

**QUALITY EVALUATION OF COW BUTTER AND GHEE VALORISED
WITH SELECT ANTIOXIDANTS OF NATURAL SOURCES**



**THESIS SUBMITTED TO THE
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE,
(DEEMED UNIVERSITY)**

**IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF**

**MASTER OF TECHNOLOGY
IN
DAIRY CHEMISTRY**

By

**Mr. Chavhan Ankit Prakashrao
B. Tech. (Dairy Technology)**

**DAIRY CHEMISTRY
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE
SOUTHERN REGIONAL STATION
ADUGODI, BENGALURU- 560030, INDIA
2022**

Reg. No. 20-M-DC-09



**QUALITY EVALUATION OF COW BUTTER AND GHEE VALORISED
WITH SELECT ANTIOXIDANTS OF NATURAL SOURCES**

By

Chavhan Ankit Prakashrao (20-M-DC-09)

Thesis Submitted to the

ICAR- NATIONAL DAIRY RESEARCH INSTITUTE

(Deemed University)

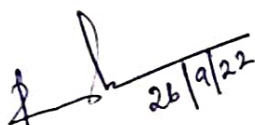
in partial fulfilment of the requirement for the degree of

MASTER OF TECHNOLOGY

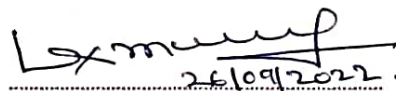
IN

DAIRY CHEMISTRY

Approved By



Dr. Rachana C. R.
(External Examiner)

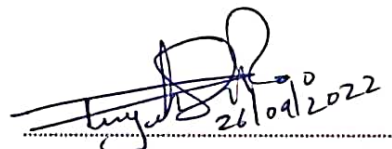


Dr. Laxmana Naik N.
(Major Advisor)

Members of Advisory Committee

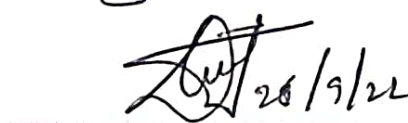
1. Dr. Priyanka Singh Rao

(Scientist, Dairy Chemistry)



2. Dr. Devaraja H.C.

(Senior Scientist, Dairy Technology)



3. Dr. Sathish Kumar M.H.

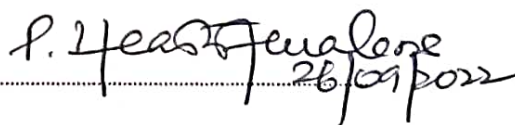
(Senior Scientist, Dairy Technology)

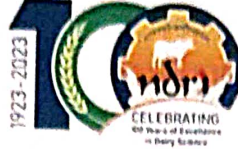


4. Dr. Heartwin A Pushpadass

(Principal Scientist, Dairy Engineering)

(JD's Nominee)





ದಕ್ಷಿಣ ಪ್ರಾದೇಶಿಕ ಕೇಂದ್ರ/ದಕ್ಷಿಣ ಕ್ಷೇತ್ರೀಯ ಕೇಂದ್ರ/
Southern Regional Station
ಭಾ.ಕೃ.ಸಂ.ಪ.-ರಾಷ್ಟ್ರೀಯ ಹೈನು ಸಂಶೋಧನಾ ಸಂಸ್ಥೆ/
ಭಾ.ಕೃ.ಅ.ಪ.-ರಾಷ್ಟ್ರೀಯ ಡೇರರಿ ಅನುಸಂಧಾನ ಸಂಸ್ಥಾನ/
ICAR-National Dairy Research Institute
ಬೆಂಗಳೂರು / ಬೆಂಗಳೂರು / Bengaluru-560 030

Dr. Laxmana Naik N, PhD, ARS.

Date: 30-09-2022

CERTIFICATE

This is to certify that the thesis entitled, "Quality evaluation of cow butter and ghee valorised with select antioxidants of natural sources" submitted by Mr. Chavhan Ankit Prakashrao, (Reg. No: 20-M-DC-09) towards the partial fulfilment for the award of the degree of M. Tech. in Dairy Chemistry of the ICAR-National Dairy Research Institute, Karnal (Haryana), India, is a bonafide research work carried out by him under my guidance at SRS of ICAR-NDRI Bengaluru campus and no part of the thesis has been submitted for any other degree or diploma.

30/09/2022

(Dr. Laxmana Naik N.)
MAJOR ADVISOR

DEDICATED TO
MY
BELOVED PARENTS,
RESPECTED GUIDE
&
ALMIGHTY GOD

ACKNOWLEDGEMENTS

This dissertation would not have been possible without the guidance and the help of several individuals who in one or another way contributed and extended their valuable assistance in the preparation and completion of this study. It is to them that I owe my deepest gratitude.

*It was a great pleasure to carry out my research work under the guidance of **Dr. Laxmana Naik N.**, Scientist, Dairy Chemistry, and Incharge Dairy Chemistry and Bacteriology Section, SRS of ICAR-NDRI, Bengaluru. He provided me with valuable and inspiring guidance, immense help, warm hearted support, humane care, and suggestions throughout the process of this research work. His continuous attention and keen observation made me to complete my work. He understood me thoroughly and has been very kind to me throughout the period. I am very thankful to him as he gave me all freedom to carry out this work on my term.*

*I express my sincere thanks to **Dr. Priyanka Singh Rao**, Scientist, Dairy Chemistry. I whole heartedly thank all the advisory committee members **Dr. Devaraja H.C.**, Senior Scientist, Dairy Technology Section, **Dr. M.H. Sathish Kumar**, Senior Scientist, Dairy Technology Section, **Dr. Heartwin A Pushpadass**, (JD's Nominee) Principal Scientist, Dairy Engineering Section, for their valuable suggestions and wholehearted support during the course of my research investigation.*

*I am extremely thankful to **Dr. M.S. Chauhan**, Former Director and Vice-Chancellor ICAR-NDRI, Karnal, **Dr. Dheer Singh**, Acting Director and Joint Director (Research), ICAR-NDRI, Karnal, **Dr. K. P. Ramesha**, Head, SRS of ICAR-NDRI, Bengaluru, **Dr. Raman Seth**, Head Division of Dairy Chemistry, ICAR-NDRI, Karnal and **all the scientist of Dairy Chemistry**, ICAR-NDRI, Karnal for their encouragement and providing all the necessary facilities during course work. I sincerely acknowledge financial assistance in the form of **ICAR-NDRI institutional fellowship** awarded to me.*

*I whole-heartedly thank to **Dr. Mukund A Kataktalware**, Incharge Education and Training Section, thanks also to **Ms. Wilma** for their kind support in academic matters and concern shown for me throughout the research work.*

*My thanks to **Mr. Ramakrishna Prasad**, Technical officer, **Mr. Sonu K.S.**, Senior technical officer, **Smt. Vimala**, Retired Senior technical officer and **Mrs. Indrani**,*

Supporting Staff for their constant help and support and possible cooperation in my work.

I gratefully acknowledge my lab mates **Mr. Sonu K. S, Ms. Aziza Begum, Mr. Manjunath, Ms. Nishigandha** and **Ashar** for all moral support, advices and many valuable discussions that helped me understanding my research area better.

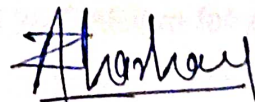
I immensely thank my classmates **Utkarsh, Mayur, Diksha, Navaneetha, Taherabbas, Sapna, Ranjit and Vivek** for their encouragement and affection. I will never forget the charishable moments shared by us in NDRI. I would like to thank **Amandeep, Abhishek, Susmita, Somveer, Abhishek, Somyadeep, Manisha** and all Dairy Technology, Dairy Microbiology and Dairy Engineering friends for being with me and supporting me in all possible way.

I am also indebted to **my seniors** for their valuable advices, support and affection towards me. I would also like to mention **my B. Tech friends Gopal, Nikhil, Sachin, Akshay, Suraj, Akash** and other classmates for constant source of love, concern and strength. A very Special thanks to **my childhood friends Pravin, Shubham, Rushikesh, Kedar, Nagorao** for always being with me whenever I found needy, encouragements and love. Without their priceless company life would have been less lively.

Words do not suffice to express my devotion and gratitude to my parents who dreamt big for me and supported me through thick and thin in my life. I would never have reached so far without the full support, strength, encouragement and love given to me by my dad **Mr. Prakashrao Chavhan** and mom **Mrs. Aruna Chavhan**, My brother **Mr. Pramod Chavhan** and Sister **Ms. Priyanka Chavhan**. They were always supporting, correcting and encouraging me. It is none else but to all in my loving family that I dedicate this thesis. I thank them for having faith in me and for their support.

Thank you all.....

Date: 30-09-2022



Chavhan Ankit Prakashrao

Reg. No. 20-M-DC-09

“Quality evaluation of cow butter and ghee valorised with select antioxidants of natural sources”

ABSTRACT

Butter and ghee are major fat rich dairy products; these fat rich dairy products undergo spoilage during prolonged storage due to autoxidation. The end products formed from the lipid oxidation results in the production of objectionable flavour and odour; some of the secondary and tertiary end products of oxidation can cause diarrhoea and the free radicals formed can be of carcinogenic in nature. Since, the addition of synthetic antioxidant(s) is prohibited by the FSSAI, an attempt was made to prepare butter and ghee enriched with carotenoids as natural sources of antioxidants. The β -carotene is a natural yellow-red coloured carotenoid pigment. It is an important precursor for vitamin A and used as a colorant in the food industry under the numbers E-160. In this study, using Box Behnken Design methodology, optimization study was carried out for extraction of the β -carotene from carrot and microbial source, and in place of chemical solvents ghee was used as a solvent in the ultrasonication extraction process. Ultrasonication power, extraction time, and carrot powder to ghee ratio were optimized for extraction of β -carotene rich antioxidant ingredient. The optimized butter and ghee were prepared by subjecting the antioxidant rich extract during the working stage in butter and after clarification process in the ghee samples. The optimized butter and ghee product were found to be superior in terms of sensory properties and colour parameter than the control samples. The physico-chemical quality indicator was found to be unaltered and their values were on par with the specifications given by FSSAI. Sensory analysis and accelerated storage study was done till the sample became unacceptable. It was observed that the total phenolic content in the optimized ghee was found to be 88.36 gallic acid equivalent (GAE)/gm fat and for control ghee it was 9.61 GAE/gm fat and for butter it was 65.21 GAE/gm fat and for control butter it was 6.26 GAE/gm fat and also exhibited enhanced antioxidant activity. This study resulted in development of a green bio-refining method for extraction of β -carotene from natural source. The extracted beta-carotene rich antioxidant ingredient is also a potential alternative to Annato as a colourant in the butter. The study also resulted in creating an ingredient with 3 functionalities as a source of provitamin of A, colourant and natural antioxidant.

"प्राकृतिक स्रोतों के चुनिंदा एंटीऑक्सीडेंट के साथ गाय के मक्खन और घी की गुणवत्ता का मूल्यांकन"

सार

मक्खन और घी प्रमुख वसा युक्त डेयरी उत्पाद हैं; ये वसा युक्त डेयरी उत्पाद लंबे समय तक भंडारण के दौरान ऑक्सीकरण के कारण खराब हो जाते हैं। लिपिड ऑक्सीकरण से बनने वाले अंतिम उत्पादों के परिणामस्वरूप आपत्तिजनक स्वाद और गंध उत्पन्न होती है; ऑक्सीकरण के कुछ द्वितीयक और तृतीयक अंत उत्पाद दस्त का कारण बन सकते हैं और बनने वाले मुक्त कण कार्सिनोजेनिक प्रकृति के हो सकते हैं। चूंकि, एफएसएसएआई द्वारा सिंथेटिक एंटीऑक्सीडेंट को शामिल करना प्रतिबंधित है, इसलिए एंटीऑक्सीडेंट के प्राकृतिक स्रोतों के रूप में कैरोटेनॉयड्स से समृद्ध मक्खन और घी तैयार करने का प्रयास किया गया था। β -कैरोटीन एक प्राकृतिक पीले-लाल रंग का कैरोटीनॉयड वर्णक है। यह विटामिन ए के लिए एक महत्वपूर्ण अग्रदूत है और खाद्य उद्योग में संख्या E-160 के तहत एक रंगीन के रूप में उपयोग किया जाता है। इस अध्ययन में, बॉक्स बेकन डिजाइन पद्धति का उपयोग करते हुए, गाजर और सूक्ष्मजीव स्रोत से β -कैरोटीन के निष्कर्षण के लिए अनुकूलन अध्ययन किया गया था, और रासायनिक सॉल्वेंट्स के स्थान पर घी का उपयोग अल्ट्रासोनिकेशन निष्कर्षण प्रक्रिया में विलायक के रूप में किया गया था। अल्ट्रासोनिकेशन पावर, निष्कर्षण समय, और गाजर पाउडर से घी अनुपात को β -कैरोटीन समृद्ध एंटीऑक्सीडेंट घटक के निष्कर्षण के लिए अनुकूलित किया गया था। मक्खन में काम करने की अवस्था के दौरान और घी के नमूनों में स्पष्टीकरण प्रक्रिया के बाद एंटीऑक्सीडेंट से भरपूर अर्क के अधीन अनुकूलित मक्खन और घी तैयार किया गया था। अनुकूलित मक्खन और घी उत्पाद नियंत्रण नमूनों की तुलना में संवेदी गुणों और रंग पैरामीटर के मामले में बेहतर पाए गए। भौतिक-रासायनिक गुणवत्ता संकेतक अपरिवर्तित पाए गए और उनके मूल्य एफएसएसएआई द्वारा दिए गए विनिर्देशों के अनुरूप थे। नमूना अस्वीकार्य होने तक संवेदी विश्लेषण और त्वरित भंडारण अध्ययन किया गया था। यह देखा गया कि अनुकूलित घी में कुल फेनोलिक सामग्री 88.36 जीएई/जी वसा और नियंत्रण घी के लिए 9.61 जीएई/जी वसा और मक्खन के लिए 65.21 जीएई/जी वसा और नियंत्रण के लिए पाया गया। मक्खन यह 6.26 जीएई/जी वसा था और यह भी बढ़ी हुई एंटीऑक्सीडेंट गतिविधि का प्रदर्शन करता था। इस अध्ययन के परिणामस्वरूप प्राकृतिक स्रोत से β -कैरोटीन के निष्कर्षण के लिए एक हरित जैव-शोधन विधि का विकास हुआ। निकाला गया बीटा-कैरोटीन समृद्ध एंटीऑक्सीडेंट घटक भी मक्खन में एक रंग के रूप में एनाटो का एक संभावित विकल्प है। अध्ययन के परिणामस्वरूप ए, कोलोरेंट और प्राकृतिक एंटीऑक्सीडेंट के प्रोविटामिन के स्रोत के रूप में 3 कार्यात्मकता वाला एक घटक तैयार किया गया।

INDEX

Chapter	Description		Page No.
1	INTRODUCTION		1-3
	1.1	Justification	2
	1.2	Title and Objectives	2
2	REVIEW OF LITERATURE		4-32
	2.1	Butter: Product Description and Specification	4
	2.1.1	Process of Butter Manufacturing	6
		<i>Fritz Process</i>	6
		<i>Concentration or Inversion Process</i>	6
		<i>Emulsification Process</i>	6
	2.1.2	Manufacturing of Butter	7
	2.2	Ghee: Product Description and Specification	8
	2.2.1	Method of Ghee Manufacturing	10
		<i>Desi or Indigenous or Traditional Method</i>	10
		<i>Creamery-Butter Method</i>	11
		<i>Direct-Cream Method</i>	11
		<i>Pre-stratification Method</i>	11
		<i>Continuous Method</i>	12
	2.2.2	Physico-Chemical Characteristics of Ghee	12
		<i>Specific Gravity</i>	12
		<i>Iodine Value</i>	13
		<i>Peroxide Value</i>	13
		<i>Richert-Meissl Value and Polenske Value</i>	13
		<i>Saponification Value</i>	14
	2.2.3	Shelf Life of Ghee	14

	2.3	Antioxidant	15
	2.4	Carotene (Carotenoids)	15
	2.5	Extraction of Bioactive Components	20
	2.6	Brief Resume of Work Done in India and Abroad	21
	2.7	Work Done at NDRI	26
	2.8	Method for Determining Polyphenols and Antioxidant Potential	30
	2.9	Accelerated Stability Tests	30
	2.10	Response Surface Methodology	31
		Summary of Review of Literature	32
3	MATERIAL AND METHOD		33-52
	3.1	Chemicals	33
	3.2	Solvents	33
	3.3	Glassware's	33
	3.4	Equipment's/Instruments and other Accessories	33
	3.5	Reagents	34
	3.6	Extraction of Antioxidant Rich Fraction from Natural Sources	36
	3.6.1	Extraction from Microbial Source	36
	3.6.2	Extraction of β -carotene from Carrots Using Ultrasonic Assisted Extraction (UAE)	36
	3.6.3	Preparation of Standard Curve	38
	3.6.4	Analysis of β -carotene Using Ultra Violet (UV) Spectroscopy	38
	3.6.5	Experimental Design	39
	3.7	Collection of Cream Sample	39
	3.7.1	Analysis of Cream Sample	39

	3.7.1.1	Determination of Fat Content in Cream	39
	3.7.1.2	Determination Of Titrable Acidity in Cream	40
	3.8	Method of Preparation of Butter	40
	3.8.1	Physico-Chemical Analysis of Butter Sample	41
	3.8.1.1	Determination of Fat Content in Butter	41
	3.8.1.2	Determination of Moisture in Butter	41
	3.8.1.3	Free Fatty Acid Value of Butter	42
	3.8.1.4	Peroxide Value of Butter	42
	3.8.1.5	Colour (L* a* b*) Value of Butter	42
	3.8.1.6	Measurement of pH	43
	3.8.1.7	Textural Analysis of Butter Samples	43
	3.8.1.9	Total Phenolic Content	44
	3.8.1.10	Estimation of Antioxidant Property of Ghee by DPPH Model System	45
	3.8.1.11	Microbiological Analysis	45
		<i>Total Plate Count of Butter</i>	45
		<i>Coliform Count of Butter</i>	46
		<i>Yeast and Moulds Count of Butter</i>	46
	3.9	Method of Preparation of Ghee (Creamery Butter Method)	46
	3.9.1	Determination of Chemical Analysis and Physico-Chemical Constants	46
	3.9.1.1	Determination of Moisture Content	47
	3.9.1.2	Estimation of Free Fatty Acid Content	48
	3.9.1.3	Determination of Butyro-Refractometer (BR) Reading	48
	3.9.1.4	Determination of Saponification Value	48
	3.9.1.5	Determination of Reichert Meissl (RM Value)	49

		and Polenske Value	
	3.9.1.6	Determination of Iodine Value	50
	3.9.1.7	Determination of Peroxide Value	51
	3.9.1.8	Total Phenolic Content	51
	3.9.1.9	Estimation of Antioxidant Property of Ghee by DPPH Model System	52
	3.10	Statistical Analysis	52
4	RESULT AND CONCLUSION		53-81
	4.1	Collection and Analysis of Cream Samples	53
	4.2	Extraction of Antioxidant Rich Fraction from Natural Sources	53
	4.2.1	Antioxidant Rich Pigment Extraction from Microbial Source	53
	4.2.2	Antioxidant Rich Pigment Extraction from Carrot	55
	4.3	Optimization of Ultrasonication Assisted Extraction (UAE) with Ghee Using Response Surface Methodology	55
	4.3.1	Effect of Carrot to Oil Ratio	56
	4.3.2:	Effect of Extraction Time	56
	4.2.3	Effect of Ultrasonic Power	57
	4.4	Yield of Extracted β -carotene Pigment	58
	4.5	Graphical Representation of UAE Parameter on β -carotene Content	59
	4.6	Modelling and Validation	60
	4.7	Standard curve of β -carotene	61
	4.8	Physico-Chemical Analysis of Prepared Pigment Extract	61
	4.9	Development of Protocol for Manufacture of Ghee with Added Antioxidant	64

	4.9.1	Addition of Natural Antioxidant According to Stage and Concentration	64
	4.9.2	Effect of Different Concentration of β -carotene Added in Ghee and Its Effect on (DPPH) RSA	64
	4.10	Physicochemical Constants Analysis of Control and Optimized Ghee	65
	4.11	Effect of Storage Study on Control and Optimized Ghee	66
	4.11.1	Effect of Storage on FFA Value of Control and Optimized Ghee	66
	4.11.2	Effect of Storage on Peroxide Value in Control and Optimized Ghee	67
	4.11.3	Effect of storage on %RSA of Control Ghee and Optimized Ghee	68
	4.11.4	Effect of Storage on Colour of Control and Optimized ghee at 80°C	69
	4.11.5	Effect of Storage at 80°C on Sensory Score of Control and Optimized Ghee	70
	4.11.6	Storage Stability Study: Rancimat Analysis	71
	4.12	Development of Protocol for Manufacture of Butter with Added Antioxidant Rich Pigment Extract	71
	4.12.1	Addition of Natural Antioxidant According to Concentration	71
	4.12.2	Addition of Antioxidant Rich Extract: Stage of Addition	72
	4.12.3	Effect of β -carotene Added in Butter and Its Effect on Sensory Analysis and (DPPH) Radical Scavenging Activity	73
	4.12.4	Composition and Chemical Analysis of Control and Optimized Butter	73
	4.13	Effect of Storage Study on Control and Optimized Butter During Storage at Refrigeration Condition.	74
	4.13.1	Effect on Free Fatty Acid Content	74

	4.13.2	Effect on Peroxide Value (PV)	75
	4.13.3	Effect on the pH	76
	4.13.4	Effect on Colour Value of Butter During Storage at Refrigeration Temperature.	76
	4.13.5	Effect on Textural Profile Analysis (TPA)	78
	4.13.6	Effect on Microbial Analysis of Control and Optimized Butter	78
	4.13.7	Effect on Sensory Analysis	79
	4.14	Total Phenolic Content of Control and Optimized Butter and Ghee	80
5	SUMMARY AND CONCLUSION		82-85
BIBLIOGRAPHY			i-xii
APPENDICES			
CURRICULUM VITAE			

LIST OF TABLES

Table no.	Description	Page no.
2.1	Standard for Butter as per FSSR (2011)	5
2.2	Codex Standard for Butter	6
2.3	Codex Standards for Ghee	9
2.4	Standards for Ghee as per FSSR (2011)	10
2.5	Physico-Chemical Constants of Cow Milk Fat	13
2.6	Permitted level of β -carotene: Food Safety and Standards Regulations, 2011	18
2.7	Properties of β -carotene	19
2.8	β -carotene Content (mg/100 gm) in Fruits and Vegetables	19
2.9	Properties of Canthaxanthin	20
2.10	Stability Test Methods and Condition of Acceleration	31
3.1	Colour Parameters Derived from Image Analysis	43
3.2	TA-XT Plus Setting for Analysis of the Butter Samples	44
4.1	Composition of Cream	53
4.2	Sets of Variables Selected in the Box Behnken Design (BBD)	56
4.3	Optimization Table for β -carotene Extraction by UAE Using Ghee as Solvent	57
4.4	ANOVA Table for UAE of β -carotene Using Ghee as Solvent	58
4.5	Physico-Chemical Analysis of Ghee and β -carotene Rich Extract (CREG)	62
4.6	Physicochemical Analysis of Control g and Optimized Ghee	65

4.7	Changes in Colour Value of Control and Optimized Ghee During Storage at 80°C	69
4.8	Proximate Composition of Control and Optimized Butter	74
4.9	Changes in Colour Value of Butter During Storage at Refrigeration	77
4.10	Changes in Texture Value of Control and Optimized Butter During Storage at Refrigeration	78
4.11	Changes in Total Plate Count of Control and Optimized Butter During Storage at Refrigeration	79
4.12	Total Phenolic Content of Control and Natural Antioxidant Added Cow Butter and Ghee	81

LIST OF FIGURES

Figure No.	Description	Page No.
2.1	Flow Chart of Butter Manufacturing	7
2.2	General Structure of alpha and beta Carotene.	17
2.3	Commercial Market Value and Significance of Carotenoids in Human Health.	18
2.4	General Structure of Canthaxanthin	20
3.1	Protocol of Pigment Extraction from Microbial Source	37
3.2	Ultrasound-Assisted Extraction (UAE) Using Ghee as Green Solvent	38
3.3	Flow-Chart for Preparation of Butter	40
3.4	Flow-Chart for Preparation of Ghee (Creamery Butter Method)	47
4.1	Pigment Extraction Process from Microbial Source	54
4.2	Ultrasound Assisted Axtraction (UAE) of Pigment from Carrot	54
4.3	RSM Graph (A) Effect of Power, (B) Effect of Time (C) Effect of Concentration Level on Extraction of β -carotene Content.	60
4.4	RSM Graph (A) Effect of Time and Concentration (B) Effect of Power and Concentration (C) Effect of Power and Time on Extraction of β -carotene Content	60
4.5	Standard Curve of β -carotene	61
4.6	Effect of Different Concentration of β -carotene on Sensory Score and %RSA	64
4.7	Comparison of Control and Optimized Ghee on Free Fatty Acid During Storage of Ghee at 80°C.	67
4.8	Comparison of Control and Optimized Ghee on Peroxide Value During Storage of Ghee at 80°C	67
4.9	Comparison of Antioxidant Activity of Control and Optimized Ghee During Storage at 80°C.	68

4.10	Changes in YI Value of Control and Optimized Ghee During Storage at 80°C	70
4.11	Comparison of Sensory Analysis of Control and Optimized Ghee During Storage at 80°C.	70
4.12	Rancimat Analysis: Induction Period of Control Ghee (A) and Optimized Ghee Sample (B)	71
4.13	Effect of Different Concentration of Adding β -carotene on Sensory Score	72
4.14	Effect of Addition of β -carotene According to Stage on Sensory Score and %RSA	73
4.15	Comparison of Control and Optimized Butter on Free Fatty Acid Value During Storage at Refrigeration.	75
4.16	Comparison of Control and Optimized Butter on Peroxide Value During Storage at Refrigeration.	75
4.17	Comparison of pH of Control and Optimized Butter During Storage at Refrigeration	76
4.18	Yellowness Index of Control and Optimized Butter During Storage.	77
4.19	Sensory Analysis of Control and Optimized Butter During Storage at Refrigeration.	80
4.20	Standard Curve for Total Phenolic Content	81

LIST OF ABBREVIATION AND SYMBOL

a*	Red-Green
A.O.A.C	Association of Official Agricultural Chemists.
ABTS	(2,2'-Azino-bis (3-Ethylbenzothiazoline-6-Sulfonic Acid
ADI	Acceptable Daily Intake
AGMARK	Agricultural Marketing
ANOVA	Analysis of Variance
b*	Blue-Yellow
BBD	Box-Behnken Design
BHA	Butylated Hydroxy Anisole
BHT	Butylated Hydroxy Toluene
BOD	Biochemical Oxygen Demand
BR	Butyro-Refractometer
C	Concentration
CAC	Codex Alimentarius Commission
CAGR	Compound Annual Growth Rate
CD	Conjugated Dienes
CE	Conventional Extraction
CLA	Conjugated Linoleic Acid
CO	Carrot to oil Ratio
CREG	β -carotene Rich Extract Ghee
DPPH	2,2-Diphenyl-1 Picrylhydrazyl
DRP	Dehusked Ragi Powder
EAE	Enzyme Assisted Extraction
Eos	Essential Oils
EPP	Erythropoietic Protoporphyria
EU	European Union
FAO	Food and Agricultural Organization
FDA	Food and Drug Administration
FFA	Free Fatty Acids
FRAP	Fluorescence Recovery After Photobleaching
FSSAI	Food Safety Standards Authority of India
FSSR	Food Safety and Standards Regulations

FTIR	Fourier-Transform Infrared Spectroscopy
GAE	Gallic Acid Equivalent
GRAS	Generally Recognized as Safe Substance
HCl	Hydrochloric Acid
HSD	High Shear Dispersion
IARC	International Agency for Research on Cancer
ICAR	Indian Council of Agricultural Research
IP	Induction Period
ISI	Indian Standard Institute
IV	Iodine Value
KI	Potassium Iodide
KOH	Potassium Hydroxide
L*	Lightness
MAE	Microwave Assisted Extraction
MCFAs	Medium Chain Fatty Acids
MS	Magnetic Stirring
Na₂CO₃	Sodium Carbonate
NaOH	Sodium Hydroxide
NDRI	National Dairy Research Institute
OGE	Oregano Extract
OO	Olive Oil
OP	Olive Pomace
OPE	Orange Peel Extract
P	Power
PAV	P-anisidine Value
PDA	Potato Dextrose Agar
PEP	passion fruit peel
PG	Propyl gallate
pH	Power of Hydrogen
PP	Pomegranate Peels
ppm	Part Per Million
PS	Peanut Skins

PV	Peroxide Value
Pv	Polenske Value
RDA	Recommended Dietary Allowance
RM	Reichert-Meissl
RME	Rosemary Extract
RSA	Radical Scavenging Activity
RSM	Response Surface Methodology
SC-CO₂	Super Critical Carbon Dioxide
SD	Standard Deviation
SNF	Solids-Not-Fat
SO	Salvia Officinalis
SO	Sunflower Oil
SPA	Standard Plate Agar
SRS	Southern Regional Station
SSHE	Scraped Surface Heat Exchanger
SV	Saponification Value
T	Time
TBA	Thio-Barbituric Acid
TBHQ	Tertiary Butyl Hydroquinone
TPA	Textural Profile Analysis
TPC	Total Phenolic Content
TPC	Total Plate Count
UAE	Ultrasonication Assisted Extraction
UV	Ultra-Violet
VRBA	Violet Red Bile Agar
WHO	World Health Organization
YI	Yellowness Index
λ max	Maximal Absorption

Chapter-1

INTRODUCTION

INTRODUCTION

Milk fat is the third main source of lipids for human nutrition. Historically, milk fat and milk fat-based products have found a special place in food habits of all cultures; as people all over the world have been consuming them. It is widely acknowledged that milk fat imparts excellent flavour and superior mouth feel to prepared products. Cream, butter and ghee are the major fat rich dairy products; these are valuable for the dairy industry due to their distinct flavour, textural quality, and nutritional property.

