The development of extruded meat alternatives using Maillard-reacted beef bone hydrolysate and plant proteins

A thesis presented in partial fulfilment of the requirements for the degree of

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ABSTRACT

This research thesis aimed to process beef bone extract into a flavoursome protein ingredient to be added to extruded meat analogues to form meat alternatives and study their impact on the structural, textural, and sensory properties of meat alternatives. The thesis consists of three main parts. In the first part, two methods namely enzymatic hydrolysis and Maillard reaction (MR) treatments were evaluated for their suitability of modifying the flavour character of beef bone extract to become flavoursome protein ingredients. The second part studied the effects of soy protein concentrate (SPC) to wheat gluten (WG) ratio as a way of improving the structural and textural properties of current extruded meat analogues. The third part studied the effects of flavoursome protein ingredient (i.e. Maillard-reacted beef bone hydrolysate) with plant proteins on extruded meat alternatives. It also investigated the effects of moisture contents on extruded meat alternatives and their application in sausages.

To begin, an experimental study on the effects of enzymatic hydrolysis treatments (i.e. single, simultaneous and sequential) on the physicochemical properties of beef bone extract using Protamex®, bromelain, and Flavourzyme® was conducted. Next, the changes in the physicochemical properties and volatile compounds of beef bone hydrolysates during heat treatment as a result of the MR were investigated. Beef bone hydrolysates were combined with ribose in aqueous solutions and heated at 113°C to produce Maillard reaction products (MRPs). Results showed that Flavourzyme® was the most effective in increasing the proportion of low *Mw* peptides, reducing viscosity and enhancing the flavour intensity of beef bone extract. Concurrently, the effects of SPC to WG ratio at a constant mass of SPC and WG on the physicochemical properties of extruded meat analogues were studied. Meat analogues containing 30%WG showed the highest degree of texturisation, fibrous structure, hardness and chewiness using instrumental and sensory analysis.

For the third part of this research thesis, the effects of flavoursome protein ingredient (i.e. Flavourzyme®-MRP) at different concentrations (0, 10, 20, 30 and 40% wet weight) with plant proteins on extruded meat alternatives were investigated. Meat alternatives containing 20%MRP obtained the highest sensory scores for appearance, meaty aroma, meaty taste, and overall

acceptability. Results showed that the addition of MRP with soy protein concentrate and wheat gluten to produce meat alternatives changed the textural, structural, and sensory properties significantly. The effects of moisture content (MC) on the physicochemical properties of extruded meat alternatives made from Flavourzyme®-MRP and plant proteins were studied. Samples were extruded at different dry feed rate of 1.8, 2.2, 2.6 and 3.0 kg/h to obtain MC of 60%MC, 56%MC, 52%MC and 49%MC, respectively. Meat alternatives at 49%MC were the closest in terms of both textural and microstructural properties to reference sample, boiled chicken breast. Results showed that the change in MC as a process parameter played an important role in the formation of fibrous structure in extruded meat alternatives. Lastly, the physicochemical properties of sausages made from extruded meat alternatives at different MC were conducted. Five sausages made from meat alternatives (S49%MC, S52%MC, S56%MC and S60%MC) and chicken breast (SCB) as a reference sample were prepared. Results showed that S49%MC had the highest sensory scores among all sausages made from meat alternatives. However, SCB obtained the highest sensory scores for all attributes except for appearance among all sausages at a 95% confidence level.

Overall, the present work demonstrated that a flavoursome protein ingredient (i.e. Flavourzyme®-MRP) from low-value meat by-product (i.e. beef bone extract) can be successfully incorporated into extruded meat analogues to form meat alternatives with high aroma and taste quality while maintaining fibrous structure. However, further work needs to be done to improve the textural and sensory properties of sausages made from extruded meat alternatives.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

%	Percent
°C	degree Celsius
2-ME	2-mercaptoethanol
AA	Amino acids
AAS	α-amino adipic semialdehyde
ACE	Angiotensin-converting enzyme
B	Bromelain
BCAA	Branched-chain amino acids
BE	Bone extract
BHA	Butylated hydroxyanisole
BSA	Bovine serum albumin
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
DH	Degree of hydrolysis
DNPH	2,4-dinitrophenylhydrazine
DoE	Design of Experiments
DSF	Defatted soy flour
DT	Degree of texturisation
DTNB	2,2'-dithio-5,5'-dithio-dibenzoic acid
DTT	1,4-dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
E/S	Enzyme-substrate
F	Flavourzyme®
FAA	Free amino acids
FD	Flavour dilution
FT-IR	Fourier-transform infrared spectroscopy
F_L	Lengthwise strength
F_V	Crosswise strength
GC	Gas chromatography
GC-O	Gas chromatography-olfactory

GC-MS	Gas chromatography-mass spectroscopy
GGS	γ-glutamic semialdehyde
GRAS	Generally Recognised as Safe
H-bonds	Hydrogen bonds
HAA	Heterocyclic aromatic amines
HPLC	High performance liquid chromatography
H&E	Haematoxylin and eosin
K_M	E/S ratio which the reaction rate was 50% of V_{max} after 120 min
kPa	kiloPascal
LM	Light microscopy
MC	Moisture content(s)
MFT	2-methyl-3-furanthiol
MR	Maillard reaction
MRP	Maillard reaction product
Mw	Molecular weight
NA	Not available
NTSB	2-nitro-5-thiosulfobenzoate
NTB	2-nitro-5-thiobenzoate
OAV	Odour activity value
OCT	Optimal cutting temperature
OD	Optical density
OPA	*Ortho*-phthaldialdehyde
P	Protamex®
PA	Papain
PB	Potassium phosphate buffer
PL	Lipase
PPI	Pea protein isolate
ppb	parts per billion
RTD	Residence time distribution
rpm	Revolutions per minute
RSM	Response Surface Methodology

SCB	Chicken breast sausage
SDS	Sodium dodecyl sulphate
SEC	Size exclusion chromatography
SEC-HPLC	Size exclusion chromatography-high performance liquid chromatography
SEM	Scanning electron microscopy
SH	Sulfhydryl
SME	Specific mechanical energy
SN-TCA	Soluble nitrogen after trichloroacetic acid
SPC	Soy protein concentrate
SPI	Soy protein isolate
SS	Single-screw
S-S bonds	Disulphide bonds
TNBS	2,4,6-trinitrobenzenesulfonic acid
TPA	Texture profile analysis
TS	Twin-screw
TU	Thiourea
TX	TritonX-100
U	Urea
UV	Ultraviolet
V	The degree of hydrolysis after 120 min
V_{max}	The maximum degree of hydrolysis after 120 min
WG	Wheat gluten
WS	Wheat starch

LIST OF PUBLICATIONS AND CONFERENCE PROCEEDINGS

(a) Publications

1. **Chiang, J.H.**, Hardacre, A.K., & Parker, M.E. (2020). Effects of Maillard-reacted beef bone hydrolysate on the physicochemical properties of extruded meat alternatives. *Journal of Food Science, 85(3), 567-575.* doi: https://doi.org/10.1111/1750-3841.14960

2. **Chiang, J.H.**, Hardacre, A.K., & Parker, M.E. (2020). Extruded meat alternatives made from Maillard-reacted beef bone hydrolysate and plant proteins. Part II - Application in sausages. *International Journal of Food Science and Technology, 55(3), 1207-1217.* doi: https://doi.org/10.1111/ijfs.14362

3. **Chiang, J.H.**, Hardacre, A.K., & Parker, M.E. (2020). Extruded meat alternatives made from Maillard-reacted beef bone hydrolysate and plant proteins. Part I - Effect of moisture content. *International Journal of Food Science and Technology, 55(2), 649-659.* doi: https://doi.org/10.1111/ijfs.14319

4. **Chiang, J.H.**, Eyres, G.T., Silcock, P.J., Hardacre, A.K., & Parker, M.E. (2019). Changes in the physicochemical properties and flavour compounds of beef bone hydrolysates after Maillard reaction. *Food Research International, 123, 642-649.* doi: https://doi.org/10.1016/j.foodres.2019.05.024

5. **Chiang, J.H.**, Loveday, S.M., Hardacre, A.K., & Parker, M.E. (2019). Effects of soy protein to wheat gluten ratio on the physicochemical properties of extruded meat analogues. *Food Structure, 19, 100102.* doi: https://doi.org/10.1016/j.foostr.2018.11.002

6. **Chiang, J.H.**, Loveday, S.M., Hardacre, A.K., & Parker, M.E. (2019). Effects of enzymatic hydrolysis treatments on the physicochemical properties of beef bone extract using endo- and exo-proteases. *International Journal of Food Science and Technology, 54(1), 111-120.* doi: https://doi.org/10.1111/ijfs.13911

(b) Conference proceedings

1. **Chiang, J.H.**, Hardacre, A. & Parker, M. (2019). *Effects of moisture contents on extruded meat alternatives made from Maillard-reacted beef bone hydrolysate and plant proteins*. Oral presentation presented at the meeting of the 13th International Congress on Engineering and Food (ICEF13). 23-26 September, Melbourne, Australia.

2. **Chiang, J.H.**, Hardacre, A. & Parker, M. (2019). *Interactions between Maillard-reacted beef bone hydrolysate and plant proteins on extruded meat alternatives.* Poster session presented at the meeting of the 8th International Symposium on Delivery of Functionality in Complex Food Systems. 7-10 July, Porto, Portugal.

3. **Chiang, J.H.**, Loveday, S., Eyres, G., Hardacre, A., & Parker, M. (2018). *Changes in meat flavour volatile profiles of beef bone hydrolysates during Maillard reaction.* Poster session and short paper presented at the meeting of the 64th International Congress of Meat Science and Technology (ICoMST). 12-17 August, Melbourne, Australia. http://icomst-proceedings.helsinki.fi/papers/2018_06_38.pdf

4. **Chiang, J.H.**, Parker, M., Loveday, S., & Hardacre, A. (2017). *Effects of enzymatic hydrolysis treatments on the physicochemical properties of beef mixed bone extract using endo- and exo-proteases.* Poster session presented at the meeting of the 7th International Symposium on Delivery of Functionality in Complex Food Systems. 5-8 November, Auckland, New Zealand.

5. **Chiang, J.H.**, Parker, M., Hardacre, A., & Loveday, S. (2017). *Effects of wheat gluten levels on the physicochemical properties of extruded soy-based meat analogues.* Poster session presented at the meeting of the 54th Annual New Zealand Institute of Food Science and Technology (NZIFST) Conference: Produced Locally - Consumed Globally - Shaping the Future. 4-6 July, Nelson, New Zealand.

6. Deb-Choudhury, S., Bah, C., **Chiang, J.H.**, Parker, M., & Day, L. (2017). *Protein hydrolysates from low-value meat processing streams as potential functional ingredients in textured meat analogues.* Oral presentation presented at the meeting of the 54th Annual New Zealand Institute of Food Science and Technology (NZIFST) Conference: Produced Locally - Consumed Globally - Shaping the Future. 4-6 July, Nelson, New Zealand.

Chapter 1: Introduction

1.1 Background information

Over 20 million animals are slaughtered annually in New Zealand, where low-value meat products such as waste meats for rendering, desinewed minced meats, mechanically separated meats and bones can be recovered from the animals' carcases. These products are rich in proteins, polyunsaturated fatty acids, calcium and iron (Henckel, Vyberg, Thode, & Hermansen, 2004; Mayer, Smith, Kropf, Marsden, & Milliken, 2007; Püssa et al., 2009). The ingredients make them attractive for a variety of applications in food product development. Hence, there is an interest in converting these low-value meat products into high-value functional ingredients through enzymatic hydrolysis and Maillard reaction (MR). The aim of this study was to develop flavoursome protein ingredients by converting commercial beef bone extract into soluble proteins and large peptides with optimal hydrolysis so that these peptides and amino acids are free to participate in MR, without damaging the nutritionally valuable amino acids.

In addition, the thesis also aimed to understand how animal proteins can be intimately associated with plant proteins to make structures simulating fibrous tissues. Therefore, these flavoursome protein ingredients were used as an ingredient in the production of extruded meat analogues to have enhanced flavour and meat-like texture. Meat analogues are a type of food product which are made from plant proteins. These meat analogues are supposed to resemble meat in terms of their appearance, taste, texture, and mouthfeel. However, current meat analogues exhibit very weak aroma and are almost tasteless which has resulted in limited market success. To date, meat analogues are not only consumed by vegetarians; but are also gaining popularity in the diet of flexitarians due to their nutritional benefits and potential to provide lower-cost protein alternatives to meat. A potential solution to the weak flavour of current meat analogues was to add a flavoursome protein ingredient to an analogue to improve the sensory properties of extruded meat alternatives while maintaining its fibrous texture.

The main aim of this research thesis was to process beef bone extract into a flavoursome protein ingredient to be added to extruded meat analogues to form meat alternatives. The impact

on the structural, textural and sensory properties of meat alternatives were investigated. Therefore, in this thesis, the development of flavoursome protein ingredients prepared using firstly enzymatic hydrolysis followed by MR was evaluated by incorporating the flavoursome protein ingredient to meat analogues through extrusion processing. A flow diagram of project overviews with research questions, aim and objectives are shown in **Figure 1-1**.

The objectives of the thesis were:

1. To investigate the effects of enzymatic hydrolysis treatments (i.e. single, simultaneous and sequential) on the physicochemical properties of beef bone extract using Protamex®, bromelain and Flavourzyme®.
2. To compare the effects of single and simultaneous enzymatic hydrolysis treatments using Protamex®, bromelain and Flavourzyme® on the physiochemical properties and flavour compounds of beef bone hydrolysates with ribose after MR.
3. To study the effects of soy protein concentrate (SPC) to wheat gluten (WG) ratio (89:0, 79:10. 69:20 and 59:30% w/w dry ingredient) on the physicochemical properties of extruded meat analogues.
4. To study the effects of Maillard-reacted beef bone hydrolysate at different concentrations (0, 10, 20, 30 and 40% wet weight basis) with plant proteins (a combination of SPC and WG) on the physicochemical properties of extruded meat alternatives.
5. To investigate the effects of moisture content (MC) on the physicochemical properties of extruded meat alternatives by varying the dry feed rate at a constant liquid feed rate.
6. To characterise the physicochemical properties of sausages made from extruded meat alternatives at different MC.

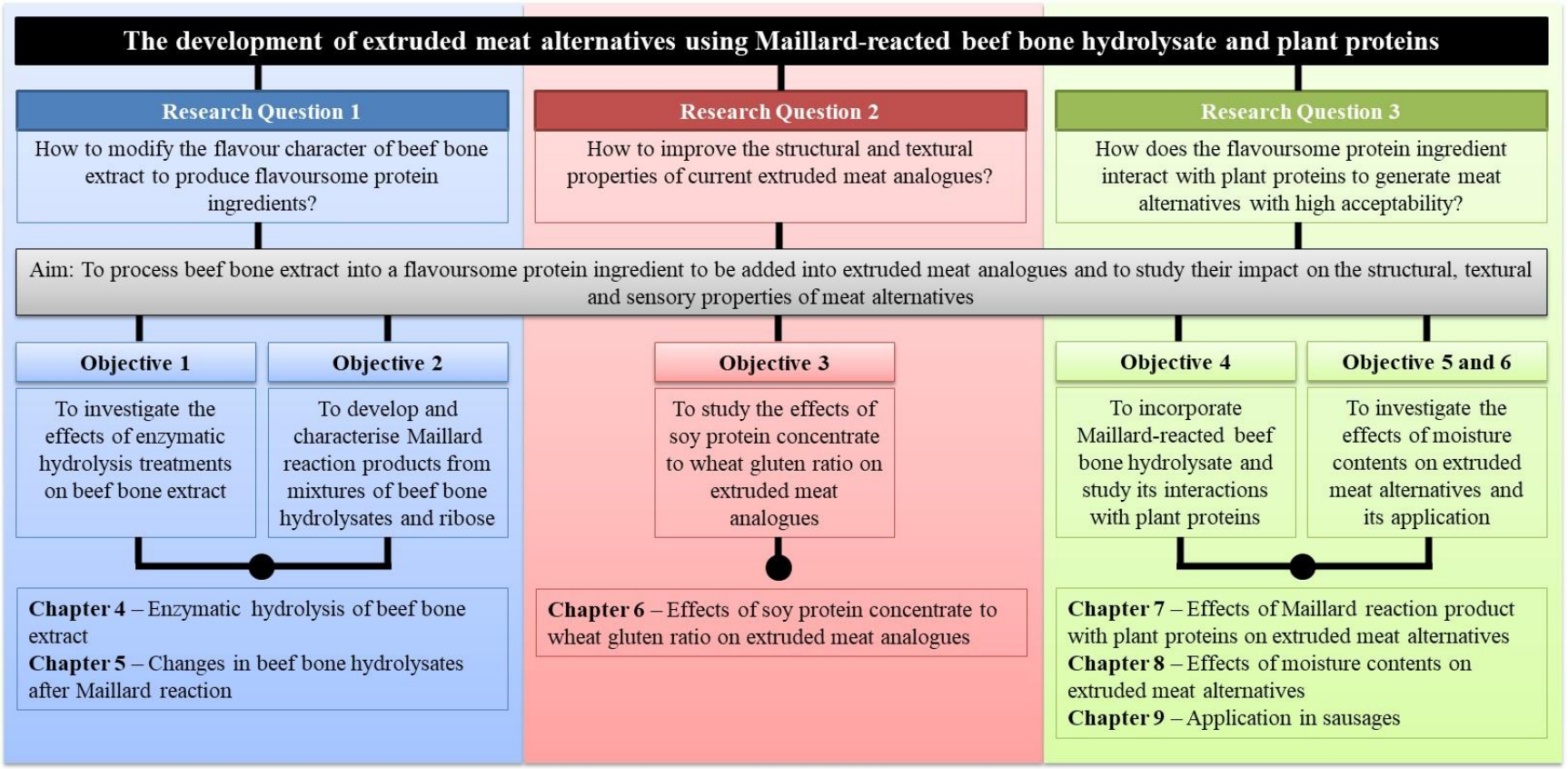

Figure 1-1 A diagram of thesis overviews with research questions, aim and objectives, and the chapters where they are addressed.

1.2 Overview of thesis

This study evaluated the incorporation of Maillard-reacted beef bone hydrolysate as part of the ingredients in extruded meat analogues to form meat alternatives. The thesis consists of ten chapters briefly described as follows:

Chapter 1 describes the rationale for developing this thesis and outlines the main framework of the research thesis. **Chapter 2** is a literature review of current methods used in developing meat flavours and meat analogues. **Chapter 3** discusses some major experimental techniques used in this research thesis. **Chapter 4** shows the enzymatic hydrolysis kinetics of Protamex®, bromelain and Flavourzyme® on beef bone extract using the Michaelis-Menten model. The hydrolysis efficiency between single, simultaneous, and sequential hydrolysis treatment on the three enzymes was studied and compared. **Chapter 5** compares the effects of single and simultaneous hydrolysis treatments on the physicochemical properties and flavour compounds of beef bone hydrolysates with ribose after MR. **Chapter 6** shows the effects of varying SPC to WG ratio on the physicochemical properties of extruded meat analogues. **Chapter 7** reports on the incorporation of Maillard-reacted beef bone hydrolysate (MRP) at different concentrations into meat analogues to form extruded meat alternatives. **Chapters 8 and 9** discuss the effects of MC on the physicochemical properties of extruded meat alternatives and their application in sausages. The overall conclusions of all experimental chapters and some directions for future work are presented in **Chapter 10**.

The thesis follows a logical flow from modifying the flavour character of beef bone extract using enzymatic hydrolysis and MR treatments, to finally incorporating Maillard-reacted beef bone hydrolysate in extruded meat analogues to form meat alternatives. The physicochemical properties of extruded meat alternatives were further characterised by varying its MC and made into sausages to determine consumers' acceptability (**Figure 1-2**).

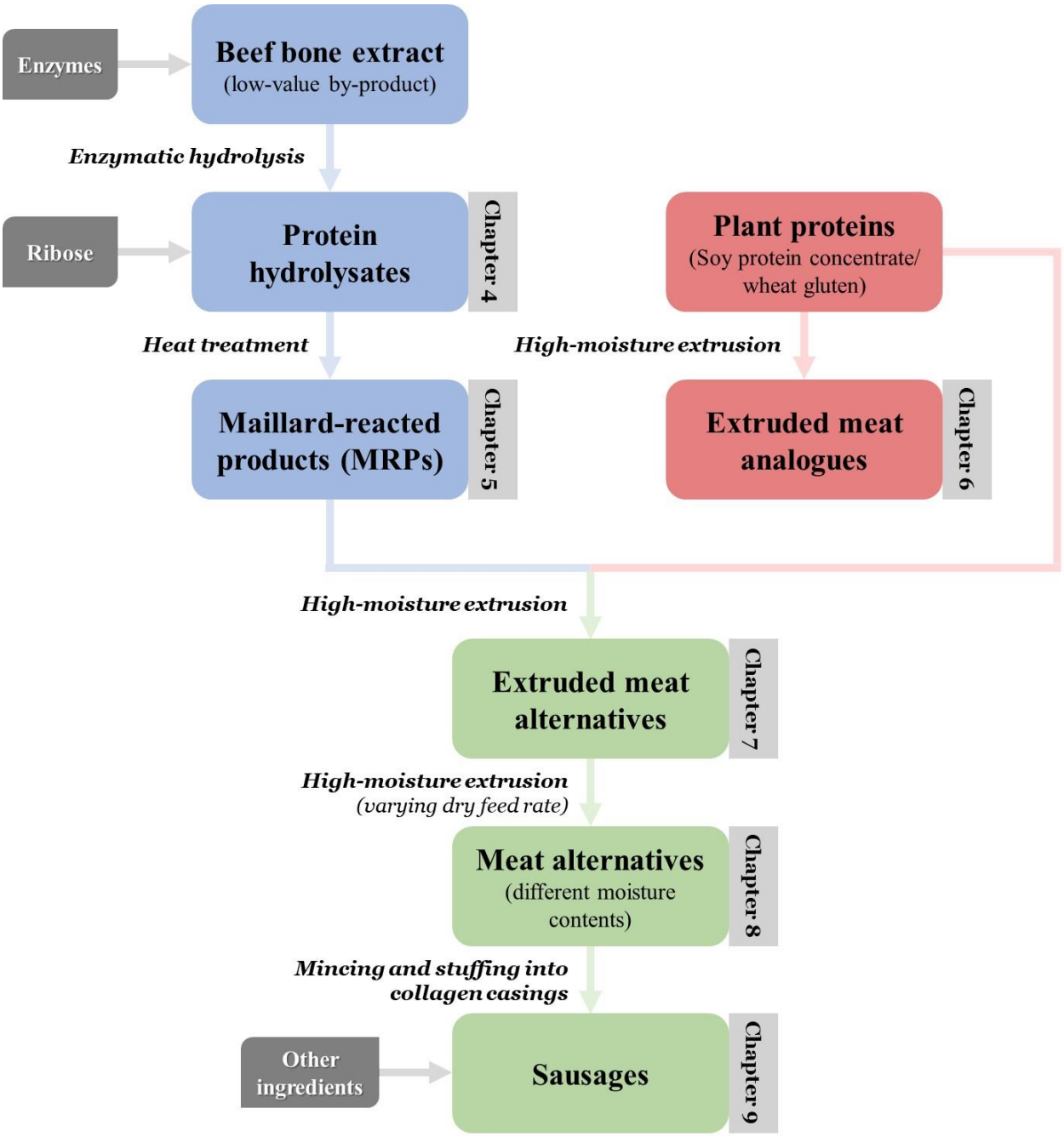

Figure 1-2 A flow diagram of showing an overview of the thesis with the links between each experimental chapter.

Chapter 2: Literature review

This literature review provides background information and previous studies on meat flavours and meat analogues. The first section covers enzymatic hydrolysis and MR of protein hydrolysates to generate meat flavours, while the second section is on meat analogues. This review aims to highlight recent advances and discuss the implications for the development of meat flavours and meat analogues. The knowledge gained through the literature review has been used to formulate the aim and objectives of this study.

2.1 Meat flavours

2.1.1 Introduction

Meat flavour is one of the most important attributes for consumers' eating quality and food purchasing decision (Mottram, 1994, 1998; Van Ba, Touseef, Jeong, & Hwang, 2012; Khan, Jo, & Tariq, 2015). Numerous research studies on meat flavour chemistry have resulted in the discovery of thousands of volatile compounds from meat or model systems consisting of meat ingredients (Bailey, 1994; Mottram, 1994, 1998). Meat flavour is a mixture of taste and aroma; however, sensory characteristics such as mouthfeel and juiciness of meat products also influence individual flavour perception and acceptability. Raw fresh meat has little aroma and metallic taste, and cooked meat aroma flavour is only developed after heat treatment. Primary precursors such as sugars (e.g. monosaccharides), nucleotides, amino acids (e.g. cysteine), peptides and lipids are responsible for desirable meat flavour compounds such as pyrazines and thioethers. MR, fermentation and lipid oxidation are the main reactions that convert these precursors into volatiles flavour compounds through heat degradation (Bailey, 1994; Sucan & Weerasinghe, 2005; Khan et al., 2015). The evaluation of the development of meat flavours has been the purpose of several studies in the literature. The general process starts with the generation of protein hydrolysates through enzymatic hydrolysis of animal or plant sources such as chicken breast meat, chicken bone extracts, beef bone protein, sheep bone protein, sunflower protein isolate and *Brassica napus* seed. These protein hydrolysates are then reacted with reducing sugars (e.g. ribose, xylose, glucose and fructose) with a heat treatment to generate meat flavours due to the MR (Guo, Tian, & Small, 2010;

Zhan, Tian, Zhang, & Wang, 2013; Sun et al., 2014; Karangwa et al., 2015; Liu, Liu, He, Song, & Chen, 2015; Song et al., 2016).

