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RESEARCH ARTICLE

Development of process protocol to enhance the phospholipid content of ghee residue

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Abstract: Many by-products of the dairy industry contain nutritive and commercial value, which with appropriate technological interventions can be exploited for improving the profitability of the industry. Ghee residue (GR) is one such by-product reported to contain considerable amount of polar lipids dominated by phospholipids (PLs). An attempt was made to develop a stepwise protocol to enhance the PL content in ghee residue, to facilitate its efficient and economical extraction from the residue matrix. As a first step, remnant ghee (about 13%) in the residue was expelled by pressing the ghee residue matrix at 5 kg/cm^2 for 5 min. This was followed by treating the ghee residue with two solvents, namely n-hexane and water. Treatment with n-hexane at solvent to solid ratio of 1:4, 1:3 and 1:2, for a contact time of 30 min, resulted in a PL content 26.22, 24.68 and 21.29% (on lipid basis), respectively, in the ghee residue samples. Steeping of the ghee residue in hot water (boiled to 100°C) at a solid to water ratio of 1:4 for 60 min resulted in retention of 27.03% lipids in the residue, corresponding to a removal of 16.93% fat with the solvent stream. This resulted in an appreciable enrichment of phospholipids in the ghee residue matrix to the tune of 30.56% on lipid basis; corresponding to the initial PL content of 8.26%. Increase of surface area of the solid matrix through size comminution was also explored to enhance the efficacy of solvent treatment. When the ghee residue was ground to a particle size of 0.25 mm and 0.30 mm and then subjected to hot water treatment, its PL content was found to increase to 9.56% and 9.32% (ghee residue basis), respectively. Thus, the physical and chemical interventions significantly improved the phospholipid content of ghee residue.

Key words: Ghee residue, boiling water treatment, polar lipids, phospholipids, neutral lipids

Introduction

Milk lipids are globular macrostructures composed of triglycerides with different melting points and covered by three layers of milk fat globular membrane (MFGM) (Martini et al. 2016). Out of the total lipids present in milk, phospholipids (PLs) represent up to 1% of the composition (Lopez et al. 2017) and variations in their percentage are attributed to season, lactation stage and type of feed (Liu et al. 2017). Many dairy by-products are reported to have considerable amounts of polar lipids (Ravindra et al. 2022) such as beta serum, whey protein phospholipids concentrate, butter, milk, cream and ghee residue.

Polar lipids from biological matrices can be extracted using conventional techniques but the process is reported to have challenges such as excess time, expensive solvents and energy consumption (Traversier et al. 2018). These processing hurdles have been eased by adopting assisted extraction techniques to separate polar lipids from non-polar fraction across different dairy products. Some of the assisted techniques reported for dairy products include ultrasonication, filtration, supercritical fluid extraction, switchable solvent extraction and solid phase extraction. Literature reports on extraction of dairy based polar lipids are majorly focussed on buttermilk as the substrate, due to the presence of significant amounts of phospholipids (polar) in its composition. In this context, ghee residue can also be considered as a dairy by-product with significant polar lipids in its matrix.

Ghee residue is a dark brown residue separated during melting of butter or cream while manufacturing ghee. Compositionally, it is rich in lipids, proteins with fractions of lactose and minerals and negligible amount of moisture (Wani et al. 2022). While

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manufacturing ghee, butter or cream is subjected to different temperature protocols, leading to varied proportion of polar lipids (Krishnegowda et al. 2022). Butter heated at 110 to 120°C leads to generation of volatile aromatic compounds which is attributed to heat catalyzed reaction (Aneja et al. 2002). Combination of temperature and time of heating are known to influence the presence of volatile components in ghee. This is due to the fact that processing at low temperature retains more volatile compounds whereas, high temperatures and extended heating time results in loss (Sserunjogi et al. 1998). It has been reported that duration and temperature of ghee clarification alters the phospholipid content owing to the migration of phospholipids from GR to ghee at higher clarification temperatures (Santha and Narayanan, 1979).

Lipids present in ghee residue have a complex structure with both neutral and polar lipid fractions. Unlike plant based matrices, ghee residue is different in its physical features with a solidified structure in its core. Perturbing ghee residue using heat or mechanical action would facilitate loosening of the matrix and movement of its constituents. The application of solvent and hot (boiling) water treatment could be a simple approach to separate polar lipids from neutral lipids in such matrices. Since the amphiphilic activity of this polar lipid fraction could potentially aid in its utilization as an emulsifier, this could ultimately lead to the use of ghee residue as a substrate to yield a green emulsifier. Hence, the present study was undertaken to optimize different treatment interventions in ghee residue to extract its polar lipids fraction using different solvents.

