Extraction and characterisation of bioactive compounds from New Zealand black-footed abalone

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Abstract

There is expanding interest in marine organisms as a source of new bioactive chemicals for development of novel health-promoting products. Marine species account for half of the world's biodiversity making them a rich source of new chemicals and the largest remaining source of beneficial natural molecules that might be exploited as functional ingredients in various food, cosmetic and pharmaceutical products. Abalone, a marine organism, have believed to provide health benefit with consumption for many years. Recent research has shown that abalone contain physiologically active chemicals with diverse modes of action, including antioxidant, antibacterial, anticancer, anti-hyaluronidase, anti-collagenase activities, and collagen. Thus, this thesis aimed to explore the extraction of bioactive compounds from Zealand black-footed abalone using novel environmentally friendly extraction techniques in order to make it an ideal candidate as a natural active ingredient for health promoting products.

Bioactive compounds were extracted from New Zealand black-footed abalone using subcritical water extraction (SWE), an environmentally friendly extraction technique. Abalone subcritical water extracts are promising sources of bioactive compounds, including antioxidants, glycogen, phenolic compounds, carbohydrates, proteins, and amino acids, based on the overall findings. Furthermore, the findings demonstrate that the efficiency of SWE is affected by temperature, which is a critical factor in demonstrating extraction efficiency. Antioxidant and antiageing properties of bioactive compounds were recovered from wild and farmed black-footed abalone using SWE. The black-footed abalone extracts obtained by SWE, found to be rich in majority of essential and non-essential amino acids and exhibited broad antioxidant and antiageing properties such as anti-hyaluronidase and anti-collagenase activity without causing significant toxicity at the high doses evaluated. It was also demonstrated that there was a strong correlation between antioxidant, antiageing and total phenolic compounds in both farmed and wild abalone extracts. Consequently black-footed abalone extracts were found as promising sources of novel anti-aging agents due to their high antioxidant, anti-collagenase and anti-hyaluronidase activities that could be incorporated into cosmetics. In addition, collagen type I was isolated from farmed and wild black-footed abalone using ultrasound assisted extraction (UAE) and CO_2 water extraction (CO_2-WE). Both techniques yield comparable SDS-Page, FTIR, and cytotoxicity results. However, CO_2-WE had higher collagen extraction yield, shorter extraction time and used water instead of organic solvent. Therefore, CO_2-WE could be an alternative promising technique than UAE with higher sustainability for enhancing the non-toxic extraction efficiency of collagen type I from black-footed abalone with applications in the cosmetic, biomedical, and pharmaceutical industries.

The overall findings of this study, have confirmed the feasibility of using SWE and CO_2-WE to produce high-quality abalone extracts from black-footed abalone. This study has also proved that New Zealand

black-footed abalone possesses vast unexploited potential to be used to make high value products. This research resulted in a better understanding of the composition and bioactivity of black-footed abalone that can inspire to further research of this native abalone and accordingly, deriving high value products and expand it's applications.

Keywords: Antioxidants; anti-hyaluronidase; anti-collagenase; protein; collagen; subcritical water extraction; bioactive compounds; cytotoxic activity; black-footed abalone; *Haliotis iri*s; amino acids; ultrasound assisted extraction; CO_2 acidified water extraction.

List of tables

List of figures

Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly define in acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning."

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Candidate contributions to co-authored manuscript

Chapter 3 *Extraction of bioactive compounds from black-footed abalone (Haliotis iris) using subcritical water extraction* Authors: Soniya Mohammadi, Ali Seyfoddin, Saeid Baroutian and Andrea C. Alfaro	Mohammadi 80%, Seyfoddin 8% Baroutian 8%, Alfaro 4%
Chapter 4 Antioxidant and antiageing properties of bioactive compounds extracted from wild and farmed black-footed abalone using subcritical water Authors: Soniya Mohammadi, Ali Seyfoddin, Saeid Baroutian and Andrea C. Alfaro	Mohammadi 80%, Seyfoddin 8% Baroutian 8%, Alfaro 4%

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Acknowledgments

In 2012, I enrolled in the Bachelor of Chemistry programme at Auckland University of Technology after graduating from high school. Through my years of bachelor studying, became more interested in science and during my final year of study, I was employed as a teaching assistant for both chemistry and microbiology fields. After graduating, I carried out scientific research to gain more knowledge and become closer to my future career. Therefore, I studied for a postgraduate diploma (one-year course) with a project on the treatment of colon cancer with fucoidan from brown seaweed at AUT and graduated in 2016. When I worked with fucoidan as a natural product, I was so excited and was looking for a project to discover a new natural compound. In the same year, I joined a drug delivery research group at AUT. I had a project on the extraction of natural blue pigment from black-footed abalone (*paūa*) for my master's degree.

During my master's degree, the demand for natural products became more apparent to me than it had been before. Then my passion for the need to increase my knowledge and discover something new became even more intense. On the other hand, I realised the significance of black-footed abalone not only in New Zealand, but also internationally. Thus, this led me to my PhD project to extract bioactive compounds from black-footed abalone with a potential in pharmaceutical, nutraceutical and cosmeceutical industries.

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extremely support, professional recommendations, and his help in thesis writing has contributed to the successful completion of this research project. I am thankful to Prof Andrea Alfaro, my co-supervisor, for her continuous help and support in my project. I always looked forwards to her detailed feedback, which has helped me improve my technical writing skills.

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Chapter 1. Scope

1.1. Thesis introduction and framework

1.1.1. General introduction

Extensive research is being conducted on the applications of natural materials as opposed to synthetic ingredients in a variety of human health-related fields, such as the food industry, the cosmetics industry, and the medical field, because of their perceived beneficial effects (Coelho et al., 2017). Therefore, significant effort is being directed toward the extraction of biologically active substances from marine organisms. Bioactive natural compounds or secondary metabolites are produced by living organisms with variety of activities including not limited to antioxidants, antimicrobial, antiviral, anti-hyaluronidase, anti-collagenase, and collagen as a result of the organism adjusting to its surroundings or are created to potentially serve as a defence mechanism against predators to aid in the organism's survival. (Choi et al., 2012; Dias et al., 2012).

Natural products, particularly those derived from microorganisms and terrestrial plants, have been a source of drug molecules for centuries (Newman & Cragg, 2004; Nigam et al., 2019). Most of the previous investigation done on natural products have been based on terrestrial sources. However, it has been demonstrated marine creatures have unique traits and bioactive substances that set them apart from terrestrial sources because of the variety of their environments (Ghosh et al., 2022). Thus, there is a great deal of interest in the study of ingredients derived from natural marine sources (Molinski et al., 2009; Munro et al., 1999). Scientific studies have shown that the majority of marine natural products are derived from the Phyla Porifera and Cnidaria (approximately 80%), and the remaining from the Phyla Echinodermata, Chordata, and Mollucsa (

Figure 1-1), reported in a review (Nigam et al., 2019).

The phylum Mollusca is one of the largest, most diverse, and most important groups in the animal kingdom, comprising snails, slugs, and other gastropods; clams, abalone, mussels, oysters, and other bivalves; squids, octopuses, and other cephalopods; and other less well-known, but nonetheless distinctive subgroups. Due to their high nutritional value, many molluscan species are designated as commercial animals and have been artificially bred or caught from the wild for human consumption (Wang et al., 2019). As food materials, animals in this phylum are abundant and easily captured, and can therefore be processed into a variety of industrial products, as well as developed into high-value items such as bioactive compounds. More than 95% of the world's biodiversity has not been evaluated for any biological activity. The challenge is how to access and use this natural chemical diversity effectively and efficiently (David et al., 2015).

Marine bioactive compounds can be extracted using either conventional or novel alternative techniques. Many industrial extraction processes use conventional organic solvent extraction techniques to recover bioactive compounds. However conventional extraction technique involve the use of large amount of solvents which are harmful to the environment (Munir et al., 2018). Therefore, to make extracts ready for medical purposes or food, solvent removal processes are essential. Additionally, high purity solvents may be expensive and sometimes are difficult to dispose of. Consequently, non-toxic extraction methods are required (Munir et al., 2018).

Alternative extraction technique, frequently referred to as "green" extraction methods, demonstrate a number of advantages over conventional methods, such as a decrease in the

amount of solvent used, a decrease in extraction time, and an improvement in performance. These methods have improved selectivity for the extraction of desired bioactive compounds, while preventing the formation of unwanted chemicals and reactions during extraction and optimising the extraction method. Novel extraction techniques most commonly used for extracting bioactive compounds from marine organisms including fishes, echinoderms, mollusca, sponges, and bacteria are subcritical water extraction (SWE), CO_2-acidified water extraction (CO_2-AWE), ultrasound assisted extraction (UAE), supercritical fluid extraction (SFE) as well as microwave assisted extraction (MAE) (Dang et al., 2017; Getachew et al., 2019; Sousa et al., 2020; Subra-Paternault et al., 2015; Zhao et al., 2009). The current context of sustainable development encourages the emergence of new products or new processes that satisfy societal expectations while simultaneously reducing polluting waste and energy costs.

This research focuses on naturally occurring bioactive compounds from indigenous New Zealand black-footed abalone (*Haliotis.iris*) that is belongs to the Halitodiae family of the class of Gastropoda. The rocky reefs along the subtidal and sublittoral coasts of the seashore are where black-footed abalone live. They inhabit crevices around stones of rocky reefs that are sheltered with algae/seaweed which they feed on (Allen et al., 2006). Māori (indigenous population of New Zealand) have long relied on black-footed abaloneas a food supply for several hundred years (Gray & Smith, 2004). Today, Majority (95%) of the abalone produced through aquaculture, as opposed to the earlier fisheries were the main source of global abalone production (Hernández-Casas et al., 2023). China and republic of Korea are the largest world abalone producers and followed by South Africa and Australia. Additionally, Chile, the Philippines, Taiwan, and New Zealand are other countries that cultivate abalone (T. V. Nguyen et al., 2022). The black-footed abalone is the only abalone species currently farmed in New Zealand. It is thought that New Zealand black-footed abalone is a valuable species with

excellent aquaculture economic potential because of its nutritional value (Grandiosa et al., 2016).

The purpose of this study was to successfully recover bioactive chemicals contained in farmed and wild black-footed abalone tissue utilising green extraction methods, such as SWE and CO2-AWE, which only utilise water. The application of SWE, and CO_2-AWE in the study of black-footed abalone tissue and its various bioactive compounds has never been attempted, making this a novel study.

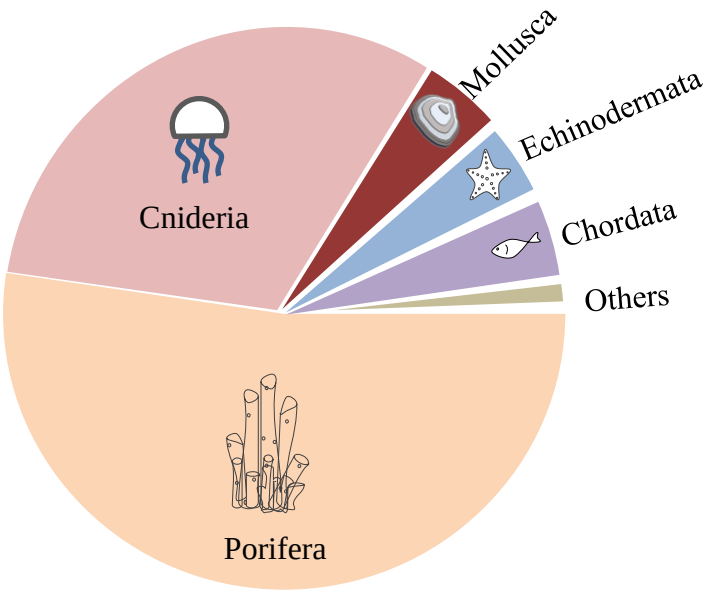

Figure 1-1. Representation of the different phyla according to the % of the existing Marine Natural Products (MNP)

1.1.2. Thesis aim

The main aim of this thesis is to discover the biological activities of compounds in New Zealand black-footed abalone extracts. The first and second sections of this thesis focuses on obtaining extracts from farmed and wild black-footed abalone using subcritical water extraction and alternative methods that are environmentally friendly and looking at various biological activities of the abalone extracts. While, the third section is about collagen extraction using another environmentally friendly technique, CO_2 acidified water extraction, which was compared with ultrasound assisted extraction (UAE) followed by characterisation of extracted collagen.

The specific objectives of this thesis are as below:

To investigate whether subcritical extraction can efficiently and selectively extract bioactive compounds from New Zealand black-footed abalone.

To determine the composition and biological activities of the black-footed abalone extracts.

To investigate the differences between biological activity of farmed and wild black-footed abalone extracts obtained by subcritical water extraction.

To evaluate whether CO_2 assisted extraction can efficiently and selectively extract collagen from New Zealand black-footed abalone

To compare properties of extracted collagen from New Zealand farmed and wild abalone

1.1.3. Thesis structure

This thesis consists of an introduction (Chapter 1), a literature review (Chapter 2), experimental studies (Chapter 3-5), a final thesis synthesis, discussion, and conclusion (Chapter 6) and a

comprehensive reference list. Chapter 2-5 are prepared as individual manuscripts, with minor modifications to conform to the postgraduate rules and regulations of the Auckland University of Technology (AUT). In the following section, the contents and rationales of core chapters are described.

1.1.4. Chapter contents and rationales

Chapter 2- Literature review: Recent studies on bioactive compounds extracted from marine organisms using a variety of extraction techniques.

A wealth of naturally occurring bioactive chemicals thought to improve human health have been produced by the great taxonomic variety of marine organisms. Bioactive compounds including, not limited to antioxidant, anti-collagenase, anti-hyaluronidase and collagen extracted from marine organisms, particularly molluscs have deposited an important and dynamic new field of research, resulting in significant advances in nutritional, medicinal and cosmetic knowledge. Predominant focus is on eco-friendly/green extraction methods that can isolate compounds with health-promoting properties. Therefore, at the beginning of this thesis, a literature review was conducted on bioactive compounds of marine organisms and their extraction techniques which were employed in the following Chapter 3.

Chapter 3- Extraction of bioactive compounds from black-footed abalone (*Haliotis iris*) using subcritical water extraction

After summarising the literature of bioactive compounds extracted from marine organisms using a variety of extraction techniques, the subcritical water extraction technique (SWE) was selected to extract bioactive compounds from New Zealand black-footed abalone (*Haliotis. iris*). Therefore, crude extracts with high biological activities were obtained from freeze-dried black-footed abalone at varying temperatures (110-280 °C) using SWE. The bioactivities of all extracts were identified by biological assays such as antioxidant (DPPH & FRAP), glycogen

content, phenolic content, carbohydrate content, protein content, and amino acids. Temperatures between 220 and 250 °C were associated with the highest levels of antioxidant activity, glycogen content, and phenolic content. Furthermore, the safety of extracts was evaluated using cytotoxicity assays. This research demonstrates that the extraction temperature at which black-footed abalone extracts were produced had a substantial effect on their bioactivity. Eventually, subcritical water extraction can be utilised to produce abalone extracts of superior quality.

Chapter 4- Antioxidant and antiaging bioactive compounds extracted from wild and farmed black-footed abalone using subcritical water extraction

Skin ageing cannot be reversed. However, it can be slowed down by therapy. Preventing the formation of wrinkles has become a crucial aspect of anti-aging skin care. This can be accomplished by limiting the breakdown of the skin's three key structural components, hyaluronic acid, elastin, and collagen, through the use of compounds with biological activity. Antioxidants, anti-hyaluronidase, anti-collagenase, and collagen are examples of biological activity that can assist in postponing skin ageing, repairing injured skin, and restoring skin. After confirming the efficiency and selectively of subcritical water extraction of bioactive compounds from black-footed abalone in Chapter 3, the temperature of 220°C with better bioactivity was selected to extract bioactive compounds from wild and farmed black-footed abalone at different extraction times (5-60 minutes). In this study, 14 extracts were analysed for their antioxidant properties (DPPH & FRAP), antiaging properties (anti-hyaluronidase & anti-collagenase activity), total phenolic content, glycogen content, protein content, and amino acids. Antioxidant capacity and antiaging properties as well as essential and non-essential amino acids were obtained at 30 and 45 minutes of subcritical water extraction, with no significant toxicity, making it a candidate for the development of new cosmetic products.

Chapter 5- Extraction and characterization of collagen from black-footed abalone (H.iris) by ultrasound assisted extraction and CO_2 water extraction

Utilization of marine-based collagen is expanding rapidly as a result of its exceptional properties including absence of disease transmission risk, low molecular weight, biocompatibility and its easy absorption by the human body (Jafari et al., 2020). Currently, marine collagen is extracted using solvent extraction techniques that are time-consuming, utilise organic solvent, and are harmful to the environment. To address this issue, this study focused on the extraction of collagen from farmed and wild black-footed abalone using CO_2-acidified water extraction (CO_2-AWE) and ultrasound assisted extraction (UAE). The result of this study showed extraction of collagen using CO_2-AWE improved the extraction efficiency in lower extraction time without using of organic solvent and toxicity.

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Chapter 2. Introduction: Recent studies on bioactive compounds extracted from marine organisms using a variety of extraction techniques

Abstract

Healthcare has become an integral part of current human society. With the ever-increasing production and consumption of numerous nutraceuticals, pharmaceuticals, and cosmeceuticals, it is imperative to find novel alternative resources to meet demands. Many of these new resources come in the form of bioactives from marine organisms. Abalone, a marine gastropod, is one such marine organism, from which bioactive compounds such as collagen possessing antioxidant, anticoagulant, anti-microbial, antibacterial, anti-viral, and anti-aging properties can be extracted. Bioactive compounds have been isolated using a variety of extraction methods, including conventional and non-conventional techniques. The New Zealand black-footed abalone is regarded as a delicacy both in New Zealand and internationally, particularly in Asian markets, where it is valued for its nutritional and health benefits.

This literature review provides a comprehensive coverage of recent research on types of bioactive compounds extracted from a range of marine organisms, predominantly molluscs. The review covers general information on various molluscan bioactive compounds, and different types of extraction techniques, including traditional and modern methods with their advantages and limitations. The literature review also provides a discussion on the identification and characterisation of value-added products. Additionally, it also reveals their possible potential in the industry.

2.1. Introduction

Consumers are increasingly preferring natural products over synthetic ones due to the numerous health benefits (Resende et al., 2021). Currently, more than 50% of marketed drugs are sourced from natural products [3]. Several natural product medicines originate from microbial and have been used clinically as anti-infectives. Many others, such as morphine, quinine, teniposide, topotecan, vincristine, and vinblastine have been derived from plants and are significant discoveries in human history. Natural product research is continuing to explore a range of lead structures that have the potential to be exploited by the pharmaceutical sector to produce new medicines. This is due to the failure of alternative drug discovery approaches to provide lead compounds in critical therapeutic areas, such as metabolic disorders, infections, and immunosuppression.

The ocean covers 70% of the earth's surface and has been shown to be a rich source of biological and chemical variety, representing a significant potential resource for innovative natural product development (Zhang et al., 2000). Marine natural compound studies only began in the last 60 years, with the exception of pigments extracted from marine molluscs as early as 1600 BC and some scents and vitamins extracted from cod fish oil (Carté, 1996). More than 10,000 compounds have been discovered from marine sources, and many of these have been utilized in clinical trials and show promise as potential medicines (Zhang et al., 2000). Consumer demand for natural products derived from marine organisms, such as seaweeds, fish, sponges, and molluscs for developing medication has risen dramatically in recent years. This is because the marine environment has an abundant natural supply of numerous physiologically active substances, including polyunsaturated fatty acids (PUFAs), sterols, proteins, polysaccharides, antioxidants, and pigments. On the other hand, with technological

advancements, the oceans have become more accessible for research, and the richness of its resources has become apparent.

2.1.1. Marine bioactive ingredients

Unique secondary metabolites (biologically active) are produced by numerous marine species inhabiting complex ecosystems that are not produced by any other organisms. Additionally, given the high taxonomic variety of the marine environment, research into the discovery of novel bioactive chemicals is an almost limitless field (Lordan et al., 2011). Molluscs, including mussels, clams, oysters, abalones, squids, bivalves, snails, octopus and squids are important sources of marine bioactives (L. C. Wang et al., 2019). They are constantly exposed to chemical and physical stress, wounds, microbial attacks, and intra/inter-species competition, therefore are biologically diverse (Suarez-Jimenez et al., 2012). Several molluscan species have been classified as commercial animals and are intentionally raised or harvested for human consumption due to their high nutritional content and health benefits. As food resources, organisms in this phylum are abundant and can be easily acquired and may thus be processed into a range of industrial goods, as well as developed into high-value products, such as nutritious foods, functional meals, and feed additives. Apart from being used as food, some species have a high medicinal value and have been utilised as folk remedies in a variety of places since ancient times long (L. C. Wang et al., 2019).

In traditional ways, marine organisms such as molluscs have been used to treat neoplasm, phlogosis, and digestive problems based on their active components such as sterols, nucleosides, mineral elements, carbohydrates (polysaccharides), lipids, and proteins, These compounds possess antioxidant, antimicrobial/antibacterial, anticoagulation, antiviral, anti-hyaluronidase, and anti-collagenase activities , which have enabled the organisms to adapt to

different types of stresses (Benkendorff et al., 2011; Pati et al., 2015; Shanmugam et al., 2013; L. C. Wang et al., 2019; Zou et al., 2017).

Antioxidant activity of compounds extracted from marine organisms

A substrate that inhibits molecular oxidation inside a cell is referred as an antioxidant. During biological oxidation reactions, free radicals are produced as by-products of metabolism. Their production is increased by exposure to toxic chemicals, environmental pollutants, sunlight, and ionizing radiation (Rao, 2016). Free radicals start chain reactions that lead to DNA, lipid and protein damage, and cell damage or death (Webb, 2017). Therefore, antioxidant agents are able to stop a chain reaction by free radical elimination. Antioxidants are also reducing agents, such as polyphenols, thiols and ascorbic acid. Antioxidants act in various ways, depending on their components: 1) prevent photo-oxidation by competing with the polymer in reaction with peroxide radicals, 2) entrap peroxide radicals and alkyl without letting the polymer enter the photodegradation propagation stage, and 3) inhibit hydro peroxide destruction during photodegradation reactions by breaking down peroxides in photo-excited polymer (Webb, 2017). Many chronic diseases such as heart disease or cancer as well as aging processes are caused by free radicals. Antioxidants are generally used as supplements in foods to decrease the production of free radicals and reduce cellular damage (Webb, 2017). In discovery of marine antioxidants, bioactive compounds with antioxidant properties have been extracted from various marine species such as snail (Gayathri et al., 2017), abalone (Herath et al., 2017), squids (Nakchum & Kim, 2016), echinoderms (Soleimani et al., 2016) and algae (B. Li et al., 2013). Marine antioxidants are mostly found in polysaccharides with high radical scavenging activity, which is due to sulphate and phosphate acting as electrophiles and enhancing intermolecular hydrogen abstraction (B. Li et al., 2013; Shanmugam et al., 2013; Tsiapali et al., 2001; Z. L. Wang et al., 2014; Zhu et al., 2008).

For example, water soluble sulphated polysaccharides conjugates derived from abalone (*Haliotis discus hannai Ino*) possess radical scavenging activity (Zhu et al., 2008). Conversely, the antioxidant activity of the purified sulphated polysaccharides conjugates exhibited less

antioxidant activity in compare with crude extracts which contained other components such as protein and lipids. In another study tissue extract of freshwater Ampullariidae snail *Pila virens* has novel bioactive compounds with antioxidant potential which could scavenged free radicals of Diphenylpicrylhydrazyl (DPPH) (Gayathri et al., 2017). Therefore, sulphation is not the only factor involved in the radical scavenging activity (Zhu et al., 2008).

Accordingly, it is further reported that the radical scavenging activity is also related to conjugated polysaccharides implying peptide involvement (X. Li et al., 2008). In another study, peptide (BNH-P7) was isolated from a blue mussel (*Mytilus edulis*) and its amino acid sequence was identified as YPPAK (Tyr-Pro-Pro-Ala-Lys), which was found to exhibit excellent free radical scavenging (B. Wang et al., 2013). It has been reported that glutathione reductase homolog (AbGSR) compounds , a peptide found in abalone (*Haliotis discus*), have a significant function in antioxidant-mediated defence and immunological mechanism (Herath et al., 2017). It is further reported that there is a high antioxidant activity with peptides that contains high amount of free amino acids such as alanine, leucine, methionine, valine, proline, and histidine (Guo et al., 2009; Wu et al., 2003). However, other study suggested that there is no correlation between protein content (peptide and amino acids) or polysaccharides (Zhu et al., 2008). On the other hand, Wang et al. (2008) hypothesised that antioxidant activity may involve a combination of factors and cannot be the result of a single factor.(J. Wang et al., 2008).

Therefor these positive activities have led to abalone receiving particular attention in drug discovery research. **Error! Reference source not found.**. The preceding discussion illustrates the several potent and significant antioxidant compounds that can be derived from marine microorganisms.

1.1.1

Antimicrobial/antibacterial activity of compounds extracted from marine organisms

Antimicrobial peptides have a significant function in the natural systems of both vertebrates and invertebrates. Antimicrobial peptides are small, cationic, and amphipathic in different sequences and structures that create powerful interactions with anionic molecules (Reddy et al., 2004). They have a wide range of activities against many organisms such as fungi, yeast, gram-positive and gram-negative bacteria (Peng et al., 2010). Many antimicrobial peptides/proteins have been isolated from marine organisms, especially molluscs, and arthropods. It was thought that the initial interaction between the peptide and the bacterial membrane depended heavily on the nature of the peptides. The majority of peptides showed a variety of microbial activity against gram positive and gram negative bacteria (Dolashka et al., 2011).

Antibacterial proline-rich peptides isolated from the haemolymph of marine snail *Rapana venosa* showed gram-positive and gram-negative bacterial antimicrobial activity(Dolashka et al., 2011). Moreover, carcinin (whey acidic protein) proline-rich peptide have been isolated from shore crab exhibited antibacterial activity (Brockton et al., 2007). Histone antimicrobial peptides has also been shown to have potent antimicrobial activity in marine organisms such as pacific salmon *Salmo salar* (Richards et al., 2001) pacific white shrimp *Litopenaeus vannamei* (Patat et al., 2004), scallop *Chlamys farreri* (C. Li et al., 2007) *Haliotis discus hannai and Haliotis discus discus* (De Zoysa et al., 2009). In the previous studies, histone antimicrobial peptides possessed antimicrobial activity, represented defence mechanisms and were active against gram positive and negative bacteria (Patat et al., 2004). Beside antimicrobial peptide, glycoprotein with antimicrobial activity was found in the mucin isolated from the body mucus of Giant African snail *Achatina fulica* containing two subunits (Iguchi et al., 1982). The extracted mucin showed positive antibacterial activity against both gram positive and gram-

negative bacteria. Hemocyanin (a copper-containing extracellular protein) found in the haemolymph of African Giant Snails*Lissachatina fulica* was found to have antibacterial activity against bacteria (Jummai & Okoli, 2013).

Corresponding to the previous studies, histone-derived peptides were the most peptides with antimicrobial activity in marine organisms. Therefore, marine organisms with limited studies (such as abalone) . could be a valuable source of antibacterial compounds and immunological histone peptides for the development of new drugs to combat a variety of infectious diseases.

Anticoagulation activity of compounds extracted from marine organisms

In normal homeostatic conditions of the human body, there is a constant balance between the production and elimination of clots, which are maintained by interaction between the coagulation (clotting) pathways, vascular endothelium, fibrinolytic system, and platelets. The coagulation pathway is a combination of intrinsic and extrinsic processes that result in the conversion of prothrombin to thrombin, which then results in the creation of fibrin. The platelets are stabilised, and a continuous clot is formed as a result of this development. Some conditions, such as hypertension, diabetes, and smoking, can cause an abnormal homeostatic condition (thrombosis), which is associated with a high mortality rate. The thrombosis mechanism begins with damages to the vessel wall which causes the rupture of atherosclerotic plaques. This rupture leads to aggregation of high amount of platelets and poses a significant risk of developing cardiovascular disease and strokes (Battinelli et al., 2013). To overcome the thrombosis disorder, anticoagulant drugs have been discovered and used such as warfarin, heparins, factor Xa inhibitors, direct thrombin inhibitors (DTIs), and Fibrinolytics. However, These anticoagulants were associated with a plethora of side effects including risk of haemorrhages potentially occurring in people with a prior history of stroke, chronic kidney disease, arterial hypertension, liver dysfunction, cancer, gastrointestinal bleeding, and intracranial haemorrhage (Harter et al., 2015). Compounds with anticoagulant activities have

been extracted from various marine sources. A previous study group successfully isolated heparin with a high molecular weight and anticoagulant activity from clams (*Anomalocardia brasiliana*) (Dietrich et al., 1985). Corresponding to Li et al. (2013), disc abalone (*Haliotis discus Hannai Ino)* extract improved Pt, APTT and TT. However, requires 2-3 times higher concentration (on a sulphated polysaccharide basis) to maintain the same as heparin activity (J. Li et al., 2013). In similar separation study, a sulphated polysaccharide fraction was isolated with anticoagulant activity from the gonads of abalone (*haliotis discus hannai Ino*) having high molecular weight and high anticoagulant activity (J. Zhao et al., 2016). According to Suleria et al, (2017), sulphated polysaccharides with anticoagulant potential were extracted from Blacklip abalone which could inhibit thrombin through cofactor II with concentration of 50% of 16 µg/ml compared to heparin at 2 µg/ml (Suleria, Masci, Zhao, et al., 2017).. . The above cited examples clearly shown that marine organisms can be an ideal source of anticoagulation ingredients in future.

Anti-viral effect of compounds extracted from marine organisms

Viruses are widespread in the oceans, with about 10^9 viruses per litre of water, vastly outnumbering bacteria and Archaea. Viral infections are common in marine environments, with an estimated 1023 infections every second (V. T. Dang, Benkendorff, et al., 2011). Marine viruses belonging to the *Herpesviridae* family have a morphology and protein structure that is comparable to the human herpes simplex virus 1 (HSV-1) (Defer et al., 2009). Antiviral compounds may have developed in marine organisms defend themselves from viruses because of natural selection. Secondary metabolites, bioactive peptides, and proteins are examples of natural defences against viruses produced by marine organisms, extraction of which can be used to generate novel medicines to treat human diseases. Nucleoside analogue inhibitors, such as acyclovir, famciclovir, valacyclovir, and the second line of antiviral medications for resistant virus, foscarnet, and cidofovir, are the most used antiviral treatments for herpes simplex virus

(HSV) infections. Thus, enhancing the efficiency of existing HSV treatment requires the development of novel antiviral drugs that target a variety of viral activities, particularly viral attachment (glycoprotein interaction with heparan sulphate proteoglycans HSPG) and entrance, which are regulated by glycoprotein (Talaei Zanjani et al., 2016). Hemocyanins are large copper-containing glycoproteins found in the haemolymph of most molluscs and arthropods. The main function of these glycoproteins is to take in, transport, and release oxygen into the right tissues. The immunogenic and antigenic character of these proteins, on the other hand, has made them fascinating targets in biological and clinical research (Zanjani et al., 2014). Various marine organisms have been shown to have antiviral effect against the HSV virus in previous studies (V. T. Dang, Benkendorff, et al., 2011; V. T. Dang, Speck, et al., 2011; Defer et al., 2009; Green et al., 2014; Talaei Zanjani et al., 2016). In a recent study by Green et al. (2014) antiviral activity of the pacific oyster *Crassostrea gigas* was investigated. N-linked glycoprotein isolated from haemolymph of pacific oyster could inhibit viral activity of herpes simplex virus type 1. They also discovered that antiviral activity is associated with cavortin, a major haemolymph protein. Cavortin is a monomer which may form intermolecular disulphide bonds with another peptide to form a multiprotein complex, and the antiviral activity may be attributed to this accessory peptide.

Dang et al. (2011) found that haemolymph extract of abalone *Haliotis laevigata* could inhibit herpes simplex virus at an early stage. They also discovered that the antiviral activity of abalone was likely due to protein and carbohydrates rather than lipophilic active compounds like fatty acids/aliphatic/aromatic alkaloid.

Abalone *Haliotis rubra* in a similar research was found to have a unique mechanisms of antiviral activity against herpes simplex virus 1 (HSV-1) infections (Talaei Zanjani et al., 2016). Furthermore, abalone hemocyanin impeded viral attachment and entry binding to the viral surface glycoproteins by imitating their receptors. Conversely, hemocyanin had no effect

on post entry occurrence and could not prevent infection by binding to the HSV cellular receptor.

In another study, antiviral activity of abalone *Haliotis laevigata* (greenlip), *Haliotia rubra* (blacklip) and their hybrid/farmed were investigated (V. T. Dang, Speck, et al., 2011). It was shown that both greenlip and blacklip abalone have similar antiviral activity against herpes virus. The hybrid abalone, on the other hand, had less antiviral activity. Various factors such as immunodepression, artificial diet of farmed abalone could influence their immune response and lead wild greenlip/blacklip abalone have higher antiviral activity than farmed abalone (V. T. Dang, Speck, et al., 2011). Therefore, these studies indicate that hemocyanin may be used to develop antiviral drugs to counteract HSV-1 infection from marine organisms. Additionally, more in-depth molecular analysis are required to discover a smaller hemocyanin domain that interacts with HSV-1.

