PULSED ELECTRIC FIELD, MICROWAVE AND ULTRASONICATION ASSISTED EXTRACTION OF PHOSPHOLIPIDS FROM GHEE RESIDUE

THESIS SUBMITTED TO THE

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(DEEMED UNIVERSITY)

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE AWARD OF DEGREE OF

DOCTOR OF PHILOSOPHY

IN

DAIRY ENGINEERING

By

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M. Tech. (FOOD & AGRIL. PROCESS ENGINEERING)

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DEDICATED TO
MY
BELOVED FAMILY
AND
FRIENDS

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ABSTRACT

Ghee residue is the dark brown sediment obtained as a by-product during the heat clarification of butter or cream. It is often discarded as waste, causing environmental concern, or used as animal feed by most ghee manufacturing dairy plants. It is found to be good source of lipids including phospholipids (PLs), which is reported to have good emulsifying property. The present study is an attempt to obtain a PLs enriched extract from ghee residue using assisted extraction techniques such as microwave (MW), ultrasound (UL) and pulsed electric field (PEF). The PLs in ghee residue was enriched to 9.56% from 4.98% through series of pre-treatments viz., hydraulic pressing, boiling water treatment and comminution to 0.25 mm particle size. This pre-treated ghee residue was subjected to the assisted extraction process with different solvents like water, ethanol and enzyme treatment and water as solvent was deduced to yield better yield of PLs in the extract. Taguchi orthogonal design T9 was used to optimize process parameters for the three assisted extraction techniques based on yield of PLs and antioxidant activity of extract. Extraction at optimized combination of power (540 W), time (60 s), solvent to solid ratio of 10 (S:S-v/w) resulted in a yield of 21.96% PLs in the microwave treated extract. Ultrasound assisted extraction was optimized at power (80%), time (4 min.) solvent temperature (80^{o}C) and S:S ratio 15 and reported a yield of 24.12% PLs in the extract. PEF treatment reported PLs yield of 18.14% at optimal levels of voltage (60 kV/cm), time (5 min.) and S:S ratio of 7.5. The antioxidant activity of the extracts obtained by microwave, ultrasound and PEF assisted techniques was found to be 29.89, 51.94 and 37.01% radical scavenging activity for extract, respectively, when determined at the optimal process conditions for maximising the activity. The extract was evaluated for the classes and species of PLs present by LC- MS and results indicated presence of 61, 118 and 31 species across 5 classes of PLs in the extract obtained by MW, UL and PEF assisted process, respectively. Kinetics of extraction in the MW assisted technique followed Peleg's model whereas, ultrasound and PEF assisted extraction process was best described by the parabolic model. Evaluation of the extract from ghee residue exhibited good emulsion capacity and stability at concentration of 5% for extract obtained with MW and UL assistance, while a concentration of 10% was required for similar results for extract obtained with PEF assistance. Analysis of hydrophilic-lipophilic balance (HLB) of extract from all three assisted techniques deduced the values to be close to 10. Replacement of guar gum and glyceryl monostearate in different proportion with the extract obtained with MW and UL assistance in ice cream mix resulted in comparable attributes for textural, fat destabilization, overrun and melting properties of ice cream mix and hardened ice cream, to the respective control sample. The study demonstrated the efficacy of obtaining a PLs rich extract from ghee residue using assisted extraction techniques and the scope of utilizing the extract as replacement for conventional emulsifier in dairy products such as ice cream.

सारांश

घी अवशिष्ट एक प्रकार का गहरे भूरे रंग का नीचे जमा हुआ पदार्थ होता है, जो है जो मक्खन या क्रीम को गर्म करने के पश्चात उप-उत्पाद के रूप में प्राप्त होता है। इसे अक्सर कचरे के रूप में छोड़ दिया जाता है, जो पर्यावरण से संबंधित चिंता का कारण हो सकता है अथवा ज्यादातर घी बनाने वाले डेयरी संयंत्रों द्वारा पशु आहार के रूप में उपयोग किया जाता है। इसमें लिपिड के रूप में फास्फोलिपिड्स (पीएल) का अच्छा स्रोत होता है, जिसके बारे में माना जाता है कि, इसमें काफी अच्छे पायसीकारी गुण पाए जाते हैं। इस अध्ययन में माइक्रोवेव (MW), अल्ट्रासाउंड (UL) और स्पंदित विद्युत क्षेत्र (PEF) जैसी सहायक निष्कर्षण तकनीकों का उपयोग करके घी अवशेषों से PLs समृद्ध अर्क प्राप्त करने का प्रयास किया गया है। पूर्व-उपचारों जैसे की हाइड्रोलिक प्रेसिंग, उबलते पानी के उपचार एवं 0.25 मिमी कण संचार की श्रृंखला के माध्यम से, घी अवशेषों में उपस्थित पीएल को 4.98% से 9.56% तक बढ़ाया गया था। इस पूर्व-उपचारित घी के अवशेषों को पानी, इथेनॉल और एंजाइम उपचार जैसे विभिन्न सॉल्वैंट्स के साथ सह-निष्कर्षण प्रक्रिया के द्वारा किया गया था और अर्क में पीएल की बेहतर लब्धि प्राप्त करने के लिए विलायक के रूप में पानी की मात्रा को कम किया गया था। PLs की उपज और अर्क की एंटीऑक्सीडेंट गतिविधि के आधार पर तीन सह-निष्कर्षण तकनीकों के लिए प्रक्रिया मापदंडों को अनुकूलित करने के लिए, टैगुची ऑर्थोगोनल डिज़ाइन T9 का उपयोग किया गया था। एक्सट्रैक्शन के परिणामस्वरूप, पॉवर (540 W), समय (60 s), सॉल्वेंट टू सॉलिड रेश्यो 10 (S:S-v/w) के ऑप्टिमाइज्ड कॉम्बिनेशन पर माइक्रोवेव ट्रीटेड एक्सट्रेक्ट में 21.96% PLs का उत्पादन हुआ। अल्ट्रासाउंड की सहायता से निष्कर्षण शक्ति (80%), समय (4 मिनट।) विलायक तापमान (80oC) और S:S अनुपात 15 पर अनुकूलित किया गया था और अर्क में 24.12% PLs की उपज की सूचना उपलब्ध कराई। पीईएफ उपचार ने वोल्टेज (60 केवी/सेमी), समय (5 मिनट) और एस:एस अनुपात 7.5 के इष्टतम स्तर पर 18.14% की पीएल उपज की सूचना उपलब्ध कराई। निष्कर्ष की एंटीऑक्सीडेंट गतिविधि, माइक्रोवेव, अल्ट्रासाउंड और पीईएफ सहायक तकनीकों द्वारा प्राप्त हुई जो की 29.89%, 51.94% और 37.01% थी| जब गतिविधि को अधिकतम करने के लिए इष्टतम प्रक्रिया स्थितियों पर निर्धारण किया जाता है, तब यह मूलतः सफाई करने वाली गतिविधि थी| अर्क का मूल्यांकन, एलसी-एमएस द्वारा मौजूद पीएल की कक्षाओं और प्रजातियों के लिए किया गया था एवं प्राप्त परिणामों ने क्रमशः मेगावाट, यूएल और पीईएफ सहायता प्राप्त प्रक्रिया द्वारा उपलब्ध अर्क में पीएल के 5 वर्गों में 61, 118 और 31 प्रजातियों की उपस्थिति का संकेत दिया गया। MW सह-तकनीक में निष्कर्ष की गतिकी ने, पेलेग के मॉडल का अनुसरण किया, जबकि अल्ट्रासाउंड और PEF असिस्टेड एक्सट्रैक्शन प्रक्रिया को, परवलयिक मॉडल द्वारा सबसे अच्छे तरीके से समझाया गया। घी अवशेषों के निष्कर्ष के मूल्यांकन ने, 5% सघनता पर, MW और UL सहायता से प्राप्त अर्क के लिए अच्छी पायस क्षमता और स्थिरता का प्रदर्शन किया, जबकि PEF की सहायता से प्राप्त अर्क के समान परिणामों के लिए 10% सघनता की आवश्यकता थी। सभी तीन सहायक तकनीकों से प्राप्त, अर्क के हाइड्रोफिलिक-लिपोफिलिक बैलेंस (HLB) के विश्लेषण की वैल्यू 10 के आस-पास पाई गयी| आइसक्रीम मिश्रण में, MW और UL सहायता से प्राप्त निष्कर्ष को ग्वार गम और ग्लाइसेरिल मोनोस्टियरेट के साथ के अलग-अलग अनुपात में प्रतिस्थापन करने पर के परिणामस्वरूप नियंत्रण नमूने की बनावट, वसा अस्थिरता, एवं कठोर आइसक्रीम के पिघलने और पिघलने के गुणों के लिए तुलनात्मक गुण प्राप्त हुए|इस अध्ययन के माध्यम से, सहायक निष्कर्षण तकनीकों का उपयोग करते हुए, घी के अवशेषों से पीएल समृद्ध अर्क प्राप्त करने की प्रभावकारिता और आइसक्रीम जैसे डेयरी उत्पादों में पारंपरिक पायसीकारी के प्रतिस्थापन के रूप में उपयोग करने की संभावना को बड़े अच्छे तरीके से प्रस्तुत किया गया है|

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LIST OF SYMBOLS AND ABBREVATIONS

%	Percent
~	Nearly equal to
±	Plus or minus
<	Less than
>	Greater than
µ	Mu
^{o}C	Degree centigrade
a	Amplitude
AFM	Atomic fore microscopy
ARE	Average relative error
ANSA	1-amino-2-napthol-4-sulfonic acid
ANOVA	Analysis of variance
AOAC	Association of official analytical chemists
CAGR	Compounded annual growth rate
CBSW	Clarified butter sediment waste
CLSM	Confocal laser scanning microscopy
CWFGM	Cheese whey fat globule membrane
CyNMe2	N,N-dimethylcyclohexylamine
Da	Dalton
DSLR	Digital single-lens reflex
DoE	Design of experiments
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
EC	Electrical conductivity
FFA	Free fatty acids
G	Giga
GAE	Gallic acid equivalent
g	Gram
GMS	Glyceryl monostearate
GR	Ghee residue
h	Hour
HIP	Hexane and isopropanol
HLB	Hydrophilic and lipophilic balance

HPLC	High performance liquid chromatography
HYBRID	Hybrid fractional error function
Hz	Hertz
IDF	International Dairy Federation
L:S	Liquid to solid ratio
LC-MS	Liquid chromatography and mass spectrometry
LMWE	low molecular weight emulsifier
LRS	Livestock Research Station
M	Mega
mg	Mili gram
min	Minutes
MCLP	Mexican Manchego cheese-like product
MOSFET	Metal oxide semiconductor field effect transistor
MAE	Microwave assisted extraction
MFGM	Milk fat globular membrane
MPSED	Marquardt's percent standard deviation
MPLs	Milk phospholipids
MTBE	Methyl-tert-butyl ether
MW	Microwave
N	Newton
NMR	Nuclear magnetic resonance
NDRI	National Dairy Research Institute
nm	Nano meter
NR	Not reported
OFAT	One-factor-at a-time
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEF	Pulsed electric field
PFN	Pulse forming network
PHSCG	Post-hydrolyzed spent coffee grounds
PI	Phosphatidylinositol
PLs	Phospholipids

PS	Phosphatidylserine
R^2	Regression co-efficient
RMS	Root mean square
RMSD	Root mean square deviation
RPM	Revolutions per minute
SCFE	Supercritical Fluid Extraction
SDS-PAGE	sulphate-polyacrylamide gel electrophoresis
SD	Standard deviation
SEE	Standard error of estimation
SEM	Scanning electron microscopy
SHS	switchable hydrophilicity solvents
SCG	spent coffee grounds
S:L	Solid to liquid ratio
SM	Sphingomyelin
SNF	Solid not fat
SRS	Southern Research Station
SSE	Sum of the squares of the error
SPE	Solid phase extraction
SSR	Sum of Squares of Error
STEP	Simultaneous Texturization and Extraction of Phospholipids
TAG	Tri acyl glycerol
TDS	Total dissolved solids
TEM	Transmission electron microscopy
TPA	Texture profile analyser
TPC	Total phenolic compound
TLC	Thin layer chromatography
UAE	Ultrasound assisted extraction
UL	Ultrasonication
USDA	United State Department of Agriculture
V	Volts
v/v	Volume by volume
v/w	Volume by weight
Viz	Namely

WPPC	Whey protein phospholipids concentrate
WPI	Whey protein isolate
W	Watt
w/w	Weight by weight basis
wt.	weight
ZP	Zeta potential

Chapter-1

INTRODUCTION

1.0 INTRODUCTION

Milk lipids are macrostructures of globules with major constituent of triglycerides having different melting points and covered by three layers of milk fat globular membrane (MFGM) (Martini *et al*., 2016). These MFGM comprises constituents such as sialic acid, lactoferrin and range of polar lipids (Dewettinck *et al*., 2008). The major polar lipids present in globular layer are milk phospholipids (MPLs). Out of total lipids present in milk, MPLs represent up to 1% (Lopez *et al*., 2017) and variation in its percentage was attributed to season, lactation stage and its type of feed (Liu *et al.*, 2017).

Phospholipids are constituents of biological membrane, known for their amphiphilic property due to the presence of hydrophobic tail and hydrophilic head. They are complex polar lipids with phosphate group linked to serine, ethanolamine, inositol or choline (Contarini and Povolo, 2013). Egg yolk, vegetable oils, milk, fish, meat, brain and some nuts were reported to be rich in phospholipids (Weihrauch and Son, 1983).MPLs are broadly classified into two major groups namely,the glycerol containing phospholipids, commonly referred to glycerophospholipids and sphingophospholipids (Sikorski and Kolakowska, 2003). Glycerophospholipids contains glycerol as backbone with polar head group and two fatty acids esterified at *sn*-1 and *sn*-2 positions. Under this group, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) are present as sub classes (Huang, 2001). Sphingophospholipids contains sphingoid as structural backbone with a set of amino alcohols (aliphatic). Sphingomyelin (SM) contained phosphorylcholine head-group and fatty acid which is linked to amide nitrogen (Cevc and Paltauf, 1995).

High content of SM (24%) and PS (12%) were reported in dairy products which was found to be less or absent in vegetable source or egg (Gassi *et al.*, 2016; Burling *et al*., 2012).MPLs were reported to have health benefits on heart, gastrointestinal systems and neurological system. Due to its amphiphilic property, MPLs also exhibit technical functionalities such as emulsifiers, surfactants and foaming agent. Dairy by-products such as buttermilk, butter serum, whey protein phospholipids concentrate (WPPC) and ghee residue were reported to be good stock for MPLs.

Ghee residue (GR) is a dark brown residue obtained as sediment after melting and straining of butter or cream. This granular material is dark brown in colour, comprises mainly of solids not fat (SNF) which is coagulated during heat clarification of ghee or cream. Conservative

estimates indicate that roughly 30-35% of milk produced in India is converted to ghee (Pawar *et al*., 2014). This indicates production of significant quantum of residue, depending on the ghee making process adopted.For example, preparation of ghee using the creamery butter process resulted in yield of 12% GR (Santha and Narayanan, 1978).Presently, GR is often discarded as waste causing environmental concern or used as animal feed by most of the ghee manufacturing dairy plants. Few studies were conducted to incorporate ghee residue as an ingredient in some food products. In addition to phospholipids, GR is also known to be a good source of protein (Arumugam *et al.*, 1989). Hence, there is potential to use GR as a valuable source of MPLs after employing suitable processes to eliminate neutral lipids and proteins from the matrix.

For extraction of phospholipids from plant and animal sources, conventional solvents were used with or without assistance of pre-treatments. Super critical fluid extraction, switchable hydrophilicity solvents (SHS), ultrasound assistance, microfiltration/ultrafiltration assistance were used to improve extraction efficiency of MPLs from dairy by-products. Attempts were also made to treat dairy products with select enzymes to loosen protein and lipid bonds to improve extraction efficiency (Muniratnamma *et al*., 2017a). Major focus of these extraction studies was devoted to extract MPLs from dry buttermilk powder, whey protein phospholipids concentrate (WPPC), butter serum, fresh cream, buttermilk, concentrated buttermilk and whey buttermilk powder. However, in all attempts, inferred results indicated incomplete extraction of MPLs from milk by-productand inadequacy of method to fit to all dairy products.

Process technology adopted for disruption or destabilization of MFGM will influence extraction efficiency. Attempts made to extract MPLs from butter serum using ultrasonication assistance prior to SHS extraction resulted in improved extraction of lipids and phospholipids. As a processing technique, microwave (MW), pulsed electric field (PEF), Ultrasonication (UL)were reported improved yield of extract from different matrices. These are less expensive techniques involves simple operations procedures and are user friendly.

UL is conventional technique used in food preservation by killing microorganisms and improving extraction of target compounds from biological matrix.Frequencies of UL waves higher than 100kHz and intensity less than 1 W/cm^2 were used for low energy application whereas, high frequency (20-500kHz) with greater than 2 W/cm^2 intensity are used for high energy application (Mason *et al.*, 2011). Acoustic cavitation, which is due to formation,

growing and collapse of small size bubbles are fundamentals of UL application. When UL waves are generated, it propagates to instigate bubble formation, oscillation and collapse leading to mechanical, thermal and chemical effects. Collapse pressure, shear stress and turbulence are attributed to mechanical effect (Yusaf and Al-Juboori, 2014) and free radical generation to chemical effect (Lateef *et al.*, 2007). Generation of temperature in continuous medium due to collapse of bubbles are associated to thermal effect(Soria and Villamiel, 2010).

MW are having frequency between 300 MHz to 300 GHz and found application in preservation by drying and extraction through heat assistance. This processing principle is applied to material having capacity to absorb energy from microwave and convert to heat. Dipolar rotation and ionic movement are two major contributors for heating material by microwave. Depth of penetration is considered as major limitation factor in MW application. Pulse electric field (PEF) involves exposure of food to high electric field strength (0.1 to 80 kV/cm) for very short period of time (nanoseconds to milliseconds) (Bhat *et al*., 2019). When an electrostatic attraction between membranes exceeds elastic resistance, small pore will be formed. These pores facilitate movement of substrate from cell or matrix to solvent. Applied voltage, waveform, and application time are found to be influencing factors in controlling electroporation.

From an engineering point of view, scale up of extraction techniques requires understanding of process parameters. For optimal operating conditions, effect of process parameters on mass transfer kinetics of extract is important. The kinetics of extraction process and knowledge of extraction rate also play an important role in comparing influence of assisted extraction technique with control/ conventional processes. Mathematical models help in scaling up of process by providing information on process controlling parameters on extraction yield. Many models in the form of theoretical, empirical and semi- empirical have been applied in studying extraction kinetics of bioactive material.

Owing to structure of matrix present in sample, complex phenomena of extraction be observed. Though solid-liquid extraction phenomena are simple, it poses challenge to explain mechanism of extraction by single theory. Many defined models were used to compare extraction of target material with experimental data. In solid liquid extraction, initial period extraction is fast and difficult to explain by diffusion equations. Two stage mechanisms were used to explain extraction phenomena which include washing stage and diffusion stage. In

case of washing, solute transfer to solvent from surface and process is very fast. During second stage of extraction, solute transfers to solvent by slow diffusion from matrix of sample. Parabolic diffusion model and power law model for medicinal fungi (Cheung *et al*., 2013b), second-order mechanism model for jatropa seeds oil extraction (Sayyar *et al*., 2009), Peleg's model for polyphenol extraction of grapes (Bucić-Kojić *et al.*,2007) and Elovich's model for resionoid extraction from St. John's wort are some of the models that showed a good mathematical description of the extraction kinetics.

There is growing demand for green emulsifiers to improve value addition and marketability of processed food products. Proteins, polysaccharides, phospholipids and saponins are natural emulsifier groups used in food and beverage industries. Emulsions are reported as versatile system with various sizes, surface structure and electrochemical properties. They are known to be unstable over a period of time and tend to separate when they were allowed to stand for long time. Physicochemical mechanisms involved in separation of emulsion due to thermodynamic instability includes gravitational separation, flocculation, coalescence, Ostwald ripening and phase separation (Israelachvili, 2011). Ability of phospholipids to perform as an emulsifier depends on head and tail group attached to glycerol (McClements and Jafari, 2018).Depending on pH of solution and nature of head group present in phospholipids, electrical charge may vary between positive and negative. Degree of unsaturation of fatty acid chains will alter chemical stability as it influences its oxidation susceptibility (Cui and Decker, 2016).

Characterization of emulsion to different stress or storage time is essential to know its efficacy. Study of emulsion matrix and droplet characteristics helps to measure its influence on physico-chemical and sensory properties of the target product. Emulsion characteristics are often described in terms of physical separation, morphology, surface charge, pH, electrical conductivity, resistance to centrifugation and rheology. Some of the desirable characteristics of an emulsifier include lowest achievable size, good stability and surface characteristics when added in the target food (McClements, 2015).

Primarily, the industrial source for natural emulsifiers are based on lecithin sourced from soybean, sunflower, egg yolk and rapeseed kernels. In this context, ghee residue is an untapped source of phospholipids that could be suitably extracted and enriched by eliminating neutral lipids and proteins, making it a potential food emulsifier.

In light of the above points, the present study is proposed with the following specific objectives.

1. Optimization of process parameters for extraction of phospholipids from ghee residue using pulsed electric field, microwave and ultrasonication - assisted techniques
2. Modelling of extraction kinetics of phospholipids from ghee residue using pulsed electric field, microwave and ultrasonication-assisted treatments
3. Characterization of the extracted phospholipid rich fraction for compositional and techno-functional properties

Chapter-2

REVIEW OF LITERATURE

2.0 REVIEW OF LITERATURE

Ghee residue (GR) is a by-product obtained during ghee preparation, separated as sediment during butter or cream fat clarification. It is also termed as Clarified Butter Sediment Waste (CBSW) mainly consisting of solids-not-fat (SNF) with fat entrapped in solid matrix. It is considered as good source of fat, protein, lactose and minerals with minimal moisture content (Janghu *et al*., 2014).Compounded annual growth rate (CAGR) for preparation of butter and ghee over the past five years (2014-2019) in India is reported to be 7.1%. This reflects the potential for generation of high volume of GR as a dairy by-product. The average particle density of GR was reported as 1.10g/cm^3 however, the value may vary with the method of ghee preparation (Galhotra and Wadhwa, 1993).Notably, it is reported to be a good source of phospholipids (PLs) and natural antioxidants owing to presence of phosphorus (Wani *et al.,* 2022). Considering that PLs exhibit amphiphilic property due to the presence of hydrophobic tail and hydrophilic head, its utilization as a suitable emulsifier provides scope for its commercial exploitation. So far, the reported work on utilization of GR is restricted to either animal feed or as an ingredient in food processing applications. Hence, there is scope for exploring this valuable by-product in alternate applications such as, a source for PLs and its use as a food grade emulsifier.

2.1 Production of ghee and ghee residue

Clarified butter fat, prepared from milk of cow, buffalo or mixed milk is called as ghee in Indian context (Rajorhia, 1993).The International Dairy Federation defined ghee as product obtained from processing of milk, cream or butter from different species to remove moisture leading to particular structure for retained SNF.Different names are used for ghee in different parts of world, namely, *maslee and samna* (middle east), *roghan* (Iran), *meshho* (Aramea), *Samin* (Sudan), *Samuli* (Uganda) (Kumbhare *et al*., 2021). It is a liquid mass composed of free fatty acids (FFA), triacylglycerols, sterols, PLs, caseins and minerals. Unique flavour profile differentiates ghee from anhydrous fat and butter. Though ghee stored under ambient condition is consumed by most of the population, the presence of unsaturated fatty acids makes it sensitive to oxidative changes (Kumar *et al*., 2010).

Butter and cream are used as raw material for production of ghee. Thermal treatment during the preparation of ghee is very important because butter heated at 110 to 120^{o}C leads to generation of volatile aromatic compounds which is attributed to heat catalysed 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 of volatile compounds (Sserunjogi *et al*., 1998). The consumers' expectation for flavour of ghee in India is also different, as some regions prefer ghee with lower volatiles, while other regions may prefer ghee with higher volatiles.

2.1.1. Methods of ghee preparation

Preparation of ghee is obviously an essential step for the generation of GR. Many methods are followed for preparation of ghee which involves the concentration of milk fat from different raw materials, namely cream and butter. The composition and quality of the residue is reported to vary with method employed for preparation of ghee (Wani *et al*., 2022). In the Indian context, three methods are widely reported for preparation of ghee viz., *desi* method, direct cream method and creamery butter method. The complete flow of process for all three methods of ghee preparation is depicted in Fig. 2.1.

2.1.1.1. *Desi* method of ghee preparation

Indian households follow this method for domestic or small-scale preparation of butter and ghee (Halder *et al*., 2021). Milk collected from source is subjected to heating to inactivate microbes, followed by cooling before the addition of lactic acid bacteria to initiate fermentation. The curd is allowed to set during overnight incubation with development of typical flavour compounds. Usually, separation of a top layer of curd (rich in fat content) is observed by virtue of density difference. Post fermented curd is then cold-churned, traditionally using a wooden churner, preferably during morning hours. Churning yields a soft butter and sour buttermilk with pleasant aroma. Butter is manually separated out from the buttermilk and butter mass is hand-pressed to remove residual buttermilk. Heating of the butter under controlled conditions (<120^{o}C) till the end-point described as manual observation of disappearance of surface bubbles results in ghee. On cooling, the GR settled at the bottom is separated from ghee by straining to get clarified ghee.

2.1.1.2. Direct cream method of ghee preparation

In this method, cream is used as the raw material for ghee preparation. Milk from source is initially subjected to centrifugal separation cream and skim milk. Depending on milk source, fat content present in the separated cream will vary. This fat-rich cream is transferred to a heating kettle to evaporate moisture from cream by direct heating (at temperature maintained between 115 to 118^{o}C) till SNF present in cream turns light brown in colour. Ghee is then separated from the GR (sediment) by straining through muslin cloth. This method yields less

amount of ghee as the major amount of moisture evaporation leads to less conversion ratio. It is reported that the direct cream method consumed more energy for processing; however, the process results in higher yield of ghee residue (Kumbhare*et al*., 2021). High serum solid content in cream leads to intensive caramelized flavour in ghee prepared by this method. It was considered as less laborious compared to *desi* method but fails to recover complete fat present in cream (Deosarkar *et al*., 2016). Nearly 4-6% of butterfat was reported to be lost in this method along with GR (Kumbhare *et al*., 2021).

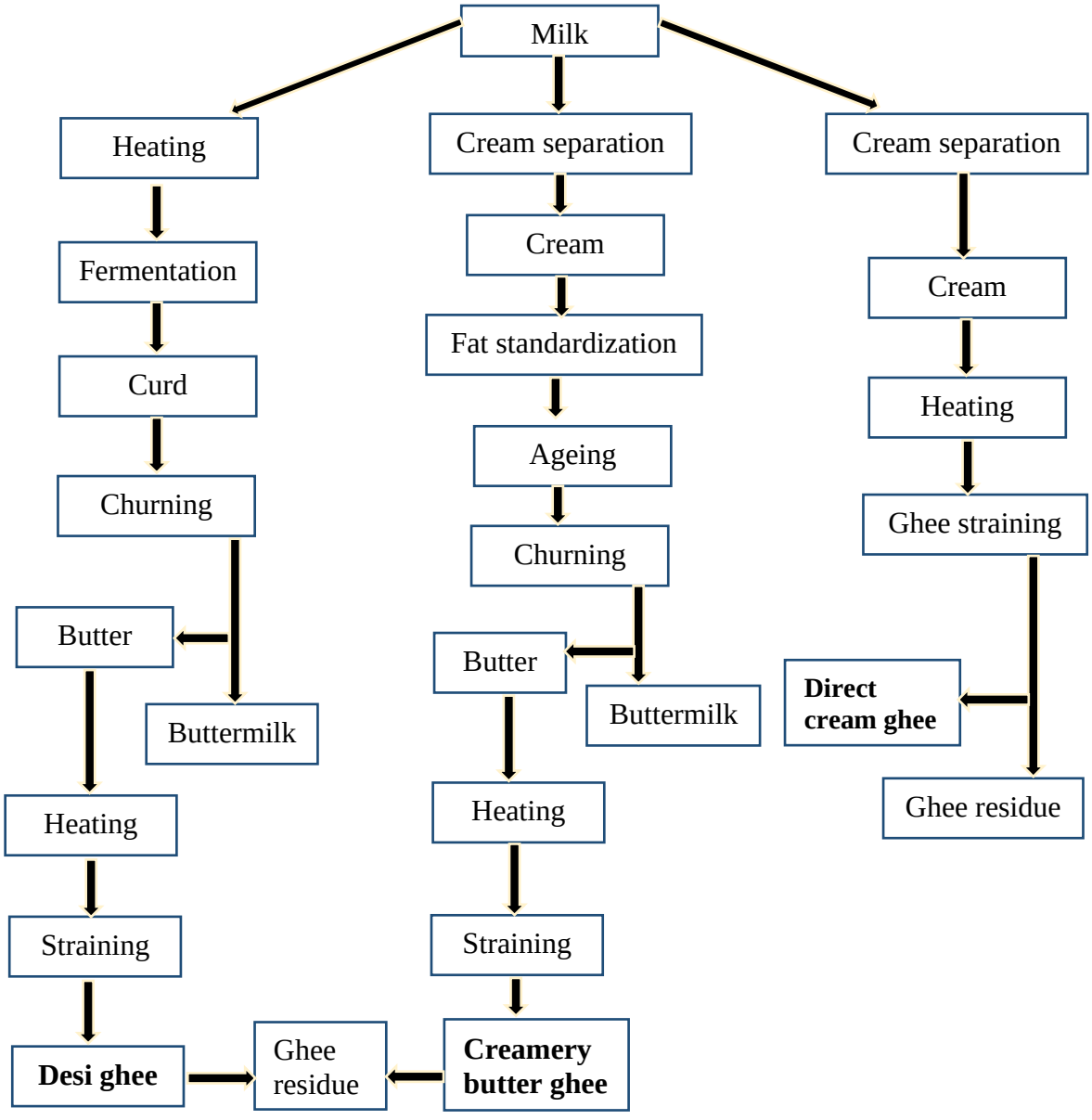


Fig. 2.1 Detailed flow chart for preparation of ghee from *desi*, direct cream and creamery butter method

2.1.1.3. Creamery butter method of ghee preparation

Similar to direct cream method, cream from raw milk is separated under centrifugation, followed by standardization to pre-defined fat content using toned milk. The standardized cream is aged under refrigeration for 12-16h. The aged cream is then churned in a cold jacketed vessel to yield white butter and plain buttermilk. Similar to the process in *desi* method, butter is separated from buttermilk with gentle pressing. Butter is then heated under controlled temperature and for duration till bubbles disappears from surface. Ghee is then separated from GR using muslin cloth (Santha, 1977).

2.1.2. Composition of ghee and ghee residue

Ghee is composed of glycerides, FFA, sterols, esters, PLs, hydrocarbons, carbonyls, fat soluble vitamins, carotenoids, traces of iron, phosphorus and calcium. It contains very negligible amount of moisture (<0.3%) with a major composition of glycerides (~98%). In the remaining 2% of total matter, most part is composed of sterol (cholesterol) and some amount of conjugated linoleic acid. Carbonyls, FFA and lactones were reported to be key components for imparting flavour to ghee (Wadhwa and Jain, 1990). Shelf stability of ghee is attributed to itsPLs content, low moisture and free amino acids (liberated into fat phase from phospholipid-protein complex) (Achaya, 1997).It was also reported that ghee obtained from fresh cream or butter exhibits longer shelf life than ripened cream or butter (Ganguli and Jain, 1972). As per the compositional analysis reported by Pena and Restrepo,(2020) details of composition of ghee from cow and buffalo milk are listed in Table 2.1.

Table 2.1. Composition of cow and buffalo ghee

Sl. No.	Component	Species	
		Cow	Buffalo
1	Protein (%)	0.78±0.02	0.81±0.04
2	Moisture (%)	0.3±0.01	0.3±0.022
3	Lipids (%)	98±0.50	98.8±0.80
4	Ash (%)	0.03±0.002	0.09±0.028
5	Free fatty acids (%)	0.01±0.005	0.1±0.01
6	Energy (kcal/kg)	9305±230	9483±44.5

Source:Pena and Restrepo, (2020)

Composition of GR is reported to be dependent on the raw material and the time-temperature combination of heating. The reasons attributed for change in composition of GR reported by various workers are heating mechanism, time-temperature combination, raw material composition, source of raw material. The proximate composition of GR compiled from different sources is listed in Table2.2. A close perusal of the data revealed that fat and protein

are the major components of GR. The methodologies adopted for preparation of ghee in the listed references (Table 2.2) were also not identical, and this could also have contributed to the differences in the composition. Fat content reported is between 33 and 59%, which is indicative of the quantum of fat present in GR. Protein is reported as second largest composition with range of 18 to 33%, mainly due to SNF fraction. Further, some references described some techniques (extended time of heating) to reduce the fat content present in GR.

Table 2.2. Composition of ghee residue from different raw material

Raw material	Composition (%)					Reference
	Fat	Protein	Moisture	Lactose	Ash	
Creamery butter	47.12	19.86	12.10	NR	3.90	Ramesh *et al.*(2018)
Ghee residue*	35.99	NR	21.04	17.88	3.81	Muniratnamma *et al*.(2017b)
Sweet cream	33.13	30.91	26.64	NR	3.27	Janghu *et al*.(2014)
Creamery butter	41.83	31.69	17.71	NR	2.56	
Unripen cream	59.50	18.60	14.10	7.90	1.30	Santha, (1977)
Ripen cream	57.00	19.80	13.60	7.10	2.20	
Creamery butter	36.20	27.50	25.50	5.50	4.60	
Desi butter	36.80	33.50	17.00	10.40	3.10	

NR-Not reported, * Ghee residue from dairy industry

2.1.3. Yield of ghee residue from different processing methods

No extensive studies were conducted for reporting yield of GR from different raw material and method of preparation. One of the most comprehensive reports is presented by Santha, (1977) who indicated yield and composition of GR obtained from different methods in her dissertation work. The study considered four methods of ghee preparation viz., heat clarification of creamery butter, *desi* butter, ripened cream and unripened cream. Highest amount of GR was reported from unripened cream (13.5%) followed by ripened cream (11.1%), prepared using direct cream method. For the *desi* method and creamery butter method, relatively lower yield of GR i.e., 2.8 and 5.2%, respectively, was reported. The study emphasised that maximum yield of GR can be obtained from direct cream method.

Janghu *et al.* (2014) attempted to prepare GR by direct cream and creamery butter methods, for its use as an ingredient in confectionary products. Under direct cream method of preparation, cream (40±2% fat) was heated till its temperature reached 110 to 120^{o}C. This

method resulted in a yield of 93-94% for ghee and 6-7% for GR. For a kg of raw material used in the study, 444 g of ghee and 131.6 g of GR was reported for direct cream method. While, in case of creamery butter method, 760 g of ghee and 49.69 g GR were reported.

2.2. Phospholipids derived from dairy products and techniques of extraction

PLs belongs to lipids class which contains phosphorus in their structure, chemically described as esters of phosphoric acid or esters of phosphonic acid. PLs are the main components of cell membrane and exhibit good biocompatibility, amphiphilicity and wetting characteristics (Xu *et al*., 2020). They are distributed in animal-based food (egg yolk, bovine brain, milk and by-products) and plant (soybean, sunflower, rapeseed, cottonseed and corn) based products. Biologically derived PLs are built from combination of apolar group and a glycerol backbone moiety.

Even though PLs have been classified in literature on various criteria, the major classification is based on alcohol backbone. Glycerol containing PLs (glycerophospholipids) and sphingolipids (sphingophospholipids) are the two major types of PLs reported (Erickson, 2008; Sikorski and Kolakowska, 2003). PC, PE, PI and PS are subclasses of glycerophospholipids (Huang, 2001). In case of glycerophospholipids, O-acyl, O-alkyl or O-alk-1'-enyl residue are bond on glycerol backbone at sn-1 position and O-acyl residue at the sn-2 position. Phosphate residue with combination of different polar heads at sn-3 position differentiates the kind of PLs. Sphingophospholipids consists of sphingoid base, a long chain aliphatic amino alcohols of two to three hydroxyl group and fatty acid (long chain) linked to polar head and amide group (Contarini and Povolo, 2013; Parodi, 2004).

Basically, milk fat is composed of triglycerides which originate in endoplasmic reticulum of the mammary glands. After its synthesis, it is accumulated in the cytoplasmic lipid droplets followed by transportation to secreting cell (Danthine *et al*., 2000). Before secretion from mammary glands, the fat droplet is enveloped by a membrane (Keenan, 2001). PLs in milk are present in this epithelial plasma membrane in the form of complex structure called as milk fat globular membrane (MFGM). This MFGM is composed of an exterior double layer of protein, PLs (synthesised in the secreting cells of mammary glands), interior single layer of protein and PLs derived from cytoplasmic lipid droplets. The typical arrangement of the MFGM enables the fat to be emulsified and dispersed in continuous phase (Costa *et al*., 2010).

2.2.1. Milk fat globular membrane

It is a triple layer membrane with total thickness of 10 to 20 nm and acts as natural emulsifier (Walstra *et al*., 2006). Protein and lipids together account for 90% of dry weight of MFGM with minor amounts of minerals and enzymes (Danthine *et al*., 2000). PLs present in milkwas determined as 3.59 mg/g of fat compared to 1.95 mg/g of fat in butter and 44.85mg/g of fat inbuttermilk (Avalli and Contarini, 2005). The relative position of specific classes of PLs in MFGM is reported to be as follows. PC and SM positioned outside of membrane whereas PE, PS and PI rests inside of MFGM (Contarini and Povolo, 2013). Table 2.3 presents the composition of MFGM reported in literature.

Table 2.3. Composition of milk fat globular membrane

Protein (mg/100g)	Lipids (mg/100g)	Phospholipids (mg/100g)	Cholesterol (mg/100g)	Reference
1800	770	730	40	Walsta *et al*. (2006)
900	740	700	40	Goff and Hill, (1993)

2.2.2. Phospholipids rich dairy by-products

PLs in milk and dairy products are mainly present in the MFGM (60-65%) and the rest (35-40%) is dispersed in the continuous phase. They are mainly concentrated in external leaflets of the structure as liquid disordered phase co-existing with the ordered phase lipids (Lopez *et al.,* 2010; Gallier *et al*., 2010). The content of PLs in milk ranges from 0.5 to 1.0% of total lipids (Patton and Jensen, 1976). Variation in the composition of subclasses of PLs has been observed in milk and dairy products which are widely attributed to the differences in the methods of analysis, feed of animal and lactation season (Lopez *et al*., 2008). As PLs are mainly concentrated in MFGM, any unit operation intended for disruption of membrane or its fractionation may affect PLs profile in derived products (Rombaut, *et al*., 2006b). As a consequence of processing, PLs expressed on total lipids was lower in cream compared to skim milk. Similarly, PLscontent was more in buttermilk and whey compared to butter and cheese, respectively (Rombaut *et al*., 2006a).

2.2.2.1. Raw milk

The composition of raw milk is variable due to many factors such as animal species, feed, season of lactation, lactation stage and history of cow. Similarly, PLs present in raw milk is also susceptible for variation (Bitman and Wood, 1990). Rombaut *et al*.(2006a)conducted extensive work on the disparity in phosphor and sphingolipid distribution during processing. The investigation started with raw milk and extended to different products derived from milk

during processing. The study reported that raw milk contained 0.04% polar lipids which was equivalent to 0.98% of total fat content. The subclasses of PLs quantified were PE (31.5%), PC (26.0%) and SM (23.8%). The same sample of raw milk was subjected to cream-separation to separate the skim milk and cream. Skimmed milk contained 0.02% of polar lipids where the same was equated to 19.06% on fat basis. The results indicated that PLs is leached to the polar side, as skimmed milk contains more water than cream.

In a study conducted by Avalli and Contarini, (2005) bulk milk was sampled to analyse PLs and its subclasses. The experimental work focused on the evaluation of different solid phase extraction (SPE) columns to quantify total PLs. They reported fat content of 2.6% with PLs of 3.59 mg/g of fat, which included 32.3% of PE fraction. A summary of total PLs content and its subclasses in different reports is compiled in Table 2.4.

Table 2.4. Composition of phospholipids and its classes in bovine milk

Milk type	Total PLs (mg of PLs/100mg total lipids)	Phospholipids subclasses (% of total PLs)					Reference
		PE	PI	PS	PC	SM	
Bulk milk from vat	0.40	32.3	9.3	10.5	27.3	20.5	Avalli and Contarini, (2005)
Raw milk	0.71	31.5	4.9	8.8	26.0	28.73	Rombaut *et al.* (2005)
Skim milk	10.7	NR	NR	NR	NR	31.16	Gallier *et al.*(2010)
Raw milk	NR	26.4	3.4	2.0	42.8	25.5	Avalli and Contarini, (2005)
Raw milk	0.7	42.0	4.8	6.7	19.1	17.9	Rombaut *et al.*(2005)
Semi skimmed	1.3	35.0	7.9	8.9	20.2	27.9	
UHT full fat	0.6	34.0	7.9	9.1	20.5	28.5	
UHT semi skimmed	0.9	33.0	4.8	7.9	22.0	32.3	
UHT skimmed	10.7	38.2	5.5	9.9	19.6	26.8	
Sterilized semi-skimmed	1.0	34.3	5.1	7.7	24.2	28.7	

UHT- Ultra high temperature treated; PLs-Phospholipids; NR-Not reported

2.2.2.2. Buttermilk

Buttermilk resembles skim milk as far as its composition, except for the fat content (reported to be high in buttermilk). Buttermilk is also characterized by the presence of fragments of MFGM, attributed to the membrane rupture during churning**.** Avalli and Contarini, (2005)

used buttermilk as one of the selected product to test efficacy of different SPE cartridges for separation of PLs. They reported PLs content of 44.85 mg/g of fat in buttermilk. Rombaut *et al*. (2005) used buttermilk as a base-sample to analyse the PLs and sphingolipids distribution among different dairy products using high performance liquid chromatography (HPLC).The study revealed that buttermilk contained 21.85% of PLs on fat basis. The classification of PLs reported in the study included PE (42.90%), PI (8.91%), PS (8.55%), PC (19.10%) and SM (12.83%).

Rombaut*et al.* (2007) mapped the changes in PLs and sphingolipids content when milk was converted to different dairy products. Cream obtained from raw milk was pasteurized and churned to get buttermilk. The study estimated a fat content of 0.49%, polar lipids of 33.05% on fat basis and 19.06% of sphingolipids on polar lipid basis in buttermilk. Spence *et al.*(2009)applied filtration techniques (microfiltration and ultrafiltration) followed by spray drying to concentrate the PLs content in buttermilk. The spray dried buttermilk powder was treated with SCFE to eliminate non-polar lipids before the estimation of PLs. The result of experiment indicated an increase in PLs concentration by five folds with a reduction in total fat content from 55% to 38% due to the elimination of neutral lipids.

Costa *et al*.(2010) explored the option of ultrafiltration and diafiltration of whey buttermilk to concentrate the content of MFGM and PLs in the retentate. The collected retentate (10kDa) was subjected to spray drying, followed by SCFE at 350 bar and 50^{o}C to eliminate non-polar lipids. The resultant powder contained 21% of lipids which included 61% of PLs. Li,(2017) also worked on SCFE for separation of non-polar lipids from buttermilk powder. Results revealed that under optimal conditions (550 bar and 60^{o}C) of SCFE process, 16.88 mg PLs/100mg fat was reported for the butter milk powder. Ubeyitogullari and Rizvi, (2020) conducted experiments to enrich PLs concentration in buttermilk powder using food grade ethanol-coupled with supercritical carbon dioxide extraction. Sequential extraction of PLs using pure ethanol andmodified ethanolin SCFE resulted in lipids yield of 6.3% which includes 49% of PLs.

2.2.2.3. Whey protein phospholipids concentrate

Whey protein phospholipids concentrate (WPPC), a by-product of cheese processing industry, is reported as a co-product during production of whey protein isolate. It is reported to have functional attributes such as good water holding capacity, heat stability, gelling property and low foamability (Merrill and Singh, 2012). The concentrate was used as an ingredient in a cheese product and observed to improve cheese properties such as firmness,

melt appearance and water holding. The proximate composition of WPPC was determined by Bund and Hartel, (2013) as 50% protein, 12% of lipids, 8% of ash and 6% moisture content; with 20% of fat containing in the WPPC estimated to be PLs.

STEP (Simultaneous Texturization and Extraction of Phospholipids) method of PLs extraction designed for egg yolk was attempted for WPPC by Prince *et al*. (2018). This method helped in precipitation of the protein present in WPPC and extracted lipids with ethanol as solvent. The results reported in the study indicated total lipid content of 40.7% of which 58.1% were PLs. Sprick*et al*. (2019) used SCFE for enrichment of PLs in WPPC using a two-step approach. The results indicated significant improvement in PLs content in the final product. Under optimized condition of SCFE, the average PLs content reported were 26.26g per 100g fat with SM(11.07 g/100g fat), PC (10.07 /100g fat) and PE (7.2/100g fat). In order to completely remove lipids from the dairy matrix, extraction using a total of 20 mass equivalent of solvent was optimised by Ferraris and Qian, (2021) with minimal apparatus. The resultant experiment obtained 37.7% of PLs in the final mass of lipids.

2.2.2.4. Butter serum

Butter serum is the aqueous phase of butter and derived as a by-product during production of anhydrous milk fat. In the process of manufacturing pure butterfat, butter is melted to get butterfat and butter serum (Rombaut *et al*., 2006a). Composition of butter serum is reported to be similar to buttermilk with the exception of its fat content (McPherson and Kitchen, 1981). Butter serum contains higher amount of glycerophospholipids and sphingolipids compared to buttermilk. Buttermilk contained 91.7 g of dry matter per kg of sample compared to 105.6 g per kg of butter serum. However, the dry matter of butter serum was 2.5 times more in total fat as compared to buttermilk (Bourlieu *et al*., 2018). Butter serum reported a composition of 35% protein, 26.9% lipids, 9.3% polar lipids and 7.1% of ash (Gassi *et al*., 2016). Rombaut *et al*. (2006a) determined that butter serum comprised of 28.4% of polar lipids originally present in the raw milk.

Rombaut *et al*. (2006b) presented a detailed profile of the major PLs present in butter serum as PC (33.5±0.2 g/100g of PL), sphingomyelin (27.9±1.8 g/100 g of PL) and PE (18.7±2.0 g/100 g of PL). Minor PLswere determined as lactosylceramide (8.0±0.4 g/100 g of PL), PI (6.3±0.7 g/100g of PL), PS (3.6±0.9 g/100 g of PL) and glucosylceramide (2.0±0.3 g/100 g of PL). Total sphingolipid content (the sum of glucosylceramide, lactosylceramide, and sphingomyelin) was reported to account for 37.9±1.9 g/100 g of PLs. Lipids and PLswere observed to show significant increase in PLs to protein ratio in buttermilk and butter serum

after washing. The process was reported to act as an excellent technique for PLs enrichment in serum and buttermilk (Britten *et al*., 2008).

2.2.3. Techniques for extraction of phospholipids from dairy matrix

Isolation and fractionation are reported as the two important steps in quantification of PLs from dairy matrix. Milk has been denoted as a complex matrix compared to other foods, mainly due to high content of water and uneven distribution of protein, fat and lactose content (Contarini and Povolo, 2013). Also, structurally it has been observed that PLs attach to both proteins and lipids due to its amphiphilic characteristic. Therefore, additional care is recommended to ensure recovery of whole fat fraction. Neutral lipids are commonly reported to be extracted using organic solvents such as hexane, chloroform or solvents of similar group, while for the extraction of polar lipids use alcohols has been cited (Lopez *et al*., 2014).

2.2.3.1. Isolation of lipids from dairy matrix

Rose-Gottlieb method of lipid extraction is reported as one of the commonly used technique for lipids extraction. Under this method, samples are digested with 25% ammonia followed by extraction with different proportion of ethanol, diethyl ether and petroleum ether. The advantages cited for this method are its simplicity, as it requires only few solvents and minimal equipment. However, this method is said to result in incomplete extraction of polar lipids. On the other hand, Folch method, which originated for lipid extraction from muscles was modified by various authors and adopted for extraction in dairy products. The merits of this method include use of simple solvents like chloroform and methanol. This method is considered as most adopted technique for estimation of PLs. One of the demerits of this technique is use of chloroform (toxic) as solvent (Pimentel *et al*., 2016).

Bligh and Dyer, (1959) slightly modified Folch method of extraction by specifying solvent proportions and taking water present in sample into consideration. Later, Iverson *et al*.(2001) compared Folch and Bligh and Dyer methods to understand their isolation efficiency. Results indicated that at sample fat content <2%, there was no significant difference between two methods studied. However, Bligh and Dyer method showed underestimates of lipid content when lipid content in the sample increased beyond 2%.

Hara and Radin,(1978) used hexane and isopropanol (HIP) as a replacer to chloroform. The advantages of this method over Folch method include its lesser toxicity, non-interference with proteolipid matrix and rapid separation of solvent due to density gradient. The methodology involved a washing step that eliminated all non-lipid components that aided in

avoiding the clogging of columns. Cequier-Sánchez *et al.* (2008) explored the use of dichloromethane/methanol as a substitute for chloroform/methanol in fat extraction. The results were found to be comparable with Folch method; only minor differences were observed in total fat estimates. Also, no significant variation in lipid classes was reported in the study when analysed using the modified technique.

Matyash *et al*.(2008)replaced chloroform with methyl-tert-butyl ether (MTBE) to extract lipids from brain tissue and egg. Synthetic lipid standards, under fully controlled experimental settings, were used to validate the recovery of fat that was quantified using the proposed method. Results concluded that MTBE as solvent helped in recovery of lipids at a faster rate with cleaner fractionation. Finally, the extensive analysis conducted in this study demonstrated the efficacy of MTBE as a replacer to chloroform, by delivering similar or better recovery of the lipid species. This research work suggested the developed method as a replacement for the Folch and Bligh and Dyer methods.

2.2.3.2. Fractionation of lipids

Many methods have been reported on the fractionation and classification of PLs from bulk lipids. The earlier methods attempted for the separation are based on open column chromatography, thin layer chromatography (TLC) and solid phase extraction. Conventionally, column chromatography has been applied to separate PLs from lipids by eluting desired lipids under varying polarity and strength of column. The TLC method reported disadvantages of low sample load capacity, scratching of component and extraction using solvents (Pimentel *et al*., 2016). These limitations in TLC methods led to inaccurate fractionation and quantification of PLs classes.

The advent of commercial columns preloaded with solid phase material, also called as solid phase extraction (SPE) columns, helped to fractionate desired components based on polarity. These columns are merited with enhanced ease for handling lab scale samples. Among the first attempts to prove efficacy of PLs fractionation, silica gel bonded column were used. This solid phase extraction technique for dairy matrix reported good separation of polar lipids (Avalli and Contarini, 2005). The detailed flow of process for solid phase extraction followed by the authors is presented in Fig. 2.2. Ferraris *et al*. (2020) also followed the same sequence of elution to quantify PLs from whey protein concentrate. Rathnakumar *et al*. (2021) slightly modified the sequence of solvents used in elution of PLs as detailed in Fig.2.3. which resulted in quantification of PLs in beta serum.

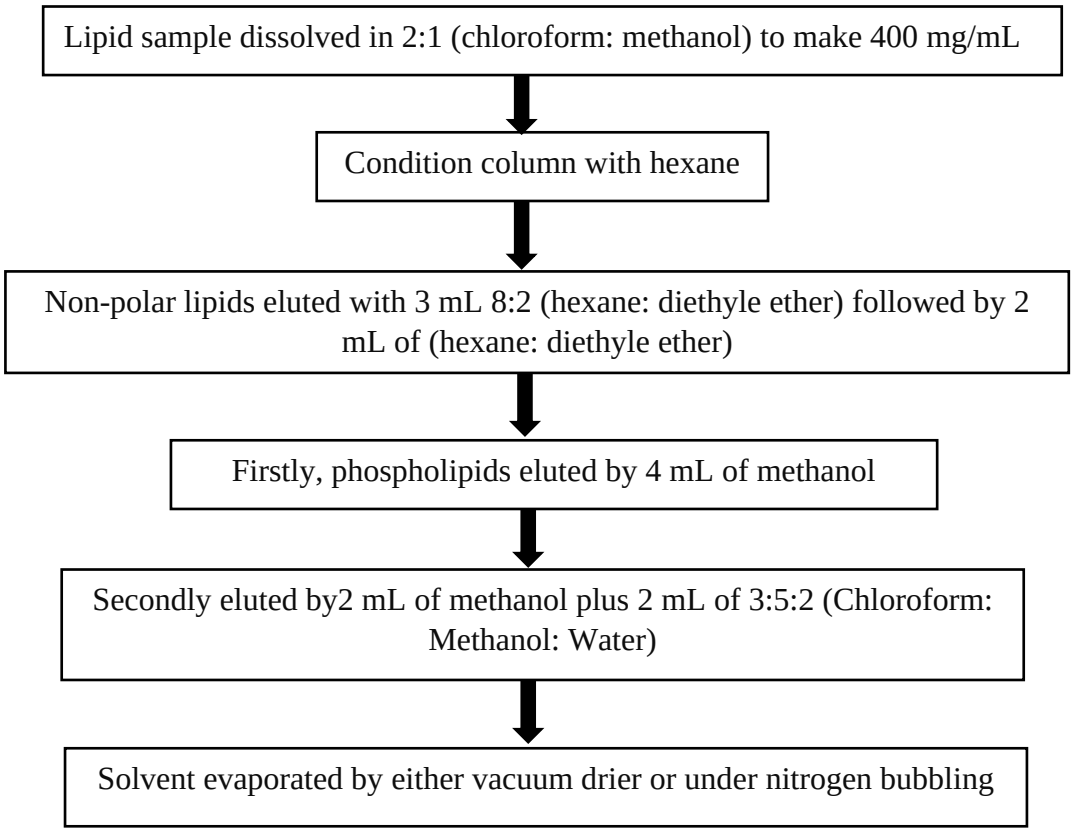


Fig. 2.2. Steps followed for elution of neutral and polar lipids from dairy product

Source: Avali and Contarini, (2005)

Cheng *et al*. (2019) studied the feasibility of extracting dairy PLs using a switchable solvent called N,N-dimethylcyclohexylamine (CyNMe2). Raw cream, buttermilk, concentrated buttermilk, beta serum was used as the test samples of dairy matrix to evaluate the efficacy of the extraction. The detailed description of the process is depicted in Fig. 2.4. Results from the experiment were compared with conventional extraction methods such as Folch and Mojonnier methods. Among the chosen dairy by-products, only buttermilk showed a recovery of 99% of PLs, whereas other dairy products failed to report significant extraction.

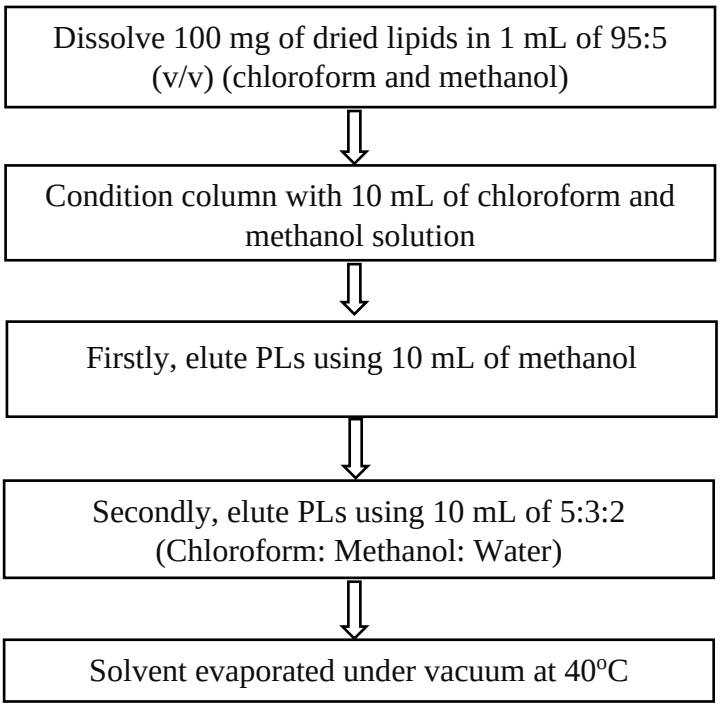


Fig. 2.3. Steps followed for elution of polar lipids from beta serum

Source: Rathnakumaret al. (2021)

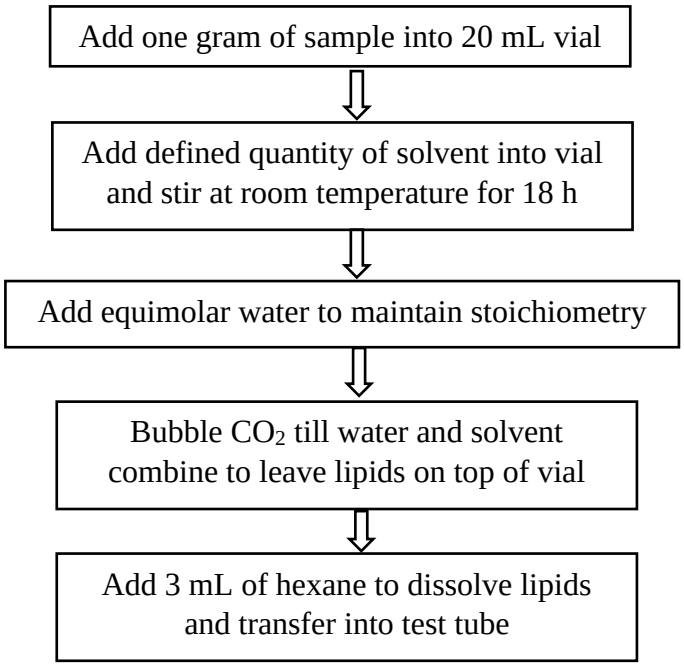


Fig. 2.4. Flow chart for extraction of dairy PLs using switchable solvent

*Source:*Cheng et al. (2019)

2.2.3.3. Concentration and purification of phospholipids by filtration and SCFE

Astaire *et al*. (2003) attempted to improve PLs concentration in samples of buttermilk by subjecting it to microfiltration, diafiltration and two steps SCFE. Using a 2n two factorial

experimental designs, microfiltration was optimized for temperature and source of buttermilk by passing the samples through a ceramic membrane (0.8 µm pore size). The retentate was spray dried before SCFE (at 375 bar, 77^{o}C extraction temperature, 20 g/min flow rate) with three cycles of extraction for 75 min. Results revealed that the milcrofiltrate fraction powder after SCFE showed increase in polar lipids from 9.5 mg/g dry powder to 19.54 mg/g of dry powder. Concurrently, triglycerides decreased from 21.33 to 3.98 mg/g of dry powder.

Buttermilk powder, regular buttermilk and whey buttermilk was opted as dairy matrix for microfiltration and SCFE by Spence *et al.* (2009). Through microfiltration, buttermilk was concentrated till a volumetric concentration factor of 2X was reached. In diafiltration, chilled tap water was circulated till 2x dilution factor was reached in the permeate. The retentate was subjected for spray drying followed by SCFE (at flow rate of 20g/min. for 86 min.) and resultant powder was stored at 4^{o}C. Non-polar lipids in the total lipids were brought down to 38% from 55% and 5-fold increase in PLs was reported.

As milk by-products are rich in MFGM, especially PLs, Costa *et al*. (2010) applied SCFE for the enrichment of PLs in whey buttermilk. Prior to SCFE, whey buttermilk was concentrated to 10X by ultrafiltration followed by a 5X diafiltration process. The retentate of this filtration was subjected for spray drying to obtain whey buttermilk powder. The powder was subjected to SCFE at 350 bar and 50^{o}C using carbon dioxide. Results of this experiment revealed that ash and lactose were removed by filtration and non-polar lipids by SCFE. The resultant powder reported a composition of 73% proteins and 21% lipids, of which 61% were classified as PLs.

Barry *et al*. (2017) integrated enzymatic hydrolysis, ultrafiltration and SCFE to enrich PLs in buttermilk powder. Buttermilk powder was reconstituted to 10% total solids, sample pH was adjusted to 8.0, before it was treated with alcalase enzyme (at 1:100(w/w) enzyme:substrate ratio on protein basis). The resultant hydrolysate was subjected to ultrafiltration at 50^{o}C to attain a concentration factor of 11 by volume. The retentate and permeate were evaporated to 40% total solids through a falling film single stage evaporator followed by spray drying. The retentate dry fraction was subjected to SCFE at 40^{o}C and 300 bar by using only carbon dioxide to remove non-polar lipids. In the second step of purification, carbon dioxide along with ethanol (co-solvent) was used at different percentage to concentrate PLs in the sample. Results reported in the study indicated increase in PLsin the retentate after ultrafiltration from 1.3% to 11.05%. Total lipids also reported a significant increment at 60.07%, compared to

6.84% estimated for the untreated buttermilk powder. After the second step of SCFE, PLs were reported as 56.24% on dry weight basis.

2.2.4. Dairy phospholipids as an emulsifier

Food, pharmaceutical and cosmetic industry requires emulsifiers to ensure uniform distribution of the constituents of product throughout the continuous phase. In food applications, natural food grade emulsifier is gaining traction. Presently, this need is primarily met using soy derived lecithin that is commercially produced and used in the food industry (Bueschelberger, 2004). Single type of emulsifiers is common, however, to improve stability and functional attributes, combination of emulsifiers is also reported to be effective (McClements *et al*., 2016). Dairy by-products which are rich in PLs can be concentrated by appropriate techniques and incorporated in food as a replacer to conventional emulsifiers. PLs from dairy origin are rich in SM which are absent in most of plant derived PLs.

Wong and Kitts, (2003) compared two kinds of isolates obtained from buttermilk viz., whole isolate (protein, casein, protein of MFGM) and isolate of MFGM alone, and the two fractions were analysed for composition. The results indicated a high cholesterol and polar lipid composition in whole isolate whereas, PC, PE and SM showed higher proportion in MFGM isolate. Resultant fractions of buttermilk were used to formulate an emulsifier with 10% soyabean oil to form oil in water emulsion at different proportion. A larger amount of MFGM isolate was required to form droplet size distribution compared to emulsion prepared using 1-2% (w/v) whole isolate. It was further suggested that heat treatment and churning process of cream affected the emulsifying behaviour of MFGM protein.

To evaluate efficacy of MFGM isolate as an emulsifier, Roesch *et al*. (2004) considered reconstituted buttermilk powder as the test sample. Buttermilk was reconstituted as per the method referred by Corredig *et al*. (2003). Sodium citrate was added to reconstituted buttermilk, MFGM to proteins ratio was improved and made as MFGM isolate. Other fraction, i.e. butter milk concentrate was retained during the membrane separation process at 70% w/w proteins. The emulsion properties were expressed in terms of creaming profile and particle size distribution at various concentrations. Results proved that MFGM isolate at concentration of 0.25 to 10% showed small particle size distribution. The droplet size reduced with increase in MFGM isolate in the emulsion. On the other hand, emulsion prepared using butter milk concentrate showed flocculation and lower surface coverage. Study concluded that selective isolation of MFGM led to better emulsifying properties compared to buttermilk concentrate.

Zhu and Dhamodaran, (2013) used extract of cheese whey fat globule membrane (CWFGM) to study its oxidative stability and emulsifying property. Two step extraction using ethanol as solvent was carried at 6.5 and 4.5 pH to result in a lipid extract of 17.2%. Using TLC and NMR, neutral and PLs from purified lipids were estimated as 69% and 31%, respectively. The extract was reported to be a semisolid mass composed of 31% SM, 27% PC, 4.6%PS and 3.1% PI. Oil in water emulsion prepared by using dairy lecithin at <2% were deemed unstable, whereas an emulsion made at >4% were reported to be stable for 60days at room temperature. Average particle size of emulsion were analysed and a decrease in droplet size from 1µm to 0.25µm were reported for increased dairy lecithin concentration.

MFGM isolated from reconstituted buttermilk powder and buttermilk whey was studied to evaluate emulsifying properties by Phan *et al*. (2014). Six samples were prepared in combination namely, buttermilk-MFGM at 100%, whey-MFGM at 100%, Lacprodan at100% and other three were blends with butter milk powder at 4:6 ratios. Among the six samples, the whey-based (whey-MFGM-100 and whey with BMP) stabilized emulsions showed aggregation of droplets, high protein load and polar lipid load. The remaining four samples showed similar emulsion behaviour such as narrow particle distribution, smaller droplet size, low viscosity and also exhibited Newtonian flow behaviour. It was concluded that behaviour of emulsifier was dependent on constituents of MFGM.

2.2.5. Nutraceutical aspects of phospholipids

Reports on dietary intake of glycerophospholipids are sparse, while an intake of 2-8 g of PC was reported as intake in normal diet. One third of the lipid intake by humans is credited to milk fat (USDA, 2017). The initial application of milk PLs turned liposomes to encapsulate bioactive compounds spiked scholarly interest in use of PLs in diet. Thereafter, a wide range of health benefits have been reported for milk PLs based on the work undertaken by different clinical trials. The nutraceutical effects of PLs reported in literature is summarised below.

2.2.5.1. Inhibition of intestinal cholesterol absorption

Zierenberg and Grundy, (1982) reported that in humans, half of the cholesterol consumed is absorbed in gut lumen and rest is excreted via faeces. However, the exact proportions are dependent on individual. As milk SM contains fatty acids with longer chain length and higher degree of saturation, this trait is reported to be beneficial in inhibiting cholesterol absorption compared to egg SM. A study revealed that more than 90% of dietary PC was completely absorbed by intestine and appeared in lipoproteins (plasma) and red blood cells (Phan and Tso, 2001).

2.2.5.2. Neurological and neurocognitive diseases

The PLs constituents, mainly SM,are attributed with a structural role in brain cell development by acting as effective carriers of essential fatty acids(Noh and Koo, 2004).A reduction in the endoplasmic reticulum-stress-induced cell death using commercially available fraction of MFGM PLs was demonstrated in-vitro (Küllenberg *et al*., 2012). Modulation of cell signalling and simulation of auto phagocytosis were ascribed for protective action of dietary PLs. In another study, bovine SM promoted neurobehavioral development when tested on premature babies (Tanaka *et al*., 2013). It was elaborated that all measured neurodevelopment parameters were positively associated with the MFGM-sphingomyelin treatment.

2.2.5.3. Phospholipids as anticancer agent

PLs are reported to have beneficial effect against colon cancer. In a study, Fischer-344 rats were administered with MFGM which was rich in PLs and bioactive proteins (Snow *et al*., 2010). The treatment was proved to reduce colon cancer. However, it was cautioned that MFGM is highly susceptible to heat treatment. Hence, proper care should be taken in designing the formulation.

PLs is also known to play significant role in prevention of liver cancer, as it readily incorporates omega 3-fatty acids, reported to be lipogenesis inhibitor (Lamaziere *etal*., 2013). By using PLs and sphingolipid fractions of buttermilk obtained from membrane filtration, growth modulatory effect on SW480 colon cancer cells was studied (Kuchta-Noctor *et al*., 2016). Results confirmed anti-proliferative effect of buttermilk, particularly due to SM and glycosphingolipids. In line with this study, Castro-Gómez *et al*. (2016) isolated lipid fractions using food-grade and non-food grade solvents. Results confirmed that food grade solvents possessed rich content of phosphor- and sphingo- lipids, capable of showing anti-proliferative activity against human colon and ovary cancer. Anti-cancerous activity of sphingolipids was ascribed to its retention in entire length of small intestine and colon owing to slow digestion.

2.2.5.4. Effect against infections

Administration of PC and PI to rats led to protection against degeneration of the enteric mucosa and bacterial translocation (Wang *et al*., 1994). Further, rats administered with butter milk rich in PLs exhibited better resistance against *Listeria monocytogenes* by preventing its adhesion to intestinal mucosa (Sprong *et al*., 1998).

2.2.5.5. Effect against hepathopathies

It has been proved that PLs have hepatoprotective properties. Experiments conducted on the effect of administering PC on rats prevented uncoupling of mitochondria and reduced alcohol-induced hike in Tri acyl glycerol (TAG) and cholesterol esters (Navder *et al*., 1997). Gundermann *et al*. (2011) established, through human studies, the hepatoprotective properties of soy PC. The influence of PLs on hepatoprotective effect was stronger when it was derived from milk and its by-products. It was also perceived that the administration of complex PLs exert stronger protective action than isolated PC.

2.3. Assisted extraction techniques

Extraction of compounds from food and other natural materials generally require large quantity of solvent, extraction time and capital investment (Garcia-Vaquero *et al*., 2020). Hence, there is now a great focus on exploring sustainable and scalable technologies which influence the yield of extraction from food matrix. Assisted extraction techniques include thermal and non-thermal extraction methods which are based on different principles of operation. Among these sustainable extraction techniques, microwave assisted extraction (MAE), ultrasound assisted extraction (UAE), PEF extraction techniques have evolved as promising methods in enhancing extraction of target compounds from food matrix. Under the assisted extraction process, the target molecule is often expelled out of biological web due to supplemental physical, chemical or thermal effects. It leads to concurrent benefits of enhanced yield of target molecule, reduced solvent utilized and lower time of extraction when compared to conventional extraction.

2.3.1. Microwave assisted extraction

Electromagnetic spectrum with wavelength of 1mm to 1m and frequency range of 300 MHz to 300 GHz are termed as microwaves. Unlike conventional heating, microwaves act at molecular level through interaction with electromagnetic field. Microwave is a unique heating mechanism as the waves can penetrate in all directions resulting in heat generation throughout the sample volume (Venkatesh and Raghavan, 2004).