Materials and Methods

Preparation of ghee and collection of ghee residue

Cow milk was procured from the Livestock Production Centre (LRC) of ICAR - National Dairy Research Institute (NDRI), Southern Research Station (SRS), Bangalore. Cream separation from milk was carried using centrifugal cream separator. Fat content of cream was determined using the standard Gerber test (IS: SP-18 1981)) and found to vary between 52 to 60% across different batches. Ghee was prepared by direct cream and creamery butter methods as outlined in Aneja et al. (2002). Ghee was carefully decanted from the kettle through muslin cloth to separate the ghee residue. Adhered ghee in residue was expressed by gentle hand press. Resultant ghee residue was stored under refrigeration (4±2°C) until it was used for further experiments. As a comparative study, ghee residue was also prepared by direct cream method (Santha and Narayanan, 1979) to evaluate the influence of preparation method on yield of lipids and PLs.

Mechanical pressing of ghee residue

Ghee residue obtained from previous step contained higher and varied proportion of lipids due to its particulate structure and multiple batch processing. The stored (4°C) ghee residue was heated (tempered) to 40±2°C in a hot air oven (Falcon Scientific Co., Bangalore, India) . To remove excess surface lipids and to account for the uneven draining of ghee from residue during straining, it was subjected to compression using a mechanical press. Ghee residue was firmly confined inside a muslin cloth and pressed between the plates of a hydraulic press (Multipurpose machine, Milk Tech Engineers, Bangalore, India). This helped to eliminate loosely adhered lipids in the particulate material. Compression pressure was evaluated at 3 levels (3, 4, and 5 kg/cm^2) for duration of 5 min. The pressed ghee residue was removed from muslin cloth and stored at 4±2°C. The hydraulic press used to apply the pressure on the residue is depicted in Figure 1.

Pre-treatment with n-hexane

Ghee residue obtained from creamery butter method contained larger proportions of lipids which include neutral and polar lipids. In order to eliminate the neutral lipids and concentrate the proportion of polar lipids in the matrix, the ghee residue was pre-treated with two solvents.

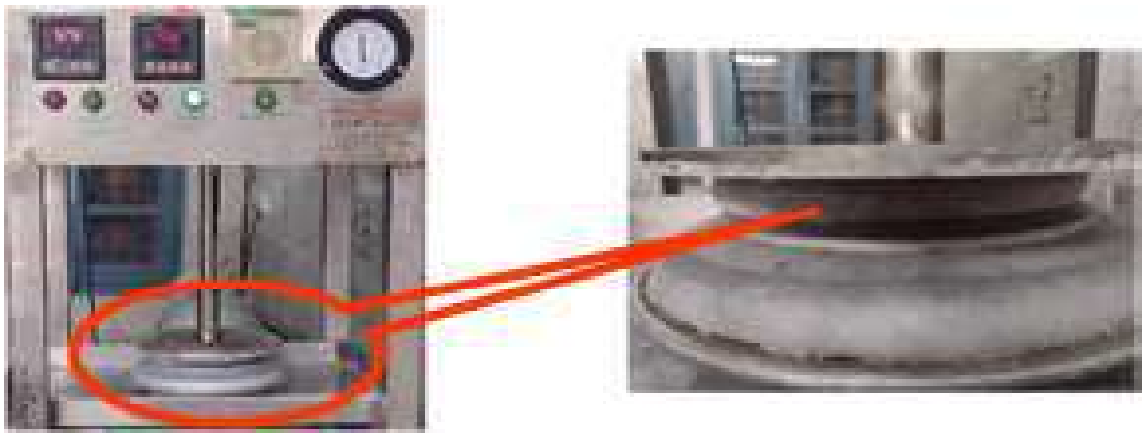

Fig. 1 Hydraulic press used for extraction of lipids from ghee residue

Ghee residue stored after pressing was tempered at 40±2°C for 4 h to melt lipids entrapped in the matrix. Tempered ghee residue was mixed with n-hexane at 1:2, 1:3 and 1:4 solid to solvent ratio (w/v) in a wide mouth glass beaker (15 cm dia.). The beaker was covered with aluminium foil and agitated frequently (every 5 min.) for better solid-solvent contact for varied time periods (20, 30 and 40 min.). After a defined time of contact, the solvent was drained out through Whatman filter paper 1 (185 mm Ø). The retentate residue was then analysed for its lipids and PLs content using phosphorus estimation methods (Sharma et al. 2007). Time of exposure and solid to solvent ratio were considered as the independent parameters. The experiment was aimed to optimize combination of time and solid to solvent ratio to eliminate maximum non-polar lipids.

