Extraction of Betalains and Beta-carotene from Beetroot and Carrot: A Practical Approach to Food Fortification

A Thesis Submitted to the University of Reading in Fulfilment of the Degree of Doctor of Philosophy (PhD)

Rahul Kumar B. Tech, M. Tech

Department of Food and Nutritional Sciences

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Declaration

I confirm that this is my own work and the use of all materials from other sources have been properly and fully acknowledged.

Rahul Kumar

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Abstract

Betalains and beta-carotene extracted from beetroot and carrot wastes, respectively, are natural pigments and offer health benefits. This research primarily focuses on the technological and mechanistic aspects of extracting these bioactives. The extraction of betalains (betacyanin and betaxanthin) is investigated in citric acid and ethanol solutions, whereas the extraction of beta-carotene is studied in sunflower oil. Since the rates of extraction were generally found to be slow due to the use of relatively low temperatures and the possibility of betalains degradation, a mechanistic approach was taken to mathematically model the kinetics of extraction, which was also experimentally validated. This study showed the viability of using elevated temperature short time extraction processes. A modified and generic version of the above model was then developed for the extraction of betalains in aqueous-ethanolic solution, and beta-carotene in sunflower oil and experimentally validated. Finally, the thermal degradation of beta-carotene in sunflower oil was modelled after adopting a schematic reaction network, and experimentally investigated in the temperature range 150-220 $^{\circ}$C, in order to explore the possibility of using beta-carotene enriched edible oil for frying, and using the fried product as a vehicle for delivering beta-carotene. The results of this study showed that beta-carotene containing oil can be used as a frying medium to produce fortified potato crisps. Finally, a systematic human trial was undertaken, which demonstrated effective absorption and increased blood plasma concentration of beta-carotene in volunteers who were fed with the fortified potato crisps. In summary, the research presented in this thesis provides considerable new insights into: 1) the mechanisms of extracting betalains and beta-carotenes into solvents, 2) the thermal stability of betalains and beta-carotene in these solvents, and 3) the possibility of using beta-carotene fortified edible oil as a frying medium to produce bioactive fortified fried products which can potentially be used to alleviate vitamin A deficiency.

Keywords: Betalains; Betacyanin; Betaxanthin; Beta-carotene; Extraction; Modelling, Beetroot; Carrots.

List of Publications

Chapter 2: Kumar, R., Oruna-Concha, M. J., Niranjan, K., & Vimaleswaran, K. S. (2024). A Review on Vitamin A deficiency and Depleted Immunity in South Asia: From Deficiency to Resilience. *Nutrition*, 112452.

Chapter 4: Kumar, R., Methven, L., & Oruna-Concha, M. J. (2023). A comparative study of ethanol and citric acid solutions for extracting betalains and total phenolic content from freeze-dried beetroot powder. *Molecules*, *28*(17), 6405.

Chapter 5: Kumar, R., Oruna-Concha, M. J., Methven, L., & Niranjan, K. (2023). Modelling extraction kinetics of betalains from freeze dried beetroot powder into aqueous ethanol solutions. *Journal of Food Engineering*, *339*, 111266.

Chapter 6: Kumar, R., Oruna-Concha, M. J., Balagiannis, D. P., & Niranjan, K. (2024). Elevated temperature extraction of β-carotene from freeze-dried carrot powder into sunflower oil: Extraction kinetics and thermal stability. *Journal of Food Science*.

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Chapter 1: Introduction

Beetroot and carrot wastes are rich in health promoting bioactive ingredients. The valorisation of these vegetables wastes can deliver two classes of compounds namely betalains and beta-carotene, respectively. Betalains have the ability to counter many chronic diseases and acts as an antioxidant. On the other hand, beta-carotene is a pre-cursor to Vitamin A and also acts as an antioxidant. Further, betalains and beta-carotene are the two most industrially sought after natural pigments.

In South Asia, valorisation of food and vegetable waste to extract these bioactive compounds followed by their incorporation into the food systems can reduce malnutrition and prevalence of Vitamin A deficiency. Young age children (6-59 months of age) and pregnant women in this region are at the highest risk of mortality due to vitamin A deficiency. Thus, valorisation would not only contribute to ameliorate the malnutrition problem in this region but also reduce the burden of waste management.

This thesis represents an in depth study into the technological and mechanistic aspects of extracting betalains and beta-carotenes by developing mathematical models for the phenomena involved and validating the models. It also focuses on the thermal degradation of these bioactives, which establishes the feasibility of using beta-carotene enriched edible oil for frying to yield fried products fortified with beta-carotene. Finally, the thesis also contains the results of a human trial conducted to ascertain the absorption of beta-carotene into blood plasma after consuming fortified potato crisp produced by frying in beta-carotene enriched oil.

Chapter 2 reviews vitamin A deficiency and depleted immunity in South Asia in order to set the context of this research. Chapter 3 reviews extraction technologies used for the extraction of betalains and beta-carotene particularly focusing on the pros and cons of different extraction approaches. A key conclusion of this review is that elevated temperature extraction of betalains

and beta-carotenes is viable and the use of high temperature short extraction times, similar to HTST processes employed by the food industry, can be practically developed.

Chapter 4 addresses the challenge of lowering the environmental impact of the extraction processes by employing benign solvents and focuses on a comparison between the extraction of betalains (betacyanin and betaxanthin) and phenolics in citric acid solutions and aqueous ethanol solutions.

Chapters 5 explores the use of relatively elevated temperatures to extract betalains by developing a mathematical model which, at a given temperature, takes into account the rate of transfer of the bioactive from the solid phase and its rate of degradation in the extract phase. This chapter also includes a detailed experimental study to validate the model and explore the variation of model parameters with extraction operating parameters.

Chapter 6 extends the above model for beta-carotene extraction into edible oil by including terms to represent possible degradation of beta-carotene in the solid and liquid phases, which is also validated experimentally. This chapter also includes a detailed study of the thermal degradation kinetics of beta-carotene at high temperatures in the range 160-220 °C which represent typical deep fat frying temperatures. The study of degradation kinetics at these temperatures is aimed to explore the possibility of using beta-carotene enriched oil as a frying medium.

Chapter 7 investigates the efficacy of beta-carotene enriched oil as a frying medium, by producing potato crisps fortified with this bioactive and using it in human trials. The study involves monitoring with time the absorption of beta-carotene into blood plasma of volunteers who were fed with a fixed quantity of the crisps.

Chapter 8 discusses the overall conclusions of this research and summarises the contribution to knowledge of each chapter. Recommendations for future work are also made in this chapter.

It may be noted that the main chapters of this thesis are written in the form of research publications; therefore, each of these chapters has a separate introduction and conclusion sections, in addition to experimental methods, and results and discussions. Chapters 2, 4, 5 and 6 are already published as indicated on the page following the abstract. Chapters 3 and 7 will be shortly submitted for publication to suitable journals.

Overall Aim: The overall aim of this thesis is to develop and optimize efficient and sustainable methods for extracting betalains from beetroot and beta-carotene from carrot, with a focus on maximizing yield and extraction rates by elevating the extraction temperature. This includes characterizing the chemical composition, and stability of the extracted compounds, and evaluating their efficacy in fortifying food products to enhance nutritional content. Additionally, the thesis aims to investigate the potential health benefits associated with the consumption of these enriched foods for industrial application.

Objectives:

1. Using citric acid solutions of different pH range to extract betalains as a sustainable alternative solvent and compare the extraction efficiency with conventional aqueous ethanolic solution.

2. To develop a mechanistic kinetic model to determine the optimal conditions for the extraction process for betalains, such as solvent concentration, temperature, and time, to maximize the yield and extraction rate of betalain.

3. To investigate the extraction kinetics of beta-carotene from freeze-dried carrot powder into sunflower oil at elevated temperatures, and thermal stability of beta-carotene with the goal of

optimizing extraction conditions for maximum yield and maintaining the integrity of beta-carotene for potential food industry applications.

4. To develop a method for fortifying potato crisps with beta-carotene to effectively enhance vitamin A status. This includes ensuring high retention of beta-carotene during processing at industrial frying conditions, and assessing the fortified crisps' potential to contribute to improved dietary pro-vitamin A intake.

Chapter 2

A Review on Vitamin A Deficiency and Depleted Immunity in South Asia: From Deficiency to Resilience

This chapter has been published in journal *Nutrition*: Kumar, R., Oruna-Concha, M. J., Niranjan, K., & Vimaleswaran, K. S. (2024). A Review on Vitamin A deficiency and Depleted Immunity in South Asia: From Deficiency to Resilience. *Nutrition*, 112452.

Abstract: In the developing world, the twin challenges of depleted health and growing issue of food waste management loom large, demanding simultaneous attention and innovative solutions. This review explores how these issues can be effectively mitigated while shedding light on the transformative impact of food waste valorisation on health management. A spotlight is cast on vitamin A deficiency (VAD), an acute public health concern, especially prevalent in South Asia, driven by economic constraints, sociocultural factors, inadequate diets, and poor nutrient absorption. VAD's devastating effects are exacerbated by limited education, lack of sanitation, ineffective food regulations, and fragile monitoring systems, disproportionately affecting children and women of childbearing age. Recent studies in South Asian countries have revealed rising rates of illness and death, notably among children and women of childbearing age, due to VAD. To address inadequate dietary intake in children utilizing vegetable waste, particularly from carrots and beetroot, which are rich in beta-carotene, and betalains, respectively, offers a sustainable solution. Extracting these compounds from vegetable waste for supplementation, fortification, and dietary diversification could significantly improve public health, addressing both food waste and health disparities economically. This approach presents a compelling avenue for exploration and implementation. In summary, this review presents an integrated approach to tackle health and food waste challenges in the developing world. By tapping into the nutritional treasure troves within vegetable waste, we can enhance health outcomes while addressing food waste, forging a brighter and healthier future for communities in need.

Keywords: Vitamin A Deficiency (VAD); Food Waste Management; Carrot; Beetroot; Vitamin A; Immunity.

2.1 Introduction

Vitamin A (VA), also known as retinol, plays a crucial role in human physiology (Huang et al., 2018), and maintains innate immunity, defence against infections, and growth promotion (Almagro et al., 2022; Clark, 2007). It also protects against night blindness, a common issue in young children and pregnant women. The main sources of VA include meat products, dairy products, and plants like fruits and vegetables (Clark, 2007; Fragoso et al., 2012; Strobel et al., 2007). Provitamin A (PVA), a precursor to VA, is primarily found in leafy vegetables and yellow fruits (Imdad et al., 2022; Strobel et al., 2007). Currently, the health status of pregnant women, lactating mothers, newborns, and young children below the age of 5-6 have recorded the highest mortality, morbidity, blindness, and sickness rate due to the vitamin A deficiency (VAD) and depleted immunity (FAO, 2020; Imdad et al., 2022). To collect such health-related information in India from different sections of the population by sex, caste, creed and age, the government conducts the National Family Health Survey (NFHS) every four years. The NFHS-5 phase 1 survey in India revealed that 16 out of 22 states have increased malnutrition, VAD, and iron deficiency rates compared to NHFS-4. The report also suggests that the government needs to rethink its nutritional programs to meet nutritional targets for the coming decade. The immunization ratios are only 70% against infections and diseases. VA and iron deficiency are prevalent among pregnant, lactating women and young children, leading to reduced immunity, anaemia, and mortality risk (Akhtar et al., 2013; Ministry of Health and Family Welfare. Government of India., 2019; Sommer et al., 1986; United Nations Conference on Trade & Asian Development Bank, 2015).

Pakistan also grapples with severe subclinical VAD, contributing to child mortality, especially in cases of diarrhoea and pneumonia (Khan et al., 2007). Measles-affected children in Pakistan are

concurrently experiencing VAD, emphasizing the need for effective VA supplementation. Karachi city reported avoidable cases of child blindness (Khan & Baseer, 1996), with a majority preventable by meeting the daily requirement of VA. Sri Lanka and Bangladesh also face significant challenges, with studies indicating widespread VAD among preschool children and pregnant women, respectively (Nair et al., 2012). In Bangladesh, 51% of pregnant women had dietary deficiencies, leading to 18.5% exhibiting VAD (Akhtar et al., 2013). Addressing the pervasive nature of VAD requires a multifaceted approach, including awareness campaigns, dietary diversification, fortification programs, and enhanced healthcare infrastructure in South Asian countries. The data underscores the urgency of comprehensive strategies to combat VAD and its adverse effects on public health in the region.

On the other hand, vegetable waste represents a significant global challenge, with staggering facts and figures highlighting the scale of the issue. Each year, it's estimated that approximately one-third of all food produced for human consumption, which includes a substantial portion of vegetables, goes to waste. This amounts to over 1.3 billion metric tons of food wasted annually, with vegetables comprising a substantial portion of this figure that is approximately 30% (Porat et al., 2018; United Nations Environment Programme, 2019). Beyond the immense environmental toll, as food waste generates substantial greenhouse gas emissions, it also poses a severe economic burden, with food waste costs reaching hundreds of billions of dollars each year. Vegetable waste is particularly concerning due to its role in perpetuating malnutrition, as the discarded portions often contain valuable nutrients and bioactive compounds like beta-carotene, as well as the potential to alleviate VAD in many regions (Carrillo et al., 2022; West, 2012). Consequently, addressing the issue of vegetable waste on a global scale is not only an environmental imperative but also a crucial step towards improving food security and public health worldwide (Authors et

al., 2018; Le Mouël & Forslund, 2017). The volume of vegetables wasted in the India and neighbouring countries, per capita per household, is immense and it is more than 50% of total (United Nations Environment Programme, 2021). The need to utilise the waste must therefore be explored. Simultaneously, it is also necessary to ensure that the utilisation process is economically viable. Unfortunately, there are many utilisation routes described in published literature, but there are no studies examining economic viability. It is therefore difficult to justify a specific waste utilisation approach – be it in terms of fortification of food or any waste valorisation option. . The feasibility of fortification is rooted in comprehending the diversity of food vehicles, and ensuring technological adaptability to existing production processes, and seamless integration into supply chains. Simultaneously, resilient waste valorization relies on the identification of viable waste streams, economic feasibility, and compliance with regulatory standards. The economic sustainability of fortification is crucial, encompassing cost-effective nutrient addition, affordability for end consumers, and an evaluation of the long-term financial impact. By harmonizing these factors, a resilient approach ensures that fortification and waste valorization strategies not only align with industrial structures but also contribute to sustainable health outcomes and the economic viability of the food industry (Strotmann et al., 2017).

Hence, these leading malnutrition and depleted health status related problems can be mitigated by the use of bioactive components found in various food sources such as beta-carotene as a precursor of VA and betalains. Beta-carotene derived from natural sources undergoes conversion into two molecules of vitamin A within the animal body, subsequently storing it as retinyl ester. Similarly, the obtained betalains from beetroot waste can improve heart health, blood pressure, some types of cancer, hyperlipidaemia and also acts as an antioxidant and one of the most sought pigments in several industries (Clifford et al., 2015; O'Byrne & Blaner, 2013; Tang et al., 2005). The

bioavailability of betalains is well proven to mitigate the heart and blood pressure associated with beetroot juice consumption (Zamani et al., 2020). Considering the current status of heart-related and other cancerous diseases on rise in South Asian countries, it was also justified to incorporate them in this review (Friese, & Yang, 2019). In addition, the amount of waste created from fresh produced beetroot was one of the factors for its addition in this review (Frankowska et al., 2019). The primary aims and objectives of this review are to underscore the significant dual challenge posed by food waste, particularly vegetable waste, and the widespread issue of VAD and weakened immunity, prevalent in Asian and other developing countries. We aim to emphasize the often-overlooked opportunity for solving both problems simultaneously by taking a scientific approach to the valorisation of vegetable waste. Through this review, we intend to establish the scientific foundation for this approach, shedding light on how vegetable waste, such as beetroot and carrot remnants, which is rich in bioactive compounds like beta-carotene and betalains, holds the potential to combat nutritional deficiencies, ultimately enhancing public health. By reviewing existing research, proposing sustainable solutions, and raising awareness about the importance of integrating food waste management and nutritional strategies, our review seeks to inspire further research in this crucial area and encourage future endeavours to delve deeper into the valorisation of vegetable waste for holistic health and environmental sustainability.

2.2 Methodology

A literature search was conducted with following key words such as VAD , Vitamin A precursors, Vitamin A, Waste valorization, Food waste valorization, vegetable waste valorization, health status of Asian and Indian population, immunity supported by VA, malnutrition, hunger, and health benefits of betalains and beta-carotene. The search results shown by Web of Science, PubMed, Google Scholar, and Scopus database resulted in 492 articles, and of these, 89 articles

were identified which focused either on food waste or vegetable waste management and only one article (Torres-León et al., 2018) focused on mitigating malnutrition and waste management related problems with well-established scientific approach as shown in Figure 2.1. However, this one article (Torres-León et al., 2018) did not focus on the type of food waste that could be targeted and the strategies to prevent malnutrition. Titles of all the studies were first read to determine their relevance to the topic. Full text of those found to be relevant (either on the basis of vegetable waste or malnutrition/VAD) were then read in full detail to determine eligibility for inclusion based on the above provided key words as inclusion criteria. Hence, to address this review gap, authors have selected 89 independent articles related to food waste or vegetable waste management and depleted health status and approaches taken to improve the health status in South Asian (SA) populations. As this is not a systematic review and meta-analysis, literature was reviewed as available and applicable information about the VAD, impaired immunity, and health benefits of betalains to mitigate several non-communicable diseases were reviewed. For this review, articles that were published after 2010 were included (Figure 2.1).

2.3 Direct influence of vitamin A and betalains on function of the immune system

Clinical trials, including those by Shankar et al., (1999) (Shankar et al., 1999), West et al., (1991) (West et al., 1991), and Christian et al., (2000) (Christian et al., 2000), showcased the varied impact of VA supplementation on health outcomes, particularly in reducing morbidity and improving immune response. In addition, Tang et al., (2005) (Tang et al., 2005) and Qi et al., (2016) (Qi et al., 2016) demonstrated the importance of VA in immune health, emphasizing its diverse dietary sources, through dietary interventions. Together, these studies underscore the multifaceted significance of VA in promoting overall health and immune function across different populations and interventions. While carotenoids have been linked to health benefits like reduced risk of certain

diseases, it's the converted form, VA, that significantly affects the immune system (Clark, 2007). Beetroot, once known for its vitamin C, iron, and folate content, is gaining attention for its immune-boosting potential, as it is attributed to the synthesis of nitric oxide from betalains and nitrates in beetroot, and its role in immune regulation and anti-inflammatory effects (Kaur et al., 2018). Beetroot extract is considered a promising functional food for health promotion and disease prevention due to its various therapeutic properties, including oxidative stress reduction and immune system support (Clifford et al., 2015; Stanaway et al., 2017).

2.4 Vitamin A deficiency: a South Asian perspective

VAD is a significant global health issue (Souganidis et al., 2013) (Souganidis et al., 2013), affecting 120-170 million school-going children and 7-8 million pregnant women in developing and low-income South Asian countries. This deficiency leads to malnutrition, death, impaired health status, lower tissue development rate, slow metabolism, and vulnerability to infectious diseases (Humphrey et al., 1996; Sommer, 2008; West, 2002). South Asian countries make up one-fifth of the global population and are severely affected by VAD (Akhtar et al., 2013). Nearly 30-50% of preschool children in South Asia suffer from malnutrition and VAD, leading to the major cause of mortality in India and Bangladesh (World Health Organization, 2009).

VAD is the most severe deficiency among other diseases in 1.02 billion people globally suffering from chronic malnutrition (FAO, 2020). Studies have shown that 85% of children suffering from xerophthalmia reside in India (Bastos Maia et al., 2018; FAO, 1992). The number of individuals suffering from VAD concerns health and safety points for the South Asian population. Immediate action through existing food regulations and new strategies to counter the deficiency on a mass scale is needed (Bhutta, 2012; Nair et al., 2012). India has the highest proportion of VAD in

preschool-going children among South Asian countries, at 62%, which is either clinical or subclinical as shown in Figure 2.1S (Supplementary materials). This substantial figure of deficiency leads to the death of 330,000 children annually in India alone and data for other South Asian countries is given in Table 2.1S (Supplementary materials). Subclinical VAD is also widespread in childbearing young mothers, with 5% suffering from night blindness during pregnancy. The highest proportion of pregnant women suffering from night-blindness are from rural areas (Aguayo & Baker, 2005; Mayo-Wilson et al., 2011; Ministry of Health and Family Welfare. Government of India., 2019).

VAD is a significant health issue in Pakistan, with diarrhea and pneumonia being major causes of child mortality. Measles-related VAD is prevalent among children under 6 years old, and the need for effective supplementation systems with high dose levels of VA is crucial. Nearly 53% of avoidable cases of child blindness were reported, with 58% preventable by meeting the daily requirement of VA in a blind and deaf school in Karachi city, Pakistan (Khan & Baseer, 1996; Khan et al., 2007). VAD is also prevalent among young people under 6 years of age in major regions of Pakistan. The prevalence of VAD among pregnant and non-pregnant women is a concern from a health perspective, but the sample size is small to accurately reflect the true extent of VAD in the region (Marjan et al., 2021).

VAD is also recognized as a general health issue in Sri Lanka with 30% of the children of age less than 6 years are suffering from this deficiency (Liyanage et al., 2021), with a public overview led by the Medical Research Institute (MRI). A VAD control program was created to improve the VA status of preschool and elementary younger students, pregnant and lactating mothers. Food-based methods continued in Sri Lanka to control VAD, including dietary enhancement, development of VA-rich foods in home backyards, promotion of breastfeeding, and improving fat and VA-rich

nourishments (Akhtar et al., 2013; Nair et al., 2012). In Bangladesh, studies have revealed that 51% of pregnant women had a deficiency in the diet to meet the RDA for VA, and 18.5% showed VAD (serum retinol <0.70 µmol/L) (Akhtar et al., 2013; Marjan et al., 2021). Some of the factors such as diet and gestational age were shown to contribute to VAD in Bangladeshi women (Ahmed et al., 2012; Lee et al., 2008).

Considering all the data concerning VAD in South Asian countries, two factors were observed to be predominant; these include the early death of the new-borns or those aged less than 5-6 years, and blindness among the child-bearing women or girls. The epidemiological surveys have concluded that all the deaths and side effects of VAD were indirectly related to anemia, and reduced absorption of iron which ultimately leads to iron deficiency anemia and increased rate of mortality. Anaemia is prevalent in non-industrial nations; about 42% of preschool children, 53% of young school-age children, 44% of women of childbearing age, and 56% of pregnant women are affected by anemia (Sommer & Vyas, 2012). The reasons for anaemia are different, yet among the main etiologies in non-industrial nations are iron insufficiency, intestinal sickness, some unavoidable illnesses, and poor health that ultimately affects hemoglobin, and VA level in human body. Insufficient intake of VA was recognized among the reasons for anemia (Khor, 2005; Nguyen et al., 2007), and it was attributed that VAD impacts anemia through regulation of hematopoiesis, by the improvement of insusceptibility to unavoidable illnesses (Semba et al., 1992; Semba & Bloem, 2002).

The major factors for the prevalence of VAD in South Asian population are insufficient intake and poor absorption, which are impacted by socioeconomic status and socio-cultural limitations (Akhtar et al., 2013). The conversion of beta-carotene to VA, and storage in the body is shown in Figure 2.2 (Desobry et al., 1998). One of the predominant dietary factors is a low intake of animal-

based foods (meat and dairy) which are good sources of fat-soluble vitamins such as A, D, E, and K. There are several vegetable sources of VA (please refer section 2.5), but the consumption needs to be in large volumes, or from a concentrated source, which is difficult to afford by the lower socioeconomic group, and difficult for young children to consume the volume of vegetables needed to meet their VA requirements (Augusto et al., 2015).

2.5 Direct fortification of food items to counter VAD in South Asia

While VAD remains a significant public health concern in South Asia, various intervention programs have been implemented over time to address this issue. These programs target specific populations and utilize different strategies to combat VAD. Here are some of the current/past intervention programs in South Asian countries:

1. Fortification of vegetable vanaspati ghee: The India, Malaysia and Pakistan in South Asia have initiated programs to fortify vegetable vanaspati ghee, aiming to increase the VA content in this widely used cooking fat/oils (Vir et al., 2011).

2. Fortification of vegetable oils with retinol: Fortifying commonly used vegetable oils with synthetic retinol (a form of VA) is another intervention strategy (Borguini et al., 2020). This approach helps enhance the nutritional content of cooking oils that are widely consumed in the region.

3. Fortification and genetic modification of rice (yellow rice): Yellow rice, a genetically modified variety, has been introduced as part of fortification efforts (Wu et al., 2021). This type of rice is enriched with beta-carotene as a precursor of VA, addressing the deficiency in regions where rice is a staple food .

4. VA supplementation as retinol: Specific intervention programs focus on providing VA supplementation in the form of synthetic chemical retinol. This is particularly targeted at vulnerable populations, including young children (6-59 months old) and pregnant girls and women (Sommerburg et al., 2015).

5. Supplementation via sugar and flour: Some intervention programs involve the fortification of sugar and flour with VA. This ensures that these commonly consumed food staples contribute to the daily VA intake of the population (Dwyer et al., 2015).

The major target audiences for these intervention programs include:

- Young children (6-59 months old): These programs aim to address VA deficiency in the early stages of life when nutritional support is crucial for growth and development (Aguayo et al., 2015).
- Pregnant girls and women: Recognizing the increased nutritional needs during pregnancy, intervention programs focus on providing VA supplementation to pregnant women (Ahmed et al., 2012), reducing the risk of deficiency-related complications.

By targeting these specific demographic groups and employing various strategies such as fortification and supplementation, South Asian countries aim to alleviate the burden of VAD and improve the overall health and well-being of their populations. Ongoing monitoring and assessments are essential to gauge the effectiveness of these intervention programs and make necessary adjustments as needed.

It is evident from the above information that fortification of other food products has been also practiced but failed. It could be attributed to the studying fortification at an ideal and controlled condition that is otherwise not possible in real life of a common person. For example, when yellow

rice or any other fortified food products were given to the participants, they were supplemented with the required amount of butter/fat to help with the incorporation of the VA sources in the human body, which was otherwise not possible in real scenarios. Hence, later many of the studies were focused on extraction and stability of beta-carotene/VA products in various vegetables oils, which are also a major part of the diet by frying and cooking in South Asian countries (Bhardwaj et al., 2016). Another reason for using sunflower/soybean oils in most of studies is the stability of beta-carotene in these oils and higher bioavailability due to the higher degree of unsaturation of these oils (Gizir et al., 2008).

To support the above hypothesis about the use of sunflower oil and ability to enhance the absorption of carotenoids, Linvy et al., (2003) (Livny et al., 2003) reported that the inclusion of sunflower oil in meals significantly improved the absorption of beta-carotene, particularly when consumed with cooked, pureed carrots compared to raw, chopped carrots. Sunflower oil played a crucial role in enhancing beta-carotene bioavailability through several mechanisms. Firstly, its fat content facilitated the solubility of beta-carotene during digestion, making it more accessible for absorption. Additionally, sunflower oil promoted the formation of micelles, which served as carriers for fat-soluble beta-carotene, aiding its transport across the intestinal lining. Furthermore, the presence of antioxidants in sunflower oil protected beta-carotene from degradation during digestion, preserving its bioactivity. Moreover, sunflower oil may have contributed to a shorter lag phase in beta-carotene absorption, optimizing transit time through the gastrointestinal tract. Overall, the study underscores the importance of sunflower oil in enhancing the bioavailability of beta-carotene from vegetable sources, particularly when consumed in processed forms like cooked, pureed carrots. Hence, sunflower oil would be even more effective if it was consumed with extracted beta-carotene.

2.6 Identification of the potential dietary bioresources to combat VAD and boosting health status

South Asian countries are rich in plant sources of provitamin A (PVA), such as carrots, amaranth, and leafy vegetables, which play a vital role in providing essential nutrients for health. Additionally, yellow vegetables like tomatoes, pumpkins, squash, and spinach serve as substantial sources of PVA as shown in Table 2.1.

In South Asian countries, the substantial production of vegetables is hampered by inadequate processing facilities, leading to a significant waste issue. This problem is particularly pronounced in Asian countries, where 35-55% of fresh produce is wasted due to factors like a lack of cold supply chains, storage, and proper handling (Porat et al., 2018; Rahiel et al., 2018; Raut et al., 2019). In India, vegetable production in the fiscal year 2019-20 reached 189 million metric tons, representing a considerable portion of global vegetable production, the data for vegetable production by other South Asian countries are also given in Table 2.2S (a) – (d) (Supplementary materials) (Narayanamoorthy et al., 2018). Hence, this amount of waste not only squanders energy, water, labor, and resources involved in the entire value chain but also exacerbates issues like VAD and immune health in the South Asian community. Utilizing these discarded bioresources could provide an indigenous solution to these problems while also contributing to economic recovery and reducing the environmental impact associated with waste and global warming.

Food waste is a pressing global issue, with approximately one-third of the world's food production going to waste, a statistic that, if represented as a country, would rank it as the third-largest contributor to global warming, following China and the United States (Food and Agriculture Organization of the United Nations (FAO), 2015). Various factors contribute to this problem,

including household waste as shown in Table 2.3S (Supplementary materials), overproduction, inadequate storage and preservation facilities, the lack of cold supply chains, losses in the food processing industry and trade, post-harvest waste due to mechanical inefficiencies, and a lack of automation in handling and packaging (Iordachescu et al., 2019). This not only has significant economic implications but also poses a considerable threat to global warming. Importantly, the global warming potential (GWP) of imported vegetables is higher than domestically grown ones, largely due to long-distance transportation. Processing steps, such as packaging and on-farm production, each contribute 16% to the GWP, with processing itself accounting for 13% (Frankowska et al., 2019). Canning, due to heavy machinery and heating requirements, is a major contributor to GWP in the packaging phase (Etzel et al., 2015). Furthermore, growing conditions, such as greenhouse cultivation, can require more energy than the imported produce. In terms of carbon footprint (CFP), cereals are the highest CFP producers quantitatively, followed by vegetables and then meat. Despite its lower volume, meat and animal product waste has the highest CFP per kilogram (Food and Agriculture Organization of the United Nations (FAO), 2015). In India, an average of 7% of domestic vegetable supply goes to waste, an amount sufficient to meet the United Kingdom's needs (Sahu, 2004). This excessive waste generation not only challenges existing systems but also raises concerns about the associated environmental impact. Food waste contributes to various ecological problems, including climate change, eutrophication, acidification, ozone layer depletion, resource depletion, and biodiversity loss (Garnett, 2006; Majeau-Bettez et al., 2011; Pretty et al., 2010). With global food demand projected to increase by 70% by 2050, addressing food waste is crucial for both food security and reducing unnecessary economic costs, all while considering the moral imperative, as millions of people around the world suffer from undernourishment (FAO, 2015).

2.7 Innovative fortification approaches and health benefits of beta-carotene and betalains

In Kenya, Nderitu et al., (2018) (Nderitu et al., 2018) studied the fortification of beta-carotene in sunflower and palm oil to increase the beta-carotene level. Similarly, Gurumeenakshi et al., (2019) (Gurumeenakshi et al., 2019), Arumugam et al., (2014) (Arumugam et al., 2014) and Borguini et al., (2020) (Borguini et al., 2020) fortified vegetable oil with beta-carotene extracted from carrot (Olive, Soybean, Sunflower, Palm and other vegetable oils). Nderitu et al., (2018) reported that, after 6 months of storage, mean levels of beta-carotene were reduced significantly ($p<0.001$) by over 65%, however the remaining concentration of beta-carotene was still under the recommended level of RDA. On the other hand, Nderitu et al., (2018) and Gurumeenakshi et al., (2019) reported that due to the fortification with beta-carotene, the oil quality was reduced in terms of rancidity and peroxide values compared to the initial quality. In conclusion, it could be implied that beta-carotene can be fortified in sunflower and palm oils to address the nutritional problem despite antagonism of its storage and degradation as shown in Table 2.2.

Dutra-de-Oliveira et al., (1998) (Dutra-de-Oliveira et al., 1998) and Akhtar et al., (2012) (Akhtar et al., 2012) used the synthetic form of VA (retinyl palmitate) to fortify soybean oil and evaluated the bioavailability of beta-carotene after heating at different temperatures and cooking conditions. Dutra-de-Oliveira et al., (1998) & Dutra-de-Oliveira et al., (2013) (Dutra-de-Oliveira et al., 2013) reported that, after heating at 100 °C, there was no deterioration of beta-carotene, and, at higher temperature (170 °C), beta-carotene was available up to 65% of initial concentration. On the other hand, Akhtar et al., (2012) observed that retinyl palmitate that was added to refined soybean oil was stable when fortified into recommended dose and cooking techniques at a given temperature. This study encourages fortification in soybean oil because this oil has a strong hold in the market of developing countries and is consumed for meeting the fat requirements as well. It was reported

that the bioavailability of the retinyl palmitate was completely intact throughout normal cooking. However, conducting the heating for 20 min reduced the biological availability by 50% and was observed to be temperature sensitive. Despite the instability and sensitivity to the high temperature treatment, the biological availability of the fortified VA source is encouraging and the use of potential bioresources from natural vegetable waste could be even more promising.

The rising trends of using beta-carotene and betalains to prevent and mitigate several non-communicable diseases are shown in Table 2.3. Similar to beta-carotene, betalains are also important bioactive compounds, which are proven bioactives to mitigate hypertension, colorectal cancer, hyperlipidemia, and other non-communicable diseases (Hobbs et al., 2014; Razzaq et al., 2023; Saber et al., 2020; Sarfaraz et al., 2021). Razzaq et al., (2023) studied the effect of carrot-beet based beverages to modulate hypertension, where two kinds of beverage combinations were used ((1) carrot juice 20% and beetroot juice 80% and (2) carrot juice 40% and beetroot juice 60%). The study demonstrated that betalains, present in both beverages, showed a positive influence on hypertension and can be considered for inclusion in dietary therapy to address this condition (Razzaq et al., 2023). However, the specific mechanisms by which betalains affect blood pressure and lipid profiles may require further research for a more comprehensive understanding of their potential benefits. In the colorectal cancer cell lines study conducted by Saber et al., (2020) reported that betanin and the hydro-alcoholic extract of red beetroot demonstrated effectiveness in inhibiting the growth of colorectal cancer cells, inducing apoptosis in these cells, and did so with minimal harm to normal epithelial cells. The findings support the potential of betanin as a chemopreventive and anticancer agent, although further research is needed to fully understand the underlying mechanisms of its anticancer effects (Saber et al., 2020). In another study, Sarfaraz et al., (2021) evaluated the effect of lyophilized beetroot powder at different doses for its

hypolipidemic effects and showed that the presence of betalains in beetroot was found to have a positive impact on lipid profiles, suggesting that beetroot powder may be a beneficial dietary intervention for individuals with hyperlipidaemia or those at risk of cardiovascular diseases (Sarfaraz et al., 2021). Hence, it could be concluded that beetroot is a nutritionally dense, natural food with a range of potential health benefits (Hobbs et al., 2014) and it might have a positive impact on cardiovascular health, hypertension, hyperlipidaemia, and potentially even certain types of cancer.

2.8 Conclusions

In conclusion, the significance of harnessing the potential of bioactives like beta-carotene and betalains from vegetables such as carrots and beetroots is undeniable, given their extensive health benefits and readily availability of potential bioresources in South Asian countries. Waste generation, stemming from farm to household, is intrinsically linked to fresh produce, highlighting the need for a more scientific approach in utilizing these resources efficiently. By extracting these valuable compounds, we not only address deficiencies like VAD which is highly prevalent in South Asian countries but also provide protection against various non-communicable diseases.

The development of sustainable solutions for managing byproducts can be done by food waste management. The nexus between food waste management, waste valorization, and addressing VAD is intricately tied to the principles of reduce, reuse, and recycle (Pinotti et al., 2020). By strategically implementing these waste management practices, we can not only minimize the overall food waste footprint but also capitalize on the nutritional potential of discarded by-products. The reduction of food waste at the source is complemented by the reuse of specific by-products rich in provitamin A carotenoids, which may be traditionally discarded. Through

innovative recycling processes, these by-products can be transformed into biofortified ingredients, contributing to the alleviation of VAD. This integrated approach underscores the potential for sustainable solutions that simultaneously enhance nutritional outcomes and minimize the environmental impact of food waste (Mahawar et al., 2020).

Hence, these solutions should tap into the inherent nutritional and functional value of biomaterials, yielding economic, social, and environmental advantages. In an era where nutritional problems and the ever-growing South Asian population pose serious challenges, repurposing food waste for human consumption should be a top priority. By leveraging the nutritional potential of waste and byproducts in South Asian countries, we not only enhance food security but also create opportunities for livelihoods, offering a compelling social benefit. In essence, the efficient use of bioresources and the reduction of food waste are essential components of a healthier, more sustainable future for our planet and its people.

While fortification of VA is a proven strategy to combat deficiency, challenges such as infrastructure, distribution, awareness, quality control, and economic factors can hinder successful implementation (Dwyer et al., 2015). Addressing these limitations requires a multifaceted approach, involving collaboration across sectors, investment in capacity building, robust monitoring systems, community engagement, and adherence to quality standards. By systematically addressing these challenges, fortification programs can be more effective in improving micronutrient status and promoting public health.

2.9 References

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Table 2.1: Potential food commodities containing VA/beta-carotene including plant and animal sources.

Sl. No.	Food Commodities	Beta-Carotene	Amount of beta-carotene found	References
1	Carrot	Beta-Carotene	52 - 60 mg/100 g dry carrot	(Gul et al., 2015)
2	Tomatoes	Beta-Carotene	3.8-7.03 mg/100 g of dry tomato	(Toor & Savage, 2005)
3	Amaranth (Red or Green)	Beta-Carotene	300.2 µg/g fresh	(Pritwani & Mathur, 2017)
4	Spinach	Beta-Carotene	6.29 mg/100 g fresh	(Tang et al., 2005)
5	Orange-fleshed sweet potatoes	Beta-Carotene	11.5 mg/100 g fresh	(de Andrade Lima et al., 2019)
6	Squashes/pumpkins	Beta-carotene	4.57 mg/100 g fresh	(Chandra et al., 2014)
7	Yellow maize	Beta-Carotene	0.38 mg/300 g cooked serving	(Muzhingi et al., n.d.)
8	Mangoes	Beta-Carotene	0.55-3.21 mg/100g	(Rezaei & Liu, 2017)
9	Papayas	Beta-Carotene	2.14-2.74 mg/100g of raw papaya	(Schweiggert et al., 2014)
10	Liver	Beta-Carotene	58.28-203 µg/g fresh	(Strobel et al., 2007)
11	Eggs	Beta-Carotene	5.19-200 mg/100 g of eggs	(Volk, 2009)
12	Milks (including breast milk)	Beta-Carotene	1.45-3.60 µg/g of fat	(Ramalho et al., 2012)
13	Red palm oil	α and Beta-Carotene	500 µg/g of palm oil	(Dong et al., 2017)

Table 2.2: List of studies that focused on fortification approaches.

Sl. No.	Food bio-products used/ source of VA used	Role of beta-carotene as a fortificant/ extract	Foods fortified	Trials/experiments conducted	Main findings	References
1.	*Solanum nigrum* and *Asystasia mysorensis*	Beta-carotene as precursor to VA	Sunflower and palm oils	Extraction of beta-carotene in oils. Storage study of beta-carotene in vegetable oils for 180 days.	Beta-carotene was fortified in sunflower and palm oils to address the antagonism of its use in food and application to elevate beta-carotene levels.	(Nderitu et al., 2018)
2.	Fresh Carrot	Beta-carotene as precursor to VA	Gingelly and Mustard oils	Extraction, encapsulation, and fortification of oils with storage stability of 60 days.	Cold press extraction with hexane was the best solvent. Most suitable wall material for encapsulation was lecithin. Beta-carotene was stable in the mix.	(Gurumeenakshi et al., 2019)
3.	Beta-carotene	Beta-carotene for meeting daily requirements and extending the shelf-life of oil	Cold pressed virgin coconut oil	Shelf-life of oil and stability of beta-carotene was assessed.	Produced oil was a suitable medium for producing value added functional oil.	(Arumugam et al., 2014)
4.	Dried Carrot	Aimed to meet the Recommended Daily Intake for VA	Soybean and Olive oils	Enrichment of the oils with beta-carotene and shelf-life study.	10 mL of soybean oil or olive oil supplied the Recommended Daily Intake for VA for an adult (equivalent to 600 µg retinol).	(Borguini et al., 2020)
5.	Dried Carrot	Extraction of beta-carotene as a precursor for VA	Sunflower oil	Enrichment of the sunflower oil for better bioavailability of beta-carotene and understanding the thermal stability as higher temperature.	Beta-carotene was efficiently extracted in shorter time compared to ultrasound and supercritical fluid extraction.	(Kumar et al., 2024)

6.	VA (Retinyl Ester)	Meeting the daily requirements of VA after heating/cooking/deep frying.	sunflower oil, soybean oil, corn oil and vegetable ghee	Heated different oil at temperature range of 100-175 ºC for 5-30 minutes and VA level was evaluated.	More than 60% of the VA was retained in the oils even after heating at 175 ºC. And this concentration which was enough to meet the daily requirements.	(Akhtar et al., 2012)
s7.	Peach palm fruit by-products	ultrasound-assisted extraction of total carotenoids obtained from dried peach palm by-products using sunflower oil as extraction solvent at mild temperatures to incorporate beta-carotene.	Sunflower oil for enrichment	Ultrasonic-assisted extraction (UAE) was performed in a sonication cleaning bath with varying intensity, temperature, and extraction time.	Maximum extraction of total carotenoids was 163.47 mg/100 g of dried peel. The experimental values under optimal condition were consistent with the predicted values.	(Ordóñez-Santos et al., 2015)
8.	Dry Tomato Waste	Enriching the vegetable oil and extending the shelf-life.	Extra virgin sunflower, unrefined corn, refined rapeseed, extra virgin olive, olive pomace, soybean, refined sunflower, Peanut, Rice, and Grape seed oil	Dried tomato waste samples (5 g) were subjected to each of the following extraction procedures: (a) ultra-sound-assisted extraction in 100 mL oil at 20 ºC for 50 min; (b) microwave-assisted extraction in 100 mL oil for 5 min; (c) maceration at 20 ºC in 100 mL oil for 7 days.	Carotenoids were extracted in oils in significant amounts from tomato waste. Extraction of dry tomato waste improved the oxidative and thermal stability of oil and vice-versa.	(Nour et al., 2018)

Table 2.3: List of studies focusing on the health benefits of beetroot.

Sl. No.	Source of betalains	Aims	Food fortified/designed	Trials/experiments conducted	Notable findings	References
1.	Beetroot	To modulate hypertension in hypertensive participants	Beverage prepared with mix of Carrot and beetroot	The study participants (n=24) were given 250 ml of the beverage for 60 days before assessment.	Beverage influentially reduced the pulse rate, systolic and diastolic pressure, triglycerides, and LDL levels	(Razzaq et al., 2023)
2.	Beetroot Powder	To control hypertension as well as hyperlipidemia.	500-1000 mg of powder was dissolved in water with cholesterol rich diet.	This dose was given for 60 days to 24 participants.	Beetroot powder lowered the lipid profile and hence it could be used for the prevention of hyperlipidemia.	(Sarfaraz et al., 2021)
3.	Alcoholic Extract of Beetroot (Mostly betanin)	To assess the anticancer effects on human colorectal cancer.	Freeze dried beetroot extract	Two human colorectal cancer cell lines and normal epithelial cell line of same embryonic origin were treated with extract of beetroot.	The extract was able to induce apoptosis in the cancer and normal cells like anticancer drugs.	(Saber et al., 2020)
4.	Beetroot	To increase the vegetable consumption for better health and meet the requirements.	100 g of sample bread contained 40 g of white or red beetroot.	120 participants of different origin and age group.	The likeness of bread was not affected by addition of vegetables in the bread. Acceptability of vegetable enriched	(Hobbs et al., 2014)

					bread as good as normal bread.	
5.	Study 1: Beetroot Juice. Study 2: Beetroot powder	Study 1: To study the dose-dependent effects of beetroot juice in 18 healthy normotensive men. Study 2: to investigate the effects of red or white beetroot enriched bread products on blood pressure in 14 healthy men.	Study 1: Beetroot Juice. Study 2: Breads enriched with white and red beetroot.	Study 1: 18 participants sample 1 = 500 g water sample 2 = 100 g Juice + 400 g water sample 3 = 250 g Juice + 250 g water Sample 4 = 500 g juice. Study 2: 14 participants Sample 1 = 0 g Beetroot in 200 g bread. Sample 2 = 50% beetroot in 200 g bread.	Study 1: With increase in the beetroot juice consumption, there was significant reduction in SBP and DBP after 90 minutes of drinking. Study 2: Postprandial SBP and DBP started to decrease after 60 mins of the consumption of breads incorporated with white or red beetroot.	(Hobbs et al., 2012)

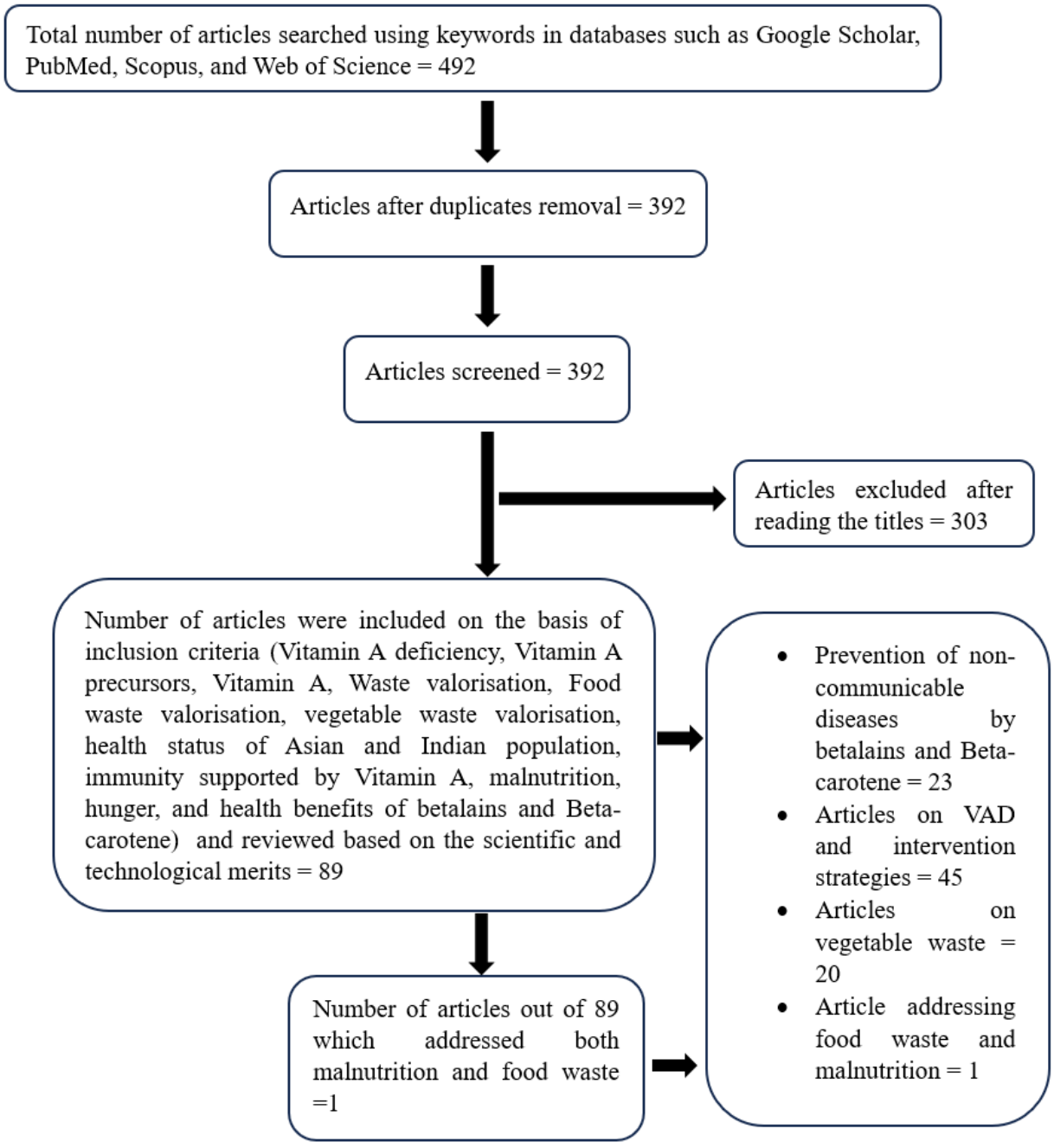

Figure 2.1: Flowchart showing the steps involved in the selection of articles focusing on the relationship between food waste and malnutrition in South Asian population.