Anti-hyaluronidase activity of compounds extracted from marine organisms

The extracellular matrix (ECM) contains an active and complex network of macromolecules encompassing and providing structure to the tissues and cells in the body. The ECM plays a crucial role in controlling several cellular mechanisms, such as gene regulation, migration, adhesion, and proliferation (Bissell et al., 1982). The most important constituents of ECM are the elastin, fibrous protein collagen, and proteoglycans (PGs). Hyaluronic acid (HA) is a linear polysaccharide that consist of alternating units D-glucuronic acid and N-acteyl-D-glycosamine via alternating β-1.3 and β-1.4 glycosidic linkages (Laurent & Fraser, 1992). Hyaluronic acid has an excellent hydration capability due to its hydrophilic property; thus, it also provides viscoelastic characteristics for the skin. Skin tissue contains almost 50% of hyaluronic acid (0.5-1mg/g) in the dermis (Laurent & Fraser, 1992). However, HA concentrations in the skin are reduced by aging processes that result in loss of laxity and skin moisture (Buhren et al., 2016). HA is produced by dermal fibroblast and epidermal keratinocytes, and HA fragments

are synthesized by HA synthase enzymes -1, -2, and -3 (HAS1-3), that link to the plasma membrane, and extrude hyaluronic acid into the extracellular cavity. In the skin, the HA chain has a lifespan of one day or less and is broken down into smaller chains by an enzyme (hyaluronidase). Hyaluronidase enzyme is located in the dermis and can attack HA and depolymerize it, causing dehydration of skin, wrinkling and sagging (Ratnasooriya et al., 2014). On the other hand, free radical and reactive oxygen species (ROS) can also interact with HA, causing its degradation (Buhren et al., 2016). The skin's moisture can be maintained by preventing matrix protein-degrading hyaluronidase and promoting the production of matrix proteins in the skin (Sutthiwanjampa & Kim, 2015a). In a study that extracted collagen from the skin of squid, it identified it to have a high antioxidant and inhibitory activities against hyaluronidase, which has the potential to enhance human skin morphology and delay ageing (Nakchum & Kim, 2016). The Alcalase hydrolysates of fresh and boiled Venus clams displayed the greatest hyaluronidase inhibitory action among the hydrolysates generated by five different proteases, according to in vitro scientific investigations. After fractionation, one of the fractions had the greatest specific activity for hyaluronidase inhibition with 141.15 percent mL/mg. As a result, it was recommended that Alcalase hydrolysate from cooked Venus clams can be employed as a cosmetics agent (Sutthiwanjampa & Kim, 2015a). A bioactive complex was isolated from small sea fish in a different investigation. Sulphated glycosaminoglycans (44-60%), essential amino acids (2-6.5%), essential fatty acids (1-2 percent, -3, -6) and mineral salts are all abundant in the complex. The complex was found to inhibit hyaluronidase activity as well as collagenase activity. Due to the high concentration of glycosaminoglycans GAG, the collagenolytic activity of cartilage degradation processes is interrupted, extracellular matrix is recovered, and a significant antioxidant activity is manifested (Roçoiu et al., 2010) **Error! Reference source not found.**. The existence of metabolites in marine species indicates their efficacy in medicinal applications.

Anti-collagenase activity of compounds extracted from marine organisms

UV exposure leads to a variety of physical alterations in the skin that include reactive oxygen species (ROS), matrix metalloproteinases (MMPs) and elastase secretion. The oxidative damage of skin lipids, proteins, and DNA that is caused directly by ROS leads to skin aging. Additionally, ROS can indirectly stimulate MMP production through the MAP-kinase activation. MMPs are zinc-dependent extracellular proteinases classified into five groups based on their substrates: stromelysins, gelatinase, collagenase, membrane-type MMPs (MT-MMPs) and others (Pientaweeratch et al., 2016). Collagenases are transmembrane zinc endopeptidase enzymes which play an important role in variety of biological processes, such as tissue homeostasis, tissue repair after wounding and tissue remodelling during development. Nevertheless, over activation of collagenases, which is due to chronical aging and photoaging, results in remodelling of extracellular matrix (ECM) including the breakdown of collagen, finally causing sagging, laxity and wrinkles of the skin (Hartmann et al., 2015). Therefore, an agent is required to inhibit collagenase activity to maintain healthy skin by preventing degradation of dermal matrix (Bo & Hyun, 2005). Marine organisms are a promising source of novel bioactive compounds since they are known to create powerful photoprotective metabolites as a defence mechanism (Hartmann et al., 2015). Investigations have shown that natural compounds extracted from marine sources, may limit collagenase expression via inhibiting MMPs expression (N. T. Nguyen et al., 2013). There was study on inhibitory effects of phlorotannins in brown algae (*Ecklonia cava*) on MMP activities by Kim et al. (2006). They investigated that brown algae extract could specifically inhibit MMP-2 and MMP-9. However, the brown algae extract inhibited MMP-2 expression and activity at higher levels than MMP-9. The high concentration of phlorotannin in the extract caused the inhibitory effects on MMP activities (M. M. Kim et al., 2006).

Nguyen et al. (2013) found that, octameric oligopeptide isolated from abalone *Haliotis discus Hannai* have MMPs inhibitory effect. They found that the extracted abalone oligopeptide (AOP) from *H.discus hannai* intestine could inhibit MMP-2/-9 expression. The data implied that AOP may be able to treat and MMPs-related disorder such as cardiovascular and angiogenesis diseases. Additionally, due to the direct involvement of MMPs during ageing, the development of MMP inhibitors has been seen as a promising strategy for the prevention of wrinkle formation. Therefore, based on the previous studies mentioned above, marine organisms can be a good source of anti-collagenase activities.

Collagen extracted from marine organisms

Collagen is the main component of extracellular matrix (ECM) of all connective tissues, such as cartilage, ligaments, bone, skin, and tendons. Collagen maintains structural and biological integrity of ECM and delivers physiological function and mechanical strength to the body. It gels because of being hydrophilic with high water absorption capacity. Tropocollagen (collagen basic unit) is made up of three polypeptide chains that are twisted in a left-handed helix (a-chain) and intertwine around one another forming a right-handed triple super helix. Collagen forms when tropocollagen polymerizes and are covalently crosslinked (Uriarte-Montoya et al., 2010). Collagen is used in pharmaceutical, biomedical industries and tissue engineering as drug delivery system, injectable dispersion, microparticles, and scaffolds intended for bone regeneration (Silvipriya et al., 2015). Collagen has been extracted from porcine and bovine for many years. However, their use has become limited in the last few decades due to the massive risk of transmitting different diseases, such as foot and mouth disease (FMD), bovine spongiform encephalopathy (BSE), and transmissible spongiform encephalopathy (TSE). Previous observations have identified that at least 2-3% of the population develops immune and allergenic reactions towards animal-derived collagen (Davison-Kotler et al., 2019; Rodríguez et al., 2017). Due to these limitations, scientists are

looking towards alternative sources of collagen, such as from marine organisms. Marine sources are safe to use in applications, as they do not pose any risk of disease transmission, are environmentally suitable and readily available, have exceptionally low immunogenicity, and have a higher collagen yield when compared with other sources (Silvipriya et al., 2015). Collagen has been extracted from fish, squids, mussels, sharks, and many others by various extraction techniques which are mentioned in **Table** 2-2. Uriarte-Montoya et al. (2010) have isolated collagen from jumbo squid (*Dosidicus gigas*) with 15% yield for the total protein. High transition temperature (123 °C) was observed by the extracted collagen. The obtained collagen consisted of two α-chains α 1 α 2 chain bands in which the proportion of α 2 chain band was greater than α 1 chain band. A detected band with a molecular weight of 190 kDa may be related to the β-chain, which is thought to be a dimer of the α-chains and it is typical of type I and V collagens found in squid mantle (Uriarte-Montoya et al., 2010).

There was a study on squid (Teuthoidea: Cephalopoda), particularly the Antarctic squid *Kondakovia longimana* and the Sub-Antarctic squid *Illex argentinus* as a potential collagen source (Coelho et al., 2017). Collagen has been isolated and purified from an Antarctic squid and Sub-Antarctic squid species to provide an alternative to the more popular bovine and porcine origins. It was discovered that the collagen extracted from *I. argentinus* had highest yield (about 3%), with high purity, and more preserved structure, showing an SDS-PAGE pattern and amino acid profile that are compatible with type I collagen.

In another study collagen has been extracted successfully from byssus (by-product) of Chilean mussels (*Mytilus Chilensis*) (Vallejos et al., 2014).The amino acid profile of the Chilean mussel Byssus revealed a high concentration of imino acids including hydroxyproline (considering the hydroxyproline concentration as indicator of collagen content) was 43 g/100 g protein and proline was 7 g/100 g protein, and it is almost entirely made up of proteins (82 percent dry

basis). The obtained collagen was highly cross-linked due to presence of only β and γ bands. Accordingly, the findings of earlier studies indicated that the Chilean mussel byssus is a reliable source of collagen that can be extracted for a variety of uses.

Kittiphattanabawon et al. (2010) had a research on extraction of collagen from skin of brown banded bamboo shark *(Chiloscyllium punctatum*) as an alternative to collagen production form cattle bone and skin, and pig skin. They could successfully isolate collagens (acid soluble and pepsin soluble) from the brown banded bamboo shark skin. The yields of isolated acid soluble and pepsin soluble were 9.38% and 8.86% (wet weight basis) respectively. The main component of extracted collagens were α- and β-chains. Both collagens were characterised as type I collagen with the cross-link of α 2-chain to β chain. The transition temperature of acid soluble and pepsin soluble extracts were around 34 °C. As a result, the skin of brown banded bamboo sharks may be used as a substitute source of collagen for various purposes.

According to the previous studies, marine organism's collagen can be an alternative to land based for food and pharmaceutical application. Moreover, there have been limited studies on molluscs, significantly abalone. It is possible that abalone can be used to extract collagen for therapeutic benefit.

Table 2-1. Isolated marine bioactives and their related activity.

Source	Active component	Bioactivity	Reference
Abalone (Haliotis discus hannai Ino)	Sulphated polysaccharides	Antioxidant	(Zhu et al., 2008)
Donacid clam (*Donax scortum*)	Sulphated Chitosan	Antioxidant	(Shanmugam et al., 2013)
Blue mussel (*Mytilus edulis*)	Peptide	Antioxidant	(B. Wang et al., 2013)
Abalone (Haliotis discus hannai)	polysaccharide	Antioxidant	(Z. L. Wang et al., 2014)
Green Algae (Enteromorpha prolifera)	Polysaccharide	Antioxidant	(B. Li et al., 2013)
Sea urchin	Pigment	Antioxidant	(Soleimani et al., 2016)
ampullariidae snail Pila virens	-	Antioxidant	(Gayathri et al., 2017)
Squid (Todarodes pacificus)	Collagen	Antioxidant, anti-hyaluronidase, anti-tyrosinase	(Nakchum & Kim, 2016)

Sea fish (Sprattus sprattus sprattus, Odontogadus merlangus euxinus and Engraulis encrassicolus ponticus)	glycosaminoglycans	Anti-collagenase, anti-hyaluronidase,	(Roçoiu et al., 2010)
Brown algae (Ecklonia cava)	Phlorotannins	Anti-collagenase	(Choi et al., 2012)
Algae (Palmaria and Porphyra sp)	Mycosporine-like Amino Acids	Anti-collagenase	(Hartmann et al., 2015)
Abalone (Haliotis laevigata)	haemolymph	Anti-viral	(V. T. Dang, Benkendorff, et al., 2011)
Pacific oyster (*Crassostrea gigas*)	haemolymph protein	Anti-viral, anti-bacterial	(Green et al., 2014)
Bivalve molluscs (Cerastoderma edule, Ostrea edulis and Ruditapes philippinarum)	-	Anti-viral, anti-bacterial	(Defer et al., 2009)
gastropods (Buccinum undatum and Crepidula fornicate)	-	Anti-viral, anti-bacterial	(Defer et al., 2009)

Abalone (Haliotis discus hannai Ino)	Polysaccharide	Anti-Coagulant	(J. Zhao et al., 2016)
Blacklip Abalone (*Haliotis rubra*)	-	Anti-Coagulant and Anti-Thrombotic	(Suleria, Masci, Zhao, et al., 2017)
Seahorse (Hippocampus kuda bleeler)	Peptide	Anti-inflammatory	(Y. J. Yang et al., 2012)
Molluscs; Donax faba	Shell extract	Antibacterial	(Giftson & Patterson, 2014)[20]
Green macroalgae (*Ulva armoricana)*	Polysaccharide	Antibacterial	(Berri et al., 2016)
molluscs (Rapana venosa and Helix aspersa)	Haemolymph	Antimicrobial	(Dolashka et al., 2016)
Scolopendra subspinipes mutilans	Peptide	Antimicrobial	(Peng et al., 2010)
Abalone (Concholepas concholepas)	Haemolymph	Antimicrobial	(Jummai & Okoli, 2013)

Table 2-2. Collagen extraction from various marine sources

Source	Collagen	technique	Solvent	Yield	Reference
Jumbo squid	Acid soluble	Solvent extraction/acidic extraction	Acetic acid	Low	(Uriarte-Montoya et al., 2010)
Chilean Mussel	Acid soluble	Acidic extraction	H_2O- HCL	Low	(Vallejos et al., 2014)
		Acidic extraction with the aid of pepsin-substrate enzyme	Pepsin-substrate	High	
Mussel	Acid soluble	Acidic extraction with the aid of pepsin-substrate enzyme	Acetic acid	High	(Rodríguez et al., 2017)

Squid	Acid soluble	Acid-based and pepsin-based extraction	Acetic acid	High	(Coelho et al., 2017)
(Sting ray and shark)	Acid soluble	Acidic extraction with the aid of trypsin- substrate	Acetic acid	-	(Z. Li et al., 2013)
Spanish mackerel (fish)	Acid soluble	Acidic extraction with the aid of pepsin	Acetic acid	-	(Chi et al., 2014)
Tuna skin	Acid soluble	Acidic extraction with the aid of pepsin	Acetic acid	-	(Ahmed & Chun, 2018)
Shark skin	Acid soluble- pepsin soluble	Acidic extraction	Acetic acid	-	(Kittiphattanabawon et al., 2010)
		Acidic extraction with the aid of pepsin	Acetic acid	-	

The significance of New Zealand Black-footed abalone

Abalone first appeared during the Cretaceous period (66 million years ago) and retains several ancestral characteristics. Abalone are sedentary, typically moving just short distance at night to feed on drifting seaweed and clamp to the rocks to avoid predators or being dislodged by wave movement. Due to their limited mobility, they have evolved a variety of morphological and metabolic approaches to utilise of their surroundings, some of which may appear ineffective but have shown to be extraordinarily beneficial (Hahn, 1989).

The term "Abalone" is an American English version of the Spanish term "Abulón," which refers to a variety of species of single-shelled molluscs belonging to the Haliotidae family (*genus Haliotis*) of the Class Gastropoda and the Phylum Mollusca. Linnaeus gave the genus name *Haliotis,* which means "sea ear," (Lopez et al., 1998). The family is distinguished by its rounded to oval shell, two to three whorls, and the final auriform whorl that grows into a huge 'ear,' giving it the name "ear-shell." It has a single convex shell that ranges from highly arched to exceedingly flat. The shell's muscles keep it attached to the body. It can stick to rocky surfaces at a variety of subtidal depths along the coast because to its large muscular foot. The foot is enclosed by an epipodium that contains sensory tentacles that enable the abalone to sense its surroundings. Internal organs (three chambered heart, digestive gland, reproductive organ, and kidney) are spiralled beneath the shell around a strong adductor muscle that connects the shell to the foot. The mantle, which is also connected to the adductor muscle, covers the organs, and secretes material for shell formation. The mantle is transparent and opens around the head, revealing the enormous bipectinate gills underneath the respiratory pores (Hahn, 1989). The respiratory system is controlled by a series of holes on the anterior edge of the body whorl (four to ten, depending on the species). Water flows over the gills where it meets the anus at the posterior end, picks up waste, and leaves via the posterior pores. The colours of the

internal and exterior shells, vary considerably from species to species, ranging from silvery white to green, blue, and red.

There are more than 100 species of abalone in the world. Three of the species are endemic to New Zealand including white-footed abalone (*Haliotis virginea*), yellow-footed abalone (*Haliotis australis*) and the commonly found black-footed abalone (*Haliotis iris*) (Tuterangiwhiu, 2015). *Haliotis iris* are usually situated in low sedimentations (low intertidal and subtidal zone) along the coasts of both main islands of New Zealand and the Chatham Islands, Stewart Island, and the Snares Islands. New Zealand black-footed abalone is the largest species (7-18 cm maturity) with a rainbow-like shell, adductor tissue and foot Figure 2-1.

Black-footed abalone is an edible sea snail that is called pāua by Maori who have been harvesting this species of abalone for centuries (Behrens et al., 2002). The active components of abalone, such as protein, carbohydrates, and fats, are influenced by whether the abalone is harvested in the wild or on a farm. (Bullon et al., 2022). Wild abalone (adductor and foot) comprises of 14-18% protein, and 0.2-1% lipid (HATAE et al., 1995; C. H. Tung & Alfaro, 2011). On the other hand, farmed abalone contains 40-56% protein and 5-7% lipid. In general, black-footed abalone contain 0.5-7% carbohydrates. Both lipid and carbohydrate contents are highly variable depending on the season, and are higher in the summer (HATAE et al., 1995; Shi et al., 2020). Due to the higher levels of dietary protein and lipids in formulated feed of farmed abalone, they have a higher nutritional value than wild abalone.

Total export value of abalone was $35 million with the export volume 674 tones p.a in 2019 (Seafood New Zealand, 2019a; Statistics, 2020). Black-footed abalone is the only abalone cultured for the export market and its farming industry first began in the mid 1980's (Setyono, 1997).

Aquaculture in New Zealand is putting in a lot of effort to reach its $3 billion yearly sales target by 2035, as well as having a greater significance in a lower-emissions economy. This aim can be reached through three crucial factors, which includes extending aquaculture into the open ocean, adding value to existing farms through innovation, and expanding high-value aquaculture on land (Ministry for Primary Industries, 2019). Deriving products of great value from New Zealand aquaculture such as extracts, oils and powders, can deliver premium and high-value products throughout the world.

Black footed abalone has gained international recognition as one of the most expensive and premium food products due to its high nutritional value particularly in Asian countries. However, more research is required on isolation and identification of high-quality compounds with pharmacological, nutraceutical, or cosmeceutical applications that will deliver premium, high value products to the world.

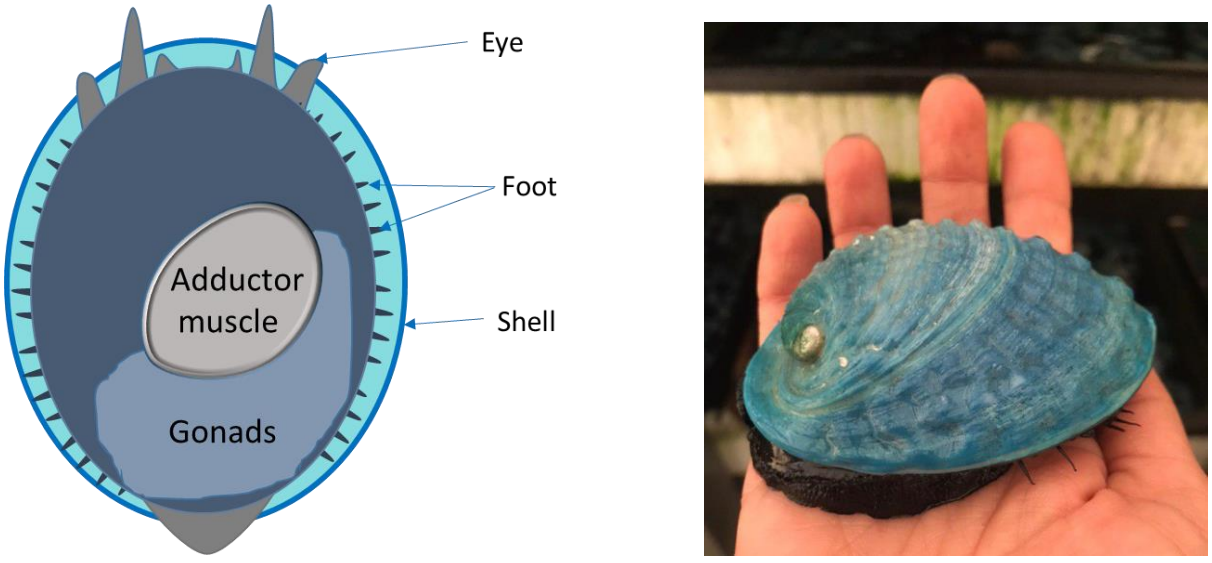

Figure 2-1. Body structure of black-footed abalone

2

2.2. Extraction of valuable products from marine organisms

Valuable marine products can be recovered using extraction techniques. Extraction is the process of using a solvent to separate soluble molecules from insoluble components in a solid or liquid mass (Mendiola et al., 2013). Extraction occurs in four steps: 1) solute desorption from active site of sample matrix; 2) diffusion of extraction fluid into the matrix; 3) spread of separated solute into the extraction fluid and 4) analysis of obtained analytes (Heng et al., 2013). Extraction techniques continue to be refined to provide better techniques to achieve higher yield from natural sources. There are two general extraction techniques namely conventional and non-conventional.

2.2.1. Conventional solvent extraction

Conventional solvent extraction technique (traditional method) is a simple method that has been used in laboratories for many years. Solvent extraction is liquid/liquid extraction that separates compounds based on their solubility and polarity of targeted compounds. It has been classified into; Soxhlet extraction, maceration (soaking), percolation and counter current extraction (Roselló-Soto et al., 2016). However, conventional extraction techniques are frequently limited by mass transfer resistance caused by the presence of more than one phase

in the system. These techniques may require more time to extract depending on the solvent diffusion rate and time. They consume a lot of energy. Moreover, conventional extraction techniques are manual operations, making repeatability difficult. Furthermore, these techniques are expensive with poor selectivity, and require high amounts of pure organic solvents. Use of these techniques can potentially result in the formation of organic waste, which could be harmful to the environment and have the possibility of decomposing thermos labile compounds (Azmir et al., 2013; Mendiola et al., 2013; Roselló-Soto et al., 2016).

2.2.2. Non-conventional extraction

A range of alternative techniques have been created in response to rising awareness of the need for environmentally friendly and cost-effective techniques (or for production sustainability). To overcome the limitations of traditional methods, novel non-conventional extraction techniques including subcritical water extraction (SWE), ultrasound assisted extraction (UAE), microwave assisted extraction (MAE), supercritical fluid extraction (SC-CO_2) and CO_2 assisted extraction have been developed. These techniques are fast, safe, and cost effective, generate high yields with improved quality for bioactive compounds recovery (Vernès et al., 2019). These methods will be discussed using their mechanisms, principles, advantages, and disadvantages in the following sections.

Subcritical water extraction (SWE)

A green extraction technique that gives high yields with less organic solvent usage in a short period is subcritical water extraction. SWE is an attractive system for extraction of substances from biologically active compounds (Nastić et al., 2018). This technique uses water at high temperatures (100-374°C) and high pressure to conserve the liquid state. Some properties of water such as high polarity, high dielectric and high boiling point for its mass have made SWE distinct from other extraction techniques (Haghighi & Khajenoori, 2013). The method of SWE

is based on how greater temperature affect the characteristics of water. In SWE process, by increase in temperature, the permittivity decreases, which leads to an increase in the rate of diffusion and a decrease in both viscosity and surface tension of water. This enhances water mass transfer resulting in higher extraction rate and yield. The dielectric constant is another feature that gets altered during subcritical water extraction. Water is strongly polar and has a high dielectric constant in its natural state as a liquid. When the temperature of water increases in a subcritical condition, the dielectric constant drops to levels comparable to organic solvents. Lowering the dielectric constant improves selectivity for bioactive chemicals with lower polarity, particularly those in the middle polarity range (Gan & Baroutian, 2022). Therefore, slightly polar and non-polar substances need a less polar medium that is influenced by high temperatures whereas materials with high polarity and solubility in water are extracted at lower temperatures (Smith, 2006). Organic components in subcritical water have high solubility due to high temperature that causes an increase in solubility and makes the water less polar (Khajenoori et al., 2009).

SWE consists of pumps (for water and extract), tank (organic solvent), extraction vessel, oven (to heat extraction vessel), pressure restrictor (to maintain pressure), heat exchanger (to decrease temperature of the extract), and sample collection. Subcritical water extraction has six steps: 1) quick entry of fluid, 2) matrix active site absorbs the solute from the sample, 3) solutes dispersion in organic materials, 4) solute dispersion in static fluid through permeable materials, 5) solute dispersion in stationary fluid layer, outside particles and 6) solute elusion by flowing fluid (Haghighi & Khajenoori, 2013). Thus, this technique is fast, inexpensive, and clean providing higher yields than conventional methods (solvent extraction).

As shown in Table 2-1 a few studies have been conducted on extraction of bioactive compounds from marine sources using SWE. Antioxidant was the dominant bioactivity that was obtained from marine organisms by SWE. Antiinflammation, antimicrobial,

antihypertensive and angiotensin-converting enzyme (ACE), and acetylcholinesterase (AChE) inhibitory were other bioactivities that were obtained by this extraction technique. Differences in achieved optimal yields of target bioactivity, may be attributable to polarity or stability of the compounds, extraction parameters as well as different species. Furthermore, temperature has a significant effect on total yield of bioactive compounds in SWE. This is due to the fact that the solubility and mass transfer of SWE are temperature dependent. The dielectric constant decreases with increasing temperature, allowing for increased solubility of medium polarity compounds. However, higher temperatures can lead to the degradation of certain substances. Therefore, the extraction temperature must be optimised to minimise degradation (Gan & Baroutian, 2022).

Ultrasound assisted extraction (UAE)

Ultrasound assisted extraction is another useful non-conventional technique requiring less extraction time and provides with higher extraction yields of products in comparison with the solvent extraction technique. This technique is suitable for bioactive compound extraction as it can obtains high quantities of compounds in a short extraction time (Prakash Maran et al., 2017).

Ultrasonic-assisted extraction involves the use of sound waves with frequencies between 20 to 100 kHz (Lazarjani et al., 2021). Passing waves create zones of high and low pressure, and the amount of energy delivered to the system is proportional to the change in acoustic pressure (Zou et al., 2017). Traditional methods for extracting compounds from biological materials, such as simple maceration, require a long time and use a large quantity of solvents. Ultrasound-assisted extraction (UAE) is a modern technique being investigated to solve these difficulties by increasing extraction efficiency, selectivity, and kinetics. UAE is a simple technology for extracting bioactive compounds from a wide range of sources (Falleh et al., 2012). Ultrasound improves extraction effectiveness by employing ultrasonic waves to increase solvent

penetration into materials and the contact surface area between solid and liquid phases (T. T. Dang et al., 2017). Furthermore, the enhancement of mass transfer through the cell wall due to the bursting of bubbles formed by cavitation is related to the destruction of cell walls, reduction of particle size, and enhancement of the extraction process utilising ultrasound (T. T. Dang et al., 2017). There are two types of ultrasound instruments, ultrasonic probe (direct sonification) and ultrasonic bath (indirect sonification). They are differentiated by way of the wave affecting the materials and operating condition. Ultrasonic probe operates at the frequency of 20 kHz, but the ultrasonic bath operates at frequency of 40–50 kHz. Ultrasonic probe is placed onto the sample, but in the ultrasonic bath, samples are placed into the ultrasonic bath (Ciko et al., 2018). These techniques operates with low temperature which preserve thermolabile compounds (Oh et al., 2011). It has been used to extract antioxidants, polyphenols compounds with various solvents such as distilled water, methanol, ethanol and others (T. T. Dang et al., 2017; S. H. Lee et al., 2013; Rodrigues et al., 2015). It has also been used to extract collagen with acetic acid (Petcharat et al., 2021). By increasing the temperature, the yield of product (phenolic) increases due to transfer of a larger amount of mass and high rate of solvent diffusion (Ciko et al., 2018). Elevated power increases yield of the product by causing intense damage to the cell wall of the material, which result in higher penetration to the solid. This approach has previously been used to extract bioactive compounds from marine organisms, **Error! Reference source not found.**.

Microwave-Assisted Extraction (MAE)

Microwave-assisted extraction (MAE) is an effective extraction technique which uses microwave irradiation for removal of different compounds from natural sources in a short time. This technique is economical and environmentally friendly compared with conventional techniques (Balasubramanian et al., 2011). MAE is a simple technique having a large number of applications, and requires low consumption of organic solvent and molecules. However, in

conventional extraction, heat and mass are quickly processed generating high extraction yield and good reproducibility. (L. Zhao et al., 2009). Mass and heat gradients work in the same direction in MAE, where heat generates within particles by interactions between polar compounds in opposite direction, following which the surface of the particles is heated and transmitted to the core. MAE contains an oscillating electric filed with frequencies 300MHz-300GHZ or wavelength ranging from 1mm-1m in MAE. These frequencies cause oscillation of polar molecules that lead to intra and inter molecular friction. Rapid heating occurs by the collision of these frictions and ion charges. Extra heating causes pressure to breakdown the cell wall and the membrane. This pressure increases in the sample cells, then the compounds transfer faster into extracting solvent (Grosso et al., 2015; Kaufmann & Christen, 2002; Rostagno et al., 2009; L. Zhao et al., 2009). In MAE technique, polar solvents are better than non-polar, as polar solvents are high dielectric solvents which absorb higher energy and results in the solvent reaching the extraction temperature faster. Dielectric constant solvents decrease in the following sequence: water, methanol, ethanol, acetone, ethyl acetate and hexane (Heng et al., 2013).

1.1

1.2

Supercritical fluid extraction (SFE)

Supercritical fluid extraction is the process of using supercritical fluid as an extracting solvent to isolate one component from a matrix component. A supercritical fluid is any materials that is at a temperature and pressure higher than critical point. Critical point is the specific pressure and temperature above which liquid and gas phases do not exist (Azmir et al., 2013). Supercritical properties is between liquid and gas where the density and viscosity of the SFE are comparable to those of a liquid and a gas, respectively, and the dispersion of the fluid is intermediate between the two states (Roselló-Soto et al., 2016). SFE penetrates as a gas through

the solid and it dissolves components as a liquid. In 1970s, supercritical fluids have been assessed with supercritical water, supercritical toluene in petroleum, shale oil cleansing and supercritical carbon dioxide (CO_2). Application of Supercritical CO_2 was so significant in the last decade due to its critical temperature of 31 °C that allows the biological compounds to be processed around 35 °C. Its critical pressure is above 74 bar and acts as a nonpolar solvent. Thus, it is suitable for the extraction of fat, lipid and non-polar compounds (Azmir et al., 2013). A product can be obtained by this technique without any solvent residues. Supercritical extraction usually uses CO_2 for extraction of high value products from natural sources at high pressure. Supercritical CO_2 sometimes requires a co-solvent such as methanol or ethanol. Supercritical CO_2 has low solubility that specifies low polarity extraction using organic solvents as a co-solvent can vary solvent polarities leading to more selection of extraction (Roselló-Soto et al., 2016). Supercritical CO_2 extraction is inexpensive, odourless, tasteless, non-flammable and non-toxic. Dispersion in supercritical fluids is faster than in liquids, as viscosities are lower than liquids and there is no surface tension. Thus, solvents can pass through small pores of matrix unreachable to liquids, which enable this technique to be fast. This technique has high application in nutraceutical, aromas, essential oils, food and fuel industries (Mendiola et al., 2013). As **Error! Reference source not found.** shows,

CO_2 acidified water extraction (CO_2-AWE)

CO_2 acidified water extraction is a new green extraction technology that uses less organic solvent and takes less time to extract substances. Carbon dioxide has recently gained popularity because to its appealing features, which includes minimal flammability, toxicity, and cost, as well as high stability, availability, and environmentally friendly. This technique is based on water pressurized with carbon dioxide (Silva et al., 2016a). Carbon dioxide is utilised as a reversible acidifying agent that is removed from the aqueous media following extraction, resulting in the extraction of the product. The use of CO_2-acidified water allows for a single

extraction step with mild operating conditions and avoids the use of any organic solvent (Sousa et al., 2020). Recently this extraction technique has been used to extract collagen in a few research, which are summarised in Table *2-3.* Novel extraction techniques for marine bioactive . Barros et al. (2014) for the first time developed an innovative green technology for CO_2 acidified water extraction of collagen/gelatine from marine sponges (*Thymosea sp, Chrondrila nuculla and Chondrosia reinformis*). The collagen extraction was processed over 16 hrs at 37°C and 50 bar. The intended technique enabled an extraction of nearly 50% of the collagen/gelatine content of the sponges, which indicating an improvement of more than 30% over traditional extraction method using acetic acid. The extracted collagens had a high degree of purity, and an analysis of their amino acid composition revealed common characteristics to collagen derived from other marine sources. Thus, the result presented, suggest that using water and carbon dioxide for extraction of sponge-origin collagen, is a promising alternative technique to traditional methodology.

In another study, Silva et al. (2016) could optimise the CO_2 acidified water extraction process operating conditions to produce high yields and high-quality collagen while also reducing extraction procedure duration and energy consumption. They extracted collagen at different processing times (3, 13.5 and 24 hrs) and different pressure (10, 30 and 50 bar) with a constant temperature (37 °C). The sponge dry mass yield was about 10% under mild operating conditions (10 bars, 3 hours), which corresponds to a process extraction efficiency of more than 50%. In addition, it was established that the suggested methodology, improved extraction yield by 30% compared to conventional acid/enzymatic method. The obtained extract contained a highly pure blend of collagen and gelatine that had similarities to those of collagen obtained from other marine organisms. (Silva et al., 2016b). Furthermore, the outcomes show how effective this strategy is and how much industrial potential this technology has for producing marine collagen/gelatine with qualities appropriate for biomedical applications.

Similar research involved extracting collagen from the skin of Atlantic cod (*Gadus morhua*) using CO_2-acidified water (Sousa et al., 2020). The collagen extraction was processed in a similar condition to the previous research at 37 °C, 50 bar for 3 hours. The yield of extracted collagen was 13.8% (w/w) which was significantly higher than previous studies using traditional method. The authors suggested that the extraction yield improvement in this study could be due to single step with mild condition using acidified and pressurised water in compare with traditional extraction which requires multiple steps. Based on characterisation of extracted collagen, the properties of obtained collagen in this study showed that the extracted collagen is type I which is compatible with extracted collagen by traditional methodology.

Therefore, the result support the efficiency of the proposed approach for collagen extraction. Additionally, only three studies have utilised this novel extraction method to extract collagen from Atlantic cod and sponges. Other marine organisms with greater availability and higher extraction yields can be used with this.