Hot water boiling

An alternate pre-treatment approach for separation of neutral lipids from ghee residue evaluated in the study was by using boiling water treatment. The ghee residue sample obtained from the creamery butter ghee was pressed using the hydraulic press as mentioned in the above section and tempered to 40°C before mixing with boiling water in solid to solvent ratio of 1:2, 1:3 and 1:4 in a glass beaker. The beaker was immersed in boiling water bath for varied time periods of 20, 30 and 40 min. After the scheduled time, the beaker (with the contents) was placed in a deep freezer (-4 to -12°C) for 5 h to facilitate solidification of lipids separated from ghee residue during the boiling water treatment. The solidified fat layer was carefully skimmed off the surface using a sharp-edged knife. The residual mixture (ghee residue and water) was poured into a stainless-steel tray and dried at 43±1°C for 48 h. Resultant dry fraction was coarsely powdered using pestle and mortar followed by the estimation of its lipids and PLs. Solid to solvent ratio and time of contact were considered as independent factors in this analysis. The data for both the pre-treatments (using n-hexane and boiling water) were compared for removal of neutral lipids from the ghee residue.

Comminution of ghee residue particles

Ghee residue obtained from the solvent treatment method (boiling water) was subjected to size comminution to pass through two sieve sizes (0.25 mm and 0.30 mm). Yield of lipids and PLs from the comminuted samples were analyzed.

Lipid estimation by gravimetric method

Lipids from the ghee residue samples after treatment was recovered using method detailed by Cheng et al. (2019) with few modifications. Ghee residue was taken in Mojonnier flask and added with 2 mL ammonia solution (30%) and 1 g of NaCl, followed by light mechanical shaking. After few minutes, 10 mL of ethyl alcohol (95%) was added and mixed thoroughly to facilitate better access to solvent. Diethyl ether and petroleum ether 15 mL each was added into the flask and plugged with rubber stopper to agitate the contents. The flask was allowed to rest for 60 min. and the solvent was carefully transferred to a pre-weighed glass beaker. In the second stage of solvent extraction ethyl alcohol (5 mL), diethyl ether (10 mL) and petroleum ether (10 mL) were added to flask and allowed to rest for 60 min. Solvent was pooled to previously collected sample followed by one more extraction with same proportion of solvents. Solvent obtained in all three steps were pooled and solvent was evaporated by hot water bath maintained at 75°C. After major quantity solvent was evaporated, beaker was placed in hot air oven maintained at 90±2°C till constant weight was obtained.

Estimation of Phospholipids content

Phospholipids content of ghee residue after two different treatments was estimated based on phosphorus content in fat extracted using organic solvents in previous step. The method used by Murthy and Narayanan (1966) with slight modifications was adopted for estimation of PLs. The lipids obtained were digested with nitric acid and sulphuric acid (5 mL each) using a heating mantle till the colour of the mix turned light yellow or colourless. Digestion flask was cooled and added with 10 mL of distilled water and heated till the evaporation of water. Further, 10 ml water was added and heated till its evaporation. After cooling, 5 mL of this aliquot was mixed with 0.44% ammonium molybdate and 0.4 mL of reducing agent (1-amonia-2-napthol-4 sulphonic acid, sodium sulphite, sodium bisulphite) in a test tube and immersed in boiling hot water bath for 7 min. For blank, 0.5% sulphuric acid was used whereas, 1 µg/mL potassium dihydrogen phosphate was used as standard. Optical density was measured at 720 nm using UV/VIS spectrophotometer (LABINDIA Analytical UV 3200XE, India). PLs were estimated as the ratio of optical density of sample and standard by multiplying with 25.9 as conversion factor for phosphorus to PLs.

Table 1: Effect of ghee preparation method in the yield, lipids and phospholipid content of the ghee residue

Method	GR yield (%)	Lipids (%)	Phospholipids (% GR basis)	Phospholipids (% on lipids basis)
Creamery butter method	5.16±1.53[b]	54.55±6.03[a]	4.98±1.26[a]	9.08±1.88[a]
Direct cream method	13.56±2.52[a]	35.90±3.83[b]	0.95±0.08[b]	2.68±0.47[b]

Same alphabet in column indicates no-significant difference ($p<0.05$) through Tukey's test.