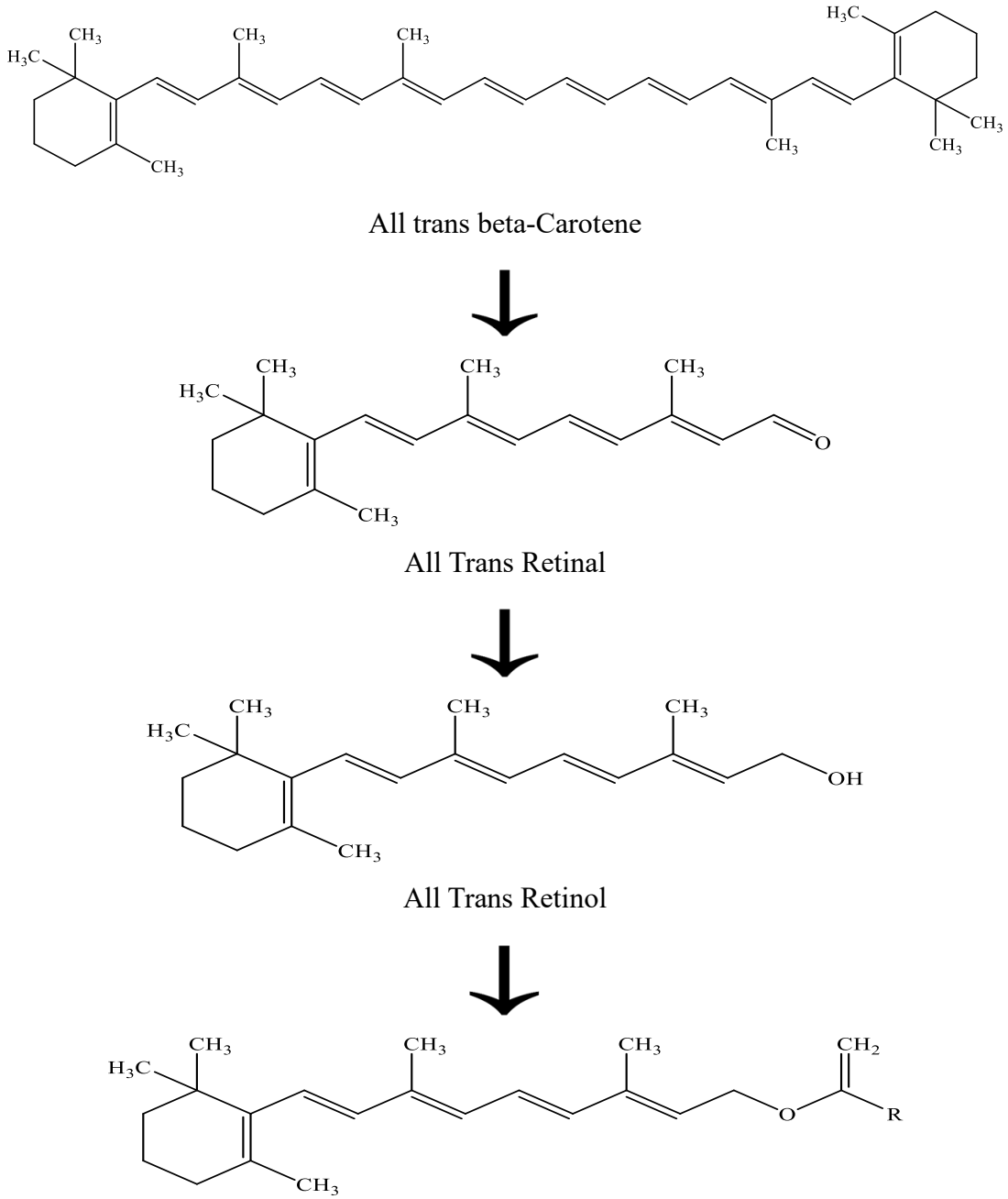

Figure 2.2: Steps involved in the conversion of all-trans beta-carotene to retinyl ester (storage form in body). Retinoid is originally derived from proretinoid carotenoids such as beta-carotene. Retinal can be formed by the central cleavage of beta-carotene by the enzyme beta-carotene 15,15'-monoxygenase. Retinol is formed by the reversible reduction of retinal by one of the retinal reductase family members. The enzyme lecithin retinol acyltransferase synthesizes

retinyl esters by transferring a fatty acyl moiety from the sn-1 position of membrane phosphatidyl choline to retinol.

Supplementary materials

Table 2.1S: Number of deaths per year due to vitamin A deficiency in South Asian countries

Sl. No.	Country	No. of deaths recorded/Year
1	Afghanistan	50,000
2	Bangladesh	28,000
3	Bhutan	600
4	India	3,30,000
5	Nepal	6,900
6	Pakistan	56,000
7	Total South Asian	4,71,500
8	World as total	11,50,000

Source: (Akhtar et al., 2013)

Table 2.2S (a): Crop-wise production of horticulture crops for three years in India

Sl. No.	Commodity	2015-16	2016-17	2017-18
1	Beans	2334	2012	2277
2	Brinjal	12515	12510	12801
3	Cabbage	8806	8807	9037
4	Carrot	1338	1350	1648
5	Tomato	18732	20708	19759
6	Okra	5849	6003	6095
7	Cauliflower	8090	8557	8668
8	Cucumber	1202	1142	1260
9	Mushrooms	436	411	487
10	Potato	43417	48605	51310
11	Onion	20931	22427	23262

Source: (Jha et al., 2019); Horticultural Statistics at a Glance, Government of India (2018). Data represented in this table are in metric tons (MT) for each commodity in respective year.

Table 2.2S (b): Crop-wise production of horticulture crops for three years in Sri-Lanka

Sl. No.	Commodity	2015-16	2016-17	2017-18
1	Other Vegetables	7849543	10582864	9027708
2	Manioc	5164241	4470003	5036134
3	Mushroom	545030	696930	834217
4	Chilies	465166	419899	416788
5	Gherkins	1225760	1061070	1608039
6	Kiri Ala	250198	278436	303805
7	Onions	20353	34786	65189
8	Potatoes	1830	55851	91289
9	Sweat Potatoes	29812	48779	25305
10	Carrots	-------	1032	102476
11	Tomato	600	63964	13769
12	Garlic	55363	8757	934
13	Cabbage	17	2	567

Source: Sri Lanka Export Development Board (2019). Data represented in this table are in kilograms (kgs) for each commodity in respective year.

Table 2.2S (c): Crop-wise production of horticulture crops for year 2019 in Bangladesh.

Sl. No.	Crop (Winter)	2015-16	2016-17	2017-18
1	Rabi Brinjal	310	348	356
2	Rabi Pumpkin	186	191	191
3	Cauliflower	268	278	274
4	Cabbage	296	312	322
5	Watergourc	218	226	232
6	Tomato	368	389	385
7	Radish	281	280	281
8	Beans	129	137	135
9	Carrot	16	16	19
10	Palongsak	51	66	55
11	Lalsak	52	54	59
12	Lausak	25	25	29

Source: Bangladesh Bureau of Statistics (BBS) (2020), Yearbook of Agricultural Statistics 2018.

Production of each commodity in year 2019 is shown in million metric tons (MT).

Table 2.2S (d): Crop-wise production of horticulture crops for three years in Pakistan per production season.

Sl. No.	Crops	2016-17	2017-18	2018-19
1	Radish	159193	156677	157526
2	Turnip	264261	258308	255191
3	Carrot	236583	240203	247813
4	Spinach	110348	109236	110554
5	Cauliflower	217470	211998	211930
6	Cabbage	79796	79451	79603
7	Sweet Potato	12478	12477	16174
8	Peas	165226	1465098	170985
9	Garden Peas	982	993	820
10	Knol Kohl	396	354	393
11	Fenugreek	471	475	527
12	Lettuce	441	432	420
13	Sugar beet	160301	292100	363733
14	Tomatoes	439254	428275	442637
15	Beans	5489	5559	5557

Source:- Provincial Crop Reporting Service Centers

Table 2.3S: Household food waste estimates in South Asian countries.

Sl. No.	Country	Household Food Waste Estimates (Waste Per Capita/Year)	Household Food Waste Estimates (Tonnes/Year)
1	Afghanistan	82	3109153
2	Bangladesh	65	10618233
3	Bhutan	79	60000
4	India	50	68760163
5	Pakistan	79	15947645
6	Sri Lanka	74	1617738
7	Nepal	76	2249412

Source: Food Waste Index Report, UN Environment Programme (2021)

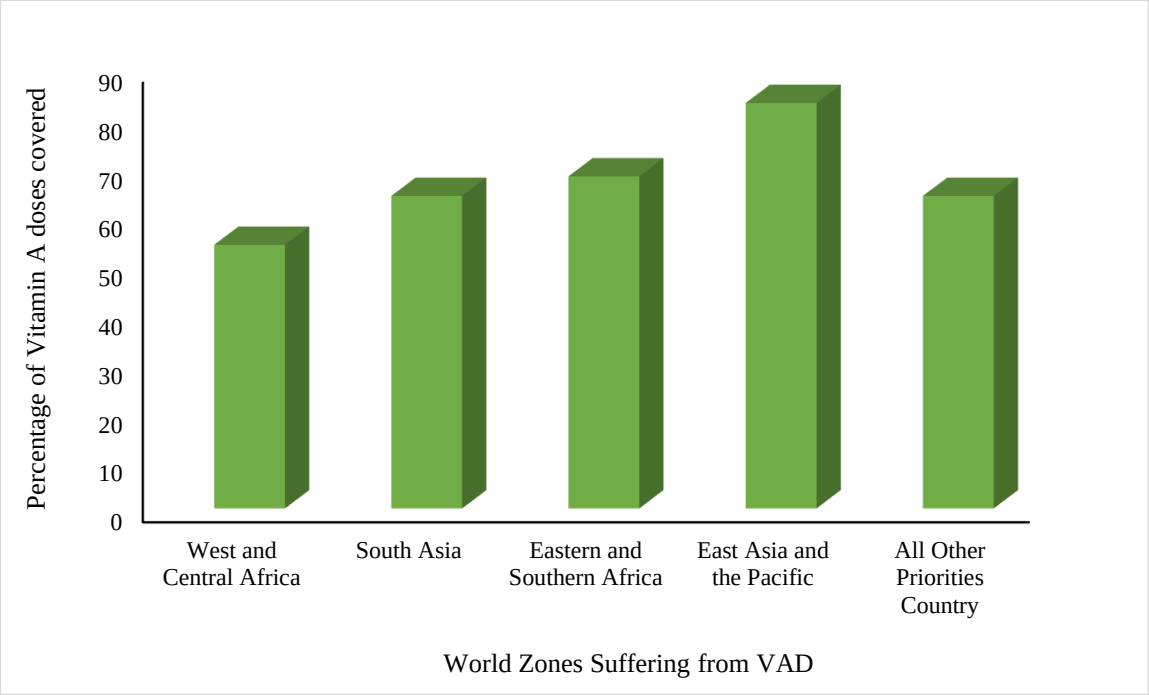

Figure 2.1S: Vitamin A doses covered across the world up to 2018 (Source: UNICEF Data, 2018).

Chapter 3

A Review of Extraction Methods for Recovering Betalains and Beta-carotene

This chapter is prepared as per the author guidelines to be submitted for publication to *Comprehensive Reviews in Food Science and Food Safety.*

Abstract: Betalains and beta-carotene are two most sought after and used natural pigments. They have several potential health benefits. The waste stream created from beetroot and carrot processing industries, production farm, and households have posed serious environmental challenges for vegetable waste management. Extraction of these industrially useful pigments can help to valorise these wastes. The drive to harness the bioactive properties and vibrant colors of betalains and beta-carotene for commercial use has spurred researchers to seek out extraction techniques, which are user friendly and scalable, but not labour intensive, exhaustive, time consuming or expensive. Researchers have tried to intensify the extraction process by using ultrasound, microwave or supercritical fluid extraction systems. Although ultrasound and microwave assisted extraction has been studied on a laboratory scale for past 20-30 years, there are no reported commercial developments of these technologies for betalains and beta-carotenes. On the other hand, supercritical fluid extraction is a scalable technology, but it can be an expensive strategy for the valorisation of vegetable waste. This comprehensive review aims to critically evaluate solvents and technologies which can be potentially applied to recover these bioactives from vegetable processing wastes like beetroot and carrots. In particular, the review will focus on the extraction mechanisms, factors influencing the efficiencies of various technologies. The review will specifically cover supercritical fluid extraction, ultrasound-assisted extraction, microwave-assisted extraction for betalains and beta-carotene extraction, and explore the possibility of using elevated temperature extraction (pressurised or otherwise) - which seems promising for the extraction of thermolabile compounds due to shorter extraction time, use of eco-friendly solvents and ease to scale up.

Keywords: Betalains; Beta-carotene; Vegetable Waste; Extraction; Ultrasound; Microwave; Supercritical; Pigments.

3.1 Introduction

Vegetable waste refers to the parts of vegetables like peels, stems, leaves, whole vegetables and other portions (Garnett, 2006) that are discarded or not used in cooking or consumption. This waste can come from various sources, such as households, restaurants, food processing facilities, and agricultural operations. It is important to note that managing vegetable waste responsibly is a step towards reducing overall food waste and its environmental impact. Additionally, reducing the amount of vegetable waste generated in the first place through better meal planning and utilizing the entire vegetable can further contribute to sustainability efforts (Pinotti et al., 2020).

Plants produce phytochemical compounds as secondary metabolites to protect themselves from pests and ailments, but they can also help with health promotion and disease prevention in human body (Ivanović et al., 2020). Hence, discarded vegetables are an important source of health-promoting components such as antioxidants, phenolics, flavonoids, terpenes, betalains, carotenoids, and others (Arscott & Tanumihardjo, 2010; Dias, 2012). Sustainable extraction of these active ingredients could be a major step in the direction of valorising vegetable waste. Their demand as raw materials for nutraceutical industries is growing every year because of consumer awareness of the adverse health effects of synthetic compounds (Álvarez et al., 2017).

In the United Kingdom, the production and consumption of carrots is only next to potatoes (Frankowska et al., 2019). The amount of carrots wasted happens to be more than half of the total production. About 4.5 million tonnes of the UK grown fresh fruits and vegetables are lost during grading. Selecting carrots for retail, based on its appearance, means that upto 50% of carrots produced will never hit the markets. Similarly, the waste creation from one of the other

root vegetables, beetroot, is similar to carrot in every reported index shown in Figure 3.1 (i.e., waste generated during pre-processing: farm, grading, storage and post-processing: packaging/processing and consumption) (Frankowska et al., 2019). The nutrient/chemical composition of carrot and beetroot are given in Table 3.1, showing these materials to be significant sources of several valuable colouring compounds. In particular, carrots and beet root are the major sources of beta-carotene and betalains, which can fulfil the increased demand for natural colorants, in the light of health concerns and greater regulatory scrutiny of artificial food dyes (Celli & Brooks, 2017).

The reasons behind this change in consumer preference and the surge in demand for natural colorants are multifaceted. Firstly, there's growing concern among consumers regarding the potential health risks associated with synthetic additives, notably artificial food dyes. Natural colorants are viewed as a safer alternative. Secondly, the "clean label" movement, which champions transparent and easily recognizable ingredient lists on product labels, aligns well with the use of natural colorants. These ingredients, often sourced from fruits, vegetables, and plants, are familiar to consumers and are considered more trustworthy. Additionally, consumers associate natural ingredients with higher quality and authenticity. They perceive products featuring natural colorants as being closer to nature and, therefore, healthier and more wholesome. Sustainability and environmental considerations also play a role. Natural colorants are typically derived from renewable sources and are seen as more environmentally friendly compared to synthetic alternatives.

Thus, the demand for natural colorants has prompted companies to explore innovative methods for isolating color from plant sources while maintaining stability, safety, and regulatory compliance. Nevertheless, it is crucial to note that not all natural ingredients are inherently safe or suitable for all individuals. Proper research and testing are essential to ensure product safety

and quality. Regulatory agencies, such as the Food and Drug Administration (FDA) in the United States, are adapting to these changing consumer preferences by revising guidelines for food additives, including colorants. As consumer preferences continue to evolve, the market for natural colorants is expected to expand, driving innovation within the food and cosmetic industries (Celli & Brooks, 2017).

In order to recover these bioactive compounds from natural sources, several steps namely extraction, isolation, and purification are necessary (Ameer et al., 2017). In the previous reviews, most of the articles have explained the extraction process with their advantages and disadvantages without shedding any light on the impact of extraction solvent, sustainability and scalability. A major limitation of the previous reviews is the unidirectional analysis of sustainability merely based on the total carbon footprint of the process rather than solvents and equipment. Furthermore, mechanistic studies in order to thoroughly understand the mechanisms governing the kinetics of extraction methods and addressing the technical barriers to scale up the extraction process are the major research gap. The market demand for both these natural pigments are high and, if this demand can be met by the utilization of carrot and beetroot wastes, the carbon footprint and environmental burden can be significantly lowered. Hence, this review aims to compare the contemporary extraction processes employed to recover betalains and beta-carotene from beetroot and carrots and explore the opportunity to scale up the process. The mechanistic design of the experiments and kinetic study of the extraction process at elevated temperature will further the objective of this review. This is particularly significant as betalains are the most sought-after natural pigment, and 99.8% of which are produced from beetroot worldwide.

3.2 Overview of beta-carotene and betalains

3.2.1 Beta-carotene

Beta-carotene, an isoprenoid and a member of the tetraterpene family, functions as an antenna pigment in plants, aiding in light absorption and acting as an antioxidant to protect against UV-B light damage. Plants deficient in beta-carotene are more susceptible to light damage after being grown in darkness (Meléndez-Martínez et al., 2022). Carotenoids, including beta-carotene, contribute to the vibrant yellow to red pigments seen in autumn leaves and the orange coloration in carrots, pumpkins, and oranges (Kumar et al., 2023). These compounds also play roles in seed dispersal, pollination, scent precursor formation, and light protection.

In the food industry, beta-carotene's versatility in providing color, stability, and health benefits has boosted its popularity. The global market for nutraceuticals, known for their health benefits, has seen significant growth, particularly in carotenoids like beta-carotene, canthaxanthin, lutein, astaxanthin, and lycopene. The international carotenoid market was valued at 1.3 billion euros in 2017, with projections to reach 1.8 billion euros by 2022, reflecting a compound annual growth rate of 5.7% (Saini & Keum, 2018; Almagro et al., 2022). Fruits and vegetables are major sources of carotenoids, underscoring their rising demand and recognized health benefits.

3.2.1.1 Structure and types of beta-carotene

Beta-carotene features a distinctive structure composed of 40 carbon atoms, arranged in eight isoprene units. This arrangement forms a linear backbone with cyclic rings at both ends, which is also devoid of oxygen atoms (Bogacz-Radomska & Harasym, 2018). The molecule belongs to the tetraterpene family and stands out due to its conjugated double-bond system, which imparts stability and heightened electron resonance (Almagro et al., 2022). This structure

enables electron transfer to other molecules, contributing to its diverse functions. In terms of types, beta-carotene is a member of the carotenoid family, which includes a range of pigments responsible for the red, orange, and yellow hues in various plants. Within the carotenoid family, beta-carotene is one of the most abundant compounds, existing in different isomeric forms, including the all-trans form which is a linear arrangement of the double bonds which are responsible for the unique colour of the beta-carotene, and various *cis* isomers, where some of the double bonds have a bent or kinked configuration (Clark, 2007; Lavelli & Sereikaitė, 2022).

3.2.1.2 Distribution of beta-carotene in nature

Beta-carotene is widely distributed in nature and can be found in various plants, fruits, and vegetables. It serves multiple functions, including contributing to the color of these foods and acting as a precursor to vitamin A. In nature, beta-carotene is present in fruits and vegetables (including carrots, sweet potatoes, pumpkins, butternut squash, bell peppers, mangoes, apricots, cantaloupe, and papayas (Ngamwonglumlert et al., 2017), leafy greens (spinach, kale, collard greens, and Swiss chard) (Durante et al., 2014), tropical fruits (mangoes, papayas, and guavas) (Rodriguez-Amaya, 1990), herbs such as oregano (Damechki et al., 2001), Algae (Fatima et al., 2023) and seeds such as rosemary gourmet (Damechki et al., 2001). The distribution of beta-carotene in nature is closely linked to its role in photosynthesis, coloration, and the nutritional needs of plants and organisms. The specific concentration of beta-carotene in these sources can vary based on factors such as plant variety, ripeness, and growing conditions.

3.2.1.3 Importance of beta-carotene in daily diet and health

Incorporating beta-carotene into one's daily diet holds significant importance for overall health and well-being. Serving as a precursor to vitamin A, beta-carotene plays a crucial role in

maintaining vital bodily functions (Vimaleswaran, 2024). This compound is converted into active vitamin A, crucial for maintaining optimal vision, particularly in low-light conditions, and preventing night blindness (Arlappa, 2011). Beyond its role in eye health, beta-carotene acts as a potent antioxidant, effectively combating harmful free radicals that can lead to chronic illnesses such as heart disease, cancer, and age-related conditions (Mäki-Arvela et al., 2014). Moreover, its contribution to immune system support, skin health, cell growth, and differentiation further accentuates its significance. Diets rich in beta-carotene-rich foods have been associated with a lowered risk of certain cancers, and its potential positive impact on heart health and healthy aging underlines its multifaceted benefits (Meléndez-Martínez et al., 2022). However, achieving these advantages requires the incorporation of a diverse array of colourful fruits and vegetables into daily eating habits, ensuring a holistic intake of nutrients that support overall wellness. Beta-carotene finds a multitude of applications within the food industry. Its versatility makes it a popular natural ingredient with various uses as shown in Table 3.2.

3.2.1.4 Availability of beta-carotene in different forms in market

Beta-carotene is commonly available in various forms to cater to different industries and needs. In the supplement market, it can be found as capsules, powders, liquids, and tablets, serving as a convenient means to fulfil daily nutritional requirements. Within the food industry, powdered forms and concentrates of beta-carotene are prevalent, often used to enhance color and nutritional content. The pharmaceutical sector primarily opts for capsule and tablet formats, leveraging beta-carotene's benefits, while the cosmetics industry tends to incorporate it in liquid form, often combined with other active ingredients. It's important to note that the recommended dietary allowance (RDA) for vitamin A is 900 µg, a value that can be achieved by consuming 10.8 mg of beta-carotene per day (Saini et al., 2022).

3.2.2 Betalains overview

3.2.2.1 Betalains

Betalains constitute a group of naturally occurring pigments primarily found in selected plant families such as Amaranthaceae and Caryophyllaceae (Castro-Enríquez et al., 2020). These intricate molecules are accountable for the striking spectrum of colors exhibited by specific flowers, fruits, and vegetables. Betalains are categorized into two principal classes: betacyanins, which give rise to captivating red to violet shades, and betaxanthins, responsible for generating captivating yellow to orange hues (Kayın et al., 2019) as shown in Figure 3.2. One of their distinctive attributes is their solubility in water, setting them apart from other prominent plant pigments like chlorophyll and carotenoids. Unlike the latter, betalains do not play a pivotal role in photosynthesis but seem to serve as antioxidants, potentially conferring protection against adversities such as intense light exposure and certain pathogens (Liliana & Oana-Viorela, 2020). Moreover, betalains have garnered noteworthy interest within the food industry, appreciated for their application as natural colorants that circumvent the use of synthetic dyes (Sivakumar et al., 2009). While ongoing research delves into the potential health benefits of betalains, including their antioxidant prowess and capacity for mitigating inflammation, a more comprehensive understanding necessitates further investigation. It's imperative to recognize that the presence and concentrations of betalains within plants are subject to a gamut of factors, encompassing genetic composition, cultivation circumstances, and the developmental stage of the plant (Liliana & Oana-Viorela, 2020).

3.2.2.2 Betalains as an antioxidant and source for nitrates

Betalains, vibrant pigments in select plants, offer significant health benefits beyond their visual appeal. These compounds are potent antioxidants, protecting cells from free radical damage,

which can lead to chronic diseases and premature aging (Tumbas Šaponjac et al., 2016; Clifford et al., 2015). Betalains, especially abundant in beetroots, also provide dietary nitrates, which are converted into nitric oxide (NO) in the body. NO improves blood flow and circulation, contributing to better cardiovascular health (Liliana & Oana-Viorela, 2020; Hobbs et al., 2012; Fu et al., 2020).

The dual benefits of antioxidation and cardiovascular support have made betalains a subject of scientific interest, highlighting their potential in health and nutrition. Ongoing research is necessary to fully understand their effects on human health, considering individual and physiological differences. Betalains represent a promising area in nutritional science, with the potential for new therapeutic applications.

3.2.2.3 Betalains structure and types of betalains

Betalains, a captivating group of pigments, infuse specific plants with their vibrant colors and distinctive chemical structures. These pigments are divided into two primary categories: betacyanins, which infuse the spectrum with red to violet hues, and betaxanthins, which impart an array of yellow to orange shades. Both classes share a common structural core – betanidin for betacyanins and betaxanthin for its counterpart – adorned with sugar molecules that lend individuality to their solubility and color variations (Skalicky et al., 2020). This water-solubility sets betalains apart from conventional lipid-soluble pigments, such as chlorophyll and carotenoids. This exceptional property has positioned betalains as sought-after natural alternatives for food coloring and has also aroused curiosity regarding their potential health benefits. Beyond their aesthetic contributions, the amalgamation of betacyanins and betaxanthins within plants creates a mesmerizing tapestry of colors, highlighting the remarkable role these pigments play in nature's palette.

3.2.2.4 Distribution of betalains in nature

Betalains, though less widespread than other pigments, are intriguingly distributed across various plant species in nature, predominantly within the Amaranthaceae and Caryophyllaceae families. In the Amaranthaceae family, beetroots stand out as a well-known source of vibrant red and purple betalain pigments (Liliana & Oana-Viorela, 2020). Similarly, Swiss chard showcases an array of colors in its leaves, stems, and veins, while amaranth plants contribute to nature's palette with their ornamental and edible varieties (Lee et al., 2014). Additionally, betalains have been discovered in a select few cacti species and other plants, although their occurrence remains less prevalent (Herbach et al., 2006). Amidst this varied distribution, betalains lend their captivating colors to the natural world, illuminating the intricate interplay of pigments across diverse plant families.

3.2.2.5 Importance of betalains in daily diet and athletes' health

Betalains are important in daily diets and for athletes due to their antioxidant properties and potential to enhance exercise performance (Zamani et al., 2020). In everyday diets, betalains combat oxidative stress from free radicals, supporting overall health and potentially reducing chronic disease risk (Carreira-Casais et al., 2021). Foods rich in betalains, like beetroots, add nutritional value and visual appeal to meals.

For athletes, betalains' dietary nitrates can convert to nitric oxide, improving blood flow and enhancing oxygen and nutrient delivery to muscles. This can increase endurance and reduce fatigue during exercise (Zamani et al., 2020). Studies suggest beetroot juice, high in nitrates, may boost exercise efficiency and performance (Stanaway et al., 2017). Betalains' antioxidants also help with recovery and minimizing exercise-induced oxidative damage. However, individual responses vary, so athletes should consult healthcare professionals before changing

their diet or supplementation. Betalains' dual benefits make them valuable for both everyday nutrition and athletic performance.

3.2.2.6 Betalains demand as a colouring matter by the food industry

Betalains, known as "beetroot red," are significant in the food, pharmaceuticals, cosmetics, and dye industries (Sadowska-Bartosz & Bartosz, 2021). In the food industry, beetroot extracts serve as natural colorants, enhancing the visual appeal of fruit juices, bakery products, yogurts, dairy products, pasta, salad dressings, sauces, and confectionery items like candies and gummies (Khan, 2016; Luzardo-Ocampo et al., 2021). These extracts are particularly useful in plant-based and vegan foods as alternatives to synthetic dyes. Betalains are stable between pH 4-5, but their use in high-temperature processed products is limited (Huang and Elbe, 1987).

The North American Betanin Food Colors Market was valued at USD 30.3 million in 2021 and is expected to grow at a CAGR of 5.7% from 2022 to 2032. The U.S. leads this market with sales of 1581 metric tons valued at over USD 26 million in 2021, reflecting a strong preference for natural food colorants. North America accounts for 36.4% of the global market. The European Union's market was valued at USD 22 million in 2021, representing 24.1% of the global share, with Germany and France leading with USD 6.6 million and USD 4 million, respectively. Regulatory flexibility worldwide supports the growing influence of Betanin food colors.

3.2.2.7 Availability of betalains in market

Betanin is primarily available in two forms: powder and liquid, offering versatility in its applications. It finds substantial use across various industries due to its vibrant color and potential health benefits. The food and feed industry, which places emphasis on natural and visually appealing products, incorporates betanin to enhance the color of foods and animal

feed. Similarly, the beverage industry benefits from betanin's ability to lend attractive hues to beverages, particularly those focusing on natural ingredients. The cosmetics industry capitalizes on betanin's natural pigment to add color to cosmetics and personal care products. Furthermore, the healthcare industry, recognizing betanin's potential health-enhancing properties, explores its use in dietary supplements and nutraceuticals. In each of these industries, betanin plays a role in meeting consumer demands for natural, visually appealing, and potentially beneficial products.

3.3 Advantages of vegetable waste management

Extraction of valuable compounds like beta-carotene and betalains from vegetable waste is a promising upcycling strategy that can help reduce waste and generate value-added products as discussed below. It is also worth noting that it can be good strategy to reduce environmental impacts (Renita et al., 2023).

3.3.1 Waste reduction

The extraction of beta-carotene and betalains from vegetable waste offers an effective solution to reduce the accumulation of organic waste in landfills. Rather than discarding nutrient-rich parts of vegetables, this strategy transforms waste into valuable resources. By diverting waste from landfills, this approach mitigates methane emissions and reduces the strain on waste management systems.

3.3.2 Sustainable development and resource conservation

Embracing the extraction of beta-carotene and betalains aligns with the principles of sustainable development (i.e., Sustainable Development Goal 12) through resource recovery from waste streams. This goal reduces the reliance on virgin resources used in synthetic

alternatives and promotes the efficient use of existing materials. It exemplifies a circular economy model, where waste is repurposed into valuable products, contributing to the overall resilience of ecosystems and industries.

3.3.3 Diversification of revenue streams and economic growth

Industries engaged in the extraction of beta-carotene and betalains from vegetable waste open doors to new revenue streams. The sale of these valuable compounds to various sectors, including food, cosmetics, supplements, and pharmaceuticals, diversifies income sources. This economic diversification can stimulate local and regional economic growth, fostering innovation and job creation.

3.3.4 Community and agricultural benefits

The upcycling strategy positively impacts communities and agriculture in multiple ways. First, it encourages collaboration between waste processors, farmers, and local industries, promoting a symbiotic relationship within the community. Second, farmers can benefit from access to nutrient-rich compost generated from residual waste (Bain et al., 2010).

3.3.5 Soil enrichment and agricultural sustainability

After extraction, the left over material for composting enhances soil fertility, structure, and water retention, ultimately leading to improved crop yields and reduced dependence on synthetic fertilizers. The strategy promotes sustainable agricultural practices that prioritize soil health and long-term productivity (Pergola et al., 2018).

3.3.6 Educational and awareness initiatives

The implementation of this upcycling strategy provides opportunities for educational programs and awareness campaigns. Communities can learn about waste reduction, sustainable practices, and the value of repurposing resources. These initiatives foster a sense of environmental stewardship, encouraging individuals to make more conscious choices in their daily lives (Thomas & Sharp, 2013).

3.3.7 Mitigation of greenhouse gases release in the environment

The extraction of bioactive compounds from vegetable waste offers a promising avenue for mitigating greenhouse gas (GHG) emissions. This process minimizes the generation of methane, a potent GHG associated with organic decomposition. Furthermore, utilizing plant waste for bioenergy production, such as through anaerobic digestion, not only prevents methane emissions but also provides a renewable energy source that can substitute for fossil fuels (Ometto et al., 2007).In summary, the extraction of beta-carotene and betalains from vegetable waste is a multifaceted upcycling strategy and it is consistent with sustainable development goal 12 (SDG 12). with far-reaching benefits. It addresses waste reduction, landfill diversion, sustainable development, revenue diversification, economic growth, community empowerment, agricultural sustainability, and environmental conservation. By embracing this approach, societies can move towards a more circular and responsible way of managing resources, fostering a balanced relationship between human activities and the environment.

3.4 Application of different solvent types for the extraction of beta-carotene and betalains

The choices of solvents for the extraction of beta-carotene and betalains from vegetable waste is crucial as it significantly influences the efficiency and selectivity of the extraction process. Different solvents have varying polarities and affinities for these compounds, leading to

different extraction yields. This is because the extraction of bioactive compounds relies primarily on their solubility in the chosen solvent, with bioactives dissolving in solvents of similar polarity; non-polar solvents extract non-polar compounds like lipids, while polar solvents are suitable for polar compounds such as phenolics and alkaloids. The process begins with the solvent penetrating the plant matrix, allowing the bioactives to diffuse into the solvent, a rate influenced by factors like temperature, solvent concentration, and the plant material's physical structure. Following diffusion, mass transfer occurs, moving the bioactives from the plant matrix to the bulk solvent phase, and this is further influenced by agitation, temperature, and solvent properties. Additionally, with aqueous solvents, bioactives may undergo hydrolysis or other chemical reactions, breaking down complex molecules into simpler, more extractable forms. Beta-carotene can be efficiently extracted by organic non-polar and non-aqueous solvents that includes vegetable oils. Betalains require more polar solvents including aqueous ones and supercritical fluids in nature. Table 3.3 (a) and (b) shows the different kinds of solvents with their specific properties used currently in the extraction of beta-carotene and betalains .

3.4.1 Aqueous solvents

Aqueous solvents are solutions primarily composed of water, showcasing characteristics of safety, versatility, and environmental friendliness. Water's polar nature enables it to dissolve a wide range of polar and ionic compounds, making it a crucial medium for chemical reactions, cleaning, and extractions of hydrophilic substances. Aqueous solvents like hydrogen peroxide, sodium hydroxide, ammonia, and acid solutions play essential roles as disinfectants, cleaning agents, and reactants in various industries. Moreover, salt solutions, sugary syrups, and buffered solutions find applications in food preservation, sweetening, and maintaining stable pH levels in biological experiments. Overall, the use of aqueous solvents offers a sustainable

and safe approach across diverse fields due to water's inherent properties as a universal solvent and its eco-friendly attributes.

3.4.2 Non-aqueous solvents

Non-aqueous solvents encompass a diverse array of substances, that includes mostly organic solvents and vegetable oils, that offer compelling alternatives to water-based solutions. Organic solvents like ethanol, methanol, chloroform, acetone, hexane, and diethyl ether find utility across industries and laboratories, serving as effective solvents for diverse chemical processes and extractions. Meanwhile, vegetable oils derived from plants such as olive, coconut, and sunflower oil possess inherent lipophilic properties that make them valuable in dissolving hydrophobic compounds. These non-aqueous solvents are especially useful for applications where water-based solutions prove less effective, playing vital roles in areas spanning chemistry, industrial processes, and cosmetics. Nonetheless, prudent attention to safety, environmental considerations, and proper handling remains paramount when working with these solvents.

3.4.3 Supercritical fluid solvent

A supercritical fluid solvent exhibits unique state of matter that exhibit the properties of both liquid and gas provided the critical temperature and pressure. This solvent dissolves the material by behaving like a liquid, while it can also diffuse through the solids like a gas. One of the most commonly used supercritical solvent is carbon dioxide (CO_2) which operates in the mild critical conditions (31.1 C, and 73.8 atm) and it is non-toxic in nature. In critical state, CO_2 can effectively extract compounds that are typically challenging to isolate using traditional solvents. This solvent also offers several advantages, such as being a green and sustainable method as it utilizes non-toxic and easily recoverable solvents. It also eliminates the need for

residual solvent removal. Moreover, SFE can be highly selective, targeting specific compounds while avoiding co-extraction of unwanted components.

3.4.4 Ionic liquids and deep eutectic solvents

Deep eutectic solvents (DES) and ionic liquids are innovative solvent alternatives, both offering low volatility, tunable properties which offers to extract both polar and non-polar bioactives using same solvent compositions, and potential sustainability benefits. However, DES, which are formed by mixing hydrogen bond donor and acceptor components, often stand out for their environmental friendliness, low toxicity, and biodegradability. These qualities make DES particularly appealing in applications where safety, eco-friendliness, and sustainability are priorities, such as in the pharmaceutical, food, and biotechnology industries. While ionic liquids possess similar advantages, DES's inherent attributes make them a preferred choice in scenarios demanding minimal environmental impact and enhanced biodegradability.

It is evident from the provided context that the extraction of bioactive compounds like beta-carotene and betalains is predominantly carried out using aqueous, non-aqueous, and supercritical fluid solvents. However, emerging trends highlight the potential of pressurized liquid solvents and deep eutectic solvents as future options. The choice of solvent significantly influences the extraction process for these compounds from plant materials. While previous academic and industrial research has explored various extraction technologies, the number of applicable methods remains limited. In the last five years, the most prominent extraction technologies for beta-carotene and betalains include Ultrasound Assisted Extraction (UAE), Microwave Assisted Extraction (MAE), Supercritical Fluid Extraction (SFE). However, Pressurised and elevated Temperature extraction (PETE) techniques has garnered substantial

attention based on the number of published articles, underscoring their relevance and effectiveness in extracting these specific bioactive compounds. These technologies and their applications are further discussed below.

3.5 Application of different extraction technologies for betalains and beta-carotene

3.5.1 Ultrasound assisted extraction

Ultrasound-Assisted Extraction (UAE) employs high-frequency sound waves to create cavitation bubbles in the extraction solvent. When these bubbles collapse, they generate intense localized pressure and temperature, which enhance the penetration of the solvent into the plant matrix and improve the release of bioactive compounds. There are two main types of UAE: probe-type and bath-type. Probe-type ultrasound involves direct immersion of an ultrasound probe into the extraction solvent, providing higher energy intensity and efficiency. Bath-type ultrasound involves placing the extraction container in an ultrasonic bath, offering more uniform energy distribution but generally lower intensity than probe-type.

Key parameters influencing UAE include the frequency and power of the ultrasound, the temperature and duration of the extraction process, the solvent type, and the solid-to-solvent ratio. Adjusting these parameters can optimize the extraction efficiency and yield for specific bioactive compounds. For instance, Maran and Priya (Maran & Priya, 2016) conducted a study aimed at optimizing the extraction of aqueous ultrasound-assisted betalains from discarded red beet stalks. The study revealed that the highest extraction yield was achieved by increasing temperature (up to 55 °C), ultrasonic power (up to 100 W), extraction time (up to 38 min), and solid-liquid ratio (up to 1:25 g/mL). This could be attributed to higher power intensities, which generally result in more effective cavitation, enhancing extraction, while higher temperatures can accelerate solvent diffusion but may degrade thermolabile compounds.

The advantages of UAE include its ability to enhance mass transfer and significantly reduce extraction time compared to traditional methods. For example, UAE reduced the extraction time of betalains from beetroot peels from hours to just 30 minutes (Šeremet et al., 2020). The results indicated that ultrasound-assisted extraction outperformed conventional maceration extraction, resulting in approximately 4.5 and 2-times higher yields for betacyanins and betaxanthins, respectively. It operates at lower temperatures, which helps preserve heat-sensitive bioactives, and can reduce the amount of solvent required. UAE is relatively low-cost and straightforward to implement on a small scale. However, it has disadvantages such as challenges in scaling up for industrial applications due to non-uniform energy distribution in larger volumes and potential equipment wear from prolonged use. Additionally, UAE is less effective for very dense or highly compact plant materials where cavitation might not penetrate effectively.

UAE is highly productive for small to medium-scale applications, offering rapid extraction times and high yields. However, scalability is moderate, as ensuring uniform cavitation in large volumes is difficult, and industrial-scale ultrasound equipment needs to be durable and efficient. Despite these challenges, UAE has been successfully industrialized in some sectors, such as the extraction of essential oils and polyphenols, but its widespread adoption is hindered by issues related to energy distribution and equipment maintenance. Relevant challenges include maintaining uniform energy distribution in larger scales, developing robust industrial-grade ultrasound equipment, and managing energy efficiency and operational costs. Addressing these challenges is crucial for optimizing the industrialization and scalability of UAE, making it a viable method for large-scale bioactive compound extraction. Table 3.4 (a) & Table 3.4 (b) has summarised the extraction of betalains and beta-carotene using ultrasonication.

3.5.2 Microwave assisted extraction

Microwave-Assisted Extraction (MAE) uses microwave energy to heat solvents and plant materials, leading to rapid heating and efficient extraction of bioactive compounds. There are two main types of MAE: closed-vessel and open-vessel systems. Closed-vessel systems operate under controlled pressure and temperature, allowing for higher efficiency and faster extraction. Open-vessel systems are simpler and operate at atmospheric pressure, but they may be less efficient and have longer extraction times compared to closed-vessel systems.

Key parameters influencing MAE include microwave power, extraction time, temperature, solvent type, and the solid-to-solvent ratio. For example, Sharma et al., (2022) & Elik et al., (2020) reported the studies of extracting beta-carotene in vegetable oils while manipulating various microwave parameters, they concluded that employed variables had their significant effect in the order of microwave power followed by extraction time and solid to solvent ratio. In these studies, the higher extraction efficiency reported by MAE over conventional extraction could be due to application of high temperature, microwave penetration into the plant cells, and solubility of beta-carotene in vegetable oils. The choice of solvent and its volume also play a crucial role in the efficiency and selectivity of the extraction. However, an anomaly was reported by Pinna et al., (2022) in which maceration yielded more beta-carotene than MAE.

MAE offers several advantages, including significantly reduced extraction times and higher efficiency compared to traditional methods. For instance, MAE of carotenoids from carrots yielded varying results (Kaur et al., 2022). The highest carotenoid yield was achieved at an extraction temperature of 50 °C, a 10-minute extraction time, and a solid-to-solvent ratio of 1:46.8 (w/v), resulting in 301.28 mg carotenoids/100 g dried weight., compared to hours extraction using conventional methods. MAE is also more energy-efficient and can reduce solvent usage, contributing to lower operational costs and environmental impact. However, MAE has some disadvantages, such as the high initial cost of microwave extraction equipment

and potential non-uniform heating in larger volumes, which can lead to inconsistent extraction and possible degradation of bioactives. Moreover, localized overheating in the sample can cause selective degradation of sensitive compounds.

MAE demonstrates very high productivity for small to medium-scale applications due to its rapid extraction times and high yields. For example, (Hiranvarachat & Devahastin, 2014) and (Chumnanpaisont et al., 2014) investigated the effects of continuous and intermittent microwave on the carrot peels and rate of beta-carotene extraction which was mostly completed within 5 minutes, a process that traditionally takes several hours. Scalability of MAE is moderate to high, with successful scale-up in some industrial applications such as the extraction of bioactive compounds from herbs and spices.

Industrialization of MAE is increasingly seen in sectors like pharmaceuticals, food processing, and natural product extraction, driven by the method's efficiency and reduced processing times. However, challenges remain in achieving uniform heating in large-scale applications, managing the high costs of microwave systems, and ensuring consistent product quality. Addressing these challenges involves developing advanced microwave technologies with better control mechanisms, optimizing process parameters for large-scale operations, and ensuring that the benefits of MAE are maintained during scale-up.

In conclusion, while MAE offers significant advantages in terms of efficiency, reduced extraction times, and lower environmental impact, its widespread industrial adoption is contingent on overcoming challenges related to uniform heating, equipment costs, and maintaining product quality at scale. With ongoing advancements in microwave technology and process optimization, MAE holds promise as a viable method for large-scale extraction of

bioactive compounds. Table 3.5 (a) & Table 3.5 (b) illustrates the extraction of beta-carotene using MAE with different sources and conditions.

3.5.3 Supercritical fluid extraction

Supercritical Fluid Extraction (SFE) utilizes supercritical fluids, most commonly carbon dioxide (CO_2), to extract bioactive compounds from various matrices. In its supercritical state, CO_2 exhibits both gas-like and liquid-like properties, allowing it to penetrate materials like a gas and dissolve substances like a liquid. SFE is categorized mainly into two types: supercritical fluid extraction with CO_2 (SC-CO_2) and co-solvent or modifier-assisted SFE. SC-CO_2 is widely used due to its non-toxicity, low critical temperature (31.1°C), and moderate critical pressure (73.8 bar). Co-solvent SFE involves adding a small amount of another solvent, like ethanol, to enhance the solubility of more polar compounds.

Key parameters influencing SFE include pressure, temperature, flow rate of CO_2, extraction time, and the use of co-solvents. For example, Fathordoobady et al., (2016, 2019) extracted betalains from red pitaya fruit using SFE technology. The results of the experiment revealed that a significant portion of the pigment extract was obtained during the initial 90 minutes of the dynamic extraction process. The study's findings indicate that employing Supercritical Fluid Extraction (SFE) led to the extraction of betacyanins from both the peel and flesh of the sample. The total betacyanins content in the peel extract was measured at 24.58 mg/100 ml, while the flesh extract contained a significantly higher concentration of 91.27 mg/100 ml. Notably, this process used a lower quantity of organic solvent compared to traditional solvent extraction methods, suggesting that SFE might be a more environmentally friendly and acceptable approach for betacyanin extraction. It's noteworthy that regardless of the pressure levels applied, the extraction yield values remained constant after the 90-minute mark. The

flesh of the sample had a significantly higher yield than the peel, which was linked to the higher levels of total soluble solids and total betacyanins content found in the flesh as compared to the peel. Higher pressures and temperatures can increase the solubility of bioactive compounds but may also lead to the degradation of sensitive compounds. The addition of co-solvents, typically in the range of 5-10%, can significantly improve the extraction efficiency of polar compounds.

The advantages of SFE are numerous. It offers high selectivity by adjusting pressure and temperature, producing high-purity extracts without solvent residues. SFE is environmentally friendly, utilizing non-toxic CO_2, and is suitable for thermolabile compounds like betalains and beta-carotene due to the relatively low temperatures involved. For instance, Ludwig et al., (2021) extracted beta-carotene using SFE technology from *Dunaliella salina,* an algal source. it was found that temperature and pressure were the two most influential operating parameters. The optimal extraction temperature, at 70 °C, resulted in the highest final beta-carotene extraction efficiency and that rose from 25% to 60%. Moreover, at the 70 °C constant temperature, increase in the pressure from 300 to 500 bar, shown a continuous increase in the beta-carotene yield. However, SFE has some disadvantages, including high initial setup and operational costs, complexity in maintaining optimal conditions, and the need for specialized high-pressure equipment and technical expertise.

SFE is highly productive for both small and large-scale applications. For example, the extraction of carotenoids from corn cob waste SC-CO_2 achieved a yield of greater yield than compared to conventional methods requiring multiple hours (Lau et al., 2019). Scalability of SFE is high, as evidenced by its successful industrial-scale application in extracting high-value compounds like caffeine from coffee beans and decaffeinated tea.

Industrialization of SFE is well-established, particularly in the food, pharmaceutical, and cosmetic industries. For instance, SFE is widely used for extracting flavors and fragrances, bioactive compounds, and nutraceuticals. However, the challenges include managing the high costs associated with high-pressure equipment, ensuring consistent product quality during scale-up, and the technical complexity of maintaining precise control over extraction conditions. A study on the industrial-scale extraction of polyphenols from grape seeds highlighted the importance of optimizing pressure and temperature to balance yield and quality, with costs managed through process efficiency and equipment maintenance.

Overall, while SFE offers significant advantages in producing high-purity extracts with minimal environmental impact, its widespread adoption is influenced by the ability to manage high operational costs and technical complexities. Ongoing advancements in equipment design, process optimization, and co-solvent utilization continue to enhance the viability of SFE for large-scale applications. With these improvements, SFE remains a promising and versatile extraction method for a wide range of bioactive compounds. Table 3.6 (a) & Table 3.6 (b) demonstrates the extraction of beta-carotene at various conditioned applied and raw materials used.

3.5.4 Pressurised and elevated temperature extraction (PETE)

PETE is a technique that uses elevated temperatures and pressure to increase the solubility of target compounds in the extraction solvent and reducing the extraction time significantly (Kumar et al., 2024; Kumar et al., 2023). This can be an effective way to extract bioactive compounds that are heat-labile, such as polyphenols, betalains, flavonoids, and carotenoids (Kumar et al., 2023; Maisurah Zakaria et al., 2022; Santoyo et al., 2012; Xu et al., 2015). The benefits of using PETE includes, 1) Increased solubility: As temperature increases, the

solubility of most compounds in water also increases. This means at higher temperatures can lead to higher extraction rate and yields of heat-unstable bioactive compounds, 2) Reduced extraction time: High temperatures can also speed up the extraction process. This can be beneficial for extracting bioactive compounds from large quantities of plant material, as it can reduce the overall processing time, 3) Improved selectivity: In some cases, high temperatures can also help to improve the selectivity of the extraction process. This means that the desired bioactive compounds can be extracted more efficiently, while unwanted compounds are left behind. However, there are also some limitations to PETE. Some bioactive compounds are sensitive to heat when exposed for prolong period and can degrade, if time was not controlled precisely. This means that PETE is suitable for all types of bioactive compounds provided the extraction time and temperature combination is supported by food engineering and precisely controlled. Overall, PETE can be an effective way to extract bioactive compounds from plant material. The PETE process's most notable aspect is not its high pressure. In actuality, increasing the pressure is usually just necessary to maintain the liquidity of solvent; pressure seldom ever affects the extraction procedure itself. It is a potential alternative to microwave and other expensive extraction technologies because it is scalable, extremely rapid process which completes within 0-5 minutes, efficient and cost effective, and reproducible.

The utilization of PETE extraction technology for valorising beetroot vegetable waste has been explored by Kumar et al. (2023), who investigated the extraction of betalains from freeze-dried beetroot powder using an aqueous mixture of ethanol and water at elevated temperatures of up to 85°C. This approach deviates from the usual extraction temperature range of 50°C, known to cause betalain degradation. The study elucidated the kinetics of interfacial extraction and post-extraction solvent phase degradation through a mechanistic model, experimentally validated with various ethanol concentrations and extraction temperatures. Results revealed

that betalain extraction rates were highest at 75°C and 85°C in ethanol solution, completing within minutes. Despite betalains' thermolabile nature, careful control of reaction time yielded greater betalain yields, suggesting potential productivity enhancement without significant degradation risks, akin to the Ultra-High Temperature (UHT) processing in the food industry.

Supporting this notion, Zakaria et al. (2022) investigated phenolic compound extraction from Chlorella sp. microalgae using pressurized hot water extraction at temperatures up to 250°C, showcasing its superiority over conventional methods due to reduced solvent usage and higher yields. Xu et al. (2015) further corroborated this trend with subcritical water extraction of phenolic compounds from marigold flower residues at 220°C, yielding promising results. Conversely, Santoyo et al. (2012) tackled the challenge of carotenoid extraction, known for instability at high temperatures, by employing accelerated solvent extraction methods. By tailoring extraction conditions for specific biomass sources, they demonstrated the efficacy of pressurized hot water extraction for obtaining phenolic compounds, flavonoids, and carotenoids, with varying solvents and optimized temperatures yielding high extraction yields. Table 3.7 illustrates the application of pressurised and elevated temperature extraction of bioactives from different sources under various conditions.

3.5.5 Overall productivities, scalability, industrialisation of these UAE, MAE, SFE and PETE

In terms of productivity, UAE is highly productive in small-scale applications but limited by equipment capacity at larger scales. MAE offers extremely high productivity due to rapid extraction times and is scalable with solutions for uniform heating. SFE, with the ability to fine-tune extraction conditions, is highly productive and suitable for high-value extracts. Scalability is moderate for UAE due to challenges in uniform energy distribution and equipment durability, while MAE shows moderate to high scalability with ongoing

technological advancements. SFE boasts high scalability, established in several industrial applications despite higher costs.

Industrialization of these methods varies: UAE is used in some industries but struggles with uniform energy distribution and equipment wear. MAE is increasingly applied industrially, with improvements addressing uniform heating and scalability. SFE is widely adopted for high-value products, notwithstanding its cost and technical complexity. The challenges for each method include achieving uniform energy distribution and heating at larger scales, managing high costs, requiring specialized expertise and maintenance, ensuring energy efficiency, and maintaining consistent product quality during scale-up. Addressing these challenges is crucial for optimizing industrialization and scalability, ensuring efficient and sustainable extraction processes. Consequently, the exploration of pressurized and elevated temperature extraction (PETE) methods emerges as a prospective avenue for transformative advancement in bioactive compound extraction processes as discussed below.