Advantages and disadvantages of non-conventional extraction techniques

One of the big challenges in pharmaceutical, nutraceutical and cosmeceutical industries is replacing of onerous solvents such as methanol, hexane, acetonitrile etc. with green solvents such as water, CO_2, and ethanol (Roselló-Soto et al., 2016). In comparison to conventional techniques, non-conventional methods are primarily focused on solvent replacement as well as a reduction in temperature and treatment duration. Non-conventional extraction techniques including UAE, MAE and SC-CO_2 are affordable, effective, inexpensive, reproducible and decrease the extraction time for recovery of valuable products. These methods can be used alone or in conjunction with traditional methods to reduce solvent consumption. However, the effect of UAE is highly dependent on the substance and the presence of a dispersal phase, which might enhance wave attenuation, lowering the method's effectiveness. In addition, MAE technique can cause thermolabile substances to degrade. The polarity of the compounds and

the solvent both play a role, making non-polar target compound recovery more challenging. Furthermore, large-scale development of microwave equipment can result in substantial investment expenditures (Roselló-Soto et al., 2016). Compounds with low polarity can be extracted by SC-CO_2. However, they require co-solvents for targeted compounds. When compared to other non-conventional extraction processes, the use of SWE and CO_2-AE for the extraction and recovery of high-added value products result in a "clean" extract. Such methods using water as a solvent result in gaining extracts that are free of residual organic solvents and interfering compounds.

Table 2-3. Novel extraction techniques for marine bioactive

Source	Bioactivity	Technique	Yield %	Solvent	T (ºC)	Time (min)	P (bar)	Power (W)	Amplitude (%)	Frequency (kHz)	Reference
Abalone	Antioxidant	SWE	46	H_2O	170	60	-	-	-	-	(Hao et al., 2019a)
Tuna skin	Antioxidant	SWE	21	H_2O	280	5	80	-	-	-	(Ahmed & Chun, 2018)
Shrimp	Antioxidant	SWE	88	H_2O	200	10	30	-	-	-	(Cho et al., 2019)
Pacific oyster	Antioxidant, antihypertensive	SWE	18.6	H_2O	125	14.93	-	-	-	-	(Getachew et al., 2019)

microalga	Antioxidant-antimicrobial	SWE	30	H_2O	200	20	103	-	-	-	(Rodríguez-Meizoso et al., 2010)
Oyster	Antioxidant, antihypertensive	SWE	-	H_2O	225	5	100	-	-	-	(H. J. Lee et al., 2018)
Cordyceps *militaris*	Antioxidant, antiinflammation	SWE	7	H_2O	180	13	51	-	-	-	(Luo et al., 2017)
Blue mussel	Antioxidant angiotensin-converting enzyme (ACE), and acetylcholinesterase (AChE) inhibitory	SWE	1	H_2O	200	60	-	-	-	-	(Han et al., 2018)
Brown seaweed (Himanthalia elongate)	Antioxidant, anti-elastase, anti-tumor	SWE	70	H_2O	180	-	-	-	-	-	(Cernadas et al., 2019)

Undaria pinnatifida, Cystoseira abies-marina, Sargassum vulgare, Sargassum muticum		SWE	6.7, 4.8, 7, 5.8	H_2O	200	20	100	-	-	-	
algae Hormosira banksii	Antioxidant	UAE	-	Ethanol	-	-	-	250	-	50	(T. T. Dang et al., 2017)[54]
red, brown, and green seaweeds	Antioxidant	UAE	-	H_2O	-	60	-	400	-	50/60	(Rodrigues et al., 2015)[55]
clown featherback (*Chitala ornata*) skin	Collagen	UAE	-	Acetic acid	-	10	-	750	80	20	(Petcharat et al., 2021)
sea bass Lateolabrax japonicus	Collagen	UAE	-	Acetic acid	-	24	-	-	60	20	(H. K. Kim et al., 2012)
soft-shelled turtle calipash	Collagen	UAE	-	Acetic acid	-	24	-	200	-	24	(Zou et al., 2017)
Marine sponge (*Porifera*)	Collagen	CO_2	36	H_2O	37	960	50	-	-	-	(Barros et al., 2015)

Marine sponge (*Porifera*)	Collagen	CO_2	22	H_2O	37	180	10	-	-	-	(Silva et al., 2016a)
Fish (*Gadus morhua*) skins	Collagen	CO_2	13.7	H_2O	37	180	50	-	-	-	(Sousa et al., 2020)

2.3. Identification and characterization of marine valuable products

To identify and characterise valuable product extracts, a variety of methods, including chromatographic and non-chromatographic techniques, are required. Chromatographic methods play an important role in natural product and substantially contribute to the development of new and revolutionary pharmacological and biological substances (Bajpai et al., 2016). They are separation techniques based on distribution or partitioning of a sample between a mobile phase (moving) and a stationary phase (fixed). A liquid (in liquid chromatography, LC), a gas (in gas chromatography, GC), or a supercritical fluid (SF) can be used as the mobile phase (in superfluid chromatography, SFC). A liquid or, more commonly, a solid might be used as the stationary phase. The chromatography can be divided into several techniques such as Liquid chromatography mass spectrometry (LC/MS) according to the physicochemical principles involved in the separation (Koyama et al., 2015). Non-chromatographic techniques are various biochemical characterizations such as biological activity screenings and cytotoxicity evaluations. In addition, Fourier-transform infrared spectroscopy (FTIR), Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-Page) and Rheology can also be used to acquire and make the identification of the valuable products easier.

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2.3.1. Chromatographic techniques

Liquid Chromatography-Mass Spectrometry (LC/MS)

The hyphenated analytical technique Liquid Chromatography-Mass Spectrometry (LC-MS) is a combination of Liquid Chromatography (LC) and Mass Spectrometry (MS). By passing mixtures over a chromatographic column, High Performance Liquid Chromatography (HPLC) a type of LC, separates the components. In most cases, LC alone will not be able to positively identify the separated components. Mass spectrometry is also used to identify new and recognised chemicals, as well as to deduce their structures. Because a mass spectrum mixture is essentially a complex of overlapping spectra from separated individual components, mass spectrometry alone is ineffective for detecting mixes. Liquid chromatography (LC) and mass spectrometry (MS) are challenging to link. The liquid eluents are transferred from LC to MS through an interface. This technique is useful for dissolution, bioavailability, bioequivalence, and pharmacodynamics studies. Preparative LC-MS systems may be used to purify particular chemicals from mixtures that are significant in fundamental research, pharmaceutical, agrochemical, culinary, and other sectors (Pratima, 2018).

2.3.2. Non-chromatographic techniques

1.1.1

Biological activity assay

Biological activity assay is a reagent-based analytical measuring method that generates a detectable signal for quantifying a biological process. The robustness and repeatability of the signal in the absence of a test substance are used to determine the assay's quality which is based on the type of signal measured including but not limited to radioactivity, absorbance, luminescence, and fluorescence. Assay quality is further determined by reagents, reaction conditions, automated equipment, and statistical models for data interpretation. The quality of

the assay and the data generated have an impact on the predictive analysis of a compound's biological and pharmacological activity. Assays can be either cell-based or cell-free (biochemical) procedures. Cell-based assay provides physiologically relevant information to anticipate a drug's reaction on an organism by evaluating a compound's effects on cell viability, cell growth, or cytotoxicity. Biochemical assays, such as antioxidant activity assays (DPPH, FRAP), anti-collagenase activity (MMP), and anti-hyaluronidase, on the other hand, detect, measure, and/or investigate the binding or activity of biological molecules like enzymes.

Fourier transform infrared (FTIR)

Fourier transform infrared (FTIR) has been shown to be an extremely useful technique for characterising and identifying chemicals or functional groups (chemical bonds) present in an unknown extract (Duistermaat & Kolk, 2000). In addition, FTIR spectra of pure compounds are usually so unique that they are like a molecular "fingerprint". For most common plant compounds, the spectrum of an unknown compound can be identified by comparison to a library of known compounds. Samples for FTIR can be prepared in several ways. For liquid samples, the easiest is to place one drop of sample between two plates of sodium chloride. The drop forms a thin film between the plates. Solid samples can be milled with potassium bromide (KBr) to and then compressed into a thin pellet which can be analysed. Otherwise, solid samples can be dissolved in a solvent such as methylene chloride, and the solution then placed onto a single salt plate. The solvent is then evaporated off, leaving a thin film of the original material on the plate (Duistermaat & Kolk, 2000).

Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS- PAGE) is the most widely used method for obtaining high resolution analytical separation of protein mixtures. Initially, component proteins are denatured using an anionic detergent that also binds to them, giving all

proteins a negative charge proportionate to their molecular mass. Following this, proteins are separated with great resolution based on molecular mass using electrophoresis via a porous acrylamide gel matrix. This technique, which has remained mostly unaltered since its inception in the early 1970s, works well in applications that do not need the preservation of natural protein structure or function. SDS-PAGE is used in a variety of procedures including for determining the purity of protein samples, determining protein expression, and immunochemical identification and quantification of proteins (Nowakowski et al., 2014).

The identification and characterisation of valuable products, like the bioactive compounds found in marine organisms, is difficult due to their multi-component mixture which requires a combination of chromatographic and nonchromatographic approaches. These techniques together, help in the understanding of valuable product potential in various industries including pharmaceutical, nutraceutical and cosmeceutical.

2.4. Potentials of valuable marine products

2.4.1. Pharmaceutical applications

Derivatives of secondary natural products/metabolites, the end products of gene-expression from marine organisms, have been used as the most successful drugs to treat many illnesses and diseases. Marine organisms are adapted to different biotic and abiotic stresses resulting in the formation of many bioactive secondary metabolites. Drug development is a process leading from simple research through a sequence of developmental stages till the commercial product. The US Food and Drug administration (FDA) initiated a Critical Path, a project designed to improve development of drug and medical devices for marketing approval. Along the path to commercialisation, safety and efficacy of the drugs are predicted through a series of evaluations that enable mass production (Woodcock & Woosley, 2008).

2.4.2. Nutraceutical applications

Increasing consumer demand for nutraceuticals as a result of their health benefits and disease prevention has accelerated the expansion of the nutraceutical market. Nutraceuticals are combination of pharmaceutical and nutrition sciences, a moderately new field. Nutraceutical is any substance that is a food/part of the food with health/medical benefits. They may consist of extracted nutrients such as minerals, fatty acids, amino acids, vitamins and dietary supplements (enzymes, antioxidant, pre and probiotics)(Yusof et al., 2015). With novel product development strategies in recent years, food industries have utilized marine organism as gelling agents, stabilizers, pigments and preservatives (Achterberg, 2014). To develop a nutraceutical product for the current market, some stages need to be further researched such as natural requirement, cultural requirement, innovative delivery format and health benefits (Yusof et al., 2015). Currently most nutraceutical products are in the form of beverages, tinctures, capsules, and tablets.

2.4.3. Cosmeceutical applications

The cosmeceutical product development process is faster and cheaper than pharmaceutical and nutraceutical development. That is because the human testing, if required, has a small sample size and usually no animal study is involved. For cosmeceutical products to get into the market, a bioactive compound, fraction, or extract requires isolation and justification of their use using screening methods. Safety, efficacy, and constant supply of the products are the main concerns for success in the market. The safety and efficacy of the product are usually performed in vitro by using 3D models and/or cell-based models to evaluate skin penetration, toxicity to the skin, heart, liver, and eye, as well as efficacy and bioavailability. After passing through safety tests, the product is characterized biochemically, chemically, and physically. In the final stage, the

product will be tested on volunteers for a short period of time (14-52 days) where efficacy and the performance of the product will be evaluated (Achterberg, 2014).Summary of chapter and conclusion

The review demonstrates a knowledge gap in terms of understanding the superior prospects of compounds that can be extracted from underutilized marine organisms for both human health and commercial applications. The potential of black-footed abalone has not yet been discovered due to various factors discussed in the literature review. Most researchers focused on biological activities of other marine organisms, because of their higher availability in the past. Several investigations have indicated that antioxidant, antiviral, antibacterial, and anticoagulant bioactive chemicals have been extracted from molluscs other than black-footed abalone using traditional extraction techniques (solvent extraction). The most common bioactive chemicals that are frequently mentioned in the literature relative to the recovery of compounds with beneficial qualities are also evaluated. Overall, this chapter provides information that will aid in the extraction of bioactive compounds, particularly black-footed abalone, and the potential of its products. It is suggested that researchers look into extracting these compounds utilising ecologically friendly technology for use in the cosmeceutical, pharmaceutical, and nutraceutical industries.

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Chapter 3. Extraction of bioactive compounds from black-footed abalone (*Haliotis iris*) using subcritical water extraction

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3.1. Chapter Preface

Knowledge gap regarding marine bioactive compounds, notably molluscs, have been reviewed in Chapter 3. It was discovered that there was a minimal investigation in extraction of abalone bioactive compounds. Moreover, bioactive compounds have been extracted mostly using conventional solvent based extraction. Based on the previous chapter, bioactive compounds of New Zealand black-footed abalone, had not been investigated. Therefore, this chapter present an effort to extract bioactive compounds from black-footed abalone by subcritical water extraction (non-conventional) for a clean, efficient, effective and environmental and economical friendly product. In addition, the best extraction temperature was further investigated for recovery of bioactive compounds with antioxidant activity, total phenolic content, glycogen, protein, and amino acids.

Abstract

Owing to biodiversity, many valuable natural compounds have been extracted from marine resources and used to develop pharmaceutical, nutraceutical, and cosmeceutical. Pāua, New Zealand black-footed abalone (*Haliotis iris*) is rich in proteins, carbohydrates and lipids preparation of which are often used in chinese medicine. Pāua is known to host a variety of bioactive compounds in its flesh and blood.

In this study, water-soluble bioactive compounds were extracted from *H. iris* by subcritical water extraction technique, and the effect of subcritical water temperature (110-280℃) on the extraction performance was studied. The obtained extracts were screened for their antioxidant activity and subsequently glycogen and phenolic content. The highest biological activity and concentration of bioactives, were found at temperatures between 220-250 °C. The carbohydrate content of the extracts peaked at 25 °C, which was then degraded at higher temperatures. Protein and amino acid contents of *H. iris* extracts were also decreased as the temperature increased over 160 °C. Furthermore, the *H. iris* extracts found to be non-toxic. The results indicate that the extraction temperature has a significant impact on the bioactivity of *H. iris* extracts. Subcritical water extraction can be used in place of more traditional techniques to create high-quality abalone extracts.

Keywords: Bioactive compounds; antioxidant; subcritical water extraction; *Haliotis iris*; phenolic compounds; glycogen content

3.2. Introduction

Pharmaceutical, nutraceutical, and cosmeceutical industries have shown significant interest in natural compounds derived from plants, bacteria and marine organisms. Marine organisms, notably molluscs, are an important natural source of bioactive compounds (Gayathri et al., 2017). Molluscs have active components, including sterols, nucleosides, mineral elements, carbohydrates (polysaccharides), lipids and proteins which have biological activities such as antioxidant, anti-inflammatory and antimicrobial/antibacterial properties. These biological activities arise from secondary metabolites, enabling them to adapt to different biological and environmental stress factors (Benkendorff et al., 2011; Pati et al., 2015; Shanmugam et al., 2013; L. C. Wang et al., 2019). One type of marine gastropod, which is endemic to New Zealand, is the black-footed abalone (*Haliotis iris*), known as pāua by Māori people, who are the tangata whenua (the indigenous people) of New Zealand. Abalone inhabit the low intertidal and subtidal zones in the coastline throughout New Zealand and feed primarily on seaweed. *H. iris* is the only New Zealand abalone species for the export market with an export volume and value of 674 tonnes p.a. and $35 million, respectively (Seafood New Zealand, 2019b; Statistics, 2020). With COVID-19 implications on exports, it is evident that the development of high-value export products is necessary to safeguard the future of this primary production sector.

Abalone are known to have compounds with high biological activities such as antithrombotic, anticoagulant, antimicrobial, antioxidant, anti-ageing skin as well as potential anti-inflammatory activity (Hao et al., 2019a; Joung et al., 2014; Seo et al., 2016; Suleria, Masci, Addepalli, et al., 2017). Marine-derived bioactives can play an essential role in the treatment of inflammation as alternative therapies. It has been reported that indole alkaloids from marine invertebrates such as tunicates, ascidians and sponges have anti-inflammatory properties (Senthilkumar & Kim, 2013). Previous researchers have found that glycogen extracted from

New Zealand green-lipped mussels (*Perna canaliculus* has anti-inflammatory properties, which could reduce the foot-pad oedema model in rats (T. B. Ahmad et al., 2018). In another study, glycogen metabolism was shown to regulate macrophage-mediated acute inflammatory responses (Ma et al., 2020). Wen et al. (Wen et al., 2019) found that a decrease in glycogen content of rats with diabetes resulted in a significant increase in necroinflammation.

The overproduction of reactive oxygen species (ROS) in biological systems destroys cellular structures, such as nucleic acids, lipids, and proteins (Pizzino et al., 2017). However, this can be managed by counterbalancing ROS production with the aid of antioxidants, which inhibit the oxidation of molecules (Webb, 2017). It has been reported that sulphated polysaccharide conjugates that have been derived from abalone (*Haliotis discus hannai* Ino) have antioxidant properties (Zhu et al., 2008). Other antioxidants have also been identified and extracted from other marine sources, such as the freshwater ampullariidae snail, *Pila virens* (Gayathri et al., 2017). In addition, many studies have reported that bioactive compounds are derived from polysaccharides, polypeptides, polyphenols and taurine (H. J. Lee et al., 2018; Son et al., 1998; Tanaka et al., 2003).

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The extraction of bioactive compounds is normally performed by conventional methods using either water or organic solvents, depending on the type of compound and final purpose. However, these techniques have disadvantages, such as requiring high amounts of pure solvents, being expensive and time-consuming, requiring the disposal of chemical solvents and the possibility of decomposition of thermolabile compounds (Azmir et al., 2013; Roselló-Soto et al., 2016; Sapkale, Patil, Surwase, & Bhatbhage, 2010). Novel methods have been introduced to overcome the mentioned disadvantages. Subcritical water extraction (SWE) is one of the most efficient novel extraction techniques for this purpose. This ‘green extraction’ technique is fast, safe, cost-effective and provides high yields of the extract. SWE is a popular system for the extraction of substances from biologically active natural resources (Nastić et al., 2018). This technique uses temperatures (100-374 °C) and pressure high enough to conserve the water in a liquid state (Essien et al., 2020). Water has unique characteristics, such as high polarity, high dielectric constant, high boiling point for its mass and high selectivity. In the SWE

process, the permittivity decreases with an increase in temperature, which leads to a rise in diffusion rate and a decline in both viscosity and surface tension (Munir et al., 2018). Lower temperatures are best for extracting more polar target compounds that have a high solubility in water under ambient conditions (Smith, 2006). On the other hand, slightly polar and non-polar substances need a less polar medium that is influenced by high temperatures (Haghighi & Khajenoori, 2013).

Subcritical water extraction has been used to extract bioactive compounds from marine sources such as microalgae (Rodríguez-Meizoso et al., 2010), tuna skin (Ahmed & Chun, 2018), and shrimp (Cho et al., 2019). They found that high bioactivity (antioxidant and antimicrobial) can be obtained at high temperature by subcritical water extraction. They were also could recover protein, amino acids and phenolic compounds at higher temperature than lower temperature of SWE . A few studies have also been made on the extraction of bioactive compounds from molluscs, such as abalone and oyster using SWE (Hao et al., 2019a; H. J. Lee et al., 2018). Their research demonstrated that high temperatures of SWE could recover high levels of antioxidant activity, protein, glycogen, and phenolic content.. However, bioactive extraction studies using SWE have not been carried out for *H.iris* to date.

The aim of this study was to extract substances with high bioactivity from *H.iris* flesh by implementing the subcritical water extraction technique and to investigate the effect of temperature on extraction to achieve a high yield of bioactive compounds.

3.3. Materials and Methods

3.3.1. Materials

Live adult abalone were purchased from Moana Blue Abalone (Ruakaka, New Zealand). Folin-Ciocalteu’s phenol reagent, sodium carbonate reagent, gallic acid, 2,2-diphenyl-1-

picrylhydrazyl (DPPH), Trolox standard solution (6-hydroxy-2,- 5,-7,-8-tetramethylchroman-2-carboxylic acid), Lugol solution (I_3K), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), mannose, rhamnose, galactose, glucose, arabinose, sodium acetate, Vero cell (monkey cell line) and thiazolyl blue tetrazolium bromide (MTT), were purchased from Sigma-Aldrich (New Zealand). Purified oyster glycogen, ferrozine, dimethyl sulfoxide (DMSO), Bradford assay kit and Dulbecco's modified eagle medium (DMEM), were purchased from Thermofisher Scientific (New Zealand). Hydrochloric acid (HCl), acetic acid, ferric chloride ($FeCl_3$), potassium hydroxide (KOH), iron chloride ($FeCl_2$) and methanol (CH_3OH) were purchased from ECP Labchem (New Zealand). Phosphate buffer solution (PBS) and fetal calf serum (FBS) were purchased from medi'Ray (New Zealand)All the chemicals, reagents, and standards used in this study were analytical grades.

Abalone soft bodies were separated from the shells, minced with a grinder (1000Y XICHU food grinder, China), frozen at -20 °C, and then freeze-dried using a laboratory-scale freeze dryer (Martin Christ, model Alpha 1-2 LD, Germany) at -75 °C and 1 mbar vacuum pressure for two days. The freeze-dried abalone samples were pulverised with a kitchen grinder and stored at -20 °C.

3.3.2. Subcritical water extraction

Duplicate subcritical water extractions were performed using a high-pressure autoclave extractor (Amar Equipment, India) equipped with a PID controller shown in An aqueous mixture of abalone powder, with a solid-to-liquid ratio of 1:10 (mass/volume), was placed into the extraction vessel and was tightly sealed. The extraction vessel was pressurised with nitrogen gas (30 bar) to keep the aqueous mixture in the liquid state during the extraction process. Extractions were performed at temperatures ranging between 110 °C and 280 °C, using an agitation speed of 500 rpm. The reaction time was set to 10 minutes after attaining the

desired temperature. An average of 20 minutes was taken to reach the desired temperature. After 10 minutes of extraction, the reaction was stopped by cooling down the reactor to 25°C with a circulating bath attached to the reactor. Then, the extract samples were collected, centrifuged at 4000 rpm at 4°C for 5 minutes and stored at 4°C for further analysis. Extracts were then freeze-dried at -75°C and 0.001 mbar and stored at -20°C.

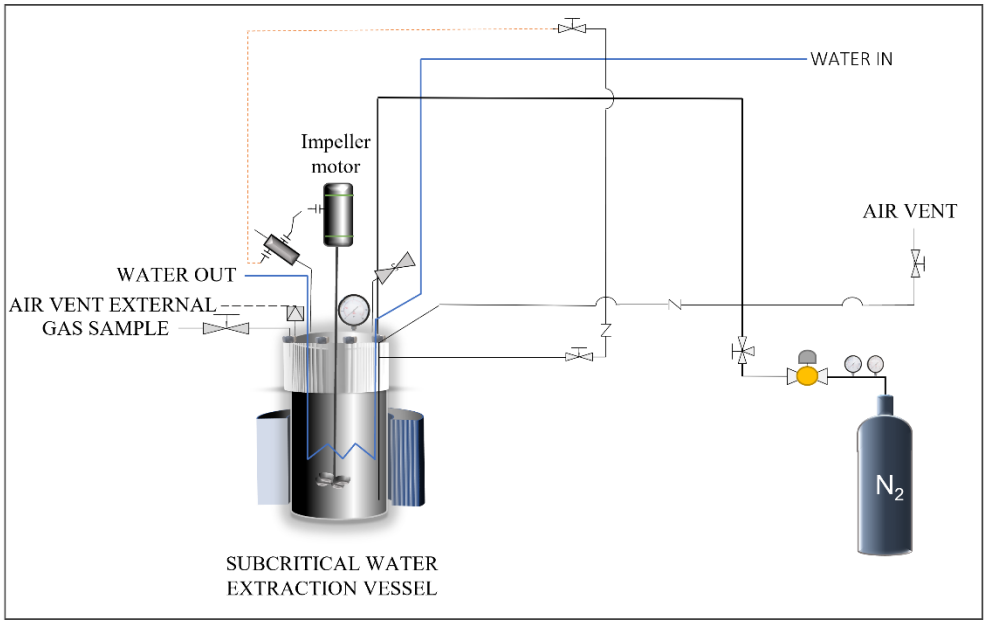

Figure 3-1. Schematic representation of the subcritical water extraction system

Determination of total phenolic content (TPC)

TPC was determined in abalone extracts using the technique reported by Kheirkhah et al. (Kheirkhah et al., 2019). A total of 25 µL of each sample, standard and blank were introduced into wells within a 96-well plate. Folin-Ciocalteau Reagent (125 µL) was then added to the wells, and after 10 minutes, 125 µL of sodium carbonate (750 mM) were added to every well. Then, the plate was incubated at room temperature and in the dark for one hour. The absorbance was detected at 765 nm. The results were expressed as milligrams gallic acid equivalent (GAE) per gram of dry abalone tissue (mg GAE/g dry tissue).

Determination of DPPH radical scavenging activity

Free radical scavenging activity of abalone extracts were determined following modified methods of Luther et al. (Luther et al., 2007). Trolox standard (20 µL), sample (20 µL), blank (200 µL 70% methanol) and control (100 µL 70% methanol) were added to 96-well microplates. Then 200 µL DPPH solution (0.1 mM, dissolved in methanol) were added to the standard, sample, and control, and incubated in the dark for 30 minutes at room temperature. The absorbance was obtained at 517 nm. The DPPH results of abalone extracts were expressed as milligram Trolox equivalent (TE) per gram of dry abalone tissue (mg TE/g dry tissue).

The radical scavenging activity was calculated by the following equation:

% DPPH scavenged = x **Error! Reference source not found.**)

where A_{sample} is the absorbance of the sample and DPPH. $A_{control}$ is the absorbance of the solvent and DPPH. A_{Blank} is the absorbance of the solvent without the sample and DPPH.

Determination of Ferric reducing antioxidant power (FRAP)

The FRAP of the abalone extracts were determined based on the procedure described in Rajurkar et al. (Luther et al., 2007). A total of 10 μL of Trolox standard, sample, and blank (ethanol) were added to individual wells in a 96-well microplate. Then, 200 μL of FRAP reagent (1:10:1, 10 mM TPTZ in HCl, acetate buffer, and 20 mM $FeCl_3$) were added to each well and incubated at room temperature for 60 minutes in the dark. The absorbance was detected at 593 nm with a UV spectrophotometer. The results were expressed as milligrams Trolox equivalent (TE) per gram dry abalone tissue (mg TE/g dry tissue).

Determination of glycogen content

The iodine glycogen method was used to determine the glycogen content of abalone extracts following the previously established procedure reported elsewhere (H. J. Lee et al., 2018). The freeze-dried extracts were mixed with 30% KOH with the solid-to-liquid ratio of 1:60 (mass/volume) and saponified by heating at 100 °C for 30 minutes. Then 0.2 mL of standard (purified oyster glycogen) and sample were transferred into an Eppendorf tube and mixed with 1.3 mL iodine reagent (1.92 mL of 0.68 M I_3K with 500 mL of saturated $CaCl_2$ solution). The samples were centrifuged at 10,000 rpm for 8 minutes, transferred to a 96-well microplate and incubated at 20 °C for 40 minutes. Then, the absorbance was detected at 460 nm with a UV spectrophotometer (Tecan Spark 10M, Switzerland).

Determination of carbohydrate content

The carbohydrate content of abalone extracts were determined corresponding to the procedures previously described (Dai et al., 2010). To 20 μL of the sample, 100 μL PMP (4 g phenylmethylpyrazolone in 20 mL DMSO) reagent were added and incubated at 70 °C for 100 minutes. Samples were cooled at room temperature, and 100 μL of Milli-Q water was added. Excess PMP reagent was removed from the sample by the addition of 0.5 mL of ethyl acetate, which was then vortexed, removed, and the ethyl acetate layer was discarded (repeated twice

more). Then 20 µL of the lower aqueous phase from derivatisation was mixed with 980 µL of 1% (v:v) formic acid. The mixture was vortexed and injected into the liquid chromatography-mass spectroscopy (LC-MS). LC-MS analyses were conducted using an Agilent 1260 Infinity Quaternary LC System (Santa Clara, CA 95051 USA). The system consisted of the following components: 1260 quaternary pump (model number: G1311B), 1260 infinity ALS sampler (model number: G1329B), 1260 infinity TCC column component (model number: G1316A), 1260 infinity diode array detector (DAD) (model number: G4212B), connected to a 6420 triple quadrupole LC/MS system with electrospray ionisation (ESI) source (model number: G1948B). Phenomenex Kinetex Evo C18 (2.1 x 150mm, 1.7µm) was used for this analysis. The mobile phases were composed of water containing 0.1% (v/v) formic acid (A) and acetonitrile containing 0.1% (v/v) formic acid (B). The initial gradient condition was 83:17 (A:B). From 13 to 14 min, the B was increased to 80%, and from 14 to 16 min, the B was decreased to 17%. The flow rate was 0.25 mL/min and the total run time was 22 min. The injection volume was 2 µL for each run. The MS ionisation source conditions were as follows: capillary voltage of 4 kV, drying gas temperature of 300 °C, drying gas flow of 10 L/min, nebuliser pressure of 30 psi. Protein analysis of abalone extracts

Protein concentration was determined by the Bradford assay (Thermo Fisher). In Bradford assay, Coomassie Brilliant Blue G-250 dye binds to the proteins via electrostatic attraction of the dye's sulphonic group with amino acids, such as arginine and lysine, resulting in a change in colour from brown to blue. The acidic dye solution's peak absorbance changes from 465 to 595 nm when binding to proteins occur. A set of eight standards up to 1000 µg/mL was prepared. Then, 10 µL of each standard, sample, and blank were added into a 96-well microplate. Then, 300 µL of Pierce Detergent Compatible Bradford Assay Reagent (Thermo Fisher) was added to each well. After 10 minutes of incubation at room temperature, the absorbance was detected at 595 nm.

Amino acids analysis

Analysis of amino acids of abalone extracts were conducted accordance with previously described methods (Salazar et al., 2012). Amino acid standards and samples (40 µL) were mixed with 40 µL internal standard-spiked methanol [ISSM] (methanol containing 10 mg L·1 d4-alanine). Samples were centrifuged at 10,000 rpm for 5 minutes at 4 °C. The supernatants were used for derivatisation. Then, 70 µL borate buffer were added to 10 µL samples, standard and blank. The blank was prepared in the same manner with distilled water instead of the sample. Then 10 mL Accutag reagent (2.8 mg 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate [accutag] in 1 mL of QrY acetonitrile) were added to the samples and vortexed immediately. The samples and standards were incubated at 55 °C for 15 minutes. After this, 400 µL of neutralising solution (10% (v:v) formic acid) were added to each vial. Samples were transferred into autosampler vials and placed in the LC-MS for analysis. LC-MS analyses were conducted using an Agilent 1260 Infinity Quaternary LC System (Santa Clara, CA 95051 USA). The system consisted of the following components: 1260 quaternary pump (model number: G1311B), 1260 infinity ALS sampler (model number: G1329B), 1260 infinity TCC column component (model number: G1316A), 1260 infinity diode array detector (DAD) (model number: G4212B), connected to a 6420 triple quadrupole LC/MS system with electrospray ionisation (ESI) source (model number: G1948B). Phenomenex Kinetex Evo C18 (2.1 x 150mm, 1.7µm) was used for this analysis. The mobile phases were composed of water containing 0.1% (v/v) formic acid (A) and acetonitrile containing 0.1% (v/v) formic acid (B). The flow rate was 0.225 mL/min. The initial gradient condition was 95:5 (A:B) and held for 0.5 min. From 0.5 to 2 min, the B was increased to 15%, from 2 to 9 min the B was increased to 20%, from 9 to 11 min, the B was increased to 50% and held for 2 min. From 13 to 14 min, the B was increased to 80% and held for 2 min. From 16 to 17 min B was decreased to 5%. The total run time was 23 min. The MS ionisation source conditions were as follows: capillary

voltage of 4 kV, drying gas temperature of 300 °C, drying gas flow of 10 L/min, nebuliser pressure of 40 psi. Both positive and negative scan modes were performed with a scan range of 100-1000 m/z. The wavelengths selected for DAD were 254 nm, 278 nm and 300 nm.

Cytotoxicity evaluation

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) test was used to assess the effect of abalone extracts on the proliferation of the Vero cell line. A 100-µL Vero cell suspension ($10x10^4$ cells/mL) in growth media (DMEM, 5 % and 1 % P/S) was seeded into all wells (10,000 cells/well). The plates were then incubated at 37 °C with 5 % carbon dioxide for 24 hours. The growth media were removed and replaced with fresh growth media containing abalone extracts at different concentrations (3000 µg/mL, 1500 µg/mL, 750 µg/mL and 375 µg/mL) to obtain the maximum level of cell death. The plates were then incubated at 37 °C for 48 hours. After incubation, all the growth-media-containing extracts were removed and replaced with 100 µL of fresh growth media. Then, 10 µl of MTT were added to all wells and incubated at 37 °C with 5 % carbon dioxide for four hours. After incubation, 80 µL was removed from each well, and 100 µl of DMSO were added to dissolve the precipitate. The absorbance of cell viability was measured at 450 nm by a UV spectrophotometer (SpectraMax iD3 Multi-Mode, USA).

Statistical analysis

Extractions were performed in duplicates. All expressed values are the means ± SD of three experiments. All statistical analyses were performed using Microsoft Excel package. To evaluate significant ($p < 0.05$) differences between extracts, analysis of variance (ANOVA) was performed.

3.4. Results and discussion

The biological activity of *H.iris* extracts was achieved by subcritical water extraction and the effect of temperature on the bioactive compounds of abalone extracts were studied. The indicators of bioactive compounds of the extracts were the total phenolic content (TPC), antioxidant capacity (DPPH and FRAP), glycogen content, carbohydrate content, amino acids, protein content and cytotoxicity.

