DESIGNING SQUALENE LOADED BIOPOLYMER MICRO PARTICLES WITH EMPHASIS ON STABILITY, BIOAVAILABILITY AND APPLICABILITY AS A FUNCTIONAL FOOD INGREDIENT

Thesis submitted in partial fulfillment
of the requirements
for the degree of

Ph.D. (Post Harvest Technology)

By

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December, 2019

Lekshmi R.G.Kumar., 2019. Designing squalene loaded biopolymer micro particles with emphasis on stability, bioavailability and applicability as a functional food ingredient. Ph.D. Thesis, ICAR- Central Institute of Fisheries Education (University under sec. 3 of UGC Act), Panch Marg, Off Yari road, Versova, Mumbai – 400 061

Dedicated to,

MY BELOVED FAMILY

DECLARATION

I hereby declare that the thesis entitled **"DESIGNING SQUALENE LOADED BIOPOLYMER MICRO PARTICLES WITH EMPHASIS ON STABILITY, BIOAVAILABILITY AND APPLICABILITY AS A FUNCTIONAL FOOD INGREDIENT"** is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

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1. INTRODUCTION

Marine flora and fauna are considered as excellent sources of bioactive compounds having immense therapeutic benefits. Owing to their unique bioactivities, marine bioactive compounds are currently being used in various areas such as biomedical, pharmaceutical and nutraceutical industries. One such bioactive compound of marine origin having potential health significance is squalene. Squalene (2, 6, 10, 15, 19, 23-hexamethyl-2, 6, 10, 14, 18, 22-tetracosahexane), a naturally occurring triterpenoid compound, has garnered considerable attention in the recent past because of its diverse pharmacological activities and remarkable health benefits. It is an important precursor in cholesterol biosynthesis and is mainly synthesized in the liver and skin of humans. The synthesized squalene is transported in blood by low density lipoproteins (LDL) and very low density lipoproteins (VLDL) and is secreted in large quantities by sebaceous glands (Koivisto and Miettinen, 1988; Stewart, 1992). Apart from the endogenous squalene, the dietary squalene is also reported to be absorbed well and transported in blood by chylomicrons. This absorbed squalene is then rapidly up taken by liver where further cyclization to sterols and bile acids occurs.

The numerous health benefits of squalene such as cardioprotective, antioxidant, chemopreventive, anticancerous, antilipidemic, membrane stabilizing properties etc. (Smith, 2000; Ko *et al*., 2002; Hashim *et al*., 2005) make its wide applications in areas such as drug delivery systems, cosmetics and as food supplements. It also helps in prevention of acidotic cell syndrome, strengthening body's immune system, detoxification of xenobiotics, lowering cholesterol content etc. (Aguilera *et al*., 2005). It is reported to have the ability to neutralize the harmful effects of excessive free radicals produced in the body. Moreover, it functions as a quencher of singlet oxygen and protects human skin surface from lipid peroxidation from sources that cause oxidative damage. Kohno *et al*. (1995) have reported that the rate constant of quenching of singlet oxygen by squalene is comparable that of Butylated hydroxytoluene (BHT). It also offers considerable protection against cardiovascular diseases and aging, act as a potential cytoprotective agent against chemotherapy-induced toxicity, protects the biomembranes from oxidative stress and in case of patients with atopic dermatitis offers protection against bacterial and fungal infections. Taking into account the numerous health benefits of squalene

such as its intrinsic biochemical and antioxidant properties, it can be surmised that daily dietary squalene supplementation can help in improving the overall health as well as immune status of human. Günes (2013) have reported that daily consumption of squalene in amounts of around 500 mg per day can help in maintaining the nutritional health of humans.

Extraction and consumption of squalene has increased considerably due to the increased consumer awareness of its health benefits. Squalene is widely present in nature in many compounds such as wheat germ, rice bran, shark liver and olive oils. Among all, shark liver oil is considered to be the richest source of squalene accounting for about 40% of its weight (Gershbein and Singh, 1969). Several methods such as solvent extraction, silver ion complexation, molecular distillation and high-speed countercurrent chromatography have been in use for squalene extraction (Lu *et al*., 2003). However, there is an increasing global concern on the use of organic solvents for extraction as they are not eco-friendly methods. Hence, innovative and sustainable extraction technologies such as supercritical fluid extraction (SFE) were introduced as an alternative to conventional separation methods (Randolph, 1990). The most commonly used solvent for supercritical extraction is CO_2 as it is non-toxic, non-flammable, inexpensive, and environmentally friendly solvent (Létisse *et al*., 2006). Another advantage is that SFE helps in the efficient extraction of thermo labile compounds as the process is often carried out at lower temperature. Rizvi (1986) reported that SFE is most suitable for extraction of non-polar compounds with molecular weights of less than 500. Squalene being a non-polar compound having molecular weight slightly above 400, it is expected that SFE will be most suitable for extraction of squalene. The use of SFE for squalene extraction from various sources such as olive oil deodorizer distillates, vegetable oil, amaranth seed oil, olive oil and lotus bee pollen has already been reported (Bondioli *et al*., 1993; Westerman *et al*., 2006; Vazquez *et al*., 2007; Fornari *et al*., 2008; Xu *et al.,* 2011).

Though squalene is reported to have so many health benefits, its direct application is limited because of its high oxidative instability. Because of the high degree of unsaturation and due to the presence of many other factors that can promote oxidation such as oxygen, temperature, moisture content, metals and light, squalene might easily undergo oxidation. The oxidation products thus produced such as free radicals, peroxides, aldehydes and ketones can in turn reduce the

nutritional quality and bioavailability of squalene. Hence a suitable strategy should be adopted to retain its oxidative stability. Encapsulation can be an effective method to address this problem because the technology is being successfully applied to protect many biologically sensitive products such as fish oil, essential oil, vitamins, probiotics, flavor, aroma etc. (Fang and Bhandari, 2010).

Encapsulation is the process of entrapping an active compound inside another material. The material which is entrapped inside is known as core material and the outer protective material is referred to as wall material or encapsulant. The wall material protects the core material from environmental factors such as light, oxygen, humidity, moisture etc. and thereby offering better oxidative stability, shelf life, easy handling and storage. The wall material governs the physical and chemical properties of encapsulate produced (Nesterenko*, et al*., 2012). Hence, the success of encapsulation often depends on the appropriate selection of wall materials and encapsulation methods. Though a wide variety of substances are being used as wall materials, gum Arabic still remains as the most commonly used wall material for microencapsulation owing to its superior properties. But the fluctuations in its availability along with the higher price have fostered researchers in identifying newer wall materials that are relatively cheaper, but of superior quality (Charve and Reineccius, 2009). Several food grade substances such as chitosan, maltodextrin, sucrose, gum Arabic, modified starch, corn syrup solids, agar, alginates, carrageenan, pectin, whey protein isolate, skimmed milk powder, gelatin, sodium caseinate etc. has been used by several researchers (either singly or in combination) for encapsulation of various substances. A number of encapsulation technologies are currently being used such as spray drying, freeze drying, coacervation, extrusion, insitu polymerization, liposome entrapment etc. Among this, encapsulation by spray drying is the simplest, efficient and economical method for encapsulation.

It has been reported that the type of wall material can play an important role in the in-vitro release of the core material. Furthermore, if the encapsulated samples are meant for food applications as a nutraceutical, it is imperative to test the stability and release behavior of these microcapsules during gastrointestinal tract transit. Hence, it is very essential that the in-vitro release properties of the encapsulated sample have to be carried out to establish their bioavailability, bio accessibility and controlled release prior to proceeding to *in-vivo* trials. Carrying out

in-vivo experiments are considered very essential to elucidate the mechanisms by which the sample modulate the metabolic pathways. Nutritional and metabolic studies based on animal model can assist in understanding the physiological effects of the encapsulated samples before validation in humans. However, an important concern in such studies is the appropriate selection of animal models, so as to translate the information obtained from experimental animals to humans (Chalvon-Demersay *et al*., 2017).

With this background, the present study is proposed with the following four objectives:

- To optimize the extraction protocol for squalene rich oil using supercritical fluid extraction
- To screen various biopolymers as wall materials for encapsulation of squalene
- To study the influence of microencapsulated squalene on nutritional aspects based on mouse model study
- To evaluate the applicability of microencapsulated squalene as a functional ingredient in food systems

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CERTIFICATE

Certified that the thesis entitled **"DESIGNING SQUALENE LOADED BIOPOLYMER MICRO PARTICLES WITH EMPHASIS ON STABILITY, BIOAVAILABILITY AND APPLICABILITY AS A FUNCTIONAL FOOD INGREDIENT"** is a bonafide record of independent research work carried out by **Ms. Lekshmi R.G. Kumar** during the period of study from August 2013 to December, 2019 under our supervision and guidance for the degree of **Doctor of Philosophy (Post Harvest Technology)** and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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ACKNOWLEDGEMENT

I would like to express special appreciation and sincere thanks to my Chair Dr. Suseela Mathew for her immense support, guidance, encouragement, relentless care, timely help, support. I would like to thank you for constant encouragements and allowing me to grow as a research scientist. Your advice on both research as well as on my career have been invaluable. Thank you for giving me the boundless freedom to research.

I consider it as my greatest privilege to have worked under the guidance of Dr. Sanath Kumar. H, Senior Scientist, FRHPHM Division CIFE, Mumbai. I am indebted to him for his excellent academic guidance, patience, unhindered support, suggestions, critical evaluations, timely help in planning and execution of my work and valuable moral support throughout my research work.

I would like to express my sincere gratitude to my co-advisor, Dr.Niladri Sekhar Chatterjee for his immense support, meticulous planning, guidance, suggestions and corrections.

I would like to express my sincere thanks to my co-advisor, Dr. B.B. Nayak, HoD, FRHPHM Division & Principal Scientist, for his constant support, help and valuable suggestions during my academic and research work.

I am extremely grateful to Dr. C.N. Ravishankar, Director, CIFT, Cochin for providing all possible facilities to complete this research work successfully

I am extremely grateful to Dr. Gopal Krishna, Director, CIFE for providing all possible facilities to complete this research work successfully

Special thanks to Dr. Manjusha L, Scientist, CIFE for her constant support, timely help, and encouragement.

I am extremely thankful to my teachers, Dr. G Venkateshwarlu, ADG, Dr.Amjad Balange, Dr.Martin Xavier, Dr.Nagalakshmi.K., Dr.Manjusha L at CIFE, for their valuable advice and support. I would like to express sincere thanks to teachers from my school and College of fisheries, Panangad, for their encouragement and moral support throughout my study period.

My sincere thanks and gratitude to my colleagues Anandan Sir, Anas K.K., Rehana Raj and Asha Madam for their support. Special Thanks to Tejpal Sir for his brotherly care, support and guidance, throughout my time at CIFT. Thank you for the effort you have put in for critically going through and correcting the thesis.

I would like to extend my sincere thanks to Dr.B. Ganesan for all his help for crrying out the animal experiments. My sincere thanks to Dr. Joshy C.G. for helping in carrying out the statistical analysis. My deep sense of gratitude to the entire staffs of B & N family, CIFT. Special Thanks to Vishnu K.V., Aneesh (QAM), Lekha chechi, Fazil, Suresh Ettan, Jaya chechi for their help, support.

My deep sense of gratitude to all the staff members of the PHT Division, Dr. Chouksey, Mr. Avinash, Mr. Phande, Pramod, and Bola for their kind cooperation, support and affectionate dealing during my academic programme at CIFE.

I am thankful to the Librarian and Library Staff of CIFT, Cochin & CIFE, Mumbai, for extending support for the library facilities.

I sincerely thank ICAR, CIFT and CIFE for the financial assistance and facilities provided to me.

My affectionate thanks to all my UG, PG and PhD batch mates for their help and support

I extend my sincere thanks to my seniors Sarika, Greeshma, for all their moral support. It was because of their company and warmth I was able to endeavour my goal.

I am indebted to my good friends Dr.Arya K.S, Meenu, Ramya Raj, Sangeetha, Roshan, Ammu for their love, care, support. Special thanks to my juniors cum friends, Dona and Vishnu for their support.

Words are short to express my deep sense of gratitude towards my ever-dearest Sandhya and Anupama chechi for their unconditional love, support, encouragement, understanding and precious friendship and prayers throughout my life.

It was the love, blessings, prayers and encouragements of my Amma, Achan, Paras, Sithu, Chettan, Arya, my Ammumma, Nunakaka, Maa, Mukund, Resmi chechi, Ponnu, Arjun, Rajesh

ettan, Kichu, Deepu and all my relatives who made it possible to complete this work and pursue my educational and professional career. Thank you for all the sacrifices you all have made for me. I owe everything to them. I lovingly dedicate this work to them.

सारांश

स्क्वैलीन, एक बायोएक्टिव आइसोप्रेनॉइड ने अपनी व्यापक श्रेणी की जैवसक्रियताओं के कारण, दवा, दवा वितरण प्रणाली, कोस्मैसिटिकल, नैदानिक क्षेत्रों आदि में एक अद्‌वितीय रुचि पैदा की है। वर्तमान अध्ययन में, ब्राम्ब्ल शार्क (*Echinorhinus brucus*) के यकृत से स्क्वैलीन समृद्‌ध तेल निकालने में सुपरक्रिटिकल CO_2 की क्षमता एवं विभिन्न कारकों जैसे निष्कर्षण दबाव, तापमान, प्राप्ति समय, स्क्वैलीन सामग्री और प्रमुख फैटी एसिड के प्रभाव को ध्यान में रखकर जांच की गई। स्क्वैलीन समृद्‌ध यकृत तेल के अलगाव के लिए 400 बार दबाव, 5.45 घंटे की अवधि एवं 43.34° सेल्सियस तापमान को निष्कर्षण स्थिति के लिए अनुकूलित किया गया। निष्कर्षण के बाद, अलग-अलग बायोपॉलिमर का उपयोग करके स्क्वैलिन के एनकैप्सुलेशन का प्रयास किया गया। स्क्वैलीन को एनकैप्सुलेशन दक्षता प्रदान करके करने के लिए सबसे अच्छी दीवार सामग्री के रूप में माल्टोडेक्सट्रिन-मट्‌ठा प्रोटीन आइसोलेट (एमडी-डब्ल्यूपीआई) पाया गया। जिसकी दक्षता लगभग 96.50$\pm$0.06% पायी गयी। इसने बेहतर विलेयता, ऑक्सीडेटिव एवं थर्मल स्थिरता प्रदर्शित किया, जिसके कारण इसमें प्रशीतित स्थिति में एक बेहतर शेल्फ अवधि पाया गया। एमडी-डब्ल्यूपीआई आधारित स्क्वैलीन का वृद्‌धि प्रदर्शन, लिपिड प्रोफाइल एवं प्रमुख एंटीऑक्सिडेंट एंजाइमों के प्रभाव का विश्लेषण विस्टार स्ट्रेन एल्बिनो चूहों में किया गया। स्क्वैलिन अनुपूरक ने कोलेस्ट्रॉल, ट्राइग्लिसराइड्स, एलडीएल और वीएलडीएल स्तर को कम कर दिया और उनके प्रभाव पूरी तरह से खुराक पर निर्भर थे। इसी तरह स्क्वैलिन पूरकता ने भी एंटीऑक्सिडेंट एंजाइमों के स्तर को बहाल करने में मदद की जिससे इसके एंटीऑक्सिडेंट क्षमता का परिचय मिलता है। एक कार्यात्मक खाद्‌य सामग्री के रूप में एन्कैप्सुलेटेड स्क्वैलिन की व्यवहार्यता का अध्ययन बेकरी उत्पाद, मफिन में इसके समावेश द्‌वारा किया गया। स्क्वैलीन समृद्‌ध खाद्‌य पदार्थों की भौतिक-रासायनिक विशेषताओं से पता चलता है कि इसमें नियंत्रण की तुलना में बेहतर बनावट, रंग विशेषताएँ, ऑक्सीडेटिव स्थिरता, बढ़ी हुई शेल्फ अबधि और स्वीकार्यता है। अध्ययन के महत्वपूर्ण निष्कर्षों के आधार पर, यह निष्कर्ष निकाला जा सकता है कि एन्कैप्सुलेटेड स्क्वैलीन को आहार अनुपूरक के रूप में या एक कार्यात्मक खाद्‌य घटक के रूप में बहुत अच्छी तरह से इस्तेमाल किया जा सकता है जो कोलेस्ट्रॉल, ट्राइग्लिसराइड्स, एलडीएल, वीएलडीएल के स्तर में कमी और एचडीएल कोलेस्ट्रॉल के स्तर में वृद्‌धि करता हैं।

ABSTRACT

Squalene, a bioactive isoprenoid, have evoked an unparalleled interest in pharmaceutical, drug delivery, cosmeceutical, clinical areas etc. by virtue of its wide range of bioactivities. In the present study, the potential of supercritical CO_2 in extracting squalene rich oil from Bramble shark (*Echinorhinus brucus*) liver was investigated by taking into account the influence of factors such as extraction pressure, temperature and time on yield, squalene content and major fatty acids. Extraction conditions of 400 bar pressure, 43.34 ºC temperature for a duration of 5.45 h was optimized for isolation of squalene rich shark liver oil. Followed by extraction, encapsulation of squalene was attempted using different biopolymers. Maltodextrin-whey protein isolate (MD-WPI) was found as the best wall material for encapsulating squalene by providing an encapsulation efficiency of 96.50 ± 0.06 %. It also exhibited better solubility, oxidative and thermal stability attributing it a better shelf life at refrigerated conditions. Effect of MD-WPI based squalene supplementation on growth performance, lipid profile, major antioxidant enzymes in wistar strain albino rats were analysed. Squalene supplementation lowered cholesterol, triglycerides, LDL and VLDL level and their effects were entirely dosage dependent. Similarly, squalene supplementation also helped in restoring the level of antioxidant enzymes suggesting their antioxidant potential. The feasibility of encapsulated squalene as a functional food ingredient was studied by its incorporation in a bakery product, muffins. The physico-chemical attributes of squalene enriched foods show that they have better textural, color attributes, oxidative stability, enhanced shelf life and acceptability than the control. Based on the significant findings of the study, it can be concluded that encapsulated squalene can be very well used as a dietary supplement or as a functional food ingredient which can exert some beneficial physiological effects such as lowering of cholesterol, triglycerides, LDL, VLDL and increasing the HDL cholesterol level.

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2. REVIEW OF LITERATURE

2.1. Squalene

Squalene, (2, 6, 10, 15, 19, 23-hexamethyl-2, 6, 10, 14, 18, 22-tetracosahexane), a polyunsaturated triterpenoid compound, is a biochemical precursor for cholesterol and many other steroid compounds. The 30 carbon compound ($C_{30}H_{50}$) was first isolated and named by an industrial Engineer, Dr.Mitsumaru Tsujimoto from the liver oil of shark, *Squalus* spp (Latin :squalus). The biochemical structure of squalene was first elucidated by Nobel laureate, Paul Karrer in the year 1936. In humans, squalene is synthesized in the liver and the skin, and secreted mostly by the sebaceous glands. Reports shows that the daily squalene production in human averages about 1.5g/day with the greatest concentration in skins (500 μg/g), followed by adipose tissues (300 μg/g) (Liu *et al*., 1976; Tsimidou, 2010). It has been already reported that the squalene secretion of skin ranges from 125 - 475 mg daily (Langdon and Bloch, 1953). It is transported in the blood by very low density lipoproteins (VLDL) and low density lipoproteins (LDL). Studies have demonstrated that 60 to 80% of dietary squalene is absorbed from dietary intake.

2.2. Structure of squalene

Squalene, $C_{30}H_{50}$, 30-carbon compound possessing 6 prenyl groups is categorised as an isoprenoid. Structurally, squalene possesses six unsaturated double bonds at C2, C6, C10, C14, C18 and C22 positions (Plate 1). The substituted methyl groups are positioned at C2, C6, C10, C15, C19, and C23 (Lu *et al*., 2003). The stability and antioxidant potential of squalene is by virtue of its six isoprenoid units. Structurally, it is quite similar to other isoprenoid compounds such as vitamin A and E, coenzyme Q10, β-carotene, lycopene etc. Squalene have two different forms, cyclic and noncyclic. The noncyclic form is entirely hydrophobic and is not engaged to the biological membrane. It is attracted by the band between both lipidic layers, the intermembrane. It accumulates there and performs its antioxidant task, capturing free radicals. However, it has been reported that under certain conditions squalene loses its non-cyclic form, curls and in the due process loses part of its antioxidant potential.

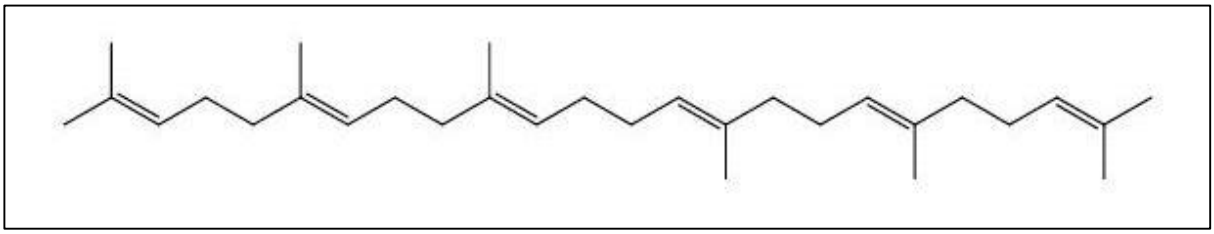


Plate 1 Structure of squalene

2.3. Sources and Properties of squalene

Squalene is widely present in nature, such as shark liver oil, olive oil, rice bran oil, wheat germ oil, amaranth oil etc. Among all the sources identified, shark liver oil remains as the abundant source of squalene accounting for at least 40% of its weight. Apart from the conventional sources, several microorganisms such as yeast (*Saccharomyces cerevisiae, Torulaspora delbrueckii*), fungus (*Aurantiochytrium* sp.), archaea are reported to have the ability to produce squalene (Gohil *et al*., 2019). Though several plant and animal sources for squalene have been reported, they are not being explored for biomedical and industrial applications citing productivity and scale - up as serious constraints. Different available sources of squalene are given below (fig. 1, Table 1, 2).

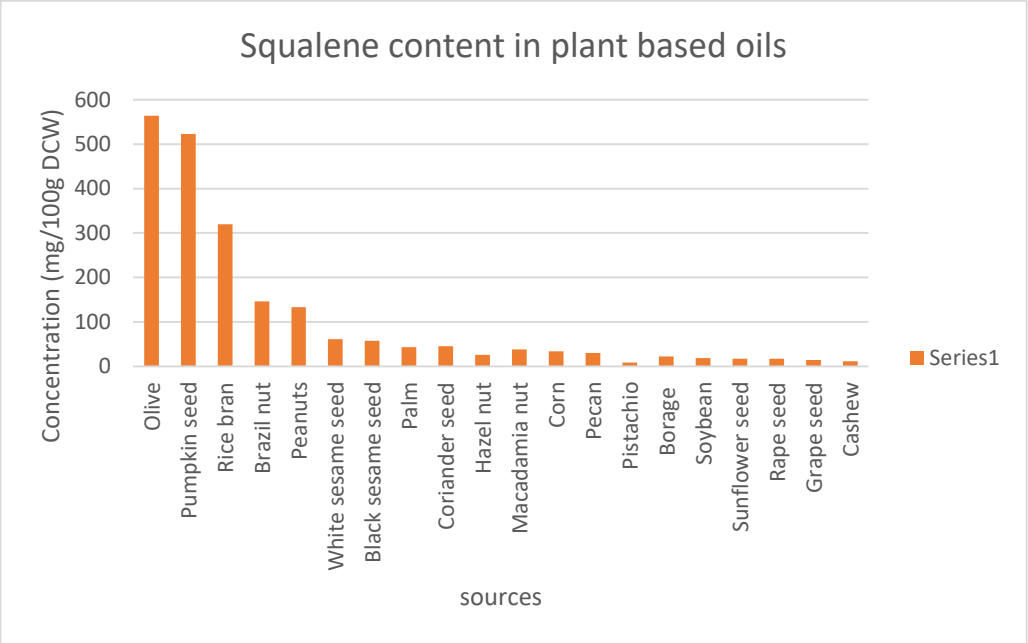


Fig. 1 Squalene content in plant based oils (Source: Gohil *et al*., 2019)

Table 1 Microorganisms as source of squalene

Source	Concentration (mg/g DCW)
Saccharomyces cerevisiae	1.6
Torulaspora delbrueckii	0.24
Aurantiochytrium sp. 18 W-13a	198
Aurantiochytrium sp. Yonez 5–1	317.74
Aurantiochytrium sp. BR-MP4-A1	0.57
Aspergillus nidulans	0.3
Halobacterium cutirubrum	1
Methylococcus capsulatus	5.5
Pseudomonas sp.	0.76
Methylomonas methanolica 1.16	1.16
Rubritalea squalenifaciens sp. Nov	15
Pseudozyma sp. JCC	70.32

(Source: Gohil *et al*., 2019, DCW: Dry cell weight)

Among all the sources available naturally, till now, deep sea shark liver is considered as the richest source of squalene. It has been reported that the high content of low-density squalene enables sharks to maintain neutral buoyancy by providing hydrostatic lift. Apart from squalene, lipid compositional studies shown that deep sea shark liver oils also possess certain major lipids such as diacyglyceryl ethers and triacylglycerols and small amounts of free fatty acids, pristane, cholesteryl esters wax esters, sterols etc. The squalene content in various shark liver oils is summarised in table 2.

Table 2 Squalene content in various shark liver oil

Species (Common name)	Scientific name	Squalene content (% of liver oil)
Gulper shark	*Centrophorus granulosus*	74.5 ± 2.9
Leafscale gulper shark	*Centrophorus squamosus*	67.9 ± 0.23
Kitefin shark	*Dalatias licha*	48.1 ± 5.2
Birdbeak dogfish	*Deania calceus*	52.5 ± 9.5
Smooth lantern shark	*Etmopterus pusillus*	44.3
Portuguese dogfish	*Centroscymmus coelolepls*	333 ± 6.73
Velvet belly	*Etmopterus spinax*	26.2
Knifetooth dogfish	*Scymnodon ringens*	0.8 ± 0.6
Bramble shark	*Echinorhinus brucus*	38.5

(Source: Batista and Nunes, 1992; Vishnu *et al*., 2016)

Among all the listed sources available, shark liver oil still remains as a rich resource for squalene (Popa *et al.,* 2015). It has been reported that for the preparation of 1 ton of squalene, about 3000 sharks are being utilised (Ciriminna *et al*., 2014). The increasing demand for squalene had led to reckless fishing of sharks leading to the extinction of many species (Tsoi *et al*., 2016). Hence, in an attempt to conserve them, shark killing has been banned in many countries (Dulvy *et al*., 2008). Hence many of the shark species cannot be utilized for squalene extraction or any other commercial/industrial purposes.

2.3.1. Bramble shark

Bramble shark (Scientific name: *Echinorhinus brucus)* are deep sea sharks mainly found in the continental or insular slopes at depths of 200–900 m and sea mounts of tropical and temperate seas in the Atlantic, Western Indian Ocean (Compagno *et al*., 2005). The scientific classification of bramble shark is given in table 3. As per the IUCN Red List of Threatened Animals, it has been given the status of data deficient (Paul, 2003) because of paucity of available information. Vishnu *et al.* (2016) reported that that bramble shark liver oil exhibits in vitro cytotoxic effect against the human neuroblastoma cell lines and suggests

that it can be used as a dietary supplement. Since they are classified under the data deficient category, there are no much restrictions on their fishing as of now.

Table 3 Scientific classification of Bramble shark

Kingdom	Animalia
Phylum	Chordata
Class	Chondrichthyes
Order	Squaliformes
Family	Echinorhinidae
Genus	*Echinorhinus*
Species	***E. brucus***

(Source: https://www.iucnredlist.org/species/41801/10563978)

2.3.2. Physico-chemical properties of squalene

Squalene is a colourless, odourless, tasteless, transparent, stable, inert, homogenous liquid oil. Physico-chemical properties of squalene are given in table 4.

Table 4 Physico-chemical properties of squalene

Properties	Value
Molecular weight	410.7 $gmol^{-1}$
Melting point	−75° C
Refractive index	1.499
Viscosity at 25° C	12 cP
Density	0.858 g/mL
Boiling point	285° C
Flash point	110° C
Iodine number	381 g/100 g
Surface tension	~32mN/m
Octanol/water partitioning coefficient (log P)	10.67
Solubility of squalene in water (mg/L)	0.124

(Spanova and Daum, 2011; Popa *et al*., 2014)

2.4. Endogenous synthesis of squalene

Squalene and its associated compounds, oxidosqualene and bis-oxidosqualene acts as precursors of several triterpenoid compounds (Xu *et al*.,

2004). The endogenous synthesis of squalene begins with the conversion of acetyl CoA to 3-hydroxy-3-methyl glutaryl coenzyme A (HMGCoA). HMGCoA is then reduced to mevalonate via a niacin-dependent reaction and mediated by mediated by 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase). HMG CoA reductase was the target of a class of cholesterol-lowering drugs. Further, mevalonate is phosphorilised in three steps via magnesium-dependent catalase enzyme. These steps include 5-phosphomevalonic acid, 5-pirophosphomevalonic acid, and isopentenyl pyrophosphate mevalonate. Mevalonate is eventually decarboxylated into 3 isopentenyl diphosphate, the donor of overall polyprenyl compounds. Successive addition of prenyl groups results in formation of 15-carbon farnesyl diphosphate. Two molecules of farnesyl diphosphate are then combined via enzymatic way to form squalene in a niacin-dependently reduced reaction (Slakey *et al*., 1973; Laaksonen *et al*., 1994). After its biosynthesis, squalene can be transported to various regions of the body or will be metabolized into cholesterol and other steroid metabolites. When compared to cholesterol, the absorption of squalene into systemic circulation is rapid. It is then transported by chylomicrons and is rapidly taken up by the liver, where it is cyclized to sterols and bile acids. Post absorptive plasma studies reported the distribution of squalene in various lipoproteins with the highest content in VLDL (50.8%), followed by LDL (25.6%) and HDL (23.6%). The efficient absorption of squalene suggests its potential as a carrier/delivery system to many therapeutic molecules.

2.5. Bioactivities of squalene

2.5.1. Anticancerous properties

Reports shows that the regular consumption of squalene can prevent/inhibit the proliferation of tumor cells (Newmark, 1997; Cirmena *et al*., 2018). The mechanisms underlying the anticancer properties of squalene is as follows: (1) Inhibition of Ras oncoprotein farnesylation and preventing the conversion of transformation of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) into mevalonate; (2) modulating functions and synthesis of xenobiotic metabolizing enzymes, (3) free radical scavenging action (Smith, 2000). When used in conjunction with anticancer drugs, squalene exhibited a potentiation effect in inhibiting tumor growth. Nakagawa (1985) reported that squalene had increased

cytotoxicity of anticancer agents such as adriamycin,5-fluorouracil, bleomycin, and cisplatin. Smith *et al.* (1998) have reported that squalene when administered orally reduced lung tumor multiplicity and hyperplasia by 58% and 70% respectively. Squalene is reported to possess cytoprotective effect in protecting only the normal cells from chemotherapeutic toxicity (Das *et al*., 2003). The same protective effect was not shown towards tumor cells proving its efficacy in anticancerous applications. The cytoprotective effect was evidenced in a cisplatin induced toxicity study wherein squalene protected the normal bone marrow cells but not the neurobalstoma cells (Das *et al*., 2008). Similar results have reported by Senthilkumar *et al*. (2006) wherein oral administration of squalene offered protection against cyclophosphamide-induced toxicity in heart, liver, and kidney. Evidences from several studies shows that squalene is having potent effect in overcoming chemotherapy induced side-effects and hence can used in conjunction with anticancerous drugs.

2.5.2. Cholesterol metabolism

Dietary squalene is absorbed well with substantial amount gets converted to cholesterol. Dietary squalene supplementation at the rate of 3.5 mg/day has been reported to decrease the lower total and LDL cholesterol in rats fed with cholesterol rich diet. Cholesterol lowering can be due to bile synthesis and cholesterol excretion by the liver into bile, mediated by changes in gene expression of key enzymes involved (Janevski *et al*., 2006). It is reported that in case of humans, daily supplementation of squalene at the rate of 900 mg/day for one month didn't make any significant increase in the serum cholesterol and triglyceride levels (Strandberg *et al*., 1990). Squalene when administered along with pravastatin was observed to lower the LDL cholesterol and increased the HDL cholesterol levels. In a similar kind of study, the antilipidemic action of squalene against isoprenaline induced hypercholesterolemia was reported (Farvin *et al*., 2009).

2.5.3. Antioxidant and other protective properties

Being an effective singlet oxygen quencher, squalene protects skin from lipid oxidation related damages due to ultraviolet radiation exposure. The singlet oxygen quenching ability is by virtue of its structural configuration comprising of six conjugated double bonds. Methyl groups at the 2-position contributes effectively to the reactive oxygen species (ROS) quenching ability

(Narayan Bhilwade *et al*., 2010; Kelly, 1999). Furthermore, the mass spectrometric data related to the photoluminescence study confirmed that the double bonds of squalene helps in preventing the photooxidation by UV rays (Mudiyanselage *et al*., 2003). It has been already reported that the singlet oxygen scavenging activity of squalene is comparable with the synthetic antioxidant, BHT (Kohno *et al*.,1995)

Squalene exhibits oxygenation effect by binding hydrogen ions from water and releases three oxygen molecules. In the due process, cellular oxygen level increases leading to an efficient metabolic process. It has the capability to take up fourth of its weight of oxygen, and thereby proving itself beneficial in prevention of wrinkles, acnes (Huang *et al*., 2009). Studies have shown that daily intake of squalene (>13.5 g) can reduce wrinkling of skin, enhance the type I procollagen expression, decrease facial erythema and reduce UV-induced skin damage (Cho *et al*., 2009). Similarly, squalene protects against age related disorders of liver mitochondrial function by exerting its ROS scavenging activity and maintains the activity of tricarboxylic acid and respiratory marker enzymes (Buddhan *et al*., 2007). It is also reported to improve the immune system of humans. Apart from this, squalene is also reported to be important in the health of retina, especially, with rod photoreceptor cells and rod outer segment disk membranes. Besides, squalene also exhibits radioprotective properties as evidenced in animal model studies (Hashim *et al.*, 2005). Because of its potent antioxidant activities, squalene is widely used in many personal care products. It has been reported that squalene can reverse the elevated transepidermal water loss when used along with riboflavin (Okuda *et al*., 2002). Moreover, squalene based topical applications are gaining increasing momentum in the treatment of skin related disorders such as acne, psoriasis, skin lesions, seborrheic and atopic dermatitis (Wołosik *et al*., 2013; Hon *et al*., 2018). Delivery of psoralen, an anti-psoriatic medicine, in a nanostructured lipid carrier based on squalene ensured better skin permeability and controlled drug release (Fang *et al*., 2008). Taking into account of its better antioxidant properties, it can be concluded that squalene can be used as a suitable candidate in cosmetic applications.

2.5.4. Drug and gene delivery

Efficient drug delivery systems are very significant as they offer better release, protection from various extrinsic and intrinsic factors, improving their bioavailability and bio accessibility, reduction of toxicity and most importantly

aids in targeted release. Taking into consideration the inertness, biocompatibility, nontoxicity, lipids are the most preferred drug carriers. Squalene, either as emulsions or as such is employed in drug delivery systems and as a vaccine adjuvant candidate. The surface tension and lipophilicity of squalene contributes in the better stabilization of oil-in water emulsions intended for delivery of lipophilic compounds (Chung *et al*., 2001; Naziri *et al*., 2011). Squalene emulsions stabilized with phosphatidylethanolamine or pluronic F68 were reported to prolong the in vitro release of a morphine prodrug (Wang *et al*., 2008). Similarly, squalene and precirol based nanostructured lipid carrier were found to increase skin permeability and aid in the control delivery of an anti-psoriatic medicine (Fang *et al*., 2008). Squalene and dioleoyl-3-trimethylammonium-propane (DOTAP) stabilized emulsions intended for gene transfection were reported to have better stability with smaller particle size and had least cytotoxicity (Chung *et al*., 2001; Kim *et al*., 2003). Squalene based emulsions were observed to offer efficient DNA protection in serum. The positive effects of squalene-based emulsions in delivery systems shows that it has got promising potential as a non-viral gene carrier candidate.

2.5.5. Detoxification of xenobiotics

Being non polar in nature, squalene shows high tendency to attach to non-ionic compounds and thereby facilitates in their removal. This property of squalene is made use for the removal of xenobiotics from body. Dietary supplementation of squalene was found to improve the elimination of the organochlorine xenobiotic, hexachlorobenzene (Richter, and Schäfer, 1982). However, the detoxifying action of squalene was found to be dose dependent (Richter and Schäfer, 1982). Dibenzofurans, hexachlorobifenyl, 12-o-tetradekanoilforbol-13-acetate, and 4(methylnitrozamino)-l-(3-pyridyl)-l-(butanone) are also reported to be detoxified by squalene (Murakoshi *et al*., 1992). It can be summarised that squalene can be used as an antidote in reducing toxicity of accidentally ingested drugs (Kamimura *et al.,* 1989).

2.6. Extraction of squalene

In nature, squalene is present in many sources and among all, shark liver is considered as its richest repository. However, owing to the environmental concerns and other sustainability issues, squalene extraction from sharks has

been facing challenges. In lieu of this, several other sources are being exploited currently for squalene extraction. Phytosqualene, extracted from plants, is reported to be very stable, non-toxic, odorless, and colorless than its shark counterparts (Popa *et al*., 2014). However, plants cannot be suggested as an ideal source of squalene as the content might get affected with seasonality, geographical variation, irrigation, edaphic factors, temperature, humidity, rainfall, agricultural management practices etc. Apart from shark and plants, several microorganisms are also reported to possess squalene. Though they are not rich in squalene when compared to shark and plant sources, they can be considered as a feasible alternative source because of its fast and remarkable growth rate. Researchers have tried to augment squalene production in microorganism by applying several techniques such as fermentation under optimized conditions, addition of inhibitor to block the competitive pathway and thereby favouring squalene accumulation (Chen *et al.,* 2010; Nakazawa *et al*., 2012), genetic engineering etc. (Singh *et al*., 2017, Valachoviˇc and Hapala, 2017).

The extraction protocols for squalene can be categorised under two major heads; (1) Conventional solvent based extraction (2) sustainable methods. Solvent based extraction is the most commonly employed technique as it is very efficient and comparatively economical. Being non-polar in nature, squalene can be extracted using non-polar solvents like hexane. However, the methods have its own setback in terms of safety, regulatory and sustainability issues. Bakes and Nichols (1995) have studied the lipid, fatty acids and squalene content in shark liver oils obtained from southern Australian waters. Higher squalene content (50-82% of oil) suggesting the potential of shark liver oils as a good repository. Gunawan *et al*. (2008) have employed a modified soxhlet extraction protocol for isolation of squalene using hexane as the solvent from soybean oil deodorizer distillate. In industries, short path distillation is often employed for obtaining squalene. Adequate temperature and vacuum have to be maintained during the process. Pietsch and Jaeger (2007) have employed a short path distillation method for squalene isolation and the optimized condition for obtaining higher concentration was 0.1 mbar at 215–230° C and 4 kg/h of feed.

Supercritical fluid extraction is a sustainable method often employed for isolation of oils and other biomolecules at a fairly low temperature. The supercritical fluid extraction is explained in detail below:

2.6.1. Supercritical fluid extraction (SFE)

Supercritical fluid extraction, a sustainable and environmental friendly technique, is often considered as an excellent alternative to conventional solvent based methods for extracting various bioactive compounds (Wang and Weller, 2006). SFE employs solvent in its supercritical state to extract bioactive compounds. Solvents/fluids at pressure and temperature above its critical point are referred to as supercritical fluids (SCFs). Such liquids have characteristics of both liquid and gases. For instance, its density is similar to that of liquids and viscosity and diffusivity are comparable to gases. In simpler terms, supercritical fluid can be defined as that form of matter in which the liquid and gaseous phases are indistinguishable. The properties of SCFs are listed below:

(1) Highly compressed gas having properties of both gas and liquids
(2) Have low viscosity, high diffusivity and hence enhanced transport properties
(3) Can fine tune the solvating properties, density and solubility by changing the pressure/temperature accordingly

Doane-Weideman and Liescheski (2004) have explained the properties of SCFs on the basis of kinetic and potential energy. Potential energy is related to the Van der Waal force, showing how close the molecules are to each other. The solvating property in turn depends upon the attractive interaction between solvent and solute molecules. In case of normal liquid solvents, molecules are close to each other causing higher surface tension, viscosity and lower diffusion properties. However, these properties can impede the extraction process. In case of SCFs, the solvent molecules move very fast due to the higher temperature provided which in turn increase their kinetic energy. This higher kinetic energy reduces the potential energy and as a result, the molecules have lower surface tension, viscosity, and faster diffusion allow supercritical fluids to perform better during which helps in efficient extraction. The lower surface tension helps the solvent molecules to rapidly penetrate into the sample matrix assisting in faster extraction process. The most commonly used solvent in SFE is carbon dioxide (CO_2) as its critical conditions (critical pressure, P_c – 74 bar, critical temperature, T_c – 31.1° C) are easily achievable at ambient conditions. Non-toxicity, safety, non-flammable, inexpensive, availability, inertness, high diffusion coefficient, lower density, increased mass transfer makes it as an excellent candidate for SFE. Furthermore, such lower temperatures make it favourable for

extraction of many thermolabile compounds. Being non polar in nature, supercritical CO_2 (SC-CO_2) can extract only non-polar compounds or substances of lower polarity. For this reason, certain solubility enhancers known as co-solvents/modifiers can be added to increase the polarity of SC-CO_2. Methanol, ethanol, chloroform, ethane etc. are some of the other co-solvents being used in SFE. Among the available co-solvents, ethanol is the most recommended it is non-toxic, miscible in CO_2 and food-grade (Liza *et al*., 2010). The advantages, disadvantages and critical conditions of solvents/co-solvents used in SFE experiments are listed in table 5 and 6.

