

ल्यूटिन के जैव बहुलक आधारित सूत्रण की प्राकृतिक खाद्य रंग के रूप में
उपयोगिता

**Biopolymeric formulation of lutein for its application as
natural food colorant**

SOUMYAJIT GHOSHAL



**DIVISION OF AGRICULTURAL CHEMICALS
ICAR-INDIAN AGRICULTURAL RESEARCH INSTITUTE
NEW DELHI-110012**

2022

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**Biopolymeric formulation of lutein for its application as
natural food colorant**

By

SOUMYAJIT GHOSHAL

A Thesis

Submitted to the Faculty of Post-Graduate School,
Indian Agricultural Research Institute, New Delhi
in partial fulfillment of the requirements
for the award of the degree of

MASTER OF SCIENCE

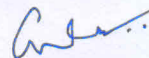
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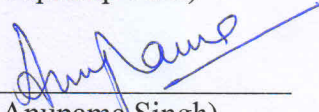
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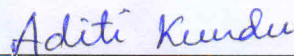
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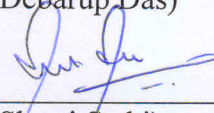
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CERTIFICATE

This is to certify that the thesis entitled, “**Biopolymeric formulation of lutein for its application as natural colorant**” submitted to the Faculty of the Post-Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfilment of the requirements for the award of degree of **MASTER OF SCIENCE IN AGRICULTURAL CHEMICALS**, is a record of bona fide research work carried out by **Mr. Soumyajit Ghoshal, Roll No. 21381** under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

It is further certified that all the assistance and help availed during course of investigation as well as all sources of information have been duly acknowledged by him.

Date: 06-08-2022

Place: New Delhi

(Dr. Anirban Dutta)

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Acknowledgement

“Gratitude, like faith, is a muscle. The more you use it, the stronger it grows, and the more power you have to use it on your behalf. If you do not practice gratefulness, its benefaction will go unnoticed, and your capacity to draw on its gifts will be diminished. To be grateful is to find blessings in everything. This is the most powerful attitude to adopt, for there are blessings in everything.”

Alan Cohen

When my mind attempts to come up with the appropriate words to express the deepest thoughts of the heart, I usually end up scratching the sheets. I want to express my gratitude to the Almighty for enabling me to complete both my Master's research programme and the entirety of my course work before beginning my thanks giving. I consider myself extremely fortunate to have this chance to study, educate, and do research in a universe with billions of people. I owe a debt of appreciation to everyone who helped make this thesis possible and who made my experience one I will always treasure.

The adage that a wonderful teacher understands how to create the ideal environment conducive to his pupil's learning process is true; he or she knows how to balance the appropriate quantity of chalk with the appropriate percentage of obstacles. The level of appreciation I feel in my heart for all the people who have helped with this work is beyond words. I would like to express my sincere gratitude and appreciation to respected Chairperson Dr. Anirban Dutta, Scientist (Sr. Scale), Division of Agricultural Chemicals, ICAR- Indian Agricultural Research Institute, New Delhi, for his sage advice, priceless guidance, subtle and expert help, and unwavering encouragement throughout my master's programme. I will always be grateful to him from the bottom of my heart because his encouragement, academic advice, words of wisdom, and constructive criticism inspired every part of my brain to think creatively and never give up until I reached my goal. I feel really fortunate to have the chance to work under his direction. My sincere appreciation will always be with him.

I would like to extend my heartfelt gratitude to Dr. Supradip Saha, Principal Scientist, Division of Agricultural Chemicals, ICAR-IARI, New Delhi, for serving as Co-Chairman of my advisory group and also for his helpful attitude, affectionate behaviour, fruitful discussion, and offering his insightful recommendations throughout my study. I consider myself really fortunate to have met such a kind individual whose unceasing support helped my M.Sc. programme.

I feel immense pleasure to convey my heartfelt sense of gratitude to Dr. Anupama Singh, Head, Division of Agricultural Chemicals, Dr. Aditi Kundu, Senior Scientist, Division of Agricultural Chemicals, Dr. Shruti Sethi, Principal Scientists, Division Of Food Science & Postharvest Technology and Dr. Debarup Das, Scientist, Division of Soil Science and Agricultural Chemistry, ICAR-IARI, New Delhi for their invaluable guidance, sustained interest, sagacious suggestions, constant encouragement and timely help as and when needed during the course of the study.

I would like to extend my heartfelt gratitude to Dr. Anupama Singh, Head, Dr. Neera Singh, Professor, and all the faculty members of the Division of Agricultural Chemicals, ICAR-IARI, New Delhi for their support and provision of the essential resources throughout my studies.

I am especially thankful to Dr. Arpan Bhowmik, Senior Scientist, IARI Assam; Dr. Rajesh Kumar, Principal Scientists, Division of Agricultural Chemicals, ICAR-IARI, New Delhi; Dr. Gautam Chawla, Principal Scientist, Division of Nematology, ICAR-IARI, New Delhi; Dr. Sonali Gangwar, Senior Project Scientist, Thermal Series laboratory, IIT Delhi Sonipat Campus; and Dr. Reeta Bhatia Dey, Senior Scientist, Division of Floriculture and Landscaping ICAR-IARI, New Delhi for their kind and self-less help during the study.

I am thankful to Sajjan ji, Akshay ji, Nabin ji, Division of Agricultural Chemicals and Baljeet ma'am, Division of Plant Pathology for technical help.

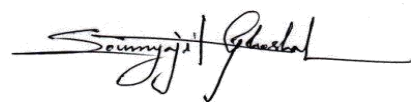
I consider myself to be extremely fortunate to have had guidance and assistance from cherished elders like Anindita di, Rohan da, Arka da, Debdas da, Sutanwa di, Debabrata da, Partha da, Shreosi di, Shyam sir, Anirban da, Sumit sir for guiding my research work and counselling my waves of disturbance whenever I felt weak or uncertain. If I didn't include my outstanding classmates Atanu, Joydeep, Jeetram, Brendon, Dharini, my bag of praise would be empty. It's difficult to find individuals you can depend on at any moment, therefore I guess I am the lucky one to have you all in one place at once. Every time, your company has always taught me to cherish the positive and keep going in the face of setbacks. For the most part, memories would endure for all time.

Finally, the endless love, affection, sacrifice and constant inspiration from my Maa, Baba, Didibhai and Dadabhai have enabled me to walk in the footsteps of my long-held ambition. My vocabulary is completely inadequate to express my gratitude to my loving parents, for their unwavering support. Without them I am nothing in this world.

I'm also grateful to the Director of the ICAR-Indian Agricultural Research Institute in New Delhi and the Dean of the P.G. School for giving the resources I needed to complete my research. I am grateful to the Indian Council of Agricultural Research for providing financial and technical assistance in furthering my studies and research.

Date: 06-08-2022

Place: New Delhi



(SOUMYAJIT GHOSHAL)



*Dedicated to
My Beloved
Parents &
Chairman*



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List of Abbreviations

%	Percent
°C	Degree Celsius
µg g ⁻¹	Microgram per gram
et al	et al. (co-authors)
etc	et cetera (and others)
Fig.	Figure
HPLC	High Performance Liquid Chromatography
mg	Miligram
min.	Minutes
mL	Milliliter
nm	Nanometer
SEM	Scanning electron microscopy
S. No.	Serial number
ΔE	Difference between colors of sample and control
h	Hour
M	Molarity
N	Normality
RGB	Red, green, and blue
GA	Genetic algorithm
RSM	Response surface methodology
<i>p</i> -value	Probability value
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
CUPRAC	Cupric reducing antioxidant capacity
FRAP	Ferric reducing ability of plasma
<i>m/z</i>	Mass/ charge
R _t	Retention time
d.w.	Dry weight
HDES	Hydrophobic deep eutectic solvent
DES	Deep eutectic solvent
DSC	Differential Scanning Calorimetry
H ¹ -NMR	Proton - Nuclear Magnetic Resonance spectroscopy
FT-IR	Fourier transform infrared
D ₅₀	Median particle diameter
P	Powder form
UPLC	Ultra-high performance liquid chromatography
Q-TOF	Quadrupole time-of-flight
ESI	Electrospray Ionisation Process
MS	Mass spectrometry

1. INTRODUCTION

The aesthetic appearance of foods has a significant impact on consumer acceptance. Most of the consumers judge foods by their visual appearance, where color is considered as the first parameter to determine quality and freshness. Therefore, food industries are using huge amount of food colorants in various processed and packaged food products to influence their perceived color to the consumers. The global market of food colorants has reached a value of USD 4.42 billion in 2021 and is expected to reach a value of USD 6.78 billion by 2027 at a compounded annual growth rate (CAGR) of 7.15% during 2022-2027 (<https://www.imarcgroup.com/food-colors-market>). At present, synthetic food colorants dominate this global market. Though these artificial colors are cheaply available and provide vibrant appearance to the foods and food products, the risks of associated side effects on health such as hyperactivity in children, cancer and allergies make their use highly controversial (Amchova *et al.*, 2015; Gautam, 2015). The global regulatory authorities like the United States Food and Drug Administration (USFDA), and the European Food Safety Authority (EFSA), therefore, have set up stringent acts and legislations to regulate the use of artificial food colorants. Despite the available legislations, in developing countries, lack of a coordinated monitoring system, sluggish implementation, and crooked intention of a section of traders have made human foods laden with illegal artificial colorants. However, an increased consumer awareness and demand for clean-label products are forcing the manufacturers to replace the synthetic food dyes with natural alternatives (Rodriguez-Amaya, 2016; Sigurdson *et al.*, 2017). Reports suggested that the global market size of natural food colorants was USD 1.86 billion in 2021, which is estimated to reach USD 2.77 billion by 2027 at a CAGR of 6.9% during the forecast period of 2022-2027 (<https://www.researchandmarkets.com/reports/5546181/global-natural-food-colors-market-size-forecast>). Environment-friendly and non-toxic nature of the natural pigments along with their additional health benefits in terms of nutraceutical values are the major driving forces to push up the future of the market of food colorants. Despite being in its infancy, the Indian food colorant market, dominated majorly by small-scale food industries and a few multinational companies, is also projected to witness a CAGR of 5.3% over a forecast period of 2020-2025 (<https://www.mordorintelligence.com/industry-reports/india-food-colorants-market-industry>). Presently, the Food Safety and Standards Authority of India (FSSAI) only permits eight synthetic food colorants to be used in food products below their acceptable daily intake (ADI) levels. FSSAI also allows use of certain natural colorants in foods (<https://fssai.gov.in/upload/uploadfiles/files/FSSAI-regulations.pdf>). Therefore, the scope of

natural food colorants in the Indian market is too promising. In this context, research focus on natural food colorants is very pertinent.

Carotenoids are one of the most diverse and wide spread lipophilic pigments found in nature. These tetraterpenes are made up of eight isoprene units with an extended conjugated double bond structure responsible for their yellow, orange and red coloration (Britton, 2020). Carotenoids are generally classified into two major classes: carotenes (hydrocarbons), and xanthophylls (oxygenated derivatives of carotenes). Lutein (β,ϵ -carotene-3,3'-diol), a yellow pigment from xanthophyll group, has the highest growth potential among all carotenoids with 23% market share (BCC Research, 2018). This natural pigment is commonly found in egg yolk, certain microalgae, and in many plants like marigold, corn, spinach, pumpkin, celery, Brussel sprouts, zucchini, orange, mango etc., either in free or esterified forms (Sommerburg *et al.*, 1998; Sowbhagya *et al.*, 2004). Marigold (*Tagetes* sp.) flower is the most common commercial source of lutein as the pigment naturally occurs in the petals of this flower in the acylated form as lutein esters (Bhattacharyya *et al.*, 2010; Sandmann, 2015). According to the National Horticulture Board estimate, India produces on an average 0.6-0.7 million tons of fresh marigold flowers annually. The fresh flowers are mainly used in festivals and rituals. The flowers after use are generally thrown away and the pigments are lost by decomposition. However, this non-food flower can be used for extraction of the pigment as natural alternative to the common synthetic yellow food dyes like tartrazine, sunset yellow and quinolone yellow. Additionally, the pigment is considered as a health beneficial nutraceutical due to its potential role in improving visual and nervous systems and antioxidant properties (Granado *et al.*, 2003). Lutein also helps in preventing the development of age-related macular degeneration, which is the major cause of blindness in persons over 65 years of age in developed nations (Frede *et al.*, 2017). An average lutein supplement of around 6 mg per day per person has been recommended to be necessary for lowering the incidence of cataracts and macular degeneration (Tan *et al.*, 2008). However, clinical trials have demonstrated that it can be safely ingested at three times the recommended dose with no adverse effects (Wallace *et al.*, 2015). As humans cannot synthesize carotenoids *de novo*, the primary source of their intake is only through oral route (Costa *et al.*, 2015).

Therefore, extensive research has been carried out to incorporate lutein into food matrices. Xanthophylls (lutein esters and lutein) from marigold have been used as food coloring agents and nutrient supplements in a variety of foods and beverages at different concentration. Nevertheless, limited water solubility, chemical instability, and poor oral bioavailability make

them difficult to incorporate into foods (Becerra *et al.*, 2020). To circumvent these challenges, colloidal encapsulation systems can be developed to include the pigments of marigold as natural colorant and nutraceutical into functional foods. Various encapsulation systems, such as micro- and nano-emulsions, liposomes, hydrogels, coacervates, molecular complexes etc. are now being extensively investigated to encapsulate lutein (Steiner *et al.*, 2018). Techniques including spray drying (Wang *et al.*, 2012; Álvarez-Henao *et al.*, 2018; Ding *et al.*, 2020), antisolvent precipitation (Li *et al.*, 2020), complex coacervation (Qv *et al.*, 2011), inclusion complex (Zhao *et al.*, 2018a), biogel formation (Jiang *et al.*, 2019), amphiphilic nanoparticles (Chang *et al.*, 2017) etc. have been explored for encapsulation of lutein to improve its solubility, stability, bioavailability and shelf-life at variable magnitude.

However, recent discoveries concerning difficulties in making intact lutein bioaccessible, have prompted the development of novel methods for delivering lutein into the systemic circulation (He *et al.*, 2016). Moreover, delivery of total xanthophyll complex of marigold including both free lutein and lutein esters is challenging as their solubility, stability and bioavailability vary at a large scale. Development of water-soluble formulations of this group of pigments is therefore become necessary to improve their bioavailability. Furthermore, the water-soluble formulation can easily be incorporated in various food matrices to impart color.

Based on the above-mentioned scope and envisaging the development of a safe, sustainable and efficient formulation of the pigment from marigold to use as natural food colorant and nutraceutical, the present research work has been undertaken with the following objectives:

- To develop and characterize encapsulated biopolymeric formulation(s) of lutein
- To evaluate the stability and antioxidant activity of prepared formulation(s)

2. REVIEW OF LITERATURE

2.1. Lutein – chemistry and distribution

Carotenoids are one of the most ubiquitous and diverse lipophilic groups of natural pigments with color shades varying from yellow to orange and red. The name of this group of pigments has been derived from the earliest known work of Wackenroder, who for the first time isolated ‘carotin’ (β -carotene) crystals from carrots in 1831 (Johnson, 2007). Since then, many compounds of this versatile group have been isolated from natural sources and structurally elucidated. This group of 40-carbon (C_{40}) tetraterpenoids is made up of eight isoprene units with an extended conjugated polyene backbone with terminal cyclic structures. Based on the chemical structures of the member compounds, carotenoids have been broadly classified into two major classes: carotenes, which are strictly hydrocarbons; and xanthophylls, the oxygenated derivatives of carotenes. Out of over 700 known carotenoids, the xanthophyll lutein has received notable attention in both academic and industrial research domains (Sun *et al.*, 2015). Lutein, the dihydroxy derivative of α -carotene, along with its structural isomer zeaxanthin (dihydroxy derivative of β -carotene) are unique in humans due to their presence in specific eye tissues (Alves-Rodrigues and Shao, 2004). Lutein ($C_{40}H_{56}O_2$) has a backbone made up of conjugated carbon-carbon double bonds with β -ionone ring (double bond between C_5 and C_6 carbons) at one terminal, and ϵ -ionone ring (double bond between C_4 and C_5 carbons) at the other. Dihydroxy substitution of the ionone rings takes place at 3 and 3' carbons (Krinsky *et al.*, 2003). The conjugated polyene system allows free electron movement, which results in its capacity to absorb light in the blue region of the visible spectrum with absorption maxima (λ_{max}) at 446 nm and appears as bright yellow-orange crystals (Yi *et al.*, 2016; Mora-Gutierrez *et al.*, 2018). Due to the presence of three chiral centers, lutein can exist in possible eight stereoisomeric forms, but in nature it exists mainly in all-*trans* and all-*cis* forms (Abdel-Aal *et al.*, 2013). As the all-*trans* isomer of lutein is thermodynamically more stable than the *cis*-isomer, the all-*trans* isomer of lutein is the most prevalent geometric isomer in nature (Yang *et al.*, 2018).

Dietary lutein can be most abundantly found in leafy green vegetables, yellow-orange colored fruits and vegetables, and egg yolk (Sowbhagya *et al.*, 2004). Corn is one of the richest source of lutein along with spinach and kale. Lutein can also be found in high concentrations in fruits like kiwi, red seedless grapes, orange, mango, papaya etc. and in vegetables like zucchini, pumpkin, broccoli, Brussels sprouts, green peas etc. (Sommerburg *et al.*, 1998; Alves-

Rodrigues and Shao, 2004). Microalgae, a sustainable source of lutein, are reported to contain 0.5-1.2% of lutein on dry weight basis (Sun *et al.*, 2015; Lin *et al.*, 2022). Recently, Zheng *et al.* (2022) has summarized a comprehensive information about cultivation of microalgae for lutein production. Different species of *Chlorella* namely, *C. minutissima*, *C. sorokiniana*, *C. vulgaris*, *C. protothecoides*, *C. salina*, *C. pyrenoidosa* etc. have been reported to produce lutein under various conditions (Zheng *et al.*, 2022). Due to high lutein content, *Chlorella* has been proposed as a dietary source to increase carotenoid levels in human blood. A daily dose of 9 g of *Chlorella* was found to supply 32 mg lutein (Miyazawa *et al.*, 2013). Apart from *Chlorella*, many others like *Asterarcys quadricellulare*, *Coccomyxa onubensis*, *Parachlorella* sp., *Scenedesmus almeriensis*, *Scenedesmus incrassatulus*, *Cladophora* sp. etc. are high lutein content microalgae (Sánchez *et al.*, 2008; Ma *et al.*, 2010; Heo *et al.*, 2018; Prazukin *et al.*, 2021). Among the non-dietary sources, marigold flowers (African marigold or *Tagetes erecta*, French marigold or *Tagetes patula*, and pot marigold or *Calendula officinalis*) contain highest amount of lutein in its esterified forms and are the main source of commercial production of lutein (Lin *et al.*, 2015). Dried marigold flowers reported to contain around 0.1–0.2% of carotenoids on dry weight basis, out of which 80% are lutein diesters (Šivel *et al.*, 2014). However, the total content of lutein and lutein esters in marigold varies from cultivar to cultivar and a total content of pigments ranging from 17 to 570 mg per 100 g of the petals has been reported (Piccaglia *et al.*, 1998). Recently, Rodrigues *et al.* (2019) showed that apart from having a large quantity of mono- and di-esters of lutein, petals of marigold flower also contain a wide array of carotenoids, mainly esters of xanthophylls other than lutein.

2.2. Health benefits of Lutein

The importance of lutein in human health, particularly for eyes have been clearly demonstrated by several epidemiological, clinical, and interventional studies. The biological mechanisms underlying carotenoids' protective effects could include powerful blue-light filtering and antioxidant properties (Ma *et al.*, 2010). Bone *et al.* (1988) identified presence of two xanthophylls (lutein and zeaxanthin) in human macula. These two primary pigments in the human retina's yellow spot protect the eyes from blue light damage, improve visual acuity and scavenge harmful reactive oxygen species (Nwachukwu *et al.*, 2016; Panova *et al.*, 2017), thus reduce the risk of age-related macular degeneration and cataracts (Krinsky *et al.*, 2003; Buscemi *et al.*, 2018). Vitamin A (retinol) insufficiency is a serious health problem in underdeveloped nations which affect particularly children and pregnant women. This leads to blindness, poor development, and mortality (Gurmu *et al.*, 2014). Aside from the benefits of

lutein consumption on various vision tests, research suggested that lutein can influence immune responses and inflammation (Kijlstra *et al.*, 2012). Wang *et al.* (2006) found that the performance of lutein in antioxidant activity was similar to that of the other two common carotenoids, β -carotene and lycopene. Lutein along with zeaxanthin provided the highest level of protection against UV irradiation in the skin (Palombo *et al.*, 2007). It's also proved to be the most effective antioxidant in preventing lipid peroxidation and scavenging hydroxyl radicals (Bhattacharyya *et al.*, 2010). The role of lutein and zeaxanthin for brain development has also been reported in literature (Barker *et al.*, 2011; Tanaka *et al.*, 2012). Lutein has been found to provide protection against heart diseases by preventing the production of LDL cholesterol (Yoshida *et al.*, 2010; Eggersdorfer and Wyss, 2018). Several factors, including oxidative stress, inflammation, and dyslipidemia, have been linked to the development of cardiovascular diseases, and there are ample evidences which suggest that lutein may help in their alleviation (Böhm, 2012; Müller *et al.*, 2016). According to van Lent *et al.* (2016), increased lutein consumption and blood concentrations are linked to a decreased risk of coronary heart disease and stroke. One study looked on the effect of lutein in preventing atherosclerosis in the neck carotid arteries. The clinical study reported less fat deposition inside the artery walls of lutein treated individuals which ultimately reduced the risk of heart attacks and strokes (Moeller *et al.*, 2000). Furthermore, research showed that diets high in carotenoids such as lutein, zeaxanthin etc. can help to prevent breast cancer (Kim *et al.*, 2002a; Sato *et al.*, 2002). According to one research, the consumption of lutein through green leafy vegetables by the natives of Fiji Islands (18-23 mg per day) might be responsible for the decreased incidence of lung cancer cases in that population (Batista *et al.*, 2006). Chemoprotective activity of lutein against colon cancer induced by dimethylhydrazine was evaluated and lutein was found to significantly reduce tumor formation (Reynoso-Camacho *et al.*, 2011). Toniolo *et al.* (2001) reported that individuals with high levels of lutein, cryptoxanthin, and carotene in blood had a decreased incidence of breast cancer. They also discovered that lutein could make malignant cells more sensitive to chemotherapy. Lutein's chemotherapeutic action on tumour cells was validated in another investigation where it was found to cause apoptosis in tumour cells but not in normal cells (Sumantran *et al.*, 2000).

2.3. Bioavailability of lutein

As humans can not synthesize lutein in their body, the only source to increase its concentration in serum is through dietary intake (Calvo, 2005). Due to the associated health benefits of lutein intake in terms of amelioration of many chronic diseases, such as age-related macular

degeneration (AMD), cancer, and cardiovascular diseases, maintaining lutein integrity during food processing, and increasing accessibility in the food matrix are two key aspects for consideration (Becerra *et al.*, 2020). As food containing lutein is generally thought to be safe (Wang *et al.*, 2006), consumption of lutein rich fruits and vegetables in large quantities are being recommended. A daily dose of 6 mg lutein is generally recommended to reduce the risk of AMD. However, this is difficult to achieve in a regular human diet due to the low concentration and bioavailability of lutein in fruits and vegetables (de Quirós and Costa, 2006). The poor aqueous solubility of lutein, further reduces its bioavailability. The high lipophilicity of lutein's C₄₀ isoprenoid carbon skeleton makes it only slightly soluble in digestive fluids, resulting in poor oral absorption (de Sousa Anselmo *et al.*, 2016). Use of lutein in the food industry is also limited due to its instability and the chemical changes caused during food processing (Qv *et al.*, 2011). Factors like high temperatures, pH, oxidation and light during food processing affect the integrity of lutein severely (Boon *et al.*, 2010; Weigel *et al.*, 2018). Therefore, over the last few decades, researchers have focused on developing lutein-rich foods to increase lutein intake, particularly among the elderly (Abdel-Aal *et al.*, 2013).

2.4. Extraction of lutein

Presently, marigold flowers are the main commercial source for extraction of lutein. The age-old commercial process of extraction of marigold pigments included uncontrolled fermentation, followed by removal of moisture by pressing and hot air drying, milling and subsequent extraction with hexane. This crude technique resulted in a considerable loss of carotenoids (Delgado-Vargas and Paredes-López, 1997). Therefore, solvent extraction and simultaneous saponification by alkaline hydrolysis has been recommended by many researchers to extract de-esterified lutein from fresh or dried marigold petals (Hojnik *et al.*, 2008; Vechpanich and Shotipruk, 2010; Kumar *et al.*, 2010; Boonnoun *et al.*, 2012). Generally organic solvent like hexane, acetone, xylene, THF, and chloroform were confirmed to be suitable for extraction of lutein from marigold. However, toxicity, availability, and viscosity characteristics of the solvent should be considered for the selection of solvents in extraction process (Surendranath *et al.*, 2016). Soxhlet extraction of lutein esters from marigold with methanol has been reported in literature (Bhattacharyya *et al.*, 2010; Saisugun *et al.*, 2019). Pressurized liquid extraction of lutein from microalgae through an accelerated solvent extractor has also been successfully employed (Casella *et al.*, 2021). Enzymatic treatment before solvent extraction has been reported to enhance extraction yield of xanthophylls/carotenoids from

marigold flower meal by several researchers (Delgado-Vargas and Paredes-López, 1997; Barzana *et al.*, 2002; Navarrete-Bolaños *et al.*, 2005; Andrea, 2008; Kaimainen *et al.*, 2015).

However, these organic solvent-based methods are time consuming and environmentally unsuitable. Therefore, in recent times, environmentally benign greener technologies are being used for extraction and purification of lutein from marigold. Recently, Manzoor *et al.* (2022) optimized the ultrasound assisted extraction of lutein from marigold petals using soybean oil as solvent. Maximum yield of lutein reported in that study was 21.23 mg g⁻¹ under the optimized conditions. Lutein esters from marigold have also been extracted with medium-chain triacylglycerols (MCT) and orange oil by ultrasonication with equal efficiency as that of conventional solvent (Khalil *et al.*, 2012). Kashyap *et al.* (2022) described an efficient green process of extraction of lutein esters from marigold with 2-methyltetrahydrofuran and subsequent hydrolysis for a significantly improved yield (2.56%) and purity (97.33%) of lutein over the conventional methods. A very high lutein recovery (99.85%) from marigold was reported in a study by developing a microwave and enzyme co-assisted aqueous two-phase extraction method (Fu *et al.*, 2018). Under optimal conditions, use of 30% (w/w) ethanol/19% (w/w) ammonium sulfate and 4.2 U g⁻¹ pectinase, yielded 5.59 mg g⁻¹ of lutein by the method. Microwave assisted green extraction technique has also been used to extract lutein from microalgae (Leema *et al.*, 2022).

One of the most efficient techniques to extract xanthophylls from marigold is by using supercritical fluids (Gong *et al.*, 2011). It improves the mass transfer coefficient in the solid phase. An ultrasound assisted supercritical CO₂ extraction technique has been reported to produce a maximum yield of 690 mg lutein esters per 100 g of dried marigold petals (Gao *et al.*, 2009). Use of soybean oil as co-solvent was found to improve the extraction yield of lutein esters from marigold petals by supercritical CO₂, with a maximum reported yield of 10397 µg g⁻¹ (Ma *et al.*, 2008). Similarly, use of medium-chain triglyceride as co-solvent in supercritical CO₂ extraction of lutein esters was found to be the best with an estimated yield of 12636.2 µg g⁻¹ (Gao *et al.*, 2010). However, Palumpitag *et al.* (2011) found 16% more recovery of marigold xanthophylls by using palm oil as co-solvent as compared to soybean oil, olive oil and ethanol in an extraction experiment by supercritical CO₂.

Recently, a novel microemulsion based technique was utilized to extract lutein from marigold petals (Jivan and Abbasi, 2019). The combination of sucrose monopalmitate and 1-propanol at 1:5 ratio showed the highest efficiency of lutein extraction in the presence of 42% water. The extraction efficiency was found to vary with the combination of different surfactant

and co-surfactant in the microemulsion. Similarly, 1:5 mixture of Span-20 as surfactant and 1-propanol as co-surfactant has also been found to increase the extraction efficiency of lutein from marigold petals (Jalali-Jivan *et al.*, 2021).

2.5. Deep eutectic solvents (DES) for green extraction

In green chemistry research, the quest for new type of green solvents has lately become significant due to their limited availability. Ionic liquids (ILs) have received a lot of attention as one of the most important and innovative green solvents to replace existing hazardous organic solvents. The majority of ILs, made up of bulky organic cations and small inorganic anions, have distinctive characteristics compared to ordinary solvents (Zhao *et al.*, 2005). ILs are liquid at room temperature, have low vapour pressure, moderate volatility, high thermostability, and recovery and reuse property (Zhang *et al.*, 2006). However, the green solvent status of ILs became questionable when some of them were found to be toxic and poorly biodegradable apart from being expensive and difficult to purify (Zhao *et al.*, 2007; Pham *et al.*, 2010; Flieger and Flieger, 2020). Fortunately, a new class of IL like solvents, known as deep eutectic solvents (DES), have evolved during this time period, which not only have the fascinating properties of ILs but also provide different advantages. Negligible toxicity, high biodegradability, sustainability, and ease of preparation of DES made them suitable in food applications (Zainal-Abidin *et al.*, 2017). In 2003, Abbott *et al.* found the deep eutectic phenomenon by mixing choline chloride (m.p. 302 °C) and urea (m.p. 133 °C) in a 1:2 molar ratio. The resultant mixture was liquid at ambient temperature, melted at 12 °C, and had unusual solvent characteristics. Thereafter, several similar DES were prepared by combining choline chloride with carboxylic acids like oxalic, malonic, succinic acids etc., consecutively (Abbott *et al.*, 2004). Another well-studied DES family is the combination of a carbohydrate with a urea derivative (mainly *N,N'*-dimethylurea and ammonium chloride). A novel class of DES has recently been developed by combination of a long chain saturated fatty acids with quaternary ammonium salts (van Osch *et al.*, 2015), other natural acids (Ribeiro *et al.*, 2015), or terpenoids (van Osch *et al.*, 2019). In contrast to the earlier hydrophilic DES, this novel class of DES exhibited a strong hydrophobic behaviour (van Osch *et al.*, 2015). Due to their propensity to extract hydrophobic compounds, these hydrophobic DES has made many extraction techniques more successful. One of the advantages of DES is the simplicity of their preparation without any complicated purification steps by simple heating (Imperato *et al.*, 2005) or freeze-drying (Gutierrez *et al.*, 2009). The components of a DES do not undergo any chemical reaction with each other, but act as hydrogen bond donor (HBD) and hydrogen bond

acceptor (HBA) as observed from the formation of hydrogen bonds between them (Tang *et al.*, 2015). This phenomenon in turn influences the physical qualities such as melting point, density, miscibility, polarity, viscosity, and conductivity of DES to determine their suitability as extraction solvents (Zhang *et al.*, 2012). The inverse relationship between viscosity and conductivity of DES in association with temperature was found to have direct influence on the H-bonding between HBD and HBA (Abbott *et al.*, 2011; Zhang *et al.*, 2012; Tang *et al.*, 2015). This in turn controls the extraction efficiency of DES.

Several studies on extraction of phytochemicals using DES have been reported in recent times. A DES composed of choline chloride and maltose (1:2) was found to be highly efficient for extraction of phenolics from *Cajanus cajan* leaves as compared to conventional solvents in a microwave assisted extraction technique (MAE) (Wei *et al.*, 2015). Similarly, another DES prepared in combination of choline chloride and oxalic acid was found to have better extraction efficiency than conventional solvents to extract grape skin phenolics by both MAE and UAE (ultrasound assisted extraction) (Bubalo *et al.*, 2016). The MAE technique based on DES (1:1 mixture of choline chloride and glycerol) was extremely efficient, environmentally benign and more effective than traditional solvent in extracting phenolic components from olive leaves and drupes (Bonacci *et al.*, 2020). A MAE based approach was used to extract genistin, apigenin and genistein from pigeon pea roots using DES (1:7 ratio of choline chloride and 1,6-hexanediol) with 30% water in order to establish an ecologically friendly extraction and separation technique of those phytochemicals (Cui *et al.*, 2015). A ternary DES composed of glycerol, l-proline, and sucrose at 9:4:1 ratio was reported to be a sustainable and efficient extraction medium for ginsenosides, the key medicinal component from ginseng (Jeong *et al.*, 2015). Lactic acid-based DES were found to be exceptionally efficient solvent for UAE of polyphenols from medicinal plants like dittany, fennel, marjoram, mint and sage (Bakirtzi *et al.*, 2016). Eutectic mixture of lactic acid and glucose was found to have similar extraction potential as that of the DES composed of choline chloride and 1,2-propanediol for extraction of anthocyanins from flower petals of *Catharanthus roseus* (Dai *et al.*, 2016). The lactic acid-based DES was also found to show three times more stabilizing capacity of anthocyanidins than acidic ethanol. Anthocyanin extraction efficiency of a ternary DES composed of 1:1:1 mixture of choline chloride, citric acid and glucose was also found to be excellent in terms of 1.24-fold increased yield from mulberry than conventional solvents (Guo *et al.*, 2019). Choline chloride, glycerol and citric acid at a molar ratio of 0.5:2:0.5 was found efficient for blueberry anthocyanins extraction comparing with organic solvent (da Silva *et al.*, 2020). When DES

were used to extract flavonoids namely, quercetin, naringenin, kaempferol, and isorhamnetin from Pollen Typhae, they outperformed traditional solvents such as methanol and 75% aqueous ethanol. Eutectic mixture of choline chloride and 1,2-propanediol (1:4) with a 30% water content exhibited the highest extraction efficiency with recovery rates ranged from 86.87 to 98.89% (Meng *et al.*, 2018).

