Deodorisation of Protein Hydrolysate and Extraction of Proteins from Hoki (*Macruronus novaezelandiae*) skin

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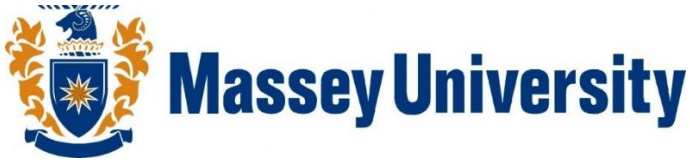

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Abstract

The present study had two main objectives. The first objective was to identify a suitable deodorisation treatment for pre-prepared Hoki skin protein hydrolysate (HSPH) and the second objective was to investigated suitable pre-treatment and extraction processes for collagen and gelatine from Hoki *(Macruronus novaezelandiae)* skin which resulted in low odour extracts. The off-odour in HSPH post-deodorisation treatments and in the Hoki collagen and gelatine post-extraction processes were assessed by determining the total volatile base nitrogen (TVB-N) and trimethylamine (TMA) concentrations. The sensory technique of flash profiling was employed to determine the odour attributes in all HSPH, gelatine and collagen samples after treatment and extraction processes. In addition to the respective off-odour assessments, the extracted collagen and gelatine were evaluated in terms of total protein content (g protein/100g dry sample), moisture content (w/w%), and yield (w/w% of dry sample). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to determine the molecular weight (kDa) of extracted collagen and gelatine. Amino acid profile analysis was performed to identify the extracted samples.

In the first part of this study, dried green tea leaves (GT), powdered tea polyphenol (TP) and dried olive leaves (OL) investigated for deodorisation of HSPH. Using an orthogonal design, three factors (concentration, temperature and time) and three corresponding levels were used in the design. The two most suitable deodorisation treatments for pre-prepared HSPH were 1) deodorant: powdered tea polyphenol; concentration: 0.04 g/ml hydrolysate; temperature: 50˚C; time: 20 min, and 2) deodorant: powdered tea polyphenol; concentration: 0.04 g/ml hydrolysate; temperature: 80˚C; time: 60 min. For a more economical solution, GT was determined to be a possible alternative deodorant to TP by manipulating the total phenolic content prior to deodorisation. For a secondary deodorisation treatment, preliminary results on strong acid hydrogen form ion exchange resin (Dowex G-26) reduced the TMA concentration in partially deodorised HSPH sample significantly (p-value<0.05) from 3.4±0.1 deodorisation to 0.8±0.1 mg of nitrogen/100g wet sample.

In the second part of this study, Hoki skins were pre-treated using 0.2 M NaOH solution (1:6 w/v) for 60 min at 18±2^{o}C and then extraction with distilled water (1:10 w/v) for 60 minutes at 50±2˚C. This treatment produced gelatine product with the highest protein content (41.3±0.9 g of protein/100g dry sample) and reduced off-odour based on TMA content (0.9±0.1 mg of nitrogen/ 100 g wet sample). However, a lower gelatine yield recovery of 61.0±1.7 % was determined in this gelatine sample. SDS-PAGE and amino acid profile tests concluded that all pre-treatment and extraction processes successfully extracted gelatine samples as the final product. In contrast, collagen samples were not confirmed as pure collagen in this study.

The current findings for both objectives of this study has shown that pre-treating the raw material using acid or alkali prior to subsequent processes is more efficient in reducing the off-odour in the final products rather than employing deodorisation processes as a subsequent countermeasure after hydrolysis and extraction.

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List of Abbreviations

AC	Activated carbon
AMP	Adenosine monophosphate
ASC	Acid-solubilised collagen
C	Catechin
DH	Degree of hydrolysis
DMA	Dimethylamine
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
FHP	Fish protein hydrolysate
GC	Gallocatechin
GC	Gas chromatography
GPA	Generalized Procrustes Analysis
GT	Dried green tea leaves
HMF	Hydroxymethylfurfural
HSPH	Hoki skin protein hydrolysate
LOX	Lipoxygenases
MRP	Maillard reaction product
NPN	Non-protein-nitrogen

OL	Dried olive leaves
PSC	Pepsin soluble collagen
PUFA	Poly-unsaturated fatty acids
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPME	Solid-phase micro-extraction
SSC	Salt-solubilised collagen
TMA	Trimethylamine
TMAO	Trimethylamineoxide
TP	Powdered tea polyphenol
TVB-N	Total base volatile nitrogen
YE	Dried active yeast
YPC	Yeast plate count

Chapter 1: General Introduction

The fish industry is a major economic source for many countries worldwide including New Zealand (Chalamaiah et al., 2012; Ministry for Primary Industries [MPI], 2016). Recently, there has been much interest in investigating possible means of utilizing fish wastes (Nagai and Suzuki, 2000). A number of researchers have investigated methods to produce fish protein hydrolysates (FPH), a breakdown product from the enzymatic conversion of fish proteins into smaller peptides resulting in a product with increased functional properties (Chalamaiah et al., 2012; Chi et al., 2014; Intarasirisawat et al., 2007; Kumar, Nazeer and Jaiganesh, 2012; Shahidi, Han and Synowiecki; 1995). There are a number of applications of FPH in food products which include emulsifying, foaming properties, antioxidant and cryoprotective properties (antifreeze compound that inhibit ice crystal formation in ice cream) (Chi et al., 2014; Kristinsson and Rasco,2000). Besides, FPH is also used in pharmaceutical and nutraceutical applications (Kristinsson and Rasco, 2000). Bioactive peptides from fish origin are found to reduce the risk of various diseases such as lowering cholesterol level and reduces pain in patients with osteoarthritis (Kristinsson and Rasco, 2000). The fish processing industry produces considerable waste which includes fish heads, fins, frames and skins (Chalamaiah et al., 2012). The skins of fish in particular, contain a large amount of protein, called collagen which can also provide the source for gelatine manufacture (Mohtar, 2013). Despite the promising possibilities in the utilization of fish by-products, one of the major setbacks in the application of marine based products is the formation of off-odours (Benjakul et al., 2014; Yarnpakdee et al., 2012). Several techniques have been employed to either, reduce, eliminate or prevent the generation of off-odours in marine based products such as deodorisation methods and pre-treatments using acid and alkali prior to hydrolysis or extraction processes (Benjakul et al., 2014; Chen et al., 2016).

Hoki constitutes New Zealand's largest commercial fish harvested (Mohtar, 2013). Hoki (*Macruronus novaezelandiae*) is a cold water fish species related to cod and hake (Mohtar, 2013). From 2010 to 2011, New Zealand recorded a total export of frozen Hoki worth NZ$ 188 million (Mohtar, 2013). The present study is focusing on the investigation

of deodorisation of pre-prepared Hoki skin protein hydrolysate and on the extraction of collagen and gelatine from Hoki skins, with the overall goal to provide more usable industrial by-products from fish waste. Therefore, the present study was divided into two parts:

1. The aim of the first part was to determine suitable deodorisation methods that reduce or remove off-odour in pre-prepared Hoki skin protein hydrolysates (HSPH) using selected deodorants which included dried green tea leaves (GT), dried olive leaves (OL), and powdered tea polyphenol (TP).
2. The aim of the second part was to determine suitable pre-treatment and extraction processes of Hoki skin collagen and gelatine that reduce or remove off-odour in the extracted collagen and gelatine products, and to identify the extraction processes that produce collagen and gelatine with high protein contents and high yields.

This thesis will be presented in six chapters. *Chapter 2* presents a general review of collagen and gelatine, protein hydrolysis processes which includes a general review on chemical and enzymatic hydrolysis processes. The review will also cover the limitations on the application of marine-based products, mainly focusing on the generation of off-odours and the methods used to overcome the problems. *Chapter 3* outlines the methods and materials used in this study. *Chapter 4* presents results concerning the deodorisation of pre-prepared Hoki skin protein hydrolysates and identifies the suitable deodorisation treatments that reduce the off-odours in the final product. *Chapter 5* describes the results obtained from the pre-treatment and extraction processes for Hoki collagen and gelatine. This chapter also identifies the final products produced after the extraction procedures employed. *Chapter 6* provides general discussions based on the findings made from both parts of the studies. *Chapter 7* concludes the thesis by highlighting the overall conclusions and future direction of work based on the results obtained from this study.

Chapter 2: Literature Review

The literature review comprises four sections: firstly, a brief overview on the sources, structure and amino acid compositions of collagen and gelatine protein molecules; secondly, a review on fish protein hydrolysis and protein hydrolysates, limitations in the applications of fish hydrolysate proteins and methods to overcome the limitations; thirdly, a review of the procedures for collagen and gelatine extraction, and lastly, the analytical methods to provide qualitative and quantitative analyses of the collagen and gelatine samples.

2.0 Utilising Fish Waste

The world fisheries and fish processing industries have 60% waste from the fish catch which consists of heads, skin, fins, frames and trimmings (Chalamaiah et al., 2012). In New Zealand alone, this waste was as much as 59,900 tonnes in 2011 and was worth $173.7 million (Field, 2012). For a developed country, these large quantities of fish by-product waste could cause a severe pollution and disposal problem if they were concentrated in a few places and left untreated (Chalamaiah et al., 2012). Current industrial practices often further process this waste into low value products such as fish meal and natural fertiliser (Mohtar, 2013). Fish waste by-products contain useful nutrients such as omega-3 fatty acids and collagen that are reported to help maintain human health (Chalamaiah et al., 2012; Shirai and Ramírez-Ramírez, 2011). In recent years, fish protein hydrolysates (FPH) in particular, have attracted much attention from researchers due to the availability of essential amino acids and high protein contents (Chalamaiah et al., 2012; Kongruang and Wisuthiphaet, 2015). Several techniques including chemical and enzymatic hydrolysis have been developed and applied in an attempt to recover the nutritional and physiologically important peptides present with a view to utilising fish by-products and to overcome the pollution and disposal problems (Shirai and Ramírez-Ramírez 2011). Hydrolysis techniques are employed to produce high value FPH (Chalamaiah et al., 2012).

However, during hydrolysis lipid oxidation and other degradative reactions can occur producing off-odour volatile compounds in marine hydrolysates causing fishy odour (Yarnpakdee et al., 2012). These off-odours in FPH can result in a major reduction in consumer acceptability and decreases the hydrolysate's commercial value for food applications (Benjakul et al., 2014). Many deodorisation treatments have been developed to remove the existing off-odour compounds in pre-prepared FPH (Chen et al., 2016). Pre-treatment processes are also often employed to overcome these problems prior to hydrolysis (Benjakul et al., 2014).

Hoki constitutes New Zealand's largest commercial fish harvested (Mohtar, 2013). In 2014, Hoki exports contributed more than $200 million to the New Zealand economy (Ministry for Primary Industries [MPI], 2016). In the same year, around 57 000 tonnes of processed Hoki was exported (MPI, 2016). However, in New Zealand the total catch is limited by quota (MPI, 2016). One of the few ways left for a fishing company to increase the returns (income) on their current catch is by utilising Hoki waste and by-product more profitably.

2.1 Hoki fish

Hoki (*Macruronus novaezelandiae*) is a cold water fish species related to cod and hake (Mohtar, 2013). Hoki live in a cold water habitat from a depth of 200 to 600 m (Connell et al., 2010). Hoki fishing takes place at several fishing grounds around New Zealand including the Stewart-Snare shelf south of Stewart Island, the west coast of the South Island and in the Cook Strait (MPI, 2016). The fish is known to be a good source of omega-3 making it an excellent dietary choice and it has a delicate white flesh without any lateral bone making it a safe product for consumption (Johnson, 2010; Mohtar, 2013). From 2010 to 2011, the total export of frozen Hoki was worth NZ$ 188 million, with the majority of exports to the USA, Japan, Europe and Australia (Mohtar, 2013).

The skins of fish usually contain a large amount of protein, called collagen (Mohtar, 2013). Collagen is the raw material required for gelatine manufacture (Mohtar, 2013).

2.1.1 Fish Skin

The skin shapes the external form of a fish providing a physical barrier and protecting it from pathogens, osmotic pressure and physical injury (Paterson, 2006). The structures of

the fish skin are shown in Figures 1 and 2. Fish skin is made up of two basic layers, the epidermis and the dermis (Figure 1). The outer layer of the epidermis is the cuticle (Figure 2) which consists of mucus and cell debris containing antibodies and enzymes to prevent abrasion (Paterson, 2006). The epidermis, mainly keratinocytes (also known as malpighian cell – a layer where mitosis occurs) consists of layers of moist and flattened cells (Mohtar, 2013; Paterson, 2006). The dermis is comprised of the upper layer called stratum spongiosum and a deeper layer called the stratum compactum (Paterson, 2006). The stratum spongiosum contains loose connective tissue and collagen fibres, while the stratum compactum contains dense collagen fibres that provide structural strength to the skin (Mohtar, 2013). The scales are developed in scale pockets within the stratum spongiosum that produces bony ridges which later are deposited on the surface of the growing scale (Mohtar, 2013).

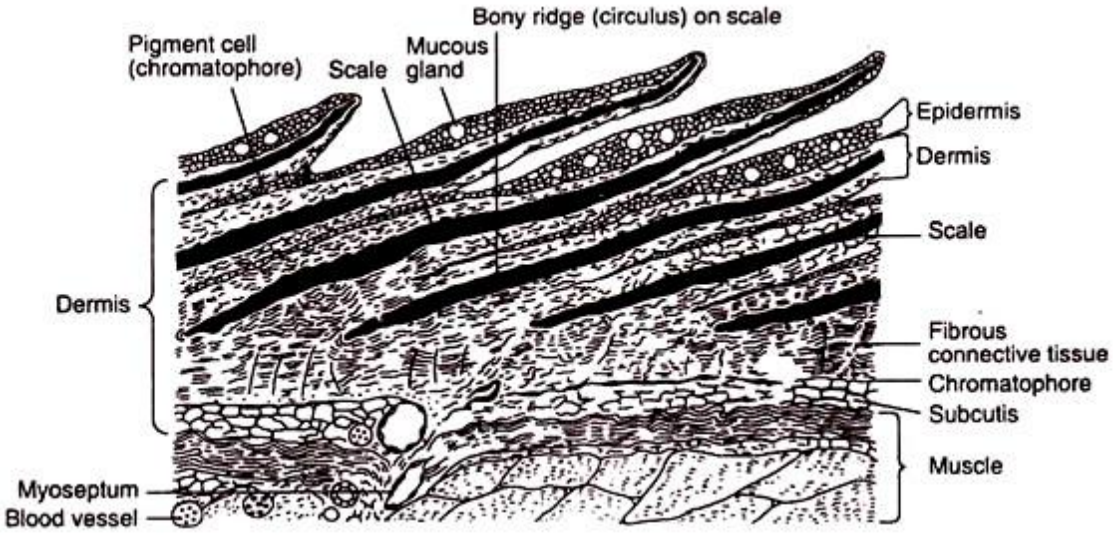


Figure 1: *General section of fish skin* (Mohtar, 2013).

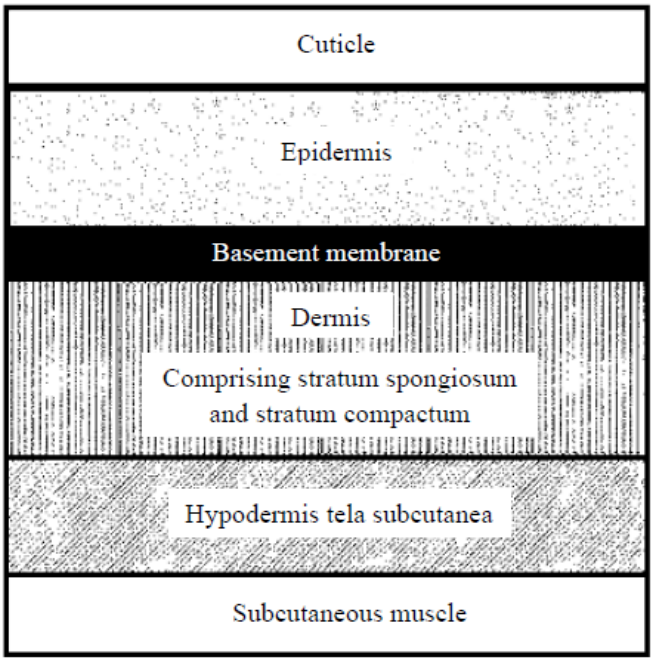


Figure 2*: Schematic illustration of a fish skin layer* (Mohtar, 2013).

2.1.2 Fish Collagen

Collagen is a major structural protein in the connective tissues of vertebrates (Li et al., 2013). Collagen proteins are composed of three polypeptide chains known as α chains, that assemble to form a stable triple helix with varied sizes (Freudenberg et al., 2007). Each of these polypeptide chains contain the repeating unit of Gly-X-Y; where Gly is glycine, X is proline, and Y corresponds to any amino acid but commonly hydroxyproline (Freundenberg et al., 2007; Mohtar, 2013). The structure of collagen and the arrangement of the amino acid sequences are shown in Figure 3. Compared to amino acid compositions from bones, collagen from skin contains higher amounts of hydroxyproline (Hyp) and proline (Pro) (Kittiphattanabawon et al., 2005). Proline and hydroxyproline control the rotation of the polypeptide bond thus contributes to the stability of the structure (Mohtar, 2013). The basic unit of collagen is tropocollagen and the molecules are stabilized by hydrogen bonds and intermolecular bonds (Schmidt et al., 2016). A tropocollagen consists of three domains; (i) the N-terminal that carries the free amine group ($-NH_2$), (ii) the central triple helix consisting of more than 300 repeating units of Gly-X-Y known as telopeptides and (iii) the C-terminal that carries a free carboxyl group (-COOH) (Figure 4) (Mohtar, 2012; Gorgieva and Kokol, 2011).

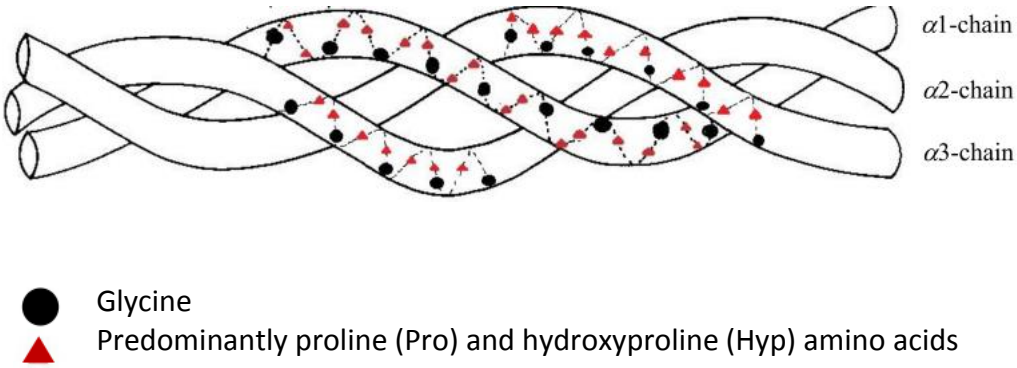


Figure 3*: Schematic drawing of the collagen triple helix structure and the arrangement of amino acids in the collagen molecule* (Mohtar, 2013).

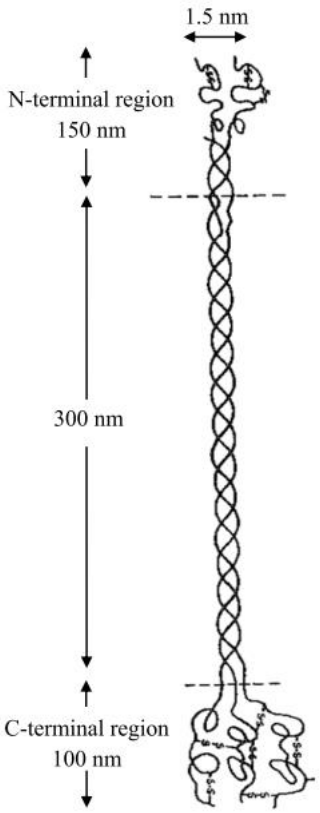


Figure 4*: The general structure of tropocollagen consisting of the N- and C- terminal region* (Mohtar, 2012)

At least 29 different types of collagen have been reported and they are characterized according to, (i) structure, (ii) splice variants, (iii) the presence of additional non-helical domains, and (iv) their assembly and functions (Gelse et al., 2003; Schmidt et al., 2016). Among these different collagen types, the type I collagen is the most common and

primarily found in the connective tissue in skin, tendons and bones (Gelse et al., 2003; Schmidt et al., 2016). This collagen type predominantly consists of Gly (approximately one third of the total amino acids content) (Gorgieva and Kokol, 2011). It is comprised of two identical α1 and one α2 subunits (Kimura et al., 1987). Type II collagen occurs mainly in cartilage tissue and is composed of three α1 subunits (Gorgieva and Kokol, 2011). The α1 subunit is similar to that of type I collagen (Schmidt et al., 2016). Type III collagen is dependent on age; young skin can contain approximately 50% type III collagen and the percentage appears to reduce to 5 to 10% of the total collagen present, over time (Schmidt et al., 2016). In general, collagen has a molar mass of 360 kDa and is approximately 280 nm in length (Schmidt et al., 2016).

Collagen is considered one of the most useful biomaterials in the food industry because of its functional properties including water absorption capacity, gel formation ability and the ability to stabilize emulsions (Schmidt et al., 2016). Recently, collagen was extracted from fish by-products, mainly skins as an alternative to the skins of land animals due to the higher yield of collagen recovered and minimum risk of disease transmission (Li et al., 2013). Pre- treatment using acid or alkali was necessary to remove covalent intra- and intermolecular cross links including ester bonds with saccharides and lysine residues before collagen extraction (Schmidt et al., 2016). The pre-treatments as well as the collagen extraction processes are further discussed in Section 2.4.

The extracted collagens can then be converted into collagen peptides by enzymatic hydrolysis (Cho et al., 2014). Collagen peptides are commonly extracted by chemical hydrolysis, however, enzymatic hydrolysis can produce higher quality products with improved functionality (Schmidt et al., 2016). Some collagen peptides produced by enzymatic hydrolysis also showed high antioxidant activities (Feng-Chi et al., 2014).

2.1.3 Fish Gelatine

Gelatine is not a naturally occurring protein as it is the product of thermal denaturation and disintegration of insoluble native collagens (Gorgieva and Kokol, 2011; Karim and Bhat, 2009). Unlike collagens that exist in many different types, gelatine is derived only from the type I collagen (Gorgieva and Kokol, 2011). Gelatines are composed of three α-chains intertwined in a similar fashion to that of the collagen structure (Gomez-Guillen

et. al., 2002). Once set as a gel, gelatine has the similar repeating unit of Gly-X-Y as collagen but the N- and C-terminal regions do not form a triple helical structure (Gomez-Guillen et al., 2002). The amino acid compositions of gelatines depends on the sources of their collagen parents (Gorgieva and Kokol, 2011; Karim and Bhat, 2009). According to Zhang et al. (2010), gelatine from pig's skin and bone do not contain cysteine (Cys) whereas the same amino acid is found to be present in the gelatine from fish scale. The molecular weight of gelatine is highly dependent on their collagen source and can vary between 40 kDa to 700kDa (Gorgieva and Kokol, 2011). Hoki skin gelatine in particular has a molecular weight of 100 – 190 kDa (Mohtar, 2013).

Gelatines have broad applications in the food, pharmaceutical and medical industries and researchers have long strived for high quality gelatines (Jamilah and Harvinder, 2002). The main attributes that define the quality of gelatine products are the gel strength which is expressed in grams and the thermal stability (Gomez-Guillen et al., 2011; Mohtar, 2012). The mechanism and extraction procedure for gelatines are discussed in further detail in Section 2.4.2. The extracted gelatines can then be hydrolysed, generally by enzymatic hydrolysis to produce gelatine peptides (Cho et al., 2015).

2.2 Protein Hydrolysis

Protein hydrolysis is a process involving the breakdown of protein molecules into shorter peptides. This resulting peptide mix is known as a protein hydrolysate (Sujith and Hymavathi, 2011). Generally, protein hydrolysis can be achieved by chemical processes or using protease enzymes (He et al., 2013). Typical process of protein hydrolysis from raw materials using either a chemical process or enzymatic protease is shown in Figure 5.

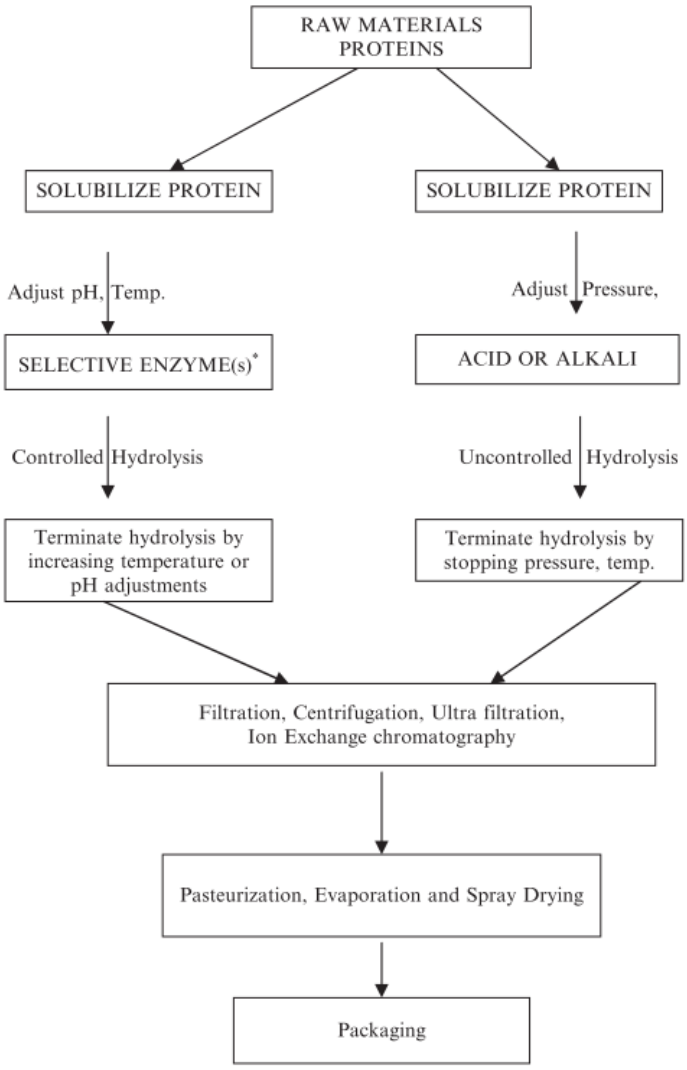


Figure 5*: Protein hydrolysis process showing procedure for enzymatic and chemical hydrolysis process* (Pasupuleti and Braun, 2010).

2.2.1 Chemical hydrolysis methods

Chemical hydrolysis can be performed under acidic or alkali conditions, requiring a short processing time at lower cost (Fountoulakis and Lahm, 1998; Wisuthiphaet and Kongruang, 2015). However, the quality of hydrolysates produced by chemical hydrolysis reactions are difficult to control due to the cleaving of unspecified peptide bonds and some of the amino acids are destroyed during the process (Wisuthiphaet and Kongruang, 2015). The hydrolysate will require neutralising once hydrolysis is complete. This will leave a salt residual within the hydrolysate.

2.2.2 Acid Hydrolysis

Acid hydrolysis is a conventional method in the production of protein hydrolysates in which a strong acid is used to convert peptides into free amino acids under high pressure and temperature (Wisuthiphaet and Kongruang, 2015). In a study on fish protein hydrolysate, fish wastes were hydrolysed in several concentrations of hydrochloric acid (HCl) at high pressure (103.4 kPa) at 121 ^{o}C for 90 minutes. (Wisuthiphaet and Kongruang, 2015). Hydrochloric acid (HCl) treatment is commonly employed in acidic hydrolysis as HCl can be applied in both its liquid- and gas-phase (Fountoulakis and Lahm, 1998). The conventional acid hydrolysis uses 6M HCl for 20 to 24 hours at 110 ^{o}C resulting in the complete conversion of asparagine and glutamine into aspartic acid and glutamic acid, respectively (Fountoulakis and Lahm, 1998). However, tyrosine was completely destroyed and there was reported to be a 5 - 10% loss of serine and threonine during the process (Fountoulakis and Lahm, 1998). Total hydrolysis of FPH was achieved with 6M HCl for 18 hours at 118 ^{o}C followed by neutralization of the hydrolysates to pH 7 before they were concentrated into a paste or dried into powder form (Kristinsson and Rasco, 2000). As a consequence of the chemical process, the hydrolysates contained high amounts of NaCl making them unpalatable thus limiting their application in food products (Kristinsson and Rasco, 2000). In addition, FPH produced by this process often lead to bitter compounds due to the exposure of hydrophobic residues of the polypeptide chains during the reaction process (Wisuthiphaet and Kongruang, 2015). Acid hydrolysis also oxidises cysteine and methionine, lowers protein quality and the biological value of hydrolysates produced (Sujith and Hymavathi, 2011).

2.2.3 Alkali Hydrolysis

Alkali hydrolysis is predominantly used to determine tryptophan which is stable in basic conditions (Fountoulakis and Lahm, 1998). Alkali hydrolysis is also employed for protein samples containing a high percentage of monosaccharides (Fountoulakis and Lahm, 1998). Generally, alkali treatments are applied using sodium hydroxide (NaOH), potassium hydroxide (KOH) or, occasionally, barium hydroxide (Fountoulakis and Lahm, 1998). Most often, alkali treatments are performed in the food industry to recover alkali-soluble proteins (Kristinsson and Rasco, 2000). For example, mechanically deboned turkey meat residue contains a significant amount of alkali-soluble protein that can be

recovered by alkali hydrolysis treatment for various food applications (Kristinsson and Rasco, 2000). On the other hand, production of fish protein hydrolysate by alkaline treatment often uses fish protein concentrate (FPC) as the starting substrate (Kristinsson and Rasco). FPCs are extracted by chemical solvents, primarily alcohol such as ethanol and propanol that concentrate fish protein into a more stable form and the resultant product is available for human consumption (Hale, 1974; Winsor, 2001). In a study that utilized high pH (12.5) at 95 ^{o}C for 20 min, it was shown that alkaline conditions aided in improving the solubility and dispersibility of FPC in food applications (Kristinsson and Rasco, 2000). Increased collagen solubility was also observed after alkali hydrolysis (Kristinsson and Rasco, 2000).

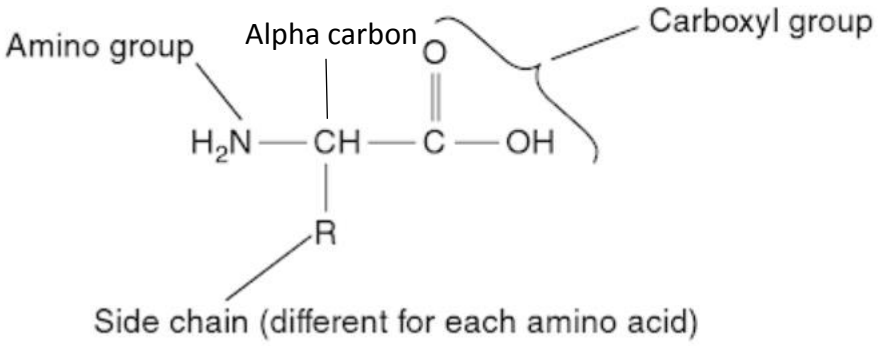


Figure 6*: Basic structure of an amino acid (Dunford and Doyle, 2015)*

However, there are drawbacks from alkaline treatment causing the method to be minimally used as it often results in hydrolysates with poor functionality and reduced nutritional value (Kristinsson and Rasco, 2000). Several detrimental reactions can occur during alkali hydrolysis and are instigated by the removal of hydrogen from the alpha carbon of the amino acid (Figure 6) (Dunford and Doyle, 2015; Krinstinsson and Rasco, 2000). This process generates racemization of amino acids forming D-form amino acids that cannot be utilised by humans and animals (Wisuthiphaet and Kongruang, 2015). Alkali hydrolysis can also form toxic substances like lysinoalanine (Kristinsson and Rasco, 2000). Amino acids such as serine, threonine, arginine and cysteine are also destroyed during the alkali process (Fountoulakis & Lahm, 1998).

2.3.1 Enzymatic Hydrolysis Method

Enzymatic hydrolysis employs enzymes such as carbohydrases, proteases and lipases (Benjakul et al., 2014; Kristinsson and Rasco, 2000; Wong, 1995). Proteases are categorised based on the principle functional group and action mechanism (Wong, 1995). Enzymatic hydrolysis employs proteolytic enzymes, both endopeptidases, which hydrolyse peptide bonds of non-terminal amino acids, and exopeptidases, which cleave peptides bonds from either the N or C terminal of amino acids (Figure 7) (Benjakul et al, 2014). Using enzymes to hydrolyse food proteins can improve and modify the functional, physiochemical and sensory properties of the protein while retaining their nutritional value (Kristinsson and Rasco, 2000; Sujith and Hymavathi, 2011). As a result, hydrolysates produced by this method are used in various applications in food, pharmaceutical and cosmetic products (Wisuthiphaet and Kongruang, 2015).

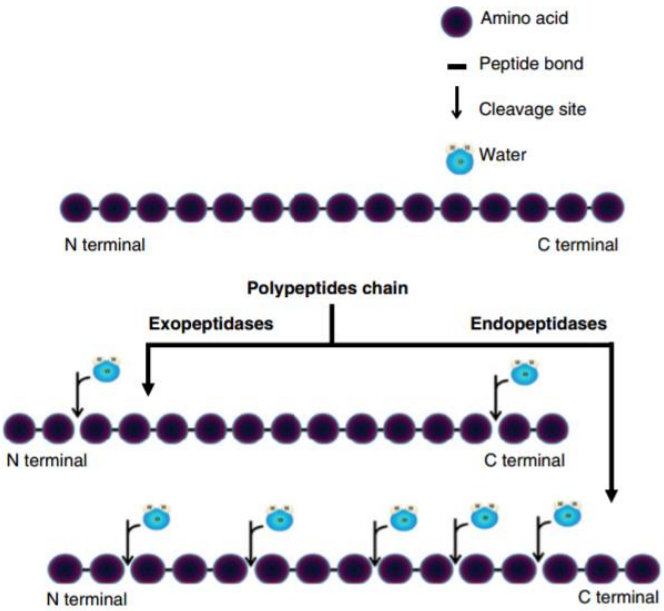


Figure 7: Enzymatic hydrolysis by endopeptidases and exopeptidases *(Benjakul et al., 2014).*

Enzymatic hydrolysis takes advantage of the substrate specificity of protease thereby allowing specific production of hydrolysates with designated functionalities (Benjakul et al., 2014). Enzymatic processes are performed under controlled conditions, taking into

account several factors including the physicochemical properties of the final product, enzymes employed, their optimum temperatures, time and pH of enzymatic activity (Kristinsson and Rasco, 2000; Muzaifa, Safriani and Zakaria, 2012). The method may involve multi-step reactions in which several proteolytic enzymes are used in the cleavage of polypeptides to produce the desired peptides and amino acids, and therefore can be time consuming (Kristinsson and Rasco, 2000). Despite this, enzymatic treatment is widely used because it produces high quality products with a wide range of applications under mild reaction conditions (Fallah et al., 2015; Kristinsson and Rasco, 2000). In enzymatic hydrolysis, temperatures ranging between 55 to 60 ˚C are required for optimal proteolytic enzyme activity (Yasothai and Giriprasad, 2015). In a study by Perez-Galvez et al. (2011), protein from porcine blood was hydrolysed by exopeptidase (Alcalase®) in pH 7.5 at 58.8 ˚C. In the enzymatic hydrolysis, proteolytic enzymes are inactivated by heating at high temperatures between 75 to 100 ˚C for 5 to 30 minutes depending on the type of enzymes used (Kristinsson and Rasco, 2000; Muzaifa et al., 2012). Different conditions of enzymatic hydrolysis are shown in Table 1.

Table 1*: Different conditions of enzymatic hydrolysis for different marine raw materials.*

Raw material	Enzymatic hydrolysis	Reference
Northern Whiting fish (*Sillago sihama*) muscle	Enzymatic hydrolysis was performed at room temperature for 24 h using protease enzymes (pepsin, trypsin and α-chymotrypsin) in different enzyme/substrate ratios (1:100, 1:500 and 1:1000). Enzymes were inactivated by heating the solution at 100 ^{o}C for 10 min.	(Venkatesan and Nazeer, 2014)
Horse mackerel (*Magalaspis cordyla*) skin	Enzymatic hydrolysis was performed using pepsin at an enzyme/substrate ratio of 1/100 (w/w) at 37 ^{o}C for 2 h. The sample pH was adjusted to 6.5 prior to storage at -80 ^{o}C.	(Kumar, Nazeer and Jaiganesh, 2012)
Nile tilapia (Oreochromis niloticus) flesh	Enzymatic hydrolysis was conducted using Alcalase at an enzyme/substrate ratio of 1/100 (w/v) at 50 ^{o}C for 2h.	(Yarnpakdee, Benjakul and Kristinsson, 2013)
Tilapia fish scale	Enzymatic hydrolysis was performed using 0.5% (w/w) Alcalase at temperature maintained between 55-60 ^{o}C for 20-24h.	(Cho et al., 2015)
Black pomfret viscera	Enzymatic hydrolysis was performed using pepsin at an enzyme/substrate ratio of 1/100 (w/w) at 37 ^{o}C for 2h.	(Jaiganesh, Nazeer and Sampath Kumar, 2011)
Spanish mackerel (Scomberomorous niphonius) skin	Enzymatic hydrolysis was conducted using trypsin at an enzyme/substrate ratio of 1/2 (w/w) at 37 ^{o}C for 3h.	(Li et al., 2013)

Enzymatic hydrolysis has several advantages over the chemical hydrolysis:

- Enzymatic hydrolysis provides higher yields with minimal hydrolytic degradation of products via racemization (Kristinsson and Rasco, 2000).
- Enzymatic hydrolysis offers higher selectivity at lower costs due to its low energy requirements (Yang et al., 2011).
- Enzymatic hydrolysis allows the production of hydrolysates in controlled conditions (Benjakul et al., 2014).

Recently, enzymatic hydrolysis has been employed as a primary alternative to utilize fish by-products (Kristinsson and Rasco, 2000). Numerous studies have been conducted to demonstrate the potential of converting fish skin into fish protein hydrolysates from different fish species. This includes the use of alcalase to hydrolyse grass carp skin in a study by Wasswa, Tang and Gu (2008); production of gelatine hydrolysates from skin of sole and squid by Gimenez et al. (2009); and production of protein hydrolysates from black pomfret using gastrointestinal proteases in a study by Sampath Kumar et al. (2011).

2.3 Protein hydrolysate

Protein hydrolysates have been reported to possess certain functional properties (Neklyudov, Ivankin & Berdutina, 2000). One of the functions of protein hydrolysates in biotechnology applications is to provide a nitrogen source for microbial, animal and plant culture in laboratory and industrial practices (Pasupuleti and Braun, 2010). Hydrolysates may also possess desirable physiochemical properties such as emulsification and water binding capacity, bio-active properties such as anti-oxidative and anti-microbial properties (He, Franco and Zhang, 2013). Physiochemical properties of hydrolysates are strongly determined by the initial substrate used for hydrolysis and the original amino acid compositions (Neklyudov, Ivankin and Berdutina, 2000). For example, a study found that protein hydrolysates from salmon had 95% solubility in aqueous solution after hydrolysis with alcalase at 61 ^{o}C at pH 7.5 for 2 hours, meanwhile blue whiting was only able to achieve 70% solubility under similar conditions (He, Franco and Zhang, 2013).

According to Pasupuleti and Braun (2010), there are several aspects to be taken into account to manufacture high quality consistent protein hydrolysate:

1. Strictly following the Good Manufacturing Practice (GMP) procedure and maintain a hygienic manufacturing environment.
2. Screen raw materials to obtain high quality product.
3. Constantly monitor the protein, enzyme and water sources as these determine the quality of hydrolysate.

2.4 Factors Affecting Protein Hydrolysate Production

Several factors affect the production of hydrolysates including the type of enzyme used, type of initial substrate for hydrolysis, enzyme and substrate concentration, temperature and pH (Fountoulakis and Lahm, 1998).

