Microencapsulation of Flaxseed Oil for Fortification of Milk with Omega- 3 Fatty Acids

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IN

DAIRY TECHNOLOGY

By

Tambade Pramod Bhivasen

B. Tech. (Dairy Technology)

DAIRY TECHNOLOGY SECTION

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CERTIFICATE

This is to certify that the thesis entitled, **"MICROENCAPSULATION OF FLAXSEED OIL FOR FORTIFICATION OF MILK WITH OMEGA- 3 FATTY ACIDS"** submitted by **Mr. Tambade Pramod Bhivasen** towards the partial fulfillment for the award of the degree of **M. Tech.** in **DAIRY TECHNOLOGY** of the **ICAR-NATIONAL DAIRY RESEARCH INSTITUTE**, Karnal (Haryana), India, is a bonafide research work carried out by him under my guidance and no part of the thesis has been submitted for any other degree or diploma.

Dated: 7/7/2018

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DEDICATED TO

MY

RESPECTED GUIDE

AND

BELOVED FAMILY

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ABSTRACT

Milk is often referred as a complete food, but it lacks certain essential fatty acids like omega-3 fatty acids. Omega-3 fatty acids have been associated with reduced risk of several health diseases. Milk is being widely consumed throughout the masses; it would serve as an ideal vehicle for omega-3 fatty acid fortification. Flaxseed oil is one of the richest vegetarian sources of omega-3 fatty acids. However, due to high susceptibility to oxidation, the use of flaxseed oil is limited in food and dairy products. But this problem can be overcome by encapsulating flaxseed oil where a protective coat is formed on oil droplets. Thus in the present study, effect of soy protein isolate (SPI) and modified starches as coating material for flaxseed oil emulsions and microcapsules was evaluated. For emulsion preparation, flaxseed oil level was varied at 25, 30 and 35% of total solids (TS) while TS was maintained at 20, 25 and 30%. Emulsions were prepared by homogenization using high shear mixer at 18000 RPM for 5 minutes. Among all the emulsion samples, the emulsion with 30% oil load and 30% TS was found most stable in terms of low creaming index (2.673%) and highest zeta potential. Emulsion prepared from NC 46 showed narrowest particle size distribution and higher zeta potential (38.5mV) than NC 180 emulsions. All the emulsion samples were subjected to atomization in a spray dryer for preparation of flaxseed oil microcapsules. Maximum microencapsulation efficiency was 95.84% and 90.76%, while average particle size was 37.917µm and 87.307µm for NC 46 and NC 180 microcapsule, respectively. The samples with 30% oil load and 30% TS were selected for storage study and evaluation of selected characteristics. In terms of oxidative stability, the microcapsules were oxidatively stable for 3 months at 4-7°C. Surface morphology illustrate that the microcapsules prepared from NC 46 starch were spherical in shape with smooth surface, while for NC 180 microcapsules, uneven shape was observed. *In-vitro* release study under simulated gastro-intestinal conditions revealed that heating at 85°C/5 min before enzymatic treatment improves the release of oil from the microcapsules. Based upon better sensory acceptability, microcapsule addition to provide 25% RDA of α-linolenic acid (ALA) was selected to fortify milk. Although, the sensory scores of plain fortified milk were non-significantly($p>0.05$) different from that of control milk. However, the fortified milk was also flavoured for wider consumer acceptance especially in children and adolescent group. The pH and viscosity of the fortified flavoured milk samples differ significantly ($p <0.05$) from the control. The fortified pasteurized and sterilized milk were evaluated for pH, acidity, viscosity and sensory characteristics during 6 and 28 days of storage, respectively. There was no yeast and mold, coliform count in the samples during storage. Also, the total bacterial count was within the permissible limits for both the fortified pasteurized and sterilized milk. The moisture, fat, protein, ash and total carbohydrates for fortified sterilized milk and pasteurized milk were 87.30 and 87.23%, 3.42 and 3.37%, 3.57 and 3.62%, 0.85 and 0.79%, 4.85 and 4.99%, respectively. Therefore, it can be concluded that prepared microencapsulated flaxseed oil powder showed excellent storage stability without any off-flavour for the studied storage period, and can be suitably used to fortify milk with omega-3 fatty acids. Three grams of microcapsules would provide 0.612 g of ALA in one serving of milk.

सारांश

दूध को अक्सर पूर्ण आहार कहा जाता है, परन्तु इसमें कुछ आवश्यक तत्वों की कमी भी पाई गई है, जैसे ओमेगा-३ वसीय अम्ल| ओमेगा-३ वसीय आम्ल कई रोगो के जोखिम से बचाने में लाभदायक भूमिका निभाते है | क्यूंकि दूध का उपयोग व्यापक स्तर पर सभी आयु के लोगो द्वारा किया जाता है, अतः यह ओमेगा-३ वसीय अम्ल के संवर्धन का एक का आदर्श जरिया बनने में सक्षम है | अलसी का तेल ओमेगा-३ वसीय अम्ल का सबसे समृद्ध शाकाहारी स्त्रोत है | हालांकि, ऑक्सीकरण के प्रति अतिसंवेदनशीलता के कारण, अलसी के तेल का खाद्य और डेयरी उत्पादों में सीमित उपयोग होता है | लेकिन अलसी के तेल के सूक्ष्मकैप्सूलीकरण के द्वारा इस समस्या को दूर किया जा सकता है | इस अध्ययन में सोया प्रोटीन आइसोलेट और संशोधित स्टार्च के उपयोग के द्वारा अलसी के तेल का इमल्शन और सूक्ष्मकैप्सूलीकरण किया गया | इमल्शन बनाने के लिए अलसी के तेल को कुल ठोस के २५, ३०, और ३५ प्रतिशत स्तर पर और कुल ठोस (टी एस) के स्तर को २०, २५, और ३० प्रतिशत निर्धारित किया गया | हाई शीयर मिक्सर को १८००० आर पी एम पर पांच मिनिट तक चलाकर इमल्शन को होमोज़िनाइज़ किया गया | ३० प्रतिशत टी एस और ३० प्रतिशत तेल भार वाले इमल्शन में सबसे कम क्रिमिंग इंडेक्स (२.६७%) और सबसे अधिक भौतिक स्थिरता पाई गयी | एन सी ४६ स्टार्च से बने इमल्शननो में एन सी १८० स्टार्च की तुलना में अधिक ज़ेटा पोटेंशियल और संकुचित कण आकार वितरण देखा गया | सभी इमल्शनो को सूक्ष्मकैप्सूलीकरण के लिए स्प्रे ड्रायर से सुखाया गया | एन सी ४६ और एन सी १८० के सूक्ष्मकैप्सूलो में सर्वाधिक माइक्रोइनकेप्सूलेशन दक्षता; क्रमशः ९५.८४ और ९०.७६ % पाई गयी और इन नमूनों का औसत कण आकार ३७.९१७ और ८७.३०७ माइक्रोमीटर था | भंडारण अध्ययन और चयनित विशेषताओं के मूल्यांकन हेतु ३० % तेल भार और ३०% टी एस वाले नमूनों को चुना गया | चयनित सूक्ष्मकैप्सूल तीन माह के लिए ४-७° सेल्सियस तापमान पर ऑक्सीडेटिव स्थिरता के आधार पर स्थिर पाए गए |सतह की आकृति अनुसार एन सी ४६ स्टार्च से तैयार सुक्ष्मकैप्सूल गोलाकार एवं हमवार थे | जबकि एन सी १८० स्टार्च सूक्ष्मकैप्सूलो में असमान आकार के साथ कुछ गड्ढे भी पाए गए | सिमुलेटिड आंतो की स्थितियों के तहत इन - विट्रो रिलीज़ अध्ययन से पता चला की एन्ज़ाइमेटिक उपचार से पहले ८५ डिग्री सेल्सियस पर ५ मिनिट तक गरम करने से सूक्ष्मकैप्सूलों में से अधिक तेल का निष्काशन होता है | बेहतर संवेदी स्वीकार्यता के आधार पर दूध के सवंर्धन के लिए सूक्ष्मकैप्सूलो की वह मात्रा मिलाई गयी जिससे दूध में ओमेगा-३ वसीय अम्लों का कम से कम २५ % आर डी ए प्रदान किया जा सके | हालांकि सवंर्धित दूध संवेदी स्वीकार्यता के आधार पर सादे दूध जैसा ही पाया गया परन्तु बच्चो और किशोरों में अधिक स्वीकार्यता हेतु सुगन्धित संवर्धित दूध बनाया गया | सुगन्धित दूध चिपचिपाहट और पि एच के आधार पर सादे दूध से काफी भिन्न पाया गया | सुगन्धित निर्जीवीकृत और विसंक्रमित दूध के विभिन्न भौतिक - रासायनिक एवं स्वेन्दि गुणों की जाँच क्रमशः ६ और २८ दिनों तक की गयी | सुगन्धित निर्जीवीकृत और विसंक्रमित दूध में नमी, वसा, प्रोटीन, कुल खनिज और कुल कार्बोहायड्रेट की मात्रा क्रमशः ८७.३० और ८७.२३ % , ३.४२ और ३.३७, ३.५७ और ३.६२, ०.८५ और ०.७९, ४.८५ और ४.९९ प्रतिशत पाई गयी | २४० मि. ली. संवर्धित दूध में ०.६१२ ग्राम ओमेगा - ३ वसीय अम्ल की मात्रा पाई गयी | अतः इस शोध से यह निष्कर्ष निकला जा सकता है की तैयार अलसी के तेल के सूक्ष्मकैप्सूल अध्ययन भंडारण अवधि के दौरान बिना किसी खराब महक और स्वाद के दूध में ओमेगा - ३ वसीय अम्लों के संवर्धन हेतु उपयोग में लाए जा सकते हैं | तैयार अलसी के तेल के सूक्ष्मकैप्सूलों की तीन ग्राम मात्रा ही ओमेगा - ३ वसीय अम्ल का ३८.२५ % आर डी ए उपलब्ध करवाने के लिए पर्याप्त है |

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List of abbreviations

ALA	Alpha linolenic acid
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists Approx. Approximately
AR	Analytical reagent
cfu	Colony forming unit
CHD	Coronary heart diseases
CLA	Conjugated linoleic acid
cm	Centimetre
Conc.	Concentrated
CVD	Cardiovascular disease
DHA	Docosahexaenoic acid
DW	Distilled water
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid
etc.	Etcetera
EU	European Union
FAME	Fatty acid methyl esters
FAO	Food and Agricultural Organization
FCC	Foods Chemicals Codex
FDA	Food and Drug administration
FFA	Free fatty acids
FID	Flame Ionization Detector
FT-IR	Fourier Transform Infrared Spectrometer
GC-MS	Gas Chromatography Mass Spectroscopy
GLC	Gas Liquid Chromatography
GRAS	Generally Recognized as Safe
h	Hour
IUPAC	International Union of Pure and Applied Chemistry
KDa	Kilodalton
Kg	Kilogram
LA	Lactic acid
LAB	Lactic acid bacteria
LC	Liquid Chromatography
M	Molar
MFOP	Microencapsulated flaxseed oil powder
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimetre

MSNF	Milk solids-not fat
MUFA	Monounsaturated Fatty acid
N	Normality
NC 46	N Creamer 46 (OSA Modified Starch from Waxy maize)
NC 180	N Creamer 180 (OSA Modified Starch Tapioca maltodextrin)
nm	Nanometre
No.	Number
OSA	Octenyl succinic anhydride
o/w	Oil-in-water
^{o}C	Degree Celsius
PUFA	Polyunsaturated fatty acid
PV	Peroxide value
RI	Refractive Index
s	Second
SD	Standard Deviation
SNF	Solids not fat
SPC	Standard plate count
UHT	Ultra high temperature
v/s	Versus
w	Weight
w/v	Weight/volume
WHO	World Health Organization
µEq	Microequivalent
µg	Microgram

Chapter 1

Introduction

1.0 Introduction

Milk is very nutritious and perhaps requisite food for human being. Milk is fundamental contributor to improve food security and nutrition throughout the world. Predominantly in developing countries, it may serve as a promising food source in reducing malnutrition. However, milk is devoid of omega-3 (ω-3) fatty acids and alpha linolenic acids (ALA), which are considered to be functional ingredients owing to several physiological health benefits.

There are numerous health benefits of omega-3 fatty acids especially in prevention of cardiovascular disease (Etherton *et al.*, 2002), hypertension, hypocholesterolemic effect (Bhardwaj, *et al.*, 2015), anti-inflammatory properties. Evidence shows that those who eat a lot of ALA are less likely to suffer a heart attack. ALA are also helps to reduce the possibility of cancer, and is beneficial in the growth of healthy hair and nails, reduces menopause symptoms, and play a role in burning excess body fat. ALA are also beneficial for those who suffer from Crohn's Disease and Colitis (Rubilar. *et al*, 2010). Thus, ALA and Omega-3 fatty acids serve as vital ingredient in developing the nutraceutical and functional food.

The Indian Council of Medical Research (ICMR, 2010) recommends 1.6 g/ day of ALA and 250 mg of EPA plus DHA per day. This can be achieved through fortification of food products. The rich sources of omega-3 fatty acids includes fish oil, flaxseed oil, algal oil, canola oil etc.

Although fish is the greatest contributor of ω-3 (EPA and DHA) but the Indian diets do not include enough oily fish to meet dietary recommendations of ω-3 fatty acids. Moreover, addition of fish oil preparations (in the form of emulsions or spray dried powder) in food is literally impossible for vegetarians due to their religious beliefs and practices. In such a situation, it is very difficult to meet recommended intakes of ω-3 for vegetarians. It has been reported that ω-6: ω-3 ratio in current Indian urban and Western diets is 38-50:1 and 20:1, respectively (Singh *et al*., 2011; Singh 2011. Simopolulos, 2011) which appears to be very high as compared to the recommended ratio, i.e. 5:1 (FAO/WHO 2010)

Flaxseed (*Linum usitatissimum*) oil, also known as linseed oil, is the rich source of ω-3 fatty acids, having 50-60% α-linolenic acid (ω-3, C18:3). Flaxseed oil contains both omega-3 and omega-6 fatty acids, which are needed for health. Flaxseed oil comprises the essential fatty acid; alpha-linolenic acid (ALA) which the body converts into eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). As a source for vegetarian population, flaxseed oil can be used to substitute to fish oil, for those who cannot consume fish and other marine products due to various religious belief and practices. Due to its highly polyunsaturated nature, flaxseed oil is highly susceptible to oxidation and leads to production of off-flavors and toxic peroxides during heating, processing and handling of the products. Hence, flaxseed oil cannot be used for cooking. So, working stabilization of the flaxseed oil for its food applications is a challenging job.

Microencapsulation is the most commonly used technique for the stabilization of active compounds. Spray drying is a common method of encapsulation in the food industry (Gouin, 2004 and Phisut, 2012). Maltodextrins and cyclodextrins are also used for microencapsulation, but due to their non-emulsifying properties, they are used in combination with other emulsifiers such as proteins. Protein, carbohydrates, gums, cellulosic materials are used as coat material. Proteins are more preferably used as coat material over carbohydrates for microencapsulation of core material due to their superior emulsifying and stabilizing properties. It offers the advantages like more payload capacity, efficient upscale process, etc. Modified starches, SPI & Maltodextrins (5%) have better encapsulating capacity (Renata *et al.,* 2014*)*. Modified starches form uniform film around the core material thus stable emulsion. Octenyl succinylated starches are amphiphilic in nature because of hydrophobicity introduced by octenylsuccinic anhydride (OSA) group in the hydrophilic starch backbone. These starches can thus be suitably used as efficient emulsifiers (Trubiano, 1995). Thus, it is a promising replacement for gum Arabic. Both SPI and modified starches are quite stable, easily available, cheap, effective functional properties with potential application for microencapsulation of essential fatty acids.

Thus, SPI and modified starches should explored for preparation of stable emulsion and also microencapsulation of flaxseed oil. Also, they have the potential to produce microcapsules with higher oil load capacity (>20%), when compared to

maltodextrin, whey proteins, gum arabic, skim milk powder, lactose etc. (9-15% oil load) as studied by various authors (Goyal *et al.*, 2014; Tonan, 2012). As milk and milk products are widely consumed by all age group of people and by various standard of livings they play a vital role in nutritional security. So, milk can serve as an ideal vehicle for fortification of omega-3 fatty acids or alpha linolenic acids. Further, limited information is available on fortification of omega 3 fatty acids by microencapsulated flaxseed oil powder prepared using soy protein isolates and modified starch. Thus, the present study was planned to microencapsulate flaxseed oil using modified starch and SPI and its utilization in milk fortification with following objectives:

1. To optimize the process for microencapsulation of flaxseed oil using soy protein isolate and modified starches

2. To fortify milk with omega-3 fatty acids using developed microencapsulated flaxseed oil

3. To characterize the fortified milk for physicochemical properties and shelf life evaluation

Chapter 2

Review of Literature

2.0 Review of Literature

This review outlines the latest status of our knowledge with regard to the microencapsulation of flaxseed oil for fortification of omega-3 (ω-3) fatty acids; particularly α-linolenic acid, their importance in imparting health benefits and major challenges in its fortification in various dairy and non-dairy products.

2.1 Polyunsaturated fatty acids (PUFAs)

Polyunsaturated fatty acids (PUFAs) are fatty acids that contain more than one double bond in their methyl end. This class includes many important compounds, such as essential fatty acids (Gunstone *et al.,* 2006). Polyunsaturated fatty acids can be classified in various groups based on their chemical structure like methylene-interrupted polyenes, conjugated fatty acids, other PUFAs

Methylene-interrupted polyenes are fatty acids that have 2 or more cis double bonds that are separated from each other by a single methylene bridge (-CH2-). This form is also sometimes called a divinyl-methane pattern. (Gunstone *et al.*, 2006). They contain essential fatty acids which are alpha linolenic acids (omega-3 and -6 methylene-interrupted fatty acids). Conjugated fatty acids have two or more double bonds and other polyunsaturated fatty acids include podocapric acids and pinolenic acid. With respect to the present study, alpha linolenic acid, omega-3 and omega-6 fatty acids are discussed in detail in the following sections.

2.1.1 Omega-3 fatty acids

Fats and oils have fatty acids as their basic structural components. Based on the presence of double bonds in fatty acid chain, fatty acids are classified into saturated fatty acids (no double bond), monounsaturated fatty acids (MUFAs) (one double bond) and polyunsaturated fatty acids (PUFAs) (>1 double bond) (Surette, 2008). An ω-3 fatty acids or n-3 fatty acids (Gunstone and Frank, 2006) are polyunsaturated fatty acids (PUFAs) with a double bond (C=C) at the third carbon atom free at the end of the carbon chain. The fatty acid chain has two ends, the carboxylic acid (-COOH) end (alpha) and the methyl (-CH3) end (omega). One way in which a fatty acid is named by the location of the first double bond or counted from the omega (ω-) or the n- end. Thus, in omega-3 fatty acids, the first double bond is between the third and fourth position from methyl end. However, the standard IUPAC chemical nomenclature system starts from the carboxyl

end. There are three types of omega−3 fatty acids which is involved in human physiology. They are α-linolenic acid (ALA), which is mainly found in plant oils, and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), both are commonly found in marine oils**.**

2.1.2 Omega-6 fatty acids

Omega-6 fatty acids (also referred to as ω-6 fatty acids or n-6 fatty acids) are composed of polyunsaturated fatty acids in which the first double bond in the hydrocarbon chain occurs between the sixth and seventh carbon atoms from the end of the molecule most distant from the carboxylic acid group (Kuang, 2001). Chemical structure of ω-3 and ω-6 fatty acids are presented in Figure 2.1. Members of PUFA family have pro-inflammatory or anti-inflammatory effects (Nowak, 2010). Linoleic acid (18:2, n−6), is the shortest-chained omega-6 fatty acid, which is one of the essential fatty acids because it cannot be synthesize in the human body. As mammalian cells lack the enzyme omega-3 denaturises and therefore cannot convert omega-6 fatty acids to omega-3 fatty acids. Closely related omega-3 and omega-6 fatty acids act as competing substrates for the same enzymes **(**Bibus *et al.*, 2001; Lands *et al*., 2015). This explains the significance of the metabolic conversion of omega-3 to omega-6 fatty acids in a food. Omega-6 fatty acids are precursors to endocannabinoids, lipoxins, and specific eicosanoids.

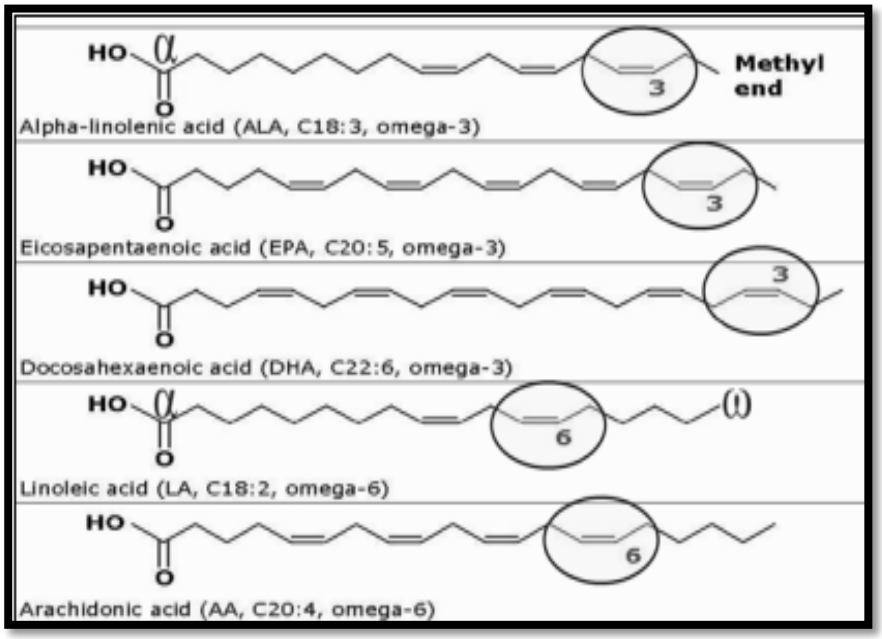

Figure 2.1 Chemical structure of ω-3 and ω-6 fatty acids (Adopted from: Etherton *et al*., 2002)

2.1.3 Metabolic conversion and physiological functions of ω-3 and ω-6 fatty acid

Metabolic conversion of parent ω-3 and ω-6 fatty acid is shown in Figure 2.2. Both ω-6 and ω-3 PUFA are important structural components of cell membranes. LA, which is the parent ω-6 fatty acid, gets converted in to 2- and 4-series prostaglandins and prostacyclin compounds. Omega-6 fatty acids are pro-inflammatory and promote platelet aggregation; while ω-3 fatty acids are anti-inflammatory and inhibit platelet aggregation. The metabolism of ω-6 fatty acids is associated with production of a number of eicosanoids like thromboxanes, leukotrienes and prostaglandins which are known to trigger inflammation in the blood vessels causing atherosclerosis (Ghafoornissa, 1998). Arachidonic acid which is the metabolic end product of linoleic acid (ω-6) is the most inflammatory agent, which stimulates the production of glutamate, a neurotransmitter which initiates the destruction of neurons by excess production of oxygen free radicals.

Alternatively, ω-3 fatty acids, when incorporated into phospholipids, affect cell membrane properties such as the fluidity, flexibility, permeability, and activity of membrane-bound enzymes (Stillwell and Wassall, 2003). Omega-3 and DHA decreases cellular and vascular inflammation in the brain, and ensure integrity of brain cell membranes to keep them soft and pliable. Fish oil and DHA are credited to reduce the level of thromboxane (TXA2) and increase prostacyclin (PGI2) level leading to enhanced tissue perfusion and oxygen delivery due to vasodilation and decreased blood viscosity. Apart from its role in fabrication of synaptic communication centres, DHA is credited to increase the level of “feel good” neurotransmitter serotonin and “memory boosting” chemical acetylcholine. DHA is also credited to neutralize oxygen-free radicals.

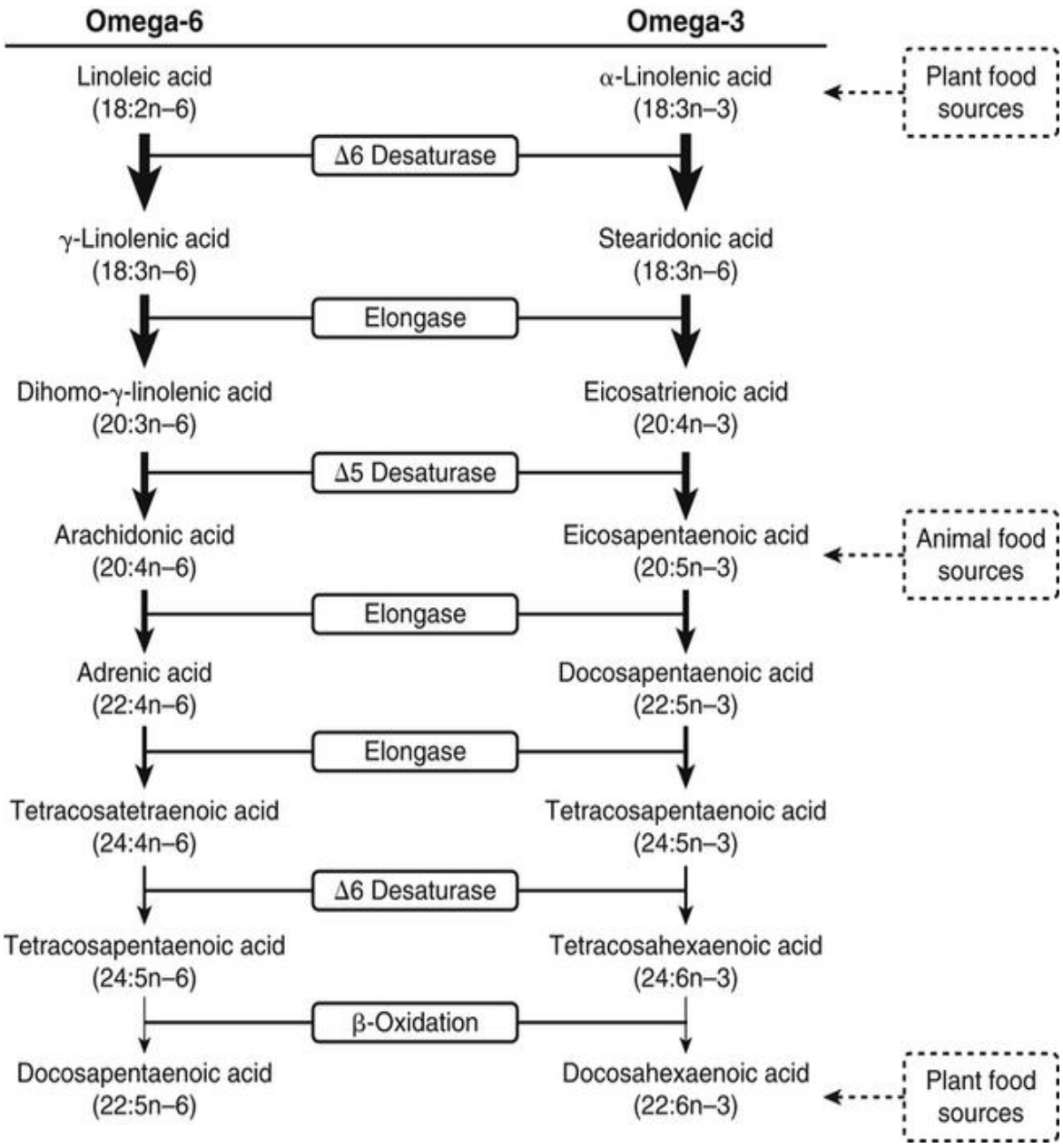

Figure 2.2 Metabolic conversion of ω-3 and ω-6 fatty acid (Adopted from: Ibarguren *et al*., 2014)

2.2 Health benefits of omega-3 and omega-6 fatty acids

2.2.1 For treatment of Diabetes mellitus

According to the data, in India around 62 million diabetic's patients are present and by 2030 nearly 9% of the total population of India is likely to be affected by the diabetes, in which many of them are overweight and/or obese. Because of this, they are at three time's higher risk for developing type 2 diabetes than normal weight people (Jeppesen *et al*., 2013). Diabetes mellitus (DM), is commonly called as diabetes, which is

a group of metabolic disorders due to high blood sugar levels over a prolonged period. Diabetes is characterized by hyperglycemic index and associated with abnormalities in the metabolism of carbohydrate, protein, and lipid that result in development of secondary complications (Mani *et al*., 2011). Kelley *et al*., (2009) studied that when conjugated linoleic acid (0.5%) and flaxseed oil (0.5%) was supplemented in diet of rats susceptible to plumpness and diabetic tumors, a 20% reduction in glycemia was observed. Kapoor *et al*., (2011) examined the effect of flaxseed powder supplementation on diabetic human females. Patients were provided with 15 and 20 g/day of flaxseed powder for two months. The blood glucose levels were found to be decreased by 7.9 and 19.1%, respectively. Nazni *et al*., (2006), who conducted a study on 25 diabetic subjects and supplemented flaxseed powder in bread form for 90 days and reported a significant reduction in blood glucose levels after feeding the fortified bread. Insulin production by modulation of phospholipid membrane in diabetic patient was affected by ALA (omega-3 and omega-6 fatty acids) (Borkman *et al*., 1993).

2.2.2 Tumor and cancer reducing effects

Awareness in investigation on the association between flaxseed oil ingestion and risk of cancer emerged when epidemiologic evidences suggested a beneficial relationship. Research in laboratories has shown that flaxseed inhibits the formation of colon, breast, skin, and lung tumors and also reduces blood vessel cell formation in female rats, all suggesting a protective effect against breast, colon and ovarian cancer (Truan *et al*., 2012). Higher levels of insulin and insulin-like growth factor 1 (IGF-1) increase cancer risk by stimulating cell proliferation and increasing survival of DNA damaged cells through antiapoptotic mechanisms (Sturgeon *et al*., 2011). Blood insulin has also been associated with increased risk of pancreatic and colorectal cancers (Pisani, 2008). Flaxseed oil with its exceptionally high ALA content was also shown to reduce human estrogen receptor-positive breast tumors (MCF-7) growth by 33% compared to control. (Truan *et al*., 2010). Chen *et al*., (2007) did *in vivo* study with groups of mice that were fed with 5% and 10% flaxseed in the diet for 2 months and reported inhibition of tumor growth by 26% and 38%, respectively.

2.2.3 Prevention of kidney diseases

Chronic kidney disease (CKD) is common health problem among older adults and can lead to end-stage renal disease (Lauretani *et al*., 2009; Coresh *et al*., 2007). Due to the

anti-inflammatory properties of omega-3 fatty acids, it has been suggested that these nutrients may protect the kidneys from damage in adults. Baggio *et al*., (2005), Gopinath *et al*., (2011) reported PUFA reduces renal inflammation and fibrosis in animal models and showed that increased dietary intake of long-chain omega-3 PUFA was inversely associated with the prevalence of CKD. Similarly, Cicero *et al*., (2010) showed that long-term supplementation of omega-3 fatty acids was associated with a significant reduction in systolic and diastolic blood pressure. Hypertension is a risk factor for CKD; hence, the influence of long-term supplementation of omega-3 PUFA on blood pressure may be a potential mechanism by which it protects the kidneys.

2.2.4 Reduction of cardiovascular diseases (CVDs)

Serum lipid profile has direct relation with risk factors of cardiovascular diseases. It is the most hugely studied in animals and humans after supplementation of flax in diet. Rats, mice, and rabbits presented positive responses for biochemical parameters, indicating the hypocholesterolemic activity of flaxseed, generally related to the greater faecal content of lipids (Kristensen *et al*., 2011, 2012; Hassan *et al*., 2012; Khalesi *et al*., 2011; Mani *et al*., 2011; Cardozo *et al*., 2010; Barakat and Mehmoud, 2011; Leyvaa *et al*., 2011). Dietary flaxseed may also offer protection against ischemic heart disease by improving vascular relaxation responses and by inhibiting the incidence of ventricular fibrillation (Jennifer *et al*., 2010). However, Vedtofte *et al*., (2011) reported that higher intake of ALA was not significantly decreasing the risk of ischemic heart disease among women or men.

2.2.5 Prevention and treatment of obesity

Simulated laxatives in fitness and weight loss have more negative impact on human body than benefits. Flaxseed oil is a natural laxative, as it helps to keep the digestive system in excellent condition and rids the body of toxins that lead to weight gain and bloating. Traditionally, obesity, diabetics were often treated and/or prevented using many plant materials including flax (Santos *et al*., 2010; Singh *et al*., 2011).

2.3 Sources of omega-3 and omega-6 fatty acids

There are numerous source of omega-3 fatty acids (Table 2.1) the existing rich sources of ALA are classified as animal origin sources and plant sources based upon their origin in following section.