3.4.1. Total phenolic content of abalone extracts

The results of TPC of abalone extracts are shown in

Figure 3-2. As can be seen, the extraction temperature had a substantial effect on total phenolic content (TPC). The TPC of the abalone extract was increased as the extraction temperature increased from 110 °C to 280 °C. The highest TPC was achieved at 250 °C. However, there were no significant differences between the TPC of the extract at 220 °C and 250 °C ($p<0.05$). However, there was a slight decrease in TPC when the temperature reached 280 °C due to the degradation of TPC at higher temperatures. By increasing the temperature of the extraction, the viscosity of water and surface tension of water is decreased, which can result in higher mass transfers and better diffusion of the solvent into the sample and ultimately increased phenolic content (Haghighi & Khajenoori, 2013). These results concur with the previous studies on shrimp (H. J. Lee et al., 2018), green kiwifruit (Actinidia deliciosa) (Kheirkhah et al., 2019) and brown macroalgae (*Ecklonia maxima*) (Bordoloi & Goosen, 2020).

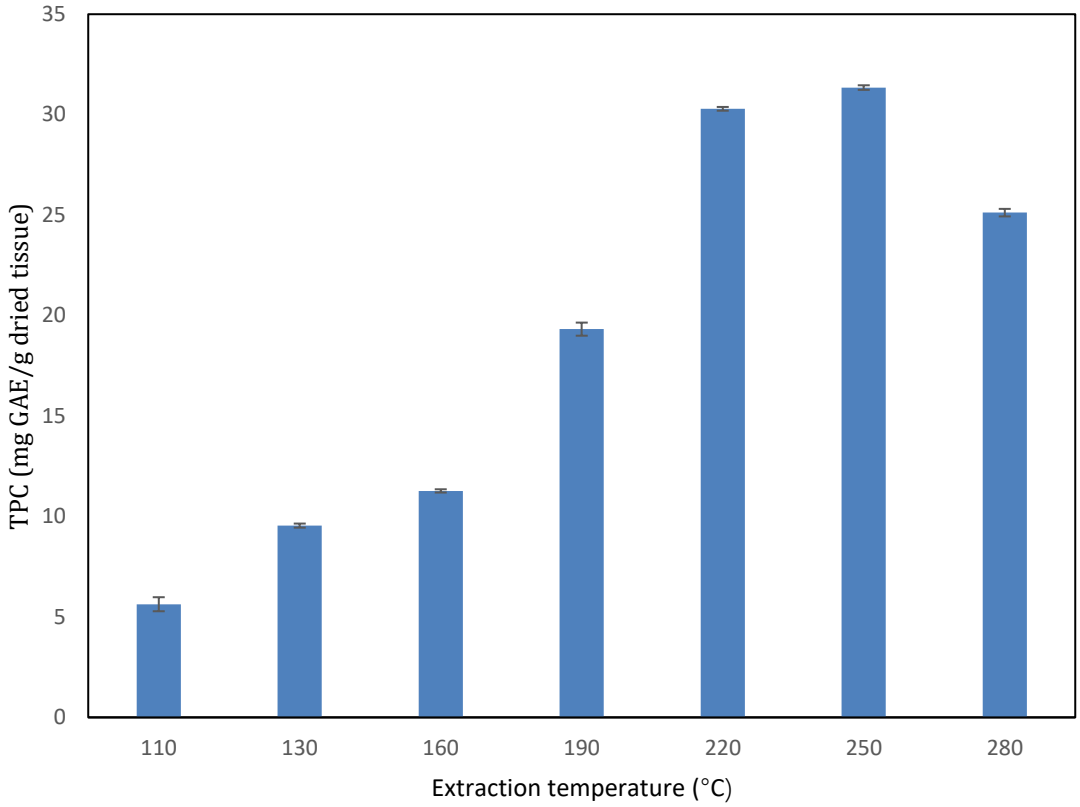

Figure 3-2. Total phenolic content (TPC) of *H.iris* extracts obtained at different temperatures

3.4.2. Antioxidant capacity of abalone extracts

In this study, the antioxidant activity of the subcritical water extracts of abalone were assessed by DPPH and FRAP assays. DPPH assay is generally used for assessing the antioxidant's ability to scavenge free radicals produced from DPPH reagent which results in a decrease in absorbance at 517 nm (Hajji et al., 2015). FRAP is typically used to assess the antioxidant compounds' ability to donate hydrogen/electrons. DPPH and FRAP radical assays reflected the antioxidant properties of abalone extracts in **Figure 3-3** (A) and (B), respectively. Abalone extracts which were obtained by varying the extraction temperature were tested by their relative antioxidant capacity equivalents using Trolox. In both antioxidant scavenging activities (DPPH and FRAP), by increasing the extraction temperature from 110°C to 280°C, the antioxidant activity increased, which were statistically significant ($p<0.05$). The highest DPPH activity of abalone extracts was found at 220°C with the concentration of 9.53±0.2 mg TE/g dry abalone tissue. In the FRAP assay, the ability of abalone extracts to reduce $Fe3^{+}$ to $Fe2^{+}$ was determined (Hajji et al., 2015). The FRAP of abalone extracts showed the highest activity at 250°C with the concentration of 19.62± 1.52 mg TE/g dry abalone tissue. At temperatures higher than 220°C and 250°C, the antioxidant activity decreased for both DPPH and FRAP, respectively, which indicates that a further increase in temperature results in the degradation of bioactive compounds. These results indicate that temperatures less than 250°C notably impacted the power of water solvation. The antioxidant result obtained from abalone extracts is in accordance with a previous study on abalone viscera, in which the best antioxidant recovery was achieved at higher temperatures (>170°C) (Hao et al., 2019a). Furthermore, the results concur with another study on Kiwi fruit (Kheirkhah et al., 2019) in which the best antioxidant activity was achieved when the temperature increased from 170°C to 200°C, and decreased after exceeding the thermal degradation capacity at 220°C.

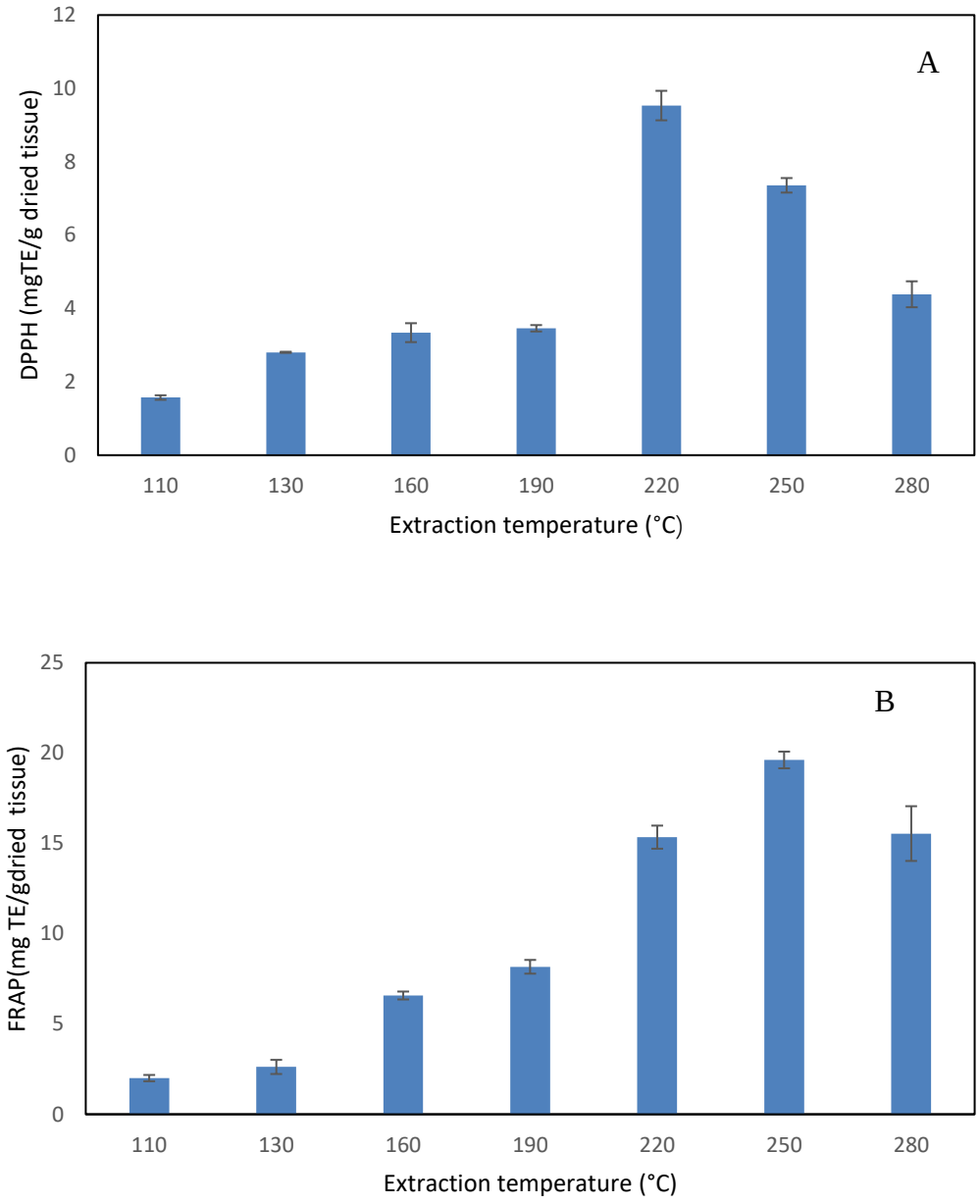

Figure 3-3. Radical scavenging activity (DPPH) of *H.iris* extracts obtained at different temperatures (A). Ferric reducing antioxidant power (FRAP) of abalone extracts obtained at different temperatures (B)

3.4.3. Glycogen content of abalone extracts

The glycogen (polysaccharide) content of abalone extracts obtained by subcritical water extraction was investigated, and the results are provided in

Figure 3-4. By increasing the extraction temperature to 220°C, the glycogen content of the abalone extracts increased considerably ($p<0.05$). Surface tension reduced at higher extraction temperatures, resulting in a faster diffusion rate and, eventually, the release of glycogen-containing extract. However, the glycogen content of abalone extracts slightly decreased with further increments of temperature due to the degradation of glycogen at higher temperatures. The glycogen content of abalone extracts differed from those reported in previous work by Lee et al. (2018), who found that the glycogen content of oysters decreased gradually by increasing the temperature and pressure of the extraction. In the aforementioned study, both parameters were changed simultaneously, and it cannot be confirmed that reducing glycogen content alone was due to the high temperature. The different results from these studies may be due to the different extraction and sources parameters used. In contrast, another study by Yang et al. (2013), indicated that out of a range of 100-230°C, subcritical water extraction at 210°C was the optimal temperature for extracting polysaccharides, which is similar to the findings of this study. Subcritical water extraction at 210°C was the best temperature for extracting polysaccharides out of a range of 100-230°C, which is consistent with the findings of this study (L. Yang et al., 2013). Glycogen has been shown to decrease inflammation in several studies (Ma et al., 2020; Mani & Lawson, 2006; Miller & Ormrod, 1980; Wen et al., 2019). The aim of the present study was only to confirm the presence of glycogen in abalone extracts. However, further research is required to confirm the anti-inflammatory effects of *H.iris* extracts.

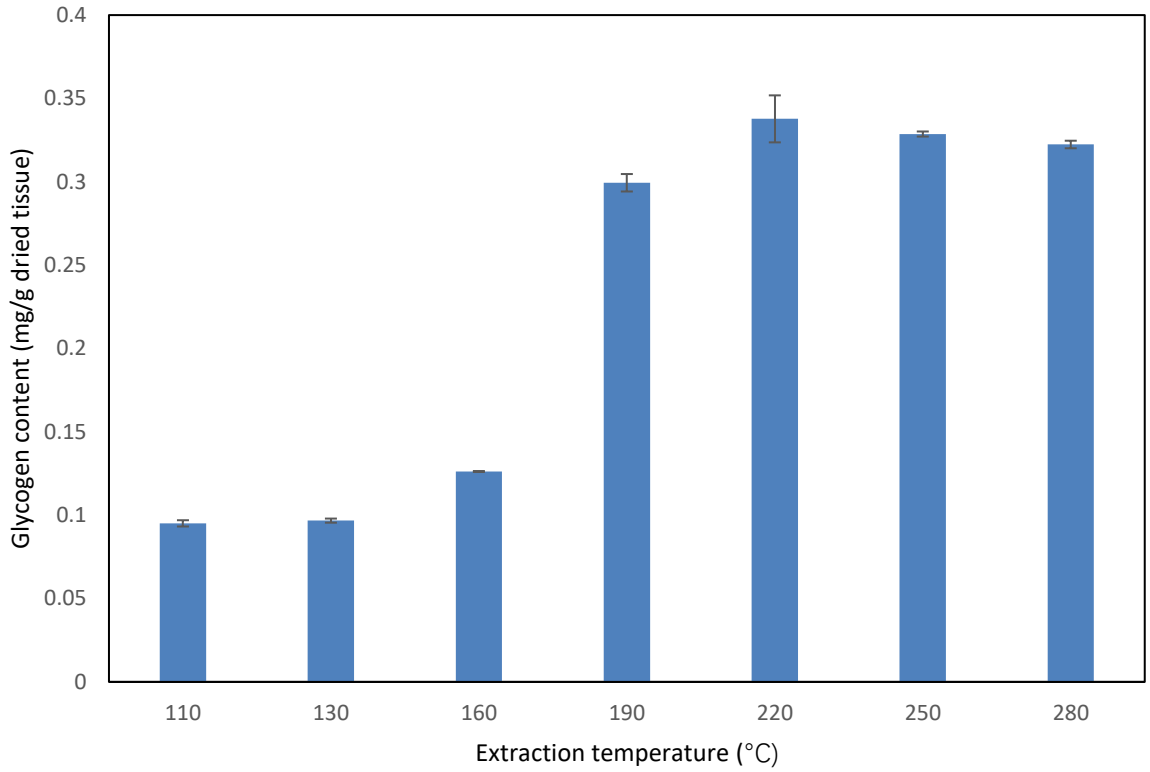

Figure 3-4. Glycogen content of *H.iris* extracts obtained at different extraction temperatures *(values are mean ± SD, n = 3)*

3.4.4. Carbohydrate content of abalone extracts

Abalone extracts were found to contain three reducing sugars: glucose, mannose, and arabinose, as shown in Figure 3-5. The temperature had a substantial impact on the extracts, according to the findings. By increasing the temperature of extraction, glucose with the highest concentration of 4.26 ± 1.12 mg/g of dried abalone at 110°C decreased to 0.01 ± 0 mg/g of dried tissue at 280°C. Mannose and arabinose with the concentration of 0.06 ± 0.01 and 0.01 ± 0 mg/g of dried abalone at 110°C, decreased to 0 ± 0 and 0 ± 0 with increasing temperature to 280°C, respectively. Rhamnose and galactose were not detected. These results were in agreement with previous study on abalone which reported that carbohydrate decreased with increasing temperature over 200 °C (Hao et al., 2019a). At subcritical temperatures,

carbohydrates have a similar polarity to water, indicating that they will dissolve fully in the water. The proton donation phase is made easier by a homogenous mixture of water and carbohydrates, which allows for rapid hydrolysis (conversion of complicated chain carbohydrates to simple sugars or depolymerization) (Ravber et al., 2015). However, the breakdown cycle does not end here since sugars can react with water molecules even more to breakdown of sugar-contributing compounds and their degradation to other products, such as organic acids, ketones, and aldehydes by isomerization.

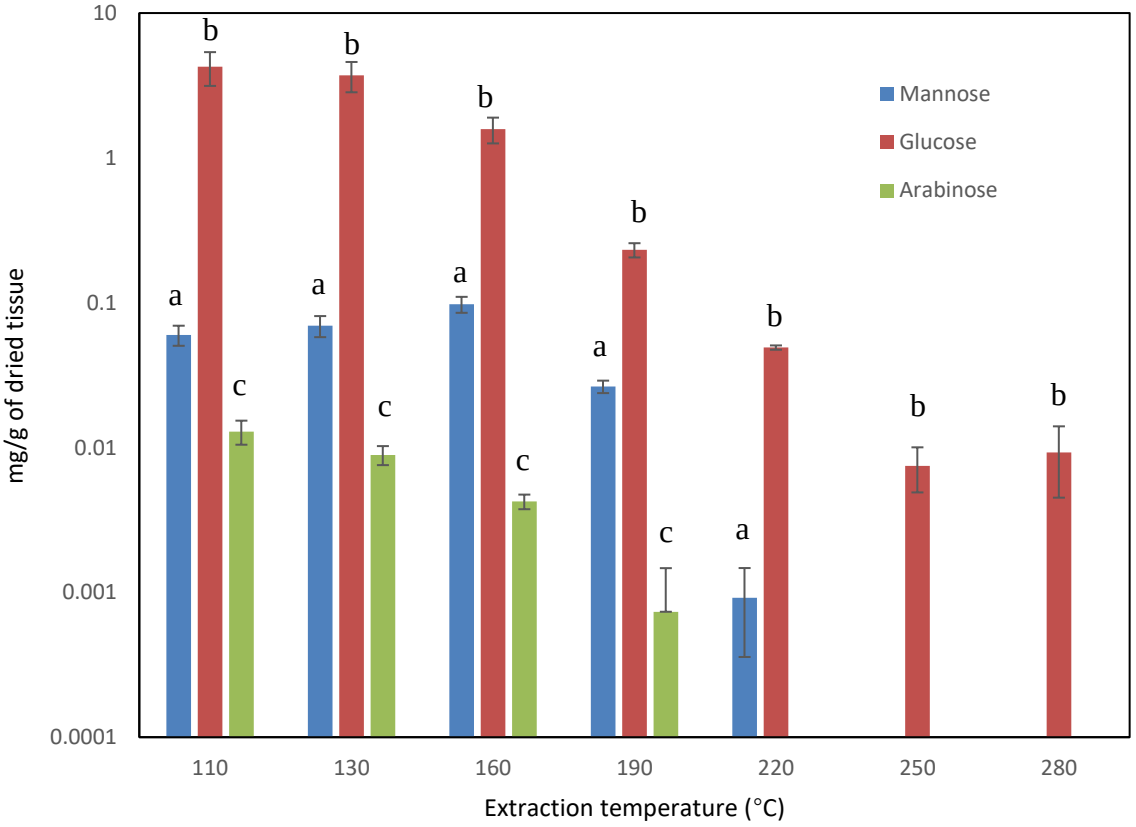

Figure 3-5. Carbohydrate of *H.iris* extracts obtained at different temperatures. Values with different letters are significantly different from each other (values are mean ± SD, n = 3, $p \leq 0.05$)

3.4.5. Protein content of abalone extracts

Proteins are highly hydrophobic, particularly those containing high amounts of arginine and lysine, which have a higher absorbance than less hydrophobic amino acids (Bunney et al., 2017). Wolfenden et al. (2015) reported that the hydrophobicity of amino acids increases with increasing temperature (Wolfenden et al., 2015). It can be seen in

Figure 3-6 the greatest protein concentration (51.42 1.63 mg/g dried tissue) was achieved from the 160°C abalone extract. During subcritical water extraction, the polarity of water changes as the temperature rises, resulting in changes in the protein content of the extracts and, ultimately, increases in the hydrophobicity of the amino acids, leading in increased absorbance (Wolfenden et al., 2015). After 160°C, however, there was a drop in protein because proteins can be denatured by high temperatures if their thermal denaturation resistance is exceeded (H. J. Lee et al., 2018). These results are in accord with previous study on macroalgae (Fl et al., 2022). This finding suggests that raising the extraction temperature to 160°C has a considerable impact on the protein concentration of abalone extracts ($p<0.05$).

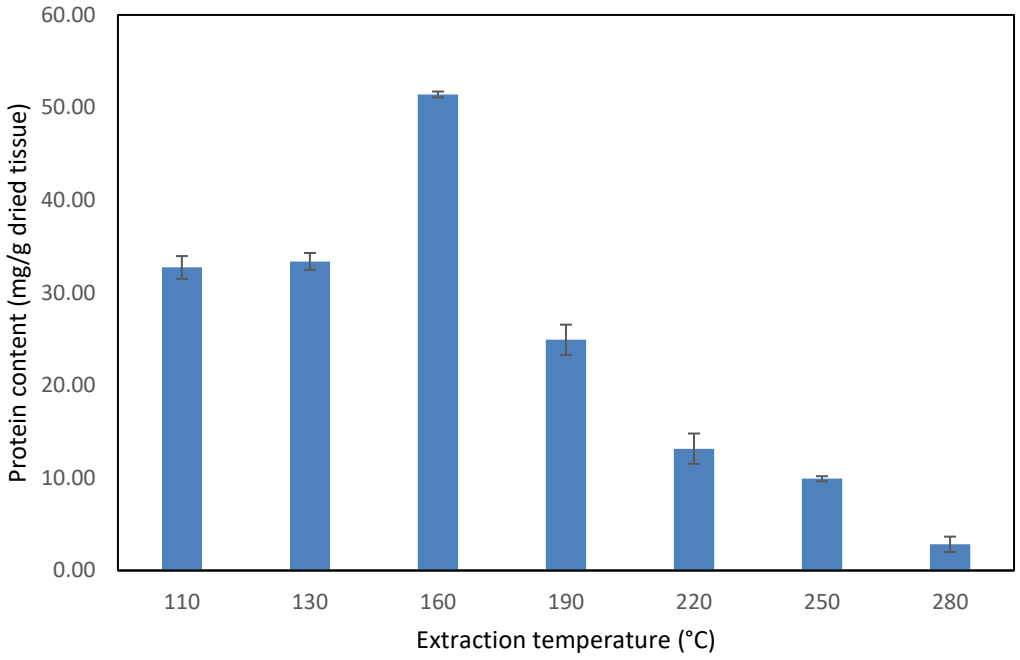

Figure 3-6. Proteins of *H.iris* extracts obtained at different temperatures (values are mean ± SD, n = 3)

3.4.6. Essential and non-essential amino acids of abalone extracts

Amino acid quantification is a technique that can be used to describe the intensity of the subcritical water extraction at various temperatures in breaking down proteins' peptide bonds to amino acids. The results of 25 amino acid (essentials and non-essentials) profiles from abalone extracts at different temperatures were obtained and are presented in ***Error! Reference source not found.***. The LC-MS results of the abalone extracts revealed the presence of all essential amino acids (L-Histidine, L-Leucine, L-Isoleucine, L-Lysine, L-Methionine, L-Phenylalanine, L-Tryptophan, L-Threonine, L-Valine). With the rise in temperature from 110 to 160°C, any of the essential amino acids (EAA) had a slight increase in the amino acid content of abalone, which is consistent with earlier research (Hao et al., 2019b). In subcritical water temperatures ranging from 160°C to 280°C, however, there was a steady decline in total EAA content. The impacts of Temperature on essential amino acids of abalone extracts were statistically significant ($P<0.05$). At temperatures of 250 and 280°C, methionine was undetectable. Citrulline, a non-essential amino acid (NEAA), was found to have similar

tendencies. As a result, these amino acids appear to be unstable at high subcritical water temperatures. The highest EAA/TAA and EAA/NEAA value of abalone subcritical extracts were obtained at 160°C which decreased with increasing subcritical water temperature which is in agreement with previous study (Hao et al., 2019b). The predominant NEAA present at the various temperatures was taurine with an average of 76.24 mg/g of dried abalone, which could be due to abalone's nature rather than the abalone diet (Bewick et al., 1997). Taurine decreased from 99.93±2.99 to 44.56± 0.27 mg/g dried tissue by increasing temperature from 110C to 280°C which could be the result of exceeding thermal denaturation capacity. After taurine, glutamic acid, L-alanine, glycine, L-proline and tyrosine were found to be the highest-occurring NEAA (6.11±1.73, 5.96±0.24, 5.24±0.3, 2.99±0.8, and 1.77±0.02 mg/g dried tissue) respectively. Most of NEAA decreased by increasing subcritical water temperature. On the other hand, most of the hydrophobic amino acids showed an increased in content by increasing subcritical water temperature. This is due to higher temperature can help breakdown of amino acids in subcritical water (Hao et al., 2019a).

Table 3-1. Amino acid composition of *H.iris* extracts obtained at different temperatures in mg/g dried tissue (values are mean ± SD, n = 3)

	110	130	160	190	220	250	280
Histidine	0.7±0	0.69±0.2	0.68±0.2	0.44±0	0.23±0.1	0.19±0	0.19±0
* Leucine	0.89±0.1	0.94±0	1.01±0.2	0.74±0	0.48±0	0.74±0	0.72±0.1
*Isoleucine	1.9±0.5	2.26±0.6	1.88±0.7	1.1±0	0.74±0.1	1.22±0.1	1.06±0.2
Lysine	0.83±0.8	1.19±0.9	2.29±0.4	1.07±0	0.36±0	0.38±0	0.61±0
*Methionine	0.94±0.3	1.09±0.4	0.85±0.3	0.35±0	0.13±0	0±0	0±0
*Phenylalanine	1.06±0.2	1.17±0.3	1.21±0.3	0.77±0.1	0.38±0.1	0.55±0	0.64±0
*Tryptophan	0.43±0	0.46±0.1	0.48±0.1	0.33±0	0.06±0	0.11±0	0.13±0
*Threonine	1.2±0	1.22±0.3	1.24±0.1	0.67±0	0.3±0	0.05±0	0.03±0
*Valine	1.26±0.2	1.34±0.1	1.41±0.2	1.15±0	0.75±0.1	0.89±0	0.95±0.2
Arginine	0.3±0.1	0.37±0	0.51±0.2	5.02±0.1	0.95±0.3	1.47±0.2	1.23±0.1
Hydroxy-L-Proline	0.06±0	0.07±0	0.08±0	0.09±0	0.09±0	0.19±0	0.09±0
Ethanolamine	0.2±0	0.23±0	0.3±0	0.23±0	0.37±0	0.54±0	0.7±0

*Serine	0.11±0.1	0.45±0.5	0.41±0.1	1.67±0.1	1.2±0.1	0.47±0.1	0.07±0.1
*Glycine	3.84±0.1	3.97±0.3	4.39±0.1	3.55±0.2	3.33±0.1	4.28±0.1	5.24±0.3
Aspartic acid	0.62±0.4	0.76±0.5	1.31±0.6	2.16±0	2.48±0.3	0.94±0.1	0.19±0
Taurine	99.93±3	92.9±6.5	92.32±5.1	75.13±6.3	71.23±1.7	57.62±7.4	44.56±0.3
b-Alanine	0.26±0.1	0.23±0.1	0.32±0.2	0.28±0	0.14±0	0.1±0	0.11±0
*Glutamic acid	6.11±1.7	5.4±0.5	4.12±1.1	0.55±0	0.32±0	0.34±0	0.4±0
Citrulline	0.36±0.2	0.22±0.2	0.26±0.1	0.01±0	0±0	0±0	0.01±0
*Alanine	3.14±0.6	3.38±0.3	3.57±0.6	2.95±0.1	3.3±0.1	4.75±0.1	5.96±0.2
*Proline	2.32±0.7	2.14±0.1	2.99±0.8	2.56±0.1	1.01±0.1	1.19±0	0.97±0.1
Cystathionine	0.15±0	0.06±0.1	0.06±0	0.02±0	0.03±0	0.06±0	0.17±0
Anserine	0.04±0	0.03±0	0.06±0	0.03±0	0.02±0	0.07±0	0.03±0
*Tyrosine	1.35±0	1.46±0.2	1.77±0	0.92±0.1	0.4±0.1	0.56±0	0.64±0
TAA	128.01	122.05	123.55	101.79	88.36	76.98	65.19
EAA	9.20	10.37	11.06	6.61	3.43	4.12	4.32
EAA/TAA (%)	7.19	8.50	8.95	6.50	3.88	5.35	6.63
NEAA	118.81	111.68	112.49	95.18	84.93	72.87	60.87
EAA/NEAA (%)	7.75	9.29	9.83	6.95	4.04	5.65	7.10

*: hydrophobic amino acids; TAA: total amino acids

3.4.7. Cytotoxicity activity of abalone extracts

The cytotoxicity activity of abalone extracts by subcritical water extraction was evaluated in vitro by the MTT assay. To analyse the cytotoxicity, two extracts that were obtained at 220 °C and 250 °C were used due to their high bioactivity compared with extracts obtained at other temperatures. The MTT assay measured the reduction of yellow MTT to an insoluble purple formazan by succinate dehydrogenase existent in mitochondria of viable cells (Udhayakumar et al., 2017). The cytotoxic effect of abalone extracts was determined on the Vero cell line derived from a monkey. According to

Figure 3-7, after 48-hour exposure of the cells to the abalone extracts, cell viability was more than 100 % in response to 375 µg/ml for both extracts. However, by increasing the extract's concentration to 3000 µg/ml, cell viability decreased to 78 % and 74 % at 220 °C and 250 °C, respectively. The IC50 of 220 °C and 250 °C abalone extracts were 4358.72 and 4336.98 µg/ml, respectively. This indicates that the high concentrations of abalone extract that were obtained at high temperatures (220 °C and 250 °C) by subcritical water extraction are non-toxic to the Vero cells.

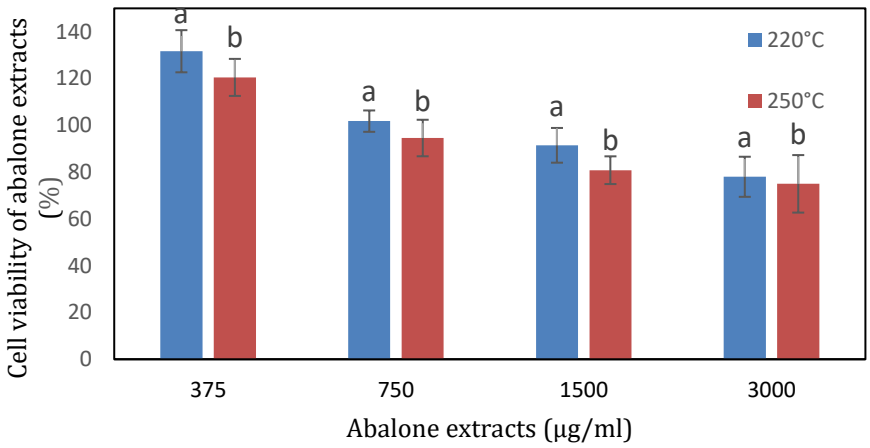

Figure 3-7. Effect of *H.iris* extracts obtained at 220 °C and 250 °C on Vero cells. Values with different letters are significantly different from each other (values are mean ± SD, n = 3)

3.5. Conclusions

Bioactive compounds from abalone (*Haliotis iris*) were successfully extracted using subcritical water extraction (SWE). The results of this study indicate that the effectiveness of the SWE is affected by temperature, which is a crucial temperature in proving extraction efficiency. The overall results show that subcritical water extracts of abalone are promising sources of bioactive compounds, such as antioxidants, glycogen, phenolic compounds, carbohydrates, protein and amino acids. There was a strong relationship between the total phenolic compounds, glycogen content, and antioxidant activity of abalone extracts, which at temperatures of 220-250 °C, had the highest biological activity. As a result, considering the importance of energy conservation as well as the potential of antioxidant degradation higher than 220 °C, the optimal temperature for extracting bioactive compounds from abalone was found to be 220 °C. Therefore, subcritical water extract of abalone can be used as a reliable and valuable source of high-quality bioactive compounds from a natural source. Further studies will therefore be directed at the analysis of the antiaging properties of the abalone extracts as well as the effect of another significant parameter (time) on the efficiency of SWE.

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Chapter 4. Antioxidant and antiageing properties of bioactive compounds extracted from wild and farmed black-footed abalone using subcritical water

Content of this chapter is submitted as a journal paper in Sustainable Chemistry and Pharmacy

4.1. Chapter Preface

Extraction of bioactive compounds from black-footed abalone by subcritical water extraction have been investigated in Chapter 3. The subcritical water extraction could recover the highest bioactive compounds such as antioxidant activity, total phenolic content, glycogen content, protein, and amino acids. Furthermore, it has been reported that temperature is an important factor in the subcritical water extraction of bioactive compounds, with the highest bioactive compounds of black-footed abalone being obtained at temperatures between 220-250 °C. In consideration of the need for energy saving and bioactive degradation, the temperature of 220 °C was chosen for this chapter to extract bioactive compounds from farmed and wild black-footed abalone at different extraction times (5-60 min). Moreover, the extracts were investigated in terms of antiaging properties such as anti-hyaluronidase and anti-collagenase, as well as antioxidants, phenolic compounds, proteins, and essential and non-essential amino acids. In addition, further research was conducted on the bioactive chemicals of both farmed and wild abalone extracts to determine whether or not wild abalone extracts would have higher biological activities than farmed abalone extracts.

Abstract

New and novel ingredients for product development are always in demand in the pharmaceutical and cosmeceutical industries. Due to their numerous benefits, both cosmetic enterprises and customers are particularly interested in compounds derived from natural sources in the context of ongoing renovation. In this study, 14 new extracts were obtained from farmed and wild abalone tissue, using a novel and green extraction technique, subcritical water extractions at 220 °C temperature and 30 bar pressure and different extraction times (5-60 min). The amino acid profile, total phenolic compounds, glycogen content, and protein content of the extracts were obtained. Multiple bio-screening techniques were employed to determine the antioxidant, anti-hyaluronidase, and anti-collagenase properties of the extracts. The extracts obtained with subcritical water extraction had 24 essential and non-essential amino acids and showed a broad antioxidant capacity and antiageing properties, making it a potential new ingredient to consider in the development of new cosmetic products.