3.6 Conclusions

Undoubtedly, the vegetable waste of beetroot and carrot are significant sources of the health promoting pigments beta-carotene and betalains. However, the extraction of these bioactives have been evolutionary. Traditional extraction processes which generally employ lower temperatures and longer extraction times are laborious, exhausting and time consuming. To intensify and accelerate the extraction process, methods like ultrasound, microwave, and supercritical fluid extraction have been explored. However, although these techniques are in existence for more than 20-30 years, scalability and energy efficiency are still questionable. Elevated temperature extraction for relatively shorter times is a plausible strategy which can be scaled up and operated on an industrial scale.. The parameters of this process are scale-

independent and the solvents used are mostly water and vegetable oil based which are also safe to use.

3.7 References

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Table 3.1: Phytochemical compositions of beetroot and carrot with their functionality and colouring properties.

Phytochemicals of Beetroot	Description and Function
Betalains	Responsible for the red and yellow-orange colors of beetroot. Powerful antioxidants with potential health benefits.
Anthocyanins	Present in some beetroot varieties, contributing to red and purple colors. Known for their antioxidant properties.
Phenolic Compounds	Includes compounds like rutin and others, with antioxidant and anti-inflammatory properties.
Dietary Fiber	While not a phytochemical, dietary fiber in beetroot supports digestive health and helps regulate blood sugar levels.
Phytochemicals of Carrot	**Description and Function**
Carotenoids	Carrots are especially rich in carotenoids, including beta-carotene, alpha-carotene, and others. Beta-carotene, in particular, is converted into vitamin A in the body and is essential for vision, immune function, and skin health. Carotenoids also act as antioxidants, helping to protect cells from damage.

Polyacetylenes	These compounds are responsible for the earthy aroma of carrots. Some polyacetylenes have been studied for their potential anti-inflammatory and anti-cancer properties.
Phenolic Compounds	Carrots contain various phenolic compounds, including quercetin and kaempferol, which have antioxidant and anti-inflammatory properties.
Dietary Fiber	Dietary fiber in carrots supports digestive health, helps maintain regular bowel movements, and may aid in weight management.

Table 3.2: Use of beta-carotene as a colouring matter across the food industries.

Application	Description
Colorant	Used to add vivid orange hue to various food products such as beverages, baked goods, cereals, snacks, and dairy items, replacing synthetic colorants.
Food Coloring Stability	Maintains color integrity under heat and light, making it suitable for processed and stored food products.
Fruit and Vegetable Juices	Enhances the color of fruit and vegetable juices, aligning with the demand for clean label ingredients.
Confectionery	Provides colourful appeal to confectionery items like candies, gummies, and chocolates without using artificial dyes.
Dairy and Dairy Alternatives	Enhances the appearance of yogurt, cheese, and dairy-alternative products, associating vibrant colors with freshness and quality.
Prepared Foods	Improves the visual appeal of sauces, dressings, soups, and ready-to-eat meals, maintaining desired color during processing.
Functional Foods	Added to fortified cereals, energy bars, and beverages to enhance nutritional profile, given its potential health benefits as an antioxidant and precursor to vitamin A.
Packaging	Used in packaging films to protect food products from light-induced degradation, contributing to extended shelf life.
Pet Food	Incorporated into pet foods to enhance the appearance of pet kibble and treats, appealing to pet owners seeking natural ingredients.

Table 3.3 (a): Application of various solvent types for the extraction of beta-carotene.

Solvent Class	Solvent name	Properties	Carbon Foot Print (kg of CO_2 equivalent/kg of solvent)	References
Organic solvent	Hexane	Higher Solubility, miscible with water, toxic, hydrogen bonding, volatile and flammable	0.62	Carboncloud.com
	Petroleum Ether		1.30	(Hamieh et al., 2022)
	Acetone		2.55	(Liew et al., 2022)
	Isopropanol		1.85	(Liew et al., 2022)
	Ethanol		0.96	(Müller et al., 2020)
	Methanol		2.20	(Jong, 2022)
	Diethyl ether		3.60	(Nguyen et al., 2023)
	Tetrahydrofuran		5.65	(Stewart Slater et al., 2012)
	Dichloroethane		7.14	(D. Y. Lee et al., 2018)
Super critical solvents & Pressurized/Subcritical solvents	Carbon dioxide (CO_2)	Green extraction method	-----	-----
	Ethanol		0.96	(Müller et al., 2020)
	Ethanol	Rapid extraction	0.96	(Müller et al., 2020)
	Water		-----	-----

Ionic liquids	Ionic liquids, Deep Eutectic solvents	Applicable for both polar and non-polar solutes	----- -----	----- -----
Vegetable oils	Sunflower oil, groundnut oil, Coconut oil, gingelly oil, Soyabean oil, mustard oil, Rice bran oil, soy oil Groundnut oil, Canola oil, Palm oil, flaxseed oil	Green, Bio-based, cheaper and extensively available	3.5	(Alcock et al., 2022)

Table 3.3 (b): Application of various solvent types for the extraction of betalains.

Solvent Class	Solvent name	Properties	Carbon Foot Print (kg of CO_2 equivalent/Unit)	References
Aqueous Solvent	Water	Highly polar, non-toxic, hydrogen bonding and ionic interaction	------	(Maran & Priya, 2016)
Organic solvents	Ethanol: water	Higher Solubility, miscible with water, toxic, hydrogen bonding, volatile and flammable.	0.96	(Müller et al., 2020)
	Acetic acid in water		1.60	(Medrano-García et al., 2019)
	Hexane		0.62	Carboncloud.com
	Methanol: water		2.20	(Jong, 2022)
	Formic acid		2.20	(Rumayor et al., 2018)
	Phosphate buffer		5.1	Yara.co.uk
	Citric acid		16.78	(Nica & Woinaroschy, 2010)
	B-cyclodextrin solution		-----	-----
Super critical solvent &	Carbon dioxide (CO_2)	Green extraction method	-----	-----

	Ethanol		0.96	(Müller et al., 2020)
Pressurized/Subcritical solvents	Ethanol	Rapid extraction	0.96	(Müller et al., 2020)
	Ethanol: water		0.96	(Müller et al., 2020)

Table 3.4 (a): Extraction of betalains from various sources using ultrasound and its operating parameters.

Source of betalains	Ultrasound Equipment Specifications	Solvent and Extraction Conditions used	Maximum recovery based on the source of betalains	Estimated g of solvent / g of betalains	Notable Findings	References
Beetroot Stalks, Air dried before extraction	Probe Type Ultrasonicator	Solvent = water Temperature = 40-60 °C; Ultrasonic Power = 60-120 W; Extraction Time = 15-45 min; liquid/solid = 10-30 ml/g of dried powder	Betaxanthin = 5.18 mg/g powder; Betacyanin = 1.23 mg/g powder	Betaxanthin = 3861.00 Betacyanin = 16260.16	Optimized condition was found to be: extraction temperature of 53 °C, ultrasonic power of 89 w, extraction time of 35min and S/L ratio of 1:19 g/ ml.	(Maran & Priya, 2016)
Beetroot peels, dried and milled before extraction	Bath type ultrasonicator	Solvent = water Power : 200 W; Frequency = 200 Hz; Time: 30-60 min; solid/liquid: 1:20	Total Betacyanin = 3.87 mg/g dry matter. Total Betaxanthin = 8.61 mg/g of dry matter	Betacyanin = 5291.01 Betaxanthin = 2322.88	Ultrasound extraction for 30 minutes reported here to be more recovery of betalains than 60 min of extraction.	(Šeremet et al., 2020)
Dried whole beetroot powder.	Bath type ultrasonicator	Solvent 1= Citric acid Solvent 2= Ethanol Power = 100 W Time = 5-30 min Temperature = 20-40 °C Ethanol = 10, 20, 30% (v/v) Citric acid pH = 4, 5, and 6	Ethanol: Betacyanin = 4.38 ± 0.17 mg/g Betaxanthin = 3.95 ± 0.22 mg/g Citric acid: Betacyanin = 3.98 ± 0.21 mg/g Betaxanthin = 3.64 ± 0.26 mg/g	Ethanol BC = 18013.05 Ethanol BX = 19974.46 Citric acid BC = 25125.68 Citric acid BX = 27472.53	This study demonstrates the potential of citric acid as a viable alternative to polar organic solvents for better sustainability.	(R. Kumar, Methven, et al., 2023)

Table 3.4 (b): Extraction of beta-carotene from various sources using ultrasound and its operating parameters.

Food sources	Ultrasound Equipment Specifications	Solvent and Extraction Conditions used	Maximum Extraction Yield	Estimated g of solvent / g of beta-carotene	Notable Findings	References
Carrot pomace, hot air drying and milled to uniform particle size before extraction	Electrohydrodynamic treatment (EHD) + Probe Type Ultrasound	Solvent = Ethanol EHD time = 2.5 to 25 min; EHD voltage = 0 to 22 kV at fixed ultrasound condition: temperature = 30 °C, Power = 500 W, frequency = 20 kHz, time = 80 min.	Highest values of beta-carotene concentration (415.28 ± 0.56 mg/L	1901.21	EHD pretreatment method leads to higher extraction of beta-carotene content	(Salehi & Taghian Dinani, 2020)
Fresh carrot pomace, dried and milled to uniform particle size before extraction	Bath type ultrasonicator and Probe type ultrasonicator used separately	Solvent = Ethanol 0.3g of pomace powder was extracted with 10 ml of ethanol for 25 mins at 50 °C. Probe type: Power 100 W and 50% duty cycle. Bath type: Power 180 W and frequency 40 kHz.	Using ultrasonic probe type, maximum extraction yield of beta-carotene = 80.32%. Using ultrasonic probe type, maximum extraction yield of beta-carotene = 64.66%.	NA	Probe type was more efficient than bath type. Organic solvents were more efficient for extraction than vegetable oils as solvent.	(Purohit & Gogate, 2015)
Tomato pomace, dried and milled to uniform particle	Probe type ultrasonicator was used in the pressurised chamber by nitrogen gas	Solvent = hexane – ethanol (50:50); Ultrasound amplitude = 58 - 94 µm; Static pressures = 0 = 100 kPa;	Maximum yield of carotenoids content = 17.37 mg/ 100 g dry	134615.4	It was demonstrated that the external pressure significantly increased the effect	(Luengo et al., 2014)

size before extraction		Time = 0-10 min at 25 °C. Solvent = 100 ml Power = 125 W	weight of tomato pomace powder.		of ultrasound on yield.	
Fresh carrot, dried and milled to uniform particle size before extraction	Probe type ultrasonicator	Solvent = Sunflower oil Sample = 200 g Solvent volume = 1 litre solid/liquid ratio = 1:10 – 3:30; Power intensity = 9.5, 16, and 22.5 W/cm^{-2}; Temperature = 20-60 °C; Time = 5-35 min.	Maximum reported yield = 334.7 mg of beta-carotene per Liter of sunflower oil	2748.73	Sunflower oil extract had higher extraction efficiency than conventional solvent extraction using hexane as solvent.	(Li et al., 2013)
Peach palm peel, dried and pulverised to obtain uniform particle size before extraction	Bath type ultrasonicator	Solvent = Soy oil Ultrasonic power = 150 W, Frequency = 40 kHz, Temperature = 28-62 °C, Time = 13-47 min, ration of solid/liquid (g/mL) = 0.0005-0.006	Total carotenoids = 166.43 mg/100 g of dried peach powder	846843.4	Soy oil was considered to be an strategic solvent for sustainable development. Temperature and solid/liquid ratio was more effective parameter.	(Ordóñez-Santos et al., 2021) (ordonez)
Tomato processing waste, Tray drying and grinding before extraction	Bath type ultrasonicator	5 g of tomato powder into 100 ml of tested oil; Ultrasound power = 480 W, Temperature = 25 °C, Time = 50 min.	Highest carotenoid content was reported in grape seed oil = 16.7 mg/kg of powder.	1077844.01	Carotenoids were able to improve the physico-chemical properties of oils. The 50 min time was an optimum condition for the extraction.	(Nour et al., 2018)

Table 3.5 (a): Extraction of betalains from various sources using microwave and its operating parameters.

Food sources	Microwave Equipment Specifications	Solvent used Extraction Conditions	Maximum Extraction Yield	Estimated g of solvent / g of betalains	Notable Findings	References
B. glabra branches were collected, forced air dried and sieved through 0.5mm sieve before extraction.	Conventional microwave (Electrolux, ME-F41) at 2450 MHz	Solvent = Ethanol S/L ratio = 1:20 Microwave Power: 100, 200, 400, 600, 800 and 1000 W, until temperature reached to 60 °C. Solvent = ethanol/water (70/30)	The best condition for MAE treatments was obtained using the power of 100 W for 70 min, with contents of 4.78 ± 0.03 mg g^{-1} (d.b) of BC and 1.03 ± 0.03 mg g^{-1} (d.b) of BX.	For BC = 3301.25 For BX = 15320.39	Exhaustive solvent extraction shown the highest extraction yield of betalains followed by MAE and Ultrasound.	(Kuhn et al., 2021)
Fresh diced beetroot (0.1 x 0.1 x 0.1 cm^3)	Conventional Defy microwave (1000 W)	Extraction 1 Sample = 100 g of diced beetroot Solvent = 100 ml distilled water. Extraction 2= Sample = 100g of diced beetroot Solvent = 100 ml of distilled water with 5ml of 5% ascorbic acid.	Total content was = 71.71 mg/100 g	For ascorbic acid experiment = 138888.91	Addition of ascorbic acid gave more yield compared to extraction without ascorbic acid.	(Sigwela et al., 2021)

		Heating time = 10 s Microwave power = 1000 W Time = 30 s				
Prickly pear (Opuntia streptacantha) peel, hot air oven drying before extraction	NEOS microwave extraction system (Milestone, Italy) with an output power of 900 W (100% of power) and a frequency of 2450 MHz	Sample = 100g powder Solvent = 750 ml (70% of ethanol). Microwave power = 180 W Average vessel temperature = 60 °C. Time = 5-10 min	Betacyanin = 25.21 ± 2.85 mg/g of extract. Betaxanthin = 17.76 ± 1.88 mg/g of extract.	For betacyanin = 234.73 For Betaxanthin = 333.19	The extract obtained with conventional method showed better extraction yield than microwave. However, time taken by MAE was shortest.	(Gómez-Salazar et al., 2022b)
Cylindra beetroot (Beta vulgaris L.) stalk, flesh, and peel.	Domestic microwave oven (Specs Electrolux EMM 2005)	Solvent = water Microwave power = 100-800 W Time = 30-150 s S/L ratio = 0.1 - 0.2	From peel: BC = 7.06 mg/g DM BX = 5.25 mg/g DM From stalk and Flesh: BC = 0.54 mg/g DM BX = 3.82 mg/g DM	For peel = BC = 2832.86 BX = 3809.52 For flesh BC = 37037.04 BX = 5208.33	MAE was influenced mostly by the solvent ratio due to microwave efficiency. Peel had higher betalains content than other parts of the beetroot.	(Zin & Bánvölgyi, 2022)
Crown parts of beetroots (2.5 cm) were separated and cleaned properly	Pretreatment with laboratory-scale microwave oven (EMM2005; Electrolux, Sweden)	Solvent = water S/L ratio = 0.1 Time = 3 mins Solvent contains = 50% ethanol	Maximum extracted betacyanin = 4.87 mg/g of dry matter	1745.38	Process was not optimized. Highlight was that microwave as pre-treatment	(Zin et al., 2022)

before grinding without water by a pulveriser.		Microwave power = 800 W			improved the yield of Betacyanin.	
Fresh beetroot peels	Domestic microwave oven (Specs Electrolux EMM 2005)	Solvent = Ethanol Microwave power = 100-800 W Time = 30-150 s S/L ratio = 0.1 - 0.2 Solvent used: Acidified water, ethanol-water. Acidified-ethanol-water	BC = 115.89 ± 1.08 mg/100 g FW BX = 86.21 ± 1.16 mg/100 g FW	BC = 13721.74 BX = 18348.84	Acidified extraction medium of pH 3.5 adjusted by ascorbic acid upgraded the yield of betanin to the highest.	(Zin & Bánvölgyi, 2023)
Amaranthus tricolour leaves, dried at 40 °C for 24 h before grinding to get uniform size.	Microwave reactor by Nutech Analytical Technologies Pvt. Ltd., India (Model: NuWav Pro 2450 MHz frequency)	Solvent = water Duty cycle of microwave = 50% On time = 0.5 s Off time = 0.5 s S/L ratio = 1:80	BC = 71.95 ± 0.33 mg/g BX = 42.30 ± 0.29 mg/g	BC = 1112.81 BX = 1904.76	The mass transfer, i.e. diffusion of the betacyanin and betaxanthin pigments was influenced by microwaves thus enhancing the extraction.	(Sharma et al., 2023)
Juice from the discarded red beetroot	Closed microwave assisted reaction system (Multiwave PRO SOLV reactor 50 Hz with a Rotor type 16HF100, Anton Paar GmbH, Austria, Europe).	Solvent = PEG4000 S/L ratio = 1:10 Solvent used was PEG 4000 temperature = 100–160 ◦C, time = 5–15 min, and PEG4000 concentration = 2–10 g/L.	Betalains = 1426 ± 24 mg/L	841.51	It was observed that discarded red beetroot was not a waste after all. Waste materials are a potential source of bioactive compounds.	(del Amo-Mateos et al., 2023b)

Table 3.5 (b): Extraction of beta-carotene from various sources using microwave and its operating parameters.

Food sources	Microwave Equipment	Solvent used Extraction Conditions	Maximum Extraction Yield	Estimated g of solvent / g of beta-carotene	Notable Findings	References
Fresh carrot waste, Diced, frozen, freeze dried and sieved with 300 µm sieve for homogeneous sample	Closed-vessel energy-intensive microwave	Solvent = Hexane, ethanol, ethyl acetate and acetone Power = 1000 W Experiment 1: Hexane ration = 25-100%; Temperature = 40-70 °C; Time = 5-15 min; S/L ratio = 1:20-1:40 Experiment 2: Temperature = 40-60 °C; Time = 5-15 min; S/L ratio = 1:20-1:40	Experiment 1: Carotenes = 172.88 ± 3.07 mg/100 g dry weight. Experiment 2: Carotenes = (254.248 ± 3.89 mg/100 g dry weight.	Experiment 1 = 16744.18 Experiment 2 = 11338.58	Optimized conditions = temperature 50 °C, time 5 min, and solid to the solvent ratio (1:40); At this condition MAE recovered highest yield compared to UAE and conventional solvent extraction.	(Kaur et al., 2022)
Seabuckthorn pomace, Freeze dried followed by grinding and storage	Closed-vessel energy-intensive microwave	Solvent = Olive oil, Corn oil, and hexane. 5 g of sample into 50 ml of respective oil as solvent. pre-optimized conditions (130 W for 30 min)	Total carotenoids content = 34.35 ± 0.94 mg/100 g of extract	26470.58	Microwave assisted extraction with Olive oil had the highest extraction yield compared to corn oil and conventional solvent of hexane.	(Sharma et al., 2022)s
N. pulmonarioides (NP) fresh aerial parts were dried in the shade for	Multimodal household microwave oven (Panasonic	Solvent = petroleum ether, acetone, and methanol. Power = 900 W Solvent vol. = 2500 ml Sample = 250 g	Total carotenoids = 16.64 g	105.17	Microwave assisted extraction with methanol had the highest extraction at lower temperature	(Mohammed & Abdullah, 2022)

2 weeks and then ground to a fine powder with an electric grinder	P90N28AP-S3) at 900 W	Time =5 min Irradiation cycle = 20 s intervals.			range. However, flavonoids were more in acetone extract.	
Fresh pumpkin waste, air dried at 40°C and sieved with 300 µm sieve for homogeneous sample	Closed vessel system microwave (Model Initiator 2.0, version 2.3, Biotage AB, Uppsala, Sweden)	Solvent = Hexane/acetone/ethanol (50:25:25) S/L ratio = 1:20 Time = 15 or 30 min Temperature = 45°C Microwave Power = 40 W Pressure = 5 bar	Total carotenes = 1468.91 ± 112.79 µg/g	9333.33	Mixture of hexane and Isopropanol shown lower recovery than mixture of hexane, acetone, and ethanol.	(Pinna et al., 2022)
Chayote leaves, tray drying at 35 °C for 12 h, ground and sieved through a 0.75 mm stainless steel sieve	Microwave Accelerated Reaction System for Extraction and Digestion, CEM, Mathews, NC, USA	Solvent = acetone, ethanol Microwave power = 300 W S/L ratio = 1:30 Time = 30 minutes Temperature = 55 °C	Total carotenoids = 9.01 ± 1.70%	NA	MAE was compared against UAE and similar yield was reported for both. However, MAE extracted more TPC than UAE.	(Pinna et al., 2022)
Chlorella vulgaris, dried powder was purchased for extraction.	MAS-II Plus microwave synthesis/extraction reaction workstation (Sineo Microwave Chemistry Technology Co. Ltd., Shanghai, China)	Solvent = ethanol S/L ratio = 1:20 to 1:90 Extraction temperature = 40-60 °C. Extraction time = 5 – 25 minutes Microwave power = 300- 800 W	Total carotenoids = 23.09 mg/g of extraction powder	1543.696	MAE was exceptionally efficient in reducing the extraction time. However, solid-liquid conventional system showed higher extraction than MAE.	(Georgiopoulou et al., 2023)

Table 3.6 (a): Extraction of betalains from various sources using supercritical fluid extraction and its operating parameters.

Food sources	SCFE Equipment Specifications	Extraction Conditions	Maximum Extraction Yield	Estimated g of solvent / g of betalains	Notable Findings	References
Pitaya fruit pulp and pomace, dried at 42 °C and ground before extraction.	SFE system comprised of CO_2 cylinder, chiller ,CO2 HPLC pump (Jasco PU-1580, Tokyo, Japan), and back pressure regulator (Jasco BP-1580-81, Tokyo, Japan)	CO_2 flow rate = 2 ml/min Ethanol/water = 10:90 Sample loading = 5 g Pressure = 25 MPa Temperature = 50 °C Vessel volume = 50 ml Time = 90 min	BC = 28.44 mg/100 ml	3515.625	Ethanol to water ratio 50:50 resulted in the highest extraction yield. Red Pitaya fruit provided the higher yield and betacyanins than its peel.	(Fathordoobady et al., 2016b)
Red pitaya fruits, dried, stored and ground before extraction.	A laboratory scale system of a SFE (Jasco BP-1580-81, Tokyo, Japan) was used.	CO_2 flow rate = 2 ml/min Ethanol/water = 10:90 Sample loading = 5 g Pressure = 25 MPa Temperature = 50 °C Vessel volume = 50 ml Time = 90 min	BC = 25.74 ± 0.48 mg/100 ml	3883.495	Maximum betacyanin extraction was achieved at 25 MPa pressure, 50 °C temperature at 15% ethanol as co-solvent.	(Fathordoobady et al., 2019b)
Lyophilised beetroot material	Semi-continuous SCFE high-pressure flow apparatus designed for a maximum	Solvent feed (F/S)= 8.17 Pressure = 100, 300 bar	BC = 4.17 mg/100g BX = 3.41 mg/100g		Highest value was obtained with isopropanol as a co-solvent under the pressure of	(Borjan et al., 2022)

	pressure of 500 bar and a temperature of 100 ◦C	Temperature = 40, 60 °C Sample amount = 15 g Reactor volume = 60 ml Time = 120 minutes			300 bar and a temperature of 60 ◦C.	
Pitaya peel fruit was freeze dried and ground before extraction	Extraction was done before SFE was used for the fractionation of betalains.	Solvent = ethanol: water (50:50) Temperature = 40 °C Pressure =10-30 MPa Extraction vessel = 500 ml Time = 24 h	Total betalains = 30.67 mg/100 g dw	13714285.7	SCFE was a great technology for the fractionation of betalains.	(Yu et al., 2023)

Table 3.6 (b): Extraction of beta-carotene from various sources using supercritical fluid extraction and its operating parameters.

Food sources	SCFE Equipment Specifications	Extraction Conditions	Maximum Extraction Yield	Estimated g of solvent / g of betalains	Notable Findings	References
All vegetable samples were frozen, freeze dried, ground and sieved for particle size of bigger than 750 µm.	Supercritical fluid extractor (SciMed, Stockport, UK)	Sample loading = 5 g Glass beads = 95 g Extraction vessel = 100 ml CO_2 flow = 15 g/min Time = 30 min Temperature = 59.0 ◦C Pressure = 350 bar Ethanol = 15.5% (v/v)	Total carotenoids content = 430.6 ± 27.7 µg/dry matter.	209302.33	Out of 15 waste vegetable matrices selected, sweet potato showed the highest carotenoids recovery.	(de Andrade Lima et al., 2019)
Freeze dried melon powder of 500 µm particle size was used.	Automated SFE system (Model 7100, Thar Technologies Inc., USA)	Sample loading = 50 g Ethanol = 5% (v/v) CO_2 flow = 15-55 ml/min Temperature = 50-90 °C Extraction vessel = 1 L Time = 45–225 min	Maximum beta-carotene recovery was = 76.99 mg/100 g of powder	7039.51	Maximum beta-carotene % yield was obtained by extracting at 69.15 °C temperature, 393.31 bar pressure, 36.98 mL/min flow rate for 190.36 min.	(Patel et al., 2019)
Freeze dried peels, and skins of discarded tomatoes were pulverised and 350 µm particle	Laboratory apparatus (Speed SFE system, Applied Separations,	Sample loading = 18 g Time = 20-80 min Extraction vessel = 25 ml Temperature = 60 °C CO_2 flow = 2 ml/min	Beta-carotene = 1.5 ± 0.4 g	106.67	After 80 minutes, the achievement of a plateau with the maximum yield at 550 bar was	(Pellicanò et al., 2020)

size was selected for extraction.	Allentown, PA, USA)	Pressure = 350-550 bar			observed. Also, pressure was the most significant factor.	
Mixture of stem and leaves dried at 55 °C before grinding.	Waters Prep Supercritical Fluid Extraction system (SFE-100 Waters®, Thar SFC, Thar Technologies, Inc., Pittsburgh, PA, USA)	Sample loading = 15 g Extraction vessel = 100 ml Time = 20 min	Beta-carotene = 254.8 mg g^{-1} extract	NA	The highest total content was observed in the mixture of leaf/stem = 3:1	(Borja-Martínez et al., 2020)
Spray-dried Dunaliella salina powder (supplied by Denk Ingredients GmbH, Germany, Art. no: 967996) was	NA	Sample loading = 100 g Extraction vessel = 2 L Pressure = 300-500 bar Ethanol = 0-10% CO_2 flow = 4-5 kg/h Temperature = 50-70 °C Time = 180 min	Beta-carotene = 19 µg/g	473684210.5	Energy demand and operating cost calculations, it was concluded that SCFE extraction consumes two fold the energy of conventional n-hexane extraction.	(Ludwig et al., 2021)
Commercial *C. vulgaris* biomass was purchased for extraction.	SFE-500, SEPAREX CHIMIE FINE, Champigneulles, France	Sample loading = 80 g CO_2 flow = 20-40 g/min Ethanol = 10% (v/v) Temperature = 40-60°C Pressure = 110-250 bar Time = 3.33 – 6.67 h	Carotenoids extracted = 22.51 mg/g extract	6667.037	SCFE showed lower yield compared to conventional solvent extraction.	(Georgiopoulou et al., 2022)

		Extraction vessel = 400 ml			However, evaporation was no needed in SCFE.	
Commercially available biomass of *Chlorella vulgaris* was used for extraction.	Bench scale SCFE apparatus (SFE-500, SEPAREX CHIMIE FINE, Champigneulles, France)	Sample loading = 80 g CO_2 flow = 40g/min Ethanol = 10% (v/v) Temperature = 60°C Pressure = 250 bar Time = 3.33 h Extraction vessel = 400 ml	Total carotenoids extracted = 6.70 % (w/w)	NA	SFE employed with ethanol as co-solvent shown lowest recovery compared to MAE and conventional solvent extraction.	(Georgiopoulou & Tzima, 2023)

Table 3.7: Pressurised and elevated temperature extraction of bioactive components using relatively elevated temperature and assisted with pressure to accelerate the extraction process.

Sample	Extraction Equipment	Solvent used & Extraction Parameters	Maximum extraction yield	Estimated g of solvent / g of beta-carotene	Notable findings	References
Freeze dried beetroot powder	Magnetic stirrer with hot plate	Solvent = ethanol and water (20:80) S/L ratio = 0.02 Temperature = 55-85 °C Particle size = 0.12-0.30 mm	Betacyanin = 0.0044 kg/kg of dry powder Betaxanthin = 0.0049 kg/kg of dry powder	3586.36	Elevation in temperature accelerated the extraction process and completed in less than a minute.	(Kumar, et al., 2023)
Freeze dried carrot powder	Magnetic stirrer with hot plate	Solvent = sunflower oil S/L ratio = 0.02 Temperature = 90-150 °C Particle size = 0.35-1.4 mm	Beta-carotene 8.03×10^{-4} kg of beta-carotene/kg dry matter	56039.85	Elevation in temperature accelerated the extraction process and completed in less than 4 minutes.	(Kumar et al., 2024)
Chlorella sp. Microalgae was purchased in powder form.	Batch pressurized hot fluid bath system (Thomas Kogaku Co Ltd.)	Solvent = water Temperature = 100-250 °C Pressure = 0.10–3.97 MPa S/L ratio = 0.2	Phenolic compound yield = 58.15 mg GAE/g	NA	Highest obtained yield was reported at 175 °C and that was equilibrium value.	(Zakaria et al., 2020)

Marigold (*Tagetes erecta L.*) flower granules were dried to moisture content of 7.11% and crushed to the particle size of 0.25–0.35 mm before extraction.	The subcritical water extraction apparatus (300mL sample capacity, Model CWYF- 2, Nantong, China).	Solvent = ethanol& water (70:30) Temperature = 80-260 °C S/L ratio = 0.05-0.016 Time = 15-90 min	TPC = 116.61±1.92 mg GAE/g TFC = 138.59±2.03 mg RE/g	NA	Highest value of extraction was reached at 150 °C in 45 minutes, after this peak degradation started.	(Xu et al., 2015)
1. Haematococcus pluvialis (BNA 10/024, National Bank of Algae, Canary Islands, Spain). 2. *D. salina* sample consisted on freeze-dried microalgae supplied by NBT Ltd. (Jerusalem, Israel)	Accelerated solvent extractor (ASE 200, Dionex, USA)	Solvent = Ethanol, water and hexane Temperature = 100 & 160 °C; Time = 15 & 20 min Sample = 1.5 g in ethanol. Sample = 1 g in water Sample = 0.8 g in hexane	*Haematococcus pluvialis* yield = 21.19 % *D. salina* yield = 31.4 %	NA	1. From *H. pluvialis* biomass maximum when the extractions were carried out with water. 2. From *D. salina* the highest yield was obtained with ethanol.	(Santoyo et al., 2012)

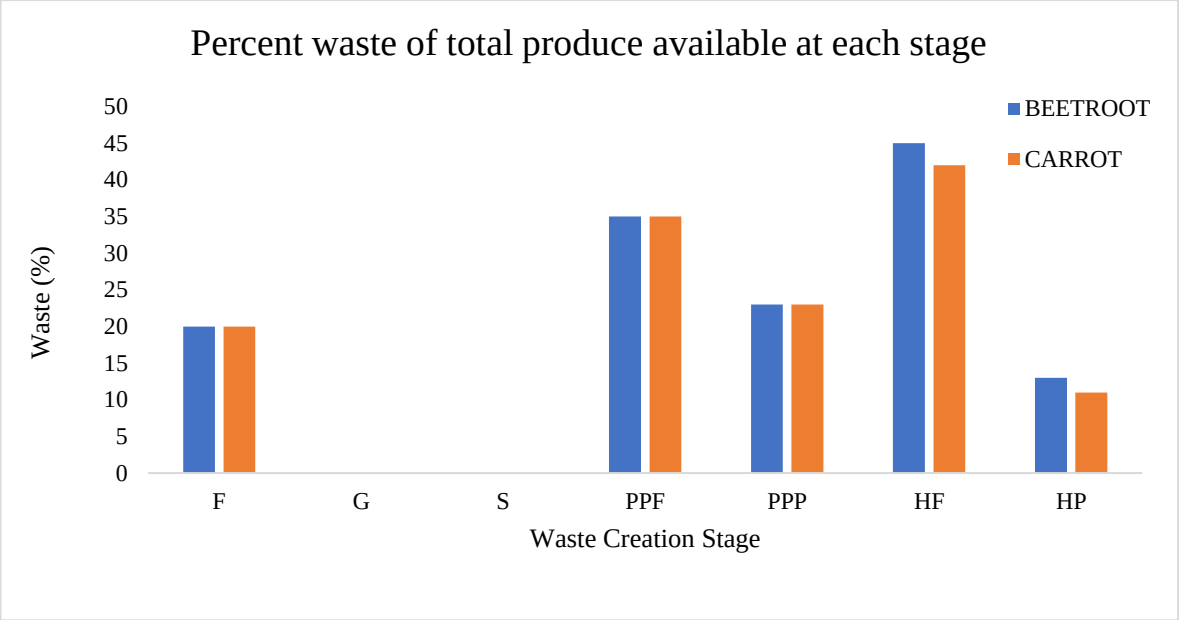
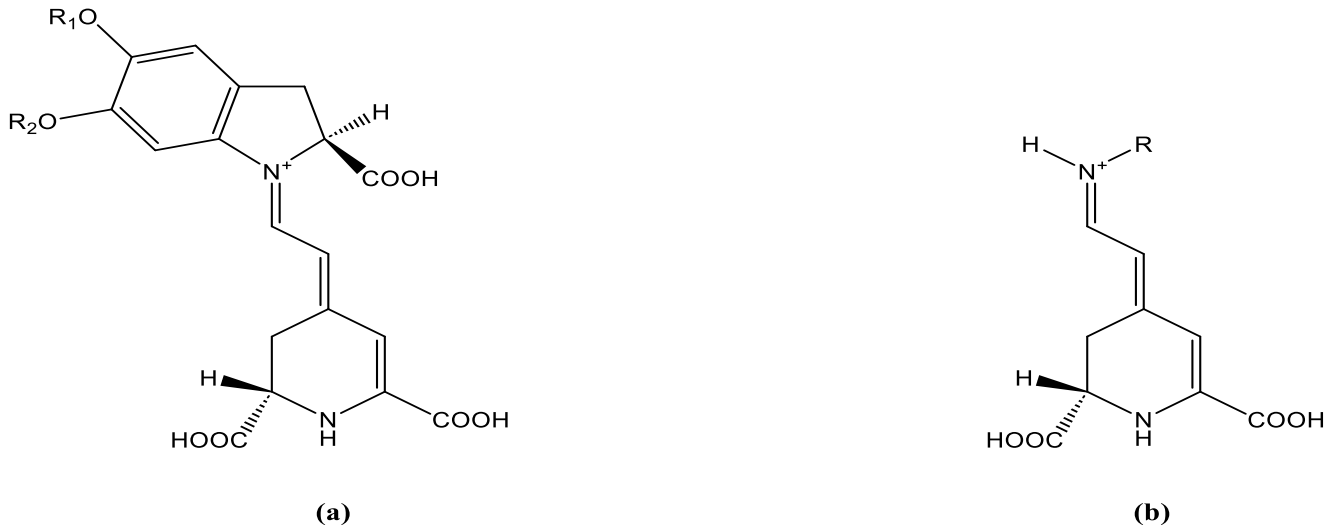


Figure 3.1: The percent of waste created at different stages from production to consumption for beetroot and carrot. F-farm waste, G-grading waste, S-storage waste, PPF-packaging/processing of fresh waste, processing/packaging of processed waste, HF-household fresh waste, HF-household processed waste (Frankowska et al., 2019).



(a) **(b)**

Figure 3.2: Chemical structure of betalains: (**a**) betacyanins and (**b**) betaxanthins.

Chapter 4

A Comparative Study of Ethanol and Citric Acid Solutions for Extracting Betalains and Total Phenolic Content From Freeze Dried Beetroot Powder

This chapter has been published in journal *Molecules*: Kumar, R., Methven, L., & Oruna-Concha, M. J. (2023). A comparative study of ethanol and citric acid solutions for extracting betalains and total phenolic content from freeze-dried beetroot powder. *Molecules*, *28*(17), 6405.

Abstract: This research compares the extraction of betalains (betacyanin and betaxanthin) and total phenolic content using citric acid and aqueous-ethanol solutions. The aim is to find an environmentally sustainable alternative solvent for extracting these compounds from dried beetroot powder. Using citric acid solution as a solvent offers several benefits over ethanol.. Citric acid is a weak organic acid found naturally in citrus fruits, making it a safe and environmentally friendly choice for certain extraction processes. Moreover, the use of citric acid as solvent offers biodegradability, non-toxicity, non-flammability, and is cost effective. A full factorial design and response surface methodology (RSM) were employed to assess the effects of extraction parameters (extraction time (5-30 min), extraction temperature (20, 30, 40 °C), pH of citric acid solution (3, 4, 5) and ethanol concentration (10, 20, 30 % v/v)). The yield was determined spectrophotometrically and expressed as mg/g of dry powder. Results showed that citric acid solution yielded 85-90% of the ethanolic extract under identical conditions. The maximum yields of betacyanin, betaxanthin, and total phenolic content in citric acid solution were 3.98 ± 0.21 mg/g dry powder, 3.64 ± 0.26 mg/g dry powder, and 8.28 ± 0.34 mg/g dry powder, respectively, while aqueous-ethanol yielded 4.38 ± 0.17 mg/g dry powder, 3.95 ± 0.22 mg/g dry powder, and 8.45 ± 0.45 mg/g dry powder. Optimization resulted in maximum extraction yields of 90% for betalains and 85% for total phenolic content. The study demonstrates the potential of citric acid as a viable alternative to polar organic solvents for extracting phytochemicals from plant material, providing comparable results to aqueous-ethanol. Artificial Neural Network (ANN) models outperformed RSM in predicting extraction yields. Overall, this research highlights the importance of exploring bio-solvents to enhance the environmental sustainability of phytochemical extraction.

Keywords: Extraction; Betalain pigments; Total phenolic content; Ultrasound; Optimization; Response Surface Methodology; Artificial Neural Network.

4.1 Introduction

Beetroot (*Beta vulgaris L.*) is an herbaceous blooming biennial plant native to Asia and Europe that belongs to the Chenopodiaceae family (Nirmal et al., 2021) and can be grown across the seasons (Hobbs et al., 2012). It is widely consumed as a salad, as a juice, or after pickling. It is known to contain high levels of nutritional and bioactive compounds including nitrates, phenolics, ascorbic acid, and water soluble pigments called betalains (Azeredo, 2009; Sawicki et al., 2016; Verónica Fernández et al., 2017). Beetroot and its juice consumption have been clinically proven to provide protection against non-communicable diseases (NCDs). The claimed health benefits of beetroot include functioning as an antioxidant, anti-depressant, anti-microbial, anti-fungal, anti-inflammatory, diuretic, expectorant, and in preventing liver and cardiovascular damage (Clifford et al., 2015).

Industrial scale production, processing, packaging, retail market and household consumption of beetroot leads to a wastage of more than 50% across the United Kingdom (UK) (Frankowska et al., 2019). The valorisation of beetroot and its wastes can be achieved by extracting the natural pigment betalains and total phenolic compounds (Celli & Brooks, 2017). Betalains are classified into two different classes namely betacyanins (BC) and betaxanthins (BX) (Figure 4.1). These two nitrogeneous compounds can be of significant importance to food, pharmaceuticals, cosmetics and dye industries, where it is also known as “beetroot red” (Sadowska-Bartosz & Bartosz, 2021). The use of beetroot extracts as natural colorants in the food industry offers a variety of benefits. Fruit juices can be enhanced with a visually appealing color, while bakery products like cakes and pastries can achieve vibrant reddish or pinkish hues in the dough or frosting. Yogurts and dairy products can achieve a pink or red tint naturally, and pasta can be made in colorful variations such as red or purple. Beetroot extracts are also incorporated into salad dressings and sauces to enhance their appearance, and they are used in

confectionery products like candies and gummies to achieve red or purple colors without artificial additives. Furthermore, in the context of plant-based and vegan food products, beetroot extracts serve as an alternative to synthetic dyes for adding color (Khan, 2016; Luzardo-Ocampo et al., 2021). The stability of this colourant is pH and temperature dependent, and its application in high temperature processed products is limited. Betalains are stable between the pH range of 4-5 (Huang & Elbe, 1987), with betacyanin remaining unchanged for at least 20 days at 4 °C and over 275 days when frozen at -30 °C. However, betalains are sensitive to heat and start degrading at temperatures above 50 °C. Boiling betanin-containing material causes a gradual color change from red to yellowish-brown at 100 °C, leading to a decrease in both betacyanins and betaxanthins content. Higher temperatures and longer heating times result in more significant degradation of betalains. Overall, temperature and pH play crucial roles in determining the stability of betalains during storage and food processing (Sadowska-Bartosz & Bartosz, 2021). However, its ready availability and low price has driven large scale applications in the food industry (Azeredo, 2009).

Betalain pigments are mostly extracted from the whole tuber rather than just from the peels by various methods including the use of aqueous-ethanol, supercritical fluids and other organic solvents (Bengardino et al., 2019; Singh et al., 2017). More recently, the extraction process has been intensified by using pulsed electric field, ultrasound and microwave technology, in order to avoid higher consumption of solvents, shorten the extraction time, and lowering of the extraction temperature (Chemat et al., 2020). The use of organic solvents has been heavily questioned in recent years due to the environmental impact of the solvents, such as volatile organic compound (VOC) emissions, hazardous waste generation, and non-renewable resource depletion as well as the safety concerns associated with their handling. Additionally, traditional extraction methods are energy-intensive. Hence, there is a growing interest in the development of extraction procedures using alternative solvents which are perceived to be greener, cleaner,

safer, and easier to adopt (Chemat et al., 2019), and citric acid solution meets all the requirements aforementioned to be a greener and cleaner solvent because it is easy to obtain, get rid of, safer and easier to handle (Lazăr et al., 2021; Singh et al., 2017; Troter et al., 2016). In this context, citric acid solutions have been added to aqueous-ethanol solutions to extract betalains and total phenolic compounds. Lazăr et al., (2021) (Lazăr et al., 2021) have used aqueous-ethanol acidified with citric acid; however, they did not control pH of the solvent mixture, and yet the stability and extraction yield of betalains are known to be highly dependent on pH (Wong & Siow, 2015). On the other hand, Singh at. al., (2017) (Singh et al., 2017) extracted betalains employing a similar mixture in the pH range 4-6 without varying ethanol concentration. While these earlier publications show the addition of citric acid to be promising, it is difficult to ascertain the individual contribution of the two components, citric acid and ethanol, in the mixture. The present study aims to overcome the limitations of earlier studies by investigating extraction in citric acid solution and aqueous-ethanol solutions separately, and comparing the extracts obtained under otherwise identical conditions. This approach offers valuable insights into their solvent selectivity, yield of extraction, environmental impact, and process optimization. This knowledge is essential for advancing sustainable extraction practices and enhancing the utilization of betalains in various industries. The use of ultrasound to intensify extraction has also been explored. Ultrasound-assisted extraction (UAE) is an innovative and environmentally friendly extraction technique that has gained significant attention in recent years. This non-invasive method utilizes high-frequency sound waves to enhance the extraction process, making it more efficient and effective compared to traditional extraction methods. UAE offers several advantages, including reduced extraction times, lower energy consumption, and decreased reliance on organic solvents, making it a greener alternative. The ultrasound waves create cavitation bubbles in the solvent, causing rapid changes in pressure and temperature, which facilitate the release of bioactive compounds from

the source material. This technology has been successfully applied to extract various bioactive compounds, such as carotenoids, betalains, and polyphenols, from different plant and food matrices (Chemat et al., 2011). The independent and interactive effects of operating parameters such as strength of the ethanol solution and pH of citric acid solution, extraction time, extraction temperature and ultrasound application will be evaluated using response surface methodology (RSM) as well as artificial neural networks (ANN). ANN architecture was developed on the basis of optimized number of hidden neurons, least mean square error (MSE), least root mean square error (RMSE), and highest coefficient of determination (R^2). These statistical and model parameters played an extensive role in ANN design completing the aim of the work.

4.2 Materials and methods

4.2.1 Experimental design

A full-factorial design was implemented for extraction using different concentrations of ethanol in water (10, 20, and 30% v/v) and citric acid solutions of variable pH (3, 4, 5) as solvents, coupled with ultrasonic parameters as shown in Table 4.1. The ethanol concentration range was in line with previous studies, where findings have reported that if too high the concentration of ethanol has a negative effect compound recovery; this was confirmed in our preliminary study (data not reported) and would also lead to higher solvent consumption (Cardoso-Ugarte et al., 2014; Celli & Brooks, 2017; Fernando et al., 2021; Nutter et al., 2021; Tumbas Šaponjac et al., 2016). The pH range of citric acid solutions was favourable for the stability of betalains (Bastos & Gonçalves, 2017; Celli & Brooks, 2017; Singh et al., 2017). Extraction time with ultrasound above 30 minutes was observed to have a negative effect in the preliminary study and in previous literature (Nutter et al., 2021). Hence, the time increased in 5 minute interval up to 30 minutes. The extraction temperature was at three levels of 20, 30 and 40 °C, and not higher to

minimise the risk of thermal degradation (Celli & Brooks, 2017; Righi Pessoa da Silva et al., 2018).

All experiments were carried out in triplicate. Means and standard deviation of the data were calculated for each treatment. Analysis of variance (ANOVA) was carried out to determine any significant differences ($p<0.05$) between treatments and multiple pairwise comparisons were carried out using Tukey's HSD test, using XLSTAT 2021.2 (Addinsoft, Paris, France).

4.2.2 Chemicals

Ethanol (purity >99%), citric acid (purity >95%), sodium hydroxide pellets, sodium phosphate dibasic (purity ≥99 %), formic acid (Purity ≥98 %), acetonitrile (LC/MS grade) and sodium carbonate were supplied by Fisher Scientific (UK). Folin-Ciocalteau reagent (2M) was purchased from Scientific Laboratory Supplies Ltd (UK). Standards of betanin (purity >99%) and gallic acid (purity ≥98%) to measure betacyanin, betaxanthin and total phenolic content were purchased from Merck Chemicals Limited (UK).

4.2.3 Sample preparation

Fresh beetroot was purchased from a local supplier in Reading, UK. The beetroot was washed, cleaned, wiped, and chopped in a food processor (Kenwood Blend-X Fresh BLP41.A0GO). It was then transferred to an aluminium tray and subjected to blast freezing at –80 °C, for 24-36 hours. It was subsequently freeze dried (VirTis SP Scientific, UK) for 70-72 hours until the moisture content dropped below 3% (dry weight basis). After freeze drying samples were ground (Kenwood Prospero AT286 KW714229 Spice Mill) and sieved. The extraction experiments were performed using particles of average diameter 230 μm based on our previous research (Kumar et al., 2023) as this particle size achieved the maximum extraction efficiency

4.2.4 Extraction of betalains

4.2.4.1. Ultrasound assisted ethanolic extraction

Betalains were extracted according to the method described by Silva et al., (2018) (Righi Pessoa da Silva et al., 2018) with some modifications. Freeze-dried beetroot powder (0.2 g, 230 µm) was extracted with 25 ml of aqueous-ethanol solvent (10%, 20% and 30 % v/v ethanol), under continuous ultrasonication (Power 100 W; Frequency 42 kHz) at different time and temperature combinations as per the design given in Table 4.1. After ultrasound treatment, the mixture was centrifuged (SIGMA, Laborzentrifugation 3K10, Germany) twice at 9384 × g for 30 min to obtain a clear supernatant. The extract was then stored at 4 °C until analysis.

4.2.4.2 Preparation of citric acid solution

A 1mM solution of citric acid solution was prepared using food grade crystalline citric acid. The pH of the obtained solution was 3.2-3.3, and further adjustment of the pH for extraction was attained by addition of 1M solution of sodium hydroxide.

4.2.4.3 Ultrasound assisted citric acid extraction

Betalains were extracted according to the method described by Singh et. al., (2017) and Silva et. al., (2018) (Righi Pessoa da Silva et al., 2018; Singh et al., 2017) with some modifications. Freeze-dried beetroot powder (0.2 g, 230 µm) was extracted with 25 ml of citric acid solution (of varied pH), and continuous ultrasonication (Power 100 W; Frequency 42 kHz) at different time and temperature combinations as per the design given in Table 4.1. After ultrasound treatment the mixture was centrifuged (SIGMA, Laborzentrifugation 3K10, Germany) twice at 9384 × g for 30 min to obtain a clear supernatant. The extract was then stored at 4 °C until analysis.

4.2.5 Analysis of betalains

4.2.5.1 Spectrophotometric analysis of total betalains

Betalains were determined spectrophotometrically according to the method described in previous literature (Bhagya Raj & Dash, 2020b; Wong & Siow, 2015). The sample extract (section 4.2.4) was diluted 5 times before the spectrophotometer measurement (Cecil CE1011 Spectrophotometer) using McIlvaine buffer, which was prepared by mixing 30 mL of 0.1 M citric acid with 70 mL of 0.2 M sodium phosphate dibasic. The wavelengths used were 480 nm (for BX), 538 nm (for BC), and 600 nm (in order to account for any impurities). The measurement of BX and BC at 480 nm and 538 nm represents more than 95% of betalains present in beetroot sample. The expression used for the calculation of betalains is given by the equation (4.1) below.

$$\text{Betalains (mg of BX or BC/g of dried beetroot)} = \frac{A*DF*V*MW}{E*L*M} \quad (4.1)$$

Where $A=A_{538}-A_{600}$ for betacyanins (BCs) or $A_{485}-A_{600}$ for betaxanthins (BXs); DF=dilution factor; MW (Molecular Weight) = 550 g/mol for betacyanin and 339 g/mol for betaxanthin; E=molar extinction co-efficient in $Lmol^{-1}cm^{-1}$, and the values for betacyanins and betaxanthins are 60,000 and 48,000, respectively; V=volume of the extract; L= path length of quartz cuvette in cm and M= mass of dried sample taken for extraction.

4.2.5.2 Identification and quantification of betacyanin and betaxanthin by high performance liquid chromatography (HPLC)

The analysis and quantification of betalains was adapted from Nestora et al. (2016) into a HPLC with DAD method (Nestora et al., 2016) with some modifications. The detection of the betacyanins and the betaxanthins was done at 540 nm and 480 nm, respectively (Chethana et al., 2007), and betanin (betacyanin) was used as the reference. The HPLC system (Agilent Technologies) consisted of diode array detector (DAD) with quadrupole solvent system. HPLC

analyses were performed on a C18 reverse phase (RP) column (ZORBAX Eclipse XDB-C-18; 4.6 x 150 mm, 5 µm). The mobile phase consisted of 0.1% formic acid (eluent A), and HPLC grade acetonitrile (eluent B). The gradient program was as follows: 0 min 0% B, 13% B at 21 min, held at 13% B for 4 min, increased to 80% B at 30 min and held for 5 min. The flow rate was set to 1 ml/min, and the detection was monitored at 485 nm for BX and 538 nm for BC. The injection volume was 10 µl. Commercially available betanin containing a mixture of BC and BX was used to quantified BC and BX present in the extracts using an external calibration curve (concentration from 10 to 200 mg/ml; R^2=0.99).