Butter is essentially a milk fat concentrate; it is prepared from milk, dahi or cream by churning or agitating it until the fat separates as semisolid mass from the liquid portion (serum) of milk. Butter, according to the Food Safety Standards Authority of India (FSSR, 2011), is a fatty product produced exclusively from cow and/or buffalo milk or its products, primarily in the form of a water-in-oil emulsion. Butter has unique functional, nutritional and sensory properties. Butter is easily spread and readily melts on the tongue and should have a uniform colour, and a smooth consistency.

Ghee means the pure clarified fat derived solely from milk or dahi or from desi (cooking) butter or from cream to which no colouring matter and preservative has been added. Ghee as a product exclusively obtained from milk, cream or butter, by means of processes, which result in almost total removal of water and solids-not-fat, with an especially developed flavour and physical structure.

As per the literature data, fat rich dairy products spoilage may occur during prolonged storage due to autoxidation. The oxidized fat produces objectionable flavour and odour. The end products formed from oxidation of lipids can cause diarrhoea and the free radicals formed can be of carcinogenic in nature. Since the addition of synthetic antioxidant(s) to extend the shelf life of such products is prohibited by the Food Safety Standards Authority of India (FSSAI). Considering the socio-economic value of fat-rich products, few studies have been carried out to add natural extracts to increase antioxidant activity. Few reports suggest that

natural pigment can be used to extend shelf life without affecting sensory attributes.

Natural pigments are believed to enhance the functional property of the product. Carotenoids are the foremost common bioactive terpenoid pigments, which are commonly produced by a variety of plants and microbes. Because of their various biological properties like antioxidant, anticancer, anti-inflammatory and antimicrobial activities; have attracted the eyes of researchers in the development of nutraceuticals and functional foods. Owing to the increase in consumer tendency to buy natural products, therefore, this work was formulated with a hypothesis that the use of selected natural pigments in butter and ghee may enhance the shelf life, sensory properties and nutraceutical benefits without affecting the quality parameters.

1.1: Justification:

- **Context:**

Effect of incorporation of natural antioxidant to butter and ghee enhances the antioxidant activity, sensory properties and shelf life.

- **Complication:**

The stability and compatibility of added natural antioxidant on the butter manufacturing and clarification of ghee.

- **Question:**

Are there any green method for extraction of natural antioxidant ingredients and its effect on the quality, sensory property and antioxidants activity of butter and ghee?

- **Hypothesis:**

H₀: There is no effect of natural antioxidants on quality, sensory property and antioxidant activity of butter and ghee.

H₁: There is the effect of natural antioxidants on quality, sensory property and antioxidant activity of butter and ghee.

1.2: Title and Objectives

“Quality evaluation of cow butter and ghee valorised with select antioxidants of natural sources”

Objectives:

1. Extraction of antioxidant rich fraction from select natural sources and characterization for antioxidant activity,
2. Optimization for the preparation of valorised cow butter and ghee with extracted antioxidant ingredient, and
3. Assessment of quality indicators and antioxidant properties of prepared butter and ghee.

Chapter-2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

2.1 Butter: Product Description and Specification:

Butter may be defined as a fat concentrate which is obtained by churning cream, gathering the milk fat into a compact mass, and then working it. Butter, according to the Food Safety Standards Authority of India (FSSAI, 2011), is a fatty product generated exclusively from cow and/or buffalo milk or its products, primarily in the form of a water-in-oil emulsion.

Butter comprises more than 80% of milk fat, water, and trace amounts of calcium, phosphorus, protein, and several fat-soluble vitamins (A, D, and E) in addition to 1.5 to 2.0% of salt (in case of Table butter). The density of butter is 0.911 kg/m³; it is considered as high calorie food as 100 gm of butter provides almost 700 kcal. Butter contains conjugated linoleic acid (CLA), which may help to protect against cancer. Approximately 67% saturated fat, 29% monounsaturated fat and 4% polyunsaturated fat contain in butter. Phospholipids, hydrocarbons, sterols and sterol esters, complex glycerides, free fatty acids, fat-soluble vitamins, and minerals are also included in the composition of butter (Kumar *et al.*, 2016). Butter is rich in minerals like calcium, phosphorus and potassium. It has good amount of sodium and small amounts of fluoride, selenium, zinc and magnesium.

As per ideal features, butter should have natural golden or creamy yellow colour, smooth and slightly matt surface (absence of greasiness, oiling off and leakiness), firmness to prevent sagging under its own weight, softness to be easily spreadable (not too short and crumbly) and ease of deformation in the mouth without being greasy (Walstra *et al.*, 2005). Good butter should be of fine and close texture; have a firm, waxy body; and be sufficiently plastic to be spreadable at cold temperatures.

The distinctive flavour of butter is contributed by the short-chain fatty acids of milk fat, ketones, lactones and phospholipids. Diacetyl, lactic acid, acetic acid, and acetic aldehyde play vital role in butter (Mortensen & Denmark, 2011). Butter flavour is made up of many volatile and non-volatile compounds. Researchers

have identified more than 40 neutral volatiles, of which the most prominent are lactones, ethyl esters, ketones, aldehydes, and free fatty acids (Siek, 1970). The non-volatiles, of which salt (sodium chloride) is the most prominent, contribute to a balanced flavour profile. Diacetyl and dimethyl sulphide also contribute, especially in cultured butter flavour (Kinsella, 1975).

Butter is generally used as a spread and a condiment, as well as in cooking applications, such as baking, sauce making, and pan frying. Butter remains a firm solid when refrigerated, but softens to a spreadable consistency at room temperature, and melts to a thin liquid consistency at 32 to 35°C.

Annatto is often used to add colour to dairy products such as butter, cheese, or puddings. In India usage of annatto is restricted to butter and cheese, however there are no tailor-made formulations available to obtain standardized colour shades for the products (Mala *et al.*, 2015).

As per the various literature data, butter can have a few days of shelf life at room temperature; one to two months when stored in the refrigerator; six to nine months if stored frozen. The Products taste can turn rancid or sour, spoilage may occur during prolonged storage due to autoxidation. Oxidized fat Produce objectionable flavour and odour. For a short period, butter can be stored at about 4°C but if longer storage is involved it must be deep frozen at -23°C and only the best quality butter should be selected for deep freezing.

Table 2.1 Standard for Butter as per FSSR (2011)

Parameter	Table Butter	White Butter
Moisture, maximum, %, (m/m)	16.0	-
Milk fat, minimum, %, (m/m)	80.0	76.0
Milk solids-not-fat, maximum, %, (m/m)	2.0	-
Common salt, maximum, %, (m/m)	3.0	-

The worldwide market for butter is valued at 54710 million USD in 2020 is expected to reach 67440 million USD by the end of 2026, growing at a compound annual growth rate (CAGR) of 3.0% during 2021-2026. (<https://www.marketwatch.com/press-release/butter-market>).

In India, the standards for butter (Table 2.1), are laid down by the food safety and standards Act (FSSA, 2006), which are mandatory in nature. Butter means the fatty product derived exclusively from the milk of cow and/or buffalo or its products principally in the form of water-in-oil type of an emulsion (FSSR 2011).

As per Codex standard (Table 2.2), butter is a fatty product derived exclusively from milk and/or products obtained from milk, principally in the form of an emulsion of the type water-in-oil. It contains minimum of 80% (m/m) fat and maximum of 16% (m/m) water and 2% (m/m) non-fat milk solids (CODEX standards 279-1971).

Table 2.2 Codex Standard for Butter.

Parameter	Values (%)
Milk fat, minimum, %, (m/m)	80
Moisture, maximum, %, (m/m)	16
Milk solids-not-fat, maximum, %, (m/m)	2

2.1.1: Process of Butter Manufacturing:

Fritz process: In this process high-speed mixers used to create butter grains in a matter of seconds. The buttermilk is drained out, and the washed or unwashed grains are handled in a kneading area. Contimab, Westfalia, Silkeborg, and a few other manufacturers' machines used this principle.

Concentration or Inversion Process: The technique in which 30-40% fat cream is concentrated to 80-82% fat in a particular separator according to the fat content necessary in butter. The concentrated butter fat is chilled and worked once it has been standardized. Alfa-Laval and Meleshin processes utilize this principle.

Emulsification Process: Creamery Package and Cherry Burrel are two machines that use this technique. To the concentration of 30-40% fat cream; the emulsion is disrupted during concentration, and the fat, water, and salt content are standardized, followed by re-emulsification, cooling, and functioning.

2.1.2: Manufacturing of Butter

General flow chart for butter making from cream is depicted in the Fig. 2.1. Commercial butter can be prepared from both sweets as well as cultured cream. Butter is made from milk fat (cream) usually separated from the milk to a 40% fat and then aged overnight (Apprx. 10-12 h) at a controlled temperature in ageing tank at Apprx. 8-10°C. The cream ageing process allows the fat to partially crystallize. During the crystallization process a chemical reaction takes place and heat is released causing a temperature rise in the cream (Latent heat of crystallization).

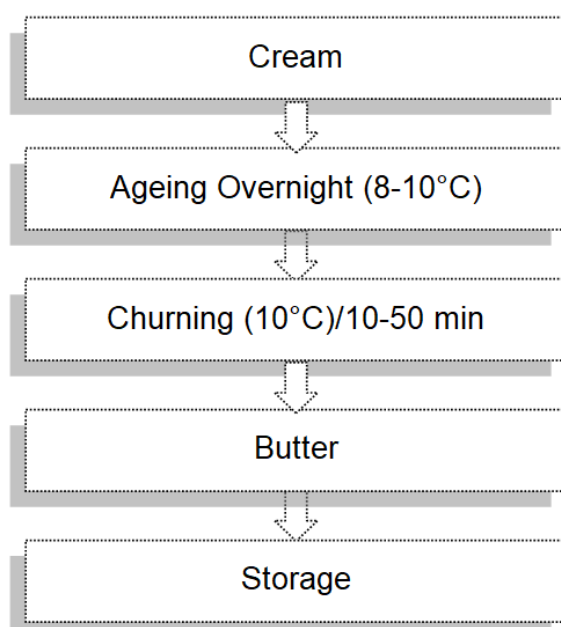


Fig. 2.1: Flow Chart of Butter Manufacturing

Churning refers to the process of agitation of cream (fat) at a suitable temperature until the fat globules adhere with each other forming a larger mass and leading to almost complete separation of fat and serum. At this stage, cold water at 10°C is added and agitation continued. The addition of water is necessary to help the cream to 'break' and hence it is known as breakwater. Churning is initiated with the agitation of cream causing incorporation of air bubble into the cream. Surface- active forces like frictional forces cause partial disruption of the fat globule membrane as a result; a part of the liquid fat leaves the globule and spreads over the surface of air bubble in the form of a thin layer. The fat film thus formed serves as a foam depressant causing the air bubble to

burst. Also, the liquid fat serves as cementing material causing fat globules to join together. Eventually butter grains are formed which float in the plasma, i.e., buttermilk.

2.2 Ghee: Product Description and Specification:

Ghee is defined as a pure clarified fat made purely from milk or curd, desi (cooking) butter, or cream, with no colouring or preservatives added. Ghee is derived from the Sanskrit word '*Ghrita*,' which signifies bright or brightening. Ghee is a product made only from milk, cream, or butter, using techniques that result in the almost complete elimination of water and solids-not-fat, and has a distinctive flavour and physical structure.

Ghee is complex lipid composed of triglycerides (~98%), derived from cow or buffalo milk and also contains diglycerides (1-2%), monoglycerides (0.1-0.2%), free fatty acids (1-10 mg/100 gm), phospholipids (0 to 80 mg/100 gm), sterols (cholesterol), fat soluble vitamins, carbonyl (4-6 µg/g), glyceryl ethers (0.8 µM /gm) and alcohols (1.8-2.3 µM/gm) in trace amounts. The major component of ghee is glycerides which constitute 98% of total composite materials and the remaining 2% consist of sterols most commonly cholesterol (Kumar *et al.*, 2016). Ghee is nutritionally superior to other oils/fats because it contains medium chain fatty acids (MCFAs), which are directly absorbed by the liver and used for energy. Carbonyls, lactones, and free fatty acids have been described as the main flavour agents in ghee (Wadhwa and Jain, 1990).

The Codex Alimentarius Commission (CAC) under the joint Food and Agricultural Organization (FAO) and World Health Organization (WHO) has established standards for milk fat products intended for further processing or culinary use. Ghee comes under the CODEX STAN: 280-1973 standard (Table 2.3) as fatty products exclusively obtained from milk, cream or butter, by means of processes, which result in almost total removal of water and not-fat-solids, with an especially developed flavour and physical structure (Codex Alimentarius).

In India, the standards for ghee (Table 2.4) are laid down by the Food Safety and Standards Act (2006), which are mandatory in nature. The ghee of cow milk has a distinct golden yellow colour, attributable to carotene. On the

other hand, the ghee of buffalo milk is noted for its whitish appearance with a greenish tinge, attributed to bilirubin and biliverdin pigment (Battula *et al.*, 2020).

Table 2.3 Codex Standards for Ghee.

Composition and Quality Parameters	Values
Minimum milk fat (% m/m)	99.6
Maximum water (% m/m)	--
Maximum Free fatty acids (% m/m as oleic acid)	0.4
Maximum Peroxide Value (Milli-equivalents of oxygen/kg fat)	0.6
Taste and odour	Acceptable for market requirements after heating a sample to 40–45°C
Texture	Smooth and fine granules to liquid, depending on temperature
Copper, Maximum level mg/kg	0.05
Iron, Maximum level mg/kg	0.2

Ghee is the most widely used milk product in the Indian sub-continent and primarily used for cooking and frying and as dressing or toppings for various foods. According to Food Safety and Standards Regulations (FSSR 2011), Ghee means the pure clarified fat derived solely from milk or curd or from desi (cooking) butter or from cream to which no colouring matter and preservative have been added (FSSAI).

In India, 80% of ghee produced is used for culinary purposes. The remaining 20% is used for confectionery, including a small amount consumed on auspicious occasions like religious ceremonies (Rajorhia *et al.*, 1993). The highly prized characteristic flavour of ghee is due to a complex mixture of compounds produced during the various stages of processing as well as those naturally present in milk (Achaya *et al.*, 1997). Ghee is also used in *Ayurveda*, for making herbal formulations. It is the perfect vehicle to carry herbal medicine, which is termed Medicated or herbal ghee. Herbal ghee carries properties of the added substance; hence ghee with more potency is developed (Battula *et al.*, 2020). Around 28% of milk produced in India is converted to ghee (Bhavaniramya *et al.*, 2018). The global ghee market size was 6.1 million tonnes in the year 2019 and

is expected to grow at a CAGR of 4.3% over the forecast period 2020-2025. (<https://www.expertmarketresearch.com/reports/gheemarket>).

Table 2.4 Standards for Ghee as per FSSR (2011).

Parameter	Values
Moisture, Maximum, %, (m/m)	0.5
Milk Fat, Minimum, %, (m/m)	99.5
Butyro-refractometer reading at 40 °C	40.0-44.0
Reichert Meissl Value, minimum	24
Polenske Value	0.5-2.0
Iodine Value	25-38
Saponification value	205-235
Free fatty acids as Oleic acid, maximum, %	2.0
Peroxide Value (Milli equivalent of Oxygen/Kg fat), maximum	-
Baudouin Test	Negative

2.2.1 Method of Ghee Manufacturing

Ghee is also popular with some other names in many other tropical and sub-tropical countries. The quality of ghee made in any of these methods is determined by the quality of milk, cream, dahi, butter, technique of preparation, temperature, storage conditions, and animal feed.

The different methods of ghee manufacture are:

- I. Desi or Indigenous or Traditional Method
- II. Creamery-Butter Method
- III. Direct-Cream Method
- IV. Pre-stratification Method
- V. Continuous Method

Desi or Indigenous or Traditional Method:

This technique is mainly in practice in rural areas where extra milk would be cultured and fermented overnight. To separate the milk fat in the form of desi butter, the dahi will be churned with hand-driven wooden beaters. The desi butter is made by churning the *malai*. After collecting desi butter, it is melted over an

open fire in a metal pan or earthenware vessel. When abrupt foaming emerges, the heating should be turned off and can carefully decant the clear fat. Only 75-85% fat recovery can be achieved with this strategy.

Creamery-Butter Method:

This strategy is used by the majority of organised dairies. In butter melter, butter is melted at 60 to 80°C. The molten butter is pumped into the ghee boiler, where it will be heated with steam. When the effervescence vanishes, finer air bubbles develop on the surface of the fat, and the browning of the curd particles indicates to stop heating. The aroma is formed at this step. Upon adjusting the final heating temperature to around 112 to 116°C, the product is transferred to the ghee settling tank, then it is filtered and stored.

Direct-Cream Method:

Fresh cream is heated to 112 to 116°C in stainless steel, jacketed ghee kettle which comprises centrifugation separation of $65 \pm 5\%$ fat cream from milk. When the ghee residue turns golden yellow or light brown in colour, the heating is turned off. In the early stages of boiling, there is usually a lot of effervescence and a crackling sound, but both of these things fade away as the moisture level drops. The development of a second effervescence, which is subtler than the first, together with the browning of curd particles, signals the end point. The typical ghee flavour emerges at this point, signalling the completion of the ghee processing process.

Pre-stratification Method:

In this method, butter is made from old cream with a fat content of 38 to 40% using a continuous butter production machine. Then the butter is placed in a butter melter and melted at around 80°C. The molten butter is held at a temperature of 80-85°C for 30 min in a ghee kettle or boiler. Stratification occurs in the ghee kettle, with the product stratifying into three different layers. The top layer collects denatured protein particles (curd particles) and contaminants, which float on the surface. The middle layer is transparent fat, and the bottom layer is buttermilk serum, which contains around 80% moisture and 70% solids-not-fat in butter. The bottom layer is removed away from the top and middle layers without causing any damage. The middle layer, which is mostly fat, and the top layer of

floating curd particles and denatured protein are both heated to 112 to 116°C. This stage is required to generate the distinctive ghee aroma.

Continuous Method:

Butter is heated in a butter melter and then transferred to a balancing tank before being pumped to a scraped surface heat exchanger (SSHE) and flashed in a vapour separator. To minimise the moisture level, heating and flashing is repeated in the heat exchanger in the next two steps. The ghee is then sent through a centrifugal clarifier to remove the residue. Clarified ghee is stored for filling and packing.

2.2.2 Physico-Chemical Characteristics of Ghee

Fats and oils exhibit certain physical and chemical characteristics that are employed for their characterization and differentiation. These characteristics are reflections of the nature of the constituents i.e., the fatty acids that make up the fat. Milk fat is unique among natural fats it contains a large proportion of fatty acids of chain length lower than 12 carbon atoms. The characteristics show variation due to factors such as species, breed, feed, and stage of lactation as the fatty acid composition is affected by these factors. Physico-chemical constants of cow milk reported by Sharma *et al.*, (1981); Sindhu *et al.*, (2000); and Battula *et al.*, (2020) are given in (Table 2.5).

Certain well-known chemical and physical constants have been used for the purpose of characterization of cow ghee. Among these physico-chemical constants, the Saponification value and Butyro-refractometer (BRR) reading, gives an overall average nature of the constituent fatty acids present in milk fat while other constant, Reichert-Meissl value (RM), the Polenske value (PV) and the Iodine value (IV), measure certain specific constituents of milk fat.

Specific Gravity: The specific gravity of milk fat is the ratio of the density of milk fat (ghee) to the density of reference substances (water). Specific gravity is one of the important criteria for the quality and purity of ghee. Density or Sp. gr. of ghee is a temperature dependent property. Generally, it increases or decreases with the rise or fall of each degree of temperature. It is also observed that the specific gravity of fats or fatty acids at a given temperature in the solid state is

higher than in the liquid state. Specific gravity of ghee varies directly with molecular weight and unsaturation.

Table 2.5: Physico-Chemical Constants of Cow Milk Fat

Sr. No.	Characteristics	Cow milk fat
1	Specific gravity	0.9390
2	Solidifying Point (°C)	15.0-23.5
3	Meting Point(°C)	28.5-41.0
4	B.R. reading at 40°C	41.20
5	Saponification Value	227.3
6	Reichert Meissl Value	28.5
7	Polenske Value	1.8
8	Iodine Value	33.8
9	Acid Value	0.374
10	Colour (Yellow units/g) Tintometer	8.8

Iodine Value: The iodine value is used to determine the degree of unsaturation of constituent fatty acids; thus, it measure the unsaturated bond present in the sample. It measures the number of grams of iodine consumed by 100 gm of sample.

Peroxide Value: The peroxide value is used to determine the oxidative rancidity in ghee. The peroxide value helps in determining the age of the ghee sample. The older the ghee is more rancid in nature. Higher the peroxide value, the higher the rancidity of ghee. It is express in milli-equivalents of peroxide oxygen per kilogram of samples.

Richert-Meissl Value and Polenske Value: Ghee is distinguished from other fats by the presence of glycerol esters of fatty acids especially butyric acids, caproic acid, capric acid, caprylic acid and myristic acid. The Richert value reflects the amount of butyric and caproic acids in the sample. These acids are wholly or partially soluble in steam. The Polenske value reflects the caprylic acid, capric acid and lauric acid and myristic acid. These values detect the presence of margarine or other vegetable oils in ghee samples.

Saponification Value: The Saponification value is the number of milligrams of potassium hydroxide required to saponify 1 gm of ghee. The Saponification value is the index of the mean molecular weight of fatty acids of glycerides comprising fat. Lower is the Saponification value, larger the molecular weight of fatty acids and triglycerides and vice-versa.

In 2020, the worldwide ghee market was estimated to be worth about 6.1 million tonnes. The industry is projected to expand at a compound annual growth rate (CAGR) of 4.3% between 2021 and 2026, reaching a volume of more than 7.8 million tonnes by 2026 (<https://www.expertmarketresearch.com/reports/ghee-market>). In 2020, the Indian ghee market was valued at Rs 2374 billion (<https://www.imarcgroup.com/ghee-market-india>).

The health benefits of ghee have been scientifically established. It is considered as a good source of important essential fatty acids and fat-soluble vitamins. Components of ghee such as CLA, sphingomyelin, butyric acid and other lipids have been reported to have anti-carcinogenic potential (Akalln & Tokusoglu, 2003). The digestibility of cow ghee has been reported to be 96% which is much higher as compared to other fats and oils. Ghee slows down the ageing process, improving the body's immune system, facilitating bowel movement, improving the health of the teeth and gums, preventing chronic cough problems and treating eye diseases (Mahakalkar *et al.*, 2014).

Products similar to ghee are also available (Battula *et al.*, 2020) and are known as *Samna* in Egypt (Abou-Dhonia and El-Agamy, 1993), *Meshho* in ancient Assyrian empire (Abdalla, 1994), *Samin* in Sudan (Hamid, 1993), *Maslee* or *Samn* in Middle East, *Rogan* in Iran (Urbach and Gordon, 1994), and *Samuli* in Uganda (Sserunjogi *et al.*, 1998).

2.2.3: Shelf Life of Ghee:

Ghee can be stored at ambient temperature for 4-6 months, but spoilage may occur due to autoxidation. Contact with atmospheric oxygen, dissolved oxygen, presence of peroxides, water activity (a_w), light, the temperature of storage, presence of metal catalysts are the factors responsible for autoxidation. Ghee undergoes oxidative degradation processes such as hydrolysis, oxidation and polymerisation followed by the formation of desirable and undesirable

secondary products that affects both, ghee and finished product quality (Tamime 2009). Guriye *et al.*, (1949) studied the effect of storage in ghee and reported higher the storage temperature higher will be the rate of deterioration. Primary and secondary oxidation products are formed which not only affect the nutritional and economic value of the product but also spoil its appetizing flavor, making it unpalatable and may possess toxic compounds (Mehta *et al.*, 2015). Gosewade *et al.*, (2017) studied the effect of storage at 37⁰ C for 8 months and reported, an increase in RM, PV, and Saponification value and loss in iodine and BRR, but these changes were very low for ghee added with butylated hydroxyanisole (BHA) than control ghee samples in which antioxidant was not added.

2.3: Antioxidant

Antioxidants: Any substance that when present at relatively low concentrations, compared with those of the oxidisable substrate significantly delays or inhibits oxidation of that substrate (Halliwell *et al.*, 1995).

There are two types of antioxidants: Natural antioxidants: Natural antioxidants are naturally occurring additives that aim to delay the oxidative rancidity of the product to make its conditions optimal for a longer period of time. Some of the most commonly used natural antioxidants are tocopherols, rosemary extract or ascorbic acid, phytosterols, phenolic acids, flavonoids, carotenoids. Synthetic antioxidants: Some of the commonly used synthetic antioxidant are Butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT), Propyl gallate (PG), Tertiary butyl hydroquinone (TBHQ). Toxicity of synthetic antioxidants are carcinogenic, tumor promotor (International Agency for Research on Cancer (IARC,1987)), impaired adipogenesis at adipocytes, internal and external hemorrhage (high dosage), tumor promote (Chevallard *et al.*, (2010), precursor to stomach tumor, and damage to DNA (Kashanian and Dolatabadi, (2009), liver toxicity and carcinogenic and local anesthetic action (Rangan and Barceloux, (2009) are also reported similar findings.

2.4: Carotene (Carotenoids)

Carotenoids are a natural pigment found in a plants, animals, and microbes. The origin name of carotenoid is from carrot (*Daucus carota*). Carotenoids are categorized in oxygen containing (xanthophylls) and non-oxygen

containing (carotene) molecules (Domke *et al.*, 2004). The β -carotene belongs to the part of carotenoids family that together with xanthophyll belongs to the upper-level grouping of carotenoids (Bergmann, 2004). The β -carotene is a natural yellow-red pigment with the chemical formula $C_{40}H_{56}$. Its sources are plants, vegetables and fruits.

Carotenoid pigments are also present in a wide variety of bacteria, algae, fungi, and plants. Microbial colorants and pigments have attracted much attention in recent years. The microorganism can grow rapidly in cheaper media which might result in high productivity with high efficiency. *Micrococcus*, genus is spherical bacteria in the family Micrococcus that is widely disseminated in nature. *Micrococci* are microbiologically characterized as gram positive cocci, 0.5 to 3.5 μm (micrometers; 1 $\mu m = 10^{-6}$ meter) in diameter. *Micrococci* are usually not pathogenic. They are normal inhabitants of the human body and may even be essential in keeping the balance among the various microbial flora of the skin. In the genus Micrococcus, the mesophilic species *M. roseus* produces the pigments which were found to be mainly α or β carotene derivatives, with canthaxanthin as the main pigment (Jagannadham *et al.*, 1991).

Chemically β -carotene is classified as tetraterpene (Koskinen, 2012). It is a precursor for vitamin-A and it's utilized in the food sector as colorant under the numbers E-160a (Domke *et al.*, 2004). The β -carotene is hydrophobic because it lacks a hydroxyl group and has eight isoprene-units, linked to the two cyclic rings at either end of the molecule chain ($C_{5}H_8$). The colour effect is caused by a large number of conjugated double bonds, which are known as chromophors (Bergmann, 2004), and this β -carotene is absorbed in the visible light at 450 nm. Carotene has the potential to dissolve in fat, and they affect a variety of biological functions such as photosynthesis, eyesight, and the elimination of free radicals and singlet oxygen.

In *Latin*, the word '*carotene*' stands for carrot (Jeyakodi *et al.*, 2018). The β -carotene is a secondary metabolite synthesized by plants and belongs to an unoxidized compound group of carotenoids. Being a polyene compound, derived from the acyclic structure, $C_{40}H_{56}$ possesses a long chain of conjugated double bonds. The general structure of β -carotene is presented in Fig. 2.2.

The high temperature promotes the isomerization of double bonds, which results the brightening of the resulting colour (Fratianni *et al.*, 2010 and Meléndez-Martínez *et al.*, 2010). The β -carotene is partially converted to vitamin-A, the remaining amount of unconverted β -carotene and retinal esters is incorporated in chylomicrons, secreted into the lymph, and then transported to the liver. Carotenoids in which molecules do not show the presence of the β -ion ring do not exhibit pro-vitamin activity (Eroglu and Harrison, 2013). It is an antioxidant that can be found in yellow, orange and green leafy vegetables and fruits.

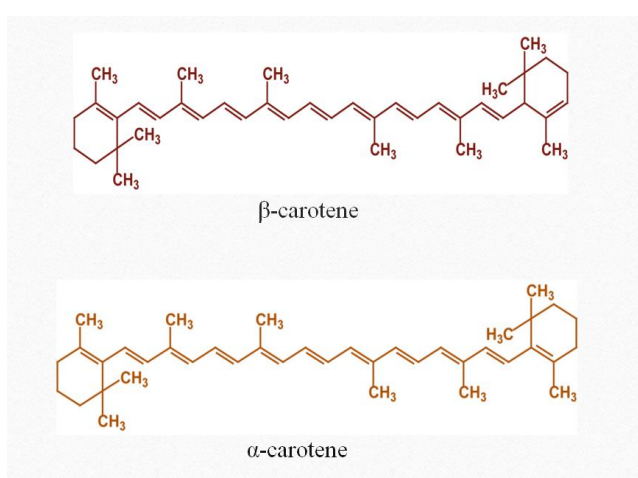


Fig. 2.2: General Structure of alpha and beta Carotene.

Under the Food and Drug Administration (FDA), beta-carotene is considered as a generally recognized as safe substance (GRAS). According to FSSR 2011, carotene is added in different Fat rich dairy products with recommended maximum level as shown in (Table 2.6).

Carotenoids have the ability to dissolve in fat, carotenoids affect biological processes, including photosynthesis, vision process, or sweeping of free radicals and singlet oxygen (Geens *et al.*, 2009; Odriozola-Serrano *et al.*, 2009; Widomska *et al.*, 2009). Carotenoids are used as natural food colourants, cosmetics, phytonutrients, and animal feed supplements. In humans, carotenoids reduce the risk of developing chronic diseases such as cancer, cardiovascular diseases, high levels of cholesterol, cataract, and macular degeneration, aside from the provitamin-A activity of some of these compounds (Woodside *et al.*, 2015).