2.1.2 Enzymatic hydrolysis

Protein hydrolysis is often carried out to solubilise the protein source to improve its biological and nutritional value, to obtain hydrolysates of high-added value and commercial interest. Proteolysis cleaves the peptide bonds of proteins to produce free amino acids (FAA) and low molecular weight (*Mw*) peptides (**Figure 2-1**), through chemical or enzymatic reactions (Benjakul, Yarnpakdee, Senphan, Halldorsdottir, & Kristinsson, 2014; Villamil, Váquiro, & Solanilla, 2017). Acid, alkaline and enzymatic hydrolysis are some common processes used to hydrolyse proteins. Both acid and alkaline hydrolysis are difficult to control as they are performed at extreme temperatures (95-121°C) and pH (≤pH 1 or ≥pH 12.5), and generally yield products with reduced nutritional qualities (e.g. destruction of tryptophan) and poor functionality (e.g. limited to use as flavour enhancers) (Kristinsson & Rasco, 2000). Whereas enzymatic hydrolysis uses mild conditions and is easy to control. Enzymatic hydrolysis typically uses temperature in the range of 40 to 80°C. Specific enzymes cleave specific peptide bonds and can be selected to hydrolyse and solubilise proteins while maintaining the nutritional value (Villamil et al., 2017). Protein hydrolysates are commonly used as flavour enhancers, functional ingredients, or simply as nutritional additives to improve low protein quality foods.

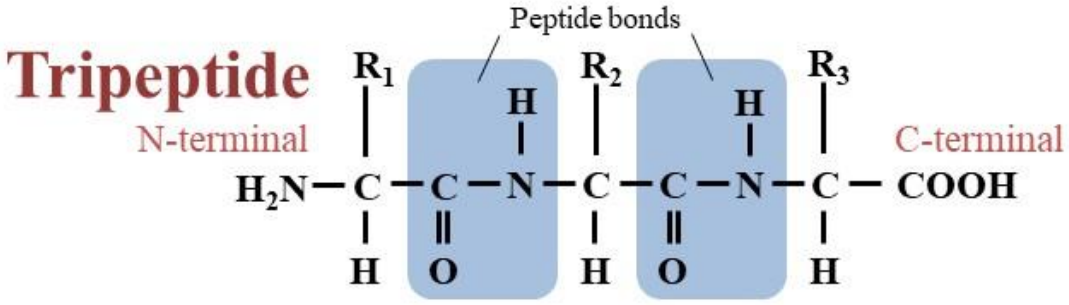

Figure 2-1 Illustration of a tripeptide with two peptide bonds showing N- and C-terminals.

Table 2-1 Proteases classified as Generally Recognised as Safe (GRAS), its temperature effects on enzyme activity, pH range of enzyme activity, and its strength in the hydrolysis of myofibrillar proteins and collagen (Calkins & Sullivan, 2007)

Protease	**Papain**	**Bromelain**	***Bacillus* protease**	**Aspartic protease**
Type	Vegetable	Vegetable	Bacterial	Fungal
Source	Papaya	Pineapple	*Bacillus*	*Aspergillus*
Protease class	Cysteine	Cysteine	Serine	Aspartic
Active temperature	50-80°C	50-80°C	50-65°C	40-60°C
Active pH	4.0-9.0	4.0-7.0	5.0-9.0	2.5-7.0
Hydrolysis of myofibrillar proteins	Excellent	Moderate	Poor	Moderate
Hydrolysis of collagen	Moderate	Excellent	Excellent	Poor

The characteristics of proteases from plant, bacterial and fungal sources are summarised in **Table 2-1**. Proteases are enzymes that catalyse the hydrolysis of proteins into smaller peptides and FAA. Proteases are classified as endo- or exo-proteases. Endo-proteases cleave the peptide bonds within protein molecules whilst exo-proteases hydrolyse peptide bonds from either the N or the C-terminal, respectively (**Figure 2-2**) (López-Otín & Bond, 2008; Benjakul et al., 2014). Proteases are also categorised into six different classes, namely, aspartic, glutamic, metallo, cysteine, serine, and threonine proteases, based on the mechanism of catalysis. For instance, cysteine, serine, and threonine proteases utilise an amino acid residue (Cys, Ser, or Thr, respectively) located in the active site from which the class name derives, as a nucleophile to attack the peptide bond of the substrate.

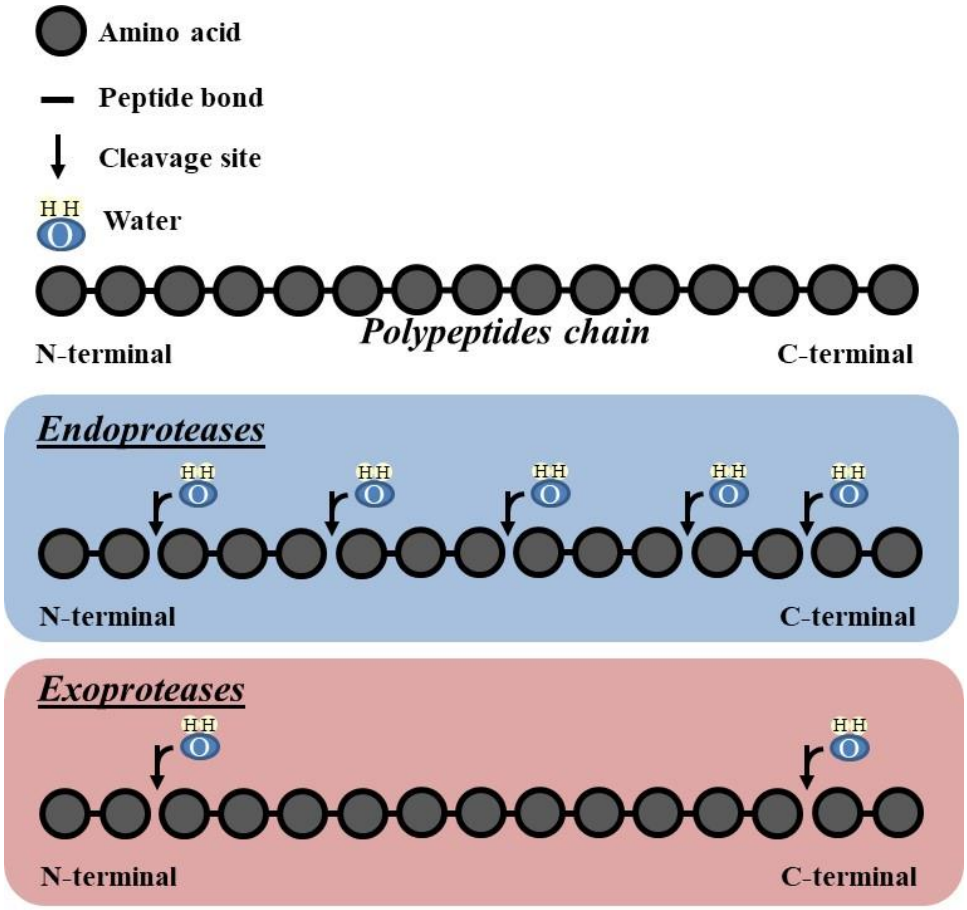

Figure 2-2 Enzymatic hydrolysis of proteins by endo- and exo-proteases (Benjakul et al., 2014).

Enzymatic hydrolysis is usually conducted in a reaction system with controlled temperature, pH, agitation and time (Villamil et al., 2017). The temperature and pH of the system are adjusted to optimise the working conditions of the enzyme. Upon adding the enzyme, the spontaneous reaction between the enzyme and substrate causes the pH of the mixture to change. This is due to the cleavage of peptide bonds, forming new amino or carbonyl groups. In some studies, the optimal pH of enzyme activity is maintained by the constant addition of neutralising solution during the hydrolysis process (Kurozawa, Park, & Hubinger, 2008; Nchienzia, Morawicki, & Gadang, 2010; Pagán, Ibarz, Falguera, & Benítez, 2013; Liu et al., 2015). At the end of the hydrolysis, the mixture is deactivated by changes in temperature, pH or both variables concurrently.

Table 2-2 List of meat protein sources, proteases and reaction systems' parameters used to obtain protein hydrolysates

Protein sources	Proteases	Parameters			References
		E/S[a]	Temperature	pH	
Chicken bone extracts	Protamex®	0.5% w/w	53±1°C	6.8±0.2	Sun et al. (2014)
Chicken bone extracts	Flavourzyme®	0.5% w/w	53±1°C	7.0±0.2	Dong et al. (2014)
Chicken breast meat	Alcalase®	4.2% w/w	52.5°C	8.0	Kurozawa et al. (2008)
Chicken breast meat	Protamex® (P) + Flavourzyme® (F)	900U/g (P) 200U/g (F)	55°C	6.5	Liu et al. (2015)
Poultry meal	Alcalase® (A) → Flavourzyme® (F)	0.25% v/v (A) 0.50% v/v (F)	50°C	8.0 (A) 7.0 (F)	Nchienzia et al. (2010)
Pig bones	Neutrase®	0.1-2.5% w/w	55°C	7.0	Pagán et al. (2013)
Beef bone protein	Papain	1.0% w/w	60°C	6.0	Song et al. (2016)
	Porcine pancreatic lipase (PL) → Papain (PA)	1.5% w/w (PL) 1.0% w/w (PA)	35°C (PL) 60°C (PA)	7.0 (PL) 6.0 (PA)	
	Lipase (L) → Papain (PA)	1.5% w/w (L) 1.0% w/w (PA)	35°C (L) 60°C (PA)	7.5 (L) 6.0 (PA)	
	Protamex®	1.0% w/w	40°C	6.5	
	Porcine pancreatic lipase (PL) → Protamex® (P)	1.5% w/w (PL) 1.0% w/w (P)	35°C (PL) 40°C (P)	7.0 (PL) 6.5 (P)	

[a] E/S denotes enzyme-substrate ratio.

There are numerous studies on optimising the hydrolysis conditions for meat proteins, which involved several different proteases such as Flavourzyme®, Alcalase®, Protamex®, Neutrase®, papain and lipase as shown in **Table 2-3** (Kurozawa et al., 2008; Nchienzia et al., 2010; Pagán et al., 2013; Dong et al., 2014; Liu et al., 2015; Song et al., 2016). The selection of proteases for hydrolysis is critical to the physicochemical (e.g. amino acid composition) and functional (e.g. solubility, emulsifying, etc.) properties of protein hydrolysates, as some enzymes have preferences for the cleavage of certain peptide bonds (Villamil et al., 2017). The choice of medium (e.g. acidic, alkaline or neutral pH) used for enzymatic hydrolysis is vital (Kristinsson & Rasco, 2000). For instance, acidic (e.g. aspartic and glutamic) proteases at its optimum pH can inhibit bacterial growth but have low protein recovery and decreased nutritional and functional value as compared

to alkaline and neutral proteases. Hence, bacterial proteases with high proteolytic activity are commonly used and are suitable to produce meat hydrolysates.

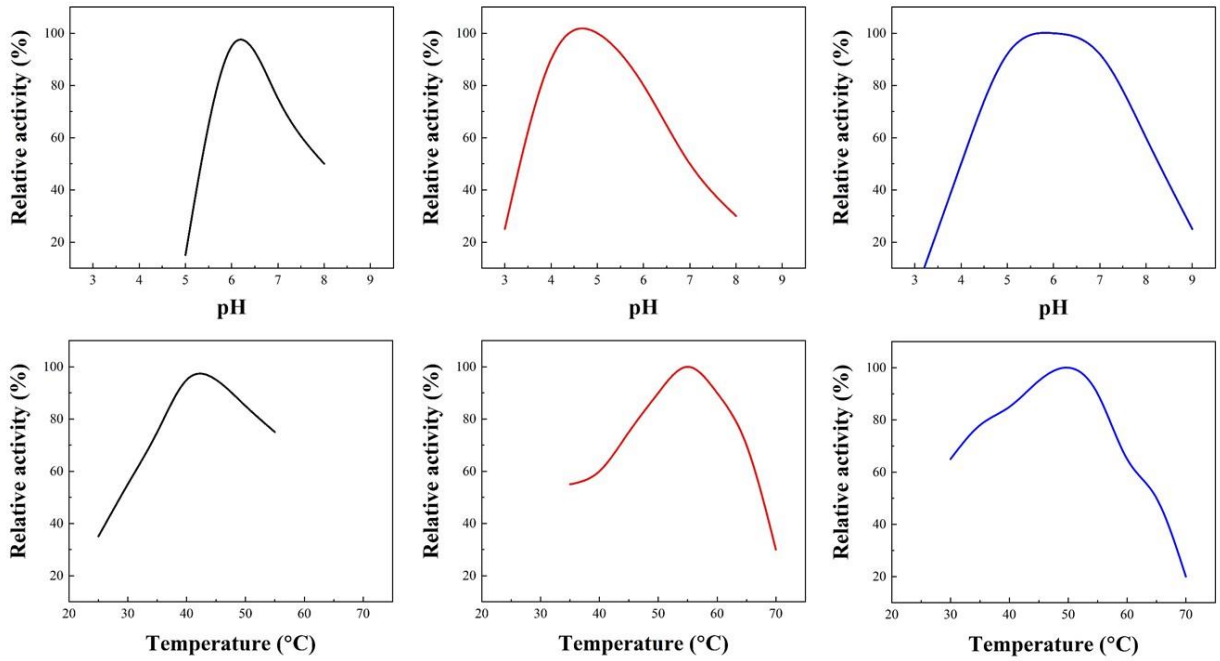

Figure 2-3 Effects of pH and temperature on the activity of Protamex® (—), bromelain (—) and Flavourzyme® (—) (obtained from product specification given by the suppliers).

Enzymatic hydrolysis is mostly influenced by factors such as choice of enzyme, hydrolysis conditions (e.g. pH and temperature), enzyme-substrate (E/S) ratio and reaction time (Benjakul et al., 2014). The choice of enzyme employed to hydrolyse a protein usually affects the physicochemical properties (e.g. degree of hydrolysis (DH), amino acid composition, *Mw* distribution, etc.) of the resulting hydrolysates. The extent of protein hydrolysis achieved within a specified time is dependent on the E/S ratio (Klompong, Benjakul, Kantachote, & Shahidi, 2007). The relationship between E/S ratio and enzyme activity is typically affected by pH and temperature (Linder, Fanni, Parmenter, Sergent, & Phan-Tan-Luu, 1995). Enzymatic hydrolysis of proteins is characterised by an initial rate of reaction where a large number of peptide bonds are hydrolysed. Subsequently, the rate of enzymatic hydrolysis decreases and reaches a stationary phase where no apparent hydrolysis takes place (Shahidi, Han, & Synowiecki, 1995). For instance, Kurozawa et al. (2008) used the Response Surface Methodology (RSM) design to optimise the enzymatic hydrolysis of chicken breast meat with commercial protease (i.e. Alcalase®). Factors such as temperature, pH and E/S ratio were used as independent variables, while the DH was one of the

dependent variables. It was reported that all three factors were significant with respect to the DH, where optimised conditions at 52.5°C, pH 8 and 4.2% w/w (E/S), with a hydrolysis duration of four hours, obtained a DH of 31% using the pH-stat method.

2.1.3 Maillard reaction

The MR, also known as Maillard browning or non-enzymatic browning, plays an important role in the development of volatile flavour compounds and the appearance of cooked food (Van Ba et al., 2012). The MR was discovered by French chemist Louis Maillard (1912) when he investigated the browning reaction between lysine and glucose. The MR takes place with the participation of primary precursors such as free amino compounds (e.g. amines, amino acids, peptides or proteins) and reducing sugars (e.g. ribose, xylose, glucose or fructose) at a specific heating condition to produce Maillard reaction products (MRPs) (Reineccius, 2005; Van Boekel, 2006; Van Ba et al., 2012). These precursors react during heating in primary reactions to form intermediate products. The intermediate products further react with other degradation products to form a complex combination of volatiles responsible for flavour, aroma, and dark-coloured pigment. The MR is usually divided into three main phases.

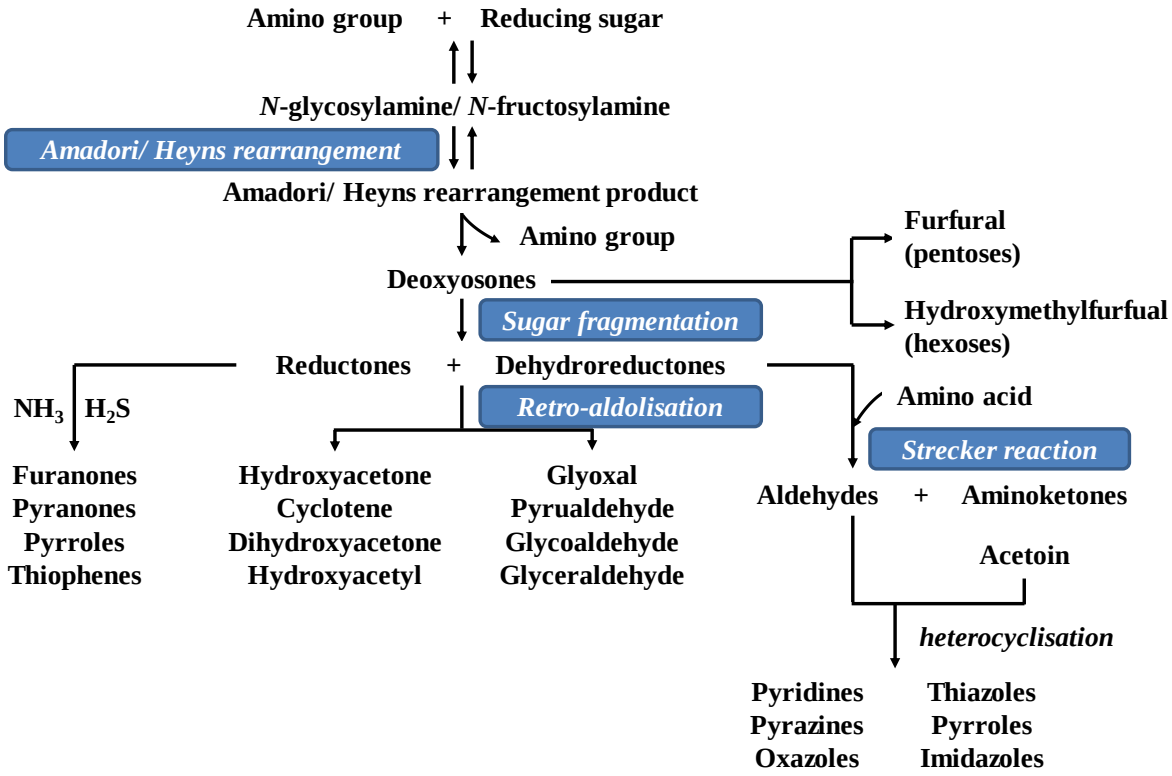

Figure 2-4 Overview of Maillard reaction showing the development of flavour compounds as end products (Van Boekel, 2006; Van Ba et al., 2012).

The initial phase starts with a reversible condensation between the carbonyl group of the α-amino group and reducing sugar (**Figure 2-4**). The rapid loss of water from the amino group produces an amine that can cyclise, resulting in the formation of an *N*-substituted glycosylamine (a sugar attached to NR_2 group). *N*-substituted glycosylamine is formed in the case of an aldose that rearranges into an Amadori product (e.g. 1-amino-1-deoxy-2-ketoses), while *N*-substituted fructosylamine or Heyns product (e.g. 2-amino-2-deoxy-aldoses) is formed if the reducing sugar is a ketose. The intermediate phase involves the rearrangement and decomposition of the Amadori/ Heyns product as the *N*-substituted glycosylamine/ fructosylamine is very unstable. The *N*-substituted glycosylamine/ fructosylamine will undergo a transition which leads to the release of the amino group and sugar fragmentation. The final phase of MR leads to dehydration, fragmentation, polymerisation and cyclisation reactions in which the amino groups take part once again (Bailey, 1994; Izydorczyk, 2005; Van Boekel, 2006; Van Ba et al., 2012).

Strecker degradation is a chemical reaction which converts the α-amino acid into an aldehyde containing the side chain. Strecker degradation is crucial in the context of flavour development, as amino acids are degraded via deamination and decarboxylation in the presence of dicarbonyl compounds that formed in the MR (Van Boekel, 2006; Van Ba et al., 2012). The various possible reaction pathways depend on temperature, pH and the nature of the reactants (e.g. reducing sugar, amino acid or protein). For proteins and peptides, the reactive amino group is the ε-amino group of lysine, because the α-amino groups are tied up in the peptide bond and are not available for MR or Strecker degradation. Thus, there are differences in the behaviour of amino acids compared to proteins and peptides.

2.1.4 Meat flavour development via Maillard reaction

The MR plays an important role in meat flavour development, along with caramelisation or lipid oxidation (Reineccius, 2005). For the MR, the most abundant flavour compounds formed are aliphatic aldehydes, ketones, diketones, and lower fatty acids. However, heterocyclic compounds containing oxygen, nitrogen, sulphur, or combinations of these atoms are much more numerous and play a significant role in the flavour development of thermally processed foods. The development of meat flavour is often influenced by reacting sulphur-containing amino acids (e.g. cysteine) with reducing sugars, where pentoses such as ribose or xylose are preferably used (Kerler, Winkel, Davidek, & Blank, 2010).

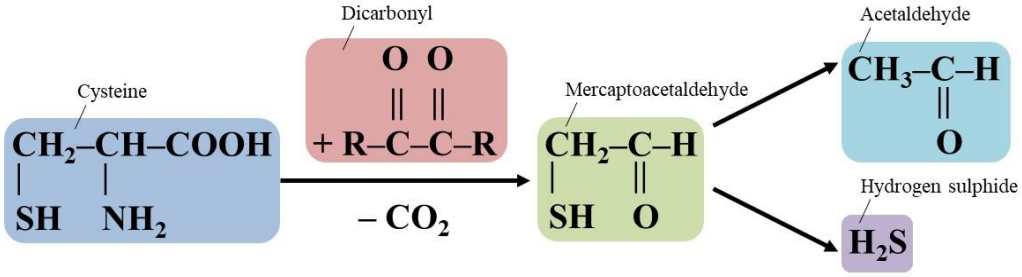

Figure 2-5 Primary products resulting from Strecker degradation of cysteine (de Roos, 1992).

The MR between cysteine and reducing sugars is believed to be the main pathway for the formation of meat flavour for most food products. The dicarbonyl compounds formed during the MR catalyse the Strecker degradation of cysteine to generate mercaptoacetaldehyde, acetaldehyde and hydrogen sulphide as the primary degradation products (**Figure 2-5**) (de Roos, 1992). These

Strecker degradation products then start a series of reactions that lead to the formation of meat flavour compounds. Most of the research on the formation of Maillard-based flavour compounds are based on mixtures of FAA and reducing sugars and with little research on protein-sugar or peptide-sugar mixtures (Van Boekel, 2006). Meat hydrolysates or meat extracts have been used as condiments to impart the same flavour as meat stock (Varavinit, Shobsngob, Bhidyachakorawat, & Suphantharika, 2000). The authors also highlighted that meat extracts impart inferior flavour and odour characteristics when compared with meat hydrolysates.

2.1.5 Factors influencing the Maillard reaction

Flavour development via MR largely depends on the reactants (e.g. nature of amino acids and reducing sugars), and the reaction conditions (e.g. pH, temperature and time) (Jousse, Jongen, Agterof, Russell, & Braat, 2002). The reactants, environment and heating conditions must be chosen wisely in order to produce the desired flavour, as the pathways leading to flavour are very specific (Reineccius, 2005). However, the choice of sugar type is of minimal importance in determining flavour character while the choice of the amino acid is very important when making a processed flavour. The type of amino acids determine the kind of flavour compounds formed, for instance, sulphur-containing compounds are usually generated from MR between cysteine and ribose (Elmore, Campo, Enser, & Mottram, 2002; Cerny & Davidek, 2003). The reaction conditions influence the kinetics of flavour development by MR (Van Boekel, 2006). The following factors have been found to affect the reactivity rate of MR.