2.3.1.1. Mechanism of microwave heating

Whenever an electromagnetic wave is confronted with a medium, a combination of three interactions is possible viz., absorption, reflection and transmittance. The interaction between matter and microwave lead to dielectric loss, magnetic loss and conductive loss. Dielectric heating, Joule heating and induction heating phenomena are encountered due to these mechanisms and they are dependent on the nature of electromagnetic field and material

property. Heating by microwave is often ascribed to the synergistic effect of electric and magnetic field. Dipolar polarization and ionic conductivity are the two fundamental mechanisms that are observed when a food is exposed to the electrical field component of microwave. As dipoles are sensitive to electric field, they tend to realign by shifting polarity of sinusoidal field rotation leading to heat generation. Electron, ions or any charge carriers also get influenced by the electric field of the microwave. This leads to random motion, ultimately resulting in the heating of the sample due to collisions between the atoms/molecules in the food matrix. Under the influence of a magnetic field, eddy current loss, magnetic resonance loss and hysteresis loss are reported. Eddy current loss is reported to be observed when there is a relative motion between magnetic field and a conductive material (Aguiar *et al.,* 2009). The influence of magnetic field on food heating is not yet extensively reported whereas; it has been studied in depth in applications related to conductor and semiconductor heating. Fig.2.5. illustrates the components of a typical domestic microwave oven and functionality of microwave components are enlisted in Table 2.5.

2.3.1.2. Assisted extraction of lipids by microwave

Assisted extraction of lipids using microwave has gained importance due to the advantages related to solvent use, energy and time. Many solvents have been explored in association with microwaves for the extraction of lipids from different biological matrices. The low specific heat associated with lipids is advantageous. This property renders the lipids to be susceptible to microwave radiation, improving their solubility into the extract. Lipid groups in a solid matrix, when subjected to microwave radiation, experience localized pressure and thermal stress causing the matrix to rupture, ultimately leading to the leaching of the lipids into the solvent (Shams *et al.,* 2015). The work reported by scholars for microwave assisted extraction of lipids from different matrices has been compiled in Table 2.6.

2.3.2. Ultrasound assisted extraction

Application of ultrasonication in food industry dates back to 1927, when it was reported for the mixing of oil-in-water emulsion. With its advent into commercial scale food processing applications, ultrasonication expanded to areas such as homogenization, extraction, microbial inactivation, degassing, cleaning and enzyme inactivation (Majid *et al*., 2015). Fundamentally, sound propagates as a wave through a material medium in a range of frequency. Ultrasound is a sound wave at a frequency greater than 20 kHz whereas, human audible range is reported as 20 to 20 kHz (Dong *et al*., 2019). Based on end use of ultrasound, it is classified as high frequency - low energy US (intensity <1 W/cm^2 and frequency >100

kHz) and low frequency- high energy US (intensity >1 W/cm^2 and frequency 20 to 500 kHz) (Jiang *et al*., 2019).

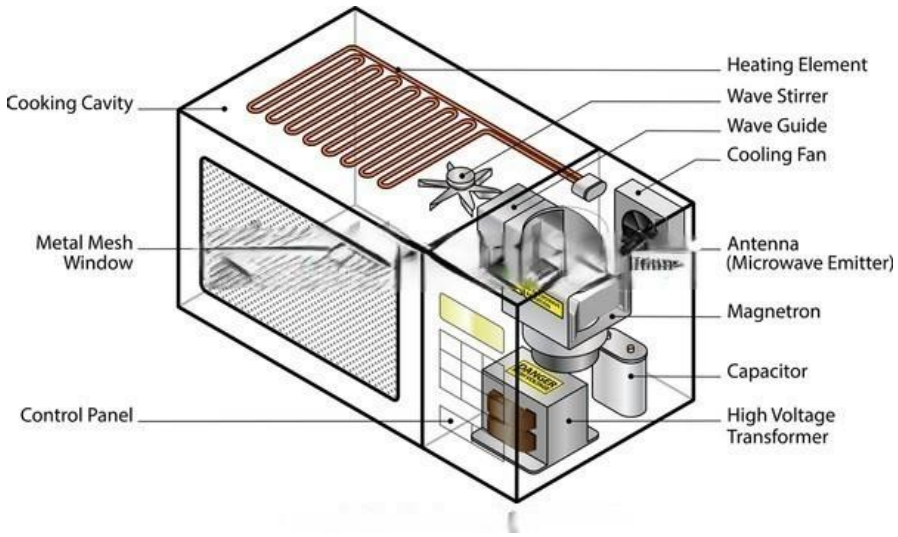


Fig. 2.5. Line diagram of domestic microwave oven depicting different components
Source:https://rethority.com/parts-of-a-microwave

Table 2.5. List of domestic microwave oven components and its function

Sl. No.	Component	Function
1	Cooking cavity	Space where food material placed for heating
2	Metal mesh window	To reflect microwaves and facilitate view
3	Control panel	Setting provision for power, time and other heating features
4	Heating element	As an hybrid in microwave to create convective heating
5	Wave stirrer	Facilitate even distribution of generated waves in cavity
6	Wave guide	Conduit to covey wave from magnetron to cooking cavity
7	Microwave antenna	Facilitate emission of microwave complex
8	High voltage transformer	Facilitate in transforming power to desired voltage
9	Magnetron	Self-excited microwave oscillator to generate microwave field
10	Cooling fan	To exhaust heat generated in microwave generation unit

Table 2.6. Microwave assisted extraction of lipids from different biological matrices using diverse solvents

Sl. No.	Lipid source	Solvent	Remark	Reference
1	Chinese tallow tree seeds	Ethanol	Continuous mode reported 35.32% of lipids and batch mode with 32.51% with microwave assistance	Boldor *et al*. (2010)
2	Soybean and rice bran	Ethanol	Microwave reported 17.3% for soy bean and 17.2% rice bran oil yield compared to 11.3 and 12.4%, respectively by conventional method	Kanitkar *et al*. (2011)
3	*Isatis indigotica* seeds	Water and enzyme cocktail (cellulase, pectinase and proteinase)	Oil recovery of 59.27% was reported at optimal conditions by microwave	Gai *et al*. (2013)
4	Pumpkin seeds	Water and enzyme cocktail (cellulase, pectinase and proteinase)	Under optimal conditions, 64.17% of oil recovery was observed with better oxidative stability	Jiao *et al*. (2014)
5	Microalgae	1-butyl-3- 45 methylimidazolium hydrogen sulfate	10-15 times increase in extraction rate compared to conventional method	Pan *et al*. (2016)
6	Rice bran	Petroleum ether, hexane and methanol	MAE reported 85 ppm of γ-oryzanol compared to 82.0 and 73.5 ppm for ultrasonication assisted and conventional method, respectively	Kumar *et al*. (2016)
7	Fish waste	Isopropanol/hexane and water	Lipid yield was 80.5 mg/g from MAE whereas, 46.6 and 15.8 mg/g for Soxhlet and Hara and Radin method, respectively	Rahimi *et al*. (2017)
8	Rice bran	Hexane	More than 95% of oil recovery was reported in the study with extraction time of 8-10 min.	Pandey and Srivastava (2018)
9	Microalgae paste	chloroform/methanol	Reported higher lipid yield by microwave assisted extraction against conventional method	de Moura *et al*. (2018)
10	*Nannochloropsis oceanica*biomass	Tetra methyl ammonium chloride	Reported total lipids yield as 19.58 g/g of total biomass using microwave assistance	Rezaei *et al*. (2021)
11	Coconut	Water	Coconut oil yield was ~20% for MAE whereas 10-16% reported for UAE	Martínez *et al*. (2022)

The basic principle and mechanism of ultrasonication is elucidated as follows. Sound waves travel as sinusoidal waves in solid or fluid media, causing elastic vibrations. When the waves pass through a liquid medium, the pressure created by ultrasound waves causes acoustic cavitation (Majid *et al*., 2015). Further, the wave also results information, growth and collapse of bubbles, leading to localized pressure and thermal effects (Soria and Villamiel, 2010). These effects are manifested as mechanical, chemical and biological changes in the liquid medium. Shear stress, instantaneous collapse of bubble and turbulence in fluid were the reported mechanical effects (Yusaf and Al-Juboori, 2014). Sonochemical generation and emergence of free radicals have been listed as the chemical changes. Whereas, destruction of microbes and enzymes are the noticeable biological effects due to ultrasonication (Sivagami *et al*., 2019).

2.3.2.1. Components of ultrasonication unit

Horn type and bath type systems are two kinds of ultrasound systems used in the food industry. During the initial days of ultrasound application, bath type systems were used for most of food treatments due to its easy availability. Eventually, due to availability of varied size ultrasound horns and mobility, horn type ultrasonicators were adopted for food applications. Electrical power generator, transducer and emitters are reported as the major components of ultrasonicator (Povey and Mason, 1998). In case of the liquid whistle type ultrasonicator, ultrasound is generated by pure mechanical energy. The electrical generator could be omitted from the system (Mason *et al*., 1996).

Electrical generator acts as the tool for driving the transducer in ultrasonic system. It generates electrical current of required power rating by indirectly setting the power with current and voltage. Low frequency electrical generators (10-40 kHz) are used in industrial cleaning, welding and disinfection. Transducers act as bridge between generator and emitter by facilitating ultrasound generation. Electrical generator of specific frequency attached with transducer leads to transform electrical energy into ultrasound of same frequency (Lee *et al*., 2003). Magnetostrictive and piezoelectric are two types of transducer commonly used due to its ability to convert electrical and magnetic energy into mechanical energy and ultrasound.

The emitter radiates ultrasound waves into medium and also meet requirement of amplification of ultrasound from transducer to medium. Replaceable emitter tips available in some models are called as sonotrode. Shape of sonotrode determines intensity of radiation. Emitters are susceptible to wearing and hence, commercial food processing units uses robust

tips which are wear resistant. Fig.2.6. representsthe line diagram of a horn type ultrasonication unit.

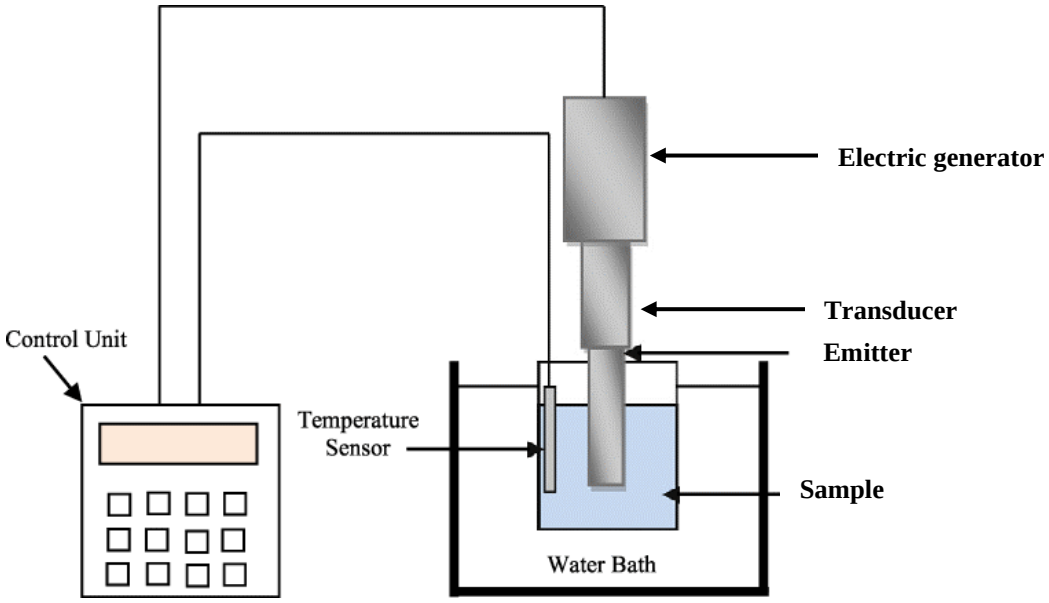


Fig. 2.6. Line diagram of horn type ultrasonication unit

Source: Mondal and Mandal, (2019)

2.3.2.2. Mechanism of extraction by ultrasonication

Many theories have been proposed to explain the extraction of target compound using ultrasonication. Ultrasound is believed to increase the hydration of matrix by fragmentation, thereby improving extraction (Toma *et al*., 2001). Physical impact phenomena such as erosion, fragmentation, detexturization, sonoporation and capillarity have been ascribed to be main guiding factors in extraction. Fragmentation imparts rapid access of solvent to target molecule within a minute of sonication. Fragmentation also leads to reduction in particle size and increase in surface area. Cavitation bubble implosion induces erosion of matrix leading to release of component into extraction medium. Sonoporation causes pores in membrane of solid matrix which hastens release of component to solvent. This phenomenon could be reversible or irreversible based on intensity of ultrasonication (Chemat *et al*., 2017).

Additionally, shear forces are said to be involved when liquid with particulate material are subjected to ultrasound treatment. Turbulence in the liquid medium created due to formation, growth and collapse of bubble leads to shear in particulate material. Mixing and emulsification using ultrasound is said to be the resultant effect of streaming and microstreaming (Vilkhu *et al*., 2011). Chemat *et al*. (2004) postulated detexturization of the solid matrix based on the investigation on ultrasound assisted oil extraction using caraway

seeds. They observed that even though conventional and ultrasound assisted extraction reported similar yields, the caraway seeds exhibited physical modifications after ultrasonication. Table 2.7. enlists the reports by various scholars on ultrasound assisted extraction of lipids from various matrices.

2.3.3. Pulsed electric field assisted extraction

PEF, as an unconventional processing technique, has gained importance in the past few years. It has been reported as a process to extract components from solid matrix along with microbial inactivation. Diffusion, pressing, drying and osmosis phenomena using PEF per-treatment on food substrates are few applications listed (Bansal *et al*., 2015). The technique has also been reported as an alternative to other contemporary processing techniques and successfully used for separation, intensification and stabilization (Bozinou *et al*., 2019). The ability of this technique to electroporate membranes led its usage to induce stress in biological matrix to extract target compounds. PEF was used in utilization of food by-products and extraction of valuable compounds from industrial waste (Oroian and Escriche, 2015). PEF treatment was also reported as pre-treatment step before extraction by other traditional techniques.

2.3.3.1. Principle of working PEF-assisted extraction

The underlying mechanism of PEF treatment and its effect on assisted extraction at cellular level is not yet elaborated in reported research works. However, the electromechanical model reported by Zimmerman for cell electroporation is the most acceptable theory (Zimmerman *et al*., 1974). The study compared cell membrane to capacitor of low dielectric constant. Charges of opposite polarity were assumed to be present on both sides to create natural transmembrane potential. The membrane exhibits elastic resistance till certain threshold level. When the electrostatic attraction between membranes exceeds this elastic resistance, pore formation was observed. Geometry and size of cellular particle plays significant role in membrane permeabilisation. For cell size of 40-200 µm, 1-2 kV/cm and for micro-organisms of 1-10 µm size, 12-20 kV/cm of intensity is recommended for effective poration (Heinz *et al*., 2002).

Table 2.7. Ultrasound assisted extraction of lipids from different biological matrices using diverse solvents

Sl. No.	Lipid source	Solvent	Remark	Reference
1	Soybean	hexane, isopropanol and combination	Among different factors, combined solvent extraction with ultrasonication treatment reported highest yield	Li *et al*. (2004)
2	Flax seed	n-Hexane	At 50 W power, 85% of flaxseed yield was reported	Zhang *et al.* (2008b)
3	Grape seed	n-Hexane	Ultrasound treatment at 20 kHz frequency, 150 W power for 30 min. yielded 14% extract which was similar to Soxhlet extraction for 6 h	Da Porto *et al*. (2013)
4	*Chlorella vulgaris*	1-butyl-3-methylimidazolium methylsulfate	Lipid yield was 1.6 times more with assisted ultrasonication than solvent alone	Kim *et al*. (2013)
5	Papaya seed	n-Hexane	Papaya seed oil was light in colour with lower unsaponified matter, higher oxidative stability by ultrasonication compared to Soxhlet and conventional method of extraction	Samaram *et al*. (2014)
6	Pomegranate seed	Hexane	Oil yield was 27.99% for 20 min. exposure against 30.19% and 22.73% by other conventional methods for larger exposure time	Barizão *et al*. (2015)
7	Rice	Hexane, petroleum ether, isopropanol	hexane as solvent reported 77.31% lipid yield by ultrasound assistance	Xu *et al*. (2016)
8	Canola seed	Hexane and 3:2 hexane isopropanol	Extraction efficiency of 22.39% for hexane and 30.66% for hexane–isopropanol solvent was reported	Jalili *et al*. (2018)
9	*Mortierella isabellina*	chloroform:methanol:water (2:1:0.8)	Reported yield of 14.46 wt.% and 19.49 wt.% for ethanol alone and solvent combination (chloroform: methanol: water), respectively	Sallet *et al*. (2019)
10	Favela seeds	Ethanol	Oil yield of 46.9 wt.% reported for 5 min. against 50 wt.% for Soxhlet extraction for few hours	Santos *et al.* (2021)

It was reported that PEF assisted extraction at 100-300 V/cm for batch and 20-80 kV/cm for continuous mode of extraction are the suggested process conditions (Ranjha *et al*., 2021). Target substrate placed between electrodes connected to high voltage electric field is subjected to high voltage pulses for short time to cause hydrophilic pores leading to extraction of target compounds. The mechanism of cell pore formation is detailed in Fig. 2.7. To accomplish extraction of a target compound from matrix, specific heat of 1-10 kJ/kg of energy for nano to milli seconds of time was sufficient (Koubaa *et al*., 2015). Physical properties of the substrate also influenced the extraction process. For example, soft material required lesser electrical field strength of 0.1 to 10 kV/cm whereas, hard material necessitated high intensities of 10 to 60 kV/cm (Sarkis *et al*., 2015). Electrical field strength was proportional to magnitude of damage where high intensity caused irreversible damage and low intensity caused reversible damage (Teh *et al*., 2015).

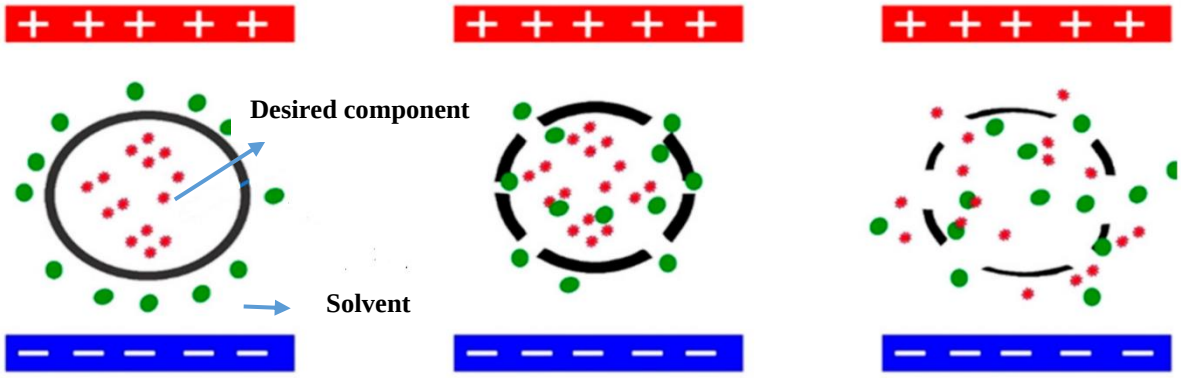


Fig.2.7. Illustration of cell pore formation and extraction of components from cell membrane by PEF treatment

Source: Ranjha *et al.* (2021)

2.3.3.2. Pulsed electric field applicator

Two modes of PEF equipment are reported for assisted extraction applications namely, batch and continuous systems. Three components are considered as main parts of PEF viz., pulse generator, treatment chamber and control cum monitoring system. Pulse generator acts as essential component to generate dedicated pulse wave and to impart the desired field strength on substrate. It is basically designed to generate and deliver high voltage pulses to the treatment chamber containing sample (Sack and Mueller, 2017). Pulse generator combines passive discrete elements such as resistive, capacitive and inductive elements along with transformers and power switches (Elgenedy *et al*., 2017).

The layout of a typical pulse generator is divided into three sections based on working objectives namely, generation of direct current, storage and release. Power generation is achieved by transformers, storage by capacitors and release of power in desired wave form by pulse forming network (PFN). For operation of a conventional PEF system, electricity requirement varies from 20 to 100kV/cm depending on the specific requirement. However, voltage of power supply from utility is reported to be far lesser than requirement therefore, power amplification is achieved by using transformers and rectifiers (Ranjha *et al*., 2021). Control panel in PEF system helps to set required inputs such as frequency, treatment time, voltage and amplitude. Wave form monitors are also attached to PEF unit to extract wave flow with respect to time. In case of continuous system, flow rate of food substrate is monitored through the control panel. Treatment chamber usually comprises two electrodes with specific gap in between to pass food for PEF treatment. One of the electrodes is connected to high voltage generator and other to ground. The strength of electric field generated is based on electric field potential on both sides, which in turn is dependent on type of electrode, distance and sample. Pulse form, treatment chamber configuration and conductivity of product also influence the intensity of PEF treatment (Puértolas *et al*., 2016). Fig.2.8. illustrates the basic components of a batch type PEF unit.

2.3.3.3. Pulsed electric field application for extraction

PEF treatment has been reported as an effective method to improve extraction yield, efficiency and quality of lipids obtained from substrate. It is described as non-thermal process that could accomplish extraction in short span of time by minimizing energy loss for heating of stuff (Yu *et al*., 2015). Formation of pores in substrate matrix when exposed to high voltage pulses is the basic concept behind this enhanced extraction. The increased permeability of matrix owing to the developed pores facilitates diffusion and release of target compound into solvent (Boussetta *et al*., 2014). Table 2.8. enlists the various attempts for PEF assisted extraction of lipids from different biological matrices reported in literature.

2.4 Factors affecting assisted extraction techniques

Assisted extraction techniques are governed by equipment and product related factors and optimization of these two groups of factors is important for obtaining best possible combination to attain the desired maximization or minimization objective. A summary of the commonly considered factors to evaluate the performance of microwave, ultrasonication and PEF assisted extraction from biological matrices is presented in Table 2.9.

Table 2.8. Pulsed electric field assisted extraction of lipids from biological matrices using diverse solvents

Sl.No.	Lipid source	Solvent	Remark	Reference
1	Rapeseed oil	Soaked in water	Hulled rapeseed reported 55% permeabalization and the same was 17% for non-hulled rapeseed	Guderjan *et al*. (2007)
2	Olives	no solvent	Application of PEF to olive paste reported increased oil yield of 13.3% compared to control	Puértolas and de Marañón, (2015)
3	*Salmonella typhimurium*	no solvent	PEF induced treatment modified membrane lipid composition by decreasing ratio of unsaturated fatty acids to saturated fatty acids	Yun *et al.* (2016)
4	Cannabis seeds	no solvent	PEF treatment at voltage intensity of 3 kV/cm was considered as optimal for pre-treatment before pressing	Haji-Moradkhani *et al*. (2019)
5	Chlorella pyrenoidosa	Chloroform/methanol Hexane/ethanol	PEF pre-treatment reported 12.0% higher fatty acid methyl ester compared to ultrasound pre-treatment	Han *et al*. (2019)
6	Pacific white shrimp	Water	PEF pre-treatment followed by ultrasonication resulted highest lipid yield of 30.34 g/100g solids	Gulzar and Benjakul, (2020)
7	Sunflower cake	no solvent	At 7 kV/cm of electrical field strength 2.3% increase in extraction yield was reported compared to non-treated sample	Shorstkii *et al*. (2020)
8	Chlorella	Water	An increase in lipid yield of 166.67% was reported compared to non-treated sample	Zhang *et al*. (2021)
9	Pecan nut	Water	At 0.5 kJ/kg of specific energy input 21.4% oil extraction yield reported compared to non-treated sample	Rábago-Panduro *et al*. (2021)
10	Hoki roe	no solvent	All high PEF inputs resulted in maximum yield (16.2 w/w) of lipids compared to non-treated samples	Ahmmed *et al*. (2022)

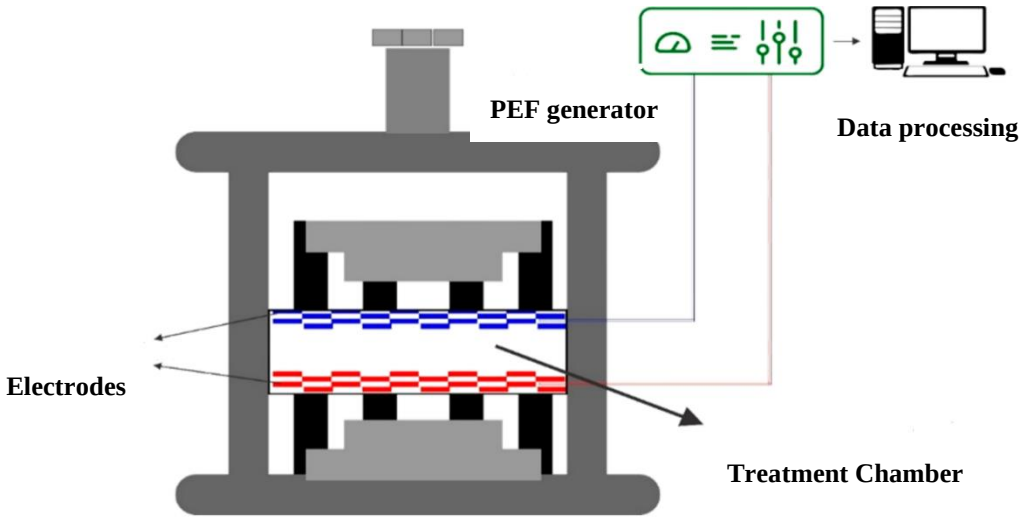


Fig.2.8. Batch type pulsed electric field unit describing components

Source: Ranjha *et al.* (2021)

Table 2.9. Machine factors considered for optimization in assisted extraction studies

Sl. No.	Microwave	Utrasonication	PEF
1	Position of sample	Ultrasound time	Treatment chamber
2	Microwave power	Ultrasound power	Electric field intensity
3	Microwave temperature	Ultrasound frequency	Time, temperature
4	Extraction time	Ultrasound temperature	Pulse type, frequency

*Compiled from (*Kumari *et al.*, 2018*;* Bakhshabadi *et al.*, 2018)

2.4.1. Factors affecting microwave assisted extraction

Extraction of target compounds using microwave assistance depends on many factors as they have different degrees of influence on mass transfer. Microwave for scientific and industrial application operates on a set frequency (915Hz; Osepchuk, 1984). Power applied to substrate can be controlled by the amount of energy imparted to the food material. Time of exposure and temperature are also considered important factors influencing yield of extract. Solvents are of great importance as they influence dielectric constant and dielectric loss factor by governing rate of extraction (Singh *et al*., 2014).

2.4.1.1. Solvent type and solid to solvent ratio

Characteristics such as solubility, dielectric constant, penetration or diffusion capacity play key role in selection of solvent. Depending on component to be extracted, water or organic based solvents can be opted as solvent for extraction (Mandal and Mandal, 2010). Affinity of

solvent towards target compounds and ease of separation of compound from solvent mixture is also considered while selecting solvent. Ethanol, hexane, petroleum ether, methanol, isopropanol and chloroform are some of the solvents used for lipid extraction as reported in Table 2.6. To increase heating efficiency of solvent, modifiers may be added, for example, water added to diethyle ether for extraction of steroids from *Rodgersia aesculifolia* Batal (Casazza *et al*., 2010). Ionic liquids bearing good solvent properties such as thermal stability, miscibility, small vapour pressure and extractability render them suitable as an alternative solvent (Du *et al.,* 2007). After selection of desired solvent, optimal proportion of feed and solvent is an important consideration to maximise the extraction of target compounds from substrate (Zheng *et al*., 2009). Reduced quantity of solvent in extraction process will lead to incomplete extraction whereas, excessive solvent leads to wastage of solvent and energy (Wang *et al*., 2009). Organic solvents pose environmental challenges therefore; there is a focus on minimizing organic solvents by mixing with greener solvents (Chen *et al.,* 2007a).

2.4.1.2. Treatment time

It was observed in many studies that yield of target compound during microwave assisted extraction increased with increase in treatment time.However, increase in time beyond certain level did not exhibit proportional increase in the amount of extract (Wang *et al*., 2008). Long exposure to microwaves with lower boiling point of solvent also reduced yield of compounds owing to loss of chemical structure (Wang *et al*., 2009). As extraction time ranged between few min. to 30 min. across different experiments, excessive thermal exposure resulted in thermal degradation; which could be avoided by replenishing solvent in the form of multiple cycles (Chen *et al*., 2007b). Generally, it was observed that extraction time had positive impact on yield of desired compound (Lucchesi *et al.,* 2007).

2.4.1.3. Temperature

The stability of desired compound at elevated temperature guides the selection of maximum temperature recommended for microwave assisted extraction (Xiao *et al*., 2008). Elevated temperatures are reported to increase yield of compounds due to interaction of solvent and compound at intermolecular level. Solvent characters like decreased viscosity, increased mobility and solubility resulted in improved extraction. Also, build-up of cellular pressure triggers membrane rupture, causing cell contents to ooze out into solvent. However, in a study on extraction of flavonoids at 1000W using microwave, temperature beyond 110^{o}C led to degradation of compound (Xiao *et al*., 2008). In most of the cases, it was observed that extraction increased up to certain temperature. When considered in combination with other

parameters, increase of temperature beyond certain limit had reduced extraction yield in microwave assisted processes.

2.4.1.4. Microwave power

Amount of energy supplied to the sample is controlled by changing the intensity of microwave radiation. Intensity increases the temperature, thereby, rate of extraction of compound. Extraction time can be reduced with increased microwave power to a certain limit, beyond which degradation of compounds were reported. It has been discussed that microwave power influence interaction, analyte separation and equilibrium rate (Ma *et al.,* 2009).

2.4.1.5. Pre-treatment of sample

Surface area of sample will influence extraction efficiency due to increase in area of contact between solvent and matrix. Generally, increased surface area will have positive effect on rate of extraction when substrate is well disbursed in solvent. In order to maintain uniform characteristic of sample, many studies used sample blending and passing through defined sieve size (Kothari and Seshadri, 2010). Apart from surface area, pre-treatment of sample before subjecting for microwave extraction improved yield. Pan *et al*., (2003) pre-treated tea sample in solvent for five min. before subjecting for microwave treatment. Dried samples were sometimes soaked before treatment as it heats moisture available in matrix to develop inner pressure (Wang *et al*., 2006). Optimized factors for extraction of different compounds in biological matrices in microwave treatment are illustrated in Table 2.10.

2.4.2. Factors affecting ultrasound assisted extraction

Ultrasound assisted extraction is influenced by medium parameters and physical or machine parameters. Ultrasound characteristics such as intensity, frequency, time and amplitude are considered as machine parameters whereas, solvent, temperature, matrix morphology are considered as medium/process parameters. Medium parameters are also called as intrinsic characteristics which influences extraction of compound from matrix. Few machine parameters are fixed whereas; few can be varied to different levels. Based on mathematical relationships, physical parameters or machine parameters can be converted into different forms.

Table 2.10. Optimized parameters reported for microwave assisted extraction of different compounds from biological matrices

Sl. No.	Matrix	Desired compound	Optimal parameters				Results	Reference
			Power	Time	Temp.	S:S ratio		
1	Peanut hull	Soluble dietary fibre	700W	6 min.	90^{o}C	1:16 w/w	Yield of 2.68% soluble dietary fibre reported	Yu *et al*. (2011)
2	Potato peel	Phenolic compounds	14.67%	15 min.	-	67.33% v/v	Total phenolic compound of 3.94 mg/g reported	Singh *et al*. (2011)
3	Potato	Total phenolic content	300W	2 min.	80^{o}C	-	Reported better yield than conventional method	Wu *et al*. (2012)
4	Tomato	Total phenolic content	100W	5 min.	96.5^{o}C	66.2% v/v	489.30–997.45 mg GAE/100 g dry weight was reported	Li *et al*. (2012)
5	Orange peel	Pectin	422W	169 s	-	1:16.9 g/mL	Reported yield of 19.24% at optimal levels	Maran *et al*. (2013)
6	Cotton seed	Oil	900W	3.57min.	-	1:4 w/w	Extraction efficiency of 32.6% was reported	Taghvaei *et al*. (2014)
7	Pumpkin seeds	Oil	419W	66 min.	44^{o}C	-	Linoleic acid of 57.33% reported against 53.72% by soxhlet extraction	Jiao *et al*. (2014)
8	Neem seed	Oil	1000W	24 min.	80^{o}C	1:3 w/w	Extracted 80% oil from seed	Nde*et al*. (2015)
9	Tomato	Lycopene	400W	1 min.	-	-	13.59 mg/100g of wt. was reported	Ho *et al*. (2015)
10	Pomegranate seed	Oil	220W	5 min.	-	1:10 w/w	Extraction yield was 35.19%	Keskin Çavdar *et al*. (2017)
11	Tiger nut	Oil	420W	55 min.	72^{o}C	7.0 mL/g	Oil yield of 24.12% reported	Hu *et al*. (2018)
12	Carob bark	Phenolic compound	-	29.5 min.	80^{o}C	35 mL/g	Yield of 66.5% was reported	Quiles-Carrillo *et al*. (2019)
13	Avocado seeds	Bioactive compounds	-	19.01 min.	72.18^{o}C	-	The extract exhibited highest antioxidant activity	Araújo *et al*. (2020)

W-watts; s-second; Temp.-Temperature; S:S-Solid to solvent ratio; v/v-volume by volume; w/w-weight by weight; min.-minute; GAE-Gallic acid equivalent

2.4.2.1. Ultrasound power and frequency

Actual acoustic power applied during ultrasonication process was indirectly determined using physical methods. The amount of applied energy was measured by gauging physical and chemical changes in matrix or medium. Methods like calorimetric method, optical microscopy and aluminium foil methods were reported (Chivate and Pandit, 1995; Margulis and Margulis *et al*., 2003; Martin and Law *et al*., 1983). Ultrasound power at higher levels was reported to induce shear force, thereby resulting in increased extraction yield. To increase yield and composition of extract by ultrasonication, higher ultrasound power, lower moisture and optimal temperature was preferred. Contrarily, few studies indicated that selective extraction of target molecules was influenced by ultrasound power (Chemat *et al*., 2004).

Commonly used frequency for ultrasound assisted extraction ranges between 20 and 100 kHz. Very limited studies are reported on the influence of frequency on extraction yield. Ultrasonication at higher frequencies resulted in reduced physical impact when compared to low frequency treatment (Toma *et al.*, 2001). Selective extraction of compounds at different frequency from peanuts was reported with longer exposure time (Chukwumah *et al*., 2009). Higher frequency treatment also inversely impacted cavitation due to the time lag required between compression and rarefaction (Mason *et al*., 2002). Length of rarefaction was inversely proportional to frequency, demanding larger amplitude and intensity.

2.4.2.2. Intensity of ultrasound energy

Intensity is described as a measure of energy transmitted per unit time per unit surface area (Tiwari, 2015). Increase in ultrasound intensity was linked to improved sonication effect and reduced transducer efficiency leading to agitation instead of cavitation. Li *et al*. (2004) experimented with different intensities (16.4, 20.9 and 47.6 W/cm^2) at 20 kHz frequency using hexane and isopropanol as solvents. The study reflected that between 16.4 and 20.9 W/cm^2, there was an increase in yield of soybean oil and reduced when intensity increased from 20.9 to 47.6 W/cm^2. However, Wang *et al*. (2015) reported that the influence of ultrasound intensity on increasing yield of pectin was irrelevant.

2.4.2.3. Shape and size of ultrasonication unit

Keeping in view the reflection and attenuation of ultrasound waves, a flat based vessel with minimal thickness is preferred as the treatment unit (Kadam *et al*., 2015a). In case of fixed emitter, it is recommended that the position of emitter be chosen such that it should transmit waves uniformly throughout matrix (Sun *et al*., 2011). Ultrasound vessel base and emitter tip

should be placed at minimal distance to ensure no physical contact. Shape of probe chosen for ultrasonication also influences the distribution pattern of ultrasound waves. The exponential probe with small tip diameter is used in micro-applications whereas, stepped probe is used for macro-applications (Chemat *et al*., 2017).

2.4.2.4. Temperature and type of solvent

Viscosity, surface tension and vapour pressure are interlinked with solvent and its temperature. A decrease in surface tension, viscosity and increase in vapour pressure of solvent has been reported with increase in temperature of extraction, (Hemwimol *et al*., 2006). At a higher vapour pressure, solvent vapour intrudes into cavity of bubble potentially leading to violent collapse. It is recommended that the temperature of solvent should not exceed boiling point as it reduces solvent solubility and diffusivity. Increase in temperature is also associated with improved yield mainly due to increase in bubble formation and surface area. In any ideal solvent opted, breaking of cohesive force between molecules is required to induce cavitation. Viscosity of solvent can be overcome by using high intensity ultrasound to induce vibration in the medium (Kadam *et al*., 2015b). Lower vapour pressure solventsare opted over high vapour pressure as bubble collapse was predominant (Flannigan and Suslick, 2010).

2.4.2.5. Substrate matrix

The type of raw material used for ultrasoinication reported in literature varied from dry to pre-treated samples. The force that binds the target compound in the matrix and the ability of the solvent to infringe into the matrix structure influences extract yield. Solid to solvent ratio, particulate size, porosity and surface structure of matrix also determines the rate of extraction. Plasticity of material, material structure, composition were reported as factors affecting cavitation (Vilkhu *et al*., 2011). Table 2.11 summarises the different factors optimized for extraction of compounds from varied matrix using ultrasonication.

Table 2.11. Optimized parameters reported for ultrasound assisted extraction of different compounds from biological matrices

Sl. No.	Matrix	Desired compound	Optimal parameters	Results	Reference
1	Grape seed	Total phenolic compounds Antioxidant activity Anthocyanin	S-53.15%; T-56.03^{o}C; t-29.03 min. S-53.06%; T-60.65^{o}C; t-30.58 min. S-52.35%; T-55.13^{o}C; t-29.49 min.	5.44 mg GAE/100 mL 12.31 mg/mL 2.28 mg/mL	Ghafoor *et al*. (2009)
2	Mulberry	Anthocyanin	S-63.8%; T-43.2^{o}C; L:S-23.8 v/w; t-40 min.	64.7mg/g of anthocyanin yield at optimal condition	Zou *et al*. (2011)
3	Marjoram	Antioxidants	a-61µm; T-35^{o}C; t-15 min.	Improved total phenol content and individual phenols	Hossain *et al*. (2012)
4	Perilla seed meal	Protein	T-40^{o}C; P-61W; t-12 min.; L:S-40 mL/g	10.77% of protein yield at optimal conditions	Zhu and Fu, (2012)
5	Chinese star anise	Shikimic acid	P-480W; L:S-15mL/g; t-16 min.	Shikimic acid was 1.367% by conventional method against 2.75% in microwave	Cai *et al*. (2014)
6	Pomegranate peel	Pectin	S:L-1:17.52 g/mL; pH-1.27; T-61.9^{o}C	Optimal yield and predicted values were very close	Moorthy *et al*. (2015)
7	*Bougainvillea glabra* flower	Pigment	T-55^{o}C; P-88 W; t-37min.; S:L-1:17g/mL	Betacyanin of 1.72 and Betaxanthin of 5.78 mg/g was reported	Maran *et al*. (2015)
8	Pomegranate peel	Phenolic	S-59%; T-80^{o}C; t-25min.; S:S-1:44	190.94 TPC (mg GAE/g dry wt.,) was reported as highest	Živković, *et al*. (2018)
9	*Moringa peregrina*	Oil	T-30^{o}C; t-26.3min.; L:S-17.8mL/g; P-348W	53.10% of oil yield at optimal condition	Mohammadpour *et al*. (2019)
10	Dragon fruit peel	Phyto compounds	S-60%; T-60^{o}C; S:S^{1}-25:1mL/g; t-20 min.	Mass transfer co-efficient was maximum at optimized levels	Raj and Dash, (2020)

T- temperature; S-solvent concentration; a-amplitude; t-time; S:S-solid to solvent ratio; S:S^{1}-Solvent to solid ratio; S:L solid to liquid ratio; L:S Liquid to solid ratio; P-Power; TPC-Total Phenolic Compound; GAE-Gallic Acid Equivalent P-Power; v/w- volume by weight;

2.4.3. Factors affecting pulse delectric field assisted extraction

Electroporation is considered as the driving factor for enhancing rate of extraction due to pulsed electric field treatment. Extraction of the desired compound from biological matrix is possible through influence of solvent and high intensity electric field (Amiali *et al*., 2007). It is postulated that as high voltage is passed between two electrodes, ions present in the biological matrix will induce certain degree of electrical conductivity. The biological matrix experiences force per unit charge leading to electroporation (Puértolas *et al.*, 2013; Roselló-Soto *et al*., 2015). Process parameters, medium parameters and characteristics of target compounds are considered as main factors influencing the efficacy of pulsed electric field treatment (Niu *et al*., 2020). Factors such as field strength, shape of pulse, number of pulse, length of pulse, treatment time are major process parameter considered in PEF assisted extraction. As the food matrix is complex and heterogeneous in nature, electrical conductivity and pH of food is also reported to influence the efficiency of extraction.

2.4.3.1 Electric field intensity

The intensity of the electrical field is considered as critical factor due to its influence on distribution of power throughout the substrate matrix. Electric field should be evenly distributed across matrix to achieve complete extraction of compounds. Type of matrix and bonding force of desired component inside matrix may alter amount of field intensity required by the sample. Field intensity ranges between 10 to 45 kV/cm for different objectives like microbial inactivation, compound extraction and pre-treatment process (Michalac *et al*., 2003). For extraction of betalains from beetroot Nowacka *et al*., (2019) evaluated PEF pre-treatment at different field strength, pulse number and energy input. The outcome of the results showed that effective extraction was possible even at low electrical field intensity of 4.34 kV/cm.

2.4.3.2. Type of waveforms

Opting suitable wave form to match the substrate matrix was paramount in effective application of PEF for extraction. The biological matrix responds differently to varied kinds of wave forms with respect to the degree of disruption and electroporation. To induce electroplasmolysis in fruits using PEF, monopolar and bipolar waves have been attempted (Ngadi *et al*., 2003). Square pulse and exponential decay pulse have also been widely used in PEF treatment (Barbosa-Canovas *et al*., 2000). Gulyi *et al*. (1994) reported 1-2% purity in juice (ratio of sugar to dry solids in juice expressed in percentage) with bipolar waves compared to 3-5% using mono polar waves. Knoor *et al*. (1994) reported square waves as

more efficient than exponential or oscillatory waves. The advantage of square wave was attributed to high rise time of square wave compared to exponential wave.

2.4.3.3. Treatment time and pulse width

In order to avoid excessive heating and electrolytic reactions during PEF treatment, short pulses are usually applied. The duration of treatment is computed as the product of pulse width and number of pulse applied. Electrodeposits and electrolytic reactions were considered to have detrimental effect of PEF due to application of long pulses (Zhang *et al*., 1995). Repeated pulse application increases effectiveness of treatment by inducing stress, reorienting flaws in electrical field. Also, repeated pulse helps in synergic effect with matrix components to cause disruption of cell.

2.4.3.4. Design of treatment chamber

As per the process requirement and need of sample, many designs are reported for treatment chamber with associated merits and limitations. Batch type treatment chamber requires long processing times due to loading and unloading of samples whereas, it is avoided in continuous process. Some of the design precautions to be taken while opting for good design of chamber are intrinsic electrical resistance, homogeneity of electric field and reduction or generation of field area. Consumption of electric power inside treatment chamber depends on many factors like, electrical resistance of product, resistance of conduction line and design of chamber. Typical value of energy consumed was reported as 100 A to 10 kA.

2.4.3.5. Frequency of pulse

Frequency of pulse has both positive and negative influence on assisted extraction by PEF treatment. Much importance was not given to this factor in earlier studies for optimization (Vorobiev and Lebovka, 2009). Studies conducted on lipid vesicles and animal membrane concluded that low frequency caused more damage than high frequency. This was because of more time available for charging the cell membrane between pulses leading more pore-formation (Loghavi *et al*., 2008). In another study, Vernhes *et al*. (1999) explored work of testing cell pulse frequency on viability of animal tissues. When frequency was increased from 0.5 to 10 Hz, the viability of cell also increased.

2.4.3.6. Substrate matrix characteristics

Electrical conductivity, pH, dielectric property and composition of biological matrix play influential role in extraction efficiency. The effect of these factors on extraction may either be due to mutual interaction or in isolation or singular effect (Tedjo *et al*., 2022). Low ionic

strength in the matrix is desirable to cause compression and electroporation while pH influence contents of cell. Electric field conductivity of the matrix is important to pass energy within the system (Parniakov *et al*., 2014). Table 2.12 enlists the influence of factors associated with PEF treatment during extraction of different compounds from a biological matrix.

2.5. Optimization and model fitting techniques

Appropriate design of experiments (DoE) and application of suitable optimization tools play an important role in obtaining statistically proven results in any experiment. Many statistical tools are available to optimize process parameters with associated advantages and limitations. Meticulously executed DoE facilitate in planning, experimenting, analysing and data interpretation. The main objective of selecting a suitable DoE is to correctly investigate effect of input variable (independent) on the output (dependent) variable. For carrying out any experimental study, the following steps are generally adopted (Krishniah and Shahabudeen, 2012). Statement of problem →Factor and level selection→ Response variable selection→ Selection of DoE→ Conduct of experiment→ Analysis of data→ Drawing interpretations and conclusions. Some of the commonly used designs adopted by different experiments are listed in Table 2.13 (Guo and Mettas, 2010).

2.5.1. Taguchi method for optimization studies

Variability in experimental data is cause of concern in most of optimization studies, which could alter the outcome significantly. Dr. Genichi Taguchi proposed a robust design called Taguchi design to minimize this variability in experimental outcome. Under variability terms, they are divided into controllable (determined by manufacturer and not influenced by user) and non-controllable variables (according to usage of consumer and environment). Non-controllable factors or noise factors are further categorised into external, internal and product specific factors. As per the Taguchi approach, quality of product is important and any loss in its originality is considered as loss. Three types of targets are adopted to establish quality characteristics namely, nominal the best, smaller the best and larger the best. Fig.2.8. illustrate steps followed in optimization of process parameters using Taguchi design.

Table 2.12. Optimized parameters reported for pulsed electric field assisted extraction for different compounds from biological matrices

Sl. No.	Matrix	Desired compound	Optimal parameters	Results	Reference
1	Emblica juice	Quercetin and ellagic acid	V-22 kV/cm; t-500µs	Cell disintegration of 0.79 was reported at optimal level	Bansal *et al*. (2014)
2	Blueberry by-product	Anthocyanin	S-60%; L:L-1:6; N-10; V-20kV/cm	Reported 223.13mg/L of blueberry by-product at optimal condition	Zhou *et al*. (2015)
3	Red prickly pear	Colorants	V-20 kV/cm; N-50	PEF pre-treatment reported 75mg/100g of fresh material	Koubaa *et al*. (2016)
4	Patchouli	Oil	V-2000; F-1874; t-8h	8mL of oil was extracted from 300 g of dry material at optimal condition	Irawan and Argo, (2017)
5	Blackcurrant	Bioactive compounds	V-1318V/cm; N-315	Total phenolic compound (19%), 45% antioxidant activity was reported at optimal condition	Gagneten *et al*. (2019)
6	Potato peel	Polyphenols	S-52%; t-230min.; T-50^{o}C	Extract from PEF resulted in 10% increase in total phenolic compound	Frontuto *et al*. (2019)
7	Cinnamon	Functional compounds	V-5.12 kV/cm; N-40	Extract yield was 5.06% with DPPH of 91.7% at optimal condition	Pashazadeh *et al*. (2020)
8	Carrot	Sugar	V-750V/cm; N-10; T-45^{o}C	Significant increase in sugar was observed due to permeability of cells	Dastangoo *et al*. (2020)
9	Brewers' spent grain	Bioactive compounds	V-2.5kV/cm; F-50Hz; t-14.5 s	Reported 2.7 and 1.7 times more free and bound phenolic compounds, respectively	Martín-García *et al*. (2020)
10	Chubak root	Natural saponins	V-6.4 kV/cm; N-80	Improved extraction of saponins with emulsifying properties	Khosrow *et al*. (2021)

V-Voltage intensity; N-Pulse number; T-Temperature; t-time; F-frequency; L:L-Liquid to liquid ratio; S-Solvent concentration; h-Hours; s-Seconds; kV/cm-Kilo volts per centimetre

Table 2.13. Summary of types of experimental designs and its utility

Sl. No.	Design type	Utility
1	Comparison	t-test, Z-test and F-test to compare one factor among multiple factors
2	Screening of variable	Two level factor design intended to select most influencing parameters among many in process, product or system
3	Transfer function identification	Relationship between input and output variables were explored to further explain system, product and process
4	Optimization	Based on input interactions on output, transfer functions are used to improve performance of system or product
5	Robust design	By including cause of variation in design, process optimization was carried

Source: Guo and Mettas, (2010)

2.5.2. Mathematical models and evaluation of goodness of fit

Many mechanisms are associated with extraction of compounds from a substrate matrix which contribute to the yield of desired compound. Mechanistic and empirical models are two approaches used to describe the kinetics of extraction. The mathematical relation between the quantities is often evaluated using empirical models fit to the experimental data. Empirical models facilitate determination of parameter values and identification of best model among those considered. They also play pivotal role in mapping of input values without showing explicit description of process (Radosavljevic *et al*., 2018).

Experimental data are compared with defined models to understand and explain how experimental outcome behaved with time. To establish a suitable model which adequately describes the experimental data in kinetic study, the coefficient of determination (R^2 value) are calculated and compared. It is defined as ratio of explained variation to total variation expressed based on magnitude of values. Closeness of value to unity is considered as practical validity of model to predict experimental values (Gharibzahedi *et al*., 2013).

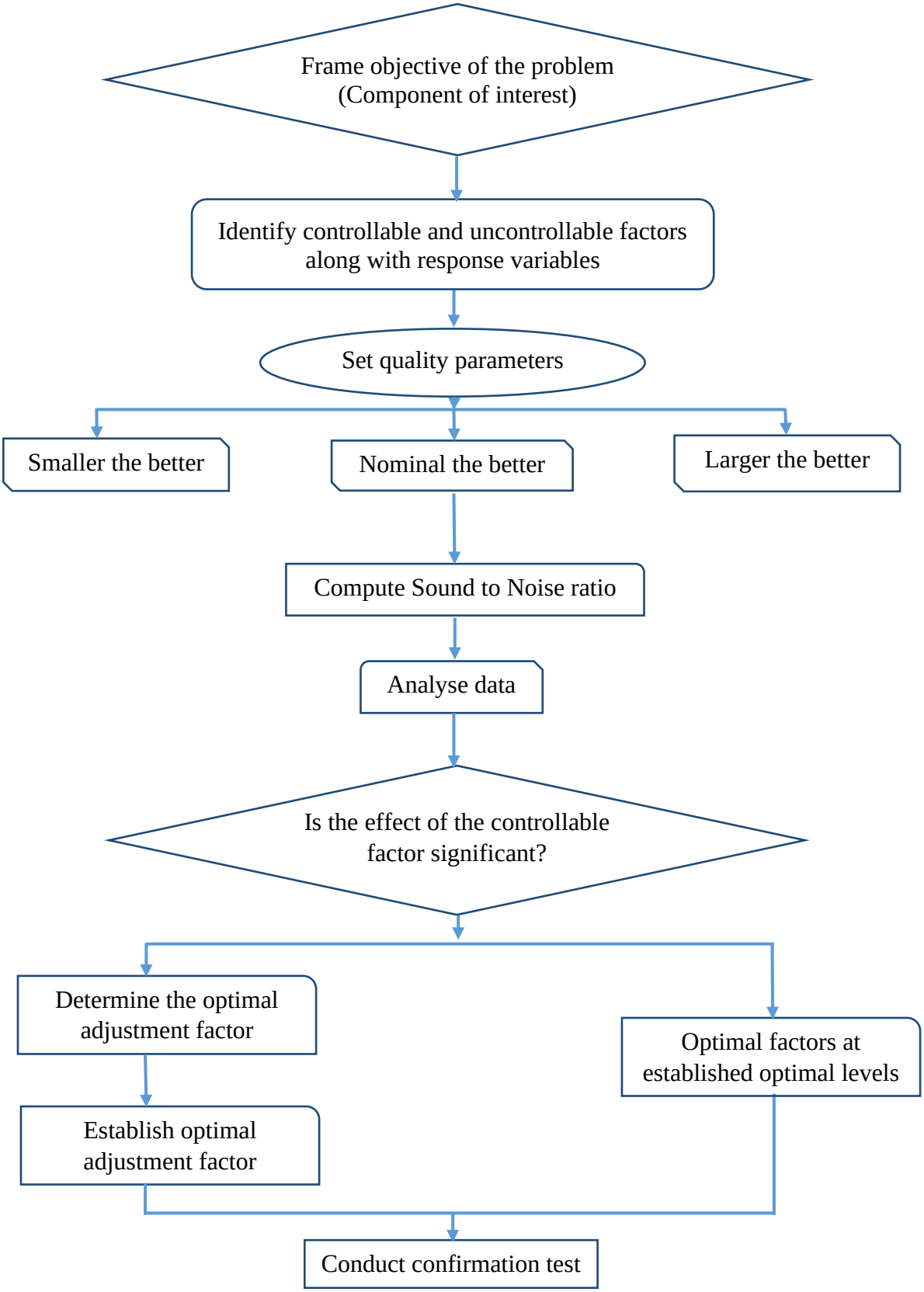


Fig. 2.9. Flow chart for Taguchi optimization technique adopted for optimization problems

Source: Asghar *et al.* (2014)

2.5.2.1. Fitting of kinetic models

To fit kinetics of extraction in experimental data, non-linear regression has been employed by different researchers using different software's. Usually, the liquid phase concentration of desired component as a function of time is generated to compute the extraction rate. Physical laws applied to extraction process are successfully evaluated using solver tool available in Microsoft Excel. This is considered to be most extensively used tool for comparing many models. However, the data fitting software Origin Pro (Originlab Corp, USA) is also considered to be versatile tool for evaluation of empirical model. The software is reported to be handy in areas like graphing, peak analysis, data exploration, signal processing, data processing and compatible to other software's. Minimum sum of square and maximum R^2 and adj. R^2 values are considered desirable for identifying the model with the best fit to experimental data. To collectively evaluate kinetics of extraction, regression analysis is used with the objective of establishing the relation between variables. It also facilitates the analysis of the impact of one or many variables on each other. Linear, semi linear or non-linear equations are often chosen to know the relation of variables in experimental data (Graybill and Iyer, 1994).

If data follows a linear trend, obtaining parameters of the equation may be simple by using smallest squares method. Otherwise, extra caution might be required to select appropriate model with suitable parameters to fit experimental data. Different quantitative indicators are available to evaluate or determine conformity of model with experimental data (Cichosz, 2014). By calculating minimum squares of the distance of experimental points from points of model, models are evaluated on the basis of "lower the better".

2.6. Kinetics of extraction and empirical models

Prediction of extraction behavior enables quantifying the efficacy of extraction process, which in turn would aid in scaling up of the process. Also, parameters of a process such as rate constant are often used to compare process performance of different experimental treatments and the control. Basically, any solvent extraction comprises of two steps, namely, the washing stage (fast extraction) and diffusion stage (slow extraction). The process of extraction is initiated when the solvent penetrate into solid matrix, causing constituents to be in physical contact with the solvent. Washing and diffusion steps also depend on pre-processes such as sample preparation (like grinding and soaking in solvent) that enhance sample surface area and improve solvent penetration. Some of the key influencers impacting the extraction process include the type of solvent, solvent to feed ratio and sample particle

size. Obviously, temperature plays a significant role in extraction under both conventional and non-conventional techniques.

Rakotondramasy-Rabesiaka*et al.* (2007) studied solid–liquid extraction of protopine from *Fumaria officinalis* and used second-order rate law to understand the extraction kinetics. The study demonstrated that the extraction rate was faster in the beginning and progressively slowed down. The authors proposed that the extraction happened in two stages, viz. solvent induced dissolution and external diffusion of protopine into extract. The initial extraction rate (h), extraction capacity (C_s) and the second-order rate constant (k) increased with temperature for studies conducted with two solvents.

Kitanovic *et al.* (2008) used six empirical kinetic models to describe the extraction kinetics of resinoid from aerial parts of St. John's wort. Parabolic diffusion model, power law equation, hyperbolic equation, an exponential Weibull type equation and logarithmic Elovich type models were considered. This study also discussed the fast washing action in the beginning, followed by slow diffusion-controlled extraction in the succeeding extraction time. All models were found to be suitable for describing the extraction of resinoid in water-ethanol solvent. Statistical analysis using root mean square (RMS) and linear correlation coefficient were employed to rank the model. RMS decreased and the linear correlation coefficient increased in the following order: Parabolic Diffusion model->Power Law model>Hyperbolic model>Weibull's model>Elovich's model.

Second-order law was adopted to understand solid-liquid extraction process in ultrasound-assisted extraction of pomegranate seed oil (Goula, 2013). The extraction capacity and extraction rate constant were calculated from the linearized equation of the model. Based on the experimental results, it was observed that kinetic parameters decreased with increase of particle size in the substrate. The optimal extraction was obtained at 20^{o}C temperature, solvent-seed ratio of 20:1 and pulse duration-pulse interval ratio of 5:15. At these optimal experimental conditions, the extraction yield reported was 60%.

Optimization of process condition using the kinetics of extracting for antioxidants from forestry biomass was reported (Piwowarska and Gonza´lez-Alvarez, 2012). Methanol-water solution was used as solvent in the solid liquid ratio of 1:10 (w/v) for all experiments. Elovich's, Peleg's and Page's equations were applied to model the experimental data. Peleg's model with highest R^2 (0.901-0.971) value showed better goodness of fit to the experimental data irrespective of extraction temperature. To understand the effect of process variables

(temperature, time and methanol concentration) on extraction kinetics, response surface methodology enabled evaluation was performed. Initial extraction rate, maximum total phenols content and antioxidant capacity were studied as the responses in the study.

Cheung *et al*.(2013) used parabolic diffusion, power law, Weibull's exponential, Elovich's logarithmic, unsteady diffusion equation and Peleg's hyperbolic models to describe the solid–liquid extraction of polysaccharides and water-soluble components from medicinal fungi. Five fungal species were studied under ultrasound assisted extraction. For two species, the power, Weibull and Elovich's models were close to experimental data with R^2 value 0.964-0.984. The hyperbolic model reportedlow R^2 value, indicating its non-suitability for ultrasound-assisted extraction. However, for the remaining three species of fungal mycelia (all polysaccharides) the parabolic model fitted best to the experimental data with R^2 value of nearly 0.98.

Parabolic diffusion, power law, hyperbolic, Elovich's and pseudo second order models were applied to investigate extraction kinetics of *Colocynthis vulgaris Shrad*seeds oil (Agu *et al.,* 2018). Oil extraction was carried using hexane in a Soxhlet extractor stabilized at different temperatures using a thermostatic bath. The models were evaluated using statistical parameters such as coefficient of determination (R^2), RMS, standard error of estimation (SEE), average relative error (ARE), sum of the squares of the error (SSE), hybrid fractional error function (HYBRID), Marquardt's percent standard deviation (MPSED) and standard deviation (SD). Other than the power model, remaining four models gave a good fit to the experimental data. The predicted oil yield in all models was very close to the yield obtained experimentally. Hyperbolic and pseudo second order model exhibited least difference between model and experimental values.

Kaderides *et al.* (2019) studied optimization and extraction kinetics of microwave assisted extraction of phenolics from pomegranate peel. Aqueous ethanol (50%), solvent/solid ratio (60mL/g) and power at 600 W were found to be optimal combination of process parameters for microwave assisted extraction. At optimal condition of microwave treatment, the yield was 1.7 times higher than ultrasonication pre-treatment. To understand the extraction kinetics, three mathematical models were used, namely, the Peleg's model, the first-order kinetic model and the second-order kinetic model. Among the models studied, the Peleg's model fitted best to experimental values with R^2 value of 0.924-0.991and lowest standard error of 0.889–1.322. The two step kinetic parameters of the model included two specific rate

constants to quantify the process rate and yield. The study also reported that the predicted values of yield fell in the region where 95% of experimental data points were observed.

Extraction of lipids from post-hydrolyzed spent coffee grounds (PHSCG), spent coffee grounds (SPG) for biodiesel production using hexane as solvent was studied (Go *et al.*, 2020). The experiments were conducted at solvent to solid ratio of 4-20 mL/g and temperature 30-60^{o}C. Theoretical and semi-empirical models applied in the study included modified Fick's law, Patricelli model, So and Macdonald, modified Peleg and Linares model. Results indicated that for SCG and PHSCG, Patricelli and Linares model adequately described kinetics of lipids extraction.

Rakshit *et al.* (2020) used ultrasound assistance to extract punicalagin from pomegranate peel and modelled the process kinetics. Ultrasonication parameters like amplitudeduty cycle well and thesolvent volume were studied at varied levels. The experimental data were compared among Peleg's model, mass transfer model, first order kinetics and power law model. Amongthe models studied, first order kinetics was best in explaining experimental values with R^2 value of 0.940-0.985.The mass transfer model reported to be least adoptable with lack good fit to experimental values. Peleg's model showed R^2>0.9 and its fit was good for solvent volume range 20-30 mL, amplitude 30-60% and duty cycle 60-90%.

Six mathematical models (parabolic diffusion, power law, Weibull's equation, Elovich's equation, second order rate, two-site kinetic models) were employed to investigate kinetics of conventional and ultrasound assisted extraction of polyphenols from fresh and distilled grape marc (Natolino and Da Porto, 2020). The ultrasound power was fixed at 200W and extraction was carried for 1, 5, 10, 15, 20, and 30 min. Kinetic behavior between conventional and US assisted extraction showed different trends at initial stage of the process. Two-site kinetic model best described the extraction kinetics of polyphenols of grape for both conventional and US assisted extraction. Kinetic co-efficient of washing and diffusion stages of extraction in two-site kinetic model stood high for US assisted extraction than conventional extraction.

Brewer's spent grain was chosen as raw material to extract polyphenol compounds using ultrasound assistance with water as solvent by Alonso-Riaño *et al*. (2020). Power law and Weibull models were chosen to establish solvent extraction kinetics of phenolic compounds. The yield of polyphenolic compounds was expressed in mg of GAE per gram of dry brewers spent grain. The study used Marquardt algorithm to estimate kinetic parameters. Models were

compared with experimental results using root mean square deviation (RMSD). Temperature, solvent, solid-solvent ratio and kind of extraction were used as parameters to evaluate kinetics. Results indicated Weibull and power law model as best fit to experimental results with RMSD values 7.45 to 7.5, respectively. Weibull extraction rate constant (k) was reported high for all considered parameters indicating it as better model.

2.7 Characterization of emulsion property

An emulsion system contains two immiscible liquids which are dispersed in each other. They were considered thermodynamically unstable due to high surface energy (Tesch *et al*., 2002). Generally, emulsions are formed in the presence of a surface active agents or stabilizer which includes chemical surfactants or solid particles. Two basic forms of emulsion are:

a. Oil in water emulsion where oil droplets are dispersed or encapsulated within water column
b. Water-in-oil emulsion in which water dispersed within the oil phase

Emulsifying agents or emulsifiers are included in formulation to keep oil and aqua phases in dispersed condition. Selection of emulsifiers depends on emulsion preparation methodology, phase properties and final product. An ideal emulsifier must assist in forming stable emulsion by reducing surface tension at oil-water interface. Emulsifiers act through hydrophilic head attracted to aqueous phase and lipophilic tail attached to oil phase (Hasenhuettl, 2008). Hence, theoretically, dairy PLs are capable of exhibiting good emulsion behaviour owing to its amphiphilic nature. However, the claim needs to be substantiated by testing its efficacy as an emulsifier.

An emulsion can be kept in stable condition if surfactant or solid particles obey favouring conditions such as wettability, surface charge, particle dimension and continuous-dispersed phase influence (Low *et al*., 2020). To evaluate quality of an emulsion, certain procedures or methodologies are laid down in literature. Some of the evaluation methods for emulsion study are discussed below.

2.7.1. Distribution and size of emulsion particles

Potential demulsification of a well dispersed emulsion is dependent on droplet size and its distribution in emulsion. In case of oil in water emulsion, viscous energies contribute by inertial force as dispersed phase has more viscosity than continuous phase. Many analytical instrument methods have been adopted to estimate size and distribution of emulsion droplets in an emulsion. As the emulsion was bound to have different range particles distributed in

continuous phase,it is imperative that the equipment should be able to precisely measure size and distribution in an emulsion.

2.7.1.1 Microscopic observation

Optical microscopy has been used to identify particle size of an emulsion. The method uses visible light rays to obtain or capture images of desired magnitude (10-1000 times). Imaging software was utilized by many researchers to analyse average droplet size and its distribution. Ferreira *et al.* (2010) had used optical microscope of 400x to analyse droplet size in HLB studies of andiroba oil. Similarly, optical microscope of 40x was used by Meher *et al.* (2013) to evaluate particle size distribution in citronella oil. Electronic microscopy and atomic force microscopy were also used for particle size characterization in many studies. However, they require highly sophisticated sample preparation and instrument handling skills. They also find application in generation of information on surface morphology and particle interfacial properties.

2.7.1.2. Laser diffraction measurement

This technique was used to estimate droplet size distribution by measuring change in intensity of light due to angular variation when it was passed through emulsion (ISO 13320, 2009).It is known that change in droplet size of emulsion will directly affect angle of diffraction (smaller sized emulsion leads to higher diffraction angle) (Syvitski, 1991). The pattern of diffraction is mathematically analysed to calculate mean diameter by surface weight ($d_{3,2}=\sum n_i d_i^3/\sum n_i d_i^2$) or volume weight ($d_{4,3}=\sum n_i d_i^4/\sum n_i d_i^3$)(where n_i is the number of emulsion droplets with the diameter d_i). Pickering emulsionis often analysed with $d_{4,3}$ methods to describe average diameter (Low *et al*., 2020).

2.7.1.3. Mathematical characterization methods

It is an indirect approach wherein characterization of emulsion is carried by using mathematical methods. Emulsification parameters are assessed through correlation with change in droplet size. This method is having an advantage of analysing emulsion in deep sense which is lacking in experimental methods. Semi-empirical approach by using surface-weighted mean diameter was evaluated in Pickering emulsion (Tsabet and Fradette, 2015). The steps followed in evaluation of droplet mean size were as follows: Calculate interface generation and coverage potential→ Deduct theoretically covered interface→ Stabilization of efficiencies → Calculate effectively covered surface→ Deduct mean droplet size. This method was ratified by using laser diffraction technique and concluded with slight variation. The accounted variation was adjusted by moderating parameters co-efficient. Golemanov *et*

al. (2006) explained that solubility of particle in continuous or dispersed phase determines two types of emulsion. If the emulsifier was more soluble in stable solution of continuous phase it is called Bancroft emulsion, otherwise it is designated as anti-Bancroft emulsion. Following equation was derived to describe variation of stable droplet size for anti-Bancroft emulsion

$$d_s \approx 8r_p\Phi_a\rho_{pd}/\Phi_p \text{----------} 2.1$$

Where ρ_{pd}- Ratio of density of particle and dispersed phase

Φ_a - Mass fraction of area covered by particles

Φ_p- Mass fraction of solid particles in the drop of a specific radius

r_p - Radius of a particle

2.7.2. Morphology and interfacial properties

Surface morphology of emulsion undergoes series of changes from synthesis, development to destabilization. Visualization of changes acts as an effective tool to characterise the emulsion. Human eyes without any aid can gauge object size up to 100µm (Aguilera and Stanley, 1999) and beyond it would be difficult to visualize. However, structural components of emulsion are far lower than the threshold limit of human eye. Microscopic techniques such as optical, electron, confocal and atomic force microscopy are used to visualize these features (Egerton, 2008).

2.7.2.1. Electron microscopy

This technique was used to examine nano-emulsion, nano-particles and Pickering emulsions. The technique uses electron beam of wavelength smaller than light to examine material (McClements, 2004). Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are reported to examine the emulsion sample. SEM requires fixation and dehydration of sample to make it suitable for analysis. However, Pickering emulsions can be polymerized to maintain sample integrity (Xu *et al*., 2007). Interestingly, cryogenic SEM was used to characterise interfacial thickness and other properties in-situ. Following steps were reported to know morphology of specimen (Xiao and Huang, 2016): Rapid freeze of emulsion by liquid nitrogen→ Freeze fracture by cold knife→ Controlled sublimation to remove water or oil→ Coat with thin layer of gold to visualise emulsion. This method not only visualize interfacial layer but also reveal internal structure and particle orientation. TEM technique sends electron bean directly into sample to transmit electron beam and magnify

captured image. TEM images without and with staining showed wide variation. Stained sample showed clear distinction of continuous and dispersed phase (Zhai *et al*., 2018).

2.7.2.2. Optical microscopy

As explained for study of particle size and its distribution, the magnification limit of optical microscopy is limited. The image from this method could not provide detailed information of emulsion structural features. Major location of oil and water phase cannot be distinguished in this method due to similar refractive indices. Confocal laser scanning microscopy (CLSM), which emits narrow laser beam has been used in microscopy technique to study interfacial structure (Dinsmore *et al.,* 2001). Raman spectroscopy can also be used to measure differences in characteristic spectra of chemical bonds to distinguish particles (Kwok and Ngai, 2016).

2.7.2.3. Atomic force microscopy

This acts as an advanced technique wherein a probe of little micrometre size is introduced near targeted sample surface. A contriver attached to the probe bends when it comes in contact with surface of sample. The deflection of this stylus can lead to measurement by detector followed by conversion into images (McClements, 2015). The sensitivity of process can be envisioned from the working principle of AFM as it requires tiny probe and needs careful placement near to sample. Also, it implies that the probe used in this method was very small and fragile. Thus, force acting on probe needs to be maintained constant to avoid possible damage.