2.4.1 Type of Enzyme

Enzymes are biochemical catalysts that aid in the acceleration of chemical reactions between organic compounds within a biological cell (Kristinsson and Rasco, 2000). In food science and technology applications, enzymes are important to assist in the conversion of raw materials to produce improved high quality food products (Frey and Hegeman, 2007; Kristinsson and Rasco, 2000). Enzymes have active sites that catalyse specific reactions by forming enzyme-substrate complexes (Frey and Hegeman, 2007; Kristinsson and Rasco,2000; Wong, 1995). Enzymatic catalysis occurs in three consecutive steps; 1) formation of enzyme-substrate complex (also known as Michealis complex), 2) polypeptide bond cleavage liberating one of the two peptides, 3) nucleophilic attack where the other enzyme-substrate complex splits to liberate free amino acids (Copeland, 2000; Frey and Hegeman, 2007; Kristinsson and Rasco, 2000). According to Kristinsson and Rasco (2000) the mechanism can be depicted as shown in Equation 2.1, where E is enzyme, ES is enzyme-substrate complex, P is product:

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} ES \xrightarrow{k_2} EP + H - P'$$

$$\xrightarrow[H_2O]{k_3} E + OH - P + H - P'$$

Equation 2.1

Where k_1 = the rate of the forward reaction to ES; k_{-1} = the rate of the reverse reaction from ES; k_2 = the rate of the forward reaction to EP + H-P'; and k_3 = the rate of the forward reaction to form a product.

The principal functional group in the active site defines the functional group of an enzyme and this can be serine, thiol or carboxyl (Kristinsson and Rasco, 2000; Wong, 1995). The enzymes action mechanism can be either endopeptidase or exopeptidase (Kristinsson and Rasco, 2000). It is crucial to select the most suitable enzyme in order to produce hydrolysates with the desired functional properties (He, Franco and Zhang, 2013).

Particularly in hydrolysing fish protein, proteolytic enzymes from plants, microorganisms and animals can be employed (Kristinsson and Rasco, 2000). Among the proteolytic enzymes from microorganisms, fungal proteases are more commonly used as they provide broader substrate specificity because they often contain a mixture of several enzymes (Kristinsson and Rasco, 2000). Several types of enzymes that are widely established in the production of fish protein hydrolysate include (i) alcalase – enzyme produced from *Bacillus licheniformis,* an alkaline bacterial protease and determined to be one the best enzymes for fish protein hydrolysis, (ii) flavourzyme – an enzyme produced by the fermentation of non-genetically modified *Aspergillus oryzae* that is readily used under neutral or acidic conditions and reported to produce hydrolysates with desired functional properties, (iii) papain – an endopeptidase from papaya often used to produce hydrolysates ideal for biomedical applications (Muzaifa, Safriani and Zakaria, 2012; Wisuthiphaet and Kongruang, 2015).

2.4.2 Type of Initial Substrate

Functional properties of hydrolysates produced can vary depending on the type of initial substrate for hydrolysis (He, Franco and Zhang, 2013). In a study on cod protein hydrolysate, it was found that the enzyme Flavourzyme® produced protein hydrolysates with higher oil binding capacity than hydrolysates produced using the enzyme Neutrase®, containing 4.1 g oil /g protein and 3.1 g oil/g protein, respectively (He, Franco and Zhang, 2013). However, better emulsifying capacity was shown in the cod protein hydrolysates hydrolysed by Neutrase® than Flavourzyme® (He, Franco and Zhang, 2013). The impact of the enzyme and the different initial substrates of various fish species on the functionality of the hydrolysate produced is summarised in Table 2.

***Table 2**: Summary of the effect of enzyme type and initial fish substrate on the functional properties of hydrolysates produced.*

Fish Species	Enzymes employed	Functional properties of hydrolysates	Reference
Red salmon	Alcalase	High solubility (easily dissolved in water)	He, Franco and Zhang (2013)
Hake	Alcalase	High solubility (easily dissolved in water)	Kristinsson & Rasco (2000)
Tuna	Flavourzyme®	Antioxidant activities	Chalamaiah et al. (2012)
Tuna	Neutrase®	DPPH scavenging activities	Chalamaiah et al. (2012)
Grass carp	Alcalase	Oil binding capacity, emulsifying capacity and water holding capacity	He, Franco and Zhang (2013)
Mackerel	Protease N	High anti-oxidative activity	He, Franco and Zhang (2013)

2.4.3 Enzyme and Substrate Concentration

The effectiveness of the hydrolysis process is measured by the degree of hydrolysis (DH), defined as the proportion of peptide bonds cleaved in the protein hydrolysate (Rutherford, 2010). Equation 2.2 shows the general equation for calculating DH (Nielsen, Peterson and Dambmann, 2001).

$$DH = \frac{The\ number\ of\ hydrolyzed\ bonds, h}{The\ total\ number\ of\ peptide\ bonds\ per\ protein\ equivalent, h_{tot}} \times 100\% \quad \text{Equation 2.2}$$

The h_{tot} depends on the amino acid composition of the raw material used in the hydrolysis (Nielsen, Peterson and Dambmann, 2001).

Enzyme concentration is one of the factors that affects DH during protein hydrolysis and is reported to have a greater effect on reducing hydrolysis time than increasing temperature (Karamac, Amarowicz and Kostyra, 2002; Salwanee et al., 2013). Results

obtained in a study on tuna protein hydrolysis using alcalase showed that the DH was significantly increased from approximately 80% to 90% as the enzyme concentration was increased from 1.0% to 1.5%, respectively (refer Equation 2.2; Salwanee et al., 2013). Higher alcalase concentrations resulted in more peptide bond cleavage resulting in an increased number of amino acids and small peptides in the resulting hydrolysates (Salwanee et al., 2013). However, there was no significant increase in the DH between 1.5% and 2.0% enzyme concentration with alcalase (Salwanee et al., 2013). Enzymatic hydrolysis is characterized by an initial rapid phase where a large number of peptide bond cleavages takes place (Kristinsson and Rasco, 2000). After this, the rate of hydrolysis reduces, entering a period when increasing enzyme concentration does not increase the hydrolysis rate as it is now no longer the limiting factor (Kristinsson and Rasco, 2000). Although increasing the enzyme concentration has been proven to increase the DH value to a certain level, it may not be cost effective for industrial applications (Kristinsson and Rasco, 2000). Substrate concentration can also affect DH value in hydrolysis. In a study by Shahidi, Han and Synowiecki (1995), high concentrations of substrate released during the initial phase reduced the hydrolysis rate and protein recovery.

2.4.4 pH and Temperature

Environmental factors such as pH and temperature can significantly affect the enzymatic activity and these factors are specific to each type of enzyme employed in the hydrolysis process (Kristinsson and Rasco, 2000). Each enzyme has an optimum pH and temperature range where enzymatic reaction is most active, whereas extreme pHs and temperatures will denature the enzyme thus inhibit enzymatic activity (Bayindirli, 2010).

2.5 Limitations in the applications of fish protein hydrolysate

There can be limitations to the wide application of the protein hydrolysates in food products due to bitterness and undesirable odour. In fish hydrolysis fishy odour can be a problem (Dauksas et al., 2004; Yarnpakdee et al., 2012). These problems cause the hydrolysates to be unpalatable and can be unacceptable due to the undesirable aroma thus reducing the commercial value of the product (Thiansilakul, Benjakul & Richards, 2010). In this present literature review, factors affecting the undesirable fishy odour of

fish protein hydrolysate are mainly focused on and it is beyond the scope of this study to cover the limitation in the application of hydrolysates associated with bitterness.

2.5.1 Lipid oxidation in fish hydrolysates

According to Maqsood and Benjakul (2011), pro-oxidants such as unstable lipid substrates and haem proteins in hydrolysates, prepared from fish flesh, are among the major contributors to lipid oxidation. This lipid oxidation results in undesirable odours, predominantly fishy odour. Fish muscle has a higher content of unsaturated fatty acids than those of mammals making it more susceptible to oxidation (Yarnpakdee et al., 2012). The formation of off-odours are contributed to by the haem proteins and other forms of iron as well as lipoxygenases (LOX) (Fu, Xua and Wang, 2009). The haemoglobin in the blood and muscle of the fish contains iron that can readily change from its ferrous form (Fe^{2+}) into its ferric form (Fe^{3+}) through auto-xidation (Maqsood and Benjakul, 2011; Tsuruga et al., 1998). During this process, a superoxide anion radical is produced and is converted into hydrogen peroxide that will generate ferryl haemoglobin, a pro-oxidant that induces lipid oxidation (Tsuruga et al., 1998; Yarnpakdee et al., 2012). Meanwhile, LOX enzyme has positional specificity for deoxygenation of poly-unsaturated fatty acids (PUFA) (Fu, Xua and Wang, 2009). LOX is responsible for part of the fish aroma due to the formation of carbonyls and alcohols, the compounds that contribute to the special odour of fish (Fu, Xua and Wang, 2009). In addition, myoglobin promotes the generation of hydroperoxides that are readily decomposed into volatile compounds such as alcohols, aldehydes and ketones causing the development of fishy odour (Thiansilakul, Benjakul and Richards, 2010). A study on yellowtail flesh found that the high contents of PUFA and myoglobin in the fish muscle were the major contributors to the formation of rancid off-odour during refrigeration (Sohn et al., 2005). Yarnpakdee et al. (2012) observed lipid oxidation during the hydrolysis of Nile tilapia at 50 ^{o}C. This suggested that at higher temperatures accelerated lipid oxidation will occur (Gunstone, 2008; Luten, 2009; Yarnpakdee et al., 2012). In another study on protein hydrolysate from brownstripe red snapper (*Lutjanus vitta*) muscle, it was reported that a pre-treatment process for fish mince was recommended to reduce pro-oxidants and phospholipids prior to hydrolysis (Khantaphant, Benjakul and Ghomi, 2011).

2.5.2 Generation of off-odour volatile compounds

Enzymatic reactions, lipid oxidation and microbial degradation can generate the formation of off-odour volatile compounds that contribute to the fishy odour in marine hydrolysates (Chen et al., 2016). The volatile compounds known to be the contributors to the fishy odour in aquatic products are n-hexanal, n-heptanal, 2,4-heptadienal, 2,4-decadienal, 1-octen-3-ol and 2, 3-octanedione (Chen et al., 2016). In a study on sea brim fish, volatile compounds such as E-2-hexenal, Z-4-heptenal, and (E,E)-2,4-heptadienal were described as rancid, painty, fishy and cod-liver like (Iglesias et al., 2009). In another study on jelly fish hydrolysate, volatile aldehydes such as alkanals, 2-alkenals, trans-2,4-heptadienal, 2-alkanones, 1-octen-3-one, trans-2,4,7-decatrienal and 1-penten-3-one were described as fishy (Kromfang et al., 2015).

Some proteolytic enzymes can hydrolyse polypeptides into volatile amines including ammonia, dimethylamine (DMA) and trimethylamine (TMA) (Etienne and Ifremer, 2005). Total base volatile nitrogen (TVB-N) is used to represents these volatile amines and other basic nitrogenous compounds in marine fish (Etienne and Ifremer, 2005). TVB-N is often used as a quantitative parameter for the assessment of fish product quality (Etienne and Ifremer, 2005). Ammonia is formed by deamination of proteins, peptides and amino acids by bacteria and can also be produced by autolytic breakdown of adenosine monophosphate (AMP), a nucleotide used as monomer in RNA (Etienne and Ifremer, 2005). Fish flesh contains the non-protein-nitrogen (NPN) fraction including free amino acids and nucleotides which serve as the readily available bacterial growth substrate (Gram and Huss, 1996). Trimethylamineoxide (TMAO) is part of the NPN fraction and found in marine fish (Gram and Huss, 1996). The TMAO is readily reduced to TMA, resulting in off-odours in fish products (Chen et al., 2016; Gram and Huss, 1996). TMA has a pungent, strong fishy ammonia-like odour and is often associated with a rotting fish smell (Boraphech and Thiravetyan, 2015). The formation of TMA is highly dependent on the TMAO content (Gram and Huss, 1996; Thiansilakul, Benjakul and Richards, 2010). On the other hand, DMA is also produced from TMAO found in fish tissues, and often associated with a fish-like odour (Gill and Paulson, 1982; Gram and Huss, 1996). The reduction of TMAO to DMA can be catalyzed non-enzymatically by iron and various reductants or enzymatically by TMAO-ase (Dong et al., 2013). TMAO-ase enzyme can be

found in the viscera and red muscle of marine fish (Gill and Paulson, 1982). The non-enzymatic decomposition of TMAO into DMA was due to the reaction of TMAO with cysteine in the presence of Fe or haemoglobin as a catalyst at a temperature between 22 and 24 ^{o}C (Dong et al., 2013). In a study by Etienne and Ifremer (2005), increasing levels of DMA were observed in fish including cod, haddock and whiting after a week of storage due to the presence of TMAO-ase, whereas no DMA formation was detected in fish species which lack this enzyme. The odour thresholds of ammonia, DMA and TMA are 110 000, 30 000 and 600 ppb, respectively (Niizeki et al., 2003).

2.6 Deodorisation methods for fish protein hydrolysates

Over the years, several methods and techniques have been employed to eliminate and mask the fishy-odour components in fish protein hydrolysates (Chen et al., 2016). There are three different types of deodorisation methods commonly used to modify the odour in hydrolysates which are biological, chemical and physical methods.

2.6.1 Lactic Acid Bacterial Fermentation as a Biological Method in Hydrolysate Deodorisation

Biological methods for hydrolysate deodorisation use lactic acid bacteria fermentation (Jung et al., 2004). Lactic acid bacteria such as *Leuconostoc, Streptococcus, Lactobacillus, Enterococcus, Aerococcus and Pediococcus spp.* produces different compounds during lactic acid fermentation which include (i) organic acids including palmitic, pyruvic, lactic and acetic acids; (ii) alcohols, mainly ethanol; and (iii) aldehydes and ketones such as acetaldehyde, acetoin and 2-methylbutanol (Chelule, Mokoena and Gqaleni, 2010). In a study on raw heads of African river prawn (*Macrobrachium vollenhovenii*), lactic acid fermentation of the prawn heads with *Lactobacillus plantarum* for 7 days at 30 ^{o}C, with an addition of trona (source of sodium carbonate), had restricted the protein hydrolysis by endogenous enzymes (Fagbenro, 1996). In another study by Jung et al. (2004), lactic acid fermentation was used for red crab shell waste using *Lactobacillus paracasei* subsp. *tolerans* KCTC-3074. However, there is limited information regarding the mechanism of hydrolysate deodorisation using lactic acid bacterial fermentation thus limiting further review on the method.

2.6.2 Maillard Reaction as a Chemical Method in Hydrolysate Deodorisation

One of the chemical methods employed in the deodorisation of FPH is achieved by adding sugar to the initial substrate during pre-treatment before enzymatic hydrolysis to promote Maillard reactions (Kouakou et al., 2014). This improves the sensory quality of the product as it produces flavours associated with roasted caramel (Kouakou et al., 2014). The Maillard reaction is a chemical reaction that occurs between amino acids and reducing sugars producing Maillard reaction products (MRPs) including volatile compounds, non-volatile coloured compounds and brown substances (Kouakou et al., 2014; Tamanna and Mahmood, 2015). The process of forming MRPs is divided into three stages (Figure 8); 1) condensation of amino acid and sugar into 1-amino-1-deoxy-2 ketose, 2) dehydration and fragmentation of sugars producing hydroxymethylfurfural (HMF) fission products such as pyruvaldehyde and diacetyl, 3) condensation of aldol forming melanoidins, a brown coloured compound (Tamanna and Mahmood, 2014). In a study on salmon hydrolysates, sugar was added to mask the fish odour and off-flavours generated in the product (Kouakou et al. 2014). In addition, the Maillard reaction also produces volatile aldehydes (Valet, Prost and Serot, 2007). The process consists of the non-enzymatic binding between the carbonyl group of an aldose or ketose and the free amino group of an amino acid producing aromatic aldehydes including 2-furancarboxaldehydes and 5-methyl-2-furancarboxaldehyde, both described as having sweet aroma (Guillen, Manzanos and Zabala, 1995; Varlet, Prost and Serot., 2007). However, this method of deodorisation of FPH often produces glycated proteins such as fructosamine and 2,3-enediol that are unavailable for human metabolism (Friedman, 1996; Kouakou et al., 2014). There is also limited information on the mechanism of Maillard reaction particularly during protein hydrolysis thus limiting further review on the method for deodorisation.

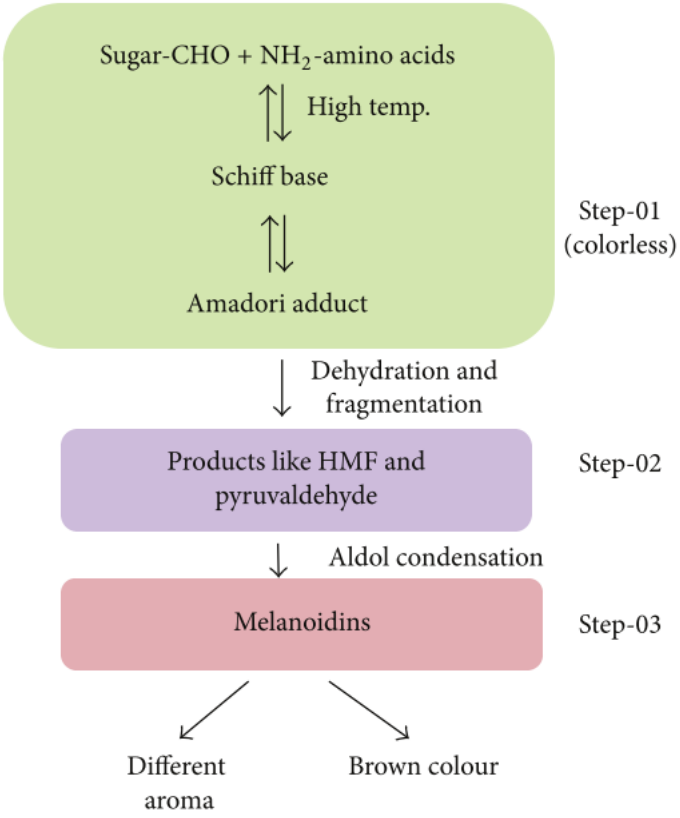


Figure 8*: Schematic representation of three stages in Maillard reaction* (Tamanna and Mahmood, 2014)

2.6.3 Physical Methods in Hydrolysate Deodorisation

Physical methods in hydrolysate deodorisation employ additional material known as deodorants to remove undesirable odours in the product (Chen et al. 2016).

2.6.3.1 Activated carbon adsorption

Activated carbon (AC) is a versatile adsorbent often used to remove undesirable odour, colour, taste and impurities (Aspevik, 2016). Activated carbon is characterised by different pore sizes which are micro-, meso-, and macro-pores with diameters of <2nm, 2 - 50nm and >50nm, respectively (Iyobe et al., 2003). Its surface is non-polar and hydrophobic, increasing its performance for adsorption of volatile organic compounds (Iyobe et al., 2003).

Carbon containing materials are processed to make them extremely porous with large available surface areas and then carbonized using either chemicals or gas (Aspevik (2016). In chemical activation, direct carbonation of raw material is performed under high temperature ranging between 500^{o}C and 900^{o}C in the presence of catalysts such as phosphoric acid and zinc chloride (Aspevik, 2016). Meanwhile, gas activation is achieved by carbonation at a lower temperature ranging between 400^{o}C and 500^{o}C and then it undergoes partial gasification by carbon dioxide and steam at 800^{o}C to 1000^{o}C (Aspevik, 2016). According to Aspevik (2016), AC adsorption of volatile compounds is a spontaneous process and can be presented as Equation 2.3:

$$\mathbf{\Delta G_{ads} = \Delta G_{non\text{-}elect.} + \Delta G_{elect.}} \qquad \text{Equation 2.3}$$

ΔG_{ads} represents adsorption free energy, ΔG_{elect} is the electrostatic contribution to the adsorption free energy and $\Delta G_{non\text{-}elect.}$ is specific to the system. Newcombe & Drikas (1997) stated that factors affecting $\Delta G_{non\text{-}elect.}$ include:

- The pore size and the pore size distribution of the carbon and the relationship between the size of the pores and the size of the adsorbing material.
- The hydrophobic interaction between the graphitic surface of the carbon and hydrophobic parts of the adsorbing species.
- The interaction between aromatic rings on the adsorbant and carbon.

Due to its high microporosity, activated carbon offers advantages for industrial applications including gas purification, metal extraction, water and chemical purification (Desilva, 2000).

In food industries, activated carbon acts as an excellent deodorant and is often used to improve the odour and appearance of food products (Desilva, 2000). In a study on clam (*Paphia undulate*) hydrolysates, AC adsorption effectively removes the off-odour volatile compounds n-hexanal, n-heptanal, 2,4-heptadienal and 2,3-octanedione (Chen et al., 2016). In addition, the clam hydrolysates treated with AC were described to have low intensity fishy odour by trained sensory panellists during organoleptic assessment (Chen et al., 2016). The AC deodorisation treatment also reduced the trimethylamine (TMA) content in the clam hydrolysates studied (Chen et al., 2016). However, once the AC was

filtered out of the sample the reduction in fish odour or TMA was no longer observed and the TMA content increased significantly after 72 hours of storage at 4°C (Chen et al., 2016).

2.6.3.2 Hydrogen Form Strong Acid Ion Exchange Resin for Adsorption of Amines

Ion exchange resins are polymers with capability of exchanging specific ions within the polymer with ions in a solution that is flushed through them (Zaganiaris, 2016). Ion exchange resins allow reversible interchange of ions without permanently changing the structure of the resin polymers (Dowex Chemical Co., 1974; Zaganiaris, 2016). Yoshida and Ruthven (1989) had investigated the adsorption of amines using a dry hydrogen form (H-form) strong acid ion exchange resin, which was claimed to have a favourable equilibrium relation and easy desorption compared to activated carbon adsorption. According to Yoshida and Kataoka (1986), upon contact with a H-form ion exchange resin, volatile amines are immobilised on the resin by the acid-base neutralization reaction (Equation 2.4).

$$R - H + R'NH_2 \leftrightarrow R - NH_3 - R' \qquad \text{(Equation 2.4)}$$

where R'-NH_2 denotes the amine and R-NH_3-R' denotes the amine-resin complex. The amines are recovered using caustic soda (NaOH) solution followed by regeneration of the resins by strong acid (Yoshida and Kataoka, 1986; Yoshida and Ruthven, 1989). The reactions for the amine recovery (Equation 2.5) and the regeneration of resins are presented (Equation 2.6) as follow:

$$R - NH_3 - R' + NaOH \rightarrow R - Na + R' - NH_2 + H_2O \qquad \text{(Equation 2.5)}$$

$$R - Na + HX \leftrightarrow R - H + NaX \qquad \text{(Equation 2.6)}$$

According to Yoshida and Kataoka (1986), volatile amines and ammonia are strongly adsorbed on the resins according to the previously stated acid-base neutralisation, suggesting that this method is technically and economically feasible for industrial practice.

2.6.3.3 Active Yeast masking

Active yeast is commonly used as a food additive and flavouring (Lin et al., 2013). They can contribute to the flavour and aroma of many food products due to their ability to

ferment different sugars and produce flavour compounds (Lin et al., 2013). Yeast was found to be associated with the reduction of carbonyls to alcohols, to 2-methyl-1-propanol, a volatile compound with sweet odour as the main compound to be produced (Chen et al., 2016).

Yeast masking was studied as a deodorising method for clam protein hydrolysates (Chen et al., 2016). It was determined that yeast significantly decreased off-flavour aldehydes with approximately 98% reduction of n- hexanal, 36% reduction of n-heptanal, 67% reduction of 2,4-heptadienal and 54% reduction of 2,4-decadienal in the hydrolysates (Chen et al., 2016). One of the major contributors to the off-flavour odour, 1-octen-3-ol, was also effectively decreased after the deodorisation method was employed (Chen et al., 2016). In contrast, aromatic flavour volatile compounds such as 2-ethylfuran, 2-pentylfuran and benzaldehyde were increased. These results suggested that yeast masking can modify the odour profile by increasing other aromatic compounds thus masking the fishy odour in hydrolysates (Chen et al., 2016). However, the method was found to have no effect on TMA content in the hydrolysates samples tested (Chen, et al., 2016).

2.6.3.4 Tea polyphenol treatment

Over the years, tea catechins have been employed as food antioxidants due to their antioxidant activities (Cabrera, Artacho and Gimenez, 2006; Graham, 1992; Gramza et al., 2006). These include; (i) inhibition of 'pro-oxidant' enzymes such as lipoxygenases and xanthine oxidase, and (ii) interruption of chain oxidation reactions by donating a hydrogen atom as a replacement for free radical compounds (Cabrera, Artacho and Gimenez, 2006; Lorenzo and Munekata, 2016). Tea catechins are also thought to have a deodorizing effect on amines including ammonia, dimethylamine and trimethylamine due to the ammonium salt formation between amine and polyphenols. (Dong et al., 2013; Kida et al., 2002). Polyphenols have been reported to have a neutralizing effect on amines in the same way as the addition of acid (Kida et al., 2002). It was found that the pH of a reaction mixture of TMA and polyphenols in solution was lowered as a result from neutralization reactions on amines by tea catechins acting as acids, thus decreasing the volatility of amines (Kida et al., 2002).

Tea leaves are a good source of polyphenolic compounds particularly green tea leaves which contain 30% flavonoids and phenolic acids while black tea only contains 10% flavonoids (by dry weight) (Gramza et al., 2006). According to Gramza et al. (2006), the three basic polyphenol groups in tea leaves are catechin, theaflavins and thearubigenes. Catechins are colourless, astringent and water soluble compounds predominantly found in green tea leaves (Graham, 1992). The main catechins of green tea includes (+)-catechin (C), (-)-epicatechin (EC), (-)-gallocatechin (GC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), and (-)-epigallocatechin gallate (EGCG) (Figure 9) (Gramza et al., 2006). Among these catechin compounds, EGCG has the highest antioxidant activity due to its highest number of free hydroxyl (-OH) groups (Gramza et al., 2016).

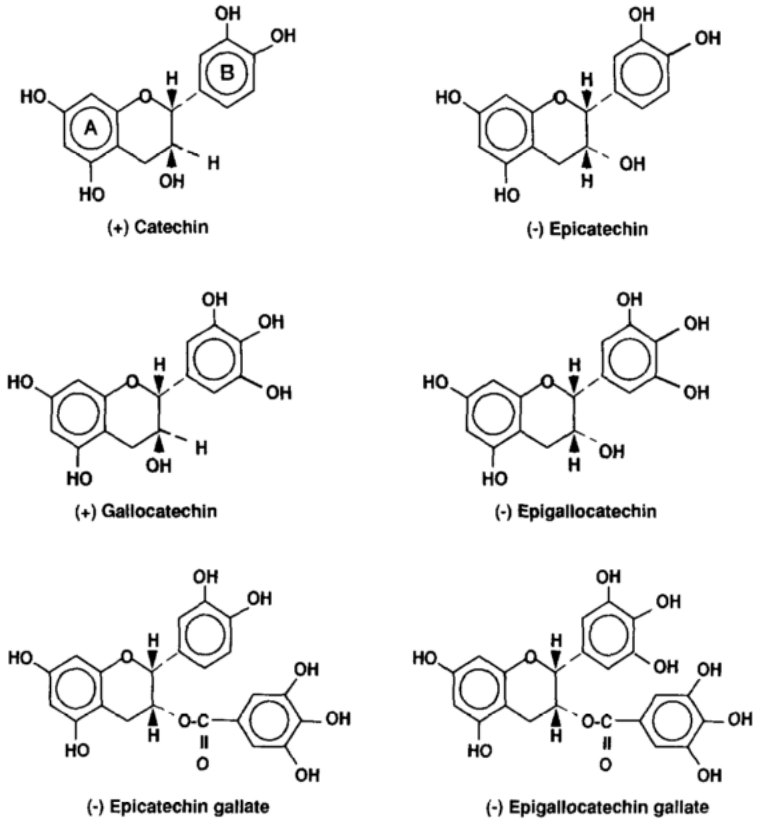


Figure 9*: Chemical structure of six main catechin coumpounds found in gree tea leaves; (+)-catechin (C), (-)-epicatechin (EC), (-)-gallocatechin (GC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), and (-)-epigallocatechin gallate (EGCG)* (Graham, 1992)

Chen et al. (2016) used the quantitative analysis technique SPME-GCMS in a study to investigate the effect of tea polyphenol (TP) treatment in clam protein hydrolysate deodorisation. In the corresponding study, the variation in the headspace was evaluated by comparing the peak area of each identified compound integrated from the total ion chromatogram, then the absolute peak area was converted to an arbitrary unit area (aua) (Chen et al., 2016). They reported that the percentage of peak area (aua) of 1-octen-3-ol in TP treated hydrolysate decreased to 4.65% from 6.07% which was found in the original untreated hydrolysate. It was found that TP significantly decreased TMA content in clam protein hydrolysates after deodorisation treatment and reduced it slightly further after 72 hours of storage at 4 °C (Chen, et al., 2016). In another study on dried-seasoned squid, the DMA content in the dried squid dipped in TP was lower (180 mg/kg) compared to the dried squid sample without TP dip treatment (270 mg/kg), after 90 days of storage at 25 ^{o}C (Dong et al., 2013). As previously discussed in Section 2.3.3.2, the formation of TMA and DMA are affected by the TMAO content in raw material. In the same study on the dried seasoned squid by Dong et al. (2013), it was found that TP has a capability to restrain the non-enzymatic reaction of TMAO thus inhibit the formation of DMA. It was reported that polyphenol tannic acid which is abundantly found in TP could inhibit hydroxyl radical formation by forming a stable complex with Fe^{2+}, consequently resulting in the inhibition of DMA formation in the squid product (Dong et al., 2013).

2.6.4 Pre-treatment process minimizes off-odour production in hydrolysate

When fishy odour in fish protein hydrolysate is due to lipid oxidation, pre-treatment is employed to remove pro-oxidants and lipids prior to enzymatic hydrolysis (Yarnpakdee et al., 2012). During pre-treatment, fish muscle protein is solubilized with acid or alkali at pHs approximately 2.5 or 11, respectively. To separate undesirable contaminants from soluble proteins the soluble proteins are precipitated at their isoelectric points at approximately pH 5.5 and recovered by centrifugation (Yarnpakdee et al, 2012). In a study on blue mussel protein isolate, it was found that samples pre-treated with 10 mmol/l calcium chloride solution and 5 mmol/l citric acid had lower concentrations of phospholipid, thereby reducing the fishy odour in the final product (Vareltris and Undeland, 2008). In another study, Indian mackerel mince was prepared by acid and alkaline solubilisation processes where 8 mmol/l calcium chloride solution was used for

the alkaline pre-treatment process and 5 mmol/l citric acid solution were used for an acidic pre-treatment process (Yarnpakdee et al., 2012). Results showed that pre-treatment processes decreased myoglobin, prooxidative haem and non-haem iron content in Indian mackerel mince (Yarnpakdee et al., 2012). Negligible fishy odour in fish protein hydrolysates allows for their wider application in food systems (Yarnpakdee et al., 2012).

2.6.5 Storage Conditions for Protein Hydrolysate

Storage conditions for protein hydrolysates is important in order to prevent secondary lipid oxidation in the product (Chen et al., 2016). Typically, dried fish hydrolysates are stored at 4 ^{o}C or lower in vacuum packaging (He, Franco and Zhang, 2013). In a study on by fish protein hydrolysates from Pacific Whiting, samples were stored at -20 ^{o}C in vacuum packed polyethylene bags and were maintained until used (Mazorra-Manzano et al., 2012). In another study, herring protein hydrolysates were stored in plastic containers at 4 ^{o}C until used (Liceaga-Gesualdo and Li-Chan, 1999). Lower storage temperature and oxygen removal prior to storage can reduce the oxidation of hydrolysate thus, extending the shelf life of the product (He, Franco and Zhang, 2013). Temperatures below 4 ^{o}C can effectively reduce oxidation regardless of the oxygen levels (He, Franco and Zhang, 2013).

2.7 Fish Collagen and Gelatine Extraction

This section discusses the extraction procedures of both collagen and gelatine from raw fish materials.

2.7.1 Collagen Extraction

Collagen extraction from marine fishes often involves two main steps; the pre-treatment of raw materials and the extraction of collagen (Pal and Suresh, 2016). The pre-treatment processes, prior to the extraction of collagen, to remove impurities such as non-collagenous proteins, calcium and lipids are necessary to increase the yield and quality of the final extracted collagen (Pal and Suresh, 2016; Schmidt et al., 2016). Collagen proteins in the connective tissue of marine fishes have covalent intra- and intermolecular cross links including ester bonds with saccharides and lysine residues that dissolve at a very slow rate even in boiling water (Schmidt et al., 2016). Because of this, chemical treatment

is essential to break these cross-links prior to collagen extraction and hydrolysis (Schmidt et al., 2016). Current industrial practices employ diluted acids and alkalis to cleave the cross-links without disturbing the collagen chains (Schmidt et al., 2016). During acidic pre-treatment, raw materials are immersed in acid solution allowing the solution to penetrate throughout the materials causing them to swell to two or three times their initial volume before breaking the non-covalent inter- and intramolecular bonds (Ledward, 2000). Acidic pre-treatment is more suitable for raw materials with less inter-twined collagen fibres such as fish skins (Schmidt et al., 2016). Alkali pre-treatment is usually employed when more aggressive penetration by the chemical reagent is required for thicker raw materials such as bovine ossein and bovine shavings (Schmidt et al., 2016). Sodium hydroxide is typically used as it causes significant swelling of raw materials which facilitates the extraction of collagen protein (Schmidt et al., 2016). According to Liu et al. (2015), sodium hydroxide concentrations between 0.05 to 0.1M effectively removed non-collagenous proteins without losing the acid soluble collagen and collagen structure of grass carp skin. The use of acids and alkalis can assist in the cleavage of cross-links to produce products with different characteristics (Schmidt et al., 2016).

There are three common methods of collagen extraction; (i) salt solubilisation extraction that produces salt-solubilised collagen (SSC), (ii) acid solubilisation extraction that produces acid-solubilised collagen (ASC) and (iii) enzymatic solubilisation extraction that often uses pepsin to produce pepsin soluble collagen (PSC) (Kumar and Suresh, 2016; Schmidt et al., 2016). Collagens are resistant to most proteases and special enzymes are essential for their enzymatic extraction (Gorgieva and Kokol, 2011). Very often, pepsin is used for the extraction of collagen (Kittiphattanabawon et al., 2015; Kumar and Suresh, 2016; Schmidt et al., 2016; Wang et al., 2014). The extraction of collagen from marine fish is generally achieved at low temperature (often at 4 ^{o}C) to avoid denaturation of the collagen structure (Kumar and Suresh, 2016). Collagen is highly sensitive to temperature mainly due to its helical structure maintained by the conformational restrictions of hydroxyproline and proline as well as the inter-chain hydrogen bonding between the hydroxyl groups of hydroxyproline (Liu et al., 2015). Generally, collagens from cold water fishes have lower contents of these amino acids compared to those of warm water fishes indicating that the warm water fishes have higher heat resistance (Liu et al., 2015).

Kumar and Suresh (2016) reported that increasing the concentration of chemical reagent used in the extraction process can increase the collagen yield, however, it is less desirable as this can cause the degradation of the final collagen. After the extraction process, collagens are recovered by salt precipitation followed by centrifugation. In general practices, collagens are precipitated using 2.6M NaCl in the presence of tris(hydroxymethyl)aminomethane at pH 7.5 (Kumar and Suresh, 2016; Li et al., 2013). The precipitated collagens are then collected by centrifugation at 10,000 to 20,000 rpm for 60 minutes at 4 ^{o}C (Kumar and Suresh et al., 2016).

2.7.2 Gelatine Extraction by Thermal Denaturation of Collagen

As discussed in Section 2.1.2, gelatine is the product of thermal denaturation and disintegration of insoluble native collagen (Gorgieva and Kokol, 2011; Karim and Bhat, 2009). Gelatine extraction involves acid or alkali pre-treatment to breakdown the intra- and intermolecular covalent crosslinks present in the native collagen, resulting in the loss of the ordered structure of the insoluble native collagen into swollen but still insoluble collagen (Karim and Bhat, 2009; Mohtar, 2012). Later heat treatment destabilises the triple helix structure of the collagen, resulting in gelatine (Mohtar, 2012; Gorgieva and Kokol, 2011). According to Gomez-Guillen et al. (2011), heat treatment of gelatine is normally conducted at temperatures higher than 45 ^{o}C. Gelatines are divided according to the pre-treatment procedures; (i) Type A which is derived from acid pre-treatment (isoelectric point at pH ranges between 8 and 9), (ii) Type B which is derived from alkali pre-treatment (isoelectric point at pH ranges between 4 and 5) (Gomez-Guillen et al., 2011; Gorgieva and Kokol, 2011). Compared to collagen (refer to Section 2.1), gelatines are more susceptible to most proteases and can be broken down into peptides chains containing only 20 amino acids (Gorgieva and Kokol, 2011). Upon cooling, gelatine chains form triple helices that are stabilised by intermolecular hydrogen bonds forming gels (Figure 10 and 11) (Mohtar, 2012). The rearrangements of these gelatine chains occur in a random manner and the gels formed are often referred to as renaturated gelatines (Gorgieva and Kokol, 2011).

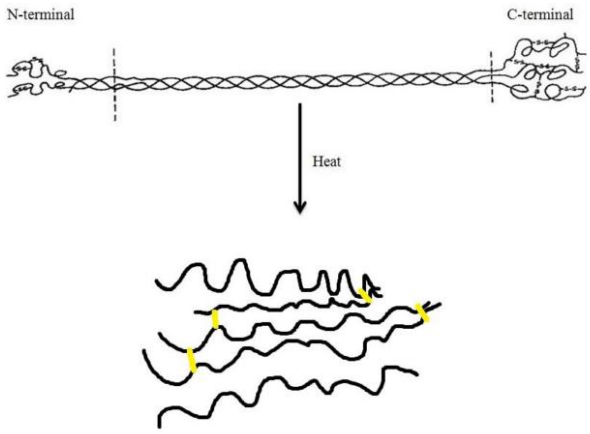


Figure 10*: Illustration of the thermal denaturation of collagen to gelatine* (Mohtar, 2012).

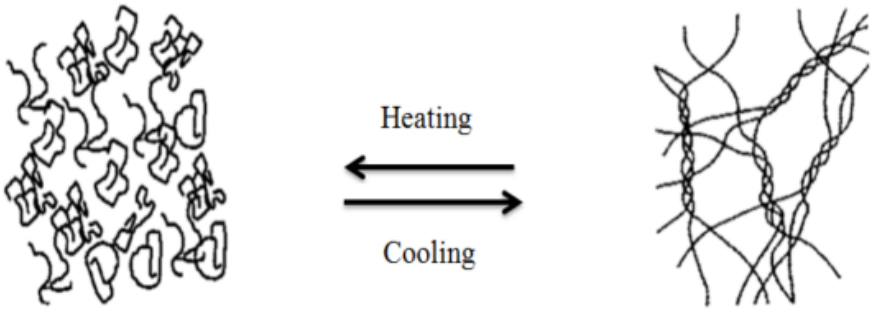


Figure 11*: Illustration of the gels formation from gelatine solution. Triple helix structure is regained due to the randomly-coiled gelatine peptides stabilised by the intermolecular hydrogen bond after cooling. The process is reversible* (Mohtar, 2012).

2.7.3 Pre-treatments and Extraction Procedures for Collagen and Gelatine

The pre-treatment and extraction procedures of both collagen and gelatine from various raw materials are summarised in Table 3. Attributes of final products manufactured after the extraction processes are also provided. Note the pre-treatment and extraction processes of collagen and gelatine in Table 3 are a summary of laboratory processes which do not reflect the full size of industrial practice, thus these may not be practicable at full scale.