2.1.5.1 Reactants

Both amino acids and reducing sugars influence the rate of the MR. In general, the reducing sugar has less influence on the sensory quality of the final flavour than the amino acids (de Roos, 1992; Kerler et al., 2010). Hence, the use of reducing sugars appears to be an attractive approach to enhance the rate of meat flavour development without compromising too much on flavour quality.

(a) Amino acids

Amino acid selection generally plays a much greater role in flavour character than the reducing sugar which may only have some influence (Reineccius, 2005). The possible range of

flavours that can be realised by changing the amino acid heated with glucose under acidic condition is listed in **Table 2-3**. Amino acids have two different roles during the MR. The first role is to promote the first step in the reaction (e.g. sugar-amino condensation), while the second role is to generate specific aromas via Strecker degradation (Parker, 2015). Cysteine, an important precursor of meat flavour, is often being used in precursor systems for the industrial production of meat process flavouring (de Roos, Wolswinkel, & Sipma, 2005). Meat flavour development in these systems is usually based on the MR of cysteine as the favoured amino acid by heating with reducing sugars such as ribose at 100°C to produce compounds with meaty and roast beef flavour description (Lane & Nursten, 1983; de Roos et al., 2005).

Table 2-3 Possible flavours generated from heating different amino acids with glucose under acidic conditions (Wong, Abdul Aziz, & Mohamed, 2008; Newton, Fairbanks, Golding, Andrewes, & Gerrard, 2012)

Amino acid	Odour generated on heating with glucose
Indispensable amino acid	
Arginine	Bitter, sour, fruity
Histidine	Sour
Isoleucine	Burnt, caramel
Leucine	Burnt, caramel
Lysine	Pleasant/ sweet, caramel, cardboard, herbal tea
Methionine	Potatoes, prawn crackers
Cysteine	Sulphur, meaty
Phenylalanine	Flowery, almond, bitter
Tyrosine	Fruity, flowery, tea-like
Threonine	Sweet, fruity, astringent
Valine	Caramel, biscuit, malty, chocolate, bitter
Non-indispensable amino acid	
Alanine	Fruity, flowery, sweet
Aspartic acid	Fruity, sweet
Glutamic acid	Sour
Glycine	Caramel, sweet, flowery
Proline	Fruity, bitter
Serine	Fruity, sweet

In a study on meat flavour generation in Maillard complex model systems using xylose, glucose, cysteine, glycine and glutamic acid, Martins, Leussink, Rosing, Desclaux, and Boucon (2010) reported that the formation of sulphur-containing meat flavour compound such as 2-methyl-3-furanthiol (MFT) was highly dependent on cysteine content of the protein. The authors explained

that 0.05% w/v of cysteine in a pH 6 buffer heated at 100°C for 3 hours generated only ~10 ppb of MFT, while 0.75% w/v of cysteine in similar condition generated ~410 ppb of MFT. The authors also reported that synergistic effects occurred between cysteine and glutamic acid. The sensory profile of the product changed from burnt, roasted meat to bouillon-like, boiled meat, when glutamic acid was added to cysteine and xylose buffer.

(b) Reducing sugars

Reducing sugars are essential in the initial phase of the MR. The main role of reducing sugars is to supply precursors for flavour formation but using a different type of reducing sugar can produce subtle differences in flavour (Parker, 2015). The rate of the initial phase depends on the kinetics of the sugar ring opening. The order of reactivity is greater for aldopentoses than for aldohexoses and relatively low for reducing disaccharides (Izydorczyk, 2005). The rate of reaction is influenced by sugar composition as follows: pentoses ($C_5H_{10}O_5$, e.g. ribose, xylose or arabinose) > hexoses ($C_6H_{12}O_6$, e.g. glucose or fructose) > disaccharides ($C_{12}H_{22}O_{11}$, e.g. maltose or lactose) > trisaccharides ($C_{20}H_{35}O_{16}$) > maltodextrins ($C_{6n}H_{(10n+2)}O_{(5n+1)}$) > starches ($(C_6H_{10}O_5)_n$), where smaller sugar molecules react at a faster rate (Reineccius, 2005). Ribose, a pentose sugar, which is known to be associated with ribonucleotides in meat muscle, is highly involved in MR during thermal processing of meat flavour (Jayasena, Ahn, Nam, & Jo, 2013).

In a study to assess the ability of reducing sugars to induce the carbonylation of myofibrillar proteins of porcine meat through the Maillard pathway, Villaverde and Estévez (2013) reported ribose as the most reactive reducing sugar as it generates the highest amount of α-amino adipic semialdehyde (AAS) and γ-glutamic semialdehyde (GGS), followed by fructose, glucose, galactose, maltose and lactose. The authors explained that the rate of the MR is usually dependent on the number of carbon atoms and the chemical nature of the reducing carbonyl moiety of the carbohydrate. The reaction is generally faster and more intense when the reacting sugar is small. Therefore, ribose was expected to yield more dicarbonyl compounds than hexoses and disaccharides, which was eventually reflected in a more effective formation of protein semialdehydes.

2.1.5.2 pH

The pH is also known to influence the reaction rate of specific Maillard pathways by changing the balance of volatiles formed. Most of the steps within the MR are sensitive to pH, and small changes in pH can alter the aroma profile of the final product (Parker, 2015). In general, carboxylic acids formed during the MR, which lead to a decrease in pH in the final product (Newton et al., 2012). Maillard browning is mostly favoured by high pH (e.g. maximum at pH 10) based on browning intensity and gas chromatography-mass spectrometry (GC-MS) analysis, where the pH affects flavour by influencing the yields of the various flavour compounds in different ways (Ashoor & Zent, 1984; de Roos, 1992). However, amino acids such as lysine, alanine and arginine reacted with glucose or fructose were only studied. Meat flavours are preferably prepared at low pH of 4.0-5.5, whereas roast and caramel flavours are obtained under neutral or slightly basic conditions (Ames, 1990; Kerler et al., 2010), where a large number of sulphur-containing compounds like MFT and 2-furfurylthiol form at lower pH condition (i.e. pH 3-4) (Madruga & Mottram, 1995; Van Ba et al., 2012).

In a study on the generation of meat-like flavourings from a mixture solution of protein hydrolysates of *Brassica sp.* with cysteine and xylose in reaction vessels between 100 and 180°C, Guo et al. (2010) reported that MRPs were strongly affected by pH (between pH 4 and 8). The authors stated that the number of volatile compounds obtained at pH 4 was greater than at pH 6 and pH 8, at high temperatures (i.e. 160 and 180°C). Based on the effects of pH and reaction temperature, the authors reported that flavour generated at 160°C and pH 4 had more roasted meat aroma characteristics and these were strongly preferred, while flavour generated at 180°C and pH 8 produced burnt odour which was the least preferred. The results correlated well with literature that meat flavours are preferably prepared at low pH. In another study on the formation of volatile compounds in meat-related model systems by Meynier and Mottram (1995), the effects of pH (i.e. 4.5, 5.0, 5.5, 6.0 and 6.5) on the cysteine-ribose model system was studied. It was reported that the MRPs were strongly affected by pH. The authors described the cysteine-ribose model system as strongly sulphurous and unpleasant at pH 4.5 but became more roasted meat-like and less sulphurous at higher pH values. Pyrazines such as methylpyrazine and dimethylpyrazine in the cysteine-ribose model system were only detected at pH 6.0 and 6.5. This indicates that the control of pH is crucial for MR in foods and model systems.

2.1.5.3 Temperature and time

Both the reaction temperature and time are known to have a significant impact on the formation of flavour through the MR systems (de Roos, 1992; Izydorczyk, 2005). Achieving a temperature (above 100°C) considerably impacts the initiation of the MR. Reineccius (2005) observed that stewed meat lacked the flavour characteristics of a roasted product as measured by sensory analysis. This is because the stewed product has a water activity of approximately 1.0 and never exceeds a temperature of 100°C. In contrast, roasting at 175°C on the same product causes drying of the surface, resulting in a surface water activity less than 1.0. Therefore, the surface temperature rises above 100°C. This indicates that lower water activity and higher surface temperatures favour the production of flavour compounds giving the product roasted notes from the same basic reactants. As for processing time, increasing the reaction time enables a greater extent of reaction and is one of the methods to get more of the flavour product in many situations. By increasing the reaction time of a MR does not necessarily increase the flavour intensity of the product but it changes the final balance of flavour compounds and thereby changes the flavour character indicating there may be an optimum time for the desired flavour (Reineccius, 2005).

In a study on the effect of thermal treatment on the flavour generation from the MR of xylose and chicken peptides dissolved in deionised water, Liu et al. (2015) used reaction temperatures of 80, 100, 120 and 140°C for 30, 60, 90 and 120 min in a high-pressure stainless steel reactor. Based on the sensory evaluation results, it was reported that the flavour of the Maillard reacted products were strongly affected by reaction temperature and time. The intensity of basic meaty and roast aroma was slowly increased with heating times at 80 and 100°C treatments. While, at 120°C, the aroma attributes increased rapidly along with the increase of heating time. The results demonstrated that the higher temperature (i.e. >100°C) could increase the formation and concentration of meat flavour volatile compounds. In a different study on the changes of flavour compounds of hydrolysed chicken bone extracts during MR in a high-pressure stainless steel reactor at 105°C, Sun et al. (2014) analysed the volatile compounds at the different heating time of 0, 30, 60 and 90 min. It was reported that pyrazines (i.e. 3-ethyl-2,5-dimethylpyrazine, 2,3-dimethyl-5-methylpyrazine and 3,5-dimethyl-2-methylpyrazine) detected in the MRPs increased in concentration from 0 to 90 min of MR. The authors also commented that the volatile compounds

of hydrolysed chicken bone extracts increased with longer heating times. This shows that heating time strongly influences the formation of meat flavour during the MR.

2.2 Meat analogues

2.2.1 Introduction

Meat analogues, also known as meat mimics, meat substitutes or imitation meats, are food products that are designed to have similar properties and sensory experiences to meat but are made from non-meat ingredients (Wild et al., 2014; Malav, Talukder, Gokulakrishnan, & Chand, 2015). The breakthrough in extruded chunked products with spongy meat-like structure was started in Western markets during the early 1960s at Wenger's research laboratory (Sadler, 2004; Strahm, 2005), but research on high-moisture meat analogues using extrusion processing only began in the early 1990s (Wild et al., 2014). Meat analogues resemble meat in terms of its aesthetic properties (i.e. structure, texture and appearance) and chemical characteristics (i.e. nutritional profile) (Strahm, 2005; Asgar, Fazilah, Huda, Bhat, & Karim, 2010; Kumar et al., 2017). Meat analogues also offer a cheaper alternative, that is nutritious with potential health benefits over meat (Malav et al., 2015). Plant proteins are currently used as the primary source of ingredients to produce meat analogues. Examples of such plant proteins include soy flour, soy proteins, modified defatted peanut flour, WG, pea protein, lupin protein, etc. (Manski, van der Goot, & Boom, 2007; Osen, Toelstede, Wild, Eisner, & Schweiggert-Weisz, 2014; Wild et al., 2014; Jones, 2016; Palanisamy, Franke, Berger, Heinz, & Töpfl, 2018a).

2.2.2 Preparation of meat analogues

The conventional development of meat analogues consists of two main steps, which are mixture preparation and chunk formation (Orcutt et al., 2006; Malav et al., 2015). The mixture is prepared either prior to extrusion or within the extruder by blending, chopping and emulsifying the proteins, fat, salts, and other ingredients to form a matrix of proteins that encapsulates the fat and the non-soluble components. The mixture is then heated under pressure inside an extruder as it travels in the direction of the screw. The pressure orients the protein chains and forms a three-dimensional network. The heat denatures the proteins and sets the matrix irreversibly, so the final chunk products retain their desired shape. Typical meat analogue ingredients and their functionalities are shown in **Table 2-4**.

Table 2-4 Typical meat analogues ingredients and their functionalities (Egbert & Borders, 2006)

Ingredient	Functionality	Usage level (%)
Water	Ingredient distribution, emulsification, juiciness	50-80
Textured vegetable proteins • *Soy flour, soy protein concentrate, wheat gluten, protein combinations such as soy and wheat*	Water binding, texture/ mouthfeel, appearance, protein fortification/ nutrition, source of insoluble fibre	10-25
Non-textured proteins • *Soy protein isolate, functional soy concentrate, wheat gluten, egg whites, whey proteins*	Water binding, emulsification, texture/ mouthfeel, protein fortification/ nutrition	4-20
Flavour/ spices	Flavour enhancement (i.e. salt), mask cereal notes	3-10
Fat/ oil	Flavour, texture/ mouthfeel, succulence, Maillard reaction/ browning	0-15
Binding agents • *Wheat gluten, egg whites, hydrocolloids, enzymes, starches*	Texture/ "bite," water binding, may contribute to fibre content	1-5
Colouring agents • *Caramel colours, malt extracts, beet powder, FD&C colours*	Appearance/ eye appeal	0-0.5

Extrusion processing will be used to produce meat analogues in this research thesis. Traditional meat analogues can be produced at lower (<35%, wet weight basis) MC with the use of a single-screw (SS) extruder. Fibrous and non-expanded high-moisture meat analogues can be produced at higher (>50%, wet weight basis) MC with the utilisation of a twin-screw (TS) extruder fitted with a long cooling die.

2.2.3 Characteristics of meat analogues

Meat analogues can be formulated to have protein, fat and MC that resemble whole muscle meats (i.e. chicken breast meat), which are lean or have a low-fat content (Lin, Huff, & Hsieh, 2002; Ranasinghesagara, Hsieh, & Yao, 2005). They possess striated, layered and anisotropic structures which are similar to whole muscle meats in terms of visual appearance and taste sensation. Meat analogues can be made into different shapes such as chunks, sheets, disks, patties, strips and others. They imitate coarse ground meat and other products that are available in various colours and sizes (Manski et al., 2007; Asgar et al., 2010) as listed in **Table 2-5**. Meat analogues

provide a high amount of protein, and design as low calorie/ high nutrient food products for the human diet (Riaz, 2004). They can be widely used in school luncheon program, hospital meals, nursing homes, prisons or by worldwide relief agencies if they have enhanced nutrients (i.e. vitamins and minerals).

Table 2-5 Possible applications of meat analogues (Riaz, 2004; Sadler, 2004; Wild et al., 2014; Malav et al., 2015)

Coarse ground meat analogues	Emulsified meat analogues	Loose-fill	Others
Hamburgers Steak Chicken patties Sausages Battered/ breaded nuggets Meatballs	Deli "meats" (sliced lunch meats/ meatloaves) Frankfurters/ hot dogs Spreads	Taco fillings Chilli mixes Sloppy Joe Stuffing Bacon bits	Casseroles Stews Sauces

2.2.4 Global meat analogues market

In recent years, there has been a decline in meat consumption per head of population due to the sustainability of farming, environmental and animal welfare issues, along with increasing meat prices (Euromonitor, 2011; Mintel, 2015). Meat analogues provide an alternative to meat and other food products. It was reported that the market for meat analogues in the US and the UK has boomed over the past decade, as consumers have moved away from meat-centred diets. There was an increase of 18.2% in the global retail sales of meat analogues from 2005 to 2010 as shown in **Table 2-6**. Of all variety of meat analogues, ready meals had the strongest growth (43.5% growth from 2005 to 2010), as they were developed by high profile manufacturers such as Kraft, Kellogg and Quorn Foods.

Table 2-6 Global retail sales (US$ million) of meat analogues by sector from 2005 to 2010 (Euromonitor, 2011)

Type of meat analogues	2005	2010	% growth
Soy-based frozen meat analogues	446	497	11.5
Soy-based meat analogues ready meals	161	231	43.5
Other soy-based meat analogues	71	105	48.4
Non soy-based meat analogues	331	357	7.8
Non soy-based meat analogues ready meals	14	19	33.1
TOTAL	1023	1209	18.2

2.2.4.1 Meat analogues market in New Zealand

In New Zealand, meat analogues products are usually sold in chilled, frozen or shelf-stable form (Euromonitor, 2017). The actual and forecast sales of meat analogues are shown in **Figure 2-6**. It indicates that the sales for all three types of meat analogues increased from 2012 to 2017, and it is expected to grow further from 2017 to 2022. However, it is observed that the sales for chilled meat analogues are more than three times that for frozen or shelf-stable meat analogues. This could be due to consumer desire for products perceived as "fresh", "natural" and of high quality (Sadler, 2004).

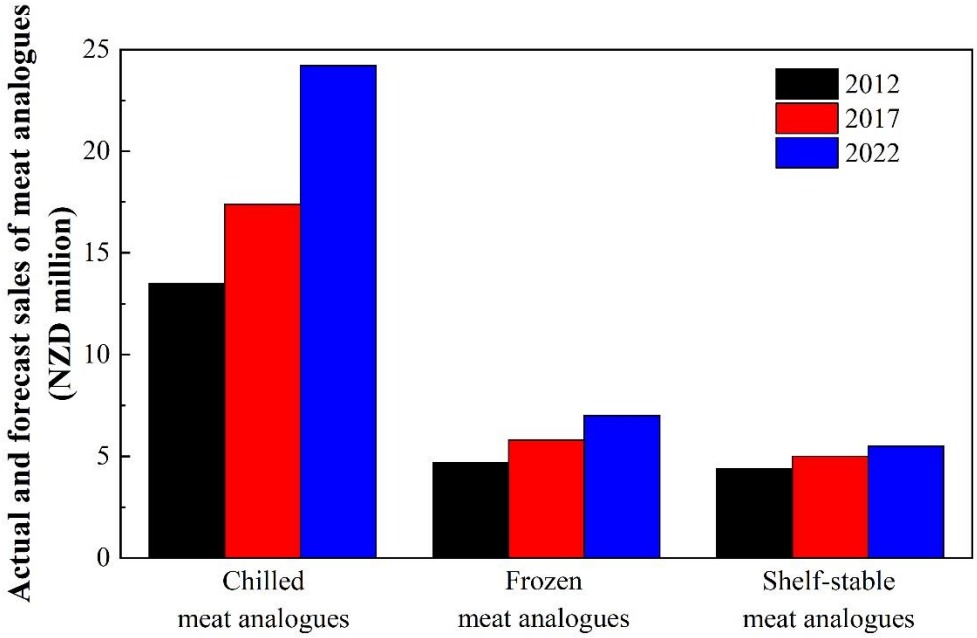

Figure 2-6 Actual and forecast sales (NZD million) of meat analogues in New Zealand from 2012 to 2022 (Euromonitor, 2017).

2.3 Extrusion processing

2.3.1 Introduction

Extrusion is defined as a process in which molten or dough-like material is formed and shaped by forcing the mixture through a restriction or die at a predetermined rate to produce various products (Riaz, 2013; Bouvier & Campanella, 2014; Alam, Kaur, Khaira, & Gupta, 2016). Rossen and Miller (1973) defined food extrusion as a process in which food material is forced to flow, under one or more of a variety of conditions of mixing, heating and shear, through a die which is designed to form and/ or puff-dry the ingredients. Food extrusion, introduced in the late

1950s, is an emerging and promising technology for the agri-food processing industries as it is possible to manufacture and market a large number of new and novel products of varying size, shape, texture, and taste as shown in **Figure 2-7** (Riaz, 2013; Alam et al., 2016). Extrusion processing is preferred over other conventional thermal processing (e.g. in-container sterilisation such as canning) as the operation is automated, continuous, versatile, energy-efficient, has a low operating cost, and has high capacity and productivity. In addition, extrusion processing can produce a broad range of high-quality finished products with minimum processing time using inexpensive ingredients.

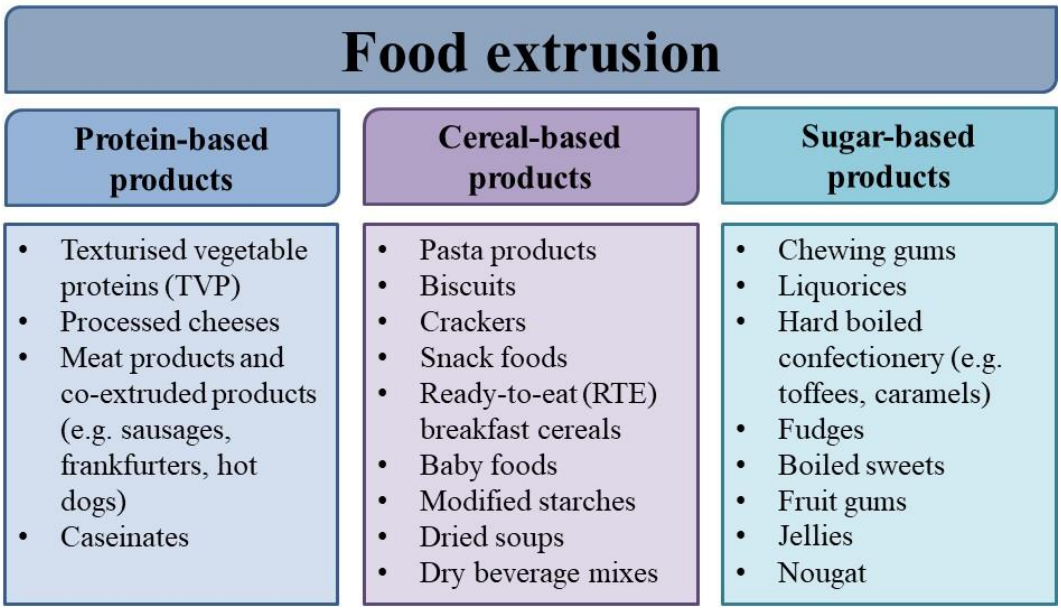

Figure 2-7 An example of products for human consumption produced using extrusion processing (Best, 1994; Fellows, 2009; Moscicki & van Zuilichem, 2011; Alam et al., 2016).

2.3.2 Principles of extrusion cooking

An extruder can be divided into five different components, which include (1) feeding system, (2) pre-conditioning system, (3) screws, (4) barrel, (5) die and cutting mechanism as shown in **Figure 2-8** (Steel, Schmiele, Leoro, Ferreira, & Chang, 2012; Riaz, 2013). At the beginning of an extrusion process, dry ingredients (~15-30%MC) are transported from the feeding system to pre-conditioning system. The role of the feeding system is to provide consistent and uniform feeding, by mixing the ingredients using a screw feeder. The ingredients are then pre-conditioned with water for hydration.

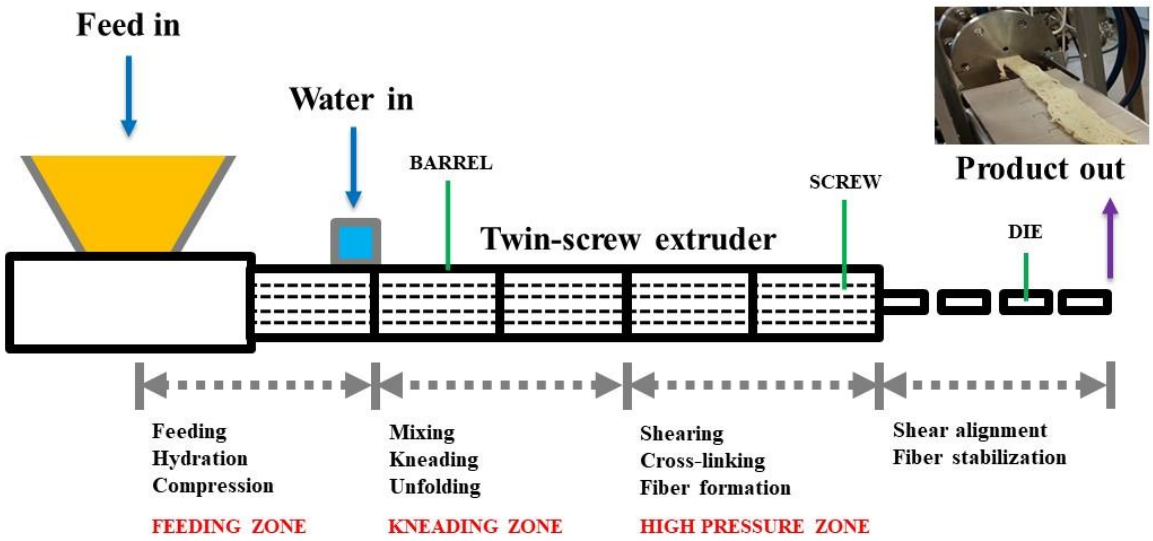

Figure 2-8 Schematic illustration of a twin-screw extruder for the production of high moisture meat analogues (Liu & Hsieh, 2008; Steel et al., 2012).

The material is then transported from the feeding zone to the kneading zone. In the kneading zone, the screw depth and pitch are reduced, increasing shear rate, temperature (110-180°C) and pressure (2026.5-3039.75 kPa). Under these conditions, solid material starts to convert into molten or dough-like material. The screw depth and pitch is even more reduced at the subsequent high-pressure zone, resulting in high shear and maximum heat generation causing the material to reach its maximum temperature and pressure. A reduction in apparent viscosity immediately before exiting the extruder. The material is expelled through the die under high pressure and expands to its final format when in contact with ambient pressure.