Because of the over exploration of hydrophilic DES, majority of the reports on DES based phytochemical extraction techniques have been concentrated around hydrophilic compounds. However, limited research progress has also been made in extraction of lipophilic phytochemicals like carotenoids using hydrophobic DES (Yu *et al.*, 2022). Though eutectic mixture of choline chloride and tartaric acid was used for extraction of carotenoids from apricot and shrimp waste (Koutsoukos *et al.*, 2019), Leite *et al.* (2021) reported the inability of choline chloride-based DES to improve carotenoid yield from *Mauritia flexuosa* fruits as compared to ethanolic extraction. Menthol-based DES (with lauric or acetic acid) as a co-solvent with ethanol was found to increase β -carotene yield from crude palm oil (Manurung and Siregar, 2020). Eutectic combination of menthol and lactic acid (1:2) was found to be better solvent for carotenoid extraction from sunflower waste (Ricarte *et al.*, 2020). The eutectic mixture of menthol and lactic acid at 8:1 molar ratio was also found suitable for extraction of lycopene from tomato processing wastes (Silva *et al.*, 2019). However, Lazzarini *et al.* (2022) reported better extraction efficiency of the DES prepared with ethyl acetate and ethyl lactate for lycopene and β -carotene from tomato pomace as compared to the eutectic mixture of menthol and lactic acid. Hydrophobic DES prepared by combination of octanoic and deconoic acid has recently been reported to enhance extraction efficiency of β -carotene from pumpkin (Stupar *et al.*, 2021). Very recently, Fan *et al.* (2022) with the help of mathematical model demonstrated that a DES composed of equimolar mixture of fenchyl alcohol and thymol can effectively extract lutein from the microalgae *Scenedesmus* sp.

2.6. Formulation of lutein

Lutein is being frequently utilized as a natural food colorant because of the strong yellow color it produces. The additional health benefits offered by lutein and its derivatives made them more attractive as natural food colorants in food industries. However, solubility and stability remained the key obstacles for their use in the food sector. To mitigate this difficulty, several approaches of increasing lutein stability have been recommended (Rodriguez-Amaya, 2016). Isomerization of lutein at pH levels below 4.0 or over 8.0, de-esterification of lutein esters, and degradation to low molecular weight compounds are common phenomena during processing

of lutein containing foods (Boon *et al.*, 2010; Weigel *et al.*, 2018; Cheng *et al.*, 2019). As a result, maintaining lutein integrity throughout food processing and enhancing accessibility in the food matrix are considered as two most important factors. Therefore, formulations must be developed to maintain lutein stability throughout storage of the finished product (Boon *et al.*, 2010).

Freezing, addition of antioxidants, and storing foods in vacuum-sealed and air-tight containers to reduce loss due to degradation during preparation and storage are the most common practices to improve shelf-life of carotenoid rich food products (Gouveia and Empis, 2003). Nevertheless, to overcome the limitations of low-water solubility, chemical instability, and poor oral bioavailability of lutein/lutein esters as natural food colorant, different colloidal encapsulation systems have been developed (Steriner *et al.*, 2018; Bhat *et al.*, 2020). The use of micro- and nano-emulsions for carotenoid distribution has been widely reported in various literature (Losso *et al.*, 2005; Batista *et al.*, 2006; Mao *et al.*, 2009). Microemulsions prepared using a food-grade non-ionic surfactant (tween 80) have been shown to be effective at encapsulating both lutein and lutein esters (Amar *et al.*, 2004). Lutein containing extract of *Rhinacanthus nasutus* was emulsified using a surfactant mixture (Capryol, Transcutol, and tween 80) with droplet diameter varied between 10.4-10.7 nm (Ho *et al.*, 2016). A 6.25% increase in bioavailability of lutein was observed from the microemulsion as compared to aqueous suspension of the extract. Biosurfactants like Quillaja saponins were also found to increase the stability of lutein emulsion (Weigel *et al.*, 2018). Stable nanoemulsion of lutein could be obtained by dissolving it in medium chain triglyceride, followed by emulsification using tween 80 (Surh *et al.*, 2017). A beverage containing lutein encapsulated oil-in-water (O/W) nanoemulsion made up of 5% soybean oil and 4% phospholipid as an emulsifier was found to deliver lutein in greater quantity than typical nutritional supplements as observed from a clinical trial (Vishwanathan *et al.*, 2009). Mitri *et al.* (2011a) used high-pressure homogenization to make lutein-loaded miglyol nanoemulsions, which showed greater photostability and penetration as compared to suspension of pure lutein in corn oil. Further, the nanoemulsions were shown to be extremely stable across a variety of storage settings, including refrigeration, ambient temperature, and 40 °C. A lutein loaded nanoemulsion prepared using whey protein isolate (WPI) showed higher stability of lutein at 4 °C (Zhao *et al.*, 2018b). However, Yi *et al.* (2016) found that sodium caseinate performed better than WPI for protecting lutein from oxidative stress. Stabilization of lutein microemulsion with various polymers like WPI (Teo *et al.*, 2017), corn fibre gum (Feng *et al.*, 2017), polyvinylpyrrolidone

(Zhao *et al.*, 2014), dextran-caseinate conjugate (Davidov-Pardo *et al.*, 2016) etc. has been reported to improve the solubility, stability, and bioaccessibility of lutein. Multilayered emulsions prepared with chitosan, flaxseed gum and WPI was reported to improve stability of lutein (Xu *et al.*, 2016). Similarly, WPI, fish gelatin and dodecyltrimethylammonium bromide were used to prepare multilayered emulsion containing lutein for its improved release profile (Beicht *et al.*, 2013).

Apart from nanoemulsions, other nano delivery system for lutein have also been explored by various researchers. A supercritical antisolvent precipitation approach was employed to prepare nanocapsules of lutein using hydroxypropylmethylcellulose phthalate to protect it against light and heat degradation with 88.41% encapsulation efficiency (Milo *et al.*, 1998). Nanosuspension of lutein developed by high-pressure homogenization technique was found to have improved saturation solubility (26 times more) of lutein than coarse powder. Further, converting the nanosuspension to pellets followed by filling in gelatin capsule improved the release profile of lutein (Mitri *et al.*, 2011b). Lutein was loaded in zein nanoparticles and stabilized by the combined lecithin and pluronic F127 surfactants for improved chemical stability under different temperatures and in the presence of UV (Chuacharoen and Sabliov, 2016). Self-aggregated nanoparticles of an amphiphilic polymer of octenyl succinic anhydride modified short glucan chains were reported to have 85% encapsulation efficiency of lutein and sustained release property (Chang *et al.*, 2017). Lutein loaded lipid nanocarriers were synthesized by melting emulsification coupled with high shear homogenization technique using fish oil, carnauba wax, and glycerol stearate for 88.5% lutein encapsulation and sustained release profile (Lacatusu *et al.*, 2013). Nanocapsules of lutein prepared with poly-(lactic-co-glycolic acid) (PLGA)-phospholipid showed enhanced bioavailability from *in vivo* studies in rat model (Kamil *et al.*, 2016; Ranganathan *et al.*, 2016). Thermo and photo stability of lutein has been improved by encapsulating it in a ferritin-chitosan nanocomposite (Yang *et al.*, 2016).

Liposomal delivery systems have also been fabricated to improve solubility, stability and bioavailability of lutein. A comparison of carotenoid encapsulation efficiency of liposomes prepared with egg yolk phosphatidylcholine and Tween 80 showed strongest incorporation of lutein as compared to β -carotene, lycopene, and canthaxanthin (Xia *et al.*, 2015). Lutein loaded liposomes have been prepared using supercritical CO₂ method with reported particle size of 147.6-195.4 nm, and an encapsulation efficiency of 56.7-97.0% (Zhao *et al.*, 2017). A composite phospholipid-chitosan vesicle (chitosome) was prepared by thin-film evaporation

method which showed highest encapsulation efficiency for lutein than other carotenoids with enhanced stability and improved release profile (Tan *et al.*, 2016). Supercritical antisolvent technology was used to make proliposome containing lutein (55 mg g⁻¹) and hydrogenated phosphatidylcholine (Xia *et al.*, 2012). Hydration of the proliposome resulted in formation of lutein liposome suspensions with an encapsulation efficiency of more than 90%.

Supramolecular complexes of lutein and zeaxanthin were prepared with glycyrrhizic acid, glycyrrhizic acid disodium salt, and arabinogalactan. These complexes increased the aqueous solubility of the carotenoids 1000 times (Apanasenko *et al.*, 2015). An attempt was made to enhance lutein solubility by formation of inclusion complex with cyclodextrins and methyl-cyclodextrin was found to greatly increase the solubility and consequently bioavailability of lutein (Nalawade and Gajjar, 2015). Encapsulation of lutein by spray drying has also been reported by some researchers in various matrices like mixture of gum Arabic, maltodextrin and modified starch (Álvarez-Henao *et al.*, 2018) or combination of gelatin and porous starch (Wang *et al.*, 2012).

Out of all these efforts to encapsulate lutein/lutein esters, development of an efficient, effective and practical delivery system still remains to be elusive for its use as natural food colorant. There is ample scope to utilize biopolymers as carrier for developing a novel delivery system. Moreover, an efficient technology to extract lutein/lutein esters from marigold and subsequent encapsulation in biopolymers for effective delivery is the need of the hour.

3. MATERIALS AND METHODS

3.1. Chemicals and reagents

Standard lutein (xanthophyll from marigold; CAS no. 127-40-2) was purchased from Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India. Solvents namely hexane, acetone and methanol used for extraction of xanthophylls from marigold were of analytical grade and supplied by Merck Life Science Pvt. Ltd., New Delhi, India. Capric (*n*-decanoic) and caprylic (octanoic) acids were procured from SRL Pvt. Ltd., Mumbai, India. HPLC grade methanol and methyl *tert*-butyl ether (MTBE) were obtained from Merck Life Science Pvt. Ltd. and SRL Pvt. Ltd., respectively. Thermo Fisher Scientific India Pvt. Ltd., Powai, Maharashtra, India supplied the LC-MS grade acetonitrile, water and formic acid. All other chemicals used in this work were procured from Sigma-Aldrich Chemicals Pvt. Ltd., Merck Life Science Pvt. Ltd., HiMedia Laboratories Pvt. Ltd., Nashik, India and CDH Pvt. Ltd., New Delhi, India and used without any further purification. Double distilled water was used for carrying out the experiments wherever required.

3.2. Glassware and plastic ware

All the glassware like Erlenmeyer flasks, round-bottom flasks, beakers, funnels, separatory funnels, test tubes, volumetric flasks, measuring cylinders, culture tubes etc. and plastic ware like centrifuge tubes, micropipette tips, droppers etc. were purchased locally.

3.3. Plant material

Fully grown bright orange flowers of a hybrid genotype of African marigold (*Tagetes erecta* L.), namely DAMH-39 were freshly collected from experimental farm of Division of Floriculture and Landscaping, ICAR-Indian Agricultural Research Institute, New Delhi during the months of October and November in 2021. Florets were separated from the bracts and frozen at -20°C before freeze drying in a lyophilizer (FreeZone 6 Plus, Labconco, Kansas City, MO, USA) to remove moisture. The lyophilized florets were coarsely ground and passed through a BSS16 mesh sieve to obtain powdered sample. The sample was then stored in air-tight containers at -20°C until further use.

3.4. Extraction of xanthophylls from marigold florets

3.4.1. Extraction using conventional solvents

3.4.1.1. Conventional solid-liquid extraction (CE)

Lyophilized marigold powder (5 g) was taken in an Erlenmeyer flask (250 mL) and soaked in excess (150 mL) of extraction solvent (1:1 hexane-acetone, v/v). Continuous stirring (500 rpm; 6 h) was carried out at ambient temperature (20 ± 1 °C) for extraction of xanthophylls (lutein esters and free lutein) from the sample. The solvent fraction was collected by vacuum filtration and the process was repeated for four more times adding fresh solvent to the residue until it became colorless. The pooled solvent fractions were partitioned using double distilled water (3×150 mL). The solvent fraction was then passed through anhydrous sodium sulfate (Na_2SO_4) and collected in a pre-weighed round bottom flask. Solvent was completely evaporated to dryness using a rotary vacuum evaporator (Laborota, Heidolph, Germany). Physical weight of the extracted material was noted and it was stored in dark at -20 °C until analysis by high performance liquid chromatography (HPLC).

3.4.1.2. Ultrasonic bath extraction (UBE)

Lyophilized powder of marigold florets (5 g) was soaked in 150 mL of extraction solvent (1:1 hexane-acetone, v/v) and subjected to ultrasonication in a bath sonicator (PCI Analytics Pvt. Ltd., Mumbai, India; 250W) for 50 min. Supernatant was separated by vacuum filtration and the process was repeated by adding fresh solvent into the same matrix. A total of 5 cycles were repeated until the residue became colorless. The solvent fractions were pooled together, partitioned with double distilled water (3×150 mL), and passed through anhydrous Na_2SO_4 . Complete drying of solvent was done by rotary evaporation. Physical weight of the dried extract was recorded and it was stored in dark at -20 °C before HPLC analysis.

3.4.1.3. Ultrasonic probe extraction (UPE)

3.4.1.3.1. Preliminary extraction

In a beaker (500 mL), 5 g of lyophilized marigold powder was weighed and 150 mL of extraction solvent (1:1 hexane-acetone, v/v) was added. The mixture was then exposed to the titanium horn (13 mm diameter) of an ultrasound probe sonicator (VCX 750, Sonics, Sonics & Materials Inc., CT, USA) having maximum frequency of 20 kHz. The sample was treated for 10 min at 20% amplitude of ultrasonic power keeping in an ice bath. After the ultrasound treatment, the extract was vacuum filtered, partitioned with double distilled water (3×100 mL), collected through anhydrous Na_2SO_4 , and evaporated to dryness by a rotary vacuum evaporator. A separate experiment was carried out using similar process with 250 mL extraction solvent, 60% amplitude of ultrasonic power, and 30 min of ultrasound treatment (5 min interval was given after every 10 min of ultrasonication to dissipate the heat generated

from the continuous process). Physical yield was recorded for both the experiments and HPLC analysis was carried out.

3.4.1.3.2. Optimization of extraction protocol by response surface methodology (RSM)

To extract maximum amount of xanthophylls (lutein esters) using minimal quantity of solvent in least time, the extraction procedure was further optimized using response surface methodology (RSM). A Box-Behnken design (BBD) of the RSM was adapted to optimize the extraction protocol with three independent variables which reported to have significant impact on ultrasound assisted extraction of phytochemicals (Sarkar *et al.*, 2021). Three different levels of each of the variables namely, solvent to solid ratio (30:1, 40:1 and 50:1; mL g⁻¹), ultrasonication amplitude (20, 40 and 60; %), and ultrasonication time (10, 20 and 30; min) were selected to generate a factorial design of 17 experimental runs/treatments including five replicates at the central point using the Design Expert software (version 9, Stat-Ease Inc., Minneapolis, MN, USA). The coded forms of the independent variables and the layout of the experimental design have been presented in Tables 3.1 and 3.2, respectively.

Table 3.1. Variables (actual and coded) used for experimental design of UPE

Independent variable	Coded level of the variable		
	-1	0	1
Solvent:solid (mL g ⁻¹)	30	40	50
Ultrasonication amplitude (%)	20	40	60
Time (min)	10	20	30

Table 3.2. Layout of the UPE experiment for optimized extraction by RSM

Run	Solvent:solid	Ultrasonication amplitude	Time
1	1	0	1
2	0	0	0
3	1	0	-1
4	-1	1	0
5	0	0	0
6	1	-1	0
7	0	1	-1
8	1	1	0
9	-1	0	1
10	0	-1	-1
11	0	0	0
12	0	0	0
13	-1	-1	0
14	0	1	1
15	-1	0	-1
16	0	-1	1
17	0	0	0

The experiment was carried out by keeping the samples in an ice bath and 5 min interval was given after every 10 min of ultrasonication cycle. Extracts obtained from the experimental runs were processed individually as mentioned in section 3.4.1.3.1. Physical yield obtained from each treatment was recorded and total xanthophyll content was calculated after analyzing in HPLC. The data were fitted to a second order response surface model (quadratic polynomial) to get the optimum values of the independent variables for maximum output. The results were further validated by conducting the experiment in the optimized conditions.

3.4.1.3.3. Optimization using genetic algorithm (GA)

More accurate optimization of the extraction variables was carried out by fitting the non-linear exponential form of the quadratic polynomial equation obtained from the BBD experiment into a genetic algorithm (GA) based mathematical model. This robust optimization approach, inspired by Darwin's theory of natural selection to preserve only the fittest individuals over different generations, has the advantage to select the best solution from a set of random prior solutions through an artificial intelligence-based algorithm (Sarkar *et al.*, 2021; Keerthiraj *et al.*, 2022). The idea of this evolutionary algorithm is to combine the different solutions generation after generation to extract the best information from each one. In the present study, the data obtained from the BBD experiment were used as seed values (starting solutions) for the GA to produce more unique random solutions in consecutive generations by applying genetic operators (cross over and mutation) until the most optimal solution was reached. The non-linear model fitting was carried out using SAS 9.3 and the GA based optimization was executed in R software (version 4.1.0). A validation experiment of the most optimal solution was conducted in real time conditions.

3.4.1.4. Comparison of antioxidant activity of xanthophylls extracted by different techniques

The quality of xanthophylls extracted by different techniques was compared by carrying out antioxidant assays (free radical scavenging activity and reducing power). Ascorbic acid (6.25-100 $\mu\text{g mL}^{-1}$) was chosen as standard antioxidant in all the assays. Samples (500-4000 $\mu\text{g mL}^{-1}$) were prepared by dissolving required quantity of xanthophyll extracts in 1:1 (v/v) mixture of methanol and MTBE. For all the assays, blank samples were maintained. Absorbance of the samples was measured by a UV-vis spectrophotometer (SPECORD 200, Analytik Jena, Jena, Germany) at assay specific absorption maxima (λ_{max}). Vitamin C equivalent antioxidant capacity (VCEAC) of the samples for every assay was calculated by putting the antioxidant

value of the samples to the standard curve obtained with ascorbic acid/vitamin C (Kim *et al.*, 2002b). The following methodologies were adapted for the antioxidant assays:

3.4.1.4.1. DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The ability of antioxidants to scavenge the stable DPPH free radicals was assessed by this test (Brand-Williams *et al.*, 1995). DPPH solution (0.5 mM) was prepared by dissolving exact amount of the reagent in methanol. In a test tube, standard/sample solution (1 mL) was mixed with 2 mL of DPPH solution and 4 mL of methanol, vortexed, and incubated in dark at room temperature for 40 min. Absorbance was recorded at the wavelength of 517 nm. The antioxidant activity was expressed in terms of per cent inhibition of the reagent as per the following equation:

$$\text{Inhibition (\%)} = \left[1 - \left(\frac{A_{\text{sample}}}{A_{\text{blank}}} \right) \right] \times 100 \quad (\text{eq. 3.1})$$

Where, A is the absorbance at 517 nm. IC₅₀ values (concentration at 50% inhibition) were calculated from the inhibition (%) vs. concentration curve of the samples.

3.4.1.4.2. ABTS (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) assay

This assay measured the capacity of the antioxidants to scavenge ABTS^{•+} free radicals (Re *et al.*, 1999). For this assay, 7 mM ABTS solution (25 mL) and 2.45 mM potassium persulfate (K₂S₂O₈) solution (14.9 mL) were mixed together and kept for 30 min until a greenish-blue color appeared. The mixture (20 mL) was then diluted with ethanol (300 mL) to reduce the color intensity. In a test tube, 0.3 mL standard/sample was added to 2.7 mL of the prepared reagent solution and absorbance was recorded at 734 nm. Antioxidant activity expressed in terms of inhibition (%) of the reagent was measured as per equation 3.1 (λ_{max} 734 nm) and IC₅₀ values were calculated.

3.4.1.4.3. FRAP (ferric reducing antioxidant power) assay

The ability of the antioxidants to convert ferric ion (Fe³⁺) to ferrous ion (Fe²⁺) was measured by this assay (Benzie and Strain, 1996). The FRAP reagent was prepared by mixing aqueous ferric chloride (FeCl₃) solution (20 mM), TPTZ (2,4,6-tripyridyl-s-triazine) solution (10 mM) in 40 mM HCl, and acetate buffer (300 mM; pH 3.6) at the ratio of 1:1:10 (v/v/v). This reagent (3 mL) was added to 0.1 mL of sample/standard, incubated for 40 min, and absorbance was recorded at 593 nm. Antioxidant activity was expressed in terms of EC₅₀ (Effective concentration to reduce 50% of ferric ion) values by calculating the concentration of samples at 0.5 absorbance.

3.4.1.4.4. CUPRAC (cupric ion reducing antioxidant capacity) assay

This test is based on the ability of the copper-neocuproine reagent to oxidize the antioxidants (Apak *et al.*, 2004). In this assay, 1 mL sample/standard was mixed with 1 mL each of aqueous cupric chloride (CuCl_2) solution (1mM), ethanolic neocuproine solution (7.5 mM), and ammonium acetate buffer (1 M) with vigorous shaking, followed by volume make up to 4.1 mL with distilled water. After 30 min of incubation, absorbance was recorded at 450 nm wavelength. Effective concentration to reduce 50% of cupric ion (EC_{50}) was calculated to express the antioxidant activity.

3.4.2. Extraction using green solvents

3.4.2.1. Synthesis of hydrophobic deep eutectic solvents (HDES)

Hydrophobic deep eutectic solvents (HDES) were synthesized by combining two saturated fatty acids (Figure 3.1) namely caprylic acid (octanoic acid, M.W. 144.22) and capric acid (*n*-decanoic acid, M.W. 172.27) at different molar ratios (3:1, 2:1, 1:1, 1:2 and 1:3). Required quantities of the two fatty acids were precisely weighed in a glass beaker and stirred continuously at 50 °C until clear homogeneous liquids were formed. The prepared eutectic mixtures were brought to room temperature and stored in air-tight glass vials until further use.

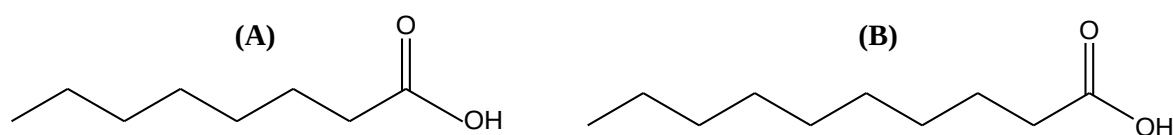


Figure 3.1. Chemical structures of caprylic acid (A) and capric acid (B)

3.4.2.2. Characterization of the prepared HDES

3.4.2.2.1. Density

Density of the prepared HDES was measured at room temperature (30 ± 2 °C) using a specific gravity bottle (10 mL). Volume calibration of the specific gravity bottle was done before recording any measurements.

3.4.2.2.2. Differential Scanning Calorimetry (DSC)

Thermal analysis of the prepared HDES along with caprylic and capric acid were conducted using a differential scanning calorimeter (DSC 2500, TA Instruments, New Castle, DE, USA) coupled with TRIOS processing software (version 5.1.1). About 5-10 mg of sample was loaded into a hermetically sealed aluminium pan and cooled to -50 °C. The samples were scanned

within the temperature range from -50 to 150 °C with a heating rate of 10 °C min^{-1} . Inert environment (N_2 with flow rate of 50 mL min^{-1}) was maintained throughout the analysis.

3.4.2.2.3. Fourier Transform Infrared (FT-IR) spectroscopy

FT-IR spectra of the HDES, caprylic acid and capric acid were recorded in a FT-IR spectrometer (ALPHA, Bruker, Billerica, MA, USA) operated in the ATR (attenuated total reflection) mode and coupled with OPUS processing software (version 7.0.122). The samples were scanned (typical 24 scans) over a range of 600 - 4000 cm^{-1} at a resolution of 2 cm^{-1} .

3.4.2.2.4. Nuclear Magnetic Resonance (NMR) spectroscopy

The ^1H NMR spectra of the eutectic mixtures and the constituent fatty acids were recorded in a 400 MHz nuclear magnetic resonance spectrometer (JNM-ECZ400R, JEOL, Tokyo, Japan) coupled with the processing software Delta (version 5.3.1). Each sample was dissolved in deuterated chloroform (CDCl_3) and 0.6 mL of the solution was loaded in sample probe for analysis.

3.4.2.3. Ultrasound assisted HDES based extraction of lutein esters

3.4.2.3.1. Single factor optimization

Ultrasound assisted extraction process was adapted for extracting the xanthophylls from marigold florets using HDES. Initially, to understand the extraction efficiency of the prepared HDES, a single factor optimization experiment was carried out. Three factors namely, molar ratio of caprylic acid (C_8) and capric acid (C_{10}), volume of HDES, and ultrasonication time were taken into consideration. At first, 0.1 g of the lyophilized marigold powder was taken in a glass vial and 5 mL of the HDES ($\text{C}_8\text{:C}_{10}$ of $1\text{:}1$ molar ratio) was added to it. After closing the vial, it was exposed to a bath sonicator (PCI Analytics Pvt. Ltd., Mumbai, India; 250W) for 15 min maintaining the temperature at 30 ± 2 °C. The extract was collected immediately by centrifugation at 3000 rpm for 10 min in a centrifuge (Z 326 K, Hermle Labortechnik GmbH, Wehingen, Germany). The supernatant was diluted 10 times with HPLC grade solvent ($1\text{:}1$ mixture of methanol-MTBE; v/v), filtered through a syringe filter (0.45 μm) and analyzed by HPLC. The same process was repeated for extraction with the other HDES ($\text{C}_8\text{:C}_{10}$ of $1\text{:}2$, $2\text{:}1$ and $3\text{:}1$ molar ratio). Further, to observe the effect of ultrasonication time on extraction, the process was carried out for 15 , 30 , 45 , 60 , 75 and 120 min using 5 mL of HDES ($\text{C}_8\text{:C}_{10}$ of $2\text{:}1$). Similarly, the effect of volume of extraction solvent was studied by carrying out the experiment with 5 , 7.5 and 10 mL of HDES ($\text{C}_8\text{:C}_{10}$ at $2\text{:}1$) for 45 min of ultrasonication.

3.4.2.3.2. Optimization of extraction protocol by response surface methodology (RSM)

The ultrasound assisted extraction procedure of xanthophylls using HDES was further optimized by a Box-Behnken design (BBD) of response surface methodology (RSM). The design involved three independent factors namely, molar ratio of fatty acids (C₈:C₁₀) constituting the HDES, volume of HDES, and ultrasonication time, each at three levels. The coded forms of the independent variables and the layout of the experimental design have been presented in Tables 3.3 and 3.4, respectively.

Table 3.3. Variables (actual and coded) used for experimental design of ultrasound assisted extraction using HDES

Independent variable	Coded level of the variable		
	−1	0	1
C ₈ :C ₁₀ (molar ratio)	0.5	1.25	2.0
Volume of solvent (mL g ^{−1})	50	75	100
Time (min)	15	30	45

Table 3.4. Layout of the experiment of ultrasound assisted extraction using HDES for optimized extraction by RSM

Run	C ₈ :C ₁₀	Volume	Time
1	1	0	−1
2	0	0	0
3	−1	−1	0
4	0	0	0
5	0	0	0
6	0	1	1
7	−1	0	−1
8	0	0	0
9	−1	0	1
10	1	−1	0
11	0	−1	1
12	0	1	−1
13	0	0	0
14	1	1	0
15	−1	1	0
16	0	−1	−1
17	1	0	1

Design Expert software (version 9, Stat-Ease Inc., Minneapolis, MN, USA) was used to generate the experimental layout which involved a total of 17 runs/treatment including five replicates at the central point. Extracts obtained from the experimental runs were processed individually as mentioned in section 3.4.2.3.1. Total xanthophyll content was calculated by analysis in HPLC. The data were fitted to a second order response surface model (quadratic

polynomial) to get the optimum values of the independent variables for maximum output. The results were further validated by conducting the experiment at the optimized conditions.

3.4.2.3.3. Polarity switch for separation of extracted xanthophylls

High viscosity and low volatility of the DES makes them difficult to remove from solution to recover the extracted compounds. Therefore, the concept of polarity switch (Sed *et al.*, 2018) of the DES was introduced in the present study to convert the hydrophobic DES into hydrophilic solvent, which enabled precipitation of the dissolved xanthophylls. To execute the concept, 15 mL of xanthophyll extract obtained from the optimized extraction procedure as described in section 3.4.2.3.2 was taken in a round-bottom flask (250 mL capacity) and 50 mL of 40% aqueous potassium hydroxide (KOH) solution was added to it. The mixture was refluxed on a heating mantle with occasional stirring for 2 h followed by slow cooling to room temperature. Dissolved xanthophylls started to precipitate out, which were collected by centrifuging at 5000 rpm for 15 min. The precipitate was washed with double distilled water several times until the washing became pH neutral. Then the residue was air dried in dark and stored in air-tight glass vial at $-20\text{ }^{\circ}\text{C}$ until being analyzed. Quantification was done in HPLC by dissolving the precipitate in HPLC grade solvent (1:1 mixture of methanol-MTBE; v/v).

3.5. Saponification of lutein esters

The marigold floret extract containing xanthophylls (lutein esters) was saponified to get de-esterified lutein. In a 250 mL round-bottom flask, 0.5 g of dried extract was taken and 50 mL of 40% methanolic KOH was added to it. The content was refluxed for 3 h and cooled to room temperature. Then the content was partitioned with hexane ($3 \times 100\text{ mL}$) and the methanolic fraction was collected. This methanolic fraction was mixed with 20 mL of water and further partitioned with ethyl acetate ($3 \times 50\text{ mL}$). The ethyl acetate fractions were pooled together, dried over anhydrous Na_2SO_4 and concentrated to dryness under reduced pressure by rotary vacuum evaporator. The dried residue was stored at $-20\text{ }^{\circ}\text{C}$ until further analysis.

3.6. Characterization of surface morphology of marigold powder by scanning electron microscopy (SEM)

Surface morphology of the lyophilized marigold powder before and after extraction was analyzed using a Tescan Vega-3 LMU scanning electron microscope (Brno-Kohoutovice, Czech Republic) operated at 5.0 kV HV and average working distance of 19 mm. Samples were prepared by mounting on metal stubs with a double-sided tape followed by thin-layer sputter coating of gold using Denton Vacuum Desk II sputter coater (Moorestown, NJ, USA).

3.7. Characterization of xanthophylls

3.7.1. UPLC-QToF-ESI-MS analysis

Profiling of the xanthophylls in the marigold extract was carried out in an ultra-performance liquid chromatography (UPLC) system (ACQUITY, Waters Corporation, Milford, MA, USA) coupled with a SYNAPT G2 HDMS (high-definition mass spectrometer), equipped with an electrospray ionization (ESI) source and hybrid quadrupole-time of flight (Q-ToF) technology. Sample was dissolved in LC-MS grade acetonitrile, filtered through 0.45 μm syringe filter, and injected (injection volume 10 μL) to the UPLC system equipped with binary pump, degasser, autosampler and diode-array detector (DAD) for chromatographic separation on an ACQUITY UPLC BEH C18 column (2.1 mm $\times$ 100 mm, 1.7 μm ; Waters India Pvt. Ltd., New Delhi, India) at 25 $^{\circ}\text{C}$. The mobile phase, consisted of water (phase A) and acetonitrile (phase B), both containing 0.1% formic acid, was used with the following gradient at a flow rate of 0.3 mL min^{-1} : 0-0.5 min, 50% B; 0.5-3.0 min, 80% B, and 3.0-120 min, 100% B. The ESI source was operated in the positive mode. The optimized ion source parameters included ion source temperature (120 $^{\circ}\text{C}$), dissolution temperature (350 $^{\circ}\text{C}$), dissolution gas flow (500 L h^{-1}), cone gas flow (50 L h^{-1}), capillary voltage (3 kV), sampling cone voltage (30 V), and extraction cone voltage (5 V). Mass data were acquired in continuum mode within the range of m/z 50-1200 with the MSE function which provided the full scan MS data (low energy, 6 V) and MS/MS data (high energy, 20-60 V ramping), simultaneously. Mass spectrometer calibration was done with 0.5 mM sodium formate solution, whereas, leucine enkephalin (2 $\mu\text{g mL}^{-1}$) was used as the reference standard (m/z 556.2771 in positive and m/z 554.2670 in negative polarity) in lock spray at the flow rate of 20 $\mu\text{L min}^{-1}$ for mass correction at every 10 s interval. Data analysis was done with the help of the MassLynx software (version 4.2).

3.7.2. HPLC analysis

Lutein and lutein esters were quantified by an Agilent 1260 Infinity II high performance liquid chromatography (HPLC) system (Santa Clara, CA, USA) equipped with quaternary pump, degasser, auto-sampler and variable wavelength detector (VWD). Chromatographic separation was carried out at 35 $^{\circ}\text{C}$ on a YMC Carotenoid C30 column (4.6 mm $\times$ 250 mm, 5 μm ; YMC Co. Ltd., Kyoto, Japan). The mobile phase, consisted of methanol (phase A) and MTBE (phase B), was used as per the gradient program represented in Table 3.5., with a total run time of 85 min. Required quantity of samples were dissolved in 1:1 mixture (v/v) of methanol and MTBE, filtered through syringe filter (0.45 μm), and 20 μL injection volume was introduced into the

HPLC. Major peaks detected at 446 nm wavelength were integrated, summed together, and quantified by comparing with the calibration curve prepared with standard lutein solution (3.125-50 $\mu\text{g mL}^{-1}$). Total xanthophyll content of the samples was expressed in terms of lutein equivalent (Piccaglia *et al.*, 1998).

Table 3.5. Gradient program for HPLC analysis of xanthophylls

Time (min)	Solvent A (%)	Solvent B (%)	Flow rate (mL min ⁻¹)
0.00	80.0	20.0	0.8
9.00	80.0	20.0	0.8
10.00	80.0	20.0	1.2
80.00	55.0	45.0	1.2
83.00	55.0	45.0	1.2
85.00	80.0	20.0	0.8

3.8. Preparation of water-soluble powder formulation of xanthophylls

Freeze drying technique of biopolymer stabilized oil-in-water (O/W) microemulsion was employed for preparation of water-soluble powder formulation of xanthophylls (lutein esters) with the objective to overcome the impediment of their water solubility.

