Influence of processing techniques on quality and nutritional composition of the tropical sea cucumber *Holothuria scabra*

Thesis submitted by

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Statement of contributors of authors

Publication details

Chapter No	Details of publications	Nature and intellectual input of each author, including the candidate
2	Ram, R., Chand, RV., Zeng, C., & Southgate, PC. (2016). Recovery rates for eight commercial sea cucumber species from the Fiji Islands. *Regional Studies in Marine Science, 8, Part 1*, 59-64.	Ram and Southgate co-developed the research question. Ram and Chand performed the experiment, collected the data and Ram performed statistical tests. Ram wrote the first draft of the paper which was revised with editorial inputs from Zeng and Southgate.
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Abstract

Recent years have seen increasing demand for consumer-friendly, non-dried, ready to cook sea cucumber products in Asian markets along with greater awareness on the potential health benefits of seafood consumption. On this basis, there is opportunity to develop new sea cucumber products that are more user friendly and with improved nutrient content. However, a major determinant of processed sea cucumber quality and value is the texture of the consumed product and so any future development in the processing and packaging of sea cucumber must account for texture as well and nutrient content. This study addressed issues relating to the impact of processing techniques on the quality and nutritional composition of the sea cucumber *Holothuria scabra* (Sandfish). The overall objectives of this study was to to generate improved information on the processing of sandfish relating to nutrient composition and texture, and the potential and implications of novel processing and packaging methods for this species. The study determined the recovery rates for eight commercial sea cucumber species from the Fiji Islands (Chapter 2); the effect of different processing techniques on the nutrient composition of bêche-de-mer produced from sandfish (Chapter 3); the effect of processing method on quality, texture, collagen and amino acid composition of sandfish (Chapter 4); isolation and characterization of collagen from the body wall of sandfish (Chapter 5); and assessed novel processing and packing methods on the quality and the nutrient composition of sandfish (Chapter 6).

Determination of the original weight and length of sea cucumbers processed and dried to become bêche-de-mer (BDM), is an important tool in sea cucumber fishery management. The only management mechanism for the sea cucumber fishery in the Fiji Islands is a minimum length

prescribed for BDM for export. However, different commercial species have different shrinkage rates during processing and previous studies have suggested modification of fisheries management for sea cucumbers to include species-specific minimum harvest size limits. Chapter 2 determined weight-based and length-based recovery rates (i.e. the length/weight of BDM recovered after processing from the initial length/weight of fresh sea cucumber), for eight commercial sea cucumber species following processing to BDM; White Teatfish (*Holothuria fuscogilva*), Black Teatfish (*Holothuria whitmaei*), Tigerfish (*Bohadschia argus*), Surf Redfish (*Actinopyga mauritiana*), Hairy Blackfish (*Actinopyga miliaris*), Stonefish (*Actinopyga lecanora*), Prickly Redfish (*Thelenota ananas*) and Sandfish (*Holothuria scabra*). Length and weight recovery rates varied between species and ranged from the highest recovery values of 54.9% for length and 11% for weight in Black Teatfish, to the lowest recovery values of 32.6% for length and 3.0% for weight in Sandfish and Tigerfish, respectively. Length-based and weight-based relationships were generated for each species through the various stages of processing from fresh to dried (BDM) allowing estimation of initial fresh weight/length from partially or fully processed BDM and *vice versa*. Information generated in this study provides a basis for developing more species-specific harvest size restrictions for sea cucumbers in the Fiji Islands, and has application in stock assessment studies, estimation of harvest data, monitoring of harvest size limits and standardizing catch data.

Proximate and fatty acid compositions of fresh and processed sandfish, and the effects of salting on product composition, were determined in Chapter 3. Processing using dry salting (kenching) for up to 72 h and immersion in 15-25% brine solutions for 48 h produced significantly reduced protein contents in resulting BDM compared to non-salted BDM (845.90 ± 9.28 mg g^{-1}). BDM

produced by 48 h kenching (717.52 ± 10.54 mg g^{-1}) and by 15% brine salting (713.93 ± 11.98 mg g^{-1}) had the highest protein contents among salted treatments. The lipid contents of BDM processed using salting did not differ significantly from that of the control non-salted treatment (13.89 ± 2.89 mg g^{-1}), but ash contents of salted BDM were generally significantly greater than that of the control product (111.13 ± 15.73 mg g^{-1}). Carbohydrate contents of BDM increased with increasing duration of kenching to 119.15 ± 36.16 mg g^{-1} (72 h kenching). Inclusion of a salting step in BDM processing resulted in reduced levels of EPA in some treatments but did not significantly reduce ∑n-3 PUFA levels. Results provide a basis for further development of processing methods for *H. scabra* that optimize the nutritional characteristics of resulting BDM.

Textural properties and collagen and amino acid contents of fresh (raw) and processed sandfish were compared in Chapter 4. Several processing procedures using different salting methods (brine and kench salting) were tested, and the resulting processed BDM were compared with partially processed tissue and BDM processed without salting and by smoke drying. Weight and length based recovery rates did not differ significantly across salting treatments or from the non-salted control treatment. There was a general trend of decreasing collagen content with increasing brine strength in the brining treatments, and sequential increases in the force required to shear reconstituted BDM processed with increasing brine strength. This has implication for BDM processing because the quality of reconstituted BDM is judged primarily by texture, not flavor, with softness and elasticity being of prime importance. BDM from most treatments was significantly less firm than cooked, partially processed tissue. The most abundant protein-bound amino acids in sandfish BDM were glycine, glutamic acid, proline, arginine, aspartic acid, alanine and hydroxyproline, but their levels did not vary significantly across treatments. Our

results provide a basis for improvements to sandfish processing that optimize textural properties of resulting BDM.

The body wall of sea cucumbers is rich in collagen but there is limited knowledge of its structure, particularly in tropical species. Chapter 5 of this study focused on the isolation and characterization of collagen and pepsin-soluble collagen from the body wall of sandfish. The crude collagen fibrils were extracted using acid extraction and then digested using the porcine pepsin to extract the pepsin solubilized collagen. Resulting collagen was characterized using UV-vis spectrophotometer, Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), V8 protease enzyme digested peptide maps, Fourier transform infrared spectroscopy (FTIR) and solubility tests. Maximum absorbance of the collagen samples was at 240 – 250 nm. The triple helix structure of the collagen molecule was determined by FTIR and SDS-PAGE and the 3 α_1 chain with a molecular weight of 133.2 kDa. The maximum and minimum solubility was observed at pH 2 and 4 respectively. A sharp decrease in collagen solubility was found in between 2% and 4% NaCl concentration. The findings show that the collagen from *Holothuria scabra* was Type I and this result is consistent with those of other studies that have characterized collagen from sea cucumbers.

Research in Chapter 6 of this study assessed the potential of novel packaging for partially processed (cooked at 80-90^{o}C [control], cooked and treated with essential oils, and cooked and cold smoked) as well as processed and reconstituted sandfish. Sandfish products were subject to modified atmospheric packaging (MAP), with a gas ratio of 60%CO_2/40%N_2 and 5%0_2/50%CO_2/45%N_2, and also vacuum packaging prior to shelf-life assessment tests. Microbial

examination, nutrient composition, collagen content and texture were assayed for the samples. The control, essential oil treatment and the reconstituted samples were discarded from the shelf after 7 days because of high microbial and yeast and mold counts. The cold smoked samples, packaged using MAP and vacuum-packing, recorded 0 CFU/g at Day 0 for SPC, yeast and mold and this remained consistent throughout the shelf life. The cold smoked sandfish (MAP and vacuum-packed) recorded 0 CFU/g bacterial, yeast and mold counts until day 30. There were no significant differences between initial and 7 days samples for moisture, protein, lipid or NFE contents, across treatments. There were no significant differences in the levels of moisture, protein, lipid, ash and NFE between initial treatment samples. There were no significant differences ($P>0.05$) in the levels of the metabolically important fatty acids C20:4n-6, 20:5n-3, 22:6n-3 between samples from either day, across all treatments after 7 days of storage. This was also true of the total level of n-3 polyunsaturated fatty acids (PUFA) in the samples. No significant differences were observed in collagen composition or texture, however, the tissue toughened with the storage time. Successful use of MAP and vacuum-packing, combined with the preservative action of cold-smoking, demonstrates the potential of novel packaging methods for sea cucumber products.

This study assessed the effects of processing on yield, nutrient composition and texture of sandfish for the first time. It also assessed the potential of novel packaging methods for sandfish products for the first time. The new information generated has the following broad applications: (1) sea cucumber processing data have application in stock assessment studies, estimation of harvest data, monitoring of harvest size limits and standardizing catch data, and they provide a basis for developing species-specific harvest size restrictions for tropical sea cucumbers in Fiji

Islands; (2) where salting is use to process BDM, the results of this study provide a basis for further development of processing methods that optimize both physical and nutritional characteristics of resulting BDM; (3) preliminary examination of the potential of novel packaging for sandfish products provide a basis for further development of these methods such as examination of different gas mixtures during MAP and the effectiveness of other preservative treatments/materials combined with MAP or vacuum-packing; and (4) characterization of the collagen of sandfish creates an opportunity for commercial production/processing of collagen from this species that is of high value compared to that of temperate species.

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Chapter 1

Sea cucumber processing in the Pacific Islands

1.1 Introduction

Sea cucumbers are marine organisms belonging to the Class Holothuroidea in the Phylum Echinodermata. Around 58 species (Table 1.1) are harvested commercially and this practice has existed for over 1000 years (Conand and Byrne, 1993; Purcell et al., 2012b). Sea cucumbers and are either consumed raw, dried or boiled in many tropical and subtropical countries (Conand, 1990b). Dried sea cucumber is known as bêche-de-mer (BDM) and it is consumed as a delicacy and for its perceived medicinal value (SPC, 1997). There is high demand for BDM and sea cucumber products in south-east Asian countries and at least 15,000 tonnes of BDM are traded annually in S.E. Asia (Qao, Xu, & Yang, 2011), with major markets in China, Hong Kong, Taiwan, Singapore and Malaysia (Eriksson & Clarke, 2015; Ferdouse, 2004). The major suppliers to these markets are Pacific island countries such as Fiji, Tonga, Solomon Islands and Vanuatu (Conand, 2004a; Lovatelli et al., 2004; Purcell, 2014b) and recent years have seen increasing exploitation of the sea cucumber resources of developing tropical countries in the Pacific (Eriksson and Clarke, 2015; Mangubhai et al., 2016). The current value of the global BDM market is estimated at around $US 15-385 kg^{-1} (Purcell, 2014b), with high grade well-dried 'A' grade product commanding prices of $US 70-190 kg^{-1} depending on the species used, size and quality (Purcell et al., 2012b). *Holothuria* species such as *H. scabra* (Sandfish), *H. fuscogilva* (White teatfish) and *H. whitmaei (*former *H. nobilis)* (Black teatfish) are among the higher value species (Holland, 1994b) in Asian markets (Purcell et al., 2012a).

Although consumers in Western countries are well aware of the significant nutritional and health benefits of seafood associated with 'omega-3 fatty acids' and lean protein (Olsen, 2004; Pieniak et al., 2008), the major factors affecting the quality and value of BDM (i.e. species used, appearance, odour, colour, thickness of the body wall and market demand), do not include nutrient composition and nutritional quality (Wen et al., 2010). But despite this, 'nutrition and health' is a major motivation for consuming seafood in China (Fabinyi et al., 2016). Body wall thickness of BDM greatly influences commercial value (Skewes et al., 2004) with thicker body wall flesh generally resulting in better texture and improved eating quality (Lo, 2004). The body wall of sea cucumbers contains a high proportion of collagen (Chen et al., 2015a; Xia and Wang, 2015; Zhong et al., 2015) that has a major influence on BDM firmness and texture quality, and therefore, product value.

1.2 Sea cucumber fisheries

The sea cucumber fishery and BDM production provide an essential source of income for artisanal and small-scale fishers in coastal communities in developing countries of the Pacific. Some countries fish sea cucumbers as a major source of income where opportunities such as crop farming may be limited (Preston, 1990). The harvesting and processing of sea cucumbers requires low input and simple technology and the dried product can be stored for long term providing permanent income to the fishers (Bumrasarinpai, 2006b). However, the value of this income depends on the grade of BDM produced and the resource is not infinite. Sea cucumber stocks are at a risk from over–exploitation due to their limited dispersal patchy distribution, ease of collection, slow recovery from over-fishing and limited information on biological and spatial distribution available to guide management practices (Bumrasarinpai, 2006b). Sea cucumber fisheries generally reflect "boom

and bust" cycles and many have collapsed because of overfishing (Anonymous, 2004). Sea cucumbers are vitally important component of their marine ecosystems as they remove nutrients and 'clean' ocean sediments and recycle nutrients back into the food web.

A recent survey of sea cucumber stocks in the Fiji Islands reported that there is imminent risk of over-exploitation and a need for strict management interventions such as a five year moratorium (Jupiter et al., 2013). A similar moratorium on the sea cucumber fishery in Papua New Guinea was introduced for similar reasons in 2009 and continues today (Hair et al., 2016). More recently, a study by Nautilus Consulting on behalf of the Secretariat of the Pacific Community (SPC) reported that Fiji had never placed a ban on the sea cucumber fishery and this contributed to a sharp decline in sea cucumber stocks (Carleton et al., 2012), increasing risk from over exploitation. Despite the limitations of available literature on sea cucumber processing methods, this review focuses on BDM processing methods used by Pacific islanders and the potential for developing or introducing new techniques that could eliminate the dry processing chain and increase product value and revenue for processors.

1.3. Sea cucumber processing techniques

1.3.1. Sea cucumber habitat and harvesting

Sea cucumbers are usually found scattered over the reefs and sandy parts of the ocean floor (Conand, 2004b; Purcell et al., 2016). It is usually associated with seagrass beds or buried in the sand, and hidden in the corals or crevices within the corals (Purcell et al., 2012b). Most commercially fished species can be found intertidally, but as fishing pressure increases, fishers are forced into deeper waters. Harvesting sea cucumbers involves collection by hand in the

intertidal zones and pools or by diving in deeper waters within the reefs. Fishing using the fishing gear usually damages the skin of sea cucumbers in the deep water resulting in degraded quality of the final product (Buckworth and Sly, 2004; Mathews et al., 1990). It is important that only larger individuals are collected during harvest while smaller ones are left behind to mature and grow to the required length for harvest (Purcell et al., 2012b; SPC, 1994).

Deep sea divers, usually store the harvested sea cucumbers in the sacks, while the reef gleaners store the sea cucumbers in containers or buckets with clean sea water. The sea cucumbers are kept away from the sunlight to avoid drying of the skin that would downgrade the final product. The high value species such as White Teatfish (*Holothuria fuscogilva*), Black Teatfish (*Holothuria whitmaei*) (Table 1.1) are generally kept on flat surfaces in a single layer away from the sunlight until processing. If stacked on top of each other, the outer skin of the body wall tends to break and after processing these lesions appear as tear marks resulting in a lower grading. Sandfish (*Holothuria scarba*) is an exception. Since they have tougher body wall with many spicules, these animals can be kept on top of each other as they will flatten out remaining alive (Sachithananthan et al., 1985; SPC, 1994). Prickly redfish (*Thelenota ananas*) also requires care as the tubercles (teats) can be damaged if not handled properly resulting in product with reduced quality.

Table 1.1. List of sea cucumbers traded globally (adapted from Purcell, 2012)

Scientific name	Common Name	Retail Price (US$/kg)
Actinopyga agassizii (Selenka, 1867)	Pepino de mar (Costa Rica).	*NA*
Actinopyga echinites (Jaeger, 1833)	Deep water redfish (Pacific)	20 – 65
Actinopyga lecanora (Jaeger, 1835)	Stonefish (Pacific)	79 – 108
Actinopyga mauritiana (Quoy and Gaimard, 1833)	Surf redfish (Pacific)	57 – 145
Actinopyga miliaris (Quoy and Gaimard, 1833)	Hairy blackfish (Pacific)	63 – 92
Actinopyga palauensis Panning, 1944	Deepwater blackfish (Pacific)	95 – 116
Actinopyga spinea (Cherbonnier, 1980)	Burying blackfish	63 – 95
Actinopyga sp. affn. *flammea* (Cherbonnier, 1979)	Spiky deepwater redfish	5 – 15
Bohadschia argus (Jaeger, 1833)	Leopard fish (Pacific)	49 – 63
Bohadschia atra (Massin, Rasolofonirina, Conand and Samyn, 1999)	Falalijaka madarasy and Papiro (Madagascar), Dole (Zanzibar, Tanzania).	Singapore unknown
Bohadschia marmorata Jaeger, 1833	Brown spotted sandfish (Pacific)	1.4 – 2.0
Bohadschia subrubra (Quoy & Gaimard, 1833	Falalyjaka (Madagascar)	Unknown
Bohadschia vitiensis (Semper, 1868)	Brown sanfish (Pacific)	103 – 167
Pearsonothuria graeffei (Semper, 1868)	Flowerfish (Pacific)	2 – 5
Holothuria arenicola (Semper, 1868)	Tripang kappallah poetih (Indonesia)	2
Holothuria atra (Jaeger, 1833)	Lollyfish (Pacific)	63 – 210
Holothuria cinerascens (Brandt, 1835)	Zanga fleur (Madagascar)	Unknown
Holothuria coluber (Semper, 1868)	Snakefish (Pacific)	4 – 38
Holothuria lessoni (Massin, Uthicke, Purcell, Rowe and Samyn, 2009)	Golden sandfish (Pacific)	242 – 787
Holothuria flavomaculata (Semper, 1868)	Red snakefish (Pacific)	2
Holothuria fuscocinerea (Jaeger, 1833)	Labuyo (Philippines)	3
Holothuria fuscogilva (Cherbonnier, 1980)	White teatfish (Pacific)	128 – 274
Holothuria fuscopunctata (Jaeger, 1833)	Elephant trunkfish (Pacific)	11 – 19
Holothuria hilla (Lesson,1830)	Tiger Tail, Mani-mani (Peanut-like), Bat-tuli (Philippines)	3
Holothuria impatiens (Forsskål, 1775)	Brown spotted sea cucumber	2
Holothuria kefersteini (Selenka, 1867)	Sea cucumber	126

Table 1.1 continued next page

Holothuria edulis (Lesson, 1830)	Pinkfish (Pacific)	4 – 20
Holothuria leucospilota (Brandt, 1835)	White threadfish (Pacific)	5
Holothuria mexicana (Ludwig, 1875)	Pepino de mar (Latin American countries)	64 – 106
Holothuria nobilis (Selenka, 1867)	Black teatfish (Pacific)	106 – 139
Holothuria notabilis (Ludwig, 1875)	Dorilisy, Tsimihoke (Madagascar)	Unknown
Holothuria sp. (type 'Pentard')	Flower teatfish	17 – 26
Holothuria pardalis (Selenka, 1867)	Sea cucumber, Bantunan (Indonesia)	Unknown
Holothuria pervicax (Selenka, 1867)	Unknown	3
Holothuria scabra (Jaeger, 1833)	Sandfish (Pacific)	115 – 1668
Holothuria spinifera (Théel, 1886)	Brownfish	160 – 188
Holothuria whitmaei (Bell, 1887)	Black teatfish (Pacific)	25 – 116
Apostichopus japonicus (Selenka, 1867)	Japanese sea cucumber	970 – 2950
Apostichopus parvimensis (Clark, 1913)	Warty sea cucumber	1 – 9
Astichopus multifidus (Sluiter, 1910)	Furry sea cucumber	Unknown
Australostichopus mollis (Hutton 1872)	Brown mottled sea cucumber	275
Isostichopus badionotus (Selenka, 1867)	Four-sided sea cucumber	203 – 402
Isostichopus fuscus (Ludwig, 1875)	Brown sea cucumber, Giant sea cucumber	1.4 per unit fresh
Parastichopus californicus (Stimpson, 1857)	Giant red sea cucumber	3.7 wet
Stichopus chloronotus (Brandt, 1835)	Greenfish (Pacific)	63 – 95
Stichopus herrmanni (Semper, 1868)	Curryfish (Pacific)	79 – 159
Stichopus horrens (Selenka, 1868)	Selenka's sea cucumber (Pacific)	56 – 83
Stichopus monotuberculatus (of authors, not *S. monotuberculatus* (Quoy and Gaimard, 1833))	Selenka's sea cucumber (Pacific)	111 – 133
Stichopus naso (Semper, 1868)	Selenka's sea cucumber (Pacific)	39
Stichopus ocellatus (Massin, Zulfigar, Tan Shua Hwai and Rizal Boss, 2002)	Curryfish	35 – 111

Table 1.1 continued next page

Stichopus pseudohorrens (Cherbonnier, 1967)	Unknown	Unknown
Stichopus vastus (Sluiter, 1887)	Curryfish (Pacific)	35 – 58
Thelenota ananas (Jaeger, 1833)	Prickly redfish (Pacific)	22 – 184
Thelenota anax (Clark, 1921)	Amberfish (Pacific)	14 – 32
Thelenota rubralineata (Massin and Lane, 1991)	Lemonfish	13
Athyonidium chilensis (Semper, 1868)	Sea cucumber	10
Cucumaria frondosa (Gunnerus, 1767)	Orange footed sea cucumber	0.25 fresh
Cucumaria japonica (Semper, 1868)	Japanese cucumaria	Unknown

Harvested sea cucumbers are kept alive and clean until processing. While harvesting, pieces of sand and coral gets stuck to the skin that must be removed by washing with clean seawater. Sometimes the sea cucumbers stick to each other with dried up slime. In the boat after a few hours of collection, leaves the boat bottom full of sea cucumbers mixed up with their eviscerated guts, respiratory trees, sticky threads and water thrown out of the respiratory trees (Sachithananthan et al., 1985). In this case, the gut contents and the sticky threads are disposed of keeping the sea cucumbers clean until processing.

1.3.2 Traditional processing techniques for sea cucumbers

Bêche-de-mer processing is done by boiling, cleaning, smoke drying and sun drying (Conand, 1990b; SPC, 1994a) before storage (Fig. 1.1). The traditional process has been well documented (Ram, 2008; Sachithananthan et al., 1985; SPC, 1994) and with minimum modification, has been in use in the Pacific since the 1800's. Sea cucumber processing is time specific with dependent and cooking sea cucumbers depends on the species that is species with thick flesh takes more

time to process than the species with thin flesh. Species with thick flesh such as (White teatfish, Black teatfish, Amberfish, Tigerfish, Prickly redfish and Tigerfish) require a longer boiling time during the initial cook than other species harvested in the Pacific. The cooking time ranges from 15-25 minutes at water temperature of below 50^{o}C to avoid damages to the skin if introduced in water at 100^{o}C. The species with thinner flesh are cooked approximately 5-10 minutes at water temperature of >50^{o}C but <90^{o}C until the sea cucumber becomes cylindrical, tough and less rubbery.

After the first cook, depending on the species, the sea cucumbers are cooled and then gutted to remove the visceral organs. The larger and high value species are generally cut on the dorsal side of the sea cucumber while medium and low value species are cut at the anal side slicing about one and half inches at the dorsal surface. Improper cutting and low product value due to poor quality issues has been well documented (Ram, 2008).

Bêche-de-mer is graded according to the species, size, appearance, odour, colour, moisture, spoilage and content of extraneous matter (McElroy, 1990; Ozer et al., 2004). The larger the size of the dried product, the better the grade and higher the price (McElroy, 1990). A species that has been handled and processed properly would demonstrate a smooth surface, a uniform shape and clean body wall body cut with a pleasant smell would also mean that the product was stored properly(McElroy, 1990). The final dried product is stored in 50 kg polyethylene sacks in a cool dry place or exported to Asian countries. Inadequate handling and processing methods can also reduce nutrients in the product that leads to nutritional loss (Ram, 2008; Wen et al., 2010).

Previous studies have indicated that poor handling and poor processing technique can lead to nutritional loss from sea cucumbers. A study was conducted (Wen et al., 2010) on the nutrient content (moisture, ash, protein, minerals, fat, and amino acids) of 10 different species of tropical sea cucumbers. The study revealed that season and processing technique had a significant effect on the nutrient composition of holothurian body wall. This study was also supported further (Ozer et al., 2004) where similar data for nutrient compositions of sea cucumbers to different treatment levels (processing techniques) was reported. Other studies conducted (Dong et al., 2011) revealed that collagen was also vastly affected at different boiling temperatures during sea cucumber processing. In the Pacific Islands, however, people are unaware of the chemical changes that occur during sea cucumber processing therefore, a better technique based on scientific analysis needs to be developed to help indicate the proper way and duration of processing sea cucumbers to produce a product with optimal physical characteristics, retain essential nutrients in the final product and maximize income for processors. For example, the level of 'beneficial nutrients' in BDM (e.g. omega-3 fatty acids) could be a useful marketing point but this has not yet been considered because of lack of information on the influence of processing method on nutrient composition and on the potential to manipulation processing methods to optimize both physical and nutritional characteristics of BDM.

1.4. Nutritional value of sea cucumbers and bêche-de-mer

Sea cucumbers are regarded as a high quality food with medicinal value and are generally consumed raw, dried or boiled (Conand, 1990b) to optimize their medicinal properties (Lou et al., 2012). Sea cucumbers are rich in protein (ca. 43%) and low in fat (ca. 2%) (Table 1.2). Sea cucumber also contain high levels of micronutrients such as minerals (ca. 21%) (SPC, 1994)

including copper, magnesium and potassium (Wen and Hu, 2010), amino acids (Wen et al., 2010), collagen (Dong et al., 2011) and fatty acids including those considered essential for human wellbeing the highly unsaturated eicospentaenoic acid (20:5n-3, EPA), docosahexaenoic acid (C22:6n-3, DHA) and arachidonic acid (C20:4n-6, AA) (Bordbar et al., 2011). The presence of fatty acids in the sea cucumbers range from 5–61% depending on the species (Wen et al., 2010) and have been associated with a reduced risk of coronary heart disease and cancer (Harper and Jacobson, 2005; Roynette et al., 2004).

Table 1.2. Proximate composition of bêche-de-mer processed from various species of sea cucumber.

Proximate composition (%)	**Moisture (%)**	**Ash (%)**	**Total protein (%)**	**Total fat (%)**	**Carbohydrates (%)**
Holothuria scabra (Omran, 2013)*	85.76	2.26	43.43	5.66	48.65
Holothuria fuscogilva (Wen et al., 2010)**	11.6	26.4	57.8	0.3	
Actinopyga mauritiana (Haider et al., 2015; Omran, 2013; Wen et al., 2010)	84.71[a]	2.12[a]	48.27[a]	4.99 [a]	44.62 [a]
	76.54[b]	31.81[b]	66.86[b]	0.76[b]	
	11.6[c]	15.4[c]	63.3[c]	1.1[c]	
*Parastichopus californicus (Bechtel et al., 2012; Liu et al., 2010)**	85.66[d]	1.72[d]	9.46[d]	0.44[d]	
	4.03[e]	25.73[e]	47.03[e]	8.19[e]	15.02[e]
Apostichopus japonicus (Lee et al., 2012)*	80.26 – 91.49%	2.57 – 6.85%	1.13 – 3.99%	0.14 – 2.12%	
Thelenota ananas (Wen et al., 2010)**	15.1	25.1	55.2	1.9	
Bohadschia marmorata (Omran, 2013)*	83.17	6.03	43.23	4.83	45.91
Holothuria leucospilota(Omran, 2013)*	81.41	4.3	45.71	4.60	44.96

Table 1.2 continued next page

Stichopus hermanni (Wen et al., 2010)	10.2	37.9	47.0	0.8	
Thelenota anax (Wen et al., 2010)**	1.2	39.2	40.7	9.9	
Holothurian fuscopunctata (Wen et al., 2010)**	7.0	39.6	50.1	0.3	
*Bohadschia argus (Wen et al., 2010)***	13.0	17.7	62.1	1.1	
Actinopyga caerulea (Wen et al., 2010)**	0.81	28.4	56.9	10.1	
Holothuria arenicola (Haider et al., 2015)*	72.12	31.81	66.86	0.76	

a- Omran (2013)* b-(Haider et al., 2015)* c-(Wen et al., 2010)** d-(Liu et al., 2010)* e-(Bechtel et al., 2012)*

* Authors researched with raw freeze dried tissue samples
** Authors researched with processed dried tissue samples

Very few research studies have attempted to correlate the relationship between varying processing techniques and nutrient composition of bêche-de-mer (Aydın et al., 2011; Ozer et al., 2004; Skewes et al., 2004; Wen and Hu, 2010; Wen et al., 2010). For example, spoilage of unprocessed sea cucumber tissue is likely to occur rapidly in the tropics and could affect the quality of resulting BDM. Amino acids impart flavour to sea food products and can rapidly leach from tissues when immersed in liquid (Bremner, 2012). Furthermore, long-chain highly unsaturated fatty acids can rapidly oxidize to reduce the content of 'beneficial' nutrients and potentially produce toxic by-products (Chow, 2008). Because BDM undergoes multi-step processing that potentially includes multiple cooking, salting and drying (Fig. 1.1), the texture and nutrient composition of the product is likely to be modified during processing. Despite this, few studies have reported on such changes that could influence the nutrient content and nutritional value of BDM.

Previous studies have indicated that, poor handling and poor processing technique can lead to nutritional loss from the sea cucumbers. Wen et al. (2010) analyzing the nutrient content (moisture, ash, protein, minerals, fat, and amino acids) of 10 species of sea cucumbers and reported that season and processing technique had a significant effect on the nutrient composition of holothurian body wall. This study was also supported by Ozer et al. (2004)

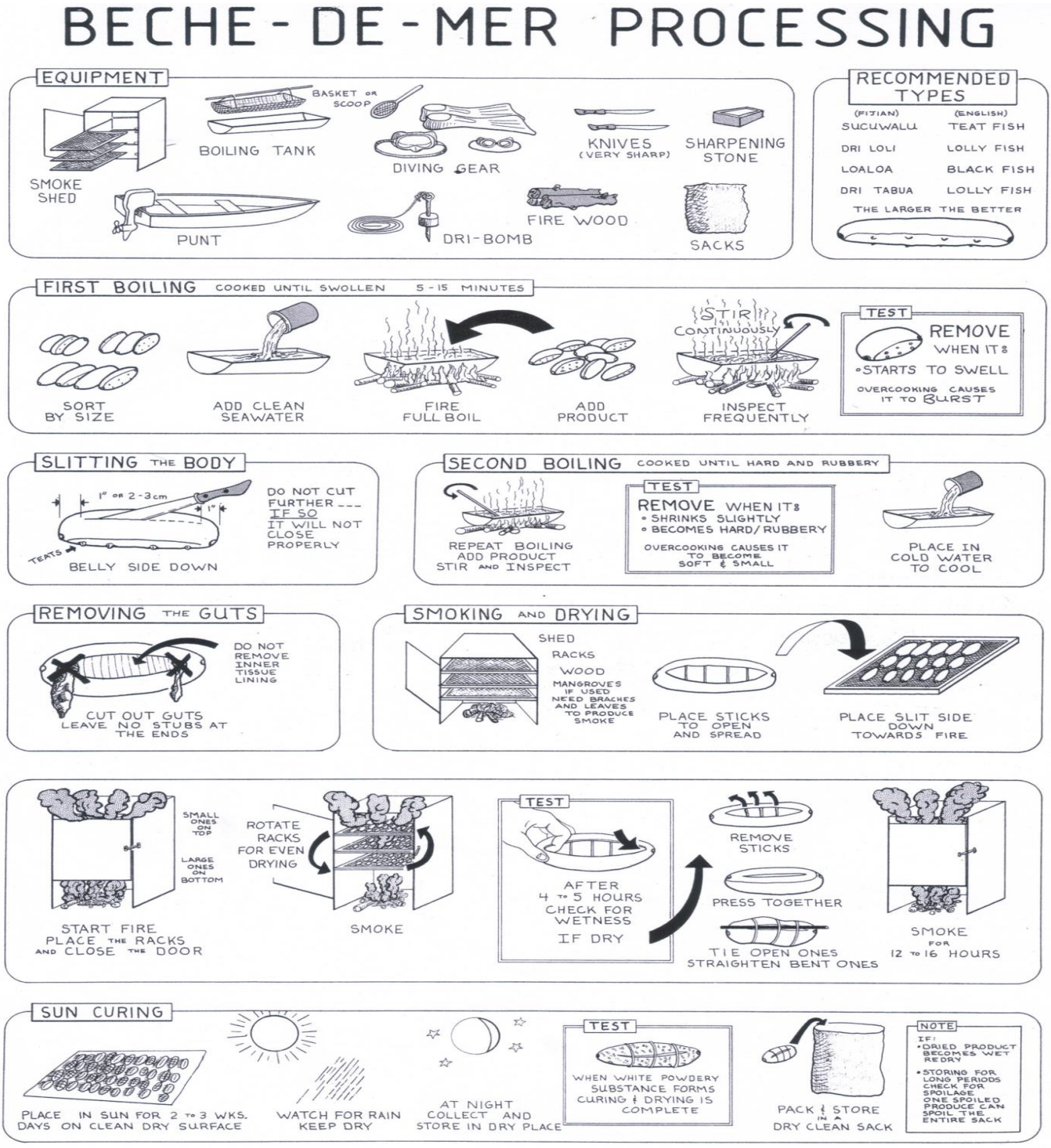

Fig. 1.1. Steps in bêche-de-mer processing; traditional technique. *Source: Ministry of Fisheries, Fiji. (Ram, 2008)*

showing similar data for nutrient composition of sea cucumbers to different treatment levels (processing techniques). Other studies conducted by Dong et al. (2011) revealed that collagen was also vastly affected at different boiling temperatures during sea cucumber processing.