4.2.6 Total phenolic content

Total phenolic content was estimated using previously reported methods (Malencić et al., 2007; Rani et al., 2018; Righi Pessoa da Silva et al., 2018) with some modifications. As a result of the lower temperature used, incubation time was increased by 30 min instead of the standard 40 - 45 min. A standard calibration curve of gallic acid (GA) was prepared using a stock solution of 1000 mg of GA/L (0.2 to 1.0 mg of GA/ml, R^2 = 0.99). The procedure was as follows: 0.2 ml of the extracted sample was diluted with 2.8 ml of double distilled water, and 0.25 ml of Folin-Ciocalteau Reagent (FCR) was added. After 5 min of incubation, 0.75 ml of 20% Na_2CO_3 was added to the mixture and stirred using an auto stirrer for 30 sec. After mixing the solution was stored for 90 min and measured at 760 nm using a spectrophotometer (Cecil CE1011 Spectrophotometer). The calculation for total phenolic content was done as per the equation (4.2) given below. The blank for the reference measurement was prepared with 0.2 ml of water instead of sample.

$$\text{Total phenolic content (mg of GA/g of dried beetroot)} = \frac{C*V}{M} \quad (4.2)$$

where, C=concentration of GA per ml of extract; V=volume of extract (ml); and M=amount of sample taken for extraction (g).

4.2.7 Predictive modelling and optimization

The general method employed for the prediction and optimization of the process parameters was RSM, and the predicted results of RSM were compared with ANN (Bhagya Raj & Dash, 2020a).

4.2.7.1 Response surface methodology (RSM)

RSM was applied to develop the model, investigate the effect of process parameters and their interaction on the response variable which are yield of betalains and total phenolic content. The variables used for the optimization of the amounts of betalains and total phenolics extracted are mentioned above. Design Expert (Version 11.0.0, Stat-Ease Inc, Minneapolis, USA) was used to estimate the constants in the second-order polynomial given by equation (4.3) below and draw the relevant surface response plots:

$$Y = Bo + \sum_{i=1}^{k} B_i x_i + \sum_{i=1}^{k} B_{ii} x_i x_i + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} B_{ij}\, x_i\, x_j \quad (4.3)$$

where Y is the predicted response; Bo is the constant term; Bi is the linear coefficient; Bii the squared coefficient; Bij is the cross-product coefficient; i and j are the indices; $x_i \; and \; x_j$ are the independent predictors and k is the number of factors.

4.2.7.2 Artificial neural network (ANN)

Feed forward architecture, where information flows layer wise in the forward direction, was used for predictive modelling by ANN (Lawal et al., 2021). The general structure of ANN model consists of three basic layers knowns as input layer, hidden layer, and output layer (Bhagya Raj & Dash, 2020a). The independent variables were input parameters such as

extraction time (Ut), extraction temperature (UT), and ethanol concentration (EC) in the case of extraction with ethanol; and Ut, UT and pH in the case of extraction with citric acid solution. The amounts of BC, BX, and TPC in the extract were the output parameters. ANN network has multiple internal parameters and one of these are weights, which is a real variable associated with two neurons in a network depending on the other parameters of the network, like number of iterations, and number of neurons (Elkatatny et al., 2016). The number of neurons in the input layer is simply the number of input or independent variables of the study, and it propagates the information to the hidden layer by scaling the input information via weights (Arab et al., 2016; Kumar et al., 2020). Consequently, the information received from the input layer into the hidden layer is processed in two steps. Firstly, the summation of the weighted input information of neurons that also sums bias as given by the equation (4.4) below (Desai et al., 2008).

$$\text{Sum} = \sum_{i=1}^{n} X_i W_i + b \tag{4.4}$$

where, W_i is the weight function of the network, x_i is the input variables of the study, *i* denoting the indices, *n* is the number of input variables, and b is the bias of the network. The next step in the processing of hidden layer is to pass the weighted output through activation function whose role is to shift the space in non-linearity of input data (Desai et al., 2008). The implementation of the logistic output function is given in equation (4.5) below.

$$f(sum) = \frac{1}{1+exp\ (-sum)} \tag{4.5}$$

The output produced by the hidden layer becomes the input for the output layer, the process to get the output from output layer is similar to the process of obtaining output from the hidden layer. To minimize the error between the experimental value and predicted, an error function is calculated as mean squared error (MSE). As training of an ANN model is an iterative process

where these pre-defined model adequacies check error, and minimize it, by adjusting the weights and bias of the network appropriately. The formula for the calculation of error is given below in equation (4.6).

$$MSE = \frac{1}{n}\sum_{i=1}^{n}(y_{exp} - y_{pred})^2 \tag{4.6}$$

where, MSE is mean squared error; n is the number of total datasets; y_{exp} stands for experimental dataset used for making predictions; and y_{pred} represents the values predicted by the model. On the other hand, to check the deviation of the predicted values from the experimental dataset, root mean squared error (RMSE) and coefficient of determination (R^2) were estimated for the entire dataset. R^2 and RMSE are important parameters to establish the statistical deviation of the data across the central prediction line and reflect the accuracy of predictive modelling as given below in equations (4.7) and (4.8).

$$RMSE = \sqrt{\frac{\sum_{1}^{n}(E_{pre} - E_{exp})}{n}} \tag{4.7}$$

$$R^2 = 1 - \frac{\sum_{i=1}^{n}(E_{pre} - E_{exp})}{\sum_{i=1}^{n}(E_m - E_{exp})} \tag{4.8}$$

where, n is the number of experimental or predicted data; Epre is the predicted value for each experimental results; E_{exp} is the experimental results; E_m is the average value of the observed experimental data; i denotes the index for each passing data. The training of the ANN model was done using neural network feed-forward back-propagation algorithm, which is expected to take less time and memory for iterations (Ouma et al., 2020). The back-propagation training is based on the adjustments of two key network parameters, namely, the learning rate ($0<\varepsilon<1$), and momentum co-efficient ($0<\gamma<1$). The number of neurons in the hidden layer was optimized considering the lowest RMSE between the predicted and experimental values. Total number of

neurons set for the optimization was 1 to 60, and RMSE was measured for each increase in the number of neurons.

4.3 Results and discussion

A full factorial design was implemented for aqueous-ethanol extraction as well as extraction with citric acid solution, as shown in Table 4.1. Since betalains are readily water soluble, extraction was also done using pure water as the solvent, however, the yields were significantly ($p<0.05$) lower than with aqueous-ethanol and citric acid solution (data not reported). The stability of betalains is also likely to be reduced at the higher pH of water. Moreover, during preliminary study, extraction was performed with aqueous-ethanol and citric acid solution without the application of ultrasound and the yield was significantly lower ($p<0.05$) (data not reported), as similarly reported before (Nutter et al., 2021). Hence, data are only reported for aqueous-ethanol and citric acid solutions with ultrasonic extraction.

4.3.1 Effect of extraction time on betalains and total phenolic content

Figure 4.2 shows the effect of time, temperature and pH on the extraction of betalains and total phenolic content in citric acid solutions, whereas, Figure 4.3 shows data for extraction using aqueous-ethanol solutions as the solvent.

Extraction time was not a significant factor for extraction of betalains and total phenolic content when citric acid was used as the solvent (Table 4.2), and this is well illustrated by the response surface plots in Figure 4.2. This conclusion applies to the time range covered in this work (5-30 min), which is expected given the ready solubility of the betalains in citric acid solution and cavitational effect of ultrasound to perform a quick and effective extraction (Gaete-Garretón et al., 2011). Short extraction times will only have an effect if there are mass transfer limitations (Lazăr et al., 2021; Nutter et al., 2021). Long extraction times, such as over 50 min, can result

in lower yields due to degradation of the extract caused by free radical formation in the presence of ultrasound (Lazăr et al., 2021; Nutter et al., 2021). Using citric acid solutions as a solvent, the maximum levels extracted per g of dried beetroot powder were 3.98 mg BC, 3.64 mg BX and 8.28 mg GA/g as a measure of TPC. There were not significant interactions of time with temperature, nor of pH with time on the extraction of betalains and TPC. Therefore, it can be concluded that for extraction of betalains and TPC from dried beetroot using citric acid solution as the solvent, time can be kept to a minimum in order to optimize the process.

However, when aqueous-ethanol was used as solvent then extraction time was significant for BC and TPC, but not for BX (Table 4.2). Figure 4.3 illustrates that betalain content increased with time, which could be attributed to the cavitational and thermal effect of ultrasound treatment (Fernando et al., 2021; Gaete-Garretón et al., 2011; Nutter et al., 2021; Silva et al., 2020). Using aqueous-ethanol as a solvent, the maximum amounts extracted per g of dried beetroot powder were 4.38 mg BC, 3.95 mg BX and 8.45 mg GA/g as a measure of TPC. The yield obtained in this study was comparable or better than previous findings (Fernando et al., 2021; Lazăr et al., 2021; Nutter et al., 2021; Righi Pessoa da Silva et al., 2018). The interactive effect of time and ethanol concentration had significant positive effect on extraction of BC and BX at lower temperature range and this could be easily depicted from Figure 4.3, which was dominated by time. It can be concluded that time had positive effect on the extraction using aqueous-ethanol as solvent and the process should be optimized for optimum time to enable maximum yield of betalains and TPC. In addition, when comparing the extraction efficiency for both solvents the percentage yield using citric acid as a solvent in comparison to aqueous was over 91% for BC, 92% for BX and 98% for TPC, respectively.

4.3.2 Effect of extraction temperature on betalains and total phenolic content

Temperature had a significant effect on the extraction of BC, BX and TPC using either solvent (citric acid or aqueous-ethanol) (Table 4.2). Using either solvent, increasing temperature from 30 to 40 °C tended to decrease the amount of betalains (BC and BX) in the resulting extract (Figures 4.2 and 4.3 (a) to (f)). However, the effect of temperature on TPC varied between the solvents; where the effect of temperature was limited with citric acid but substantial with ethanol. When using aqueous-ethanol increasing temperature from 30 to 40 °C increased the yield of TPC substantially, especially at the higher ethanol concentrations (20%, 30%) (Figure 4.3).

The decrease in betalain (BC and BX) and TPC content in the citric acid extracts with increasing temperature is due to their heat sensitivity. It is evident from Figures 4.2 (a) – (i), (b). This observation is consistent with previously published data (Janiszewska-Turak et al., 2021; Lazăr et al., 2021; Stintzing et al., 2003). Janiszewska-Turak et al., (2021) (Janiszewska-Turak et al., 2021) observed first order degradation kinetics for betalains in the temperature range between 60-90 °C. At higher temperature and low pH betalain content decreased due to the instability of BC and BX at higher temperature and lower pH, as expected from previous studies (Castro-Enríquez et al., 2020; Dumbravă et al., 2012; Skalicky et al., 2020). The negative effect of higher extraction temperature on phenols and betalains was in good agreement with previously reported studies (Kannan, 2011; Righi Pessoa da Silva et al., 2018).

Using aqueous-ethanol as the solvent, temperature again had a significant negative effect on recovery of BC (Figure 4.3 (a), (b), and (c)) and BX (Figure 4.3 (d), (e), and (f)), explained by the thermal degradation of betalains (MERIN et al., 1987). However, extraction temperature significantly (increased the TPC content of the extract (Figure 4.3 (g), (h), and (i)). This increased extraction could be attributed to the enhanced damage to cell membranes of the beetroot powder by temperature and greater permeability for the solvent, coupled with the

greater thermal stability of phenolics compared to betalains (Fernando et al., 2021; Nutter et al., 2021; Righi Pessoa da Silva et al., 2018).

4.3.3 Effect of solvent type on extraction of betalains and total phenolic content

Table 4.2 shows pH of the citric acid solution was the most influential parameter for the extraction of betalains and TPC by citric acid, as previously reported (Lazăr et al., 2021; Singh et al., 2017). Increasing pH significantly increased the yield of BC, BX and TPC (Table 4.2, and Figure 4.2 (a) to (i)). The results of this study are in agreement with previous studies that the concentration of BC extracted from beetroot was higher than of BX (Cardoso-Ugarte et al., 2014; Fernando et al., 2021; Nutter et al., 2021; Silva et al., 2020), The results of this study are in agreement with previous studies that the concentration of BC extracted from beetroot was higher than of BX (Sawicki et al., 2016), which may be due to BC naturally occurring at higher levels than BX in beetroot, or due to the greater stability of BC than BX on extraction.

Using aqueous-ethanol as the solvent, the ethanol concentration had a significant effect (Table 4.2) and was the second most influential parameter for the extraction of BC, BX, and TPC after temperature. The increase in ethanol concentration tended to reduce the extraction of BC and BX (Figure 4.2 (a) to (f)), but it increased the extraction of TPC (Figure 4.3 (g) to (i)). The negative effect of ethanol concentration on betalains was similar to the findings of previous trials (Righi Pessoa da Silva et al., 2018) and may be to the ability of ethanol to extract multiple components at a time at higher concentration. Roriz et al., (2017) (Roriz et al., 2017) reported that an ethanol concentration above 20% compromised extractability of betalains and they attributed this to the increased affinity of other ethanol soluble substances at the solvent levels. The combined effect of increasing ethanol concentration and time tended to reduce the concentration of betalains in the extract (Figure 4.3 (a) to (f)), whereas no combined effect on the total phenolic content was observed. On the other hand, it was observed that the

concentration of betalains in the extract increased with ethanol concentration until at temperature of 30 °C was reached and then decreased with increase in temperature (above 30 °C), which is well illustrated in Figure 4.3. This could be explained by swelling of the cellular structure with an initial increase in temperature, whereas further increase led to thermal degradation of betalains (Cardoso-Ugarte et al., 2014; Righi Pessoa da Silva et al., 2018; Roriz et al., 2017). These findings illustrated that for process optimization the concentration of ethanol could be reduced and this could be environmentally and economically beneficial by reducing solvent consumption .

4.3.4 Modeling, prediction and optimization by response surface methodology (RSM)

4.3.4.1 Citric acid solution as an extraction solvent

The response models obtained from RSM analysis for BC, BX, and TPC is given below in equations (4.9), (4.10), and (4.11). The same models were also used for performing prediction, optimization of the extraction process, and comparing the prediction ability of RSM against ANN.

Betacyanin (BC) = -9.18 + (0.323 * Temperature) + (2.637 * pH) – (0.0342 * Temperature * pH) – (0.045 * Temperature^2) (4.9)

Betaxanthin (BX) = -4.69537 + (0.063 * Temperature) + (2.793 * pH) - (0.043 * Time * Temperature) + (0.002 * Temperature * pH) – (0.018 * Temperature^2) (4.10)

Total phenolic content (TPC) = -16.7401 + (0.536 * Temperature) + (7.073 * pH) – (0.078 * Temperature * pH) – (0.005 * Temperature^2) (4.11)

Correlations between experimental and predicted results by RSM models given in equations (4.9), (4.10), and (4.11) can be seen in Figure 4.4. Coefficient of determination (R^2) between predicted and experimental values were 0.78, 0.89, and 0.79 for BC, BX, and TPC, respectively, indicating a good fit of the model as previously reported (Cardoso-Ugarte et al., 2014; Chen et al., 2015; Singh et al., 2017). Furthermore, RSME was also calculated to quantify the deviation of predicted values from the experimental values. The RMSE values for BC, BX, and TPC was computed to be 0.60, 0.28, and 0.67, respectively. The RMSE values are relatively high, and it was evident from the scattered pattern of predicted and experimental data across the central prediction line in Figures 4.4 (a), (b), and (c).

For performing optimization, equations (4.9), (4.10), and (4.11) were used, which leads to the maximum yield of betalains and TPC with tested variable range. The resulting optimized conditions were 10 min of extraction time at 30 °C of extraction temperature and pH 5, with yields of BC, BX, and TPC of 3.95 mg of BC/g, 3.54 mg BX/g, and 7.17 mg of GA/g of dried beetroot powder, respectively, with RSM desirability value of 0.928. The optimized condition and responses were validated by real time experiment in triplicate as shown in Table 4.3. The significance of the interaction between the variables evaluated in the experimental design was used to define this condition.

4.3.4.2 Aqueous-ethanol as an extraction solvent

The response models obtained from RSM analysis for BC, BX, and TPC is given below in equations (4.12), (4.13), and (4.14), respectively. The same models were also used for performing prediction, optimization of the extraction process, and comparing the prediction ability of RSM against ANN.

Betacyanin (BC) = 2.97675 + (0.029 * Time) + (0.096 * Temperature) - (0.043 * Ethanol) – (0.0711 * Time * Ethanol) + (0.089*Temperature * Ethanol) – (0.019 * Temperature^2) (4.12)

Betaxanthin (BX) = 2.20591 + (0.092 * Temperature) - (0.013 * Ethanol) – (0.007 * Time * Ethanol) – (0.002 * Temperature^2) (4.13)

Total Phenolic Content (TPC) = 9.1896 + (0.062 * Time) - (0.165 * Temperature) +(0.004 * Ethanol) + (0.004 * Temperature * Ethanol) - (0.002 * Time^2) + (0.002 * Temperature^2) - (0.003 * Ethanol ^2) (4.14)

Figure 4.4 shows the correlation between the predicted values by RSM models given in equations (4.12), (4.13), and (4.14) and experimental results. The values of co-efficient of determination for BC, BX and TPC was calculated to be 0.88, 0.79, and 0.86, respectively. The obtained values were greater than those reported before (Singh et al., 2017), but are in close agreement with other authors (Chen et al., 2015; Righi Pessoa da Silva et al., 2018). The RMSE for BC, BX, and TPC was calculated to be 0.21, 0.30, and 0.26, respectively. The lower values of RMSE are well illustrated by less scattering of data across the central prediction line as shown in Figure 4.4 (d), (e), and (f), and this could be attributed to the low range of variation within the data (Zafar et al., 2012).

For performing optimization, equations (4.12), (4.13), and (4.14) were used. The resulting optimized conditions were 15.8 min of extraction time at 20.1 °C of extraction temperature and 10% of ethanol concentration in water, with yields of BC, BX, and TPC as 4.15 mg of BC/g, 3.52 mg of BX/g, and 7.71 mg of GA/g of beetroot powder, respectively, with RSM desirability value of 0.679. The optimized condition and responses were validated by real time experiment in triplicate as shown in Table 4.3. The significance of the interaction between the variables evaluated in the experimental design was used to define this condition.

4.3.5 Artificial neural network (ANN) modelling, prediction and comparison with response surface methodology (RSM)

4.3.5.1 Predictive model development with artificial neural network

The design of experiments of RSM with responses were adopted for developing an additional predictive model with ANN to compare with RSM. The total number of datasets for this ANN based machine learning approach was equal to the number of experimental results shown in Table 4.4. To train the model partitioning of the data was important to avoid overfitting of the model and overparameterization of the functions (Desai et al., 2008). It was partitioned as 70%, 15% and 15% for training, testing, and validation, respectively (Bhagya Raj & Dash, 2020a; Desai et al., 2008; Kumar et al., 2020). Training was done for 1-60 neurons in series, for each increase in number of neurons the predicted value was compared with experimental results and RMSE was calculated. The predicted yields by RSM and ANN are shown in Table 4.4. The selection of the optimized number of neurons in hidden layer was based on the least RMSE value.

For citric acid solution as extraction media the ANN based prediction illustrated promising results in terms of RMSE, MSE and R^2, and the predicted values by ANN model had great accuracy as evidenced by the scattering of the data across the central prediction line. The obtained values of RMSE, MSE and R^2 are shown in Table 4.5. The total number of optimized neurons in the hidden layer for citric acid solution as extraction media was 6 with lowest possible value of RMSE and it was 0.0043 as shown in Figure 4.5 (i) (a). If number of neurons are very low or very high it may cause underfitting or overfitting of the model (Desai et al., 2008). The correlation between the predicted and experimental values for BC, BX, and TPC are shown in Figure 4.5 (i) (b).

Predicted yields by the ANN models for aqueous-ethanol as extraction media are shown in Table 4.3 (b). The obtained values of RMSE, MSE and R^2 are shown in Table 4.5. The number of optimized neurons in the hidden layer for aqueous-ethanol as extraction media was 8 with lowest possible value of RMSE and it was 0.0042 as shown in Figure 4.5 (ii) (a). The correlation between the predicted and experimental values for BC, BX, and TPC are shown in Figure 4.5 (ii) (b).

4.3.6 Prediction performance comparison for artificial neural network (ANN) with response surface methodology (RSM)

The prediction performance of ANN and RSM were compared in terms of their capacity to predict data as closely as possible to the original dataset. In terms of all statistical characteristics obtained from Table 4.5 (a) and 4.5 (b), it was discovered that the ANN tool was preferable. The R^2 for ANN predicted data was found to be close to 0.99 for both type of the solvents and for their respective responses. On the other hand, the R^2 for RSM predicted data for both type of the solvent varied between 0.78-0.89. R^2 is not the only parameter to be checked, but it was the first check point for the comparison. Additionally, it was observed that ANN had 10-fold less error in terms of the MSE and RMSE compared to RSM. The value of RMSE and MSE for ANN ranged between 0.02 - 0.05 and 0.000049 - 0.0927, whereas, for RSM the range was 0.20 - 0.67 and 0.1011 - 0.2111, as illustrated in Table 4.5 (a), and 4.5 (b). The better accuracy of the former tool could be attributed to its universal ability to approximate non-linearity of the system, whereas RSM is restricted to a second-order polynomial (Desai et al., 2008; Kumar et al., 2020; Pires dos Santos et al., 2019). Moreover, many studies reported that ANN modelling is a useful and flexible tool to generate models and to calculate the multiple responses in a single run (Bhagya Raj & Dash, 2020a). This concludes that ANN could be a useful alternative predictive tool over conventional RSM.

4.3.7 High performance liquid chromatography (HPLC) analysis

The individual betalains present in the optimised beetroot extract were identified against standards of betanin (Chethana et al., 2007; Nestora et al., 2016; SlAvov et al., 2013). Figure 4.6 (A) shows the HPLC elution profile at 538 nm of BC (13.85 min) and iso-betacyanin (IBC) (15.67 min) standards. Figure 4.6 (B) and 4.6 (C) show the HPLC elution profiles for the optimized samples extracted using aqueous-ethanol and citric acid solution, respectively.

4.4 Conclusions

Extraction of betalains and the determination of total phenolic content from beetroot powder was performed using ultrasonication technology with conventional organic solvent of aqueous-ethanol and citric acid solution, as solvents. Extraction using citric acid solution demonstrated a great potential for the extraction of polar compounds like betalains, and phenolics. Comparing the extraction efficiency of the both solvents, it could be concluded that the percentage yield using citric acid as a solvent in comparison to aqueous-ethanol was over 91% for BC, 92% for BX and 98% for TPC, respectively. Which is sufficiently high to be considered as a potential solvent for the future extraction of such bioactive compounds. To optimize the extraction process for ethanol and citric acid solution as solvents a full factorial RSM design was implemented. The optimization secured more than 90% of the betalains and 85% of the total phenolics in the extract for both solvent types. The resulting optimized conditions for citric acid solution as solvent was 10 min of extraction time at 30 °C of extraction temperature and pH 5, with yields of BC, BX, and TPC of 3.95 mg of BC/g, 3.54 mg BX/g, and 7.17 mg of GA/g of dried beetroot powder, respectively, with RSM desirability value of 0.928. Whereas, for aqueous ethanol optimized conditions were 15.8 min of extraction time at 20.1 °C of extraction temperature and 10% of ethanol concentration in water, with yields of BC, BX, and TPC as 4.15 mg of BC/g, 3.52 mg of BX/g, and 7.71 mg of GA/g of beetroot

powder, respectively, with RSM desirability value of 0.679. Therefore, the method developed can be successfully utilized for the efficient extraction of betalains and phenolics from beetroot to enable economic utilization. The developed models by RSM and ANN were used to forecast future data and ANN proved to be a better predictive tool than RSM.

In summary, extraction of betalains and total phenolic compounds using citric acid as an alternative solvent approach opens a new possibility of performing extraction. In addition, it also opens other possibilities of exploring options available with ionic liquids (ILs) and natural deep eutectic solvents (NADES). Citric acid and other such food grade acids, which are commonly present in plant tissues, could be explored to develop NADES with the aim of optimising extraction procedures.

4.5 References

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Table 4.1: Factors applied for the ultrasonic extraction of betalains using aqueous-ethanol and citric acid solution as solvents.

Sl. No.	Variable Name	Variable Coding*	Range
1	Extraction Time (min)	Ut	5-30
2	Extraction Temperature (°C)	UT	20-40
3	Ethanol (%)	EC	10-30
1	Extraction Time (min)	Ut	5-30
2	Extraction Temperature (°C)	UT	20-40
3	Citric Acid Solution (pH)	pH	3-5

*****Ut – Extraction Time (min); UT - Extraction Temperature (°C); EC - Ethanol (%); pH - Citric Acid Solution (pH).

Table 4.2: ANOVA table showing significance (p-values) of the different treatment parameters and solvents on betalains and total phenolic compounds.

	Solvent: Aqueous ethanol solutions			**Solvent: Citric Acid**		
Parameters	Betacyanin	Betaxanthin	TPC	Betacyanin	Betaxanthin	TPC
A-Extraction Time (min) (Ut)	0.023	---	0.002	---	---	---
B- Extraction Temperature (°C) (UT)	< 0.000	0.003	0.004	< 0.000	< 0.000	<0.000
Ethanol Concentration (%) (EC)/pH	0.000	0.036	<0.000	< 0.000	< 0.000	<0.000
AB	---	---	---	---	---	---
AC	0.001	0.003	---	---	---	---
BC	0.000	---	<0.000	0.015	0.015	0.015
A^2	---	---	0.000	---	---	---
B^2	< 0.000	< 0.000	0.000	0.022	0.022	0.022
C^2	---	---	<0.000	---	---	---
R^2	0.89	0.80	0.86	0.78	0.89	0.79

AB – Extraction Time * Extraction Temperature

AC – Extraction Time * Ethanol Concentration or pH

BC - Extraction Temperature * Ethanol Concentration or pH

A^2 - Extraction Time * Extraction Time

B^2 - Extraction Temperature * Extraction Temperature

C^2 - Ethanol Concentration or pH * Ethanol Concentration or pH

Table 4.3: Optimization table and validation with real time experiment for citric acid solution and aqueous-ethanol as solvent.

Sl. No.	Responses of citric acid	Optimized Response of citric acid	Average Real Time experimental Value of citric acid
1.	BC	3.95	3.91±0.12
2.	BX	3.54	3.59±0.23
3.	TPC	7.17	7.06±0.36
Sl. No.	Responses of ethanol	Optimized Response of ethanol	Average Real Time experimental Value of ethanol
1.	BC	4.15	4.07±0.15
2.	BX	3.52	3.68±0.13
3.	TPC	7.71	7.65±0.41

Responses are expressed as mg/g of beetroot powder for betacyanin (BC) and betaxanthin (BX), and as mg of gallic acid (GA)/g of beetroot powder for total phenolic content (TPC).

Table 4.4(a): Full factorial design matrix of independent variables and their corresponding experimental and predicted yields of total phenolic content betacyanin, and betaxanthin for citric acid solution as solvent.

				Experimental Responses			Predicted Responses by RSM			Predicted Responses by ANN		
Sl. No.	Time (min)	Temperature (°C)	pH	BC (mg/g)	BX (mg/g)	TPC (mg of GA/g)	TPC (mg of GA/g)	BC (mg/g)	BX (mg/g)	TPC (mg of GA/g)	BC (mg/g)	BX (mg/g)
1	5	40	5	3.17	3.14	5.26	5.51	2.88	3.13	5.31	2.39	2.41
2	10	40	5	3.04	3.02	5.30	5.32	2.67	2.99	5.34	2.43	2.34
3	15	40	5	2.96	2.97	5.30	5.08	2.56	2.91	5.25	2.35	2.28
4	20	40	5	2.81	2.90	4.82	4.82	2.55	2.86	4.76	2.20	2.23
5	25	40	5	2.79	2.86	4.83	4.53	2.64	2.83	4.74	2.11	2.20
6	30	40	5	2.77	2.82	4.66	4.22	2.83	2.83	4.74	2.11	2.17
7	5	40	4	1.53	2.21	5.17	5.68	1.71	2.4	5.23	1.08	1.64
8	10	40	4	1.01	2.02	4.86	5.52	1.61	2.27	4.84	0.80	1.45
9	15	40	4	0.87	1.71	4.53	5.33	1.61	2.16	4.48	0.79	1.36
10	20	40	4	0.80	1.65	4.67	5.12	1.71	2.08	4.69	0.71	1.30
11	25	40	4	0.79	1.59	4.65	4.87	1.89	2.02	4.66	0.59	1.26
12	30	40	4	0.73	1.59	4.43	4.62	2.18	1.99	4.40	0.58	1.22
13	5	40	3	0.57	1.43	5.20	4.94	0.33	1.22	5.11	0.48	1.07
14	10	40	3	0.43	1.31	5.05	4.83	0.33	1.05	4.93	0.31	1.05
15	15	40	3	0.33	1.13	4.85	4.68	0.43	0.92	4.88	0.26	0.97
16	20	40	3	0.31	1.25	5.33	4.51	0.63	0.80	5.21	0.23	0.94
17	25	40	3	3.01	1.10	4.54	4.31	0.93	0.72	4.65	2.34	0.90
18	30	40	3	2.89	1.09	4.47	4.07	1.32	0.66	4.35	2.02	0.88
19	5	30	3	0.88	1.59	5.23	5.22	1.21	1.79	5.14	0.69	1.13
20	10	30	3	0.57	1.01	4.26	5.14	1.13	1.65	4.42	0.50	0.82
21	15	30	3	0.43	1.10	4.62	5.03	1.14	1.53	4.43	0.39	0.77
22	20	30	3	0.34	0.86	3.90	4.89	1.26	1.44	4.08	0.37	0.68
23	25	30	3	0.32	0.85	3.86	4.72	1.47	1.38	4.06	0.26	0.65
24	30	30	3	1.30	0.83	3.80	4.52	1.77	1.34	3.85	0.89	0.63
25	5	30	4	3.59	3.23	7.08	6.73	2.84	2.95	6.99	2.70	2.48
26	10	30	4	3.44	3.19	7.12	6.61	2.65	2.84	7.09	2.56	2.42
27	15	30	4	3.28	3.09	7.14	6.46	2.56	2.75	7.03	2.41	2.40
28	20	30	4	3.35	3.17	7.26	6.28	2.57	2.69	7.15	2.46	2.42
29	25	30	4	3.26	3.09	7.17	6.07	2.67	2.66	7.30	2.46	2.40
30	30	30	4	3.21	3.09	7.12	5.83	2.88	2.65	7.23	2.47	2.34
31	5	30	5	3.98	3.64	8.13	7.34	4.24	3.63	8.13	3.20	2.75
32	10	30	5	3.96	3.63	8.28	7.18	3.95	3.54	8.17	3.20	2.79
33	15	30	5	3.88	3.60	8.20	6.98	3.76	3.48	7.96	3.22	2.80
34	20	30	5	3.86	3.61	5.23	6.76	3.66	3.45	5.35	3.36	2.78
35	25	30	5	3.77	3.55	5.08	6.51	3.66	3.44	5.11	3.36	2.75
36	30	30	5	3.75	3.55	5.03	6.23	3.76	3.46	4.97	3.18	2.72

37	5	20	3	1.74	2.22	5.42	4.56	1.29	2.01	5.56	1.42	1.63
38	10	20	3	1.39	2.02	5.21	4.52	1.12	1.89	5.37	1.28	1.57
39	15	20	3	1.17	1.85	4.52	4.44	1.05	1.79	4.56	1.05	1.51
40	20	20	3	1.18	1.92	4.28	4.34	1.07	1.72	4.26	0.88	1.47
41	25	20	3	1.14	1.84	4.22	4.21	1.23	1.68	4.30	0.84	1.43
42	30	20	3	1.10	1.83	4.22	4.05	1.42	1.66	4.16	0.80	1.41
43	5	20	4	3.14	3.12	5.81	6.86	3.15	3.15	5.72	2.39	2.43
44	10	20	4	3.01	3.02	6.12	6.77	2.88	3.06	6.01	2.37	2.37
45	15	20	4	2.97	3.03	6.60	6.66	2.75	2.99	6.44	2.34	2.32
46	20	20	4	2.90	2.94	6.28	6.51	2.63	2.95	6.22	2.30	2.28
47	25	20	4	2.89	2.89	6.18	6.34	2.65	2.94	6.13	2.24	2.29
48	30	20	4	2.86	2.87	6.19	6.13	2.76	2.95	6.19	2.21	2.30
49	5	20	5	3.92	3.58	7.26	8.25	4.80	3.8	7.23	3.37	2.74
50	10	20	5	3.95	3.63	8.13	8.12	4.42	3.74	7.99	3.34	2.73
51	15	20	5	3.97	3.60	8.28	7.96	4.14	3.7	8.42	3.32	2.74
52	20	20	5	3.97	3.62	8.05	7.78	3.96	3.69	8.16	3.32	2.78
53	25	20	5	3.88	3.60	7.95	7.56	3.88	3.75	7.73	3.37	2.78
54	30	20	5	3.84	3.56	7.67	7.32	3.89	3.75	7.59	3.38	2.75

*RSM-Response Surface Methodology; ANN-Artificial Neural Network; TPC – Total Phenolic Compounds; BC-Betacyanin; BX- Betaxanthin; Responses are expressed as mg/g of beetroot powder for betacyanin (BC) and betaxanthin (BX), and as mg of gallic acid (GA)/g of beetroot powder for total phenolic compounds (TPC).

Table 4.4(b): Full factorial design matrix of independent variables and their corresponding experimental and predicted yields of total phenolic content betacyanin, and betaxanthin for aqueous ethanol as solvent.

				Experimental Data			Predicted Responses by RSM			Predicted Responses by ANN		
Sl. No.	Time (min)	Temperature (°C)	EC (%)	BC (mg/g)	BX (mg/g)	TPC (mg of GA/g)	BC (mg/g)	BX (mg/g)	TPC (mg of GA/g)	BC (mg/g)	BX (mg/g)	TPC (mg of GA/g)
1	5	40	30	4.04	3.61	7.92	3.95	3.43	8.09	3.96	3.57	7.93
2	10	40	30	3.81	3.43	8.16	3.91	3.39	8.30	3.91	3.51	8.15
3	15	40	30	3.98	3.45	8.31	3.87	3.35	8.43	3.88	3.43	8.30
4	20	40	30	3.81	3.38	8.32	3.83	3.31	8.49	3.84	3.37	8.33
5	25	40	30	3.77	3.30	8.30	3.78	3.27	8.47	3.79	3.31	8.31
6	30	40	30	3.72	3.23	8.25	3.73	3.23	8.37	3.68	3.26	8.27
7	5	40	20	3.65	3.16	7.85	3.80	3.33	7.83	3.68	3.17	7.88
8	10	40	20	3.69	3.19	8.02	3.80	3.33	8.04	3.72	3.20	8.09
9	15	40	20	3.77	3.29	8.18	3.79	3.33	8.17	3.74	3.24	8.17
10	20	40	20	3.71	3.29	8.21	3.78	3.32	8.22	3.74	3.29	8.18
11	25	40	20	3.76	3.35	8.17	3.77	3.32	8.19	3.77	3.34	8.17
12	30	40	20	3.79	3.40	8.13	3.75	3.31	8.09	3.79	3.42	8.15
13	5	40	10	3.88	3.34	7.34	3.75	3.27	7.07	3.88	3.41	7.31
14	10	40	10	3.89	3.34	7.39	3.78	3.31	7.27	3.90	3.35	7.43
15	15	40	10	3.82	3.28	7.44	3.81	3.34	7.40	3.91	3.33	7.46
16	20	40	10	3.89	3.34	7.51	3.84	3.37	7.44	3.91	3.33	7.47
17	25	40	10	3.86	3.31	7.50	3.86	3.46	7.41	3.87	3.32	7.47
18	30	40	10	3.86	3.31	7.49	3.88	3.43	7.3	3.81	3.31	7.46
19	5	30	30	4.03	3.39	7.56	4.10	3.58	7.28	4.04	3.40	7.56
20	10	30	30	4.03	3.39	7.76	4.08	3.56	7.49	4.06	3.38	7.75
21	15	30	30	4.17	3.51	7.87	4.05	3.53	7.62	4.11	3.38	7.89
22	20	30	30	4.04	3.39	7.89	4.03	3.5	7.68	4.13	3.41	7.90
23	25	30	30	4.12	3.46	7.85	4.00	3.47	7.66	4.12	3.45	7.84
24	30	30	30	4.14	3.47	7.80	3.96	3.43	7.56	4.10	3.50	7.76
25	5	30	20	4.12	3.57	7.49	4.03	3.53	7.41	4.14	3.63	7.48
26	10	30	20	4.20	3.69	7.70	4.05	3.54	7.62	4.09	3.60	7.69
27	15	30	20	4.06	3.51	7.78	4.06	3.55	7.75	4.02	3.54	7.78
28	20	30	20	3.97	3.44	7.79	4.07	3.55	7.87	3.98	3.47	7.79
29	25	30	20	3.94	3.41	7.77	4.07	3.55	7.77	3.98	3.40	7.78
30	30	30	20	3.88	3.35	7.74	4.07	3.55	7.67	3.99	3.35	7.77
31	5	30	10	3.95	3.54	6.96	4.07	3.51	7.04	3.98	3.53	6.97
32	10	30	10	4.00	3.58	7.03	4.12	3.56	7.24	4.07	3.60	7.09
33	15	30	10	4.21	3.78	7.12	4.17	3.62	7.37	4.16	3.67	7.11
34	20	30	10	4.16	3.74	7.13	4.22	3.64	7.41	4.24	3.76	7.11
35	25	30	10	4.29	3.87	7.11	4.26	3.68	7.38	4.29	3.87	7.10
36	30	30	10	4.38	3.95	7.10	4.29	3.72	7.27	4.35	3.95	7.10
37	5	20	10	3.84	3.24	7.53	4.01	3.41	7.38	3.98	3.31	7.53

38	10	20	10	4.22	3.52	7.71	4.08	3.46	7.58	4.14	3.44	7.68
39	15	20	10	3.99	3.33	7.78	4.14	3.52	7.71	4.07	3.43	7.75
40	20	20	10	4.22	3.48	7.78	4.26	3.57	7.75	4.16	3.48	7.78
41	25	20	10	4.26	3.52	7.76	4.26	3.62	7.72	4.24	3.54	7.76
42	30	20	10	4.32	3.58	7.72	4.31	3.66	7.62	4.31	3.62	7.72
43	5	20	20	3.96	3.42	7.49	3.88	3.38	7.36	4.01	3.46	7.48
44	10	20	20	4.08	3.57	7.60	3.91	3.44	7.57	4.06	3.50	7.62
45	15	20	20	4.05	3.51	7.67	3.94	3.42	7.74	4.08	3.53	7.67
46	20	20	20	4.02	3.49	7.69	3.97	3.44	7.75	4.07	3.55	7.69
47	25	20	20	4.05	3.53	7.68	3.99	3.45	7.72	4.06	3.56	7.69
48	30	20	20	4.06	3.55	7.66	4.01	3.46	7.62	4.05	3.55	7.66
49	5	20	30	3.88	3.47	6.86	3.85	3.4	6.84	3.88	3.43	6.85
50	10	20	30	3.75	3.37	6.91	3.85	3.38	7.05	3.82	3.41	6.92
51	15	20	30	3.76	3.36	6.96	3.85	3.37	7.18	3.78	3.38	6.99
52	20	20	30	3.78	3.37	6.99	3.84	3.35	7.24	3.75	3.35	6.99
53	25	20	30	3.74	3.31	7.00	3.82	3.32	7.21	3.73	3.33	6.99
54	30	20	30	3.73	3.28	6.99	3.81	3.34	7.12	3.72	3.30	7.01

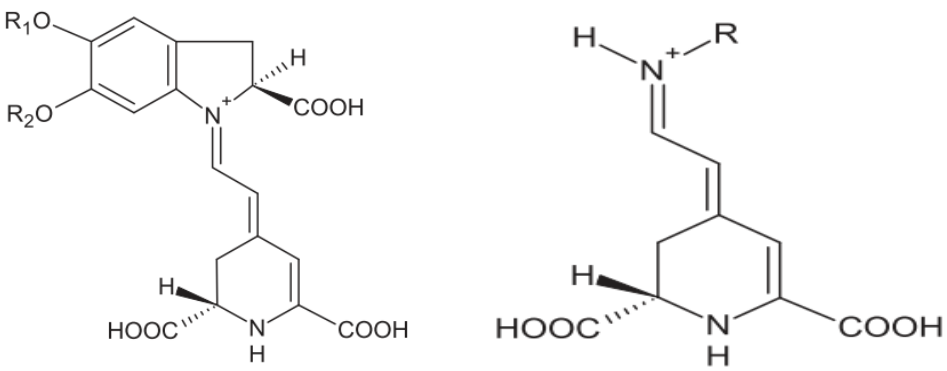
Table 4.5(a): Statistical parameters to assess the predictive capability of the ANN for betacyanin (BC), betaxanthin (BX) and total phenolic content (TPC).

1. Citric Acid solution as Solvent				
Sl. No.	Responses	RMSE	MSE	R^2
1	BC	0.032	0.092	0.99
2	BX	0.052	0.002	0.99
3	TPC	0.023	0.055	0.99
2. Aqueous-ethanol as solvent				
1	BC	0.051	0.003	0.99
2	BX	0.047	0.002	0.99
3	TPC	0.020	0.001	0.99

*RMSE-root mean square error; MSE – mean squared error; R^2 – correlation coefficient

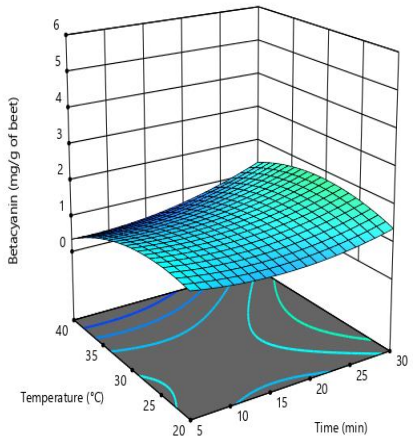
Table 4.5(b): Statistical parameters to assess the predictive capability of the RSM models for betacyanin, betaxanthin and total phenolic content.

1.Citric acid solution as solvent				
Sl. No.	Responses	RMSE	MSE	R^2
1	BC	0.600	0.101	0.78
2	BX	0.282	0.126	0.89
3	TPC	0.671	0.138	0.79
2. Aqueous-ethanol as solvent				
1	BC	0.471	0.211	0.88
2	BX	0.206	0.193	0.79
3	TPC	0.262	0.164	0.86

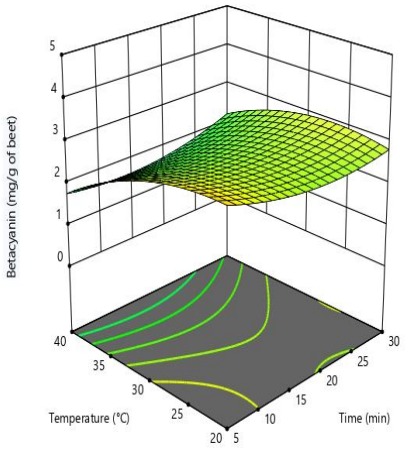


(a) Betacyanins (b) Betaxanthins

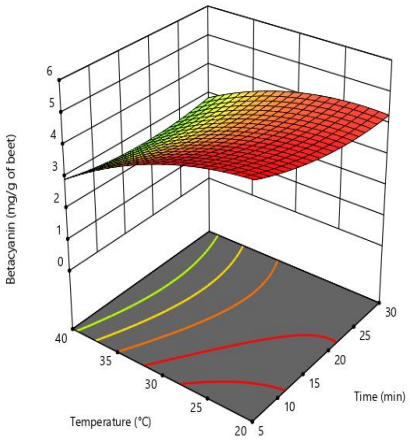
Figure 4.1: Chemical structure of betalains: (a) betacyanins and (b) betaxanthins



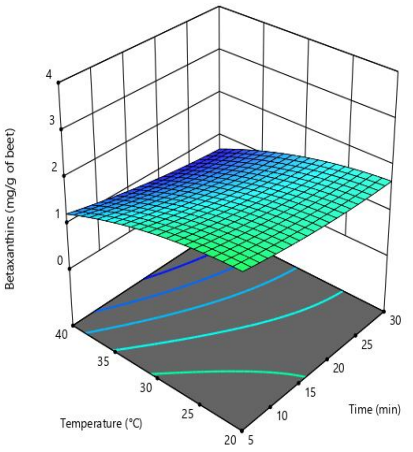
(a) Betacyanin at pH 3



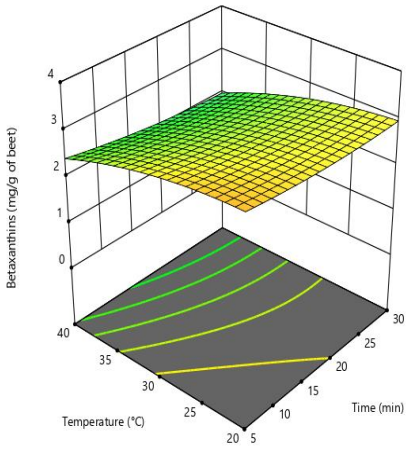
(b) Betacyanin at pH 4



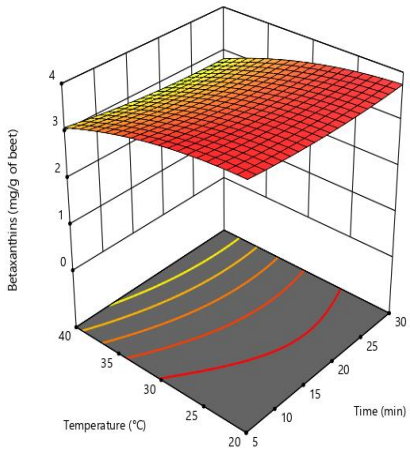
(c) Betacyanin at pH 5



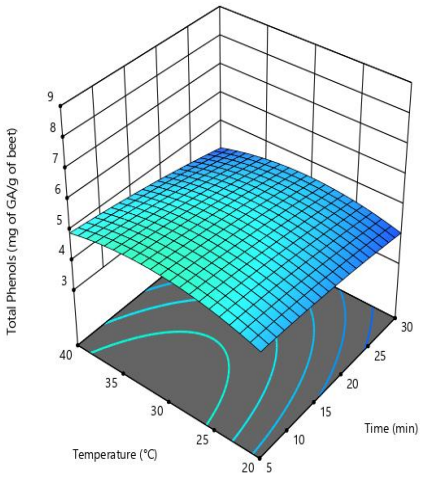
(d) Betaxanthin at pH 3



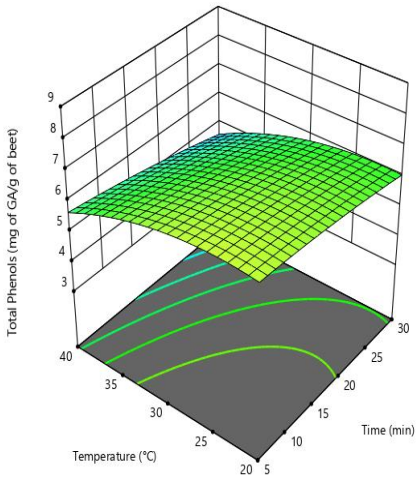
(e) Betaxanthin at pH 4



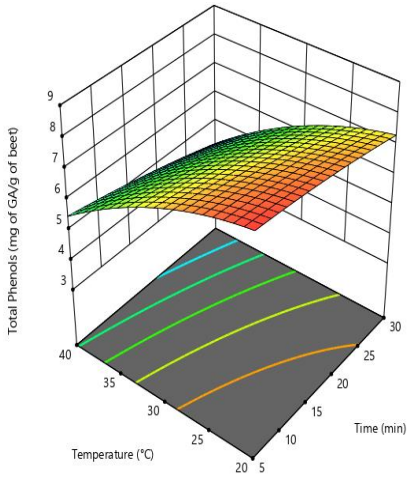
(f) Betaxanthin at pH 5



(g) TPC at pH 3

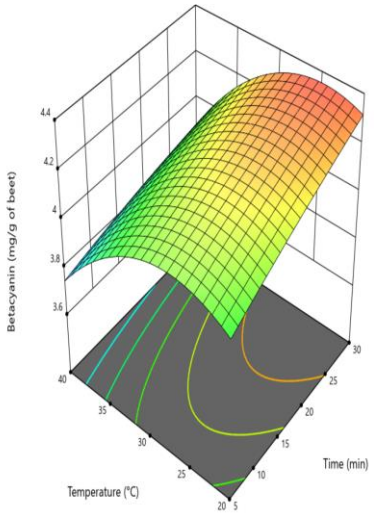


(h) TPC at pH 4

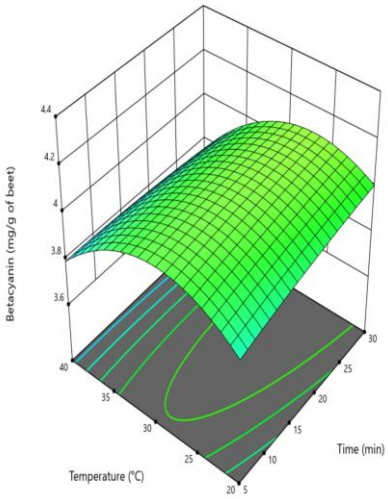


(i) TPC at pH 5

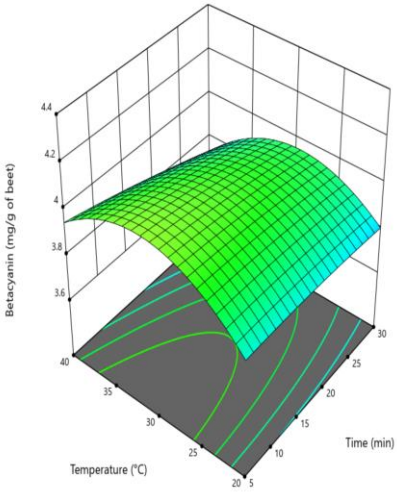
Figure 4.2: Response surface model plots for citric acid solutions as solvent; (a), (b), and (c) show the effect of time and temperature on betacyanin at fixed pH; (d), (e), and (f) show effect of time and temperature on betaxanthin at fixed pH; and (g), (h), (i) show the effect of time and temperature on total phenolic content (TPC) at fixed pH.