Keywords: antioxidant; amino acids; black-footed abalone; anti-collagenase; anti-hyaluronidase

4.2. Introduction

In developed countries, life expectancy is steadily increasing (Krutmann, 2003). However, ageing is still one of biology's mysteries. Life expectancy has increased significantly because of medical advancements, particularly throughout the twentieth century. Skin ageing is a topic of increasing importance in today's society (Krutmann, 2003). The appearance of age, in addition to actual age, predicts crucial aspects of health and well-being (Ganceviciene et al., 2012). Extrinsic, genetic, and stochastic factors all contribute to the ageing process (Krutmann, 2003). Skin ageing is a biological process that is impacted by both intrinsic or endogenous factors (e.g., hormones, cellular metabolism, metabolic processes, and heredity) and extrinsic or exogenous factors (e.g., toxins, chemicals, pollutants, ionising radiation, and prolonged light exposure) (Ganceviciene et al., 2012). These variables cause physiological and structural changes in all layers of the skin significantly Extracellular matrix (ECM) the outermost part of the skin which is vital for the skin growth and elasticity. Skin ageing has been correlated with ECM degradation and is linked to an increase in the activity of some skin-aging enzymes, such

as elastase, collagenase, and hyaluronidase (Ndlovu et al., 2013). The appearance of the skin is affected by the activity of these enzymes, which can result in skin damage, deep wrinkles, roughness, sagging, discoloration, dullness and laxity(Resende et al., 2021).

Cosmeceuticals are cosmetic products that contain biologically active compounds and are intended to have a medicinal impact on the skin (J. H. Kim et al., 2018). Over the last several decades, increased focus has been dedicated to identifying bioactive compounds from marine species for cosmeceutical applications, due to their greater biological activity compared to compounds isolated from terrestrial sources. While terrestrial organisms continue to be a significant source of promising compounds, marine organisms are a unique yet abundant source of the physiologically and pharmacologically active compounds. For instance, bioactive compounds isolated from marine sources have demonstrated a range of biological activities, including antioxidant, antiviral, anti-inflammatory, anticancer, anticoagulation, antimicrobial, and immunomodulatory properties, and are widely used in nutraceutical, cosmeceutical, and pharmaceutical products (Hamed et al., 2015; Jung & Kim, 2009; Suleria, Addepalli, et al., 2017; B. Wang et al., 2013). Marine bioactive components can be derived from a variety of marine animals, plants, and bacteria, with even greater compound diversity when the organism is exposed to different environmental conditions, such as temperature, pressure, light, and salinity (Lordan et al., 2011).

Black-footed abalone (*Haliotis iris)* are large edible molluscs in the family Haliotidae (J. Zhao et al., 2016). Black-footed abalone are present across New Zealand, including the Chatham Islands and Stewart Island (Somerville et al., 2014). The term pāua is used locally to refer to these endemic abalone. This iconic name originates from the Māori language of New Zealand, where they have been a significant traditional resource for at least 600 years. At present, in addition to considerable cultural, recreational, and illegal catches, this shallow water delicacy

plays an important role in New Zealand's commercial fisheries, supplying a mostly international market (Somerville et al., 2014).

The nutritional value of abalone varies depending on whether the abalone are wild or farmed. This is because wild abalone consume on variety of seaweed species, whereas farmed abalone are fed formulated feeds with higher nutritional value (Chiou et al., 2001; Shi et al., 2020; Stone et al., 2013; Thongrod et al., 2003; C.-H. Tung & Alfaro, 2011). The protein, carbohydrate, and lipid profiles of farmed abalone are higher than those of wild abalone (Bullon et al., 2022). Due to its great nutritional content, especially in Asian countries, black footed abalone has achieved worldwide prominence as one of the most expensive and exclusive seafood. However, its health benefits within nutraceutical, pharmaceutical and cosmeceutical applications have received little attention. Developing innovative, powerful, and safe cosmeceuticals or supplementary products from marine materials as anti-ageing agents is unquestionably necessary.

Several studies have been conducted recently on the nutritional and medicinal properties of abalone extracts. Previous research on sulphated polysaccharides from Blacklip Abalone (*Haliotis rubra*) are being explored as a source of anti-thrombotic and anti-coagulant molecules due to its structurally varied bioactive compounds (Suleria, Masci, Zhao, et al., 2017). They discovered that extracted sulphated polysaccharides from *Haliotis rubra* showed higher thrombin inhibition than standard heparin. According to previous study sulphated polysaccharides extracted from abalone (*Haliotis Discus Hannai Ino*) gonad contain free radical scavengers (antioxidant activities) (Zhu et al., 2010). Furthermore, Nguyen et.al (2013) found that peptides extracted from abalone (*Haliotis discus hannai*) have anti-matrix metalloproteinases which are responsible for degradation of components of skin extracellular matrix (V. T. Nguyen et al., 2013).

Currently, natural compounds are extracted using organic solvents in standard extraction methods. To get natural components for the food, pharmaceutical and cosmeceutical industries, however, it is preferable to obtain extracts using green methods, avoiding the use of toxic solvents (Rodríguez-Meizoso et al., 2010). Subcritical water extraction (SWE) has gained significant interest in recent decades as a green and ecologically friendly method for recovering biofunctional materials. Subcritical water is water that is maintained at a temperature between its ordinary boiling point and 374 °C and under sufficient pressure to remain liquid. Water exhibits unusual behaviour under these conditions, and its physical characteristics, such as diffusion, viscosity, and dielectric constant may be altered by adjusting the temperature. As a result, this can aid the SWE process in effectively extracting biofunctional compounds with a range of polarities (Getachew et al., 2019). As mentioned previously, t bioactive compounds with the antioxidant, anti-coagulant and anti-thrombotic activity have been extracted from abalone viscera extracts (J. Li et al., 2012; Suleria, Addepalli, et al., 2017; Suleria, Masci, Addepalli, et al., 2017). However, only one of the studies used subcritical water extraction to isolate bioactive compounds from abalone that were responsible for antioxidant activity (Hao et al., 2019a). Moreover, other studies have shown that bioactive compounds can be extracted from other marine resources such as brown macroalgae (*Ecklonia maxima*), pacific oyster (*Crassostrea gigas*) and microalgae (*Haematococcus pluvialis*) with subcritical water extraction (Bordoloi & Goosen, 2020; Getachew et al., 2019; Han et al., 2018; H. J. Lee et al., 2018; Rodríguez-Meizoso et al., 2010). At high temperatures of subcritical water extraction (125- 200°C), they were able to recover a significant amount of antioxidant activity from obtained marine extracts. However, the increased temperature had no impact on the antimicrobial activity. According to published literature, there have been no studies on the antiaging properties of marine subcritical water extracts, including their ability to inhibit hyaluronidase and collagenase. To the best of our knowledge, no investigation has been

conducted on the antioxidant and antiageing potential of bioactive compounds, such as anti-hyaluronidase and anti-collagenase isolated from black-footed abalone using subcritical extraction. Thus, the purpose of this study was to compare the antioxidant and antiageing activities of bioactive components isolated from wild and farmed black-footed abalone by subcritical water extraction. It is envisaged that the findings from this work will lead to novel uses of seafood products and contribute to the growing New Zealand abalone industry.

4.3. Materials and Methods

4.3.1. Materials

Live adult abalone (farmed) were purchased from Moana Blue Abalone (Ruakaka, New Zealand), and wild abalone were caught from Chatham Islands, New Zealand. Abalone (farmed and wild) soft bodies were separated from the shells, minced with a grinder (1000Y XICHU food grinder, China), frozen at -20°C, and then freeze-dried using a laboratory-scale freeze dryer (Martin Christ, model Alpha 1-2 LD, Germany) at -75°C and 1 mbar vacuum pressure for two days. The freeze-dried abalone samples were pulverised with a kitchen grinder and stored at -20°C.

Folin-Ciocalteu's phenol reagent, sodium carbonate reagent, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Trolox standard solution (6-hydroxy-2, - 5,-7,-8-tetramethylchroman-2-carboxylic acid), Lugol solution (I3K), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), hyaluronidase, bovine serum albumin, sodium phosphate monobasic, sodium phosphate dibasic, sodium chloride (NaCl), sodium hydroxide (NaOH), acetic acid, mannose, rhamnose, galactose, glucose, arabinose, Vero cell (monkey cell line) and thiazolyl blue tetrazolium bromide (MTT), were purchased from Sigma-Aldrich (New Zealand). Purified oyster glycogen, ferrozine, formic acid, dimethyl sulfoxide (DMSO), Bradford assay kit, Dulbecco's modified eagle medium (DMEM) and sodium acetate trihydrate were purchased from

Thermofisher Scientific (New Zealand). Hydrochloric acid (HCl), acetic acid, ferric chloride ($FeCl_3$), potassium hydroxide (KOH), iron chloride ($FeCl_2$) and methanol (CH_3OH) were purchased from ECP Labchem (New Zealand). Hyaluronic acid was purchased from Lifecore biomedical. Inhibitor Screening Kit (Abcam, ab211108) was purchased from Abcam. Phosphate buffer solution (PBS) and fetal calf serum (FBS) were purchased from medi'Ray (New Zealand). All the chemicals, reagents, and standards used in this study were of analytical grades.

4.3.2. Subcritical water extraction

Subcritical water extractions were performed using a 1.0 L high-pressure autoclave extractor (Amar Equipment, India) equipped with a PID controller and magnetic stirrer (**Error! Reference source not found.**). An aqueous mixture of abalone powder (farmed and wild), with a solid-to-liquid ratio of 1:10 (mass/volume), was placed in the extraction vessel and was tightly sealed. The extraction vessel was pressurised with nitrogen gas (30 bar) to keep the aqueous mixture in the liquid state during the extraction process. Extractions were performed at a temperature of 220°C, using an agitation speed of 500 rpm. The reaction time points were set to 5-60 min after reaching the desired temperature. An average of 20 min was needed to reach the desired temperature. Samples were taken in predetermined intervals beginning when the interior temperature of the extractor reached the set-point up to 60 min. After 60 min, the extraction was stopped by rapidly cooling down the vessel to 25°C. Then, the collected extracts were centrifuged at 4000 rpm at 4°C for 5 min and stored at 4°C for further analysis. Extracts were then freeze-dried at -75°C and 0.001 mbar and stored at -20°C. Extraction experiments were repeated twice.

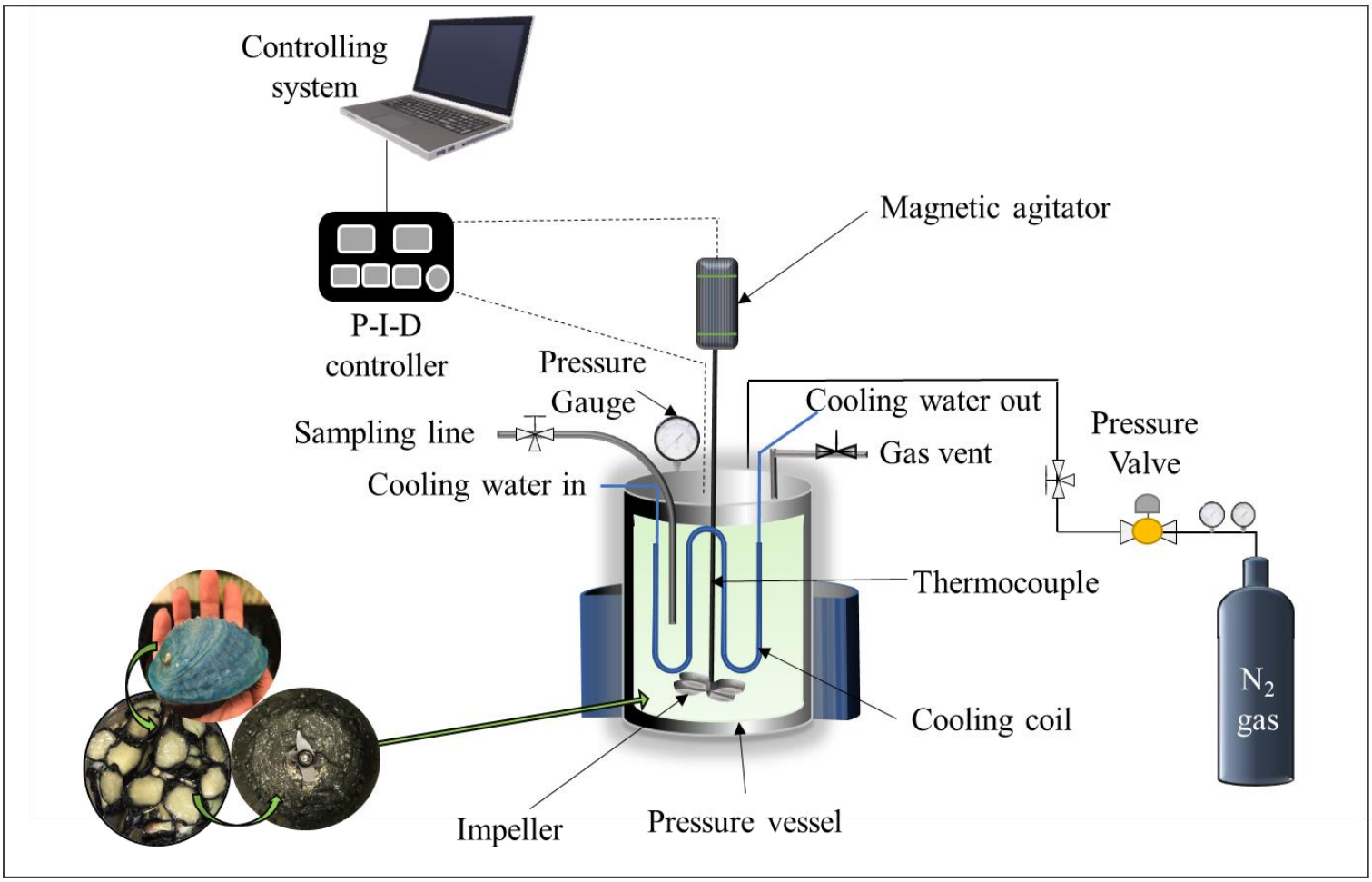

Figure 4-1. Schematic representation of the subcritical water extraction system

4.3.3. Analysis of farmed and wild black-footed abalone extract

Total phenolic content of farmed and wild black-footed abalone extract

TPC was determined in abalone extracts (farmed and wild) according to the procedure reported by Kheirkhah et al. (Kheirkhah et al., 2019). A total of 25 µL of each extracted sample, standard and blank, were placed into wells within a 96-well plate. Folin-Ciocalteau Reagent (125 µL) was then added to the wells, and after 10 min, 125 µL of sodium carbonate (750 mM) was added to every well. Then, the plate was incubated at room temperature and in the dark for one hour. The absorbance was detected at 765 nm with a UV spectrophotometer (Tecan Spark 10M, Switzerland). The total phenolic content results were expressed as mg of gallic acid equivalents (GAE) per gram of dry abalone tissue (mg GAE/g dry tissue).

Glycogen content of farmed and wild black-footed abalone extract

The glycogen content of farmed and wild abalone extracts from subcritical water extraction was determined by the iodine glycogen method, according to the previously established procedure reported by (H. J. Lee et al., 2018). The freeze-dried extracts were mixed with 30% KOH with the solid-to-liquid ratio of 1:60 (mass/volume) and saponified by heating at 100°C for 30 min. Then, 0.2 mL of standard (purified oyster glycogen) and sample were transferred into an Eppendorf tube and mixed with 1.3 mL of iodine reagent (1.92 mL of 0.68 M I_3K with 500 mL of saturated $CaCl_2$ solution). The samples were centrifuged at 10,000 rpm for 8 min, transferred to a 96-well microplate and incubated at 20°C for 40 min. Then, the absorbance of the samples was detected at 460 nm with a UV spectrophotometer (Tecan Spark 10M, Switzerland).

Protein content of farmed and wild black-footed abalone extract

Bradford assay was used to determine the protein concentration of the abalone extracts (farmed and wild). The Coomassie Brilliant Blue G-250 dye binds to proteins in the Bradford assay by electrostatic interaction of the dye's sulphonic group with amino acids like arginine and lysine, resulting in a colour change from brown to blue. The acidic dye solution's peak absorbance changes from 465 to 595 nm when binding to proteins occurs. A set of eight standards up to 1000 µg/mL was prepared. Then, 10 µL of each standard, sample, and blank were added into a 96-well microplate. Then, 300 µL of Pierce Detergent Compatible Bradford Assay Reagent was added to each well. The absorbance was measured at 595 nm after 10 min of room temperature incubation.

Amino acids profile of farmed and wild black-footed abalone extract

Analysis of amino acids of abalone extracts (farmed and wild) was conducted by previously described methods (Salazar et al., 2012). Amino acid standards and samples (40 µL) were mixed with 40 µL internal standard-spiked methanol [ISSM] (methanol containing 10 mg L^{-1} d4-alanine). Samples were centrifuged at 10,000 rpm for 5 min at 4°C. The supernatants were used for derivatisation. Then, 70 µL borate buffer were added to 10 µL samples, standard and blank. The blank was prepared in the same manner as the samples with distilled water instead of the sample. Then, 10 mL Accutag reagent (2.8 mg 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate [accutag] in 1 mL of QrY acetonitrile) were added to the samples and vortexed immediately. The samples and standards were incubated at 55°C for 15 min. After this, 400 µL of neutralising solution (10% (v: v) formic acid) was added to each vial. Samples were transferred into autosampler vials and placed in the LC-MS for analysis. LC-MS analyses were conducted using an Agilent 1260 Infinity Quaternary LC System (Santa Clara, CA 95051 USA). The system consisted of the following components: 1260 quaternary pump (model number: G1311B), 1260 infinity ALS sampler (model number: G1329B), 1260 infinity TCC

column component (model number: G1316A), 1260 infinity diode array detector (DAD) (model number: G4212B), connected to a 6420 triple quadrupole LC/MS system with electrospray ionisation (ESI) source (model number: G1948B). Phenomenex Kinetex Evo C18 (2.1 × 150mm, 1.7µm) was used for this analysis. The mobile phases were composed of water containing 0.1% (v/v) formic acid (A) and acetonitrile containing 0.1% (v/v) formic acid (B). The flow rate was 0.225 mL/min. The initial gradient condition was 95:5 (A: B) and held for 0.5 min. From 0.5 to 2 min, the B was increased to 15%, from 2 to 9 min, the B was increased to 20%, from 9 to 11 min, the B was increased to 50% and held for 2 min. From 13 to 14 min, the B was increased to 80% and held for 2 min. From 16 to 17 min, B was decreased to 5%. The total run time was 23 min. The MS ionisation source conditions were as follows: capillary voltage of 4 kV, drying gas temperature of 300°C, drying gas flow of 10 L/min, and nebuliser pressure of 40 psi. Both positive and negative scan modes were used with a scan range of 100-1000 m/z. The wavelengths selected for DAD were 254 nm, 278 nm and 300 nm.

Antioxidant capacity of farmed and wild black-footed abalone extract

Free radical scavenging activity (DPPH) of abalone extracts (farmed and wild) was determined following the modified methods of Luther et al. (Luther et al., 2007). In a 96-well microplates, Trolox standard (20 µL), sample (20 µL), blank (200 µL of 70% methanol) and control (100 µL of 70% methanol) were added. Then, 200 µL of DPPH solution (0.1 mM, dissolved in methanol) were added to the standard, sample, and control and were incubated in the dark for 30 min at room temperature. The absorbance of the samples was detected at 517 nm using a spectrophotometer. The DPPH results of abalone extracts were represented as milligram Trolox equivalent (TE) per gram of dry abalone tissue (mg TE/g dry tissue).

The following equation was used to determine radical scavenging activity:

$$\% \text{ DPPH scavenged } = \left[1 - \frac{A_{\text{sample}} - A_{\text{Blank}}}{A_{\text{Control}} - A_{\text{Blank}}}\right] \times 100$$ **Error!**

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where A_{sample} is the absorbance of the sample and DPPH. A_{control} is the absorbance of the solvent and DPPH. A_{Blank} is the absorbance of the solvent without the sample and DPPH.

The FRAP of the abalone extracts (farmed and wild) was determined using the procedure described in Rajurkar et al. (Luther et al., 2007). In a 96-well microplate, 10 µL of Trolox standard, sample, and blank (ethanol) were introduced to individual wells. Then, 200 µL of FRAP reagent (1:10:1, 10 mM TPTZ in HCl, acetate buffer, and 20 mM $FeCl_3$) were added to each well and incubated for 60 minutes in the dark at room temperature. The absorbance was detected at 593 nm with a UV spectrophotometer. The results were expressed as milligrams of Trolox equivalent (TE) per gram of dry abalone tissue (mg TE/g dry tissue).

Antiageing capacity of farmed and wild black-footed abalone

The hyaluronidase inhibitory activity of abalone extracts (farmed and wild) was performed using the spectrophotometric method described by Ratnasooriya et al. with slight modifications (Chaiyana et al., 2020). The experiment was carried out on a 96-well plate with a clear flat bottom. Abalone extracts of (5 µl) with a concentration 50000 µg/mL in water were preincubated with 25µL hyaluronidase (15 U/mL) in 20 mM phosphate buffer at pH 5.35 containing Bovine serum albumin (0.01% w/v) and NaCl (77 mM) for 10 minutes at 37°C. Subsequently, the reaction was initiated by adding 25µL hyaluronic acid (0.03% in 300 mM sodium phosphate, pH 5.35) to the incubation mixture, which was then incubated further for 45 min at 37°C. Undigested hyaluronic acid was precipitated with the addition of 250 µL

acid albumin solution (bovine serum albumin 0.1%, sodium acetate (24 mM) and acetic acid (79 mM), pH 3.75) and incubated at room temperature for 10 min. Then, the absorbance was

measured at 600 nm. The assay was performed with oleanolic acid (1000 µg/mL) in DMSO as a positive control under exactly the same experimental condition.

Inhibition of hyaluronidase was calculated as follows:

$$\left[\text{Hyaluronidase inhibition (\%)} = \mathbf{1} - \frac{(\text{A}-\text{B})-(\text{C}-\text{D})}{(A-B)}\right] \times 100 \quad \textbf{Error! Reference source not found.})$$

where A is absorbance of mixture with enzyme without sample, B is the absorbance of mixture without enzyme and sample, C is the absorbance of mixture with enzyme and sample, and D is the absorbance of mixture with sample and without enzyme.

To assess the ability of the obtained abalone extracts (farmed and wild) to inhibit collagenase activity, a Collagenase Inhibitor Screening Kit (Abcam, ab211108) was used. The abalone extracts at the concentrations of 500 µg/mL were used for the analysis. Following the manufacturer's instructions and the procedure previously reported by Zagórska-Dzioket al. (Zagórska-Dziok et al., 2021) with slight modification, the experiment was carried out on a white fluorometric 96-well plate with a clear flat bottom. Firstly, collagenase (COL) and collagenase substrate were diluted and dissolved in collagenase assay buffer (CAB), respectively. Abalone extracts (1 µL) and inhibitor control 1,10-phenanthroline (1 µL) were mixed with diluted COL (5 µL) and CAB (44 µl). Enzyme controls (EC) were prepared by mixing diluted COL (5 µL) and CAB (45 µL). The CAB (50µl) was used as a background control. The samples were then incubated for 15 min at room temperature. Moreover, the dissolved collagenase substrate was diluted with CAB to make a reaction mixture which was then added to the samples and mixed well. The fluorescence was then measured immediately at 490 nm excitation and 520 nm emission on a microplate reader in kinetic mode for 30-60 min at 37°C. Measurements were carried out in duplicate for each sample, as directed by the manufacturer.

Inhibition of the collagenase was calculated from the equation:

$$\%\ \text{Relative inhibition} = \frac{RFU(EC) - RFU(S)}{RFU(EC)} \times 100 \quad (3)$$

where *RFU* is relative fluorescence unit, *EC* is enzyme control, and *S* is sample.

Biocompatibility of farmed and wild black-footed abalone extract

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) test was used to assess the effect of abalone extracts on the proliferation of the Vero cell line. A 100-µL Vero cell suspension (10×10^4 cells/mL) in growth media (DMEM, 5% and 1%P/S) was seeded into all wells (10,000 cells/well). The plates were then incubated at 37°C with 5% carbon dioxide for 24 hours. The growth media were removed and replaced with fresh growth media containing abalone extracts at different concentrations (3000 µg/mL, 1500 µg/mL, 750 µg/mL, and 375 µg/mL) to obtain the maximum level of cell death. The plates were then incubated at 37°C for 48 hours. After incubation, all the growth-media-containing extracts were removed and replaced with 100 µL of fresh growth media. Then, 10 µL of MTT were added to all wells and incubated at 37°C with 5% carbon dioxide for four hours. After incubation, 80 µL was removed from each well, and 100 µL of DMSO was added to dissolve the precipitate. The absorbance of cell viability was measured at 450 nm by a UV spectrophotometer (SpectraMax iD3 Multi-Mode, USA).

4.3.4. Statistical analysis

Extractions were performed in duplicates and the expressed values of each extraction are the means ± SD of three samples. The correlation between phenolic compounds and antioxidant activity was analysed. To evaluate significant ($p < 0.05$) differences between extracts, an analysis of variance (ANOVA) was performed. All statistical analyses were performed using the Microsoft Excel package.

4.4. Results and discussion

Discovery of new chemicals with antioxidant and antiaging properties is of great interest in pharmaceutical and cosmeceutical industries.The biological activities of black-footed abalone extracts was obtained through subcritical water extraction and the effect of time on the bioactivity of the black-footed abalone extracts was investigated. The indicators of bioactive compounds of black-footed abalone extracts were total phenolic compounds, Glycogen content, protein content, amino acid profile, antioxidant capacity (DPPH and FRAP), antiaging capacity (anti-collagenase and anti-hyaluronidase) and biocompability.

4.4.1. Total phenolic content of farmed and wild black-footed abalone extract

The results of TPC of abalone extracts (farmed and wild) are shown in Figure 4-2. As can be seen, the extraction time at 220°C had a significant effect on the TPC of both farmed and wild abalone extracts. The highest TPC values were achieved at 45 min after extraction. As the time of extraction increased from 5 to 45 min, the TPC values of the farmed abalone and wild abalone extracts increased 29.64 and 29.09% respectively. This behaviour might be due to hydrogen bond breakage occurring at higher extraction times, resulting in increased phenolic compounds solubility in water phase extracts (Kheirkhah et al., 2019). However, when the time exceeded 45 min, the TPC content slightly decreased with increasing extraction time. Prolonged exposure of the compounds to high temperature resulted in a decrease in total phenolic compounds, which is associated with the degradation of phenolic compounds (Essien et al., 2020). These findings are in accordance with a previous study on green kiwifruit (*Actinidia deliciosa)* (Kheirkhah et al., 2019) and brown macroalgae (*Ecklonia maxima*) (Bordoloi & Goosen, 2020) in which the highest TPC level was achieved at 90 min

in green kiwifruit and 23.75 min in brown algae, and the TPC decreased by increasing temperature to 180 min of extraction. On the other hand, there were no significant differences in TPC between the obtained extracts of farmed and wild abalone (p>0.05).

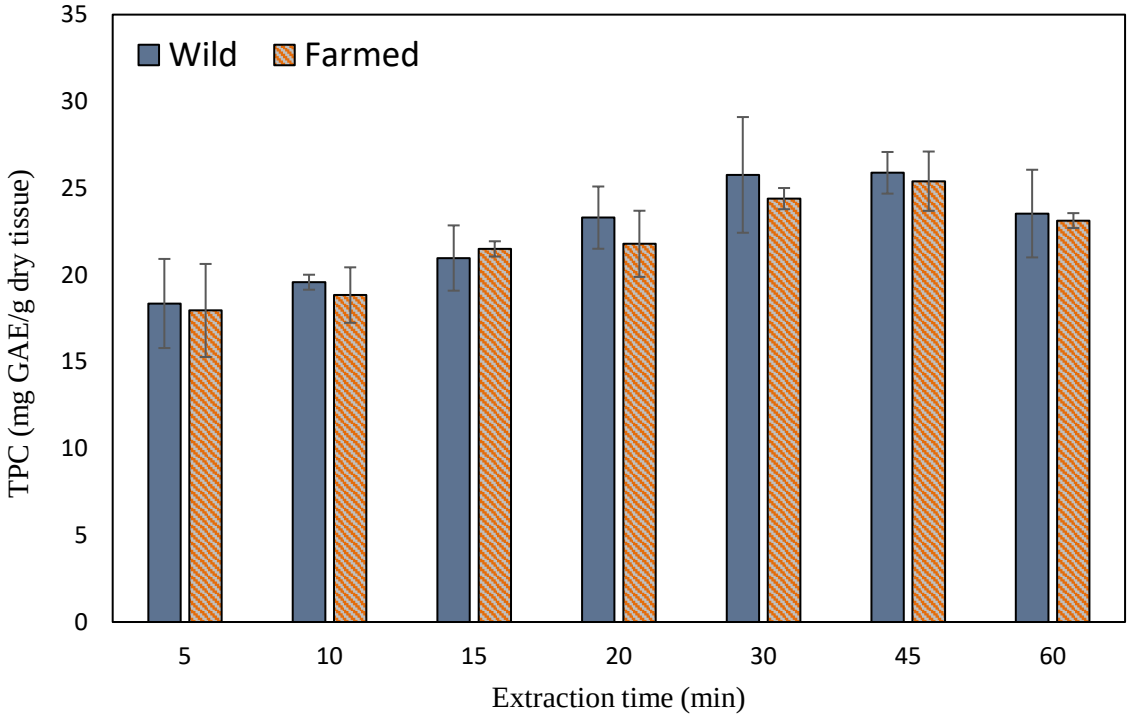

Figure 4-2.Total phenolic content (TPC) of abalone extracts obtained at 220 °C at different extraction times *(values are mean ± SD, n = 3)*

4.4.2. Glycogen content of farmed and wild black-footed abalone extract

The effect of extraction time on the glycogen (polysaccharide) content of abalone extracts (farmed and wild) obtained by subcritical water extraction was investigated, and the results are illustrated in Figure 4-3. By increasing the extraction time from 5 to 15, the glycogen content of farmed and wild abalone extract increased slightly 8% and 1.21 %, respectively and decreased as the extraction time increased. The thermal breakdown of polysaccharides would be accelerated by prolonged exposure to high temperatures (Getachew et al., 2019). However,

this increase was not statistically significant (p>0.05). These results concur with the results of a previous study on Pacific oyster polysaccharides which From 5 to 15 minutes of extraction, the yield of polysaccharide increased, and then it began to decrease. (Getachew et al., 2019). On the other hand, there were no significant differences between glycogen contents of farmed and wild abalone extracts obtained at different times (p>0.05). To the best of our knowledge, no studies has has compared glycogen content of farmed and wild abalone obtained by subcritical water extraction.

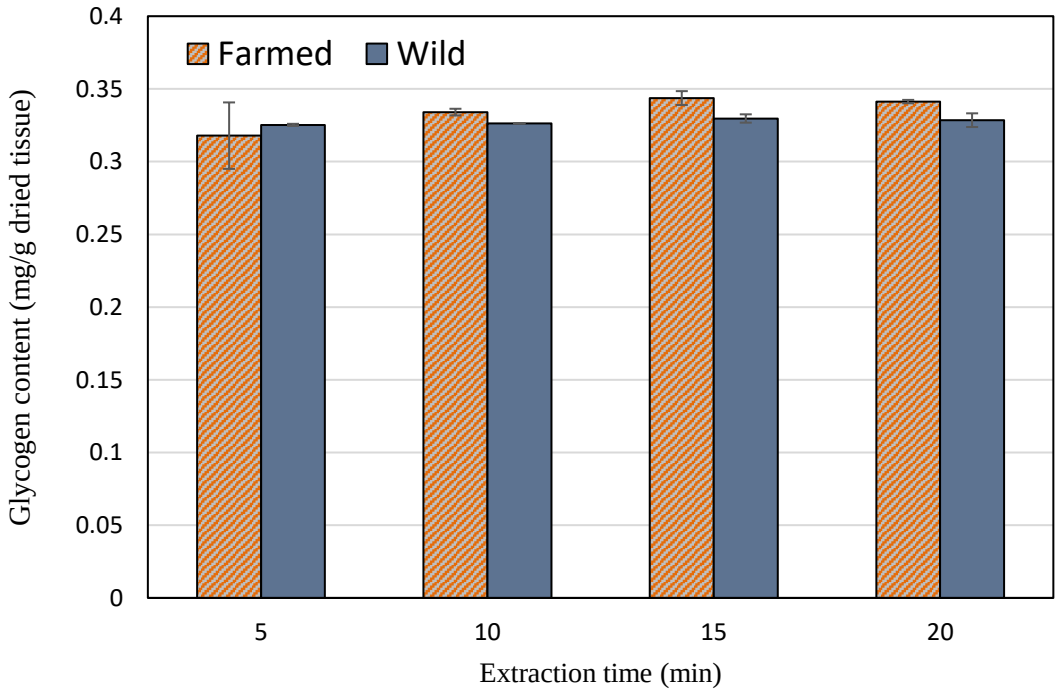

Figure 4-3. Glycogen content of abalone extracts obtained at 220 °C at different extraction times *(values are mean ± SD, n = 3)*

4.4.3. Protein content of farmed and wild black-footed abalone extract

The protein content of farmed and wild abalone extracts is presented in **Error! Reference source not found.** Increasing the time of extraction from 5 to 30 min resulted in an increase in the protein contents of farmed and wild abalone extracts by 29.37 and 42.41%, respectively,

and they were statistically significant. Differences were considered statistically significant when the *p*-value was smaller than 0.05. The protein content of the abalone extract was reduced after 30 min because the protein was hydrolysed more into water-soluble compounds under subcritical water conditions. Water acts as a reactant and catalyst because of the decreasing dielectric constant that enhances the solubility of the organic compounds (Phusunti et al., 2017). The present findings also support previous studies, which concluded that by increasing the time of extraction, the protein content of the green microalgae *Chlorella. vulgaris* extract decreased (Phusunti et al., 2017). They found that at 200°C of extraction, the protein content decreased from 28.90% to 14.75% by increasing the time of extraction from 90-180 min.