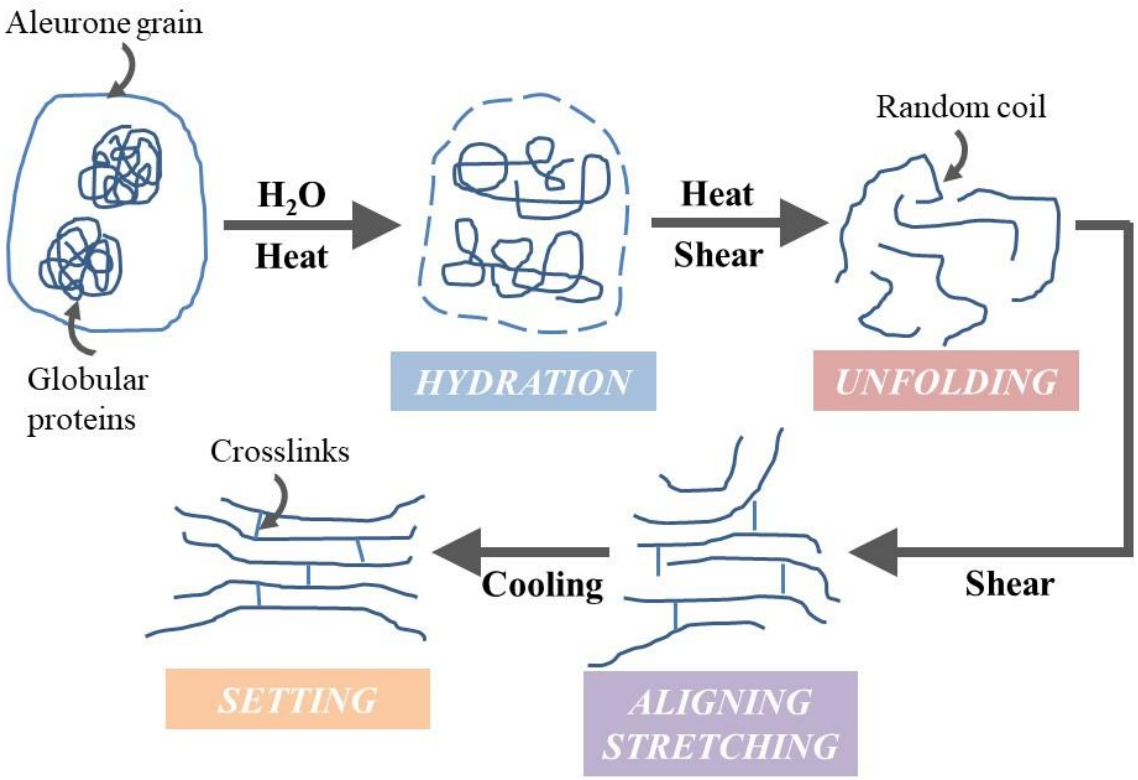

Figure 2-9 Schematic illustration of the mechanism of protein texturisation (Joshi & Kumar, 2015).

For high-moisture meat analogues, a long cooling die is attached at the extruder outlet to prevent the expansion of the meat analogue and to allow texturisation to take place (Cheftel, Kitagawa, & Queguiner, 1992; Lin, Huff, & Hsieh, 2000). After the material exits the extruder, a cutting machine is used to cut the product to the desired length. The shape of the die determines the cross-sectional size and shape (Steel et al., 2012; Riaz, 2013). Extrusion processing is widely used to achieve the restructuring of protein-based products such as meat analogues (Strahm, 2005). The macromolecules in the proteinaceous ingredients lose their native and organised structure when mechanical and thermal energy is applied during extrusion. This results in the formation of a continuous, viscoelastic mass. In addition, the extruder barrel, screws and die also align the molecules in the direction of flow, resulting in cross-linking and texturisation (**Figure 2-9**).

2.3.3 Twin-screw (TS) extruder

Extruders exist in a wide range of sizes, shapes and operation modes. Food extruders operated in HTST (high temperature, short time) processing method and are capable of carrying out cooking under high pressure (Moscicki & van Zuilichem, 2011). This is beneficial for

vulnerable food, as exposure to high temperatures for only a short time is sufficient to cause the necessary cooking reactions. Yet, the short time at high temperatures limits unwanted detrimental effects on the nutritional and functional properties of foods. Three major types of extruders are being used for food processing, screw extruders, roller-type extruders, and piston extruders (Steel et al., 2012; Alam et al., 2016). Screw extruders are most commonly used and can be categorised as SS extruders and TS extruders. SS extruders are the most conventional extruders used in the food industry, while TS extruders are usually used for high moisture extrusion, whereby the products have higher quantities of components such as fibre, fat, etc. The role of the screw in these extruders is to convey, compress, melt and plasticise the ingredients and force it under pressure through small die holes located at the barrel end (Moscicki & van Zuilichem, 2011).

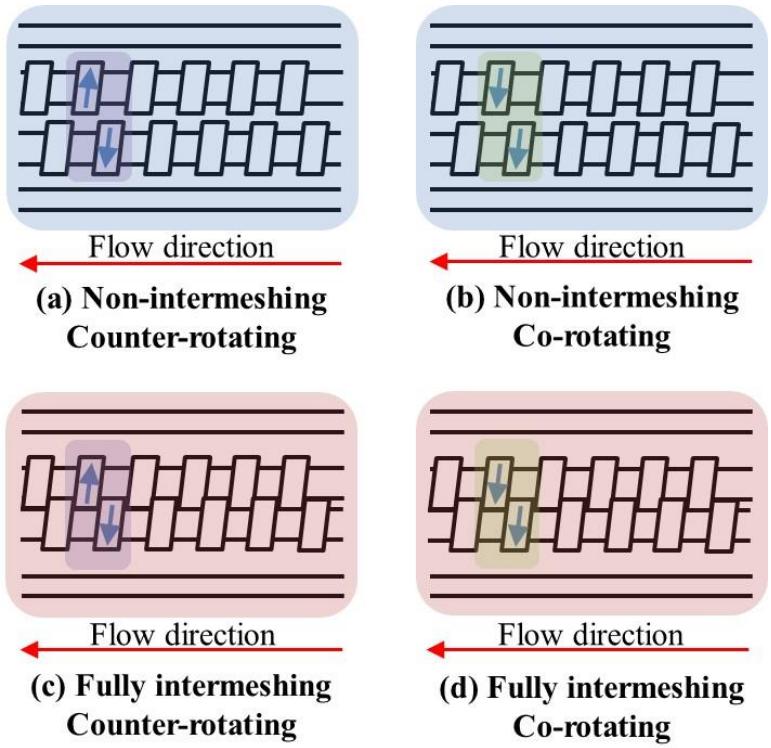

Figure 2-10 Basic screw configuration of twin-screw extruders (a) non-intermeshing, counter-rotating, (b) non-intermeshing, co-rotating, (c) fully intermeshing, counter-rotating, and (d) fully intermeshing, co-rotating (Bouvier & Campanella, 2014).

A TS extruder is comprised of two screws that rotate inside a single barrel. The internal surface of the barrel is usually smooth (Steel et al., 2012). TS extruders can be grouped according to their screw positions and screw-rotation direction (Fellows, 2009; Riaz, 2013; Bouvier &

Campanella, 2014). TS extruders can have either intermeshing screws where the flight of one screw penetrates the channels of the other screw or non-intermeshing screws where the screws do not engage each other's threads, allowing one screw to turn without interfering with the other. For screw-rotation direction, the screws can either rotate in the same direction with the screw crests (co-rotating) or opposite directions (counter-rotating). Hence, there are four different combinations of screw configurations, which are (1) non-intermeshing, co-rotating, (2) non-intermeshing, counter-rotating, (3) fully intermeshing, co-rotating, and (4) fully intermeshing, counter-rotating as shown in **Figure 2-10**.

The most common form of co-rotating TS extruder is the fully intermeshing, self-wiping style according to Frame (1994). The author reported that this type of extruder could be operated at higher screw speeds than counter-rotating TS extruders as the radial forces are more uniformly distributed. A fully intermeshing, co-rotating TS extruder (Clextral BC21, Firminy Cedex, France) with a long cooling die was used to produce high-moisture meat analogues in this research thesis. Several previous studies on meat analogues have been manufactured using this type of equipment (Lin et al., 2000, 2002; Yao, Liu, & Hsieh, 2004; Ranasinghesagara et al., 2005; Ranasinghesagara, Hsieh, & Yao, 2006; Liu & Hsieh, 2007, 2008; Chen, Wei, Zhang, & Ojokoh, 2010; Chen, Wei, & Zhang, 2011; Osen et al., 2014; Osen, Toelstede, Eisner, & Schweiggert-Weisz, 2015).

TS extruders offer numerous advantages as compared to SS extruders (Frame, 1994; Fellows, 2009; Riaz, 2013). TS extruders provide excellent mixing capability, due to the interpenetration of the screws and broad diversity of screw designs, which allow the extent of mixing to be precisely adjusted. TS extruders also have greater process flexibility and productivity, because it can handle a wider variety of ingredients or mix formulations which may be viscous, oily (18-27% fat), sticky (up to 40% sugar), or wet (up to 65% water). The positive pumping action of the screws in the TS extruder produces higher production rates and better mixing. This results in a narrower residence time distribution (RTD) and a smaller temperature gradient within the extruded mass. Hence, this leads to much better control of shear-time-temperature histories in the extruder and consistency in final product quality. TS extruder has less wear in the smaller part of the machine than in SS extruder (Riaz, 2013). However, the disadvantages of TS extruder include

a sophisticated design and higher cost of acquisition compared to SS extruder (Moscicki & van Zuilichem, 2011).

2.3.4 Variables and parameters involved in extrusion cooking

Food extrusion is a reactive process which involves several biochemical reactions such as protein denaturation, starch gelatinisation, etc. (Campanella, Li, Ross, & Okos, 2002). In the case of meat analogues, the aim of extrusion is to produce meat analogues which have appearance, texture and sensory properties that are similar to meat. The quality of meat analogues is commonly determined by measuring properties such as degree of texturisation, texture profile analysis (TPA), product morphologies and sensory characteristics such as hardness and chewiness (Lin et al., 2002; Fang, Zhang, & Wei, 2014; Grabowska et al., 2016). Degree of texturisation is used to indicate the fibrous structure formation of meat analogues, which is expressed as a ratio of lengthwise strength, F_L and crosswise strength, F_V, using a texture analyser. A high degree of texturisation is one of the main characteristics required from the extrusion process for meat analogues. TPA examines the chewing properties of meat analogues which correlates with the sensory evaluation. Product morphologies such as light microscopy (LM), scanning electron microscopy (SEM) and confocal scanning laser microscopy are used to investigate the fibrous microstructure of meat analogues.

A list of variables and parameters that can affect the extrusion process is shown in **Figure 2-11**. Variables such as ingredient characteristics and extruder operational conditions are regarded as *input* variables. Interactions among these input variables result in another group of variables, known as *process* variables, which include specific mechanical energy (SME), RTD, product temperature, and melt viscosity. These process variables will influence product quality such as texture properties, sensory properties, and product morphologies. These variables used to define the quality of extruded meat analogues can be regarded as *output* variables. It is noted that the extrusion process can be affected by external disturbances such as ambient conditions and equipment wear, particularly of the screws.

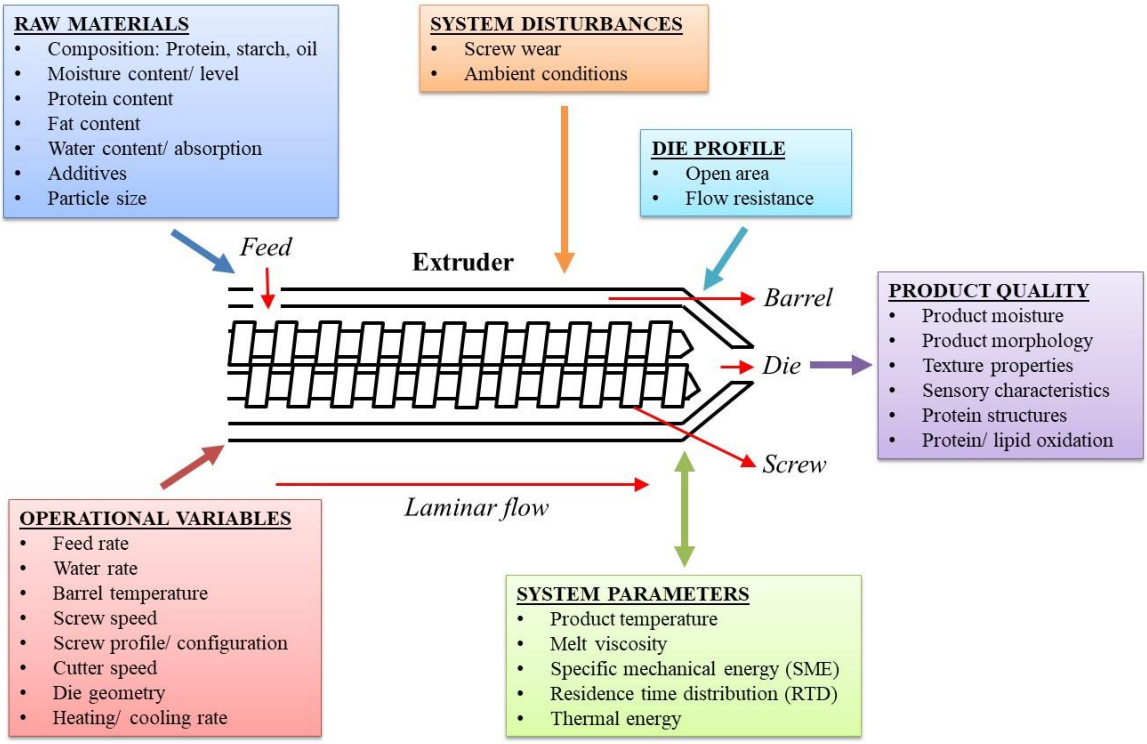

Figure 2-11 List of variables and parameters involved in extrusion processing (Chessari & Sellahewa, 2001; Campanella et al., 2002).

The numerous variables involved and the complex interactions that occur in extrusion processing make it difficult to establish relationships for researchers to understand the process and the effect of variables on product quality (Campanella et al., 2002). However, in many studies, the effect of several variables using Design of Experiments (DoE) has been widely used to account for the quality of finished products. Rehrah, Ahmedna, Goktepe, and Yu (2009) used RSM design to optimise the extrusion process for peanut-based meat analogues. Input variables such as protein content, moisture level, barrel temperature and screw speed were used to achieve optimised peanut-based meat analogues with the highest consumer acceptance. In another study, Chen et al. (2010) used a 5×3 factorial experimental design to investigate extrusion of soybean protein meat analogues using TS extruder at relatively wide moisture range spanning low moisture and high moisture (i.e. 28, 36, 44, 52, 60% wet weight basis). The authors studied the effect of MC and cooking temperature on system parameters (i.e. SME, RTD and in-line viscosity) and product properties (i.e. tensile strength, hardness, chewiness, and shear stress), and analysed their interrelationship. These experimental design approaches involve a large number of experiments which may be disadvantageous, but the approach has been widely used and accepted in extrusion

research. It was said that the relationship between input and output variables could be studied by defining the key variables and also establishing the basis of the interactions between these key variables and product quality (Campanella et al., 2002).

2.3.4.1 Factors influencing the extrusion process

As mentioned in the previous section, the changes in product quality of meat analogues depend on variables such as extruder type, screw configuration, feed MC, barrel temperature profile, screw speed, feed rate and die profile (Ding, Ainsworth, Plunkett, Tucker, & Marson, 2006). In this section, variables such as MC, extrusion temperature and screw speed are reviewed.

(a) Moisture content

MC has been widely studied for its effects on the product quality of meat analogues. It was reported that MC >50% is required for extrusion of high-moisture meat analogues to obtain fibrous structure (Cheftel et al., 1992).

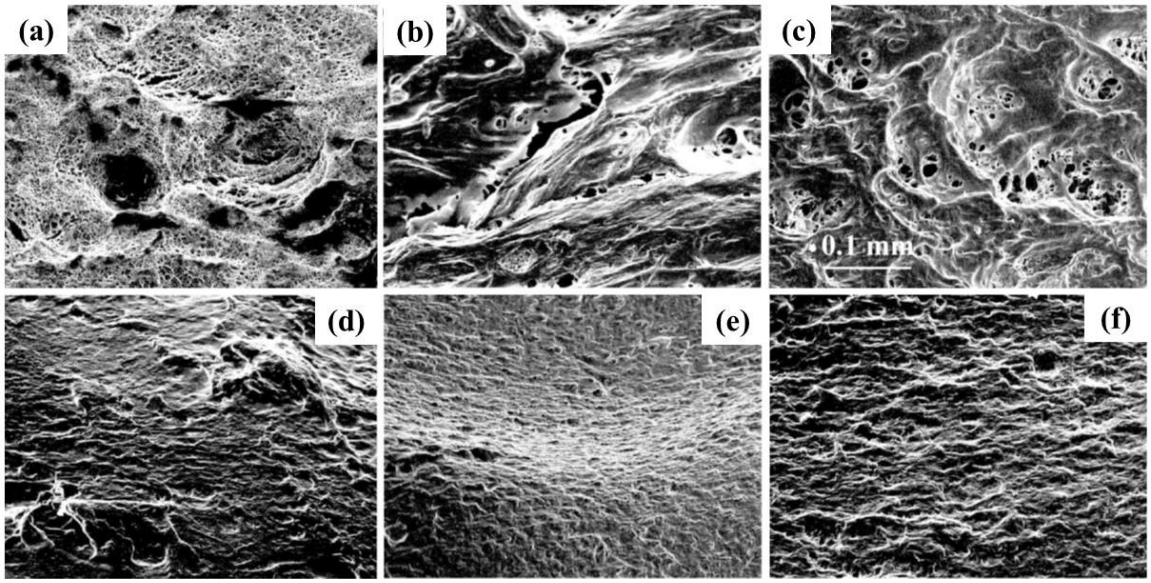

Figure 2-12 Scanning electron micrographs of samples extruded at 70% moisture at cooking temperatures of (a) 138°C, (b) 149°C and (c) 160°C, and 60% moisture at different cooking temperatures of (d) 138°C, (e) 149°C and (f) 160°C at 200× magnification. Reproduced with permission from John Wiley and Sons; Lin et al. (2002).

In a study on the effect of moisture (i.e. 60, 65, 70%) on the qualities (i.e. microstructure, texture and sensory properties) of soy protein isolate (SPI) meat analogues, Lin et al. (2002) found

that product extruded at 60% moisture gave the highest score on “layered” attribute in descriptive sensory analysis. The layered microstructure was observed at 60% moisture using SEM (see **Figure 2-12**), which corresponded with the sensory results. It appears the “layered” descriptor was considered as desired structure. In another study on the effect of water at different MC (i.e. 28, 36, 44, 52, 60%) on the quality (i.e. the degree of texturisation) of soybean protein meat analogues, Chen et al. (2010) reported that MC had significant effects on the degree of texturisation, where samples extruded at 60%MC had the best fibrous structure. Lastly, a study by Rehrah et al. (2009) on extruded peanut-based meat analogues, reported that fibrous texture was more apparent when the moisture level was about 55%, from the range of moisture levels between 40 and 60%. This indicates a MC of 55-60% is required to obtain fibrous structures in extruded meat analogues.

(b) Extrusion temperature

Extrusion temperature is one of the other important parameters which determine the quality of the extruded product. The barrel is segmented into different temperature-controlled zones within the extruder, and the temperature for each barrel zone can be adjusted by the extruder control system. The location of the thermocouple at each barrel zone may vary due to the design of the extruder. In some studies, the cooking phase of meat analogue production occurred in the middle or last zone of the extruder barrel (Lin et al., 2000, 2002; Chen et al., 2010; Osen et al., 2014). In a review paper, Cheftel et al. (1992) reported that a minimum temperature of 150°C was required to plasticise soy proteins at 60% moisture levels. The authors also stated that the barrel required a minimum of 140°C for fibre formation of soy proteins.

Figure 2-13 Digital image of extruded pea protein isolate of 55% moisture content at a cooking temperature of 160°C, exhibiting predominant lengthwise fibrous structures. Reproduced with permission from Elsevier; Osen et al. (2014).

In a study by Osen et al. (2014), the authors examined the product texture (e.g. texturisation of three types of pea protein isolates when heated to different extrusion barrel (last zone) temperatures (i.e. 100, 120, 140, 160°C)). The authors observed a soft dough-like texture without any fibrous structure at temperatures below 120°C due to incomplete melting and partial unravelling of macromolecules. At higher temperatures, samples displayed multi-layered structures with layers parallel to the die wall, and fine fibres appeared upon tearing. The authors proposed that the extra energy resulting from the increase in temperature caused the macromolecules to unravel, thus making bonding sites available for further crosslinking which were previously buried. The authors reported that the macrostructure of samples became more homogenous with a smooth surface when the cooking temperature was ≥160°C. Predominantly, lengthwise oriented fibres only appeared upon tearing as shown in **Figure 2-13**. In another study, Chen et al. (2010) evaluated the product quality (e.g. degree of texturisation) of soybean protein meat analogues when cooked at different middle barrel zone temperatures (i.e. 140, 150, 160°C). A high degree of texturisation was observed from response surface plot when samples with a 60%MC were extruded at cooking temperatures of 150 and 160°C. Lastly, a study by Lin et al. (2002) who investigated the effect of cooking temperatures for the last two-barrel zones (i.e. 138, 149, 160°C) on the quality of SPI meat analogues found that as the cooking temperature was increased from 138 to 160°C at 70%MC, the structure of the resulting samples became more organised, which was due to the increase in product temperature causing greater protein texturisation.

(c) Screw speed

Screw speed is another important factor that affects the product quality of meat analogues. Based on previous studies, the screw speeds used for developing meat analogues by TS extruder were reported to be between 150 and 250 rpm (Lin et al., 2000, 2002; Rareunrom, Tongta, & Yongsawatdigul, 2008; Chen et al., 2010; Chen et al., 2011; Osen et al., 2014; Osen et al., 2015). Studies on the effects of screw speed are found in the literature on developing snack foods. In a review paper by Cheftel et al. (1992), screw speed (not stated) affects the residence time of meat analogues inside the barrel. They reported that a minimum residence time of about 150 seconds was necessary for protein plastification. Rehrah et al. (2009) investigated the effects of extrusion parameters. Screw speed was one of the variables the group studied when developing peanut-based

meat analogues. A screw speed of 80-90 rpm was optimal to produce most fibrous meat analogues from the preliminarily tested range of 60-200 rpm.

2.3.5 Ingredients for extrusion to produce meat analogues

Soy proteins are widely used in the development of meat analogues (Lin et al., 2000, 2002; Rareunrom et al., 2008; Chen et al., 2010; Krintiras, Göbel, Van der Goot, & Stefanidis, 2015; Grabowska et al., 2016). Other sources of proteins such as pea, peanut and lupin have also been used to produce meat analogue (Rehrah et al., 2009; Osen et al., 2014; Palanisamy et al., 2018a; Zhang, Liu, Zhu, & Wang, 2018). Other ingredients that are reviewed in this section include WG and starch.

2.3.5.1 Soy proteins

Soy proteins are extracted from soybeans (*Glycine max*) of a leguminous plant related to peas, lentils, and peanuts (Asgar et al., 2010). The use of soy food around the world varies widely. For example, soymilk, tofu and fermented products are commonly consumed in Asia. Western countries consume soybeans in the form of refined soy protein ingredients that are used in food processing. In recent decades, soybeans have attracted the attention of the general public as an economical and high-quality plant protein source for the human diet. Furthermore, in 1999, the U.S. Food and Drug Administration (FDA) confirmed that 25g of soy protein a day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease in “Soy Protein Health Claim”. Soy protein products, such as defatted soy flour, SPC, and SPI, have been developed from whole soybeans.

Table 2-7 Amino acid composition (mg/g protein) of plant proteins from legumes and cereal grains (Day, 2013) and an average of four raw beef cuts consist of the hind shin, oyster blade, rump centre and striploin (Purchas, Wilkinson, Carruthers, & Jackson, 2014)

	Amino acid content (mg/g protein)			
	Soybean	**Pea**	**Wheat (grain)**	**Beef cuts**
Indispensable amino acid				
Histidine	26	25	24	36
Isoleucine	46	46	34	41
Leucine	79	73	69	75
Lysine	65	81	30	78
Methionine	13	10	16	32
Phenylalanine	50	49	47	38
Threonine	39	44	30	36
Tryptophan	13	10	11	10
Valine	49	51	46	44
Dispensable amino acid				
Alanine	43	44	57	51
Arginine	73	102	48	65
Aspartic acid [a]	119	118	51	81
Cysteine	13	12	26	12
Glutamic acid [a]	190	174	309	138
Glycine	42	44	41	54
Proline	56	42	103	32
Serine	52	47	48	35
Tyrosine	32	29	31	33

[a] Including asparagine and glutamine respectively.

