EVALUATION OF THE FEASIBILITY OF ULTRASOUND ASSISTED EXTRACTION METHOD FOR THE EXTRACTION OF CARNOSINE FROM FRESH CHICKEN BREAST MEAT

SUBMITTED TO

DEPARTMENT OF FOOD PROCESSING TECHNOLOGY

ST. TERESA'S COLLEGE (AUTONOMOUS), ERNAKULAM

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

M.VOC FOOD PROCESSING TECHNOLOGY

SUBMITTED BY,

AMANA RASHEED.V. A

UNDER THE GUIDANCE OF

DR. INDIRA DEY PAUL

SCIENTIST

DEPARTMENT OF MEAT AND MARINE SCIENCE

CSIR- CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE

MYSURU, KARNATAKA

APRIL 2025

ಸಿ.ಎಸ್.ಐ.ಆರ್–ಕೇಂದ್ರೀಯ ಆಹಾರ ತಂತ್ರಜ್ಞಾನ ಸಂಶೋಧನಾಲಯ, ಮೈಸೂರು – 570020, ಭಾರತ
सीएसआईआर-केन्द्रीय खाद्य प्रौद्योगिक अनुसंधान संस्थान, मैसूरु 570 020, भारत
CSIR - Central Food Technological Research Institute, Mysuru - 570 020, India
Cheluvamba Mansion, Opp. Railway Museum, KRS Road, Mysuru - 570 020
Website: http://www.cftri.res.in

cftri
Giving Food a Future

An ISO 9001:2015 & 14001:2015 & 17025:2017 (NABL) Organization

Dr. Indira Dey Paul
Scientist (Assistant Professor, AcSIR)
Department of Meat and Marine Sciences.
CSIR-CFTRI, Mysore
Email: indira27dp@cftri.res.in

CERTIFICATE

I certify that the project entitled **'Evaluation of the feasibility of ultrasound-assisted extraction method for the extraction of carnosine from fresh chicken breast meat'** submitted to the Department of Food Processing Technology, St. Teresa's College, Ernakulum, Kerala, India, for the award of Master of Vocation (M.Voc.) by **Ms. Amana Rashid V. A.**, is the result of the work carried out by her under the guidance of **Dr. Indira Dey Paul, Department of Meat and Marine Sciences, CSIR-Central Food Technological Research Institute, Mysore-570020**, from 20th December 2024 to 20th April 2025.

Date: 21/04/2025

Place: Mysore, Karnataka

Signature of Supervisor

Dr. Indira Dey Paul
वैज्ञानिक / Scientist
मांस एवं समुद्री विज्ञान
Meat & Marine Sciences
केंद्रीय खाद्य प्रौद्योगिक अनुसंधान संस्थान
Central Food Technological Research Institute
सीएफटीआरआई / CFTRI
मैसूर / Mysore – 570 020

HEAD/MMS

प्रधान / Head
मांस एवं समुद्री विज्ञान
Meat & Marine Sciences
केंद्रीय खाद्य प्रौद्योगिक अनुसंधान संस्थान
Central Food Technological Research Institute
मैसूर / Mysore – 570 020

HEAD/HRD

Dr. Revathy Baskar
प्रमुख / HEAD
मानव संसाधन विकास विभाग
Human Resource Development
सीएसआईआर-सीएफटीआरआई / CSIR-CFTRI
मैसूर-570 020

ST.TERESA'S COLLEGE, (AUTONOMOUS)
ERNAKULAM

Re- Accredited by NAAC in the 4th cycle with grade A++ and CGPA 3.57
Ranked 41st in National Institutional Ranking Framework (NIRF 2023)

ANNEXURE I

UNDERTAKING BY THE SELECTED STUDENT
FOR PROJECT WORK AT CSIR-CFTRI

The project students and their college / staff members shall have no claim whatsoever on the results of the project work. CSIR-CFTRI retains all Intellectual Property Rights in patents, designs, software, copyright and publications, if any that may be generated during the course of project work. In the event of any dispute arising, the decision of Director, CSIR-CFTRI, is final and is binding on the student(s) and college. (Sl. No. 17 of Annexure I)

The college certificate in the project report prescribed by college / University shall categorically reflect that the place of project work is CSIR-CFTRI and acknowledge the name of the staff member who has guided. (Sl. No. 18 of Annexure I)

The successful execution of the project work by the students is solely under the guidance of scientific/technical guide of CFTRI. **No interference from the College/University is entertained.**

We have read the prescribed rules and regulations (Sl. Nos. 1 to 18 of Annexure I) and we agree to abide by them during our project work at CFTRI, Mysore and to the Conduct and Discipline mandate of the Institute.

26/09/2024

AMANA RASHEED V A

ST. TERESA'S COLLEGE AUTONOMOUS ★ ERNAKULAM-682 011 ★

Dr. ALPHONSA VIJAYA JOSEPH
PRINCIPAL
ST. TERESA'S COLLEGE
Autonomous
ERNAKULAM

DECLARATION

I, Ms. **Amana Rasheed V A**, a student of M.Voc Food Processing Technology at St Teresa's college, Ernakulam, hereby declare that the project thesis titled **" EVALUATION OF THE FEASIBILITY OF ULTRASOUND - ASSISTED EXTRACTION METHOD FOR THE EXTRACTION OF CARNOSINE FROM FRESH CHICKEN BREAST MEAT** " was carried out by me during the year 2024-2025, in partial fulfillment of the award of the degree of Master of Vocation in Food Processing Technology. The project has been carried out under the guidance of **Dr. Indira Dey Paul**, Scientist, **Department of Meat and Marine Sciences, CSIR-Central Food Technological Research Institute, Mysuru**. I declare that this work has not been previously presented for the award of any Degree/Diploma/Fellowship or any other similar title to any candidate of any Institute/University.

Date:

Place: Mysore

Amana Rasheed. V.A

ACKNOWLEDGEMENT

First and foremost, I would like to thank God for giving me the strength and wisdom to complete my thesis. I would like to express my deepest gratitude **to Dr. Indira Dey Paul**, Scientist, Meat and Marine Sciences Department, **CSIR-Central Food Technological Research Institute (CFTRI)**, Mysuru, for their invaluable mentorship, continuous support, and insightful guidance throughout my research journey. Their patience, expertise, and constructive feedback have played a crucial role in shaping this thesis, and I am truly grateful for their unwavering belief in my capabilities.

I express my sincere thanks to **Dr. Suresh P. V**., Head of the Department, Meat and Marine Sciences, **CSIR-CFTRI**, for availing me to pursue my project work in the Department.

I would like to sincerely thank to my esteemed mentor, **Mr. Shinde Vijay Sukhdeo**, Sr. Technical Officer**, CSIR-CFTRI**, for his support and guidance throughout my research work. His willingness to always help me out, regardless of how busy he was, truly made a difference in my journey.

My special thanks to the Director, **CSIR-CFTRI**, **Dr. Sridevi Annapurna Singh**, for allowing me to do my project at **CSIR-CFTRI**, Mysuru. I would like to thank **Head Human Resource Development, CSIR-CFTRI**, Mysuru, for giving me the opportunity to work in this prestigious institute.

I would like to express my deepest gratitude to **Dr. Tanaji. G. Kudre**, Principal **Scientist, CSIR-CFTRI**, for his unwavering support throughout my research work. His valuable assistance in providing all the necessary equipment has been instrumental in the successful completion of this study.

I also extend my heartfelt gratitude to my family for their unwavering support, patience, and sacrifices. Their constant encouragement has been my greatest source of strength. Finally, I am grateful to everyone who has supported me in any way throughout this journey. Your encouragement, patience, and kindness have made this achievement possible. Thank you all.

CONTENT

LIST OF TABLES

LIST OF PLATES

LIST OF FIGURES

LIST OF SYMBOLS, ACRONYMS AND ABBREVIATIONS

ABBREVIATIONS	FULLFORM
DW	Distilled water
mL	Milliliters
LDPE	Low-Density Polyethylene
wb	Wet basis
db	Dry basis
%	Percentage
°C	Degree celcius
g	Grams
rpm	Revolutions Per Minute
UAE	Ultrasound-assisted extraction
KHz	Kilohertz
mm	Millimetre
AC	Alternating current
µm	Micrometer
h	Hour
µL	microliter
RSA	radical scavenging activity
RCCD	rotatable central composite design
RSM	response surface methodology
mg/g	Milligrams per gram
db	Dry basis
nm	Nanometer
g/mol	Grams per mole
mM	Millimolar
µg	microgram
CC	Carnosine content
PC	Protein content
ND	Not deproteinized
WMT	With methanol treatment
WHT	With heat treatment
ANOVA	Analysis of variance

ABSTRACT

Carnosine, a bioactive dipeptide of β-alanine and L-histidine, is renowned for its antioxidant, anti-glycation, and neuroprotective action. Dominantly present in skeletal muscle tissue, chicken breast meat is a prime dietary source of carnosine. However, traditional extraction techniques generally result in substantial losses of carnosine through thermal breakdown. This research investigates the effectiveness of ultrasound-assisted extraction (UAE) as an environmentally friendly and cost-effective option for carnosine recovery from chicken breast muscle, in contrast to conventional hot water extraction (HWE). The present study examined how UAE parameters—sonication time (10–50 min), temperature (4–40°C), and deproteinization approaches (heat or methanol)—influence the carnosine yield, antioxidant activity (% radical scavenging activity, %RSA), and total protein content.

Rotatable central composite design (RCCD) and response surface methodology (RSM) were used to maximize the extraction conditions. Outcome indicated that UAE without deproteinization (ND) showed the maximum carnosine content (19.121 mg/g dry basis, db) and %RSA (38.23%), performing better compared to UAE with heat treatment (WHT) and HWE. UAE-ND also maintained higher antioxidant activity, although it increased protein content to 237.84 mg BSA equivalent/g db, indicating possible selectivity issues. Key results show that UAE-ND at 25 min and 4°C optimized carnosine extraction with retention of its bioactivity, providing a green and cost-effective alternative to traditional methods. The research highlights UAE's industrial application prospects for nutraceuticals and functional foods in response to the emerging need for environmentally friendly extraction technologies. Additional studies are suggested to improve selectivity and limit protein co-extraction.

Keywords: Carnosine, ultrasound-assisted extraction (UAE), chicken breast meat, antioxidant activity, response surface methodology (RSM), green extraction.

CHAPTER 1

INTRODUCTION

1.1. INTRODUCTION

Carnosine is a physiologically active dipeptide that is mostly found in skeletal muscle, the heart, and the central nervous system. It is composed of β-alanine and L-histidine (Boldyrev & Severin, 1994). L-histidine's imidazole ring provides metal-chelating and antioxidant qualities, and β-alanine controls the rate of production. Carnosine gets its name from the Latin word "carnis," which means meat or flesh, and was first identified in 1900 by the Russian scientist Vladimir Gulevich (Boldyrev, 2007). It is a natural antioxidant and strong non-enzymatic free-radical scavenger that is essential to cellular defense systems (Boldyrev et al., 2013). Additionally, its ability to chelate metal ions such as Zn2+ and Cu2+ strengthens its defense against oxidative damage (Chan & Decker, 1994). Interestingly, polaprezinc, its zinc-bound version, is therapeutically utilized to heal stomach ulcers, demonstrating its therapeutic significance.

Beyond its well-documented antioxidant function, carnosine has also been widely studied for its physiological functions, including pH buffering, anti-glycation, anti-inflammatory, and neuroprotective effects (Boldyrev et al., 2013). It plays a crucial role in protecting cells from oxidative stress. It has been involved in delaying cellular aging processes, including protein cross-linking and glycation, which are associated with chronic diseases such as diabetes, neurodegenerative disorders, and cardiovascular diseases (Tian et al., 2007). Its high concentration in skeletal muscle suggests that it significantly contributes to muscle function and exercise performance by buffering hydrogen ions, delaying muscle fatigue, and enhancing anaerobic capacity (Hazell, 1982). Furthermore, emerging evidence suggests that carnosine exhibits neuroprotective properties by preventing the aggregation of amyloid-beta peptides, which are linked to Alzheimer’s disease, making it a potential therapeutic candidate for age-related cognitive decline (Guimarães et al., 2021). Considering these diverse health benefits, there is increasing interest in optimizing carnosine extraction methods for applications in functional foods, nutraceuticals, and pharmaceuticals (Zhang et al., 2018).

Chicken meat has been recognized as a rich dietary source of carnosine, containing significantly higher concentrations compared to other white muscle tissues (Kim et al., 2012). Owing to its bioactive compounds, chicken has been used traditionally as a functional food that possesses possible beneficial effects on human health, including antioxidant and anti-glycation properties (Li et al., 2012). Unlike red meat, chicken contains lower levels of myoglobin, making it a preferred protein source with minimal lipid oxidation risks (Hazell,

1982). However, conventional cooking and processing methods often lead to significant losses in carnosine content due to heat-induced degradation, highlighting the need for optimized extraction techniques that maximize its recovery while preserving bioactivity (Mora et al., 2008). Traditional methods for extracting bioactive compounds, such as maceration, Soxhlet extraction, and solvent-based techniques, often suffer from several limitations, including low extraction efficiency, prolonged processing times, and the excessive use of organic solvents, which pose environmental and health concerns (Zhang et al., 2018). These conventional methods also risk thermal degradation of heat-sensitive compounds, resulting in reduced bioactivity and functional properties. As consumer demand for natural, minimally processed, and sustainable food ingredients increases, there is a growing emphasis on developing green extraction technologies that enhance yield, efficiency, and bioavailability (Guimarães et al., 2021).

For the extraction of bioactive substances, ultrasound-assisted extraction (UAE) has become a potential green method due to its ability to enhance mass transfer, reduce solvent consumption, and minimize thermal degradation of sensitive biomolecules (Zhang et al., 2018). UAE utilizes acoustic cavitation, which disrupts cellular structures and facilitates the release of intracellular compounds, improving extraction efficiency (Guimarães et al., 2021). This method has demonstrated effectiveness in extracting phenolic compounds, flavonoids, and proteins from various food matrices, yet limited research has focused on its application for extracting carnosine from chicken breast meat (Kim et al., 2012).

UAE presents several advantages over conventional extraction techniques, including enhanced extraction efficiency due to improved solvent penetration into cellular structures, minimal thermal degradation preserving the structural integrity and bioactivity of sensitive molecules like carnosine, an eco-friendly approach that reduces the need for hazardous organic solvents, and scalability for industrial applications (Guimarães et al., 2021). While the advantages of UAE are well-documented for other bioactive compounds, the specific effects of ultrasound processing on carnosine extraction remain underexplored. In particular, factors such as sonication power, frequency, solvent composition, temperature, and extraction duration may significantly impact both the yield and functional properties of carnosine. Additionally, the potential synergistic effect of UAE with heat treatment has not been extensively studied, despite its potential to further enhance extraction efficiency (Li et al., 2012). Heat-assisted UAE has been shown to improve the release of intracellular compounds by breaking down

protein structures and improving solvent penetration. However, excessive heat may lead to the degradation or modification of bioactive peptides, necessitating careful optimization of processing conditions (Zhang et al., 2018).

To address these research gaps, this study aims to investigate the effect of ultrasound-assisted extraction with and without heat treatment on the total carnosine content extracted from chicken breast meat. By comparing different UAE parameters, this study seeks to determine optimal conditions that maximize carnosine yield while preserving its functional properties. Additionally, this study will evaluate the antioxidant activity of carnosine extracted using UAE under different processing conditions. Given the role of carnosine as a potent antioxidant, assessing its bioactivity post-extraction will provide insights into the effectiveness of UAE in preserving its health-promoting properties.

The results of this study will help develop sustainable and effective extraction methods that maximize carnosine recovery while preserving its useful qualities for possible uses in the pharmaceutical, food, and nutraceutical sectors. Additionally, this work addresses the growing need for bioactive peptides and natural antioxidants in the production of functional foods, which is in line with the global trend toward environmentally friendly food processing methods. This study opens the door for future developments in peptide-based bioactive molecule recovery by investigating UAE as a novel extraction technique, which may have advantages for both industrial and human health.

CHAPTER 2

REVIEW OF LITERATURE

2.1. Carnosine: Structure and Bioactivity

Carnosine is a naturally occurring, water-soluble dipeptide composed of β-alanine and L-histidine, predominantly present in skeletal muscle, brain tissue, and other excitable tissues (Boldyrev et al., 2013). Its molecular structure features an imidazole ring from L-histidine that imparts antioxidant and metal-chelating properties, while β-alanine controls its synthesis rate (Schmid, 2010). In addition to carnosine, related dipeptides such as anserine (β-alanine–3-methyl-L-histidine) and homocarnosine (γ-glutamine–L-histidine) contribute to the overall bioactivity found in muscle tissues (Aristoy & Toldrá, 2004).

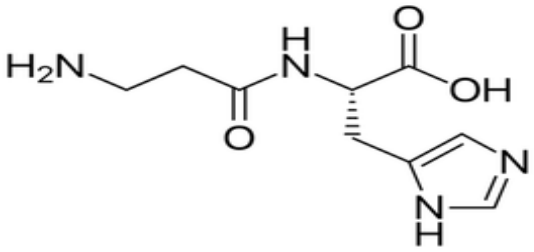


Figure 1: Molecular Structure of Carnosine (source : Cesak et al.,2023

By scavenging reactive oxygen species (ROS) and chelating metal ions, carnosine inhibits lipid peroxidation and stops the production of advanced glycation end-products (AGEs), acting as an endogenous antioxidant (Hipkiss, 2010; Chan & Decker, 1994). According to Decker et al. (2001), carnosine can prevent low-density lipoprotein (LDL) from oxidizing, which is the initial stage of atherosclerosis development. By stabilizing neuronal membranes and preventing neurotoxic damage in neurodegenerative diseases, carnosine has been demonstrated to increase cell longevity, preserve cellular homeostasis, and provide neuroprotective effects (Boldyrev et al., 2013).