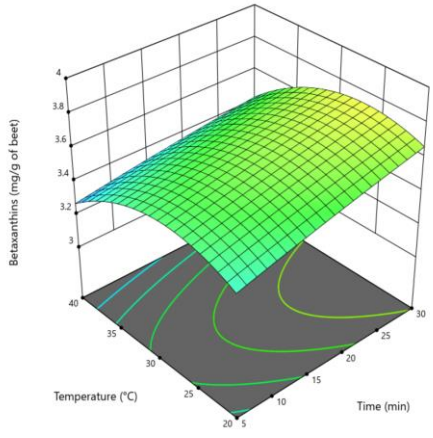
(a) Betacyanin at 10% Ethanol



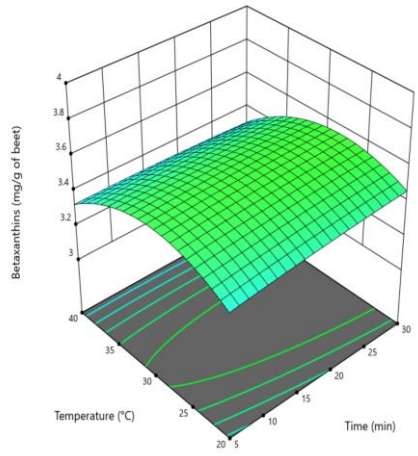
(b) Betacyanin at 20% Ethanol



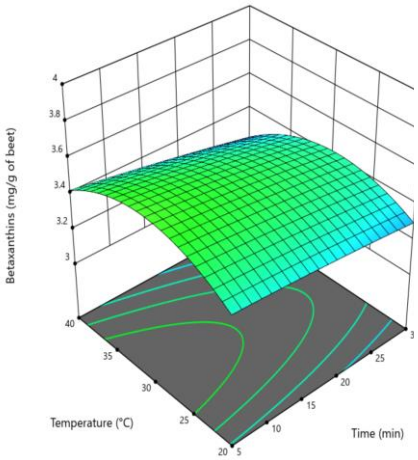
(c) Betacyanin at 30% Ethanol



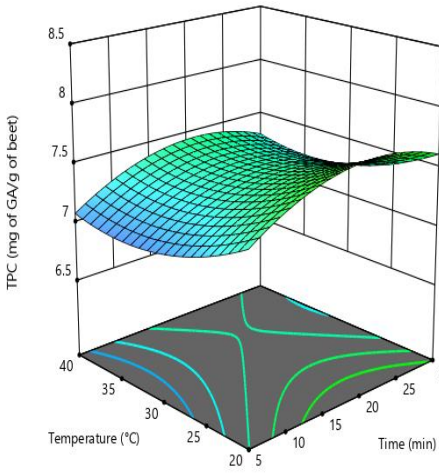
(d) Betaxanthin at 10% Ethanol



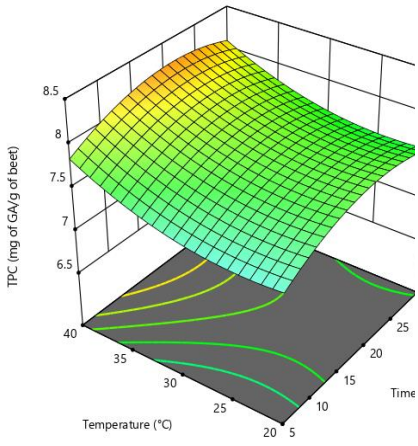
(e) Betaxanthin at 20% Ethanol



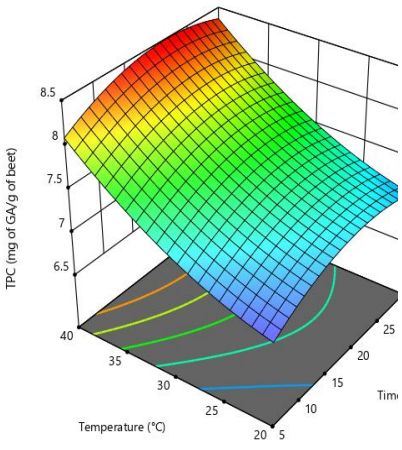
(f) Betaxanthin at 30% Ethanol



(g) TPC at 10% Ethanol



(h) TPC at 20% Ethanol



(i) TPC at 30% Ethanol

Figure 4.3: Response surface model plot where aqueous-ethanol was used as the solvent; (a), (b), and (c) show the effect of time and temperature on betacyanin at fixed ethanol concentration; (d), (e), and (f) show the effect of time and temperature on betaxanthin at fixed ethanol concentration; and (g), (h), (i) show the effect of time and temperature on total phenolic content (TPC) at fixed ethanol concentration.

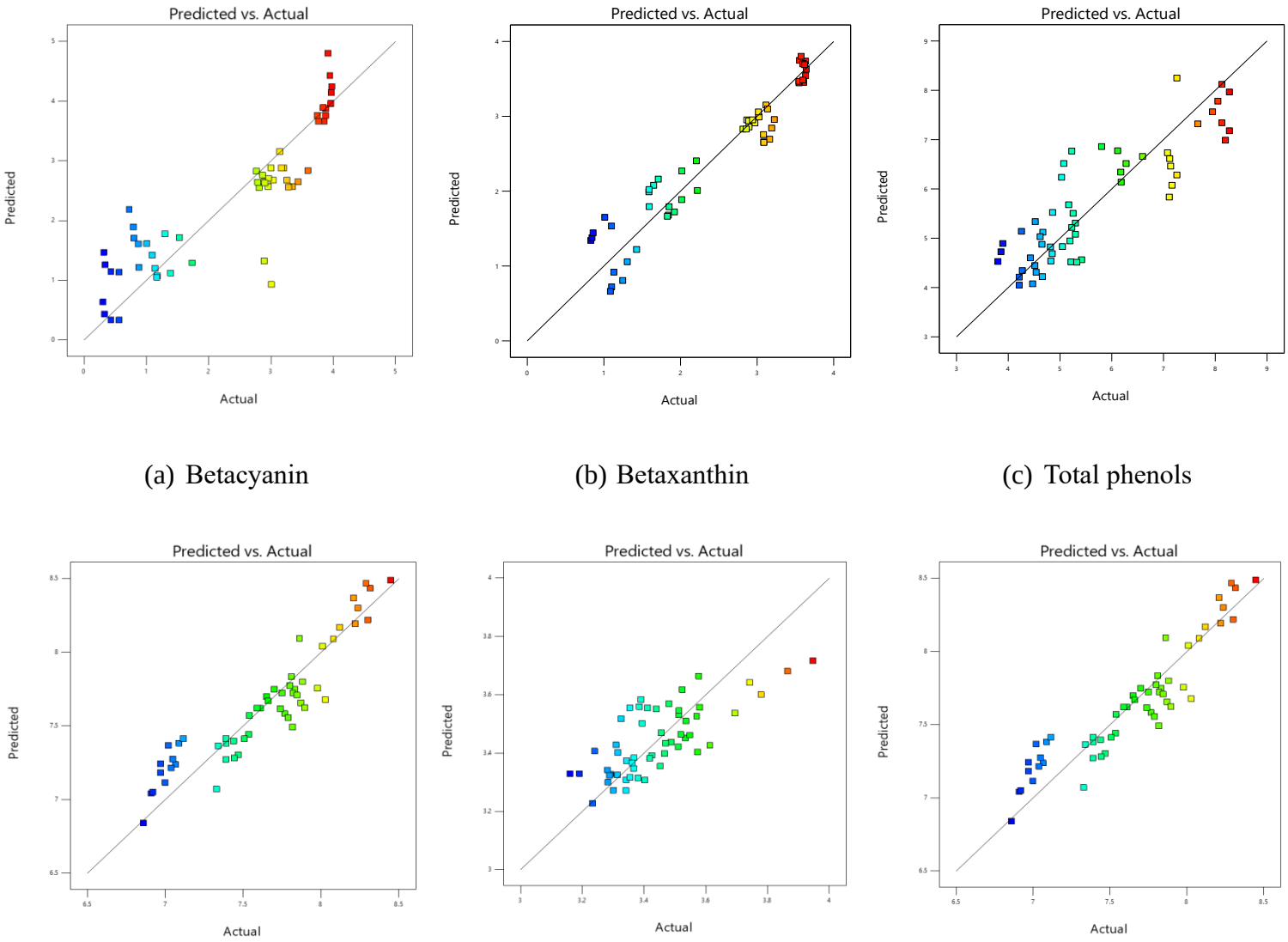
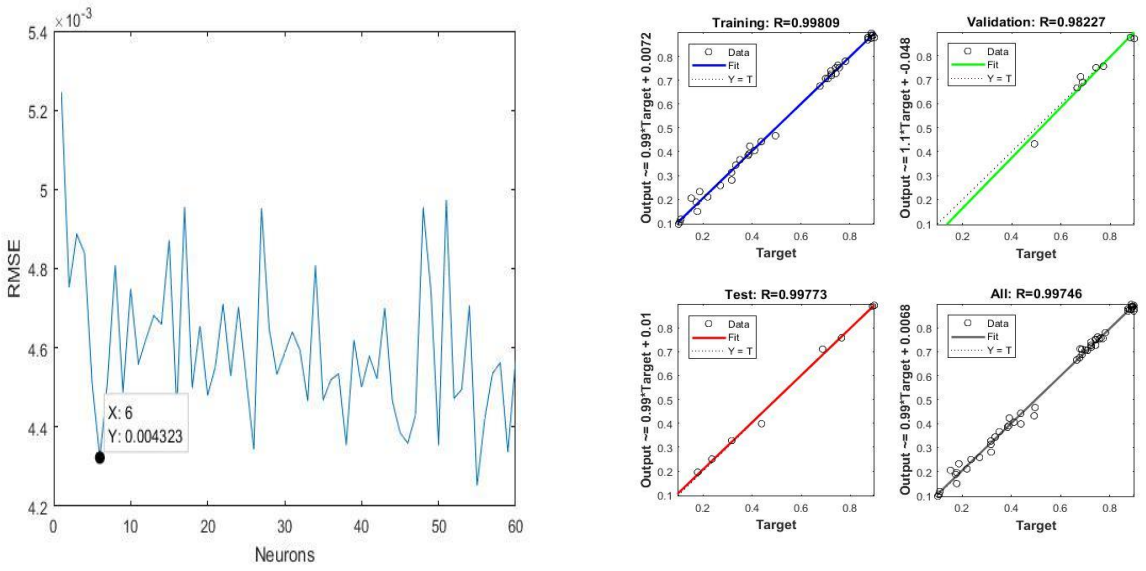
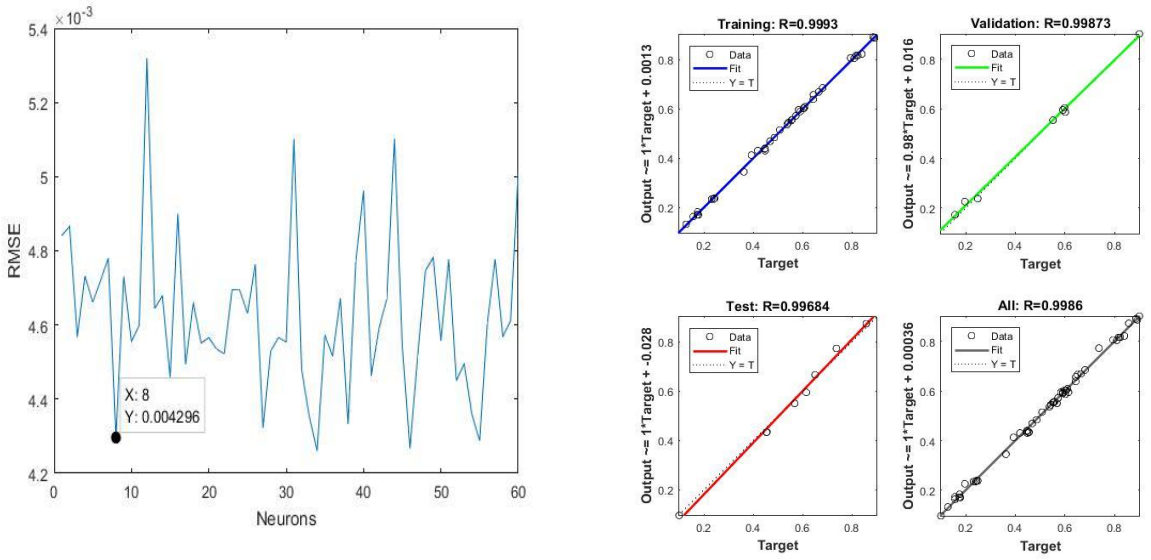


Figure 4.4: The correlation between predicted and actual values by response surface methodology (RSM), (i) for citric acid as the solvent (a), (b), (c); (ii) ethanol as the solvent (d), (e), and (f). The change in colour from blue to red indicates an increase in the concentration value.



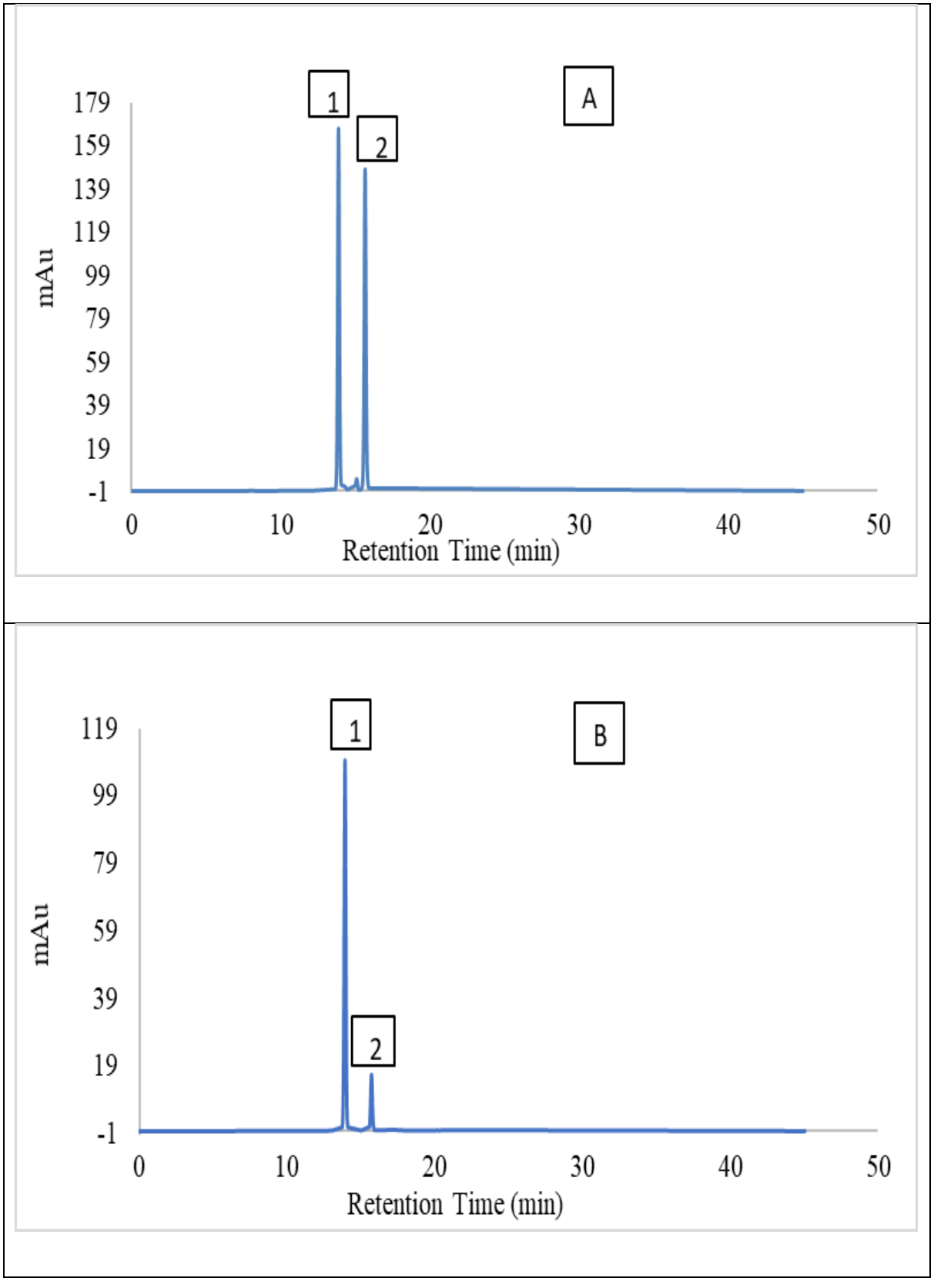
(a) (b)

Figure 4.5(i): Optimization of the number of neurons against root mean square error (RMSE); (b) regression between predicted and experimental data where citric acid solutions were used as the extraction solvent.



(a) (b)

Figure 4.5(ii): Optimization of the number of neurons against root mean square error (RMSE); (b) regression between predicted and experimental data where aqueous-ethanol was used as the extraction solvent.


A
1
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99
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59
39
19
-1
mAu
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Retention Time (min)
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Retention Time (min)

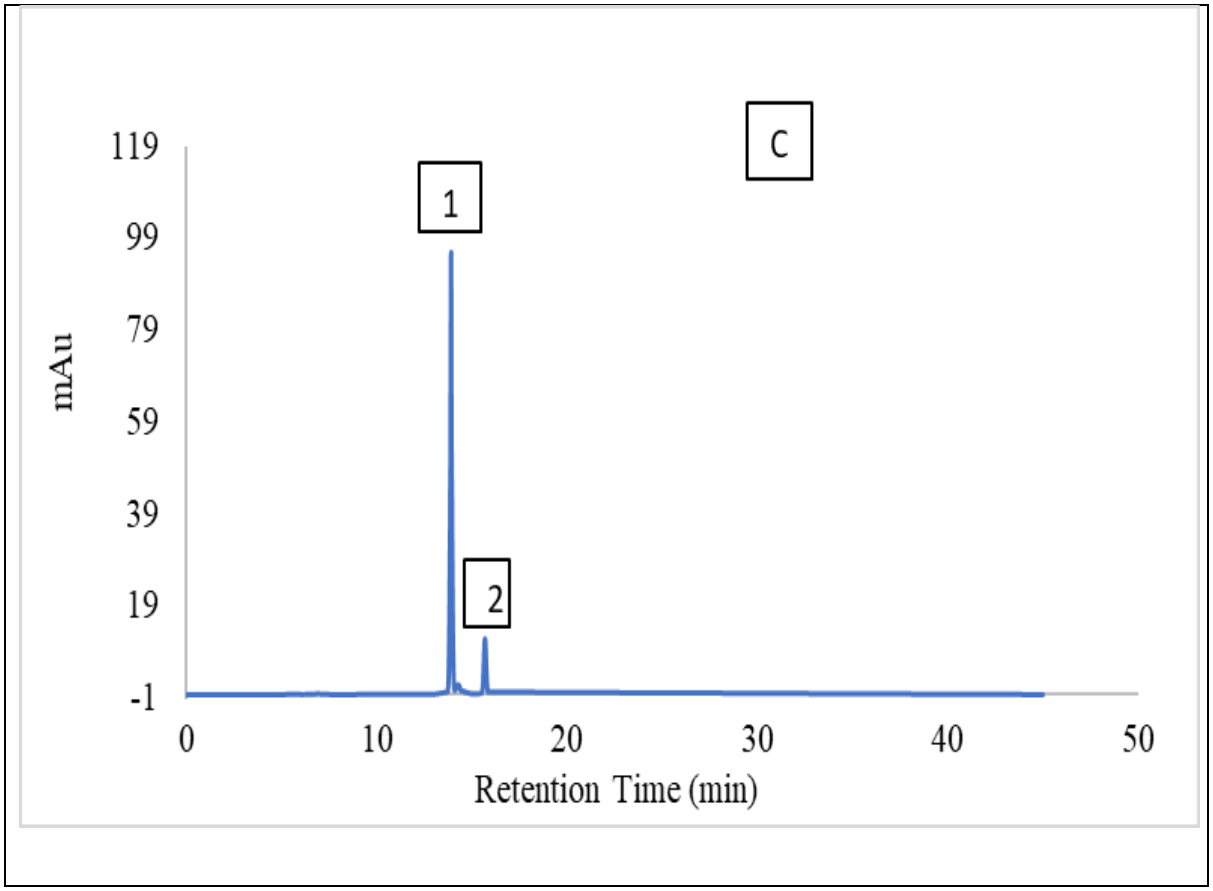

Figure 4.6: HPLC Chromatogram at 538 nm for betanin (1) and iso-betanin (2) (betacyanin and iso-betacyanin) in (A) Standard; (B) Ethanolic extract of optimized sample; (C) Citric acid extract of optimized sample.

Chapter 5

Modelling Extraction Kinetics of Betalains From Freeze Dried Beetroot Powder into Aqueous Ethanol Solutions

This chapter has been published in the *Journal of Food Engineering*: Kumar, R., Oruna-Concha, M. J., Methven, L., & Niranjan, K. (2023). Modelling extraction kinetics of betalains from freeze dried beetroot powder into aqueous ethanol solutions. *Journal of Food Engineering*, *339*, 111266.

Abstract: The extraction kinetics of betalains (betacyanin and betaxanthin) from freeze dried beetroot powder into aqueous ethanol solutions is modelled by considering the concentration of a given betalain at any given time to result from a balance between the rate of its release from the solid phase and the rate of its chemical degradation in the extract phase. The mathematical model obtained shows that the concentration of the betalain peaks before progressively decreasing with time. The model was experimentally validated for various combinations of temperature (55-85 °C), ethanol concentration (10-30%) and particle size (120-300 μm). The ratio of betacyanin to betaxanthin in the liquid phase was approximately 1 over the duration of extraction at 55 and 65 °C. However, the ratio decreased at the higher temperatures of 75 and 85 °C. A maximum productivity rate of a given betalain was defined as its peak concentration divided by the time taken to reach the peak concentration, which was found to be relatively insensitive to the ethanol concentration below 75 °C.

Keywords: Beetroot; Betalain; Betaxanthin; Betacyanin; Extraction; Modelling

Nomenclature

A	Pre-exponential factor, Equation 5.8, s^{-1},	L	Path length of cuvette, cm
A_{480}	Absorbance measured at 480 nm	MW	Molecular weight, g mol^{-1}
A_{538}	Absorbance measured at 538 nm	n	Number of observations for each experiment
A_{650}	Absorbance measured at 650 nm	p	Total number of predicted results from model
Adj-R^2	Adjusted coefficient of determination,	**P**	Betalain productivity rate, kg m^{-3} s^{-1}
BC	Betacyanin	R^2	Coefficient of determination
BX	Betaxanthin	**RMSE**	Root mean squared error
C_L	Concentration of betalain in the extract, kg m^{-3}	R	Universal gas constant, 8.314 $J\ mol^{-1}\ K^{-1}$
$(C_L)max$	The maximum or peak concentration of the extracted betalain, kg m^{-3}	RSS	Residual sum of square
C_s	Betalain concentration in the solid phase at any time, kg betalain (kg dry solid)$^{-1}$	S	Solid loading, kg dry powder m^{-3} extract
C_{si}	Initial concentration of betalain that is extractable, kg m^{-3}.	SSE	Sum of squared error
DF	Dilution Factor, Equation 5.7	t	Time, s
T	Extraction Temperature, K	t^*	The time when C_L peaks, s
E_a	Activation Energy, Equation 5.8, J mol^{-1}	TSS	Total sum of square
E	molar extinction co-efficient, Equation 5.7, L mol^{-1} cm^{-1}	y_{exp}	Experimental results
k	First order rate constant for betalain degradation, s^{-1}	y_{model}	Predicted results from model
k_m	First order rate constant for exhaustion of the given betalain from the solid phase, s^{-1}		

5.1 Introduction

Beetroot (*Beta vulgaris L.*) is an herbaceous blooming biennial plant, native to Asia and Europe, that belongs to the Chenopodiaceae family and grown across seasons (Nirmal et al., 2021). It is widely consumed as a salad, as a juice, after pickling and as a cooked vegetable. It is known to contain high levels of nutritionally beneficial and bioactive compounds, such as nitrate, phenolics, and ascorbic acid, as well as vitamins, minerals, carbohydrates, fibre, protein, essential amino acids, fatty acids, phytosterols, alkaloids, steroids, carotenoids, and a significant level of pigments called betalains soluble in polar solvents (Fernandez et al., 2017a).

Industrial scale primary production, processing, packaging, retail market and household consumption of beetroot leads to a wastage of more than 30-50% across the world (Nirmal et al., 2021). One predominant approach to valorising beetroot waste is to extract the betalain pigments (Celli and Brooks, 2017). Betalains mainly consist of two nitrogenous components betacyanin (BC) and betaxanthins (BX). These two nitrogeneous compounds are of significant importance to food, pharmaceuticals, cosmetics and dye industries, where it is also known as "beetroot red" (Stintzing et al., 2003). Natural extracts from beetroot are also good for replacing synthetic colours in products such as confectionery and bakery, ice creams, yoghurts, and sweets (Azeredo, 2009a). However, the application of betalains, especially betaxanthin is limited due to limited production (Nestora et al., 2016). The stability of this colorant is pH and temperature dependent, and its application in high temperature processed products is limited. However, the ready availability of beetroot and low price seem to be driving forces for large scale applications of betalains in the food industry.

There have been many published reports on the extraction and analysis of betalains from beetroot. In addition, extraction, degradation, and stability of betalains from sources other than beetroot has been studied; (Merin et al., 1987) studied the stability of betacyanin as color

extracted from prickly pear fruit and it was observed to be highly sensitive to temperature. (Wong and Siow, 2015) investigated the effect of heat, pH, antioxidant, agitation and light on betacyanin present in red-fleshed dragon fruit (*Hylocereus polyrhizus*) juice and concentrate. On the other hand, the stability of betalain pigments has been studied in a variety of food matrices such as milk, gummies, and beverages (Bassama et al., 2021). In general, these studies concluded that the stability of betalains during any processing such as extraction, storage, and thermal treatment was dependent on several factors including; genotype, part of the plant used, concentration of betalains, solvent employed and its pH, temperature, and water activity (Rodríguez-Sánchez et al., 2017). However, reports of the kinetics of extraction and degradation from beetroot are scarce. (Saguy, 1979) studied the thermostability of betanine (Betacyanin) and vulgaxanthin- I (betaxanthin) in blended beet juice at 61.5, 75.5 and 85.5 °C and pH range 4.8-6.2, and modelled the results using first order kinetics. The two pigments were found to exhibit maximum stability at pH of 5.8 , and betanine was found to be more stable than vulgaxanthin-I (Saguy, 1979). (Silva et al., 2020a) reported the extraction kinetics of the betalains from dried beetroot powder using Fick's first law of diffusion, and noted that higher temperature and exposure time had a negative effect on the extraction rates. The temperature of extraction is critical in this process because of the location of the betalains within the beetroot tissue architecture. The betalains are mainly present inside vacuoles of the tissue, and the membrane protecting the vacuoles has to be broken to release the betalain (Nutter et al., 2021). A thermal shock is needed to rupture the membrane. Thus, a high temperature is needed in the process, however, the use of high temperature is also detrimental to the stability of the betalain released.

It is clear from a review of literature that there are only isolated studies providing insights into the extraction of betalains and their subsequent stability, but no systematic study which explores the kinetics of extraction in specified solvents and links it to the operational

parameters such as the temperature. Since betalains are soluble in polar solvents, water and other aqueous solutions are effective media for extraction. Ethanolic solutions are usually preferred to water due to relatively lower extraction efficiency and stability of betalains (Roriz et al., 2017). The main objective of this paper is to develop a mechanistic model for the extraction of betalains from freeze dried beetroot into ethanolic solutions which takes into account: 1) the mechanisms operating during interfacial mass transfer of the betalains into a solvent phase and 2) the post-extraction chemical degradation in the solvent. The paper then reports extensive experimental data which validates the model, and discusses the effects of a range of operating parameters such as temperature, ethanol concentration and the particle size on extraction kinetics.

5.2 Modelling the transient concentration of betalains in the extract

If C_L (kg m^{-3}) is the concentration of a given betalain in the extract (betacyanin or betaxanthin) at any time, then the net rate of change of this concentration is given by the balance between: i) the rate at which the betalain is released from the solid phase and ii) the rate at which the betalain decomposes in the extract phase. The former rate depends on the intraparticle mass transfer characteristics, the liquid film mass transfer coefficient around the particles as well as the partition characteristics between the solid and extract phases. In addition, there could be other factors influencing the rate of release such as the location of betalain and the nature of its affinity within the particle phase cellular architecture. Thus, a detailed quantitative description of all such factors and how they influence the rate of release is expected to be complicated. However, as a simplified empirical description, it would be reasonable to assume that, at a given temperature, the rate of release of betalains is first order with respect to the concentration of betalain in the solid phase C_S (mg betalain per g dry matter). A key justification for this assumption is earlier well-documented experimental observations: e.g. in the case of sugars

(Appiah-Nkansah et al., 2019), pectins (Leach et al, 1995) and total phenolic content (Bengardino et al., 2019), where the solid-phase concentrations have been reported to vary in this manner. Thus, if C_{si} is the mean initial concentration of betalain that is extractable into a liquid medium, the mean extractable concentration at any time (C_s) will be given by:

$$C_S = C_{Si}e^{-k_m t} \tag{5.1}$$

where k_m (s^{-1}) is the first order rate constant for exhaustion of the given betalain from the solid phase. If S is the solid loading (g dry matter in the solid per m^3 of extraction medium), the rate at which the liquid phase gains betalain per unit volume is given by:

$$-S\frac{dC_S}{dt} = SC_{Si}k_m e^{-k_m t}. \tag{5.2}$$

It is necessary to note that C_{Si} is the initial mean concentration of betalain that is extractable into a given extraction medium, and not the initial solid phase concentration *per se*. Its value will depend on the nature of the solid phase, the chemical nature of the extraction medium as well as the temperature of extraction. C_{Si} is therefore a model parameter which must be experimentally determined. Likewise, the exhaustion rate constant k_m is not the mass transfer coefficient because it is not based on a driving force, but a mere rate constant. It may include within it, a measure of the solid phase resistance and the liquid film resistance to the transfer of the solutes.

At a given temperature, the rate at which the betalain degrades in the extraction medium can also be assumed to be a first order with respect to its concentration in the liquid phase i.e., kC_L where k is the rate constant for betalain degradation. Therefore, the net rate of change of a betalain concentration in the liquid phase is given by:

$$\frac{dC_L}{dt} = SC_{Si}k_m e^{-k_m t} - kC_L \tag{5.3}$$

which can be analytically solved with the initial condition: C_L=0 at t=0, to give:

$$C_L = \frac{k_m S C_{si}}{(k-k_m)}\left[e^{-k_m t} - e^{-kt}\right] \quad (5.4)$$

Experimentally determined C_L versus t data for a range of different conditions (described in the materials and methods section), will be fitted to equation (5.4) to validate the model, as well as determine the best-fitting values of the parameters k_m, k and C_{Si}.

Given the inflow of the betalain into the extract phase from the solid phase and its inherent decomposition in this phase, C_L goes through a maximum, and the time when the maximum value occurs, t^*, can be determined by differentiating equation (5.4) and setting $\frac{dC_L}{dt} = 0$,

whence:

$$t^* = \frac{1}{(k-k_m)} \ln\left(\frac{k}{k_m}\right) \quad (5.5)$$

Therefore, the maximum concentration of the extracted component is:

$$(C_L)_{max} = \frac{k_m S C_{si}}{(k-k_m)}\left[e^{-k_m t^*} - e^{-kt^*}\right] \quad (5.6)$$

where t^* is given by equation (5.5). The maximum rate of productivity of betalain under any given set of solvent (i.e., liquid phase) and operating conditions can be approximated to $[(C_L)_{max}/t^*]$ and this value will be used to compare the productivity rate observed under different conditions of temperature and solvent composition.

5.3 Materials and methods

5.3.1 Experimental design

A random design was implemented for performing the extraction using different concentrations of ethanol in water (10, 20, and 30%) as the solvent phase. For the purpose of comparison, extraction was also undertaken using distilled water. The extraction temperatures investigated were: 55, 65, 75, and 85 °C. The ethanol concentration range employed was consistent with earlier studies (Celli and Brooks, 2017). The temperature range employed served to understand the kinetics of betalain decomposition (Bengardino et al., 2019).

All extraction experiments were carried out in triplicate. Means and standard deviations of the data were calculated for each extraction condition. Data analysis was performed using XLSTAT version 2021.1 (AddinSoft, Paris, France). Fitting of the equations to the model and determination of the model constants were performed using MATLAB 2022a Academic version (Mathworks Inc., USA); further details are given below in section 5.3.7.

5.3.2 Preparation of beetroot powder

Fresh beetroot was purchased from a local supplier in Reading, United Kingdom. The beetroot was washed, cleaned, and chopped in a food processor (Kenwood Blend-X Fresh BLP41.A0GO). It was then transferred to an aluminium tray and subjected to blast freezing at –80 °C, for 24-36 hours. It was subsequently freeze dried (VirTis SP Scientific, UK) for 70-72 hours until the moisture content dropped below 3% (dry weight basis). After freeze drying samples were ground (Kenwood Prospero AT286 KW714229 Spice Mill) and sieved to obtain different particle sizes. Most of the experiments were performed using particles of average diameter 300 µm. To study the effect of particle size on extraction kinetics different sieved fractions were used, with average particle diameter of 300 ± 12.1, 230 ± 8.6, 180 ± 5.1 and 120 ± 3.3 µm.

It may be noted that freeze drying is an expensive process, and in practice, it would be better to use beetroot in its harvested form. However, for the purpose of this research work, a starting

material was needed which had uniform and consistent betalain composition, so that the model developed could be validated over the range of operating conditions. Hence, it was decided to freeze dry the beetroot which avoided processing and storage losses of betalain and also yielded consistent initial concentration. The model developed above (section 5.2) can also be applied to extraction from beetroot in its harvested form. However, the model parameters may have to be experimentally determined.

5.3.3 Chemicals and reagents

Analytical grade ethanol, citric acid, sodium phosphate dibasic, and betanin standard were purchased from Merck Chemicals Limited (UK).

5.3.4 Extraction of betalains in aqueous ethanol solutions

All experiments were performed by contacting the solid and liquid phases in closed beakers and agitating these in a hot water shaking-bath operating at a frequency of 1.6 Hz. For each time point, a separate extraction was performed to determine the extract concentration. Arbitrarily 22 time points were selected so that sufficient concentration versus time data points could be obtained to fit the model. Each of these 22 extractions were performed in triplicate in order to determine the mean and standard deviation for each time point. Each extraction batch was prepared by adding 1 g of dehydrated beetroot powder to 100 ml of the solvent phase which was already pre-heated to the desired extraction temperature. After the desired extraction time, the extract was collected, immediately cooled to 4 ^{o}C, and centrifuged at 14000 rpm for 40 mins at 5 ^{o}C to obtain a clear supernatant. The extract was then stored at 4 °C for further analysis.

5.3.5 Spectrophotometric measurement of betalains

Betalains were determined using the methods described in literature (Wong and Siow, 2015). McIlvaine buffer was prepared by mixing 30 mL of 0.1 M citric acid with 70 mL of 0.2 M sodium phosphate dibasic. The clear extract from the centrifuge was diluted 10 times using McIlvaine buffer before the spectrophotometer measurement. The concentration of betalains was spectrophotometrically determined (Cecil CE1011 Spectrophotometer). Betaxanthin (BX) absorbance was measured at wavelength of 480 nm (A_{480}) and Betacyanin (BC) absorbance was measured at 538 nm (A_{538}). In addition, a measurement was also taken at 650 nm (A_{650}) to remove the effect of any impurities. The measurement of BX and BC at 480 nm and 538 nm, together account for more than 95% of the betalains present in beetroot (Janiszewska-Turak et al., 2021; Stintzing et al., 2002). The concentration of the betalain was determined as:

$$\text{Betalains (mg of BX or BC/litre of extract)} = \frac{A \times DF \times MW \times 1000}{E \times L} \quad (5.7)$$

where A=(A_{538}-A_{650}) for betacyanins (BC) or (A_{485}-A_{650}) for betaxanthins (BX); DF=dilution factor; MW (Molecular Weight) = 550 g/mol for betacyanin and 339 g/mol for betaxanthin; E=molar extinction co-efficient in L $mol^{-1}cm^{-1}$, and the values for betacyanins and betaxanthins are 60,000 and 48,000, respectively; L= path length of quartz cuvette in cm.

5.3.6 Activation energy (E_a) calculation for solid phase exhaustion and degradation of betalains

The rate constants k_m and k, obtained at any given temperature by fitting the experimentally obtained C_L *versus* t data to equation (5.2), are temperature dependent. An Arrhenius type equation was used to correlate the variation of the rate constants with temperature (equation 5.6 below), which involved plotting ln(k_m) and ln (k) separately against 1/T, where T is the extraction temperature in K, and determining the gradient and intercept of the best fitting line to yield activation energy E_a (J mol^{-1}) and pre-exponential factor, A (s^{-1}) (Zin and Bánvölgyi, 2021).

$$\ln(k)\ or\ \ln(km) = \ln(A) - \frac{E_a}{RT} \tag{5.8}$$

5.3.7 Estimation of model parameters and goodness of fit

Experimentally determined C_L versus t data for a range of different conditions was fitted to equation (5.4) to validate the model, as well as determine the best-fitting values of the parameters k_m, k and C_{Si}. A regular curve fitting tool from the toolbox of MATLAB 2020b was used (Mathworks Inc., USA). The curve fitting tool works on the principle of reducing the sum squared error (SSE) and minimizing the root of mean squared error (RMSE) and requires a reasonable initial guess for k_m, k and C_{si} to obtain their best fit values.

MATLAB 2020b uses Levenberg–Marquardt (LM) estimation algorithm with 95% confidence interval. This is the most significant method used in high accuracy software packages for model parameter optimization. The Levenberg-Marquardt algorithm is an iterative technique that locates the minima of error function and optimizes the model parameters. It is a standard technique for nonlinear least-squares problems and can be thought of as a combination of steepest descent and the Gauss-Newton methods.

The best-fit values of the three model parameters were based on 22×3 data points for each experimental condition. The 95% confidence interval for each model parameter was estimated to locate the parameter values precisely and obtain a unique set of values. The narrow range of joint confidence interval obtained (see Tables 5.1(a) and 5.1(b)) established the precision in estimating the parameters, and also reinforces the adequacy of the number of experimental data points used in the fitting exercise.

SSE and RMSE indicate model validity and goodness of fit between the experimental data and the proposed model. Further, the co-efficient of correlation, R^2 and adjusted R^2 were

determined to indicate whether an adequate number of parameters have been used for fitting the model to the experimental data.

$$\text{Sum of squarred error (SSE)} = \sum(y_{exp} - y_{model})^2 \quad (5.9)$$

$$\text{Root mean squarred error (RMSE)} = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(y_{exp} - y_{model})^2} \quad (5.10)$$

$$\text{Coefficient of determination } (R^2) = 1 - \frac{RSS}{TSS} \quad (5.11)$$

$$\text{Adjusted coefficient of determination } (Adj - R^2) = 1 - \frac{(1-R^2)(n-1)}{(n-p-1)} \quad (5.12)$$

where, n=number of observations for each experiment; y_{exp} – Experimental results; Y_{model} – Predicted results from model; *RSS* – Residual sum of square; *TSS* – Total sum of square; *p*-total number of predicted results from model.

5.4 Results and discussion

5.4.1 Validation of model

The transient variation in the extract or liquid phase concentration of betacyanin and betaxanthin is given by equation (5.4). Experimental data on the concentrations of betacyanin and betaxanthin, extracted from dehydrated beetroot powder at different temperatures (55, 65, 75 and 85 °C), into aqueous ethanol solutions (concentration: 10, 20, and 30%), were fitted to equation (5.4) to test the validity of the model. Figures 5.1 (a) and 5.1 (b) demonstrate the model fit with the experimental data at four specific temperatures; the best fitting values of the model parameters are also stated in the caption. Tables 5.1 (a) and 5.1 (b) confirms the validation of equation (5.4) for all the experimental conditions investigated in this study. The best-fit model parameters as well as statistical parameters indicating the goodness of fit are also given in Tables 5.1 (a) and 5.1 (b) for each set of experimental conditions. Even though

the high R^2 value illustrates a good fit between model and experimental data, other fitness parameters such as sum of squared error (SSE), root mean squared error (RMSE), and Adjusted R^2 were also calculated (Equations 5.7, 5.8 and 5.10). The distinctly evident lower values of error functions and higher values of determination coefficients (Tables 5.1 (a) and 5.1 (b)) enhances model validity.

It is clear from Figure 5.1 that the concentration of the extracted component rises sharply at the start of the extraction, goes through a maximum value, and then gradually decreases with time. This is consistent with the model, which hypothesises that the concentration of the betalain at any time in the liquid phase is given by a balance between the rates of inflow from the solid phase and the rate of chemical degradation of the component. It is also clear from the caption of Figure 5.1 that the first order rate constant for solid exhaustion of a given betalain, i.e., k_m, is orders of magnitude greater than the rate constant for its chemical degradation. This indicates that the components are readily released from the solid phase but its subsequent degradation in the liquid phase is relatively slower. The fact that the betalain concentration peaks soon after commencement of the extraction indicates that short-time extraction is preferable to longer times. If this extraction is to be carried out continuously, then a reactor with tubular configuration will be effective in controlling residence times at such low values. Sivakumar et al. (2009) studied the extraction of beetroot coloring matter (combined betacyanin and betaxanthin) from fresh beetroot into 50% ethanol solution at 45 °C, collecting extract samples for analysis every 30 mins for 3 h. They found that the combined concentration of betalains increased progressively before attaining a uniform value. Bengardino et al. (2019) studied the extraction kinetics of betacyanin and betaxanthin separately from dehydrated beetroot leaves which was cut into an average size of 1 mm^2 at 30 and 80 °C, over a period of 24 hours. The betalain extraction profile was similar to the one reported in this study at 80 °C, except that the maximum concentration was observed after around 10 minutes which is considerably longer

than the time observed in this study, which is due to the significantly larger particle size employed. In another study extraction from dehydrated beet leaves a similar concentration profile was observed for betaxanthin, but the extraction was assisted by ultrasound and the temperature was not precisely controlled (Nutter et al., 2021).

Unlike earlier studies, the present study reports for the first time the transient concentration profiles of betaxanthin as well as betacyanin when extracted from beetroot powders into ethanolic solutions. Moreover, a model is also proposed and experimentally validated for the concentration profile observed.

5.4.2 Values of the model parameters k, k_m, and C_{si}

As mentioned in section 5.2, the value of k_m represents the rate constant for solid phase exhaustion of betalains and k represents the degradation rate constant. It is evident from Tables 5.1 (a) and 5.1 (b) that k_m is significantly greater than k for betacyanin as well as betaxanthin. A possible explanation for the rapid exhaustion of betalains from solid or particulate phase is the short diffusion path length resulting from the use of relatively small particle sizes (Alsaud and Farid, 2020). The betalains are mainly present in vacuoles of the beetroot cellular structure (Nutter et al., 2021). The protecting membranes of the vacuoles can be easily broken by heat in the presence of the solvent to release the betalains (Nutter et al., 2021). It is also possible that the freezing and freeze drying may have altered the cellular architecture to facilitate betalains release. The rapid release of betalains from particle have been reported in number of earlier studies (Silva et al., 2020a). The temperature dependence of k_m value for a given particulate phase and solvent can be expressed by Arrhenius model; the constants of the model for different particulate and solvent combinations is given in Table 5.2.

Betalains are thermolabile compound and their stability is known to decline considerably between 50-80 °C (Herbach et al., 2006). Betacyanin degrades by decarboxylation and

dehydrogenation to produce stable yellow colorants known as neo-betacyanins (Herbach et al., 2006). Betaxanthin degrades by hydrolysis and isomerisation (Herbach et al., 2006). The *k* value for betacyanin and betaxanthin degradation are close to the rate constant values reported at 50 °C by (Rodríguez-Sánchez et al., 2017) . It is also evident from Table 5.1 that the *k* values for betacyanin increased more sharply with temperature than betaxanthin, irrespective of the ethanol concentration. The higher sensitivity to thermal degradation of betacyanin has also been reported by two previous studies (Herbach et al., 2006).

C_{si} as mentioned earlier in section 5.2, maybe considered to indicate the amount of betalain extractable under a given set of operating conditions. From Tables 5.1 (a) and 5.1 (b) it is evident that concentration of extractable betalains (betacyanin and betaxanthin) is influenced by solvent and temperature. In a given solvent the value of C_{si} was greater at 65 °C than 75 °C but its value at 85 °C was lowest. One possible reason for this observation is the thermal degradation of betalain in particulate phase itself due to the higher temperature.

5.4.3 Effect of Ethanol concentration on extraction kinetics and model parameters

From Tables 5.1 (a) and 5.1 (b), it can be observed that ethanol does not only plays an important role in the extraction of betalains but it also controls the maximum extractable betalains (C_{si}). It was observed that for both betacyanin and betaxanthin C_{si} values were higher in the case of 20% solution, than in 10 and 30%. The highest C_{si} value for the betacyanin and betaxanthin was observed at 65 °C in 20% ethanol solution, and the values for betacyanin and betaxanthin were 0.0044 and 0.0049 kg/kg of dried beetroot powder, respectively. At the same temperature, C_{si} value for extraction in pure water were determined as being 0.0037 kg/kg for betacyanin and 0.0035 kg/kg for betaxanthin; these values are significantly ($p<0.05$) lower than the corresponding values for ethanol solutions. In addition, the solid phase exhaustion rate constant for pure water was also lower than for the ethanol solutions. The rate constants for degradation

of betacyanins in water are similar to the constants for ethanol solutions. Thus, we can reinforce the conclusion that ethanol solutions act as better solvents than pure water for betalains (Roriz et al., 2017). As mentioned earlier, ln(k) and ln(k_m), for each solvent, can be correlated with temperature by employing Arrhenius type of equation. For the range of temperatures employed in this study, the activation energy E_a varied with ethanol concentration, and the relevant values are illustrated in Figure 5.2. The activation energy for k_m increased sharply with ethanol percentage for both betacyanin and betaxanthin. On the other hand, the activation energy for k was observed to be lower for 20% ethanol solution than for 10% and 30% solutions. It is therefore clear that the use of a 20% ethanol solution as the extraction medium not only facilitates mass transfer from the particulate phase, but also results in lesser post-extraction degradation. Literature reports on the activation energy for betalain extraction are scarce, but the values are extensively reported for other solutes such as polyphenols. Balyan and Sarkar (2017) reported an activation energy for polyphenols from extraction jamun seeds in the range of 5.45-12.1 kJ/mol for the temperature range of 34.8-85.2 °C. Hobbi et al. (2021) reported a value of 12.4 kJ/mol for the extraction of polyphenols from apple pomace in the temperature range 40-85 °C. The values of activation energy for betaxanthin and betacyanin shown in Figure 5.2 are consistent with the values reported in literature. For example, Güneşer (2016) reported E_a value of 42.449 kJ/mol for betalain degradation from beetroot extracted into milk. Rodríguez-Sánchez et al. (2017) reported E_a values of 66.25 kJ/mol for the degradation of betaxanthin extracted from *S. pruinosus*. Kayın et al. (2019) reported E_a values of 66.13 and 92.04 kJ/mol for the degradation of betacyanin and betaxanthin, respectively, in red beet juice, which are consistent with the values given in Figure 5.2(b).

5.4.4 Variation of the ratio of betalains (betacyanin and betaxanthin) with time in the extract phase

It is interesting to note from Figures 5.1 (a) and (b) that the extraction profile for betaxanthin mirrors that for betacyanin, with the solid exhaustion rate constant values (i.e., k_m) being similar. However, the rate constant for betaxanthin degradation is somewhat lower than that for betacyanin. In other words, the betacyanin released by the solid is expected to suffer greater levels of degradation over time. Figure 5.3 shows the variation of the ratio of the concentrations of betacyanin to betaxanthin with time at four different temperatures. At 55 and 65 °C, the ratio does not vary significantly with time ($p<0.05$). However, at the higher temperatures of 75 and 85 °C, a linear decreasing trend is observed that is consistent with the fact that betacyanin degrades much faster, especially at higher temperatures. The ratio of the concentrations of betacyanin to betaxanthin is important from the point of view of extract composition. Even though the concentration of betacyanin is greater than betaxanthin in beetroot powder (Fernandez et al., 2017), this ratio is maintained at the lower temperatures of 55 and 65 °C, and for shorter extraction times at the higher temperatures of 75 and 85 °C (Figure 5.3).

5.4.5 Effect of particle size on the extraction kinetics and model parameters

Effect of particle size was investigated by separating four sieve fractions in the range of 120-300 µm to give average particle sizes of 300 ± 12.1, 230 ± 8.6, 180 ± 5.1, and 120 ± 3.3 µm. The model parameters as a function of mean particle size are given in Table 5.3. The values of C_{si} and the rate constant for betalain degradation k are not expected to vary significantly with particle size. Table 5.3 confirms this fact for betacyanin and betaxanthin, except that the value of k for 300 µm particle size is somewhat higher. Although a specific reason for this observation has not been identified, a similar observation has been made by (Alsaud and Farid, 2020) who noted that the bioactive degradation rate is higher in the case of very fine particles (<200 µm).

The critical parameter influenced by the particle size is the rate constant for solid exhaustion (k_m). As mentioned in section 5.4.1, the value of this parameter is strongly influenced by the

solute diffusion path length, which drops sharply with particle size. Thus, k_m is expected to increase with a decrease in particle size, which is observed in Table 5.3. However, the data for the smallest mean particle size, i.e., 120 µm, shows an anomaly. The value of K_m for this particle size is lower than the value for the next higher mean particle size, i.e., 180 µm. In other words, the solute transfer rate from 180 µm particles is faster than the transfer from 120 µm. This observation is not uncommon when very fine particle sizes are employed. Such fine particles tend to increase the effective suspension viscosity of the liquid phase and slow down mass transfer. Similar observations have been reported earlier (Asai et al., 1988). To conclude, it is worth noting that smaller particle sizes lower diffusion path length and increase rates of mass transfer from the particulate phase; however, when the particle size falls below a critical value, the particle hold-up for a given solid loading becomes very high and tends to increase the suspension viscosity which adversely affects mass transfer rates.