Table 2.6: Food Safety and Standards Regulations, 2011

Product	Permitted Content	Recommended Maximum Level
Cream	Beta-Carotene	20 mg/kg
Butter	Beta-Carotene	600 mg/kg
	Annatto	20 mg/kg
Ghee	No synthetic antioxidant	--

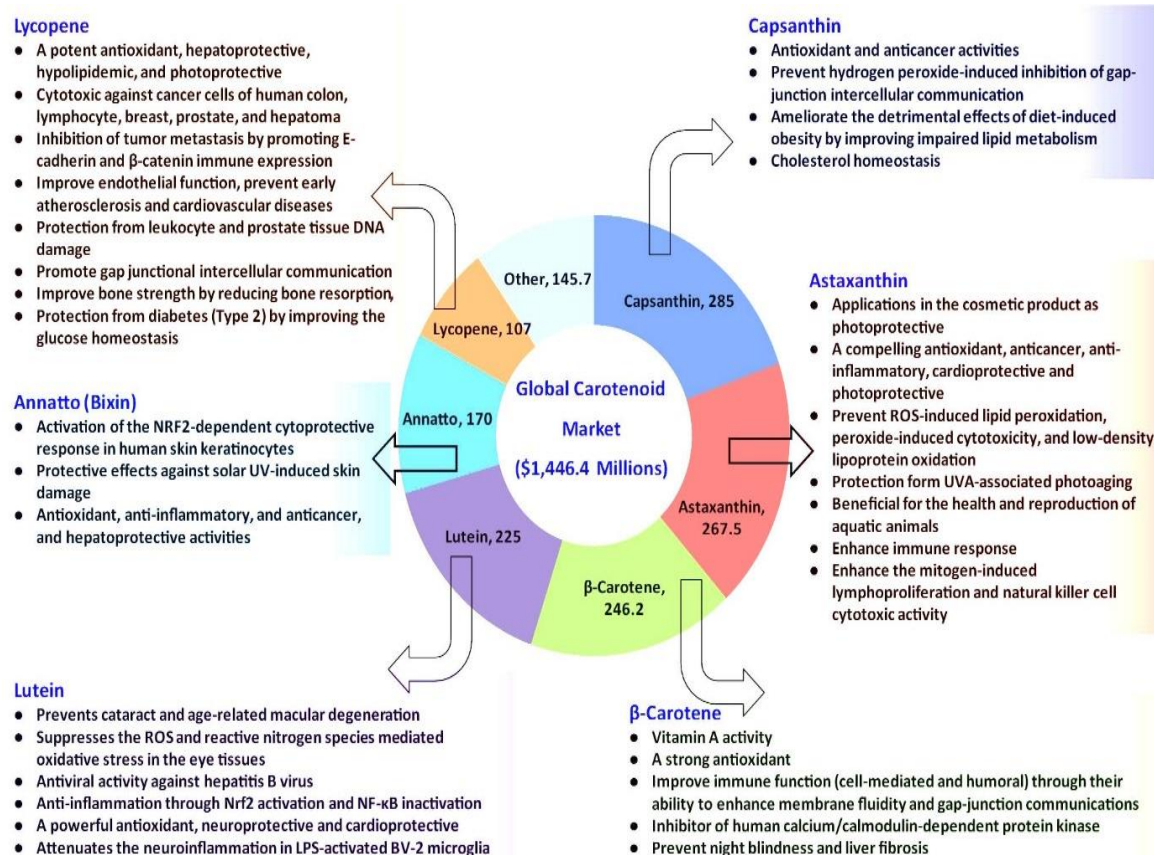


Fig. 2.3: Commercial Market Value and Significance of Carotenoids in Human Health.

Beta-carotene is a normal constituent of the human diet and is commonly ingested over the entire lifespan. Its biological importance rests on the provitamin-A function. The average daily intake of carotene from natural sources is estimated to be about 2 mg per day which is equivalent to approximately 3300 IU of vit-A. The larger amounts may be ingested in diets rich in coloured vegetables. The Recommended Dietary Allowance (RDA) of vit-A from all

sources is 5000 IU for adults. Consumption information from various sources, suggests that the per capita daily intake of β -carotene added to foods is 0.2 to 0.3 mg. (FDA 2009). The estimate of acceptable daily intake (ADI) for humans is 0-5 mg/kg body weight.

The properties of carotene are depicted in Table 2.7. In humans, 30-90% of ingested beta-carotene is excreted and concomitant intake of fat does not improve absorption. According to Harasym *et al.*, 2018 the β -carotene content (mg per 100 g) in fruits and vegetables is shown in (Table 2.8).

Table: 2.7: Properties of β -carotene:

Sr. No.	Properties	
1	Chemical formula	$C_{40}H_{56}$
2	Molar mass	536.888 g/mole
3	Appearance	Dark orange crystals
4	Density	1.00 g/cm ³
5	Melting point	183°C
6	Boiling point	654.7 °C
7	Solubility in water	Insoluble
8	Refractive index (n_D)	1.565

Table 2.8: β -carotene Content (mg per 100 g) in Fruits and Vegetables.

Sr. No.	Fruits and Vegetables	Values (mg/100 g)
1	Carrot	9.938
2	Tomato	0.64
3	Spinach	4.243
4	Squash	2.974
5	Broccoli	0.92
6	Red paprika	3.165
7	Strawberry	0.014
8	Raspberry	0.016
9	Orange	0.114
10	Watermelon red	0.23

Canthaxanthin is a carotenoid pigment, also sourced from microbial origin. These are authorized by the EU as food additive. It is mainly composed of all-trans β -carotene-4,4'-dione with other minor carotenoids. The ADI dose of canthaxanthin is established as 0.03 mg/kg bw/day. The General Structure of Canthaxanthin is shown in Fig 2.4 and the properties of Canthaxanthin are depicted in (Table 2.9). Canthaxanthin (β , β' -carotene-4, 4' - dione) is one every of the foremost important xanthophylls from a commercial point of view because it is extensively applied in medicine, pharmaceuticals, cosmetics, poultry, fishery and food industries. Canthaxanthin is employed to scale back sensitivity to sunlight (photosensitivity) experienced by folks that have a rare inherited disorder called erythropoietic protoporphyria (EPP).

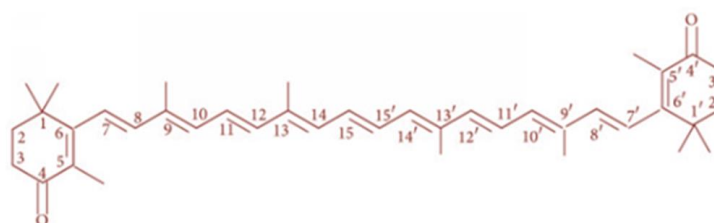


Fig. 2.4: General Structure of Canthaxanthin

Table 2.9: Properties of Canthaxanthin

Sr. No.	Properties	
1	Chemical names	Canthaxanthin β - β -Carotene-4,4'- dione 4,4'-Dioxo- β - carotene
2	Molecular formula	C ₄₀ H ₅₂ O ₂
3	Molecular weight	564.86 g/M
4	Melting point	217°C
5	Boiling point	716.95°C at 760 mmHg
6	Density	1g/cm ³ (predicted)
7	Color	Deep violet or red crystals or crystalline powder
8	Solubility	Insoluble in water and ethanol

2.5. Extraction of Bioactive Components

For the recovery of bioactive compounds, extraction is a key step. Extraction is achieved through different physical and chemical methods such as mechanical expelling, solvent assisted extraction, supercritical extraction, and microwave assisted extraction process. The recoveries of compounds through

these methods have limitations such as the use of extra solvent in solvent extraction, low yield in mechanical expelling, large capital in supercritical fluid extraction, and requirement of an aqueous phase in microwave assisted extraction (Kumar *et al.*, 2021).

Compared to these methods, Ultrasound-assisted extraction (UAE) has advantages such as less time and energy requirement, extraction at low temperature and retention of the quality of the extract. The ultrasonication extraction process involves subjecting the product to a high intensity sound wave. The ultrasound waves cause disruption in the plant tissue through physical forces developed during acoustic cavitation and help in the release of extractable components in the solvent in very less time by enhancing the mass transport (Ashok kumar, M. (2015).

2.6: Brief Resume of Work Done in India and Abroad:

Mehta *et al.*, (2006) reported that addition of methanol pre-extract of dehusked ragi powder (DRP) at the rate of 0.1%, 0.25% and 0.5% of methanol extract, the corresponding increase (over control) in phospholipids content observed was about 2.15, 4.53 and 8.15 mg/100 g) and water extractable phenolics content was about 0.52, 1.25 and 2.78 mg/100 g) of ghee respectively. The anti-oxxygenic indexes calculated from the induction periods of ghee samples stored at $80 \pm 2^{\circ}\text{C}$ in comparison with control were in order: 1.47 (0.1% pre-extract) < 1.89 (0.02% BHA) < 1.93 (0.25% pre-extract) < 2.75 (0.5% pre-extract) suggesting that the phospholipids and the phenolic compounds of DRP enhance its oxidative stability of ghee.

Liu *et al.*, (2011) reported that ultrasound significantly increased the extraction yield when the particle sizes were greater than 0.28 mm compared to ultra-sonication (CE) extraction. Dichloromethane caused degradation of all-*trans*- β -carotene extracted under ultrasound treatment. Ethanol, generally recognized as a safe (GRAS) solvent, can be considered as an alternative solvent for use in ultrasound extraction, although it showed a poor extraction yield under CE extraction. The proper ultrasonic intensity and duty cycle were also necessary to optimize extraction yield, given the optimum conditions of particle size and solvent. Low extraction efficiency and degradation during ultrasound-assisted extraction may occur if conditions are not optimized.

Konwarh *et al.*, (2012) reported that the sonication and biocatalysis are improving the extraction of lycopene from tomato peel, generated as a by-product of the food-processing industry. The use of sonication resulted in a decrease in the requisite optimal enzyme concentration and incubation period. Derivative spectra of the first and the second order, FTIR analysis and free radical scavenging test vouched for the retention of the structural integrity and bioactivity of lycopene, extracted using the developed process.

Li *et al.*, (2013) concluded the UAE produced the maximum beta-carotene production (334.75 mg/l) in 20 minutes using sunflower as the solvent, whereas CSE obtained a similar yield (321.35 mg/l) in 60 minutes using hexane as the solvent. The green UAE yielded the most b-carotene when extracted under ideal conditions (carrot to oil ratio of 2:10, ultrasonic intensity of 22.5 Wcm^{-2} , the temperature of 40°C , and sonication time of 20 min).

El-Shourbagy *et al.*, (2014) concluded that bioactive compounds found in peanut skins (PS), pomegranate peels (PP) and olive pomace (OP) were extracted using ethanol (80%), ethyl acetate and n-hexane. Ethanol extract showed slightly better antioxidant characteristics compared with ethyl acetate and hexane extracts. Ethanol extracts of different by-products were added to ghee at concentrations of 200, 400 and 600 ppm, respectively. BHA was also added to ghee at a concentration of 200 ppm for comparison. All samples were incubated at 63°C for 21 days. Ethanol extracts of PS, OP and PP gave good antioxidant activity during accelerated oxidative incubation of ghee. The results revealed that ethanolic extracts under study, at a concentration of 200 ppm, could be used to retard fat auto-oxidation.

Sharmin *et al.*, (2016) concluded that beta-carotenes are extracted from carrots under different conditions involving different temperatures, treatment of samples, and solvents (ethanol, methanol). Carrot roots were tested for the extraction yields of carotenes at temperatures 20°C , 40°C , and 60°C , the samples having been examined after harvest, after cold storage (stored at 5°C), and after freezing (-5°C). The best extraction efficiency was achieved with the samples treated by freezing and using the extraction at 60°C for 2-4 hours at 20°C , the yield of carotenes from the fresh after-harvest sample was slightly affected by the time and temperature. After 5 hr of extraction, the fresh sample

showed 1.58 mg/100 gm of carotene yield, the velocity of extraction being very slow. With the extraction at 40°C, the yield of carotenes (2.45 mg/100 gm) was higher compared to that at 20°C while the highest extraction yield was found at 60°C. At 60°C, the extraction maximum was found in the second hour of extraction (4.28 mg/100 gm).

Goula *et al.*, (2017) concluded that extracting carotenoids from pomegranate peels using green solvents and ultrasounds successfully retrieved between 85.7 and 93.8% of the total carotenoids present in the waste material. The optimal extraction time for the highest yield was around 30 min. Extraction yield improved as the extraction temperature was increased from 20 to 40°C, the amplitude level was increased to roughly 40%, and the peels/solvent ratio was increased to around 0.2. The best extraction yield was 0.3255 mg carotenoids per 100 gm dried peels.

Singh *et al.*, (2017) reported that oregano extract (OGE) can protect ghee against oxidative degradation during accelerated storage and frying. The addition of 1.0% OGE significantly inhibited the formation of peroxides and FFA and lowered the p-anisidine value (PAV) during accelerated storage and frying. The 1.0% OGE showed higher antioxidant activity than the synthetic antioxidants BHA and BHT under accelerated storage conditions. Rancimat revealed that there was no significant difference in the induction period (IP) values of ghee samples with added 1.0% OGE or 0.02% BHA until 18 min of frying, but thereafter the IP value of BHA decreased throughout the remaining frying time.

Liquorice contained 1030.00 ± 69.76 mg GAE/100 gm total phenolic content and gave $83.20 \pm 1.29\%$ DPPH radical scavenging activity. The addition of liquorice at initial stage of heat clarification was found more effective than at final stage of heat clarification. The optimum rate for use of liquorice in the treatment of ghee was found 0.3%. Liquorice was found to be capable of retarding oxidative degradation in ghee and even more effective than BHA. Bipinbhai *et al.*, (2017) reported curry leaves were analysed for their total phenolic content and radical scavenging activity. Curry leaves contained 1913.33 ± 57.35 mg GAE/100 gm total phenolic content and gave $84.07 \pm 0.93\%$ DPPH radical scavenging activity. The addition of curry leaves at the final stage of heat clarification was found more effective than at initial stage of heat clarification. Curry leaves were found to be

capable of retarding oxidative degradation in ghee but were less effective than BHA.

Numan *et al.*, (2018) reported that bacterial pigments are actually coloured secondary metabolites secreted during stress conditions. Due to its potential activity against many human pathogenic bacterial strains, it can be a potential drug to cure patients infected with these pathogenic bacterial strains. These bacterial pigments are active against some cell lines which mean if further research is performed on these pigments, they can help in the treatment of cancer. Bacterial pigments also showed strong antioxidant activity helpful in the scavenging of free radicals. Despite of therapeutic applications, bacterial pigments also have applications in the food and textile industries. In the food and textile industries, these pigments are used as colorants.

Pajohi-Alamoti *et al.*, (2019) reported ghee containing essential oils (EOs) was reported with a higher level of tolerance in increasing the peroxides, TBA, and FFA than the sample without EO. The results of the present study showed that among the evaluated EOs, *Salvia officinalis* (SO) can be considered a good natural antioxidant for the reduction of the oxidation rate in fat-rich foods.

Deshmukh *et al.*, (2019) reported antioxidant activities of Shatavari (*Asparagus racemosus*) and Ashwagandha (*Withania somnifera*) extracts were evaluated and compared with BHA using β -carotene bleaching assay, DPPH. The total phenolic content of *Shatavari* extract was significantly higher. Higher antioxidant activity was noticed in *Ashwagandha*. The free radical-scavenging potential of *Shatavari* extract was higher (62.85%). The peroxide value (0.32) of *Ashwagandha* extract was lower. Antioxidant activity of the herbs decreased in the order *Ashwagandha* > *Shatavari*.

Miekus *et al.*, (2019) suggested carotenoid extraction from carrots with the use of two “green chemistry” methods, super-critical carbon dioxide (SC-CO₂) and enzyme assisted extraction (EAE). The author reported the main benefits of using SC-CO₂ for isolation of carotenoids are solvent-free products, no co-products, and low temperatures in the separation process. SC-CO₂ is non-carcinogenic, non-toxic, non-mutagenic, non-flammable, and thermodynamically stable and CO₂ does not usually cause oxidization of the analytes that are very

important for the carotenoids. EAE offers a faster extraction, higher recovery, reduced solvent usage, and lower energy consumption when compared to non-enzymatic methods. For the carotenoid's extraction from carrots, EAE improves the extraction yields by disrupting the cell walls of the carrot's tissues. The SC-CO₂ method requires important technological investments, while EAE is a more convenient solution for the application in developing countries.

Study conducted by da Silva *et al.*, (2020) to optimise the ultrasonic-assisted extraction (UAE) of β -carotene for sunflower oil by modifying the operational factors of temperature, duration, and oil to carrot ratio, as well as obtaining extraction kinetics. The characteristics of enriched sunflower oil and commercial sunflower oil were investigated. Temperature (55°C), time (60 min), and oil to carrot ratio (30 ml/gm) at their maximums resulted in the most β -carotene extraction.

Maiza *et al.*, (2020) study to characterize the effects of aromatic plants addition (rosemary and clove) on the nutritional, volatile and oxidative profile of cow ghee and to evaluate the effect of flavoured ghee. Two flavoured ghee products were obtained by adding clove (CG) and rosemary (RG) as aromatic plants through maceration in cattle traditional ghee (BT). It was revealed that enriched ghee samples had significantly lower peroxide values (6.76 and 6.80 meqO₂ /kg) compared to control samples (8.20 meqO₂ /kg). Moreover, the addition of rosemary and clove change the volatile profile, and increased the retinol levels of ghee (BT: 1.3 mg/kg; CG: 1.9 mg/kg; and RG: 3.05 mg/kg).

Elik *et al.*, (2020) investigated the impact of microwave technology on carotenoid extraction yield from carrot juice processing waste utilising flaxseed oil as the solvent. The influence of microwave power, extraction duration, and oil to waste ratio on percentage carotenoid recovery was optimised using response surface approach. The optimal microwave-assisted carotenoid extraction variables were 165 W of microwave power, 9.39 min of extraction time, and 8.06:1 gm/gm of oil to waste ratio, yielding carotenoid recovery of 77.48%. The enriched flaxseed oil was found to be of good quality, with a high phenolic content (214.05 ± 1.61 gm GAE/ g oil) and antioxidant activity (DPPH inhibition % = $70.67 \pm 0.85\%$).

Chutia *et al.*, (2021) investigated the extractability of carotenoids from passion fruit peel (PEP) that is discarded as waste using green procedures such as ultrasonication assisted extraction (UAE) and microwave assisted extraction (MAE) using olive oil (OO) and sunflower oil (SO) as solvents. The optimal parameters for UAE utilising olive oil as the solvent to achieve optimum extraction efficiency were 39 min, 47°C, and 30 gm/100 mL S/L ratio, while those for MAE were 200 W microwave power, 25 min, and 10 gm/100 mL S/L ratio. 91.4% (OO) and 86.7% (SO) of the carotenoids present were extracted under UAE-optimized conditions, while MAE extracted 86.9% in OO. Both procedures have the potential to improve the extraction efficiency.

Sharma *et al.*, (2021) studied the effects of green extraction procedures on carotenoids, polyphenols, and antioxidant activities of pulp and peel of two types of pumpkin (*Cucurbita maxima*). The extractions that are green conventional extraction (CE; hexane/isopropyl alcohol; 60:40, v/v) were compared to ultrasound and microwave-assisted extractions synergized with corn oil. When IGE was used instead of conventional extraction, the total carotenoids were substantially doubled. Polyphenolic contents in IGE ranged from 510.69 ± 5.50 to 588.68 ± 7.26 mg GAE/100 gm of extract, compared to conventional extracts (269.50 ± 2.17 to 318.46 ± 6.60 mg GAE/100 gm), and % inhibition of 2,2-Diphenyl-1 picrylhydrazyl (DPPH) in IGE was between 88.32 ± 1.51 and $93.53 \pm 0.30\%$, compared to conventional extraction (50.61 ± 1.44 to $57.79 \pm 2.09\%$).

2.7: Work Done at NDRI

Pawar *et al.*, (2012) reported that the potential of *Asparagus racemosus* (Shatavari) extract was evaluated in comparison with natural (rosemary, green tea) and synthetic (BHA and TBHQ) antioxidants, in ghee using accelerated oxidation tests. Aqueous and ethanolic extracts of shatavari were found to be capable of retarding oxidation in ghee but were less effective than other natural (rosemary and green tea extracts) and synthetic (BHA and TBHQ) antioxidants.

Gandhi *et al.*, (2013) reported that antioxidant activities of vidarikand (*Pueraria tuberosa*) extracts were evaluated and compared with BHA, TBHQ, rosemary and green tea using a β -carotene bleaching assay, DPPH assay and the Rancimat method. Aqueous and ethanolic extracts of vidarikand were found

to be capable of retarding oxidative degradation in ghee but were less effective than natural (rosemary and green tea) and synthetic (BHA and TBHQ) antioxidants.

Parmar *et al.*, (2013) reported that the addition of ethanolic extract of *Terminalia Arjuna* bark at 7% by the weight was highly effective in retarding the auto-oxidation of both cow and buffalo ghee during storage. The shelf life of the Arjuna herbal ghee at $80 \pm 1^\circ\text{C}$ was 8 days as compared to 2 days at $80 \pm 1^\circ\text{C}$ in control ghee samples. The findings suggested that ethanolic extract of *T. Arjuna* could be used as a natural antioxidant in ghee and enhance the phytosterol content in ghee. Freshly prepared cow milk Arjuna ghee possesses the good potential to act as a free radical scavenger and thus could help in prevention of many free radical related disorders.

Pawar *et al.*, (2014) reported that antioxidant activities of vidarikand (*Pueraria tuberosa*), shatavari (*Asparagus racemosus*) and ashwagandha (*Withania somnifera*) extracts (aqueous and ethanolic) were evaluated and compared with BHA using β -carotene bleaching assay, DPPH assay and Rancimat method. Aqueous and ethanolic extracts of herbs were found to be capable of retarding oxidative degradation in ghee but were less effective than BHA. Antioxidant activity of the herbs decreased in the order vidarikand > ashwagandha > shatavari. Thus, the ethanolic extract of vidarikand was having the maximum antioxidant activity among all the herbs.

A study conducted by Patel *et al.*, (2014) to evaluate antioxidant potential of Ashwagandha, clove, coriander, green tea, Shatavari and Vidarikand extracts as compared to BHA. Clove, coriander and green tea extracts showed significantly higher antioxidant activity than Vidarikand, Shatavari and Ashwagandha extracts. Radical-scavenging activity by DPPH model system revealed that clove and green tea extracts were superior to other extracts. All the natural antioxidants significantly reduced the formation of peroxides, FFA, thiobarbituric acid value (TBA) and conjugated dienes (CD).

Siwach *et al.*, (2016) reported the incorporation of lycopene in cow ghee; at all levels ranging from 30 ppm to 150 ppm significantly ($p < 0.05$) reduced the development of rancidity as compared to control. Lycopene incorporation at the

the highest level of 150 ppm, in this study, was found less effective than BHA (at a level of 200 ppm) in controlling oxidative rancidity in terms of free fatty acids content, peroxide value and thiobarbituric acid value.

Asha *et al.*, (2015) reported that the ghee incorporated with orange peel extract (OPE) showed stronger activity in quenching DPPH radicals and least development of PV, TBA and FFA than ghee incorporated with BHA and control. The study revealed that orange peel could be a good natural source of antioxidants which can be used in fat rich food products like ghee to retard oxidative deterioration.

Rahila *et al.*, (201) reported that the addition of rosemary extract (RME), a source of natural antioxidants, @ 0.1%, increased the antioxidant potential of ghee in terms of radical scavenging activity (DPPH assay) without affecting sensory and physicochemical properties. Based on peroxide and TBA value determinations, the shelf life of ghee added with RME was significantly higher than that of control and ghee added with BHA. The addition of RME has also imparted thermal stability to ghee and delayed the onset of cholesterol oxidation, when used as frying medium.

Lodh *et al.*, (2018) reported that anti-oxidant rich ghee uses curcumin (yellow pigment in turmeric) as the natural anti-oxidant. Effects of varying curcumin levels (160-350 ppm), heating temperature (110-120°C) and duration (16-22 min) on anti-oxidant, chemical and sensory attributes of ghee were studied. The increasing level of curcumin increased the DPPH free radical scavenging activity and decreased the amount of conjugated dienes formation. Increasing heating time and temperature significantly decreased the anti-oxidant activity in ghee, but their combination significantly increased the activity. Increase in curcumin level and heating temperature improved the sensory attributes of ghee, but the longer duration of heating decreased the same.

Baria *et al.*, (2019) used magnetic stirring (MS), ultrasonication (US), microwave (MW), and high shear dispersion (HSD) techniques to extract carotenoids for various time intervals in different green solvents at different pulp to vegetable oil ratios. On the basis of the highest total carotenoid content (TCC), one combination was chosen among the rest. Using the HSD approach,

maximum extraction was achieved in flaxseed oil. TCC, antioxidant activity tocopherol content and colour value (L, a, b) were all higher in the selected combination than in the corresponding vegetable oil (control).

Tiwari *et al.*, (2019) used to recover carotenoids from carrot pomace utilising ultrasonication and high shear dispersion techniques, with flaxseed oil as the green solvent. The final selection was determined based on the highest recovery of carotenoids after experimenting with various combinations of time and temperature. Maximum carotenoids were recovered (recovery $94.8 \pm 0.08\%$) using a high shear disperser. When compared to the extracting medium, the total carotenoid content, antioxidant activity as ABTS, DDPH, and FRAP, and carotene of carotenoid rich extract from carrot pomace (CREP) were 82.66 ± 0.06 $\mu\text{g/gm}$, 1596.04 ± 69.45 $\mu\text{g Trolox eq./ml}$, 380.21 ± 39.62 $\mu\text{g Trolox eq./ml}$, 941.20 ± 19.91 $\mu\text{g Trolox eq./ml}$, 78.37 $\mu\text{g/gm}$, respectively were significantly higher when compared with the extracting medium.

Hazra *et al.*, (2019) reported that the addition of tomato skin, as a source of natural antioxidants, @ 0.4%, increased the antioxidant potential of ghee in terms of without affecting sensory and physicochemical properties. Peroxide value analysis revealed that the shelf life of ghee added tomato skin was significantly higher than that of control ghee. Thus, the study suggested that tomato skin could be used as a source of natural antioxidants to increase the oxidative stability of ghee.

A recent study carried out in our lab by Jha (2021) concluded that the natural antioxidants added to ghee in the form of herbs and spices (Betel leaves, fenugreek seeds and turmeric) to the ghee improved sensory quality (in color, texture and flavor) and other functional properties. These ingredients were added either directly to butter and then it is subjected to a clarification process or after preparation of ghee by holding at 80°C for 30 min. The methodology has resulted in a significant increase in total phenolic content (159.9 mg GAE/gm ghee) and enhanced the antioxidant activity of the developed product (approx. 4 times than the control ghee) and also improved the shelf life in comparison to control ghee because of the synergistic potential of the bioactivities without effecting the physico-chemical constant of ghee.

2.8. Methods for Determining Polyphenols and Antioxidant Potential

Herbs, spices and other plant materials rich in phenolic content are being widely studied for their use in the food industry as they retard oxidative degradation of lipids and thereby improving the quality and nutritional attributes of food. Generally, Folin-Ciocalteu colorimetric method (1999); is used for the estimation of total polyphenol content. Quantification is done with respect to Gallic acid standard curve and results are expressed as Gallic acid equivalent (GAE), mg per 100 gm of dry weight.

The most commonly used assay for evaluation of antioxidant activity is ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)), DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (Fluorescence recovery after photobleaching). ABTS and DPPH are characterized by excellent reproducibility under certain assay conditions, but they also show significant differences in their response to antioxidants. The relatively stable organic radical, DPPH, has been widely used in the determination of the antioxidant activity of single compound, as well as of different plant extracts (Katalinic *et al.*, 2006). DPPH is a relatively stable Nitrogen centered free radical that easily accepts an electron or hydrogen, it reacts with suitable reducing agents as a result of which the electron becomes paired off and the solution loses color depending on the number of electrons taken up (Blois, 2001). Leontowicz *et al.*, (2003) reported that a close correlation ($R^2 = 0.935$) was obtained between the quantity of polyphenols in fruit extracts measured by the Folin-Ciocalteu method and the antioxidant capacity results obtained in this test.

2.9 Accelerated Stability Tests

To estimate the stability of fat to oxidation, the sample is subjected to an accelerated oxidation test under standardized conditions and a suitable endpoint is chosen to determine signs of oxidative deterioration (Frankel, 1993). Several parameters (e.g., temperature, metal catalysts, oxygen pressure, shaking) are manipulated to accelerate oxidation and the development of rancidity in oil and emulsions (Frankel, 1993). The degree of lipid oxidation can be measured by chemical and physical methods as well as stability tests, which measure the stability of oil under conditions that attempt to accelerate the normal oxidation

processes (Gray, 1978). Increasing temperature is the most common and effective type of accelerated storage study method. Conventional stability tests are listed below in (Table 2.10).

Table 2.10. Stability Test Methods and Condition of Acceleration

Test	Condition
Ambient Storage	Room temperature Atmospheric Pressure
Light	Room temperature Atmospheric Pressure
Metal Catalysts	Room temperature Atmospheric Pressure
Weight-gain method	30-80°C Atmospheric Pressure
Schaal Oven test	60-70°C Atmospheric Pressure
Oxygen uptake	80-100°C Atmospheric Pressure
Active oxygen method (AOM)	98°C Air bubbling
Rancimat	100-140°C

(Frankel,1993)

2.10. Response Surface Methodology

Response Surface Methodology (RSM) is a useful technique for the investigation of complex processes. The RSM approach was developed by Box and colleagues in the 1950s, with the major advantage being the reduced number of experiments that are required to appraise multiple parameters and their interactions (Bruns *et al.*, 2006; Ge *et al.*, 2002; Naik *et al.*, 2013). The term “Response Surface Methodology” was derived from the graphical view created after fitting the mathematical model. RSM depicts the effect of the independent variables on the dependent variables. In addition, the approach also generates an empirical model (Yolmeh and Jafari, 2017).