Furthermore, there were significant differences between the protein content of farmed abalone extract and wild abalone extract ($p < 0.05$). As stated previously, farmed abalone extracts had more protein content than wild abalone extracts, which could be linked to the abalone diet. Unlike farmed abalone, which are fed a high-protein formulated diet, wild abalone feed on seaweeds (Bullon et al., 2022). A previous study has reported that the protein content of farmed abalone fed higher protein diets was higher than that of juveniles fed macroalgae (Stone et al., 2013). Therefore, the findings of this study are consistent with the results of previous studies.

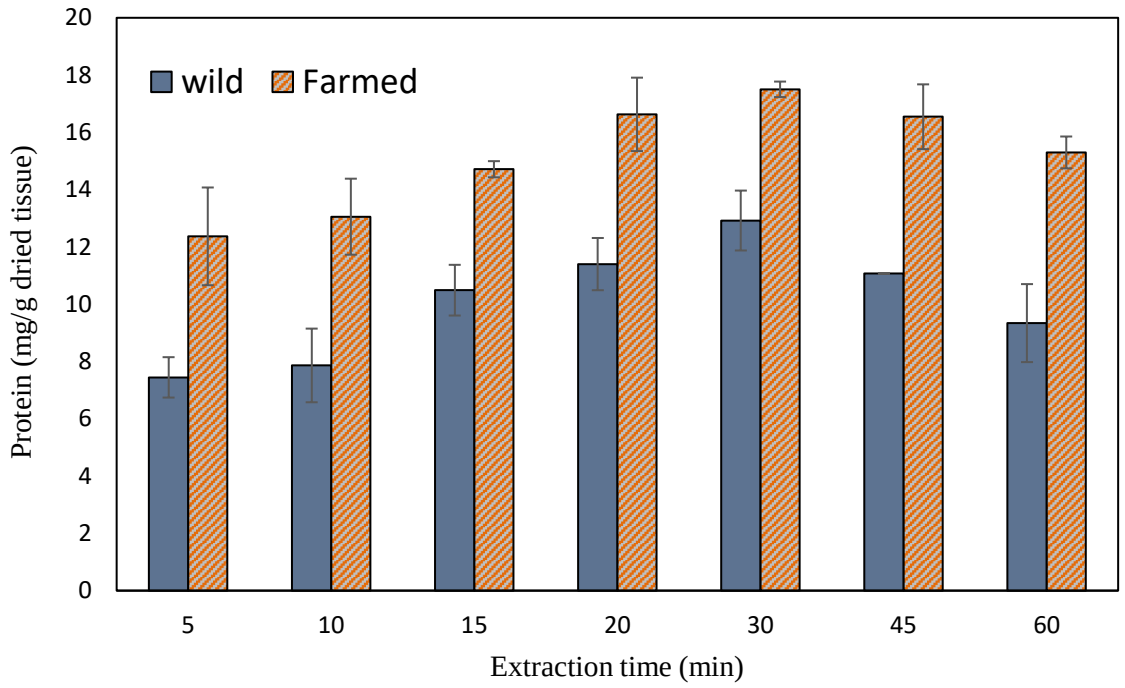

Figure 4-4. Proteins of abalone extracts obtained at 220 °C at different extraction times *(values are mean ± SD, n = 3)*

4.4.4. Amino acids content of farmed and wild black-footed abalone extract

The total amino acid (TAA) profiles, including essential (EAA) and non-essential (NEAA) amino acids of abalone extracts for both wild and farmed abalone, are presented in **Error! Reference source not found.**.

Extraction time had an effect on the TAA content of both farmed and wild abalone extracts. By increasing the time of extraction from 5 to 60 min, the TAA of farmed and wild abalone increases by 33.68 and 10.65%, respectively. However, this effect is not statistically significant (p>0.05). Furthermore, there was a significant difference between the TAA of farmed and wild abalone (p<0.05). Farmed abalone extracts showed higher TAA than wild abalone extracts, which could be due to variations between farmed abalone's high protein formulated diets and wild abalones fed seaweed (Stone et al., 2013). Both farmed and wild abalone extracts contained all EAA, including histidine, leucine, lysine, methionine, phenylalanine, tryptophan,

threonine, valine, and isoleucine. The results indicate that the prolonged exposure time (5-60 min) to heating temperature (220°C) affected the EAA of extracts which caused the EAA of farmed and wild abalone to increase by 54 and 45% significantly (p<0.05). This finding agrees with a previous study on scallop viscera waste, that a longer reaction time yields a higher amount of amino acids (Kang et al., 2001; Tavakoli & Yoshida, 2006) However, because most EAA are hydrophobic non-polar, such as leucine, isoleucine, methionine, phenylalanine, tryptophan, and valine, proteins require a high temperature and longer reaction time to unfold and release (Hao et al., 2019a) (Kang et al., 2001; Tavakoli & Yoshida, 2006).Therefore, a higher amount of EAA is obtained at a longer time of exposure to high temperatures. Alternatively, the physical-chemical properties of water can be altered dramatically with temperature in subcritical circumstances. In the subcritical range, the ionic product of water increases, raising the concentration of H^+ and OH^-, favouring biomass hydrolysis. Furthermore, as the dielectric constant lowers, water can interact with non-polar compounds, reducing their binding force and allowing them to dissolve. As a result, biopolymers, such as proteins, are released from the matrix and degraded into amino acids (Alonso-Riaño et al., 2021). This could also be owing to the fact that simple amino acids, such glycine and alanine are stable at high temperatures and pressures of water which require more time to be realised. Complex amino acids may also break down to produce simple amino acids (Quitain et al., 2001). Moreover, there were no significant differences between the EAA of farmed and wild abalone extracts.

Furthermore, prolonged exposure of farmed and wild abalone extracts to high temperatures slightly affected total NEAA, but their differences were not statistically significant (p>0.05). This could be due to predominant NEAA like taurine, which had a high yield of 41.98 and 33.09 mg/g of dried abalone tissue (farmed and wild, respectively) during 10 min of extraction that affected all NEAA. Taurine is a polar amino acid which was not affected by more prolonged exposure to high temperatures. Polar amino acids are present on the surface of

proteins and interact with solvent molecules more quickly than non-polar amino acids, which are situated inside proteins and are protected from direct contact with water (Abdelmoez & Yoshida, 2013). As a result, non-polar amino acids require a longer extraction time to be released than polar amino acids. There were also no significant differences between NEAA of farmed and wild abalone extract obtained at different times. The highest EAA/TAA % and NEAA/TAA % of both farmed and wild abalone were obtained at 60 and 10 min of extraction, respectively. At 60 min of extraction time, the highest EAA/NEAA of farmed and wild abalone extracts were likewise achieved.

Table 4-1. Amino acids profile of farmed black-footed abalone extracts *(values are mean ± SD)*

Amino acids	Extraction time (min)						
	5	10	15	20	30	45	60
Essential amino acids (EAA)							
L-Histidine	0.22±0.03	0.17±0.01	0.22±0.07	0.29±0.03	0.39±0.01	0.34±0.07	0.5±0
*L-Leucine	0.45±0.03	0.43±0.03	0.65±0.03	0.77±0.08	0.96±0.03	1.17±0.12	1.58±0.01
*L-isoleucine	0.32±0.02	0.26±0.02	0.38±0.02	0.44±0.04	0.54±0.02	0.64±0.05	0.81±0.01
L-Lysine	0.31±0.02	0.2±0.01	0.28±0	0.28±0	0.32±0.01	0.36±0.03	0.45±0.04
*L-Methionine	0.02±0	0.01±0	0.04±0	0.04±0.01	0.03±0	0.01±0	0±0
*L-Phenylalanine	0.32±0.01	0.25±0.03	0.35±0.02	0.42±0.07	0.5±0.01	0.58±0.08	0.75±0.03
*L-Tryptophan	0.11±0	0.08±0.01	0.09±0	0.1±0.01	0.1±0.01	0.11±0.01	0.14±0.01
L-Threonine	0.27±0.01	0.18±0.01	0.24±0.01	0.25±0.03	0.25±0.01	0.2±0.01	0.19±0.01
*L-Valine	0.51±0.03	0.4±0.03	0.58±0.03	0.66±0.06	0.79±0.03	0.91±0.1	1.15±0.01
Non-essential amino acids							

L-Arginine	2.3±0.12	1.33±0.01	1.47±0.04	1.42±0.22	1.36±0.11	1.01±0.08	1.16±0.35
*Hydroxy-L-Proline	0.04±0.01	0.05±0.01	0.07±0.01	0.1±0.02	0.13±0	0.17±0.03	0.25±0.02
Ethanolamine	0.16±0.02	0.15±0.01	0.22±0.01	0.26±0.03	0.32±0.01	0.37±0.04	0.45±0.01
L-Serine	0.85±0.03	0.7±0.06	0.97±0.07	1.09±0.13	1.18±0.06	1.11±0.09	1.19±0.05
*Glycine	1.67±0.1	2.5±0.1	3.04±0.16	2.84±0.23	3.53±0.14	4.51±0.14	5.58±0.1
L-Aspartic acid	1.96±0.14	1.55±0.14	2.12±0.06	2.09±0.16	1.89±0.07	1.56±0.11	1.46±0.08
Taurine	28.47±0.11	41.99±2.31	37.67±0.43	39.41±1.07	37.79±1.09	36.75±0.56	37.08±0.43
*b-Alanine	0.07±0.01	0.04±0	0.05±0	0.06±0	0.06±0	0.05±0	0.05±0
L-Glutamic acid	0.11±0.01	0.09±0.01	0.14±0.01	0.14±0.01	0.17±0.01	0.18±0.01	0.21±0
L-Citrulline	0±0	0±0	0±0	0±0	0±0	0±0	0±0
*L-Alanine	1.8±0.26	2.56±0.18	3.13±0.12	3.2±0.2	3.99±0.01	5.16±0.48	6.79±0.03
*L-Proline	0.93±0.05	0.62±0.04	0.84±0.04	0.91±0.08	1.07±0.04	1.24±0.15	1.61±0.01
Cystathionine	0±0	0±0	0±0	0±0	0±0	0±0	0±0

L-Anserine	0±0	0±0	0±0	0±0	0±0	0±0	0±
L-Tyrosine	0.36±0.01	0.26±0.03	0.36±0.02	0.46±0.08	0.52±0.01	0.61±0.09	0.8±0.03
TAA	41.25	53.82	52.91	55.23	55.89	57.04	62.20
EAA	2.53	1.98	2.83	3.25	3.88	4.32	5.57
EAA/TAA (%)	6.13	3.68	5.35	5.88	6.94	7.57	8.95
NEAA	38.72	51.84	50.08	51.98	52.01	52.72	56.63
NEAA/TAA (%)	93.87	96.32	94.65	94.12	93.06	92.43	91.05
EAA/NEAA=	6.53	3.82	5.65	6.25	7.46	8.19	9.84

*: hydrophobic amino acids; TAA: Total amino acids; EAA: Essential amino acids; NEAA: Non-essentila amino acids

Table 4-2. Amino acids profile of wild black-footed abalone extracts *(values are mean ± SD, n = 3)*

Amino acids	Extraction time (min)						
	5	10	15	20	30	45	60
Essential amino acids							
L-Histidine	0.18±0.04	0.16±0.01	0.19±0.06	0.18±0.03	0.29±0.07	0.5±0.01	0.58±0.58
*L-Leucine	0.33±0.03	0.43±0.09	0.59±0.06	0.61±0.07	0.74±0.05	0.91±0.02	1.1±0.02
*L-isoleucine	0.27±0.03	0.29±0.07	0.39±0.02	0.38±0.03	0.44±0.03	0.5±0	0.59±0.02
L-Lysine	0.24±0	0.22±0.04	0.25±0.02	0.22±0.03	0.23±0.03	0.25±0	0.27±0
*L-Methionine	0.02±0	0.03±0.02	0.05±0.01	0.04±0	0.03±0	0.01±0	0±0
*L-Phenylalanine	0.25±0.03	0.25±0.06	0.32±0.03	0.31±0.04	0.37±0.02	0.43±0.01	0.51±0.03
*L-Tryptophan	0.09±0.01	0.07±0.02	0.08±0.01	0.07±0.01	0.07±0.01	0.07±0.01	0.09±0
*L-Threonine	0.34±0.03	0.28±0.07	0.32±0.03	0.26±0.04	0.22±0.02	0.16±0	0.14±0

*L-Valine	0.54±0.05	0.53±0.11	0.67±0.06	0.63±0.06	0.7±0.05	0.77±0	0.88±0.01
Non-essential amino acids							
L-Arginine	1.95±0.05	1.57±0.43	1.48±0.2	1.2±0.28	1.16±0.25	1.13±0.11	1.18±0.11
Hydroxy-L-Proline	0.03±0.01	0.04±0.01	0.06±0.01	0.06±0.01	0.08±0	0.11±0	0.15±0
Ethanolamine	0.15±0.01	0.2±0.03	0.26±0	0.26±0	0.33±0.01	0.44±0	0.44±0.01
*L-Serine	0.82±0.06	0.88±0.18	1.08±0.07	0.96±0.11	0.95±0.08	0.9±0.02	0.84±0.01
*Glycine	2.09±0.06	1.7±0.46	2.21±0.22	2.76±0.23	3.02±0.05	3.61±0.04	3.97±0.16
L-Aspartic acid	2±0.16	2.1±0.45	2.6±0.13	2.1±0.16	1.75±0.1	1.4±0.01	1.09±0.01
Taurine	32.63±0.69	33.09±0.06	31.43±0.18	30.99±0.01	33.33±1.45	29.75±1.59	28.9±1.59
b-Alanine	0.07±0.01	0.07±0.01	0.09±0	0.08±0	0.08±0	0.08±0	0.06±0
*L-Glutamic acid	0.13±0.03	0.11±0.02	0.16±0	0.15±0.01	0.16±0	0.18±0.01	0.18±0
*L-Citrulline	0±0	0±0	0±0	0±0	0±0	0±0	0±0
*L-Alanine	2.04±0	1.83±0.49	2.46±0.03	3.19±0.34	3.19±0.05	4.22±0.09	5.03±0.14

*L-Proline	0.75±0.06	0.67±0.16	0.76±0.08	0.7±0.07	0.77±0.06	0.88±0.01	1.02±0
Cystathionine	0±0	0±0	0±0	0±0	0±0	0±0	0±0
L-Anserine	0±0	0±0	0±0	0±0	0±0	0±0	0±0
*L-Tyrosine	0.28±0.03	0.27±0.07	0.36±0.03	0.35±0.05	0.41±0.03	0.48±0.02	0.55±0.02
TAA	45.20	44.79	45.81	45.50	48.32	46.78	47.57
EAA	2.26	2.26	2.86	2.70	3.09	3.60	4.16
EAA/TAA (%)	5.00	5.05	6.24	5.93	6.39	7.70	8.75
NEAA	42.94	42.53	42.95	42.80	45.23	43.18	43.41
NEAA/TAA (%)	95.00	94.95	93.76	94.07	93.61	92.30	91.25
EAA/NEAA=	5.26	5.31	6.66	6.31	6.83	8.34	9.58

*: hydrophobic amino acids; TAA: Total amino acids; EAA: Essential amino acids; NEAA: Non-essentila amino acids

4.4.5. Antioxidant capacity of black-footed abalone extract

The 2-diphenyl-1-picrylhydrazyl (DPPH) and Ferric reducing antioxidant power (FRAP) tests were used to determine the antioxidant activity of farmed and wild abalone extracts at different extraction temperatures at 220°C in this study. The DPPH test is commonly used to evaluate an antioxidant's capacity to scavenge free radicals generated by the DPPH reagent, which causes a decrease in absorbance at 517 nm (Hajji et al., 2015). Ferric reducing antioxidant power (FRAP) is a common method for determining the ability of antioxidant compounds to donate hydrogen or electrons. The antioxidant capabilities of farmed and wild abalone extracts are represented in Figure 4-5. In both antioxidant scavenging activities (DPPH and FRAP), by increasing the extraction time from 5 to 60 min, the antioxidant activity increased, which was statistically significant (p<0.05). The highest DPPH activity of farmed and wild abalone extracts was found at 30 min with the values of 10.49 ± 0.27 and 9.93 ± 0.20 mg TE/g dried abalone tissue, respectively. At extraction time longer than 30 min, the antioxidant activity decreased for DPPH, which indicates that a further increase in the time of extraction results in the degradation of bioactive compounds. The capacity of abalone extracts to decrease Fe^{3+} to Fe^{2+} was determined using the FRAP assay (Hajji et al., 2015). The FRAP result of farmed and wild abalone extracts showed the highest activity at 60 min with the concentrations of 36.15 ± 1.37 and 32.79 ± 2.13 mg TE/g dried abalone tissue, respectively. However, there was no significant difference between 30-, 45-, and 60-min of both abalone extracts (farmed and wild). Therefore, the best recovery of antioxidants can be achieved at 30 min with energy-saving. The antioxidant outcome obtained from abalone extracts is consistent with a previous study on blue mussel, in which the best antioxidant recovery was achieved at a higher extraction time (Han et al., 2018). Furthermore, the findings are consistent with those of another study on Kiwi fruit [13], which the maximum antioxidant activity was obtained at 90 min, but it decreased once the thermal degradation capacity was exceeded at 120 min. The longer contact between the abalone and water permitted more diffusion of the extracted

compounds, resulting in higher extraction yields over time (B. Li et al., 2020). The finding of this study is consistent with those of a previous study on the effect of subcritical water treatment on the antioxidant activity of golden oyster mushroom, which found that antioxidant activity increased with extraction time at high temperatures (Jo et al., 2013). Furthermore, there was a significant difference between antioxidant activity (DPPH and FRAP) of wild and farmed abalone extract. Farmed abalone extract had higher antioxidant activity than wild abalone (p<0.05). This could be related to aformentiond reason which the diet farmed abalone has a higher nutritional profile than wild abalone (Bullon et al., 2022).

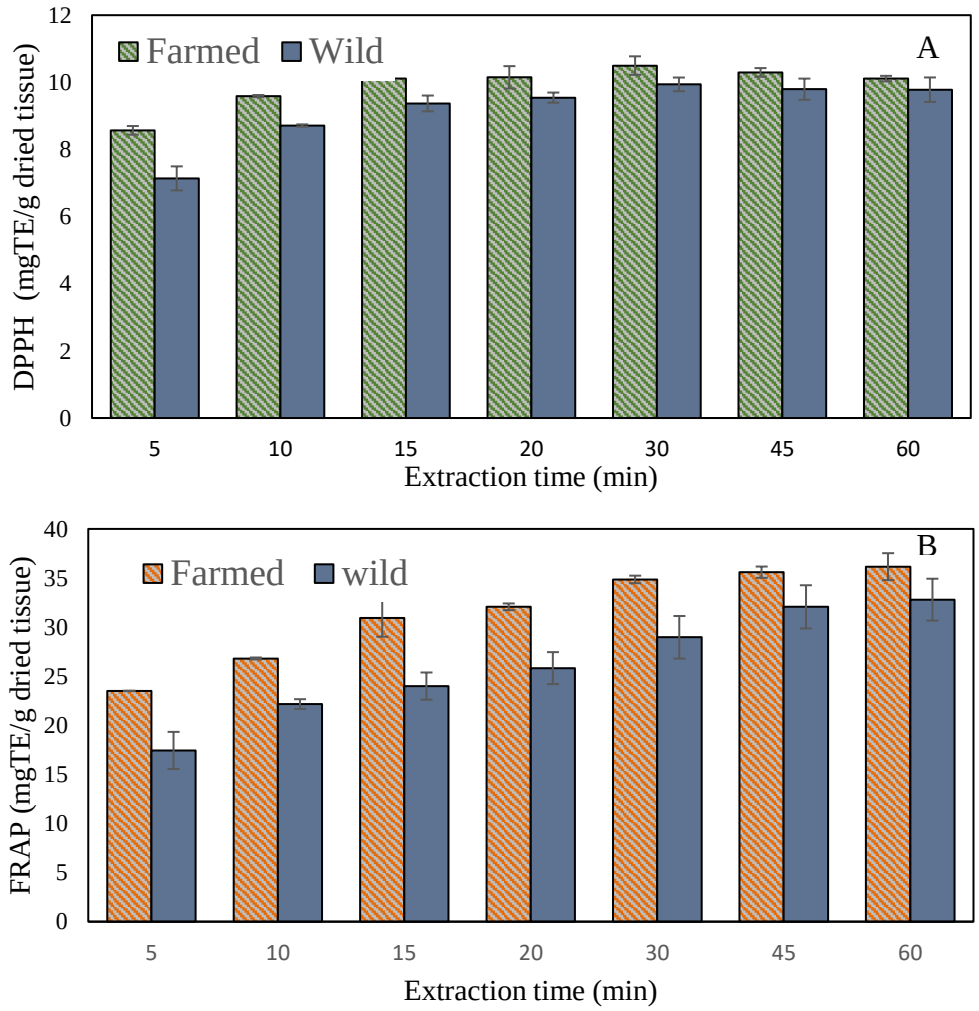

Figure 4-5. Radical scavenging activity (DPPH) of abalone extracts obtained at 220 °C at different times (A), Ferric reducing antioxidant power (FRAP) of abalone extracts obtained at 220 °C at different times (B) *(values are mean ± SD)*

4.4.6. Antiageing capacity of black-footed abalone extract

Hyaluronic acid (hyaluronan) is a component of the skin's extracellular matrix (ECM) that has certain elastin characteristics. Hyaluronidase is an enzyme that can degrade hyaluronic acid into small fragments by hydrolysing disaccharides at the β (1–4) linkages causing skin ageing (Chaiyana et al., 2020). Anti-hyaluronidase and anti-collagenase activity of both farmed and wild abalone were obtained and are presented in **Error! Reference source not found.**. According to the result of hyaluronidase inhibition, by increasing the time of extraction from 5 to 60 min, hyaluronidase inhibition increased significantly in both farmed and wild abalone extracts ($p<0.05$). The highest hyaluronidase inhibition was achieved by 31% and 32 %, with a 50000 µg/mL concentration of farmed and wild abalone extracts, obtained at 60 min of extraction, respectively. However, this is a low inhibition compared to the 84 % inhibition by oleanolic acid at 1000 µg /mL. There are some factors including sulphate content, molecular weight, carbohydrate content, and phenolic compounds which might contribute to anti-hyaluronidase activity (Pozharitskaya et al., 2020) (Ciko et al., 2018).

There were no significant differences between the anti-hyaluronidase activity of farmed and wild abalone extracts ($p>0.05$). To our knowledge, no research has been conducted on the anti-hyaluronidase activity of New Zealand black-footed abalone extract obtained by subcritical water extraction. However, there have been a few investigations on other marine organisms, such as venus clam (*Gomphina melanaegis*), seaweed (*Fucus vesiculosus*) and sea cucumber (*Stichopus japonicus*) that possess anti-hyaluronidase activity (Ding et al., 2018; Pozharitskaya et al., 2020; Sutthiwanjampa & Kim, 2015b).

Collagen is a protein that helps the skin maintain its strength, suppleness, and moisture. It is involved in ageing process, skin regeneration and wound healing. Protein reduction in the skin may contribute to wrinkle formation by weakening the link between the dermis and the epidermis, which has a substantial impact on the skin’s look. Collagenase is an enzyme that aids in the

breakdown of the collagen structure in the skin. Free radicals and UV radiation, as well as internal and genetic factors, may all contribute to its heightened activity (Zagórska-Dziok et al., 2021). The ability of extracts from farmed and wild black-footed abalone to inhibit the activity of the collagenase enzyme was tested to determine the possible application of the studied extracts in the battle against skin ageing. The ability of extracts from farmed and wild abalone to inhibit collagenase was examined as part of this study, and all of the extracts tested were able to inhibit this enzyme, indicating its potential anti-ageing capabilities (Figure 4-66).

All farmed and wild abalone extracts obtained at different extraction times (5-60 min) exhibited significant anti-collagenase activity (ranging from 40 and 41% to 48 and 46%) with a concentration of 500 µg/mL, respectively. The greatest bioactivity was exhibited at 45 and 60 min of extraction with 48 and 46% of collagenase inhibition by wild and farmed abalone extracts, respectively.

There were no statistically significant changes with the extraction times (p>0.05). As a result, prolonged exposure of the compounds to high temperatures did not affect the abalone extracts' anti-collagenase action. The collagenase inhibition of abalone extracts was found even higher than 1,10 phenanthroline (500 µg/mL) with 37% collagenase inhibition. This high anti-collagenase activity may contribute to phenolic compounds that are present in the extracts. Many previous studies have stated that hyaluronidase and collagenase inhibition are due to the contribution of phenolics, terpenoids or steroids (e.g., hydroxycinnamic acids, ellagic acid, catechins, curcumin and carnosic acid) (Agulló-Chazarra et al., 2020; Zagórska-Dziok et al., 2021). Additionally, there were no significant differences between collagenase inhibition of farmed and wild abalone (p<0.05). To the best of our knowledge, this is the first study on New Zealand black-footed abalone serving as collagenase inhibition, and there was no other study on the anti-collagenase activity of molluscs. Additionally, there were no significant differences between collagenase inhibition of farmed and wild abalone (p<0.05). To the best of our knowledge, this is the first study on New

Zealand black-footed abalone serving as collagenase inhibition, and there was no other study on the anti-collagenase activity of molluscs. . Additionally, there were no significant differences between collagenase inhibition of farmed and wild abalone ($p<0.05$). To the best of our knowledge, this is the first study on New Zealand black-footed abalone serving as collagenase inhibition, and there was no other study on the anti-collagenase activity of molluscs.

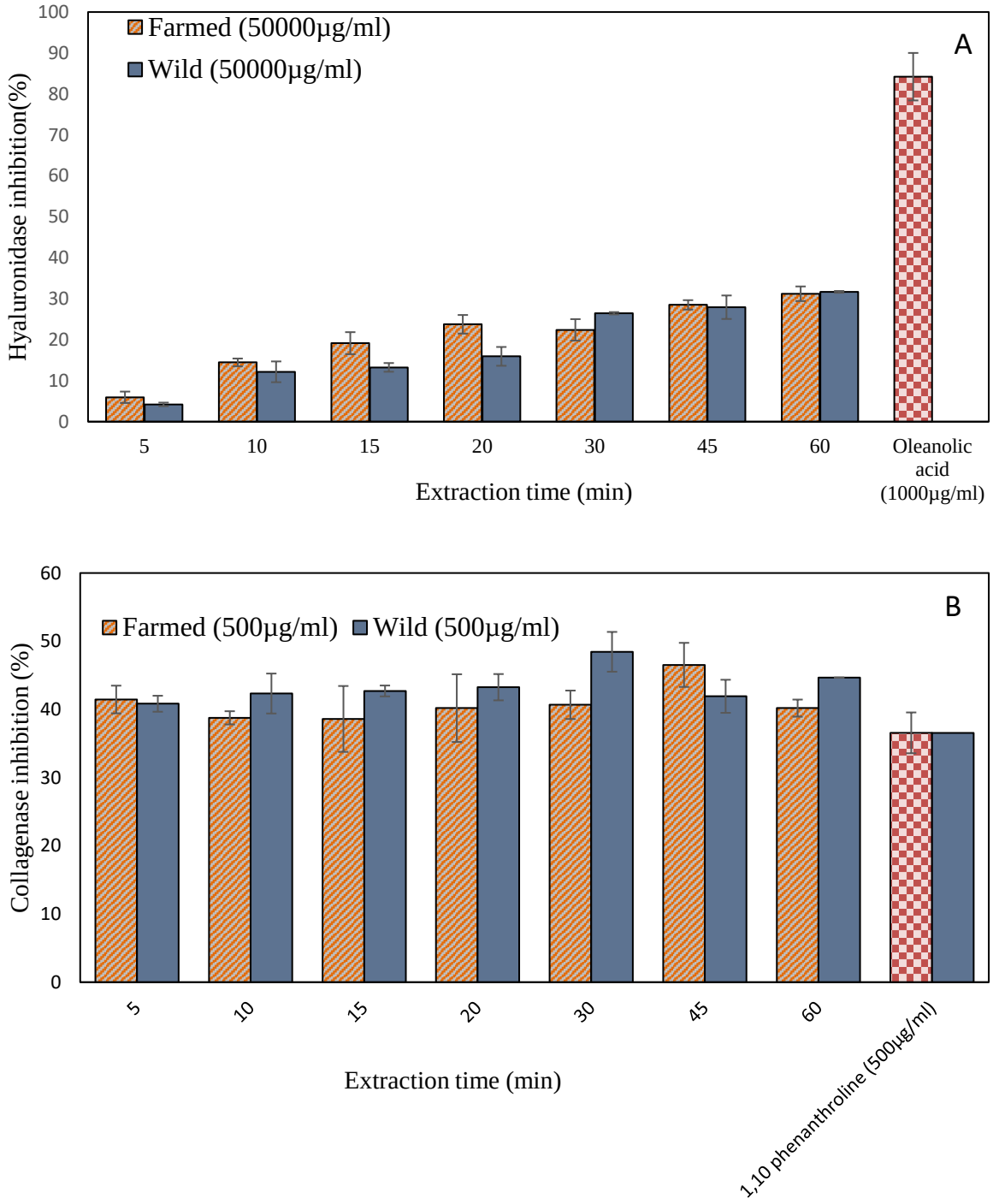

Figure 4-6. Hyaluronidase (A) and collagenase inhibition (B) of abalone extracts obtained at 220 °C at different times (5-60 minutes) ***(values are mean ± SD, n = 3)***

4.4.7. Biocompatibility of black-footed abalone extract

The cytotoxicity of farmed and wild abalone extracts obtained by subcritical water extraction was evaluated in vitro by the MTT assay. Farmed and wild extracts that were obtained at a temperature of 220°C at 45 min were used for cytotoxicity analysis due to the high bioactivity that obtained between 30 and 60 min compared with extracts obtained at lower extraction times. The MTT assay evaluated the conversion of yellow MTT to an insoluble purple formazan by succinate dehydrogenase found in live-cell mitochondria (Udhayakumar et al., 2017). The cytotoxic effect of abalone extracts was determined on the Vero cell line derived from a monkey. As can be seen in figure **Error! Reference source not found.**, after 48-hours of exposure of the cells to the abalone extracts, cell viability was more than 100% in response to 375 µg/mL for both extracts. However, by increasing the extract's concentration to 3000 µg/mL, cell viability decreased to 82% and 87% for farmed and wild abalone extracts, respectively. The IC50 of farmed and wild abalone extracts were 5950.46 and 6433.63 µg/mL, respectively. This indicates that the high concentrations of abalone extracts are non-toxic to the Vero cells.

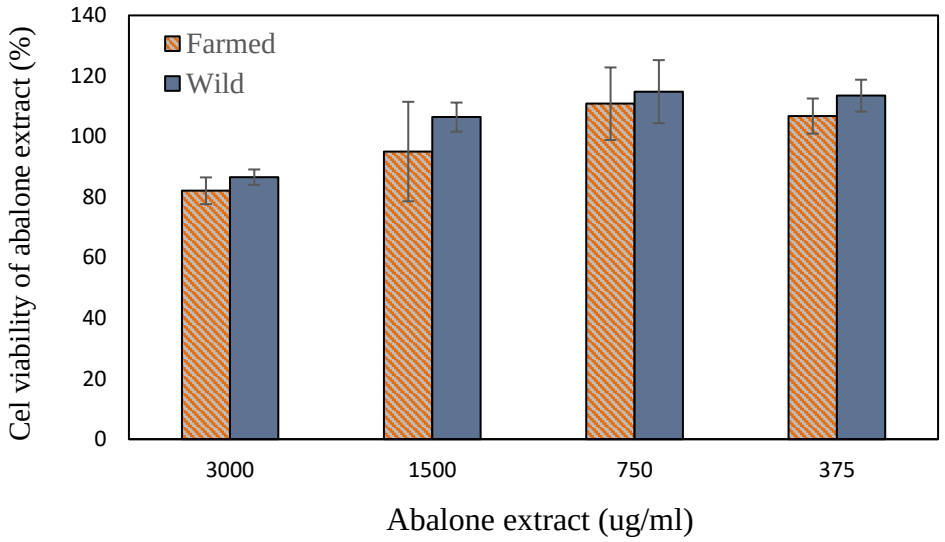

Figure 4-7. Effect of abalone extracts obtained at 220 °C at 45 minutes on Vero cells ***(values are mean ± SD, n = 3, p ≤ 0.05).***

4.5. Conclusions

The extracts obtained from black-footed abalone were found to be rich in protein, lipids, and carbohydrate, showing several biological activities, including antioxidant, anti-hyaluronidase, and anti-collagenase activities. These activities are influenced by the extraction process. A green extraction technique based on subcritical water was used in this study. Both farmed and wild abalone extracts obtained at different extraction times had a correlation between anti-hyaluronidase, antioxidants, TPC, protein and glycogen when the extraction time increased. Both farmed and wild abalone extracts showed a link between anti-collagenase, antioxidants and total phenolic compounds. In conclusion, this study demonstrates that extracts from abalone (farmed and wild) possess strong antiageing properties with no significant toxicity at the high doses assessed. As a result, these extracts are promising sources of novel antiageing agents that might be used in cosmeceutical products.

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Chapter 5. Extraction and characterisation of collagen from black-footed abalone by CO_2 acidified water extraction

5.1. Chapter Preface

Subcritical water extraction of bioactive compounds with antiaging properties from farmed and wild black-footed abalone have been studied in Chapter 4. Abalone extract derived from both farmed and wild sources have shown high biological activities, including but not limited to antioxidant, anti-hyaluronidase, anti-collagenase with no notable harm at high concentrations, making it a promising potential for use in antiaging products as a source of novel antiaging compounds. Furthermore, this chapter attempts to extract collagen (bioactive compounds with pharmaceutical, biomedical and cosmeceutical applications), from farmed and wild black-footed abalone using another environmentally friendly extraction method, CO_2 acidified water extraction (CO_2 AWE). Differences in the collagen extracts of farmed and wild abalone, obtained by CO_2 AWE were further investigated and compared to the collagen extract using ultrasound assisted extraction.