3.8.1. Preparation of biopolymer stabilized oil-in-water (O/W) microemulsions

3.8.1.1. Primary coarse emulsions

As the first step to prepare the O/W emulsion, two phases (water and oil) were prepared separately. The water phase was prepared by dissolving a water dispersible biopolymer (a polysaccharide of approximately 500 kDa molecular weight) in double distilled water (10%; w/v). The biopolymer solution was centrifuged at 13000 rpm for 1 h to remove any insoluble and extraneous particles. The supernatant was collected and mixed with a defined amount of surfactant. Three different surfactants (S1, S2, and S3) were separately used in the study at three different concentrations to observe their effects on microemulsion formation. Surfactants S1 and S2 were used at 2.5, 5.0, and 7.5% (w/v) level; whereas, level of surfactant S3 was 1.0, 2.0, and 3.0% (w/v), depending upon their physico-chemical properties and solubility parameters. The oil phase was prepared by dissolving the extracted xanthophylls (lutein esters) in a green lipophilic solvent at the concentration of 5% (w/v). The green solvent used in this study was non-toxic, environmentally safe, and of food grade. Initially, a primary coarse emulsion was prepared by addition of the oil phase (0.1 mL) into the water phase (9.9 mL) maintaining the concentration of oil phase at 1% (v/v). The mixture was then vortexed for 5 min to prepare the primary O/W emulsion. A total of nine sets of primary emulsions were

prepared using three levels of each of the surfactants separately. The chemical identities of the formulation ingredients are not being disclosed here due to a possible chance of IPR filling.

3.8.1.2. Secondary microemulsions

Emulsion dilution technique was used to prepare secondary O/W microemulsions from individual primary emulsion. Exact volume of the primary emulsion was pipetted out and mixed with the same water phase (containing biopolymer and surfactant) which was used to prepare the primary emulsion. Three sets of secondary microemulsions containing 0.1, 0.25, and 0.5% oil phase were prepared from each set of primary emulsion. Then they were subjected to ultrasonication in a bath sonicator (PCI Analytics Pvt. Ltd., Mumbai, India; 250W) for 2 h at room temperature. Thereafter, the secondary microemulsions were kept for ageing at ambient temperature (25 ± 1 °C) overnight and were subjected to further characterizations.

3.8.2. Physico-chemical characterization of microemulsions

3.8.2.1. Particle size analysis

The average droplet diameter and size distribution of the prepared microemulsions were determined using a particle size analyzer (Zetatrak, Microtrac, Montgomeryville, PA, USA) using the laser scattering principle. The polydispersity index (PDI) was calculated using the following formula:

$$\text{Polydispersity index (PDI)} = (\text{Standard deviation}/\text{Mean})^2 \quad (\text{eq. 3.2})$$

3.8.2.2. Transmission Electron Microscopy (TEM)

The droplet morphology and droplet size of the microemulsions were further analyzed in a transmission electron microscope (JEM-1011, JEOL, Tokyo, Japan) operated at an accelerated voltage of 80 kV. Sample was impregnated on a carbon coated 400 mesh copper grid and kept for 10 min for partial drying. Then the grid was stained with uranyl acetate solution (2%) after washing with double distilled water and kept for 3 h for complete drying. Micrographs were visualized at 30kx-120kx magnifications.

3.8.2.3. Thermodynamic stability

The microemulsion samples were subjected to thermodynamic stability studies by exposing to subsequent stress tests as per the following steps described by Shafiq *et al.* (2007):

- i) *Heating-cooling cycle*: The samples were exposed to six consecutive cycles of refrigeration (4 °C) and heating (45 °C) for 48 h at each temperature. Those samples

which did not show any phase separation or sedimentation after completion of the heating-cooling cycles were chosen for the second stress test.

- ii) *Centrifugation*: The samples were subjected to centrifugation at 3500 rpm for 30 min and those for which any phase separation or sedimentation was not observed, were selected for the third stress test.
- iii) *Freeze-thaw cycle*: The samples were exposed to three consecutive cycles of freezing ($-21\text{ }^{\circ}\text{C}$) and thawing ($25\text{ }^{\circ}\text{C}$) for 48 h at each temperature. The samples which passed this third test too were chosen for dispersibility test for efficiency of self-emulsification.

3.8.2.4. Dispersibility

Dispersibility test was performed by dropping 0.1. mL of the microemulsion sample into 50 mL of deionized water with gentle agitation (50 rpm) on a magnetic stirrer at $30 \pm 1\text{ }^{\circ}\text{C}$. The self-emulsification efficiency of the microemulsion samples was visually assessed as per the following grading system:

- *Grade A*: Rapid formation of transparent emulsion
- *Grade B*: Rapid formation of slightly less clear emulsion
- *Grade C*: Quick formation of milky emulsion
- *Grade D*: Slow formation of dull grayish emulsion with slightly oily appearance
- *Grade E*: Poor emulsification with large oil globules at the surface

The microemulsions which passed the thermodynamic stability tests and scored A or B grade in dispersibility test, were considered as stable microemulsions.

3.8.2.5. Color analysis of the microemulsions

Color of the prepared microemulsions have been assessed using a RGB color-cube concept depending on camera-based image technique (Joshi *et al.*, 2016; Minz and Saini, 2021). Each sample (3 mL) was placed individually in a central sample holder kept inside a cabinet with neutral gray internal walls. Images of the sample were captured in the dark condition using a 48-megapixel digital camera placed exactly vertical to the sample holder at a fixed distance of 6 inches (Thippeswamy *et al.*, 2022). Intensities of the primary colors (red-R, green-G, and blue-B) within the standard range of 0-255 at three different points of the photograph of the sample were calculated using an RGB color-cube (<https://www.rapidtables.com/web/color/color-tester.html>). The RGB values (mean values of three different points) were then converted into Hunter's L^* (whiteness/darkness), a^* (redness/greenness), and b^* (yellowness/blueness) coordinates

(<http://colormine.org/convert/rgb-to-hunterlab>). These values were further used for determination of hue angle and chroma of the samples using the following formulae (McLellan *et al.*, 1995):

$$\text{Chroma} = \sqrt{(a^{*2} + b^{*2})} \quad (\text{eq. 3.3})$$

$$\text{Hue angle} = \tan^{-1}\left(\frac{b^*}{a^*}\right) \quad (\text{eq. 3.4})$$

Equation 3.4 was used as such when the values of both a^* and b^* were positive. With the negative value of a^* and positive value of b^* , 180° was added with the arc tangent value in the equation 3.4 to change the quadrant, and the following equation was used for such cases:

$$\text{Hue angle} = 180^\circ + \tan^{-1}\left(\frac{b^*}{a^*}\right) \quad (\text{eq. 3.5})$$

The color difference of the samples from the control *i.e.* microemulsions without xanthophylls (ΔE) was also calculated using the following formula:

$$\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2} \quad (\text{eq. 3.6})$$

Where, $\Delta L = L^*_{\text{sample}} - L^*_{\text{control}}$; $\Delta a = a^*_{\text{sample}} - a^*_{\text{control}}$; and $\Delta b = b^*_{\text{sample}} - b^*_{\text{control}}$.

3.8.3. Preparation of water-soluble powder

Based on the physico-chemical characteristics, three best performing microemulsions were selected for preparation of water-soluble solid powders. It was done by simple freeze-drying technique. The selected biopolymer stabilized microemulsions were frozen at -20°C , and subjected to lyophilization in a lyophilizer (FreeZone 6 Plus, Labconco, Kansas City, MO, USA) for complete removal of water. The obtained solid masses were crushed to obtain free flowing powders, which were stored in air-tight containers at -20°C away from direct light.

3.9. Physico-chemical characterization of solid powder formulations

3.9.1. Fourier Transform Infrared (FT-IR) spectroscopy

FT-IR spectra of the powder formulations, the biopolymer, and xanthophylls were recorded in a FT-IR spectrometer (ALPHA, Bruker, Billerica, MA, USA) operated in the ATR mode and coupled with OPUS processing software (version 7.0.122). The samples were scanned (typical 24 scans) over a range of $600\text{--}4000\text{ cm}^{-1}$ at a resolution of 2 cm^{-1} .

3.9.2. Scanning Electron Microscopy (SEM)

Surface morphology of the powder formulations were analyzed using a Tescan Vega-3 LMU scanning electron microscope (Brno-Kohoutovice, Czech Republic) operated at 10.0 kV HV and average working distance of 14 mm. Samples were prepared by mounting on metal stubs with a double-sided tape followed by thin-layer sputter coating of gold using Denton Vacuum Desk II sputter coater (Moorestown, NJ, USA).

3.9.3. Xanthophyll content

Total amount of xanthophylls loaded in the solid powder formulations was measured by extracting them followed by their analysis in HPLC. Formulation sample (0.5 g) was taken in a glass vial and 10 mL of extraction solvent (1:1 mixture of methanol and MTBE) was added to it. After closing with screw cap, the vial was placed in a bath sonicator and sonicated for 15 min. The supernatant was filtered immediately and fresh volume of solvent (10 mL) was again added to the residue and sonicated for further 15 min. This process was repeated until no color was observed in the supernatant. The supernatant was evaporated to dryness by a rotary vacuum evaporator and final volume was made up to 5 mL with HPLC grade methanol-MTBE mixture (1:1), filtered through syringe filter (0.45 μ m), and analyzed by HPLC.

3.9.4. Accelerated storage stability

The solid powder formulations were stored separately in air-tight glass containers away from light at 54 ± 2 °C, 26 ± 2 °C (room temperature), and 0 ± 2 °C continuously for 14 days. The formulations were removed from the storage after the specified period, brought to ambient temperature, extracted as per section 3.9.3, and analyzed in HPLC to record any changes in total xanthophyll content.

3.9.5. Color analysis

The color intensities of aqueous solution of the readily soluble powder formulations were assessed as per the methodology described in section 3.8.2.5. Specified quantities (0.25, 0.50, 0.75, and 1.00 g) of the formulations were separately dissolved in 10 mL of water, vortexed for complete dissolution, and analyzed for hue angle, chroma, and ΔE (intensity difference from control *i.e.* formulations without xanthophyll).

3.9.6. Antioxidant activity

Antioxidant activity (by DPPH, FRAP and CUPRAC assays) of the aqueous solutions of solid powder formulations (2.5, 5, 7.5, and 10%) were carried out as per the methods described in section 3.4.1.4., and VCEAC values of the samples were calculated and compared.

3.9.7. *In vitro* bioavailability

An *in vitro* experimental setup was established to mimic the gastrointestinal absorption of xanthophylls from the ingested formulations (Gil-Izquierdo *et al.*, 2002). As the first step to mimic the digestion in stomach, 1 g of sample was taken in a beaker (100 mL capacity) and 20 mL of acidified water (pH 1.7, adjusted with conc. HCl) was added to it, followed by addition of 25.2 mg of pepsin. The mixture was incubated for 2 h at room temperature (35 ± 2 °C) with continuous stirring at slow speed (100 rpm). After 2 h of incubation, to mimic absorption in small intestine, 18 mg pancreatin (4.5 mL of 4 mg mL⁻¹ solution) and 500 mg bile salt were added to the digested sample. A piece of dialysis tube (MWCO 12400, 40 mm flat width with 99.99% retention, utilized as a mimic of the microvilli present in small intestine) was filled with 5.6 mL of 0.75 M NaHCO₃ solution and tightly closed at both ends. It was then placed inside the digested sample and incubated for another 2 h. At the end of the incubation period, total xanthophyll content in solutions inside and outside of the dialysis tube was analyzed by HPLC.

3.10. Statistical analysis

RSM based optimization experiments were carried out using Design Expert software (version 9), whereas, SAS 9.3 and R software (version 4.1.0) were used for GA based optimization experiment. For all other data analysis like comparison of mean, test of significance, post-hoc analysis etc. an open-source statistical program JASP (version 0.16.3.0) was used.

4.1. UPLC-QToF-ESI-MS analysis of xanthophylls extracted from marigold

The first step before quantification of the xanthophylls extracted from marigold florets, was to identify the chemical constituents. Therefore, the xanthophyll extract from the florets of the hybrid genotype (DAMH-39) of African marigold (*Tagetes erecta* L.) was first subjected to UPLC-QToF-ESI-MS analysis. The total ion chromatogram (TIC) of the xanthophyll extract has been presented in Figure 4.1. Total thirteen compounds were tentatively identified based on their molecular mass within the acceptable mass error of 3 ppm, and fragmentation pattern. The compounds have been listed in Table 4.1 based on their retention time in UPLC-DAD.

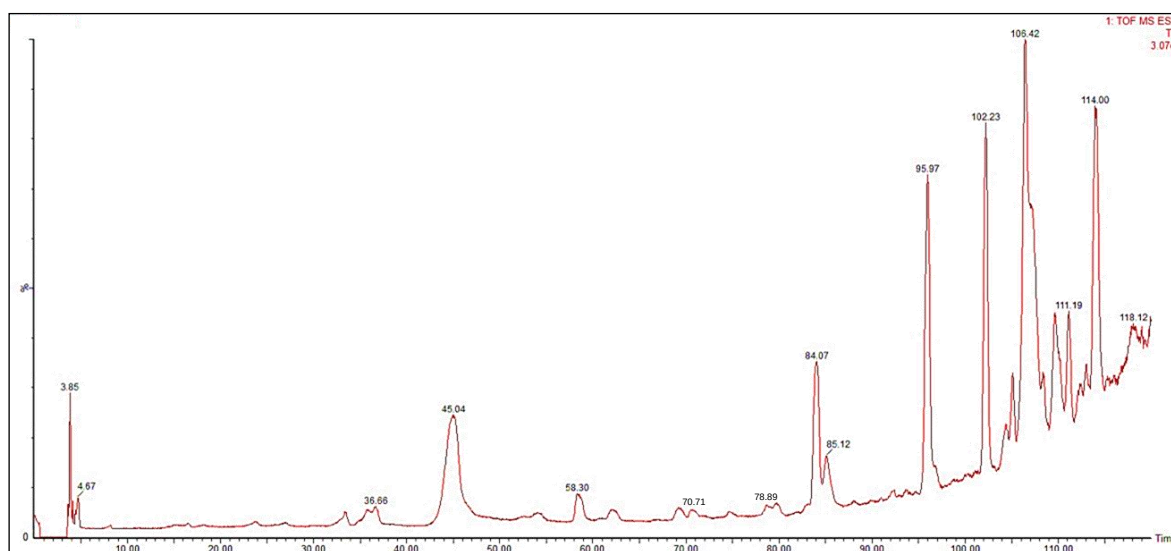


Figure 4.1. Total ion chromatogram (TIC) of xanthophyll extract of marigold (DAMH-39) from UPLC-DAD

The first compound eluted with a minor peak at R_t (retention time) 70.71 min was identified as lutein myristate ($C_{54}H_{82}O_3$) from its protonated molecular ion peak at m/z 779.6351 (relatively less abundant), and the most abundant daughter ion peak at m/z 378, due to loss of cyclic rings at both ends (Figure 4.2). Another minor compound at R_t 78.89 min was identified as lutein palmitate ($C_{56}H_{86}O_3$) with protonated molecular ion peak at m/z 807.6662. Fragmentation of the molecular ion resulted in formation of a daughter ion peak with m/z 610 due to loss of tetradecane moiety from the palmitate chain (Figure 4.3). The first major compound at R_t 83.87 min, was recognized as lutein stearate ($C_{58}H_{90}O_3$) from the accurate molecular ion peak of its protonated adduct at m/z 835.6952. Sequential loss of stearic group followed by subsequent trimethyl cyclohexenol moiety resulted in formation of daughter ion

peaks at m/z 567 and 406, respectively (Figure 4.4). Lutein linoleate ($C_{58}H_{86}O_3$), an ester of lutein with unsaturated fatty acid was closely eluted along with lutein stearate at R_t 83.97 min. This compound was identified from its protonated molecular ion peak at m/z 831.6656, and daughter ions at m/z 567 and 406 due to subsequent de-esterification and loss of cyclic ring structure (Figure 4.5). Lutein laurate ($C_{52}H_{78}O_3$) was found to be eluted at R_t 85.39 min, which was identified from the daughter ion fragments of m/z 390 and 338, originated from the peak of the proton adduct of the molecular ion at m/z 751.6025 (Figure 4.6). The sharp peak eluted at R_t 95.91 min was figured out to be violaxanthin palmitate stearate ($C_{74}H_{120}O_6$), diester of another natural xanthophyll named violaxanthin (di-epoxide form of zeaxanthin). Its protonated adduct exhibited accurate molecular mass at m/z 1105.9095, which was de-esterified at the palmitate end to produce daughter ion peak at m/z 868. The peak at m/z 434 was further generated by the loss of one cyclic ring at the stearate end (Figure 4.7). Interestingly, the peak eluted at R_t 102.23 min was identified as chlorophyll-a ($C_{55}H_{72}MgN_4O_5$) from its molecular ion peak at m/z 893.5439.

Table 4.1. Chemical composition of extract of marigold (DAMH-39) florets as analyzed in UPLC-QToF-ESI-MS

R_t (min)	Compound (tentative)	Molecular formula	Neutral mass	Observed m/z ([M+H] ⁺)	Mass error (ppm)
70.71	Lutein myristate	$C_{54}H_{82}O_3$	778.6267	779.6351	0.77
78.89	Lutein palmitate-I	$C_{56}H_{86}O_3$	806.6577	807.6662	0.86
83.87	Lutein stearate	$C_{58}H_{90}O_3$	834.6894	835.6952	-2.39
83.97	Lutein linoleate	$C_{58}H_{86}O_3$	830.6577	831.6656	0.12
85.39	Lutein laurate	$C_{52}H_{78}O_3$	750.5954	751.6025	-0.93
95.91	Violaxanthin palmitate stearate	$C_{74}H_{120}O_6$	1104.9090	1105.9095	-1.81
102.23	Chlorophyll-a	$C_{55}H_{72}MgN_4O_5$	892.5353	893.5439	0.89
106.46	Lutein dilinoleate	$C_{76}H_{116}O_4$	1092.8874	1093.8956	0.36
106.73	Lutein dibutyrate	$C_{48}H_{68}O_4$	708.5120	709.5191	-0.98
107.30	Lutein dipalmitate	$C_{72}H_{116}O_4$	1044.8874	1045.8966	1.33
111.25	Violaxanthin dipalmitate	$C_{72}H_{116}O_6$	1076.8777	1077.8845	-0.92
112.54	Lutein dilaurate	$C_{64}H_{100}O_4$	932.7622	933.7690	-1.07
114.20	Lutein palmitate-II	$C_{56}H_{86}O_3$	806.6577	807.6656	0.12

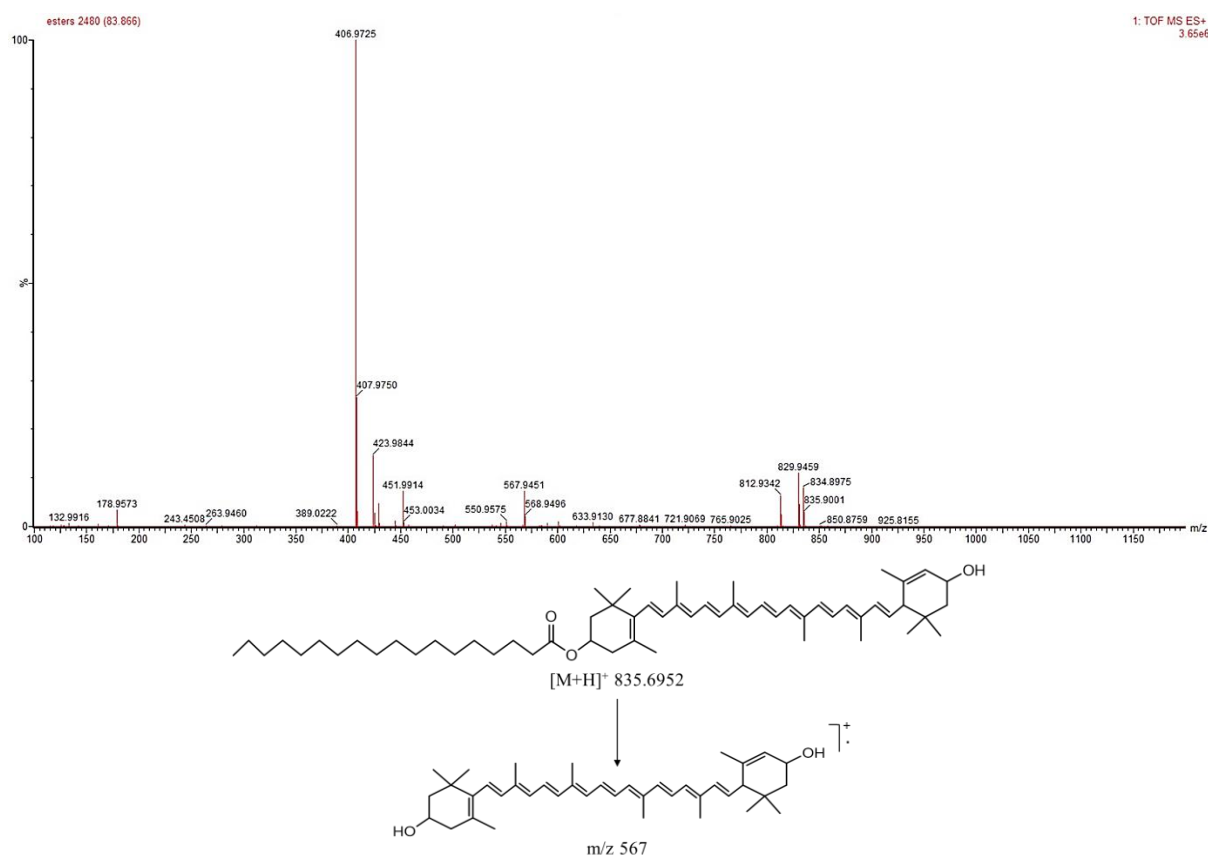


Figure 4.4. Proposed mass fragmentation pattern of lutein stearate in QToF-ESI-MS

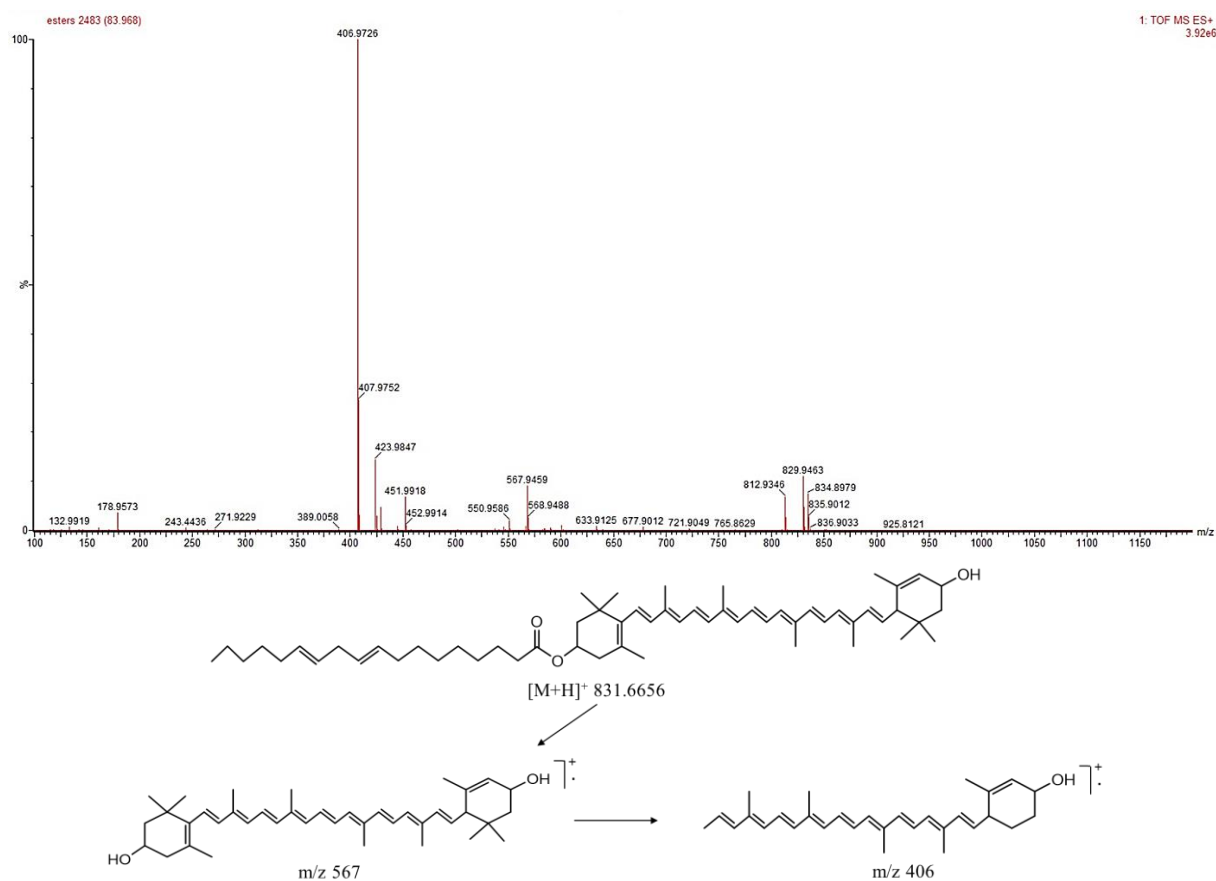


Figure 4.5. Proposed mass fragmentation pattern of lutein linoleate in QToF-ESI-MS

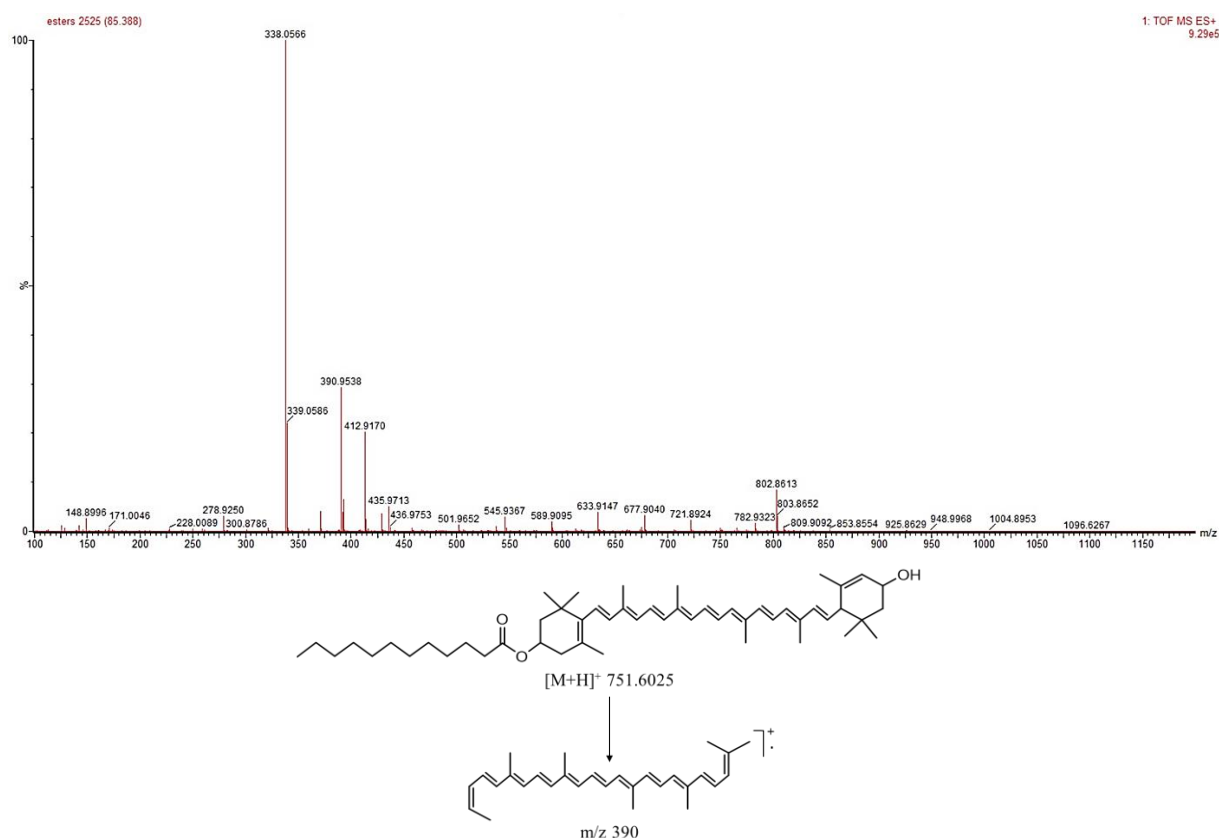


Figure 4.6. Proposed mass fragmentation pattern of lutein laurate in QToF-ESI-MS

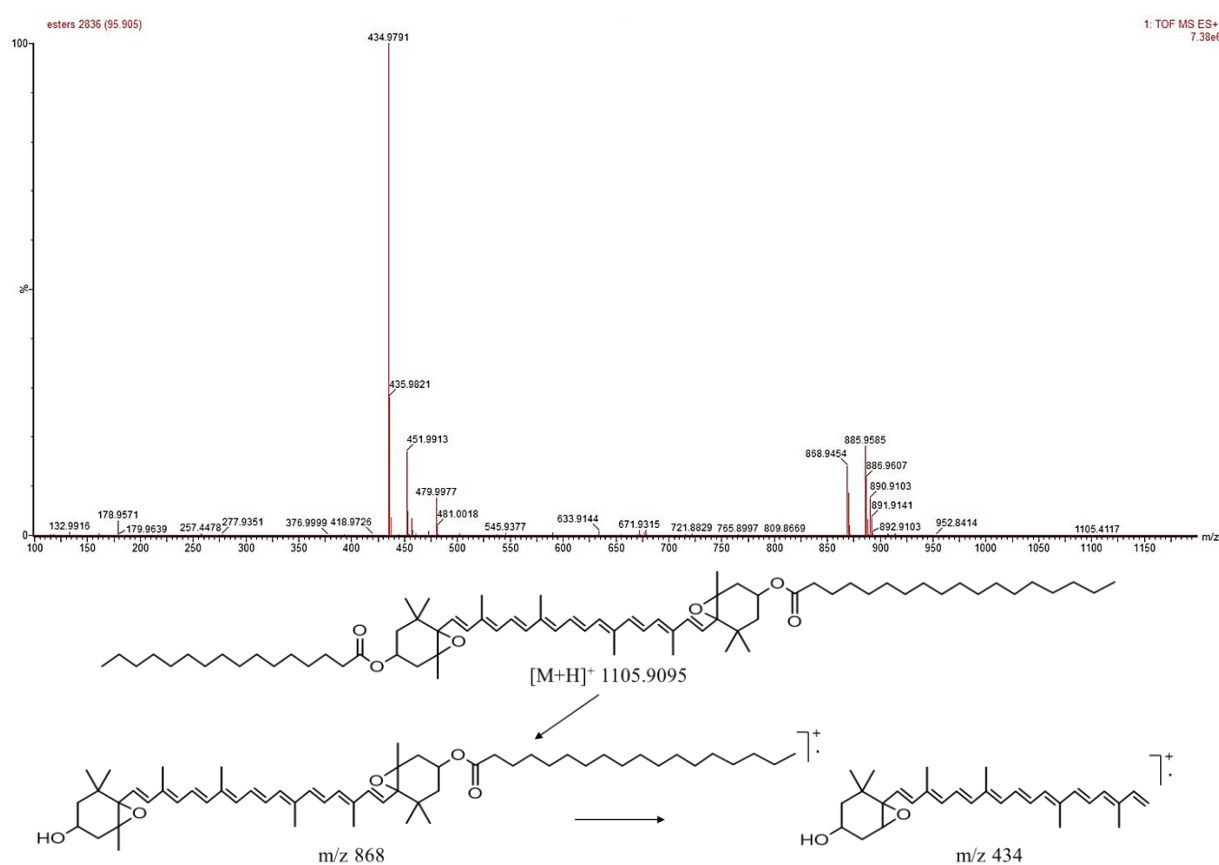


Figure 4.7. Proposed mass fragmentation pattern of violaxanthin palmitate stearate in QToF-ESI-MS

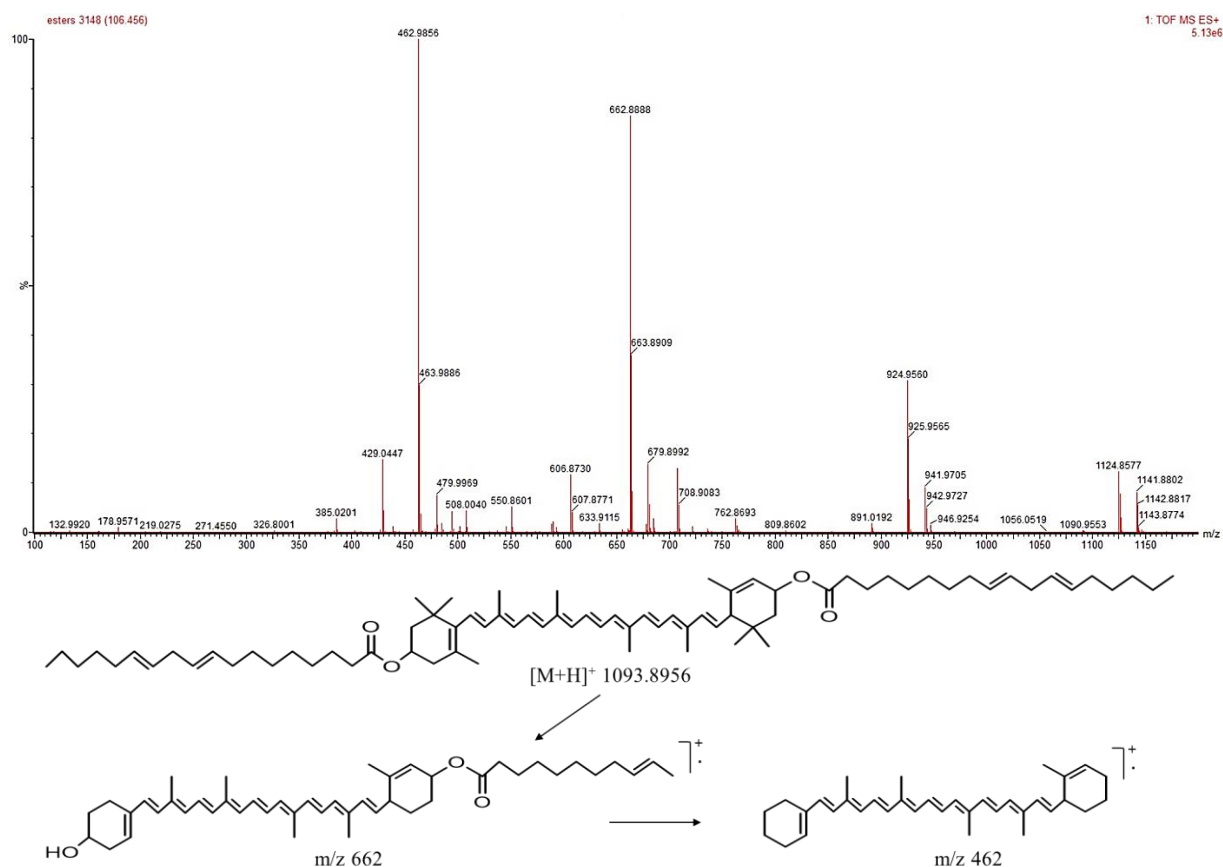


Figure 4.8. Proposed mass fragmentation pattern of lutein dilinoleate in QToF-ESI-MS