Table 5 Advantages and disadvantages of solvents/co-solvents used in SFE

Co-solvents	Advantages	Disadvantages
Carbon dioxide	Non-toxicity, non-flammable, expensive, non-corrosive	Asphyxiant at very high concentrations
	Easy to convert into supercritical fluid at ambient conditions	Can extract only polar compounds
Nitrous oxide	----	Strong oxidizing agent, Possess narcotic properties
Propane	----	Highly flammable
Ammonia	Polar in nature	Toxic in nature
	Practically achievable critical conditions	Corrosive base, obnoxious
Fluoroform	Polar in nature	Expensive, Non-availability
	Practically achievable critical conditions	Green house and hence not environmental friendly
Water	Polar in nature	Very high critical temperature
	Environmental friendly	Supercritical water is found to be corrosive to steel

Table 6 Critical conditions of solvents/co-solvents used in SFE

Solvent	Critical temperature, k (T_c)	Critical pressure, Bar (P_c)	Critical density, g/mL (D_c)	Molecular weight (g/mol)
Carbon dioxide (CO_2)	304.1	73.8	0.469	44.01
Ethanol (C_2H_5OH)	513.9	61.4	0.276	46.07
Methanol (CH_3OH)	512.6	80.9	0.272	32.04
Ammonia (NH_3)	405.6	113.99	0.24	17.031
Benzene (C_6H_6)	562.1	48.93	0.3	78.11
Methane (CH_4)	190.4	46	0.162	16.04
Ethane (C_2H_6)	305.3	48.7	0.203	30.07
Propane (C_3H_8)	369.8	42.5	0.217	44.09
Acetone (CH_3COCH_3)	508.1	47	0.278	58.08
Ethylene (C_2H_4)	282.4	50.4	0.215	28.05
Propylene (C_3H_6)	364.9	46	0.232	42.08
Water (H_2O)	647.096	220.64	0.322	18.015
Nitrous oxide (N_2O)	309.85	72.65	0.46	44.013
Chloroform ($CHCl_3$)	299.3	48.53	0.62	119.38

SFE have several advantages over the conventional methods and some are listed below:

(1) Lower viscosity and high diffusion coefficient attributes SCFs enhanced transport properties, facilitating efficient extraction
(2) Solvent strength of SCF can be modified by changing the extraction pressure and temperature
(3) SFE leaves no chemical residue making the process sustainable
(4) Lower energy requirements
(5) Highly efficient process in terms of increasing yield and lower extraction times
(6) No degradative changes to the bioactive compounds extracted using SFE

However, the technology has its own limitations such as high cost of the equipment in the production scale. The sample for SFE should be dried in such a way that its moisture content should be below 20%. In case of thermolabile substances, freeze drying has to be done which will in turn increase the operating cost (Rubio-Rodríguez *et al*., 2008).

2.6.1.1. Supercritical fluid extractor unit – Instrumentation

The supercritical fluid extractor unit consists of fluid source (CO_2 cylinder), pumps, extraction chamber, heat exchangers, collection chamber/separator, restrictors.

2.6.1.1.1. Supercritical fluid source

The most commonly employed supercritical fluid (SCF) is CO_2 which is provided either in stainless steel or aluminium cylinders. Normally this will be high pressure cylinders containing 40 pounds (18 kg) of liquid CO_2. The vapour pressure at room temperature is about 60 atm. Generally, cylinders are equipped with a dip tube to ensure the delivery of liquid at bottom of tank rather than gas at top. Previously, cylinders with helium-head pressure were in use to improve the efficiency of pumps. However, helium tainted CO_2 were reported to affect the solvating power and extraction efficiency.

2.6.1.1.2. Pumps

CO_2 is usually supplied as a liquid and temperature is maintained at 5° C. The vapour pressure of CO_2 at room temperature is well below its critical pressure. The purpose of pump is to increase the pressure of liquid above its critical pressure. Most commonly employed pumps in SFE are syringe and

reciprocating piston pumps. The advantages with syringe pumps are its smoother pressure control, high flow rate, precise volumetric delivery. However, for refilling, syringe pumps may interrupt the extraction process. Reciprocating pumps are smaller, inexpensive. Whenever high flow rates are employed, its pump head has to be cooled to prevent any vapour lock.

2.6.1.1.3. Extraction vessel

Extraction vessel serves 2 purposes.

(1) It holds the sample to be analysed and allows the SCF to flow through the sample matrix

(2) It controls the temperature of the extraction process by maintaining the temperature of the fluid and sample. For this, the extraction vessel must be equipped with a means of heating, usually an electrically heated jacket.

The chamber is constructed in such a manner that it can withstand the maximum allowable extraction pressure with a factor of four safety margin at the maximum operating temperature (usually 150°C). The extraction chamber is also equipped with a rupture disk/pressure relief value to relieve the pressure if it increases more than 150% of the maximum operating pressure. High pressure seals are another requirement. O-rings were mostly employed previously, however, their interaction with SFC CO_2 made them less preferable. Presently, Teflon lip seals are mostly employed due to its reusability and durability at elevated temperatures. Moreover, they are chemically resistant and clean, and do not suffer from the bends.

2.6.1.1.4. Restrictor

The purpose of restrictor is to control the flow of SCF after it has passed through the sample loaded in the extraction vessel. Pressure change of SCF to prevailing atmospheric conditions is mediated by the restrictor. Two types of restrictors are available commercially (1) fixed and (2) variable restrictor. Back pressure regulator, a variable type restrictor, is most commonly employed in SFE operations. Ultimately, the extracted compounds will be collected in the collection vessel which can be then subjected to further characterization/quantification.

2.6.1.2. SFE – operation

The SFE operation starts with liquid CO_2 contained in the high-pressure cylinder. It will be then increased above its critical pressure by the help of pumps. The working pressure will be decided by taking into account the type of

raw material, the target analyte etc. The pressurised fluid will be then bought to the extraction vessel where in it will be heated to a temperature above its critical point. The SCF is then allowed to interact with the sample loaded in the extraction chamber for the required time of experiment and at the desired pressure. SCF along with the target analyte then moves to the restrictor, which maintains the high pressure of fluid when inside the extraction vessel. At the restrictor, SCF pressure is brought down to the atmospheric pressure and as a result, loses its solvating power. The analyte will be collected inside the collection vessel. The complete block diagram of SFE is shown below (plate 2):

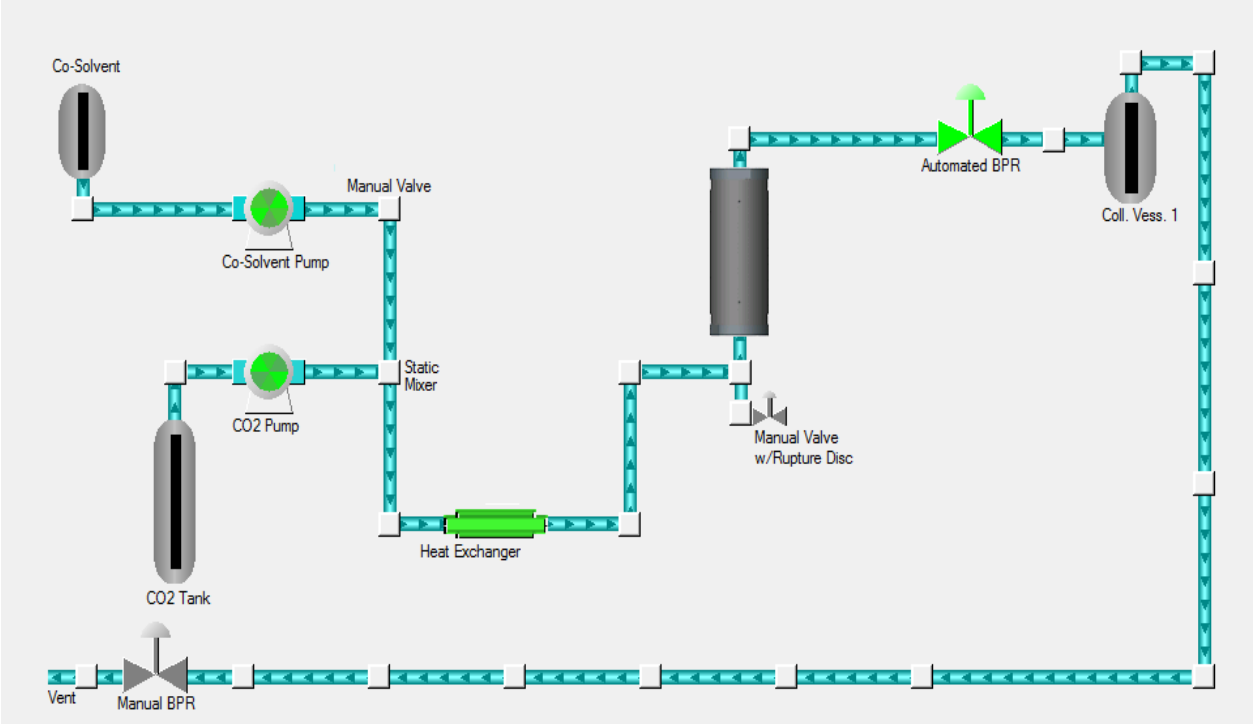


Plate 2 Schematic representation of supercritical fluid extractor unit

2.6.1.3. SFE – Extraction curves

The extraction process of SFE can be represented in the form of extraction curves. The extraction curve can be divided into three major regions based on the mass transfer phenomena (Mezzomo *et al*., 2009; Ferreira *et al*., 2002). The extraction curve is explained below:

The initial linear region represents the Constant Extraction Rate (CER) period, which represents the equilibrium solubility phase. At this point, the analyte present at the surface will be extracted by the SCF. The mode of mass transfer at this region is predominantly convention. As extraction progresses, the concentration of analyte drops and mass transfer will be predominantly controlled

by the process of diffusion. This region is the Falling Extraction Rate (FER) period. When the concentration of analytes at the surface drops down to ZERO, SCF penetrates in to the matrix, solubilizes the material and diffuses out. This phase is entirely diffusion controlled and is referred to as the Low Extraction Rate (LER) or Diffusion-Controlled (DC) period, where the external layer of oil practically disappeared and the mass transfer occurs mainly by diffusion inside the solid particles. However, depending on the operative parameters such as solvent/co-solvent flow rate, temperature, pressure etc. there can be changes in the trend of extraction curves.

2.6.1.4. Operative parameters in SFE

Several factors need to be considered in the successful and efficient extraction of bioactive compounds, viz, nature of raw materials, physico-chemical properties of co-solvent employed, pressure, temperature, flow rate of both main and co-solvent used etc. Some of these factors may have significant effects on the extraction efficiency. Different experimental designs can be employed to determine the most important factors that affect the process of extraction, either positively or negatively. Researchers have employed several designs to study such aspects. The most commonly employed screening design includes two-level full factorial and Plackett-Burman design (Dejaegher and Vander Heyden, 2009; Sharif *et al*., 2014).

Sizes of particles are reported to have serious implications on the extraction efficiency. Samples having particles of smaller size can result in better yield and process efficiency than their large sized counterparts. The larger size area along with their reduced diffusion resistance might have been the reason for better results with smaller sized samples (Park *et al*., 2007). However, too much reduction in particle size is also not preferred as it can cause agglomeration, thereby affecting the CO_2 flow rate and consequently lesser yields (Nejad-Sadeghi, 2015).

The solubility of water in carbon dioxide is at the rate of 0.3% (v/v). It has been reported that the presence of water can either assist or impede the process of extraction. Moisture content upto 5-20% were reported to have no negative effects on the lipid extraction efficiency as reported by Balasubramanian *et al*., (2013) and Crampon *et al*., (2013). Another key parameter that affect the SFE process are temperature and pressure. The density and solvating power of

SCFs are reported to increase with increase in pressure. An increase in pressure results in an increase in fluid density and enhanced solubility of the solute

2.6.1.5. Economical aspects of SFE

The high investment cost along with the high operating cost makes SFE less economically viable when compared to the conventional solvent based extraction. However, certain benefits of SFE can outweigh their cost issues. For instance, less solvent requirement, less cost of CO_2 as solvent, operation time, ease in solvent recovery, the environmental impact of the method and most importantly, the quality of the extracted compound. Perrut (2000) has reported that as per the process economics, SFE of higher capacity which works with the optimized protocols have almost same operating cost as that of the conventional methods. Manjare and Dhingra (2019) has reported that to make the process more economically viable, more research should be carried out at pilot scale by employing high capacity SFE (upto 10L capacity). This may also help in scaling up of the processes for commercial applications.

2.6.2. Extraction of squalene using SFE

SFE with supercritical CO_2 alone can extract non-polar compounds with molecular weights of less than 500 (Rizvi *et al*. 1986). Squalene being a non-polar compound can very well be extracted using SFE. It has been already reported that squalene in soluble in supercritical CO_2 at pressure ranging from 100–250 bar. The extraction of squalene from different sources using SFE technology is given in table 7.

Table 7 Extraction of squalene using SFE

Source	Extraction condition	Yield	Analytical technique	References
Yeast (*Torulaspora delbrueckii)*	60° C, 250–255 bar, 2 h, 0.2 l/minCO_2 flow rate	1.12 g/µg	Densitometric assay	Bhattacharjee and Singhal, 2003
Olive-oil deodorizer distillate	52.058 °C, 104.8 bar, 180 min extraction time	5 %	GC - FID	Akgün, 2011
Amaranthus paniculatus	40g sample size, particle diameter - 0.75 mm, 100 °C, 550 bar, 1,5 h, 0.2 l/min	36 mg/g	Densitometric assay	Bhattacharjee *et al*., 2012
***Terminalia catappa* senescent leaves**	40 °C, 3000 psi,	51 µg/g	GCMS	Ko *et al*., 2002
Lotus (*Nelumbo nucifera* Gaertn) bee pollen	38.2MPa pressure and 49.7 °C	4.94 mg/kg	GC - FID	Xu *et al*., 2011

Extraction using minimum solvents for a shorter duration are positive attributes of supercritical fluid-based isolation protocols. Pre-treatments such as sample preparation, grinding of sample to lower particle size can also found to have profound influence on squalene yield. Bhattacharjee and Singhal (2003) have reported that lyophilisation of samples prior to SFE was found to have significant improvement in the recovery of squalene from yeast. Akgün (2011) have employed a novel strategy for isolation of squalene from olive oil deodorizer distillate. Esterification of fatty acids from olive distillate in supercritical methanol and followed by extraction using SC-CO_2 was found effective for squalene extraction.

2.7. Encapsulation

Squalene obtained even after the most efficient extraction process cannot be used as such in many applications, especially in oral delivery systems. The one probable reason attributed for the limited application of squalene in processed food can be its high oxidative instability during processing and storage. Owing to the high degree of unsaturation, squalene is easily acted upon by factors such as oxygen, temperature, moisture content, metals and light to undergo oxidation (Shiota *et al*., 1999). The oxidation products thus produced can in turn reduce the nutritional quality and bioavailability. Hence, a suitable strategy should be adopted to retain its oxidative stability. Encapsulation can be an effective method to address this problem, as the technology is being successfully applied to protect many biologically sensitive products such as fish oil, essential oil, vitamins, probiotics, flavor, aroma etc. (Fang and Bhandari, 2010). Encapsulation is a rapidly expanding technology that involves the entrapment of an active compound within another polymeric substance. The active compound that will be entrapped is usually referred to as the core material and the polymeric substance surrounding it is known as the wall material or the shell material. The technology finds it applications in many areas such as pharmaceutical, nutraceuticals and even in cosmetic industries (Sanguansri *et al*., 2013). The important benefits associated with the encapsulation of bioactive compounds are as follows:

(1) For better handling of the bioactive components. Eg: It transforms the oil into a powder, where the small droplets of oil are surrounded by a dry matrix of proteins and/or carbohydrate coating materials (Heinzelmann., 2000; Keogh., 2001).

(2) Protection of active components from factors that can cause oxidation and hence a prolonged shelf life.

(3) The microencapsulation process can also help to mask undesirable fishy odors and flavors in the final product.

(4) For the controlled release of active components, especially in the case of drug delivery systems.

(5) For enhanced bioavailability and efficacy

The microcapsules thus produced can be categorized according to their morphology such as mononuclear, polynuclear and matrix. The mononuclear

microcapsules contain the wall material layer around the core, polynuclear microcapsules will be having number of cores enclosed within the wall material. In matrix type, there will be a homogenous distribution of the core material in the wall material. Since the wall material has an important role to play in many aspects such as encapsulation efficiency, stability as well as the protection of the core compound, a proper selection of the wall material is an important task.

The most commonly used wall materials include:

- Polysaccharides – Chitosan, maltodextrin, sucrose, Gum Arabic, Modified starch, corn syrup solids, Agar, Alginates, carrageenan, pectin etc.
- Proteins – Whey protein isolate, skimmed milk powder, gelatin, sodium caseinate

The wall material selection depends on number of factors such as solubility, molecular weight, glass transition temperature, diffusibility, film forming and emulsifying properties etc. Moreover, the main role of wall material is to protect the core material from factors that can affect its oxidative stability and the controlled release of core material under the desired conditions. Apart from this, the cost of wall material also has to be taken into account because the total process should be economical. Apart from the wall materials used, another important criterion that determines the success of encapsulation is the method of encapsulation. There are a number of encapsulation technologies now being used to attain a better encapsulation process and efficiency. The technologies that are presently being used for fish oil encapsulation are discussed here.

2.7.1. Encapsulation Technologies

2.7.1.1. Spray drying

One of the most commonly used microencapsulation technology is spray drying which finds wide range of applications in food and pharmaceutical industries. It is a very economical, flexible, efficient, easy to scale-up technology which produces good quality powder with low water activities that can be easily stored and transported (Arshady, 1993). The process of spray drying involves the dissolution of wall material and core material resulting in the formation of an emulsion, followed by proper homogenization, pumping of the emulsion, atomization of the emulsion and the subsequent dehydration of the atomized droplets to yield microcapsule. The size of microcapsules formed will depend on the concentration of solids content in the dispersion content and accordingly can

vary from smaller to larger particles. Apart from this, the viscosity of the emulsion, feed rpm, inlet and outlet temperatures also have an influence on the particle size as well as the oxidative stability of the particles.

2.7.1.2. Freeze drying

Freeze drying is widely accepted as one of the best methods for production of superior quality dried products (Calvo *et al.,* 2011). Though spray drying is the most widely accepted technology for encapsulation, a lower oxidative stability of spray dried products has also been reported. Because of the low temperature employed in freeze drying process and removal of about 97-98% moisture content and oxygen, this technology is often reported to produce good quality products than spray drying (Minemoto *et al*., 2001). The process of freeze drying involves three processes, freezing at a lower temperature of −90 and −40 °C, followed by primary and secondary drying under low pressure. But one limitation of this technology is that it is an expensive process requiring high energy consumption and higher processing time. Moreover, certain researchers have reported spray drying as a better process than freeze drying for fish oil encapsulation (Chen *et al*., 2013). Some others have reported that freeze drying produces microencapsulated powder with porous, irregular, and flake-like structure that can accelerate the oxidation process (Heinzelmann *et al*., 2000 and Anwar and Kunz, 2011). Taking into consideration the limitations of both freeze drying and spray drying, it can be concluded that the freeze drying can be employed to encapsulate products that are highly sensitive to heat.

2.7.1.3. Coacervation

Coacervation, also known as phase separation is the separation of two liquid phases in a colloidal solution. The phase which is rich in polymer is known as the coacervate phase and that is devoid of polymer is known as equilibrium solution. There are two kinds of coacervation, simple and complex. In case of simple coacervation, there will be only one polymer whereas in complex coacervation, the interaction between oppositely charged polymers is made use of. The biopolymers that are being widely employed in the complex coacervation process are gelatin or whey protein and oppositely charged gum arabic, sodium polyphosphate or carboxy methyl cellulose. This method produces microcapsules that are having better and controlled release activities along with heat resistant properties (Jun-xia *et al*., 2011). The microcapsules thus produced are collected

by centrifugation or filtration and further dried by either spray or fluidized bed drying. The size of microcapsules produced depends on a number of factors such as temperature, stirring speed, viscosity and pH (Carvalho *et al*., 2016). One limitation with this technology is that the coacervates produced are stable over a narrow range of pH and ionic strength. Fish oil has been encapsulated by complex coacervation using hydroxypropyl methylcellulose with maltodextrin; sugar beet pectin and glucose syrup; whey protein and gum arabic etc. (Wu and Xiao, 2005; Drusch, 2007).

2.7.1.4. Extrusion

Extrusion process involves mixing of the molten wall material with the core material which is then allowed to pass through a nozzle under high pressure to produce microcapsules of higher density and less porosity (Serfert *et al*., 2009). But the technology is more expensive than spray drying and moreover, the generation of high shear forces during the extrusion process affects the stability of the microcapsule. The extrusion microencapsulation includes 3 processes such as centrifugal extrusion (coextrusion), melt injection and melt-extrusion. Centrifugal extrusion, also known as co-extrusion is another extrusion technology that is commonly used for microencapsulation can produce microcapsules in the size range of is 500–1000µm. Since the particle size of the extruded powder is more, it can impact the mouth feel. Melt injection process involves dispersion of the core material in a matrix containing starch, anti-oxidants, sugars, emulsifiers and water at about 130°C, extruded thorough a die or filter into a bath filled with organic solvent such as isopropanol which solidifies the sugar matrix. The microcapsules thus formed are collected by filtration or centrifugation (Valentinotti *et al*., 2006). The melt-extrusion process and melt-injection is almost similar, where melt-injection is a vertical screw less process with surface-washed particles, while the melt extrusion is a horizontal screw process with particles that are not surface-washed.

2.7.1.5. In situ polymerization

In situ polymerization is commonly used for the preparation of microcapsules and functional fibres. The process doesn't include any reactants in the core material and polymerization occurs in the continuous phase itself. By adjusting pH and temperature, the wall material precipitates and distributes evenly over the surfaces of core material. Particles produced by this technology is found

to have better encapsulation efficiency, good chemical, thermal and storage stability and controlled release.

2.7.1.6. Inclusion complexation

In this method, a cyclic oligosaccharide cyclodextrin is used as a main encapsulant which can form complexes with fish oil and thereby conferring it better oxidative stability. The formation of an inclusion complexation was observed by mixing fish oil and gamma cyclodextrin in the presence of nitrogen at 45 °C for 24 h and the resultant product contained fish oil of 15–40%. Similarly, Choi *et al.* (2010) reported that fish oil encapsulated in beta cylodextrin had an 84.1% encapsulation efficiency.

2.7.1.7. Liposome entrapment

Liposomes are microscopic, spherical lipid bilayers that can enclose a number of aqueous compartments. The most commonly used encapsulating agent in this method is phospholipids and they are bio compatible and bio degradable substances (Kim and Baianu, 1991). The formation of a lipid bilayer is mainly attributed to the amphiphilic nature of phospholipids. These liposomes can be used to encapsulate omega-3 fatty acids by dissolving them in phospholipid before addition of water. This mixture of phospholipid, omega-3 oil and water are then sonicated to form encapsulated products and oil encapsulated in liposomes is said to have better oxidative stability (Kubo *et al*., 2003). But the limitation of this technology is its high cost and low stability.

2.7.1.8. Fluidized bed drying

Fluidized bed drying is the method that is restricted mainly to the encapsulation of solid core materials where a coating is applied on the powder particles (Rumpler and Jacob, 1998). Hence this method cannot be used for the direct encapsulation of fish oil, instead it can be considered as a secondary method to provide an additional coating on the already microencapsulated fish oil for better oxidative stability and physicochemical properties. In one of the patented technologies for double encapsulation of fish oil, corn starch was used for coating the already spray dried powder (Skelbaek and Andersen, 1994). In another method, molten hydrogenated palm wax (30% w/w) was used to coat over the already spray dried fish oil powder (Ponginebbi and Publisi, 2008). Apart from this, several other methods are also being used for encapsulation of marine lipophilic

compounds. Other emerging methods for the encapsulation of fish oil includes electrospraying, spray granulation etc.

2.7.2. Characterisation methods for encapsulated particles

Microencapsulated oil produced by different encapsulation technologies using wide variety of wall materials has to be characterized for studying its physicochemical properties and its oxidative stability. The important parameters that have to be taken into account for its characterisation are described below:

2.7.2.1. Encapsulation efficiency

The encapsulation efficiency (EE) is the ratio of oil entrapped inside the wall material to the initial concentration used. For better encapsulation efficiency the content of surface oil, that is the oil that is not entrapped inside the wall material, should be as low as possible. This is because of the fact that the surface oil content remains unprotected and can easily undergo oxidation, resulting in off-flavour generation and poor oxidative stability. For safe storage and better oxidative stability, ideally the surface oil should be less than 0.1% (w/w).

Encapsulation efficiency = (TO-SO/SO)*100

Where, TO - Total oil content; SO- Surface oil content

2.7.2.2 Loading capacity (LC)

Loading capacity is the measure of the amount of oil encapsulated per unit mass of the encapsulation material.

LC = (Mass of encapsulated oil / Mass of wall material)*100

2.7.2.3. Percentage yield (PY)

The percentage yield (PY) is a measure of the encapsulation process to produce the encapsulated powders.

PY = (Mass of loaded encapsulation system/ Mass of oil and wall material used)*100

2.7.2.4. Particle size

The size of microcapsule has an important role in the oxidative stability of the product and its size range should be of a uniform in nature in order to maintain the product consistency. The size of the microcapsules can be measured using laser scattering or particle size imaging using microscopy. For the detailed study of the morphology of microcapsules, high resolution imaging using electron microscopy or confocal laser scanning microscope (CSLM) can be used.

2.7.2.5. Bulk Density and tapped density

Bulk density and tapped density are important parameters from and economic point of view. These two parameters play an important role in the packaging, storage and transport of powdered products. Based on these two parameters, the flowability and cohesiveness of the encapsulated material can be measured.

2.7.2.6. Moisture content

The moisture content of the encapsulated powder plays an important role in determining the flowability, stickiness, cohesiveness, hygroscopicity and storage life. The encapsulation method adopted is also found to have a profound influence on the moisture content. The maximum moisture content allowed for dried powders of food industry application was found to be around 3 - 4 %.

2.7.2.7. Hygroscopicity

Hygroscopicity is the capacity of food to contain occluded moisture and this can be affected by inherent product composition and the concentration of the carrier material used (Ferrari *et al*., 2011). This also plays an important role in the product reconstitution since it can lead to caking and thereby reducing dispersibility.

2.7.2.8. Oxidative Stability

The main application of encapsulation technology is to protect the bioactive material against oxidation by providing an oxygen barrier in the form of wall materials. Currently, a number of substances are being used as the wall material for encapsulation depending upon the nature of substance to be encapsulated and the type of encapsulation technology used. Oxidative stability of the encapsulated powder is measured by storing the microcapsules under a set of temperature and relative humidity for a definite period and quantifying the type and amount of oxidative products formed. There are different methods employed for the measurement of oxidative stability of the encapsulated powders. Peroxide value, TBARS, acid value, propanal and rancimat test are some of the commonly used tests to assess the oxidative stability of the product.

2.7.2.9. Other Indices

Apart from this, many other characterizations are being used for the encapsulated product such as Scanning electron microscopy measurements, FTIR, Zeta potential, water activity, colour, true density, wettability, solubility are also being used nowadays for complete analysis. In addition, the thermal stability of the encapsulated materials can be studied with the help of differential scanning calorimetry, thermogravimetric analysis, accelerated rancimat analysis too. The changes in the structural crystallinity can be studied with the help of X-ray diffraction analysis too. A wide range of characterization techniques are presently employed for establishing the characteristics of the encapsulates

2.7.2.10. Bioavailability studies

It is important to study the bioavailability of encapsulated material to see how the process of encapsulation, type of wall materials employed is affecting its release properties, bioaccesibility etc. However, studies using human subjects are labor intensive, time-consuming, expensive and have ethical issues. An alternative to human models is in-vitro digestion model studies as they don't have any ethical issues and are less time consuming. Of late, the use of in-vitro digestion model in studying bioavailability of encapsulated materials is escalating owing to the ease in carrying out experiments when compared to *in-vivo* trials (Calvo *et al*., 2012).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Shark sample

Bramble shark (*Echinorhinus brucus*) was collected from Thoppumpady fishing harbour, Cochin (Plate 2). Sharks were eviscerated, livers were excised, collected carefully placed in polyethylene bags and kept in ice boxes (1–5 ºC) and transferred to the laboratory. This was then stored at -80 ºC until use.

Plate 3 Bramble shark (*Echinorhinus brucus*)

3.1.2. Biopolymers used

Chitosan (medium molecular weight, degree of deacetylation: 85%), Maltodextrin (degree of Dextrose equivalents (DE) 16.5 – 19.5), gum Arabic were obtained from Sigma –Aldrich (USA). Whey protein isolate (protein content - >80%) was obtained from Sattvic foods, Goa.

3.1.3. Chemicals

All the chemicals used in the study were of analytical grade and were obtained from Sigma-Aldrich (USA).

3.1.4. Ingredients used for functional food preparation

All-purpose flour, powdered sugar, vanilla essence, butter, egg, baking powder. All the ingredients were procured locally.

3.1.5. Instruments

Supercritical fluid extractor unit (Waters, USA), HPLC with UV-VIS detector, Fluorescence detector (FLD), Gas Chromatography (Thermo Trace GC Ultra), Scanning Electron Microscopy (SEM, JEOL Model JSM - 6390LV, Tokyo, Japan), FTIR (Thermo Nicolet, Avatar 370), Thermogravimetric analyser (TGA), X-Ray Diffraction Instrument (XRD), (Bruker KappaApex II, Massachusetts, USA), DV-III UltraTM programmable rheometer (Brookfield Engineering Laboratories INC, Middleboro, USA), Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, England), High speed homogenizer, Tall type spray drier (SM Scientech, SMST), water activity meter (Novasina IC – 500 AW – LAB, Novasina), Hunter lab colorimeter (Hunter lab, Reston, VA, USA), UV-VIS spectrophotometer, texture analyser (TA Plus, Lloyd instruments-Ametek, Hampshire, UK), soxhlet extraction apparatus, Muffle furnace, pH meter, weighing balance.

3.1.5.1. Supercritical fluid extractor

Supercritical fluid extractor was employed for optimization of a sustainable extraction protocol for isolation of squalene rich shark liver oil (plate 4). Supercritical fluid extractor system (Waters, USA) was equipped with an extraction cell (1 litre capacity), collection vessel (1 litre capacity), Waters P-200 CO_2 pump, Waters P-50 co-solvent pump, heat exchangers, back pressure regulator and chiller. The effect of 3 variables, *viz,* extraction pressure, time and temperature on the yield, fatty acid composition, squalene, fat soluble vitamins A and E was analysed to optimize the best extraction conditions. Different extraction conditions were obtained by giving temperature, pressure and time as input variables. The conditions employed for the extraction include pressure in the range of 150 – 400 bar, 120 – 240 minutes time and 40 - 60° C temperature. The flow rate of carbon di oxide and the modifier (ethanol) was kept constant at the rate of 50 g/min and 1g/min (2%) respectively for all the extraction conditions.

Plate 4 Supercritical fluid extractor unit

3.2. Methods

3.2.1. Optimization of squalene rich shark liver oil using supercritical carbon dioxide extraction

3.2.1.1. Sample preparation

Samples meant for SFE has to be dried adequately prior to extraction. However, removal of moisture from shark liver was not possible with the available techniques such as oven drying or freeze drying because of the high lipid content. Hence, a novel sample preparation protocol was employed. Shark liver was mixed with anhydrous sodium sulphate in the ratio of 1:5 and was used for extraction.

3.2.1.2. Extraction of squalene rich oil from shark liver

About 125 g sample (25 g shark liver + 100 g anhydrous sodium sulphate) was loaded into the extraction vessel of 1L capacity. Cotton was placed at both ends of the extraction vessel to prevent entrainment of sample. Followed by loading, samples were subjected to extraction. All extractions were performed in a dynamic mode at a constant total CO_2 flow (50 g/min), pressure (150 to 400 bar), Temperature (40 to 60 ºC), time (2 to 6 h) and the experimental conditions were

varied according to the experimental design (Table 8). After the scheduled time of operation, the extractor vessel was depressurized at ambient temperature and pressure. The extracts were then collected, evaporated and stored at -20 °C till analysis.

3.2.1.3. Experimental design

Response surface methodology (RSM) by Box-Behnken design was employed for the optimization of squalene rich oil from shark liver. Three variables (pressure, temperature and time) at three levels were considered in the optimization of processing variables. Three different levels of the three variables were coded as -1, 0, +1 (Table 8). The design comprised of 15 experiments with three variables and is given below (Table 9). The single and combined effects of different variables were accordingly studied with the help of this experiments. The major responses analysed were yield of oil, squalene content, DHA, vitamin A and E.

Table 8 Experimental design in terms of original and coded levels of independent variables

Independent variable	**Coded variables**	**-1**	**0**	**1**
Pressure	X1	150	275	400
Temperature	X2	40	50	60
Time	X3	2	4	6

Table 9 Experimental design

Expt No	Pressure X_1 (bar)	Temperature X_2 (°C)	Time X_3 (h)
1	150	40	2
2	150	60	2
3	150	50	4
4	150	40	6
5	150	60	6
6	275	40	2
7	275	50	2
8	275	45	4
9	275	60	4
10	275	40	6
11	400	40	2
12	400	60	2
13	400	50	4
14	400	40	6
15	400	60	6

3.2.1.4. Analysis of squalene rich shark liver oil extracted using SFE

Squalene rich shark liver oil collected after the extraction were rotary evaporated to remove ethanol. The weight of oil thus obtained were noted down and subjected to various analysis.

3.2.1.4.1. Fatty acid analysis of shark liver oil

Fatty acid methyl esters (FAME) were prepared from the extracted lipids by following the method of Metcalfe *et al*. (1966). A fraction of the lipid extract was saponified with 0.5N NaOH in methanol followed by methylation in 14% boron trifluoride in methanol (BF3/MeOH). Methyl esters of the fatty acids thus obtained were separated by gas chromatography (Thermo Trace GC Ultra) equipped with a Perkin Elmer capillary column, SP^{TM}- 2560 (100m × 0.25mm × 0.20 μm) and a flame ionization detector. The carrier gas was nitrogen and the flow rate were 0.7

mL/min. The initial temperature of column oven was set as 140°C which increased at the rate of 4°C/min until 240° C, followed by a hold for 5 min. This resulted in a total run time of 62.15min. Injector and detector temperature was kept at 300 ºC. Hydrogen and air flow rates were adjusted to 45 mL/min and 450 mL/min, respectively. The individual components were identified by comparing retention times with those obtained from the FAME mixture standard (supelcoTM 37 component FAME mix, 100 mg neat (Sigma Aldrich, catalog No: 18919-1AMP). The results were expressed as weight percentage.

3.2.1.4.2. Squalene determination by gas chromatography (GC)

The squalene content in the extracted oil was analysed using gas chromatography. 0.5 g of oil was saponified using methanolic KOH. The non-saponifiable matter (NSM) was collected and a fraction of it (10 ml) was evaporated using a rotary flash evaporator, made to 1ml using petroleum ether. From this, 0.1 ml of solution was taken and further diluted to 5 ml using petroleum ether and used for squalene estimation by gas chromatography. Squalene (5mg/ml, sigma, 99% purity) dissolved in acetone was used as standard.

3.2.1.4.3. Estimation of fat-soluble vitamins A and E using HPLC

Fat soluble vitamins were extracted from the oil using the following procedure. To 5g of oil, 50 ml of ethanol was added. To this, 0.5 g ascorbic acid, 0.25 g pyrogallol and 50 ml KOH Solution (25g in 50 ml ethanol) were added and refluxed for 45 minutes in boiling water bath under nitrogen. This was then cooled slightly and made upto 100 ml using distilled water. This was then transferred to separating funnel. The fraction was then extracted petroleum ether and the extraction was repeated thrice for maximising the extraction efficiency. The petroleum extracts were then pooled. This was then evaporated using flash evaporator and made up to a known volume using HPLC grade methanol. Fat soluble vitamins were estimated with the help of HPLC with specifications as follows: Shimadzu high pressure gradient system consisting of DGU-20A5R degasser, LC-20AD pump, CTO-10AS column oven and SPD-M10A diode array detector (DAD) was employed for detection and quantification of Vitamin A.

Chromatographic analyses of Retinol and cis-Retinal were performed on a C_{18} stationary phase (150 × 4.6 mm, 3 µ, Purospher® STAR) with a column temperature of 24°C and a flow rate of 1 mL/min. Elution was carried out using the following gradient of 0.1% TFA in Acetonitrile (eluent A) and 0.1% TFA in methanol (eluent B): 0-5 min 80% B, 5-16 min 80-22% B, 16-17 min 22-80% B, 17-18 min 80% B. The injection volume was 20 µL. ergocalciferol, retinol and cis-Retinal were detected and quantified at 335 nm wavelength. The gradient elution was for 18 minutes to ensure full separation.

Shimadzu high-pressure gradient system (Shimadzu, Kyoto, Japan) equipped with a DGU-14A degasser, LC-20AT pumps, CTO- 10AS column oven and RF-10AXL fluorescence detector (FLD) was employed for the analysis for vitamin E in the samples. Chromatographic analyses of tocopherols were performed on a PFP stationary phase (150 × 4.60 mm, 3µ, Phenomenex, Aschaffenburg, Germany) with a column temperature of 24°C and a flow rate of 1.3 mL/min. Elution was carried out using the following gradient of methanol/water (85:15, v/v; eluent A) and tert-methylbutylether/ methanol/water (80:18:2, v/v/v; eluent B): 0–5 min 0% B, 5–25 min 0–14% B, 25–30 min 40–0% B, 30–35 min 0% B. The injection volume was 20 µL. Tocopherols were detected and quantified at 295 nm excitation and 330 nm emission wavelengths, respectively. The total separation time was 22 minutes and the gradient elution was for 30 minutes to ensure full separation.

3.2.1.5. Statistical modelling for optimization using RSM

Second order response surface regression model was used to predict and optimise the response surface variables. The quadratic model which was used to break up the total variability into variability due to linear, quadratic and interaction effect of process parameters and error (Myers and Montgometry, 2002). The performance of the fitted model was assessed by Coefficient of determination (R^2) and mean square error (MSE). Significance of the regression coefficients was determined at 5% level of significance ($p<0.05$). A software Design expert 7.0 was used to fit the models.

3.2.2. Screening various biopolymers as wall materials for encapsulation of squalene

3.2.2.1. Establishing the core to wall material ratio

A preliminary study was carried out to establish the core to wall material ratio using chitosan as the wall material. Briefly, an oil- in- water (O/W) emulsion was prepared using chitosan and squalene as wall and core material respectively. Chitosan solution (1%) was prepared by dissolving adequate amount in glacial acetic acid with continuous stirring for 24 h at room temperature using a magnetic stirrer. After complete dissolution, the solution was filtered through a nylon cloth to remove impurities and to this filtered solution, Tween-80 at the rate of 5% was added. Squalene was then added to this solution at different core to wall material ratios (1:1, 0.5:1 and 0.3:1, w/w) so as to make three different treatments. The respective treatments were subjected to high speed homogenization at 10,000 rpm for 10 min using a high-speed homogenizer. Emulsions thus prepared were transferred to 25 mL graduated test tubes, sealed and subjected to emulsion stability studies. The emulsion stability expressed as percentage of separation, was calculated as the ratio of height of cream layer to initial emulsion height using Eq. (1).

$$Percentage\ of\ separation = \frac{Height\ of\ cream\ layer}{Emulsion\ initial\ height} \times 100 \quad (1)$$

The particle size distribution, zeta potential and polydispersity index (PDI) of the emulsions were measured using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, England). Particle size was expressed as Z average hydrodynamic diameter. Rheology of the emulsions was studied using Brookfield DV-III Ultra^{TM} programmable rheometer with a cone and plate geometry. The viscosity of emulsion was measured using a steady state flow program and the flow curves were obtained by plotting the shear stress against shear rate. The data thus obtained were fitted to power law (Eq. 2) to study the emulsion rheology.

$$\eta = K\gamma^{n-1} \quad (2)$$

where, η is the viscosity (cP), γ, shear rate (s^{-1}), *K*, the consistency and n is the flow index that provides information about the flow behaviour related to the effect of shear rate.