2.7.3. Surface coverage

To achieve stabilized emulsion, emulsion droplets are covered by surfactants (Helgason *et al*., 2009). This logic suits for surfactant induced emulsion whereas, Pickering emulsion showed no inclination to surface coverage (Salari, 2011). However, Vignati *et al*. (2003)reported no considerable relationship between emulsion stability and surface coverage by surfactants. Through visualisation of droplets covered by particles or surfactants, characterization of surface coverage can be obtained. Using low temperature field emission SEM, Binks and Whitby, (2004) observed structure of silica colloids at interface of oil and water. Using technique of epifluorescence microscopy Vignati *et al*., (2003) visualised coverage of silica colloids to oil droplets in water. However, images of these methods were elaborated through software and depicted partial information on surface coverage.

2.7.4. Surface charge

It is an important tool to evaluate emulsion characteristics based on charge held by droplets in continuous medium. The behaviour is influenced by chemical reactions, strength of ions and pH (McClements, 2015). These factors influence stability of emulsion by changing strength of surface charge. Magnitude of this surface charge is usually measured by recoding it in terms of Zeta Potential (ZP). It was also called as electro kinetic potential used to measure potential at shear plane which are under electric field influence (Kaszuba *et al*., 2010). Technically ZP is defined as potential difference between electric double layer of mobile particle (electrophoretic) and dispersant layer around slipping plane.

2.7.4.1. Electric double layer and slipping plane

When a charged particle is dispersed in continuous phase, an electric double layer can be observed on surface. It is postulated that among these two layers, the inner layer contains ions exhibiting opposite charge of particle (stern layer). As per Debye's law, electrostatic effect beyond stern layer decreases by a factor of 1/e (Chen *et al*., 2013). Owing to electrostatic field of nanoparticles, a layer of similar or opposite ions grows beyond stern layer forming the electric double layer. This double layer is known to be highly dynamic and depends on ionic strength, pH, concentration etc. When such dispersions are subjected to influence of electric field, particles move to opposite electrode. There will be a hypothetical plane in the diffuse layer to act as interface between moving particle and dispersant layer around it. This plane is called as slipping plane or shear plane, where ZP can be measured at particle and fluid interface (Bhattacharjee, 2016).

2.7.4.2. Instrumentation for Zeta potential measurement

Doppler shift phenomena areused to analyse the change in beam frequency when it passed through emulsion under electrophoresis. The beam focussed on an emulsion containing particles is split into two streams. One stream is passed through sample and other used for reference. The light obtained after scattering from sample is combined or optically mixed with reference beam to estimate Doppler shift (Li and Tian, 1997). Before measuring ZP of sample, magnitude of particle velocityneeds to be deduced from Doppler shift. Following are mathematical formulas used in calculation of ZP.

a. Electrophoretic mobility (μe)= V/E----------2.2

Where, V-Particle velocity ($\mu m/s$)

E-Electric field strength (V/cm)

b. Henry equation (μe)=$2\varepsilon_r\varepsilon_o\zeta f(Ka)/3\eta$----------2.3

Where, ε_r-Relative permittivity/dielectric constant

ε_o- Permittivity of vacuum

η-Viscosity at experiment temperature

f (Ka)-Henry's function

ζ-Zeta potential

2.7.5. Colour of emulsion

The colour of an emulsion depends on absorption and scattering efficiency in broth. Absorption efficiency is dependent on colouring agents and scattering efficiency depends on characteristics of droplet (McClements, 2002). Colorimetry is used for colour measurement with 'L' indicating lightness and 'a', 'b' indicating actual emulsion colour characteristics (Chantrapornchai, *et al*., 2008). With increase in size of droplets, decrease in scattering efficiency was reported which led to lower lightness and enhanced colour (Chantrapornchai, e*t al*., 1998). Also, with increase in concentration of droplets (density), increase in brightness was observed by McClements, (2015).

2.7.6. Physical stability of an emulsion

Stability of an emulsion is quantified by measurement of resistance offered by emulsion to influencing factors over a period of time (McClements, 2007). Over storage period, the emulsion should retain its physical characteristics like shape, size, morphology and non-chemical properties intact. Gravitational settlement, coalescence, flocculation, Ostwald ripening and phase separation are series of changes reported during storage of an emulsion. Coalescence was considered as starting cause for an emulsion instability which leads to next sequence in emulsion destabilization (Marruchi, 1969). After coalescence, droplets colloid to from floc that eventually leads to Ostwald ripening (steady growth of large droplets at the cost of small droplets). Based on density of droplets, after Ostwald ripening, the droplets either settle or float due to gravitational influence.

2.7.6.1. Emulsion storage properties

Change in droplet size of emulsion is monitored over a period of time in emulsion storage studies. By using microscopic technique, size and distribution of particles inside emulsion were analysed at frequent intervals. Sequence of changes in emulsion behaviour was observed during the course of storage. Flocculation was reported in a study without undergoing coalescence in a Pickering emulsion for a pharma based study (Low *et al*., 2017).

2.7.6.2. Micromanipulation technique

Film trapping technique was used to evaluate coalescence in emulsion (Tcholakova *et al*., 2002). For this method, the setup consisted of capillary tube where oil was entrapped between oil phase and water phase with pressure control system. Capillary pressure in the setup was increased till oil and water coalesce and pressure required for the process was noted. In another study using Pickering emulsion, capillary tube and microscopy were used to establish coalescence and partial coalescence. The setup consisted of two micropipettes (capillary fashion) connected to separate water reservoir. The emulsion droplets held in micropipettes were pressurized to move together and its behaviour was observed under microscopy (Pawar *et al.,* 2011).

2.7.6.3. Accelerated coalescence test

By acceleration of the coalescence process by mechanical intervention, the stability of emulsion was assessed for shorter duration. Mechanical agitation and centrifugation were two methods used to evaluate coalescence stability of an emulsion. Particle size, appearance, morphology and phase separation were monitored after accelerated tests (Low *et al*., 2018). Under centrifugation, force was applied to droplets to cause collision, to evaluatequalitative and quantitative information. By using transparent (optically) centrifuge tubes, the movement of oil droplets towards axis of centrifuge was observed (Tcholakova *et al*., 2002). Critical osmotic pressure for disruption of oil droplets were calculated with the following formula.

$$\mathbf{P^{cr}_{osm} = \Delta\rho_c g_{ca} (V_{total} - V_{released})/A_{cross}} \quad \text{----------2.4}$$

Where, P^{cr}_{osm}- Critical osmatic pressure

$\Delta\rho_c = \rho c_2 - \rho c_1$- Density difference between continuous and dispersed phase

g_{ca}- Centrifugal acceleration

V_{total} -The total added volume of the dispersed phase for emulsification

$V_{released}$- Centrifuge-induced released volume of the dispersed phase

A_{cross}-Cross-sectional area of the centrifuge tube

2.7.7. Gravitational settlement

Emulsions can be described as dispersal of two immiscible liquids to attain homogeneous mixture of dispersed phase in continuous phase. Two phases exhibit different density by virtue of their chemical nature and composition. Influence of gravitational force was considered as tool to evaluate stability of emulsion during storage period (Low *et al*., 2018). In most cases, continuous phase has higher density than dispersed phase making it to move towards bottom.

2.7.7.1. Creaming index

It is a versatile and simple technique to evaluate emulsion without using any instruments. Based on Stoke's law, the settlement of denser particles over a period of time due to density gradient can be contemplated. Creaming index is the ratio of height of cream layer to total height of emulsion expressed in percentage. Many surfactants induced emulsions have been evaluated by this simple technique (Meher*et al*., 2013) and found to establish emulsion stability of samples over storage time. To evaluate HLB values of unknown emulsions, this method acts as a tool to ascertain emulsion stability.

2.7.7.2. Analytical method using centrifugation

Using light scattering approach, particle separation of the emulsion can be determined. Shimoni *et al*. (2013) subjected an emulsion sample placed in glass tube to near infrared light of monochromatic beam. The beam was passed through the height of emulsion, and the scattering and backscattering was recorded to know change in dispersed phase density. Through this method, velocity of separation, profile of creaming or sedimentation over wide range of centrifugal forces can be evaluated.

2.7.7.3. Turbidity measurement

To establish stability of an emulsion, turbidity is used as another simple tool. Turbidity differentiates emulsions based on concentration and size of droplets (Reddy and Fogler, 1981). The principle is based on the presence of various particle size leading to fluctuation in pattern of absorbance and scattering. When parameters like number of droplets, radius of droplet, volume of dispersed phase, gravity and viscosity were kept constant, creaming rate is dependent on diameter of droplet (Bechner, 1965). Presence of bigger droplets leads to more creaming compared to smaller droplets. The sample was highly turbid when droplet size was small and vice-versa. This tool was used to assess the coalescence of droplets over storage period (Mirhosseini *et al.*, 2008).

2.7.7.4. Electrical conductivity

In case of phase inversion, emulsion changes from oil in water to water in oil phaseleading to change in electrical conductivity (Allouche *et al*., 2004). Oil in water emulsion has more continuous phase compared to water in oil emulsion (Gu *et al*., 2000). Invariably, former exhibit better electrical conductivity compared to latter. In emulsion stability evaluation experiments, change in dispersed and continuous phase were considered as electrical conductivity dependent. Hence, this parameter could be considered as an indicator of the stability of the emulsion.

2.7.7.5. pH for emulsion stability

Dependency of physical stability of emulsion on the sample pH was explored by many scholars for protein based emulsifiers (Demetriades *et al*., 1997). When lipids were used as an emulsifier, its oxidation leads to change in composition and structure of interface. Oil and water emulsion stabilized by 5-30% of protein isolate showed increased stability with decrease in pH (Hu *et al*., 2003). It was suggested that at low pH, owing to positive surface charge in dispersed oil droplets, repulsion of transition metal ion was observed. Stable emulsion was reported at 3 and 7 pH using locust bean gum and xanthan (Owens *et al*., 2018). Table 2.14 outlines the details on different techniques used to ascertain emulsion properties compiled from various sources.

Table 2.14. Evaluation of emulsion stability using different tests

Sl. No	Tool/ Parameter	Equipment	Reference
1	Oxidative stability	pH meter	Owens *et al*. (2018)
2	Zeta potential	Zetasizer	Hannisdal *et al*. (2006)
3	Bottle test	ASTM	Kokal, (2005)
4	Turbidity	Turbidity meter	Kundu *et al*. (2013)
5	Electrical Conductivity	Conductivity meter	Almeida *et al*. (2017)
6	Particle size and distribution	Transmitted-light Microscope	Alvarado *et al*. (2011)
7	Image of particle interface	SEM	Binks and Kirkland, (2002)
8	Light scattering and absorption	NIR Spectrometer	Kallevik *et al*. (2000)
9	Scattering and diffraction of light	Malvern diffractometer	Binks *et al*. (1999)
10	Acoustics and Electroacoustic	Zeta analyser and conductivity meter	Dukhin and Goetz, (2005)

2.8. Green emulsions of dairy origin and its characteristic evaluation

Emulsions are derived from many sources which include proteins, PLs, polysaccharides, biosurfactants and bioemulsifiers. Lecithin derived from soybean, egg, milk, sunflower, cotton and canola seeds are commonly used in the food industry. Enzymatically modified lecithin has also found application in food industry. Saponins (extract from bark of tree) (Mitra and Dungan 1997), Sophorolipids (by microbial fermentation process) (Robertode Oliveira *et al.*, 2015), Rhamnolipids (microbial fermentation under controlled condition) (Deepika *et al*., 2016) and Mannoproteins (cell wall of baker or wine yeast) (Alcantara *et al*.,

2014) are categorized as biosurfectants and bioemulsifiers. Protein source emulsifiers like whey protein, lactoferrin, soy protein, egg protein, pea protein etc. have been used in isolation or combination.

2.8.1. Phospholipids as an emulsifier

The advantage of PLs as an emulsifier lies in its structural characteristic of the existence of polar and non-polar part in same molecule (Pichot *et al*., 2013).The fatty acid tail will be attached to oil droplet and water is bound to the hydrophilic head (Liu *et al*., 2015). Lecithin is the popular category under this class of emulsifiers used in food industry (Bueschelberger, 2004). From source, lecithin comprises of various forms of lipids like glycolipids, triglycerides and sterols which can be fabricated to get lecithin of varied purity, functionality and composition (Guiotto *et al*., 2013). For food grade emulsification, lecithin mainly comprises of PC, PI, PE and phosphatidic acid (PA) (Erickson, 2008). As the non-polar tail is composed of two fatty acids by its nature, it can be cleaved chemically or enzymatically to get lysolecithin with unique functional properties (Casado *et al.,* 2012). The HLB number, which is derived to numerically grade emulsion, is governed by type of head and tail group.

2.8.2. Hydrophilic and lipophilic balance of an emulsifier

Griffin (1954) introduced the concept of HLB number to quantitatively express emulsifier behavior. It indicates the characteristic of emulsifier as a lipophilic or hydrophilic molecule, based on its affinity towards the phases. To select any emulsifier, HLB values acts as ready reckoner to choose suitable combination. Based on HLB value of the ingredient combination, similar valued emulsifier or combination of emulsifiers are chosen to create stable emulsion. To evaluate HLB value of an emulsifier, series of experiments have been designed to evaluate the HLB value.

2.8.3. Evaluation of HLB values of an emulsifier

To characterize an emulsion, many methods were discussed in preceding section. Similar tools have been adopted to evaluate HLB values of an emulsifier or an ingredient mixture. Ferreira*et al*.(2010) evaluated HLB value of andiroba oil using short term and long term stability studies. For the long term study, oil was mixed with water, Tween 20 and Span 80 to get defined HLB. The emulsion mixture was evaluated for its creaming index, pH, electrical conductivity and macroscopic observation for 120 days at different time intervals. By adopting micro-emultocrit and centrifugal stress techniques, short term stability tests were

conducted by measuring the creaming index. The result in the study was able to reveal the HLB values of tested sample as 16.7.

Similarly, in a study to determine required HLB of citronella oil, Meher *et al.* (2013) evaluated the emulsion for the droplet size, creaming index, physical examination and turbidity analysis. To evaluate the validity of the methodology, this study has used light liquid paraffin emulsion (whose HLB was known) as a reference. The study concluded that lowest mean droplet diameter and high turbidity was observed at 12.6 HLB.

2.8.4. Phospholipids as an emulsifier in different foods

Goff and Jordan (1989) compared six emulsifiers by examining the destabilizing power in ice cream processing. Ice cream mix for the study was formulated using milk fat (10%), SNF (11%), sucrose (10%), corn syrup solids (5%), stabilizer (1%) and emulsifier (0.08%). The interfacial tension between butter oil and SNF in presence of emulsifiers was measured using Fisher surface tensiometer. Three pairs of emulsifiers (monoglycerides, sorbitan and polyoxyethylene sorbitan esters) were studied for HLB, unsaturation and polyoxyethlene group. It was reported that higher fat destabilization over a course of 15 min. in the barrel of batch freezer was observed for glycerol monoleate. Shearing and ice crystallization alone were insufficient to cause the destabilization encountered in barrel freezer. Further, the results emphasised that addition of the emulsifier reduced interfacial tension between serum and lipid phase.

Desrumaux and Marcand (2002) used whey protein concentrate as an emulsifier and studied its emulsifying characteristics for sunflower oil. An emulsion containing 20% of sunflower oil by mass and 80% of whey protein concentrate was prepared at $4^{o}C$ using a high pressure homogenizer. Size distribution of droplets were measured using laser light scanning method, rheology by rheometer, electrophoresis by sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and degree of denaturation of the whey protein by micro-differential scanning calorimetry. The results indicated that the droplet diameter decreased when pressure increased from 20 to 100 MPa, whereas at pressure >100MPa, the sample showed four zone behaviour for $d_{32,}$ viscosity and adsorbed protein.

Lobato-Callero*set al.*(2003)studied emulsifier blends to evaluate cheese product. Mexican Manchego cheese-like product (MCLP) was added with three different emulsifier blends such as polyoxyethylene sorbitan monostearate, glycerol monostearate and sorbitan monostearate along with canola oil; the control sample of MCLP was prepared from only milk. Using

Malvern droplet & particle size analyzer, volume surface average ($D_{2,3}$), and using dynamic shear rheometer dynamic oscillatory were measured. The results indicated that with high HLB and high concentration of polyoxyethylene sorbitan monostearate, lower droplet size was observed, whereas high sorbitan monostearate concentration led to higher droplet size. Sorbitan monostearate concentration in MCLP led to higher values for rheological parameters showing solid viscoelastic behavior.

Firebaugh and Daubert, (2005) evaluated emulsifying and foaming properties of derivatized whey protein ingredient. Derivatized powder was produced by dilution, thermal treatment and spray drying of whey protein isolates (WPI) powder. The properties were analysed based on its emulsifying capacity, emulsion stability and foam characterization (drainage, overrun and yield stress). Results indicated that there was no significant difference between derivatized and non-derivatized whey protein isolate for emulsion capacity. Derivatized whey ingredient showed apparent viscosities more than twice those of unmodified WPI. Drainage study for foam characterization indicated 80% foam volume drainage in 60 min. for unmodified WPI, whereas derivatized WPI showed 5% drainage for same period. Thus, the study established the superiority of derivatized WPI over native WPI.

Miura*et al*. (2006) evaluated emulsifying properties of PLs derived from soybean and bovine milk. Cream was reconstituted with 40% butter oil, 56% water, 3% buttermilk powder and 1%PLs derived from two sources. The two kinds of PLs added in the emulsion were evaluated with differential scanning calorimetry at crystallization temperature. Nuclear magnetic resonance was used for solid fat content and gas chromatography for fatty acid composition. Results indicated that sample added with bovine milk PLs stabilized the cream whereas, soybean derived PLs solidified the cream. Addition of soybean PLs in cream delayed crystallization (onset) upon cooling which led to low fat content inside fat globules.

Ice-cream formulation requires emulsifier to keep constitutes intact and in good dispersion. It comprises of a colloidal system including fat, air, ice crystal, protein, salt and polysaccharides dispersed as freeze concentrate. Rinaldi *et al*. (2014) worked on gelato, an Italian ice cream, to replace conventional emulsifiers (Mono- and diglycerides of fatty acids) with PLs derived from rice, milk and soy. The study adopted evaluation of overrun, melting rate, hardness, colour, moisture content, thermal and volatile compound analysis to ascertain suitability of the emulsifiers. The results inferred that conventional emulsifiers still held good results on overrun and better fat destabilization. However, soy and rice PLs emulsifiers performed well in most of the parameters assessed like textural and colorimetric values. PLs

extracted from milk failed to exhibit significant emulsifying property by reporting lower overrun, green color tones and undesirable hardness values.

Kasinos*et al*. (2014) worked toevaluate the influence of PLs fraction of dairy byproducts on heat stability of recombined evaporated milk emulsions. Two dairy byproducts which are rich in PLs were chosen for study viz., cream residue powder and sweet buttermilk powder. Skim milk powder (16.5%), hazel oil (6.5%), dairy byproducts 0-6% (w/w) were mixed in 0.02% NaN_3 (Acros Organics) aqueous solution. The samples were homogenized using a micro fluidizer before subjecting for sterilization at different time intervals. Viscosity, particle size analysis and protein load assessment were conducted after heat treatment. Results confirmed that dairy byproducts used in the study were effective in preventing heat induced viscosity change and particle size distribution. At the highest byproduct concentration (6%), the viscosity of heat treated sample was on par with non-thermal treated samples. Assessment of protein load highlighted decreased heat induced protein interaction due to alteration in protein surface load. Cream butter powder was slightly better than sweet buttermilk powder in terms of its effect on viscosity and particle size distribution.

Stability of whipped cream playan important role in maintaining the texture of product. Combination of low molecular weight emulsifier (LMWE) and protein (sodium caseinate and casein peptides) were tried on whipped cream by Ihara *et al*. (2015). Degree of fat globule aggregation and shape retention ability (reduction in firmness after whipping and 1-day storage under refrigeration) were evaluated in the study. Samples added with LMWE in isolation exhibited stability of fat globules but failed to retain shape. Addition of LMWE into continuous phase of cream led to more stability than original cream. Also, shear stability, shape retention ability and serum viscosity reduced with LMWE concentration. The protein component had little effect on the measured characteristics of whipped cream. The presence of protein along with LMWE reduced effect of LMWE on shape retention and stabilization of fat globule.

Based on the above review it is deduced that

1. Ghee residue is potentially good source of PLs that is not explored for emulsifying properties. Very limited studies are undertaken on separation of constituents of ghee residue for analyzing functionalities.
2. As such, investigations on extraction of PLs from dairy products are very limited. Most reported studies have focused on buttermilk solids, whey protein phospholipids concentrate, butter serum as the substrate matrix. Also, extraction or separations of

PLs from dairy products are limited to SCFE, modified solvent extraction, filtration and enzyme treatments.

3. PLs fraction from dairy matrix is found to have health benefits over plant based PLs.
4. Conventional solvent extraction methods, even with pre-processing have not achieved full extraction efficacy for PLs. Assisted extraction using interventions such as PEF, MW and US have potential to improve the extraction efficacy.
5. PLs from dairy matrices have great commercial potential as naturally sourced food grade emulsifiers. Proper characterization of the extract for its techno-functional attributes will aid in strengthening this claim.

Chapter-3

MATERIALS AND METHODS

3.0 MATERIALS AND METHODS

The objective of proposed research work is to utilize ghee residue extract as PLs enriched fraction to use it as replacement for conventional emulsifiers. In line to this, ghee residue was subjected to series of pre-treatments followed by assisted extraction using engineering interventions. This chapter is detailed with material and methods used for pre-treatments, assisted extraction and evaluation of emulsifying characteristics of ghee residue extract.

Details enlisted in this chapter will give an insight into equipment and methodology followed. Statistics tools used to optimise process parameters and kinetics of extraction are also elaborated. Details of process flow of work in the proposed research work are detailed in Fig. 3.1.

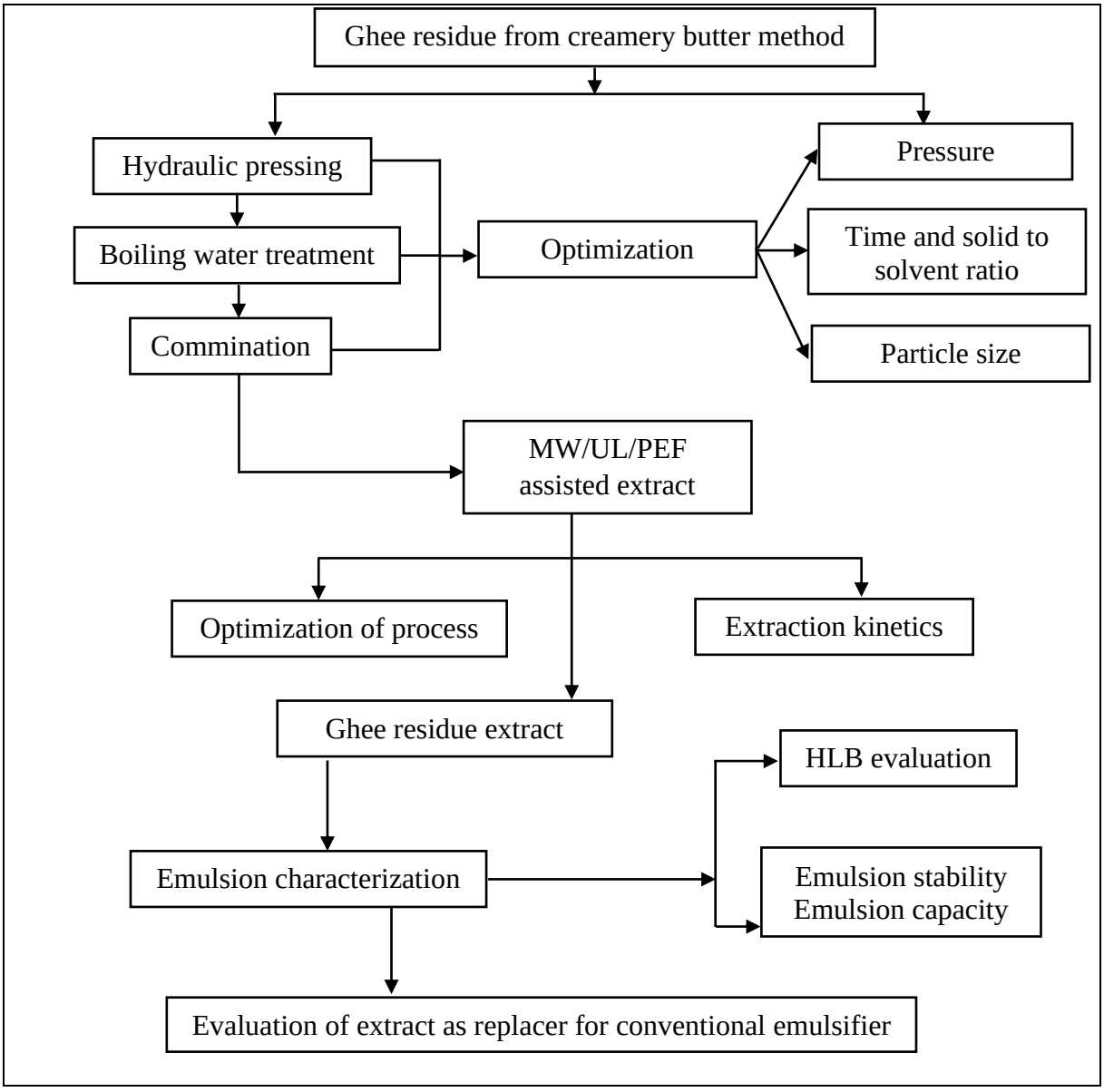


Fig.3.1. Flow chart depicting work executed in the proposed work

3.1. Process for preparation of ghee residue

Various methods have been reported for the preparation of ghee, which results in different quantities of ghee residue. Predominantly, three methods are commonly practiced, which includes *desi*, direct cream and creamery butter method. Ghee residue obtained by creamery butter method was reported to have higher PLs yield compared to direct cream method (Santha, 1977). Hence, as a prelude to the study, PL content of ghee residue obtained using both creamery butter and direct cream method was evaluated and the creamery butter method which yielded better PL content in the residue was adopted for preparation of ghee residue in the study.

3.1.1. Preparation of ghee residue by creamery butter method of ghee making

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 a centrifugal cream separator in the Experimental Dairy of the station. Fat content of the fresh cream was determined using the standard Gerber test and found to vary between 52to 60%across different batches. By admixing with toned milk, the fat content of cream was standardised to 40%, followed by overnight ageing at 4±2^{o}C. Cream was subjected to churning using a previously custom assembled batch type universal dispenser with a high-speed impeller in the Dairy Engineering section of the station. The vessel containing cream was concentrically placed inside another container. The annular space was filled with chilled water (4±2^{o}C) to ensure uniform temperature during churning for the phase inversion of cream. Butter obtained in the process was carefully separated from buttermilk by manual draining. Gentle pressing was then applied to remove the residual buttermilk from the butter mass which was then stored at 4±2^{o}C until sufficient stock of butter was prepared before converting it to ghee.

The prepared butter was converted to ghee by heat clarification following the methodology presented in (Santha, 1977). The butter was heated to melting in a steam jacketed kettle in the Experimental Dairy of the station. Temperature of the steam kettle was monitored using a digital thermometer (LCD Food Thermometer Cooking Probe, B07WJ6N555, China) and the steam pressure was periodically adjusted so that the temperature did not exceed 118^{o}C. The material inside the kettle was regularly stirred and scrapped from the sides with a long handle spatula to avoid material adhesion to inner surface and consequent burning. Heating was stopped when bubbles disappeared from surface and the golden-brown residue separated to settle at the bottom of the vat. Ghee was carefully decanted from the kettle with simultaneous

filtering using a muslin cloth to separate the ghee residue. Residual ghee in ghee residue was expressed by gentle pressing. The collected ghee residue was stored under refrigeration (4±2^{o}C) until it was used for further treatments and experiments.

3.1.2. Mechanical pressing of ghee residue

Ghee residue obtained through the procedure described in section 3.1.1. contained higher and varied proportions of lipids due to its particulate structure and multiple batch processing. The cold stored ghee residue was equilibrated to room temperature in an oven (Falcon Scientific Co., Bangalore, India) maintained at 40±2^{o}C for 4 h. To eliminate the excess lipids and 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 jaws 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^{o}C.

3.2. Pre-treatment of ghee residue

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 further concentrate the proportion of polar lipids in the matrix, the ghee residue was pre-treated with two solvents viz., n-hexane and boiling water.

3.2.1. Pre-treatment with n-hexane

Ghee residue obtained after pressing was again tempered at 40±2^{o}C for 4 h to melt lipids entrapped in ghee residue. The tempered ghee residue was mixed with n-hexane at 1:2, 1:3 and 1:4 solid to solvent ratio (w/v) in a glass beaker. The beaker was lidded with aluminium foil and agitated frequently for better solid-solvent contact for varied time periods (20, 30 and 40 min.). After a defined time of exposure, the solvent was drained out of ghee residue, which was filtered using Whatman paper 1 (185 mmØ). The retentate residue was then analysed for its lipids and PLs content using standard procedure described in the succeeding sections. Time of exposure and solid to solvent ratio were considered as the independent parameters to optimize these pre-treatment steps for elimination of maximum non-polar lipids and retention of maximum PLs.

3.2.2. Pre-treatment with boiling water

An alternate pre-treatment approach for the ghee residue by using hydrothermal treatment with boiling water instead of n-hexane was also evaluated. Tempered ghee residue was mixed with boiling water in different solid to solvent ratio (1:2, 1:3 and 1:4) in a glass beaker. The beaker was immersed in boiling water bath for varied time periods (20, 30 and 40 min.). After the scheduled time, the beaker (with the contents) was placed in a deep freezer (-4 to -12^{o}C) for 5 h to facilitate solidification of lipids separated from ghee residue during the hydrothermal treatment. The solidified fat layer was carefully skimmed off the surface using a sharp-edged knife. The residual mixture of ghee residue and water was poured into a stainless-steel tray and dried at 43±1^{o}C for 48h. Resultant dry fraction was coarsely powdered using pestle and mortar followed by the analytical estimation of its lipids and PLs content. Solid to solvent ratio and time of exposure was considered as experimental factors. The data for both the pre-treatments (using n-hexane and boiling water) were compared to select the better pre-treatment protocol for removing the neutral lipids from the ghee residue.

3.2.3. Comminution of ghee residue particles

Ghee residue obtained from the selected method of pre-treatment was subjected to size comminution so as to pass through two different sieve sizes (0.25 mm and 0.30 mm). Yield of lipids and PLs from the two comminuted samples were analysed. Particulate material which yielded maximum PLs was selected for further studies on assisted extraction techniques. Fig. 3.2 shows the pictogram of ghee residue after pressing and size reduction to 0.25 and 0.3 mm.

Fig.3.2. Ghee residue (a) after boiling water treatment; (b) particle passing through 0.25 mm sieve; (c) particle passing through 0.30 mm sieve

3.3. Materials

Chemicals, reagents, equipment and glassware used in the study are enlisted herewith.

3.3.1. Chemicals

Chemicals used in the experiment were of analytical grade and were procured from reputed firms such as HiMedia Laboratories Pvt. Ltd., SD Fine Chem. Ltd., Thermo Fisher Scientific Ltd., and Fisher Scientific Ltd.

3.3.1.1. Reducing reagent for phosphorus estimation

For the estimation of phosphorus content in the samples, reducing reagent was required, which was prepared with 0.25 g of 1-amino-2-napthol-4-sulfonic acid (ANSA), 15 g of sodium bisulphite and 0.5 g of anhydrous sodium sulphite mixed in 100 mL of distilled water. Resultant mixture was thoroughly stirred followed by filtration through Whatman 1 filter paper.

3.3.1.2. DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) solution

For analysis of antioxidant activity, the standard DPPH solution was prepared as follows. DPPH (39.4 mg) was added to a beaker containing 50 mL of methanol followed by thorough mixing using stirrer. The dissolved methanol extract was transferred to 100 mL volumetric flask. The same beaker was added with two spells of methanol and mixed using the stirrer followed by transfer to volumetric flask to adjust total volume for 100 mL. The DPPH solution was stored in deep freezer maintained at -18^{o}C until used.

3.3.2. Glassware

Glassware used in the experiments were, unless otherwise mentioned, of the standard make of the brand Borosil. Design and volume of the glassware were as per the requirement for the analysis and methodology recommended in the references.

3.3.3. Solid Phase Extraction (SPE) cartridge

To separate lipids into polar and non-polar fractions, SPE cartridges of 6mL capacity were used. Supelclean^{TM} disposable SPE tubes (Brand Supelco®, Sigma Aldrich, USA) of dimension 60 mm length, 8mm diameter and containing silica gel (1g) based adsorbent were used for this purpose. The cartridge was conditioned with different solvents to activate the packing before elution of lipids. A thin film of water miscible solvent could be seen in the packing after adding aqueous solvent for conditioning. This indicated better contact between an aqueous sample matrix and hydrophobic solid phase. Fig. 3.3presents the Supeclean disposable SPE tubes with eluted samples in vial.

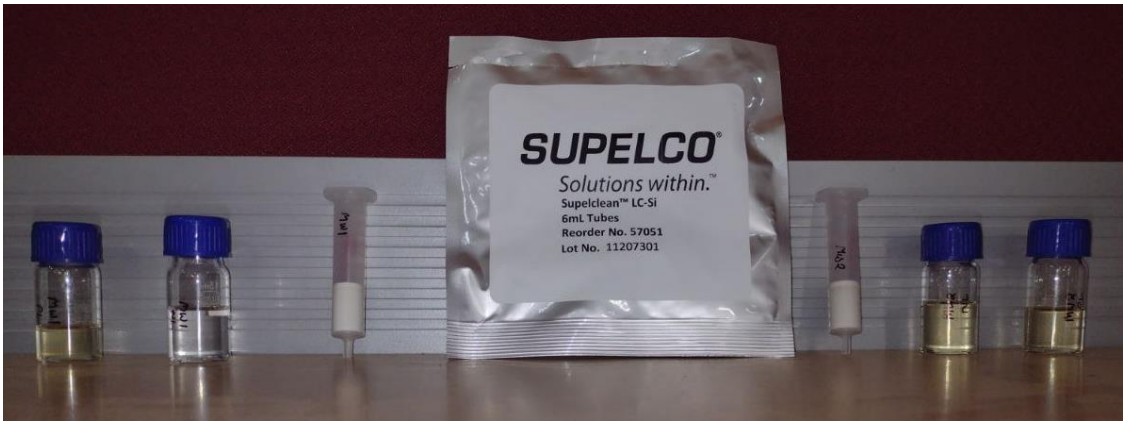


Fig. 3.3. Supelco disposable SPE tubes with eluted solvents in vials

3.3.4. Equipment and instruments

3.3.4.1. Heating mantle

To digest lipids obtained in extraction process, a heating mantle (Rivotek, Riviera, Duran Group GmbH., Germany) made out of mild steel powder coated aluminium metal body with manual energy regulator (0-100) was used. The mantle capacity was 1 L which accommodated the flat bottom flask to improve heat contact surface during heating.

3.3.4.2. Hydraulic press

To compress and squeeze the loosely adhering fat from the ghee residue, a multipurpose machine (Milk Teck Engineers, Bangalore, India) designed to develop hydraulic pressure was used. The machine consists of a solid platform and vertical axle connected with a circular plate to transfer pressure to material. The axle was operated vertically by moving up and down to create compression pressure. Equipment was provisioned with time and analogue pressure gauge to monitor operation.

3.3.4.3. Spectrophotometer

UV/VIS spectrophotometer (UV 3200 XE) was used to measure optical density of extract to evaluate phosphorus content and antioxidant activity. UV/VIS spectrophotometer (Labindia, Labindia Analytical Instruments Pvt. Ltd., Thane, India) is designed with photometric range of -4 to 4.0A with Czerny-Turner monochromator. It is equipped with a dual silicon photodiode detector with xenon lamp light source and integrated with a PC interface. The instrument consisted of four basic components namely, a light source, a monochromator, a sample holder and detector (Fig. 3.4).

Fig 3.4. UV/VIS spectrophotometer (UV 3200 XE) used for measuring optical density

3.3.4.4. Centrifuge

To consolidate ghee residue and separate extract from treated sample, centrifuge (Remi, R8C^{+}, Remi Elektrotechnik Ltd., Palghar, India) was used. Centrifuge was designed to operate at maximum speed of 4500 rpm. The instrument had the provision to set time and speed of rotation. It was installed with a wing out rotor to accommodate 24 test tubes of 15 mL capacity each.

3.3.4.5. Water bath

A double jacketed hot water bath equipped with two heaters and temperature control was used for hot water treatment and solvent evaporation. The equipment (Excel Scientific, Channasandra, Bangalore, India) is rectangular in cross-section and made of stainless steel with drain facility and a heater guard. The water bath is also equipped with a control panel to set temperature and transparent window pane for sample monitoring.

3.3.4.6. Hot air oven

Hot air oven (Falcon Scientific Co., Heat Control Instruments and Services, Peenya, Bangalore, India) was used for drying of samples. The equipment is designed to operate at 8A, 220V with single phase supply.

3.3.4.7. B.O.D. incubator

B.O.D incubator was used to condition the sample to desired temperature and for temperature control during experiments. The incubator (M K Scientific Instruments, MKSI-109-SERIES, New Delhi) is designed to operate between 5 to 60^{o}C with ±0.5^{o}C accuracy and ±1^{o}C uniformity in temperature distribution within chamber. It is provided with stainless steel

grilled shelves, a digital display and temperature control panel in the front panel of the incubator.

3.3.4.8. Analytical weighing balance

Two types of analytical balances were used for weighing different quantity of material which includes weighing balance (Sartorius Lab Instruments GmbH & Co. KG, Goettingen, Germany) with maximum weighing capacity of 200 g at 0.1 mg precision. The second scale (Essae-Teraoka Pvt. Ltd. Bangalore, India) was used for maximum of 6 kg with minimum scale reading of 20 g.

3.3.4.9. Multipurpose electrical conductivity meter

Digital water TDS EC & Temperature meter (Uniglobal multiparameter meters, Jaipur, Rajasthan, India) was used for measurement of electrical conductivity of emulsions. The instrument measures temperature between 1 and 80^{o}C, conductivity between 0-9999 µs/cm and TDS upto 9999 ppm.

3.3.4.10. Vacuum tray dryer

A custom designed vacuum tray dryer (Falcon Scientific Co., Bangalore, India), present in the Dairy Engineering laboratory, was used for drying the lipid extract for the solid phase extraction studies. The drying chamber of the unit is integrated with a vacuum pump that can generate vacuum up to 600 mm Hg. Drying chamber of the unit is also designed to operate at elevated temperatures up to 150^{o}C. The circular cross section of dryer accommodates two perforated trays for placement of the samples (Fig. 3.5).

3.3.4.11. pH meter

The pH of emulsion and ice cream samples was measured using a bench top pH meter (M/s Eutech, Laboratory Instruments, Bangalore). The cyberscan pH meter is installed with glass electrode facilitated with power adapter.

3.3.4.12. Texture analyser

Texture profile analysis of hardened ice cream and back extrusion of ice cream mix was carried using texture analyser (Stable microsystems, Model: TAXT plus, UK) (Fig. 3.6).

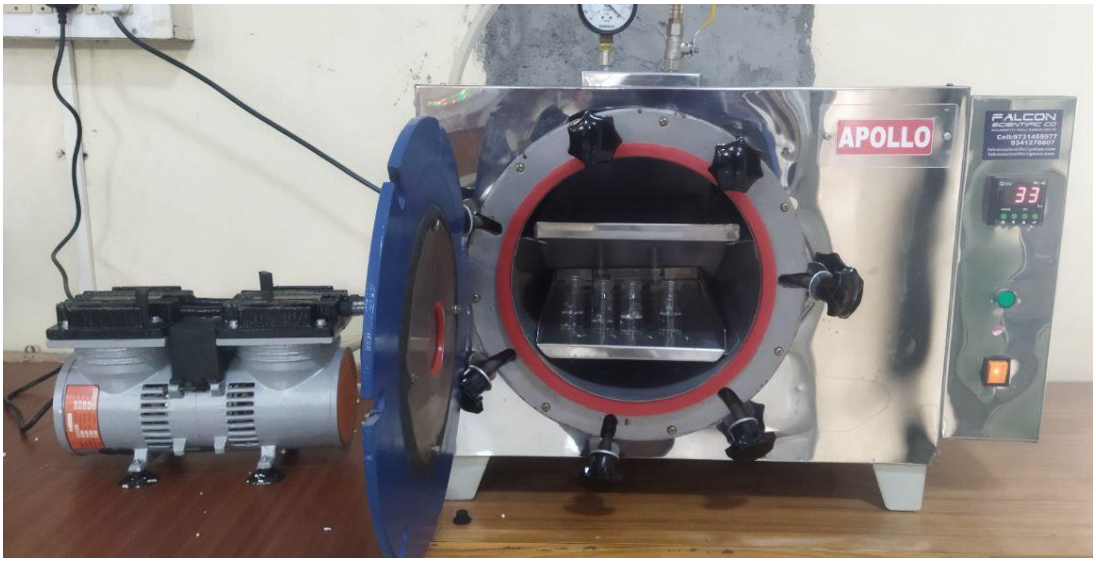


Fig. 3.5. Custom design vacuum tray dryer integrated with vacuum pump

Fig. 3.6. Texture analyser used for texture profile analysis and back extrusion of ice cream

3.3.4.13. Scanning Electron Microscopy (SEM)

The microstructure of the untreated and assisted extraction treated samples of the ghee residue was investigated using a Scanning Electron Microscope (Make: Carl Zeiss AG,

Gemini® Technology, Model: Ultra 55). The microscope (Fig. 3.7) created high resolution images of the samples using angle selective back scattered electron detector to elucidate the surface morphology. The specifications of the microscope are given in Table 3.1.

Table 3.1. Technical specifications of the scanning electronmicroscope

Parameter	**Specifications**
Equipment name	Carl Zeiss Ultra 55 SEM
X-ray generator	PhotonMax high-flux 9 kW rotating anode
Acceleration voltage	0.1 – 30 kV
Detector	Backscattered electron (EsB) detector
Probe current	4 pA – 10 nA
Resolution	1.0 nm @ 15 kV 1.7 nm @ 1 kV 4.0 nm @ 0.1 kV
Magnification	12 – 900000x in SE mode 100 – 900000x in EsB detector
Maximum Resolution	3072 *2304 pixel

Prior to imaging processing, ghee residue samples were sputtered with a gold coating, an additional thin layer (10 nm) of conductive material, to facilitate capturing of good quality images. The samples were spread as a thin layer over the base plate of the instrument and scanned under microscope at different magnifications to obtain the micrographs.

Fig. 3.7. Carl Zeiss SEM

3.4. Proximate analysis of ghee residue and ice cream

Ghee residue was analysed forlipids, PLs, protein, lactose and ash content using the standard methodology as described below.

3.4.1. Lipid content by Rose Gottileb method

Lipids from the dried extract were recovered using method detailed by Cheng*et al*., (2019) with few modifications. Dry fraction of the ghee residue extract or pre-weighed ghee residue was taken in a beaker and mixed with 2 mL ammonia solution (30%) and 1g of NaCl, followed by light stirring with glass rod. After few minutes, 10 mL of ethyl alcohol (95%) was added and again stirred with glass rod to facilitate dry matter access to solvent. Diethyl ether and petroleum ether 15 mL each was added into beaker followed by stirring with glass rod. Entire content of the beaker was transferred to Mojonnier flask. Using rubber stopper, neck of Mojonnier flask was closed and the contents were thoroughly mixed. The flask was allowed to rest for 60 min., and the solvent was carefully transferred to a pre-weighed glass beaker. Ethyl alcohol (5 mL), diethyl ether (10 mL) and petroleum ether (10 mL) were added to beaker containing traces of residual extract, swirled carefully to collect the residues and then transferred to Mojonnier flask. Solvent was pooled to previously extracted fraction followed by one more extraction with same proportion of solvents. Extract obtained in all three steps were pooled and solvent was evaporated using hot water bath maintained at 75^{o}C. After major amount of solvent was evaporated, beaker was placed in hot air oven maintained at 105±2^{o}C till constant weight was obtained. In case of lipid estimation of ghee residue, the sample was directly added to Mojonnier flask. All the chemicals and solvents were added in the sequence followed for the dry fraction of the extract.

3.4.2 Phospholipids by phosphorus estimation method

PLs content of sample was estimated based on phosphorus content in fat extracted using organic solvents. The method used by Murthy and Narayanan, (1966) with slight modifications was adopted for estimation of PLs in the study. The lipids obtained by the methodology as described in section 3.4.1. was digested with nitric acid and sulphuric acid using a heating mantle till the colour of the mix turned light yellow or colourless. The process for obtaining light yellow or colourless was optimized to 20 min. for lipid extract of 1-5 g of ghee residue. Digestion flask was cooled and added with 10mL of distilled water and again heated on the mantle till added water evaporated. The cycle of addition of distilled water and heating to its evaporation was repeated one more time. After cooling, 5 mL of this aliquot was mixed with 0.44% ammonium molybdate and 0.4mL of reducing agent (1-amonia-2-napthol-4 sulphonic acid, sodium sulphite, sodium bisulphite), followed by boiling in 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.

3.4.3. Antioxidant activity of the extract

Antioxidant activity of the extract was measured using DPPH assay as detailed by Huang *et al*., (2020). One hundred eighty µL of DPPH solution (1 mM methanolic stock prepared and stored at -18^{o}C), 60µL of extract and 2760µL of methanol were added to test tube followed by incubation in dark for 30 min. at ambient condition. Later, absorbance was measured at 515nm with UV-Visible spectrophotometer (LABINDIA Analytical UV 3200XE, India). DPPH free radical scavenging activity was measured using the following formula

$$\text{DPPH activity} = \frac{Absorbance\ of\ blank - Absorbance\ of\ sample}{Absorbance\ of\ blank} * 100 \text{----------(3.1)}$$

3.4.4. Lactose content of ghee residue

Lane Eynon method (ISI, 1981) was followed for estimation of lactose content. Approximately, 10g of ghee residue was weighed and 30 to 40 mL of distilled water was added. Samples were mixed and warmed to 40-45 °C followed by addition of 1.5 mL of 10% acetic acid. Volume was made up to 100 mL by adding distilled water. The contents were filtered through Whatman filter paper (42 grade) and collected in a conical flask. Burette filled with standard lactose solution / ghee residue filtrate was titrated against solution containing 5mL of Fehling A and Fehling B with methylene blue as an indicator. Lactose (%) was calculated by following equation.

$$\%\ \text{lactose} = 5\ \text{X}\frac{V1}{V2} \text{----------(3.2)}$$

V1 (mL) = Standard lactose solution taken to reduce 10mL of Fehling's solution

V2 (mL) = Prepared ghee residue filtrate taken to reduce 10mL of Fehling's solution

3.4.5. Ash content of ghee residue

Ghee residue samples (5 g) were taken in dried and pre-weighed silica crucibles The samples were dried in open flame to remove excess moisture before placing in muffle furnace maintained at temperature 550±10^{o}C for 4h. Muffle furnace was switched off to bring down temperature followed by collection of samples into 77esiccators. Ash content of the sample was estimated using the equation as given below.

$$\text{Ash}\ (\%\ \text{weight}) = \frac{\text{Weight of ash (g)}}{\text{Weight of sample (g)}} \text{x}100 \text{----------3.3}$$

3.4.6. Protein estimation

Protein content of ghee residue, extract and sediment was estimated by Kjeldahl method. Sample of extract and sediment was subjected for digestion and distillation using Kjeldahl analyser unit (Make: Gerhardt, Germany, Tubotherm Model: TT 125 for digestion and VAPODEST for distillation).

3.4.6.1. Digestion

Samples of the extract and sediment was weighed and added into 300 mL Kjeldahl digestion tubes. Care was taken to completely add sample into digestion tube to avoid contact with lateral lining of tube. Digestion tube was added with 5g of digestion mixture (K_2SO_4:$CuSO_4$=100:2) and 15mL of concentrated sulphuric acid. Contents in Kjeldahl digestion tubes were loaded to digestion assembly to digest for 3-4 h till clear and colourless solution was obtained.

3.4.6.2. Distillation

Distillation was carried in the Kjeldahl distillation assembly which is designed to take in constituents automatically through input commands. Cooled digestion tubes were added with 40% NaoH to make the solution alkaline and inserted into distillation unit. Tube contents were steam distilled and the ammonia liberated in the process was collected into boric acid solution containing 2-3 drops of the mixed indicator (methyl red and methylene blue). The distillation was continued until about 65 to 75 mL of distillate was collected.

3.4.6.3. Titration

Distillate obtained in previous step was titrated against 0.01N H_2SO_4 until end point (purple colour) appeared. A blank test was carried out simultaneously using all the reagents except the test material and the percent protein was calculated as follows:

$$\text{Protein } (\%) = \frac{F \times 1.4007 \times (S-R) \times N}{W} \times 100 \text{----------} 3.4$$

Where,

F-Conversion factor of nitrogen into protein (6.38)

S-Sample reading (mL)

R-Blank reading (mL)

N-Normality of H_2SO_4 (N)

W-Weight of sample (g)

3.4.7. Analysis of extract by Liquid Chromatography and Mass Spectrometry (LC-MS) for lipids classes and species

Subclasses of PLs and its species from the derived extract were evaluated using LC-MS (Thermo Scientific, Q Exactive Mass spectrometer coupled to a Thermo Scientific UltiMate 3000 UHPLC system, Fig.3.8). Extract obtained from different assisted extraction techniques were evaluated as follows. An aliquot of 2 µL sample (extract) was re-suspended in 1 mL of methanol followed by centrifugation. A volume of 20 µL of supernatant was taken for extraction with 10 µL of the splash standard addition. It was added with 200µL of ice-cold MeOH, 200 µL of ice-cold $CHCl_3$ and 200 µL of 0.89% KCl. The whole mixture was vortexed for 1 min. and incubated at 37°C for 10 min. followed by centrifugation at 10000 rpm for 5 min. at 4^{o}C. The lower organic phase was separated into clean test tube (1.5 mL capacity).Upper layer of Eppendorf tube was added with 200 µL of ice-cold $CHCl_3$ and 200 µL of 0.89% KCl followed by a repetition of the vortexing cycle. Lower fraction was added to 1.5 mL test tube containing previous solvent extract. All the solvents were evaporated to dryness in gentle nitrogen stream or under vacuum. The residue obtained was reconstituted with 200 µL of MeOH and 5µL of this was used for feeding into column.

Fig.3.8. Thermo Scientific-Q ExactiveMass spectrometer coupled to a Thermo Scientific UltiMate 3000 UHPLC system

The extract obtained after the preparatory steps of lipid preparation process (described above) was fed into LC analytical column (Acclaim C30, Themo fisher, 3 µm; 2.1 X 100 mm). The mobile phase used in the process was (A) Acetonitrile and water with 10mM ammonium formate and with 0.1% formic acid and (B) Isopropanol and acetonitrile with 10 mM ammonium formate and 0.1% formic acid. The LC gradient of mobile phase followed in the elution process is detailed in Table 3.2.

Table. 3.2. Gradient of solvent A* and B* with flow rate used for lipids elution in LC-MS

Time (min.)	% A	% B	Flow Rate (uL/min)
0:00	80	20	300
2:00	80	20	300
5:00	60	40	300
45:00	20	80	300
52:00	0	100	300
55:00	80	20	300
60:00	80	20	300

Detection of lipids was performed through Orbitrap Elite mass spectrometer having ion polarity of positive and negative. Resolution was set at 140000 for both positive and negative modes.

3.5. Enzyme treatment of ghee residue

It was reported that the yield of PLs could be improved with enzymatic treatment prior to extraction techniques (Barry *et al*., 2017). Two enzymes were evaluated in the study for treatment of ghee residue to test their efficacy in improving the PL content of the extract. The selected enzymes, i.e.,Trypsin and Alcalase (HiMedia Laboratories Pvt. Ltd., Nasik, India) were used in the experiments.

3.5.1. Trypsin

Trypsin enzyme was used with sodium citrate as buffer by adjusting pH to 8.0 using HCl or NaOH. Enzyme to substrate ratio for treatment was maintained at 1:25 on protein basis. After adding enzyme and substrate into the buffer, the container was incubated at 37^{o}C in a B.O.D incubator with frequent manual agitation (every 15 min.) for varied period of time (2, 4, 8 and 12 h). At every scheduled interval, the enzyme was inactivated by thermal treatment of contents at 80^{o}C for 15 min. The time for trypsin treatment was optimized for maximum PLs yield.

3.5.2. Alcalase

As followed for trypsin enzyme, alcalase was used for treatment of ghee residue using water as buffer at pH 8.0. Enzyme to substrate ratio of 1:25 on protein basis was maintained during experiment. Enzyme and substrate were incubated at 50^{o}C in an incubator for varied time period (2, 4, 8 and 12 h). During incubation, the substrate was agitated manually for better

enzyme and substrate contact. PLs were evaluated after every time schedule by inactivating the enzyme with thermal treatment at 80^{o}C for 30 min.

3.6. Comparison of PLs estimation between spectrometric method and solid phase extraction

Many elution techniques have been reported for separation of polar and neutral lipids from dairy products using solid phase extraction techniques. Hence, an attempt was made in this study to compare the PL content reported by standard spectrometric method with that of sample extracted through solid phase extraction methodology.

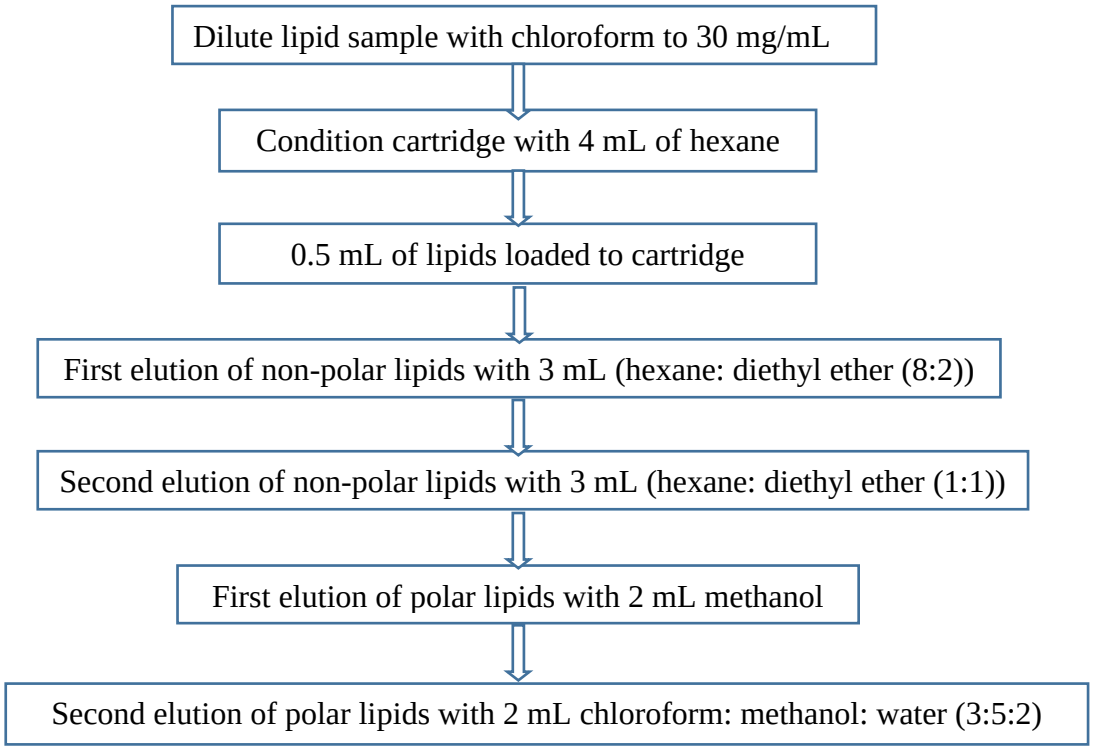


Fig. 3.9. Flow of process for elution of non-polar and polar lipids from lipids fraction of ghee residue (Ferraris *et al*., 2020)

Methods adopted by two scholarsCheng *et al*., (2019) and Ferraris *et al.*, (2020) were used for experimentation. Precautions adopted in the study included dissolution of lipids extract with solvents and centrifugation to remove particulate material co-extracted with lipids during lipid extraction process. This helped the lipid sample to get rid of large particulate material which could have blocked column material. The detailed steps followed for elution of polar lipids by Ferraris *et al.*, (2020) and Cheng*et al*., (2019) methods are depicted in Fig. 3.9 and 3.10.

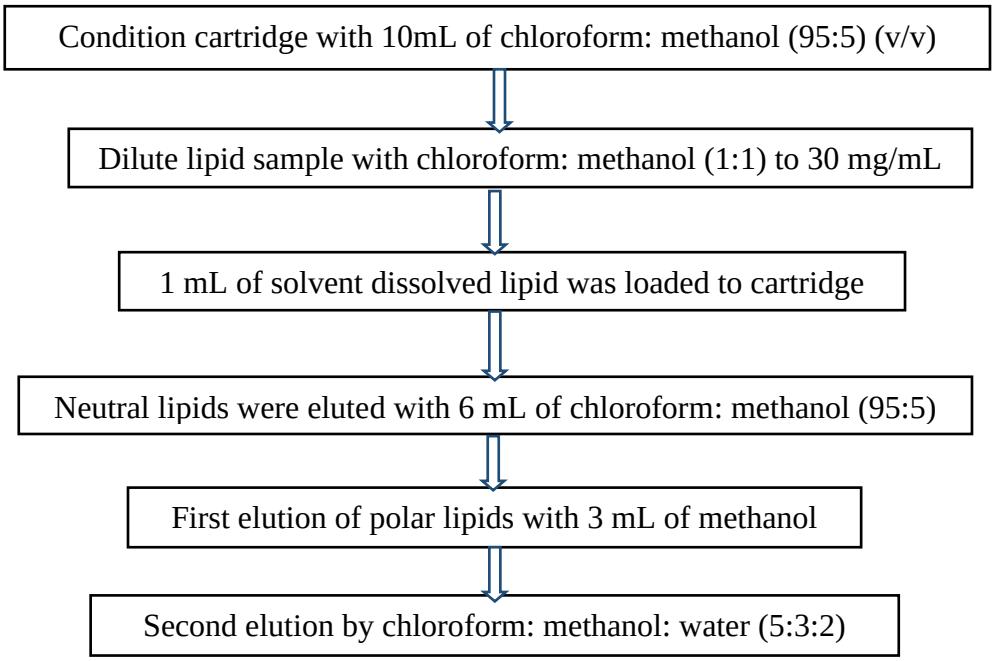


Fig. 3.10. Flow of process for elution of non-polar and polar lipids from lipids extracted from ghee residue (Cheng *et al*., 2019)

3.7. Assisted extraction techniques

Three methods of engineering assisted extraction of PLs from ghee residue were considered in the study. All methods adopted were of batch type with variables associated with machine and process.

3.7.1. Microwave assisted extraction

A domestic microwave oven (M/s L G Electronics inc., New Delhi, India) was used for assisted extraction of ghee residue. The equipment has a capacity of 28 L with a provision for convective heating in addition to microwave heating. It operates at maximum output of 900 W with defined frequency of 2450 Hz. To facilitate easy access for sample loading and cleaning, a rim was placed in cavity to accommodate a glass tray. This removable glass tray seated on rotating wheel helps to expose the sample uniformly to microwaves during treatment (Fig.3.11). Designed dimension of microwave is 30.5 cm height, 49.5 cm depth and 51.0 cm width.

While using microwave for assisted extraction, the unit was operated for 1 min. with a beaker containing water to warm up the cavity. Beaker containing the sample and solvent was placed in centre of the cavity after warm up. Microwave unit was operated at defined power level for a time frame of10s spells to avoid splashing of sample from the container. Equipment was operated only with microwave energy and interference of convection heating was avoided.

Fig. 3.11. Cavity of microwave unit with sample placed in beaker

3.7.1.1. Independent factors for microwave treatment

Three independent factors were considered for the study of microwave assisted extraction process, namely, power (W), time (s) and solvent to solid ratio (v/w). The position of sample in the cavity was maintained at the centre of the platform and same volume container was used for all experiments to maintain uniformity. Range of power was opted based on trials conducted on ghee residue sample before the final design of experiments. Equipment was designed to set power output in five levels (900, 720, 540, 360 and 180 W) whereas, time was adjusted with minimum multiples of10s. Initial two levels of power i.e., 900 and 720 W led to spontaneous heating and splashing of ghee residue from beaker. Also, the solvent evaporated very fast at very short period of exposure which resulted in choosing levels as 540, 360 and 180W for further experiments.

3.7.2. Ultrasound assisted extraction

Ultrasonic cell disrupter BK-650E (Biobase Biodustry, Shandong, China) was used for imparting ultrasound for the ultrasound assisted extraction studies on ghee residue (1 g sample in each trial). Power supply for the unit was set at 220V±5% AC voltage with frequency 50Hz. It consisted of ultrasonic generator, transducer and sound proof box with provision for lifting platform. Horn used in experimentation was 6 mm diameter which was optimal to process 10-100mL of sample. The pulse mode ultrasonicator had an adjustable output of 6.5 to 60W with working frequency of 20-25 kHz. Touch screen with LCD display screen integrated in the equipment allowed to set power, time, probe type, on-off time and

temperature detection. The unit also has a provision to display programme error, overload and over temperature protection (Fig. 3.12).

Fig. 3.12. Ultrasonication unit used for extraction of phospholipids from ghee residue

While operating the equipment, sample was placed on lifting platform which is attached to a vertical shaft inside sound proof box. Lifting platform was raised till the probe immersed into the container by leaving clearance of 1 cm from bottom and confined with screw. Instrument was operated for on and off time of 2 and 3 seconds, respectively in every experimental run. For a minute of operation under such condition, effectively 24 s will be the exposure time on sample.

3.7.2.1. Independent factors for ultrasonication treatment

Four factors were considered as independent in the study which includes ultrasound power (%), time (s), solvent temperature (^oC) and solvent to solid ratio (v/w). As power and time of operation are equipment factors, they were adjusted using equipment settings. Temperature of solvent and solvent to solid ratio were the process parameters. To maintain defined temperature in the experimental setup, dry sample was added into 50 mL beaker having diameter of 40 mm. The beaker was immersed inside another beaker of diameter 50mm having a capacity of 100 mL. The annular space between the beakers was filled with hot water maintained at defined temperature level with a variation of $\pm 2^oC$. Simultaneously water (solvent for extraction) was added in defined quantity maintained at defined temperature into the 40 mm diameter beaker. This process was followed by adjusting beaker inside sound proof box to subject sample for ultrasonication.

3.7.3. Pulsed electric field assisted extraction

The pulsed electric field (PEF) treatment unit used for the study was table-top batch type equipment designed and developed at ICAR-National Dairy Research Institute, Bangalore for generating square waves. The PEF unit comprised of power supply section, power switching system, treatment chamber and control unit. Equipment was designed to alter process parameters such as voltage (25-60 kV), pulse duration (1-1000 µs), pulse frequency (5-100 Hz) and pulse treatment time (0-5 s). Pulse generator was designed to convert line voltage of 230 V/50 Hz by using bridge diode. Electrical signals were converted into square waves using metal oxide semiconductor field effect transistor (MOSFET).

The treatment chamber of the unit was designed as a co-axial cylinder comprising of inner anode and outer cathode. Treatment chamber was designed such that the annular space between anode and container was 1 cm with height of 8 cm with the capacity to contain 240 mL of sample. An oscilloscope was also attached to the unit to visualize pulse output. To adjust different factors, control panel was facilitated with manual knob and trigger to impart electric field to treatment chamber.

Dry sample (10 g) was thoroughly mixed with defined quantity of solvent before filling into the annular space of the treatment chamber to avoid clumps. After positioning container in housing, it was covered with anode cup followed by adjustment of process parameters. The trigger was pressed in multiples of 5 s to attain defined time of exposure.

3.7.3.1. Independent factors for pulsed electric field treatment

Through preliminary trials, power levels of the unit were selected based on better output of PLs extract. Three factors were considered for the study which included voltage (kV/cm), time (s) and solvent to solid ratio (v/w). Though factors like frequency and pulse width was available for alteration in experimental setup, it was set at highest level to induce better impact on extract. Trials conducted at three levels of frequency and pulse width (pulse duration) indicated better outcome at higher treatment levels. Hence, frequency of 90 Hz and pulse width of (900 µs) were set constant for the optimization studies.

3.8. Treatment trials for setting protocol for optimization studies

To develop a protocol and to select best solvent for assisted extraction, three protocols were experimented. Distilled water, ethanol (95%) and enzyme treatment were considered in three protocols. However, factors considered for optimization of protocol was kept constant during experimentation. All experiments were conducted with ghee residue derived after pre-

treatment from same batch; the pre-treatment process included treatment with boiling water (section 3.2.2) and size comminution to 0.25 mm (section 3.2.3). Flow chart for different protocols followed in PLs extraction from ghee residue is shown in Fig.3.13.

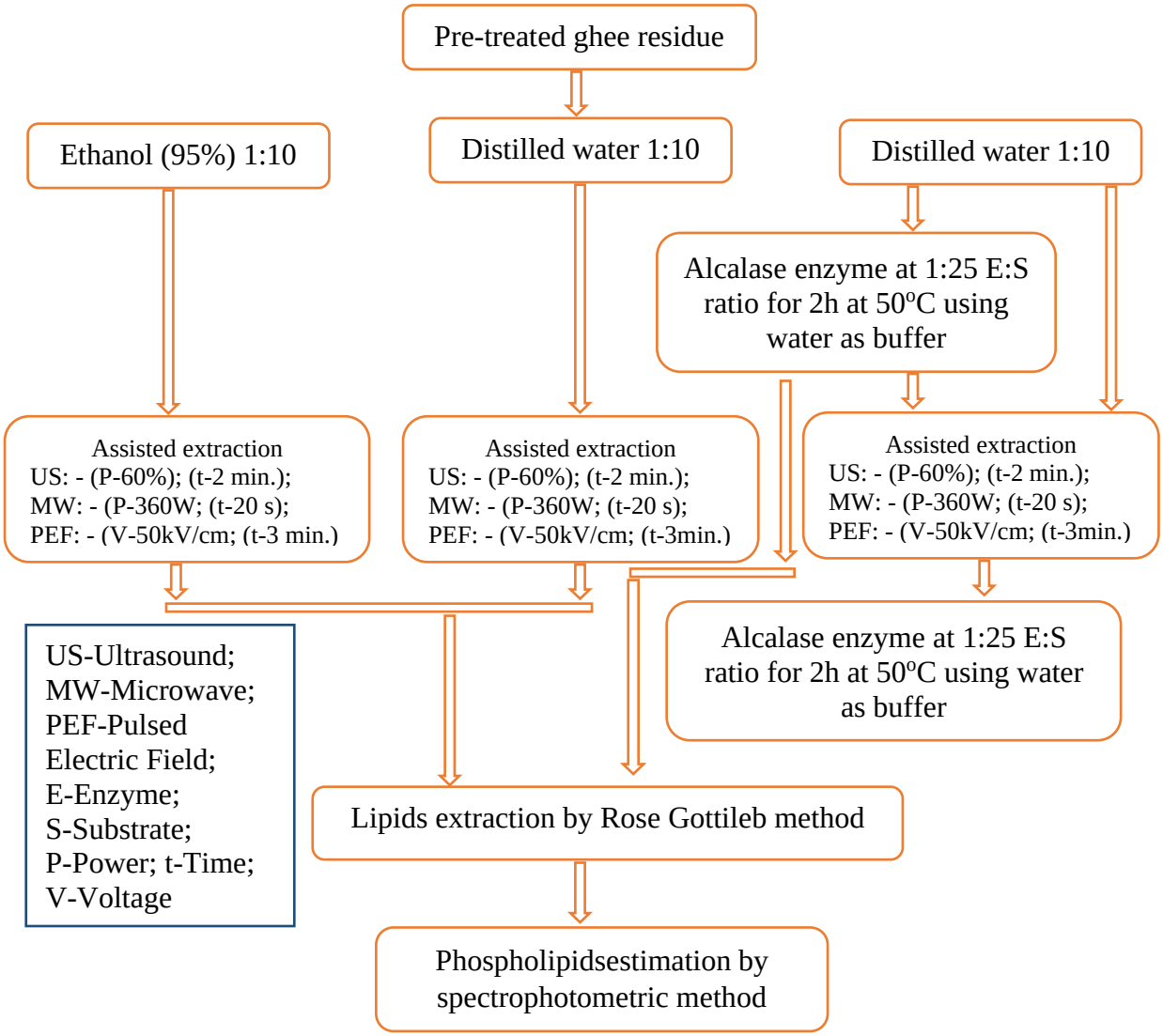


Fig.3.13. Flow chart of three protocols evaluated for phospholipids in ghee residue

3.8.1. Ethanol assisted extraction

Ghee residue obtained after pre-treatment was mixed with ethanol (95%) in 1:10 ratio (w/v). The beaker was closed with aluminium foil and subjected to each of the assisted extraction interventions. In case of microwave treatment, ghee residue and ethanol were mixed in 1:10 ratio and transferred to 50 mL vial and closed with cap. Vial was placed at the centre of microwave cavity followed by treatment at 360 W for 20 s. For the ultrasound assisted process, the ultrasonication probe was inserted into the beaker covered by aluminium foil by forming small hole to avoid solvent splashing during ultrasonication.

The sample was ultrasonicated at 60% power for 2 min. in triplicates. Similarly, PEF treatment was given at voltage of 50 kV/cm for 180s at 1:10 S:S ratio (90 Hz frequency, 900 µs pulse width). Samples treated from all extraction techniques were subjected to solvent evaporation using hot water bath at 75^{o}C. After major amount of solvent was evaporated, beaker was placed in hot air oven maintained at 105±2^{o}C till constant weight was obtained. PLs present in the sample was estimated using spectrophotometric method and expressed on lipids weight basis.