2.3.1 Animal sources

The richest sources of omega-3 fatty acids are fishes and seafood, such as salmon, oysters and sardines. The results of investigations on the use of food products enriched with fish oil, as an easy delivery system of long-chain omega-3 PUFA into the human organism, might encourage efforts towards the production of such enriched foods (Kolanowski, 2005) Atlantic mackerel, salmon fish oil, cod liver oil, Alaskan salmon (wild-caught), tuna, white fish, egg yolks are also sources of ALA in human diet from animal sources.

2.3.2 Plant sources

There are a number of foods that are rich in omega 3 fats and they are as follows: soybeans (roasted), walnuts, canola oil, chia seeds and flaxseeds.

Table 2.1 Comparison of ω-3 and ω-6 fatty acid composition (%) of some dietary oils

Oil	ALA	EPA	DHA	LA	AA	w-6:w-3
Flaxseed	58	-	-	13	-	0.2:1
Canola	10	-	-	26	-	2.6:1
Soya bean	7	-	-	54	-	7.7:1
Sunflower	-	-	-	69	-	-
Walnut	10	-	-	50	-	5:.1
Milk Fat	2	-	-	2	-	1:1
Cod liver	1	6	27	2	-	0.1:1
Menhaden	1	14	12	2	4	0.1:1
Shark liver	-	4	30	4	4	0.2:1

2.3.2.1 Flaxseed and Flaxseed oil

Flax *(Linum usitatissimum)* is an annual plant of the linaceae family. This plant grows to a height up to 60 cm, with slender and very fibrous stems, lanceolate leaves having three veins, up to 4 cm long and 4 mm wide, and its bright blue flowers are of up to 3 cm in diameter. The fruit contains a seed known as flaxseed or linseed (Pradhan *et al.,* 2010). Major flaxseed growing countries in the world are Canada, China, United

States, India and Ethiopia. Canada is the largest producer in the world with a production of 0.42 million tonnes in 2010 (FAO, 2012) and accounts for nearly 80% of the global trade in flaxseed (Oomah and Mazza, 1998). India ranks 4th with 0.15 million tonnes of total flaxseed production (FAO, 2012). The global linseed oil market size was valued at USD 720 million in 2016 (Market research report, Oct 2017). Flaxseed is considered as a functional food or source of functional ingredients, because it contains alpha-linolenic acid (Bozan and Temelli, 2008), lignans and polysaccharides (other than starch), all of which have positive effects in disease prevention. Although scientific evidence supports flaxseed consumption, many people are still unaware of the benefits provided by this product and its possible applications in the production of foodstuffs (Udenigwe, *et al.*, 2009).

Flaxseed oil is known as linseed oil. It is colourless to yellowish oil extracted from the dried seeds of the flax plant. The oil is obtained by pressing, often followed by solvent extraction. Flaxseed oil is an edible oil in demand as a nutritional supplement. Flaxseed oil is the richest plant source of the α-linolenic acid (ALA) (Gebauer *et al.*, 2006).

2.3.2.1.1 Fatty acid profile of flaxseed oil

Flax oil is a triglyceride, like other fats. Flaxseed oil is distinctive for its unusually large amount of α-linolenic acid (ALA), which has a distinctive reaction with oxygen in air. Specifically, the fatty acids in a typical flaxseed oil are of the following types (Vereshagin, and Novitskaya, 1965):

- The triply unsaturated α-linolenic acid (51.9–55.2%),
- The saturated acids palmitic acid (about 7%) and stearic acid (3.4–4.6%),
- The monounsaturated oleic acid (18.5–22.6%),
- The doubly unsaturated linoleic acid (14.2–17%).

2.4 Dietary recommendations for ω-3 fatty acids

Linoleic acid (LA) and ALA are essential as they cannot be synthesized in human body. According to ICMR (2010), the minimum intake levels for essential fatty acids to prevent deficiency symptoms are estimated to be 2.5% of total energy from

LA and 0.5% of total energy from ALA. Different agencies have recommended different levels of ALA, EPA and DHA, which is shown in Table 2.2

Table 2.2 Recommended daily intake for ALA, EPA and DHA by various health authorities

Expert committee	**RDA**
Indian Council of Medical Research (ICMR) (2010)	ALA 1.6 g/d and >250 mg EPA & DHA per day
International Society for the Study of Fatty Acids and Lipids (ISSFAL, 2004)	ALA 2.2 g/d plus EPA/DHA 500 mg/day

One major problem in Indian diet is lack of fish and marine foods which are rich source of ω-3 fatty acids (EPA and DHA). And on other hand, the consumption of refined vegetable oil is more thus ω-6 fatty acids intake is increasing day-by-day. This subsequently has led to the imbalance of the ratio of ω-6 and ω-3 fatty acids in human body. It has been reported that ω-6:ω-3 ratio in current Indian urban, Indian rural and Western diets is 38-50:1, 5-6:1 and 16:1, respectively (Singh *et al*., 2010; Singh, 2011). The ratio of ω-6:ω-3 fatty acids in current Indian and Western diet appears to be very high while the recommended ratio is 5:1 (FAO/WHO, 2010). This recommended ratio can be achieved by fortification of food rich sources of omega-3 fatty acid.

2.5 Microencapsulation: An approach for omega-3 fatty acids fortification

Microencapsulation is a process in which active compound or tiny droplets are coated in a homogeneous or heterogeneous matrix, to give small capsules (Gharsallaoui, *et al.,* 2007). The material inside the microcapsule is referred to as the core, internal phase, or fill, whereas the matrix is sometimes called a shell, coating, wall material or membrane. The diameter of microcapsules is in a range of few micrometers. Every class of food ingredient has been encapsulated; flavours are the most common encapsulated. The technique of microencapsulation depends on the physical and chemical properties of core and coat material (Jackson and lee, 1991). Encapsulation has several benefits such as providing environmental protection, converting liquids to solids, improved material handling properties, separating reactive compounds. Spray drying is the most important

technique, among the different techniques available for the encapsulation of bioactive food ingredients. (Desai and Park, 2005).

2.5.1. Encapsulating Material

Microencapsulation is the process in which individual particles or droplets of solid or liquid material (the core) are surrounded or coated with a continuous film of polymeric material (the shell) to produce capsules in the micrometer to millimeter range, known as microcapsules. There are two basic ingredients required for microencapsulation; one is core and other is coat.

2.5.1.1. Core Material

The material to be coated or the active ingredient to be protected from the external environment is said to be the core. It may be liquid or solid or gas. Liquid core may be dissolved or dispersed material. The core material is active material which may be flavours, essential oil, microorganism, drug, stabilizer, etc.

2.5.1.2. Coat Material

Inert substance which coats or covers the core with desired thickness are said to be coat materials. It includes inert polymer plasticizer, starch, protein, colouring agent etc. The coat/ shell material should be compatible with the core material, inert towards active ingredients, and should offer controlled release under specific conditions. The coating can be flexible, brittle, hard, thin, abundantly and cheaply available. Mainly used coat materials are as mentioned in Table 2.3

Table 2.3 Coat materials for microencapsulation of functional food additives

Category	Coat materials	Widely used methods	Reference
Carbohydrate	Starch, maltodextrins, chitosan, corn syrup solids, dextran, modified starch, cyclodextrins	Spray- and freeze-drying, extrusion, coacervation, inclusion complexation	James, S (2003)
Cellulose	Carboxy methyl cellulose, methyl cellulose, ethylcellulose, cellulose acetate-phthalate,	Coacervation, spray-drying, and edible films	Leon *et al.*,(1990)
Gum	Gum acacia, agar, sodium alginate, carrageenan	Spray-drying, syringe method (gel beads)	Deasy PB. (1984).
Lipids	Wax, paraffin, beeswax, diacylglyerols, oils, fats	Emulsion, liposomes, film formation	Kreitz (1999)
Protein	Gluten, casein, gelatin, albumin, peptides	Emulsion, spray-drying	James, S (2003)

2.5.1.2.1 Soy proteins isolate as coat material

Soy proteins comprise mainly of glycinin and conglycin (50-90% of total proteins) (Ruiz-Henestrosa *et al*., 2007). The glycinin fraction (11S globulin) has a molecular weight of about 350 kDa while, conglycin (7S globulin 10 fraction) is about 70 kDa. Isolated and purified soy proteins show interesting physicochemical and functional attributes in particular gel-forming, emulsifying and surfactant properties (Gu *et al*., 2009). The protein characteristics and their solubility are strongly dependent on pH, heat treatment, and the presence and concentration of salts or other ingredients (oil, carbohydrate, and surfactant).

Soy protein isolate (SPI) has been used as a coat material in microencapsulation (Table 2.4) by various researchers(Augustin *et al*., 2006; Charve and Reineccius, 2009; Favaro-Trindade *et al*., 2010; Kim *et al*., 1996; Rascon *et al*., 2010; Rusli *et al*., 2006; Yu *et al*., 2007), SPI is generally used as an individual coating material, but can also be mixed with polysaccharides (Augustin *et al*., 2006; Rusli *et al*., 2006; Yu *et al*., 2007). The combination of proteins with carbohydrates as a carrier material favours better protection, oxidative stability and drying properties (Augustin *et al*., 2006). Due to SPI hydrosolubility, microparticles are mainly produced using the spray-drying technique but

coacervation and gelation have also been investigated (Chen and Subirade, 2009; Mendanha *et al*., 2009; Nori *et al*., 2010).

Table 2.4 Microencapsulation with SPI as a wall material

Core material	Coat material	Microencapsulation technique	Reference
Orange oil	SPI	Spray drying	Kim *et al*., (1996)
Fish oil	SPI and polysaccharides	Spray drying	Augustin *et al*.,(2006)
Stearin, Palm oil	SPI	Spray drying	Rusli *et al*., (2006)
α-tocopherol	SPI	Spray drying	Nesterenko *et al*., (2012)
Casein hydrolysate	SPI/pectin	Complex coacervation	Mendanha *et al*., (2009)
Fish oil	SPI	Simple coacervation	Gan *et al*., (2008)
Paprika oleoresin	SPI	Spray drying	Rascon *et al*., (2010)
Flavours	SPI	Spray drying	Chavre and Reineccius, (2009)

2.5.1.2.2 Modified starch as coat material

Modified starches are among many functional biopolymers used in microencapsulation technologies. Well-established enzymatic, chemical, and physical modification or combination of different modification methods allow tailor-made molecular structures for optimized technical performance (Márquez-Gómez. M. *et el.*,, 2017). In particular, starches modified with hydrophobic groups can act as surface active agents. They are highly effective and economical ingredients with consistent and versatile functionality in encapsulation of oil-based flavours, micronutrients, fragrances, agri-chemicals, and pharmaceutical actives. Modified starches have been used in a wide range of the encapsulation technologies such as spray-drying, spray congealing, extrusion. Specific to spray-drying, modified starches offer high oil load, high volatile retention, long shelf life, and high manufacturing efficiency.

Zhiping, *et al.*, (2010) studied oxidative stability of microencapsulated fish oil powders stabilized by blends of chitosan, modified starch, and glucose found that the stability is more than the gum arabic. Similarly pomegranate seed oil was encapsulated

using modified starch by Bustamante *et al.*, (2016), and reported that modified starch has a potential applications for microencapsulation in the food industry.

2.5.2 Microencapsulation techniques

Encapsulation of bioactive food material can be done by various methods. The selection of technique for microencapsulation process is governed by the physical and chemical properties of coat and core material. However, the microencapsulation processes that are generally used to encapsulate food ingredients are given in Table 2.5.

Table 2.5 Various microencapsulation techniques and the processes involved in it

Sr.no	Microencapsulation technique	Major steps in encapsulation
01	Spray drying	a. Preparation of dispersion. b. Homogenization of dispersion c. Atomisation of the feed (dispersion) d. Dehydration of atomised particles
02	Spray chilling	a. Preparation of dispersion. b. Homogenization of dispersion c. Atomisation of the feed (dispersion)
03	Spray-cooling	a. Preparation of the dispersion b. Homogenization of the dispersion c. Atomization of the in feed dispersion
04	Coacervation	a. Formation of three immiscible phases b. Deposition of coating c. Solidification of coating
05	Extrusion	a. Preparation of core solution b. Preparation of coating solution c. Co-extrusion of core and coat solution
06	Centrifugal extrusion	a. Preparation of core solution b. Preparation of coating material solution c. Co-extrusion of core and coat solution through nozzles
07	Fluidized bed coating	a. Preparation of coating solution b. Fluidization of core particles c. Coating of core particles
08	Liposomal entrapment	a. Microfludisation b. Ultra-sonication c. Reverse phase evaporation
09	Co-Crystallization	a. Preparation of supersaturated sucrose solution b. Adding core into supersaturated solution c. Emission of substantial heat after solution reaches sucrose crystallisation temperature
10	Inclusion complexion method	a. Preparation of complexes by mixing / grinding / stirring which could be easily filtered and dried
11	Lyophilisation	a. Mixing of core and coat materials b. Freeze drying of mixture

An extremely wide variety of functionalities can be achieved through microencapsulation as sophisticated shell materials and technologies have been developed. Any kind of trigger can be used for release mechanism of the encapsulated ingredient, such as pH change (enteric and anti-enteric coating), mechanical stress, temperature, enzymatic activity, time, osmotic force, etc. The selection of microencapsulation method and coating materials are interdependent. Based on the coating material or method applied, the appropriate method or coating material is selected. Coating materials, which are basically film-forming materials, can be selected from a wide variety of natural or synthetic polymers, depending on the material to be coated and characteristics desired in the final microcapsules. The composition of the coating material would determine the functional properties of the microcapsule and how it may be used to improve the performance of a particular ingredient. An ideal coating material should exhibit the following characteristics:

1. Good rheological properties at high concentration and easy workability during encapsulation.

2. The ability to disperse or emulsify the active material and stabilize the emulsion produced.

2.5.2.1 Spray-drying for microencapsulation of flaxseed oil

Microencapsulation by spray-drying is an economical process as compared to other method, thus widely used for the encapsulation of starter cultures, oils and flavours. Spray drying is a technique in which continuous transformation of feed emulsion is dried in particulate (powder) form by spraying the feed into a hot drying medium. Emulsion is prepared by homogenisation of aqueous solution of core and coat material. The resultant emulsion is atomized by pneumatic nozzle into a heated compartment of spray drier. There the water portion of the emulsion is evaporated in dryer, yielding dried microcapsules of variable shape mostly in spherical shape containing scattered drops of core material. The capsules are collected through continuous discharge from the spray dryer. Spray drying process also used to dry small microencapsulated materials from aqueous slurry that are produced by chemical methods.

2.6. Emulsion and its properties

Emulsion is mixture of three components or phases, an oily phase, an aqueous phase and a surface active species, so called surfactants. Sometimes, co-surfactants are used for preparation of emulsion (Saito and Shinoda, 1967. Saito and Shinoda, 1970). Depending on the ratio of phases of emulsion, type of emulsion is determined as emulsions vary from a very tiny water droplets dispersed in oil phase (w/o emulsion) to an oil droplets dispersed in water phase (o/w emulsion). The possibility and easiness of the tuning of emulsion properties with different parameters has allowed the scientists to use them in many interdisciplinary fields of research and applications. The future scope for the emulsion systems is to develop improved stable emulsion with less coat material. (Najjar, 2012)

2.6.1. Preparation of emulsion

The methods which are commonly used to produce emulsions include ultrasonication, homogenization, and high shear mixing.

2.6.1.1. Homogenization

To form a fine stable emulsion using high-pressure homogenization, the coarse dispersion of the oil and aqueous phase and emulsifier is passed through a small inlet orifice at pressures in the range of 500-5000 psi.

2.6.1.2. Ultrasonication

The mechanism of emulsion generation using ultrasonication is attributed to bubble cavitation. The ultrasound waves (at ultrasonic frequencies typically 20 kHz or larger and high power intensity) result in sequential formation, growth and collapse of microscopic vapour bubbles in the liquid. Subsequently, the collapse of these cavities provides sufficient energy to increase surface area of droplets (Patil and Pandit, 2007). It is widely used technique for preparation of nanoemulsions (Kentish *et al*., 2008; Leong *et al*., 2009)

2.6.1.3. High shear mixing

High shear mixing is suitable for standardized applications. The close proximity of the rotor and stator provides excellent dispersion of samples with varied miscibility. This method is able to homogenize, emulsify or suspend samples at high circumferential

speeds even with small rotor diameters. The minimal gap between the rotor and stator produces an extremely strong shear force that expels the medium being processed through the slots of the rotor-stator. High shear mixer has the power and versatility to accomplish a variety of formulation development applications.

2.7 Physico-chemical characterisation of emulsions

2.7.1. Physical stability

Coat: core material ratio and amount of emulsifier added plays a fundamental role in stabilizing the emulsion. Similarly method of homogenisation or preparation of O/W emulsion paly crucible role in developing stable emulsion. Dickinson (2001) reported that lack of the encapsulating material (i.e., insufficient concentration) causes sharing of the active material between adjacent droplets and leads to irreversible bridging flocculation. However McClements (2004) reported that excess of the encapsulant/emulsifier, above that required level to complete oil droplet covering, may increase its surface load and negatively influence emulsion properties.

Pedro *et al*., (2011) observed no separation in flaxseed oil emulsions (10–30 % oil) stabilized by gum arabic till 24 h at room temperature. However, Carneiro *et al*., (2012) reported a small separation (16.8 %) and a foam phase, after 24 h homogenization in flaxseed oil emulsions encapsulated by maltodextrin: WPC80 (25:75).

Similarly, Kuhn and Cunha (2012) studied the effect of homogenization pressure (20 to 80 MPa) on the stability of flaxseed oil-whey proteins isolate emulsions and reported that none of the emulsions showed creaming till 9 days of storage; but the emulsions homogenized at 80 MPa showed high molecular weight aggregates, thus it could be concluded that 20 MPa (3,000 psi) was the optimum homogenization pressure.

Goyal *et al.*, (2014) has made attempt to find optimum whey protein concentration to stabilize the flaxseed oil emulsion. The stability study revealed that the emulsions were kinetically stable when homogenized at 20 MPa (3,000 psi) pressure and stored at 7–8 °C for 28 days. There was no separation observed of phases in emulsions produced with different concentration of WPC-80. In case of emulsion prepared by 12.5 % WPC-80, gelling was observed (visually) just after the homogenization at 4,500 psi. It could be explained by the fact that at such a high pressure, temperature of emulsion increased

rapidly and caused droplet coalescence and the formation of high molecular weight protein aggregates due to shear and increase in temperature.

2.7.2 Oxidative stability

Encapsulated oil in the form of emulsion is more oxidative stable as compared to bulk oil. The progress of lipid oxidation was monitored by measuring the formation of primary oxidation products (lipid hydroperoxides) in the emulsions.

Kuhn and Cunha (2012) reported about 41.70 % increase in PV of free flaxseed oil, which increased from 0.420 to 0.714 meq peroxides/kg oil during 30 days of storage. They also reported a significant increase in PV from 0 to1.777 meq peroxides / kg oil of flaxseed oil emulsion homogenized at 80 MPa.

Grattard *et al*., (2002), Partanen *et al*., (2008) and Karaca *et al*., (2013) concluded that improved oxidative stability of encapsulated flaxseed oil (in powder form) by different proteins. Peroxide value of flaxseed oil emulsions prepared with 7.5–12.5%WPC remained well within the limit of upto 5 meq peroxide/kg oil under the Codex Alimentarius Commission (1999) standard for cold-pressed and virgin oils (Choo *et al*., 2007). The high stability of emulsions containing higher WPC concentrations (7.5, 10 and 12.5 %) may also be attributed to their antioxidative properties and their ability to bind some pro-oxidant impurities (such as transient metals) due to presence of histidine, glutamic acid, aspartic acid, and phosphorylated serine and threonine residues (Mcclements and Decker, 2000;Tongetal, 2000); thus protecting oil against oxidation.

Similarly, Ma *et al*., (2012) reported improved oxidative stability of flaxseed oil emulsions encapsulated by sodium caseinate cross-linked by transglutaminase during 30 days of storage. Carneiro *et al*., (2012) reported peroxide values 22.6 and 24.8 meq peroxides/kg oil, respectively after 1 week of storage for flaxseed oil emulsion prepared with Maltodextrin:Hi Cap (modified starch) and Maltodextrin: Gum arabic, respectively

Goyal *et al.*, (2014) studied oxidative stability by peroxide values of different flaxseed oil emulsions stored at low temperature for 4 weeks. The initial PV of flaxseed oil (control) was 12.20, which increased to 17.60 meq peroxide/kg oil during storage. There was no significant difference between PV of samples and control on zero day (just after the preparation of emulsions), suggesting that homogenization did not affect PV of flaxseed oil significantly ($p < 0.05$). Highest PV or lower oxidative stability of 5 % WPC

emulsion could be attributed to the thinner layer or lower amount of encapsulating agent around the oil droplets, leading to higher susceptibility to the oxidation. Data showed that there was only ~20.98% increase in PV of all the emulsions (except prepared with 5 % WPC), as compared to free flaxseed oil (~44.26 %) after 4 weeks of storage.

2.7.3 Zeta potential and emulsion droplet size

The charge on droplet influences the rheological properties and stability of an emulsion. Emulsions with high zeta-potential (negative or positive) are electrically stabilized while emulsions with low zeta-potential tend to coagulate or flocculate. The charge of the droplets with adsorbed protein and/or biopolymer can be represented by ζ potential, plus the charge associated with any ions that move along with the droplet in the electric field (Surh *et al*., 2006).

Sharma *et al.,* (2012) summarizes the ζ –potential of the emulsion droplets as a function of concentration of the encapsulating agent (WPC-80). Whey proteins concentrate being negatively charged at neutral pH, showed negative ζ-potential on emulsion droplets, and ranged from −28.6 to −33.5 mV. There was no significant difference between the ζ-potential of 7.5 and 10 % WPC emulsions, which was comparatively higher than that of other emulsions. However, on increasing the WPC concentration from 5 to 7.5 or 10 %, a significant increase was observed ($p < 0.05$). Higher ζ-potential in 7.5 and 10 % WPC emulsions could be explained by the maximum utilization of whey proteins for the coverage of oil droplets, leaving very non-significant amount of unabsorbed protein or uncovered oil droplets.

Similar findings have been reported by other researchers (Nikovska 2012; Chanamai and McClements 2004; Saglam *et al*. 2013) who studied different oil-in-water emulsions and observed negative zeta potential on emulsion droplets stabilized by whey proteins. Their data suggested that 7.5 and 10% WPC emulsions were the most stable systems among all emulsions in terms of ζ –potential. Similar results were reported by Wang *et al*., (2010) for the soybean oil flaxseed protein emulsions. They reported that ζ-potential of the emulsions varied from −30.7 to −49.5mV, with significantly increasing with flaxseed protein concentration.

Khalloufi *et al*., (2008) reported around -50 mV ζ–potential on soybean oil based emulsions droplets stabilized by WPI. The ζ-potential results lead to the hypothesis that electrostatic repulsion occurs between the oil droplets covered by negatively charged

whey proteins. The relatively higher negative ζ-potential of whey protein concentrate coated droplets may account for greater intensity of the electrostatic repulsion force and superior stability of emulsion (Taherian *et al.*, 2011). It can be concluded from the results of particle size distribution and ζ–potential that flaxseed oil emulsion produced by using 7.5 % WPC-80 was the most stable showing narrowest particle size distribution and highest zeta potential.

2.7.4. Rheological properties

Depending upon the composition of emulsion, particle size, charge on it and viscosity, emulsions show different rheological properties like Newtonian (ideal fluid) and Non-Newtonian (shear thinning, shear thickening, bingham, plastics, etc.) behaviour. Rheological properties play a vital role during the process condition like pumping, atomising, mixing and flow in pipe or in designing a delivery mechanism for a particular food application. Certain food systems like juices and beverages, which have very low viscosity, should not be changed during mixing with other ingredients, or during flowing & filling operations. Other food systems are highly viscous or gel like (for example, dressings, desserts) and in the second cases, the delivery system should not decrease the viscosity or disrupt the gel network (McClements *et al*., 2007).

Laplante *et al.*, (2005) and Zinoviadou, (2012) observed high viscosity values irrespective of composition of emulsion. Similarly, high viscosities at low-shear rates have been reported for emulsions stabilized by whey protein isolate. Shear-thinning may occur for a variety of reasons in food emulsions (Hunter, 1993). Such as Sun and Gunasekaran (2009) observed shear thinning behaviour, who worked on whey protein isolate stabilized oil-in water emulsions. However, Saglam *et al*., (2013) reported shear thickening behaviour of emulsions stabilized by whey protein isolates. Pseudoplastic behaviour is the most common type of non-ideal behaviour exhibited by food emulsions. For most non-Newtonian liquids, the viscosity decreases with an increase in shear rate, giving rise to what is known as pseudoplasticity or shear thinning behaviour (Rao, 1977).

Dybowska (2011) and Wang *et al*., (2010, 2011) also reported shear thinning behavior at lower shear rate (<100 s^{-1}) for rapeseed oil and soybean oil emulsions, respectively. Same finding was given by Taherian *et al*., (2011) and Dybowska (2011) shear thinning behaviour in O/W fish oil and rapeseed oil emulsion, respectively.

However, Lizarraga *et al*., (2008) found that corn oil-in-water emulsions (50 g oil/ 100 g) stabilized by WPC presented a Newtonian behaviour.

However, Kuhn and Cunha (2012) studied the flaxseed oil emulsions stabilized by whey protein isolates (total solids: 33 %) and reported that all O/W emulsions showed very low pseudoplasticity with flow behaviour index in the range of 0.78–0.95. It was observed that consistency index (k) (Pa.sn) increased from 0.154 to 0.511 Pa.sn with increase in concentration of whey proteins, suggesting the increase in viscosity and droplets concentration. Similar increase in consistency index (from 0.012 to 0.472 Pa.sn) was reported by Wang *et al*., (2011) in soybean oil emulsions stabilized by flax proteins and soy protein isolates.

Goyal (2014) studied the emulsion's apparent viscosity (cP) under the shear rate (5–150 s^{-1}) for emulsions having different concentration of WPC-80 during storage of 28 days. All the emulsions showed non-Newtonian, shear thinning (Pseudoplastic) behaviour as viscosity decreased with increase in shear rate. Viscosity increased with increase in protein concentration, highest and lowest for emulsions containing 12.5 and 5 % WPC, respectively. Viscosity also increased during the storage period ranging from 7.85 to 23.7 cP at 150 s^{-1} shear rate.

2.8. Microencapsulated flaxseed oil and its properties

2.8.1. Moisture content & Water activity (a_w)

Moisture content and water activity determines the shelf life of powders. More the moisture and a_w leads to fungal growth and possibility of caking during storage; thus both the parameters affect microcapsule's physical as well as chemical stability and overall acceptability. In general, Gallardo *et al*., (2013) recommended moisture content of 3-4% is the minimum specification for most dried powders used in the food industry. Generally, moisture content depends upon the compositional changes of wall & core materials, inlet/outlet temperature & flow rate of spray dryer, dryer design, etc. (Re, 1998). Klaypradit and Huang (2008) reported that dry foods with moisture content between 3 and 10 g per 100 g have good stability during storage. Quispe-Condori *et al*., (2011) and Aghbashlo *et al*., (2013) developed flaxseed and fish oil powder and reported 3.88-5.06% and 1.41-4.36% moisture content, respectively.

2.8.2 Particle size

Powder particle size governs the dispersion behaviour after rehydration. It is an important property for the solid fortificants because it may adversely affect the stability of food products in which it is added. Reconstitution property depends upon the particle size whether microcapsules would sediment or float, or remain dispersed in continuous phase. The particle size distribution of an emulsion gives idea about the fraction of particles present in different size classes (McClements, 2005). Particle size and particle size distribution of microcapsules influence the textural as well as sensory properties of added food products. Particle size distribution is depending upon mode of dispersion, total solids content, wall material, and processing parameter. It may be monomodal or bimodal distribution. Monomodal distribution shows more homogenous nature of particles as compared to bimodal distribution. Bimodal and polydispersed distribution was reported by various authors, who encapsulated fish oil using milk proteins (Chen *et al*., 2013), chia essential oil using WPC-polysaccharides (Rodea-Gonzalez *et al*., 2012) and D-limonene using modified starch (Jafari *et al*., 2007). Polydispersity index (PDI) is another term to describe the dispersity of the particles. PDI is a dimensionless scale between zero and one, which indicates the polydispersity of the particles. In fact, PDI is more appropriate in explaining bimodal distribution profiles than average particle size. A lower PDI indicates narrower particle size distribution and vice versa. In general, PDI >0.7 indicates more heterogeneous nature and wide distribution of particles (Nidhin *et al*., 2008). Chen *et al*., (2013) microencapsulated fish oil using WPC and sodium caseinate (wall:core ratio 1:1), and reported 0.32-1.00 PDI of the microcapsules.

2.8.3 Scanning Electron Microscopy

The scanning electron microscope (SEM) is the most versatile instrument available for the analysis of the surface morphology, microstructure and chemical composition characterizations. The topography (surface characteristics) of microencapsulated and spray dried powders are commonly studied by scanning electron microscopy. Presence of cavities, dents on the surface of microcapsules is a typical characteristic of spray dried powder. (Gallardo *et al*., 2013).

Similar morphology was observed by Tonan *et al*., (2011, 2012) who microencapsulated flaxseed oil using WPC, gum arabic and modified starch. Presence of dents on surface of microcapsules could also be attributed to high total solids in the

formulations as reported by Faldt and Bergenstahl (1996), Anandharamakrishnan *et al*., (2007), Gallardo *et al.*, (2013), Tang and Li (2013) and Aghbashlo *et al.*, (2013) who microencapsulated omega-3 oils using skim milk & whey proteins, observed spherical shaped microcapsules with no cracks and pore on the surface. Another characteristic which is generally seen in microencapsulated spray dried powders is the aggregation of microcapsules. This aggregation of the microcapsules can be attributed to the presence of surface (free) fat in the powder (Shivakumar *et al*., 2012). Similar type of aggregation in microcapsules was observed by Onwulata and Holsinger (1995) and Hogan *et al*., (2001b).

2.8.4. Oxidative stability of microcapsules

Oxidative stability of any food material or product can be described in terms of peroxide value, p-Anisidine value and TBA value. Peroxide value truly indicates the oxidation as it determines the amount of hydroperoxides formed during the oxidation of fats and oil. The peroxide value indicates the extent of oxidation and formation of primary oxidation products. Although these oxidation products, particularly hydroperoxides are colourless, odourless and produce no off-flavours, but are highly toxic and reduce the bioavailability of fatty acids.

Codex Alimentarius Commission (1999) prescribed the maximum permissible limit of peroxide value up to 5 meq peroxides/ kg oil for edible fats and oils. It is reported that ALA is 20 times more susceptible to oxidation as compared to oleic acid (Decker *et al*., 2012).

Kagami *et al*., (2003) studied the oxidative stability of microcapsules formed by spray drying of fish oil with protein and dextrin wall materials. They observed that highly branched cyclic dextrin or maltodextrin with sodium caseinate improved the oxidative stability of encapsulated fish oil.

Similarly, Serfert *et al*., (2009) observed that the hydroperoxide content of microencapsulated fish oil was three times higher for the powder produced at inlet/outlet temperatures of 210/90°C, when compared to that produced at 160/70°C.

In another study, Tonon *et al*., (2011) studied the influence of emulsion composition and inlet air temperature on the microencapsulation of flaxseed oil by spray drying. They reported that peroxide value increased with increasing inlet spray drying

temperatures. The use of higher inlet air temperatures provides more energy available for the lipid oxidation process, which occurs more intensely, favouring the formation of peroxides. Thomsen *et al*., (2005) also observed strong temperature dependence for quality deterioration of milk powders, verifying an Arrhenius type dependence of lipid oxidation on temperature.

Decker *et al*., (2012), Carneiro *et al*., (2012) studied the oxidative stability of flaxseed oil microencapsulated by spray drying using different combinations of wall materials such as maltodextrin (MD) with gum arabic (GA), whey protein concentrate (WPC) and two types of modified starch (MD-Hi-Cap 100TM and Capsul TA). They reported that at zero day, all the samples showed a low level of oxidation, ranging from 6.12 to 8.77 meq peroxide/kg oil. The samples encapsulated with MD:Hi- Cap and MD:GA presented higher peroxide concentration after one week, reaching values of 22.6 and 24.8 meq peroxide/kg oil, respectively. Samples encapsulated with Hi-Cap, GA or capsul with maltodextrin suffered a significant increase in oxidation at the third week of storage.

2.8.5. Microencapsulation Efficiency

Microencapsulation efficiency (ME) of microcapsules depends upon the nature of encapsulating agent, pH of the system, core: wall material ratio, homogenization pressure, method of preparation etc. In general, proteins are better microencapsulating agents than that of carbohydrates because of amphiphilic nature of proteins may be attributed to their better emulsifying and stabilizing properties thus they are good micro-encapsulating agents. The ability of proteins to generate repulsive interactions (steric and electrostatic) between oil droplets, and at the same time form an interfacial membrane that is resistant to rupture, plays an important role in stabilizing the droplets against flocculation and coalescence during long-term storage (McClements, 2004). Owing to its hydrophobic and hydrophilic regions, whey proteins are most commonly used as microencapsulating agents. However in case of octenyl succinylated starch; both hydrophobic and hydrophilic groups are introduced thereby improving their emulsifying capacity.