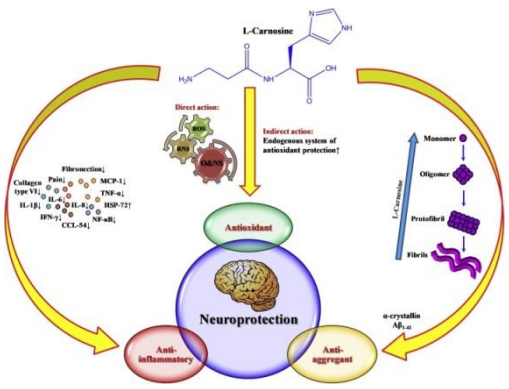


Figure 2: Bioactivity of Carnosine in Cellular Protection

2.2. Carnosine Content in Meat: Focus on Chicken Breast

Chicken meat, particularly chicken breast, is identified as one of the richest dietary sources of carnosine. Studies have reported that chicken breast contains approximately 180 mg of carnosine per 100 g, significantly higher than the 63 mg per 100 g found in chicken thigh muscle (Hu et al., 2009; Aristoy & Toldrá, 2004).This disparity is partly attributed to differences in muscle fiber type and metabolic activity. The high concentration of carnosine in chicken breast not only enhances its nutritional value but also makes it a prime candidate for extraction and further application in functional foods and nutraceuticals (Hu et al., 2009).

2.3. Extraction Methods for Carnosine

For bioactive substances, traditional extraction techniques include solvent extraction, Soxhlet extraction, and maceration. However, these techniques frequently have drawbacks, including the possibility for thermal degradation of heat-sensitive chemicals like carnosine, low yields, high solvent consumption, and lengthy extraction durations (Zhang et al., 2018; Li et al., 2012).

These limitations have prompted the search for more efficient and environmentally friendly extraction techniques that maximize carnosine recovery while preserving its functional integrity.

A green technology that addresses many of the shortcomings of conventional extraction techniques is ultrasound-assisted extraction (UAE). In order to improve extraction efficiency, UAE uses acoustic cavitation—the creation and dissolution of microbubbles—to break down cellular structures and increase solvent penetration (Chemat et al., 2017).UAE has been effectively applied to the extraction of polyphenols, flavonoids, and proteins from various food matrices (Guimarães et al., 2021)

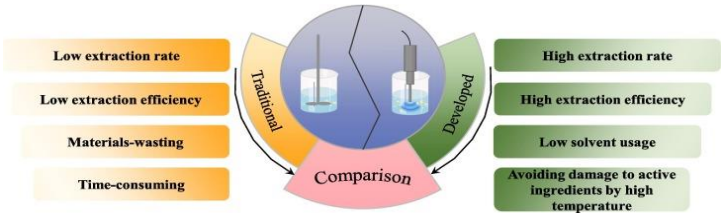


Figure 3: comparison between traditional extraction technique and UAE

Ultrasound is Sound waves with frequencies higher than 20 kHz are inaudible to humans.(Sanderson et al., 2004). Ultrasonic waves are defined by parameters like power, frequency, and intensity (UI), whereas equipment-specific characteristics include emitter type (ET), shape, and size (Carreira-Casais et al., 2021). Ultrasound power denotes the intensity of the rate of sound energy over time and is dependent on mass of solvent (m), its specific heat (Cp), and variation of temperature (T) (Toma et al., 2011). Frequency is of pivotal importance in ultrasound-assisted extraction (UAE), since it dictates physical as well as biochemical action due to collapse of bubble, diminishes refraction phase, and accelerates cavitation (Tiwari, 2015). Ultrasound intensity (UI), or power delivered per unit area, is another critical parameter; to maximize cavitation, UI must be kept at a minimum. Above the cavitation threshold, acoustic pressure is increased, resulting in excessive agitation, which distorts wave propagation and decreases cavitation efficiency (Santos et al., 2009). Increased ultrasound power enhances mechanical vibrations, which in turn increases the contact area between the solvent and plant tissue, leading to improved solvent penetration and finally increased extraction yield. Of all the parameters, the interaction among ultrasound power, treatment time, and solvent type is regarded as The most crucial elementaffecting UAE efficiency (Garcia-Castello et al., 2015)

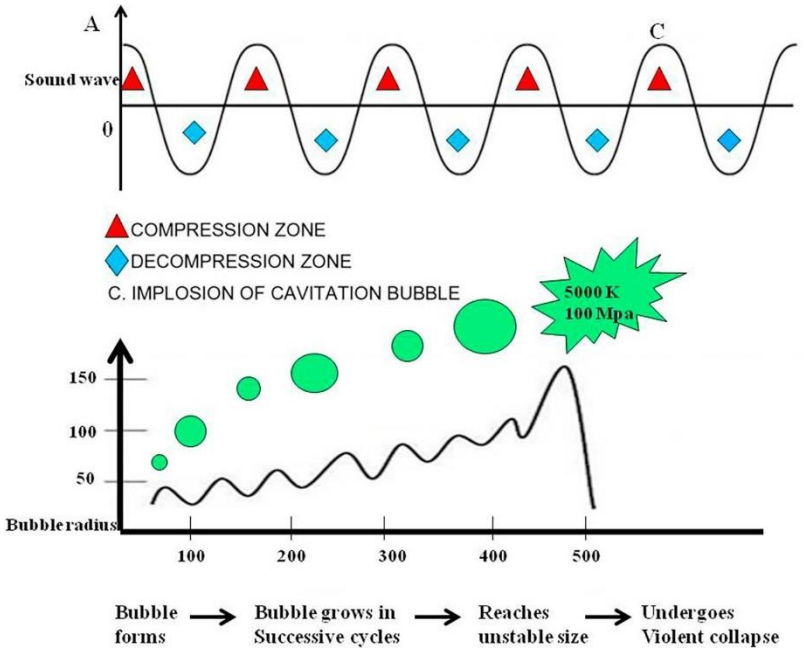


Figure 5: Principle of Ultrasound-Assisted Extraction

Advantages of UAE include:

1. Higher Efficiency: The cavitation effect enhances solvent access to intracellular compounds, leading to higher yields (Zhang et al., 2018).

2. Reduced Thermal Degradation: Operating at lower temperatures helps preserve the stability of heat-sensitive molecules like carnosine (Chemat et al., 2017).

3. Eco-Friendly Processing: UAE minimizes the use of hazardous organic solvents, aligning with green chemistry principles (Guimarães et al., 2021).

Despite these advantages, optimal UAE parameters—such as ultrasound power, frequency, extraction time, and solvent composition—require further investigation, particularly for carnosine extraction from chicken breast meat.

2.4. Heat Treatment in Ultrasound-Assisted Extraction

Heat treatment significantly affects **protein denaturation, aggregation, and solubility** in meat extracts. According to **Zhang et al. (2019),** proteins in meat extracts undergo structural changes due to elevated temperature, which ultimately alters the functional properties and s olubility. Mild heat treatment (50–70°C) may increase the extractability of proteins, while severe heat (over 90°C) will lead to the precipitation of proteins and low solubility.

2.5. Antioxidant Activity of Carnosine Extracted via UAE

Carnosine's antioxidant activity is central to its health-promoting effects, as it neutralizes ROS and chelates metal ions (Chan & Decker, 1994).Evaluating the antioxidant capacity of carnosine extracted using UAE is essential to determine its suitability for use in nutraceuticals and functional foods.Studies indicate that the reduced thermal degradation achieved by UAE may help preserve carnosine's antioxidant properties better than conventional extraction methods (Guimarães et al., 2021).

Carnosine is a multifunctional dipeptide with significant antioxidant and health-promoting properties, predominantly sourced from chicken breast meat. While conventional extraction methods are hindered by low efficiency and thermal degradation, ultrasound-assisted extraction offers a promising alternative that enhances yield and preserves bioactivity. The

incorporation of controlled heat treatment may further optimize extraction, though it requires careful calibration to prevent degradation. This study aims to evaluate UAE—with and without heat treatment—to determine optimal conditions for maximizing carnosine extraction and retaining its antioxidant properties.

CHAPTER 3

MATERIALS AND METHODS

This chapter describes the materials and methods applied for the ultrasound-assisted extraction of carnosine from chicken breast meat. It describes the collection of samples, preparation, extraction conditions, and analytical methods used to determine carnosine content. It also outlines the statistical analysis procedures applied to the data to confirm the accuracy and reliability of the results.

3.1 Materials, chemicals, and equipment

3.1.1 Raw material

Broiler chicken breast meat (skinless and boneless) was purchased directly from a single local chicken shop in Mysore, Karnataka, India (Plate 3.1). An ice box was used to carry the chicken from the shop to the laboratory, in order to avoid any kind of contamination.

Plate 3.1: Procurement of chicken from a local shop in Mysore

3.1.2 Chemicals

All the chemicals used for various property measurements were of analar/extrapure grade. Chemicals, whether used as additives or in analyses, were employed as received; their names and manufacturers are provided in Appendix A. Unless stated otherwise, glass distilled water (dw) was utilized in all analyses.

3.1.3 Equipment

The details of the various instruments, equipment, and accessories used in this study are enlisted in Appendix B.

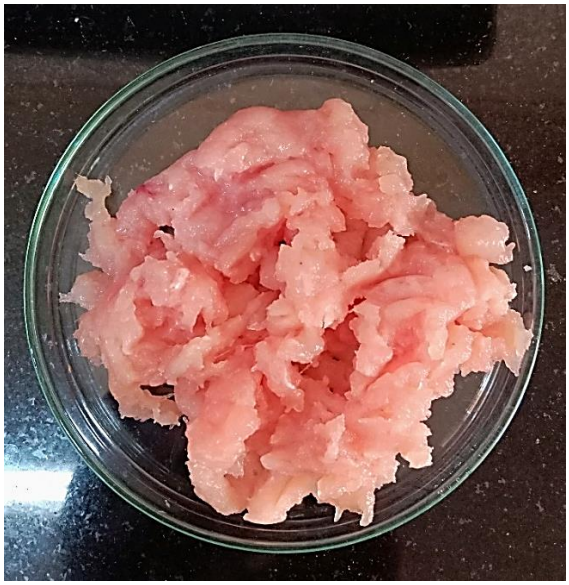
3.2 Methods

3.2.1 Mincing of chicken breast meat

About 500 g of the breast meat was minced twice using a meat mincer (vide Appendix B; Plate 3.2). The minced meat was packed in an LDPE pouch and kept in the freezer (0 °C) until further use. The minced meat was thawed in warm water for 50 minutes before being used for extraction and analysis purposes.

(a)

(b)



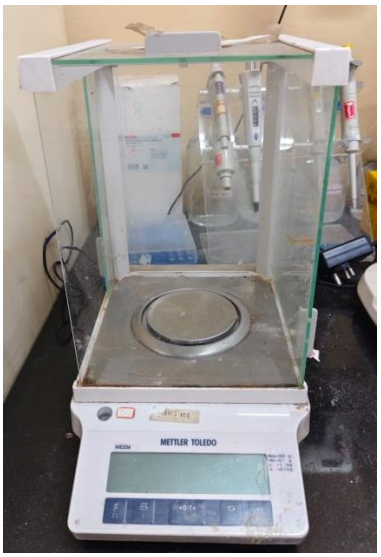
(c)

Plate 3.2: (a) Meat mincer, (b) Mincing of chicken breast meat; LDPE pouches were used for sample collection and storage, (c) Minced chicken breast meat.

3.2.2 Determination of the moisture content of the minced meat

The determination of moisture content was conducted using hot air oven drying method. About 5 g of minced breast meat sample (in triplicate) was taken in a pre-weighed glass petri plate and placed in a hot air oven (vide Appendix B; Plate 3.3a) set at 105°C. The drying was carried out until the attainment of constant weight.

(a)



(b)

(c)

(d)

Plate 3.3: (a) Hot air oven, (b) Weighing balance, (c) minced chicken meat taken for moisture determination, (d) chicken meat after drying at 105 °C in the hot air oven.

To measure weight, the samples were carefully transferred from the hot air oven to a desiccator to bring down the heated sample to room temperature and prevent moisture absorption from the surrounding environment. Once cooled, the final weight of each sample was recorded using a weighing balance (vide Appendix B; Plate 3.3b). The moisture content (%, wet basis or wb) was calculated using a standard equation that quantifies the percentage of water lost relative to the initial weight of the sample (Equation 3.1).

$$moisture\ content\ (\%) = \frac{initial\ weight\ of\ the\ sample - final\ weight\ of\ the\ sample}{initial\ weight\ of\ the\ sample} \times 100 \qquad (3.1)$$

3.2.3 Determination of pH of the minced meat

A five-gram minced meat sample was taken, and an equal volume of distilled water was added. The mixture was then homogenized using a vortex machine (vide Appendix B).

The pH meter (vide Appendix B; Plate 3.4) was calibrated with standard buffer solutions at pH 4.0, 7.0, and 10.0. The electrode was rinsed with distilled water and gently blotted dry with tissue paper after calibration with each of the three buffers. Next, the electrode of the pH meter was inserted directly into the homogenized meat sample. The pH reading was allowed to stabilize before being recorded. After the measurement, the electrode was rinsed with distilled water to remove any residue from the sample and stored in an appropriate storage solution as given in the manufacturer's instructions.

Plate 3.4: pH meter

3.2.4 Extraction of carnosine from minced chicken breast meat

The extraction of carnosine was conducted using four methods: (1) Hot water extraction, (2) Ultrasound-assisted extraction, (3) Ultrasound-assisted extraction using methanol as a deproteinizing agent, (4) Ultrasound-assisted heat extraction. The detailed procedures of each of the above-mentioned methods are described in this subsection.

3.2.4.1 Hot water extraction

Chicken extract was performed following the procedure of Maikhunthod & Intarapichet (2005, with slight modification (Figure 3.1). The meat was minced twice using a meat mincer. 10 g of

minced meat was homogenized with 100 ml of precooled (4 °C) deionized distilled water in a mixer grinder for 8 min. The homogenate was centrifuged at 10,000 rpm for 30 min at 4 °C, and then, the supernatant was filtered through Whatman filter paper no. 4. The filtrate was subjected to heat treatment at 80 °C for 10 min in a water bath for protein precipitation, and cooled in an ice bath. The heated extract was centrifuged at 10,000 rpm for 20 min to remove precipitated proteins. The supernatant was filtered through Whatman filter paper no. 4. The filtrate was then stored at 4 °C until further analyses.

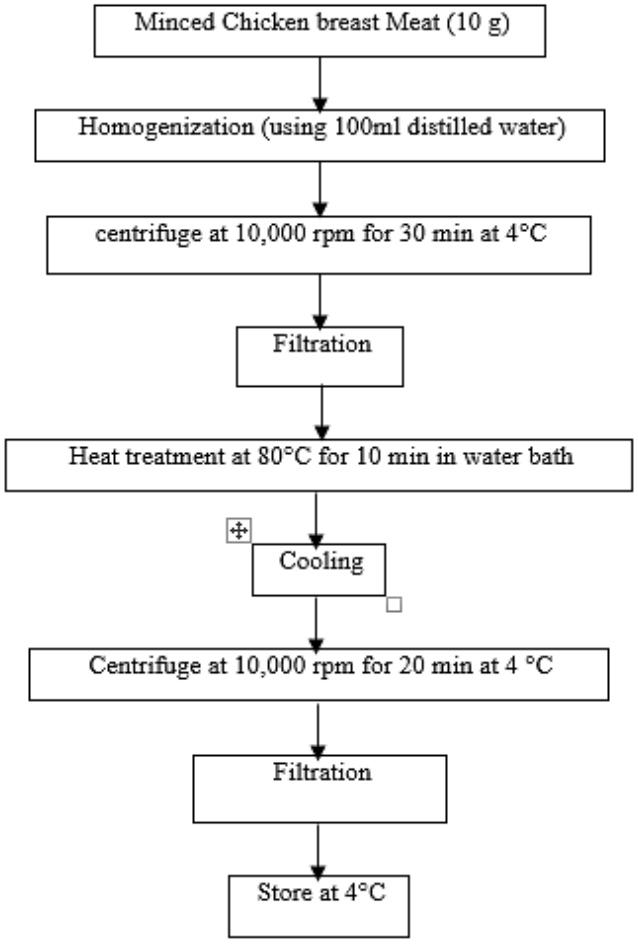


Figure 3.1: Flowchart of hot water extraction method.

3.2.4.2 Ultrasound-assisted extraction

Ultrasound-assisted extraction (UAE) is based on acoustic cavitation, which produces microscopic bubbles that violently collapse and create high shear forces and microjets. Cell wall disruption increases the release of various intracellular bioactive compounds (Plate 3.5). UAE integrates thermal, mechanical, and cavitation effects to enhance solvent penetration and mass transfer, thus increasing extraction efficiency. The mechanical stress induced by ultrasound decreases particle size, raises surface area, and enhances diffusion, thereby making

the process more energy-efficient and faster than the traditional approach. UAE also facilitates selective extraction by optimizing parameters like amplitude, pulse duration, and solvent type, which helps to minimize degradation of heat-sensitive compounds.

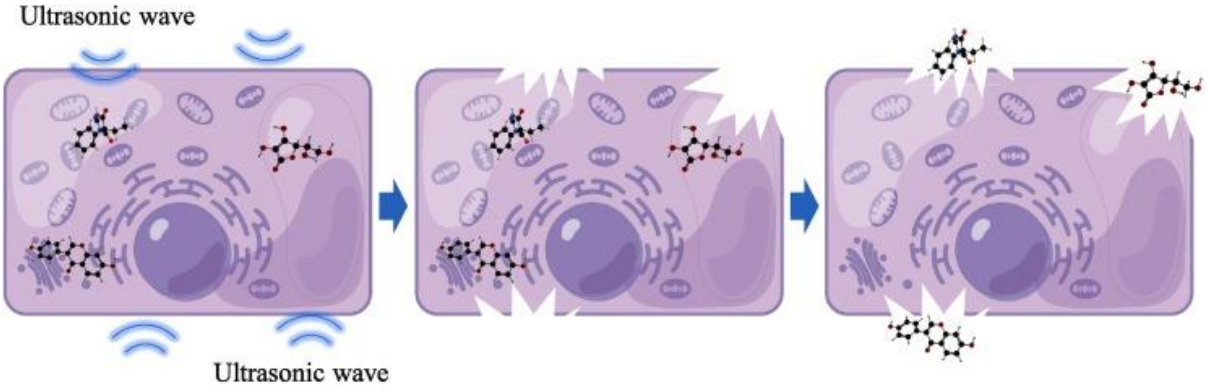


Plate 3.5: Mechanism of ultrasound induced cell disruption.