3.8.2. Water assisted extraction

For the second protocol, pre-treated ghee residue was added with distilled water on 1:10 ratio (w/v). As indicated for ethanol treatment, the mixture was treated for ultrasonication, microwave and PEF at same operational conditions. After treatment, the sample was cooled and centrifuged at 4000 rpm for 30 min. to separate ghee residue from extract. The extract was dried at 60^{o}C in a hot air oven till constant weight was obtained, and the dry fraction weight was calculated. Lipids from dry extract were extracted using Rose Gottileb method followed by PLs estimation using spectrophotometric method.

3.8.3. Enzyme treatment and assisted extraction

To evaluate efficacy of assisted extraction treatment on PLs extraction along with enzyme treatment, two-way enzyme treatment was followed i.e., enzyme treatment before assisted treatment and after assisted treatment. The outcome reported comparatively better results for enzyme treatment followed by assisted extraction treatment.

For both cases, the ghee residue was treated with enzyme for optimized time period. For the pre-extraction enzyme treated samples, it was followed by making up of solvent to solid ratio. Enzyme treated extract was subjected to all assisted extraction treatment as mentioned above with water as solvent. The fraction was centrifuged at 4000 rpm for 30 min. to separate extract followed by drying at 60^{o}C till constant weight was obtained. Lipids were extracted from dried fraction of extract using Rose Gottileb method and PLs estimated by spectrophotometric method.

3.9. One-factor-at-a-time for selection of levels

One-factor-at a-time (OFAT) approach was adopted to select levels of independent variables for all assisted extraction techniques. Under this technique, one factor was varied at larger frequency by keeping other factors constant. Based on yield of PLs from the treatment lower, middle and higher levels were chosen for optimization studies.

3.9.1. Microwave assisted extraction technique

Power levels for microwave assistance were fixed based on practical feasibility of the experiment. Through preliminary trials, power levels for microwave assisted treatment were selected at 180, 360 and 540W. By keeping microwave power at middle level i.e. 360 W, solvent to solid ratio was varied at 5, 10 and 15. Also, from selected solid to solvent ratio and power levels, time of treatment was varied between 30, 50 and 70 s. Based on yield of PLs, lower, middle and higher levels were selected.

3.9.2. Ultrasound assisted extraction technique

Ultrasonication power output levels of the unit are designed to vary between 10 to 100% (6.5 to 650W). However, the operation manual of the instrument recommends that the instrument be operated between 20 to 80% to avoid critical operation conditions. Hence, power levels were operated at permissible limit with 80% as highest levels and 70 and 60% as middle and lower level.

By keeping power level at 70%, solvent temperature at 50^{o}C, S:S ratio of 12.5, treatment time was varied at 2, 4 and 6 min. PLs yield of all these three levels were calculated in triplicates. In the second set of study, power level at 70%, solvent temperature at 50^{o}C and selected time levels were kept constant and S:S ratio was varied at 7.5, 12.5 and 17.5. Based on the best results obtained for PLs yield, S:S ratio was selected. In similar lines, solvent temperature was varied between 30, 50 and 70^{o}C by keeping other selected factors constant.

3.9.3. Pulsed electric field assisted extraction technique

Independent factors considered for evaluation of the assisted extraction process in this study were voltage, treatment time and solvent to solid ratio. To effectively utilize operational parameters, pulse frequency and pulse duration was fixed at 900 Hz and 900 µs, respectively. Treatment chamber of the PEF applicator is designed to accommodate 240 mL of sample with minimal of 100 mLto keep treatment effective. Hence, at least 100 mL was maintained as sample size during PEF treatment throughout experiments.

Keeping voltage at 50 kV/cm and S:S ratio at 10, treatment time was varied at 120, 240 and 360 s. PLs yield in three levels were calculated in triplicate. Similarly, voltage at 50 kV/cm, treatment time selected in previous step were set constant by varying S:S ratio at 5, 10 and 15. Based on PLs yield in trials lower, middle and higher levels of the selected parameters were chosen for optimization studies. The range of process parameters trailed and selected for microwave, ultrasound and PEF treatment are listed on Table 3.3.

Table 3.3. Range of factors selected for assisted extraction treatments

Sl. No.	Type of treatment	Factors	levels studied	Levels considered
1	Microwave treatment	Power (W)	180; 360; 540	180; 360; 540
		Time (s)	30; 50; 70	40; 50; 60
		S:S Ratio (w/v)	5; 10; 15	5; 7.5; 10
2	Ultrasound treatment	Power (%)	60; 70; 80	60; 70; 80
		Time (min.)	2; 4; 6	3; 4; 5
		S:S Ratio (w/v)	7.5; 12.5; 17.5	10; 12.5; 15
		Solvent temp. (^{o}C)	30; 50; 70	60; 70; 80
3	PEF treatment	Voltage (kV/cm)	40; 50; 60	40; 50; 60
		Time (min.)	2; 4; 6	3; 4; 5
		S:S Ratio (w/v)	5; 10; 15	7.5; 10; 12.5

3.10. Empirical models to study kinetics of extraction

For modelling of solvent extraction from various matrix, different empirical models have been reported. These models were either developed from fundamental principles or adapted models of mass transfer equations. Empirical models are more suitable for extraction processes involving assisted means such as microwave, ultrasound and electrical as the phenomena cannot be adequately described theoretically. Knowledge and understanding of extraction kinetics of natural products from biological matrix are also of economic importance in view of the fact that extraction process is a major industrial operation. Empirical kinetic models describe mathematical variations in amount of target compounds in solid matrix or liquid extract with time. These kinetic models are typically simpler than physical models but, are still suitable for engineering purposes (Kitanovic *et al*. 2008).

Four empirical models were chosen for the study to understand the extraction kinetics of PLs from ghee residue extracted during the three engineering assisted treatments. The experiments were conducted at levels optimized for extraction of PLs. All experiments in the study were conducted in triplicates and mean values of yield of PLs for the respective process were fitted to the selected models. The following general assumptions were made when considering the models:

1. All solid particles are spherical in shape with a uniform size
2. The extractable component is evenly distributed in the solid

3. The diffusion co-efficient of extractable component is constant
4. Solid particles are well dispersed in the extracting solvent

3.10.1. Parabolic model

Thismodel successfully describes the two-step extraction process consisting of washing followed by diffusion of extractable substances. This model agrees with washing of weakly bound material, which is instantaneously leached, followed by the diffusive release (Galgano *et al*., 2021). The washing coefficient A_0 represents the extraction yield recovered instantaneously (i.e., for t = 0), while the constant A_1 is the diffusion rate constant.

$$C_t = A_0 + A_1 * t^{1/2} \text{----------} 3.5$$

Where: C_t = Phospholipids yield

A_0= Initial yield

A_1=Diffusion co-efficient

3.10.2. Power law model

Diffusion of compounds from a non-swelling material is the extraction mechanism described under power law model (Cheung *et al*. 2013). This model has successfully explained extraction kinetics of different compounds from biological material (Sant'Anna *et al*., 2012; Cheung *et al*., 2013; Dong *et al*., 2014; Patil and Akamanchi, 2017).

$$C_t = B * t^n \text{----------} 3.6$$

Where: C_t = Phospholipids yield

B= Constant related to extraction rate

n= Power law exponent

B is a constant which quantifies the characteristics of the particle-active substance in the system. Whereas, n is the diffusion exponent, characterizing the mass transfer mechanism. In most vegetable materials, its value is lower than 1.

3.10.3. Peleg's model

It is also termed as hyperbolic model andhas been applied in food engineering science.

$$C_t = C_o + \frac{t}{K_1 + K_2 t} \text{----------} 3.7$$

Where, K_1 is model rate constant

K_2 is model capacity constant

C_t is Phospholipidsyield at time t

C_o is the initial concentration of the phospholipids in the sample

However, in this case, the extraction rate at the very beginning K_1 and constant related to maximum extraction yield K_2 were taken into consideration. Thus, the hyperbolic model used to describe the PLs extraction from ghee residue is expressed as.

$$C_t = \frac{K_1 t}{1+K_2 t} \text{----------3.8}$$

From the above equation, it is important to state that the extraction is first-order at the very onset, and drops to zero-order in the latter phase. As such, when $K_2 t \ll 1$, then above equation reduces to $y \approx K_1 t$

By linearizing above equation

$$\frac{1}{C_t} = \frac{1}{K_1} \text{x} \frac{1}{t} + \frac{K_2}{K_1} \text{----------3.9}$$

Where, K_1 is constant related to extraction rate at the beginning

K_2/K_1 is Peleg's capacity constant

3.10.4. Elovich's equation

This equation is reported to describe the kinetics of extraction process when the extraction rates of substance from solid declines exponentially with extraction yield.

$$C_t = E_o + E_1 \ln (t) \text{----------3.10}$$

Where, E_0 is the initial yield and E_1 is the initial extraction rate

3.10.5. Phospholipids extraction and quantification

The extraction kinetics of PLs from ghee residue using the engineering assisted extraction was studied at optimized levels of parameters for each of the three assisted processes. Ghee residue was mixed with the solvent at ratio optimized for different assisted treatments. The treatment was terminated at 5, 10, 15, 20, 25, 30, 40, 45, 50 55 and 60 s for microwave; 30, 60, 90, 120, 150, 180, 210, 240, 270 and 300 s for ultrasonication, and 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275 and 300 for PEF extraction. After treatment at defined timings, the extract was centrifuged and dried to calculate dry fraction of extract. Lipids present in dried fraction were estimated by Rose-Gottileb method, followed by PLs estimation by spectrophotometric method. Data of PLs yield against time for each of the assisted extraction techniques was compiled to compare model feasibility for extraction studies.

3.10.6. Fitting of kinetic models

The experimental data obtained through trials conducted as described in section 3.10.5, were fitted to the various models considered in the study. The software Origin 19B (OriginLab Corporation, USA) was employed for the data fitting and model parameters, R^2 and adj. R^2 values were calculated. Non-linear fit tool was used for estimation of model parameters and statistical characteristics. This non-linear fit tool is inbuilt with 170 fitting functions which can be used as ready reckoner for defined equations. The considered model was also be incorporated into the tool using user defined function, when the library lacked the desired function. Graphs pertaining to the models were extracted from the software with defined model parameters and statistical characteristics. Models were compared based on R^2 and adj. R^2 reported for each model and best model was selected based on highest R^2 values.

3.10.7. Statistical analysis of experimental data for process optimization

To optimize process and machine parameters for extraction of PLs from ghee residue, L_9 orthogonal array was employed. Analysis was carried out using the statistical software Minitab 18 (Minitab Inc., State College, Pennsylvania, USA). Dependent factors evaluated for the study were PLs yield and antioxidant activity of the extract. Both dependent variables were evaluated independently for optimal process conditions.

Taguchi method uses 'loss function' to calculate deviation between experimental and predicted values. This loss function was further converted into signal-to-noise (S/N) ratio, where 'S' denotes signal (desirable value) and 'N' denotes noise (undesirable value). Optimization of process was run for maximization of PLs yield and antioxidant activity of extract independently. Most influential factors and degree of influence of each factor was evaluated by S/N ratio reported in the experiment. Influence of each factor on maximization of response variable was analysed by ANOVA. Regression equation including all dependent factors was evaluated and R^2 values were estimated.

3.11. Characterization of phospholipids extract

As PLs exhibit emulsion behaviour due to the presence of hydrophilic head and hydrophobic tail, its emulsion characteristics were studied.

3.11.1. Evaluation of hydrophilic-lipophilic balance (HLB)

Emulsions are heterogeneous mixtures formulated by mixing droplets of one liquid in another immiscible liquid. As interfacial tension between two immiscible liquids creates thermodynamic instability, surfactants were added to the system. For creating stable

emulsion, one or combination of emulsions having defined HLB values were added. To analyse the required HLB values of PLs extract, it was subjected for different tests during storage.

3.11.1.1. Preparation of samples of PL-rich extracts from ghee residue for HLB studies

The PL-rich extract from ghee residue obtained from the optimised assisted extraction techniques were dried till constant weight was obtained. Tween 20 having HLB value of 16.7 was selected as hydrophilic surfactant and Span 80 with 4.3 HLB value was used as lipophilic surfactant. Emulsions were prepared by adding 5% (w/w) of dried PLs extract, 3% (w/w) of surfactants and 92% (w/w) of water. The detailed list of HLB values acquired with combination of two surfactants is enlisted in Table 3.4.

Table 3.4. Proportions of span 80 and tween 20 added to obtain different HLB values

Sl. No.	HLB	Span 80 (%)	Twine 20 (%)
1	6	13.7	86.3
2	7	21.8	78.2
3	8	29.8	70.2
4	9	37.9	62.1
5	10	46.0	54.0
6	11	54.0	46.0
7	12	62.1	37.9
8	13	70.2	29.8
9	14	78.2	21.8
10	15	86.3	13.7

3.11.1.2. Centrifugal stress induced separation

Centrifugal stress was induced to investigate the resistance offered by emulsions to different speeds of rotation. Wintrobe tubes were filled with emulsions prepared as described in section 3.11.1.1 and Table 3.4 and inserted into 15 mL centrifuge tubes. They were subjected to different speeds of rotation (430, 860, 1280, 2135, 2562, 3000, 3500 and 4000) for 5 min. By visually inspecting the layers of the emulsion in the graduated Wintrobe tubes,the separation of emulsion was noted and thickness of creamy layer was measured (Ferreira *et al*., 2010).

3.11.1.3. Droplet size analysis by optical microscopy

By using optical microscope (Olympus, U-TV1X-2, T2, Tokyo, Japan) fitted with 40x objective and 10x ocular micrometre scale, emulsion droplet size was observed. To ensure uniform dispersion, gently shaken samples were transferred to a sample container. Each time sample was diluted to 100 times using distilled water before observing droplet size. Using a

DSLR camera (Canon EOS 3000D DSLR, 18 - 55 mm Lens), the images observed on the microscope slide were captured and transferred to larger screen. Growth in droplet size between 1st and 30th day of storage was observed and recorded.

3.11.1.4. Creaming index

Rate of creaming was calculated by measuring creaming index during storage. Creaming index was calculated as ratio of total height of cream layer (CC) and total height of emulsion layer (CT). Heights of CC and CT were measured directly from graduated scale of glass jar.

$$\%CI = (CC/CT) \times 100 \quad \text{----------3.11}$$

3.11.1.5. pH measurement

By using pre-calibrated pH instrument (M/s Eutech, laboratory instruments, Bangalore), emulsion prepared by ghee residue extract was characterised. The probe end of pH meter was inserted into emulsion and its pH was measured without disturbing emulsion in container.

3.11.1.6. Conductivity of emulsion

It was reported that water in oil emulsion exhibit lower conductivity compared to oil in water emulsion. Conductivity of emulsion was measured using portable conductivity meter (AMPEREUS 3 in 1 digital water TDS EC and temperature meter) during storage and expressed in µS/cm.

3.11.1.7. Measurement of average particle size

From the previously discussed methods, emulsions exhibiting favourable attributes were screened. The selected emulsions were tested for droplet size distribution and average particle size using a laser light diffraction instrument (Zen 3600, Malvern) with a dual-wavelength detection system. The measurements were done on the day of emulsion preparation and 60th day of storage. Emulsion samples (0.1 mL) were diluted in the glass cell filled with distilled water (100 mL) before measurement.

3.11.2. Emulsifying properties

To evaluate quantum of extract required to be added to get stable emulsion, extract was evaluated for emulsifying properties. Extracts obtained at optimized levels from all assisted extraction treatments were dried till constant weight was obtained and the dry fraction was used as an emulsifier.

3.11.2.1. Emulsifying capacity

Emulsion or hydrocolloid suspensions was prepared by adding 3, 4 and 5% of dry extract from microwave and ultrasound extract and 5, 7.5 and 10% of PEF extract into 30mL of water (continuous medium). The suspension was mixed with 3mL of corn oil followed by homogenization using ultrasound disruptor for 15min. Resultant suspension was subjected for centrifugation at 800 g for 10 min. Emulsifying capacity of suspension was calculated as follows (Sciarini *et al*., 2009)

$$Emulsion\ Capacity = \frac{e_v}{t_v} \times 100 \text{----------3.12}$$

Where, e_v-Emulsion volume

t_v-Total volume

3.11.2.2. Emulsion stability

The stability of the emulsion was expressed in terms of its emulsifying capacity after heat treatment (Sciarini *et al*., 2009). Emulsion or hydrocolloid suspensions prepared as outlined in section 3.11.2.1. Suspensions were heated in hot water bath maintained at 80^{o}C for 30 min. Heat treated suspensions were centrifuged at 800g for 10 min. to calculate emulsion capacity as follows.

$$Emulsion\ Stability = \frac{f_{ev}}{i_{ev}} \times 100 \text{----------3.13}$$

Where, f_{ev}-Final emulsion volume

i_{ev}- Initial emulsion volume

3.11.2.3. Zeta potential and average particle size

Emulsions selected at levels where highest emulsion capacity and stability was observed were further evaluated for zeta potential and particle size analysis. Using particle electrophoresis instrument (Zetasizer, Zen 3600, Malvern) electrical charges on emulsion droplets were examined. The emulsion samples were diluted 1:200 using distilled water in an electrophoresis cell that had electrodes at both ends. The measurements were done on 1st, 15th and 30th day of storage at 4±1^{o}C.

3.12. Selection of dairy product to replace conventional emulsifier with the PL–rich extract from ghee residue

Extract obtained from ultrasound and microwave assistance was experimented as replacer for conventional emulsifiers. The analysis was based on the methodology reported by El-Aziz *et al*., (2015) for comparing the performance of flaxseed and cress seed mucilage extract with

commercial guar gum (Based on the HLB values obtained for the extract, ice cream was identified as the suitable product). Two sets of experiments were designed for the study. In the first set of experiments, guar gum was replaced at the levels of 25%, 50%, 75% and 100% with the obtained extract in the ice cream mix formulation. In the second set of experiments, ice cream mix formulation with glyceryl monostearate (GMS) as emulsifier and sodium alginate as stabilizer was considered and GMS in the formulation was replaced at 50 and 100% levels with ghee residue extract. Ice cream mix and hardened ice cream was evaluated for different attributes and compared with control sample prepared using the conventional formulation with guar gum and GMS, respectively.

3.12.1. Titratable acidity of ice cream

Acidity of ice cream mix was measured as per AOAC (2005). Ice cream mix was thoroughly mixed and 10g of sample was collected into a beaker. Sample was titrated against 0.1N NaoH after addition of 2-3 drops of phenolphthalein indicator. First appearance of faint pink colour was considered as end point of titration. Acidity of ice cream mix was expressed as % lactic acid by weight with following formula

$$\text{Acidity (\% Lactic acid)} = \frac{9xOxP}{W} \text{----------3.14}$$

Where,

O: Volume of NaOH (mL)

P: Normality of NaOH (N)

W: Weight of sample

3.12.2. Total solids of ice cream mix

Total solids in ice cream mix were evaluated using following procedure. Approximately, 5g of sample was taken in a tared moisture dish followed by evaporation in water bath. Moisture dishes were transferred to hot air oven maintained at 98-100^{o}C for 3 h followed by cooling by transferring into desiccator. The procedure of heating, cooling and weighing was repeated till successive weight difference not exceeded 0.5mg. Total solids in ice cream mix were calculated by using equation.

$$\text{Total solids} = \frac{\text{weight of sample after drying}}{\text{weight of sample taken}} \text{x } 100 \text{----------3.15}$$

3.12.3. pH of ice cream mix

The pH of the ice cream mix was measured by immersing the probe end of the pH meter into ice cream mix. Before testing for pH values, instrument was calibrated using standard solutions of known pH values.

3.12.4. Textural characteristics of ice cream mix and ice cream

The textural properties of the ice cream mix and hardened ice cream was analysed using the texture analyser (Stable microsystems, Model: TAXT plus, UK). Firmness (N), consistency (N.s), cohesiveness (N) and index of viscosity (N.s) of the ice cream mix were determined by using back extrusion with designated test rig (A/BE) attached to a 5kg load cell.

Texture profile analysis (TPA) of ice cream sample was performed on ice cream samples using a 3 mm- cylindrical probe attached to 30 kg load cell. The test speed was 3 mms^{-1} with trigger force of 5 g, penetration depth of 25 mm and collection rate was 200 points per second. TPA analysis was carried after hardening of ice cream at -18^{o}C for 24 h and cooling arrangements were maintained till sample was placed on texture analyser platform. At least three measurements were recorded for each category samples measured from different containers. TPA results were expressed as hardness (N), cohesiveness (N.s), gumminess and adhesiveness values.

3.12.5. Colour measurement of ice cream mix and ice cream

Using computer-based image analysis, colour values of both mix and hardened ice cream was calculated. Samples were transferred into 50 mm glass petri plate and spread uniformly throughout surface. Petri plate was placed on flatbed of scanner (Canoscan 9000 F, Mark II) and scanned at 600 dpi resolution. Captured images were imported to Adobe Photoshop 7.0 software and ‘L’, ‘a’ and ‘b’ values obtained from histogram window. Colour values were defined by three parameters namely, L*, a* and b* (International Commission of Illumination (CIE, 1986). Where, L* is a measure of lightness or illuminance which ranges between 0 (black) to 100 (white). A* is one of the two chromatic components range from -120 to 120 (green to red) and b* (blue to yellow). In the software measuring scale ranged between 0 and 255 to characterise L (lightness), ‘a’ and ‘b’. These values were converted into CIELAB values using following formula (Yam and Papadakis, 2004).

$$L^{*}= \left[\frac{L}{255}\right] x100 \text{----------} 3.16$$

$$a^* = \left[\frac{240a}{255}\right] - 120 \text{----------} 3.17$$

$$b^* = \left[\frac{240b}{255}\right] - 120 \text{----------} 3.18$$

Each sample was measured in triplicate and total change in colour was calculated by using equation (Demir, *et al*., 2021)

$$\Delta E = \sqrt{(L - L_0)^2 + (a - a_0)^2 + (b - b_0)^2} \text{----------} 3.19$$

Where, L_0, a_0 and b_0 were values of control samples

3.12.6. Overrun

Overrun of ice cream mix was measured as per method provided by Bolliger *et al*., (2000). Increase in volume of ice cream mix during ice cream making process was calculated by following formula

$$\% \text{ Overrun} = \frac{W_1 - W_2}{W_2} \text{----------} 3.20$$

Where, W_1 is weight of unit volume of ice cream mix and W_2 is weight of unit volume of ice cream.

3.12.7. Fat destabilization

Fat destabilization during ice cream making was calculated as per the method reported by Goff and Jordan, (1989). Aliquot of ice cream mix (40 mL) was removed from ice cream freezer every 5 min. from starting of freezing. Whole sample was thawed, gently mixed and 3 g of sample was weighed into beaker. By adding distilled water, the ice cream mix was diluted to 1:500 dilutions. The diluted sample was transferred into a spectrophotometer cuvette and placed in centrifuge tube. Tube was subjected to centrifugation at 1000 rpm and allowed to stand for 10 min. Absorbance of sample in cuvette was measured using spectrophotometer at 540 nm and distilled water was used as blank. Percentage fat destabilization was calculated using following formula.

$$\% \, of \, fat \, destabilization = \frac{\text{Absorbance (unfrozen mix)} - \text{Absorbance (sample)}}{\text{Absorbance (unfrozen mix)}} \text{----------} 3.21$$

3.12.8. Melting properties of ice cream

First dripping time (min.), complete melting time (min.) and melting rate (g/min.) of ice cream sample was measured by keeping hardened ice cream on wire mesh (25 holes/cm^2)

above graduated cylinder. Evaluation of melting properties was carried by keeping samples in BOD chamber at 20±2^{o}C. Dripping weight of ice cream sample was measured at interval of 5 min. During the course of test, first drip falling into the cylinder was recorded as first dripping time (Goral *et al*., 2018). The data of time recorded after first drip was counted and measured for melting rate as g/min. (Kurt and Atlar, 2018).

3.12.9. Sensory analysis

Sensory evaluation of the ice cream was measured according to Khalil and Blassey, (2019). A panel composed of 15 members were selected based on their interest in the sensory evaluations of ice cream and were trained by testing commercial ice cream. Samples (50 g) were given to a group of 15 test panellists. The selected panellists ranged in age from 25 to 55 years. The evaluated attributes include external colour and appearance (CA), structure and consistency (SC), taste and odour (TO), icy structure (IS), melt in mouth (MM), gummy structure (GS), and total acceptability (TA) under 9-point hedonic scale. The samples were randomly coded and presented to each panellist immediately after taking away from deep freezer.

Chapter-4

RESULTS AND DISCUSSIONS

4.0 RESULTS AND DISCUSSION

This chapter presents the various results obtained for the experiments conducted as per the objectives defined for the study. The details include development of process protocols for the assisted extraction methods and optimization of process parameters for extraction of phospholipids (PLs). Defined kinetic models were compared to understand pattern of PLs movement into the solvent. Detailed investigation of the extract for its characterization and identification of suitable dairy productforutilization as a replacer to conventional emulsifying agentis also reported and discussed herewith.

4.1. Preparation of ghee residue and its pre-treatments

Ghee residue was prepared using two methods to compare the yield of lipids and PLs. Ghee residue prepared using the creamery butter method(Section 3.1.1) was subjected to pre-treatments to reduce neutral lipids. The observations recorded with regard to the effect of method of preparation of ghee residue, its pre-treatments and the resultant lipid and PLsare discussed below.

4.1.1. Preparation of ghee residue using creamery butter and direct cream method

Ghee residuewas collected after the preparation of ghee using creamery butter method by straining the ghee through muslin cloth. As very limited pressure was applied in this straining process, obviously the amount of lipids adsorbed in the matrix at this stagewas expected to be high.As a comparative study,ghee residuewas also prepared by direct cream method (Santha, 1977) to evaluatethe effect of method of preparation on yield of lipids and PLs.The results of this evaluation are tabulated in Table 4.1. It can be seenthat even though there was a significant increase (2.62 folds) in ghee residue obtained in the direct cream method, there was 51.94% decrease in total lipids compared to creamery butter method. Low PLs content in direct cream method (0.95±0.08%) indicated the influence of method of preparation on the fat profile of the residue (Table 4.1).

Table. 4.1. Yield of ghee residue, lipids and phospholipids from two methods of ghee preparation

Sl. No	Method	GR yield (%)	Lipids (%)	Phospholipids (% GR basis)	Phospholipids (% on lipids basis)
1	Creamery butter method	5.16 ± 1.53^{b}	54.55 ± 6.03^{a}	4.98 ± 1.26^{a}	9.08 ± 1.88^{a}
2	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.

The observed values were compared with the data published in literature. The amount of ghee residue and PLs reported in Table 4.1 were comparable to the data reported by Santha, (1977). Similarly, Janghu *et al*. (2014) also reported the yield of ghee residue obtained from direct cream methodas 13.1% and from creamery butter method as 4.96%. It is postulated that the high serum solids present in ghee residue contributed to lesser lipids and PLs yield when expressed on total ghee residueweight basis. While working with different fractions of fat in cream, Pal and Rajorhia,(1975) observed that the increase in ghee yieldcorresponded with a reduction in amount of ghee residue. This was attributed to the yield of ghee residue being 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 of ghee preparation yielded higher PLs content in the residue. Hence, this method was adopted for preparationof ghee residue throughout study.

4.1.2. Application of pressure to remove excess lipids from ghee residue

The manual pressure applied on ghee residue as it was strained through the muslin cloth to remove residual lipids was very low. Hence, ghee residue was subjected to varied pressure levels using a hydraulic press to remove loosely adhering lipid fraction. With increase in applied pressure, more lipidswere observed to be removedfrom the residue resulting in a reduction in the lipids present in ghee residue. Based on this preliminary investigation, pressing at 5kg/cm^2 for 5 min. was adopted for the ghee residue, which resulted in reduction to the tune of 13%. The total lipid content in the pressed ghee residue was recorded to be 41%±0.28% (Fig.4.1).

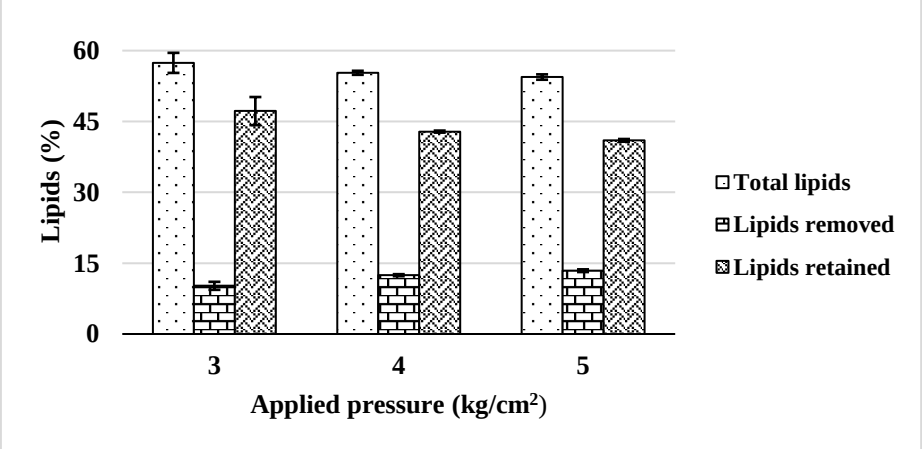


Fig. 4.1. Lipid content of pressedghee residue at different pressure levels

For the record, pressing at pressure beyond 5 kg/cm^2 did not show any considerable change in lipids content of the residue. Hence, pressure of 5 kg/cm^2 for 5 min. was considered optimal

for expulsion of loose fat fromghee residue. The hydraulic press used to apply the pressure on the residue is depicted in Fig.4.2.

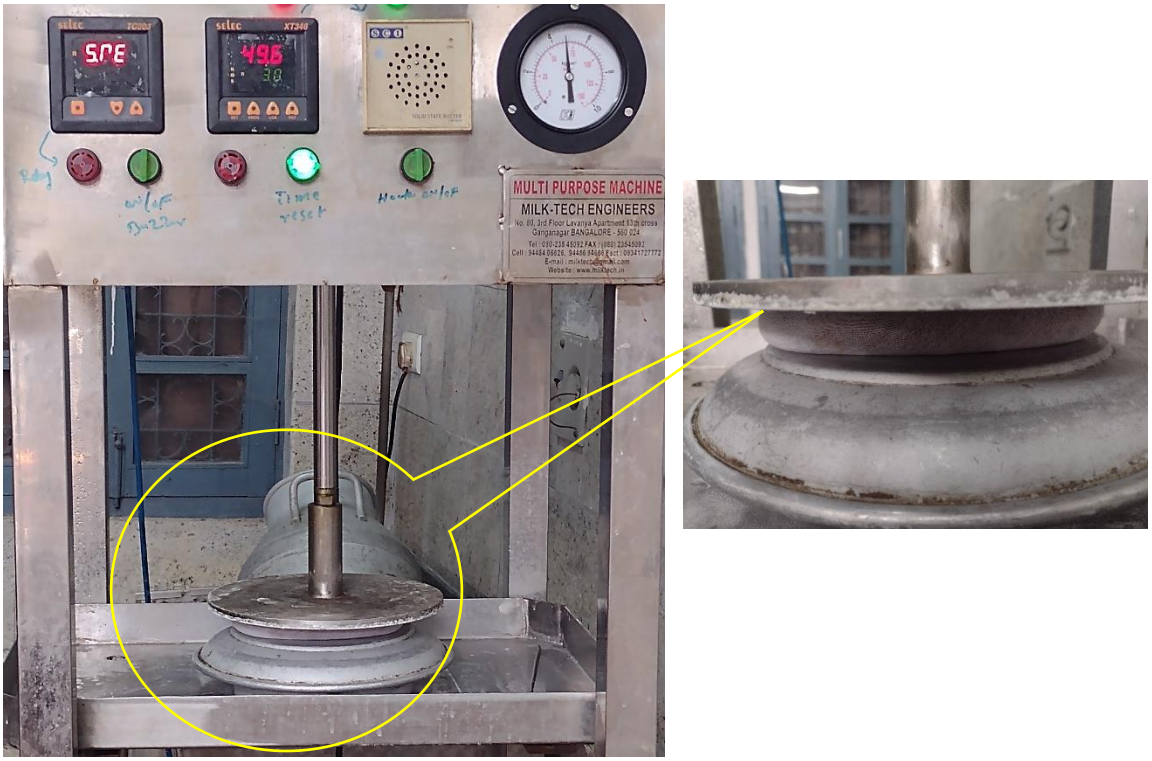


Fig.4.2. Hydraulic press used for expression of lipids from ghee residue

4.1.3. Pre-treatment with n-hexane to remove neutral lipids

Ghee residue subjected to hydraulic pressure at 5 kg/cm^2 for 5 min., was subjected to solvent treatment using n-hexane to separate 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 in this study and the results are presented in Fig. 4.3. Both factors were observed to positively influence the separation and removal of lipids from the residue (Fig. 4.3a). At a solid to solvent ratio of 1:2% (w/v), 8.21% of the lipids were removed after a contact period of 20 min. against 11.32% for 60 min. of contact. However, when solids to solvent ratio was increased to 1:4 (w/v) and the contact period was held at 60 min., 17.04% lipids were removed from the residue and the resultant ghee residue retained 27.78% of lipids after solvent extraction (Fig. 4.3b). The above observations affirmed the influence of contact time and solid to solvent ratio on the mass transfer phenomena i.e.lipids movement from the residue.

Weller and Hwang, (2005) also emphasised on the improvement in lipid yield with increase in solvent to solid volume (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. Further, evaluation of lipid quality as criteria for selection of optimal time and concentration was mooted in this study.

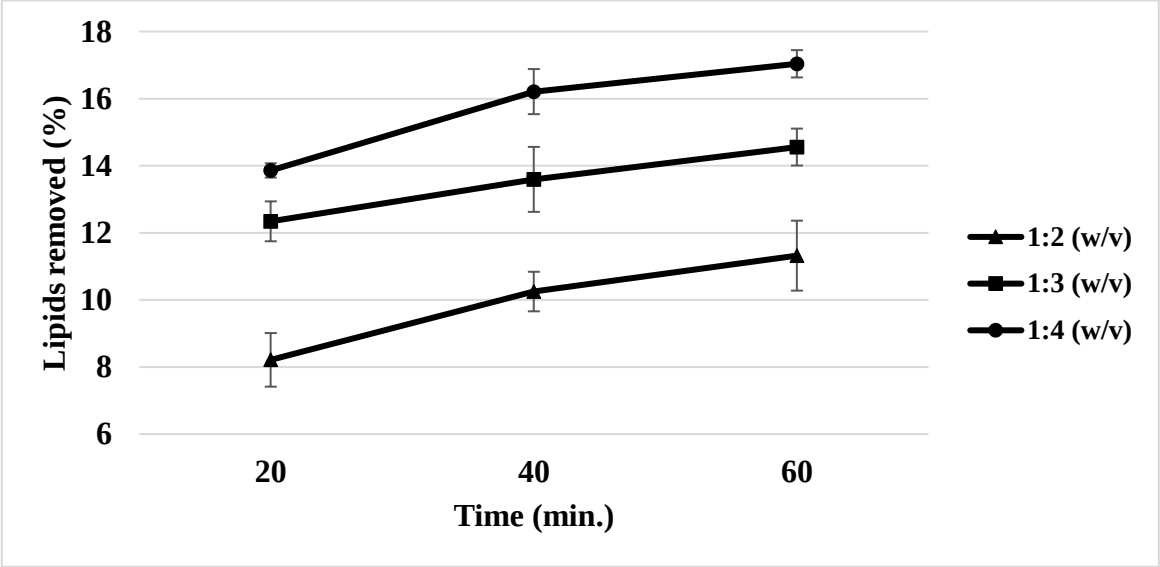


Fig. 4.3. (a) Lipids (%) removed in ghee residue by n-hexane treatment at different time and solids to solvent ratio

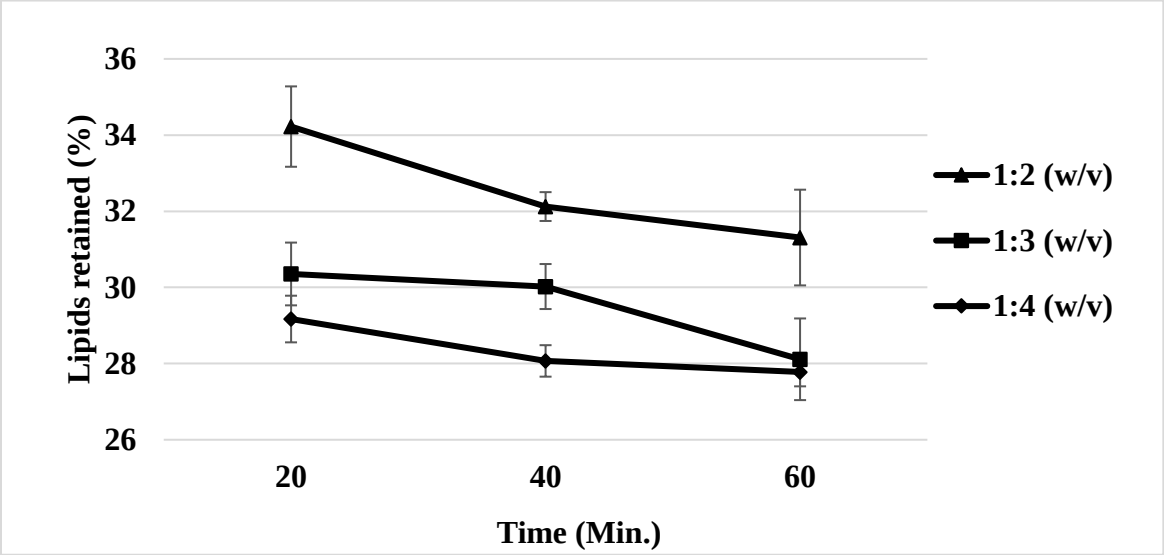


Fig. 4.3. (b) Lipids (%) retained in ghee residue by n-hexane treatment at different time and solids to solvent ratio

The ghee residue post-treatment with n-hexane was also evaluated for its phospholipids content, which was expressed on ghee residue and lipid wt. basis (Fig. 4.4 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 main reason attributed

to this increase in PLs content is due to the movement of neutral lipids from the ghee residue matrix in to 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 also demonstrated the efficacy of the solvents in classifying the neutral lipids in upper phase and polar lipids in middle phase of the solvent.

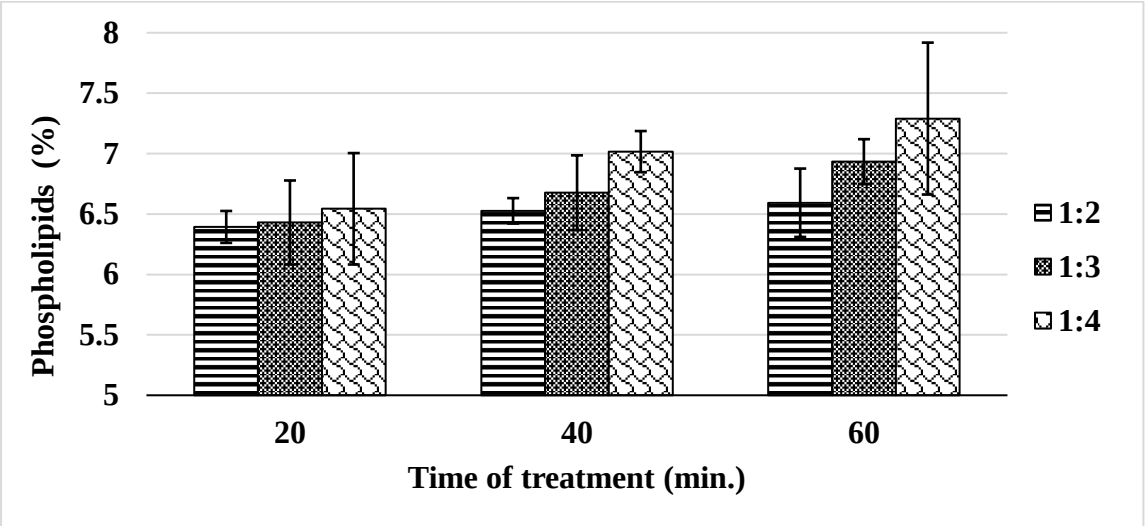


Fig.4.4. (a) Phospholipids expressed on dry weight after n-hexane treatment

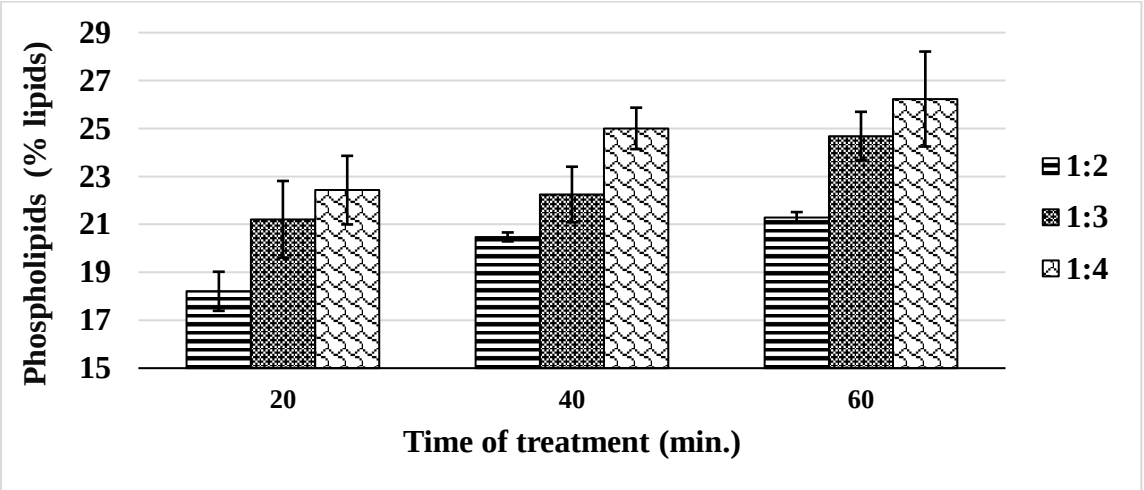


Fig.4.4. (b) Phospholipids expressed on lipids weight after n-hexane treatment

Statistical analysis of the data indicated significant difference among the PLs content in the 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 (Table 4.2). The study concludedthat there was significant improvement in the PLs content ofghee residue with increase in time and solid to solvent ratio when treated with n-hexane.

Table 4.2. Analysis of variance for phospholipidsretention in ghee residueaftern-hexane as solvent

Source of Variation	SS	df	MS	F	P-value	F_{crit}
Time	1.0622	2	0.5311	4.7971	0.0213	3.5546
Solid solvent ratio	0.9067	2	0.4533	4.0948	0.0342	3.5546
Interaction	0.2363	4	0.0590	0.5336	0.7127	2.9277
Error	1.9928	18	0.1107			
Total	4.1981	26				

4.1.4. Pre-treatment with boiling water

Boiling water was also evaluated as a solvent for pre-treatment of the pressed ghee residue considering the contact time (20, 40 and 60 min.) and solids to solvent ratio (1:2, 1:3 and 1:4) as factors. The total lipids removed and retained in ghee residue after pre-treatment with boiling wateris depicted in Fig. 4.5. Nearly 17% of lipids were removed from the residue at solids to solvent ratio of 1:2 for 60 min. (Fig. 4.5 (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 (Fig. 4.5(b)).

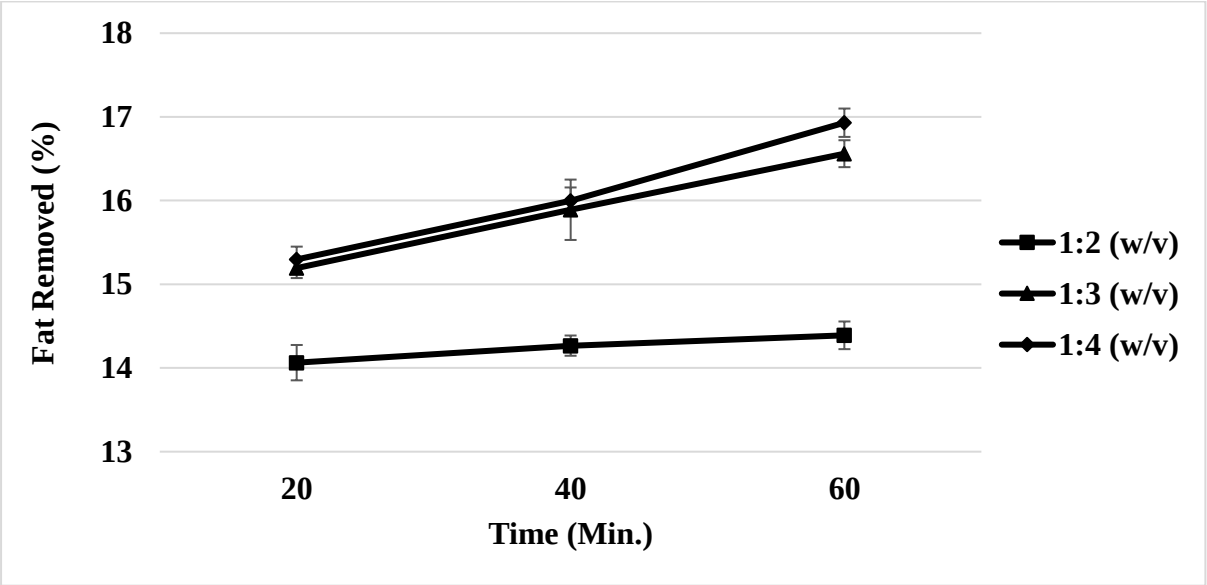


Fig.4.5. (a) Lipids (%) removed in ghee residue by boiling water treatment at different time and solids to solvent ratio

Chemat, (2015) highlighted that water could be employed as a good solvent to remove polar lipids from a mix at high temperature (100 and 374 ^{o}C) and pressure (22.1 MPa). At elevated temperature and pressures, water undergoes self-ionisation 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.

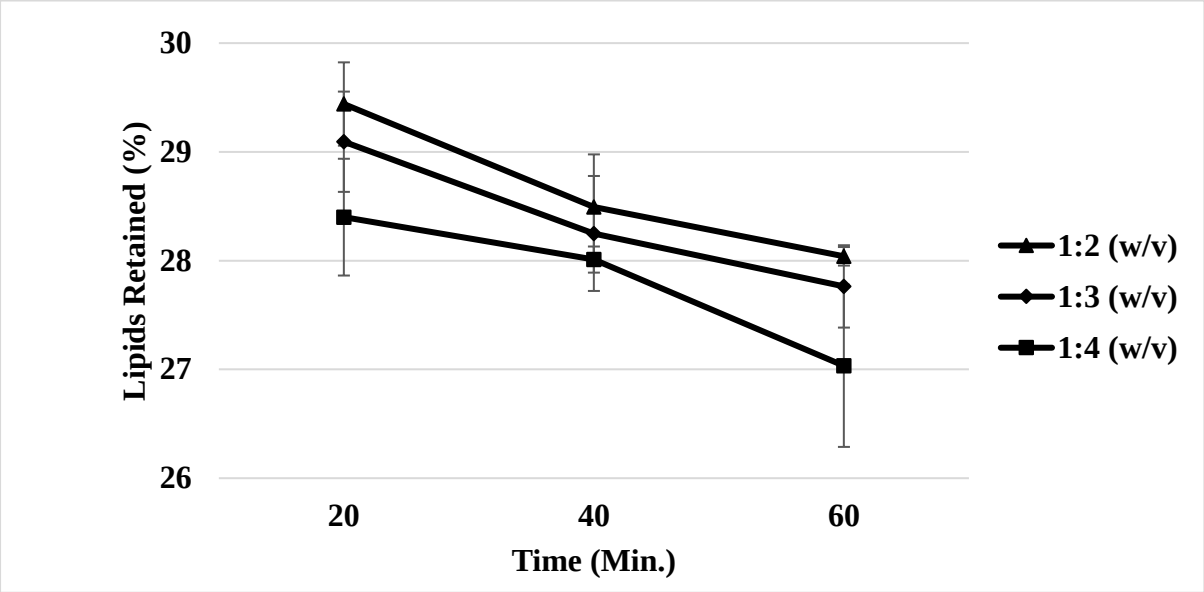


Fig.4.5. (b) Lipids (%) retained in ghee residue by boiling water treatment at different time and solids to solvent ratio

Based on the above points, water was considered as a solvent to diffuse polar components. It was hypothesised that this would facilitate free movement of non-polar fraction as a floating layerwhich could then be separated due to density gradient.

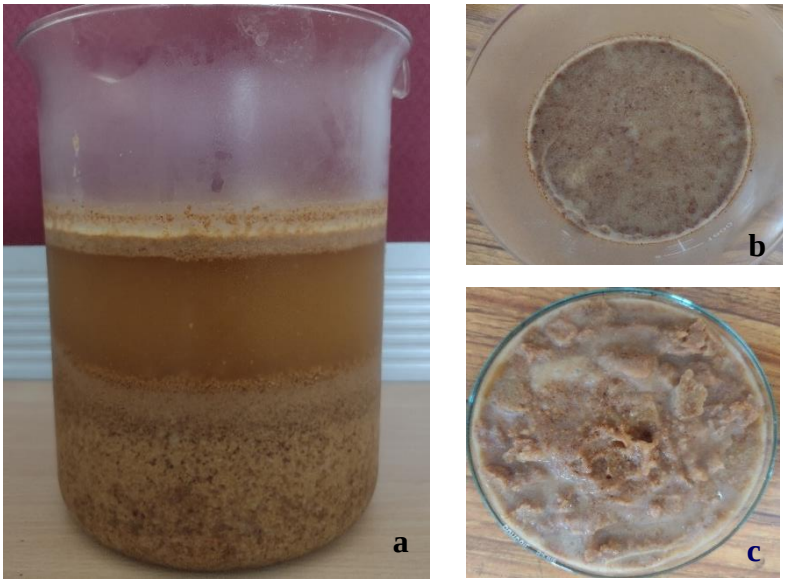


Fig. 4.6 (a) Separation of lipids after freezing of boiling water treated sample; (b) Top view of beaker with solidified lipids after freezing; (c) Lipids removed from top layer after freezing

It is assumed during this treatment that 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 (Fig. 4.6 (a)). Lipids were carefully removed along with little fraction of ghee residue after freezing for easy skimming (Fig. 4.6 (c)).

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 (Fig. 4.7 a & b). PLs retained in ghee residue reported an increasing trend with increase in time and solids to solvent ratio. Maximum PLscontent (8.26% ghee residue basis) was reported with contact time of 60 min. at solids to solvent ratio of 1:4. This corresponded to a PLs content of 30.56% when reported on lipid weight basis.

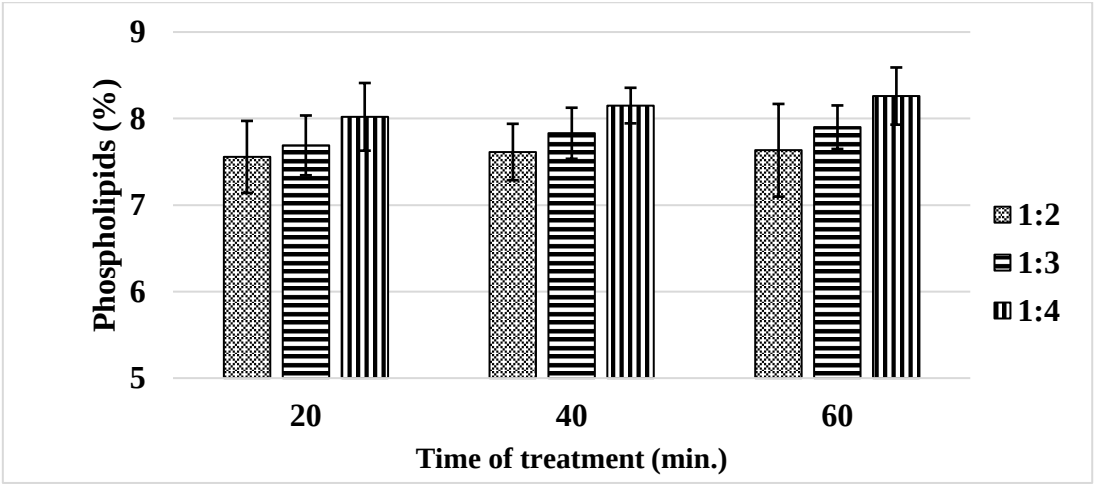


Fig.4.7. (a) Phospholipids expressed on dry weight after boiling water treatment

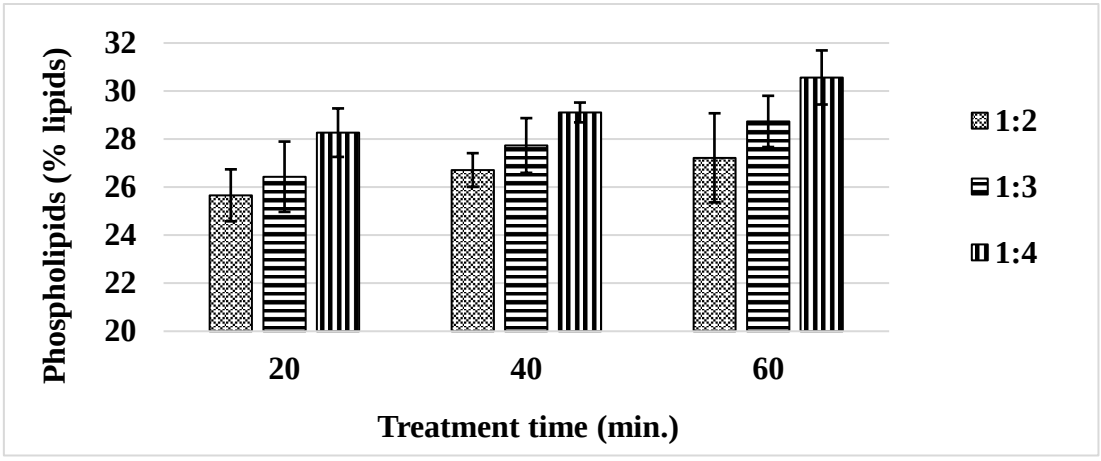


Fig.4.7. (b) Phospholipids expressed on lipids weight after boiling water treatment

The outcome of this experiment was analysed for ANOVA using the two-factor test with replications and the results are depicted in Table 4.3. No significant influence 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 theF value for this factorwas greater that F-critical value, two factor comparison was analysed by Tukey's test at 20 min. contact time. Results from this post-hoc test indicated no significant difference among treatments. Hence, solid to solvent ratio at the lower level of 1:2 was preferred over extended solvent ratio for the remaining investigations.

Table 4.3. Analysis of variancefor phospholipids retention in ghee residue afterboiling water treatment

Source of Variation	SS	df	MS	F	P-value	F crit.
Time	0.1806	2	0.0903	0.8115	0.4597	3.5545
Solid to solvent ratio	1.3497	2	0.6748	6.0650	0.0096	3.5545
Interaction	0.0369	4	0.0092	0.0829	0.9866	2.9277
Error	2.0029	18	0.1112			
Total	3.5702	26				

4.1.5. Selection of pre-treatment of pressed ghee residue with suitable solvent

Comparison of n-hexane and boiling water treatments were made based on the yield of PLsobtained and feasibility of treatment. From Fig. 4.4 and 4.7, it is evident that retention of PLsin the ghee residue was better under boiling water treatment. Also, from solvent point of view, water is non-toxic, environment friendly and cheaply available compared to n-hexane. Though n-hexane is widely used in the extraction of edible lipids from biological matrix, apprehensions on consumer health still persist (Pan *et al*., 2017). Hence, boiling water treatment was selectedfor pre-treatment of the pressed ghee residue instead of n-hexane.

4.1.6. Comminution of the pre-treated ghee residue

The pressed ghee residue after boiling water treatment and drying was subjected to size reduction or comminution 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 ofits lipids and PLs content.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 4.4.

A perusal of the data clearly indicates a definite improvement in the PLs yield with each step of pre-treatment with a slightly higher yield of PLs when the particle size 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 simultaneous elimination of non-polar lipids. Between boiling water treatment and comminution to 0.25 mm particle size, sizable increase in PLs were noticed. This could be due to increased surface area which facilitated gradient for movement of lipids from particle surface to solvent. From overall pre-treatment, there was an increase of 91.96% in PLs content expressed on ghee residue basis. Overall, this increment could be mainly due to elimination of non-polar lipids fraction and physical processing.

Table.4.4. Lipids and phospholipids of ghee residue with respect to different pre-treatments

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}

*Different alphabets in same column indicates significant difference ($p<0.05$)

4.1.7. Enzyme treatment of pre-treated ghee residue

It was hypothesized that loosening protein-lipid complex in the ghee residue would assist the process of extraction of the PLs. Hence, two proteolytic enzymes were consideredto treat the pressed and pre-treated ghee residue. Further, it has also been reported that enzymatic hydrolysis will bring down allergenicity with induced bitterness (Muniratnamma *et al.*, 2017a). Partially hydrolysed proteins are also reported to exhibit good emulsifying property and have demonstrated suitability for use in meat industry (Neklyudov *et al*., 2000). Results obtained by treating the ghee residue with trypsin enzyme at pH of 8.0 using sodium citrate buffer for varied time periods at incubation temperature 37^{o}C is depicted in Fig. 4.8 (a). It was deduced that with increase in time of enzyme treatment, there was decrease in PLscontent of the residue.Enzymatic treatment for 2h reported a PLs content of 9.23% in the extract which was a slight decrease from raw material PLs content (9.54%). This value further declined to 9.07% at 4h and resulted in 9.19% of PLs at the end of 12h of enzymatic treatment. Also, the resultantghee residue after treatment with trypsin enzyme was gummy which rendered the difficulty in extraction of lipids for the sample. It was also observed that with increase in duration of treatment, the structure of ghee residue became very fragile.

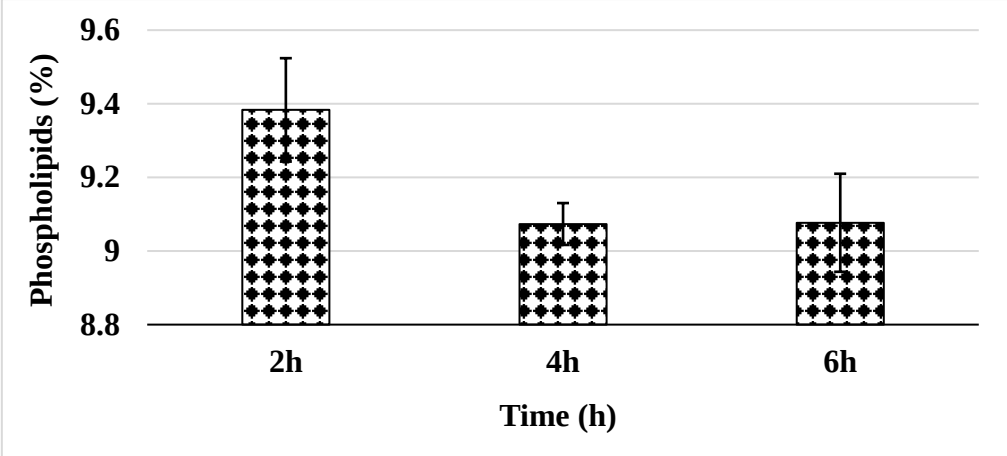


Fig.4.8. (a) Phospholipids yield from trypsin enzyme treatment

In a study by Mutilangi*et al.* (1995) on enzyme treatment of heat denatured whey protein isolate, the enzymes trypsin, chymotrypsin, alcalase and neutrase were evaluated. It was reported that with increase in degree of hydrolysis from 2.8 to 8%, protein and moisture contents showed decreased values whereas, ash content showed increased trend. The inconsistency in protein content in hydrolysates observed in the studywas attributed to corresponding increase in non-protein nitrogen toprotein nitrogen ratio. In similar lines, the inconsistent change in PLs content in the ghee residue observed in the present study after trypsin treatment could be attributed to theincomplete separation of lipids and PLs due to the fragility of the hydrolysate after the pre-treatments.

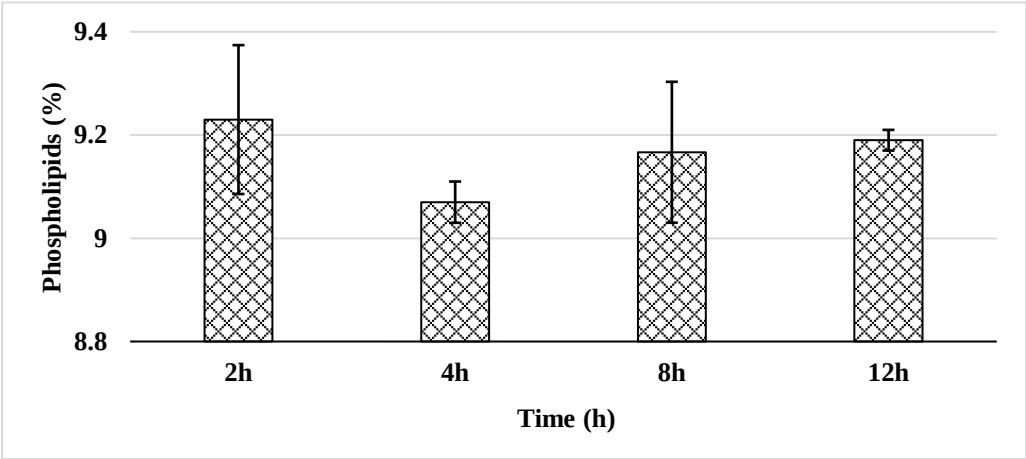


Fig. 4.8. (b) Phospholipids yield from alcalase enzyme treatment

The second enzyme considered, namely, alcalase was evaluated to hydrolyse the ghee residue samples for duration of 2, 4 and 6 h at 8.0 pH using water as buffer at an incubation temperature of 50^{o}C. Experiments were conducted at 1:25 enzyme to substrate ratio and results indicating behaviour similar to hydrolysis using trypsin enzyme (Fig. 4.8(b)). The

phospholipid content in the extract obtained after 2 h of enzyme treatment was highest (9.8%) and this subsequently decreased to 9.07% after 4 h and thereafter remained nearly unchanged. Compared to trypsin enzyme, alcalase treatment reported slightly higher values for PLs after the hydrolysis.

In an experiment to extract virgin coconut oil from coconut milk, Senphan and Benjakul, (2017) used protease (hepatopancreas of Pacific white shrimp) and alcalase enzymes. During evaluation of oil yield for hydrolysis over varied period of time, it was observed that maximum yield of oil was 93.83% after 90 min. of hydrolysis. Further, increase in enzyme treatment reported slight decrease in oil yield at different concentration of protease. In the same study, optimized protease enzyme was compared with alcalase enzyme at same enzyme concentration and time. The study inferred that alcalase enzyme treatment resulted in oil yield of 98.25%, which was higher than yield reported for protease enzyme and control.Theinfluence of enzyme concentration, pH, temperature, and incubation time on the yield of oil was highlighted in this study.

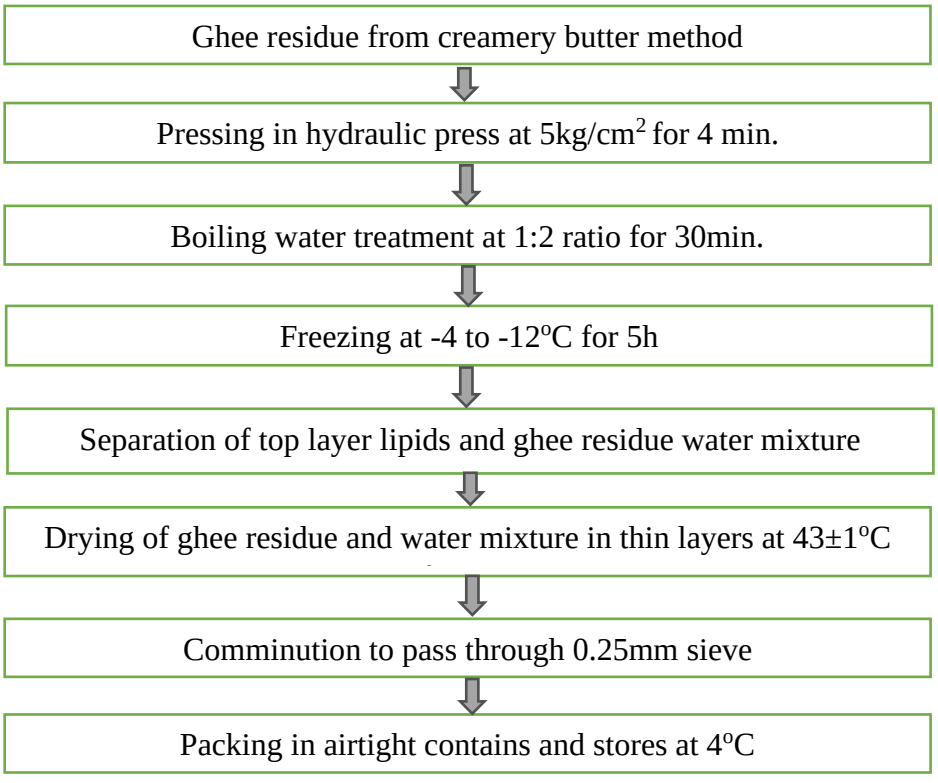


Fig. 4.9. Detailed flow chart for pre-treatment of ghee residue

In another study, Barry *et al.* (2017) treated reconstituted buttermilk with alcalase enzyme at an enzyme to substrate (protein basis) ratio of 1:100 at 50°C. The resultant hydrolysate was passed through an ultrafiltration unit having molecular weight cut off of 50 kDa. The

retentate was found to be composed of lipids and PLs at yield levels of 60.07% and 11.05%, respectively. The increase in PLs yield was attributed to deproteinising due to the synergetic effect of enzyme treatment and ultrafiltration. Based on the above preliminary experiments, the final protocol for pre-treatment of the ghee residue for assisted extraction process in presented in Fig 4.9.

4.2. Setting protocol for assisted extraction treatment

The ghee residue obtained after comminution was studied with different sequence of operations (as outlined in Fig. 3.11) to select best combination of process parameters for the assisted extraction processes based on PLs yield in the extract. As a precursor to the actual extraction trials, experiments were designed to select the suitable extraction medium based on ethanol, water and enzyme treatment. The respective treatment protocols evaluated for treating the ghee residue samples with microwave, ultrasonication and pulsed electric field, by keeping the machine factors and solvent to solid ratio constant is presented in Table 4.5. For enzyme treatment, the ghee residue was treated with alcalase for 2 h with conditions as mentioned in section 4.1.7 before the assisted extraction process.

Table 4.5. Operating conditions evaluated for assisted extraction treatment to select protocol for solvent and enzyme treatment

Microwave	Ethanol	Microwave @ 360 W power, 20 s treatment time and 1:10 ghee residue to ethanol ratio
	Water	Microwave @ 360 W power, 20 s treatment time and 1:10 ghee residue to water ratio
	Enzyme	Alcalase treated and microwave @ 360 W power, 20 s treatment time
Ultrasonication	Ethanol	Ultrasonicated @ 60% power (max: 650 W) and 2 min. (2 s on and 3 s OFF) @ 1:10 ethanol ratio
	Water	Ultrasonicated @ 60% power (max: 650 W) and 2 min. (2 s ON and 3 s OFF) @ 1:10 water ratio
	Enzyme	Alcalase treated and Ultrasonicated @ 60% power (max: 650 W) and 2 min. (2s ON and 3s OFF)
PEF	Ethanol	PEF @ 50 kV/cm for 180 s and 1:10 ghee residue to ethanol ratio (90 Hz frequency, 900 µs pulse width)
	Water	PEF @ 50 kV/cm for 180 s and 1:10 ghee residue to water ratio (90 Hz frequency, 900 µs pulse width)
	Enzyme	Alcalase treated and PEF @ 50 kV/cm for 180 s and 1:10 ghee residue to water ratio (90 Hz frequency, 900 µs pulse width)

4.2.1. Assisted extraction with ethanol as solvent

Ethanol as solvent reported highest yield of lipids from microwave assisted extraction at 27.96%. Precaution was taken to seal the solid-solvent mix in microwavable bottle before subjecting to microwave treatment. Ultrasound assisted extraction reported second highest value with 23.85%. However, PLs expressed on lipids basis were highest in PEF treated sample with 61.79% of lipids estimated to be PLs (Fig. 4.10). This could be due to selective impact of pulsation on the ghee residue during treatment. Ultrasound reported next highest PLs on lipid basis reporting 50%, while the microwave treatment exhibited good influence on lipids extraction but proved to be less effective in terms of PLs.

Ratnakumar *et al*.(2021) used ultrasonication technique to improve PLs extraction from beta-serum using tertiary amine as a solvent. Experiments were conducted on beta-serum by subjecting it to intensities of 15.53, 31.76 and 44.56 W/cm^2 for 4 min. prior to solvent extraction. Ultrasonication prior to solvent extraction reported phenomenal increase in PLs at 69.07±3.45% at 44.56 W/cm^2 intensity and 12:1 solvent to solid ratio. The study corroborated improved results for ultrasound assisted extraction triggering breakage of membrane complex of protein and PLs leading to release of PLs into solvent.