Table 3*: Pre-treatments and extraction procedures for collagen and gelatine from different raw materials.*

Raw Material	Pre-treatment	Extraction Procedure	Final product	Reference
Yellowfin tuna swim bladder	Skins were treated with 0.15M NaOH at a ratio of 1:10 (w/v) for 2 hours at 4°C then defatted with 10% butyl alcohol at a ratio of 1:10 (w/v) for 12 hours before washed with cold distilled water three times.	Extraction with 0.5M acetic acid at a ratio of 1:10 (w/v) for 48 hours at 4°C. Collagens were precipitated with 2.6 M NaCl in the presence of 0.05 M tris (hydroxymethyl) amino-methane at pH 7.5.	Acid-solubilised collagens (ASC) were successfully isolated from the raw material. The ASC extracted were mainly composed of type I collagen and glycine was the predominant amino acid present. The product showed high solubility at pH 4.	Kaewdang et al., (2014)
Spanish mackerel skin	Cleaned skins were treated with 0.1M NaOH at ratio of 1:10 (w/v) for 2 days at 4°C before washed with cold water until neutral pH is achieved to remove fat.	Defatted skins were soaked in 0.5 M acetic acid at a ratio of 1:15 (w/v) for 24 hours then filtered through cheesecloth. Collagens were precipitated with 2.6 M NaCl in the presence of 0.05 M tris (hydroxymethyl) amino-methane at pH 7.5.	ASC were successfully isolated from the raw material. The ASC extracted were mainly composed of type I collagen and glycine was the predominant amino acid present. The product showed high solubility in the acidic pH ranges between 1 and 4.	Li et al. (2013)
Japanese sturgeon skin	Skin was homogenized with 20% (w/v) NaCl three times at 4°C to remove non-collagenous substances.	Salt-solubilised collagen (SSC) was extracted with 0.45M NaCl at ratio of 1:100 (w/v) for 24 hours followed by extraction with 0.5M acetic acid twice for 24 hours. Further extraction was done using 0.1% (w/v) pepsin in 0.01M HCl for 48 hours. All extraction processes were done at 4°C.	SSC collagen were succesafully isolated. The SSC extracted were composed of type I collagen with a molecular weight of approximately 120 kDa. Glycine was the predominant amino acid present. The thermal stability, T_m = 116.62 ˚C.	Wang et al. (2014)
Hoki skin	Cleaned and minced skins were treated with 0.75M NaCl solution (1:6 w/v) for 9 minutes at 4°C, then rinsed with tap water. The steps were repeated twice. The skins were stirred in Milli-Q water (1:6 w/v) for 60 minutes at 49.3°C in shaking water bath followed by centrifugation at 10000 rpm for 30 minutes at 15°C.	Gelatine was obtained by filtering the centrifuged clear extract using filter paper.		Mohtar (2013)
Frozen African catfish skin	Cleaned skins were treated with 0.2N NaOH solution (1:6 w/v) for 60 min at room temperature then drained and rinsed with tap water.	Pre-treated skins were mixed with distilled water (1:10 w/v) and gelatine was extracted at 50 °C for 180 min.	Gelatine was successfully extracted from the raw material. Percentage of yield of protein recovery was 34.73 %. The product showed gel strength ranging between 179.42g and 187.76g. The molecular weight determined was 120 kDa.	See et al. (2013)
Frozen African catfish skin	Cleaned skins were treated with 0.5N acetic acid solution (1:6 w/v) for 60 min at room temperature then drained and rinsed with tap water.	Pre-treated skins were mixed with distilled water (1:10 w/v) and gelatine was extracted at 50 °C for 180 min.	Gelatine was successfully extracted from the raw material. Percentage of yield of protein recovery was 68.97 %. The product showed gel strength of 202.68 g. The molecular weight determined was 97.4 kDa.	See et al. (2013)

2.6 Analytical Methods for Analysing Hydrolysates Post-Deodorisation Treatment and Collagen Post-Extraction Processes

Analytical methods are employed to provide qualitative and quantitative analyses of the hydrolysates after deodorisation treatments and collagen extracts after extraction processes.

2.6.1 Steam Distillation for the Determination of Total Volatile Base Nitrogen (TVB-N) and Trimethylamine (TMA)

Total volatile basic nitrogen (TVBN) encompasses ammonia, dimethylamine (DMA) and trimethylamine (TMA) (Etienne and Ifremer, 2005). One of the analytical methods to analyse amine compounds in a fish hydrolysates sample is the steam distillation method (Kirk & Sawyer, 1991). Protein hydrolysate is treated with trichloroacetic acid, a deproteinizing agent that causes the protein to precipitate (Rajalingam et al., 2009). The sample is then mixed with alkali, typically sodium hydroxide, to facilitate the isolation of volatile amines (Kirk & Sawyer, 1991). The sample is then distilled and the distillate is collected in boric acid solution before being titrated with sulphuric acid (Kirk & Sawyer, 1991, Malle and Tao, 1987). The TVBN content in the sample is calculated using Equation 2.7 (Malle and Tao, 1987).

$$TVBN = \frac{n \times 16.8\, mg\ of\ nitrogen}{100 g\ of\ sample} \qquad (2.7)$$

Where n = volume of sulfuric acid used for titration (ml).

The same procedure as TVBN determination is applied for the quantitation of TMA except that formaldehyde solution is added to the protein hydrolysate sample prior to titration to block the primary and secondary amines as presented in the following reactions:

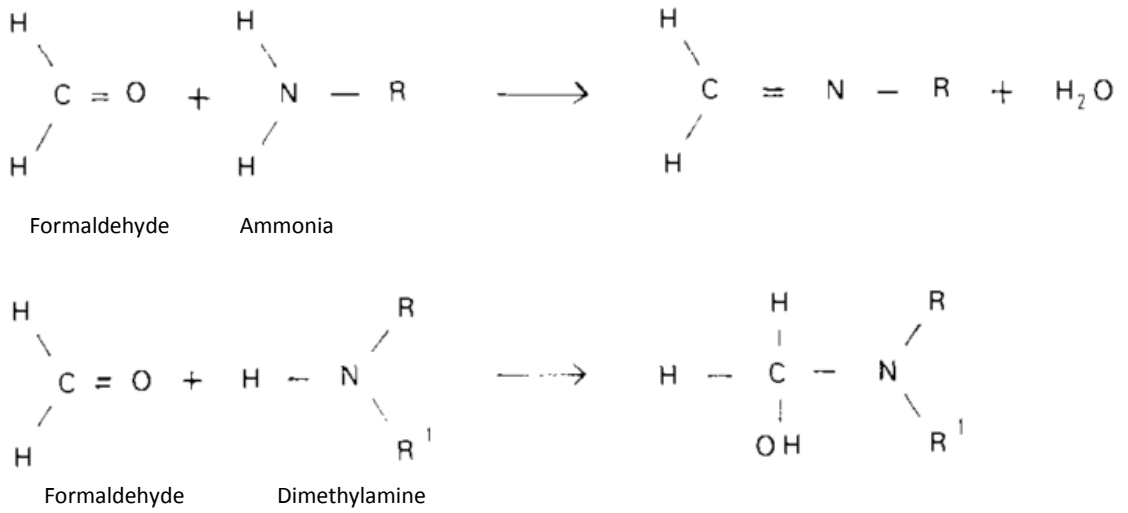


Figure 12*: The chemical reactions for the blocking of primary and secondary amines by formaldehyde* (Malle and Tao, 1987).

The resulting compounds were not volatile and thus will not interfere in the quantitation of TMA (Malle and Tao, 1987). The TMA content is determined using Equation 2.8 (Malle and Tao, 1987).

$$TMA = \frac{n \times 16.8\, mg\ of\ nitrogen}{100g\ of\ sample} \qquad (2.8)$$

Where n = volume of sulfuric acid used for titration (ml).

Malle and Tao (1987) also stated that the steam distillation method provides simplicity, efficiency and rapidity in determining the TVB-N and TMA content in fish samples.

2.6.2 Solid-phase Microextraction Gas Chromatography (SPME- GC) analysis for volatile compounds

Recently, the solid-phase micro-extraction (SPME) headspace method in combination with gas chromatography (GC) has been developed to quantify volatile amines in fish samples (Dehaut et al., 2015). SPME is an adsorption-desorption technique used to quantify volatile compounds (SUPELCO, 1998). SPME uses fused silica fibers coated with a polymer to extract compounds from samples or the headspace above samples allowing

organic analytes to be adsorbed onto the fibers around the needle (SUPELCO, 1998). The needle is then introduced to the gas chromatography injector and adsorbed analytes are thermally desorbed and delivered to the GC column (SUPELCO, 1998). There are several factors that affect the optimization of SPME headspace method including polarity and thickness of the coating fibre, sample agitation, extraction time and sampling method (SUPELCO, 1998). Different conditions for SPME-GC analysis of volatile compounds are summarised in Table 4.

Table 4*: Summary of the different conditions for SPME-GC analysis of various volatile compounds.*

Instrument	Oven temperature	Injector	Mass Spec	Automated SPME Conditions	Heated Headspace	Sample	References
The GC-MS system consisted of an Agilent 6890 gas chromatograph equipped with a Supelco PTA-5 (30 m × 0.25 mm i.d. based deactivated polydiphenyl and polydimethylsiloxane) column.	Oven temperature was initially kept at 35 ◦C for 3 min, then ramped at 40 ◦C min−1 to 200 ◦C and kept for 2 min to remove other volatile or semi-volatile interference before the next injection. Flow of helium carrier gas was set at 1.3 ml min−1 under splitless mode.	Injector: 200°C	Identification of volatile amines in samples was confirmed by the presence of ion fragments (at m/z = 30 for MA, 44 for DMA, 58 for TMA and 59 for NPA, respectively	Four different commercial SPME fibres: carbowaxdivinylbenzene (CW/DVB) with 70 µm film thickness; carboxen/divinylbenzene/polydimethylsiloxane (CAR/DVB/ PDMS) with 50/30 µm film thickness; PDMS with 100 µm film thickness and PDMS/DVB with 65 µm film thick	The detector and transfer line were set at 250 and 280 ◦C, respectively. 2 min for desorption.	Determination of volatile amines in snapper and freshwater grouper samples.	Chan et al. (2006)
Analyses were carried out on a Thermo Finnigan ThermoQuest (San Jose, CA) gas chromatograph equipped with a 30m x 0.32mm x 1 µm film thickness, fused silica DB-1701 (Folsom CA) capillary column.	The oven temperature was kept at 35 ˚C for 3 min, followed by an increase of 3 ˚C/ min to 70 ˚C; then an increase of 10˚C/min to 200 ˚C and finally an increase of 20 ˚C/min to a final temperature of 260 ˚C, held for 5 min. Flow of helium gas was set at 1.5 ml/min.	Injector:270 ˚C	All the analyses were performed at settings: ionisation energy at 70 eV, filament emission current at 150 lA and the electron multiplier voltage at 500 V. The identification of the volatile compounds was achieved by comparing their mass spectra with those stored in the National Institute of Standards and Technology (NIST) US Government li- brary. Pure standards were also injected to confirm MS identification.	The fibres: carboxen/polydimethylsiloxane 75 lm(CAR–PDMS), polydimethylsiloxane/divinylbenzene 65 lm (PDMS–DVB), poly-dimethylsiloxane 100 lm (PDMS) and carboxen/polydimethyl-siloxane/divinylbenzene 2 cm 50/30 lm (CAR–PDMS–DVB).	The fibre was exposed to headpace for for 30 min at 40 ˚C. Desorption was carried out at the temperature of 260 ˚C for 2 min.	The determination of volatile compounds in gilthead sea bream.	Iglesia et al. (2009)
GC–MS analysis was performed in a HP 5890 series II gas chromatography (GC) coupled with HP 5972 mass-selective detector equipped with a HP-Innowax capillary column (Hewlett Packard, Atlanta, GA, USA) (30m x 0.25mm ID, with film thickness of 0.25 µm).	The GC oven temperature program was: 35 ˚C for 3 min, followed by an increase of 3 ˚C/min to 70 ˚C, then an increase of 10 ˚C/min to 200 ˚C, and finally an increase of 15 ˚C/min to a final temperature of 250 ˚C and holding for 10 min. Helium was employed as a carrier gas, with a constant flow of 1 ml/min.	Injector: 270 ˚C.	All the analyses were performed with ionisation energy of 70 eV, filament emission current at 150 lA, and the electron multiplier voltage at 500 V.	The SPME fibre was (50/30 µm DVB/Carboxen™/ PDMS StableFlex™) (Supelco, Bellefonte, PA, USA)	The fibre was exposed to headspace for 1 hour at 60˚C.	The determination of volatile compounds in Nile tilapia protein hydrolysate samples.	Yarnpakdee et al. (2012).

The GC system was equipped with with a SolGel Wax capillary column (60m x 0.25mm i.d., 0.25mm film thickness	GC oven temperature was initially held at 35 ˚C for 5 min, followed by an increase of 2.5 ˚C/min to 90 ˚C, an increase of 4 ˚C/min to 180 ˚C and finally an increase of 10 ˚C/min to a final temperature of 200 ˚C, held for 4 min. Helium was used as carrier gas at a flow rate of 1.3 ml/min.	Injector: 250˚C	Identification was confirmed by both NIST (National Institute of Standards and Technology) library searches and comparison with the retention time of external standards.	SPME fibre used was carboxen/polydimethylsiloxane fibre (CAR/PDMS 75-lm; Supelco, Bellefonte, PA, USA).	The fibre was exposed at 60 ˚C for 30 min.	Identification of volatile compounds formed due to lipid oxidation of fish oil.	Serfert, Drush and Schwarz (2010)
A gas chromatography system (TRACE MS, Finnegan, USA) equipped with silica gel capillary column.	The GC oven temperature programe was set from 35 to 120 ˚C at a rate of 8 ˚C/min, from 120 to 220 ˚C at a rate of 12 ˚C/min, then kept at 220 ˚C for 12 min. Identification	Injector: 230˚C	Identification of constituents was based on comparison with pure external references. and on computer matching with commercial mass spectra libraries (Nist98, Wiley610 Library).	SPME fiber used was divinylbenzene/Carboxen/poly (dimethyl-siloxane) (DVB/Carboxen/PDMS)	The fibre was exposed to headspace for 60 min at 25 ˚C	Identification of volatile compounds formed due to lipid oxidation of silver carp.	Fu, Xu and Wang (2009)

2.6.3 Sensory Evaluation

Characterization of perceived flavour in food products is known as sensory analysis (Stone et al., 2004). There are numerous methods used for sensory evaluation with each method have different procedures in understanding the organoleptic profiles of various food products (Lawless and Heymann, 2010). The methods include; (i) screening test that allows the screening for possible respondents in sensory analysis, (ii) descriptive analysis that measures the type and intensity of attributes in a product based on the descriptions and intensity determinations by respondents using scaling procedure, (iii) forced choice discrimination method that is used to confirm suspected small differences in product attributes or product quality and (iv) threshold method which is a method designed to determine the strength or concentration of a stimulus required to produce a minimal detectable effect (minimum threshold) in a food product (Lawless and Heymann, 2010). Methods used for sensory evaluation of fish and fish protein hydrolysates are summarised in Table 5.

Table 5: *Sensory evaluation methods used for fish and fish protein hydrolysates.*

Sample	Sensory Evaluation Method	Procedure	Reference
Silver carp hydrolysate	Descriptive analysis	Hydrolysate quality was evaluated by trained panellists. Colour, odour, flavour and general acceptability and texture attributes of the products are scored on a nine-point hedonic scale.	Fan, Chi and Zhang (2007)
Clam *(Paphia undulata)* hydrolysate	Descriptive analysis and threshold method	Aroma that characterized the hydrolysates were described by trained panellists. Characteristics associated with fishy odour were scored to determine the minimum threshold for fishy odour in the product.	Chen et al. (2016)
Yellow croaker (*Pseudosciaena crocea)*	Quality index method (QIM)	Sensory scores between 0 to 3 for quality parameters that characterize the fish were assigned by trained panellists.	Li et al. (2012)
Nile tilapia hydrolysate	Descriptive analysis	The fishy odour of the hydrolysates were evaluated by untrained panellists using a nine-point hedonic scale.	Yarnpakdee et al. (2012)
Rainbow trout	Scaling test and descriptive analysis	Panellists assessed the odour intensity of the fish extract by marking the scale line. A list of attributes that characterize the aroma of cooked trout was assessed by panellists on an unstructured scale of 100mm anchored.	Selli et al. (2006)
Liver pate	Flash profiling method	Respondents generated their own free vocabulary to describe the sensory variations in the nine liver pate samples. Then, respondents were asked to rank the liver pate samples according to the intensities of each attribute assigned beforehand.	Dehlholm et al. (2012)

2.7 Conclusions

Chemical hydrolysis requires a short processing time for fish protein hydrolysate but can generate the formation of toxic compounds and causing the loss of valuable amino acids.

Enzymatic hydrolysis provides mild conditions for fish protein hydrolysates with minimum loss in physiochemical properties of the product.

Physicochemical properties and production yield of protein hydrolysates are affected by the type of enzyme used, initial substrate, enzyme and substrate concentration and pH and temperature used during enzymatic hydrolysis.

Off-odour volatile compounds associated with fish odour in fish protein hydrolysate may potentially be caused by the following compounds: n-hexanal, n-heptanal, 2,4-heptadienal, 2,4-decadienal, 1-octen-3-ol, 2, 3-octanedione and TMA.

Activated carbon adsorption can remove some off-odour volatile compounds in fish protein hydrolysate but not amines.

Hydrogen form strong acid ion exchange resin can be employed for the adsorption for volatile amines but not neutral or acidic organic compounds.

Yeast fermentation can modify the odour profile by increasing the production of aromatic compounds and metabolise some of the precursors of off-flavour aldehydes and ketones hence masking the fishy odour in fish protein hydrolysate.

Tea polyphenol treatment can remove fishy odour in fish protein hydrolysate and can suppress trimethylamine formation in hydrolysate solution for a certain period of time.

Collagen and gelatine proteins contains covalent intra- and intermolecular cross links including ester bonds with saccharides and lysine residues that are essential to be removed prior to collagen and gelatine extractions.

Acid and alkali pre-treatments can be employed to remove non collagenous proteins prior to collagen and gelatine extraction procedures.

Collagen extraction is conducted at low temperature, generally at $4^{o}C$.

Gelatine extraction involves thermal denaturation of collagen protein and generally occurs at temperatures above $40^{o}C$.

Analytical methods including steam distillation, solid-phase microextraction gas chromatography (SPME- GC) and sensory analysis can be used to provide qualitative and quantitative analyses to evaluate the collagen and gelatine samples after deodorisation treatments and extraction procedures.

Chapter 3: Materials and Methods

3.0 Introduction

This chapter describes materials and methods used in this study. This study was divided into two major parts:

1. The deodorisation of Hoki skin protein hydrolysate. The first part of the study was carried out with the aim of determining the suitable deodorisation treatments that reduce or remove off-odour in pre–prepared Hoki skin protein hydrolysates. Experiments were conducted with previously reported deodorants such as dried active yeast, powdered activated carbon, dried green tea leaves, powdered tea polyphenol, ground dried olive leaves and hydrogen-form-strong-acid ion exchange resins.
2. The extraction of collagen and gelatine from raw Hoki skins. The second part of the study was conducted to (i) determine suitable extraction processes that reduce or remove off-odour in the extracted collagen and gelatine products, and (ii) to identify the extraction processes that produce collagen and gelatine with high protein contents and high yields.

The key indicators in the assessments of the off-odour in Hoki skin protein hydrolysates post-deodorisation treatments and gelatine samples post-extraction processes are the total volatile base nitrogen (TVB-N) and the trimethylamine (TMA) contents. The flash profiling method for sensory analysis were used to provide the quantitative and qualitative analysis.

The collagen and gelatine samples obtained after extraction processes were subjected to the Kjeldahl method for total protein, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for determination of molecular weight. Amino acid testing was also conducted to provide identification of the final products.

3.1 Deodorisation of Hoki Skin Protein Hydrolysate

This section discusses the materials and methods used for the deodorisation of the pre-prepared Hoki skin protein hydrolysates.

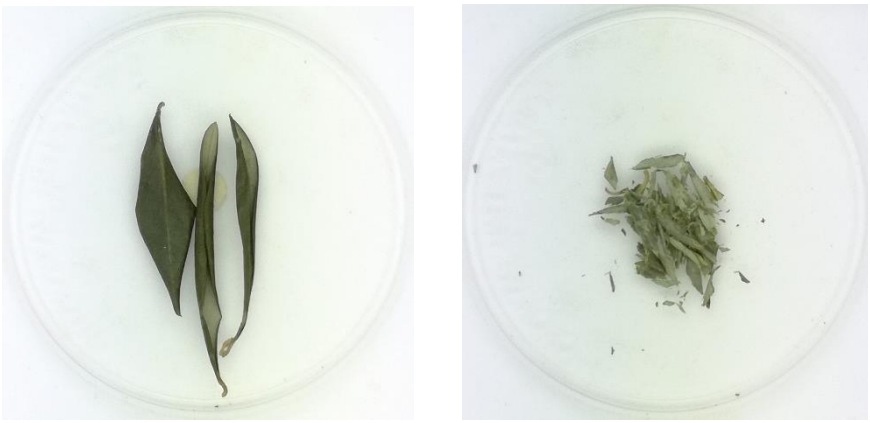
3.1.1 Hoki Skin Protein Hydrolysate

Hoki skin protein hydrolysate was pre-prepared by Sanford Ltd by enzymatic hydrolysis and received in February, 2016 after storage at -20^{o}C. The hydrolysate was thawed at room temperature (18±2^{o}C) and decanted into in 500 ml sealed plastic bottles and stored at -20^{o}C until used.

3.1.2 Deodorants

Powdered activated carbon (coconut shell, NZ Commodities Ltd., Auckland, New Zealand), dried active yeast (Prime, Auckland, New Zealand), powdered tea polyphenol (98%, Shanghai Pengwan Technology Co. Ltd., Sichuan, China), and dried green tea leaves (Jade green sencha, Tea Total Ltd., Auckland, New Zealand) were stored in closed containers at room temperature (18±2^{o}C).

Olive leaves were collected from an olive tree at the Mt Albert Research Centre, Auckland in March 2017. The olive leaves were dried at 30^{o}C for 48 hours, then ground into approximately 1 cm^{2} in size (Figure 13). The leaves were packed in resealable plastic bags and stored at room temperature (18$\pm$2^{o}C).



(a) (b)

Figure 13: *(a) Olive leaves after drying at 30 °C for 48 hours. (b) Dried olive leaves were ground to approximately 1 cm² pieces.*

Dowex G-26 (hydrogen form) resin was purchased from Sigma-Aldrich (Auckland, New Zealand). Regenerated resins were soaked in distilled water and stored at room temperature (18±2°C) until used.

3.1.3 Screening of Deodorants for Hoki Skin Protein Hydrolysates

In the preliminary studies, six different deodorants were identified from the published literature. Experiments were conducted according to the optimum deodorisation conditions recommended in the literature for each deodorant (Chen et al., 2016; Luo, 2011).

The deodorisation treatments with dried active yeast, powdered activated carbon, powdered tea polyphenol, dried green tea leaves and dried olive leaves are summarised in Table 6.

Table 6*: Summary of the deodorisation treatments using different deodorants identified in the literature.*

Deodorant	Treatment			Reference
	Concentration (g/ ml hydrolysate)	Temperature (^{o}C)	Time (min)	
Dried active yeast (YE)	0.001	45	30	(Chen et al., 2016)
	0.003			
	0.010			
Powdered activated carbon (AC)	0.019	80	40	(Chen et al., 2016)
	0.035			
	0.051			
Powdered tea polyphenol (TP)	0.004	40	50	(Chen et al., 2016)
	0.020			
	0.040			
Dried green tea leaves (GT)	0.030	90	10	(Luo, 2011)
	0.050			
	0.100			
Dried olive leaves (OL)	0.030	90	10	(Luo, 2011)
	0.050			
	0.100			

For all deodorisation treatment, 50 ml of pre-prepared HSPH was transferred into a plastic bottle and then treated with respective deodorants in shaking water bath pre-set at the specified temperatures for respective times (Table 6). All treated samples were then centrifuged at 4100 rpm (Multifuge 1 S-R, Heraeus, Kendro Laboratory Products, Germany) for 30 minutes before they were filtered using Whatman filter paper (No. 5). All experiments were carried out in triplicate.

3.1.3.1 Deodorisation with Ion Exchange Resin

The adsorption of volatile amines on hydrogen-form-strong-acid ion exchange resins (Dowex G-26) was performed according to the method of Yoshida and Ruthven (1989) in a glass column with a sintered glass plate to support the resin. The glass column was filled with 30 g of ion exchange resin. Distilled water was flushed through the column twice to rinse the resin at 2.1 ml/min. Hoki skin protein hydrolysates of different volumes (30 ml, 90 ml and 150 ml) were then allowed to pass through the column to obtain different ratios of

hydrolysate to resin (1:1, 1:3 and 1:5 w/v respectively) at a flow rate of 2.1 ml/min. The hydrolysates were passed through the resin once.

The adsorption of amines is expressed by the acid-base neutralization reaction (Equation 2.4):

$$R.H + R' - NH_2 \leftrightarrow R.NH_3 - R' \qquad (2.4)$$

where $R' - NH_2$ denotes the amine, and $R.NH_3 - R'$ denotes the amine-resin complex.

To recover the amines, 0.1 M NaOH was passed through the column at 2.1 ml/min. The reaction is expressed by the following irreversible neutralization reaction (Equation 3.1):

$$R.NH_3 - R' + NaOH \rightarrow R.Na + R' - NH_2 + H_2O \qquad (2.5)$$

The resins were regenerated with 1 M HCl at 2.1 ml/min and the reversible reaction is expressed in Equation 3.2.

$$R.Na + HCl \leftrightarrow R.H + NaCl \qquad (2.6)$$

pH values were monitored throughout the process. The experiment was conducted at room temperature (18±2^{o}C). The resin was regenerated before each batch of hydrolysate was passed through the column. The experiment was conducted in triplicate.

3.1.4 Assessment of Deodorized Hydrolysates

Suitable deodorants were determined based on an evaluation of the aroma using a scoring system as shown in Table 7. The odour profile of treated HSPH samples were self-evaluated in a well-ventilated laboratory.

Table 7*: Description of sensory scores used to assess odour profiles of hydrolysate samples after deodorisation treatment.*

Sensory score	***Description***
0	No fishy odour
1	A hint of fishy odour, almost undetectable
2	Slight detectable fishy odour
3	Detectable fishy odour
4	Strong fishy odour
5	Very strong fishy odour, similar to untreated HSPH

Sensory scores of 3 and below are considered to be the acceptable level, thus respective deodorisation treatments are selected to be further investigated to determine the suitable deodorisation conditions for pre-prepared HSPH samples. The sensory scores for all samples post deodorisation were self-assessed.

3.1.5 Experimental Design for Deodorisation Treatments using Selected Deodorants

Three suitable deodorants were identified from the screening studies. Based on the method of Chen et al., (2016), three experiments with orthogonal designs $L_9(3^4)$ for dried green tea leaves (GT), powdered tea polyphenol (TP), and dried ground olive leaves (OL) were generated using IBM SPSS Statistics Software 21.0 (IBM®, United States) to determine the deodorisation treatments which were able to decrease or remove off-odour in Hoki skin protein hydrolysates. Three factors (concentration, temperature and time) and three corresponding levels were used in the design, as shown in Table 8.

Table 8: *Orthogonal table for experimental design to test dried green tea leaves (GT), powdered tea polyphenol (TP) and dried olive leaves (OL) for deodorisation of Hoki skin protein hydrolysates.*

Deodorant	Level	Factors		
		Concentration (g mL^{-1})	Temperature (^oC)	Time (min)
Dried green tea leaves (GT)	1	0.030	30	10
	2	0.050	50	20
	3	0.100	90	30
Powdered tea polyphenol (TP)	1	0.004	40	20
	2	0.020	50	50
	3	0.040	80	60
Dried olive leaves (OL)	1	0.030	30	10
	2	0.050	50	20
	3	0.100	90	30

For each deodorisation treatment, 50 ml of pre-prepared HSPH was transferred into a plastic bottle and then treated with respective deodorants in shaking water bath pre-set at the specified temperatures for respective times (Table 8). All treated samples were centrifuged at 4100 rpm (Multifuge 1 S-R, Heraeus, Kendro Laboratory Products, Germany) for 30 minutes at 15±2^{o}C before they were filtered using Whatman filter paper (No. 5). The supernatant was collected and stored at -20^{o}C before further analysis. Each deodorisation treatment was conducted in triplicate.

3.2 Extraction of Hoki Collagen and Gelatine

3.2.1 Hoki Skin

Hoki skins were obtained as a 20 kg frozen block from Sanford Ltd. in September 2017. The skins were stored at -20^{o}C freezer prior to their preparation.

3.2.2 Preparation of Raw Hoki Skin

The frozen block of Hoki skins were thawed at 4^{o}C for 72 hours. The skins were washed in tap water twice and were then cut into 3 x 3 cm pieces. The skin pieces were packed into (14 x 20 cm size) resealable plastic bags and stored at -20^{o}C until used.

Figure 14*: Cleaned Hoki skins were cut into 3 x 3 cm pieces before packed and stored at -20* ^{o}C*.*

3.2.3 Extraction of Hoki Collagen

Three pre-treatment and extraction procedures were conducted using modified methods from published literature to extract the collagen from Hoki skins (Table 9).

Table 9*: Table of extraction procedures of collagen from Hoki skins.*

Method	Pre-treatment and extraction procedure	Reference
A	Cleaned raw Hoki skins were pre-treated with 0.5M citric acid solution (1:6 w/v) for 60 min at 18±2°C. Pre-treated skins were rinsed with distilled water. Clear collagen sample was collected by sieving the treated skins using a domestic kitchen sieve.	(Babji et al., 2015)
B	Cleaned raw Hoki skins pre-treated with 0.2M NaOH solution (1:6 w/v) for 60 min at 18±2°C. Pre-treated skins were rinsed with distilled water. Clear collagen sample was collected by sieving the treated skins using a domestic kitchen sieve.	(Babji et al., 2015)
C	Cleaned raw Hoki skins pre-treated with 0.75M NaCl solution (1:6 w/v) for 10 min at 18±2°C. Pre-treated skins were rinsed with distilled water. Clear collagen sample was collected by sieving the treated skins using a domestic kitchen sieve.	(Mohtar, 2012)

pH values of all extracted collagen samples were recorded. All collagen samples were stored in glass bottles at -20°C until used. Experiments were conducted in triplicate.

3.2.3 Extraction of Hoki Gelatine

Five pre-treatment processes were conducted using modified methods from published literature to extract the gelatine from Hoki skins (Table 10).

Table 10*: Table of pre-treatment and extraction methods of gelatine from Hoki skins.*

Method	Pre-treatment	Extraction procedure	Reference
A	The washed and pre-cut Hoki skins were pre-treated with 0.75 M NaCl solution (1:6 w/v) for 10 minutes at 18 ± 2°C (room temperature). The pre-treated skins were then rinsed with distilled water. These steps were repeated twice.	The pre-treated skins were stirred in distilled water (1:6 w/v) for 60 minutes at 50 ± 2°C in water bath (Pura, Julabo, Germany).	(Mohtar, 2012)
B	The washed and pre-cut Hoki skins were pre-treated with 0.75 M NaCl solution (1:6 w/v) for 10 minutes at 100 ± 2°C. The pre-treated skins were then rinsed with distilled water. These steps were repeated twice.	The pre-treated skins were stirred in distilled water (1:6 w/v) for 60 minutes at 50 ± 2°C in water bath (Pura, Julabo, Germany).	(Ito et al., 2011; Mohtar, 2012)
C	The washed and pre-cut Hoki skins were boiled in distilled water (1:6 w/v) for 10 minutes at 100 ± 2°C. The pre-treated skins were then rinsed with distilled water. These steps were repeated twice.	The pre-treated skins were stirred in distilled water (1:6 w/v) for 60 minutes at 50 ± 2°C in water bath (Pura, Julabo, Germany).	(Ito et al., 2011; Mohtar, 2012)
D	The washed and pre-cut Hoki skins were treated with 0.2 M NaOH solution (1:6 w/v) for 60 minutes at 18 ± 2°C (room temperature), which after they were drained. These steps were repeated twice.	The pre-treated skins were stirred in distilled water (1:10 w/v) for 60 minutes at 50 ± 2°C in water bath (Pura, Julabo, Germany).	(Babji et al., 2015)
E	The washed and pre-cut Hoki skins were treated with 0.5 M citric acid solution (1:6 w/v) for 60 minutes at 18 ± 2°C (room temperature), which after they were drained. These steps were repeated twice.	The pre-treated skins were stirred in distilled water (1:10 w/v) for 60 minutes at 50 ± 2°C in water bath (Pura, Julabo, Germany).	(Babji et al., 2015)

After all extraction procedures, the samples were centrifuged (Multifuge 1 S-R, Heraeus, Kendro Laboratory Products, Germany) at 10,000 *g* for 30 minutes at 15 ± 2°C. The clear extracts obtained were then filtered using Whatman filter paper (No. 5). pH values of all extracted gelatine samples were recorded. All gelatine samples were stored in glass bottles at -20 °C until used. Experiments were conducted in triplicate.

3.3 Analytical Methods

Analytical methods were performed to provide quantitative and qualitative analysis of all samples after the deodorisation treatments and the extraction processes. The analytical methods used in this study are discussed in the following subsections.

3.3.1 Determination of Total Volatile Base Nitrogen (TVB-N) and Trimethylamine (TMA) Content by Steam Distillation

The total volatile base nitrogen (TVBN) content in Hoki skin protein hydrolysates and gelatine samples was determined using the modified method of Malle and Tao, (1986). Twenty millilitres of 5% trichloroacetic acid (TCA) solution was added into 10 mL of sample and continuously stirred using magnetic stirrer for 2 - 3 minutes. Twenty-five millilitres of the TCA-hydrolysate mixture was loaded into the distillation tube followed by 5 mL of 10% (w/v) NaOH solution. Steam distillation was carried out using Kjeltec 8100 Distillation Unit (Foss Analytical AB, Höganäs, Sweden). The distillate was received in a conical flask containing 10 ml of 4% (w/v) boric acid solution (with added 0.1% (w/v) bromocresol green and 0.1% (w/v) methyl red solution made up with alcohol). Distillation was continued until a final volume of 50 ml was obtained in the conical flask. Titration was then performed against 0.1 M sulphuric acid solution to a pale-pink end point. The quantity of TVB-N was determined using Equation 2.7, where *n* is the volume of the sulphuric acid used in the titration.

$$TVBN = \frac{n \times 16.8\, mg\ of\ nitrogen}{100g\ of\ sample} \qquad (2.7)$$

To determine the trimethylamine (TMA) content in Hoki skin protein hydrolysates and gelatine samples, 20 ml of formaldehyde was added to 24 mL of TCA-hydrolysate mixture to block the primary and secondary amines, leaving only tertiary amines to react. The same procedure of TVB-N was then followed and the quantity of TMA was determine using Equation 2.8, where *n* is the volume of sulphuric acid used in the titration.

$$TMA = \frac{n \times 16.8\, mg\ of\ nitrogen}{100g\ of\ sample} \qquad (2.8)$$

3.3.2 Sensory Analysis

Sensory analysis was carried out using the flash profiling method of Delaru (2015) to determine the odour attributes of Hoki skin protein hydrolysate samples after deodorisation treatments. Ten untrained panellists consisting of students and professionals, age ranging between 18 to 33 years old, without nasal congestion were recruited. The sensory analysis was performed in individual sensory booths with white fluorescent lighting at room temperature (18^{o}C). The sensory analysis consisted of three separate sessions as shown in Figure 15 conducted over a week. To assist the vocabulary development, panellists were allowed to discuss in group prior to the first session. The group discussion took about 20 minutes. A list of common attributes generated was included in the forms for reference (Appendix 1; Ethics Approval Number: 4000017709).

In each session, nine Hoki skin protein hydrolysate samples treated with different deodorisation treatments were presented individually in 20 ml brown glass bottles to respective panellists. Glass bottles were used to avoid odour transfer that may influence the final results. Each session lasted about 30 minutes.

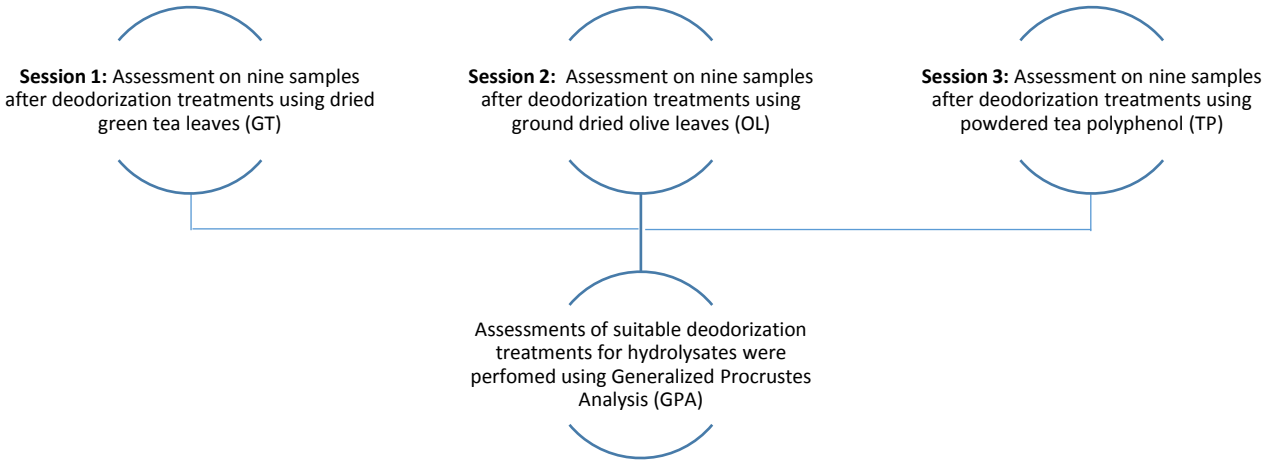


Figure 15: *The flowchart of three separate sessions for sensory assessment of Hoki skin protein hydrolysate samples after deodorization treatments using flash profiling method.*

Panellists were not informed regarding the experimental approaches and the samples were blind-coded with random letters and numbers to avoid bias. Panellists were asked to identify all odour attributes by which the samples could be discriminated and then asked to rank the samples on each of these attributes on an ordinal line scale provided, from the weakest intensity on the left-hand side to the strongest intensity on the right-hand side (Figure 16).

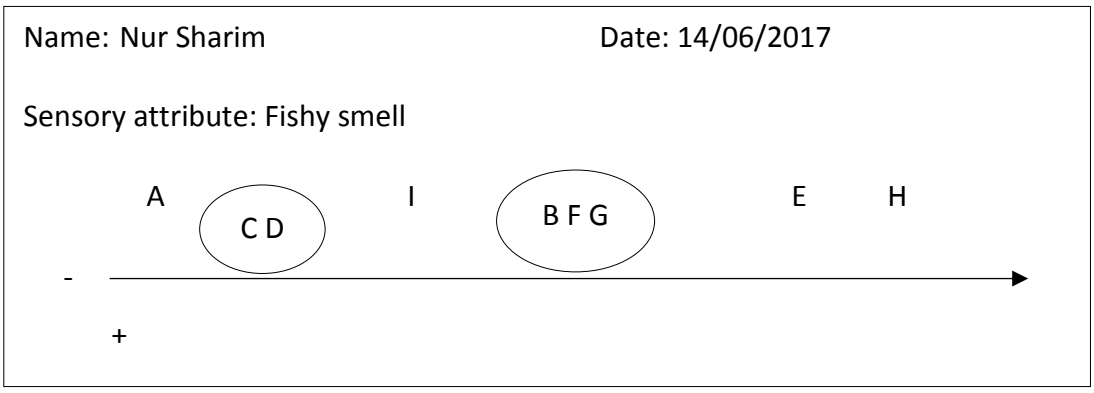


Figure 16*: Example of the transcription of the ranking of the hydrolysate samples for one odour attribute. The panellists were asked to circle ties to avoid confusion. Note that the letter codes were used to identify the samples. The relative distance on the scale is not relevant and only the ranking scores would be used for data analysis.*

For each session, all nine samples were arranged according to ranking scores assigned by panellist (1 being the weakest odour intensity and 9 being the strongest odour intensity) and analysed using Generalized Procrustes Analysis (GPA). Tied samples were grouped into the same ranking scores.

The same procedure of rapid profiling method was carried out on three collagens and five gelatine samples after extraction processes to determine the odour attributes. The sensory analysis was conducted in one session involving the same panellists. The session lasted about 30 minutes.