Summary of Review of Literature

From the above review, it is evident that β -carotene seems to be a potential and important source of natural antioxidant. Few literature reports that β -carotene can be extracted using chemicals and solvents, therefore, in this study we are attempting to extract using green technology from carrot and microbial source. Therefore, this work is proposed with a hypothesis that beta-carotene being a provitamin-A, pigment, and antioxidant; its fortification to butter and ghee may enhance the shelf life, sensory properties and nutraceutical benefits without affecting the quality parameters.

Chapter-3

MATERIALS

AND

METHODS

MATERIALS AND METHODS

This chapter deals with the materials and methodologies followed in this research. The preliminary experiments for product optimization and evaluation were carried out in Dairy Chemistry and Bacteriology Section, SRS of ICAR-National dairy research institute (NDRI), Bengaluru.

3.1: Chemicals:

All the chemicals used during the course of investigation were AR grade and procured from standard suppliers (Hi-Media and Sigma-Aldrich). The chemicals and reagents used during research work are potassium hydroxide pellets, sodium hydroxide pellets, sodium thiosulfate pentahydrate, sodium carbonate, potassium Iodate, starch, potassium dichromate, acetic anhydride, phenolphthalein, oxalic acid, Trolox (6 hydroxy- 2,5,7,8 tetramethyl chroman-2-carboxylic acid), β -carotene, Folin's reagent and DPPH, etc.

3.2: Solvents:

The solvent used during research work are sulphuric acid, ethyl alcohol, glacial acetic acid, concentrated hydrochloric acid, concentrated ammonia, gallic acetic acid, methanol, ethanol, ethyl acetate, chloroform, cyclohexane, acetone, diethyl ether and petroleum ether, carbon tetrachloride and Wij's reagent, etc.

3.3: Glassware's:

The glassware used during research work are volumetric flask, conical flask, measuring cylinder, beaker, pipettes, flat bottom boiling flask (RM flask), still head, condenser, receiver, glass funnel, glass beads, test tubes, centrifuge tubes, burette, desiccators, aluminium dish, spatula, etc.

3.4: Equipment's/Instruments and other Accessories

1. **Butyrometer:** Cream butyrometer (scale range 0-70) was taken in study. This was checked for their scale accuracy as per ISI procedure (IS: 1233 Part 1, 1970).

2. **Milk Pipette:** Pipette of 10.75 mL volume was used for the determination of fat in milk. The Pipettes were calibrated as per the ISI procedure for its specifications as per IS: 1223 (2001).
3. **Thermometer:** The thermometers were evaluated under the specification of ISI (IS: 1223 -1970).
4. **Boiling water-bath:** (The Laboratory Glassware Co., Ambala Cantt., India).
5. **Butyro-Refractometer:** (Atago India Instruments Pvt. Ltd., New Delhi, India)
6. **Centrifuge:** (Remi Udyog, Mumbai, India).
7. **Ultrasonic Cell Crusher:** (Biobase Biodustry Shandong Co., Ltd China)
8. **Gerber Centrifuge:** Used for estimation of fat, (Astell laboratory service Co. Ltd London), 1500-2000 rpm is used.
9. **Digital temperature controller oven:** (Narang Scientific Works Pvt. Limited, New Delhi, India).
10. **Electric heater:** (Vikrant, Jain Enterprises, India).
11. **Electronic balance:** (Precisa XB 220A, Switzerland).
12. **Hot air oven:** Used for determination of Moisture. (Peenya Industrial area, Bangalore)
13. **Magnetic stirrer:** (Tarsons products Pvt. Ltd., Kolkata, India).
14. **Refrigerator:** Used for low temperature storage of cream, butter samples for further analysis.
15. **UV-Visible spectrophotometer:** UV-3200, XE LABINDIA
16. **Vortex mixer:** (Spinix, Tarsons Products Pvt. Ltd., Kolkata, India).
17. **Water bath:** It was used for heating, having a temperature range from 30°C to 110°C (Rivotek).
18. **Weighing Balance:** Kanchan Instrument Rajajinagar, Bangalore
19. **pH meter:** Servewell instruments Pvt. Ltd., Rajajinagar, Bangalore
20. **Freezer:** Falcon scientific co Nagashetty halli Bangalore-94
21. **BOD Incubator:** Excel Scientific Bangalore, Karnataka.

3.5: Reagents:

1. **Alcoholic Potassium Hydroxide (0.5N):** Apprx. 35 to 40 gm KOH was dissolved in 20 mL distilled water and add aldehyde free rectified spirit used to make up volume to 1000 mL. then allowed to stand overnight and decanted the clear liquid and stored in an airtight bottle.

2. **Dilute Sulphuric Acid (0.1N):** Approximately 25 mL of concentrated sulphuric acid was diluted to 1000 mL with distilled water.
3. **DPPH 0.1 mM solution:** Exactly 0.394 mg DPPH was taken in an ambered colour conical flask and made-up volume 100 mL with ethyl acetate and kept overnight at refrigerated temperature.
4. **Ethyl Alcohol (neutralized):** Ethyl alcohol was neutralized using 0.1N sodium hydroxide solution in presence of phenolphthalein indicator before its use.
5. **Folin's Reagent (10%):** Commercially available Folin's reagent 10 mL was diluted in 100 mL of distilled water.
6. **Gallic Acid (0.1mg/mL):** Exactly 10 mg of gallic acid was dissolved in 100 mL distilled water.
7. **Hydrochloric Acid (0.5N):** Exactly 42 mL of concentrated Hydrochloric acid was diluted to 1000 mL with distilled water.
8. **Mixed Solvent:** Prepared by 2 parts of glacial acetic acid mixed in 1 part chloroform i.e., 2:1 (v/v).
9. **Oxalic Acid Solution (0.1N):** Exactly weighed 0.63 gm of oxalic acid, then dissolved in distilled water and made-up volume up to 100 mL in a volumetric flask.
10. **Phenolphthalein Indicator (1%):** Exactly 1 gm of phenolphthalein was dissolved in ethyl alcohol and then volume was made to 100 mL.
11. **Potassium Iodide Solution 10%:** Taken 10 gm of potassium Iodide free from Iodine and make up volume 100 mL with distilled water.
12. **Saturated Potassium Iodide Solution:** Boiled some water for about 5 min and allowed cooling. To a portion, added enough potassium iodide to ensure a saturated solution (10.0 gm KI in 6.0 mL water). Stored it overnight to ensure that un-dissolved crystals remain there.
13. **Sodium Carbonate (7.5%):** Exactly 7.5 gm of Na_2CO_3 was dissolved in 100 mL of distilled water.
14. **Sodium Hydroxide Solution (0.1N):** Exactly 4.0 gm m of sodium hydroxide was dissolved in distilled water and volume was made up to 1000 mL in a volumetric flask. The strength of the solution was adjusted to 0.1N with the help of 0.1N standard oxalic acid solution.

15. **Sodium Hydroxide Solution (50%; w/w):** Exactly 50 gm of sodium hydroxide pellets were dissolved in equal weight of distilled water and kept in a bottle protected from CO₂.
16. **Sodium Thiosulphate Pentahydrate Solution (0.01N):** 0.1N solution of sodium thiosulphate was prepared and dilution was made to get 0.01N.
17. **Sodium Thiosulphate Pentahydrate Solution (0.1N):** Dissolved approximately 24.8 gm of sodium thiosulphate crystals in previously boiled and cooled distilled water and made the volume to 1000 mL. Stored the solution in a cool place in a dark coloured stock bottle. After storing the solution for about two weeks, filtered if necessary and standardized the solution.
18. **Starch Indicator Solution 1% (W/V):** Exactly 1 gm of soluble starch was weighed and paste was made in 30 mL of water. The paste was transferred to 80 mL boiling water and heated until a clear solution was obtained.
19. **Wijs' Reagent:** Dissolved 10 mL of iodine monochloride in about 1800 mL of glacial acetic acid and after shaking vigorously. Pipetted 5 mL of this, added 10 mL of potassium iodide solution and titrated with 0.1N sodium thiosulphate solution, using starch as indicator. Adjusted the volume of the solution till it was approximately 0.2N.

3.6: Extraction of Antioxydant-Rich Fraction from Natural Sources

3.6.1: Extraction from Microbial Source

Bacteria *Micrococcus* was isolated from the soil near the open ground of SRS of ICAR-NDRI campus. Soil temperature was around 29°C; sample was then serially diluted till 10⁻⁶ with 0.9% of saline. From each Dilution 1 mL of sample was pour plated with 25 mL media with one mL of the culture in nutrient agar and incubated for 24 hr for isolation of pure colony. Protocol of pigment extraction from microbial source is shown in Fig. 3.1.

3.6.2: Extraction of β-carotene from Carrots Using Ultrasonic Assisted Extraction (UAE):

Collection of Carrots Sample:

Fresh carrots (Arka Breed) were purchased from the local market of Bengaluru. Samples were cleaned to remove dirt and dust. The carrots used for

extraction were previously sliced and dried using oven dryer followed by grinding in a laboratory grinder. The powder sample were packed in bottles and kept in refrigeration.

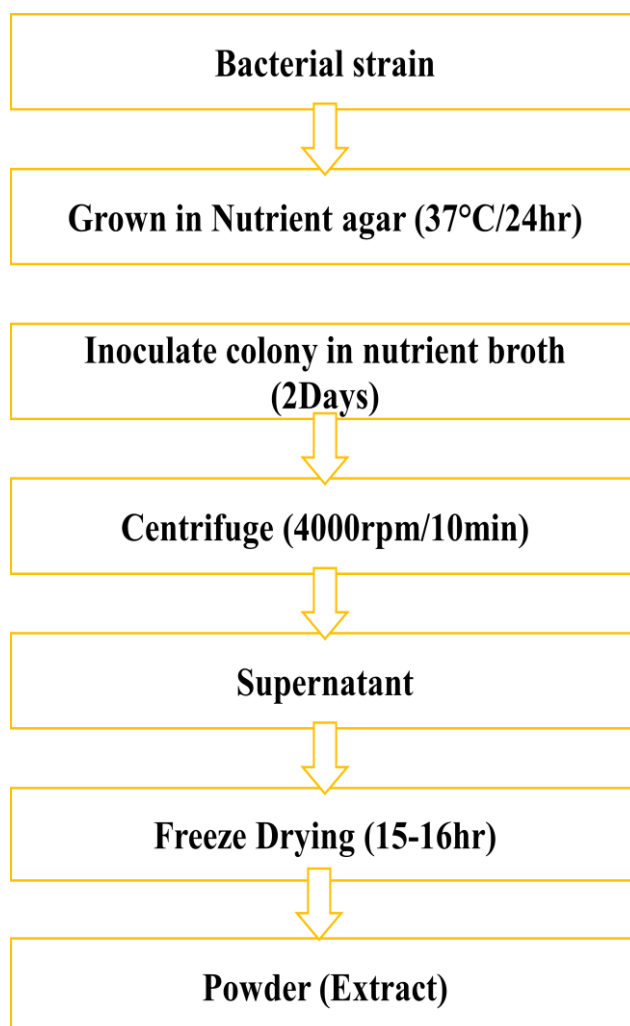


Fig. 3.1: Protocol of Pigment Extraction from Microbial Source.

Ultrasound-assisted extraction (UAE) method was utilised to facilitate the green-extraction of carotene which are depicted in Fig. 3.2. To achieve UAE extraction (Biobase Biodustry Shandong Co., Ltd China) of carotene, (1 to 5 gm) of carrot powder samples was weighed in beaker followed by addition of 10 mL of ghee. The ultrasonic probe of 6 mm (Biobase Biodustry Shandong Co., Ltd China) was immersed in the sample (carrot powder sample and ghee) at a power of (40 to 80%) for (10 to 40 min). The pulse duration was adjusted to “on” (2 sec) and “off” (3 sec) mode during extraction process. Ghee was used as alternative green solvent for extraction. The extracts were collected after extraction and

centrifuged (Remi Centrifuge Ltd Mumbai-India) at 4000 rpm for 10 min for complete separation of ghee and residue, and later on stored for further analysis.

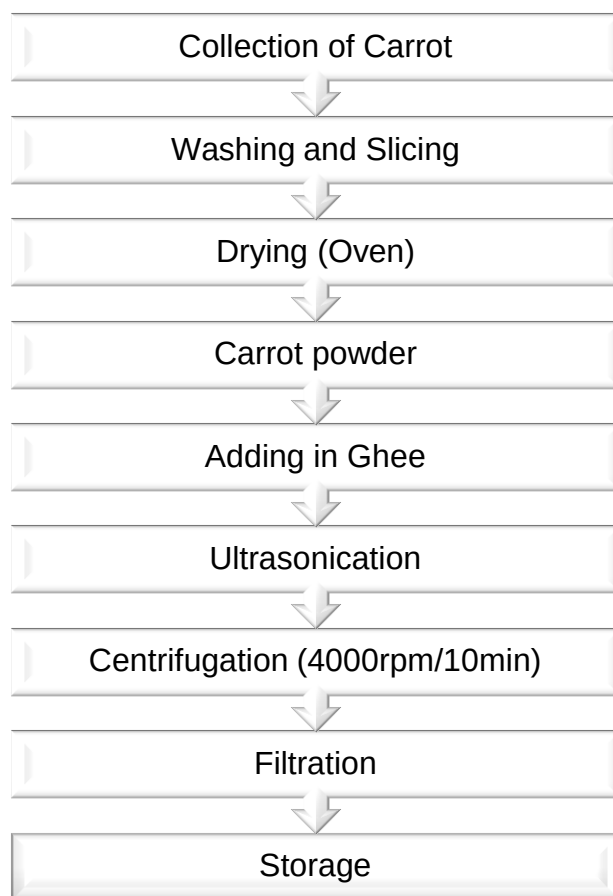


Fig. 3.2: Ultrasound-Assisted Extraction (UAE) Using Ghee as Green Solvent.

3.6.3: Preparation of Standard Curve:

Standard curve was prepared using 2 μg , 4 μg , 6 μg , 8 μg and 10 μg of dissolving standard carotene in cyclohexane and the absorption spectra of the carotene content were measured in a 1 cm optical path-length quartz cell using a spectrophotometer at 455 nm wavelength.

3.6.4: Analysis of β -carotene Using Ultra Violet (UV) Spectroscopy:

The absorption spectra of the extracted carotene content were measured in a 1 cm optical path-length quartz cell using a spectrophotometer at 455 nm for β -carotene in each extract against the cyclohexane used as a blank. The Beer-Lambert law was used to determine the β -carotene concentration in enriched ghee from a calibration curve prepared using the β -carotene standard. The

straight calibration curve of absorbance versus β -carotene concentration (mg/L) was reliant on the Beer-Lambert law.

3.6.5: Experimental Design:

The examined performance of UAE was obtained by the response surface methodology (RSM), using the Design Software. In order to examine the influence of the operating parameters in extractions, a Box Behnken design (BBD) was used to analyze the main extracted β -carotene. Three controlled factors namely ultrasonic power (P), sonication time (T) and carrot powder to oil ratio (CO) were selected to evaluate. The limit values of each variable range were chosen as function of limitations of ultrasonic apparatus, Ultrasonic power (40-80 W), Ultrasonic time (10-40 min) and carrot to oil ratio (0.1-0.5) for β -carotene extraction. The Values are presented in Table 4.3. and involved a total of 19 experiments. The surface response of data obtained were analysed by ANOVA. The experimental and predicted values of β -carotene were compared in order to determine the validity of the model.

3.7: Collection of Cream Sample:

Raw cream sample was collected from the Experimental Dairy Plant, SRS of ICAR-National Dairy Research Institute, Bengaluru (2 kg/batch). Cream samples were analysed for fat (Gerber méthodes) SNF (Gravimetric Methods) and acidity (Titration Method).

3.7.1 : Analysis of Cream Sample :

3.7.1.1: Determination of Fat Content in Cream:

Fat content of cream samples was determined by the method IS:3509 (1966) as described below.

Procedure: First mixed the sample well and weighed accurately 5 gm of cream sample in the butyrometer. Added 10 mL of sulphuric acid with the help of tilt measure. Inserted the rubber stopper and mixed the content well. Then 1 mL of amyl alcohol and water added to bring the volume up to 70 marks of butyrometer. Replaced rubber stopper tightly and mixed the content well. Centrifuged the butyrometer in centrifuge machine at 1000-1200rpm for 5 min. Then transferred

the butyrometer in the water bath at 65°C. and read the percentage fat by adjusting lower layer of fat column to zero mark.

3.7.1.2: Determination of Titrable Acidity in Cream:

Acidity of cream samples was determined by the method IS:3509 (1966) as described below.

Procedure: Exactly weighed 10 gm cream sample in conical flask, then added 10 mL of water, and stirred to disperse the cream. Then added 2 mL phenolphthalein solution into the conical flask and titrated against 0.1N sodium hydroxide solution until the pink colour appeared.

Titration acidity (% lactic acid) = $9VN/W$

Where, V = volume in mL of the standard sodium hydroxide required for titration

N= Normality of the standard sodium hydroxide solution, and

W = weight in gm of cream taken for the test.

3.8: Method of Preparation of Butter:

Cream was aged overnight at 8-10°C. Properly aged cream was then churned into butter using butter churner. The preparation of butter is depicted in Fig.3.3. and the final products (control butter and Optimized butter) were subjected to sensory evaluation judging panel.

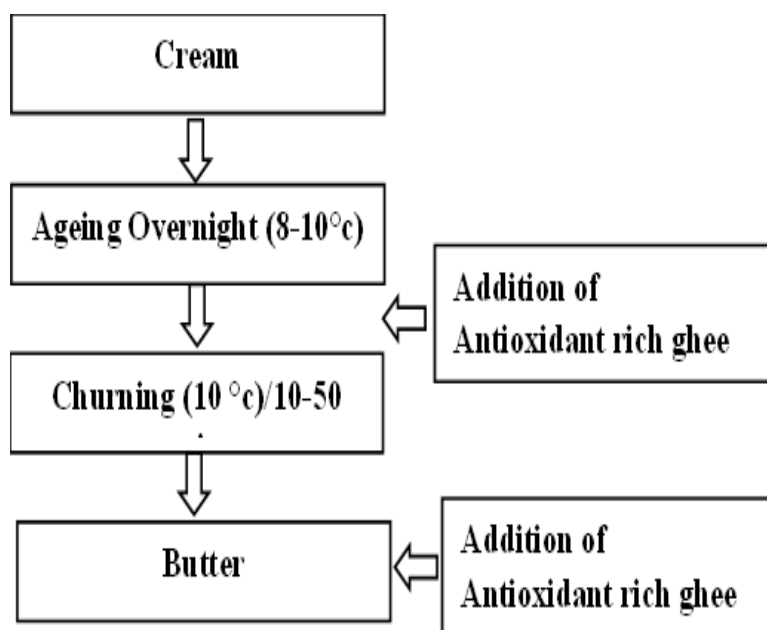


Fig. 3.3: Flow-Chart for Preparation of Butter

3.8.1: Physico-Chemical Analysis of Butter Sample

3.8.1.1: Determination of Fat Content in Butter:

Fat content of butter samples was determined by the method IS:3507 (1966) as described below.

Procedure: First mixed the sample well and weighed accurately 5 gm of butter sample in the butyrometer. Then added 10 mL of sulphuric acid with the help of tilt measure. Inserted the rubber stopper and mixed the content well. Added 1 mL of amyl alcohol and water to bring the volume up to 70 marks of butyrometer. Replaced the rubber stopper tightly and mixed the content well. Centrifuged the butyrometer in centrifuge machine at 1000-1200rpm for 5 min. transferred the butyrometer in the water bath at 65°C. then read the percentage fat by adjusting lower layer of fat column to zero mark.

3.8.1.2: Determination of Moisture in Butter: (IS 3507 (1966))

Moisture content of butter samples was determined by the method IS:3507 (1966) as described below.

Procedure: Clean the aluminium dishes were taken and dried in the oven maintained at 100°C for at least 1 hr. Allowed to cool to the room temperature in desiccators and weighed the dish. Accurately 3 to 4 gm of the butter sample were taken into each dish. Placed the dish on a steam-bath for at least 20 min, upon stirring at frequent intervals until no moisture can be seen at the bottom of the dish. Wiped the bottom of the dish and transferred to the oven maintained at $100 \pm 1^\circ\text{C}$ and kept it for 90 min. Allowed the dish to cool in the desiccator as before and weighed, Heated the dish again in the oven for 30 min. Repeated the process of heating, cooling and weighing until the difference between two consecutive weights did not exceed 0.1 mg. Recorded the lowest weight. Preserved the residue for the determination of curd.

$$\text{Moisture (\%weight)} = 100 \times (W_1 - W_2) / W$$

Where W_1 = weight in gm of the dish with the sample before heating

W_2 = weight in gm of the dish with the sample after heating

W = weight in gm of sample.

3.8.1.3: Free Fatty Acid Value of Butter:

The free fatty acids (FFA) content of butter was determined as per the method of AOAC (1995) with some modification

Procedure: The sample of butter were placed in a 50°C water bath for 8 min. the samples were centrifuged for 12 min at 3000rpm at 50°C. The top oil layers were pipetted off and combined. An aliquot of oil (5 gm) was placed into 250 mL flasks. 50 mL of neutralized alcohol and 1mL of indicator were added to the flask. The solution was titrated with 0.05N NaOH until a faint pink colour was maintained for 1 min.

Free fatty acid value (mg of NaOH/gm of oil) = (mL of NaOH × Normality of NaOH × 40)/gm of oil.

3.8.1.4: Peroxide Value of Butter:

Peroxide value was estimated by a protocol from (Chen *et al.*, 2004) with some modification.

Procedure: The samples of butter were placed in a 50°C water bath for 8 min. the samples were centrifuged for 12 min at 3000rpm at 50°C. The top oil layers were pipetted off and combined. An aliquot of oil (1 gm) was placed into each of three 250 mL Erlenmeyer flasks. 20 mL of 3:2 acetic acid: chloroform and 0.5 mL of saturated potassium iodide solution was added. Boiled the sample for 30 sec. then added 30 mL of deionized water. The flasks were titrated with 0.01N sodium thiosulfate until disappearance of yellow colour. Starch solution (1%) was added to the flask (0.5 mL). The titration was continued until the blue colour disappeared.

The PV (mEq of peroxide/kg of oil) = (mL of sodium thiosulfate) × (molarity of sodium thiosulfate) × 1,000/ (sample weight in gm).

3.8.1.5: Colour (L* a* b*) Value of Butter and Ghee

The colour parameters of butter and ghee samples were derived using image analysis. The samples taken into sterile petridish (50 mm X 15 mm), placed on the flat bed of the scanner (Canoscan 9000F mark II) and the scanned images of the samples were acquired following the protocol standardized by Wasnik *et. al.*, (2017). The images were imported to Adobe Photoshop and the

lightness (L) and chromatic parameters (a and b) of the image were obtained through the histogram method in Lab mode as described in Yam and Papadakis, (2004). The value obtained was converted in terms of CIELAB, lightness (L*) ranging from zero (black) to 100 (white), a* ranging from +60 (red) to -60 (green) and b* ranging from +60 (yellow) to -60 (blue) using Eq. 1 to 3. These values were used to derive colour descriptors like yellowness index by using Eq. 4 (Pathare *et. al.*, 2013).

Table 3.1: Colour Parameters Derived from Image Analysis

Colour parameter	Formula	Equation No.
L* (lightness)	$L^* = 100 \times L / 255$	1
a*	$a^* = (240 \times a / 255) - 120$	2
b*	$b^* = (240 \times b / 255) - 120$	3
Yellowness index (YI)	$YI = 142.86 \times b^* / L^*$	4

3.8.1.6: Measurement of pH

Procedure: Digital p^H meter was used to measure pH of the samples during production, formulation and shelf-life study. The electrode assembly was calibrated against standard buffer of pH 9.2, 7.0 and 4.0 before it was used for estimation of pH of the samples.

3.8.1.7: Textural Analysis of Butter Samples

Hardness and fracturability of the particulate fraction were determined using Texture Analyser by the method of Meullenet *et al.*, (2002).

The firmness and stickiness value of the representative particulates of the butter samples were determined using the wire cutter probe. The probe was calibrated to the distance of 50 mm, from the platform of the machine. The samples for measurement (1 Cube) were placed at the centre of the platform. The computer integrated with the analyser was allowed to execute the program to run a test, where in the sample was cut by the wire cutter and the return back was sensed by the machine giving a peak force-time curve. A TA-XT Plus setting for analysis of the butter samples is represented in (Table 3.2). and the texture

descriptors, namely firmness and stickiness of the samples were determined. The textural parameters were worked out from the force-time curve and expressed as Newtons thus obtained for each sample with force experienced by the probe on Y-axis and time on X-axis.

Table 3.2. TA-XT Plus Setting for Analysis of the Butter Samples

Mode	Measure Force in Compression
Option	Return to Start
Pre-Test Speed	1.0 mm/s
Test Speed	0.5 mm/s
Post-Test Speed	10 mm/s
Distance	10 mm
Trigger Type	Auto (Force)
Trigger force	0.049 N

Firmness: The maximum positive peak force value was referred to as the firmness and is expressed in Newtons (N).

Stickiness: The maximum negative peak force which indicated the stickiness and expressed in Newtons (N).

Work of shear: Correspondingly, the area under the penetration cycle (down stroke) in the force distance curve represented the amount of energy required to perform the shearing process, which is referred as ‘work of shear’ (N.s).

Work of adhesion: Area of negative peak force i.e., energy required to remove the probe (N.s).

3.8.1.8: Total Phenolic Content

Determination of total polyphenol content was done by Folin Ciocalteu method as described by (Wang *et al.*, 2014) with slight modification.

Procedure: Standard curve was prepared by making different concentrations of 0 µg, 25 µg, 50 µg, 75 µg, 100 µg and 125 µg. Exactly 500 µL of each concentration to the corresponding test tube were added. Then freshly made 2.5

mL of Folin's reagent was added to each test tube. After addition of 2 mL of Na₂CO₃ and vortexed the solutions and incubated the test tubes in dark for 30 min at room temperature and then spectral absorbance at 760 nm was recorded.

Sample (1 gm) was extracted with 10 mL of Methanol: Water (80:20) for 1 hr with occasional stirring using a vortex mixer. The extract was centrifuged at 4000 rpm. Bioactive compounds such as phenolic compounds were determined in the supernatant.

3.8.1.9: Estimation of Antioxidant Property of Ghee by DPPH Model System

The capacity of antioxidants to quench DPPH radical in ghee was determined as per the procedure described by Epsin *et al.*, (2000) with slight modification.

Procedure: The samples of butter were placed in a 50°C water bath for 8 min. the samples were centrifuged for 12 min at 4000 rpm at 50°C. The top oil layers were pipetted off and combined. An aliquot of oil 0.2 mL was added to 3.8 mL of ethyl acetate to obtain 4 mL mixture. Then 2 mL DPPH solution of 0.1 mM was added. After 10 min absorbance was measured at 517 nm using spectrophotometer. The control was prepared using 2 mL of DPPH solution and 4 mL ethyl acetate. Percent radical scavenging activity was calculated using the formula.

% Radical Scavenging Activity (RSA) or % Inhibition = {(Control OD - Sample OD)/ Control OD}*100

3.8.1.10: Microbiological Analysis:

Microbial analysis of butter was determined as per the method of AOAC (1995).

Sample Preparation:

Prepared butter samples were analysed for total bacterial count, Coliform count, yeast and mould count in every 10 days interval. Phosphate buffer was used for the serial dilution. 11 gm butter sample was taken into a sample bottle and dissolved with 99 mL of phosphate buffer.

Total Plate Count of Butter:

Procedure: Prepared 1st, 2nd & 3rd dilution sample for determination of total plate count. Transferred 1 mL of these serial dilutions to duplicate Petri dish. Then 10-

15 mL of melted and cooled standard plate count agar was poured in the plates. Then content of the Petri dishes was mixed and allowed to cool and solidify. Then the plates were covered and incubated the plates at 37°C for 24-48 hr.

Coliform Count of Butter:

Procedure: For determination of coliform count in butter, sample was prepared as 1st, 2nd & 3rd dilutions. Then transferred 1 mL of this dilution to Petri dish. Then 10-15 mL of melted and cooled standard plate count agar was poured in the plates. Then content of the Petri dishes was mixed and allowed to cool and solidify. Then the plates were covered and incubated the plates at 37°C for 24-48 hr.

Yeast and Moulds Count of Butter:

Procedure: For determination of Yeast and moulds count in butter samples. Transferred 1 mL of 1st & 2nd dilution in to Petri dish and poured 10-15 mL of melted and cooled PDA agar to pH 3.5 by adding calculate amount of tartaric acid solution (10%), mixed the content in Petri dishes. Allowed to cool and solidify. Then poured another layer of 10-12 mL of same agar and spread evenly to cover the first layer. Then the plates were allowed to cool and solidify, then covered and incubated the plates at 25°C for 2-3 days.

3.9: Method of Preparation of Ghee (Creamery Butter Method):

The ghee samples were prepared by using creamery butter method as depicted in Fig. 3.4.