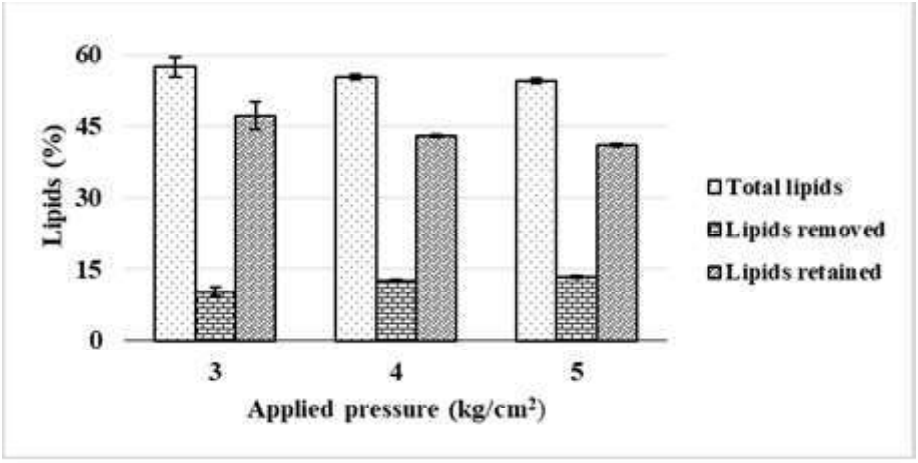

Fig. 2 Lipid content of pressed ghee residue at varied pressure levels

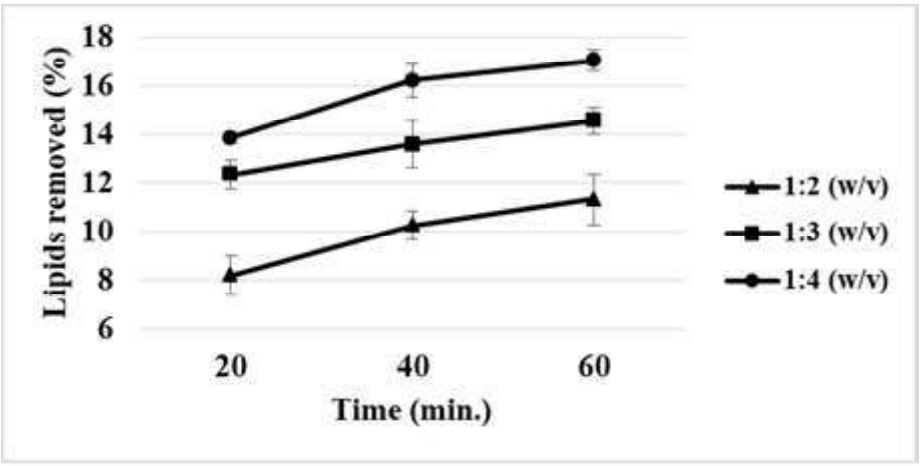

Fig. 3 (a) Lipids (%) removed in ghee residue by n-hexane treatment at different time and solids to solvent ratio

Results and Discussion

Comparison of ghee residue yield by creamery butter and direct cream method

The results for the PL content of ghee residue samples prepared by different methods are tabulated in Table 1. It can be seen that though there was 2.62 folds increase in the yield of ghee residue in the direct cream method, a 51.94% decrease in total lipids can be seen compared to creamery butter method. The PLs content was lower (0.95±0.08%) for the GR obtained from direct cream method than the creamery butter method (Table 1). Thus, it can be observed that the method of preparation also affected the PL content. The similar findings for higher PL content in the GR obtained from creamery butter method than direct cream were also reported by Sangma et al. (2023). The results are also supported by the findings of Santha (1977) and Janghu et al. (2014). It is postulated that the high serum solids present in ghee residue contributed to lesser lipids and PLs yield when expressed on total ghee residue weight basis. While working with different fractions of fat in cream, Pal and Rajorhia (1975) observed increase in ghee yield corresponded with a reduction in amount of ghee residue. This was attributed to the yield of ghee residue proportional to solids-not-fat (SNF) fraction in the raw material. Based on the above findings, it was deduced that the ghee residue obtained from the creamery butter method yielded higher PLs content in the residue.

Optimization of pressure to remove excess lipids from ghee residue

The pressure applied on ghee residue when it was strained through the muslin cloth to remove residual ghee was very low. Hence, ghee residue was subjected to varied pressure levels using a hydraulic press. With increase in applied pressure, more lipids were observed to be removed from the residue matrix resulting in a reduction of lipids present in ghee residue. However, pressing beyond 5 kg/cm^2 pressure did not show any considerable change in lipids content of the residue Based on this investigation, pressing at 5 kg/cm^2 for 5 min. was adopted for the expulsion of ghee from ghee residue. The total lipid content in the pressed ghee residue was recorded to be 41%±0.28% after hydraulic pressing (Figure 2). Similar methods are often used in the extraction of fat/oil from the oilseeds by pressing the flaked/ grounded oilseeds in a hydraulic or screw press. The application of pressure is known to create a driving force that squeezes and facilitates in oozing out of loosely held oil in the matrix (Savoire et al. 2013).