Soybeans typically contain 35-40% protein, 15-20% fat, 30% carbohydrate, 10-30% moisture, and around 5% minerals and ash (Golbitz & Jordan, 2006; Day, 2013). Soybeans may vary in nutrient content based on their specific variety and growing conditions. Soybeans also contain the highest amount of protein of any legume or grain. Plant storage globulins are the major protein fraction in soybean, ranging between 40 and 80% of total soybean proteins (Day, 2013). It was reported that globulin proteins from plants contain relatively low levels of sulphur-containing amino acids such as cysteine and methionine. Soybean globulins consist of two major components, *β*-conglycinin and glycinin. *β*-conglycinin has a sedimentation coefficient (the rate of sedimentation of the molecule in a unit gravitational field) of 7S whereas glycinin has a sedimentation coefficient of 11S (greater sedimentation rate) which is the major protein in soybean. *β*-conglycinin is a trimeric protein composed of three subunits with a molecular mass ranging between 150 and 200 kDa, while glycinin is a hexameric protein with a molecular mass ranging

between 300 and 380 kDa. Soybean proteins contain all eight amino acids essential for human health. However, it has been reported that soy protein has a lower quality than many animal proteins (Golbitz & Jordan, 2006). The amino acid compositions of soybean, pea, wheat and average of four beef cuts are summarised in **Table 2-7**. Soy proteins receive scores of between 0.95 and 1.00 for *Protein Digestibility-Corrected Amino Acid Score* (PDCAAS), a routine assay for protein quality evaluation, while beef protein, casein, and egg white have a PDCAAS value of 0.92, 1.00 and 1.00, respectively (Singh, Kumar, Sabapathy, & Bawa, 2008; Day, 2013).

Table 2-8 Nutritional composition and favouring attributes of soy proteins on meat analogues (Malav et al., 2015)

Characteristics	Soy protein concentrate	Soy protein isolate
Nutritional composition (per 100g product)		
Total fat	1.8 g	3.3 g
Protein	70.2 g	87.4 g
Moisture	4.8 g	4.8 g
Ash	4.4 g	3.8 g
Carbohydrate	18.8 g	<1 g
Favouring attributes		
Flavour	Low	Low
Flatulence	No	No
Form/ shape	Granules or chunks	Fibres
Cost (dry basis)	Low	High
Recommended hydration level	3:1	4:1
Cost of hydrated protein	Low	High
Fat retention	High	Moderate
Optimum usage level in meat extension (% hydrated level)	30-50	35-50

SPC is comprised of 65-70% soy protein, with trace amounts of fat and 5-6% crude fibre (Golbitz & Jordan, 2006; Day, 2013). SPC is produced from defatted soy flakes that have been treated with either alcohol or water to remove the soluble sugars. The end-product is a concentrated form of soy flour with improved flavour and functional characteristics. Due to the alcohol washing step used to reduce the sugars, most of the isoflavones are removed during processing, though the quality of the protein is not reduced. It is also more easily digested than soy flour as most of the sugars responsible for creating flatulence are removed during the processing. SPI contains >85% protein and is produced by alkali extraction and isoelectric precipitation, to remove the fat, soluble sugars, insoluble sugars and dietary fibre. SPI is very low in flavour, highly digestible, and easy

to use in food, beverage, and baking formulations. They disperse easily in water and work well as emulsifiers, helping to bind water and fat together. Several favouring characteristics of soy proteins on meat analogues are shown in **Table 2-8**. SPC and SPI have been widely used in the production of extruded meat analogues throughout the years (Lin et al., 2000, 2002; Liu & Hsieh, 2007, 2008; Rareunrom et al., 2008; Chen et al., 2010; Fang et al., 2014). Cheftel et al. (1992) reported that SPC was easier to extrude and texturise compared to SPI under the same extrusion conditions. SPI exhibited homogenous structure while those that contained only SPC demonstrated an anisotropic structure with layers or coarse fibres in the direction of flow through the die. It was reported that the addition of WG to SPI enhanced the formation of fibrous structure. This confirmed the conclusion from previous studies from Lin et al. (2000) and Chen et al. (2010), as WG was mixed with SPI to form fibrous meat analogues during extrusion.

2.3.5.2 Wheat gluten

WG is the main storage protein in wheat (*Triticum* spp.) grains (Asgar et al., 2010; Day, 2013). It forms a cohesive, viscoelastic proteinaceous network when mixed with water and this has a unique ability to produce leavened products. WG contains a protein content of 75-80%, through simple physical separation of wheat flour. The gluten protein consists of gliadins and glutenins, which make up approximately 80% of the protein contained in wheat seed. Gliadins are monomeric proteins with intramolecular S-S bonds with low or medium *Mw*, while glutenins contain different polypeptides connected by intermolecular S-S bonds, with size ranging from about 500,000 to more than 10 million Da (Wieser, 2007) (**Figure 2-14**).

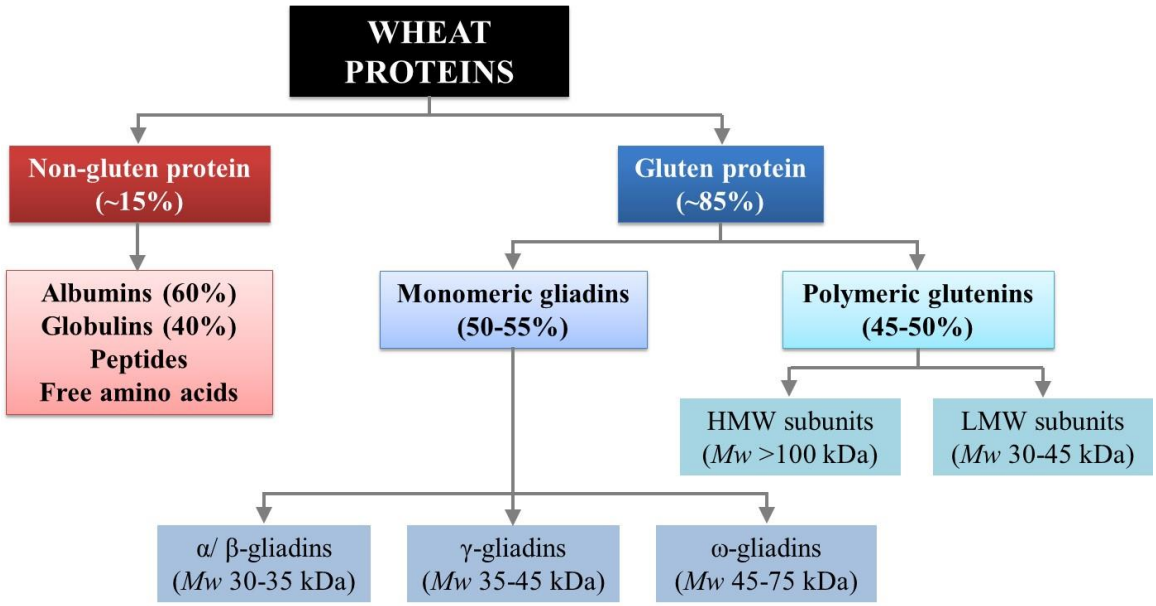

Figure 2-14 Composition and classification of wheat proteins (Day, 2011).

When WG is added as an ingredient in the manufacture of meat analogues, it has a supplementary role in holding the fibre together in the matrix for meat analogues (Rizvi, Blaisdell, & Harper, 1980), by serving as the main binding agent in the system to stick the product together and thus remain stable. Kumar, Sharma, Kumar, and Kumar (2012a) reported that with the increment of WG content from 10-18% in analogue meat nuggets, improved the texture and binding attributes. Harper and Clark (1979) reported that the presence of WG resulted in harder products, which was also observed by Ding et al. (2006), who reported that wheat-based extruded expanded snacks were harder than rice-based snacks.

2.3.5.3 Starch

Starch has a wide range of roles in a variety of foods such as binding and moisture retention in meat applications (Mason, 2009). Wheat starch (WS) is used as one of the ingredients for extruded meat analogues in many studies (Lin et al., 2000, 2002; Yao et al., 2004; Ranasinghesagara et al., 2005; Ranasinghesagara et al., 2006; Liu & Hsieh, 2007, 2008). WS comprises 54-72% of the dry weight of its kernels (Maningat, Seib, Bassi, Woo, & Lasater, 2009). It can help to improve the shelf-life of a product, for example providing freeze-thaw stability for frozen foods (Satin, 2014). WS has a bland flavour as compared to other cereal starches, and thus will not interfere with the desired end-product taste. It is useful when developing products that

make use on one of the many other properties of starch without influencing the product's overall taste. Maningat et al. (2009) stated that WS is added mainly to bind water in meat applications. The increase in WS level decreased the firmness of meat products due to the increase in water retention.

2.4 Gaps in the literature

Numerous studies on the enzymatic hydrolysis of meat, fish or plant proteins using different types of proteases have been conducted. Many of these studies have subsequently undergoing MR through heat treatment to form MRPs, also known as flavoursome protein ingredients. The outcome of these studies has provided good insights in the materials and methodologies used to generate these flavoursome protein ingredients. However, the gaps in the present literature are clear; and these are listed as follow:

1. Various studies on enzymatic hydrolysis have used either single, simultaneous, or sequential treatments to obtain protein hydrolysates. However, all of these studies have assessed the differences between two treatments such as single *vs*. simultaneous (Song et al., 2016) or single *vs.* sequential (Nchienzia et al., 2010; Liu, Zhu, Peng, Guo, & Zhou, 2016). Therefore, it is hard to justify which treatment is the most effective and efficient for producing desirable protein hydrolysates for the production of meat flavouring.

2. Several studies determined the hydrolysis kinetics and efficiency, for instance, by investigating the effect of E/S ratio (e.g. 0.1-2.5% w/w) on the DH at different hydrolysis duration (e.g. 0, 15, 30, 45, 60, 90 and 120 min) (Pagán et al., 2013). The technique that these authors used to determine the optimal E/S ratio is limited to the range of E/S ratio they set, which may not fully utilise the proteases. Thus, the hydrolysis kinetics and efficiency of the proteases may not be completely true.

3. Many studies used reflux in water bath, oil-water bath, or water bath systems as heat treatments for MR to produce flavoursome protein ingredients. However, these studies investigated extremely long times to produce MRPs. For example, reflux in water bath system took 2 or 4 hours (Varavinit et al., 2000), oil-water bath system used 1.5 to 2 hours (Karangwa et al., 2015;

Song et al., 2016), while water bath system utilised between 0 and 6 hours (Liu, Niu, Zhao, Han, & Kong, 2016).

Many studies have been carried out to investigate the development of extruded meat analogues using different protein sources, by either altering the composition of the raw materials (e.g. protein content) or by changing the operational variables (e.g. screw profiles, temperature, etc.) to generate meat analogues with fibrous meat-like structure. The outcome of these studies provided good insights on the impact of ingredients and extrusion operation of variables on meat analogues. However, the gaps in the present literature are clear, and these are listed as follow:

4. The main aim of developing meat analogues is to produce a meat alternative to real meat. However, there is no use of real meat (e.g. chicken breast) as a reference food to compare the non-meat analogues in terms of their textural, structural, and sensory properties.

5. Some studies have investigated the interactions between two different plant proteins (Liu & Hsieh, 2007, 2008; Zhang et al., 2018). However, there are no published studies completed on the interactions between meat and plant proteins on the development of meat alternatives with fibrous meat-like texture and natural meat flavour.

6. There have been many studies on the development of meat analogues using different types of plant proteins. In the literature, it was reported that meat analogues can be made into different types of food products (**Table 2-5**). However, there is a lack of published work using these meat analogues in food applications.

With all of the above, it is clear from the literature review that a systematic study is needed to compare the different enzymatic hydrolysis treatments, determine their hydrolysis kinetics and efficiency. MR should be used to process the flavour of the hydrolysates, which will be added into meat analogues to improve flavour. A reference food should be included to compare with meat analogues. Finally, the study should investigate interactions between meat and plant proteins in the production of meat alternatives that have the texture and flavour profile of real meat.

Chapter 3: Experimental techniques

3.1 Characterisation of meat hydrolysates/ flavours

3.1.1 Degree of hydrolysis

The DH is used to follow the reaction kinetics and get a measure for the extent of the hydrolytic degradation during enzymatic hydrolysis (Adler-Nissen, 1986; Kristinsson & Rasco, 2000). DH is defined as the proportion of the total number of peptide bonds that are cleaved during protein hydrolysis (Rutherfurd, 2010). There are several methods in the literature being developed and used to determine DH; such as pH-stat, *ortho*-phthaldialdehyde (OPA), 2,4,6-trinitrobenzenesulfonic acid (TNBS), formol titration, and soluble nitrogen after trichloroacetic acid precipitation (SN-TCA) methods. The pH-stat method is one of the simplest and most commonly used methods. It is based on the number of protons released during hydrolysis. The OPA, TNBS and formol titration methods are based on the measurement of free amino groups generated from hydrolysis. While the SN-TCA method measures the amount of TCA-soluble nitrogen, rather than DH in the protein hydrolysate. In this research thesis, the method used to determine the DH of protein hydrolysates after enzymatic hydrolysis was the OPA method. It is difficult to compare directly on the values of DH if the methods used to analyse the protein hydrolysates were different. The DH can only be compared by looking at the trend rather than comparing the actual values.

Table 2-2 summarised the results from a range of studies that had examined hydrolysis of a range of proteases using different enzymes. Two of these studies used the pH-stat method (Kurozawa et al., 2008; Pagán et al., 2013), one study used the TNBS method (Nchienzia et al., 2010), while three studies used the formol titration method (Dong et al., 2014; Sun et al., 2014; Song et al., 2016) to determine the DH of the meat protein hydrolysates. The pH-stat method is straightforward, eliminates derivatisation steps and allows real-time monitoring (Rutherfurd, 2010). However, the relationship between the DH and base consumption used to maintain the pH at the optimum for the enzymes is complex and may not be accurate for all proteins. The TNBS method directly determines the free N-terminal amino groups in a hydrolysate. Although the method is considered accurate, there is no real-time monitoring, it uses a toxic compound (e.g. 2-

mercaptoethanol, 2-ME), and there is interference with the ε-amino groups of lysine which leads to an overestimation of the DH (Rutherfurd, 2010). Formol titration method is a rapid and real-time monitoring method; however, it provides variable results depending on whether the direct or indirect methods are used. For the direct method, formaldehyde is added directly to the test solution which is then titrated with an alkali to end-point. While for the indirect method, the test solution is adjusted to a preselected pH, and the final pH is adjusted after the addition of formaldehyde.

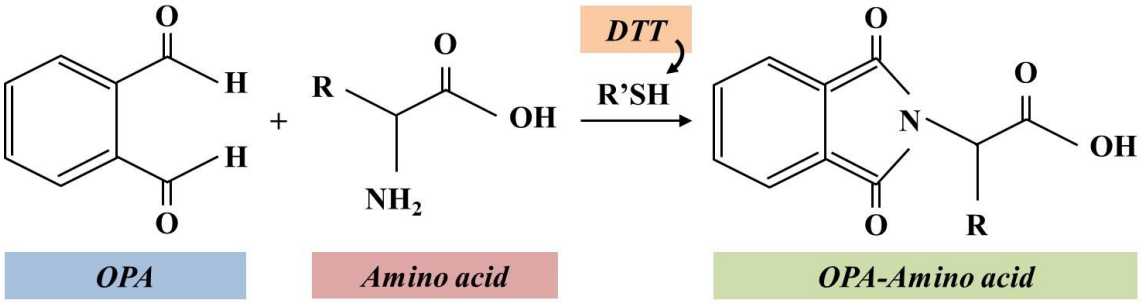

Figure 3-1 Reaction of *ortho*-phthaldialdehyde (OPA) with amino acids and an SH-compound (e.g. dithiothreitol, DTT) to form a compound that will absorb light at 340 nm (Nielsen, Petersen, & Dambmann, 2001; Rutherfurd, 2010).

The OPA method has been used to determine amino acids and is well known for being a derivatizing agent for the amino group (**Figure 3-1**). This method is described as a reaction between amino groups and OPA in the presence of dithiothreitol (DTT; a thiol group) forming a fluorescent compound detectable at 340 nm using a UV spectrophotometer (Nielsen et al., 2001). Serine (7075 OD/*m*mol/100*m*L) was selected as the standard due to it showing a response very close to the average response of amino acids (7088 OD/*m*mol/100*m*L) when OPA is reacted with amino acids and peptides under the absorption at 340 nm. The advantages of the OPA method are that the derivatization is rapid and allows real-time monitoring of the protein hydrolysis (Nielsen et al., 2001; Rutherfurd, 2010). Rutherfurd (2010) mentioned that the method is also more accurate, easier and faster to carry out (results available 2 min after the sample is taken), has a broader application range, and is environmentally safer (less toxic, eliminate the use of *β*-mercaptoethanol) than other methods.

3.1.2 Molecular weight distribution

Enzymatic hydrolysis of proteins changes the *Mw* distribution. In general, the hydrolysis results in a reduction of higher *Mw* components and an increase in lower *Mw* components. *Mw* distribution is carried out using a size exclusion chromatography-high performance liquid chromatography (SEC-HPLC). SEC-HPLC is a high-throughput analytical technique for separating proteins and other biological macromolecules according to their size under isocratic condition (Schrag, Corbier, & Raimondi, 2014). The separation is done by the differential exclusion from the pores of the packing material in the SEC column, of the sample molecules as they pass through a bed of porous particles. The pores on the surface of the packing material work as a molecular sieve to separate proteins, peptides or amino acids based on their sizes. The largest components in the hydrolysates such as protein aggregates, penetrate the matrix particles to a lesser extent and are therefore eluted from the column ahead of smaller components, such as peptides and amino acids. The smaller components penetrate the matrix more readily and are therefore eluted after the protein aggregates. The components are identified by their typical retention time and position, relative to molecular markers (**Figure 3-2**). The proportion of the sample components is determined by calculating the peak areas of each component relative to the total integrated peak area.

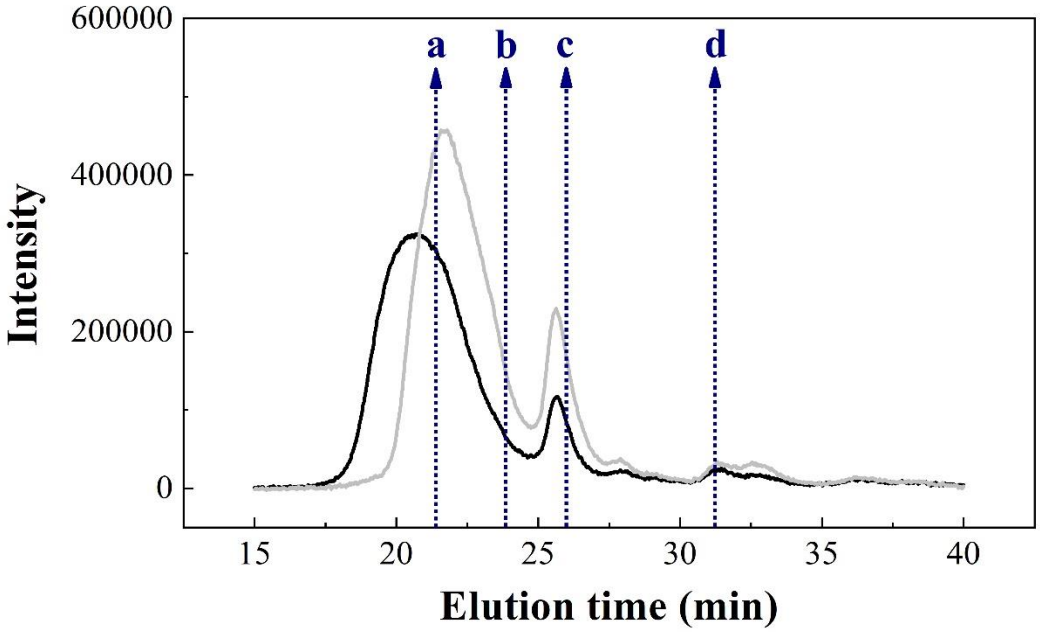

Figure 3-2 Example of size exclusion chromatography (SEC) elution profiles of beef bone extract (—) and an example of hydrolysate produced through enzymatic hydrolysis treatment (—). The *Mw* markers are as follows: a, cytochrome C (12400 Da; 21.498 min); b, aprotinin (6511 Da; 23.892 min); c, insulin chain B (3495 Da; 25.987 min); and d, leucine enkephalin (555 Da; 31.329 min).

Based on previous studies, there were observations of an increase in low *Mw* peptides after enzymatic hydrolysis. For instance, in a study on the influence of lipase pre-treatment on beef bone hydrolysates, Song et al. (2016) reported that the hydrolysates were composed of a series of low *Mw* peptides, especially lower than 1000 Da. The authors also reported that amino acids and peptides of *Mw* less than 180 Da increased (proportion of 87.36% to 94.70%) as the DH increased (12.71% to 23.17%). This observation was also reported by Pagán et al. (2013) and Dong et al. (2014). Pagán et al. (2013) investigated the enzymatic hydrolysis kinetics of pig bones hydrolysates using Neutrase® at different E/S ratio. The authors reported that hydrolysates with higher DH (12.14% at E/S ratio of 2.5%) had a higher proportion of peptides (36.3%) with *Mw* less than 10 kDa, while hydrolysates with lower DH (3.76% at E/S ratio of 0.1%) showed a higher proportion of peptides (41.8%) with *Mw* higher than 20 kDa. Dong et al. (2014) studied on the effect of enzymatic hydrolysis using Flavourzyme® on chicken bone extracts at different time intervals of 0, 1, 3, 5, 8, 12, 17 and 24 hours. The authors reported that the content of peptides with

Mw between 400-1000 Da increased greatly (peak area of 1593.20 to 136489.41) when the DH of chicken bone hydrolysates increased from 16.58% to 37.92%.

3.1.3 Free amino acid composition

Protein hydrolysates obtained after enzymatic hydrolysis include FAA and short-chain peptides that provide functional properties beyond basic nutrition due to their amino acid profile (Chalamaiah, Hemalatha, & Jyothirmayi, 2012). Amino acids are organic compounds that contain at least one amine group and at least one carboxyl group (Wade, 2009). Amino acids have side chains (symbolised by R), the simplest amino acid being glycine where the side chain is hydrogen, whereas other amino acids have a more complex side chain. For instance, cysteine is the amino acid with a thiol (i.e. sulphur) side chain (**Figure 3-3**). Amino acids have a substantial role in protein synthesis as compound carriers and also influence bioactive and functional properties (Villamil et al., 2017). Most protein hydrolysates from meat protein sources have all the essential and non-essential amino acids, which is why they are usually considered to be high in nutritional values.

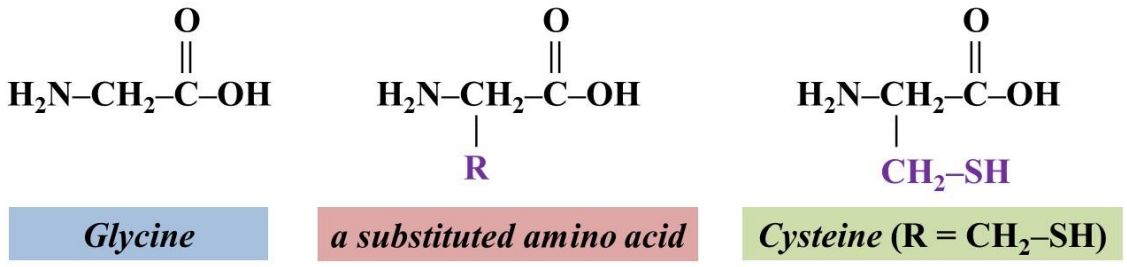

Figure 3-3 Illustration of the simplest α-amino acid (i.e. glycine) and amino acid with side-chain substituted on the carbon atom (e.g. cysteine) (Wade, 2009).

Meat protein hydrolysates have been reported to exhibit variation in their amino acid composition. These variations in the amino acid composition depend on several factors such as raw material, enzyme source and hydrolysis conditions (Klompong et al., 2007; Villamil et al., 2017). In most studies, the proportion of FAA increased after enzymatic hydrolysis. In a study on the effect of enzymatic hydrolysis using Flavourzyme® on chicken bone extracts at different time intervals of 0, 1, 3, 5, 8, 12, 17 and 24 hours, Dong et al. (2014) observed total FAA content of the hydrolysate at 24 hours of hydrolysis (5752.78 mg/100 mL) increased, as compared with at 0 hour

of hydrolysis (652.01 mg/100 mL). The authors also reported that the increase of FAA appeared to be rapid during the first 8 hours of hydrolysis and then slowed down which was in accordance with the DH results. In another study, Song et al. (2016) reported that prior lipase pre-treatment on beef bone protein had a significant influence on the content of FAA. The proportion of total FAA of beef bone proteins hydrolysed by a combination of lipase and protease (porcine pancreatic lipase + papain, lipase + papain and porcine pancreatic lipase + Protamex®) was significantly increased compared to that hydrolysed by protease alone (papain and Protamex®). The total FAA of porcine pancreatic lipase + papain, lipase + papain, porcine pancreatic lipase + Protamex®, papain and Protamex® were 534, 505, 506, 152 and 105 mg/g, respectively.