3.9.1: Determination of Chemical Analysis and Physico-Chemical Constants

The physico-chemical constants such as Reichert-Meissl (RM) value, Polenske value (PV) and Butyro-refractometer reading of above said samples were determined by the methods IS:3508 (1966). The details of the procedures for these physico-chemical constants are as follows:

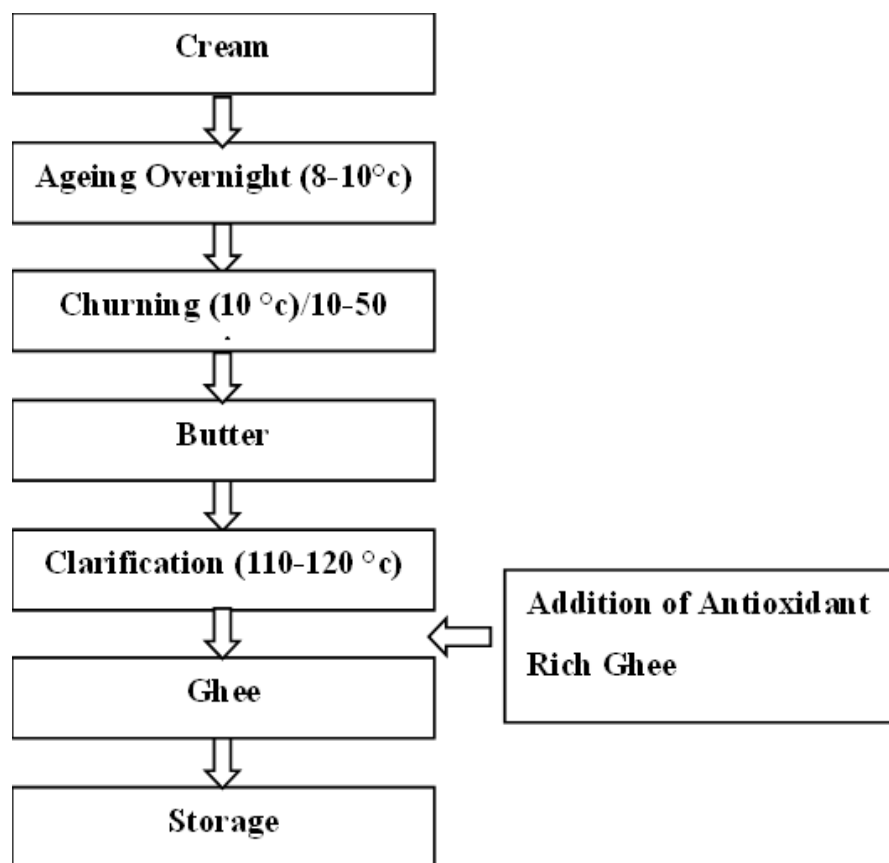


Fig. 3.4: Flow-Chart for Preparation of Ghee (Creamery Butter Method)

3.9.1.1: Determination of Moisture Content

Moisture content of ghee samples was determined by the methods as described in IS:3508 (1966). The details of the procedure are as follows:

Procedure: About 5 gm of the ghee sample was weighed into a moisture dish which has been dried previously, cooled in desiccators and then weighed. The dish was then placed in the hot air oven for approximately one hour at $105 \pm 1^\circ\text{C}$. The dish was then removed from the oven, cooled in desiccators to room temperature and weighed. The procedure of heating at 105°C for 30 min, cooling and weighing was repeated until the difference between the two successive weighing did not exceed 1 mg. The moisture content was calculated by the following formula and by the difference fat content in the ghee samples were calculated.

$$\text{Moisture content (\% by weight)} = 100 * (W_1 - W_2) / W$$

Where; W_1 =Weight in gm of the dish with ghee before drying,

W_2 = Weight in gm of the dish with ghee after drying, and

W =Weight in gm of sample

3.9.1.2: Estimation of Free Fatty Acid Content

Free fatty acid (FFA) levels, expressed as % oleic acid, of ghee samples were determined by the method IS:3508 (1966), which is described below:

Procedure: Exactly 5 gm of ghee sample was accurately weighed in a 250 mL conical flask. 50 mL of neutralized alcohol (at 70°C) was added into the flask containing ghee. The contents were boiled on a boiling water bath. The solution, while hot, was titrated against 0.1N sodium hydroxide solution, shaking vigorously during titration. The end point of the titration was perceived when the addition of a single drop produced a slight but definite colour change (pink colour) for at least 15 sec. The FFA levels were expressed as per percent oleic acid.

$$\text{FFA (\% oleic acid)} = (2.82 \times V) / W$$

Where; V = volume (mL) of NaOH required for titration of sample

W = weight in gm of the sample

3.9.1.3: Determination of Butyro-Refractometer (BR) Reading

Butyro-Refractometer (BR) reading of ghee samples were determined by the method as described in IS:3508 (1966).

Procedure: Before determining the BR reading of a sample, prism of the instrument was cleaned by ethyl alcohol and dried with tissue paper. The zero point of the instrument was made with the distilled water and temperature was set to 40°C. A drop of the molten fat sample was placed on the prism such that it covers the prism surface evenly and BR reading of the fat was recorded.

3.9.1.4: Determination of Saponification Value

Saponification value of ghee samples was determined by the method IS:3508 (1966) described below.

Procedure: Weighed accurately 2 gm of ghee into a flask. Accurately added 25 mL by measuring of the alcoholic potassium hydroxide solution. Then added 1 or 2 glass beads and boiled continuously under a reflux condenser for half an hr to 1

hr swirling the contents at the frequent intervals determined the excess of alkali while the solution was still hot by titration with 0.5N hydrochloric acid using 0.5 mL of phenolphthalein indicator. Made a blank determination upon the same quantity of the potassium hydroxide solution at the same time under the same conditions.

$$\text{Saponification value} = 28.05 (T_2 - T_1) / W$$

Where; T_2 = volume in mL of 0.5N acid required for the blank,

T_1 = volume in mL of 0.5N add required for the sample and

W = weight in gm of the sample taken.

3.9.1.5: Determination of Reichert Meissl (RM value) and Polenske Value

Reichert Meissl (RM) value of ghee samples was determined by the method IS:3508 (1966) as described below.

Procedure: Accurately 5 gm of sample was weighed in a Polenske flask and then saponified with 20 gm of glycerol and 2 mL of 50% (w/w) sodium hydroxide solution on a direct flame. Then 93 mL of freshly boiled distilled water were added followed by 50 mL of dilute sulphuric acid. The flask was immediately connected with the distillation apparatus and 110 mL of the distillate was collected within 21 min. The flask was replaced with 25 mL cylinder and the flame was removed. The collected distillate was cooled in a water bath maintained at 15°C for 10 min. Then, it was filtered through a dry Whatman No.4 filter paper and 100 mL of the filtered distillate were titrated against 0.1N sodium hydroxide solution using phenolphthalein as an indicator. Similarly, a blank test was also done by using all reagents in similar fashion. From this, the RM value was calculated as follows:

$$\text{Reichert-Meissl Value} = 1.10 (T_1 - T_2).$$

Where; T_1 = Volume (mL) of 0.1N NaOH solution used for sample titration

T_2 = Volume (mL) of 0.1N NaOH solution used for blank titration

For Polenske value (PV), the condenser, 25 mL cylinder, 110 mL flask and the filter paper were washed with three successive washings of 15 mL portions of cold water and discarded, followed by three successive washings of 15 mL with

neutralized alcohol. The washings with neutralized alcohol were collected and then titrated against 0.1N sodium hydroxide solution using phenolphthalein as an indicator. Similarly, a blank was also done. From this, the Polenske value was calculated as follows:

$$\text{Polenske Value} = T_3 - T_4$$

Where; T_3 = Volume (mL) of 0.1N NaOH solution used for sample titration

T_4 = Volume (mL) of 0.1N NaOH solution used for blank titration

3.9.1.6: Determination of Iodine Value

Iodine value of ghee samples was determined by the method IS:3508 (1966) as described below.

Procedure: Accurately 0.4 to 0.45 gm of ghee sample was weighed in a clean dried Erlenmeyer flask. To this 20 mL cyclohexane (Cyclohexane seems to solubilize the fat) was added and swirled to dissolve the sample. By means of a burette exactly 25 mL of the Wijs' reagent was added. Closed the flask with its stopper, mixed carefully and leave it standing for 1 hour in the dark. At the end of the hold, added 20 mL potassium iodide solution. Then immediately added approximately 150 mL of distilled water, swirl lightly to mix, and then promptly titrated with vigorous shaking then titrated with 0.1N sodium thiosulphate solution using 50 mL burette. Continued titration until the yellow brown colour almost disappeared, then added 1-2 mL starch indicator and continued to titrate until the blue colour just disappeared. Addition of water forces the fat into the cyclohexane and the excess iodine monochloride moves into the water, and can be titrated with sodium thiosulfate. Potassium iodide solution converts the excess iodine monochloride to free iodine (blue) which was titrated to a colourless end point with sodium thiosulfate. Recorded the volume of sodium thiosulfate used in the titration. Carried out a blank test, using the same quantities of the reagents. Calculated the iodine value by means of the following formula

$$\text{Iodine value} = 12.69 (a - b) N / W$$

Where; a = Volume in mL of 0.1N sodium thiosulfate used in the blank

b = Volume in mL of 0.1N sodium thiosulfate used in the ghee sample

N = Normality of sodium thiosulfate

W = Weight in gm of ghee sample taken for analysis

3.9.1.7: Determination of Peroxide Value:

Peroxide value of ghee samples was determined by the method IS:3508 (1966) as described below with slight modification.

Procedure: Weight 1 gm of sample of ghee in a 250 mL glass stoppered conical flask and then add 20 mL of the Glacial acetic acid-chloroform solution. Swirl the flask until the sample is dissolved. Add 0.5 mL of saturated potassium iodide solution. Allow the solution for heating exactly 1 min. with occasional shaking and then add 30 mL of distilled water. Add 0.5 mL of starch solution and continue titration using 0.01N sodium thiosulphate solution till the blue colour just disappear. Conduct a blank determination of the reagents in the same way. The titration in blank determination should not exceed 0.1 mL of the 0.1N sodium thiosulphate solution.

Peroxide value as mEq/Kg sample = $(S-B) \times N \times 1000 / \text{gm of sample}$

Where, S = volume in mL of sodium thiosulphate solution used for sample.

B = volume in mL of the sodium thiosulphate solution used for blank

N = normality of the sodium thiosulphate solution and

g = weight in gm of the sample.

3.9.1.8: Total phenolic Content

Determination of total polyphenol content was done by Folin Ciocalteu method as described by (Wang *et al.*, 2014) with slight modification.

Procedure: Standard curve was prepared by making different concentrations of 0 µg, 25 µg, 50 µg, 75 µg, 100 µg and 125 µg. Add 500 µL of each concentration to the corresponding test tube. Add freshly made 2.5 mL of Folin's reagent to each test tube. Add 2 mL of Na₂CO₃ and vortex the solutions. Incubate the test tubes in dark for 30min at room temperature. Take the absorbance at 760 nm.

Sample (1 gm) were extracted with 10 mL of Methanol: water (80:20) for 1 hr with occasional stirring using a vortex mixer. The extract was centrifuged at 4000 rpm. Bioactive compounds such as phenolic compounds were determined in the supernatant.

3.9.1.9: Estimation of Antioxidant Property of Ghee by DPPH Model System

The capacity of antioxidants to quench DPPH radical in ghee was determined as per the procedure described by Epsin *et al.*, (2000) with slight modification.

Procedure: Ghee sample of 0.2 mL was added to 3.8 mL of ethyl acetate to obtain 4 mL mixture. 2 mL DPPH solution of 0.1 mM was added. After 10 min absorbance was measured at 517 nm using spectrophotometer. The Control was prepared using 2 mL of DPPH solution and 4 mL ethyl acetate. Percent radical scavenging activity was calculated.

% Radical Scavenging Activity (RSA) or % Inhibition = $\{(Control\ OD - Sample\ OD) / Control\ OD\} \times 100$

3.10 Statistical Analysis

The data obtained in the present study was subjected to one-way analysis of variance (ANOVA) for the significant difference in the samples via Duncan's Multiple Comparison test performed at 95% confidence interval with SPSS software and comparison between means was made by critical difference (CD) value. The results were expressed as means $\pm$ standard deviation.

Chapter-4

RESULTS

AND

DISCUSSIONS

RESULT AND DISCUSSION

The results obtained from different sets of experiments carried out according to the objectives mentioned in chapter 1 and the methods described in chapter 3 are discussed in this chapter.

4.1: Collection and Analysis of Cream Samples

As per the requirements, mixed cow milk cream samples were collected from the Experimental Dairy Plant of SRS of ICAR-NDRI, Bengaluru. The representative cream samples were analyzed for fat, solids-not-fat (SNF), and titratable acidity parameters as mentioned in the section 3.7.1. The average values of cream composition with respect to fat and SNF content are shown in (Table 4.1). As reported by Deosarkar *et al.*, (2016) and Jha (2021), the cream samples used in this work falls under the category of double cream (based on fat content) and the cream acidity value indicates that it is fresh cream samples.

Table 4.1: Composition of cream

Sr. No	Parameter	Values
1	Cream Fat (%)	62.74 $\pm$ 0.61
2	Cream SNF (%)	5.23 $\pm$ 0.18
3	Cream Acidity (% lactic acid)	0.12 $\pm$ 0.01

Note: Data are presented as means $\pm$ SD (n=3)

4.2: Extraction of Antioxidant-Rich Fraction from Natural Sources

4.2.1: Antioxidant-Rich Pigment Extraction from Microbial Source

The process of pigment extraction from the microbial source is shown in Fig. 4.1. The *Micrococcus* bacterial strain isolated from the soil sample was grown on nutrient agar plates is shown in part 1 (the right-side plate is experimental and the left side plate is control). the part 2 of the figure is an image of the stain grown in the nutrient broth (left side bottle is experimental and the right side one is control). The Petri plates and broth were subjected to morphological study by focusing under the light for examination of the external character of the organism. Further, the study was confirmed using microscopic

examination and Gram's staining. The result indicates that the isolated organism is *Micrococcus roseus*.



Fig. 4.1: Pigment Extraction Process from Microbial Source



Fig. 4.2: Ultrasound-Assisted Extraction (UAE) of Pigments from Carrot

In part 3, the extraction and recovery of the pigment are presented. The cells were initially ruptured by ultrasonication process for 5 min to recover the pigment components. The mixture was then centrifuged at refrigerate temperature at 4000 rpm for 10 min. pellets were collected and the freeze dried (part 4) and the dried powder was stored in an airtight glass bottle. The ultrasonication process resulted in the destruction of the cell membrane, thus it could have enhanced the release of antioxidant-rich pigment from the cells. It was noticed that the yield of the pigment was 0.5 to 1%. Care was taken to avoid the cross-contamination and aseptic condition was maintained during the entire process of extraction.

4.2.2 Antioxidant-Rich Pigment Extraction from Carrot

The process of antioxidant-rich pigment extraction from carrots is shown in Fig. 4.2. Fresh carrots (Arka Suraj Breed) was cleaned to remove dirt and dust (part 1). The carrots used for extraction were sliced (part 2) into fine threads and dried using an oven dryer under low intensity temperature followed by grinding in a laboratory grinder (part 3). The powder sample was packed in glass bottles. From the carrot powder samples, pigment extraction was carried out using the ultrasound-assisted extraction (UAE) method (part 4).

Since carotene is water-insoluble, most of the methods mentioned in the literature have used chemical solvents or other physical processes. But in the present study, a green process was adopted for the extraction process. Milk fat in the form of ghee was added to the carrot powder and Ultrasonicated. The extract was collected after centrifugation at 4000 rpm for 10 min for complete separation of the pigments and later stored in a glass bottle. The yield of the pigment extraction was found to be around 20-25%.

4.3: Optimization of Ultrasonication Assisted Extraction (UAE) with Ghee Using Response Surface Methodology

Response Surface Methodology (RSM) experiment was used to study the effect of selected process variables on the extraction of β -Carotene. Box-Behnken design (BBD), of the experimental design matrix was opted for this activity. The coded and actual values of three design factors are presented in (Table 4.2).

Table 4.2: Sets of Variables Selected in the Box Behnken Design (BBD)

Variables	Ultrasonic Power (P)			Time (T)			Carrot to oil Ratio		
Coded Value	-1	0	1	-1	0	1	-1	0	1
Actual Value	40	60	80	10	25	40	0.1	0.3	0.5

4.3.1: Effect of Carrot to oil Ratio

The UAE efficiency in using different carrot to oil ratios was compared in (Table 4.3). It represented that ultrasonicate could effectively increase the β -carotene enriched ghee. This can be attributed to the fact that the collapse of the cavitation bubbles caused by ultrasonicate will destroy the membrane that will release the extract into the surrounding medium. The concentration of extracted β -carotene from carrot to oil ratio increased from 1:10 to 5:10, while this increase was limited when the carrot to oil ratio subsequently rose up to 6:10, This may be due to the fact that the ghee became gradually more saturated so that the solubility of ghee decreased. Thus, a carrot to oil ratio of 5:10 was selected as the referenced optimum ratio for the experiments. Similarly, Goula *et al.*, (2017) and Li *et al.*, (2013) also found the same trends while extracting carotenoids in vegetable oils (solvent) from pomegranate and carrot wastes, respectively.

4.3.2: Effect of Extraction Time

When the time (factor 2) was increased from 10 to 40 minutes, the amount of β -carotene was raised as well. The level varied from 98.8 to 116.6 ppm in the experimental runs 16 and 11 (Table 4.3). This could be due to the extraction time lengthens, the contact time between the target compounds and the solvent lengthens, resulting in increased diffusion. Mirheli and Dinani (2018) reported that the longer the ultrasonic wave duration, the larger the alterations in the cellular structure of the plant tissue, this result in more cell wall breakage and increased porosity in the carrot structure, allowing for a higher β -carotene concentration. Similar observations are also reported by Sharayei, *et al.*, (2019).

Table 4.3: Optimization Table for β -carotene Extraction by UAE Using Ghee as Solvent

		Factor 1	Factor 2	Factor 3	Response 1
Std	Run	A: Power	B: Time	C: Concentration	Content
		Watt	Minute		Ppm
12	1	60	40	0.5	213.5 $\pm$ 0.12
14	2	60	25	0.3	117.62 $\pm$ 0.09
8	3	80	25	0.5	194.43 $\pm$ 0.13
10	4	60	40	0.1	64.47 $\pm$ 0.08
4	5	80	40	0.3	140.76 $\pm$ 0.07
18	6	60	25	0.3	117.62 $\pm$ 0.09
17	7	60	25	0.3	117.62 $\pm$ 0.09
19	8	60	25	0.3	117.62 $\pm$ 0.09
5	9	40	25	0.1	47.68 $\pm$ 0.16
13	10	60	25	0.3	117.62 $\pm$ 0.12
3	11	40	40	0.3	116.6 $\pm$ 0.10
16	12	60	25	0.3	117.62 $\pm$ 0.09
2	13	80	10	0.3	115.33 $\pm$ 0.13
6	14	80	25	0.1	60.65 $\pm$ 0.09
9	15	60	10	0.1	48.19 $\pm$ 0.15
1	16	40	10	0.3	98.8 $\pm$ 0.11
15	17	60	25	0.3	117.62 $\pm$ 0.09
7	18	40	25	0.5	205.36 $\pm$ 0.17
11	19	60	10	0.5	173.06 $\pm$ 0.14

Note: Data are presented as means $\pm$ SD (n=3)

4.3.3. Effect of Ultrasonic Power

The effect of ultrasonic power (factor 1) on the response of carotene content was studied to see the cavitation effect. When the power was increased from 40 to 80 W the response carotene content was increased up to the threshold value and then declined, as depicted in (Table 4.3). As per Chemat *et al.*, (2017) and Wen *et al.*, (2018), this is due to the increased intensity caused by ultrasonic waves, including cavitation, vibration, and turbulence, which caused

particle fragmentation, cell wall breaking, and increased rate mass transfer. V. Saez *et al.*, (2005) discovered that when the ultrasonic intensity rises, the yield of reaction increases up to the threshold value and then declines so as to get the optimal ultrasonic intensity. V. S. Sutkar *et al.*, (2009) reported that a rise in ultrasonic power could lead to increase cavitation effects and an optimum power should be selected for a single frequency operation. Therefore, based on these reports the ultrasonication power was optimized.

4.4: Yield of Extracted β -carotene Pigment

According to the UAE experiment RSM planning, 19 experimental points were randomly run; the coded values of independent variables and responses obtained in the multivariate study for each experiment are shown in (Table 4.3).

Table 4.4: ANOVA Table for UAE of β -carotene Using Ghee as Solvent

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	42056.18	9	4672.91	169.57	<0.0001	Significant
A-Power	228.23	1	228.23	8.28	0.018	
B-Time	1248.75	1	1248.75	45.31	<0.0001	
C-Conc.	39953.99	1	39953.99	1449.83	<0.0001	
AB	14.55	1	14.55	0.52	0.485	
AC	142.80	1	142.80	5.18	0.048	
BC	145.93	1	145.93	5.30	0.046	
A ²	6.87	1	6.87	0.24	0.629	
B ²	4.36	1	4.36	0.15	0.700	
C ²	299.13	1	299.13	10.85	0.009	
Residual	248.02	9	27.56			
Lack of Fit	248.02	3	82.67			
Pure Error	0.00	6	0.00			
Cor Total	42304.20	18				
R ²				0.99		
Adj R ²				0.98		
C.V. %				4.33		
Std. Dev.				5.25		
Adeq Pre.				44.36		

The responses varied widely from 47.68 to 213.5 mg of β -carotene per liter of ghee in function of 3 parameters setting of experiments. Using the optimized conditions, the highest extraction of β -carotene in ghee was 213.5 ppm of dry carrot powder. Nour *et al.*, (2018), reported that the extraction efficiency of β -carotene depended on factors including oil, their polarity, phospholipid presence in the oil, and chain length of fatty acids. Therefore, selected oil with low phospholipid and higher short chain fatty acids gave the best results. Li *et al.* (2012) conclude that UAE combined with sunflower oil as a green solvent was used to extract β -carotene from fresh carrots, which was about 334 mg/L after 20 min, as compared to conventional 321 mg/L after 60 min. Since in the present study maximum β -carotene was achieved in standard 12, run 1, accordingly, these 3 factors were adopted for the extraction of β -carotene content.

The significance and suitability of the design were then studied using variance analysis (ANOVA Table 4.4). With a confidence level of 95%, F-ratio significance could be evaluated using the p-value column. Six effects were found significant (typed in bold) at a 95% confidence level in this ANOVA table.

The Model F-value of 169.57 implies the model is significant. P-values less than 0.05 indicate model terms are significant. In this case, A, B, C, AC, BC and C^2 are significant model terms. Values greater than 0.1 indicate the model terms are not significant.

4.5: Graphical Representation of UAE Parameter on β -carotene Content

The Design-Expert software diagram obtained upon optimization of 3 factors versus the β -Carotene content is presented in Fig. 4.3. A, B and C represents the effect of ultrasonication power, time, and concentration level, respectively. From this graph, we conclude that the concentration shows the maximum effect on carotene extraction followed by the other two factors.

The three-dimensional response surface plots (Fig. 4.4) show the effect of independent variables on the extraction efficiency of β -carotene. Figure 4.4(A) indicates that the extraction efficiency was highly affected by the concentration and ultrasonication power. Fig. 4.4(B) shows that the linear increase in extraction efficiency of β -carotene is affected by time and ultrasonication power. Fig. 4.4(C) shows the interaction of the extraction time and concentration. It was found that

the maximum extraction yield was achieved when the extraction time was 40 min and the concentration was 0.5.

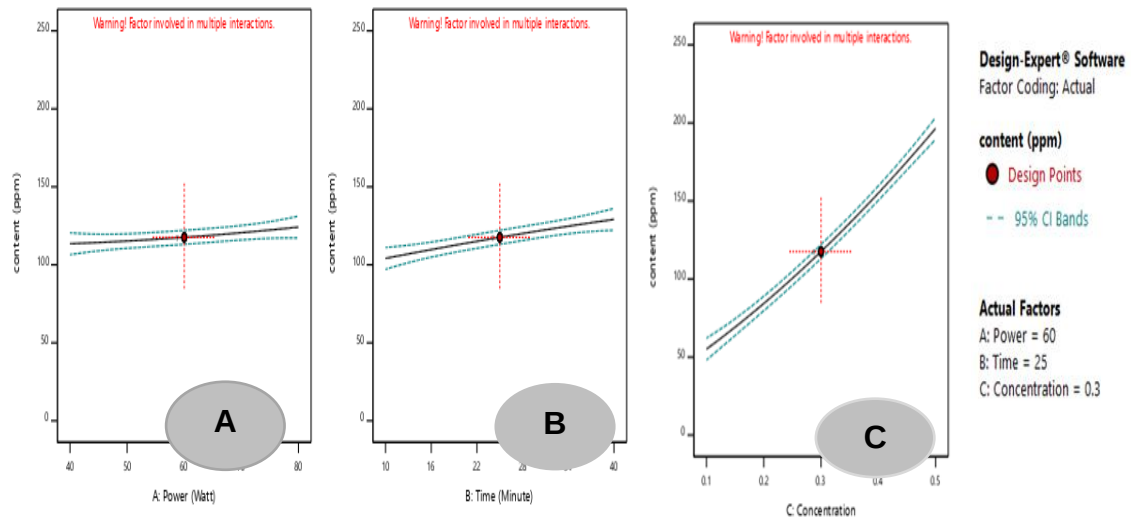


Fig. 4.3: RSM Graph (A) Effect of Power, (B) Effect of Time, and (C) Effect of Concentration Level on Extraction of β -carotene Content.

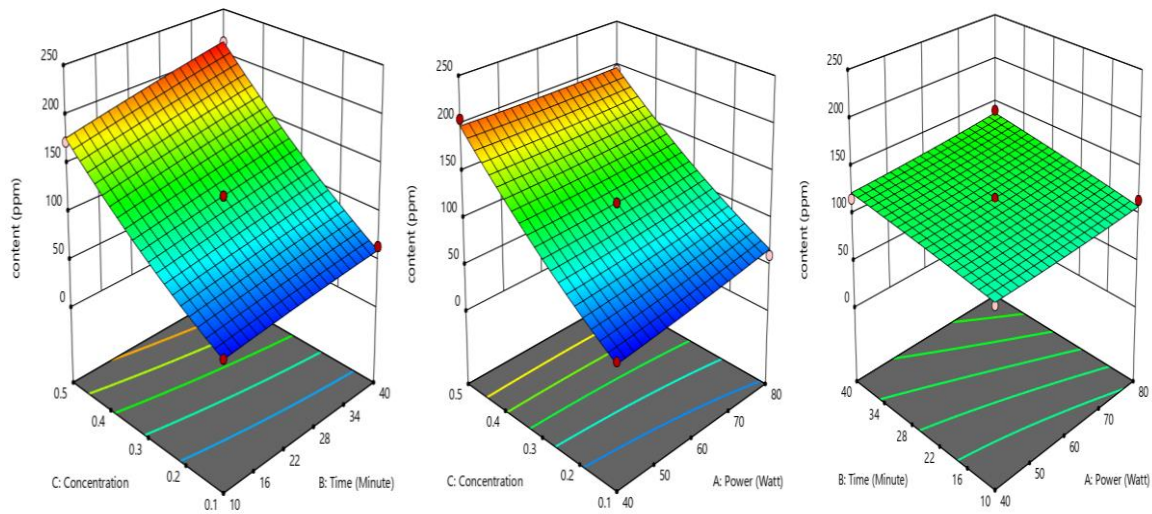


Fig. 4.4: RSM Graph (A) Effect of Time and Concentration, (B) Power and Concentration and (C) Power and Time on Extraction of β -carotene Content

4.6: Modelling and Validation

The quadratic polynomial equations fitted with all the experimental data explain the effect of parameters on β -carotene extraction and are presented in eq. (1).

Total β -Carotene (mg/L):

$$5.34A + 12.49B + 70.67C + 1.90AB - 5.975AC + 6.04BC + 1.23A^2 - 0.986B^2 + 8.17C^2 \dots (1)$$

Where, A= Power, B= Time and C= Carrot to oil ratio

The R^2 values obtained were 0.99 (Table 4.4) for the yield of carotene extraction using ghee of UAE process. Higher R^2 of the quadratic model indicates a good correlation between the input parameters and response variables. CV % values being less than 10% indicate good fitting of the model. The non-significant lack-of fit for responses indicated the validation of models. The model was highly significant ($p < 0.0001$ for ghee), which indicates that the model could explain the experimental data with high efficiency.

4.7: Standard Curve of β -carotene

A standard curve was prepared using 2 μg , 4 μg , 6 μg , 8 μg and 10 μg of dissolving standard β -carotene in cyclohexane and the absorption spectra of the β -carotene content were measured in a 1 cm optical path-length quartz cell using a spectrophotometer at 455 nm wavelength and the standard curve plotted is shown in Fig. 4.5.

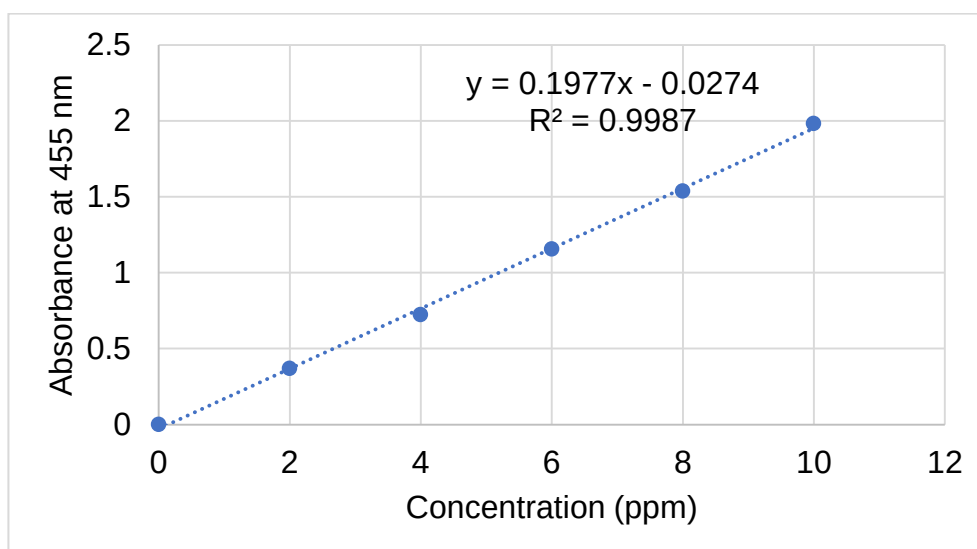


Fig. 4.5: Standard Curve of β -carotene

4.8: Physico-Chemical Analysis of Prepared Pigment Extract

In the present study, microbial pigment extract yield was not up to the mark and the yield was not sufficient to carry out the lab-scale production. Hence,

carotene extract was used as a source of antioxidant ingredient base material in the preparation of butter and ghee products.

Since ghee was used as a solvent, hence ghee sample was taken as a control and the carotene rich ghee extract (CRGE) samples were analyzed for the physico-chemical quality parameters including the β -carotene content and antioxidant activities (6 parameters) as per the standard protocol mentioned in chapter 3. The results of these parameters are presented in (Table 4.5) after statistical analysis using ANOVA.