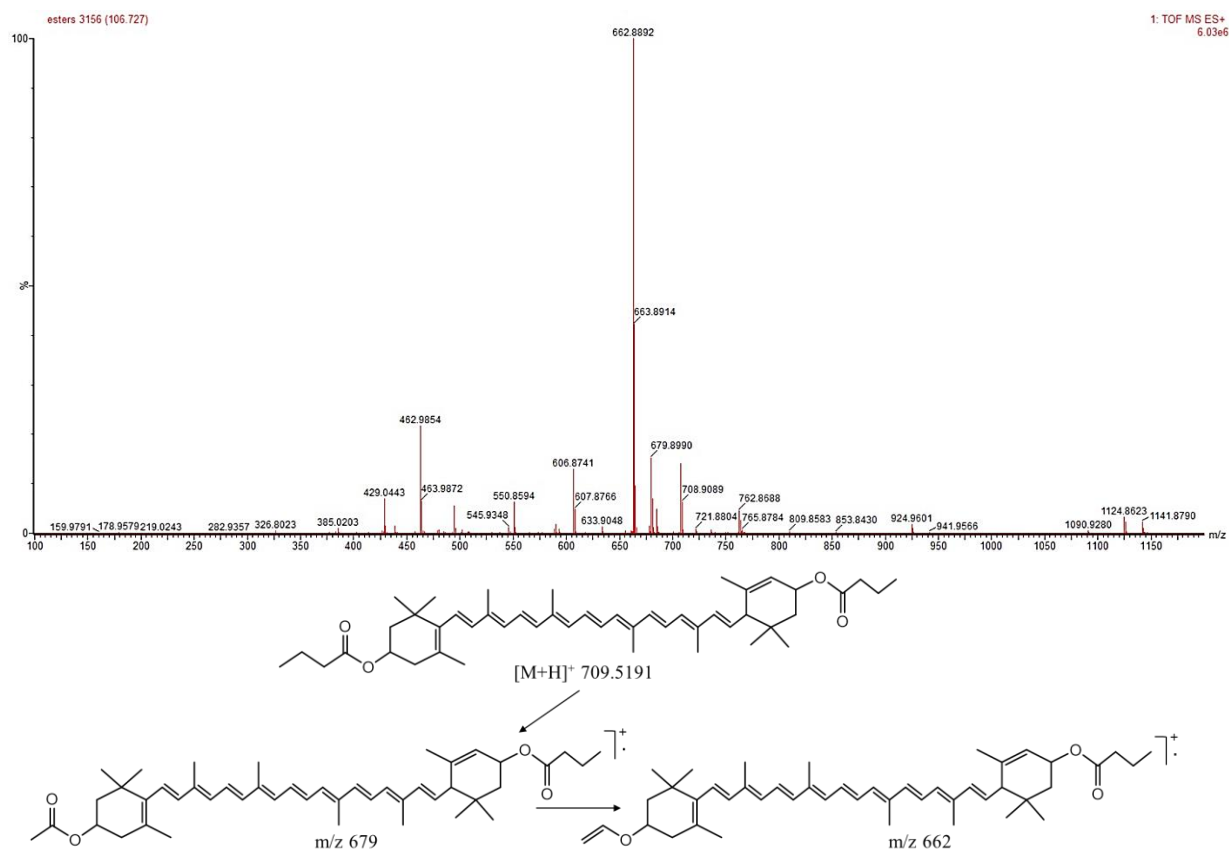


Figure 4.9. Proposed mass fragmentation pattern of lutein dibutyrate in QToF-ESI-MS

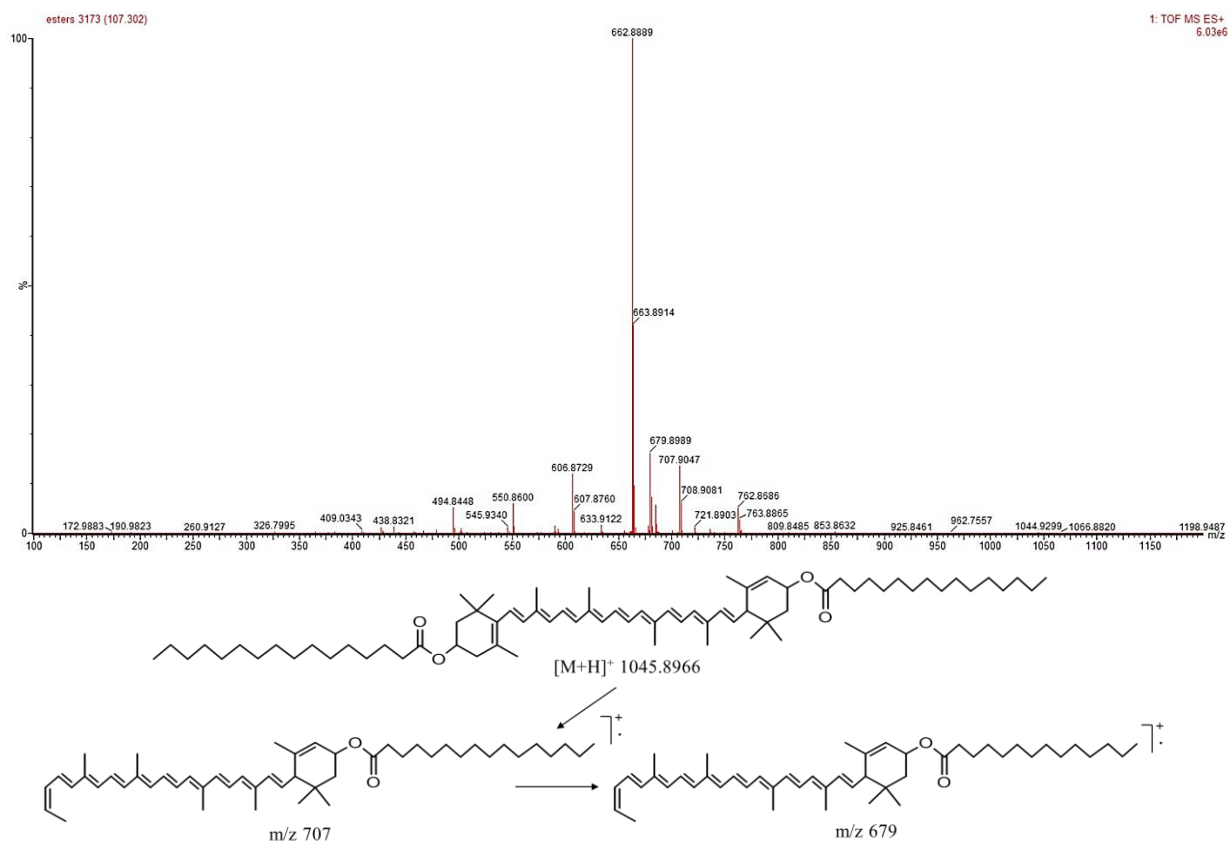


Figure 4.10. Proposed mass fragmentation pattern of lutein dipalmitate in QToF-ESI-MS

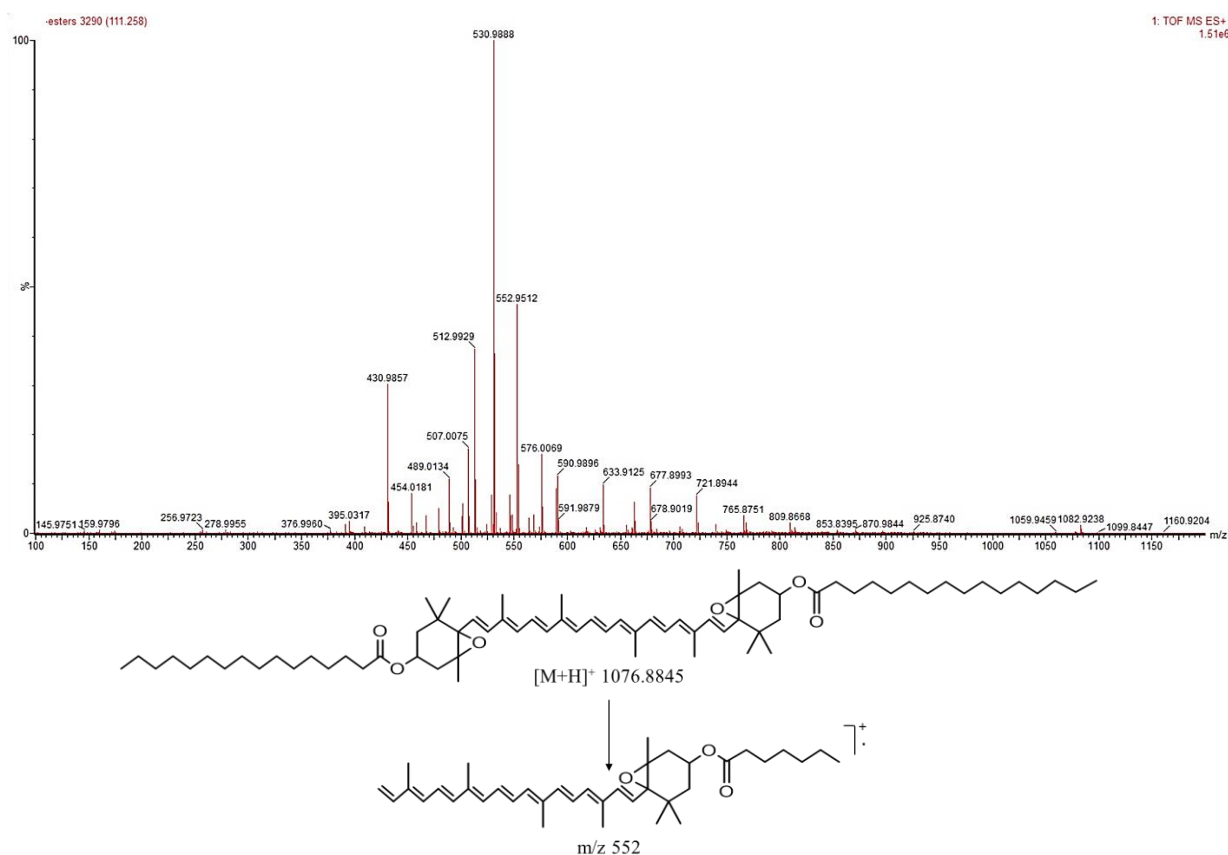


Figure 4.11. Proposed mass fragmentation pattern of violaxanthin dipalmitate in QToF-ESI-MS

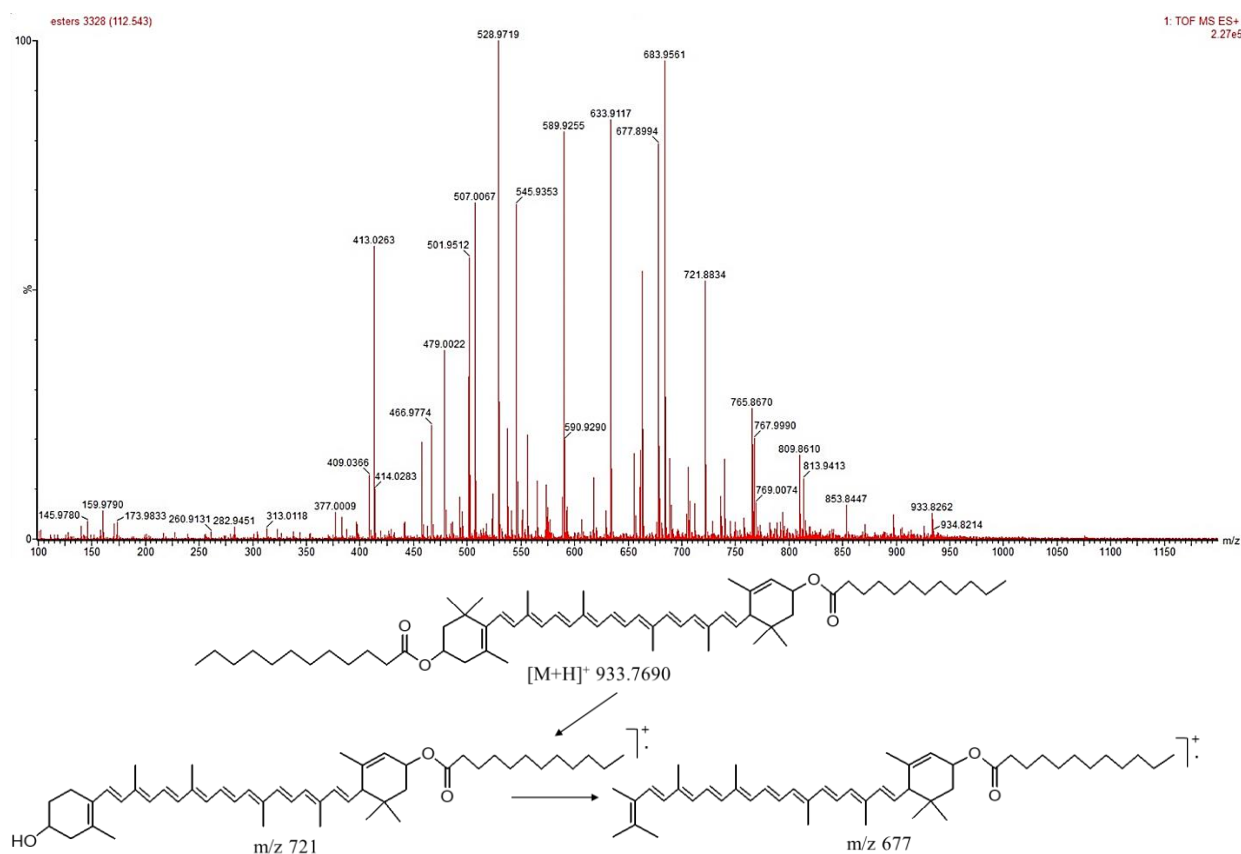


Figure 4.12. Proposed mass fragmentation pattern of lutein dilaurate in QToF-ESI-MS

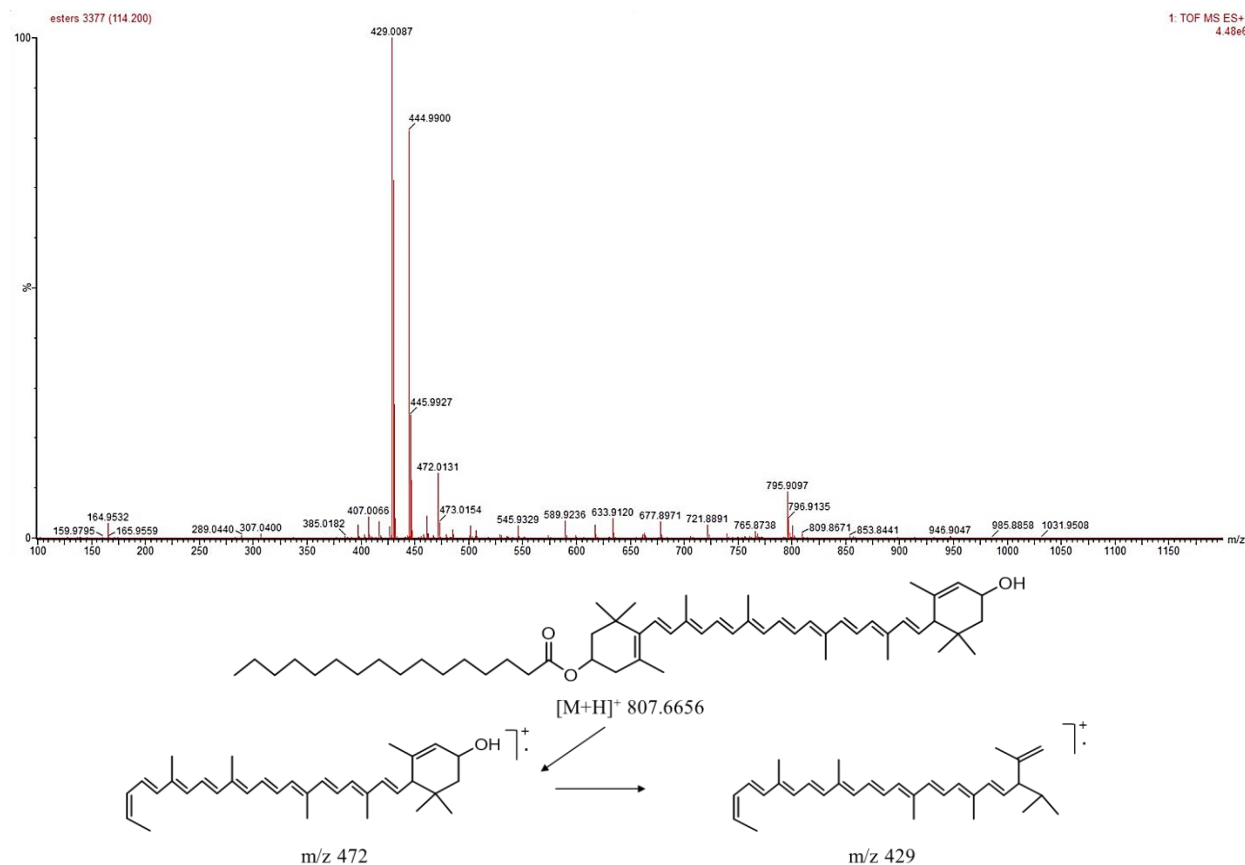


Figure 4.13. Proposed mass fragmentation pattern of lutein palmitate-II in QToF-ESI-MS

Three diesters of lutein namely, lutein dilinoleate, lutein dibutyrate, and lutein dipalmitate were eluted together with very close R_t . lutein dilinoleate ($C_{76}H_{116}O_4$) was detected at R_t 106.46 min and characterized from its protonated molecular ion peak at m/z 1093.8956. Fragmentation of the molecular ion resulted in daughter ion peaks at m/z 662 and 462 (Figure 4.8). Lutein dibutyrate ($C_{48}H_{68}O_4$) was recognized at R_t 106.73 min from its protonated molecular ion peak at m/z 709.5191, and daughter ion peaks at m/z 679 and 662 due to subsequent loss of ethyl and hydroxyl group (Figure 4.9). Similarly, the peak at R_t 107.30 min was assigned to lutein dipalmitate ($C_{72}H_{116}O_4$), which showed peak of a relatively less abundant protonated adduct of the molecular ion at m/z 1045.8966. Further fragmentation of the long alkyl chains generated daughter ions with m/z 707, 679 and 662 (Figure 4.10). Another diester of violaxanthin, namely violaxanthin dipalmitate ($C_{72}H_{116}O_6$) was also identified at R_t 111.25 min. The protonated adduct of the molecular ion (m/z 1077.8845) was dissociated from both terminals to produce daughter ion of m/z 552 (Figure 4.11). Lutein dilaurate ($C_{64}H_{100}O_4$) with protonated molecular ion peak at m/z 933.7690 was found to be eluted at R_t 112.54 min. De-esterification at one end followed by ring opening resulted in formation of daughter ions at m/z 721 and 677, respectively (Figure 4.12). The last peak at R_t 114.20 min was again identified as lutein palmitate ($C_{56}H_{86}O_3$) from the exact mass value (m/z 807.6656). The fragmentation pattern suggested the formation of daughter ions at m/z 472 and 429, due to loss of palmitate moiety and possible ring opening (Figure 4.13). The two peaks of lutein palmitate identified in the TIC might be due to the possible presence of regioisomers of the molecule (Mellado-Ortega and Hornero-Méndez, 2012).

4.2. Quantification of xanthophylls by High Performance Liquid Chromatography (HPLC)

Xanthophylls were quantified by HPLC and expressed in terms of lutein equivalence (Piccaglia *et al.*, 1998) considering that the chromophore properties of the carotenoid molecules remained unchanged by esterification (Pérez-Gálvez and Mínguez-Mosquera, 2005). An 85 min long run was standardized for the HPLC method of analysis of xanthophylls. Standard lutein was eluted at R_t 6.7 min as a sharp peak with λ_{max} of 446 nm (Figure 4.14A). HPLC chromatogram of the extracted xanthophylls has been represented in Figure 4.14B, in which 12 major peaks were identified including trace amount of free lutein (R_t 6.4 min) with the detector wavelength set at 446 nm (Gombač *et al.*, 2021). All those 12 peaks were invariably observed in the HPLC profile of the xanthophyll extracts of the DAMH-39 marigold genotype every time, irrespective of the method of extraction. Therefore, area under curve (AUC) of those 12 major peaks were

integrated, combined, and used to quantify the total xanthophyll concentration from the calibration curve of lutein standards. The calibration curve of the standard lutein solutions ($3.125\text{--}50\text{ }\mu\text{g mL}^{-1}$) was found to be linear with R^2 value of > 0.99 (Figure 4.15).

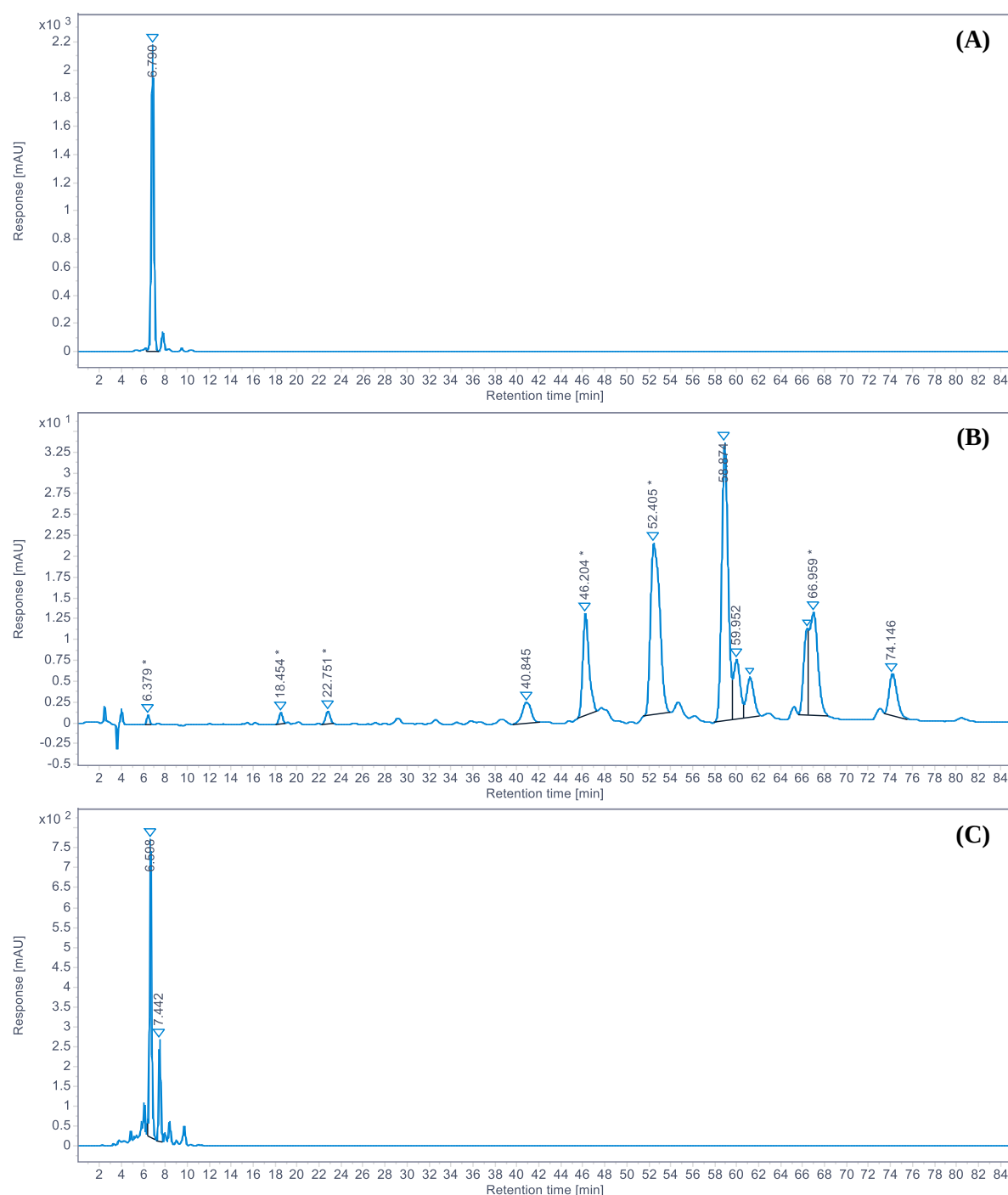


Figure 4.14. HPLC chromatograms of standard lutein (A), extracted xanthophylls/lutein esters (B), and saponified xanthophylls/lutein esters (C)

The HPLC profile of the saponified xanthophylls (Figure 4.14C) suggested the presence of lutein (Rt 6.6 min) along with another sharp peak at Rt 7.4 min. Based on literature reports

this peak was assigned to zeaxanthin, a principal isomer of lutein (Liu *et al.*, 2011). The HPLC profile of the saponified xanthophylls further reinforced the presence of esters of lutein and its isomers in the non-saponified extracts.

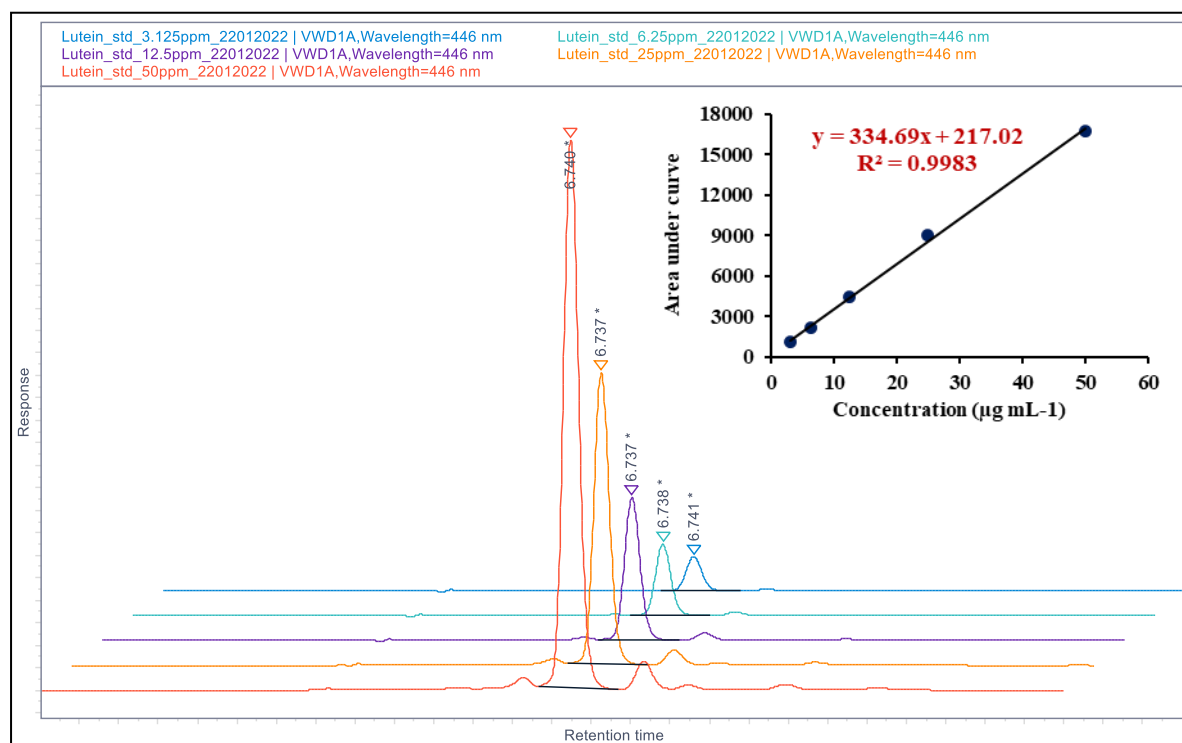


Figure 4.15. Calibration of standard lutein in HPLC at 3.125-50 $\mu\text{g mL}^{-1}$ level with calibration curve (first 12 min of the total run has been highlighted)

4.3. Extraction of xanthophylls using conventional solvents

4.3.1. Comparison of extraction techniques

Xanthophyll extraction efficiency of three different techniques namely, conventional solid-liquid extraction (CE), ultrasonic bath extraction (UBE), and ultrasonic probe extraction (UPE) was compared in terms of physical weight of extract (mg g^{-1} of dry weight) and total xanthophyll content ($\mu\text{g g}^{-1}$ of dry weight). UPE was carried out at two different conditions – UPE1: 10 min of ultrasonication at 20% amplitude with 150 mL of extraction solvent; and UPE2: 30 min of ultrasonication at 60% amplitude with 250 mL of extraction solvent. Comparative results have been represented in Table 4.2, which suggested that though physical weight of extracts obtained by CE and UBE was significantly more than UPE, total xanthophyll content by both those techniques was statistically at par with UPE2. Use of large volume of solvent and prolonged time of extraction in CE and UBE might have resulted in extraction of compounds other than xanthophylls which increased the physical weight of the extracts. For

UPE, increase in solvent volume, ultrasonication amplitude and time significantly increased xanthophyll extraction from dry marigold florets. Nevertheless, UPE2 extracted highest quantity of xanthophylls using much less volume of solvent in very less time as compared to CE and UBE. Therefore, the UPE technique was further optimized using statistical designs.

Table 4.2. Comparison of extraction efficiency of xanthophylls by different extraction techniques

Extraction technique	Physical weight (mg g ⁻¹ d.w.) ± SD	Total xanthophylls (µg g ⁻¹ d.w.) ± SD
CE	161.32 ± 12.81 ^a	11405.36 ± 632.37 ^a
UBE	160.55 ± 7.59 ^a	10635.02 ± 734.44 ^a
UPE1	90.12 ± 4.74 ^b	5961.62 ± 413.33 ^b
UPE2	113.86 ± 8.03 ^b	12828.18 ± 270.16 ^a

Tukey's HSD test was performed to flag significant differences among the treatment means within a column. Different alphabets within a column suggested significant difference at 95% confidence limit

4.3.2. Optimization of xanthophyll extraction by response surface methodology (RSM)

The conditions of UPE for maximum xanthophyll extraction from the florets of DAMH-39 genotype of marigold was statistically optimized using the Box-Behnken design (BBD) of RSM. This factorial design is used to understand the interactive influence of the independent variables to get the optimal response. Considering the second order quadratic polynomial model, the three-factor design in the present study took the form of the following equation:

$$y = \beta_0 + \beta_1A + \beta_2B + \beta_3C + \beta_{11}A^2 + \beta_{22}B^2 + \beta_{33}C^2 + \beta_{12}AB + \beta_{13}AC + \beta_{23}BC \quad (\text{eq. 4.1})$$

Where, A = solvent to solid ratio, B = ultrasonication amplitude (%), C = ultrasonication time (min), and y = total xanthophyll (µg g⁻¹ d.w.).

Dataset obtained from the 17 experimental runs/treatments was fitted to equation 4.1 and the ANOVA (analysis of variance) of the model has been presented in Table 4.3. The response surface quadratic model was found to be highly significant at 95% confidence interval with the *p*-value of 0.011. The model predicted significant influence of ultrasonication amplitude (B) on xanthophyll content as the linear (*p* = 0.0388) and quadratic (*p* = 0.0066) effects of the term were found highly significant at 5% and 1% significance level, respectively. Though the linear effects of solvent to solid ratio (A) and ultrasonication time (C) did not significantly impact the model, but their interaction had a significant influence on the model output. On a similar note, the interaction between ultrasonication amplitude and time, and the

quadratic effect of solvent to solid ratio imposed significant impact on the quadratic model at 5% significance level.

The *p*-value of the overall lack of fit of the model was 0.3625 (non-significant at 5% level), suggesting a perfect fit and that the model could able to describe the functional relationship between the experimental factors and the response variable adequately. The R^2 value of the model was calculated as 0.8930, indicating the model's ability to explain 89.30% variation. The adjusted R^2 value (0.7555) was also close enough to explain a considerably significant portion (75.55%) of variation by the model. Based on the model fitting, the final form of the quadratic equation was established as:

$$y = -24635.29 + 1317.65A + 322.52B + 388.39C - 2.73AB - 12.32AC + 5.72BC - 12.69A^2 - 3.65B^2 - 2.96C^2 \quad (\text{eq. 4.2})$$

Table 4.3. ANOVA of the response surface model for the UPE optimization experiment

Source	Sum of square	Degrees of freedom	Mean square	F value	<i>p</i> value
<i>Model</i>	3.625×10 ⁷	9	4.028×10 ⁶	6.49	0.0110*
A (Solvent:solid)	2.239×10 ⁶	1	2.239×10 ⁶	3.61	0.0992
B (Amplitude)	3.993×10 ⁶	1	3.993×10 ⁶	6.44	0.0388*
C (Time)	28335.86	1	28335.86	0.046	0.8369
AB	1.193×10 ⁶	1	1.193×10 ⁶	1.92	0.2080
AC	6.068×10 ⁶	1	6.068×10 ⁶	9.78	0.0167*
BC	5.235×10 ⁶	1	5.235×10 ⁶	8.44	0.0228*
A ²	6.778×10 ⁶	1	6.778×10 ⁶	10.93	0.0130*
B ²	8.996×10 ⁶	1	8.996×10 ⁶	14.50	0.0066**
C ²	3.700×10 ⁵	1	3.700×10 ⁵	0.60	0.4652
<i>Residual</i>	4.342×10 ⁶	7	6.203×10 ⁵		
Lack of fit	2.233×10 ⁶	3	7.444×10 ⁵	1.41	0.3625
Pure error	2.109×10 ⁶	4	5.273×10 ⁵		
<i>Corrected total</i>	4.059×10 ⁷	16			

*Significant at 5% level of significance; **Significant at 1% level of significance

Solving equation 4.2 in all possible combinations, the model predicted the optimum values of the independent variables as 32.80 solvent to solid ratio, 55.05% ultrasonication amplitude, and 29.43 min of ultrasonication time for a maximum xanthophyll extraction of 12923.40 µg g⁻¹. The model prediction (Figure 4.16) suggested that at a low solvent to solid

ratio, higher ultrasonication amplitude and higher extreme values of ultrasonication time would extract maximum amount of xanthophylls from the marigold florets (between 11407.2 to 14439.6 $\mu\text{g g}^{-1}$ at 95% confidence interval) with highest desirability (1.00). The laboratory validation of the experiment with the model predicted values of the independent variables resulted in xanthophyll yield of 12879.39 $\mu\text{g g}^{-1}$ (dry weight basis), which was very close to the predicted response.

