procedure

Dry whey products	Total lipids[a] (% w/w)	
	Method of Clark *et al.* (Ref. 10)	Present procedure
Whey powder, 12% protein	0.89 ± 0.03	0.89 ± 0.03
Whey protein concentrates, 34% protein	3.60 ± 0.18	3.60 ± 0.18
Whey protein concentrates, 75% protein	7.87 ± 0.25	7.87 ± 0.25

[a]Reported values are means ± standard deviations.

TABLE 4
Lipid Composition of Anhydrous Butter Fat and Dry Whey Products Using Amino Propyl Solid Phase Extraction (SPE) Compared with Those Reported in the Literature Using Thin-Layer Chromatography (TLC)

Lipid classes	Total lipids (% w/w)[a]				
	Anhydrous milk fat		WPC-75	WPC-34	WP
	Procedure				
	SPE	TLC[b]	SPE	SPE	SPE
Free fatty acids	0.22 ± 0.03	0.28	2.29 ± 0.10	3.36 ± 0.52	4.95 ± 0.26
Phospholipids	1.30 ± 0.14	1.11	23.64 ± 1.15	17.53 ± 1.63	18.04 ± 2.55
Neutral lipids	98.48 ± 0.21	98.61	74.08 ± 1.45	79.11 ± 1.12	77.01 ± 2.69
Cholesterol ester	0.22 ± 0.05	0.02	1.52 ± 0.11	1.7 ± 0.37	1.84 ± 0.57
Triacylglycerols	95.44 ± 0.22	95.8	61.12 ± 2.44	64.10 ± 3.86	60.82 ± 2.74
Cholesterol	0.22 ± 0.05	0.46	2.41 ± 0.20	2.54 ± 0.30	3.21 ± 0.53
Diacylglycerols	2.17 ± 0.06	2.25	5.46 ± 0.16	4.87 ± 0.49	5.32 ± 0.38
Monoacylglycerols	0.07 ± 0.01	0.08	3.30 ± 0.04	5.70 ± 0.05	5.83 ± 0.10
% Total lipids recovery	99.64 ± 1.22	—	99.74 ± 1.42	99.80 ± 0.97	99.01 ± 1.51

[a]Reported values are means ± standard deviations. WPC-34 and WPC-75 (Commonwealth Scientific and Industrial Research Organization, Australia, New Zealand Milk Products, CA, and New Zealand Dairy Research Institute, New Zealand, respectively). WP, dry whey powder.
[b]From Reference 23.

classes, except C and CE, using modified SPE procedure were comparable with that using TLC procedure. However, when the sum of C and CE contents was compared with that of TLC value, it was similar. This could be due to the variation in the proportion of C and CE contents of the sample used. Similar variation in the proportion of C and CE contents has been reported (24). The lipid class values for FA, PL, and DG for all whey products were lower than the reported values of 7 to 15%, 27 to 28%, and 6 to 15%, respectively (25). On the other hand, values for TG for all whey products studied were higher than the reported value of 48 to 53%. These variations could be due to the source of the whey and processing conditions used for the manufacture of these products. The other lipid class values for whey products are not available for comparison. The recoveries of the total lipids after SPE from all samples were gravimetrically determined (Table 4), and were more than 99%.

As demonstrated in this report, total lipids from high protein containing products like WPC can be extracted rapidly, efficiently, and reliably. This method can be applicable to any low-moisture products. If changes are made in accordance with the moisture content of the product, this modified method can be used with practically any sample. Total lipids also can rapidly and efficiently be separated into lipid classes using the SPE method described. As there is minimal exposure of lipids to air or high temperature, extracted lipid samples are compatible for subsequent analysis of fatty acids or molecular species distribution. We believe, due to its versatility, rapidity, and preparative-scale separation of lipid classes, this method will prove very useful for all scientists working with lipids in general and for food scientists specifically.

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