1.4.1 Protein and amino acids

The protein contents of BDM processed from various species of sea cucumbers is shown in Table 1.2. Crude protein ranged from 1.13% to 66.86% (Bechtel et al., 2012; Haider et al., 2015; Liu et al., 2010; Omran, 2013; Wen et al., 2010). Significantly, given the importance of BDM body wall texture in the value of BDM, collagen has been reported to make up at least 70% of the protein content of BDM (Saito et al., 2002), and, individually, between 3.4% and 24.3% of BDM dry weight (Liu et al., 2010; Zhong et al., 2015).

Collagens belong to a family of extracellular matrix protein that maintains the integrity of various tissues. There are approximately 27 types of collagen found with 42 distinct types of polypeptide chains and about 20 additional proteins and collagen – like domain and 20 other collagen modifying enzymes (Kivirikko and Prokop, 1995; Myllyharju and Kivirikko, 2004). Collagen molecules are composed of three α – chains that is mainly stabilized by intra and inter chain hydrogen bonds known as collagen triple helix (Zhang et al., 2013). It contains a repeat of the amino acids glycine, proline and hydroxyproline (Ichikawa et al., 2010). The usage of collagen include leather products, biomedical products such as wound dressings, implants and drug carriers as well as the food industries in the production of gelatin (Nam et al., 2008). Gelatin is produced from partial hydrolysis of collagen and the extraction of gelatin is done by a chemical pre-treatment with an acid or an alkali (Zhang et al., 2013). This treatment however,

breaks the non-covalent bonds in collagen and destabilizes the structure of collagen (Djabourov et al., 1993) and however, heating at the temperatures greater than 45^{o}C denatures the collagen triple helix and converts the collagen into gelatin (Gomez-Guillen et al., 2002).

Few studies have reported on isolation and analysis of pepsin-soluble collagen (PSC) and acid-soluble collagen (ASC) from sea cucumbers; however, some literature is available that have analysed the collagen content in various species of fish and shrimps (Kittiphattanabawon et al., 2005 ; Nagai et al., 2001). Liu et al. (2010) conducted a study on purification and characterization of pepsin-soluble collagen from skin and connective tissues of the sea cucumber (*Parastichopus californicus*). In the study the maximum transition temperature for gel formation and denaturation temperature of collagen was also studied. The findings revealed that the collagen extracted from *P. californicus* was type I and gel forming ability at pH 6. The denaturation time and maximum transition time was determined to be 18.5 and 33.2^{o}C respectively. The researchers concluded that the collagen content from the giant sea cucumber could be used in foods and for pharmaceutical purposes. Cui et al. (2007) also analysed the pepsin – soluble collagen by characterizing the subunit of the collagen from the body wall of *Stichopus japonicus*. The thermal stability temperature of the collagen was also determined by the differential scanning calorimetry (DSC) that was determined to be 57^{o}C. The PSC isolated from *S. japonicus* was found to be type I and of the molecule structure composed of three α – chains.

Limited literature is available for sea cucumber relating to possible changes in collagen content during processing and the effects of particular processing steps and conditions (e.g. temperature)

on collagen content of products. Niamnuy et al. (2008) studied the changes on the protein composition and its effect on the physical changes on the shrimp during boiling at different salt solutions. The findings revealed that rising salt concentration affected the protein quality and texture of the shrimp. With an increased boiling time and salt concentration the protein denaturation was more common and the shrimp became harder due to the decrease in myofibrillar, sarcoplasmic and stroma protein. Niamnuy et al. (2007) reported that boiling time affected the shrimp quality through cooking loss and texture loss due to protein denaturation as well as the salt, moisture and protein content. The final product quality can only be determined through sensory evaluation and be known the extent of quality loss due to boiling time and various salt concentrations that affect the texture and overall acceptance of the final product (Tapaneyasin et al., 2005).

Amino acids play a significant role in metabolic process of a human body. Research by Wen et al. (2010) determined that the most abundant amino acids in BDM processed from eight species of sea cucumbers imported to China were glycine, glutamic acid, aspartic acid, alanine and arginine that together constituted between 58-65% of the total amino acids. Previous studies indicated the usefulness of amino acids such that the serum cholesterol level is reduced by the amino acid glycine (Aljawad et al., 1991; Ikeda et al., 1993). Having a low level of lysine:argine ratio reduces the concentrations of cholesterol in the serum and aorta and suggests that low lysine/arginine ratio exerts a hypocholesterolemic effects (Rajamohan and Kurup, 1997; Sugano et al., 1984).

1.4.2 Lipids and fatty acids

The lipid contents of BDM processed from various species of sea cucumbers is shown in Table 1.2. Lipid ranged from 0.3%-9.9% of BDM dry weight in these studies (Bechtel et al., 2012; Liu et al., 2010; Wen et al., 2010). Various studies have reported on the lipid and fatty acid profiles of sea cucumbers in either dried or fresh form. Svetashev et al. (1991) studied the lipid and fatty acid composition in 12 tropical and temperate water species; 10 tropical and two temperate species. The findings revealed that lipid content in tropical holothurians (range 0.25%-2.60%) were generally lower than the temperate holothurians (range 4.9%-9.9%), but that in the winter months, lipid content was greater in tropical holothurians (12.5%-29%). Levels of the metabolically important fatty acid arachidonic acid (C20:4n-6) were also reported to be higher in tropical holothurians than the temperate species (Svetashev et al., 1991).

Aydın et al. (2011) studied the proximate composition and fatty acid profile during drying process of three temperate holothurians in Turkey; *Holothuria tubulosa, H. polii* and *H. mammata*. The holothurians were cut and gutted followed by boiling and drying in the sun and the fatty acid profile was determined for fresh wet and after drying. The analysis showed that saturated fatty acids, monounsaturated fatty acids and polyunsaturated made up 15.48-19.21%, 13.92-15.01% and 53.38-57.76% of total fatty acids in fresh sea cucumbers, respectively, and 12.93-18.78%, 14.30-19.81% and 41.66-50.54% of total fatty acids in dried sea cucumbers, respectively. Saturated and polyunsaturated fatty acids decreased during the drying process but the authors noted higher levels of arachidonic acid and docosahexaenoic acid (22:6n-3, DHA) and lower amount of eicosapentaenoic acid (20:5n-3, EPA) after drying. The results of this study were supported by those of Wen et al. (2010) who reported on the fatty acid composition of eight

dried tropical holothurians. The analysis showed that saturated fatty acids, monounsaturated fatty acids and polyunsaturated made up 31.23-61.60%, 27.0-45.64% and 5.10-16.0% of total fatty acids in dried sea cucumbers, respectively. Palmitic acid (C16:0) (5.91-31.86% total fatty acids) and stearic acid (C18:0) (7.14-14.13% total fatty acids), were the major saturated fatty acids in the eight species tested, while arachidonic acid AA (C20:4 n-6) (1.82-14.42% total fatty acids) and eicosapentaenoic acid EPA (C20:5n-3) (0.32-3.84% total fatty acids), were the major polyunsaturated fatty acids (Wen et al., 2010). Information on the loss of fatty acids from the holothurian body wall during processing is extremely limited and recommendations on processing methods that help retain levels of metabolically important fatty acids in BDM have not yet been considered.

1.4.3 Carbohydrates

Limited literature is available reporting the levels of carbohydrates in sea cucumber tissue or BDM. Carbohydrates are an essential part of the human diet and they are used primarily as an energy source. Lawrence (1972) analysed the carbohydrate content in freshly harvested *H. atra* using tissue samples from small intestine, large intestine and *rete mirabile*. The collection was done in summer and winter months to determine the variation. The findings revealed that carbohydrate content in the large intestine was significantly higher (4.9% of dry weight) than in the small intestine (2.6%) and *rete mirabile* (4.3%) and did not vary seasonally.

1.5. Medicinal value of sea cucumbers and bêche-de-mer

Extracts from sea cucumbers serve as a tonic and traditional remedy for many ailments (Anderson, 1988; Bordbar et al., 2011; Chen, 2003; Jilin and Peck, 1995; Weici, 1987; Wen et al., 2010; Yaacob et al., 1997) and for their unique biological and pharmacological properties including anti-angiogenic (Tian et al., 2005), anticancer (Roginsky et al., 2004), anticoagulant (Chen et al., 2011; Nagase et al., 1995), anti-hypertension (Hamaguchi et al., 2010), anti-inflammatory (Collin, 1998, 1999, 2004), antimicrobial (Beauregard et al., 2001; Hing et al., 2007), antioxidant (Althunibat et al., 2009), antithrombotic (Mourao et al., 1998; Pacheco et al., 2000), antitumor (Tong et al., 2005; Zou et al., 2003) and wound healing (Miguel-Ruiz and García-Arrarás, 2007) activities. Such medicinal properties are related to the presence of bioactive compounds such as triterpene glycosides (saponins) (Aminin et al., 2010; Kerr and Chen, 1995; Miyamoto et al., 1990), chondroitin sulfates (Vieira et al., 1991), glycosaminoglycan (Nagase et al., 1995; Pacheco et al., 2000), sulfated polysaccharides (Mourao and Pereira, 1999), sterols (glycosides and sulfates) (Goad et al., 1985), phenolics (Mamelona et al., 2007), specific peptides (Rafiuddin et al., 2004), cerberosides (Sugawara et al., 2006) and lectins (Mojica and Merca, 2004; Mojica and Merca, 2005a; Mojica and Merca, 2005b). Consumption of sea cucumbers is thought to aid growth, blood clotting and wound healing (Gil, 2002; Mat et al., 1994) supporting their use as a traditional remedy for burns and cuts (Fredalina et al., 1999).

Pepsin-Solubilized Collagen (PSC) extracted from *Stichopus japonicus* has been shown to improve proliferation of human keratinocytes (Park et al., 2011). So far, researches on sea cucumber collagen have been mainly focused on functions of hydrolytic bioactive peptides,

including damaged tissue repairing (Park et al., 2012), antitumor (Zhou et al., 2012b), antioxidant (Zhou et al., 2012a), and angiotensin-converting enzyme inhibitory activity (Forghani et al., 2012; Zhao et al., 2009). Investigations on isolation, purification, and characterization of PSC have been carried out in some sea cucumber species, such as *S. japonicus* (Cui et al., 2007; Saito et al., 2002), *Parastichopus californicus* (Liu et al., 2010; Liu et al., 2011), *S. vastus* (Abedin et al., 2013), *Bohadschia bivitatta* (Siddiqui et al., 2013) and *Holothuria parva* (Adibzadeh et al., 2014), but similar research with tropical species or those from the south Pacific has not yet been reported.

1.6. Advancements in processing and drying

Traditional processing of seafood constitutes a wide variety of techniques used to preserve food, either by salting and/or drying to increase shelf life (Fig. 1.2).

Fig 1.2. Dried sea cucumbers in Hong Kong market

1.6.1 Salting

Salting is one of the common traditional processes (Wang et al., 2011) that increases the shelf life by reducing the water activity of the drying product and produces a high processing yield and better quality of the final product. There are generally three different types of salting used to process seafood: dry salting (kench), wet salting and the slurry (intermediate of the dry and wet). For wet salting the brine needs to be about 80-100 degree saline for its effectiveness as it makes water molecules to move out of the tissues and salt to penetrate. The findings (Wang et al., 2011) revealed that salt uptake depends on the protein and the concentration of water phase in fish muscle. At lower concentrations (0.1 M) fish muscle swells but maximum swelling (water holding capacity) occurs at 1 M (5.8% salt). However, at higher concentrations that is above 9-10% the proteins tend to have a greater protein to protein bond resulting in shrinkage and dehydration of the fish muscle. The researchers also indicated that certain factors affect drying and final product quality such as type of salting, brine concentrations, salting time, temperature and material species and sizes.

The effect of dry salting and wet salting in 21% saline solution at 20^{o}C for moisture loss and salt uptake by the tissues of sardines was studied (Bellagha et al., 2007). This study however, was evaluated for 48 hours at 40^{o}C. The findings from the study revealed that after drying, (48 hours) the water activity (a_w) of the sardine tissue accounted at 0.698 for brining and 0.661 for dry salting. The study indicated that brining was more preferred over dry salting for its appearance, texture and acceptability. Dry salting however, makes the fish more brittle and affects the overall quality and acceptance.

In addition, a similar study on the effect of different brine concentrations (4, 10, 15, 18 and 25% salt weight by weight (w/w) and dry salting on the salting kinetics and the yield of smoked Atlantic salmon fillets during a period of 14 days at 4^{o}C was done (Gallart-Jornet et al., 2007b). The findings revealed that the fillet weight increased with decreasing saline concentration and dry salting resulted in lowest process yield. The texture, elasticity and hardness however, also decreased with increasing saline concentration. This study suggested that the use of 10, 15 and 18% saline gave the best and highest yield after the smoking process and the use of lower saline concentrations (4-18%) increased the water holding capacity thus lowering the protein denaturation and resulting in better yield of salting process. However, higher salting that is 25% w/w and dry salting lead to further protein denaturation, resulting in rapid loss of water and thus affected the texture and reduced the water holding capacity. Salting process has been a traditional process but today, modern technology is incorporating this process in current food preservation techniques to improve the texture and quality (Shi et al., 2008).

However, before drying the raw material it is necessary to eliminate the microorganism, enzymes, water activity that spoil the dried product and increase the shelf life of the product. The effects of salt concentration and boiling time on the quality (colour and toughness) of dried shrimp was studied (Niamnuy et al., 2007). The findings from the research revealed that higher concentrations of salt solutions prolonged boiling times. Higher salt concentrations lowered moisture and proteins leading to higher value of hardness, roughness, shrinkage and colour change of shrimp.in contrary, a novel nanoscale silver coating sterilization technique was developed where sea cucumbers were immersed in a nanoscale silver solution of 0.045 mg/L for

one minute to effectively reduce the microorganism in the dried sea cucumbers (Duan et al., 2010b).

1.6.2 Drying methods

The traditional technique of drying sea cucumbers uses solar drying. However, use of continuous solar drying technique is weather unfriendly and prolonged rain can spoil the product due to insect manifestations and poor sanitary conditions (Ram, 2008). Various types of solar dryers such as vacuum assisted solar dryers (Rajkumar et al., 2007), unglazed transpired solar dryers (Hassanain, 2010), mixed mode solar dryers (Phoungchandang et al., 2009) and solar cabinet or tunnel dryers (Kituu et al., 2010; Sankat and Mujaffar, 2004) are used to concentrate solar radiation to effectively dry sea food and other commodities. Those used for sea cucumber processing include solar drying, solar cabinet and tunnel drying.

Another form of drying technique is the superheated steam drying that saves energy and the oxygen free atmosphere improves quality (Martinello et al., 2003). This type of drying involves the use of superheated steam in a direct dryer instead of hot air, combustion, or flue gases as the drying medium to supply heat for drying and to remove the evaporated moisture. The drying characteristics of shrimp in a drying media at a temperature range of 120–180^{o}C for superheated steam and of 70 –140^{o}C for hot air was examined for this study (Prachayawarakorn et al., 2002). The findings revealed that temperature had an important effect on drying rate and effective diffusion. Compared to the use of hot air, superheated steam showed a lower rate of shrinkage in shrimps and the product colour slightly different from other commercial sources. The findings also revealed that using superheated steam, there was a very little loss of valuable omega-3-fatty

acids due to reduced drying time (Bo´rquez et al., 2008). However, superheated steam drying is not suitable for heat sensitive products (Devahastin, undated). Careful analysis needs to be done before this technique can be used. For sensitive products a low pressure superheated steam can be used (Thomkapanich et al., 2007).

Moreover, microwave assisted drying is a more rapid method of moisture removal based on the microwave heating or conduction heat transfer (Wang et al., 2011). Microwave freeze drying technique was developed for drying sea cucumbers (Duan et al., 2010b). Microwave freeze dryer reduces drying time by half of conventional freeze drying and provides a similar good quality product as freeze dried. Freeze drying under vacuum conditions is the best method of water removal for all kinds of foods because the primary flavor, colour, structure, and the nutrient compositions are maintained to great extent and final products have better rehydration capacity than those produced using other drying methods. Freeze drying technique however, is a very expensive preservation technique due to higher operation cost (Duan et al., 2010b) that might not be favourable for Pacific Islands. This study also indicated that microwave freeze drying assisted with vacuum packaging with sea cucumbers impregnated with nanoscale calcium carbonate was found to be an efficient drying technique. Regular vacuum freeze drying can be improved by improving the heat transfer to aid sublimation and using the absorbent to replace the condenser and also optimizing process parameters to minimize consumption loss such as freeze temperature and time, vacuum, thickness of material and mass load (Ratti, 2001).

1.7. Potential for future development

For over two centuries, the peoples of the Pacific Islands have used the sea cucumber drying techniques that had been handed to the people from the Chinese communities trading in the South Pacific for sea cucumbers in the early 1800's. People espoused to the traditional drying techniques better than now, but over time, increasing global demand and better prices on offer for the sea cucumbers have led to insufficient dried products of low grade price and quality (Ram, 2008). The sea cucumber products that are now processed using the traditional techniques are of poor quality and inferior priced. Bulk of the studies that had been conducted in the Pacific Islands has reported to improving the processing techniques to improve the return of sea cucumber exports from Asian markets (Carleton et al., 2012; Purcell et al., 2009a; Purcell et al., 2013; Purcell et al., 2012a; Ram, 2008). Previous sections have looked at the drying techniques of sea cucumbers through traditional drying and through some of the advanced techniques such as modified vacuum packaging and freeze drying. The advanced techniques improve the quality, however come at a higher cost of operation. In addition to the techniques used above, a number of packaging systems have also been developed (Duan et al., 2008a, b; Duan et al., 2007; Duan et al., 2010b), that could be used to package sea cucumbers without drying the final product (Fig. 1.3).

Fig. 1.3. Vacuum packaged (A) and frozen (B) sea cucumbers in Hong Kong market

Recent years have seen increasing demand for consumer-friendly, non-dried, ready to cook sea cucumber products (Purcell et al., 2014). These include cooked and frozen and cooked and vacuum-packed products that are refrigerated or frozen. This has required development of novel packaging methods for non-dried sea cucumbers that are significantly more perishable than BDM. The following section discussed some of the techniques current employed (i.e. vacuum-packaging) as well as those with potential for use with sea cucumbers.

1.7.1. Vacuum packing

Vacuum packaging removes the air surrounding the product slowing the growth of spoilage bacteria and extending shelf-life; however, shelf life extension of 'fresh' vacuum-packed products that have not been otherwise cured (i.e. by salting, smoking or use of other curatives) may be limited as the main spoilage organisms are not removed (Bremner, 2012). A processor in

the Kingdom of Tonga (South Pacific Resource Limited; SPRL) is now using vacuum packaging in processing local sea cucumber species for the South Korean market. The processor deals with the low value sea cucumber species *H. atra* (Lollyfish) that are boiled, shredded, vacuum packed and refrigerated to prevent spoilage (Fig. 1.4). There is limited literature available on vacuum-packed sea cucumbers; the shelf life has to be determined for prolonged storage at the refrigerated temperatures. SPRL however, receives better revenue than the other Chinese sea cucumber exporters from Tonga. Purcell et al. (2014) reported broad availability of vacuum-packed sea cucumber products throughout Asia, where improved retail infrastructure (i.e. refrigerators and freezers) as well as improved cold-chain shipping and transportation methods, support development of such products.

Fig. 1.4. Shredded and whole freeze dried and vacuum packed Lollyfish, from clockwise direction (A& B) Shredded dried Lollyfish, (C) Vacuum packed whole Lollyfish (first boiled) and (D) Shredded wet Lollyfish. The products are currently trading at USD 50–65/kg in the Korean market.

1.7.2 Modified Atmospheric Packaging (MAP)

Modified atmospheric packaging utilizes the use of three main gases nitrogen, oxygen and carbon dioxide. Altering the atmospheric conditions of the food being packed using these three gases increases the shelf life of the resulting product. Nitrogen is a tasteless gas that displaces

oxygen, is not reactive with the food but is highly soluble in water. Oxygen however, promotes the growth of aerobic microorganisms while inhibiting the growth of anaerobic microorganisms (Mastromatteo et al., 2010a). The presence of oxygen in the MAP is also responsible for a number of oxidation reactions and rancidity of lipids and fats and is generally avoided during the packaging. Carbon dioxide gas is soluble in both water and lipids and acts as a bacteriostatic agent in the growth of microorganisms and slows down the respiration of many products. All the three gases are safe, economical and readily available for packaging. The advantages of MAP is that the product has a longer shelf life without affecting its freshness like characteristics, reduces economic losses and produces a high quality product. The sealed MAP product provides a barrier against the environment and the contaminants thus improving the overall presentation of the packaged product.

The disadvantages of having the MAP product is that it requires different gas combination for each of the different food product and requires expensive equipment for packaging (Mastromatteo et al., 2010a). The packaged product also requires space for storage and cannot be stacked on top of each other. The product also requires specific temperature controls for safety purpose and long-term storage. MAP packed sea cucumber products if managed properly could become one of high earning commodity for the sea cucumber industries in the Pacific as MAP will add value to the product.

The indispensable part of MAP packaging is the use of preservative or an antimicrobial product to reduce the risk of microbial growth in the package. A study (Mastromatteo et al., 2010a), to improve the shelf life of shrimps through modified atmospheric packaging was conducted. The

findings revealed that MAP alone did not improve the shelf life of the shrimps. However, coating the shrimps in a range of thymol concentrations (500-1500 mg/L) before packing, significantly improved the shelf life of the shrimps. At a concentration of 1000 mg/L thymol, it reduced the mesophilic bacteria, *Pseudomonas* spp. and hydrogen-sulphide producing bacteria as key microbes in spoilage. The results indicated that microbial load in the shrimps after coating in thymol reduced significantly by 2log cycle and prolonged the shelf life of the shrimps by further six days.

A number of studies have been conducted on the preservation of fish, meat and vegetables using the antimicrobial agents and through MAP packaging. A study (Kostaki et al., 2009), on the combined effect of MAP using two different gas mixtures (40% CO_2/50% N/10% O_2 and 60% CO_2/30% N/10% O_2) and thyme oil (0.2% v/w) on the quality of filleted sea bass during storage was carried out. Unpacked Sea bass were used as a control experiment. It was found out that, fillets packed in MAP reached 7-logCFU/g after several days than the unpacked fillets that reached the same level in 7 days. Similar research (Kykkidou et al., 2009) on combined effect on thyme essential oil (0.1% v/w) and packaging on microbial and sensorial characteristics of fresh Mediterranean swordfish fillets was also done. Air, MAP (5%O_2/50%CO_2/45%N_2), air with thyme oil and MAP with thyme oil were used. The findings revealed that MAP and MAP combined with thyme oil were most effective against the growth of Pseudomonads and Hydrogen Sulfide (H_2S) producing bacteria. The shelf life of the swordfish under aerobic and MAP was determined to be 8 and 13 days respectively. Further addition of 0.1% thyme essential oil extended the shelf life of the swordfish fillets by another five days in aerobic conditions and by seven and half days under combination of MAP with thyme oil as compared to the control.

The overall acceptance and the quality of the swordfish decreased with storage time stored both under aerobic conditions and MAP either with or without essential thyme oil.

1.7.3. Use of essential oils in seafood preservation

The use of essential oils have also shown to improve the shelf life in MAP of the unprocessed food (Mastromatteo et al., 2010a; Mastromatteo et al., 2010b). It has been used for flavoring in the food as well as acting as an antimicrobial agent and display more inhibitory against Gram-positive bacteria than the Gram-negative bacteria. Another group of natural antimicrobial agent is bacteriocins such as Nisin as a food preservative with MAP. Nisin combines with the bacterial cell Lipid II and inserts in the plasma membrane of the bacteria causing it to become porous thus causing cell death and improving the shelf life of the food. In addition, organic acids such as acetic acid, latic acid are also an ideal choice of antimicrobial agents that cause cell death by damaging the plasma membrane and making the bacterial cell porous. Chitosan is another antimicrobial agent that is derived through deacetylation of chitin. Chitosan has the ability to form film formation or coating that enables the foods shelf life extension of raw food.

1.7.4 Other potential packaging methods

Other packaging techniques such as the high-pressure packaging (HPP) of the food have also shown bacterial inhibition of food borne pathogens (Caner et al., 2004; Garriga et al., 2004; Jofr et al., 2007; Marcos et al., 2008). HPP techniques have shown to have inhibited *Listeria monocytogenes* in the food after food was packed with preservatives. A study (Jofr et al., 2007), on inhibition of *Listeria monocytogenes* in cooked ham through active packaging with natural

antimicrobials and high-pressure processing was done. This study involved, the use of natural preservatives enterocins A and B and sakacin K at 200 and 2,000 activity units (AU)/cm, nisin at 200 AU/cm, 1.8% potassium lactate, and a combination of 200 AU/cm of nisin and 1.8% lactate were incorporated into interleavers and the effectiveness were tested against the *L. monocytogenes*. The ham was later subjected to HPP at 400Mpa. In non-pressurised ham nisin plus lactate containing interleavers, were most effective in inhibiting *L. monocytogenes* for almost 30 days. In the other antimicrobial preservatives, *L. monocytogenes* were found to be the lowest by the end of storage in bacteriocins that reduced the bacterial load by 4 log CFU/g.

A similar study was also done on high-pressure processing and antimicrobial biodegradable packaging to control *L. monocytogenes* during storage of cooked ham (Marcos et al., 2008). Three different lots of ham were prepared control, packaging with alginate films, and packaging with antimicrobial alginate films containing enterocins. After packaging half of the samples were pressurized. The findings revealed that both antimicrobial packaging and pressurization delayed the microbial growth. At 6^{o}C, the antimicrobial packaging cooked ham showed *L. monocytogenes* levels below the detection limit at day 90. In non-pressurized ham stored at 1^{o}C, the pathogen growth was controlled until day 39, while the antimicrobial packaging with HPP exerted a bacteriostatic effect for 60 days. After a cold chain break *L. monocytogenes* were undetected in the antimicrobial packaging HPP ham that showed the efficiency of the combined packaging technique.

HPP has not been performed on sea cucumbers but has been successful with other marine organisms and in maintaining a longer shelf life (Galotto et al., 2008; Garriga et al., 2004;

Jantakoson et al., 2012; Jofr et al., 2007; López-Rubio et al., 2005; Marcos et al., 2008; Norton and Sun, 2008). In addition, sea cucumbers have also not packed in a MAP before. Numerous studies using MAP package with vegetables and poultry and sea food excluding holothurians were conducted (Bhat et al.; Debevere and Boskou, 1996; Fernández et al., 2009; Jayathunge and Illeperuma, 2005; Lu, 2009; Mastromatteo et al., 2010a; Mastromatteo et al., 2010b; Özogul et al., 2004; Pesis et al., 2000; Tucker, 2008). The studies showed that MAP alone did not increase the shelf life, however, when combined with preservatives such as essential oils prolonged the shelf life of the product as well as inhibited a number of food spoilage microorganisms.

Canning trials with sea cumber have been conducted in Asian countries (Pan et al., 2014), however, the proteins of the sea cucumber body wall are unstable at high temperatures and the product breaks apart after the can is opened for further cooking.

1.8. Overall objective, aims and statement of organisation

Recent years have seen increasing demand for consumer-friendly, non-dried, ready to cook sea cucumber products in Asian markets along with greater awareness on the potential health benefits of seafood consumption (Fabinyi, 2012; Fabinyi et al., 2016; Purcell, 2014b). On this basis, there is opportunity to develop new sea cucumber products that are more user friendly and with improved nutrient content. However, a major determinant of processed sea cucumber quality and value is the texture of the consumed product and so any future development in the processing and packaging of sea cucumber must account for texture as well and nutrient content. These issues provide the basis of this study.

The main objective of this study was to generate improved information on the processing of sandfish (*Holothuria scabra*) relating to nutrient composition and texture, and the potential and implications of novel processing and packaging methods for this species. This objective was addressed in five research chapters:

- **Chapter 2** - Recovery rates for eight commercial sea cucumber species from the Fiji Islands;
- **Chapter 3** - Effect of different processing techniques on the nutrient composition of bêche-de-mer produced from Sandfish, *Holothuria scabra*;
- **Chapter 4** - Effect of processing method on quality, texture, collagen and amino acid composition of sandfish (*Holothuria scabra*);
- **Chapter 5** - Isolation and characterization of collagen from the body wall of the tropical sea cucumber *Holothuria scabra* (Sandfish) Jaegar 1833; and
- **Chapter 6** - Assessing novel processing and packing methods on the quality and the nutrient composition of Sandfish (*Holothuria scabra*).

This thesis is presented in a Thesis-by-Publication format. Each research chapter represents a succinct study that has either been published or submitted. The status of each chapter at the time of thesis submission is indicated using footnotes associated with chapter titles. On this basis, there is some repetition of content between chapters.

Chapter 2

Recovery rates for eight commercial sea cucumber species from the Fiji Islands[1]

2.1 Introduction

Sea cucumber fisheries are an important source of income for coastal communities in the Pacific (Conand, 1990a). Sea cucumber are usually processed into a dried product called bêche-de-mer (*iriko* in Japanese, *hai – som* in Chinese or *trepang* in Indonesian) (Bumrasarinpai, 2006a; Ferdouse, 1999; McElroy, 1990) that is consumed as a delicacy and for perceived medicinal benefits (Bordbar et al., 2011; Esmat et al., 2013). The major markets for bêche-de-mer (BDM) are China, Hong Kong, Taiwan, Singapore and Malaysia (Ferdouse, 2004), and around 58 species of sea cucumber are commercially exploited as BDM in Asian markets (Conand, 1990a; Li, 2001; McElroy, 1990). The majority of these belong to the genera *Actinopyga*, *Bohadschia*, *Holothuria, Stichopus* and *Thelenota*, with Asian buyers particularly targeting species from the genus *Holothuria* (Li, 2001). The Sandfish, *Holothuria scabra* (Jaeger 1833), White Teatfish, *H. (Microthele) fuscogilva* (Cherbonnier 1980) and Black Teatfish, *H. (Microthele) whitmaei* (Bell 1887) are among the highest value species (Holland, 1994a) and well-dried 'A' grade product may command a price of $US 70-190 per kg depending on size and quality (McElroy, 1990; Purcell et al., 2012b). Papua New Guinea (PNG), the Solomon Islands, Australia and the Fiji Islands were the leading suppliers of BDM to Asian markets from the Pacific (Ferdouse, 2004) but a moratorium on the fishery has prevented supply from PNG since 2009 (Carleton et al.,

[1] Data from this chapter has been published as: Ram, R., Chand, R.V., Zeng, C., Southgate, P.C. 2016. Recovery rates for eight commercial sea cucumber species from the Fiji Islands. *Regional Studies in Marine Science* **8(1)**:59-64.

2013; Hair et al., 2016). Fiji currently exports around 243 tonnes of BDM per year (Carleton et al., 2012; Ram et al., 2016) composed of at least 27 species ranging from very high to low value species.

BDM processing entails an uncomplicated sequence of actions (Fig. 2.1) resulting in a product that is non-perishable if stored in dry, dark conditions. The BDM processing method currently used in the Fiji Islands was developed in the 1800s and has changed little since. Post-harvest steps include first boiling, slitting and gutting, second boiling, smoking and finally sun-drying (Purcell, 2014a; Ram et al., 2014a). Each step in this process contributes to the resulting quality of the final product which determines the suitability of processed products for Asian markets (Conand, 1990a; Purcell, 2014b; Sachithananthan et al., 1985; SPC, 1994), and their value (Ram et al., 2014b). Although these steps are uncomplicated, it requires continuous attention to obtain a high quality dry product of consistent quality. Failure to do so can result in reduced quality and value of the final product (Sachithananthan et al., 1985; SPC, 1994). Nevertheless, because of a general lack of equipment required to optimise BDM quality (e.g. kerosene burners, smoking sheds and drying amenities), BDM production in Fiji uses simple customary methods described above (Ram et al., 2014a; Seeto, 1999; SPC, 1994). Processing BDM may also involve a 'salting' step where a saline solution or coarse salt is used to draw water from sea cucumber tissues (Lavitra et al., 2008) to facilitate dehydration and shrinking of the tissue.