3.3.3 Folin-Ciocalteu Method for Total Phenolic Content

Based on the results obtained from the sensory analysis, three Hoki skin protein hydrolysate samples treated with powdered tea polyphenol (HSPH-TP8: concentration = 0.04 g/ml; temperature = 50 ^{o}C; and time = 20 min and HSPH-GT3: concentration = 0.03 g/ml; temperature = 30 ^{o}C; time = 20 min) were identified to be treated with the suitable deodorisation treatments. Two ml of each HSPH samples (HSPH-TP8, HSPH-TP9 and HSPH-GT3) were mixed with 16 ml of methanol. The solution was left at 18±2^{o}C (room temperature) for 2 hours to precipitate the protein. The resulting supernatant was subjected to Folin-Ciocalteu method. The total phenolic content of these Hoki skin protein hydrolysate samples was determined using Folin-Ciocalteu method of Waterhouse (2012) with modification. Gallic acid was used as the standard. A 20 µl hydrolysate sample was pipetted into a cuvette. Distilled water (1.58 ml) and 100 µl Folin-Ciocalteu reagent were added into the cuvette followed by thoroughly mixing with a vortex mixer. Three hundred microliters of 1.8 M sodium carbonate solution was added after 30 seconds or no later than 8 minutes. The solution was left at 18±2^{o}C (room temperature) for 2 hours and the absorbance of each solution was determined using a UV spectrophotometer (Shimadzu, Japan) at a wavelength of 765 nm. The standard curve was prepared using 0.05, 0.25, 0.5, 3.0, 5.0 and 10.0 g/l solutions of gallic acid in absolute ethanol.

The total phenolic content was calculated using Equation 2.9 for the linear standard curve obtained by plotting the absorbance values at 765 nm against the concentration of gallic acid.

$$Absorbance\ of\ standard\ gallic\ acid = slope \times concentration\ of\ standard\ gallic\ acid + intercept \quad (2.9)$$

The results were presented as gram of gallic acid equivalent (GAE) per millilitre of hydrolysate.

$$The\ total\ phenolic\ content\ (g\ GAE/ml\ hydrolysate) = \frac{(Absorbance\ of\ hydrolysate\ sample - Intercept) \times volume\ of\ hydrolysate\ sample}{Slope \times Volume\ of\ pre-prepared\ hydrolysate\ used\ in\ deodorization\ treatment} \quad (3.0)$$

3.3.4 Protein Analysis of Hoki Collagen and Gelatine

Protein analysis on collagen samples after extraction processes were conducted using Kjeldahl method in accordance with AOAC Official Method 2001.11 (AOAC, 2005). A 5 g collagen sample was weighed into the digestion tube followed by two Kjeltabs (containing 3.2 g K_2SO_4 and 0.00035g Se, Foss Analytical, United Kingdom) and 25 ml concentrated sulphuric acid. The digestion was carried out using Buchi K424 Digestion Unit (Buchi labortechnik, Switzerland) with selected mode (Processing mode 3). The collagen sample was digested at 420°C for 1 hour until clear solutions were obtained. The digest was left to cool to room temperature. Steam distillation was carried out using a Kjeltec 8100 Distillation Unit (Foss Analytical AB, Höganäs, Sweden). Seventy millilitres of 40% NaOH solution was automatically added into the digestion tube. The distillate was received in a conical flask containing 50 ml of 4% (w/v) boric acid solution [with added 0.1% (w/v) bromocresol green and 0.1% (w/v) methyl red solution made up with alcohol]. Distillation was continued for 4 minutes until a final volume of 50 ml was obtained in the conical flask. Titration was then performed against 0.1 M HCl solution to a grey-mauve end point.

The crude protein content of gelatine was calculated using Equations 3.1 and 3.2, using a conversion factor of 5.55 (AOAC, 2005).

$$Nitrogen\ (\%) = \frac{(A \times B) \times 14 \times 100}{1000 \times C} \quad (3.1)$$

$$Crude\ protein\ (\%) = \%\ nitrogen \times F \quad (3.2)$$

Where A = volume (ml) of HCl used; B = exact molarity of HCl; C = weight (g) of gelatine sample used; and F = conversion factor (AOAC, 2005).

3.3.5 Determination of Total Moisture and Solid Contents of Gelatine

Determination of moisture and total solids of gelatine samples were conducted using an air-oven method (Bradley, 2010). Aluminium moisture dishes with well-fitting covers were dried at 105 ± 0.5°C and kept in a desiccator at room temperature prior to use. The aluminium moisture dish fitted with lid was weighed to 4 decimal places. Approximately 50 g of liquid gelatine sample was placed into the dish and reweighed. The dish was placed in the air oven (Contherm, New Zealand) set at 105 ± 0.5°C for 12 hours with lid opened. The dish was covered before it was removed from the oven using tongs and left to cool in the desiccator. The dried gelatine sample and dish was re-weighed. The total moisture content was calculated using the following equation:

$$Total\ moisture\ content\ (\%) = \frac{w_2 - w_3}{w_2 - w_1} \times 100 \quad (3.3)$$

Where w_1 = weight (g) of moisture dish + lid; w_2 = weight (g) of moisture dish + lid + liquid gelatine sample; and w_3 = weight (g) of moisture dish + lid + dried gelatine sample.

The total solids content was determined using Equation 3.4.

$$Total\ solids\ content\ (\%) = 100 - Total\ moisture\ content\ (\%) \quad (3.4)$$

3.3.6 Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) for Determination of Molecular Weight Distribution of Hoki Collagen and Gelatine

Protein patterns of collagen and gelatine samples were determined using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Anema (2009) and Mohtar (2012) with modifications.

Preparation of sample

Collagen and gelatine samples were diluted with SDS sample buffer [SDS sample buffer was prepared using 0.4% bromophenol blue solution (Sigma Aldrich, NZ), glycerol (Sigma Aldrich, NZ), 0.5M Tris-HCl buffer (pH 6.8; Sigma Aldrich, NZ), 10% w/v SDS, distilled water at ratios of 1:4:5:8:20 (v/v) respectively] to a final concentration of 1 mg protein per ml.

Heat treatment of sample to denature protein

Diluted collagen and gelatine samples (1 ml) were transferred to small sealable vials and 20 µl of β-ME (Sigma Aldrich, NZ) added to each. The subsamples of collagen and gelatine were then heated at 90 ± 2^{o}C in a thermostatically controlled water bath for 10 min. After heating, the samples in the vials were cooled to room temperature (18± 2^{o}C).

Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) was performed using a Bio-Rad mini-gel slab electrophoresis unit (Bio-Rad Laboratories, USA). Collagen and gelatine samples (10µl) were loaded into each well of pre-cast gels (Mini-PROTEAN® TGX, Bio-Rad Laboratories, USA). A marker standard [Natural standard (unstained) SDS-PAGE broad range 161-0317, Bio-Rad Laboratories, USA] was also loaded into one of the well of pre-cast gels. The outer chamber of the gel cassette was then filled with SDS electrode buffer (Sigma Aldrich, NZ). The electrophoresis apparatus was connected to a power supply (Bio-Rad power supply unit, Model 1000/500, Bio-Rad Laboratories, USA) and operated at a constant voltage of 210 V and a current of 70mA for 1 hour. The gels were then removed from the casting assembly and stained with 0.1% w/v amido black 10B in 25% v/v isopropanol and 10% v/v acetic acid for 12 hours. After staining, the gels were destained using 10% v/v acetic acid solution until a clear background was achieved. The photographs of protein

patterns were scanned using a photo scanner (Bio-Rad ChemiDoc™ MP Imaging System, Bio-Rad Laboratories, USA), and analysed using Image Lab software (Version 6.0, Bio-Rad Inc., USA).

3.3.7 Amino Acid Testing for Identification of Final Products

Amino acid analysis was conducted to determine the amino acid compositions of collagen and gelatine samples. The collagen and gelatine samples were sent to an external laboratory (Nutrition Laboratory, Massey Institute of Food Science and Technology, Massey University, Palmerston North, New Zealand) for acid stable amino acid profile test (Appendix 2; quotation number: 2017219). All samples were delivered in frozen wet form.

3.4 Statistical Analysis

All measurements were carried out in triplicate. Data were subjected to analysis of variance (ANOVA). The results from sensory evaluation were analysed through Generalized Procrustes Analysis (GPA). All data analysis was performed using statistical software and data analysis XLSTATS (Microsoft Excel®, Microsoft Co., 2016) and IBM SPSS Statistics Software 21.0 (IBM®, United States). Protein patterns were analysed using Image Lab Software (Version 6.0, Bio-Rad Inc., USA).

Chapter 4: Deodorisation of Hoki Skin Protein Hydrolysate

This chapter presents and discusses the results obtained from the experiments for the deodorisation of pre-prepared Hoki skin protein hydrolysates (HSPH).

4.1 Preliminary Studies of Hoki Skin Protein Hydrolysate

Preliminary studies were conducted with six different deodorants identified from the available literature to determine suitable deodorants for the treatment of pre-prepared HSPH. Deodorisation treatments using dried active yeast (YE), powdered activated carbon (AC), powdered tea polyphenol (TP), dried green tea leaves (GT), and dried olive leaves (OL) at different concentrations were conducted at various temperature and time combinations recommended in the literature (Chen et al., 2016; Luo, 2011). The adsorption of volatile amines on hydrogen-form-strong-acid ion exchange resins (Dowex G-26) was also performed as part of the preliminary studies for the deodorisation of pre-prepared HSPH according to the method of Yoshida and Ruthven (1989), as described in Section 3.1.3.1.

All odour scores assigned to each treatment was self-assessed and self-scored as shown in Table 11 and 12 using the method described in Section 3.1.4. Self-assessed odour scores for untreated HSPH (scored 5, indicating a very strong fishy odour) was also included. Table 11 shows self-assessed odour scores for deodorisation treatments using YE, AC, TP, GT and OL.

Table 11*: Self-assessed odour scores assigned for untreated HSPH and HSPH samples after deodorisation treatments using five different deodorants (dried active yeast, powdered activated carbon, powdered tea polyphenol, dried green tea leaves, dried olive leaves).*

Deodorant/hydrolysate	Treatment			Odour score
	Temperature (°C)	Time (min)	Concentration (g/ml hydrolysate)	
Untreated HSPH	--	--	--	5
Dried active yeast (YE)	45	30	0.001	5
			0.003	4
			0.010	5
Powdered activated carbon (AC)	80	40	0.019	5
			0.035	5
			0.051	4
Powdered tea polyphenol (TP)	40	50	0.004	3
			0.020	3
			0.040	2
Dried green tea leaves (GT)	90	10	0.030	3
			0.050	2
			0.100	2
Dried olive leaves (OL)	90	10	0.030	3
			0.050	3
			0.100	3

All experiment was conducted in triplicate.
Odour scores represent means (n=3)

Based on results presented in Table 11, odour profiles for all HSPH samples treated with YE and AC at the specified deodorisation conditions were scored between 4 and 5 indicating strong fishy odour. Particularly for HSPH treated with AC, an off-odour associated with rancid fat was also detected in all of the samples after centrifugation and filtration. As previously discussed in Section 2.6.3.1 the adsorption of organic volatile compounds by AC is influenced by: (i) the size of the pores in the AC and the size of the adsorbed odour compounds, (ii) the hydrophobic interactions between the carbon and hydrophobic parts of the odour compounds, and (iii) the interaction between aromatic rings on the odour compounds and the carbon resulting in the specific AC adsorption mechanism (Newcombe and Drikas, 1997).

Several studies found that charcoal and activated carbon changes structurally and chemically as a result of carbonizing temperatures used during manufacturing processes (Abe et al., 2000; Asada et al., 2002; Hitomi et al., 1992; Iyobe et al., 2004). In a study to examine the relationship between carbonizing temperatures of charcoal made from Maso bamboo (*Phyllostachys pubescens)* and the removal effects of harmful volatiles and odorants, it was found that bamboo charcoal carbonized at 500^{o}C possessed higher adsorption ability to ammonia gas (NH_3) than those carbonized at 700^{o}C and 1000^{o}C (Asada et al., 2002). Based on this study, Asada et al. (2002) concluded that the effective carbonizing temperature varies for each chemical compound, thus charcoal must be specifically selected to use as deodorant. In another study to investigate the comparison of removal efficiencies of amine gases between woody charcoal and activated carbon, it was found that the activated carbon made from woody material (type was not specified in the study) using a chemical activation process with zinc chloride solution at 700^{o}C showed higher capacity for trimethylamine adsorption due to a larger specific surface area and pore volume compared to that of woody charcoal (Iyobe et al., 2004). Hence, the reappearance of off-odour in HSPH after filtration and centrifugation may have been due to off-odour volatile compounds present in the HSPH samples that were not adsorbed during the deodorisation treatment with AC powder made from coconut shell used in this study (refer to Section 3.1.2). This result may also be due to the particular specificity of the AC powder used in this study, resulting in poor adsorption of at least some off-odour volatile compounds present in the HSPH. However, no further investigation was conducted as the results obtained from the preliminary study on AC were not promising, thus no further conclusion can be drawn.

As shown in Table 11, odour profiles for HSPH treated with YE at specified deodorisation conditions were scored between 4 and 5 indicating strong fishy odour. Further investigation was then conducted by adding 2% (w/w) granulated sugar (sucrose) prior to the treatment to observe the yeast growth during the deodorisation treatment and the effects it had on improving the odour profile of the pre-prepared HSPH. Sucrose is often used as a carbohydrate adjunct for yeast fermentation (D'Amore, Russell and Stewart, 1989). To determine the yeast growth, yeast plate counts (YPC) was conducted for all HSPH samples.

The results from YPC were expressed in colony forming unit (cfu/g sample). All YE-treated HSPH samples were incubated at 25±1^{o}C and was evaluated at time intervals of 1, 4 and 24 hours. The treated sample was then labelled as HSPH-YE1 (for 1 hour interval), HSPH-YE4 (for 4 hour interval) and HSPH-YE24 (for 24 hours interval) for easy differentiation. Table 12 shows the YPC and self-assessed odour scores of HSPH-YE1, HSPH-YE4 and HSPH-YE24.

Table 12: *Yeast Plate Count (YPC) of the YE-treated HSPH samples at time interval of 1, 4 and 24 hours in the incubator (temperature = 25±1 ^{o}C). YPC of untreated HSPH sample is included for comparison.*

Sample	Time (hour)	Yeast Plate Count (cfu/g sample)	Odour Score
Untreated sample	--	2.7 x $10^{4\ a}$	5
HSPH-YE1	1	3.2 x $10^{4\ a}$	5
HSPH-YE4	4	4.1 x $10^{4\ a}$	5
HSPH-YE24	24	5.2 x $10^{5\ b}$	4

HSPH-YE1, HSPH-YE4 and HSPH-YE24 are all the same sample. The numbering represents the time interval in which the data collections were made.
All experiment was conducted in triplicate.
Yeast Plate Count (YPC) and odour scores represent means (n=3)
Values in same columns that do not share the same letters are significantly different at p < 0.05.

Based on Table 12, the yeast plate count (YPC) in HSPH-YE1 and HSPH-YE4 were not significantly increased compared to that of untreated HSPH (*p*-value > 0.05). Meanwhile, there was a significant increase in YPC for HSPH-YE24 with a value of 5.2 x 10^5 cfu/g sample (*p*-value < 0.05) suggesting that there was yeast growth. However, the odour scores between 4 and 5 were assigned for all treated HSPH samples, indicating strong fishy odour. This suggests that the deodorisation treatments using YE with added sucrose incubated at 25±1 ^{o}C for 1, 4 and 24 hours were insufficient in removing the off-odour of pre-prepared HSPH. No further investigation was conducted as the results obtained from the preliminary study on YE were not promising.

Meanwhile, all samples treated with TP, GT and OL achieved odour scores of 3 and below (Table 11). As described in Section 3.1.4, self-assessed odour scores of 3 and below were considered to be the acceptable levels of odour profile for treated pre-prepared HSPH. The respective deodorants would be selected to be further investigated to determine the

suitable deodorisation conditions in removing off-odour volatile compound in pre-prepared HSPH samples.

The results obtained from the deodorisation of pre-prepared HSPH using Dowex G-26 (hydrogen form) strong-acid ion exchange resins is presented in Table 13. Chemical reactions involved in adsorption of volatile amines on hydrogen-form-strong-acid ion exchange resins were discussed in Section 3.1.3.1. One of the key indicators in the assessments of the off-odour in Hoki skin protein hydrolysates post-deodorisation is the trimethylamine (TMA) contents (Section 3.0). pH values throughout the deodorisation treatment were monitored as an indicator for the adsorption of TMA onto the resin.

In contact with hydrogen-form-strong-acid ion exchange resins (Dowex-G26), the trimethylamine was immobilized on the resin by the acid-base neutralization reaction, forming a trimethylamine-resin complex ($(CH_3)_3NH-R$) as shown in Equation 2.4:

$$(CH_3)_3N + R \cdot H \leftrightarrow (CH_3)_3NH - R \qquad (2.4)$$

Consequently, the pH of HSPH dropped from 6.58±0.02 (pH value of untreated HSPH) to pH values of about pH 4 (Table 13). This indicated that TMA was adsorbed onto the hydrogen-form-strong-acid ion exchange resin and the immobilization of the strong base TMA compound causes the pH of the solution to drop. The HSPH samples collected after this reaction were then assessed and assigned odour scores (Table 13).

To determine whether the TMA present in HSPH was removed by the adsorption onto the resin, it was eluted with 0.1M sodium hydroxide solution to recover the immobilised TMA. The reaction process is expressed in Equation 2.5:

$$(CH_3)_3NH - R + NaOH \rightarrow R \cdot Na + CH_3N + H_2O \qquad (2.5)$$

As shown in Table 13, the pH values of the solutions collected after the chemical reaction ranged between pH 8 and 9. The alkaline pH values indicated that TMA may have been recovered from the reaction. Odour scores between 4 and 5 were assigned to all HSPH samples treated at with the various Dowex G-26 resin to sample ratios (resin:HSPH samples = 1:1, 1:3 and 1:5), indicating strong residual fishy smell (Table 13). Although the elution

process using sodium hydroxide solution performed for all treatments may indicate that TMA present in HSPH could be removed by resin adsorption, the sensory assessment results suggested that ion exchange resin alone was insufficient for the deodorisation of pre-prepared HSPH. Based on the preliminary results, ion exchange resin was excluded from further analysis as a primary deodorant for HSPH samples. No further investigation was conducted to determine the TMA content in the eluent to confirm that deodorisation with hydrogen-form-strong acid resin (Dowex G-26) was conducted as the results obtained from the preliminary study on resins were not promising. Hence, no further conclusion can be made. The hydrogen-form-strong acid resin was reintroduced as secondary deodorant and will be discussed later in in this chapter.

Table 13: *pH values measured throughout the deodorisation treatment using Dowex G-26 ion exchange resins. Self-assessed odour scores were assigned following the adsorption of the amines. The pH value of untreated HSPH = 6.58±0.02.*

Sample ratio (resin:HSPH)	**pH**		
	After adsorption of amines onto resins[1]	After amines recovery with 0.1M NaOH[2]	Odour Score[3]
1:1	4.13±0.05	8.10±0.06	4
1:3	4.22±0.06	8.11±0.07	4
1:5	4.15±0.05	9.12±0.05	5

[1]pH values of HSPH sample collected after it was passed through the column containing Dowex-G26 resins.
[2]pH values of solution collected after 0.1M NaOH was passed through the column containing Dowex-G26 resins.
[3]Self-assessed odour scores assigned to HSPH samples collected after adsorption of amines onto resins.
Values represent means ± standard deviation (n=3)

Based on the results obtained from the preliminary experiments, GT, TP and OL were selected as deodorants to be further analysed to determine the suitable deodorisation treatments that effectively remove or decrease off-odour in pre-prepared HSPH.

4.2 Experimental Design for Deodorisation Treatment

Based on the results obtained from the preliminary studies, orthogonal design experiments for GT, TP and OL were conducted as shown in Table 14, using the method described in Section 3.1.5.

All samples were analysed to determine the suitable deodorisation treatments for the pre-prepared HSPH.

Table 14*: Orthogonal design experiments generated using IBM SPSS Statistics Software 21.0 for suitable deodorants identified in the preliminary studies.*

Deodorant	Level	Sample	Factors		
			Concentration (g mL^{-1})	Temperature (˚C)	Time (min)
Dried green tea leaves (GT)	1	HSPH-GT1	0.030	30	20
	2	HSPH-GT2	0.030	50	10
	3	HSPH-GT3	0.030	90	30
	4	HSPH-GT4	0.050	30	30
	5	HSPH-GT5	0.050	50	20
	6	HSPH-GT6	0.050	90	10
	7	HSPH-GT7	0.100	30	10
	8	HSPH-GT8	0.100	50	30
	9	HSPH-GT9	0.100	90	20
Powdered tea polyphenol (TP)	1	HSPH-TP1	0.004	40	20
	2	HSPH-TP2	0.004	50	60
	3	HSPH-TP3	0.004	80	50
	4	HSPH-TP4	0.020	40	60
	5	HSPH-TP5	0.020	50	50
	6	HSPH-TP6	0.020	80	20
	7	HSPH-TP7	0.040	40	50
	8	HSPH-TP8	0.040	50	20
	9	HSPH-TP9	0.040	80	60
Dried olive leaves (OL)	1	HSPH-OL1	0.030	30	20
	2	HSPH-OL2	0.030	50	10
	3	HSPH-OL3	0.030	90	30
	4	HSPH-OL4	0.050	30	30
	5	HSPH-OL5	0.050	50	20
	6	HSPH-OL6	0.050	90	10
	7	HSPH-OL7	0.100	30	10
	8	HSPH-OL8	0.100	50	30
	9	HSPH-OL9	0.100	90	20

4.3 Compositional Analyses for Hoki Skin Protein Hydrolysate Post-Deodorisation Treatments

4.3.1 Determination of Total Volatile Base Nitrogen (TVBN) and Trimethylamine (TMA) Content by Steam Distillation

To investigate suitable deodorisation treatments for reducing or removing the off-odour in pre-prepared HSPH, the total volatile base nitrogen (TVB-N) and trimethylamine (TMA) concentrations in HSPH samples post deodorisation treatments were determined using the steam distillation method by Malle and Tao (1987), as described in Section 3.3.1.

The TVB-N and TMA concentrations in HSPH samples after deodorisation treatments using dried green tea leaves (GT), powdered tea polyphenol (TP) and dried olive leaves (OL) are presented in Table 15, 16 and 17, respectively. The TVB-N and TMA concentrations in the untreated HSPH are also included to provide comparison.

The TVB-N concentrations in HSPH-GT2, HSPH-GT5 and HSPH-GT8 (Table 15) were significantly reduced after the deodorisation treatments with the same value of 11.8±0.1 (mg of nitrogen/100 g wet sample) compared to that of the HSPH-untreated (TVB-N concentration = 15.9±0.1 (mg of nitrogen/100 g wet sample)) (p-value < 0.05). There was no significant decrease in the TVB-N concentrations in any of the other samples treated with GT (p-value > 0.05). The TMA concentrations in all of the samples treated with GT decreased, but not significantly (p-value > 0.05) when the concentrations were compared to that of the untreated HSPH (TMA concentration = 7.6±0.1 mg of nitrogen/ 100 g wet sample).

Table 16 shows that the TVB-N concentrations in 8 out of 9 samples (all samples except HSPH-TP3) treated with TP decreased compared to the untreated HSPH, but not significantly (p-value > 0.05). On the other hand, the TMA concentrations in HSPH-TP7 and HSPH-TP9 decreased significantly to the same value of 3.4±0.1 (mg of nitrogen/100 g wet sample) (p-value< 0.05). There was no significant decrease in TMA concentrations in all of the other samples treated with TP (p-value > 0.05).

The TVB-N concentrations in HSPH-OL1, HSPH-OL3, HSPH-OL6 and HSPH-OL9 (Table 17) decreased compared to that of the untreated HSPH, but not significantly (*p*-value > 0.05). The TVB-N concentration in HSPH-OL2 remained constant after the deodorisation treatment. Interestingly, the TVB-N concentrations in HSPH-OL4, HSPH-OL5, HSPH-OL7 and HSPH-OL8 increased after the treatment, but not significantly (p-value > 0.05). As discussed in Section 2.3.3.2, TVB-N is used as a quantitative parameter that represents volatile amines including ammonia, dimethylamine (DMA) and trimethylamine (TMA) (Etienne and Ifremer, 2005). Ammonia is formed by deamination of proteins, peptides or amino acids in the HSPH by bacteria already present in the sample (refer to Section 2.5.2).

Table 15: *Summary of the pH values, total volatile base nitrogen (TVB-N) and trimethylamine (TMA) contents of hydrolysate samples before and after deodorisation treatment using dried green tea leaves.*

Parameter	HSPH-untreated	Deodorisation Treatment								
		HSPH-GT1	HSPH-GT2	HSPH-GT3	HSPH-GT4	HSPH-GT5	HSPH-GT6	HSPH-GT7	HSPH-GT8	HSPH-GT9
Concentration (g/ml)	--	0.030	0.030	0.030	0.050	0.050	0.050	0.100	0.100	0.100
Temperature (˚C)	--	30	50	90	30	50	90	30	50	90
Time for reaction (min)	--	20	10	30	30	20	10	10	30	20
TVB-N[1]	15.9±0.1^{b}	13.4±0.1^{ab}	11.8±0.1^{a}	12.6±0.1^{ab}	15.9±0.1^{b}	11.8±0.1^{a}	12.6±0.1^{ab}	13.4±0.1^{ab}	11.8±0.1^{a}	13.4±0.1^{ab}
TMA[1]	7.6±0.1^{a}	5.9±0.1^{a}	4.2±0.1^{a}	4.2±0.1^{a}	4.2±0.1^{a}	5.0±0.1^{a}	5.0±0.1^{a}	4.2±0.1^{a}	5.0±0.1^{a}	4.2±0.1^{a}
pH	6.±0.02^{f}	8.17±0.02^{g}	6.16±0.01^{d}	6.27±0.03^{e}	8.14±0.03^{g}	5.50±0.02^{a}	6.20±0.02^{d}	5.54±0.02^{a}	5.67±0.04^{b}	6.02±0.01^{c}

[1]The TVB-N and TMA contents are presented as mg of nitrogen/100 g wet sample.
Values represent means ± standard deviation (n=3)
Values in same rows that do not share the same letters are significantly different at p < 0.05.

Table 16*: Summary of the pH values, total volatile base nitrogen (TVB-N) and trimethylamine (TMA) contents of hydrolysate samples before and after deodorisation treatment using powdered tea polyphenol.*

Parameter	HSPH-untreated	Deodorisation Treatment								
		HSPH-TP1	HSPH-TP2	HSPH-TP3	HSPH-TP4	HSPH-TP5	HSPH-TP6	HSPH-TP7	HSPH-TP8	HSPH-TP9
Concentration (g/ml)	--	0.004	0.004	0.004	0.020	0.020	0.020	0.040	0.040	0.040
Temperature (˚C)	--	40	50	80	40	50	80	40	50	80
Time for reaction (min)	--	20	60	50	60	50	20	50	20	60
TVB-N[1]	15.9±0.1^{ab}	14.3±0.1^{ab}	11.8±0.1^{a}	16.8±0.1^{a}	12.6±0.1^{a}	10.9±0.1^{a}	11.8±0.1^{a}	11.8±0.1^{a}	10.1±0.1^{a}	10.1±0.1^{a}
TMA[1]	7.6±0.1^{b}	5.0±0.1^{ab}	4.2±0.1^{ab}	5.9±0.1^{ab}	4.2±0.1^{ab}	4.2±0.1^{ab}	4.2±0.1^{ab}	3.4±0.1^{a}	4.2±0.1^{ab}	3.4±0.1^{a}
pH	6.58±0.02^{d}	7.94±0.08^{f}	6.45±0.02^{c}	6.56±0.01^{d}	6.21±0.01^{b}	6.78±0.02^{e}	6.21±0.02^{b}	6.04±0.02^{a}	5.60±0.05^{a}	6.19±0.02^{b}

[1]The TVB-N and TMA contents are presented as mg of nitrogen/100 g wet sample.
Values represent means ± standard deviation (n=3)
Values in same rows that do not share the same letters are significantly different at p < 0.05.

Table 17: Summary of the pH values, total volatile base nitrogen (TVB-N) and trimethylamine (TMA) contents of hydrolysate samples before and after deodorisation treatment using dried olive leaves.

Parameter	HSPH-untreated	Deodorisation Treatment								
		HSPH-OL1	HSPH-OL2	HSPH-OL3	HSPH-OL4	HSPH-OL5	HSPH-OL6	HSPH-OL7	HSPH-OL8	HSPH-OL9
Concentration (g/ml)	--	0.030	0.030	0.030	0.050	0.050	0.050	0.100	0.100	0.100
Temperature (˚C)	--	30	50	90	30	50	90	30	50	90
Time for reaction (min)	--	20	10	30	30	20	10	10	30	20
TVB-N[1]	15.9±0.1^{ab}	15.1±0.1^{ab}	15.9±0.1^{ab}	15.1±0.1^{ab}	20.2±0.1^{b}	17.6±0.1^{ab}	14.3±0.1^{ab}	16.8±0.1^{ab}	18.5±0.1^{ab}	12.6±0.1^{a}
TMA[1]	7.6±0.1^{a}	6.7±0.1^{a}	6.7±0.1^{a}	5.9±0.1^{a}	5.9±0.1^{a}	6.7±0.1^{a}	6.7±0.1^{a}	6.7±0.1^{a}	7.6±0.1^{a}	5.0±0.1^{a}
pH	6.58±0.02^{f}	6.35±0.04^{de}	6.49±0.04^{ef}	6.37±0.02^{de}	6.42±0.06^{e}	6.24±0.10^{cd}	6.08±0.04^{ab}	8.56±0.06^{g}	6.20±0.03^{bc}	6.05±0.04^{a}

[1]The TVB-N and TMA contents are presented as mg of nitrogen/100 g wet sample.
Values represent means ± standard deviation (n=3)
Values in same rows that do not share the same letters are significantly different at $p < 0.05$.

According to a study conducted to investigate the effects of olive products on amine accumulation in fish muscle samples, it was found that the effect of olive leaves on ammonia production varied depending on specific bacterial strains presented in the samples (Kuley et al, 2017). Based on the study, it was determined that olive leaves effectively reduced the ammonia production by bacteria such as *Photobacterium damselae, Pontea spp., Vibrio vulnificus, Stenotrophomonas maltophilia, and Pasteurella spp*. in the marine samples (Kuley et al., 2017). Meanwhile, there was a significant increase of ammonia production by bacterium *Yersinia enterocolitica* from 73.0 ± 4.46 to 88.4 ± 3.03 mg/L in the presence of olive leaves (Kuley et al., 2017). Hence, it is assumed that the increased in TVB-N concentrations in HSPH-OL4, HSPH-OL5, HSPH-OL7 and HSPH-OL8 post-deodorisation treatment may be due to the accumulation of ammonia production by specific bacteria already present in the HSPH sample, in the presence of olive leaves used during deodorisation. Moreover, based on the results obtained, it was found that deodorisation treatments by OL were overall less effective in reducing the TVB-N concentrations in HSPH samples compared to that by GT and TP. Thus it was decided not to investigate the bacteria present in the HSPH prior to deodorization.

Similar to the results obtained after the deodorisation using GT and TP, there was no significant decrease in the TMA concentrations of all of the samples treated with OL (*p*-value > 0.05).

Figure 17 and 18 represent the TVB-N and TMA concentrations in all HSPH samples to compare of the two amine concentrations between the three sets of the deodorisation treatments (treatment using dried green tea: GT; powdered tea polyphenol: TP; and dried olive leaves: OL).

Figure 17 shows that the TVBN contents in all HSPH samples treated with GT and TP were reduced to below the TVB-N value of the untreated HSPH. Meanwhile, the TVB-N contents in only 4 out of 9 HSPH samples treated with OL were reduced below the initial TVB-N content in the untreated HSPH. Figure 18 shows that TMA values of all samples treated with GT, TP and OL were reduced to below the initial TMA value of the untreated HSPH. It can be seen that the TMA contents in TP-treated samples decreased to a greater degree than for the GT- and OL-treated samples suggesting that TP was a more efficient deodorant in reducing the off-odour in HSPH compared to GT and OL.

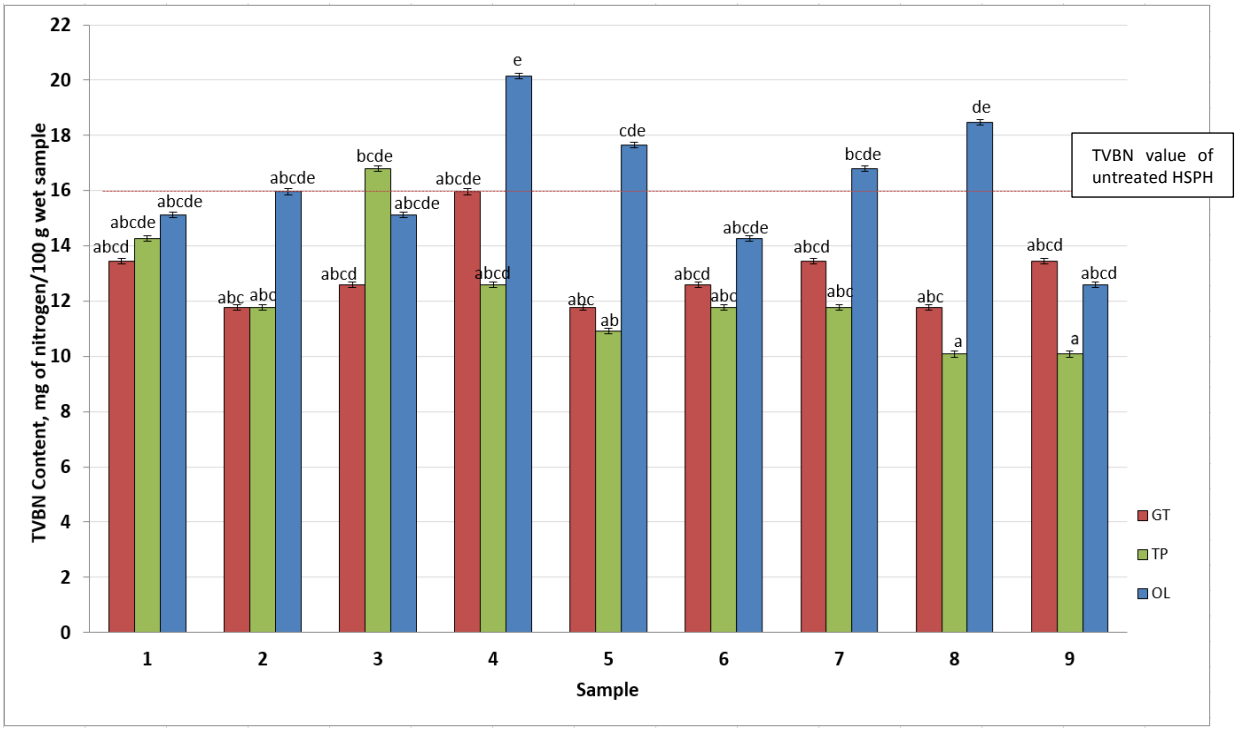


Figure 17: *Total volatile basic nitrogen (TVBN) concentration in Hoki skin protein hydrolysate (HSPH) samples treated using three different deodorants; dried green tea leaves (GT), powdered tea polyphenol (TP) and dried olive leaves (OL) at nine different conditions of time, concentration and temperature combination. Red line indicates the TVBN concentration in untreated HSPH sample. Samples that do not share the same letters are significantly different at p-value < 0.05.*

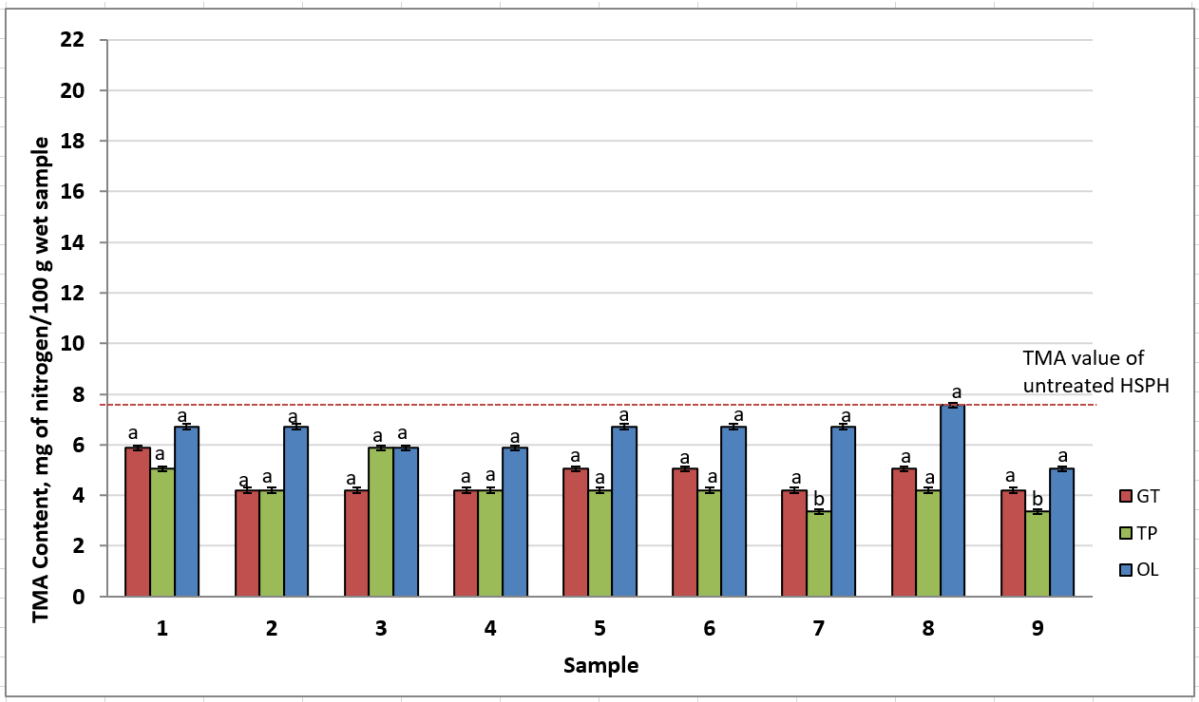


Figure 18*: Trimethylamine (TMA) content in Hoki skin protein hydrolysate (HSPH) samples treated using three different deodorants; dried green tea leaves (GT), powdered tea polyphenol (TP) and dried olive leaves (OL) at nine different conditions of time, concentration and temperature combination. Red line indicates the TMA content in untreated HSPH sample. Samples that do not share the same letters are significantly different at p-value < 0.05.*

As mentioned in Section 2.6.3.4 polyphenols present in tea and olive leaves have neutralizing effects on ammonia, dimethylamine and trimethylamine in a similar way to acids (Dong et al., 2013; Kida et al., 2002). Neutralization of the amines with polyphenols are indicated by the drop in pH of the reaction mixture (Kida et al., 2002). Hence, to determine whether the neutralization reaction takes place during the deodorisation treatment using GT, TP and OL, pH values of HSPH samples were measured after each treatment and presented in Table 15, 16 and 17 respectively. The pH value of the untreated HSPH was also included to provide comparison.

As shown in Table 15, 16 and 17 the pH values of all samples except HSPH-GT1, HSPH-GT4, HSPH-TP1, HSPH-TP3, HSPH-TP5, and HSPH-OL2 decreased significantly after the deodorisation treatments (p-value < 0.05). This indicated that the neutralization reactions occurred between the phenolic compounds of the deodorants used and the amines present in the HSPH samples during the deodorisation treatments. These results were in agreement with the decreased TMA concentrations in all of the samples after

each deodorisation treatment as previously discussed. However, it is important to note that only the TMA contents in HSPH-TP7 and HSPH-TP9 were significantly decreased (*p*-value < 0.05) suggesting that the neutralizing effects of the phenolic compounds in all other samples were insufficient in reducing or removing the TMA contents.