Table 4.5: Physico-Chemical Analysis of Ghee and β -carotene Rich Ghee Extract (CRGE)

Sr. No	Parameter	Control ghee sample	carotene rich ghee extract
1	Moisture (%)	0.139 ± 0.01^a	0.142 ± 0.013^a
2	B.R Reading	42 ± 00^a	42 ± 00^a
3	FFA (%oleic acid)	0.28 ± 0.01^a	0.24 ± 0.01^a
4	β -Carotene (mg/kg)	21.98 ± 0.10^a	213.5 ± 0.12^b
5	Antioxidant Activity (% RSA)	24.20 ± 0.82^a	56.76 ± 1.08^b
6	Colour values		
	L*	61.49 ± 0.19^a	54.80 ± 0.17^b
	a*	1.23 ± 0.06^a	8.10 ± 0.17^b
	b*	37.91 ± 0.13^a	56.59 ± 0.10^b

Note: Data are presented as means $\pm$ SD (n=3). The Identical Superscript (on same line) do not differ statically ($p > .05$), L*=lightness, a* = greenness, and b* = yellowness.

There was no significant difference with respect to the moisture content, B.R reading and the free fatty acid level among the control and optimized product extract (Table 4.5). Moisture content in ghee samples plays significance with respect to the keeping quality and sensory properties. Higher moisture may result in faster spoilage related to the hydrolytic rancidity. The optical property; refractive index is characteristic of the particular liquid which can be converted to Butyro-Refractometer (BR) reading. It signifies that the degree of bending of light

waves upon passing through the liquid or transparent solids. The BR value is a basic value that relates to molecular weight, fatty acid, chain length, the degree of unsaturation and the degree of conjugation (Jha, 2021). Free fatty acids (FFA) are produced by the hydrolysis of oils and fats.

The level of FFA in ghee depends on time, temperature and moisture content because ghee is exposed to various environments such as storage, processing, heating or frying. The value is a measure of the type of reaction leading to the splitting of glycerides, such as hydrolytic rancidity, and can be used to follow the progress of such rancidity in milk fat products. Further, the reported values on moisture, BR and FFA contents of the extract are within the FSSAI and AGMARK specifications.

With respect to the β -carotene content, there was, a significant increase (10 times higher) was noticed in the prepared extract base sample as compared to that of the control sample. Similarly, the antioxidant activity was also found to be 2.5 times higher than the control samples. It is evident that the increased antioxidant property is attributed to the β -carotene content in the prepared sample. Studies show that β -carotene is a strong antioxidant and also a scavenger of singlet oxygen. Hence it can be concluded that the addition of the β -carotene pigment extract has resulted in significant increase in the antioxidant activity of the prepared ghee.

The colour of the ghee largely depends on the quality of milk and the temperature as well as the time of post-clarification. Milk collected from the green fodder, and open grazing animals may result an increased quantity of beta carotene (Nandini. 2019). With respect to the colour value of the prepared samples, the L^* value (lightness) was significantly reduced while the a^* (greenness), and b^* values (yellowness) were found to be enhanced due to the incorporation of pigment components. Similar observations are reported by Jha (2021); hence our findings are in agreement with the reported literature.

4.9: Development of Protocol for Manufacture of Ghee with Added Antioxidant

4.9.1: Addition of Natural Antioxidant According to Stage and Concentration

Adding β -carotene enriched ghee extract before the clarification of ghee at the higher temperature of 110-120°C (Clarification temperature) may reduce the β -carotene content in ghee due to their destruction. To avoid this destruction, it was added directly after the clarification step. The work was done to decide on the β -carotene to be added to ghee, based on its effect on sensory, colour enhancement and antioxidant activity. The Sensory scores of ghee are depicted in Fig. 4.6. The score obtained ranges from 87.81 to 93.62.

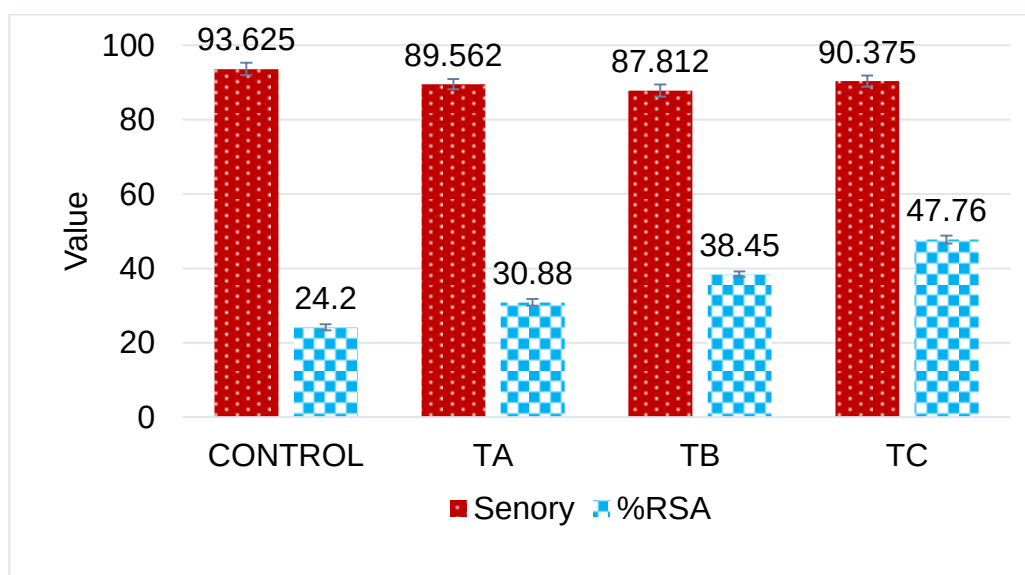


Fig. 4.6: Effect of Different Concentration of β -carotene on Sensory Score and %RSA (Control, TA= Trial A, TB= Trial-B and TC= Trial C).

4.9.2: Effect of Different Concentration of β -carotene Added in Ghee and Its Effect on DPPH Radical Scavenging Activity.

The antioxidant activity of the sample prepared was measured by the DPPH method and results were reported in terms of % radical scavenging activity. The inhibition process of the autoxidation in lipids by antioxidants is linked to the ability of antioxidants to break the radical formation reaction. The DPPH radical scavenging assay is the most accepted method to primarily evaluate the free radical scavenging activity. In the present study, the ability to

trap the DPPH radicals of ghee samples treated with natural antioxidants ranged from 30.88 to 47.76% (Fig. 4.6). The radical scavenging activity for control ghee was 24.20%. The sample which showed maximum radical scavenging ability was Trial C was 47.76%. The minimum radical scavenging activity shown by sample Trial A was 30.88%. From this plot, it is evident that increase in the concentration of carotene had positive effect on the radical scavenging activity. In other words, with the increase in carotene concentration, an increase in antioxidant activity was observed. Findings of this study was in agreement to Asouri *et al.*, (2013), the author reported that concentration of antioxidant and radical scavenging activity is directly proportional to each other.

4.10: Physicochemical Constants Analysis of Control and Optimized Ghee

The compositional profile of the milk product is the major factor that determines the nutritional content, physicochemical attribute and shelf life of the product. Fats and oils exhibit certain physical and chemical characteristics as quality and purity indicators, which have long been employed for their characterization and differentiation. Therefore, some of its characteristics like RM value and BR reading find a place in the FSSAI standards. In addition to these two characteristics, AGMARK has specifications for PV also in its standards for ghee.

Table 4.6: Physico-Chemical Analysis of control and Optimized Ghee.

Parameters	Control ghee	Optimized ghee
Moisture (%)	0.138 ± 0.01 ^a	0.140 ± 0.02 ^a
Fat content (%)	99.86 ± 0.012 ^a	99.85 ± 0.02 ^a
Free fatty acid (% oleic acid)	0.28 ± 0.014 ^a	0.237 ± 0.012 ^a
BR reading @40 ^o C	42 ± 0 ^a	42 ± 0 ^a
Reichert Miessl value	28.68 ± 0.12 ^a	28.3 ± 0.12 ^a
Polenske value	1.07 ± 0.04 ^a	1.08 ± 0.07 ^a
Saponification Value	222.73 ± 0.15 ^a	222.51 ± 0.12 ^a
Iodine value	33.86 ± 0.12 ^a	33.66 ± 0.11 ^a

Note: Data are present as means ± Sd (n=3). The identical Superscript on the same line indicates that they do not differ statically (p > 0.05).

Therefore, to ascertain the effect of the β -carotene on the composition and quality of optimized ghee was studied in comparison to the control ghee. The results of different tests are reported in (Table 4.6). The moisture and fat content of control ghee was $0.138 \pm 0.01\%$ and $99.86 \pm 0.01\%$ respectively and for optimized ghee it was $0.140 \pm 0.02\%$ and $99.85 \pm 0.02\%$ respectively. The study indicates that the addition of β -carotene does not affect the composition of the product.

Similar observations were noticed for the various physicochemical constants of ghee. It is noticed that the free fatty acid level in the optimized ghee product is slightly lower than the control ghee. The antioxidant activity of β -carotene might have contributed to lowering the free fatty acid level. Further, the reported values are within the FSSAI and AGMARK specifications and the control ghee values are matching to the report of Sindhu *et al.*, (2000), Sharma, (1981) and Nandini (2019). Since there is no significant difference in the composition and physico-chemical constants of the prepared, it can be concluded that the method of addition of the β -carotene will not have an effect on the quality of ghee.

4.11: Effect of Storage Study on Control and Optimized Ghee

The Storage study was evaluated for control ghee and natural antioxidant added ghee by accelerated storage study method. The optimized ghee sample and control ghee sample was prepared and packed in a glass jar. The samples were incubated at a BOD Incubator maintained at 80°C . Sensory analysis, FFA value, Peroxide value, colour value and antioxidant activity were done in an interval of 7 days and result was depicted below.

4.11.1: Effect of Storage on FFA Value of Control and Optimized Ghee

The increasing FFA value in both control and optimized ghee samples has shown an increase with the storage period at accelerated condition due to the hydrolytic decomposition of fat into free fatty acids. The FFA value of control and optimized ghee was depicted in Fig. 4.7. The β -carotene added ghee was lower in FFA value during the storage period and its increment was much lower than the control ghee. The β -carotene along with the total phenolic content might have contributed to the lower FFA content in the prepared sample as compared to that of the control sample. Similar observations are reported by Jha (2021).

4.11.2: Effect of Storage on Peroxide Value in Control and Optimized Ghee

The peroxide value of both control and optimized ghee samples has shown to increase with the storage period at accelerated condition due to oxidative rancidity of fat (Fig. 4.8). Hydroperoxides are the main product of lipid oxidation. The optimized ghee shows a low peroxide value compared to the control ghee owing to the antioxidant activity of β -carotene and it was depicted in Fig. 4.8. Burton and Ingold (1984) concluded that carotenoids, a strong antioxidant that can prevent vegetable oils from being oxidized. Therefore, our findings are in accordance with the reported literature.

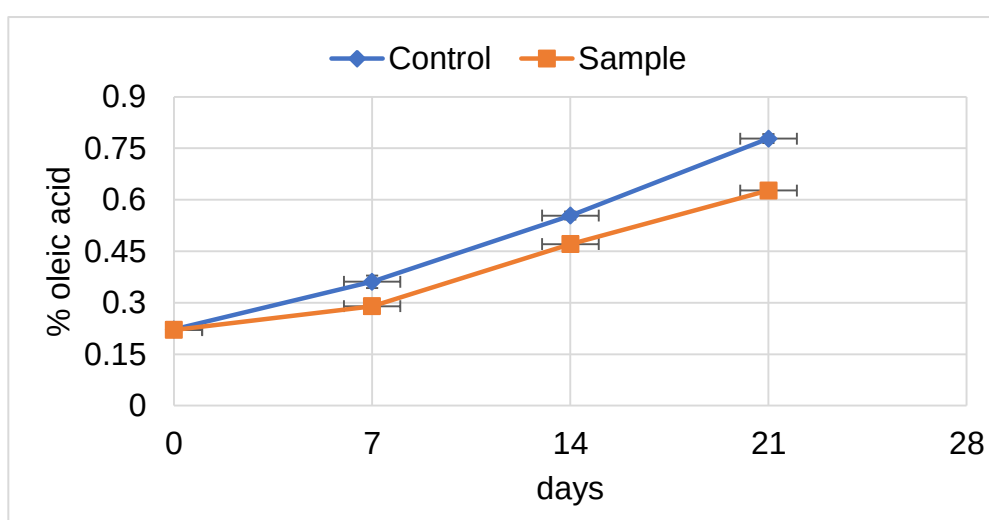


Fig. 4.7: Comparison of Control and Optimized Ghee on Free Fatty Acid During Storage of Ghee at 80°C.

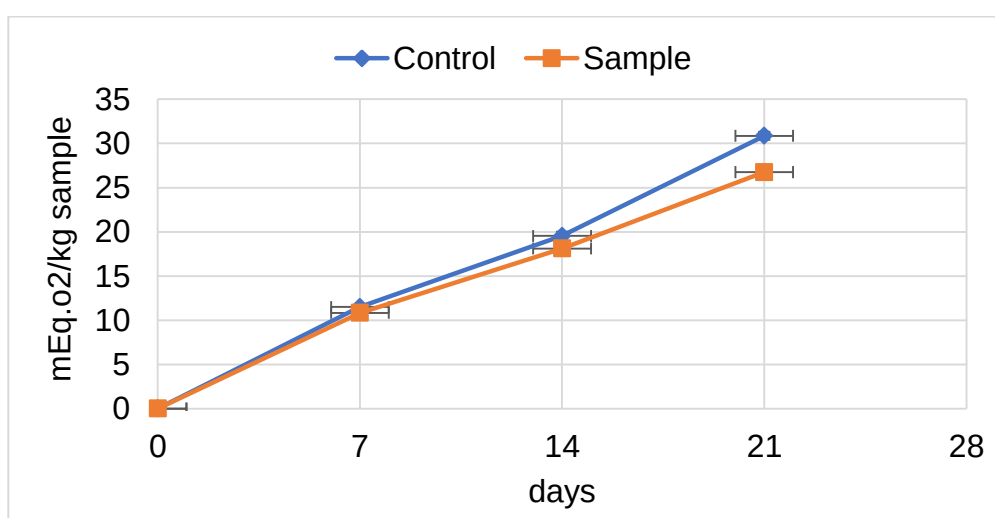


Fig. 4.8: Comparison of Control and Optimized Ghee on Peroxide Value During Storage of Ghee at 80°C.

4.11.3: Effect of Storage on %RSA of Control and Optimized Ghee

Antioxidant activity was assessed by the ability of added β -carotene to quench the free DPPH radicals. The extent of reduction in absorbance of DPPH determines the free radical scavenging potential of the antioxidant. The results were determined as radical scavenging activity (% inhibition). Antioxidant activity was done in an interval of 7 days. The effect of storage on the antioxidant activity of ghee is depicted in Fig. 4.9. The results show that the addition of β -carotene shows significant improvement in the antioxidant activity of ghee. Approximately two-fold increase is observed in the radical scavenging activity of natural antioxidant added cow ghee before storage at 80°C. After storage at 80°C for 21 days of both control and optimized ghee, the antioxidant activity of both ghee decreases but β -carotene added ghee had six times higher antioxidant activity than the control ghee. Hence it can be concluded that the addition of these β -carotene extracts significantly increased the antioxidant activity of the ghee. The reducing nature of the conjugated double bond system, the ring structure of terminal ends and the electron rich conjugated structure of polyene are responsible for antioxidant activities of carotenoids, which quenches singlet oxygen (Conn *et al.* 1991) and also scavenges radicals to stop chain reactions (Everett *et al.*, 1996). Hence it can be concluded that our findings are in accordance with the reported literature.

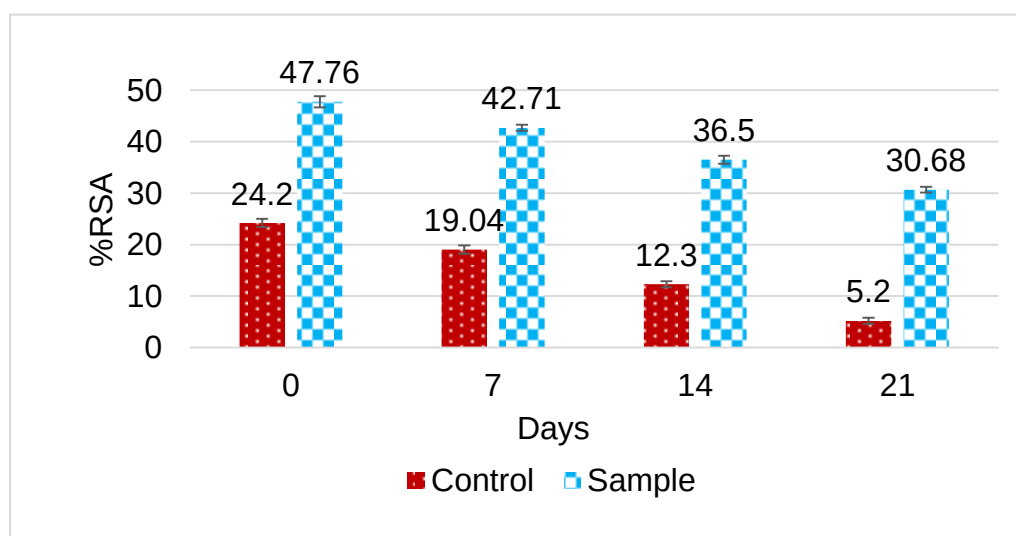


Fig. 4.9: Comparison of Antioxidant Activity of Control and Optimized Ghee During Storage at 80°C.

4.11.4: Effect of Storage on Colour of Control and Optimized Ghee at 80°C.

The color value of the control ghee and optimized ghee upon storage period at accelerated condition was measured using the image analysis method and profiled in terms of L^* , a^* , b^* and derived as yellowness index (YI).

The colour profile data is tabulated in (Table 4.7) and the effect of storage period on the YI value of control and optimized ghee is shown in Fig. 4.10. The b^* value of control and optimized ghee was 30.88 and 58.57 respectively from this we conclude that the increase in b^* value is due to the β -carotene content. Holstun, & Zetocha (1994) reported that the β -carotene present in prepared oil sample is responsible for the yellow colour of oil, which protects oil rich in unsaturated fatty acids from autoxidation.

Table 4.7: Changes in colour Value of Control and Optimized ghee During Storage at 80 °C.

Days	Sample	L^*	a^*	b^*
0 th	Control	59.27 $\pm$ 0.35	0.778 $\pm$ 0.10	30.88 $\pm$ 0.30
	Optimized	59.05 $\pm$ 0.168	0.398 $\pm$ 0.08	58.57 $\pm$ 0.18
7 th	Control	61.52 $\pm$ 0.00	1.678 $\pm$ 0.00	30.86 $\pm$ 0.05
	Optimized	60.96 $\pm$ 0.17	0.812 $\pm$ 0.03	38.68 $\pm$ 0.20
14 th	Control	61.39 $\pm$ 0.06	1.98 $\pm$ 0.01	35.08 $\pm$ 0.00
	Optimized	60.55 $\pm$ 0.02	1.345 $\pm$ 0.02	37.83 $\pm$ 0.02
21 th	Control	61.89 $\pm$ 0.50	2.05 $\pm$ 0.11	23.43 $\pm$ 0.09
	Optimized	62.23 $\pm$ 0.05	1.41 $\pm$ 0.00	32.95 $\pm$ 0.07

Note: Average of triplicate analysis

It was observed that the YI values recorded a decrease from an initial value of 81.63 to 54.08 and 141.7 to 75.63 for the control and optimized ghee sample respectively, over the storage period of 21 days at 80°C. The decrease in the YI value may be due to the loss of β -carotene during accelerated storage at 80°C.

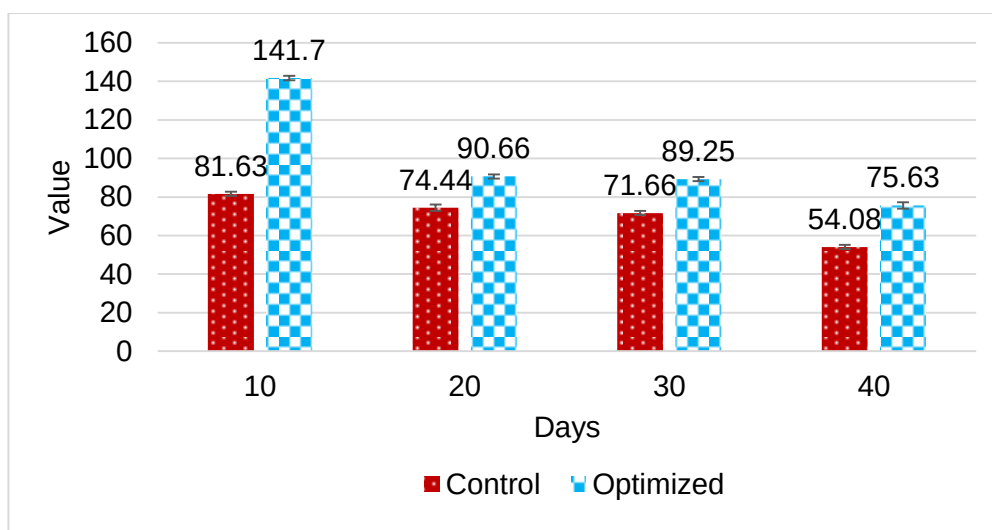


Fig. 4.10: Changes in YI value of Control and Optimized Ghee During Storage at 80°C.

4.11.5: Effect of Storage at 80°C on Sensory Score of Control and Optimized Ghee

Sensory analysis of control ghee and optimized ghee upon storage period at accelerated condition was analysed. The overall acceptability score on days 0 was almost similar for both samples. The result of sensory evaluation at day 0 to 21 days was depicted in Fig. 4.11. On 21st day, the sensory evaluation was not done for control ghee as it was already unacceptable. The Sensory score for optimized ghee on day 21st is found to decrease, but it is acceptable. The reason is ascribed to the effect of added β -carotene in optimized ghee samples.

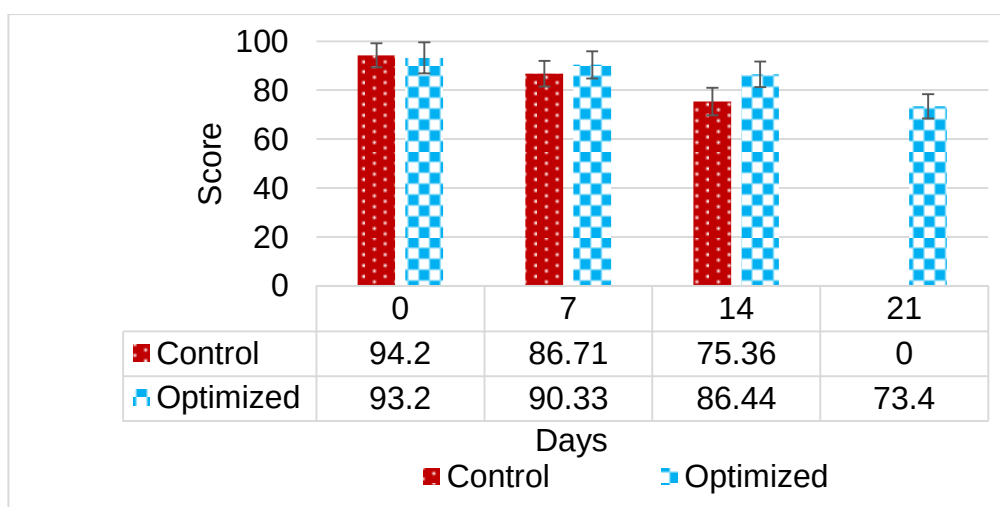


Fig. 4.11: Comparison of Sensory Analysis of Control and Optimized Ghee During Storage at 80°C.

4.11.6: Storage Stability Study: Rancimat Analysis

Rancimat analysis was carried out for 4-month-old optimized ghee samples and the control samples were stored at room temperature. Professional Rancimat (Model 892) was used in the accelerated study to measure the induction period. The result of the analysis is presented in Fig. 4.12, which depicts that the 4-month-old control ghee sample induction time was found to be 2.06 h while the optimized ghee sample was almost 2.1 times higher at 140°C upon recording the endpoint conductivity ($\mu\text{S}/\text{cm}$). Higher stability in the case of optimized ghee is due to the β -carotene and other polyphenol pigments, and our results are in agreement with the reports of Nandini (2019).

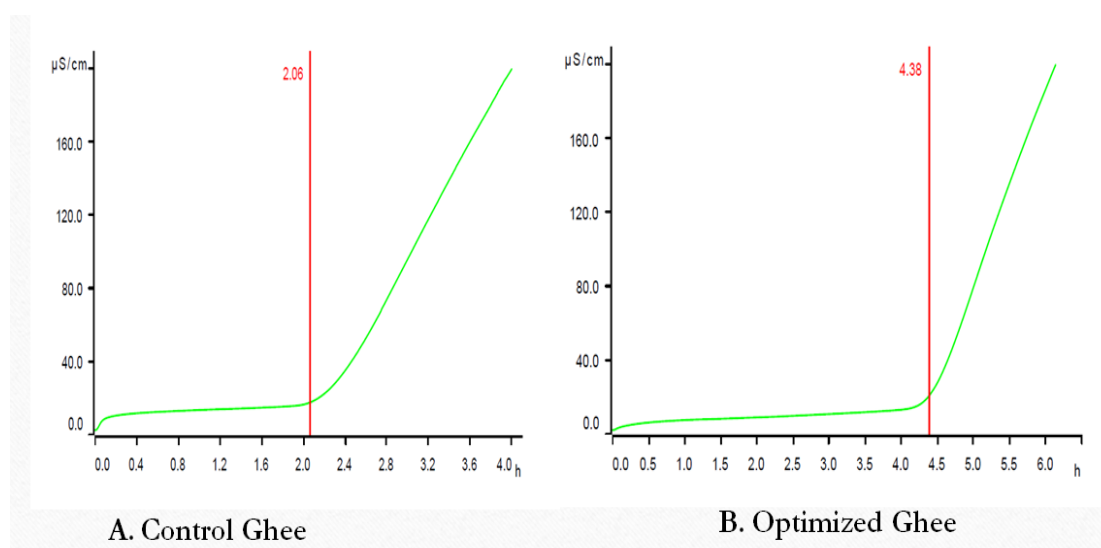


Fig. 4.12: Rancimat Analysis: Induction Period of Control Ghee (A) and Optimized Ghee Samples (B).

4.12: Development of Protocol for Manufacture of Butter with Added Antioxidant Rich Pigment Extract

4.12.1: Addition of Natural Antioxidants According to Concentration:

The β -carotene rich extract was added to the butter during the working stage, at three different concentrations. Control butter sample (without the addition of the extract) along with the three trial samples (Trial A, B and C) were subjected to the sensory evaluation.

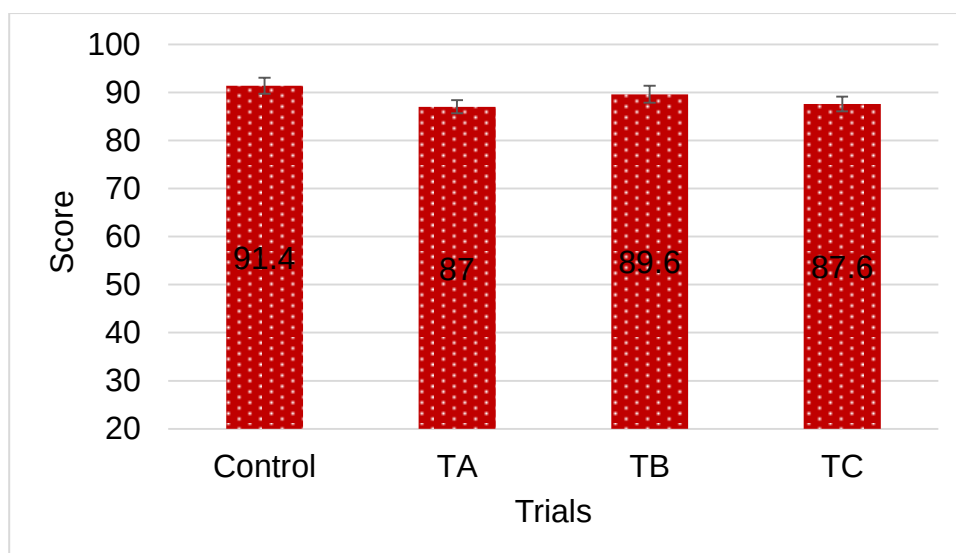


Fig. 4.13: Effect of Different Concentration of Adding β -carotene on Sensory Score (TA= Trial A, TB= Trial-B and TC= Trial C)

The sensory scores of these butter trials are depicted in Fig. 4.13. The score obtained range from 87 to 91.4. the control sample scored highest in which no β -carotene was added, the reason for the highest score of control butter was the indigenous diacetyl flavour of butter. Moreover, the ghee used in the extraction process as the solvent, has also resulted in softness. However, trial B appeared to be in close comparison with the control butter sample.

4.12.2: Addition of Antioxidant-Rich Extract: Stage of Addition

In the preliminary study, it was noticed the addition of extract in butter before the churning process resulted in the reduction of the β -carotene content through the buttermilk. Therefore, to avoid these losses, it was suggested to add β -carotene directly during the working step. The product was prepared as per the standard protocol and the sensory analysis was carried out and the result is depicted in Fig. 4.14. This study was done to decide the β -carotene to be added to butter, based on its effect on sensory, colour enhancement and antioxidant activity.