Several workers have worked on the microencapsulation of omega-3 rich oils and reported different ME for different wall materials. Tonon *et al*., (2011) worked on the microencapsulation of flaxseed oil by spray drying and reported that ME was significantly influenced by total solid contents and oil concentration. The higher the oil

concentration, the lower is the encapsulation efficiency, i.e., the higher the amount of surface (free) oil. The same behaviour was observed by Huynh *et al*., (2008) in the microencapsulation of lemon myrtle oil, using modified starch + maltodextrin and whey protein concentrate + maltodextrin as wall materials. Tan *et al*., (2005) also verified that high oil loadings resulted in lower process yield and lower encapsulation efficiency for microencapsulated fish oil by spray drying.

According to Jafari *et al*., (2008a), the poor retention or lower encapsulation efficiency related to higher oil loads can be attributed to the greater amount of core material close to the drying surface, which makes the diffusion path length shorter to the air/particle interface, thus increasing the surface oil content.

Minemoto *et al*., (2002), working with microencapsulation of linoleic acid, also observed that encapsulation efficiency decreased when the weight ratio of core to wall material increased. At higher ratios, the amount of wall material can be insufficient for fully covering the oil droplets and this insufficiency might result in a decrease in the encapsulation efficiency (Hogan *et al*., 2001a; Jafari *et al*., 2008b; Tonon *et al*., 2011).

Various researchers have reported different microencapsulation efficiency for Chia essential oil (70.70-80.70%; Rodea Gonzalez, 2012), flaxseed oil (62.3%; Carneiro *et al*., 2012) and algal DHA oil (82.16%; Karthik and Anandharmakrishnan, 2013), who applied whey proteins as a wall material. In the same way, Young *et al*., (1993) showed improved microencapsulation efficiency of anhydrous milk fat microcapsules when stabilized by a blend of whey protein and carbohydrate compared with whey protein alone.

Quispe-Condori *et al*., (2011) investigated microencapsulation of flax oil with zein (corn protein) using spray and freeze drying. The maximum microencapsulation efficiencies observed were 93.26 and 59.63% for spray drying and freeze drying, respectively.

Anwar and Kunz (2011) compared the influence of various drying methods (Spray granulation, spray drying and freeze drying) on the stabilization of fish oil microcapsules. The results indicated that microcapsules produced by spray granulation having a very low propanol content and a shelf life of 5 weeks at 21±1°C. In conclusions, spray drying is an economical and flexible process for microencapsulation (Velasco *et al*., 2003).

2.8.6 Powder flow properties

There are limited studies on powder flow properties of microcapsules of oils. Goyal, (2014) prepared microcapsules using WPC and sodium caseinate found poor flow properties of microencapsulated flaxseed oil powder. Tonon *et al*., (2012) also found less bulk density and tapped density of microencapsulated flaxseed oil powder and interpreted microcapsules being a fortificant are added in very less amount hence, the flow properties of powder do not affect more of its use.

2.9 Food fortification

Fortification as part of a country's nutrition strategy is supported by global organizations such as UNICEF, the World Health Organization (WHO), and the U.S. Centres for Disease Control and Prevention (CDC), the Global Alliance for Improved Nutrition (GAIN), and Nutrition International. In India, food fortification offers a tremendous opportunity towards improving the health and productivity of populations. Food fortification involves the identification of commonly eaten foods that can act as vehicles for one or more nutrients and lend themselves to centralized processing on an economical scale. Over the last 40 years, food fortification has played a major role in the human health and several nutritional deficiencies have been eliminated The Codex General Principles for the Addition of Essential Nutrients to Foods (61) defines "fortification", or synonymously "enrichment", as "the addition of one or more essential nutrients to a food whether or not it is normally contained in the food, for the purpose of preventing or correcting a demonstrated deficiency of one or more nutrients in the population or specific population groups" (FAO, 1996).

Foods that are consumed by all population groups regularly in predictable amounts, and are affordable, are the best vehicles for fortification. These are: wheat flour, oil, milk, salt, rice and legumes. With the expanding range of fortificant compounds available and the need to use various vehicles according to the designated target groups, there is need to consider the technologies best suited to achieve a fortified product with the desired properties.

2.9.1 Requirements for a food vehicle for fortification

- Commonly consumed by the target population
- Constant consumption pattern with a low risk of excess consumption

- Good stability during storage
- Relatively low cost
- Centrally processed with minimal stratification of the fortificant
- No interactions between the fortificant and the carrier food
- Contained in most meals, with the availability unrelated to socio-economic status
- Linked to energy intake (FAO, 1996)

2.10. Uses of Flaxseed or flaxseed oil in food and dairy products

Flaxseed is gaining the status of a functional food after centuries of use as natural medicine. Flaxseeds can be used as roasted and milled seeds, while flaxseed oil can be used in various food formulations in the form of neat oils, stable emulsions and micro- and nano-encapsulated powder. Bakery sector in the west has resorted to the method of adding ground flax seed into mixed grain bread for the purpose of meeting customer demands. Flax or flaxseed oil has been incorporated into baked foods (Payne, 2000; Pohjanheimo *et al*., 2006), juices, milk and dairy products (Ivanov *et al*., 2011; Dodin *et al*., 2008), muffins (Ramcharitar *et al*., 2005; Aliani *et al*., 2011), dry pasta products (Hall *et al*., 2005; Marconi, 2001 and Sinha and Manthey, 2008), macaroni (Hall *et al*., 2005) and beef patties (Bilek and Turhan, 2009).

2.10.1. Milk and dairy products

Milk and milk products are consumed by all age group on regular basis; thus, fatty acids. Nielsen *et al*., (2007) studied the oxidative stability of fish oil enriched drinking yoghurt. Studies showed enriched milk had a very good oxidative stability. Let *et al*., (2004) found that oxidative flavour of omega-3–enriched milk could be prevented by using a mixture of fish oil and rapeseed oil (1:1). A sensory panel was clearly able to distinguish between milk emulsions produced with fish oil and rapeseed oil mixture with a PV of 0.1 meq O_2/kg and those produced with oils of peroxide value 0.5, 1.0, or 2.0 meq O_2/kg (Let *et al*., 2005). Throughout storage, the milk emulsion with oil of PV 0.1 meq O_2/kg was perceived as being less fishy and rancid and could not be discriminated from milk without fish oil. Veena (2014) developed omega-3 enriched milk using flaxseed oil emulsion, and reported that milk was oxidatively stable for up to 5 days at 4-7°C temperature. Effect of ω-3 fatty acids fortification on sensory characteristics of various dairy products as studied by different authors are mentioned in Table 2.6.

Table 2.6 Sensory characteristics of dairy products fortified with ω-3 fatty acids

Dairy product	ω-3 fatty acid source	Effects on sensory characteristics	Reference
Milk	Flaxseed oil and DHA	Rancid flavour after 3 days of storage	Divya *et al.*, (2013)
Milk Butter	Fish oil in cow feed	Significant difference between flavour of samples and control during the storage	Baer *et al.*, (2001)
Milk	Algal oil emulsion	No significant difference between control	Gallaher *et al.*, (2005)
Milk Butter Cheddar cheese	Fish meal in cow feed	Fish meal added cheese had softer/smoother texture and stronger cheddar lavour after 6 months ripening	Avramis *et al.*, (2003)
Milk	Rumen protected tuna oil	Rating: no difference, no signs off lavour/fish oil lavour Triangle test: no significant difference	Kitessa *et al.*, (2004)
Milk and *dahi*	Flaxseed oil	No significant difference in sensory characteristics except flavour, which got lower scores	Veena (2014)
Ice-cream	Flaxseed oil	Decreased flavour scores with increased levels of flaxseed oil	Lim *et al.*, (2010)

2.10.2. Bakery products

Bread is a very popular food as delivery vehicle for a number of functional omega-3 fatty acids. Omega-3 oil ingredient should be protected from the liquid environment, shear, and heat during the bread making process. The incorporation of omega-3 oils into bread has been made possible by microencapsulation without affecting sensory properties and shelf life. The partial substitution of soy oil with flaxseed oil (25, 50 and 75%) in bread formulations resulted in an increased ALA content and the gradual reduction of the omega-6:omega3 ratio without negative effects on bread technical quality or sensorial attributes (Aguiar *et al*., 2011). Gokmen *et al.*, (2011) also developed functional bread fortified with omega-3 fatty acids. High amylose corn starch was used to form nano sized complexes with flax seed oil that was converted to powder of microparticles by spray drying. It was observed that encapsulation significantly decreased lipid oxidation as measured by the formation of hexanal and nonanal in breads during baking. Scanning electron microscopic analysis of bread demonstrated that particles added to dough remained intact in the crumb, but partially destroyed in the crust. Comparing to its free form, addition of nanoencapsulated flax seed oil increased final

product quality and safety by lowering lipid oxidation and formation of harmful compounds in breads during baking. A number of other cereal-based products such as breakfast bars and muesli bars fortified with microencapsulated omega-3 oils are available worldwide (Bagdan, 2000).

2.10.3 Infant formula

Omega-3 fatty acids are fortified in infant formula because of their benefits to early childhood development. Fortification of infant food with omega-3 fatty acids is quite challenging, due to the longer shelf life of the product (usually 1 year from manufacture) and the limited number of ingredients allowed in it. Omega-3 oil was added to infant formula without affecting its odour, flavour, and shelf life during storage. The first successful commercial entry into this market was seen in Australia and New omega-3 oils that had been specially formulated using ingredients already in use for infant formula manufacture. Omega-3 encapsulated powder was incorporated without affecting the sensory properties, shelf life, and the ingredient listing of the original product. There is now a number of commercial infant formula products fortified with omega-3 fatty acids using a whole range of incorporation technologies, such as Nestle, Similac, Enfamil A+, Dexolac, etc.

2.11. Milk - an ideal vehicle for fortification

Milk provides a convenient and useful vehicle for addition of certain nutrients to human diet. Milk is a popular food for fortification because of its wide availability and acceptance. It is economical when compared with, for example, supplements, and is a commonly used food in the home. The stability and bioavailability of added micronutrients in the milk remain high (Mannar, 2003). Secondly, milk and milk products can easily fulfil the requirements for the three main drivers of functional foods, i.e. health, taste and convenience. Though milk has the major nutrients required by the human body, it lacks adequate levels of omega-3 fatty acids. Therefore, fortification of milk with these substances will greatly enhance the nutritive and therapeutic values. Based on these considerations, milk appears to be an ideal vehicle for omega-3 fatty acid/alpha linolenic acid fortification. Therefore the present study was aimed to fortify milk with omega-3 fatty acids, so as to increase health benefits and to provide recommended daily intake of the omega-3 fatty acids.

Chapter 3

Scope and Plan of Work

3.0 Scope and Plan of Work

3.1 SCOPE

Keeping in view the need of functional foods in human life, the products rich in various functional ingredients such as omega-3 and omega-6 fatty acids are being developed by several approaches. Preparing emulsions of high-value oils and ingredients and microencapsulating the bioactive components for sustained release in the human body is one way of developing functional foods. Considering the importance of omega-3, omega-6 and alpha-linolenic acids (ALA) in the human health, the present investigation was planned with the aim of using soy protein isolate and modified starches as coating materials to encapsulate flaxseed oil by using spray drying technique and then followed by fortification of milk with the developed microcapsules. The objectives of the present investigation are:

1. To optimize the process for microencapsulation of flaxseed oil using soy protein isolate and modified starches
2. To fortify milk with omega-3 fatty acids using developed microencapsulated flaxseed oil
3. To characterize the fortified milk for physicochemical properties and shelf life evaluation

3.2 PLAN OF WORK

3.2.1. Preliminary trails

Preliminary trails were taken for preparation of emulsion with the objective of fixing the level of ingredients in emulsion and microcapsules. The quantity of soy protein isolate was fixed at the level of 5%W/W of total solids of emulsion based on the viscosity of the emulsion. The oil load of the emulsion was varied with 25%, 30%, and 35% of total solids (TS) while, TS was maintained at 20%, 25%, and 30% by varying the percentage of modified starch. For homogenization, depending upon the particle size of the emulsion, the RPM and time for homogenization were fixed at 18000 rpm for 5 min.

For preparation of microencapsulated flaxseed oil powder by spray drying, the inlet and outlet temperatures were prefixed by analysing the final moisture content and microencapsulation efficiency.

For the preparation of fortified milk, the level of addition of microcapsules was decided for providing at least 25 % of the recommended dietary allowance of alpha linoleic acid and sensory acceptability of the fortified milk.

3.3 Process optimization for preparation of emulsion, microencapsulation and fortification of milk with microcapsules

3.3.1. Emulsion

The emulsions were prepared by initial mixing of soy protein isolate, modified starch and flaxseed oil using hand blender for proper mixing of phases and then subjected to ultra -turrax (IKA T18, Germany) with batches (500mL) at 18000 rpm for 5 min. The prepared emulsion was analyzed for the following properties

Characterisation	**Analytical method**
Oxidative stability	Peroxide value
Creaming stability	Method given by Kuhn and Cunha (2012)
Droplet size and zeta potential	Zetasizer (Malvern Instruments)
Colour measurement	Colorimeter (Hunter Colour)
Rheological properties	Rheometer (Anton paar)

3.3.2. Microencapsulated flaxseed oil powder (MFOP)

The microcapsules were prepared from emulsion using spray drying technique. Following conditions of spray dryer were maintained while drying the emulsions:

- Inlet air temp: 180 ± 5˚C
- Outlet air temp.: 80 ± 5˚C
- Feed flow rate: 65 ± 5 ml/min

Microcapsules were collected after drying and stored in aluminium foil laminates at refrigeration temperature (4-7˚C) for storage study. The prepared microencapsulated flaxseed oil powder were characterized for

Parameters/Properties	**Analytical method**
Microencapsulation efficiency	Method described by Bae and Lee (2008)
Particle size and morphology	SEM (Cornell EDS)
Moisture content	AOAC Method
Colour measurement	Colorimeter (Hunter Colour)
Bulk density	BIS Method
Solubility	Method described by Anema *et al.,* (2006)
In-vitro release study	Simulated gastric and intestinal method Burgar *et al.,* (2009)

3.3.3. Fortified Milk

Standardized cow milk (3.0% Fat & 8.5% SNF) was used for fortification with MFOP. The microcapsules were added in particular quantity so as to meet RDA of ALA at least 25, 37.5 and 50% in to milk. The samples were then pasteurized/sterilized. To increase the acceptability of fortified milk vanilla flavoured milk was prepared by addition of artificial vanilla flavour and sugar for selected level of microcapsules in milk

3.3.3.1. Physicochemical properties of fortified milk

Properties	**Analytical method**
Proximate composition	IS: SP-18 (1981)
Titratable acidity	IS 11766 – 1986
Colour	Adobe Photoshop
Viscosity	Ostwald viscometer (make)
Free fatty acids content	Method described by Deeth *et al.,* (1975)
Sensory evaluation	Nine point hedonic scale

3.3.3.2. Shelf life evaluation of fortified milk

Shelf life of fortified milk was determined by evaluating the following properties for pasteurised milk. The shelf life study of pasteurised milk was carried out for 6 days with interval of 2 days while, for sterilized milk, shelf life study was carried out for 28 days with interval of 7 days.

Analysis/Property	**Analytical method**
Microbiological analysis	Standard plate count
	Yeast and mould
	Coliform
Titrable acidity	IS 11766 – 1986
Free fatty acids content	Method described by Deeth *et al.*, (1975)
Sensory evaluation	Nine point hedonic scale

3.4 Statistical Analysis

The statistical analysis was performed using SPSS software.

Chapter 4

Materials and Methods

4.0 Materials and Methods

This chapter encompasses details of materials and experimental methodologies, procedure, techniques employed during the investigation. Methodologies related to the technological, physico-chemical and statistical aspects are delineated here under.

4.1 Materials/ Ingredients:

4.1.1. Flaxseed oil

Cold pressed flaxseed oil was received from AAK Kamani Pvt. Ltd. Andheri, Mumbai (Maharashtra). The physicochemical parameter of the oil as provided by company are given in following Table 4.1.

Table 4.1 Technical specifications of commercial flaxseed oil

Sr. No.	Parameters	Results
01	Colour(1”Cell) Lovibond	3.0Y+0.4R=5.0
02	Moisture content (In %)	0.015%
03	Refractive Index (at 40^{o}C)	1.4725
04	Free fatty acids	0.06%
05	Acid value	0.12
06	Iodine value	173.25
07	Peroxide value	0.44
08	Sap value	191.58
09	Unsaponifiable value (in %)	0.29%

4.1.2. Soy protein isolate (SPI)

SPI was procured from Shridurga Sales Corporation, Bangalore (imported from China Shandong Yuxin Biotech Cp. Ltd.). The specification of SPI as provided in the certificate of analysis are mentioned in Table 4.2.

Table 4.2. Technical specifications of commercial soy protein isolate

Sensory organ index		
Item	**Standard**	**Result**
Appearance	Light yellow powder	Light yellow powder
Odour	Neutral to nutty	Neutral to nutty
Flavour	Pleasant nutty	Pleasant nutty
Physical and chemical index		
Crude protein	90.00% min	90.50
pH value	7.00±0.5	6.75
Moisture %	7.0 max	5.65
Ash(dry basis)%	6.0 max	5.10
Functional index		
Dispersability	Within 25 sec.	Within 20 sec.
Microbial index		
TPC	20000 max	1000
E.coli	Negative	Negative
Conclusion	**Eligible**	

4.1.3. Modified starches

4.1.3.1. N creamer 46

N-CREAMER 46 (NC 46) was procured from Ingredion India Private Ltd, Thane Maharashtra. It is a unique, octenyl succinic anhydride modified food starch based on waxy maize. It is characterized by excellent emulsion stabilizing and encapsulating ability. NC 46 is cold water soluble. Spray-dried emulsions reconstitute immediately without lumping NC 46

4.1.3.2. N Creamer 180

N-CREAMER 180 (NC 180) was procured from Ingredion India Private Ltd, Thane Maharashtra. It is an octenyl succinic anhydride modified tapioca starch designed primarily for the dairy industry to replace milk solids non fat (MSNF) typically in dairy products. NC 180 has been formulated to be extremely bland and hence will not contribute to flavour. It is white to off-white in colour.

4.1.4. Milk

The cow milk received from the experimental dairy of National Dairy Research Institute, Bangalore was standardized to 3% fat, and 8.5% SNF with the help of skim milk and cream obtained from the same whole milk.

4.1.5. Skim milk

Skim milk separated from fresh cow milk at SRS-ICAR, NDRI Bengaluru experimental dairy plant was taken for standardisation of milk.

4.1.6. Cream

Cream (50-60% fat) was separated by open bowl centrifuge from fresh cow milk at SRS-ICAR, NDRI Bengaluru Experimental dairy plant was used for standardisation of milk

4.1.7. Skimmed milk powder

Nandini brand spray dried skim milk powder manufactured by Mother Dairy, Yelahnka, Karnataka Milk Federation, (KMF) Bangalore was used to increase the SNF content of milk. The powder as declared by the manufacturer contained less than 3% moisture and less than 1% fat.

4.1.8. Packaging material

Pasteurised milk was packed in mixed polymer film provided by Experimental dairy SRS-NDRI plant. Metalized polyester-LDPE laminate pouches for packaging of flaxseed oil microcapsules were provided by Experimental dairy ICAR-NDRI, Karnal.

4.1.9. Glass Bottles

Glass bottle (250mL) and cork were purchased from local Bangalore market for filling sterilized milk.

4.2. Equipment

4.2.1. High shear mixer

Emulsion of flaxseed oil, soy protein isolate and modified starches was prepared by using high shear mixer (IKA ULTRA-TURRAX T-18, Germany). It is a high shear mixer with speed range: 3000 - 25000 rpm and volume range: 1mL to 1500 mL.

4.2.2. Spray Drier

Microencapsulated flaxseed oil powder was obtained by spray drying the emulsion. The pneumatic nozzle type spray dryer (Technosearch Instrument, Thane,

Mumbai, Maharashtra), installed in Technology Business Incubator (SINED-TBI) at National Dairy Research Institute, Karnal, Haryana was used in the present investigation with a feed rate of 40-60mL/min and water evaporation capacity of approx. 5 kg/h.

4.2.3. Cream separator

Open bowl cream separator (Kamdhenu, New Delhi, India) was used to separate cream and skim milk from whole milk.

4.2.4. Homogeniser

Laboratory scale homogeniser (H-102, LAB-03, 20-50 LPH, 200 Kg/cm^2; GOMA, Mumbai, India) was used to homogenize milk samples.

4.2.5. Zetasizer

A Zetasizer nano series (Ver 6.30, Malvern Instruments Ltd., UK) was used to measure zeta potential of emulsion and also for droplet size of emulsion.

4.2.6. Particle size analyser

A Malvern 3000 particle size analyser (Malvern Instruments Ltd., UK) was used to measure microcapsules size and particle size distribution in prepared samples by dry method.

4.2.7. Water activity meter

Water activity meter (AQUA Lab, Decagon Devices, WA, USA) was used to measure a_w of microcapsules after preparation.

4.2.8. Scanning electron microscope

For surface morphology, Scanning electron microscope (ZEISS, EVO 55 Ultra, UK) was used, while for gold plating Sputter Coater (Quorum Q 150 R ES) was used. SEM analysis was performed using facilities at Micro and Nano Characterization Facility (MNCF), CeNSE, IISc, Bengaluru.

4.2.9. Hunter Lab Colorflex Colorimeter

Colorflex- colorimeter, of Hunterlab (Hunter Associates Laboratory, Reston, VA, USA) was used to measure colour of emulsion and microcapsules.

4.2.10. Gas chromatography–mass spectrometry (GC-MS)

Fatty acids profile of selected microcapsules were analysed by GC-MS (Agilent 5977E GC/MS) to measure the level of ALA in developed microcapsules.

4.2.11. Autoclave

Autoclave (Excel Scientific, Bangalore, India) was used for sterilization of milk samples and microbiological media.

4.2.12 Fourier Transfor-Infrared (FT-IR) Spectroscopy

FT-IR (Shimadzu IR Affinity-1S, Japan) was used to identify chemical bonding in developed microcapsules

4.2.13. Other equipment

a) Hand blender: Bajaj Electricals, India

b) Vacuum packaging machine: Indvac, Saurabh Engineers, Ahmedabad, India

c) BOD incubator: Metrex Scientific Instruments Ltd., New Delhi, India

d) pH meter: EUTECH Instruments, India

e) Magnetic stirrer: Remi Stirrer, Mumbai, India

f) Hot air oven: Falcon Scientific Instruments, India

g) Laboratory centrifuge: REMI Laboratory Instruments, Mumbai, India

h) Weighing balance: Shimadzu corporation, Japan

i) Vortex shaker: Remi Laboratory Instruments, Mumbai, India

j) Sealing Machine: Pactec India, Bangalore

4.3 Apparatus and glassware

All volumetric flasks, burettes, conical flasks pipettes and other glassware were washed, cleaned and dried before use. Glassware were procured from Borosil India Ltd., Mumbai, India; parafilm from Sigma Aldrich, St. Louis, MO, USA; and filter papers (HIMEDIA filter paper no. 1, 4 and 41) from HiMedia Laboratories Pvt.

Limited, Mumbai. Packaging material- Aluminium foil laminates having thickness 80-100 µm were purchased from local market, Bangalore.

4.4. Chemicals and Reagents:

All the chemicals used in the study were of AR grade and were procured from standard companies. The reagents required for analysis were freshly prepared adopting standard procedures.

4.4.1. Preparation of 0.01 N solution of $Na_2S_2O_3$

To prepare a 0.1 eq/L (or 0.1 mol/L) sodium thiosulphate solution, 24.818 g of $Na_2S_2O_3.5H_2O$ was dissolved in 500 mL of freshly distilled water (or freshly boiled and cooled deionised water) and 2 or 3 drops of $CHCl_3$ (or also 0.4 g of NaOH) and volume was made up to 1000 mL using a volumetric flask.

4.4.2. Saturated potassium iodide (KI) solution

Some quantity of water was boiled for 5 minutes and allowed to cool. To a portion, enough potassium iodide was added to ensure a saturated solution (~10 g KI in 6 mL water).

4.4.3. Starch indicator (1%)

One g of soluble starch was weighed and paste was made in 30 mL of water. The paste was transferred to 80 mL boiling water and heated until a clear solution was obtained. The contents were cooled and stored in a tight stoppered bottle.

4.4.4. Phenolphthalein indicator solution

One g of phenolphthalein was weighed and taken in a 100 mL volumetric flask containing about 50 mL of 95% ethanol. The flask was stoppered and shaken vigorously for few minutes. 20 mL more ethanol was then added and shaken until a clear solution was obtained and volume was finally made to 100 mL with 95% ethanol.

4.4.5. Standard aqueous sodium hydroxide solution (0.1N)

0.1 N aqueous NaOH solution was prepared and stored in coloured glass bottle.

4.4.6. Methanolic KOH solution (0.005 N)

0.1 N methanolic KOH was prepared by dissolving 5.8 g of KOH in 1000 mL methanol and was standardised against 0.1 N oxalic acid. Suitable volume of this solution was diluted to 100 mL using methanol to get 0.005 N methanolic KOH.

4.5 Optimization of flaxseed oil emulsions

For preparation of emulsion the quantity of soy protein isolate is fixed at level of 5%W/W of total solids of emulsion based on viscosity of emulsion. The oil load of emulsion was 25%, 30% and 35% of TS while, TS was maintained at 20%, 25% and 30%. The sample preparation were as given in Table 4.3.

Table 4.3 Oil load and total solid content for preparation of emulsion samples

Sr. No.	Oil Load (% of T.S.)	Total Solids (%)	SAMPLE NAME	
			NC 46 starch	NC 180 Starch
1	25	20	A1	B1
		25	A2	B2
		30	A3	B3
2	30	20	A4	B4
		25	A5	B5
		30	A6	B6
3	35	20	A7	B7
		25	A8	B8
		30	A9	B9

4.5.1. Preparation of emulsion

For preparation of flaxseed oil emulsion, flaxseed oil, soy protein isolates (90%) and modified starches were mixed in calculated amount by using hand blender (Phillips, India) for approximately 5 minutes and then prepared solution was homogenised using high shear mixture (IKA T-18, Germany) in order to obtain stable emulsion.

The prepared emulsions were then stored at refrigerated condition (4-7˚C) and further analysed for various properties.

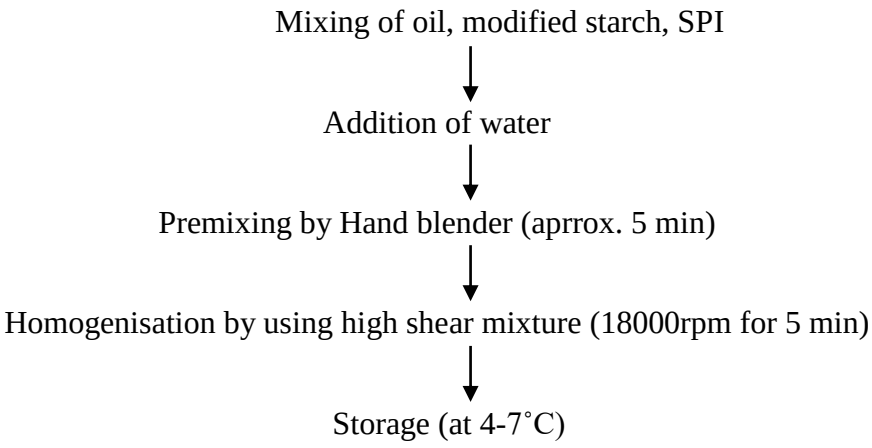

Figure 4.1 Flow chart for preparation of flaxseed oil emulsions

4.5.2. Physico- chemical analysis of emulsion

4.5.2.1. Creaming index

Immediately after preparation, 15 mL of each emulsion were poured into a centrifuge tube (internal diameter = 11 mm, height = 94 mm), sealed with a plastic cap and centrifuged at 2000rpm for 10 min. The emulsion stability was measured by the change in height of the bottom serum phase (H) with storage time. The creaming index (CI) was determined according to Eq. (1). The analyses were carried out in triplicate.

$$Creaming\ index = \frac{H}{H_0} \times 100 \quad (1)$$

Where,

H represents the separated phase of emulsion

H_0 represents the initial height of the emulsion

4.5.2.2. Physical stability

Samples of each emulsion were stored in 50 mL glass bottles (internal diameter = 45 mm, height = 70 mm) stored at refrigerated temperature (4-7^{o}C) for 28 days and sample were visually observed for any separation of oil. To facilitate visualization of the phase separation, Sudan III (reddish dye) was added to the flaxseed oil before preparing the emulsion.

4.5.2.3. Zeta (ζ) – potential and droplet size

The electric charge on the particles of flaxseed oil emulsion and droplet size of emulsion were measured by using Zetasizer nano series ZS90 (Malvern Instruments Ltd., UK). About 1 mL of the emulsion was added to 99 mL of distilled water at 25 °C to measure the particle size. The emulsions were analyzed 1 day after their preparation. Emulsion droplet size is expressed as Z-average diameter (nm) and ζ-potential in mV.

4.5.2.4.Rheological measurements

Steady shear measurements were performed using a dynamic rheometer (Anton Paar Rheometer, MCR-52, Austria, Europe). The probe with 75 mm dia, 1° cone angle having cone-and-plate geometry (CP 75/1°) was used for viscosity measurements. Emulsion viscosity was measured at 25±0.1 °C, over a shear rate range of 5-100/s. Viscosity was measured every 7th day till 28 days of storage at low temperature (4–7 °C).

Figure 4.2 Rheometer

4.5.2.5. Oxidative stability

The selected emulsion samples were evaluated for oxidative stability every 7th day till 28 days of storage at low temperature (4–7 °C).

4.5.2.5.1.Peroxide value

To determine the peroxide value, oil was extracted from emulsion by the method of Folch *et al.,* (1957) with slight modifications. Twenty grams of sample was mixed in 200 mL cold mixture of chloroform: methanol (2:1) in a separating funnel. After shaking gently for 3 min, mixture was allowed to stand for 10 min. A lower chloroform layer was removed separately. Upper layer was washed with 100 mL of chloroform: methanol (2:1) mixture and again lower chloroform layer was removed and mixed with previous one followed by mixing with 40 mL distilled water. After phase separation, lower chloroform layer was collected, and evaporated by using water bath which was pre-maintained at 40-60°C. Peroxide value of extracted oil was evaluated at every week during storage of emulsion for 28 days by the standard iodometric method of AOAC (2005)

About 5±0.5 g extracted oil was taken in a flask and mixed with 30 mL of mixed solvent (acetic acid: chloroform, 3:2) followed by the addition of 0.5 mL of saturated KI solution. The contents of the flask were heated for one minute on boiling water bath with occasional shaking. After cooling the contents of the flask, 30 mL of water was added. The solution was then titrated with 0.01 N Sodium thiosulphate solutions with vigorous shaking until yellow colour disappeared. 0.5 mL of starch solution (1%) was then added and titration was continued to release all I_2 (Iodine) from chloroform layer until blue colour disappears. Blank determination was also conducted. Peroxide value was calculated according to the following formula (2).

$$Peroxide\ value = \frac{V \times N \times 100}{Wt.of\ sample} \quad (2)$$

4.5.2.5.2. Free fatty acids (FFA) content of emulsion

An extraction titration method devised by Deeth *et al*., (1975) was followed for the determination of FFA content of emulsion samples. Thirty millilitres of emulsion sample was taken in a 500 mL glass stoppered conical flask. To this 100 mL of freshly prepared extraction mixture (isopropanol, petroleum ether and 4 N sulphuric acid in the ratio of 40:10:1, respectively) was added followed by 50 mL petroleum ether. The conical flask was stoppered and shaken vigorously for 25-30 s and then it was transferred to separating funnel allowed to settle for 10-15 min or till the two layers get clearly separated. Solvent was evaporated in waterbath at 50^{o}C. Separated fat was taken for titration by noting the weight, six drops of 1% methanolic phenolphthalein were added to it and titrated against 0.005 N methanolic potassium hydroxide (KOH) solution. A blank using 5 mL of water instead of milk was used to obtain the blank titre value. The FFA content in micro equivalents per mL emulsion was obtained using the following formula (3):

$$\text{FFA (}\mu\text{Eq/ mL)} = \frac{N}{5P} \times 1000 \times (V2 - V1) \quad (3)$$

Where,

N=Normality of KOH solution

P=Proportion of upper layer in separating funnel

V2=Volume of standard KOH solution used for the milk sample

V1=Volume of standard KOH solution used for the blank

4.6. Method of preparation flaxseed oil microcapsules (MFOP)

Emulsion prepared as per the section 4.5.1 were preheated in water bath up to 40˚C in order to decrease the viscosity for proper atomisation of flaxseed oil emulsion in drying chamber of spray dryer. The drying conditions were maintained as Inlet hot air temp.: 180±5˚C and outlet hot air temperature 85±5˚C, while, flow rate was maintained from 40-60 mL/min to control the outlet air temperature.