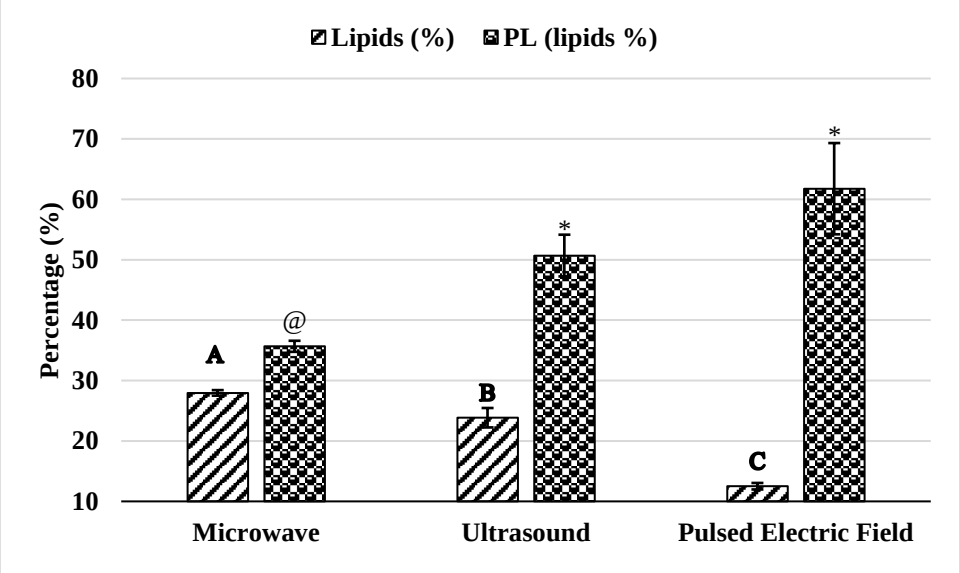


Fig.4.10. Yield of lipids and phospholipids (lipids basis) from ethanol solvent by assisted extraction treatments

*****Same alphabet on lipids yield bars indicate no-significant difference ($p<0.05$). Same symbols on phospholipids yield bars indicate no-significant difference ($p<0.05$) through Tukey's test.

Single factor ANOVA of the data indicated significant difference between assisted treatments in the extraction of lipids ($p<0.05$). However, PLs reported from ultrasonicated and PEF treated sample showed no significance difference ($p<0.05$). The microwave treated extract

showed significant difference in the lipid profile when compared to ultrasound and PEF extract.

4.2.2. Assisted extraction with water as solvent

As a polar solvent, water was usedfor extraction of PLs aided by the selectedengineering interventions. In case of ethanol, non-polar lipids were also extracted from the sample as it was used as solvent for lipids extraction. Extract, lipids and PLs expressed on lipids and ghee residue wt. basis is reported in Fig. 4.11.

Among three treatments, ultrasonication treatment resulted in maximum extract content compared to other two treatments by reporting 48.4% of ghee residue constituents. Comparatively, PEF treatment reported least constituent extract with 22% whereas, microwave reported 26.79% of ghee residue constituents in its extract. This implies that ultrasound treatment through its shearing action eroded most of the ghee residue constituents into the solvent within very short time. Though heat and mass transfer is an effective phenomenon in microwave treatment, limited treatment time and solvent evaporation led to lesserextract. No significant difference was observed in the quantity of extract reported between microwave and PEF extract ($p<0.05$).

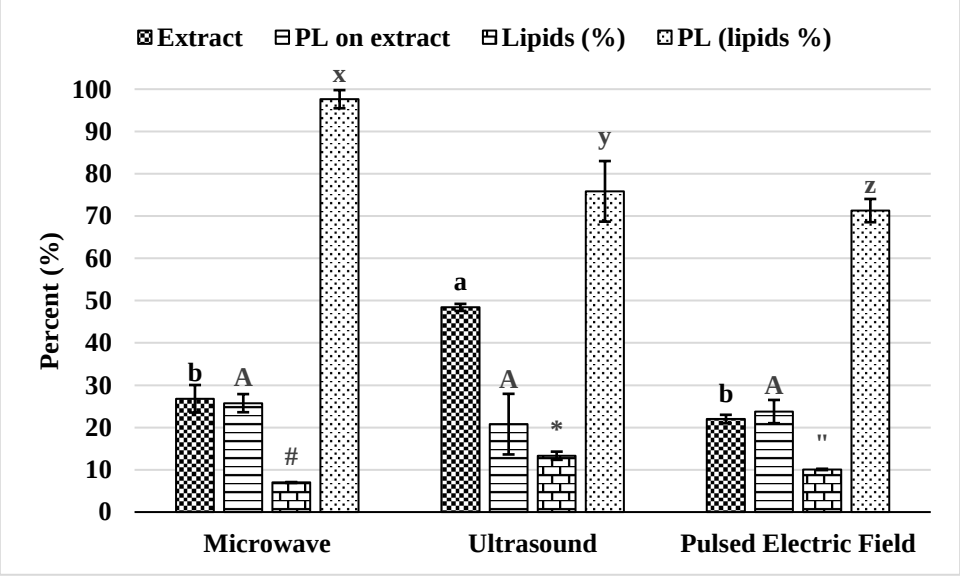


Fig.4.11. Yield of extract, lipids and phospholipids expressed on ghee residue and lipids basis from water as solvent by assisted extraction treatments

*Same alphabet or symbol referred incommonpattern bars indicate no significant difference ($p<0.05$).

Lipids content extracted by assisted treatment through water as solvent was also highest in ultrasonication with 13.32% lipids. Microwave reported least value for lipids yield at 6.99%, while PEF treatment resulted in a lipid content of 10.08% in the extract. Ultrasonication

treatment extractedPLs along with other constituents reiterating its ability for effective extraction. Through a pilot study reported by Puertolas *et al*. (2016), it was deduced that PEF assisted technique extracted 13.3% more lipids from olive mill against control. Electric field of 2 kV/cm and 65 J energy was applied in this study to report increased yield of polyphenols, phytosterols and tocopherols.Similarly, *Moringaoleifera*seed as substrate yielded 91.35% oil recovery by ultrasound assisted extraction (Zhong*et al*., 2018). However, in the same study microwave assisted process reported 94.21% of oil recovery. Lipids extracted from different assisted extraction techniques were reported to be significantly different ($p<0.05$).

Interestingly, PLs expressed on extract weight basis reported nosignificant difference among different treatments ($p<0.05$). This trend implies that PLs also proportionately transferred into solvent along with non-lipid fraction. Microwave assistance reported 25.75% of PLs in the extract followed by PEF treatmentat 23.76%. Ultrasonication facilitated non-polar lipids extraction in larger proportion among all treatments through its mechanical, chemical and biological action. When the PLs content was expressed on lipids basis, microwave extraction stood highest with majority of lipids as PLs (97.63%). Ultrasound treated ghee residue extractreported 75.84% of lipids as PLs. This indicated aselective extraction of polar lipids during short span of operation. PLs extracted on lipids basis reported significant difference among all treatments ($p<0.05$).

4.2.3. Assisted extraction with enzyme treatment

Ghee residue was treated with alcalase enzyme at optimized treatment time before subjecting to assisted extraction. Buffer used for enzyme treatment was used as solvent for all assisted extraction at operational conditions mentioned in Table 4.5. It was also noted that the enzyme treated ghee residue was very loose in structure and gummy to handle compared to water and ethanol treated samples. The yield of extract and lipids withcorrespondingPLs expressed on extract and lipid basis is depicted in Fig. 4.12.

Results indicated that large amount of ghee residue constituents migrated from the sample to solvent during the extraction process. Ultrasound assisted extraction process recorded a composition of 72.94% due to this movement into solvent. PEF treated sample reported least extract movement (64.07%) and microwave treatment reported 70.58% of extraction of constituents into solvent. Due to this effect, PLs expressed on extract basis reported very less values for all treatments. Single factor ANOVA indicated no significant difference between microwave and ultrasound treated sample ($p<0.05$). Also, no significant difference was

reported between PEF and microwave treated samples. The trend was similar for PLs expressed on extract basis. However, no significant difference was reported for lipids expressed on ghee residue basis among all treatments ($p<0.05$).

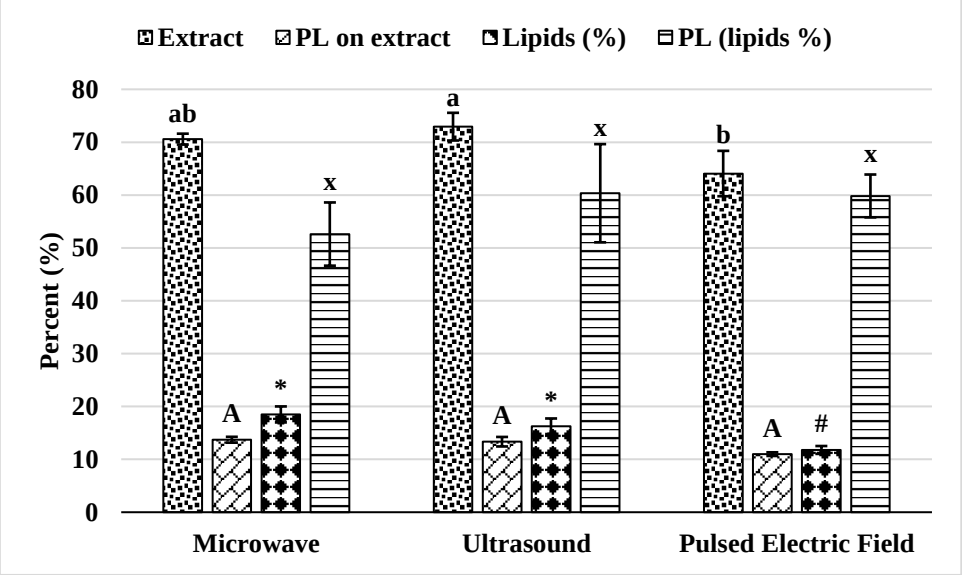


Fig.4.12. Yield of extract, lipids and phospholipids expressed on ghee residue and lipids basis from enzyme pre-treated and assisted extraction treatments

Same alphabet or symbol referred for common pattern bars indicate no significant difference ($p<0.05$).

It has been reported that enzyme aides in hydrolysing of the substrate matrix leading to disintegration of cell wall structure to facilitate solute extraction. Extraction yield is dependent on various factors such as pH, enzyme type, temperature, particle size and reaction time (Poojary, *et al*., 2017). However, in the present study, ghee residue subjected to enzyme treatment resulted in disintegration of structure. Subsequent assisted extraction treatment further aided in leaching of the constituents into the solvent matrix, hence reporting higher extract yield.

When lipids were extracted from dried fraction for assisted treatments, i.e. microwave, ultrasonication and PEF assisted treatment; the study reported 18.5, 16.26 and 11.78% of lipids, respectively. PLs expressed on lipids basis reported highest value of 60.35% for ultrasound treatment and 59.83% for PEF treated sample. Single factor ANOVA showed no significant difference between microwave and ultrasound for PLs and total lipids extracted. Also, no significant difference was reported for all samples when PLs were expressed on lipids basis ($p<0.05$).

Hu *et al*.(2019) in an experiment used simultaneous ultrasound-microwave assisted extraction using double distilled water as solvent. Resultant extract was treated with different group of

enzymes under optimal conditions to select best enzyme based on recovery of oil. It was found that enzyme cocktail (cellulase/hemicellulsse/pectinase = 1/1/1, w/w/w) reported nearly 40% recovery of oil. Authors attributed enhanced extraction to decomposed cell wall due to enzyme treatment.

In another study, *Scenedesmusalmeriensis* was used as raw material to extract protein by using PEF pre-treatment before subjecting to enzyme treatment (Akaberi*et al*., 2019). The process of PEF pre-treatment was also compared with high pressure homogenization to reduce enzyme hydrolysis time. The study concluded that both methods did not show any influence of enzyme hydrolysis on processing time. ButPEF treatment played significant role in achieving better degree of hydrolysis and improving the kinetics of hydrolysis.

4.2.4. Selection of protocol for optimization studies

Before undertaking the optimization trials, yield of PLs obtained from different protocols studied during the preliminary experimentswere compared. As ethanol treatment did not result in any extract per se, the yield of PLs for the said protocol was compared only on fat basis. Table 4.6 presents the yield of PLs on extract and lipids weight basis to compare and identify the better protocol.

Table.4.6. Yield of phospholipids expressed on extract and lipid basis from different assisted extraction and protocols

Assisted treatment/Protocol	Ethanol	Water	Enzyme
	Phospholipids expressed on extract weight basis		
Microwave	-	25.75±3.49^{a}	13.71±0.55^{b}
Ultrasonication	-	20.79±1.28^{a}	13.34±0.90^{b}
Pulsed Electric Field	-	23.76±2.67^{a}	10.99±0.34^{b}
Phospholipids expressed on lipids weight basis			
Microwave	35.67±0.91^{c}	97.63±2.14^{a}	52.62±5.98^{b}
Ultrasonication	50.69±3.45^{b}	75.84±7.16^{a}	60.35±9.28^{ab}
Pulsed Electric Field	61.76±7.55^{b}	71.29±2.75^{a}	59.83±4.05^{b}

*Same alphabets in a row indicate no-significant difference ($p<0.05$) (one-way ANOVA Tukey's test)

Results indicated that different solvents and enzyme treatment showed significant difference in its efficiency to extract PLs ($p<0.05$). Among the treatments, water as a solvent reported higher PLs in the extract (expressed on extract weight basis) for all the assisted extraction treatments evaluated. Similarly, PLs expressed on lipids basis also indicated similar trend showing good results in treatment where water used as solvent.

The superior extraction of PLs by using water as solvent may be ascribedto its selective extraction. Though the yield of total lipids from water assisted extraction was lower compared to enzyme and ethanol treatment, it was observed that the PLs content of this extract was higher in this protocol. In addition, water as a solvent is eco-friendly and cost effective than the other solvents, which presents theadvantage of usage in food products for further studies. Considering the above points, the extraction protocol using water as a solvent was finalised for further detailed study and optimization.

4.2.5. Selection of levels for machine and process parametersfor optimization of the assisted extraction processes

To select the range of machine and process parameters for the optimization studies, experiments on the assisted extraction techniques based on the protocol finalised during the preliminary trials (section. 4.2.4.) were carried out. The ghee residue obtained after pre-treatment was subjected to one factor at a time analysis (i.e. levels of different factors were varied by keeping one of the factors constant). Maximizing the yield of PLs was considered as the criteria for identifying the range of factors for each of the assisted extraction process.

4.2.5.1. Microwave assisted extraction process

Microwave assisted extraction is a heat and mass transfer technique where heat (temperature) plays significant role in driving the PLs and other fractions into the solvent matrix i.e. water. Heat generation would be dependent on amount of power supplied to generate microwaves (Sturm *et al*., 2013). The microwave unit used in the study was designed to operate at 900, 720, 540, 360, 180 and 90 W. Based on initial experiments, it was decided to restrict the operation to under 540 W power level, as higher-level operation conditions led to splashing of contents from beaker.

Fig 4.13 (a & b) illustrated the yield of extract and PLs on extract and lipids weight basis for the “one factor at a time approach” analysis. The extraction of PLs at microwave operated at 360 W and solvent to solid (S: S) ratio 10 resulted in highest yield at time factor 50 s with value 14.28% (Fig. 4.13 (a)). With further increase in time factor, PLs extraction slightly decreased to 13.95%. However, there was no significant difference between time factors considered in the study at 50 and 70s ($p<0.05$). Hence, equidistant points of time (40 and 60 s) from the centre (50 s) were selected as low and high levels for optimization studies.

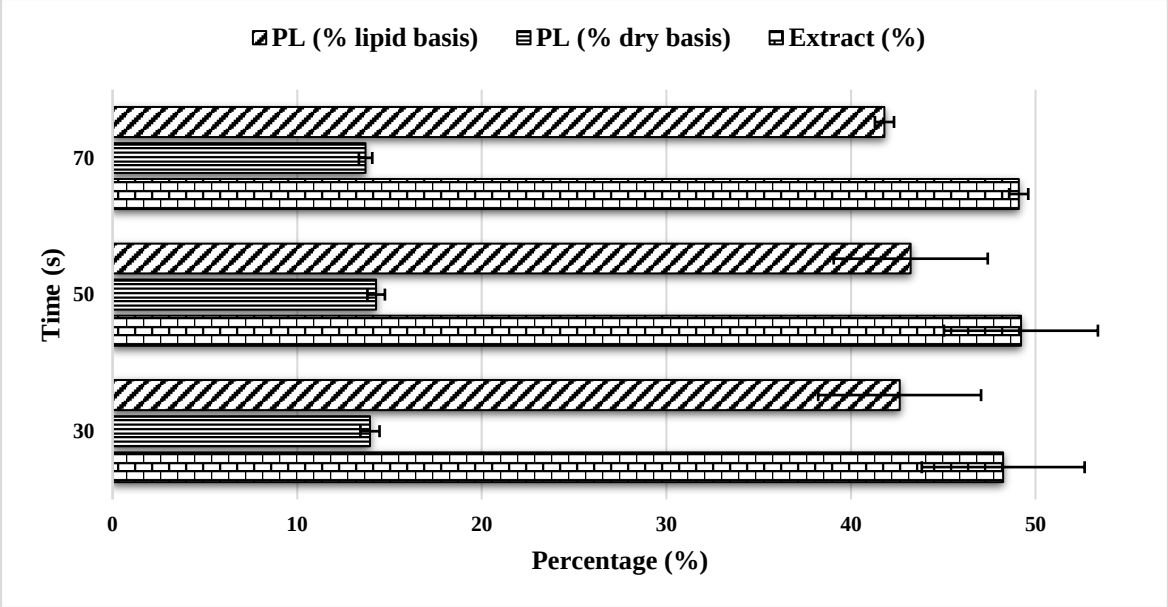


Fig.4.13. (a) Yield of extract, lipids and phospholipids by microwave operated at 360 W power and 10 solvent to solids ratio

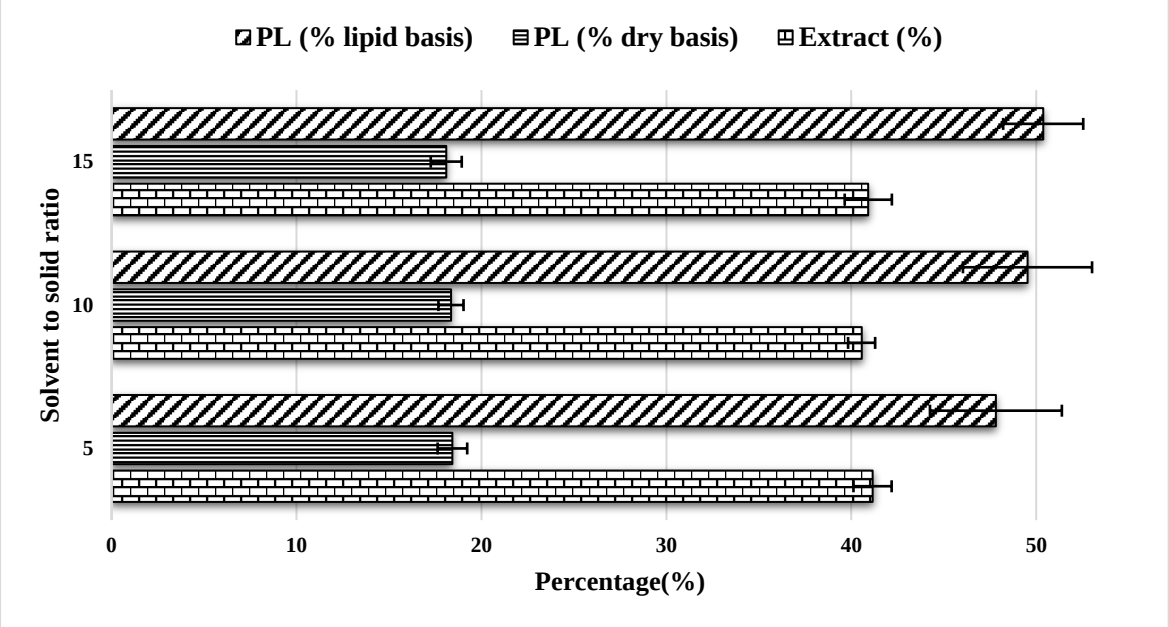


Fig.4.13. (b) Yield of extract, lipids and phospholipids by microwave operated at 360 W power and 50 s treatment time

Similarly, keeping power level at 360 W and time factor at 50 s microwave assisted extraction was studied for solvent to solid ratio of 5, 10 and 15. PLs yield was highest at a ratio of 5 (18.43%) and further decreased with increase in the solvent to solid ratio (Fig. 4.13 (b)). There was no significant difference in PLs yield with increase in the solvent to solid ratio ($p<0.05$). It was further observed during these experiments that a minimal solvent to solid ratio of 5:1 was required to keep the ghee residue in suspension during the extraction process. Also, at lower levels of treatment, water evaporated from the matrix, leading to

residue remaining in the minimal volume of solvent. Hence, this point was chosen as low level for the solvent to solid ratio, with two equidistant points (7.5 and 10) opted for middle and high levels.

4.2.5.2. Ultrasound assisted extraction process

Four factors were considered for the optimization trials for the ultrasound assisted extraction viz., power levels, solvent to solid ratio, time and temperature of solvent. Though the ultrasound generator was designed to operate between 0-100% power level, to avoid wear and tear, it was advised in the operation manual of the instrument to operate at a power rating between 20-80%. Initial extraction experiments were conducted by keeping power level (70%), solvent to solid ratio (10 w/v) and solvent temperature (70^{o}C). Three treatment times (2, 4 and 6 min.) were evaluated and PLs yield from the ghee residue was comparatively higher (21.80% on extract weight basis) at operation time of 4 min. (Fig. 4.14 (a)). Though PLs yield on lipids basis increased with increase in time,there was no significant difference in the yield recorded at different treatment times ($p<0.05$). Hence, 4 min. as the midpoint with equidistant points 3 and 5 min. were considered as the parameter values to be evaluated for timefor optimization of the process parameters for ultrasound assisted extraction studies.

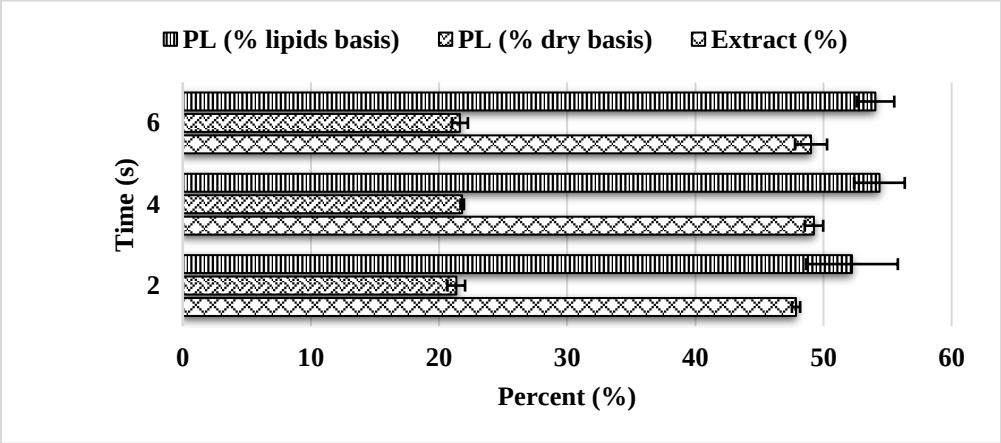


Fig.4.14. (a) Yield of extract, lipids and phospholipids by ultrasonication operated at 70% power, 60^{o}C solvent temperature and solvent to solid ratio of 10

The next set of "one factor at a time" experiments was conducted at 4 min. treatment time with power level (70%) and solvent to solid ratio (10%) at different solvent temperatures. The results are presented in Fig.4.14 (b) and it can be observed that the yield of PLs in the extract increased with increase in solvent temperature. However, when the PLs were expressed on lipids basis, the yield was maximum at 50^{o}C and decreased slightly at 70^{o}C (However, no statistical difference was deduced in the yield of PLs at different solvent temperatures ($p<0.05$)). Hence, 70^{o}C was considered middle level and equidistant

temperature 60°C and 80°C were opted as low and high levels, respectively for further optimization trials. The temperature was restricted to 80°C, as solvent temperature above this level raised concerns regarding operational difficulty and potential susceptibility of the ultrasound probe to wear and tear.

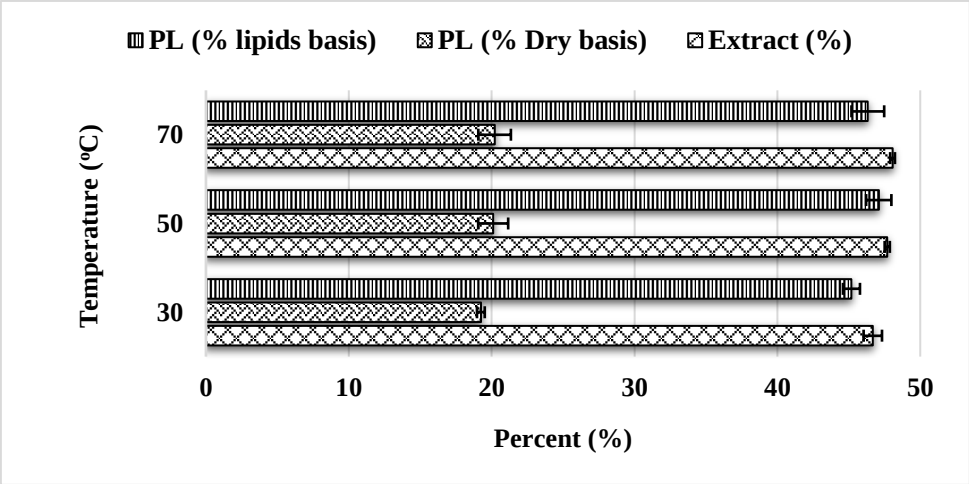


Fig.4.14. (b) Yield of extract, lipids and phospholipids by ultrasonication operated at 70% power, 60°C solvent temperature and treatment time of 4 min.

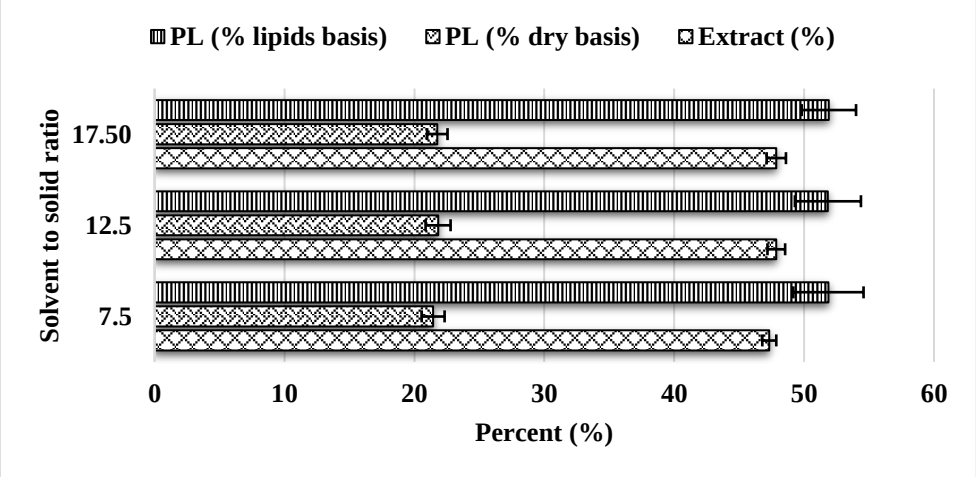


Fig.4.14. (c) Yield of extract, lipids and phospholipids by ultrasonication operated at 70% power, 4 min. operation and 70°C solvent temperature

During third set of experiments, treatment time 4 min., power level 70% and solvent temperature 70°C were kept constant and the effect of different solvent to solid ratiowas analysed. Extraction experiments were conducted at solvent to solidratios of 7.5, 12.5 and 17.5 (the levels were selected based on preliminary trials). Minimum of 7.5 mL of solvent was needed to keep the ghee residue in dispersion to facilitate extraction. Among the different solvent proportions evaluated, PLs yield (% extract weight basis) was relatively higher when extracted at a solvent to solid ratio of 12.5 (Fig.4.14(c)). Hence, the solvent to

solid ratio of 12.5 opted as the midpoint level with 10 and 15 as equidistant points for low and high levels of this parameter in the optimization trials.

4.2.5.3. Pulsed electric field assisted extraction process

Three factors were considered for optimization under PEF treatment for the assisted extraction process, viz., voltage, solvent to solid ratio and treatment time. The pulse generator of the PEF set up was designed to generate square pulse waves and operate at maximum voltage of 60 kV/cm. Initially, the pulsed electric voltage at50 kV/cm and solvent to solid ratioof 10 was maintained constant to conduct experiments at varied treatment time levels (180–360 s) and the results are presented in (Fig. 4.15(a)).

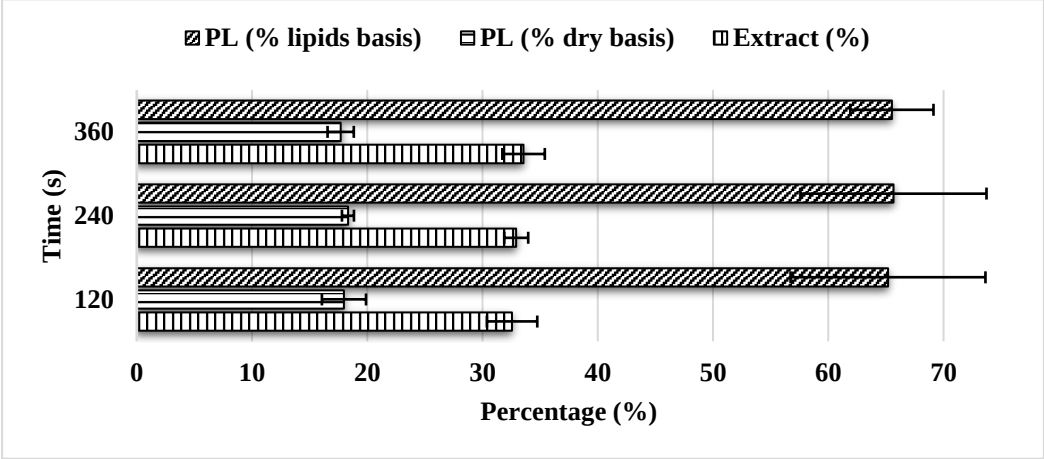


Fig.4.15. (a) Yield of extract, lipids and phospholipids by PEF unit operated at 50 kV/cm voltage and solvent to solids ratio of 10

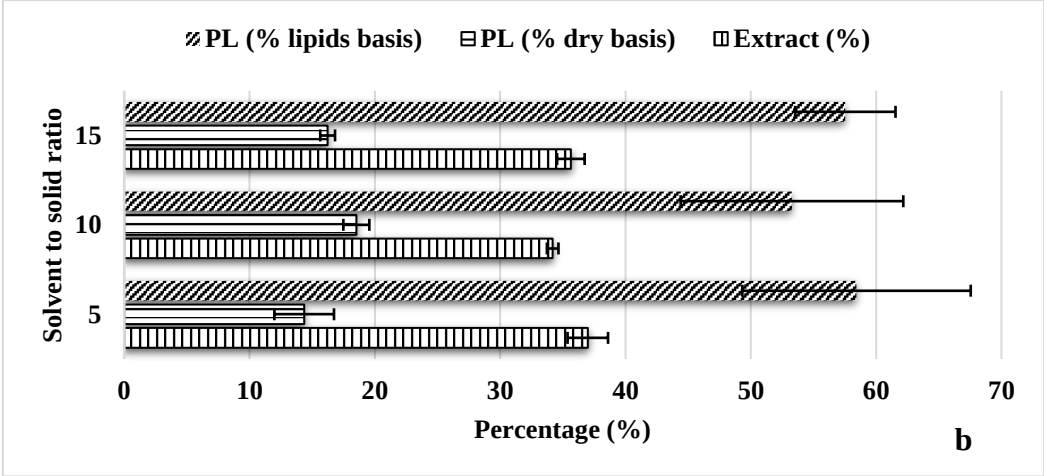


Fig.4.15. (b) Yield of extract, lipids and phospholipids by PEF unit operated at 50 kV/cm voltage and 240 s extraction time

The yield of PLs was found to be highest at 240 seconds (18.2%) and it decreased with further increase in time (17.68% at 360 s). Also, even though the yield of PLs (when expressed on % lipids basis) increased proportionately with treatment time, the effect was

statistically not significant ($p<0.05$).Therefore, PEF operated at 240 s was taken as the middle level for this parameter for the optimization trials, with 180 and 300 s as low and high levels of treatment time, respectively.

Next, the PEF unit was operated at 50 kV/cm for 240 s at varied solvent to solid ratios and the effect on the yield of PLs was recorded (Fig. 4.15 (b)). It was observed that the yield of PLs was highest (18.52%) at the solvent to solid ratio of 10, whereas when the PLs were expressed on lipids basis, it was lowest (53.28%) at same treatment.A significant difference in the yield of PLs among different solvent to solid ratios ratio ($p<0.05$) was deduced during the statistical analysis of the data. Hence, a value of 10 was finalised- as the middle level for the range for solvent to solid ratios, with 7.5 and 12.5 as low and high level, respectively for optimizing the PEF assisted extraction process in this study.The final range of various process and machine parameters finalised for the optimization trials of the three assisted extraction processes is summarised in Table 4.7

Table 4.7. Range of process and machine parameters finalized for different assisted extraction techniques

Assisted treatment techniques	Parameters	Levels		
		Low	Middle	High
Microwave	Power (W)	180	360	540
	Time (s)	40	50	60
	Solvent to solid ratio (v/w)	5	7.5	10
Ultrasonication	Power (%)	60	70	80
	Time (min.)	3	4	5
	Solvent temperature (^oC)	60	70	80
	Solvent to solid ratio (v/w)	10	12.5	15
PEF	Voltage (kv/cm)	40	50	60
	Time (s)	180	240	300
	Solvent to solid ratio (v/w)	7.5	10	12.5

4.3. Optimization of process parameters by assisted extraction techniques

4.3.1. Optimization of process parameters for microwave assisted treatment

To optimize process parameters, Taguchi orthogonal array L9 (3^4) was applied to analyse the influencing factors and its levels. While treating the ghee residue samples with solvent, microwave treatment was given in incremental spells of 10 s, as continuous treatment led to

splashing of contents. A small quantity of water was used to remove residual ghee residue after decanting of treated samples.

4.3.1.1. Optimization of process parameters for microwave assisted extraction process based on yield of PLs

The experiments for the optimization were designed as per the Taguchi L_9 orthogonal array is depicted in Table 4.8. The extract obtained after each treatment run was analysed for its PLs content andantioxidant activity. The yield of PLs in the extract obtained bythe microwave assisted process was calculated on extract weight basis.The antioxidant activity of extract was directly expressed on percentage basis using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay.

Table.4.8. Phospholipids yield and antioxidant activity of extract from ghee residue treated with microwave assisted extraction based on Taguchi L_9 orthogonal array design

Run	Power (W)	Time (s)	S:S Ratio (v/w)	Phospholipids (% extract wt. basis)	Antioxidant Activity (%)
1	180	40	5.0	17.27±1.69	26.98±4.14
2	180	50	7.5	18.04±2.45	28.24±2.27
3	180	60	10.0	18.82±2.02	29.78±2.26
4	360	40	7.5	19.18±0.76	27.89±2.03
5	360	50	10.0	19.30±0.47	29.02±1.89
6	360	60	5.0	20.38±0.53	25.12±0.59
7	540	40	10.0	21.29±1.51	27.14±1.45
8	540	50	5.0	20.96±1.23	24.44±1.76
9	540	60	7.5	21.84±0.49	26.01±1.34

The PLs content in the extract varied from 17.27 to 21.84% for different combination of power, time and S:S (solvent to solid) ratio. Microwave was reported to have good penetration ability throughout volume of sample (Li *et al*., 2013) which aids in time saving during extraction processes and lesser bioactive degradation (Venkatesh and Raghavan, 2004). Results obtained in the L_9 orthogonal array was analysed to convert the effect of factors at each level into signal to noise ratio. The effect of three factors at different levels on yield of PLs in the extract expressed as signal to noise ratio is shown in Fig.4.16. It is evident from the figure that operation power played a significant role in assisting the extraction of PLs during the treatment ($p<0.05$). The delta values for means reported from signal to noise ratio for different factors were ranked in the order: power>time>S:S ratio.

It is evident from the results that the yield of PLs increased with increase in power level with the highest yield reported at power level 540 W. Due to operational difficulty (overheating and splashing of extract) the higher level of power factor was restricted to 540 W. Extraction time also had a positive influence on the yield of PLs, with a significant influence at time >

50 s. This could be due to the effective heat transfer from microwaves in assisting component diffusion into solvents during the extended extraction time compared to initial time of treatment. Among the three factors considered, the solvent to solid ratio exhibited the least influence on the extraction of PLs in to the extract, with the maximum yield being reported at the solvent to solids ratio of 10.

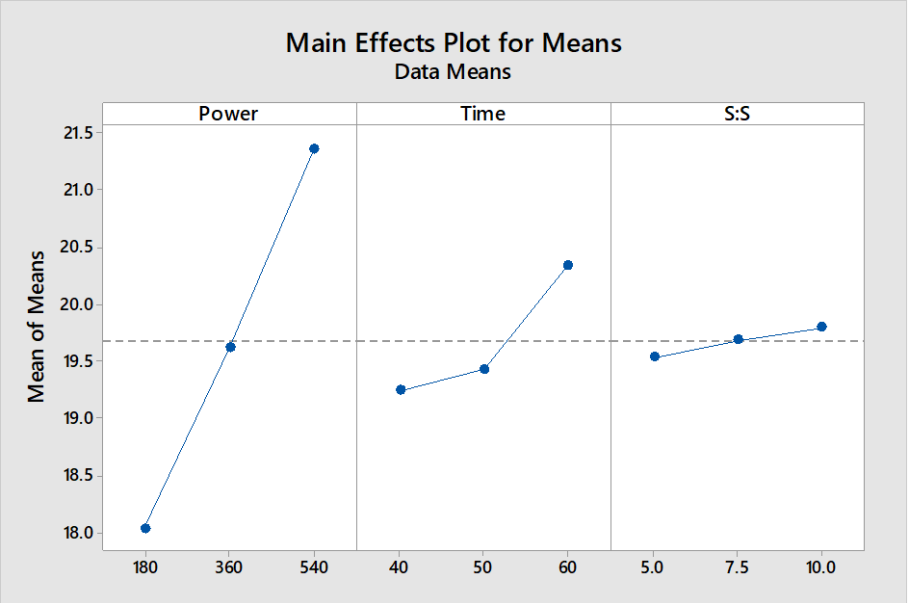


Fig.4.16. Effect of power, time and S:S ratio at different levels expressed in S/N ratio for phospholipids yield from microwave assisted extraction

Qv *et al*. (2014) used *Dunaliellatertiolecta* as raw material to extract lipids using microwave and ultrasound assisted techniques. The study optimized lipids extraction rate at the process parameters of power at 490 W, time 160 s and liquid to solid ratio of 100 mL/g. In the analysis, microwave power was established as extremely significant and time as second significant factor on extraction rate of lipids. However, solvent to solids ratio stood insignificant ($p<0.05$). This study is in agreement with the observations in the present study with regard to microwave assisted extraction of PLs from ghee residue, where the influence of higher power level at lower solvent to solid ratio had better impact on yield of PLs.

Statistical analysis using ANOVA for PLsyield forprocess factors is depicted in Table.4.9 ($p<0.05$). From the data, it is confirmed that microwave power was highly significant in influencing extraction of PLs from the ghee residue samples ($p<0.01$).The optimizedlevels of factors for maximum yield of PLs were obtained as power (540 W), time (60 s) and solvent to solid ratio (10).

Table.4.9. Analysis of variance of process parameters for phospholipids yield from gheeresidueusing microwave assisted extraction

Source	DF	Adj. SS	Adj. MS	F-Value	P-Value
Regression	3	18.4690	6.1563	54.60	0.000
Power	1	16.5515	16.5515	146.78	0.000
Time	1	1.8125	1.8125	16.07	0.010
S:S	1	0.1049	0.1049	0.93	0.379
Error	5	0.5638	0.1128		
Total	**8**	**19.0328**			

The regression equation derived to describe the influence of the process parameters on the yield of PLs in the extract is presented as eq.4.1. The linear equation was in good fit with data by reporting good R^2 value (97.04%). While extracting oil from tiger nuts (*Cyperusesculentus L.*) using microwave assistance, Hu *et al*. (2018) used microwave power, temperature, solvent to solid ratio and time as optimizing factors. In line with the trend observed in the present study, the yield of lipids was reported to increase with increase in power level with the highest value recorded at 420W. The study also concluded that except temperature, all other factors were highly significant in defining the mathematical model for extraction ($p<0.01$). Increase in the yield of PLs in the extract with increasing microwave power may be attributed to increase in temperature of solvent, which in turn may induce better diffusion co-efficient.

Phospholipids yield = 13.20 + 0.009 Power + 0.05 Time + 0.05 S:S----------(4.1)

4.3.1.2. Confirmation test for validating optimized factors for phospholipids yield by microwave assisted extraction

Experiments were conducted in triplicates at theoptimized levels of the process parameters to compare results for the yield of PLs in the extract derived fromthe Taguchi analysis. Central levels of factors were chosen as initial parameters to quantify percentage increase in PLs yield. Yield of PLs at optimized level was reported as 21.96% against yield of 19.26% at middle level (Tables 4.10).

Table.4.10. Confirmation test for yield of phospholipids in the extract under optimized combination for microwave assisted extraction

	Initial process parameter	Optimal process parameter	
		Prediction	Experiment
Level	P_2-Ti_2-S:S_2	P_3-Ti_3-S:S_3	P_3-Ti_3-S:S_3
PL yield	19.26	--	21.96
S/N ratio	25.69	26.95	26.83
Improvement in S/N ratio	1.14		
Percentage increase in phospholipids yield	14.01%		

The increase in yield of PLs was to the tune of 14.01%, which indicated the effectiveness of microwave assisted extraction as validated by the Taguchi design. Further, comparison of S/N ratio reported for predicted and experimental value was very close, which proved therobustness of the design and prediction. Improvement of 1.14 units' in S/N ratio between middle and optimized levels of factors reaffirms the validity of the prediction model.

4.3.1.3.Interactive effect of factors on phospholipids yield in the extract obtained by microwave assistance

The various factors influencing the microwave assisted extraction process exhibited different degree of interaction at the different levels studied during the optimization analysis. The interactive effect of power and time factors is shown in Fig. 4.17 (a). From the figure it is confirmed that with increase in microwave power and time the yield of PLs also in the extract also increased.

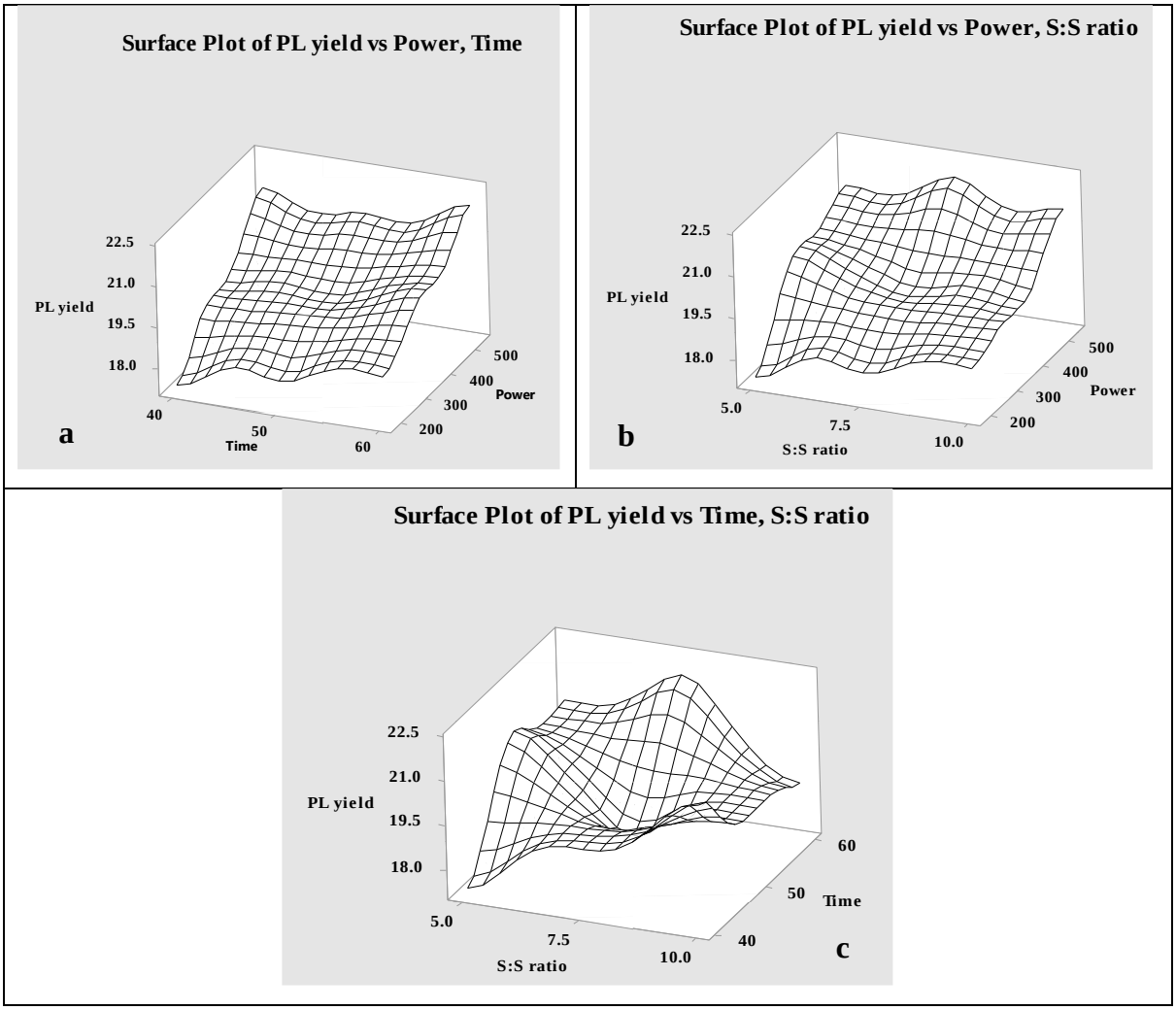


Fig.4.17. Interactive effects of process factors influencing microwave assisted extractionon phospholipids yield (a) time and power; (b) power and S:S ratio; (c) time and S:S ratio

This trend of extraction is in agreement with study conducted by Maran *et al*. (2014) on extraction of pectin from *Citrulluslanatus* fruit rind. The study reported increase in pectin yield with increase in microwave power and time to report highest yield of pectin (25.79%) at 477 W microwave power, 128 s extraction time and 1:20.3 g/mL solid-solvent ratio. The study attributed the observed results on enhanced extraction to rise in temperature and internal pressure in solvent.

Similarly, from Fig. 4.17 (b), it is deduced that amongst power and solvent to solids ratio, powerplayed a significant role in the extraction of PLs. Though no considerable improvement can be seen at lower and middle power levels (180, 360 W), the interactive effect was prominent at higher power level across all S:S ratios considered in the study. This could be due to inadequacy of lower power levels in influencing the pressure gradient to drive the extract into solvent. Contradictorily, time and solvent to solids ratio exhibited an inconsistent behaviour in their interaction to enable maximum PLs yield (Fig. 4.17 (c)).

4.3.1.4. Optimization of process parameters for microwave assisted extraction process based on antioxidant activity of extract

The extract obtained during the trials conducted as per the experimental design outlined in Table 4.7. was also analysed for its antioxidant activity. The DPPH assay for radical scavenging activity (RSA) of extract was evaluated and expressed in percentage.The influence of the process parameters on the antioxidant activity of extract were different to that observed for its effect on the yield of PLs in the extract (Fig.4.18). The solvent to solids ratio depicted highest influence on antioxidant activity of extract by reporting a mean delta value of 3.13 for S/N ratio. Microwave power was deduced as the second important influencing factor with delta values of 2.47 followed by time factor contributing the least influence with a delta value of 0.37.

The optimized levels for the process parameters for microwave assisted extraction from ghee residue when analysed for maximizing the antioxidant activity was deduced as 180W power, 40s time at a solvent to solids ratio of 10. Kumar *et al.* (2019) conducted a study for extraction of bioactive phytoceuticals and evaluation of its antioxidant activity and reported similar results. The study showed inverse relation of microwave power and extraction time on antioxidant activity of extract (expressed on µmol Trolox equivalents). However, in contrast to the findings of the present study, the solvent to solids ratio was reportedas insignificant in its effect on antioxidant activity of extract. The study projected the higher recovery of total phenolic compound at lower power level as the reason for higher antioxidant activity.

The mathematical model obtained from the data analysis to predict the antioxidant activity of the extract as a linear function of the process parametersis presented as eq. 4.2.

Antioxidant activity=25.87 – 0.006 Power – 0.02 Time + 0.63 S:S ratio----------(4.2)

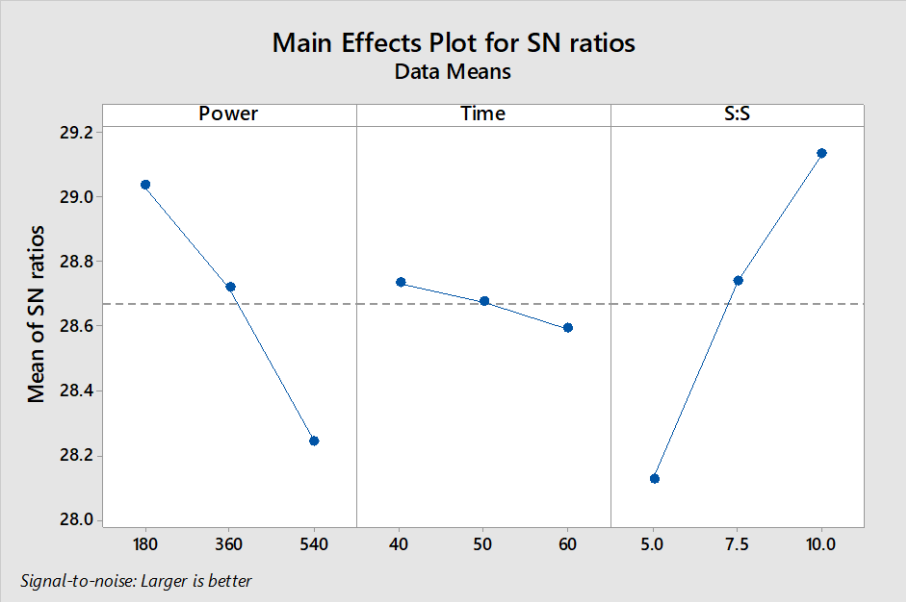


Fig.4.18. Effect of power, time and S:S ratio at different levels expressed in S/N ratio for antioxidant activity of extract from microwave assisted extraction

ANOVA of the data obtained for antioxidant activity of extract affirmed the significant influence of solvent to solids ratio and power level on antioxidant activity (Table. 4.11). However, treatment time remainedinsignificant in its effect on antioxidant activity of extract ($p<0.05$). The regression equation derived (eq. 4.2) was in good fit with the experimental data by reporting R^2 value of 96.57% ($p<0.05$). Huang *et al.*(2017)evaluated the extraction of flavonoids from pomegranate peel and inferred similar results. Long extraction time by microwave led to degradation of flavonoids which had a bearing on antioxidant activity of the extract. Further, the study also confirmed that higher solvent to solids ratio resulted in increased extraction of flavonoids.

Table 4.11.Analysis of variance of process parameters for antioxidant activity of extract from ghee residue using microwave assisted extraction

Source	DF	Adj. SS	Adj. MS	F-Value	P-Value
Regression	3	24.0797	8.0266	46.92	0.000
Power	1	9.1513	9.1513	53.50	0.001
Time	1	0.2017	0.2017	1.18	0.327
S:S	1	14.7267	14.7267	86.09	0.000
Error	5	0.8553	0.1711		
Total	**8**	**24.9350**			

4.3.1.5. Confirmation test for validating optimized factors for antioxidant activity of extract by microwave assistance

Experiments were conducted at middle and optimized levels of the parameters to evaluate the validity of optimized levels of the factors. Antioxidant activity of the extract obtained when extracted at the optimized combination of parameters was 29.89%, with a S/N ratio of 29.51. Improvement in S/N ratio from mid-point level combination of the parameters to optimized levels was 0.57. Similarly, S/N ratio of experimental and predicted value was very close (29.57 and 29.51), which confirmed the validity of the analysis. The extract obtained at the optimized combination of parameters reported an increase of 6.67% of antioxidant activity compared to that extracted at mid-point levels of the process parameters (Table.4.12)

Table.4.12. Confirmation test for antioxidant activity of extract under optimized combination for microwave assisted extraction

	Initial process parameter	**Optimal process parameter**	
		Prediction	**Experiment**
Level	P_2-Ti_2-S:S_2	P_1-Ti_1-S:S_3	P_1-Ti_1-S:S_3
Antioxidant activity	28.02	--	29.89
S/N ratio	28.94	29.57	29.51
Improvement in S/N ratio	0.57		
Percentage increase in antioxidant activity of extract	6.67%		

4.3.1.6. Interactive effect of factors on antioxidant activity of extract obtained by microwave assistance

The interactive effect of treatment time with lower levels of microwave power did not influence the antioxidant activity of the extract (Fig. 4.19 (a)). However, increased treatment time at high power levels led to lower antioxidant activity. This could be due to denaturation of compounds in the extract. The resultsare in agreement with studies conducted on *Vernoniaamygdalina* leaf for flavonoids and antioxidants using microwave assistance by Alara *et al*. (2018). The study found that antioxidant activity of extract decreased with increasing microwave power and time of treatment.

The interactive plots of the effect of solvent to solids ration and microwave power (Fig. 4.19 (b)) revealed that the effect of power was nearly flat at all levels of the solvent to solids ratios investigated in this study. No specific trend could be deduced on the interactive effect of treatment time and from the solvent and solids ratio (Fig. 4.19 (c)).

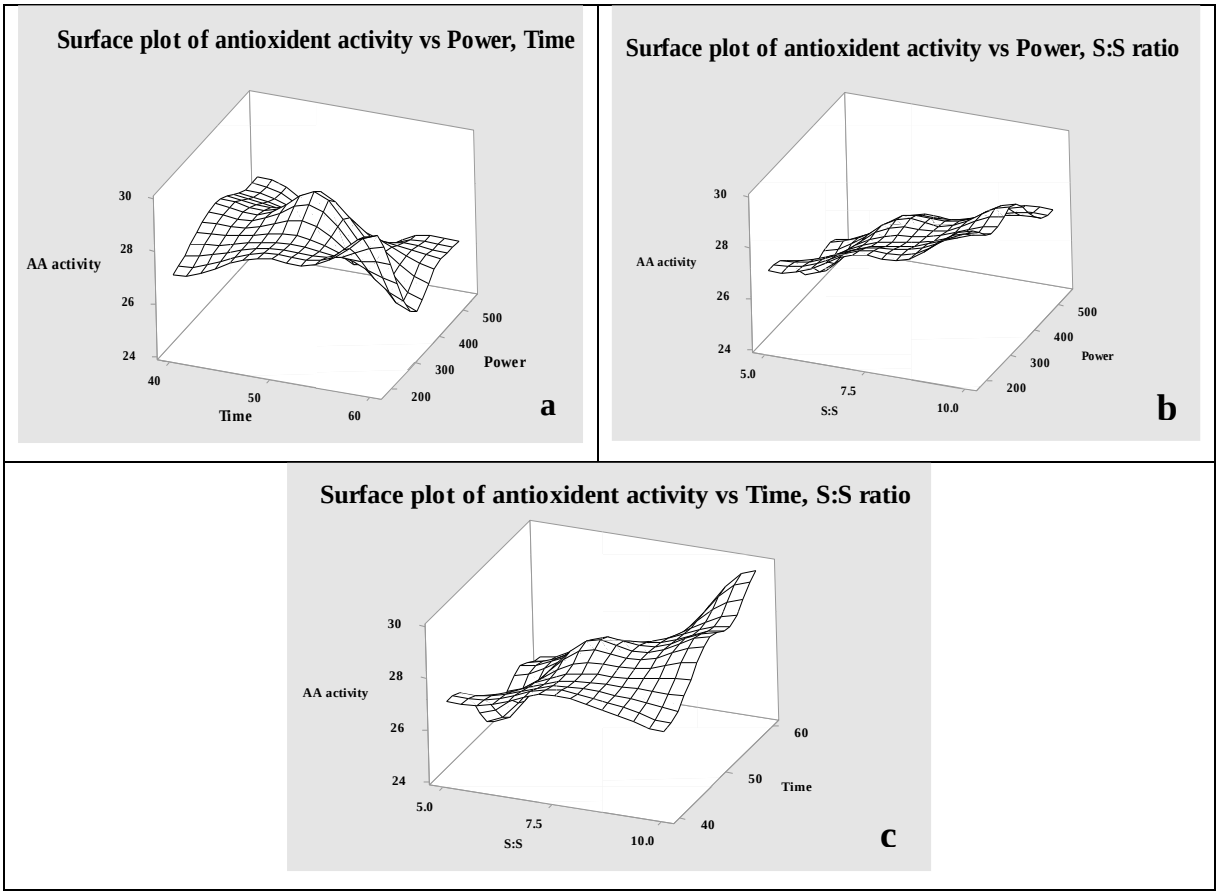


Fig. 4.19. Interactive effects of process factors influencing microwave assisted extraction on antioxidant activity of extract (a) time and power; (b) power and S:S ratio; (c) time and S:S ratio

4.3.2. Optimization of process parameters for ultrasound assisted treatment

The efficacy of ultrasonication as a treatment technique to assist the extraction of PLs from ghee residue was evaluated with four independent factors at three levels and the data obtained is tabulated in Table 4.13. It was observed during the experiments that the ultrasonication of ghee residueup to 6 min. did not change temperature of sample. Hence, the treatment was undertaken by adding the solvent (water) maintained at different temperatures to establish the influence of temperatureon the yield of PLs yield and antioxidant activity of the extract.

4.3.2.1. Optimization of process parameters for ultrasound assisted extraction process based on yield of PLs

During ultrasound assistedextraction, the pre-treated ghee residue sample was well dispersed in solvent leading to mild light colour in extract compared to microwave treated sample. To remove the residual ghee residue from the sample container, a small quantity of water was used. The yield of PLs and antioxidant activityrecorded for the obtained extract at different levels of factors under Taguchi L_9 orthogonal array design is presentedin Table 4.13.

Table.4.13. Phospholipids yield and antioxidant activity of extract from ghee residue treated with ultrasound assisted extraction based on Taguchi L_9 orthogonal array design

Run	Power (%)	Time (min.)	Temp (°C)	S:S Ratio (w/v)	Phospholipids (% dry basis)	Antioxidant Activity (%)
1	60	3	60	10	18.54±0.29	47.01±1.23
2	60	4	70	12.5	19.78±1.25	47.94±1.51
3	60	5	80	15	20.19±0.90	49.63±0.85
4	70	3	70	15	21.89±0.73	49.99±0.72
5	70	4	80	10	22.01±0.60	47.06±1.57
6	70	5	60	12.5	20.82±0.58	48.45±0.96
7	80	3	80	12.5	23.89±0.72	49.00±1.04
8	80	4	60	15	22.67±0.93	50.64±0.38
9	80	5	70	10	22.78±0.78	47.47±0.99

Ultrasound assisted extraction resulted in a yield of PLs in the range of 18.54 to 23.89% across different treatment combinations studied in the design. Among the factors considered, ultrasound power exhibited most influence on means of S/N ratio.The yield of PLs in the extract was found to increase with increase in ultrasound power levels (Fig. 4.20).

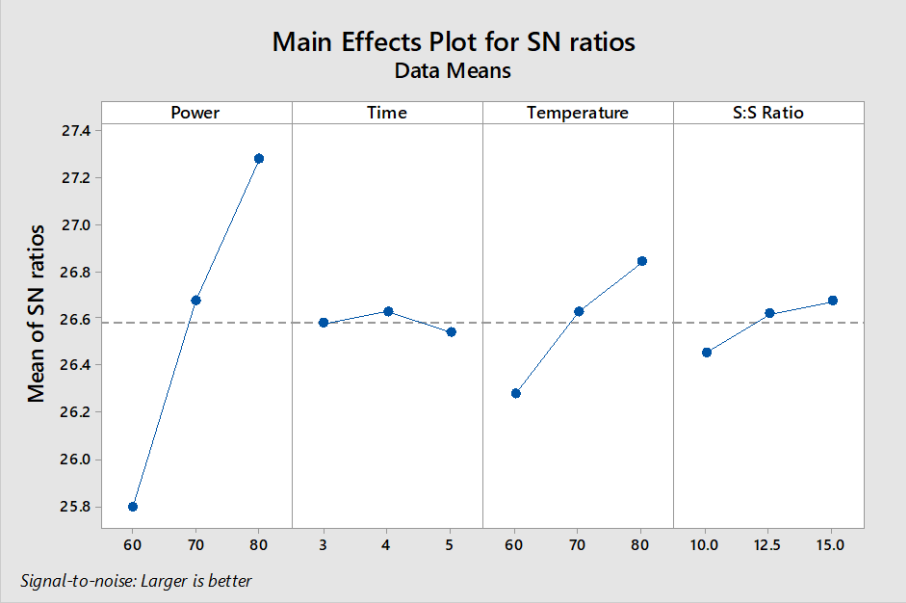


Fig.4.20. Effect of power, time, solvent temperature and S:S ratio at different levels expressed in S/N ratio for phospholipids yield from ultrasound assisted extraction

Temperature also followed similar trend by reporting better S/N ratio at 80°C. Due to practical operational difficulties, the higher level for temperature and power levels were restricted to 80°C and 80%, respectively. Time and solvent to solids ratio exhibited least influence on extraction of PLs from the ghee residue during the ultrasound assisted process. Based on rankings for delta values emulated from analysis, factors influencing the yield of

PLs in the extract can be placed in the order power> temperature> solvent to solids ratio>time.

Through ANOVA the influence of different factors on yield of PLs in the extract during the ultrasound assisted extraction process was reaffirmed (Table. 4.14). From the table, it is deduced that ultrasound power was highly significant in maximizing PLs yield ($p<0.01$). Temperature was also found to be significant ($p<0.05$) but, solvent to solids ratio and time factors were insignificant in its effect on the yield of PLs in the extract ($p<0.05$). The regression equation (eq-4.3) developed by including all main factors to predict the yield of PLs in the extract obtained by ultrasound assisted process demonstrated a good fit to the data (R^2 98.89).

Table.4.14. Analysis of variance of process parameters for phospholipids yield in extract from ghee residue using ultrasound assisted extraction

Source	DF	Adj. SS	Adj. MS	F-Value	P-Value
Regression	4	22.6783	5.6696	88.69	0.000
Power	1	19.5482	19.5482	305.80	0.000
Time	1	0.0468	0.0468	0.73	0.440
Temperature	1	2.7473	2.7473	42.98	0.003
S:S ratio	1	0.3361	0.3361	5.26	0.084
Error	4	0.2557	0.0639		
Total	8	22.9340			

PL yield= 3.19+0.18 Power-0.08 Time + 0.06Temperature+0.09 S:S ratio---------- (4.3)

Thefindings in this study are in agreement with the results reported for ultrasound assisted extraction of oil from papaya seeds by Samaram *et al*. (2015). The study concluded that ultrasound power, temperature, time and solvent to sample ratioinfluenced the extraction process, with the results indicating a positive influence of power and temperature on the oil yield. In the present study, softening of ghee residue matrix at elevated temperatures was noted and could be considered as one of the reasons for the improved yield of PLs in the extract. Further, the mechanical vibrations generated in the matrix at higher power level may also have assisted in facilitating better penetration of solvent and wider material contact within the matrix to improve the yield (Patist and Bates, 2008).

4.3.2.2. Confirmation test for validating optimized factors for phospholipids yield by ultrasound assisted extraction

The data for the validation of the performance under optimized combination of parameters is presented in Table 4.15. It can be seen that the yield of PLs in the extract reported an increase of 9.53% from the value obtained at the mid-level combination of parameters. The validity of

the prediction model was reaffirmed by comparing S/N ratios reported for predicted and experimental values. Predicted S/N ratio (27.65) was in agreement with the experimental value (27.64). The increase in S/N ratio for the yield of PLs when compared between the results for the mid-level combination of parameters and the optimized level of parameters was only 0.79.

Table.4.15. Confirmation test for yield of phospholipids in the extract under optimized combination for ultrasound assisted extraction

	Initial process parameter	**Optimal process parameter**	
		Prediction	**Experiment**
Level	P_2-Ti_2-Te_2-S:S_2	P_3-Ti_2-Te_3-S:S_3	P_3-Ti_2-Te_3-S:S_3
PL yield	22.02	--	24.12
S/N ratio	26.85	27.65	27.64
Improvement in S/N ratio	0.79		
Percentage increase in phospholipids yield	9.53%		

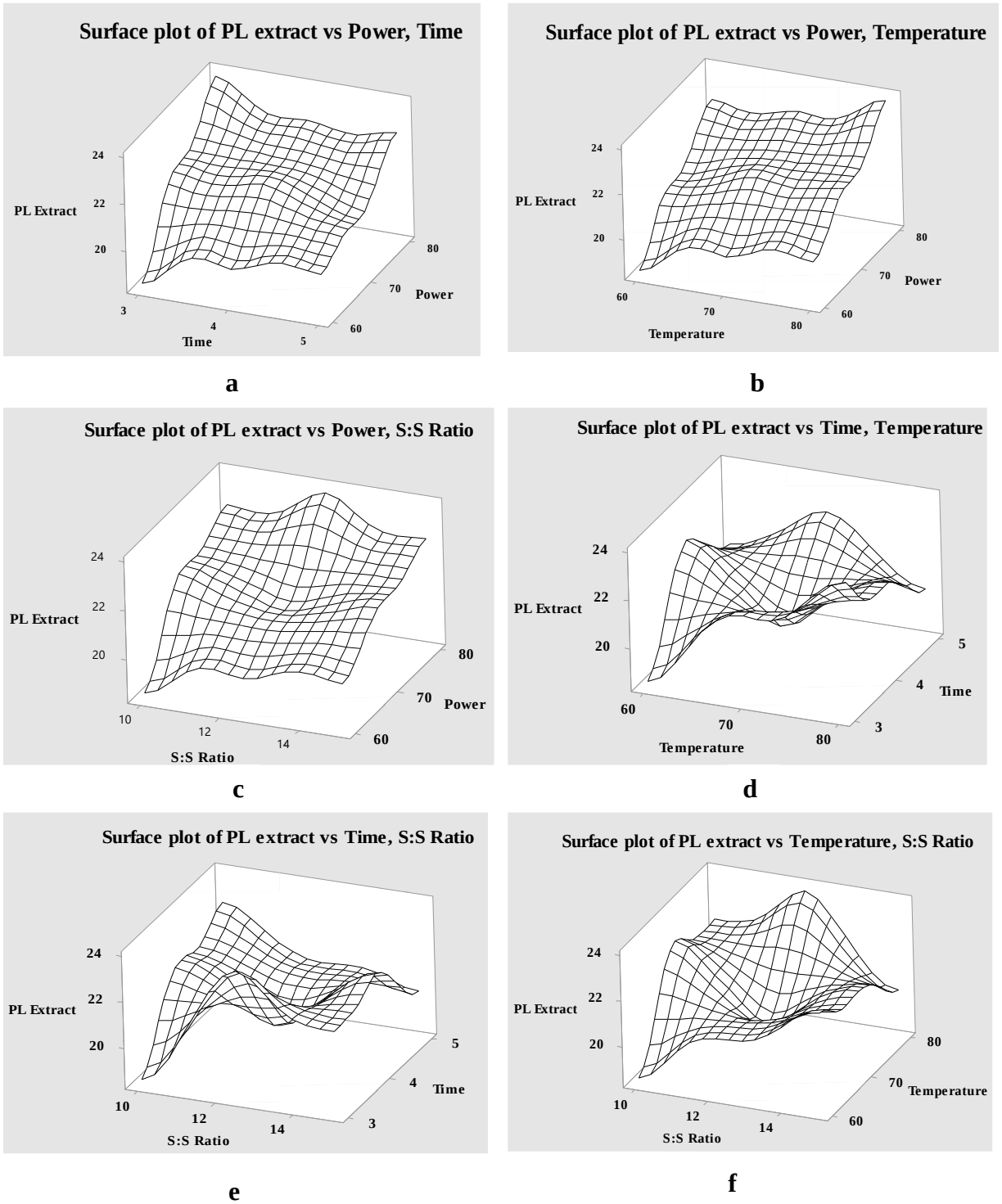
4.3.2.3. Interactive effect of factors on phospholipids yield in the extract obtained by ultrasound assistance

The interactive effect of the different process parameters evaluated for the ultrasound assisted extraction process for yield of PLs from ghee residue is played in Fig 4.20. Though both ultrasound power and extraction time positively influenced the yield of PLs, influence of power was highly significant compared to that of treatment time (Fig. 4.21 (a)).Time factor at higher levels (5 min.) had the least influence on yield of PLs at lower power level (60 and 70%).

In a study on extraction of lipids from oleaginous yeast biomass, Kumar and Banerjee, (2019) reported the optimal time for extraction as 20 min. This was based on their study where ultrasound power was applied at 40 W and 35 °C for different timings (5, 10, 15, 20, 30 and 60 min.), and maximum lipids content (37±0.38%) was obtained at 20 min. The enhanced extraction of lipids at lower time of exposure in the study was ascribed to mechanical shear caused by high ultrasound intensity.

The interactive effect of temperature and time was also found to exert a positive influence on the yield of PLs in the extract (Fig. 4.21 (b)). The synergistic effect of both these factors was found to facilitate the movement of PLs into solvent. Gam *et al*. (2020) reported increased destruction of cells at higher ultrasound power levels which enhanced the extraction of total phenolic compounds from *epigallocatechin gallate*. Interactive effect of temperature and ultrasound power was positive and maximum yield was reported at 80.2^{o}C (tried between 50 to 90^{o}C). Solvent to solids ratio in interaction with ultrasound power was effective at higher

power levels. At lower power level, this interaction was very limited and only a small rise in the yield of PLs was observed when the solvent to solids ratio ranged between 10 and 15 (Fig. 4.21 (c)).



a **b** **c** **d** **e** **f**

Fig. 4.21. Interactive effects of process factors influencing ultrasound assisted extraction on phospholipids yield (a) power and time (b) power and temperature (c) power and S:S ratio (d) temperature and time (e) time and S:S ratio (f) temperature and S:S ratio

The interactive effect of time with temperature and solvent to solids ratio during the ultrasound assisted extraction of PLs from ghee residue is depicted in Fig. 4.21 (d-e). From the plot it can be deduced that the interaction between these factors was inconsistent with no specific trend depicted with respect to the yield of PLs in the extract.