The deodorisation treatments were performed using different deodorants at different concentration, temperature and time combinations (Table 14). To understand the relationship between the variables of the deodorisation treatment and the pH drop in HSPH post-deodorisation, linear regression analysis was conducted using IBM SPSS Statistics Software 21.0 (refer to Appendix 5). As discussed previously, the neutralization reactions occurred between the phenolic compounds present in all deodorants used in this study and amines present in the HSPH samples during deodorisation treatment. Thus, the linear regression analysis excluded the type of deodorant as variable. It was found that 41% of the variance in the pH changes in HSPH corresponded to the concentration of the deodorant used, the temperature and the time for the treatment (Adjusted R-Square = 0.41). It was determined that the pH changes in all HSPH samples after deodorisation treatments depend significantly on the concentration of the deodorant used and the treatment temperature (*p*-value < 0.05). The correlation between the time for the treatment and the pH changes in HSPH after deodorisation was found to be not significant (*p*-value>0.05). The correlations between the three variables of the deodorisation treatments and the pH changes in HSPH can be presented in the following regression equation (Equation 3.5):

$$pH = -16.26C - 0.02\,T + 0.04\,t + 7.488 \qquad (3.5)$$

Where C = concentration of deodorant (g/ml); T = temperature (^{o}C); and t = time (min).

The linear equation (Equation 4.1) can be used to estimate the pH values of HSPH samples after deodorisation treatment and to determine whether or not neutralization reaction takes place between the phenolic compounds present in deodorants used and pre-prepared HSPH.

4.3.2 Sensory Analysis of Hoki Skin Protein Hydrolysates

Sensory analysis of treated HSPH samples was conducted using the flash profiling method by Delarue (2015) to determine the odour attributes of the HSPH samples after

deodorisation treatments. Data obtained were analysed using generalized procrustes analysis (GPA). GPA is used to describe a consensus configuration in describing the sensory profiles of multiple products based on all individual configurations generated by different panellists (Tomic, 2013).

Is this particular study, all 10 panellists used several terms to describe the odour attributes of all HSPH samples post deodorisation treatment. The number of the attributes varied from 2 to 5. The statistics used in assessing the overall perception of the odour profiles of all treated HSPH samples across all panellists included, i) residual values by configuration of panellists, ii) R_c value obtained from permutation test and iii) scaling factors for configuration of panellists. Figure 19 illustrates the residual values of all panellists varying from 168.76 (Panelist4) to 436.42 (Panelist7). Residual values indicated a general perception on the odour profiles of all treated HSPH by each panellist. The similar trend of the residuals with no notable high value shown in Figure 19 indicated that there was consensus between the panellists in describing the odour attributes of the treated HSPH samples.

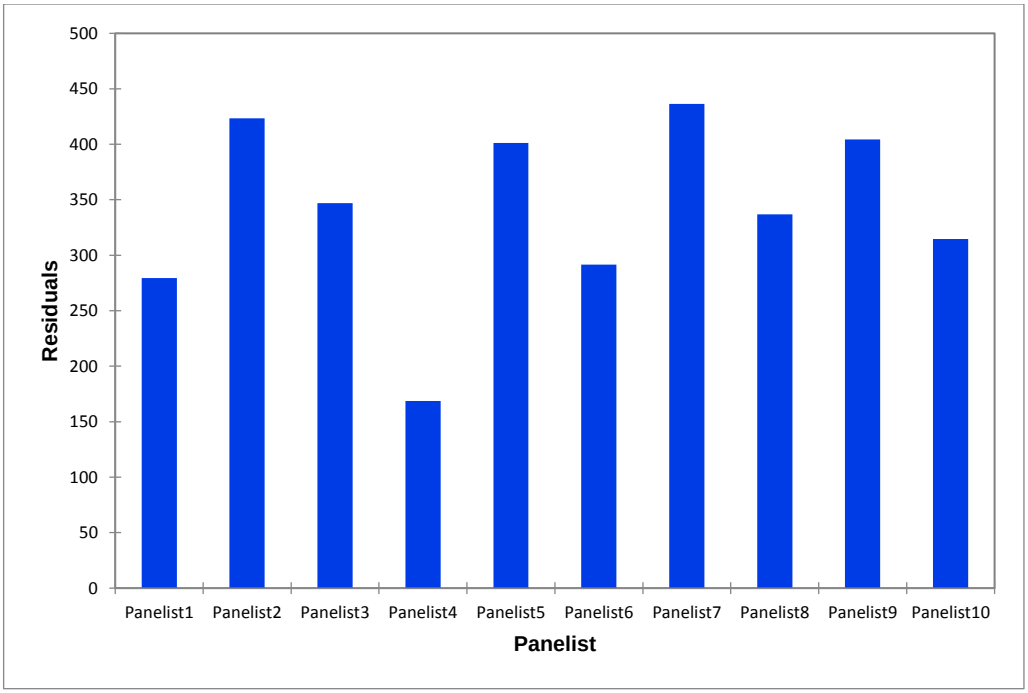


Figure 19: *Distribution of the residuals by configuration of the panellists to indicate a general perception on the odour profiles of all HSPH samples by each panellist.*

Despite thorough measurements implemented in ensuring the quality of results obtained from the sensory analysis, there is still a probability that the consensus may have been a product of chance (Tomic, 2013). To validate that the consensus between the panellists obtained in this study was real and not acquired by chance, a permutation test was conducted. The level of consensus was presented by R_c value (Gower, 1975; Tomic, 2013). The R_c value can also be presented in percentage (King and Arents, 1990). Table 18 presents the results obtained from the permutation test with 300 permutations, automatically generated by the statistical software (XLSTAT, Microsoft Excel®, Microsoft Co., 2016).

Table 18: *Results obtained from the permutation test automatically generated by XLSTAT (Microsoft Excel®, Microsoft Co., 2016).*

Permutations	300
R_c value	0.229
Percentile	100

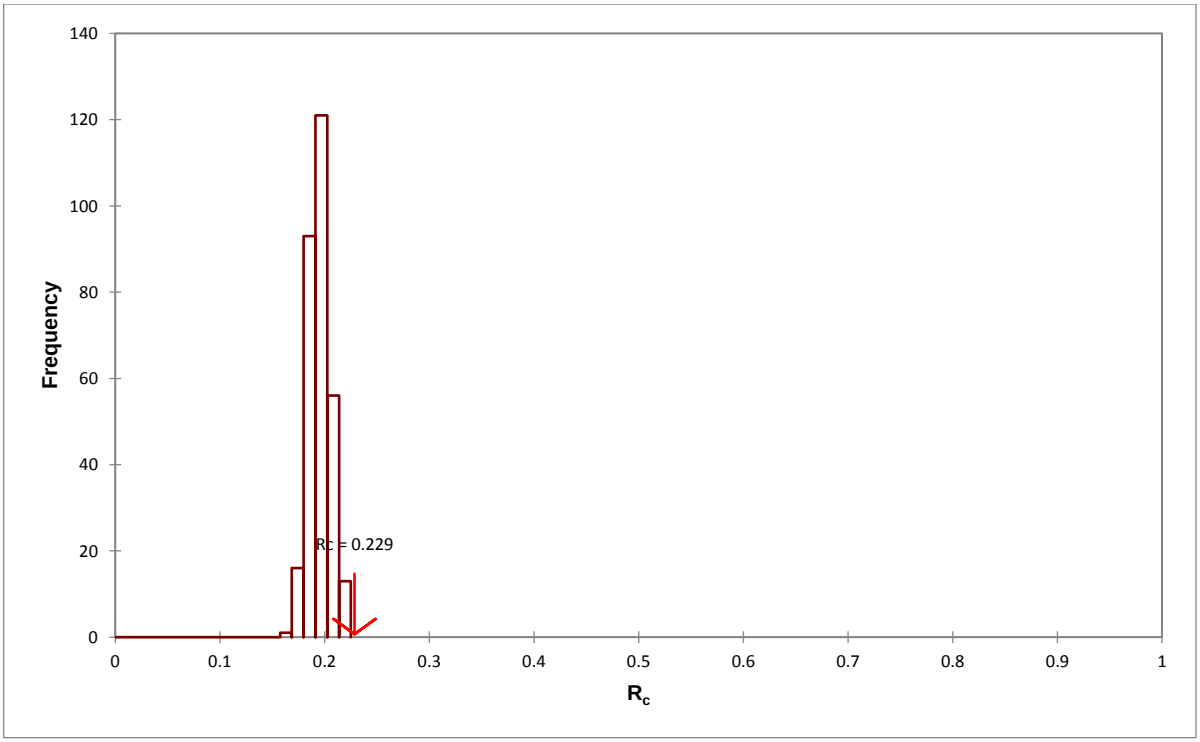


Figure 20*: Histogram showing the R_c distribution from 300 permutations. Note that the R_c value of the data obtained from the sensory analysis of this particular study was marked by the red arrow.*

The R_c value for the consensus configuration was relatively low with a value of 0.229 (Table 18). This suggested that there were some difficulties across the panellists in describing the odour profiles of all HSPH samples and to clearly differentiate them according to the attributes generated. A similar pattern was presented in a study comparing the consensus configurations of different food products in which, the R_c value for wine was relatively low compared to that of apple juice with values of 0.304 and 0.713, respectively (Tomic, 2013). The corresponding R_c values were due to wine being a more complex product in terms of its sensory profile compared to that of an apple juice (Tomic, 2013). The percentile was computed from the permutation test with 300 permutations and it indicated at which level the R_c of the treated HSPH samples data compared to the distribution of 300 R_c from the permutation test. The R_c value with a level of significance below 95th percentile may have been a product of chance, thus compromising the reliability of the consensus between the panellists (Tomic, 2013).

Figure 20 illustrates the R_c distribution from 300 permutations. The R_c value of the treated HSPH samples data obtained from the sensory analysis of this particular study was marked by the red arrow (Figure 20). The R_c value from the treated HSPH samples data was larger than the R_c distribution from the permutation test and is considered significant in which the R_c was at 100th percentile (refer Table 18). The corresponding statistics suggested that the consensus between the panellists in describing the odour profile of all treated HSPH samples was not acquired by chance, despite being relatively low.

On the other hand, the behaviour of the panellists in describing the odour profiles of all HSPH samples can be explained from the scaling factors obtained as shown in Figure 21. It was found that 7 out of 10 panellists had a scaling factor of higher than 1, indicating that the corresponding panellists did not use a wide scale when describing the odour attributes of all treated HSPH samples.

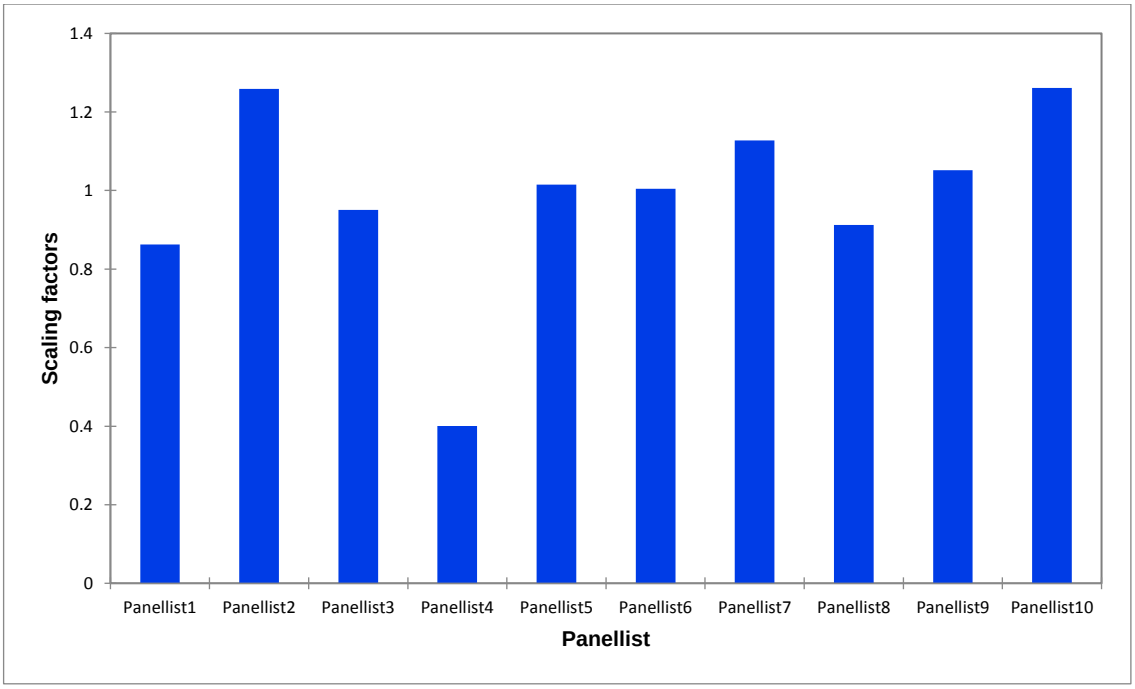


Figure 21: *The scaling factors of all panellists from the sensory analysis for Hoki skin protein hydrolysate samples after deodorisation treatments.*

The behaviour of the panellists was analysed through the first two dimensions (F1 and F2) responsible for 84.82% of the variation as presented in Table 19 (eigenvalue > 1.0).

Table 19: *Explanation of two-dimensional solution.*

	F1	F2
Eigenvalue	2.83	1.52
Variability (%)	55.17	29.64
Cumulative %	55.17	84.82

The procrustean analysis also produced a biplot as shown in Figure 22 to illustrate the correlation between the two dimensions (F1 and F2) based on the consensus of the panellists and the attributes generated. The correlation module higher than or equal to 0.4 was used as a criterion to determine the odour attributes relevant to describe the HSPH samples. Note that the correlation module explained a cut-point to indicate that there was at least 50% overlap between the constructed attributes and the two dimensions generated (Grice, 2007). The first dimension(F1) was primarily related to the odour attributes of tea and earthy (positive) in contrast to fishy and foul (negative). The

second dimension (F2) was related to the odour attribute of grassy. The small angle (angle < 90˚) for the vectors of tea and earthy indicated similar positive response patterns between the two odour attributes meanwhile the small angle for the vectors of fishy and foul indicated similar negative response patterns between the two specified attributes.

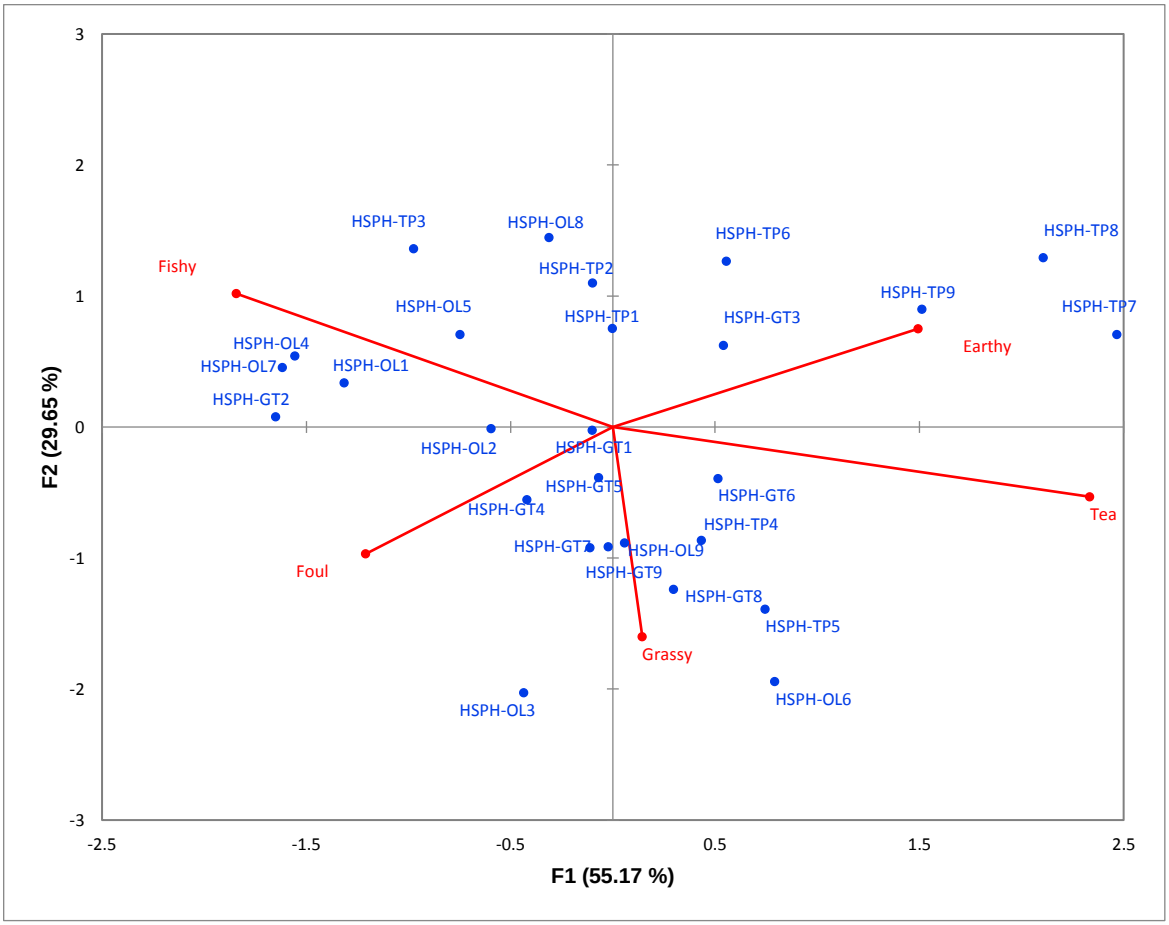


Figure 22*: The biplot of the two-dimensional solutions. The percentages indicate the amount of total variance explained by each dimension.*

Based on an overall observation of the biplot in Figure 22, it showed that the majority of the HSPH samples were not very clearly separated according to the attributes generated. This may be due to the scaling factors of higher than 1 obtained for all panellists, as previously discussed based on Figure 21. Despite that, it can be seen that samples HSPH-TP7, HSPH-TP8 and HSPH-TP9 were notably characterised by the earthy attribute. On the other side of the biplot in the first dimension were samples HSPH-OL1, HSPH-OL4, HSPH-OL7 and HSPH-GT2 characterised by the fishy odour attribute, thus indicating that HSPH-

TP7, HSPH-TP8 and HSPH-TP9 had less fishy odour compared to these latter samples. HSPH-OL3 and HSPH-OL6 were characterized by the grassy attributes compared to samples HSPH-OL8, HSPH-TP2, and HSPH-TP6 with an opposite response pattern. The samples HSPH-GT1, HSPH-GT5 and HSPH-OL2 were positioned close the origin indicating that these samples are equally balanced for all of the attributes and were thus difficult to describe using this technique and no further conclusion was drawn.

4.3.3 The Correlation Between the Sensory Analysis and Trimethylamine Content

A correlation analysis was performed to determine the correlation between the sensory analysis data and the trimethylamine concentrations in reducing the fishy odour in HSPH samples after deodorisation treatments. Figure 23 illustrates a slight positive relationship between the two variables (R-squared = 0.48) and the p-value obtained was 0.01 indicating a statistically significant correlation between them (p-value < 0.05).

As previously discussed in Section 4.3.2, samples HSPH-TP8 and HSPH-TP9 are characterized by the earthy attribute and are negatively characterized to the fishy attribute indicating that these samples had less fishy odour. This result showed an agreement with the TMA concentrations determined using the distillation method as discussed in Section 4.3.1 in which the samples HSPH-TP8 and HSPH-TP9 have low TMA concentrations with the values of 4.2±0.1 and 3.4±0.1 mg of nitrogen/100g wet sample, respectively (Table 16). On the other hand, the samples HSPH-OL1, HSPH-OL4, HSPH-OL5 and HSPH-OL7 were characterized by the fishy attribute (Section 4.3.2; Table 17). This is correlated to the high TMA concentrations of the four corresponding samples with the values of 15.1±0.1, 20.2±0.1, 17.6±0.1 and 16.8±0.1 mg of nitrogen/100g wet sample, respectively (Table 16).

The positive correlation between the trimethylamine concentrations and sensory analysis of the HSPH samples post deodorisation suggests that the trimethylamine concentration is a potential marker to determine the off-odour profile of the HSPH.

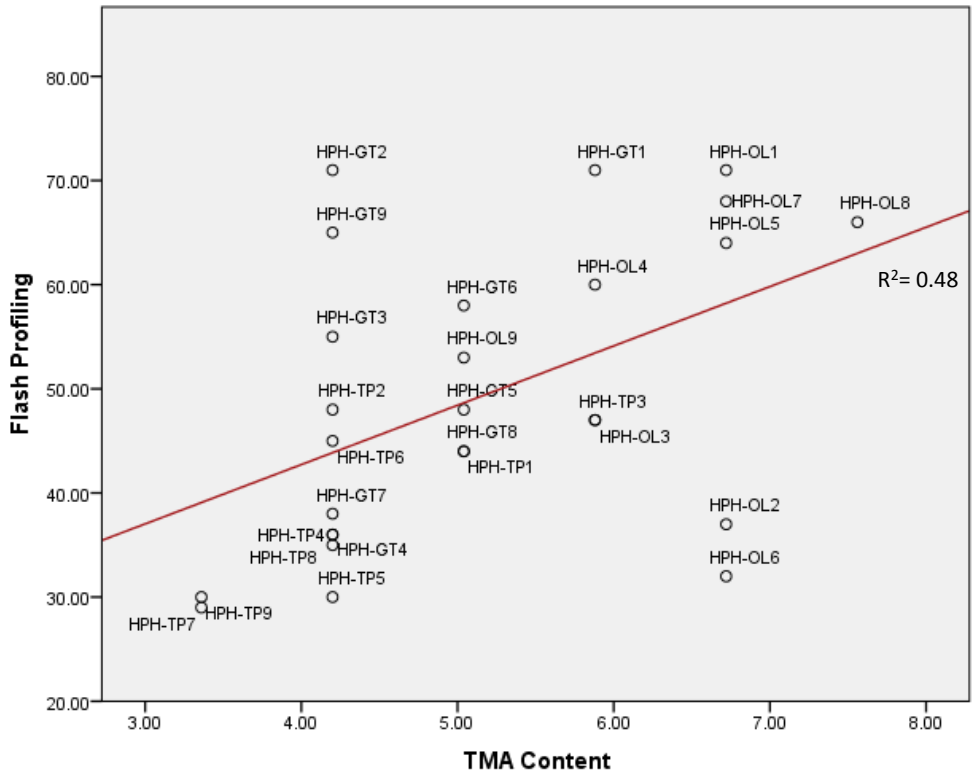


Figure 23*: The scatterplot for the correlation between the sensory analysis (flash profiling method) and trimethylamine content of HSPH samples after deodorisation treatments.*

4.3.4 Determination of Phenolic Content using Folin-Ciocalteu Method

As previously discussed in Section 4.3.1., polyphenolic compounds appear to have neutralizing effects on trimethylamine and subsequently reduce the off-odour in treated HSPH samples. It was determined that the successful deodorisation treatments were HSPH-TP7, HSPH-TP8 and HSPH-TP9. As dried green tea leaves are considerably cheaper than powdered tea polyphenols, a deodorisation treatment was carried out using dried green tea leaves with the same phenolic content of HSPH-TP8.

Based on the results from the sensory analysis, HSPH-GT3 was found to have a similar sensory response pattern to HSPH-TP8 and HSPH-TP9 but to a smaller magnitude as it was positioned closer to the origin in the biplot (Section 4.3.2; Figure 22). The total phenolic contents in HSPH-TP8, HSPH-TP9 and HSPH-GT3 were determined using Folin-Ciocalteu reagent using the method of Waterhouse (2001) (refer Section 3.3.3). The absorbance values obtained at 760 nm of different concentrations of gallic acid solutions

(100, 200, 300, 400, 500, 1000, 3000 and 5000 mg/ml) were used to construct a calibration curve (refer Appendix 7).

The total phenolic contents are expressed as mg gallic acid equivalent (GAE) per millilitre of hydrolysate (mg GAE/ml) as presented in Table 20.

Table 20: *Total phenolic contents of HSPH samples treated with powdered tea polyphenol (TP) and dried green tea leaves (GT) at specified treatment conditions (HSPH-TP8: concentration = 0.04 g/ml of hydrolysate; temperature = 80˚C; time = 20 min, HSPH-TP9: concentration = 0.04 g/ml of hydrolysate; temperature = 80˚C; time = 60 min, HSPH-GT3: concentration = 0.03 g/ml of hydrolysate; temperature = 90˚C; time = 30 min).*

Sample	Total phenolic content (mg GAE/ml of hydrolysate)[1]
HSPH-TP8	0.49±0.01
HSPH-TP9	0.33±0.01
HSPH-GT3	0.10±0.01

[1] *Data of total phenolic contents are expressed as milligrams of gallic acid equivalents (GAE) per millilitre of hydrolysate sample.*
Values represent means ± standard deviation (n=3)

As shown in Table 20, the highest amount of phenolic was detected in HSPH-TP8 followed by HSPH-TP9 and HSPH-GT3 with values of 0.49, 0.33 and 0.10 mg GAE/ml of hydrolysate respectively.

A deodorisation treatment was then conducted on the untreated HSPH sample with a modified condition. The concentration for HSPH-GT3 was increased to 0.08 g/ml of hydrolysate to match the total phenolic content in HSPH-TP8 (total phenolic content = 0.49 mg GAE/ml of hydrolysate). The time and temperature remained the same (temperature = 90˚C; time = 30 min) (Table 20).

The TMA concentration of the modified sample HSPH-GT3 was significantly decreased to a value of 1.1±0.4 mg of nitrogen/100g wet sample compared to that of the untreated pre-prepared HSPH (TMA concentration = 7.6±0.1) (p-value < 0.05). This suggested that dried green tea leaves may be used as an alternative deodorant to the powdered tea polyphenol in deodorizing pre-prepared HSPH for a more economical solution.

4.4 Secondary Deodorisation of Treated Hoki Skin Protein Hydrolysate (HSPH) Sample using Strong Acid Hydrogen Form Resin

Multiple steps processes for deodorisation treatments were found to be employed in a few studies to reduce or completely eliminate off-odours in protein peptides (Cho et al., 2015; Ito et al., 2011). In a study on the collagen peptides from tilapia scale, two-step deodorisation treatments were used in which the protein peptides were first biologically deodorized by microbial fermentation using various microbial species followed by secondary deodorisation treatment using activated carbon (Cho et al., 2015). As discussed in Section 4.1, it was found that the ion exchange resin alone was insufficient for the deodorisation of pre-prepared HSPH. Thus, further investigation was conducted to determine if secondary deodorisation by ion exchange resin can further reduce or completely eliminate off-odours in treated HSPH. An experiment was conducted on HSPH-TP9 sample in which successive secondary deodorisation treatment by hydrogen-form-strong-acid resin (G-Dowex 26) was performed on the sample. Secondary treatment using resin was performed according to the method of Yoshida and Ruthven (1989), as described in Section 3.1.3.1. A ratio of hydrolysate to resin, 1:1 was used for the secondary treatment. The treated sample was then labelled as HSPH-TP9- II for easy differentiation. Trimethylamine (TMA) concentration of all the samples were determined using the method described in Section 3.3.1.

The TMA concentrations in all HSPH samples were presented in Table 21. The TMA concentration in the untreated HSPH was also included to provide comparison.

Table 21: *The trimethylamine (TMA) concentrations of hydrolysate samples after primary deodorisation treatment using powdered tea polyphenol and secondary deodorisation treatment using hydrogen-form-strong-acid resin (G-Dowex 26). The TMA concentration of HSPH before deodorisation treatment was also included. Self-assessed odour score was included as an indication for off-odour.*

Parameter	HSPH-untreated	Primary Deodorisation Treatment	Secondary Deodorisation Treatment[1]
		HSPH-TP9	HSPH-TP9-II
Concentration (g/ml)	--	0.004	--
Temperature (˚C)	--	80	--
Time for reaction (min)	--	50	--
TMA[2]	7.6±0.1^{c}	3.4±0.1^{b}	0.8±0.1^{a}
Odour score	5	2	1

[1]Secondary deodorisation treatment was conducted using a ratio of hydrolysate to resin, 1:1.
[2]The TMA contents are presented as mg of nitrogen/100 g wet sample.
Values represent means ± standard deviation (n=3)
Values in same rows that do not share the same letters are significantly different at $p < 0.05$.

As shown in Table 21, the TMA concentration in HSPH-TP9 was reduced from 7.6±0.1 mg of nitrogen/100g wet sample (HSPH-untreated) to 3.4±0.1 mg of nitrogen/100g wet sample after the primary deodorisation treatment (*p*-value<0.05; refer to Section 4.3.1.). The TMA concentration was significantly reduced after the secondary deodorisation treatment with a value of 0.8±0.1 mg of nitrogen/100g wet sample in HSPH-TP9-II (*p*-value < 0.05; Table 21). Self-assessed odour scores were assigned to each sample (refer Section 3.1.4; Table 7) after the primary and secondary deodorisation treatment to indicate the fishy odour in HSPH samples. As shown in Table 21, an odour score of 1 was assigned to HSPH-TP9 II indicating a hint of fishy odour, almost undetectable. This suggested that secondary deodorisation may be employed to further reduce the TMA concentration in HSPH sample thus consequently reducing off-odours in the sample.

However, no further experiment was conducted to determine the effect of secondary deodorisation by resin on all primarily deodorized HSPH due to insufficient volume of samples. Hoki skin protein hydrolysate (HSPH) was pre-prepared by the supplier as discussed in Section 3.1.1. It was decided that the two-steps deodorisation treatments should be conducted using HSPH samples from the same batch to provide consistent results.

4.5 Conclusions and Recommendations

Based on the results obtained from the qualitative and quantitative analyses, it can be concluded that the suitable deodorisation treatments that reduce the fishy odour in the pre-prepared HSPH samples are 1) deodorant: powdered tea polyphenol; concentration: 0.04 g/ml hydrolysate; temperature: 50˚C; time: 20 min, and 2) deodorant: powdered tea polyphenol; concentration: 0.04 g/ml hydrolysate; temperature: 80˚C; time: 60 min.

Dried green tea leaves can be an alternative deodorant to the powdered tea polyphenol for a more economical solution by manipulating the total phenolic content of the HSPH prior to deodorisation.

Trimethylamine (TMA) concentration can be used to represent the quantitative analysis on the fishy odour in Hoki skin protein hydrolysate samples (R-squared = 0.48, p-value < 0.05).

Secondary deodorisation using resin may further reduce the trimethylamine (TMA) concentration in Hoki skin protein hydrolysate samples. Further investigation may be conducted based on the promising results obtained from the experiment discussed to determine the effective deodorisation steps for Hoki skin protein hydrolysate.

Chapter 5: Extraction of Hoki Collagen and Gelatine

5.0 Introduction

The previous chapter investigated on the methods to deodorize pre-prepared Hoki skin protein hydrolysate (HSPH). The aim of this chapter is to investigate methods to pre-treat Hoki skins prior to extraction to recover collagen and gelatine. Two sets of separate experiments were conducted to determine suitable pre-treatments and extraction procedures (i) collagen and (ii) gelatine from Hoki skins as described in Section 3.2. In addition, this chapter also investigates the pre-treatment and extraction procedures that produce collagen and gelatine with minimum off-odour and high value protein content.

5.1 Hoki Collagen Extraction

Three extraction procedures were conducted using modified methods of Babji et al. (2015) and Mohtar (2012) to extract collagen from Hoki skins as described in Section 3.2.2. Collagen extraction procedures often involve elaborate steps consisting of i) pre-treatment of raw material using acid or alkali solutions, ii) extraction process in acidic or alkaline conditions at low temperature (e.g. at 4^{o}C) and iii) precipitation of collagen protein using tris(hydroxymethyl)aminomethane at a neutral pH followed by centrifugation (refer Section 2.5.1). However, simpler procedures were employed for this particular study using modified methods from the respective published literatures by focusing on the pre-treatments of Hoki skins. Rather than using the extraction process with chemicals, successive physical processes by manually sieving the pre-treated skins was employed to extract the clear collagen samples after pre-treatment procedures. In this thesis, the extracted collagen samples are referred as Col-A (extraction using 0.5M citric acid solution), Col-B (extraction using 0.2M NaOH solution) and Col-C (extraction using with 0.75M NaCl solution).

5.1.1 Compositional Analyses of Hoki Collagen

To determine suitable pre-treatment processes that reduce or remove off-odours in extracted collagen and to identify the extraction processes that produce collagen with

high protein contents and high yields, compositional analyses were performed on all extracted collagen samples using methods described in Section 3.3. Analyses conducted were total protein analysis, moisture content analysis, steam distillation for total volatile base nitrogen (TVB-N) and trimethylamine (TMA) concentrations, gelatine yield and pH determination. All results obtained from the corresponding analyses are summarised in Table 22.

Table 22: *Summary of results obtained from compositional analyses of collagen extracted using three different processes.*

Parameter	Collagen Sample		
	Col-A	**Col-B**	**Col-C**
Pre-treatment procedure for raw Hoki skins prior to extraction	Treated with 0.5M citric acid solution (1:6 w/v) for 60 min at 18±2^{o}C.	Treated with 0.2M NaOH solution (1:6 w/v) for 60 min at 18±2^{o}C.	Treated with 0.75M NaCl solution (1:6 w/v) for 10 min at 18±2^{o}C.
Protein ***(g of protein/100g dry sample)***	37.4±0.8^{b}	52.3±1.0^{c}	8.3±1.2^{a}
Moisture content ***(w/w %)***	94.4±0.1^{a}	94.4±0.4^{a}	96.2±0.1^{b}
TVBN ***(mg of nitrogen/100g wet sample)***	7.0±0.4^{a}	5.0±1.7^{a}	5.0±1.7^{a}
TMA ***(mg of nitrogen/100g wet sample)***	1.7±0.1^{a}	1.1±0.5^{a}	1.1±0.5^{a}
Collagen yield ***(w/w % of dry sample)***	76.8±2.9^{c}	25.1±4.1^{b}	1.8±0.2^{a}
pH	2.3±0.0^{c}	11.9±0.2^{c}	7.1±0.1^{b}

Col-A, Col-B, and Col-C refer to the collagen samples from various pre-treatment and extraction processes.
Values represent means ± standard deviation (n=3)
Values in same columns with the same letters are not significantly different at p-value > 0.05

Data obtained from each analysis were subjected to analysis of variance (ANOVA). ANOVA compares between all the mean values to determine whether they are statistically different than each other and is presented as a p-value. The p-value of lower than 0.05 indicates that the means compared are statistically significant.

Protein analysis was conducted using the method described in Section 3.3.4, to determine the total protein contents in all collagen samples. As shown in Table 22, Col-B had the highest total protein content of 52.3±1.0 g of protein/100g dry sample followed by that of Col-A and Col-C with total protein contents of 37.4±0.8 and 8.3±1.2 g of

protein/ 100g dry sample, respectively (p-value < 0.05). The lower total protein content in the collagen sample treated with citric acid (Col-A) compared to that in the sample treated with NaOH (Col-B) may be due to the loss of collagen protein during the extraction procedure due to higher solubility of collagen in acidic conditions The effect of pH on collagen solubility could be explained by the isoelectric point, p*I* value (Woo et al., 2008). According to Woo et al. (2008), the p*I* value of collagen ranged between pH 6 to 9. When the pH of the collagen is higher or lower that the p*I* value, the repulsive forces between the charged residues of the protein molecules of collagen increases, thus increasing the solubility of the collagen (Woo et al., 2008). Inversely, as the pH reaches a value closer to p*I,* the interactions between the hydrophobic sites increase, thereby resulting in the almost zero total charge of collagen protein (Woo et al., 2008). The respective interaction induces protein aggregation and precipitation, causing the collagen solubility to decrease (Woo et al., 2008). As described in Section 3.2.3, all pre-treated Hoki skins were rinsed with distilled water prior to extraction process by manually sieving the pre-treated skins after pre-treatment procedures. Rinsing the pre-treated skins may have caused the collagen to solubilise and was lost during the process, consequently, decreasing the total protein content in Col-A (pH = 2.3). Collagen sample, Col-B, had a pH value above the p*I* of a collagen, suggesting that some collagen could be lost during the rinsing process. However, the higher total protein content in Col-B compared to Col-A may be due to the pH of Col-B being closer to the p*I* value of the collagen (p*I* value = between 6 - 9) compared to that of Col-A. Despite that the pH of Col-C (pH = 7.1) is within the range of p*I* value of a collagen, the lowest total protein content in Col-C (Table 22), may be explained by the lowest yield of collagen extracted using the respective procedure employed in this current study. The respective result shown by sample Col-C will be discussed later in this subsection. It is important to note that the total protein contents represent both collagenous and non-collagenous proteins in the extracted samples. Hence, further investigation was conducted to identify the extracted protein from the respective processes used in this study.

Based on methods summarised in Table 22, Col-A had the highest yield of collagen with a value of 76.8±2.9 % followed by Col-B and Col-C with collagen values of 25.0±4.1 and 1.8±0.2% respectively (p-value < 0.05). The significantly low collagen yield percentage of

Col-C compared to that of Col-A (p-value < 0.05) may be due to the different effects of NaCl and citric acid solutions on Hoki skin during extraction processes. Visual observations made during this study found that there was a significant swelling of Hoki skins treated with 0.5 M citric acid solution; meanwhile only slight swelling was observed in the skins treated with 0.75 M NaCl (visual images not presented). As described in Section 2.7.1, acid or alkali pre-treatments are essential as they allow the protein to unfold due to the disruption of non-covalent bonds of the insoluble native collagen, exposing the protein to subsequent extraction processes. The corresponding observations suggested that the apparent swollen skin caused by the treatment with citric acid, resulted in a looser protein structure compared to that treated with NaCl, resulting in a better collagen extraction and thus, producing a higher yield. A similar result was found in a study on the extraction of collagen from bigeye snapper (*Priacanthus tayenus*) in which the pre-treatment process with 0.5 M acetic acid for 48 hours had maximised the collagen yield (Nalinanon et al., 2007). In addition to the lowest yield in Col-C, the not swollen Hoki skins pre-treated with NaCl may also result in the lowest total protein content in Col-C as previously discussed (Table 22). On the other hand, mincing and homogenising the skins may also influence the collagen yield (Sadowska et al., 2003). To avoid the denaturation of collagen that can reduce the quality of the final product (refer to Section 2.7.1) and to reduce lipid oxidation which can contribute to off-odour in the extracted collagen (refer to Section 2.5.1), the skins should be ground frozen (Sadowska et al., 2003). In this particular study however, the Hoki skins were cleaned and cut into 3 x 3 cm pieces before being pre-treated (refer Section 3.2.2).

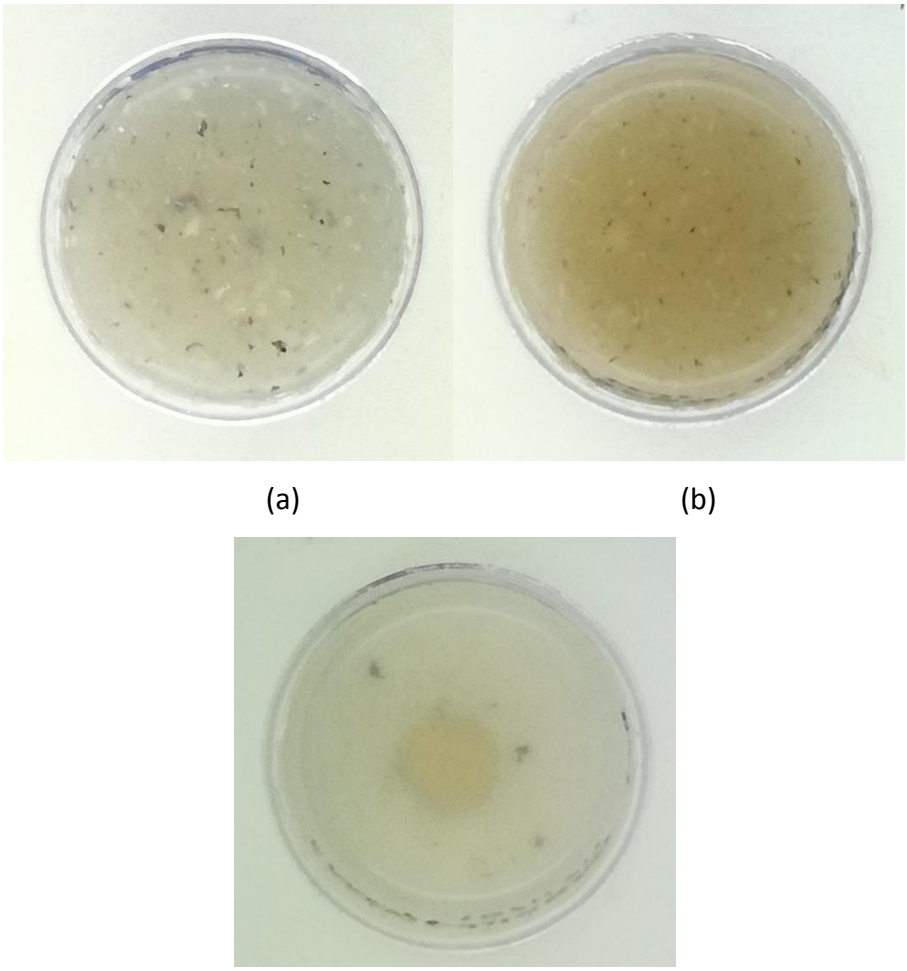
The key indicators in the assessments of the off-odour in collagen samples after extraction processes are the total volatile base nitrogen (TVB-N) and trimethylamine (TMA) concentrations (Section 3.0). Col-A had a higher TVBN concentration of 7.0±0.4 mg of nitrogen/100 g wet sample compared to that of both Col-B and Col-C which both had the same value of 5.0±1.4 mg of nitrogen/100 g wet sample (Table 22), but none of the TVB-N concentrations obtained were significantly different (p-value > 0.05). A similar pattern was also seen for TMA concentrations of all collagen samples (Table 22). There was a higher TMA concentration in Col-A (TMA = 1.7±0.1 mg of nitrogen/100g wet sample) compared to that of both Col-B and Col-C (same TMA for both corresponding

samples = 1.1±0.5 mg of nitrogen/100g wet sample), but it was not significantly different (p-value > 0.05). TMA is often associated with pungent and strong fishy ammonia-like odour, thus eliminating or reducing the concentration of the volatile compound is crucial to ensure the consumers' acceptability towards marine based products (refer Section 2.3.3.2). In this chapter, the low TMA concentrations in all collagen samples were less than the deodorized samples in Chapter 4.3.1. The lowest TMA concentration in Hoki hydrolysate sample post-deodorisation was 3.3±0.1 mg of nitrogen/100g of wet sample, indicating that all extraction procedures resulted in the final products with reduced off-odours.