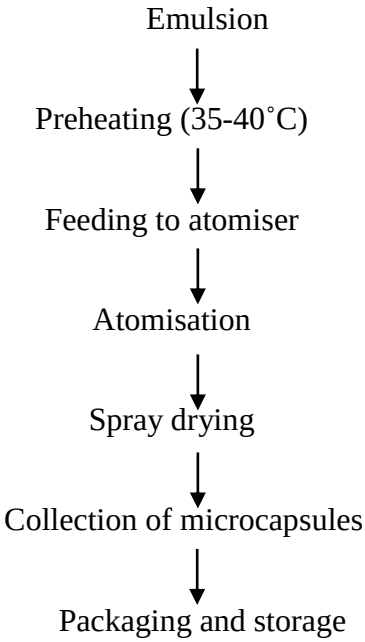

Figure 4.3 Process flow chart for preparation of flaxseed oil microcapsules using spray dryer

4.7. Physico-chemical characterization of microencapsulated flaxseed oil powder (MFOP)

4.7.1. Moisture content

The moisture content of MFOP was determined gravimetrically as per the method of BIS (2001a). Each analysis was repeated three times. The clean and washed aluminium dish was kept in the oven at 102±2°C for 1 h. The dish was allowed to cool in desiccator for 30 to 40 min. A known quantity of the sample (4 to 5 g) was weighed in the dish (also noting down the empty weight of the dish). The dish was placed in the oven maintained at 102±2°C for 3 h. The dish was removed, placed in the desiccator, allowed to cool and weighed. The dish was put back in the oven for another 1 h, placed

in the desiccator, cooled and weighed again. The process of heating, cooling and weighing was repeated every 1 h till consecutive readings with a difference of no more than 0.5 mg was obtained. From the amount of the residue left in the dish after evaporation of moisture, total solids content was calculated using the following formula:

$$\text{Moisture } (\%) = \frac{(\text{M2}-\text{M1})}{(\text{M})} \times 100 \quad (4)$$

Where,

M2 = Mass in g of the contents of dish after drying

M1 = Mass in g of empty dish taken for the test

M = Mass in g of material taken for the test

4.7.2. Water activity (a_w)

Water activity (a_w) of spray dried MFOP was measured using water activity meter Aqua lab (Model Series 3 TE) supplied by M/s Decagon Devices, WA, USA. First, the instrument was calibrated with charcoal powder at 25°C and then triplicate measurements were performed with the sample.

4.7.3. Bulk (ρB) and tapped (ρT) density

Bulk (ρB) and tapped (ρT) densities were calculated by the method given by Chinta *et al.,* (2009). Five gram of each powder sample (m) was filled in 25 mL measuring glass cylinder (Borosil) and the cylinder was slightly tapped to remove the powder sticking to the walls. The Volume (Vo) was read directly from the cylinder and bulk density was calculated by using following formula (Eq. 5). For tapped density (ρT), the cylinder was tapped until a constant volume (Vn) was reached calculated by using following formula (Eq. 6).

$$Bulk\ density(\rho B) = \frac{Wt.of\ sample(m)}{Volume\ of\ sample(\text{Vo})} \quad (5)$$

$$Tapped\ density(\rho\text{T}) = \frac{Wt.of\ sample(m)}{Volume\ of\ sample\ after\ tapping\ (\text{Vn})} \quad (6)$$

4.7.4. Flowing properties

The flowing characteristics of powder were evaluated by using Carr's Index (Compressibility Index: C) and Hausner Ratio (HR) by the method given by Turchiuli *et al.*, (2005). Carr's Index indicates the compressibility or free-flowing property; while HR indicates the cohesiveness of powder. The Carr's Index (C) and Hausner Ratio (HR) were calculated using bulk density and tapped density by the following equations:

$$Carrs\ index = \frac{Tapped\ density - Bulk\ density}{Bulk\ density} \quad (7)$$

$$Hausner\ index = \frac{Tapped\ density}{Bulk\ density} \quad (8)$$

4.7.5. Particle size distribution and average particle size of powder

Particle size and Particle distribution profile was measured by Mastersizer 3000 (Malvern Instruments Ltd., UK) at Aimil Ltd., Bengaluru. The microcapsule size was analysed by dry method with feed rate range 40ms^{-2}. Different parameters related to particle size such as specific surface area (SSA, m^2/kg), median particle size distribution (d_{10}, d_{50} and d_{90}), Volume mean diameter, ($D_{4,3}$) and ($D_{3,2}$) were determined. Use of particle size distribution and volume mean diameter has been reported in the literature to characterize the particle size of microcapsules (Kelly *et al.*, 2015 and Crowley *et al.*, 2014).

4.7.6. Colour measurement

A Tristimulus spectrophotometer Hunter Lab model Colour Flex® (MiniScan XE plus, Hunter Associates Laboratory Inc. Reston, Virginia, U.S.A.) along with the software (version 4.10) was used to measure the colour of the flaxseed oil microcapsules and the results were expressed in terms of the CIE-LAB system. The instrument was standardized in day light at reflectance angle 10° (i.e. illuminate D65/10° standard observer). Before the test, the instrument was calibrated with standard black and white tiles as specified by the manufacturer (i.e. L* 50.83, a* -26.27 and b* 12.12). The light source was dual beam xenon flash lamp. Measurements were then made

Figure 4.4 Hunter colour lab

on the sample taken in a glass sample cup (10 cm height and 6 cm diameter) supplied with the instrument by filling it to a fixed level (up to 3 cm) for each sample. Data were received through the software in terms of L* (lightness), ranging from 0 (black) to 100 (white), a* (redness), ranging from +60 (red) to -60 (green), and b* (yellowness), ranging from +60 (yellow) to -60 (blue) values. Three random readings of colour for each sample were recorded and averaged. Further Hue angle (9) and Chroma (10) are determined by using following formula.

$$Hue\ angle = \frac{ATAN(\frac{B*}{A*})\times 180}{3.14159} \quad (9)$$

$$Chroma = SQRT(A *\times A * + B *\times B *) \quad (10)$$

4.7.7. Morphology of microcapsules by Scanning Electron Microscopy (SEM)

Microstructure of spray dried MFOP microcapsules was studied by Scanning Electron Microscopy (SEM). The dry microcapsules were sprinkled onto circular aluminium stubs with double sticky tape. The excess powder was properly brushed to get uniform layer of sparsely scattered powder particles and coated with gold nano-particles (25Å) in a sputter coater (Q150R Rotary-Pumped Sputter Coater). The ion current was maintained at 6 mA with a fine vacuum of 0.07 Torr for 4 min. The observations were made using Zeiss ultra 55 Special edition Electron Microscope at 1000, 2000 and 5000X magnification at a working distance of 8 mm with an accelerating potential of 15 kV. The images of selected area were recorded with the help of Smart SEM software.

4.7.8 Fourier Transform Infrared (FTIR) Spectroscopy

Electromagnetic radiations that interact with a substance can be absorbed, transmitted, reflected, scattered, or have photoluminescence (PL), which provides significant information on the molecular structure and the energy level transition of that substance. Samples placed in the path of an infrared beam will absorb and transmit light and then the light signal will penetrate through the sample and finally to the detector. The detector measures the intensity of the radiation moving into a sample and the intensity of the radiation transmitting through a sample. Spectra is output as a function of time is converted into a plot of absorption against wavenumber by a computer using a Fourier transform method. In this experiment, the application of an ALPHA FTIR spectrometer was used to measure infrared spectra of the functional groups of transformer flaxseed oil

microcapsules. FTIR using an attenuated total reflection (ATR) technique was used in this experiment to investigate the structural changes of microcapsules by obtaining its infrared spectra. The penetration depth of a light beam using an ATR technique into a sample is about 0.5–3 µm. Each sample was analysed twice to ensure the infrared spectra of the investigated microcapsules samples. The observed spectra are the absorbance of the different samples versus the wavenumber range 4000–400 cm^{-1}.

4.7.9. Total oil & Surface (free) oil

Total oil (TO) content was calculated using gravimetric method given by AOCS (2000) with slight modifications. In brief, 5 g powder was taken in beaker and oil was extracted by 60mLof chloroform: methanol (40:60) at 50°C for 2 h on magnetic stirrer. After extraction of oil, the solvent was evaporated using waterbath at 60-80°C temperature and the extracted oil was weighed.

Free or surface oil was calculated by the method given by Hogan (2001b). 100mL of petroleum ether was added to 5 g of spray dried powder in a conical flask and mixed well at room temperature for 15 minutes to extract the surface oil. The solvent mixture was filtered through Whatman filter paper 41. The powder collected on the filter paper was again rinsed with 50 mL petroleum ether, which was mixed with previous filtrate. The filtrate was evaporated using rotary flash evaporator under vacuum at 60°C. The free oil was weighed by the following formula (11):

$$free\ oil(\%) = \frac{Wt.of\ beaker\ with\ oil(g) - Wt.of\ empty\ beaker(g)}{Wt.of\ sample\ (g)} \times 100 \qquad (11)$$

4.7.10. Microencapsulation efficiency (ME %)

Microencapsulation efficiency was calculated by the method given by Hogan (2001b) using the following Equation (12):

$$Microencapsulation\ efficiency(\%) = \frac{Total\ oil - Surface\ oil}{Total\ oil} \times 100 \qquad (12)$$

4.7.11. Peroxide value (PV)

Peroxide value of microencapsulated flaxseed oil was evaluated at every month during storage by the standard AOAC (2005) Iodometric method. To determine the peroxide value, oil was extracted from emulsion by the method of Folch *et al.,* (1957)

with slight modifications. Thirty g of sample was mixed in 200 mL cold mixture of chloroform: methanol (2:1) in a separating funnel. After shaking gently for 3 min, mixture was allowed to stand for 10 min. A lower chloroform layer was removed separately. Upper layer was washed with 100 mL of chloroform: methanol (2:1) mixture and again lower chloroform layer was removed and mixed with previous one followed by mixing with 40 mL distilled water. After phase separation, lower chloroform layer was collected, and evaporated in water bath the obtained fat was analysed for the peroxide value by method described in (4.5.2.5.1.)

4.7.12 *In-vitro* release behaviour

In-vitro release behaviour of flaxseed oil microcapsules was investigated using a simulated gastrointestinal model according to the method given by Burgar *et al.,* (2009) with slight modifications. Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) were prepared by the method given below:

- **Preparation of Simulated Gastric Fluid (SGF)**

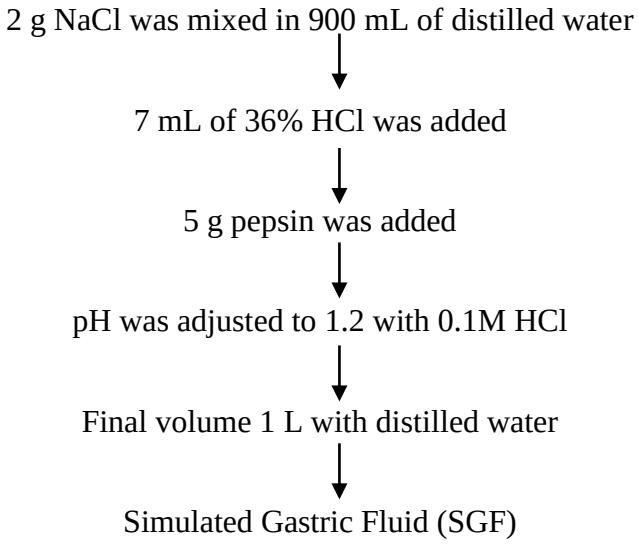

Figure 4.5 Process flow chart for preparation of Simulated Gastric Fluid (SGF)

- **Preparation of Simulated Intestinal Fluid (SIF)**

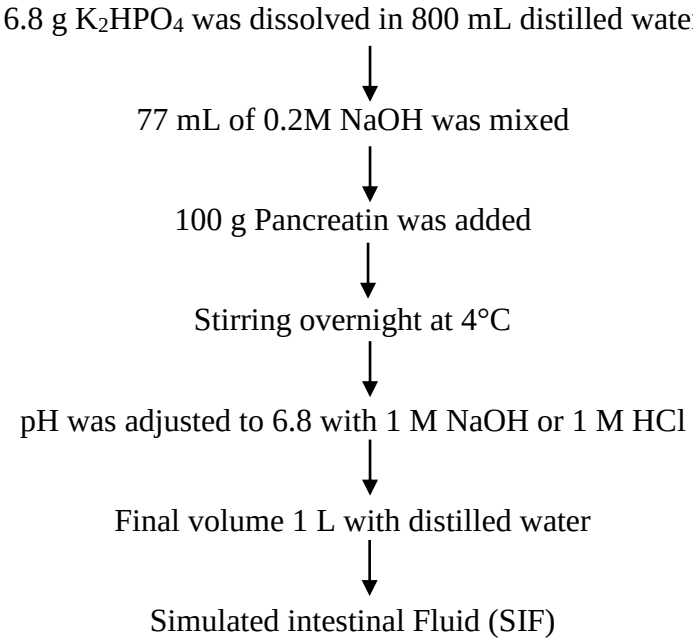

Figure 4.6 Process flow chart for preparation of Simulated Intestinal Fluid (SIF)

4.7.12.1. *In-vitro* release of flaxseed oil from microcapsules exposed to SGF conditions

Five g microencapsulated flaxseed oil powder was mixed with 50 mL of distilled water followed by the addition of 50 mL SGF and incubated at 37°C for 2 h (conditions for incubation were created in a conical flask. The contents were continuously mixed using magnetic beads and magnetic stirrer. Released flaxseed oil was extracted using 30 mL petroleum ether and 30 mL di-ethyl ether in a separating funnel. Extraction was repeated two times with 40 mL of mixture of petroleum ether and di-ethyl ether (1:1). Solvent was evaporated at 80°C and extracted oil was dried in hot air oven maintained at 100±2°C for 30 min and the quantity of released oil was determined gravimetrically.

4.7.12.2. *In-vitro* release of flaxseed oil from microcapsules exposed to SGF + SIF

For sequential exposure to SGF and SIF, 5 g flaxseed oil powder was mixed with 50 mL of SGF and incubated under the same conditions as mentioned above (Section 4.7.12.1.). After 2 h, pH of the sample was adjusted to 6.8 using 1 M NaOH, followed by addition of 50 mL of SIF and the sample was incubated for 3 h. The amount of flaxseed oil released from the microcapsules was extracted in petroleum ether and di-ethyl ether as described above (Section 4.7.12.1.) and determined by gravimetric analysis.

4.7.13 Fatty acids profile of microencapsulated flaxseed oil using GC-MS

4.7.13.1. Extraction of fat

Oil was extracted from microcapsules by the method of Folch *et al.,* (1957) with slight modifications. Twenty grams of microcapsules were mixed in 200 mL cold mixture of chloroform: methanol (2:1) in a separating funnel. After shaking gently for 3 min, mixture was allowed to stand for 10 min. A lower chloroform layer was removed separately. Upper layer was washed with 100 mL of chloroform: methanol (2:1) mixture and again lower chloroform layer was removed and mixed with previous one followed by mixing with 40 mL distilled water. After phase separation, lower chloroform layer was collected, and evaporated by using water bath which was pre-maintained at 40-60°C.

4.7.13.2. Methylation

Extracted fat was derivatized Fatty Acid Methyl Esters Fatty acids following the Official Methods of the American Oil Chemists' Society (AOAC, 2005a) with slight modifications. The oil sample was suspended in 2 mL hexane prior to derivatization. To the flask containing the fat, 5 mL of 0.5 N methanolic sodium hydroxide was added with a few boiling chips (anti bumping granules). Water cooled condenser was attached and refluxed on a heating plate for 1 hr. To the boiling mixture, 5 mL of boron tri fluoride (BF3)-Methanol (10% vol/vol) was added through the top of the condenser then refluxed for 30 minutes. About 5 mL of hexane was then added and refluxed for 1 min. While still attached to the condenser, the round bottom flask was raised above the heating plate and allowed to cool for 15 minutes. To the cooled extract, 10 mL of saturated sodium chloride (NaCl) solution was added to the flask and stoppered after disconnecting the condenser. The contents were shaken vigorously for 2 min and left to stand for 10 min followed by additional 3 mL of saturated sodium chloride (NaCl) to float the hexane layer. The hexane layer containing the methyl esters was transferred into a test tube with a small scoop of anhydrous sodium sulfate to dry the esters and the test tube was then capped. The dried sample was transferred into a 2 mL vial and kept at −4˚C until GC-MS analysis.

4.7.13.3. Method

All samples were analysed on Agilent Technologies 5977E GC/MS system (Agilent Technologies, Santa Clara, CA) equipped with a pulsed split/splitless injector

and hydrogen flame ionization detector. SP2560 fused silica capillary column 100 m × 0.25 mm (inner diameter.) with 0.20 μm film thickness (Agilent Technologies, USA) was used to separate fatty acid methyl esters. The temperature programme for separation began at 150oC, which was maintained for 2 min. Then the oven temperature increased to 230ºC at 3ºC/min (ramp) and held at 230ºC for 10 min. Injector temperatures were maintained at 250ºC. Carrier gas, helium, with flow rate 1ml/min was used. The injection volume was 3 μl. Fatty acids were identified by comparison of elution times with standard FAMEs from library of GCMS and were estimated as area per cent. Data was collected and analyzed using the GC/MSD program (Agilent Technologies, Inc.).

4.8. Fortification of microencapsulated flaxseed oil powder in milk

4.8.1. Preparation of flaxseed oil microcapsules fortified pasteurised milk

Cow milk was standardized using Pearson square method in such a way that it contained total 3% fat and 8.5% SNF. Fresh standardized milk was warmed to 55-60°C and then homogenized (double stage) at 2500 and 500 psi pressure. After that, the MFOP were added at a level to meet 25 and 50% RDA of α-linolenic acid per serving of milk. For a particular fortificant level, the whole homogenized milk was divided into four batches. First batch was used as control and rest three batches were fortified with different level of MFOP preparations. All the batches were pasteurized at 74°C for 15s and then cooled to 4°C, followed by packaging and storage at refrigerated temperature (4-7°C).

4.8.2. Preparation of flaxseed oil microcapsules fortified sterilised milk

The omega -3 fortified sterilised milk was prepared by addition of flaxseed oil microcapsule to meet atleast 25% RDA per serving. For preparation of sterilised fortified milk, milk was standardised to 3.0% fat and 8.5% SNF and then preheated (65ºC) for homogenisation. After homogenisation, the microcapsules were added (at 40ºC) and mixed properly. Bottle were filled with some headspace and corked. This was followed by sterilisation (In bottle sterilisation at 121°C for 15 min) and cooling to room temperature slowly. The sterilised milk was then stored (25ºC) for storage study and further analysis.

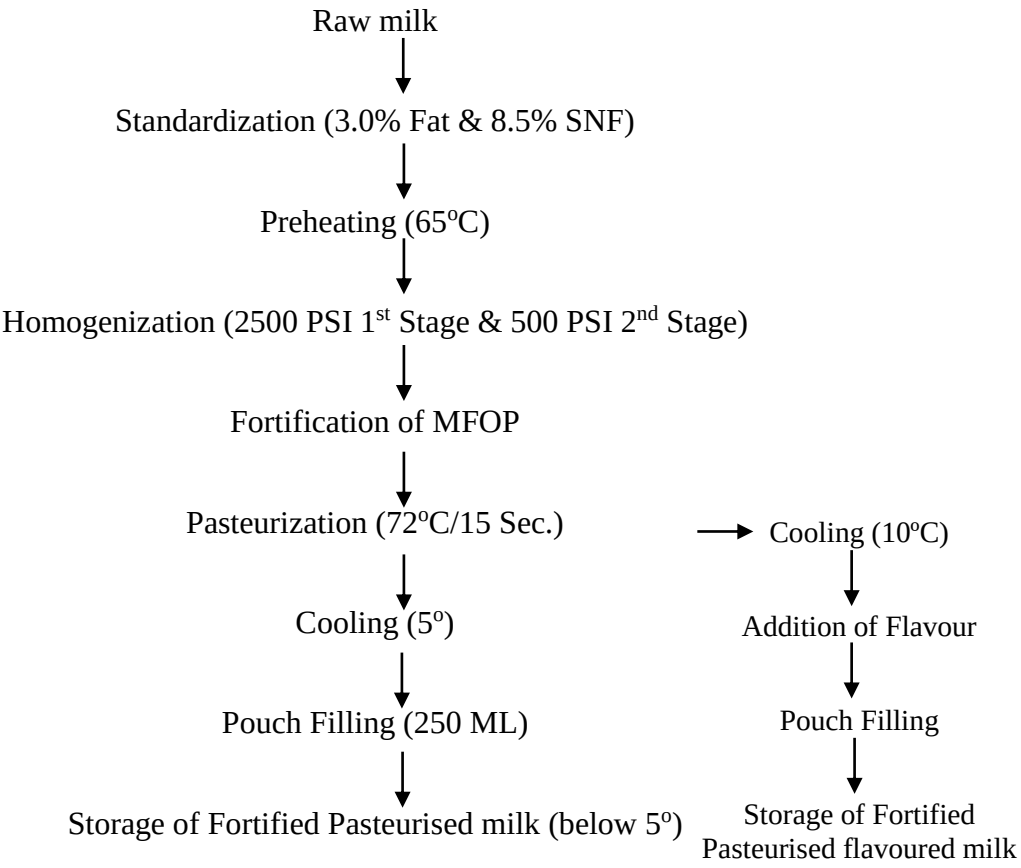

Figure 4.7 Process flow chart for preparation of flaxseed oil microcapsules fortified pasteurized milk

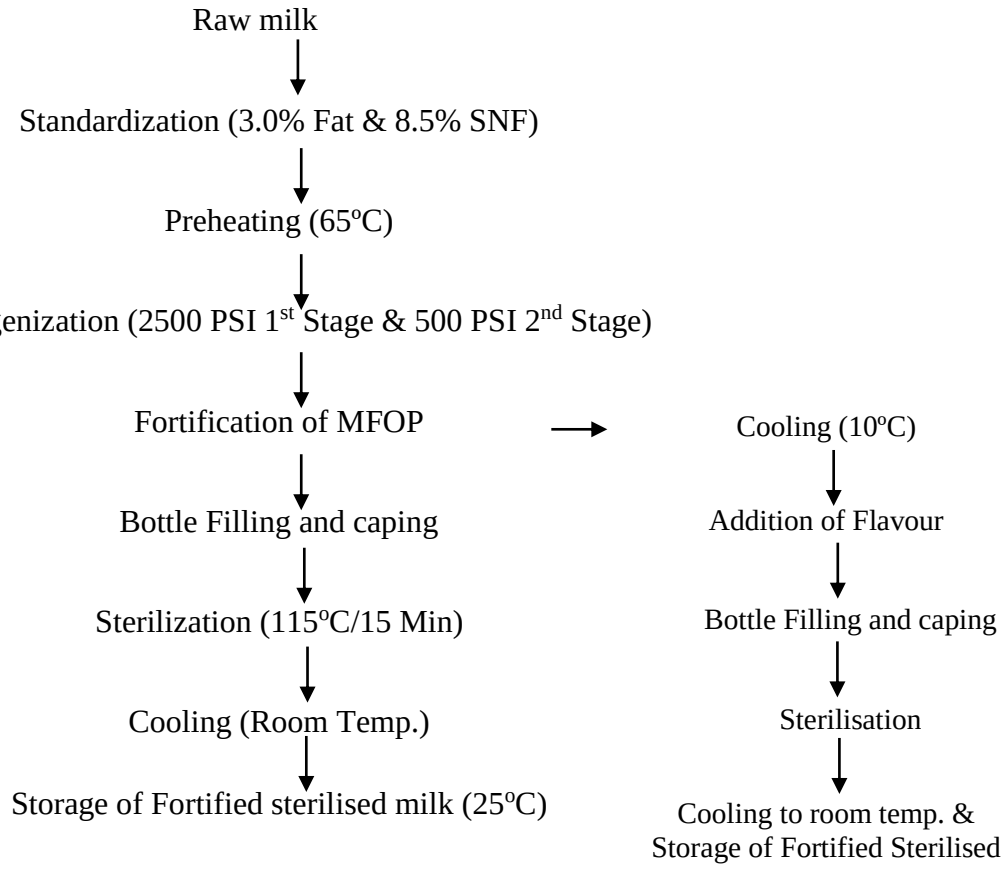

Figure 4.8 Process flow chart for preparation of flaxseed oil microcapsules fortified sterilized milk

4.9. Analysis of fortified milk

4.9.1. Titratable acidity

Titratable acidity of control and fortified milk was determined as per IS: SP: 18, Part XI (1981). Ten mL of thoroughly mixed milk was pipetted into 150 mL conical flask. Three to four drops of phenolphthalein indicator solution (1.0%) were added to the flask. The contents of flask were titrated against standard sodium hydroxide solution (0.1 N) to the end point i.e., appearance of persistent pink colour. The volume of 0.1 N NaOH consumed was noted down as titre value. The acidity of the milk was calculated according to the following formula:

$$Acidity = \frac{9 \times A \times N}{10} \quad (13)$$

Where,

A = Volume (mL) of 0.1N NaOH required for titration,

N= Normality of NaOH used

V= Volume (mL) of milk taken for test (1 mL of 0.1 N NaOH = 0.009 g lactic acid)

4.9.2. Colour characteristics of fortified milk

Colour parameters of the fortified milk samples were measured by HP colour scanner. Colour of fortified milk samples was measured by scanner Adobe Photoshop (SAP) method (Vyawahare and Rao, 2011). All the fortified milk samples were tempered at room temperature before measurement. The samples of fortified milk were poured to scratch less pertripalte (Borosil, diameter 35 mm,) which has been thoroughly cleaned and dried earlier. The perti dish containing samples were placed on the bed of the scanner scanned and were saved as JPGE file. Scanning was performed under the following parameter; resolution 75 dpi; sharpness: medium; background of the sample: white. The scanned image was opened in colour analysis software namely Adobe Photoshop version CS3 running under the Microsoft windows XP environment. Colour information was analysed from the scanned images in LAB mode: Lightness (L), Redness (a) and Yellowness (b)

Lightness to darkness (L*), Redness to greenness (a*) and yellowness to blueness (b*) colour parameters were measured after processing and during and storage and for sterilised milk

$$L* = \frac{L}{255} \times 100 \quad (14)$$

$$a* = \frac{a}{255} \times 240 - 120 \quad (15)$$

$$b* = \frac{b}{255} \times 240 - 120 \quad (16)$$

4.9.3. Free fatty acids (FFA) content

An extraction titration method devised by Deeth *et al.,* (1975) was followed for the determination of FFA content of milk samples. Fifty millilitres of milk sample was taken in a 250 mL glass stoppered conical flask. To this 150 mL of freshly prepared extraction mixture (isopropanol, petroleum ether and 4 N sulphuric acid in the ratio of 40:10:1, respectively) was added followed by 50 mL petroleum ether and 25 mL distilled water. The flask was stoppered and shaken vigorously for 25-30 s. Then transferred to separating funnel and was allowed to separate for 10-15 min or till the two layers get clearly separated. Volume of upper layer was removed, and the fat solvent is evaporated in waterbath at 40-50^{o}C. Further titration was carried out in same way as described in 4.5.2.5.2.

4.9.4. pH

pH of milk samples was determined by potentiometric method using digital pH meter (Eutech, India). The pH meter was first calibrated using standard buffers of pH 4.0 and 9.2 and standardized using pH buffer of 7.0 at 25.0 ± 0.1^{o}C.

4.9.5. Viscosity

Viscosity of fortified milk was measured by the capillary viscometer (Ostwald viscometer) Roy and Sen (1994). Thirteen mL of distilled water was tempered to 27^{o}C temperature and taken into the arm A. Then time for flowing of distilled water through capillary of arm B was determined. Similarly, then time for fortified milk was determined at the same temperature. Viscosity is calculated by the formula:

$\eta = k \times t$ (17)

$(k = \frac{\eta}{t}\ of\ water)$

Where,

η = Viscosity

K = conversion factor

T = time (seconds)

4.9.6. Sensory evaluation

Sensory evaluation was done by a semi-trained panel of ten judges of Dairy Technology Division, Dairy Chemistry and Dairy Engineering of SRS-NDRI, Bangalore, India including scientist, technical associates and students. Omega-3 fortified milk was evaluated by the panelists for colour and appearance, odour, taste and mouthfeel in comparison to control milk. Nine point hedonic scale was used (Annexure I). Milk was served at 10-15°C temperature for sensory evaluation.

4.9.7. Microbiological evaluation of fortified milk

Pasteurized milk samples packed in LDPE pouches and in-bottle sterilized milk samples were stored at refrigerated temperature (5-7^{o}C) and room temperature, respectively. The samples were evaluated for standard plate, coliform, yeast and mold and spore counts. Microbiological analysis of control and fortified milk were performed on 0, 2, 4 and 6 days of storage for pasteurized milk and on 0, 7, 14, 21 and 28 days of storage for in-bottle sterilized milk.

All test tubes were autoclaved (15 min at 121^{o}C) prior to plating, and sterile disposable tips were used. Eleven millilitres of sample was taken and mixed with 99 mL of dilution blank (0.85% NaCl) to get the first dilution. It was vortexed and 1 mL of first dilution was taken and mixed with another 9 mL dilution blank to get the second dilution. Likewise different dilution were made.

Standard plate count was enumerated using nutrient agar. Plating of serially diluted fortified and control milk samples was done and plates were incubated at 37^{o}C for 24-48 hours. Coliform count of fortified and control milk samples was enumerated using

VRBA agar and plates were incubated at 37^{o}C for 24-48 hours and Yeast and mold count of fortified and control milk sample was enumerated using PDA agar and plates were incubated at 30^{o}C for 48-72 hours. Spore count of fortified and control sterilised milk sample was enumerated using spore count agar and plates were incubated at 30^{o}C for 24-48 hours. Prior to spore count sterilised milk was incubated at 63^{o}C for 24 hours

4.10. Proximate composition of fortified milk

4.10.1. Total solids

Total solids (TS) content of milk samples was determined by the gravimetric method as described in IS: SP-18 (1981). A dry, empty and clean dish was weighed with cover. About 10 mL of milk sample was pipetted into the dish and weighed accurately. The dishes were placed on a boiling water bath for 30 min. All the dishes were kept in an oven at 100±2^{o}C for 3 h. They were then cooled in a desiccator for 30 min and weighed accurately with cover. Heating at 100±2^{o}C for 30 min, cooling and weighing were repeated until the loss in weight was not more than 0.5 mg.

$$\% \, TS \; in \; Milk = \frac{(W3 - W1)}{(W2 - W1)} \times 100 \qquad (18)$$

Where,

W1 = Weight of empty dish

W2 = Weight of empty dish + weight of sample

W3 = Weight of dish + weight of sample after drying

4.10.2. Fat

Fat content of milk samples was determined using gravimetric method as per IS: SP-18 (1981). About 10 g of milk sample was weighed into a Mojonnier extraction flask and 1 mL of concentrated ammonia solution (sp gr. 0.88) was added to it and mixed well in the bulb. 10 mL of ethyl alcohol (sp gr. 0.720) was added and the contents were mixed again. 25mL of diethyl ether (peroxide free) was poured into the flask, closed with a bark cork which is wetted with water before insertion and mixed the contents vigorously for one minute. This was followed by addition of 25mL petroleum ether (boiling point: 40 to 60^{o}C) and the contents were mixed vigorously for about a min. The contents were left

undisturbed for 1 h until a clear upper ethereal layer was separated completely. The clear ethereal layer was decanted off into a previously weighed 100 mL beaker with a few glass beads. The outside of the neck of the tube and the cork were washed with a mixed solvent (equal volumes of the ether and light petroleum) and the washings were added to the beaker. Extraction of the liquid remaining in the extraction tube was repeated twice using 15 mL of each solvent every time. The ethereal extract was added to the same container and was evaporated off completely on a water bath. The beaker was dried in a hot air oven at 100±2^{o}C for one hour, cooled to room temperature in desiccator and weighed. This process was repeated with 30 min of heating, cooling and weighing until the difference between two successive weights did not exceed 1 mg. The difference in weights before and after the ethereal extractions represented the weight of fat extracted from the milk.

$$Fat(\%) = \frac{Wt.\ of\ fat}{Wt.of\ sample\ taken} \times 100 \quad (19)$$

4.10.3. Protein

Nitrogen content in milk samples was estimated by Kjeldahl method as per IS: SP-18 (1981). Milk samples were digested in H_2SO_4, using $CuSO_4.5H_2O$ as catalyst with K_2SO_4 as boiling point elevator, to release nitrogen from protein and retain nitrogen as ammonium salt. Concentrated NaOH was added to release NH_3, which is distilled, collected in H_3BO_3 solution, and titrated.