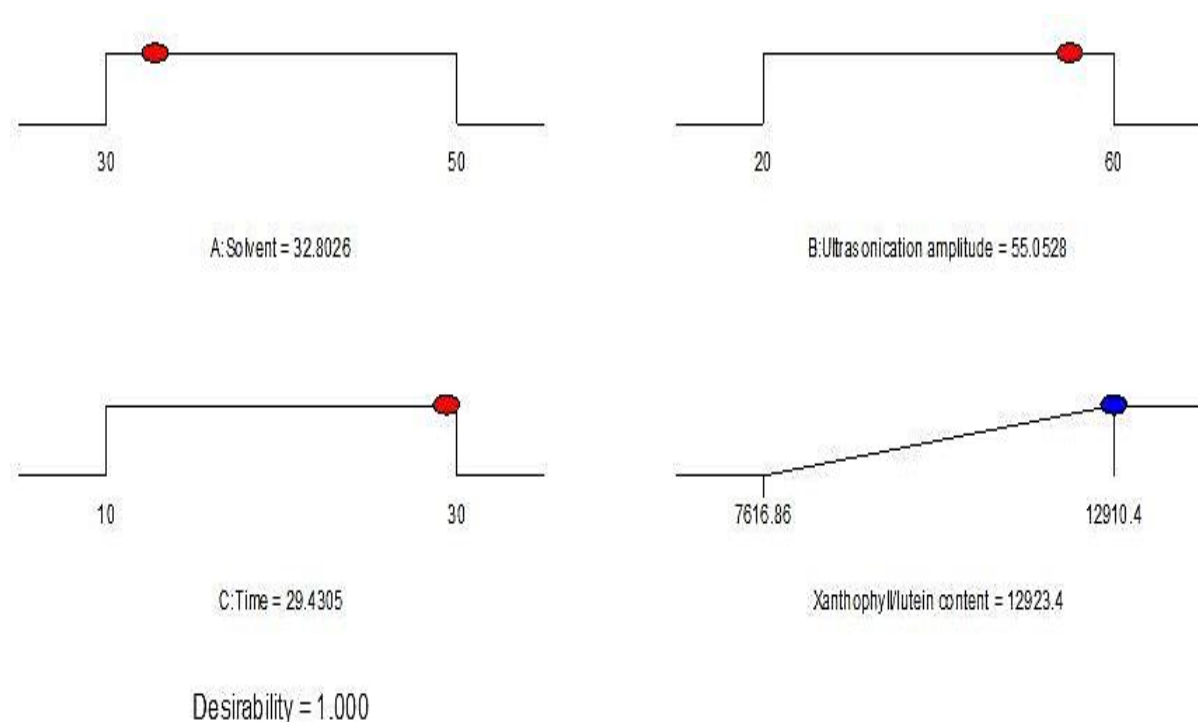


Figure 4.16. Model predicted optimum values of the independent variables of UPE for maximum output of the response variable

The three-dimensional (3D) response surface plots and contour plots in Figure 4.17 depicted the effect of interactions between two independent variables (keeping the third one constant) on the response variable. Figure 4.17A and B indicated that keeping ultrasonication time constant at 29.43 min, the maximum desirability of response variable inclined towards lower values of solvent to solid ratio and higher values of ultrasonication amplitude. Similarly, at a constant value of 55.05% ultrasonication amplitude, the desirability maxima inclined towards lower values of solvent to solid ratio and higher extreme values of ultrasonication time (Figure 4.17C and D). However, keeping the solvent to solid ratio constant at 32.80, higher values of ultrasonication amplitude along with higher extreme values of ultrasonication time was suggested to maximize the response variable (Figure 4.17E and F).

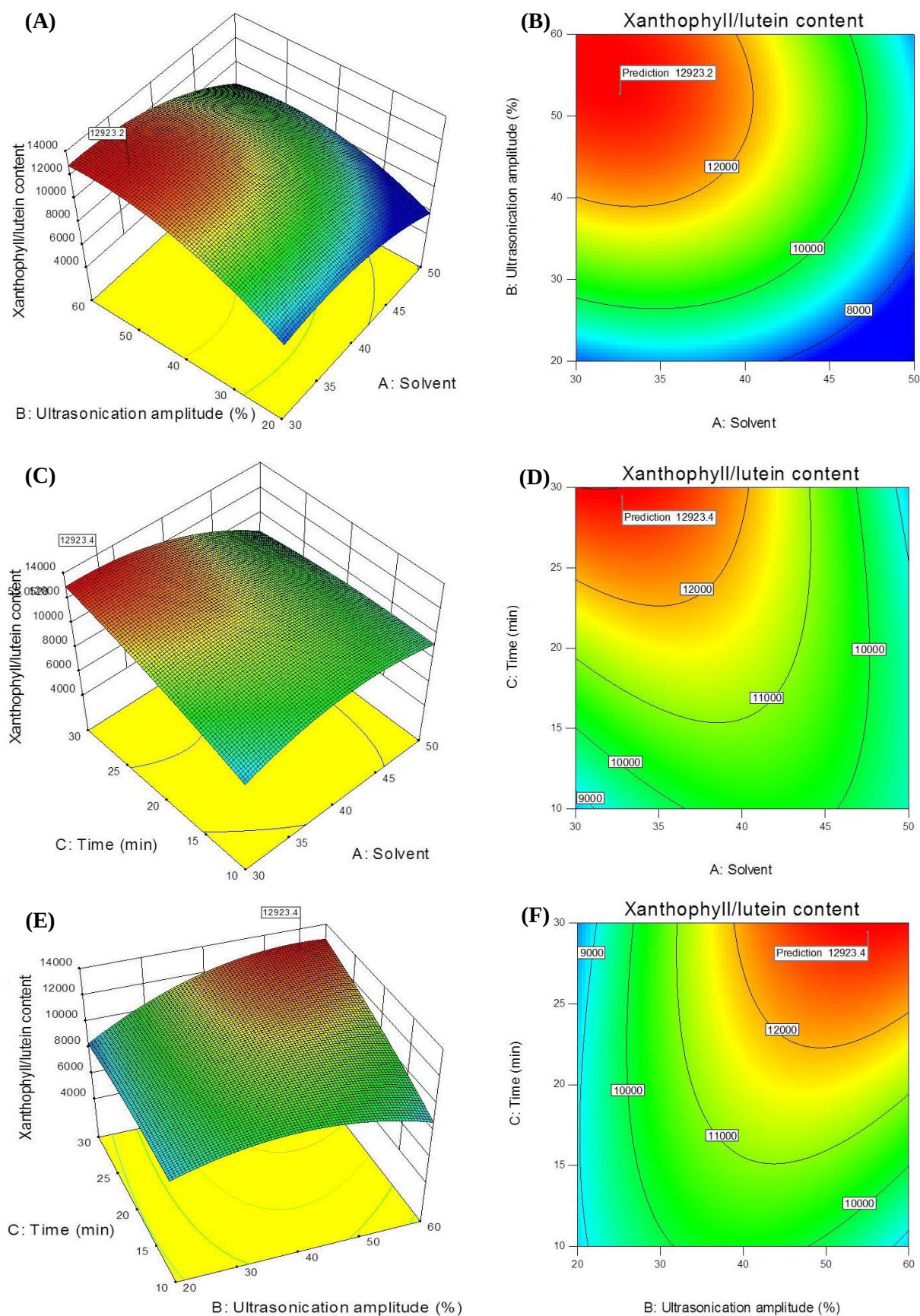


Figure 4.17. 3D response surface plots (A, C, E) and corresponding contour plots (B, D, F) for the interaction between independent variables in the UPE experiment

4.3.3. Optimization of xanthophyll extraction by genetic algorithm (GA)

Though RSM based optimization of the UPE experiment was in good agreement with the experimental data, GA based optimization was carried out with the same dataset to further improve the accuracy of the prediction model. In order to perform the GA based optimization, the exponential form of the second order quadratic polynomial equation from the response surface model was taken into consideration. The non-linear form of the equation was given as:

$$y = e^{(\beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC)} \quad (\text{eq. 4.3})$$

The Gauss-Newton method of non-linear least squares was used in an iterative process to fit the non-linear model. After 8 iterations, the convergence criteria were satisfied. The model was found to be significant at 1% level of significance. The ANOVA (analysis of variance) of the model has been presented in Table 4.4. Based on the model fitting, the final form of the non-linear equation was established as:

$$y = e^{(5.7253 + 0.1334A + 0.0325B + 0.0357C - 0.00028AB - 0.00122AC + 0.000567BC - 0.00129A^2 - 0.00036B^2 - 0.00024C^2)} \quad (\text{eq. 4.4})$$

Table 4.4. ANOVA of the genetic algorithm model for the UPE optimization experiment

Source	Sum of square	Degrees of freedom	Mean square	F value	p value
Model	1.8523×10 ⁹	10	1.8523×10 ⁸	307.02	<0.0001**
Error	4223300	7	603329		
Uncorrected total	1.8565×10 ⁹	17			

**Significant at 1% level of significance

Considering equation 4.4 as objective function, the GA optimization of the UPE experiment was carried out for 1000 generations with a population size of 50 and elitism of 2. The model predicted the optimum values of the independent variables as 31.38 solvent to solid ratio, 56.56% ultrasonication amplitude, and 30 min of ultrasonication time for a fitness function value of 13350.25, which was nothing but the predicted maximum xanthophyll yield (in µg g⁻¹) at the optimal solution points. The optimum solution was obtained with quite higher value of crossover probability (0.8) and lower value of mutation probability (0.1), which met the desirability criteria. The laboratory validation of the experiment with the model predicted values of the independent variables resulted in xanthophyll yield of 13099.05 µg g⁻¹ (dry weight basis), which was very close to the predicted response. The iteration result of the GA optimization has been graphically represented in Figure 4.18.

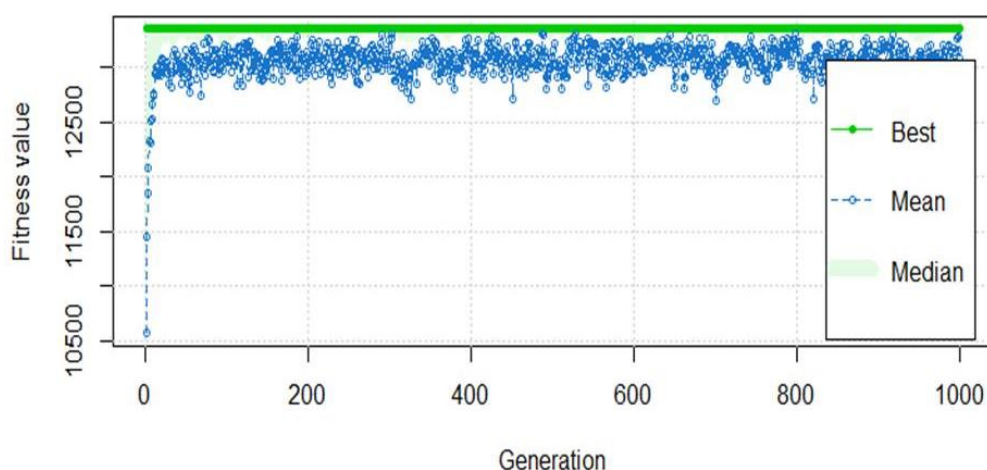


Figure 4.18. Interaction between fitness value and number of generations for the GA based optimization of the UPE experiment

4.3.4. Antioxidant activities of xanthophylls

Xanthophylls extracted from the marigold (DAMH-39) florets by different techniques including the optimized RSM and GA based techniques were evaluated for their antioxidant activity by analyzing their radical scavenging capacity (DPPH and ABTS) and reducing power (FRAP and CUPRAC).

Table 4.5. Antioxidant properties of xanthophylls extracted by different techniques

Sample	IC ₅₀ (mg mL ⁻¹)		EC ₅₀ (mg mL ⁻¹)	
	DPPH	ABTS	FRAP	CUPRAC
Ascorbic acid	0.08	0.04	0.24	0.31
Xanthophylls extracted by different techniques				
CE	5.35	0.56	7.46	1.87
UBE	3.40	0.57	15.84	1.69
UPE2	3.23	0.50	9.89	1.77
RSM	3.73	0.57	5.27	1.88
GA	3.45	0.52	2.52	1.76

The relative antioxidant activity has been assessed by comparing the IC₅₀ values (for DPPH and ABTS assays) and EC₅₀ values (for FRAP and CUPRAC assays) of the samples with that of the ascorbic acid standard (Table 4.5). It was observed that the concentration of xanthophylls required to inhibit 50% of the free radicals (IC₅₀) remained almost same irrespective of the technique of extraction as assessed by ABTS method. However, a slightly higher value was observed in DPPH assay for xanthophylls extracted by conventional

technique. Almost similar concentration ($1.69\text{--}1.88\text{ mg mL}^{-1}$) of the extracted xanthophylls were found to reduce 50% of cupric ion (EC_{50}) irrespective of the extraction technique, but their ability to reduce ferric ion varied. It was observed that xanthophylls extracted by the GA optimized technique has the lowest EC_{50} values in FRAP assay, followed by RSM optimized technique.

For a better comprehension, vitamin C equivalent antioxidant capacity (VCEAC) values of the samples were calculated and visualized in Figure 4.19, which reinforced the observations of Table 4.5. Though a conclusive difference of VCEAC of the samples extracted by different techniques were not visible in DPPH, ABTS and CUPRAC assays, the FRAP assay clearly indicated higher VCEAC of xanthophyll samples extracted by the optimized techniques (GA and RSM).

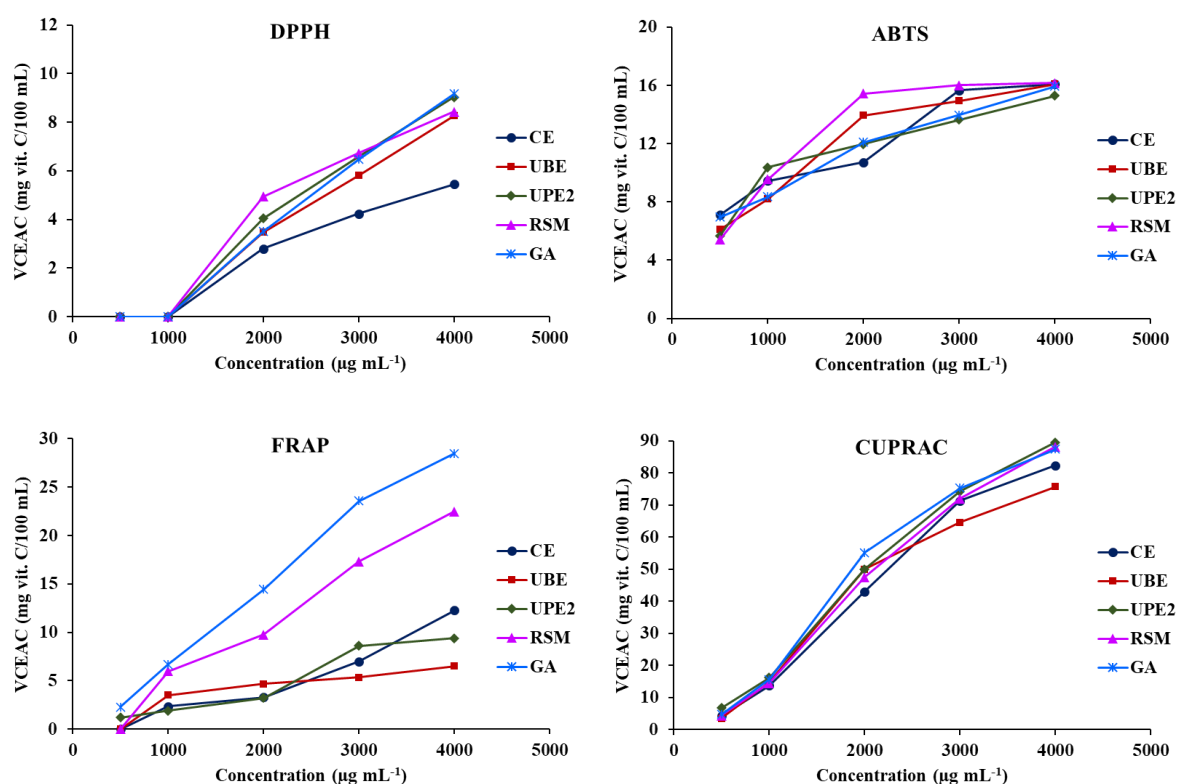


Figure 4.19. Comparison of antioxidant activity of xanthophylls extracted by different techniques

4.4. Extraction of xanthophylls using green solvents

4.4.1. Preparation and characterization of hydrophobic deep eutectic solvents (HDES)

Combination of caprylic acid (octanoic acid; C_8) and capric acid (*n*-decanoic acid; C_{10}) at different molar ratios produced five sets of hydrophobic deep eutectic solvents (HDES). The

physico-chemical properties of the prepared HDES have been presented in Table 4.6. At ambient temperature (20 ± 1 °C), caprylic acid is liquid but capric acid is solid in nature. During the process of preparation, all the eutectic mixtures became transparent liquids by thorough heating. However, upon cooling back to ambient temperature, the resultant eutectic mixtures remained as liquids, except the one prepared at 1:3 molar ratio of C₈ and C₁₀ acids. The densities of the prepared HDES were found to be decreasing with increased amount of capric acid in the mixture.

Table 4.6. Physico-chemical properties of prepared HDES

C ₈ :C ₁₀ (molar ratio)	Physical state (at 20 ± 1 °C)	Density (g cm ⁻³) at 30 ± 2 °C	Melting point (°C)	Enthalpy (J g ⁻¹)
3:1	Liquid	0.9077	-15.22	30.10
2:1	Liquid	0.9061	-13.96	34.78
1:1	Liquid	0.9016	-6.89	214.91
1:2	Liquid	0.8976	16.08	74.86
1:3	Solid	0.8964	19.99	103.04

4.4.1.1. Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry (DSC) of the prepared HDES was performed to understand their thermal behavior within the temperature range of -50 to 150 °C. The thermograms (heat flow vs. temperature) of the eutectic mixtures along with their constituents (capric and caprylic acid) have been showcased in Figure 4.20. The endothermic events in terms of melting point and glass transition were observed in the eutectic mixtures. A single sharp endothermic peak was observed for caprylic acid (Figure 4.20A) and capric acid (Figure 4.20G) indicated their melting. The enthalpy of melting for both the fatty acids were determined as 93.27 and 185.78 J g⁻¹, respectively. The melting points and corresponding enthalpies of the eutectic mixtures have been reported in Table 4.6. A trend of increasing melting point was observed with the increase in relative amount of capric acid in the eutectic mixture. Maximum enthalpy at melting was observed for 1:1 eutectic mixture. Further, glass transition phase was observed for the eutectic mixtures with higher content of capric acid (Figure 4.20E-F). This referred to the transition of the amorphous solid mixtures into a soft waxy phase before the onset of the melting temperature. However, this transition was not prominent for the eutectic mixtures with higher content of caprylic acid, because of the onset of melting temperature at an early stage.

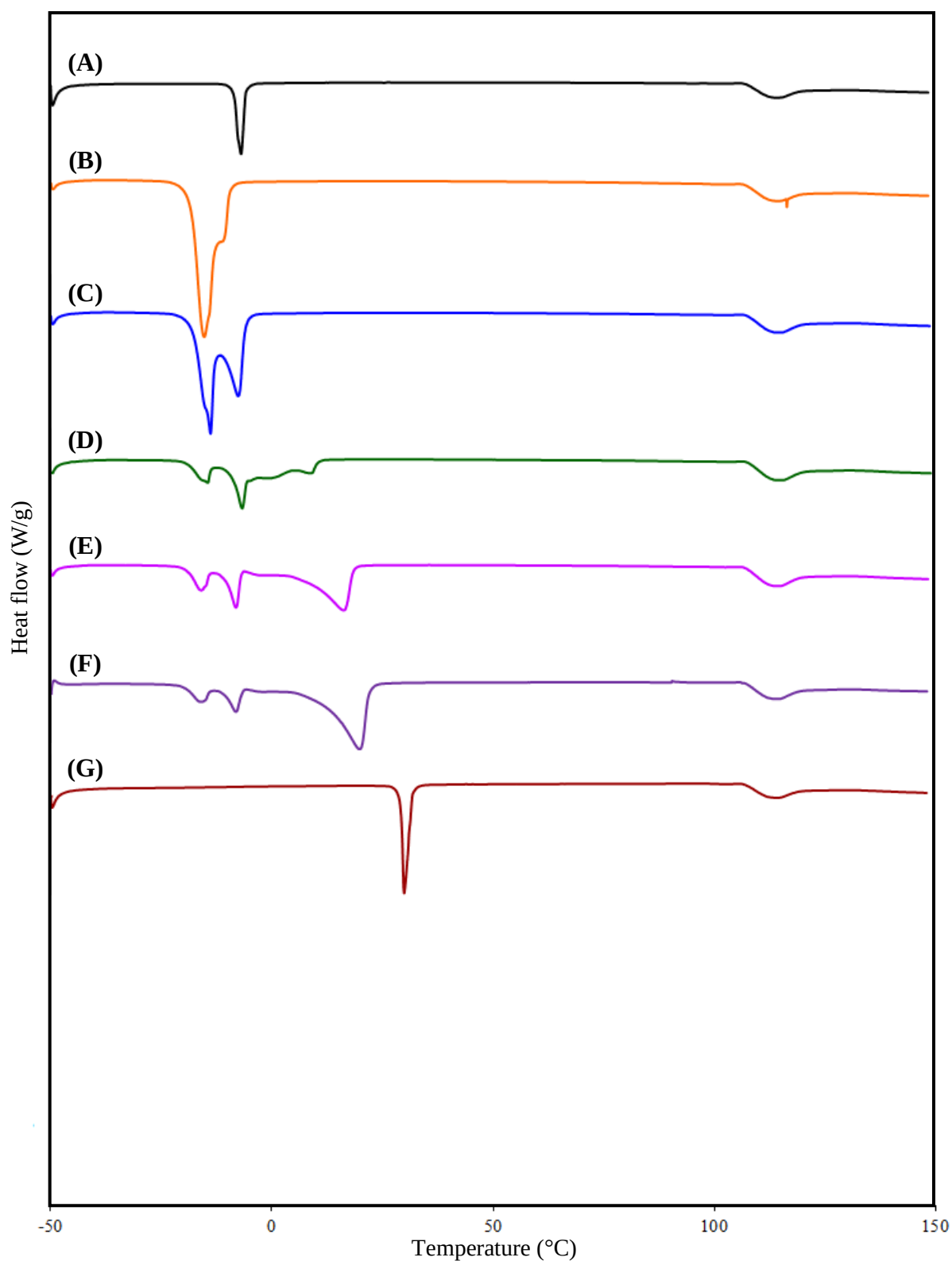


Figure 4.20. DSC spectra of C_8 (caprylic) acid (A), C_{10} (capric) acid (G), HDES prepared with different ratio of C_8 and C_{10} acids – 3:1 (B), 2:1 (C), 1:1 (D), 1:2 (E), and 1:3 (F)

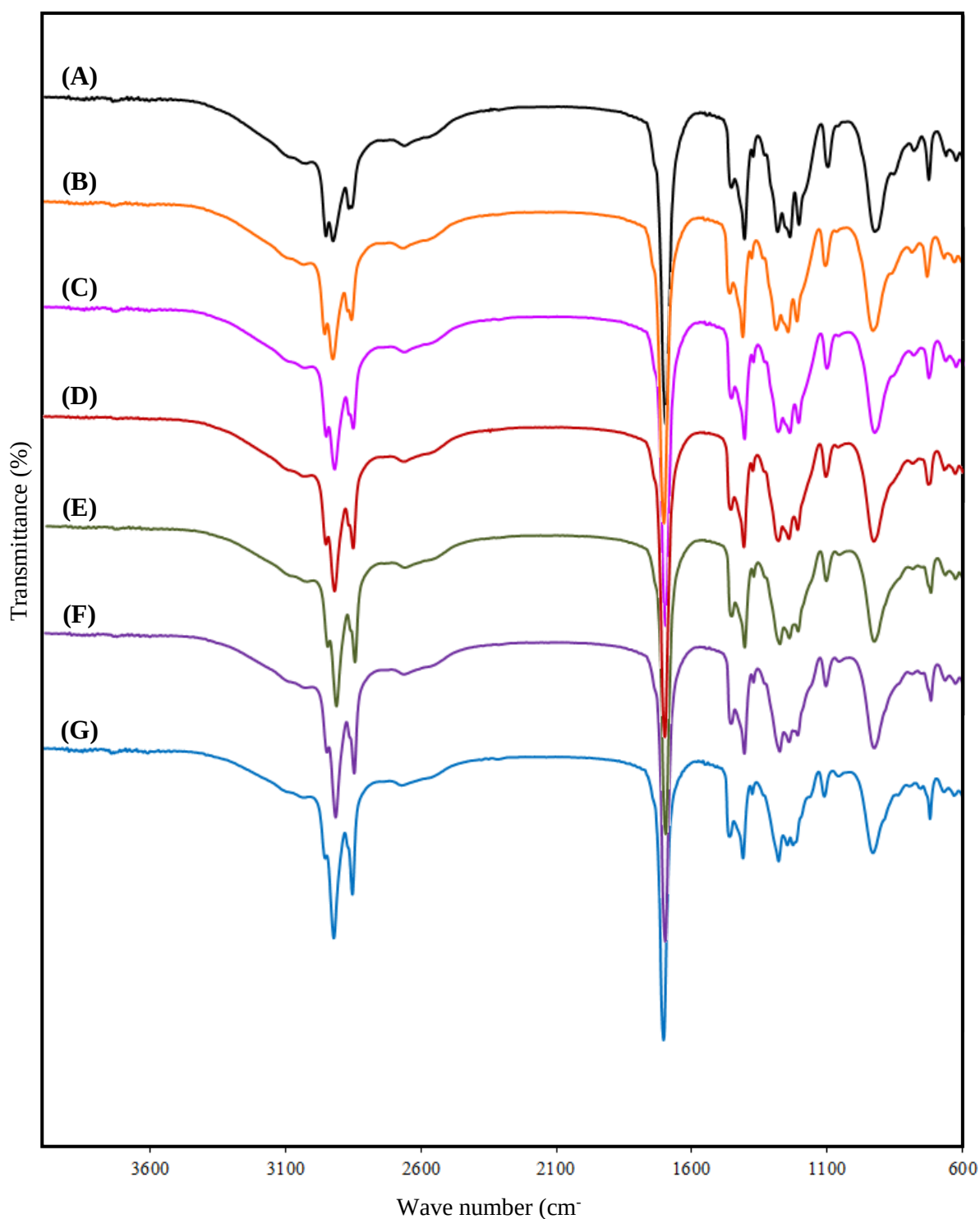


Figure 4.21. FT-IR spectra of C₈ (caprylic) acid (A), C₁₀ (capric) acid (G), HDES prepared with different ratio of C₈ and C₁₀ acids – 3:1 (B), 2:1 (C), 1:1 (D), 1:2 (E), and 1:3 (F)

4.4.1.2. Fourier Transform Infrared (FT-IR) spectroscopy

Figure 4.21 represents the FT-IR spectra of the HDES along with their constituent fatty acids. All the samples were showing very similar pattern suggesting non-occurrence of any chemical reaction between capric and caprylic acid. The typical sharp band at 1704 cm^{-1} corresponding

to the C=O stretching vibration of the carboxylic group was prominent in all the samples. The characteristic asymmetric and symmetric C–H stretching vibrations were present in all the samples at ~ 2923 and ~ 2855 cm^{-1} , respectively. A broad band at around 3035 cm^{-1} , indicated the O–H stretching vibration of the carboxyl group.

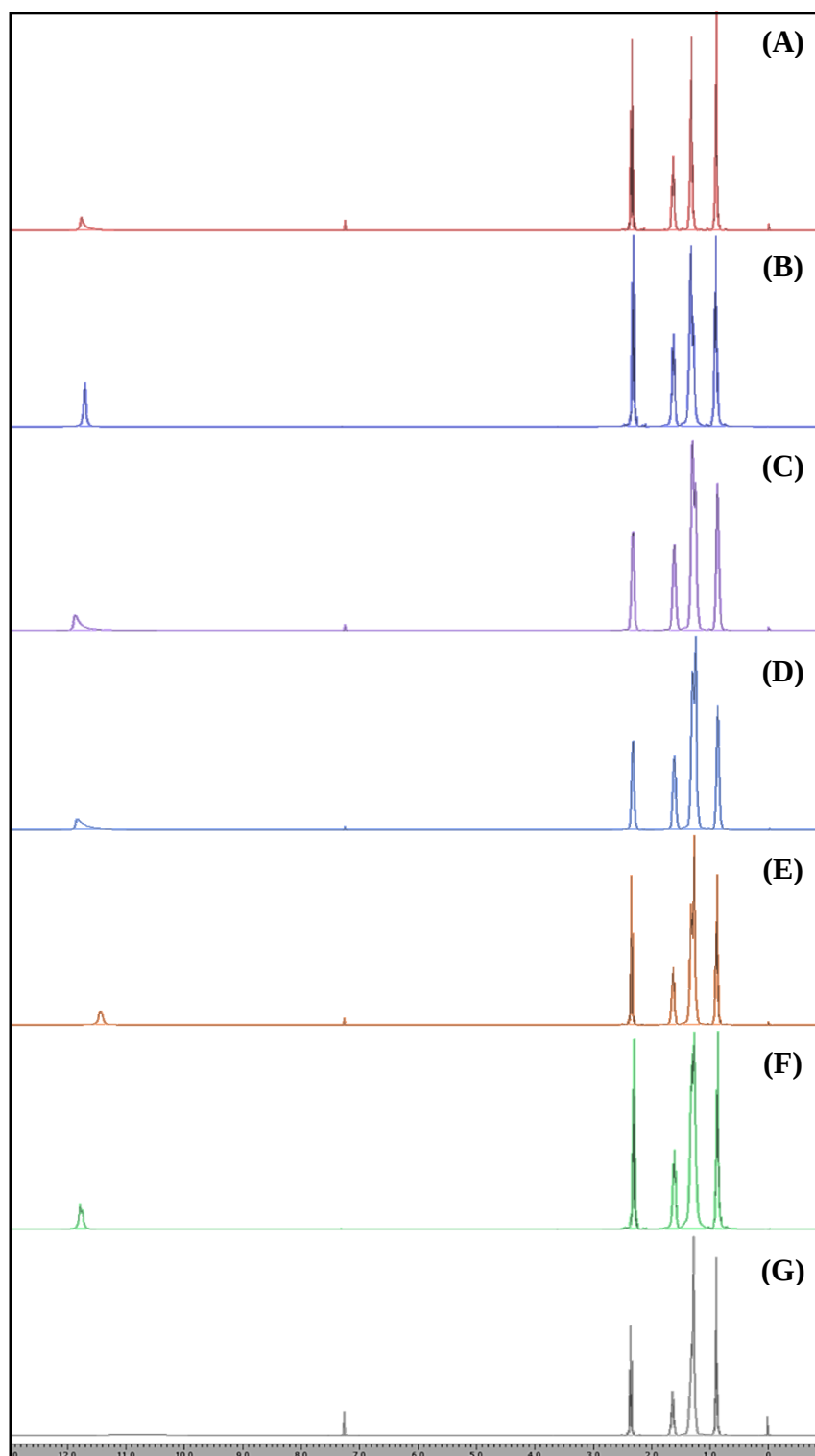


Figure 4.22. ^1H -NMR spectra of C₈ (caprylic) acid (A), C₁₀ (capric) acid (G), HDES prepared with different ratio of C₈ and C₁₀ acids – 3:1 (B), 2:1 (C), 1:1 (D), 1:2 (E), and 1:3 (F)

The only difference between the FT-IR spectra of caprylic (Figure 4.21A) and capric acid (Figure 4.21G) was the intensities of the asymmetric and symmetric C–H stretching vibration bands. The intensities of these bands were more in case of C₁₀ acid as compared to the C₈ acid, due to the presence of two extra methylene (–CH₂–) groups. With increase in the relative ratio of C₁₀ acid in the eutectic mixture, the intensities of those two bands were found to be increased gradually.

4.4.1.3. Nuclear Magnetic Resonance (NMR) spectroscopy

Proton NMR spectra of the C₈ and C₁₀ fatty acids, and their eutectic mixtures have been displayed in Figure 4.22. The pattern of the spectra for all the samples was almost similar and no chemical shift was observed for the methyl and methylene protons of the fatty acids. No additional peak was observed for the eutectic mixtures confirming that chemical reaction did not happen due to mixing. Intriguingly, on close observation of the spectra, small chemical shift of the hydroxyl proton was observed in the eutectic mixtures. The δ value of the hydroxyl proton in the eutectic mixtures was recorded to vary from 11.454 to 11.881 ppm, as compared to 11.159 and 11.796 ppm for capric and caprylic acid, respectively. This observation suggested the formation of hydrogen bonds between fatty acid molecules. The hydroxyl protons of the carboxyl moiety got de-shielded due to formation of H-bonds with electronegative oxygen atom of adjacent carboxylic groups. The extent of the chemical shift varied with the relative molar ratio of C₈ and C₁₀ fatty acids in the eutectic mixtures.

4.4.2. Ultrasound assisted HDES based extraction of xanthophylls

4.4.2.1. Single factor optimization

As the physical state of the HDES prepared at 1:3 molar ratio of C₈ and C₁₀ acids was solid at ambient temperature, it was not included in further study due to difficulty of handling. Ultrasound assisted extraction of xanthophylls from marigold (DAMH-39) florets was carried out using the other four HDES which were liquid at ambient temperature.