Equipment

An ultrasonic processor, with a power of 130 watts and 20 kHz frequency, was used for the present study (vide Appendix B; Plate 3.6). The processor was equipped with a 0.5-inch titanium probe having a tip diameter of 1/4" (6mm); this tip diameter was capable of processing sample volumes ranging from 10 mL to 50 mL. The piezoelectric transducer of the ultrasonic processor converts the electrical signal (20 kHz; derived from an AC power supply) into a mechanical vibration; which is then amplified and transmitted down the length of the probe through the longitudinal expansion and contraction of the probe tip. The distance the tip travels is dependent on the selected amplitude. The distance of one movement up and down is called its amplitude. The amplitude is adjustable. The maximum amplitude value for 1/4" probe is 120 µm. This equipment displays the amplitude in percentage (%). For example, at 100% setting, the probe will achieve an amplitude of 120 µm, and at 50% setting, the amplitude will be 60 µm. The minimum amplitude setting for this equipment is 20%. Amplitude and intensity have a direct relationship. The increase in the amplitude increases the sonication intensity within the sample. Power has a variable relationship with amplitude/intensity; its value depends on the viscosity of the sample. At a constant amplitude value, less power (in terms of wattage) is required for sonicating samples of lower viscosity compared to the power needed for highly viscous samples. Heat is generated during ultrasonication, especially after a prolonged exposure. Pulsing ultrasonics on and off helps to prevent heat build-up in temperature-sensitive samples. Moreover, pulsing may enhance processing efficiency by allowing the material to settle back under the probe after each burst. The ON and OFF pulse duration of the equipment

can be set independently from 01 second to 59 seconds. The total processing time will be different from the elapsed time in the pulsed mode as the processing time function monitors and controls only the ON portion of the duty cycle. For example, for 30 min processing time, the elapsed time will be 1 h if the ON and OFF cycle are set for 1 second.

Plate 3.6: Probe-type ultrasonic processor

Experiments for ultrasound-assisted extraction (UAE) of carnosine from chicken breast meat were conducted at various processing time and temperature levels. The other process parameters, viz., the amplitude and the ON and OFF pulse durations, were kept fixed at 80% (96 μm) and 15 s, respectively. To study the effect of temperature on the carnosine extraction, a circulator water bath (vide Appendix B) with an adjustable temperature set-up was assembled with the ultrasonic processor (Plate 3.6). Five grams of minced chicken breast meat was put in a 100 mL glass beaker, and 50 mL of DW was added to it. The beaker was then covered with a transparent stretch wrap. Next, the beaker was placed in the water bath (set at a particular temperature), and the ultrasonic probe was inserted into the beaker by piercing through the stretch wrap. The sides and end of the probe must not come in contact with anything but the sample solution. The depth of the probe within the liquid is a critical parameter. The probe should be immersed approximately halfway into the sample. Probe immersion near the solution surface will lead to air injection within the solution, resulting in foam formation. If the probe is immersed too deep, it may sonicate against the bottom of the vessel and the sample won't be homogenized effectively. The sample must flow freely below the tip to be mixed effectively.

Methodologies

a) UAE without application of heat and/or alcohol for deproteinization

The minced chicken breast meat was mixed with DW in a 1:10 (w/v) ratio (5 g of meat in 50 mL of DW). The mixture was placed in a 100 mL beaker and subjected to ultrasound-assisted extraction (UAE) using an ultrasonic probe (the method of probe insertion has been described above) at a pre-determined time-temperature combination. After ultrasound treatment, the homogenized mixture was centrifuged at 10,000 rpm for 30 minutes at 4 °C to separate the solid residues from the liquid extract. Next, the supernatant was collected and filtered through a Whatman no. 4 filter paper to obtain a clear chicken extract. The extract was immediately stored at 4°C until further analysis to prevent microbial growth and enzymatic degradation. The flowchart of this method is given in Figure 3.2.

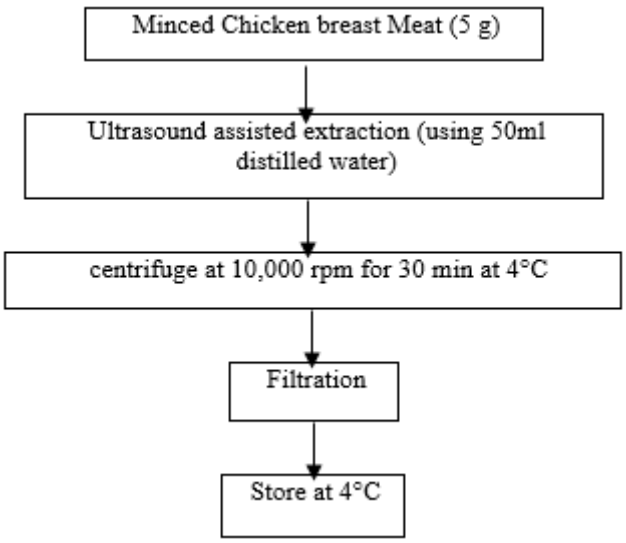


Figure 3.2: Flowchart of UAE without application of heat and/or alcohol for deproteinization.

b) UAE with the application of methanol for deproteinization

Here the extract was prepared as described in the previous section. The UAE extract was then further deproteinized by the method followed by Jozanović *et al.* (2017). After filtration of the extract, 300 µL of the aliquot (filtrate) was taken and mixed with 900 µL of methanol. The mixture was shaken well and kept at 4 °C for 15 min. It was then centrifuged at 10,000 rpm for 10 min to remove precipitated protein and particulate matter. Subsequently, 0.1 mL and 0.2 mL of the supernatant were taken and evaporated to dryness in a water bath at 80–85°C. The evaporated residue was supplemented with 0.5 mL of Milli-Q water, shaken well, and placed

in an ultrasonic bath for 10 minutes. Finally, the extract was stored at 4°C until further analysis. The flowchart of this method is given in Figure 3.3.

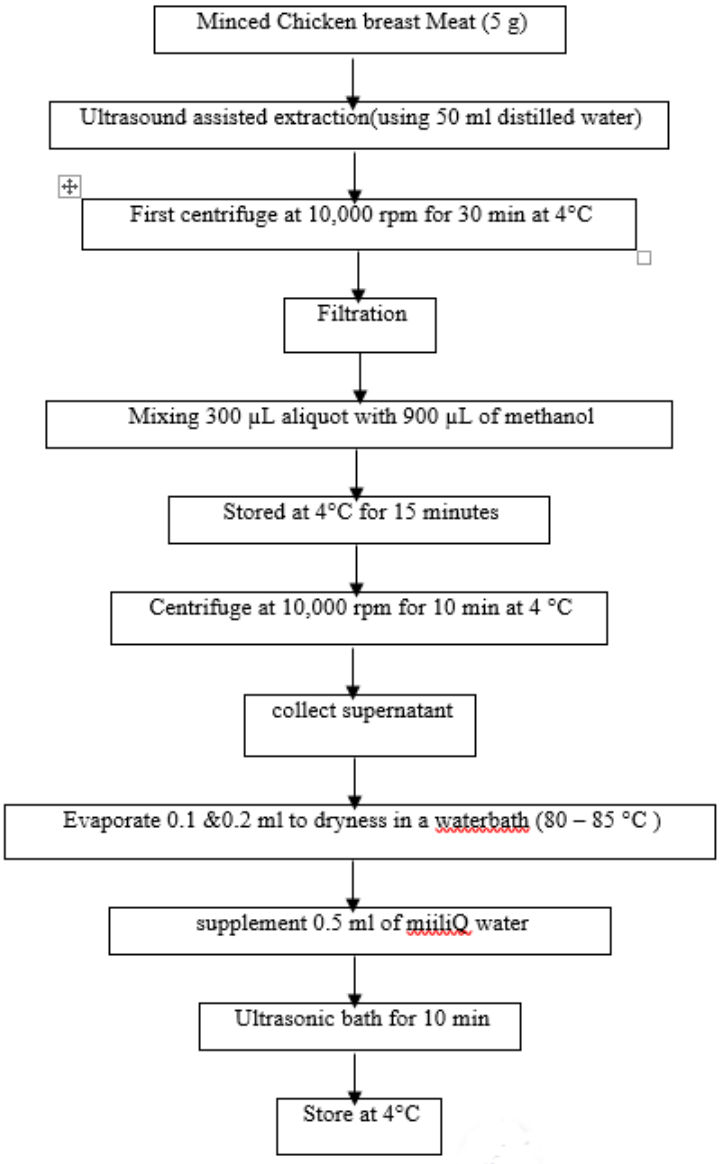


Figure 3.3: Flowchart of UAE with methanol application for deproteinization.

c) UAE with the application of heat for deproteinization

The filtered UAE extract, prepared as described in the sub-section 3.2.4.2a, was deproteinized by heating the extract at 80°C for 10 min. It was then cooled in an ice chamber, followed by centrifugation at 10,000 rpm for 20 minutes and subsequent filtration. The filtrate was stored at 4°C until further analysis. The flowchart of this method is given in Figure 3.4.

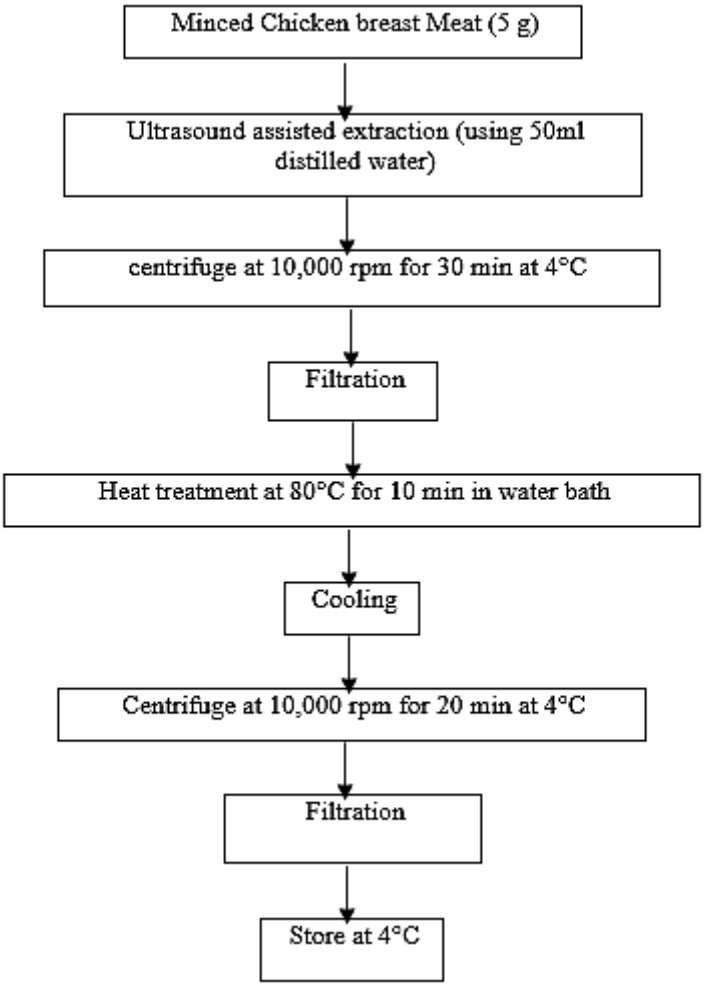


Figure 3.4: Flowchart of UAE with heat application for deproteinization.

3.2.5 Conduction of trials for fixing the range of process parameters of UAE methods

Several trial experiments were conducted to optimize and finalize the range of process parameters for the ultrasound-assisted extraction (UAE) process, specifically for extracting carnosine from chicken breast meat. Based on the results of these trials, the process optimization of UAE was conducted. The extract was subjected to ultrasound treatment at different levels of time, temperature, pulse, and amplitude.

3.2.5.1 Selection of the most effective sample-to-solvent ratio for UAE

The sample-to-solvent ratio is a critical parameter for ensuring efficient homogenization of the sample, which in turn directly affects the extraction efficiency of any extraction method.

Hence, in the first set of trials (Trial 1), three sample solutions were prepared with different sample-to-solvent ratios (Table 1). Fresh chicken breast meat was taken as the sample, and distilled water (DW) was used as the solvent. The three solutions were subjected to ultrasound treatment with 70% amplitude, at 25 °C for 30 min; the pulse rate was 30 s ON 30 s OFF. After treatment, they were analysed for antioxidant activity in terms of % radical scavenging activity (RSA) and total protein content (mg/g, db). The best sample-to-solvent ratio was selected based on the maximum retention of %RSA.

Table 3.1: Sample-to-solvent ratios of the samples taken for Trial 1

Wt. of sample (g)	Vol. of solvent (mL)	Sample-to-solvent	Sample %
1	50	1 : 50	2
5	50	1 : 10	10
10	50	1 : 5	20

3.2.5.2 Selection of the ranges of time and temperature for the process standardization of UAE

Two set of trial experiments were conducted for finalizing the ranges of time and temperature of UAE method. These ranges will be utilized for the statistical optimization of the time-temperature combination of UAE for carnosine extraction form chicken breast meat. During the first trial of this set (Trial 2), UAE with no deproteinization (sub-section 3.2.4.2a) was conducted. The objective was to check the effect of UAE method on the protein removal and retention of DPPH activity. Carnosine content was not measured in this trial. The details of trial 2 is presented in Table 3.2. The sample-to-solvent ratio was selected from the results of Trial 1 (vide sub-section 3.2.5.1).

Table 3.2: The independent and dependent variables, and the fixed parameters of Trial 2 of UAE method

Method(s)	UAE without application of heat and/or alcohol for deproteinization
Fixed parameters	• Pulse: 30 s ON 30 s OFF • Amplitude: 80%
Independent variables	• Time (min): 5 – 30 • Temperature (°C): 20 – 80 The rotatable central composite design (RCCD; an explanation of the design is given in subsection 3.2.6) was used for designing experiments using these independent variables. A total of 13 experiments were developed in the following manner:

Experiment no.	Time (min)	Temperature (°C)
1	8.66	28.79
2	26.34	28.79
3	8.66	71.21
4	26.34	71.21
5	5	50
6	30	50
7	17.5	20
8	17.5	80
9	17.5	50
10	17.5	50
11	17.5	50
12	17.5	50
13	17.5	50

Dependent variables	• Total protein content (mg/g, db) • % Radical scavenging activity (RSA) through DPPH assay

The second trial of this set (Trial 3) was conducted with a longer exposure time and lower temperature level. Most of the earlier studies on UAE indicated that high processing temperatures (> 70 °C) result in a higher loss of antioxidant activity. Longer exposure time allows for better extraction of the bioactives. The pulse was also reduced to 15 s ON 15 s OFF for better disintegration of the muscle tissue. In this trial, two methods of UAE were applied, (i) UAE with no deproteinization and (ii) UAE with the application of methanol for deproteinization. The details of Trial 3 are presented in Table 3.3.

Table 3.3: The independent and dependent variables, and the fixed parameters of Trial 3 of UAE method

Method(s)	• UAE without application of heat and/or alcohol for deproteinization • UAE with the application of methanol for deproteinization
Fixed parameters	• Pulse: 15 s ON 15 s OFF • Amplitude: 80%
Independent variables	• Time levels (min): 10, 30, 50 • Temperature levels (°C): 20, 40, 60 All the combinations of time and temperature were applied to develop following experiments:

Experiment no.	Time (min)	Temperature (°C)
1	10	20
2	30	20
3	50	20
4	10	40
5	30	40
6	50	40
7	10	60
8	30	60
9	50	60

Dependent variables	• Total carnosine content (mg/g, db) • Total protein content (mg/g, db) • % Radical scavenging activity (RSA) through DPPH assay

To check whether application of heat along with UAE can enhance the extraction of carnosine or not, another small trial (Trial 4) was conducted. In this set, all three variations of UAE treatment (sub-sections 3.2.4.2a, 3.2.4.2b and 3.2.4.2c) were attempted on the fresh chicken breast meat. The time of exposure was fixed at 50 min (highest level from Trial 3), and temperature was varied at two levels, 20 °C (lowest level from Trial 3) and 60 °C (highest level from Trial 3). The samples were analysed for carnosine content (mg/g, db), % RSA, and total protein content (mg/g, db). The details of Trial 4 are presented in Table 3.4.

Table 3.4: The independent and dependent variables, and the fixed parameters of Trial 4 of UAE method

Method(s)	• UAE without application of heat and/or alcohol for deproteinization • UAE with the application of methanol for deproteinization • UAE with the application of heat for deproteinization
Fixed parameters	• Pulse: 15 s ON 15 s OFF • Amplitude: 80%
Independent variables	• Time levels (min): 50 • Temperature levels (°C): 20, 60 All the combinations of time and temperature were applied to develop following experiments: Experiment no. | Time (min) | Temperature (°C) 1 | 50 | 20 2 | 50 | 60
Dependent variables	• Total carnosine content (mg/g, db) • Total protein content (mg/g, db) • % Radical scavenging activity (RSA) through DPPH assay

During each of the above trials, the chicken extracts obtained from hot water extraction (HWE; vide sub-section 3.2.4.1) were also evaluated for the selected responses. This method was considered as the reference method of carnosine extraction. Therefore, the results of the samples obtained UAE extracts were always compared with the HWE extracts to draw a definite conclusion. All the experiments were conducted in triplicates.

3.2.6 Process optimization of the UAE method for extraction of carnosine from the chicken breast meat using rotatable central composite design (RCCD) and response surface methodology (RSM)

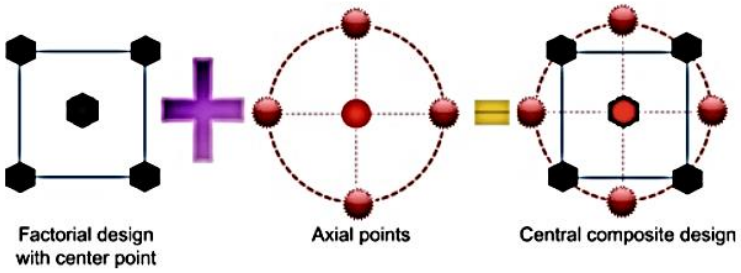
To find out the optimal process condition of the UAE method for extraction of carnosine from the chicken breast meat, process standardization of the UAE was conducted using rotatable central composite design (RCCD), a second-order design, and response surface methodology (RSM) through Design Expert Software Version 11.1.2.0 (Stat-Ease Inc., Minneapolis, USA).

3.2.6.1 Design of experiments using RCCD, their execution and analysis of the samples

Two independent variables/ factors viz., time of exposure (min) and temperature (°C) were chosen for RCCD experiments. The ranges of exposure time (10 – 50 min; real values) and temperature (4 – 40 °C min; real values) were determined based on the results of the trials described in sub-section 3.2.5.