The core to wall material ratio was then optimised by taking into account the emulsion stability index (percentage of separation), particle size, zeta potential, PDI, microstructural analysis, viscosity and other rheological parameters. The optimized core to wall material ratio was then utilized in the entire study for microencapsulation of squalene.

3.2.3. Encapsulation of squalene using various biopolymers

3.2.3.1. Biopolymer dispersion

Protein-polysaccharide combinations were chosen for squalene encapsulation as there are reports stating their advantages as wall materials. Accordingly, chitosan-whey protein isolate (CS-WPI, 1:10), maltodextrin-whey protein isolate (MD-WPI, 7:1), Gum Arabic (GA) were selected as different wall materials for squalene encapsulation. The ratios for individual protein-polysaccharide combinations has been optimized previously by taking into account the various emulsion stability parameters. Gum Arabic was selected as a standard for comparison as it is the most commonly employed wall material in encapsulation studies. Wall materials of the required weight were dissolved in distilled water and after complete dissolution was added to squalene at the ratio of 1: 0.3 (wall material to squalene on w/w basis). The entire solution was then subjected to high speed homogenization at the rate of 10, 000 rpm for 10 minutes after the addition of sodium azide (0.01% w/w basis) as an antimicrobial agent. The emulsions thus prepared were subjected to emulsion stability studies, rheological analysis, particle size, zeta potential and microstructural analysis before spray drying.

Followed by emulsification, spray drying of the emulsions was carried out in Tall type spray drier (SM Scientech, SMST). The spray drier operating conditions were: inlet temperature 170 ± 2°C, outlet 90 ± 2°C, feed flow 17 rpm and blower speed 3600 rpm. The spray dried powder was then collected in an amber colored glass bottle, flushed with nitrogen gas, sealed and stored at 4°C for further studies. Yield of encapsulated squalene was calculated as the ratio of total weight of microcapsules obtained after spray drying to the amount of total solids in the initial feed emulsion. The microcapsules thus produced were subjected to various physico-chemical and instrumental analysis.

3.2.4. Characterization of encapsulated squalene

3.2.4.1. Encapsulation efficiency

The encapsulation efficiency is the oil content that is entrapped into the biopolymer matrix and is calculated from the total and surface oil content of the microcapsule using the equation, Eq (3):

$$Encapsulation\ efficieny = \frac{TO-SO}{TO} \times 100 \quad (3)$$

Where,

TO - Total oil content

SO - Surface oil content

Total oil content was measured by soxhlet extraction with petroleum ether for 5 h (AOAC, 1990) whereas surface oil was estimated by hexane extraction (Bae and Lee, 2008). The oil extracted using the above methods were then vacuum evaporated and subjected to analysis. Squalene content in TO and SO was then quantified using GC (Method No:3.2.1.4.2.).

3.2.4.2. Water activity

The water activity of the encapsulated squalene was measured at 25^0C using IC – 500 IC – 500 Novasina water activity meter (Lachen, Germany).

3.2.4.3. Hygroscopicity

One gram of the encapsulated sample was placed in a dessicator with saturated NaCl solution at 25^0C for a period of one week. The samples were taken out after one week and weighed. Hygroscopicity of the microcapsule was expressed as the amount (g) of moisture adsorbed per 100 g dry solids.

3.2.4.4. Bulk Density, Tapped density, flowability and cohesiveness

Bulk density (g/mL) was determined by placing 2 g of powder in a 10 mL graduated cylinder and volume occupied was noted down (Eq.4). The same sample was then tapped manually until negligible difference in volume between succeeding measurements was observed and accordingly tapped density was calculated (Eq.5). Flowability and cohesiveness of the powders were represented as Carr Index (CI) and Hausner ratio (HR), respectively. CI and HR were calculated from the bulk and tapped densities of the powder using the Eq. (6) and (7):

$$Bulk\ Density = \frac{Mass}{Volume} \quad (4)$$

$$Tapped\ Density = \frac{Mass}{Tapped\ volume} \quad (5)$$

$$CI\ (\%) = \frac{TD-BD}{TD} \times 100 \quad (6)$$

$$HR = \frac{TD}{BD} \quad (7)$$

3.2.4.5. Solubility study

For measuring the solubility, 2 g of sample was reconstituted in 30ml of distilled water with continuous stirring for 30 min at 700rpm on a magnetic stirrer. This was then centrifuged at 6000 rpm for 30 min. The supernatant collected was dried in an oven at 105ºC for 5 h and its weight was noted down. The wet weight of the sediment in the tube after centrifugation was also measured (Choi *et al*., 2010). Water absorption index, water solubility and swelling power were then determined using the following equations (Eq.8, 9, 10).

$$Water\ absorption\ index\ (WAI) = \frac{Wet\ sediment\ weight}{sample\ weight} \quad (8)$$

$$Water\ solubility\ (WS) = \frac{Dried\ supernatant\ weight}{sample\ weight} \times 100 \quad (9)$$

$$Swelling\ power = \frac{Wet\ sediment\ weight}{Sample\ weight\ (1-\frac{WS}{100})} \quad (10)$$

3.2.4.6. Morphological and structural characterization of encapsulated squalene

Scanning electron microscopy (JEOL Model JSM - 6390LV, Tokyo, Japan) was employed to study the external morphology of the encapsulated sample. The FTIR spectra of pure squalene and encapsulated squalene were acquired at wave number 400–4000 cm^{-1} with the help of FTIR spectrophotometer (Thermo Nicolet, Avatar 370). The thermal analysis was carried out from a temperature range of 40°C to 740°C under nitrogen atmosphere at a heating rate of 20°C min^{-1}. X-ray diffraction (XRD) patterns were recorded over a 2θ range from 3° to 40° at a scan rate of 0.04° min^{-1}. The analyses were performed at Sophisticated Test and Instrumentation (STIC) Centre of Cochin University of Science and Technology, Cochin, Kerala, India.

3.2.4.7. Peroxide value

The AOAC Official Method 965.33 (22) was employed to measure the peroxide value of encapsulated squalene (Eq. 11).

$$PV = \frac{(S-B)}{W} \times N \times 1000 \quad (11)$$

Where, S is titre value for sample, B titre value of the blank; N is the normality of $Na_2S_2O_3$, and W is the weight of sample (g).

3.2.5. In-vitro release pattern of the microcapsules

The effect of different wall materials on the squalene release pattern was studied with the help of an in-vitro static model that simulates digestion in the mouth, stomach and intestines (Hur *et al*., 2009). The composition of simulated saliva, gastric, duodenal, and bile juices is given in table 9 (Flores *et al*., 2014). The pH adjustments of the simulated digestive juices were done using 1 M HCl or 1 M NaOH. Briefly, 3 g of the encapsulated powder were taken in a 100 ml flask and incubated at 37ºC in a shaking water bath with an agitation of 200 rpm. The in-vitro digestion of the samples was carried as per the method of Flores *et al.* (2014). Briefly, 6 ml of salivary juice was added to the sample and kept mixing for 5 min. This was followed by the addition of 12 ml of gastric juice to simulate the digestion in stomach and mixing for 2 h. After 2 h, 12 ml of duodenal juice and 6 ml of bile juice was added to simulate intestinal digestion and again mixing for 2 h. Throughout the process, aliquots (2.5 ml) were collected at regular intervals of 1 h for a total of 4 h. Aliquots thus collected were stored at -20°C until further analysis.

Table 10 Composition of simulated digested fluids

	Saliva stock solution	Gastric stock solution	Duodenal stock solution	Bile stock solution
Constituents	Deionized water (100 ml)	Deionized water (100 ml)	Deionized water (100 ml)	Deionized water (100 ml)
	NaCl (11.7 mg)	NaCl (550.4 mg)	NaCl (1.4024 g)	NaCl (1.0518 mg)
	KCl (14.9 mg)	KCl (164.8 mg)	KCl (112.8 mg)	KCl (75.2 mg)
	$NaHCO_3$ (210 mg)	NaH_2PO_4 (53.2 mg)	$NaHCO_3$ (677.6 mg)	$NaHCO_3$ (1.157 g)
		$CaCl_2.2H_2O$ (79.8 mg)	KH_2PO_4 (16 mg)	Con. HCl (30 µl)
		NH_4Cl (61.2 mg)	$MgCl_2$ (10 mg)	
		Con. HCl (1.3 ml)	Con. HCl (36 µl)	
	Urea (40 mg)	Urea (17 mg)	Urea (20 mg)	Urea (50 mg)
Adjuncts	Mucin (100 mg)	Pepsin (500 mg)	Pancreatin (1.8 g)	Bile salts (6 g)
	α- amylase (200 mg)	Mucin (600 mg)	Lipase (300 mg)	
pH	6.8 ± 0.2	1.30 ± 0.02	8.1 ± 0.2	8.2 ± 0.2

(Source: Flores *et al.,* 2014)

3.2.5.1. Particle size, zeta potential, PDI, Microstructure

The frozen sample was thawed before analysis. An aliquot of the sample was subjected to particle size, zeta potential and PDI analysis using Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, England). The sample was adequately diluted before measurement. The readings were taken in triplicates. The microstructure of sample was captured using optical microscope (Leica ICC50 HD) equipped with digital camera an objective magnification of 40X. An image of the emulsion was acquired using digital image processing software (image-pro plusTM, version6).

3.2.5.2. Squalene release pattern

Squalene was extracted from the digested sample using the following method. Aliquot of the sample (1.5 ml) was mixed with 20 ml of chloroform –methanol mixture (2:1 on v/v basis) and 3 ml of water. This was then transferred to a separating funnel and the lower layer was collected by passing through anhydrous sodium sulphite. Solvent (chloroform) was then evaporated and made upto 1 ml using HPLC grade acetone. The squalene thus extracted was quantified using GC. An external standard (Squalene, 98% purity, sigma) was (concentration of 5mg/ml) was used as standard.

3.2.6. Nutritional evaluation of encapsulated squalene based on animal study

Among the different squalene microcapsules prepared, the one which gave the highest encapsulation efficiency and oxidative stability was selected for nutritional evaluation studies and functional food preparation.

3.2.6.1. Experimental animals

Wistar strain albino rats (male), weighing 133–158 g, were selected for the study (Plate 5). Animals were housed in polypropylene cages (with stainless steel grill top) under hygienic and standard environmental conditions (28±2°C, humidity 60–70%, 12 h light/dark cycle). The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and approved by the Institutional Animal Ethics Committee of the Central Institute of Fisheries

Technology, Cochin for a period of 45 days. The animals were allowed a standard diet (M/s Sai Foods, Bangalore, India; the diet composition is as follows: carbohydrate 56.2%, crude protein 22%, ash 7.5%, total fat 4.2%, crude fibre 3%, glucose 2.5%, vitamin 1.8%, sand silica 1.4%, calcium 0.8%, phosphorus 0.6%, and provide metabolizable energy of 3600 KCal) and water ad libitum during the acclimatization period.

Plate 5 Wistar rats used for the experimental study

3.2.6.2. Experimental design for animal study

After seven days' acclimatization period, the experimental animals were divided into four groups of six rats each. Group I were normal control rats which received the standard diet for a period of 45 days. Group II, III and IV experimental animals received the standard diet and were orally administered with encapsulated squalene powder (MD-WPI based squalene powder) at the rate of 150, 300 and 450 mg/kg body weight respectively for a period of 45 days. The animals were weighed once in every seven days during the entire experiment period of 45 days. Weight gain (%) and specific growth rate (SGR) was calculated (Eq 12, 13) to monitor the growth performance.

$$Weight\ gain = \frac{(Final\ weight - Initial\ weight)}{Initial\ weight} * 100 \quad (12)$$

$$Specific\ growth\ rate\ (SGR) = 100\frac{(Log\ average\ final\ weight - \log average\ initial\ weight)}{Number\ of\ days} \quad (13)$$

3.2.6.3. Sample preparation for haematological parameters and enzyme assays

At the end of the 45 days' experiment, animals were sacrificed after anesthetized by chloroform and blood was collected. Blood collected was transferred immediately to a clean test tube without anti-coagulant and kept in slanting position for 3 h. Further, serum was separated from blood by centrifuging at 6,000 rpm for 20 min at 4 ºC. Serum was then carefully separated using a micropipette and stored at -20 °C until further analysis. Heart, brain, liver and kidney were dissected out immediately and washed in physiological saline. The weight of organs was noted separately. Further, tissues were homogenized in chilled sucrose solution (0.25 M) using a high-speed homogenizer followed by centrifugation at 6000 g for 20 minutes at 4° C. The supernatants were collected and stored at -20° C till analysis. A 5% homogenate was prepared for liver, kidney, brain and heart.

3.2.6.4. Serum lipid profiling

Serum was used for analysis of biochemical parameters such as serum cholesterol, serum triglycerides, LDL, VLDL and HDL. Total cholesterol, HDL (High density lipo-protein), LDL (Low density lipo-protein) and TG (Triglycerides) were determined using automated lipid analyzer (Abacus-250).

3.2.6.5. Enzyme analysis

3.2.6.5.1. Catalase activity (CAT) [EC 1.11.1.6]

Catalase activity of the tissue homogenates were measured by employing the method of Takahara *et al.* (1960). To 2.45 ml of 50 mM phosphate buffer (pH 7.0), 50 µl of sample were added and the reaction was started by the addition of 1.0 ml of 30 mM H_2O_2. The decrease in the absorbance was measured at 240 nm for 3 min. The catalase activity was expressed as nmol of H_2O_2 decomposed min^{-1} mg^{-1} protein.

3.2.6.5.2. Superoxide dismutase (SOD) [EC 1.15.1.1]

Superoxide dismutase activity was estimated as per the method of Misra and Fridovich (1972) based on the oxidation of epinephrine-adrenochrome transition by the enzyme. To, 50 µl of sample, 1.5 ml of buffer (carbonate-bicarbonate buffer,

0.1M, pH-10.2 with 57mg/dL EDTA) and 0.5 ml 3mM Epinephrine was added. This was then mixed well. The change in the absorbance was measured at 480 nm for 3 min and 1 U of SOD activity is calculated as the amount of protein required to give 50% inhibition of epinephrine autoxidation.

3.2.6.5.3. Aspartate amino transferase (AST) (L- aspartate: 2oxaloglutarate aminotransferase, E.C.2.6.1.1)

The AST activity was assayed in different tissue homogenates as described by Wooten (1964). The substrate comprised of 0.2M D, L- aspartic acid and 2mM α-ketoglutarate in 0.05M phosphate buffer (pH 7.4). In the experimental and control tubes, 0.5ml of substrate was added. The reaction was started by adding 0.1ml of tissue homogenate. The assay mixture was incubated at 370C for 60 minutes. The reaction was terminated by adding 0.5ml of 1mM 2,4 dinitrophenyl hydrazine (DNPH). In the control tubes the enzyme source was added after DNPH solution. The tubes were held at room temperature for 20 minutes with occasional shaking. Then 5ml of 0.4ml NaOH solution was added, the contents were thoroughly mixed. After 10 minutes, the OD was recorded at 540nm against blank.

3.2.6.5.4. Alanine amino transferase (ALT)

(L- alanine: 2 oxaloglutarate aminotransferase; E.C.2.6.1.2)

The procedure adopted for ALT activity was same as for AST activity except the substrate comprised of 0.2 M D, L- alanine instead of aspartic acid.

3.2.6.6. Mineral composition analysis of liver tissue using ICP – OES

The samples were analyzed as per AOAC (2003) method quantitatively for Aluminium, Arsenic, Boron, Barium, Beryllium, Bismuth, Calcium, Cadmium, Cobalt, Chromium, Copper, Iron, Gallium, Mercury, Potassium, Lithium, Magnesium, Manganese, Sodium, Phosphorus, Lead, Selenium, Strontium, Tellurium, Tin and Zinc using inductively coupled plasma optical emission spectrophotometer (ICP OES- Thermo scientific). A closed microwave digestion unit (Milestone) equipped with Teflon vessels was used to mineralize sample, to which 8 ml of ultrapure nitric acid and 2 ml of hydrogen peroxide were added, in order to determine the dissolved heavy metals and minerals. The sample solution was made up to the exact volume of 50 ml and filtered through a syringe filter of pore size 0.45 µm before being introduced to ICP-OES.

3.2.7. Functional food application based on encapsulated squalene

A preliminary study was carried out to establish the serving size by incorporating squalene in a bakery product. Based on sensory acceptance, textural and color changes, among the different doses tested 350 mg was found sensory acceptable and used for further studies. The feasibility of encapsulated squalene as a functional ingredient was then tested by incorporation in muffins. Three different treatments were made for comparative analysis of the physico-chemical properties. The different treatments are coded as follows: Muffins without squalene of any form (encapsulated/pure) – A (Control); Muffins enriched with non – encapsulated (pure) squalene – B; Muffins enriched with encapsulated squalene - C The content of squalene in treatments B and C were fixed at the level of 350 mg per 100 g of muffins. The ingredients used for the preparation of muffins are shown in table 10. For the preparation of muffins, a standardized method was followed and it is given below:

Table 11 Standardized formula used for Muffins preparation

Ingredients	Quantity
Refined flour	125 gm
Sugar	125 gm
Butter	125 gm
Baking Powder	5 gm
Vanilla essence	7.4 mL
Egg white	9.5 g
Milk	11.1 mL

Butter and sugar of required amounts were taken in a bowl and mixed well for about 3 minutes using a food processor (Philips intelligent food processor, HL 1659, India). To the homogenized mixture, refined flour and baking powder was added and blended well for 2 min. In case of treatments B and C, squalene (pure and encapsulated) was added after this step. This was followed by the addition of vanilla essence and milk and mixed well for another 1 min. Once the batter formulation was made ready, it was poured into a metallic muffin mould which was greased well with butter to prevent any batter adhesion to the sides. This was

baked in a microwave oven (IFB microwave oven, Model:30SC4) at 150 ºC for 18 min. After the baking time, the pans were taken out and allowed to cool to room temperature (Plate 6). This was then subjected to further characterization such as proximate analysis, baking loss, crust and crumb moisture content estimation, texture analysis, color, oxidative stability, fatty acid analysis microbial analysis. All the analyses were performed for a storage period of 7 days with daily sampling interval.

Plate 6 Muffins

3.2.7.1. Proximate composition

Proximate analysis of the muffins (moisture, protein, fat, and ash content) was measured as per the standard procedures (AOAC, 2003). The Muller and Tobin (1980) method was used to calculate the crude carbohydrate content (Eq.14). The total caloric intake per 100 g of muffins was also determined based on the percentage of proteins, carbohydrates and fat (Eq.15).

$$Total\ crude\ carbohydrate\ (\%) = 100 - [Moisture\ (\%) + crude\ protein((\%) + crude\ lipid\ (\%) + Total\ ash\ (\%)] \quad (14)$$

$$Total\ calories = (Fat * 9) + (Protein * 4) + (Total\ carbohydrate * 4) \quad (15)$$

3.2.7.2. Fatty acid composition of muffins

Lipid extraction of muffins were carried out as per Folch *et al*. (1957). Briefly, 30 g of muffin samples was homogenized in the organic solvent mixture (chloroform:methanol 2 : 1), keeping the solvent/tissue ratio 20 : 1, and filtered.

The extraction and filtration procedure were repeated thrice with fresh solvent mixture. The organic fractions were collected, pooled, dried in a rotary evaporator and made up to a known concentration in chloroform, and stored in graduated test tubes at 4ºC. The same methodology employed for fame preparation from shark liver oil was used for analysing the fatty acid composition of muffins (Method No:3.2.1.4.1.).

3.2.7.3. Baking loss

Weight loss happened during the process of microwave baking was estimated by taking into account the percentage difference between initial (Wi) and final product (Wf) weight (Eq. 16, wet basis).

$$Baking\ loss\ (\%) = \frac{(Wi-Wf)}{Wi} * 100 \quad (16)$$

3.2.7.4. Analysis of other physico-chemical qualities

Moisture content of both crust and crumb of muffins were analysed and for this, both crust and crumb were taken separately. The crumb portion of muffin was exposed by removing the upper part (crust) using a serrated knife. Moisture content were determined by following the AOAC method (AOAC, 2003). The color coordinates (L* a* b*) of the muffins was measured using Hunter lab colorimeter (Hunter lab, Reston, VA, USA). The range of color coordinates are as follows: L*=100 for absolute white, L*=0 for absolute black, +a$*$ denotes redness whereas −a$*$ denotes green and +b$*$ for yellowness and −b$*$ for blueness. Colour of both crust and crumb of different treatments was determined. Based on the colour parameters (L*, a*, b*), browning intensity (BI), hue angle and chroma were computed using the equations (17, 18, 19) respectively.

$$Browning\ Intensity\ (BI) = \frac{100\ (X-0.31)}{0.17} \quad (17)$$

Where, $x = \frac{(a^* + 1.75L^*)}{(5.645\ L^* + a^* - 3.012\ b^*}$

$$Hue\ angle, h^o = \tan^{-1}(\frac{b}{a}) \quad (18)$$

$$Chroma, C = \lfloor a^{*2} + b^{*2} \rfloor^{1/2} \quad (19)$$

The oxidative stability of different treatments was measured by determining the primary oxidation products (peroxides). The AOAC Official Method 965.33 was employed to measure the peroxide value of different treatments (Eq. 11). The

external morphology of the baked muffins was analysed using scanning electron microscopy.

3.2.7.5. Texture profile analysis

Texture profile analysis (TPA) of different treatments was carried out after equilibrating the samples to room temperature. Two bite TPA method was followed to assess the textural quality of muffins using texture analyser (TA Plus, Lloyd instruments-Ametek, Hampshire, UK) equipped with a load cell of 50 kg. For texture analysis, samples were cut from the center into square cubes of about 2.5 cm × 2.5cm. The texture analyser settings used were as follows: pre-test speed: 5mm/s, test speed - 5 mm/s, post- test speed - 10 mm/s, trigger force of 10 g and compression distance - 60 %. A cylindrical probe of radius 75 mm was used for the analysis. With the help of Nexygen software the textural parameters were tabulated.

3.2.7.6. Microbiological analysis

Total plate count and fungus counts (Yeast and mold) were carried out for batter A, B, C and muffins on alternate days of storage period to check the microbial load as per American Public Health Association (APHA).

3.2.8. Statistical analysis

All the measurements were carried out in triplicates. The data obtained were subjected to one-way analysis of variance (ANOVA) using the statistical software, SPSS version 16.0, Chicago IL. USA. Differences among means were evaluated by Duncan's multiple range test at a significant level of $p \leq 0.05$.

4. RESULTS

Squalene (2,6,10,15,19,23-hexamethyltetracosa-2,6,10,14,18,22-hexane) is an isoprenoid compound and precursor for cholesterol, steroids and many other hormones. Squalene cannot be used directly in any applications because of its inherent oxidative instability. Hence, the aim of the present study was to optimize a sustainable extraction protocol for obtaining squalene rich shark liver oil, encapsulation of squalene to retain its oxidative stability, improving its bioavailability etc., nutritional evaluation of the encapsulated squalene based on an animal model study, followed by investigating its feasibility as a functional food ingredient.

4.1. Optimizing a sustainable extraction protocol for isolation of squalene rich shark liver oil

In the present study, the potential of supercritical CO_2 in extracting squalene rich oil from Bramble shark (*Echinorhinus brucus*) liver was investigated. Influence of factors such as extraction pressure, temperature and time on yield, squalene content, major fatty acid content such as Docosahexaenoic acid (DHA), Eicosapentaenoic acid (EPA), Arachidonic acid (AA) and fat-soluble vitamins (A and E) were investigated. In addition, the fatty acid composition of the extracted shark liver oil was also studied. Response Surface Methodology (RSM) was used to optimize the response as a function of the input variables. Box-Behnken response surface design with three process variables with three levels and three points at the center was used for the study. Pressure (X_1), temperature (X_2) and time (X_3) were considered as independent variables and the levels were chosen based on the results of preliminary experiments. Single and combined effects of the variables on the responses were studied by formulating experimental units. Experimental data from 15 experimental units on response variables were measured. The detailed experimental set up with corresponding values of response variables is presented in Table 11. Contour plots and response surface plots were drawn using predicted values of response variables. Desirability score was computed for multi response optimization of response variables for finding the optimum extraction conditions for obtaining squalene rich shark liver oil.

Table 12 Experimental design and responses of the dependent variables for the optimization of extraction of squalene rich shark liver oil

Design points	X_1	X_2	X_3	Yield (%)	Sq (mg/g oil)	DHA (mg/g oil	EPA (mg/g oil	AA (mg/g oil	VA (µg/g oil)	VE (µg/g oil)
1	150	40	2	79.36	634.23	12.08	3.23	2.66	2.29	32.97
2	150	60	2	42.68	910.84	4.43	1.50	1.25	72.71	67.15
3	150	50	4	46.62	830.20	12.38	1.75	2.20	501.45	243.85
4	150	40	6	73.62	835.80	8.05	1.22	1.57	377.14	42.57
5	150	60	6	57.16	840.20	14.88	2.02	2.51	567.16	169.99
6	275	40	2	68.64	665.53	15.26	3.88	3.22	45.33	24.96
7	275	50	2	43.46	833.17	56.46	13.58	11.52	16.18	26.56
8	275	45	4	64.16	810.80	35.28	10.12	7.31	27.18	14.26
9	275	60	4	65.20	832.80	42.13	10.62	9.39	51.13	29.74
10	275	40	6	67.67	848.20	13.34	1.86	2.24	427.46	81.04
11	400	40	2	71.36	711.97	34.98	8.55	7.24	187.48	79.42
12	400	60	2	52.28	822.88	8.05	1.11	1.13	165.07	55.71
13	400	50	4	67.87	835.60	20.08	2.85	3.57	348.39	80.28
14	400	40	6	77.57	840.20	29.87	3.96	4.94	242.45	138.71
15	400	60	6	63.75	860.20	19.72	2.85	3.56	47.43	75.41

(Note:[a] Experiments were run at random, X_1: Pressure (bar), X_2: temperature (°C) X3: Time (h) Y: Yield, (%), Sq: Squalene, DHA: Docosahexaenoic acid, EPA: Eicosapentaenoic acid, AA:Arachidonic acid, VA: Vitamin A, VE: Vitamin E**)**

Table 13 Regression coefficients of fitted model with R^2 values for the optimizing extraction conditions for squalene rich shark liver oil

Response	β0	X_1	X_2	X_3	X_1*X_2	X_1*X_3	X_2*X_3	X_1^2	X_2^2	X_3^2	R^2
Y	493.2606	0.000984	-17.3074	1.91634	-0.00127	0.004467	0.33528	0.00013	0.15667	-2.24851	0.7752
Sq	-1530.06	0.85018	77.35157*	77.17512*	-0.01413	0.01731	-2.39029*	-0.00036	-0.583	7.08902	0.9408
DHA	-333.526	0.67464	11.71827	-9.47688	-0.00226	6.71E-05	0.096914	-0.00093	-0.1154	0.57197	0.5226
EPA	-65.1376	0.20228	1.99173	-1.70789	-0.0006	-0.00068	0.037874	-0.00029	-0.0198	-0.05305	0.5201
AA	-57.7101	0.15184	1.88062	-1.60246	-0.00051	-2E-05	0.028008	-0.00021	-0.0185	0.007876	0.761
V A	-2437.29	-3.37864	96.59376	383.4261*	-0.00978	-0.4659*	-2.96748	0.00965	-0.8769	-6.43581	0.8573
V E	-588.582	-2.70871	33.91143	114.2218	0.000623	-0.01672	-1.08449	0.004805	-0.3191	-5.6631	0.6491

(Note: Experiments were run at random, X_1: Pressure (bar), X_2: Temperature (°C), X_3: Time (h)

*: Shows the significant values with respect to $P<0.05$)

4.1.1. RSM based optimisation of process variables

The optimization based on process variables like pressure (X_1), temperature (X_2) and time (X_3) on extraction of squalene rich shark liver oil, was determined using RSM. The response variables considered for optimization were yield, squalene content, major fatty acids such as Docosahexaenoic acid (DHA), Eicosapentaenoic acid (EPA), Arachidonic acid (AA), vitamin A (VA) and vitamin E (VE). Second order response surface regression model was used to predict and optimise the response variables in terms of pressure, temperature and time.

The functional form of regression model is given in equation (20)

$$Y = \beta_o + \sum_i \beta_i x_i + \sum_{ii} \beta_{ii} x_{ii}^2 + \sum_i \sum_{j,i<j} \beta_{ij} x_i x_j + e, i \neq j \qquad (20)$$

Where, "Y" is response variable, "β_o" is intercept, "β_i" is linear, 'β_{ii}" is quadratic and "β_{ij}" is interaction regression coefficients and "e" is error term.

Second order regression model was used to estimate the linear (X_1, X_2, X_3), quadratic (X_1^2, X_2^2, X_3^2) and interaction (X_1X_2, X_2X_3 and X_1, X_3) coefficients using ordinary least square (OLS) method. Table 11 shows the regression coefficients of second-order response regression model for all the response variables. The fitted model produced significant R^2 for most of the response variables. The optimization of response variables was done based on the evaluation of response surface plot and desirability score. The detailed analysis of each response variable is explained follows.

4.1.2. Effect of SFE conditions on yield of squalene rich shark liver oil

The parameters of fitted model for yield is given in Table 12. Second order response surface model was used to explain the variations in yield of extracted oil with an R^2 value of 0.78. The precision measures of S/N (signal to noise ratio) was greater than 4 (4.528) indicating adequate model discrimination (Myers *et al*., 2009). The response surface plot of predicted values of yield at a fixed CO_2 flow rate (50 g/min) is depicted in fig. 2. The linear, quadratic and interaction effects of the input variables were found to have no-significant effect on the yield of oil obtained.

When the effect of different extraction conditions on the yield of squalene rich oil was analyzed, it was found that extraction pressure doesn't have much significance in the yield. When a lower pressure of 150 bar was employed, yield has ranged from 57.16 to 79.36%, whereas a medium pressure of 275 bar gave a yield in the range of 43.46 to 68.64%. The high pressure-based extraction at 400 bar resulted in an extraction yield of 52.28 to 77.57%. The results of the study showed that pressure had no much significance in the yield squalene rich shark liver oil. The highest yield was obtained when an extraction condition of 150 bar pressure, 40˚ C temperature was maintained for 2h. However, it was observed that when the temperature was increased to 60˚C keeping the pressure and time constant, the yield has decreased considerably. Similarly, the yield was 71.36% when an extraction condition of 400 bar pressure, 40˚ C temperature was employed for 2h time, however, when the temperature was increased to 60˚C at the same pressure, the yield has decreased considerably to 52.28 %. It was very clear from the results that increase in temperature had a lowering effect on yield. However, time and pressure, doesn't seem to have much effect on the yield.

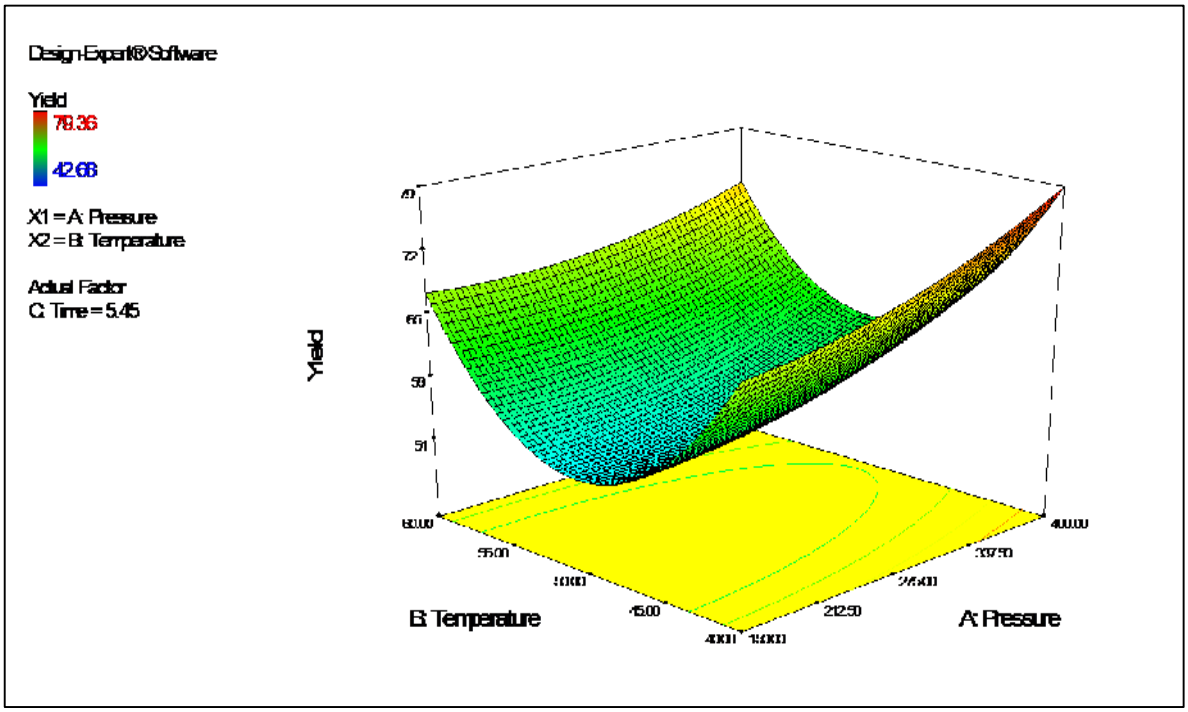


Fig. 2 Response surface plot for the effect of SFE conditions on yield

4.1.3. Effect of extraction conditions on squalene content

Second order polynomial regression model was fitted ($p < 0.05$) to predict the squalene yield at different levels of process parameters with an R^2 value of 0.9408. The adjusted R^2 of the fitted model was 0.83 and analysis of lack of fit was not significant ($p > 0.05$) indicating the suitability of the model. The response surface plot of predicted values of yield is depicted in Fig. 3. The analysis of variance of fitted model showed that the linear effect of temperature and time was found to be significant ($P<0.05$). The interaction effects of temperature and time (X_2*X_3) was only found to be significant whereas the quadratic effects were found to be statistically non-significant. The signal to noise ratio(S/N) was greater than 4 (9.237) indicating adequate model discrimination.

The effect of extraction conditions showed that squalene content has ranged from 634.23 to 910.84 mg/g of shark liver oil. Interestingly, the result was just the reverse as obtained for yield of squalene rich shark liver oil. An experimental condition of 150 bar pressure, 60 °C temperature and 2 h extraction time gave the highest squalene content and 150 bar pressure, 40 °C temperature for 2 h gave the lowest yield. Similar to yield, the extraction pressure was also found to have negligible effect on squalene content whereas temperature was found to have a positive effect on squalene content. An increase in temperature favoured squalene extraction positively. Temperature of 60 °C was found to favour the extraction of squalene giving the highest yield.

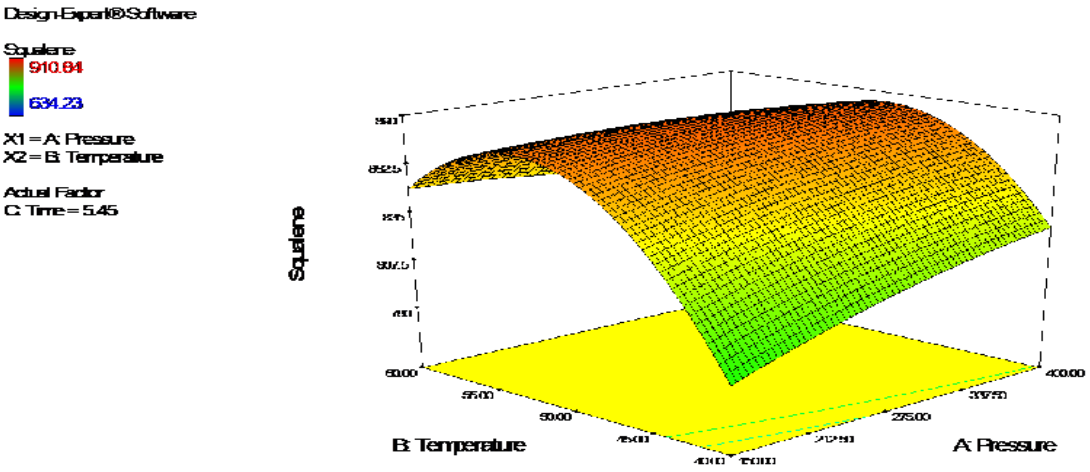


Fig. 3 Response surface plot for the effect of extraction conditions on squalene content

4.1.4. Effect of extraction conditions on fatty acid composition

Fatty acid content and composition of shark liver oil obtained by different extraction conditions were analyzed and shown in table 13 and fig. 4 respectively. Fatty acid content obtained by different extraction conditions ranged from 24.93 to 243.99 mg/g of oil. The highest fatty acid content was observed when an extraction condition maintained at 275 bar pressure, 50° C temperature was employed for 2h and the lowest yield was obtained at 150 bar pressure, 60 °C temperature, 2 h time. Employing a moderate pressure (275 bar) was found to favor the fatty acid extraction than high- or low-pressure conditions. Increase in temperature was found to have a negative effect on the fatty acid extraction except in case of moderate pressure.

The fatty acid composition of squalene rich shark liver oil obtained using various extraction conditions is shown in table 14. The saturated fatty acid content has ranged from 9.23 to 77.35 mg/g oil with the highest value obtained at an extraction condition of 275 bar pressure, 50 °C temperature for 2h. The monounsaturated fatty acid content (MUFA) has ranged from 8.20 to 80.22 mg/g oil. The highest MUFA was noticed when an extraction condition of 400 bar pressure, 40 °C temperature for duration of 6h. The PUFA content has varied from 7.50 to 89.37 mg/g of oil and the highest value was obtained when an extraction condition of 275 bar pressure, 50 °C temperature for 2h. The fatty acid composition revealed that the most dominant saturated fatty acids were C16 and C18 (Palmitic acid and stearic acid). The most dominant monounsaturated fatty acids were C18:1 and C16:1 (Oleic acid and palmitoleic acid) and the most dominant PUFA were DHA (C22:6), EPA (C20:5), AA (C20:4).