5.4.6 Maximum productivity rate of betalains extraction into the liquid phase

As mentioned in section 5.2, the concentration of a given betalain peaks at a given time (t*) and a betalain productivity rate, P, can be evaluated as the maximum concentration of the betalain divided by the time taken to reach this maximum value. Figure 5.4 plots the productivity rate of betacyanin and betaxanthin against temperature for different ethanol concentrations. It is interesting to note that similar productivity values can be achieved in all ethanol solutions at 55, 65 and 75 °C. However, at 85 °C, the betalain productivity rate in 20 and 30 % ethanol solutions are significantly higher than the values at other temperatures, with the value in 20% ethanol solution being higher than in 30% solution. This graph shows that similar betalain productivity values can be achieved at different temperatures and ethanol concentrations. In fact, a very high productivity can also be achieved at a temperature as high as 85 °C, provided the residence time can be accurately controlled. In practice, achieving

precise control of residence times is challenging and deviations from t* will result in betalain degradation due to the high temperature.

5.5 Conclusions

Based on the results obtained in this study and discussed above, the following conclusions can be derived.

1. A three-parameter model representing the balance between; 1) the rate of betalain inflow into a solvent phase and 2) the rate of betalain degradation in the solvent by a first order reaction, has been developed. The three model parameters are solid phase exhaustion rate constant (k_m), the first order betalain degradation constant (k) and the concentration of extractable betalain in the solid phase (C_{si})
2. The model has been experimentally validated for the extraction of betacyanin and betaxanthin into 10, 20 and 30% ethanol solutions at 55, 65, 75 and 85 °C.
3. The rate constants for solid phase exhaustion (k_m) and liquid phase degradation of betaxanthin and betacyanin (k) were correlated with temperature by an Arrhenius type equation.
4. The ratio of betacyanin to betaxanthin in the extract phase did not vary with extraction time significantly at 55 and 65 °C, but it decreased with time at higher temperatures due to the more thermolabile nature of betacyanin.
5. The rate constant for solid phase exhaustion (k_m) increased with decrease in particle size for a given solid loading, except for the smallest particle size i.e., 120 µm, where k_m was lower, probably due to increase in suspension viscosity.
6. The betalain productivity rate at t* - the time when the concentration peaks - did not vary significantly at temperatures of 55, 65 and 75 °C in all ethanol solutions studied. However, at 85 °C, the productivity value in 20 and 30% ethanol solutions was

significantly higher. Therefore, similar betalain productivity values can be achieved at different temperatures and ethanol concentrations provided the residence time for extraction can be precisely controlled.

7. It is clear that betalains are thermolabile; and high temperature processes, no doubt, pose a risk. But these processes are not beyond the realm of possibility. For example, UHT processes work very satisfactorily even at significantly higher temperatures and at shorter residence times than the values reported in this study. Thus, Food Engineering Operations are well-tuned to deal with such time-temperature combinations.

5.6 References

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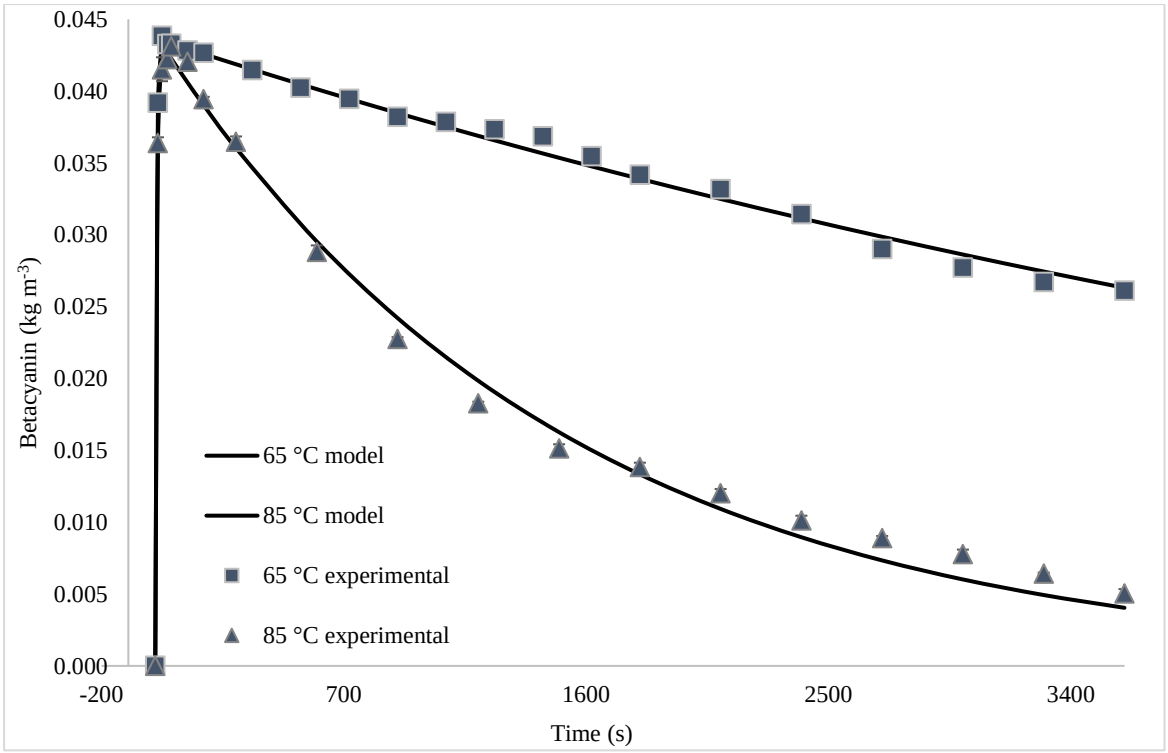
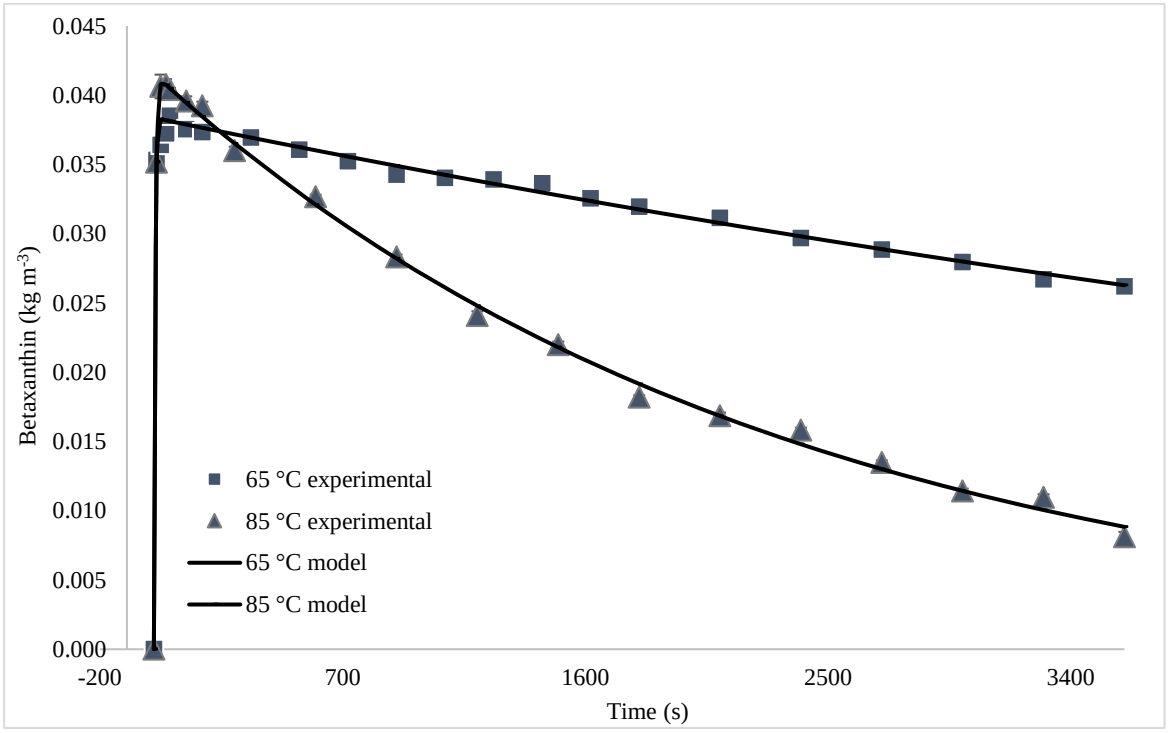


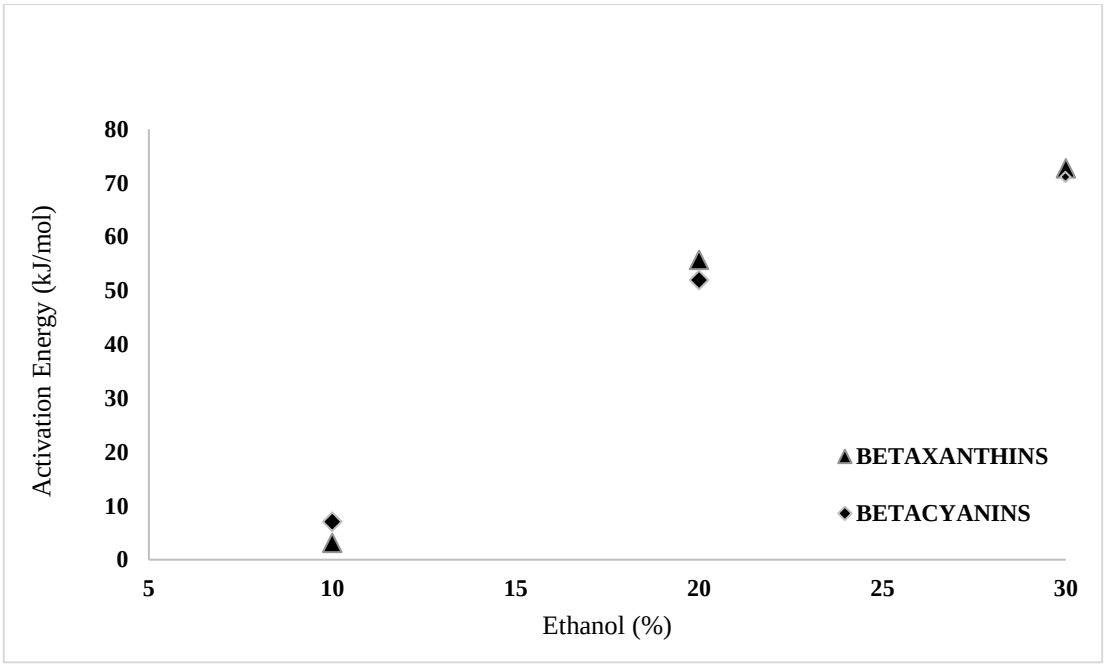
(a)



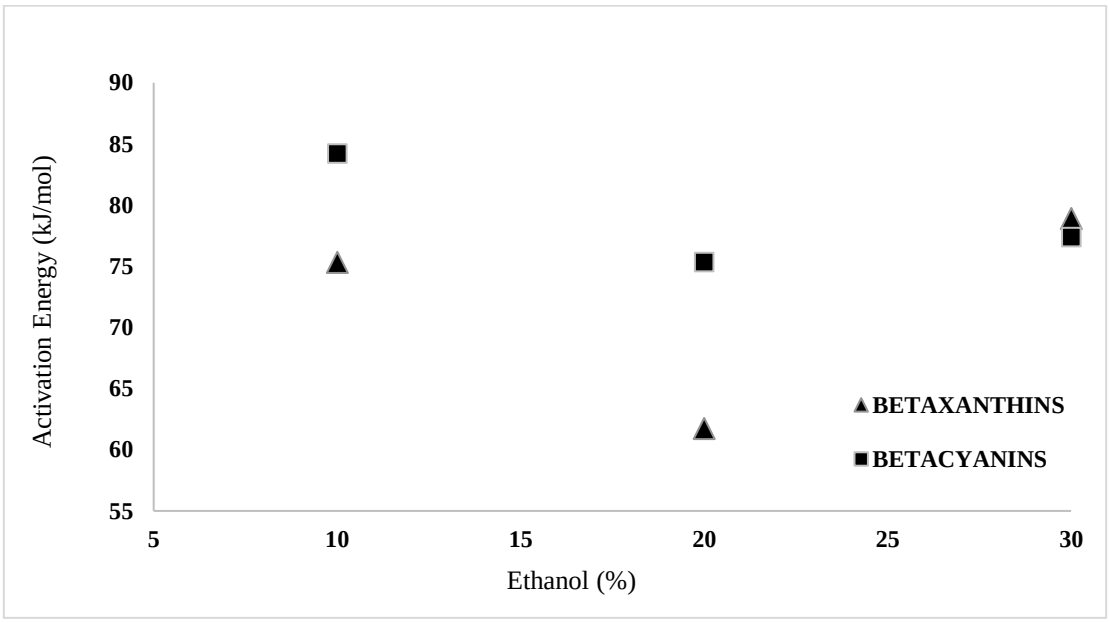
(b)

Figure 5.1: Extraction of betalains from beet root powder at 65 and 85 °C into 10% ethanol water solution (a) Betacyanin concentration, (b) Betaxanthin concentration. Solid loading

=20 kg/m^3, Particle size – 300±12.1 µm. Values of model parameters for betaxanthin at (i) 65 °C: k = 1.411 × 10^{-4} s^{-1}, K_m= 0.275 s^{-1}, C_{si} = 0.0042 kg/kg, R^2 = 0.996, and at (ii) 85 °C: k = 4.29 × 10^{-4} s^{-1}, K_m= 0.290 s^{-1}, C_{si} = 0.0041 kg/kg, R^2 = 0.954; Values of model parameters for betacyanin (i) 65 °C: k = 1.052 × 10^{-4} s^{-1}, K_m= 0.271 s^{-1}, C_{si} = 0.0038 kg/kg, R^2 = 0.997, and at (ii) 85 °C: k = 6.64 × 10^{-4} s^{-1}, K_m= 0.297 s^{-1}, C_{si} = 0.0040 kg/kg, R^2 = 0.988. The points indicate experimental values of the concentration and the solid line represents the model, i.e., concentration given by equation 5.4. Values of the model parameters for the other temperature range and ethanol-water solution are shown in Tables 5.1 (a) and 5.1 (b).



(a)



(b)

Figure 5.2: Effect of ethanol concentration on (a) Activation energy for k_m; (b) Activation energy for k. The experiments were performed with particle size of 300 ± 12.1 µm and solid loading of 20 kg m^{-3}.

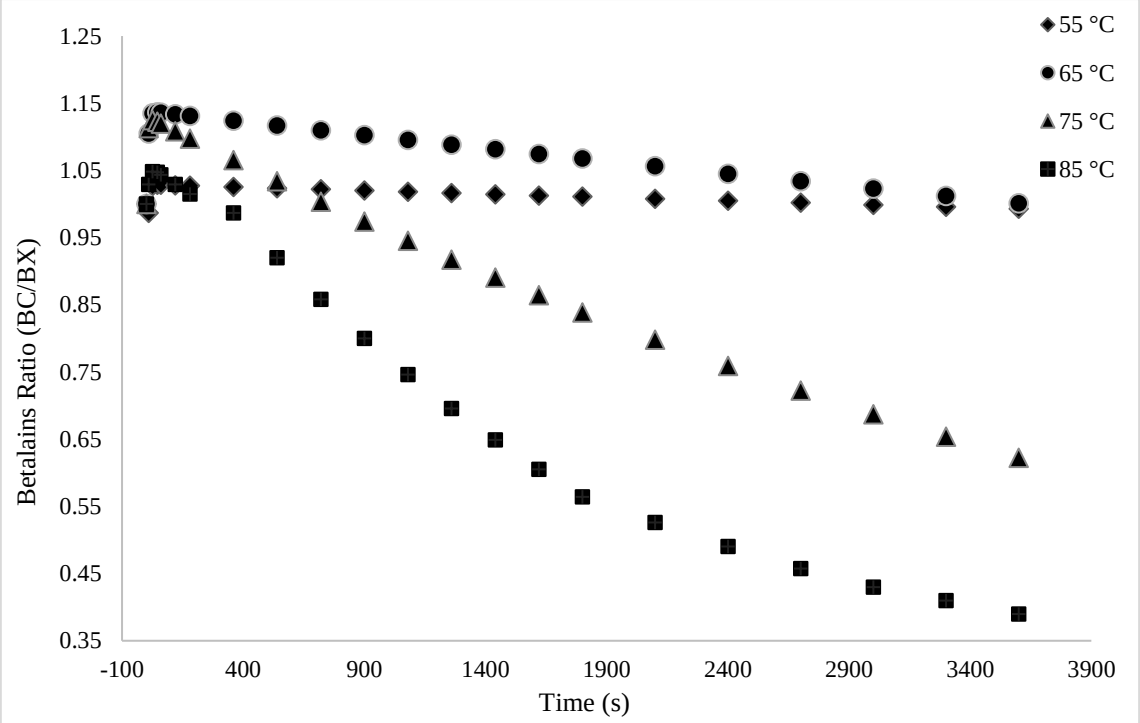
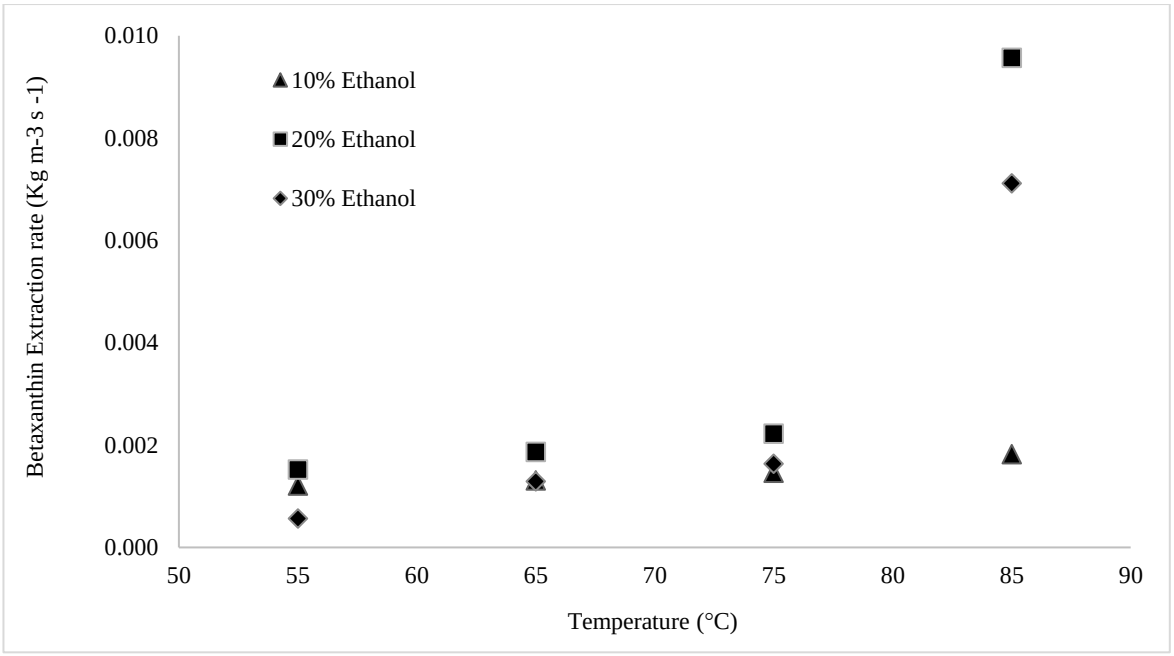
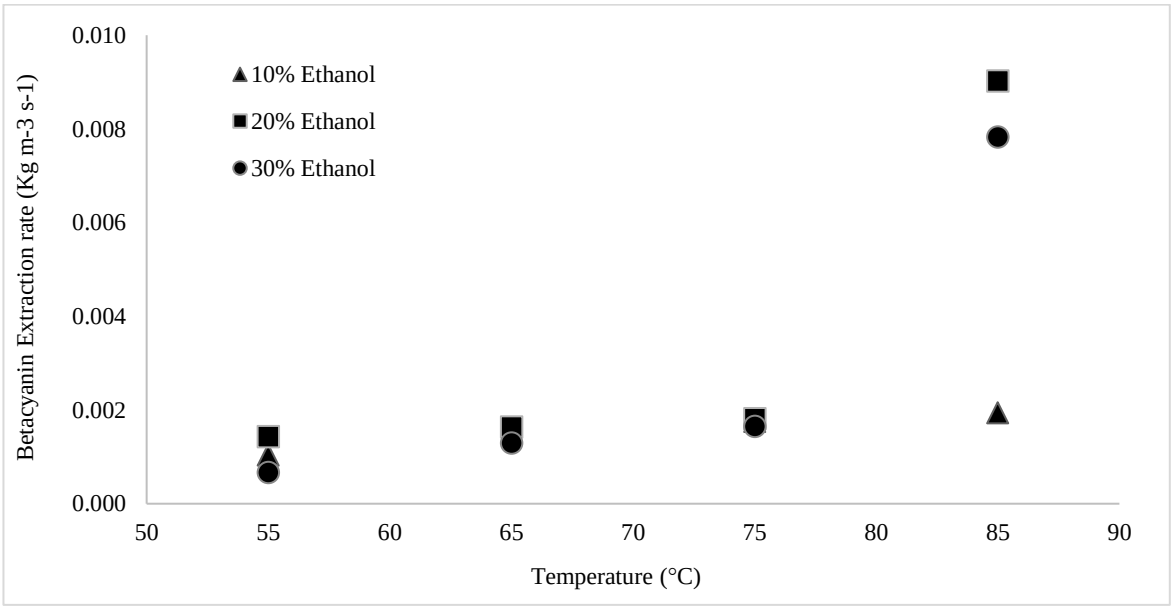


Figure 5.3: Variation of betalains ratio (i.e., concentration of betacyanin/concentration of betaxanthin) with time at 55, 65, 75, 85 °C into 10% ethanol in water. The experiments were performed with particle size of 300 ± 12.1 µm and solid loading of 20 kg m^{-3}.



(a)



(b)

Figure 5.4: Variation of betalain productivity rate with temperature for different ethanol concentrations (a) betaxanthin and (b) betacyanin. The experiments were performed with particle size of 300 ± 12.1 µm and solid loading of 20 kg m^{-3}.

Table 5.1(a): Values of model parameters fitting equation (5.4) for Betacyanin. Experiments performed with solid to liquid ratio 20 kg m^{-3} and particle size 300±12.1 μm.

Sl. No.	Temperature (°C)	Ethanol (%)	$k \times 10^{-4}$ (s^{-1})	$k \times 10^{-4}$ (s^{-1}) with 95% CI	k_m (s^{-1})	k_m (s^{-1}) with 95% CI	C_{si} (kg/kg)	C_{si} (kg/kg) with 95% CI	SSE × 10^{-5} (Eqn 5.9)	R^2 (Eqn 5.11)	Adj. R^2 (Eqn 5.12)	RMSE (Eqn 5.10)
		10	0.541	0.440-0.642	0.234	0.216-0.252	0.0037	0.0035-0.0039	1.022	0.985	0.983	0.00073
1.	55	20	0.823	0.792-0.854	0.278	0.227-0.329	0.0042	0.0041-0.0043	1.071	0.993	0.992	0.00081
		30	0.692	0.618-0.766	0.146	0.122-0.170	0.0035	0.0033-0.0037	0.166	0.875	0.859	0.00322
		10	1.411	1.011-1.811	0.275	0.245-0.305	0.0042	0.0039-0.0045	6.787	0.996	0.995	0.00065
2.	65	20	1.576	1.470-1.670	0.281	0.269-0.293	0.0044	0.0041-0.0047	5.373	0.997	0.996	0.00057
		30	1.934	1.791-2.077	0.206	0.152-0.261	0.0043	0.0042-0.0044	2.049	0.991	0.990	0.00113
		10	4.311	3.669-4.953	0.277	0.265-0.289	0.0041	0.00405-0.00415	7.842	0.997	0.996	0.00070
3.	75	20	3.913	2.927-4.899	0.285	0.278-0.292	0.0042	0.0040-0.0044	1.375	0.994	0.993	0.00092
		30	4.595	4.152-5.038	0.279	0.217-0.341	0.0039	0.0035-0.0043	1.187	0.996	0.995	0.00086
		10	6.647	4.892-8.402	0.297	0.199-0.395	0.0040	0.0038-0.0042	4.793	0.988	0.987	0.00173
4.	85	20	7.934	6.534-9.334	1.692	1.452-1.932	0.0041	0.0037-0.0045	0.244	0.958	0.953	0.00390
		30	7.238	6.167-8.309	1.544	1.348-1.741	0.0039	0.0038-0.0040	0.187	0.961	0.956	0.00342

C_{si} – Maximum extractable betalains (kg of dried betalains/kg of dried beetroot powder).

k – Degradation rate constant (s^{-1})

k_m – Solid exhaustion rate constant (s^{-1})

SSE – Sum of squared errors

R^2 – Co-efficient of determination

Adj. R^2 – Adjusted Co-efficient of determination

RMSE – Root mean squared error

CI – Confidence Interval

Eqn. – Equation

Table 5.1(b): Values of model parameters fitting equation (5.4) for Betaxanthin. Experiments performed with solid to liquid ratio 20 kg m^{-3} and particle size 300±12.1 µm.

Sl. No.	Temperature (°C)	Ethanol (%)	$k \times 10^{-4}$ (s^{-1})	$k \times 10^{-4}$ (s^{-1}) with 95% CI	k_m (s^{-1})	k_m (s^{-1}) with 95% CI	C_{si} (kg/kg)	C_{si} (kg/kg) with 95% CI	SSE × 10^{-5} (Eqn 5.9)	R^2 (Eqn 5.11)	Adj. R^2 (Eqn 5.12)	RMSE (Eqn 5.10)
		10	0.544	0.511-0.577	0.261	0.252-0.271	0.0041	0.0039-0.0045	0.725	0.995	0.994	0.00061
1.	55	20	0.823	0.684-0.962	0.297	0.273-0.321	0.0042	0.0040-0.0044	0.417	0.997	0.997	0.00051
		30	0.275	0.245-0.305	0.133	0.121-0.145	0.0037	0.0036-0.0038	0.165	0.899	0.887	0.00321
		10	1.052	0.917-1.187	0.271	0.255-0.287	0.0038	0.0035-0.0041	0.411	0.997	0.996	0.00050
2.	65	20	0.956	0.776-1.136	0.307	0.291-0.323	0.0049	0.0046-0.0052	0.469	0.997	0.996	0.00054
		30	1.038	0.901-1.175	0.214	0.156-0.272	0.0047	0.0045-0.0049	0.902	0.995	0.995	0.00075
		10	2.659	2.132-3.186	0.274	0.249-0.299	0.0037	0.0035-0.0039	1.545	0.991	0.989	0.00098
3.	75	20	2.314	1.965-2.663	0.405	0.312-0.498	0.0042	0.0039-0.0045	2.045	0.989	0.987	0.00113
		30	2.187	1.857-2.517	0.286	0.266-0.306	0.0041	0.0040-0.0042	1.943	0.991	0.989	0.00110
		10	4.291	3.845-4.737	0.290	0.265-0.315	0.0041	0.0039-0.0043	0.138	0.954	0.948	0.00294
4.	85	20	5.094	4.458-5.730	1.87	1.473-2.267	0.0043	0.0040-0.0046	0.228	0.952	0.946	0.00378
		30	0.356	0.312-0.400	1.484	1.221-1.747	0.0040	0.0036-0.0044	0.203	0.947	0.941	0.00356

Table 5.2: Variation of Arrhenius parameters (Equation 5.8) with ethanol concentration for betacyanin and betaxanthin. Experiments performed with solid loading of 20 kg m^{-3} and mean particle size 300 ± 12.1 µm.

	Betaxanthin (BX)						Betacyanin (BC)					
	k_m			k			k_m			k		
Ethanol (%)	A (s^{-1})	E_a (kJ mol^{-1})	R^2	A (s^{-1})	E_a (kJ mol^{-1})	R^2	A (s^{-1})	E_a (kJ mol^{-1})	R^2	A (s^{-1})	E_a (kJ mol^{-1})	R^2
10	1.66	3.14	0.93	2.87	75.32	0.98	0.15	7.06	0.87	3.05	84.20	0.98
20	2.93	55.76	0.87	2.57	61.73	0.92	2.85	52.00	0.89	2.91	75.35	0.99
30	3.19	72.72	0.94	2.99	78.90	0.96	3.17	71.14	0.91	2.93	77.37	0.98

A – Pre-exponential factor (s^{-1})

E_a – Activation energy (kJ mol^{-1})

R^2 – Coefficient of Determination

Table 5.3(a): Effect of particle size on the values of model parameters of equation (5.4) at 65 °C and ethanol Concentration of 20% in water for betacyanin.

Average Particle size (µm)	k_m (s^{-1})	k x 10^{-3} (s^{-1})	C_{si} (kg/kg)	SSE x 10^{-5}	R^2	Adj. R^2	RMSE
300±12.1	0.28	0.161	0.0044	0.531	0.997	0.996	0.0006
230±8.6	0.627	0.255	0.0041	3.728	0.984	0.982	0.0015
180±5.1	1.758	0.265	0.0039	5.362	0.975	0.972	0.0018
120±3.3	0.522	0.256	0.0038	2.762	0.986	0.984	0.0013

C_{si} – Maximum extractable betalains (kg of dried betalains/kg of dried beetroot powder).

SSE – Sum of squared errors

k – Degradation rate constant (s^{-1})

k_m – Solid exhaustion rate constant (s^{-1})

R^2 – Co-efficient of determination

Adj. R^2 – Adjusted Co-efficient of determination

RMSE – Root mean squared error

Table 5.3(b): Effect of particle size on the values of model parameters of equation (5.4) at 65 °C and ethanol concentration of 20% in water for betaxanthin.

Average Particle size (μm)	k_m (s^{-1})	k x 10^{-3} (s^{-1})	C_{si} (kg/kg)	SSE x 10^{-5}	R^2	Adj. R^2	RMSE
300±12.1	0.307	0.095	0.0049	0.469	0.997	0.996	0.000541
230±8.6	0.474	0.146	0.0037	2.551	0.982	0.980	0.00126
180±5.1	1.411	0.145	0.0034	2.594	0.978	0.975	0.00127
120±3.3	0.412	0.156	0.0033	1.413	0.988	0.986	0.00093

Chapter 6

Elevated Temperature Extraction of Beta-carotene from Freeze Dried Carrot Powder into Sunflower Oil: Extraction Kinetics and Thermal Stability

This chapter has been published in the Journal of Food Science: Kumar, R., Oruna-Concha, M. J., Balagiannis, D. P., & Niranjan, K. (2024). Elevated temperature extraction of beta-carotene from freeze-dried carrot powder into sunflower oil: Extraction kinetics and thermal stability. *Journal of Food Science*.

Abstract: Beta-carotene, a precursor of vitamin A, can alleviate the deficiency of this vitamin prevalent worldwide. Earlier research studies have addressed the extraction of beta-carotene at relatively low temperatures (up to 70 °C) due to its perceived instability at higher temperatures, as a result of which extraction rates recorded are relatively low. This study models the net rate of beta-carotene extraction by considering both extraction and degradation kinetics. The model developed, which accounts for degradation occurring in solid and extract phases, has been experimentally validated for the extraction of beta-carotene from freeze dried carrot powder into sunflower oil over a range of temperatures 90-150 °C. This study also gives insights into the application of sunflower oil as a carrier for beta-carotene during cooking and food processing, by monitoring and modelling the thermal degradation and isomerisation of beta-carotene at temperatures up to 220 °C. The modelling of extraction kinetics shows that it is possible to achieve viable extraction rates by employing temperatures in the range (90-150 °C) for relatively short times (< 5 mins). The degradation kinetics shows that almost 75% of the beta-carotene can survive heating at 180 °C for 10 mins – indicating the possibility of using beta-carotene enriched edible oils for frying. This study also reports on the formation of three isomers of beta-carotene identified using HPLC: *trans*-, 9-*cis* and 13-*cis*. The reaction network model developed in this study was able to account for the transient variation of the concentration of all three isomers.

***Keywords*:** Beta-carotene; Extraction; Sunflower oil; Kinetics; Modelling.

Nomenclature

A	Pre-exponential factor, s^{-1},	p	Total number of predicted results from model
AIC	Akaike information criterion	R^2	Coefficient of determination
Adj-R^2	Adjusted coefficient of determination,	rpm	Revolution per minute, min^{-1}
C_0	Concentrations of beta-carotene initially, µg/ml	**RMSE**	Root mean squared error
C_L	Concentration of beta-carotene in the extract, kg m^{-3}	R	Universal gas constant, 8.314 $J\ mol^{-1}\ K^{-1}$
C_s	beta-carotene concentration in the solid phase at any time, kg betalain (kg dry solid)$^{-1}$	RSS	Residual sum of square
C_{si}	Initial concentration of beta-carotene that is extractable, kg m^{-3}.	SSE	Sum of squared error
c_t	Concentrations of beta-carotene at any time t, µg/ml	THF	Tetrahydrofuran
E_a	Activation Energy, Equation 6.6, J mol^{-1}	t	Time, s
k_1	First order rate constant for beta-carotene degradation in the solid phase, s^{-1}	t^*	The time when C_L peaks, s
K_2	First order rate constant for beta-carotene degradation in the extract phase, s^{-1}	TSS	Total sum of square

k_m	First order rate constant for exhaustion of the given beta-carotene from the solid phase, s^{-1}	T	Extraction and degradation temperature, °C
k	First order isothermal degradation rate constant for *b*-Carotene, s^{-1}	V	Volume of the solvent, m^3
$(k_2)_{exp}$	Experimentally determined first order rate constant for beta-carotene degradation in the extract phase, s^{-1}	X_{dm}	Dry matter content of the carrot powder, kg
k_{ref}	Rate constant at reference temperature (s^{-1})	y_{exp}	Experimental results
M_s	instantaneous rate of transfer of beta-carotene to the liquid phase, kg s^{-1}	y_{model}	Predicted results from model
n	Number of observations for each experiment		

6.1 Introduction

Beta-carotene is a pigment found in fruits and vegetables that can be converted to vitamin A in the body (Rodriguez-amaya, 1999; Marty and Berset, 1986). It has antioxidant properties that can also protect against damage from harmful molecules (Elik et al., 2020). Consuming foods high in beta-carotene is reported to have health benefits, such as reducing the risk of certain types of cancer, improving immune function, and protecting against cardiovascular disease (Gul *et al.*, 2015). Beta-carotene is widely used as a colouring agent and a natural preservative in the food industry (Yilmaz *et al.*, 2017). It is also used as a natural colourant and skin conditioning agent in the cosmetics industry (Strati & Oreopoulou, 2011). Vitamin A deficiency, which can be mitigated by consuming beta-carotene, is a major public health concern worldwide, particularly in Asia and Africa. Worldwide, particularly in Asia and Africa, it is known to be one of the three most chronic deficiencies, along with zinc and iron deficiencies (Harika et al., 2017). Globally, an estimated 250 million preschool children are vitamin A deficient (Chen et al., 2021; Tang et al., 2005).

Considering the health benefits, societal impact and industrial application, extraction of beta-carotene from natural plant sources has attracted considerable attention employing methods such as microwave assisted extraction (Hiranvarachat & Devahastin, 2014), supercritical fluid extraction (M. Sun & Temelli, 2006a), ultrasound assisted extraction (Saini & Keum, 2018), pulsed electric fields (Roohinejad et al., 2014), and others. The extraction of beta-carotene from natural sources requires the use of nonpolar solvents (Hiranvarachat & Devahastin, 2014), some of which are not environmentally friendly and can also leave behind harmful residues in the extract (Elik et al., 2020). Further, beta-carotene is also sensitive to light, heat, and oxygen, and can degrade during extraction, resulting in a loss of its nutritional and functional properties (Gul *et al.*,

2015). Due to its significant bioactivity, there has been considerable interest in extracting beta-carotene into solvents that are efficient, safe, and environmentally friendly.

Vegetable oils can be effective solvents due to their low cost and abundant availability all over the world. Moreover, vegetable oils are biodegradable, non-toxic and do not leave any other harmful residues in the product. In addition, the absorption of beta-carotene in human body can be enhanced between 4-12 fold by consuming it with edible oils and fats (Hornero-Méndez & Mínguez-Mosquera, 2007). Earlier research has shown that vegetable oils can extract beta-carotene from a range of sources, including fruits, vegetables, and microorganisms, while oils can provide other health benefits such as unsaturated fatty acids and other nutrients (Chen and Meyers, 1982; Sachindra and Mahendrakar, 2005; Sun and Temelli, 2006; Elik *et al.*, 2020). The use of vegetable oils for extracting beta-carotene also provides the opportunity to use the extract directly as a food ingredient or for cooking processes such as frying.

A number of papers are available on the kinetics of beta-carotene extraction in various organic solvents (Chumnanpaisont et al., 2014; Hiranvarachat & Devahastin, 2014; Humayoun Akhtar & Bryan, 2008; Purohit & Gogate, 2015a). These papers generally report on the use of relatively low temperatures due to the nature of the solvent but more importantly due to the tendency of beta-carotene to degrade during extraction (Gul *et al.*, 2015). The common degradation pathways include oxidation, thermal, and photochemical degradation (Achir et al., 2011; Gul et al., 2015a). Photochemical degradation of beta-carotene can also lead to the formation of products, such as apocarotenoids (Mięku̇s et al., 2019). The excentric cleavage of beta-carotene produces apocarotenoids and they cannot turned into vitamin A (Caris-Veyrat et al., 2001). Isomerization of beta-carotene can form various geometric and structural isomers, such as 9-*cis*- and *13-cis* (Achir *et al.*, 2011; Gul *et al.*, 2015). They show potential bioactivity and colouring properties like *β*-

Carotene. The use of low extraction temperatures to avoid such degradation reactions inevitably results in low extraction rates being encountered and poor extraction efficiencies. Some researchers have attempted to overcome this problem by superimposing ultrasound (Purohit & Gogate, 2015a), microwaves (Hiranvarachat & Devahastin, 2014), and pulsed electric field (Roohinejad et al., 2014) which are capital intensive technologies and not easily scalable. Moreover, all these technologies are claimed to be "green" in literature without any substantive analysis of their environmental impacts.

In this paper, we hypothesize that the time-temperature conditions to be used for the extraction of beta-carotene in any appropriate solvent can be rationally deduced by modelling the kinetics of extraction. The net rate of extraction at any given temperature will be determined by a balance between 1) the rate of transfer from the solid phase into the extraction medium and 2) the rate of loss of beta-carotene due to degradation. The specific aims of this research are therefore 1) to develop for the first time a model which accounts for the transfer of beta-carotene from the solid phase as well as its degradation in solid and extract phases during extraction in sunflower oil, 2) to experimentally test the validity of the model over a range of temperatures, including high temperatures not investigated in the literature so far, and 3) to investigate the thermal stability and isomerization of beta-carotene in edible oil, particularly at high temperatures such as those encountered during frying. The last aim of the research will inform on the possibility of using beta-carotene enriched oil for cooking, which, if possible, will help considerably in alleviating vitamin A deficiency, especially in significant parts of Africa and Asia.

6.2 Modelling the extraction kinetics of beta-carotene in sunflower oil

If C_s ((kg (kg dry matter)$^{-1}$) is the average concentration of beta-carotene in the solid phase at any time *t*, the instantaneous rate at which this changes is a balance between the rate at which beta-carotene degrades in the solid phase and the rate at which the solute is transferred to the liquid phase. If M_s represents the instantaneous rate of transfer of beta-carotene to the liquid phase (kg s^{-1}), and the rate of degradation in the solid phase is assumed to be first order (Achir et al., 2010; Mba et al., 2017), i.e. proportional to the concentration of beta-carotene, we have:

$$-\frac{dC_s}{dt}X_{dm} = M_s + k_1 C_s X_{dm} \quad (6.1)$$

where X_{dm} is the dry matter content of the carrot powder and k_1 is the rate constant for beta-carotene degradation in the solid phase (s^{-1}). It is reasonable to hypothesize that C_s is an exponential function of time. This assumption is supported by previous experimental observations that have been well-documented. For instance, in the case of sugars (Appiah-Nkansah et al., 2016), pectins (Leach et al., 1994), and total phenolic content (Bengardino et al., 2019), solid-phase concentrations have been reported to exhibit this type of release kinetics. Therefore:

$$C_s = C_{si}e^{-k_M t} \quad (6.2)$$

where C_{si} is the initial average concentration of beta-carotene in the solid phase and k_M (s^{-1}) is a rate constant for solid phase exhaustion of beta-carotene. It is arguable whether C_{si} represents the initial concentration of beta-carotene *per se* in the solid phase. Experiments were conducted to determine the total mass of beta-carotene that could be extracted from freeze dried carrot powder into different solvents such as tetrahydrofuran, hexane and coconut oil. These experiments involved extraction over very long periods of time (4 h) at 25 °C and repeated extractions using fresh solvents until no more beta-carotene extraction was possible. The amount extracted into each

solvent was different. For example, the maximum amount of beta-carotene extracted into tetrahydrofuran – in which beta-carotene is known to be most soluble (Purohit & Gogate, 2015b) – was 865.68 µg g^{-1} powder. Likewise, the maximum amount extracted into hexane was 752.54 µg g^{-1} ; and that extracted into coconut oil was 722.36 µg g^{-1} This suggests that C_{si} represents the concentration of beta-carotene that is extractable into a given solvent under a given set of operating conditions. It is therefore reasonable to hypothesize that C_{si} is a model parameter which can potentially be estimated from the experimental data. By differentiating equation (6.2) and substituting the values of the derivative and C_s into equation (6.1), we get:

$$M_s = X_{dm} C_{si} e^{k_m t} (k_m - k_1) \tag{6.3}$$

which is the net rate of transfer of beta-carotene to the liquid or extract phase.

A mass balance equation for beta-carotene in the liquid phase can also be developed by assuming that the rate of change of beta-carotene concentration in the liquid phase C_L (kg m^{-3}) is the difference between the rates of transfer from the solid phase (i.e. M_s) and the rate at which beta-carotene degrades in the liquid phase. The latter can also be assumed to follow first order kinetics with a rate constant given by, say, k_2 (s^{-1}). Thus, we have:

$$V \frac{dC_L}{dt} = M_s - k_2 C_L V \tag{6.4}$$

where V is the volume of the extraction medium, in this case, the volume of sunflower oil taken (m^3). By substituting for M_s from equation (6.4), a first order ordinary differential equation is obtained which can be solved using the initial condition $C_L = 0$ at $t = 0$, to give:

$$C_L = \frac{SC_{si}(k_M - k_1)}{(k_2 - k_M)} [e^{-k_M t} - e^{-k_2 t}] \tag{6.5}$$

where, $\frac{X_{dm}}{V} = S$ represents the solid loading in the extractor (kg of carrot powder per m^3 of sunflower oil).

For the developed model the experimental conditions can be variable given the degradation kinetics of beta-carotene in solid and liquid phases during extraction. Hence, there could be two special cases for the model apart from the basic assumption of degradation of beta-carotene differently in different phases.

It is interesting to note that the rate constant for beta-carotene degradation in the solid and extract phases, i.e. k_1 and k_2, have been assumed to take different values in the model. It is known that beta-carotene degradation may be attributable to temperature (Achir et al., 2011) and oxidation (Burton et al., 2014). If beta-carotene degradation is induced by both these factors, i.e. temperature and oxidation, the values of k_1 and k_2 will be different because the oxidative environments in the solid and oil phases are different. If, on the other hand, temperature induced degradation dominates, then one expects k_1 and k_2 values to be the same since the temperatures in the solid and extract phases are not different. Thus $k_1$and k_2 can be set to equal to k in equation (6.5), to yield:

$$C_L = SC_{si}[e^{-kt} - e^{-k_M t}] \qquad (6.6)$$

In this study, k_1 and $k_2$will initially be assumed to take different values; the outcome of the analysis of experimental data will inform whether k_1 and k_2 are the same or different. Regardless, it is interesting to note that the model (i.e. equations 6.5 and 6.6) predict that the plot of C_L versus t goes through a turning point, which is a maxima, when $dC_L/dt = 0$. The time t^* at which this maximum value occurs is given by:

$$t^* = \frac{1}{(k_M - k_2)} \ln\left(\frac{k_M}{k_2}\right) \quad (6.7)$$

If the experimental conditions are such that there is no significant degradation of beta-carotene either in the solid or liquid phases, e.g. extraction at relatively low temperatures, then k_1 and k_2 can both be set equal to zero in equation 6.5, to yield:

$$C_L = SC_{si}(1 - e^{-k_M t}) \quad (6.8)$$

Thus, the plot of C_L versus t will increase monotonically before asymptotically converging to a value of $C_L = SC_{si}$.

Experimentally determined C_L versus t data for a range of different conditions (described in materials and method section), will be fitted to equation (6.5) or equation (6.6) or equation (6.8) to deduce the best-fitting values of parameters C_{si}, k_M, k_1, and k_2. Under experimental conditions resulting in beta-carotene degradation, the values of k_2 can also be directly determined at different temperatures by dissolving a known quantity in oil and monitoring its transient concentration. Thus, the experimentally determined value of k_2 can also be compared with the values indirectly deduced from the model.

6.3 Materials and methods

6.3.1 Design of experiments

Extraction of beta-carotene was performed by implementing a random design using sunflower oil as solvent phase. The extraction temperatures employed were: 90, 115, 135 and 150 °C. The use of higher extraction temperatures than those employed by earlier researchers, aimed to accelerate the extraction process and investigate the extent to which beta-carotene degradation occurred

under such conditions. All extraction experiments were carried out in triplicate to estimate means and standard deviations. Data analysis was performed using XLSTAT version 2021.1 (AddinSoft, Paris, France). Fitting of the experimental data to the model (equation (6.5) and (6.8)) and the determination of the model constants were undertaken using MATLAB 2022a Academic version (Mathworks Inc., USA); further details are given below in section 3.6.

6.3.2 Preparation of freeze-dried carrot powder and purchase of sunflower oil

Fresh carrots (*Daucus carota L.*), purchased from a local supplier in Reading (United Kingdom) were washed, cleaned, and chopped in a food processor (Kenwood Blend-X Fresh BLP41.A0GO) and subjected to blast freezing at –80 °C, for 24-36 h. The frozen material was subsequently freeze dried at pressure 0.420 mbar and temperature -35 °C (VirTis SP Scientific, UK, Pressure range: 0.001-6.11 mbar; Temperature range: 0.01 to -76 °C) for 70-72 h until the moisture content dropped below 3% (dry weight basis). The freeze dried material was ground using a spice mill (Kenwood Prospero AT286 KW714229) and sieved to obtain three cuts with mean particle size of 0. 35, 0.75 and 1.40 mm. Sunflower oil, Flora (100% Natural, Suitable for All Cooking, Made with pure sunflower oil) was purchased from a local supermarket in Reading (United Kingdom).

6.3.3 Determination of extraction kinetics

Extraction kinetics was determined by measuring the concentration of beta-carotene dissolved in the oil phase at different time points. A separate extraction was performed for each time point. The time points were arbitrarily selected so that sufficient concentration versus time data points could be obtained to fit the model. Each of these extractions were performed in triplicate in order to determine the mean and standard deviation for each time point. Each extraction batch was prepared by adding 2 g of dehydrated carrot powder sample to 100 ml of the solvent phase (sunflower oil)

which was already pre-heated to the desired extraction temperature using magnetic heating and stirring plate. The beaker was then placed on a hot plate to control the temperature and constantly agitated using a magnetic stirrer operating at 300 rpm. After the desired extraction time, the beaker and its content were immediately cooled to 4 ^{o}C in an ice-bath. The cooled mixture was then centrifuged (Eppendorf MiniSpin Plus Centrifuge, fisher scientific, UK) at 14000 rpm for 40 mins, whilst maintaining its temperature at 4 ^{o}C, to obtain a clear supernatant which was then stored at 4 °C until further analysis.

6.3.4 Measurement and characterization of beta-carotene in sunflower oil

The concentration of beta-carotene in the extract phase was determined by taking 0.25 ml of the stored oil extract, mixing it with 3.75 ml of hexane and measuring the absorbance of the mixture against a blank solution of hexane and plain sunflower oil at 450 nm using a spectrophotometer (Cecil CE1011 Spectrophotometer) (Li et al., 2013a). A standard calibration curve ($R^2 = 0.99$) was prepared by dissolving pure beta-carotene (Tokyo Chemical Industry UK Ltd, 98 %) at various concentrations (0.5 µg/ml to 12 µg/ml) in a mixture of 8:1 (v/v) hexane and plain sunflower oil and measuring the absorbance at 450 nm.

In general, the beta-carotene extract can consist of *cis* and, *trans* isomers due to the high temperature applied during extraction (B. H. Chen & Liu, 1998). The extract solutions were therefore characterized by using a HPLC based method described by Achir *et al.*, (2010) and Syamila *et al.*, (2019). This procedure involved crystallizing out the sunflower oil triglycerides by mixing 0.5 ml of stored extract with 4.5 mL acetone, vortexing the mixture for 10 s and leaving it overnight at –20 °C. The triacylglycerols were separated by rapid sampling and filtration through a 0.2 µm PES filter (Fisher Scientific, China). The triacylglycerol-free mixture was then directly

injected into the HPLC column - a polymeric YMC-30 (4.6 mm id ×250 mm, 5 mm particle size) (YMC, Wilmington, NC, USA). Elution was performed with a quaternary pump. The mobile phase consisted of methanol, tert-butyl-methyl-ether (TBME), and milli-Q water (50 : 45 : 5, v/v/v at a flow rate of 1 mL/min under isocratic conditions. A UV- visible photodiode array detector (Dionex UVD 340U) was used to analyze the chromatograms at a detection wavelength of 450 nm. Analysis were done in triplicate. The quantification was done against a standard calibration curve (R^2 = 0.99) in a concentration range between 0.5 µg/ml to 12 µg/ml in a mixture of 8:1 (v/v) hexane and plain sunflower oil.