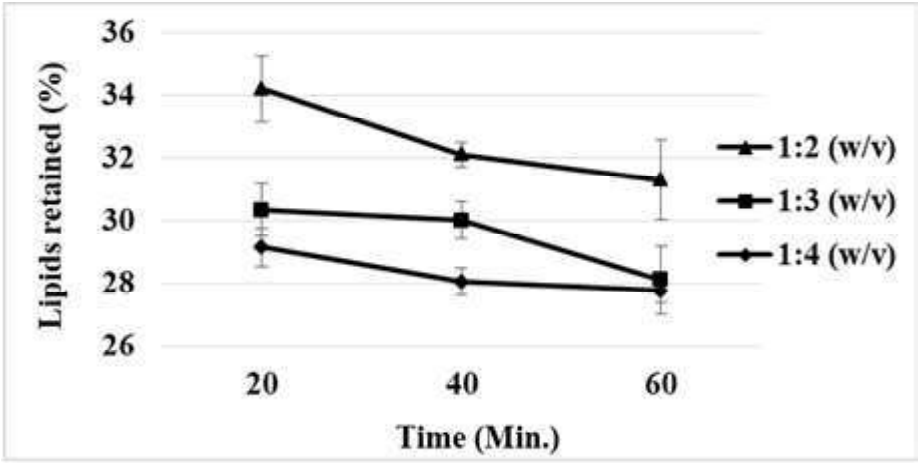

Figure 3 (b) Lipids (%) retained in ghee residue by n-hexane treatment at different time and solids to solvent ratio

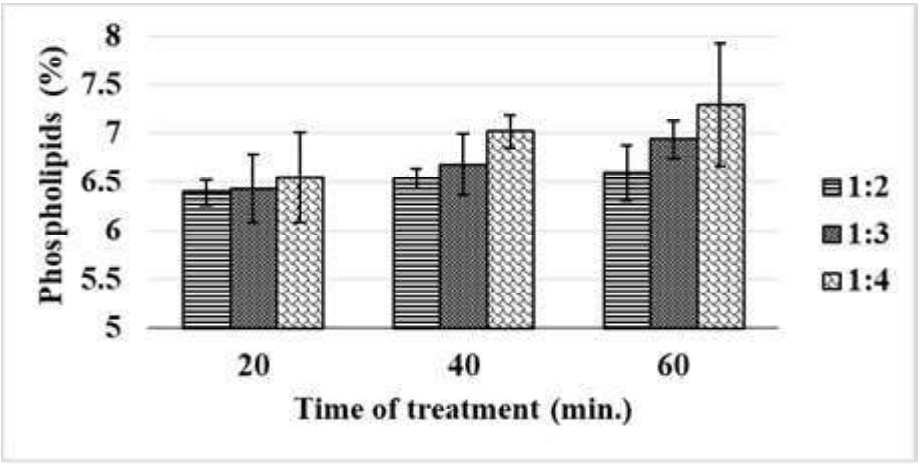

Fig. 4 (a) Phospholipids expressed on dry weight basis after n-hexane treatment

Treatment with n-hexane to remove neutral lipids

Two factors, namely contact period (20, 40 and 60 min.) and solids to solvent ratio (1:2, 1:3 and 1:3) were considered and the results are presented in Figure 3 and 4. Both factors were observed to positively influence the separation and removal of lipids from the residue through n-hexane. Solids to solvent ratio of 1:2% (w/v) led to removal of 8.21% of lipids after a contact period of 20 min. against 11.32% for 60 min. However, when solids to solvent ratio were increased to 1:4 (w/v) and the contact period was 60 min., 17.04% lipids were removed from the residue and the treated ghee residue retained 27.78% of lipids (Figure 3 a & b). The above observations affirmed the influence of contact time and solids to solvent ratio on the mass transfer phenomena i.e. lipids movement from the residue.

Weller and Hwang (2005) also emphasized on the improvement in lipid yield with increase in solvent to solid volume from 3 to 5 mL/g while extracting lipids from sorghum using n-hexane as solvent. In the same experiment, increase in time of exposure from 1 to 6 h also resulted in enhanced lipid extraction. This was attributed to higher contact time and concentration gradient for diffusion of lipids into solvent.

Phospholipids in ghee residue after n-hexane treatment

The ghee residue after treatment with n-hexane was evaluated for PLs content, which was expressed on ghee residue and lipid wt. basis (Figure 4 a & b). From the figure, it is evident that PLs content in the residue also improved with increase in solids to solvent ratio. Short exposure time (20 min.) resulted in 6.39% of PLs in ghee residue and it was 21.20% on lipid basis. As time of treatment increased to 60 min, PLs present in sample also increased to 7.29% which amounts to 26.22% on lipids basis. The reason attributed to increase in PLs content is due to the movement of neutral lipids from the ghee residue matrix into the solvent (n-hexane). Vale et al. (2019) studied the separation and extraction of lipids using a three-phase lipid extraction protocol which included hexane, methyl acetate, acetonitrile and water in 4:4:3:4 ratios. The study demonstrated the efficacy of the solvents in classifying the neutral lipids in upper phase and polar lipids in middle phase of the solvent.