Table 14 Fatty acid content of shark liver oil obtained using various extraction conditions

Sl No	Pressure/Temp/Time (bar/°C/h)	FA Content (mg/g oil)
1	150/40/2	54.66
2	150/60/2	24.93
3	150/50/4	102.27
4	150/40/6	85.41
5	150/60/6	67.16
6	275/40/2	63.54
7	275/50/2	243.99
8	275/45/4	161.71
9	275/60/4	200.89
10	275/40/6	86.63
11	400/40/2	165.47
12	400/60/2	48.61
13	400/50/4	148.53
14	400/40/6	189.07
15	400/60/6	133.75

(Note: FA content indicates fatty acid content)

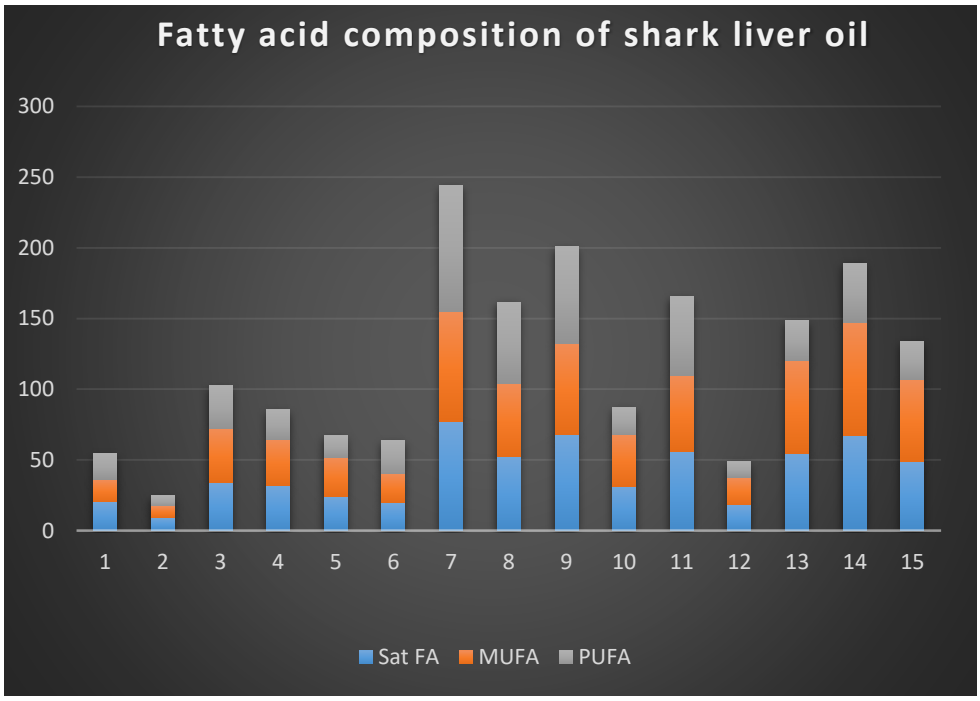


Fig. 4 Proportion of SFA, MUFA, PUFA in shark liver oil as effected by various extraction conditions

Table 15 Fatty acid composition of shark liver oil extracted under different conditions

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
SFA	C11	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.38	0.56	0.00	0.34	0.00	0.00	0.00	0.00
	C12	0.00	0.00	0.00	0.00	0.00	0.00	0.15	0.13	0.13	0.00	0.09	0.00	0.00	0.00	0.00
	C14	1.62	1.20	1.28	1.33	1.01	2.10	6.98	4.98	6.69	1.26	4.95	1.08	2.08	2.50	1.75
	C15	0.40	0.25	0.65	0.65	0.50	0.44	1.63	1.07	1.63	0.63	1.29	0.43	1.11	1.32	0.97
	C16	13.32	6.07	22.41	20.65	16.25	12.57	47.07	32.98	40.03	20.62	33.37	11.91	37.40	44.28	32.43
	C17	0.65	0.27	1.89	1.85	1.40	0.61	2.62	1.54	2.35	1.85	2.17	1.01	3.31	4.01	2.94
	C18	4.01	1.36	6.36	5.62	4.35	3.81	13.97	8.42	12.40	5.47	11.24	2.95	9.50	11.77	8.25
	C20	0.00	0.00	0.00	0.00	0.00	0.00	0.45	0.28	0.72	0.00	0.36	0.00	0.00	0.00	0.00
	C22	0.00	0.00	0.00	0.00	0.00	0.00	0.36	0.25	0.36	0.00	0.28	0.00	0.00	0.00	0.00
	C23	0.40	0.09	1.34	1.54	0.82	0.49	3.56	2.21	2.87	1.38	2.18	0.74	1.02	3.25	2.39
	C24	0.00	0.00	0.00	0.00	0.00	0.00	0.24	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Total	20.41	9.23	33.92	31.63	24.33	20.02	77.35	52.24	67.75	31.20	56.27	18.13	54.42	67.12	48.74
MUFA	C14:1	0.00	0.00	0.15	0.00	0.00	0.21	0.00	0.00	0.00	0.24	0.22	0.00	0.00	0.00	0.00
	C16:1	2.67	4.52	10.61	4.41	4.41	13.14	2.22	9.41	3.54	15.30	10.23	6.87	3.40	7.39	4.43
	C17:1	0.33	0.59	1.30	0.60	0.35	1.98	0.15	1.32	0.28	2.25	1.54	0.94	0.44	0.97	0.58
	C18:1	14.88	29.47	36.25	23.31	14.43	44.19	5.52	60.01	11.73	53.83	37.38	44.71	21.30	51.34	27.57
	C20:1	1.74	3.66	2.34	3.80	1.14	3.55	0.32	8.34	0.10	4.07	2.90	5.34	2.47	5.72	3.79
	C22:1	0.00	0.00	0.32	0.47	0.00	0.41	0.00	1.14	0.00	0.59	0.39	0.00	0.00	0.71	0.45

	C24:1	0.00	0.00	0.58	0.00	0.00	1.14	0.00	0.00	0.00	0.98	0.70	0.00	0.00	0.00	0.00
	Total	19.63	38.24	51.55	32.59	20.33	64.63	8.20	80.23	15.66	77.26	53.35	57.86	27.61	66.13	36.82
PUFA	C18:2	0.00	0.00	2.59	0.00	0.00	3.30	0.00	0.00	0.00	3.86	2.69	0.00	0.00	0.00	0.00
	C18:2	0.55	13.77	0.47	1.58	0.84	0.40	0.33	2.96	0.63	0.58	0.33	1.02	4.26	1.48	1.18
	C18:3	0.00	0.00	0.42	0.20	0.00	0.41	0.00	0.00	0.00	0.41	0.28	0.00	0.13	0.00	0.00
	C20:2	0.00	0.00	0.63	0.00	0.00	0.83	0.00	0.00	0.00	1.07	0.69	0.00	0.00	0.00	0.00
	C20:3	0.00	0.00	0.50	0.00	0.00	0.67	0.00	0.00	0.00	0.84	0.53	0.00	0.00	0.00	0.00
	C20:3	0.00	0.00	0.60	0.00	0.00	0.76	0.00	0.00	0.00	1.05	0.56	0.00	0.00	0.00	0.00
	C20:4	1.13	2.20	7.31	2.51	3.22	9.39	1.25	4.94	2.66	11.52	7.24	3.56	1.57	3.57	2.24
	C20:5	1.11	1.75	10.12	2.02	3.88	10.62	1.50	3.96	3.23	13.58	8.55	2.85	1.22	2.85	1.86
	C22:6	8.05	12.38	35.28	14.88	15.26	42.13	4.43	29.87	12.08	56.46	34.98	19.72	8.05	20.08	13.34
	Total	10.85	30.11	57.92	21.19	23.20	68.52	7.50	41.72	18.60	89.37	55.85	27.15	15.23	27.99	18.61

(Note: SFA: Saturated Fatty acid; MUFA : Mono Unsaturated Fatty Acid; PUFA: Poly Unsaturated Fatty Acid

1-15: represents different experiments)

4.1.4.1. Effect of extraction conditions on major fatty acids

The effect of extraction conditions on major fatty acid contents such as EPA, DHA, AA were analyzed and given in table. DHA content has varied from 43 to 56.46 mg/g oil of whereas the EPA and AA content ranged were 1.11 to 13.58 and 1.13 to 11.52 mg/g oil respectively. The variations in DHA, EPA and AA as affected by various extraction conditions was explained by second order response surface model ($p < 0.05$) with an R^2 value of 0.55, 0.57 and 0.52 respectively. The estimated regression coefficients of DHA, EPA and AA are depicted in Table 12. The response surface plot of predicted values of DHA, EPA and AA are shown in fig. 5. The analysis of variance of fitted model showed that linear, quadratic and interaction effect of the processing variables were found to be non-significant. The signal to noise ratio was less than 4 for all the process variables indicating their inadequate model discrimination.

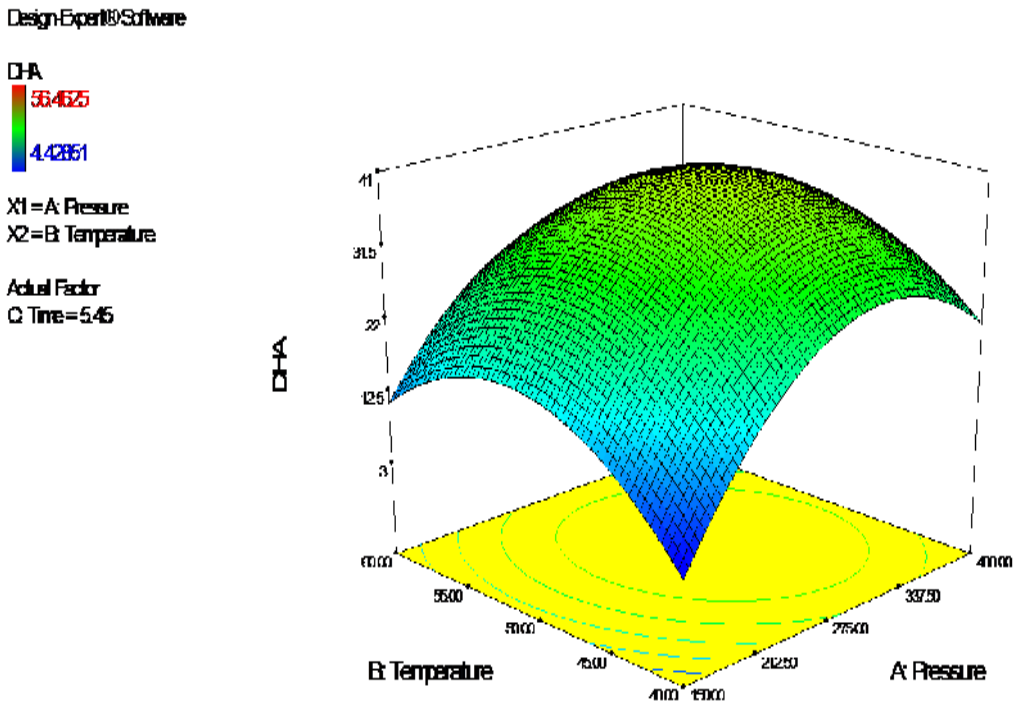


Fig. 5a

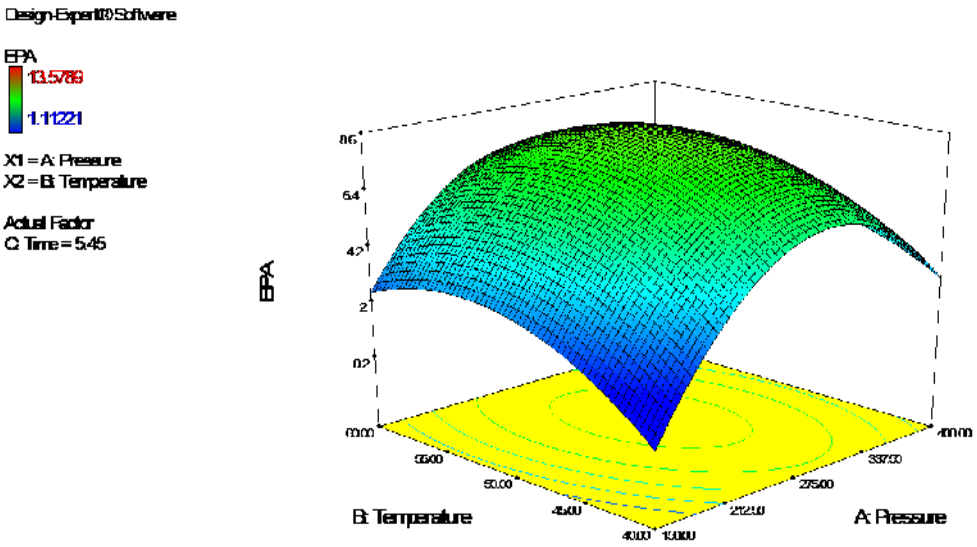


Fig. 5b

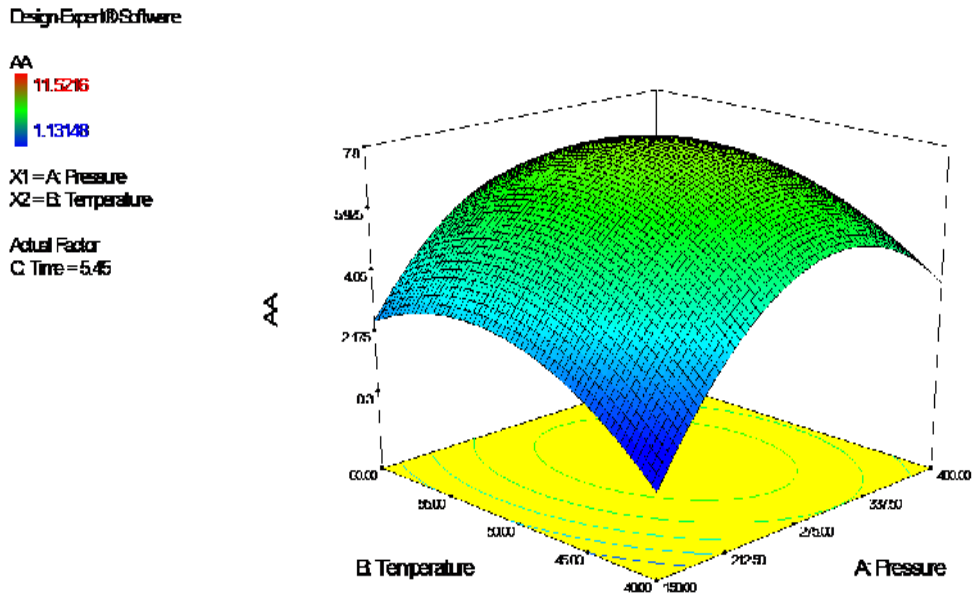


Fig. 5c

Fig. 5 Response surface plot for the effect of extraction conditions on DHA, EPA and AA

(Note: a, b, c represents the effect on DHA, EPA and AA respectively)

4.1.5. Effect of extraction conditions on fat soluble vitamins (A and E)

4.1.5.1. Effect on vitamin A and E

Second order response surface model with an R^2 value 0.857 and (Table 12) was used to explain the variation in vitamin A content. The response surface plot of predicted values of springiness is depicted in fig. 6. The analysis of variance of fitted model showed that only the linear effect of time (X_3) was found to be significant ($P<0.05$), whereas the quadratic of X_1, X_2 and X_3 was found to be non-significant. Except X1*X3, the interaction effects of the other response variables were found to be non-significant. The precision measures of S/N (signal to noise ratio) was greater than 4 (6.506) indicating adequate model discrimination (Myers e*t al*. 2009).

The variations in vitamin E as effected by extraction conditions was explained by second order response surface model ($p < 0.05$) with an R^2 value of 0.65. The analysis of variance of fitted model showed that linear, quadratic and interaction effects of different process variables, X_1, X_2 and X_3 were found to be non-significant.

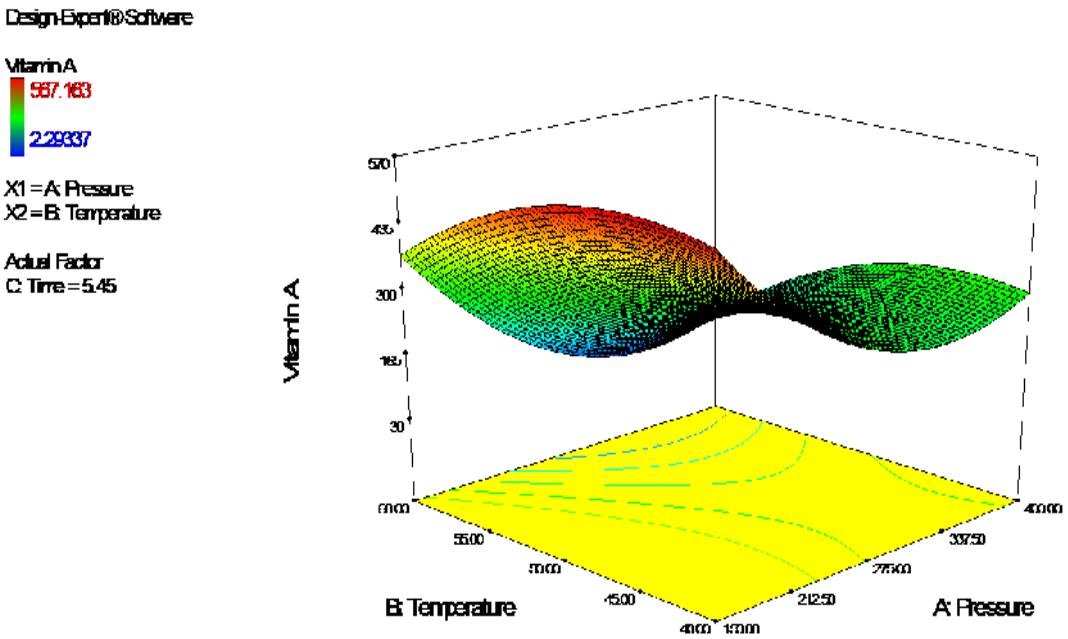


Fig. 6a

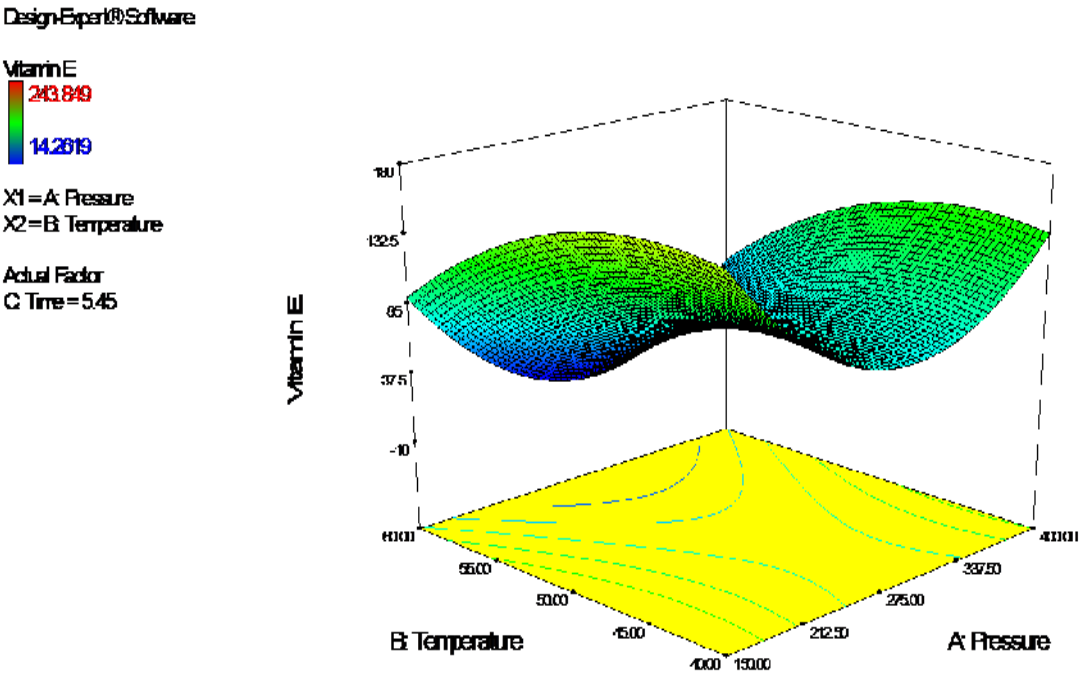


Fig. 6b

Fig. 6 a and b Response surface plot for the effect of different extraction conditions on vitamin A and E respectively

From the contour plot (Fig. 7), the desirability score obtained for the optimized parameter is 0.59. The optimized extraction conditions were found to be 400 bar pressure, 43.34 ºC temperature, 5.45 h (Table 15) for isolation of squalene rich shark liver oil.

Table 16 Response variable of optimized process parameters for extraction of squalene rich shark liver oil

Pressure (bar)	Temperature (° C)	Time (h)	Yield (%)	Squalene (mg/g oil)	DHA (mg/g oil)	Vit A (µg/g oil)	Vit E (µg/g oil)
400	43.34	5.45	69.1964	859.08	27.3097	306.829	139.515

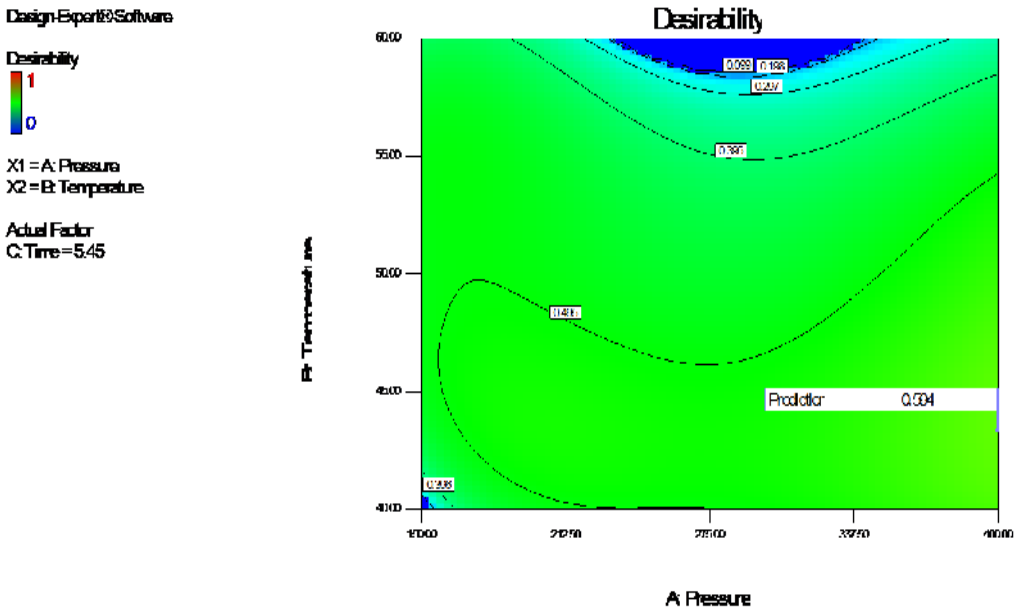


Fig. 7 Contour plot of desirability score for optimized process variables

Three experiments were performed under the optimized conditions to validate the conditions. It was found that when extraction was carried out at the optimized conditions, the yield of squalene, DHA, vitamin A and vitamin E were 838.21 mg/g oil, 24.18 mg/g oil, 305.23 µg/g oil and 140.12 µg/g oil respectively.

4.2. Encapsulation of squalene

Encapsulation is a promising technique to protect unsaturated fatty acids against oxidation and thereby increasing their shelf life (Carneiro *et al*., 2013). Hence, a preliminary study was carried out to establish the core to wall material ratio using chitosan as the wall material, before proceeding to the final encapsulation experiments.

4.2.1. Optimizing core to wall material ratio

Three different treatments were made by varying the core to wall material ratio (squalene to chitosan ratio: 0.3:1, 0.5:1, 1:1 w/w) to optimize the emulsion formulation. It was observed that as the ratio of squalene to chitosan (core material: wall material) increased from 0.3:1 to 1:1, the emulsion became more unstable which was clearly evident in the form of an upper creamy and a clear lower serum layer. The highest emulsion stability was observed in the treatment prepared with squalene to chitosan ratio of 0.3:1. There was a gradual decrease in the particle size and increase in zeta potential values with the decrease in the core to wall material ratio. All the emulsions had PDI value above 0.45 showing that they all had a broader size distribution. The lowest PDI value was observed for emulsion with squalene to chitosan ratio 0.5:1 (table 16). There was a significant change in the rheological properties of the emulsion with decrease in core to wall material ratio ($p<0.05$). It can be inferred from the viscosity data that when the core to wall material ratio was decreased, there was a considerable increase in the viscosity (table 17).

The microscopic images revealed that the droplets seem to be more flocculated and the individual droplets were not visible in case of emulsion with high squalene to chitosan ratio (1:1 and 0.5:1), Whereas, there was less evidence of flocculation and aggregation in emulsions with lower squalene to chitosan ratio (0.3:1) and the oil droplets seemed to be separated from each other (Fig. 8). It is speculated that inadequate wall material might have facilitated electrostatic attraction and flocculation between the droplets.

Table 17 Effect of different core to wall material ratio on emulsion stability, particle size, zeta potential, PDI and viscosity

Squalene to chitosan Ratio	% separation	Z - average (nm)	Zeta potential (mV)	PDI	Viscosity (cP)
1:01	4.5 ± 0.1[c]	489.9	41.8	0.826	34.6 ± 0.02[a]
0.5:1	3.63 ± 0.152[b]	352.6	51.4	0.48	36.659 ± 0.03[b]
0.3:1	2.43 ± 0.05[a]	344.6	52.1	0.52	38.33 ± 0.01[c]

(All data are given as mean ± SD, n=3; [a–c] Different letters with mean value indicate differences (p≤0.05) between treatments)

Table 18 Rheological parameters obtained from power law model for emulsions prepared using different core to wall material ratio

Squalene to chitosan Ratio	Flow index (n)	Consistency Index (cP)	Correlation index (R^2)
1:01	0.97 ± 0.02[ab]	38.5 ± 0.15[a]	0.999
0.5:1	0.99 ± 0.01[b]	38.2 ± 0.20[a]	0.999
0.3:1	0.94 ± 0.02[a]	47.7 ± 0.2[b]	0.998

(All data are given as mean ± SD, n=3; [a–b] Different letters with mean value indicate differences (p≤0.05) between treatments)

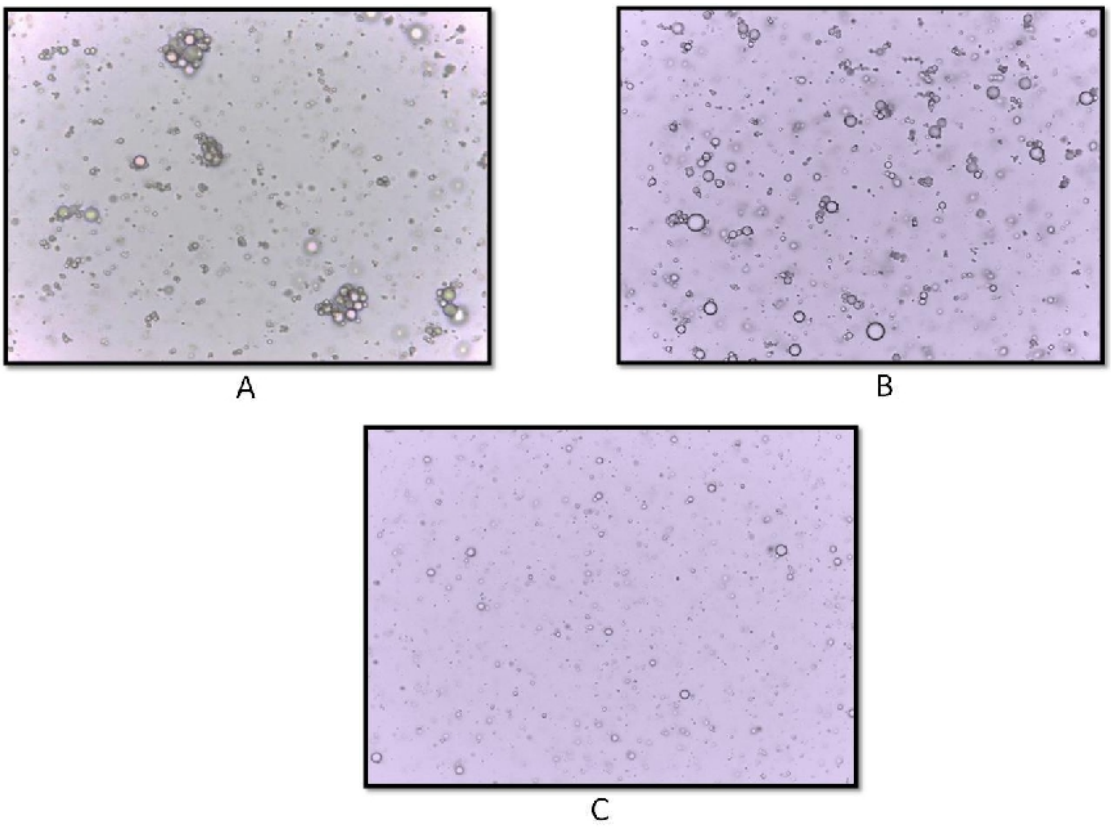


Fig. 8 Microstructure of emulsion prepared with different squalene to chitosan ratio (Note: A, B and C represents emulsion prepared with squalene to chitosan ratio 1:1, 0.5:1 and 0.3:1 respectively, taken at an objective magnification of 10X

Taking into account the results obtained, it was concluded that a lower core to wall material ratio (0.3:1) was most effective in producing stable squalene emulsion with lowest percentage separation and particle size, higher zeta potential, viscosity and other rheological parameters. However, the encapsulation of squalene with chitosan by spray drying didn't give satisfactory results. The encapsulation efficiency of squalene was found to be 26 ± 0.577 %. The average particle size of the spray dried microcapsule was around 6.796 µm whereas before spray drying it was 344.6 nm. Peroxide value of the sample increased during the storage period and it crossed the level of acceptability after 3rd week of storage. The initial peroxide value of the sample was around 6 meq/O_2/Kg of oil and after the 4th week it was 30 meq peroxide/Kg oil. It can be inferred from the results that microencapsulation with chitosan could not provide any additional protection to squalene against thermal degradation. Hence, it can be inferred that chitosan alone cannot function as better encapsulant for squalene and combination of wall materials such as chitosan-whey protein complex, chitosan-sodium alginate can be tried for better results.

4.2.2. Screening of various biopolymers for squalene encapsulation

Protein-polysaccharide complexes such as chitosan-whey protein isolate (CS-WPI, 1:10), maltodextrin (degree of dextrose equivalents – 16.5-19.5)-whey protein isolate (MD-WPI, 7:1) and gum Arabic (GA) were attempted for squalene encapsulation. The protein polysaccharide ratios used in the different combinations were optimised previously by taking into account all the emulsion stability parameters. Squalene emulsions were prepared with various biopolymers by high speed homogenization.

4.2.2.1. Emulsion stability analysis

Emulsion stability analysis revealed that the lowest percentage of separation (1.18 ± 0.14) was observed in gum Arabic based emulsion followed by MD-WPI. Viscosity also followed the same trend with gum Arabic having the highest viscosity of 9.16 ± 0.05 cP.

Power law model was used to elucidate the flow properties of the emulsions. Rheological parameters of the emulsions fitted using power law is shown in Table 17. There was a significant change in the rheological properties of the emulsion stabilized with various wall materials ($p < 0.05$). The square of correlation index (R^2) was ≥ 95.0 in all the cases showing the suitability of power law for the present work. The consistency coefficient, K was found to be highest in MD-WPI stabilized emulsions. The flow index, n was found to vary significantly with different wall materials (table 18).

Table 19 Stability parameters of squalene emulsion prepared using various biopolymers

Emulsion stability parameters	CS-WPI	GA	MD-WPI
Emulsion stability (% separation)	7.92 ± 0.18^{c}	1.18 ± 0.14^{a}	2.14 ± 0.07^{b}
Viscosity (cP)	5.43 ± 0.03^{a}	9.16 ± 0.05^{c}	7.7233 ± 0.04^{b}
K (cP)	5.85 ± 0.01^{a}	0.77 ± 0.01^{b}	8.34 ± 0.01^{c}
n	1 ± 0.01	1.54 ± 0.01	0.98 ± 0.01
R^2	99.8	95.8	99.9

(All data are given as mean ± SD, n=3; $^{a–c}$ Different letters with mean value indicate differences ($p \leq 0.05$) between treatments)

4.2.2.2. Encapsulated powder properties influenced by biopolymers

The physico-chemical properties of squalene encapsulated using various wall materials were studied in detail (table 19). There was significant difference in the encapsulation efficiency of squalene coated using different wall materials with values ranging from 75.08 ± 0.77 to 96.50 ± 0.06 %. The MD-WPI coated squalene was having the highest encapsulation efficiency (96.50 ± 0.06) followed by gum Arabic coated squalene. The lowest surface oil content was also observed in the MD-WPI coated squalene (4.17 ± 0.06 mg/g powder). The total oil content has ranged from 118.75 ± 1.29 to 86.3 ± 0.07b mg/g powder with MD-WPI coated squalene exhibiting the highest total content.

Table 20 Physico-chemical properties of squalene encapsulated using various wall materials

Encapsulated powder data	CS-WPI	GA	MD-WPI
Encapsulation efficiency (%)	75.08 ± 0.77^{a}	75.44 ± 0.09^{a}	96.50 ± 0.06^{b}
Surface oil (mg/g of encapsulated powder)	18.21 ± 0.53^{b}	21.2 ± 0.92^{c}	4.17 ± 0.06^{a}
Total oil (mg/g of powder)	73.06 ± 0.25^{a}	86.3 ± 0.07^{b}	118.75 ± 1.29^{c}
Water activity	0.41 ± 0.01^{c}	0.16 ± 0.00^{a}	0.36 ± 0.00^{b}
Bulk density (g/ml)	0.58 ± 0.01^{b}	0.31 ± 0.02^{a}	0.31 ± 0.00^{a}
Tapped density (g/ml)	0.70 ± 0.00^{c}	0.61 ± 0.01^{b}	0.43 ± 0.00^{a}
Carr Index (%)	16.78 ± 1.61^{a}	49.20 ± 3.34^{c}	28.90 ± 0.01^{b}
Hausner ratio	1.20 ± 0.02^{a}	1.97 ± 0.13^{b}	1.3923 ± 0.01^{c}
Hygroscopicity (%)	8.81 ± 0.16^{a}	10.94 ± 0.52^{b}	12.45 ± 0.12^{c}
Water absorption Index	2.14 ± 0.07^{b}	0.15 ± 0.01^{a}	0.23 ± 0.01^{a}
Swelling power	3.38 ± 0.03^{c}	1.18 ± 0.25^{a}	1.58 ± 0.01^{b}
Water solubility	74.86 ± 0.73^{a}	86.55 ± 1.63^{b}	87.41 ± 1.52^{b}

(All data are given as mean ± SD, n=3; $^{a–c}$ Different letters with mean value indicate differences ($p \leq 0.05$) between treatments)

There was a significant change in the water activity of squalene encapsulated using different wall materials. Lowest water activity was observed in GA coated squalene. The highest bulk and tapped density were observed for CS-WPI based squalene whereas the MD-WPI based squalene had the lowest value. There was a significant difference in the hygroscopic nature of squalene

encapsulated using different biopolymers. It was observed that MD-WPI based squalene was having the highest hygroscopic value followed by gum Arabic based squalene. Flowability properties of squalene encapsulated using various wall materials as expressed by Carr index (CI) and Hausner ratio (HR) showed significant differences. The highest CI and HR was observed in the case of GA based squalene and lowest in CS-WPI based squalene. Solubility is an important factor as it affects the sensory quality and this depends to a greater extent on the type of wall material used. CS-WPI coated squalene exhibited the highest water absorption index and swelling power whereas gum Arabic had the lowest values. However, the highest water solubility was exhibited by MD-WPI coated squalene and lowest in CS-WPI coated squalene.

Peroxide value is one of the most widely used tests for measuring peroxide and hydroperoxide concentrations, which are the primary oxidation product of oils. Peroxide value of the squalene encapsulated with different wall materials for one-month period is given in table 20. Irrespective of the wall material used, peroxide value increased significantly during the storage period. The highest oxidative stability was exhibited by MD-WPI based squalene followed by gum Arabic based squalene. Though the peroxide values of the encapsulated squalene showed an increasing trend, values of all the samples were within the acceptable range (20 meq peroxide/kg oil) as proposed by CODEX/FAO standards.

Table 21 Peroxide value of squalene encapsulated using different wall materials

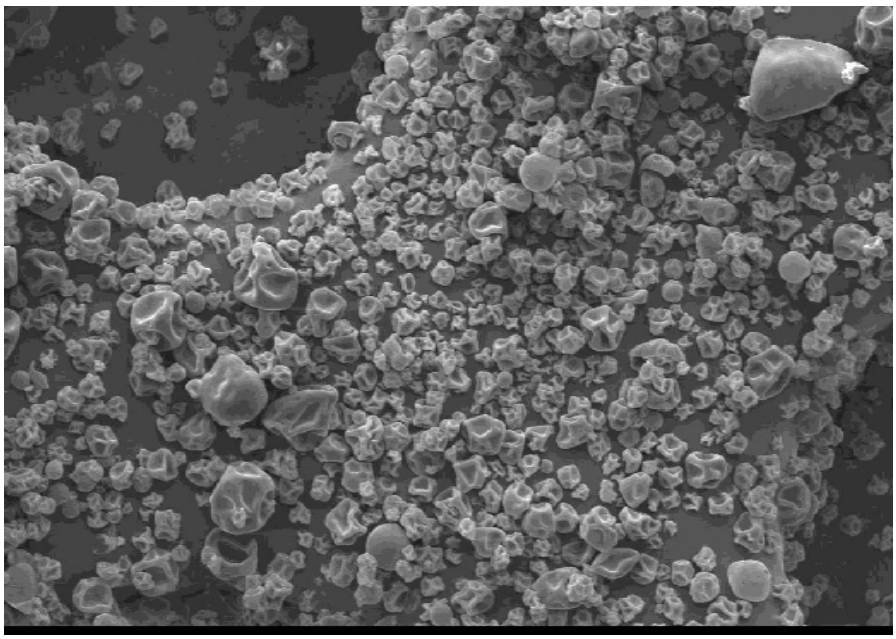
Treatments	CS-WPI	GA	MD-WPI
1st week	1.31 ± 0.02^{a}	1.15 ± 0.03^{a}	0.50 ± 0.01^{a}
2nd week	1.65 ± 0.03^{b}	1.51 ± 0.05^{b}	0.53 ± 0.01^{b}
3rd week	2.58 ± 0.07^{c}	2.37 ± 0.15^{c}	0.55 ± 0.01^{c}
4th week	3.41 ± 0.02^{d}	3.23 ± 0.07^{d}	0.62 ± 0.02^{d}

(All data are given as mean ± SD, n=3; $^{a–c}$ Different letters with mean value indicate differences ($p \leq 0.05$) between treatments)

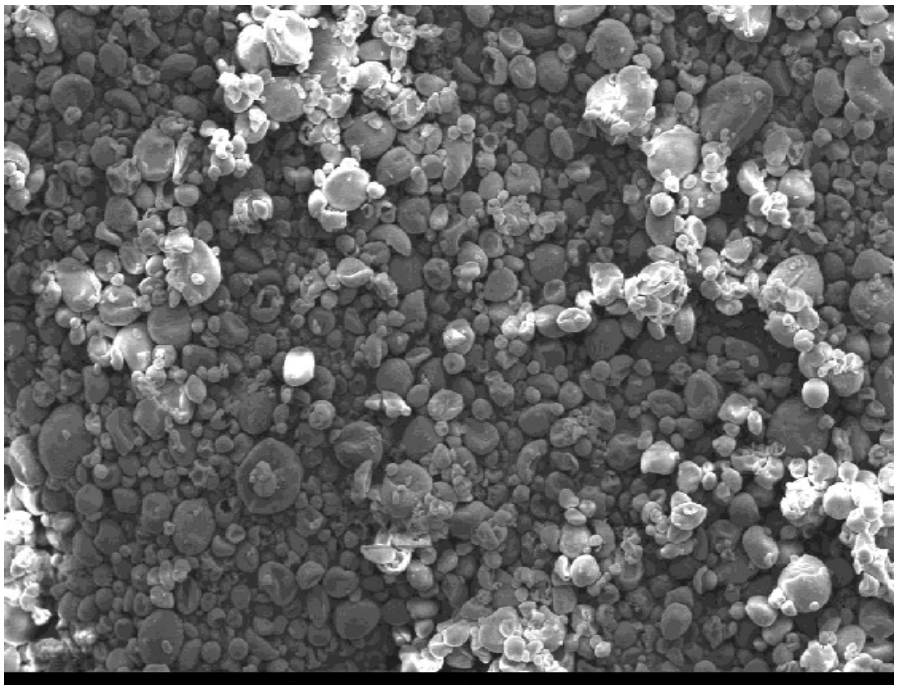
4.2.2.3. Morphological and structural characterization of encapsulates

4.2.2.3.1. External morphology of encapsulated squalene

Scanning electron microscopy helps to reveal the morphological characteristic such as size and shape of the encapsulates. The morphological characteristics of squalene encapsulated using different wall materials are shown in fig. 9. From the SEM photographs it is clear that the spray dried powder was having spherical and more or less a regular shape. It was visually free from any dents and fissures. Interestingly, the CS-WPI coated squalene was found to have a shrivelled surface. All the samples exhibited a wide variation in the size too.