It can be seen in Table 22 that there was no significant difference in the moisture content in all collagen samples (p-value > 0.05). The low pH value of Col-A (2.3±0.0) and the high pH value of Col-B (11.90±0.2) were expected as this corresponded to the acidic and alkaline conditions employed in the collagen extraction of both samples, respectively. Meanwhile, Col-C had a neutral pH value of 7.1±0.1.

One of the most important features of collagen with regards to consumers' acceptability and its applications in food products, is colour (Sadowska, Kolodziejska and Niecikowska, 2003). Figure 24 shows all collagen samples after the different extraction processes to provide visual presentations of the extracted products. It can be seen that Col-A and Col-B had clear-colour with notable grey pigments [Figure 24(a) and 24(c)]. On the other hand, the extraction process using NaOH solution produced a collagen sample, Col-B, with a slightly brown coloured solution and also, with a notable grey pigments [Figure 24(b)]. Pure collagen is generally devoid of colour (Sadowska, Kolodziejska and Niecikowska, 2003). It is however, difficult to achieve due to the presence of pigments in fish skins (Sadowska, Kolodziejska and Niecikowska, 2003). Similar occurrences were observed in this particular study for all extracted collagens in which grey pigments were observed in all samples as described earlier based on Figure 8. In a study on the removal of pigments from plaice skins, it was found that homogenization at high speed (speed was not specified) in 0.4M NaCl solution effectively removed pigments in collagen samples (Montero et al., 1995). The neutral saline solution caused the swelling of collagen fibres and the breaking down of hydrogen bonds, loosening the fiber mesh, thus resulting in the solubilisation of pigment proteins from the collagen (Montero et al.,

1995). The bleaching of pigments can also result in the loss of soluble collagen (Kolodziejska, Sikorski and Niecikowska, 1999; Montero et al., 1995; Sadowska, Kolodziejska and Niecikowska, 2003). In a study on the bleaching effect of collagen from squid (*Illex argentinus*) skins, it was found that bleaching the collagen in alkaline H_2O_2 solution (bleaching solution used: 1% H_2O_2 in 0.01 M NaOH) effectively removed pigments with minimal loss of soluble collagen (Kolodziejska, Sikorski and Niecikowska, 1999). These two corresponding procedures were then employed in a study on isolation of collagen from Baltic cod skins, but with dissimilar outcomes as pigments were only partially removed (Sadowska et al., 2003). The respective findings suggest that the procedures for removal of pigments from collagen differ depending on the source of the collagen. No further investigations in this current study were conducted to determine the bleaching procedures that effectively remove pigments in Hoki collagen.



(a) (b)

(c)

Figure 24*: Collagen samples extracted using different processes. (a) Col-A: Collagen extracted using 0.5M citric acid at 18±2˚C. (b) Col-B: Collagen extracted using 0.2M NaOH at 18±2˚C. (c) Col-C: Collagen extracted using 0.75M NaCl at 18 ±2°C.*

5.1.2 Sensory Analysis of Hoki Collagen

Sensory analysis of Hoki collagen samples was conducted using the flash profiling method by Delarue (2015), as described in Section 3.3.2, to determine the odour attributes of all collagen samples after extraction processes. Data obtained were analysed using generalised procrustes analysis (GPA). Based on the initial procrustes analysis performed, Panelist 10 had the highest residual value indicating that they differed the most from the consensus opinion in describing the odour attributes of all collagen samples. The data from Panellist 10 was therefore eliminated from the analysis to avoid inconsistent results (data not included). The results presented in this chapter were the data obtained from Panelist 1 to 9.

From the sensory analysis conducted, all nine panellists used several terms to describe the odour attributes of all extracted HHoki collagen samples. The number of attributes varied from 2 to 3. The statistics used in assessing the overall perception of the odour profiles of all collagen samples across all panellists are described in more detail in Section 4.3.2. In brief, statistics used in assessing the data obtained from the sensory analysis in this study were, i) residual values by configuration of panellists to indicate a general perception on the odour profiles of all extracted collagen by each panellist, ii) R_c value obtained from permutation test to indicate the level of consensus between the panellists and iii) scaling factors to describe the behaviour of the panellists in describing the odour profile of collagen samples. Figure 25 presents the residual values of all panellists varying from 0.452 (Panelist 2) to 1.422 (Panelist 1, 3, 4, 6 and 7). The similar trend of the residuals with no notable high value indicated that there was consensus between the panellists in describing the odour attributes of the collagen samples (Figure 25).

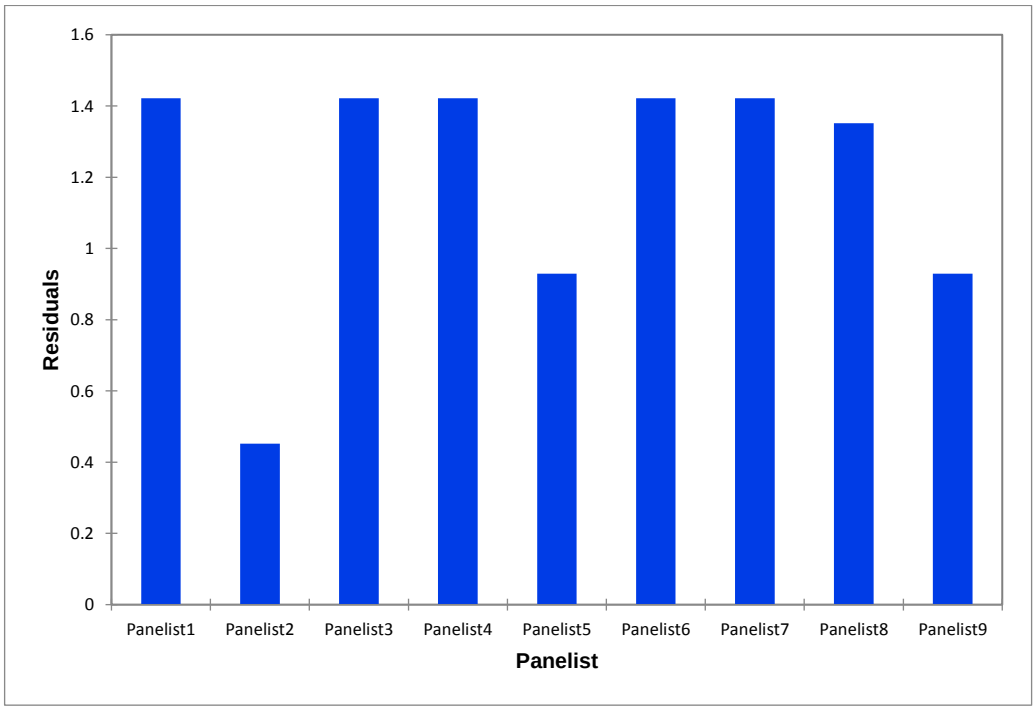


Figure 25: *Distribution of the residuals by configuration of the panellists to indicate a general perception on the odour profiles of all extracted collagen by each panellist.*

A permutation test was then conducted to validate that the consensus between the panellists obtained in this study was real and not acquired by chance. Table 23 presented

the results obtained from the permutation test with 300 permutations which were automatically generated by the statistical software (XLSTAT, Microsoft Excel®, Microsoft Co., 2016).

Table 23: *Results obtained from the permutation test automatically generated by XLSTAT (Microsoft Excel®, Microsoft Co., 2016).*

Permutations	300
R_c value	0.322
Percentile	62.33

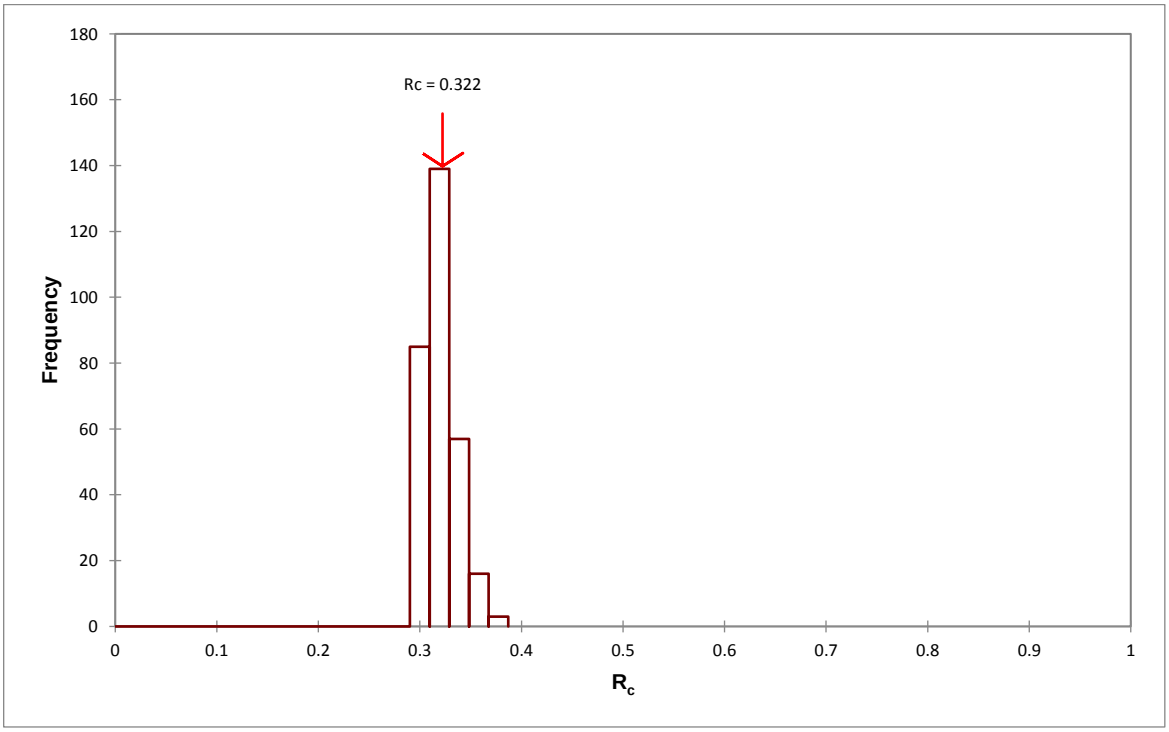


Figure 26: *Histogram showing the R_c distribution from 300 permutations. Note that the R_c value of the data obtained from the sensory analysis of this particular study was marked by the red arrow.*

The R_c value for the consensus configuration was relatively low with a value of 0.322 (Table 23). This suggested that there were some difficulties across the panellists in describing the odour profiles of all collagen samples. Figure 26 illustrates the R_c distribution from 300 permutations. The R_c value of the extracted collagen samples data obtained from the sensory analysis of this particular study was marked by the red arrow (Figure 26). The R_c value from the collagen samples data was not significant in which the

R_c was in the 62nd percentile (P>0.05) (refer Table 23). As described in Section 4.3.2, the R_c value with a level of significance below 95th percentile may have been a product of chance, thus compromising the reliability of the consensus between the panellists (Tomic, 2013). The analysis of the data in this study (R_c at 62nd percentile) suggests that the consensus obtained between the panellists in describing the odour profile of all collagen samples may have occurred by chance.

The behaviour of the panellists in describing the odour profiles of the collagen samples can also be explained by scaling factors obtained from the procrustean analysis as shown in Figure 27. It was found that 7 out of 9 panellists had a scaling factor of higher than 1, indicating that the these panellists did not use a wide scale when describing the odour attributes of all collagen samples. This may have contributed to the compromised consensus opinions as described based on Table 23 and Figure 26.

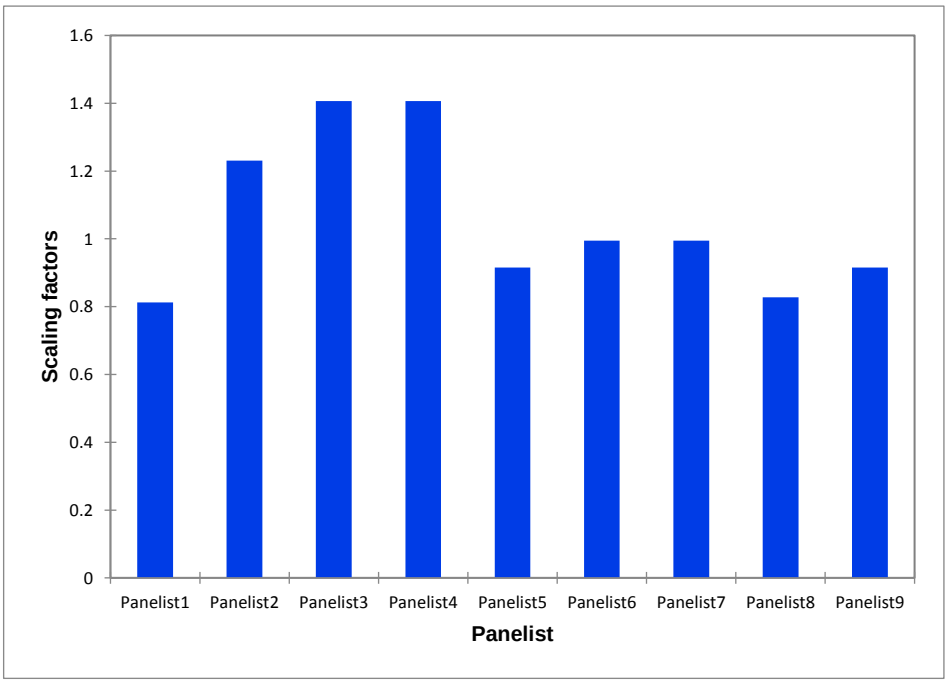


Figure 27: *The scaling factors of all panellist from the sensory analysis for HHoki collagen samples after extraction processes to explain the behaviour of the panellists in describing the odour profiles of the collagen samples.*

The procrustean analysis also produced a biplot as shown in Figure 28 to illustrate the correlation between the two dimensions (F1 and F2) based on the consensus of the panellists and the attributes generated. An overall observation presented by the biplot

showed that all three collagen samples were clearly separated according to the attributes generated (Figure 28). The behaviour of the panellists was analysed through the first two dimensions (F1 and F2) accounted for 100% of the variations. The correlation module higher than or equal to 0.7 was used as a criterion to determine the odour attributes relevant to describe the collagen samples. Note that the correlation module explained a cut-point to indicate that there was at least 50% overlap between the constructed attributes and the two dimensions generated (Grice, 2007).

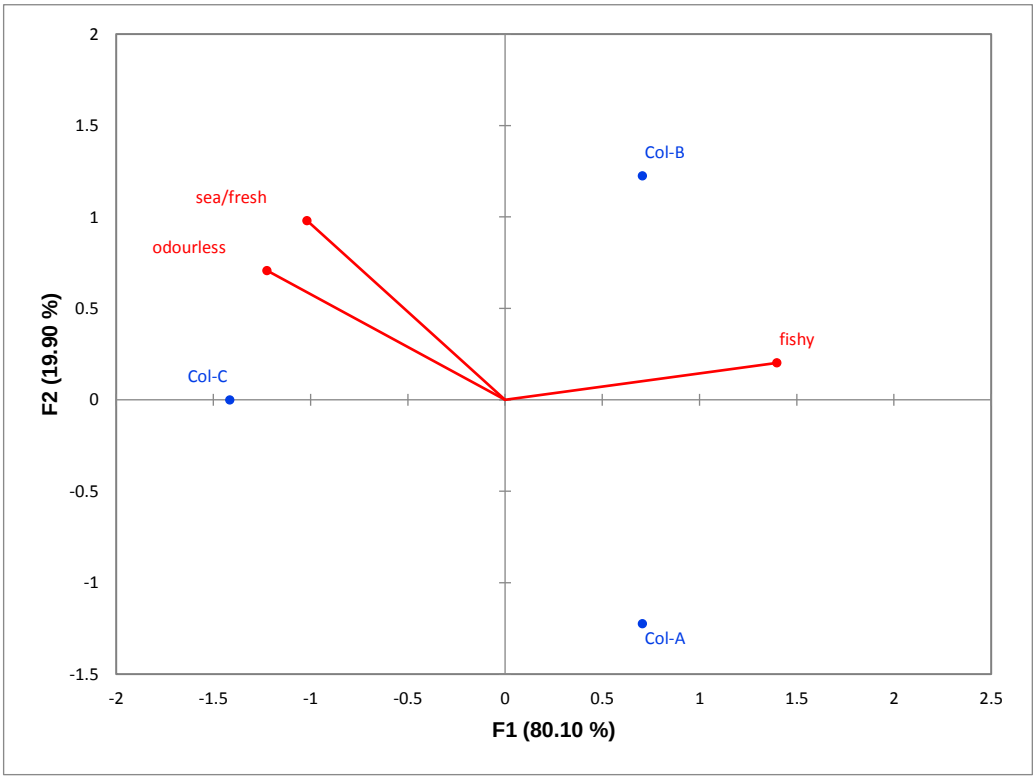


Figure 28*: The biplot of the two-dimensional solutions that accounts for 100% of the variation.*

The first dimension (F1) was primarily explained by the odour attribute fishy (positive for first dimension) in contrast to odourless (negative for first dimension). The second dimension (F2) was related to the sea/fresh odour attribute (positive). It can be seen that that Col-A and Col-B was characterised by the fishy attribute.

Based on the first dimension (F1), Col-C, sample had a less fishy odour and was characterised by the odourless attribute. Meanwhile, all collagen samples were not primarily characterised by the sea/fresh attributes. It can be seen that all collagen

samples were positioned equally further apart from the sea/fresh attribute vector. This may be due to the relatively low percentage of variance shown by the second dimension (F2), which accounted for only 20% of the total variations. No further conclusion was made regarding the perceptions of panellists on the collagen samples based on the sea/fresh attributes.

5.1.3 The Correlation Between the Sensory Analysis and Trimethylamine Content

A correlation analysis was performed to determine the correlation between the sensory data and the trimethylamine concentrations in reducing the fishy odour in collagen samples after pre-treatment and extraction procedures. Figure 29 illustrates a slight positive relationship between the two variables (Pearson r = 0.66) and the p-value obtained was greater than 0.5 indicating the correlation is not statistically significant between the sensory data and the TMA concentrations (p-value > 0.05). This indicated that the data obtained from the flash profiling method for sensory analysis could not be correlated to TMA concentration to help determine the suitable extraction method for Hoki collagen with reduced off-odour. As discussed in Section 2.3.3, factors contributing to off-odours in marine products include lipid oxidation and the generation of off-odour volatile compounds. Particularly for the later factor, some of the volatile compounds associated with off-odours are n-hexanal, n-heptanal, 2,4-heptadienal, 2,4-decadienal, 1-octen-3-ol and 2, 3-octanedione (Chen et al., 2016). Hence, this suggests that off-odour volatile compounds other that TMA may be the potential off-odour marker in extracted Hoki collagen in this particular study. In addition, more panellists or trained panellists may also assist in providing better descriptions on the odour profile of the extracted Hoki

collagen. Further investigation is to be conducted to determine the suitable sensory analysis technique in assessing the odour attributes of Hoki collagen samples.

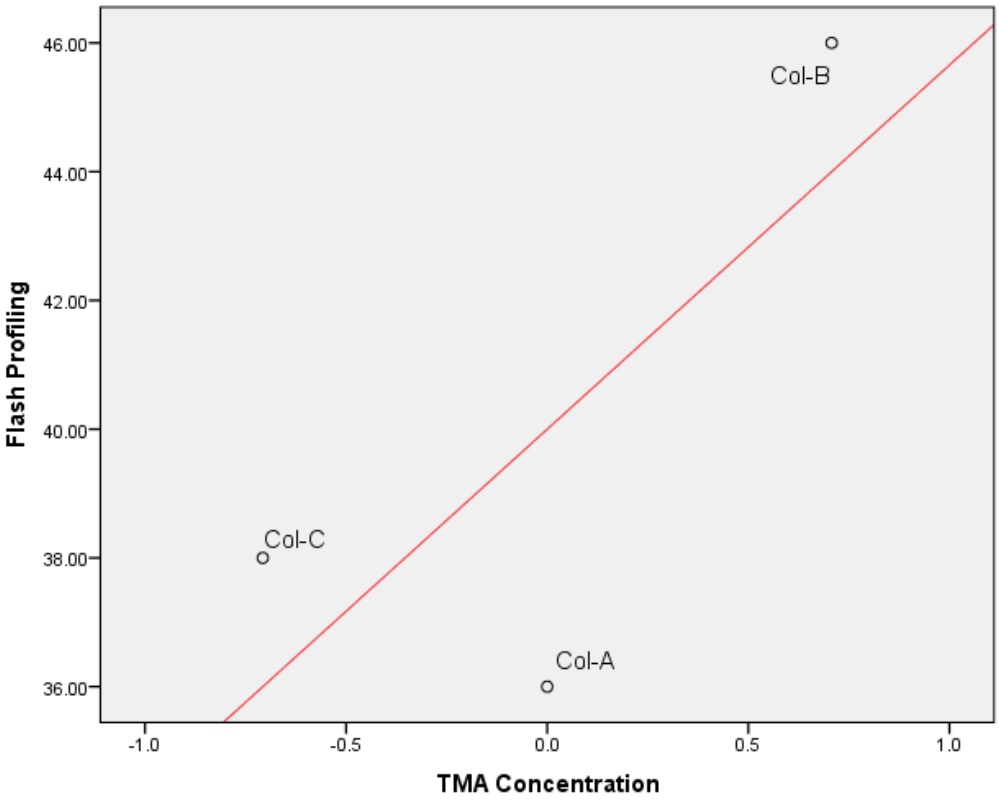


Figure 29*: The scatterplot for the correlation between the sensory analysis (flash profiling method) and trimethylamine content of collagen samples after extraction processes.*

5.2 Hoki Gelatine Extraction

Five pre-treatment and extraction procedures to extract Hoki gelatine were conducted using modified methods of Babji et al. (2015), Ito et al. (2011) and Mokhtar (2012) as described in Section 3.2.3 to extract gelatine from Hoki skins. Pre-treatments using salt, acid and alkali were employed to treat Hoki skins, with and without heat treatment, followed by aqueous extraction at 50^{o}C (Table 24). In this thesis, the extracted gelatine samples are referred as Gel-MA (pre-treatment using 0.5M citric acid solution at 18±2 ^{o}C), Gel-MB (pre-treatment using 0.5M citric acid solution at 100±2 ^{o}C), Gel-MC (pre-treatment using distilled water at 100±2 ^{o}C), Gel-MD (pre-treatment using 0.2M NaOH

solution at 18±2 ^{o}C) and Gel-ME (pre-treatment using 0.5M citric acid solution at 18±2 ^{o}C).

5.2.1 Compositional Analyses for Hoki Gelatine

All gelatine samples were subjected to analyses as described in Section 3.3 in order to determine the suitable pre-treatment and extraction processes that reduced or removed off-odours in extracted gelatine. The results were required to help determine extraction yields and final protein content after pre-treatment and extraction.Results obtained from the corresponding analyses of all gelatine samples recovered after all pre-treatment and extraction procedures are summarised in Table 24.

Table 24: *Summary of analyses of Hoki skin gelatine pre-treated and extracted using different methods.*

Parameter	Gelatine Sample				
	Gel-MA	Gel-MB	Gel-MC	Gel-MD	Gel-ME
Pre-treatment for raw Hoki skins	Treated with 0.75M NaCl solution (1:6 w/v) for 10 min at 18±2°C.	Treated with 0.75M NaCl solution (1:6 w/v) for 10 min at 100±2°C.	Boiled in distilled water (1:6 w/v) for 10 min at 100±2°C.	Treated with 0.2M NaOH solution (1:6 w/v) for 60 min at 18±2°C.	Treated with 0.5M citric acid solution (1:6 w/v) for 60 min at 18±2°C.
Extraction process of pre-treated Hoki skins	Extracted with distilled water (1:6 w/v) for 60 minutes at 50±2°C.	Extracted with distilled water (1:6 w/v) for 60 minutes at 50±2°C.	Extracted with distilled water (1:6 w/v) for 60 minutes at 50±2°C.	Extracted with distilled water (1:10 w/v) for 60 minutes at 50±2°C.	Extracted with distilled water (1:10 w/v) for 60 minutes at 50±2°C.
Protein (***g of protein/100g dry* sample**)	40.5±0.9^{d}	3.7±1.0^{a}	7.4±1.0^{b}	41.3±0.9^{d}	20.3±1.0^{c}
Moisture content (***w/w%)***	99.4±0.1^{a}	99.1±0.1^{a}	99.8±0.0^{a}	99.4±0.1^{a}	98.9±0.2^{a}
TVBN (***mg of nitrogen/100g wet sample***)	7.2±0.1^{b}	4.1±0.1^{a}	4.1±0.1^{a}	9.1±0.0^{d}	8.7±0.1^{c}
TMA (***mg of nitrogen/100g wet sample***)	0.9±0.1^{a}	1.0±0.1^{a}	0.9±0.1^{a}	0.9±0.1^{a}	0.9±0.1^{a}
Gelatine yield recovery (***w/w% of dry sample***)	23.5±1.1^{b}	37.6±2.8^{c}	7.5±0.3^{a}	61.0±1.7^{d}	83.1±5.0^{e}
pH	6.9±0.1^{b}	6.9±0.0^{b}	7.6±0.1^{c}	11.8±0.0^{d}	2.6±0.0^{a}

Gel-MA, Gel-MB, Gel-MC, Gel-MD and Gel-ME refer to the gelatine samples from various pre-treatment and extraction processes.
Values represent means ± standard deviation (n=3)
Values in same columns with the same letters are not significantly different at p-value > 0.05.

As can be seen in Table 24, Gel-MD had the highest total protein content of 41.3±0.9 g of protein/100g dry sample while Gel-MB had the lowest protein content with a value of 3.4±1.0 g of protein/100g dry sample. The lower total protein content in Gel-ME (protein content = 20.3±1.0 g of protein/100g dry sample) compared to that in Gel-MD may be

due to the removal of some of the collagen protein during the pre-treatment process due to higher collagen solubility in acidic condition, thus reducing the amount of protein for extraction of gelatine in the subsequent thermal denaturation step. As described in Section 5.1.1, when the pH of the collagen is higher or lower than the p*I* value, the repulsive forces between the charged residues of the protein molecules of collagen increases, thus increasing the solubility of the collagen (Woo et al., 2008). Rinsing the pre-treated skins prior to gelatine extraction may have caused the collagen to solubilise and was lost during the process, consequently, decreasing the total protein content in Gel-ME (refer Section 3.2.3). Similar results were reported in a study on African catfish, in which pre-treatment using acetic acid solution was found to remove a small amount of collagen protein (See et al., 2015). In the study by See et al. (2015), it was reported for total protein content that a pre-treatment solution with 0.05 N acetic acid solution had the highest total protein content compared to pre-treatment solutions with various concentrations of NaOH solution (concentrations ranging between 0.01 – 0.5 N NaOH) (See et al., 2015). The corresponding results indicated that the acid pre-treatment had removed some of the collagen protein (See et al., 2015). It was deduced by See et al (2015) that the removal of collagen protein may be due to the acids ability to crosslink with less cross-linked material presented in the catfish skin (See et al., 2015). In a study by Mohtar (2012), it was reported that the same pre-treatment and extraction processes as the method used to extract Gel-MA (pre-treatment with 0.75M NaCl at 18±2^{o}C followed by an extraction process for 60 minutes at 50±2˚C) produced a gelatine with a total protein content of 90.25g of protein/100g dry sample which was higher compared to that of Gel-MA (refer Table 24). Despite the same procedure employed for the pre-treatment and extraction of the corresponding two separate Hoki gelatine samples, the discrepancy between the two results suggested that the protein content in extracted Hoki gelatines may also be influenced by other factors such as initial protein content in Hoki skins and the fishing season of Hoki fish.

Based on Table 24, Gel-ME had the highest yield of gelatine with a value of 83.1±5.0 % (of dry sample), then the recovery of gelatine decreased based on treatment in the following order Gel-MD, Gel-MB, Gel-MA and Gel-MC, respectively. The p-value of lower than 0.05 indicated that all values for gelatine recovery in all gelatine samples were

significantly different when compared between each other (Table 24). The pre-treatment using distilled water (Gel-MC) had a different effect on the Hoki skins compared to the Hoki skins pre-treated with salt, alkali or acid. It was observed that, pre-treatment using NaCl, NaOH and citric acid solutions at specified concentrations and temperatures caused the Hoki skins to swell during the processing, whereas pre-treatment with distilled water resulted in no swelling of the Hoki skin (visual images not presented). It is to note that, although NaCl caused the Hoki skins to swell during the pre-treatment, however it was not as apparent as the swelling effect caused by acid or alkali pre-treatments. Similar result was observed in the extraction of Hoki collagen as described in Section 5.1.1. Swelling is essential as it allows the protein to unfold due to the disruption of non-covalent bonds of the insoluble native collagen, exposing the protein to subsequent solubilisation for the extraction of gelatine (See et al., 2015). As described in Section 2.7.2, swelling also permits the gelatine to be extracted with lower heat treatment due to the destabilisation of hydrogen and covalent bonds of the swollen insoluble collagen (Mohtar, 2012; See et al., 2015). Similar observations were reported in the studies on African catfish and pollock skins pre-treated with acetic acid and NaOH solutions (See et al., 2015; Zhou and Regenstein, 2005). There was a significantly higher gelatine yield for Gel-MB compared to that of Gel-MA (Table 24; p-value < 0.05). This showed that, for the same concentration of NaCl solution, a higher extraction temperature resulted in an increased gelatine yield. But when the gelatine yield was compared between Gel-MB and Gel-MC, both extracted at 100±2^{o}C, there was a significantly lower gelatine yield in Gel-MC where the skins did not swell in distilled water. (Table 24; p-value < 0.05).

To study the effect of different pre-treatment and extraction processes on the odour profiles of the gelatine samples, TVB-N and TMA contents were determined to provide quantitative parameters for the assessments of the final products. Table 24 shows that Gel-MD had the highest TVB-N concentration with a value of 9.1±0.0 mg of nitrogen/100 g wet sample, followed by that of Gel-ME, Gel-MA, Gel-MB and Gel-MC in decreasing order based on the respective treatment. The significantly low TVB-N concentrations of Gel-MB and Gel-MC compared to Gel-MA, Gel-MD and Gel-ME (p-value < 0.05) suggested that pre-treatments at higher temperature can help reduce volatile amine compounds compared to pre-treatments at lower temperature. These results are in line with results

reported by Ito et al. (2013) who reported on a method to deodorize collagen peptides by double step heating processes. Ito et al (2013) carried out a preliminary heat process at 50 - 90^{o}C by, followed by heating at 100 ^{o}C, which resulted in the removal of the off-odour in the collagen peptides. On the other hand, Table 24 also shows that there were no significant differences (p-value>0.05) in TMA concentrations of all gelatine samples with values ranging between 0.9±0.1 (Gel-MA, Gel-MC and Gel-MD) and 1.0±0.1 mg of nitrogen/100g wet sample (Gel-MB). The low TMA concentrations indicated that all pre-treatments successfully reduced the off-odours contributed by TMA in all gelatine samples.

There was no significant difference (p value>0.05) in the moisture content in all gelatine samples (Table 24). As for pH value of the gelatine samples after different pre-treatment and extraction processes, Gel-MA, Gel-MB and Gel-MC had neutral pH of 6.85 – 7.59 (Table 24). Gelatine sample, Gel-MD had a high pH value of 11.8±0.2 while Gel-ME had a low pH of 2.6±0.1 corresponding to the alkali and acidic pre-treatment conditions employed for both of the samples, respectively.

5.2.2 Sensory Analysis of Hoki Gelatine

Sensory analysis of Hoki gelatine samples was conducted using the flash profiling method by Delarue (2015) to determine the odour attributes of all gelatine samples after pre-treatment and extraction processes, as described in Section 3.3.2. Data obtained was analysed using generalized procrustes analysis (GPA). Only seven out of 10 panellists had succeeded in completing the sensory analysis. Thus, only the data obtained from the seven panellists was analysed and presented in this thesis. The number of attributes used by panellists to describe the odour attributes of all gelatine samples varied from 2 to 4. The statistics used to assess the overall perception of the odour profiles of all gelatine samples across all panellists was described in Section 5.1.2.

Figure 14 presents the residual values for all seven panellists varying between 4.97 (Panelist 2) to 9.16 (Panelist 3). The similar values for residuals for each panellist with no notable high value indicated that there was consensus between the panellists in describing the odour attributes of the gelatine samples (Figure 30). A permutation test was then conducted to validate that the consensus between the panellists obtained in

this study was real and not acquired by chance. Table 25 presents the results obtained from the permutation test with 300 permutations which were automatically generated by the statistical software (XLSTAT, Microsoft Excel®, Microsoft Co., 2016).

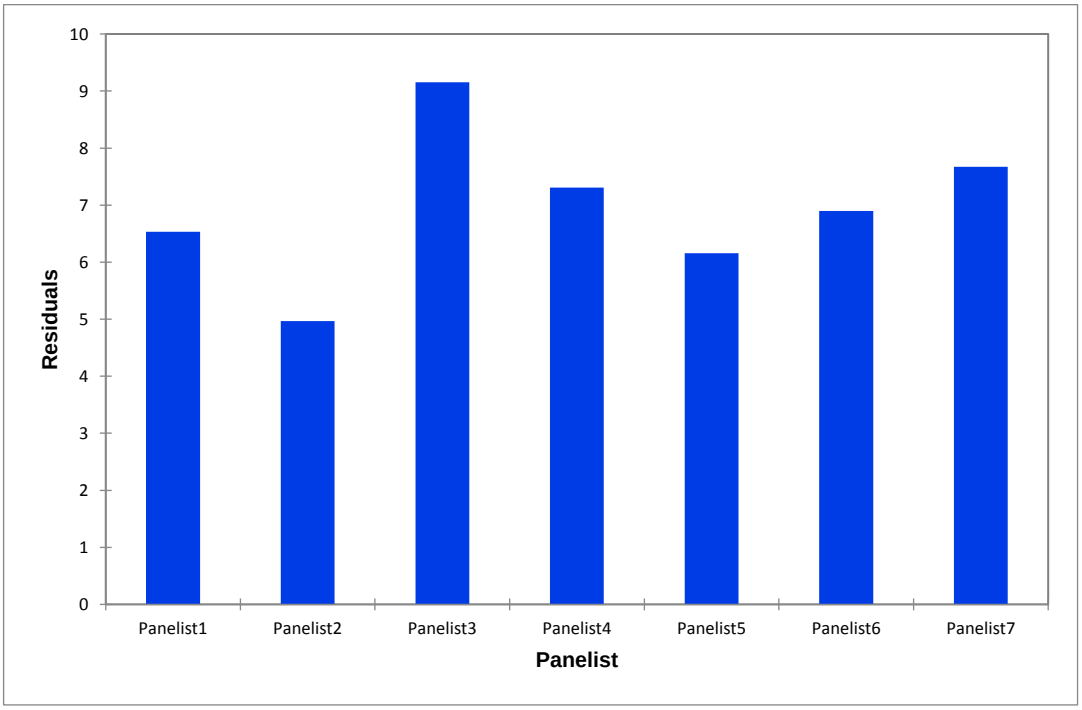


Figure 30*: Distribution of the residuals by configuration of the panellists to indicate a general perception on the odour profiles of all extracted gelatine by each panellist.*

Table 25: *Results obtained from the permutation test automatically generated by XLSTAT (Microsoft Excel®, Microsoft Co., 2016).*

Permutations	300
R_c value	0.231
Percentile	97.33

The R_c value obtained from the permutation test was relatively low with a value of 0.231 (Table 25). This suggested that there were some difficulties across the panellists in describing the odour profiles of all gelatine samples. Figure 31 illustrates the R_c distribution from 300 permutations. The R_c value of the gelatine samples data obtained from the sensory analysis of this particular study was marked by the red arrow (Figure

31). The percentile was computed from the permutation test with 300 permutations and it indicated at which level the R_c of the extracted gelatine samples data compared to the distribution of 300 R_c from the permutation test. The R_c value with a level of significance below 95th percentile may have been a product of chance, thus compromising the reliability of the consensus between the panellists (Tomic, 2013). Based on Table 25, it can be seen that the R_c value from the gelatine samples data was considered significant in which the R_c was at 97th percentile. The corresponding statistics validated that the consensus between the panellists in describing the odour profile of all gelatine samples was real and not acquired by chance, despite being relatively low.

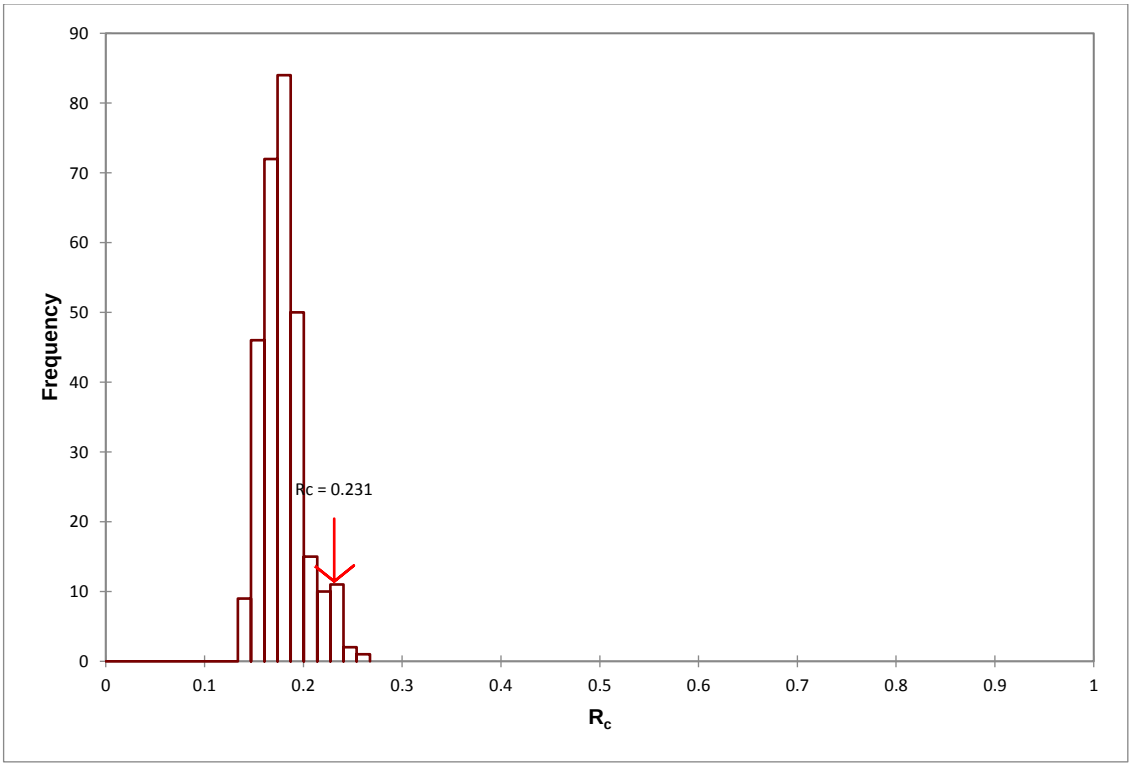


Figure 31*: Histogram showing the R_c distribution from 300 permutations. Note that the R_c value of the data obtained from the sensory analysis of this particular study was marked by the red arrow.*

The behaviour of the panellists in describing the odour profiles of all gelatine samples can also be explained from the scaling factors obtained as shown in Figure 32. It was found that 4 out of 7 panellists had a scaling factor of higher than 1, indicating that the corresponding panellists did not use a wide scale when describing the odour attributes of all gelatine samples.