Meat protein hydrolysates, when heated with reducing sugar, exhibited a decrease in total FAA after the MR. In the MR, FAA reacts with reducing sugars resulting in the formation of volatile compounds and hence results in a reduction in FAA (Lan et al., 2010; Sun et al., 2014). In a study on the changes of the physicochemical properties of hydrolysed chicken bone extracts using Protamex® and Flavourzyme® in sequential treatment during MR, Sun et al. (2014) reported that the total FAA first increased and then decreased, where the highest proportion of total FAA was obtained at 60 min of heating (MR). The authors also noted that the proportion of total FAA increased first (before 60 min) and then decreased (at 90 min). They suggested that the increase of FAA in the hydrolysate before 60 min of heating could be due to thermal degradation of protein or peptides, while the decrease in FAA at 60 min could be associated with the formation of volatile compounds from the amino acids. The content of cysteine in the hydrolysate kept on decreasing from 3.14, 0.78, 0.61 and 0.42 mg/g at 0, 30, 60 and 90 min of heating, respectively. This indicated that the sulphur-containing peptides were involved in the production of meat flavour compounds.

3.1.4 Gas chromatography-mass spectroscopy (GS-MS)

GC-MS is used as a qualitative/ quantitative technique for volatile compounds analysis. GC is used in flavour chemistry to separate a sample into its individual chemical components, while MS is an analytical technique used to produce spectrum on each of the individual components of the sample (Reineccius, 2005). A typical GC system consists of a gas control unit that supplies a carrier gas to the column, a sample injection system, an analytical column, and an MS detector with associated data acquisition/ processing (Flanagan, Taylor, Watson, & Whelpton,

2008b; McNair & Miller, 2011). Sample preparation is performed to make the sample suitable for GC chemical analysis. After the sample preparation, an aliquot is injected into the injection port of the GC device using an injection needle, where it is immediately volatilised and mixed with the carrier gas. The gas serves to move or push the solute forward down the capillary column and is known as the mobile phase. The column provides a surface for components in the sample to interact with, which is known as the stationary phase. The solute interacts with the stationary phase depending on molecular mass where heavier components take a longer time to pass through the capillary column than lighter component, allowing for separation to occur. The different chemicals in the sample separate based on their volatility and mass. Mass spectrometry is used to separate molecular species according to their mass-to-charge ratio (*m/z*) (Flanagan, Taylor, Watson, & Whelpton, 2008a). Fragments of ionized species such as intact atom or molecule or a group of ions of different masses are formed when the solute is ionized. The ions are separated by magnetic or electrostatic fields in a high vacuum typically 10^{-5} Pa, and the plot of their relative abundance versus the *m/z* of each ion constitutes a mass spectrum.

Gas chromatography-mass spectrometry (GC-MS) or gas chromatography-olfactory-mass spectrometry (GC-O-MS) are commonly used in the determination of volatile compounds of MRPs (**Table 3-1**). MRPs for GC analysis can be prepared using solvent extraction, solid-phase microextraction, or purge and trap concentration. Internal standards such as 1,2-dichlorobenzene or 2-methyl-3-heptanone are often used. The most commonly used mobile phase is helium, which is an inert carrier gas. A capillary column is often used as the stationary phase. The oven in GC is usually maintained at a lower temperature (e.g. 35-50°C) for 2 to 10 min, followed by raising to a higher temperature (e.g. 160-280°C) for an extended period with constant increasing rate (e.g. 2-6°C/min) (Elmore et al., 2002; Guo et al., 2010; Sun et al., 2014; Liu et al., 2015; Song et al., 2016).

Table 3-1 Various conditions used in gas chromatography-mass spectrometry to determine volatile compounds of Maillard reacted products

Variables	Reference				
	Elmore et al. (2002)	Guo et al. (2010)	Sun et al. (2014)	Liu et al. (2015)	Song et al. (2016)
Method [a]	GC-MS	GC-MS	GC-MS	GC-O-MS	GC-MS
Extraction method [b]	SPME	Solvent extraction using dichloromethane and sodium sulphate	SPME	Purge and trap concentrator	SPME
Internal standard	1,2-dichlorobenzene in methanol	Dodecane	2-methyl-3-heptanone	2-methyl-3-heptanone in n-pentane	1,2-dichlorobenzene in methanol
Carrier gas (Mobile phase)	Helium (16 psi) at 1.0 mL/min at 40°C	Nitrogen	Helium at 1.01 mL/min	Helium at 1.2 mL/min	Helium at 1.8 mL/min
Column type (Stationary phase)	Capillary (60 m × 0.25 mm × 0.25 μm)	Capillary (30 m × 0.32 mm)	Capillary (30 m × 0.25 mm × 0.25 μm)	Capillary (30 m × 0.32 mm × 0.25 μm)	Capillary (60 m × 0.25 mm × 0.25 μm)
Oven operating conditions	40°C for 2 min, then increased at 4°C/min to 280°C	40°C for 10 min, then increased at 2°C/min to 160°C and held for 50 min	40°C for 3 min, then increased at 5°C/min to 120°C, followed by 10°C/min to 230°C and held for 5 min	35°C for 2 min, then increase at 6°C/min to 230°C and held for 20 min	50°C for 3 min, then increased at 3°C/min to 230°C and held for 7 min
Electron ionisation mode	70 eV, 35 μA	70 eV	70 eV	70 eV	70 eV
m/z scan range	29 to 400 at 1.9 scan/s	Not stated	50 to 450	40 to 500	Not stated
MS source temperature	170°C	Not stated	Not stated	230°C	230°C

[a] GC-MS = gas chromatography-mass spectrometry, GC-O-MS = gas chromatography-olfactometry- mass spectrometry.
[b] SPME = solid phase microextraction.

In a study on the use of lipase-pre-treated beef bone protein hydrolysates in MR, Song et al. (2016) studied five MRPs prepared using a xylose/ cysteine/ hydrolysate model in an oil-bath at 110°C for 90 min. The authors reported that lipase pre-treatment affected both the number of volatile compounds and the aroma intensity, where a total of 64 compounds (i.e. furans, pyrazines,

thioethers, aldehydes, etc.) were detected and identified. Furans and pyrazines were the dominant volatile compounds in the MRPs. Sulphur-containing volatiles such as dimethyl disulphide, dimethyl trisulphide and furfuryl methyl disulphide were detected. Protein hydrolysate prepared using Protamex® was reported to contain the highest concentration of essential meat flavour compounds in GC-MS, but the overall flavour was not favoured by sensory panellists, which might be due to the high content of furans (caramel-like flavour), decreasing the meaty aroma.

In another study on the effect of thermal treatments on the flavour generation from MR of chicken peptides and xylose, Liu et al. (2015) heated the mixture at different temperatures (80, 100, 120, 140°C) and time (30, 60, 90, 120 min) in a high-pressure stainless reactor to produce MRPs. The authors reported that pyrazines, ketones, furans and Strecker aldehydes were the dominant compounds in the MRPs, while pyrazines and Strecker aldehydes were the key aroma-active compounds that contributed to the aroma of MRPs by GC-O technology. There was an increase in the generation of meaty aroma (i.e. thiophene and thiazoles) at 100, 120 and 140°C, where higher temperatures were required to generate these heterocyclic compounds (Jayasena et al., 2013). Pyrazines, the main contributor of nutty and roast meat-like odorant in the MRPs, is one of the common volatile compounds identified in the peptide MR systems (Van Lancker, Adams, & De Kimpe, 2012). The formation of pyrazines was due to the interaction of α-dicarbonyls and amines through Strecker degradation.

In this research thesis, the DH of beef bone extract and hydrolysates (**Chapter 4**) was determined using the OPA method. The *Mw* distribution of beef bone extract, hydrolysates, and Maillard-reacted hydrolysates (**Chapter 4 and 5**) were determined using SEC-HPLC. Lastly, the amount of FAA before and after MR, and the type and amount of volatile compounds generated after MR (**Chapter 5**) were determined using HPLC and GC-MS, respectively.

3.2 Characterisation of meat analogues/ alternatives

From the literature, different methods are used to characterise the textural and structural properties of meat analogues. The research work has shortlisted the following characterisation techniques as shown in **Table 3-2**.

Table 3-2 Types of methods used to characterise the textural and structural properties of meat analogues

Reference	Characterisation of meat analogues					
	Cutting force	**TPA**	**SEM**	**Light microscopy**	**Protein solubility**	**Sensory evaluation**
Azzollini, Wibisaphira, Lakemond, and Fogliano (2019)		√	√		√	
Chen et al. (2010)	√	√				
Chen et al. (2011)					√	
Fang et al. (2014)	√	√			√	
Krintiras, Göbel, Bouwman, Van Der Goot, and Stefanidis (2014)			√	√		
Krintiras et al. (2015)			√		√	
Lin et al. (2000)		√				
Lin et al. (2002)			√	√		√
Liu and Hsieh (2007)		√			√	
(Liu & Hsieh, 2008)					√	
Osen et al. (2014)	√					
Osen et al. (2015)					√	
Palanisamy et al. (2018a)	√		√			
(Palanisamy, Töpfl, Aganovic, & Berger, 2018b)	√		√			√
Rareunrom et al. (2008)	√				√	√
Rehrah et al. (2009)		√				√
Zhang et al. (2018)	√	√	√			

3.2.1 Cutting force

The four main quality attributes of foods are appearance, flavour, texture and nutrition (Bourne, 2002). Texture is the primary response of the tactile senses to physical stimuli that result from contact between some part of the body and the food. The sense of touch is the key method to evaluate the texture and sensory properties of a food product. Textural analysis is the practice of testing physical properties of food products, through compression (e.g. cutting force, TPA) or tension (e.g. tensile strength). Cutting force is performed to determine the degree of texturisation of meat analogues using a texture analyser (Chen et al., 2010). Based on previous studies by Chen et al. (2010), Fang et al. (2014) and Osen et al. (2014), the meat analogues were cut into the shape as shown in **Figure 3-4**, and cut using a knife blade (A/CKB or A/LKB) probe along the direction vertical (F_L) and parallel (F_V) to the direction of meat analogues outflow from the extruder, respectively. The degree of texturisation is used to indicate the fibrous structure formation of meat analogues, which is expressed as a ratio of F_L and F_V. Based on the literature results, a good degree of texturisation ranged between 1.2 and 1.7, while a poor degree of texturisation is 1.0. The value of F_L should be higher than F_V, as F_L requires more force to cut the fibres of the meat analogues, whereas F_V is cutting parallel to the fibres which tend to separate the fibres rather than cutting them as shown in **Figure 3-4a and b**.

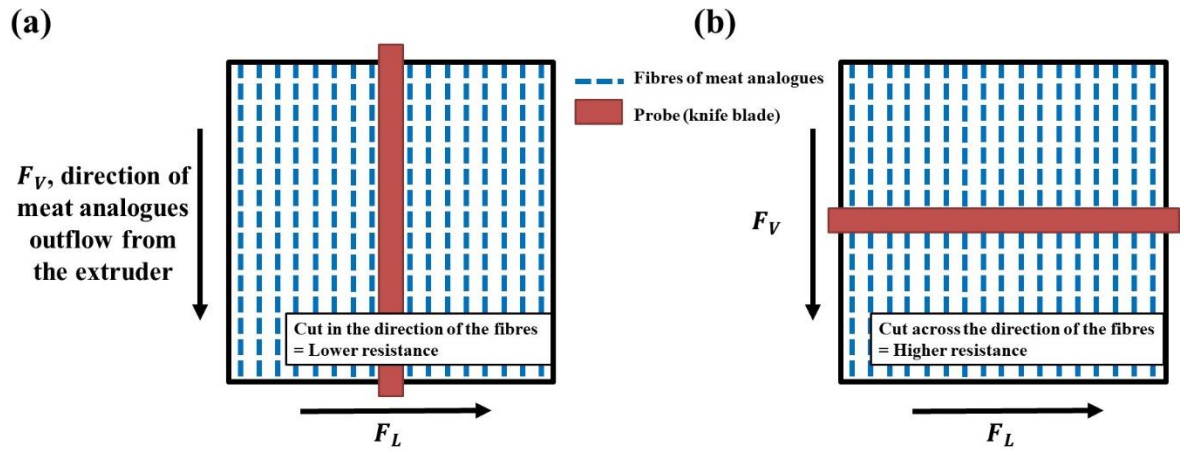

Figure 3-4 Sampling drawing for degree of texturisation test, (a) knife blade cutting in the direction of the fibre and (b) knife blade cutting across the direction of the fibres (Osen et al., 2014).

Previous studies by Fang et al. (2014) and Osen et al. (2014) reported that extrusion parameters had significant effects on the degree of texturisation. In the study by Fang et al. (2014)

who investigated the effects of extrusion SME on texturised soy protein, reported that there was a drop in the degree of texturisation when SME increased. The degree of texturisation decreased from 1.17 to 1.06, when SME rose from 819.70 to 1258.70 kJ/kg. The authors suggested that a higher SME input generated poorer fibrous structure formation. In another study by Osen et al. (2014) on high moisture extrusion of three different pea protein isolates, it was reported that cooking temperature significantly affected fibre formation. An increase in cooking temperature from 100 to 160°C resulted in an increased cutting force in F_L whereas F_V remained constant. Further increase in cooking temperature (i.e. ≥120°C) produced meat analogues with multi-layered structures with layers parallel to the die wall and, fine fibre appeared upon tearing. The authors explained that the energy input during this stage might have caused macromolecules to unravel making bonding sites available for further crosslinking that were previously buried within the macromolecules.

3.2.2 Texture Profile Analysis (TPA)

TPA is one of the destructive force/ deformation methods that measure the textural properties of foods which are solid or semi-solid (Lu & Abbott, 2004). The method directly measures either single or composite mechanical properties of food and is widely preferred as it can be related to the sensory perception of texture by humans in the hand or mouth (Bourne, 2002). TPA involves two complete cycles of compression and decompression of a food product, where the degree of compression can be as high as 90%. During the analysis, the samples are compressed twice using a texture analyser to provide insights into how sample behave when chewed. It is often called the “two-bite test” where the texture analyser simulates the biting action of the jaws. The force/ time relationship is usually recorded during the cycles of compression and decompression. From the force/ time curve, several texture parameters such as hardness, fracturability, cohesiveness, springiness, gumminess, chewiness and resilience are calculated as shown in **Table 3-3**, and are closely related to sensory evaluation results (Trinh & Glasgow, 2012). The main advantage of TPA is that it can determine and quantify multiple texture parameters in just one experiment.

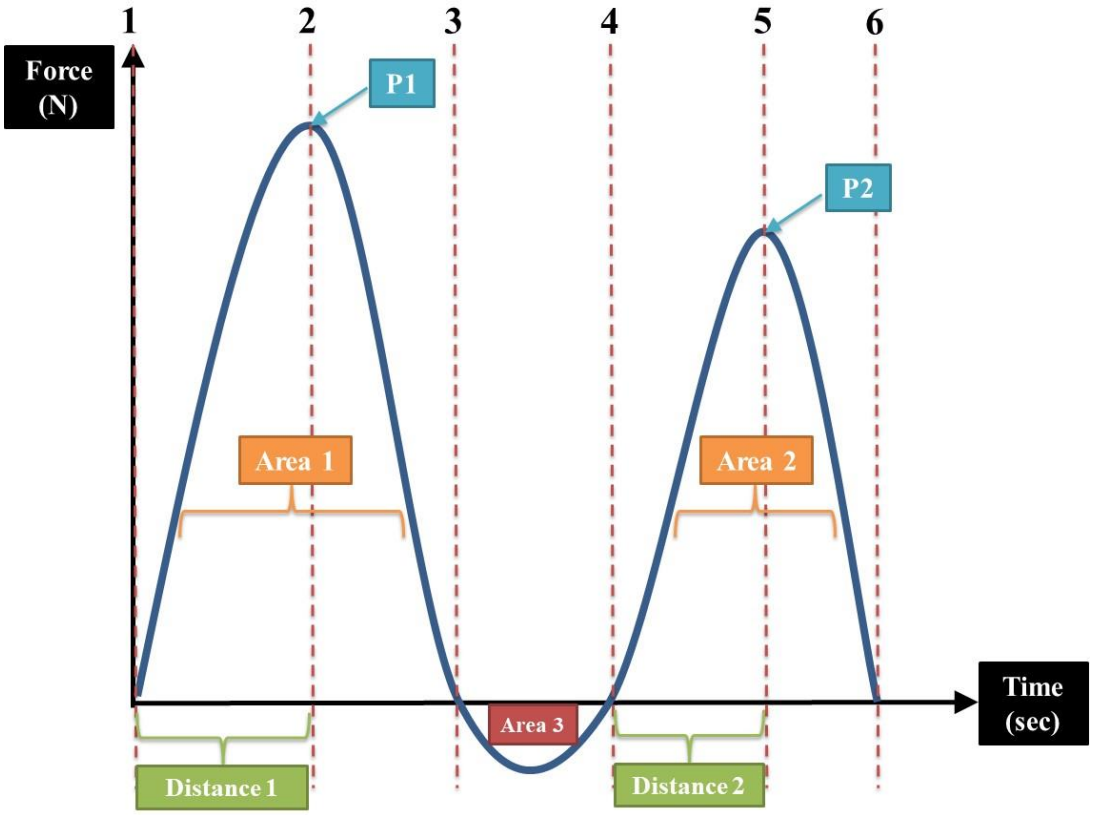

Figure 3-5 Schematic illustration of a general texture profile (force/ time) curve (Szczesniak, 2002).

Table 3-3 Texture profile analysis (TPA) parameters and its definitions (Szczesniak, 2002)

TPA parameter	Definition	How to measure?
Hardness (N)	The maximum force of the first compression	Force at P1
Cohesiveness	How well the sample withstands a second deformation relative to its resistance under the first deformation	Area 2/Area 1
Adhesiveness (N.mm)	Work required to overcome the sticky forces between the sample and the probe	Area 3
Springiness	How well a sample physically springs back after it has been deformed during the first compression and has been allowed to wait for the target wait time between strokes	Distance 2/ Distance 1
Chewiness (N)	The energy needed to chew a solid food until it is ready for swallowing	Hardness × Cohesiveness × Springiness

Previous studies on soy-based meat analogues by Lin et al. (2000), Chen et al. (2010) and Fang et al. (2014) reported that extrusion parameters had significant effects on textural properties such as hardness and chewiness. In a study on the texture characteristics of soy protein meat analogues at 60, 65 and 70%MC, Lin et al. (2000) reported that both MC and cooking temperature (137.8, 148.9 and 160°C) affected the gumminess, hardness and chewiness of meat analogues significantly, but not their cohesiveness or springiness. Cooking temperature had a significant effect on hardness and chewiness at lower MC, but not at higher MC. Meat analogues extruded at 70%MC had the lowest hardness, chewiness, and gumminess, which could be due to more water contained within the samples. The authors reasoned that the lower viscosity at higher MC might be a result of an incomplete texturisation process which led to a product with softer texture (Kitabatake, Megard, & Cheftel, 1985; Noguchi, 1989).

Chen et al. (2010) studied the effect of MC and cooking temperature for extruded soybean protein by measuring textural properties. They reported that hardness and chewiness of the extruded soybean protein analogue decreased greatly as MC increased from 28% to 60%. Samples extruded at 60%MC had the lowest hardness and chewiness. This was attributed to a higher proportion of water contained within the samples, which was similar to the findings of Lin et al. (2000). However, the cooking temperature had no significant effect on hardness and chewiness. In a study by Fang et al. (2014) when investigating the effects of extrusion SME on texturised soy protein, it was reported that there was a significant increase of 22.47% and 17.01% in hardness and chewiness, respectively, when SME increased from 819.70 to 1258.70 kJ/kg.

3.2.3 Scanning electron microscopy (SEM)

An understanding of the relationship between food texture and food structure is necessary so that texturally attractive products can be developed (Wilkinson, Dijksterhuis, & Minekus, 2000). The use of microscopy analysis helps to increase the researchers' understanding of microstructural changes that occur during processing and the role of different ingredients, allowing better control of the structure, manipulation, and regulation of texture. These microscopy techniques (e.g. SEM, confocal scanning laser microscopy, LM) enable examination of the food structure and provide a clearer understanding of food texture.

SEM involves scanning the surface of a sample with a focused beam of electrons, whereby several interactions occur and generate a variety of signals that can be captured to create images (Aguilera & Bouchon, 2008). SEM mainly capture the secondary or backscattered electrons. One of the advantages of SEM is its capability to obtain a three-dimensional image of the surface of a wide range of materials, with excellent resolution (1-5 nm) and large depth of field. SEM was used by Lin et al. (2002) and Krintiras et al. (2015) of soy protein meat analogues, though each group used different sample preparation methods. Lin et al. (2002) cut the samples into small pieces of 2 mm thick, 4 mm wide and 6 mm height, and then freeze-dried at -60°C, 10 μm Hg vacuum for 72 hours in a freeze dryer. The freeze-dried sample was then fixed onto an aluminium holder with the cutting side facing up and coated with gold at 2.5 kV and 20 mA for 1.5 min. Krintiras et al. (2015) cut their samples into 5×5×5 mm cubes parallel to the fibres and then dried them for 24 hours in an oven set at 60°C to reduce the MC. However, their samples were not coated with gold or any other coating prior to analysis due to the fact they were using a different type of scanning electron microscope. There is a risk that the removal of water by either oven-drying or freeze-drying may alter the microstructure of the samples. Another sample preparation method was used by Takei, Hayashi, Umene, Kobayashi, and Masunaga (2016) when preparing enzyme-treated chicken breast meat for microscopy (**Figure 3-6d**). The authors fixed the samples in 2.5% glutaraldehyde in 0.1 M phosphate-buffered saline and post-fixed with 1% osmium tetraoxide ($OsO4$) buffered with 0.1 M PB. The samples were then dehydrated in a graded series of ethanol solutions and dried in critical point drying equipment with liquid carbon dioxide (CO_2). Platinum/ gold was spatter-coated onto the samples before evaluation using the microscope.

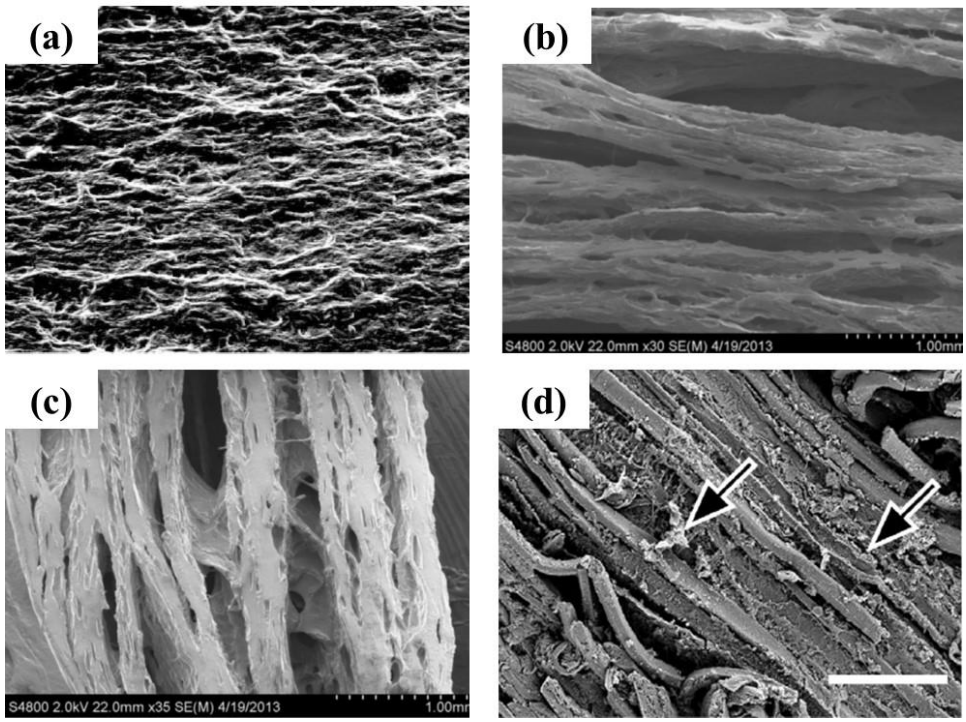

Figure 3-6 Scanning electron micrographs of samples extruded at (a) 60% moisture and 160°C at 200× magnification (fibrous structure), (b) 95°C at 30 rpm for 15 min (fibrous structure), (c) 100°C at 30 rpm for 15 min (layered structure) and (d) chicken breast, parallel to myofibril, at 100× magnification. Reproduced with permission from Elsevier; Krintiras et al. (2015), and John Wiley and Sons; Lin et al. (2002) and Takei et al. (2016).