(a)



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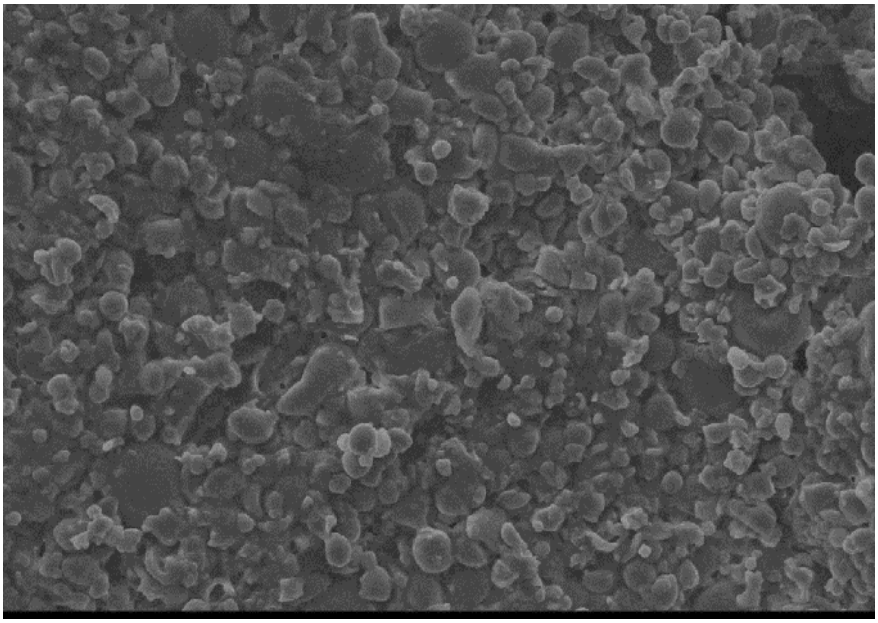
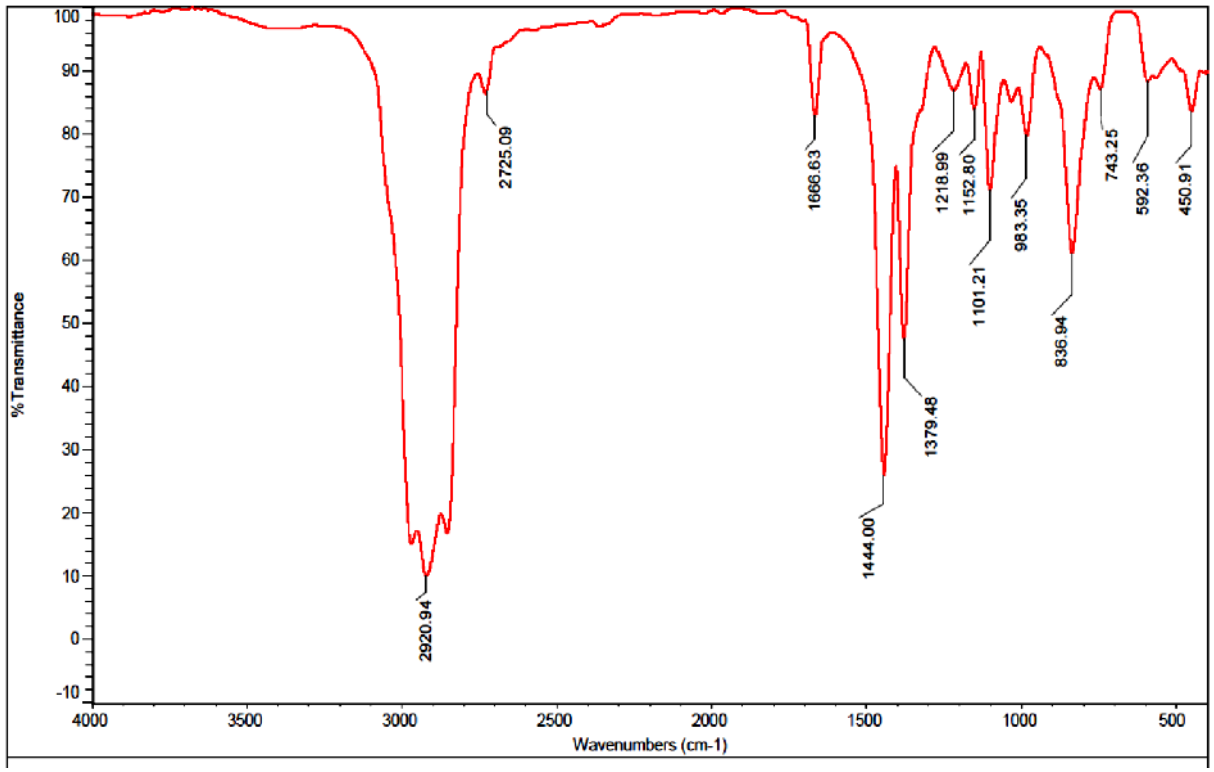


(c)

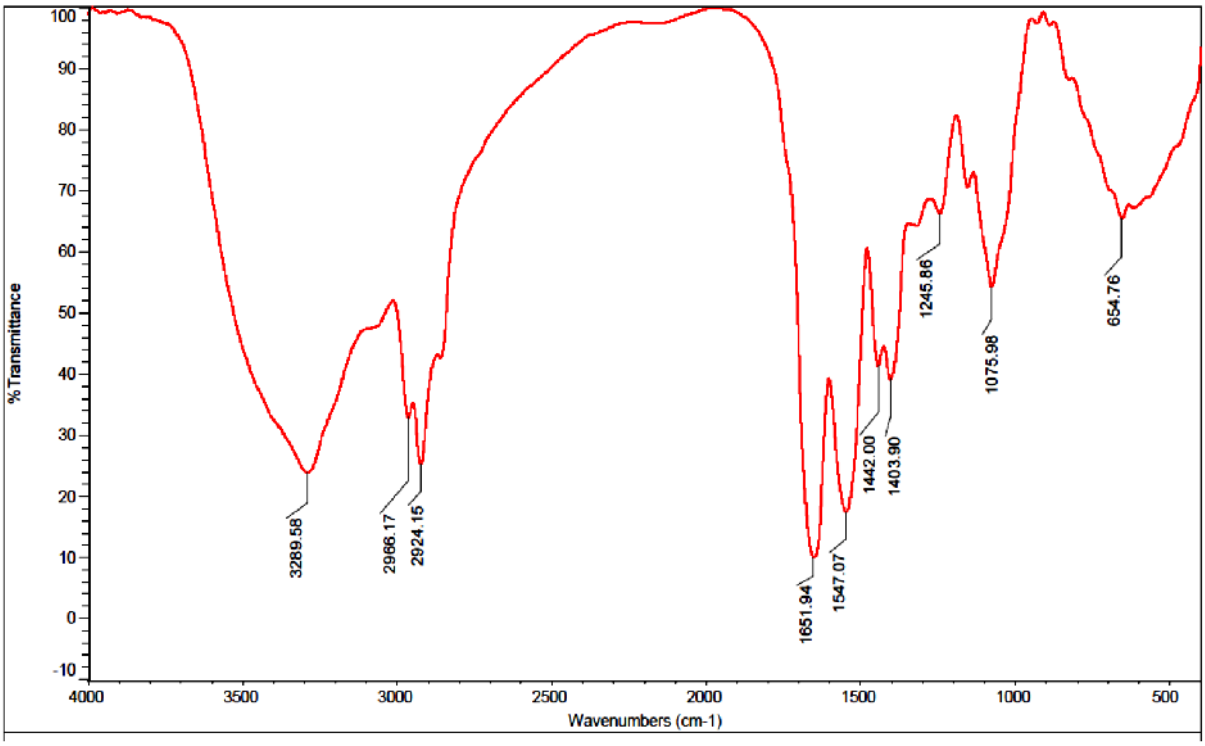
Fig. 9 Scanning electron micrographs of encapsulated squalene (a, b and c represent SEM images of squalene encapsulated using CS-WPI, gum Arabic and MD-WPI respectively)

4.2.2.3.2. Fourier transform infrared spectroscopy (FTIR)

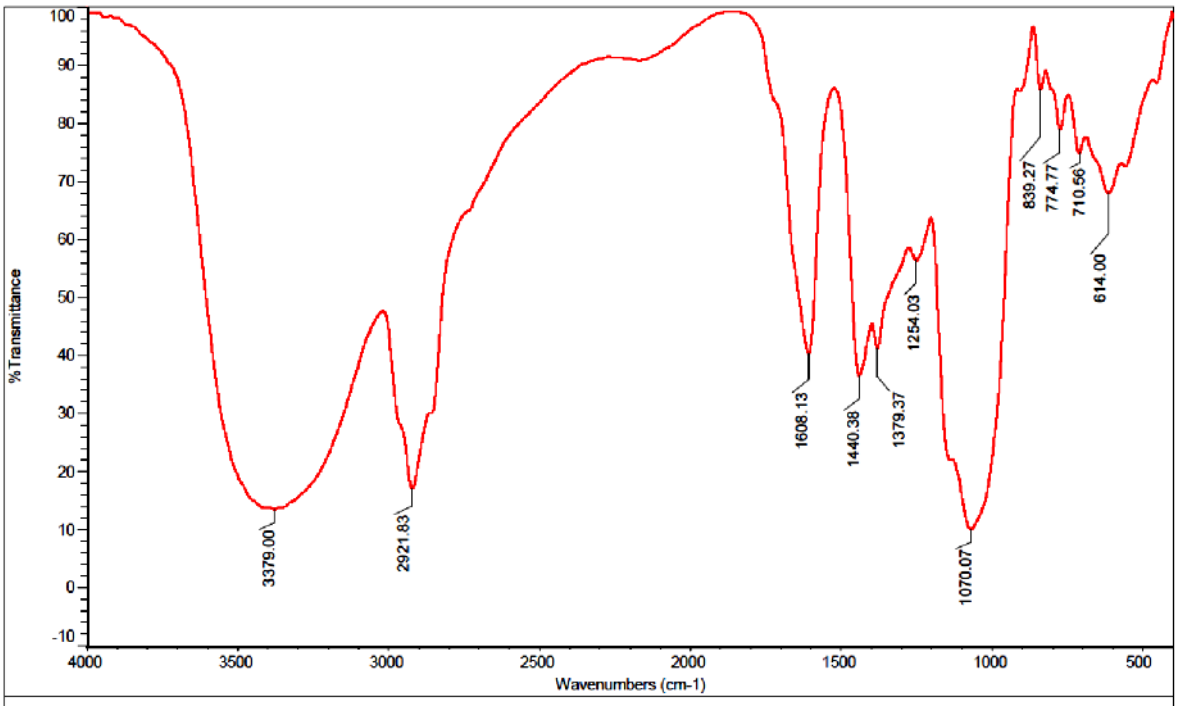
FT-IR spectroscopy can be employed for the structural and intermolecular characterization of complex substances. FTIR spectra of pure and encapsulated squalene are shown in fig. 10. Pure squalene exhibits characteristic bands in the wave number range of 2911- 3024 cm^{-1} due to CH_3 stretching. The characteristic wave numbers of squalene and the wall materials were observed in FTIR spectra of all the encapsulated preparations. The nature of peaks of pure squalene and wall materials did not vary much indicating the absence of interaction between core and wall material.



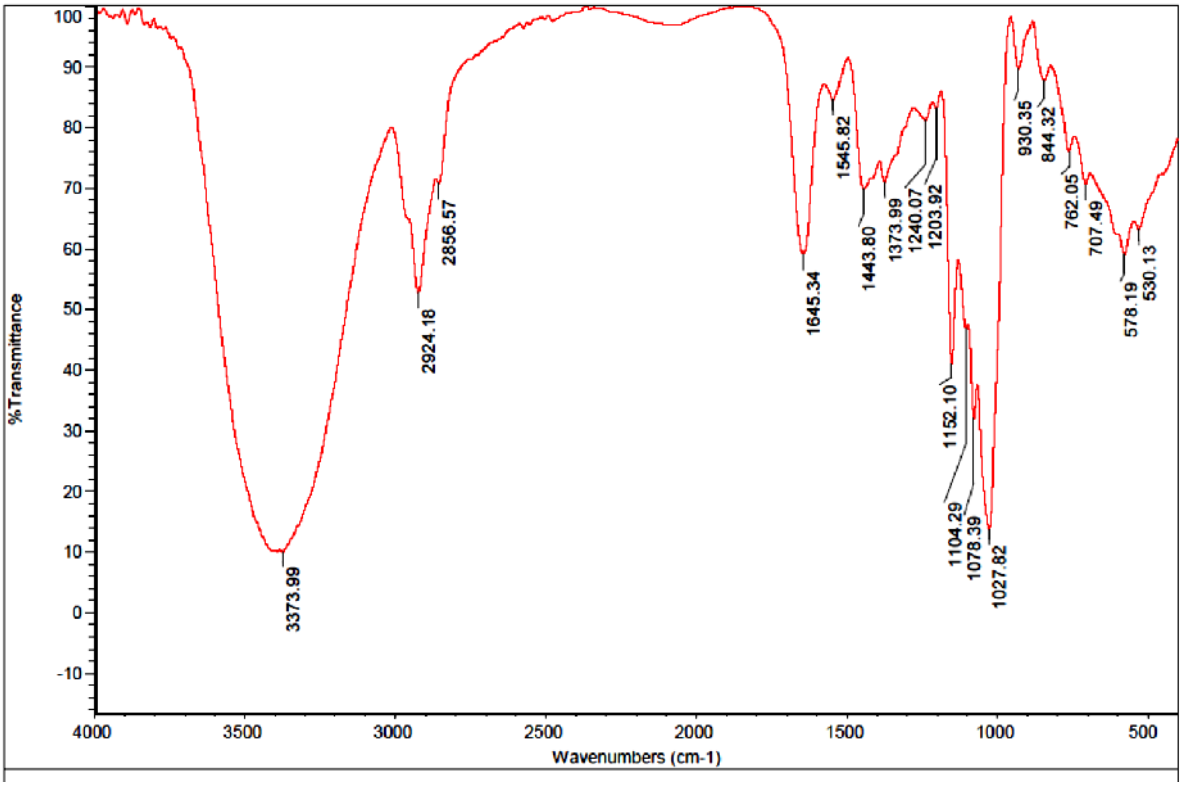
(a) Pure Squalene



(b) CS-WPI FTIR



(c) Gum Arabic FTIR

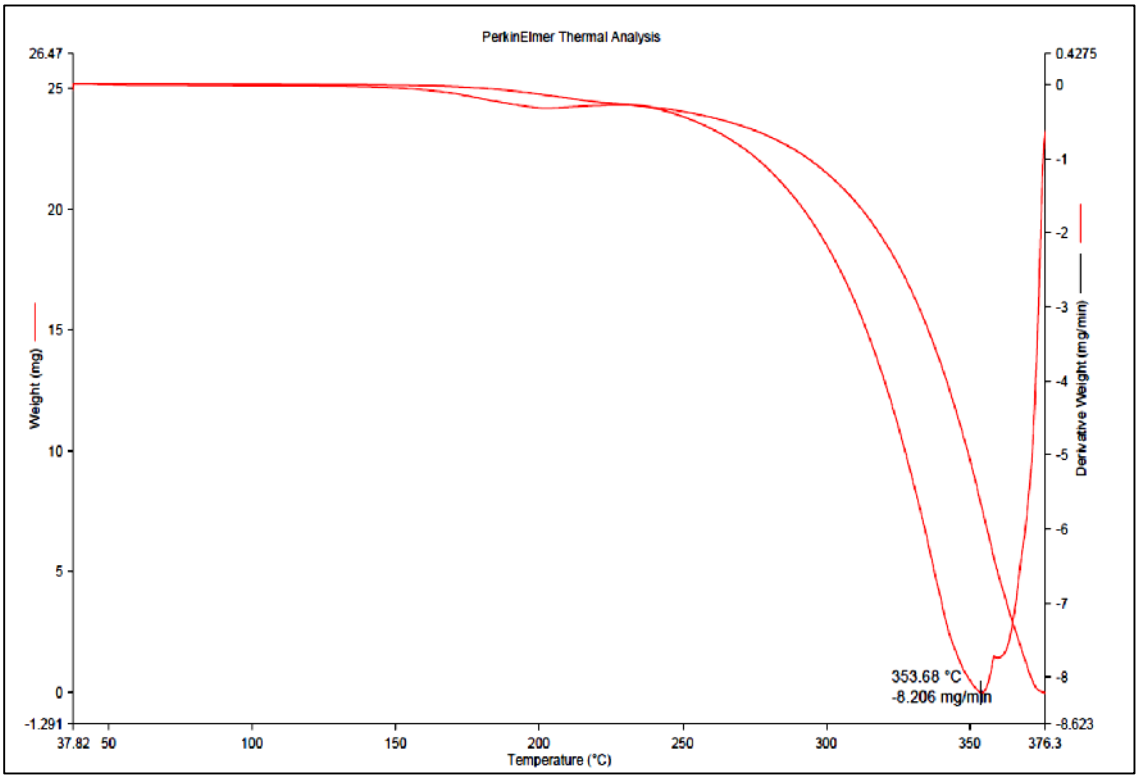


(d) MD-WPI FTIR

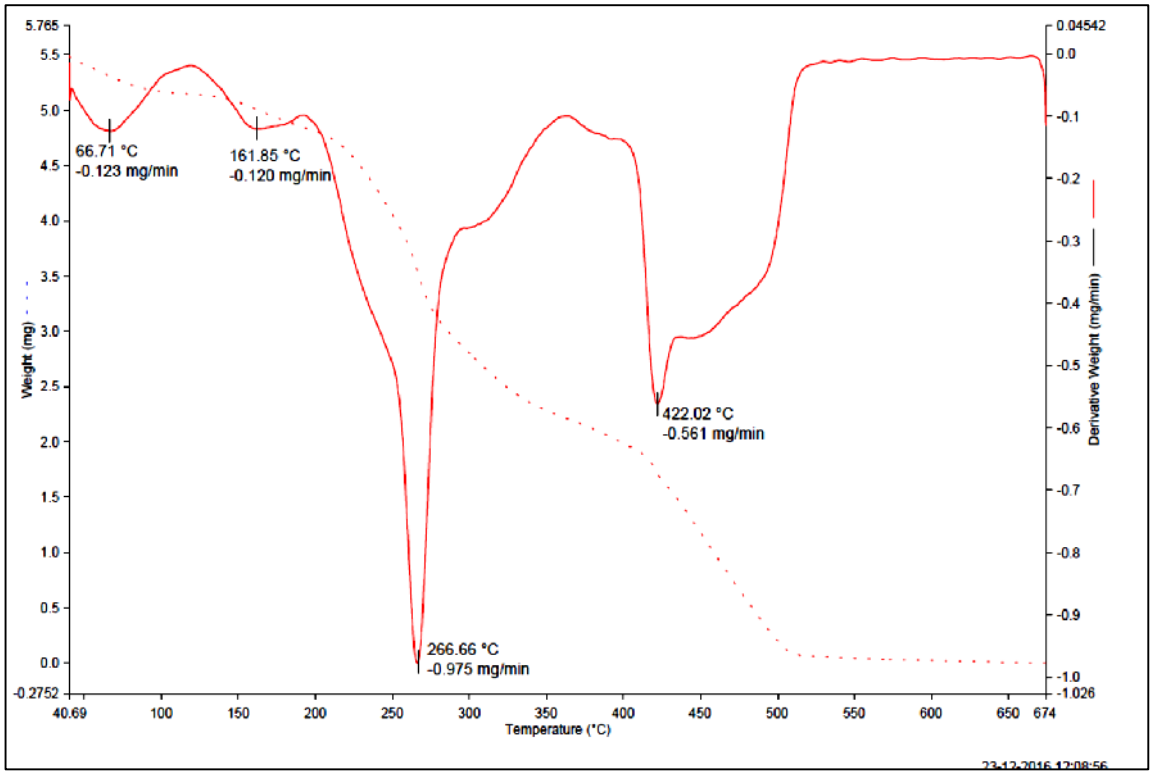
Fig. 10 FTIR spectra (a, b, c and d represent FTIR spectra of pure squalene, squalene encapsulated using CS-WPI, gum Arabic and MD-WPI respectively)

4.2.2.3.3. Thermogravimetric analysis

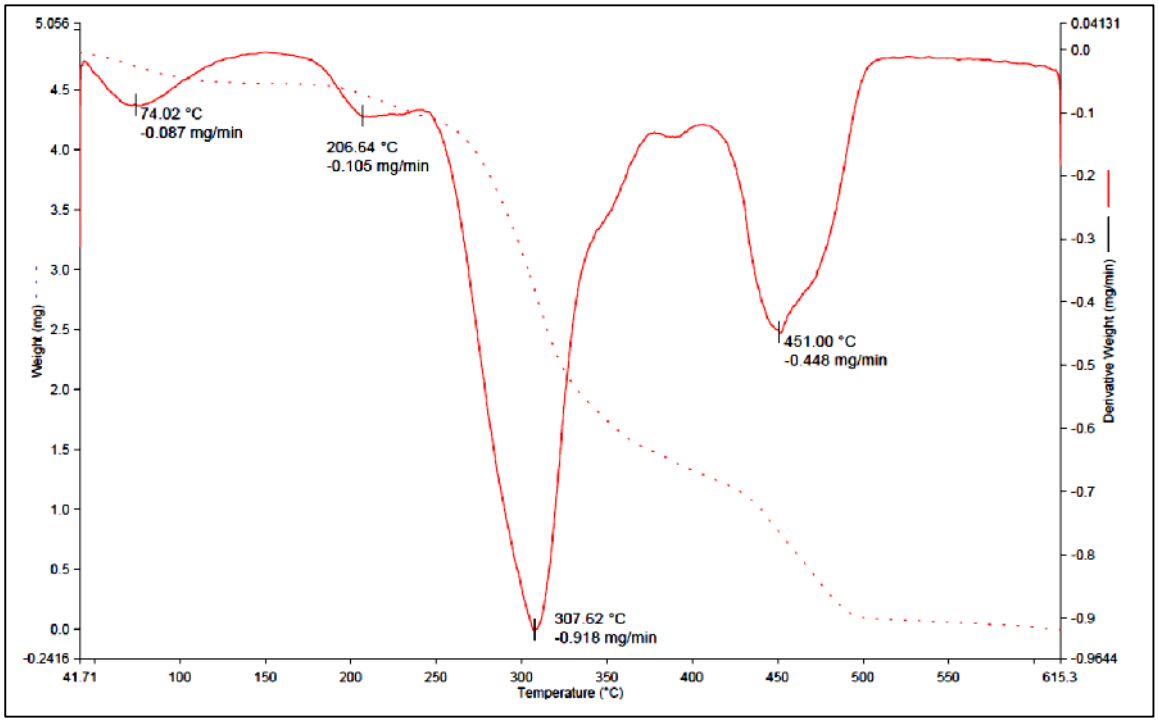
Thermogravimetric analysis (TGA) is an analytical technique to study the weight loss of materials with respect to increasing temperature. Thermal stability of pure squalene and encapsulated squalene with different wall materials is shown in fig. 11. Thermogram of pure squalene shows sharp weight loss at around 350°C. The highest stability was exhibited by gum Arabic coated squalene with thermal stability up to 451 °C. CS-WPI coated squalene conferred thermal stability up to 422.02 °C whereas MD-WPI exhibited thermal stability up to 372.58 °C. It was clear from the TGA results that thermal stability of the different encapsulated material differed each other and the difference can be attributed to the type of wall materials used since all other conditions such as core to wall material ratio, speed and duration of emulsion homogenization, spray drying conditions were constant for all the materials.



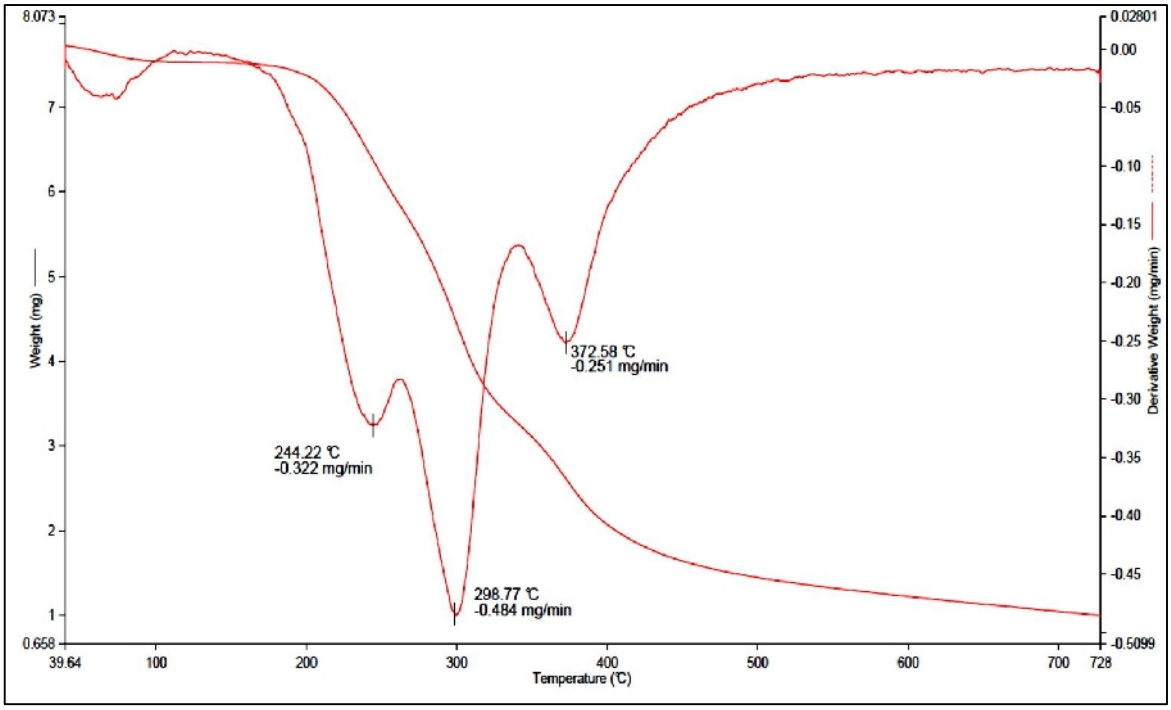
(a) Pure Squalene


66.71 °C
-0.123 mg/min
161.85 °C
-0.120 mg/min
266.66 °C
-0.975 mg/min
422.02 °C
-0.561 mg/min
Weight (mg)
Derivative Weight (mg/min)
Temperature (°C)
5.765
-0.2752
0.04542
-1.026
40.69
674

(b) CS-WPI


74.02 °C
-0.087 mg/min
206.64 °C
-0.105 mg/min
307.62 °C
-0.918 mg/min
451.00 °C
-0.448 mg/min
Weight (mg)
Derivative Weight (mg/min)
Temperature (°C)
5.056
-0.2416
0.04131
-0.9644
41.71
615.3

(c) GA

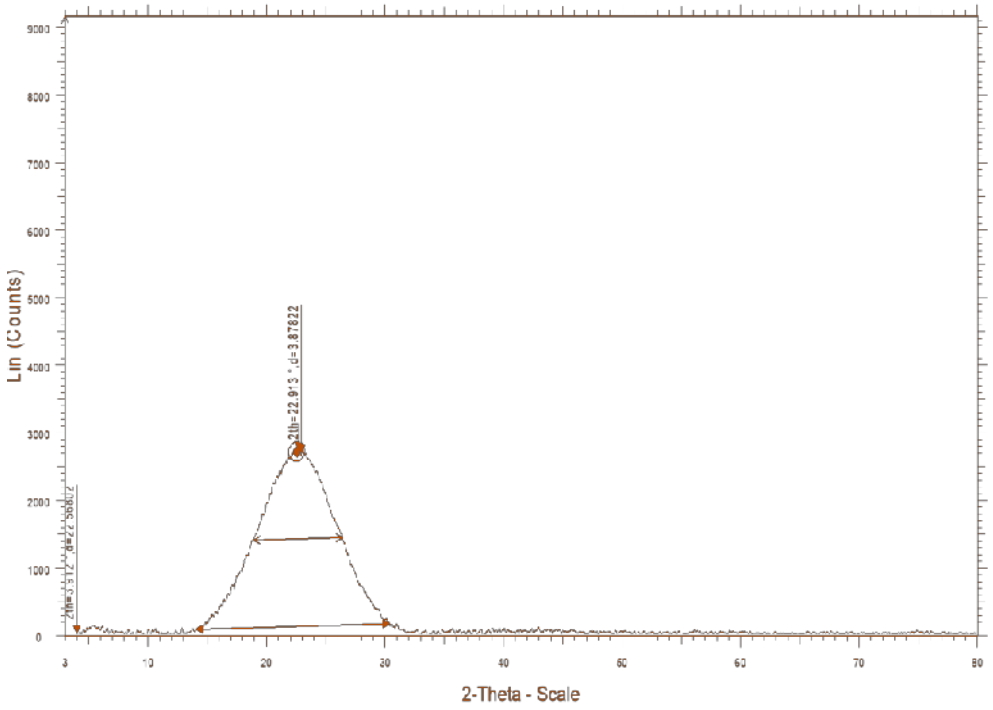


(d) MD-WPI

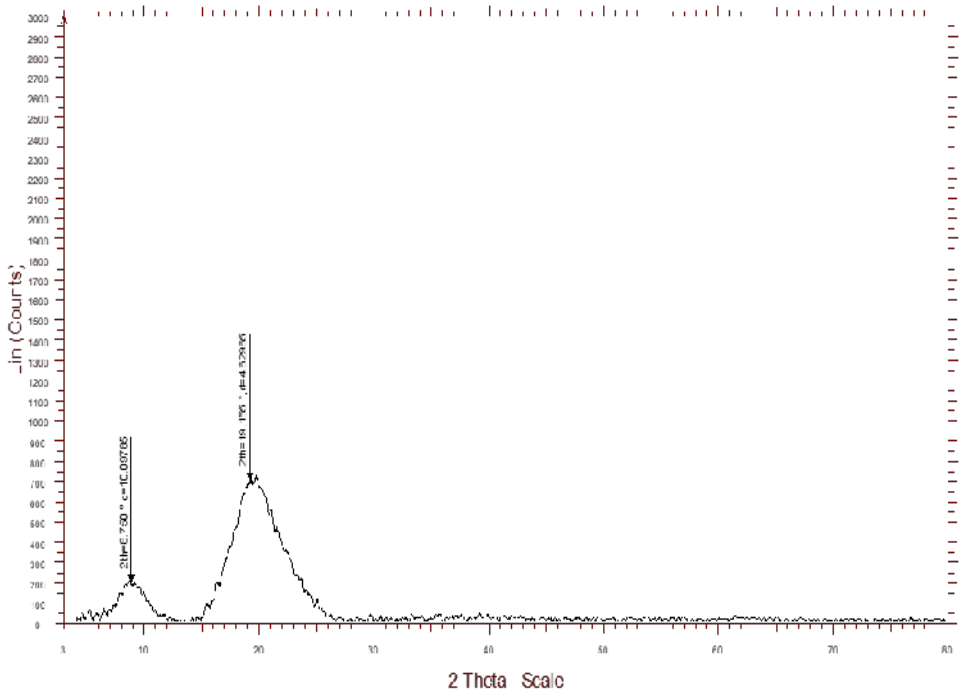
Fig. 11 Thermogravimetric analysis (a represent pure squalene and b, c and d represents squalene encapsulated using CS-WPI, GA and MD-WPI respectively)

4.2.2.3.4. X-ray diffraction analysis (XRD)

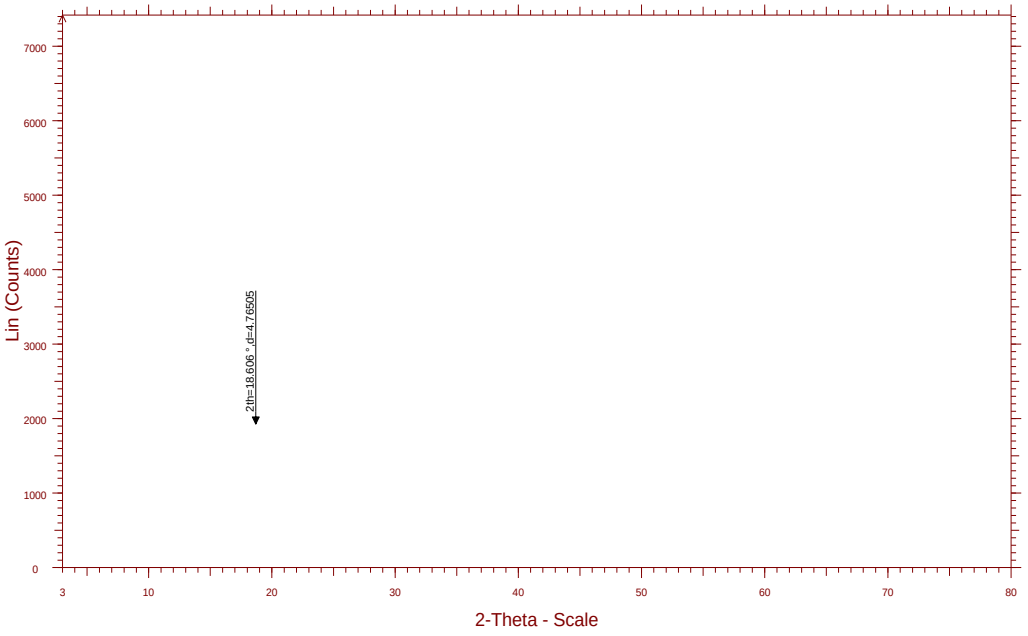
XRD is commonly employed to analyse the degree of crystallinity of the encapsulating material. The X-ray diffractograms of pure squalene and squalene encapsulated using different wall materials is given in fig. 12. From the XRD diffractograms, it is clear that all the encapsulated samples except MD-WPI had almost similar patterns. X-ray diffractograms of CS-WPI and GA coated squalene had broad and diffuse peaks. The broad and diffuse peaks are characteristic of amorphous materials indicating they are in a highly disordered state to increase the diffraction band pattern. In the case of MD-WPI, peaks were less broad than the other materials.



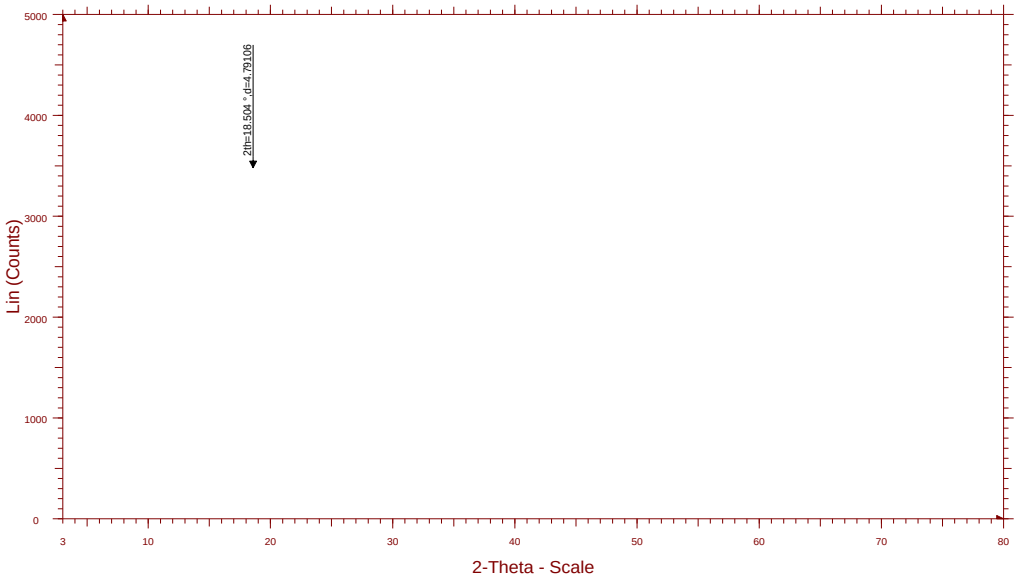
(a) Pure squalene



(b)



(c)



(d)

Fig. 12 X-ray Diffractograms (a, b, c and d represent pure squalene and squalene encapsulated using CS-WPI, GA and MD-WPI respectively)

4.2.2.4. In-vitro digestion of encapsulated materials

Release of squalene from the different encapsulated formulations were studied by an in-vitro digestion model and results are given in table 21. It was clear from the in-vitro release profile, the maximum squalene release has happened at the 4th h of digestion. Maximum release has observed in MD-WPI coated squalene and minimum in CS-WPI coated squalene. The invitro digestion results showed that the release exhibited a slow and sustained pattern

Table 22 In-vitro release profile of squalene from different encapsulated preparations

Duration	CS-WPI	GA	MD-WPI
1st h	27.12799	34.71885	44.65918
2nd h	44.20938	47.94228	56.52194
3rd h	57.52301	57.46808	60.87121
4th h	59.17395	62.1187	84.21843

4.2.2.4.1. Particle size, zeta potential and PDI changes during in-vitro digestion

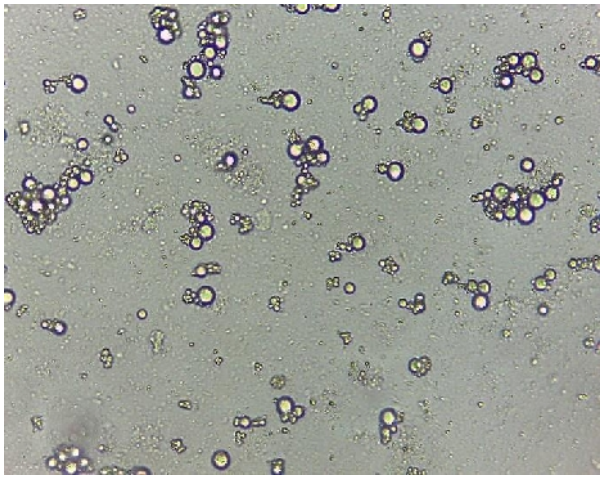
The changes in particle size, zeta potential and PDI of emulsions when exposed to the in-vitro digestion is given in table 22. There was a significant difference in the size distribution among the different samples when exposed to in-vitro digestion studies. The particle size of CS-WPI and MD-WPI coated squalene showed a decrease in particle size with the exception in the 3rd h. However, an increase in particle size was observed after 2nd h of digestion in case of squalene coated with gum Arabic. Similarly, zeta potential of different samples also showed a significant difference. In case of gum Arabic and MD-WPI coated squalene zeta potential showed an increasing trend with maximum values noted at the 4th h. However, in case of CS-WPI there was a decrease in zeta potential values after the 1st h of digestion. Among the three encapsulated squalene preparations, MD-WPI coated squalene exhibited a higher zeta potential value (-48.07 ± 1.95 mV). The lowest zeta potential was exhibited by CS-WPI coated squalene. There was a significant

decrease in the PDI of CS-WPI and MD-WPI. The PDI of MD-WPI coated squalene were observed to show an increasing trend with the digestion time whereas CS-WPI showed a decreasing trend. PDI of GA stabilized emulsions did not show any significant change with its value ranging from 0.42 ± 0.09 to 0.49 ± 0.02. The microstructure of the emulsions at different stages of digestion is shown in fig. 13. The microstructure of the emulsions shows the particle morphology and size during various stages of digestion. It can be clearly seen from the microscopic images that the particle size increased as digestion progressed. The maximum increase was observed at the 3rd h of digestion with the exception in GA coated squalene. The significant decrease in particle size at the 4th h of digestion can be clearly understood from the microscopic images.