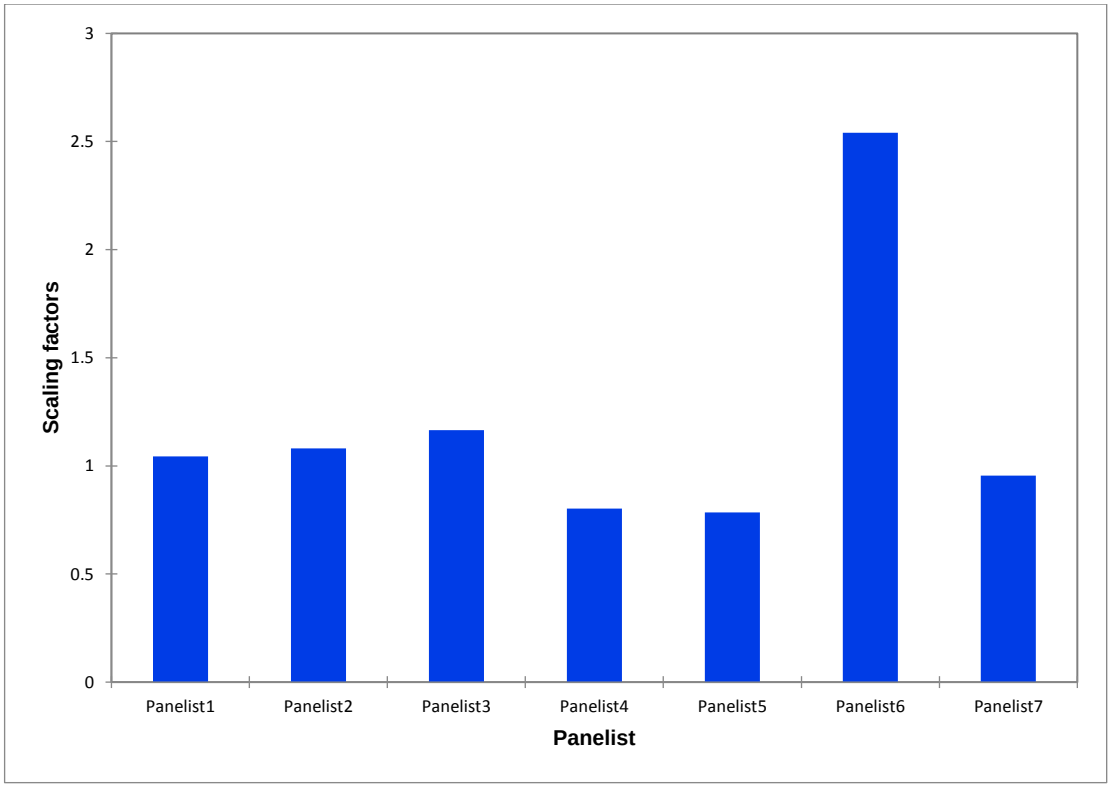


Figure 32*: The scaling factors of all panellist from the sensory analysis for HHoki gelatine samples after pre-treatment and extraction processes to explain the behaviour of the panellists in describing the odour profiles of all gelatine samples .*

The behaviour of the panellists was analysed through the first two dimensions (F1 and F2) responsible for 98.66% of the variation. The biplot produced from the procrustes analysis is shown in Figure 33 to illustrate the correlation between the two dimensions (F1 and F2) based on the consensus of the panellists and the attributes generated. The correlation module higher than or equal to 0.7 was used as a criterion to determine the odour attributes relevant to describe the gelatine samples. Note that the correlation module explained a cut-point to indicate that there was at least 50% overlap between the constructed attributes and the two dimensions generated (Grice, 2007). The first dimension (F1) was primarily related to the odour attributes of fishy and foul (positive for F1) in contrast to odourless (negative for F1). The second dimension (F2) was related to the odour attribute of sea/fresh (positive for F2). The small angle for the vectors (angle < 90˚) of fishy and foul indicated similar positive response patterns between the two odour attributes.

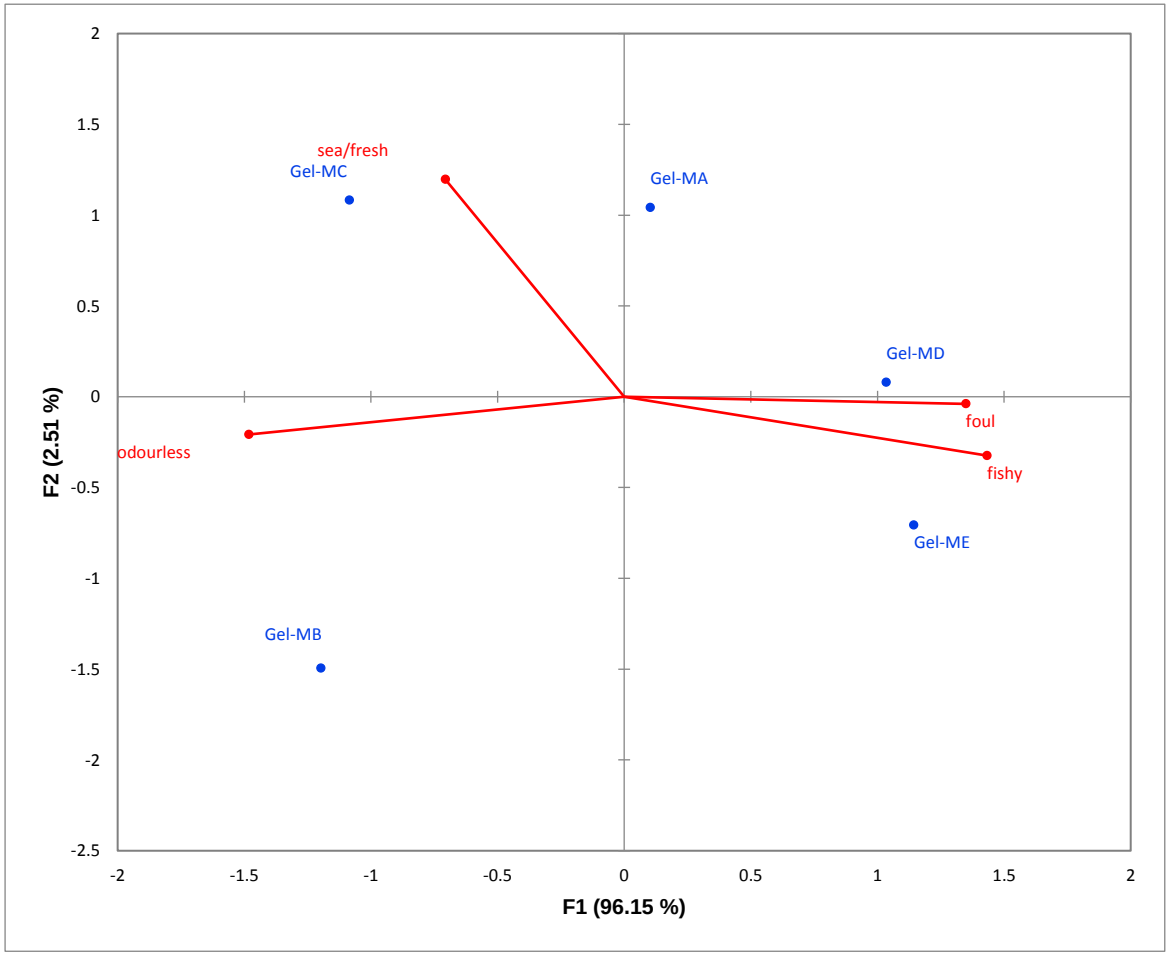


Figure 33*: The biplot of the two-dimensional solutions. The percentages indicate the amount of total variance explained by each dimension.*

Based on the overall observation of the biplot in Figure 33, it showed that all gelatine samples were clearly separated according to the attributes generated. It can be seen that Gel-MD and Gel-ME were characterised by both the fishy and foul attributes. The corresponding samples were opposite on the first dimension indicating that Gel-MB gelatine sample had less fishy odour and was characterized by the odourless attribute. In the second dimension (F2), it can be seen that Gel-MC was primarily characterized by the sea/fresh attribute. Based on the first dimension, Gel-MB and Gel-MC were positioned in the left hand side, indicating that both of the samples had less fishy odour. Meanwhile, Gel-MA was positioned close to the origin in the biplot, indicating that the gelatine sample was equally balanced for all of the attributes.

5.2.3 The Correlation Between the Sensory Analysis and Trimethylamine Content

A correlation analysis was performed to determine the correlation between the sensory analysis data and the trimethylamine concentrations in reducing the fishy odour in gelatine samples after pre-treatment and extraction processes. Figure 34 illustrates a slight positive relationship between the two variables (R-squared = 0.67) and the *p*-value obtained was 0.2 indicating no significant correlation between them (p-value > 0.05). This may be due to the insignificant difference in TMA concentrations of all gelatine samples (refer Section 5.2.1; Table 13), obstructing the use of sensory data for further statistical analysis to help determine the suitable method for the extraction of Hoki gelatine with reduced off-odour. On the other hand, as discussed in Section 5.1.3, the corresponding results obtained from the correlation analysis suggested that off-odour volatile compounds other that TMA may be the potential off-odour markers in extracted Hoki collagen in this particular study.

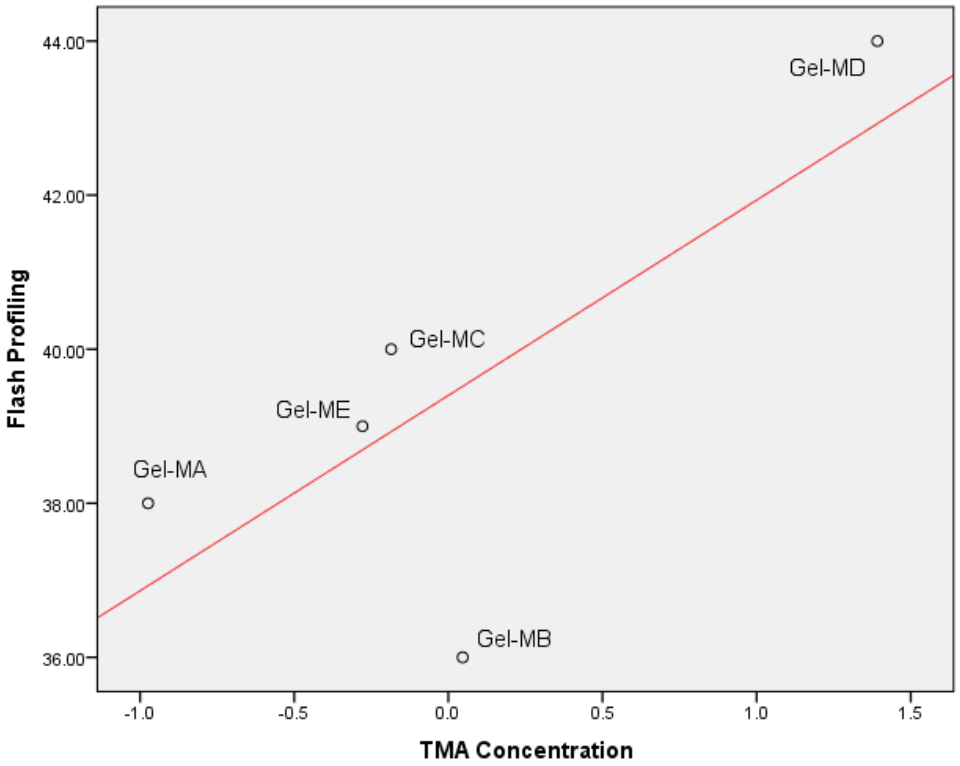


Figure 34: *The scatterplot for the correlation between the sensory analysis (flash profiling method) and trimethylamine content of gelatine samples after pre-treatment and extraction processes.*

5.3 Determination of Protein Molecular Weight Distribution of Hoki Gelatine and Collagen using Sodium-dodecyl-sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

As discussed in Section 2.1.1, collagen proteins are composed of three polypeptide chains known as α chains, that become entangled to form a stable triple helix with varied lengths. Meanwhile, gelatine is a protein derived from type I collagen, making gelatine unique according to the source of its collagen parents (Section 2.1.3). In general, collagen has a molecular weight of 360 kDa (Schmidt et al., 2016). The molecular weight of gelatine however, depends highly on their collagen source and can vary between 40 kDa to 700 kDa (Gorgieva and Kokol, 2011). Hoki skin gelatine in particular has a molecular weight of 100 – 190 kDa (Mohtar, 2013). In order to determine the molecular weight distributions of collagen and gelatine samples extracted using different procedures described in Section 3.2.2 and Section 3.2.3, respectively, sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was employed to determine the molecular weight of the corresponding collagen and gelatine samples using the method described in Section 3.3.6. The deodorized Hoki skin protein hydrolysate sample, HSPH-TP8 was also included in the electrophoresis study as a representative of all hydrolysate samples post-deodorisation treatments (Section 4.2). Figure 35 illustrates the SDS-PAGE profile of Hoki gelatine and collagen samples pre-treated and extracted using specified procedures. Image in Figure 35 is the representative of duplicate gels.

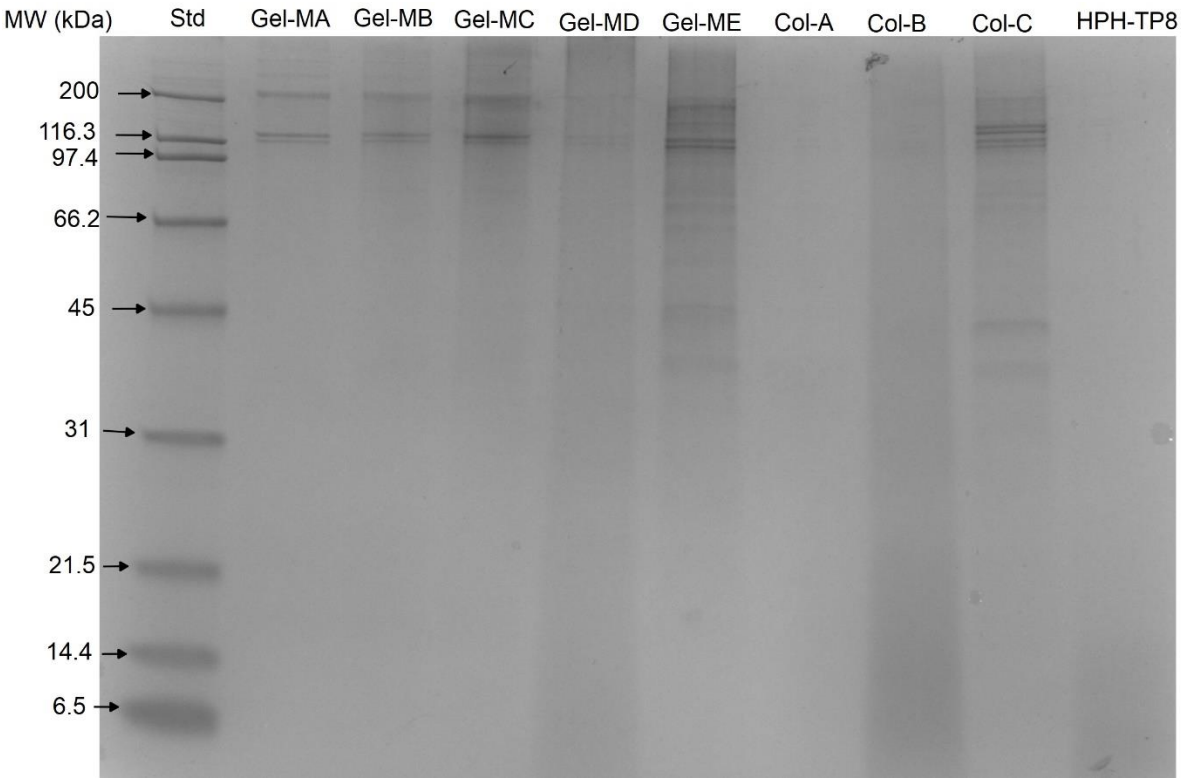


Figure 35*: SDS-polyacrylamide gel electrophoresis of gelatine and collagen samples treated using various pre-treatment and extraction processes. Std: Natural standard (unstained) SDS-PAGE broad range 161-0317 (Bio-Rad Pte. Ltd, New Zealand), Gel-MA: Gelatine pre-treated with 0.75M NaCl at 18±2°C, Gel-MB: Gelatine pre-treated with 0.75M NaCl at 100 ±2°C, Gel-MC: Gelatine pre-treated in boiling water (100 ±2°C), Gel-MD: Gelatine pre-treated with 0.2M NaOH at 18±2°C, Gel-ME: Gelatine pre-treated with 0.5M citric acid, Col-A: Collagen extracted using 0.5M citric acid at 18±2°C, Col-B: Collagen extracted using 0.2M NaOH at 18±2°C, Col-C: Collagen extracted using 0.75M NaCl at 18 ±2°C, HSPH-TP8: Hoki skin protein hydrolysate deodorized using powdered tea polyphenol (concentration: 0.040 g/L hydrolysate; time: 20 min; temperature: 50±2°C). The image is representative of duplicate gels.*

The molecular weight marker was automatically generated using Image Lab software (Version 6.0, Bio-Rad Inc., USA) specified for the marker standard [Natural standard (unstained) SDS-PAGE broad range 161-0317, Bio-Rad Laboratories, USA] used in this study. The molecular weight range generated was marked by the respective arrows as shown in Figure 35. The SDS-PAGE profile of all Hoki gelatine and collagen samples were compared against the standard marker to provide estimation of the molecular weight for protein bands of the respective collagen and gelatine samples tested.

As shown in Figure 35, for Gel-MA, clear bands were observed at molecular weight subunits of approximately 200 and 116.3 kDa. Faint bands with molecular weight subunits below 97.4 kDa were also detected in the gelatine samples. Similar band patterns were observed in Gel-MB and Gel-MC. Faint protein bands with molecular subunits below 66.2kDa were observed in Gel-MD and Gel-ME. The appearance of smaller band subunits in the corresponding gelatine samples may be due to the protein degradation by endogenous enzymes present in the Hoki skins. In the production of collagen or gelatine, endogenous proteases naturally present within the fish skins and muscles can promote protein autolysis that can further break down collagen and gelatine proteins into smaller molecules (Mukundan et al., 1986; Zhou and Regenstein, 2005). Since autolysis of endogenous enzymes arises from within the fish skins, complete prevention and control is difficult to achieve (Mukundan et al., 1986). Recently, various studies have been conducted to investigate the effect of different pre-treatments on enzymatic degradation (Intarasirisawat et al., 2007; Nalinanon et al., 2007; See et al., 2013; Zhou and Regenstein, 2005). According to See et al. (2013) and Zhou and Regenstein (2005), it was found that in both studies, alkali pre-treatments had resulted in the formation of protein subunits with higher molecular weights, indicating that the respective pre-treatments may had inhibited endogenous enzyme activity, thus decreasing enzymatic degradations of gelatine proteins. However, an opposite result was obtained in this study, in which pre-treatment using NaOH solution(Gel-MD) produced a gelatine sample with protein subunits of smaller molecular weights suggesting that alkali pre-treatment may not be effective in the process carried out to inhibit endogenous enzyme activity present in Hoki skins. No further experiments were conducted to investigate the factors affecting the discrepancy between the results obtained from the respective studies, thus no further conclusion was made. On the other hand, for Gel-ME, similar to Gel-MA, Gel-MB and Gel-MC, intense bands are visible at molecular weight subunits of approximately 200, 161.3 and 97 kDa (Figure 35). Faint bands between 45 to 66 kDa were detected in this gelatine sample. When the pH value is below the isoelectric point, an enzyme has a positive charge (Intarasirisawat et al., 2007). The repulsion between charged residues of protein molecules may results in conformational changes, leading to the denaturation of the endogenous enzymes present within the fish skin (Ahmad et al., 2011; Intarasirisawat et al., 2007). Hence, the predominant bands

exhibited at higher molecular weight subunits in Gel-ME indicated that the pre-treatment with citric acid may have inhibited enzymatic activity in Hoki skins, thus decreasing enzymatic degradation due to its low pH value. Similar findings were reported in the study by See et al. (2015) on gelatine from African catfish skin pre-treated using 0.5M acetic acid and the autolysis study by Intarasirisawat et al. (2007) of gelatine from bigeye snapper skin at various pH values.

In gelatine manufacture, the conversion of collagen to gelatine during the thermal denaturation yields protein molecules of varying molecular weights as a result of the intra- and intermolecular chain cleavage (See et al., 2015). The specific chain subunits unique to gelatine are the two α-chain subunits (α1 and α2 chains) and β chain (Kimura et al., 1987; Kumar and Suresh, 2016; Mohtar, 2012; See et al., 2015). According to Duan et al. (2009), the molecular weights of the α- and β-chains are approximately 116 kDa and 205 kDa, respectively. Based on Figure 35, all extracted gelatine samples (Gel-MA, Gel-MB, Gel-MC, Gel, MD and Gel-ME) exhibited bands at molecular weight subunits of approximately 200 and 116.3 kDa indicating the presence of α- and β-chains. This also suggested that these samples can be identified as gelatines indicating that the pre-treatment and extraction processes employed in this study were successful in producing gelatines as final products (refer Section 5.2.3; Table 24). In addition, this is also supported by the results obtained regarding the molecular weight ranges of Hoki skin determined in the study by Mohtar (2012).

The SDS-PAGE profile of Hoki collagen samples are represent by Col-A, Col-B and Col-C in Figure 35. A faint band around 200 kDa can be seen and more intense bands at molecular weight subunits of approximately 161.3 and 97 kDa were observed in Col-C. Apparent protein degradation can also be observed in this collagen sample, indicating possible enzymatic degradation caused by the autolysis of collagen molecules. The results suggested that pre-treatment using 0.75 M NaCl solution did not successfully inhibit endogenous enzyme activity. A similar result was also reported in a study on the skins of unicorn leatherjacket (*Alutherus monoceros*) (Ahmad et al., 2011). A faint band around 200 kDa with no small molecular weight subunits was seen for Col-B (Figure 35). No protein bands were observed for Col-A and HSPH-TP8. The electrophoresis study was conducted in duplicate and similar SDS-PAGE results were obtained for all collagen,

gelatine and HSPH sample tested. This indicated that the negative results obtained for Col-A and HSPH-TP8 were not due to an incorrect analysis. No further conclusion was made. Further investigation using SDS-PAGE was not conducted in response to the mentioned results due to time constraints.

5.4 Amino Acid Composition of Hoki Collagen and Gelatine

Amino acid profiling was performed to determine the amino acid compositions of collagen and gelatine samples pre-treated and extracted using various procedures (Section 3.3.7). The amino acid composition of the collagen, gelatine and hydrolysate samples tested is presented in Table 25.

The amino acid composition of collagen consists of all 20 essential amino acids (Karim and Bhat, 2009). Glycine is the major amino acid in every collagen, thus it can be used to identify a collagen protein (Karim and Bhat, 2009). Table 15 shows that Col-A, Col-B and Col-C had low glycine content with values of 15.3, 9.9 and 2.2 g/100g wet sample, respectively. It can be seen that the aspartic acid, glutamic acid and lysine were present in higher concentrations than that of glycine in all three collagen samples. These suggested that Col-A, Col-B and Col-C samples could not be confirmed as collagen based on amino acid composition, after the extraction procedures using 0.5 M citric acid, 0.2 M NaOH and 0.75 M NaCl solutions, respectively. In addition, collagen is also uniquely characterized by the large amount of hydroxyproline and proline content (Intarasirisawat et al., 2007; Karim and Bhat, 2009; Sotelo et al., 2016). The triple helix structure of collagen is maintained via hydrogen bond formation through the hydroxyl groups of the hydroxyproline and proline (Mohtar, 2013). From the results obtained, a very low hydroxyproline concentration was found in Col-A, Col-B and Col-C with the values of 3.0, 0.9 and 0.3 g/100g wet sample, respectively (Table 25). Similar patterns were also found with the proline concentration of the three respective samples (Col-A = 9.3; Col-B: 6.2; and Col-C: 2.3 g/100g wet sample). Hence, these results indicated that it was difficult to confirm that the extracted samples, Col-A, Col-B and Col-C were pure collagen sample. To further investigate the identification of the samples extracted using the methods used in this study for Hoki collagen extraction, other analyses can be employed. In a study by Sotelo et al. (2016), an analytical method based on ultraviolet visible (UV-VIS)

spectrophotometry was used to estimate the hydroxyproline content presented in collagen extracted from different discarded fish species. In another study, liquid chromatography-mass spectrometry (LC-MS) was used to identify the peptide sequencing in a collagen sample extracted from porcine skin (Li et at., 2007).

Table 26*: Amino acid composition of gelatine and collagen samples pre-treated and extracted using various procedures. Hoki skin protein hydrolysate, HSPH-TP8 was included as a representative of all HSPH samples post-deodorisation treatments.*

Amino Acid	Amino acid concentration (g/100g wet sample)							
	Gel-MA	**Gel-MB**	**Gel-MC**	**Gel-MD**	**Gel-ME**	**Col-A**	**Col-B**	**Col-C**
Aspartic Acid	1.51	0.14	0.07	1.82	1.53	18.74	19.45	3.87
Threonine	0.72	0.06	0.03	0.66	0.70	8.26	5.41	1.63
Serine	1.22	0.10	0.05	0.95	0.90	8.44	6.97	1.74
Glutamic Acid	2.50	0.25	0.11	2.86	2.32	27.62	31.54	4.82
Proline	1.91	0.13	0.07	1.51	1.39	9.30	6.19	2.31
Glycine	4.65	0.29	0.17	3.21	2.83	15.25	9.90	2.22
Alanine	2.07	0.15	0.08	1.79	1.45	11.63	11.33	2.19
Valine	0.62	0.06	0.03	0.91	0.67	8.77	10.10	1.82
Methionine	0.53	0.04	0.02	0.65	0.48	5.86	6.61	0.96
Isoleucine	0.38	0.04	0.02	0.65	0.49	7.33	8.28	1.53
Leucine	0.76	0.08	0.03	1.23	0.92	13.14	15.69	2.56
Tyrosine	0.19	0.02	0.01	0.47	0.35	5.93	6.92	1.27
Phenylalanine	0.55	0.05	0.02	0.72	0.56	6.78	7.25	1.48
Histidine	0.30	0.02	0.01	0.23	0.28	3.52	1.73	0.72
Lysine	0.99	0.11	0.05	1.39	1.15	15.61	16.79	2.49
Arginine	1.79	0.14	0.07	1.73	1.45	13.13	12.28	1.88
Hydroxyproline	1.37	0.08	0.05	0.74	0.76	2.98	0.92	0.30

Values are the mean of triplicate samples.

As described in Section 2.1.3, the amino acid composition of gelatine is very close to that of its parent collagen and has similar repeating unit of Gly-X-Y; where Gly is glycine, X is

proline, and Y corresponds to any amino acid but commonly hydroxyproline (Freundenberg et al., 2007; Karim and Bhat, 2009; Mohtar, 2013). Similar to collagens, glycine is the major amino acid in gelatines. As shown in Table 25, all gelatine samples Gel-MA, Gel-MB, Gel-MC, Gel-MD and Gel-ME had the highest glycine concentration with the values of 4.7, 0.3, 0.2, 3.2 and 2.8 g/100g wet sample, respectively. These indicated that all of the respective samples could be confirmed as gelatine. Meanwhile, lower hydroxyproline and proline concentrations were observed in Gel-MA, Gel-MB, Gel-MC, Gel-MD and Gel-ME when compared to the other amino acids particularly aspartic acid, serine, glutamic acid, alanine and arginine. This may suggest that all of the gelatine samples have low denaturing temperature and gel strength (Montero et al., 1990; See et al., 2015; Subhan et al., 2015). No further research was conducted to investigate the effect of denaturing temperature on the hydroxyproline and proline contents in the gelatine samples as it was beyond the objective of this study, thus no further conclusion can be made.

Figure 36, 37 and 38 represent the amino acid compositions of all extracted collagen and gelatine samples based on total protein content of each sample (refer Section 5.11; Table 22 and Section 5.21; Table 24). The primary pie charts represent the total protein and non-protein contents in all extracted collagen and gelatine samples based on the total protein analyses conducted as described in Section 3.3.1. Secondary pie charts represent the total amino acid compositions in all samples based on the results obtained from the amino acid profiling (Table 26). Sum of amino acids tested in Table 23 (17 amino acids were tested) provide estimations of the total protein contents in all extracted collagen and gelatine samples. These respected values were then expressed on a dry basis (g of protein/100g dry sample) to be compared with the total protein contents (Table 22 and 24) of all extracted samples. All values are presented in percentage. The results on total protein contents of all extracted collagen and gelatine samples were discussed in detail in Section 5.2.1 and 5.2.2. Briefly, an overall observation from Figure 36 showed that Col-B has the highest total protein content followed by that of Col- A and Col-C. Meanwhile, based on Table 37 and 38, Gel-MD had the highest total protein content with a value of 41%, then the protein contents decreased based on treatment in the following order Gel-MA, Gel-ME, Gel-MC and Gel-MB, respectively. Based on Figure 36, 37 and 38, the total

percentage of amino acid compositions were slightly lower than the total percentage of protein contents in all extracted collagen and gelatine samples. The respective results were expected as the amino acid profiling tested for only 17 out of 20 types of amino acids. It is therefore, assumed that the remaining values of the total amino acid compositions in all samples were regarded as tryptophan, asparagine and cysteine. Despite that the amino acid profiling can provide a good estimation of the total protein content in all samples, it is to note that results obtained from the test also indicated that it was difficult to confirm that the extracted samples, Col-A, Col-B and Col-C were pure collagen sample, as previously discussed. This indicated that non-collagenous proteins may have been extracted instead using the procedures employed. No further investigation was conducted hence no further conclusion can be drawn.

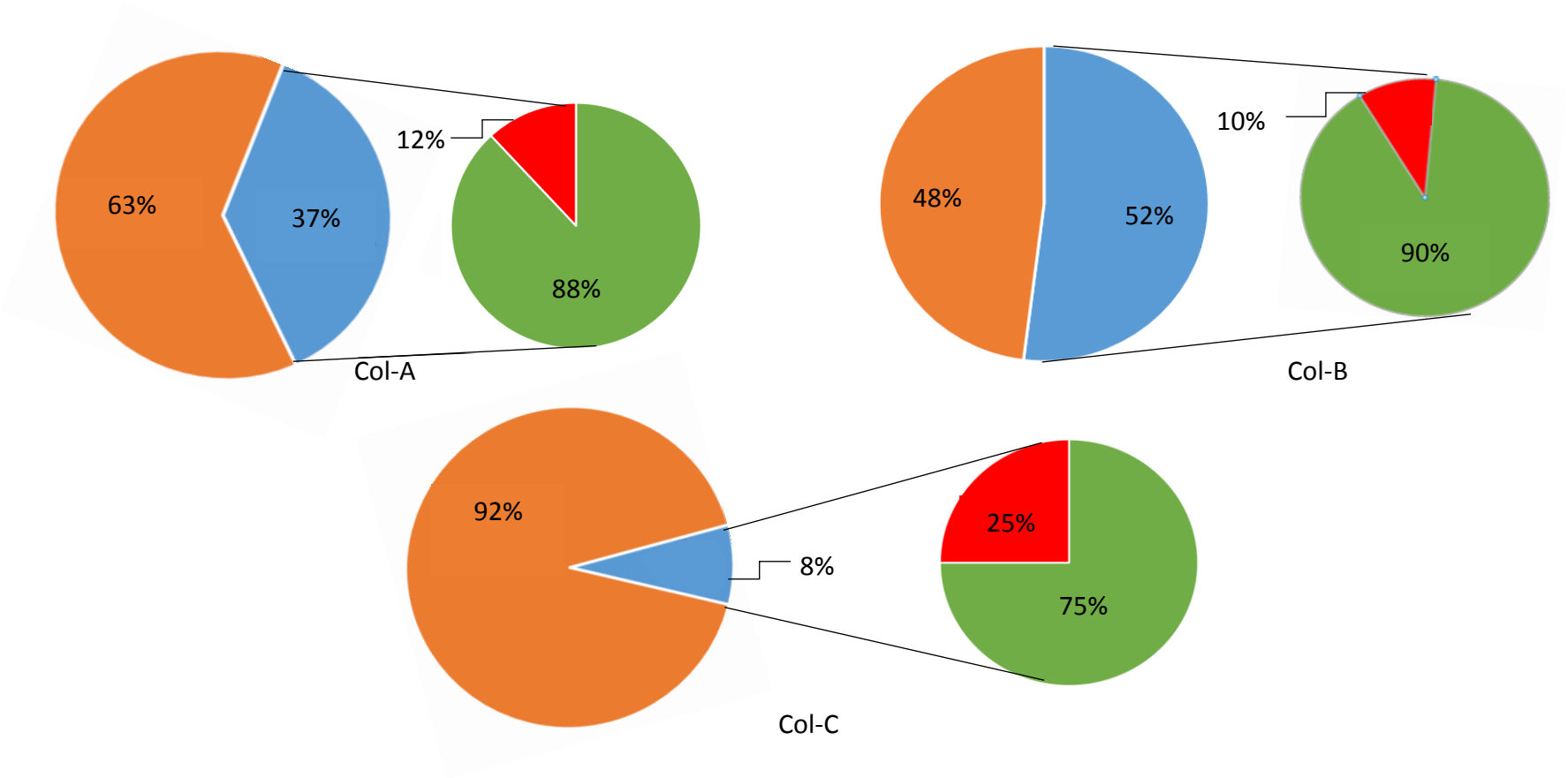


Figure 36: *Pie charts representing the amino acid compositions based on the total protein contents in all extracted collagen samples (Sample Col-A, Col-B and Col-C). The primary pie charts represent the total protein and non-protein contents in all extracted collagen and gelatine samples based on the total protein analyses (LHS). Secondary pie charts represent the total amino acid compositions in all samples based on the results obtained from the amino acid profiling (RHS). All values are expressed in percentage.* *Total protein content in collagen sample* *Non-protein in collagen sample* *Total percentage of amino acid as tested in amino acid profiling (17 types amino acid; refer Table 26)* *Total percentage of amino acid tryptophan, asparagine and cysteine.*

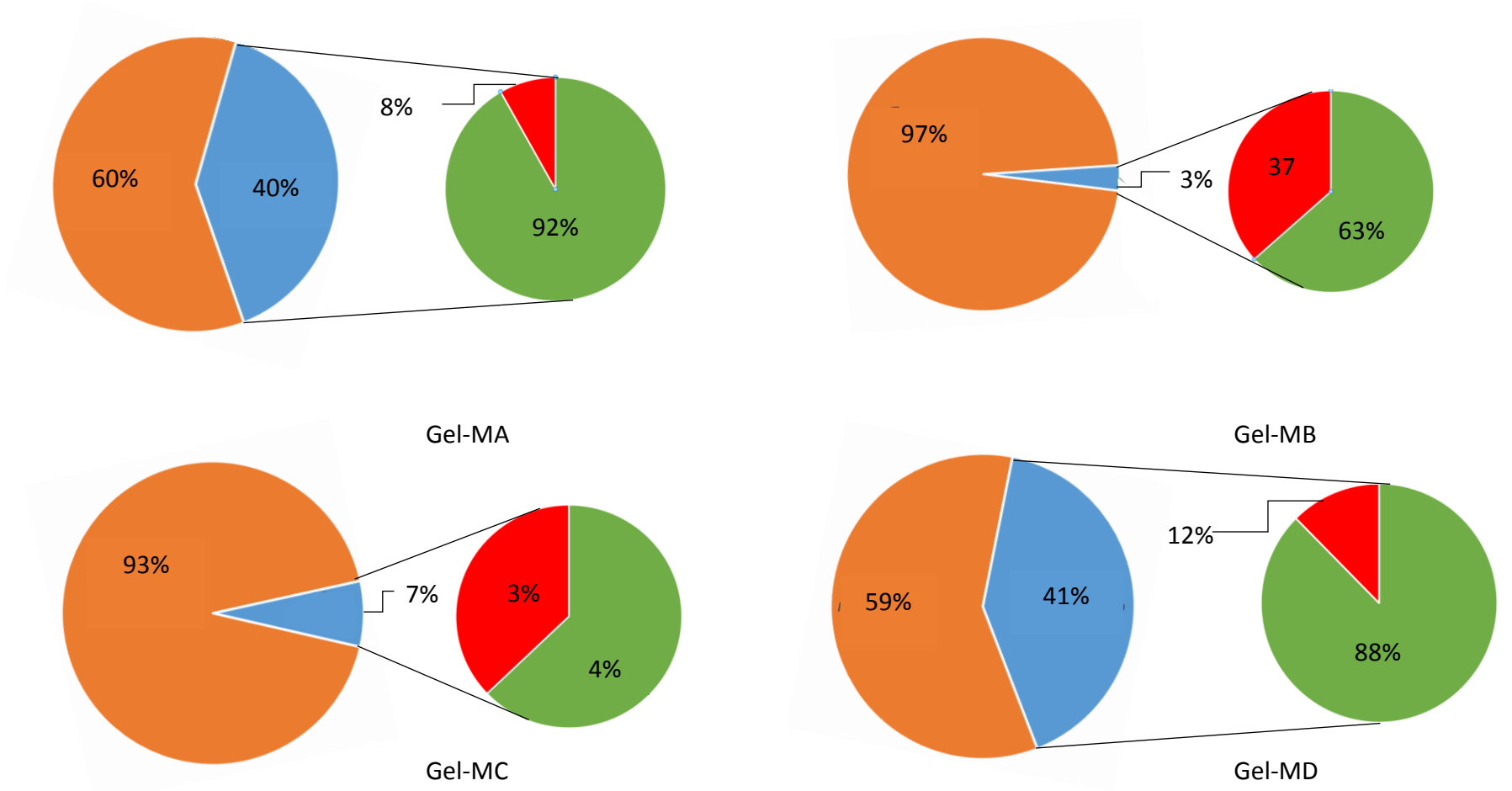


Figure 37: *Pie charts representing the amino acid compositions based on the total protein contents in all extracted gelatine samples (Sample Gel-MA, Gel-MB, Gel MC and Gel-MD). The primary pie charts represent the total protein and non-protein contents in all extracted collagen and gelatine samples based on the total protein analyses (LHS). Secondary pie charts represent the total amino acid compositions in all samples based on the results obtained from the amino acid profiling (RHS). All values are expressed in percentage.* *Total protein content in gelatine sample* *Non-protein in gelatine sample* *Total percentage of amino acid as tested in amino acid profiling (17 types amino acid; refer Table 26)* *Total percentage of amino acid tryptophan, asparagine and cysteine.*

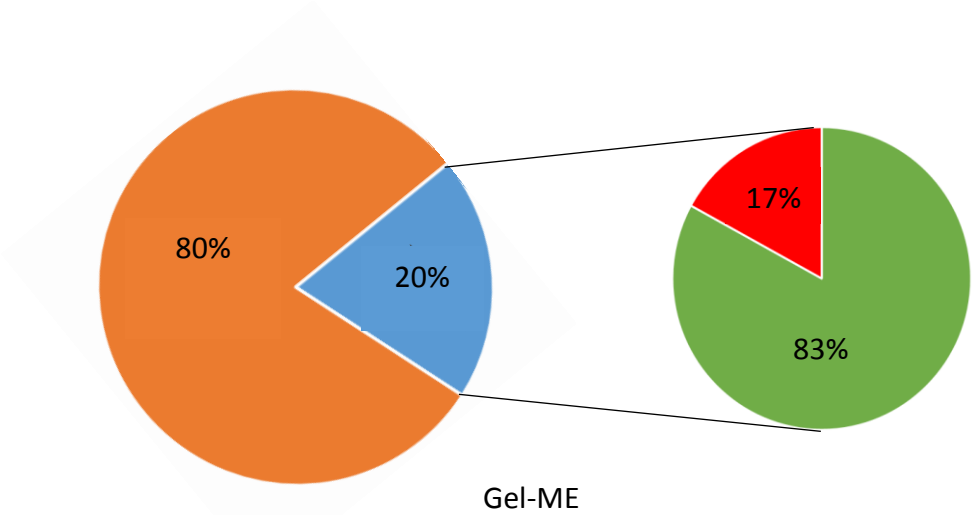


Figure 38: *Pie chart representing the amino acid compositions based on the total protein content in extracted gelatine sample Gel-ME. The primary pie charts represent the total protein and non-protein contents in all extracted collagen and gelatine samples based on the total protein analyses (LHS). Secondary pie charts represent the total amino acid compositions in all samples based on the results obtained from the amino acid profiling (RHS). All values are expressed in percentage.* *Total protein content in gelatine sample* *Non-protein in gelatine sample* *Total percentage of amino acid as tested in amino acid profiling (17 types amino acid; refer Table 26)* *Total percentage of amino acid tryptophan, asparagine and cysteine.*

5.5 Conclusions and Recommendations

Based on results from the SDS-PAGE and amino acid profile test, it can be concluded that all pre-treatment and extraction procedures employed successfully produced gelatine samples as the final products. However, collagen cannot be confirmed using the amino acid profiling employed in this study. Alternative analytical techniques need to be used to identify the presence of collagen.