Initial experiments suggested that the eutectic mixture of 2:1 C₈ and C₁₀ acids at 50 mL g⁻¹ volume extracted maximum amount of xanthophylls from marigold florets in 15 min of ultrasonication as compared to the other eutectic mixtures (Figure 4.23A). Increase in ultrasonication time up to 45 min increased the extraction capacity of the 2:1 HDES. Further increase in extraction time did not significantly increase the extraction yield as saturation was observed at 60 min (Figure 4.23C). However, further prolonging the ultrasonication time decreased the yield due to possible degradation of xanthophylls. Keeping the ultrasonication

time constant at 45 min, an increase in the volume of the 2:1 HDES from 50 to 75 mL g⁻¹ did not significantly improve the extraction yield, but a further increment to 100 mL g⁻¹ increased the yield 1.14 times (Figure 4.23B). Based on the results of the preliminary experiments, optimization of these three factors was carried out by response surface methodology to extract maximum amount of xanthophylls by this technique.

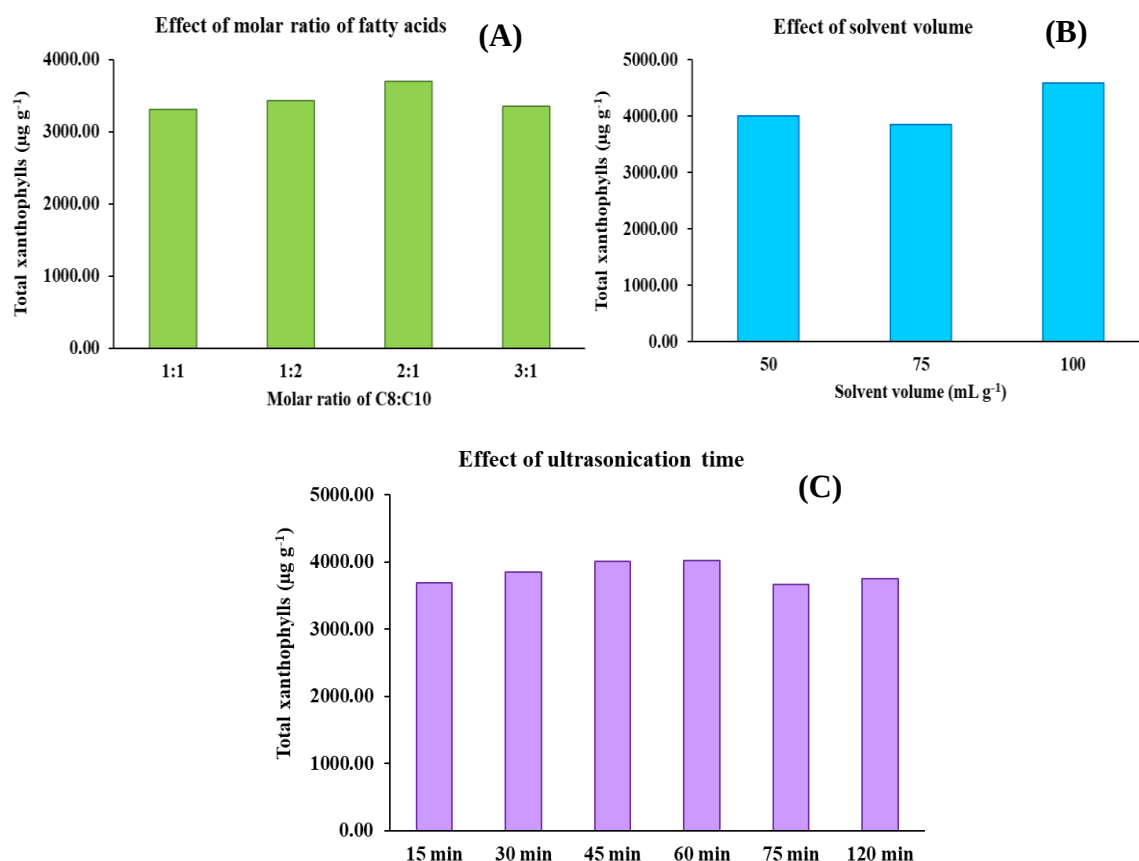


Figure 4.23. Effect of molar ratio of fatty acids (C₈: C₁₀) to prepare HDES (A), its volume (B), and ultrasonication time (C) on extraction yield of xanthophylls

4.4.2.2. Optimization of ultrasound assisted extraction of xanthophylls using HDES by response surface methodology (RSM)

Ultrasound assisted extraction of xanthophylls from the marigold (DAMH-39) florets using HDES was statistically optimized by the Box-Behnken design (BBD) of RSM. The effect of the interactions of the independent variables namely, molar ratio of fatty acids (C₈:C₁₀) constituting the HDES, volume of HDES, and ultrasonication time was assessed on the optimal output of the response variable (*i.e.* xanthophyll yield in $\mu\text{g g}^{-1}$). Data obtained from the 17 experimental runs/treatments were fitted to a quadratic polynomial model of second order

(equation 4.1), where the independent variables were denoted as: A = molar ratio of fatty acids (C₈:C₁₀) constituting the HDES, B = volume of HDES, and C = ultrasonication time.

The ANOVA (analysis of variance) of the quadratic model has been presented in Table 4.7, which showed that the proposed model was highly significant at 1% level of significance ($p = 0.0039$). Simultaneously, the overall lack of fit remained to be non-significant at 5% level of significance ($p = 0.4751$) as desirable. Interestingly, the linear effects of the independent variables were observed to have significant impact on the model (at 1% level of significance). However, their interactions and quadratic terms did not influence the model significantly. Close agreement between the R^2 (0.9226) and adjusted R^2 (0.8231) values suggested the model's ability to explain a high proportion of variation in the data. Based on the model fitting, the final form of the quadratic equation was established as:

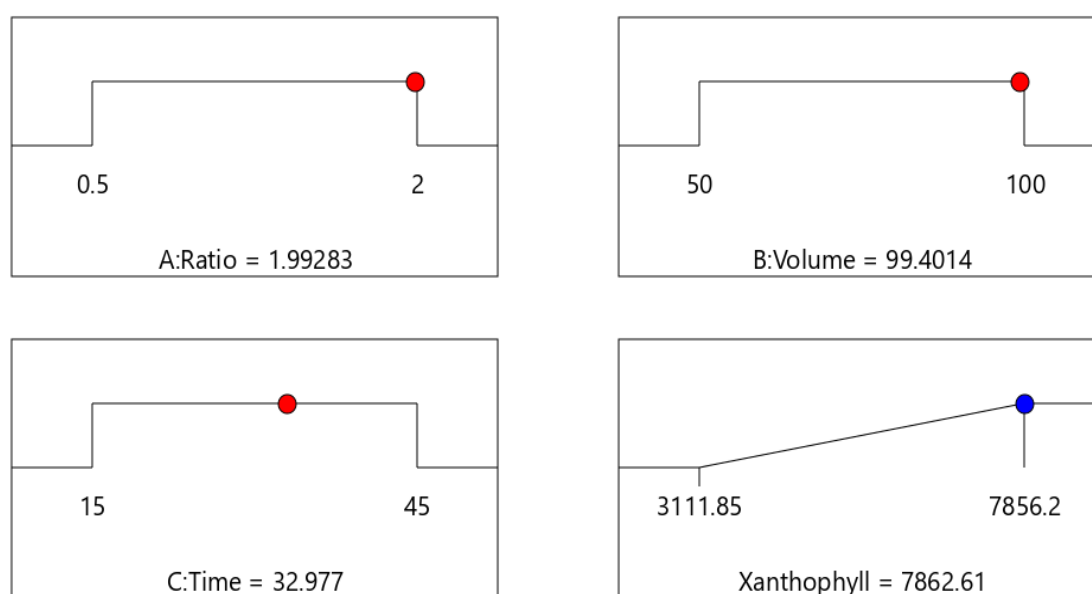
$$y = -193.10 + 2697.45A - 20.00B + 156.31C - 4.04AB - 23.83AC - 0.30BC - 55.86A^2 + 0.39B^2 - 0.74C^2 \quad (\text{eq. 4.5})$$

Table 4.7. ANOVA of the response surface model for the HDES based extraction optimization experiment

Source	Sum of square	Degrees of freedom	Mean square	F value	p value
<i>Model</i>	2.095×10^7	9	2.327×10^6	9.27	0.0039**
A (C ₈ :C ₁₀)	1.067×10^7	1	1.067×10^7	42.53	0.0003**
B (Volume)	3.110×10^6	1	3.110×10^6	12.39	0.0097**
C (Time)	6.446×10^6	1	6.446×10^6	25.68	0.0015**
AB	22935.04	1	22935.04	0.0914	0.7712
AC	2.874×10^5	1	2.874×10^5	1.15	0.3201
BC	50316.77	1	50316.77	0.2005	0.6679
A ²	4157.34	1	4157.34	0.0166	0.9012
B ²	2.541×10^5	1	2.541×10^5	1.01	0.3478
C ²	1.159×10^5	1	1.159×10^5	0.4620	0.5185
<i>Residual</i>	1.757×10^6	7	2.510×10^5		
Lack of fit	5.578×10^5	3	2.526×10^5	1.01	0.4751
Pure error	9.992×10^5	4	2.498×10^5		
<i>Corrected total</i>	2.270×10^7	16			

*Significant at 5% level of significance; **Significant at 1% level of significance

The model predicted (Figure 4.24) the optimum values of the independent variables as 1.99 molar ratio ($C_8:C_{10}$) for HDES, 99.40 mL of HDES per gram of marigold powder, and 32.98 min of ultrasonication time for a maximum xanthophyll extraction of $7862.61 \mu\text{g g}^{-1}$ at the highest level of desirability (1.00). The laboratory validation of the experiment with the model predicted values of the independent variables resulted in xanthophyll yield of $7570.80 \mu\text{g g}^{-1}$ (dry weight basis), which was very close to the predicted response. Same volume of conventional solvent (hexane-acetone 1:1) yielded only $5706.90 \mu\text{g g}^{-1}$ xanthophylls from the marigold powder for the same time of ultrasonication.



Desirability = 1.000

Figure 4.24. Model predicted optimum values of the independent variables of the HDES based extraction experiment for maximum output of the response variable

Interactions between two variables have been depicted by three-dimensional (3D) response surface plots and contour plots, keeping the third variable constant (Figure 4.25). Figure 4.25A and B indicated that keeping ultrasonication time constant at 32.98 min, the maximum desirability of response variable could be obtained with higher extreme values of both volume of HDES, and the $C_8:C_{10}$ molar ratio to prepare it. On the other hand, the relationship between ultrasonication time and $C_8:C_{10}$ molar ratio of HDES or its volume was very similar. Higher extreme values of either of these two parameters could extract xanthophyll at almost equally in moderate time keeping the other one constant (Figure 4.25C-F).

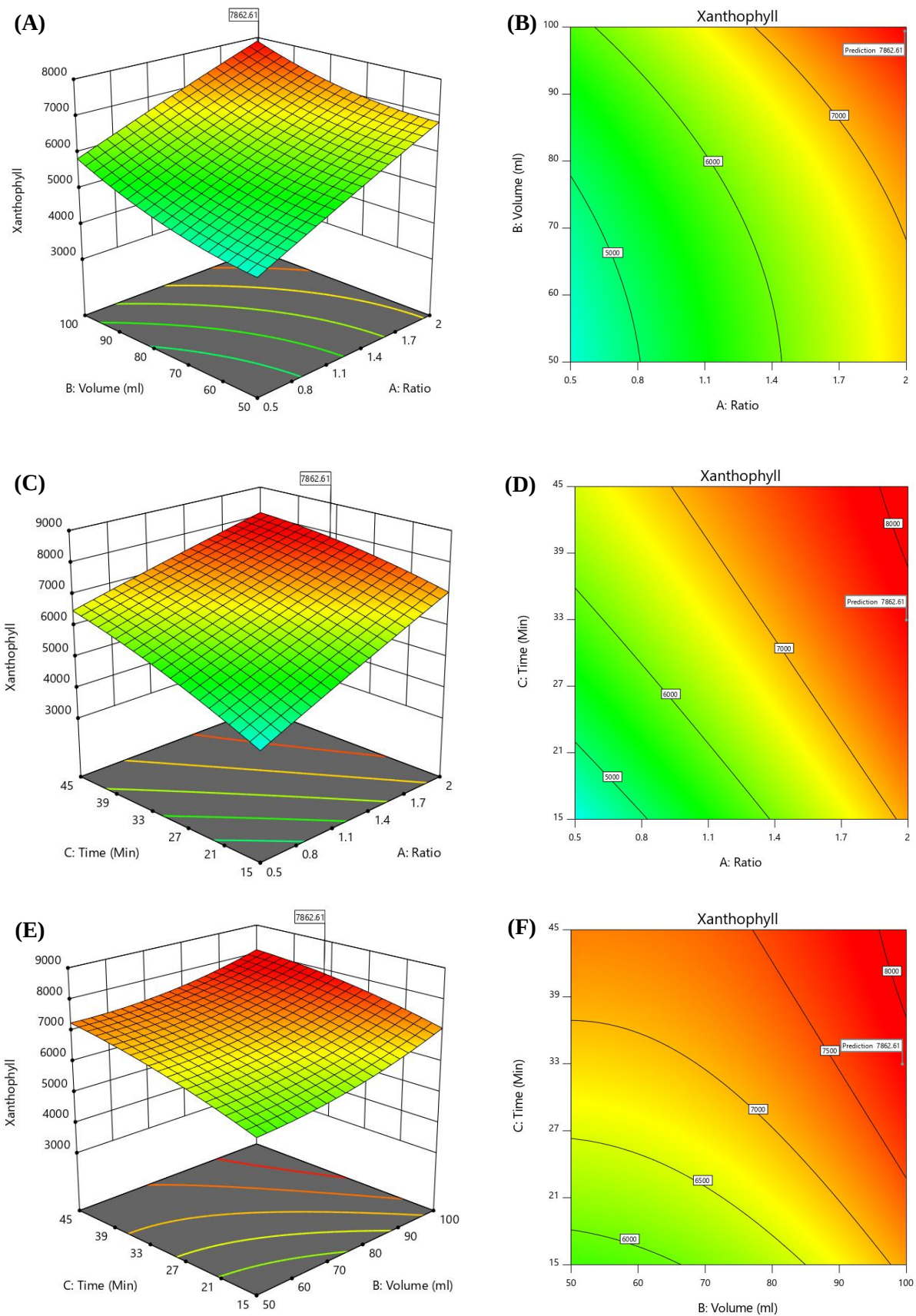


Figure 4.25. 3D response surface plots (A, C, E) and corresponding contour plots (B, D, F) for the interaction between independent variables in the ultrasound assisted HDES extraction of xanthophylls

4.4.3. Polarity switch of HDES for separation of xanthophylls

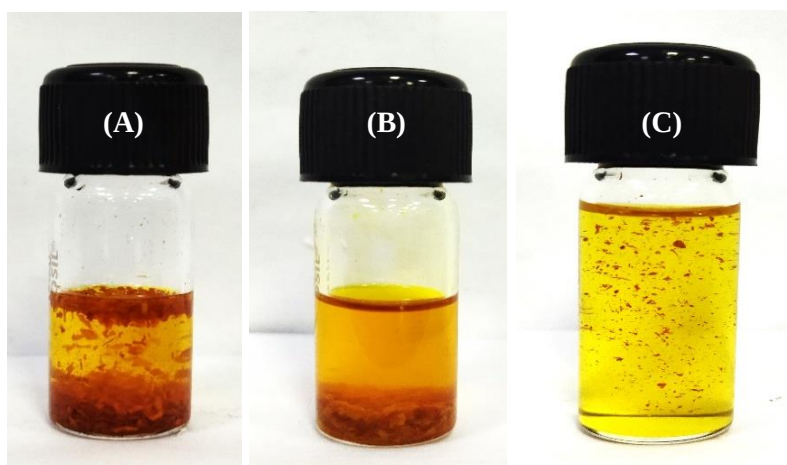


Figure 4.26. Pictorial presentation showing (A) addition of HDES to marigold floret powder for xanthophyll extraction, (B) HDES after extraction of xanthophylls from marigold floret powder, and (C) precipitation of lutein esters from the supernatant after polarity switch

Heat treatment of the HDES (2:1 molar ratio of C₈ and C₁₀ fatty acids) with 40% aqueous KOH solution made it hydrophilic. This polarity switch enabled the dissolved lipophilic xanthophylls (lutein esters) to precipitate (Figure 4.26). Analysis of the precipitated material by HPLC confirmed their identities as lutein esters. Absence of the peak of lutein in the HPLC chromatogram suggested that saponification of the esters did not occur due to the alkali treatment for polarity switch. Further, quantification of lutein esters in the precipitate suggested 72.34% conversion of the dissolved amount of xanthophylls to its precipitated form by the polarity switch.

4.5. Surface morphology of marigold powder by scanning electron microscopy (SEM)

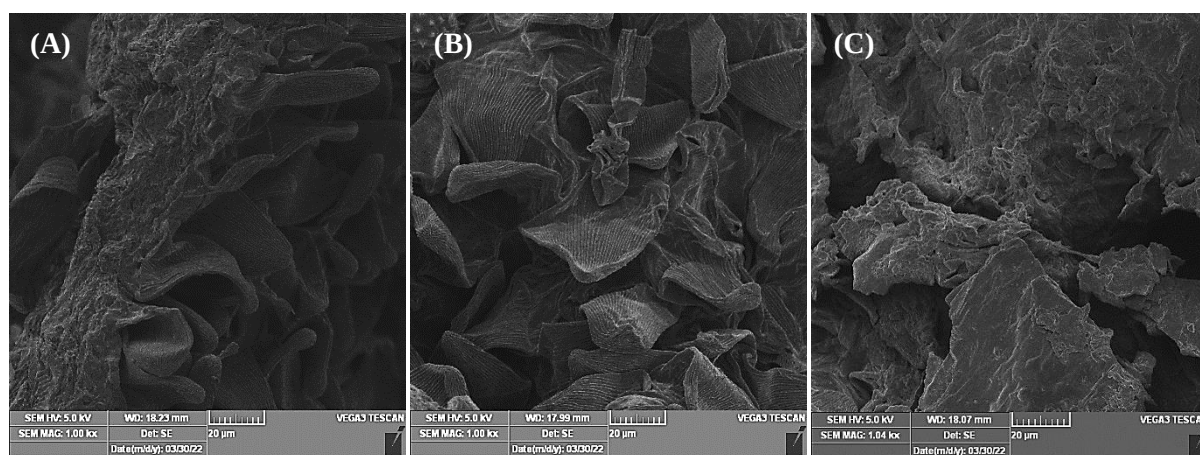


Figure 4.27. SEM images of lyophilized marigold powder before extraction (A), after extraction by conventional solvent (B), and after extraction by HDES with ultrasonication (C)

SEM images of lyophilized marigold powder before and after extraction have been presented in Figure 4.27 at 1000x magnification. Conventional extraction technique did not change the surface morphology significantly as the texture of the surface remained almost identical. However, ultrasound assisted extraction of xanthophylls using HDES destroyed the smoothness of the surface of the powder. The texture of the surface became rough due to cell rupturing.

4.6. Preparation of water-soluble powder formulation of xanthophylls

4.6.1. Preparation of biopolymer stabilized oil-in-water (O/W) microemulsion

Biopolymer stabilized oil-in-water (O/W) microemulsions of xanthophylls were prepared initially before freeze drying to get the water-soluble powder formulations. A total of 9 sets of primary emulsions were prepared using three different surfactants at three different levels of concentration. Further, from each primary emulsion three sets of microemulsions were prepared by emulsion dilution technique, which gave rise to a total 27 sets of O/W microemulsions (Table 4.8, Figure 4.28).



Figure 4.28. Physical appearance of the prepared microemulsions

It was observed from Figure 4.28 that all the microemulsions prepared with the surfactant S1 had transparent appearance (the texts behind the microemulsions could be clearly readable through them). Most of the microemulsions prepared with surfactant S2 were also transparent, but few of them were found to be translucent (texts behind diffused). In contrary, all the microemulsions prepared with surfactant S3 were completely opaque (texts behind not visible). Though the concentration of surfactants S1 and S2 used was up to 7.5%, S3 could not be used beyond 3% due to its poor water solubility. No phase separation was noted for any of the prepared microemulsions on standing.

4.6.2. Physico-chemical characterization of microemulsions

4.6.2.1. Particle size analysis

The particle size distribution of the oil droplets in the prepared O/W microemulsions was determined based on the laser scattering principle keeping the refractive indices of fluid and particles as 1.33 and 1.47, respectively. Mean particle diameter (hydrodynamic diameter based on intensity distribution), median particle diameter (D_{50}), and polydispersity index (PDI) of the microemulsion samples have been represented in Table 4.8. It was observed that the mean droplet diameter of the microemulsions prepared with surfactant S1 was within nano range (50.5-86.9 nm) irrespective of the oil concentration and the concentration of the surfactant used.

Table 4.8. Particle size indices of the O/W microemulsions

Microemulsion sample				Particle size indices		
Sample code	Surfactant	Conc. of surfactant (%)	Oil conc. (%)	Mean droplet diameter (nm)	D_{50} (nm)	PDI
S1L1	S1	2.5	0.1	51.3	50.5	0.076
S1L2	S1	2.5	0.25	73.3	71.3	0.118
S1L3	S1	2.5	0.5	65.5	65.3	0.031
S1M1	S1	5.0	0.1	57.0	56.0	0.074
S1M2	S1	5.0	0.25	72.7	70.1	0.137
S1M3	S1	5.0	0.5	83.1	79.8	0.138
S1H1	S1	7.5	0.1	61.1	59.8	0.113
S1H2	S1	7.5	0.25	88.7	86.9	0.096
S1H3	S1	7.5	0.5	66.9	63.8	0.180
S2L1	S2	2.5	0.1	32.2	32.3	0.104
S2L2	S2	2.5	0.25	141.9	97.6	0.536

S2L3	S2	2.5	0.5	267.8	95.8	1.133
S2M1	S2	5.0	0.1	25.9	27.0	0.480
S2M2	S2	5.0	0.25	65.2	38.3	0.171
S2M3	S2	5.0	0.5	68.6	48.5	0.312
S2H1	S2	7.5	0.1	1252	685	0.526
S2H2	S2	7.5	0.25	135.2	41.1	0.905
S2H3	S2	7.5	0.5	89.5	50.6	0.457
S3L1	S3	1.0	0.1	2238	2096	0.212
S3L2	S3	1.0	0.25	1883	1804	0.215
S3L3	S3	1.0	0.5	2373	1632	0.457
S3M1	S3	2.0	0.1	1938	1289	0.302
S3M2	S3	2.0	0.25	1944	1717	0.068
S3M3	S3	2.0	0.5	2960	1603	0.626
S3H1	S3	3.0	0.1	1725	1247	0.063
S3H2	S3	3.0	0.25	2164	2047	0.155
S3H3	S3	3.0	0.5	2202	2074	0.105

The droplet size distribution was unimodal and of narrow spectrum as suggested by very low PDI (0.074-0.180) for all the microemulsions prepared with S1 (Figure 4.29A-B). This observation explained the transparent appearance of the microemulsions prepared with S1. Mean droplet diameter was found to be variable for the microemulsions prepared using S2 surfactant. Nano sized droplets were formed at 5% concentration of the surfactant irrespective of the concentration of oil. The mean droplet diameter of samples S2L1 (2.5% surfactant, 0.1% oil) and S2H3 (7.5% surfactant, 0.5% oil) was also below 100 nm, but with little change in the oil concentration at these surfactant levels, a considerable change in the droplet diameter took place, resulted in translucent appearance of the microemulsions. Interestingly, PDI of the microemulsions prepared with S2 was found highly variable due to bimodal droplet size distribution in many of them (Figure 4.29C-D). The average droplet size of the microemulsions prepared with surfactant S3 never reached below 100 nm. Therefore, transparent microemulsions were not obtained with this surfactant. The mean droplet diameter for these microemulsions varied from 1.73 to 2.96 μm . Though the general trend of PDI was at higher side for the microemulsions prepared with S3 due to bimodal droplet size distribution, lower PDI values were also observed for few cases (Figure 4.29E-F).

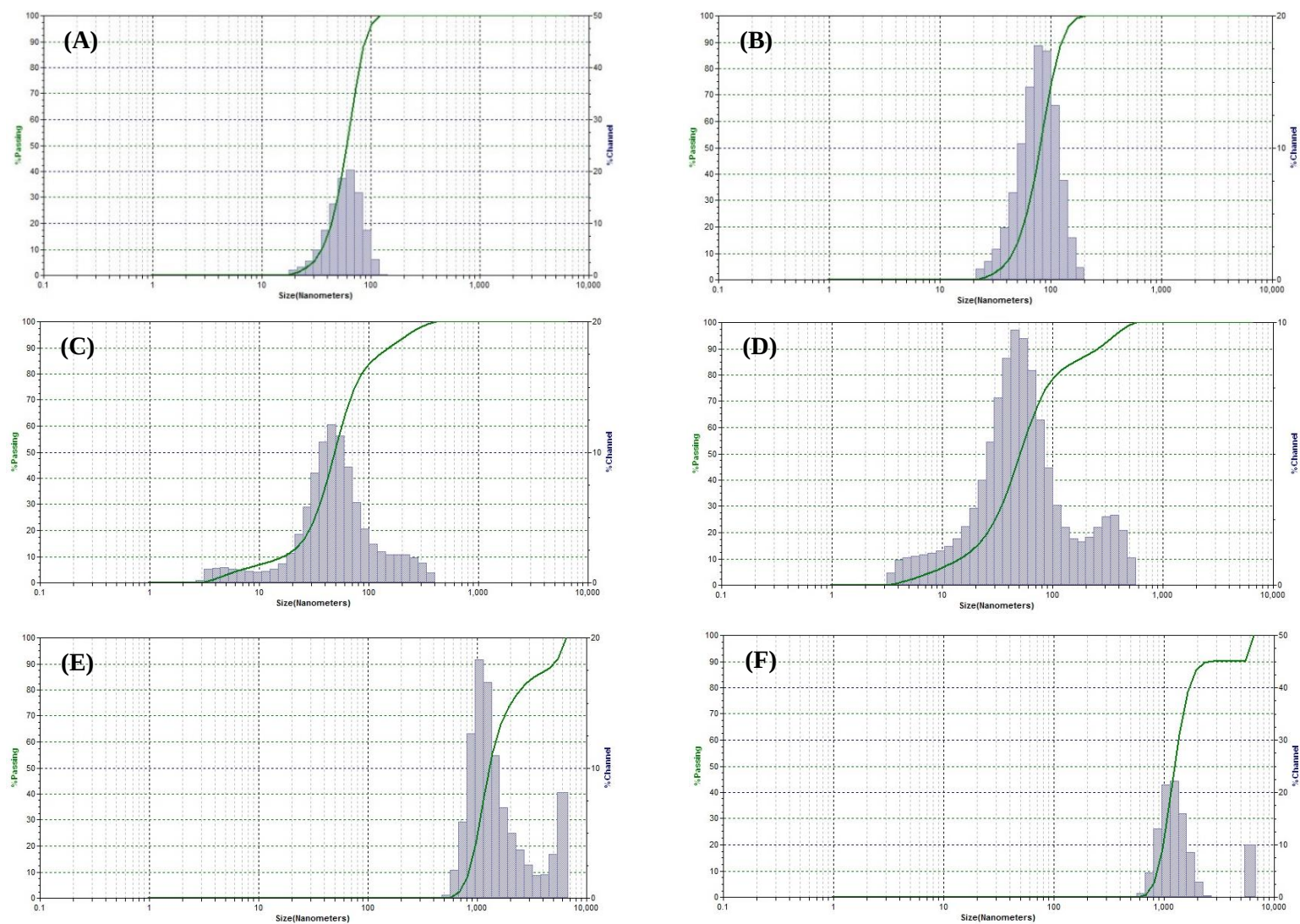


Figure 4.29. Droplet size distribution of microemulsions – S1H1 (A), S1M3 (B), S2M3 (C), S2H3 (D), S3M1 (E), and S3H1 (F)

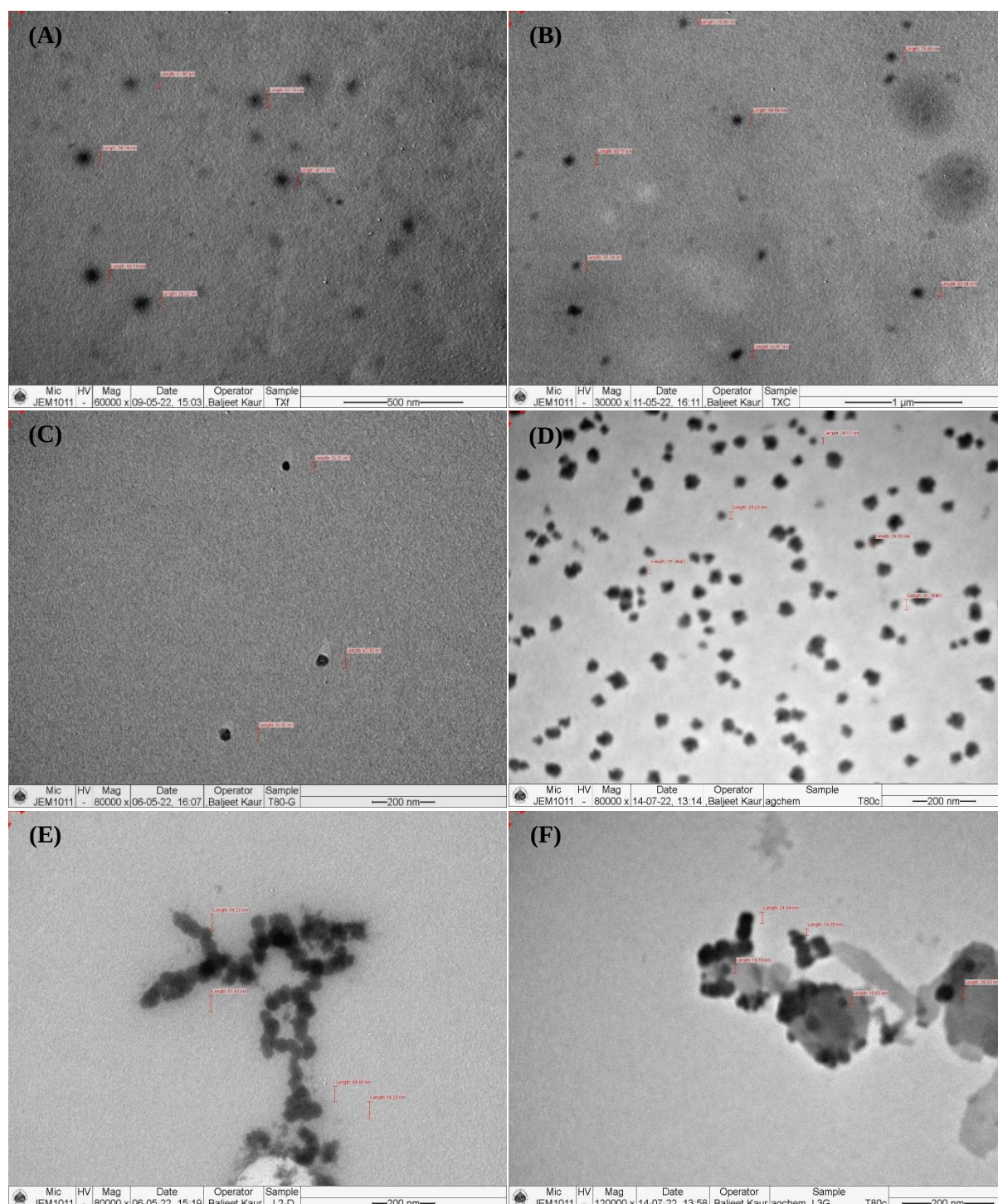


Figure 4.30. Transmission electron micrograph of microemulsions – S1M3 (A), S1H3 (B), S2L1 (C), S2H3 (D), S3M1 (E), and S3H3 (F)

4.6.2.2. Transmission Electron Microscopy (TEM)

The droplet size of the microemulsions was further confirmed by visualization in transmission electron microscope. Representative TEM images of the microemulsions have been illustrated in Figure 4.30. Oil droplets in the microemulsions prepared with S1 surfactant appeared to be

globular in shape and of nano size (Figure 4.30A-B). Aggregation of the droplets was not observed in any microemulsion sample prepared with S1. Micrographs of microemulsions prepared with S2 also suggested formation of nano sized droplets (Figure 4.30C-D). However, Figure 4.30D indicated possible aggregation of oil droplets in few samples which resulted in translucent appearance of the affected microemulsions. Contrastingly, aggregation of oil droplets was observed in all the microemulsion samples prepared with S3 (Figure 4.30E-F). The micrographs hinted at conglomeration of individual nano droplets which might have formed initially during the process. The observations obtained from the particle size analyzer were supplemented with the visuals from TEM.

4.6.2.3. Thermodynamic stability and dispersibility

All the microemulsions had undergone thermodynamic stability tests including heating-cooling cycle, centrifugation, and freeze-thaw cycle. Dispersibility test was performed on those microemulsion samples which successfully passed the thermodynamic stability tests. The findings of the tests have been depicted in Table 4.9.

Results suggested that all the microemulsions prepared with S1 passed all the stress tests successfully and graded A in the dispersibility test. Though all the S2 microemulsions survived the heating-cooling test, only three samples (S2L1, S2M3 and S2H3) were able to endure the remaining stress tests and scored grade A in the dispersibility test. Sedimentation was observed in the remaining S2 samples during the centrifugation test. The scenario was almost similar for the S3 microemulsions. Most of the samples failed in the centrifugation test, despite managed to get through the heating-cooling test. Even after surviving the first two stress tests, sample S3L1 could not overcome the hurdle of freeze-thaw test. Only S3M1 and S3H1 passed all the tests with grade A in the dispersibility test. In brief, two microemulsions prepared with S3, three microemulsions prepared with S2, and all nine microemulsions prepared with S1 were found to be thermodynamically stable and rapidly dispersible in water.