About 60% of the sea cucumber body wall is composed of water (SPC, 1994) and most of this is lost during processing. The remainder is composed primarily of protein that accounts for the high

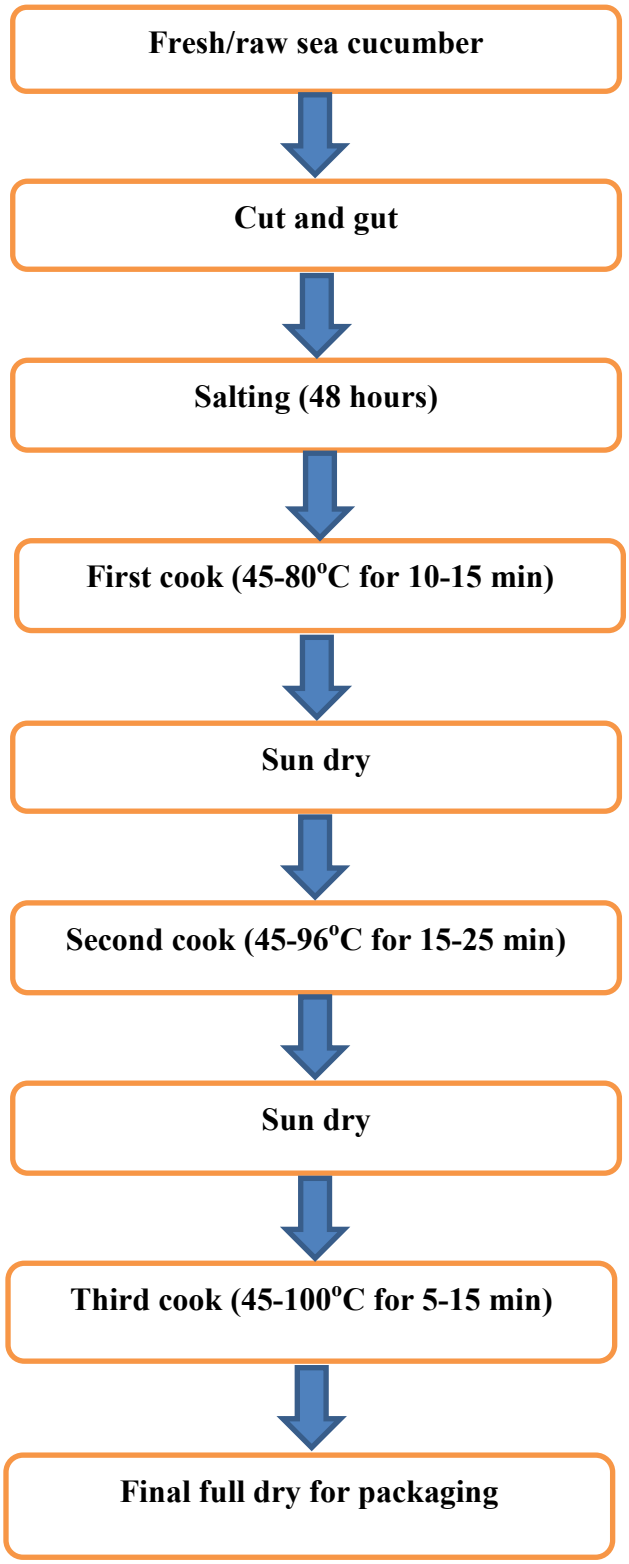

Figure 2.1. Steps used by processors in Fiji for production of bêche-de-mer (BDM) from fresh sea cucumbers.

protein content of BDM (Dong et al., 2011). Much of the protein content of the body wall of sea cucumbers, and resulting BDM, is composed of collagens; structural proteins that provide sea cucumbers with their body shape and form, and assist during feeding, respiration, burrowing and in defence (Yamada et al., 2010). If BDM processing includes salting, salt soluble proteins are generally leached from the tissues during this process, but salt also enters the tissues and binds to the triple-helix collagen structure (Duerr and Dyer, 1952; Gómez-Guillén et al., 2011), where it contributes to the weight of the final BDM product (Bao et al., 2010; Dong et al., 2008; Dong et al., 2011), helps minimize weight and length loss during processing (Lavitra et al., 2008), protects from spoilage and prolongs shelf life.

Yield of BDM is generally expressed as a 'recovery rate' that determines the relationship (usually percentage) between fresh weight of sea cucumbers and the dry weight of resulting BDM (Skewes et al., 2004). Recovery rates vary between species and this has implications for fisheries management. The Fijian sea cucumber fishery, for example, is in decline (Pakoa et al., 2013) and there is a push towards stricter management (Ram et al., 2016). Fiji's only management mechanism for this fishery is a minimum length of 7.63 cm (or 3 inches) prescribed for BDM for export (Carleton et al., 2012; Pakoa et al., 2013; Ram et al., 2016), regardless of species. Because different commercial sea cucumber species have different shrinkage rates during processing, previous studies have suggested modification of fisheries management for sea cucumbers in Fiji to include species-specific minimum harvest size limits (Purcell et al., 2009b; Seeto, 1999; Skewes et al., 2004; Vuki and Viala, 1989). However, the data on which such species-specific management protocols for sea cucumbers in the Pacific could be based are limited to a small number of reports in regional bulletins and unpublished sources (Ngaluafe and

Lee, 2013; Purcell et al., 2009b; Shelley, 1981; Vuki and Viala, 1989), and information in the primary literature on recovery rates for the highest value commercial species from the Pacific (Sandfish, *Holothuria scabra*) is limited to studies in Papua New Guinea (Shelley, 1985) and northern Australia (Skewes et al., 2004). Reported recovery rates for *H. scabra* in both studies are based on mass, not length, providing limited application in Fiji where the major criterion defining the suitability of BDM for export is minimum length.

There is therefore an immediate need for research into the processing yield of the major species of sea cucumbers utilized for BDM production in Fiji. The data generated would provide a basis for developing more focused fishery management protocols where, for example, species-specific minimum harvest sizes could be determined to ensure that resulting BDM is of an appropriate length for export. Such data would assist responsible fisheries agencies in obtaining more accurate estimates of the fresh weight of sea cucumbers processed by fishers and exporters (Skewes et al., 2004), and facilitate enforcement of size limits for harvest. The aim of this study was to determine both mass-based and length-based recovery rates of eight high-value to medium-value commercial species from Fiji's sea cucumber fishery.

2.2 Materials and Methods

This study was carried out with a commercial seafood processor in the Fiji Islands and live sea cucumbers were supplied to the processor by local fishermen. The recovery rates of eight sea cucumber species; Surf Redfish (*Actinopyga mauritiana*, n=77), Hairy Blackfish (*Actinopyga miliaris*, n=28), Stonefish (*Actinopyga lecanora*, n=73), Tigerfish (*Bohadschia argus*, n=117), White Teatfish (*Holothuria fuscogilva*, n=10), Sandfish (*Holothuria scabra*, n=51), Black

Teatfish (*Holothuria whitmaei*, n=46) and Prickly Redfish (*Thelenota ananas*, n=19), were determined in this study. These species represent the most important commercial species harvested for BDM export in Fiji. Mean length and weight of each species prior to processing is shown in Table 2.1.

Sea cucumbers were processing using the method normally used by BDM processors in Fiji as outlined by Purcell (2014a) and represented in Fig. 2.1. Briefly, sea cucumbers were laid on a table for approximately five minutes to allow them to relax before the length and weight of each was determined to the nearest 10 g. Individual sea cucumbers were than gutted and the viscera removed before length and weight were determined for a second time. The sea cucumbers were then cooked for 10-15 min. (at 45^{o}C rising to 80^{o}C) and cooled before being salted using a coarse 'grade 11' solar salt for 48 h. After salting the sea cucumbers were again measured and weighed before being cooked for the second time for 15-25 min. (at 45^{o}C rising to 96^{o}C) and then solar dried for 2 to 3 weeks. A third cook for 5-15 min. (at 45^{o}C rising to boiling) was then followed by shape correction (straitening and closure of body cut) to assist market acceptability, before the product was finally dried using solar and oven drying, then weighed. Approximately one kilogram of sea cucumbers are processed for $AUD 20.00.

Recovery rate for length (RRL) and recovery rate for weight (RRW) were calculated for each species as:

RRL = *Mean length after processing/mean fresh length x 100*

RRW = *Mean weight after processing/mean fresh weight x 100*

2.3 Results

Changes in the mean lengths and weights of the eight species of sea cucumbers after each of the main processing stages (fresh, gutted, salted and fully dried) are shown in Table 2.1, and RRL and RRW for dried product from all species are shown in Fig. 2.2 Weight recovery after gutting was around 95% for all species, while length recovery ranged from 94.4% for Prickly Redfish to 99.3% for Tigerfish at the same stage. Weight recovery after salting ranged from 20.7% for Hairy Blackfish to 73.7% for Sandfish, while length recovery after salting ranged from 58.9% for Hairy Blackfish to 99.4% for Tigerfish (Table 2.1). RRL of fully dried BDM ranged from 32.6% for Sandfish to 54.9% for Black Teatfish while RRW ranged from 3.0% for Tigerfish to 11.0% for Black Teatfish (Fig. 2.2). Biometric relationships between length and weight of the eight target species at different points in the processing sequence are shown in Table 2.2.

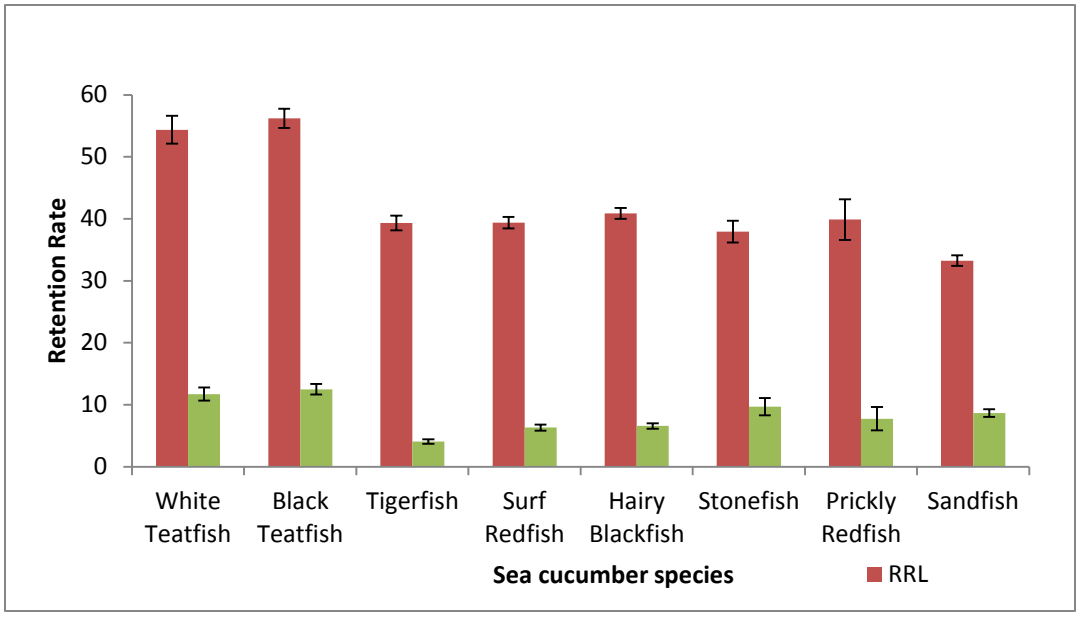

Figure 2.2. Retention rate for length and weight of eight BDM species of commercial value

Table 2.1. Mean (± SD) length (L, cm) and weight (W, g) of eight commercial sea cucumber species after each of the main stages (Fresh, Gutted, Salted, Fully Dried) of processing to bêche-de-mer (BDM) in Fiji. Weight losses as a proportion of prior processing stage weight (%) are shown in brackets.

Species	**Length (L) and Weight (W)**				
	Fresh		**Gutted**	**Salted**	**Fully dried**
Actinopyga lecanora (Stonefish)	L	24.55 ± 4.85	24.18 ± 4.51	15.93 ± 2.81	9.09 ± 3.35
	W	709.45 ± 181.42	673.98 ± 172.35 (95%)	224.04 ± 74.08 (33%)	67.17 ± 74.97 (30%)
Actinopyga mauritiana (Surf Redfish)	L	25.12 ± 4.61	24.12 ± 4.25	16.08 ± 1.95	9.68 ± 1.69
	W	635.39 ± 205.24	603.62 ± 194.98 (95%)	254.16 ± 74.30 (42%)	36.05 ± 18.03 (14%)
Actinopyga miliaris (Hairy Blackfish)	L	29.29 ± 3.03	28.18 ± 2.95	17.25 ± 2.82	11.86 ± 0.76
	W	1079.00 ± 287.35	1025.05 ± 272.98 (95%)	223.46 ± 131.22 (22%)	67.89 ± 22.29 (30%)
Bohadschia argus (Tigerfish)	L	30.85 ± 5.38	30.63 ± 5.20	30.50 ± 8.49	11.81 ± 3.45
	W	1016.57 ± 569.89	965.74 ± 541.40 (95%)	435.64 ±250.07 (45%)	30.49 ± 21.69 (7%)
Holothuria fuscogilva (White Teatfish)	L	36.40 ± 2.42	35.40 ± 2.80	21.75 ± 4.37	19.70 ± 2.04
	W	1532.00 ± 763.67	1455.40 ± 25.49 (95%)	694.70 ± 235.37 (48%)	162.12 ± 40.33 (23%)
Holothuria scabra (Sandfish)	L	18.60 ± 3.03	18.22 ± 2.94	12.18 ± 1.36	6.06 ± 0.82
	W	128.95 ± 45.29	122.50 ± 43.02 (95%)	95.02 ± 28.99 (78%)	10.40 ± 5.13 (11%)
Holothuria whitmaei (Black Teatfish)	L	27.93 ± 4.74	27.17 ± 4.51	23.35 ± 4.24	15.34 ± 1.99
	W	1084.24 ± 367.18	1030.03 ± 48.82 (95%)	570.67 ± 150.37 (55%)	119.06 ± 36.24 (21%)
Thelenota ananas (Prickly Redfish)	L	50.68 ± 12.22	47.84 ± 10.51	45.74 ± 8.03	18.80 ± 3.59
	W	2145.53 ± 737.43	2038.25 ± 700.56 (95%)	1136.32 ± 275.79 (56%)	139.87 ± 121.81 (12%)

Table 2.2. Biometric relationships for eight species of fresh and processed of sea cucumbers from Fiji where FL = length of fresh sea cucumber (cm), FW = weight of fresh sea cucumber (g), GW = gutted weight of sea cucumber (g), DL = length of dried sea cucumber (cm) and DW = weight dried sea cucumber (g).

Species	**X**	**Y**	**R^2**	**Equation**
Actinopyga lecanora	FL	FW	0.3557	$Y = 0.0159X + 13.246$
	FL	GW	0.3557	$Y = 21.214X + 153.21$
	FL	DW	0.0159	$Y = 1.9527X + 19.231$
	FL	DL	0.0572	$Y = 0.1652X + 5.0356$
	DL	DW	0.8415	$Y = 0.041X + 6.3399$
Actinopyga mauritiana	FL	FW	0.1165	$Y = 0.0077X + 20.241$
	FL	GW	0.1165	$Y = 14.419X + 241.45$
	FL	DW	0.0721	$Y = 1.0493X + 9.6904$
	FL	DL	0.1192	$Y = 0.1268X + 6.4982$
	DL	DW	0.7621	$Y = 0.0821X + 6.7252$
Actinopyga miliaris	FL	FW	0.3478	$Y = 0.0062X + 22.567$
	FL	GW	0.3478	$Y = 53.067X - 529.06$
	FL	DW	0.2021	$Y = 3.3029X - 28.843$
	FL	DL	0.0459	$Y = 0.0539X + 10.286$
	DL	DW	0.4024	$Y = 0.0217X + 10.391$
Bohadschia argus	FL	FW	0.4037	$Y = 0.006X + 24.757$
	FL	GW	0.4037	$Y = 63.939X - 1007.1$
	FL	DW	0.0084	$Y = 0.3692X + 19.098$
	FL	DL	0.0088	$Y = 0.0602X + 9.9564$
	DL	DW	0.4988	$Y = 0.1125X + 8.3856$
Holothuria fuscogilva	FL	FW	0.2332	$Y = 0.0015X + 34.059$
	FL	GW	0.2332	$Y = 144.97X - 3821.6$
	FL	DW	0.0003	$Y = 0.2675X + 152.38$

Table 2.2 continued next page

	FL	DL	0.0007	$Y = -0.0223X + 20.51$
	DL	DW	0.2037	$Y = 0.0228X + 15.999$
Holothuria scabra	FL	FW	0.3016	$Y = 0.0368X + 13.859$
	FL	GW	0.3016	$Y = 7.7969X - 22.502$
	FL	DW	0.0758	$Y = 0.4658X + 1.7355$
	FL	DL	0.0335	$Y = 0.0494X + 5.1452$
	DL	DW	0.7695	$Y = 0.1401X + 4.6079$
Holothuria whitmaei	FL	FW	0.0678	$Y = -0.0034X + 31.582$
	FL	GW	0.0678	$Y = -19.153X + 1565.1$
	FL	DW	0.1514	$Y = 2.973X + 36.009$
	FL	DL	0.0809	$Y = 0.1194X + 12.005$
	DL	DW	0.4404	$Y = 0.0364X + 11$
Thelenota ananas	FL	FW	0.4438	$Y = 0.011X + 27.004$
	FL	GW	0.4438	$Y = 38.199X + 102.17$
	FL	DW	0.0047	$Y = -0.6816X + 174.42$
	FL	DL	0.0172	$Y = -0.0386X + 20.755$
	DL	DW	0.6043	$Y = 0.0229X + 15.595$

2.4 Discussion

This study determined mass-based and length-based recovery rates of eight high-value to medium-value species from Fiji's sea cucumber fishery after processing to BDM. Our results show that recovery rates varied between the eight species and were species-specific. Table 2.3 shows the recovery rates reported in this study and compares them with those of prior relevant studies for the same target species.

The most valuable species from the Fijian sea cucumber fishery is Sandfish which has a broad Indo-Pacific range and is the focus of research to develop commercial scale mariculture enterprises in east Africa, Vietnam, Philippines, Papua New Guinea and the Pacific islands (Hair et al., 2012). Given the importance of this high value species it is surprising that RRL has not been reported prior to this study. The RRW for Sandfish in this study was 8.1%, and this is higher than previously reported values of 5.0% (Conand, 1979) and 5.1% (Skewes et al., 2004). BDM processed from Sandfish earns around $US115-1,668 per kg in S.E. Asian markets (Purcell et al., 2012b). This high value has resulted in over-exploitation by the Fijian sea cumber fishery and, in 1989, the Fijian Government imposed a complete ban on the export of this species that is still in place. The ban applies to both fresh and dried products (Ram et al., 2016).

The second most valuable sea cucumber species in Fiji, White Teatfish, had a RRL of 54.1% in this study which is slightly higher than that of 53% reported by Vuki and Viala (1989) and 51% reported by (Shelley, 1981). Weight recovery for this species was 10.6% after processing in this study which is again slightly more than the values previously reported for this species of 9.8%

Table 2.3. Recovery rates based on weight (RRW) and length (RRL) for tropical sea cucumbers from the Fiji Islands from this study and from previous studies targeting the same species.

Species	Common Name	RRW (%)	RRL (%)	Author(s)
Actinopyga lecanora	Stonefish	17.2	-	Ngaluafe and Lee (2013)
Actinopyga lecanora	Stonefish	9.5	37	This study
Actinopyga mauritiana	Surf Redfish	6.7	44	Zoutendyk (1989)
Actinopyga mauritiana	Surf Redfish	4.9	46	Vuki and Viala (1989)
Actinopyga mauritiana	Surf Redfish	5.7	38.5	This study
Actinopyga miliaris	Hairy Blackfish	5.6	-	Harriot (1984)
Actinopyga miliaris	Hairy Blackfish	9.7	52	Vuki and Viala (1989)
Actinopyga miliaris	Hairy Blackfish	11.5	-	Skewes (2004)
Actinopyga miliaris	Hairy Blackfish	6.3	40.5	This study
Bohadschia argus	Tigerfish	14.3	-	Ngaluafe and Lee (2013)
Bohadschia argus	Tigerfish	3.0	38.3	This study
Holothuria fuscogilva	White Teatfish	7.6	51	Shelley (1981)
Holothuria fuscogilva	White Teatfish	9.8	53	Vuki and Viala (1989)
Holothuria fuscogilva	White Teatfish	18.6	-	Ngaluafe and Lee (2013)
Holothuria fuscogilva	White Teatfish	10.6	54.1	This study
Holothuria scabra	Sandfish	5.0	-	Conand (1979)
Holothuria scabra	Sandfish	5.1	-	Skewes (2004)
Holothuria scabra	Sandfish	8.1	32.6	This study
Holothuria whitmaei	Black Teatfish	8.7	-	Harriot (1984)
Holothuria whitmaei	Black Teatfish	9.8	44	Shelley (1981)
Holothuria whitmaei	Black Teatfish	8.1	55	Vuki and Viala (1989)
Holothuria whitmaei	Black Teatfish	11.6	-	Purcell (2009)
Holothuria whitmaei	Black Teatfish	11.0	54.9	This study
Thelenota ananas	Prickly Redfish	3.0	-	Harriot (1984)
Thelenota ananas	Prickly Redfish	4.6	38	Shelley (1981)

Table 2.3 continued next page

Thelenota ananas	Prickly Redfish	5.6	36	Vuki and Viala (1989)
Thelenota ananas	Prickly Redfish	8.0	-	Parrish (1978) cited in Conand (1990)
Thelenota ananas	Prickly Redfish	6.7	-	Skewes (2004)
Thelenota ananas	Prickly Redfish	5.1	-	Purcell (2009)
Thelenota ananas	Prickly Redfish	6.5	37.1	This study

(Vuki and Viala, 1989) and 7.6% (Shelley, 1981) (Table 2.3). Given the high value of White Teatfish, and the export moratorium for Sandfish, the former is now preferentially targeted by the Fijian sea cucumber fishery (Ram et al., 2016).

Black Teatfish is a relatively high value species that has been the subject of a number of prior similar studies to determine sea cucumber recovery rates. Our results show that the RRL of Black Teatfish was 54.9% and this is similar to the RRL values reported for this species in prior studies that range from 55%-44% (Shelley, 1985; Vuki and Viala, 1989) (Table 2.3). The RRW of Black Teatfish in this study was 11.0% and this is also similar to the value of 11.6% reported for this species by (Purcell et al., 2009b), but less than values of 8.1-9.8% reported in some prior studies (Shelley, 1985; Vuki and Viala, 1989).

Prickly Redfish lost 93.5% of its fresh weight during processing with a RRW of only 6.5% after final drying. Similarly, length was reduced by 62.9% during processing resulting in a RRL of only 37.1% and this value compares well to those of 38% and 36% reported for this species by Shelley (1981) and Vuki and Viala (1989). Prior studies have also reported low RRW values for Prickly Redfish of 3.0% (Harriott, 1984), 4.6% (Shelley, 1981), 5.6% (Vuki and Viala, 1989), 8.0% (Parrish 1978; cited in Conand 1990), 6.7% (Skewes et al., 2004) and 5.1% (Purcell et al., 2009b) (Table 2.3).

The Surf Redfish had RRL of 38.5% and RRW of 5.7% in this study. Our results for RRL are slightly less than those reported in prior studies of 46% (Vuki and Viala (1989) and 44% (Zoutendyk, 1989) and possibly result from differences in the processing methods between

studies. The RRW of Surf Redfish in the current study (5.7%) was slightly higher than that of 4.9% reported by (Vuki and Viala, 1989), but lower than that of 6.7% reported by Zoutendyk (1989) (Table 2.3). Surf Redfish has a skin thickness of around 6 mm that renders this species susceptible to significant moisture loss during processing.

When comparing the results of this and prior studies, some noticeable differences are apparent between our values for RRW for Stonefish and Tigerfish and those of others. Ngaluafe and Lee (2013) reported RRW values for Stonefish and Tigerfish of 17.2% and 14.3%, respectively, while equivalent values in the current study were 9.5% and 3.0%, respectively. The reason for these differences is unclear but they are likely to be influenced by factors including differences in initial sea cucumber size, variations in processing techniques (e.g. method and duration of drying), when measurements were taken, ambient conditions and the degree of BDM dryness (Skewes et al. 2004; Purcell et al. 2009). In this particular case, Ngaluafe and Lee (2013) mentioned that some of the fresh sea cucumbers used in their study, including Stonefish, had already eviscerated prior to the start of the study and so accurate determination of initial fresh weight was not possible under these circumstances.

Fiji's only management mechanisms for the sea cumber fishery is a minimum length of 7.63 cm prescribed for BDM for export (Carleton et al., 2012; Pakoa et al., 2013; Ram et al., 2016), regardless of species, and a moratorium on the export of Sandfish. Regulations relating to BDM export have not been revised in the past 25 years despite a number of studies suggesting a need for further research that would provide a basis for such revision. A key issue in this regard is improved knowledge of the reproductive biology of target species (i.e. knowledge of minimum

reproductive size), to provide a basis for replacing the current 'catch-all' export size limit with species-specific minimum harvest sizes that account for size at maturity (Ram et al., 2016). Another key research issue is improved knowledge of the recovery rates of target species following processing to BDM (Carleton et al., 2013; Pakoa et al., 2013; Ram et al., 2014a, b; Ram et al., 2014c; Skewes et al., 2004), and this is addressed in the current study.

This study reports on the length-based and weight-based recovery rates of eight key species from the Fijian sea cucumber fishery for the first time. The data generated provide a crucial source of information for sea cucumber fishery management in Fiji and for developing policy relating to quality control for BDM exports. We report length-based and weight-based relationships through the various stages of processing from fresh sea cucumbers to dried BDM. These relationships allow conversion between processing stages and estimation of initial fresh weight/length from partially of fully processed BDM or estimation of BDM yields from fresh weight measurements. These data will have application in stock assessment studies, estimation of harvest data, monitoring of harvest size limits and standardizing catch data, and provide a basis for developing more species-specific harvest size restrictions for sea cucumbers in the Fiji Islands.

The Sea Cucumber Fishery Act in Fiji has not been revised since it was implemented in 1984 and, in contrast to some other South Pacific countries, the Fijian sea cucumber fishery has not been closed, even temporarily, in response to over-harvesting (Ram et al., 2016). However, the Fijian government is currently drafting the Fiji Sea Cucumber Management Plan (Mangubhai et al., 2016) that should be completed in 2017. The Plan will outline future management strategies for the sea cucumber fishery in Fiji that will hopefully incorporate recommendations from prior

reviews of the fishery (e.g. Carleton et al. (2012); Pakoa et al. (2013); Ram et al. (2016)), and utilise the data generated in this study as a basis for developing species-specific management strategies supporting sustainability in the future fishery.

Chapter 3

Effect of different processing techniques on proximate and fatty acid composition of bêche-de-mer produced from the tropical sea cucumber, *Holothuria scabra*[2].

3.1 Introduction

Sea cucumbers are typically processed into a dried product called bêche-de-mer (*iriko* in Japanese, *hai–som* in Chinese or *trepang* in Indonesian) (Bumrasarinpai, 2006a; Ferdouse, 1999; McElroy, 1990) that is consumed throughout south-east Asian as a delicacy and for a broad range of perceived medicinal benefits (Bordbar et al., 2011; Esmat et al., 2013), where well-dried 'A' grade product may command $US 70-190 kg^{-1} depending on size and quality (Purcell et al., 2012b). BDM processing entails an uncomplicated sequence of actions (Fig. 3.1) resulting in a product that is non-perishable if stored in dry, dark conditions. The BDM processing method was developed in the 1800s and has changed little since. Traditional post-harvest steps include first boiling, slitting and gutting, second boiling, smoking and finally sun-drying (Chapter 2) (Purcell, 2014a; Ram et al., 2014a). Each contributes to the resulting quality of the final product which determines the suitability of processed products for Asian markets (Conand, 1990a; Purcell, 2014b; Sachithananthan et al., 1985; SPC, 1994) and their value (Ram et al., 2014b). Processing BDM may also involve a 'salting' step (Fig. 3.1) that uses salt to draw water from sea cucumber tissues (Lavitra et al., 2008) in order to facilitate dehydration and shrinking. Salting can be achieved by immersing sea cucumber tissue in a brine solution (brining), or by dry salting where

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the tissue is covered in coarse salt and developing brine is allowed to drip away (kenching) (Chapter 4) (Sampels, 2015). If BDM processing includes salting, salt soluble proteins are generally leached from the tissues during the process, while salt enters the tissues and binds to the triple-helix collagen structure (Duerr and Dyer, 1952; Gómez-Guillén et al., 2011), where it contributes to the weight of the final BDM product (Bao et al., 2010; Dong et al., 2008; Dong et al., 2011), protects the product from spoilage and prolongs shelf life.

Sea cucumbers are regarded as a high quality seafood (Gómez-Guillén et al., 2011). Previous studies have shown that sea cucumber products are rich in protein, with an average composition of 40-70% (dry weight) depending on species (Haider et al., 2015; Omran, 2013; Wen et al., 2010). Sea cucumber tissue is also low in fat (ca. 2%) but contains fatty acids considered essential for human well-being, including eicosapentaenoic acid (EPA; 20:5n-3), docosahexaenoic acid (DHA; 22:6n-3) and arachidonic acid (AA; 20:4n-6, AA) (Bordbar et al., 2011) that have been associated with a reduced risk of coronary heart disease and cancer in humans (Harper and Jacobson, 2005; Roynette et al., 2004). Sea cucumber tissue is also known for containing high levels of essential amino acids and essential minerals (Bordbar et al., 2011; Pasig et al., 2006; Wen and Hu, 2010; Wen et al., 2010), and is considered to have broad medicinal value (Bordbar et al., 2011).

As BDM undergoes multi-step processing, potentially including multiple cooking, salting and drying steps (Fig. 3.1), the texture and nutrient composition of the product is likely to be modified during processing. For example, the body wall of sea cucumbers contains a high proportion of collagen (Chen et al., 2015a; Zhong et al., 2015) that has a major influence on BDM firmness and

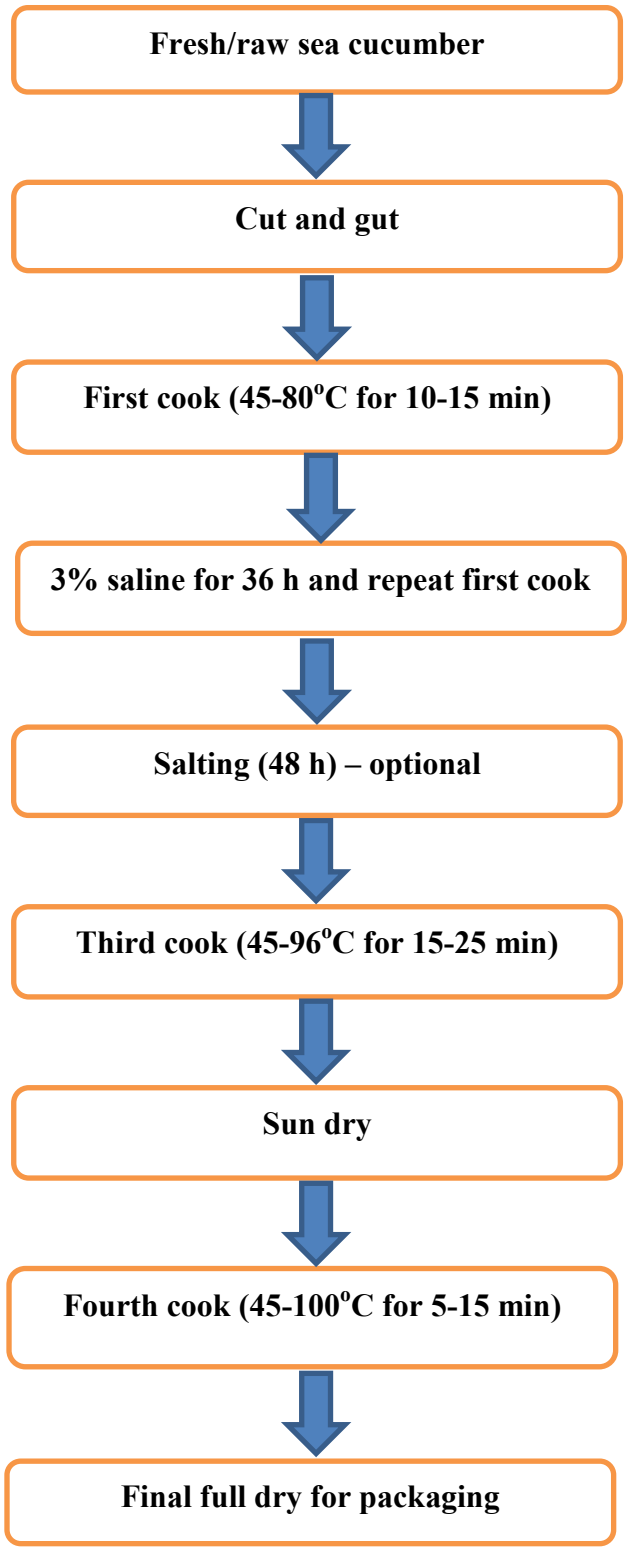

Figure 3.1. Generalised sequence for production of bêche-de-mer (BDM) from fresh sea cucumbers specific to sandfish processing

texture quality, yet collagen composition and structure is influenced by both cooking temperature and cooking duration during BDM processing (Dong et al., 2011). Amino acids impart flavour to seafood products but can rapidly leach from tissues when immersed in liquid (Bremner, 2012), while a loss of micronutrients is likely to be accelerated when immersed in a salt or brine solution as commonly occurs during BDM processing (Fig. 3.1). Furthermore, heating may have a significant negative impact on the fatty acid composition of seafood (Bhouri et al., 2010) and long-chain polyunsaturated fatty acids can rapidly oxidize to reduce the content of 'beneficial' nutrients and potentially produce toxic by-products (Chow, 2008). A number of studies have reported the nutrient composition of both fresh (Haider et al., 2015; Lee et al., 2012; Omran, 2013) and processed (Ozer et al., 2004; Wen et al., 2010) sea cucumbers, but only one prior study has assessed the effects of variations in processing method on nutrient composition of resulting BDM (Chapter 4).

Although consumers in Western countries are well aware of the significant nutritional and health benefits of seafood associated with 'omega-3 fatty acids' and lean protein (Olsen, 2004; Pieniak et al., 2008), the major factors affecting the quality and value of BDM (i.e. species used, appearance, odour, colour, thickness of the body wall and market demand), do not include nutrient composition and nutritional quality (Wen et al., 2010). This is perhaps the reason that interest in the nutrient compositions of both fresh sea cucumbers (e.g. Svetashev et al. (1991); Drazen et al. (2008) and BDM (e.g. Wen et al. (2010); Bechtel et al. (2012) is a relatively recent development. Despite this, 'nutrition and health' is a major motivation for consuming seafood in China (Fabinyi et al., 2016) and, with increasing awareness, the nutrient content of BDM may become an increasingly important consideration in S.E. Asian markets. On this basis there is

scope to develop processing methods for sea cucumbers that optimize both physical and nutritional characteristics of BDM.