In a central composite design (CCD), there are three different points (levels), namely factorial points $(-1, +1)$, central points (0) and axial points $(-a_m, +a_m)$. These levels are known as the coded values of the independent variables.

- **Factorial points** are the vertices of the n-dimensional cube that come from the full or fractional factorial design.
- **Central point** is the point at the center of the design space.
- **Axial (or star) points** are located on the axes of the coordinate system symmetrically with respect to the central point at a distance a_m from the design center.



There are two main varieties of CCD namely face-centred CCD or FCCD ($a_m = 1$) and rotatable CCD or RCCD ($a_m > 1$). As mentioned in the beginning, RCCD was used for our experiments. Now the total number of experiments (N) required by RCCD is the sum total of the 'factorial point' experiments (n_f) carried out at +1 and -1, 'axial point' experiments (n_a) carried out at levels $+a_m$ and $-a_m$, and 'center point' experiments (n_c) carried out at level 0 (given in Equation 3.2).

$$N = n_f + n_a + n_c \tag{3.2}$$

The required number of n_f is given by,

$$n_f = v^k \tag{3.3}$$

where, k is the number of independent variables, v is the number of levels or steps of variations for each of the independent variables. In RCCD, the value of v is kept fixed at 2 $(-1, +1)$. Therefore, $n_f = 2^k$.

Number of n_a to be carried out at $+a_m$ and $-a_m$ is given by,

$$n_a = 2k \tag{3.4}$$

where, k is the number of independent variables.

To find out the significance of the developed model for its adequacy in relating response y and independent variables x, 'pure experimental error' is required to be determined. For this, certain additional experiments are carried out at the 'center point' where the coded values of x are zero.

According to Myers (1971), the minimum number of n_c required for two independent variables is 5.

Therefore, for our study with two independent variables, $N = (2^2) + (2 \times 2) + (5) = 13$ experiments were generated.

As discussed till now, RCCD represents the real variable X in coded form x. The five levels of RCCD include the two primary factorial levels at the coded values of –1 and +1, one centre point (coded value 0) and two axial levels having coded values of $-a_m$ and $+a_m$. The coded value of a_m depends upon the number of variables.

$$a_m = \left(2^{number\ of\ variables}\right)^{0.25} \tag{3.5}$$

Here, two independent variables have been chosen. Therefore the coded value of $a_m = (2^2)^{0.25} = 1.4142$. The real values of $-a_m$ and $+a_m$ for the UAE exposure time are 10 min and 50 min, respectively. Similarly, the real values of $-a_m$ and $+a_m$ for temperature are 4 °C and 40 °C, respectively. The real values of –1 and +1 can be calculated using the relationship between the coded value x and real value X of the variables as given in the following equations:

$$X_M = \frac{X_{max} + X_{min}}{2} \tag{3.6}$$

$$X_D = \frac{X_{max} - X_M}{a_m} \tag{3.7}$$

$$x = \frac{X - X_M}{X_D} \tag{3.8}$$

where, X_{max} is the maximum value of X and $+a_m$ is the coded value associated with X_{max}, X_{min} is the minimum value of X and $-a_m$ is its coded value. X_M is the arithmetic mean of X_{max} and X_{min}. The real values of –1 and +1 for both the independent variables are calculated and presented in Table 3.5.

Table 3.5: Calculation of the real values of the coded forms –1 and +1

Independent variable	X_{min}	X_{max}	X_M	X_D	**Real value (X) when $x = -1$**	**Real value (X) when $x = +1$**
UAE exposure time (min)	10	50	$\frac{10 + 50}{2} = 30$	$\frac{50 - 30}{1.4142} = 14.14$	$\{(-1) \times 14.14\} + 30 = 15.86$	$\{(+1) \times 14.14\} + 30 = 44.14$
Temperature (°C)	4	40	$\frac{4 + 40}{2} = 22$	$\frac{40 - 22}{1.4142} = 12.73$	$\{(-1) \times 12.73\} + 22 = 9.27$	$\{(+1) \times 12.73\} + 22 = 34.73$

The five levels of the coded and real (actual) values of the two independent variables are tabulated in Table 3.6.

Table 3.6: Coded and actual values of the independent variables used in the experimental design

Symbol	Independent Variables	Coded values				
		-1.4142	-1	0	+1	+1.4142
		Real (Actual) values				
X_1	UAE exposure time (min)	10	15.86	30	44.14	50
X_2	Temperature (°C)	4	9.27	22	34.73	40

Table 3.7: RCCD experiments designed by Design Expert Software for the optimization of the process parameters of UAE for carnosine extraction from chicken

Std	Run	Experiment type	UAE exposure time (min)	Temperature (°C)
1	3	Factorial	15.86 (-1)	9.27 (-1)
2	8	Factorial	44.14 $(+1)$	9.27 (-1)
3	11	Factorial	15.86 (-1)	34.73 $(+1)$
4	6	Factorial	44.14 $(+1)$	34.73 $(+1)$
5	13	Axial	10 $(-a_m)$	22 (0)
6	9	Axial	50 $(+a_m)$	22 (0)
7	4	Axial	30 (0)	4 $(-a_m)$
8	12	Axial	30 (0)	40 $(+a_m)$
9	10	Center	30 (0)	22 (0)
10	7	Center	30 (0)	22 (0)
11	5	Center	30 (0)	22 (0)
12	1	Center	30 (0)	22 (0)
13	2	Center	30 (0)	22 (0)

The coded values of the independent variables are shown in the brackets beside the actual values.

The 13 RCCD experiments, as generated by the Design Expert software (Table 3.7), were conducted for the UAE methods selected from the Trial 4 results. The sample-to-solvent ratio was selected based on the result obtained from Trial 1. The amplitude and pulse rate of the ultrasonic processor were kept fixed at 80% and 15 s ON 15 s OFF, respectively. Each of the 13 experiments was conducted in triplicates. After UAE, the extracts were processed as

described in the sub-section 3.2.4.2, and stored at 4 ºC in a refrigerator before conducting evaluation tests.

The HWE and UAE chicken extracts were evaluated based on the carnosine content (mg/g of sample, db), % RSA and total protein content (mg BSA equivalent/g of sample, db). The details of the analytical procedures are discussed in the sub-section 3.2.7.

3.2.6.2 Determination of the best fit model

The three dependent variables (or responses) were expressed by second-order polynomial equation as a function of the two independent variables (Equation 3.9).

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (3.9)$$

where, β_0, β_1, β_2, β_{11}, β_{22} and B_{12} are model constants. β_1 and β_2 are the coefficients of the linear terms (viz., X_1 and X_2), β_{11} and β_{22} are the coefficients of the quadratic terms (viz., X_1^2 and X_2^2) and β_{12} is the coefficient of the interaction term (i.e., $X_1 X_2$). Equation 3.9 is called the regression equation or response surface methodology (RSM) model. The validation of the overall RSM model (Equation 3.9) was done through ANOVA on the basis of R^2 (coefficient of determination) and lack of fit data. Moreover, the experimental values of the responses were compared with their predicted values with the help of R^2 of actual versus predicted plot as well as residual plot (plot of the difference between actual and predicted values versus actual values). In ANOVA, the software also indicated if certain terms in overall equations are insignificant. Moreover, for any term in a model with coded value, a higher regression coefficient and a small p value indicates higher significant effect on the concerned dependent variable. Three-dimensional (3D) response surface plots of the fitted polynomial equations were also studied. Each response surface plot showed the response as a function of the interaction of the two independent variables.

3.2.6.3 Optimization of the process parameters

The optimum UAE exposure time (min) and temperature (min) was achieved following numerical optimization technique by applying desirability function approach. Each response is converted into desirability index at a range from "0" to "1." Concerning the aim of the study, "Goal" was set for each of the independent variables (in range) and responses (maximize). The target was to obtain maximum carnosine content and %RSA, and minimum total protein content; carnosine content and %RSA were assigned "++++" whereas total protein content was

assigned "+++" importance. Next the geometric mean of the individual desirability values was determined (overall desirability) to find out the effect on combined responses. The combination of independent variables generating highest overall desirability was selected as the "optimum" formulation. To validate the optimization process, a sample of minced chicken meat was subjected to UAE at the optimum levels of independent variables and analyzed for the selected responses. Absolute residual error (%) was calculated using the experimental and predicted (through best fit polynomial model) data by following Equation 3.10.

$$Absolute\ residual\ error\ (\%) = \frac{|Experimental\ value - Predicted\ value|}{Experimental\ value} \times 100 \qquad (3.10)$$

3.2.7 Evaluation tests of for the chicken breast extracts obtained from HWE and the UAE methods

3.2.7.1 Protein estimation using Lowry's method

Lowry protein assay, also known as the Lowry's method, was used for protein estimation. It involves combination of the Biuret reagent with the Folin-Ciocalteau phenol reagent, which interacts with the tryptophan and tyrosine residues present in protein. This reaction imparts bluish color to the solution, whose intensity can be measured in a UV-Vis spectrophotometer (Plate 3.7; vide appendix B) at a wavelength of 750 nm.

Plate 3.7: UV-Vis spectrophotometer

Preparation of the reagents

Four main reagents are required for the estimation of protein using Lowry's method. The composition and combination of the reagents are explained in Table 3.8.

Table 3.8: Preparation of reagent I and reagent II

Reagents	Composition
A	2% Na_2CO_3 dissolved in 0.1 N NaOH
B	2% Na-K Tartarate prepared in DW
C	1% $CuSO_4$. $5H_2O$ prepared in DW
D – ***Reagent I***	48 mL of A + 1 mL of B + 1 mL of C
E – ***Reagent II***	Folin-Ciocalteu reagent (5 mL diluted with 6 mL of DW)

Preparation of standard curve using bovine serum albumin (BSA) solution

A standard curve was prepared by dissolving 20 mg of bovine serum albumin (BSA) in 100 mL of distilled water (DW), giving a stock solution of 0.2 mg/mL strength. A series of dilutions were made with DW to obtain BSA solutions of different strengths (Table 3.9). The final volume of the BSA solution was made up to 1 mL. Next, 0.7 mL of Reagent I was added to 1 mL of the BSA solution and mixed properly using a vortex shaker (Plate 3.8). The solution was incubated at room temperature for 20 minutes. Then, 0.1 mL of Reagent II was added to the solution and mixed properly using a vortex shaker. The final solution was incubated for 30 minutes in the dark at room temperature. The blank solution was prepared by mixing 1 mL of DW with 0.7 mL of Reagent I and 0.1 Reagent II in the above-explained manner. The absorbances of the standard and blank solutions were read at 750 nm. The blank OD was subtracted from the OD of each of the standard solutions to cancel out the effect of color given out by the reagents alone. These final absorbances were plotted against the corresponding BSA concentrations to get a standard calibration curve. This standard graph was utilized for the estimation of the total protein present in the chicken extract.

Plate 3.8: Vortex shaker

Table 3.9: Preparation of different concentrations of BSA solution

BSA Concentration (mg)	Vol. of stock solution (strength 0.2 mg/mL) taken (mL)	Vol. of DW added (mL)	Total volume of BSA solution (mL)	Vol. of Reagent I	Vol. of Reagent II	Final vol. of solution taken for absorbance measurement at 750 nm (mL)
0	0	1	1	0.7	0.1	1.8
0.02	0.1	0.9	1	0.7	0.1	1.8
0.04	0.2	0.8	1	0.7	0.1	1.8
0.06	0.3	0.7	1	0.7	0.1	1.8
0.08	0.4	0.6	1	0.7	0.1	1.8
0.1	0.5	0.5	1	0.7	0.1	1.8
0.12	0.6	0.4	1	0.7	0.1	1.8
0.14	0.7	0.3	1	0.7	0.1	1.8
0.16	0.8	0.2	1	0.7	0.1	1.8
0.18	0.9	0.1	1	0.7	0.1	1.8
0.2	1	0	1	0.7	0.1	1.8

Estimation of total protein content in the chicken extracts

Precisely, 0.5 mL of chicken extract was taken in test tubes and diluted to 1 mL with DW. To this, 0.7 mL of Reagent I was added. Then the solution was mixed thoroughly by using a vortex shaken and incubated in dark at room temperature for 20 minutes. Then, 0.1 mL of Reagent II was added, mixed properly by the vortex shaker, and incubated in dark for 30 minutes at room temperature. Duplicate test tubes were prepared for each sample. The reading of the absorbance of unknown samples was taken at 750 nm. The total protein content (mg BSA equivalent/g of sample, db) was calculated using the regression equation obtained from the standard curve of BSA.

3.2.7.2 Determination of carnosine content in the chicken breast extracts

Carnosine content was determined using the spectrophotometric method described by Parker (1966). Here pure carnosine ($C_9H_{14}N_4O_3$) was used as standard.

Preparation of standard curve of pure carnosine

Carnosine solutions of different concentrations ranging from 0 – 0.3 mM were prepared using DW as the diluent (Table 3.10).

Table 3.10: Preparation of carnosine solutions of different concentrations

Concentration of carnosine (mol. wt. 226.23 g/mol) in			Vol. of DW mixed
mM	**mg**	**µg**	**(mL)**
0	0	0	1
0.05	0.0113	11.3	1
0.1	0.0226	22.6	1
0.15	0.0339	33.9	1
0.2	0.0452	45.2	1
0.25	0.0565	56.5	1
0.3	0.0678	67.8	1

Next, 1 mL of each of the carnosine solutions was mixed with 1 mL of 0.04 M Versene (EDTA in phosphate buffer saline), 1 mL of 20% Na_2CO_3, and 2 ml of diazotized p-bromoaniline (4-bromoaniline, 97%). The diazotization of p-bromoaniline is explained in Table 3.11. The reaction was stopped exactly 5 minutes after adding the diazonium salt by introducing 2 ml of 95% ethanol. The absorbance of the solutions was measured in a spectrophotometer (Plate 3.7) at 500 nm. The blank solution was prepared by mixing 1 mL od DW with the above-mentioned reagents. The blank OD was subtracted from the OD of each of the standard solutions to cancel out the effect of color given out by the reagents alone. These final absorbances were plotted against the corresponding carnosine concentrations (mM/mL converted to µg/mL) to get a standard calibration curve. This standard graph (µg/mL vs. OD) was utilized for the estimation of the carnosine content in the chicken extract.

Table 3.11: Preparation of diazotized p-bromoaniline

Reagents	Composition & preparation method
Stock solution of p-bromoaniline (x)	4.5 g of para-bromoaniline was dissolved in 45 ml of 37% HCl. The solution was diluted to 500 mL with DW.
Sodium nitrite solution (y)	25 g of 90% sodium nitrite was dissolved in DW and the volume was made up to 500 mL.
Diazotized of p-bromoaniline	• 1.5 mL of x and 1.5 mL of y was mixed in a 50 mL volumetric flask. • The flask was immersed in a water bath for 5 minutes.

- 6 mL of *y* was again added to the mixture in the flask.
- The flask was placed in an ice bath for 5 minutes.
- DW was used to make up the solution volume to 50 mL.
- The diluted solution was kept in the ice bath for at least 15 minutes before use.

Determination of carnosine content in the chicken extracts

To determine the amount of carnosine present in the chicken breast extract, 0.1 ml of the extract was mixed with 0.9 mL of DW, 1 ml of 0.04M Versene, 1 ml of 20% Na_2CO_3, and 2 ml of diazotized p-bromoaniline. The reaction was stopped exactly 5 minutes after adding the diazonium salt by introducing 2 ml of 95% ethanol. Duplicate test tubes were prepared for each sample. The reading of the absorbance of unknown samples was taken at 500 nm. The total carnosine content (mg/g of sample, db) was calculated using the regression equation obtained from the standard curve of pure carnosine.

3.2.7.3 Determination of the antioxidant activity of pure carnosine and the chicken extracts using DPPH Assay

The antioxidant activity of pure carnosine and the chicken extract was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical assay. The procedure described by Wu *et al.* (2003) was followed with few modifications. The antioxidant activity was expressed in terms of %RSA.

Determination of %RSA of pure carnosine

The %RSA of pure carnosine was evaluated to facilitate comparison with the %RSA of the unknown extracts. Carnosine solutions of different concentrations (0 – 120 mM) were prepared for the experiment (Mol. Wt. of carnosine is 226.23 g/mol) using Milli-Q water. Preparation of different concentrations of carnosine solution is explained in Table 3.12.

Table 3.12: Preparation of carnosine solutions of different concentrations

Concentration of carnosine (mol. wt. 226.23 g/mol) in		Vol. of DW mixed (mL)
mM	Mg	
0	0	1.5
5	1.697	1.5
10	3.393	1.5
20	6.787	1.5
30	10.18	1.5
40	13.574	1.5
50	16.967	1.5
60	20.36	1.5
70	23.75	1.5
80	27.14	1.5
90	30.54105	1.5
100	33.9345	1.5
110	37.32795	1.5
120	40.7214	1.5

About 1.5 mL of each of the carnosine solutions was mixed with 1.5 mL of 0.1 mM DPPH solution (prepared by dissolving 0.059 mg of DPPH in 1.5 mL of 99.99% ethanol; Mol. Wt. of DPPH is 394.32 g/mol). The mixtures were shaken and incubated in the dark for 30 min at room temperature. The absorbances of the resulting solutions were measured using spectrophotometer (Plate 3.7) at 517 nm against Milli-Q water as blank. The absorbance of a negative control sample containing 1.5 mL of milli-Q water and 1.5 mL of 0.1 mM DPPH was also measured. The DPPH % radical scavenging activity (RSA) of the pure carnosine solution was calculated using Equation 3.11.

$$\%\ Radical\ Scavenging\ Activity\ (\%RSA) = \frac{absorbance\ of\ control\ at\ 517\ nm - absorbance\ of\ sample\ at\ 517\ nm}{absorbance\ of\ control\ at\ 517\ nm} \times 100 \quad (3.11)$$

Determination of %RSA of chicken breast extracts

Precisely, 0.2 ml of the chicken extract was taken and diluted with 1.3 ml of Milli-Q water. The diluted extract (1.5 mL) was mixed with 1.5 ml of 0.1 mM DPPH radical, shaken well, and incubated for 30 min in dark at room temperature. The absorbances of the resulting solutions were measured using spectrophotometer (Plate 3.7) at 517 nm against Milli-Q water as blank. The sample containing 1.5 mL of milli-Q water and 1.5 mL of 0.1 mM DPPH was taken as negative control. The DPPH % radical scavenging activity (RSA) of the chicken extracts was calculated using Equation 3.11.