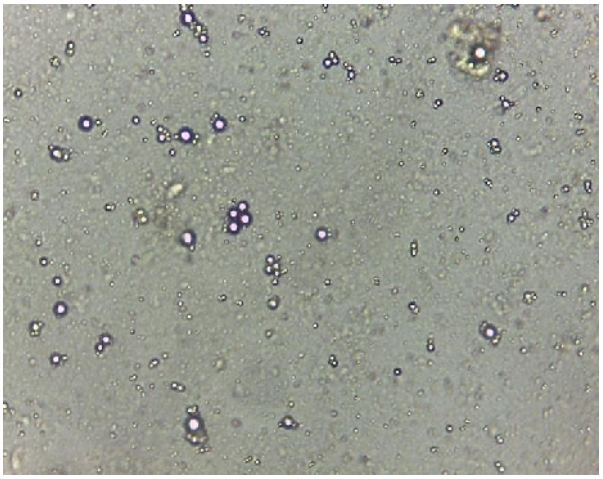
Table 23 Particle size, zeta potential and PDI of encapsulated squalene at various stages of digestion

Particle size (µm)			
Duration	**CS-WPI**	**GA**	**MD-WPI**
1st h	4.47 ± 0.25^{b}	3.77 ± 1.82^{a}	3.33 ± 0.15^{b}
2nd h	3.82 ± 0.27^{a}	3.58 ± 0.25^{a}	3.51 ± 0.05^{b}
3rd h	5.19 ± 0.33^{c}	4.77 ± 0.11^{a}	5.07 ± 0.37^{c}
4th h	3.72 ± 0.16^{a}	5.26 ± 0.40^{a}	2.35 ± 0.19^{a}
Zeta potential (mV)			
1st h	+1.68 ± 0.29^{a}	-8.00 ± 0.35^{b}	-6.77 ± 0.55^{a}
2nd h	+3.79 ± 0.32^{c}	-6.52 ± 0.42^{a}	-9.81 ± 0.14^{b}
3rd h	-3.71 ± 0.25^{bc}	-16.87 ± 1.14^{c}	-40.7 ± 0.5^{c}
4th h	-3.10 ± 0.45^{b}	-17.67 ± 0.83^{c}	-48.07 ± 1.95^{d}
PDI			
1st h	0.339 ± 0.13^{ab}	0.42 ± 0.09^{a}	0.27 ± 0.02^{a}
2nd h	0.42 ± 0.01^{b}	0.46 ± 0.11^{a}	0.35 ± 0.14^{a}
3rd h	0.45 ± 0.03^{b}	0.52 ± 0.01^{a}	0.51 ± 0.04^{b}
4th h	0.27 ± 0.05^{c}	0.49 ± 0.02^{a}	0.92 ± 0.02^{c}

(All data are given as mean ± SD, n=3; $^{a–c}$ Different letters with mean value indicate differences (p≤0.05) between treatments)



(a)



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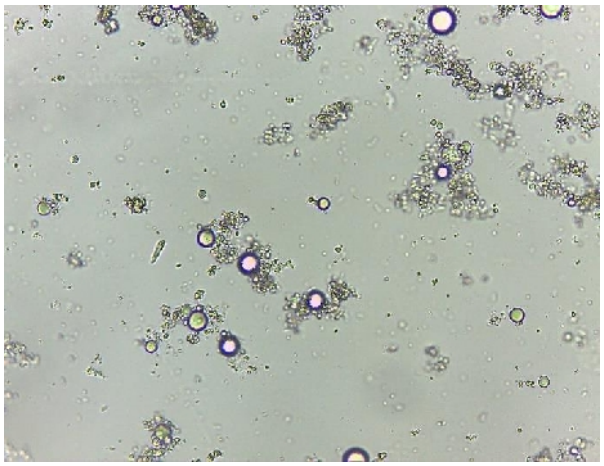

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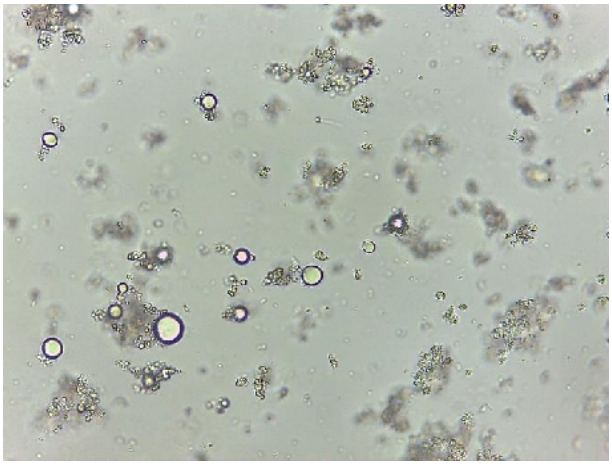

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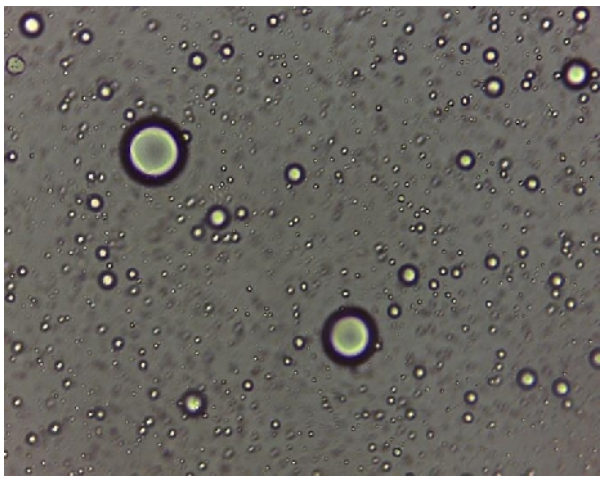
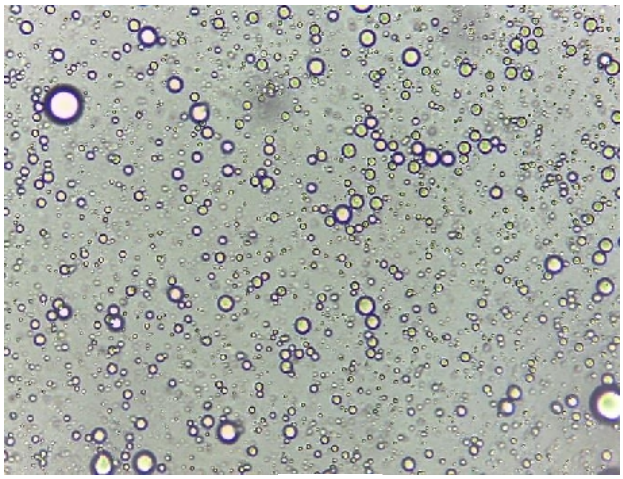
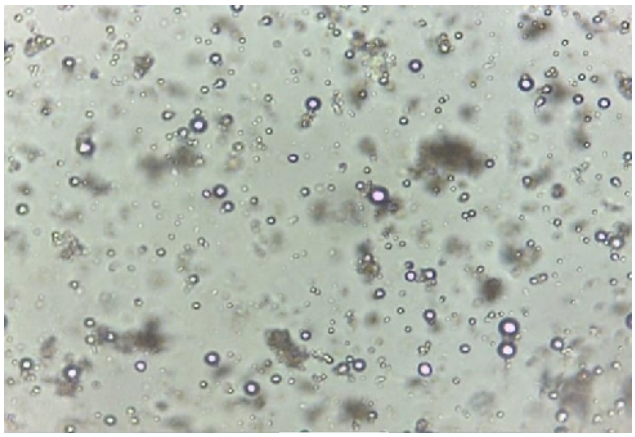


(e)



(f)



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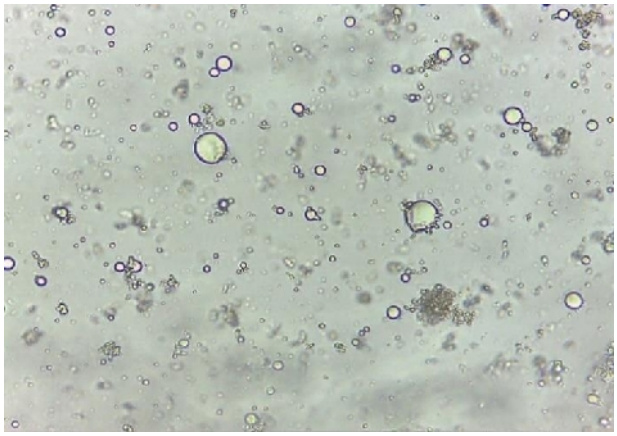

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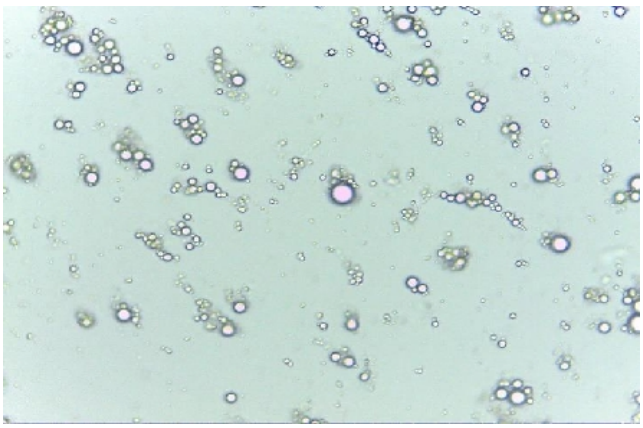

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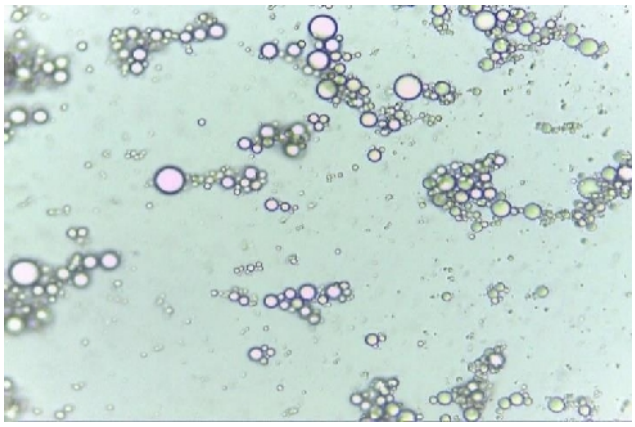
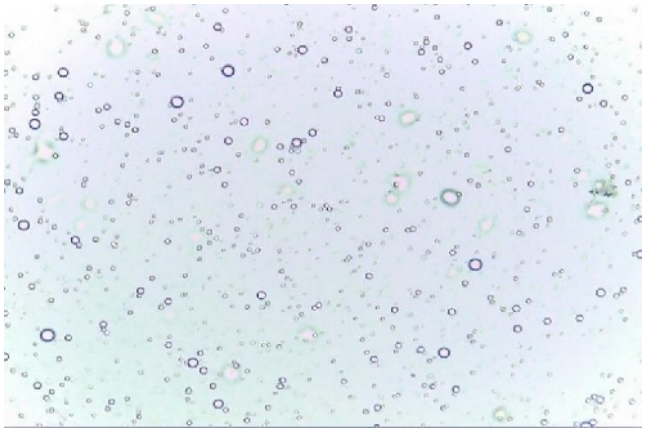
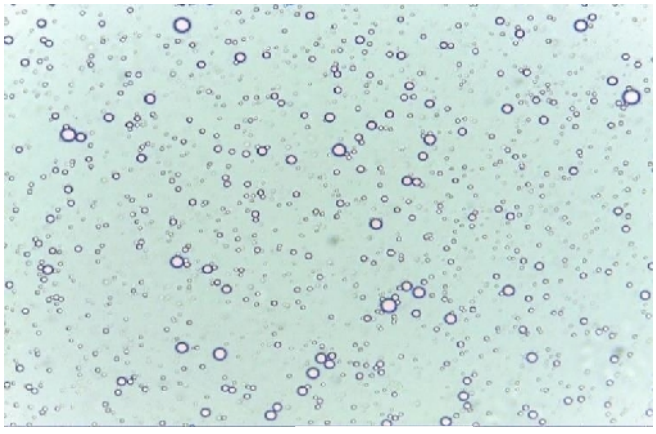


(J)



(k)



(l)

Fig 13 Microstructure of encapsulated squalene at various stages of digestion

[Microstructure of digested emulsions (a, b,c, d represents the digested squalene emulsions stabilized by CS-WPI at 1st, 2nd, 3rd, 4th h of digestion respectively; e, f ,g, h represents the digested squalene emulsions stabilized by GA at 1st, 2nd, 3rd, 4th h of digestion respectively; i, j, k, l represents the digested squalene emulsions stabilized by MD-WPI at 1st, 2nd, 3rd, 4th h of digestion respectively]

4.3. Nutritional evaluation of encapsulated squalene based on animal study

Among the different wall materials prepared, MD-WPI coated squalene was found to have superior properties in terms of its encapsulation efficiency, oxidative and thermal stability and other physico-chemical properties. Hence, the same combination was optimized and was subjected to nutritional evaluation study. The effect of dietary supplementation of squalene encapsulated using MD-WPI on growth performance and lipid profile in wistar strain albino rats has been studied and the results are shown below.

4.3.1. Effect of encapsulated squalene on growth performance

The weight gain % and specific growth rate (SGR) of rats fed with encapsulated squalene are given in Table 23. When compared to control, animals fed with squalene encapsulated using MD-WPI showed improved weight gain % and SGR. There was a non-significant increase in the weight of experimental animals fed with encapsulated squalene when compared to that of control. Specific growth rate also showed an increasing trend with squalene supplementation. Weight gain and SGR% was found to vary with squalene dosage. The highest weight gain and SGR was observed in rats fed with the highest dose of encapsulated squalene.

Table 24 Effect of encapsulated squalene dietary supplementation on weight gain and SGR

Treatments	Wt gain	SGR
T1	43.3 ± 3.77[a]	0.8 ± 0.06[a]
T2	44.93 ± 6.31[a]	0.83 ± 0.10[a]
T3	50.02 ± 8.57[a]	0.90 ± 0.93[a]
T4	50.68 ± 9.82[a]	0.91 ± 0.15[a]

(All data are given as mean ± SD, n=3; [a] Letters with mean value indicate there was no significant differences (p>0.05) between treatments)

4.3.2. Squalene supplementation on tissue weight and their protein content

It was clear from the results the tissue weights have decreased with increasing dose level of squalene supplementation except in the case of heart tissue (table 24). When the effect of squalene supplementation on tissue protein content was analysed, it was found that there was a significant difference in the protein content

of various tissues. With the increase in squalene content in the diet, the tissue weights have increased considerably with the exception in liver tissue. However, the highest protein content was observed in liver followed by kidney and the lowest was noticed in brain and heart (table 25)

Table 25 Effect of squalene supplementation on weight of different tissues

Treatments	Liver	Kidney	Brain	Heart
T1	5.0 ± 0.38^{a}	1.45 ± 0.23^{a}	1.65 ± 0.05^{ab}	0.54 ± 0.02^{a}
T2	5.22 ± 0.16^{a}	1.26 ± 0.02^{ab}	1.78 ± 0.10^{b}	0.58 ± 0.01^{a}
T3	5.11 ± 0.70^{a}	1.13 ± 0.17^{a}	1.59 ± 0.04^{ab}	0.55 ± 0.01^{a}
T4	5.11 ± 0.17^{a}	1.22 ± 0.03^{ab}	1.56 ± 0.17^{a}	0.64 ± 0.05^{b}

(All data are given as mean ± SD, n=3; $^{a–b}$ Different letters with mean value indicate differences ($p \leq 0.05$) between treatments)

Table 26 Effect of squalene supplementation on protein content of various tissues

Treatments	Liver	Kidney	Brain	Heart
T1	129.16 ± 5.19^{c}	100.91 ± 1.31^{a}	65.18 ± 0.79^{a}	59.35 ± 1.03^{a}
T2	113.8 ± 2.07^{a}	102.18 ± 5.48^{a}	70.55 ± 2.39^{c}	68.41 ± 1.21^{b}
T3	122.07 ± 3.01^{b}	114.09 ± 5.48^{c}	67.77 ± 0.28^{b}	75.3 ± 1.33^{c}
T4	113.83 ± 0.87^{b}	105.31 ± 6.16^{ab}	70.89 ± 0.13^{c}	70.49 ± 0.76^{b}

(All data are given as mean ± SD, n=3; $^{a–c}$ Different letters with mean value indicate differences ($p \leq 0.05$) between treatments)

4.3.3. Effect of squalene supplementation on lipid profile

The effect of squalene supplementation on serum lipid profile has been analysed and the results are shown in table 26. The serum lipid profiling has showed a significant difference in the lipid profile among various treatments Serum cholesterol level was found to decrease with increase in squalene dosage. Interestingly, it was observed that an initial lower dose of squalene supplementation increased the

cholesterol content to 94 mg/dL than that of the control groups (74 mg/dL). However, the increased squalene dosage in the diet has lowered the cholesterol content from 94 mg/dL to 58 mg/dL.

Triglyceride level have also followed a similar trend with the squalene supplementation at increasing dosage reducing their levels. When compared to control (52 mg/dl), there was an initial increase in triglyceride level when squalene was fed at lower doses when compared to the control. However, with increasing dosage of squalene supplementation, the value has decreased to 42 mg/dl which is much lower than the control. The LDL and VLDL has also decreased significantly with increased dosage of squalene supplementation. The LDL and VLDL has decreased from 73 mg/dl to 41 mg/dl and 10.4mg/dl to 8 mg/dl with increased squalene supplementation. Unlike cholesterol, HDL was found to increase with the increase in squalene dosage. The HDL level has increased from 1.7 mg/dl in case of control to 9 mg/dl in squalene fed groups.

Table 27 Effect of squalene supplementation of lipid profile

Treatment	Cholesterol (mg/dl)	Triglycerides (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)	HDL (mg/dl)
T1	74	52	61.9	10.4	1.7
T2	94	84	73	16.8	4.1
T3	88	72	67.6	14	6
T4	58	42	41	8	9

4.3.4. Effect of squalene supplementation on catalase and superoxide dismutase activity

The effect of squalene supplementation on the major antioxidant enzymes, catalase and SOD were studied. Catalase activity of liver and brain exhibited a significant change, whereas no significant change was observed in the kidney and heart activity. Among all the tissues, kidney exhibited the highest catalase activity followed by liver whereas, the lowest activity was noticed in brain. The catalase activity was found to decrease with the increase in squalene supplementation (table 27). Effect of squalene supplementation on SOD activity showed that there was a significant difference in the activity of liver, kidney, heart. The highest SOD activity was

exhibited by liver tissues followed by kidney and the lowest activity in heart and brain. Similar to catalase, SOD activity was also found to decrease with increase in squalene supplementation (table 28).

Table 28 Effect of squalene supplementation on catalase (EC 1.11.1.6)

Treatments	Liver	Kidney	Brain	Heart
T1	19.94 ± 2.04^{a}	39.15 ± 1.08^{a}	9.09 ± 1.61^{b}	14.51 ± 1.85^{a}
T2	37.52 ± 1.80^{b}	42.08 ± 2.43^{a}	9.45 ± 0.14^{a}	9.27 ± 0.75^{a}
T3	39.84 ± 0.77^{b}	38.78 ± 1.22^{a}	3.94 ± 1.50^{ab}	12.54 ± 3.17^{a}
T4	30.17 ± 0.18^{b}	41.39 ± 0.63^{a}	2.70 ± 4.91^{ab}	7.43 ± 0.23^{a}

(All data are given as mean ± SD, n=3; $^{a–b}$ Different letters with mean value indicate differences (p≤0.05) between treatments)

Table 29 Effect of squalene supplementation on SOD (EC 1.15.1.1)

Treatment	Liver	Kidney	Brain	Heart
T1	258.25 ± 1.39^{b}	201.36 ± 5.11^{b}	63.19 ± 5.^{43a}	58.63 ± 2.70^{a}
T2	219.5 ± 5.6^{a}	170.87 ± 1.046^{a}	87.44 ± 2.21^{a}	71.99 ± 2.20^{b}
T3	247.06 ± 1.08^{b}	224.91 ± 1.19^{c}	84.86 ± 3.48^{a}	85.98 ± 4.70^{c}
T4	220.07 ± 1.25^{a}	193.31 ± 1.73^{ab}	81.22 ± 6.65^{a}	85.24 ± 4.72^{c}

(All data are given as mean ± SD, n=3; $^{a–c}$ Different letters with mean value indicate differences (p≤0.05) between treatments)

4.3.5. Effect of squalene supplementation on aspartate aminotransferase (AST, EC 2.6.1.1) and alanine aminotransferase (ALT, EC 2.6.1.2)

The effect of dietary supplementation of encapsulated squalene on AST and ALT has been studied and the results are given in table 29. It was clear from the results that there was a significant change in the AST activity of different tissues. With the increase in dosage, the AST activity was found to decrease in all the tissues. The highest activity was noticed in brain and heart and the least in However, the ALT activity has increased with increase in dosage of squalene supplementation with the exception in brain. Interestingly, the higher ALT activity was noticed in the liver tissue and comparatively lower levels in kidney.

Table 30 Effect of encapsulated squalene supplementation on AST and ALT

		AST		
Treatments	Liver	Kidney	Brain	Heart
T1	4.14 ± 0.20^{b}	7.82 ± 0.21^{c}	10.98 ± 0.10^{c}	13.98 ± 0.86^{c}
T2	5.58 ± 0.49^{d}	6.98 ± 0.55^{b}	8.90 ± 0.51^{a}	10.60 ± 1.22^{b}
T3	4.66 ± 0.10^{c}	6.36 ± 0.35^{ab}	9.63 ± 0.08^{b}	6.93 ± 0.12^{a}
T4	3.53 ± 0.07^{a}	5.60 ± 0.45^{a}	9.46 ± 0.03^{b}	8.17 ± 0.29^{a}
		ALT		
T1	5.19 ± 0.15^{a}	1.22 ± 0.11^{b}	6.27 ± 0.08^{a}	3.77 ± 0.05^{a}
T2	5.19 ± 0.33^{a}	0.89 ± 0.02^{a}	3.33 ± 1.24^{a}	4.26 ± 0.12^{a}
T3	5.79 ± 0.19^{b}	2.07 ± 0.29^{c}	3.82 ± 0.63^{a}	4.67 ± 1.04^{a}
T4	5.48 ± 0.32^{ab}	1.37 ± 0.13^{b}	4.10 ± 0.39^{a}	4.14 ± 0.18^{a}

(All data are given as mean ± SD, n=3; $^{a–c}$ Different letters with mean value indicate differences ($p \leq 0.05$) between treatments)

4.3.6. Mineral profiling of liver

The effect of squalene supplementation on the overall mineral status in liver has been analysed and given in table 30. When compared to control, the levels of major elements such as sodium, potassium, calcium, phosphorous, magnesium were found to decrease considerably. Similarly, iron and zinc content were also found to decrease with increase in squalene supplementation. Concentrations of the heavy metals were very below the limits.

Table 31 Mineral profiling as affected by squalene supplementation

Mineral content in liver				
Minerals	T1	T2	T3	T4
Na	1370.94	1164.32	1043.58	935.76
K	5346.01	4510.17	5292.76	4284.64
Ca	146.813	106.83	111.08	138.4
P	1489.45	1472.21	1117.82	1149.58
Mg	156.8	158.9	125.84	131.88
Fe	125.94	139.39	80.36	52.64
Zn	30.49	27.57	20.43	26.41
Cu	3.7	4.12	2.8	6.34
Se	1.09	1.25	0.83	0.13
Cd	0.02	0.02	0.022	0.12
Sn	0.02	ND	0.021	0.1
Be	0.02	ND	0.031	0.035
Co	0.035	0.03	0.03	0.04
Hg	ND	ND	ND	ND
As	0.09	0.11	0.07	0.097
Cr	0.2	0.11	0.11	0.15
Pb	0.07	0.04	0.04	0.05

(Note: ND indicates not detected)

4. 4. Feasibility of squalene as a functional food ingredient

In the present study the functional food application of encapsulated squalene has been attempted by incorporating it in a bakery product, muffins. Based on the physico-chemical characterization, it was found that squalene encapsulated in maltodextrin-whey protein isolate was having an encapsulation efficiency of 96.50 ± 0.06 % with a total oil content of 118.75 ± 1.29 mg/g of encapsulated material. A preliminary study has been carried out to fix the serving size by taking into account the textural, color and most importantly, the sensory acceptance. Accordingly, a serving size of 350 mg squalene per 100g of muffin has been fixed for the product formulation. For the preparation of cake, squalene powder was freshly prepared without the addition of sodium azide as it is not considered as a food grade antimicrobial agent.

4.4.1. Nutritional composition, baking loss and fatty acid composition of muffins

The nutritional analysis revealed that there was a significant difference in the moisture content of the treatments with values varied from 8.16 ± 0.09 to 11.25 ± 0.05 with the lowest content in control and highest in encapsulated squalene enriched muffins. The highest fat and ash content were observed in muffins with encapsulated squalene whereas the highest protein content was observed in muffins with pure squalene. Differences in the proximate composition of the muffins has also reflected in their total calorie content. The total calorific value of muffins showed that the control sample had the highest value as the highest carbohydrate content was observed in the same (table 31)

There was no significant change in moisture loss upon baking across the treatments. Interestingly, the highest baking loss was observed in the control sample followed by muffins with pure squalene. The least baking loss was observed in encapsulated squalene enriched muffins. Irrespective of the treatments, the crust of muffins exhibited lower moisture content than the crumb. Crust and crumb fractions of the encapsulated squalene enriched muffin had the highest moisture content. Lower moisture content of the crust can be due to the dehydration that occurred at the surface layer during the baking process. Water activity was found to vary significantly among the treatments. The lowest water activity was observed in

control muffins and the least value was observed in muffins with encapsulated squalene. The highest water activity can be correlated to the inclusion of maltodextrin.

Table 32 Nutritional composition, Baking loss of various treatments

Properties	A - Control	B - Pure	C - Encapsulated
Moisture (%)	8.16 ± 0.09^{a}	10.31 ± 0.06^{b}	11.25 ± 0.05^{c}
Protein (%)	7.03 ± 0.02^{a}	8.33 ± 0.04^{c}	7.57 ± 0.05^{b}
Fat (%)	25.78 ± 0.03^{a}	26.35 ± 0.03^{b}	27.43 ± 0.02^{c}
Ash (%)	0.73 ± 0.02^{a}	0.75 ± 0.01^{b}	0.78 ± 0.01^{c}
CHO (%)	56.64 ± 0.06^{c}	54.25 ± 0.06^{a}	54.62 ± 0.11^{b}
Total energy (Kcal)	501.61 ± 0.38^{c}	487.47 ± 0.38^{b}	480.78 ± 0.10^{a}
Baking Loss (%)	11.49 ± 3.40^{a}	15.25 ± 5.59^{a}	8.22 ± 4.43^{a}
Crust Moisture (%)	9.54 ± 0.02^{b}	8.09 ± 0.02^{a}	12.53 ± 0.05^{c}
Crumb Moisture (%)	10.57 ± 0.02^{b}	9.67 ± 0.04^{a}	13.90 ± 0.01^{c}
Water activity	0.55 ± 0.002^{a}	0.64 ± 0.01^{b}	0.75 ± 0.002^{c}

(All data are given as mean ± SD, n=3; $^{a–c}$ Different letters with mean value indicate differences ($p \leq 0.05$) between treatments)

Fatty acid composition of different treatments was analyzed and is shown below (table 32). The highest fatty acid content was observed in muffins enriched with encapsulated squalene. The predominant saturated fatty acids were palmitic (C16), stearic (C18) and mystric acid (C14). The predominant monounsaturated and polyunsaturated fatty acids were oleic (C18:1) and linoleic acid (C18:2) respectively.

Table 33 Fatty acid composition of muffins

	Muffins - A		Muffins - B		Muffins - C	
Fatty acids	mg/100g	FAME (%)	mg/100g	FAME (%)	mg/100g	FAME (%)
C6	128.69	1.92	341.03	5.08	841.75	12.53
C8	382.20	5.69	372.79	5.55	806.00	12.00
C10	0.00	0.00	661.60	9.85	73.87	1.10
C11	0.00	0.00	0.00	0.00	0.00	0.00
C12	859.12	12.79	678.70	10.11	1819.47	27.09
C13	0.00	0.00	0.00	0.00	0.00	0.00
C14	3573.71	53.21	2868.37	42.71	7934.57	118.15
C15	418.76	6.24	315.90	4.70	829.20	12.35
C16	11950.76	177.95	10592.40	157.72	28737.50	427.91
C17	0.00	0.00	201.96	3.01	0.00	0.00
C18	5583.07	83.13	4554.54	67.82	24303.54	361.89
C22	35.59	0.53	33.12	0.49	86.18	1.28
C23	0.00	0.00	0.00	0.00	91.73	1.37
C24	20.41	0.30	18.35	0.27	41.56	0.62
ΣSFA	**22952.31**	**341.76**	**20638.76**	**307.31**	**65565.37**	**976.29**
C14:1	324.07	4.83	262.71	3.91	712.25	10.61
C16:1	660.70	9.84	541.76	8.07	1287.67	19.17
C18:1	10727.07	159.73	8881.55	132.25	15704.10	233.84
C22:1	42.39	0.63	34.28	0.51	99.84	1.49
C24:1	128.22	1.91	0.00	0.00	2133.45	31.77
ΣMUFA	**11882.45**	**176.94**	**9720.30**	**144.74**	**19937.31**	**296.88**
C18:2	1148.32	17.10	778.35	11.59	2624.28	39.08
C18:3	87.81	1.31	0.00	0.00	165.14	2.46
C20:4	81.00	1.21	61.42	0.91	201.55	3.00
C22:6	14.11	0.21	0.00	0.00	36.30	0.54
ΣPUFA	1331.24	19.83	839.77	12.50	3027.27	45.08
Total	**36246.23**	**539.72**	**31273.64**	**465.67**	**88723.99**	**1321.13**

4.4.2. Color kinetics of muffins upon storage

4.4.2.1. Crust and crumb color kinetics

Color is a significant parameter that determines the consumer acceptability. Color development usually happens at the end of the baking process (Mundt and Wedzicha 2007). The crust color of different treatments were analysed and presented in table 33. A significant difference was observed in the various color attributes of treatments. Irrespective of the treatments, color parameters, L^*, a^* and b^* of crust has decreased during the storage period. When compared to control,

lightness value has observed to be decreased by squalene addition. Muffins enriched with encapsulated squalene scored the lowest L^* value. Similarly, the a^* and b^* values also showed a decreasing trend with the addition of squalene, either in its pure or encapsulated form. Browning intensity which indicates the crust brown color was highest in muffins enriched with encapsulated squalene. Chroma values, which indicates the color intensity or saturation, were highest in the control samples.

When the crumb region was analysed, higher L^*, a^* and b^* values than that of the crust region was observed. This signifies that the inside temperature might not be enough to accelerate a maillard reaction and hence the crumb color can be attributed to the intrinsic parameters such as the ingredients. Among the different treatments, muffins with pure squalene had the highest lightness value and least in encapsulated squalene enriched muffins. All the samples exhibited a positive a* values, indicating hue on red axis. Similarly, all the samples exhibited a positive b* values indicating the yellow hue. Similar to the crust color kinetics, highest browning intensity was observed in the encapsulated squalene enriched crumb region (table 34).

Table 34 Crust color analysis of muffins

	Storage days	Lightness	Redness	Blueness	BI	Chroma
A	1	39.463 ± 1.25^{c}	15.16 ± 0.27^{c}	23.20 ± 1.29^{b}	112.51 ± 1.82^{b}	27.71 ± 1.22^{b}
	2	36.3 ± 0.48^{ab}	14.75 ± 0.12^{ab}	22.22 ± 0.91^{a}	119.16 ± 5.12^{a}	26.67 ± 0.69^{a}
	3	37.52 ± 2.03^{b}	14.46 ± 0.13^{a}	22.08 ± 1.48^{a}	113.63 ± 15.53^{a}	26.39 ± 1.28^{a}
	4	37.58 ± 0.22^{b}	15.41 ± 0.29^{b}	22.00 ± 0.12^{a}	113.74 ± 1.511^{a}	26.86 ± 0.23^{a}
	5	37.30 ± 0.70^{b}	15.11 ± 0.30^{ab}	20.03 ± 1.77^{a}	103.84 ± 8.56^{a}	25.11 ± 1.39^{a}
	6	34.96 ± 0.16^{a}	14.98 ± 0.72^{ab}	19.54 ± 2.27^{a}	109.9 ± 13.62^{a}	24.64 ± 2.05^{a}
	7	34.89 ± 0.18^{a}	14.84 ± 0.27^{ab}	21.41 ± 0.67^{a}	120.76 ± 4.44^{a}	26.05 ± 0.41^{a}
B	1	37.04 ± 0.83^{bc}	14.52 ± 0.09^{a}	22.26 ± 0.20^{b}	115.82 ± 4.54^{a}	26.57 ± 0.13^{b}
	2	36.44 ± 0.69^{bc}	14.33 ± 0.25^{a}	22.26 ± 0.20^{b}	117.94 ± 1.47^{a}	26.47 ± 0.27^{b}
	3	37.27 ± 0.08^{bc}	14.26 ± 0.22^{a}	21.44 ± 1.26^{b}	109.83 ± 7.05^{a}	25.75 ± 1.17^{b}
	4	37.65 ± 1.11^{c}	14.47 ± 0.46^{a}	21.48 ± 1.95^{b}	108.97 ± 8.31^{a}	25.91 ± 1.75^{b}
	5	37.63 ± 0.90^{c}	14.40 ± 0.32^{a}	21.99 ± 0.79^{b}	111.7 ± 5.42^{a}	26.29 ± 0.57^{b}
	6	35.73 ± 0.12^{ab}	15.10 ± 0.10^{b}	21.08 ± 1.58^{b}	115.7 ± 9.12^{a}	25.94 ± 1.34^{b}
	7	34.31 ± 1.35^{a}	14.30 ± 0.13^{a}	18.94 ± 1.33^{a}	107.37 ± 2.47^{a}	23.74 ± 1.01^{a}
C	1	32.74 ± 1.19^{c}	15.84 ± 0.19^{c}	19.92 ± 0.44^{ab}	123.82 ± 8.52^{b}	25.45 ± 0.35^{ab}
	2	32.11 ± 0.50^{bc}	15.79 ± 0.30^{bc}	21.39 ± 1.60^{bcd}	137.41± 14.45^{ab}	26.59 ± 1.45^{bcd}
	3	31.9 ± 0.81^{bc}	15.73 ± 0.21^{bc}	22.10 ± 0.19^{d}	143.53 ± 6.67^{b}	27.13 ± 0.18^{d}
	4	30.97 ± 0.06^{ab}	15.82 ± 0.30^{c}	21.74 ± 0.34^{cd}	146.68 ± 2.65^{b}	26.89 ± 0.257^{cd}
	5	31.71 ± 0.47^{bc}	15.39 ± 0.22^{ab}	19.45 ± 0.34^{a}	124.79 ± 4.39^{b}	24.81 ± 0.17^{a}
	6	31.47 ± 0.06^{b}	15.26 ± 0.12^{b}	20.65±0.43^{abcd}	134.16 ± 3.28^{ab}	25.68 ± 0.39^{abc}
	7	30.16 ± 0.48^{a}	14.39 ± 0.18^{a}	20.18 ± 1.54^{abc}	136.97± 11.7^{ab}	24.80 ± 1.15^{a}

(Values are indicated as Mean ± standard error with n=3.
$^{a–d}$ Different letters with mean value indicate differences (p≤0.05) between treatments)

Table 35 Crumb color parameters of muffins

Treatment	Storage days	Lightness	Redness	Blueness	BI	Chroma
A	1	72.86 ± 0.950^{bc}	2.41 ± 0.46^{a}	29.7 ± 0.38^{ab}	53.34 ± 0.88^{ab}	29.80 ± 0.40^{ab}
	2	71.54 ± 0.40^{abc}	2.61 ± 0.21^{a}	30.66 ± 1.29^{b}	57.02 ± 3.08^{b}	30.77 ± 1.3^{b}
	3	73.41 ± 1.08^{d}	2.32 ± 0.06^{a}	30.01 ± 0.01^{ab}	53.42 ± 0.97^{ab}	30.10 ± 0.00^{ab}
	4	71.24 ± 1.98^{abc}	2.08 ± 0.45^{a}	28.5 ± 0.81^{a}	51.86 ± 2.10^{a}	28.58 ± 0.79^{a}
	5	70.14 ± 0.22^{a}	2.16 ± 0.35^{a}	28.50 ± 1.24^{a}	52.99 ± 2.83^{ab}	28.59 ± 1.23^{a}
	6	69.22 ± 2.24^{a}	2.27 ± 0.17^{a}	28.74 ± 0.69^{a}	54.61 ± 3.11^{ab}	28.83 ± 0.69^{a}
	7	70.81 ± 0.90^{ab}	2.15 ± 0.17^{a}	28.79 ± 0.26^{a}	52.96 ± 0.48^{ab}	28.87 ± 0.25^{a}
B	1	73.55 ± 0.48^{c}	1.33 ± 0.1^{a}	29.56 ± 0.39^{b}	51.23 ± 1.33^{a}	29.59 ± 0.40^{b}
	2	73.55 ± 0.48^{c}	1.62 ± 0.35^{ab}	29.56 ± 0.39^{b}	51.55 ± 1.62^{a}	29.60 ± 0.40^{b}
	3	72.52 ± 0.88^{bc}	1.59 ± 0.18^{ab}	28.47 ± 0.60^{ab}	50.01 ± 1.79^{a}	28.51 ± 0.59^{ab}
	4	72.20 ± 1.84^{bc}	1.71 ± 0.18^{b}	28.07 ± 0.79^{a}	49.72 ± 3.46^{a}	28.13 ± 0.80^{a}
	5	72.22 ± 0.49^{bc}	2.41 ± 0.15^{c}	27.64 ± 0.64^{a}	49.45 ± 0.98^{a}	27.75 ± 0.64^{a}
	6	71.09 ± 0.45^{ab}	2.17 ± 0.08^{c}	27.46 ± 1.16^{a}	49.78 ± 2.32^{a}	27.54 ± 1.16^{a}
	7	69.93 ± 0.10^{a}	1.81 ± 0.07^{b}	27.45 ± 0.80^{a}	50.40 ± 1.90^{a}	27.51 ± 0.80^{a}
C	1	70.52 ± 1.47^{a}	3.27 ± 0.11^{e}	32.28 ± 0.58^{c}	62.69 ± 1.75^{c}	32.44 ± 0.59^{c}
	2	72.19 ± 1.11^{a}	3.56 ± 0.15^{f}	32.29 ± 0.58^{c}	61.13 ± 1.79^{c}	32.48 ± 0.57^{c}
	3	72.26 ± 0.83^{a}	2.88 ± 0.02^{d}	30.83 ± 1.09^{b}	56.99 ± 3.07^{b}	30.97 ± 1.09^{b}
	4	71.24 ± 1.68^{a}	2.41 ± 01^{c}	28.83 ± 0.52^{a}	52.99 ± 2.90^{a}	28.93 ± 0.54^{a}
	5	72 ± 0.09^{a}	2.44 ± 0.08^{c}	29.18 ± 0.75^{a}	53.03 ± 1.74^{a}	29.28 ± 0.75^{a}
	6	70.10 ± 1.38^{a}	2.13 ± 0.07^{b}	28.53 ± 0.45^{a}	53.03 ± 0.85^{a}	28.61 ± 0.46^{a}
	7	70.11 ± 1.26^{a}	1.66 ± 0.06^{a}	28.65 ± 0.57^{a}	52.74 ± 0.60^{a}	28.69 ± 0.5^{8a}

Values are indicated as Mean ± standard error with n=3.

$^{a–c}$ Different letters with mean value indicate differences (p≤0.05) between treatments

4.4.3. Textural quality of muffins

Textural parameters of muffins stored at room temperature is given in the table 35. The hardness of the control and muffins with pure squalene were found to increase during the storage period. Interestingly, it was observed that the inclusion of encapsulated squalene had decreased the hardness. Hardness of control and pure squalene enriched muffins has increased from 11.57 ± 1.77 to 17.98 ± 4.77 N and 11.16 ± 1.36 to12.79 ± 1.58 N respectively upon storage. In case of muffins with encapsulated squalene the hardness has decreased from 7.39 ± 0.02 to 5.69 ± 0.83 N. In contrast to hardness, other textural parameters such as cohesiveness, springiness, springiness index, gumminess, chewiness and adhesiveness has decreased throughout the storage period. Among different treatments, cohesiveness, springiness, springiness index and chewiness were highest in muffins with encapsulated squalene followed by muffins with pure squalene. Whereas chewiness and gumminess value were lowest in muffins with encapsulated squalene. In general, muffins prepared with encapsulated squalene were cohesive, springier and chewy with less gumminess and stiffness than the other treatments indicating their efficacy in improving the textural quality.

4.4.4. External morphology of muffins

The SEM images of various treatments had shown significant variations visually. Left and right images correspond to100 X and 500 X magnification respectively (fig.14). Cracks and discontinuous networks were observed in the control muffins. The discontinuous networks indicated the non-homogenous distribution. When compared to control, muffins with pure and encapsulated had a denser network. The denser network in the encapsulated enriched muffins can be due presence of whey proteins as wall materials. Whey proteins might have facilitated in the formation of crosslinks to form a denser network. From the results, it can be surmised that incorporation of squalene in its pure or encapsulated form had a significant effect in forming a denser network. Several small or medium sized pores were visible in muffins with squalene (pure of encapsulated), showing the addition had a significant effect.