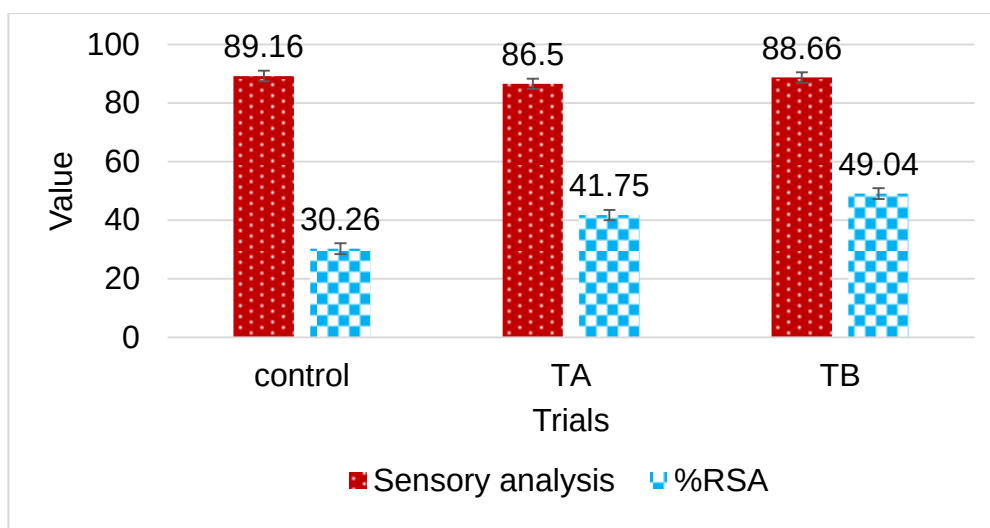


Fig. 4.14: Effect of Addition of β -carotene According to Stage on Sensory Score and %RSA (Control, TA= Trial A, TB= Trial B)

4.12.3: Effect of β -carotene Added in Butter and Its Effect on Sensory Analysis and DPPH Radical Scavenging Activity.

The DPPH radical scavenging assay is the most accepted method to primarily evaluate the free radical scavenging activity. The inhibition process of the autoxidation in lipids by antioxidants is linked to the ability of antioxidants to break the radical formation reaction. The antioxidant activity of the sample prepared was measured by the DPPH method and results were reported in terms of %RSA. Antioxidant activity of control butter was 30.26% and for optimized butter it came out to be 49.04%. The optimized butter which shown maximum radical scavenging ability than that of control butter which was depicted in Fig. 4.14. this may be due to the antioxidant activity of β -carotene.

4.12.4: Composition and Chemical Analysis of Control and Optimized Butter

To ascertain the effect of the β -carotene on the composition and quality of optimized butter was studied in comparison to the control butter. The results of different tests are reported in (Table 4.8). The moisture and fat content of control butter was 14.87% and 83.36% respectively and for optimized butter it was 14.91% and 83.42% respectively. The study indicates that the addition of β -carotene does not affect the composition of the product. The reported values in (Table 4.8) are within the FSSAI specifications. Since there is no significant the

difference in the composition and physico-chemical constants of the prepared, it can be concluded that the addition of the β -carotene will not have an effect on the quality of butter.

Table 4.8: Proximate Composition of Control and Optimized Butter

Sr. No	Parameter	Control Butter	Optimized Butter
1	Moisture (%)	14.87 $\pm$ 0.22 ^a	14.91 $\pm$ 0.15 ^a
2	Fat (%)	83.36 $\pm$ 0.18 ^a	83.42 $\pm$ 0.18 ^a
3	Curd (%)	1.68 $\pm$ 0.15 ^a	1.64 $\pm$ 0.15 ^a
4	FFA (% oleic acid)	0.148 $\pm$ 0.012 ^a	0.149 $\pm$ 0.017 ^a
5	Colour value		
	L*	79.32 $\pm$ 0.04 ^a	73.15 $\pm$ 0.04 ^b
	a*	-1.18 $\pm$ 0.00 ^a	-2.09 $\pm$ 0.30 ^b
	b*	13.63 $\pm$ 0.04 ^a	20.57 $\pm$ 0.08 ^b

Note: Data are present as means $\pm$ Sd (n=3). The identical letters on same line indicate that they do not differ statically (p > 0.05).

4.13: Effect of Storage Study on Control and Optimized Butter During Storage at Refrigeration Condition.

The storage study was evaluated for control butter and antioxidant rich extract added to butter during storage at refrigeration temperature. The optimized butter sample and control butter sample was prepared and packed in parchment paper and kept at refrigeration temperature of 4-6°C. The sensory analysis, FFA value, Peroxide Value, Colour value, PH value, TPA analysis and microbial analysis was done in an interval of 10 days and the result was depicted below.

4.13.1: Effect on Free Fatty Acid

The increasing FFA value in butter is due to the hydrolytic decomposition of fat into free fatty acids. The FFA value in control and optimized butter was depicted in Fig. 4.15. The β -carotene added butter resulted in a lower FFA value during the storage period and lower increment than the control butter. So, this analysis was shown that the preservative action of β -carotene in the butter sample.

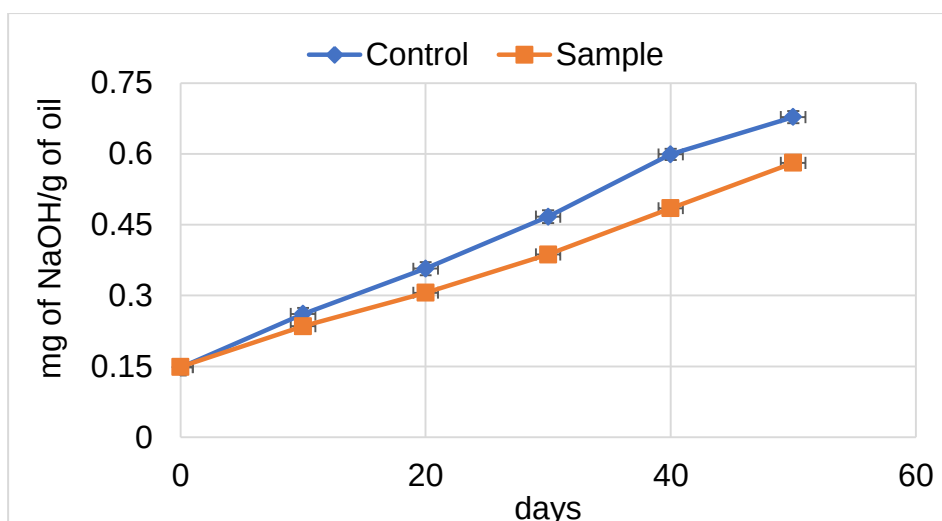


Fig. 4.15: Comparison of Control and Optimized Butter on Free Fatty Acid Value During Storage at Refrigeration.

4.13.2: Effect on Peroxide Value

Hydroperoxide is the main product of lipid oxidation. Hence, the peroxide value can be employed as an oxidative index for the initial stage of lipid oxidation (Ramadan & Mörsel, 2004). The Peroxide value in the optimized butter and control is depicted in Fig. 4.16. The PV of control and optimized butter rose in a classical manner during the storage period of 50 days at refrigeration temperature, but with different levels. Control butter had shown high Peroxide value than the optimized butter during 50th days of storage. This may be due to the antioxidant property of β -carotene.

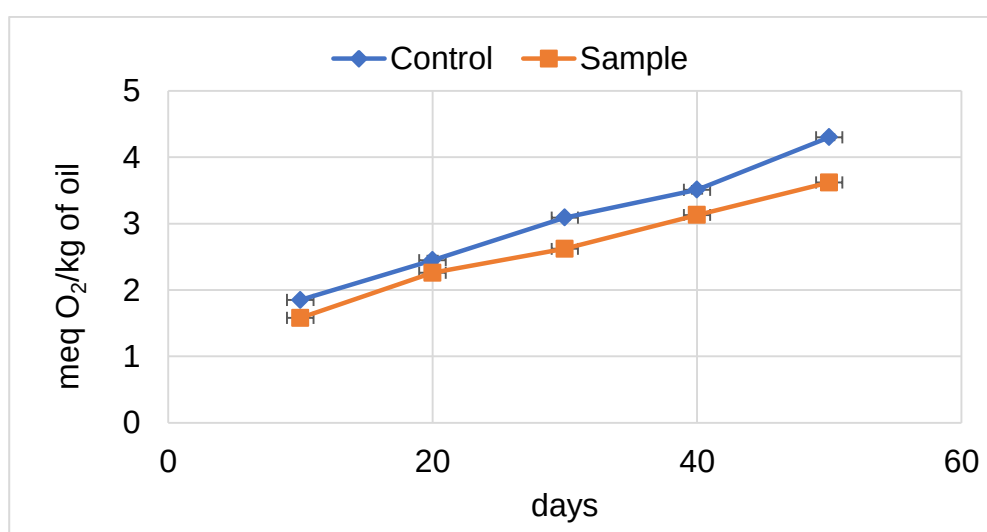


Fig. 4.16: Comparison of Control and Optimized butter on Peroxide Value During Storage at Refrigeration.

4.13.3: Effect on the pH

The difference in pH change was observed throughout the storage period at refrigeration. The changes in pH during refrigeration temperature of control and optimized butter samples were depicted in Fig. 4.17.

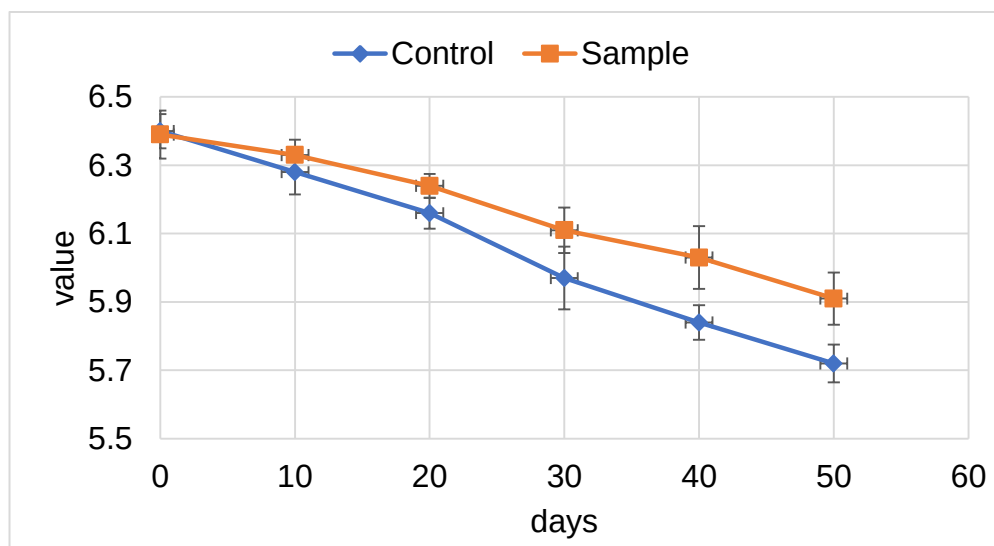


Fig. 4.17: Comparison of pH of Control and Optimized Butter During Storage at Refrigeration.

According to the results, there was a decreasing trend was seen on pH value for both samples corresponding to an increase acidity of butter samples. The pH reduced as increasing the free fatty acid value (Fig. 4.15). So, the decreasing pH value also was confirmed by increasing free fatty acid value and vice versa. The graph has shown β -carotene added butter was characterized by higher pH value when compared to the control butter sample.

4.13.4: Effect on Colour Value of Butter During Storage at Refrigeration Temperature.

The color value of the control butter and optimized butter was measured using the image analysis method and profiled in terms of L^* , a^* , b^* and derived as the Yellowness index (YI). The recorded data is tabulated in (Table 4.9) and the effect of the storage period on the YI value of control and optimized butter is shown in Fig 4.18. The yellowness of the optimized butter sample (b^*) is increased due to its richness in yellow pigments coming from β -carotene. Whereas, Samet-Bali *et al.*, (2009) conclude that an increase in lightness and decrease in yellowness of butter oil after storage at 60°C which can be due to the

loss of β -carotenes (yellow pigment). However, this effect is not noticed as the product is different.

Table 4.9: Changes in Colour Value of Butter During Storage at Refrigeration.

Days	Sample	L*	a*	b*
0 th	Control	79.32 $\pm$ 0.04	-1.19 $\pm$ 0.00	13.64 $\pm$ 0.04
	Optimized	73.16 $\pm$ 0.03	-2.10 $\pm$ 0.31	20.57 $\pm$ 0.08
10 th	Control	76.83 $\pm$ 0.07	-1.13 $\pm$ 0.01	11.11 $\pm$ 0.06
	Optimized	75.91 $\pm$ 0.15	-2.42 $\pm$ 0.02	24.51 $\pm$ 0.21
20 th	Control	76.24 $\pm$ 0.02	-1.22 $\pm$ 0.00	12.38 $\pm$ 0.07
	Optimized	73.66 $\pm$ 0.09	-2.33 $\pm$ 0.03	24.98 $\pm$ 0.28
30 th	Control	79.44 $\pm$ 0.24	-1.17 $\pm$ 0.01	17.75 $\pm$ 0.01
	Optimized	77.12 $\pm$ 0.08	-2.11 $\pm$ 0.03	32.07 $\pm$ 0.09
40 th	Control	71.30 $\pm$ 0.32	-1.91 $\pm$ 0.00	16.37 $\pm$ 0.21
	Optimized	71.68 $\pm$ 0.33	-2.69 $\pm$ 0.02	32.06 $\pm$ 0.47

Note: Average of triplicate analysis

In butter, it was observed that, the YI values recorded an increase from an initial value of 20.67 to 32.81 and 40.18 to 63.9 for the control and optimized butter sample respectively, over the storage period of 50 days at refrigeration temperature. The increase in the YI value may be due to the β -carotene content.

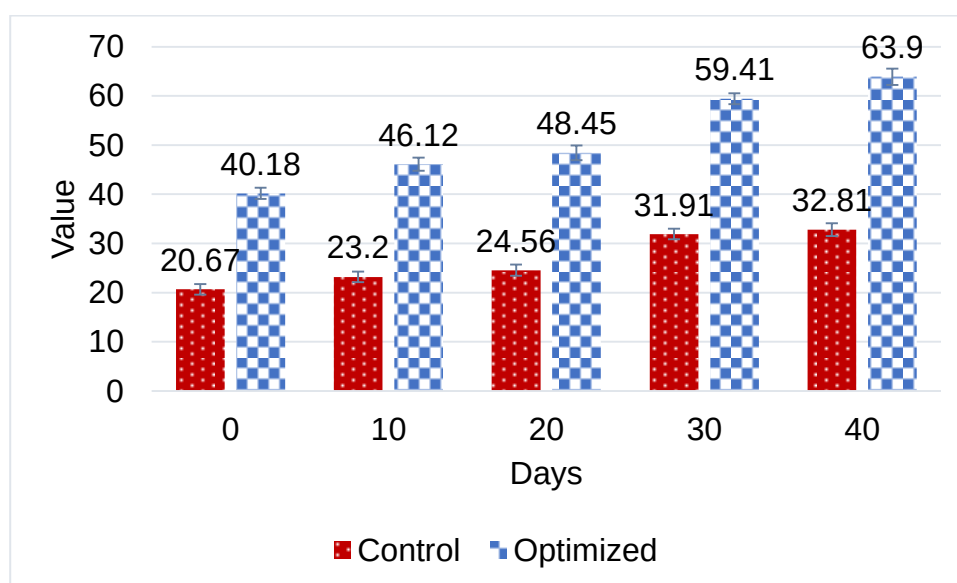


Fig. 4.18: Yellowness Index of Control and Optimized Butter During Storage

4.13.5: Effect on Textural Profile Analysis (TPA)

Texture profile analysis of the control and optimized butter sample stored at refrigeration samples was carried out using Texture Analyzer. The gradual decrease in the firmness, stickiness, work of shear and adhesion values was noticed. The control sample had better textural parameters as compared to that of the developed butter. This could be due to the dilution effect of the presence of ghee in the extract base material. Thus, the antioxidant rich extract addition improved the spreadability as evidenced by the slight variation in the composition (Table 4.10) and the sensory graphs (Fig. 4.19). Since the refrigerated temperature stored samples were analyzed by maintaining the cold condition in the texture analyzer, however the atmospheric temperature might also have affected these textural parameter values.

Table 4.10: Changes in Texture Value of Control and Optimized Butter During Storage at Refrigeration.

Days	Sample	Firmness (N)	Work of Shear (N.S)	Stickiness (N)	Work of Adhesion (N.S)
0	Control	2.513	43.16	-1.30	-0.59
	Optimized	2.354	43.41	-1.27	-0.613
10	Control	1.910	30.93	-0.841	-0.396
	Optimized	1.93	30.18	-0.975	-0.44
20	Control	1.17	24.23	-0.797	-0.354
	Optimized	1.73	24.712	-0.926	-0.338
30	Control	1.15	23.66	-0.767	-0.22
	Optimized	1.64	23.98	-0.667	-0.303
40	Control	1.12	19.62	-0.724	-0.129
	Optimized	1.51	19.61	-0.593	-0.15

4.13.6: Effect on Microbial Analysis of Control and Optimized Butter

Control and optimized butter sample stored at refrigeration samples were subjected to microbiological analysis.

Table 4.11: Changes in Total Plate Count of Control and Optimized Butter During Storage at Refrigeration.

Days	Sample	TPC (cfu/gm)
0	Control	4.67×10^1
	Optimized	3.33×10^1
10	Control	9.36×10^1
	Optimized	6.30×10^1
20	Control	2.43×10^2
	Optimized	1.23×10^2
30	Control	4.77×10^2
	Optimized	2.47×10^2
40	Control	1.63×10^3
	Optimized	3.86×10^2

The total plate count (TPC) data showed a trend toward an increase in the count with time for both control and developed butter samples (Table 4.11). However, due to the antibacterial properties of β -carotene, the extract containing β -carotene added butter has shown a low microbiological count in comparison to control butter. It was noticed that both the butter sample did not contain any yeast and mould (ND) during the storage period. Coliform counts were also found nil during 40 days of storage indicating the sample was prepared aseptically and a better microbial quality product.

4.13.7: Effect on Sensory Analysis

Control and optimized butter sample stored at refrigeration samples were subjected to sensory analysis. The overall acceptability score at day 0 was almost similar for both the control and optimized butter sample. The result of sensory evaluation at day 0 to 40th days was depicted in Fig. 4.19. On the 40th day, the control sample indicated unacceptability and thus scored 76.8 as the overall score due to the pH, FFA values and textural attributes. However, the developed sample was shown to be acceptable due to the preservative effect due to the β -carotene and its antioxidant activity. It can be concluded that the overall acceptability score during the storage conditions is dependent on the spoilage

related changes and the presence of natural antioxidants might help in providing the preservative effect.

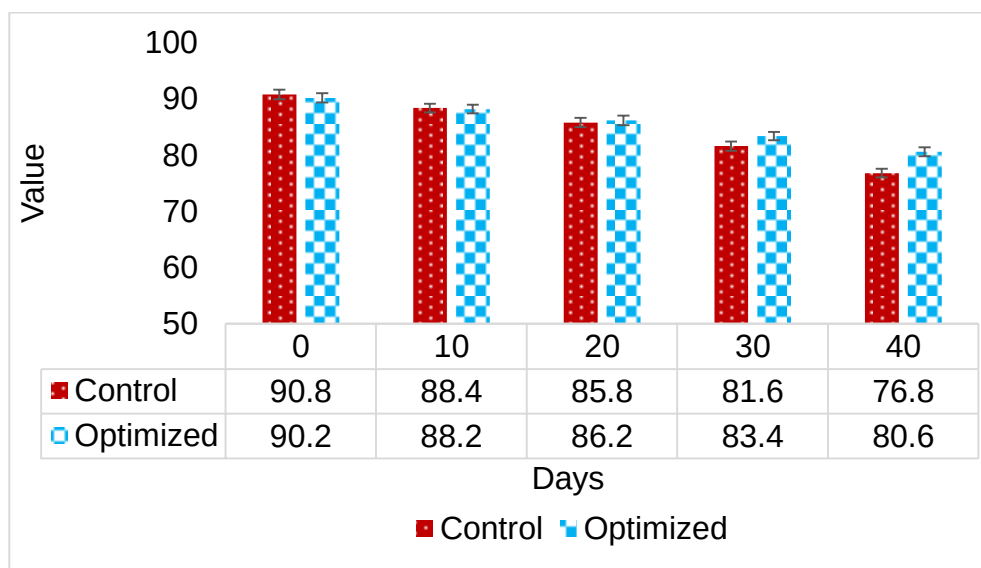


Fig. 4.19: Sensory Analysis of Control and Optimized Butter During Storage at Refrigeration.

4.14: Total Phenolic Content of Control and Optimized Butter and Ghee

The total phenolic content for cow butter and ghee and natural antioxidant rich β -carotene extract added to cow butter and ghee was determined by the Folin-Ciocalteu method and the standard curve of gallic acid (25-150 mg/ mL) was used (Fig. 4.20). The results were expressed as mg gallic acid equivalent (GAE) per gm on the (Table 4.12). the addition of carotene showed a highly significant increase in total phenolic content in optimized butter and ghee compared to control butter and ghee samples.

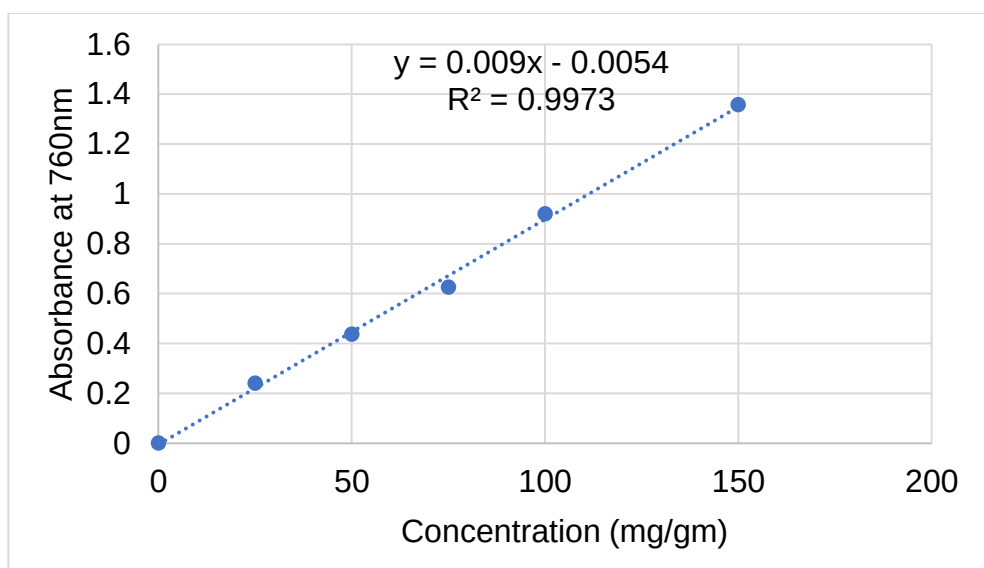


Fig. 4.20: Standard Curve for Total Phenolic Content.

Table 4.13: Total Phenolic Content of Control and Natural Antioxidant Added Cow Butter and Ghee.

Sample	mg GAE/gm ghee
Control butter	6.26 ^a
Optimized butter	65.21 ^b
Control ghee	9.61 ^a
Optimized ghee	88.36 ^b

Note: Data is presented as Average $\pm$ SD (n=3) Terms with different superscript shows significant difference($p < 0.05$).

From (Table 4.12) it is evident that there is a 10-fold increase in the total phenolic content in the butter sample as compared to that of the control sample. In the case of the ghee sample, a 9-fold increase was observed. It has been reported that the antioxidant, and radical activity of plant extracts or natural antioxidants depends on their phenolic content. There is a positive relationship was reported in total phenolic content and radical scavenging activity. Asha *et al* (2015); Rahila *et al*, (2017); Nandini and Jha (2021), hence our study outcomes are in agreement with the similar studies carried in the other fat rich products.

Chapter-5

SUMMARY

AND

CONCLUSION

SUMMARY AND CONCLUSION

India is the largest producer and consumer of butter and ghee. Since milk fat is the third main source of lipid for human nutrition; it is being harvested for human use from the time of domestication of dairy animals. As a practice, the surplus milk fat is conserved either in the form of butter or ghee. As per the published report, these fat rich dairy products undergo spoilage during prolonged storage due to autoxidation. The end products formed from the lipid oxidation results in the production of objectionable flavour and odour; some of the secondary and tertiary end products of oxidation can cause diarrhoea and the free radicals formed can be of carcinogenic in nature. According to the food safety standards regulations of FSSAI, addition of synthetic antioxidant(s) to extend the shelf life of such fat rich product is prohibited.

Considering the socio-economic value of the fat rich products, researchers are shifting towards natural antioxidants source. As per our literature survey, few reports suggest that natural pigment can be used to extend shelf life without affecting the sensory attributes of the food. Carotenoids are the foremost common bioactive terpenoid pigments. These are commonly produced by a variety of plants and microbes. Selected antioxidants such as β -carotene and many other extracts possess diverse biological properties and health benefits, therefore researchers are developing various nutraceuticals and functional foods from these ingredients.

The present work was undertaken with a hypothesis that use of β -carotene pigment extract in butter and ghee may enhance the shelf life, sensory properties and nutraceutical benefits without affecting the quality parameters.

The study was conducted in three phases, first being optimization of the β -carotene pigment extraction from 2 different sources. For the first time, ghee as an alternate to the chemical solvent was used (bio-refining) and the extraction process was optimized with respect to the ultrasonic processing parameters adopting Response surface methodology.

In the second phase, butter and ghee samples were prepared, β -carotene rich ghee extract as a natural source of antioxidants. Products were optimized on the basis of sensory analysis, antioxidant activity and the quality indicators of the products. In the third phase, prepared products were analyzed for composition including content of β -carotene; physicochemical constants, and the storage study. The summary of the work is reported below:

- 👍 Source of β -carotene extract: Carrot was found to be better over the microbial (*Micrococcus roseus*), with respect to the yield and the extraction process.
- 👍 Based on the Response Surface methodology, the ultrasonication parameters at 60-watt power, 40 min of extraction time and carrot extract powder to ghee ratio of 0.5 resulted in highest β -carotene: (213.5 $\pm$ 0.12 ppm).
- 👍 At a concentration of 20 and 30 ppm of β -carotene rich extract added to the butter and ghee, respectively resulted in the highest antioxidant and sensorily acceptable product.
- 👍 The β -carotene rich extract was found to be stable at room temperature for 6 months and its addition to butter and ghee has only 10% loss in their by-products.
- 👍 Total phenolic content in the optimized butter was found to be 10-times higher (65.21 GAE/gm fat) than the control butter. Similarly, the antioxidant activity was also found to be 1.5 times higher than the control butter.
- 👍 Total phenolic content in the optimized ghee was found to be 10-times higher (88.36 GAE/gm fat) than the control ghee. Similarly, the antioxidant activity was also found to be 2.0 times higher than the control ghee.
- 👍 Addition of carotene had no effect on the physico-chemical analytical parameters.
- 👍 During the storage study, developed butter and ghee were found to be more stable than the control samples

- 👉 Accelerated storage study indicates that control ghee stored at 80°C, shown a gradual decrease in score on 21th day, while its antioxidant and sensory properties were retained in the developed product.
- 👉 Significant effect ($p < 0.05$) of added β -carotene was shown on its sensory analysis, when ghee was stored at 80°C, minute decrease in overall acceptability score was reported and the sample remained fresh and acceptable till the end of the storage period (21 days).
- 👉 After accelerated storage study, extreme change in colour and appearance was observed in control ghee, from pale yellow to dark brownish colour due to formation of oxidative products and Maillard browning. The β -carotene added cow ghee maintained its appetizing colour and flavour even after completion of storage study.
- 👉 Rancimat analysis on the accelerated study showed that the 4-month-old control ghee sample induction time was found to be 2.06 hr while the optimized ghee sample was almost 2.1 times higher (4.38 hr).
- 👉 Both products showed enhanced shelf life in comparison to the control product because of the increased antioxidant activity (Butter 49.04 and ghee 47.76% RSA over its control 30.26 and 24.2% RSA).

It can be concluded that the extraction of carotene from fresh carrots using ultrasound-assisted extraction has been developed and shown to be a green bio-refining method with a number of benefits in terms of cost, time, energy, and yield. Ghee as a solvent has been shown to be a safe and environmentally friendly alternative to petroleum-based solvents that can be utilised in extraction process.

The addition of natural antioxidant β -Carotene extract in the butter and ghee has significantly improved the antioxidant activity and total phenolic content. The β -carotene has helped in extending the shelf life, enhanced sensory properties and colour of the prepared butter and ghee, thus, making it a value-added product. The conclusion from the work is reported below:

- ⌚ A green bio-refining method was optimized for extraction of β -carotene from natural source: Eco friendly method (Ghee as medium in place of solvents and chemicals),

- ⌚ The extract can be a potential alternative to *Annato* as a colourant in the butter, and
- ⌚ An ingredient with 3 functionalities: As a provitamin of A, colourant and antioxidant is developed for the preparation of functional butter and ghee.

BIBLIOGRAPHY

BIBLIOGRAPHY

- A.O.A.C *Official Methods of Analysis*. Association of Official Analytical Chemists. 16th edition. Washington D.C; 1995
- Abdalla, M. (1994). Milk in the rural culture of contemporary Assyrians in the Middle-East. *Milk and Milk Products from Medieval to Modern Times*, 27-39.
- Abou-Donia, S. A., & El-Agamy, S. I. (1993). Ghee. *Encyclopaedia of food science, food technology and nutrition*. London (UK): Academic Press Ltd, 3992-3994.
- Achaya, KT (1997). Ghee, vanaspati and special fats in India. In , *Lipid Technologies and Applications*, eds F.D. Gunstone and F.B. Padley. Marcel Dekker Inc., New York: 369-390.
- Agmark (1981). Ghee Grading and Marking Rules, 1938 (as amended). Government of India, Ministry of Food and Agriculture, Department of Agriculture, New Delhi, India.
- Akalln, A. S., & Tokusoglu, Ö. (2003). A potential anticarcinogenic agent: conjugated linoleic acid (CLA). *Pakistan Journal of Nutrition*, 2(2), 109-110.
- Alimentarius, C. (2011). Codex standard for butter, codex standard 279-1971. *Milk and milk products*, 36e37.
- Asha, A., Manjunatha, M., Rekha, R. M., Surendranath, B., Heartwin, P., Rao, J., & Sinha, C. (2015). Antioxidant activities of orange peel extract in ghee (butter oil) stored at different storage temperatures. *Journal of food science and technology*, 52(12), 8220-8227.
- Ashokkumar, M. (2015). Applications of ultrasound in food and bioprocessing. *Ultrasonics sonochemistry*, 25, 17-23.
- Asouri, M., Ataee, R., Ahmadi, A. A., Amini, A., & Moshaei, M. R. (2013). Antioxidant and free radical scavenging activities of curcumin. *Asian J. Chem*, 25(13), 7593-7595.
- Baria, B., Upadhyay, N., Singh, A. K., & Malhotra, R. K. (2019). Optimization of 'green' extraction of carotenoids from mango pulp using split plot design and its characterization. *LWT*, 104, 186-194.