To express the antioxidant activity of the extracts in equivalence of the pure carnosine (mg carnosine equivalent/g of sample, db), the regression equation obtained from the standard curve of carnosine was used.

CHAPTER IV
RESULT AND DISCUSSION

This chapter reports and discusses the results of the experiments outlined in Chapter 3.

4.1 Determination of the moisture content and pH of the minced chicken breast meat

The moisture content of the minced chicken meat was found to be 74.90 ± 0.05%. Ahmad *et al.* (2018), stated that chicken meat is a perishable product containing more than 70% moisture.

The pH plays a vital role in determining meat quality, affecting its appearance, moisture retention, and microbial growth potential; higher pH levels favor microbial growth. The pH of the minced meat was calculated to be 5.69 ± 0.03. Hassanin *et al.* (2017) reported that the maximum pH of the chicken breast ranged from 5.7 to 5.96. The pH value is influenced by many factors including the different meat handling practices, storage conditions, and the time since it has been slaughtered.

4.2 Computation of the standard curves for the calculation of the total protein content, carnosine content and %RSA of the chicken extract samples

The standard curves, obtained from the analyses mentioned in the sub-sections 3.2.7.1, 3.2.7.2 and 3.2.7.3, were utilized for the generation of regression equations which helped in the calculation of the total protein content, carnosine content and %RSA of the chicken extract samples.

4.2.1 Standard curve of BSA

The plot of BSA concentration (mg) vs. the average OD of the BSA solutions at 750 nm is given in Figure 4.1. The trendline generated a high R^2 value of 0.9755, which indicated good linear fit. The regression equation $y = 4.7036x + 0.0885$ was utilized for the calculation of total protein content in chicken extract. Here, y denotes the OD value and x denotes the BSA concentration in mg. Hence, if the OD value of any unknown substance (in this case chicken extract) falls within the range of the standard curve, the concentration of protein in terms of mg BSA equivalent can be estimated by replacing the y in the regression equation with the OD of the unknown substance.

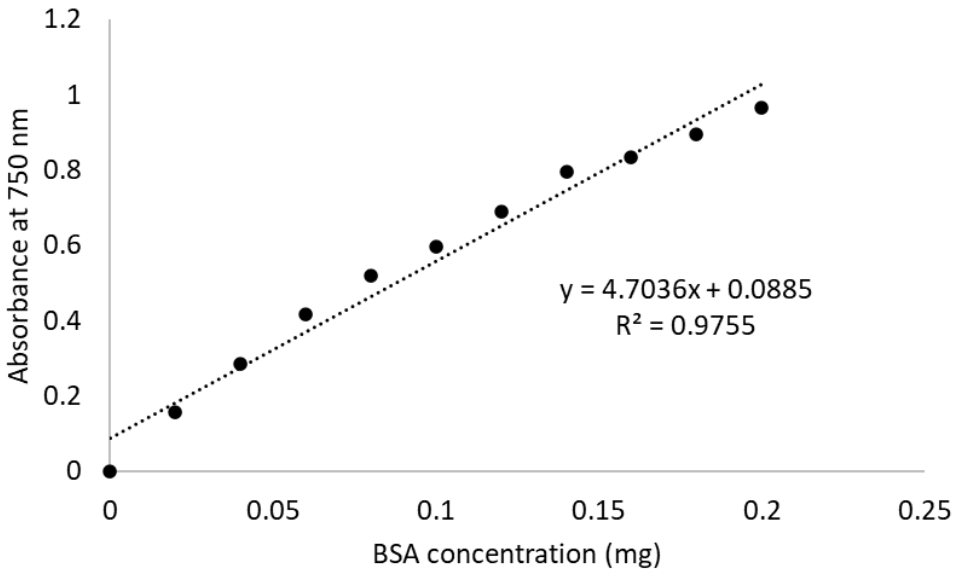


Figure 4.1: Standard curve of BSA.

The total protein content of the chicken extracts (both HWE and UAE extracts) in terms of mg BSA equivalent/ g of sample (wb) was calculated using Equation 4.1.

$$Total\ protein\ content\ (mg\ BSA\ equivalent/g\ of\ sample, wb) = \frac{\{(sample\ OD - Blank\ OD) - 0.0885\} \times total\ vol.of\ extract\ recovered\ (mL)}{4.7036 \times Vol.of\ extract\ taken\ (mL) \times wt.of\ the\ chicken\ sample\ (g)} \quad (4.1)$$

The dry basis (db) calculation was done taking into account the average moisture content of the chicken breast, 74.90 ± 0.05%; rounded off as 75%. It implies that 1 g of chicken breast contains 0.75 g of moisture and 0.25 g of bone dry material. Hence, the total protein content of the chicken extracts (both HWE and UAE extracts) in terms of mg BSA equivalent/ g of sample (db) was calculated using Equation 4.2.

$$Total\ protein\ content\ (mg\ BSA\ equivalent/g\ of\ sample, db) = \frac{Total\ protein\ content\ (mg\ BSA equivalent/g of\ sample, wb)}{0.25} \quad (4.2)$$

4.2.2 Standard curve of carnosine for quantitative determination of carnosine content

The plot of carnosine concentration (µg) vs. the average OD of the carnosine solutions at 500 nm is given in Figure 4.2. The trendline generated a high R^2 value of 0.9951, which indicated good linear fit. The regression equation $y = 0.0213x - 0.016$ was utilized for the calculation of total carnosine content in chicken extract. Here, y denotes the OD value and x denotes the carnosine concentration in µg. Hence, if the OD value of any unknown substance (in this case

chicken extract) falls within the range of the standard curve, the concentration of carnosine in terms of µg can be estimated by replacing the y in the regression equation with the OD of the unknown substance.

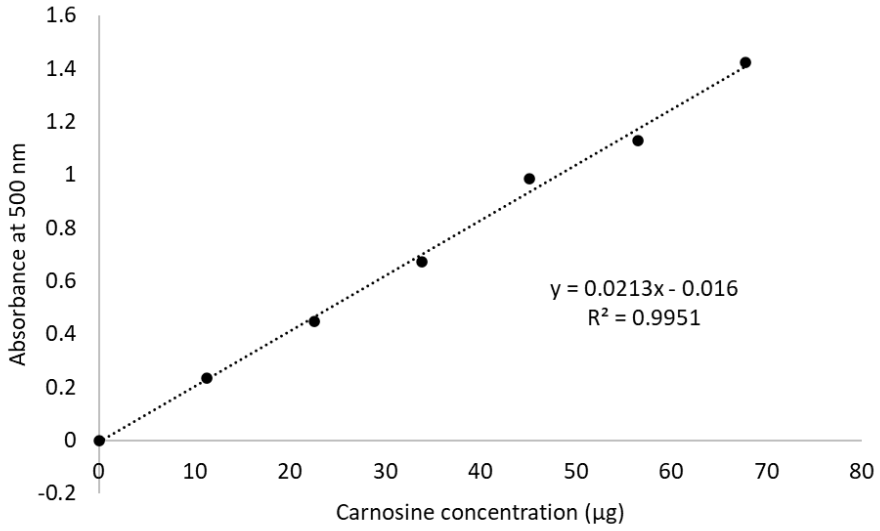


Figure 4.2: Standard curve of carnosine for determination of total carnosine content.

The total carnosine content of the chicken extracts (both HWE and UAE extracts) in terms of mg/ g of sample (wb) was calculated using Equation 4.3.

$$Carnosine\ content\ (mg/g\ of\ sample, wb) = \frac{\{(sample\ OD - Blank\ OD) + 0.016\} \times total\ vol. of\ extract\ recovered\ (mL)}{0.0213 \times Vol. of\ extract\ taken\ (mL) \times wt. of\ the\ chicken\ sample\ (g) \times 1000} \quad (4.3)$$

The dry basis (db) calculation was done taking into account the average moisture content of the chicken breast, 74.90 ± 0.05%; rounded off as 75%. It implies that 1 g of chicken breast contains 0.75 g of moisture and 0.25 g of bone dry material. Hence, the total protein content of the chicken extracts (both HWE and UAE extracts) in terms of mg / g of sample (db) was calculated using Equation 4.4.

$$Carnosine\ content\ (mg/g\ of\ sample, db) = \frac{Carnosine\ content\ (mg/g\ of\ sample, wb)}{0.25} \quad (4.4)$$

4.2.3 Standard curve of carnosine for determination of the antioxidant activity of the chicken extracts

The plot of carnosine concentration (mg) vs. the %RSA at 517 nm is given in Figure 4.3. The trendline generated a high R^2 value of 0.9847, which indicated good linear fit. The regression equation $y = 1.3064x + 4.4392$ was utilized for the determination of the antioxidant activity

of the chicken extract. Here, y denotes %RSA and x denotes the carnosine concentration in mg. Hence, if the %RSA value of any unknown substance (in this case chicken extract) falls within the range of this curve, the concentration of carnosine in terms of mg can be estimated by replacing the y in the regression equation with the %RSA of the unknown substance.

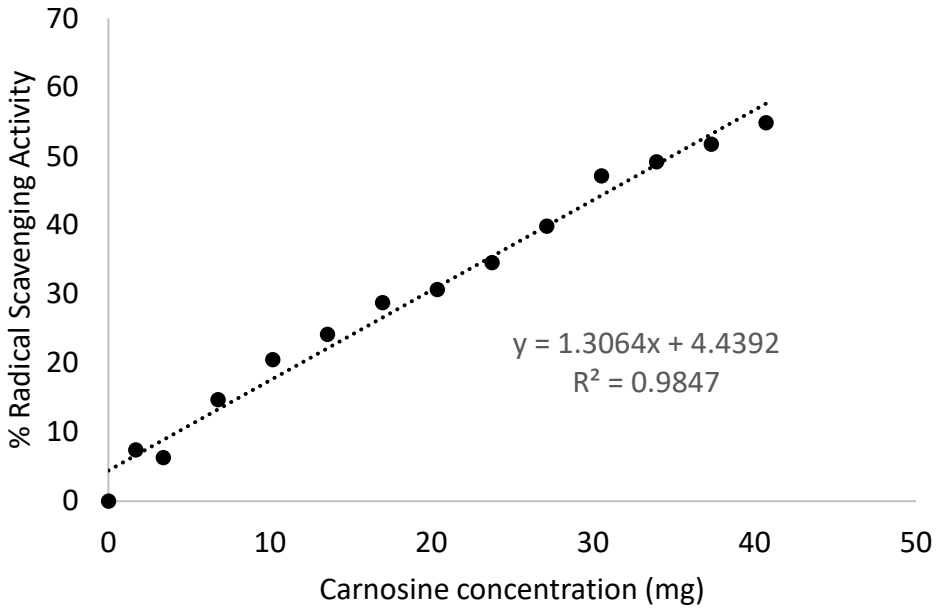


Figure 4.3: Plot of carnosine concentration (mg) vs %RSA.

The antioxidant activity of the chicken extracts (both HWE and UAE extracts) of the pure carnosine (mg carnosine equivalent/g of sample, wb) was calculated using Equation 4.5.

$$Antioxidant\ activity\ (mg\ carnosine\ equivalent/g\ of\ sample, wb) = \frac{(\%RSA - 4.4392) \times total\ vol.of\ extract\ recovered\ (mL)}{1.3064 \times Vol.of\ extract\ taken\ (mL) \times wt.of\ the\ chicken\ sample\ (g)} \quad (4.5)$$

The dry basis (db) calculation was done taking into account the average moisture content of the chicken breast, 74.90 ± 0.05%; rounded off as 75%. It implies that 1 g of chicken breast contains 0.75 g of moisture and 0.25 g of bone dry material. Hence, the antioxidant activity of the chicken extracts (both HWE and UAE extracts) in terms of mg carnosine equivalent/g of sample (db) was calculated using Equation 4.6.

$$Antioxidant\ activity\ (mg\ carnosine\ equivalent/g\ of\ sample, db) = \frac{Antioxidant\ activity\ (mg\ carnosine\ equivalent/g\ of\ sample, wb)}{0.25} \quad (4.4)$$

4.3 Selection of the most effective sample-to-solvent ratio for UAE

The mean values of % RSA and total protein content (mg BSA equivalent/g of sample, db) of the chicken extracts containing different sample-to-solvent ratios (Trial 1) are given in Table 4.1.

Table 4.1: Mean values of % RSA and total protein content () of the chicken extracts containing different sample-to-solvent ratios (Trial 1)

Wt. of sample (g)	Vol. of solvent (mL)	Sample-to-solvent	Sample %	%RSA	Total protein content (mg BSA equivalent/g of sample, db)
1	50	1 : 50	2	4.08	22.97
5	50	1 : 10	10	40.91	214.16
10	50	1 : 5	20	42.86	381.88

It was observed that 1:50 sample-to-solvent ratio exhibited very low % RSA. However, when the sample-to-solvent ratio was raised to 1 : 10, the antioxidant activity (%RSA) was increased by 36.83%. Further increase of sample-to-solvent ratio (1 : 5) increased %RSA by 1.95% only. Regarding total protein content, the increase in sample-to-solvent ratio from 2% to 10% , increased total protein extraction from 22.97 to 214.16 mg BSA equivalent/g of sample, db. At 1 : 5 ratio, protein extraction was increased by 167.72 mg BSA equivalent/g of sample, db. Our objective was to obtain maximum % RSA keeping the total protein yield low; this was necessary to maintain the selectivity of UAE treatments towards maximum carnosine extraction. Hence, 10% sample concentration (1 : 10 sample-to-solvent ratio i.e., 5 g minced chicken meat homogenized in 50 mL DW) was selected for all the subsequent UAE experiments. Plate 4.1 shows the UAE treated chicken solution containing 10% sample concentration.

Plate 4.1: UAE treated chicken solution containing 10% sample concentration.

4.4 Selection of the ranges of time and temperature for the process standardization of UAE

4.4.1 Observations from Trial 2

The %RSA (obtained from the mean value of the triplicate ODs at 517 nm) and the total protein content (obtained from the mean value of the triplicate ODs at 750 nm) of the 13 UAE extracts (no deproteinization step included) of Trial 2 are given in Table 4.2. The primary analysis of the RCCD experiments of Trial 2 gave significant models for both the responses, but the lack of fit came significant too. Hence, we did not proceed for optimization. The trend of the results indicated that %RSA increased negligibly with increase in UAE exposure time, but decreased sharply with increase in the extraction temperature. In case of total protein extraction, both time and temperature negatively impacted the protein yield, temperature having the more severe effect. This may have happended due to the tendency of protein denaturation at higher temperatures.

Table 4.2: %RSA and total protein content of chicken extract obtained from Trial 2

Experiment no.	Time (min)	Temperature (°C)	%RSA	Total protein content (mg BSA equivalent/g of sample, db)
1	8.66	28.79	25.139	217.92
2	26.34	28.79	25.556	167.94
3	8.66	71.21	11.806	19.55
4	26.34	71.21	13.056	21.08
5	5	50	14.514	109.23
6	30	50	14.514	42.57
7	17.5	20	22.10	236.61
8	17.5	80	5.46	22.67
9	17.5	50	15.000	55.39
10	17.5	50	14.899	58.43
11	17.5	50	15.012	60.03
12	17.5	50	15.245	54.39
13	17.5	50	14.765	55

4.4.2 Observations from Trial 3

The carnosine content (CC; obtained from the mean value of the duplicate ODs at 500 nm), %RSA (obtained from the mean value of the duplicate ODs at 517 nm) and the total protein content (PC; obtained from the mean value of the duplicate ODs at 750 nm) of the 27 UAE extracts (9 extracts of UAE without application of heat and/or alcohol for deproteinization and

18 extracts of UAE with the application of methanol for deproteinization) of Trial 3 are given in Table 4.3a, 4.3b and 4.3c.

Table 4.3a: Carnosine content, %RSA and total protein content of chicken extract obtained from UAE without application of heat and/or alcohol for deproteinization

Time (min)	Responses at different temperatures								
	CC (OD at 500 nm)*			%RSA			PC (mg BSA eqv/g, db)		
	20 °C	40 °C	60 °C	20 °C	40 °C	60 °C	20 °C	40 °C	60 °C
10	2.198	2.265	2.209	18.92	27.74	22.04	236.97	165.13	25.72
30	2.151	2.2495	2.2025	16.02	21.83	18.71	212.29	93.44	8.69
50	2.199	2.3075	2.1725	20.75	16.24	22.69	214.39	125.25	2.22

*the ODs were out of the range of standard curve, so carnosine content could not be calculated.

Table 4.3b: Carnosine content, %RSA and total protein content of chicken extract obtained from UAE with the application of methanol for deproteinization (0.1 mL extract taken for the analyses)

Time (min)	Responses at different temperatures								
	CC (mg/g, db)			%RSA			PC (mg BSA eqv/g, db)		
	20 °C	40 °C	60 °C	20 °C	40 °C	60 °C	20 °C	40 °C	60 °C
10	0.54	0.61	0.80	34.41	22.80	0.43	-31.91	-32.59	-31.87
30	0.50	0.51	0.73	13.44	14.19	-4.52	-35.45	-29.47	-31.80
50	0.50	0.78	0.73	13.33	6.34	-0.97	-31.39	-29.70	-29.94

Table 4.3c: Carnosine content, %RSA and total protein content of chicken extract obtained from UAE with the application of methanol for deproteinization (0.2 mL extract taken for the analyses)

Time (min)	Responses at different temperatures								
	CC (mg/g, db)			%RSA			PC (mg BSA eqv/g, db)		
	20 °C	40 °C	60 °C	20 °C	40 °C	60 °C	20 °C	40 °C	60 °C
10	0.87	0.85	1.03	26.77	13.87	2.04	-30.93	-28.44	-31.31
30	0.86	0.86	1.02	13.44	13.33	1.08	-32.04	-27.53	-28.11
50	0.56	1.00	0.92	17.20	7.63	2.58	-35.17	-21.95	-29.20

It was observed from Trial 3 that carnosine yield was much more in case of UAE without deproteinization compared to that obtained in the extracts of UAE with application of methanol for deproteination. This is evident from the high OD values of the UAE extracts without deproteination. Addition of methanol, stripped off the protein content drastically from the extracts (UAE with application of methanol for deproteination), which may have also resulted in the destruction of carnosine or loss of carnosine along with the precipitated proteins. Table 4.3a shows that %RSA increased till 40 °C at exposure times of 10 and 30 min; at 60 °C the value of RSA dropped a bit. For 50 min exposure, %RSA decreased at 40 °C and again increased at 60 °C; this trend seemed erroneous as carnosine content and total protein content decreased at 60 °C. Tables 4.3b and 4.3c showed that %RSA decreased both with increasing exposure time as well as increasing temperature; combination of longer time and higher temperature yield negative values in case of lower volume of extracts (Table 4.3b). Overall, it was observed that the extracts of UAE with no deproteinization step retained more antioxidant activity than the extracts of UAE with application of methanol for deproteination. This result is in agreement with the effect of methanol on the loss of carnosine and total protein content of the chicken breast extracts. The change of pulse rate from 30 s ON 30 s OFF to 15 s ON 15 s OFF increased the values of all the three responses indicating better extraction. Here after, 15 s ON 15 s OFF was kept fixed for all the experiments.