Similar graphs were reported by Yue *et al*. (2012) during their studies on ultrasound assisted extraction of polyphenols from unripe apples. Interestingly, the authors reported that ultrasound parameters influenced total polyphenols content of the extract in the sequential order of ethanol concentration>power>time>temperature. In another study, the interactive effect of solvent to solids ratio and time was reported to be insignificant while extracting oil from papaya seeds in an ultrasound assisted process (Samaram *et al*., 2015).

No significant interaction could be deduced between solvent to solids ratio and temperature during the extraction process (Fig. 4.21 (f)). This trend is in agreement with study reported by Zivkovic *et al*. (2018) on ultrasound assisted extraction of punicalin content from pomegranate peel. Only temperature and solvent concentration were reported as significant parameters influencing the ultrasound assisted extraction of punicalin content from the selected matrix.

4.3.2.4. Optimization of process parameters for ultrasound assisted extraction process based on antioxidant activity of extract

The process of ultrasound assisted extraction from the ghee residue sample was significantly influencedby the solvent to solids ratio and power (Fig. 4.22). The antioxidant activity of the extract was observed to increase with increase in solvent to solids ratio.The highest antioxidant activity reported for the extract obtained when the solvent to solids ratio was maintained at a value of 15. Ultrasound power was found to be the second highest influencing factor with a trend of increase in antioxidant activity of extract with increase in power levels. Based on rankings secured by factors (means), antioxidant activity of the extract wasinfluenced in the ordersolvent to solids ratio>power>temperature >time. Vuong *et al*. (2014) studied the antioxidant activity of extract from *uphorbiatirucalli* using ultrasound assisted process and reportedthat the treatment time was insignificant in its effect. The study reported influence of factors in the order of temperature > power > time by reporting maximum value of 66.5% DPPH at optimal conditions.

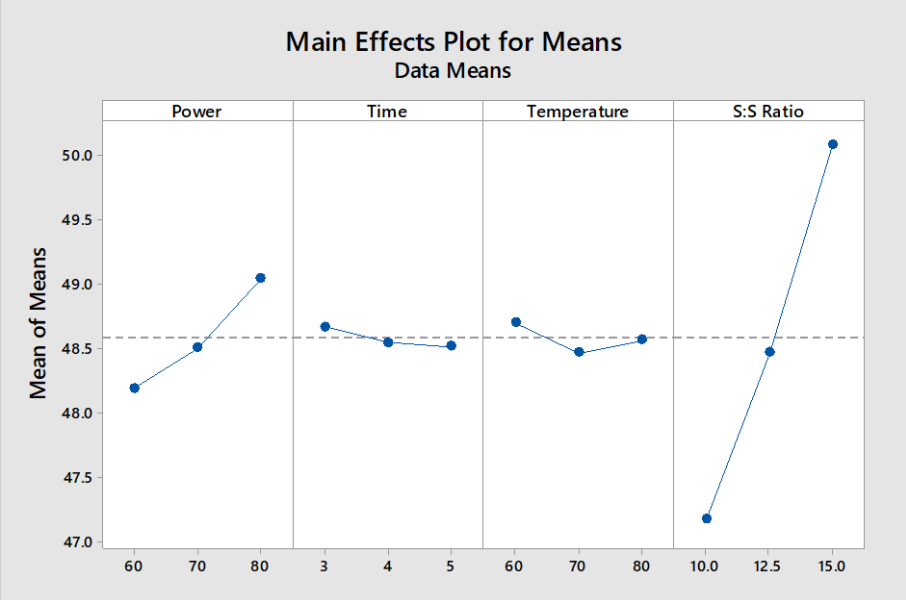


Fig. 4.22. Effect of power, time and S:S ratio at different levels expressed in S/N ratio for antioxidant activity of extract from ultrasound assisted extraction

The ANOVAof the experimental results indicated significant effect of solvent to solids ratio and power factors ($p<0.05$) on the antioxidant activity of the extract. Temperature and time were found to exert nosignificant influence on antioxidant activity of the extract (Table 4.16). Regression equation (eq. 4.4) developed to describe the effect of the main factors on the antioxidant activity of the extract reported a R^2 value of 98.98%. While studying antioxidant activity of extract from wild garlic using ultrasonication technique Tomsik*et al.* (2016) reported similar results. The study reported a mathematical model with good R^2 value by eliminating the time factor, whereas, temperature factor showed a negative influenceon the antioxidant activity of extract.

Table.4.16. Analysis of variance of process parameters for antioxidant activity of extract from ghee residue using ultrasound assisted extraction

Source	DF	Adj. SS	Adj. MS	F-Value	P-Value
Regression	4	13.8017	3.4504	96.68	0.000
Power	1	1.0668	1.0668	29.89	0.005
Time	1	0.0337	0.0337	0.95	0.386
Temperature	1	0.0280	0.0280	0.79	0.426
S:S ratio	1	12.6731	12.6731	355.11	0.000
Error	4	0.1428	0.0357		
Total	8	13.9444			

Antioxidant activity= 39.13 + 0.04 Power - 0.07 Time - 0.006 Temp. + 0.58 S:S ratio----(4.4)

4.3.2.5. Confirmation test for validating optimized factors for antioxidant activity of extract by ultrasound assistance

Test to confirm the findings were conducted at optimized conditions for antioxidant activity of the extract and the results are tabulated in Table 4.17. The experimental values obtained at the combination of mid-point levels and optimized levels of the parameters reported change in S/N ratio of 0.35 units with the antioxidant activity of the extract estimated as 48.79 and 51.94%, respectively. This change was equivalent to a rise of 6.45% in antioxidant activity of the extract. Further, it was also observed that the predicted and experimental values were very close to each other indicating validity of optimization studies.

Table.4.17. Confirmation test for antioxidant activity of extract under optimized combination for ultrasound assisted extraction

	Initial process parameter	**Optimal process parameter**	
		Prediction	**Experiment**
Level	P_2-Ti_2-Te_2-S:S_2	P_3-Ti_1-Te_1-S:S_3	P_3-Ti_1-Te_1-S:S_3
Antioxidant activity (%)	48.79	--	51.94
S/N ratio	33.76	33.17	34.11
Improvement in S/N ratio	0.35		
Percentage increase in antioxidant activity	6.45%		

4.3.2.6. Interactive effect of factors on antioxidant activity of extract obtained by ultrasound assisted extraction

Two-way interactive effect of the main factors on antioxidant activity of the extract for the ultrasound assisted extraction from ghee residue is depicted in Fig. 4.23 (a-f). The synergistic effect of power and time (Fig 4.23 (a)), temperature and power (Fig. 4.23 (b)) and time and temperature (Fig. 4.23 (d)) reported no correlation with each other for antioxidant activity of the extract. As time and temperature stood insignificant in influencing antioxidant activity of the extract, their interaction amongst each other did not show any trend. However, interaction between power and solvent to solids ratio on antioxidant activity of the extract depicted a linear behaviour. Antioxidant activity of the extract increased with increase in solvent to solids ratio and power (Fig.4.23 (c)). This trend is in agreement with DPPH activity (mg TE/g) of *Catharanthusroseus* reported by Pham *et al*. (2018) using ultrasound. The study attributed this behaviour to the saturation of solutes at lower solvent concentration, limiting the movement of the bioactive compound into solvents.

In case of the interactive effects of time and solvent to solids ratio, the ratio showed influence while the time stood inexpressive (Fig. 4.23 (e)). Results on olive leaf extract by ultrasound assisted extraction reported similar trend for solvent to solids ratio and time (Sahin and

Samli, 2013). The study discussed the possibility of overexposure up to a threshold time enhancing the movement of constituents in to the solvent, beyond which the molecules degraded leading to lower antioxidant activity of the extract.

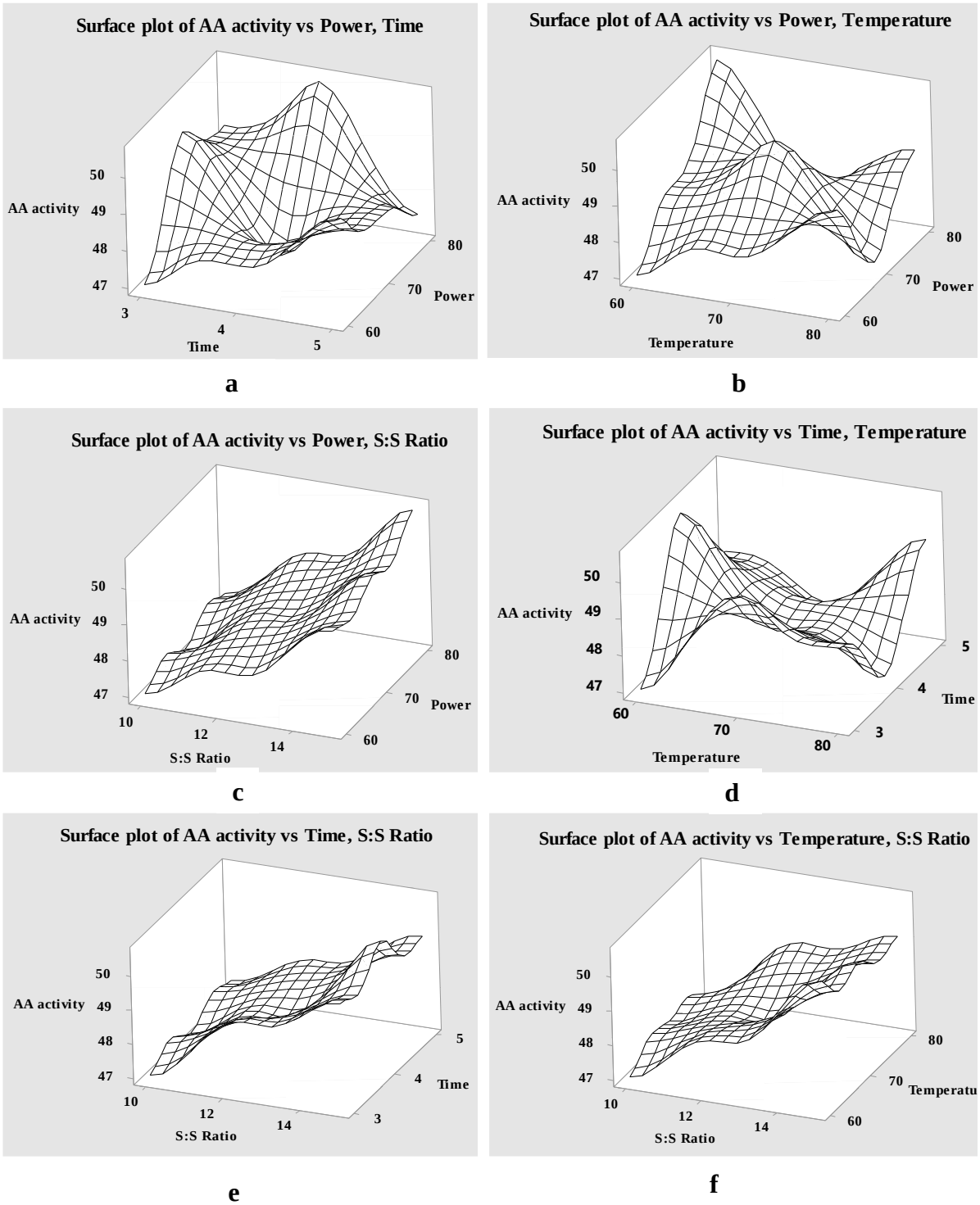


Fig. 4.23. Interactive effects of process factors influencing ultrasound assisted extraction on antioxidant activity of extract (a) power and time (b) power and temperature (c) power and S:S ratio (d) temperature and time (e) time and S:S ratio (f) temperature and S:S ratio

Interaction between temperature and solvent to solids ratio also followedsimilar trend reported for time and solvent to solids ratio. Though the effect of temperature on the antioxidant activity of the extract remained more or less similar across all levels studied, influence of solvent to solids ratio was linearly linked to antioxidant activity of extract. Samaram *et al*. (2015) reported similar influence of solvent to solidsratio, alongwith a negative influence of temperature on antioxidant activity of the extract.

4.3.3. Optimization of process parameters for PEF treatment

The experimental design to evaluate the effect of the parameters along with the yield of PLs and antioxidant activity of the extract using PEF assisted extraction process from ghee residue and its optimization is presented in Table 4.18. A comparative analysis of the data indicated that the yield of PLs was lower in PEF treated samples compared to ultrasound and microwave assisted extraction samples. The yield of PLs in the extract obtained in the PEF assisted process varied between 11.85 to 18.13% on extract weight basis, while the antioxidant activity of the extract obtained by the PEF assisted process ranged between 31.05 to 37.05%.

Table.4.18. Phospholipid yield and antioxidant activity of extract from ghee residue treated with PEF assisted extraction based on Taguchi L_9 orthogonal array design

Run	Voltage (kV/cm)	Time (min.)	S:S Ratio (w/v)	Phospholipids (% extract weight basis)	Antioxidant Activity (%)
1	40	3	7.5	11.85±1.04	30.05±0.49
2	40	4	10	12.38±1.46	31.72±0.11
3	40	5	12.5	12.62±0.93	32.50±0.10
4	50	3	7.5	14.85±1.07	33.05±0.31
5	50	4	10	14.88±0.39	33.98±0.53
6	50	5	12.5	15.93±0.92	35.62±0.56
7	60	3	7.5	15.42±1.04	34.01±0.71
8	60	4	10	18.13±1.48	36.52±0.78
9	60	5	12.5	17.54±0.50	37.05±1.06

4.3.3.1. Optimization of process parameters for PEF assisted extraction process based on yield of PLs

Among the process parameters for the PEF assisted extraction process, the process voltage played a significant role in increasing the yield PLs yield in the extract with increasing values reported with increase in voltage level (Fig. 4.24). The next significant factor influencing the extraction of PLs from the ghee residue samples was the treatment time with a mean delta value of 1.32 followed by solvent to solids ratio with mean delta value of 1.0. The optimal combination of process parameters deduced for PEF assisted extraction process for

maximizing the yield of PLs from the ghee residue samples was found to be power (60 kV/cm), time (5 min.) and solvent to solid ratio (7.5).

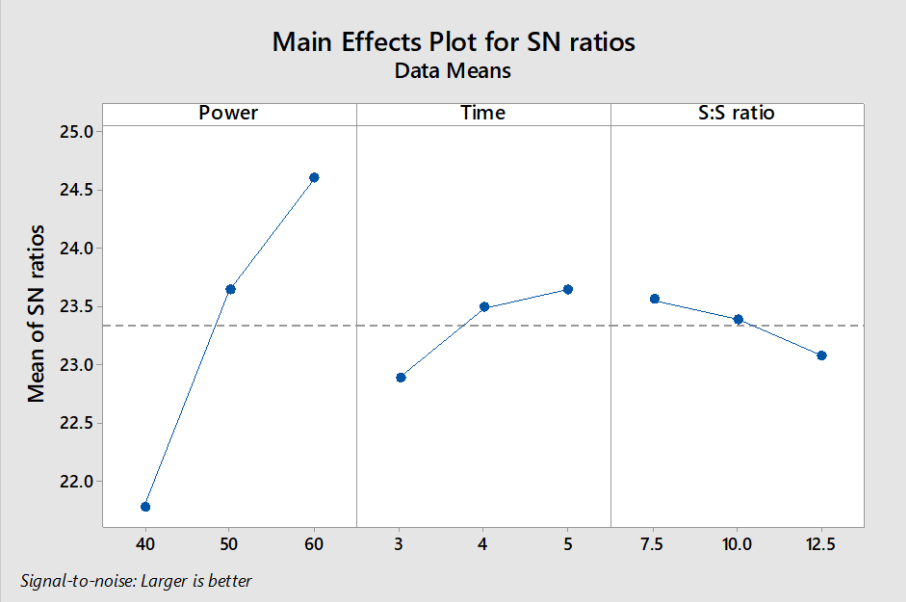


Fig.4.24. Effect of voltage, S:S ratio and time at different levels expressed in S/N ratio for phospholipids yield from PEF assisted extraction

Statistical analysis of the effect of the main factors through ANOVA established significant influence of voltage on the yield of PLs ($p<0.01$). Though time was observed to be marginally influential in its effect on the yield of PLs in the extract, the effect of solvent to solids ratio was deemed to be insignificant in this analysis (Table 4.19). Further, it was noted that the ghee residue samples dispersed in solvent settled at the bottom of treatment chamber, leaving mostly fine particulate material for contact with the solvent, hampering the overall extraction yield. Regression equation (eq.4.5) generated for the yield of PLs in the extract as a function of the main factors was in good agreement with experimental data with an R^2 value of 95.83.

PLs yield=2.32 + 0.24 Voltage + 0.66 Time – 0.20 S: S ratio---------- (4.5)

Table.4.19. Analysis of variance of process parameters for phospholipids yield fromghee residue using PEF assisted extraction

Source	DF	Adj. SS	Adj. MS	F-Value	P-Value
Regression	3	37.938	12.6459	38.31	0.001
Voltage	1	33.817	33.8165	102.43	0.000
Time	1	2.627	2.6268	7.96	0.037
S:S ratio	1	1.494	1.4943	4.53	0.087
Error	5	1.651	0.3301		
Total	8	39.588			

The extraction of polysaccharides from corn silk was increased from 5.23% to 7.59% when PEF voltage intensity was increased from 10 to 30 kV/cm (Zhao *et al*., 2011). This reference corroboratedthe hypothesis that higher field strength aids to drive solute from the sample matrix into the solvent. As the ghee residue was only partially dispersed in water, higher voltage levels induced better movement of PLs from matrix, improving the yield.

4.3.3.2. Confirmation test for validating optimized factors for phospholipids yield by PEF assistance

Evaluation of factors at optimized levels for PEF assisted extraction of PLs from ghee residue resulted in good correlation between experimental and predicted values. Significant rise of 21.25% in the yield was observed when experiments were conducted at optimized levels of the process parameters when compared to the mid-point combination. A close relation between predicted and experimental values for S/N ratio was also noted (Table. 4.20), indicating validity of regression model developed during the optimization process. Comparative evaluation of the S/N values between optimized and mid-point levels of the process parameters indicated an improvement of 1.68 units, affirming the robustness of the optimized results.

Table.4.20. Confirmation test for yield of phospholipids in the extract under optimized combination for PEF assisted extraction

	Initial process parameter	Optimal process parameter	
		Prediction	Experiment
Level	V_2-Ti_2-S:S_2	V_3-Ti_3-S:S_1	V_3-Ti_3-S:S_1
PL yield	14.96	--	18.14
S/N ratio	23.49	25.12	25.17
Improvement in S/N ratio	1.68		
Percentage increase in phospholipids yield	21.25%		

4.3.3.3. Interactive effect of factors on phospholipids yield in the extract obtained by PEF assisted extraction

The two-way interactive effect of the main factors during the PEF assisted extraction of PLs rich extract from ghee residue is plotted in Fig. 4.25. The interactive effect of voltage and time indicated the greater influence of voltage on the yield of PLs in the extract at mid-level for treatment time (Fig.4.25 (a)). However, this did not translate into significant output and no considerable increase in yield of PLs was recordedacross different time levels in interaction with voltage. This is in agreement with observations reported by Liu, *et al*.(2018) on extraction of phenolic compounds from onion using PEF assisted process. The interactive effect of solvent to solids ratio and PEF voltage depicted the positive effect of voltage on the

yield of PLs in the extract (Fig.4.25 (b)). However, extract yield was slightly better at lowersolvent and solids ratio compared to middle and higherlevels. Increase in this ratio did not show any raise in PLs yield, probably owing to settlement of the ghee residue in treatment chamber rather being in dispersion. No consistent interaction was noted between time and solvent to solids ratio on the yield of PLs in the extract as is evident from Fig. 4.25 (c). This could again be attributed to the negative effect of solvent to solids ratio coupled with very moderate influence of time factor on the yield of PLs in the extract.

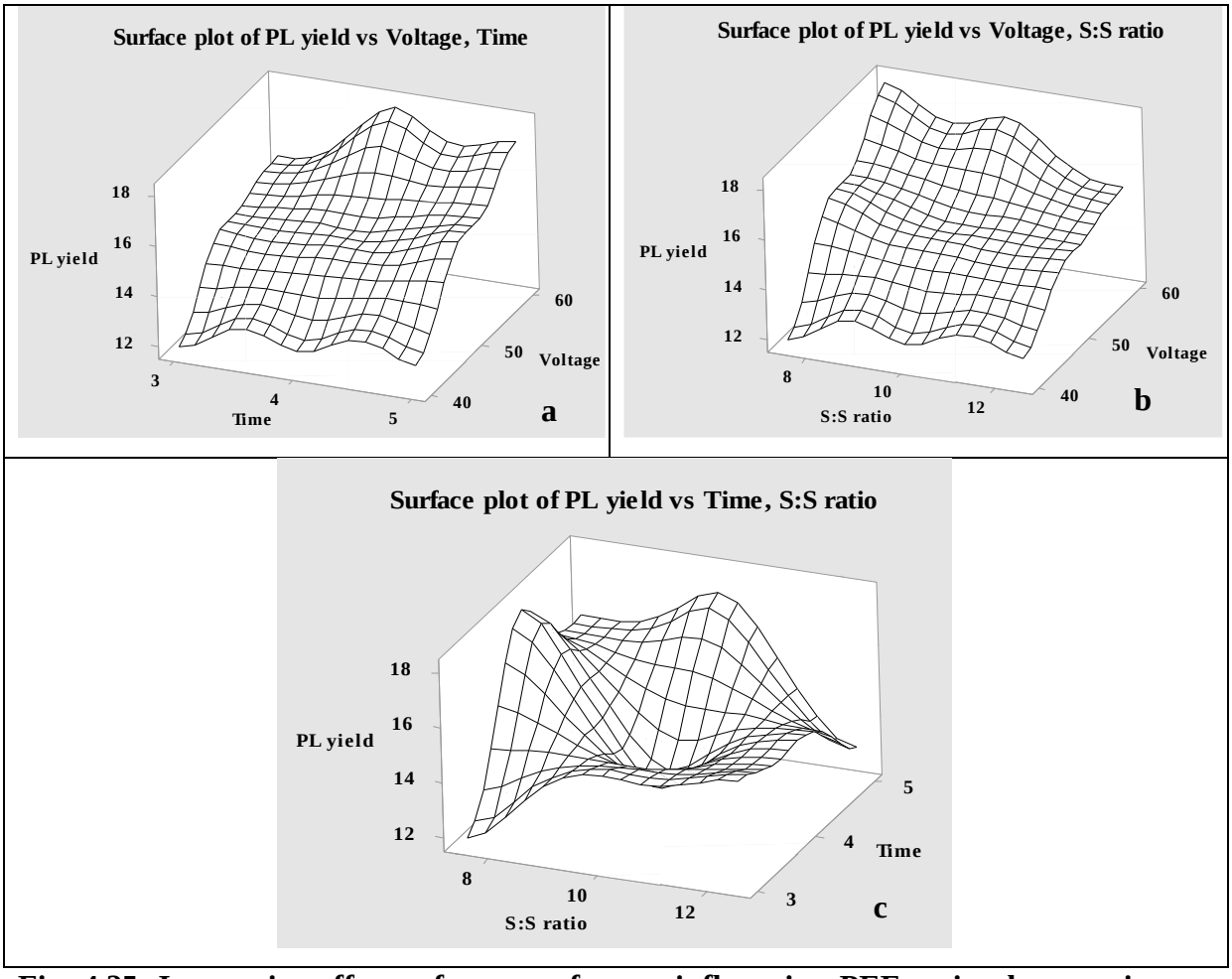


Fig. 4.25. Interactive effects of process factors influencing PEF assisted extraction on phospholipids yield (a) voltage and S:S ratio; (b) voltage and time; (c) S:S ratio and time

4.3.3.4.Optimization of process parameters for PEF assisted extraction process based on antioxidant activity of extract

The antioxidant activity of the extract obtained by the PEF assisted extraction process is compiled in Table 4.18. It can be seen that overall, the extract from the PEF assisted process exhibited better antioxidant activity than extract obtained from the microwave assisted process but lesser than the extract obtained by the ultrasound assisted process. The effect of

the main factors on the antioxidant activity of the extract obtained by the PEF assisted process followed a similar trend to that of the results obtained for yield of PLs in the extract by this technique (Fig. 4.26). However, the influence of time on antioxidant activity of extract was of higher degree compared to that observed for the yield of PLs in the extract. Optimized levels of factors for maximum antioxidant activity of the extract were determined as voltage (60 kV/cm), time (5 min.) and solvent to solids ratio (7.5). Based on delta rankings recorded for S/N ratios of means, the sequential order of influence of different factors was deemed to be PEF voltage>time>solvent to solids ratio.

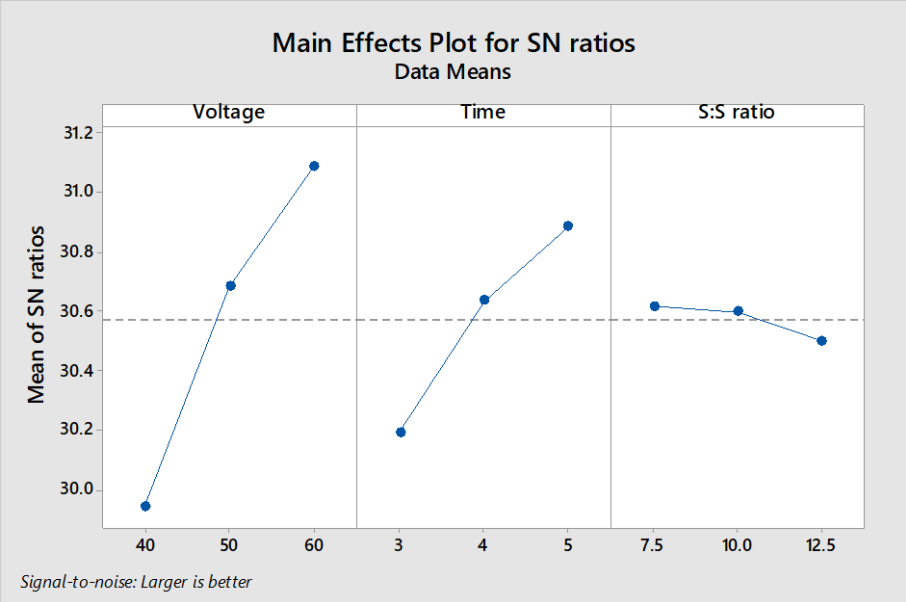


Fig.4.26. Effect of voltage, time and S:S ratio at different levels expressed in S/N ratio for antioxidant activity of extract from PEF assisted treatment

Voltage was highly significant ($p<0.01$), time was significant ($p<0.05$) and solvent to solids ratio was insignificant ($p<0.05$) in influencing antioxidant activity of the extract (Table 4.21). Regression equation (eq.4.6) to describe the antioxidant activity of the extract with the main factors influencing the PEF assisted process was found to provide a good fit to the experimental data (R^2 value of 92.27%).

Table.4.21. Analysis of variance of process parameters for antioxidant activity of extract from ghee residue using PEF assisted extraction

Source	DF	Adj. SS	Adj. MS	F-Value	P-Value
Regression	3	40.8349	13.6116	59.37	0.000
Voltage	1	29.5260	29.5260	128.79	0.000
Time	1	10.8273	10.8273	47.23	0.001
S:S ratio	1	0.4817	0.4817	2.10	0.207
Error	5	1.1463	0.2293		
Total	8	41.9812			

Antioxidant activity= 18.50 + 0.22 Voltage + 1.34 Time - 0.11 S:S ratio ---------- (4.6)

A similar revelation was made by Gagneten *et al.* (2019) during their study to optimize process parameters of PEF for extraction of bioactive compounds from blackcurrant, Antioxidant activity of extract increased with increase in field intensity from 650 to 1300 V/cm. Though a model was developed with two process factors (electric field and number of pulses) for the PEF process, R^2 value reported was only 0.724.

4.3.3.5. Confirmation test for validating optimized factors for antioxidant activity of extract by PEF assistance

The validity of the developed mathematical model for the antioxidant activity of extract from ghee residue obtained by PEF assisted extraction in terms of the main factors was tested and found to be in good conformity with experimental data. This is evident from close values reported for predicted and experimental values for antioxidant activity of extract. Considerable improvement in the antioxidant value of extract (15.62 % points) was also reported between the extracts obtained at optimized levels and the mid-point level combination of the parameters (Table 4.22).

Table.4.22. Confirmation test for antioxidant activity of the extract under optimized combination for PEF assisted extraction

	Initial process parameter	**Optimal process parameter**	
		Prediction	**Experiment**
Level	V_2-Ti_2-S:S_2	V_3-Ti_3-S:S_1	V_3-Ti_3-S:S_1
Antioxidant activity (%)	32.01	--	37.01
S/N ratio	30.10	31.45	31.36
Improvement in S/N ratio	1.26		
Percentage increase in antioxidant activity	15.62%		

4.3.3.6. Interactive effect of factors on antioxidant activity of extract obtained by PEF assisted extraction

Interactive effect of PEF voltage and time exhibited positive impact on antioxidant activity of the extract. With increase in voltage and time of treatment, antioxidant activity of extract also increased considerably (Fig. 4.27 (a)). However, influence of voltage was significantly higher compared to extraction time. In a study conducted by Pashazadeh *et al*. (2020) on PEF treatment of cinnamon, interaction between voltage and pulse number on the quality of the extract was evaluated. Influence of voltage on antioxidant activity of extract reported in this reference is in agreement with the observations in the present study. However, the influence of number of pulses was negligible at lower levels of voltage. High voltage for comparatively

longer time is expected to result in disruption of solid matrix. However, due to sedimentation of ghee residue during the PEF treatment, the impact was not fully experienced.

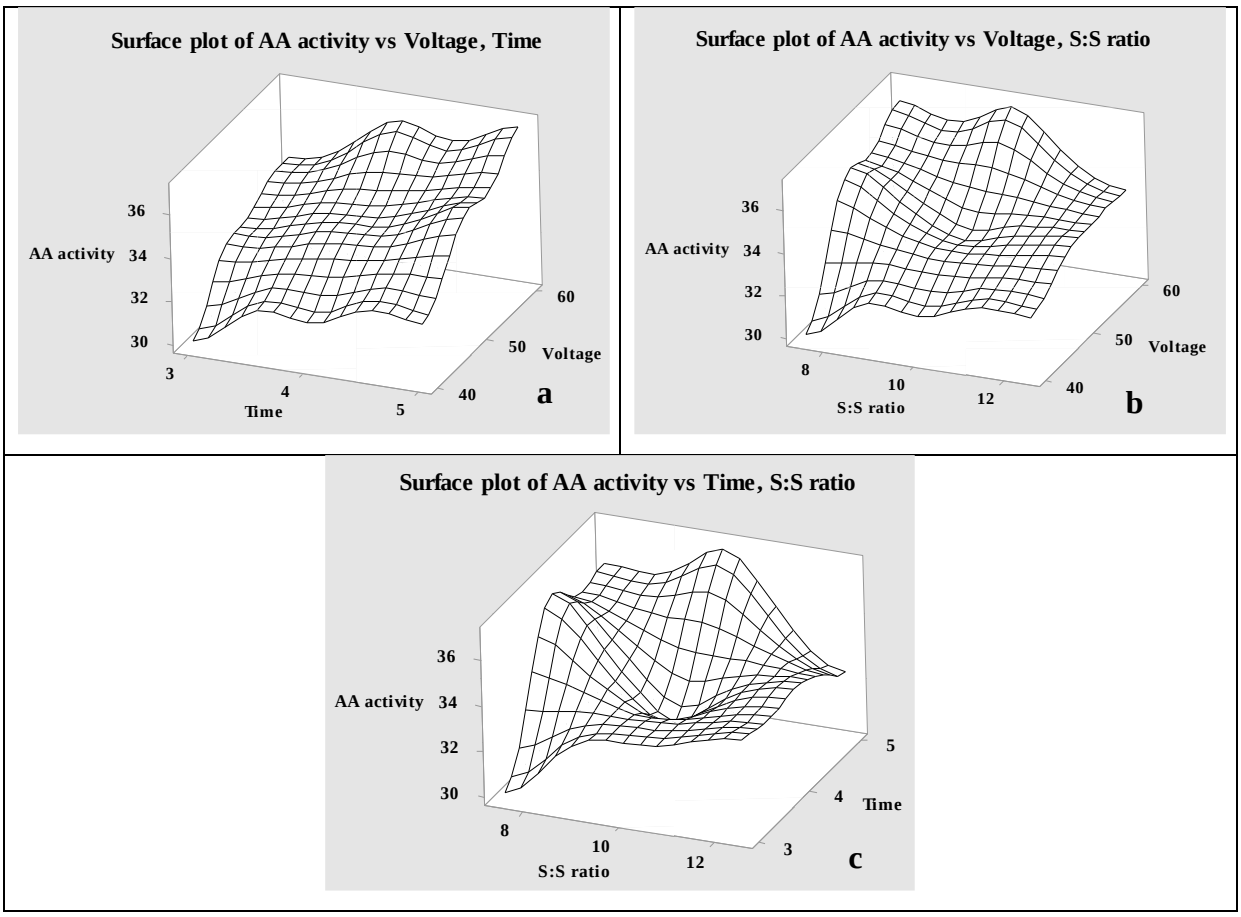


Fig.4.27. Interactive effects of process factors influencing PEF assisted extraction on antioxidant activity of extract (a) voltage and S:S ratio; (b) voltage and time; (c) S:S ratio and time

At lower solvent to solids ratio, the effect of PEF voltage showed increasing trend for antioxidant activity of extract. At higher level of solvent to solids ratio, the effect of voltage was negligible with no considerable difference in antioxidant activity of the extract (Fig. 14.27(b)). This could be due to the poor dispersal of ghee residue in solvent at different ratios. For the same reason, the interactive effect of solvent to solids ratio with time followed a similar trend. At low and mid-levels of solvent to solids ratio and higher level of time factor, the antioxidant activity of the extract was considerably better. Excluding these two points of interaction, no consistent behaviour was observed for the interactive effect of solvent to solids ratio and time.

4.4. Scanning Electron Microscopy (SEM) of ghee residue

The ghee residue samples that were subjected to the three assisted extraction techniques under the optimized process conditions were analysed through SEM for evaluating the

respective morphological changes. The post-extraction residue sediments were centrifuged and carefully collected without mechanically disturbing its structure and dried. The sample obtained after drying was subjected to SEM analysis to observethe surface morphology. The pre-treated ghee residue samples (before subjecting to any assisted extraction technique) were also evaluated as a reference (control sample).

4.4.1. SEM analysis of sample subjected to microwave assisted extraction

SEM images of ghee residue samples prior to microwave treatment and after microwave treatment at optimized conditions is shown in Fig. 4.28 (a-d). In the untreated sample, a defined structure of the ghee residue particles with small crevices on the surfacecan be evidently noted inFig. 4.28 (a). The same image magnified at 25kX indicated helical structure like appearance on the surface of the residue particles (Fig. 4.28 (b)). Also, seen in the magnified image is the presence of a cylindrical shape running through the particulate matrix giving the untreated ghee residue particles its definite structure and shape.

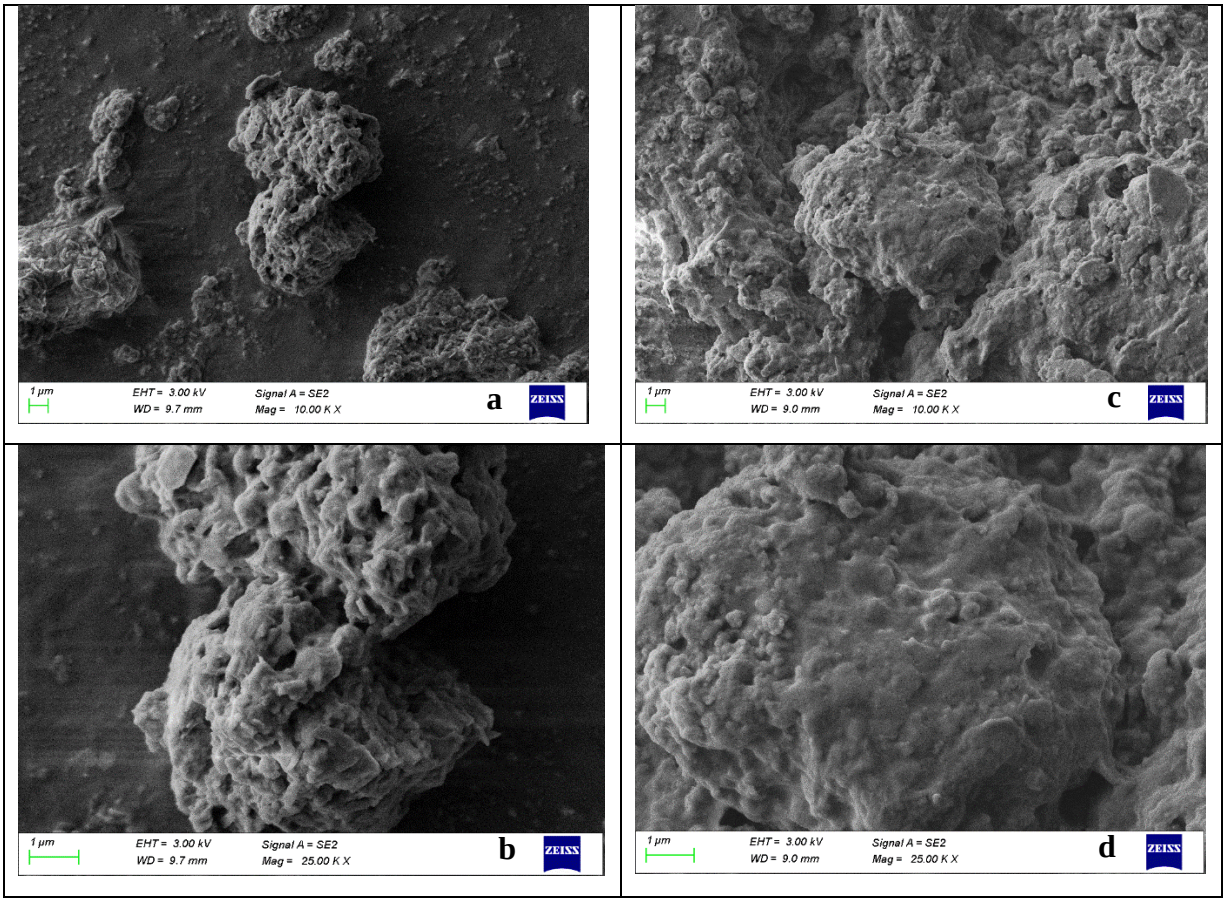


Fig.4.28. Scanning Electronic Microscopy (SEM) images of ghee residue. (a) prior to microwave treatment (10 kX) (b) prior to microwave treatment(25kX) (c) Microwave treated(10 kX) (d) Microwave treated(25 kX)

When the same matrix was subjected to microwave treatment, collapse of defined structure was observed (Fig. 4.28 (c)). Surface structure exhibiting crevices got decimated to give a

smoother surfaced body. This could be due to the impact of heat and mass transfer during microwave treatment. Also, solvent in contact with the ghee residue softened structure, which probably led to formation of the observed continuous structure of irregular shape as seen in Fig. 4.28 (d).

In a study conducted by Zhang *et al*. (2008a) on extraction of chromogenic acid from flower buds of *Lonicera japonica* Thunb, similar results were enlisted. Images of untreated, heat flux extracted sample and microwave treated samples indicated visible results on surface morphology. The study attributed the change in morphology of sample to sudden rise in temperature and rise in internal pressure of cells. Images observed in the present study are in agreement with changes of sample observed in flower buds of *Lonicera japonica* Thunb.

4.4.2. SEM analysis of sample subjected to ultrasound assisted extraction

It is well established that the waves during ultrasound treatment disrupts physical structure of sample through its mechanical action. The same was evident from the SEM images recorded for untreated and ultrasound treated sample at optimized process conditions (Fig. 4.29 (a-d)). The particulate morphology of the ghee residue samples were observed to be of different size with irregular shape and rough surface (Fig. 4.29 (a)). Further, magnification of particles at 50kX indicated a nestled structure with continuous cylindrical connectors (Fig. 4.29 (b)). The structure was continuous with different sized particulate material gluing to each other.

After ultrasound treatment, the particulates seemed more de-structured to show rock like surface (Fig. 4.29 (c)). This could be attributed to the interactive effect of ultrasonication and solvent contact, with size commination and softening due to ultrasounds may have led to the compaction of particulate material during its contact with solvent. Porous surface observed in the untreated sample was replaced with more compact surface indicating impact of ultrasonication. Also, from Fig. 4.29 (d) it can be seen that cylindrical connectors seen in the untreated samples were absent, indicating significant change in surface morphology.

Zhang *et al*. (2008b) used ultrasound technique for extraction of oil from flaxseeds and studied the SEM images of the matrix. Images of flaxseeds prior to extraction, maceration extraction for 30 min. and ultrasound treated sample were compared. Through the obtained images, the study concluded that oil from the flaxseed was completely expelled leading to 84.9% yield. Visible changes of flaxseed morphology were reported which are in agreement with structural changes observed for ghee residue after ultrasonication in the present study.

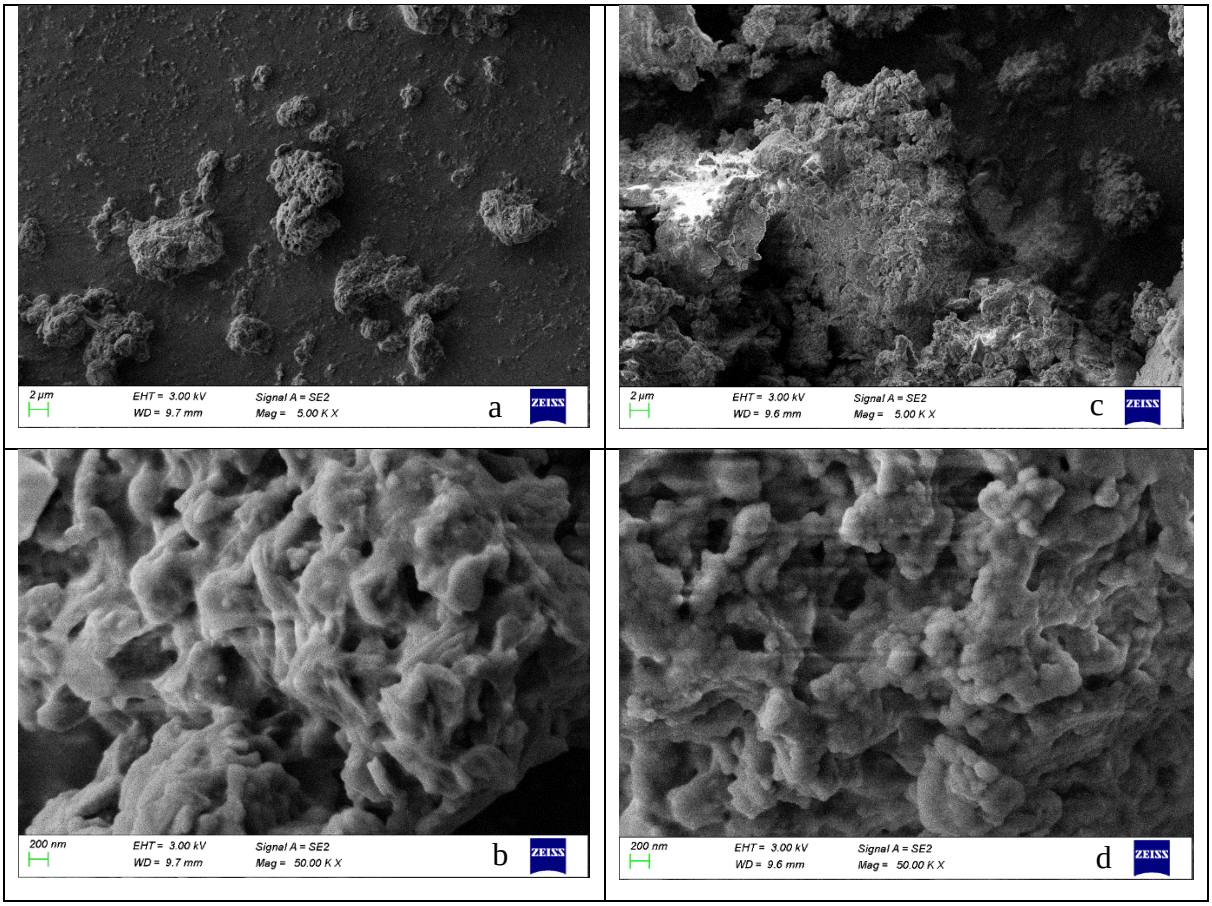


Fig.4.29. Scanning Electronic Microscopy (SEM) images of ghee residue (a) prior to ultrasound treatment(5 kX); (b) prior to ultrasound treatment(50 kX); (c) Ultrasound treated GR(5 kX); (d) Ultrasound treated GR(50 kX)

4.4.3. SEM analysis of sample subjected to PEF assisted extraction

Compared to ultrasound and microwave treatment, PEF caused less change in morphology of the ghee residue samples. SEM images captured at 50 kX and 25 kX for untreated and treated sample is shown in Fig. 4.30 (a-d). Structure of untreated ghee residue sample is similar in morphology as explained for microwave and ultrasonication. Upon PEF treatment, surface underwent slight modification with fragmented rod structures (Fig. 4.30 (d)). The magnified image of PEF treated ghee residue particulate material showed partial destruction after PEF treatment (Fig. 4.30 (c)). This could be due to uneven exposure of residue in treatment chamber as the ghee residue had settled in the bottom during treatment.

Frontuto *et al*. (2019) while working for extraction of phenols from potato peel using PEF technique reported similar results. Through SEM analysis, results confirmed the damage to the cells in potato peel to facilitate the movement of phenols into solvent. Though the sample in case of potato peel is a thin layer, the present study also noted similar structural destruction in the ghee residue matrix.

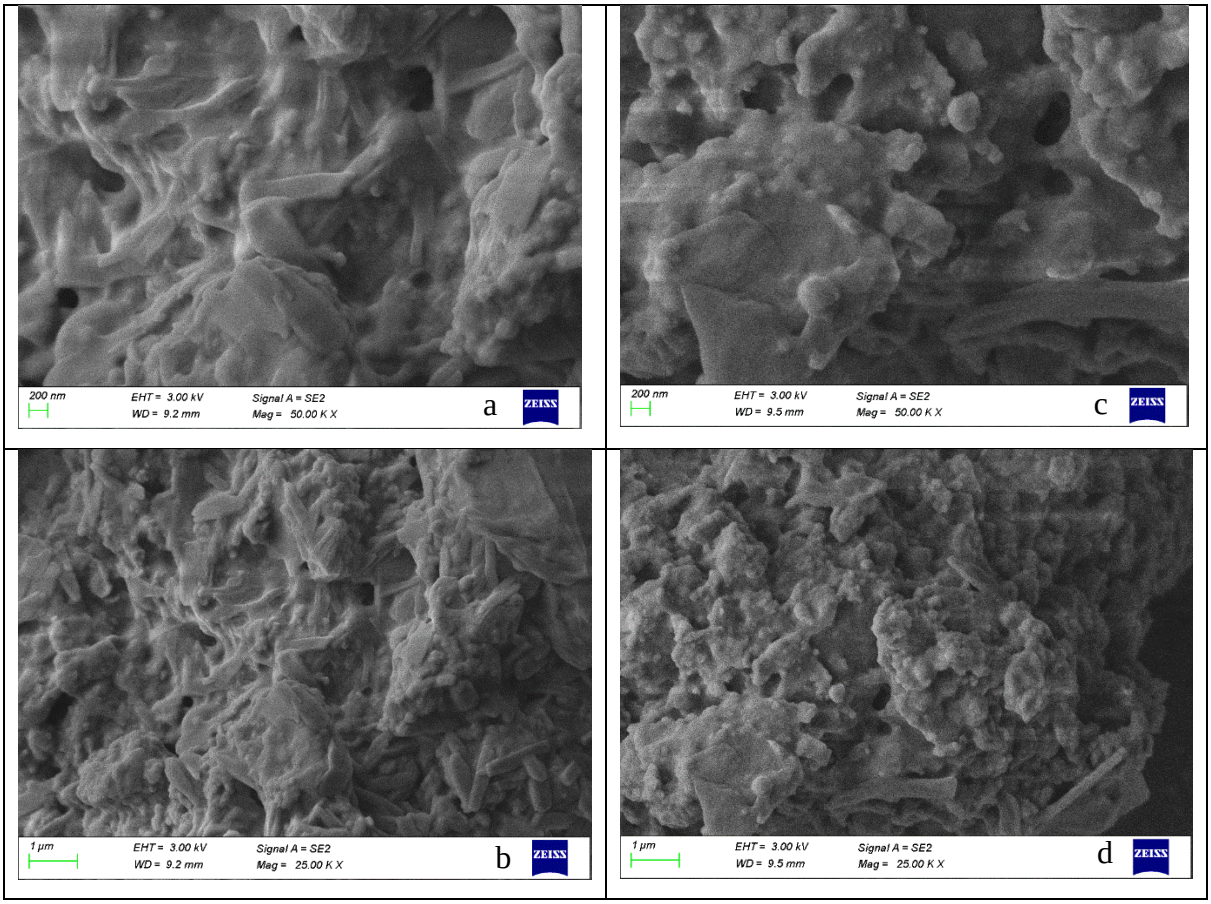


Fig.4.30. Scanning Electronic Microscopy (SEM) images of ghee residue (a) prior to PEF treatment (50 kX); (b) prior to PEF treatment (25 kX); (c) PEF treated GR(50 kX) magnification; (d) PEF treated GR(25 kX)

4.4.4. Comparison of spectrophotometric method with solid phase extractionfor estimation of PLs in the extract

The quantification of PLs in the ghee residue samples in the present study was by using the spectrophotometric method proposed by Murthy and Narayanan, (1966).The estimationis based on indirect measure of phosphorus content in sample and its conversion to quantity of PLs based on a mathematical conversion factor. The quantity of PLs estimated using spectrophotometric method could be liable for deviation due to various factors such as method of lipids extraction, sample preparation, source of lipids and machine error. Therefore, to address these concerns and to validate spectrophotometric method, a comparative estimation of PLs with solid phase extraction (SPE) of polar lipidswas carried using two methods of elution. The results of experiments for PLs quantification by two elution methods is reported in Table 4.23.

Table.4.23. Phospholipids quantified by spectrophotometry and solid phase extractionfor treated and untreated sample

Treatment	Wt. of extract (per g.)	Lipids (per g.)	PL on dry wt. basis (%)	PL on lipid basis (%)
MW1	0.39±0.01	0.17±0.01	9.22±0.82[a]	21.39±1.25[a]
MW2	0.39±0.01	0.16±0.01	12.14±1.07[b]	28.35±0.71[b]
MW3	0.39±0.02	0.17±0.00	20.26±0.87[c]	50.25±1.92[c]
UL1	0.47±0.01	0.20±0.01	8.88±0.92[a]	20.57±1.50[a]
UL2	0.46±0.01	0.20±0.01	13.10±1.32[b]	30.17±2.06[b]
UL3	0.47±0.01	0.19±0.01	23.89±0.99[c]	49.52±1.41[c]
PEF1	0.32±0.01	0.11±0.01	3.36±0.62[a]	9.61±0.98[a]
PEF2	0.32±0.02	0.11±0.02	4.29±0.73[a]	12.25±1.60[a]
PEF3	0.32±0.01	0.11±0.01	16.33±0.61[b]	38.56±1.55[b]
Untreated1	-	0.29±0.02	7.20±0.65[a]	24.70±2.17[a]
Untreated2	-	0.29±0.03	5.72±0.98[a]	19.60±1.79[b]
Untreated3	-	0.29±0.03	9.56±0.98[b]	32.47±2.14[c]

MW1, UL1, PEF1= quantification of phospholipids by Ferrari method for microwave, ultrasound, PEF treatment; MW2, UL2, PEF2= quantification of phospholipids by Cheng method for microwave, ultrasound, PEF treatment; MW3, UL3, PEF3=quantification of phospholipids by spectrophotometric method for microwave, ultrasound, PEF treatment

*Different alphabets in same column indicates significant difference by Tukey's test ($p<0.05$)

From the data presented in Table 4.23 it is evident that there was an overestimation of PLs by spectrophotometric method across all treatments. Amongst different methods of elution, Ferrari's method reported higher values compared to Cheng's elution method for all treated sample. The PLs estimated using the spectrophotometric method were approximately 66.88% and 119.73% more PLs than Ferrari's and Cheng's elution method, respectively (on extract wt.). Similarly, over estimation of PLs were reported for values expressed on lipids weight basis for microwave treated sample. Further, a significant difference was deduced by statistical analysis between the different methods of quantification for PLs ($p<0.05$). While comparing spectrophotometric method and colorimetric method for PLs quantification, Pimentel *et al*. (2016) reported overestimation for spectrophotometric method. The study

revealed overestimation of PLs by spectrophotometric method was due to co-extraction of organic and inorganic compounds along with lipids.

Similarly, for ultrasound assisted extract, overestimation of PLs by spectrophotometric method was observed. The Ferrari's method quantified the result as 8.88% and Cheng method as 13.10% of PLs against spectrophotometric method of 23.89% on extract wt. basis. A significant difference between methods compared was observed for the yield of PLs on extract weight basis and lipids basis ($p<0.05$). It was also noted that during the course of sample preparation for elution, gravimetrically separated lipids contained non-lipids fraction. This was evident when lipids were re-dissolved in solvent before centrifugation to further purify the sample.

PEF treated sample was found to be in agreement with trend followed for microwave and ultrasound PLs quantification. Ferrari's elution method reported 3.36 and 9.61% PLs on extract wt. and lipids basis, respectively. While corresponding amounts of 20 and 24% of PLswere quantified by spectrophotometric method. The lower quantities observed for the PEF assisted process could be ascribedto uneven exposure of ghee residue particulate material in treatment chamber due to sedimentation. No significant difference was surmised between Cheng and Ferrari's method of elution for the quantification of PLs in the extract ($p<0.05$).

Conversely, when the pre-treated samples of ghee residue (before assisted extraction) were subjected for quantification through the SPE methods, Ferraris' method of elution reported higher values compared to Cheng's elution method. However, values reported for spectrophotometric method were comparatively close to SPE elution. It can be concluded from the study that assisted extraction sample facilitated more organic and inorganic compound movement into extract. This led to overestimation of PLs in assisted extraction sample compared to untreated samples.

4.5. Modelling the kinetics of extraction by the assisted extraction techniques

The assisted extraction techniques adopted for improving the yield of PLs in the extract and its antioxidant activity were evaluated by comparing the extraction kinetics using defined empirical models. Four empirical models reported with good fit for extraction of different constituents from biological matrix were employed to describe the experimental data.

4.5.1. Modelling the kinetics of extraction of PLs by microwave assisted extraction

The optimal conditions for the microwave assisted extraction for maximizing the yield of PLs in the extract were obtained as 540 W power at a solvent to solid ratio of 10 for 60 s time. Experiments to evaluate the kinetics of extraction were conducted at these factors for a duration of 60s, with samples extracted and evaluated for its PLs content at a frequency of 5 s time interval. The experimental data along with the plot of the empirical models is presented in Fig.4.31.

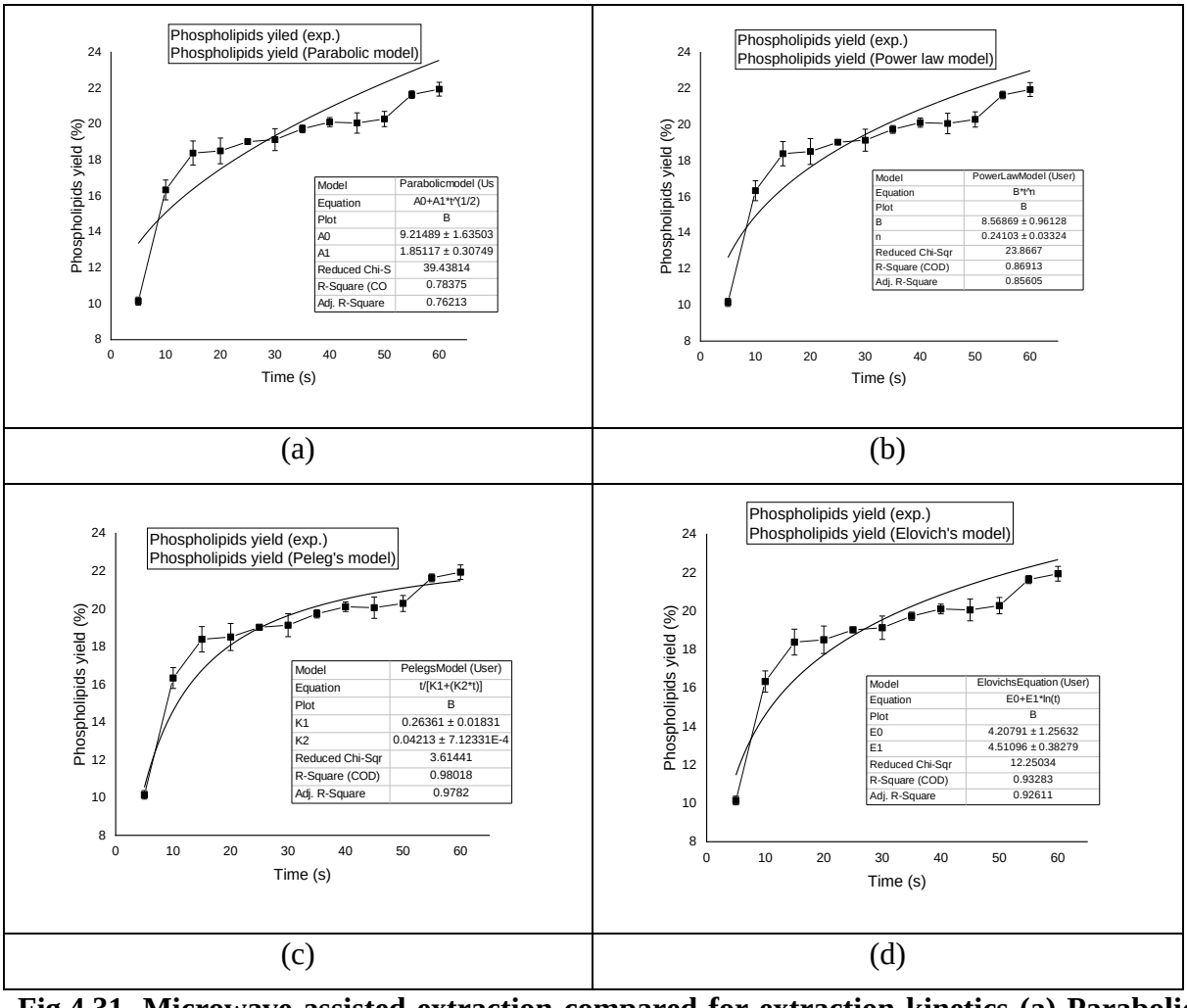


Fig.4.31. Microwave assisted extraction compared for extraction kinetics (a) Parabolic model; (b) Power law model; (c) Peleg's model; (d) Elovich's model

From a perusal of the plot, it is evident that all models were relatively in good agreement with experimental data. The Peleg's model reported highest R^2 of 0.98 which was trailed by Elovich's model with R2 value of 0.93. Whereas, power model and parabolic model reported lesser R^2 value of 0.87 and 0.78, respectively. Among the model constants, it is understood that a better value for K_1 is linked with better process rate while lower K_2 values implies maximum yield. In the present study, very low K_2 (0.042x10-4) value and better K_1 (0.26)

indicated good rate of process and maximum yield. While modelling the kinetics of extraction of pomegranate peel using microwave assistance, Kaderides *et al*. (2019) reported good fit for Peleg's model.

From the Fig. 4.31 (a) it can be implied that parabolic model was not in alignment with the experimental data. Similarly, from Fig. 4.31 (c & d) the experimental data points were observed to be in good agreement with model plot, which reiterates good fit to Peleg's and Elovich's model. It has been postulated that the rate of extraction is not only restricted to resistance of mass transfer but also inter-particulate diffusion (Ji *et al*., 2006). Thus, the kinetic model constants for the present study indicated that the optimized level of solvent to solid ratio must have ensured good mixing and penetration of solvent into the ghee residue matrix during the microwave assisted extraction.

4.5.2. Modelling the kinetics of extraction of PLs by ultrasound assisted extraction

Experiments were conducted at optimized levels of the process parameters (in triplicate) for ultrasound assisted extraction to monitor the kinetics of extractionusing established empirical mathematical models. Yield of PLs was evaluated at every 30 s to assess the model constants and statistical parameters for the regression (Fig. 4.32).

Three of the evaluated models reported good R^2 values indicating good fit to experimental data. However, Elovich's model reported low R^2 value of 0.69 which was far lower than other models (Fig.4.32 d). Parabolic model with R^2 value of 0.95 was comparatively highest among the models (Fig.4.32 a). Power law model reported the second highest R^2 value (Fig.4.32b).

The yield of PLs increased steadily with respect to time till 210 s and the yield stagnated to a near constant value between 210 and 300 s. This could be due to saturation of extract and PLs content in solvent which caused no concentration gradient beyond 210 s to induce diffusion. Parabolic and power law models reported nearly same R^2 values against experimental results. Peleg's and Elovich's models reported R^2 values of 0.88 and 0.62, respectively which was relatively low compared to other two models. The results could be linked to the well-disbursement of sample in water and its non-swelling nature. Cheung and Wu (2013) evaluated the kinetics of ultrasound assisted extraction from a fungi species *Cordycepssinensis* (C_S-HK1)and reported a similar trend for kinetics of extraction, with a high R^2value of 0.995.

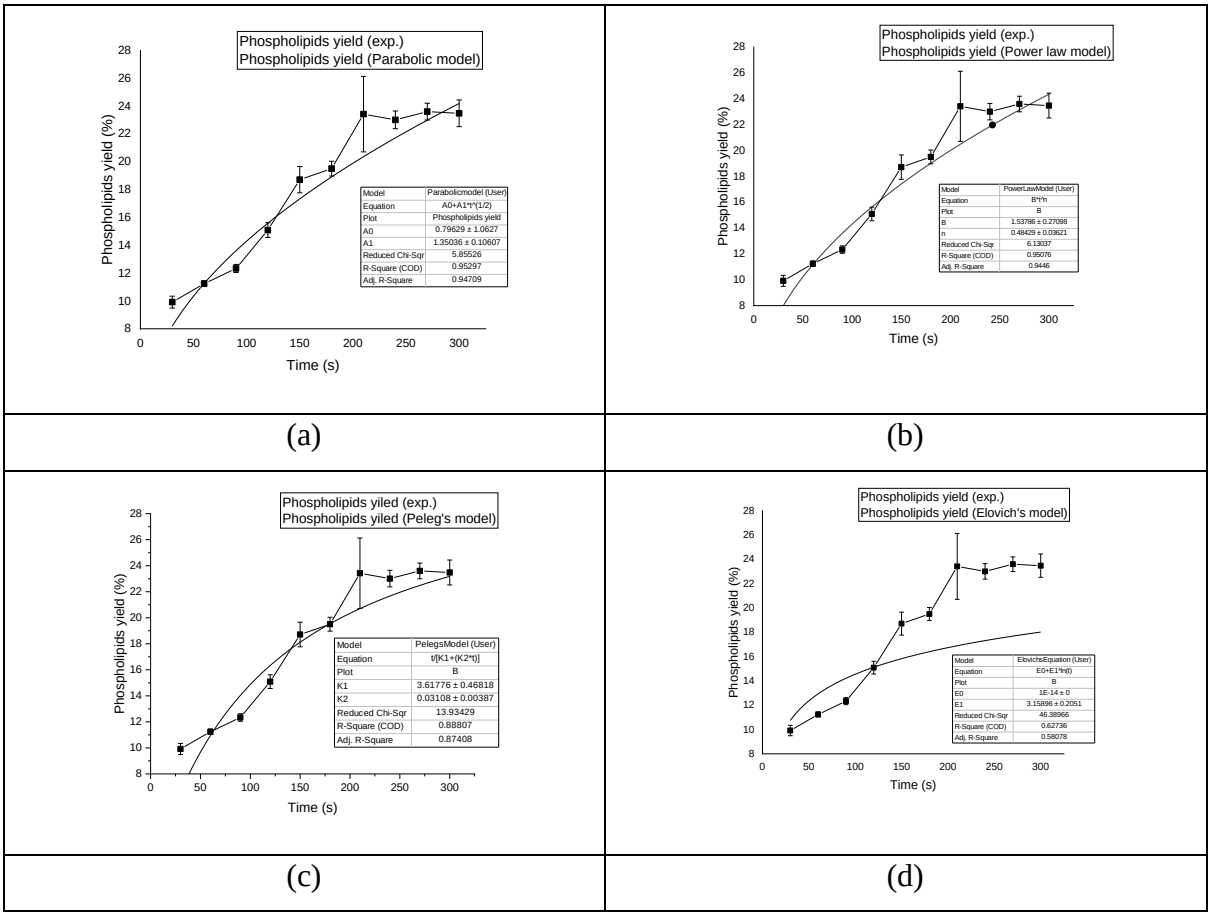


Fig. 4.32. Ultrasound assisted extraction compared for extraction kinetics (a) Parabolic model; (b) Power law model; (c) Peleg's model; (d) Elovich's model

4.5.3. Modelling the kinetics of extraction of PLs by PEF assisted extraction

Assisted extraction of PLs at optimized factors for the PEF assisted extraction process and comparison of the kinetics of extraction using the empirical models is depicted in Fig. 4.33. Parabolic and power law model was found to be in good agreement with experimental data by reporting R^2 value very close to 0.95 (Fig. 4.33 (a), (b)). Peleg's model also reported relatively good fit to the experimental data by reporting R^2 value of 0.91 (Fig. 4.33 (c)). Only Elovich's model reported poor R^2value of 0.55 (Fig. 4.33 (d)); thus it may be inferred that this model failed to describe the extraction kinetics of PLs from ghee residue. Pataro*et al*. (2020) extracted lycopene from tomato fruit and reported that Peleg's model was in agreement with extraction data with good R^2 value.

Time frame between 25 and 125 s, there was a steady increase in the yield of PLs in the extract followed by a sudden spike in the yield of PLs from 125 to 175 s. almost constant values of PLs in the extract were reported at times greater than 175 s.

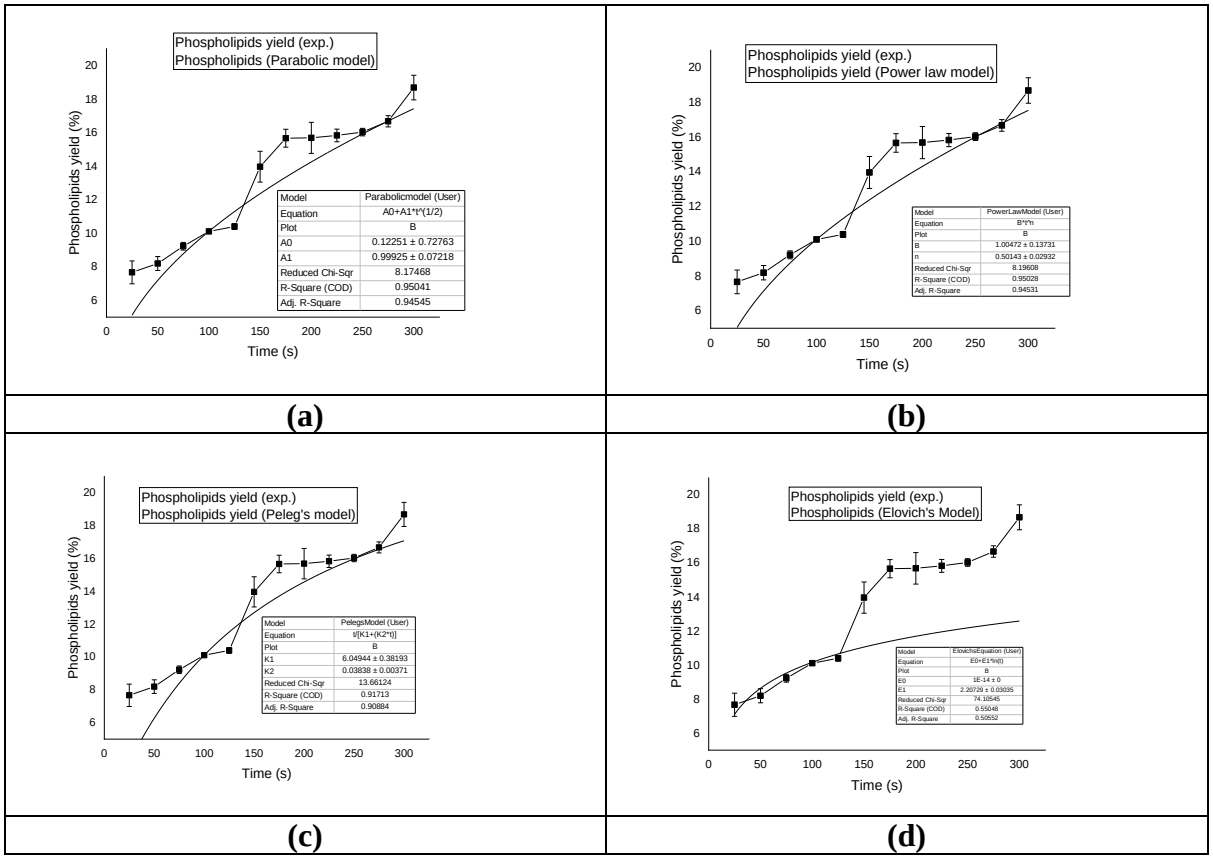


Fig.4.33. PEF assisted extraction compared for extraction kinetics (a) Parabolic model; (b) Power law model; (c) Peleg's model; (d) Elovich's model

4.5.4. Modelling the kinetics of antioxidant activity of extract by the assisted extraction techniques

The antioxidant activity of the extract obtained under optimized process conditions for the different assisted extraction techniques were also attempted to describe by the empirical models. The antioxidant activity was evaluated using DPPH assay and the antioxidant activity of the extract was expressed on percentage basis against time. The details of models, model parameters and statistical parameters obtained in this analysis are summarized in Table 4.24.