Table 4.9. Thermodynamic stability and dispersibility tests of microemulsions

Sample code	Observations				Inference
	Heating-cooling	Centrifugation	Freeze-thaw	Dispersibility	
S1L1	✓	✓	✓	Grade A	Passed
S1L2	✓	✓	✓	Grade A	Passed
S1L3	✓	✓	✓	Grade A	Passed
S1M1	✓	✓	✓	Grade A	Passed

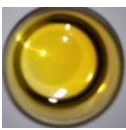
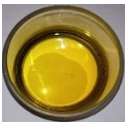
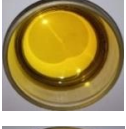
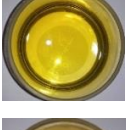
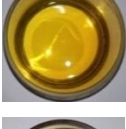
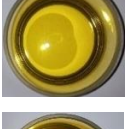
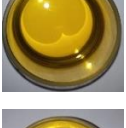
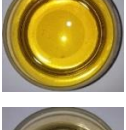
S1M2	✓	✓	✓	Grade A	Passed
S1M3	✓	✓	✓	Grade A	Passed
S1H1	✓	✓	✓	Grade A	Passed
S1H2	✓	✓	✓	Grade A	Passed
S1H3	✓	✓	✓	Grade A	Passed
S2L1	✓	✓	✓	Grade A	Passed
S2L2	✓	×	-	-	Failed
S2L3	✓	×	-	-	Failed
S2M1	✓	×	-	-	Failed
S2M2	✓	×	-	-	Failed
S2M3	✓	✓	✓	Grade A	Passed
S2H1	✓	×	-	-	Failed
S2H2	✓	×	-	-	Failed
S2H3	✓	✓	✓	Grade A	Passed
S3L1	✓	✓	×	-	Failed
S3L2	✓	×	-	-	Failed
S3L3	✓	×	-	-	Failed
S3M1	✓	✓	✓	Grade A	Passed
S3M2	✓	×	-	-	Failed
S3M3	✓	×	-	-	Failed
S3H1	✓	✓	✓	Grade A	Passed
S3H2	✓	×	-	-	Failed
S3H3	✓	×	-	-	Failed

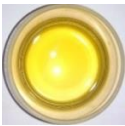
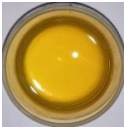
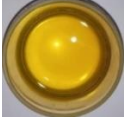
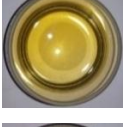
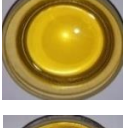
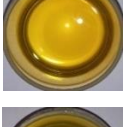
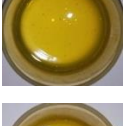
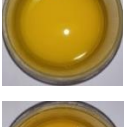
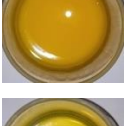
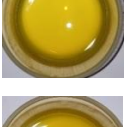
4.6.2.4. Color analysis

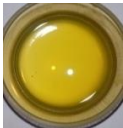
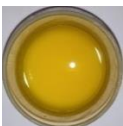
Color is an important parameter for materials to be used as colorants. Therefore, Hunter's L^* , a^* , b^* , chroma and hue angle of the microemulsions were calculated from image analysis of the samples. The results have been portrayed in Table 4.10. All the microemulsions produced different shades of yellow color as observed from the RGB color shade reference. In the Hunter's color scale, all the shades demonstrated a high L^* values (55.26-88.94) to indicate neutral to higher degree of darkness. Positive b^* values were obtained for all the samples, whereas, few positive values were observed for a^* amidst a general negative trend. Determination of hue angle in the Hunter L, a, b color space gave a clear picture about the yellowness of the samples. For all the samples, the hue angle remained close to 90° (pure

yellow) either in the 1st or 2nd quadrant. The highest deviation of hue angle in the 1st quadrant occurred for sample S3L3 (78.40°) with negligible shift towards red (0°), whereas, in the 2nd quadrant for sample S2L2 (105.52°) with minor shift towards green (180°). High chroma (32.03-55.02) implied vividness or saturation of the yellow color in the samples.

Table 4.10. Color expression of microemulsions

Sample	Image	RGB color shade (3-points ave.)	L*	a*	b*	Hue angle (°)	Chroma	ΔE
S1L1			70.39	-1.53	42.89	92.05	42.92	19.62
S1L2			70.07	-1.61	42.76	92.15	42.79	19.32
S1L3			73.39	-1.09	44.69	91.39	44.70	22.60
S1M1			73.13	-6.08	44.50	97.79	44.91	19.69
S1M2			68.62	-1.69	41.90	92.31	41.93	18.06
S1M3			74.51	-2.38	45.37	93.01	45.43	22.93
S1H1			72.43	-8.11	43.99	100.45	44.73	18.08
S1H2			67.56	-0.89	41.26	91.24	41.27	17.84
S1H3			73.71	-5.17	44.92	96.56	45.22	20.74
S2L1			64.23	-5.53	31.55	99.95	32.03	11.21

S2L2			88.94	-14.80	53.30	105.52	55.32	42.23
S2L3			67.54	1.98	41.22	87.25	41.27	21.66
S2M1			67.57	-4.91	37.92	97.38	38.24	17.69
S2M2			62.67	-1.49	38.19	92.24	38.22	17.95
S2M3			65.54	2.05	39.98	87.07	40.03	20.32
S2H1			67.39	-4.31	40.01	96.16	40.24	19.70
S2H2			70.22	-4.06	42.55	95.45	42.74	22.71
S2H3			67.76	-0.63	41.35	90.87	41.35	21.29
S3L1			58.29	2.86	35.63	85.41	35.74	25.09
S3L2			61.17	1.63	37.39	87.50	37.42	26.74
S3L3			55.35	6.95	33.85	78.40	34.55	24.90
S3M1			67.12	-4.34	40.86	96.06	41.09	30.96
S3M2			58.29	2.85	35.67	85.44	35.78	25.12

S3M3			55.26	6.09	33.84	79.79	34.39	24.57
S3H1			63.72	-4.22	38.83	96.21	39.06	28.12
S3H2			62.31	1.20	38.07	88.20	38.09	27.49
S3H3			61.11	3.29	37.40	84.98	37.54	27.10
S1 Control			58.10	-14.75	35.19	112.74	38.16	-
S2 Control			64.76	-3.71	20.50	100.25	20.84	-
S3 Control			57.86	-3.41	11.34	106.74	11.84	-

4.6.3. Freeze drying of biopolymer stabilized oil-in-water (O/W) microemulsions

Based on the thermodynamic stability, dispersibility, droplet dimensions, color expression and surfactant concentration, three microemulsions (S1M3, S2M3 and S3M1) were selected for preparation of powder formulations (Fig 4.31) separately by simple freeze-drying technique. This technique removed moisture from the outer aqueous phase of the microemulsions at very low temperature (-70°C) under vacuum. The biopolymer which was at the aqueous phase, encapsulated the oil droplets without disturbing the microemulsion structure at these conditions. The freeze-dried powders were named as P_{S1M3} , P_{S2M3} , and P_{S3M1} , respectively depending on the microemulsions from which they have been prepared. Comparative visuals have been presented in Fig 4.31, which showed that the xanthophylls/lutein esters remained insoluble in water, but their powder formulations readily got solubilized in water and produced transparent/translucent yellow-colored solutions immediately.

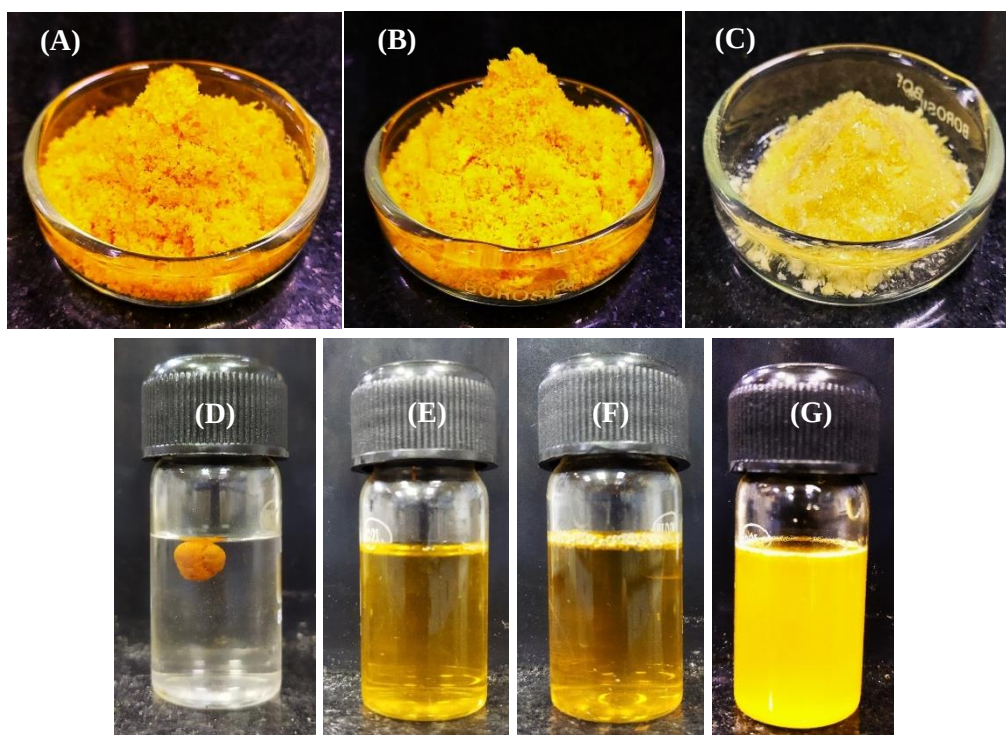


Figure 4.31. Solid powder formulations of xanthophylls/lutein esters and their respective water-soluble forms: P_{S1M3} (A, E), P_{S2M3} (B, F), and P_{S3M1} (C, G); Aqueous insolubility of xanthophylls/lutein esters (D)

4.6.4. Physico-chemical characterization of the soluble powder formulations

4.6.4.1. Fourier Transform Infrared (FT-IR) spectroscopy

FT-IR spectra of extracted xanthophylls, the biopolymer and the three powder formulations have been represented in Figure 4.32. Xanthophyll extract was characterized by a sharp band at 1730 cm^{-1} for C=O stretching of the esters, two sharp bands at 2920 and 2852 cm^{-1} for asymmetric and symmetric C–H stretching of the alkyl chain(s), a broad band at 3394 cm^{-1} for O–H stretching the free hydroxyl groups, a sharp band at 1460 cm^{-1} for C=C stretching of the aromatic ring, and a sharp band at 965 cm^{-1} for C–H bending vibration (Figure 4.32E). No sharp bands were observed in the spectra of the biopolymer. However, broad bands at 3744 cm^{-1} (O–H stretching), 2863 cm^{-1} (C–H stretching), and 1026 cm^{-1} (C–N stretching) hinted at the typicality of a polysaccharide (Figure 4.32A). Domination of the bands of C–H and C–N stretching vibrations in the FT-IR spectra of the powder formulations, indicated the encapsulation of xanthophylls within the biopolymer. Furthermore, the absence of the typical C=O stretching of the esters in any of the spectra of the formulations reinforced the concept.

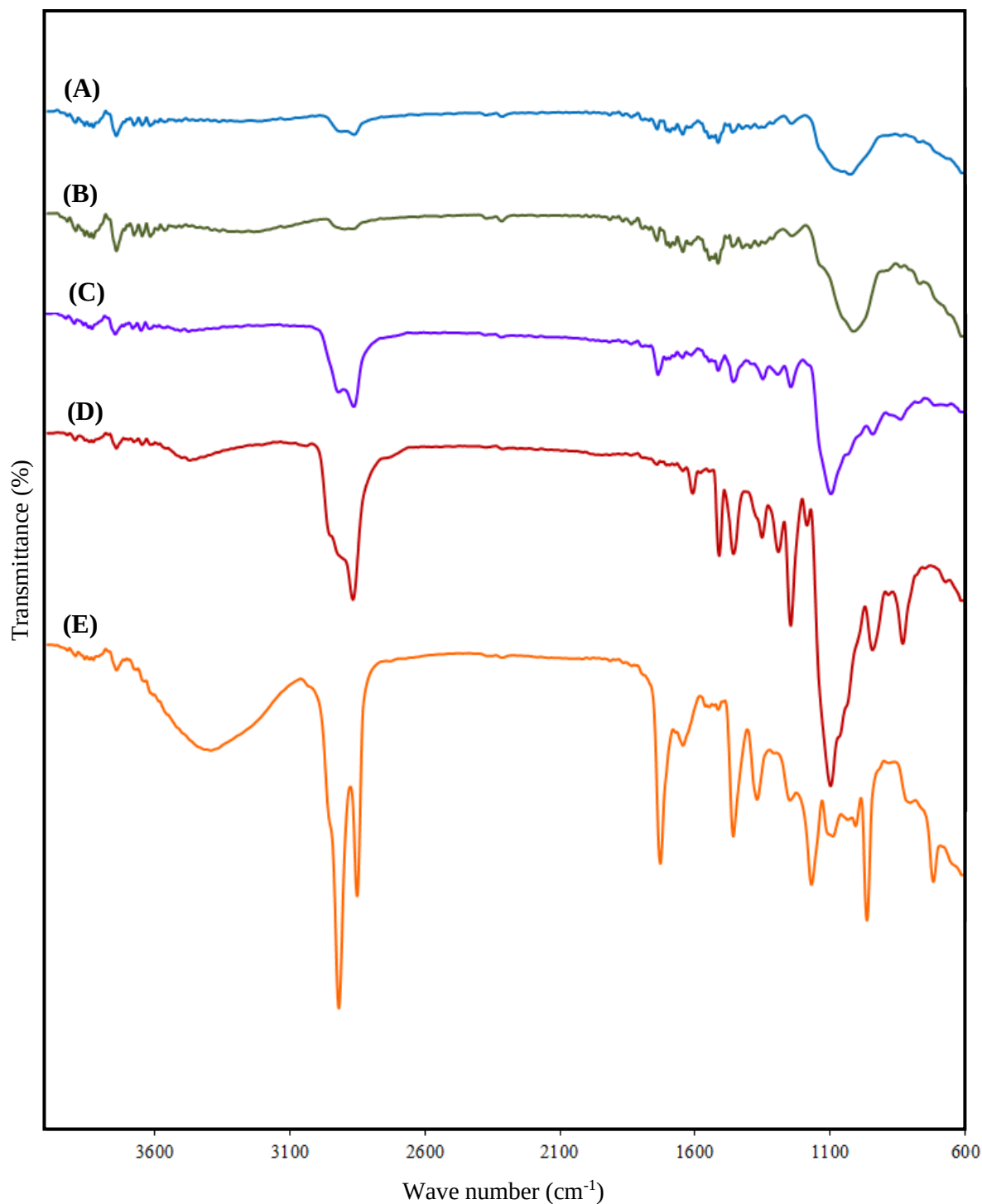


Figure 4.32. FT-IR spectra of biopolymer (A), P_{S3M1} (B), P_{S2M3} (C), P_{S1M3} (D), and extracted xanthophylls (E)

4.6.4.2. Scanning Electron Microscopy (SEM)

Scanning electron micrographs of the powder formulations indicated rough surface morphology for P_{S1M3} (Figure 4.33A) and P_{S2M3} (Figure 4.33B). Porous nature of both the powders suggested the fluffiness of their structure. In contrary, smooth surface morphology

was observed for P_{S3M1} (Figure 4.33C), suggesting glossy appearance of the powder formulation.

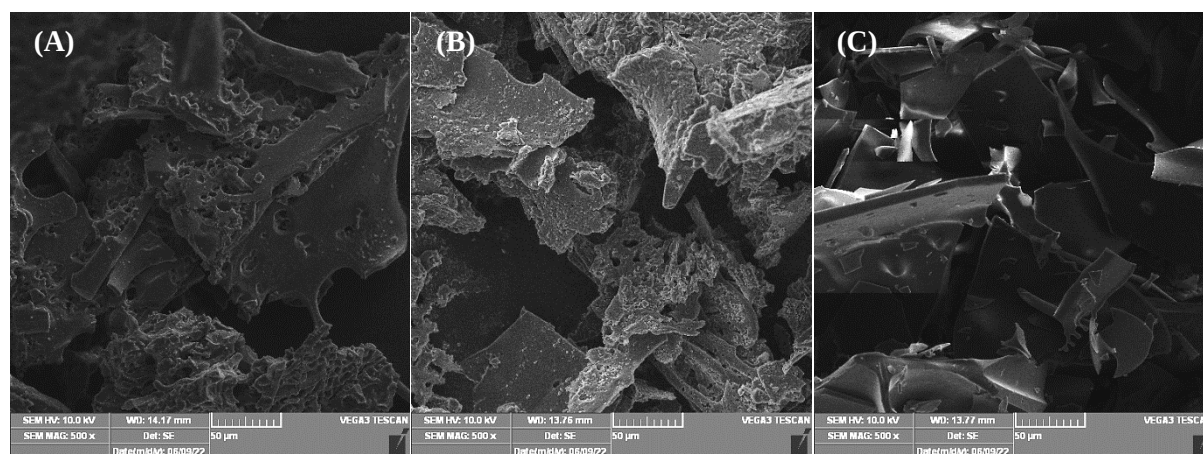


Figure 4.33. Scanning electron micrographs of P_{S1M3} (A), P_{S2M3} (B), and P_{S3M1} (C)

4.6.4.3. Storage stability

Table 4.11. Effect of storage temperature on stability of xanthophylls in the powder formulations

Parameter	Temperature	Formulation		
		P _{S1M3}	P _{S2M3}	P _{S3M1}
Total xanthophyll content (µg g⁻¹)	-	245.46 ± 5.27 ^a	247.79 ± 4.18 ^a	175.61 ± 3.89 ^a
Xanthophyll content after storage (µg g⁻¹)	0±2 °C	239.47 ± 8.63 ^a	236.55 ± 6.23 ^a	171.24 ± 5.47 ^a
	26±2 °C	121.04 ± 2.96 ^b	150.74 ± 4.75 ^b	47.33 ± 1.87 ^b
	54±2 °C	54.19 ± 2.59 ^c	34.70 ± 0.59 ^c	23.66 ± 1.82 ^c
Change in xanthophyll content after storage (%)	0±2 °C	2.44	4.54	2.49
	26±2 °C	50.69	39.17	73.05
	54±2 °C	77.92	86.00	86.53

Tukey's HSD test was performed to flag significant differences among the treatment means within a column. Different alphabets within a column suggested significant difference at 95% confidence limit

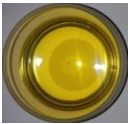
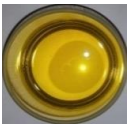
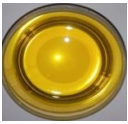
Analysis of the powder formulations suggested that P_{S1M3} and P_{S2M3} contained almost same amount of total xanthophylls, because both of them were prepared from the microemulsions containing same amount of oil phase (0.5%). For the same reason, xanthophyll content of P_{S3M1} was lower than the other two, as it was prepared from the microemulsion containing 0.1% oil phase. Storage temperature has been found to have significant impact on the stability of the encapsulated xanthophylls in the powder formulations (Table 4.11). Storage of the formulations at 0 ± 2 °C for 14 days degraded only insignificant amount of xanthophylls (2.44-

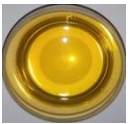
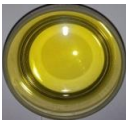
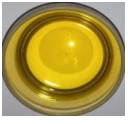
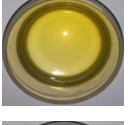
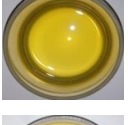
4.54%). However, at accelerated storage conditions (54 ± 2 °C for 14 days), degraded the encapsulated xanthophylls to a great extent (77.92-86.53%) in all the formulations. Even storage at room temperature (26 ± 2 °C) for 14 days degraded almost 50% encapsulated xanthophylls in P_{S1M3}, 39% in P_{S2M3}, and 73% in P_{S3M1}. Highest degradation was observed for P_{S3M1}. These observations suggested that the prepared formulations were not heat stable and storage above the temperature of 0 °C might significantly impact the stability of the encapsulated xanthophylls. Therefore, the formulations should be used and stored at subzero temperature.

4.6.4.4. Color intensity

Color expression of the aqueous solutions of the final products were of utmost importance for their further use as food colorants. Therefore, the color parameters namely, Hunter's L*, a*, b*, chroma and hue angle of the aqueous solutions (2.5, 5, 7.5 and 10%) of all the three powder formulations were analyzed from their respective images (Table 4.12). All the samples except 2.5% P_{S3M1}, produced vivid yellow color of the aqueous solutions (chroma of 40.53-56.00). The hue angle for all the samples was near about 90° (87.09°-106.26°). Interestingly, for P_{S1M3} and P_{S2M3}, the hue angle was found to be shifted from 2nd quadrant (> 90°) to 1st quadrant (< 90°) of the color space with an increase in the concentration of aqueous solution. The deviation of the color of the samples from the control (ΔE) was sufficient enough to distinguish the color of the aqueous solutions as unique yellow. The exact color of the aqueous solutions as observed from the color shade provided in Table 4.12 suggested that with little change in the concentration of the powder formulations in aqueous solution, a wide array of shades of yellow color can be produced as per requirement.

Table 4.12. Color expression of aqueous solutions of the powder formulations

Sample	Image	RGB color shade (3-points ave.)	L*	a*	b*	Hue angle (°)	Chroma	ΔE
P _{S1M3} (2.5%)			65.89	-5.18	40.20	97.35	40.53	30.48
P _{S1M3} (5%)			71.35	-2.75	43.51	93.62	43.60	35.30
P _{S1M3} (7.5%)			69.20	0.29	42.26	89.60	42.26	37.34

P _{S1M3} (10%)			71.93	2.24	43.93	87.09	43.98	35.25
P _{S2M3} (2.5%)			89.02	-15.68	53.76	106.26	56.00	59.26
P _{S2M3} (5%)			71.73	-12.18	43.48	105.65	45.15	40.05
P _{S2M3} (7.5%)			75.92	-5.11	46.25	96.30	46.53	46.63
P _{S2M3} (10%)			69.60	0.21	42.48	89.72	42.48	41.30
P _{S3M1} (2.5%)			61.88	-5.38	23.07	103.13	23.68	21.77
P _{S3M1} (5%)			69.87	-7.81	41.57	100.64	42.29	42.60
P _{S3M1} (7.5%)			68.76	-4.19	41.84	95.72	42.05	36.59
P _{S3M1} (10%)			87.35	-11.96	52.78	102.77	54.12	47.55

4.6.4.5. Antioxidant activity

Apart from providing color, the aqueous solutions of the powder formulations exhibited antioxidant properties in terms of free radical scavenging activity (DPPH assay) and reducing power (FRAP and CUPRAC assays). The antioxidant activity of the prepared formulations has been depicted in Figure 4.34 as vitamin C equivalent antioxidant capacity (VCEAC). The free radical scavenging activity of P_{S1M3} and P_{S2M3} was comparable as observed from the curves of DPPH assay. As xanthophyll content of P_{S3M1} was less, VCEAC of the formulation was also less than the other two formulations in that particular assay. However, the less xanthophyll content of P_{S3M1} did not significantly impact its ferric ion reducing power at all the concentrations and cupric ion reducing power at lower concentrations.

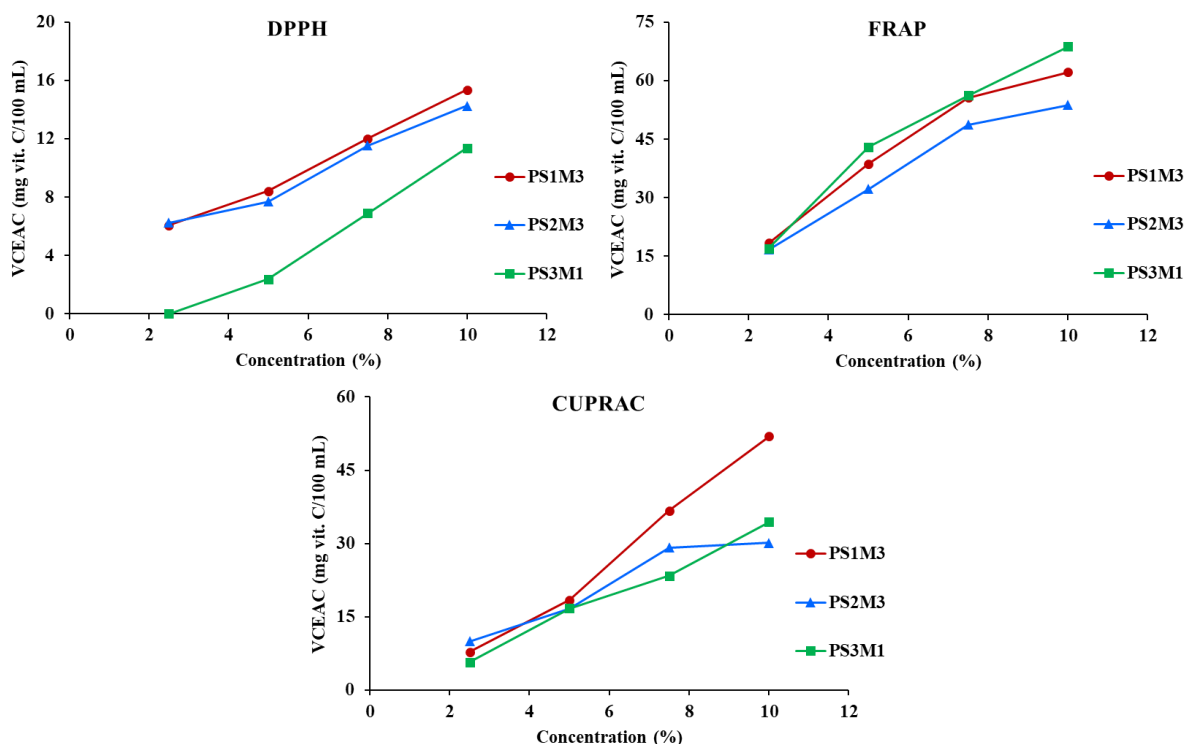


Figure 4.34. Antioxidant activity of solid powder formulations

4.6.4.6. *In vitro* bioavailability

The results of the simulated *in vitro* bioavailability study of the powder formulations have been reported in Table 4.13. Major portion of the encapsulated xanthophylls was available in the simulated gastric phase. Around 31% xanthophylls was detected in the simulated intestinal phase from P_{S1M3}. Whereas, only 9% xanthophylls from P_{S2M3} crossed the microvilli membrane and reached to the intestinal phase. Xanthophylls were not detected in the intestinal phase for P_{S3M1}. The results suggested that the surfactant had a major role to play in crossing the membrane barrier to enable the xanthophyll molecules to reach the intestine from stomach.

Table 4.13. Bioavailability of xanthophylls from powder formulations in a simulated gastro-intestinal condition

	Formulation		
	P _{S1M3}	P _{S2M3}	P _{S3M1}
Xanthophyll content in simulated gastric condition (%)	68.33	90.89	98.25
Xanthophyll content in simulated intestinal condition (%)	31.67	9.11	nd

5. DISCUSSION

The main pigment responsible for the bright yellow to orange coloration of marigold flowers has been identified as lutein, a xanthophyll, which naturally occurs in its esterified forms. Early literature reported preponderance of lutein in pigment complex of both *Tagetes erecta* and *Tagetes patula* petals (Quackenbush and Miller, 1972). Later it was established that lutein esters invariably account for more than 70% of the total xanthophyll pigments present in marigold florets (Sowbhagya *et al.*, 2004; Šivel *et al.*, 2014), along with some other minor carotenoids like zeaxanthin, cryptoxanthin, rhodoxanthin, canthaxanthin, violaxanthin, antheraxanthin, carotenes etc. (Zonta *et al.*, 1987; Scalia and Francis, 1989). Previously, various mono- and di-esters of lutein were tentatively identified and quantitated from marigold petal extracts directly by high performance liquid chromatography (HPLC) or indirectly by gas chromatography (GC) following hydrolysis (Gregory *et al.*, 1986; Rivas, 1989; Piccaglia *et al.*, 1998). The list of identified compounds included monoesters of lutein like palmitate, myristate, stearate, and diesters like dipalmitate, dimyristate, distearate, myristate-palmitate, palmitate-stearate etc.

With the advent of mass spectrometry (MS) and introduction of high-resolution MS techniques, complete profiling of the extract could be possible with identification of the major and minor compounds (Petry and Mercadante, 2016). Two separate studies identified six major lutein diesters namely, laurate-myristate, dimyristate, myristate-palmitate, dipalmitate, palmitate-stearate, and distearate in marigold flower extract by LC-MS analysis (Breithaupt *et al.*, 2002; Tian *et al.*, 2003). Researchers further went on to separate and identify *cis*- and *trans*-isomers of those six lutein diesters from marigold petals by LC-MS (Tsao *et al.*, 2004; Li *et al.*, 2007). Along with the previously mentioned diesters, Tsao *et al.* (2004) also reported the presence of *cis*- and *trans*- isomers of lutein laurate-palmitate and myristate-stearate in marigold extract. Abdel-Aal and Rabalski (2015) identified regioisomers of mono- and di-esters of lutein from seven marigold cultivars including both *T. erecta* and *T. patula*. Recently, in a comprehensive analysis by LC-MS/MS, a total of 56 carotenoids including 6 free carotenoids, 20 monoesters and 30 diesters were identified from marigold petals (Rodrigues *et al.*, 2019). In that study, apart from mono- and di-esters of lutein, esters of zeaxanthin, β -cryptoxanthin, violaxanthin, zeinoxanthin, and auroxanthin were also identified. In all the above-mentioned MS based techniques, atmospheric pressure chemical ionization (APCI) mode was used in ionization source to obtain abundant molecular ion peaks. Carotenoid profiling with electrospray ionization (ESI) mode in LC-MS is not uncommon though (Wada

et al., 2011; Pop *et al.*, 2014). In the present study, UPLC-QToF-ESI-MS was used for profiling of xanthophylls from the DAMH-39 genotype of *T. erecta*. Five monoesters and four diesters of lutein were successfully identified along with two diesters of violaxanthin. Interestingly, lutein linoleate and lutein dilinoleate were reported to be present in marigold for the first time, which are commonly abundant in cereal grains like wheat, canary, and tritordeum (Mellado-Ortega and Hornero-Méndez, 2012; Ziegler *et al.*, 2015; Abdel-Aal *et al.*, 2022). During the HPLC quantification of total xanthophylls in the marigold extract, trace amount of free lutein was detected along with the fatty acid esters. Saponification of the extract confirmed that the peaks obtained in the native extract were indeed of esters of lutein and its isomers (Vechpanich and Shotipruk, 2010).

As compared to ultrasonic probe extraction (UPE), both ultrasonic bath extraction (UBE) and conventional solid-liquid extraction (CE) techniques consumed 3-times more extraction solvent to extract almost same amount of xanthophylls from the marigold florets. Furthermore, to do so only 30 min time was found to be sufficient for UPE in comparison to 4 h and 30 h for UBE and CE, respectively. Clearly, UPE emerged out as a more efficient technique than CE and UBE for xanthophyll extraction from marigold florets in terms of solvent usage and time management. Nevertheless, prolonged extraction time with large volume of solvent resulted in more physical weight of the extract in CE and UBE which in turn reduced the purity of the extracted xanthophylls. The better extraction efficiency of ultrasound assisted techniques can be attributed to the phenomenon of acoustic cavitation, where ultrasound produces fast moving micro-bubbles in solvent, which propagate and eventually collapse on the solid surface producing powerful shock waves to rupture the cells (Chemat *et al.*, 2008). However, direct contact of the ultrasonic probe with the sample generated 100 times more power to extract xanthophylls in shorter time as compared to ultrasonic bath, where indirect irradiation was supplied through the walls of container (Pico, 2013). In a similar study to extract phenolic acids from *Quercus infectoria* galls, UPE technique was found to be 15% more efficient than UBE, and required much less time to obtain same amount of yield as compared to conventional solvent extraction (Sukor *et al.*, 2018). Martín-García *et al.* (2022) compared the efficiency of ultrasonic probe and ultrasonic bath to extract oleuropein and hydroxytyrosol from olive leaves and also found no significant difference in their recovery by both the techniques, but UPE required much less time (8 min) than UBE (180 min) to obtain the same yield.

Optimization of the UPE technique by Box-Behnken design (BBD) of response surface methodology (RSM) predicted the optimum values of the factors namely, solvent to solid ratio, ultrasonication amplitude, and time, which were considered responsible for maximization of the xanthophyll yield. Fine-tuning of the RSM based optimization process using genetic algorithm (GA) further improved the extraction yield. As compared to the predicted xanthophyll yield of 12923.40 $\mu\text{g g}^{-1}$ by RSM, the GA coupled model estimated maximum xanthophyll yield of 13350.25 $\mu\text{g g}^{-1}$. Validation experiment yielded 12879.39 and 13099.05 $\mu\text{g g}^{-1}$ of xanthophyll by RSM and GA optimized conditions, respectively. The closeness of the observed values with the predicted values indicated the success of the statistical models for process optimization. Previously, mathematical/statistical models have been used for optimization of various extraction process of lutein/xanthophylls from marigold petals in several studies. The process kinetics of a single-step solvent extraction and simultaneous alkali hydrolysis to obtain lutein from marigold petals has been optimized by mathematical modelling (Hojnik *et al.*, 2008). The optimized process was reported to extract about 80% of total lutein within 4 min. RSM was used to optimize solid to solvent ratio, extraction time and temperature for lutein extraction from marigold petals with a maximal reported yield of 5670.5 $\mu\text{g g}^{-1}$ (Mai and Ta, 2020). A yield of 6900 $\mu\text{g g}^{-1}$ for lutein esters from marigold petals by ultrasound assisted supercritical CO_2 extraction technique was reported by Gao *et al.* (2009), where Sovová model was used to optimize various extraction parameters. The BBD or CCD (central composite design) of RSM has been used in process optimization for ultrasound assisted solvent extraction of lutein/lutein esters/xanthophylls from various other sources like microalgae (Deenu *et al.*, 2013; Priyanka *et al.*, 2021), oil palm fronds (Ofori-Boateng and Lee, 2013), spinach (Altemimi *et al.*, 2015), pumpkin peel (Song *et al.*, 2018), corn meal (Wang *et al.*, 2019), kinnow peel (Saini *et al.*, 2021) etc. However, process optimization of ultrasound assisted extraction of xanthophylls through the RSM model coupled with GA approach has been reported for the first time in the present study.