6.3.5 Degradation kinetics of beta-carotene in sunflower oil under frying conditions

As mentioned earlier, a key purpose of this research is to explore the possibility of using beta-carotene enriched oil in cooking and food processing. It was therefore thought desirable to investigate the degradation kinetics of beta-carotene at different temperatures which included common frying temperatures (135, 150, 160, 180, 200 and 220 ^{o}C), by measuring the concentration of beta-carotene remaining in the sunflower oil after exposure to the temperature for a stipulated time. At each temperature, the concentration of beta-carotene was measured after 5, 10, 15, 20, 25, and 30 mins, in addition to the initial concentration. A separate batch of beta-carotene in oil, contained in a heat stable test tube (Pyrex, UK), was used for each time point. 9 ml of commercially available sunflower oil (Flora, United Kingdom) was first heated to the desired temperature and 1 ml of beta-carotene enriched sunflower oil was added to it, so as to result in an initial beta-carotene concentration of 200 mg kg^{-1}. This procedure ensured that the beta-carotene attained the pre-determined temperature in the shortest possible time, which was less than 10 s. The test tube was then maintained at this temperature for the desired time. It was then rapidly cooled to in an ice bath, and stored at 4 °C temperature in an amber vial which protected it from light degradation

until further analysis . The transient concentrations of beta-carotene, determined at each temperature, were fitted to the first order equation to deduce the rate constant:

$$\ln\left(\frac{C_0}{C_t}\right) = kt \tag{6.9}$$

where, c_t and c_0 are the concentrations of beta-carotene at any time t and initially, respectively, and k is the first order isothermal rate constant, assumed to vary with temperature (T) according to the well-known Arrhenius equation: $k = A\ exp(\frac{-E_a}{RT})$ where, A is the pre-exponential factor (s^{-1}); E_a is the activation energy (J mol^{-1}); and R is the universal gas constant (8.314 J mol^{-1} K^{-1}).

6.3.6 Statistical analysis

The validity of the model was tested by fitting equations (6.5) and (6.8) to the experimentally determined c_L versus t data using MATLAB 2020b's curve fitting tool for 95% confidence interval. The tool works by minimizing the sum squared error and root mean squared error, and requires an initial guess for the model parameters. The Levenberg-Marquardt algorithm is used to optimize the model parameters, and the best-fit values were based on 15×3 data points (in triplicates) for each experimental condition. This article also explains that the SSE and RMSE values indicate model validity and goodness of fit, and the co-efficient of correlation and adjusted R^2 are determined to ensure an adequate number of parameters have been used. The narrow range of joint confidence intervals obtained reinforces the precision in estimating the parameters and the adequacy of the number of experimental data points used in the fitting exercise.

$$\text{Sum of squarred error (SSE)} = \sum(y_{exp} - y_{model})^2 \tag{6.10}$$

$$\text{Root mean squarred error (RMSE)} = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(y_{exp} - y_{model})^2} \tag{6.11}$$

$$\text{Coefficient of determination } (R^2) = 1 - \frac{RSS}{TSS} \quad (6.12)$$

$$\text{Adjusted coefficient of determination } (Adj - R^2) = 1 - \frac{(1-R^2)(n-1)}{(n-p-1)} \quad (6.13)$$

where, n=number of observations for each experiment; y_{exp} – Experimental results; Y_{model} – Predicted results from model; *RSS* – Residual sum of square; *TSS* – Total sum of square; *p*-total number of predicted results from model.

6.4 Results and discussion

6.4.1 Validation of the model

Experimentally determined C_L versus t data were fitted to equation (6.5). At temperatures of 90 and 115 °C, the C_L values increased with time before reaching asymptotic values ($p<0.05$) – as shown in Figure 6.1. This trend suggests that the degradation of beta-carotene during extraction is negligible at these temperatures. In other words, k_1 and k_2 can be considered to be negligible in equation (6.5), and equation (6.8) represents the variation of concentration with time. Figure 6.1 also shows the fit between the experimental data at these temperatures with equation (6.6) and (6.8), and the model constants are reported in the caption of Figure 6.1.

At the higher temperatures of 135 and 150 °C, the concentration goes through a maximum value which is consistent with equation (6.5) and also confirms the occurrence of beta-carotene degradation during extraction. The acceptable fit between the equation (6.5) and the experimental data at these temperatures is shown in Table 6.1S (Appendices 2), along with the corresponding best-fit values of the model constants as well as goodness of fit. Even though the high R^2 value illustrates a good fit between model and experimental data, other fitness parameters such as sum

of squared error (SSE), root mean squared error (RMSE), and adjusted R^2 were also estimated (Equations 6.9, 6.10 and 6.12). The distinctly lower values of the statistical error and higher values of determination coefficients (Table 6.1S) enhance the model validity.

The values of the constant k_2 – which represents the first order rate constant for the degradation of beta-carotene in the oil phase – were estimated by fitting the C_L versus t data as mentioned above, as well as by undertaking separate beta-carotene degradation experiments, already stated earlier under materials and methods (section 6.3.6). ANOVA (pair comparison test) was run to check the null hypothesis of a significant difference existing between k_2 values given by the model and the experimentally determined values of k_2; the p value obtained was greater than 0.05 which negates the null hypothesis. Thus, k_2 values deduced from equation (5) and experimental values are statistically the same – which further reinforces the model hypothesis that degradation kinetics of beta-carotene in oil follows first order between 135-220 ℃.

It is evident from Table 6.1S that the values of k_1 and $(k_2)_{exp}$ are very close. An ANOVA was therefore run to check whether k_1 and $(k_2)_{exp}$ were significantly different or not, which resulted in a p value for the null hypothesis greater than 0.05 suggesting the rejection of the hypothesis. Thus, k_1 and $(k_2)_{exp}$ can be assumed to be equal in equation (6.5), which indicates that the general variation of beta-carotene concentration in oil is given by equation (6.6) where k_1 and k_2 are considered to be equal and both replaced by k. The insignificant difference between k_1 and k_2 values also suggests that the degradation is predominantly thermal by nature, and any differences in the structural environments of the two phases do not play a significant role in the degradation process. Thus, the experimentally determined C_L versus t data were fitted to equation (6.6) to generate Table 1 which shows the best fit model parameters as well as the goodness of fit. Figure

6.1 illustrates the fit of the experimental data against equations 6.6 and 6.8, for all the temperatures and particle sizes investigated in this work.

As mentioned in section 6.2, the value of k_m represents the rate constant for solid phase exhaustion of beta-carotene and k represents its degradation rate constant. It is evident from Table 6.1 that k_m is significantly greater than k which suggests that chemical degradation of beta-carotene in sunflower oil is relatively slow in comparison with its transfer from the solid phase, even at temperatures as high as 135 or 150 ^oC, which enables rapid and efficient extraction to be carried out at such high temperatures. If this extraction is to be carried out continuously, then a reactor with tubular configuration will be effective to control residence times.

Earlier work on beta-carotene extraction has largely been undertaken using organic solvents such as hexane (Y. Sun et al., 2010), Tetrahydrofuran (Y. Sun et al., 2010), ethyl acetate (Y. Sun et al., 2010), dichloromethane (Y. Sun et al., 2010) and ethanol (Purohit & Gogate, 2015a) where rapid degradation of beta-carotene has been noted, prompting the use of relatively low temperatures (30-60 ^oC) and, in some cases, the use of devices such as microwaves or ultrasound (Chutia & Mahanta, 2020; Demiray & Tulek, 2017; Stupar et al., 2021). The rates of extraction observed in the present study are significantly greater than those observed in some earlier studies. For example, Purohit and Gogate (2015) have reported an extraction time of around 50 mins to attain a yield of 70% (based on the total extractable beta-carotene) in ultrasound assisted ethanol solutions at temperature of 30^oC, when using carrot particles of sizes comparable with the sizes used in the present study. By employing higher temperatures such as those used in this work, similar yields can be obtained in a matter of 5-6 mins. Chumnanpaisont et al (2014) have also reported extraction times of 2 -5 mins for the extraction of beta-carotene from carrots using microwave power, operating either continuously or intermittently. Table 6.2 shows a comparison between the net rate

of extraction determined using various extraction methods and the values observed in this work employing solely thermal heating. It is clear that the extraction rates at 135 and 150 °C are higher or comparable with the values obtained employing energy intensive extraction methods such as microwave, pulsed electric field and electrohydrodynamic combined with ultra sound.

6.4.2 Composition of the sunflower oil extract

Beta-carotene can exist in three isomeric forms in oil: *trans,* 9-*cis and* 13-*cis* (Achir et al., 2011). HPLC analysis was performed for each extract and the concentrations of the three isomers in the extract are shown as a function of time in Figures 6.2 (a)-(d). The concentration of 9-*cis* in the extract was below the detection limit, therefore the concentrations of only the other two isomers are shown. A similar result was reported earlier by Achier et. al., (2011). It is also interesting to note that the sum of the concentrations of the two isomers is the total beta-carotene concentration determined spectrophotometrically; this is also shown in Figures 6.2 (a)-(d). At higher extraction temperatures the concentration of 13-*cis* increases initially, but decreases to virtually zero soon after the peak concentration is reached. Therefore, longer extraction durations only result in *trans* isomers. In general, the extract is dominated by the *trans*-isomer with its percentage varying between 70-87% of the beta-carotene in the extract. This implies that the percentage of cis isomers ranged between 13-30%, which is somewhat lower than the value of 40% reported for copra fat and palm olein by Achir et al (2011). could the higher value for these materials may be attributed to the higher concentration of beta-carotene used and the application of more severe treatment. It may be noted that these observations are valid for all the particle sizes employed in this study (data not shown).

The three isomers have been reported to possess similar vitamin A forming potentials and colouring attributes (Rodriguez-amaya, 1999). Therefore, the relative concentrations of the isomers may not be critical from applications point of view. However, Figures 6.2 (a)-(d) provide insights into the distribution of the isomers in the extract phase under different operating conditions.

6.4.3 Effect of temperature and particle size on the extraction kinetics

Beta-carotene is mainly present in chromo- and chloroplast, and protected by the cellulose and pectin layers of the cellular structure (Thürmann et al., 2002). Smaller particle sizes imply shorter diffusion path length and greater accessibility of the beta-carotene. Therefore, k_m increases with decrease in particle size, which is confirmed in Table 6.1. Higher temperatures, on the other hand, improve accessibility by rupturing the protecting membranes (Nutter et al., 2021). Therefore k_m also increases with temperature, but, as Table 6.1 shows, the increase is not as marked as the effect of particle size.

6.4.4 Degradation kinetics of beta-carotene in sunflower oil, especially at normal frying temperatures

Beta-carotene degradation experiments were performed by dissolving commercially available *trans*-beta-carotene in sunflower oil and allowing the beta-carotene to degrade at the desired temperatures (section 5.3.6). For all heating treatments, the concentration of *trans*-beta-carotene decreased as a function of the heating time. This disappearance was visible macroscopically by a loss of color, and it was more rapid as the temperature increased around 200 °C. Figure 6.3 shows a semi-log plot of normalized beta-carotene concentration against time over a range of temperatures between 135 and 220 °C. The linear nature of the plots confirm that the degradation

follows first order isothermal kinetics; the rate constants values are given in Table 6.3, which also reports the Arrhenius constants: activation energy and pre-exponential factor. Table 6.3 shows that the activation energy value over the temperatures 135-220 °C is 56.65 kJ/mol (R^2=0.91), which is consistent with the reported values of 48 kJ/mol for beta-carotene degradation in palm olein (Achir et al., 2010).

The choice of temperature and time employed in this study were intended to cover values encountered during the use of sunflower oil for deep fat frying (Totani et al., 2013). The values of degradation rate constant given in Table 6.3 are in close agreement with the values previously reported by (Achir et al. 2011). But the values of the rate constants observed in this study are significantly lower than the values reported by Sun et al., (2010) for trans beta-carotene degradation in dichloromethane under the influence of ultrasound at temperatures in the range -5-25 ^{o}C. It is unclear whether the chemical nature of the solvent medium plays a role in influencing kinetics, but these studies suggest that there is a role played by the solvent. Further experiments are needed to confirm solvent effects. Regardless, it is clear that in sunflower oil, beta-carotene undergoes degradation at frying temperatures, the extent depending on the time-temperature combination employed. If we assume a typical frying temperature of 180 °C for 10 mins (e.g. for frying French fries), the percentage of beta-carotene remaining in the oil, based on the rate constant values reported in this work is 75%, which suggests that beta-carotene fortified vegetable oils can be used, in practice, for frying and other food processing applications. At such high temperatures, the heating time needs to be over 30 minutes for 90% of beta-carotene to be destroyed (Achir et al., 2010).

During heating, beta-carotene degradation is reported to be accompanied by concomitant isomerization, as well as oxidation to produce epoxy- and hydroxy-*β*-Carotene, and cleavage products such as apocarotenals and apocarotenones (Mordi, 1993); Caris-Veyrat *et al.*, 2001). In this study, the development of *trans*-, 9-*cis* and 13-*cis* isomer concentrations were monitored with time, at various temperatures, using HPLC-DAD (section 6.3.4). Achir et al (2011) have proposed plausible reaction networks leading to the formation of the isomers and thermal degradation products. A simplified network model scheme is presented in Figure 6.4, which assumes that, at any given temperature, the trans isomer can reversibly change either to 9-*cis* or13-*cis* isomer, each of which can also undergo subsequent thermal degradation. Each reaction in the network shown in Figure 6.4 is also assumed to be first order with corresponding rate constants. It is also reasonable to assume that the thermal degradation rate constants for all three isomers are the same at a given temperature, as suggested by Achir (2011). Based on these assumptions, an instantaneous mass balance can be written for each of the isomers as follows:

$$\frac{\mathrm{d}C_{trans}}{\mathrm{dt}} = -(k_1 + k_3 + k_5) \times C_{trans} + k_2 \times C_{9-cis} + k_4 \times C_{13-cis} \quad (6.14)$$

$$\frac{\mathrm{d}C_{9-cis}}{\mathrm{dt}} = k_1 \times C_{trans} - (k_2 + k_5) \times C_{9-cis} \quad (6.15)$$

$$\frac{\mathrm{d}C_{13-cis}}{\mathrm{dt}} = k_3 \times C_{trans} - (k_2 + k_5) \times C_{13-cis} \quad (6.16)$$

The above set of differential equations was used to model the concentration of the three isomers with respect to reaction (processing/cooking) time and temperature, the initial conditions being $C_{\text{trans}} = C_{\text{beta-carotene}}$ and $C_{\text{9-cis}} = C_{\text{13-cis}} = 0$. Multiresponse modelling to obtain the best estimates of the rate constants from k_1 through to k_5 and their corresponding activation energies was performed by non-linear regression using the Bayesian approach and the determinant criterion (van Boekel

2008), included in the modelling software Athena Visual Studio software package (Athena Visual Software Inc., Naperville, IL). The minimisation of the determinant criterion (Stewart, Caracotsios, & Sørensen, 1992) is ideal for multiresponse studies since it removes the need for the statistical compliance that is required for the typical minimization of the sum of squares (van Bokel, 2008). Each of the rate constants from k_1 through to k_5 was assumed to follow Arrhenius behaviour with respect to temperature and was reparametrized as follows:

$$k = k_{\mathrm{ref}} \exp\left(\frac{-E_a}{\mathrm{R}}\left(\frac{1}{\mathrm{T}} - \frac{1}{\mathrm{T_{ref}}}\right)\right)$$

where:

k: rate constant at any temperature T (^{o}K)

k_{ref}: rate constant at reference temperature T_{ref} (set at 473 ^{o}K, i.e. 200 ^{o}C)

Ea: activation energy (Joules/mole)

R: Universal gas constant (8.314 J mol^{-1} K^{-1})

Three models were compared, which differ on the number of parameters employed: The first model had explicit activation energies attributed to each rate constant, in the second model k_1 and k_2 shared the same activation energy, and the same applied to k_3 and k_4, while in the third candidate model all rate constants shared the same activation energy. The sum of squares of the residuals (RSS) is a measure of the discrepancy between the experimental and model data, with a lower value indicating a better fit between the two. In addition, the Akaike information criterion (AIC) was also employed to discriminate between the different candidate models. Out of the three models, the first one was the best since it had the lowest RSS as well as the lowest AIC value. The

best estimates of the parameters of the first model - i.e. the rate constants at 200 °C and their activation energies - along with their 95% confidence intervals are presented in Table 6.4.

A graphical comparison between the experimentally determined concentrations of different beta-carotene isomers and the values predicted by the model is shown in Figures 6.5 (a)-(d). The rate constants for back isomerization of the trans isomer from 9-*cis* and 13-*cis* are greater than the corresponding values for the forward reaction, which accounts for the significantly higher concentrations of the *trans* isomer in the mixture at all the temperatures, except 220 ^{o}C where the concentrations become comparable. This observation of generating higher concentrations of trans isomers at higher temperatures is consistent with Achir et al (2011), who reported lower rate constants for the back isomerization of *cis*. The rate constant k_5 representing irreversible thermal degradation of all three isomers can be compared with the rate constant values obtained by measuring absorbance values as a function of time (section 5.3.4) and a parity plot of values obtained at different temperatures is shown in Figure 6.6.

The significance of isomer formation during degradation to potential bioactivity is not conclusive. According to Rodriguez-Amaya (1999), all three isomers are capable of synthesizing vitamin A, and can impart coloration to food materials. However, Castenmiller & West (1998) has stated that the *trans*-isomer is more active at synthesizing vitamin A than the *cis*-isomers. It is therefore evident that further research is needed to conclusively establish the role played by each isomer in this regard.

6.5 Conclusions

1. Elevated temperatures (upto 150 °C) can be used viably to extract beta-carotene in edible oil. Despite thermal degradation of beta-carotene at high temperatures, the net rates of

extraction observed in this study were found to be significantly higher or comparable with the rates observed in earlier studies using energy intensive technologies such as pulsed electric field, microwave and electrohydrodynamic in combination with ultrasound.

2. A model developed to determine the transient concentration of beta-carotene in sunflower oil, which accounts for thermal degradation occurring in the solid and extract phases, gave a good fit with the experimental data. This kinetic model can potentially be used to design and size extractors.
3. beta-carotene enriched sunflower can be used as a frying medium to enrich the nutritional value of fried products.
4. A reaction network model was developed to explain kinetics of formation and degradation for each beta-carotene isomer during thermal degradation.

6.6 References

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Table 6.1: Values of model parameters fitting to equations (6.6) and (6.8). Experiments performed with solid to liquid ratio 20 kg m^{-3} and for different particle sizes at different temperatures.

Sl. No.	Particle size (mm)	Temperature (°C)	$C_{si} \times 10^{-4}$ (kg of BC/kg dry matter)	$k \times 10^{-4}$ (Degradation rate constant in the solid phase, s^{-1})	$k_m \times 10^{-3}$ (Exhaustion rate constant of BC in the liquid phase, s^{-1})	SSE × 10^{-6} (Eqn. 6.10)	R^2 (Eqn. 6.12)	Adjusted-R^2 (Eqn. 6.13)	RMSE × 10^{-4} (Eqn. 6.11)
1	0.350	90	7.52±0.16	---	8.0±0.71	17.60	0.94	0.94	10.83
		115	7.61±0.19	---	8.0±0.77	14.07	0.95	0.95	9.68
		135	8.03±0.15	5.70±0.11	18.0±1.22	20.17	0.89	0.87	36.67
		150	6.67±0.13	4.64±0.07	22.0±1.31	62.18	0.92	0.90	20.36
2	0.750	90	4.98±0.09	---	10.0±0.83	5.92	0.96	0.95	6.28
		115	6.27±0.11	---	16.0±1.09	90.82	0.99	0.99	2.46
		135	5.77±0.12	0.51±0.03	17.0±1.04	3.73	0.97	0.97	5.16
		150	6.82±0.17	0.62±0.07	20.0±1.53	35.95	0.99	0.99	16.03
3	1.400	90	4.68±0.02	---	6.0±0.21	0.99	0.99	0.99	2.57
		115	5.05±0.06	---	9.0±0.55	5.88	0.96	0.96	6.26
		135	4.51±0.08	1.35±0.09	13.0±0.33	86.01	0.98	0.98	2.47

150	4.68±0.02	4.08±0.11	15.0±1.19	14.74	0.92	0.90	10.26

Note: the model parameters were predicted by using average value of triplicate experimental dataset (n=3).

C_{si} – Maximum extractable betalains (kg of dried β-*Carotene*/kg of dried carrot powder).

k – Degradation rate constant (s^{-1})

k_m – Solid exhaustion rate constant (s^{-1})

SSE – Sum of squared errors

R^2 – Co-efficient of determination

Adj. R^2 – Adjusted Co-efficient of determination

RMSE – Root mean squared error

Eqn. – Equation

Table 6.2: Comparison of maximum extraction rates reported in literature with values observed in this research.

Authors	Solvent Used	Extraction method	Operating parameters	Maximum extraction rate (kg of beta-carotene (kg of dry matter)$^{-1}$ s^{-1})	Comment
(Li et al. 2013)	Sunflower oil	Ultrasound Extraction	Solid/liquid = 1/20, Time = 30 min	2.0×10^{-6}	Particle size was not mentioned
(Roohinejad et al., 2014)	Glycerol monocaprylocaprate+Posphate Buffer + Tween 20	Pulsed Electric Field Treatment as pre-treatment	Solid/liquid = 1/30, Time = 60 min	2.0×10^{-6}	Particle size was not mentioned
(Hiranvarachat & Devahastin, 2014)	Hexane (50%), acetone (25%), Ethanol (25%)	Microwave Extraction	180 W/75 ml, Time = 4 min	4.6×10^{-6}	Particle size was not mentioned

(Salehi & Taghian Dinani, 2020)	Ethanol	Ultrasound-electrohydrodynamic	Solid/liquid = 1/10, Time = 60 min	3.5×10^{-6}	Particle size was not mentioned
This Study	Sunflower oil	Hot plate Stirring Extraction	(a) 90-150 °C, 4 min extraction time	3.1×10^{-6}	Particle size = 350 µm.

Table 6.3: Effect of temperature on the rate constants for thermal degradation of beta-carotene in sunflower oil, and Arrhenius constants.

Temperature (°C)	Degradation rate constants, k (s^{-1})	Half-life, ($t_{1/2}$) (min)	R^2	Activation Energy, E_a (kJ/mol^{-1})	Pre-exponential Factor, A (s^{-1})
135	0.0001	115.5	0.87	56.65	7.6
150	0.0002	57.75	0.99		
160	0.0004	28.87	0.96		
180	0.0006	19.25	0.96		
200	0.0009	12.83	0.98		
220	0.0022	5.25	0.97		

Note: the model parameters were deduced by plotting average value of triplicate experimental dataset (n=3).

k – First order isothermal degradation rate constant of beta-carotene (s^{-1})

$t_{1/2}$- Half life time for degradation of beta-carotene (min)

R^2- Co-efficient of determination

E_a – Activation energy of degradation for beta-carotene ($kJ/mole^{-1}$)

A - Pre-exponential Factor, (s^{-1})

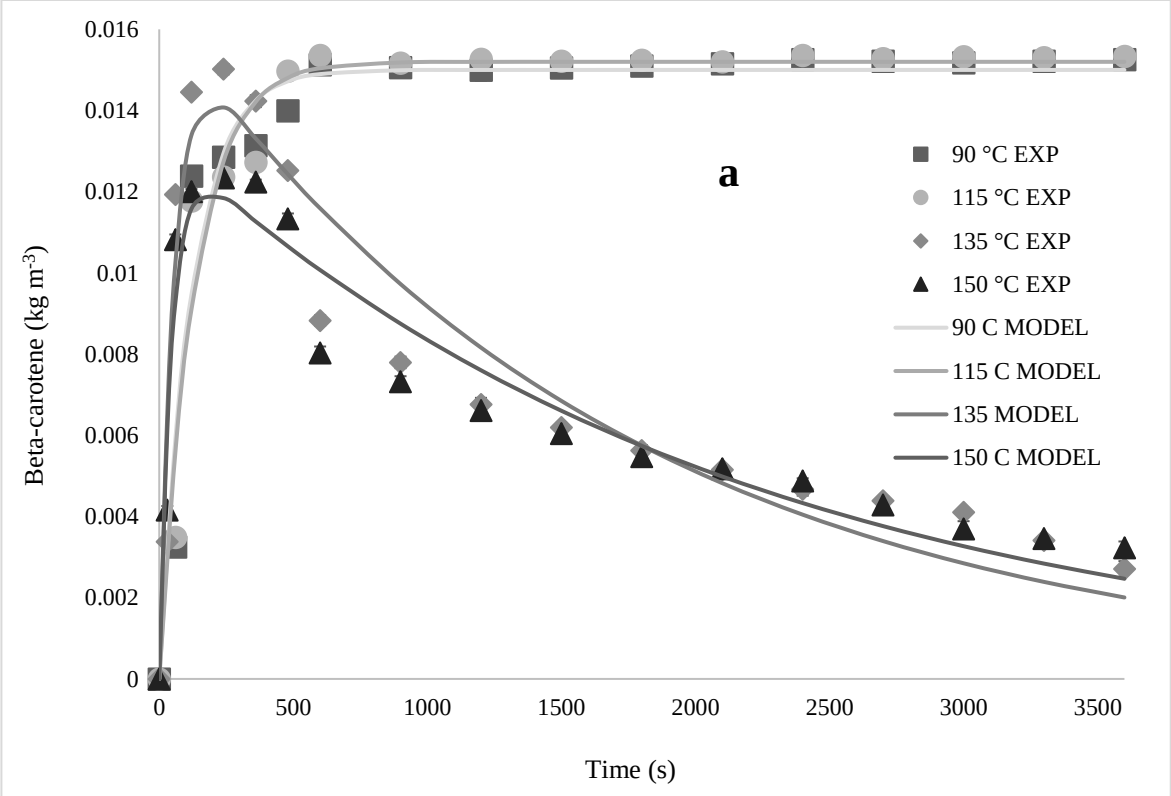
Table 6.4: Rate constant values and Arrhenius parameters for the reaction network described in Figure 6.4.

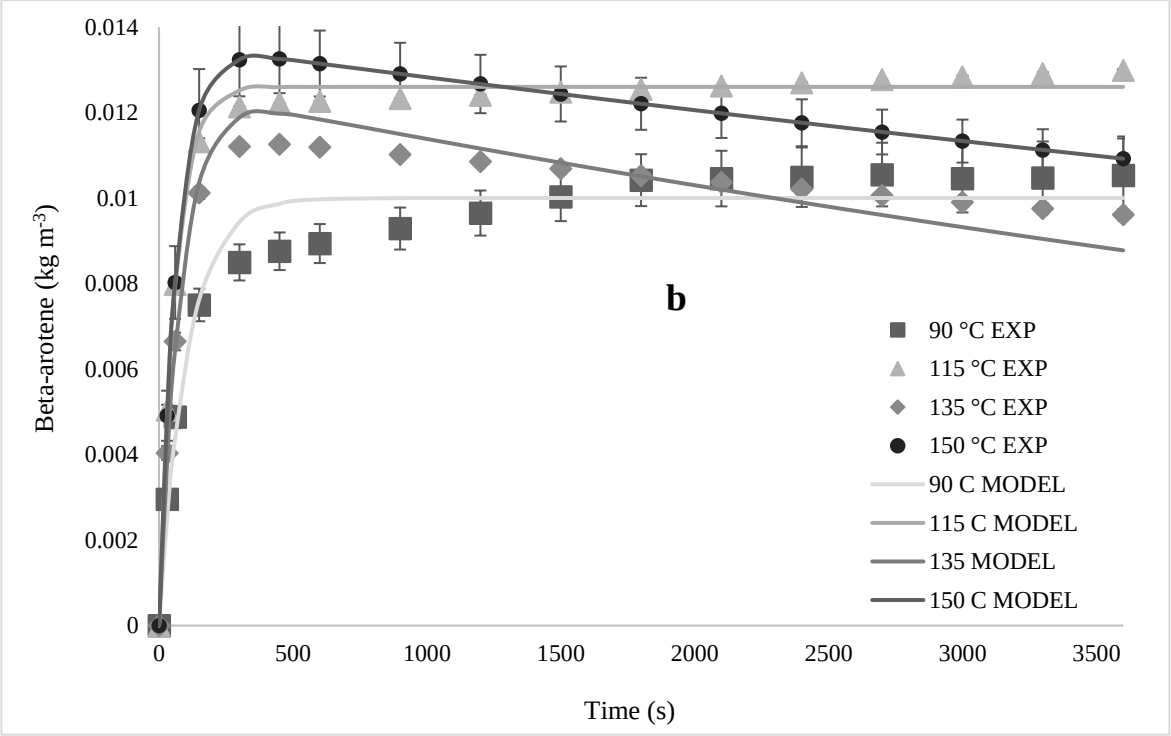
Reaction rate constants (min^{-1})	Temperatures (°C)				Activation Energy (kJ mol^{-1})	Goodness of fit	
	160	180	200	220		RSS	n
k_1	0.079±0.001	0.188±0.002	0.415±0.002	0.860±0.006	70.51		
k_2	0.148±0.001	0.330±0.002	0.689±0.005	1.356±0.010	65.52		
k_3	0.166±0.001	0.232±0.001	0.316±0.001	0.418±0.003	27.24	0.005	288
k_4	0.461±0.002	0.569±0.003	0.689±0.001	0.821±0.005	17.04		
k_5	0.011±0.001	0.027±0.001	0.060±0.004	0.125±0.002	70.16		

Note: the model parameters were predicted by using average value of triplicate experimental dataset (n=3).

RSS – Sum of square of the residuals ($RSS = \sum_{i=1}^{n}([X_{optipred}] - [X_{exp}])$), where n is the number of data points, $[X_{exp}]$ the experimental result, and $[X_{optipred}]$ the optimized simulated result.

n – no. of datapoints model was evaluated.


a
Beta-carotene (kg m-3)
Time (s)
90 °C EXP
115 °C EXP
135 °C EXP
150 °C EXP
90 C MODEL
115 C MODEL
135 MODEL
150 C MODEL
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
0
500
1000
1500
2000
2500
3000
3500


b
Beta-arotene (kg m-3)
Time (s)
90 °C EXP
115 °C EXP
135 °C EXP
150 °C EXP
90 C MODEL
115 C MODEL
135 MODEL
150 C MODEL
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
0
500
1000
1500
2000
2500
3000
3500

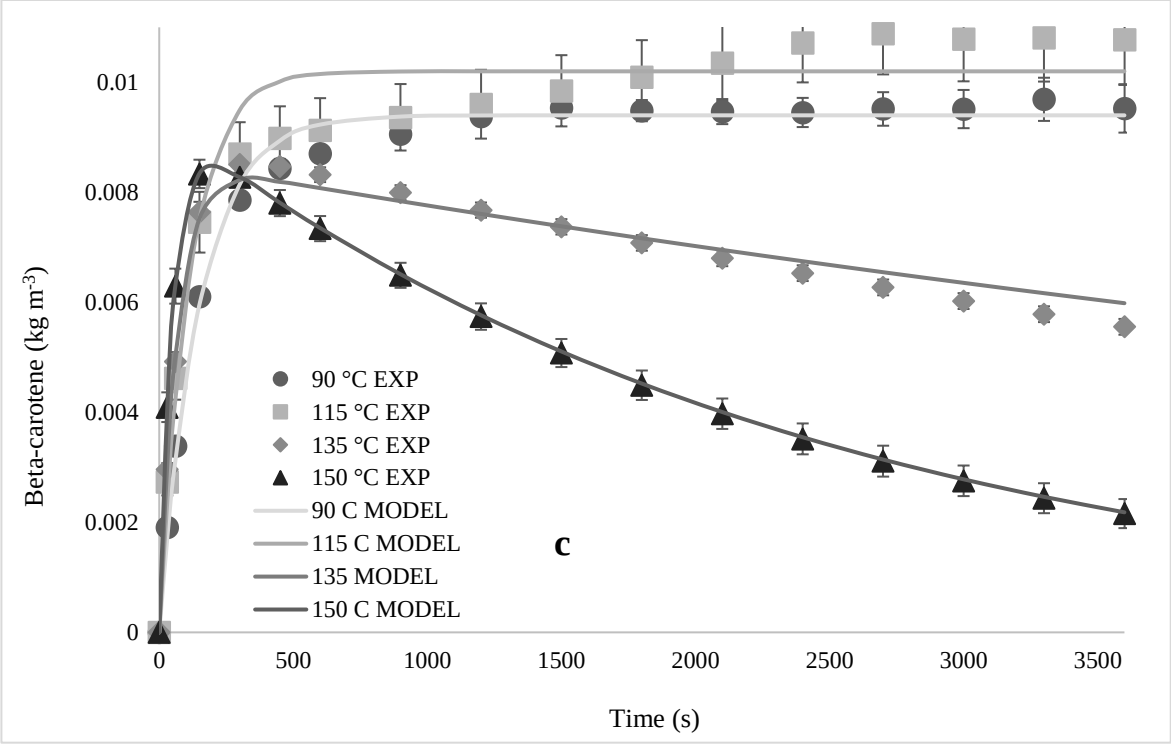
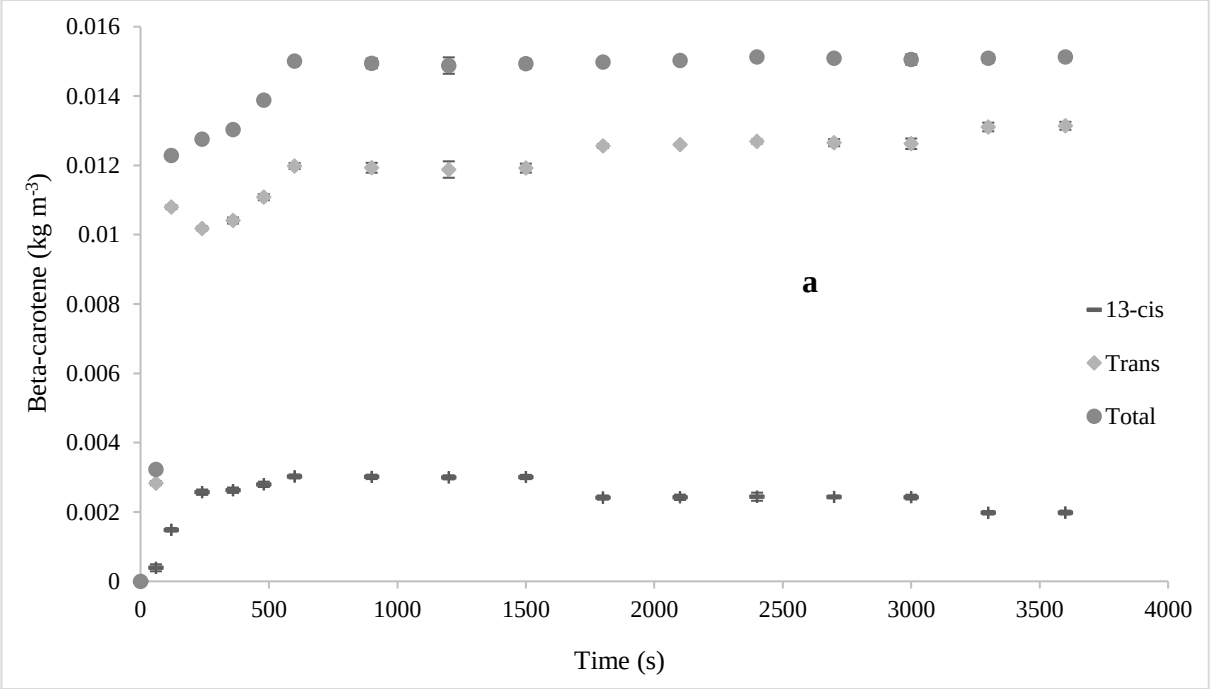
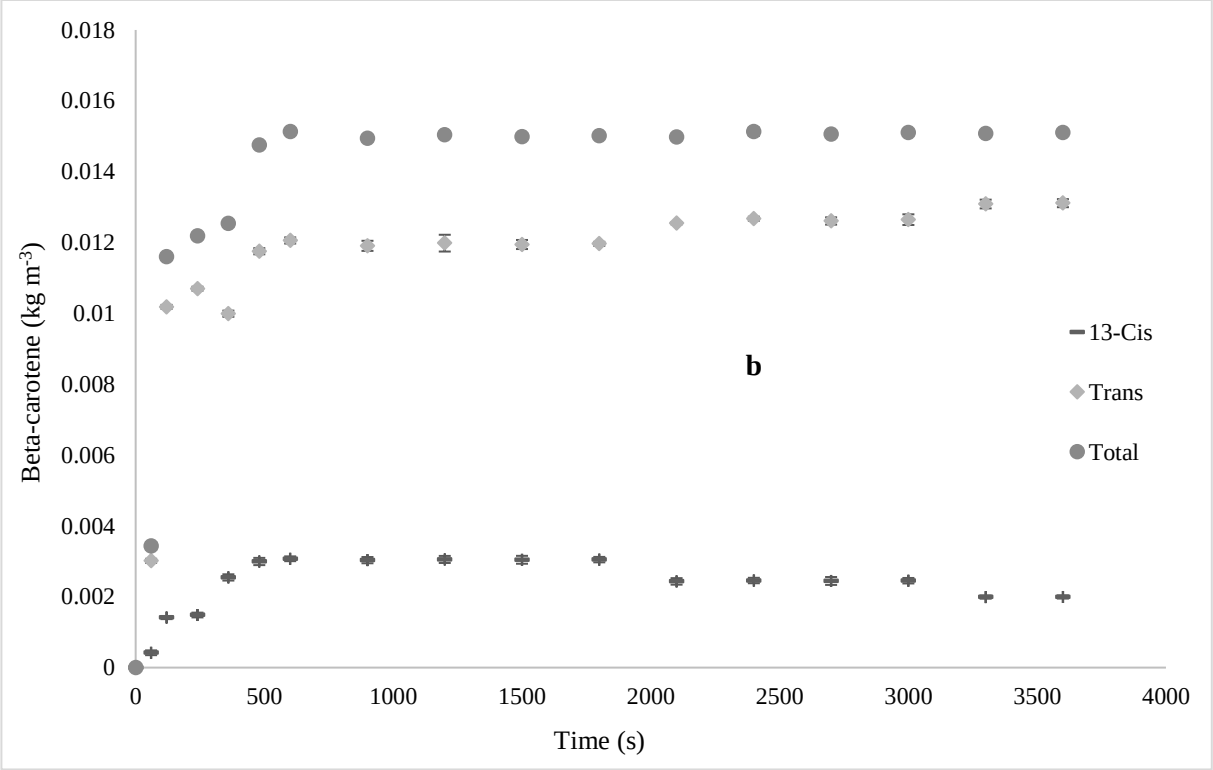


Figure 6.1: Extraction of beta-carotene from carrot powders at 90, 115, 135 and 150 °C into sunflower oil, Solid loading =20 kg/m^3, a) Particle size – 0.35 mm, b) Particle size – 0.75 mm, (c) Particle size – 1.40 mm. The points indicate experimental values of the concentration and the solid line represents the model, i.e., concentration given by Equations (6.6) and (6.8). Values of the model parameters for the other particle sizes with temperature range in sunflower oil are shown in Table 6.1. Standard deviation was included for triplicates (n=3).


0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
Beta-carotene (kg m-3)
0
500
1000
1500
2000
2500
3000
3500
4000
Time (s)
a
13-cis
Trans
Total


0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
Beta-carotene (kg m-3)
0
500
1000
1500
2000
2500
3000
3500
4000
Time (s)
b
13-Cis
Trans
Total

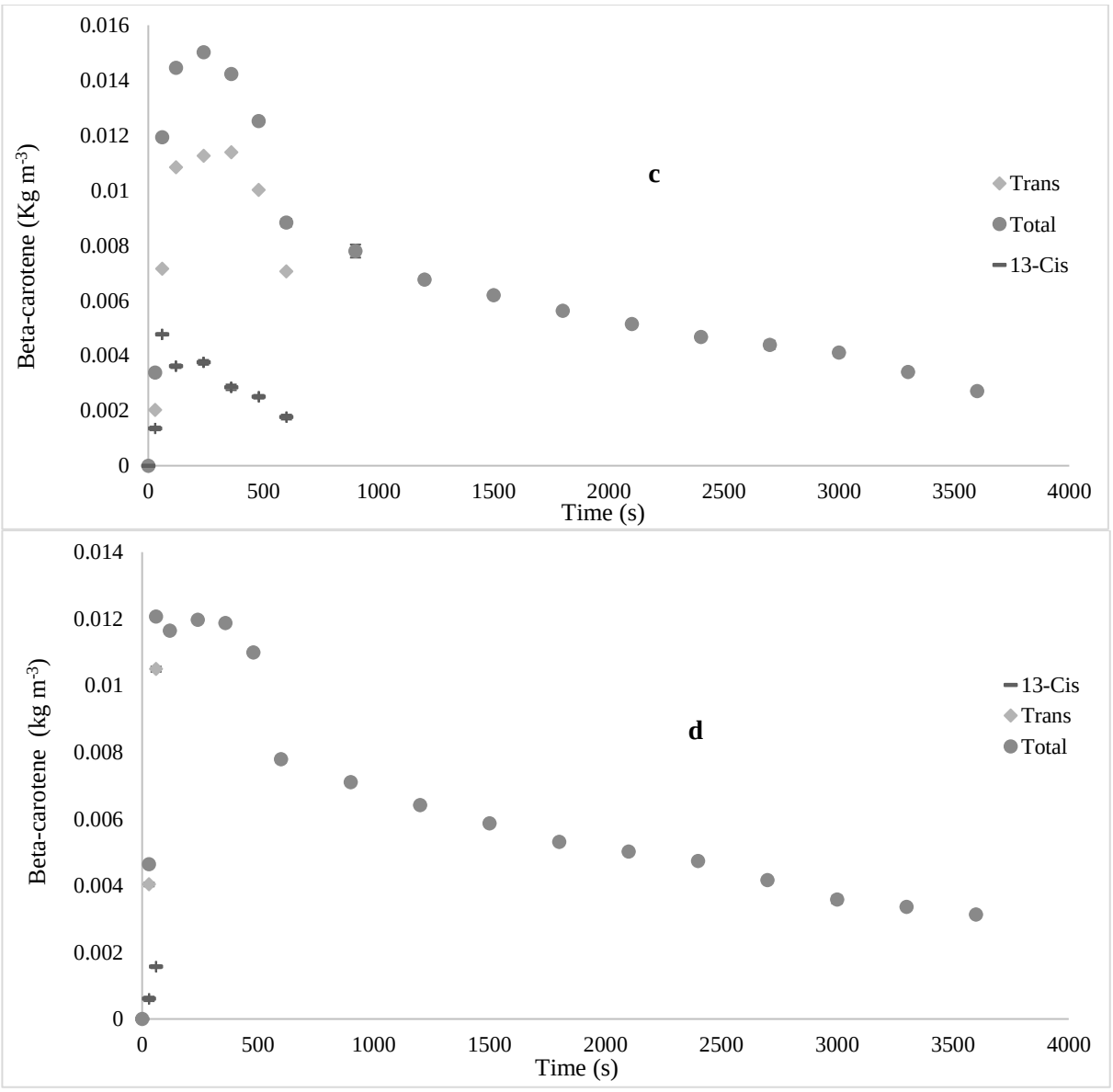


Figure 6.2: Composition of beta-carotene isomers, *trans* and 13-*cis*, in sunflower oil extract at different extraction temperatures (a) 90 °C, (b) 115 °C, (c) 135 and (d) 150 °C. It may be noted that 13-cis isomer was only observed in the initially stages of extraction at higher temperatures of 135, and 150 °C (Figures c and d). 9-*cis* isomer was not detected in the extracts. Standard deviation was included for triplicates (n=3).

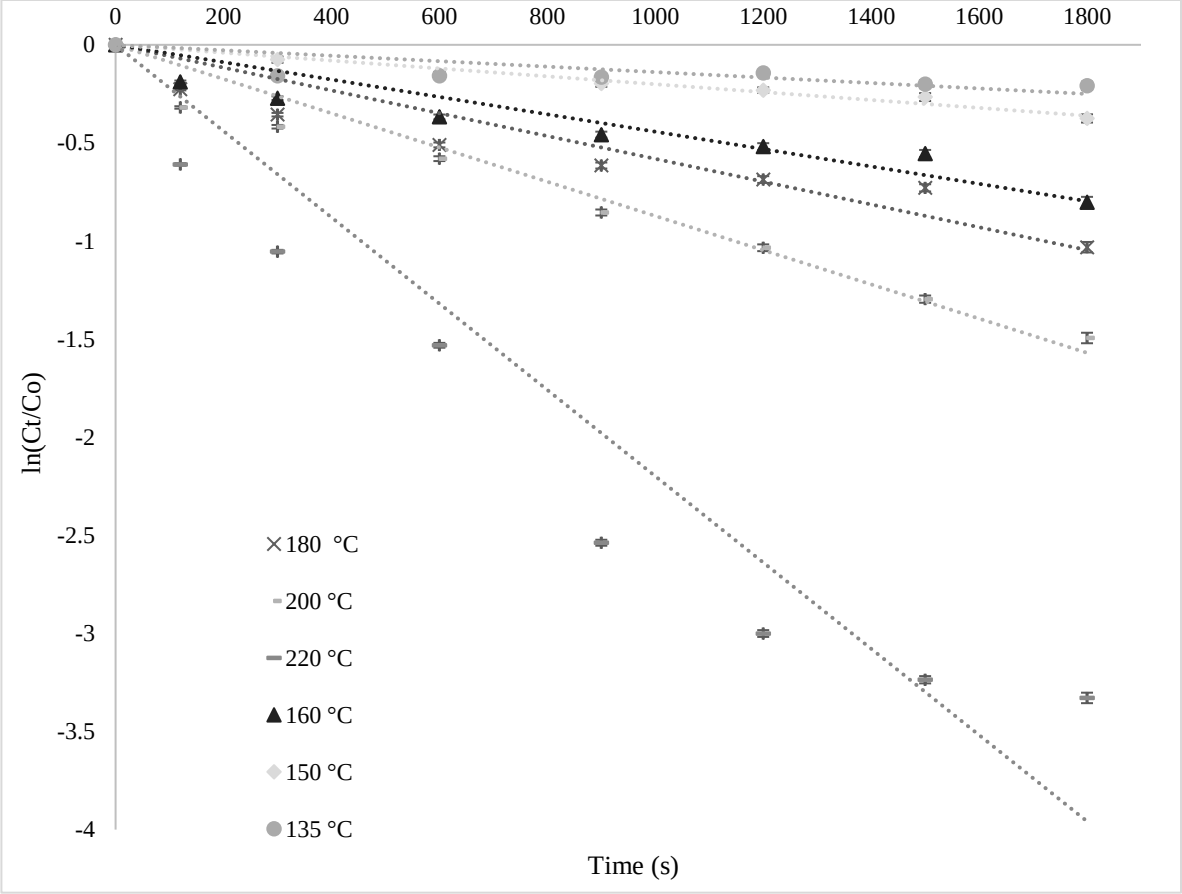


Figure 6.3: Degradation kinetics of beta-carotene in sunflower oil at different temperatures. Temperature range was selected to reflect normal frying and cooking conditions. Solid lines indicate first-order kinetic fit. The rate constant at different temperatures are reported in Table 3. Note: the model parameters were deduced by plotting average value of triplicate experimental dataset (n=3).