4.4.3 Observations from Trial 4

Since, 50 min exposure time gave fairly good yield of carnosine and retention of %RSA, the next trial (Trial 4) was conducted for this exposure time. Both 20 and 60 °C gave similar results for all the three responses. Therefore, Trial 4 was conducted at these two temperature levels under the same exposure time for drawing a robust conclusion. Again, application of methanol resulted in very low to negative values of carnosine and total protein content. Hence, for Trial 4, in addition to the two methods tested in Trial 3, UAE with application of heat for deproteinization was also tested. The temperature and time of heating was kept at 80 °C and 10 min, similar to that of HWE. The objective was to find out whether the combination of UAE and HWE can yield better results or not. The carnosine content (CC; obtained from the mean value of the duplicate ODs at 500 nm), %RSA (obtained from the mean value of the duplicate ODs at 517 nm) and the total protein content (PC; obtained from the mean value of the duplicate ODs at 750 nm) of the UAE extracts (UAE without application of heat and/or alcohol for deproteinization, UAE with the application of methanol and UAE with the application of heat for deproteinization) of Trial 4 are given in Table 4.4.

Table 4.4: Carnosine content, %RSA and total protein content of chicken extract obtained from UAE without heat (ND) with methanol (WMT), and with heat (WHT)

Temperature (°C)	Observations for 50 min UAE exposure								
	CC (mg/g, db)			%RSA			PC (mg BSA eqv/g, db)		
	ND	WMT	WHT	ND	WMT	WHT	ND	WMT	WHT
20	3.48	0.03	1.32	35.542	-3.441	12.41	230.40	-30.56	46.30
60	2.13	0.04	1.41	9.043	-4.301	9.15	16.62	-34.34	18.27

It was clear from the results of Trial 4 that, the application of methanol with UAE won't be helpful for significant extraction of carnosine from the chicken breast meat. Application of heat gave comparable results w.r.t. the results of UAE without heat (ND).

4.5 Process optimization of the UAE (both ND and WHT) of chicken breast meat using rotatable central composite design (RCCD) and response surface methodology (RSM)

The trials of sub-section 4.4 helped to find out the range of UAE time and temperature for carnosine extraction and its antioxidant activity. It also helped to shortlist the type of UAE treatment needed. Based on these understanding, statistical optimization of the UAE (ND and WHT) was conducted. The optimized results of the UAE extracts were compared with that of the HWE extracts.

The 13 RCCD experiments for each of the two UAE treatments (ND and WHT) were conducted in an ascending order and in three blocks i.e., the factorial point $(+1, -1)$ experiments were done first, followed by the axial point $(+a_m, -a_m)$ experiments and then center point (0) experiments. The RCCD samples were stored in the refrigerator (4 °C) after UAE (both ND and WHT) until the conduction of the evaluation tests. The values of the responses obtained from the 26 experiments conducted according to the RCCD design are given in Table 4.5.

Table 4.5: Effect of UAE process variables on the major responses

Std.	Level	Independent variables		Dependent variables					
		UAE exposure time (min)	Temperature (°C)	Carnosine content (mg/g, db)		RSA (%)		Total protein content (mg BSA eqv./g, db)	
				ND	WHT	ND	WHT	ND	WHT
1	Factorial	15.86 (-1)	9.27 (-1)	19.33	10.99	37.31	11.64	220.79	29.63
2	Factorial	44.14 $(+1)$	9.27 (-1)	21.43	11.43	34.63	11.54	259.64	37.25
3	Factorial	15.86 (-1)	34.73 $(+1)$	16.77	10.62	31.58	12.46	238.4	35.62
4	Factorial	44.14 $(+1)$	34.73 $(+1)$	17.3	11.63	25.4	13.78	194.92	21.46
5	Axial	10 $(-a_m)$	22 (0)	17.46	10.23	39.68	10.72	249.94	26.04
6	Axial	50 $(+a_m)$	22 (0)	20.17	11.38	28.82	11.31	246.7	28.95
7	Axial	30 (0)	4 $(-a_m)$	21.77	11.77	39.6	11.72	236.39	38.53
8	Axial	30 (0)	40 $(+a_m)$	16.58	11.4	25.23	13.99	183.92	29.07
9	Center	30 (0)	22 (0)	19.96	12.37	33.79	10.24	242.92	38.38
10	Center	30 (0)	22 (0)	19.53	12.16	31.35	11.24	234.47	41.36
11	Center	30 (0)	22 (0)	19.95	12.27	33.81	10.28	242.89	38.4
12	Center	30 (0)	22 (0)	19.54	12.30	31.36	11.21	234.37	41.33
13	Center	30 (0)	22 (0)	19.53	12.21	31.26	11.23	234.69	38.44

- For each of the experimental runs, three samples of 5 g of minced chicken breast meat were taken and homogenized in 50 ml of DW i.e., S1, S2 and S3. Therefore, the above-listed values are obtained from the mean of the ODs of the three replicate samples.
- The volume of extracts taken for carnosine content determination, DPPH assay (to calculate %RSA) and total protein content were 0.1 mL, 0.2 mL and 0.02 mL, respectively.
- The amplitude and pulse rate were kept constant at 80% and 15 s ON 15 s OFF, respectively (for both ND and WHT). The temperature and time for heating in case of WHT were 80 °C and 10 min.

Taking into account the actual values of responses obtained (Table 4.5), the RSM developed analysis of variance (ANOVA) data of the regression parameters, and coefficients of overall model(s) along with R^2 and adjusted R^2 values (for both ND and WHT), as presented in Table 4.6 and 4.7, respectively. For any of the terms of the model, a large regression coefficient β and a small p-value would indicate more significant effects on the respective response variables. The R^2 and adjusted R^2 values (Table 4.7) more than 0.8 and 0.75, respectively, and nonsignificant lack of fit (Table 4.6) ensure the goodness of fit of the model(s) for each of the responses. Using these overall models, the predicted values of the responses were evaluated. The R^2 of actual values versus predicted values plot, as well as the random residual plots further supported the adequacy of the developed model(s). Effect of independent variables significantly controlling the responses can be pictured clearly in the response surface plots (shown in terms of actual values) as discussed below.

Table 4.6: Analysis of variance (ANOVA) data for the regression parameters of the predicted response surface models (for both UAE-ND and UAE-WHT)

UAE – ND					
Source	**Sum of squares**	**Degree of freedom (df)**	**Mean square**	**F-value**	**p-value Prob > F**
Carnosine content (mg/g of sample, db)					
Model	32.80	5	6.56	74.99	< 0.0001
Residual	0.6123	7	0.0875	-	-
Lack of fit	0.3988	3	0.1329	2.49	0.1994
Pure error	0.2135	4	0.0534	-	-
Corrected total	33.41	12	-	-	-
RSA (%)					
Model	228.92	2	114.46	44.41	< 0.0001
Residual	25.77	10	2.58	-	-
Lack of fit	18.41	6	3.07	1.67	0.3234
Pure error	7.37	4	1.84	-	-
Corrected total	254.69	12	-	-	-
Total protein content (mg BSA equivalent/g of sample, db)					
Model	5252.28	5	1050.46	41.31	< 0.0001
Residual	177.66	7	25.38	-	-
Lack of fit	93.04	3	31.01	1.47	0.3503
Pure error	84.63	4	21.16	-	-
Corrected total	5429.95	12	-	-	-

UAE – WHT					
Source	**Sum of squares**	**Degree of freedom (df)**	**Mean square**	**F-value**	**p-value Prob > F**
Carnosine content (mg/g of sample, db)					
Model	5.53	5	1.11	163.65	< 0.0001
Residual	0.0473	7	0.0068	-	-
Lack of fit	0.0210	3	0.0070	1.07	0.4572
Pure error	0.0263	4	0.0066	-	-
Corrected total	5.57	12	-	-	-

RSA (%)					
Model	14.57	5	2.91	13.63	0.0017
Residual	1.50	7	0.2138	-	-
Lack of fit	0.3742	3	0.1247	0.4445	0.7344
Pure error	1.12	4	0.2807	-	-
Corrected total	16.07	12	-	-	-
Total protein content (mg BSA equivalent/g of sample, db)					
Model	462.53	5	92.51	24.54	0.0003
Residual	26.39	7	3.77	-	-
Lack of fit	16.03	3	5.34	2.06	0.2479
Pure error	10.36	4	2.59	-	-
Corrected total	488.92	12	-	-	-

Table 4.7: Actual values of regression coefficients of the best fit model obtained through RSM for each of the responses (for both UAE-ND and UAE-WHT)

Nature of best fit model	**UAE – ND**			**UAE – WHT**		
	Y1: Carnosine content (mg/g, db)	**Y2: *RSA* (%)**	**Y3: Total protein content (mg BSA eqv./g, db)**	**Y1: Carnosine content (mg/g, db)**	**Y2: *RSA* (%)**	**Y3: Total protein content (mg BSA eqv./g, db)**
	Quadratic	**Linear**	**Quadratic**	**Quadratic**	**Quadratic**	**Quadratic**
Coefficient						
β_0	+16.260***	+46.647***	+171.674***	+7.778***	+14.432**	-9.924***
Linear						
β_1	+0.260***	-0.214***	+0.926^{ns}	+0.230***	-0.083^{ns}	+2.431^{ns}
β_2	+0.019***	-0.347***	+6.057***	+0.063*	-0.300**	+1.442**
Interaction						
β_{12}	-0.002*	-	-0.114***	+0.001*	+0.002^{ns}	-0.030***
Quadratic						
β_{11}	-0.003**	-	+0.025*	-0.004***	+0.001^{ns}	-0.030***
β_{22}	-0.002*	-	-0.087***	-0.002***	+0.007***	-0.017**
R^2	0.98	0.90	0.97	0.99	0.91	0.95
Adjusted R^2	0.97	0.88	0.94	0.99	0.84	0.91

***significant at $p < 0.001$; ** significant at $p < 0.01$; * significant at $p < 0.05$; ^{ns}insignificant

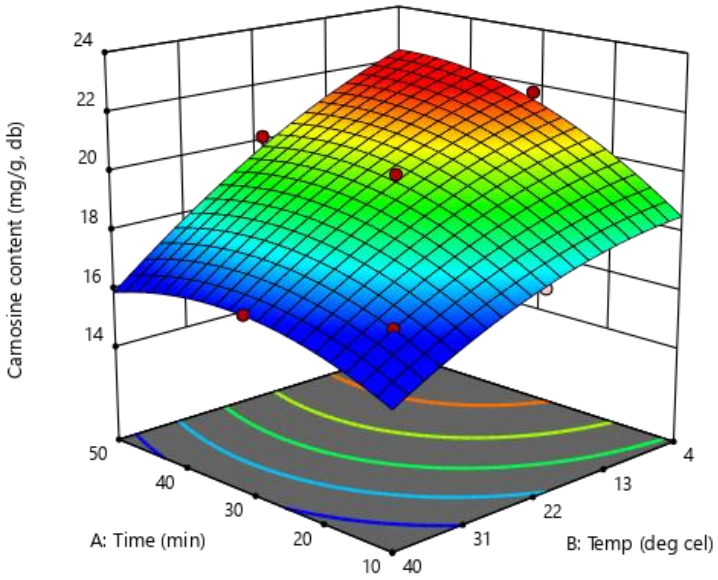
4.5.1 Effect of UAE-ND and UAE-WHT on the carnosine content, %RSA and total protein content of fresh chicken breast meat

4.5.1.1 Effect on the carnosine content

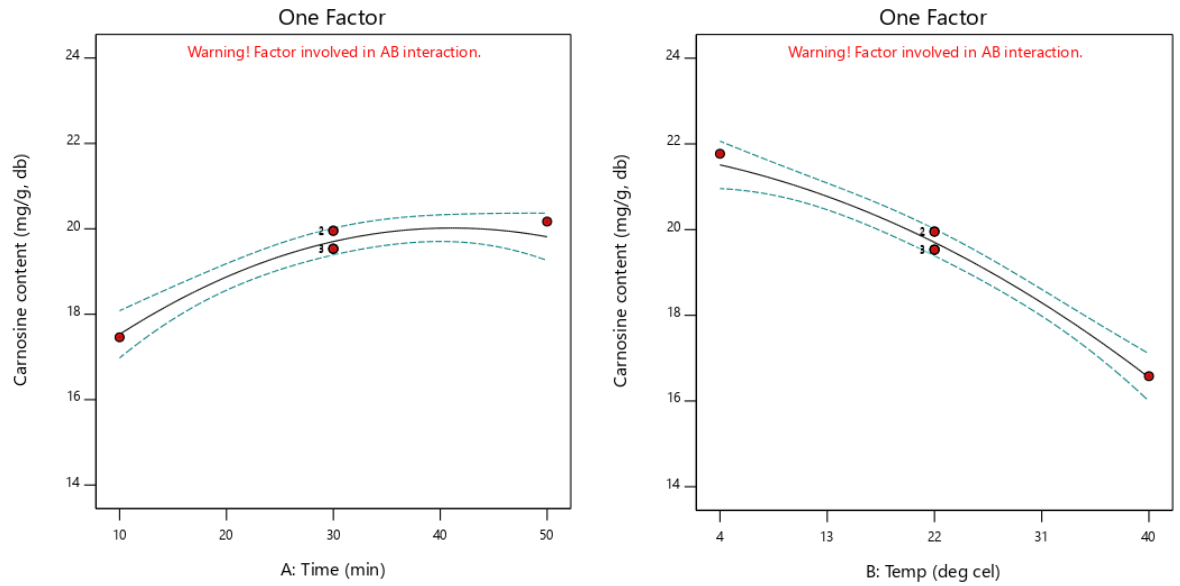
Effect of UAE with no heat treatment (ND)

The carnosine content of the UAE-ND samples varied from 16.58 to 21.77 mg/g of sample, db (Table 4.5). Figure 4.4a presents the response surface plot showing the simultaneous effect of the two independent variables on this response. According to ANOVA, the quadratic regression model suited this response. From Table 4.7, it is observed that the linear component of both the UAE exposure time and temperature have significant positive effect on the carnosine extraction

from chicken breast meat ($p < 0.001$). However, the interaction of time and temperature negatively impacted the extraction of carnosine ($p < 0.05$). The quadratic terms of time and temperature also exerted adverse effect on this response. This means that at higher values of these UAE process parameters carnosine extraction reduced. Curvature in the response surface plot also indicates the involvement of significant quadratic term. The exposure time ($p < 0.01$) have more prominent effect than temperature ($p < 0.05$). The individual effect of the process parameters of UAE-ND is explained by the One-factor plots (Figure 4.4b). It is seen from Table 4.7, that the coefficient value of the positive linear component of time is higher than the negative quadratic component of time. Therefore, exposure time has an overall positive effect on the extraction of carnosine. On the other hand, carnosine content was decreased with the increase in temperature, as depicted in the second graph of Figure 4.4b. However, the coefficient values of both the positive linear and negative quadratic components of temperature are lower than those of time. Hence, it can be said that the extraction of carnosine is more dependent on the extraction time than temperature. Looking at the overall analysis, it can be concluded that, when no heat is involved in the UAE, longer UAE exposure time at low temperature is required for achieving higher carnosine content in the chicken breast extract.



(a)



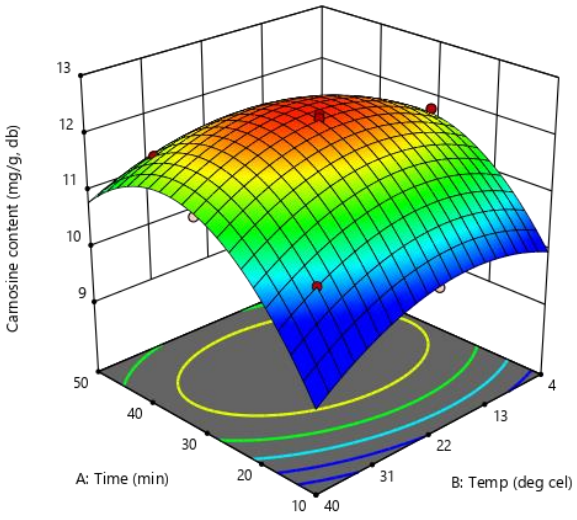
(b)

Figure 4.4: Effect of time and temperature on the carnosine content of UAE-ND chicken breast extracts.