The aim of this study was to determine the proximate composition and fatty acid profile of fresh tropical sea cucumbers, *Holothuria scabra*, and BDM processed from this species for the first time. To generate a greater understanding of the influence of processing method on BDM composition, this study also determined the effects of salting method, using brine and kench salting, and the influence of brine strength and kenching duration, on BDM composition.

3.2 Materials and Methods

3.2.1 Sample collection and processing

Holothuria scabra were collected from Tavua Bay on the north coast of the main island of Viti Levu, Fiji Islands (17°26'29.4"S 177°51'44.4"E). After collection, they were left for 5 min before length and weight was determined. Individuals were gutted and left in an insulating fish box containing ice. A total of 30 individuals were collected and subsamples of three randomly selected sea cucumbers were each subjected to nine different processing treatments. All sea cucumbers were first cooked at a water temperature of 45^{o}C for 10 min before the water temperature was gradually increased to 80^{o}C (Fig. 3.1). Sea cucumbers were cooked for a further 10 min until they were hard and springy indicating the completion of the first cook. Cooked sea cucumbers were then immersed in a 3% (w/v) saline solution for 36 h to allow the outer layer of spicules to disintegrate (Purcell, 2014a). Sea cucumbers with ossicles removed were then subject to seven different salting treatments using grade 11 coarse solar salt (Table 3.1):

Table 3.1. Chemical composition of the grade 11 coarse solar salt used in this study.

Component	**Specifications**	**Typical**
Sodium Chloride as NaCl	Min 99.5%	99.7%
Moisture Content	Max 2.5%	1.8%
Matter Insoluble in water	Max 0.1%	0.015%
Sulphate as Na_2SO_4	Max 0.30%	0.11% (1100 mg kg^{-1})
Calcium as Ca	Max 0.06%	0.04% (400 mg kg^{-1})
Magnesium as Mg	Max 0.04%	0.003% (30 mg kg^{-1})
Arsenic as As	Not specified	<0.1 mg kg^{-1}
Copper as Cu	Max 2 mg kg^{-1}	<0.5 mg kg^{-1}
Lead as Pb	Not specified	<0.1 mg kg^{-1}
Iron as Fe	Max 10 mg kg^{-1}	2.0 mg kg^{-1}
Potassium as K	Not specified	100 mg kg^{-1}
Anticaking Agent as $Na_4[Fe(CN)_6]$	Max 50 mg kg^{-1}	0 - 8 mg kg^{-1}

1) Kenching for 24 h prior to further processing;
2) Kenching for 36 h prior to further processing;
3) Kenching for 48 h prior to further processing;
4) Kenching for 72 h prior to further processing;
5) Immersion in a 15% w/v saline solution for 48 h prior to further processing;
6) Immersion in a 18% w/v saline solution for 48 h prior to further processing; and
7) Immersion in a 25% w/v saline solution for 48 h prior to further processing.

After salting the sea cucumbers were cooked a second time for 15-25 min (at 45^{o}C rising to 96^{o}C) and were then solar dried for at least 2 to 3 weeks. Any salt crystals that formed on the surface of the *H. scabra* during the drying period were washed off. After 2 to 3 weeks, a third cook for 5-15 min (at 45^{o}C rising to boiling) was then followed by shape correction (straitening and closure of body cut) to assist market acceptability, before the product was finally dried using solar drying. Sea cucumbers in two other treatments were not salted and served as controls. Following removal

of the ossicles, these sea cucumbers were not salted, but proceeded directly to the second cooking step and were then dried as follows:

8) Solar dried; and
9) Smoke dried using smoke machine (Dish–100–DX, Ikeden Seisakusho Co. Ltd, Japan) with an exhaust fan for 48 h at 45^{o}C using wood chips to generate the smoke.

3.2.2 Proximate analysis

Proximate analysis of dried BDM from each of the treatments was conducted using standard procedures (AOAC, 1990). Moisture was determined by oven drying the sea cucumber samples at a temperature of 105^{o}C until a constant weight was obtained. Protein content was determined according to the Kjeldahl method (crude protein calculated as nitrogen x 6.25) in an automated Kjeltech (Tecator, Sweden). Total lipid content was estimated according to the modified version of Folch et al. (1957). Dry samples were weighed then sonicated (Vibracell, Sonics and Materials, Newton, USA) for 10 min in a 2 mL aliquot of dichloromethane:methanol (CH_2Cl_2:CH_3OH). This mixture was then filtered and the solid residue resuspended and sonicated for a further 10 min with another 2 mL aliquot of CH_2Cl_2:CH_3OH followed by a further filtration step. This process was repeated three times. The combined filtrates (~6 mL) were then transferred into a separation funnel and combined with a 3.5 mL sample washing solution of KCl (0.44%) in H_2O/CH_3OH (3:1). The mixture was shaken vigorously and allowed to settle overnight. The following morning, the bottom layer contained the extracted lipid was recovered and the solvent was evaporated under nitrogen. The lipid content was then quantified on a micro-balance. Total ash was determined by incineration in a muffle furnace at 550^{o}C for 24 h.

3.2.3 Fatty acid analysis

Following extraction, fatty acids were esterified into methyl esters using the acid catalyzed methylation method (Christie, 2003), 100 µL of 23:0 (0.75 mg mL^{-1}) was added as an internal standard (Sigma-Aldrich, Inc., St Louis, MO, USA) alongside 2 mL of freshly prepared AcCl/MetOH (1:10) as the methylation catalyst. Sample vials were then sealed, shaken and placed in an oven at 100°C for 1 h. Once cool, 2 mL of K_2CO_3 (1 M) was added, followed by 3 mL of hexane to dissolve the fatty acids methyl esters. The sample was then centrifuged and the hexane supernatant recovered and placed in a gas chromatography (GC) vial for GC injection. Fatty acid methyl esters were isolated and identified using an Agilent Technologies 7890A GC System (Agilent Technologies; Santa Clara, CA, USA) equipped with a BPX70 capillary column (120 m x 0.25 mm internal diameter, 0.25 µm film thickness, SGE Analytical Science, Ringwood, VIC, Australia), a flame ionization detector (FID), an Agilent Technologies 7693 auto sampler, and a split injection system (split ratio 20:1). The injection volume was 1 µL and the injector and detector temperatures were 300°C and 270°C, respectively. The temperature program was 60°C held for 2 min, then from 60°C to 150°C at 20°C min^{-1}, and held at 150°C for 2 min, then from 150°C to 205°C at 1.5°C min^{-1}, then from 205°C to 240°C at 5°C min^{-1}, and held at 240°C for 24 min. The carrier gas was helium at 1.5 mL min^{-1}, at a constant flow. Each of the fatty acids was identified relative to known external standards (a series of mixed and individual standards from Sigma-Aldrich., Inc, St, Louis, MO, USA and from Nuchek Prep Inc., Elysian, MN, USA), using the software GC ChemStation (Rev B.04.03; Agilent Technologies; Santa Clara, CA, USA). The resulting peaks were then corrected by the theoretical relative FID response factors (Ackman, 2002) and quantified relative to the internal standard.

3.2.4 Statistical analysis

All analyses were conducted in triplicate. Results were expressed as means (± SE) and one-way analysis of variance (ANOVA) was carried out using SPSS version 24 statistical software. Differences in the levels of nutritional components of BDM across all treatments were tested with ANOVA followed by Tukey HSD test, and further post-hoc tests were performed to analyse differences in levels of key fatty acids within each of the two salting treatments. Differences between means were considered to be significant when $p < 0.05$. Pearson's correlation test was used to assess relationships between ash and protein content, and between ash and $\sum$ n-3 LC PUFA contents of BDM.

3.3 Results

Proximate data for fresh and processed *H. scabra* are shown in Table 3.2. Water made up 891.69 ± 4.01 mg g^{-1} of unprocessed *H. scabra* tissue, with protein, ash, and NFE making up 48.59 ± 5.96 mg g^{-1}, 43.62 ± 7.7 mg g^{-1}, and 14.67 ± 5.07 mg g^{-1}, respectively. Fresh *H. scabra* tissue contained a very low lipid content of 1.44 ± 0.29 mg g^{-1} and the major fatty acids of fresh *H. scabra* tissue were palmitic acid (16:0; 5.28 ± 1.19 mg g $lipid^{-1}$), stearic acid (18:0; 4.55 ± 0.72 mg g $lipid^{-1}$), arachidonic acid (20:4n-6; 5.10 ± 0.44 mg g $lipid^{-1}$), and EPA (20:5n-3; 3.62 ± 0.52 mg g $lipid^{-1}$) (Table 3.3). DHA made up only 0.21 ± 0.06 mg g $lipid^{-1}$ of fresh *H. scabra* tissue.

After processing using normal solar drying and smoke drying, the protein contents of resulting BDM were 845.90 ± 9.28 mg g^{-1} and 812.04 ± 7.73 mg g^{-1}, respectively, and these values did not

Table 3.2. Proximate composition of *Holothuria scabra* (fresh and processed). Means (± SE) are expressed in mg g^{-1} dry weight (n=3) and do not differ significantly if sharing the same superscript (P >0.05)

Treatment	Tissue Components				
	Protein	**Lipid**	**Ash**	**Moisture**	**NFE**
Fresh Tissue	48.59±5.96	1.44±0.29	43.62±7.70	891.69 ± 4.01	14.67±5.07
Cooked with ossicles removed	845.90±9.28^{c}	13.89±2.89^{a}	111.13±15.73^{a}	137.52 ± 17.13^{a}	29.08±9.03^{ab}
Smoke dry	812.04±7.73^{c}	15.85±1.38^{a}	133.53±5.77^{a}	190.67 ± 63.91^{a}	38.58±9.13^{ab}
Kenching -24 h	658.88±8.90^{ab}	10.22±0.33^{a}	311.87±11.37^{c}	127.00 ± 13.36^{a}	19.04±3.32^{a}
Kenching -36 h	683.25±8.98^{b}	13.59±0.71^{a}	271.61±13.47^{c}	122.00 ± 6.82^{a}	31.56±11.77^{ab}
Kenching -48 h	717.52±10.54^{b}	13.62±1.59^{a}	235.85±11.08^{bc}	134.84 ± 9.61^{a}	33.01±7.01^{ab}
Kenching -72 h	617.35±20.62^{a}	16.27±1.58^{a}	247.22±20.06^{bc}	156.33 ± 11.85^{a}	119.15±36.16^{b}
Brine -15%	713.93±11.98^{b}	15.45±0.85^{a}	181.95±15.42^{ab}	194.22 ± 60.49^{a}	88.67±23.59^{ab}
Brine -18%	660.37±2.74^{ab}	16.00±2.16^{a}	267.51±29.89^{c}	196.83 ± 49.91^{a}	56.12±31.39^{ab}
Brine -25%	674.86±21.91^{ab}	17.36±2.36^{a}	277.96±11.41^{c}	188.30 ± 11.36^{a}	29.81±17.10^{ab}

Table 3.3. Fatty acid profile of fresh and processed dried *Holothria scabra* body wall. Means (± SE) are expressed in mg g lipid^{-1} dry weight (n=3) and do not differ significantly if sharing the same superscript (P >0.05)

	Processing Treatments									
Fatty acid	**Fresh tissue**	**Cooked with ossicles removed**	**Smoke dry**	**Kenching 24 h**	**Kenching 36 h**	**Kenching 48 h**	**Kenching 72 h**	**Brine - 15%**	**Brine - 18%**	**Brine - 25%**
C6:0	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	0.23±0.11^{a}	0.51±0.15^{b}
C13:0	n.d	1.09±0.75^{a}	0.61±0.31^{a}	0.42±0.42^{a}	0.88±0.08^{a}	0.94±0.09^{a}	1.05±0.55^{a}	0.86±0.11^{a}	0.51±0.30^{a}	0.89±0.18^{a}
C14:0	1.64±0.50	6.96±2.91^{a}	8.76±0.94^{a}	8.64±0.88^{a}	12.71±2.00^{a}	10.41±1.56^{a}	8.31±0.45^{a}	12.46±1.69^{a}	7.94±0.38^{a}	7.28±0.28^{a}
C15:0	0.56±0.18	3.01±1.75^{a}	3.70±0.72^{a}	3.58±0.15^{a}	5.84±1.14^{a}	4.75±0.51^{a}	3.28±0.19^{a}	5.11±0.31^{a}	3.26±0.10^{a}	3.31±0.32^{a}
C16:0	5.28±1.19	24.66±11.75^{a}	32.53±4.82^{a}	30.33±0.78^{a}	38.88±3.52^{a}	32.69±3.49^{a}	30.16±2.22^{a}	41.52±4.13^{a}	26.99±1.01^{a}	29.21±0.10^{a}
C17:0	0.44±0.11	1.60±0.73^{a}	3.16±1.56^{a}	3.26±1.53^{a}	5.40±0.47^{a}	3.68±1.61^{a}	4.60±0.09^{a}	5.56±0.45^{a}	4.37±0.15^{a}	5.08±0.36^{a}
C18:0	4.55±0.72	27.78±6.91^{a}	30.71±4.26^{a}	27.78±1.15^{a}	32.50±3.15^{a}	28.37±0.93^{a}	25.95±2.07^{a}	32.27±3.38^{a}	23.53±1.27^{a}	25.82±1.23^{a}
C20:0	1.68±0.09	5.71±2.75^{a}	5.62±2.95^{a}	8.27±0.43^{a}	8.59±0.24^{a}	8.34±0.35^{a}	7.79±0.70^{a}	10.02±0.60^{a}	8.25±0.40^{a}	9.13±0.22^{a}
C21:0	1.02±0.06	4.82±0.67^{a}	4.06±0.40^{a}	4.51±0.37^{a}	4.96±0.24^{a}	4.49±0.08^{a}	4.56±0.41^{a}	4.83±0.03^{a}	4.79±0.18^{a}	5.08±0.12^{a}
C22:0	1.27±0.06	6.47±0.43^{a}	5.71±0.83^{a}	7.16±0.63^{a}	6.38±0.21^{a}	5.88±0.45^{a}	6.96±0.47^{a}	5.95±0.27^{a}	6.08±0.39^{a}	6.85±0.25^{a}
C24:0	0.23±0.03	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
C14:1n-5	0.42±0.12	2.32±1.02^{a}	2.99±0.84^{ab}	3.22±0.06^{ab}	5.70±0.38^{b}	4.98±0.90^{ab}	2.98±0.22^{ab}	5.34±0.71^{ab}	3.33±0.22^{ab}	3.26±0.42^{ab}
C15:1n-5	0.21±0.06	1.42±0.36^{ab}	1.31±0.26^{a}	1.55±0.03^{abc}	2.64±0.24^{c}	2.19±0.37^{abc}	1.50±0.07^{ab}	2.51±0.24^{bc}	1.63±0.09^{abc}	1.49±0.13^{ab}
C16:1n-7	2.66±0.62	11.00±6.51^{ab}	15.89±2.87^{b}	0.28±0.01^{a}	16.69±2.87^{b}	13.88±1.66^{ab}	6.83±3.31^{ab}	15.99±1.66^{b}	9.71±0.86^{ab}	9.17±0.45^{ab}
C17:1n-7	0.06±0.06	0.38±0.38^{a}	0.73±0.13^{a}	0.62±0.06^{a}	1.10±0.29^{a}	0.92±0.08^{a}	0.61±0.04^{a}	0.95±0.06^{a}	0.57±0.04^{a}	0.48±0.05^{a}
C18:1n-7	0.47±0.47	2.74±2.74^{a}	6.79±0.63^{a}	6.01±0.17^{a}	8.30±0.77^{a}	7.17±0.57^{a}	7.28±1.77^{a}	8.23±0.48^{a}	6.09±0.16^{a}	5.92±0.59^{a}
C18:1n-7 t	n.d	0.47±0.17^{a}	0.12±0.06^{a}	0.16±0.08^{a}	0.32±0.02^{a}	0.17±0.09^{a}	0.39±0.28^{a}	0.31±0.06^{a}	0.39±0.18^{a}	0.11±0.06^{a}
C18:1n-9	1.91±0.44	3.13±0.76^{a}	4.76±0.37^{a}	3.19±0.40^{a}	3.85±0.57^{a}	4.19±0.29^{a}	5.29±2.03^{a}	3.45±0.36^{a}	3.62±0.53^{a}	2.73±0.46^{a}
C18:1n-9 t	0.09±0.04	0.26±0.13^{a}	0.38±0.06^{a}	0.06±0.06^{a}	0.33±0.05^{a}	0.74±0.42^{a}	0.20±0.11^{a}	0.93±0.32^{a}	0.51±0.11^{a}	0.14±0.08^{a}
C20:1n-9	0.20±0.20	1.13±1.13^{ab}	3.31±0.21^{b}	n.d	2.40±0.22^{ab}	2.63±0.11^{ab}	2.03±1.12^{ab}	2.44±0.18^{ab}	2.40±0.13^{ab}	2.35±0.15^{ab}
C22:1n-11	0.37±0.00	2.19±0.38^{ab}	1.29±0.26^{a}	2.32±0.34^{b}	1.48±0.09^{ab}	1.87±0.12^{ab}	1.53±0.09^{ab}	1.52±0.04^{ab}	1.54±0.10^{ab}	1.81±0.00^{ab}
C22:1n-9	0.18±0.18	0.75±0.75^{a}	2.10±0.16^{a}	1.63±0.86^{a}	1.97±0.49^{a}	1.84±0.17^{a}	1.17±0.63^{a}	1.77±0.06^{a}	1.55±0.08^{a}	1.70±0.07^{a}

Table 3.3 continued next page

C24:1n-9	1.97±0.12	10.34±0.73^{a}	8.89±1.24^{a}	8.38±0.14^{a}	9.04±0.52^{a}	8.99±0.32^{a}	9.22±0.31^{a}	9.32±0.17^{a}	9.14±0.75^{a}	9.32±0.23^{a}
C18:3n-3	n.d	n.d	n.d	n.d	0.05±0.05^{a}	0.06±0.06^{a}	n.d	1.19±0.50^{b}	0.04±0.04^{a}	n.d
C18:4n-3	4.48±0.05	23.97±1.58^{ab}	18.66±1.35^{a}	20.21±0.69^{ab}	21.98±0.90^{ab}	24.30±1.01^{ab}	22.30±0.30^{ab}	23.20±0.70^{ab}	21.88±0.39^{ab}	25.21±2.37^{b}
C20:4n-3	n.d	n.d	0.10±0.10^{a}	n.d	n.d	n.d	0.29±0.29^{a}	n.d	0.15±0.15^{a}	n.d
C20:5n-3	3.62±0.52	14.45±1.21^{bc}	18.87±0.67^{c}	7.93±1.83^{a}	10.58±2.48^{ab}	9.93±0.56^{ab}	5.70±0.62^{a}	9.53±0.46^{ab}	7.26±0.84^{a}	7.67±1.19^{a}
C22:3n-3	n.d	0.42±0.42^{a}	1.25±0.65^{a}	1.66±0.16^{a}	1.81±0.06^{a}	1.51±0.20^{a}	1.64±0.33^{a}	1.53±0.05^{a}	1.67±0.29^{a}	1.55±0.06^{a}
C22:5n-3	0.15±0.01	0.49±0.04^{ab}	0.63±0.07^{ab}	0.48±0.03^{ab}	0.65±0.17^{ab}	0.89±0.26^{b}	0.27±0.05^{a}	0.51±0.14^{ab}	0.30±0.02^{a}	0.33±0.05^{ab}
C22:6n-3	0.21±0.06	0.49±0.27^{a}	1.32±0.38^{a}	0.48±0.24^{a}	0.84±0.21^{a}	0.83±0.31^{a}	0.40±0.12^{a}	1.01±0.12^{a}	0.38±0.05^{a}	0.38±0.07^{a}
C16:2n-4	1.23±0.01	2.05±0.08^{ab}	2.92±0.65^{b}	1.34±0.06^{a}	1.63±0.14^{a}	1.63±0.07^{a}	1.35±0.14^{a}	1.88±0.15^{ab}	1.72±0.10^{ab}	1.93±0.28^{ab}
C18:3n-4	n.d	n.d	0.13±0.07^{a}	0.09±0.09^{a}	0.13±0.13^{a}	0.07±0.07^{a}	n.d	n.d	n.d	n.d
C18:2n-6	0.56±0.08	3.27±0.62^{a}	3.78±0.52^{a}	3.54±0.11^{a}	2.59±0.61^{a}	3.80±0.74^{a}	3.43±0.38^{a}	3.03±0.73^{a}	3.12±0.62^{a}	3.66±0.26^{a}
C18:3n-6	0.06±0.03	0.59±0.23^{a}	0.46±0.09^{a}	0.41±0.04^{a}	0.50±0.07^{a}	0.48±0.06^{a}	0.34±0.02^{a}	0.80±0.08^{a}	0.40±0.03^{a}	0.46±0.09^{a}
C20:2n-6	0.49±0.05	2.78±0.10^{bc}	2.84±0.25^{c}	2.02±0.12^{a}	2.06±0.11^{ab}	2.29±0.02^{abc}	2.10±0.12^{abc}	2.32±0.09^{abc}	2.11±0.13^{abc}	2.29±0.23^{abc}
C20:3n-6	0.03±0.03	0.35±0.15^{a}	0.17±0.03^{a}	0.09±0.09^{a}	0.22±0.01^{a}	0.31±0.09^{a}	0.25±0.06^{a}	0.26±0.02^{a}	0.24±0.08^{a}	0.27±0.15^{a}
C20:4n-6	5.10±0.44	19.22±0.97^{ab}	24.08±1.48^{b}	16.81±1.53^{ab}	18.92±0.51^{ab}	20.97±1.09^{ab}	14.64±2.30^{a}	21.80±1.65^{ab}	17.34±1.43^{ab}	17.84±3.67^{ab}
C22:2n-6	0.03±0.03	0.20±0.10^{a}	0.28±0.02^{a}	0.24±0.12^{a}	0.29±0.04^{a}	0.30±0.03^{a}	0.07±0.07^{a}	0.27±0.01^{a}	0.29±0.06^{a}	0.17±0.10^{a}
C22:4n-6	0.21±0.11	0.80±0.08^{a}	1.11±0.26^{a}	0.63±0.10^{a}	0.78±0.04^{a}	0.92±0.05^{a}	0.57±0.12^{a}	0.95±0.04^{a}	0.90±0.21^{a}	0.73±0.15^{a}
C22:5n-6	0.31±0.03	1.15±0.15^{a}	1.77±0.34^{a}	1.11±0.23^{a}	1.34±0.19^{a}	1.44±0.08^{a}	0.98±0.21^{a}	1.39±0.04^{a}	1.39±0.12^{a}	1.17±0.22^{a}
∑Total Fatty acid	41.68±5.33	188.45±31.59^{a}	221.95±20.61^{a}	178.43±2.98^{a}	234.33±18.80^{a}	218.98±11.54^{a}	186.06±11.53^{a}	240.97±12.10^{a}	185.60±2.50^{a}	195.30±9.38^{a}
∑SFA	16.67±2.73	82.08±18.60^{a}	94.87±12.22^{a}	93.95±2.34^{a}	116.15±9.67^{a}	99.55±7.73^{a}	92.67±5.95^{a}	118.57±10.25^{a}	85.95±2.42^{a}	93.16±1.03^{a}
∑MUFA	8.17±1.68	33.95±12.78^{a}	47.36±5.76^{a}	25.12±1.13^{a}	52.33±5.32^{a}	47.70±3.72^{a}	37.79±8.36^{a}	51.24±3.74^{a}	39.09±1.17^{a}	36.67±1.64^{a}
∑PUFA	16.84±0.94	72.43±1.85^{ab}	79.72±2.69^{b}	59.37±3.93^{a}	65.85±4.34^{ab}	71.73±0.89^{ab}	55.60±3.84^{a}	71.16±2.92^{ab}	60.56±1.14^{ab}	65.47±8.77^{ab}
∑n-6 PUFA	6.47±0.36	27.22±1.04^{ab}	32.72±1.60^{b}	23.75±1.56^{ab}	25.35±0.98^{ab}	29.08±1.61^{ab}	21.42±2.39^{a}	29.42±2.02^{ab}	24.40±1.08^{ab}	25.42±4.65^{ab}
∑n-6 LC PUFA	6.17±0.42	24.51±1.25^{ab}	30.25±1.47^{b}	20.91±1.82^{ab}	23.61±0.86^{ab}	26.23±1.01^{ab}	18.63±2.66^{a}	26.98±1.61^{ab}	22.26±1.16^{ab}	22.47±4.52^{ab}
∑n-3 PUFA	8.83±0.63	42.01±0.72^{a}	42.02±2.61^{a}	33.08±2.13^{a}	37.40±3.31^{a}	39.41±0.50^{a}	31.85±1.15^{a}	38.48±1.08^{a}	33.07±0.73^{a}	36.95±3.62^{a}
∑n-3 LC PUFA	4.34±0.58	18.04±1.46^{bc}	23.37±1.35^{c}	12.87±2.35^{ab}	15.37±2.72^{ab}	15.05±0.88^{ab}	9.55±0.95^{a}	14.09±0.22^{ab}	11.15±0.65^{ab}	11.73±1.25^{ab}

differ significantly ($p>0.05$). Exposure of sea cucumbers to coarse solar salt (kenching) for up to 72 h and immersion in 15-25% brine solutions for 48 h resulted in a significant reduction in protein content of resulting BDM compared to control and smoke dry products (Table 3.2). BDM produced by kenching for 48 h (717.52 ± 10.54 mg g^{-1}) and by 15% brine salting (713.93 ± 11.98 mg g^{-1}) had the highest protein contents among the dry salted and brine salted treatments, respectively. There was a significant reduction in protein content when the duration of dry salting was increased from 48 h to 72 h.

The lipid contents of BDM from salting treatments did not differ significantly from that of BDM produced by normal solar drying (13.89 ± 2.89 mg g^{-1}) and smoke drying (15.85 ± 1.38 mg g^{-1}). Lipid contents of BDM produced by kenching ranged from 10.22 ± 0.33 mg g^{-1} (24 h salting) - 16.27 ± 1.58 mg g^{-1} (72 h salting), while those of BDM produced by brine salting ranged from 15.54 ± 0.85 mg g^{-1} (15% brine) - 17.36 ± 2.36 mg g^{-1} (25% brine) (Table 3.2). The ash contents of BDM from all salting treatments were significantly higher than those of BDM produced by normal solar drying (111.13 ± 15.73 mg g^{-1}) and smoke drying (133.53 ± 5.77 mg g^{-1}). Ash contents of BDM processed by kenching ranged from 235.85 ± 11.08 mg g^{-1} - 311.87 ± 11.37 mg g^{-1} but did not differ significantly between treatments (Table 3.2). In contrast, the ash content of BDM processed by brining with 15% brine (181.95 ± 15.42 mg g^{-1}) was significantly lower than the ash contents of BDM processed with 18% brine (267.51 ± 29.89 mg g^{-1}) and 25% brine (277.96 ± 11.41 mg g^{-1}). Pearson's correlation analysis showed that there was a non-significant relationship between ash and protein contents of BDM across treatments ($P>0.05$). Moisture contents of BDM from all treatments ranged from 122.00 ± 6.82 mg g^{-1} - 196.83 ± 49.91 mg g^{-1} and there were no significant differences between salted treatments and the normal solar dried

and smoke dried treatments (Table 3.2). Carbohydrate contents of BDM (reported as nitrogen-free extract, NFE) increased steadily in BDM processed with increasing duration of kenching from 19.04 ± 3.32 mg g^{-1} (24 h salting) - 119.15 ± 36.16 mg g^{-1} (72 h salting) and these values were significantly different ($p<0.05$; Table 3.2). The NFE contents of BDM from all other treatments, including the normal solar dried and smoke dried treatments, did not differ significantly.

The sum of total fatty acids (∑TFA), sum of saturated fatty acids (∑SFA) and sum of monounsaturated fatty acids (∑MUFA) did not differ significantly between the control, smoked and salted products (Table 3.3). However, BDM processed using 24 h and 72 h kenching had significantly lower levels of ∑PUFA than normal smoked dry BDM. The same was true for BDM processed using 72 h kenching with regard to ∑n-6 PUFA, ∑n-6 LC PUFA and ∑n-3 LC PUFA (Table 3.3). BDM processed using 72 h kenching had significantly lower levels of ∑n-3 LC PUFA than both smoked dry BDM and the control, and was the only salting treatment to differ from the control in this regard (Table 3.3).

The major fatty acids in the control product was palmitic acid (16:0) and stearic acid (C18:0) that made up nearly 30% of the total fatty acids (Table 3.3). Other major fatty acids in the control product included Arachidonic acid (20:4n-6; 19.22 ± 0.97 mg g $lipid^{-1}$) and EPA (20:5n-3; 14.45 ± 1.21 mg g $lipid^{-1}$), while DHA (22:6n-3) contributed only 0.49 ± 0.27 mg g $lipid^{-1}$ (Table 3.3). Smoke dried BDM had higher levels of arachidonic acid, EPA and DHA than the control product but these differences were not significant (Table 3.3). Smoke dried BDM did however contain significantly higher levels of EPA than BDM produced by salting but there were no significant

differences in the levels of DHA between any treatments. When fatty acid concentrations were analysed within each of the salting treatments, results showed an increase in ∑PUFA from 24 h to 48 h kenching, with the level resulting from 48 h kenching (71.73 ± 0.89 mg g lipid^{-1}) being significantly greater than that of BDM from all other kenching treatments (Table 3.4). Within BDM processed using brine treatments, the level of DHA was significantly higher following treatment with 15% brine and was almost three times the level of DHA found in BDM resulting from 18% and 25% brine treatments (Table 3.4). Pearson's correlation analysis showed that there was a non-significant relationship between ash and ∑ n-3 LC PUFA contents of BDM across treatments ($P > 0.05$).

Table 3.4. Major polyunsaturated fatty acid compositions of kenched and brine salted BDM from sandfish*. Means (± SE) are expressed in mg g lipid^{-1} dry weight (n=3) and do not differ significantly if sharing the same superscript ($P > 0.05$)

Fatty acid	**Kenching - 24 h**	**Kenching – 36 h**	**Kenching – 48 h**	**Kenching – 72 h**
C18:3n-3	n.d	0.05±0.05^{a}	0.06±0.06^{a}	n.d
C20:4n-6	16.81±1.53^{a}	18.92±0.51^{a}	20.97±1.09^{a}	14.64±2.30^{a}
C20:5n-3	7.93±1.83^{a}	10.58±2.48^{a}	9.93±0.56^{a}	5.70±0.62^{a}
C22:6n-3	0.48±0.24^{a}	0.84±0.21^{a}	0.83±0.31^{a}	0.40±0.12^{a}
∑PUFA	59.37±3.93^{ab}	65.85±4.34^{ab}	71.73±0.89^{b}	55.60±3.84^{a}
∑n-3 PUFA	33.08±2.13^{a}	37.40±3.31^{a}	39.41±0.50^{a}	31.85±1.15^{a}
	Brine – 15%	**Brine – 18%**	**Brine – 25%**	
C18:3n-3	1.19±0.50^{a}	0.04±0.04^{a}	n.d	
C20:4n-6	21.80±1.65^{a}	17.34±1.43^{a}	17.84±3.67^{a}	
C20:5n-3	9.53±0.46^{a}	7.26±0.84^{a}	7.67±1.19^{a}	
C22:6n-3	1.01±0.12^{b}	0.38±0.05^{a}	0.38±0.07^{a}	
∑PUFA	71.16±2.92^{a}	60.56±1.14^{a}	65.47±8.77^{a}	
∑n-3 PUFA	38.48±1.08^{a}	33.07±0.73^{a}	36.95±3.62^{a}	

3.4 Discussion

Inclusion of a salting step during processing of sea cucumbers facilitates dehydration and shrinking of the tissue. Extraction of water from seafood tissues decreases the water activity resulting in reduced bacterial and enzymatic activity (Oliveira et al., 2012), while an increase in the salt content of the resulting product protects from spoilage and prolongs shelf life. However, while salting is an increasingly popular step used in sea cucumber processing (Lavitra et al., 2008; Purcell, 2014a), it is not universally applied (Purcell, 2014a). Salt taken into sea cucumber tissues during the salting procedure contributes to the weight of the final BDM product (Bao et al., 2010; Dong et al., 2008; Dong et al., 2011) which is advantageous to sea cucumber processors because BDM is generally sold on a weight basis. On the other hand, salting also results in a loss of nutrients from seafood tissue (Sampels, 2015; Thorarinsdottir et al., 2004), potentially reducing the nutrient content of BDM. The quality of seafood products is influenced by salting technique (Sampels, 2015), but no prior study has reported on the influence of salting, or salting procedure, on the nutrient content of resulting BDM. Such information is of value in developing advanced and improved processing methods for sea cucumbers that not only consider the physical characteristics of the product, but also nutrient content.