Both sample preparation methods by Lin et al. (2002) and Krintiras et al. (2015) were able to obtain fibrous microstructure images as shown in **Figure 3-6**. Lin et al. (2002) stated that as the MC decreased at a fixed cooking temperature, more fibrous and directional structure were observed (**Figure 3-6a**). The authors explained that lower extrusion moisture caused an increase in friction and shear inside the cooling die, resulting in a greater velocity gradient with a higher degree of texturisation and fibre formation. Krintiras et al. (2015) reported that fibrous and layered structures were observed when using a Couette cell (**Figure 3-6b and c**). The only difference in process parameters to obtain these two structures was the cooking temperatures (i.e. 95 and 100°C). The two fibre diameters were also different, where the structures in **Figure 3-6b and c** range between 150-300 μm and 50-200 μm, respectively. The authors explained that the fibrous structures were made of smaller fibres which were interconnected with much smaller fibres (1-5 μm diameters). The authors also suggested that these interconnecting fibres were probably gluten.

3.2.4 Light microscopy (LM) (rapid freezing and cryosectioning)

LM is another well-established method of studying the microstructure of food products (Heertje, Vlist, Blonk, Hendrickx, & Brakenhoff, 1987). The light microscope is an instrument that uses visible light to produce a magnified image of a sample that is projected onto either the retina of the eye or the photosensitive surface of an imaging device such as a digital camera (Murphy & Davidson, 2013). The working principle of the microscope involves a light source that can be focused onto the sample by using a condenser lens. The light that illuminates the sample reaches the objective lens, which produces a magnified image. The eyepiece, also known as an ocular lens, produces the final magnification (about 1000×) of the image of the sample.

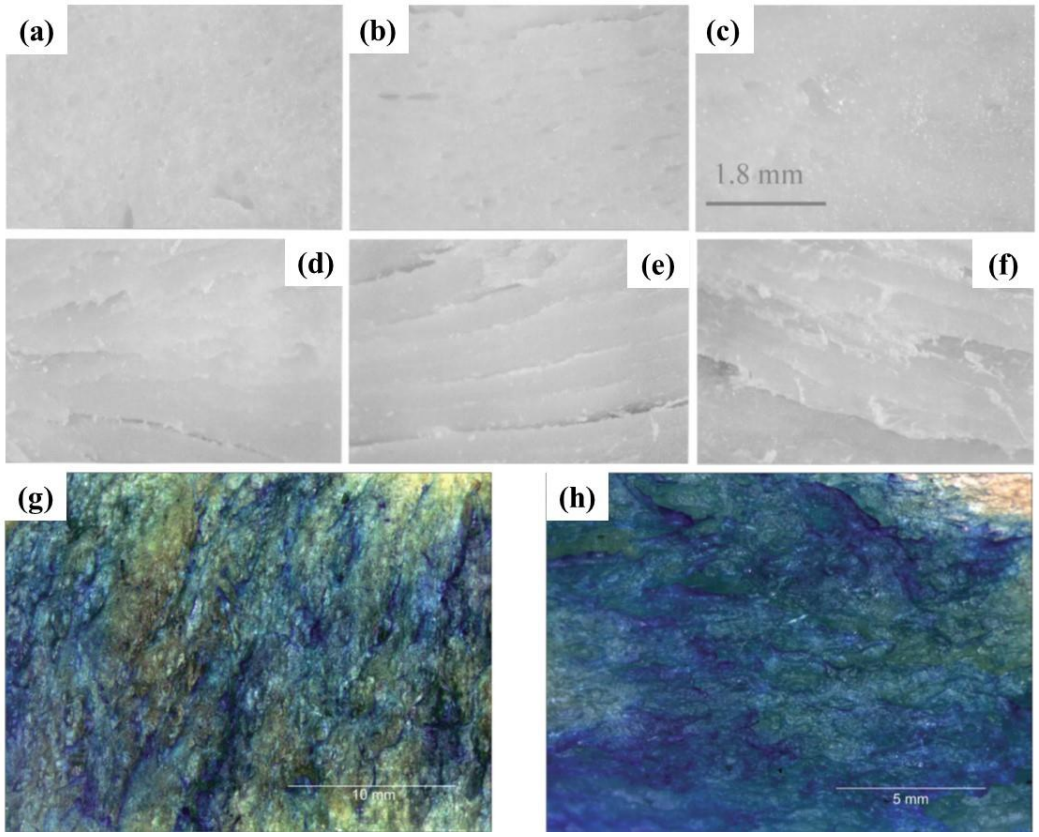

Figure 3-7 Light micrographs of samples extruded at (a) 70% moisture and 138°C, (b) 70°C moisture and 149°C, (c) 70% moisture and 160°C, (d) 70% moisture and 138°C, (e) 60°C moisture and 149°C, and (f) 60% moisture and 160°C at 100× magnification, and images of structured sample using toluidine blue stain mountant (dark purple-blue colour for SPI and pale blue-green colour for wheat gluten) at (g) 5× magnification and (h) 10× magnification. Reproduced with permission from John Wiley and Son; Lin et al. (2002), and Royal Society of Chemistry; Krintiras et al. (2014).

Lin et al. (2002) and Krintiras et al. (2014) when preparing their soy protein meat analogues for light microscopy used different methods. Lin et al. (2002) cut the defrosted samples into small cubes (about 7×7×7 mm). The images were taken on samples with the cut side facing up and the extrusion direction perpendicular to the x-axis. As for Krintiras et al. (2014), the samples were stained using toluidine blue stain mountant, to differentiate the two plant proteins, SPI and WG, dark purple-blue colour and pale blue-green colour, respectively. A few drops of stain were applied to the surface and sample left to rest for a couple of minutes before viewing under a light microscope. Based on the light micrographs (**Figure 3-7a to f**), samples were viewed under a microscope at 30× magnification and showed no difference in structure based on the effect of cooking temperatures (138, 149 and 160°C) (Lin et al., 2002). It was reported the layered structure of meat analogues became clear as the MC decreased from 70% to 60%. The layered structure at **Figure 3-7d to f** seemed to become obvious but difficult to differentiate. As for **Figure 3-7g and f**, where the samples were viewed at 5× and 10× magnification, Krintiras et al. (2014) observed that the stained proteins followed a certain direction indicating anisotropic structure formation. The authors also reported that the lighter parts in the sample were enrobed with a stranded continuous network, suggesting SPI was being dispersed in a continuous gluten matrix. However, by looking at the micrographs, the structure was not clear and the images between 5× and 10× magnification do not seem to correspond. Samples in both studies seemed to be too thick in their natural state to be examined directly in a light microscope. The authors suggested that sample preparation method such as rapid freezing and cryosectioning should be considered for viewing under a light microscope.

Microtomy is defined as the technique of cutting sections suitable for light microscopy. The microtome is capable of cutting semi-thin (0.1 to 2.5 μm thick) and thick ($\geq$ 2.5 μm thick) sections (Reid & Beesley, 1991). Cryosectioning is the process of cutting sections at low temperatures. One of its advantages is that the sample is not exposed to chemical fixation, which can modify proteins, or to liquids, which can redistribute soluble ions, and may extract proteins. Rapid freezing is an important aspect of cryosectioning as a badly frozen sample is difficult to section and the resulting sections can be damaged by the ice crystals that lead to valueless results. Currently, there are no reported studies that have examined meat analogues under a light microscope using rapid freezing and cryosectioning technique. In a study by Maeda et al. (2013)

on the development of a novel staining procedure for visualising the gluten-starch matrix in bread dough and cereal products, rapid freezing and cryosectioning technique were used. Small pieces of samples were put into embedding plastic plate moulds and embedded in OCT compound. OCT (i.e. optimal cutting temperature) compound is a formulation of water-soluble glycols and resins, providing an excellent specimen matrix for cryostat sectioning at -10°C and below. It leaves no residue during the staining procedure and eliminates undesirable background staining. The plastic moulds were placed into a shock freezer at -50°C and were rapidly frozen for 10 min. The frozen samples were then fixed onto the cryostat at -30°C for thin sectioning. The samples were sliced to 20 μm and were air-dried on the microscope slides.

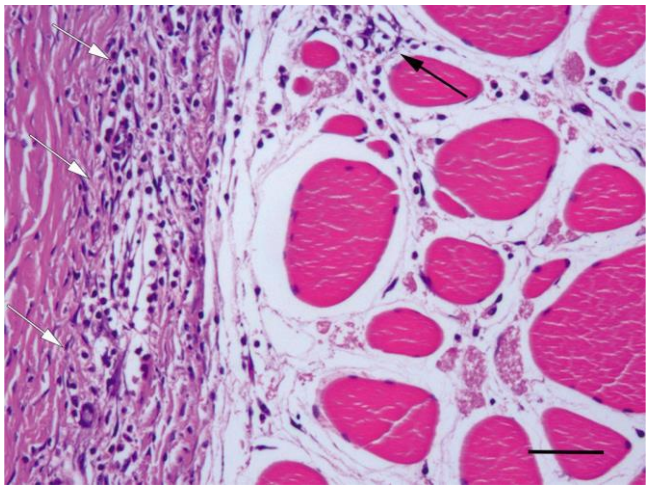

Figure 3-8 Photomicrograph of perivertebral tissues in Chinook salmon with LKS (lordosis, kyphosis and scoliosis). Section was sliced at 5 μm and stained with H&E dyes. Scale bar = 20 μm. Reproduced with permission from Inter-Research; Munday et al. (2016).

Haematoxylin and eosin (H&E) stain are regularly used for staining the sections of meat analogues. It is considered as the principal stain and the gold standard in histology. The basic staining process involves submerging the slides into a series of solvents such as xylene, alcohol and water, to give the samples an affinity for the dyes. The slides are then stained with haematoxylin (nuclear) dye and rinsed with water, then stained with eosin (counterstain) dye. The slides are then rinsed with water, followed by dehydration in different graded strengths (e.g. 50%, 70%, 80%, 95% and 100%) of alcohols, clear in xylene, and lastly being cover slipped. With the use of rapid freezing and cryosectioning technique, together with H&E staining, the sample (e.g.

perivertebral tissues in a Chinook salmon) was able to be viewed under a light microscope and captured clearly by the digital camera (**Figure 3-8**).

3.2.5 Protein solubility

Extrusion processing is widely used in food industries to alter protein structure and solubility by a combination of heat (120-200°C), pressure (1.6-6.1 MPa), and shear (Corredig, 2005; Day & Swanson, 2013). Oriented patterns are reorganised as a result of the complete restructuring of polymeric material during extrusion. Protein interactions (i.e. SS-bonding and non-covalent bonding) occur upon cooling of texturised proteins (Akdogan, 1999). Protein-protein interactions of texturised proteins may be enhanced by decreased temperature and macromolecular alignment. Parallel fibre formation of varying length and thickness is caused by crystalline aggregation. Recent research attribute S-S bonds, H-bonds and hydrophobic interactions as the main interactions responsible for protein texturisation by extrusion (Lin et al., 2000; Liu & Hsieh, 2007; Rareunrom et al., 2008; Chen et al., 2011).

Changes in protein structure can occur during extrusion. The changes were investigated by protein solubility where the forces responsible for stabilising the meat analogues during extrusion were studied (Lin et al., 2000). Besides that, protein-protein interactions formed during extrusion can be determined using protein solubility, by treating the meat analogues with various extracting buffers (Corredig, 2005). Sodium dodecyl sulphate (SDS) or urea is used to solubilise proteins that are made insoluble by non-covalent interactions, mainly hydrophobic interactions or H-bonding, respectively. DTT or 2-ME is used to cleave proteins with S-S bonds formed during extrusion. DTT will be used in this study to examine disruption of S-S bonds, as 2-ME is considered toxic. 2-ME irritates skin, eyes, and respiratory tract, and may be fatal if absorbed through the skin. However, DTT is relatively unstable due to oxidation; thus, fresh DTT solution will need to be prepared prior to protein solubility analysis.

Liu and Hsieh (2007) used three types of reagents, phosphate buffer (PB), urea, DTT and combination of the two (i.e. PB+U+DTT), to study the protein-protein interactions in high (60%) moisture-extruded SPI meat analogues and heat-induced SPI gels. It was reported that both SPI gels and meat analogues had the same type of chemical bonds (i.e. covalent S-S bonds and non-

covalent bonds). The authors further explained that both covalent S-S bonds and non-covalent bonds were important in forming the fibrous structure of soy protein meat analogues made under high moisture extrusion. In a study by Rareunrom et al. (2008), the authors investigated the chemical linkages of soy protein meat analogues containing different SPI contents (i.e. 20, 40, 60 and 80%) with defatted soy flour. Four types of selective reagents, PB, 2-ME, urea, SDS and their combinations were used for protein solubility analysis. The authors reported S-S bonds, hydrophobic interactions and H-bonds were the major linkages in SPI meat analogues structure. It was also reported that extrusion processing did not alter the type of chemical bond between the ingredient and meat analogues. In a study by Osen et al. (2015) when investigating the protein-protein interactions in three commercial pea protein isolates (PPI) before and after extrusion at 60% MC, the authors used similar extracting buffers and combinations as Liu and Hsieh (2007). The authors reported that the structural integrity of PPI meat analogues could be attributed mainly to covalent SS-bonding, and, to a smaller extent, to non-covalent interactions. This was slightly different to the finding of Liu and Hsieh (2007) on SPI meat analogues, who reported that covalent SS bridges, hydrophobic interactions and H-bonding were the forces responsible for insolubilisation and rigid structure of extruded SPI meat analogues. Liu and Hsieh (2007) also stated that no conclusion could be deduced regarding which type of bonding played a more dominant role in the structural integrity of SPI meat analogues. Based on the above findings, it could be concluded that there were slight differences in the chemical linkages between SPI and PPI meat analogues.

Table 3-4 Various extracting solvents with selective reagents and their combinations for protein solubility study of extruded meat analogues

Reagent [b]	References [a]					
	1	**2**	**3**	**4**	**5**	**6**
Phosphate buffer (PB)	√	√	√	√	√	√
PB+2ME	√		√		√	
PB+U	√	√	√	√	√	√
PB+SDS	√		√		√	
PB+DTT		√		√		√
PB+TU				√		
PB+TX				√		
PB+CHAPS				√		
PB+U+2ME	√		√		√	
PB+U+SDS	√		√		√	
PB+U+DTT		√		√		√
PB+SDS+2ME			√		√	
PB+U+2ME+SDS					√	
PB+TU+TX+CHAPS				√		
PB+DTT+TU+TX+CHAPS				√		
PB+U+TU+TX+CHAPS				√		
PB+U+DTT+TU+TX+CHAPS				√		
Main protein sources [c]	SPI	SPI	SPI, DSF	SPI	SPI	PPI
Extrusion moisture (%)	60, 65, 70	60	30	60.11, 66.78, 72.12	28, 60	60
Extrusion temperature (°C)	137.8, 148.9, 160.0	170.0	160.0	170.0	150.0	140.0

[a] 1: Lin et al. (2000); 2: Liu and Hsieh (2007); 3: Rareunrom et al. (2008); 4: Liu and Hsieh (2008); 5: Chen et al. (2011); 6: Osen et al. (2015).
[b] 2ME = 2-mercaptoethanol, U = urea, SDS = sodium dodecyl sulphate, DTT = dithiothreitol, TU = thiourea, TX = TritonX-100, CHAPS = 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.
[c] SPI = soy protein isolate, DSF = defatted soy flour, PPI = pea protein isolate.

Various extracting solvents with selective reagents and their combinations for protein solubility of extruded products are shown in **Table 3-4**. Liu and Hsieh (2008) used three other types of reagents for protein solubility of SPI meat analogues made with different MC; namely thiourea (TU), TritonX-100 (TX) and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). TU, a substituted urea, is known to break non-covalent interactions but is more efficient in breaking hydrophobic interactions than urea. TX and CHAPS are

zwitterionic and non-ionic detergents, respectively, and they also disrupt hydrophobic bonds. The authors explained that the use of these additional reagents helped to differentiate the relative importance among non-covalent interactions. Results from this study indicated that SS-bonding played a more important role than non-covalent bonds in not only holding the rigid structure of SPI meat analogues but also forming the fibrous structure. Based on the above finding, it could be suggested that subsequent protein solubility tests might not require the use of TU, TX, and CHAPS as the extracting buffers.

Different types of protein analysis were used to determine the protein content and soluble protein content of meat analogues and extracting buffers. Both Liu and Hsieh (2007) and Liu and Hsieh (2008) used the Kjeldahl method to obtain the protein contents of meat analogues by using a conversion factor of 6.25 for SPI samples and 5.70 for WG. The soluble protein content of the supernatant from extracting buffers was determined using the Bradford protein assay at 595 nm. Osen et al. (2015) used the same method for determining soluble protein content of the supernatant as both Liu and Hsieh (2007) and Liu and Hsieh (2008). Instead of using the Kjeldahl method to determine the protein contents of meat analogues, Osen et al. (2015) used the Dumas combustion method by using a conversion factor from Total Nitrogen. Rareunrom et al. (2008) used the Lowry protein assay at 750 nm to determine the soluble protein content of their supernatant.

From the previous studies, it could be concluded that meat analogues with a high degree of texturisation and fibrous structure were observed to have high levels of S-S bonds. The protein solubility results can be used to complement the results obtained from cutting force, sensory analysis and microscopy analysis. It will be interesting to investigate and understand the forces that are responsible for stabilising the meat analogues during extrusion when Maillard-reacted beef bone hydrolysate was added, or different concentrations of SPI and WG are used. The methods used to determine the protein contents and soluble protein contents were selected wisely.

In this research thesis, the cutting force, TPA, SEM, LM and protein solubility of extruded meat alternatives at different SPC to WG ratio (**Chapter 6**), minced meat alternatives at different concentrations of MRPs (**Chapter 7**) and extruded meat alternatives at different MC (**Chapter 8**) were determined and studied.

3.3 Characterisation of sausages made from meat alternatives

3.3.1 Protein oxidation

Oxidation is one of the main factors for quality deterioration of food products during processing and storage (Zhang, Xiao, & Ahn, 2013). Protein oxidation, unlike lipid oxidation, has not been comprehensively studied, and its influence and mechanisms in meat products are still mostly unknown. Protein oxidation is defined as a covalent protein modification induced either directly by reactive species or indirectly by reaction with secondary by-products of oxidative stress (Bhattacharya, Kandeepan, & Vishnuraj, 2016). Protein oxidation results in the generation of different oxidation derivatives. These protein oxidative changes take place at the side chain of amino acids and include (i) loss of sulfhydryl (thiol) groups, (ii) formation of protein cross-linking, and (iii) formation of protein carbonyl groups (Lund, Heinonen, Baron, & Estévez, 2011; Estrada et al., 2018).

The generation of protein carbonyl groups is the most commonly used measurement for protein oxidation of meat products (Lund et al., 2011; Zhang et al., 2013). Both AAS and GGS are the carbonyl compounds that are commonly identified in oxidised muscle protein due to metal ion-catalysed oxidation. They are thought to account for approximately 70% of the total protein carbonyls formed in oxidised animal proteins. They are also used as an indicator for protein oxidation in raw meat and a large variety of processed foods such as patties, frankfurters, and dry-cured meats. The quantification of protein carbonyl groups using DNPH (2,4-Dinitrophenylhydrazine) method is widely used to measure protein oxidation in food products. DNPH derivatisation method is developed as a convenient and regular technique to determine the amount of carbonyl compounds (oxidised protein) in food products (Zhang et al., 2013). DNPH reacts with the protein carbonyl groups to generate hydrazones and the absorbance reading is measured at 370 nm (Levine et al., 1990). The amount of carbonyl content in the samples is then calculated as *n*mol/mg protein using an absorption coefficient of 22000 $M^{-1}cm^{-1}$ (Levine, Williams, Stadtman, & Shacter, 1994).

Estrada et al. (2018) hypothesised that (i) process conditions, which often involves a thermomechanical process and (ii) fortification with nutritional micronutrients such as iron which have a pro-oxidant activity, may affect the chemical stability and induce protein oxidation in plant

protein-based fibrous structures. The authors found that when proteins were heated in a high-temperature shear cell (at 140°C), carbonyl content was found to increase. However, the addition of iron (free or encapsulated) did not affect the carbonyl content. Therefore, it was concluded that high-temperature process conditions induce protein oxidation in plant protein fibrous structures.

3.3.2 Sensory evaluation

Sensory evaluation is the measurement of the quality of a food product based on information received from the human senses (Bourne, 2002). Sensory evaluation can be categorised into two types of testing, namely objective and subjective testing (Kemp, Hollowood, & Hort, 2011). In objective testing, the sensory properties of a food product are evaluated by a group of trained panellists using descriptive (e.g. identify the sensory difference and the magnitude of the difference) or discrimination (e.g. sensory differences between samples) tests. While in subjective testing, the responses of untrained consumers to the sensory attributes using hedonic ratings on a food product are determined.

Descriptive sensory analysis involves the discrimination and description of both the qualitative and quantitative sensory components of a food product by a trained panel (Meilgaard, Carr, & Civille, 2006). Descriptive analysis provides a comprehensive sensory description of a food product that enables the comparison of multiple sensory characteristics within food products (Kemp, Ng, Hollowood, & Hort, 2018). However, this analysis is more time-consuming and expensive than other sensory methods. For instance, Heymann, King, and Hofer (2014) reported that panellists with good sensory abilities were usually screened, selected and trained for a few (≤6) months to rate perceived intensity and quality in a way that is consistent within themselves and the panel to produce data that could be validated as acceptable. This sensory method is expensive because of the long period of training that is required and also because a large number of sensory sessions are required.

Consumer testing measures the subjective responses to a food product (Kemp et al., 2011). The insight on consumer preferences, attitudes, opinions, behaviours, and perceptions concerning the food products can be gained using both qualitative (e.g. sensory analysis) and quantitative (e.g. one-to-one interviews or focus group) methodologies. This test is considered as a key part of the

product development process of a food product. It is also important to monitor the market position and also to find avenues for product improvement or optimisation after the launch of the product. For quantitative consumer tests, large numbers of panellists, a minimum of at least 100, are required if the results are to be meaningfully extrapolated to the larger population. However, opinions are varied on the number of consumers necessary for the sensory acceptability test. Singh-Ackbarali and Maharaj (2014) reported that there should be a minimum of 20 consumers for pilot testing, and 75-150 consumers for the acceptance test. Meilgaard et al. (2006) stated that 50-300 responses should be collected for central location tests, while 75-300 responses per city in three or four cities for home use tests. Stone, Bleibaum, and Thomas (2012) recommended 25-50 subjects per product in laboratory testing, 100 or more responses per product in central location tests, and 50-100 families for home use tests. Quantitative consumer testing is used to measure either preference or acceptance of food products. Preference testing such as paired comparison and ranking tests, suggest some sort of hierarchy in the results but does not necessarily indicate if the consumer likes the product. Acceptance testing such as hedonic rating provides an indication of the magnitude of the level of liking for the product. The test ascertains how much consumers like the concept of the new food product and compares the level of liking with a control/ standard product.

In this research thesis, the chemical stability of sausages made from extruded meat alternatives and chicken breast (**Chapter 9**) were compared. The protein carbonyl contents of sausages at chilled storage (4°C) at 0, 7, 14 and 21 days were determined using DNPH method. The sensory evaluation of beef gravies made from beef bone extract and Maillard-reacted beef bone hydrolysates (**Chapter 5**), extruded meat alternatives at different SPC to WG ratio and chicken breast (**Chapter 6**), minced meat alternatives at different concentrations of MRPs (**Chapter 7**) and sausages made from extruded meat alternatives at different MC and chicken breast (**Chapter 9**) were studied. All sensory evaluations were conducted using acceptance testing to compare the level of liking among a few food products in different chapters by untrained consumers through hedonic ratings.

Chapter 4: [1] Effects of enzymatic hydrolysis treatments on the physicochemical properties of beef bone extract using endo- and exo-proteases

4.1 Abstract

This study reported the effects of enzymatic hydrolysis treatments on the physicochemical properties of beef bone extract using endo- and exo-proteases. The hydrolysis kinetics of each enzyme were studied using the Michaelis-Menten model and the ideal E/S ratio obtained for Protamex® (P), bromelain (B), and Flavourzyme® (F) was found to be 1.10, 1.60 and 4.70% w/w, respectively. Seven hydrolysates were produced from single (P, B, F), simultaneous (P+F, B+F) and sequential (P>F, B>F) treatments, where bone extract hydrolysed by Flavourzyme® exhibited highest DH and proportion of low *Mw* peptides (<5000 Da) in single treatment. When Flavourzyme® was used with Protamex® or bromelain in simultaneous or sequential treatments, no significant differences in *Mw* distribution, exposed SH content, SS content, and viscosity was evident compared with Flavourzyme® only. This indicated that without the addition of other enzymes, Flavourzyme® was capable of increasing the proportion of low *Mw* peptides and reducing viscosity.