Statistical analysis of the data indicated significant difference among the PLs content in solvent due to solid to solvent ratio and time factors ($p<0.05$). However, interactive effect of the two factors was found to be statistically insignificant ($p<0.05$). The study concluded that there was significant improvement in the

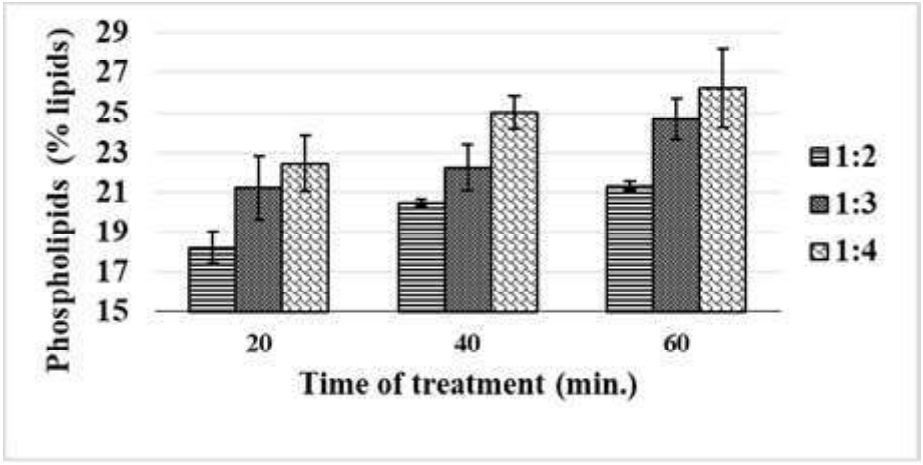

Fig. 4 (b) Phospholipids expressed on lipids weight after n-hexane treatment

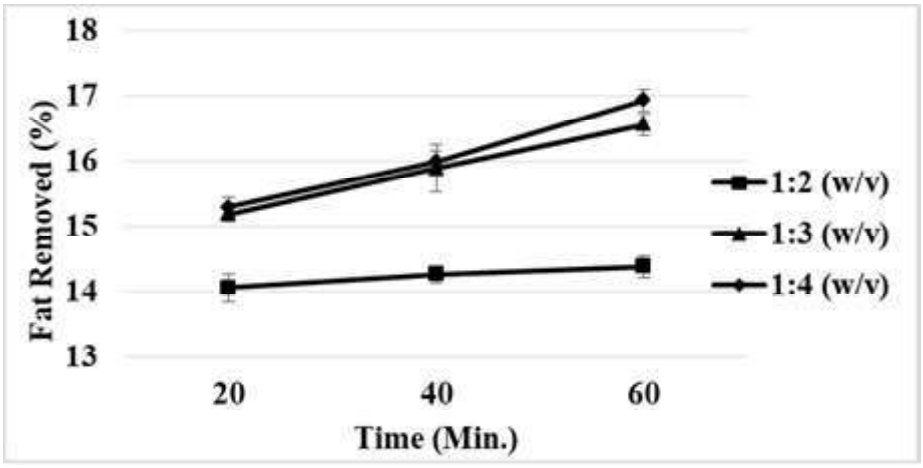

Fig. 5 (a) Lipids (%) removed in ghee residue by boiling water treatment at different time and solids to solvent ratio

PLs content of ghee residue with increase in time and solid to solvent ratio when treated with n-hexane.

Boiling water treatment of ghee residue

Boiling water treatment of the pressed ghee residue was also considered for the contact time of 20, 40 and 60 min. and solids to solvent ratio 1:2, 1:3 and 1:4. The total lipids removed and retained in ghee residue after boiling water treatment are depicted in Figure 5 a & b. Nearly 17% of lipids were removed from the residue at solids to solvent ratio of 1:2 for 60 min (Figure 5 a). Solids to solvent ratio of 1:3 and 1:4 followed similar trend lines for the removal of total lipids from the ghee residue under this treatment. The effect of solids to solvent ratio on retention of lipids in ghee residue was also found to be significant ($p<0.05$).

Chemat (2015) indicated that water could be employed as a good solvent to remove polar lipids from lipids complex at high temperature (100 and 374°C) and pressure (22.1 MPa). At elevated temperature and pressures, water undergoes self-ionization leading to decreased viscosity, surface tension and increase in diffusivity (Shitu et al. 2015). Even at normal atmospheric conditions, water demonstrates the ability to act as polar solvent; this is ascribed to its dielectric constant of 80. Based on these points, water can be considered as a solvent to diffuse polar components. It was hypothesized that this would facilitate free movement of non-polar fraction as a floating layer which could then be separated due to density gradient.