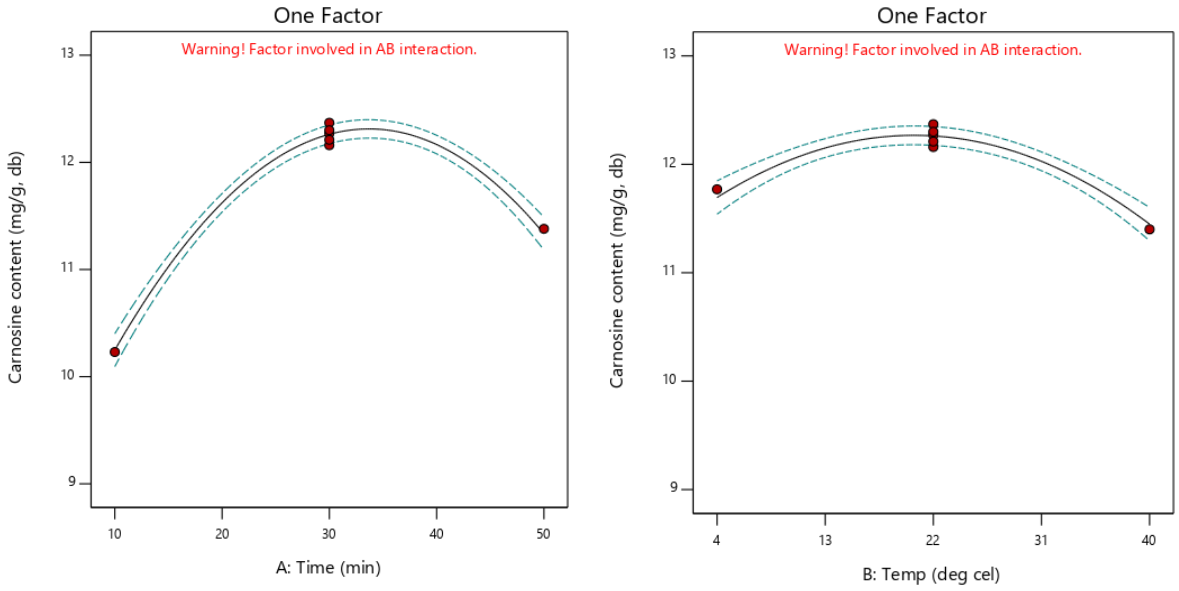
Effect of UAE with heat treatment (WHT)

The carnosine content of the UAE-WHT samples varied from 10.23 to 12.37 mg/g of sample, db (Table 4.5). Figure 4.5a presents the response surface plot showing the simultaneous effect of the two independent variables on this response. According to ANOVA, the quadratic regression model suited this response. From Table 4.7, it is observed that the linear component of both the UAE exposure time and temperature have significant positive effect on the carnosine extraction from chicken breast meat; time ($p < 0.001$) having more prominent effect than temperature ($p < 0.05$). The interaction of time and temperature also positively impacted the extraction of carnosine ($p < 0.05$), but the value of its coefficient is very low. The quadratic terms of time and temperature exerted adverse effect on this response ($p < 0.001$). This means that at higher values of these UAE process parameters carnosine extraction reduced. Curvature in the response surface plot also indicates the involvement of significant quadratic term. Like UAE-ND, in UAE-WHT the values of coefficients of the positive linear components of time and temperature are higher than the negative quadratic components of the process variables. The individual effect of the process parameters of UAE-WHT is explained by the One-factor plots (Figure 4.5b). The carnosine content increased linearly with time till 30 min of UAE-WHT. Carnosine extraction suffered a mild downward curve when the exposure time was

extended beyond 30 min. The effect of temperature on carnosine extraction was less effective than time as is clearly depicted by the one-factor plots (Figure 4.5b) and the value of its coefficients (Table 4.7). The extraction of carnosine increased slightly till around 20 - 22 °C; temperature above 22 °C led to a mild fall in the carnosine content of the extract. Looking at the overall analysis, it can be concluded that, when heat is involved in the UAE, UAE exposure time of 30 min and at temperature lower than or equal to 20 °C is required for achieving higher carnosine content in the chicken breast extract.



(a)



(b)

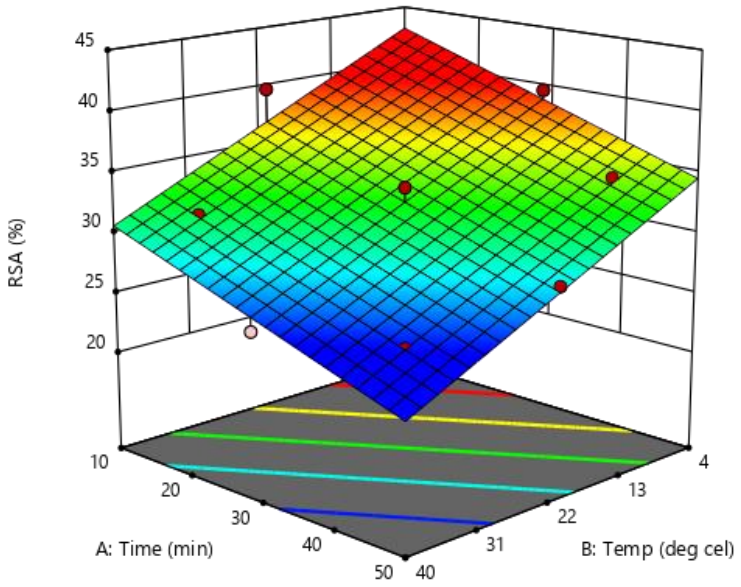
Figure 4.5: Effect of time and temperature on the carnosine content of UAE-WHT chicken breast extracts.

Comparing the values of the carnosine content of UAE-ND and UAE-WHT extracts, it was found that UAE-ND extracts have accumulated and retained higher carnosine than the UAE-WHT extracts.

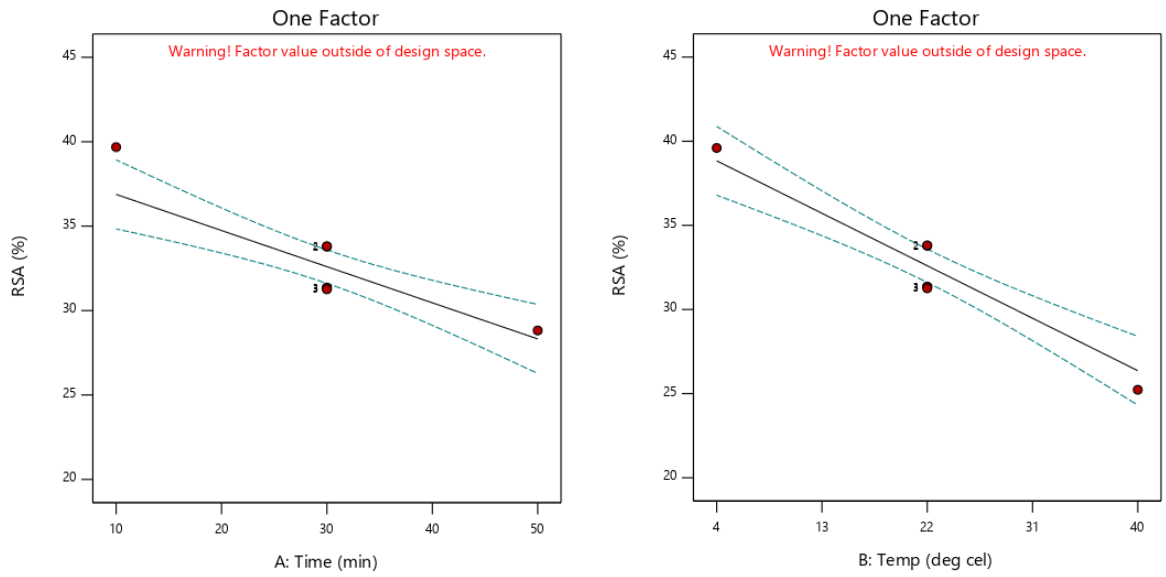
4.5.1.2 Effect on %RSA

Effect of UAE with no heat treatment (ND)

The % RSA of the UAE-ND chicken breast meat extract varied from 25.23% to 39.68% (Table 4.5). Figure 4.6a presents the response surface plot showing the simultaneous effect of the two independent variables on this response. According to ANOVA, the linear model suited this response. From Table 4.7, it is seen that the model was significant at $p < 0.001$. Both time and temperature exerted negative effect ($p < 0.001$) on the radical scavenging activity of the chicken extract. The one factor plots of Figure 4.6b, clearly shows that the %RSA linearly decreased with both extraction time and temperature. It is widely known that longer exposure under higher temperature leads to the destruction of the antioxidant activities of the bioactive compounds, especially for peptides like carnosine. Noticing the higher value of the coefficient of extraction temperature than the coefficient value of the UAE exposure time (Table 4.7), it is inferred that the *%RSA* of UAE-ND chicken extract is more dependent on the extraction temperature than the time of exposure. Overall, when no heat is involved in the UAE, shorter UAE extraction time and lower extraction temperature will help in retaining higher antioxidant activity of the chicken extract.



(a)

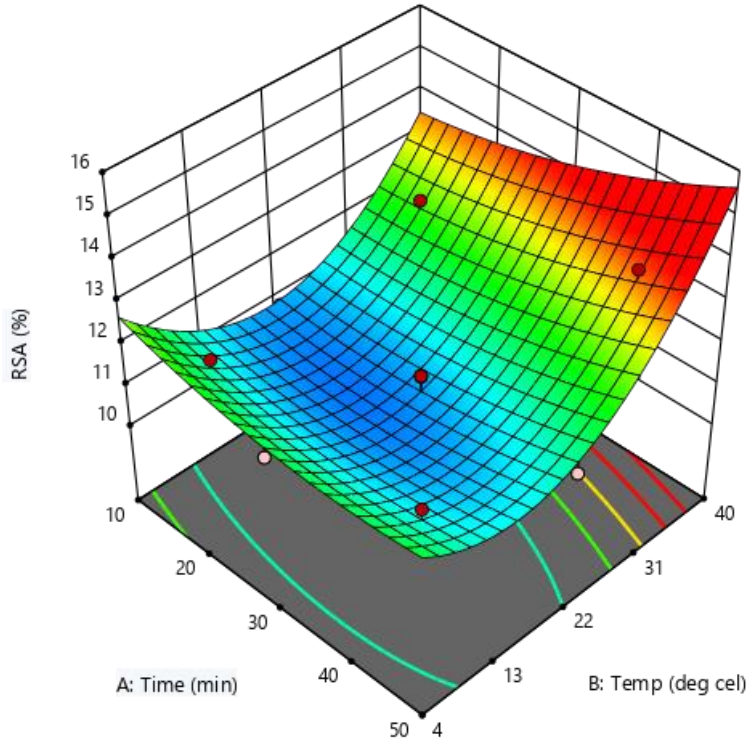


(b)

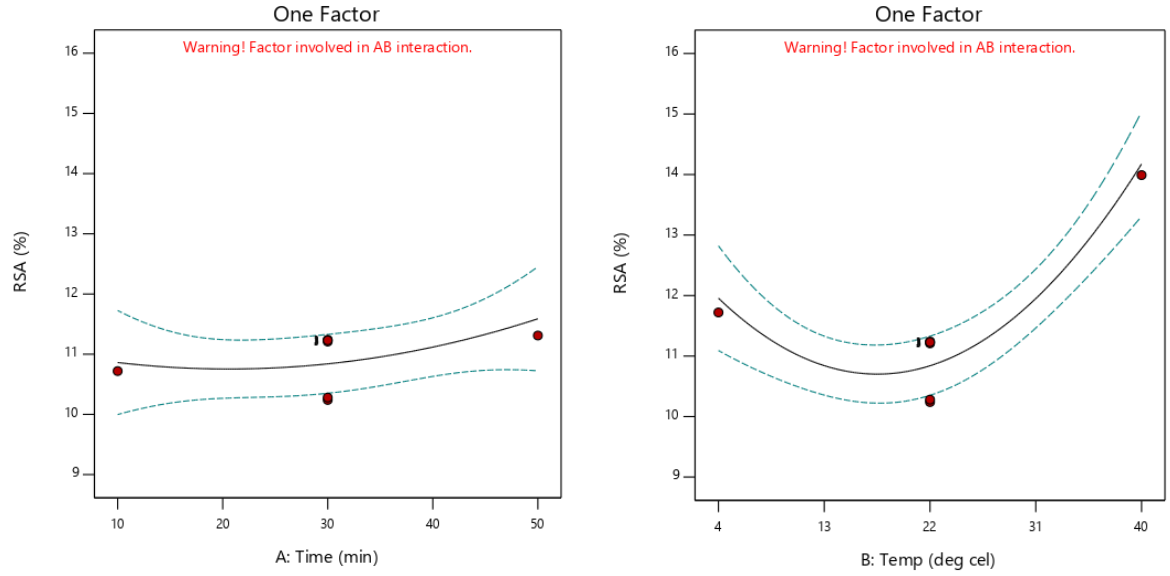
Figure 4.6: Effect of time and temperature on %RSA (antioxidant activity) of UAE-ND chicken breast extracts.

Effect of UAE with heat treatment (WHT)

The % RSA of the UAE-WHT chicken breast meat extract varied from 10.24% to 13.99% (Table 4.5). Figure 4.7a presents the response surface plot showing the simultaneous effect of the two independent variables on this response. According to ANOVA, the quadratic model suited this response. From Table 4.7, it is seen that the model was significant at $p < 0.01$. Both the linear and quadratic terms of time, and their interactive term were found to have statistically non-significant effect on the %RSA of the UAE-WHT chicken extract. The coefficient value of the negative linear component of temperature is higher than the coefficient value of the positive quadratic term of temperature, but the level of significance is less for the linear term ($p < 0.01$) than the quadratic term ($p < 0.00$). The second one factor plot of Figure 4.7b, shows that the %RSA linearly decreased with increase in temperature till 20 °C; a further increase in temperature led to an increase in %RSA. Although insignificant, extraction time also exerted a positive effect on this response. This result was completely contradictory to the results obtained for UAE-ND. If heat is involved with the proposed UAE method, %RSA was observed to increase with the increase in the extraction temperature and time; temperature being the major parameter.



(a)



(b)

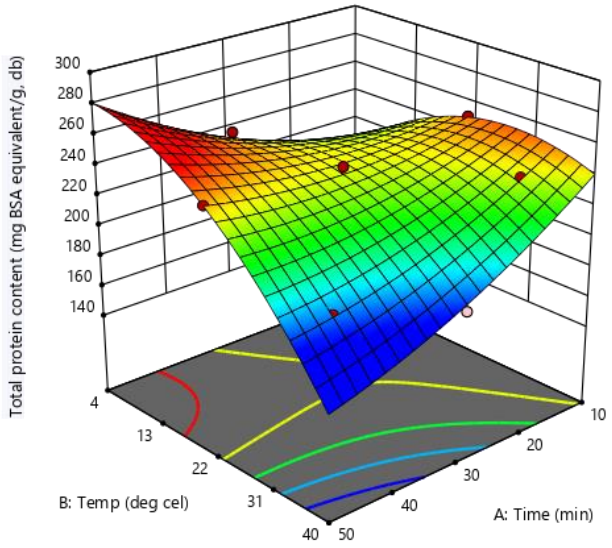
Figure 4.7: Effect of time and temperature on %RSA (antioxidant activity) of UAE-WHT chicken breast extracts.

Comparing the values of the %RSA of UAE-ND and UAE-WHT extracts, it was found that UAE-ND extracts have retained higher %RSA, thereby better antioxidant activity than the UAE-WHT extracts.

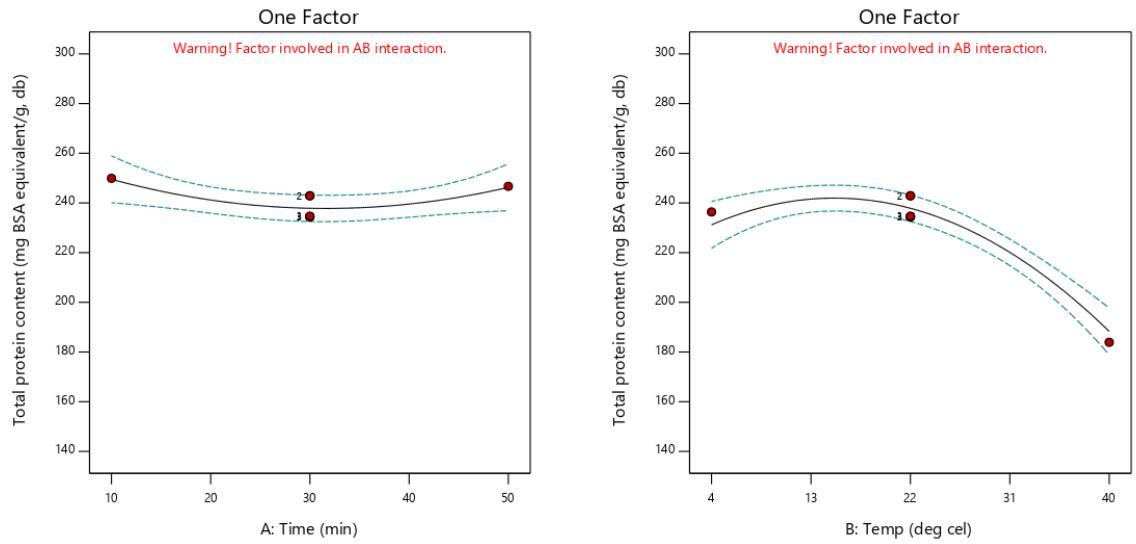
4.5.1.3 Effect on the total protein content

Effect of UAE with no heat treatment (ND)

The total protein content of the UAE-ND samples varied from 183.92 to 259.64 mg BSA equivalent/g of sample, db (Table 4.5). Figure 4.8a presents the response surface plot showing the simultaneous effect of the two independent variables on this response. According to ANOVA, the quadratic regression model suited this response. From Table 4.7, it is observed that the linear component of extraction time is statistically non-significant, whereas the quadratic component of time exerted favourable effect on the protein content at a lower significant level of $p < 0.05$. The positive linear component of temperature ($p < 0.001$) had a higher value of coefficient than the negative quadratic component of temperature ($p < 0.001$). The interaction of extraction time and temperature led to loss of total protein content from the extract ($p < 0.001$). The individual effect of the process parameters of UAE-ND is explained by the One-factor plots (Figure 4.8b). The first plot of 4.8b clearly explains that the time does not have any noticable effect on the total protein content. The second plot of 4.8b shows that the protein content is directly proportional to the extraction temperature at lower levels (< 20 °C), and becomes inversely proportional at higher temperatures. The fall in the total protein content can be attributed to the fact that protein denatures at higher temperatures. Hence, it can be concluded that the total protein content of the UAE-ND extracts can be deproteinized at higher extraction temperature for a longer extraction time.