Abstract

Collagen is an important protein with applications in biomedical, pharmaceutical, and cosmeceutical formulations. Recently, marine collagens have been proposed as a potential alternative to mammalian collagen due to their unique properties such as no risk of transmitting diseases, low molecular weight, high absorption by the human body, and biocompatibility. New Zealand black-footed abalone, a marine gastropod, has a high natural product content, including collagen. New approaches are emerging to extract collagen with the objective of enhancing process yields as well as the qualities of the final products. This study investigates collagen extraction from farmed and wild black-footed abalone using CO_2 acidified water extraction method. The effects of operation parameters (CO_2 pressure and extraction time) on the extraction yield and the extracted collagen were studied. Carbon dioxide acidified water was able to enhance collagen extraction yield in a mild operating condition of 10 bar and 3 h. The isolated collagen was evaluated by scanning electron microscopy (SEM), Dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-page), Fourier-transform infrared spectroscopy (FITR), pH, colour, appearance, and biocompatibility. The result of CO_2 acidified water extraction showed similarities with the result of ultrasound-assisted extractions. As a result, the application of CO_2 acidified water extraction could be a promising alternative technique with high sustainability for enhancing the extraction efficiency of collagen from black-footed abalone with no toxicity. **Keywords:** CO_2 acidified water extraction; green extraction; collagen; black-footed abalone; carbon dioxide; natural products

5.2. Introduction

Collagen is one of the most abundant high-molecular-weight proteins in both invertebrate and vertebrate organisms. This material is the main constituent of animal skin, bone, and connective tissue, accounting for approximately 30% of total animal proteins (Rodríguez et al., 2017). Collagen's primary biological functions are to sustain and maintain structural integrity and provide texture, shape, and durability. Collagens are extracellular proteins having a unique amino acid profile. They have polypeptide chains made up of repeated triplets of glycine and two additional amino acids, proline and hydroxyproline (Rodríguez et al., 2017). Tropocollagen is the fundamental structure of collagen comprised of three polypeptide chains, each twisted in a left-handed helix (a-chain) coiling around one another to produce a right-handed triple superhelix. Collagen is polymerised via covalent cross-links development (Uriarte-Montoya et al., 2010). The molecular weight of collagen is around 300 kDa (Felician et al., 2018). Collagen makes up around a quarter of the human body, with at least 28 genetically distinct varieties found to date, categorised based on its organisation, tissue distribution and supramolecular structure function and described as types I – XXVIII (Coelho et al., 2017; Felician et al., 2018). Collagen type I makes up about 80% of the body's collagen and is admittedly related to skin ageing (Felician et al., 2018). It has a triple helical structure with an interchain hydrogen bonding between the amide group and glycine in a neighbouring chain playing a vital role in its stability. Collagen type I has excellent biocompatibility and biodegradability, enabling it to be used broadly in pharmaceutical, biomedical, and cosmeceutical tissue engineering and food industries (Matmaroh et al., 2011).

Collagen is mostly derived from land-based animals, including bovine or porcine skin and bones, for industrial applications. Restriction of land-based animal collagen due to many issues including bovine spongiform encephalopathy, foot-and-mouth disease crisis and religious

barriers, has led to the extraction of collagen from alternative sources (Felician et al., 2018). Recently, marine collagen such as fish, sharks and molluscs are being intensively studied as novel materials for pharmaceutical and food and personal care products (Chi et al., 2014; Kittiphattanabawon et al., 2010; Petcharat et al., 2021; Rodríguez et al., 2017; Silva et al., 2016b; Uriarte-Montoya et al., 2010; Zou et al., 2017). New Zealand black-footed abalone (*Haliotis iris*) is a marine gastropod mollusc that has received attention due to its high nutritional values, including lipid, protein, collagen, carbohydrate, and mineral elements (C.-H. Tung & Alfaro, 2011; L. C. Wang et al., 2019; Wells et al., 1998). However, collagen derived from abalone is not yet available, and industrial extraction technology has yet to be developed.

Collagen in its natural form is soluble in acidic conditions. Traditionally, marine collagen has been successfully extracted by different extraction methods such as acid/enzymatic extraction. These methods are time-consuming and require a large amount of solvents, resulting in low selectivity and low extraction yields (Barros et al., 2015). Ultrasound has assisted acid extraction in recovering bioactive chemicals from a number of sources in recent years (Zou et al., 2017). Ultrasound is described as a high-frequency sound wave that exceeds the human hearing limit of 20 kHz. Its operation is dependent on passing waves that create high and low-pressure zones; the change in acoustic pressure is related to the amount of energy delivered to the system. This method still needs a considerable amount of solvents and is time-consuming with several processes. On the other hand, chemical industries are moving towards the adoption of new processing procedures which comply with the green chemistry philosophy due to environmental concerns, waste prevention, safety—no toxic as well as processing cost, and efficiency (Barros et al., 2015; Marcus, 2018). Therefore, a more efficient extraction process with fewer operating steps is required, as well as one that is more ecologically friendly and adheres to green chemistry principles. Recently, a new green extraction technique has been

developed based on water acidified with carbon dioxide (CO_2-AWE) (Barros et al., 2015; Silva et al., 2016b; Sousa et al., 2020). This extraction technique consists of a single step that takes place under controlled conditions using water as a solvent.

However, only a few investigations have been done on extracting collagen from marine sources such as sponge using carbon dioxide-acidified water (CO_2-AWE) (Barros et al., 2015; Silva et al., 2016a). On the other hand, to the best of our knowledge no investigation has been conducted on extraction of collagen from New Zealand black-footed abalone. Therefore, the purpose of this study is to compare collagen extracts isolated from black-footed abalone using Ultrasound assisted extraction (UAE) and CO_2 acidified water extraction (CO_2-AWE). The results of this study are expected to lead to new uses for seafood products and contribute to the developing abalone sector in New Zealand.

5.3. Material and method

5.3.1. Materials

Acetic acid and methanol (CH_3OH) were purchased from ECP Labchem (New Zealand), sodium hydroxide and Whatman filter paper (Grade1) were purchased from Sigma-Aldrich (New Zealand). Sodium dodecyl sulphate (SDS) (4-15%), Coomassie Brilliant Blue G-250, and protein molecular weight (MW) markers were purchased from Bio-Rad (New Zealand). SnakeSkin dialysis tube (3,500 MW) was purchased from Thermo Fisher Scientific (USA). All the chemicals, reagents, and standards used in this study were of analytical grades.

Live adult abalone (farmed) were purchased from Moana Blue Abalone (Ruakaka, New Zealand), and wild abalone were caught from Chatham Islands under a research agreement. Abalone (farmed and wild) soft bodies were separated from the shells, minced with a grinder (1000Y XICHU food grinder, China) and stored at -20 °C.

5.3.2. Acid extraction (AE)

Abalone collagen was extracted according to the modified method by Petcharat et. al (Petcharat et al., 2021). Frozen farmed abalone soft bodies (10 g) were soaked in acetic acid (0.5 mol L^{-1}) with a solid to liquid ratio of 1:15, (mass/volume) with constant stirring (500 rpm) for 24 hours. The mixture was then filtered through a double-layered cheesecloth. The filtrated solution was then precipitated by salting out with NaCl at a final concentration of 2.6 mol L^{-1}. Afterwards, the mixture was centrifuged at 10,000 × g for 60 min at 4 °C using a multipurpose, high-speed centrifuge (model 1580R; Gyrozen, Korea). The pellet was resuspended in a minimum volume of acetic acid (0.5 mol L^{-1}) and transferred into a dialysis tube and dialysed against acetic acid (0.1 mol L^{-1}) at 20 volume for 24 hours and milliQ water for another 48 hours (water being changed every day). The dialysed matter was then freeze-dried using a laboratory-scale freeze dryer (Martin Christ, model Alpha 1-2 LD, Germany) at -75 °C and 1 mbar vacuum pressure for two days. The freeze-dried extracts were stored at -20 °C for further analysis.

5.3.3. Ultrasound-assisted extraction (UAE)

Ultrasound-assisted extraction (UAE) was performed using a previously established method reported elsewhere (Petcharat et al., 2021). Frozen farmed abalone soft bodies (10 g) were soaked in acetic acid (0.5 mol L^{-1}) with a solid to liquid ratio of 1:15, (mass/volume) with continuous stirring (500 rpm) for 30 minutes at 4 °C in an ice bath. The mixtures were then subjected to ultrasonication using an ultrasonic homogeniser (BEM-150A; Bueno Biotech, China) equipped with a 6 mm diameter probe. The extraction was performed in pulse mode (5-second acting and a 5-second resting period) at 750 W with a single frequency of 20 kHz. The treatments were conducted at 80 % amplitude for 30 min. The temperature of the mixture was controlled at 4 °C in an ice bath during extraction to prevent thermal degradation. Following the treatment, extraction was carried out with constant stirring (500 rpm) until the process was

completed (24 hours). The mixture was then filtered through a double-layered cheesecloth. The filtrated solution was then precipitated by salting out with NaCl at a final concentration of 2.6 mol L^{-1}. Afterwards, the mixture was centrifuged at 10,000 × g for 60 min at 4 °C using a multipurpose, high-speed centrifuge (model 1580R; Gyrozen, Korea). The pellet was resuspended in a minimum volume of acetic acid (0.5 mol L^{-1}) and transferred into a dialysis tube and dialysed against acetic acid (0.1 mol L^{-1}) at 20 volume for 24 hours and milliQ water for another 48 hours (water being changed every day). The dialysed matter was then freeze-dried using a laboratory-scale freeze dryer (Martin Christ, model Alpha 1-2 LD, Germany) at -75 °C and 1 mbar vacuum pressure for two days. The freeze-dried extracts were stored at -20 °C for further analysis.

5.3.4. CO_2 acidified water extraction (CO_2-AWE)

Carbon dioxide acidified water extraction (CO_2-AWE) of collagen was carried out according to a previously reported method with slight modification (Silva et al., 2016b). Farmed and wild abalone soft tissue (10 g) was mixed with water at 1:20 (mass/volume) ratio, and the mixture was placed into a high-pressure extraction vessel (

Figure 5-1). The extraction vessel was sealed and heated to 37 °C, and the system was pressurised with carbon dioxide (CO_2) to acidify the water, promoting collagen's solubilisation. The extraction was performed at 10 and 50 bar pressure for 3- and 6-hours extraction times. At the end of extraction, the vessel was rapidly depressurised, and the product was collected. The obtained extract was filtered twice through a Whatman filter paper (No.1), followed by a 0.45 µm filter. The extract solution was then freeze-dried and stored at -20 °C for further analysis.

The extraction yield was determined as the ratio between the obtained extract's final dry mass and the initial abalone mass loaded in the high-pressure extraction vessel (Eq. (**Error! Reference source not found.**)).

(1)

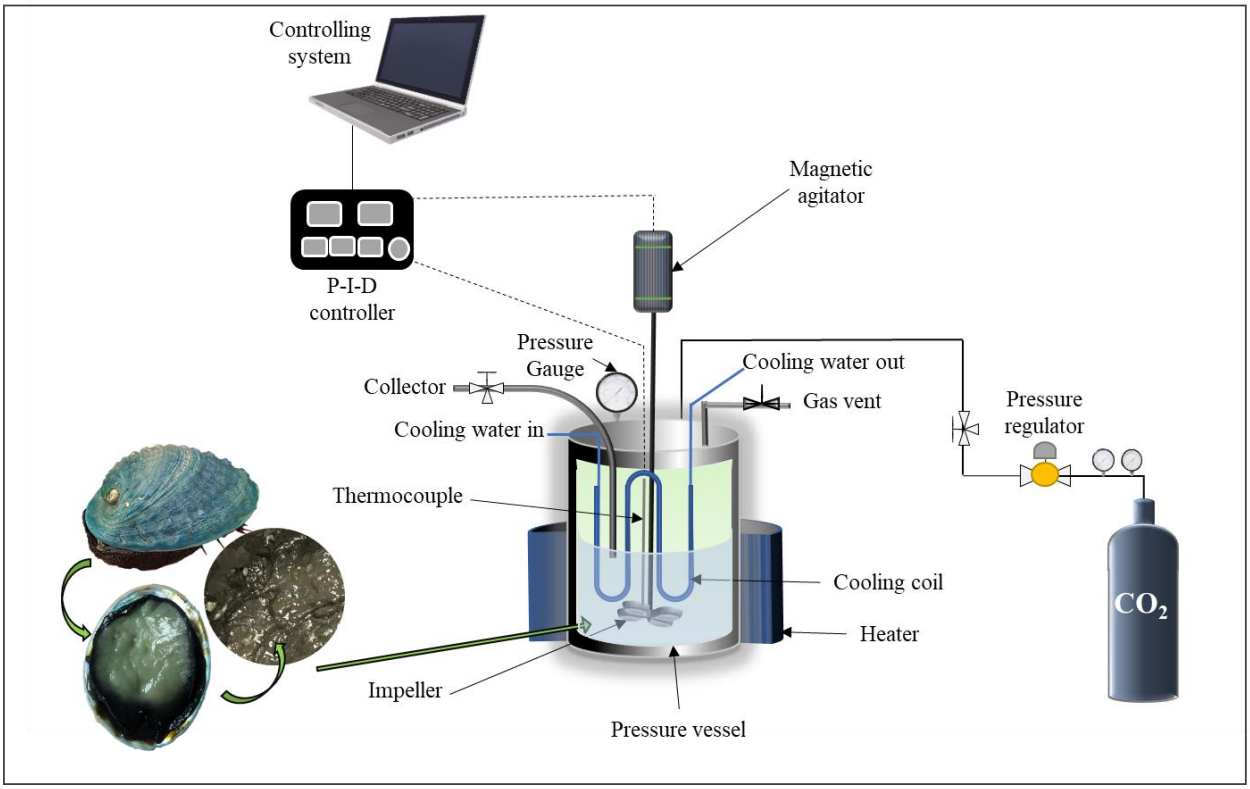


Figure 5-1. Schematic representation of the CO_2 *acidified* water extraction *(CO2-AWE)* system

5.3.5. Characterization of black-footed abalone extracted collagen

Fourier-transform infrared (FTIR) spectroscopy analysis

The representative infrared spectra of collagen extracts (farmed and wild) obtained by UAE and CO_2-AWE were determined with Fourier transform infrared spectroscopy (FTIR). Freeze-dried collagens were placed on the monolithic diamond crystal cell. The spectra were acquired over the spectral region of 4000–500 cm^{-1}, with a resolution of 4 cm^{-1} for 32 scans against a background spectrum recorded from the clean empty cell at room temperature (25 °C) using FTIR spectrometer Thermo Scientific (model: Nicolet iS10, USA). In order to identify the functional groups, present in extracted collagen, an analysis of spectra data was carried out using the Omnic software programme.

Dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Obtained collagens from abalone were subjected to Dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to evaluate the protein fractions' molecular weights (MW) released in the extraction step. SDS- PAGE was prepared using Sigma SDS-PAGE reagents and casted on a Bio-Rad Mini Protean TGX precast gel. Freeze-dried collagen samples obtained from farmed and wild abalone were dissolved in 0.5 M acetic acid (2 mg/mL) under stirred conditions. The solutions were then mixed with loading buffer in a ratio of 3:1 (v/v). The mixture was heated at 95 °C for 10 min to denature the proteins completely. It was then centrifuged at 1200 rpm for 1 min. The centrifuged samples (20 μL) and protein marker (7 μL) were loaded onto SDS-PAGE gel. Both separating and stacking gels were run at 90 V for 1 h in buffer (10X Tris glycine SDS buffer). After running, the gels were stained in a staining solution (0.500 g Coomassie Brilliant Blue G-250 (Biorad), 100 mL acetic acid, 500 mL methanol, and 400 mL deionised water) for 30 min. The stain was removed once by soaking

the gel in Destain I containing 14 mL acetic acid, 80 mL methanol, and 156 mL milliQ water, for 30 min. This process was followed by Destain II (17.5 mL acetic acid, 12.5 mL methanol, and 220 mL milliQ water) for 24 h, under stirring.

Scanning Electron Microscopy analysis

The structure of collagen extracts obtained from black-footed abalone by acid extraction (AE), ultrasound assisted extraction (UAE) and CO_2-AWE were analysed by the scanned electron microscopy (SEM) (Thermos scientific, HITACHI, SU-70). The collagen samples were fixed by mutual conductive adhesive tape on aluminium stubs and coated with platinum by ion sputter (HITACHI, E-1045) prior to microscopic analysis. Then the collagen extracts were scanned by SEM.

PH analysis

The pH of black-footed abalone collagen extracts was measured according to the previously described method with slight modification (Khong et al., 2018). Briefly, 1.0% (w/v) collagen solution was prepared in milliQ water and stirred for 2 hours with a vortex. The pH was measured with a glass electrode (Eutech pH 700 Meter With pH Electrode (ECFC7252101B), Thermofisher, USA) which was calibrated against pH 4.01, 7 and 10 buffers at room temperature.

Colour measurement

Colour measurements were made using Colorflex 45/0 V1.80 spectrophotometer (Hunter lab, US), and the results were expressed as Hunter 'L'- 'lightness', 'a'- 'redness' and 'b' – 'yellowness' values.

Biocompatibility assessment

The effect of obtained abalone collagen extracts (farmed and wild) from CO_2-AWE on the growth of the Vero cell line was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5

diphenyl tetrazolium bromide) test. In growth media (DMEM, 5% and 1% P/S), a 100µL Vero cell suspension (10×10^4 cells/mL) was prepared and seeded into all wells (10×10^3 cells/well). After that, the plates were incubated for 24 hours at 37 °C with 5 % CO_2. To achieve the highest level of cell death, the growth media was removed and replaced with fresh growth media containing abalone extracts at various concentrations (3000 g/mL, 1500 g/mL, 750 g/mL, and 375 g/mL). The plates were incubated at 37 °C for another 48 hours and after that all the growth-media-containing collagen extracts were removed after incubation and reconstituted with 100 µL of fresh growth media. Afterwards, 10 µL of MTT was added to each well and incubated for 4 hours at 37 °C with 5 % carbon. After incubation, 80 µL was removed from each well, and 100 µL of DMSO was added to dissolve the precipitate. A UV spectrophotometer (SpectraMax iD3 Multi-Mode, USA) was used to assess the absorbance of cell viability at 450 nm.

5.4. Results and discussion

5.4.1. Determination of collagen yield

The yield of collagen extracts from black-footed abalone was achieved using AE, UAE and CO_2-AWE, at different parameters (time, power and pressure) which are presented in Table 5-1. According to the UAE results, the highest collagen yield recovered at higher ultrasonication time (30 min) additional to the 96.5 hours and higher amplitude (80%), with the extraction yield of 8.93% which was comparable to the yield of acid extraction (AE) of collagen from black-footed abalone (7.74%). Ultrasound could result in great extraction efficiency, mainly through a cavitation effect, in which the strong shear gradient generated by microbubble collapse damages the cell wall in skin tissue, allowing collagen to be liberated. Furthermore, the higher amplitude and longer extraction time in ultrasonication, provided more

energy to loosen the tissue matrix, allowing for greater acetic acid penetration and release of the target components (Petcharat et al., 2021). UAE could increase efficiency of collagen extract from black-footed abalone by 15%. Although, UAE requires more time (30 min) than AE. The finding is consistent with the results of previous studies on collagen extraction from clown featherback (*Chitala ornata*) skin UAE in which a higher amplitude and longer extraction time that were used in the extraction process led to a higher extraction yield (Petcharat et al., 2021).

In the CO_2-AWE technique, CO_2 is absorbed by water, resulting in water acidification. It forms carbonic acid (H_2CO_3) when it interacts with H_2O molecules. The hydrogen ion (H^+) and bicarbonate (HCO_3^-) are formed as a result of this reaction. All of these hydrogen ions lower the pH of the water, making it acidic (Van Dien, 2018). Collagen expands under acidic conditions, increasing its solubilisation and resulting in the extraction of collagen (Vallejos et al., 2014). The extraction yield attained under different operating conditions (time and pressure) that are reported in Table 5-1, shows that collagen can be extracted by acidified water. Lower pressure (10 bar) and lower time of extraction (3 hours) in CO_2 AWE, possessed higher extraction yield (10.76 %). This result is in consonance with previous study on extraction of collagen from marine demosponge using water acidified collagen, in which higher collagen extract obtained at 10 bar and 180 min (Silva et al., 2016b). The yield of collagen extract from farmed black-footed abalone was 16 % higher than the yield of collagen extract from wild abalone obtained by CO_2 AWE at operating condition of 3 hours and 10 bar. However, this difference was not statistically significant when the *p*-value was smaller than 0.05. Additionally, CO_2-AWE technique could enhance the yield of collagen by 38 % in 3 hours as compared to AE, which required 97 hours extraction period.

Table 5-1. Yield of extracted collagens by acid extraction (AE), ultrasound-assisted extraction (UAE) and CO_2 acidified water extraction (CO2-AWE) at different operating conditions ***(values are mean ± SD, n = 3, p ≤ 0.05)***

Extraction technique	Black-footed Abalone	Temperature (°C)	Pressure (bar)	Power (amplitude)	Ultrasonication time (min)	Total extraction time (hour)	Yield (%)
AE	Farmed	4	-	-	-	96	7.74 ± 0.2
UAE	Farmed	4	-	60	15	96.25	8.59 ± 0.2
UAE	Farmed	4	-	80	15	96.25	8.70 ± 0.1
UAE	Farmed	4	-	60	30	97	8.68 ± 0.1
UAE	Farmed	4	-	80	30	97	8.93 ± 0.1
CO_2-AWE	Farmed	37	10	-	-	3	10.76 ± 0.85
CO_2-AWE	Farmed	37	50	-	-	3	8.22 ± 0.25

CO_2-AWE	Farmed	37	10	-	-	6	10.27 ± 0.08
CO_2-AWE	Farmed	37	50	-	-	6	8.48 ± 0.84
CO_2-AWE	Wild	37	10	-	-	3	9.056 ±0.49

5.4.2. Fourier-transform infrared (FTIR) spectroscopy

The obtained collagen extracts by acid extraction (AE) ultrasound assisted extraction (UAE) and CO_2 acidified water extraction (CO_2-AWE) were analysed by FTIR and are shown in Figure 5-2. The FTIR spectra of both collagens from black-footed abalones (farmed and wild) obtained using the various extraction procedures were similar to those of collagen from other marine sources such as seabass (*Lates calcarifer*) and spotted golden goatfish (*Parupeneus heptacanthus*) (Chuaychan et al., 2015; Matmaroh et al., 2011). Although the collagen extraction methods were different, the spectra showed a comparable chemical structure, implying that the extracts have similar chemical structure, and the extraction technique did not effect on the chemical structure of extracted collagens. In FTIR spectra, collagen normally has peaks/bands including Amide A, Amide B, Amide I, Amide II, and Amide III. Amide A is commonly associated with N-H stretching vibration and has a broad band which occurs in the wavenumber range of 3400-3440 cm^{-1} (Chuaychan et al., 2015). When the N-H group is involved in hydrogen bonding in a peptide chain, the frequency begins to shift to a lower position at around 3300 cm^{-1} (Chuaychan et al., 2015). The absorption peaks of AE (farmed abalone), UAE (farmed abalone) and CO_2-AWE (farmed and wild abalone) were found at 3279, 3293, 3293, and 3360 cm^{-1}, respectively. The amide B band, on the other hand, is known to be associated with the asymmetrical stretch of CH_2 and absorption due to the -CH_2 alkyl chain (Zou et al., 2017). The amide B bands of all collagens in previous studies were also found at wavenumbers around 2900 and 3100 cm^{-1} (Chuaychan et al., 2015; Matmaroh et al., 2011; Muthumari et al., 2016; Petcharat et al., 2021; Silva et al., 2016b; Sousa et al., 2020; Zou et al., 2017). According to Figure 5-2, the amide B bands of the collagen of AE (farmed abalone), UAE (farmed abalone), CO_2-AWE (farmed abalone) and CO_2-AWE (wild abalone) were observed at 2921, 2916, 2918, and 2967 cm^{-1}, respectively. The finding of this study are

consistent with those of the earlier investigation on pepsin soluble collagen (*Haliotis discus hannai*) from abalone in which amide B band was found at 2927 cm^{-1} (Dong et al., 2012). The findings demonstrated that all collagens contribute to hydrogen bonding between free NH stretch and hydrogen in polypeptide chains. However, UAE (farmed abalone) and CO_2-AWE (farmed abalone) collagen extracts had lower wave numbers than the CO_2-AWE (wild abalone) which could be due to higher NH_3^+ group interaction between peptide chains (T. Ahmad et al., 2018).

The C=O stretching vibrations along the polypeptide backbone are principally related to the amide I band, which has a strong absorbance in the region of 1600–1700 cm^{-1}, and a decrease in molecular order induces a peak shift to a lower wave number (Zou et al., 2017). The peak for amide I, from the stretching vibrations of the carbonyl groups (C=O) in the collagen of AE (farmed abalone), UAE (farmed abalone), CO_2-AWE (farmed abalone) and CO_2-AWE (wild abalone) were observed at 1643, 1646, 1615 and 1613 cm^{-1}. This finding proved that the formation of a hydrogen bond between C=O (Gly) of the fourth residue and N–H stretch (X position) is what causes the formation of the triple helix (Matmaroh et al., 2011). The absorbance of the amide II band in FTIR generally occurred in the region of 1550-1600 $cm^{-1,}$ which resulted from the combination of the in-plane NH bending and the CN stretching vibration of the peptide group (T. Ahmad et al., 2018; Chuaychan et al., 2015). The amide II bands of collagen extract of AE (farmed abalone), farmed abalone (UAE), farmed abalone (CO_2-AWE) and wild abalone (CO_2-AWE) were found at 1534,1537, 1538, and 1511 cm^{-1}, respectively. The result of amide II of this study is in accordancewith a previous study on amide II of collagen from pepsin-soluble collagen from gastrabalone (*Haliotis discus hannai*) and clown featherback (*Chitala ornata*) skin (Dong et al., 2012; Petcharat et al., 2021).

Collagen vibrations in the range of 1200–1300 cm^{-1} are typical of amide III band vibrations. (Petcharat et al., 2021). Amide III bands were found at wave numbers of 1239, 1243, 1208,

and 1209 for collagen extract of farmed abalone (AE), farmed abalone (UAE), farmed abalone (CO_2-AWE), and wild abalone (CO_2-AWE). The amide III peak in collagen is comprised components from C–N stretching and N–H in plane bending from amide bonds. There were also absorptions from wagging vibrations from CH_2 groups on proline side chains and the glycine backbone, implying that hydrogen bonds were comprised (Matmaroh et al., 2011). If the absorption ratio between the amide III and 1450 cm^{-1} peaks is 1, it indicates that the triple helix structure of collagen has been preserved. The collagen extract of farmed abalone (AE), farmed abalone (UAE), farmed abalone (CO_2-AWE) and wild abalone (CO_2-AWE) spectrum presented an absorption ratio between the amide III and 1450 cm^{-1} peaks of 1.0, 1.0, 1.1, and 1.5, respectively. Consequently, suggesting all extraction techniques, AE, UAE and CO_2-AWE did not disrupt the triple-standard helical structure in the collagen extracts.

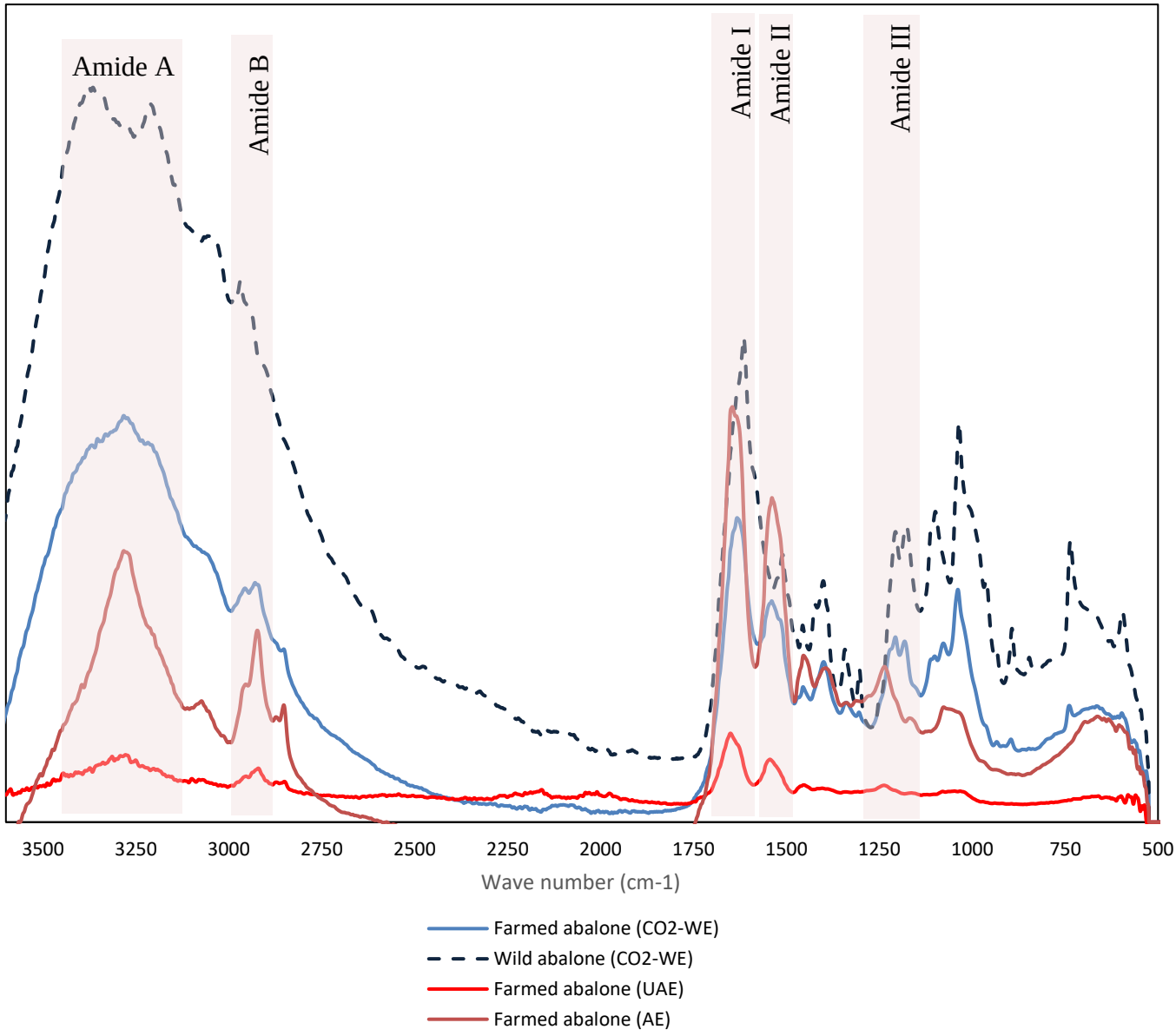


Figure 5-2 FTIR spectra of collagen extracts from abalone by ultrasound-assisted extraction (UAE) and CO_2 acidified water extraction (CO_2-AWE)

5.4.3. SDS-PAGE analysis

Protein patterns (electrophoresis results) of all four collagen extracts obtained from farmed and wild abalone by AE, CO_2-AWE and UAE were assessed by SDS-PAGE and are presented in Figure 5-3. Two alpha chains and one betta chain make up each type I collagen molecule (Chuaychan et al., 2015). Alpha chains normally have a molecular weight of ~ 100 kDa, and beta chains have a molecular weight greater than 200 kDa (Ali et al., 2018; Matmaroh et al., 2011; Petcharat et al., 2021; Sousa et al., 2020). As shown in Figure 5-3, the electrophoresis profile of collagen obtained by CO_2-AWE and UAE are comparable to that obtained by acid extraction. According to the result, farmed abalone collagen extract achieved by an acid extraction contained β-chains (dimer of the α -chains) and α-chains (α 1- and α 2-chains) as the major components. The two alpha chains and one betta chain were also present in farmed abalone collagen extract obtained by UAE as well as farmed and wild abalone collagen extracts obtained by CO_2 acidified water CO_2-AWE. The electrophoresis profile of collagen extracted by UAE from farmed abalone was similar to the electrophoresis profile of collagen extracted by CO_2-AWE from the same abalone. No differences were observed in the protein patterns of farmed and wild abalone collagen extracts that were attained by CO_2-AWE. The finding of this study was similar to the finding of pepsin soluble collagen from abalone (*Haliotis discus hannai*) and other marine species, including Atlantic cod (*Gadus morhua*) skins (Dong et al., 2012; Sousa et al., 2020). The result showed that the extraction method did not impact the molecular weight of collagen. To summarise the results of the SDS-Page analysis, our findings indicate the presence of a naturally generated collagen type I in all black-footed abalone extracts obtained by different methods.

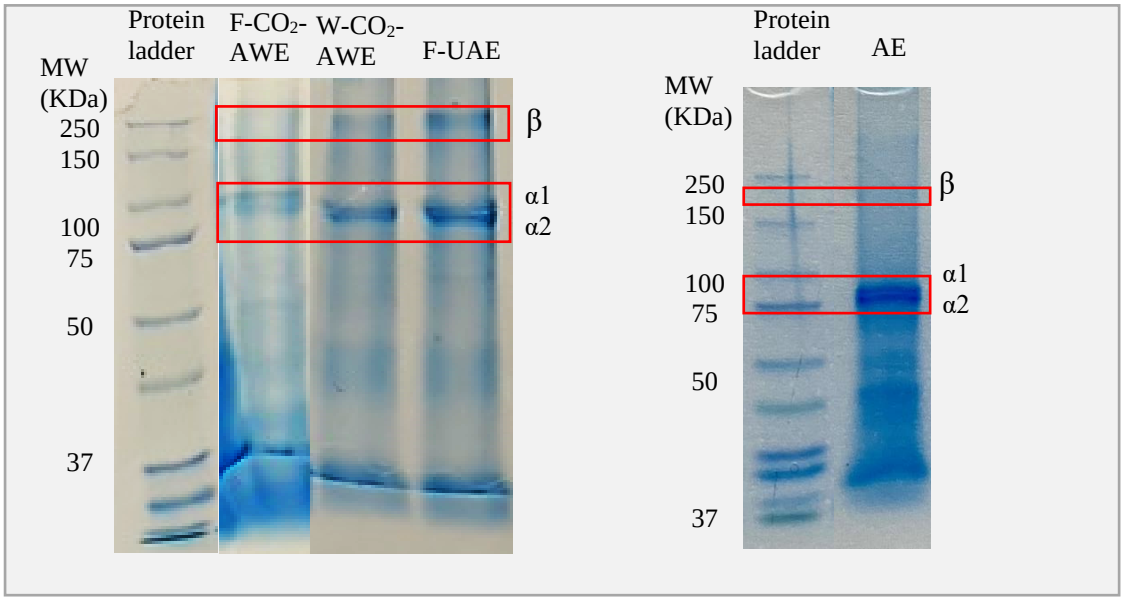


Figure 5-3. SDS-PAGE pattern of collagen extracted from farmed and wild abalone, by acid extraction (AE), CO_2 acidified water extraction (farmed: F-CO_2-AWE, wild: W-CO_2-AWE), and ultrasound-assisted extraction (UAE).