The antioxidant activity of xanthophylls extracted by different techniques gave an indirect idea about the purity of the extracts. Though no difference in the IC_{50} value of the xanthophylls extracted by various techniques was observed in ABTS radical scavenging assay, a relatively higher value for conventionally extracted xanthophylls in DPPH radical scavenging assay suggested a reduced antioxidant activity. This might be due to the increased physical yield of the extract contributed by other materials from prolonged extraction. Similarly, though the CUPRAC assay could not distinguish the reducing power of the extracts, FRAP assay

clearly indicated the purity of the extracted xanthophylls by GA optimized UPE technique over the others. This result was in agreement with a study conducted by Kazibwe *et al.* (2017), where ultrasonic probe assisted aqueous extract of marigold flower was found to have enhanced antioxidant property (in terms of DPPH radical scavenging activity) as compared to its ultrasonic bath assisted aqueous extract and the aqueous extract obtained by conventional extraction technique. Similarly, antioxidant capacity (DPPH assay) of several plant extracts obtained by ultrasonic probe extraction technique was found to be significantly higher than the same extracts obtained by other techniques like ultrasonic bath, Soxhlet extraction, pressurized liquid extraction, and supercritical fluid extraction (Bajerová *et al.*, 2014). However, Adam *et al.* (2009) could not find any difference in antioxidant activity (DPPH assay) of extracts of six different plants extracted by ultrasonic bath and probe.

Green extraction techniques are now-a-days preferred to reduce the usage of hazardous organic solvents and power consumption with minimum/no waste generation (Chemat *et al.*, 2012). Extraction of lutein/lutein esters from marigold by various techniques using green solvents have been reported in recent literature. Techniques like supercritical CO₂ extraction of lutein esters with soybean oil (Ma *et al.*, 2009) or medium-chain triglyceride (Gao *et al.*, 2010) as co-solvents; ultrasound assisted extraction of lutein using soybean oil as solvent (Manzoor *et al.*, 2022), have been successfully demonstrated as green extraction techniques. In order to employ a novel green technique for extraction of xanthophylls from marigold florets, deep eutectic solvents (DES) were explored in the present study. Till date no report is available on extraction of xanthophylls from marigold petals using DES. Only recently, the potential of fenchyl alcohol/thymol-based natural deep eutectic solvent (NADES) for lutein extraction from microalgae has been reported (Fan *et al.*, 2022). As the native pigments of marigold are lipophilic lutein esters, hydrophobic deep eutectic solvents (HDES) composed of binary mixture of two saturated fatty acids (capric and caprylic acid) were explored for their potential application as green solvent in the present study.

The melting point of the eutectic mixture was found to be decreasing with increase in molar ratio of caprylic acid. In contrary, density of the mixture was found to be increasing with increased content of caprylic acid in the mixture. This result is in agreement with previous reports where decrease of melting point of C₁₂ fatty acid was observed with increasing molar ratio of C₈, C₉ and C₁₀ fatty acids in the eutectic mixture (Florindo *et al.*, 2018; Arcon and Franco Jr, 2020). The calculated enthalpy of melting of the eutectic mixtures indicated strong interaction between the two fatty acids. As the DES system is based on the interaction between

a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA), formation of H-bonds between the participating molecules are anticipated (Abbott *et al.*, 2004). The formation of the H-bonds between C₈ and C₁₀ fatty acids in the prepared eutectic mixtures resulted in higher enthalpy (Kollau *et al.*, 2018). Interestingly, highest enthalpy was observed at 1:1 molar ratio of caprylic (C₈) and capric (C₁₀) acid, suggesting maximum interactions between the two molecules.

FT-IR and ¹H NMR are two specific techniques to characterize DES, from which the interaction between HBD and HBA can be confirmed. In the present study, all the HDES mixtures displayed the same characteristic peaks as those of the individual components in FT-IR, indicated that no chemical reaction occurred between the C₈ and C₁₀ fatty acids. These observations were in agreement with the previous reports of HDES based on binary mixture of fatty acids (He *et al.*, 2013; Ke, 2017; Arcon and Franco Jr, 2020; Haider *et al.*, 2020). Like those of the FT-IR spectra, the ¹H NMR spectra of the eutectic mixtures of the two fatty acids were almost similar to that of their individual constituents suggesting no chemical reactions (Arcon and Franco Jr, 2020). However, chemical shift of the hydroxyl proton in the eutectic mixtures clearly indicated the formation of H-bonds between the constituents.

Use of DES as solvent in ultrasound assisted extraction of natural compounds has been considered as one of the efficient green techniques (Duan *et al.*, 2016). Therefore, in the present study, efficiency of the HDES to extract xanthophylls from marigold florets by ultrasonication technique has been optimized by BBD of RSM. Three factors namely, molar ratio of C₈ and C₁₀ fatty acids, volume of the solvent, and ultrasonication time were optimized to obtain maximum yield of xanthophylls. As compared to the model predicted value of 7862.61 µg g⁻¹, the validation experiment yielded 7570.80 µg g⁻¹ total xanthophylls. Same volume of conventional solvent (hexane-acetone 1:1) yielded only 5706.90 µg g⁻¹ xanthophylls from the marigold powder for the same time of ultrasonication. Therefore, the HDES not only could extract more amount of xanthophylls, complete elimination of hazardous solvents was the added advantage of this technique. Recently, Stupar *et al.* (2021) showed the efficiency of HDES composed of C₈ and C₁₀ fatty acids (3:1) to extract β-carotene from pumpkin by the ultrasound assisted technique. In that study, RSM and ANN (artificial neural network) model were adopted to optimize the extraction process to maximize β-carotene yield. In a similar study, RSM based optimization was carried out to extract ergosterol from button mushrooms using HDES composed of menthol and pyruvic acid by ultrasonication (Khare *et al.*, 2021). RSM based optimization for ultrasound assisted HDES based extraction of natural compounds

has become quite popular in recent times and the technique was further extended for extraction of various phytochemicals, of which, artemisinin from *Artemisia annua* (Cao *et al.*, 2017), curcuminoides from turmeric (Kongpol *et al.*, 2022), taxanes from *Taxus chinensis* (Fan *et al.*, 2021) are to name a few.

High viscosity and low volatility of the HDES make them difficult to remove from extract by conventional technique like rotary evaporation and recovery of the extracted compounds remains a challenge (Mišan *et al.*, 2020). Therefore, to separate the dissolved compounds from HDES, alternative strategy like polarity switching has been adapted (Sed *et al.*, 2018; Chen and Mu, 2021). In the present study, heat treatment of the HDES with 40% aqueous KOH solution was found to effectively switch the polarity of the solvent. The lipophilic xanthophylls started to precipitate from the solvent with switched polarity (hydrophobic to hydrophilic). Earlier, Sed *et al.* (2018) demonstrated that the hydrophobicity of the HDES prepared with combination of C₈ and C₁₂ fatty acids could be altered to hydrophilicity by treatment with dilute (5%) aqueous solution of Jeffamine D-230. Later, Stupar *et al.* (2021) used ammonium hydroxide (25%) to switch the polarity of HDES prepared with combination of C₈ and C₁₀ fatty acids for recovery of β -carotene extracted from pumpkin. Probably the change of pH with addition of alkali triggered the polarity switch which in turn altered the strength of weak interaction between the HBD and HBA (Chen and Mu, 2021; Stupar *et al.*, 2021).

The SEM micrographs of marigold floret powder after ultrasound assisted extraction by HDES showed complete destruction of the surface morphology. The cavitation process of ultrasound helped the microbubbles of HDES to rupture the cells easily and thereby efficient extraction of xanthophylls. In comparison, the surface morphology of marigold floret powder after ultrasound assisted extraction by exactly same volume of conventional solvent (hexane-acetone 1:1) for same duration remained almost intact. This observation justified less efficiency of conventional solvent than HDES in extracting xanthophylls. Similar morphological condition was reported for Rhei Radix et Rhizoma powder while extracting anthraquinones with HDES composed of combination of long-chain alkanol and alkyl carboxylic acid (Huang *et al.*, 2022).

A clinical human trial study in the USA during 2002 categorically suggested that lutein bioavailability in human beings is not compromised by esterification with fatty acids (Bowen *et al.*, 2002). In fact, the study reported better bioavailability of lutein diester formulation as compared to the free lutein formulation. The only determining factor that limited the

bioavailability of esterified lutein was emerged as the dissolution. Therefore, the present study aimed to develop water-soluble powder formulation of native xanthophylls (dominated by lutein esters) of marigold.

In the present study, the water-soluble powder formulations of xanthophylls were prepared in two steps. The first step involved preparation of a biopolymer stabilized oil-in-water (O/W) microemulsions by emulsion dilution technique, followed by the second step of freeze drying of the selected microemulsions. Utmost importance was given to the first step of preparation of microemulsions and their characterization as the best performing microemulsions were subsequently chosen for the second step. The type and concentration of surfactant played a major role in the droplet size distribution of the microemulsions, which in turn effected their physical appearance/optical transparency (McClements, 2012). This correlation was found very prominent as it was observed that the microemulsions which appeared optically transparent had mean droplet diameter below 100 nm and narrow polydispersity index (PDI). Similarly, microemulsions with mean droplet diameter above 100 nm and/or high PDI appeared optically translucent or opaque. Gradual aggregation or coalescence of the oil droplets was found responsible for translucency/opacity of the microemulsions. Uniform, unimodal and narrow spectrum of droplet size distribution resulted in a thermodynamically stable microemulsion due to the reduced probability of Ostwald ripening (McClements, 2012), which provides a long-term stability of the microemulsion. In the present study, rigorous stress tests were performed to check the thermodynamic stability of the microemulsions. As expected, the microemulsions with unimodal and narrow spectrum of droplet size distribution only passed those tests and emerged as thermodynamically stable. Interestingly, the thermodynamically stable microemulsions were also found to be highly dispersible in water as on aqueous dilution transparent emulsions formed rapidly.

An early attempt was made to prepare a biopolymer stabilized O/W emulsion, where marigold oleoresin was homogenized in an aqueous solution of mesquite gum and gum Arabic blend with the help of propylene glycol (Vernon-Carter *et al.*, 1996). Though the prepared emulsions were found to be stable against drop coalescence, transparent microemulsions did not form in that study. Later, Amar *et al.* (2003) tried to prepare microemulsions of lutein esters using a composition of water, glycerol, ethanol, Tween-80, and limonene, but could achieve only limited solubilization (~60%) of lutein esters in the O/W microemulsion. In a similar study, O/W emulsion (mean droplet diameter 1.4-1.5 μm) of lutein esters was prepared by emulsifying a medium-chain triacylglycerol oil/orange oil extract of marigold in an aqueous

solution of β -lactoglobulin using an ultrasonic homogenizer (Khalil *et al.*, 2012). Recently, O/W nanoemulsions of carotenoid esters extracted from *Lycium barbarum* were prepared with olive oil and soybean oil using sucrose esters and monoacylglyceride as emulsifiers (Luo *et al.*, 2020).

All the microemulsions prepared in the present study showed vivid yellow color as assessed from the calculated parameters like hue angle and chroma. The RGB color shade produced by each microemulsions was completely distinct from the neutral greyish color of the respective controls. The distinctiveness was further substantiated by very high ΔE values for all the microemulsions, as ΔE value exceeding 5 is quite obvious to the human eyes (Obón *et al.*, 2009; Mesnier *et al.*, 2014).

Freeze drying is a common technique for encapsulation of thermolabile phytochemicals. Colloidal dispersions systems like microemulsions can easily be freeze dried without destabilizing the colloidal structure (do Vale Morais *et al.*, 2016). In the present study, freeze drying of selected microemulsions enabled encapsulation of xanthophylls within the biopolymer by sublimation of water and resulted in solid powder formulations. Previous attempt to prepare free flowing solid powder formulation of lutein esters included a crude process to mix lutein esters with rice flour (Tiwarý *et al.*, 2014). Recently, Tippel *et al.* (2016) prepared lutein ester loaded micellar solution and nanoemulsion using 1% Quillaja saponin in a microfluidizer, which were mixed with 50% glucose syrup and spray dried to prepare powder formulations. Nevertheless, the powder formulations prepared in the present study were readily water soluble and produced transparent/translucent aqueous solutions.

Complete encapsulation of xanthophylls within the biopolymer was confirmed from FT-IR spectra. Absence of the characteristic sharp bands of xanthophylls (Wang *et al.*, 2013; 2015) in the FT-IR spectra of powder formulations suggested that the xanthophylls remained trapped inside the biopolymer matrix. Similar observations were recorded for FT-IR spectra of lutein loaded zein nanoparticles, where encapsulation resulted in reduction of intensity of the major bands of lutein (Jiao *et al.*, 2018). In scanning electron micrographs, porous surface morphology was observed for two powder formulations (P_{S1M3} and P_{S2M3}), while the surface of P_{S3M1} appeared smooth. The porous powders allowed better diffusion of water to produce transparent aqueous solutions for P_{S1M3} and P_{S2M3}. Smooth surface of P_{S3M1} probably hindered water diffusion within the powder structure and thereby relatively less dissolution resulted in formation of translucent aqueous solution for that formulation.

All the three powder formulations were stable at 0 ± 2 °C as no significant change in the xanthophyll concentration took place before and after storage. However, exposure of the formulations to higher temperatures (26 ± 2 °C and 54 ± 2 °C) reduced the xanthophyll content significantly. Thermostability of lutein esters varies with their structures (Subagio *et al.*, 1999). Therefore, the constituents of the xanthophyll complex determine the overall thermal stability of the extracted xanthophylls (Hadjal *et al.*, 2013). Furthermore, thermal stability of lutein esters also depends on the physical environment of their formulation (Khalil *et al.*, 2012). Though the formulations prepared in this study improved the aqueous solubility of lutein esters, but could not improve their thermal stability apparently. Therefore, the best conditions to use the formulations as biocolorants might be for food products produced/processed/stored at subzero temperature.

Aqueous solutions of all the three powder formulations produce vibrant yellow color of different shades as observed from their hue angle and chroma. Very high ΔE values (> 5) for all the solutions suggested distinctiveness of their color from the neutral grey controls. The powder formulation of lutein esters developed by Tippel *et al.* (2016) also produced stable yellow color, but polyphenols present in Quillaja saponin extract used in preparation of the formulations severely affected the color upon storage. In the present study, the possibility of similar interactions did not arise due to absence of such components in the prepared formulations.

Significantly high antioxidant activities in terms of VCEAC were observed for the formulations by DPPH, FRAP and CUPRAC assays. The relatively high content of encapsulated xanthophylls in P_{S1M3} and P_{S2M3} resulted in their higher antioxidant activities than P_{S3M1} as clearly observed in DPPH radical scavenging assay. However, this observation was not conclusive in assessment of their reducing power.

Enhanced aqueous solubility of the prepared formulations also improved the bioaccessibility of the encapsulated xanthophylls. Interestingly, in the simulated *in vitro* study, xanthophylls from both P_{S1M3} and P_{S2M3} were detected in intestinal conditions. However, for P_{S3M1} , they remain undetected. This observation was well correlated with their surface morphology and dissolution properties. As P_{S1M3} and P_{S2M3} having porous structures, produced transparent aqueous solutions, it was expected that the droplet size of the solutions remained below 100 nm size which improved their dissolution. The smooth surface of P_{S3M1} probably hindered water diffusion and produced translucent aqueous solution. Quite naturally, the droplet distribution of its aqueous solution remained wider, resulted in poor dissolution.

Therefore, the correlation between dissolution and bioavailability of lutein esters suggested by Bowen *et al.* (2002) proved to be true in the present study.

The present study showed the path to develop water soluble formulations of total xanthophyll complex extracted from marigold flower. The versatility of the constituents in the marigold extract including free lutein, lutein esters and other xanthophylls made the prepared formulations unique as the focus was not only on a specific target molecule. Rather, the total xanthophyll complex was considered for development of formulations which might find its applications in food and beverage industries, and to some extent in pharmaceutical industries. The paradox of dissolution and bioavailability of lutein esters was successfully addressed in this study and hopefully it will make a significant way forward in biocolorant research.

6. SUMMARY AND CONCLUSION

Color has a significant impact on consumers' acceptance and perception of food products. The huge market demand of food colorants leads the food and beverage industries to use hefty amount of synthetic colorants. However, indiscriminate and illegal use of the synthetic colorants in food products resulted in serious health related issues in human beings. To address the growing concern of the consumers and to circumvent the strict regulations about the synthetic food colorants, exploration of the potential of natural colorants as safer alternatives is the need of the hour. Nevertheless, to establish a natural pigment as food colorant, many challenges like solubility, stability, bioavailability etc. need to be addressed with a scientific approach. Therefore, not only the extraction/production of natural pigments in substantial quantities from sustainable sources are important, development of suitable formulations for their versatile applications is also of paramount significance.

Carotenoids, a naturally occurring group of pigments, comprised of two major classes namely, carotenes (unsaturated hydrocarbons) and xanthophylls (oxygenated derivatives of carotenes), have been widely explored as natural food coloring agents. One of the most sustainable sources of carotenoids is the flowers of marigold (*Tagetes* sp.), which is a non-food crop and commercially cultivated in many countries. It is a major source of the yellow pigment lutein in its esterified forms. Hydrolysis of the native lutein esters of marigold to produce lutein for its commercial exploitation has been practiced since long. However, use of the total xanthophylls from marigold flowers as natural food colorant is challenging due to their limited aqueous solubility, which in turn effects their bioavailability. Hence, preparation of a suitable formulation of the native marigold pigments is the key to successfully address the issue.

In the present study, xanthophylls from the florets of a hybrid genotype (DAMH-39) of African marigold (*Tagetes erecta* L.) was extracted and profiled using UPLC-QToF-ESI-MS. Xanthophylls of that particular marigold genotype comprised mainly of monoesters (namely laurate, myristate, palmitate, stearate, and linoleate) and diesters (namely dibutyrate, dilaurate, dipalmitate, and dilinoleate) of lutein along with two diesters (dipalmitate and palmitate-stearate) of violaxanthin (diepoxide of zeaxanthin, a natural isomer of lutein). Subsequent saponification of the total xanthophylls, which resulted in formation of lutein and a little quantity of zeaxanthin, strengthened their chemical distinctiveness.

Ultrasonic probe extraction of xanthophylls from the lyophilized powder of the marigold florets was found to be the most efficient technique as compared to ultrasonic bath

extraction and conventional solid-liquid extraction in terms of solvent usage and time requirement to extract same quantity of xanthophylls. As factors like solvent to solid ratio, ultrasonication amplitude, and time were found to influence the yield of xanthophylls by ultrasonic probe extraction, process optimization using statistical models were carried out to optimize the independent variables for maximizing the yield. The output of the Box-Behnken design (BBD) of response surface methodology (RSM) was further improved by application of genetic algorithm (GA). Maximum yield of xanthophylls was obtained at the model predicted optimized conditions of the independent variables.

The extracted xanthophylls demonstrated high antioxidant activities in terms of free radical scavenging capacity (DPPH and ABTS assays) and reducing power (FRAP and CUPRAC assays). Though the DPPH, ABTS and CUPRAC assays could not significantly differentiate the quality of the xanthophylls extracted by different techniques, lowest EC_{50} and highest VCEAC values in FRAP assay for xanthophylls extracted by the GA optimized technique signified their purity.

To replace the hazardous extraction solvents with greener ones, xanthophylls from the lyophilized marigold powder was extracted by ultrasound assisted technique using hydrophobic deep eutectic solvents (HDES). HDES were prepared with different molar ratio of two saturated long chain fatty acids having 8 (caprylic acid) and 10 (capric acid) carbon atoms, respectively. Five different eutectic mixtures of the two fatty acids were prepared and characterized using differential scanning calorimetry (DSC), Fourier Transform Infrared (FT-IR) spectroscopy, and proton Nuclear Magnetic Resonance (^1H NMR) spectroscopy. Melting points of the eutectic mixtures were found to be decreasing with increasing molar ratio of caprylic acid and four out of the five HDES were found to remain liquid at ambient temperature. FT-IR and ^1H NMR spectroscopy data confirmed no chemical reactions between the two fatty acids. However, the enthalpy of melting of the eutectic mixtures and the chemical shift of the hydroxyl proton suggested the formation of hydrogen bonds between the constituents, which is a desirable characteristic for DES.

As the yield of xanthophylls by ultrasound assisted extraction technique using HDES was found to vary with the composition of the eutectic mixture ($C_8:C_{10}$), its volume, and time of ultrasonication, statistical model was implemented to optimize those process variables for yield maximization. At the RSM model predicted optimum conditions of the process variables xanthophyll yield was found to be very close to the expected value. Switching the polarity of the DES from hydrophobic to hydrophilic facilitated precipitation of the extracted lipophilic

xanthophylls and their subsequent separation. Scanning electron microscopy (SEM) revealed that ultrasound assisted extraction with HDES disrupted the cells of the marigold florets to extract more xanthophylls as compared to the conventional solvents.

On a quest to prepare water soluble powder formulations of the extracted xanthophylls, a two-step method was adapted, where firstly the xanthophylls were dissolved in a green solvent and biopolymer stabilized microemulsions were prepared using suitable surfactants, followed by subsequent freeze drying. Three different surfactants were used to prepare 27 sets of microemulsions with varying concentration of xanthophylls following emulsion dilution technique.

Majority of the microemulsions appeared transparent due to narrow distribution of the oil droplets within the nano range (< 100 nm diameter). The droplet diameter of the translucent and opaque microemulsions were found to be higher (> 100 nm) with multimodal distribution. Transmission electron micrographs (TEM) further confirmed the presence of individual nano sized droplets in transparent microemulsions and droplet aggregation in translucent and opaque ones. Color of the microemulsions appeared as different shades of yellow in the RGB color space, whereas, hue angle and chroma calculations in the Hunter L, a, b color space revealed the yellowness and vividity of the color shades. Thermodynamic stability of the prepared microemulsions was tested by subjecting them to three subsequent rigorous stress tests. Those microemulsions which passed the heating-cooling, centrifugation, and freezing-thawing stress tests without phase separation or sedimentation were further subjected to aqueous dispersibility test to check the spontaneity of dispersion.

Based on the thermodynamic stability, dispersibility, droplet dimensions, color expression and surfactant concentration, three microemulsions were individually selected for preparation of powder formulations by freeze drying. The prepared free flowing powders were found to be readily water soluble and observed to form transparent/translucent aqueous solutions. FT-IR spectra of the powder formulations suggested complete encapsulation of the xanthophylls within the biopolymer matrix. SEM images depicted the porous nature of two of the formulations which produced transparent aqueous solutions. Color of the aqueous solutions of the powder formulations at different concentration produced various shades of yellow with high saturation.

The xanthophylls encapsulated within the biopolymeric matrix in the powder formulations were found to be stable at subzero temperature as negligible degradation was

observed at or below 0 °C. However, considerable degradation of xanthophylls was observed on storage of the formulations at room temperature and elevated temperature. Additionally, all the prepared powder formulation showed superior antioxidant activities due to the encapsulated xanthophylls. A simulated *in vitro* bioavailability study revealed that though the major portion of encapsulated xanthophylls in the powder formulations became available in gastric phase after ingestion, a significant portion of xanthophylls from one particular formulation was available in the intestinal phase too.

The present study reported the preparation of water-soluble powder formulations of native xanthophyll pigments of marigold flower. The inherent insolubility issue of lutein esters has been addressed in this study and the prepared powder formulations were found to produce transparent aqueous solutions. Apart from producing vibrant yellow color, the antioxidant properties of the prepared formulations improved the nutraceutical values of the formulations. The prepared formulations may find their potential to use as water soluble natural colorants in food and beverage industries and pharmaceutical industries also.

Biopolymeric formulation of lutein for its application as natural food colorant

ABSTRACT

Growing consumer awareness about the harmful health effects of artificial food coloring agents has increased the market demand of natural food colorants in recent times. Marigold (*Tagetes* sp.) flowers are the major non-food source of lutein, a naturally occurring yellow pigment from the xanthophyll group, in its esterified forms. The additional nutraceutical values of this pigment have further augmented its growth potential. Despite the extensive commercial exploitation of this pigment from *Tagetes* sp. to incorporate in food products, aqueous insolubility, chemical instability, and poor bioavailability have limited the potential of esterified lutein. To address these challenges, the present study was carried out to develop a formulation of the pigments extracted from marigold flowers, for its use as natural food colorant.

Profiling of the xanthophyll pigments extracted from florets of a hybrid genotype (DAMH-39) of *Tagetes erecta* revealed the presence of lutein in its mono- and di-esterified forms. Comparison of extraction techniques suggested that almost same amount of xanthophylls could be extracted by ultrasonic probe extraction (UPE) using minimum solvent at relatively less time as compared to ultrasonic bath extraction and conventional solid-liquid extraction. Process optimization of the UPE technique using response surface methodology (RSM) in conjunction with genetic algorithm (GA) predicted the optimized extraction conditions (31.38 solvent to solid ratio, 56.56% ultrasonication amplitude, and 30 min ultrasonication time), at which a maximum xanthophyll yield of 13099.05 $\mu\text{g g}^{-1}$ was obtained. Comparison of antioxidant activities of the xanthophylls extracted by different techniques exhibited no significant difference in DPPH, ABTS and CUPRAC assays. However, FRAP assay suggested a relatively higher antioxidant capacity of xanthophylls extracted by the optimized process. Further, on a quest to develop a green process of extraction of xanthophylls from marigold, hydrophobic deep eutectic solvents (HDES) were explored. HDES were prepared using two long chain saturated fatty acids (C_8 and C_{10}) at different molar ratios, and were characterized by DSC, FT-IR, and ^1H NMR spectroscopy. The process of ultrasound assisted extraction technique using HDES was optimized by RSM to obtain a maximum xanthophyll yield of 7570.80 $\mu\text{g g}^{-1}$ at the model predicted optimized conditions (1.99 molar ratio of $\text{C}_8\text{:C}_{10}$, 99.40 mL g^{-1} of HDES, and 32.98 min ultrasonication time). Around 72% of the extracted xanthophylls were recovered by switching the polarity of the eutectic solvent.

Water soluble powder formulations of the xanthophylls were prepared in two steps: preparation of biopolymer stabilized oil-in-water microemulsions, followed by freeze drying. Suitable biopolymer, green solvent and three different surfactants were used to prepare 27 microemulsions with variable concentration of xanthophylls. Based on thermodynamic stability, dispersibility, droplet dimensions, and color expression, three microemulsions were chosen for preparation of powder formulations by freeze drying. The powder formulations were characterized using FT-IR spectroscopy and SEM. The readily water-soluble powder formulations were analyzed for their color expression in aqueous solution, which showed an inclination towards yellow color. Accelerated storage stability study recommended safe use and storage of the powder formulations at subzero temperature, but 39-86% degradation of xanthophylls was observed at higher temperatures. The powder formulations exhibited high antioxidant activities and considerable bioavailability at simulated *in vitro* conditions.

The powder formulations prepared in this study addressed the insolubility issue of lutein esters and were found to produce transparent aqueous solutions. The prepared formulations may find their potential to use as natural colorants for food and beverage industries and pharmaceutical industries as well.

ल्यूटिन के जैव बहुलक आधारित सूत्रण की प्राकृतिक खाद्य रंग के रूप में उपयोगिता

सार

कृत्रिम रंगों के स्वास्थ्य पर पड़ने वाले दुष्प्रभावों के बारे में बढ़ती उपभोक्ता जागरूकता के चलते पिछले कुछ समय में बाजार में प्राकृतिक खाद्य रंगों की मांग को बढ़ावा मिला है। गेंदे के फूल ल्यूटिन का प्रमुख गैर खाद्य स्रोत है, जो कि जैन्थोफिल समूह का ऐस्टरीफाईड अवस्था में पाया जाने वाला एक प्राकृतिक पीला रंगद्रव्य है। इस रंगद्रव्य में पाए जाने वाले अतिरिक्त न्यूट्रास्युटिकल गुणों ने इसके प्रयोग को और बढ़ावा दिया है। गेंदे के फूल में पाए जाने वाले इस रंगद्रव्य को खाद्य उत्पादों में शामिल किये जाने के लिए व्यापक वाणिज्यिक प्रयोग किये गए हैं परंतु इसकी जलीय अघुलनशीलता, रासायनिक अस्थिरता एवं कम जैव-उपलब्धता ने ऐस्टरीफाईड ल्यूटिन की खाद्य उत्पादों में शामिल होने की क्षमता को सीमित कर दिया है। इन चुनौतियों से निपटने के लिए, वर्तमान अध्ययन में गेंदे के फूलों से निकाले गए रंगद्रव्य का एक सूत्रण विकसित किया गया जिसका प्रयोग प्राकृतिक खाद्य रंग के रूप में किया जा सके।

गेंदा (*टैगेट्स इरेक्टा*) के हाइब्रिड जीनोटाइप (डी.ए.एम.एच.-39) के फूलों से प्राप्त किये गए जैन्थोफिल रंगद्रव्य का रासायनिक चित्रण करने पर उसमें ल्यूटिन के मोनो एवं डाई ऐस्टरीफाईड रूपों के होना पता चला। विभिन्न निष्कर्षण तकनीकों के अध्ययन पर पाया गया की न्यूनतम विलायक का प्रयोग करके अल्ट्रासोनिक प्रोब द्वारा निष्कर्षण करने पर अल्ट्रासोनिक बाथ निष्कर्षण एवं पारंपरिक ठोस-तरल निष्कर्षण की तुलना में लगभग सामान्य मात्रा में जैन्थोफिल का निष्कर्षण प्राप्त किया गया जिसमें अपेक्षाकृत कम समय लगा। अनुवांशिक एल्गोरिथम (जी.ए.) एवं प्रतिक्रिया सतह पद्धति (आर.एस.एम.) के संयोजित प्रयोग द्वारा अल्ट्रासोनिक प्रोब निष्कर्षण तकनीक के लिए आदर्श निष्कर्षण परिस्थितियों का पूर्वानुमान लगाया गया (विलायक एवं ठोस उत्पाद अनुपात 31.38, अल्ट्रासोनिक ऐम्प्लिट्यूड 56.56% एवं 30 मिनट का अल्ट्रासोनिकेशन समय)। इन परिस्थितियों में अधिकतम 13099.05 माइक्रोग्राम प्रतिग्राम जैन्थोफिल प्राप्त किया गया। विभिन्न तकनीकों द्वारा निकाले गए जैन्थोफिल की प्रतिऑक्सीकारक गतिविधियों में विभिन्न जांच विधियों डी.डी.पी.एच., ए.बी.टी.एस. एवं सी.यू.पी.आर.ए.सी. द्वारा तुलना करने पर कोई विशेष अंतर नहीं पाया गया। हालांकि, एफ.आर.ए.पी. विधि द्वारा जांचे जाने पर पूर्वानुमानित प्रक्रियानुसार निष्कर्षित किये गए जैन्थोफिल की प्रतिऑक्सीकारक क्षमता अपेक्षाकृत उच्च पायी गयी। इसके अतिरिक्त, गेंदे से जैन्थोफिल के निष्कर्षण की एक हरित प्रक्रिया विकसित करने हेतु, हाइड्रोफोबिक डीप यूटेक्टिक सॉल्वेंट्स (एच.डी.ई.एस.) को भी परखा गया। एच.डी.ई.एस. को अलग-अलग-मोलर अनुपात में दो लंबी श्रृंखला वाले संतृप्त फैटी एसिड (C_8 एवं C_{10}) का प्रयोग करके तैयार किया गया एवं इनका रासायनिक चित्रण डी.एस.सी., एफ.टी.आई.आर. एवं 1-एच-एन.एम.आर. स्पेक्ट्रोस्कोपी से निर्धारित किया गया। एच.डी.ई.एस. का प्रयोग करते हुए अल्ट्रासाउंड की सहायता से निष्कर्षण तकनीक को आर.एस.एम. विधि द्वारा मॉडल की अनुमानित अनुकूलित स्थितियों ($C_8:C_{10}$ का मोलर अनुपात 1.99, एचडीईएस 99.40 मिलीलीटर प्रतिग्राम एवं न्यूनतम अल्ट्रासोनिकेशन समय 32.98 मिनट) में जैन्थोफिल की अधिकतम मात्रा 7570.80 माइक्रोग्राम प्रतिग्राम प्राप्त करने के लिए अनुकूलित किया गया। यूटेक्टिक सॉल्वेंट्स की धुवता को बदलकर लगभग 72% निकाले गए जैन्थोफिल को पुनः प्राप्त किया गया।

जैन्थोफिल के पानी में घुलनशील पाउडर सूत्रणों को दो चरणों में तैयार किया गया: पहले बायोपॉलिमर द्वारा स्थिरता प्राप्त जल में तेल प्रकार का माइक्रोइमल्शन तैयार किया गया, इसके बाद इसे लायोफिलाईज़ेशन प्रक्रिया द्वारा सुखाया गया। उपयुक्त बायोपॉलिमर, पर्यावरण के लिए उपयुक्त विलायक एवं तीन अलग अलग-पृष्ठसक्रियकारकों का प्रयोग करके जैन्थोफिल की विभिन्न मात्रा के 27 माइक्रोइमल्शन तैयार किये गए। थर्मोडायनेमिक स्थिरता, फैलाव, बूंद के आकार के आयाम और रंग के आधार पर, लायोफिलाईज़ेशन प्रक्रिया द्वारा पाउडर सूत्रण तैयार करने के लिए तीन माइक्रोइमल्शन चुने गए। एफ.टी.-आई.आर. स्पेक्ट्रोस्कोपी और एस.ई.एम. तकनीकों द्वारा पाउडर सूत्रणों का गुण चित्रण स्थापित किया गया। आसानी से पानी में घुलनशील पाउडर सूत्रणों को जलीय घोल में उनके रंग की अभिव्यक्ति के लिए जांचा गया, जिसमें पीले रंग की ओर झुकाव पाया गया। त्वरित भंडारण स्थिरता अध्ययन में शून्य से कम तापमान पर पाउडर सूत्रीकरण के भंडारण और सुरक्षित उपयोग को अनुशंसित किया गया, लेकिन उच्च तापमान पर 39-86% जैन्थोफिल का क्षरण देखा गया। कृत्रिम परिस्थितियों में पाउडर सूत्रण ने उच्च प्रतिऑक्सीकारक गतिविधि एवं जैव उपलब्धता दर्शाई।

इस अध्ययन में तैयार किए गए पाउडर सूत्रण ने ल्यूटिन एस्टर की अघुलनशीलता की समस्या को सुलझाया फलस्वरूप इससे ल्यूटिन एस्टर के पारदर्शी जलीय घोल तैयार किये गए। विकसित किए गए सूत्रण को संभवतः खाद्य, पेय और दवा उद्योगों में प्राकृतिक रंगों के रूप में भी प्रयोग किया जा सकता है।

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