Milk samples were warmed at 38±1^{o}C and 5±0.1 g was weighed into Kjeldahl tube. Five grams of digestion mixture (potassium sulphate:copper sulphate; 100:2) and 12.5 mL of concentrated sulphuric acid were added to the flask. The contents were digested in Kjeldhal digestion unit until clear bluish-green digest was obtained. About 30 mL of distilled water was added to the tube along the sidewalls. The tube was placed in Kjeldahl distillation unit. Auto measured quantity (30 mL) of 50% (w/v) standard sodium hydroxide solution was added to it make the solution alkaline. The contents were steam distilled and liberated ammonia was collected in 25 mL of saturated boric acid solution containing 2-3 drops of mixed indicator (equal volumes of a 0.1% saturated solution of methyl red in 95% ethanol and 0.1% solution of methylene blue in 95% ethanol). After completion of distillation, the distillate was titrated against 0.1 N standard sulphuric acid to an end point of pink colour. A blank test was carried by simultaneously using all the

reagents and pure analytical grade sucrose in place of the test material. The total nitrogen content was calculated using following formula:

$$Nitrogen\ \% = \frac{14.07\times(Vs-Vb)\times Normality of\ sulphuric\ acid\times 100}{Wt.of\ sample} \quad (20)$$

Where,

Vs = mL 0.1 N H_2SO_4 titrant used for test portion

Vb = mL 0.1 N H_2SO_4 titrant used for blank

Protein content % in milk= Nitrogen content (%) × 6.38

4.10.4. Total Ash

Total ash content was determined as per the method described in IS: SP-18 (1981). About 5 g of sample was weighed accurately in a previously heated, cooled and weighed silica crucible. It was carefully charred on a heater and then the sample was kept in a muffle furnace maintained at a temperature not more than 550^0C until white ash was obtained about 5 hours. The temperature of the muffle furnace should not exceed 550^0C otherwise lower values will be obtained due to evaporation of certain metal chlorides. The crucibles were cooled and stored in a desiccator until the final weight was taken.

$$Ash\ in\ milk = \frac{W1}{W}\times 100 \quad (21)$$

Where,

W = Weight of the sample.

W1= Weight of the residue after heating.

4.10.5. Total Carbohydrate

Total carbohydrate content was determined by difference method as per IS: SP18 (1981). The sum of moisture, fat, protein and ash contents (%) were subtracted from 100 to get carbohydrate content (%).

$$\%\ Total\ Carbohydrate\ by\ weight = 100 - (A + B + C + D)\% \quad (22)$$

Where,

A = % by mass of moisture

B = % by mass of total protein

C = % by mass of fat and

D = % by mass of total ash

4.11. Statistical analysis

The entire experiments were triplicated and means and standard deviations were calculated. All statistical analyses were performed using SPSS software and statistical significance was set at $p<0.05$. The least significant difference (LSD) test was used to find out significant differences between sample means. Analysis of variance (ANOVA) was used to determine differences among treatment means using the Post Hoc Test (Dunkan). Sensory attribute of milk samples data during storage were analysed using 2-way analysis of variance (ANOVA), with main effects of treatments and day of storage.

Chapter 5

Results and Discussion

5.0 Results and Discussion

This chapter deals with the results obtained during the present investigation on 'Microencapsulation of flaxseed oil for fortification of milk with omega- 3 fatty acids'. In this chapter, the results are discussed in three segments, according to the three objectives. The first segment of the study deals with the optimization of flaxseed oil emulsion in terms of composition depending upon the emulsion stability; and then followed by spray drying to obtain microencapsulated flaxseed oil powder (MFOP). Both the emulsion and developed microcapsules were evaluated for various physico-chemical properties. In the second part, microencapsulated flaxseed oil powder was fortified in milk at different levels to meet recommended dietary allowance of alpha linolenic acid; and the optimum level of the fortificant was selected on the basis of sensory evaluation. Then both fortified pasteurized and sterilized milk samples were prepared and evaluated for various physic-chemical and sensory characteristics. In the third phase, the effect of storage on physico-chemical and sensory characteristics of fortified pasteurized and sterilized milk was studied. The final optimized product was evaluated for proximate composition. The results obtained are presented and discussed in this chapter under relevant headings and sub-headings.

5.1 Physico-chemical characteristics of flaxseed oil emulsion

The different flaxseed oil emulsion samples (as mentioned in section 4.5, chapter 4) were prepared with soy protein isolate and modified starches by homogenizing using high shear mixer (ultra-turrax, IKA T-18, Germany). The prepared flaxseed oil emulsions were stored at refrigerated temperature (4-7^{o}C) for further study. During emulsification, the small droplets of oil are covered by encapsulating agent and thus aggregation is avoided. Amount and properties of coat material play an important role to develop stable emulsion. As, in this case, a combination of soy protein isolate and modified starches was used, so it was very vital to study their effect on emulsion properties.

5.1.1. Physical stability and creaming index

The physical stability and creaming index of prepared flaxseed oil emulsions are mentioned in Table 5.1. The emulsion samples were significantly ($p<0.05$) different from each other for creaming index values. It is evident that the per cent of separated oil increases with increasing oil load for the same TS level. The creaming index values were higher for NC 180 emulsion samples as compared to NC 46 emulsions. The creaming index values for

NC 46 and NC 180 emulsion samples varied from 0.159±0.036 to 7.811±0.328% and 2.889±0.385 to 9.799±0.344%, respectively. The samples having 35 % oil load and 20% total solid were the most unstable samples in terms of oil separation. This can be due to the lack of sufficient coat material (encapsulating agent), which causes sharing of core material (active material) and leads to irreversible bridging flocculation (Disckinson *et al.*, 2001). Further, more coating material may increase surface load and adversely influences properties of emulsion (McClements, 2004), therefore very high concentrations of coat material were avoided.

5.1.2. Zeta potential and average droplet size

Zeta potential is a good indicator of stability of emulsion. Higher the value of zeta potential from zero (in positive or negative), indicates greater stability of an emulsion, but lower values indicate flocculation or coagulation of emulsion. The charge on droplet influences the rheological properties of an emulsion. The zeta potential shows the droplet with absorbed protein and/or biopolymer and charge on ions that move along with the droplet in the electric field (Surh *et al.*, 2006). Table 5.2 summarizes the zeta potential and average droplet size of NC 46 and NC 180 flaxseed oil emulsions, which varied significantly ($p<0.05$) among the samples.

It is evident that zeta potential value is higher for the sample with higher total solids (Table 5.2), this is might be because of maximum utilisation of soy protein isolates for coverage of oil droplets or uncovered flaxseed oil droplets (Dickson, 1992). The zeta potential of NC 46 emulsions ranged from -28.16±1.36 mV to -38.50±2.26 mV and the values for NC 180 emulsions varied from -28.73±3.37 to -34.80±1.25 mV. The negative values of zeta potential could be due to negatively charged soy proteins isolates used as coat material. The positive or negative values of zeta potential from zero are indicative of the charge on the emulsion droplet. Additionally these values are in agreement with creaming index values as the sample having lowest zeta potential showed maximum creaming index. It can be interpreted from the zeta potential values that all the emulsions were stable in term of zeta potential and the emulsion having 30% total solids and 30% oil load (Table 5.2) were most stable compared to others.

The particle size distribution of NC 180 emulsion is wider than NC 46. The mean droplet diameter (Z average size) was significantly ($p < 0.05$) different for various emulsion samples of NC 46 and NC 180 (Table 5.2).

Table 5.1: Physical stability and creaming index of flaxseed oil emulsions

Oil Load (% of T.S.)	Total Solids (%)	Sample (NC 46 emulsions)	Creaming index (%)	Physical stability	Sample (NC 180 emulsions)	Creaming index (%)	Physical stability
25	20	A1	0.647 ± 0.034^{g}	Stable	B1	6.697 ± 0.052^{bc}	Stable
25	25	A2	0.334 ± 0.001^{h}	Stable	B2	4.036 ± 0.031^{cd}	Stable
25	30	A3	0.159 ± 0.036^{i}	Stable	B3	2.889 ± 0.385^{d}	Stable
30	20	A4	6.889 ± 0.385^{b}	Stable	B4	8.018 ± 0.031^{d}	Stable
30	25	A5	3.991 ± 0.031^{d}	Stable	B5	6.696 ± 0.026^{bc}	Stable
30	30	A6	2.673 ± 0.010^{f}	Stable	B6	3.123 ± 0.364^{d}	Stable
35	20	A7	7.811 ± 0.328^{a}	Stable	B7	9.799 ± 0.349^{a}	Stable
35	25	A8	5.333 ± 0.000^{c}	Stable	B8	6.178 ± 4.311^{bc}	Stable
35	30	A9	3.333 ± 0.000^{e}	Stable	B9	6.667 ± 0.000^{bc}	Stable

Results are expressed as Mean±SD, n=3; Means with different small letters superscript (a,b,c) within the column differ significantly ($p<0.05$) among the samples

Table 5.2: Zeta potential and average droplet size of flaxseed oil emulsion at refrigerated storage (4-7ºC)

Oil Load (% of T.S.)	Total Solids (%)	Sample (NC 46 emulsions)	Zeta potential (mV)	Average droplet size (nm) (for NC 46 emulsions)	Sample (NC 180 emulsions)	Zeta potential (mV)	Average droplet size (nm) (NC 180 emulsions)
25	20	A1	-28.17±1.36^{f}	721.00±48.77^{a}	B1	-28.73±3.37^{a}	801.80±201.66^{cd}
25	25	A2	-34.50±5.67^{e}	1360.13±407.35^{ab}	B2	-33.73±1.27^{bc}	1837.00±223.55^{a}
25	30	A3	-35.70±2.90^{e}	888.40±221.3^{cde}	B3	-34.53±0.90^{bc}	1761.66±454.37^{a}
30	20	A4	-37.93±3.61^{c}	1151.17±346.27^{de}	B4	-31.60±1.73^{ab}	1605.00±454.71^{a}
30	25	A5	-31.57±0.97^{d}	892.43±256.95^{ab}	B5	-34.80±1.25^{c}	1417.66±326.13^{a}
30	30	A6	-38.50±2.26^{d}	1073.83±175.2^{e}	B6	-33.83±0.93^{bc}	1932.33±172.34^{ab}
35	20	A7	-35.43±1.05^{b}	1119.47±267.1^{cd}	B7	-36.30±0.95^{d}	362.03±9.14 ^{d}
35	25	A8	-32.87±1.40^{b}	1219.00±302.9^{bc}	B8	-29.73±2.84^{a}	1050.50±112.66^{bc}
35	30	A9	-32.70±2.54^{a}	782.80±128.60^{bc}	B9	-31.90±1.74^{b}	1908.00±208.97 ^{a}

Results are expressed as Mean±SD, n=3; Means with different small letters superscript (a,b,c) within the column differ significantly ($p<0.05$) among the samples

The mean droplet diameter value of NC 46 emulsion sample having 25% oil load and 20% total solids was 721.00±48.77 nm, being the lowest among the NC 46 emulsions. The NC 46 emulsion sample having 25% oil load and 25% total solid had the maximum $d_{4,3}$ value of 1360±407.35 nm. While, for NC 180 emulsion having oil load of 35% and total solid 25% had mean droplet diameter value of 362.03±9.14 nm and the one with 35% oil load and 30% total solid had mean droplet diameter value of 1908.00±208.97 nm. The mean droplet size of flaxseed oil emulsions prepared in the present study are quite lower as compared to refined vegetable oil emulsions prepared with soy protein isolate by Achouri *et al.* (2012) which had average particle droplet size of 4170 nm. However, the size is little more when compared to β-carotene emulsion ($d_{4,3}$ value of 690 nm) prepared from a combination of modified starch and soy protein isolate (Deng *et al.*, 2014).

5.1.3. Rheological properties

The rheological properties play a vital role for emulsion characteristics and in determining the optimised condition during the processing treatments (like pumping, mixing, flowing in pipe, atomising). Further, rheological characteristics have an important role in designing the equipment which involve heat treatment or cooling. In the present study, the apparent viscosity with variable shear rate (5-100/s) for the NC 46 (Figure 5.1) and NC 180 emulsions (Figure 5.2) shows the decreasing trend with increasing shear rate. This confirms the shear thinning behaviour (pseudoplastic nature) of the emulsion, which is a characteristic property of the most food emulsions.

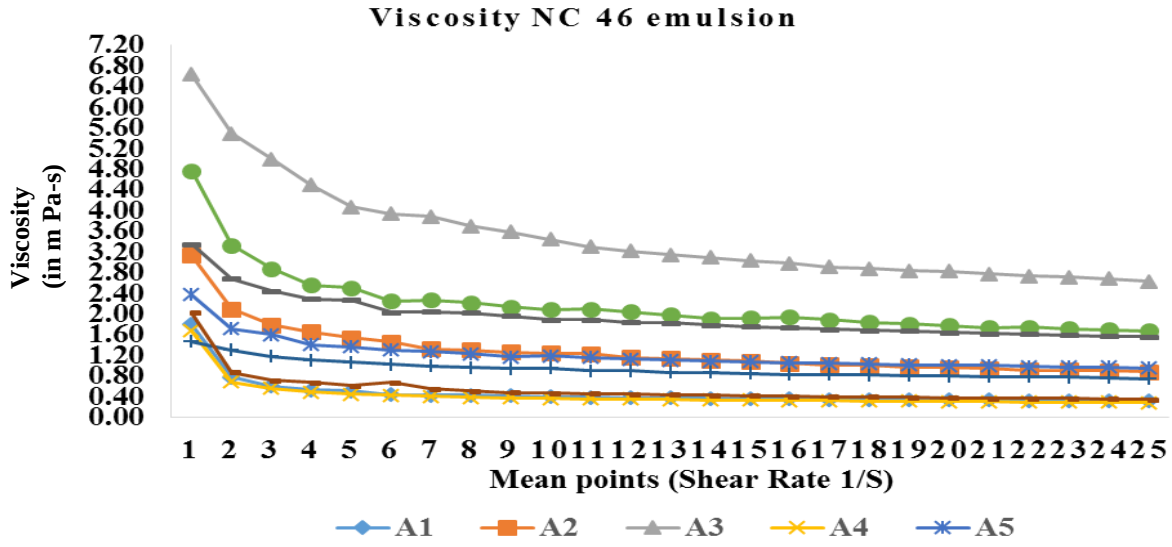

Figure 5.1: Apparent viscosity (m Pa-s) of NC 46 emulsion samples with variable shear rate (5-100 s^{-1})

The observed shear thinning may be due to spatial distribution of the particles as a result of shear field (Hunter, 1993). The results are in agreement with shear thinning behaviour of the fish oil and rapeseed oil emulsions studied by Taherian *et al.*, (2011) and Dybowska, (2011), respectively. It is also evident that with increase in the total solid level in the emulsions, the viscosity of emulsion increases but at the same time with increase in oil load at same total solid level, there is decrease in the viscosity (Figure 5.1 and 5.2).

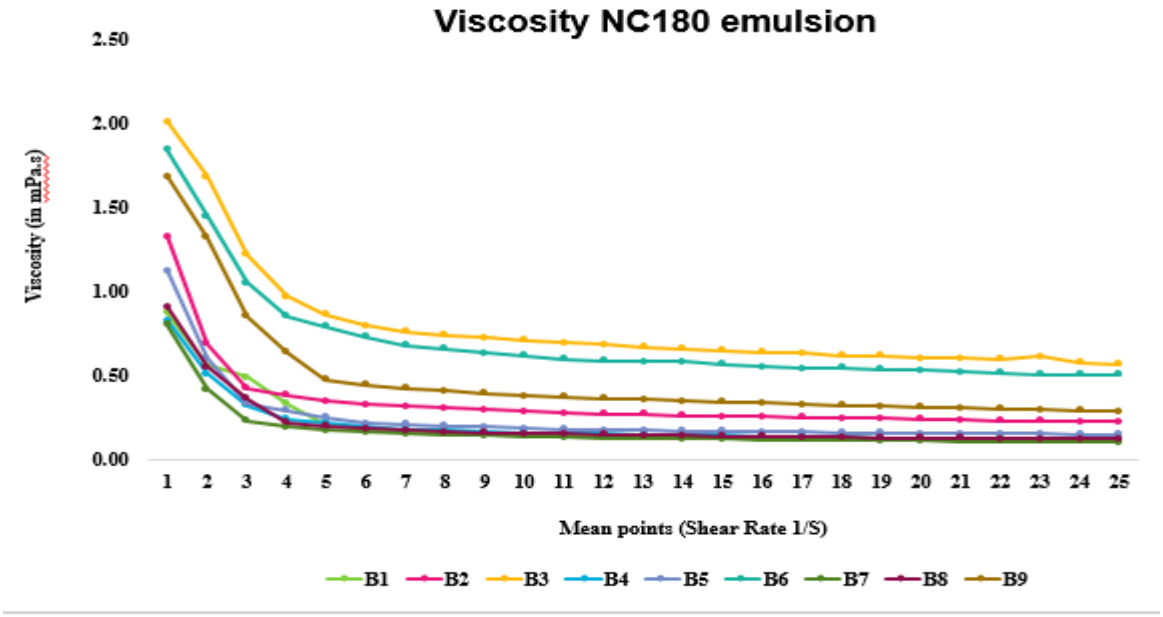

Figure 5.2: Apparent viscosity (m Pa-s) of NC 180 emulsion samples with variable shear rate (5-100 s^{-1})

The viscosity of the selected emulsion samples for both the starches having 30% total solids and 30% oil load were also studied for 28 days at 7 days interval. These samples were selected owing to their maximum zeta potential values. The viscosity values increased with storage (Figure 5.3 and 5.4). The increase in the viscosity of emulsion may be due to self-aggregation or flocculation of the emulsion particles.

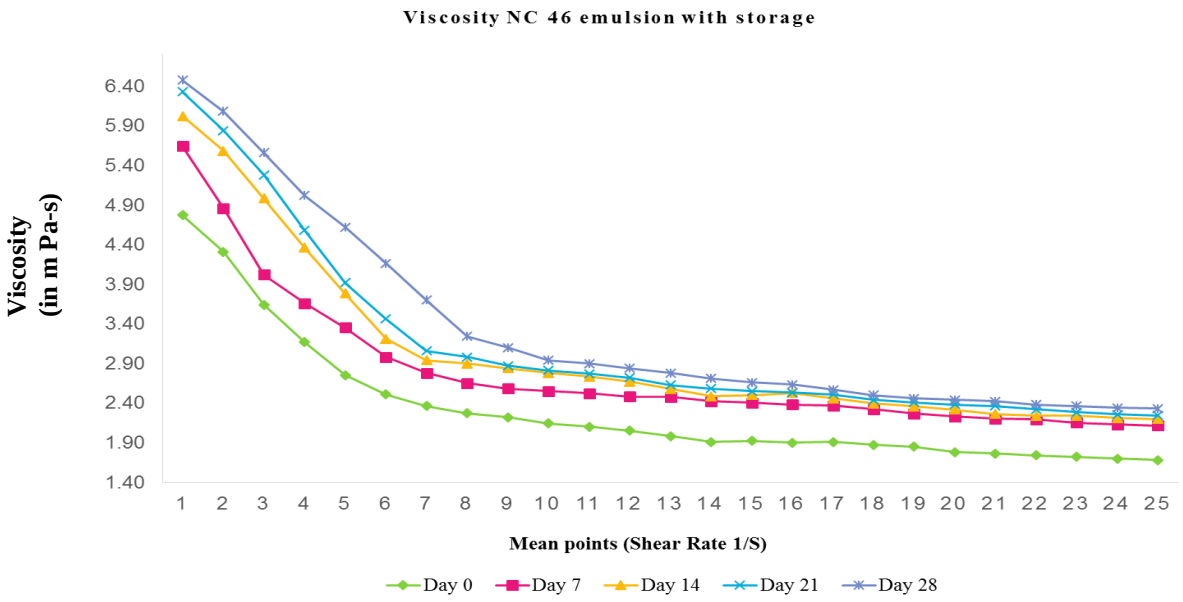

Figure 5.3: Apparent viscosity (m Pa-s) of selected (A6) NC 46 emulsion sample with variable shear rate (5-100 s^{-1})

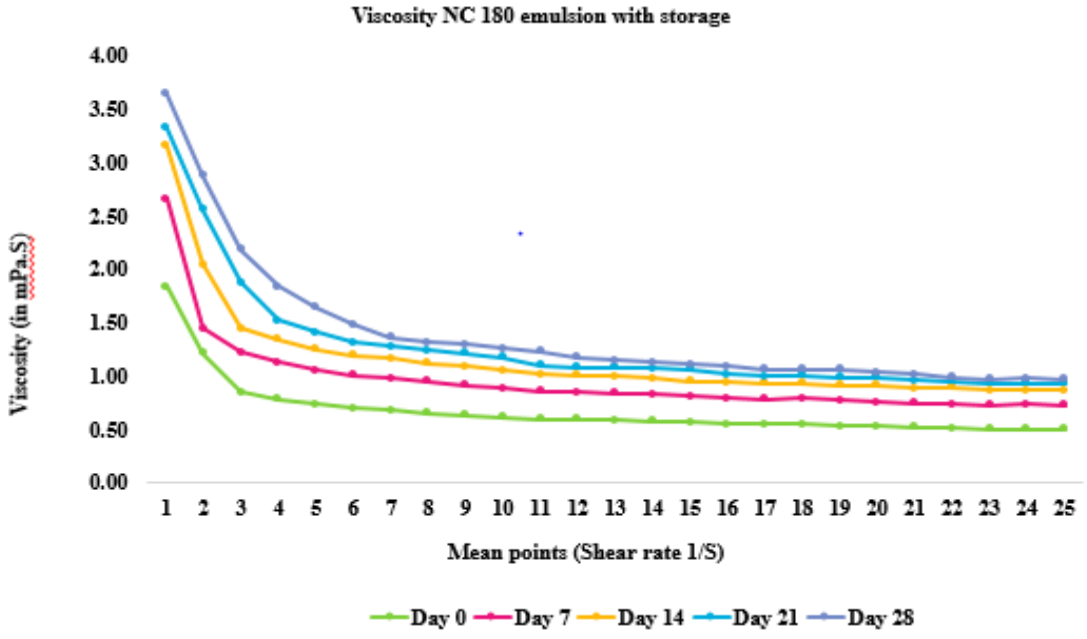

Figure 5.4: Apparent viscosity (m Pa-s) of selected (B6) NC 180 emulsion sample with variable shear rate (5-100 s^{-1})

5.1.4. Peroxide value and free fatty acid

Encapsulated oil in the form of emulsion is more stable than the free flaxseed oil. The stability of flaxseed oil in the emulsion was measured by the formation of primary oxidation products (like lipid hydroperoxide) and depicted as peroxide value (Table 5.3). The peroxide value of flaxseed oil increased from 2.61±0.06 to 6.20±0.03 mEq peroxide/kg oil at the end of 28 days of storage. There was non significant ($p > 0.05$) difference in the peroxide value of oil and the emulsions on zero day, while the period of storage exhibited significant ($p < 0.05$) difference in the peroxide value for oil as well as both the emulsions. For NC 46 emulsion, peroxide value increased from 2.599±0.033 to 4.016±0.064 mEq peroxide/kg oil, while for NC 180 emulsion, peroxide value increased from 2.61±0.02 to 5.06±0.03 mEq peroxide/kg oil. The higher peroxide value for NC 180 emulsion could be due to the insufficient coat material around the droplet, making this emulsion more suseptible to oxidative changes. The present investigation shows that similar trend was found by Kuhn and Cunha (2012) who studied oxidative stability of emulsions and reported that peroxide value increases from 0.420 to 0.714 m eq peroxide/kg of oil during 30 days of storage. The results also show complete agreement with Goyal *et al.,* (2014) who studied flaxseed oil emulsion prepared with WPC-80 and reported increase in peroxide value from 1.67 to 3.55 mEq peroxide/kg of oil.

Table 5.3: Effect of storage (at 4-7ºC) on peroxide value (mEq peroxide/kg oil) of flaxseed oil and emulsions

Sample	Storage period				
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**
Oil (Control)	2.61 ± 0.06^{eA}	3.30 ± 0.04^{dA}	4.01 ± 0.07^{cA}	5.05 ± 0.04^{bA}	6.20 ± 0.03^{aA}
N-Creamer 46 emulsion	2.60 ± 0.03^{eA}	3.07 ± 0.08^{dB}	3.35 ± 0.03^{cB}	3.70 ± 0.02^{bB}	4.02 ± 0.06^{aB}
N-Creamer 180 emulsion	2.61 ± 0.02^{eA}	3.07 ± 0.01^{dC}	3.43 ± 0.02^{cC}	3.97 ± 0.02^{bC}	5.06 ± 0.03^{aC}

Results are expressed as Mean±SD, n=3; Means with different small letters superscript (a,b,c) within the rows and capital letters (A,B,C..) within the column differ significantly ($p<0.05$) among the samples

The peroxide value of flaxseed oil emulsion prepared with NC 46 starch and soy protein isolate was within the permissible limits as per Codex Alimentarious commosion (1992) standard.

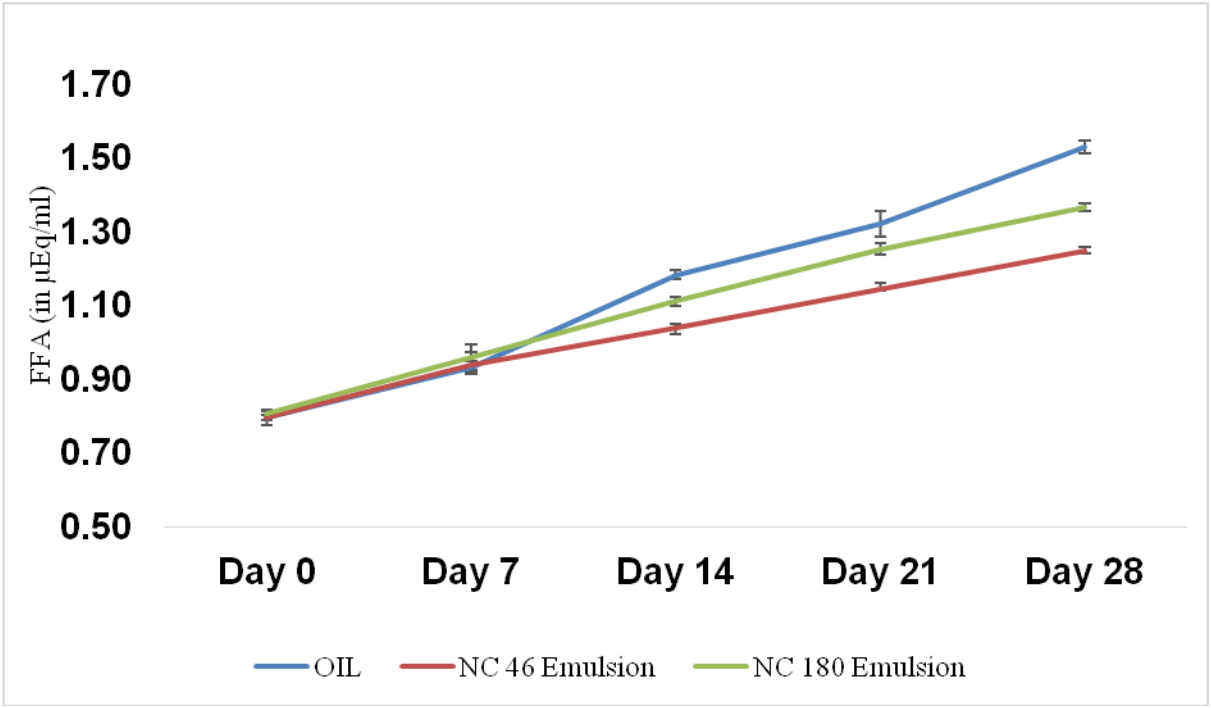

Figure 5.5: Free fatty acid content of flaxseed oil, NC 46 and 180 emulsions

The stability of flaxseed oil emulsion might have also increased due to antioxidant properties of soy protein isoates as it contains histidine, aspartic acid and glutamic acids (Ruann *et al.*, 2013). Free fatty acids are strong pro-oxidants in both bulk and emulsified oils. Addition of oil to an oil-in-water emulsion increased lipid hydroperoxide and hexanal formation at free fatty acid concentrations as low as 0.1% of the lipid (Waraho *et al.*, 2011). Thus, FFA content of emulsions was measured with storage of bulk oil and emulsion at same temperature (4-7^{o}C) as presented in the Figure 5.5. It is revealed that the free fatty acid content of flaxseed oil emulsion increases with increase in storage period. The increase in the FFA in bulk flaxseed oil was 0.75 µEq/mL, while in emulsion it was increased by 0.45 and 0.57 in NC 46 and NC 180 emulsion, respectively with storage for 28 days.

5.2. Preparation of microencapsulated flaxseed oil powder

All the emulsion samples were subjected to atomization in a spray dryer (Technosearch, Thane, Maharashtra) for preparation of flaxseed oil microcapsules. The prepared samples were packed in aluminium laminates (180 µm) and stored at refrigerated (4-7˚C) condition.

5.2.1. Physico-chemical evaluation of microencapsulated flaxseed oil powder

The developed samples were evaluated for microencapsulation efficiency, surface (free) oil, moisture, water activity, bulk density, tapped density, flowing characteristics and colour values. Selected samples were evaluated for particle size distribution, surface morphology and alpha linolenic acid content. Further, moisture content, solubility and peroxide value were determined for the selected samples during 90 days storage period at refrigerated (4-7˚C) condition.

5.2.1.1. Moisture content and water activity (a_w)

Moisture content plays a crucial role for determining the shelf life of low moisture food products. The moisture content affects the physico-chemical properties, thus in general, the maximum moisture content in dried food or powder should be lower than 3-4% (Gallardo *et al.,* 2013). In the present study, moisture content of different formulations for the NC 46 microcapsules ranged from 2.71 to 4.69 % while, for NC 180 microcapsules it varied from 3.59 to 4.54 % (Table 5.4). Both the water activity and moisture content were significantly ($p<0.05$) different for the samples. The moisture content of microcapsules varies mainly with the spray drying conditions like inlet and outlet air temperature, also on the method of atomising, design of dryer etc. The moisture content in microencapsulated flaxseed oil powder as mentioned by other researchers is also less than 5 % (Quispe-Condori *et al.,* 2011 and Goyal *et al.,* 2014).

The water activity (a_w) of dried food products is lower than 0.30, and for limiting the oxidation of lipid, a_w range from 0.2 to 0.3 (Polavarapu *et al.,* 2011) is ideal as the catalytic effect of transition metals is reduced, reduction in quenching of free radicals and hydroperoxide decomposition (Velasco *et al.,* 2003). For the prepared microcapsule samples, a_w of microcapsules prepared from NC 46 was between 0.154 to 0.199 while, 0.143 to 0.213 for NC 180 microcapsules. There was an increase in a_w for increasing moisture content. Karaca *et al.,* (2013) reported the a_w value of 0.25 for flaxseed oil microcapsules prepared using chickpea/lentil protein isolates and maltodextrin. While, Polavarapu *et al.,* (2011) reported a_w value of about 0.3 for fish oil microcapsules prepared using maltodextin and gum arabic.

Table 5.4: Moisture content and water activity of flaxseed oil microcapsules

Oil Load (% of T.S.)	Total Solids (%)	Sample (NC 46 micocapsules)	Moisture (%, w.b.)	a_w	Sample (NC 180 micocapsules)	Moisture (%, w.b.)	a_w
25	20	A1	4.692±0179^{a}	0.154±0.001^{d}	B1	4.54±0.27^{a}	0.213±0.001^{a}
25	25	A2	4.403±0134^{ab}	0.161±0.001^{d}	B2	4.40±0.13^{a}	0.153±0.000^{g}
25	30	A3	2.586±0068^{e}	0.199±0.001^{a}	B3	4.41±0.32^{a}	0.184±0.001^{c}
30	20	A4	2.716±0299^{e}	0.185±0.001^{b}	B4	3.88±0.11^{b}	0.198±0.001^{b}
30	25	A5	4.180±0263^{bc}	0.172±0.001^{c}	B5	3.59±0..33^{b}	0.143±0.002^{i}
30	30	A6	4.233±0172^{bc}	0.148±0.001^{d}	B6	3.58±0.22^{b}	0.156±0.001^{f}
35	20	A7	3.893±0080^{cd}	0.163±0.001^{d}	B7	4.42±0.15^{a}	0.168±0.001^{d}
35	25	A8	4.385±0211^{ab}	0.183±0.000^{bc}	B8	4.38±0.21^{a}	0.151±0.001^{h}
35	30	A9	3.659±0178^{d}	0.181±0.002^{bc}	B9	3.95±0.32^{a}	0.16±0.001^{e}

Results are expressed as Mean±SD, n=3; Means with different small letters within the column differ significantly ($p<0.05$) among the samples

5.2.1.2. Bulk density and tapped density

Storage stability and storage space requirement of powders (microcapsules) are dependent upon the bulk and tapped density to certain extent. The occluded air is more in case of lower bulk density samples and *vice versa*. Thus, higher the bulk density, better is the storage stability of the powder samples. Further, more storage space is required for powders having low bulk density. The bulk density and tapped density values for the developed flaxseed oil microcapsules are presented in Table 5.5. In the present study, the bulk density of flaxseed oil microcapsules was found to be more for samples having low oil load in feed emulsion. The bulk density for NC 46 microcapsules ranged from 0.32 to 0.36 g/mL, while in NC 180 samples, it varied from 0.36 to 0.39 g/mL. The values obtained are in the typical range of bulk density for microcapsules (Onwulata *et al.,* 1996).