For microwave assisted extraction, Peleg's model reported good fit for experimental data for antioxidant activity with a R^2 value of 0.81. This model also reported good K_1value of 0.1272 and low K_2 of $0.032X10^{-4}$, which implied good extraction rate and maximum yield. Elovich's model was found to be thesecond-best model in terms of the fit with the experimental data with R^2 value of 0.7080.The antioxidant activity of the extract obtained under optimized conditions for the ultrasound assisted process exhibited a good fit for Peleg's, parabolic and power law model by reporting R^2 value of 0.96, 0.93 and 0.93, respectively. However, experimental data for antioxidant activity of the extract was not in agreement with Elovich's

model with R^2 value 0.62. While comparing extraction kinetics of extract by DPPH assay for brewer's spent grain using microwave and ultrasound assisted processes, Carciochi *et al*. (2018) reported good fit for Peleg's model. However, compared to Peleg's model, Patricelli's model was found to be slightly better fit for extract obtained by microwave assistance. Authors opined better constants under washing step of extraction than diffusion step as reason for best fit of Patricelli's model. Coincidentally, parabolic and power law model reported nearly identical R^2 value for the antioxidant activity of the extract.

Table.4.24. Comparison of empirical models for antioxidant activity of extract with model constants and regressed values

Sl. No.	Model	Constant	Microwave	Ultrasound	PEF
1	Parabolic Model	A_o	18.53	10^{-14}	0.67
		A_1	1.36	3.07	0.63
		R^2	0.59	0.93	0.67
		Adj. R^2	0.55	0.92	0.63
2	Power Law Model	B	16.12	2.82	0.75
		N	0.14	0.51	0.73
		R^2	0.67	0.93	0.75
		Adj. R^2	0.64	0.92	0.73
3	Peleg's Model	K_1	0.12	1.80	0.92
		K_2	$0.03x10^{-4}$	$0.01x10^{-4}$	0.92
		R^2	0.81	0.97	0.92
		Adj. R^2	0.79	0.96	0.92
4	Elovich's Model	E_0	13.25	10^{-14}	0.80
		E_1	3.83	7.36	0.78
		R^2	0.70	0.62	0.80
		Adj. R^2	0.67	0.57	0.78

For PEF assisted extraction, the antioxidant activity of the extract was adequately described using the Peleg's model by reporting R^2 of 0.92; this was followed by Elovich's model (R^2 of 0.80). However, parabolic and power law model reported lower R^2 values, indicating a lack of fit to experimental values. Across all assisted extraction techniques, Peleg's model was found to be the best model to explain antioxidant activity of extract with high R^2 values compared to other models (Table 4.24).

4.5.5. Proximate analysis of ghee residue extract

The ghee residue samples, both before the extraction process and that obtained from three assisted extraction techniques were analysed for its protein, lactose, ash, moisture and lipids content. The data is presented in Table 4.25 and it can be seen that lipids reported highest fraction in the extract for ultrasound and microwaves assisted techniques. However,the extract obtained through the PEF assisted technique reported lower lipids fraction with

39.11% compared to protein values of 41.63%. As the yield of extract from PEF assisted technique was the lowest among the assisted treatments, further reduction in total lipids value indicated its poor PLs extraction.

Table.4.25. Proximate composition of ghee residue, extract and sediment obtained from different assisted treatment techniques

Sample#	Moisture	Lipids	Protein	Lactose	Ash
Ghee residue					
GR	3.5±0.76	30.12±1.94	47.53±1.31	8.25±0.73	10.9±0.84
Extract					
MW	2.53±0.52	45.00±1.17	**35.27±1.12**	7.53±1.06	9.56±0.66
UL	3.02±0.25	45.50±2.23	34.25±2.38	7.98±0.63	9.32±0.86
PEF	3.12±0.37	39.11±2.05	41.63±1.85	6.21±0.30	9.98±0.96
Sediment					
MW	4.15±0.43	20.10±1.93	55.78±1.62	8.73±0.62	11.80±0.67
UL	3.93±0.23	16.34±1.47	59.43±1.56	8.49±0.99	12.32±1.23
PEF	3.70±0.59	25.28±1.67	50.71±1.69	9.35±0.54	11.40±0.75

[#] GR: Ghee residue, MW: Microwave assisted extraction, UL: Ultrasound assisted extraction, PEF: PEF assisted extraction

Protein was observed to be a major component in ultrasound and microwave assisted extract with a composition of 34.25 and 35.27%, respectively. High shear in ultrasound treatment and improved heat and mass transfer in microwave assisted extraction could have contributed to the higher diffusion from the ghee residue to the solvent.In a study to extract proteins from green microalga *Chlorella vulgaris* using water with ultrasound assistance Hildebrand *et al*. (2020) reported significant recovery of 40% protein present in the sample through supernatant (water) which was lesser to the alternate solvent compared (0.4N NaOH solution). A significant portion of protein present in the ghee residue samples were also found as part of the sediment as reported in Table 4.25. The ghee residue sediment (after extraction) was also composed of significant quantity of lipids fraction, majority of which was non-polar lipids.

Lactose and ash were slightly higher in sediment compared to extract obtained using ultrasound assisted technique reporting least ash value among the samples (9.32%). Conversely, extract obtained through the PEF assisted extraction reported least lactose content (6.21%) but reported highest ash content of (9.98%) in extract. The trend was obviously reversed for the sediment samples, where the ultrasound treated sediment reported highest ash content and lowest lactose content with 12.32% and 8.49%, respectively. Similarly, the PEF treated sediment reported highest lactose and lowest ash among the treatments.

4.6. Characterization of ghee residue extract for phospholipids classes

The extract obtained under the optimized conditions for the three assisted extraction techniques were examined for various classes of PLs in its composition i.e. phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylglycerol (PG), using liquid chromatography and mass spectrometry. The extract (after standard sample preparation) obtained from the PEF assisted technique (5µL) and 2µL each of the extract obtained using the ultrasound and microwave assisted extraction from ghee residue of was fed into the column of the instrument for analysing PLs classes and species. For detection of the moleculespecies, an orbitrap with both positive and negative polarity was used. Each species of PLs class was quantified using following formula.

$$\text{Mass of PL class (µg)} = \frac{\text{area of lipid species}}{\text{area of internal standard species}} \text{X} \frac{\text{amount of internalstandard species}}{\text{volume of reconstitution}} \text{x vol. of injection} \text{-----4.7}$$

4.6.1. Phospholipids classes and species in extract obtained using microwave assisted technique

The extract obtained using microwave assisted technique was analysed for the profile of PLS and the results revealed that it was composed with 31, 11, 16, 2 and 1 species for PC, PE, PI, PS and PG, respectively. Among the identified species, no short chain fatty acids such as C4:0, C6:0 or C8:0 were observed. Majority of fatty acids across the classes of the PLs considered in the present study reported fatty acids with the structure conforming C16:1 to C18:1. Based on the cumulative total of all species studied, the major composition was identified as class belong to PCs with a reported quantity with of 14.1309 µg. The next major component was of PIs (0.6447 µg) followed by PE (0.0110 µg). Traces of PG (0.0042 µg) and PS (0.0002 µg) was also identified in the extract obtained through the microwave assisted technique. All classes together reported a composition of 14.79 µg of weight in 2 µL of the sample used in the study (Appendix I). To identify the diverse structure of polar lipids, both positive and negative modes were included in the study for all classes. Russo *et al.,* (2013) had identified 40 species of PLs in milk spread across 6 classes (PI, PE, PS, PC, SM and LPC). No studies were reported in the literature on species of PLs for either ghee residue or its extract. Thus, this study is the first of its kind which emphasizes details of PLs species found in extract from ghee residue.

4.6.2. Phospholipids classes and species in extract obtained using ultrasound assisted technique

Similar operational parameters were used for identification and quantification of PLs classes and its species in the extract obtained by ultrasound assisted technique using LC-MS. The

extract was identified with 5 PLs classes (with 118 species across the classes), namely PC (61), PE (18), PI (19), PS (15) and PG (5). However, unlike the extract obtained with microwave assistance, from a quantification point of view, the extract reported PLs mass of only 1.548 µg from 2 µL aliquot of sample analysed. Among the PLs classes, highest mass fraction was reported for PC (0.8916 µg) followed by PI (0.3597 µg); whereas, PE, PS and PG reported mass fractions of 0.1504, 0.1335 and 0.0123 µg, respectively. In this case also, no short chain fatty acids were reported in the extract and majority followed a structural conformity between C16:1 and C18:2 (Appendix II).

4.6.3. Phospholipids classes and species in extract obtained using PEF assisted technique

Based on preliminary trials, the sample preparation protocol for the extract obtained with PEF assistance was slightly modified before the analysis for better identification and quantification. The extract was subjected to lipid extraction using Mojonnier method and residue was re-suspended in chloroform and supernatant was taken as sample. The results from the analysis revealed a total mass of combined species of PL classes (PC, PE, PI and PS) of 0.1985 µg. The compositional distribution of the various classes of PLs in the extract followed the similar trend to that of the extracts obtained with microwave and ultrasound assistance. The extract obtained using PEF assisted technique reported a major composition of PCs at 0.1157 µg, followed by PE (0.0696 µg). Traces of PI (0.0103 µg) and PS (0.0029 µg) were also reported in the extract (Appendix III). Among the classes of PLs in the extract, the number of species detected was as follows: PC (16), PE (8), PI (4) and PS (3).

4.7. Characterization of the extract obtained from ghee residue for its emulsion properties

The extract obtained from ghee residue under optimized protocols for all assisted extraction techniques were evaluated for its emulsion characteristics in terms of its emulsion capacity, stability and hydrophilic-lipophilic balance (HLB) value assessment.

4.7.1. Emulsion capacity and stability

Preliminary studies were conducted at fractional levels of the extracts obtained from the assisted extraction techniques (0.5, 0.75 and 1%) in 1:10 oil to water emulsions. However, separation of the phases was observed at these levels indicating insufficiency of emulsifier (extract) to bring oil droplets in continuous phase. This could be due to lower proportions of PLs present in the whole extract (8-13% across different elution techniques). Hence, higher quantities of extract were selected as levels to analyse emulsifying capacity of extract. The higher proportions of extract used quantitatively amounted to approximately 10% of PLs.

Further, initial studies indicated poor stability of the emulsions prepared using the extract prepared using PEF assisted technique at concentrations of 3 to 5%. Hence, only for this extract the fractional levels in the emulsion were revised to 5, 7.5 and 10%.

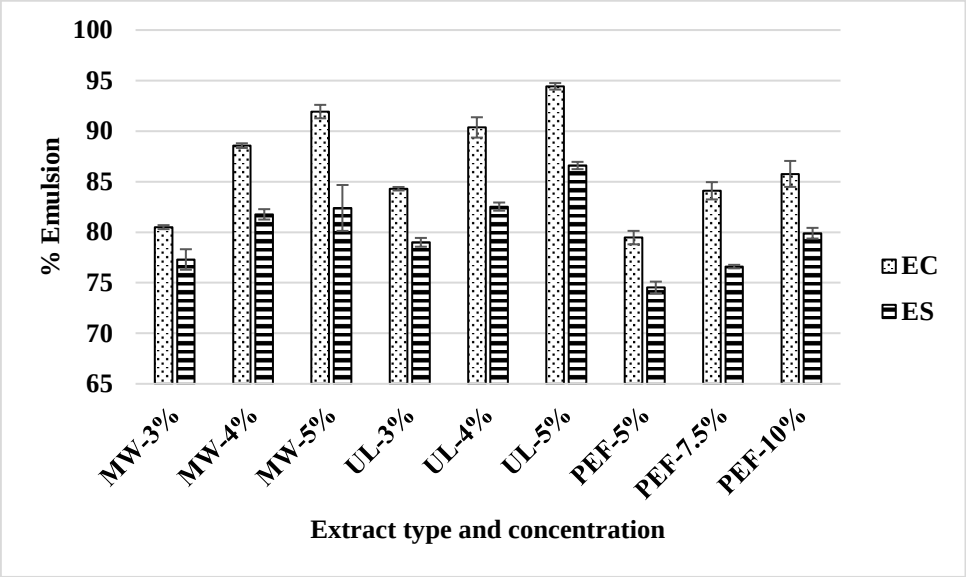


Fig.4.34. Emulsion capacity and stability of emulsions prepared using different assisted extraction at varied concentration. EC: Emulsion Capacity, ES: Emulsion Stability

Emulsion capacity and stability exhibited by different extracts at different concentration is shown in Fig. 4.34. Across all three extraction techniques, it was observed that with increase in extract quantity in the emulsion, emulsion capacity and stability improved (Fig. 4.34). This trend could be ascribed to the enhanced availability of PLs with increasing concentration enabling better binding of the oil phase with the continuous phase.

Among the three techniques evaluated to assist the extraction, the extract obtained using ultrasound assistance exhibited highest emulsion capacity of 94.43% at 5% level and 84.3% at 3% level. In similar lines, extract obtained through microwave assisted technique also demonstrated good emulsion potential with a capacity of 91.93% at 5 % level against 80.5% at 3% level. Even though the quantity of extract used in emulsion prepared using extract obtained with PEF assistance was higher than the other two samples, it did not reflect in higher value for emulsion capacity. This could be due to that fact that compositionally, the extract obtained with PEF assistance contained significantly lower PL content and this may have hampered the emulsion capacity of the extract.

The results recorded for emulsion stability (Fig. 4.34) mirrored the trend observed for emulsion capacity, with the emulsion prepared using extract obtained through ultrasound

assistance demonstrating better stability amongst the samples. The emulsion prepared using ultrasound and microwave assistance at 5% level recorded stability values of 86.6% and 82.4%, respectively. In comparison, the values recorded for the emulsion prepared using the extract obtained using PEF assistance were only 79.9% and 74.53% at 10 and 5% of concentration, respectively.

Koocheki *et al.,* (2009) evaluated emulsion capacity and emulsion stability of extract of *Alyssum homolocarpum* seed gum. Even though an improvement in the stability and capacity of emulsion with increase in gum extract content was reported, poorer surface activity due to co-extraction of proteins in the extract was also discussed. Due to significant composition of proteins in the extract obtained from ghee residue in the present study, the same inference may be drawn for the emulsion capacity and stability behaviour of the samples.

4.7.2. Particle size and zeta potential of emulsion

From the experiments conducted to evaluate emulsion capacity and stability it was confirmed that the emulsion prepared using higher concentration of extract (5% for microwave and ultrasound extract; 10% PEF extract) showed better performance for the emulsion properties. Hence, further characterization of the emulsion was performed in terms of average particle size and zeta potential of emulsion, prepared using indicated levels of the extract from the respective assisted extraction techniques. The analysis was carried out for stored emulsion samples and samples were drawn on the 0^{th}, 15^{th} and 30^{th} day of storage.

Particle size and zeta potential reported for emulsion prepared using the extract obtained with microwave assistance is shown in Fig. 4.35. From the plot, it can be noticed that particle size marginally increased over the storage duration. The emulsion prepared using the extract obtained with microwave assistance indicated zeta potential range falling in category of insipient stability. However, no considerable change in particle size and zeta potential of the sample was observed during storage. This implied that the emulsion would hold the oil and water phases in relatively strong bondage to restrict its separation. In a study to investigate effect of different concentration of soy hull polysaccharides in formulating emulsions Wang *et al.,* (2020) reported similar change during storage, with a change of 5 to 6 mV in zeta potential between 1^{st} and 30^{th} day. This change was attributed to decrease in electrostatic repulsion and interfacial film formed by emulsifiers during storage.

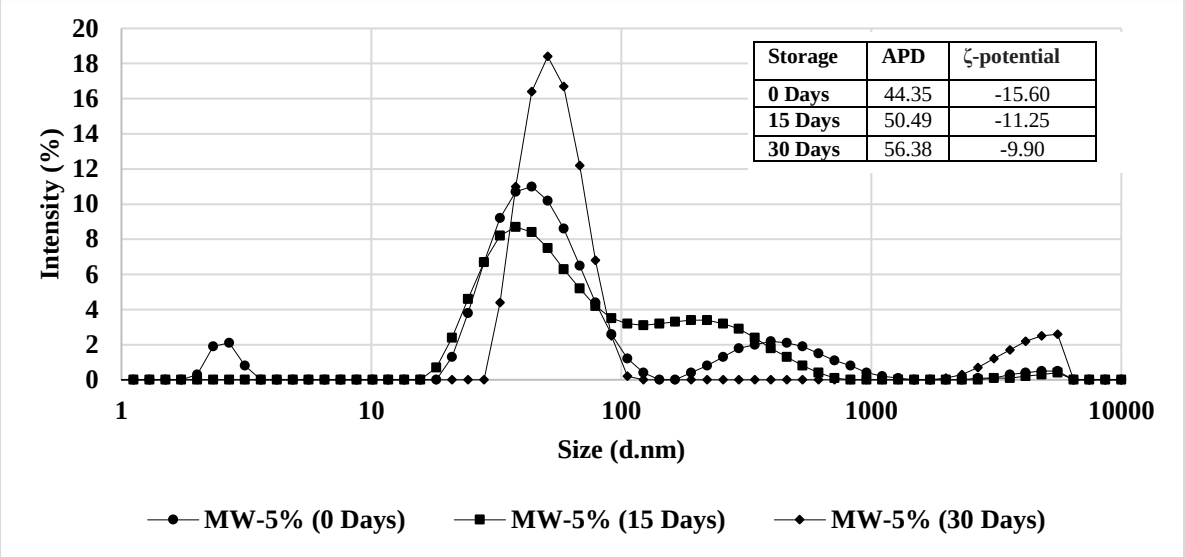


Fig.4.35. Particle size distribution in emulsion prepared by using 5% extract obtained with microwave assistance during storage at 4±1ºC

Emulsion prepared using the extract obtained with ultrasound assistance exhibited similar trend similar to the sample prepared with microwave assistance, albeit with smaller particle size (Fig. 4.36). This could be due to two-time ultrasonication of sample (during extraction and emulsion preparation) which led to good dispersion of oil droplets in continuous phase. Also, slightly better zeta potential was recorded for the ultrasonicated sample compared to emulsion prepared using the extract obtained with microwave assistance. During the course of experiments, it was visually noticed that the extract prepared using ultrasonication was slightly whitish in colour compared to the other two assisted extraction techniques. This may be attributed to a thorough dispersion of the ghee residue particles during extraction due to the mechanical action of ultrasound probe.

The observations of the present study were compared to that reported on ultrasonication and its effect on emulsion stability undertaken by Sui, *et al.*, (2017). The study involved emulsion preparation with soybean protein isolate and lecithin using ultrasonication at different powers and time with an inference that higher power of ultrasonication led to protein aggregation. In the present study, even though composition analysis revealed protein to be the second major fraction present in the ghee residue samples, the emulsification property was deemed to be on account of the PLs fraction in the extract. Even at lower treatment times, ultrasonication was found to successfully break the emulsion and achieve good dispersion in the continuous

phase.

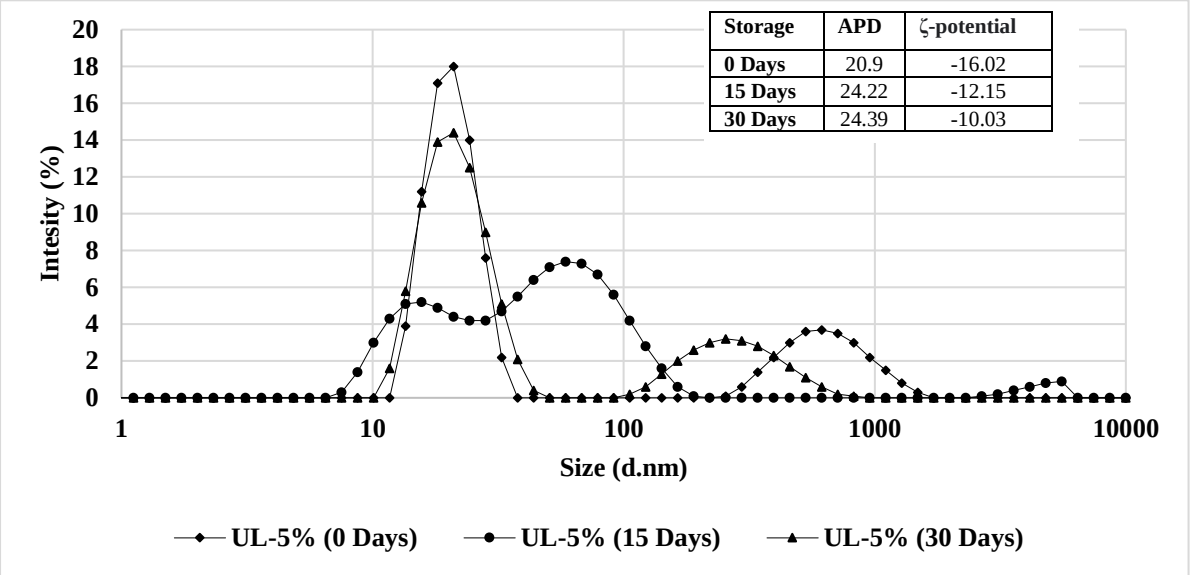


Fig.4.36. Particle size distribution in emulsion prepared by using 5% extract obtained with ultrasound assistance during storage at 4±1ºC

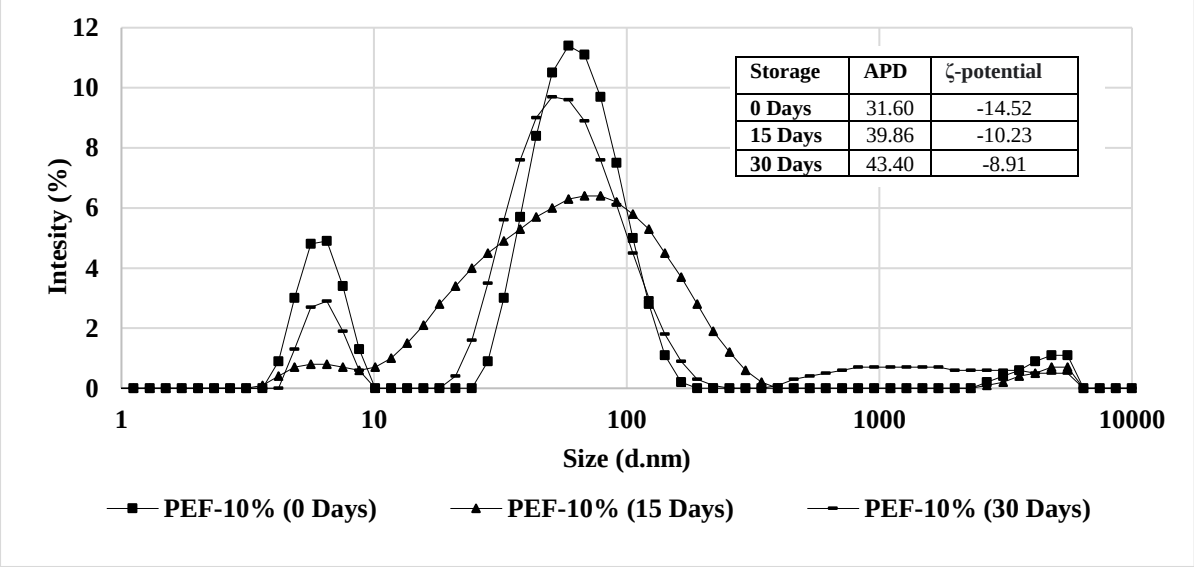


Fig.4.37. Particle size distribution in emulsion prepared by using 10% extract obtained with PEF assistance during storage at 4±1ºC

Emulsion prepared by PEF extract (10%) was able to show slightly better average particle size distribution, ranging in intermediary size, compared to the other two emulsions investigated. Zeta potential of the emulsion also indicated decreasing trend, which was on similar lines observed with the emulsion prepared using microwave and ultrasound assistance (Fig. 4.37). Though the concentration of the extract used to formulate the emulsion was higher, the particle size of the emulsion was similar to the sample prepared with ultrasound assistance. Hence, it can also be construed that rather than the concentration of the extract,

the proportion of PLs in the extract had an influence on particle size and zeta potential. Distribution of particles in the emulsion was observed to be narrow on 1st day and 30th day, whereas, they were widely distributed on 15th day. This could be due to reorientation of particles during the course of storage.

4.7.3. Hydrophilic and lipophilic balance (HLB) of extract

Extract obtained from assisted extraction techniques were subjected to different tests to assess its potential HLB value. Since the extract (PL rich fraction from ghee residue) is a fairly unreported commodity, quantifying the HLB value was important in identifying suitable products for evaluating its emulsification performance.

4.7.3.1. Centrifugal stress on emulsion

To assess the ability of emulsion to resist separation of phases, the emulsion was evaluated for its stability under different levels of centrifugal stress. The emulsion was filled in Wintrobe tubes and subjected to different speeds of rotation in a laboratory centrifuge for 10 min. The height of the creamy layer was directly read from the graduated Wintorbe tubes and expressed as creaming index (CI). The results are tabulated in Table 4.26 and from a perusal of the data it can be perceived that at lower speeds of centrifugation (427 and 854 rpm) no phase separation was observed across all samples. With increasing centrifugal stress, amongst the three assisted techniques evaluated, the emulsion prepared with PEF assistance exhibited the first instance of phase separation (at centrifugal stress of 201.6g i.e.1281 rpm), for emulsions formulated for HLB value between 6 and 7. At this point, it can be assumed that the PEF extract was unable to bind oil and water together with its capacity for emulsification. Compositionally, the formulation corresponding to the said HLB values had a higher composition of the lipophilic component (Span 80 HLB=4.3) than hydrophilic component (Tween 20 HLB=16.7).

Similarly, the emulsion formulated with the extract obtained with microwave and ultrasound assistance depicted the first instance of phase separation at 560 g (2135 rpm) indicating failure of emulsifying ability at this level of centrifugal stress. As centrifugal stress was increased to 1097.6 g (3000 rpm), samples having HLB value below 10 showed phase separation (all extracts). Amongst the samples from different assisted techniques evaluated, sample prepared with ultrasound assistance was found to be comparatively better than other two samples. It is evident from report that no phase separation was reported for the sample having HLB value of 9 for emulsions prepared with ultrasound assistance.

Table. 4.26. Creaming index of samples subjected for centrifugal stress measured in wintrobe tubes (samples stored at 4±1 ºC)

HLB	Resistance to centrifugation (RPM)																	
	427			854			1281			2135			2562			3000		
	MW	UL	PEF	MW	UL	PEF	MW	UL	PEF	MW	UL	PEF	MW	UL	PEF	MW	UL	PEF
6	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	PS	PS	PS	PS	PS	PS	PS	PS	PS	PS
7	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	PS	PS	2.0	PS	PS	PS	PS	PS	PS	PS
8	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.5	2.0	PS	PS	2.5	PS	PS	PS	PS
9	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.0	1.5	PS	2.5	2.0	PS	PS	2.5	PS
10	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
11	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
12	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
13	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
14	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
15	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

MW-Microwave assisted extract emulsion; UL-Ultrasound assisted extract emulsion; PEF-Pulsed electric field assisted extract emulsion; PS-phase separation; HLB- Hydrophilic and Lipophilic balance; RPM- Revolutions per minute

These results are in agreement with work reported by Ferreira *et al*., (2010) for evaluation of HLB value of *andiroba* oil, who reported phase separation for samples having HLB value of less than 8.7 at centrifugal stress of 1097.6 g. However, samples were stored in ambient condition during stability study, in contrast to the present study where samples were stored under refrigeration.

4.7.3.2. Change in pH values of emulsion

The emulsion samples prepared using the extract from the different assisted extraction techniques and stored, were analysed for changes in its pH values at a frequency of 5 days. It is reported that partial hydrolysis of triglycerides released from fatty acids of substrate will alter pH value of emulsions (Tadros, 2004). Lesser change in pH values of emulsions indicate the ability of emulsifier to encage oil component across different HLB values (Ferraria *et al*., 2010).

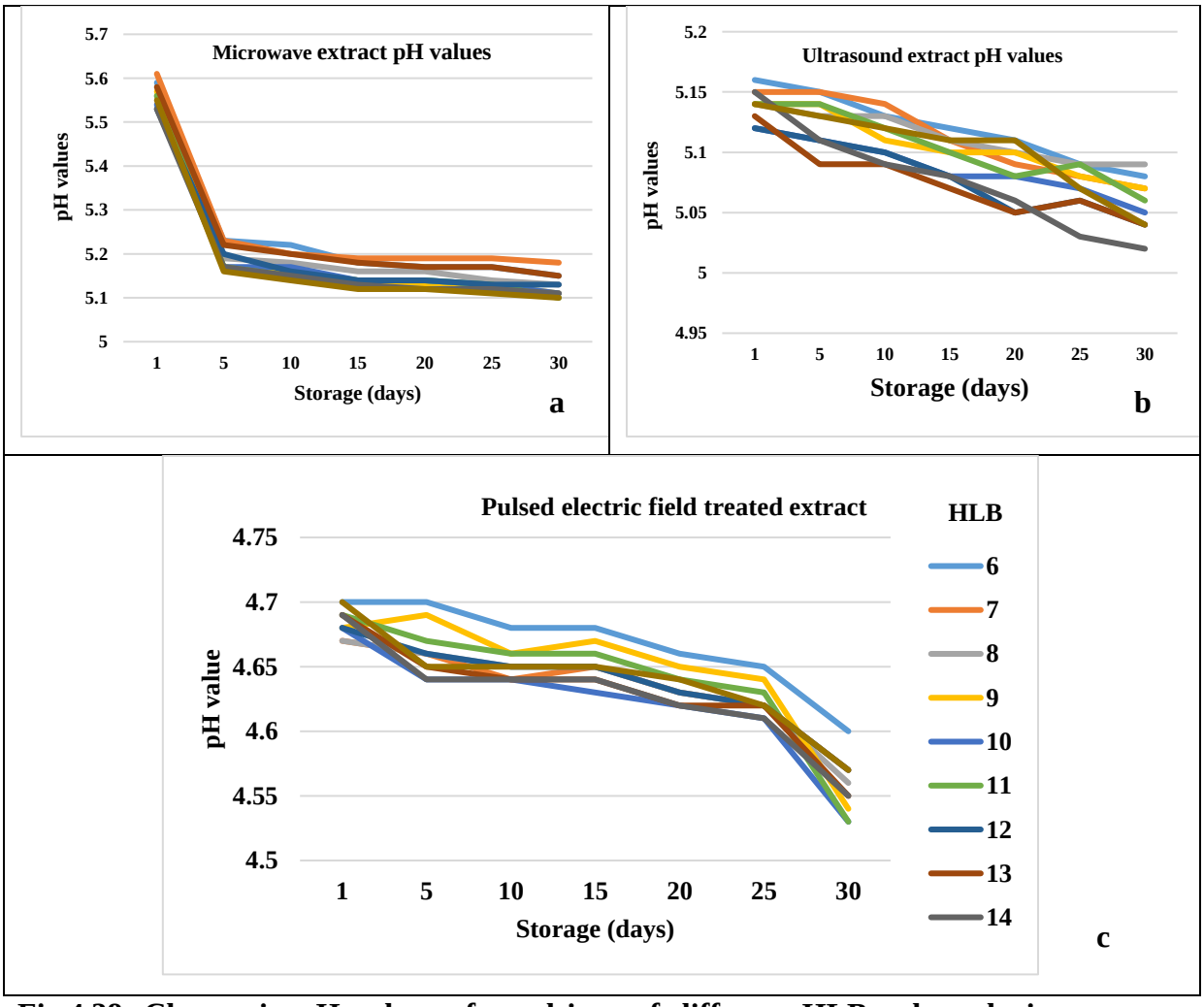


Fig.4.38. Change in pH values of emulsions of different HLB values during storage, samples prepared by extract obtained with (a) microwave assistance (b) ultrasound assistance (c) PEF assistance

Details of pH values reported for emulsions prepared for different HLB values from different extracts are shown in Fig. 4.38. There was steep fall in pH of the emulsion prepared using the extract obtained using microwave assistance across all HLB values between 1st and 5th day. Further, during course of storage for 30 days, the pH readings were fairly consistent in all samples; the narrow change in pH values, though insignificant indicated ability of extract to change pH value of emulsion. Across all HLB values, the change in pH value was limited to 5.1 (from an initial value of 5.6) after 30 days of storage.

In case of emulsions prepared using the extract obtained by ultrasound assistance, a linear change in pH values across the storage period was observed. This phenomenon is different to the results observed for the sample prepared with microwave assistance. Overall, change of pH across different HLB values ranged between 5.02 to 5.16, which reiterated the ability of the emulsifier-extract samples to contain change in pH. An important observation that was recorded while comparing the behaviour of the sample prepared using ultrasound and microwave assistance was that the latter sample took few days to stabilize to a particular pH, while the former sample was almost instantaneous in its binding action.

The emulsion prepared using the extract obtained with PEF assistance reported comparatively acidic pH than the other two treatment samples. Change in pH values across different HLB values varied between 4.7 and 4.53 during the storage. A steep drop in pH values across all HLB values was observed between 25th and 30th day. Though pH alone will not reflect the emulsion capability of sample, the lower values of pH in an emulsion could be construed for reduced ability of emulsion to bind oil and water phase.

4.7.3.3. Change in electrical conductivity of the emulsion

Though electrical conductivity (EC) of emulsion is not directly linked with its emulsion stability, it gives an indirect measure of emulsion behaviour during storage.EC values reported during the storage across different HLB values for different emulsion samples studies is shown in Fig. 4.39.

During storage, EC values reported for the sample obtained with microwave assistance showed increasing trend. Initial values of EC across different HLB values varied from 2068 to 3735 µS/cm, whereas at the end of 30 days, the values varied between 7701 to 9084 µS/cm. Samples bearing HLB values 10, 11 and 12 reported EC values less than 7700 µS/cm. Samples in this HLB range showed consistent increase in EC values during storage period. Ferreira *et al.,* (2010) evaluated emulsification properties of a formulation with *andiroba* oil

and reported almost constant values of EC for samples bearing HLB values 8 to 12 indicating comparatively good emulsifying property of sample.

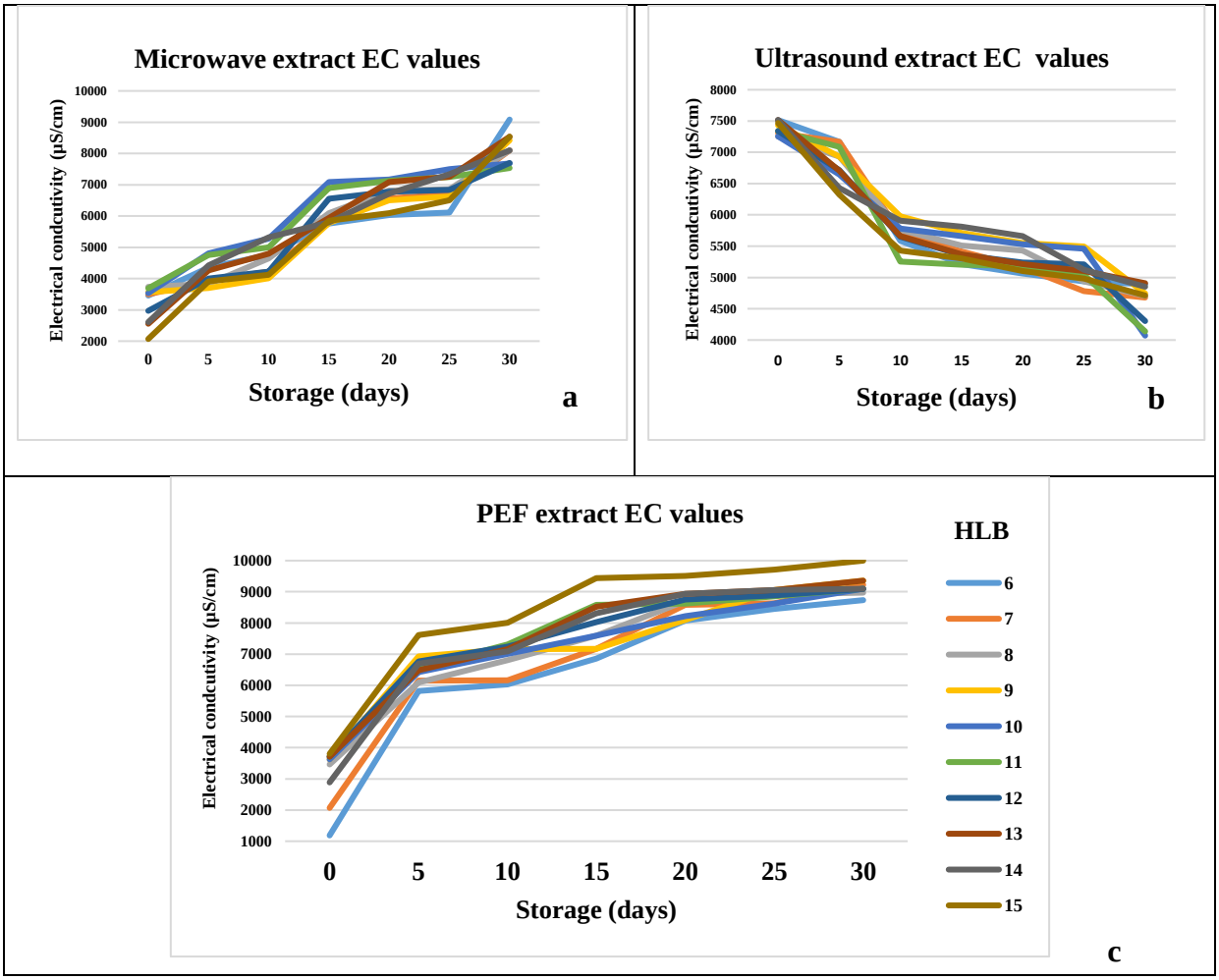


Fig.4.39. Change in electrical conductivity values of different HLB values during storage, samples prepared by extract obtained with (a) microwave assistance (b) ultrasound assistance (c) PEF assistance

Contradictorily, emulsion prepared with the extract obtained with ultrasound assistance showed decreasing EC values during storage (Fig.4.39 (b)). The range of EC values reported for the ultrasound treated samples were between 7516 and 4679 µS/cm. The trend of decrease in EC values of samples indicates better stability of emulsion. Among the reported EC values after 30 days of storage, incidentally, sample of HLB value 10 to12 maintained lesser EC value. This behaviour may be considered as one of yardstick to screen samples across different HLB values.

In case of sample prepared with PEF assistance, increasing trend for HLB values were reported during storage. However, the magnitude of change in EC was observed to be too high compared to ultrasound and microwave treated samples. In this case too, samples of

HLB values 10-12 reported lowest values. The elevated values reported for EC could be attributed to the lower PLs content in the extract obtained by PEF assistance.

4.7.3.4. Microscopic observation of emulsion droplets

Relative size of emulsion droplets is another property to indicate stability of emulsion. Droplets size in emulsions prepared with different HLB values using the extracts of different assisted extraction techniques were examined on 1st and 30th day of storage. Increased size of emulsion droplets in few samples between 1st and 30th day was noted and the images are shown in Fig. 4.39.

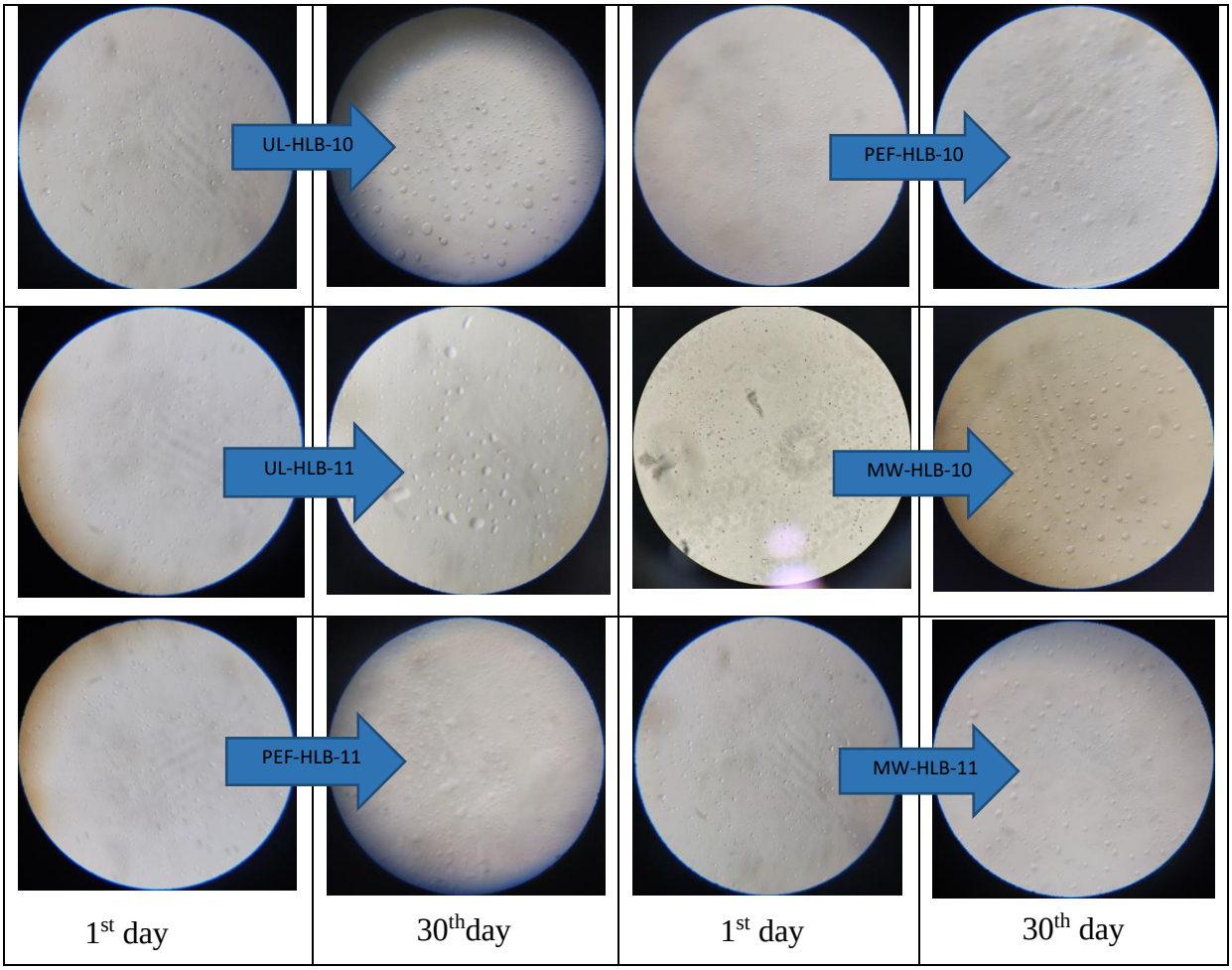


Fig.4.40. Images of change in size of emulsion droplets from different assisted extracts

The optical images indicate coalesced oil droplets after 30 days of storage across all HLB values. The flocculated oil droplets create a protective layer surrounding bigger droplets (Nesterenko *et al*., 2014). Fig. 4.40 represents only few samples of emulsion indicating clear growth of oil droplets during storage. However, on observing the emulsion droplets across all HLB values, samples bearing HLB of 10, 11 and 12 were comparatively smaller. This trend is in agreement with observations highlighted during electrical conductivity measurement

during storage. Similar results were also reported by Nesterenko *et al*., (2014) during the study of two surfactants across five HLB values.

4.7.3.5. Creaming index of emulsion

Creaming Index is an important factor to differentiate between emulsions prepared across different HLB values. The tendency of oil droplets in an emulsion to migrate to the surface and ability of the emulsifier to resist migration is practically manifested in the form of separate layer; the thickness of this layer (expressed as a %) quantifies the creaming index of sample. Table 4.27 is presented with change in %CI values of emulsion prepared with the extract obtained through the different assisted techniques.

All treatment samples showed visible creamy layer separation after 30 days of storage excluding sample bearing HLB values of 10, 11 and 12 (Fig. 4.41). No significant change in the CI values were recorded during the first five days of storage across all samples. However, the creamy layer formation was observed on 20th day, in samples prepared using microwave and ultrasound assistance. The PEF treated samples showed earlier creamy layer formation (on 15th day of storage).

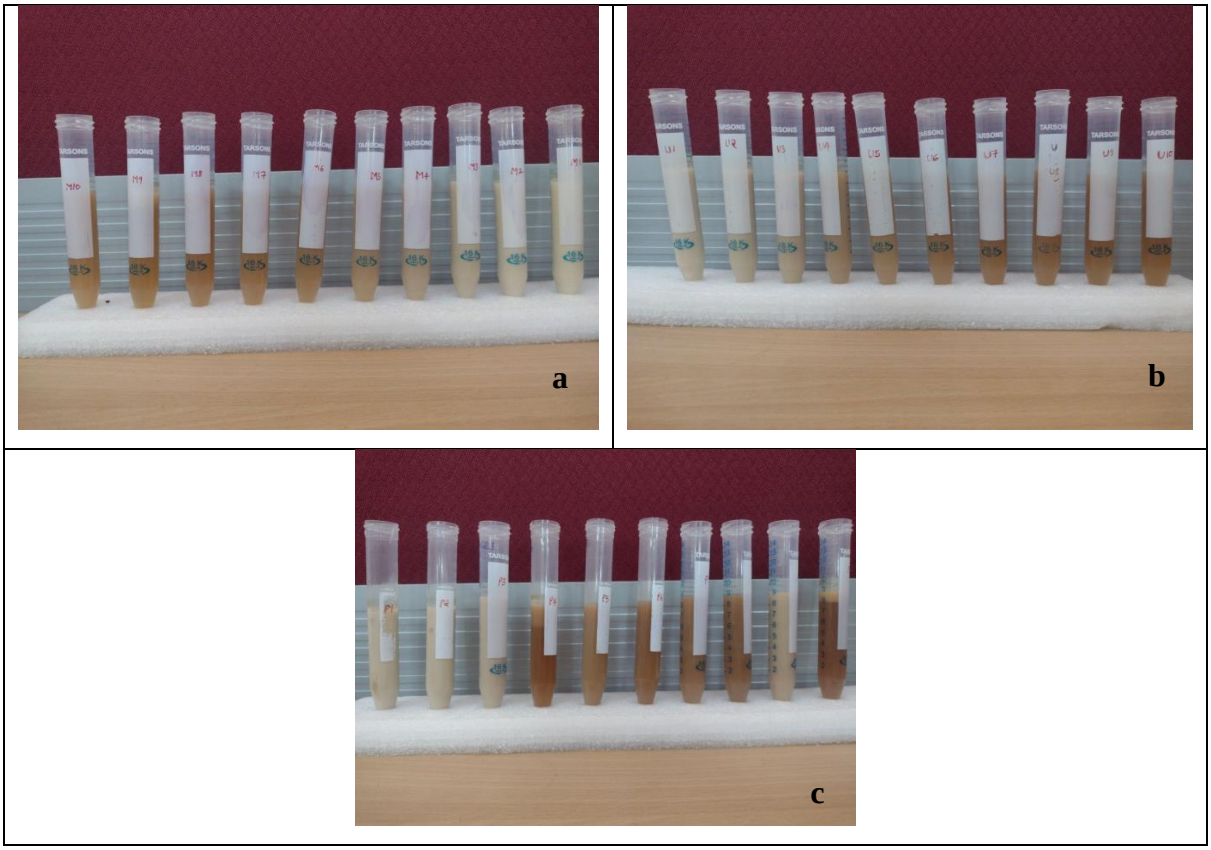


Fig. 4.41. Phase separation in emulsions of different HLB values during storage, samples prepared by extract obtained with (a) microwave assistance (b) ultrasound assistance (c) PEF assistance

Table. 4.27 Creaming Index (CI) values of emulsions prepared from extracts of microwave, ultrasound and PEF treatment during storage

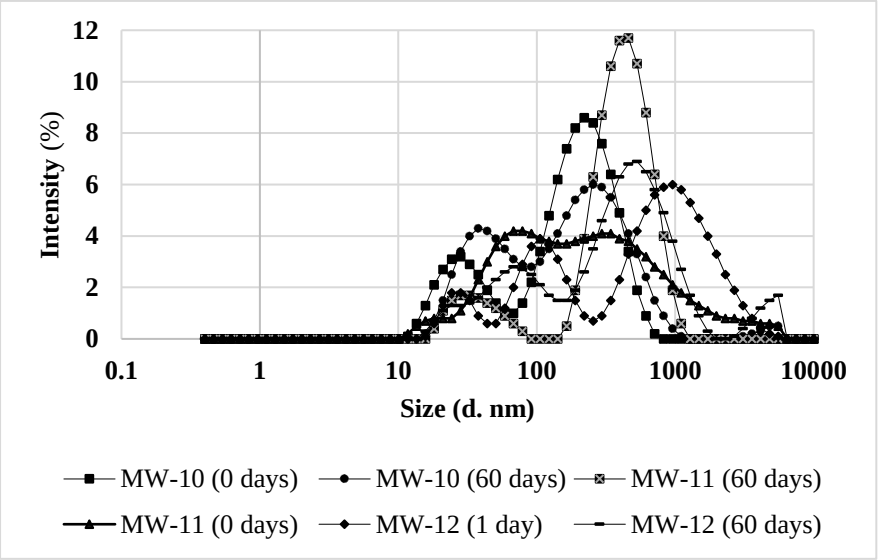
	Storage (days)																			
	Microwave							Ultrasonication							PEF					
HLB	**0**	**5**	**10**	**15**	**20**	**25**	**30**	**0**	**5**	**10**	**15**	**20**	**25**	**30**	**0**	**5**	**10**	**15**	**20**	**25**	**30**
6	1	2	3	5	P	P	P	1	1	1.5	2	P	P	P	1	1	4.5	P	P	P	P
7	1	2.5	3	4.7	6	P	P	1	1	1.5	2	3.1	P	P	1	1	2	4	5.1	7.1	P
8	1	3	3.5	4.5	5	7	P	1	1	1.5	2	3	P	P	1	1	2.5	4	7.1	8.1	P
9	1	1	1	2.5	4.5	7	P	1	1	1.5	3	5.3	7	P	1	1	2.5	3.6	4.5	7.4	P
10	1	0	1	1	1.5	2.5	3	1	1	1	1.5	1.5	1.5	2	1	1	2	3.5	4.4	4.8	5.5
11	1	0	1	1	1.5	2.5	3.5	1	1	1	1.5	1.5	2	2.5	1	1	1.5	2	3	4	5
12	1	0	1	1.5	2	3	4	1	2	2.9	3	3.1	3.8	3.9	1	1	1.5	3	4.2	5	6
13	1	2.2	4.9	6	7.1	8	P	1	1	1.5	2	3	3.9	P	1	1	3	3.5	7	7.5	P
14	1	2.5	5.3	7	8.5	P	P	1	1.5	2	3	3.5	4.1	P	1	1	3.5	4.5	5.2	6.9	P
15	1	3	6	7	P	P	P	1	1	1.5	2.5	3.5	P	P	1	1	3.5	7	8.5	P	P

P-Phase separation; HLB- Hydrophilic and Lipophilic balance

Meher *et al*., (2013) reported HLB values of a formulation prepared using citronella oil using similar methodology. The study reported the formation of creamy layer within 30 days of storage (even for samples formulated with HLB values of 11, 12 and 13).

4.7.3.6. Particle size analysis during extended storage of the emulsions

The results obtained from analysis of creaming index, electrical conductivity and particle size identified the emulsions formulated at HLB values of 10, 11 and 12 as competitive samples in terms of emulsion attributes. Lesser % CI values, lower electrical conductivity and lower particle size change rendered these samples effective compared to others. Incidentally, samples formulated with extract from all the assisted extraction techniques showed good emulsion response at similar HLB values. To further screen or narrow the HLB values of sample among selected range of HLB values, particle size analysis of the emulsion over extended storage period of up to 60 days was undertaken.



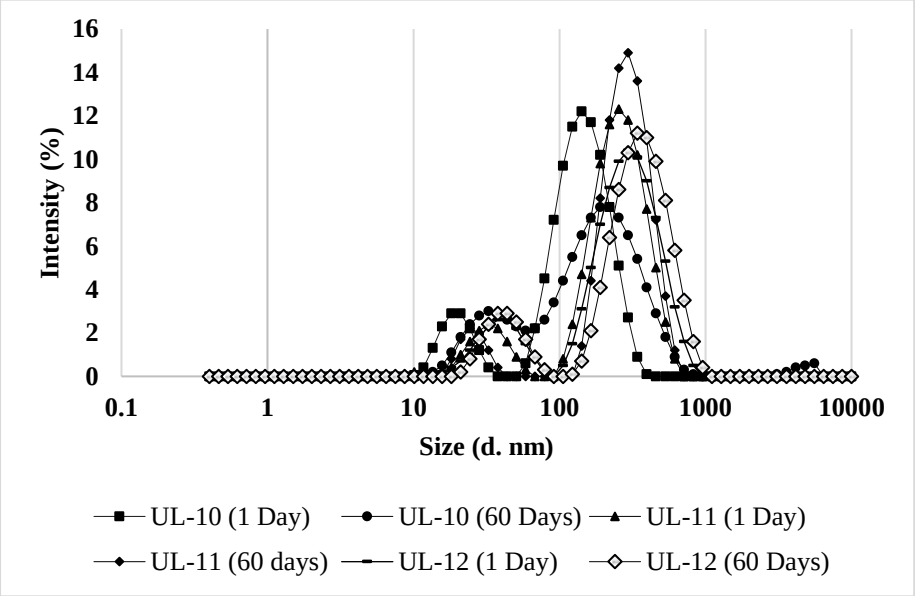
Average particle size (expressed in nm)

Days/HLB	1	60
MW-10	90.44	90.57
MW-11	125.90	227.10
MW-12	177.10	293.00

Fig.4.42. Particle size distribution of emulsion prepared from extract obtained with microwave assistance on 1st day and 60th day of storage at (4±1ºC)

The results of mean particle size of sample bearing HLB values of 10, 11 and 12 on 1st and 60th day of storage for the emulsion prepared using the extract obtained with microwave assistance is depicted in Fig. 4.42. Samples bearing HLB values of 10 showed near steady values for average particle size between 1st and 60th day compared to other two samples. Also, the change in average particle size increased with increase in HLB value of the sample. From these experiments, it can be concluded that extract obtained from microwave assisted treatment exhibited HLB value close to 10. The emulsion prepared with the extract obtained

through ultrasound assistance also showed increase in average particle size in emulsion between 1st and 60th day of storage. Compared to the sample prepared with the extract obtained with microwave assistance, the samples prepared with ultrasound assistance were found to have narrow distribution of particle size as observed in Fig. 4.43. Lower particle size was observed in sample bearing HLB value of 10 compared to other two HLB values. Also, the magnitude of change in particle size is less (84.63 nm on 1st day compared to 101.3 nm on 60th day). Sample with HLB values of 11 and 12 showed wider variation between 1st and 60th day and reported higher particle size. From this analysis, it can be concluded that the extract obtained through ultrasound treatment is closest to HLB value 10. Hong *et al*., (2018) evaluated the average particle size distribution of experimental samples to establish HLB value of two different surfactants. The study emphasised that the lesser particle size variation during storage as an indicator HLB value.

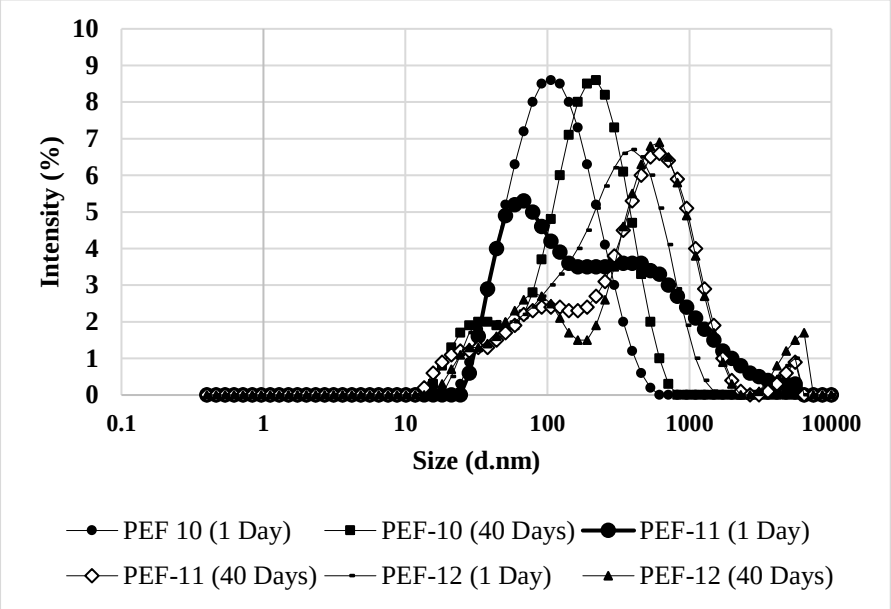


Average particle size expressed in nm)		
	1	60
UL-10	84.63	101.30
UL-11	147.20	200.90
UL-12	147.70	177.90

Fig.4.43. Particle size distribution of emulsion prepared from extract obtained with ultrasound assistance on 1st day and 60th day of storage at (4±1ºC)

The samples prepared with the extract obtained using PEF assisted extraction showed phase separation after 45 days of storage. Hence, the particle size analysis of sample was undertaken on 40th day to evaluate the change during storage. Fig. 4.44 illustrates the change in particle size between 1st and 40th day of storage. Average particle size of the particle after 40 days of storage was lower in sample bearing HLB value of 10, samples formulated for HLB value of 11 and 12 reported nearly same values for particle diameter after 40 days of storage. Though samples showed phase separation after 45 days of storage, extract exhibited

emulsion property for 40 days at refrigerated storage. From the data, it may be inferred that the HLB value of the extract was close to 10.



Average particle size expressed in nm)		
	1	40
PEF-10	105.32	139.56
PEF-11	178.52	210.09
PEF-12	172.56	222.19

Fig.4.44. Particle size distribution of emulsion prepared from extract obtained with PEF assistance on 1st day and 45th day of storage at (4±1ºC)

4.8. Evaluation of efficacy of the extract obtained from ghee residue as an emulsifier in a dairy product

Based on the results obtained for the various parameters evaluated to identify the specific HLB value of the extracts from different assisted extraction techniques, it was inferred that the extracts from all the three techniques exhibited HLB value close to 10. As per the classification of emulsifiers based on HLB values, the extract categorised to fit in oil in water type emulsion (Hong *et al*., 2018). Further, it was also deemed that the extract from ghee residue would be able to support a stable emulsion under refrigerated conditions (since the emulsion properties and storage tests were conducted under refrigerated conditions). Based on these assessments, ice cream is chosen as product to test efficacy of the extract obtained from ghee residue as an emulsifier.

Many combinations of emulsifiers and stabilizers have been reported by researchers to test the changes in ice cream mix and ice cream. Flaxseed mucilage as a replacer for commercial guar gum was evaluated by El-Aziz *et al*., (2015) by comparing physical properties of the resultant ice cream. Similarly, Loffredi *et al*., (2021) evaluated the quality of ice cream by replacing standard emulsifiers in the ice cream mix formulation with eight different substitutes. Based on the review of similar literature, the present study was envisaged with the replacement of commercial guar gum (possess both emulsifier and stabilizer property)

and glyceryl monostearate (emulsifier) in the ice cream mix with the extract obtained from ghee residue. As the extract obtained using PEF assistance fared relatively poor during the assessment of its emulsification properties, this study on emulsification behaviour of the extract in dairy product was restricted to samples obtained with microwave and ultrasound assistance.

4.8.1. Replacement of emulsifier in ice cream mix with the extract at varied proportions

Based on preliminary trials, experiments were designed to replace guar gum in four proportion (25, 50, 75 and 100%) and glyceryl monostearate (GMS) in two proportions (50 and 100%). Through trials, fat fraction and total solids in the ice cream formulation was confirmed to 8.5% and 30%, respectively. Since the extract obtained from the ghee residue had a mildly brown hue in colour, chocolate powder was used to mask the change in colour of ice cream prepared using the extract. All the samples were prepared in triplicates and the various quality attributes of the ice cream mix and hardened ice cream were measured and reported as means.

The proportion of different ingredients used in ice cream preparation was arrived after preliminary trials with different compositional combination. The relative proportions of various ingredients used for preparing various test samples are summarized in Table. 4.28. The proportions of the extract obtained from ghee residue and used in the study was calculated based on dry fraction present in solvent. Pre-treated ghee residue was subjected to assisted extraction treatment using optimized conditions for microwave and ultrasonication techniques and the resultant extract (dry fraction) was considered to be incorporated as substitute for control additives in the mix as per the composition presented in Table 4.28.

4.8.2. Effect of replacement of emulsifier in ice cream mix with the extract on textural parameters of ice cream mix

From the Table 4.29 it is evident that firmness, consistency, cohesiveness and index of viscosity of the mix was highest in control samples prepared with guar gum. With decrease in proportion of guar gum in the mix, values of all attributes showed decreasing trend. Firmness of ice-cream mix was significantly different for all samples compared to control ($p<0.05$). However, there was no significant difference in the firmness of the samples prepared by replacing guar gum with the two extracts (obtained with microwave and ultrasoundassistance) at 25%. Firmness values of the samples where the replacement was at 50% level showed significant difference between the two extracts. Overall, the samples reported firmness values of 0.95 to 0.29 (N) across different experimental treatments.

Table.4.28. Composition of ice cream mix with different proportions of ghee residue extract as replacer for guar gum and glyceryl monostearate

Ingredient	Ice cream samples													
	GC	GMSC	GMW-25	GUL-25	GMW-50	GUL-50	GMW-75	GUL-75	GMW-100	GUL-100	EMW-50	EUL-50	EMW-100	EUL-100
Pasteurized milk (3% fat)	53.94	53.94	53.94	53.94	53.94	53.94	53.94	53.94	53.94	53.94	53.94	53.94	53.94	53.94
Pasteurized milk cream (60% fat)	11.34	11.34	11.34	11.34	11.34	11.34	11.34	11.34	11.34	11.34	11.34	11.34	11.34	11.34
Skim milk powder	16.22	16.22	16.22	16.22	16.22	16.22	16.22	16.22	16.22	16.22	16.22	16.22	16.22	16.22
Sugar	14.00	14.00	14.00	14.00	14.00	14.00	14.00	14.00	14.00	14.00	14.00	14.00	14.00	14.00
Choco powder	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Guar gum	0.5	-	0.375	0.375	0.25	0.25	0.125	0.125	-	-	-	-	-	-
Sodium alginate	-	0.3	-	-	-	-	-	-	-	-	0.3	0.3	0.3	0.3
GMS	-	0.2	-	-	-	-	-	-	-	-	0.1	0.1	-	-
GR extract (MW)	-	-	0.125	-	0.25	-	0.375	-	0.5	-	0.1	-	0.2	-
GR extract (UL)	-	-	-	0.125	-	0.25	-	0.375	-	0.5	-	0.1	-	0.2

GC- guar gum as control; GMW-25, GMW-50, GMW-75, GMW-100-microwave assisted extract at 25, 50, 75 and 100% replacement, respectively; GUL-25, GUL-50, GUL-75, GUL-100-ultrasound assisted extract at 25, 50,75 and 100% replacement, respectively; GMSC-glyceryl monostearate as control; EMW-50, EMW-100- microwave assisted extract at 50, and 100% replacement, respectively; EUL-50, EUL-100-ultrasound assisted extract at 50 and 100% replacement, respectively

Addition of extract at larger proportions resulted in less firmness in ice cream mix. This could be reflective of the poorer efficacy of extract in thickening of mixture in comparison to a commercial additive such as guar gum.

The same trend was observed when the samples were evaluated for replacement of GMS with lower firmness values across different treatments. Compared to control samples prepared with guar gum, the samples formulated with sodium alginate (SA) and GMS showed lower firmness. Significant difference for firmness was observed between samples added with the extract obtained with microwave assistance at (50 and 100% replacement), while, no significant difference in the firmness was observed in samples added with extract obtained with ultrasound assistance.

Table.4.29. Textural property of ice cream mix prepared using ghee residue extract in different proportions

Texture property	Firmness (N)	Consistency (N. s)	Cohesiveness (N)	Index of viscosity (N.s)
GC	1.17 ± 0.13^{a}	9.61 ± 0.62^{a}	-0.60 ± 0.07^{e}	-0.59 ± 0.03^{f}
GMW-25	0.95 ± 0.01^{b}	8.02 ± 0.47^{b}	-0.49 ± 0.04^{cd}	-0.45 ± 0.08^{e}
GMW-50	0.62 ± 0.01^{d}	5.87 ± 0.60^{c}	-0.40 ± 0.01^{bc}	-0.31 ± 0.03^{cd}
GMW-75	0.50 ± 0.01^{d}	4.99 ± 0.95^{c}	-0.30 ± 0.01^{ab}	-0.18 ± 0.02^{ab}
GMW-100	0.29 ± 0.03^{e}	2.01 ± 0.10^{d}	-0.28 ± 0.02^{a}	-0.08 ± 0.02^{a}
GUL-25	0.88 ± 0.00^{bc}	8.26 ± 0.32^{ab}	-0.51 ± 0.04^{de}	-0.50 ± 0.03^{ef}
GUL-50	0.77 ± 0.00^{c}	6.24 ± 0.62^{c}	-0.43 ± 0.04^{cd}	-0.34 ± 0.04^{d}
GUL-75	0.57 ± 0.02^{d}	5.01 ± 0.20^{c}	-0.38 ± 0.06^{abc}	-0.21 ± 0.02^{bc}
GUL-100	0.36 ± 0.03^{e}	2.25 ± 0.28^{d}	-0.30 ± 0.03^{ab}	-0.19 ± 0.03^{b}
GMSC	0.92 ± 0.06^{a}	5.02 ± 0.60^{b}	-0.61 ± 0.03^{c}	-1.11 ± 0.13^{a}
EMW-50	0.61 ± 0.01^{b}	4.03 ± 0.63^{b}	-0.41 ± 0.04^{ab}	-0.71 ± 0.11^{a}
EMW-100	0.51 ± 0.01^{c}	1.96 ± 0.46^{a}	-0.32 ± 0.08^{a}	-0.62 ± 0.06^{a}
EUL-50	0.60 ± 0.03^{bc}	4.56 ± 0.85^{b}	-0.53 ± 0.02^{bc}	-0.76 ± 0.06^{a}
EUL-100	0.57 ± 0.01^{bc}	2.01 ± 0.10^{a}	-0.38 ± 0.05^{a}	-0.63 ± 0.15^{b}

GC- guar gum as control; GMW-25, GMW-50, GMW-75, GMW-100-microwave assisted extract at 25, 50, 75 and 100% replacement, respectively; GUL-25, GUL-50, GUL-75, GUL-100-ultrasound assisted extract at 25, 50,75 and 100% replacement, respectively; GMSC-glyceryl monostearate as control; EMW-50, EMW-100- microwave assisted extract at 50, and 100% replacement, respectively; EUL-50, EUL-100-ultrasound assisted extract at 50 and 100% replacement, respectively.

Consistency of ice cream mix plays an important role in attaining required texture in the hardened ice cream (Urkek, 2021). With increase in the levels of the extract from ghee residue in the sample, there was decrease in consistency values. An exception to this trend was the ice cream mix sample formulated by replacing guar gum with the extract obtained by ultrasound assistance at 25 % level. Complete replacement of guar gum with the extract resulted in least values for consistency of the ice cream mix. In a rheological study of yog-ice cream by El-Nagar *et al*., (2002), increased proportions of inulin reported improved consistency index of sample. They attributed the change to binding ability of inulin to form gel like network to alter consistency index of the mix. Though the PLs in the extract were expected to exhibit emulsifying action, the binding strength exhibited by guar gum was not fully compensated by the extract. Comparatively, the extract exhibited better binding when used as a replacer for GMS as reflected in the consistency values of the mix (Table 4.29).

Cohesiveness and index of viscosity of the mix followed similar trend depicted for firmness cohesiveness of samples added with both the extracts. No significant difference was reported for cohesiveness and index of viscosity ($p<0.05$) between control and sample where the replacement of guar gum was at 25% of extract obtained by ultrasound assistance. Between the experimental samples, the textural characteristics of the samples formulated with the extract obtained by microwave assistance was poorer compared to the samples formulated with the extract obtained by ultrasound assistance. Similar kind of observations were made by Sert *et al*., (2021) while studying ice cream mix attributes subjected for different pressure. Increase in pressure of homogenization led to increased cohesiveness and index of viscosity. Ghee residue extract acted efficiently in exhibiting emulsifier characteristics as observed in studies on emulsifier replacement. However, it did not report desirable results when it was replaced for guar gum at higher proportions. The attributes of thickening and stabilizing were not shown by extract which is evident from the ice cream mix texture results.

4.8.3. Effect of replacement of emulsifier in ice cream mix with the extract on Physico-chemical properties of ice cream mix

The effect of replacement of emulsifier in ice cream mix with the extract from ghee residue on the physico-chemical properties of ice cream mix was recorded in terms of pH, acidity and total solids (Fig. 4.45). No significant effect was observed on the pH of the ice cream as a result of the replacement across all treatment levels evaluated. The samples reported pH values ranging between 6.44 and 6.21. These values are in agreement with values reported for guar gum added ice cream samples by El-Aziz *et al*., (2015).