In all but one of the salting treatments in this study (15% brine), the ash content of salted BDM was significantly higher (more than double in some cases) than in non-salted BDM. Elevated ash content results from the uptake of salt by sea cucumber tissues where it binds the triple-helix collagen structure within the body wall (Duerr and Dyer, 1952; Gómez-Guillén et al., 2011). Similarly, in raw Atlantic cod, salting resulted in increased salt content (18-21%) compared to 0.8% in an unsalted control (Thorarinsdottir et al., 2004). The salt content of brine and the

amount of salt taken-up by fish tissue are positively correlated (Thorarinsdottir et al., 2004), with a similar trend indicated in this study where BDM processed using 25% brine contained a significantly greater ash content than BDM processed using 15% brine. Uptake of salt into seafood is influenced by a number of factors including the salting method used, salt concentration and the duration of the salting step (Gallart-Jornet et al., 2007a). However, there were no significant differences between the ash contents of BDM produced after varying durations of kenching in the present study. The uptake of salt into seafood during salting can also be influenced by tissue characteristics, including size, thickness, nutritional composition and species type (Gallart-Jornet et al., 2007a). The body wall of sea cucumbers contains a high proportion of collagen (Xia and Wang, 2015) that influences BDM firmness and texture quality (Hair et al., 2018) and, while the uptake of salt contributes to increased yield (weight) of resulting BDM, the influence of tissue collagen content and structure is undocumented, and the influence of increased salt content on product texture is unknown. The composition of salt used to salt seafoods may also influence the texture of seafood products (Sampels, 2015), with higher proportions of calcium and magnesium ions shown to improve texture and appearance of fish tissues (Lauritzsen et al., 2004).

The values for ash content of BDM in the present study agree with those of prior studies where Ozer et al. (2004), for example, reported the ash content of processed *H. scabra*to range from 17.9-44.5%, while Bechtel et al. (2012) reported the ash content of processed *P. californicus* to range from 12.18-25.73%. The ash content of eight other commercial species imported to China ranged from 15.4% in *A. mauritiana* to 39.6% in *H. fuscopunctata* (Wen et al. (2010). Ash content is generally higher in BDM processed from sea cucumbers with an abundance of

microscopic skeletal elements (ossicles) within the body wall (Smiley, 1994). As such, the outer body wall is generally removed during *H. scabra* processing (Rich, 1995; SPC, 1994), as was the case in the present study. Yet despite this, the ash content of BDM from the control and smoke dry treatments still made up 11-13% of dry weight.

The protein content of BDM from the control treatments in this study was significantly higher than that of salt-processed and dried BDM. Similar protein values (39.8-60.2%) for processed *H. scabra* were reported by Ozer et al. (2004), as was the case for Wen et al. (2010), who reported a similar range of protein values in eight other species of dried commercial sea cucumbers (40.7-62.1%). The protein contents of *H. scabra* from the control and smoke dried treatments in the present study were higher than those previously reported for this species. Salting significantly reduced the protein content of resulting BDM compared to non-salted BDM. However, protein loss did not increase with increasing salting duration (kenching) or with increasing brine strength. In a previous study with *H. scabra* salting did not result in any significant loss of collagen content during processing (Chapter 4). The results of the current study indicate therefore that because collagen makes up around 40-60% of the body wall protein content of sea cucumbers (ref), then the loss of protein reported here reflects losses of non-collagen protein. Loss of protein during salting has been reported previously for fish tissues (Jittinandana et al., 2002; Nketsia-Tabiri and Sefa-Dedeh, 1995) and, similarly, neither brine concentration or brining duration significantly affected tissue protein composition (Jittinandana et al., 2002). Despite a significant increase in the ash content of salt-treated BDM in most treatments in this study, presumably resulting from salt uptake by sea cucumber tissues, the relationship between protein content and ash content was non-significant. This is perhaps surprising given that, in fish,

protein extraction from tissue is a function of salt concentration, and the protein denaturing effect of salt (Sampels, 2015; Thorarinsdottir et al., 2004).

Lipid levels in BDM from all treatments in this study ranged from 10.22 ± 0.33 mg g^{-1} - 17.36 ± 2.36 mg g^{-1} and did not differ significantly between treatments. These relatively low values (< 2%) for lipid are consistent with the generally accepted view that BDM is high in protein but low in lipid content (Chang-Lee et al., 1989; Svetashev et al., 1991; Wen et al., 2010). Wen et al. (2010) reported the proximate composition of BDM produced from eight species of sea cucumbers imported into China. Lipid content ranged from 0.3%-10.1% of dry weight and, although *H. scabra* was not analysed in their study, BDM processed from *H. fuscogilva* and *H. fuscopunctata* contained a low 0.3% lipid (Wen et al., 2010). The lipid contents of BDM did not differ significantly between salted and non-salted products in the present study indicating that salting did not promote the loss of fat from sea cucumber tissue. In contrast, the fat content of rainbow trout fillets was reported to be significantly greater when salted in higher brine strength (17.4% w/w) than at a lower brine strength (8.7% w/w), although no further significant differences resulted from changes in brining duration (Jittinandana et al., 2002).

Salt is an oxidant (Sampels, 2015), and kenching allows greater access of oxygen to seafood tissue than brining, and has been thought to increase oxidation (Thorarinsdottir et al., 2004). However, this phenomenon was not well illustrated by the results of the present study in relation to fatty acid composition. While BDM processed using 72 h kenching had significantly lower levels of ∑n-3 LC PUFA than both smoked dry BDM and the control, it was the only salting treatment to differ from the control in this regard. Furthermore, although smoke dried BDM

contained significantly higher levels of EPA than BDM produced by salting, there were no significant differences in the levels of DHA between any treatments. When levels of constituent fatty acids were analysed within each of the salting treatments, results showed an increase in ∑PUFA from 24 h-48 h kenching with the level resulting from 48 h kenching (71.73 ± 0.89 mg g lipid^{-1}) being significantly greater than that of BDM from all other kenching treatments. Some brine salting treatments (24 h and 72 h) resulted in significant reductions in the EPA content of resulting BDM compared to the control treatment, but a similar effect on the levels of DHA was not evident in any of the salting treatments. Wen et al. (2010) reported levels of EPA ranging from 0.32%-3.92% of total fatty acids in BDM processed from eight species of sea cucumbers imported into China. These values are similar to those in the present study that ranged from 3%-4.5% of total fatty acids across treatments. However, in contrast to our results, Wen et al. (2010) also reported that DHA was undetected within BDM of the eight species sampled and the authors suggested that this resulted from processing methodology, including repeated boiling.

Chapter 4

Effect of processing method on quality, texture, collagen and amino acid composition of sandfish (*Holothuria scabra*)[3]

4.1 Introduction

Over 1200 species of sea cucumbers are known of which 58 are commercially exploited (Ferdouse, 2004; Purcell et al., 2012b). Sea cucumbers are usually processed into a dried product called bêche-de-mer (*iriko* in Japanese, *hai-som* in Chinese or *trepang* in Indonesian) (Bumrasarinpai, 2006a; Ferdouse, 1999; McElroy, 1990) that is consumed as a delicacy and for perceived medicinal benefits in S.E. Asian countries (Bordbar et al., 2011; Esmat et al., 2013), particularly in China, Hong Kong, Taiwan, Singapore and Malaysia (Eriksson and Clarke, 2015; Ferdouse, 2004) where well-dried 'A' grade product may command $US 70-190 kg^{-1} depending on the species used, size and quality (Purcell et al., 2012b). At least 15,000 tonnes of bêche-de-mer (BDM) are traded annually in S.E. Asia (Qao et al., 2011).

BDM processing results in a product that is non-perishable if stored in dry, dark conditions. Processing steps include first boiling, slitting and gutting, second boiling, smoking and finally sun-drying (Chapter 2) (Purcell, 2014a; Ram et al., 2014a). For example, the processing steps used for sandfish (*Holothuria scabra*) are shown in Fig. 3.1. Each contributes to the resulting quality of the final product which determines the suitability of processed product for Asian markets (Conand, 1990a; Purcell, 2014b; Sachithananthan et al., 1985; SPC, 1994) and their

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value. Processing BDM may also involve a 'salting' step (Fig. 3.1) that uses salt to draw water from sea cucumber tissues (Lavitra et al., 2008) in order to facilitate dehydration and shrinking. Salting can be achieved by immersing sea cucumber tissue in a brine solution (brining), or by dry salting where the tissue is covered in coarse salt and developing brine is allowed to drain away (kenching) (Sampels, 2015). If BDM processing includes salting, salt soluble proteins are generally leached from the tissues during the process, while salt enters the tissues and binds to the triple-helix collagen structure (Duerr and Dyer, 1952; Gómez-Guillén et al., 2011), where it contributes to the weight of the final BDM product (Bao et al., 2010; Dong et al., 2008; Dong et al., 2011), protects the product from spoilage and prolongs shelf life.

The major factors affecting the quality and value of BDM are species used, appearance, odour, colour, thickness of the body wall and market demand (Ram, 2008). Nutrient content and nutritional quality are not considered when BDM is assessed for quality and value (Wen et al., 2010) because these are generally unknown. Body wall thickness of BDM is a key determinant of quality and value (Skewes et al., 2004) because thicker body wall flesh provides better texture and improved eating quality (Lo, 2004). The body wall of sea cucumbers contains a high proportion of collagen (Xia and Wang, 2015) that has a major influence on BDM firmness and texture quality. Collagen molecules are composed of three α–chains that are stabilized by intra- and inter- chain hydrogen bonds forming the collagen triple helix (Zhang et al., 2013). It contains a repeat of the amino acids glycine, proline and hydroxyproline (Ichikawa et al., 2010). In beef tissue, collagen content is generally influenced by the age of the animal because, as the animal matures, less collagen is synthesized and more mature crosslinks occur in the collagen fibrils (Weston et al., 2002). The nature of the bonds changes over time as these reducible crosslinks

are replaced by mature thermally-stable and less soluble crosslinks (Weston et al., 2002). In livestock, the tissues of older animals develop more mature crosslinks that generate greater tension resulting in increased red meat toughness (Marsh, 1977; Weston et al., 2002), however, similar information on the influence of age on the structure of body wall collagen has not been documented in sea cucumbers. Although a number of prior studies have reported collagen contents of seafood such as fish (Liu et al., 2007 ; Matmaroh et al., 2011b; Pati et al., 2010; Singh et al., 2011), similar reports on collagen content of sea cucumber (Dong et al., 2011; Zhong et al., 2015) and the effect of processing on collagen composition and texture in sea cucumbers (Chen et al., 2015a) are few. Prior studies have shown that sea cucumber collagen is denatured around 60-100^{o}C (Dong et al., 2008; Dong et al., 2011) and the collagen fibrils were observed to be thicker and broken. But the influence of variations in processing methods on collagen content of resulting BDM, and the resulting influence on product texture and quality, has not previously been reported.

The flavours of aquatic foods are derived from non-protein nitrogen compounds including free amino acids, salts and minerals (Bremner, 2012). The flavor of BDM is generated by a range of amino acids including glycine, glutamic acid, lysine and aspartic acid that give sea cucumbers their unique taste (Zunying et al., 2011). However, amino acids are readily soluble in water and can be leached from tissue during processing (Bremner, 2012) and such losses are likely to be increased if a salting step is included in BDM processing, because this process draws water from sea cucumber tissues (Lavitra et al., 2008) to facilitate dehydration. Furthermore, excessive cooking at a temperature above 100^{o}C can also result in excessive loss of amino acids during sea cucumber processing (Dong et al., 2008). However, information on losses of amino acids from

BDM during process, and the effects of variation in processing method on such losses is very limited and has not previously been reported for tropical species.

A number of studies have reported the nutrient composition of both fresh (Haider et al., 2015; Lee et al., 2012; Omran, 2013) and processed (Ozer et al., 2004; Wen et al., 2010) sea cucumbers, but we are aware of only one prior study that determined the effects of variations in processing method on nutrient composition of resulting BDM. It reported on the effects of inclusion of a salting step, and variations in salting method, on the proximate and fatty acid compositions of resulting BDM processed from sandfish, *Holothuria scabra* (Chapter 3). However, no prior study has reported the effects of various methods. This study examined changes in yield and texture, as well as collagen and amino acid compositions, of BDM processed from sandfish, *Holothuria scabra*, using variations in processing method. The influences of brine and kench salting, as well as brine strength and kenching duration, were determined and results provide a greater understanding of the influence of processing method on BDM composition.

4.2 Materials and Methods

This study was conducted at the post-harvest processing facility of the University of the South Pacific, Fiji, and the Food Science Precinct, Department of Agriculture and Fisheries, Queensland Government, Australia.

Holothuria scabra (Sandfish) were collected from Tavua Bay, Fiji Islands (17°26'29.4"S 177°51'44.4"E). After collection, they were left for 5 min before length and weight was

determined. Individuals were gutted and held in an insulated fish box containing ice. A total of 36 individuals were collected and subsamples of three sea cucumbers were each subject to nine different processing treatments. The remaining nine individuals were used for collagen and amino acid analysis of fresh *H. scabra* tissues (3), and for partial processing through cooking at 80-90^{o}C for 15 min (3) and cooking then kenching for 48 h only (3). Sea cucumber processing followed the general method normally used by BDM processors in Fiji as outlined by Purcell (2014a). Sea cucumbers were laid on a table for approximately five minutes to allow them to relax before the length and weight of each was determined to the nearest 10 g. All sea cucumbers were first cooked at a water temperature of 45^{o}C for 10 min before the water temperature was gradually increased to 80^{o}C (Fig. 3.1). Sea cucumbers were cooked for a further 10 min until they were hard and springy indicating completion of the first cook. Cooked sea cucumbers were then immersed in a 3 g/100 mL saline solution for 36 h to allow the outer layer of spicules to disintegrate (Purcell, 2014a). Sea cucumbers with spicules removed were then subject to seven different salting treatments using grade 11 coarse solar salt:

1) Kenching for 24 h prior to further processing;
2) Kenching for 36 h prior to further processing;
3) Kenching for 48 h prior to further processing;
4) Kenching for 72 h prior to further processing;
5) Immersion in a 15 g/100 mL saline solution for 48 h prior to further processing;
6) Immersion in a 18 g/100 mL saline solution for 48 h prior to further processing; and
7) Immersion in a 25 g/100 mL saline solution for 48 h prior to further processing.

After salting the sea cucumbers were cooked a second time for 15-25 min (at 45^{o}C rising to 96^{o}C) and were then solar dried for at least 2 to 3 weeks. Any salt crystals that formed on the surface of the sandfish during the drying period were washed off. After 2 to 3 weeks, a third cook for 5-15 min (at 45^{o}C rising to boiling) was then followed by shape correction (straitening and closure of body cut) to assist market acceptability, before the product was finally dried using solar drying. Sea cucumbers in two other treatments were not salted and served as controls. Following removal of the spicules, these sea cucumbers were not salted, but proceeded directly to the second cooking step and were then dried as follows:

8) Solar dried; and
9) Smoke dried using smoke machine for 48 h (Dish–100–DX, Ikeden Seisakusho Co. Ltd, Japan) with an exhaust fan for 48 h at 45^{o}C using wood chips to generate the smoke.

4.2.1 Recovery rates

On completion of processing, recovery rate for length (RRL) and recovery rate for weight (RRW) were calculated for BDM resulting from each treatment as:

RRL = *Mean length after processing/mean fresh length x 100*

RRW = *Mean weight after processing/mean fresh weight x 100*

4.2.2 Texture determination

Prior to texture determination and amino acid analysis of BDM, processed dried BDM samples were reconstituted in fresh water for five days at 4^{o}C (Fukunaga et al., 2004). Once reconstituted,

the samples along with partially processed cooked samples were crushed using the Retch-MM301 mixer mill and lyophilized.

Texture analysis of processed and reconstituted BDM, as well as cooked (80-90^{o}C) Sea cucumber tissue was performed using an Instron Penetrometer (Model no 5543, Instron Corporation, 825 University Avenue, Norwood MA, USA) with a 500 N load cell with a Kramer-Shear cell modified to have two blades instead of five.

4.2.3 Amino acid analysis

The amino acid analysis procedure employed pre-column derivatisation with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) and ultra-performance liquid chromatography (UPLC) analysis after first performing acid hydrolysis on the sea cucumber samples to release the protein-bound amino acids. Each sample was run in duplicate. The methodology used has been described in detail by Truong et al. (2015).

The samples were mixed till uniform, then 40-50 mg was hydrolysed by adding 5.0 mL of 20 ml/100 mL HCl in a 10 mL hydrolysis vial, flushing with nitrogen then incubated at 110°C for 24 h. An internal standard (norvaline) was added to the hydrolysate and it was diluted 1 part in 25 with MQ water prior to derivatization.

Hydrolysed samples and amino acid standards (Standard H, ThermoFisher Scientific) were derivatised with AQC reagent using the AccQ-Tag Ultra derivatisation kit (Waters Corporation, Milford, MA, USA) according to the manufacturer's instructions.

Chromatographic separation and quantitation of the reported 17 acid hydrolysate amino acids was performed on an ACQUITY UPLC system (Waters Corporation). The column was a BEH RP C18 (2.1 x 100 mm, 1.7 μm, Waters Corporation). The separation of amino acids was run with a binary gradient flow rate of 0.7 mL min^{-1} at 60^{o}C and UV detection was at 260 nm with a 10.2 min. analysis time per sample. Data was acquired and quantified using Empower software (Waters) using prepared 2.5, 10 and 50 pmol per μL injection analytical standards and an internal standard calibration procedure.

BDM samples from each treatment (n=3) were analysed for their proximate amino acid compositions. For amino acid analysis including hydroxyproline and taurine determination, the samples were hydrolysed in 6 mol/L HCl at 110^{o}C for 24 h. Cysteine and tryptophan were not analysed using this method. All amino acids were analysed using a Waters AccQTag Ultra Chemistry on a Waters Acquity UPLC system. Since the limit of reporting using this method was 1 mg/g, taurine was not detected in the samples.

4.2.4 Collagen analysis

Hydroxyproline content of the BDM was used to estimate collagen content following application of a conversion coefficient (Chen et al., 2015a). Collagen analysis was carried out using the ISO method (3496: 1978). BDM was reconstituted for 3-5 days and cut into small pieces before being milled using Retch-MM301 mixer mill and lyophilised. Approximately 250 mg of dried sample was measured into hydrolysis jar and filled with 10 mL of 6 mol/L HCl and hydrolysed at 115 ^{o}C for 18 h. Hydrolysates were filtered using Whatman No. 1 filter paper and serially diluted to the ratio of 1:100 and 1:10. A 2 mL aliquot of the 1:10 diluent was transferred to a glass test tube for

analysis of hydroxyproline and diluted where necessary in duplicates and 1 mL chloramine-T reagent was added, mixed and left at room temperature for 20 minutes. An aliquot of 1 mL colour reagent was added and mixed thoroughly and heated at 60°C ± 0.5 °C for 20 min. The tubes were cooled under tap water for 3 min and left at room temperature for a further 30 min. Absorbance was read at 558 nm against a deionized water blank. Hydroxyproline content was calculated from the absorbance using a standard curve, taking into account the dilution factors. Amino acid analysis (*section 2.3*) determined that hydroxyproline contributed 64 reissues per 1000 amino acids within sandfish BDM tissues (i.e. 6.4% of all amino acids (Table 4.2). On this basis, estimation of collagen content based on hydroxyproline equivalents, employed a conversion factor of 6.4 in this study.

4.2.5 Statistical analysis

All analyses were conducted in triplicate. Results are expressed as mean values ± standard error mean (SEM) and one-way analysis of variance (ANOVA) was carried out using the SPSS Version 24 statistical software. Differences in the nutrient contents of BDM across treatments were tested with ANOVA followed by the multiple comparison test (Tukey HSD) after Levene's test for homogeneity of variance was not significant ($p>0.05$). Data for collagen content and texture (firmness and hardness) were similarly analysed, but following a significant Levene's test ($p<0.05$), data were $\log_{10}$ transformed and a non-parametric (Kruskal Wallis) test was conducted. A Pearson's correlation test between collagen content and the firmness reconstituted BDM processed using a salting step (kench and brining) was conducted. Differences were considered to be significant when $p<0.05$.

4.3 Results

Weight and length based recovery rate (RRW and RRL, respectively) of BDM from all treatments is shown in Fig. 4.1. Mean RRL ranged from 20.70 ± 0.02% - 26.40 ± 3.50% across all salting treatments but none of these values differed significantly from that of the control treatment (25.9 ± 1.30%) ($p>0.05$). Smoke dried BDM, however, had a significantly greater RRL (35.10 ± 2.80%) than all other treatments ($p<0.05$). Mean RRW ranged from 4.50 ± 1.10% (brining 25 g/100 mL) - 9.70 ± 2.20% (kenching 24 h) across salting treatments but did not differ significantly among treatments or from those of control (3.80 ± 0.60%) and smoked product (6.90 ± 1.60%) (Fig. 4.2; $p>0.05$).

Crude collagen contents and textural measurements of BDM from all treatments are shown in Table 4.1. Collagen content of BDM ranged from 81.90 ± 4.30 mg g^{-1} (brining 25 g/100 mL) to 108.20 ± 8.80 mg g^{-1} (brining 15 g/100mL) across salting treatments. These values did not differ significantly among treatments or from those of control (126.70 ± 24.20 mg g^{-1}) and smoked product (110.90 ± 17.20 mg g^{-1}) (Table 4.1; $p>0.05$). Kruskal Wallis test for collagen content (($X^2(8, N=27) = 6.466$, $p=0.595$; $p>0.05$) across all treatments was not significant. Partial

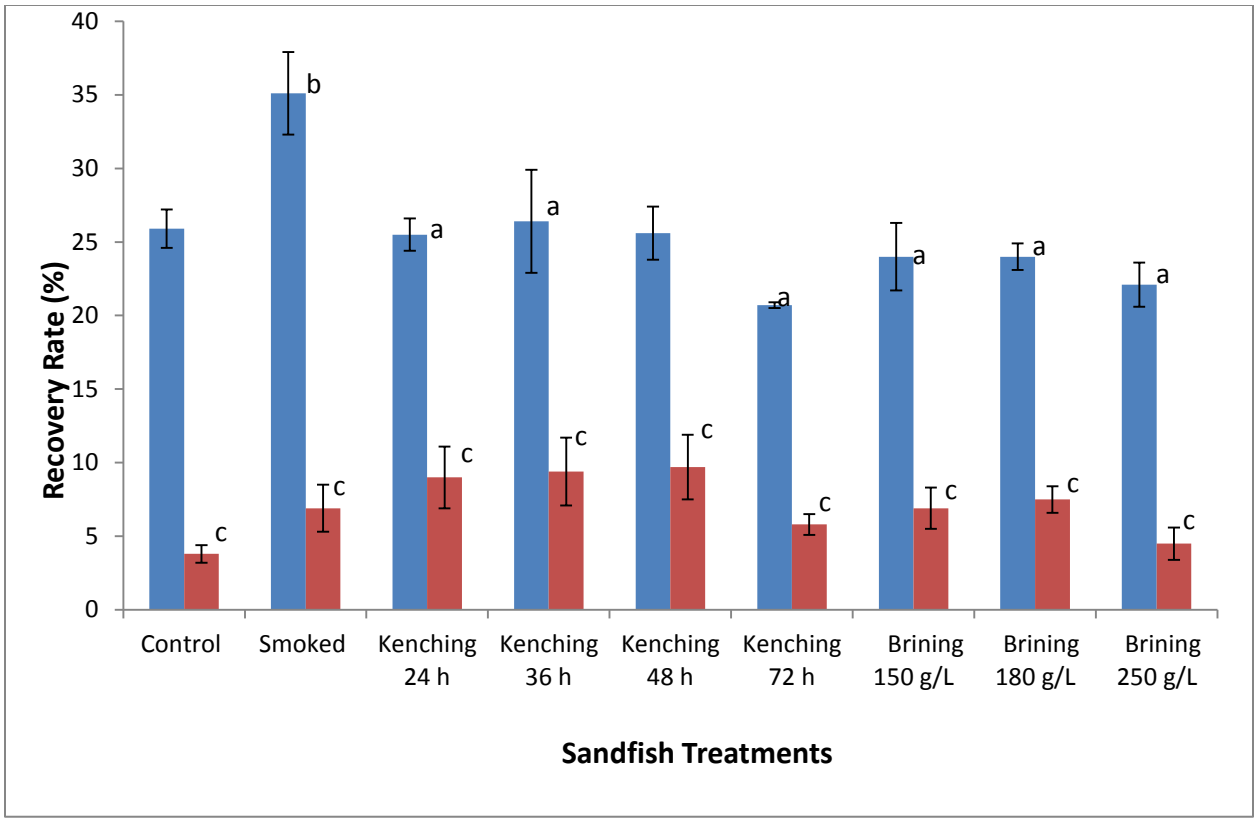

Figure 4.1. Recovery rates (RRL RRW) for bêche-de-mer processed from sandfish using different methods. Means with the same superscript are not significantly different ($p>0.05$)

processing of *H. scabra* by cooking sea cucumbers at 80-90°C, and cooking at 80-90°C followed by salting for 48 h, produced tissue that had lower crude collagen contents (38.25 ± 1.36 and 78.04 ± 15.11 mg g^{-1} dry weight, respectively) than those of BDM from any of the treatments. Increasing the duration of kenching or the strength of brine during the salting step of BDM processing did not have any significant influence on collagen content of the product.

Textural parameters for cooked and cooked and kenched sandfish, and for BDM processed using various methods assessed in this study are shown in Table 4.1. The results show that 87.37 ±

Table 4.1. Crude collagen contents and texture characteristics (mean ± SE) of fresh, cooked and processed sandfish.

Processing Method	**Crude collagen content (mg g^{-1}) dry tissue weight (except fresh)**	**Shear force (N g^{-1}) Firmness**	**Energy (mJ g^{-1}) Hardness**
Unprocessed fresh	4.34 ± 0.63	na	na
Cooked at 80-90^{o}C	38.25 ± 1.36	87.36 ± 3.32^{b}	180.19 ± 19.27^{b}
Cooked at 80-90^{o}C and salted for 48 h	78.04 ± 15.11	na	na
Normal dry (control)	126.72 ± 24.16^{a}	20.73 ± 4.48^{a}	51.39 ± 12.50^{a}
Smoked	110.87 ± 17.25^{a}	39.16 ± 17.05^{a}	90.87 ± 41.92^{ab}
Kenching 24 h	103.28 ± 22.45^{a}	8.24 ± 2.05^{a}	34.11 ± 4.72^{a}
Kenching 36 h	83.72 ± 2.49^{a}	22.99 ± 11.47^{a}	83.98 ± 42.08^{ab}
Kenching 48 h	83.20 ± 5.04^{a}	31.71 ± 8.05^{a}	96.89 ± 19.22^{ab}
Kenching 72 h	99.06 ± 16.89^{a}	8.48 ± 2.24^{a}	29.40 ± 3.97^{a}
Brining 15 g/100 mL saline	108.19 ± 8.78^{a}	6.91 ± 1.68^{a}	29.04 ± 7.66^{a}
Brining 18 g/100 mL saline	102.88 ± 15.29^{a}	26.23 ± 7.06^{a}	88.53 ± 31.74^{ab}
Brining 25 g/100 mL saline	81.89 ± 4.27^{a}	40.49 ± 17.62^{ab}	83.05 ± 27.81^{ab}

Means in the same column with different superscripts are significantly difference ($p < 0.05$)

3.32 N g^{-1} force and 180.19 ± 19.27 mJ g^{-1} of energy was required to shear sandfish flesh that has been cooked at 80-90^{o}C for 15 min. Processed, dried and reconstituted BDM showed no significant differences ($p<0.05$) for either of the textural parameters across treatments (Table 4.1); but BDM from all treatments, with the exception of 25 g/100 mL brining, was significantly less firm than flesh cooked at 80-90^{o}C. Despite the fact that dried and reconstituted BDM resulting from the 48-h and 72-h kenching, and 18 g/100 mL and 25 g/100 mL brining treatments, showed around half the hardness of sandfish flesh that was cooked at 80-90^{o}C, these differences were not significant ($p>0.05$). However, subsequent non-parametric analysis (Kruskal Wallis test) showed a significant differences between treatment for firmness ($X^2(9, N=30) = 19.942$, $p=0.018$) and hardness ($X^2(9, N=30) = 17.473$, $p=0.042$) (Table 4.1). There was a significant correlation (Pearson's correlation (*2-tailed*), $p=0.04$) between collagen content and firmness in reconstituted BDM produced across salting treatments.

The protein-bound amino acid contents of fresh sandfish flesh, cooked (80-90^{o}C) sandfish and cooked and salted sandfish are shown in Table 4.2 along with those of normally dried and smoke dried BDM, and BDM processed with 48 h kenching. Similar data for free amino acids are shown in Table 4.3. The most abundant protein-bound amino acids in fresh sandfish tissue were glycine, glutamic acid, proline, arginine, aspartic acid, alanine and hydroxyproline which made up 10.22 ± 0.80, 9.91 ± 0.51, 5.81 ± 0.48, 5.53 ± 0.36, 5.39 ± 0.32, 5.31 ± 0.37 and 3.82 ± 0.33 mg/g of tissue weight, respectively. The same amino acids dominated the BDM samples and there were no significant differences ($p>0.05$) in levels of these seven major protein-bound amino acids across the three BDM treatments. Furthermore, there were no significant differences in the levels of any of the 17 protein-bound amino acids reported between the three BDM

Table 4.2. Tissue amino acid content (mg/g ± SEM) of fresh and cooked sandfish, and normal dried, smoke dried and kench salted BDM. Means in the same row with a different superscript are significant different ($p < 0.05$)

Amino Acid residue in tissue (mg/g tissue)	Fresh	Cooked (80-90°C)	Cooked (80-90°C) and salted	Normal dried	Smoked	Kench salted 48 h
Hydroxyproline	3.82 ± 0.33	14.12 ± 0.29	4.43 ± 0.33	44.13 ± 0.56^{a}	45.53 ± 1.09^{a}	50.25 ± 3.34^{a}
Histidine	0.35 ± 0.01	1.35 ± 0.04	0.37 ± 0.02	4.35 ± 0.12^{a}	4.05 ± 0.13^{a}	4.12 ± 0.26^{a}
Serine	2.08 ± 0.12	7.10 ± 0.12	2.16 ± 0.15	23.78 ± 0.19^{ab}	23.09 ± 0.42^{a}	24.77 ± 0.24^{b}
Arginine	5.53 ± 0.36	19.58 ± 0.42	6.03 ± 0.43	63.19 ± 0.34^{a}	62.32 ± 1.57^{a}	68.94 ± 2.32^{a}
Glycine	10.22 ± 0.80	36.74 ± 0.81	11.53 ± 0.86	116.02 ± 1.10^{a}	117.66 ± 3.15^{a}	131.79 ± 7.00^{a}
Aspartic acid	5.39 ± 0.32	18.53 ± 0.39	5.70 ± 0.39	60.36 ± 0.74^{a}	60.26 ± 1.44^{a}	63.59 ± 0.54^{a}
Glutamic acid	9.91 ± 0.51	30.35 ± 0.71	9.36 ± 0.67	99.93 ± 1.09^{a}	99.43 ± 3.09^{a}	107.62 ± 2.37^{a}
Threonine	2.63 ± 0.17	9.17 ± 0.19	2.82 ± 0.20	30.77 ± 0.22^{a}	29.99 ± 0.72^{a}	32.11 ± 0.52^{a}
Alanine	5.31 ± 0.37	18.75 ± 0.41	5.85 ± 0.42	59.74 ± 0.50^{a}	60.33 ± 1.74^{a}	66.86 ± 3.00^{a}
Proline	5.81 ± 0.48	21.00 ± 0.44	6.59 ± 0.50	67.57 ± 0.39^{a}	67.63 ± 1.71^{a}	75.90 ± 3.23^{a}
Lysine	1.15 ±0.01	3.97 ± 0.13	1.11 ± 0.07	13.91 ± 0.51^{a}	12.13 ± 0.62^{a}	12.74 ± 1.07^{a}
Tyrosine	1.21 ± 0.07	4.18 ± 0.11	1.28 ± 0.08	14.60 ± 0.21^{a}	14.13 ± 0.14^{a}	14.52 ± 0.30^{a}
Methionine	0.78 ± 0.04	2.64 ± 0.08	0.78 ± 0.05	8.91 ± 0.14^{a}	8.42 ± 0.27^{a}	8.95 ± 0.18^{a}
Valine	2.16 ± 0.12	7.37 ± 0.14	2.21 ± 0.14	25.06 ± 0.20^{a}	24.25 ± 0.53^{a}	25.37 ± 0.16^{a}
Isoleucine	1.29 ± 0.06	4.32 ± 0.12	1.26 ± 0.08	15.03 ± 0.30^{a}	14.06 ± 0.47^{a}	14.43 ± 0.61^{a}
Leucine	2.29 ± 0.10	7.84 ± 0.19	2.33 ± 0.15	26.74 ± 0.36^{a}	25.46 ± 0.83^{a}	26.71 ± 0.53^{a}
Phenylalanine	1.25 ± 0.06	4.18 ± 0.08	1.22 ± 0.08	14.63 ± 0.21^{a}	13.76 ± 0.29^{a}	14.02 ± 0.54^{a}

Table 4.3. Free amino acid content (mg/g ± SEM) of fresh and cooked sandfish, and normal dried, smoke dried and kench salted BDM. Means in the same row with a different superscript are significant different ($p < 0.05$)

Free Amino Acid (mg/g tissue)	Fresh	Cooked (80-90°C)	Cooked (80-90°C) and salted	Normal dried	Smoked	Kench salted 48 h
Hydroxyproline	4.42 ± 0.39	14.06 ± 0.29	5.13 ± 0.38	51.16 ± 0.65a[a]	52.78 ± 1.27[a]	58.25 ± 3.87[a]
Histidine	0.40 ± 0.01	1.31 ± 0.04	0.42 ± 0.02	4.92 ± 0.14[a]	4.58 ± 0.15[a]	4.67 ± 0.30[a]
Serine	2.51 ± 0.14	7.36 ± 0.12	2.60 ± 0.18	28.70 ± 0.23[ab]	27.87 ± 0.51[a]	29.89 ± 0.29[b]
Arginine	6.17 ± 0.41	18.75 ± 0.40	6.73 ± 0.48	70.48 ± 0.38[a]	69.51 ± 1.75[a]	76.89 ± 2.59[a]
Glycine	13.45 ± 1.05	41.52 ± 0.92	15.17 ± 1.13	152.67 ± 1.45[a]	154.83 ± 4.14[a]	173.41 ± 9.21[a]
Aspartic acid	6.23 ± 0.36	18.41 ± 0.39	6.59 ± 0.45	69.80 ± 0.86[a]	69.69 ± 1.66[a]	73.54 ± 0.63[a]
Glutamic acid	10.38 ± 0.58	29.70 ± 0.70	10.66 ± 0.76	113.87 ± 1.24[a]	113.30 ± 3.52[a]	122.63 ± 2.70[a]
Threonine	3.10 ± 0.20	9.28 ± 0.19	3.32 ± 0.23	36.25 ± 0.26[a]	35.33 ± 0.85[a]	37.83 ± 0.61[a]
Alanine	6.66 ± 0.46	20.19 ± 0.45	7.33 ± 0.53	74.88 ± 0.63[a]	75.62 ± 2.18[a]	83.80 ± 3.76[a]
Proline	6.88 ± 0.57	21.38 ± 0.45	7.81 ± 0.59	80.10 ± 0.46[a]	80.18 ± 2.03[a]	89.97 ± 3.83[a]
Lysine	1.31 ± 0.01	3.89 ± 0.13	1.27 ± 0.08	15.87 ± 0.58[a]	13.84 ± 0.70[a]	14.54 ± 1.22[a]
Tyrosine	1.35 ± 0.07	3.99 ± 0.10	1.42 ± 0.09	16.21 ± 0.23[a]	15.69 ± 0.16[a]	16.12 ± 0.33[a]
Methionine	0.89 ± 0.05	2.57 ± 0.07	0.89 ± 0.06	10.14 ± 0.16[a]	9.58 ± 0.31[a]	10.18 ± 0.20[a]
Valine	2.55 ± 0.14	7.48 ± 0.15	2.62 ± 0.17	29.62 ± 0.25[a]	28.66 ± 0.63[a]	29.99 ± 0.19[a]
Isoleucine	1.50 ± 0.07	4.30 ± 0.12	1.46 ± 0.09	17.42 ± 0.35[a]	16.30 ± 0.55[a]	16.73 ± 0.71[a]
Leucine	2.65 ± 0.12	7.80 ± 0.19	2.70 ± 0.17	30.99 ± 0.41[a]	29.51 ± 0.96[a]	30.97 ± 0.61[a]
Phenylalanine	1.40 ± 0.07	4.03 ± 0.08	1.37 ± 0.09	16.42 ± 0.24[a]	15.45 ± 0.32[a]	15.74 ± 0.61[a]

products, with the exception of serine content that differed between BDM processed using smoked drying and 48-h kenching. The most abundant free amino acids in fresh, cooked, and process sandfish tissues were glycine, glutamic acid, proline, alanine, aspartic acid and arginine (Table 4.3). Again, there were no significant differences in the levels of any of the 17 free amino acids reported between the three BDM products, with the exception of serine content that differed between BDM processed using smoked drying and 48-h kenching.