(a)

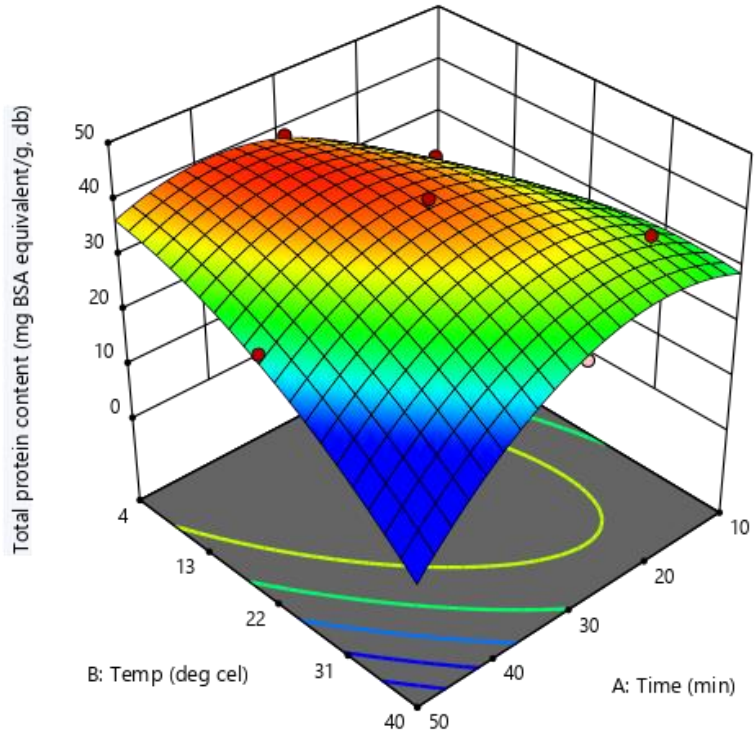


(b)

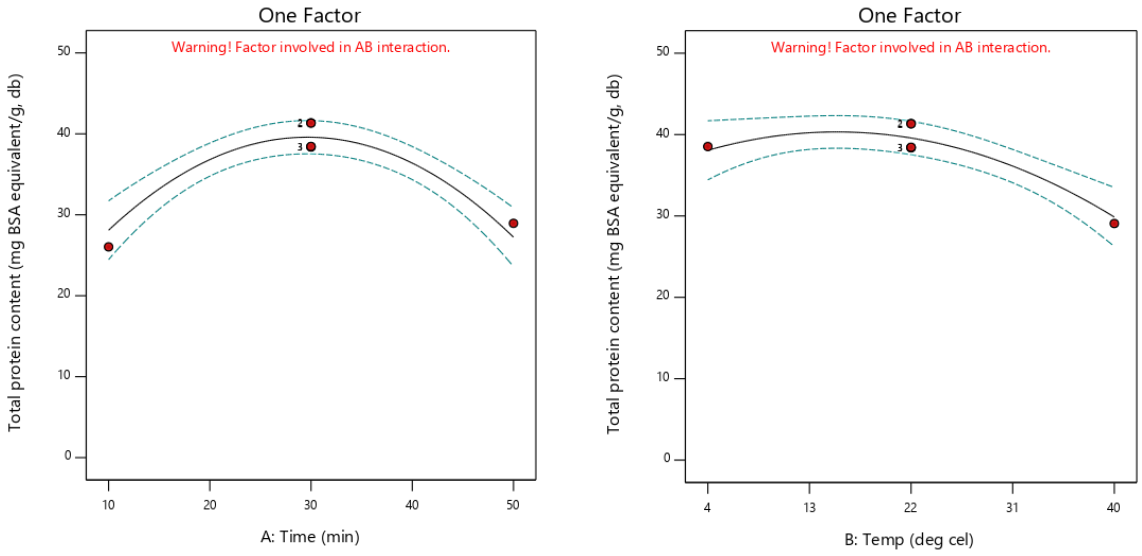
Figure 4.8: Effect of time and temperature on the total protein content of UAE-ND chicken breast extracts.

Effect of UAE with heat treatment (WHT)

The total protein content of the UAE-WHT samples varied from 21.46 to 41.36 mg BSA equivalent/g of sample, db (Table 4.5). Figure 4.9a presents the response surface plot showing the simultaneous effect of the two independent variables on this response. According to ANOVA, the quadratic regression model suited this response. From Table 4.7, it is observed that the linear component of extraction time is statistically non-significant, whereas the quadratic component of time exerted negative impact on the protein content ($p < 0.001$). The positive linear component of temperature ($p < 0.01$) had a higher value of coefficient than the negative quadratic component of temperature ($p < 0.01$). The interaction of extraction time and temperature led to loss of total protein content from the extract ($p < 0.001$). The individual effect of the process parameters of UAE-ND is explained by the One-factor plots (Figure 4.9b). The curvature of the first plot of 4.9b establishes the prominence of the quadratic effect of time on the total protein content; the total protein extraction increased with the increase in time till 30 min of exposure time, later it gradually declines. The second plot of 4.8b shows that the increase in the extraction temperature led to a steady decrease of the protein content. Hence, it can be concluded that the total protein content of the UAE-WHT extracts can be deproteinized at higher extraction temperature for a longer extraction time. This trend is in agreement with the results obtained for UAE-ND.



(a)



(b)

Figure 4.9: Effect of time and temperature on the total protein content of UAE-WHT chicken breast extracts.

Comparing the values of the total protein content of UAE-ND and UAE-WHT extracts, it was found that UAE-ND extracts have accumulated and retained higher protein content than the

UAE-WHT extracts. The higher protein content in the extract may resulted in the retention of more carnosine in the UAE-ND extracts.

4.5.2 Computation of the optimized parameters of UAE-ND and UAE-WHT and comparison of the optimized values of UAE extracts with that of HWE extracts

The above discussion indicates that both the independent variables had considerable effects on the three selected responses. And, the combination of the UAE exposure time and temperature for minimum or maximum value was response specific. Hence, optimization is needed to attain the process condition of UAE-ND and UAE-WHT required for the maximal extraction of carnosine from the chicken breast meat and the maximum retention of its %RSA with minimum total protein content in the extract . It is reiterated that the basis of followed optimization was numerical optimization with desirability function approach to attain maximum of all the responses, as has been described in section 3.2.6.3.

The optimized levels of the process parameters of UAE-ND and UAE-WHT corresponding to highest desirability, along with the experimental and software generated predicted values of the responses are presented in Table 4.8. The experiments were conducted in triplicates. The absolute residual error values (Equation 3.10) are also mentioned in the same table, which for all the three responses (for both UAE-ND and UAE-WHT) were found out to be less than 10%. This indicated precision of the regression model developed vis a vis optimized combination of the ozone inlet and time. As observed in Table 4.7, the R^2 and adjusted R^2 values of all the responses remained above 0.80 and 0.75 indicating high goodness of fit of the developed model. The non-significant lack of fit (Table 4.6) also supported the above statement for each of the responses.

Table 4.8: Validation of non-linear regression model for both UAE-ND and UAE-WHT

Extraction method	Optimum process parameters	Responses						Desirability
		Carnosine content (mg/g, db)		RSA (%)		Total protein content (mg BSA eqv/g, db)		
		Experimental value	Predicted value	Experimental value	Predicted value	Experimental value	Predicted value	
UAE-ND	**Exposure time (min): 25.099 ≈ 25 Temperature (°C): 4**	19.121	20.977	38.23	39.888	237.84	222.115	0.798
Absolute Residual Error (%)		9.71		4.34		6.60		
UAE-WHT	**Exposure time (min): 42.998 ≈ 43 Temperature (°C): 36.649 ≈ 37**	12.453	11.588	13.770	13.990	19.562	21.460	0.859
Absolute Residual Error (%)		6.95		1.60		9.70		

Table 4.9: The carnosine content, %RSA and total protein content of HWE extract

Extraction method	Process parameters	Responses		
		Carnosine content (mg/g, db)	RSA (%)	Total protein content (mg BSA eqv/g, db)
Hot water extraction	**Heating time (min): 10 Temperature of hot water (°C): 80**	10.34	10.38	9.71

About 5 g of minced chicken breast meat was homogenized in 50 mL of DW (10% sample concentration) and subjected to hot water extraction (HWE) at 80 °C for 10 min. The resultant extract was evaluated for carnosine content, %RSA and total protein content. The values of the responses are tabulated in Table 4.9. Comparing these values with the optimized values of UAE-ND and UAE-WHT, we observed that UAE-ND extracts gave highest values for all the responses, followed by UAE-WHT and HWE extracts. The carnosine content of UAE-ND, UAE-WHT and HWE extracts were estimated to be 19.121 mg/g (db), 12.454 mg/g (db) and 10.34 mg/g (db), respectively. Hence, UAE-ND and UAE-WHT led to an increase in the carnosine extraction by 8.781% and 2.114%, respectively. The %RSA of the UAE-ND and UAE-WHT extracts were also increased by 27.85% and 3.39% w.r.t. that of HWE extracts. The total protein content of the HWE was lowest (9.71 mg BSA eqv./g, db), followed by UAE-WHT (21.460 mg BSA eqv./g, db) and UAE-ND (222.115 mg BSA eqv./g, db) extracts.

Hence, it can be concluded that although UAE method (both ND and WHT) was able to extract more carnosine from the chicken breast meat than the HWE method, the large total protein content in the extract puts a hint of doubt regarding the selectivity of the ultrasound-assisted extraction method. More experiments and literature survey is needed to fix this drawback.

CHAPTER V
CONCLUSION

In this research, it was proven that ultrasound-assisted extraction (UAE) is a highly efficient and environmentally friendly technique to recover carnosine from chicken breast meat while maintaining its antioxidant activity. The best UAE conditions without deproteinization (25 minutes at 4°C) gave the highest content of carnosine (19.121 mg/g db) and antioxidant capacity (38.23% RSA) and outperformed significantly UAE with heat treatment and hot water extraction under normal conditions. The findings emphasize UAE's strengths as an efficient and environmentally friendly extraction method, with greater carnosine recovery and bioactivity preservation compared to conventional processes. Nevertheless, the increased protein content in UAE extracts indicates that further purification would be necessary to enhance selectivity, possibly via hybrid extraction processes or process optimizations. The results have significant implications for the food and nutraceutical industries, where UAE may be a scalable and environmentally friendly option for bioactive peptide extraction.

By optimizing carnosine yield and preserving its functional properties, this process is consistent with the increasing demand for natural antioxidants in functional foods and health supplements. Future studies should aim to optimize UAE parameters to reduce protein co-extraction and investigate the stability and bioavailability of UAE-extracted carnosine in food applications. In summary, this work sets UAE as a promising method for carnosine extraction, balancing efficiency with environmental friendliness. Although there are still challenges in realizing ideal selectivity, the shown advantages of UAE open the door to its possible industrial application.

More studies into process optimisation and application-oriented formulations will be essential for realising these discoveries in operational, large-scale practice. The research adds to the general topic of green extraction technologies, presenting an effective means for the recovery of high-value bioactive materials from foodstuffs.

References

Ahmad, R. S., Imran, A., & Hussain, M. B. (2018). Nutritional composition of meat. *Meat science and nutrition*, *61*(10.5772), 61-75.

Aristoy, M. C., & Toldrá, F. (2004). Essential amino acids and dipeptides in dry-cured ham. *Food Chemistry*, 84(2), 239–246.

Bellia, F., Vecchio, G., & Rizzarelli, E. (2011). Carnosine derivatives: new multifunctional drug-like molecules. *Amino Acids*, 41(2), 205–217.

Boldyrev, A. A. (2007). Carnosine and other histidine-containing dipeptides: From the past to the future. *Biochemistry (Moscow)*, 72(7), 741–750.

Boldyrev, A. A., & Severin, S. E. (1994). The histidine-containing dipeptides, carnosine and anserine: Distribution, properties and biological significance. *Advances in Enzyme Regulation*, 34, 1–17.

Boldyrev, A. A., Aldini, G., & Derave, W. (2013). Physiology and pathophysiology of carnosine. *Physiological Reviews*, 93(4), 1803–1845.

Carreira-Casais, A., Otero, P., Garcia-Perez, P., Garcia-Oliveira, P., Pereira, A. G., Carpena, M., Soria-Lopez, A., Simal-Gandara, J., & Prieto, M. A. (2021). Benefits and drawbacks of ultrasound-assisted extraction for the recovery of bioactive compounds from marine algae. *International Journal of Environmental Research and Public Health*, 18, 9153.

Chan, E. W. C., & Decker, E. A. (1994). The antioxidative properties of carnosine. *Journal of Food Biochemistry*, 18(2), 131–146.

Chan, K. M., & Decker, E. A. (1994). Endogenous skeletal muscle antioxidants. *Critical Reviews in Food Science and Nutrition*, 34(4), 403–426.

Chasovnikova, L. V., Formazyuk, V. E., Sergienko, V. I., & Boldyrev, A. A. (1990). Biological role of carnosine in the body. *Biophysics*, 35, 725–731.

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. *Ultrasonics Sonochemistry*, 34, 540–560.

Decker, E. A., Ivanov, V., Zhu, B. Z., & Frei, B. (2001). Inhibition of low-density lipoprotein oxidation by carnosine and histidine. *Journal of Agricultural and Food Chemistry*, 49, 511–516.

Garcia-Castello, E. M., Rodriguez-Lopez, A. D., Mayor, L., Ballesteros, R., Conidi, C., & Cassano, A. (2015). Optimization of conventional and ultrasound-assisted extraction

of flavonoids from grapefruit (Citrus paradisi L.) solid wastes. *LWT—Food Science and Technology*, 64, 1114–1122.

Guimarães, J. T., Silva, E. K., Costa, A. L. R., Cunha, R. L., Meireles, M. A. A., & Freitas, M. Q. (2021). Emerging technologies for extraction of bioactive compounds: Microwave, ultrasound, and supercritical fluid extraction. *Food Research International*, 147, 110552.

Guimarães, J. T., Silva, E. K., Costa, A. L. R., Cunha, R. L., Sant'Ana, A. S., & Meireles, M. A. A. (2021). Ultrasound-assisted technologies for designing healthier food products. *Food Engineering Reviews*, 13(1), 111–136.

Hassanin, F. S., Hassan, M. A., Shaltout, F. A., Shawqy, N. A., & Abd-Elhameed, G. A. (2017). Chemical criteria of chicken meat. *Benha veterinary medical journal*, *33*(2), 457-464.

Hazell, T. (1982). Meat color and myoglobin. *Meat Science*, 7(3), 209–228.

Hipkiss, A. R. (2010). The therapeutic potential of carnosine: A review of clinical studies. *Rejuvenation Research,* 13(2), 102–108.

Hu, Y., et al. (2009). Analysis of carnosine content in chicken breast and thigh muscles. *Journal of Agricultural and Food Chemistry*, 57(5), 202–208.

Jozanović, M., Hajduković, M., Galović, O., Kralik, G., Kralik, Z., Sakač, N., ... & Sak-Bosnar, M. (2017). Determination of anti-oxidative histidine dipeptides in poultry by microchip capillary electrophoresis with contactless conductivity detection. *Food chemistry*, *221*, 1658-1665.

Kang, J., Pervaiz, S., & Chun, Y. S. (2002). Carnosine inhibits pro-inflammatory cytokines and stress in macrophages. *Biochemical and Biophysical Research Communications*, 294(3), 687–691.

Kim, J., Wattanachant, S., & Jaturasitha, S. (2012). Carnosine and anserine contents in muscles of chicken breeds. *Asian-Australasian Journal of Animal Sciences*, 25(6), 861–866.

Li, X., Wu, G., Liu, Z., & Wang, C. (2012). Effects of carnosine supplementation on lipid oxidation and protein carbonyl formation in chicken breast meat. *Poultry Science*, 91(7), 1790–1795.

Li, Y., et al. (2012). Effects of thermal treatment on bioactive peptides in meat. *Meat Science*, 90(3), 275–282.

Matsukura, T., & Tanaka, H. (2000). Polaprezinc (zinc L-carnosine complex) for treatment of gastric ulcers: Clinical efficacy and in vitro characteristics. *Biochemical Pharmacology*, 59(4), 441–446.

Mora, L., Sentandreu, M. A., & Toldrá, F. (2008). Contents of creatine, creatinine and carnosine in porcine muscles of different metabolic types. *Meat Science*, 79(4), 709–715.

Parker, C. J. (1966). Spectrophotometric determination of carnosine and anserine in muscle. *Analytical Chemistry*, *38*(10), 1359-1362.

Santos, H. M., Lodeiro, C., & Capelo-Martinez, J.-L. (2009). The power of ultrasound. In *Ultrasound in Chemistry: Analytical Applications*; Wiley: Hoboken, NJ, USA, pp. 1–16.

Schmid, A. (2010). Carnosine and its related dipeptides. In *Encyclopedia of Food Chemistry*.

Tian, Y., Xie, M., Wang, W., & Sun, B. (2007). Carnosine and its role in disease prevention and treatment. *Amino Acids*, 33(3), 267–277.

Tiwari, B. K. (2015). Ultrasound: A clean, green extraction technology. *TrAC Trends in Analytical Chemistry*, 71, 100–109.

Toma, M., Fukutomi, S., Asakura, Y., & Koda, S. (2011). A calorimetric study of energy conversion efficiency of a sonochemical reactor at 500 kHz for organic solvents. *Ultrasonics Sonochemistry*, 18, 197–208.

Wu, H. C., Shiau, C. Y., Chen, H. M., & Chiou, T. K. (2003). Antioxidant activities of carnosine, anserine, some free amino acids and their combination. *Journal of Food and Drug analysis*, *11*(2), 13.

Zhang, Q. W., Lin, L. G., & Ye, W. C. (2018). Techniques for extraction and isolation of natural products: A comprehensive review. *Chinese Medicine*, 13(1), 1–26.

Appendix A

Sl. No.	Chemicals (Analar or Extrapure Grade)	Manufacturers
1	L-Carnosine (Crystalline)	Sigma-Aldrich, Co., USA
2	2,2-diphenyl-1-picrylhydrazyl (DPPH)	Sigma-Aldrich, Co., USA
3	Bovine serum albumin (BSA)	
4	Folin Ciocalteu phenol reagent	
5	2-Thiobarbituric acid (TBA)	Sisco Research Laboratories Pvt. Ltd., Mumbai, India
6	Ethanol (99.9%)	RCP Distilleries India Pvt. Ltd., Meerut, India
7	4-Bromoaniline (97%)	Sigma-Aldrich, Co., USA
8	Sodium carbonate	Sisco Research Laboratories Pvt. Ltd., Mumbai, India

Appendix B

Sl. No.	Equipment	Manufacturer
1	Hot air oven	Quesst International, Bangalore, India
2	Weighing balance	Ganathi Instruments Pvt. Ltd., Mysore, India
3	Vortex shaker (Spinix)	Tarsons, Kolkata, India
4	Meat Mincer	Koneteollisuus Oy, Klaukkala, Finland
5	Spectrophotometer	Hitachi High-Tech Science, USA
6	pH meter	Eutech Instruments, ThermoFisher Scientific, Singapore
7	Ultrasonic processor	Sonics & Materials, Inc., USA
8	Circulator water bath	Optima, Japan