Table 36 Textural quality of muffins

Results

	Days	Hardness (N)	Cohesiveness	Springiness (mm)	Springiness Index	Gumminess (kgf)	Chewiness (kgf)	Fracture Force (kgf)	Stiffness (kg/mm)	Adhesiveness (kg.mm)
A	1	11.57 ± 1.77^{a}	0.08 ± 0.02^{bc}	2.91 ± 0.35^{b}	0.23 ± 0.02^{c}	0.93 ± 0.10^{a}	2.71 ± 0.60^{a}	0.51 ± 0.01^{a}	3.75±0.39^{abc}	0.4 ± 0.23^{a}
	2	9.62 ± 1.61^{a}	0.10 ± 0.01^{c}	3.15 ± 0.09^{c}	0.23 ± 0.01^{c}	0.80 ± 0.17^{a}	2.53 ± 0.6^{a}	0.51 ± 0.01^{a}	3.21 ± 0.38^{ab}	0.66 ± 0.36^{a}
	3	9.12 ± 0.77^{a}	0.09 ± 0.02^{c}	3.11 ± 0.46^{c}	0.2 ± 0.04^{bc}	0.87 ± 0.27^{a}	2.16 ± 1.25^{a}	0.51 ± 0.00^{a}	2.77 ± 0.77^{a}	0.62 ± 0.15^{a}
	4	12.24 ± 0.93^{a}	0.06 ± 0.01^{ab}	2.44±0.33^{ab}	0.17 ± 0.01^{ab}	0.78 ± 0.09^{a}	1.88 ± 0.25^{a}	0.52 ± 0.01^{a}	5.01 ± 0.46^{c}	0.34 ± 0.50^{a}
	5	12.80 ± 0.29^{a}	0.05 ± 0.01^{a}	2.32 ± 0.29^{a}	0.14 ± 0.03^{a}	0.61 ± 0.07^{a}	2.42 ± 0.38^{a}	0.53 ± 0.02^{a}	4.16±0.97^{abc}	0.25 ± 0.25^{a}
	6	12.78 ± 1.70^{a}	0.057±0.01^{ab}	2.39±0.05^{ab}	0.19±0.05^{abc}	0.72 ± 0.08^{a}	1.85 ± 0.28^{a}	0.52 ± 0.01^{a}	4.79± 1.38^{bc}	0.22 ± 0.26^{a}
	7	17.98 ± 4.77^{b}	0.05 ± 0.02^{a}	2.51±0.21^{ab}	0.17 ± 0.03^{ab}	0.88 ± 0.31^{a}	2.25 ± 0.96^{a}	0.58 ± 0.05^{b}	7.24 ± 1.30^{d}	0.17 ± 0.21^{a}
B	1	11.16±1.36^{ab}	0.1 ± 0.04^{a}	3.04 ± 0.31^{a}	0.26 ± 0.03^{ab}	1.14 ± 0.18^{a}	3.5 ± 0.91^{a}	0.51 ± 0.00^{a}	5.64 ± 2.09^{a}	0.82 ± 0.04^{a}
	2	10.1 ± 0.88^{ab}	0.07 ± 0.0^{1a}	3.21 ± 0.35^{a}	0.24 ± 0.03^{ab}	1.0 ± 0.11^{a}	3.45 ± 0.82^{a}	0.51 ± 0.01^{a}	4.33 ± 0.21^{a}	0.70 ± 0.05^{a}
	3	11.74±1.13^{ab}	0.11 ± 0.03^{a}	3.4 ± 0.23^{a}	0.273± 0.05^{b}	1.26 ± 0.35^{a}	3.28 ± 0.07^{a}	0.51 ± 0.02^{a}	4.41 ± 0.45^{a}	0.37 ± 0.75^{a}
	4	12.40±3.44^{ab}	0.08 ± 0.02^{a}	3.48 ± 0.41^{a}	0.22 ± 0.03^{ab}	1.02 ± 0.33^{a}	3.65 ± 1.52^{a}	0.54 ± 0.01^{b}	5.10 ± 1.32^{a}	0.19 ± 0.19^{a}
	5	9.55 ± 1.67^{ab}	0.09 ± 0.03^{a}	3.24 ± 0.98^{a}	0.21 ± 0.04^{ab}	1.02 ± 0.12^{a}	2.25 ± 2.21^{a}	0.53 ± 0.01^{b}	3.99 ± 0.37^{a}	0.67 ± 0.06^{a}
	6	8.97 ± 1.98^{a}	0.077 ± 0.02a	3.18 ± 0.70^{a}	0.19 ± 0.03^{a}	0.83 ± 0.05^{a}	2.64 ± 1.02^{a}	0.54 ± 0.01^{b}	3.66 ± 1.51^{a}	0.72 ± 0.12^{a}
	7	12.79 ± 1.58^{b}	0.09 ± 0.01^{a}	3.43 ± 0.28^{a}	0.22 ± 0.02^{ab}	1.15 ± 0.22^{a}	3.96 ± 1.09^{a}	0.54 ± 0.01^{b}	5.34 ± 0.64^{a}	0.43 ± 0.35^{a}
C	1	7.39 ± 0.02^{b}	0.13 ± 0.01^{c}	4.51 ± 0.44^{a}	0.28 ± 0.01^{a}	0.91 ± 0.14^{c}	4.06 ± 0.25^{b}	0.51 ± 0.00^{a}	2.31 ± 0.45^{a}	0.97 ± 0.21^{a}
	2	7.14 ± 0.64^{b}	0.12 ± 0.01^{bc}	4.45 ± 0.27^{a}	0.27 ± 0.01^{a}	0.81±0.12^{bc}	3.61±0.38^{ab}	0.51±0.01^{ab}	2.24 ± 0.41^{a}	0.94 ± 0.09^{ab}
	3	7.89 ± 0.54^{b}	0.09 ± 0.03^{ab}	4.25 ± 0.71^{a}	0.23 ± 0.04^{a}	0.6 ± 0.15^{a}	2.63 ± 1.04^{a}	0.51±0.01^{ab}	1.92 ± 0.18^{a}	0.90 ± 0.13^{ab}
	4	7.53 ± 0.48^{b}	0.07 ± 0.01^{a}	3.48 ± 1.96^{a}	0.22 ± 0.04^{a}	0.68±0.03^{ab}	2.71 ± 0.26^{a}	0.52 ± 0.00^{b}	1.95 ± 0.16^{a}	0.94 ± 0.18^{ab}
	5	6.94 ± 0.13^{b}	0.10±0.02^{abc}	4.06 ± 0.03^{a}	0.25 ± 0.03^{a}	0.85 ± 0.08^{bc}	3.26±0.62^{ab}	0.52±0.01^{ab}	2.62 ± 0.29^{a}	0.66 ± 0.11^{b}
	6	6.97 ± 0.10^{b}	0.07 ± 0.03^{a}	4.14 ± 0.10^{a}	0.25 ± 0.04^{a}	0.72±0.04^{abc}	2.65 ± 0.05^{a}	0.51±0.01^{ab}	3.51 ± 0.85^{b}	0.75 ± 0.09^{ab}
	7	5.69 ± 0.83^{a}	0.11 ± 0.01^{bc}	4.16 ± 0.55^{a}	0.24 ± 0.03^{a}	0.69 ± 0.14^{ab}	2.90±0.97^{ab}	0.52±0.01^{ab}	1.97 0.26^{a}	0.85±0.19^{ab}

Values are indicated as Mean ± standard error with n=3.

$^{a–f}$ Different letters with mean value indicate differences (p≤0.05) between treatments

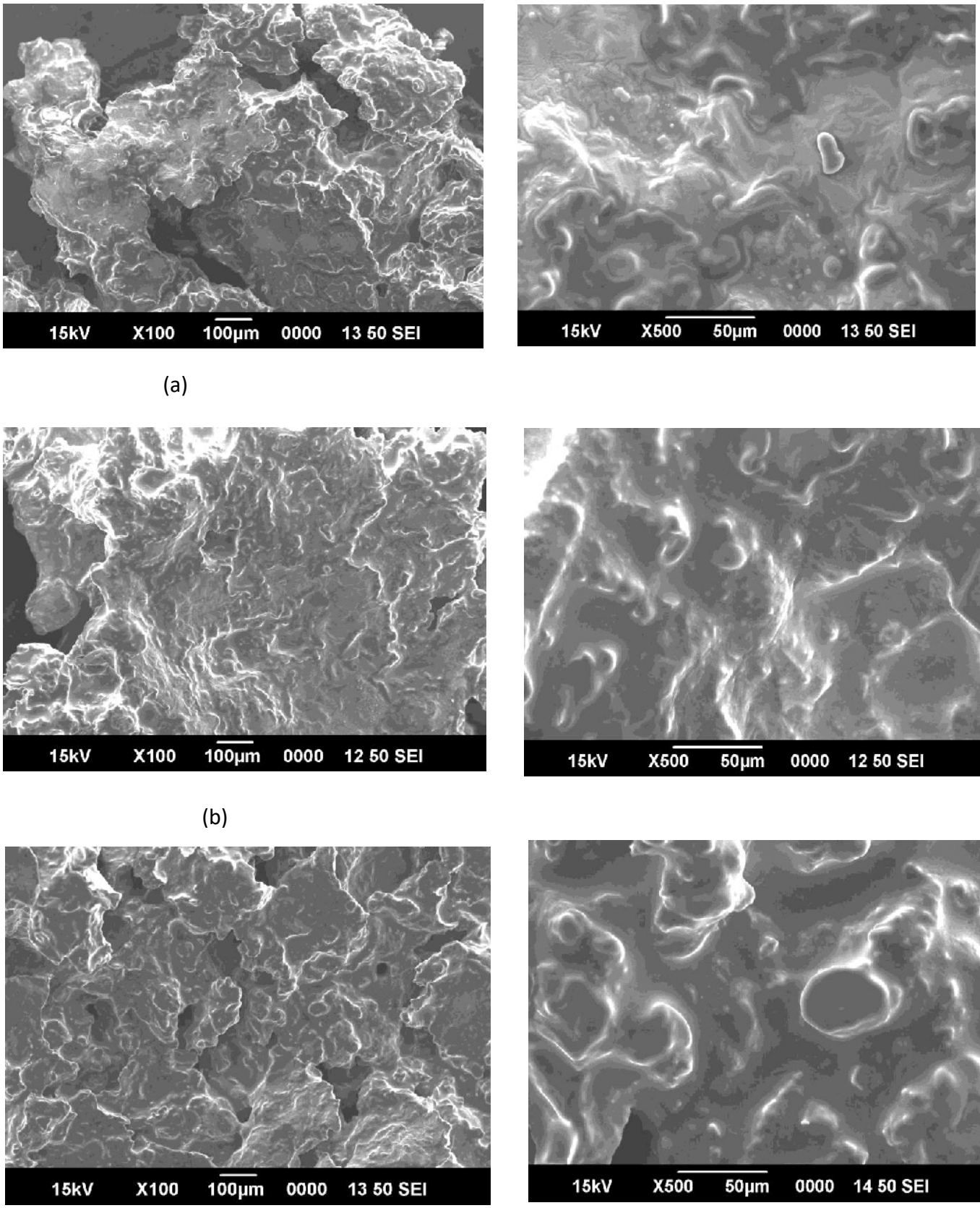


Fig. 14 External morphology of muffins (a, b, c represents control, pure and encapsulated squalene enriched muffins respectively. Left and right side represents micrographs taken at 100 and 500X magnification respectively

4.4.5. Oxidative stability of muffins

Peroxide value of the muffins stored at room temperature were analyzed and shown below (table 36). It was found that irrespective of the treatments, the values had increased throughout the storage period. The highest peroxide value was observed in control and lowest in encapsulated squalene enriched muffins. Though the peroxide value showed an increasing trend, values of all the samples were within the acceptable range (20 meq peroxide/kg oil) as proposed by CODEX/FAO standards.

Table 37 Peroxide value of muffins

Days	A	B	C
1	1.68 ± 0.03^{a}	0.97 ± 0.01^{a}	0.83 ± 0.11^{a}
2	2.38 ± 0.16^{b}	1.21 ± 0.09^{b}	0.97 ± 0.01^{b}
3	2.76 ± 0.09^{c}	1.38 ± 0.06^{c}	1.09 ± 0.01^{c}
4	3.11 ± 0.02^{d}	1.81 ± 0.03^{d}	1.36 ± 0.02^{d}
5	3.59 ± 0.03^{e}	2.10 ± 0.02^{e}	1.69 ± 0.03^{e}
6	4.05 ± 0.07^{f}	2.28 ± 0.03^{f}	1.92 ± 0.01^{f}
7	4.32 ± 0.90^{g}	2.61 ± 0.05^{g}	2.11 ± 0.02^{g}

(Values are indicated as Mean ± standard error with n=3
$^{a–g}$ Different letters with mean value indicate differences ($p \leq 0.05$) between treatments)

4.4.6. Microbiological quality of muffins

Bakery products, especially muffins are classified as intermediate moisture food and are vulnerable to physico-chemical changes and microbiological spoilage. The microbiological quality analysis of the batters prior to baking were analysed and results are shown in table 37. The initial total plate count (TPC) were found to be 8.2×10^{2} cfu/g, 4.8×10^{2} cfu/g and 1.3×10^{3} cfu/g respectively for batter A, B and C. The presence of fungus was observed in batter A and C and the counts were found to be 2×10^{1} cfu/g and 1×10^{1} cfu/g respectively. Total plate count of all the three baked muffins showed an increasing trend during the storage period (table 38). Initial TPC for Muffin - A was 4.2×10^{1} cfu/g and reached 2.7×10^{3} cfu/g on the 4th day of storage. Similarly, initial TPC for muffins B and C was found to be 2.6×10^{1} cfu/g and 3.1×10^{1} cfu/g and reached 3.6×10^{3} cfu/g and 8.1×10^{3} cfu/g on the 6th

day of storage. Fungal growth was not noticed in any of the treatments. *Bacillus* and *Staphylococcus* were the predominant ones present among the normal mesophilic flora while among pigmented mesophilic flora *Micrococcus* were found to be at the highest counts.

Table 38 Microbiological quality of batter prior to baking

Treatments	TPC (cfu/g)	Fungal count (cfu/g)
A	8.2×10^2	2×10^1
B	4.8×10^2	Nil
C	1.3×10^3	1×10^1

Table 39 Microbiological quality of muffins after baking

Days of storage	Muffins A	Muffins B	Muffins C
0th Day	4.2×10^1	2.6×10^1	3.1×10^1
2nd Day	3.4×10^2	6.2×10^1	9.3×10^1
4th Day	3.9×10^3	2.8×10^2	7.6×10^2
6th Day	2.7×10^4 (Rejected)	3.6×10^3	8.1×10^3
8th Day	Nil	3.2×10^4 (Rejected)	$6.8 \text{ X } 10^4$ (Rejected)

5. DISCUSSION

Squalene, a triterpenoid hydrocarbon, is the precursor compound for biosynthesis of cholesterol and many other steroid hormones. It is found to have health promoting properties such as cardioprotective, antioxidant, chemopreventive, anticancerous, antilipidemic, membrane stabilizing properties, acidotic cell syndrome, strengthening body's immune system, detoxification of xenobiotics, lowering cholesterol content etc. The notable health benefits and wide applications has prompted extraction of squalene from various sources using different techniques such as molecular distillation, high-speed counter current chromatography, supercritical fluid extraction etc. (Vazquez *et al*., 2007). Even after the most efficient extraction process, the direct use of squalene will still remain limited because of its oxidative instability. Hence, a suitable strategy should be adopted to address its oxidative stability issues. Encapsulation can be an effective method to address this problem as the technology is being successfully applied to protect many biologically sensitive products such as fish oil, essential oil, vitamins, probiotics, flavor, aroma etc. However, very few studies have focused on the encapsulation of squalene till date (Abd Ghani et al., 2016; Jin *et al*., 2018). If squalene can be effectively encapsulated, it can have wider applications. Hence, the present study was carried out with an aim to develop a sustainable extraction protocol for isolation of squalene rich oil from liver of Bramble shark (*Echinorhinus brucus*), encapsulation of squalene, nutritional evaluation and assessing feasibility of encapsulated squalene as a functional food ingredient. Shark liver was chosen as the raw material owing to its richness in squalene than any raw material from the marine as well as plant sources.

5.1. Optimization of an extraction protocol for isolation of squalene rich shark liver oil using supercritical CO_2 as solvent

The potential of supercritical CO_2 in extracting squalene rich shark liver oil was studied by taking into account the effect of various extraction parameters. The effects of three process variables, pressure, temperature and time on extraction yield, squalene content, major fatty acids (EPA, DHA, AA) and fat-soluble vitamins were investigated by keeping the CO_2 and ethanol flow rate constant. For obtaining a suitable combination of these process variables, Response Surface Methodology (RSM) was employed. RSM plays an important role in designing the experiment and optimizing the process parameters in order to deliver large amount of information

from a small number of experimental runs (Joseph *et al*., 2017). Responses for the process variables were determined using Box Behnken design (BBD). BBD was used to optimize extraction conditions by building models between the response variable and independent factors. BBD is a spherical, revolving RSM design which comprises of three interlocking 22 factorial design with all the points lying on the surface of a sphere surrounding the center of the design (Maran *et al*., 2013). BBD uses quantitative data obtained from an appropriate experimental design to solve multivariate equations. Single and combined effects of the variables on the responses were studied by formulating experimental units. The effect of process parameters on each response is as follows.

5.1.1 Effect of process parameters on yield

The variations in yield was explained by second order response surface regression model ($p < 0.05$) with an R^2 value of 0.78. The yield of squalene rich shark liver oil varied from 42.68 to 79.36% when various extraction conditions were employed. The linear, quadratic and interaction effects had no significant effect on the yield of oil obtained. However, at a constant pressure, increase in temperature was found to have a negative impact on yield. In contrast to our results, Asep *et al.* (2016) reported an increase in yield with increase in extraction temperature in case of cocoa butter. In line with our results, Chen *et al* (2018) have reported similar effects of decreasing yield with increase in temperature in case of extraction of volatile compounds from Finnish wild mushrooms. In accordance with our study, Zeković *et al.* (2016) have also reported the negative effect of increasing temperature on yield of lipid extracts from coriander seeds. It has been reported that the effect of temperature is complex; when the temperature is increased, the density of CO_2 decreases and reduces its solvating power. Another possibility is that with the increase in temperature, the solute vapor pressure will increase which will in turn increase the solubility (Coelho *et al.*, 2015; Jesus *et al*., 2014). In the present context, with increase in temperature, density and solvating power of supercritical CO_2 might have decreased leading to lowering of yields.

5.1.2. Effect of extraction conditions on squalene content

The effect of extraction conditions on squalene content were analyzed to optimize the best extraction conditions. The yield of squalene has ranged from 634.23 to 910.84 mg/g of liver oil under different extraction conditions. From the quadratic fitted model the linear effects of process variables, temperature and time

were observed to have significant effect ($p < 0.05$) on squalene content, whereas pressure had no-significant effects. The linear effects of temperature (X_2) and time (X_3) were found to have a positive regression coefficient value of 77.35 and 77.18 respectively. The quadratic effects of pressure (X_1), temperature (X_2) and time (X_3) showed no significant effects. The interaction effects of all the process variables except X_2*X_3 was observed to have no significant effects. The negative regression coefficient of X_2*X_3 meant that squalene yield had a decreasing effect with temperature and time.

It was found that when the pressure was kept constant, an increase in temperature has favoured the extraction of squalene positively. It was clear from the experimental results that temperature and time have profound influence on the extraction efficiency and extraction rate. Temperature is reported to have two opposing effects. CO_2 density decreases with increasing temperature, thereby reducing the solvating power. With the increase in temperature, vapor pressure of solvents increases which in turn increases the extraction efficiency. Among these two competing factors, extraction efficiency will be affected by the factor which is dominant during the extraction process (Xie *et a*l., 2019). In case of squalene extraction, an increase in temperature might have increased the vapour pressure resulting in the thermal desorption of compounds from the sample matrix. Similar effect has already been reported by Sheibani and Ghaziaskar (2009). Furthermore, Machmudah *et al*. (2006) reported that increase in temperature might have damaged the cell walls resulting in the enhanced mass transfer rate. Bhattacharjee *et al.* (2012) reported that an increase in pressure and decrease in temperature increased the solubility of squalene. However, in contrast to our results, Xu *et al.* (2011) have reported that an increase in temperature has a negative effect on the squalene yield from bee pollen. Till date, very few studies have attempted the extraction of squalene rich oil from shark liver. A lower extraction yield of 38.5% has been reported by Vishnu *et al*. (2016) when conventional lipid extraction protocol was followed for squalene isolation from Bramble shark liver. This signifies the potential of supercritical CO_2 in enhancing the extraction yield of squalene from shark liver.

5.1.3. Fatty acid composition as affected by various conditions

The fatty acid composition of squalene rich shark liver oil obtained by different extraction conditions ranged from 24.93 to 243.99 mg/g of oil. The highest fatty acid content was observed when a moderate pressure (275 bar) was employed. In general, an increase in temperature was had a negative effect on the fatty acid extraction in case of high- and low-pressure conditions. Interestingly, it was observed that in the case of moderate pressure conditions (275 bar), an increase in temperature favored the fatty acid extraction. The same effect was observed in SFA, MUFA and PUFA extraction too. The fatty acid composition revealed that the most dominant saturated fatty acids were palmitic acid (C16) and stearic acid (C18). The most dominant monounsaturated fatty acids were oleic acid (C18:1) and palmitoleic acid (C16:1) and the most dominant PUFA were DHA (C22:6), EPA (C20:5), AA (C20:4). Saturated fatty acids (SFA) such as palmitic, and stearic acid are often employed as energy source, building blocks for structural elements, protein modification, and in regulation of gene transcription. García *et al.* (2004) have reported similar results stating that palmitic (16:0) and stearic (18:0) acids were the predominant saturated fatty acid in shark liver oil. Saify *et al.* (2003) have also reported similar results in the liver oil of two marine fishes, *Eusphyra blochii* and *Carcharhinus bleekeri*. Remme *et al.* (2006) have also reported similar results stating oleic acid as the most dominant MUFA. Richness of shark liver oil in PUFA such as EPA and DHA has already been reported by Vishnu *et al*. (2016).

5.1.3.1. Effect of extraction conditions on major fatty acids composition

The effect of extraction conditions on major fatty acid contents such as EPA, DHA, AA were analyzed. Among PUFA, the most dominant fatty acid was DHA with its value ranging from 4.43 to 56.46 mg/g of squalene rich shark liver oil. The lowest DHA content was obtained at an extraction condition of 150 bar pressure, 60°C temperature for 2h. Similarly, EPA content has varied from 1.11 to 13.58 mg/g of oil and AA content ranged from 1.13 to 11.52 mg/g of squalene rich shark liver oil. The highest DHA content was obtained at an extraction condition of 275 bar pressure, 50° C temperature for 2h. The lowest EPA and AA contents were obtained when 400 bar pressure, 60° C temperature was employed for 2h.

Omega-3 polyunsaturated fatty acids, especially eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6) are reported to play an important role in prevention of many human ailments and disorders such as

coronary heart diseases, inflammation, hypertension, arthritis, cancer etc. (von Schacky, 2003). DHA, in particular, plays a significant role in the functional development of the retina and brain, especially in premature infants (Connor, 2000). In this context, DHA from marine sources can be of great value as nutraceuticals. The results of the study showed that a moderate pressure operating at moderate temperature can maximize the extraction of major PUFAs. Shark liver oil as a rich source of PUFAs has already been reported by Remme *et al.* (2006). Bergé and Barnathan (2005) reported similar EPA values in marine liver oils. Achouri *et al.* (2018) have reported the high levels of EPA and DHA in liver oil of sharks from Tunisian coast however, the values were lower when compared to our results stating supercritical CO_2 is very efficient in extracting lipophilic bioactive compounds. The lesser contribution of EPA when compared to DHA in cartilaginous species has also been reported by Dunstan *et al.* (1988). In general, it can be concluded that Bramble shark liver oil can be recommended as a rich source of PUFAs such as EPA, DHA and AA.

5.1.4. Effect of extraction conditions on vitamin A and E

Vitamin A content ranged from 2.29 to 567.16 µg/g oil under various extraction conditions. The highest and the lowest vitamin A content were observed at extraction conditions of 150 bar pressure, 60°C, 6 h and 150 bar pressure, 40°C for 2 h respectively. It may be speculated that an increase in temperature along with the increased time factor contributed for increased vitamin A extraction.

Vitamin E content ranged from 14.26 to 243.85 µg/g oil under various extraction conditions. The highest vitamin E content was obtained with an extraction condition of 150 bar pressure, 50° C for 4h and the lowest obtained at 275 bar pressure, 45° C for 4h. At lower pressures, an increase in temperature favored vitamin E extraction whereas at moderate or higher pressures an increase in temperature decreased the extraction rate of vitamin E.

From the results of the study, it can be concluded that supercritical carbon dioxide can be considered as an effective solvent for extraction of squalene rich oil from shark liver. The method is environmentally friendly, requires less amount of solvents and less time consuming. Unlike the conventional methods, oil extracted is not only rich in squalene, but also rich in beneficial long chain PUFAs such as EPA, DHA, AA and most importantly vitamin A and E in good

concentrations. Hence, it can be concluded that squalene rich oil extracted from shark liver oil is highly nutritious and can be recommended as a dietary supplement.

5.2. Encapsulation of squalene

Squalene obtained even after the most efficient extraction process cannot be used as such for any applications owing to its high degree of unsaturation. Hence, it is very essential that structural integrity of squalene should be preserved by adopting an effective strategy. Encapsulation is one such promising technique to protect unsaturated fatty acids against oxidation and thereby increasing their shelf life (Carneiro *et al*., 2013). Apart from this, encapsulation helps in masking undesirable flavors, improving stability and bioavailability and in the sustained release of the core material (Liang *et al*., 2017; Wang *et al*., 2017). However, the success of encapsulation often depends on the wall materials used, core to wall material ratio etc. Hence, a preliminary study was carried out to establish the core to wall material ratio using chitosan as the wall material, before proceeding to the final encapsulation experiments.

5.2.1. Establishing the core to wall material ratio

A preliminary trial was carried out to establish the core to wall material ratio for squalene encapsulation using chitosan. It was observed that the emulsion became more unstable with the increase in squalene to chitosan ratio. For the preparation of a stable emulsion, an important pre-requisite is the adequate amount of wall material so as to protect the core material from any external factors and to preserve its integrity and stability (McClements *et al.,* 2007). When the squalene to chitosan ratio was decreased, there will be sufficient chitosan to cover the squalene completely. This might be the reason for higher emulsion stability in such treatments. Similar findings were reported by researchers in the case of microencapsulation of fish oil, flax seed oil, linoleic acid etc. (Tan *et al*., 2005; Tonon *et al*., 2011; Minemoto *et al*., 2002). Based on the results, it was concluded that emulsion prepared with a lower squalene to chitosan ratio (0.3:1) was kinetically more stable.

Microstructure of the emulsion revealed that at higher core to wall material ratios, droplet appeared to be more flocculated and the individual droplets were not visible. Chitosan could have possibly adsorbed to the oil-water interface to form a protective coating around the droplets, reducing the interfacial tension and

thereby preventing the oil droplets from aggregation (Whitehurst, 2008). There was a gradual decrease in particle size with decrease in core to wall material ratio and this can be related to the availability of emulsifier. At a higher core to wall material ratio, there might not be enough chitosan to exhibit the adequate emulsifying activity, hence more aggregation among the droplets resulting in larger sized particles and vice-versa in case of emulsions with a lower core to wall material ratio. Zeta potential values increased with decrease in the core to wall material ratio. Malhotra and Coupland (2004) have stated that the larger the zeta potential, the more will be the repulsion and greater separation distance between particles. This will eventually lead to reduced aggregation or flocculation because of lesser Vander Waals force of attraction. This indicates that a lower core to wall material ratio can produce emulsion of adequate stability by virtue of the higher zeta potential.

It was observed that when the core to wall material ratio was decreased, there was a considerable increase in the viscosity. Higher viscosity might have helped in reducing the rate at which particles sediment or cream or coalescence and thereby increasing the emulsion stability. Therefore, it was concluded that irrespective of the core to wall material ratios, the emulsions prepared with chitosan had a shear thinning behavior. Based on the significant findings of the study, a lower core to wall material (0.3:1) was optimized for further encapsulation studies.

Squalene emulsion was prepared at the optimized core to wall material ratio (0.3:1) and encapsulated using the technique of spray drying. However, the encapsulation efficiency of sample was found to be only 26 ± 0.577 %, which is quite low. The lower encapsulation efficiency indicated the presence of higher proportion of surface oil content in the encapsulated powder (0.1095g per gram of encapsulated powder). This can be due to the fact that 1% chitosan may not be sufficient to act as a wall material for squalene encapsulation. Our findings are supported by Laplante *et al.* (2005) and Klaypradit and Huang (2008) who have reported that chitosan alone cannot produce a stable emulsion. Similar results showing an encapsulation efficiency of 7.2–26.9% was reported by Esmaeili and Asgari (2015) when chitosan nanoparticles cross linked with TPP were used to encapsulate essential oil.

It was found that the peroxide value of the sample increased during the storage period and it crossed the level of acceptability after 3rd week of storage

(30 meq peroxide/Kg oil). Based on the oxidative stability studies, it can be concluded that chitosan alone might be insufficient to maintain the structural integrity of squalene. Another possibility is that, squalene might have come in contact with atmospheric air during the process of homogenization and this might have accelerated the oxidation rate in the initial stage itself. Apart from this, the higher temperature that prevailed during the time of spray drying might be another reason for the increased peroxide value. The increase in peroxide value can be even related to the encapsulation efficiency also. Similar findings were reported by Tonon *et al.* (2012) relating the increased surface oil, lower encapsulation efficiency and the oxidation of the final product.

Based on the outcome of the study, it was surmised that chitosan alone cannot function as better encapsulant for squalene and hence, a combination of wall materials such as chitosan-whey protein complex, chitosan-sodium alginate or any other protein-polysaccharides can be tried for better results. Apart from this, structurally modified chitosan with improved functional properties or squalene loaded chitosan nanoparticles may also work well for the encapsulation of squalene.

5.2.2. Effect of various biopolymers on squalene encapsulation

5.2.2.1. Emulsion stability analysis

Squalene based emulsions were prepared using various biopolymers and the highest emulsion stability was observed in gum Arabic based emulsion followed by MD-WPI. Santiago-Adame *et al.* (2015) have reported similar behaviour of lower emulsion stability in MD-WPI coated particles and stated the inclusion of maltodextrin as the reason.

Emulsion viscosity is an important parameter that affects the encapsulation efficiency and oil retention. Interestingly, gum Arabic based emulsion had the highest viscosity followed by MD-WPI based emulsion. This can be due to the thickening property because long and complex structure of gum Arabic attributes it a better thickening property and hence an increased viscosity (Tonon *et al.,* 2012; Carneiro *et al.*, 2013). It can be concluded that there is a positive correlation between the emulsion stability and viscosity. The higher viscosity of emulsions can help in the formation of protective layer around the core material and reduced the rate of destabilization mechanisms such as flocculation, thereby increasing the emulsion stability. Also, an increased viscosity poses greater resistance to the movement of droplets and inhibits further coalescence. The comparatively lower

viscosity in MD-WPI based emulsions can be attributed to the inclusion of high dextrose equivalent maltodextrin. It has been shown that maltodextrins of shorter chain length will have better interaction with oil and aids in reducing the viscosity (Bae and Lee, 2008; Dokic-Baucal *et al.,* 2004).

It was observed that emulsions stabilized with CS-WPI had a flow index value of 1, indicating that it had Newtonian behaviour which is common for non-aggregated emulsion droplets. Whereas the MD-WPI stabilized emulsion had n value <1, showing that they possess shear thinning (pseudoplastic) behaviour. However, in case of gum Arabic stabilized emulsions, n value was greater than 1, indicating that they possessed shear thickening (dialatant) behaviour. In accordance with our study, Campelo *et al.* (2017) have reported similar flow index for squalene encapsulated using MD-WPI; however, the consistency coefficient value, K was much lower.

5.2.2.2. Encapsulation efficiency

Physico-chemical properties of the squalene encapsulated using various wall materials were analysed. There was a significant difference in the encapsulation efficiency of squalene coated using different wall materials with MD-WPI coated squalene having the highest encapsulation efficiency (96.50 ± 0.06 %). It is speculated that the lowest surface oil content in MD-WPI coated squalene (4.17 ± 0.06 mg/g powder) can be one probable reason for the highest encapsulation efficiency. Whey proteins are considered as effective emulsifiers due to its ability to form a stable layer over oil droplets by its unfolding and adsorption on the oil–water interface. In addition to this, maltodextrins can also confer excellent protection to encapsulated lipophilic materials and their efficacy can be related to its dextrose equivalents. It has been already proven that MD with higher DE can act as an impermeable barrier to atmospheric gases and thereby resulting in higher encapsulation efficiency (Campelo *et al.,* 2017; Jafari *et al.,* 2008). In the present study, MD employed had a DE of 16-5-19.5 which can substantiate the highest encapsulation efficiency. Hence, it can be concluded that the highest encapsulation efficiency along with the lowest surface oil content could be due to the combined emulsification properties of MD and WPI. Botrel *et al.* (2014) and Turasan *et al.* (2015) have reported lowest surface oil content when MD and WPI was employed in the microencapsulation of fish oil and rosemary essential oil respectively. Akhtar

and Dickinson (2007) have already reported that MD-WPI based conjugates can work as effective emulsifying agent than gum Arabic.

5.2.2.3. Water activity and hygroscopicity

Water activity is an indication of the free water available for microbial activity and biochemical degradations. Water activity of the encapsulated samples ranged from 0.16 to 0.41 with lowest water activity noted in gum Arabic coated squalene. For dried products, water activity in the range from 0.1 to 0.4 has been reported to possess greater stability (Tontul and Topuz, 2014). Chew *et al.* (2018) has reported that low water activity helps in attaining better microbial stability, reduction in caking, oxidative stability etc.

Hygroscopicity is an indication of the ability of encapsulated powders to adsorb moisture. It has significant effect on the shelf life of powders as it affects the lipid oxidation and flowability. The wall material composition was found to have a profound effect on the water adsorption properties It was observed that MD-WPI based squalene had the highest hygroscopic value followed by gum Arabic based squalene. The highest hygroscopicity in MD-WPI based squalene can be due to the inclusion of maltodextrin of high dextrose equivalents. Higher the DE, higher will be the hydrophilicity and hygroscopicity. Similar observations have been reported by Tonon *et al.* (2009). The hygroscopic value can be correlated to the moisture content, the powders with higher moisture content were found to have the highest hygroscopic value. Similar observations were already reported by Premi and Sharma (2017). The higher hygroscopicity can be even related to the particle size. Smaller the particle, greater will be the rate of moisture adsorption.

5.2.2.4. Flowability and solubility properties

Bulk and tapped density were found highest in CS-WPI based squalene and lowest in MD-WPI based squalene. Carr index (CI) and Hausner ratio (HR) shows the flowability and cohesiveness of the encapsulated powder. Samples having CI and HR value in the range of 16-20 and 1.19-1.25 are considered to have fair flow. As per the specifications, it was found that CS-WPI based squalene had fair flow whereas MD- WPI and GA had poor and very poor flow properties as their values beyond the specified ranges. The fair flow properties of CS-WPI indicates less contact surface area between particles, reducing the cohesiveness among the particles. The poor and very flow properties of GA and MD-WPI can be due to their comparatively smaller particle size. This can be further correlated to the van der

Waals forces and electrostatic forces of attraction between the particles also. The poor flowability can be due to the presence of higher water content too. Tze *et al.* (2012) reported that with the increase in moisture content, interparticle distance will be decreased leading to better van der Waals forces attraction among them. This stronger attraction might have acted in reducing the flowability of powders.

Highest water absorption index and swelling power was observed in CS-WPI coated squalene and lowest in gum Arabic based squalene. Water absorption index (WAI) indicates the amount of water absorbed by particles upon submersion in water. The higher WAI can be attributed to the type of wall material used. Water might have penetrated through whey protein layer of the microcapsule and hence higher WAI values. WAI values of 1.82 ± 0.51 and 0.73 ± 0.05 was reported in case of flax seed oil and linolenic acid encapsulated with modified starch and whey protein isolate - maltodextrin respectively (Barroso *et al.,* 2014; Choi *et al.,* 2010). Highest water solubility was exhibited by MD-WPI coated squalene. The highest solubility can be attributed to the presence of sugar and whey protein in the wall material. Better solubility power of squalene microcapsules showed its higher dispersibility in water which will favor its use in drink products such as milk, juices etc. Choi *et al.* (2010) have reported similar results stating that inclusion of whey protein as a wall material for oil encapsulation can help in attaining better swelling power.

5.2.2.5. Oxidative stability of encapsulated powders

The oxidative stability of encapsulated powders is greatly affected by the nature of wall material, process of encapsulation and the wall to core material ratio. Among all the samples, MD-WPI coated squalene had the lowest peroxide value indicating its better oxidative stability. Better oxidative stability in MD-WPI coated squalene can be attributed to the lower surface oil content. It has been reported that the presence of surface oil (non-encapsulated oil) at the surface can reduce the shelf life of encapsulated powders as it is more susceptible to oxidation (Karaca *et al.,* 2013). Highest peroxide value was observed in CS-WPI coated squalene which had the highest surface oil content. This data reaffirms that minimising the surface oil content is crucial in the process of encapsulation as it can affect the encapsulation efficiency, oxidative stability and shelf life of the final product to a greater extent (Pegg and Shahidi, 2007). The combined properties of maltodextrin and whey protein might have also helped in retaining the oxidative

stability of the encapsulates. The presence of aminoacids such as histidine, glutamic acid, aspartic acid, and phosphorylated serine and threonine residues in whey protein might have helped in giving a better antioxidative and metal ion chelation properties (Singh, 2011). Furthermore, maltodextrins of high dextrose equivalent are reported to act as effective barriers to protect the encapsulated lipophilic bioactives (Campelo *et al.,* 2017; Jafari *et al.,* 2008).

5.2.2.6. External morphology of encapsulated squalene

Analysing the morphology of encapsulated particles (micro or nano) is an important pre-requisite in the encapsulation process as it reveals presence of cracks or dents on the particle surface (Botrel *et al.,* 2014). The morphology of encapsulated samples depends to a greater extent on the type of wall material used. Though there were some shrinkage noticed in squalene prepared using CS-WPI, the samples didn't have dents or fissures on their surface. The presence of shrinkage on the surface of encapsulate can be attributed to the type of wall material and drying conditions (Alvarenga Botrel *et al.,* 2012; Carvalho *et al.,* 2014). Since the spray drying condition employed was constant for all the samples, occurrence of shrinkage can be attributed to the type of wall material only. Kim and Morr (1996) have reported that the solidification of the wall material prior to onset of expansion can contribute to the shrinkage of encapsulate. Similar morphological characteristics of encapsulates were reported in case of linolenic acid encapsulated with whey protein and maltodextrin (Choi *et al.,* 2010) and oregano essential encapsulated with modified starch and gum Arabic (Hijo *et al.,* 2015).

The absence of dents and fissures is advantageous from the stability point of view as it will be having a lower permeability to gases and can very well protect the core material from oxidation due to environmental parameters such as oxygen and moisture ingress. Among the samples analysed, MD-WPI coated squalene was observed to be more agglomerated. The agglomerated nature of particles encapsulated with maltodextrins of varying dextrose equivalence has already been reported by Campelo *et al.* (2017). The variation in size was also another feature seen in the SEM analysis. This indicates the high polydisperse nature of the encapsulate produced. High polydispersity is one of the major characteristics of spray dried particles because in spray drying the atomizer tends to produce particles of non-uniform size.

5.2.2.7. FTIR analysis

FTIR is employed to study the structural characteristics of squalene encapsulated using different wall materials. Pure squalene exhibited characteristic bands in the wave number range of 2911- 3024 cm^{-1} due to CH_3 stretching (fig 10 a). From the FTIR spectra, it is clear that the characteristic band of squalene can be clearly distinguished from both the pure and squalene encapsulated using wall materials.

FTIR spectra of squalene encapsulated with CS-WPI is shown in fig 10 b. The most distinctive spectra of whey protein appear between 1650 and 1540 cm^{-1} due to C=O and C–N stretching from amide I and II bands respectively (Silverstein *et al.,* 2000). Furthermore, in proteins there is an amide III band at 1300–1200 cm^{-1} which arises due to C–N stretching and N–H deformation. Similarly, chitosan showed characteristic peaks at 3391 (-OH and NH_2 stretching), 2920 (CH stretching) and at 1647 (amide I), 1088 (C-O-C stretching) cm^{-1}. All the above characteristic peaks of whey protein, chitosan and squalene appear in the encapsulated spectra almost at the same wave number indicating no structural interactions between the core (squalene) and wall material (whey protein and chitosan).

FTIR spectra of gum Arabic coated squalene is shown in fig 10 c. Gum Arabic shows a characteristic band at 3331 cm^{-1} indicating the presence of amino groups (Shaddel *et al.,* 2018). The characteristic peak of gum Arabic and squalene was observed in the encapsulated spectra indicating the presence in the encapsulated powder preparation.

FITR spectra of squalene encapsulated with MD-WPI is shown in fig 10 d. Maltodextrin exhibits characteristic bands at 1015 and bands between 3000 – 3600 cm^{-1} due to the presence of C-O-C and O-H bonds respectively. Characteristic bands of whey protein appear between 1650,1540 and 1300–1200 cm^{-1} due to amide I, II and III bands. Almost the same characteristic bands of core and the wall material were exhibited by the encapsulated squalene confirming their presence in the final material. Moreover, the presence of characteristic peaks of individual materials in the encapsulated spectra almost at the same wave number indicating no structural interactions between the core (squalene) and wall material.

5.2.2.8. Thermogravimetric analysis

TGA is used to study the thermal stability of encapsulated materials as a function of temperature. The thermal stability analysis is used to evaluate whether the materials can withstand higher heat treatment in processes such as pasteurization, cooking, drying etc. TGA of pure squalene showed a sharp weight loss at around 350°C. Thermograms of CS-WPI coated squalene exhibited an extended thermal stability up to 422.02 °C with weight loss occurred at three stages. An initial weight loss of 3.33 – 5.67 % was observed at a temperature range of 40.69 to 161.99 °C, followed by 29.67% weight loss at 266.68 °C and a final major weight loss of 51.69 % at 422.45 °C. Thermal stability analysis of GA coated squalene showed that it can confer thermal stability up to 451°C with three step weight loss. The initial weight loss of 2.43 to 4.98% was recorded at the temperature range of 41.71 to 206.9 °C, 36.51% loss at 307.64 °C and a major weight loss of around 72.2% at 451.98 °C. TGA of MD-WPI coated squalene showed that it can confer thermal stability up to 372.58C with three major weight loss peaks. An initial weight loss of 17.49% was noted when the temperature was increased from 39.65 to 244.15C. The second major weight loss (29.5%) was observed at temperature of 298.81° C and a final weight loss of 42% at temperature of 372.64 °C.