4.4 Discussion

This study focused on the influence of processing method on yield, collagen content and textural properties, and amino acid content of BDM processed from Sandfish using different methods, particularly the influence of salting and salting procedure.

Literature on the processing yield of sea cucumbers relates primarily to recovery rates determined for fishery management purpose (Chapter 2) (Purcell et al., 2009b; Skewes et al., 2004). Yield of BDM is generally expressed as a 'recovery rate' that determines the relationship (usually percentage) between fresh weight of sea cucumbers and the dry weight of resulting BDM (Skewes et al., 2004), but the relationship between fresh length and processed length is also meaningful (Chapter 2) . We are unaware of any previous research that has reported recovery rates for any species of sea cucumber to assess the effects of variations in BDM processing method. This study determined a maximum yield by weight (RRW) of 9.7% and of 25.6% by length (RRL) for Sandfish. This RRW value for Sandfish is higher than previously reported values of 5.0% (Conand, 1979), 5.1% (Skewes et al., 2004) and 8.1% (Chapter 2) . The maximum RRL value reported here was lower than the previous reported value for sandfish of

32.6% (Chapter 2). Use of a salting step during sea cucumber processing facilitates entry of salt into the tissues where it binds to the triple helix structure and contributes to the weight of the final BDM product (Bao et al., 2010; Dong et al., 2008; Dong et al., 2011). Prior research in this laboratory reported that the ash content of BDM produced from sandfish was significantly higher when a salting (kenching or brining) steps was included on processing (Chapter 3), presumably as a result of the uptake of salt. It is surprising therefore that BDM produced by salting did not have increased RRW values compared to the control treatment and that increased kenching duration or brine strength did not produce sequential gains in RRW in such treatments.

About 60% of the sea cucumber body wall is composed of water (SPC, 1994) and most of this is lost during processing. The remainder is composed primarily of soluble and insoluble proteins that accounts for the high protein content of BDM (Dong et al., 2011). Much of the protein content of the body wall of sea cucumbers, and resulting BDM, is composed of collagen, that provides sea cucumbers with their body shape and form, and assists during feeding, respiration, burrowing and in defence (Yamada et al., 2010). The potential effect of processing on the crude collagen composition of sandfish has not previously been reported. The collagen content of fresh tissue of sandfish was less than that of 8.16 ± 1.14% reported for *Astichopus japonicus* (Chen et al., 2015a) and 10.9% reported for snapper (*Priacanthus tayenus*) skin (Kittiphattanabawon et al. (2005). The temperature used in processing sea cucumbers can have considerable effect on structure of collagen fibrils, resulting in shortening of fibrils and complete denaturing around 90-100^{o}C (Dong et al., 2008; Dong et al., 2011).

However, despite BDM from the normal dry control and smoke-dry treatments having the two highest (respectively) collagen contents, there were no significant difference between these levels and those of BDM prepared using brine or kench salting. There was however a general trend of decreasing collagen content with increasing brine strength in the brining treatments, although these differences between treatments were non-significant. On this basis our results do not show salting-out of collagen, with increasing salt concentration, in contrasts to the results of Duan et al. (2013) who worked with the collagen from fresh calf skin. Generally, higher salt strengths during brining result in a higher degree of protein denaturisation in fish flesh (Gallart-Jornet et al., 2007b) and this may have been evident in the results of this study where we recorded sequential decreases in collagen content with increasing brine strength.

Our results also indicate sequential increases in the force required to shear reconstituted BDM processed with increasing brine strength. The force required for BDM processed with 15 g/100 mL brine (6.91 n g^{-1}) was almost six-times less than that of BDM prepared using 25 g/100 mL brine solution. Similar results have been reported for fish tissues where, for example, the hardness of fresh Atlantic salmon (*Salmo salar*) tissue increased and elasticity decreased with increasing brine concentration (Gallart-Jornet et al., 2007b). This probably results because protein denaturation increases with higher brine strength (Sampels, 2015; Thorarinsdottir et al., 2004; Thorarinsdottir et al., 2011a; Thorarinsdottir et al., 2011b), resulting in a harder product (Barat et al., 2002, 2003). Reconstituted BDM is preferred when it has softer texture (Akamine, 2007; Fukunaga et al., 2004) and, on this basis, our results indicate that inclusion of a salting step during BDM processing, and choice of salting method, does influence the quality of resulting BDM, and that lower brine strengths are likely to result in improved product quality. Further

research could determine the effect of brine strength on BDM texture at a finer scale than used in the present study (15, 18 and 25 g/100 mL), with a view to optimizing conditions for processing sandfish.

Sea cucumbers processed with a salting step lose around 90% of their original moisture content after drying (Chapter 2). Resulting BDM is rehydrated for as long as 7 – 10 days prior to consumption (Fukunaga et al., 2004). However, the quality of reconstituted BDM is judged primarily by texture, not flavor, with softness and elasticity being of prime importance (Fukunaga et al., 2004). Our results show that there were no differences in the texture (firmness and hardness) of reconstituted sandfish BDM across the salting treatments used in this study. However, all salt processed BDM was soft compared to sandfish tissue that was only cooked (80 – 90^{o}C) and not fully process and reconstituted. The softer texture that results from dehydration and reconstitution of sea cucumbers is probably the reason that rehydrated dried sea cucumbers are preferred to fresh or cooked sea cucumbers in SE Asians countries. The softer texture of reconstituted BDM may result from a greater degree of damage to the crosslinks of the collagen fibril of the tissue after rehydration (Fukunaga et al., 2004). In other animal proteins, toughness and tenderness of the meat depends on collagen crosslink maturity (Weston et al., 2002), but research in this field with sea cucumbers is lacking and the changes that occur in tissue protein as a result of processing are unknown. However, during cooking of red meat the shear forces (toughness) increases in 2 phases: (1) as temperature increases to 40-50^{o}C denaturation of the contractile proteins, actin and myosin, and an increase in fluid loss takes place; and (2) when temperature increases to 64-68^{o}C, collagen denaturation results from shrinkage in fibrils and excessive fluid loss generated by the pressure exerted during thermal contraction (Weston et al.,

2002). Similar knowledge of changes in tissue proteins during sea cucumber processing would provide a basis for optimizing BDM production methods, and is a key component of future research in this field.

Together with appropriate texture, the taste of sea cucumbers is an important factor contributing to product quality and value (Akamine, 2007). Amino acids play an important role in giving rise to sweetness, bitterness, sourness and umami taste in sea cucumbers (Sicuro et al., 2012). In this study we found that glycine, glutamic acid, proline, arginine, aspartic acid, alanine and hydroxyproline were the most abundant amino acids found in BDM processed from sandfish. Prior studies with other species of sea cucumbers have similarly reported that glycine, glutamic acid, aspartic acid were the dominant characteristic amino acid (Bechtel et al., 2012; Haider et al., 2015; Lee et al., 2012; Omran, 2013; Sicuro et al., 2012), and higher glutamic acid levels are considered responsible for the distinctive flavor (Zhong et al., 2007) (Table 4.4). Omran (2013) reported values for glycine, alanine and aspartic acid of 18.38 ± 1.10%, 6.52 ± 1.05% and 4.81 ± 0.80% of total amino acid, respectively, for fresh tissue of sandfish, and these are higher than the values reported here for the same species. Despite that fact that levels of all seven dominant amino acids (glycine, glutamic acid, proline, arginine, aspartic acid, alanine and hydroxyproline) in sandfish BDM were higher in product that was processed with kenching for 4 h than in normal dried and smoked dried BDM, these differences were not significant. Our results show that inclusion of a kenching step in sandfish processing does not negatively affect levels of tissue amino acids that contribute to product quality. This is surprising because prior research in this laboratory has that salting significantly reduced the protein content of resulting BDM processed from sandfish compared to non-salted BDM (Chapter 3) and it is reasonable to assume

Table 4.4. Major amino acids reported for various sea cucumber species. Samples reported in the table are for fresh sea cucumbers

Sea cucumber species	Amino acid	Amino acid content (%)	References
Holothuria scabra	Glycine	18.38	Omran (2013)
	Aspartic acid	4.81	Omran (2013)
	Alanine	6.52	Omran (2013)
	Hydroxyproline	6.30	This study
	Arginine	9.16	This study
	Glycine	16.90	This study
	Aspartic acid	8.93	This study
	Glutamic acid	15.11	This study
	Alanine	8.79	This study
	Proline	9.59	This study
Parastichopus californicus	Hydroxyproline	2.6	Bechtel (2012)
	Glycine	12.3	Bechtel (2012)
	Aspartic acid	11.8	Bechtel (2012)
	Glutamic acid	13.1	Bechtel (2012)
	Alanine	5.6	Bechtel (2012)
	Proline	6.7	Bechtel (2012)
Astichopus japonicus	Aspartic acid	10.8 – 11.3	Lee et al. (2012)
	Glutamic acid	4.53 – 5.12	Lee et al. (2012)
Holothuria aernicola	Aspartic acid	15.71	Haider (2015)
	Glycine	17.33	Haider (2015)
Actinopyga mauritiana	Aspartic acid	10.83	Haider (2015)
	Glycine	21.70	Haider (2015)
Holothuria tubulosa	Glycine	10.36	Sicuro (2012)
	Aspartic acid	5.60	Sicuro (2012)
	Glutamic acid	8.03	Sicuro (2012)
Holothuria polii	Glycine	7.42	Sicuro (2012)
	Aspartic acid	4.49	Sicuro (2012)
	Glutamic acid	6.18	Sicuro (2012)

that such protein loss would result in reduced tissue amino acid content when expressed on a dry tissue weight basis as it was in this study. Loss of protein as a result of salting has also been reported for fish tissues (Jittinandana et al., 2002; Nketsia-Tabiri and Sefa-Dedeh, 1995), with protein extraction from tissue being a function of salt concentration attributed to the protein denaturing effect of salt (Sampels, 2015; Thorarinsdottir et al., 2004; Thorarinsdottir et al., 2011a; Thorarinsdottir et al., 2011b).

Chapter 5

Isolation and characterization of collagen from the body wall of the tropical sea cucumber *Holothuria scabra* (Sandfish) Jaegar 1833[4]

5.1 Introduction

Over 1200 species of sea cucumbers are known of which 58 are commercially exploited (Ferdouse, 2004; Purcell et al., 2012b). Sea cucumbers are usually processed into a dried product called bêche-de-mer (BDM) (*iriko* in Japanese, *hai-som* in Chinese or *trepang* in Indonesian) (Bumrasarinpai, 2006a; Ferdouse, 1999; McElroy, 1990), and at least 15,000 tonnes of BDM are traded annually in S.E. Asia (Qao et al., 2011), with major markets in China, Hong Kong, Taiwan, Singapore and Malaysia (Eriksson and Clarke, 2015; Ferdouse, 2004). BDM is consumed as a delicacy and for broad perceived medicinal benefits (Bordbar et al., 2011; Esmat et al., 2013). For example, consumption of sea cucumbers is thought to aid growth, blood clotting and wound healing (Gil, 2002; Mat et al., 1994) supporting their use as a traditional remedy for burns and cuts (Fredalina et al., 1999). Sea cucumbers and sea cucumber extracts are consumed as traditional remedies for many ailments (Anderson, 1988; Bordbar et al., 2011; Chen, 2003; Jilin and Peck, 1995; Weici, 1987; Wen et al., 2010; Yaacob et al., 1997) and for their unique biological and pharmacological properties including anti-angiogenic (Tian et al., 2005), anticancer (Roginsky et al., 2004), anti-hypertension (Hamaguchi et al., 2010), antioxidant (Althunibat et al., 2009) and antithrombotic (Mourao et al., 1998; Pacheco et al., 2000) properties.

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The major components of the BDM body wall are collagenous fiber and mucopolysaccharides that may contribute up to 83% of BDM dry weight (Chen, 2003; Saito et al., 2002). Pepsin-solubilized collagen (PSC) from *S. japonicus* has been shown to improve proliferation of human keratinocytes (Park et al., 2012). So far, research on sea cucumber collagen have focused mainly on functions of hydrolytic bioactive peptides (Gómez-Guillén et al., 2011; Gomez-Guillen et al., 2002; Park et al., 2012). Research to isolate, purify and characterize PSC have been carried out with some sea cucumber species including *S. japonicus* (Cui et al., 2007; Saito et al., 2002), *Parastichopus californicus* (Liu et al., 2010), *S. vastus* (Abedin et al., 2013), *Bohadschia bivitatta* (Siddiqui et al., 2013) and *Holothuria parva* (Adibzadeh et al., 2014). However, little is known about the structure and composition of the collagen of sea cucumbers, particularly tropical species.

At least 27 different types of collagen have been identified and characterized in the animal kingdom. They vary in their properties and potential applications. Collagen has a triple-helix structure (Gelse et al., 2003; Gómez-Guillén et al., 2011) and is very insoluble because of covalent cross-links formed by non-helical telopeptides of adjacent fibres. However, partial hydrolysis of collagen produces gelatin that is soluble (Gómez-Guillén et al., 2011) and used broadly in the food, photographic, cosmetic, and pharmaceutical industries because of its gel-forming and water-binding properties (Gómez-Guillén et al., 2011; Gomez-Guillen et al., 2002).

The primary structure of type I collagen is characterized by continuous repeating of a Gly-X-Y sequence (where X is mostly proline and Y is mostly hydroxyproline), and the very short N- and

C-terminal regions called telopeptides (15 to 26 amino acid residues) (Abedin et al., 2013; Adibzadeh et al., 2014; Chen et al., 2015b; Cui et al., 2007; Liu et al., 2007 ; Liu et al., 2010; Saito et al., 2002; Sikorski et al., 1984). The Gly-X-Y repeating sequence in the α1 chain plays an important role in triple helix formation of secondary structure (Gelse et al., 2003; Gómez-Guillén et al., 2011). It is used in the food, cosmetic, pharmaceutical, biomedical, and tissue engineering industries because of its excellent biocompatibility and biodegradability (Liu et al., 2010). The few studies that have so far characterized the collagen content of sea cucumbers have reported that they contain type 1 collagen (Abedin et al., 2013; Adibzadeh et al., 2014; Cui et al., 2007). However, isolation and characterization of collagen from tropical sea cucumbers is scarce, with only one prior study reporting the presence of type 1 collagen in *Stichopus monotuberclatus* (Zhong et al., 2015). Prior research in this laboratory has identified the presence of collagen in the tropical sandfish, *Holothuria scabra* (Chapter 4) (Hair et al., 2018), but the type of collagen present was not determined. The body wall of *H. scabra* is a rich source of protein and collagen (Chapter 4) and collagen from tropical aquatic species has high industrial value (Zhong et al., 2015). The hypothesis tested in this study was that *H. scabra,* like all other sea cucumbers so far tested, contains type 1 collagen; it was addressed through isolation and characterization of collagen from *H. scabra* for the first time.

5.2 Materials and Methods

This study was conducted at the School of Biological and Chemical Sciences laboratory facility of the University of the South Pacific, Fiji, and the Food Science Precinct, Department of Agriculture and Fisheries, Queensland Government, Australia.

5.2.1 Materials

Tropical sea cucumbers, *Holothuria scabra*, with a mean (±SE) weight of 242.48 ± 19.55 g were harvested from Tavua Bay, Fiji Islands. Three individuals were gutted and the body wall cut into small pieces (approx. 10 x 10 mm) and kept chilled in ice in a cooler box. The samples were immediately transported to the laboratory at the University of the South Pacific and kept at -80^{o}C before being freeze-dried and crushed using the Retch-MM301 mixer mill and lyophilized. Pepsin (EC 3.4.23.1), type I collagen from calf skin (calf skin collagen, CSC), β-mercaptoethanol (β-ME), Endoproteinase Glu-C from *Staphylococcus aureus* V8 protease (EC 3.4.21.19), was purchased from Sigma-Aldrich Co. Australia. Premixed protein marker ranging from 10 to 200 kDa and 7.5% precast gels was purchased from Sigma-Aldrich Co Australia. Other reagents used in this study were all of analytical grade.

5.2.2 Preparation of crude collagen fibril (CC)

The water-EDTA-water method (Cui et al., 2007; Zhong et al., 2015) was used to extract sea cucumber crude collagen fibril (CC) with a slight modification. All operations were performed at 4^{o}C. Briefly, lyophilised *H. scabra* tissue samples were homogenized in deionized water and centrifuged at 10000 × g for 10 min. The resulting pellet was stirred with fresh deionized water for 1 h and then collected by centrifugation. Subsequently, 10 volumes of EDTA (4 mM, dissolved in 0.1 M Tris-HCl, pH 8.0) were added to the pellet. After 48 h stirring, the centrifugate was washed with deionized water for several times. Ten volumes of 0.1 M NaOH was then added and stirred for 72 h. After alkali extraction, the sample was centrifuged at 20000 × g for 30 min, and the resulting pellet was washed several times with deionized water to neutral.

Finally, the CCF of the upper layer was lyophilized and stored at −80^{o}C awaiting subsequent analysis.

5.2.3 Isolation of pepsin-solubilized collagen (PSC)

Pepsin-solubilized collagen (PCS) of *H. scabra* was isolated and purified as described previously (Saito et al., 2002; Zhong et al., 2015) and all operations were performed at 4 ˚C. Briefly, lyophilized CCF was dissolved in 500 volumes (v/w) of 0.5 M acetic acid, mixed with pepsin (3200 – 4500 Units/ mg) and stirred for 48 h. The supernatant containing pepsin-soluble collagen was collected by centrifugation at 25000 × g for 60 min., and PSC was salted out by adding NaCl to a final concentration of 0.8 M with slight stirring for 24 h. The precipitate containing PSC-Sm was collected by centrifugation at 7500 × g for 10 min and dissolved in 0.5 M acetic acid again. The sample was dialyzed against 0.02 M Na_2HPO_4 (pH 8.0), 0.1 M acetic acid, and deionized water successively. Each dialysate was stirred slightly for 48 h and changed every 6 h. The purified PSC was finally lyophilized and stored at −80^{o}C.

5.2.4 UV-vis spectra

Collagen samples were dissolved in 0.5 M acetic acid to a final concentration of 1 mg/mL, and a UV-vis spectra analysis was recorded using Pekin Elmer UV- vis spectrophotometer Lambada 365 with wavelength ranging from 190 to 400 nm using 0.5 M acetic acid as the blank control.

5.2.5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970) and Zhong et al. (2015). Collagen samples were dissolved in 0.1 M phosphate-buffered sodium (pH 7.2, with 1% SDS and 3.5 M urea) to a final concentration of 1 mg/mL, and stirred at 4^{o}C for 12 h. After centrifugation at 3000 × g for 5 min, 20 μL supernatant was taken and mixed with 5 μL 5× sample loading buffer in the presence or absence of β-ME, and boiled at 100 ˚C for 3 min, then applied to discontinuous tris-glycine buffer system electrophoresis with 7.5% precast gel. Electrophoresis was conducted at 200 V for 50 min using Power Pak Model. The gel was then stained with 0.1% Coomassie brilliant blue R-250 and imaged using a Bio-Rad Gel Doc^{TM} E2 Imager and image gel 5.0 version software.

5.2.6 Peptide mapping

Peptide mapping of the collagen samples was performed using the methods of Zhong et al. (2015) and Ahmad et al. (2010). Collagen samples (0.2 mg) were dissolved in 0.1 mL of phosphate-buffered sodium (0.1 M with 0.5% SDS, pH 7.2), and mixed with 10 μL of the same buffer containing 5 μg Endoproteinase Glu-C from *Staphylococcus aureus* V8 protease (500 to 1000 Unit/mg solid) and then incubated at 37 ˚C for 30 min. The reactions were terminated by boiling at 100 ˚C for 3 min. Protease digested peptides were separated by SDS-PAGE with 7.5% precast gel.

5.2.7 Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) spectra were measured using a Nicolet infrared spectrophotometer 5700 (Thermo Electron Cooperation) with infrared spectra recorded from 4000 to 500 cm^{-1} at a data acquisition rate of 2 cm^{-1} per point.

5.2.8 Collagen solubility test

The solubility of PSC at various pH and NaCl concentrations was determined according to the method of Zhong et al. (2015), Kittiphattanabawon et al. (2005) with slight modification. Briefly, PSC was weighed and dissolved in 0.5 M acetic acid, then stirred for 12 h. All operations were performed at 4 ˚C.

5.2.8.1 Effect of pH on collagen solubility

Aliquots (1.2 mL) of PSC solution (3 mg/mL) were transferred into individual centrifuge tubes, and the pH adjusted with 6 M NaOH or 6 M HCl to a final pH ranging from 1.0 to 10.0. Deionized water was added to adjust the solutions volume up to 1.5 mL. Solutions were stirred gently for 30 min. and centrifuged at 20000 × g for 30 min. The supernatant was 10-fold diluted with deionized water, and the hydroxyproline content of supernatant was determined according to the ISO method (3496: 1978). Relative solubility was calculated in comparison with that obtained at the pH exhibiting the highest solubility.

5.2.8.2 Effect of NaCl concentration on collagen solubility

Aliquots (0.75 mL) of PSC solution (6 mg/mL) were transferred into individual centrifuge tubes, and an equal volume of NaCl in 0.5 M acetic acid with various concentrations (0%, 2%, 4%, 6%, 8%, 10%, and 12%, w/v) was added to each to obtain final NaCl concentrations of 0%, 1.0%, 2.0%, 3.0%, 4.0%, 5.0%, and 6.0% (w/v). Solutions were stirred gently for 30 min and centrifuged at 20000 × g for 30 min. The hydroxyproline content of supernatant and relative solubility was determined as stated above.

5.3 Statistical analysis

All analyses were conducted in triplicate using tissue samples from three individuals. Least Significant Difference (LSD) test was used to compare means and significant differences between means were established at the level of $P < 0.05$. Analysis was performed using SPSS Version 24 statistical software.

5.4 Results

Collagen fibers exist as insoluble and soluble fibers with the majority being insoluble. The yield of crude collagen fibrils from the present study was 19.2% while the pepsin-soluble collagen yield was 32%. The UV-vis spectra of PSC, CC and calf skin type I collagen (CSC) exhibited the maximum absorbance at a range of 240–250 nm (Fig. 5.1). Collagen samples were separated by SDS-PAGE and are shown in Fig 5.2. The β and γ components including the α1 and α2 chains of higher molecular weight was separated during the SDS-PAGE. There were no differences

observed between the reducing and non-reducing patterns (Fig. 5.2 a, b). Collagen from the body wall of *H. scabra* was found to be type I collagen; however, the triple helix was formed by 3 homologous α1 chains as (α1)3 with a molecular weight of 133.2 kDa. With digestion by V8 protease, the α1 chain of PSC was partially degraded and the β and γ of the crude collagen was degraded (Fig. 5.2)

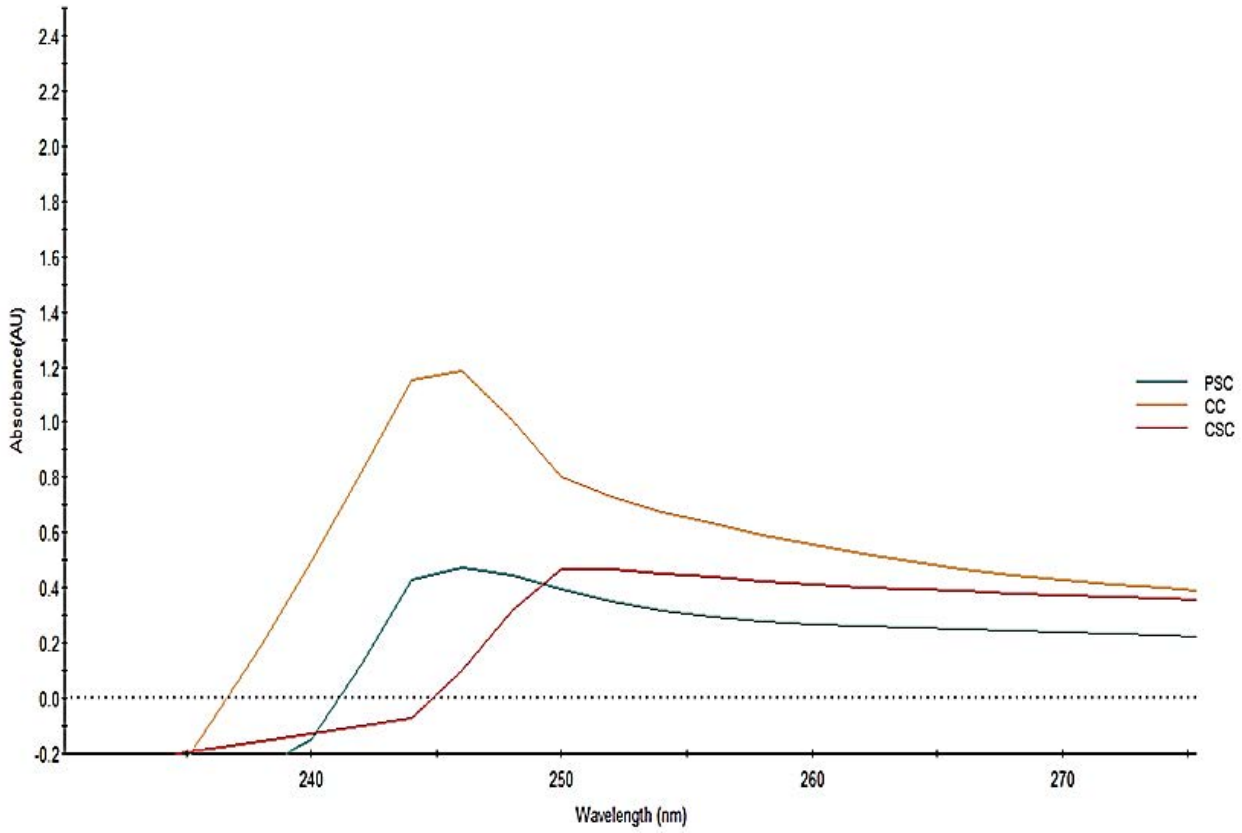

Figure 5.1. UV-vis spectrum of collagen (crude (CC) and pepsin soluble collagen (PSC)) from *H. scabra* and calf skin (CSC)

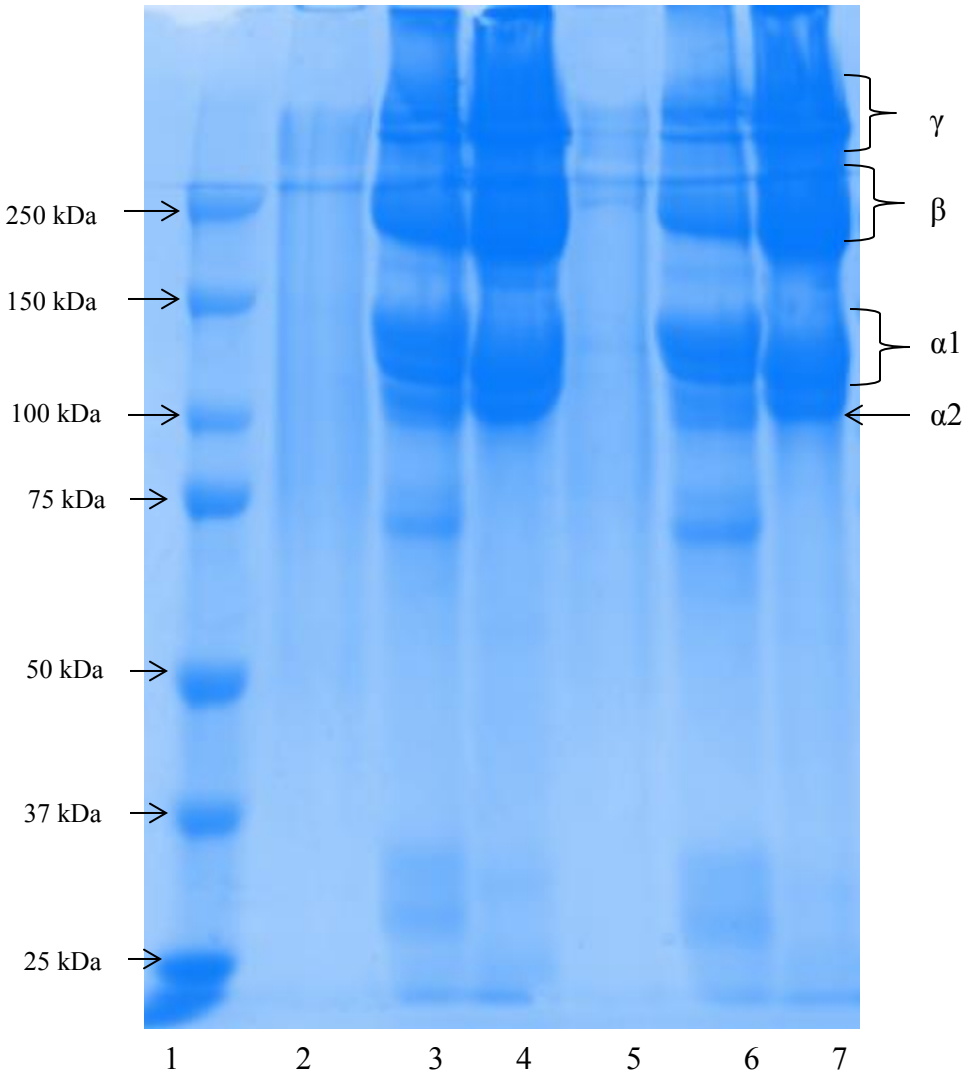

Figure 5.2a. SDS PAGE pattern (reducing with β-mercaptoethanol) of collagen (PSC and CC) from *H. scabra* and calf skin type I collagen. Lane 1: protein marker; Lane 2: CC with β-mercaptoethanol; Lane 3: PSC with β-mercaptoethanol; Lane 4: CSC with β-mercaptoethanol; Lanes 5, 6 and 7: CC, PSC and CSC digested by V8 protease.