The bulk density of different samples varied significantly ($p < 0.05$) differ from each other. The results obtained show agreement with the reported bulk density values (0.17 to 0.35 g/mL) for oil microcapsules prepared from gum arabic (Tonon *et al.,* 2009). However, Thirundas *et al.,* (2012) reported higher bulk density (0.426- 0.490 g/mL) for flaxseed oil microcapsules prepared with gum arabic, maltodextrin and tween 80.

Tapped density is also important in view of packaging, transport and commercialization of powder (Finney *et al.,* 2002). The tapped density for NC 180 microcapsules were non significantly ($p > 0.05$) different and those for NC 46 microcapsules were different significantly ($p < 0.05$) varying from 0.47 to 0.52 g/mL. These findings are similar to those reported by Fernandes *et al.,* (2013) for microencapsulated rosemary oil (0.41 to 0.52 g/mL) and Finney *et al.,* (2002) for microencapsulated orange oil powder (0.48-0.65 g/mL).

5.2.1.3. Flowing characteristics

In present study, powder flowing characteristics were given in terms of Carr's index (% compressibility) and Hausner ratio, which are deduced by formulas form bulk and tapped density values. Higher the value of carr's index, higher the compressible material and less flowable powder, while Hausner index explains the cohesive nature of a powder. Higher Hausner index indicates more cohesiveness and less flowability of the powder.

Table 5.5: Bulk density and tapped density of flaxseed oil microcapsules

Oil Load (% of T.S.)	Total Solids (%)	Sample (NC 46 microcapsules)	Bulk Density (g/ml)	Tapped Density (g/ml)	Sample (NC 180 microcapsules)	Bulk Density (g/ml)	Tapped Density (g/ml)
25	20	A1	0.32 ± 0.01^{c}	0.50 ± 0.01^{ab}	B1	0.36 ± 0.01^{de}	0.50 ± 0.01^{a}
25	25	A2	0.32 ± 0.01^{c}	0.52 ± 0.01^{a}	B2	0.36 ± 0.01^{de}	0.50 ± 0.01^{a}
25	30	A3	0.31 ± 0.00^{c}	0.47 ± 0.00^{b}	B3	0.35 ± 0.00^{e}	0.50 ± 0.00^{a}
30	20	A4	0.35 ± 0.00^{ab}	0.49 ± 0.00^{ab}	B4	0.37 ± 0.00^{abc}	0.52 ± 0.00^{a}
30	25	A5	0.34 ± 0.00^{b}	0.49 ± 0.00^{b}	B5	0.36 ± 0.00^{cde}	0.50 ± 0.00^{a}
30	30	A6	0.34 ± 0.01^{ab}	0.50 ± 0.01^{ab}	B6	0.37 ± 0.01^{bcde}	0.51 ± 0.01^{a}
35	20	A7	0.35 ± 0.00^{ab}	0.48 ± 0.00^{b}	B7	0.39 ± 0.00^{a}	0.50 ± 0.00^{a}
35	25	A8	0.36 ± 0.00^{a}	0.49 ± 0.00^{ab}	B8	0.38 ± 0.00^{ab}	0.51 ± 0.00^{a}
35	30	A9	0.35 ± 0.01^{ab}	0.49 ± 0.01^{ab}	B9	0.37 ± 0.01^{bcd}	0.50 ± 0.01^{a}

Results are expressed as Mean±SD, n=3; Means with different small letters within the column differ significantly ($p<0.05$) among the samples

In the present study, the flaxseed oil microcapsules prepared from the soy protein isolates and NC 46 modified starch had 27.61-38.58 Carr's index and 1.63-1.38 Hausner ratio values, while NC 180 microcapsules had lower values; 21.43-28.41 and 1.28-1.42 for Carr's index and Hausner ratio, respectively (Table 5.6). Thus, it can be interpreted that NC 46 microcapsules had poor flowability and NC 180 had passable flowabilty (Quispe-Condori *et al.,* 2011). However, flaxseed oil microcapsules being functional ingredient, their low flowabilty is not a cause of much concern as these microcapsules would be directly mixed with milk or other food ingredients. These results are in agreement with the finding of Kagami *et al.,* (2003), Turchiuli *et al.,* (2005), and Domian and Wasak, (2008) who reported poor or very poor flowablity for microcapsules of different oils using spray drying and/or freeze drying techniques.

5.2.1.4. Surface (free) oil and Microencapsulation efficiency (ME)

In the present study, amount of surface oil in different microcapsules preparations and microencapsulation efficiency (ME) of different wall materials were measured and represented in Table 5.7. Presence of free (surface) oil on wall materials and microencapsulation efficiency (ME) of microcapsules affect the physico-chemical stability of dry powders. Free oil leads to the aggregation of the powder particles and in turn increases the rate of oxidation.

In the present study, for NC 46 and NC 180 microcapsules, Microencapsulation efficiency ranged from 95.84 to 63.79 and 90.76 to 60.53 %, respectively (Table 5.7). There was significant difference ($p<0.05$) between the different microcapsule formulations in terms of surface oil as well as ME. The microcapsules containing 25% oil load and 30 % total solids had maximum microencapsulation efficiency for both the starches, however lesser free oil was observed in case of NC 46 microcapsules. The same trend was observed in free oil for all other concentrations of oil load and total solids, i.e. for similar composition, NC 180 samples had higher surface oil than NC 46 microcapsules. Thus, it can be interpreted that NC 46 has better encapsulation efficiency than NC 180.

Table 5.6: Flowing characteristics of flaxseed oil microcapsules

Oil Load (% of T.S.)	Total Solids (%)	Sample (NC 46 microcapsules)	Carr's index	Hausner ratio	Sample (NC 180 microcapsules)	Carrs index	Hausner ratio
25	20	A1	35.79 ± 1.58^{ab}	1.56 ± 0.04^{ab}	B1	28.41 ± 1.67^{ab}	1.40 ± 0.03^{ab}
25	25	A2	38.58 ± 1.28^{a}	1.63 ± 0.03^{a}	B2	28.90 ± 1.51^{ab}	1.41 ± 0.03^{ab}
25	30	A3	33.60 ± 3.40^{bc}	1.51 ± 0.08^{bc}	B3	29.46±1.55a	1.42 ± 0.03^{a}
30	20	A4	29.09 ± 3.55^{c}	1.41 ± 0.07^{d}	B4	27.32 ± 3.25^{ab}	1.38 ± 0.06^{ab}
30	25	A5	30.83 ± 0.72^{c}	1.45 ± 0.01^{cd}	B5	27.61 ± 0.95^{ab}	1.38 ± 0.02^{ab}
30	30	A6	30.98 ± 2.38^{c}	1.45 ± 0.05^{cd}	B6	27.38 ± 2.53^{ab}	1.38 ± 0.05^{ab}
35	20	A7	27.61 ± 0.95^{c}	1.38 ± 0.02^{d}	B7	21.79 ± 2.22^{c}	1.28 ± 0.04^{c}
35	25	A8	27.61 ± 0.95^{c}	1.38 ± 0.02^{d}	B8	25.43 ± 1.55^{bc}	1.34 ± 0.03^{bc}
35	30	A9	29.06 ± 1.78^{c}	1.41 ± 0.04^{d}	B9	25.22 ± 1.90^{bc}	1.34 ± 0.03^{bc}

Results are expressed as Mean±SD, n=3; Means with different small letters superscript (a,b,c) within the column differ significantly ($p<0.05$) among the samples

Table 5.7: Microencapsulation efficiency (ME%) and surface/ free oil content of flaxseed oil microcapsules

Oil Load (% of T.S.)	Total Solids (%)	Sample (NC 46 microcapsules)	ME (%)	Surface oil (%)	Sample (NC 180 microcapsules)	ME (%)	Surface oil (%)
25	20	A1	87.36 ± 0.21^{c}	12.640 ± 0.210^{f}	B1	83.66 ± 1.15^{c}	16.34 ± 1.15^{g}
25	25	A2	89.95 ± 0.65^{b}	10.047 ± 0.649^{g}	B2	87.12 ± 0.74^{b}	12.88 ± 0.74^{h}
25	30	A3	95.84 ± 0.26^{a}	4.160 ± 0.262^{h}	B3	90.76 ± 0.45^{a}	9.24 ± 0.45^{i}
30	20	A4	74.77 ± 0.41^{e}	25.227 ± 0.407^{f}	B4	71.18 ± 0.48^{f}	28.82 ± 0.48^{d}
30	25	A5	78.00 ± 0.50^{d}	22.005 ± 0.496^{e}	B5	77.16 ± 0.38^{e}	23.00 ± 0.38^{e}
30	30	A6	87.12 ± 0.74^{c}	12.880 ± 0.741^{d}	B6	80.32 ± 0.74^{d}	19.56 ± 1.00^{f}
35	20	A7	63.79 ± 1.33^{h}	36.210 ± 1.328^{a}	B7	60.53 ± 0.55^{i}	39.47 ± 0.55^{a}
35	25	A8	66.03 ± 0.91^{g}	33.969 ± 0.912^{b}	B8	62.68 ± 0.54^{h}	37.32 ± 0.54^{b}
35	30	A9	71.18 ± 0.17^{f}	28.816 ± 0.169^{c}	B9	64.84 ± 0.81^{g}	35.16 ± 0.81^{c}

Results are expressed as Mean±SD, n=3; Means with different small letters superscript (a,b,c) within the column differ significantly ($p<0.05$) among the samples

Further, the microencapsulation efficiency decreased with increasing oil load as the values ranged from 60.53±0.55 to 71.18±0.17 % for 35 % oil load. This may be due to the fact that the coat material might be insufficient to cover the core completely close to the drying surface, which makes the diffusion path length shorter towards the air/particle interface, thereby leading to an increase in free/surface oil (Tonon *et al.*, 2011). Similar results were observed by Minemoto *et al.,* (2002) and Tonon *et al.,* (2011) for flaxseed oil microcapsules prepared with gum arabic. The surface oil is also related to the emulsion droplet size as reported by Jafari *et al.,* (2008b), larger emulsion droplet leads to higher surface oil due to droplet breakdown during atomization.

5.2.1.5. Colour values of microcapsules

The microcapsules were prepared for fortification in milk, thus the microcapsules should not affect the colour of milk so that the acceptability of milk is not reduced after fortification. Thus, colour parameters form an important quality attribute for the developed microcapsules. According to the classification given by CIELAB system, colour of the any product can be described in terms of L* (lightness) value ranging from 0 (black) to 100 (white), a* (redness) value ranging from +60 (red) to -60 (green), and b* (yellowness) value ranging from +60 (yellow) to -60 (blue). In the present study, colour values of NC 46 ranges from 85.78 to 89.05 for L*, -0.76 to -0.12 for A* and 11.65 to 13.76 for B*, while for NC 180, L* varied from 84.04 to 88.20, A* from -0.041 to -0.40 and B* from 10.72 to 17.46. Also, the colour values for various microcapsule samples prepared from both NC 46 and NC 180 were significantly ($p<0.05$) different. The a* value significantly ($p<0.05$) decreased while b* value increased significantly ($p<0.05$) with increasing oil load from 25 to 35% in the microcapsules.

The results are in agreement with those reported by Karaca *et al.,* (2013), who found L* values ranged from 87.3 to 90.6, a* values from -0.5 to 0.3 and b* values from 11.2 to 20.3 for microencapsulated flaxseed oil employing chickpea protein isolates as coat material. Our results are also in agreement with Shivakumar *et al.,* (2012), who reported L* (83.00), a* (-3.13) and b* (15.31) values of the encapsulated flaxseed oil powder prepared using casein.

Table 5.8: Colour values of flaxseed oil microcapsules prepared from NC 46 starch

Oil Load (% of T.S.)	Total solids (% coat material)	Sample	L*	A*	B*	Hue	Chroma
25	20	A1	88.80 ± 0.17^{b}	-0.76 ± 0.16^{c}	11.88 ± 0.07^{cd}	86.388 ± 0765^{a}	11.905 ± 0.056^{cd}
25	25	A2	88.81 ± 0.22^{b}	-0.65 ± 0.09^{bc}	11.66 ± 0.09^{d}	86.388 ± 0465^{a}	11.679 ± 0.087^{d}
25	30	A3	89.42 ± 0.07^{a}	-0.61 ± 0.01^{bc}	11.65 ± 0.38^{d}	87.388 ± 0113^{ab}	11.669 ± 0.384^{d}
30	20	A4	85.78 ± 0.08^{e}	-0.29 ± 0.09^{a}	11.54 ± 0.09^{d}	88.388 ± 0429^{cd}	11.541 ± 0.095^{d}
30	25	A5	86.49 ± 0.28^{dc}	-0.12 ± 0.06^{a}	13.76 ± 0.24^{a}	89.388 ± 0246^{d}	13.761 ± 0.239^{a}
30	30	A6	87.87 ± 0.04^{c}	-0.24 ± 0.03^{a}	11.78 ± 0.08^{d}	88.388 ± 0144^{cd}	11.786 ± 0.075^{d}
35	20	A7	89.05 ± 0.06^{b}	-0.28 ± 0.14^{a}	11.74 ± 0.40^{d}	88.388 ± 0722^{cd}	11.741 ± 0.392^{d}
35	25	A8	87.68 ± 0.24^{c}	-0.50 ± 0.24^{b}	13.34 ± 0.04^{b}	87.388 ± 1032^{bc}	13.354 ± 0.051^{b}
35	30	A9	86.50 ± 0.27^{d}	-0.29 ± 0.01^{a}	12.24 ± 0.04^{c}	88.388 ± 0056^{cd}	12.240 ± 0.038^{c}

Results are expressed as Mean±SD, n=3; Means with different small letters superscript (a,b,c) within column differ significantly ($p<0.05$) among the samples

Table 5.9: Colour values of flaxseed oil microcapsules prepared from NC 180 starch

Oil Load (% of T.S.)	Total solids (% coat material)	Sample	L*	A*	B*	Hue	Chroma
25	20	B1	86.35 ± 0.07^{d}	-0.33 ± 0.06^{d}	11.18 ± 0.06^{f}	88.31 ± 0.30^{b}	11.19 ± 0.05^{f}
25	25	B2	84.04 ± 0.43^{g}	$-0.40\pm0.19^{a,b}$	17.46 ± 0.021^{a}	88.70 ± 0.63^{a}	17.47 ± 0.21^{a}
25	30	B3	84.62 ± 0.22^{f}	-0.34 ± 0.03^{a}	14.89 ± 0.02^{c}	88.72 ± 0.12^{a}	14.90 ± 0.02^{c}
30	20	B4	85.17 ± 0.54^{e}	$-0.29\pm0.03^{a,b}$	15.93 ± 0.04^{b}	88.96 ± 0.12^{a}	15.93 ± 0.04^{b}
30	25	B5	$87.93\pm0.07^{a,b}$	-0.33 ± 0.04^{a}	14.93 ± 0.10^{c}	88.75 ± 0.13^{a}	14.93 ± 0.10^{c}
30	30	B6	85.17 ± 0.04^{e}	$-0.22\pm0.07^{a,b}$	10.22 ± 0.09^{d}	89.06 ± 0.31^{a}	13.18 ± 0.09^{d}
35	20	B7	86.95 ± 0.06^{c}	$-0.04\pm0.03^{b,c}$	10.92 ± 0.04^{f}	89.82 ± 0.16^{a}	10.92 ± 0.04^{f}
35	25	B8	87.77 ± 0.05^{b}	$-0.13\pm0.04^{c,d}$	11.20 ± 0.03^{f}	89.37 ± 0.20^{b}	11.20 ± 0.03^{f}
35	30	B9	88.22 ± 0.12^{a}	-0.26 ± 0.14^{d}	12.28 ± 0.71^{e}	88.84 ± 0.57^{b}	12.29 ± 0.71^{e}

Results are expressed as Mean±SD, n=3; Means with different small letters superscript (a,b,c) within column differ significantly ($p<0.05$) among the samples

5.2.1.6. Powder particle size and Particle size distribution

Powder particle size and its distribution determines the texture and mouthfeel of fortified product thus, the powder particle size plays a significant role in its food applications. The particle size distribution of selected NC 46 and NC 180 microcapsules (containing 30% oil load and 30% TS) are presented in Figure 5.6 and 5.7, respectively. The mean particle diameter ($d_{4,3}$) for NC 46 microcapsule was 37.917±1.788, while 87.307±6.231µm for NC 180 microcapsule. The larger size of NC 180 microcapsules could be due to the higher viscosity of NC 180 emulsion as compared to NC 46 emulsion. The predominant size can be seen as one representative peak in NC 46 microcapsule distribution, while two peaks in case of NC 180 distribution (Figure 5.6 and 5.7). The presence of particles larger than 100 µm, could be due to the possible incipient agglomeration taking place during spray drying process, thereby leading to the formation of irreversible linkages between the individual particles. The particle size distribution for NC 46 microcapsules was symmetrical but it was non- symmetrical for NC 180 microcapsules. However, the powders were quite homogenous with low span values of 2.067±0.030 and 5.911±0.471 for NC 46 and NC 180 microcapsules, respectively. The results show that the target of microencapsulation in term of size is achieved successfully. The particle size obtained in the present investigation was lower than the attained by Goyal, (2014) who prepared microencapsulated flaxseed oil powder by using WPC and sodium caseinate with 424.36 and 880.30 µm, respectively. The particles exhibited the larger size 87.307±6.231 µm for NC 180 microcapsules than 37.917±1.788 µm for NC 46 microcapsules.

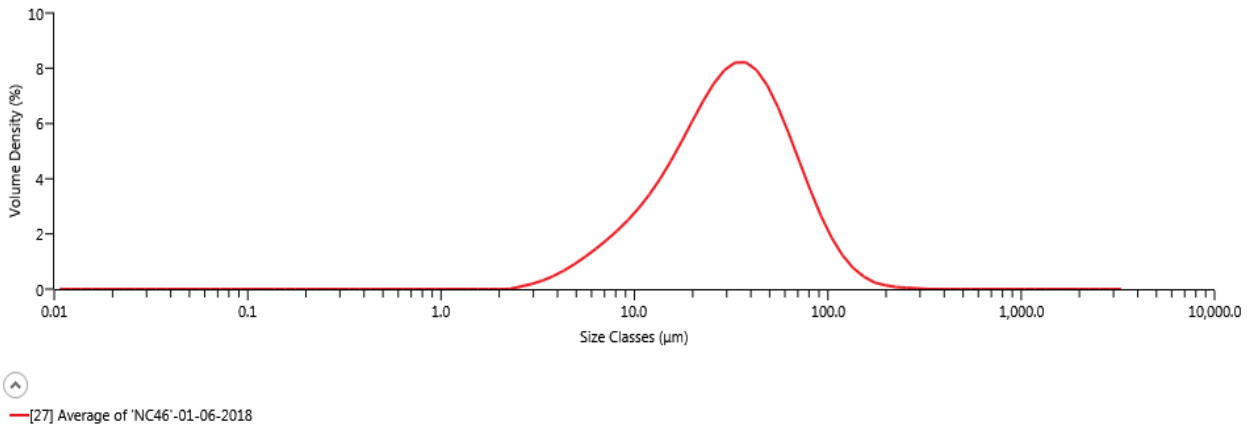

Figure 5.6: Particle size distribution of NC 46 microcapsules

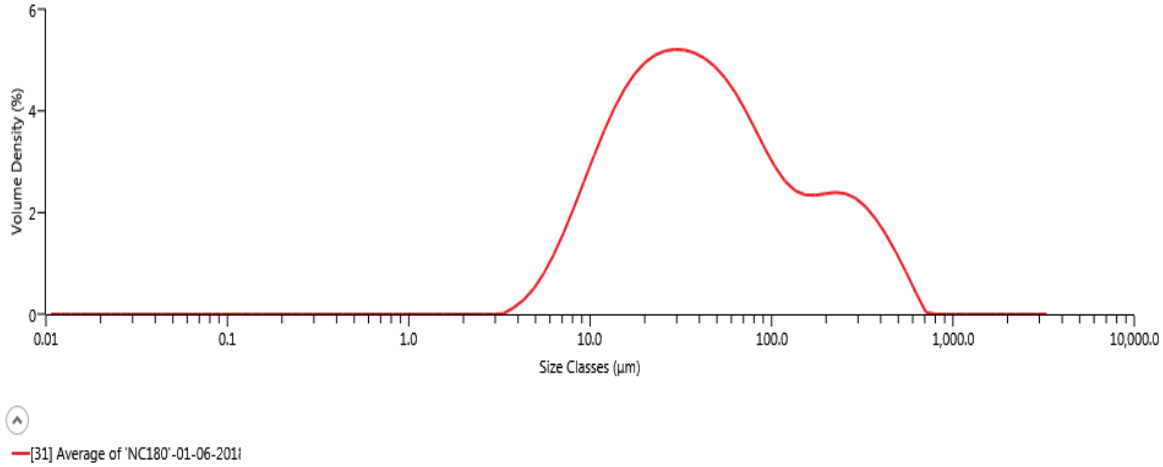

Figure 5.7: Particle size distribution of NC 180 microcapsules

5.2.1.7. Morphology of microcapsules

The scanning electron microscope (SEM) is one of the most versatile tools existing for the analysis of the microstructure and surface morphology. It is most commonly used instrument for imaging and characterization of solid microstructures. Scanning electron micrographs were used to observe the external (surface) of selected (containing 30% oil load and 30% TS) microcapsules (Figure 5.8 and 5.9).

It is evident from the SEM images that most of the microcapsules had spherical shape and smooth surface. The external morphology clearly suggests that the core material, i.e., flaxseed oil is well encapsulated with selected coat material. Further, SEM images also confirmed that there were different sized particles as observed with the particle size distribution determined by mastersizer. The electron micrographs also indicated a significant proportion of particles showing dents on the surface, which is a typical of microcapsules produced by spray drying (Gallardo *et al.*, 2013).

In this study, NC 46 microcapsules had a significant higher proportion of microcapsules with spherical shape and smoother surface than NC 180 microcapsules. The NC 46 microcapsules showed comparatively fewer dents and teeth (imperfections) probably due to faster film formation at drying stage (Tonan *et al.*, 2012). Imperfections or dents are generally formed due to droplet collapse during the initial stages of drying or uneven drying, when there is a slow process of film formation (Re, 1998; Hogan *et al.*, 2001b) presence of dents on surface of microcapsules could also be attributed to high total solids in the formulations as reported by other researchers (Faldt and Bergenstahl, 1996,

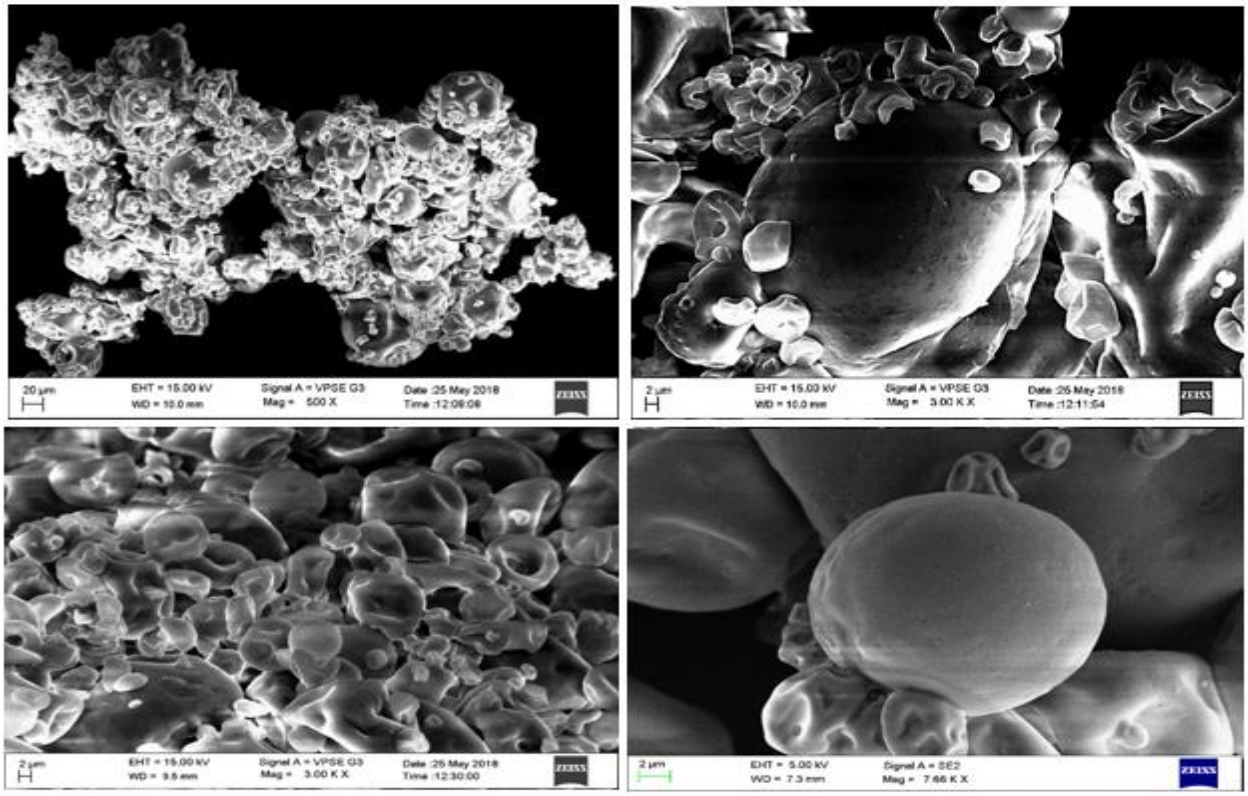

Figure 5.8: Scanning electron micrograph of flaxseed oil microcapsules prepared with NC 46 and SPI

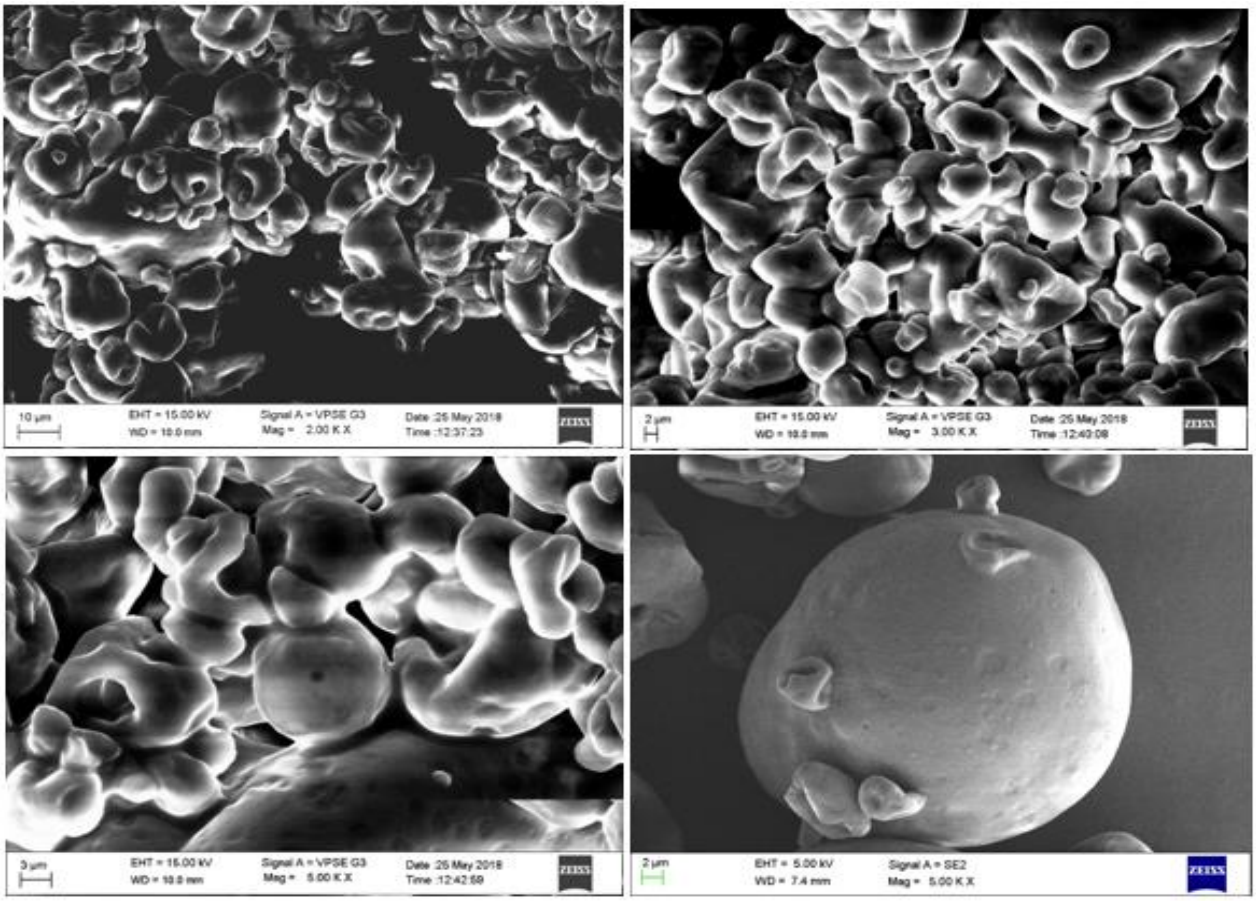

Figure 5.9: Scanning electron micrograph of flaxseed oil microcapsules prepared with NC 180 and SPI

Gallardo *et al.*, 2013, Tang and Li, 2013). Our results are in agreement with the findings of Tonan *et al.*, (2011), who observed dents on the surface of the microencapsulated flaxseed oil powder prepared using gum arabic.

In both the microcapsules, agglomeration of powder particles is visible. This agglomeration behaviour of the microcapsules might be due to presence of surface (free) fat and presence and/or absorption of moisture (Shivakumar *et al.*, 2012). Our results are in agreement with the findings of earlier researchers who reported a similar kind of aggregation in microencapsulated oil powders (Onwulata and Holsinger, 1995; Hogan *et al.*, 2001b).

5.2.1.8 FT-IR Spectroscopy of microcapsules

Spectroscopic electromagnetic radiations that interact with a substance can be absorbed, transmitted, reflected, scattered, or have photoluminescence (PL), which provides significant information on the molecular structure and the energy level transition of that substance (Baker *et al.*, 2016 and Cui *et al.*, 2017). The incorporation of flaxseed oil in the coat material could be confirmed by FT-IR spectroscopy.

To support the results of microencapsulation of flaxseed oil, studies were carried out by FT-IR analysis to know about any complex formation and interaction of major functional groups involved in microencapsulation of flaxseed oil as shown in Figure. 5.10 and 5.11. The results help in confirming the presence of flaxseed oil in microcapsules..