In general, the initial weight loss observed in all the samples can be due to evaporation of moisture from the samples (Liu *et al.,* 2010). Since the moisture content of the encapsulated powders are low, the percentage of weight loss are also less. The second weight loss can be attributed to the loss of non-encapsulated squalene deposited on the surface of microparticles. Fournier *et al.* (2006) have reported similar results stating the weight loss happened at temperature range of 100 – 300 °C can be due to the surface bound oil. Since the boiling point of squalene is 285 °C, the possibility of degradation of surface bound squalene can be reaffirmed. The final weight loss occurred at a temperature range of 300-450°C might be due to the complete degradation of wall material along with the release of encapsulated squalene. Similar results have been reported by Chatterjee and Judeh (2016) in the encapsulation of fish oil with chitosan derivative. The weight loss occurred at temperature range of 200 and 400 °C could be attributed to the reactions of wall materials, especially proteins and carbohydrates leading to decomposition, and polymer depolymerization (Fritzen-Freire *et al.,* 2012; Hosseini *et al.,* 2013). Among the three samples, the lowest weight loss was recorded in MD-WPI coated

squalene suggesting its thermal stability. It also exhibited a weight loss rate of 0.251 mg/min at 372.58 °C, which is quite low when compared to the other samples. de Barros Fernandes *et al.* (2017) have reported similar results stating the enhanced thermal stability conferred to ginger oil by MD-WPI.

5.2.2.9. X-Ray Diffraction analysis (XRD)

XRD is often employed to study the structural crystallinity of materials. In case of encapsulated powders, the analysis of structural stability by XRD is very important (Silva *et al.,* 2016). X-Ray diffractograms of squalene encapsulated using different wall materials suggests that they possess broad and diffuse peaks. As a general rule, broad peaks are characteristics of amorphous material structure as they are in highly disordered configuration resulting in the broadness of the X-ray diffraction band (Caparino *et al.,* 2012). Amorphous materials are advantageous when compared to crystalline materials as they exhibit higher solubility and hygroscopicity (Silva and Meireles, 2015). It has been already reported that the spray drying process always favour the formation of amorphous materials (Botrel *et al.,* 2014). Though the XRD patterns of all the materials had a broad and diffuse pattern, the peak shape of MD-WPI coated squalene was different. Similar diffraction patterns were reported by Matsuura *et al.* (2015) when maltodextrins of higher DE were used in the encapsulation of coconut oil. The presence of noises observed in the X-ray diffractograms of all encapsulated material indicates that the spray drying mediated encapsulation might have induced changes in the crystallinity of wall materials protein leading to their amorphous state. Similar observations have been reported by da Silva Bastos *et al.* (2012) in case of cashew apple juice.

5.2.2.10. In-vitro digestion behaviour of encapsulated squalene

In-vitro release of squalene from different encapsulated formulations were studied by employing a suitable in-vitro digestion model. It is important to evaluate the release behaviour of encapsulated squalene to establish its potential as a food supplement and its targeted delivery. MD-WPI coated squalene had the maximum release followed by gum Arabic. The maximum release of squalene indicates that the use of MD-WPI as wall material helped in increasing the bioavailability of squalene by providing a slow and sustained release. Flores *et al.* (2014) have reported similar results stating the use of whey protein-based microcapsules in providing a sustained release. Release of oil from encapsulated formulations (micro or nano) can be due to any of the following mechanisms: surface

erosion, disintegration, diffusion and desorption (Hariharan *et al.,* 2006). In vitro release profile of squalene from encapsulated preparations exhibited a two-step biphasic pattern, an initial burst release followed by subsequent slow and sustained release. The gradual and sustained release of squalene was observed in all the samples irrespective of the wall materials. The initial burst release can be due to the squalene deposited at the surface as well as near to the surface (Anitha *et al.,* 2011). It was found that the irrespective of the wall materials used, the maximum release occurred at the intestinal phase of digestion. Chatterjea and Shinde (2012) reported that the digestion and absorption of lipids mainly happens in the small intestine. Through encapsulation, oil release is controlled to prevent any degradation before it enters into the small intestine. A significant finding of the study is that encapsulation of squalene has succeeded in achieving its sustained and controlled release to the targeted site, the small intestine.

The particle size of digested emulsions has changed significantly at various stages of digestion. During the gastric phase of digestion, particle size has decreased in CS-WPI and GA coated squalene whereas MD-WPI shown an increase in particle size. However, in the intestinal digestion, the particle size of all the emulsions shown an increasing trend. The change in particle size when the emulsions moved from gastric to intestinal digestion can be due to the action of specific enzymes along with the incorporation of digested products. Pancreatin is a combination of digestive enzymes such as trypsin, amylase and lipase which hydrolyse proteins, starch and fats respectively. Hydrolytic action of pancreatin on the emulsifiers such as whey protein, chitosan, gum Arabic and maltodextrin might be the reason for an increased particle size in the first hour of intestinal digestion. Further, bile salts might have displaced the emulsifiers that were initially adsorbed on the lipid droplets. During the process of displacements, voids might appear on the oil droplets which have the tendency to combine with another void area through the mechanism of coalescence resulting in larger particles (Cheong *et al.,* 2016). Similar behaviour has been reported by Hur *et al.* (2009) who observed an increase in particle size when the particles moved from simulated stomach to small intestine. However, in contrast to our results, they have reported that particle size of all the emulsions remained almost similar from mouth to stomach. The PDI of the digested emulsions were also found to vary significantly with the digestion process. PDI of GA and MD-WPI coated squalene were found to increase significantly with the

digestion whereas in CS-WPI, the PDI has decreased after the first hour of intestinal digestion. PDI values of all the digested emulsions were above 0.3, indicating they all had a broader size distribution. The inconsistency in PDI showed that some had smaller sized and some possess larger sized droplets. This was evident from the microstructure of emulsions too. Cheong *et al.* (2016) have reported similar results of increasing PDI with increasing digestion time in case of kenaf seed oil-based nano emulsions.

Studying the electrical characteristics of digested emulsions is important as they can provide relevant information regarding their interfacial composition. It has been reported that the electrical characteristics of emulsions can be affected by a number of factors such as pH, ionic strength, competitive displacement of the emulsifiers and adsorption of charged components from the simulated fluids etc. (Hur *et al.,* 2009). When the electrical charge of the digested emulsions was studied, it was found that the zeta potential values of emulsions stabilized using various wall materials has varied significantly. In general, the zeta potential values have shown an increasing trend with the various stages of digestion, with MD-WPI coated squalene possessing the highest value followed by gum Arabic coated squalene. Gum Arabic and MD-WPI have shown negative charges during the entire phase of digestion whereas CS-WPI had shown positive value up to gastric digestion stage (2h). Following the gastric digestion its positive charge has changed to negative with an increase in magnitude. In general, the negative charge of emulsions might be due to the preferential adsorption of OH^- ions from the water by the oil droplets (McClements, 2015). The positive charge in CS-WPI can be due to the fact that less displacement of whey proteins might have happened up to gastric digestion phase. The globular proteins in WPI is reported to form a covalent crosslinked interfacial layer which is very difficult to displace unlike the other wall materials. Furthermore, they are also reported to resistant against the action of acid and enzymes (Chen and Subirade, 2005). The combined effect of crosslinked layer formed along with its resistance to acid may be the reason for positive charge. However, at a later stage it might have been displaced effectively by the cation of bile salts and phospholipids present in the digestive fluids. The adsorption of mucin and competitive displacement of emulsifiers and adsorption of substances present in the digested fluids can be the probable reason for negative charge in gum Arabic and MD-WPI coated squalene.

In conclusion, significant findings of the study suggest that maltodextrin-whey protein is an effective wall material for the encapsulation of squalene by providing it a higher encapsulation efficiency (96.5 %), higher oxidative stability, solubility, flowability, thermal stability, better morphology and other physico-chemical properties etc. The *in-vitro* release study further emphasizes that MD-WPI can help in improving the bioavailability of squalene by providing a slow and sustained release. Interestingly MD-WPI performed better than gum Arabic as a wall material indicating that the former can be used to replace gum Arabic as an efficient wall material for thermally labile lipophilic compounds. Further, the cost of maltodextrin and whey protein isolate is very less making it an economically feasible wall material.

5.3. Nutritional evaluation of encapsulated squalene

Squalene encapsulated using MD-WPI was found to have a high encapsulation efficiency of 96.50 ± 0.06 % with an increased shelf life at refrigerated conditions. Its water solubility was observed to be 87.41 ± 1.52 which was comparatively higher when compared with other wall materials. The results showed that MD-WPI coated squalene can be very well utilised as a functional food ingredient. However, the nutritional and metabolic influence of encapsulated squalene have to be evaluated based on an animal model study to establish their safety and mode of action. In the present investigation, effect of dietary supplementation of squalene encapsulated using MD-WPI on growth performance, lipid profile and antioxidant enzymes was investigated in wistar strain albino rats.

5.3.1. Effect of encapsulated squalene on weight gain and growth performance

When the weight gain % and specific growth rate (SGR) of the control and treatment groups were analysed, the groups fed with squalene encapsulated using MD-WPI showed improved weight gain % and SGR. Specific growth rate also showed an increasing trend with squalene supplementation. The weight gain % and SGR were found be dosage dependent. The highest weight gain % and SGR was observed in rats fed with the highest dose of encapsulated squalene (450 mg/kg wt). The increased weight gain indicates that the main constituents present in encapsulated particles such as squalene along with the wall materials, maltodextrin and whey protein isolate might have some potential health promoting properties.

Motawi *et al.* (2010) reported similar results stating an increased weight gain with squalene supplementation. Liu *et al.* (2018) have also reported a significant weight gain when squalene was fed at the rate of 2%. In contrast to our results Gabás-Rivera *et al.* (2014) have reported no significant weight gain with squalene supplementation suggesting that long term supplementation would be well tolerated within the bodily changes. Liu *et al.* (2009) have reported weight gain in the control groups whereas squalene fed groups exhibited weight loss. The results of the present study suggest the efficacy of encapsulated squalene as a health promoting functional food ingredient.

5.3.2. Influence of squalene supplementation on tissue weight and protein content

The tissue weights of kidney, brain has shown a decreasing trend with the increase in squalene dosage; however, the decrease was not-significant statistically. However, there was a non-significant increase in liver weight. In contrast to our results, Liu *et al.* (2018) has reported that there was no significant influence of squalene supplementation in the tissue weight of major issues except in case of liver. Kumar *et al*. (2016) have also reported similar results stating a significant increase in liver weight upon squalene supplementation. A significant difference in protein content of tissues were also observed with liver having the highest protein content followed by kidney and the lowest was noticed in brain and heart.

5.3.3. Squalene supplementation effect on lipid profile

Serum lipid profiling was found to have a significant difference among control and treatment groups. With the increase in dosage, serum cholesterol was found to decrease from 94 mg/dL to 58 mg/dL. Squalene, an isoprenoid compound, is a biochemical precursor for cholesterol and many other steroid compounds. Hence, it is expected that an oral administration of squalene may increase the serum levels of cholesterol. However, in the present study, it was noticed that there was an initial increase in serum cholesterol level when compared to control and has decreased with the increase in squalene dosage. This can be attributed to the effect of 3-hydroxy-3-methylglutaryl coenzyme A reductase which is a key enzyme involved in the biosynthesis of cholesterol. It has already been studied and established that the enzyme will be feedback inhibited in the presence of exogenous squalene thereby downregulating the conversion from acetyl CoA to cholesterol

(Newmark, 1999; Sawada *et al.,* 2001). Chan *et al.* (1996) have reported that a daily squalene supplementation at the rate of 0.86 g reduced the serum cholesterol levels. In contrast to our results, Strandberg *et al.* (1989) reported that a dietary supplementation of squalene (900 mg/day) did not make any significant increase in serum triglyceride or cholesterol levels. This can be due to the fact that the cholesterol synthesized from squalene might have been excreted in the form of cholesterol itself or as bile acids.

Similar to cholesterol, the triglycerides and LDL and VLDL have also shown a similar trend with the squalene supplementation of increasing dosage reducing their levels. The triglyceride level has increased from 52 mg/dl in control to 84 mg/dl when squalene was fed at lower doses (150 mg/kg). However, with the increase in squalene dosage, triglyceride level was shown to decrease the triglyceride levels. The LDL and VLDL also decreased significantly with increased dosage of squalene supplementation. Liu *et al.* (2009) reported that squalene supplementation reduced the serum cholesterol and triglyceride levels. Gabás-Rivera *et al.* (2014) reported similar results stating squalene supplementation has decreased triglyceride levels. Khor and Chieng (1997) have reported similar results of lower LDL and VLDL level in squalene fed groups when compared to that of control.

HDL has shown an increasing trend with the increase in squalene dosage. Previous studies have suggested an inverse correlation between HDL cholesterol levels and coronary heart diseases (Assmann *et al.,* 1996; Sharrett *et al.,* 2001). Farrer (2018) has reported that 1mg/dl increase in HDL level can reduce the risk of CVD, LDL cholesterol and triglyceride levels by 2-3%. Moreover, a higher level of HDL can have anti-inflammatory, anti-thrombotic, anti-atherosclerotic and antioxidant properties (Estrada-Luna *et al.,* 2018). The lipid profiling suggests that squalene supplementation can have significant cardioprotective properties through its mode of action. Farvin *et al*. (2004) have already reported that squalene supplementation daily has significant cardioprotective effect. Overall, the results indicated that the cholesterol, LDL and VLDL lowering effect is highly dose dependent.

5.3.4. Effect of squalene supplementation on catalase and superoxide dismutase activity

The effect of squalene supplementation on the major antioxidant enzymes, catalase and SOD were investigated. Catalase activity of liver and brain exhibited a significant change, whereas a non-significant change in the kidney and heart activity was observed between the control and experimental groups. Among all the tissues, kidney exhibited the highest catalase activity followed by liver whereas the lowest activity was noticed in brain. The catalase activity was found to decrease with the increase in squalene supplementation. Effect of squalene supplementation on SOD activity showed that there was a significant difference in the activity of liver, kidney, heart. The highest SOD activity was exhibited by liver tissues followed by kidney and the lowest activity in heart and brain. Similar to catalase, SOD activity was also found to decrease with increase in squalene supplementation.

Catalase, a common antioxidant enzyme, is highly efficient and can breakdown millions of hydrogen peroxide molecules in one second. Superoxide dismutase is another important endogenous antioxidant enzyme that acts against reactive oxygen species (ROS). Being the first line antioxidant defense enzyme, both of these are expressed mostly in stressful conditions and helps to protect the organism from cellular damage. A lower expression of catalase and SOD enzymes in squalene fed groups suggested that squalene alone was sufficient to act against the ROS. The scavenging activity of squalene can be attributed its isoprenoid structure. Moreover, the free radical scavenging activity of squalene might have helped in maintaining the cell viability and keeping the enzyme activity at its lowest level. In contradiction of our results, Kumar *et al.* (2016) have reported an increased level of antioxidant enzymes such as catalase and SOD upon squalene supplementation. Tejpal *et al.* (2017) have reported lower activities of catalase and SOD in case of rats fed with encapsulated vitamins. In general, it can be concluded that dietary supplementation of squalene itself is enough to take care of radical scavenging effects and thereby restoring the activities of antioxidant enzymes such as catalase and SOD.

5.3.5. Effect of squalene supplementation on AST and ALT

AST and ALT activities are mostly employed as biological markers for hepatic injury. There was a significant change in the AST activity of different tissues

with squalene supplementation. With the increase in dosage, AST activity was found to decrease significantly with brain having the highest value. However, the ALT activity was found to increase with increase in squalene dose except in the brain tissue. Generally amino acids are deaminated and the intermediates produced in TCA cycle are used as substrates for gluconeogenesis. AST and ALT help to reuse the amino nitrogen for the synthesis of new amino acids. Farvin *et al.* (2004) have observed higher levels of AST and ALT when the effect of squalene supplementation on isoproterenol-induced myocardial infarction was analysed. This indicates that AST and ALT enzymes are highly expressed in case of cellular damages or injuries. Hence, the lowered expression of AST is justified as the treatment groups have not been exposed to any stressful condition.

5.3.6. Mineral status in liver tissue

The effect of squalene supplementation on the overall mineral status in liver has been analysed to study the occurrence of any significant variations. Major elements such as sodium, potassium, calcium, phosphorous, magnesium was found to decrease considerably with increase in squalene concentration. When compared to control, their levels were considerably lower. It was observed that iron and zinc content were also found to decrease with increase in squalene supplementation. Interestingly, it was observed that the iron content in rats fed with highest squalene dose (450mg) were 50% lower than that of the control. It has been already reported that dietary fats play an important role in the absorption and utilization of iron (Lukaski *et al.,* 2001). However, the effect depends upon the type and amount dietary fat. In our study, it was observed when lowest dose of squalene was fed, the iron content has reduced, but that difference was not drastic. However, with the increase in dosage, iron content seems to be reduced with a pronounced effect at higher dose. It can be concluded that squalene supplementation at higher doses is having significant effect on iron absorption. In accordance with our results, Lukaski *et al.* (2001) have reported that diets rich in polyunsaturated fatty acids can impair the absorption and utilization of iron and zinc etc. Similarly, Shotton and Droke (2004) has reported that flaxseed oil and olive oil can alter tissue mineral status and affect iron utilization.

Based on the findings of animal study, it can be concluded that squalene supplementation has a lowering effect on the cholesterol, triglycerides and LDL and VLDL level and its effects are entirely dosage dependent. Similarly,

squalene supplementation also helps in restoring the level of antioxidant enzymes emphasizing their antioxidant potential. However, the effects of squalene supplementation on mineral profiling have to be studied further in detail to elucidate their mode of action.

5.4. Feasibility of encapsulated squalene as a functional food ingredient

5.4.1. Nutritional characteristics of muffins

The physiological benefits of squalene based on animal model have been already which suggests that consumption of squalene in adequate amounts can improve the overall wellbeing of humans. Hence, the feasibility of squalene as a functional food ingredient was studied by its incorporation in a bakery product, muffins. Muffins are sweet, baked products of high calorific value, with great consumer acceptance owing to their superior texture and textural attributes. Muffins batter is an oil-in-water emulsion with egg-oil-sugar-water mixture as continuous phase (Matos *et al.,* 2014). The incorporation of encapsulated squalene and its effects on the physico-chemical properties has been studied in detail.

There was a significant difference in the moisture content of the treatments with the lowest content in control and highest in encapsulated squalene enriched muffins. The high moisture content can be due to the inclusion effect of squalene encapsulated in MD-WPI. MD-WPI might have acted as an emulsifier and retained more moisture in the emulsified form. Nelson (2000) reported that the addition of bulking agents such as maltodextrin can have a significant effect on moisture retention. Fat and ash content were also found to be high in muffins with encapsulated squalene whereas the highest protein content was observed in muffins with pure squalene. Similar to our results, Umesha *et al.* (2015) have reported an increase in protein and ash content in biscuits enriched with microencapsulated garden cress oil than control. Calorific values of the treatments has ranged from 480.78 ± 0.10 to 501.61 ± 0.38 Kcal with the highest value noted for muffins. The results showed that muffins prepared can be categorised under high energy foods.

The loss of moisture during baking has implications on the weight loss and this is a less favoured aspect commercially. Baking loss of the various treatments ranged from 8.22 ± 4.43 to 15.25 ± 5.59 % with highest loss in control

samples. The baking loss can be related to the matrix and water interactions. The lower baking loss in muffins with encapsulated squalene can be attributed to the presence of maltodextrin which might have a positive impact upon water retention. Similar results have been reported by Ureta *et al.* (2014) and Martínez-Cervera (2012).

Baking is a complex process which converts dough into baked products such as cakes, muffins, breads etc. by virtue of the mass and heat transfer phenomenon. During baking, an evaporation front starts moving from the surface towards core resulting in formation of two different zones, crust and crumb. Crust is the outer, brown, dehydrated region, mainly formed through caramelization during baking whereas, crumb is the inner, humid, spongy texture region. Crust and crumb of muffins enriched with encapsulated squalene had the highest moisture content. In general, the crust fractions had the lowest moisture content than crumb. Lower moisture content of the crust can be due to the dehydration that occurred at the surface layer during the baking process. After the baking process, the dried crust layer of muffins acts as an insulator after baking and prevents further moisture loss from the inner zones. This may have some impact in reducing the crumb staling. Behaviour of crust layer as a weight loss barrier has already been studied and reported (Wahlby and Skjoldebrand, 2002). Primo-Martin *et al.* (2006) have reported an increased moisture loss in case of crustless breads. Similar behaviour of baked products has already been reported in case of certain other baked products (Ureta *et al.,* 2014; Purlis and Salvadori, 2009). Water activity was found to vary significantly among the treatments with control muffins having the lowest value. The highest water activity can be correlated to the inclusion of maltodextrin.

When the fatty acid composition of muffins was analysed, it was observed that the highest fatty acid content was recorded in muffins with encapsulated squalene and lowest in control. In general, irrespective of the treatments, the most dominant fatty acids were stearic (C18), mystric acid (C14), oleic (C18:1) and linoleic acid (C18:2). Our results fall in accordance with the findings of Pustjens *et al.,* 2017 who have reported myristic, stearic acid etc. as the dominant fatty acids in butter.

5.4.2. Kinetics of color development in muffins

Color kinetics of baked products, especially reflected in the crust and crumb regions, were studied with the help of L^*, a^* and b^* colour system. This is the

most commonly employed method in color determination because of its closeness to human reception (Pedreschi *et al.,* 2006, León *et al.,* 2006). The L^* value which represents lightness ranges from 0 to 100 while a^* and b^* values represents greenness to redness and blueness to yellowness has values in the range of -120 to 120, respectively (Papadakis *et al.,* 2000).

5.4.2.1. Crust color kinetics

Color development in muffins is influenced by various parameters such as individual ingredients or the result of interaction between the ingredients through processes like maillard, chemical or enzymatic reactions, physico-chemical characteristics, baking etc. (Gularte *et al.,* 2012; Acosta *et al.,* 2011). Color parameters, L^*, a^* and b^* of crust regions has decreased significantly throughout the storage period with encapsulated squalene enriched muffins with the lowest values. In general, the crust of all the treatments had a brown color. Maillard reactions and caramelization are the two important phenomenon that can cause browning of baked products. For maillard reactions to occur, pH should be around 4-7, temperature >50 °C and an intermediate moisture content whereas caramelization requires pH<3 or >9 and temperature >120 °C. In the present product, color development in muffins may be attributed to maillard reaction since the pH of the product was around 7 and temperature above 120 °C (150 °C). The presence of whey protein and maltodextrin in the treatments as wall materials might have accelerated the browning in muffins and hence a lower value. Similar results of Maillard reaction contributed by the reaction between free amino groups of the proteins and the reducing sugars has already been reported by Martins *et al.* (2000). Takeungwongtrakul *et al.* (2015) reported similar results in cakes fortified with microencapsulated shrimp oil and suggested the presence of sugar and proteins in the wall material as the possible reason for browning in the crust regions.

Browning Index (BI) is an useful indicator to measure the extent of color change happened due to caramelization and Maillard reactions (Isleroglu *et al.,* 2012; Ureta *et al.,* 2014). This is presently employed to study the browning variation in many bakery products (Ureta *et al.,* 2014; Yang *et al.,* 2014). BI was found to increase during the storage period with encapsulated squalene having the highest brown intensity. Similar results of increasing browning reactions in the crust of muffins have been reported by Marco and Rosell (2008). They have attributed the browning reaction due to the presence of maltose. A comparatively browning

intensity value has been reported by Shaabani *et al.* (2018) in the crust of chickpea protein isolate enriched muffins. Chroma (C) is the attribute of color which indicates the degree of departure of the color from grey of the same lightness. Chroma values of all the treatments was found to decrease throughout the storage period.

5.4.2.2. Crumb color kinetics

The crumb color of different treatments were analysed to study the color kinetic variation with respect to that of crust region. It was observed that L^*, a^* and b^* values were higher in crumb region than that of the crust portions. However, the lightness value was found to decrease with the increase in storage days. Similarly, the redness and blueness also followed a decreasing trend with the storage period. The highest lightness value was observed in muffins with pure squalene and lowest in muffins with encapsulated squalene. Marco and Rosell (2008) has reported similar results stating the increase in lightness value of crumb regions when compared to the outer crust region. Apart from the lightness value, browning intensity of the crumb region was found to differ when compared to that of crust. At the 0^{th} day, a^* and b^* values were higher in muffins enriched with encapsulated squalene. The browning intensity of muffin crust (irrespective of the treatments) on 0^{th} day ranged from 112.51 to 143.53 whereas the crumb region had exhibited a BI value of 53.03 to 62.69. This shows that browning reaction has happened mainly at the surface and has not affected the inside color. In accordance with our results, Wählby and Skjöldebrand (2002) reported browning was mainly a surface phenomenon and hence the crumb region remained unaffected compared to that of crust.

5.4.3. Textural quality of muffins

The effect of different treatments on the major textural parameters such as hardness, adhesiveness, cohesiveness, springiness, gumminess, chewiness etc. was analysed. Hardness of control, pure and encapsulated squalene enriched muffins has ranged from11.57 ± 1.77 to 17.98 ± 4.77 N, 11.16 ± 1.36 to12.79 ± 1.58 N and 7.39 ± 0.02 to 5.69 ± 0.83 respectively upon storage. From the TPA analysis, it was clear that the inclusion of encapsulated squalene gave a comparatively softer texture as it had the lowest hardness value and it has shown a decreasing trend with the increase in storage time. However, other textural parameters of muffins with encapsulated squalene such as cohesiveness, springiness, springiness index, gumminess, chewiness and adhesiveness

decreased throughout the storage period. The lower hardness in muffins with encapsulated squalene can also be related to its increased moisture content. The plasticizing effect of water and its importance in shelf life and sensory qualities has already been reported (Furlán *et al.,* 2015). Moisture content of the crust and crumb were also highest in muffins with encapsulated squalene. Water helps to make foods softer by reducing the intermolecular attraction.

Though there was a decreasing trend, among different treatments, cohesiveness, springiness, springiness index and chewiness were highest in muffins with encapsulated squalene followed by muffins with pure squalene. This shows that inclusion of squalene in its pure or encapsulated form had significant effect on the textural parameters. Cohesiveness shows the ability of a material to stick to itself and the internal resistance of food structure. The lower moisture loss can be one probable reason for the increased cohesiveness. Al-Muhtaseb *et al.* (2013) have also reported that higher the moisture content, higher the cohesiveness. Tess *et al.* (2015) have reported that the highly cohesive products would retain higher amount of gas and hence a higher volume.

Springiness shows how food reacts to the recovery between first and second compression. This is also an indicator of the strength of food network and its quality. Sanz *et al.* (2009) have reported that the springiness value is greatly associated with fresh, elastic and aerated products of high specific volume. The higher springiness noted for muffins with encapsulated squalene hence reflects its superior quality and elastic nature. On the other hand, the decrease in springiness in other treatments may be attributed to the lower number of air bubbles and a denser matrix (Sanz *et al.,* 2009). To conclude, the higher cohesiveness and springiness of muffins with encapsulated squalene indicates that it possesses higher specific volume and better aerated structure. This can be attributed to the presence of whey protein as wall material which might have helped in creating a stronger network. Similarly, the lower springiness and cohesiveness of other treatments indicated their non-homogenous distribution.

Chewiness is an important parameter which indicates the ease/difficulty in chewing the food and indicates the energy required in swallowing food. The lower chewiness value suggests the ease of chewing muffins with encapsulated squalene proving its superior textural quality. Gumminess is defined as the force required to disintegrate a food before swallowing. When the gumminess

parameter was analysed, muffins with pure squalene had the highest value, whereas muffins with encapsulated squalene had the lowest value. Stiffness was found lowest in muffins with encapsulated squalene. The lowest stiffness can also be related to the higher moisture content and similar results has been reported by Furlán *et al.* (2015). In general, muffins prepared with encapsulated squalene were cohesive, springier and chewy with less gumminess and stiffness than the other treatments indicating their efficacy in improving the textural quality.

5.4.4. External morphology of muffins

The SEM images of various treatments had significant observable variations. Control muffins exhibited many cracks and discontinuous networks with loose distribution nature. It shows that control muffins had a non-homogenous distribution whereas muffins with pure and encapsulated squalene had a denser network. The presence of whey proteins as wall materials in the encapsulated formulation might have helped in forming crosslinks in dough to form a denser network. The results show that squalene inclusion in its pure or encapsulated form had a significant effect in forming a strong and denser network. Several small or medium sized pores were visible in muffins with squalene (pure of encapsulated), suggesting that squalene addition had a significant effect. The formation of a denser network is important from the view point of muffins textural quality. From the textural study, it was clear that the control muffins had a harder texture. This can be due to the poor gas holding capacity of the dough owing to its loose and discontinuous network structure. Similar results have been reported by Cao *et al*. (2019) who showed that baked products (dough) may not be able to retain the gas in case of a lack and continuous structure and thereby leading to a harder texture. Apart from this, the micrographs showed the presence of lager number of pores in muffins with encapsulated squalene, confirming that they had a much porous and aerated structure. The results showed that muffins with encapsulated squalene were more organized structurally with larger number of gas cells contributing to superior textural attributes.

5.4.5. Oxidative stability of muffins

Peroxide value of the muffins stored at room temperature were analyzed to study their oxidative stability. Peroxide values showed an increasing trend throughout the storage period, however, values of all the samples were within the acceptable range (20 meq peroxide/kg oil) as proposed by CODEX/FAO

standards. The highest peroxide value was observed in control and lowest in encapsulated squalene enriched muffins. The absence of any preservatives or antioxidant compounds along with the storage at room temperature might be one reason for oxidation in control muffins. Only compositional difference between the control and the other samples was squalene, either in its pure or encapsulated form. Squalene might have exerted an antioxidant effect and helped in lowering the oxidation rates. In case of muffins with encapsulated squalene, the wall materials used might have also played an important role in reducing the oxidation rates. It has been reported that the inclusion of maltodextrin of higher dextrose equivalence has an important in extending the shelf life by slowing the starch retrogradation. Similar observations have been made by Witczak *et al.* (2010). Furthermore, the higher baking temperature employed in the baking process might have caused the maillard reaction. The maillard reaction products has proven to have antioxidant capability (Einhorn-Stoll *et al.,* 2005). Though maillard reaction might have happened in all the treatments, results of browning intensity show that the higher maillard reaction has happened in encapsulated squalene enriched muffins. This might be the reason for better oxidative stability in such treatments.

5.4.6. Microbiological quality of muffins

Microbiological quality of the batter and the baked muffins stored at room temperature were studied to establish their shelf life. Initial total plate counts ranged from 8.2×10^2 cfu/g, 4.8×10^2 cfu/g and 1.3×10^3 cfu/g respectively for A, B and C batters. Fungal growth was noticed in batter A and C whereas muffins with pure squalene had no fungal growth. However, fungal growth was not observed in the baked muffins signifying the potential of baking process in inhibiting their growth. TPC also had decreased after the baking process. However, upon storage it has exhibited an increasing trend. As per the TPC, the control samples were rejected on 6^{th} day whereas muffins with pure and encapsulated squalene were rejected on the 8^{th} day. Among the normal mesophilic biota, *Bacillus* and *Staphylococcus* spp. were found to be predominant, whereas in pigmented mesophilic biota, *Micrococcu*s spp. was the dominant. Ijah *et al.* (2014) has reported similar results indicating the presence of *Bacillus, Micrococcus*, and *Staphylococcus* spp. as dominant groups in breads prepared from wheat and potato flour blends. In general, gram positive bacteria were the predominant group than the gram-negative microorganisms. Similar results were reported by Pundir and Jain (2003) in case of baked products.

Bacterial contamination might have occurred from the raw materials used in the preparation of muffins such as all-purpose flour, sugar, butter etc. The presence of *Bacillus* spp. as a dominant bacterial group has already reported by Ijah *et al.* (2014). *Bacillu*s spp. or their spores might have been present in the raw material, especially in the flour, and their ability to withstand the higher temperature processes have already been studied (Saranraj and Geetha, 2012). Similarly, the ubiquitous nature of *Staphylococcus* spp. can be the reason for its presence in the final product. The presence of *Staphylococcus* spp. might have been attributed to the secondary contamination occurred during handling or packaging of the final product as it cannot withstand high temperature processes. Ogundare and Adetuyi (2003) reported the presence of *Staphylococcus* spp. in baked products.

Patriarca *et al.* (2001) have reported moisture content and pH as the two major intrinsic factors that control the microbial growth. Interestingly, it was observed in the present study there was no effect of moisture content on the microbial growth when compared among the treatments. Among the different treatments, the higher moisture content and water activity was noticed in the muffins with encapsulated squalene. However, when compared to control the treatments with squalene (pure of encapsulated) had lesser TPC counts. This signifies that squalene has some antimicrobial potential and that can be the reason for the extended shelf life.

The significant findings of the study show the emulsification of squalene in MD-WPI and its encapsulation by spray drying is a potential process to produce oxidatively stable encapsulates for the development of functional foods. Moreover, the physico-chemical attributes of squalene enriched foods show that they can have better sensory acceptability compared to normal muffins prepared without squalene.

6. SUMMARY

Squalene, the precursor compound for biosynthesis of cholesterol and many other steroid hormones, is found widely in many sources like olive oil, wheat germ oil, rice bran oil, amaranth oil, shark liver oil etc. Health promoting properties of this functional lipid compound like cardioprotective, antioxidant, chemopreventive, anticancerous, antilipidemic, membrane stabilizing properties etc. are well documented by the researchers across the globe. The notable health benefits and wide applications has prompted extraction of squalene from various sources using different techniques. The conventional extraction using solvents involves longer duration, large amounts of solvents, removal of solvents which further affects the process economy, efficiency and even the environment. Supercritical fluid extraction (SFE) has emerged as a feasible alternative to the conventional methods in extracting bioactive compounds.

In the present study, the extraction of squalene rich oil from bramble shark (*Echinorhinus brucus*) liver was investigated by taking into account the influence of extraction conditions on yield, squalene content, major fatty acids, fat soluble vitamins using Response Surface Methodology (RSM). Based on the experimental results, an extraction condition of 400 bar pressure, 43.34 ºC temperature for 5.45 h was optimized for isolation of squalene rich shark liver oil. Advantage of this method is that, in addition to squalene the extracted oil is also rich in long chain PUFAs such as EPA, DHA, AA and most importantly vitamin A and E. The presence of vitamin E is considered advantageous as it is a potent antioxidant which can even take care of the oil's oxidative stability. Moreover, the method is environment friendly, requires less amount of solvents and can be carried out at conditions (pressure and temperature) which can be easily achieved.

In spite of the potential bioactivities, squalene has not been attempted as a functional food ingredient. The one probable reason attributed for the limited application of squalene in processed food can be its high oxidative instability during processing and storage. Owing to the high degree of unsaturation, squalene is easily acted upon by various environmental factors and the oxidation products thus produced can in turn reduce the nutritional quality and bioavailability. Encapsulation of squalene can be an effective method to address this problem. A preliminary study was carried out to establish the squalene to wall material ratio for better emulsion

stability and encapsulation attributes using chitosan as the wall material. A core to wall material ratio of 0.3:1 was optimized by taking into account all the emulsion stabilization parameters. However, chitosan was found as an ineffective encapsulant for squalene and it was concluded that a combination of protein-polysaccharide combinations can work effectively for squalene encapsulation. Accordingly, the encapsulation of squalene was further carried out using various biopolymers such as chitosan-whey protein isolate, gum Arabic, maltodextrin-whey protein isolate. It was found that MD-WPI was the most effective wall material for squalene encapsulation providing the highest encapsulation efficiency (96.5%), oxidative stability, thermal stability, solubility etc. The in-vitro release kinetics also shown that MD-WPI increased the bioavailability of squalene by providing it a slow, sustained and targeted release. The findings of the study suggest that MD-WPI can be utilised as an alternative to gum Arabic as it performed better than the latter and is cost-effective also.

The effect of dietary supplementation of squalene encapsulated using MD-WPI on growth performance, lipid profile and antioxidant enzymes was studied in wistar strain albino rats. The treatment groups fed with encapsulated squalene showed improved weight gain % and SGR indicating that squalene along with the wall materials used (maltodextrin and whey protein isolate) might have some potential health promoting properties. The catalase and superoxide dismutase enzyme activity were found to decrease with the increase in squalene supplementation. Based on the results, it can be presumed that supplementation of squalene itself is enough to take care of radical scavenging effects and can restore the activities of antioxidant enzymes. The cholesterol, LDL and VLDL level of the groups fed with squalene has shown a decreasing trend whereas HDL level increased with the increased squalene dosage indicating that they have potential cholesterol lowering effect.

Further, the feasibility of encapsulated squalene as a functional food ingredient was analysed by studying the physico-chemical, textural and microbial quality after its incorporation in a bakery product, muffins. The nutritional analysis showed that squalene enriched muffins were high energy foods. There was a significant difference in the textural quality of squalene enriched foods when compared to control. In general, muffins prepared with encapsulated squalene were

cohesive, springier and chewy with less gumminess and stiffness than the other treatments indicating their efficacy in improving the textural quality. The superior textural effects can be attributed to the combined effects of squalene as well as the wall material. Similarly, the color attributes have also varied significantly among the treatments. These results were further supported by scanning electron micrographs revealing their stronger structural organization. Oxidative stability was also higher in encapsulated squalene enriched muffins and this can be due to the maillard reaction products produced during the process of baking. The microbiological analysis showed that gram positive bacteria were the predominant group than the gram-negative microorganisms in the baked muffins with *Bacillus* and *Staphylococcus* spp. as the dominant microbiota. Similar to the other parameters, microbiological quality was also high in squalene enriched foods suggesting that squalene might have some antimicrobial effects.

Overall, it can be concluded that bramble shark, which is often discarded as a by-catch, is a rich source of squalene and can be utilized for commercial based squalene extraction. The study also underpins the potentiality of supercritical fluid based extraction as an effective technique for squalene isolation in minimum time when compared to the conventional methods. The effectiveness of MD-WPI as a wall material for encapsulation of lipophilic compounds was also showed in the study. The utility of encapsulated squalene as a functional food ingredient was also proven showing that it can be very well utilised in the development of ready -to-eat or ready-to-drink functional foods as its solubility is very high. The future challenge is scaling up and sustainable transfer of these technologies to the highly competitive and consumer driven food industry.

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SYMBOLS AND ABBREVIATIONS

%	Percentage
µg	microgram
a*	Redness
b*	Yellowness
cP	Centipoise
g	gram
h	hour
Kg	Kilogram
L*	Lightness
mg	milligram
ml	millilitre
N	Newton
α	Alpha
β	Beta
AA	Arachidonic acid
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AOAC	Association of official analytical chemists
AST	Aspartate aminotransferase
BHT	Butylated Hydroxy Toluene
CAT	Catalase
CER	Constant Extraction Rate
cfu	Colony forming unit
CS-WPI	Chitosan-Whey Protein Isolate
DAD	Diode array detector
DCW	Dry cell weight
DE	Dextrose equivalents
DHA	Docosa Hexaenoic acid
DOTAP	DiOleoyl-3-Trimethyl Ammonium-Propane
EE	Encapsulation efficiency
EPA	Eicosapentaenoic acid
FER	Falling Extraction Rate

FLD	Fluorescence detector
FTIR	Fourier transform infrared spectroscopy
GA	Gum Arabic
GC	Gas Chromatography
HMGCoA	3-hydroxy-3-methyl glutaryl coenzyme A
HPLC	High performance liquid chromatography
LC	Loading capacity
LDL	Low Density Lipoproteins
MD	Maltodextrin
MD-WPI	Maltodextrin-Whey Protein Isolate
MUFA	Mono unsaturated Fatty acid
NSM	Non-Saponifiable Matter
PDI	Poly dispersity Index
PUFA	Poly Unsaturated Fatty acid
ROS	Reactive Oxygen Species
RSM	Response Surface Methodology
S/N	Signal to Noise ratio
SC-CO_2	Supercritical CO2
SCF	Super Critical Fluid
SEM	Scanning electron microscopy
SFA	Saturated Fatty acid
SFE	Supercritical Fluid Extraction
SO	Surface Oil
SOD	Superoxide Dismutase
TGA	Thermogravimetric analysis
TO	Total Oil
TPC	Total plate count
VLDL	Very Low Density Lipoproteins
WPI	Whey protein isolate
XRD	X-Ray Diffraction analysis