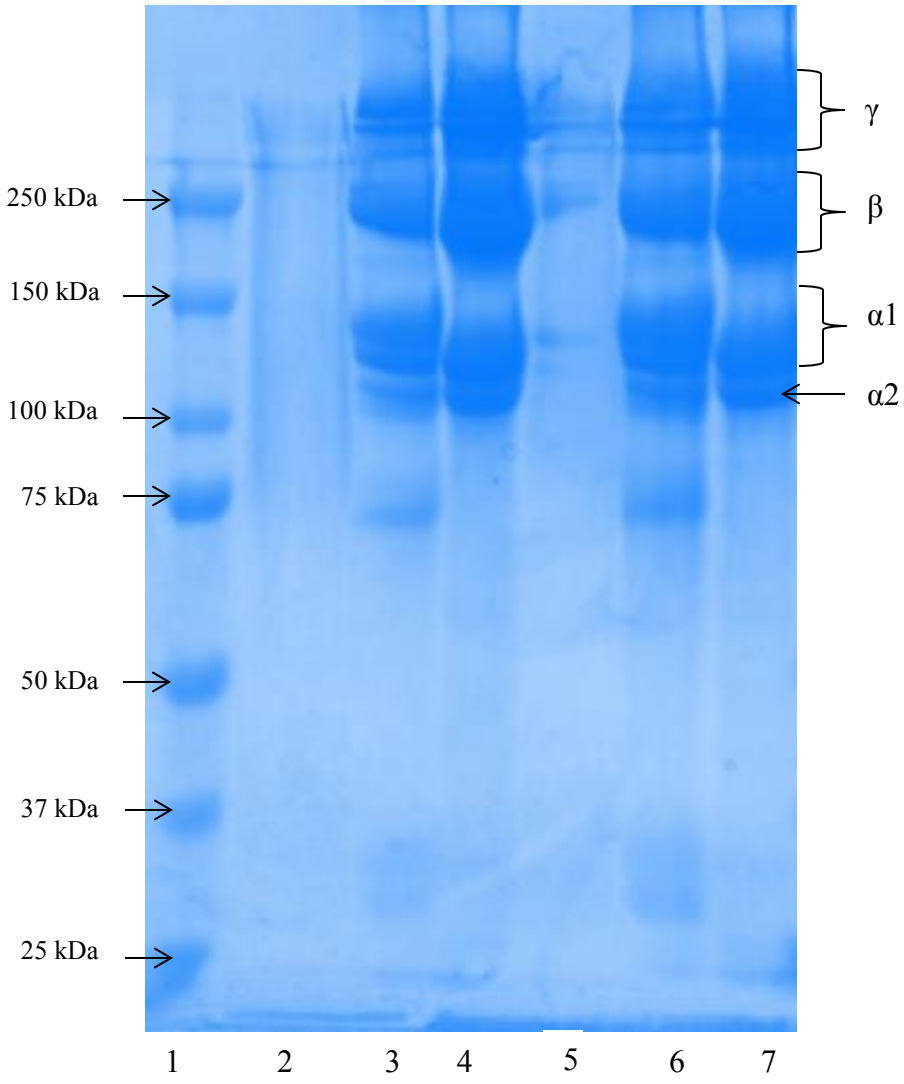

Figure 5.2b. SDS PAGE pattern (non-reducing without β-mercaptoethanol) of collagen (PSC and CC) from *H. scabra* and calf skin type I collagen. Lane 1: protein marker; Lane 2: CC without β-mercaptoethanol; Lane 3: PSC without β-mercaptoethanol; Lane 4: CSC without β-mercaptoethanol; Lanes 5, 6 and 7: CC, PSC and CSC digested by V8 protease.

The FTIR spectra of PSC-Sm and CSC are shown in Fig. 5.3. The characteristic amide bands for collagen, including amide A, amide B, amide I, amide II, and amide III, were observed in both PSC and CSC. The amide III band is related to C–N stretching vibrations, N–H deformation from amide linkages, and CH_2 group wagging vibrations of glycine and proline in α1 chain. The Amide A band was due to stretching vibrations of the N–H group. A free N–H stretching vibration occurred in the range of 3400 - 3440 cm^{-1}, and the shift of amide A to lower wavenumber was associated with the increasing of hydrogen bonding by N–H groups.

The effect of pH on the solubility of PSC is shown in Fig. 5.4a. As pH increased from 1 to 2, solubility gradually increased, with maximum solubility at pH 2 ($P < 0.05$). When pH increased to 4, however, the solubility decreased to the lowest ($P<0.05$). Further increasing of pH from 6 to 10 increased the solubility from 70% - 90%. The effect of NaCl concentration on the solubility of PSC is shown in Fig. 5.4b. A sharp decrease in pH solubility was observed between 2% and 4% NaCl concentration as collagen samples salted out.

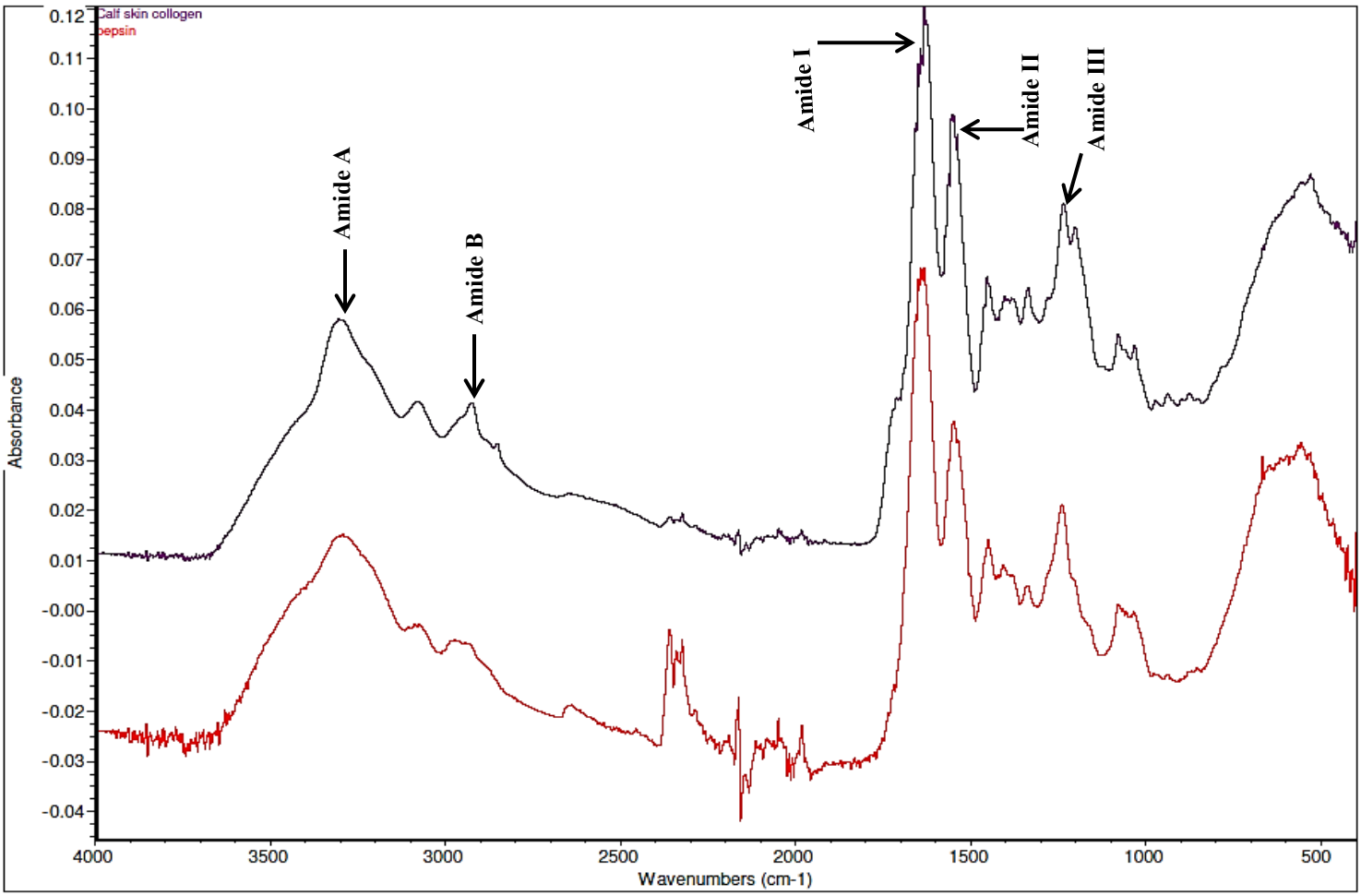

Figure 5.3. Fourier-transform infrared specra of collagen (PSC) from *H. scabra* and calf skin

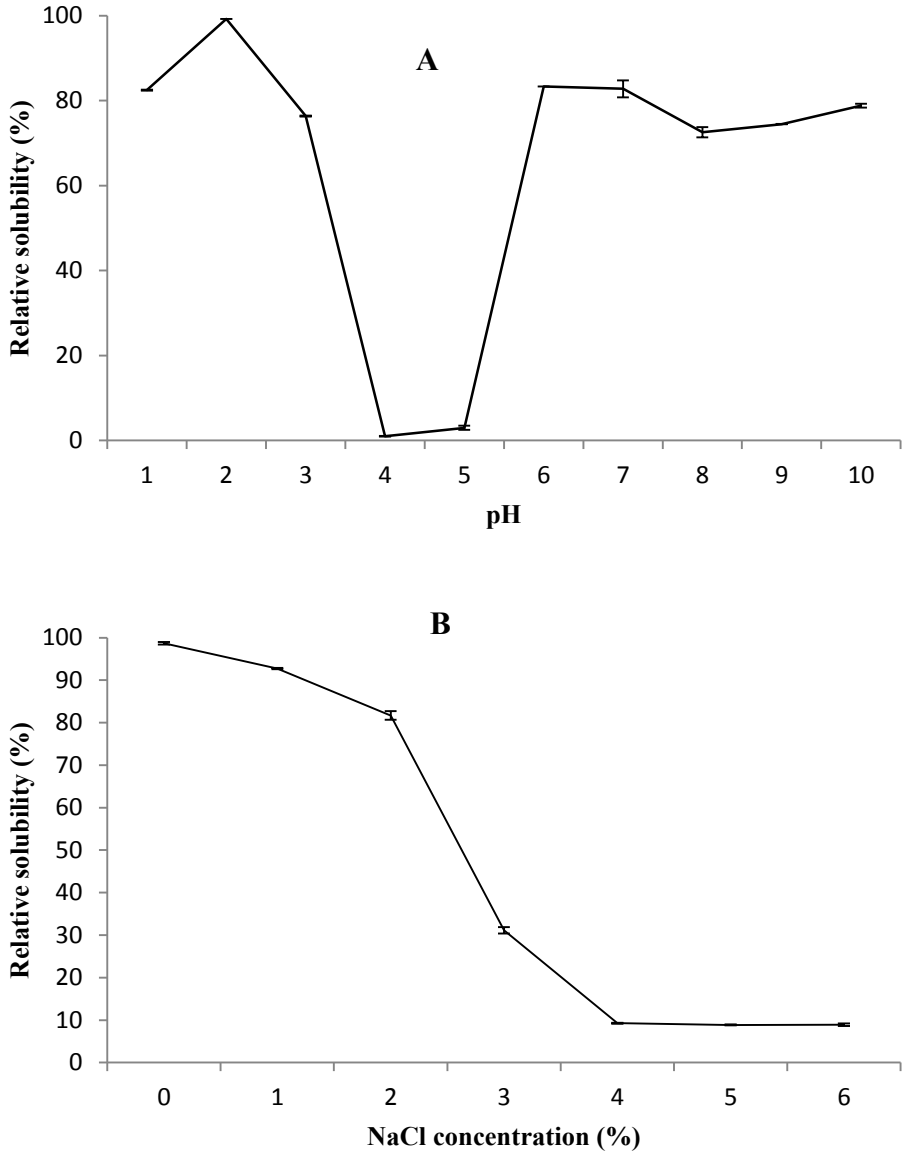

Figure 5.4. Relative solubility of PSC from H. scabra in 0.5 M acetic acid in different pH's (A) and in different NaCl concentrations (B). Error bars represent the standard deviation (n=3).

5.5 Discussion

This study focused on the isolation and characterization of collagen from a tropical sea cucumber *H. scabra*. The extraction rate of the collagen samples for *H. scabra* in this study was found to be lower than that of temperate sea cucumber species such as *S. japonicus* (Saito et al., 2002) which had a high yield of 60%. Acid-solubilized collagen (ASC) can be extracted by damaging the salt linkages and Schiff base in the cross-link structure with weak acid, whereas PSC can be extracted with the effect of pepsin on hydrolyzing nonhelical telopeptides participating in cross-links (Liu et al., 2010; Zhang et al., 2007). The PSC yield of *H. scabra* was found to be higher than that of *Parastichopus californicus* (24.3%) on dry weight basis (Liu et al., 2010). Based on research with *Stichopus monotuberculatus*, Zhong et al. (2015) indicated that collagen from tropical sea cucumbers is of high value and of pharmaceutical grade.

Triple helical structure has been reported to be proportional to the total content in pyrrolidine imino acids, given that it is the Proline + Hydroxyproline rich zones of the molecules that are most likely to be involved in the formation of nucleation zones (Ledward, 1986). Although Proline is important, Hydroxyproline plays an important role in the stabilization of the triple-stranded collagen helix due to its hydrogen-bonding ability through its -OH group (Burjandze, 1979; Ledward, 1986). The results in Chapter 4 have found higher concentrations of Proline and Hydroxyproline (5.81 and 3.82 mg/g, respectively) in the body wall of *H. scabra*. However, the total Glycine-Proline-Hydroxyproline sequence content is one of the main factors affecting collagen thermostability (Burjandze, 2000).

The UV-vis spectrum of a protein concentration is analysed by its amino acid composition (Zhong et al., 2015). The concentration of tyrosine, phenylalanine, and tryptophan, have maximum absorbance at 250-280 nm, are usually low in collagen, and as a result, the maximum absorbance of collagen is at about 230 nm (Cui et al., 2007). In this study, the wavelengths with maximum absorbance of PSC and CSC were in the range of 240-250 nm, indicating that the amino acid composition of tyrosine, phenylalanine, and tryptophan is lower in *H. scabra* as reported in Chapter 4.

Type I collagen consists of heterologous α1 and α2 chains forming triple helix as $(\alpha1)_2$ α2, whereas β and γ components of higher molecular weight are dimers and trimers of α chain, respectively (Cui et al., 2007; Zhong et al., 2015), which was observed in other sea cucumber species such as *S. monotuberclatus* (Zhong et al., 2015), *S. japonicus* (Cui et al., 2007), *Cucumaria frondosa* (Trotter et al., 1995), *P. californicus* (Liu et al., 2010), *S. vastus* (Abedin et al., 2013), and *H. parva* (Adibzadeh et al., 2014). In the present study, α1, β,and γ components were determined for the collagen samples, separated by SDS-PAGE, and the triple helix was found to be formed by α1 chains with molecular weight of 133.2 kDa, which was consistent with those of type I collagen in other sea cucumbers such as *S. monotuberculatus* (137 kDa) (Zhong et al., 2015), *S. japonicus* (135 kDa; Cui et al. (2007), *P. californicus* (138 kDa; Liu et al. (2010), and *H. parva* (130 kDa; Adibzadeh et al. (2014). This indicates that the molecular weight of collagen from sea cucumber has no difference among species (Zhong et al., 2015).

The FTIR image of the collagen samples in the present study was found to be similar to the previously extracted Type I collagen from a number of sea cucumber species including *S. monotuberculatus* (Zhong et al., 2015), *S. vastus* (Abedin et al., 2013), *H. parva* (Adibzadeh et al., 2014), *Parupeneus heptacanthus* (Matmaroh et al., 2011a) *Priacanthus tayenus* (Kittiphattanabawon et al., 2005), *Lates niloticus* (Muyonga et al., 2004) and *Ctenopharyngodon idella* (Chen et al., 2015b). The FTIR of PSC in the present study was found to be similar to the Type I calf skin collagen indicating that the collagen found in the *H. scabra* was Type I. Because the wavenumber of amide A in CSC and PSC was higher could be due to more N–H groups participating in hydrogen bond association (Zhong et al., 2015). The Amide B band is related to the asymmetric stretch vibrations of =C–H and $–NH3^{+}$, and the shift of amide B to higher wavenumber is associated with an increase of free $NH3^{+}$ groups from lysine residues of N-terminal (Doyle et al., 1975).

The glutamic acid and aspartic acid contents of the *H. scabra* body wall has was reported in Chapter 4 as 9.91 and 5.39 mg/g of fresh tissue, respectively, with glutamic acid being one of the dominant amino acid in *H. scabra*. In the present study we found that V8 protease shows a high specific preference for glutamic acid and aspartic acid residues in proteins (Vercaigne-Marko et al., 2000) which could be the reason for the difference in their V8 protease digested peptide maps. During the digestion process of proteins using V8 protease, the presence of β, and γ components (covalently linked α-chain dimers and trimers, respectively), together with higher molecular weight forms as well as low-molecular weight protein degradation fragments (Stainsby, 1987). This was evident through peaks occurring in the SDS-PAGE (25 – 37 kDa) that could be further investigated for its significance. A decrease in β, and γ components and an

increase in the presence of degradation fragments, are normally the result of the application of more intense extracting conditions (pH, temperature, time), which is normal industrial practice for yield improvement (Johnston-Banks, 1990).

Therefore, the results in wavenumbers of amide B indicated PSC had fewer free $NH3^{+}$ groups than CSC. Generally, the amide I band, occurs at the wave number ranging from 1600 - 1700 cm^{-1} due to C=O stretching vibrations accompanied with N–H bending vibrations, CN stretch and CCN deformation (Zhong et al., 2015). The amide I band is also considered as a sensitive marker of protein secondary structure (Surewicz and Mantsch, 1988). The previous studies indicate that the secondary structures was exhibited in the amide I region: β-turn (1660 - 1700 cm^{-1}), α-helix (1645 - 1659 cm^{-1}), irregular structure (1640 - 1644 cm^{-1}), and β-sheet or extended structure (1620 - 1640 cm^{-1}), and shift of amide I to lower wavenumber was associated with the conversion of secondary structures from regular to irregular form (Farrell et al., 2001). The carbonyl (C=O) group generates hydrogen bonding with neighboring chains by stretching vibrations, therefore, a shift in amide I to lower wavenumber was also related to an increasing number of hydrogen bonds, formed by the carbonyl group stretching vibrations (Li et al., 2004). The collagen samples in the present study had an identical frequency of amide I. Amide II band indicates the degree of N–H groups involved in hydrogen bonding with the neighboring α chain, therefore, the shift of amide II to lower wavenumber is associated with the increasing of hydrogen bonds by N–H groups (Jackson et al., 1995). In this study, the wavenumber of amide II in CSC was higher than that in PSC, indicating that PSC had more hydrogen bonds between adjacent α chains. Hydrogen bonds formed by the carbonyl group and N–H stretching vibrations were the main force for maintaining triple helix, and the lower energy of hydrogen bond

association indicated a more stable force between peptide chain (Payne and Veis, 1988). Since the PSC had higher hydrogen bond energy than CSC, the triple helix of PSC was not as stable as CSC.

The collagen molecule requires specific pH for solubility. When the pH of a protein molecule is at isoelectric point, a total net charge residue is zero and the hydrophobic-hydrophobic interaction increases leading to precipitation and aggregation. The repulsive forces between chains can increase solubility, which is originated from increasing net charge of protein molecules residues (Vojdani, 1996). The removal of telopeptide regions further influences the protonation and deprotonation of charged amino and carboxyl groups respectively. Thereby affecting the repulsion of molecules correlated with the different solubility (Chen et al., 2015b; Matmaroh et al., 2011a). The isoelectric point of collagen has been reported at pH 6 - 9 (Ahmad et al., 2010; Jongjareonrak et al., 2005). Collagen solubility in the present study, was lowest at pH 4 which could be due to higher glutamic acid (16.2%) content of *H. scabra* (Chapter 4) compared to those of *H. parva* (7.4% Adibzadeh et al. (2014), and *S. japonicus* (10.4% Liu et al. (2010) and other fish species such as *Priacanthus tayenus* (7.8%, Kittiphattanabawon et al. (2005) and *Parupeneus heptacanthus* (6.9%, Matmaroh et al. (2011a).

Salt also affects collagen solubility. Salting ions can competitively bind to water molecules within collagen, then decrease surface hydrated layer of collagen. Thereby, collagen may aggregate and precipitate with the increasing of hydrophobic–hydrophobic interaction when the concentration of salt ions rises (Vojdani, 1996). The salting out effect of collagen molecules

occurred at a NaCl concentration of 4.0%. Similar findings were also observed for collagens extracted from *H. parva* (Adibzadeh et al., 2014), *S. japonicus* (Liu et al., 2010) and other fish species such as *Priacanthus tayenus* (Kittiphattanabawon et al., 2005), *Aluterus Monoceros* (Ahmad et al., 2010) and *Parupeneus heptacanthus* (Matmaroh et al., 2011a). The water binding ability of the collagen molecule is influenced by its molecular weight and radius, and there were no differences observed between PSC here, and PSC extracted in previous studies; therefore, PSC in this study showed identical water binding ability and concentration of salting out phenomenon as previously investigated.

Chapter 6

Assessing novel processing and packing methods on the quality and the nutrient composition of Sandfish (*Holothuria scabra*)

6.1 Introduction

Sea cucumbers have been harvested and processed to a dry form known as bêche-de-mer (BDM) for more than two centuries. BDM is regarded as a high quality seafood with significant medicinal value (Bordbar et al., 2011). Traditional BDM processing involves an uncomplicated sequence of actions resulting in a product that is non-perishable for a year or more that does not require refrigeration (Purcell et al., 2014). Briefly, traditional post-harvest steps include first cooking, slitting and gutting, second cooking, smoking and finally sun-drying (Chapter 2) (Kinch, 2002; Mangubhai et al., 2016; Purcell, 2014a). Processing BDM may also involve a 'salting' step where a saline solution or coarse salt is used to draw water from sea cucumber tissues (Lavitra et al., 2008) to facilitate dehydration and shrinking of the tissue. This general processing method was developed in the 1800s and has changed little since. The resulting product is hard and requires soaking to reconstitute before cooking and consumption. Reconstitution involves soaking, heating in the soaking solution, cooling and repetition of this process several times (Fukunaga et al., 2004).

Recent years have seen increasing demand for consumer-friendly, non-dried, ready to cook sea cucumber products (Purcell, 2014b). These include sea cucumbers that are: (1) gutted and frozen, and available bagged or unpackaged; (2) cooked vacuum-packed but not refrigerated; and (3) cooked, vacuum-packed and frozen. Vacuum packaging removes the air surrounding the product

slowing the growth of spoilage bacteria and extending shelf-life; however, shelf-life extension of 'fresh' vacuum-packaged products that have not been otherwise cured (e.g. salting, smoking or use of other curatives) may be limited as the main spoilage organisms are not removed (Bremner, 2012). Yet despite increasing demand for ready to cook sea cucumber products and increasing reliance on appropriate packaging of these products, the potential of advanced packaging methods for sea cucumbers has received little research attention, despite being widely used in the broader seafood packaging industry (Mertens, 1993; Rastogi et al., 2007; Venugopal, 2006).

Modified atmospheric packaging (MAP) involves a vacuum being drawn and then replacement of the atmosphere with another gas or mixture of gases. MAP utilizes three main gases: nitrogen, oxygen and carbon dioxide – that are used to modify the atmospheric conditions of the food being packed. Gases used in MAP generally contain at least 40% carbon dioxide (CO_2) (Bremner, 2012). Reduction in the pH, caused by dissolution of CO_2 into product juices, forms carbonic acid which has a preservative action. Nitrogen is a tasteless gas that displaces oxygen and is highly soluble in water; it is associated with maintaining freshness of the product (Kostaki et al., 2009; Mastromatteo et al., 2010a; Wang et al., 2008). The lower pH and low oxygen tension conditions generated with MAP stimulates growth of lactobacilli which are inhibitors of Gram-negative spoilage bacteria, greatly improving chilled shelf-life. MAP supports improved shelf life without affecting product freshness, and sealed MAP products provides a barrier to the environment and potential contaminants thus improving the overall presentation and shelf life of the packaged product. MAP has been used with part-cooked seafood products, but despite its potential for packaging cooked sea cucumbers, this has not been investigated in prior studies.

The aim of this study was to assess the potential of MAP for novel processing and packaging of tropical sea cucumbers (Sandfish; *Holothuria scabra*) based on shelf stability and nutrient composition of the product. The findings of this study will assist sea cucumber processors in developing a novel processing and packaging methods that address contemporary market developments for sea cucumber products.

6.2 Materials and Methods

This study was conducted at the post-harvest processing facility of the University of the South Pacific, Fiji, and the Food Science Precinct, Department of Agriculture and Fisheries, Queensland Government, Australia. Sandfish were collected from Tavua Bay on the north coast of the main island of Viti Levu, Fiji Islands (17°26'29.4"S 177°51'44.4"E). Individuals were gutted and left in an insulated fish box containing ice. All processing and packaging treatments were conducted in triplicate.

6.2.1 Sea cucumber processing

Sea cucumbers were first cooked at a water temperature of 45^{o}C for 10 min before water temperature was gradually increased to 80^{o}C (Fig. 1). They were cooked for a further 10 min until they were hard and springy indicating completion of the first cook. Cooked sea cucumbers were then immersed in a 3% (w/v) saline solution for 36 h to allow the outer layer of spicules to disintegrate (Purcell, 2014a). Processing of sea cucumbers, with ossicles removed, was then completed using one of three methods:

- Kench salted for 48 h using grade 11 coarse solar salt; cooked for the second time for 15-25 min (Ram et al. 2014c) (at 45^{o}C rising to 96^{o}C (temperature monitored using a probe thermometer with occasional stirring of the water every 2-3 min) and then solar dried for at least 2 - 3 weeks;
- Not salted; cooked for the second time for 15-25 min (at 45^{o}C rising to 96^{o}C) and then solar dried for at least 2 - 3 weeks;

All sea cucumbers were then cooked for a third time for 5-15 min (at 45^{o}C rising to boiling), followed by shape correction (straitening and closure of body cut) to assist market acceptability, before the product was finally dried using solar drying. Processed sea cucumbers from each of these treatments were the reconstituted (Fukunaga et al., 2004) for use in the novel packaging trial.

6.2.2 Novel packaging trial

Five fresh cooked sea cucumbers and reconstituted BDM treatments were used to assess the potential of MAP and vacuum packaging for sea cucumbers.

- Cooked at 80-90^{O}C (first cook)
- Cooked at 80-90^{O}C and treated with essential oil (0.6% thymol in 1% cavacrol)
- Cooked at 80-90^{O}C and cold smoked
- Dry processed without salting and reconstituted
- Dry processed with kenching (48 h) and reconstituted

Based on prior testing of a range of essential oil combinations in minimum inhibitory dosage (MID) tests on sea cucumber extract for *Salmonella typhimurium* and *Escherichia coli* and *Stapylococcus aureus* (0.1-1.4% thymol in 1% cavacrol and 0.1-1.4% cavacrol in 1% thymol), a combination of 0.6% thymol in 1% cavacrol was selected for use in this study and was prepared according to the method of Lambert et al. (2001). Cooked sandfish was treated with approximately 2-3 mL of the essential oil prior to packaging. In the cold smoked treatment, cooked sandfish were smoked using a Mauting smoking machine 046/12 under a cold smoking computer program for 2 h at 15^{o}C. In treatments using dried sea cucumbers, BDM was reconstituted using the method of (Fukunaga et al., 2004) for 10 days prior to packaging.

The various packaging methods used for each of the five sea cucumber processing treatments (Table 6.1) were:

- MAP (60% CO_2/40% N) (Özogul et al., 2004)
- MAP (50% CO_2/45% N/5% O_2) (Kykkidou et al., 2009)
- Vacuum
- Normal atmosphere

Individual sandfish was packed in a 15 mL black modified atmospheric packaging (MAP) tray with moisture absorbent pads covered with polyethylene film. MAP packaging was performed using a Mecapack S1000 machine, and vacuum packaged using the Eazy Vac Pty Ltd vacuum machine. A control treatment was packed in normal atmosphere and stored together with the samples from all other treatments.

Table 6.1. Shelf life treatments for novel processing of Sandfish

Processing treatment	Packaging method
Cooked at 80-90^{o}C (control)	• Normal atmosphere • MAP 60% CO2/40% N • MAP 5%O2/50%CO2/45%N • Vacuum
Cooked at 80-90^{o}C; essential oil (0.6% thymol in 1% cavacrol)	• MAP 60% O2/40% N • MAP5%O2/50%CO2/45%N • Vacuum
Cooked at 80-90^{o}C and cold smoked	• MAP 60% O2/40% N • MAP5%O2/50%CO2/45%N • Vacuum
Normal dry processed and reconstituted	• MAP 60% O2/40% N • MAP5%O2/50%CO2/45%N • Vacuum
Salted dried 48h and reconstituted	• MAP 60% O2/40% N • MAP5%O2/50%CO2/45%N • Vacuum

6.2.3 Microbiological assay

Prior to microbiological assay, the gas in the MAP container was assayed using the PBI dansensor 260442-B gas analyzing instrument to determine the gas mix at the particular day of the microbiology assessment.

Microbiology assays were conducted at the Food Science Precinct, Department of Agriculture and Fisheries, microbiology unit, Australia and were performed using the ISO method 4833/1; 2013 for standard plate count and the ISO method 21527/2; 2008 95 for enumerating yeast and mould in food with water activity ranging from 0.4 - 0.95. Sea cucumber samples 10 g were taken and transferred aseptically into a stomacher bag containing 100 mL of 0.1% peptone water and homogenized for 60s using a Lab Blender 400, Stomacher at room temperature. For

microbial enumeration, 1000 µL of the serial dilutions (1:100; 1:1000, diluent, 0.1% peptone water) of the Sandfish homogenate was inoculated in 15 mL of the molten standard plate count agar (APHA CM0463, Oxoid Ltd, Basingstoke Hampshire, England) and left to settle and incubated upside down at 30^{o}C for three days. For yeast and mold enumeration, 100 µL of the serial dilutions (1:100, diluent, 0.1% peptone water) were enumerated using the dry surface of the Dichloran Rose-Bengal Chloramphenicol Agar (DRBC, CM0727 Oxoid Ltd, Basingstoke Hampshire, England) containing Chloramphenicol supplement (SR0078E). The DRBC plates were incubated right side up at 25^{o}C for five days. All plating was done with duplicates and all colonies on the APHA and DRBC plates were counted. Yeast and mold were reported separately. Microbiological data were reported as colony forming units (CFU g^{-1}). All plates were examined visually for typical colony types and morphology characteristics associated with each growth medium. When plate counts reached 10^{6} cfu (colony forming units) per gram of Sandfish tissue it was assumed to be at, or near, spoilage (El-Marrakchi et al., 1990) and was discarded. Microbiological assays were originally planned to be conducted on days 0, 7, 14, 28, 45, 60 and 90. However, samples from all processing treatments, except the cold smoked treatment, regardless of the packaging method, were discarded after 7 days following plate counts that indicated spoilage. At this point (7 days) samples were assessed for proximate and fatty acid compositions as well as collagen content.

6.2.4 Proximate analysis

(See section 3.2.2, Chapter 3)

6.2.5 Lipid class and fatty acid analysis

(See section 3.2.3, Chapter 3)

6.2.6 Collagen analysis

(See section 4.2.4, Chapter 4)

6.2.7 Texture determination

Texture analysis was performed only on the MAP (60% CO_2/40% N) packaged samples of sandfish from the cooked (80-90^oC) and cold smoked treatment because this was the only treatment that was shelf stable beyond 7 days. Texture determinations were measured using a Instron Penetrometer 5543 equipped with 500N load cell modified Kramer shear technique with 2 blade forces instead of 5 (Chapter 4). Analyses were conducted on days 0, 7, 10, 14, 17, 21, 24 and 30.

6.2.8 Statistical analysis

All samples were prepared and analyses in triplicate. Results are expressed as mean values ± standard error mean (SEM) and one-way analysis of variance (ANOVA) was carried out using SPSS version 24 statistical software. Homogeneity of variance (Levene's statistic) was conducted prior to ANOVA. The differences in the nutritional composition for *Holothuria scabra* were tested with ANOVA followed by the multiple comparison test (Tukey HSD). The differences were considered to be significant when $P < 0.05$.

6.3 Results

Microbiological assays of all samples were originally planned to be conducted on days 0, 7, 14, 28, 45, 60 and 90 of the shelf life trial, with the criterion that when plate counts reached 10^6 cfu per gram of sandfish tissue, that it was assumed to be at, or near, spoilage (El-Marrakchi et al., 1990). This point was reached by samples from all processing treatments, except the cold smoked treatment, regardless of the packaging method, after only 7 days. However, the shelf assessment was continued with the cold smoked product which showed microbial counts of zero throughout the shelf life assessment up to 30 days.

The proximate compositions of MAP (60% CO_2/40% N) packaged samples of sandfish from four treatments (cooked at 80-90^oC; salted (48 h), dried and reconstituted; normal dried processed and reconstituted; and cooked at 80-90^oC and cold smoked) at the start and end (7 days) of the shelf life assessment are shown in Table 6.2. There were no significant differences between initial and 7 days samples for moisture, protein, lipid or NFE contents, across treatments. There were no significant differences in the levels of moisture, protein, lipid, ash and NFE between initial treatment samples; however, some significant differences in the ash content between treatments was evident (Table 6.2), and of particular note is the relatively low ash content of samples from the salted (48 h), dried and reconstituted treatment. The fatty acid profile for the MAP (60% CO_2/40% N) packaged samples of sandfish from four treatments is shown in Table 6.3. Again, significant differences in the levels of specific fatty acids between initial (day 0) and final (day 7) samples, and between treatments are scarce. There were no significant differences ($P>0.05$) in the levels of the metabolically important fatty acids C20:4n-6,

20:5n-3, 22:6n-3 between samples from either day, across all treatments (Table 6.3). This was also true of the total level of n-3 polyunsaturated fatty acids (PUFA) in the samples.