Total solid content varied across samples ranging from 60.63 to 51.76% with a trend of decrease in total solids values with increase in levels of the extract added as a replacement of guar gum and GMS in the formulation of ice cream mix. The effect was statistically significant across all experimental samples with the exception to samples where the extract (microwave) was replaced at 50% GMS. This could also be due to the lack of thickening ability of the extract to conglomerate sufficient solid fraction in unit mass. The results are in line with values reported by *Guven et al.,* (2018) where 3.3% of stabilizer and emulsifier blends were evaluated on ice cream mix.

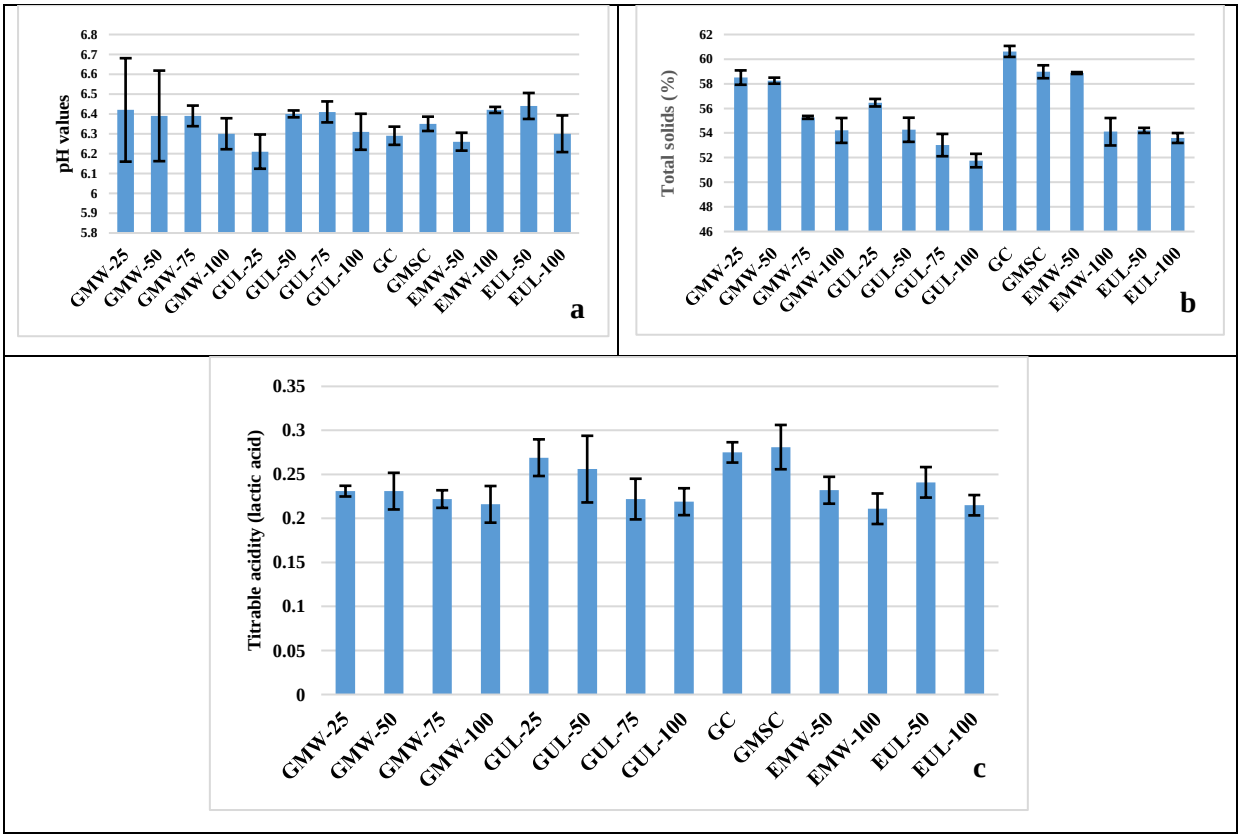


Fig.4.45.Effect of replacement of emulsifier in ice cream mix with extract from ghee residue on its physico-chemical properties (a) pH, (b) total solids, (c) acidity (%lactic acid)

GC- guar gum as control; GMW-25, GMW-50, GMW-75, GMW-100-microwave assisted extract at 25, 50, 75 and 100% replacement, respectively; GUL-25, GUL-50, GUL-75, GUL-100-ultrasound assisted extract at 25, 50,75 and 100% replacement, respectively; GMSC-glyceryl monostearate as control; EMW-50, EMW-100- microwave assisted extract at 50, and 100% replacement, respectively; EUL-50, EUL-100-ultrasound assisted extract at 50 and 100% replacement, respectively.

Titratable acidity (% lactic acid) of ice cream mix was within the range of 0.28 and 0.21 across the different treatments. No significant difference was noted in the acidity values of the samples formulated with guar gum/GMS at 50% level for both extracts (Fig. 4.45 c). The

titratable acidity values of the mix reported in the present study are in agreement with values reported by Urkek, (2021) for stabilizers substituted with chia seed powder in ice cream mix formulation.

4.8.4. Effect of replacement of emulsifier in ice cream mix with the extract on overrun and fat destabilization

Overrun is considered as one of the important attributes of good ice cream mix (Amador *et al*., 2017). Incorporation of air in the mix under influence of refrigerated atmosphere aids to enhance the consumer acceptance of the hardened ice cream. During the process of ice cream making, the mix undergoes shearing action leading to breakdown of constituent material. Fat component of ice cream mix and its destabilization is considered as a factor to understand its dispersion in the mix. High shear and time of ice cream making is known to have a bearing of fat destabilization (Goff, 1997).

Fat destabilization of ice cream mix during freezing, the experimental samples were monitored at different time intervals and is plotted in Fig. 4.46. Destabilization of fat during freezing will influence structure of ice cream by forming stabilized air cells by trapping aqueous phase ((Goff *et al*., 1999). Higher destabilization of fat during freezing favours better overrun and slow melting rate (Segall and Goff, 2002). Control sample prepared using guar gum showed highest fat destabilization compared to other treatments (Fig. 4.46 (a)). Among the experimental samples, the highest destabilization of fat (44.25%) was reported for sample microwave assistance replaced at 25%. The destabilisation index was not statistically different from the value reported at a replacement at 50% level ($p<0.05$). Complete replacement of guar gum in the formulation with 100% extract resulted in the lowest value of destabilization (25.36%).

The extract obtained from the ghee residue samples using ultrasound assistance performed marginally better for fat destabilization behaviour when compared with the extract obtained with microwave assistance. Fat destabilization index of both experimental samples were significantly different from the respective control samples. The extract obtained using ultrasound assistance as a replacer to guar gum at 25% level reported 46.77% fat destabilization compared to 61.2% reported for control sample (Fig. 4.46 (b)). No significant difference was deduced for the fat destabilization index estimated for experimental samples prepared using extract obtained by ultrasound assistance at 25% and 50% levels for guar gum. Lesser destabilization noted for the experimental samples could be due to lack of support by the extract, even with its significant protein content, to form a thin layer between

fat and serum during freezing process. A similar observation was discussed in a study reported by Segall and Goff, (2002) to compare effect of emulsifier and polysaccharide action in ice cream mix where the replacement of either of two additives in experimental samples resulted in fat destabilization value less than 20%.

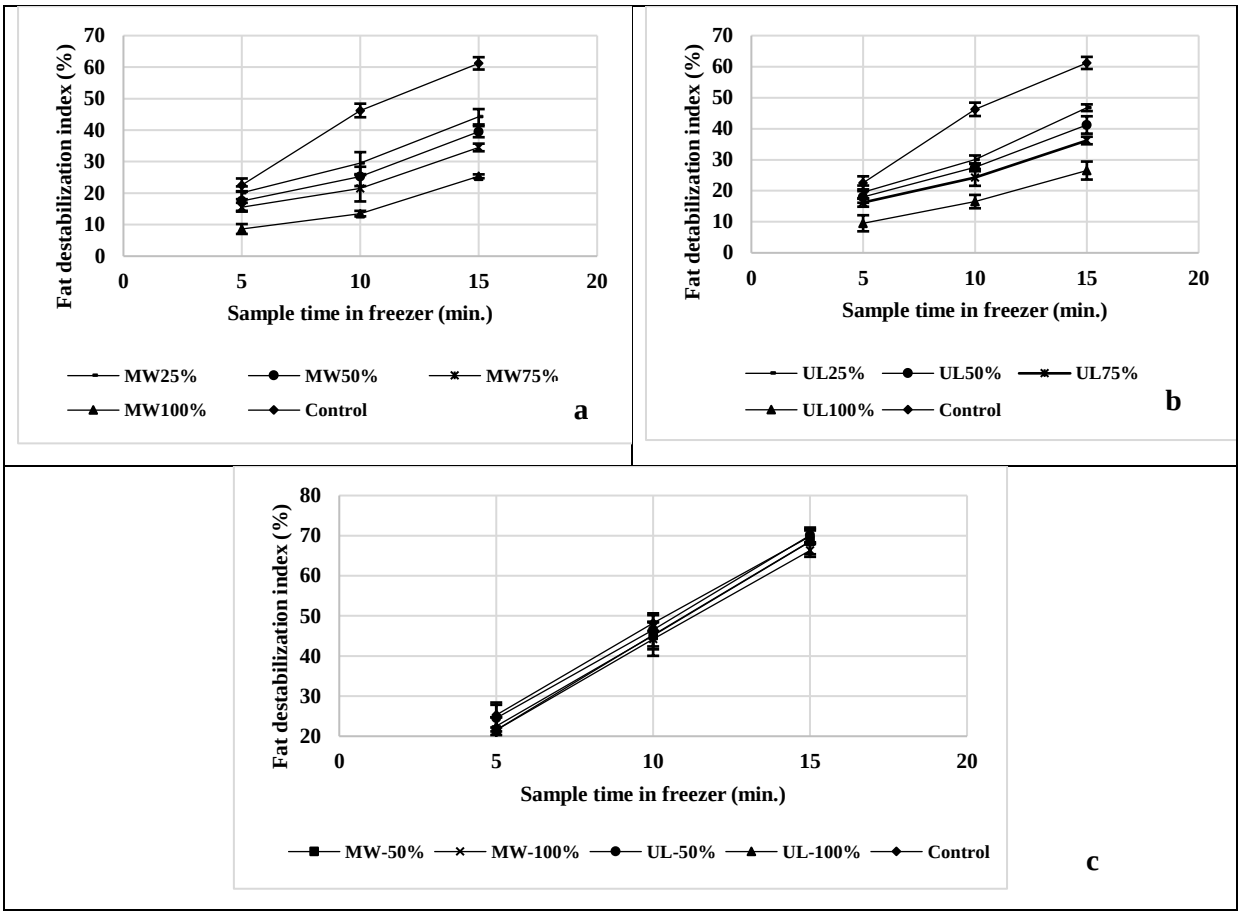


Fig. 4.46. Effect of replacement of emulsifier in ice cream mix with extract from ghee residue on freezing time and fat destabilization of ice cream mix (a) microwave assistance (replacement for guar gum) (b) ultrasound assistance (replacement for guar gum) (c) microwave and ultrasound assistance (replacement for GMS)

The experimental samples where the extracts were used as replacer for GMS showed better fat destabilization performance than the samples studied for replacement for guar gum (Fig. 4.46 (c)). Ice cream mix prepared by replacing GMS with the extract prepared by ultrasound assistance at 50% and 100% reported relatively better values compared to control. The fat destabilization index determined for the sample at a replacement level of 50 % (71.11%) was the highest value recorded in the present study. Comparatively control samples prepared with GMS reported an index of 69.82%. Also, no significant difference was deduced among all the samples studied for replacement of GMS as emulsifier ($p<0.05$). This may be inferred as indicative of the efficacy of the experimental extracts as a replacer for a conventional

emulsifier (GMS); the synergistic effect of the PLs present in extract from ghee residue in presence of stabilizer (sodium alginate) was adequate to bring required fat destabilization in the ice cream mix.

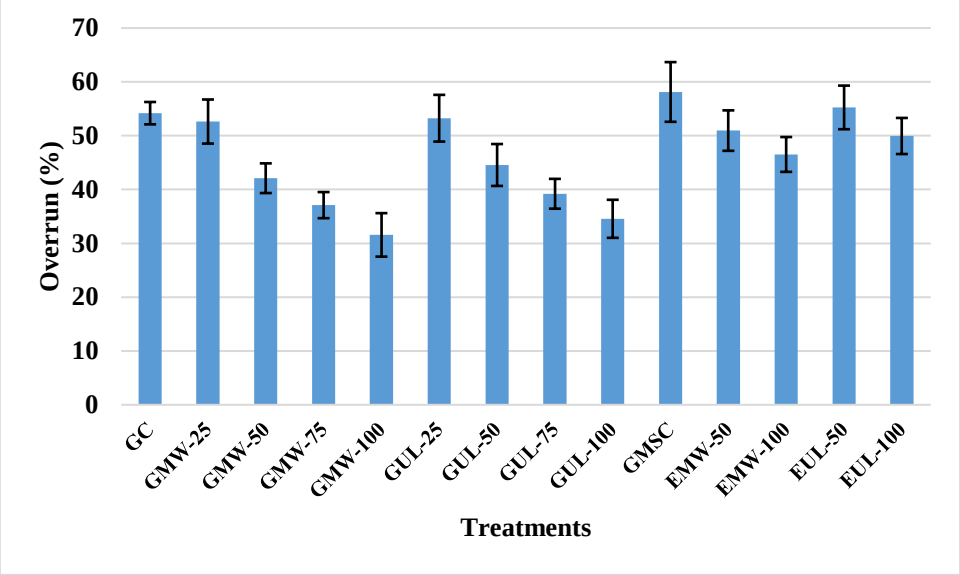


Fig.4.47. Effect of replacement of emulsifier in ice cream mix with extract from ghee residue on overrun

GC- guar gum as control; GMW-25, GMW-50, GMW-75, GMW-100-microwave assisted extract at 25, 50, 75 and 100% replacement, respectively; GUL-25, GUL-50, GUL-75, GUL-100-ultrasound assisted extract at 25, 50,75 and 100% replacement, respectively; GMSC-glyceryl monostearate as control; EMW-50, EMW-100- microwave assisted extract at 50, and 100% replacement, respectively; EUL-50, EUL-100-ultrasound assisted extract at 50 and 100% replacement, respectively.

Overrun of ice cream mix noted for the experimental samples is plotted in Fig. 4.47. Among the two control samples studied, samples with guar gum reported lesser overrun compared to GMS. Highest overrun was reported for control samples formulated with GMS (58.13%) and lowest overrun for the sample prepared with extract by microwave assistance formulated at 100% replacement for guar gum (31.57%). Replacement of guar gum in the experimental samples with both the extracts (prepared with microwave and ultrasound assistance) at 25% did not show any significant difference with the corresponding control sample ($p<0.05$). However, when the extracts were added at a level above 50% for replacement of guar gum, it resulted in significantly different overrun values than the control samples. El-Aziz *et al.*, (2015) evaluated different proportions of cress seed and flax seed mucilage as a replacer for guar gum in ice cream and reported that increase in mucilage proportion in ice cream mix led to slightly decrease in overrun. The reduced overrun was attributed to the increased viscosity of ice cream mix with increased concentration of the replacer additives.

When the extract obtained from ghee residue with microwave and ultrasound assistance was used as a replacer for GMS in the presence of sodium alginate (SA) as stabilizer, the overrun values were comparatively higher. No significant difference was reported for overrun values between control and sample prepared by extract prepared extract of ultrasound assistance used at50 and 100%. However, 100% replacement of GMS with the extract prepared with microwave assistance resulted in significantly different values for overrun from the control sample for GMS. Overall, the ice cream samples exhibited better overrun when formulated with extracts from ghee residue as a replacer for GMS than replacement of guar gum.

4.8.5. Effect of replacement of emulsifier in ice cream mix with the extract on melting properties of ice cream

The melting property of the ice cream samples were measured in terms of first drip time, total melting time and melting rate and the results are tabulated in Table 4.30. First drip time was highest in case of control sample prepared by using guar gum, the time decreased with increase in proportion of both the experimental extracts in the formulation. First drip time is an indicator for ability of ice cream to resist external atmosphere change and early first drip time is an indicator of ice cream losing its structure quickly (Guzeler *et al*., (2012).

Control and experimental samples formulated with extract prepared by ultrasound assistance as a replacer at 25 and 50% levels showed no significant difference for first drip time ($p<0.05$). Early drip time was reported for sample with complete replacement of guar gum in the formulation with extract prepared with ultrasound assistance (at 100% level). Similar values for first drip time were reported by Sert *et al*., (2021) for samples homogenized for different pressures. In another study where guar was used as stabilizer, Guzeler *et al*., (2012) reported a first drip time as 9 min 25 s. Comparatively, better performance was observed in all samples for first drip time in the present study (Table 4.30).

Samples formulated with both stabilizer (sodium alginate) and emulsifier (GMS) reported relatively lower values for first drip time compared to samples formulated with guar gum. Among the extract studied, samples where GMS was replaced with extract of ultrasound assistance performed better over samples formulated with extract by microwave assistance. Relatively better values were observed for experimental samples formulated with guar gum than samples formulated with GMS. This could be due to thickening property of guar gum which resisted melting of ice cream during the test.

Table.4.30. Melting properties of hardened ice cream formulated using extract from ghee residue in different proportions

Sample	First drip time (min.)	Total melting time (min.)	Melting rate (g/min)
GC	14.4±0.53^{a}	40.51±0.63^{abc}	0.95±0.02^{ab}
GMW-25	13.13±0.88^{ab}	42.22±1.00^{a}	0.88±0.01^{c}
GMW-50	12.01±0.83^{b}	39.15±0.94^{abcd}	0.92±0.02^{abc}
GMW-75	11.22±0.54^{bc}	38.41±0.83^{cde}	0.91±0.00^{abc}
GMW-100	9.53±0.67^{cd}	35.02±2.15^{f}	0.98±0.06^{a}
GUL-25	14.2±0.92^{a}	42.02±0.54^{ab}	0.89±0.04^{bc}
GUL-50	12.51±0.62^{ab}	39.07±0.51^{bcd}	0.94±0.00^{ab}
GUL-75	8.50±0.46^{d}	37.04±1.00^{def}	0.87±0.03^{bc}
GUL-100	8.00±0.80^{d}	35.32±1.20^{ef}	0.91±0.04^{abc}
GMSC	13.01±0.86^{a}	44.56±2.09^{a}	0.79±0.06^{c}
EMW-50	8.36±0.57^{b}	33.55±2.06^{c}	0.99±0.08^{a}
EMW-100	9.36±0.57^{b}	35.44±0.60^{c}	0.95±0.04^{ab}
EUL-50	13.59±1.46^{a}	43.17±0.94^{ab}	0.84±0.02^{bc}
EUL-100	12.56±1.00^{a}	39.53±0.99^{b}	0.92±0.06^{abc}

GC- guar gum as control; GMW-25, GMW-50, GMW-75, GMW-100-microwave assisted extract at 25, 50, 75 and 100% replacement, respectively; GUL-25, GUL-50, GUL-75, GUL-100-ultrasound assisted extract at 25, 50,75 and 100% replacement, respectively; GMSC-glyceryl monostearate as control; EMW-50, EMW-100- microwave assisted extract at 50, and 100% replacement, respectively; EUL-50, EUL-100-ultrasound assisted extract at 50 and 100% replacement, respectively.

Total melting time was found to decrease with increase in proportion of extract in the sample. However, no significant difference was deduced for experimental samples formulated with the extracts added as replacer at 25 and 50% and control samples formulated with guar gum. In case of samples with GMS as an emulsifier, replacement with extract prepared using ultrasound assistance at 50% level showed no significant difference with control for its total melting time. The experimental samples formulated with guar gum recorded total melting time ranging from 35 to 42 min. The samples prepared by ultrasound assistance reporting highest values. In samples formulated with GMS, the range of total melting time was between 33 and 44 min.

Melting rate of 0.95 to 0.88 g/min. was reported across samples formulated with guar gum, the experimental sample prepared by ultrasound assistance at 100% replacement for guar gum registering melting rate of 0.98 g/min. Samples formulated with extract prepared with ultrasound and microwave assistance at 50% replacement for GMS recorded the melting rate as 0.99 g/min. The values recorded was significantly lower than values reported by Tekin *et al*., (2017) where the melting rate was noted as 2.059 to 1.825 g/min across different fat content of ice cream for guar gum as stabilizer/emulsifier.

4.8.6. Effect of replacement of emulsifier on textural property of hardened ice cream

Increasing the proportion of the extracts as a replacer for guar gum in the experimental samples resulted in progressively reduced hardness of the samples. The experimental samples recorded hardness values ranging from 95.52 to 17.53 N for the hardened ice cream. The observation is in agreement with values reported for ice cream hardness formulated by adding different proportions of guar gum by Amador *et al*., (2017). The study corroborated the decrease in hardness values of sample to increased serum phase viscosity which necessitated more force to probe into the sample. Among the two extracts evaluated, the extract prepared with microwave assistance showed better values compared to that obtained with ultrasound assistance.

Adhesiveness is described as the resistance offered by sample for backward movement of probe and indicates bonding character of sample with contact material (Segall and Goff, 2002). With increase in proportion of the extract in ice cream mix, adhesiveness values also reported decreasing trend. This could be attributed to the lack of guar gum component in the formulation to thicken and offer resistance for free flow of probe during backward movement. Adhesiveness values ranged between -275 to -20 N.s for the experimental samples with progressive replacement of guar gum with extract from ghee residue (Table.3.30). The effect of replacement of GMS in the formulation with the extract did not significantly influence adhesiveness of the hardened ice cream ($p<0.05$). The data for adhesiveness was found to be close to the values reported by Sert *et al*., (2021).

Increasing replacement of conventional emulsifiers with the extract resulted in lower gumminess values in hardened ice cream samples. The experimental samples formulated with the extract prepared with microwave assistance as a replacer for GMS reported inconsistent behaviour for gumminess. The values indicated in this study is similar to values reported by Sabet-Sarvestani *et al* (2021) where salep was used as emulsifier and stabilizer. Synergic effect of coco powder and guar gum could be presumed to have contributed for higher

gumminess values reported in the present study. Cohesiveness values were found to be lower in experimental samples formulated with guar gum than samples formulated with GMS.

Samples added with extracts prepared by microwave and ultrasound assistance as replacers at 25 and 50% were found to be more cohesive than the corresponding control samples. This could be due to interaction between protein component in the extract and guar gum which aided in improving particulate bonding. Ice cream prepared with extract added as replacer for GMS reported higher cohesive values 1.3 to 0.59. Sodium alginate and protein content in the extracts seemed to have helped in better bonding compared guar gum (Table 4.31).

Table.4.31. Textural property of hardened ice cream formulated using extract from ghee residue in different proportions

Texture property	Hardness (N)	Adhesiveness (N. s)	Gumminess	Cohesiveness
GC	145.2±5.18^{a}	-359.1±17.8^{g}	103.21±14.49^{a}	0.71±0.08^{bc}
GMW-25	95.52±0.80^{b}	-275.49±8.83^{f}	87.42±6.33^{ab}	0.91±0.07^{a}
GMW-50	85.65±3.01^{c}	207.40±4.12^{e}	71.66±5.04^{be}	0.83±0.04^{ab}
GMW-75	26.24±2.07^{f}	-176.46±4.34^{d}	18.98±2.43^{de}	0.72±0.05^{bc}
GMW-100	19.25±0.83^{g}	-20.23±2.55^{a}	11.87±0.19^{e}	0.61±0.02^{cd}
GUL-25	63.25±0.90^{d}	-204.52±5.24^{e}	52.98±5.33^{c}	0.83±0.07^{ab}
GUL-50	43.13±1.75^{e}	-186.00±5.70^{de}	31.60±3.23^{d}	0.73±0.06^{bc}
GUL-75	22.32±1.03^{fg}	-102.42±3.76^{c}	13.33±1.18^{e}	0.59±0.02^{cd}
GUL-100	17.13±1.00^{g}	-48.30±2.62^{b}	8.29±1.50^{e}	0.48±0.06^{d}
GMSC	104.97±4.82^{a}	-285.62±8.16^{a}	137.19±9.23^{a}	1.30±0.05^{a}
EMW-50	91.33±2.25^{bc}	-236.5±26.00^{a}	54.23±5.47^{c}	0.59±0.04^{c}
EMW-100	83.06±0.50^{d}	-198.56±8.98^{a}	69.76±8.66^{c}	0.84±0.10^{b}
EUL-50	98.67±2.81^{ab}	-221.63±13.66^{a}	115.40±5.32^{a}	1.17±0.02^{a}
EUL-100	87.29±2.53^{cd}	200.15±1.99^{a}	63.00±10.49^{c}	0.72±0.10^{bc}

GC-guar gum as control; GMW-25, GMW-50, GMW-75, GMW-100-microwave assisted extract at 25, 50, 75 and 100% replacement, respectively; GUL-25, GUL-50, GUL-75, GUL-100-ultrasound assisted extract at 25, 50,75 and 100% replacement, respectively; GMSC-glyceryl monostearate as control; EMW-50, EMW-100- microwave assisted extract at 50, and 100% replacement, respectively; EUL-50, EUL-100-ultrasound assisted extract at 50 and 100% replacement, respectively.

4.8.7. Effect of replacement of emulsifier in ice cream mix with the extract on colour of ice cream mix and hardened ice cream

Change in colour of the samples was expressed in terms of variation of colour attributes such as lightness, greenness/yellowness and blueness/yellowness and the data is presented in Table 4.32. There was significant difference in L* values reported for ice cream mix prepared with different extracts. The extract prepared with microwave assistance added at 25 and 50% for guar gum did not indicate any significant effect on the colour characteristics of the samples ($p<0.05$). However, colour values for all the samples formulated with ultrasound assistance extractwas significantly different from control sample. Interestingly, in case of hardened ice cream, when the emulsifier was replaced with extract prepared with ultrasound assistance at 25, 50 and 75%, no significant difference in lightness was observed compared to control.

Table.4.32. Colour values of ice cream mix and hardened ice cream formulated using extract from ghee residue in different proportions

	Ice cream mix				Hardened ice cream			
Sample	**L***	**a***	**b***	**ΔE**	**L***	**a***	**b***	**ΔE**
GC	41.15±0.07^{a}	14.07±0.03^{cd}	17.90±0.15^{b}		42.85±0.07^{d}	13.26±0.08^{b}	18.97±0.11^{ab}	
GMW-25	35.98±0.08^{e}	16.79±0.06^{a}	17.09±0.15^{cde}	5.89±0.06^{a}	46.42±0.05^{ab}	12.75±0.08^{c}	18.28±0.12^{bc}	3.67±0.03^{bc}
GMW-50	40.20±0.20^{b}	14.51±0.04^{c}	19.87±0.02^{a}	2.23±0.22^{e}	47.29±0.15^{a}	11.91±0.14^{d}	18.00±0.21^{cd}	4.74±0.01^{b}
GMW-75	41.59±0.15^{a}	10.81±0.02^{g}	14.29±0.20^{f}	4.88±0.09^{b}	47.14±0.17^{a}	9.84±0.07^{f}	14.21±0.14^{g}	7.26±0.22^{a}
GMW-100	41.43±0.80^{a}	11.76±0.41^{f}	17.38±0.48^{bcd}	2.38±0.21^{de}	46.10±0.15^{b}	10.97±0.08^{e}	16.87±0.14^{e}	4.49±1.13^{bc}
GUL-25	37.83±0.47^{d}	15.43±0.21^{b}	17.50±0.45^{bc}	3.60±0.49^{c}	42.83±0.42^{d}	12.75±0.19^{c}	15.64±0.13^{f}	3.38±0.37^{c}
GUL-50	37.53±0.14^{d}	13.96±0.05^{d}	16.64±0.03^{e}	3.85±0.07^{c}	42.93±0.04^{d}	14.11±0.46^{a}	19.15±0.33^{a}	0.89±0.07^{d}
GUL-75	38.91±0.04^{c}	12.48±0.06^{e}	16.84±0.09^{de}	2.94±0.03^{d}	42.75±0.08^{d}	12.34±0.14^{cd}	17.42±0.28^{de}	1.80±0.24^{d}
GUL-100	40.58±0.24^{b}	11.75±0.11^{f}	17.55±0.07^{bc}	2.40±0.08^{de}	45.10±0.01^{c}	10.01±0.02^{f}	16.76±0.08^{e}	4.53±0.10^{bc}
Sample	**L***	**a***	**b***	**ΔE**	**L***	**a***	**b***	**ΔE**
GMSC	32.79±0.15^{d}	11.56±0.04^{b}	14.07±0.31^{b}		38.00±0.04^{e}	12.07±0.06^{b}	15.41±0.03^{c}	
EMW-50	37.04±0.03^{c}	12.41±0.04^{a}	14.52±0.21^{b}	4.35±0.19^{c}	40.34±0.68^{c}	13.35±0.21^{a}	16.26±0.55^{b}	2.88±0.40^{b}
EMW-100	42.58±0.31^{b}	10.79±0.11^{c}	14.31±0.19^{b}	9.81±0.20^{b}	41.87±0.07^{b}	11.40±0.03^{c}	15.60±0.13^{bc}	3.93±0.10^{b}
EUL-50	47.64±0.04^{a}	10.74±0.14^{c}	13.94±0.4^{b}	14.86±0.12^{a}	43.44±0.16^{a}	13.28±0.23^{a}	17.49±0.03^{a}	5.94±0.16^{a}
EUL-100	42.46±0.19^{b}	12.55±0.01^{a}	16.94±0.17^{a}	10.13±0.04^{b}	44.62±0.89^{a}	11.53±0.06^{c}	14.61±0.03^{d}	6.68±0.91^{a}

GC-guar gum as control; GMW-25, GMW-50, GMW-75, GMW-100-microwave assisted extract at 25, 50, 75 and 100% replacement, respectively; GUL-25, GUL-50, GUL-75, GUL-100-ultrasound assisted extract at 25, 50,75 and 100% replacement, respectively; GMSC-glyceryl monostearate as control; EMW-50, EMW-100- microwave assisted extract at 50, and 100% replacement, respectively; EUL-50, EUL-100-ultrasound assisted extract at 50 and 100% replacement, respectively.

The colour values in case of samples formulated with GMS showed increased L* values in all samples replaced with extracts by microwave and ultrasound assistance (both ice cream and ice cream mix). Overall colour change values consistently increased in case of mix formulated with extracts prepared by microwave assistance and inconsistency was observed in samples formulated with extracts prepared by ultrasound assistance. For the hardened ice cream samples, a consistent change in overall colour values with increased proportion of extracts (prepared with microwave and ultrasound assistance) was noted.

4.8.8. Effect of replacement of emulsifier in ice cream mix with the extract on Sensory quality of ice cream

Sensory analysis of ice cream with six attributes and total acceptability was performed and the results are tabulated in Table 4.33. All sensory attributes evaluated for different ice cream samples showed no-significant difference ($p<0.05$). With increase in proportion of the extract in the formulation, ice cream samples scored lower for attributes like structural consistency and icy structure. Overall acceptability of samples also showed decreasing score with increasing proportion of the extract added as a replacement for the conventional emulsifier in the ice cream formulation. Colour and appearance did not show any significant change across samples studied. Another important attribute, melt in mouth also did not report much change across different experimental samples studied. Atallah *et al*., (2022) evaluated with different sweeteners in ice cream formulation and did not report any influence of the change in ice cream formulation during the subjective evaluation for similar attributes.

Table. 4.33. Sensory scores on 9-point hedonic scale of ice cream samples formulated using extract from ghee residue in different proportions

Sample code	Sensory attributes						
	CA	SC	TO	IS	MM	GS	TA
GC	8.01±0.58	8.23±0.41	8.01±0.58	8.01±0.58	7.86±0.38	7.73±0.50	7.87±0.39
GMW-25	7.57±0.53	7.00±1.00	7.14±1.21	7.29±0.76	7.43±0.53	7.43±0.53	7.71±0.49
GMW-50	7.86±0.38	7.57±0.53	7.29±0.49	7.29±0.76	7.43±0.79	7.57±0.53	7.57±0.53
GMW-75	7.80±0.38	7.23±0.71	7.23±0.71	6.66±1.19	7.37±0.95	7.51±0.76	7.23±0.71
GMW-100	7.14±0.69	6.71±0.49	7.43±0.79	6.71±0.49	7.43±0.53	7.00±0.58	7.00±0.82
GUL-25	7.66±1.04	7.71±0.76	7.86±0.69	7.43±0.53	7.80±0.59	8.14±0.38	7.86±0.69
GUL-50	7.71±0.49	7.29±0.95	7.00±1.15	7.57±0.79	7.00±0.82	7.29±0.76	7.57±0.79
GUL-75	7.86±0.38	7.00±1.15	7.14±1.21	7.14±1.21	7.29±0.76	7.29±0.95	7.29±0.76
GUL-100	7.29±0.49	7.29±0.76	7.00±0.58	7.14±0.69	7.14±0.69	7.14±0.69	7.14±0.69
GMSC	8.00±0.00	7.86±0.38	8.14±0.38	7.57±0.79	7.57±0.79	7.86±0.38	7.86±0.38
EMW-50	7.66±0.47	7.51±0.50	7.23±0.91	6.51±2.54	6.66±2.53	7.37±0.75	7.51±0.76
EMW-100	7.71±0.76	7.86±0.69	7.43±0.95	7.29±0.98	7.43±0.95	7.43±0.98	7.43±0.98
EUL-50	7.80±0.59	7.43±0.53	7.86±0.38	7.71±0.49	7.94±0.47	8.14±0.69	7.86±0.38
EUL-100	7.86±0.69	7.43±0.98	7.57±0.98	7.14±1.07	7.71±0.49	7.71±0.76	7.57±0.53

CA-Colour and appearance; SC-Structure and consistency; TO-Taste and odour; IS-Icy structure; MM-Melt in mouth; GS-Gummy structure; TA-Total acceptability

GC-guar gum as control; GMW-25, GMW-50, GMW-75, GMW-100-microwave assisted extract at 25, 50, 75 and 100% replacement, respectively; GUL-25, GUL-50, GUL-75, GUL-100-ultrasound assisted extract at 25, 50,75 and 100% replacement, respectively; GMSC-glyceryl monostearate as control; EMW-50, EMW-100- microwave assisted extract at 50, and 100% replacement, respectively; EUL-50, EUL-100-ultrasound assisted extract at 50 and 100% replacement, respectively.

Chapter-5

SUMMARY AND CONCLUSION

5.0 SUMMARY AND CONCLUSION

Milk lipids are macrostructures of globules with major constituent of triglycerides having different melting points and covered by three layers of milk fat globular membrane (MFGM). Major polar lipids present in globular layer are milk phospholipids. Phospholipids (PLs) are constituents of biological membrane, known for their amphiphilic property due to the presence of hydrophobic tail and hydrophilic head. Egg yolk, vegetable oils, milk, fish, meat, brain and some nuts were reported to be rich in PLs. Milk PLs are broadly classified into two major groups namely, the glycerol containing phospholipids, commonly referred to glycerophospholipids and sphingophospholipids. High content of sphingomyelin (24%) and phosphatidylserine (12%) were reported in dairy products which was found to be less or absent in vegetable source or egg. Due to its amphiphilic property, MPLs also exhibit technical functionalities such as emulsifiers, surfactants and foaming agent.

Ghee residue is a dark brown residue obtained as sediment after melting and straining of butter or cream. Conservative estimates indicate that roughly 30-35% of milk produced in India is converted to ghee. Presently, ghee residue is often discarded as waste causing environmental concern or used as animal feed by most of the ghee manufacturing dairy plants.Hence, there is potential to use ghee residue as a valuable source of PLs after employing suitable processes to eliminate neutral lipids and proteins from the matrix.

Since the fat rich component of the target matrix of the study is encased within the MFGM, any process technology that causes disruption or destabilization of MFGM is expected to enhance extraction efficiency. It is with this hypothesis that the study was envisaged to investigate the extraction using microwave (MW),ultrasonication (UL) and pulsed electric field (PEF) assisted extraction from the ghee residue matrix. Further, from an engineering point of view, scale up of extraction techniques requires understanding of process parameters. Hence, empirical models such as parabolic model, power law model, Peleg's model and Elovoich's model were used to describe the kinetics of extraction of target material using experimental data.

There is growing demand for green emulsifiers to improve value addition and marketability of processed food products.Study of emulsion properties and droplet characteristics in terms of emulsion stability, hydrophilic–lipophilic balance value would aid in measuring its influence on physico-chemical and sensory properties of the end-use product.

In light of the above points, the present study was proposed with the following specific objectives.

1. Optimization of process parameters for extraction of phospholipids from ghee residue using pulsed electric field, microwave and ultrasonication - assisted techniques

2. Modelling of extraction kinetics of phospholipids from ghee residue using pulsed electric field, microwave and ultrasonication-assisted treatments

3. Characterization of the extracted phospholipid rich fraction for compositional and techno-functional properties

To achieve the envisaged objectives, experiments were planned and conducted as follows.

- Initially, preliminary studies were carried out to enrich the PLs content in the ghee residue. The approaches attempted included such as hydraulic pressing, solvent treatments to remove neutral lipids and size comminution. Based on the evaluation, a protocol for pre-treatment of ghee residue (before extraction) was finalized.
- Studies were conducted to select machine and process parameters influencing the extraction process for each of the three assisted extraction techniques considered in the study. Based on preliminary trials using the "one factor at a time" approach, the range of process variable for each of the selected parameter was identified and experiments were designed based on Taguchi orthogonal array. The data was independently analysed for maximizing the yield of PLs in the extract as well as its antioxidant activity and the optimal combination of parameters obtained was validated for its accuracy.
- The kinetics of the extraction process for microwave, ultrasound and pulsed electric field assisted techniques was studied. The experimental data was fitted to parabolic model, power law model, Peleg's model and Elovoich's model and the best-fit model was identified based on R^2 values of the fit.
- The pre – treated ghee residue sample (before extraction), the obtained extract and sediment after extraction for each of the three assisted extraction techniques were analysed for its proximate composition in terms of lipids, protein, lactose, ash and moisturecontent using standard analytical techniques.

- The extract obtained from each of the three assisted extraction techniques was subjected to a detailed lipidomic profiling using LC-MS to identify the classes and species present in the extract.
- The extract obtained from ghee residue under optimized protocols for all assisted extraction techniques were formulated in to suitable emulsions and characterized for its emulsion capacity and stability. The emulsions were also evaluated in terms of its particle size and zeta potential, microscopic evaluation and stability under centrifugal stress, change in pH, acidity and electrical conductivityunder refrigerated storage for 30 days.
- The extract obtained from ghee residue under optimized protocols for all assisted extraction techniques were formulatedin to emulsions with hydrophilic-lipophilic balance (HLB) value ranging from a value of 6 to 15 and evaluated using particle size analysis during extended storage and creaming index under refrigerated storage to identify the potential HLB value of the extracts.
- Based on the above analysis and the HLB identified, experiments were designed to tests the efficacy of using the extract obtained from ghee residue under optimized protocols forMW and UL assisted extraction techniques as a replacement for guar gum and glyceryl monostearate (GMS) in ice cream mix. The effect of the replacement in varying proportions was assessed based on the quality attributes and acceptability of ice cream mix and hardened ice cream.

The salient finding of the present study is as summarized below.

- Among the methods for preparation of ghee, even though overall yield of ghee residue was higher in direct cream method (13.56%) compared to creamery butter method (5.16%), the concentration of phospholipids in freshly prepared ghee residue was significantly higher in the latter method (4.98%) when compared to direct cream method (0.95%).
- Sequential processing during pre-treatment of the ghee residue using hydraulic pressing (5 kg/cm^2 for 5 min.), treatment in boiling water (S:S ratio 2:1) and comminution to 0.25mm particle size led to enrichment of PLs content in the ghee residue from 4.98% to 9.56% after pre-treatment.

- Water was found to be a better solvent than organic solvents for extracting PLs from ghee residue using the assisted extraction techniques.
- Based on the "one factor at a time" approach the independent factors influencing microwave assisted extraction of PL rich extract from ghee residue were identified as power (180, 360, 540W), time (40, 50, 60s) and solvent to substrate (S:S) ratio (5, 7.5, 10). Similarly, the parameters and its respective ranges identified for ultrasound assisted extraction were power intensity (60, 70, 80%), time (3, 4, 5s), solvent temperature (60, 70, 80^{o}C) and solvent to substrate ratio (10, 12.5, 15). The corresponding factors identified for the pulsed electric field assisted technique were voltage (40, 50, 60 kV/cm), time (3, 4, 5 min.) and solvent to substrate ratio (7.5, 10, 12.5).
- The optimised process conditions for microwave assisted extraction were power (540W), time (60 s), S:S ratio of (10 v/w), which resulted in 21.96% PLs in the extract. The extract obtained with microwave assisted extraction exhibited antioxidant activity of 29.89% at optimal conditionspower (180 W), time (40 s), S:S ratio of (10 v/w)
- Ultrasound assisted extraction was optimized at power intensity (80%), time (4 min.), solvent temperature (80^{o}C) and S:S ratio of (15 v/w)yielding a concentration of 24.12% of PLs in the extract. Maximum antioxidant activity (51.94%) in the extract was optimized at power intensity (80%), time (3 min.), solvent temperature (60^{o}C) and S:S ratio of (15 v/w). Ultrasound assisted extraction was found to result in the maximum yield of PLs in the extract among the three assisted techniques evaluated.
- Pulsed electric field treatment resulted in 18.14% of PLs in the extract at optimal condition of voltage (60 kV/cm), time (5min.) and S:S ratio (7.5 v/w). Extract exhibited highest antioxidant activity (37.01%) at levels optimized for its maximization.
- Micrographs of the ghee residue sediments obtained after microwave, ultrasound and PEF assisted extraction treatment at optimal combination of the process parameters revealed fragmentations in the order microwave>ultrasound>PEF treatment.
- Comparative estimation of phospholipids in the extract using spectrophotometric method and solid phase extraction technique using Ferrari and Cheng methods of elution, revealed significant overestimation of PLs in the former method, with the

content of PLs using spectrophotometric method being almost double of that estimated by solid phase extraction technique.

- Kinetics of extraction in the MW assisted technique followed Peleg's model whereas, ultrasound and PEF assisted extraction process was best described by the parabolic model. Extraction kinetics for antioxidant activity of all assisted extraction techniques was best described using the Peleg's model.
- Proximate analysis of ghee residue extract indicated enrichment of lipids content in extract with a corresponding depletion in the sediment fraction. The sediment obtained after the extraction contained significant content of proteins.
- The lipidomic profiling of the extract using LC – MS technique revealed the presence of 61, 118 and 31 species across 5 classes of PLs in the extract obtained by MW, UL and PEF assisted process, respectively. The 5 main classes of PLs present in the extract were phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylglycerol (PG).
- The extract was found to possess satisfactory emulsion capacity and stability characteristics when formulated with 5% of the extract obtained using microwave and ultrasound assistance. However, the extract obtained through PEF assistanceexhibitedsimilar emulsion capacity and stability when formulated with 10% of extract. The latter extract was deemed to be weaker among the three extracts for its emulsification characteristics.
- Detailed characterization of the extracts in terms of analysis of formulated emulsion for its particle size and stability revealed the HLB value of the extract to range between 10 -12. Evaluation of average particle diameter of the extract over extended storage period of 60 days narrowed the HLB value of extract obtained from all three assisted techniques to close to 10. Based on HLB values, it was deduced that extract belonged to the category of oil in water emulsion.
- Effect of proportional replacement of conventional emulsifiers guar gum and glyceryl monostearate (GMS) with extracts obtained through microwave and ultrasound assistance on textural and physico-chemical attributes of ice cream mix revealed its efficacy at a replacement level of up to 50% of conventional extract in both guar gum and GMS. Among the conventional emulsifiers, GMS was deduced to be better candidate for replacement with the two extracts.

- Proportional replacement of conventional emulsifiers guar gum and glyceryl monostearate (GMS) with extracts obtained through microwave and ultrasound assistance did not alter the overrun, melting characteristics as well as objective and subjective quality attributes of hardened ice cream, when the replacement was within 50%.

The study demonstrated the efficacy of obtaining a PLs rich extract from ghee residue using assisted extraction techniques and the scope of utilizing the extract as replacement for conventional emulsifier in dairy products such as ice cream.

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APPENDIX

Appendix I

A1. Phosphatidylcholine species identified in ghee residue extract obtained from microwave assistance

Class	Formula	Isomer I	Isomer-2	Mass (µg)	Molar mass
PC 26:0	$C_{35}H_{71}NPO_9$	C16:0/C10:0		0.012	680.49
PC 29:0	$C_{38}H_{75}NPO_{10}$	C15:0/C14:0		0.003	736.51
PC 30:0	$C_{38}H_{76}NPNaO_8$	C16:0/C14:0		0.093	728.52
PC 31:1	$C_{39}H_{77}NPO_8$	C19:0/C12:1		0.042	718.54
PC 31:0	$C_{39}H_{79}NPO_8$	C25:0/C6:0		0.189	720.55
PC 31:0	$C_{40}H_{79}NPO_{10}$	C15:0/C16:0		0.018	764.54
PC 32:2	$C_{40}H_{77}NPO_8$	C14:0/C18:2		0.053	730.54
PC 32:1	$C_{41}H_{79}NPO_{10}$	C18:1/C14:0	C16:0/C16:1	0.098	776.54
PC 32:3	$C_{40}H_{75}NPO_8$	C20:1/C12:2		0.093	728.52
PC 32:0	$C_{40}H_{80}NPNaO_8$	C16:0/C16:0		0.088	756.55
PC 33:2	$C_{42}H_{79}NPO_{10}$	C15:0/C18:2		0.002	788.54
PC 33:1	$C_{42}H_{81}NPO_{10}$	C15:0/C18:1	C17:1/C16:0	0.013	790.56
PC 33:0	$C_{42}H_{83}NPO_{10}$	C16:0/C17:0	C18:0/C15:0	0.023	792.58
PC 34:3	$C_{43}H_{79}NPO_{10}$	C16:0/C18:3		0.004	800.54
PC 34:3	$C_{42}H_{79}NPO_8$	C22:1/C12:2		0.051	756.55
PC 34:1	$C_{42}H_{82}NPNaO_8$	C16:0/C18:1	C23:0/C11:1	0.461	782.57
PC 34:1	$C_{42}H_{83}NPO_8$	C18:0/C16:1	C19:1/C15:0	12.368	760.58
PC 34:2	$C_{43}H_{81}NPO_{10}$	C16:0/C18:2		0.004	802.56
PC 34:2	$C_{43}H_{81}NPO_{10}$	C16:1/C18:1	C23:0/C11:2	0.012	802.56
PC 35:2	$C_{43}H_{83}NPO_8$	C24:1/C11:1		0.093	772.58
PC 35:2	$C_{44}H_{83}NPO_{10}$	C17:1/C18:1	C17:0/C18:2	0.007	816.58
PC 35:1	$C_{44}H_{85}NPO_{10}$	C17:0/C18:1		0.010	818.59
PC 36:4	$C_{45}H_{81}NPO_{10}$	C18:2/C18:2	C18:1/C18:3	0.019	826.56
PC 36:3	$C_{44}H_{82}NPNaO_8$	C26:0/C10:3		0.027	806.57
PC 36:3	$C_{45}H_{83}NPO_{10}$	C18:1/C18:2	C18:0/C18:3	0.032	828.58
PC 36:2	$C_{44}H_{84}NPNaO_8$	C18:1/C18:1	C26:1/C10:1	0.184	808.58
PC 36:2	$C_{45}H_{85}NPO_{10}$	C18:0/C18:2		0.064	830.59
PC 38:5	$C_{47}H_{83}NPO_{10}$	C18:1/C20:4		0.006	852.58
PC 38:4	$C_{47}H_{85}NPO_{10}$	C18:1/C20:3	C27:0/C11:4	0.008	854.59
PC 38:6	$C_{47}H_{81}NPO_{10}$	C18:1/C20:5		0.051	850.56
PC 38:3	$C_{47}H_{87}NPO_{10}$	C18:0/C20:3		0.002	856.61

A2. Phosphatidylethanolamine species identified in ghee residue extract obtained from microwave assistance

Class	Formula	Isomer I	Isomer-2	Isomer-3	Isomer-4	Mass (µg)	Molar mass
PE 32:0	$C_{37}H_{75}NPO_8$	C16:0/C16:0				0.001	692.522
PE 32:1	$C_{37}H_{71}NPO_8$	C18:1/C14:0				0.000	688.491
PE 34:2	$C_{39}H_{75}NPO_8$	C16:0/C18:2				0.001	716.522
PE 34:2	$C_{39}H_{73}NPO_8$	C16:1/C18:1				0.000	714.508
PE 34:1	$C_{39}H_{77}NPO_8$	C16:0/C18:1	C18:0/C16:0	C18:0/C16:1	C25:1/C9:0	0.006	718.539
PE 35:1	$C_{40}H_{77}NPO_8$	C17:0/C18:1				0.000	730.540
PE 36:4	$C_{41}H_{73}NPO_8$	C16:0/C20:4				0.000	738.508
PE 36:2	$C_{41}H_{79}NPO_8$	C18:1/C18:1	C18:0/C18:2			0.004	744.554
PE 38:3	$C_{43}H_{79}NPO_8$	C18:0/C20:3				0.000	768.557
PE 38:4	$C_{43}H_{77}NPO_8$	C18:0/C20:4				0.000	766.540
PE 42:4	$C_{47}H_{86}NPNaO_8$	C32:1/C10:3				0.000	846.596

A3. Phosphatidylinositol species identified in ghee residue extract obtained from microwave assistance

Class	Formula	Isomer I	Isomer-2	Isomer-3	Mass (µg)	Molar mass
PI 32:0	$C_{41}H_{78}PO_{13}$	C16:0/C16:0			0.01	809.52
PI 32:1	$C_{41}H_{76}PO_{13}$	C16:0/C16:1			0.00	807.50
PI 34:1	$C_{43}H_{85}NPO_{13}$	C16:0/C18:1			0.03	854.57
PI 34:1	$C_{43}H_{80}PO_{13}$	C25:1/C9:0			0.01	835.53
PI 34:2	$C_{43}H_{78}PO_{13}$	C16:0/C18:2	C16:1/C18:1		0.01	833.52
PI 34:2	$C_{43}H_{83}NPO_{13}$	C17:1/C17:1	C20:1/C14:1	C23:1/C11:1	0.12	852.56
PI 36:2	$C_{45}H_{84}PO_{13}$	C12:0/C24:2			0.04	863.56
PI 36:4	$C_{45}H_{83}NPO_{13}$	C16:0/C20:4			0.01	876.56
PI 36:2	$C_{45}H_{82}PO_{13}$	C18:0/C18:2			0.10	861.55
PI 36:2	$C_{45}H_{84}PO_{13}$	C18:1/C18:1			0.02	863.56
PI 36:3	$C_{45}H_{80}PO_{13}$	C18:1/C18:2			0.01	859.53
PI 38:3	$C_{47}H_{84}PO_{13}$	C18:0/C20:3			0.04	887.57
PI 38:4	$C_{47}H_{87}NPO_{13}$	C18:0/C20:4			0.07	904.59
PI 38:5	$C_{47}H_{80}PO_{13}$	C18:0/C20:5			0.00	883.53
PI 38:3	$C_{47}H_{89}NPO_{13}$	C18:1/C20:2			0.14	906.61
PI 38:5	$C_{47}H_{85}NPO_{13}$	C18:1/C20:4			0.01	902.58

A4. Phosphatidylserine species identified in ghee residue extract obtained from microwave assistance

Class	Formula	Isomer I	Mass (µg)	Molar mass
PS 36:2	$C_{42}H_{77}NPO_{10}$	C18:0/C18:2	0.000	786.52
PS 36:2	$C_{42}H_{77}NPO_{10}$	C18:1/C18:1	0.000	786.52

Appendix II

B1. Phosphatidylcholine species identified in ghee residue extract obtained from ultrasound assistance

Class	Formula	Isomer I	Isomer-2	Mass (µg)	Molar mass
PC 29:1	$C_{38}H_{73}NPO_{10}$	C15:0/C14:1		0.00	734.50
PC 30:0	$C_{39}H_{77}NPO_{10}$	C15:0/C15:0		0.00	750.53
PC 30:0	$C_{38}H_{77}NPO_{8}$	C16:0/C14:0		0.15	706.54
PC 31:0	$C_{39}H_{78}NPNaO_{8}$	C15:0/16:0		0.00	742.54
PC 31:0	$C_{39}H_{79}NPO_{8}$	C15:0/16:0		0.01	720.55
PC 32:4	$C_{41}H_{64}NPO_{10}D_{7}$	C18:0/C14:4		0.00	775.52
PC 32:1	$C_{40}H_{79}NPO_{8}$	C16:0/C16:1		0.07	732.55
PC 32:3	$C_{40}H_{75}NPO_{8}$	C12:0/C20:3		0.00	728.52
PC 32:1	$C_{41}H_{79}NPO_{10}$	C18:1/C14:0		0.00	776.54
PC 32:4	$C_{41}H_{66}NPO_{10}D_{7}$	C18:1/C14:3		0.00	777.54
PC 32:1	$C_{40}H_{78}NPNaO_{8}$	C9:0/C23:1		0.00	754.54
PC 32:0	$C_{40}H_{72}O_{8}D_{7}$	C18:0/C14:0		0.00	739.60
PC 33:2	$C_{41}H_{79}NPO_{8}$	C15:0/C18:2		0.00	744.55
PC 33:4	$C_{41}H_{75}NPO_{8}$	C11:0/C22:4		0.00	740.52
PC 33:1	$C_{41}H_{76}NPO_{8}D_{5}$	C16:1/C17:0		0.00	751.60
PC 33:1	$C_{41}H_{81}NPO_{8}$	C15:0/C18:1		0.01	746.57
PC 33:1	$C_{42}H_{81}NPO_{10}$	C17:1/C16:0		0.00	790.56
PC 33:0	$C_{41}H_{83}NPO_{8}$	C16:0/C17:0		0.01	748.58
PC 33:0	$C_{42}H_{83}NPO_{10}$	C18:0/C15:0		0.00	792.58
PC 34:3	$C_{42}H_{79}NPO_{8}$	C16:1/C18:2	C20:2/C14:1	0.01	756.55
PC 34:3	$C_{43}H_{79}NPO_{10}$	C16:0/C18:3		0.00	800.54
PC 34:2	$C_{42}H_{81}NPO_{8}$	C24:0/C10:2	C16:0/C18:2	0.01	758.57
PC 34:2	$C_{43}H_{81}NPO_{10}$	C16:1/C18:1		0.00	802.56
PC 34:1	$C_{42}H_{83}NPO_{8}$	C16:0/C18:1	C19:1/C15:0	0.41	760.58
PC 34:1	$C_{42}H_{82}NPNaO_{8}$	C25:1/C9:0		0.01	782.57
PC 34:1	$C_{42}H_{85}NPO_{7}$	C16:0/C18:1		0.00	746.61
PC 35:3	$C_{43}H_{81}NPO_{8}$	C17:1/C18:2		0.00	770.57
PC 35:0	$C_{43}H_{87}NPO_{7}$	C16:0/C19:0		0.01	760.63
PC 35:2	$C_{44}H_{83}NPO_{10}$	C17:1/C18:1	C17:0/C18:2	0.00	816.58
PC 35:2	$C_{43}H_{83}NPO_{8}$	C17:0/C18:2	C11:0/C24:2	0.01	772.58
PC 35:1	$C_{43}H_{85}NPO_{8}$	C16:1/C19:0		0.01	774.60
PC 35:1	$C_{44}H_{85}NPO_{10}$	C17:0/C18:1		0.00	818.59
PC 36:4	$C_{45}H_{81}NPO_{10}$	C18:1/C18:3		0.00	826.56

PC 36:6	$C_{45}H_{77}NPO_{10}$	C18:4/C18:2		0.00	822.53
PC 36:4	$C_{44}H_{81}NPO_8$	C16:0/C20:4	C22:1/C14:3	0.02	782.57
PC 36:3	$C_{44}H_{83}NPO_8$	C18:1/C18:2		0.01	784.58
PC 36:3	$C_{45}H_{83}NPO_{10}$	C16:0/C20:3		0.00	828.58
PC 36:5	$C_{45}H_{79}NPO_{10}$	C18:4/C18:1		0.00	824.54
PC 36:2	$C_{44}H_{85}NPO_8$	C18:0/C18:2		0.10	786.60
PC 36:2	$C_{45}H_{85}NPO_{10}$	C18:1/C18:1		0.00	830.59
PC 36:2	$C_{44}H_{84}NPNaO_8$	C25:0/C11:2		0.00	808.58
PC 36:0	$C_{40}H_{81}NPO_8$	C16:0/C16:0		0.00	734.57
PC 37:2	$C_{46}H_{87}NO_{10}$	C19:1/C18:1		0.00	844.61
PC 37:2	$C_{45}H_{87}NPO_8$	C19:0/C18:2		0.00	800.62
PC 38:5	$C_{46}H_{79}NPO_8$	C18:4/C20:3		0.00	804.55
PC 38:5	$C_{47}H_{83}NPO_{10}$	C20:3/C18:2		0.00	852.58
PC 38:5	$C_{46}H_{83}NPO_8$	C18:1/C20:4		0.00	808.58
PC 38:7	$C_{47}H_{79}NPO_{10}$	C20:5/C18:2		0.00	848.54
PC 38:4	$C_{47}H_{85}NPO_{10}$	C18:1/C20:3		0.00	854.59
PC 38:6	$C_{47}H_{81}NPO_{10}$	C18:1/C20:5	C18:2/C20:4	0.00	850.56
PC 38:4	$C_{46}H_{85}NPO_8$	C18:0/C20:4		0.00	810.60
PC 38:3	$C_{47}H_{87}NPO_{10}$	C18:1/C20:2	C18:0/C20:3	0.00	856.61
PC 38:3	$C_{46}H_{87}NPO_8$	C20:1/C18:2		0.00	812.62
PC 39:7	$C_{48}H_{81}NPO_{10}$	C17:1/C22:6		0.00	862.55
PC 40:5	$C_{49}H_{87}NPO_{10}$	C18:0/C22:5		0.00	880.61
PC 40:5	$C_{48}H_{87}NPO_8$	C20:1/C20:4		0.00	836.62
PC 40:8	$C_{48}H_{81}NPO_8$	C18:3/C22:5		0.00	830.58
PC 40:6	$C_{48}H_{85}NPO_8$	C18:0/C22:6		0.00	834.60
PC 42:2	$C_{50}H_{99}NPO_7$	C18:0/C24:2		0.00	856.71
PC 43:2	$C_{51}H_{101}NPO_7$	C20:2/C23:0		0.00	870.73
PC 44:2	$C_{52}H_{103}NPO_7$	C20:0/C24:2		0.00	884.74

B2. Phosphatidylethanolamine species identified in ghee residue extract obtained from ultrasound assistance

Class	Formula	Isomer I	Isomer-2	Isomer-3	Mass (µg)	Molar mass
PE 30:0	$C_{35}H_{69}NPO_8$	C16:0/C14:0			0.00	662.48
PE 32:1	$C_{37}H_{71}NPO_8$	C16:0/C16:1			0.00	688.49
PE 32:1	$C_{37}H_{73}NPO_8$	C18:1/C14:0	C16:0/C16:0		0.00	690.51
PE 32:0	$C_{37}H_{75}NPO_8$	C16:0/C16:0	C18:0/C14:0		0.01	692.52
PE 33:1	$C_{38}H_{73}NPO_8$	C15:0/C18:1			0.00	702.51

PE 34:2	$C_{39}H_{73}NPO_8$	C16:1/C18:1			0.00	714.51
PE 34:2	$C_{39}H_{75}NPO_8$	C16:0/C18:2			0.01	716.52
PE 34:0	$C_{39}H_{78}NPNaO_8$	C18:0/C16:0			0.00	742.54
PE 34:1	$C_{39}H_{76}NPNaO_8$	C11:0/C23:1			0.00	740.52
PE 34:1	$C_{39}H_{77}NPO_8$	C16:0/C18:1	C18:0/C16:1		0.04	718.54
PE 35:2	$C_{40}H_{75}NPO_8$	C17:1/C18:1	C17:0/C18:2		0.00	728.52
PE 35:1	$C_{40}H_{79}NPO_8$	C17:0/C18:1			0.00	732.55
PE 36:4	$C_{41}H_{73}NPO_8$	C18:2/C18:2	C18:1/C18:3	C16:0/C20:4	0.00	738.51
PE 36:3	$C_{41}H_{77}NPO_8$	C18:1/C18:2			0.01	742.54
PE 36:2	$C_{41}H_{79}NPO_8$	C18:1/C18:1	C18:0/C18:2		0.06	744.55
PE 38:4	$C_{43}H_{77}NPO_8$	C18:1/C20:3			0.00	766.54
PE 38:4	$C_{43}H_{79}NPO_8$	C18:0/C20:4	C18:0/C20:3		0.00	768.56
PE 40:5	$C_{45}H_{79}NPO_8$	C18:0/C22:5			0.00	792.56

B3. Phosphatidylinositol species identified in ghee residue extract obtained from ultrasound assistance

Class	Formula	Isomer I	Isomer-2	Mass (µg)	Molar mass
PI 30:0	$C_{39}H_{74}PO_{13}$	C16:0/C14:0	C18:0/C12:0	0.00	781.49
PI 32:1	$C_{41}H_{76}PO_{13}$	C18:1/C14:0	C16:0/C16:1	0.01	807.50
PI 32:0	$C_{41}H_{78}PO_{13}$	C16:0/C16:0		0.01	809.52
PI 33:1	$C_{42}H_{78}PO_{13}$	C15:0/C18:1		0.00	821.52
PI 33:0	$C_{42}H_{80}PO_{13}$	C18:0/C15:0		0.00	823.53
PI 34:2	$C_{43}H_{78}PO_{13}$	C16:1/C18:1	C16:0/C18:2	0.01	833.52
PI 34:2	$C_{43}H_{83}NPO_{13}$	C17:1/C17:1	C20:1/C14:1	0.02	852.56
PI 34:1	$C_{43}H_{82}PO_{13}$	C16:0/C18:1		0.01	837.55
PI 36:4	$C_{45}H_{83}NPO_{13}$	C16:0/C20:4		0.01	876.56
PI 36:3	$C_{45}H_{80}PO_{13}$	C18:0/C18:3		0.00	859.53
PI 36:3	$C_{45}H_{85}NPO_{13}$	C18:1/C18:2	C16:0/C20:3	0.02	878.57
PI 36:2	$C_{45}H_{84}PO_{13}$	C18:1/C18:1	C18:0/C18:2	0.03	863.56
PI 36:2	$C_{45}H_{82}PO_{13}$	C18:1/C18:1	C18:0/C18:2	0.10	861.55
PI 36:1	$C_{45}H_{85}NPNaO_{13}$	C25:1/C11:0		0.01	887.56
PI 38:5	$C_{47}H_{85}NPO_{13}$	C18:1/C20:4		0.01	902.58
PI 38:5	$C_{47}H_{80}NPO_{13}$	C18:0/C20:5		0.00	883.53
PI 38:4	$C_{47}H_{82}PO_{13}$	C16:0/C22:4	C18:1/C20:3	0.00	885.55
PI 38:4	$C_{47}H_{87}NPO_{13}$	C18:0/C20:4		0.03	904.59
PI 38:3	$C_{47}H_{89}NPO_{13}$	C18:0/C20:3		0.10	906.61

B4. Phosphatidylserine species identified in ghee residue extract obtained from ultrasound assistance

Class	Formula	Isomer I	Isomer-2	Mass (µg)	Molar mass
PS 29:0	$C_{35}H_{71}NPO_9$	C8:0/C21:0		0.09	680.48
PS 34:1	$C_{40}H_{75}NPO_{10}$	C16:0/C18:1		0.00	760.51
PS 34:2	$C_{40}H_{73}NPO_{10}$	C16:0/C18:2		0.00	758.50
PS 35:1	$C_{41}H_{77}NPO_{10}$	C19:1/C16:0		0.00	774.53
PS 36:3	$C_{42}H_{75}NPO_{10}$	C18:1/C18:2	C18:0/C18:3	0.00	784.51
PS 36:14	$C_{42}H_{73}NPO_{10}$	C18:12/C18:2		0.00	782.49
PS 36:2	$C_{42}H_{79}NPO_{10}$	C18:1/C18:1	C18:0/C18:2	0.01	788.54
PS 36:3	$C_{42}H_{77}NPO_9$	C18:2/C18:1		0.00	770.53
PS 36:1	$C_{42}H_{81}NPO_{10}$	C18:0/C18:1		0.02	790.56
PS 37:2	$C_{43}H_{79}NPO_{10}$	C19:1/C18:1		0.00	800.54
PS 38:5	$C_{44}H_{75}NPO_{10}$	C20:3/C18:2		0.00	808.51
PS 38:3	$C_{44}H_{79}NPO_{10}$	C18:0/C20:3		0.00	812.54
PS 38:4	$C_{44}H_{77}NPO_{10}$	C18:1/C20:3		0.00	810.53
PS 40:5	$C_{46}H_{79}NPO_{10}$	C18:0/C22:5		0.00	836.54
PS 40:4	$C_{46}H_{81}NPO_{10}$	C18:0/C22:4		0.00	838.56

Appendix III

C1 Phosphatidylcholine species identified in ghee residue extract obtained from PEF assistance

Class	Formula	Isomer I	Isomer-2	Mass (µg)	Molar mass
PC 16:0	$C_{24}H_{51}NPO_7$	C10:0/C6:0		0.0034	496.3403
PC 18:0	$C_{27}H_{55}NPO_9$	C8:0/C10:0		0.0020	568.3617
PC 18:1	$C_{26}H_{53}NPO_7$	C8:1/C10:0	C8:0/C10:1	0.0044	522.3571
PC 28:0	$C_{36}H_{73}NPO_8$	C6:0/C22:0		0.0024	678.5069
PC 30:0	$C_{39}H_{77}NPO_{10}$	C16:0/C14:0		0.0089	750.5291
PC 32:0	$C_{40}H_{81}NPO_8$	C18:0/C14:0		0.0202	734.5696
PC 32:0	$C_{41}H_{81}NPO_{10}$	C16:0/C16:0		0.0101	778.5612
PC 32:1	$C_{41}H_{79}NPO_{10}$	C18:1/C14:0		0.0019	776.5444
PC 34:2	$C_{43}H_{81}NPO_{10}$	C16:0/C18:2		0.0060	802.5611
PC 34:1	$C_{43}H_{83}NPO_{10}$	C16:0/C18:1		0.0247	804.5771
PC 34:0	$C_{43}H_{85}NPO_{10}$	C18:0/C16:0		0.0034	806.5920
PC 34:2	$C_{42}H_{81}NPO_8$	C20:1/C14:1		0.0115	758.5688
PC 34:1	$C_{42}H_{83}NPO_8$	C19:1/C15:0		0.0526	760.5856
PC 36:2	$C_{45}H_{85}NPO_{10}$	C18:1/C18:1		0.0091	830.5931
PC 36:1	$C_{45}H_{87}NPO_{10}$	C18:0/C18:1		0.0008	832.6081
PC 36:3	$C_{44}H_{83}NPO_8$	C22:2/C14:1	C25:0/C11:3	0.0138	784.5825

C2. Phosphatidylethanolamine species identified in ghee residue extract obtained from PEF assistance

Class	Formula	Isomer-I	Isomer-2	Isomer-3	Isomer-4	Isomer-5	Isomer-6	Mass (µg)	Molar mass
PE 34:3	$C_{39}H_{74}NPNaO_7$	C14:1/C20:2	C20:3/C14:0	C20:1/C14:2	C18:3/C16:0	C10:1/C24:2	C16:0/C18:3	0.0328	722.5061
PE 36:1	$C_{41}H_{79}NPO_8$	C18:0/C18:1						0.0001	744.5554
PE 36:2	$C_{41}H_{77}NPO_8$	C18:1/C18:1						0.0001	742.5401
PE 36:5	$C_{41}H_{73}NPO_7$	C16:1/C20:4	C18:2/C18:3					0.0077	722.5070
PE 36:2	$C_{41}H_{78}NPNaO_8$	C25:1/C11:1	C17:1/C19:1	C26:0/C10:2	C18:0/C18:2	C24:0/C12:2	C24:1/C12:1	0.0168	766.5321
PE 37:2	$C_{42}H_{80}NPNaO_8$	C24:2/C13:0						0.0004	780.5479
PE 38:5	$C_{43}H_{77}NPO_8$	C18:3/C20:2	C27:1/C11:4	C18:4/C20:1				0.0112	766.5323
PE 40:4	$C_{45}H_{82}NPNaO_8$	C22:2/C18:2						0.0005	818.5653

C3. Phosphatidylinositol species identified in ghee residue extract obtained from PEF assistance

Class	Formula	Isomer I	Isomer 2	Mass (µg)	Molar mass
PI 36:2	$C_{45}H_{82}PO_{13}$	C18:1/C18:1	C18:0/C18:2	0.002	861.55
PI 36:1	$C_{45}H_{84}PO_{13}$	C18:0/C18:1		0.001	863.56
PI 38:3	$C_{47}H_{84}PO_{13}$	C18:0/C20:3		0.000	887.56
PI 58:1	$C_{67}H_{129}PNaO_{13}$	C34:1/C24:0		0.007	1195.89

C4. Phosphatidylserine species identified in ghee residue extract obtained from PEF assistance

Class	Formula	Isomer I	Mass (µg)	Molar mass
PS 29:0	$C_{35}H_{71}NPO_9$	C8:0/C21:0	0.002	680.48
PS 31:0	$C_{37}H_{75}NPO_9$	C16:0/C15:0	0.000	708.51
PS 36:2	$C_{42}H_{79}NPO_{10}$	C26:1/C10:1	0.000	788.53

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B.Tech.(Agril. Engg)	2003-07	UAS, Bangalore	Agricultural Engineering	85.20

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