It is assumed that during boiling water treatment polar lipids of the ghee residue matrix migrate to and is held by water whereas; the non-polar lipids would be concentrated in the top layer, which could be skimmed off. Thus, evaporation of water and drying of the ghee residue after boiling water treatment was expected to result in the retention of polar lipids in ghee residue matrix. During pre-treatment, it was observed that wider diameter containers facilitated better lipids flotation than narrow diameter (Figure 5 a). Lipids were carefully removed along with little fraction of ghee residue after freezing for easy skimming (Figure 5 b).

Phospholipids in ghee residue after hot water boiling treatment

The PLs content of the dried fraction of the ghee residue was estimated post-treatment with boiling water and expressed on ghee residue and lipids basis (Figure 6 a & b). PLs retained in ghee residue reported an increasing trend with increase in time and solids to solvent ratio. Maximum PLs content (8.26% ghee residue basis) was reported with contact time of 60 min. at solids

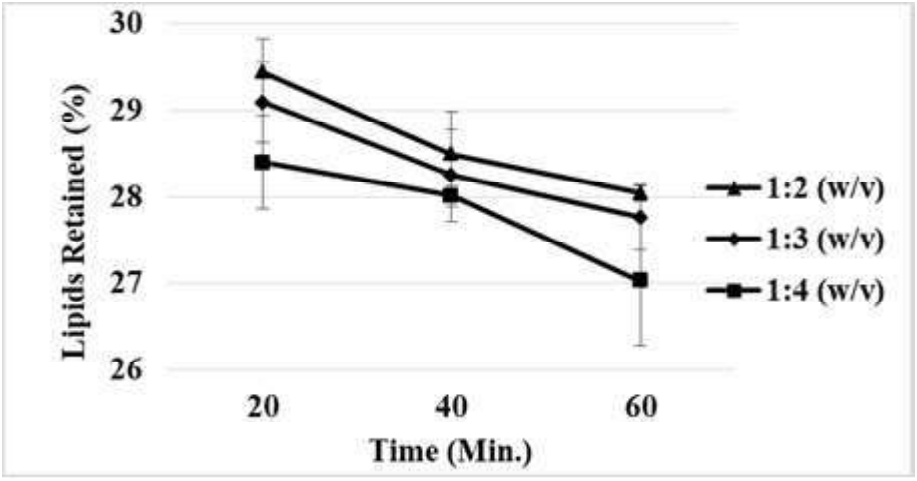

Fig. 5 (b) Lipids (%) retained in ghee residue by boiling water treatment at different time and solids to solvent ratio