Table 6.2. Proximate composition of Sandfish stored at 4^{o}C using various packaging treatments at the start of the storage period (initial) and after 7 days in storage (final). Values are means ± SEM*.

Treatment	Proximate component (mg g^{-1})				
	Moisture	Protein	Lipid	Ash	NFE
Cooked at 80-90^{o}C (initial)	765.22±11.67^{a}	211.10±13.37^{b}	4.17±0.36^{ab}	18.57±2.19^{ab}	0.94±0.94^{ab}
Cooked at 80-90^{o}C (final)	778.56±18.23^{ab}	135.69±37.18^{ab}	3.91±0.47^{ab}	71.06±14.02^{c}	10.78±5.51^{ab}
Salted dried 48 h and reconstituted (initial)	875.61±7.78^{ab}	118.31±7.04^{ab}	2.01±0.44^{ab}	3.52±0.27^{a}	0.55±0.44^{a}
Salted dried 48 h and reconstituted (final)	895.61±16.84^{b}	93.1±13.65^{a}	1.69±0.61^{a}	7.6±2.25^{a}	1.99±1.01^{ab}
Normal dried processed and reconstituted (initial)	812.11±11.01^{ab}	160.00±10.22^{ab}	3.01±0.25^{ab}	24.79±2.02^{ab}	0.87±0.87^{a}
Normal dried processed and reconstituted (final)	838.78±5.73^{ab}	143.89±4.13^{ab}	3.38±0.93^{ab}	11.84±2.67^{a}	2.12±1.48^{ab}
Cooked at 80-90^{o}C and cold smoked (initial)	775.47±34.41^{a}	152.76±27.17^{ab}	5.01±1.19^{b}	52.11±8.46^{bc}	14.57±3.58^{b}
Cooked at 80-90^{o}C and cold smoked (final)	805.47±49.55^{ab}	124.23±31.05^{ab}	3.35±0.56^{ab}	54.96±14.23^{bc}	12±3.87^{ab}

*Values in the same column with different letters are significantly different ($p<0.05$)

Table 6.3. Fatty acid content (mg g^{-1}) of Sandfish stored at 4°C using various packaging treatments at the start of the storage period (initial) and after 7 days in storage (final). Values are means ± SEM*.

Fatty acid	Treatment							
	Cooked at 80 – 90°C (initial)	Cooked at 80 – 90°C (final)	Salted dried 48 h and reconstituted (initial)	Salted dried 48 h and reconstituted (final)	Normal dried and reconstituted (initial)	Normal dried and reconstituted (final)	Cooked and cold smoked (initial)	Cooked and cold smoked (final)
C6:0	0.02±0.01^{a}	0.02±0^{a}	0.01±0^{a}	0.01±0^{a}	0.01±0^{a}	0.02±0^{a}	0.59±0.1^{c}	0.34±0.04^{b}
C8:0	0.02±0^{ab}	0.02±0.01^{ab}	0.02±0^{ab}	0.01±0.01^{ab}	0.01±0^{ab}	0.01±0^{a}	0.09±0.02^{c}	0.06±0.02^{bc}
C13:0	0.03±0.01^{a}	0.03±0^{a}	0.03±0^{a}	0.03±0.01^{a}	0.03±0^{a}	0.03±0^{a}	0.07±0.02^{a}	0.04±0.01^{a}
C14:0	1.32±0.28^{a}	1.06±0.25^{a}	0.7±0.08^{a}	1.1±0.92^{a}	1.43±0.14^{a}	1.04±0.32^{a}	1.92±1.22^{a}	1.02±0.2^{a}
C14:1n-5	0.93±0.16^{a}	0.64±0.21^{a}	0.46±0.06^{a}	0.53±0.42^{a}	0.69±0.06^{a}	0.48±0.15^{a}	1.33±0.82^{a}	0.79±0.17^{a}
C15:0	0.7±0.19^{a}	0.37±0.07^{a}	0.37±0.07^{a}	0.56±0.44^{a}	0.54±0.05^{a}	0.37±0.12^{a}	0.82±0.54^{a}	0.39±0.1^{a}
C15:1n-5	0.01±0^{a}	0.02±0.02^{a}	0.06±0.05^{a}	0.05±0^{a}	0.17±0.09^{a}	0.12±0.08^{a}	0.04±0.01^{a}	0.03±0.01^{a}
C16:0	5.5±0.95^{a}	5.15±1.05^{a}	2.39±0.2^{a}	3.32±2.58^{a}	7.21±0.4^{a}	4.75±1.34^{a}	8.24±4.71^{a}	4.01±0.82^{a}
C16:1n-7	2.39±0.56^{a}	2.17±0.44^{a}	0.96±0.1^{a}	1.87±1.62^{a}	3.15±0.25^{a}	2.11±0.66^{a}	3.52±2.29^{a}	1.73±0.38^{a}
C17:0	0.77±0.09^{a}	0.54±0.07^{a}	0.59±0.07^{a}	0.62±0.4^{a}	0.49±0.03^{a}	0.44±0.13^{a}	0.99±0.5^{a}	0.58±0.15^{a}
C16:2n-4	0.12±0.03^{a}	0.1±0.01^{a}	0.04±0^{a}	0.07±0.06^{a}	0.1±0.01^{a}	0.08±0.03^{a}	0.11±0.07^{a}	0.07±0.02^{a}
C17:1n-7	0.05±0.04^{a}	0.1±0.01^{a}	0.02±0.02^{a}	0.02±0.01^{a}	0.04±0.02^{a}	0.07±0.02^{a}	0.07±0.03^{a}	0.03±0.03^{a}
C16:3n-4	0.1±0.02^{a}	0.08±0.02^{a}	0.04±0.01^{a}	0.04±0.03^{a}	0.1±0.01^{a}	0.06±0.01^{a}	0.12±0.07^{a}	0.07±0.01^{a}
C18:0	5.01±0.81^{a}	4.69±0.72^{a}	2.65±0.34^{a}	2.9±2.13^{a}	4.2±0.08^{a}	3.93±1.13^{a}	6.3±2.85^{a}	3.85±0.69^{a}
C18:1n-9 t	0.28±0.03^{a}	0.25±0.02^{a}	0.13±0.02^{a}	0.15±0.1^{a}	0.19±0.01^{a}	0.2±0.04^{a}	0.27±0.12^{a}	0.2±0.04^{a}
C18:1n-7 t	0.06±0.01^{a}	0.03±0.01^{a}	0.02±0.01^{a}	0.03±0.01^{a}	0.03±0^{a}	0.04±0.01^{a}	0.06±0.02^{a}	0.06±0.01^{a}
C18:1n-9	0.64±0.1^{a}	1.14±0.39^{a}	0.31±0.02^{a}	0.32±0.2^{a}	0.64±0.03^{a}	0.52±0.13^{a}	0.75±0.31^{a}	0.52±0.15^{a}
C18:1n-7	1.54±0.22^{a}	1.42±0.22^{a}	0.84±0.11^{a}	0.85±0.66^{a}	1.34±0.07^{a}	1.3±0.38^{a}	1.85±0.81^{a}	1.35±0.28^{a}
C18:2n-6 t	0.05±0.03^{a}	0.06±0.03^{a}	0.04±0.03^{a}	0.02±0.01^{a}	0.08±0.01^{a}	0.02±0.01^{a}	0.08±0.03^{a}	0.07±0.02^{a}
C18:2n-6	0.24±0.03^{a}	0.23±0.07^{a}	0.1±0^{a}	0.12±0.09^{a}	0.2±0.01^{a}	0.15±0.04^{a}	0.31±0.17^{a}	0.18±0.04^{a}
C18:3n-6	0.11±0.02^{a}	0.1±0.02^{a}	0.02±0.02^{a}	0.05±0.04^{a}	0.09±0^{a}	0.08±0.02^{a}	0.14±0.08^{a}	0.06±0.03^{a}
C18:3n-4	0.06±0.02^{a}	0.05±0.03^{a}	0.05±0.02^{a}	0.03±0.01^{a}	0.06±0.01^{a}	0.08±0.01^{a}	0.03±0.02^{a}	0.02±0^{a}
C18:3n-3	0.16±0.03^{a}	0.11±0.03^{a}	0.06±0.01^{a}	0.06±0.03^{a}	0.13±0.01^{a}	0.12±0.02^{a}	0.27±0.17^{a}	0.13±0.01^{a}
C20:0	2.35±0.21^{a}	2.25±0.29^{a}	1.06±0.12^{a}	0.87±0.44^{a}	1.86±0.12^{a}	1.83±0.48^{a}	2.54±0.67^{a}	1.89±0.4^{a}
C18:4n-3	8.33±0.78^{b}	7.08±1.09^{ab}	3.33±0.58^{ab}	2.41±1.25^{a}	5.95±0.45^{ab}	6.88±1.67^{ab}	7.18±1.47^{ab}	5.62±1.35^{ab}

Table 6.3 continued next page

C20:1 (isomers)	0.11±0.01^{ab}	0.08±0^{ab}	0.04±0^{ab}	0.03±0.01^{a}	0.07±0^{ab}	0.06±0.01^{ab}	0.12±0.03^{b}	0.11±0.04^{b}
C21:0	0.08±0.01^{a}	0.08±0^{a}	0.05±0.01^{a}	0.04±0.02^{a}	0.08±0.01^{a}	0.08±0.02^{a}	0.12±0.04^{a}	0.1±0.02^{a}
C20:2n-6	0.8±0.08^{a}	0.81±0.11^{a}	0.32±0.04^{a}	0.28±0.14^{a}	0.48±0.01^{a}	0.55±0.14^{a}	0.86±0.26^{a}	0.6±0.11^{a}
C20:3n-6	0.24±0.03^{a}	0.27±0.05^{a}	0.11±0.02^{a}	0.1±0.03^{a}	0.2±0.01^{a}	0.19±0.04^{a}	0.28±0.08^{a}	0.21±0.04^{a}
C20:4n-6	12.84±1.36^{b}	10.74±1.52^{b}	2.55±0.05^{a}	2.16±0.81^{a}	7.7±0.41^{ab}	7.54±1.75^{ab}	11.13±3.27^{b}	6.65±1.28^{ab}
C22:0	1.66±0.11^{b}	1.53±0.23^{a}b	0.65±0.07^{ab}	0.57±0.24^{a}	1.12±0.04^{ab}	1.18±0.29^{ab}	1.7±0.36^{b}	1.18±0.21^{ab}
C22:1 (isomers)	1.04±0.08^{a}	0.95±0.13^{a}	0.49±0.06^{a}	0.41±0.19^{a}	0.81±0.01^{a}	0.57±0.08^{a}	0.97±0.34^{a}	0.85±0.19^{a}
C20:4n-3	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
C20:5n-3	6.2±0.64^{ab}	8.41±1.37^{b}	1.85±0.07^{a}	1.21±0.65^{a}	5.49±0.22^{ab}	6.13±1.54^{ab}	6.03±2.4^{ab}	3.18±0.64^{ab}
C22:2n-6	0.15±0.01^{a}	0.13±0.02^{a}	0.07±0^{a}	0.06±0.02^{a}	0.1±0.01^{a}	0.11±0.03^{a}	0.16±0.04^{a}	0.14±0.03^{a}
C22:3n-3	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
C22:4n-6	1.29±0.11^{b}	1.28±0.17^{b}	0.14±0.01^{a}	0.12±0.04^{a}	0.96±0.1^{b}	0.91±0.2^{b}	0.99±0.28^{b}	0.63±0.11^{a}
C24:0	0.42±0.03^{a}	0.42±0.07^{a}	0.18±0.01^{a}	0.16±0.07^{a}	0.33±0.02^{a}	0.32±0.08^{a}	0.49±0.12^{a}	0.37±0.08^{a}
C22:5n-6	0.76±0.06^{b}	0.67±0.12^{ab}	0.2±0.01^{ab}	0.15±0.06^{a}	0.45±0.02^{ab}	0.46±0.12^{ab}	0.78±0.27^{b}	0.48±0.07^{ab}
C24:1n-9	2.16±0.19^{a}	1.94±0.32^{a}	0.9±0.18^{a}	0.67±0.32^{a}	1.69±0.1^{a}	1.04±0.59^{a}	2.01±0.49^{a}	1.49±0.34^{a}
C22:5n-3	0.19±0.04^{a}	0.23±0.04^{a}	0.06±0.01^{a}	0.04±0.02^{a}	0.15±0.01^{a}	0.18±0.07^{a}	0.19±0.09^{a}	0.14±0.02^{a}
C22:6n-3	0.49±0.1^{a}	0.42±0.11^{a}	0.12±0.02^{a}	0.1±0.05^{a}	0.34±0.03^{a}	0.33±0.09^{a}	0.53±0.25^{a}	0.32±0.04^{a}
C24:5n-3	0.01±0.01^{a}	0.02±0^{a}	n.d	n.d	0.01±0^{a}	0.02±0.01^{a}	0.01±0.01^{a}	0.01±0^{a}
C24:6n-3	0.02±0^{a}	0.03±0.01^{a}	0.01±0^{a}	0.010±0^{a}	0.02±0^{a}	0.02±0^{a}	0.03±0.01^{a}	0.01±0^{a}
∑Total Fatty acid	59.25±5.15^{a}	55.76±8.44^{a}	22.03±1.89^{a}	22.14±13.93^{a}	48.74±1.9^{a}	44.43±10.67^{a}	64.1±25.22^{a}	39.55±7.98^{a}
∑SFA	17.86±2.24^{a}	16.17±2.7^{a}	8.7±0.83^{a}	10.2±7.24^{a}	17.31±0.74^{a}	14±3.77^{a}	23.86±11.13^{a}	13.82±2.71^{a}
∑MUFA	9.21±1.2^{a}	8.75±1.61^{a}	4.22±0.38^{a}	4.91±3.51^{a}	8.83±0.42^{a}	6.52±1.44^{a}	10.99±5.18^{a}	7.16±1.51^{a}
∑PUFA	32.18±2.84^{c}	30.84±4.4^{bc}	9.12±0.74^{ab}	7.03±3.28^{a}	22.61±0.93^{abc}	23.91±5.74^{abc}	29.24±8.97^{abc}	18.58±3.76^{abc}
∑n-6 PUFA	16.49±1.57^{b}	14.3±1.96^{b}	3.55±0.18^{a}	3.05±1.21^{a}	10.26±0.51^{ab}	10.01±2.32^{ab}	14.74±4.46^{b}	9.01±1.68^{ab}
∑n-6 LC PUFA	15.13±1.49^{b}	12.96±1.78^{b}	3±0.09^{a}	2.53±0.94^{a}	9.31±0.5^{ab}	9. 1±2.1^{ab}	13.18±3.9^{b}	7.96±1.5^{ab}

Table 6.3 continued next page

∑n-3 PUFA	15.4±1.41^{ab}	16.3±2.4^{b}	5.44±0.55^{ab}	3.84±2^{a}	12.08±0.44^{ab}	13.69±3.39^{ab}	14.24±4.37^{ab}	9.41±2.06^{ab}
∑n-3 LC PUFA	6.91±0.77^{ab}	9.11±1.53^{b}	2.05±0.08^{a}	1.36±0.72^{a}	6.01±0.26^{ab}	6.68±1.7^{ab}	6.79±2.74^{ab}	3.65±0.7^{ab}

*Values in the same row with different letter are significantly different ($p<0.05$)

The collagen content of the MAP (60% CO_2/40% N) packaged samples of sandfish from four treatments varied from 8.37 mg g^{-1} - 18.92 mg g^{-1} across samples (Table 6.4), but there were no significant differences between samples or between initial (day 0) and final (day 7) samples for any treatment (Table 6.4). The texture assessment for the MAP (60% CO_2/40% N) packaged cold smoked sandfish is shown in Table 6.5. While both force and energy required to penetrate the tissue increased from day 7 to day 30, indicating increasing toughness of the samples, there were no significant differences between samples on the different sampling days (Table 6.5).

Table 6.4. Collagen content (mg g^{-1}) of shelf assessed Sandfish stored at 4^{o}C using various packaging treatments at the start of the storage period (initial) and after 7 days in storage (final). Values are means ± SEM*.

Treatment	**Collagen content**
Cooked at 80 - 90^{o}C initial	18.92±1.93^{a}
Cooked at 80 – 90^{o}C final	11.5±3.4^{a}
Boiled/ salted 48 h/dried and reconstituted initial	9.54±2.16^{a}
Boiled/ salted 48 h/dried and reconstituted final	8.37±0.77^{a}
Normal dried/reconstituted initial	11.61±0.91^{a}
Normal dried/ reconstituted final	13.31±0.75^{a}
Cold smoked initial	18.06±8.84^{a}
Cold smoked final	17.09±4.29^{a}

*Values in the same column with different alphabet are significantly different ($p<0.05$)

Table 6.5. Texture of shelf stored cooked (80-90^{o}C) and cold smoked Sandfish stored at 4^{o}C for 30 days*

Treatment	Force (N g^{-1})	Energy (mj g^{-1})
Day 0	na	na
Day 7	28.71±4.94^{a}	135.78±29.07^{a}
Day 10	27.05±8.16^{a}	189.39±35.88^{a}
Day 14	29.58±8.23^{a}	237.79±53.66^{a}
Day 17	19.28±1.43^{a}	149.38±19.71^{a}
Day 21	20.55±1.94^{a}	160.63±19.48^{a}
Day 24	38.12±7.74^{a}	239.49±74.88^{a}
Day 30	38.65±8.99^{a}	256.86±72.58^{a}

*Values in the same column with different alphabet are significantly different ($p<0.05$)

6.4 Discussion

This study assessed modified atmospheric packaging (MAP) and vacuum packaging to preserve sandfish at 4^{o}C for shelf life assessment. The gas composition ratios of 60%CO_2/40%N_2 and 5%0_2/50%CO_2/45%N_2 used in this study failed to deter microbial growth on either cooked or dried and reconstituted sea cucumber products, and all MAP and vacuum-packaged products were discarded after 7 days because of high bacterial counts. The gas compositions used for MAP in the current study have been shown to inhibit microbial content of the sandfish which was also observed in prior studies on MAP of fish tissues (Özogul et al., 2004). Use of essential oils as an antimicrobial agent prior to MAP or vacuum packaging failed to improve shelf-life of sea cucumber products with high bacterial counts forcing products to be discarded after 7 days. In contrast, (Mastromatteo et al., 2010a) reported that MAP alone did not improve the shelf life of the shrimps, but coating the shrimps in thymol (500-1500 mg/L) before packing, significantly improved the shelf life by

up to 6 days by reducing the levels of mesophilic bacteria, *Pseudomonas* spp. and hydrogen-sulphide producing bacteria associated with product.

A number of studies have reported preservation of fish using antimicrobial agents and MAP packaging. Kostaki et al. (2009), assessed the combined effects of MAP using two different gas mixtures (40% CO_2/50% N/10% O_2 and 60% CO_2/30% N/10% O_2) and thyme oil (0.2% v/w) on the quality of sea bass fillets during storage. Fillets in MAP reached 7-logCFU/g after 19 days several days than the unpacked fillets that reached the same level in 7 days. Similar research (Kykkidou et al., 2009a) on the combined effect on thyme essential oil (0.1% v/w) and different type of packaging on microbial and sensorial characteristics of fresh Mediterranean swordfish fillets was also done. Air, MAP (5%O_2/50%CO_2/45%N_2), air with thyme oil and MAP with thyme oil were used. The findings revealed that MAP and MAP combined with thyme oil were most effective against the growth of Pseudomonads and Hydrogen Sulfide (H_2S) producing bacteria. The shelf life of swordfish under aerobic and MAP was determined to be 8 and 13 days respectively. Further addition of 0.1% thyme essential oil extended the shelf life of the swordfish fillets by another five days in aerobic conditions and by seven and half days under combination of MAP with thyme oil as compared to the control. The overall acceptance and the quality of the swordfish decreased with storage time stored both under aerobic conditions and MAP either with or without essential thyme oil.

Nutrient analysis of the discarded and cold smoked MAP products in the current study showed, in the majority of cases, no significant differences in the levels of key nutrients (protein, lipid, NFE, essential fatty acids) and other components (moisture and ash) between initial (day 0) and final (day 7) samples, indicating that despite the bacterial contamination of

products by day 7, microbial activity had not yet begun to affect product nutrient content. This assumption is supported by the fact that there were no significant differences in the levels of nutrient between day 7 samples of the cold smoked MAP treatment, which lacked microbial proliferation, and those of other treatments. Despite this, storage of cooked sea cucumbers at 4^{o}C has been shown to result in a degraded nutrient composition (Chen et al., 2015a) with time.

Cold smoked cooked sandfish was the only treatment tested in the current study that survived the shelf life assessment and, at day 30, recorded no bacteria, yeast or mold counts. Smoke contains a huge range of organic compounds (Daun, 1979) that are deposited on the surface of the product where show antibacterial activity (Bremner, 2012). Smoke inhibits micro-organism development (e.g. *Escherichia coli*, *Staphylococcus aureus* and *Saccharomyces cerevisiae*) in sea food products that are related to food spoilage. The results of this study clearly show the effectiveness of smoking in providing effective shelf-life stability of sea cucumber products when combined with MAP. This is the first time that cold smoking and cold-smoking combined with MAP have been investigated for preservation of sea cucumbers.

Although samples from the control treatments (cooked at 80-90^{o}C only) had to be pulled off the shelf after day 7 because of high microbial, yeast and mold counts, the cold smoked product was held with zero counts at the completion of 30 days in MAP (60%CO_2/40%N_2 and 5%0_2/50%CO_2/45%N_2) and vacuum. This indicates that advanced packaging of Sandfish with preservatives, could improve the shelf life of refrigerated product. However, the texture of cold smoked MAP product was found to toughen with increased storage time and, although this change was non-significant over the sampling period (up to 30 days), samples may have continued to toughen with increasing storage period. The reason for this is

unknown since previous studies have reported that the toughness of sea cucumber products reduces with increasing storage time (Chen et al., 2015a). Further research is required to assess the effect of cold smoking and MAP on the texture of sandfish products, particularly because Asian markets prefer sea cucumber products with soft texture (Fukunaga et al., 2004). Further treatments with enzymes and flavouring could improve product quality of either MAP or vacuum-packed product and this aspect also requires research attention as the potential of novel packaging methods for sea cucumber products is developed further.

Chapter 7

General Discussion

7.1 Introduction

This study addressed issues relating to the effects of processing technique on the quality and nutritional composition of the sea cucumber *Holothuria scabra* (Sandfish). The overall objectives of this study was to to generate improved information on the processing of sandfish relating to nutrient composition and texture, and the potential and implications of novel processing and packaging methods for this species. The major outputs of this study and their potential applications are summarised in Fig 7.1 and described below.

7.2 Effect of processing on yield of tropical sea cucumbers.

Bêche-de-mer processing in the Pacific has been done since the 1800s and the methods used have changed little. However, improper processing has resulted in poor BDM yield and unacceptably low grading of products relating to size, shape and colour of BDM that result in low income. A total of 28 sea cucumber species are heavily exploited in the Pacific, however, processing information for many of them is very limited and there are not much published literature available on processing yields that could be used as a basis to improve BDM yield and quality. Processing manuals have been designed and distributed to processors and communities in the Pacific (Purcell, 2014a); however, it is not yet apparent whether this has resulted in improved yield, quality and value of BDM processed using standard techniques.

Recovery rates for length and weight of sea cucumbers (conversion ratio, CR) were determined for eight species of tropical sea cucumber in Chapter 2. CR allows conversion between processing stages and estimation of initial fresh weight/length from partially of fully

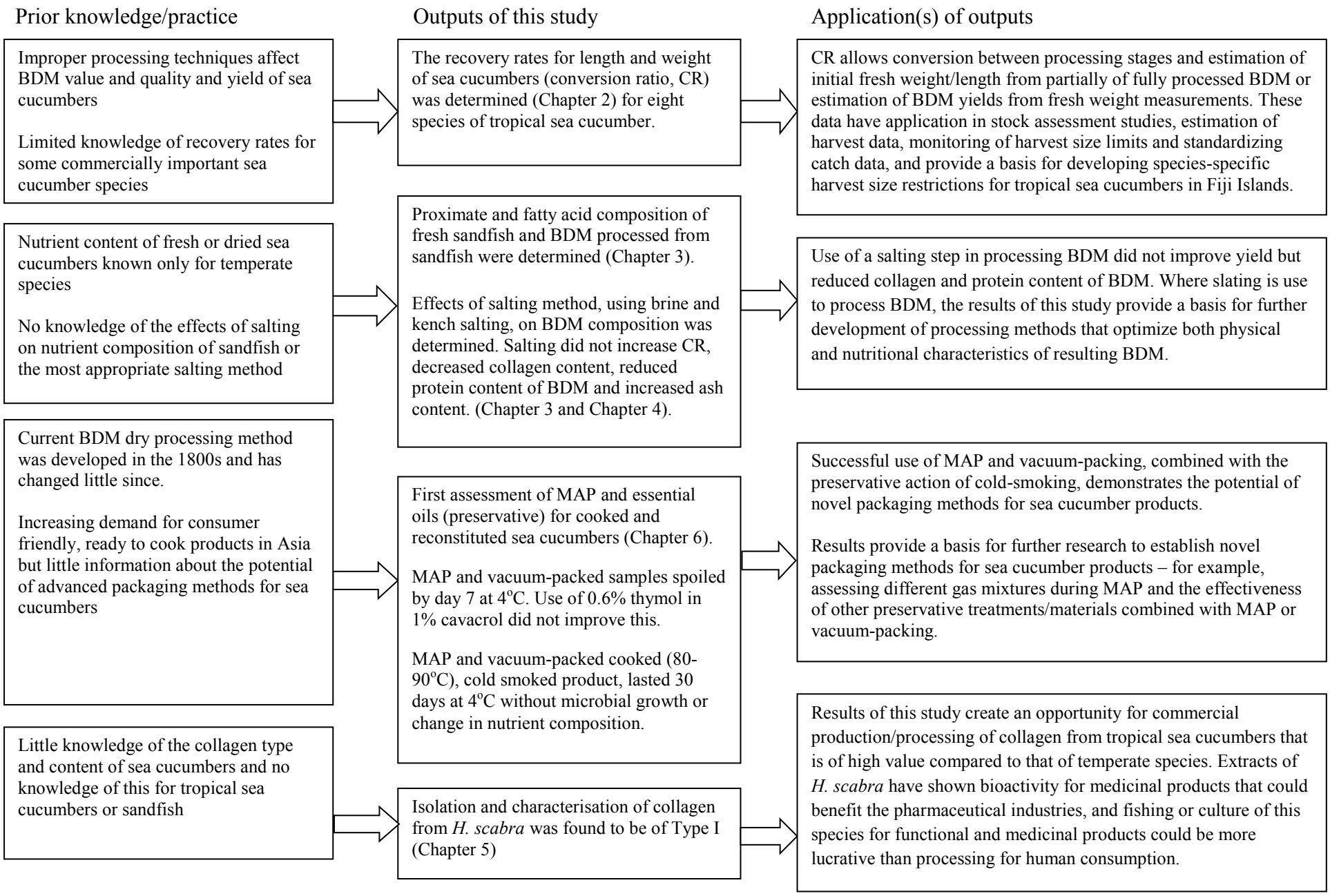

Fig. 7.1. Major outputs from this study and their potential applications

processed BDM as well as estimation of BDM yields from fresh weight measurements. These data have application in stock assessment studies, estimation of harvest data, monitoring of harvest size limits and standardizing catch data, and they provide a basis for developing species-specific harvest size restrictions for tropical sea cucumbers in the Fiji Islands. The results of this study provide a baseline for further research into the factors affecting recovery rates of sea cucumbers, such as processing technique, as well as species-specific harvesting limit for processing.

7.3 Effect of processing on nutrient content of sandfish

Sea cucumbers are regarded as a nutritious delicacy and BDM consists of high protein content, essential fatty acids, lipid content and minerals. Prior to this study, there was limited information available on the impact of processing on the nutrient composition of BDM, particularly for tropical sea cucumbers, including sandfish. Current processing methods used for sandfish in Fiji involve kenching and drying, but and no prior studies have focused on nutrient losses from sea cucumbers during processing.

Prior to this study, there was no knowledge of the effects of salting, and various methods of salting, on nutrient composition of resulting sandfish BDM, or the most appropriate salting method supporting optimal nutrient retention. Proximate and fatty acid composition of fresh sandfish and BDM processed from sandfish were determined in Chapter 3. The effects of salting method, using brine and kench salting, on BDM composition was determined. Perhaps surprisingly, salting did not increase CR (yield), but resulted in decreased collagen and protein content of BDM and increased ash content (Chapter 3 and Chapter 4). Collagen is the main constituent of the protein of sea cucumbers/BDM and is important in imparting appropriate texture to BDM. Because salting reduced collagen content of BDM it may be

counter-productive to include salting in sandfish processing. It would be interesting for future research to assess the organoleptic acceptability of sandfish BDM prepared using various salting methods to determine whether the resulting reduction in collagen content is detrimental to resulting BDM quality. Where salting is used to process BDM, the results of this study provide a basis for further development of processing methods that optimize both physical and nutritional characteristics of resulting BDM. The main advantage of salting has been found to increase the yield as well as the shelf life of BDM. The results of this study has also provided further basis on optimising the collagen content of sea cucumbers by using improved processing techniques.

7.4 Potential of novel packaging for sea cucumber products

Since the 1800s, BDM has been process dried to increase the shelf life. This practise is still used and has changed little since. The process however, in converting the perishable sea cucumber to a non-perishable product is labour intensive and requires additional resources during processing such as wood and kerosene. As the global food market is shifting towards more ready to eat products, there has been increased demand for 'consumer friendly' products in Asia which are popular with the younger generation. However, there is little information about the potential of advanced packaging methods for sea cucumbers that would support development of such products. This study assessed for the first time, modified atmospheric packaging (MAP) and the use of essential oils (preservative) for cooked and reconstituted sea cucumbers (Chapter 6).

The MAP and vacuum-packed samples spoiled by day 7 at 4^{o}C and the use of 0.6% thymol in 1% cavacrol did not improve this. MAP and vacuum-packed cooked (80-90^{o}C), cold smoked product, lasted 30 days at 4^{o}C without microbial growth or change in nutrient composition.

Successful use of MAP and vacuum-packing, combined with the preservative action of cold-smoking, demonstrates the potential of novel packaging methods for sea cucumber products and the results provide a basis for further research to investigate novel packaging methods for sea cucumber products. For example, assessing different gas mixtures during MAP and the effectiveness of other preservative treatments/materials combined with MAP or vacuum-packing, would be important follow-up steps to the research presented here.

7.5 Determining the collagen type of sandfish

Collagens belong to a family of extracellular matrix proteins that maintain the integrity of various tissues. Approximately 27 types of collagen have been reported with 42 distinct types of polypeptide chains and about 20 additional proteins and collagen – like domain and 20 other collagen modifying enzymes. Currently a number of studies on collagen extraction and characterisation have been conducted on temperate sea cucumber species and fish. Although it has been indicated that collagen from the tropical sea cucumbers is of high value and quality (Zhong et al., 2015), there is very limited knowledge of the collagen type of tropical sea cucumbers, including sandfish. Isolations and characterisation of collagen from sandfish in Chapter 5 of this study found it to be of Type I, similar to all other sea cucumbers so far investigated. Results of this study indicate that there is an opportunity for commercial production/processing of collagen from sandfish that is of high value compared to that of temperate species. In addition to collagen, extracts of sandfish have shown bioactivity for medicinal products that could benefit the pharmaceutical industries (Bordbar et al., 2011), and fishing or culture of this species for functional and medicinal products could be more lucrative than processing for human consumption.

7.6 Future research

This study has identified a number of areas that follow-on and future research should address. They include:

1. The effects of salting on recovery rate and texture quality of BDM processed from sandfish and, given the additional costs associated with purchase of salt and increased labour and time required for salting, a cost-benefit analysis would provide important information for sea cucumber processors

2. The influence of reproductive seasonality on yield and nutrient composition of sandfish and sandfish product. Reproductive seasonality has been reported in sandfish (Ramofafia et al., 2003) and, in other species of sea cucumbers, is associated with changes in tissue composition (Wen et al., 2010). Research to determine the effects of reproductive seasonality of sandfish in Fiji would help determine the best time of year to harvest to maximize nutrient composition of resulting BDM. It would also have potential implications for stock management.
3. Further research into novel product development is needed to develop optimal product processing and packaging methods that maximise shelf-life while maintaining high nutrient composition and good texture that is acceptable in Asian markets. Further studies could also focus on utilisation of the waste of sea cucumber processing (guts) that could be marketable and may have potential for extraction of compounds with nutritional or biomedical properties.
4. While the results from Chapter 5 reported successful isolation and characterisation of collagen from sandfish and confirmed it as Type I, the results of other chapters of this thesis show that processing method affects collagen recovery. Given that there may be potential for commercial collagen production from sandfish, further research could

address the relationship between processing method and collagen recovery in more detail.

5. Like all sea cucumbers, sandfish have potential use for production of bioactive compounds of medicinal value. Future research could screen sandfish for this potential, because BDM may not be the most lucrative of sandfish products.

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