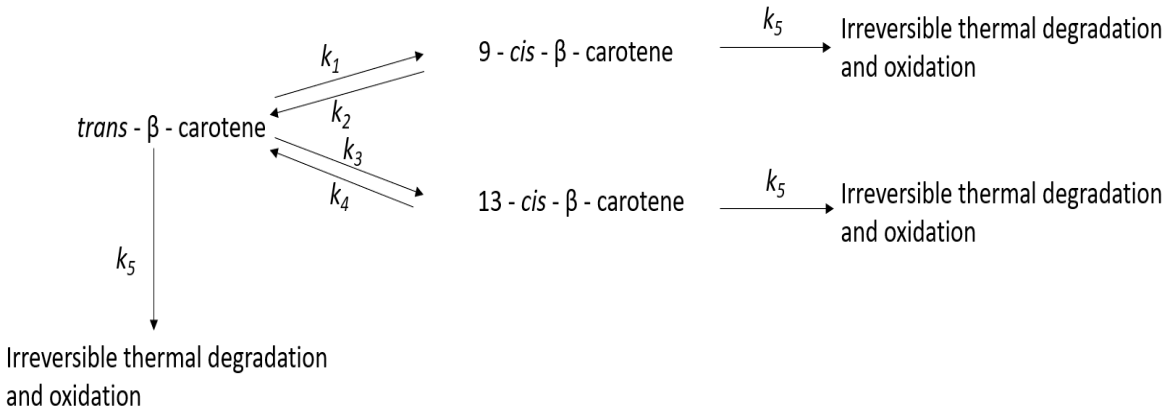
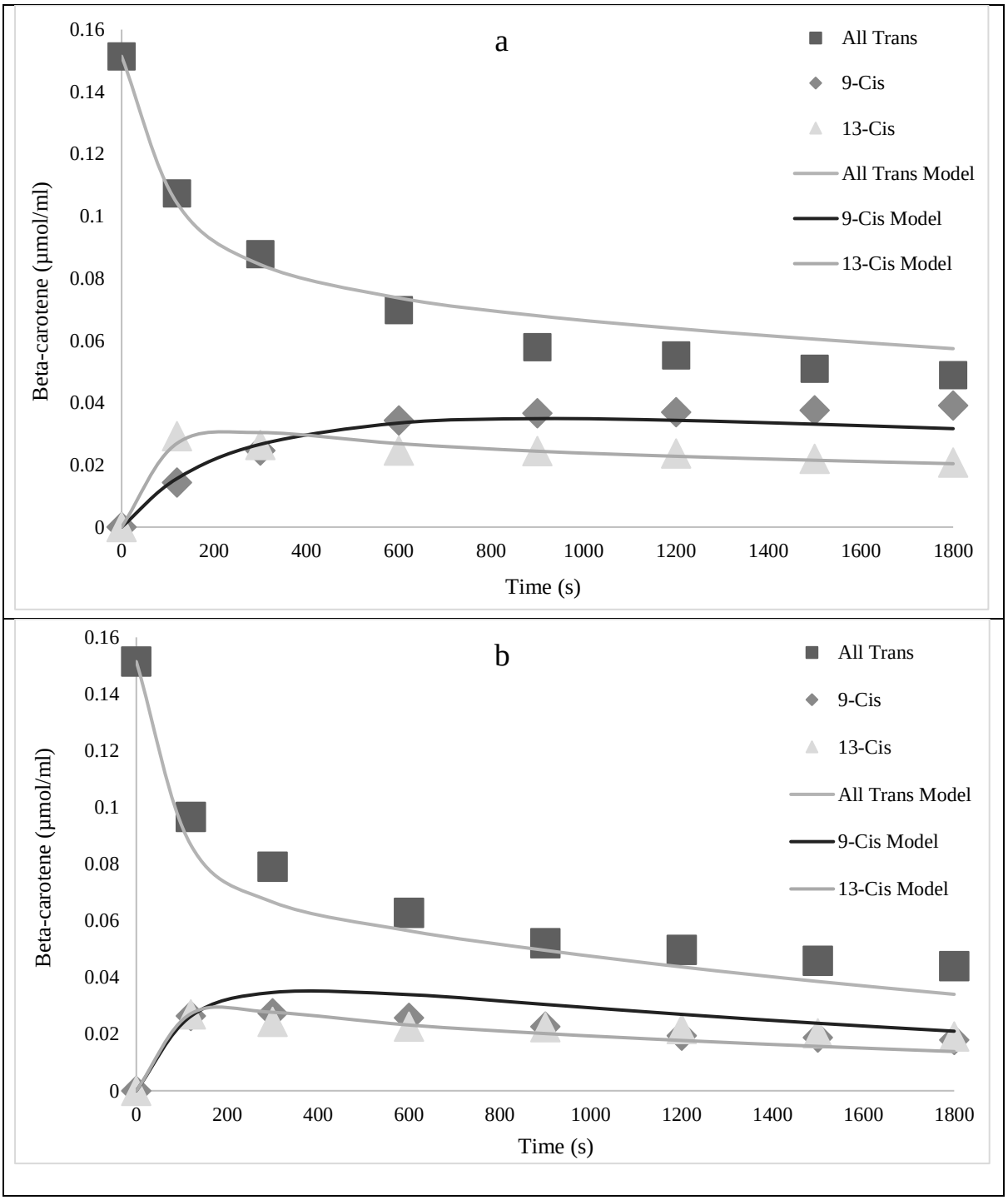


Figure 6.4: Schematic representation of the reaction network which includes degradation and isomerization of beta-carotene during heating in sunflower oil.


a
All Trans
9-Cis
13-Cis
All Trans Model
9-Cis Model
13-Cis Model
Beta-carotene (µmol/ml)
Time (s)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
200
400
600
800
1000
1200
1400
1600
1800
b
All Trans
9-Cis
13-Cis
All Trans Model
9-Cis Model
13-Cis Model
Beta-carotene (µmol/ml)
Time (s)

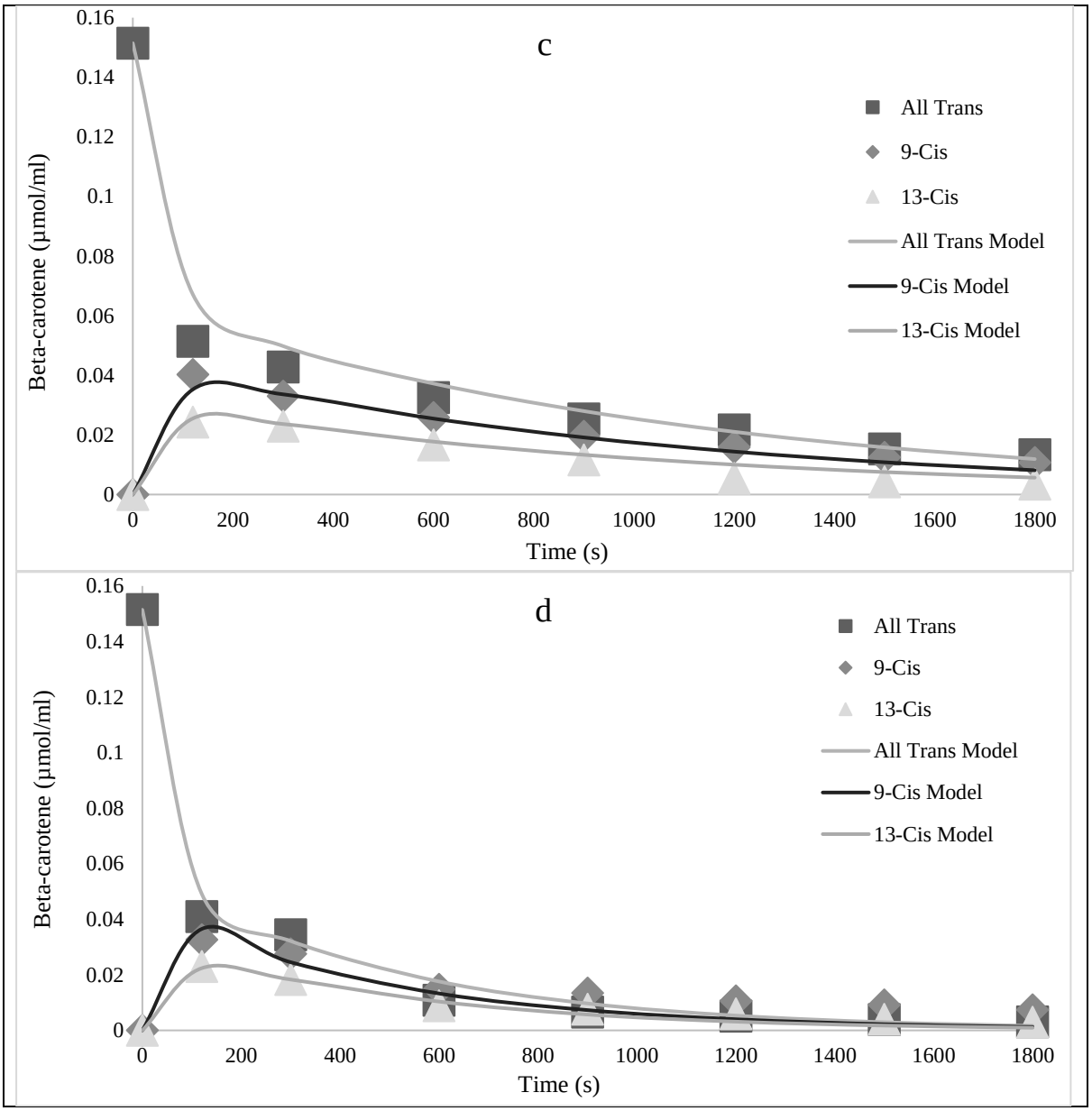


Figure 6.5: Concentration of beta-carotene isomers during thermal degradation of trans-beta-carotene in sunflower oil at different temperatures (a) 160 °C, (b) 180 °C, (c) 200 °C, and (d) 220 °C. Solid lines passing through the experimental points are deduced from indicate Athena Visual Software applied to reaction network shown in Figure 6.4. All experimental datasets were used for predictive modelling. Hence, no standard deviations applied.

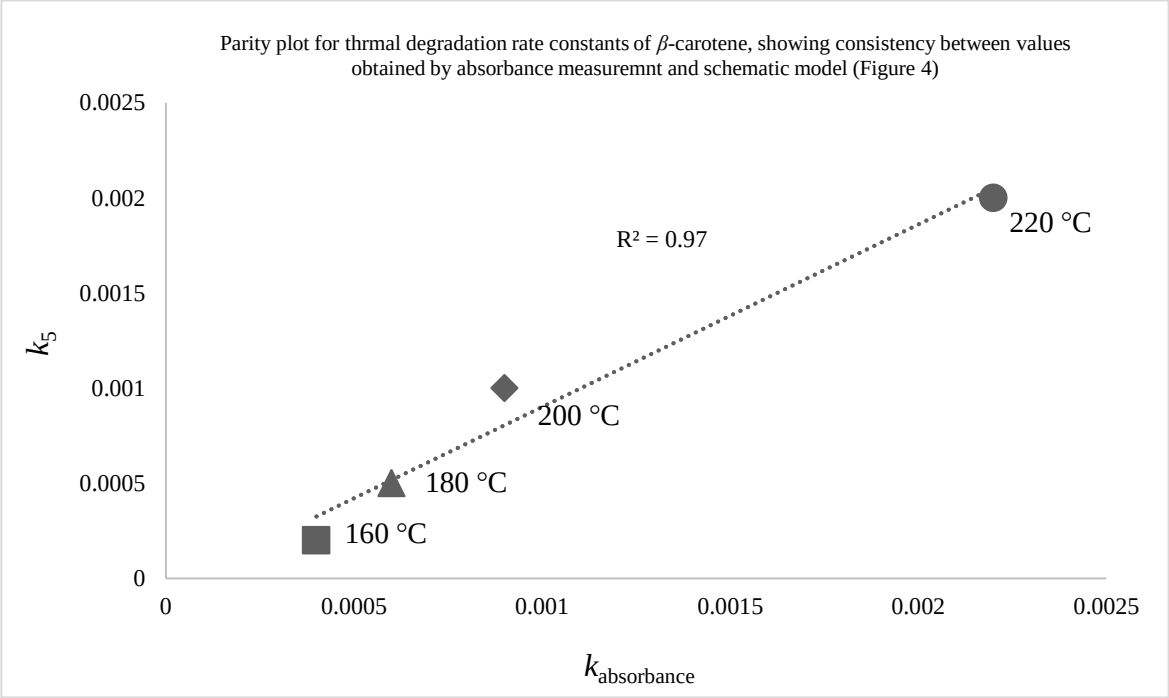


Figure 6.6: Parity plot showing consistency between the rate constant values obtained by absorbance measurement during beta-carotene degradation, and the value of k_5 estimated by Athena Visual Software.

Supplementary materials

Table 6.1S: Values of degradation rate constants in extract and solid phases for beta-carotene after fitting to equation (6.5) for beta-carotene. Experiments performed with solid to liquid ratio 20 kg m^{-3} and for different particle sizes at different temperatures.

Sl. No.	Particle size (mm)	Temperature (°C)	$k_1 \times 10^{-4}$ (Degradation rate constant in the solid phase, s^{-1})	$k_2 \times 10^{-4}$ (Degradation rate constant in the liquid phase, s^{-1})	$(k_2)exp \times 10^{-4}$ (Degradation rate constant in the liquid phase experimental, s^{-1})	SSE × 10^{-6} (Eqn. 10)	R^2 (Eqn. 12)	Adjusted-R^2 (Eqn. 13)	RMSE × 10^{-4} (Eqn. 11)
1	0.35	90	---	---	---	17.60	0.94	0.94	10.83
		115	---	---	---	14.07	0.95	0.95	9.68
		135	1.41±0.01	5.84±0.39	1.00	20.17	0.89	0.87	36.67
		150	1.33±0.02	4.68±0.21	2.00	62.18	0.92	0.90	20.36
2	0.75	90	---	---	---	5.92	0.96	0.95	6.28
		115	---	---	---	90.82	0.99	0.99	2.46
		135	1.22±0.01	0.51±0.02	1.00	3.73	0.97	0.97	5.16
		150	1.01±0.01	0.62±0.02	2.00	35.95	0.99	0.99	16.03
3	1.40	90	---	---	---	0.99	0.99	0.99	2.57
		115	---	---	---	5.88	0.96	0.96	6.26

135	1.42±0.06	1.34±0.05	1.00	86.01	0.98	0.98	2.47
150	1.45±0.07	4.08±0.10	2.00	14.74	0.92	0.90	10.26

Note: the model parameters were predicted by using average value of triplicate experimental dataset (n=3).

k_1– Degradation rate constant in liquid phase (s^{-1})

k_2– Degradation rate constant in solid phase (s^{-1})

$(k_2)_{exp}$– Degradation rate constant in liquid phase experimental (s^{-1})

SSE – Sum of squared errors

R^2 – Co-efficient of determination

Adj. R^2 – Adjusted Co-efficient of determination

RMSE – Root mean squared error

Chapter 7

Beta-carotene Fortification Through Potato Crisps: A Practical Approach to Enhance Vitamin A Status

This chapter is prepared as per the author guidelines to be submitted for publication to *Public Health and Nutrition Journal*

Abstract: South Asian countries have been facing a malnutrition challenge for decades and this has only grown further after the SARS-CoV 19 pandemic. Vitamin A deficiency is prevalent in children aged 6-59 months and in pregnant women, and is a major cause of fatalities and blindness in these groups. The objective of this study was to incorporate the precursor of vitamin A (beta-carotene) into a potential food vehicle (potato crisps) and assess the impact on plasma beta-carotene concentrations up to 7.5 h after consumption in healthy participants. Potato crisps were prepared by frying sliced potatoes in 0.037% beta-carotene enriched sunflower oil at 180 °C for 2.5 min and their moisture content, fat content and total absorbed beta carotene was measured. For the human study, 10 volunteers (n=5 males and n=5 females) were recruited with an average age of 27±3 years and a body mass index of 22.8±2.0 kg/m^2. Participants were asked to avoid eating any carotenoid-rich foods 24 h prior to the study visit, and fast overnight for 8-12 h. On the study day, blood samples were collected at regular intervals (every 1.5 h) for 7.5 h after consuming 50 g of potato crisps containing approximately 5 mg of beta-carotene. A standard low beta-carotene lunch was provided at 3.5 h. Fasting and postprandial plasma beta-carotene concentrations were measured by LC-MS. The amount of beta-carotene absorbed by the crisps was 4.86±0.21 mg/50 g of crisps, equivalent to half the daily requirements of vitamin A. A significant decrease ($p < 0.05$) was observed in the moisture content of potato slices before and after frying (75.23±0.35%, and 2.42±0.38% wet basis, respectively) whereas their fat content significantly ($p<0.05$) increased reaching a concentration of 32.3±0.9% wet basis in the final product. Following the consumption of the crisps, a significant ($p<0.05$) increase was observed in the plasma beta-carotene concentration from fasting (0.21±0.11 μg/ml) to end of study period at 7.5 h (0.45±0.17 μg/ml). The plasma concentration of beta-carotene was almost doubled after consumption of 50 g beta-carotene enriched crisps. As frying is very common in South Asian countries, incorporation of beta-carotene in potential food vehicle could help to counter vitamin A deficiency in the region.

Keywords: Beta-carotene; Potato Crisps; Bioaccessibility; Vitamin A Deficiency; Sunflower Oil.

7.1 Introduction

Beta-carotene, a natural pigment present in various fruits and vegetables, falls under the carotenoid category, contributing to the vibrant red, orange, and yellow hues in these foods (Boon et al., 2010). The isoprene structure of beta-carotene is composed of 40 carbon atoms that enables it for electron transfer to other molecules when reacting, contributing to its diverse functions. Abundant in foods like carrots, sweet potatoes, spinach, and mangoes, beta-carotene serves as a precursor to vitamin A (Mata-Gómez et al., 2014) which can be converted by humans into active vitamin A, essential for maintaining healthy skin, vision, and immune function (Strobel et al., 2007). Beyond its role as a nutrient, in an *in vitro* beta-carotene acts as an antioxidant, safeguarding cells from damage caused by free radicals (Boon et al., 2010). While supplements are available, it is generally recommended to obtain beta-carotene from a diverse diet rich in fruits and vegetables (Sharmin et al., 2016).

According to data from the World Health Organization (WHO), an estimated 250 million preschool children, particularly in low-income countries, suffer from vitamin A deficiency, which increases their vulnerability to infections and can result in higher mortality rates (Borguini et al., 2020). Moreover, its role in supporting maternal health and child development is pivotal, addressing risks associated with vitamin A deficiency during pregnancy and early childhood (Christian et al., 2013; Marjan et al., 2021). By promoting the consumption of beta-carotene-rich foods, such as leafy greens and vibrant coloured vegetables, and addressing nutritional gaps, communities in the developing world can not only combat deficiencies but also enhance overall health and well-being. This approach aligns with low-cost, sustainable interventions that leverage locally available resources and contribute to a more resilient and healthier population. To develop a low cost sustainable and indigenous intervention for developing countries, extraction of beta-carotene from the colossal amount of vegetables waste

and incorporation into any potential food as a vehicle could be one of the potential strategies to reduce the prevalence of vitamin A deficiency.

Previously, to explore the health benefits of beta-carotene, numerous food products were fortified with beta-carotene and *in vivo/vitro* studies were carried out. Showing an improvement in carotenoid bioaccessibility (Augusto et al., 2015; Eduardo Dutra-de-Oliveira et al., 1998). Furthermore, in these investigations, the food samples given to volunteers were typically prepared either at relatively lower temperatures which do not mimic the conditions in South Asian countries of frying and cooking at higher temperatures. For instance, Yao et al., (2023) provided vegetables oils with salad containing beta-carotene without any heat treatment and Livny et al., (2003) provided test samples of mildly cooked carrot and pureed carrot.

Thus, the primary objective of this investigation was to assess how beta-carotene responds to heating processes commonly encountered in industrial settings. By subjecting beta-carotene to conditions mimicking frying and cooking, it was aimed to elucidate its stability and bioaccessibility in these scenarios. The outcomes of this study are anticipated to shed light on the feasibility of using beta-carotene-enriched oils for frying, presenting a potential avenue for enhancing the nutritional content of food prepared through these methods. As it is evident, oils are abundantly used in developing countries to meet the daily needs of fats.

The overarching goal of this study was to characterize the bioaccessibility of beta-carotene in human subjects when consumed through a single portion of potato crisps, delivering an equivalent of 5 mg of beta-carotene (equivalent to 50% of vitamin A daily requirements).

7.2 Materials, subjects and methods

7.2.1 Food grade items

Food grade beta-carotene (purity, 98%) was purchased from Bio Sciences (New York, USA) for incorporation into crisps. Fresh potatoes (Maris Piper, UK) were purchased from a local grocery supermarket in the UK. Sunflower oil was purchased from local supermarket to maintain the consistency of the oil (Flora Brand, UK)

7.2.2 Chemicals

For extraction of beta-carotene from plasma methanol, tetrahydrofuran (THF), butylated hydroxytoluene (BHT), and ethanol was used of reagent grade (Fisher scientific, UK). For measurement of beta-carotene in crisps, reagent grade hexane was used.. LC-MS grade standard of beta-carotene (purity, 98%) was purchased from Sigma Aldrich (Merck, UK). Solvents used for LC-MS analysis were LC-MS grade including 0.1 M aqueous ammonium acetate, 0.33% acetic acid and 50:50 (w/w) methanol/2-propanol and were obtained from Sigma Aldrich (Merck, UK).

7.2.3 Preparation of beta-carotene enriched crisps

7.2.3.1 Potato slices preparation

Potatoes were peeled, washed and then sliced to 1.7±0.05 mm thickness by using domestic slicer (Kenwood, UK). The slices were washed to remove starch and intracellular matter released during slicing, and the excess water was removed with tissue paper.

7.2.3.2 Frying of potato slices

The potato slices were fried in beta-carotene enriched oil, which was prepared by dissolving 1 g of beta-carotene in 3L of sunflower oil using a high-speed mixer (Silverson, USA). The oil (3 liters) was transferred to a fryer (Buffalo Single Tank Single Basket 5 Litre Countertop Fryer 2.8 kW, UK) and heated to the frying temperature of 180 °C. Preliminary experiments

conducted without addition of beta-carotene in the same sunflower oil (data not shown) showed that a frying time of 2.5 minutes at 180 °C was sufficient to fry 200 g potato slices, to yield crisps with moisture content less than 3%; the average oil content of the crisps was found to be 30% on a wet basis (wb). The moisture content was determined using the standard hot air oven method and the oil content using the standard Soxhlet method (Albuquerque et al., 2012). The frying time was therefore set to 2.5 mins after which the crisps were left on the strainer of the fryer for 30-60 s to drain adhering oil. The fried crisps were then packed in air-tight containers wrapped in aluminum foil in order to prevent any direct contact with ambient air and minimize exposure to light. The crisps used in the human trials were prepared the day before each study visit. However it was observed that crisps stored under the conditions stated above could be preserved for up to 2 weeks without any significant change in sensorial properties.

7.2.3.3 Measurement of beta-carotene content of the fried crisps

Oil was extracted from the fried crisps using the soxhlet method as in section 7.2.2.2. The concentration of beta-carotene in the oil was determined by aliquoting 0.25 ml of the oil extract, mixing it with 3.75 ml of hexane and measuring the absorbance of the mixture against a blank solution of hexane and plain sunflower oil at 450 nm using a spectrophotometer (Jenway 6315, UK) as previously described (Kumar et al., 2024; Li et al., 2013). A standard calibration curve ($R^2 = 0.99$) was prepared by dissolving pure beta-carotene (Tokyo Chemical Industry UK Ltd, Purity 98 %) at a concentration range between (0.5 µg/ml and 12 µg/ml in a mixture of hexane and plain sunflower oil (8:1 (v/v)) and measuring the absorbance at 450 nm. Analysis were done in triplicate.

7.2.4 Human study approval

The study protocol was given a favourable opinion for ethical conduct by the University of Reading Research Ethics Committee (Study No UREC 23_28) and the study was performed in

accordance with the Declaration of Helsinki. Written informed consent was gained from each volunteer during the screening visit and on the study day.

Volunteer Screening: Adults aged 20-40 years and body mass index 18.5—30.00 kg/m^2 were recruited and the study was conducted at the Hugh Sinclair Unit of Human Nutrition, University of Reading between November and December 2024.. In order to participate in this study, suitable volunteers needed to: (1) be a non-smoker or have given up smoking more than 6 months prior to participation, (2) have normal blood pressure (<140/90 mm Hg), (3) not be pregnant or planning to get pregnant during the study period, (4) have no history of a heart attack, stroke, angina, thrombosis, liver or kidney diseases, diabetes, chronic gastrointestinal disorder, or cancer, (6) not be anaemic (haemoglobin > 130.0 g/L in males and >115 g/L in females), (7) not had a hysterectomy, (8) not have a history of excess alcohol intake (in excess of 14 units per week) or substance abuse, (9) not have food intolerances or allergies, (10) not be currently participating in another clinical trial, and (11) be prepared to follow dietary instructions prior to and during the study visit.

After a health and lifestyle questionnaire was completed, potentially suitable participants were invited for a screening session where the study was described in more detail. A consent form was completed before height, body weight and blood pressure were measured and a small venous blood sample was collected to measure the full blood count using a DxH520 haematology analyser (Beckman Coulter, High Wycombe UK). Height and weight of the participants were measured using a stadiometer (Seca) recorded to the nearest 0·01m and 0·1 kg, respectively. Resting blood pressure was measured three times while seated using an automated upper arm blood pressure monitor (Omron, HEM-7121) and the average values used as the final readings.

7.2.5 Main study visit

This proof of concept study included only a single visit in which the healthy males and females received 50 g of crisps fried in beta-carotene enriched oil. During the 24 h period prior to the study visit, no foods or supplements containing beta-carotene/carotenoids were allowed (an exclusion list was provided to the participants) and volunteers fasted from 8:00 pm onwards, drinking only water during this time.

On the visit day, measurements of height, weight, and blood pressure was taken. A finger prick blood sample was also collected to ensure that haemoglobin measured using a Haemocue device (HemoCue® Hb 201 DM System, Sweden) was >130 g/l for males and >115 g/L for females. A cannula was inserted into the antecubital vein of the forearm to allow blood samples to be taken over the 8 h period. The first blood sample was taken under fasting conditions, and this served as the baseline (control) sample. The volunteer was then provided with a breakfast consisting of 50 g crisps fried in beta-carotene enriched vegetable oil with a glass of water (200 ml). This meal contained approximately 5 mg of beta-carotene with the following macronutrient composition: energy 114 kcal, protein 2.1 g, carbohydrate 15.7 g, fat 5.2 g, fibre 1.2 g, and salt 0.6 g. The justification for the dose of beta-carotene provided in the test meal was twofold: firstly, to meet 50% of the daily requirements of vitamin A for the human body, and secondly, to avoid micellar loss of carotenoids which can take place when consumed beta-carotene in excess of 10 mg (Parker, 1996). After consumption of 50 g of crisps, venous blood samples were collected via the cannula every 1.5 h interval for 7.5 h. The volunteers were provided with a light lunch 4 h after the test breakfast was consumed and this meal did not contain any carotenoids/beta-carotene (white bread sandwich with chicken breast excluding all vegetables with 200 ml of water). The volunteers were allowed to drink water ad-libitum throughout the study visit.

7.2.6 Isolation of plasma

Blood samples were collected into lithium heparin tubes (VACUETTE; Greiner Bio-One) and placed on ice prior to centrifugation at 1750 x g for 15 min at 4 °C in a bench-top centrifuge. After centrifugation, plasma was aliquoted in 2 ml vials and frozen at -80 °C until analysis.

7.2.7 Extraction and determination of beta-carotene from plasma using liquid chromatography-mass spectroscopy in atmospheric pressure chemical ionization mode (LC-MS-APCI)

The extraction of plasma beta-carotene was conducted as previously described (Livny et al., 2003; Toh et al., 2021) with slight modifications to exclude the extraction of any lipid or oily materials present in plasma. Plasma aliquots (750 µl) were transferred to 15 ml plastic falcon tubes (Thermo Scientific, UK), and subsequently, 1 ml of methanol containing 0.1% (w/v) BHT was added to each tube. The resulting mixture was vortexed for 2 min to precipitate the protein within the plasma.

For the extraction of beta-carotene, 4 ml of THF was employed instead of the previously used combination of acetone and petroleum ether. This modification was based on the solubility data of beta-carotene in organic solvents, with THF demonstrating the highest solubility (Craft & Soares, 1992). The mixture was vigorously vortexed for 3-4 min before being subjected to gentle shaking at 100 rpm for 30 min at room temperature (OLS aqua pro, Grants, Fisher UK), followed by centrifugation at 8750 x g for 30 min to obtain a clear supernatant. The resulting yellowish supernatant was dried under a stream of nitrogen at room temperature until complete dryness was achieved. Prior to analysis by LCMS-APCI, the dried sample was reconstituted in 2 ml of ethanol and then filtered through a 0.2 µm polyethersulfone syringe filter (Fisher Scientific, UK).

Chromatographic separation of beta-carotene was carried out as previously described (Oxley et al., 2014) with some modifications using an Agilent series (Agilent 6546XT Q-ToF and Agilent 1290 Infinity II UHPLC) which was equipped with a Zorbax RRHD Eclipse Plus C_{18}

column (1.8 µm, 95Å; 50 mm x 2.1 mm i.d.) maintained at 20 °C. Mobile phase consisted of 0.1 M aqueous ammonium acetate, 0.33% acetic acid (A) and 50:50 (w/w) methanol/2-propanol (B) using the following gradient: 1 min linear gradient from 80% to 99% B, held for 3 min, then returned to 80% B for 3 min to re-equilibrate. Flow rate was 0.6 ml/min with an injection volume of 5 µl.

The QTOF was operating in positive ion mode with an APCI source. The source settings were Gas Temp. 350 °C, Vaporizer 400 °C, Drying Gas 7 l/min, Nebulizer 60 psi, VCap 2000V, Corona+ 4 µA, Fragmentor 175 V, Skimmer 65 V, Oct 1 RF Vpp. The instrument was calibrated immediately prior to run and a reference mass (922.009798 m/z) was infused throughout the run. The instrument was run in MS1 only mode acquiring data from 100 to 3200 m/z at 2 spectra/sec. Quantification was done against external calibration curves of beta-carotene . The data was analysed in Agilent Mass Hunter Quantitative Analysis Version 11.0.

The beta-carotene was used to assess the linear dynamic ranges, limit of detection, limit of quantification, and to construct external calibration curves. Stock solutions of beta-carotene was prepared in ethanol containing 0.1% BHT at a concentration of 0.2 mg/ml. A calibration curve for beta-carotene was prepared in ethanol at concentration range of 0.1-12.5 µg/ml. All standards and samples were run on the same day in triplicate.

7.2.8 Data analysis and statistics

The mean concentrations of beta-carotene from triplicate analyses in plasma, along with their corresponding standard deviations (±SD), were recorded. Similarly, the fat and moisture content of the potato crisps underwent the same analysis. To determine significant differences in beta-carotene levels among the study visit sampling points, a repeated measures one-way ANOVA was conducted, to determine the main effect of time. The analysis was performed using XLSTAT software version 23 (Addinsoft, Paris, France).

7.3 Results and discussions

7.3.1 Moisture, fat and beta-carotene content of potato crisps with and without beta-carotene

The data on moisture content of fried potato crisp, along with total fat and beta-carotene content can be seen in Table 7.1. It represents average of three individual samples (n = 3) with standard deviation. The moisture content for the potato slices before frying was observed to be 75.23±0.35% on wet weight basis (wb.). The moisture content of the fried crisps was noted to be on average 2.42±0.38% on wb. Moisture content is an important parameter for quality control of fried potato crisps to maintain its integrity and texture.

The absorption of oil into fried potatoes crisps occurs as the water leaves the product. For example, par-fried or completely fried potatoes have an oil content of 3–5% and 16–18%, respectively (USDA, 2011). Oil absorption primarily occurs after the product is removed from the fryer after cooking, so the oil content can be reduced by draining the product immediately after processing (Decker & Ferruzzi, 2013). In this study, the oil content was measured in triplicate using a conventional soxhlet method of fat extraction for crisps with and without beta-carotene. Significant ($p<0.05$) difference was observed in the oil content of crisps with and without beta-carotene, 32.34±0.94% and 29.87±0.41%, respectively. with those crisps fried using the enriched beta-carotene oil showing higher absorption of sunflower oil, which could be attributed to the lipophilic ability of beta-carotene. In previous studies, an analysis of 18 different commercial varieties of crisps for oil content was carried out with a reported range of oil content between 20.5-40.8% (Albuquerque et al., 2012; Mai Tran et al., 2007).

Beta-carotene content in the crisps was measured spectrophotometrically as described in our previously published article (Kumar et al., 2024) and results can be seen in Table 7.1. The average beta-carotene content of 50 g crisps was 4.86±0.21 mg.

7.3.2 Screening outcomes

The average age, BMI, systolic and diastolic blood pressure of the participants were 27±3 years, 22.80±2.0 kg/m^2 , 120±5, and 80±5, respectively.

7.3.3 Beta-carotene concentration in plasma

During and after feeding of the crisps enriched with average 5 mg of beta-carotene no adverse effects were reported by the volunteers.

The concentration of plasma beta-carotene over 7.5 h after the consumption of the breakfast test meal is shown in Figure 7.2. A monotonic increase in the plasma concentration of beta-carotene was observed. The increasing trend observed in this study, was notably more pronounced when compared to the results reported by Livny et al. (2003) and Johnson et al., (1996). Interestingly, there was no indication of a decrease in beta-carotene concentration in the plasma throughout the entire 7.5-hour duration of the study. However, peak concentration was yet to attain and a possible reason for not observing this (Van den Berg & Van Vliet, 1998; Yao et al., 2023) is due to the shorter intervention period of the current study. Because total absorption of beta-carotene virtually completes in nearly 12 hours (Van den Berg & Van Vliet, 1998). A significant rise in plasma concentration of beta-carotene was observed between the average value of baseline (t=0) and final time point (t=7.5h) with p-value of 0.003 analysed using Bonferroni correction. This demonstrates the bioaccessibility of beta-carotene consumed with potato crisps. This significant rise could be explained by the influence of simple potato crisp matrix (Van Loo-Bouwman et al., 2014) and the presence of sunflower oil during frying. Previously, it has been reported that the degree of unsaturation in fats facilitated the better absorption of carotenoids (Van den Berg & Van Vliet, 1998). The sunflower oil consist of 85% of unsaturated fatty acids and 15% of saturated fatty acids, that leads to the better absorption of the carotenoids in human body (Akkaya, 2018). Contrary to the current study, Yao et al.,

(2023), and Van den Berg & Van Vliet, (1998) reported a marginal decline in the concentration of the plasma beta-carotene after peaking between 5-7 h of the study. The plausible reason for the decline could be attributed to the chemical degradation and enzymatic action to breakdown the beta-carotene (Van den Berg & Van Vliet, 1998; Yao et al., 2023).

Yao et al. (2023) conducted a study examining the effectiveness of beta-carotene absorption from a vegetable source when co-consumed with two forms of olive oil: emulsified and natural. Their findings align with previous research indicating that co-consumption of olive oil with carotenoid-rich sources enhances carotenoid absorption (Akkaya, 2018). This correlation is well-established, reinforcing the study's observations. Prior studies have demonstrated that fats high in unsaturated fatty acids facilitate greater carotenoid absorption in the gastrointestinal tract compared to those high in saturated fats (González-Casado et al., 2018; Yi et al., 2015), supporting the bioaccessibility data presented in Yao et al.'s (2023) research.

Despite the extensive recognition of the significant impact of beta-carotene formulation on its bioaccessibility, particularly within encapsulated supplements, there remains a surprising gap in research regarding its application in industrially produced fried food/poducts, such as beta-carotene-fortified crisps. Numerous factors known to influence carotenoid bioaccessibility, including fat and fiber content, have been investigated. However, the variability in individual fat intake from meals, which can significantly affect nutrient absorption, has not been adequately considered. Additionally, critical factors such as the particle size distribution of emulsified oils, which are known to impact beta-carotene bioaccessibility, were not rigorously controlled in the current study.

The study's data, shown in Table 7.2, reveals the average baseline plasma concentrations of beta-carotene, as well as the final point and total increment over the intervention period for all volunteers, disaggregated by sex. The results indicate no observable or statistical difference

($p>0.05$) between the corresponding baseline or final time point values among males, females, or the combined group. This lack of significant change underscores the need for more precise control of variables that influence beta-carotene bioaccessibility in future research.

7.3.4 Reconciliation of data on absorption pattern of beta-carotene in *in vivo* studies

A comparative analysis of beta-carotene absorption across various dietary sources provides critical insights into its bioaccessibility dynamics. In this study, volunteers consumed crisps fried in sunflower oil enriched with beta-carotene. Figure 7.3 illustrates a progressive increase in plasma beta-carotene levels over a 7.5-hour period. This trend contrasts with earlier research, where beta-carotene levels typically plateaued five hours post-ingestion, except in studies by Van den Berg & Van Vliet (1998) and Yao et al. (2023), which noted a marginal decrease in plasma concentrations after seven hours. Notably, the volunteers in the current study consumed an average of 5 mg of beta-carotene, which is lower than the amounts in previous studies (Johnson et al., 1997; Livny et al., 2003; Van den Berg & Van Vliet, 1998; Yao et al., 2023).

The use of different food matrices likely impacted beta-carotene bioaccessibility, complicating direct comparisons. The continuous increase observed in our study suggests that crisps fried in beta-carotene-enriched oil could be a highly effective vehicle for beta-carotene delivery. Livny et al. (2003) found that pureed carrots resulted in greater beta-carotene absorption than fresh carrots, a phenomenon that might also apply to the enhanced absorption seen with sunflower oil-fried crisps. This aligns with previous findings that lipid-rich matrices can augment the absorption of fat-soluble compounds (Livny et al., 2003).

Considering the widespread consumption and accessibility of crisps, this discovery offers a practical method for beta-carotene supplementation, especially in populations with limited access to diverse nutrient sources. In contrast, Johnson et al. (1997) found slower absorption rates when beta-carotene was sourced from algae, underscoring the variability in absorption

kinetics depending on the beta-carotene source. These variations emphasize the necessity for personalized dietary recommendations that consider the nutritional compositions of different foods.

The implications of these findings are particularly significant for developing nations. They suggest that beta-carotene could be efficiently extracted from plant sources for use in cooking and frying, potentially addressing nutritional deficiencies and promoting healthier diets where nutrient diversity is limited.

Figure 7.3 also highlights differences in baseline plasma beta-carotene concentrations across studies, influenced by study-specific protocols and geographical dietary habits. For example, Van den Berg & Van Vliet (1998) conducted their study in the Netherlands, requiring participants to follow a low-carotenoid diet for a week, whereas the current study only mandated a 24-hour avoidance of carotenoid-rich foods. Johnson et al. (1997) in the United States instructed volunteers to maintain a low-carotenoid diet and avoid vitamin A and carotene supplements for two weeks, ensuring lower serum carotenoid levels at the study's start. Livny et al. (2003) and Yao et al. (2023) in Israel and Singapore, respectively, required participants to adhere to a low-carotenoid diet for three and seven days. These protocols resulted in varying baseline beta-carotene levels, with the lowest observed in Yao et al. (2023) and Van den Berg & Van Vliet (1998), and the highest in Johnson et al. (1997). This analysis underscores the importance of consistent dietary control and standardized protocols in beta-carotene absorption studies to ensure comparability and reliability of results.

The choice of crisp as a matrix stands out as a significant strength, offering a unique approach to the investigation. The selection of potato matrix was based on extremely poor source of beta-carotene (Diretto et al., 2007), easy cooking, highly popular matrix among the consumers (Mai Tran et al., 2007). Conversely, the reliance on a single study group leaves numerous avenues

for further exploration untapped. For instance, future research could encompass diverse age and sex cohorts, encompassing varying ranges of oil types and beta-carotene amounts. Furthermore, exploring the effects in populations deficient in vitamin A or implementing a pre-intervention period where participants abstain from carotenoids for 2-3 weeks could enrich the study's insights.

7.4 Conclusions

In conclusion, as part of a balance diet, potatoes fried in enriched beta-carotene sunflower oil could be a potential vehicle to increase the Vit A content in those populations where there is a deficiency in this vitamin. Furthermore, and contrary to previous findings, beta-carotene is stable at high temperature which offers the potential to generate improved beta-carotene enriched food products with enhanced nutritional profiles. This finding could be further supported by taking human trials in vitamin A deficient populations of different age and sex. The findings hold the potential to inform not only industrial applications but also public health strategies, particularly in regions where incorporating plant-based sources of beta-carotene into daily diets can offer substantial benefits.

7.5 References

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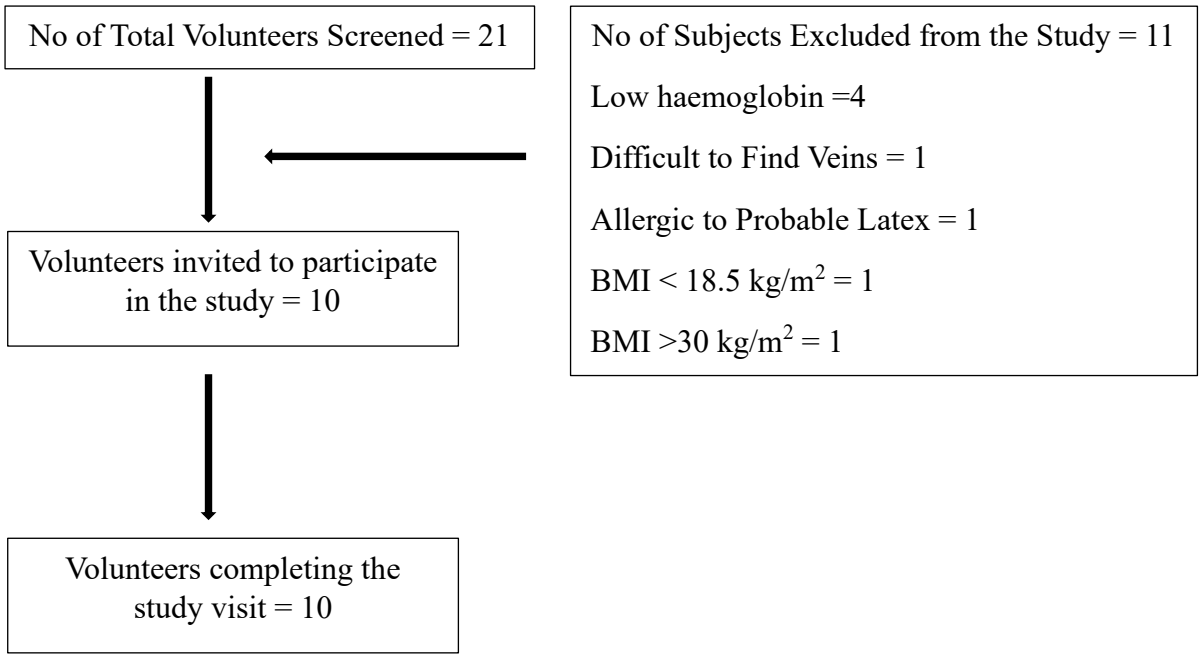

Figure 7.1: Flow diagram for the study

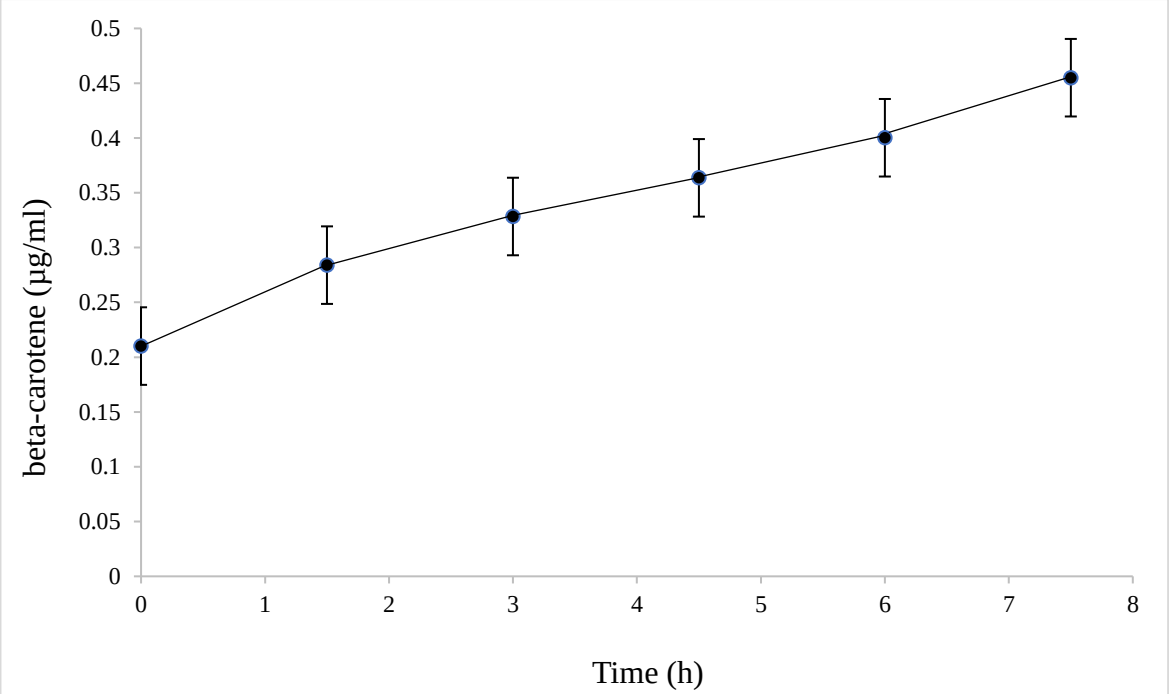

Figure 7.2: Time course of beta-carotene plasma concentration (values are given with average of n=3, with standard deviations; Total number of participants was 10; A fixed amount of 50g crisp was given to each participant).

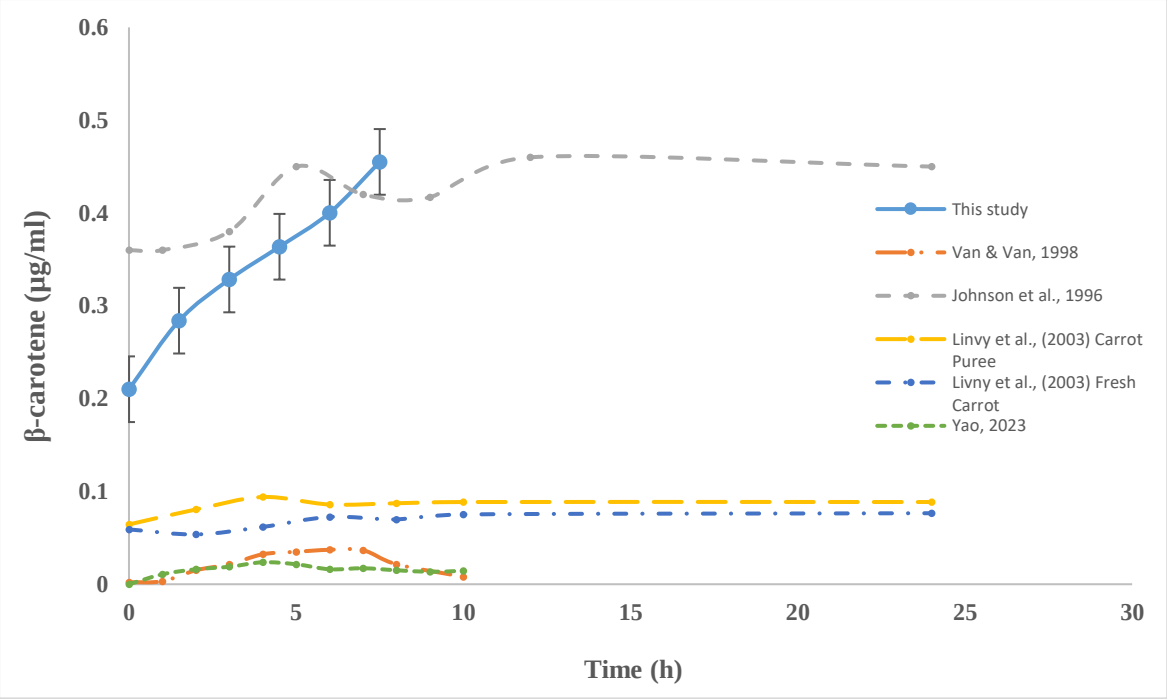

Figure 7.3: Illustration of the comparison between current and previous studies for beta-carotene uptake in plasma.

Table 7.1: Moisture (%), fat (%) and beta-carotene level (mg per serving) in fried crisps.

	Moisture content (wb, %)	Fat content (%) of crisps with beta-carotene	Fat content (%) of crisps without beta-carotene	Beta-carotene in 50 g of crisps serving (mg)
Fried crisps	2.42±0.38	32.34±0.94	29.87±0.41	4.86±0.21

Mean & standard deviation (n=3); wb-wet weight basis

Table 7.2: Illustrates the increments in beta-carotene plasma concentrations after 7.5 hours of study.

Subjects group	Total number	Average baseline beta-carotene (µg/ml)	Average final point beta-carotene (µg/ml)
All together	10	0.210±0.11[a]	0.455±0.17[b]
Male	5	0.165±0.11[a]	0.446±0.11[b]
Female	5	0.254±0.10[a]	0.472±0.22[b]

Note: Total No of subjects was 10. No of male subjects = 5; No of female subjects = 5.

Superscript a and b shows the difference between the groups value.

Chapter 8

8.1 Concluding remarks

The vegetable waste refers to the discarded part of the vegetables/whole vegetables which are not consumed. This type of vegetable waste is created throughout the food supply chain from production to processing (farm to fork). Managing vegetable waste is essential for both environmental sustainability and reducing the overall waste footprint, because the biodegradation of vegetable waste releases methane which is 25 times more harmful for the environment than carbon dioxide. Globally, one third of food produced intended for human consumption is wasted. Sustainable development and reducing food waste is one of the seventeen agendas of UNITED NATIONS. Hence, an integrative approach is needed to valorise the food and vegetable waste. In this study, beetroot and carrot were studied as a whole for the extraction of betalains and beta-carotene.

The key conclusions are as follows:

1. Chapter 4 of this thesis highlights the successful use of alternative green solvents, specifically citric acid solutions with varying pH levels, for the extraction of betalains. Given their polar nature, betalains were efficiently extracted using citric acid solutions, comparable to the performance of aqueous ethanolic solutions. This significant finding suggests that the dependency on organic solvents for the extraction of polar bioactives can be substantially reduced.

Employing such green solvents enhances the sustainability of extraction processes and aligns well with the thesis's objectives. The developed extraction process, initially tested on fresh vegetable materials, demonstrates the potential for adaptation to waste vegetables. This approach not only promotes environmental sustainability but also utilizes naturally occurring

citric acid as a solvent, creating an ideal method for sustainable bioactive extraction without compromising environmental integrity.

2. Chapter 5 addressed the limitations identified in Chapter 4 and the literature review by developing a mechanistic kinetic model to describe the solute mass balance in both the solid and liquid phases during the extraction of betalains from freeze-dried beetroot. The study revealed that betalains extraction was a rapid process at elevated temperatures without significant degradation. This approach resolved the issue of lower temperature extractions, which previously resulted in reduced extraction rates and yields.

The key model parameters obtained were found to be independent of scalability, indicating that the extraction process can be scaled up effectively. With precise control of reaction time, this scalable extraction method can minimize betalains degradation. This aligns well with established food engineering processes such as UHT and HTST, suggesting its feasibility for industrial applications.

3. The research conducted in Chapter 6 highlights the impact of elevated temperature treatment on the extraction kinetics, degradation kinetics, thermal stability, and isomer formation of beta-carotene during extraction and degradation studies. The study demonstrated that elevated temperatures (up to 150°C) can be effectively used to extract beta-carotene into edible oil. Despite some thermal degradation at high temperatures, the net extraction rates observed were significantly higher or comparable to those achieved with energy-intensive technologies such as pulsed electric field, microwave, and electrohydrodynamic combined with ultrasound.

Similar kinetic model to the one developed for betalains was used to predict the transient concentration of beta-carotene in sunflower oil, accounting for thermal degradation in both the solid and extract phases. This model showed a good fit with experimental data and has potential

applications in designing and sizing extractors. Additionally, beta-carotene-enriched sunflower oil was shown to be a viable frying medium to enhance the nutritional value of fried products.

A reaction network model was also developed to elucidate the kinetics of formation and degradation of each beta-carotene isomer during thermal degradation. This comprehensive understanding of beta-carotene behaviour under thermal conditions supports the practical application of this enriched oil in food processing.

4. Chapter 7 focused on the practical application of beta-carotene to enhance Vitamin A levels in consumers by providing a precursor to this essential nutrient. In this study, volunteers consumed potato crisps fried in beta-carotene-enriched sunflower oil, which led to a significant increase in the beta-carotene concentration in their plasma, indicating a positive response to the intervention. This approach could therefore serve as an effective method to boost Vitamin A intake, particularly in populations where there is a deficiency in this vitamin.

These findings have significant implications for both industrial applications and public health strategies. Findings also suggest that incorporating beta-carotene into everyday diets could offer substantial health benefits, especially in regions where Vitamin A deficiency is prevalent. This approach could be a valuable tool in addressing nutritional deficiencies on a broader scale.

8.2 Recommendations for future work

This thesis has primarily focused on the extraction of betalains and beta-carotene into specific solvents, but it has not address the separation and isolation of these bioactives. Also, the extraction process technology developed in this thesis was from the fresh raw materials such as carrot and beetroot, and the plausible reasoning was to maintain the uniformity in the raw material for experimental purpose. Future work must look to use waste materials and after extraction isolate the bioactives in various forms which can be used either for direct human consumption or as ingredients in food product formulations. New low environmental impact

solvents with high selectivity for these bioactives may have to be developed, from which the bioactives can also be easily separated.

1. In this study, the application of betalains was not explored. Given the diverse applications of betalains in the food and supplement industries, future work should focus on incorporating this colored substance into various food, drink, and supplement products. Studying its color stability and health effects in these contexts would be ideal, providing valuable insights into its practical uses and benefits.
2. This study employed sunflower oil for extracting beta-carotene. Other edible oils must be explored to ascertain whether the extraction kinetics and thermal decomposition kinetics are dependent on the chemical composition of the oil. Further, it is also necessary to ascertain the chemical characteristics of the oil after extraction to determine if there is a correlation between oil composition and stability.
3. Beta-carotene absorption into blood plasma was only studied after frying crisps in one oil, i.e, sunflower oil. The use of different edible oils must be explored in order to ascertain whether blood absorption of beta-carotene depends on the type of oil. Further, the human study undertaken in this research required the volunteers to consume beta-carotene devoid foods for 24 h prior to intervention. This period may be prolonged in future studies in order to minimise interference of inherent carotenoid in the blood.