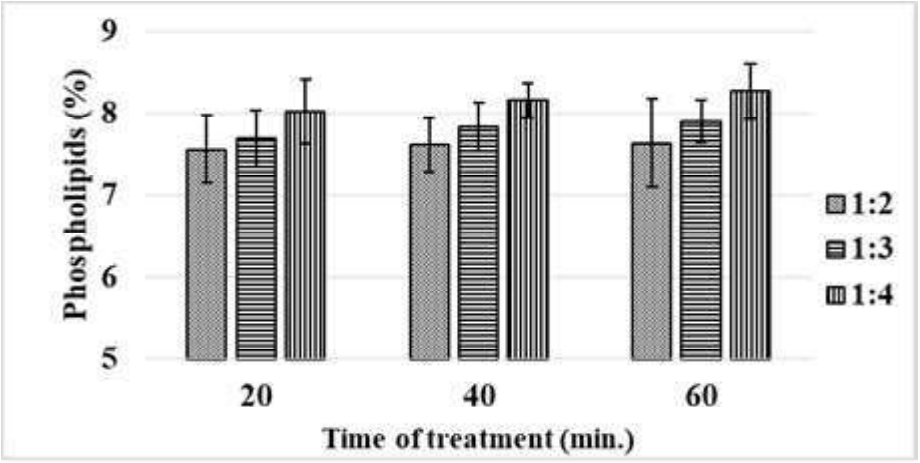

Fig. 6 (a) Phospholipids expressed on dry weight after boiling water treatment

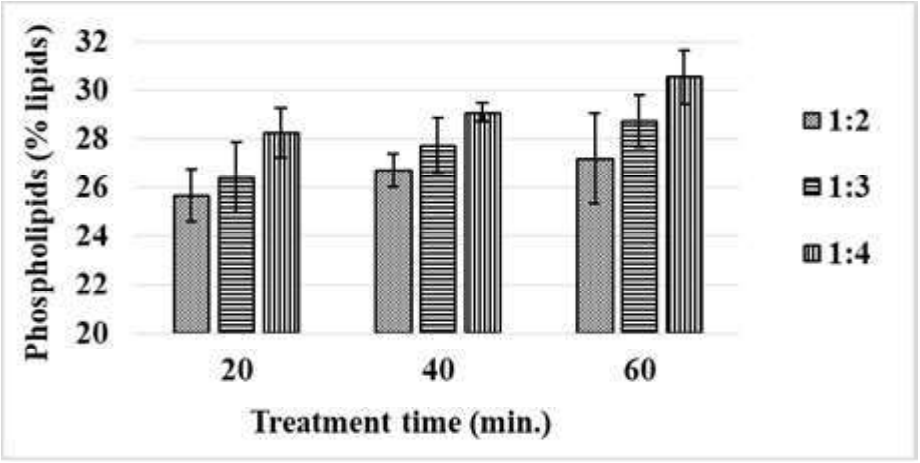

Fig. 6 (b) Phospholipids expressed on lipids weight after boiling water treatment

to solvent ratio of 1:4. This corresponded to a PLs content of 30.56% when reported on lipid weight basis.

No significant influence ($p>0.05$) was noted for contact time for retention of PLs ($p<0.05$). However, solids to solvent ratio had significant influence on retention of PLs in the treated ghee residue. As the F value for this factor was greater that F-critical value, two factor comparison was analysed by Tukey's test at 20 min. contact time. Results from post-hoc test indicated no significant difference among treatments. Hence, solid to solvent ratio at the lower level of 1:2 can be considered optimal over extended solvent ratio.

Table 2: Effect of various pre-treatments on the lipids and phospholipid content of the ghee residue

Stage of ghee residue	Lipid (%)	Phospholipids (GR wt. basis)	Phospholipids (lipids wt. basis)
After filtering	54.55±6.03[a]	4.98±1.26[b]	9.08±1.88[b]
After pressing	41.48±0.41[b]	6.29±1.32[b]	15.14±3.02[b]
After hot water treatment	29.46±0.45[c]	7.4±0.73[ab]	25.09±2.10[a]
After comminute to 0.25 mm	29.55±1.52[c]	9.56±0.82[a]	32.47±4.03[a]
After comminute to 0.30 mm	28.54±1.45[c]	9.32±1.16[a]	32.74±4.74[a]

Comminution of the pre-treated ghee residue

The pressed ghee residue after boiling water treatment and drying was subjected to size reduction by passing through a mechanical mill. The size reduced particles were then classified by passing through 0.30 and 0.25 mm sieve followed by estimation of its lipids and PLs. The progressive improvement in lipids and PLs profile of the pre-treated ghee residue, as it was subjected to different steps of the pre-treatment protocol, is depicted in Table 2.

A perusal of the data indicated a definite improvement in the PLs content with each step of treatment with a slightly higher yield of PLs when the particle size was reduced to 0.25 mm (9.56%) over 0.3 mm (9.32%). The PLs content in the ghee residue was enriched from 4.98% at freshly prepared stage to 9.56% after comminution to 0.25 mm particle size. The results indicated successive improvement in PLs content that could be ascribed to the elimination of non-polar lipids. Between boiling water treatment and comminution to 0.25 mm particle size, sizable increase in PLs was noticed. This could be due to increased surface area which facilitated gradient for movement of lipids from particle surface to solvent. From overall treatments, there was an increase of 91.96% in PLs content expressed on ghee residue basis. This increment could be mainly due to elimination of non-polar lipids fraction and physical processing.

Conclusion

Ghee residue is an untapped by-product of the dairy industry, with most avenues for its downstream utilization being restricted to as an ingredient in either the confectionary or animal feed industry. The presence of phospholipids in the residue and the potential industrial use of this valuable component as an emulsifier opens alternate avenues for the utilization of ghee residue. The present study formulated a stepwise protocol, targeting a stepwise removal of neutral lipids from the residue matrix leading to progressive improvement of the PL content in the ghee residue. The developed protocol included steps such as mechanical pressing of the fresh ghee reside matrix at 5 kg/cm^2 for 5 min., extraction with n-hexane at solids to solvent ratio of 1:4 for 30 min, treatment with boiling water at a solid to water ratio of 1:4 for contact time of 60 min. and size comminution to 0.25 mm. The progressive enhancement of PLs in the ghee residue with each treatment step ultimately resulted in a PL content of 9.56% in the residue. Thus, the developed protocol can be suggested as a preliminary treatment of ghee residue, for the effective and economical extraction of PLs from the residue for further use.

Acknowledgment

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