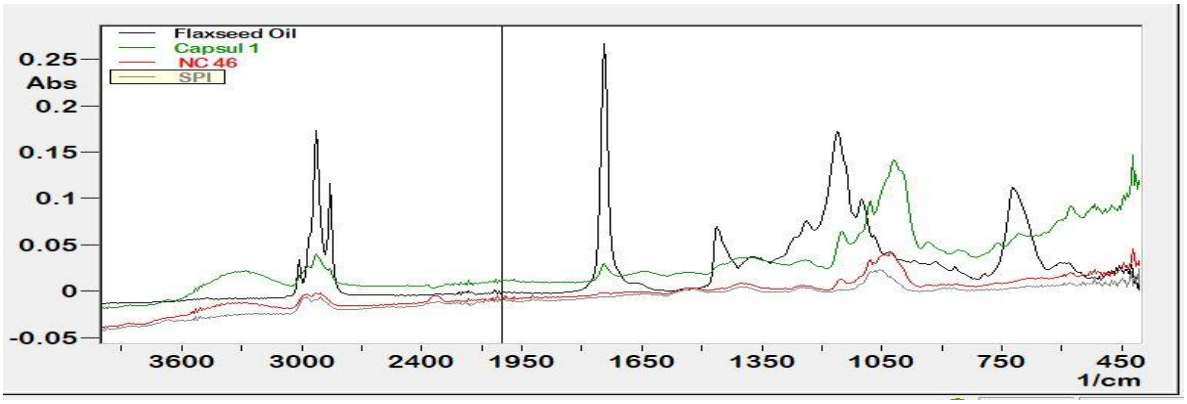

Figure 5.10: FT-IR spectra for NC 46 microcapsules with its ingredients

The spectra of microcapsules clearly revealed that the carboxylic acid (C=C.CO.OH) (C=C stretch) group interact with aldehyde group (-RCHO) to form strong bond at 2980cm^{-1} indicates the presence of carboxylic group and aldehyde groups. The

peaks at 1722.30cm^{-1} of flaxseed oil are dissolved by NC 46 microcapsule peaks. This indicates that association of flaxseed oil and microcapsules and presence of alkane group

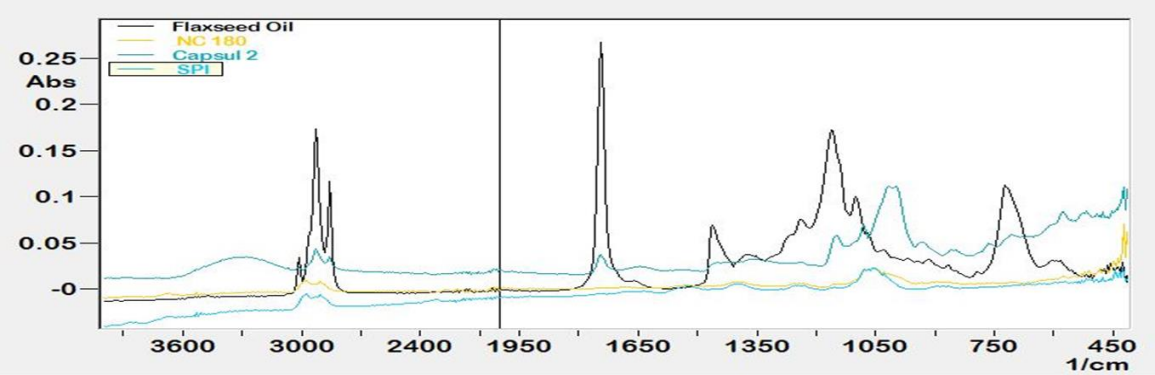

Figure 5.11: FT-IR spectra for NC 180 microcapsules with its ingredients

with C=C stretch. It is also evident that major peaks present in the spectra of coat material are also prominent in the flaxseed oil loaded capsules. However many peaks were formed probably due to the presence flaxseed oil in the capsule. In case of microcapsules spectra peak at 1052cm^{-1} is due to presence of alkyl halide C=C stretch and the peak at 1395cm^{-1} is due to carboxylic acid R-CO.O structure with C=O stretch. In the view of this spectra, it is established that core material (flaxseed oil) has been successfully encapsulated with soy protein isolated and NC 46 starch. It is visible from the FT-IR spectra of the coat material and flaxseed oil that the both are closely matching at the characteristic peak of flaxseed oil at 1656cm^{-1} (C=C stretch) and down at 1700cm^{-1} (C=N Stretch) has been absorbed by peak of microcapsules at same wavelength. The FT-IR spectrum of microcapsules is also matching with spectra reported by various authors who worked on microencapsulation of essential oils. In FT-IR spectra of both the microcapsules (Figure 5.10 and 5.11), no new bonds were formed beside the spectra of coat materials (SPI and modified starch) and core material (flaxseed oil).

The peaks of flaxseed oil at 2900cm^{-1} was corresponding to methyl group C=C stretch. Also the peak at 1160cm^{-1} is corresponding to amine group (CN) stretch. It might be due to active compound of core and coat material. Thus, it was revealed by the FT-IR spectra that all the functional group of both core and coat moiety are present in microcapsule.

5.2.1.9. Oxidative stability (peroxide value) of flaxseed oil microcapsules

Peroxide value (PV) of oil indicates the amount of oxidation and formation of primary oxidation products during storage and processing. Although these oxidation products, particularly hydroperoxides are colourless & odourless and produce no off-flavours, but are highly toxic and reduce the bioavailability of fatty acids. Flaxseed oil is highly polyunsaturated (~76% PUFA) and thus, highly susceptible to atmospheric oxygen, high temperature and metal ions. It is reported that ALA is 20 times more susceptible to oxidation as compared to oleic acid (Decker *et al.*, 2012). In the present study, peroxide value of selected (containing 30% oil load and 30% TS) microcapsules was measured at an interval of one month up to the 3 months of storage at 4-7°C. The peroxide value of refined flaxseed oil was observed to be ~2.34 mEq peroxides/kg at zero day, which continuously increased and reached to 8.68 mEq peroxides/kg at the end of three months of storage at refrigerated temperature (4-7°C) as evident from Figure 5.12.

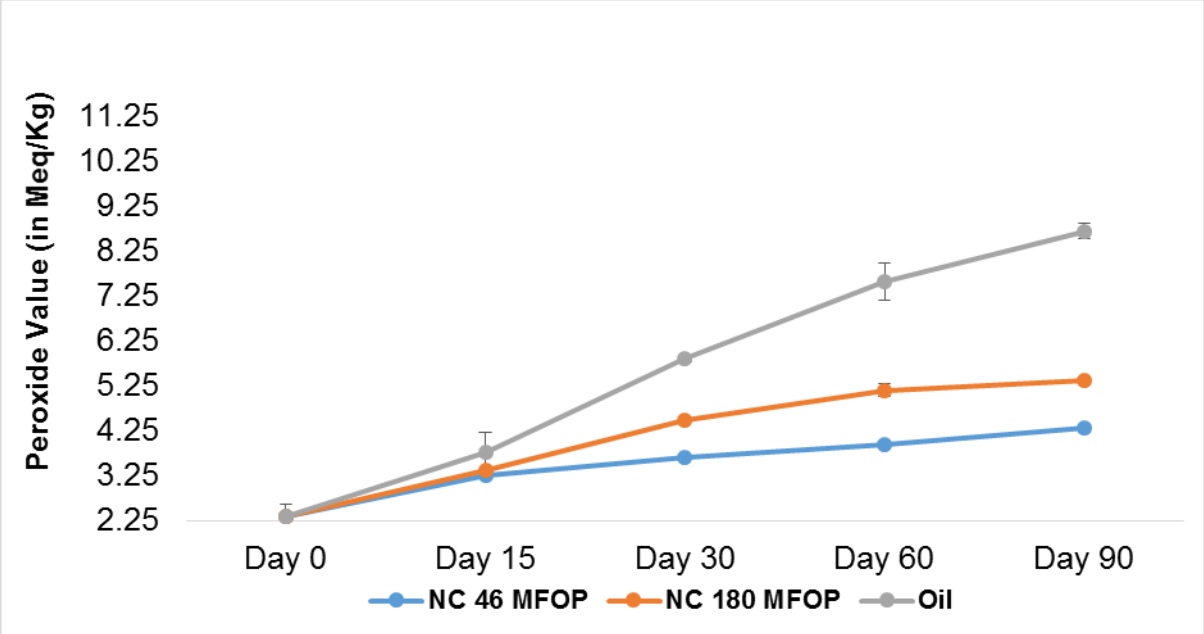

Figure 5.12: Peroxide values of microcapsules and oil during storage (4-7ºC)

It is clear from the Figure 5.12 that there was a significant difference ($p>0.05$) amongst the peroxide value of different formulations of microcapsules prepared from NC 46 and NC 180. The data also revealed that the peroxide value of flaxseed oil on zero day was almost similar to the peroxide value of microcapsules and there was statistically non-significant ($p>0.05$) difference. The data suggests that the microencapsulation followed by spray drying has not caused any oxidative damage to the flaxseed oil. Both the microcapsules

showed a gradual but significant ($p<0.05$) increase in peroxide value during the storage of three months at 4-7°C. In NC 46 microcapsules, it increased from 2.34 to 4.30 mEq peroxides/kg (Figure 5.12), while in NC 180, peroxide value increased from the 2.34 to 5.37 mEq peroxides/kg. It is also evident from the Figure 5.12 that the peroxide value of free oil increased more rapidly as compared to the encapsulated oil. The data of peroxide value of microcapsules preparations was about 69.08 and 52.20% lesser in case of NC 46 and NC 180 respectively as compared to free oil (control). It is also evident that higher peroxide value in NC 180 microcapsule could be attributed to the presence of more amount of free/ surface oil as compared to NC 46 formulations. Further, the peroxide value of free oil was more than the permissible limit of 5 mEq peroxides/kg of oil while, lesser in case of NC 46 microcapsules as per the Codex Alimentarius Commission (1999) standards.

The present results are in good agreement with the findings of Karaca *et al.,* (2013), Partanen *et al.,* (2008) and Grattard *et al.,* (2002), who reported improved oxidative stability of flaxseed oil encapsulated by different proteins. However, Tonon *et al.,* (2011) reported a very low range of PV (0.017-0.106 mEq peroxide/kg oil) in microencapsulated flaxseed oil prepared using gum arabic.

5.2.1.10. Moisture content and solubility of selected microcapsules with storage

Moisture content in dried foods plays a vital role for the stability. As microcapsules were prepared to encapsulate the highly unstable flaxseed oil as core material, the moisture content determination in capsules with storage of 90 days is an important parameter to be reported. Slight non-significant ($p>0.05$) difference in the moisture content of microcapsules was observed with storage (Table 5.10). Same observation was reported by Goyal (2014) and Tonon *et al.,* (2011) for flaxseed oil microcapsules.

Table 5.10: Moisture content and solubility of microcapsules during storage (4-7ºC)

Parameter	Day	Day 15	Day 30	Day 60	Day 90
Moisture content (%)	NC 46	3.89±0.27[a]	3.91±0.04 [a]	3.93±0.05 [a]	3.96±0.03 [a]
	NC 180	3.73±0.08 [a]	3.83±0.02 [a]	3.88±0.07 [a]	3.89±0.04 [a]
Solubility (%)	NC 46	91.65±1.10[a]	90.86±1.66 [a]	89.29±0.66 [b]	89.02±0.75 [b]
	NC 180	83.24±1.43 [a]	82.31±0.37 [b]	82.20±0.71 [b]	81.61±0.55 [b]

Results are expressed as Mean±SD, n=3; Means with different small letters superscript (a,b,c) within row differ significantly ($p<0.05$) among the samples

Solubility is one of major important parameter for fortification of microcapsules into milk. The solubility of developed microcapsules was determined for 90 days. The solubility

of microcapsules prepared from NC 46 starch was 91.65% on 0^{th} day and slightly decreases on 90th days to 89.02%. There is non-significant difference ($p>0.05$) in solubility of microcapsules up to 30 days of storage which further decreased slightly. Solubility of NC 180 microcapsules is less than that of microcapsules prepared from NC 180. It was initially 83.24% and on 90^{th} day, it was 81.61%. Solubility decreases on 30th day then after there is non significant ($p>0.05$) difference in solubility up to 60 days and further slight decrease in solubility of NC 180 microcapsules was observed.

5.2.1.11. *In-vitro* release behaviour of flaxseed oil from MFOP under simulated gastro-intestinal conditions

In-vitro systems have been extensively used by the pharmaceutical industry to check the delivery of newly formulated drugs. Food industry is now using similar approach for the design of food-grade delivery systems. In such models, samples are exposed to human gastric and intestinal conditions which are created in labwares. Samples in simulated gastric conditions are exposed to an acidic pH (1- 2), salts (such as NaCl), biopolymers (such as porcine gastric mucosa) as well as proteolytic enzymes (pepsin) (Sarkar *et al.,* 2009; Van Aken *et al.,* 2011), which mimic the human gastric conditions. While, in simulated intestinal conditions, samples are subjected to higher pH (~6.8), mixture of enzymes (such as pancreatin), salts and bile salts (Hu *et al.,* 2010). In the present study, the release behaviour of flaxseed oil was investigated through the simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) conditions.

5.2.1.11.1. Release (in %) of flaxseed oil under simulated gastric conditions

Per cent release of flaxseed oil from the microcapsules exposed to simulated gastric fluid (SGF, pH 1.2) is shown in Table 5.11. The per cent oil released was almost similar for both the microcapsules during initial 2 hours of incubation, while, later on for subsequent incubation for 4 and 6 hours, the per cent release was more in case of NC 46 microcapsules than NC 180 (15.69 and 10.69 % flaxseed oil, respectively). However, low per cent release at very low pH (~1.2) may be due to coalescence of droplet at gastric pH, protein surface remains positively charged and unable to provide sufficient electrostatic repulsion and thus favour increase in flocculation (Singh, 2011; Sarkar *et al.,* 2009). Additionally, the study reveals that the per cent oil released was comparatively higher in case of microcapsules subjected to with heating at 85°C for 5 min prior to gastric conditions. In case of NC 46 microcapsule, the per cent release increased from

15.69 to 30.69% for 6 hours incubation in gastric condition when subjected to heating at 80°C for 5 minutes. This may be due to the fact the heat treatment would have made the protein present in the coat material susceptible to pepsin action, thereby facilitating release of flaxseed oil.

Table 5.11: Release (in %) of flaxseed oil under simulated gastric conditions

Microcapsules/ treatment	Time(Hours)		
	2	4	6
NC 46 (without heating)	5.31	9.40	15.69
NC 180(without heating)	5.15	7.49	10.69
NC 46(with heating 85°C/5 min)	8.05	12.57	30.69
NC 180(with heating 85°C/5 min)	7.26	10.88	22.64

5.2.1.11.2. Release (in %) of flaxseed oil under simulated gastro-intestinal condition (Sequential exposure of SGF+SIF for 2+3 hours)

During the digestion of food, it is first digested by gastric pepsin at very low pH and then reaches the intestine (pH 6.8) having higher pH and presence of food stimulates the pancreatic enzymes for further digestion and absorption of food components. Therefore in present study, it was tried to simulate the gastro-intestinal conditions by mimicking the sequential exposure of microcapsules to SGF and SIF for evaluating the per cent release in intestine. The per cent flaxseed oil release in gastro-intestinal condition was measured by sub sequential exposure of microcapsules to SGF for 3 hours and for SIF 2 hours and the results calculated with (85^{o}C for 5 min.) and without heating of microcapsules and the values are reported in Table 5.12.

Table 5.12: Release (in %) of flaxseed oil under simulated gastro-intestinal condition

Microcapsules/ treatment	Per cent release of flaxseed from microcapsules
NC 46 Without Heating	38.24±2.4
NC 46 With Heating	60.86±1.44
NC 180 With Heating	34.69±2.42
NC 180 Without Heating	51.26±2.74

It can be clearly interpreted that there is huge increase in per cent release of flaxseed oil in gastro-intestinal conditions when compared to gastric condition. This could be due to higher degradation of the microcapsules in SGF+SIF containing pancreatin (amylase and trypsin), which hydrolyzes both proteins and carbohydrate resulting in the change in capsule structure (such as large pore formation) with subsequent oil release (Kosaraju *et al.*, 2009). Also the per cent release increases with heat treatment due to the probable unfolding of soy proteins present in the coat of microcapsules due to heat treatment. Our results are in agreement with findings of Shen *et al.*, (2011), who reported that free fatty acids release increased from 5.2±4.1 (SGF) to 78.3±1.7% (SGF+SIF) during *in-vitro* digestion of microencapsulated tuna oil powder. However, Karaca *et al.*, (2013) reported a very high % release of oil under SGF (36.6-43.4%) and SGF+SIF (84.5-92.6%) conditions in microencapsulated flaxseed oil powder. Such a high % oil release in their study may be due to the different wall material (chick pea and lentil protein isolates) and freeze drying used in the preparation of flaxseed oil powder.

5.2.1.12. Fatty acids profile of microencapsulated flaxseed oil

The fatty acids as their methyl esters (FAMEs) were resolved using the conditions given for GC-MS analysis. Fatty acids were identified by comparison of elution times with standard from library of GC-MS FAMEs and were estimated as AREA on fatty acids. A typical chromatogram obtained from standard mixture of FAMEs from NC 46 and NC 180 microcapsules are shown in Figure 5.13 and 5.14. The entire chromatographic run was for 45 min.

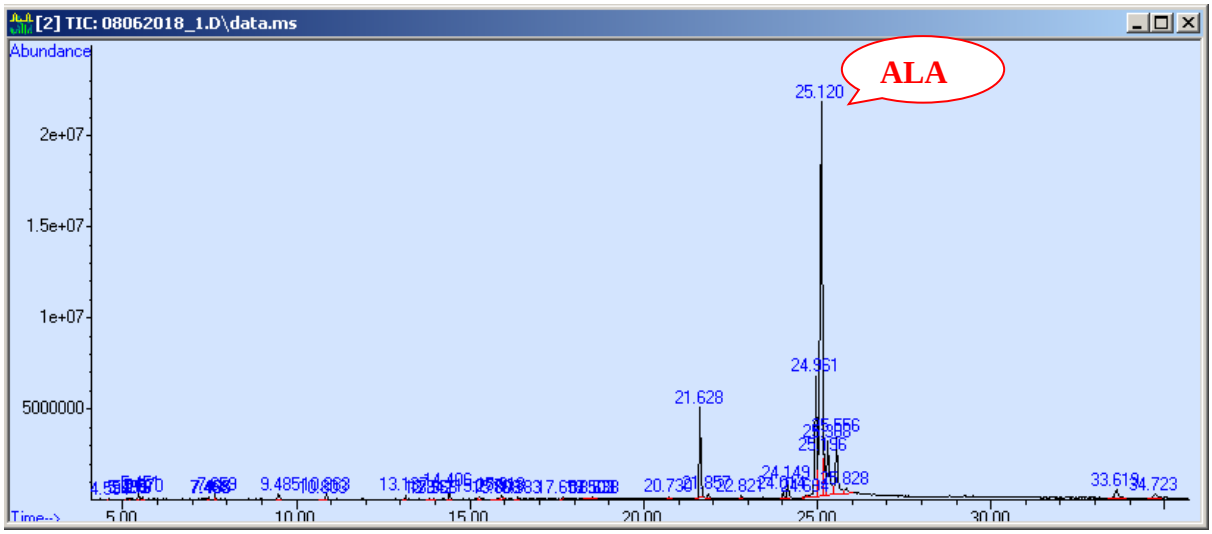

Figure 5.13: GC-MS chromatogram for NC 46 microcapsules

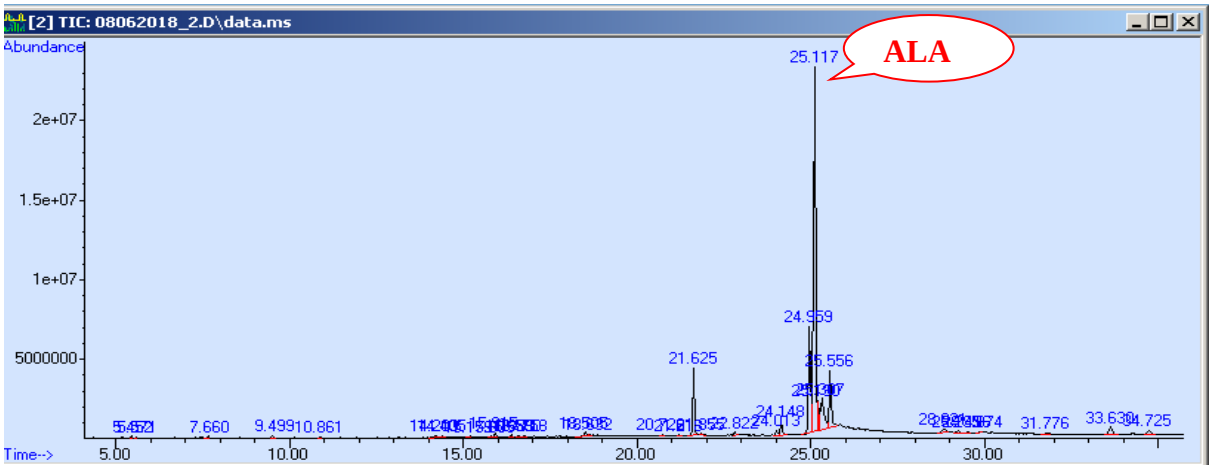

Figure 5.14: GC-MS chromatogram for NC 180 microcapsules

The retention time and peak area for alpha linolenic acid of extracted flaxseed oil from NC 46 and NC 180 microcapsules are shown in Table 5.13. The ALA content was estimated in terms of the % area occupied by the fatty acid methyl ester of alpha linolenic acid, i.e. Butyl 9, 12, 15, Octadecatrienoate in this case. There were two major peaks observed for the oil extracted from NC 46 microcapsule at the retention time of 25.120 and 25.308 minutes for the fatty acid ester of ALA i.e. Butyl 9,12, 15, Octadecatrienoate. However, in case of NC 180 microcapsule, three major peaks at 25.118, 25.189 and 25.305 min. were observed.

Table 5.13: Fatty acid profile microcapsules with specific to omega-3 fatty acid

Omega 3 fatty acid in microcapsules					
Sample	**Peak Number**	**Ret. Time (in min)**	**Database**	**Area**	**% Area**
NC 46 microcapsules	21	25.120	Butyl 9,12, 15, Octadecatrienoate	1023034109	54.11
	23	25.308	Butyl 9,12, 15, Octadecatrienoate	0133582297	07.06
Total Area of ALA				**1156616406**	**61.17**
Sample	**Peak Number**	**Ret. Time (in min)**	**Database**	**Area**	**% Area**
NC 180 Microcapsules	16	25.118	Butyl 9,12, 15, Octadecatrienoate	1082397226	55.62
	17	25.189	Butyl 9,12, 15, Octadecatrienoate	0065732020	03.38
	18	25.305	Butyl 9,12, 15, Octadecatrienoate	0127581960	06.56
Total Area of ALA				**1276711266**	**65.56**

Thus, from the results, it is evident that concentration of alpha linolenic acid (18:3) in the oil extracted from NC 46 and NC 180 microcapsules was 61.67 and 65.56%, respectively. Hence, addition of flaxseed oil microcapsules to milk must result in an increased ALA content in fortified milk. From the results obtained for quantity of ALA in the oil extracted from the microcapsule, it can be inferred that prepared flaxseed oil microcapsules had ALA content more than 20%, so just by adding two grams of microcapsules in one serving of any food or dairy product, at least 25% RDA of the ALA can be achieved.

Further, the omega-6 fatty acid present in the oil extracted from flaxseed oil microcapsules showed retention time of 24.961 minutes for both the microcapsules (Table 5.14). In this case, one major peak was observed for both the microcapsules with 13.8 and 13.34 % of linoleic acid content in the oil extracted from NC 46 and NC 180 microcapsule, respectively.

Table 5.14: Fatty acid profile microcapsules with specific to omega-6 fatty acid

Omega 6 fatty acid in microcapsules					
Sample	**Peak Number**	**Ret. Time (in min)**	**Database**	**Area**	**% Area**
NC 46 microcapsules	20	24.961	Butyl, 9 ,12, Octadecadienoate	261010325	13.80
Total area of linoleic acid				**261010325**	**13.80**
Sample	**Peak Number**	**Ret. Time (in min)**	**Database**	**Area**	**% Area**
NC 180 Microcapsules	15	24.961	Butyl, 9,12, Octadecadienoate	259554077	13.34
Total area of linoleic acid				**259554077**	**13.34**

5.3 Fortification of flaxseed oil microcapsules in pasteurized milk

As the flaxseed oil microcapsules prepared from NC 46 starch performed better than NC 180 microcapsules, it was selected for further fortification in milk. Firstly, two different levels of microcapsules were fortified for preparation of fortified pasteurized milk and then one level was selected based on sensory acceptability for further study.

5.3.1. Optimization of level of microcapsules in preparation of milk

Milk was fortified with flaxseed oil microcapsules for providing at least 25 and 50% recommended dietary allowance (RDA) of alpha linolenic acid (ALA) in one serving (240mL). The sensory acceptability of fortified samples are represented in Table 5.15.

Table 5.15: Effect of level of microcapsules on sensory acceptability of fortified milk

Sensory Parameters	control	F25	F50
Colour and appearance	8.78 ± 0.25^{a}	8.72 ± 0.56^{a}	8.81 ± 0.12^{a}
Mouthfeel	8.61 ± 0.65^{a}	8.58 ± 0.78^{a}	8.39 ± 0.77^{b}
Taste and Flavour	8.72 ± 0.23^{a}	8.47 ± 0.46^{ab}	7.39 ± 0.82^{b}
Overall Acceptability	8.67 ± 50^{a}	7.78 ± 0.72^{b}	7.28 ± 0.24^{c}

F25- sample having microcapsules providing 25% RDA of ALA, F50- sample having microcapsules providing 50% RDA of ALA, Results are expressed as Mean±SD, n=3; Means with different small letters superscript (a,b,c) within row differ significantly ($p<0.05$) among the samples

It is evident from the table data, that there was no significant ($p>0.05$) difference among the scores for colour, mouthfeel of control milk and F25 milk sample, however, the mouthfeel of F50 milk was lower than control milk. Further, the taste and flavour and overall acceptability decreased significantly ($p<0.05$) upon addition of microcapsule. Based upon higher sensory acceptability, 25% RDA level was selected.

5.3.2. Storage study of fortified pasteurized milk

In order to further increase the acceptability by a wider group of consumers, flavoured fortified milk was also developed and compared for various physic-chemical parameters, sensory acceptability, rancidity and microbiological spoilage during storage.

5.3.2.1 Sensory acceptability during storage for pasteurized fortified milk

There was no significant ($p>0.05$) difference among the sensory scores for colour and appearance of control pasteurised milk, fortified milk and fortified flavoured milk with the storage days and the variation with samples. The colour and appearance of fortified flavoured pasteurised milk shows slightly less score than the control and fortified pasteurised but there is non significant ($p> 0.05$) difference in the colour values of all the samples.

There was non-significant difference ($p>0.05$) between the sensory score for mouthfeel within the sample and over a storage period (Table 5.16). However, the slightly lower scores were observed for the plain fortified and fortified flavoured pasteurized milk as compared to the pasteurised control milk sample.

Table 5.16: Colour and mouthfeel scores of control and fortified pasteurised milk samples during storage (at 4-7 ˚C)

	Days	Sample		
		PP	FP	FFP
Colour and appearance	0	8.89 ± 0.22^{aA}	8.44 ± 0.30^{aA}	8.58 ± 0.17^{aA}
	2	8.17 ± 0.35^{aB}	8.11 ± 0.70^{aB}	8.06 ± 0.32^{aB}
	4	8.22 ± 0.26^{aAB}	8.17 ± 0.25^{aAB}	8.11 ± 0.22^{aAB}
	6	7.78 ± 0.44^{aB}	8.03 ± 0.40^{aB}	8.03 ± 0.40^{aB}
Mouthfeel	0	8.72 ± 0.06^{bA}	8.26 ± 0.25^{bA}	8.39 ± 0.39^{aA}
	2	8.44 ± 0.39^{bB}	8.89 ± 0.33^{bB}	7.94 ± 0.32^{aB}
	4	8.42 ± 0.26^{bB}	7.83 ± 0.35^{bB}	8.00 ± 0.25^{aB}
	6	8.33 ± 0.43^{bC}	7.61 ± 0.33^{bC}	7.50 ± 0.50^{aC}

PP- Plain pasteurized milk (Control), FP- fortified pasteurized milk, FFP- fortified flavoured pasteurized milk, Results are expressed as Mean±SD, n=3; Means with different small letters superscript (a,b,c) within row differ and capital letters (A,B, C) within the column significantly ($p<0.05$) among the samples

The taste and flavour scores showed a decreasing trend during storage period. The initial score were 8.50, 7.33 and 7.72 for pasteurised control, fortified pasteurised and fortified flavoured pasteurised milk respectively which became 8.00, 7.39 and 7.28, respectively after 6 days of storage (Table 5.17). The fresh taste and flavour of the fortified milk was changed from rich taste of milk to bland flavour of soy protein after 6th days. While in control milk the change in taste and flavour is negligible.

Table 5.17: Taste & flavour and overall acceptability of control and fortified pasteurised milk samples during storage (at 4-7 ˚C)

	Days	Sample		
		PP	PF	PFF
Taste & Flavour	0	8.50 ± 0.50^{bA}	7.33 ± 0.05^{bA}	7.72 ± 0.36^{aA}
	2	8.33 ± 0.50^{bA}	7.22 ± 0.57^{bA}	7.31 ± 0.70^{aA}
	4	8.00 ± 0.36^{bA}	7.24 ± 0.58^{bA}	7.26 ± 0.54^{aA}
	6	8.00 ± 0.60^{bA}	7.39 ± 0.42^{bA}	7.28 ± 0.36^{aA}
	Days	**Sample**		
		PP	PF	PFF
Overall Acceptability	0	8.57 ± 0.18^{aA}	7.43 ± 0.04^{bA}	7.88 ± 0.35^{cA}
	2	8.39 ± 0.33^{aA}	7.61 ± 0.41^{bA}	7.85 ± 0.35^{cA}
	4	8.61 ± 0.22^{aA}	7.56 ± 0.17^{bA}	7.72 ± 0.30^{cA}
	6	7.83 ± 0.35^{aB}	7.39 ± 0.42^{bB}	7.41 ± 0.40^{cB}

PP- Plain pasteurized milk (Control), FP- fortified pasteurized milk, FFP- fortified flavoured pasteurized milk, Results are expressed as Mean±SD, n=3; Means with different small letters superscript (a,b,c) within row and capital letters (A,B, C) within the column differ significantly ($p<0.05$) among the samples

The overall acceptability of fortified milk and fortified flavoured milk decreased significantly ($p<0.05$) and with the storage days, but there is no significant ($p>0.05$) difference within the sample upto fourth day, but on sixth day of storage the statistical analysis shows the difference. This might be because of increase in acidity of milk and thereby adversely affecting the taste and flavour of fortified milk.

5.3.2.2 Physico-chemical properties of pasteurized fortified milk during storage

The fortified milk was characterized for physico-chemical properties viz. acidity, pH, free fatty acid, viscosity and colour with storage for 6 days (Table 5.18). Is revealed that there is no significant difference ($p>0.05$) in the acidity of different milk samples, however, the difference was significant ($p<0.05$) with storage days. This data shows agreement with Veena (2014) who developed milk fortified with omega-3 fatty acids, phytosterols and soluble dietary fibre.

Table 5.18: Effect of storage (4-7˚C) on physico-chemical characteristics of pasteurised fortified milk

Parameter	DAY	PC	PFP	FFP
Acidity (% Lactic acid)	0	$0.165\pm0.005^{a,D}$	$0.162\pm0.000^{a,D}$	$0.168\pm0.005^{a,D}$
	2	$0.186\pm0.004^{a,C}$	$0.182\pm0.009^{a,C}$	$0.184\pm0.004^{a,C}$
	4	$0.201\pm0.005^{a,B}$	$0.201\pm0.005^{a,B}$	$0.198\pm0.009^{a,B}$
	6	$0.222\pm0.005^{a,A}$	$0.225\pm0.009^{a,A}$	$0.222\pm0.005^{a,A}$
pH	0	6.63 ± 0.01^{aA}	6.61 ± 0.01^{bA}	6.58 ± 0.01^{cA}
	2	6.57 ± 0.01^{aB}	6.51 ± 0.01^{bB}	6.47 ± 0.01^{cB}
	4	6.54 ± 0.01^{aC}	6.48 ± 0.01^{bC}	6.43 ± 0.01^{cC}
	6	6.48 ± 0.01^{aD}	6.45 ± 0.01^{bD}	6.41 ± 0.01^{cD}
Viscosity (m Pas)	0	$1.380\pm0.016^{c,B}$	$1.877\pm0.015^{b,B}$	$2.503\pm0.248^{a,B}$
	2	$1.420\pm2.016^{c,B}$	$1.997\pm2.015^{b,B}$	$2.727\pm0.284^{a,B}$
	4	$1.904\pm4.016^{c,A}$	$2.427\pm4.015^{b,A}$	$3.153\pm0.047^{a,A}$
	6	$2.083\pm6.016^{c,A}$	$2.667\pm6.015^{b,A}$	$3.280\pm0.026^{a,A}$
FFA (µEq/ml)	0	0.095 ± 0.001^{aD}	0.094 ± 0.001^{abD}	0.094 ± 0.001^{bD}
	2	0.103 ± 0.001^{aC}	0.102 ± 0.001^{abC}	0.102 ± 0.001^{bC}
	4	0.115 ± 0.001^{aB}	0.114 ± 0.002^{abB}	0.116 ± 0.001^{bB}
	6	0.136 ± 0.000^{aA}	0.136 ± 0.000^{abA}	0.136 ± 0.001^{bA}

PP- Plain pasteurized milk (Control), FP- fortified pasteurized milk, FFP- fortified flavoured pasteurized milk, Results are expressed as Mean±SD, n=3; Means with different small letters superscript (a,b,c) within row and capital letters (A,B, C) within the column differ significantly ($p<0.05$) among the samples

As the literature pertaining to microencapsulated flaxseed oil fortified milk is scanty, but these results were in agreement with those who used flaxseed oil instead of powder and reported no effect of omega-3 fortification on the acidity of fortified milk (Liutkevicius *et al.*, 2007; Lim *et al.*, 2010; Veena, 2014). Also, there is statistically significant difference ($p<0.05$) in pH value of fortified and control pasteurised milk samples with variation in sample and storage days.