5.4.4. Morphological analysis of black-footed abalone collagen extract

SEM images of abalone collagen extracted by AE, UAE and CO_2-AWE were obtained with different magnifications and are presented in

Figure 5-4. As can be seen in

Figure 5-4 cavities with fibrillar structure, rough and uneven surface were found in the abalone extracts obtained by AE and UAE. The creation of a big fibril or the organisation of molecules into fibrils characterises collagen fibrils, which are largely composed of type I collagen (Muthumari et al., 2016). Collagen pattern of AE (

Figure 5-4- A & B) was similar to collagen pattern of UAE (

Figure 5-4- C & D). However, image C & D had bigger cavities than image A & B. This was due to both AE and UAE methods used acetic acids which resulted in a comparable pattern, but larger pores sizes which may have resulted from the cavitational forces during ultrasonic treatment, as well as micro-streaming and turbulent forces (Jiang et al., 2014).

Farmed abalone collagen (

Figure 5-4-E & F) and wild abalone collagen (

Figure 5-4-G & H) obtained by CO_2-AWE had similar patterns. However, they were different from collagen obtained by AE (

Figure 5-4-A & B) and UAE (

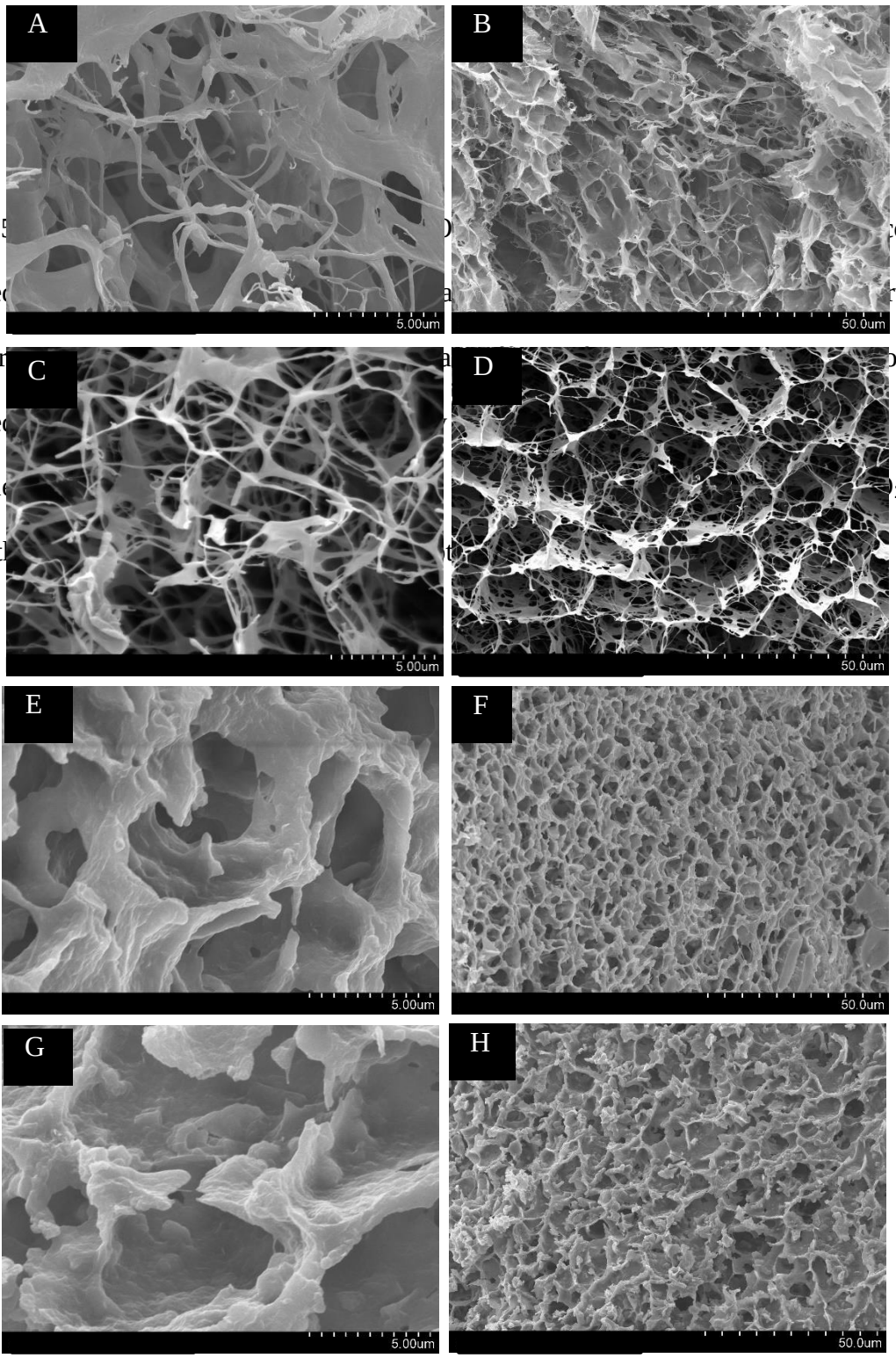


Figure 5 ollagen obtained rved for the extr f those obtained was in agreem $_2$-AWE which t

Figure 5-4. Scanning electron microscopy (SEM) of abalone collagen extracts: A & B: collagen extracted from farmed abalone by AE, C & D: collagen extracted from farmed abalone by UAE; E & F: collagen extracted from farmed abalone by CO_2-AWE, G & H: collagen extracted from wild abalone by CO_2-AWE

5.4.5. pH analysis

There was a significant difference in pH of abalone collagen extract obtained by AE, UAE and CO_2-AWE (Table 5-2). It was found that Ae and UAE collagen extracts were acidic with a pH of 4.1 ± 0.02 and 3.86 ± 0.11 compared with collagen obtained by CO_2 -AWE with a pH of 7.12 ± 0.05 and 6.85 ± 0.35 for farmed and wild abalone extracts, respectively. The acidity of the AE and UAE extract could be due to the acetic acid that were used for both extraction techniques. The pH result of the UAE collagen extract was similar to a previous study on collagen extraction from jellyfish (*Acromitus hardenbergi*) by acid extraction (Khong et al., 2018). On the other hand, in CO_2-AWE, the pH of the extract is dependent on pressure, temperature, and the amount of CO_2 ingested; in which by increasing the temperature and pressure of CO_2 gas, the pH of carbonated water decreases (King et al., 2012). However, in this

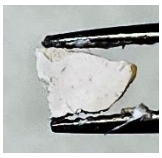
study, the temperature and pressure of the extraction was low. The pH of the collagen of black-footed abalone was close to milli Q water pH of 8 that was used in this experiment.

5.4.6. Colour measurement

The Hunter colour values of collagen extracted from black-footed abalone varied with different extraction techniques. The Hunter colour L value, which reflects lightness, was affected by the extraction technique, which were 35.97 ± 0.25, 38.04 ± 0.58, 26.04 ± 0.51, 18.78 ± 2.45, for collagen extracts of black-footed abalone obtained by AE, UAE, CO2-AWE (farmed abalone), CO2-AWE (wild abalone), respectively. The colour lightness of black-footed abalone collagen obtained was lower than other marine collagen sources such as jelly fish (*Acromitus hardenbergi*) and Skate (*Raja kenojei*) skins which L value were almost two times higher than L value of collagen of black-footed abalone (Khong et al., 2018; Shon et al., 2011). This may be attributed to the dark tissue colour of black-footed abalone. The Hunter colour a value, which represents redness, were, 0, 0, 0.69 and 0.67 for black-footed abalone collagen samples obtained by AE, UAE, CO2-AWE (farmed abalone) CO2-AWE (wild abalone), respectively. The other relevant part of Hunter colour b value which represents yellowness, for the collagen extracts obtained by AE and UAE was 1.59 and 1.46, which was lower than collagen extracts of farmed and wild abalone obtained by CO_2-AWE with 4.97 and 4.36 of b value, respectively. According to the results, collagens abtained by AE and UAE had higher lightness, less yellowness and redness compared with collagen extract from CO_2-AWE. This could be due to the use of acetic acids, which can eliminate pigment during extraction. Therefore, the extraction process resulted in the appearance of the collagen, which AE and UAE collagen extracts had greyish white colour in comparison with collagen of CO_2-AWE, which had a slightly yellowish colour.

Table 5-2. Appearance, pH and colour of extracted collagen from black-footed abalone *(values are mean ± SD, n = 3)*

Extraction technique	pH	Hunter colour value			appearance	
		L	a	b		
Acid extraction	4.1 ± 0.02	35.97 ± 0.25	0.0 ± 0.0	1.59 ± 0.03	Greyish white	
F-UAE	3.86 ± 0.11	38.45 ± 0.58	0.0 ± 0.0	1.46 ± 0.02	Greyish white	

F-CO_2-AWE	7.12 ± 0.02	26.04 ± 0.51	0.69 ± 0.09	4.99 ± 0.97	Slight yellowish	
W-CO_2-AWE	6.85 ± 0.35	17.48 ± 2.45	0.67 ± 0.0	4.36 ±0.45	Slight yellowish	

F-CO_2-AWE: Farmed abalone CO_2 acidified water extraction; W-CO_2-AWE: wild abalone CO_2 acidified water extraction; and F-UAE: Farmed abalone ultrasound-assisted extraction water

5.4.7. Biocompatibility assessment

The possible cytotoxicity of collagen extracts by CO_2-AWE was evaluated in vitro by the MTT assay. Two collagen extracts (farmed and wild abalone) obtained by CO_2-AWE were used due to the better feasibility and sustainability (energy saving, efficiency and less environmental impacts) of CO_2-AWE and their higher extraction bioactivity compared with UAE.

The MTT assay determined yellow MTT reduction to an insoluble purple formazan by succinate dehydrogenase found in mitochondria of viable cells (Udhayakumar et al., 2017). The cytotoxic effect of collagen extracts was determined on the Vero cell line derived from a monkey. According to

Figure 5-5**Error! Reference source not found.**, after 48 hrs exposure of the cells to the farmed and wild abalone' collagen extracts (375-3000 µg/mL), cells were viable more than 99 and 77%, respectively. On the other hand, there were also no significant differences in the concentration of the collagen extracts (p>0.05). Our findings support earlier research on the cytotoxicity of collagen from Atlantic cod (*Gadus morhua*) skins and Demosponge

(*Chondrosia reniformis*) extracted by CO_2-AWE (Silva et al., 2016b; Sousa et al., 2020). According to ISO 109333-5:2009, a material intended for medical purposes is toxic to cells if it causes more than 30% of death (Araújo et al., 2021). Thus, the findings demonstrate that the high concentration of collagen extracts generated using CO_2-AWE had no toxicity.

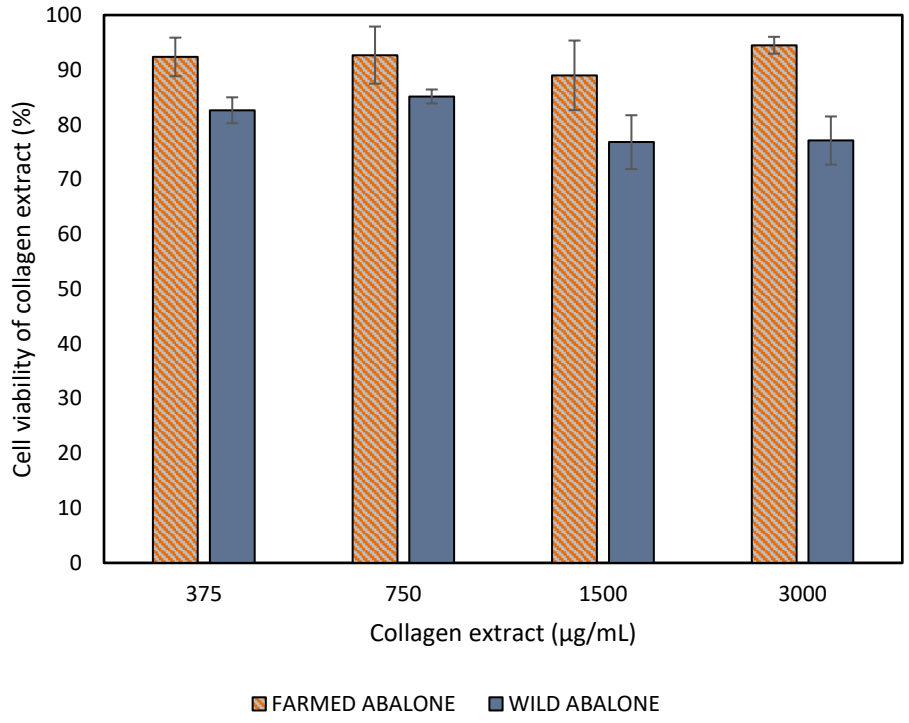


Figure 5-5 Influence of black-footed abalone collagen extracts (farmed and wild) obtained by CO2-AWE on Vero cells *(values are mean ± SD, n = 3)*

5.5. Conclusion

The extraction of collagen from black-footed abalone was successfully achieved through different techniques; Acid extraction (AE), ultrasound-assisted extraction (UAE) and CO_2 acidified water extraction (CO_2-AWE). The CO_2-AWE was found to be an effective method to

extract collagen, which allowed using only water instead of acetic acids, reducing extraction time and processing costs. Additionally, CO_2-AWE showed 38% and 20% higher extraction yield compared to AE and UAE. Collagen amides including Amide A, B, I, II and II were present in the black-footed abalone collagen extracts obtained by AE, UAE, and CO_2-AWE. The result of SDS page showed similar properties in terms of molecular weight of collagen and presence of major components including α and β bands in all collagen extracts. All methods confirmed the presence of collagen type I based on SDS-page and FTIR. Furthermore, collagen extract of UAE had better morphology and appearance in term of colour than collagen of CO_2-AWE. However, the acidity of the collagen of UAE was higher than collagen obtained by CO_2-AWE. Overall, the findings of this study suggest that non-toxic collagen of black-footed abalone obtained by CO_2-AWE might be a viable alternative to the land-based collagen. It was also opening the possibility of this easy, inexpensive, and environmentally sustainable technique to be used in biomedical, cosmeceutical, and pharmaceutical industries.

5.6. References

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Chapter 6. General discussion

6.1. Thesis overview

Many disease-causing pathogens have developed medication resistance, resulting in the emergence of their mutant forms. Coupled with such threat perceptions and a rapid decline in the availability of land-based natural resources, scientists have changed their research direction to the marine environment, which has been discovered to have an abundance of bio-molecules that can be utilised in the discovery of novel products to promote health (Kanchana et al., 2013; Pati et al., 2015; Rudd & Benkendorff, 2014; B. Wang et al., 2013).

Molluscs have a substantial diversity of natural products, such as carbohydrates, amino acids, proteins with therapeutic properties including but not limited to antibacterial, anticoagulant, antioxidant, anti-inflammatory and antiviral (Ahmad et al., 2018). Abalone are marine molluscs that play a significant role in the worldwide economy due to their high nutritional value (Dang, Speck, et al., 2011).

Abalone powder have long been a key ingredient in many Chinese medicine preparations/remedies for the treatment of various ailments as well as being used as skin enhancers, beautifiers and protectants (Q. Zhao et al., 2022). As an additional form of traditional Chinese medicine, the abalone shell (*Haliotis diversicolor*) has been used to treat eye diseases and skin injuries, especially for the treatment of traumatic wounds or poorly managed ulcers (Chen et al., 2016). Moreover, in traditional Chinese medicine abalone shell has been used with other ingredients (plants and animals) to treat Parkinson disease (Article, 2022). Nonetheless, their biological functions and their extraction methods have not been well studied.Many marine bioactive compounds have been extracted using conventional solvent extraction methods including but not limited to soxhlet extraction and maceration, which have limitations such as a long extraction time, high dependence on organic solvents during extraction creating a large amount of organic waste (being harmful for the environment), excessive costs, and potential for degradation of valuable compounds (Kheirkhah et al., 2019;

Roselló-Soto et al., 2016). In response to the demand for environmentally friendly and sustainability production, unique alternative processes such as subcritical water extraction (SWE) and CO_2 acidified water (CO_2AWE) extraction have been launched recently.

The aim of this thesis was to extract valuable chemicals with potential applications in the pharmaceutical, nutraceutical, and cosmeceutical industries from a native New Zealand black-footed abalone using SWE and CO_2AWE. Bioactive components from black-footed abalone were recovered utilising a subcritical water extraction method under subcritical conditions (high temperature and pressure) without harming the environment by employing water as opposed to organic solvents. The findings of this chapter show that temperature has an impact on the productivity of the SWE, which is a critical step in determining extraction efficiency. Overall, the findings indicate that abalone subcritical water extracts are prospective sources of bioactive substances like antioxidants, glycogen, phenolic compounds, carbohydrates, protein, and amino acids and can be used as a safe and beneficial natural source of high-quality bioactive chemicals (Chapter 3).

It has been long speculated whether or not there would be higher bioactivity in extracts obtained from wild vs farmed abalone. Bioactive compounds were extracted from wild and farmed black-footed abalone using subcritical water extraction at high temperature and different times in Chapter 4, confirming that both farmed and wild abalone extracts, have potent sources of novel anti-aging compounds such as anti-hyaluronidase and anti-collagenase with no toxicity, as well as antioxidants, phenolic compounds, proteins, and essential and non-essential amino acids. Therefore, the subcritical abalone extracts might be developed as a cosmeceutical agent because of their strong collagenase and hyaluronidase inhibitory activities. The second green extraction method (CO_2 water extraction) was utilised to efficiently extract collagen from both farmed and wild black-footed abalone. This chapter proved that in a short period of time, CO_2

water extraction might serve as an alternative to standard techniques for enhancing the efficiency of collagen extraction using water (Chapter 5).

6.2. Core chapter philosophies

6.2.1. Chapter 2- Introduction: Recent studies on bioactive compounds extracted from marine organisms using a variety of extraction techniques

This chapter was written to provide a comprehensive review of recent research on various bioactive compounds to reveal a knowledge gap in terms of available marine bioactive compounds especially abalone by various extraction techniques focusing on their potential use and health benefits.

Based on the present literature, various bioactive compounds have been extracted from marine organisms due to their secondary metabolites such as protein, carbohydrates and lipids with high bioactive components that are not produced by any other organisms which contribute to health benefits. Abalone a marine gastropod, has been used in traditional medicine to cure digestive neoplasm, diseases, and phlogosis or prevent senility since it contains a variety of active ingredients including but not limited to antioxidant, antibacterial, anti-collagenase, anti-hyaluronidase and collagen which have enabled the organisms to adapt to various stresses (Wang et al., 2019). However, there was limited studies on the recovery of active substances of abalone. There was also no study specifically on the bioactive compounds of New Zealand black-footed abalone, the species that this thesis focused on. In the past, abalone may have been less accessible than other marine organisms. However, abalone aquaculture is expanding in New Zealand, making it more available for research. Additionally, extraction of bioactive compounds from marine sources was based on conventional techniques (solvent based traditional extraction using methanol, hexane and acetonitrile which could be expensive, time consuming and not eco-friendly. In contrast to traditional extraction methods, non-conventional extraction methods such as subcritical water extraction (SWE) and CO_2 acidified

water extraction (CO_2 AWE) are effective, high-efficient, economical, environmentally friendly, and have a shorter extraction time.

It is hoped that this review is intended to help to a better understanding of extraction and recovery of bioactive compounds from black-footed abalone by novel green extraction technique including SWE and CO_2 AWE for clean, more efficient, and effective products in various industries.

6.2.2. Chapter 3- Extraction of bioactive compounds from black-footed abalone (*Haliotis iris*) using subcritical water extraction

After reviewing various studies on the extraction of bioactive compounds from marine organisms, a green extraction technique was selected to extract bioactive chemicals from black-footed abalone. Green extraction techniques, minimise or completely eliminates use of harsh organic solvents as well as reduces environmental footprints (Bordoloi & Goosen, 2020). The initial work conducted in this project was a preliminary study of 7 abalone extracts achieved at different extraction temperatures using subcritical water extraction which substitute water for organic solvent. This study determined which extraction temperature could recover the highest bioactive compounds, including antioxidant activity, total phenolic content, glycogen content, protein, and amino acids. Temperature of extraction had a significant effect on the bioactive compounds of black-footed abalone as was suggested by Hao et al. (2019). The temperature of 220-250 °C could achieve the highest bioactivity of the New Zealand black-footed abalone and temperatures higher than 250 °C, resulted in decrease in bioactivity due to thermostability of compounds, similar to the finding of previous study by Lee et al. (2018). There was a significant correlation between the antioxidant and total phenolic content of the abalone extracts. Consequently, the best temperature for extracting bioactive chemicals from abalone was discovered to be 220 °C, considering the necessity of energy conservation as well as the potential for antioxidant breakdown at temperatures greater than 220°C which are novel

observation brought about by this research . As a result, abalone subcritical water extract can be used as a reliable and important natural source of high-quality bioactive chemicals such as antioxidant, protein amino acids, due to using water instead of organic solvent for extraction of bioactive compounds. However, more research is needed to identify the substances of abalone subcritical water extract that are responsible for each bioactivity. Furthermore based on the results of Chapter 3, the next following chapter will be focused on the antiaging characteristics of abalone extracts, as well as the impact of another important element (time) on SWE efficiency (Yang et al., 2013).

6.2.3. Chapter 4- Antioxidant and antiaging bioactive compounds extracted from wild and farmed black-footed abalone using subcritical water extraction

Ageing is an unavoidable aspect of life which has an impact on all systems of the body significantly skin. Age-related/chronological ageing and premature ageing/photoaging are the two types of skin ageing (Ndlovu et al., 2013). The production of reactive oxygen species is increased during the ageing process, which leads to the activation of collagenase, and hyaluronidase, which can contribute to skin ageing. Until recently, the potential of marine-derived natural compounds as anti-aging chemical sources remained mostly unexplored (Resende et al., 2021). Although several studies have explored marine bioactives with anti-aging properties, there is still a gap in knowledge regarding the extraction of bioactive compounds from abalone in terms of antioxidant, anti-hyaluronidase, and anti-collagenase activities. Thus, this Chapter aimed to investigate the antioxidant and antiaging properties of wild and farmed black-footed abalone extracts, by following up on Chapter 3 subcritical water extraction technique. Black-footed abalone extracts (farmed and wild) with biological activities such as antioxidant, anti-hyaluronidase, and anti-collagenase activity were obtained at temperature 220 °C and different times (5-60 min). There was a correlation between antioxidant and total phenolic compounds (TPC) of black-footed abalone extracts in which,

optimum recovery of antioxidants and TPC can be accomplished in 30 minutes of extraction while conserving energy. The antioxidant and TPC results obtained from abalone extracts are consistent with previous studies on blue mussel (*Mytilus edulis*) (Han et al., 2018), green kiwifruit (*Actinidia deliciosa)* (Kheirkhah et al., 2019) and brown macroalgae (*Ecklonia maxima*) (Bordoloi & Goosen, 2020). They found that the longer exposure of bioactive compounds to high temperatures, resulted in a decrease in TPC. The antioxidant and TPC results of farmed and wild black-footed abalone extracts were also highly correlated with the obtained antiaging properties (anti-hyaluronidase and anti-collagenase activity) with the highest bioactivity obtained at higher extraction time. Prior researchers have suggested that phenolics, terpenoids, or steroids could be responsible for the inhibition of hyaluronidase and collagenase (e.g., hydroxycinnamic acids, ellagic acid, catechins, curcumin and carnosic acid) (Agulló-Chazarra et al., 2020; Zagórska-Dziok et al., 2021). Glycogen content of black-footed abalone extract (farmed and wild) was also affected by time of extraction in which prolonged exposure of glycogen (polysaccharide) to high temperature led to thermal decomposition of polysaccharides (Getachew et al., 2019). Antioxidant, TPC, anti-collagenase, anti-hyaluronidase, and glycogen contents were comparable in farmed and wild abalone extracts. However, higher protein and amino acids profile were found in farmed black-footed abalone extract than in wild black-footed abalone extracts which could be attributed to the higher protein diet of farmed abalone than the seaweed diets of wild abalone (Bullon et al., 2022). In addition to confirming the protein and amino acid profiles of farmed and wild abalone extracts, it was determined that the extraction time had an influence on the protein and amino acids of extracts, with longer extraction times yielding greater amino acid and protein content which was in agreement with previous studies (Kang et al., 2001; Tavakoli & Yoshida, 2006; ZHU et al., 2008). Moreover, the black-footed abalone extracts (farmed and wild) were non-toxic to

Vero cell lines which indicates it's safety for human consumption as well as cosmeceutical applications.

To summarise, this chapter found that extracts from abalone (both farmed and wild) have potent anti-aging activities with no notable harm at the high levels tested. As a result, these extracts hold promise as potential sources of novel antiaging compounds for use in antiaging products.

Bioactive substances have long been utilised as medications, and they still serve as a source of potential therapeutics today (David et al., 2015). Abalone (*Haliotidis*) dried muscle has a long history of use in traditional Chinese medicine as a tonic cure, including but not limited to lowering blood pressure, avoiding anaemia, stimulating blood circulation, regulating menstruation and encouraging lactation, and improving vision (De Zoysa, 2013; Q. Zhao et al., 2022). Recently, few products have been developed from abalone tissue and shell with nutraceutical, cosmeceutical applications such as "abalone essence healthy liquid" as an immunity booster, "abalone active element capsule" as a dietary supplement for controlling blood pressure, cholesterol and blood sugar, "abalone *Haliotis iris* powder" as a dietary supplement product for supporting immune system, heart and blood circulation, and preventing anemia and "paua facial scrub" as a deep cleanser (De Zoysa, 2013).

Anti-collagenase, anti-hyaluronidase, antioxidants, and amino acids derived from black-footed abalone gives the possibility to produce new products with potential applications in medicinal, nutraceutical and cosmeceutical industries to give health and medical advantages and increase the fundamental nutritional value (Grand View Research, 2017). Anti-collagenase can be applied topically and systematically in medicine to supress collagenase enzyme which is responsible for degradation of collagen in the body (Brooks & Ollivier, 2004). It can be also conjugated with anti-hyaluronidase and antioxidant as an antiaging product in form of tablet,

serum and cream. Antioxidant can also be used in conjunction with amino acids as a dietary supplement in the nutraceutical industry.

6.2.4. Chapter 5- Extraction and characterization of collagen from black-footed abalone by CO_2 water extraction

Collagen is one of the main building blocks of the skin and the primary constituent of connective tissue, nails and hair. Scientists have discovered marine collagen as an alternative source to land-based collagen with low immunogenicity, biocompatibility, and disease transmission potential (Uriarte-Montoya et al., 2010). One of the objectives of this thesis was to develop a marine collagen from New Zealand black-footed abalone with high extraction efficiency. Thus, in the final Chapter, collagen was extracted from farmed and wild black-footed abalone by CO_2 acidified water extraction (CO_2 AWE), a novel green extraction technique, ultrasound assisted extraction (UAE) as well as traditional extraction technique (acid extraction).

Traditional or conventional solvent extraction method is the most commonly used technique, due to its simplicity and extensive applicability (Kheirkhah et al., 2019). However, this approach for extracting collagen from abalone required numerous procedures, a substantial amount of organic solvent (acetic acid), and considerable time which resulted in high amount of organic solvent waste and low efficiency. UAE as an alternative technique to conventional extraction, could enhance the efficiency of abalone collagen extract; nonetheless, it required the same quantity of solvent and time as conventional extraction as well as production of organic solvent waste. In this regard, CO_2 AWE as a novel approach conforming to the principles of green chemistry and environmentally friendly alternative processes could efficiency extract collagen in a short amount of time by substituting water for organic solvent.

Collagen extracts of farmed and wild abalone were compared in terms of scanning electron microscopy (SEM), Dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-page), Fourier-transform infrared spectroscopy (FITR), PH, colour, appearance, and biocompatibility. The highest collagen yield was recovered by (CO2 AWE) in 180 min which used only water as a solvent in comparison with UAE and acid extraction which required 5760 and 5790 min of reaction with acetic acid, respectively. Furthermore, more collagen was extracted from farmed abalone compared to wild abalone which may be related to the aforementioned difference in diet. The SEM results of recovered collagens by UAE and acid extraction revealed fibrous rough structure that corresponds to type I collagen (Muthumari et al., 2016). Collagen obtained by CO2 AWE had rough structure and smaller pore sizes in compared to acid extraction and UAE. Similar FTIR and SDS-page results were observed for collagen extracts recovered using CO2 AWE, UAE, and acid extraction in which Amide A, B, I, II and III were detected by FTIR and α and β bands were obtained by SDS-page that corresponding to type I collagen. However, the PH, colour and appearance of black-footed abalone collagen obtained by CO_2 AWE were different from the collagen obtained by UAE, and acid extraction where CO_2 AWE had higher pH (more neutral), slight yellowish colour. Moreover, there were no significant differences between collagen extracted from farmed and wild abalone in terms of appearance, microscopy, spectral fingerprint and protein separation. Furthermore, the collagen extracts obtained by CO_2 AWE showed no toxicity for both farmed and wild abalone extracts.

Therefore, the use of CO_2 water extraction may be a potential technology with a high level of sustainability for enhancing the extraction efficiency of marine collagen from black-footed abalone as an alternative to land-based collagen with applications in food and biomedicine.

6.3. Study limitations and recommendations for future research

This thesis had some limitations just like all other research.

First limitation for chapter 3, 4 and 5 was the lack of research on subcritical water extraction and CO_2 acidified water extraction of bioactive compounds from other abalone species. Thus, this was difficult to locate prior literature with which to compare the findings of this study to those of others. However, this demonstrates the originality of the concept of my study.

The bioactive compounds that were in charge of various bioactivities were not identified, and the abalone extract was not fractionated. This was brought on by the high cost of bioactive standards and the constrained availability of all standards during COVID19. Furthermore, some instruments, including LC-ESI-QTOF-MS/MS and LC-ESI-QqQ-MS/MS, also were not accessible at AUT.

Another limitation was that chapter 4 of the thesis did not examine how abalone size and age affected the biological activity of the abalone extracts. This was a result of the difficult access to the wild abalone on Chatham Island and the market purchases of farmed abalone in various sizes.

The characterization of collagen extracts obtained from black-footed abalone in Chapter 5 was another limitation of this study. Differential scanning calorimetry (DSC), X-ray diffraction (XRD), and Circular dichroism spectroscopy (CD) instruments are required for additional experiments in this study. However, these instruments were not available at AUT, which can be an opportunity for future studies.

This research confirms that extracts with high biological activities can be obtained from New Zealand black-footed abalone using environmentally friendly extraction techniques, such as subcritical water extraction and CO_2 water extraction. However, this research was limited by COVID-19 lock-down by preventing access to the laboratories (level 3 & 4) resulted in degradation of extracted samples. This led spending couple of months to repeat extraction of bioactive compounds which pushed forward some experiment which it did not allow further

research on identification of extracted bioactive compounds. Covid-19 lock-down was also caused a delay in the delivery of certain chemicals for a couple of months which led to interruption of some experiments. This led to the purchase of test kits from other vendors where the price was high in comparison with other chemicals.

Therefore, additional research is required to identify the compounds responsible for bioactivities in this research. Furthermore, it is recommended to consider stability of bioactive compounds for pharmaceutical and cosmeceutical applications. Additionally, in-vivo studies and clinical research studies are necessary to determine any potential side effects of the extracts. Moreover, future studies can be used to explore the individual or synergistic effects of compounds extracted from abalone. In addition, there are other opportunities for future work, it is suggested to investigate different possibilities of formulation of the extracts for health promoting products.

6.4. Conclusion

The demanding nature of the marine environment would require the marine species to generate and accumulate a variety of secondary metabolites to survive and deal with environmental challenges. These marine derived chemicals have sparked a lot of interest for applications in food, Biomedicine and cosmetics.

The aim of this thesis was to extract water-based bioactive compounds from indigenous New Zealand black-footed abalone which is rich in secondary metabolites such as proteins and carbohydrates and maximising the value of the New Zealand black-footed abalone. For this purpose, green extraction techniques, were applied based on subcritical water extraction (SWE) and CO_2 acidified water extraction (CO_2 AWE). SWE was used to recover bioactive compounds including antioxidants, glycogen, phenolic compounds, carbohydrates, protein,

essential and non-essential amino acids, as well as anti-hyaluronidase and anti-collagenase activities. The findings of this study revealed that the effectiveness of SWE is influenced by temperature and time, both of which are important factors in determining black-footed abalone extraction efficiency. Furthermore, black-footed abalone extract can be used as a reliable and important source of high-grade bioactive chemicals from a natural source.

Additionally, collagen with high efficiency and sustainability was extracted from black-footed abalone using the second environmentally friendly extraction technique, CO_2 AWE. The extracted collagen from black-footed abalone could be a potential alternative to land-based collagen which can add value to New Zealand black-footed abalone.

Thus, the results presented in this thesis have demonstrated the great possibilities of green extraction processes, including SWE and CO_2 AWE, which can be used effectively to recover bioactive compounds from black-footed abalone, making it suitable for new potential pharmaceutical, nutraceutical, and cosmeceutical products for human health benefit.

6.5. Bibliography

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