Based on the analyses conducted on Col-A, Col-B and Col-C, it can be concluded that non-collagenous proteins may have been extracted instead using the procedures employed.

Hoki skins pre-treated using 0.2 M NaOH solution (1:6 w/v) for 60 min at 18±2˚C and the extraction procedure with distilled water (1:10 w/v) for 60 minutes at 50±2˚C was determined to produce gelatine product with the highest protein content (total protein content = 41.3±0.9 g of protein/100g dry sample) and reduced off-odour (low TMA content of 0.9±0.1 mg of nitrogen/ 100 g wet sample). However, there was a slight compromise regarding the gelatine yield as a lower gelatine yield recovery of 61.0±1.7 % was obtained in the respective gelatine sample.

The permutation test showed that the consensus between the sensory panellists in describing the odour profile of collagen samples were acquired by chance, thus compromising the reliability of the results obtained from the sensory analysis technique used in this study. In contrast, the consensus between panellists in describing the odour profile of gelatine samples was found to be statistically significant.

Chapter 6: Overall Discussion

The present study was concerned with the deodorisation of pre-prepared Hoki skin protein hydrolysate (HSPH) and the extraction of collagen and gelatine from Hoki skins. The overall findings discussed herein are based on the results obtained and are explained below.

6.1 The Deodorisation of Pre-prepared Hoki Skin Protein Hydrolysate

The first part of this study focused on determining the suitable deodorisation treatments that reduce or remove off-odour in pre-prepared Hoki skin protein hydrolysates (HSPH). Based on the results obtained from qualitative and quantitative analyses conducted, it was found that deodorisation treatments with powdered tea polyphenol (TP) under two different conditions were the most suitable in reducing the off-odour in pre-prepared HSPH. The corresponding two conditions were, 1) TP concentration: 0.04 g/ml hydrolysate; temperature: 40˚C; time: 50 min, and 2) TP concentration: 0.04 g/ml hydrolysate; temperature: 80˚C; time: 60 min. Significant reduction of trimethylamine (TMA) concentrations, supported by the findings obtained from the sensory analysis on the HSPH samples deodorized using the respective conditions confirmed that the methods could reduce off-odour in pre-prepared HSPH.

As described in Chapter 3, Section 3.1.1, the HSPH was pre-prepared and received in February, 2016. By the time it was used in this study, the HSPH was stored for more than 12 months. The determination of total volatile base nitrogen (TVB-N) is a chemical parameter commonly used to evaluate the quality of fish products (Chan et al., 2006). TVB-N represents the total amount of ammonia, dimethylamine (DMA) and trimethylamine (TMA) content of the fish or fish products and is used as an estimate of microbiological spoilage (Chan et al., 2006; Ali et al, 2010). The increased amount of TVB-N was in parallel with the increase of TMA during spoilage. This is a result of the increase in the reduction of trimethylamineoxide (TMAO) to TMA (refer to Section 2.3.3.2) (Ali et al., 2010). The off-odour TMA gradually accumulated over time during storage and the degree of deterioration was highly dependent on the storage temperature and storage

time (Chan et al., 2006). As described in Section 4.3.1, the TVB-N and TMA concentrations in the untreated HSPH were 15.9±0.1 and 7.6±0.1 mg of nitrogen/100g wet sample, respectively. These suggest that, the TVB-N and TMA may have gradually accumulated despite that the pre-prepared HSPH used in this study was stored at -20˚C until used. The already present TVB-N and TMA in the untreated HSPH may have affect the overall deodorisation treatments of the HSPH samples. Therefore, it would be interesting to conduct the deodorisation treatments on freshly prepared HSPH and compare its TVB-N and TMA concentrations to those determined in this study.

This study has revealed the potential application of dried green tea leaves as a more economical alternative for the deodorisation of HSPH in the full size industrial practice. As discussed in Section 4.3.4, increasing the total phenolic content by increasing the concentration of dried green tea leaves per ml of HSPH to match that of deodorisation treatment specified for TP described previously, had subsequently reduced the TMA concentration in HSPH sample. A study by Kida et al. (2002), has revealed the existence of another mechanism of deodorisation, other than neutralization, by identifying the reaction products of epigallocatechin gallate (EGC, one of the main catechins as described in Section 2.3.4.3) with a primary amine. Based on the respective study, it was found that the reaction products were formed by replacement of hydroxyl group with an amino group at the 4’ position of EGC (Kida et al., 2002). It would be interesting to investigate the deodorizing effect of EGC or other catechins on primary, secondary and tertiary amines to utilize the application of green tea leaves as the primary deodorant for HSPH.

6.2 The Extraction of Collagen and Gelatine from Hoki Skins

In the second part of the study (refer Chapter 5), selected pre-treatments and extraction processes were employed for the extraction of Hoki collagen and gelatine to determine which methods produced low odour extracts but still maintaining extraction yield (refer Section 3.0). Current findings have determined that it was difficult to confirm that the extracted samples, Col-A, Col-B and Col-C were pure collagen sample. To further investigate on the identification of the samples extracted, using the methods used in this study for Hoki collagen extraction, other analyses can be employed which include

ultraviolet visible (UV-VIS) spectrophotometer and liquid chromatography-mass spectrometry (LC-MS) (Li et at., 2007; Sotelo et al., 2016). According to the results obtained from the protein analysis, all samples extracted using the methods used for collagen extraction in this study had total protein contents ranging between 8 to 52g of protein/100g dry sample (refer Section 5.1.1). Pre-treatments with acid or alkali, prior to the extraction of collagen, are necessary to remove impurities such as non-collagenous proteins to increase the yield and quality of the final extracted collagen (Pal and Suresh, 2016; Schmidt et al., 2016) (Section 2.7.1). It was described in Section 5.1.1 that the lower total protein content in the collagen sample treated with citric acid (Col-A) compared to that in the sample treated with NaOH (Col-B) may have been due to the loss of collagen protein during the extraction procedure due to higher solubility of collagen in acidic conditions. It was also indicated that the total protein contents in all extracted collagen samples may have contained both collagenous and non-collagenous proteins (Section 5.4). To further investigate the removal of protein and collagen during pre-treatments, it is therefore recommended to determine the total protein and hydroxyproline contents in the pre-treatment solutions. Findings from the investigation would give better insights on whether or not the specified pre-treatment procedures conducted in this study could remove non-collagenous proteins without loss of skin collagen in the extracted samples.

In the case of gelatine extraction, it was found that pre-treatment using 0.2 M NaOH solution (1:6 w/v) for 60 min at 18±2^{o}C and extraction procedure with distilled water (1:10 w/v) for 60 minutes at 50±2^{o}C was determined to produce gelatine product with the highest protein content (total protein content = 41.3±0.9 g of protein/100g dry sample) and reduced off-odour (low TMA content of 0.9±0.1 mg of nitrogen/ 100 g wet sample). Despite that, it should be noted that NaOH pre-treatment may have compromised the yield of gelatine recovery (gelatine recovery = 61.0±1.7 w/w% of dry sample) and the quality of the gelatine produced. It was described in Section 5.2.1 that acid or alkali pre-treatments may change the isoelectric point of the collagen, increasing the collagen solubility. This may result in the loss of collagen during washing steps, consequently reducing the amount of protein for extraction of gelatine in the subsequent thermal denaturation step. The lower yield for alkali pre-treated gelatine sample may be due to the loss of collagen. On the other hand, extreme conditions, such as harsh pre-

treatments in strong acid or alkali solutions may also result in the excessive disruption of hydrogen bonds in the collagen molecules (Mohtar, 2013). As described in Section 2.4.1, alkali pre-treatment is usually employed when more aggressive penetration by the chemical reagent is required for thicker raw materials such as bovine ossein and bovine shavings (Schmidt et al., 2016). Thus, the low yield observed in gelatine sample treated with 0.2M NaOH, as described previously, could be due to the loss of soluble collagen during the washing steps due to a looser protein structure compared to that treated with NaCl and citric acid (Mohtar, 2013). However, although pre-treatments with citric acid produced a higher gelatine yield (83.05±4.97 w/w% of dry sample) than that using NaOH, a significantly lower total protein content was obtained in the gelatine samples pre-treated in the acidic solution (refer Section 5.2.1). Results obtained from the SDS-PAGE performed showed that there was formation of faint smaller bands in both gelatine samples treated with NaOH and citric acid indicating that specified alkali and acid pre-treatments used in this study may be not effective in inhibiting endogenous enzyme activity present in Hoki skins (refer Section 5.3), thus may have compromised the quality of the extracted gelatine. Therefore, when considering the production of gelatine from Hoki skins for industrial scale, it is important to strictly define the properties of the targeted product as the suitable pre-treatment and extraction processes will highly depend on the specified final product.

6.3 The Advantages of Pre-treatment

In the first part of the study, the removal of off-odour in HSPH samples was tackled by employing deodorisation treatments as countermeasure processes for the prepared final products. In the second part of the study, the removal of off-odour was addressed by implementing acid and alkali pre-treatments prior to collagen and gelatine extractions to determine the efficiency of pre-treating the raw material in reducing the off-odour in the final products. The pre-treatment is often applied as part of the major steps in the extraction of collagen and gelatine samples (Kumar and Suresh, 2016). The pre-treatment is essential to remove impurities and to increase the quality of the final extracted collagen and gelatine (Kumar and Suresh, 2016). Findings from this study have therefore provided a better understanding on the importance of pre-treating the raw material prior to subsequent extraction procedure. Based on this study, it was found that pre-treatments

could further reduce the TMA concentrations of the final products, as observed when comparing the TMA concentrations between both HSPH samples post-deodorisation treatments and the extracted collagen and gelatines samples. The respective results suggested that pre-treating the raw material using acid or alkali prior to subsequent processes is more efficient in reducing the off-odour in the final products rather than employing deodorisation processes as a countermeasure. Current findings made in this study are supported by findings made in a number of studies by Professor Soottawat Benjakul of Prince of Songkla University, Hat Yai, Thailand (Ahmad and Benjakul, 2011; Benjakul, Rawdkuen and Thitipramote, 2013; Benjakul, Sae-Leaw and O'Brien, 2016). In July 2018, from discussions with Professor Benjakul on the off-odour control in marine products, particularly marine protein hydrolysate, collagen and gelatine, the following information was obtained from his research (Benjakul, 2018) summarised:

- Pre-treating the raw material prior to successive extraction or hydrolysis processes is important to minimize the generation of off-odour in the final product. Pre-treatments with acid or/and with alkali are often used to eliminate non-collagenous proteins and lipid to prevent off-odours in the products. Alternatively, spray drying can also be incorporated as an additional step to the extraction or hydrolysis process to further reduce the off-odour in fish-derived products (Benjakul, Sae-Leaw and O'Brien, 2016). During spray drying, volatile odorous compounds can be eliminated. Hence, the appropriate pre-treatment at an optimum condition depending on the raw material used, along with an effective drying method, could be a promising means to lower off-odours in marine based products (Benjakul, Sae-Leaw and O'Brien, 2016).
- One of the major factors that contribute to the generation of off-odours in many fish-derived products is lipid oxidation. Fish skins in particular contain lipids with high degree of unsaturation (Benjakul, Sae-Leaw and O'Brien, 2016). These lipids are susceptible to oxidation during extraction process at high temperature resulting in the formation of off-odour in the final product. Therefore, the removal of lipids from skin by pre-treatment is important as a means to lower the oxidation (Benjakul, Sae-Leaw and O'Brien, 2016). The process of removing lipids, also known as defatting, can be achieved by pre-treating the raw materials with

acid and/or alkali. Alternatively, defatting seabass skins using isopropanol at a skin/solvent ratio of 1:10 (w/v) at room temperature for 1 hour was also found to be efficient in removing lipids and some pro-oxidants from the skins (Benjakul, Sae-Leaw and O'Brien, 2016).

- Working with fresh raw materials may also minimize the generation of off-odour in the final product. Lipid oxidation can also occur during storage leading to the generation of undesirable fishy odour. In a study, extended storage time of seabass skins in ice (time and temperature of storage are not specified) displayed a strong fishy odour, predominantly due to lipid oxidation (Benjakul, Sae-Leaw and O'Brien, 2016).

Besides, pre-treatment was essential as it promoted the swelling effects on the treated Hoki skins allowing a more efficient extraction, and consequently, producing higher yield of collagen and gelatine.

Chapter 7: Overall Conclusions and Recommendations

7.0 Overall Conclusions

This study presents experimental results regarding the deodorisation of pre-prepared Hoki skin protein hydrolysates (HSPH) and the extraction of collagen and gelatine from Hoki skins. Based on the results obtained from this study, it can be concluded that the suitable deodorisation treatments that reduce the fishy odour in the pre-prepared HSPH samples are 1) deodorant: powdered tea polyphenol; concentration: 0.04 g/ml hydrolysate; temperature: 50˚C; time: 20 min, and 2) deodorant: powdered tea polyphenol; concentration: 0.04 g/ml hydrolysate; temperature: 80˚C; time: 60 min. Further investigation determined that dried green tea leaves can be an alternative deodorant to the powdered tea polyphenol for a more economical solution by manipulating the total phenolic content of the HSPH prior to deodorisation. Secondary deodorisation using resin may further reduce the trimethylamine (TMA) concentration in Hoki skin protein hydrolysate samples.

Current findings concluded that all pre-treatment and extraction procedures employed successfully produced gelatine samples as the final products. However, collagen cannot be confirmed using the amino acid profiling employed in this study. Alternative analytical techniques need to be used to identify the presence of collagen and to understand the suitable extraction procedures for Hoki collagen. It was determined that pre-treating the raw materials prior to successive extraction process was more efficient in reducing the off-odour in the final products rather than employing deodorisation treatment as a subsequent countermeasure. The study revealed that pre-treatment using 0.2 M NaOH solution (1:6 w/v) for 60 min at 18±2˚C and the extraction procedure with distilled water (1:10 w/v) for 60 minutes at 50±2˚C was determined to produce gelatine product with the highest protein content (total protein content = 41.3±0.9 g of protein/100g dry sample) and reduced off-odour (low TMA content of 0.9±0.1 mg of nitrogen/ 100 g wet sample).

Overall, it can be concluded that the findings from this study can provide better understanding on the odour control of Hoki skin protein hydrolysate and Hoki gelatine. Findings from this study can also provide an initial background towards a further investigation on the suitable pre-treatment and extraction procedures of Hoki collagen and gelatine. In addition, overall findings from this study may provide a stepping stone towards the commercialization of the Hoki skin protein hydrolysate, Hoki collagen and Hoki gelatine productions.

7.1 Recommendations on Future Work

Areas on which more focus can be directed and further development can be carried out are listed below:

1. As discussed in Chapter 4.4, secondary deodorisation can be employed as part of the multiple steps deodorisation treatment to reduce off-odour in pre-prepared HSPH. Further study on secondary deodorisation using strong acid hydrogen form ion resin can be conducted to investigate the efficiency of the proposed procedure to further reduce or to completely eliminate off-odours in HSPH. However, it is to note that, looking at the perspective of implementing secondary deodorisation for full scale industrial practice this will incur additional cost due to an additional process in the processing line.
2. Further investigation on the suitable pre-treatment and extraction procedure of Hoki collagen is to be conducted. It is also important to further develop the analytical method in the identification of the final product after pre-treatment and extraction procedures based on the hydroxyproline content, which is regarded as the unique attribute of collagen and gelatine.
3. There was limitation in correlating between the results obtained from the flash profiling method conducted on the extracted Hoki gelatine and the TMA concentrations. Thus, it is recommended that further development on the sensory analysis method is to be conducted to provide further understanding on the odour profile of the extracted final product. The respective results also suggested that further investigation on alternative potential markers to assess off-odour in extracted Hoki collagen and gelatine is recommended.

4. Optimization of the pre-treatment and extraction procedure for Hoki collagen and gelatine can be further studied to determine the optimum condition for the production of Hoki collagen and gelatine with optimum yield and quality.
5. Apart from trimethylamine (TMA) concentration, lipid oxidation is also one of the factors causing the undesirable fishy odour in marine based products (refer Section 2.3.3.1 and Section 6.3). Thus, the effect of lipid oxidation on the contribution towards the formation of off-odours in Hoki skin protein hydrolysate, Hoki collagen and Hoki gelatine can be investigated to achieve further understanding on the factors affecting the sensory profile of the final products.
6. As described in Section 2.5.2, some of the volatile compounds known to be the contributors to the fishy odour in aquatic products are including n-hexanal, n-heptanal, 2,4-heptadienal, 2,4-decadienal, 1-octen-3-ol and 2, 3-octanedione (Chen et al., 2016). Hence, use of GCMS flavour profiling for extracted hoki skin protein hydrolysates, gelatine and collagen could be developed to be able to monitor other possible off-odours present in the samples.

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Appendices

Appendix 1: Sensory Analysis – Questionnaire for Hoki skin protein hydrolysate samples after deodorisation treatments.

Massey Institute of Food Science & Technology
School of Food & Nutrition
Turitea Place
Massey University

TITLE of Work: **The Deodorisation of Pre-prepared Hoki Skin Protein Hydrolysate and the Extraction of Collagen and Gelatine from Hoki Skins**

Researcher(s) Introduction

Researchers Name:	Nur Sharim	Supervisors Name:	A/P Marie Wong
Contact Details:	N.Sharim@massey.ac.nz	Contact Details:	M.Wong@massey.ac.nz

You are invited to take part in a panel sensory evaluation to evaluate odour profile of fish protein hydrolysate.

The type of activity that this work involves smelling fish protein hydrolysate samples.

Your participation in this activity will take approximately 1.5 hours and to be completed in 3 separate sessions. Each session will take approximately 30 minutes.

This work may be sponsored by a commercial company that is likely to benefit from this work. Any money that Massey University receives for such work contributes to the teaching of students.

We will ensure that there is no conflict of interest between the commercial sponsor, Massey University, the participants or a competing product used for comparative purposes.

The foods you will be evaluating contain the following components that can be harmful or cause allergic reactions with certain groups of people. You will be excluded from taking part if you are allergic, or may be adversely affected by any of the following:

- Fish and Fish derivatives

The type of food that you will be testing is: Fish protein hydrolysate

This food does not comply with specific religious, ethical or cultural beliefs.

The information collected in this study will be used to complete an assignment in partial fulfilment of the Master of Food Technology. Non-participation will not affect your academic performance. No data linked to an individual's identity will be collected. In some circumstances the research may be published.

You should not participate in this trial if you have any doubts concerning the appropriateness of this food with respect to your religious, ethical or cultural beliefs or any other reason.

You are under no obligation to accept this invitation. If you decide to participate, you have the right to:

- *decline to answer any particular question;*
- *withdraw from the study at any time;*
- *ask any questions about the study at any time during participation;*
- *provide information on the understanding that your name will not be used unless you give permission to the researcher;*
- *be given access to a summary of the project findings when it is concluded.*
- *ask for the recorder to be turned off at any time during the interview.*

If you have any questions about this work, please contact one of the people indicated above.

You are welcome to a summary of the results.
Please indicate if you wish to receive a summary of the results from this trial YES NO

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 4000017709. If you have any concerns about the conduct of this research, please contact Dr Brian Finch, Chair, Massey University Human Ethics Committee: Southern A telephone 06 350 5799 x 84459, email humanethicsoutha@massey.ac.nz.

Massey Institute of Food Science & Technology
School of Food & Nutrition
Turitea Place
Massey University

The Deodorisation of Pre-prepared Hoki Skin Protein Hydrolysate and the Extraction of Collagen and Gelatine from Hoki Skins

PARTICIPANT CONSENT FORM - INDIVIDUAL

- I have read the Information Sheet and have had the details of the study explained to me. My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.

- I agree the interview being sound recorded.

- I do not wish to have my recordings returned to me.

- I have advised and discussed the Researcher of any potentially relevant cultural, religious or ethical beliefs that may prevent me from consuming the Foods under consideration.

- I agree to participate in this study under the conditions set out in the Information Sheet.

Signature: ..... **Date:** .....

Full Name - printed .....

Massey Institute of Food Science & Technology
School of Food & Nutrition
Turitea Place
Massey University

The Deodorisation of Pre-prepared Hoki Skin Protein Hydrolysate and the Extraction of Collagen and Gelatine from Hoki Skins

CONFIDENTIALITY AGREEMENT

I ..... (Full Name - printed)

agree to keep confidential all information concerning the project
.....
.....
.....
..... (Title of Project).

I will not retain or copy any information involving this project.

Signature: ..... **Date:** .....

Full Name - printed .....

Session 1

FISH PROTEIN HYDROLYSATE QUESTIONAIRE

I; would like to ask your opinion about this fish protein hydrolysate.

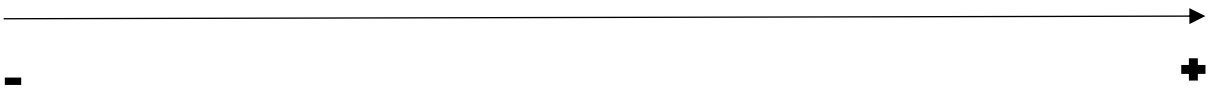
There are nine samples to evaluate. Please start with the bottle labelled **A followed with the bottles labelled B, C, D, E, F, G, H, and I.**

Please smell all the samples. List all attributes that describe the odour of the samples. Then rank each sample along the line according to the attribute assigned, from the weakest intensity on the left-hand side to the strongest intensity on the right-hand side. **Ties are allowed. Please circle ties.**

You may use but not restricted to these attributes to describe the odour attributes of the samples:

- Fishy – related to the smell of fish or seafood
- Rancid/foul – related to the smell of rotten food
- Sea/fresh – related to the smell of sea/fresh seafood
- Odourless – related to the smell of tap water
- Tea – related to the smell of tea/tea leaves

Sensory attribute 1:



Comments:

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Sensory attribute 2:

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Sensory attribute 4:

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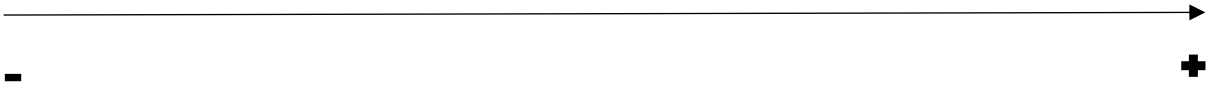
Sensory attribute 5:



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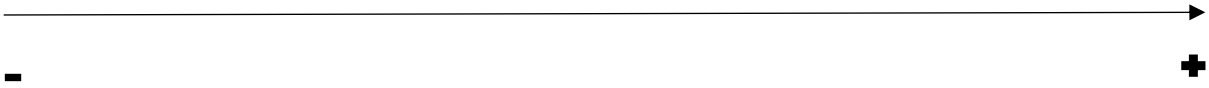
Sensory attribute 6:



Comments:

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Sensory attribute 7:

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END OF QUESTIONAIRE

Thank you for your time.

Please place back the tray and close the lid.

Appendix 2: Amino Acid Testing – Quotation Number: 2017219

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Nutrition Laboratory
Riddet Innovation
T: +64 **6 3505869**
Email: F.S.Jackson@massey.ac.nz
http://nutritionlab.massey.ac.nz

TO:	Nur Syazwana Sharim	AT:	MIFST
Quotation #:	2017219	DATE:	01/12/17
SUBJECT:	Collagen/Gelatin		

Test	Method	Price NZ$ per sample
Sample preparation	FD/Grinding	$ 18.00
*Acid stable AA profile	HCl hydrolysis, AOAC 994.12	$ 110.00
*Cys/met	Performic acid oxidation, AOAC 994.13	$ 110.00
Tryptophan	Sub-contracted	$ 275.00

Please note quote is exclusive of GST and valid for 3 months from date of issue.
Amino Acid profile; Asp, Thr, Ser, Glu, Pro, Gly, Ala, Val, Iso, Leu, Tyr, Phe, His, Lys, Arg,Tau.
Asparagine & Glutamine are not routinely analysed as part of this profile.
****These tests are currently accredited to ISO 17025***

Fliss Jackson
Manager, Nutrition Laboratory
Riddet Innovation
Massey Institute of Food Science and Technology
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DDI 06 350 5869
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Appendix 2: Tukey HSD analysis for HSPH Samples Treated with Green Tea Leaves

Tukey HSD of pH values for HSPH samples treated with green tea leaves

Tukey HSD[a]

sample	Subset for alpha = 0.05					
	a	b	c	d	e	f
GT5	5.4967					
GT7	5.5467					
GT8		5.6733				
GT9			6.0167			
GT2				6.1567		
GT6				6.1967		
GT3					6.2733	
GT4						8.1367
GT1						8.1733
Sig.	.324	1.000	1.000	.594	1.000	.691

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Tukey HSD of TVBN concentrations for HSPH samples treated with green tea leaves

sample	Subset for alpha = 0.05	
	a	b
GT2	11.7600	
GT5	11.7600	
GT8	11.7600	
GT3	12.6000	12.6000
GT6	12.6000	12.6000
GT1	13.4400	13.4400
GT7	13.4400	13.4400
GT9	13.4400	13.4400
GT4		15.9600
Sig.	.784	.138

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

Tukey HSD of TMA concentrations for HSPH samples treated with green tea leaves

Tukey HSD[a]

sample	Subset for alpha = 0.05
	a
GT2	4.2000
GT3	4.2000
GT4	4.2000
GT7	4.2000
GT9	4.2000
GT5	5.0400
GT6	5.0400
GT8	5.0400
GT1	5.8800
Sig.	.719

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

Appendix 3: Tukey HSD analysis for HSPH Samples Treated with Powdered Tea Polyphenol

Tukey HSD of pH values for HSPH samples treated with powdered tea poplyphenol

Tukey HSD[a]

sample	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
TP8	3	5.9900					
TP7	3	6.0367					
TP9	3		6.1933				
TP6	3		6.2067				
TP4	3		6.2133				
TP2	3			6.4500			
TP3	3				6.5633		
0	3				6.5767		
TP5	3					6.7800	
TP1	3						7.9367
Sig.		.768	.999	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Tukey HSD of TVBN concentrations for HSPH samples treated with powdered tea poplyphenol

Tukey HSD[a]

sample	N	Subset for alpha = 0.05	
		1	2
TP8	2	10.0800	
TP9	2	10.0800	
TP5	2	10.9200	10.9200
TP2	2	11.7600	11.7600
TP6	2	11.7600	11.7600
TP7	2	11.7600	11.7600
TP4	2	12.6000	12.6000
TP1	2	14.2800	14.2800
0	2	15.9600	15.9600
TP3	2		16.8000
Sig.		.096	.096

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

Tukey HSD of TMA concentrations for HSPH samples treated with powdered tea poplyphenol

Tukey HSD[a]

sample	N	Subset for alpha = 0.05	
		1	2
TP7	2	3.3600	
TP9	2	3.3600	
TP2	2	4.2000	4.2000
TP4	2	4.2000	4.2000
TP5	2	4.2000	4.2000
TP6	2	4.2000	4.2000
TP8	2	4.2000	4.2000
TP1	2	5.0400	5.0400
TP3	2	5.8800	5.8800
0	2		7.5600
Sig.		.350	.114

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

Appendix 4: Tukey HSD analysis for HSPH Samples Treated with Olive Leaves

Tukey HSD of pH values for HSPH samples treated with olive leaves

sample	Subset for alpha = 0.05				
	a	b	c	d	e
OL9	6.0500				
OL6	6.0767				
OL8	6.2000	6.2000			
OL5		6.2367	6.2367		
OL1		6.3467	6.3467	6.3467	
OL3			6.3733	6.3733	
OL4				6.4233	
OL2				6.4867	
OL7					8.5600
Sig.	.055	.063	.098	.085	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Tukey HSD of TVBN concentrations for HSPH samples treated with olive leaves

Tukey HSD[a]

sample	N	Subset for alpha = 0.05	
		1	2
OL9	2	12.6000	
OL6	2	14.2800	14.2800
OL1	2	15.1200	15.1200
OL3	2	15.1200	15.1200
OL2	2	15.9600	15.9600
OL7	2	16.8000	16.8000
OL5	2	17.6400	17.6400
OL8	2	18.4800	18.4800
OL4	2		20.1600
Sig.		.118	.118

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

Tukey HSD of TMA concentrations for HSPH samples treated with olive leaves

Tukey HSD[a]

sample	N	Subset for alpha = 0.05
		1
OL9	2	5.0400
OL3	2	5.8800
OL4	2	5.8800
OL1	2	6.7200
OL2	2	6.7200
OL5	2	6.7200
OL6	2	6.7200
OL7	2	6.7200
OL8	2	7.5600
Sig.		.619

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

Appendix 5: Linear Regression Analysis Between Variables of Deodorisation Treatment and pH Drop in HSPH Post-deodorisation

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.692[a]	.478	.410	.76058	.478	7.027	3	23	.002

a. Predictors: (Constant), concentration, temperature, time

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	12.194	3	4.065	7.027	.002[b]
	Residual	13.305	23	.578		
	Total	25.499	26			

a. Dependent Variable: pH

b. Predictors: (Constant), concentration, temperature, time

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B		Collinearity Statistics	
		B	Std. Error	Beta			Lower Bound	Upper Bound	Tolerance	VIF
1	(Constant)	7.488	.591		12.665	.000	6.264	8.711		
	time	.036	.018	.306	2.030	.054	-.001	.073	1.000	1.000
	temperature	-.015	.006	-.376	-2.499	.020	-.027	-.003	1.000	1.000
	concentration	-16.274	4.972	-.493	-3.273	.003	-26.559	-5.988	1.000	1.000

a. Dependent Variable: pH

Appendix 6: The Correlation Between the Sensory Analysis and Trimethylamine Content

Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.476[a]	.226	.196	12.58881	.226	7.320	1	25	.012

a. Predictors: (Constant), TMAmean

b. Dependent Variable: FPsum

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	1160.118	1	1160.118	7.320	.012[b]
	Residual	3961.956	25	158.478		
	Total	5122.074	26			

a. Dependent Variable: FPsum

b. Predictors: (Constant), TMAmean

Appendix 7: Calibration Curve for Determination of Phenolic Content using Folin-Ciocalteu Method

The total phenolic contents in the treated HSPH samples were calculated from the regression equation of calibration curve as follows:

$$y = 0.0006x + 0.1511; R^2 = 0.9456$$

The total phenolic contents are expressed as mg gallic acid equivalent (GAE) per millilitre of hydrolysate (mg GAE/ml) as presented in Table 21 (Section 4.3.4).

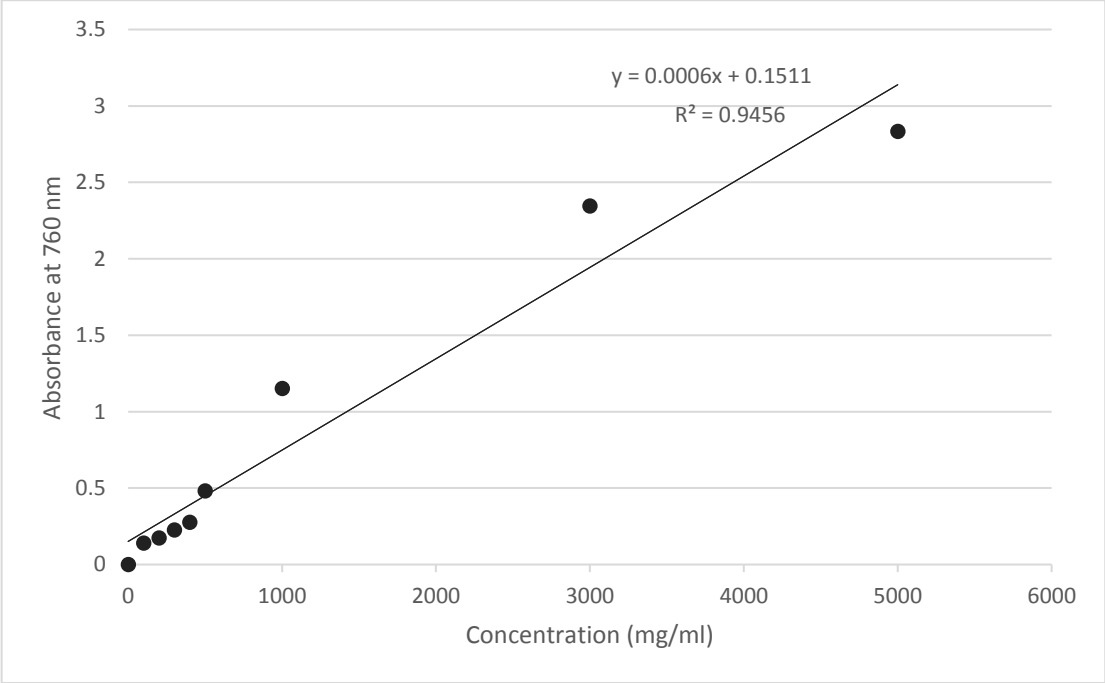

Figure 1: *The calibration curve for standard gallic acid.*

Appendix 8: Compositional analyses for Hoki Collagen Sample

Descriptives Analysis for Hoki Collagen

		N	Mean	Std. Deviation
TM	Citric acid	3	94.3959	.09552
	NaOH	3	94.3522	.43621
	NaCl	3	96.1810	.04801
	Total	9	94.9764	.93115
Proteindry	Citric acid	3	37.3801	.80569
	NaOH	3	52.3230	1.02109
	NaCl	3	11.2716	1.15952
	Total	9	33.6566	32.09722
TVBN	Citric acid	3	7.0000	.48497
	NaOH	3	5.0400	1.68000
	NaCl	3	5.0400	1.68000
	Total	9	5.6933	1.55897
TMA	Citric acid	3	1.6800	.00000
	NaOH	3	1.1200	.48497
	NaCl	3	1.1200	.48497
	Total	9	1.3067	.44272
collagenyield	Citric acid	3	76.8123	2.93972
	NaOH	3	25.0578	4.10576
	NaCl	3	1.7548	.22295
	Total	9	34.5416	33.36595
pH	Citric acid	3	2.3333	.00577
	NaOH	3	11.8767	.24214
	NaCl	3	7.1500	.03000
	Total	9	7.1200	.13425

Total Moisture (%)

Tukey HSD[a]

Sample	N	Subset for alpha = 0.05	
		1	2
NaOH	3	94.3522	
Citric acid	3	94.3959	
NaCl	3		96.1810
Sig.		.977	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Total protein (g protein/100 g dry sample)

Tukey HSD[a]

Sample	N	Subset for alpha = 0.05		
		1	2	3
NaCl	3	11.2716		
Citric acid	3		37.3801	
NaOH	3			52.3230
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

TVBN

Tukey HSD[a]

Sample	N	Subset for alpha = 0.05
		1
NaOH	3	5.0400
NaCl	3	5.0400
Citric acid	3	7.0000
Sig.		.275

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

TMA

Tukey HSD[a]

Sample	N	Subset for alpha = 0.05
		1
NaOH	3	1.1200
NaCl	3	1.1200
Citric acid	3	1.6800
Sig.		.269

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Collagen yield (%)

Tukey HSD[a]

Sample	N	Subset for alpha = 0.05		
		1	2	3
NaCl	3	1.7548		
NaOH	3		25.0578	
Citric acid	3			76.8123
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix 9: Compositional analyses for Hoki Gelatine Sample

Descriptives

		N	Mean	Std. Deviation
TM	NaCl	3	99.4416	.12963
	NaCl at 100C	3	99.0873	.10817
	Boiled	3	99.7675	.01036
	NaOh	3	99.3768	.09353
	Citric acid	3	98.8505	.15924
	Total	15	99.3047	.33812
proteindry	NaCl	3	40.4612	.88270
	NaCl at 100C	3	5.7479	.98626
	Boiled	3	10.3970	1.01580
	NaOh	3	41.2808	.88159
	Citric acid	3	20.2960	.98721
	Total	15	23.6320	32.40996
gelatineyield	NaCl	3	23.4491	1.08804
	NaCl at 100C	3	37.5858	2.79476
	Boiled	3	7.5425	.32539
	NaOh	3	60.9382	1.74431
	Citric acid	3	83.0537	4.96604
	Total	15	42.5139	27.84480
TMA	NaCl	3	.9067	.11547
	NaCl at 100C	3	.9728	.11505
	Boiled	3	.9067	.11547
	NaOh	3	.9067	.11547
	Citric acid	3	.9067	.11547
	Total	15	.9199	.10130
pH	NaCl	3	6.8533	.07506
	NaCl at 100C	3	6.9067	.03055
	Boiled	3	7.5900	.07810
	NaOh	3	11.7733	.02082
	Citric acid	3	2.5567	.00577
	Total	15	7.1360	3.02923
TVBN	NaCl	3	7.1833	.04933
	NaCl at 100C	3	4.1067	.08963
	Boiled	3	4.0600	.04583
	NaOh	3	9.0667	.03786
	Citric acid	3	8.6700	.13229
	Total	15	6.6173	2.23914

Total Moisture (%)

Tukey HSD[a]

sample	N	Subset for alpha = 0.05			
		1	2	3	4
Citric acid	3	98.8505			
NaCl at 100C	3	99.0873	99.0873		
NaOh	3		99.3768	99.3768	
NaCl	3			99.4416	
Boiled	3				99.7675
Sig.		.146	.061	.950	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Total protein (g protein/100 g dry sample)

Tukey HSD[a]

sample	N	Subset for alpha = 0.05				
		1	2	3	4	5
NaCl at 100C	3	9.7479				
Boiled	3		21.3970			
Citric acid	3			36.2960		
NaCl	3				76.4612	
NaOh	3					90.2808
Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

TVBN

Tukey HSD[a]

sample	N	Subset for alpha = 0.05			
		1	2	3	4
Boiled	3	4.0600			
NaCl at 100C	3	4.1067			
NaCl	3		7.1833		
Citric acid	3			8.6700	
NaOh	3				9.0667
Sig.		.947	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

TMA

Tukey HSD[a]

sample	N	Subset for alpha = 0.05
		1
NaCl	3	.9067
Boiled	3	.9067
NaOh	3	.9067
Citric acid	3	.9067
NaCl at 100C	3	.9728
Sig.		.951

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Gelatin yield (%)

Tukey HSD[a]

sample	N	Subset for alpha = 0.05				
		1	2	3	4	5
Boiled	3	7.5425				
NaCl	3		23.4491			
NaCl at 100C	3			37.5858		
NaOh	3				60.9382	
Citric acid	3					83.0537
Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

pH

Tukey HSD[a]

sample	N	Subset for alpha = 0.05			
		1	2	3	4
Citric acid	3	2.5567			
NaCl	3		6.8533		
NaCl at 100C	3		6.9067		
Boiled	3			7.5900	
NaOh	3				11.7733
Sig.		1.000	.711	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.