To know the effect of addition of microcapsules into the fortified milk, the viscosity of fortified and control milk was compared. It can be interpreted that the viscosity of plain pasteurised (control) < fortified pasteurised milk < fortified flavoured pasteurised milk. This is because of more total soilds in fortified and flavoured milk. The viscosity of plain pasteurised (control) was 1.380 mPa-s whereas for fortified pasteurized milk and flavoured fortified pasteurised milk was 1.877 and 2.503 mPa-s, respectively. The viscosity of all the samples was found increasing with storage as 2.083, 2.667 and 3.280 mPa-s in plain pasteurised (control), fortified pasteurised milk and fortified flavoured milk, respectively. This increase might be because of the aggregation of milk solids during storage.

Flaxseed oil contains about approximately 73% of polyunsaturation, hence the free fatty acid content (FFA) of flaxseed oil increases with small contaminations with metals and any heat treatment, thus the effect of pasteurization on FFA content was measured. From the Table 5.18, it is evident that the FFA level of control and fortified milk were statistically non significant ($p>0.05$) while, flavoured fortified pasteurised milk had shown more FFA content than the control, possibly due to interaction of added sugar and other additives with the fat. Further, the free fatty acid content increased significantly ($p<0.05$) during storage for all the studied samples.

5.3.2.3 Microbiological quality of pasteurized fortified milk during storage

Pasteurization treatment has a positive effect on the microbiological quality of milk, as it reduces the standard plate count (SPC), coliform count and other pathogens (El Zubeir *et al.*, 2007). Simon and Hansen, (2001) stated that milk processed at 76.4^{o}C had the lowest bacterial growth rate. Data on microbiological analysis of pasteurized milk samples during storage are given in Table 5.19. Standard Plate Count (SPC) in control milk was 12000 cfu/ml soon after pasteurization and reached 36000 cfu/ml at the end of storage period (6 days). For fortified milk, SPC count was 12000 cfu/ml on zero day and reached 32000 cfu/ml at the end of storage period, while in flavourd pasteurised milk it was initially 13000 and

increased to 39000 cfu/ml on 6th day. Mourgues and Auclair, (1975) showed that storage temperature had a considerable effect on growth of psychrotrophic bacteria and thus on shelf-life of pasteurized milk. The presence of psychrotrophs in cold raw milk (pre processing) could be the critical factor in undermining the keeping quality of pasteurized milk and other dairy products (Zall, 1990) Colifoms, yeasts and molds were not detected in both control and fortified milk samples throughout storage period.

Table 5.19: Effect of storage (at 4-7 ˚C) on microbial counts of pasteurized control and fortified milk samples

Microbial count (Pasteurised milk)	Days	Cfu/ml		
		Plain Pasteurised	Fortified plain pasteurised	Fortified flavoured pasteurised
Standard Plate Count	0	12×10^3	12×10^3	13×10^3
	2	15×10^3	17×10^3	18×10^3
	4	21×10^3	25×10^3	24×10^3
	6	36×10^3	32×10^3	39×10^3
Yeast and mould	0	ND	ND	ND
	2	ND	ND	ND
	4	ND	ND	ND
	6	ND	ND	ND
Coliform	0	ND	ND	ND
	2	ND	ND	ND
	4	ND	ND	ND
	6	ND	ND	ND
ND = Not Detected				

PP- Plain pasteurized milk (Control), FP- fortified pasteurized milk, FFP- fortified flavoured pasteurized milk

This suggested that pasteurisation was done efficiently and there is no post processing contamination. Thus, no potential health hazards associated with storage conditions could be seen. According to Food Safety and Standards Regulation, 2011 (FSSA, 2012), the acceptable quality of pasteurized milk should contain less than 30,000/g for total plate count and less than 10/g for coliform count. Thus, the developed fortified pasteurized samples are safe for consumption upto 4 days of storage at refrigerated conditions.

5.4. Preparation of fortified sterilised milk

As per approved technical program, the fortified sterilised milk was prepared with fortification of flaxseed oil microcapsules as a source of omega-3 fatty acids. The fortified

and control sterilised milk samples were analysed for the sensory, physicochemical properties and proximate composition of fortified and control sterilised milk.

5.4.1 Sensory acceptability of fortified sterilized milk during storage

The changes in the score of colour and appearance are shown Table 5.20. It is evident from the data that on 0^{th} day, score of plain sterilised milk was slightly higher than the fortified sterilised and fortified flavoured sterilised milk. The sensory scores for colour and appearance were statistically same for first and seventh day of storage and thereafter the score were found to decrease over the period of storage. The colour and appearance score was less for the fortified flavoured sterilised milk because of maillard browning taking place during the heat treatment as addition of sugar was done in to milk. While control and fortified sterilized milk has almost same colour and appearance score over the storage period. Also, the score for mouthfeel of fortified and control milk samples decreased with storage period. It can be seen that the mouthfeel scores followed following trend: fortified flavoured milk > Plain sterilised > fortified sterilised. The mouthfeel score for fortified flavoured sterilised milk was more may be because of smoothness in texture of fortified flavoured milk, while followed by plain sterilised and lastly fortified sterilised, because of addition of microcapsules.

Table 5.20: Colour and mouthfeel scores of control and fortified sterilized milk samples during storage (at 25ºC)

	Days	Sample		
		PS	FS	FFS
Colour and appearance	0	8.27 ± 0.26^{aA}	8.16 ± 0.25^{aA}	8.16 ± 0.25^{aA}
	07	8.05 ± 0.63^{aA}	8.05 ± 0.30^{aA}	8.12 ± 0.20^{aA}
	14	7.66 ± 0.75^{aAB}	7.55 ± 0.68^{aAB}	8.05 ± 0.05^{aAB}
	21	8.00 ± 0.43^{aB}	7.88 ± 0.22^{aB}	8.11 ± 0.22^{aB}
	28	7.20 ± 0.26^{aC}	7.22 ± 0.26^{aC}	7.22 ± 0.36^{aC}
Mouthfeel	0	7.94 ± 0.30^{bA}	7.88 ± 0.33^{bA}	8.23 ± 0.27^{aA}
	07	7.94 ± 0.39^{bA}	7.94 ± 0.30^{bA}	8.33 ± 0.25^{aA}
	14	7.55 ± 0.58^{bAB}	7.27 ± 0.66^{bAB}	8.16 ± 0.50^{aAB}
	21	7.75 ± 0.35^{bB}	7.83 ± 0.25^{bB}	7.88 ± 0.33^{aB}
	28	7.33 ± 0.35^{bC}	7.33 ± 0.35^{bC}	7.44 ± 0.46^{aC}

PS- Plain sterilized milk (Control), FS- fortified sterilized milk, FFS- fortified flavoured sterilized milk, Results are expressed as Mean±SD, n=3; Means with different small letters superscript (a,b,c) within row and capital letters (A,B, C) within the column differ significantly ($p<0.05$) among the samples

Effect of storage on sensory scores of taste and flavour and overall acceptability of fortified and control sterilized milk are given in Table 5.21. The taste and flavour scores were maximum on 0 day for all the three samples.

Table 5.21: Taste & flavour and overall acceptability of sterilised fortified milk during storage (at 25ºC)

	Days	Sample		
		PS	FS	FFS
Taste and Flavour	0	7.94 ± 0.30^{cA}	7.77 ± 0.26^{bA}	8.61 ± 0.48^{aA}
	07	7.56 ± 0.62^{cB}	7.33 ± 0.66^{bB}	8.55 ± 0.30^{aA}
	14	7.44 ± 0.39^{cAB}	6.88 ± 0.65^{bAB}	8.05 ± 0.52^{aAB}
	21	6.34 ± 0.33^{cAB}	7.22 ± 0.61^{bAB}	7.77 ± 0.66^{aAB}
	28	7.31 ± 0.45^{cC}	7.16 ± 0.35^{bC}	7.72 ± 0.44^{aC}
Overall acceptability	0	8.05 ± 0.30^{bA}	7.88 ± 0.33^{bA}	8.61 ± 0.41^{aA}
	07	7.55 ± 0.60^{bA}	7.84 ± 0.32^{bA}	8.61 ± 0.22^{aA}
	14	7.44 ± 0.46^{bB}	7.16 ± 0.55^{bB}	8.11 ± 0.48^{aB}
	21	7.27 ± 0.06^{bB}	7.38 ± 0.54^{bB}	7.83 ± 0.61^{aB}
	28	7.11 ± 0.33^{bC}	7.94 ± 0.39^{bC}	7.55 ± 0.39^{aC}

PS- Plain sterilized milk (Control), FS- fortified sterilized milk, FFS- fortified flavoured sterilized milk, Results are expressed as Mean±SD, n=3; Means with different small letters superscript (a,b,c) within row and capital letters (A,B, C) within the column differ significantly ($p<0.05$) among the samples

Acceptability of fortified milk on first day was comparable to the control milk while sterilised flavoured milk had slightly higher taste and flavour scores than the fortified milk. Upon storage for seven, fourteen and twenty-one days of storage there is non significant difference ($p>0.05$) in the table flavour score of fortified and control sterilised milk samples. But on 28th day of storage there is significant ($p<0.05$) difference with sensory score of taste and mouthfeel of fortified and control samples.

The fortified flavoured sterilised milk was liked the most by the sensory panellist as compared to control and fortified sterilised milk samples. The overall acceptability scores on the 0th day for plain sterilised and fortified sterilised milk were 8.05 and 7.88 respectively, while on 28th day, it was 7.11 and 7.94. Whereas score for the fortified flavoured sterilised was 8.61 on first day while at end of storage it was 7.55 (Table 5.21). The higher overall

acceptability may be due to the addition of sugar and artificial flavour in fortified flavoured sterilised milk.

5.4.2. Physico-chemical characterization of fortified sterilized milk with storage study

The physico-chemical properties viz. acidity, pH, viscosity and free fatty acid content of control and fortified milk samples during storage are shown in Table 5.22. Lactic acid is the principal acid produced due to which titrable acidity of milk rises. Increase in free fatty acids is also responsible for increasing the total titrable acidity of milk (Swartzel, 1983). From the results, it can be seen that titratable acidity increased significantly ($p< 0.05$) with storage in both control and fortified sample. Titratable acidity of control and fortified sterilised and flavoured fortified sterilised milk increased from 0.159 to 0.1339% LA, 0.168 to 0.369% LA and 0.168 to 0.375% LA, respectively, at 28 days of storage.

Table 5.22: Effect of storage (4-7˚C) on physico-chemical characteristics of sterilized fortified milk

Parameter	DAY	PS	FS	FFS
Acidity (% Lactic acid)	0	0.159 ± 0.005^{cE}	0.168 ± 0.005^{bE}	0.168 ± 0.005^{aE}
	7	0.180 ± 0.009^{cD}	0.192 ± 0.005^{bD}	0.204 ± 0.014^{aD}
	14	0.219 ± 0.005^{cC}	0.225 ± 0.009^{bC}	0.234 ± 0.009^{aC}
	21	0.285 ± 0.005^{cB}	0.303 ± 0.005^{bB}	0.318 ± 0.005^{aB}
	28	0.339 ± 0.005^{cA}	0.369 ± 0.016^{bA}	0.375 ± 0.010^{aA}
pH	0	6.68 ± 0.01^{aA}	6.67 ± 0.01^{bA}	6.65 ± 0.00^{cA}
	7	6.64 ± 0.00^{aB}	6.64 ± 0.00^{bB}	6.63 ± 0.02^{cB}
	14	6.57 ± 0.01^{aC}	6.55 ± 0.01^{bC}	6.51 ± 0.01^{cC}
	21	6.49 ± 0.01^{aD}	6.45 ± 0.01^{bD}	6.41 ± 0.01^{cD}
	28	6.42 ± 0.01^{aE}	6.36 ± 0.01^{bE}	6.33 ± 0.01^{cE}
Viscosity (m Pas)	0	$2.012\pm0.033^{c,D}$	$2.833\pm0.042^{b,D}$	$5.290\pm0.247^{a,D}$
	7	$2.062\pm0.01^{c,C}$	$2.883\pm0.028^{b,C}$	$5.793\pm0.028^{a,C}$
	14	$2.093\pm0.042^{C,BC}$	$2.978\pm0.047^{b,BC}$	$5.827\pm0.074^{a,BC}$
	21	$2.259\pm0.024^{c,B}$	$3.099\pm0.053^{b,B}$	$5.938\pm0.039^{a,B}$
	28	$2.509\pm0.250^{c,A}$	$3.796\pm0.070^{b,A}$	$6.009\pm0.070^{a,A}$
FFA (µEq/ml)	0	$0.247\pm0.000^{c,D}$	$0.329\pm0.005^{b,E}$	$0.351\pm0.004^{a,E}$
	7	$0.272\pm0.001^{c,D}$	$0.464\pm0.012^{b,D}$	$0.356\pm0.007^{a,D}$
	14	$0.329\pm0.004^{c,C}$	$0.498\pm0.001^{b,C}$	$0.433\pm0.004^{a,C}$
	21	$0.360\pm0.002^{c,B}$	$0.496\pm0.005^{b,B}$	$0.430\pm0.000^{a,B}$
	28	$0.417\pm0.005^{c,A}$	$0.644\pm0.003^{b,A}$	$0.517\pm0.003^{a,A}$

PS- Plain sterilized milk (Control), FS- fortified sterilized milk, FFS- fortified flavoured sterilized milk, Results are expressed as Mean±SD, n=3; Means with different small letters superscript (a,b,c) within row and capital letters (A,B, C) within the column differ significantly ($p<0.05$) among the samples

According to Cais-Sokolińska *et al.,* (2002), acidity changes in sterilised milk could be attributed to the course of enzymatic (mainly lipolytic) reactions, which result in the formation of free fatty acids. A similar phenomenon, in which pH decreased and potential acidity increased with the time of UHT milk storage, was described by Bilińska *et al.*, (1998). A certain effect on the acidity of sterilized milk (mainly milk sterilized by the long-time method) can be exerted by small amounts of formic, acetic and other acids and maillard-type reactions (Fink and Kessler, 1986). As reported in the literature, acidity of sterilised and UHT milk reflects product freshness and, to a certain extent, the quality of raw milk used for processing and the intensity of heat treatment used during the technological process (Obrusiewicz, 1993; Kruk and Czerniewicz, 1995).

It can be seen from results (Table 5.22) that the pH of the control and fortified milk decreased significantly ($p<0.05$) with storage period, which corresponds to the increasing acidity. Between zero and the 28th day of storage, significant differences ($p<0.05$) in pH value were noticed in both control and fortified milk samples. Fortified milk had the lowest pH value throughout storage period and did not differ significantly ($p>0.05$) from the control sample. The initial pH of the control and fortified sterilised and flavoured fortified sterilised milk was 6.68, 6.67 and 6.65, respectively and at the end of storage (28th day) it was 6.42, 6.36 and 6.33, respectively. Venkatachalm and McMahon, (1991) verified a drop in pH during storage of UHT milk and associated it with browning reactions. Andrews *et al.*, (1977) confirmed similar effects and concluded that the level and extent of pH decrease was related to age-gelation. When milk is heated at a temperature above 100^{o}C and subsequently stored, lactose is degraded to acids.

Polyunsaturated fatty acids are susceptible to oxidation during storage, resulting in the development of rancid off-flavours. The lipid oxidation of control and fortified milk during storage was evaluated by determining FFA content and the results are presented in Table 5.22. The level of un-esterified fatty acids in milk provides a measure of the extent of lipolysis in milk. The free fatty acids (FFA) content in milk is dependent on the changes in the lipolytic activity of sterilized milk. From the results, it can be seen that FFA content increased significantly ($p<0.05$) with storage in both control and fortified milk samples. Between control and fortified milk samples, significant ($p<0.05$) difference in FFA content was observed. The increase in level of FFA was 0.120 µEq/ml , 0.115 µEq/ml and 0.166 µEq/ml for control, fortified sterilised and fortified flavoured sterilised milk, respectively Deeth et al. (1975) reported FFA content of $\leq$ 1.0 µEq/ml for milk with low levels of

lipolysis. After 28th day of storage at 25ºC, the mean level of FFA in control and fortified milk samples was 0.417 µEq/ml, 0.644 µEq/ml and 0.517 µEq/ml and 1.456 µEq/ml, control, fortified sterilised and fortified flavoured sterilised milk respectively.

Viscosity is the most important physical change taking place during the storage of market milk. The extent depends on temperature and time of storage and history of heat treatment (Usarek *et al.,* 1997). It may be seen from the results (Table 5.22) that the viscosity of the fortified milk was significantly ($p<0.05$) higher than the control sample throughout the storage period. The viscosity of both control and fortified milk increased significantly ($p<0.05$) with increasing storage period. Cano-ruiz and Richter (1998) reported that the apparent viscosity of the samples increased as milk solids non fat increased. The highest viscosity was found in fortified flavoured sterilised milk (5.290 mPas), followed by fortified sterilised milk (2.833 mPa-s) and sterilised milk (2.012 mPa-s) while, at the end of storage it was 6.009, 3.796 and 2.509 mPa-s in fortified flavoured, fortified, plain sterilised milk, respectively. This observation indicated that the interaction of added sugar, milk proteins and milk fat caused a significant increase in viscosity of the fortified milk. A higher volume fraction of milk solids would result in greater viscosity. Thus, higher fat and total solids content in fortified milk might have contributed for a higher viscosity. Dzwolak and Ziajka, (1997) reported that storage gelification is reflected in a slight decrease in viscosity right after UHT milk production, after which it remains almost unchanged and suddenly increases. This phenomenon is the result of direct interactions between casein micelles and the formation of a three dimensional network of bonds.

5.4.3 Microbiological quality of sterilized fortified milk during storage

The application of heat at high temperatures for a sufficient time renders milk or milk products commercially sterile, thus resulting in products that are safe and microbiological stable at room temperature. The fortified sterilised milk was analysed for standard plate count, yeast and mold, coliform count and spore count during the 28 days storage period (Table 5.23). In plain sterilised and fortified sterilised milk, the standard plate count was 100 cfu/ml and with storage of 28 days it increased to 200 cfu/ml; while in fortified flavoured sterilised milk, the standard plate count was nil and with storage it increases to 200 cfu/ml. The spore count for control milk was nil on 14th day while, 100 on 21st day. For fortified sterilised milk it was not detected and for fortified flavoured milk it was nil and 100 cfu/ml on 7th and 21 days, respectively. Thus from the data, it could be interpreted that the

sterilisation treatment to milk was carried out successfully as per FSSA standard (2011) as they said that the spore count in sterilised milk should not more than 100 cfu/ml.

Table 5.23: Effect of storage (25˚C) on microbial counts of sterilised control and fortified milk samples

Microbial count (sterilised milk)	Days	cfu/ml		
		Plain sterilised	Fortified plain sterilised	Fortified flavoured sterilised
Standard Plate Count	0	1×10^2	1×10^2	0×10^2
	7	1×10^2	1×10^2	2×10^2
	14	1×10^2	1×10^2	2×10^2
	21	2×10^2	2×10^2	2×10^2
	28	2×10^2	2×10^2	2×10^2
Yeast and mold	0	ND	ND	ND
	7	ND	ND	ND
	14	ND	ND	ND
	21	ND	ND	ND
	28	ND	ND	ND
Coliform	0	ND	ND	ND
	7	ND	ND	ND
	14	ND	ND	ND
	21	ND	ND	ND
	28	ND	ND	ND
Spore count	0	ND	ND	ND
	7	0	0	0
	14	ND	ND	ND
	21	1×10^2	0	1×10^2
	28	….	….	….
ND = Not Detected				

n=3, cfu-colony forming unit

5.5 Proximate composition of developed omega- 3 fatty acid fortified milk

As described in the previous section, the process was standardized to fortify milk with omega-3 fatty acids using flaxseed oil microcapsules. The milk samples thus fortified, along with control samples were analysed for proximate composition i.e moisture content, fat, protein, carbohydrate and ash. The result for pasteurized milk samples are shown in Table 5.24. The moisture, fat, protein, carbohydrate and ash contents in fortified pasteurized milk were observed to be 87.23, 3.37, 3.62, 4.99 and 0.79%, respectively.

Table 5.24: Proximate composition of Fortified pasteurized milk

Constituents (%)	Pasteurised Milk		
	Control	Plain fortified Pasteurised milk	Flavoured fortified Pasteurised milk
Moisture content	88.47±0.02[a]	87.23±0.10[b]	79.88±0.15[c]
Fat	3.06±0.04[b]	3.37±0.04[a]	3.35±0.05[a]
Protein	3.45±0.06[b]	3.62±0.03[a]	3.65±0.01[a]
Ash	0.74±0.02[c]	0.79±0.10[b]	0.95±0.15[a]
Total Carbohydrates	4.29±0.08[b]	4.99±0.11[b]	12.20±0.15[a]
ALA, g/100mL (Calculated)	---	0.255	0.255

Results are expressed as Mean±SD, n=3; Means with different small letters superscript (a,b,c) within row differ significantly ($p<0.05$) among the samples

Significant ($p<0.05$) differences were observed in the contents of fat, total solids and carbohydrate between control and fortified milk. The higher levels of fat were observed in fortified milk, due to the addition of flaxseed oil microcapsules. The protein content slightly increased due to the soy protein isolates present in the coat of microcapsules. The carbohydrate content increased approximately by 0.7% due to starch from the microcapsules and in flavoured milk due to addition sugar by three times. As expected, total solids increased due to increase in fat and carbohydrate (modified starch) contents in fortified milk. The levels of fat and total solids were within the range for normal milk. The fat content ranges from 2.5 to 5.5% and total solids ranges from 11.3 to 14.7% in cow milk (Walstra *et al.,* 2006).

The proximate composition of fortified and control sterilized milk were analysed and the results obtained are presented in Table 5.25. From the table, it can be interpreted that the control sample and fortified sample were significantly different ($p<0.05$) from each other. The moisture, fat, protein, ash and total carbohydrates for control milk were 88.52, 3.10, 3.47, 0.81, 4.10% respectively. While the fat, protein and total carbohydrates difference in control and fortified milk sample were due to addition flaxseed oil microcapsules made from soy protein isolates and modified starch. The ash content in fortified flavoured sterilised milk was slightly higher which may be due to the presence of impurities in sugar. The moisture, fat, protein, ash and total carbohydrates for fortified

milk were 87.30, 3.42, 3.57, 0.85, and 4.85% and for flavoured fortified milk were 79.56, 3.37, 3.57, 0.99, and 12.51% respectively.

Table 5.25: Proximate composition of fortified sterilized milk

Constituents (%)	Sterilized Milk		
	Control	Plain fortified sterilized milk	Flavoured fortified sterilized milk
Moisture contents	88.52±0.13^{a}	87.30±0.21^{b}	79.56±0.47^{c}
Fat	3.10±0.03^{b}	3.42±0.02^{a}	3.37±0.02^{a}
Protein	3.47±0.05^{b}	3.57±0.03^{a}	3.57±0.03^{ab}
Ash	0.81±0.13^{c}	0.85±0.21^{b}	0.99±0.47^{a}
Total Carbohydrates	4.10±0.11^{c}	4.85±0.19^{b}	12.51±0.44^{a}
ALA, g/100mL (Calculated)	---	0.255	0.255

Results are expressed as Mean±SD, n=3; Means with different small letters superscript (a,b,c) within row differ significantly ($p<0.05$) among the samples

Hence, it can be concluded that 3 g of microcapsules added to 240 mL of milk would provide 0.612 g of ALA (Table 5.24 and 5.25), which accounts to 38.25% of RDA as per ICMR (Indian Council of Medical Research), 2010 guidelines and 27.81 % as per ISSFAL (International Society for the Study of Fatty Acids and Lipids), 2004 guidelines.

Thus, it can be inferred from the results obtained that microencapsulated flaxseed oil powder can be suitably used to fortify milk in order to provide at least 25% recommended dietary allowance of alpha linolenic acid in one serving of fortified milk with acceptable sensory characteristics. Further, encapsulated flaxseed oil powder can also be used for fortification of bakery products, confectionary, fruit juices and other dairy and food products.

Chapter 6

Summary and Conclusion

6.0 Summary and Conclusion

Flaxseed oil is a rich source of omega-3 fatty acids and gaining popularity due to its health benefits in spite of imparting so many health benefits, such as in reducing the risks of cardiovascular diseases, LDL-& total cholesterol, diabetes and cancer. The use of flaxseed oil is limited in food and dairy products. Due to its high polyunsaturation (>73%), flaxseed oil is extremely susceptible to oxidation at high temperature, which leads to the production of toxic hydroperoxides and off flavour during processing and handling of the products. Other than the flaxseed oil, other available ω-3 sources in nature are algal oil and cold water fishes. As the vegetarian diet does not include the fish products, it remains deficient in ω-3 fatty acids. Thus to fulfill this nutritional gap there is need to increase the intake of ω-3 fatty acids by various food sources. However, addition of flaxseed as such is not possible because of rancid and off-flavour in food products as discussed earlier. Microencapsulation technique, which emulsifies the bulk oil by coating a layer of wall material around the hydrophobic core, has been proved successful in protecting the oil from the oxidation at high temperatures.

The study was selected by keeping in mind that to coat the flaxseed oil droplet by soy protein isolate and octenyl anhydride modified starches (NC 46 and NC 180) to stabilize the flaxseed oil and assessment of the physicochemical properties of the developed emulsions and microcapsules. The prepared microcapsules were fortified in milk to achieve the 25% RDA level of ALA in milk as it has mass consumption among the different age-groups of people. The summary of the present study is mentioned below:

1. For the present study, oil-in-water emulsions of flaxseed oil were prepared by using soy protein isolates and modified starches. For emulsion preparation flaxseed oil level was varied at 25, 30 and 35% of total solids (TS) while TS was maintained at 20, 25 and 30%. Emulsions were prepared by homogenization by using Ultra-turrax at 18000 RPM for 5 min.
2. Among all the emulsion samples, the emulsion with 30% oil load and 30% TS was found more stable in terms of low creaming index (2.673%) and highest zeta potential. Emulsion prepared from NC 46 showed narrowest particle size distribution and higher zeta potential (38.5mV) than NC 180 emulsions. The peroxide value of the oil was significantly higher than the emulsion.

3. All the emulsion samples were subjected to atomization in a spray dryer (Technosearch, Thane, Maharashtra) for preparation of flaxseed oil microcapsules. The prepared samples were packed in aluminium laminates (180 µm) and stored at refrigerated (4-7˚C) condition. The developed samples were evaluated for microencapsulation efficiency, surface (free) oil, moisture, water activity, bulk density, tapped density, flowing characteristics and colour.
4. The amount of surface oil present in different MFOP preparations revealed that NC46 with SPI is a better microencapsulating agent as compared to NC 180 with SPI. The maximum microencapsulation efficiency was observed with NC 46 preparation. Carr's index and Hausner ratio indicate that all the formulations did not have good flowing properties, which is a characteristic of powders containing high oil content.
5. The particle size for NC 46 and NC 180 microcapsule was 38 and 87 µm, respectively. Thus it was inferred that the target of microencapsulation in terms of size was achieved. The SEM images illustrate that the microcapsules prepared from NC 46 starch were spherical in shape with smooth surface, while for NC 180 microcapsules, uneven shape was observed. Slight agglomeration of microcapsules was observed in both the starches due to high oil load.
6. *In-vitro* release behaviour of flaxseed oil from the microcapsules under simulated gastro-intestinal conditions suggested that heating of MFOP solution at 85°C/5 min before enzymatic treatment improves the release of oil from the microcapsules.
7. The microcapsules prepared from the 30% TS and 30% oil load were evaluated for oxidative stability, solubility and moisture content over a storage period. Moisture content remained less than 5% during 3 months storage. There was a slight decrease in solubility of microcapsules during storage. In terms of oxidative stability, the microcapsules were oxidatively stable for 3 months at 4-7°C and remained well below to the limit of peroxide value as prescribed by CODEX (2013).
8. The concentration of alpha linolenic acid (18:3) in the oil extracted from NC 46 and NC 180 microcapsules was 61.67 and 65.56%, respectively. The selected microcapsule was used to fortify milk with the aim of providing 25 and 50% RDA of alpha linolenic acid in the milk. Based upon better sensory acceptability, 25% RDA level was selected for further study. Although, the sensory scores of plain fortified milk were non significantly different from that of control milk. However, the fortified milk was also flavoured for wider consumer acceptance especially in children and

adolescent group. The pH and viscosity of the fortified flavoured milk samples differ significantly ($p < 0.05$) from the control.

9. The fortified pasteurized and sterilized milk were evaluated for pH, acidity, viscosity and sensory characteristics during 6 and 28 days of storage, respectively. There was no yeast and mould, coliform count in the samples during storage. Also, the total bacterial count was within the permissible limits for both the fortified pasteurized and sterilized milk.
10. The moisture, fat, protein, ash and total carbohydrates for control sterilized milk were 88.52, 3.10, 3.47, 0.81 and 4.10. The moisture, fat, protein, ash and total carbohydrates for fortified sterilized milk were 87.30, 3.42, 3.57, 0.85 and 4.85 and for sterilized flavoured fortified milk were 79.56, 3.37, 3.57, 0.99 and 12.51, respectively. The ash content in fortified flavoured sterilised milk was slightly higher which may be due to the presence of impurities in sugar.
11. Three grams of microcapsules added to 240 mL of milk would provide 0.612 g of ALA, which accounts to 38.25% of RDA as per Indian Council of Medical Research guidelines and 27.81 % as per International Society for the Study of Fatty Acids and Lipids guidelines.
12. Therefore, it can be concluded that microencapsulated flaxseed oil powder was prepared having high oil content (~30%) and improved oxidative stability. The prepared powder showed excellent storage stability without any off-flavor for the studied storage period. Approximately 3 g of the prepared flaxseed oil microcapsules are sufficient to meet 25% RDA of the α-linolenic acid from one serving. Thus, the omega-3 powder formulated in present study can be used as a fortificant in various commercial food and supplementary products.

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Annexure

Annexure I

SENSORY SCORE CARD FOR FORTIFIED MILK

Sample : **Date**

Name of Judge:……

Please rate the samples for quality attributes according to the 9 points Hedonic scale given below:

9. Liked extremely

8. Liked very much

7. Liked moderately

6. Liked slightly

5. Neither liked nor disliked

4. Disliked slightly

3. Disliked moderately

2. Disliked very much

1. Disliked extremely

Attributes / Sample Code	Colour and Appearance	Mouthfeel	Taste and flavour	Overall acceptability

Remarks if any:

Signature

Tambade Pramod Bhivasen

National Dairy Research Institute, (Ind.)

Seeking a position in a company that will give me an opportunity to positively apply my knowledge, skill in food processing industry and come up with new innovation, ideas.

pramodtambade3@gmail.com | +918482939282 | Pune, Maharashtra, India | DOB: 03 Oct 1992

EDUCATION

M.Tech
National Dairy Research Institute
08/2016 – 06/2018 Karnataka, India OGPA 8.18

- Dairy Technology
- Thesis Title: Microencapsulation of Flax-seed oil for fortification of milk with omega 3 Fatty acid.

B.Tech
College of Dairy Technology, Udgir
08/2012 – 06/2016 Udgir, India OGPA 8.62

- Dairy Technology

Diploma in Dairy Technology
Dairy Science Institute
2010 – 2012 Aarey, Mumbai Courses

- Dairy Technology
- Percentage Obtained: 80.64%

High School & Intermediate Pune Board
2008 – 2010

Courses
- 78.76%, High School
- 65.33%, Intermediate

WORK EXPERIENCE

In plant training
ICAR-National Dairy Research Institute
02/2016 – 06/2016 *Bangalore, India*
Southern Regional Station

Tasks/Achievements
- Market Milk Section, QC, Utilities and Maintenance

Hands-on Training
In 4 well established Dairy Plants
4 Months training (08/2014-12/2014)

Achievements/Tasks
- Warana Dairy, Kolhapur (Training on Condensing & Drying)
- Sonai Dairy, Indapur (Training on Fat Rich Dairy Products)
- Hutatma Dairy, Walawa (Training on Traditional Dairy Products)
- Govind Dairy, Phaltan (Training in Milk Procurement)

Parag Milk Foods, Manchar
Internship
05/2012 – 06/2012 *Manchar*

SKILLS & COMPETENCES

- Communication Skills ●●●●○
- Computer Application ●●●○○
- Quality Assurance ●●●●○
- Research & Development ●●●●●
- Team Work ●●●●●

AWARDS & FELLOWSHIPS

State University Topper (08/2012 – 07/2016)
- B.tech Dairy Technology, OGPA 8.65

Sudhakarrao Naik Gold medal (08/2012 – 07/2016)
(08/2012 – 07/2016)
- B.Tech, Dairy Technology, OGPA 8.65

Silver medallist at Dairy Science Institute, Aarey (2010 – 2012)
- Diploma in Dairy Technology, Dairy Science Institute, Aarey

Institutional Scholarship (08/2016 – Present)

Appreciation price at 8th National food and Dairy quiz competition (2015)
- Anand Gujrat

CERTIFICATIONS

Diploma in Production Management (2012)
National Institute Of Labour Education and Management, Grade 67%

REFERENCES

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LANGUAGES

Marathi
Native

Hindi
Expert Level

English
Upper-intermediate

INTERESTS

Travelling | Music | Cooking | Farming | Movies