- Battula, S. N., Naik, N. L., Sharma, R., & Mann, B. (2020). Ghee, Anhydrous Milk Fat and Butteroil. In *Dairy Fat Products and Functionality* (pp. 399-430). Springer, Cham.
- Bergmann, M. 2004. Schülerduden Chemie, Mannheim, Leipzig, Wien, Zürich, Dudenverlag. Biswas, A. K., Sahoo, J. & Chatli, M. K. 2011. A simple UV-Vis spectrophotometric method for determination of [beta]-carotene content in raw carrot, sweet potato and supplemented chicken meat nuggets. *LWT - Food Science and Technology*, 44, 1809.
- Bhavaniramy, S., Vishnupriya, S., Vijayarani, K., & Baskaran, D. (2018). A review on understanding the subterranean insights in nature of South Indian ghee with its biological and physiochemical properties. *Int J of Food Sci and Nutrition*, 3(6), 257-262.
- Bipinbhai, K. D., & Aparnathi, K. D. (2017). Evaluation of curry leaves (*Murraya koenigii*) for enhancing shelf-life of ghee against oxidative deterioration. *Journal of Pharmacognosy and Phytochemistry*, 6(6), 951-962.
- Bipinbhai, K. D., & Aparnathi, K. D. (2017). Evaluation of Liquorice (*Glycyrrhiza glabra*) for Enhancing Shelf-Life of Ghee against Oxidative Deterioration. *Int. J. Curr. Microbiol. App. Sci*, 6(12), 1455-1477.
- Blois, M. S. (2001). Antioxidant activity of grape seed extracts on peroxidation models in vitro. *J. Agric. Food Chem* 55:1018
- Bruns, R. E., Scarminio I. S., & de Barros Neto, B. (2006). Statistical design-chemometrics (vol. 25): Elsevier
- Burton, G. W., & Ingold, K. U. (1984). β -Carotene: an unusual type of lipid antioxidant. *Science*, 224(4649), 569-573.
- Chemat, F., Rombaut, N., Sicaire, A. G., Meullemiestre, A., Fabiano-Tixier, A. S., & Abert-Vian, M. (2017). Ultrasound assisted extraction of food and natural products. Mechanisms, techniques, combinations, protocols and applications. A review. *Ultrasonics sonochemistry*, 34, 540-560.
- Chen, S., G. Bobe, S. Zimmerman, E. G. Hammond, C. M. Luhman, T. D. Boylston, A. E. Freeman, and D. C. Beitz. 2004. Physical and sensory

- properties of dairy products from cows with various milk fatty acid compositions. *J. Agric. Food Chem.* 52:3422–3428.
- Chevillard, G., Nouhi, Z., Anna, D., Paquet, M., & Blank, V. (2010). Nrf3-deficient mice are not protected against acute lung and adipose tissue damages induced by butylated hydroxytoluene. *FEBS letters*, 584(5), 923-928.
- Chutia, H., & Mahanta, C. L. (2021). Green ultrasound and microwave extraction of carotenoids from passion fruit peel using vegetable oils as a solvent: Optimization, comparison, kinetics, and thermodynamic studies. *Innovative Food Science & Emerging Technologies*, 67, 102547.
- Codex Alimentarius. (2018). *Milk and milk products* (2nd ed.). Codex Standard for Milk fat Products Stan 280-1973.
- Conn, P. F., Schalch, W., & Truscott, T. G. (1991). The singlet oxygen and carotenoid interaction. *Journal of Photochemistry and Photobiology B: Biology*, 11(1), 41-47.
- da Silva, H. R. P., Iwassa, I. J., Marques, J., Postaue, N., Stevanato, N., & da Silva, C. (2020). Enrichment of sunflower oil with β -carotene from carrots: Maximization and thermodynamic parameters of the β -carotene extraction and oil characterization. *Journal of Food Processing and Preservation*, 44(4), e14399.
- Deosarkar S.S., Khedkar C.D., Kalyankar S.D. and Sarode A.R. (2016) Cream: Types of Cream. In: Caballero, B., Finglas, P., and Toldrá, F. (eds.) *The Encyclopedia of Food and Health* vol. 2, pp. 331-337. Oxford: Academic Press. (17) (PDF) *Cream: Types of cream*.
- Deshmukh, A. R., Dhadge, N. S., Desale, R. J., & Kadam, D. G. (2019). Effect of *Asparagus racemosus* (Shatavari) and *Withania somnifera* (Ashwagandha) extracts on oxidative stability of ghee, in relation to added synthetic antioxidant. *IJCS*, 7(6), 175-181.
- Domke, A., Grofklau, R., Niemann, B., Przyrembel, H., Richter, K., Schmidt, E., Weiflenborn, A., Wörner, B. & Ziegenhagen, R. (2004). Verwendung von Vitaminen in Lebensmitteln - Toxikologische und ernährungsphysiologische Aspekte, Teil I. Berlin: Bundesinstitut für Risikobewertung

- Elik, A., Yanık, D. K., & Göğüş, F. (2020). Microwave-assisted extraction of carotenoids from carrot juice processing waste using flaxseed oil as a solvent. *Lwt*, 123, 109100.
- El-Shourbagy, G. A., & El-Zahar, K. M. (2014). Oxidative stability of ghee as affected by natural antioxidants extracted from food processing wastes. *Annals of Agricultural Sciences*, 59(2), 213-220.
- Eroglu, A., & Harrison, E. H. (2013). Carotenoid metabolism in mammals, including man: formation, occurrence, and function of apocarotenoids: Thematic Review Series: Fat-Soluble Vitamins: Vitamin A. *Journal of lipid research*, 54(7), 1719-1730.
- Espin, J. C., Soler-Rivas, C., & Wichers, H. J. (2000). Characterization of the total free radical scavenger capacity of vegetable oils and oil fractions using 2, 2-diphenyl-1-picrylhydrazyl radical. *Journal of Agricultural and Food chemistry*, 48(3), 648-656.
- Everett, S. A., Dennis, M. F., Patel, K. B., Maddix, S., Kundu, S. C., & Willson, R. L. (1996). Scavenging of Nitrogen Dioxide, Thiyl, and Sulfonyl Free Radicals by the Nutritional Antioxidant β -Carotene (*). *Journal of Biological Chemistry*, 271(8), 3988-3994.
- Frankel, E. N. (1993). In search of better methods to evaluate natural antioxidants and oxidative stability in food lipids. *Trends in Food Science & Technology*, 4(7), 220-225.
- Fратиanni, A., Cinquanta, L., & Panfili, G. (2010). Degradation of carotenoids in orange juice during microwave heating. *LWT-Food Science and Technology*, 43(6), 867-871.
- FSSA. (2006). Food Safety and Standards Act 2006, Rules 2011, Regulations, 7th ed. international law book company, Delhi, 2011, p. 643.
- FSSR. (2011). *Food products standards*. In Food safety and standards (Food products standards and food additives) regulations. <http://www.fssai.gov.in>
- Gandhi, K., Arora, S., Pawar, N., & Kumar, N. (2013). Effect of Vidarikand (extracts) on oxidative stability of ghee: A comparative study. *Research and Reviews: Journal of Dairy Science and Technology*, 2(1), 1-10.

- Ge, Y., Ni, Y., Yan, H., Chen, Y., & Cai, T. (2002). Optimization of the supercritical fluid extraction of natural vitamin E from wheat germ using response surface methodology. *Journal of Food Science*, 67(1), 239–243.
- Geens, A., Dauwe, T., & Eens, M. (2009). Does anthropogenic metal pollution affect carotenoid colouration, antioxidative capacity and physiological condition of great tits (*Parus major*). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 150(2), 155-163.
- Gosewade, S., Gandhi, K., Ranvir, S., Kumar, A., & Lal, D. (2017). A study on the physico-chemical changes occurring in ghee (butter oil) during storage. *Indian J Dairy Sci*, 70(1), 81-8.
- Goula, A. M., Ververi, M., Adamopoulou, A., & Kaderides, K. (2017). Green ultrasound-assisted extraction of carotenoids from pomegranate wastes using vegetable oils. *Ultrasonics sonochemistry*, 34, 821-830.
- Gray, J. I. (1978). Measurement of lipid oxidation: a review. *Journal of the American Oil Chemists' Society*, 55(6), 539-546.
- Guriye, K. V., Srinivasan, M. R., Desai, K. K., & Ray, S. C. (1949). Studies on the direct cream heating method of ghee making. *Indian~. Dairy Sci*, 2, 77.
- Halliwel, B., Aeschbach, R., Löliger, J., & Aruoma, O. I. (1995). The characterization of antioxidants. *Food and Chemical Toxicology*, 33(7), 601-617.
- Hamid, A. D. (1993) *The Indigenous Fermented Foods of the Sudan: A Study in African Food and Nutrition*. CAB International, Wallingford, UK.
- Hazra, T., Sindhav, R., Parmar, M., Patel, M., & Aparnathi, K. D. (2019). Tomato skin: A source of natural antioxidant for ghee during storage. *Journal of Pharmacognosy and Phytochemistry*, 8(3), 718-721.
- Holstun, J., & Zetocha, D. (1994). An analysis of flaxseed utilization in the health food industry.
- <https://www.expertmarketresearch.com/reports/gheemarket>.
- <https://www.imarcgroup.com/ghee-market-india>.
- <https://www.marketwatch.com/press-release/butter-market>.

- International Agency for Research on Cancer, & International Agency for Research on Cancer. (1987). *Overall evaluations of carcinogenicity: an updating of IARC Monographs volumes 1 to 42* (p. 53). Lyon, France: IARC.
- IS: 3507 (1966) Indian standards, methods for sampling and test for butter. Bureau of Indian Standards, New Delhi
- IS: 3508 (1966) Indian standards, methods for sampling and test for ghee (butter fat). Bureau of Indian Standards, New Delhi
- IS: 3509 (1966) Indian standards, methods for sampling and test for Cream. Bureau of Indian Standards, New Delhi
- Jagannadham, M. V., Rao, V. J., & Shivaji, S. (1991). The major carotenoid pigment of a psychrotrophic *Micrococcus roseus* strain: purification, structure, and interaction with synthetic membranes. *Journal of Bacteriology*, 173(24), 7911-7917.
- Jeyakodi, S., Krishnakumar, A., & Chellappan, D. K. (2018). Beta Carotene- Therapeutic Potential and Strategies to Enhance Its Bioavailability. *Nutri Food Sci Int J Review Article*, 7(4).
- Jha et al., (2021). Evaluation of synergistic potential of select bioactives on the antioxidant properties and quality of ghee.
- Joint, F. A. O., WHO Expert Committee on Food Additives, & World Health Organization. (1974). *Evaluation of certain food additives: eighteenth report of the Joint FAO*. World Health Organization.
- Kashanian, S., & Dolatabadi, J. E. N. (2009). DNA binding studies of 2-tert-butylhydroquinone (TBHQ) food additive. *Food Chemistry*, 116(3), 743-747.
- Katalinic, V., Milos, M., Kulisic, T., & Jukic, M. (2006). Screening of 70 medicinal plant extracts for antioxidant capacity and total phenols. *Food chemistry*, 94(4), 550-557.
- Kinsella, J. E. 1975 Butter flavor. *Food Technology* 29 (5) 82, 84, 86, 88, 90, 92, 96, 98.
- Konwarh, R., Pramanik, S., Kalita, D., Mahanta, C. L., & Karak, N. (2012). Ultrasonication—A complementary ‘green chemistry’ tool to biocatalysis: A

laboratory-scale study of lycopene extraction. *Ultrasonics Sonochemistry*, 19(2), 292-299.

Koskinen, A. M. P. 2012. *Asymmetric Synthesis of Natural Products*, Hoboken, Wiley-Blackwell. LEITNER, P., FITZ-BINDER, C., MAHMUD-ALI, A. & BECHTOLD, T. 2012. Production of a concentrated natural dye from Canadian Goldenrod (*Solidago canadensis*) extracts. *Dyes and Pigments*, 93, 1416-1421.

Kumar, A., Goyal, S. K., Munesh, K. V., & Kumar, L. (2016). Study on physico-chemical analysis of ghee. *South Asian J. of Food Technol. and Environ*, 2(3&4), 448-451.

Kumar, K., Srivastav, S., & Sharanagat, V. S. (2021). Ultrasound assisted extraction (UAE) of bioactive compounds from fruit and vegetable processing by-products: A review. *Ultrasonics Sonochemistry*, 70, 105325.

Leontowicz, M., Gorinstein, S., Leontowicz, H., Krzeminski, R., Lojek, A., Katrich, E., & Trakhtenberg, S. (2003). Apple and pear peel and pulp and their influence on plasma lipids and antioxidant potentials in rats fed cholesterol-containing diets. *Journal of Agricultural and Food Chemistry*, 51(19), 5780-5785.

Li, Y., Fabiano-Tixier, A. S., Tomao, V., Cravotto, G., & Chemat, F. (2013). Green ultrasound- assisted extraction of carotenoids based on the bio-refinery concept using sunflower oil as an alternative solvent. *Ultrasonics sonochemistry*, 20(1), 12-18.

Liu, Y., Chen, T., Qiu, Y., Cheng, Y., Cao, Y., Zhao, A., & Jia, W. (2011). An ultrasonication-assisted extraction and derivatization protocol for GC/TOFMS-based metabolite profiling. *Analytical and bioanalytical chemistry*, 400(5), 1405-1417.

Lodh, J., Khamrui, K., & Prasad, W. G. (2018). Optimization of heat treatment and curcumin level for the preparation of anti-oxidant rich ghee from fermented buffalo cream by Central Composite Rotatable Design. *Journal of food science and technology*, 55(5), 1832-1839.

- Mahakalkar, A., Kashyap, P., Bawankar, R., Hatwar, B., & Id, E. (2014). The versatility of cow ghee-an ayurveda perspective. *American Journal of Drug Delivery and Therapeutics*, 1(1), 028-034.
- Maiza, A., Nzekoue, F. K., Ghazouani, T., Afif, M., Caprioli, G., Fiorini, D., & Fattouch, S. (2020). Butter oil (ghee) enrichment with aromatic plants: Chemical characterization and effects on fibroblast migration in an in-vitro wound healing model. *Arabian Journal of Chemistry*, 13(12), 8909-8919.
- Mala, K. S., Rao, P. P., Prabhavathy, M. B., & Satyanarayana, A. (2015). Studies on application of annatto (*Bixa orellena* L.) dye formulations in dairy products. *Journal of food science and technology*, 52(2), 912-919.
- Mehta, B. M. (2006). Ragi (*Eleusine coracana* L.)-a natural antioxidant for ghee (butter oil). *International journal of food science & technology*, 41, 86-89.
- Mehta, B. M., Darji, V. B., & Aparnathi, K. D. (2015). Comparison of five analytical methods for the determination of peroxide value in oxidized ghee. *Food chemistry*, 185, 449-453.
- Meléndez-Martínez, A. J., Escudero-Gilete, M. L., Vicario, I. M., & Heredia, F. J. (2010). Study of the influence of carotenoid structure and individual carotenoids in the qualitative and quantitative attributes of orange juice colour. *Food Research International*, 43(5), 1289-1296.
- Meullenet, J. F., Finney, M. L. and Gaurd, M. (2002). Measurement of biting velocities, and predetermined and individual crosshead speed instrumental imitative tests for predicting cheese hardness. *Journal of texture studies*, 33(1):45-58.
- Miękus, N., Iqbal, A., Marszałek, K., Puchalski, C., & Świergiel, A. (2019). Green Chemistry Extractions of Carotenoids from *Daucus carota* L.-Supercritical Carbon Dioxide and Enzyme-Assisted Methods. *Molecules*, 24(23), 4339.
- Mirheli, M., & Taghian Dinani, S. (2018). Extraction of β -carotene pigment from carrot processing waste using ultrasonic-shaking incubation method. *Journal of Food Measurement and Characterization*, 12(3), 1818-1828.
- Mortensen, B., & Denmark, T. (2011). Butter and other milk fat products, the product and its manufacture

- Naik, L., Mann, B., Bajaj, R., Sangwan, R. B., & Sharma, R. (2013). Process optimization for the production of bio-functional whey protein hydrolysates: adopting response surface methodology. *International Journal of Peptide Research and Therapeutics*, 19(3), 231-237.
- Nandini H. S. et al., (2019) Deoni and Malnad Gidda Cow Ghee: Characterization for Physico-chemical and Functional Properties.
- Nour, V., Corbu, A. R., Rotaru, P., Karageorgou, I., & Lalas, S. (2018). Effect of carotenoids, extracted from dry tomato waste, on the stability and characteristics of various vegetable oils. *Grasas y Aceites*, 69(1), e238-e238.
- Numan, M., Bashir, S., Mumtaz, R., Tayyab, S., Rehman, N. U., Khan, A. L., & Al-Harrasi, A. (2018). Therapeutic applications of bacterial pigments: a review of current status and future opportunities. *3 Biotech*, 8(4), 1-15.
- Odriozola-Serrano, I., Soliva-Fortuny, R., Hernández-Jover, T., & Martín-Belloso, O. (2009). Carotenoid and phenolic profile of tomato juices processed by high intensity pulsed electric fields compared with conventional thermal treatments. *Food Chemistry*, 112(1), 258-266.
- Pajohi-Alamoti, M., Khaledian, S., & Bazargani-Gilani, B. (2019). Antioxidant Effects of Aromatic Plant Essential Oils on Oxidative Stability of Ghee. *Archives of Hygiene Sciences*, 8(4), 232-244.
- Parmar, P., & Kaushik, K. (2013). The effects of alcoholic extract of Arjuna (*Terminalia arjuna* Wight & Arn.) bark on stability of clarified butterfat. *Journal of Medicinal Plants Research*, 4(35), 2545-2550.
- Patel, S., Shende, S., Arora, S., Singh, R. R. B., Rastogi, S., & Singh Rawat, A. K. (2014). Antioxidant potential of herbs and spices during deep frying of ghee. *International Journal of Dairy Technology*, 67(3), 365-372.
- Pathare, P. B., Opara, U. L. and Al-Said, F. A. (2013) Colour measurement and analysis in fresh and processed foods- A Review. *Food Bioprocess Technology*, 6(11):36-60.
- Pawar, N., Arora, S., Bijoy, R. R., & Wadhwa, B. K. (2012). The effects of *Asparagus racemosus* (shatavari) extract on oxidative stability of ghee, in

- relation to added natural and synthetic antioxidants. *International journal of dairy technology*, 65(2), 293-299.
- Pawar, N., Gandhi, K., Purohit, A., Arora, S., & Singh, R. R. B. (2014). Effect of added herb extracts on oxidative stability of ghee (butter oil) during accelerated oxidation condition. *Journal of Food Science and Technology*, 51(10), 2727-2733.
- Rahila, M. P., Surendra Nath, B., Laxmana Naik, N., Pushpadass, H. A., Manjunatha, M., & Franklin, M. E. E. (2018). Rosemary (*Rosmarinus officinalis* Linn.) extract: A source of natural antioxidants for imparting autoxidative and thermal stability to ghee. *Journal of Food Processing and Preservation*, 42(2), e13443.
- Rajorhia, G. S. (1993). Ghee. In *Encyclopaedia of Food Science, Food Technology and Nutrition*, Vol. 4, eds R. Macrae, R. K. Robinson and M. J. Sadler. Academic Press Ltd, London, pp. 2186-2192.
- Ramadan, M. F., & Mörsel, J. T. (2004). Oxidative stability of black cumin (*Nigella sativa* L.), coriander (*Coriandrum sativum* L.) and niger (*Guizotia abyssinica* Cass.) crude seed oils upon stripping. *European Journal of Lipid Science and Technology*, 106(1), 35-43.
- Rangan, C., & Barceloux, D. G. (2009). Food additives and sensitivities. *Disease-a-month*, 55(5), 292-311.
- Samet-Bali, O., Ayadi, M. A., & Attia, H. (2009). Traditional Tunisian butter: Physicochemical and microbial characteristics and storage stability of the oil fraction. *LWT-Food Science and Technology*, 42(4), 899-905.
- Sharayei, P., Azarpazhooh, E., Zomorodi, S., & Ramaswamy, H. S. (2019). Ultrasound assisted extraction of bioactive compounds from pomegranate (*Punica granatum* L.) peel. *Lwt*, 101, 342-350.
- Sharma, M., & Bhat, R. (2021). Extraction of carotenoids from pumpkin peel and pulp: Comparison between innovative green extraction technologies (ultrasonic and microwave-assisted extractions using corn oil). *Foods*, 10(4), 787.

- Sharma, S., Durand, R. M., & Gur-Arie, O. (1981). Identification and analysis of moderator variables. *Journal of marketing research*, 18(3), 291-300.
- Sharmin, T., Ahmed, N., Hossain, A., Hosain, M. M., Mondal, S. C., Haque, M. R., & Siddik, A. B. (2016). Extraction of bioactive compound from some fruits and vegetables (pomegranate peel, carrot and tomato). *American Journal of Food and Nutrition*, 4(1), 8-19.
- Siek, T. J., & Lindsay, R. C. (1970). Semiquantitative analysis of fresh sweet-cream butter volatiles. *Journal of Dairy Science*, 53(6), 700-703.
- Sindhu, J. S., Sumit, A., & Nayak, S. K. (2000). Physico-chemical aspects of indigenous dairy products. *Indian Dairyman*, 52(10), 51-64.
- Singh, A. K. (2017). Evaluation of the antioxidant potential of oregano leaves (*Origanum vulgare* L.) and their effect on the oxidative stability of ghee. *International Journal on Nutraceuticals, Functional Foods and Novel Foods*.
- Siwach, R., Tokas, J., & Seth, R. (2016). Use of lycopene as a natural antioxidant in extending the shelf-life of anhydrous cow milk fat. *Food chemistry*, 199, 541-546.
- Sserunjogi, M. L., Abrahamsen, R. K., & Narvhus, J. (1998). A review paper: current knowledge of ghee and related products. *International Dairy Journal*, 8(8), 677-688.
- Tiwari, S., Upadhyay, N., Singh, A. K., Meena, G. S., & Arora, S. (2019). Organic solvent-free extraction of carotenoids from carrot bio-waste and its physico-chemical properties. *Journal of food science and technology*, 56(10), 4678-4687.
- Urbach, G., & Gordon, M. H. (1994). Flavours derived from fats. In *Fats in food products* (pp. 347-405). Springer, Boston, MA.
- V. Saez, A. Fry´as-Ferrer, J. Gonzalez-Garcy´, A. Aldez, E. Riera, Characterization of a 20 kHz sonoreactor. Part 2: analysis of chemical effects by classical and electrochemical methods, *Ultrasonics Sonochemistry* 12 (2005) 67.

- V.S. Sutkar, P.R. Gogate, Design aspects of sonochemical reactors: techniques for understanding cavitation activity distribution and effect of operating parameters, *Chemical Engineering Journal* 155 (2009) 26–36.
- Wadhwa BK and Jain MK. (1990) Chemistry of ghee flavor: a review. *Indian J Dairy Scie*, 43: 601-607.
- Walstra, P., Walstra, P., Wouters, J. T., & Geurts, T. J. (2005). *Dairy science and technology*.
- Wang, Y., & Xu, B. (2014). Distribution of antioxidant activities and total phenolic contents in acetone, ethanol, water and hot water extracts from 20 edible mushrooms via sequential extraction. *Austin Journal of Nutrition and Food Sciences*, 2(1), 5.
- Wasnik, P. G., Menon, R. R., Surendra Nath, B., Balasubramanyam, B. V., Manjunatha, M. and Sivaram, M. (2017). Application of particle analysis and colour parameters for detection of adulteration of cow ghee with Vanaspati derived from image analysis. *Indian Journal Dairy Science*, 70(2):34-38.
- Wen, C., Zhang, J., Zhang, H., Dzah, C. S., Zandile, M., Duan, Y., ... & Luo, X. (2018). Advances in ultrasound assisted extraction of bioactive compounds from cash crops—A review. *Ultrasonics sonochemistry*, 48, 538-549.
- Widomska, J., Kostecka-Gugała, A., Latowski, D., Gruszecki, W. I., & Strzałka, K. (2009). Calorimetric studies of the effect of cis-carotenoids on the thermotropic phase behavior of phosphatidylcholine bilayers. *Biophysical chemistry*, 140(1-3), 108-114.
- Woodside, J. V., McGrath, A. J., Lyner, N., & McKinley, M. C. (2015). Carotenoids and health in older people. *Maturitas*, 80(1), 63-68.
- Yam, K. L., & Papadakis, S. E. (2004). A simple digital imaging method for measuring and analyzing color of food surfaces. *Journal of food engineering*, 61(1), 137-142.
- Yolmeh, M., & Jafari, S. M. (2017). Applications of response surface methodology in the food industry processes. *Food and Bioprocess Technology*, 10(3), 413-433.

APPENDICES

Cost of the Developed Butter and ghee

A. Ingredient cost:

- 1) Cost of raw carrot (1 Kg) = ₹. 50
- 2) Yield 250 gm carrot powder = ₹. 55

B. Extraction of carotene from Carrot powder using ghee as solvent (1:2)

- 1) Carrot powder cost (250 gm) = ₹. 55
- 2) Ghee cost (500 gm) = ₹. 275
- 3) Total Cost = ₹. 330

C. Charge of Instruments/ Equipment's = Kg (ghee)/hr = ₹. 100

D. For 40 min extraction = ₹. 66/Kg ghee

Overall cost for extraction = B (3) + D

= 330 + 33 (for 500 gm ghee)

= ₹. 363 for 500 gm Carotene extracted ghee

Cost For 100 gm Carotene rich extracted ghee = ₹. 73

Addition to 1kg ghee:

Cost of 1 Kg ghee = ₹. 550

Ingredient added for 1Kg = 140 gm = ₹. 102

Total cost for Developed ghee (1 Kg) = ₹. 652

Addition to 1kg butter:

1 Kg butter cost = ₹. 450

Ingredient added for 1Kg = 90 gm = ₹. 66

Total Cost for Developed butter (1 Kg) = ₹. 516

β-carotene content in Products:

Sr.no.	Product	β-carotene content (ppm)
1	Carrots	780 ppm
2	Carotene extracted ghee	213 ppm
3	Control Butter	16 ppm
4	Developed Butter	36 ppm
5	Control Ghee	21 ppm
6	Developed ghee	51 ppm

Development of β-carotene added Butter and Ghee



Control Butter



Developed Butter



Control Ghee



developed Ghee

BIS Score Card of Butter-Sensory Analysis

Product: **β -carotene Enriched Butter**

Panelist Name:

Date and Time:

Instruction: Taste the given sample and you are requested to give score against each parameter based on sensory perception.

Parameter/Sample	Perfect Score	Sample:	Sample:
Flavour	50		
Body and Texture	30		
Colour	15		
Package	5		
Total	100		

Comments/Remarks:

Signature:

BIS Score Card of Ghee -Sensory Analysis

Product: **β -carotene Enriched Ghee**

Panelist Name:

Date and Time:

Instruction: Taste the given sample and you are requested to give score against each parameter based on sensory perception.

Parameter/Sample	Perfect Score	Sample	Sample
Flavour	45		
Texture	10		
Acidity (oleic)	25		
Colour	10		
Freedom from suspended matter	5		
Package	5		
Total	100		

Comments/Remarks:

Signature:

Ankit Prakashrao Chavhan



CONTACT DETAILS

Address : At. Hatla, Post. Palshi District
Yavatmal, Maharashtra 445211

Contact No. : 9130539133

Email ID : ankitchavhan26@gmail.com

Date of Birth : 01/06/1998

Skills and Competences

1. Self Confidence
2. Capacity and Willingness to Hard

Work

3. Computer Proficiency

Languages

1. English
2. Hindi
3. Marathi

Hobbies

1. Playing Cricket
2. Reading
3. Listening to music

 <https://www.linkedin.com/in/ankit-chavhan-aa89a01a2>

ACADEMIC QUALIFICATIONS

Examination	Year of passing	University/ Board	OGPA / Percentage
M. Tech (Dairy Chemistry)	2022	ICAR-NDRI Karnal	7.62
B. Tech. (Dairy Technology)	2020	MAFSU Nagpur	7.76
12 th	2016	Maharashtra State Board	75.54
10 th	2014	Maharashtra State Board	88.60

PG RESEARCH

“Quality Evaluation of Cow Butter and Ghee Valorised with Select Antioxidant of Natural Sources”

TRAINING AND EXPERIENCE

- Hands on Training at Students Training Dairy Plant, Udgir CDTU from January 2020 to June 2020.
- Inplant training at Govind Milk and Milk Products Pvt. Ltd. Phaltan Maharashtra from 23rd July 2019 to 5th January 2020.
- Successfully Completed 40 days training at Natural Sugar & Allied Industries Ltd. Ranjhani Maharashtra under domain of READY PROGRAMME- II
- Successfully Completed 40 days training at Maharashtra Rajya Sahakari Dudh Mahasangh Maryadit (MRSDMM) chilling centre Loni Maharashtra and ILFC COVAS Udgir under domain of READY PROGRAMME- I

AWARDS AND ACHIEVEMENTS

- Institutional Research Fellowship by ICAR- National Dairy Research Institute, Karnal for M. Tech (Dairy Chemistry) during academic year 2020-22
- Awarded certificate of participation in the International Web-Workshop on the topic titled “Recent advances in design and emerging applications in lateral flow assay”.
- Awarded certificate of participation in the international web-workshop on the topic titled “ Innovative and Emerging Technologies for food Safety and Nutritional quality”.