Keywords: beef bone extract; enzymatic hydrolysis; Michaelis-Menten model; degree of hydrolysis; molecular weight distribution; viscosity

4.2 Introduction

Proteins from meat, milk, wheat, and soy are commonly used as ingredients in the food industry (Nielsen, 2009). In this study, meat protein (i.e. beef bone extract) obtained from meaty beef bones was pressure-cooked in water at 121°C for at least two hours, before the resulting liquid was extracted, defatted, and concentrated. However, opportunities to use beef bone extract as a food ingredient are often limited due to its high viscosity and weak flavour, which constrains its

[1] This chapter is published as Chiang, J.H., Loveday, S.M., Hardacre, A.K., & Parker, M.E. (2019). Effects of enzymatic hydrolysis treatments on the physicochemical properties of beef bone extract using endo- and exo-proteases. *International Journal of Food Science and Technology, 54(1), 111-120*. doi: https://doi.org/10.1111/ijfs.13911

application to relatively low-value ingredients such as soup- or sauce-based, sports nutrition or pet foods. Currently, there is interest in converting these low-value meat products into high-value functional food ingredients through enzymatic hydrolysis (Kristinsson & Rasco, 2000). The process reduces the viscosity by cleaving peptide bonds to release FAA and low *Mw* peptides (Villamil et al., 2017). It also enhances the flavour potential by generating meat flavour precursors and exposing the sulfhydryl groups (Lantto et al., 2009).

Enzymatic hydrolysis can be controlled to produce hydrolysates with desired compositions and properties by choosing appropriate enzymes, varying the E/S ratio, and controlling the pH, temperature and time of hydrolysis (Villamil et al., 2017). The use of commercial microbial (Alcalase®, Protamex®, Flavourzyme® and Neutrase®) or plant (papain, bromelain, actinidin) proteases have been used to hydrolyse beef, chicken and pig bones, pigskin, and marine fishes to produce hydrolysates of increased value (Hou, Li, Zhao, Zhang, & Li, 2011; Pagán et al., 2013; Dong et al., 2014; Song et al., 2016; Zhang et al., 2017). For example, a hydrolysate of chicken bone extract made using Protamex® or Flavourzyme® (Dong et al., 2014; Sun et al., 2014) and veal bone extract made using Neutrase® (Linder et al., 1997) was found to be nutritional and flavourful. This made them potential ingredients as a natural meat flavour enhancer for food products including soups, consommé, sauces, and gravies.

Proteases are classified according to the origin source (plant, animal or microbial), the site at which they hydrolyse a protein (endo- or exo-proteases) and the mechanism of enzyme catalysis (aspartic, glutamic, metallo, cysteine, serine or threonine proteases) (López-Otín & Bond, 2008; Benjakul et al., 2014). Protamex® is a microbial serine endoprotease obtained from *Bacillus sp.* that hydrolyses internal peptide bonds (mainly 'hydrophobic' -COOH) of a protein and is known to produce non-bitter hydrolysates (Liaset, Nortvedt, Lied, & Espe, 2002; Nguyen et al., 2011). Bromelain, a cysteine endoprotease derived from pineapple stems (Calkins & Sullivan, 2007), has low substrate specificity (Lys-, Arg-, Phe-, Tyr-COOH) and can hydrolyse different bonds such as peptide, amide, ester, thiol ester and thiono-ester bonds (Adler-Nissen, 1986; Cazarin, Lima, da Silva, & Maróstica Jr, 2016). Flavourzyme® is a mixture of fungal endo- and exo-proteases from *Aspergillus oryzae* strain with very broad specificity that minimises the bitterness that can occur in protein hydrolysates (Benjakul et al., 2014). The presence of exoproteases in Flavourzyme®

which cleave at the C- or N-terminal end of hydrophobic amino acid residues led to a reduction in bitterness (O'Sullivan, Nongonierma, & FitzGerald, 2017). Besides that, the extent of hydrolysis is important as excessive proteolysis reduces the *Mw* and could create unwanted flavours such as bitterness due to the formation of small peptides with a relatively high content of hydrophobic amino acids (Nielsen, 2009). The DHs directly influences the *Mw* distribution and amino acid composition of hydrolysates (Kristinsson & Rasco, 2000), whereas different DH indicate different functionality of hydrolysates such as *Mw*, where low *Mw* hydrolysates have lower viscosity (Nieto-Nieto, Wang, Ozimek, & Chen, 2014; Zhang et al., 2017).

The application of Protamex® and Flavourzyme® in single or sequential hydrolysis treatments to produce protein hydrolysates from animal bone extracts has been previously reported (Dong et al., 2014; Sun et al., 2014; Song et al., 2016). However, there are no published studies done on beef bone extract using combinations of plant and microbial proteases to hydrolyse protein. Furthermore, no work has been conducted on the hydrolysis kinetics of the three proteases, nor comparison of the hydrolysis efficiency between simultaneous and sequential hydrolysis treatments. Therefore, in this study, the objective was to investigate the effects of enzymatic hydrolysis treatments on the physicochemical properties of beef bone extract. Protamex®, bromelain and Flavourzyme® were used to investigate the extent of hydrolysis in single, simultaneous, and sequential hydrolysis treatments. The hydrolysis kinetics of each enzyme and the effects of hydrolysis treatments on the DH, *Mw* distribution, sulfhydryl and SS-bond contents, and viscosity of hydrolysates were studied. This allows us to have a better understanding of how to control and optimise the extent of hydrolysis in future meat flavour development work.

4.3 Materials and methods

4.3.1 Materials

Beef bone extract (≥44% protein, ≤55% moisture, ≤3% ash, ≤1% fat and ≥53°Brix total soluble solids) supplied by Taranaki Bio Extracts Ltd (Hawera, New Zealand) was used as the substrate for hydrolysis. Protamex® (1.5 Anson Units/g, Batch: PW2A1117) and Flavourzyme® 1000L (1000 Leucine Amino Peptidase Units/g, Batch: HPN00539) were obtained from Novozymes (Bagsværd, Denmark); and Enzidase® bromelain (1200 Gelatine Digesting Units/g, Batch: 190117) from Zymus International Ltd (Auckland, New Zealand). Sodium caseinate (92.7%

protein, 4.3% moisture, 3.6% ash, 0.2% carbohydrate and 0.7% fat) was obtained from Fonterra Co-operative Group Ltd (Palmerston North, New Zealand). Folin-Ciocalteu's phenol reagent, OPA, L-serine, glycine, SDS, trichloroacetic acid and guanidine thiocyanate were obtained from Sigma-Aldrich, New Zealand. Tris-(hydroxymethyl) aminomethane, ethylenediaminetetraacetic acid (EDTA), L-tyrosine and sodium sulphite were obtained from BDH VWR Analytical, Australia. 2,2'-dithio-5,5'-dithio-dibenzoic acid (DTNB) and DTT were obtained from Merck Life Science, New Zealand. Di-potassium hydrogen orthophosphate, potassium dihydrogen orthophosphate and copper (II) sulphate were obtained from Ajax Finechem, New Zealand. Disodium tetraborate decahydrate was obtained from Koch-Light Laboratories, U.K. Urea and ammonia solution were obtained from ThermoFisher Scientific, New Zealand. The chemicals were of ≥98-99% purity. Ultrapure water purified by treatment with a Milli-Q apparatus; Millipore Corporation (Bedford, Massachusetts, USA) was used in all experiments. All other chemicals and reagents used were of analytical grade.

4.3.2 Total amino acid composition determination

The total amino acid of beef bone extract was determined according to Wilkinson, Lee, Purchas, and Morel (2014) with slight modification. Beef bone extract was freeze-dried and ground to a particle size of <1 mm. Approximately 5 mg of the sample was mixed with 1 mL of 6 M HCl containing 0.1% phenol in glass hydrolysis tubes and sealed under vacuum to remove oxygen. Cysteine and methionine were determined using performic acid oxidation technique (AOAC 994.12). Hydrolysis was conducted to convert proteins to amino acids at 110°C for 24 h. The hydrolysate was then spiked with internal standard (50 µL of 40 mM L-Norleucine) and evaporated to dryness in a concentrator (Savant SpeedVac, Thermo Scientific, USA) to remove the acid. Then 2 mL of 0.02 N HCl with 0.1% phenol was added to the concentrated solution and filtered off using a 0.22 μm filter prior to amino acid determination using an ion-exchange HPLC system (LC-10A VP, Shimadzu, Japan) with amino acid cation exchange column (Waters, USA) and OPA post-column derivatisation, except for proline that was determined using another gradient system (Dionex RSLC3000, Thermo Scientific, USA) with pre-column derivatisation (AccQ Tag, Waters, USA) and C18 reverse-phase column (Dionex Acclaim, Thermo Scientific, USA) (AOAC, 2000). The total protein content of the beef bone extract was determined by the Kjeldahl method (N×6.25).

4.3.3 Enzyme activity assay

The proteolytic activity of each enzyme was determined according to Cupp-Enyard (2008) with slight modifications. Each enzyme was analysed under standard conditions and its recommended conditions (**Table 4-1**). One unit of the protease was defined as the amount of enzyme required to hydrolyse sodium caseinate to produce colour equivalent to 1 μM of tyrosine in 10 min. Sodium caseinate solution (0.65% w/v) was prepared in 0.05 M potassium phosphate buffer (PB) at pH 7.5. A mixture of 5 mL of the sodium caseinate solution and 1 mL of the enzyme solution diluted 1:1000 or for powdered enzyme diluted 1:5000 w/w was vortexed and incubated at 37°C for exactly 10 min. A "blank" was used by omitting the enzyme solution and replaced with ultrapure water. The enzyme activity was measured as the liberation of tyrosine from the substrate, which was measured as follows: The reaction was deactivated by adding 5 mL of 0.11 M trichloroacetic acid and held at 37°C for 30 min in a water bath. The precipitate was then removed using a 0.45 μm cellulose acetate (CA) syringe filter. Next, 2 mL of the filtrate was removed and added to 5 mL of 0.5 M sodium carbonate solution and 1 mL of 0.5 M Folin-Ciocalteu's phenol reagent. The reaction mixture was thoroughly vortexed and incubated at 37°C for 30 min and then filtered using a 0.45 μm CA syringe filter into a spectrophotometer cuvette. Absorbance was read against a blank at 660 nm using a spectrophotometer (Ultrospec II, Pharmacia, England) and triplicates of the samples analysed. Solutions of L-tyrosine at concentrations between 0.055-0.553 μM were used to generate a standard curve. One unit of the enzyme (U) was defined as follows:

$$units/mL\ enzyme = \frac{tyrosine\ equivalents\ released\ (\mu mole) \times A}{B \times C \times D} \qquad \textbf{(1)}$$

where A = total volume of assay (mL); B = volume of enzyme used (mL); C = time of assay as per the unit definition; and D = volume used in colourimetric determination (mL).

Table 4-1 Proteolytic activity of Protamex®, bromelain and Flavourzyme® at standard conditions (pH 7.5, 37°C) and recommended reaction conditions specified by the manufacturers

Enzymes	Standard condition	Total activity [1,2] (units/mg solid)	Recommended conditions	Total activity [1,2] (units/mg solid)
Protamex®	pH 7.5, 37°C	191.36 ± 30.43	pH 6.0, 40°C	240.84 ± 14.23
Bromelain		154.77 ± 10.25	pH 5.0, 55°C	258.06 ± 7.76
Flavourzyme®		290.54 ± 10.41	pH 6.0, 50°C	395.54 ± 9.85

[1] Values are expressed as the mean and standard deviation of three replicates.
[2] Enzyme activity (units/mg solid) which yielded the colour equivalent to 1 μmol of tyrosine per minute at each pH and temperature value assayed.

4.3.4 Enzymatic hydrolysis of beef bone extract

Beef bone extract was transferred into a 250-mL conical flask and pre-incubated in a temperature-controlled water bath before the enzyme was added. The enzymes were added on a weight basis rather than activity units. The native pH of bone extract was 6.68. The hydrolysis reaction was done in a shaking incubator (Multitron Standard, INFORS HT, Switzerland) at the recommended temperature of each enzyme (**Table 4-1**) for 120 min at 150 rpm. At the end of the hydrolysis, the flasks were placed in a heated water bath (85°C) for 15 min to inactivate the enzyme and then cooled in a cold-water bath for another 10 min. The hydrolysates were stored at 4°C before further analysis. The hydrolysis duration was limited to 120 min as most studies show that DH for similar enzymes started to exhibit a stationary phase at 120 min of hydrolysis (Pagán et al., 2013; Jridi et al., 2014; Shu, Zhang, Chen, Wan, & Li, 2015). The different enzyme systems used in the hydrolysis of beef bone extract is shown in **Table 4-2**.

To study the effect of E/S ratio, single-enzyme hydrolysis treatment was conducted at different E/S ratio at 0%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0% and 4.0% w/w (enzyme weight to protein weight). The DH was calculated to determinate the optimum E/S ratio for each enzyme, and single, simultaneous, and sequential hydrolysis treatments were then conducted. For single treatment, the reaction temperature was based on the recommended temperature of each enzyme. Recommended temperature of Flavourzyme® was used for simultaneous treatment. While, for sequential treatment, P>F and B>F followed the recommended temperature for Protamex® and Bromelain for the first two hours, respectively, followed by adjusting to the recommended temperature of Flavourzyme® for the next two hours.

Table 4-2 Different enzyme systems used in different hydrolysis treatment of beef bone extract

Sample description	Enzyme system [ab]	Hydrolysis treatment
P	Protamex®	Single
B	Bromelain	
F	Flavourzyme®	
P+F	Protamex® + Flavourzyme®	Simultaneous
B+F	Bromelain + Flavourzyme®	
P>F	Protamex® > Flavourzyme®	Sequential
B>F	Bromelain > Flavourzyme®	

[a] "P+F", "B+F" represent simultaneous hydrolysis using Protamex® or Bromelain with Flavourzyme®.
[b] "P>F", "B>F" represent sequential hydrolysis using Protamex® or Bromelain first followed by Flavourzyme® with intermediate temperature adjustment.

4.3.5 Degree of hydrolysis determination

The DH of beef bone hydrolysates was carried out using the OPA method as described by Nielsen et al. (2001) with slight modifications, to determine the concentration of α-amino groups in the hydrolysates. The OPA reagent was prepared as follows: 7.62 g disodium tetraborate decahydrate and 200 mg SDS were dissolved and sonicated in 160 mL ultrapure water. Fresh reagent was prepared by adding 40 mg OPA dissolved in 1 mL ethanol and 44 mg DTT dissolved in 1 mL ultrapure water for every 40 mL of borax/ SDS solution, and the mixture was made up to 50 mL with ultrapure water before analysis. L-serine standard was prepared as follows: 7 mg serine was diluted with 50 mL ultrapure water (1.332 meqv/L). The sample solution was prepared by diluting the hydrolysates with ultrapure water. Then, 1 mL OPA reagent and 100 μL sample, blank or serine standard were mixed in a 1.5 mL Eppendorf tube. The mixture was then vortexed and transferred into a semi-micro disposable cuvette. The samples were left to stand for exactly two min before reading the absorbance at 340 nm using a spectrophotometer. The values of constants, α, β and h_{tot} for meat protein were 1.0, 0.40 and 7.6, respectively. DH was calculated as follows:

$$DH(\%) = \frac{h}{h_{tot}} \times 100\% \qquad \textbf{(2)}$$

h was calculated as follows:

$$Serine\ NH_2 = \frac{A_{sample} - A_{blank}}{A_{standard} - A_{blank}} \times \frac{1.332\ meqv/L \times 0.1 \times 100}{X \times P} \qquad \textbf{(3)}$$

where serine NH_2 = milliequivalent serine NH_2/g protein; X = g sample; P = protein % in sample; 0.1 is the sample volume in litre (L). h was then calculated as follows:

$$h\ (meqv/g\ protein) = \frac{serine\ NH_2 - \beta}{\alpha} \quad \textbf{(4)}$$

4.3.6 Molecular weight distribution analysis

The *Mw* distributions of beef bone hydrolysates were determined by SEC-HPLC as described by Venuste et al. (2013), Zhang et al. (2017) and Nchienzia et al. (2010) with modifications. The system consisted of a HPLC system (LC-20AD, Prominence UFLC, Shimadzu, Japan) with an autosampler (SIL-20A HT), a column oven (CTO-20AC), together with an ultraviolet (UV) (SPD-20AV), and differential refractive index (DRI) detector (RID-20A) detectors. The eluent was prepared by dissolving 0.1 M PB (pH 7.0) and 0.02% w/v sodium azide in ultrapure water. The solution was filtered through a 0.22 μm membrane filter followed by a 0.025 μm membrane filter and was degassed before use. The hydrolysates were diluted to a concentration of 20 μL/mL with eluent and filtered through a 0.22 μm filter before sample loading. Separation of the soluble hydrolysate fraction was accomplished using an SEC column (SB-806M HQ, Shodex, Japan) connected to a guard column (OHpak SB-G 8B, Shodex, Japan). The eluent was continuously sparging with helium and pumped through the HPLC system to the SEC column at a flow rate of 0.5 mL/min at 1.5 MPa. The eluent from the SEC column flowed through the UV detector at 214 nm and the DRI detector. The hydrolysates (50 μL) were loaded into the column through an injection port and were separated at 35°C, over an elution time of approximately 45 min. Calibration curves were obtained using four molecular standards from Sigma-Aldrich: cytochrome C (12400 Da), aprotinin (6511 Da), insulin chain B (3495 Da) and leucine enkephalin (555 Da). The data was analysed using LabSolutions software (version 5.73, Shimadzu Corporation, Japan) to determine the *Mw* distribution. The *Mw* was calculated as follows:

$$\log Mw = -0.1385T + 7.1047, R^2 = 0.9967 \quad \textbf{(5)}$$

The equation was obtained from the calibration data where *Mw* represents the molecular weight, while T represents elution time.

4.3.7 Sulfhydryl content determination

The sulfhydryl (SH) contents of the hydrolysates were determined according to Chan and Wasserman (1993) and Yin, Tang, Wen, and Yang (2010) with slight modifications. Ellman's reagent was prepared by dissolving 4 mg of DTNB reagent in 1 mL of Tris-glycine buffer (0.086

M Tris, 0.09 M glycine and 4 mM EDTA, pH 8.0). Next, 30 μL of bone hydrolysates was mixed with 10 mL of Tris-glycine buffer with (total SH) or without 8 M urea and 1% SDS (exposed SH). Then, 100 μL of the Ellman's reagent was added. The resultant solution was incubated for an hour at 25±1°C in a water bath, with occasional shaking at 10 min intervals, it was then filtered using a 0.45 μm CA syringe filter. The absorbance of the filtered solution was determined at 412 nm against the reagent buffer as the blank. The total protein contents of the hydrolysates were determined by Kjeldahl method (N×6.25). The SH contents were calculated by using the extinction coefficient of 2-nitro-5-thiobenzoate (NTB) at 412 nm (13,600 $M^{-1}cm^{-1}$) and expressed as μmol/g protein.

4.3.8 Disulphide bond content determination

The SS-bond contents of the hydrolysates were determined according to Thannhauser, Konishi, and Scheraga (1987) and Yin et al. (2010) with slight modifications. The synthesis of 2-nitro-5-thiosulfobenzoate (NTSB) was performed by dissolving 0.1 g DTNB in 10 mL of 1 M sodium sulphite (Na_2SO_3). The pH of the reaction mixture was adjusted to 7.5, and 50 μL of 0.1 M ammoniacal solution copper (II) sulphate ($CuSO_4$) (three parts of NH_4OH mixed with one part of $CuSO_4$) was added. The reaction mixture was magnetically stirred at 38±1°C in a water bath for approximately 45 min until more than 99% of DTNB was transformed into NTSB. The reaction was followed by measuring the concentration of NTB by its absorbance at 412 nm. The NTSB test solution was prepared by diluting the reaction mixture (1:100 w/w) with fresh 0.2 M Tris-base buffer containing 0.1 M Na_2SO_3, 10 mM EDTA, and 3 M guanidine thiocyanate ($C_2H_6N_4S$). The NTSB test solution was then adjusted to pH 9.5 with 1 M HCl. Aliquots (200 μL) of bone hydrolysates were mixed with 6 mL of the NTSB test solution prepared just before use. Absorbance at 412 nm was determined using the NTSB test solution as the reference. The SS contents were calculated by using the extinction coefficient of NTB at 412 nm (13,600 $M^{-1}cm^{-1}$) and expressed as μmol/g protein.

4.3.9 Viscosity measurement

The viscosity of the hydrolysates was determined using a Paar Physica controlled-stress rheometer (MCR 301, Anton Paar Austria) fitted with concentric cylinder geometry (CC27 and C-

PTD 200). Steady-state viscosity measurements were carried out at shear rates ranging from 1.0 to 100 s^{-1} at 20±0.1°C with 5 measurements per decade. The measurements were performed in three replicates, and the results were expressed as an average value.

4.3.10 Data analysis

All experiments were carried out in three replicates, on new, freshly prepared samples and the results were reported as means ± standard deviations of the measurements. Data were analysed using Minitab® 16.2.1 statistical software (Minitab Inc, USA). Statistical analyses of observed differences among means consisted of one-way analysis of variance (ANOVA), followed by Tukey's pairwise comparison of means ($p \leq 0.05$). Figures were exported from Origin Software 8.5 (OriginLab Corp., MA, USA).

4.4 Results and discussions

4.4.1 Total amino acid composition of beef bone extract

The total amino acid composition of beef bone extract is shown in **Table 4-3**. The total protein content of bone extract from Kjeldahl method was 47.36±0.54%. The essential, non-essential and hydrophobic amino acid contents of bone extract were 19.49, 80.51 and 53.64% of the total amino acids, respectively.

Table 4-3 Total amino acid composition of beef bone extract

Amino acids	Amino acids content [1]		Predominant taste [a,b]
	mg/100 mg product	mg/100 mg protein	
Essential amino acids			
Histidine	0.47 ± 0.01	0.99 ± 0.03	Bitter
Isoleucine [#]	0.77 ± 0.01	1.63 ± 0.03	Bitter
Leucine [#]	1.72 ± 0.01	3.63 ± 0.05	Bitter
Lysine	1.75 ± 0.10	3.70 ± 0.21	Sweet and bitter
Methionine	0.81 ± 0.01	1.70 ± 0.03	Bitter
Phenylalanine	1.09 ± 0.04	2.30 ± 0.09	Bitter
Threonine	0.96 ± 0.02	2.03 ± 0.05	Sweet
Valine [#]	1.32 ± 0.02	2.79 ± 0.05	Bitter
Non-essential amino acids			
Alanine	3.83 ± 0.13	8.09 ± 0.28	Sweet
Arginine	3.47 ± 0.03	7.33 ± 0.10	Bitter
Aspartic acid	2.81 ± 0.16	5.93 ± 0.34	Sour
Cysteine	0.07 ± 0.01	0.14 ± 0.02	NA
Glutamic acid	4.81 ± 0.15	10.16 ± 0.33	Sour
Glycine	9.61 ± 0.25	20.29 ± 0.59	Sweet
Proline	5.32 ± 0.06	11.23 ± 0.19	Sweet and bitter
Serine	1.48 ± 0.01	3.13 ± 0.05	Sweet
Taurine	0.02 ± 0.01	0.04 ± 0.02	NA
Tyrosine	0.54 ± 0.02	1.14 ± 0.05	Bitter
Hydroxyproline	4.77 ± 0.21	10.07 ± 0.46	NA
Total amino acid content	**45.62 ± 0.44**	**96.32 ± 0.99**	
Essential amino acids	8.89 ± 0.11	18.77 ± 0.25	
Non-essential amino acids	36.73 ± 0.42	77.55 ± 0.95	
Hydrophobic amino acids[c]	16.89 ± 0.15	35.67 ± 0.37	

[1] Values are expressed as the mean and standard deviation of three replicates.
[a] Li-Chan and Cheung (2010).
[b] NA = Not available
[c] Hydrophobic amino acids (AA): Alanine, Cysteine, Histidine, Isoleucine, Leucine, Methionine, Phenylalanine, Proline, Threonine, Tyrosine, Valine (Damodaran, 2008).
[#] Branched-chain amino acids (BCAAs).

Bone extract contained all essential amino acids other than tryptophan (not analysed), making it nutritionally beneficial. Clemente (2000) reported that the hydrophobic amino acid content contributed to the bitter flavour of peptides, which could be an issue when used in foods. The use of enzymes such as Flavourzyme®, which cleave at the C- or N-terminal end of hydrophobic amino acids, could be used to reduce the bitterness of bone extract. The predominant amino acids in bone extract were glutamic acid, glycine, proline and hydroxyproline. Bone extract contained 10.16 mg/100 mg protein of glutamic acid, the sweet-umami amino acid used in the

food industry as a flavour enhancer in the form of monosodium salt (Sukkhown, Jangchud, Lorjaroenphon, & Pirak, 2017). Bone extract contained 10.07 mg/100 mg protein of hydroxyproline, which constituted 0.75 mg/mg protein of collagen, which gelled at chilled condition (≤4°C). In order to obtain protein hydrolysates with low bitterness, high umami taste, low viscosity and non-gelling at chilled condition, enzyme selection is critical. Results from the amino acid composition showed that bone extract was a good source of collagen for various food applications.

4.4.2 Enzyme-substrate (E/S) ratio of each enzyme on beef bone extract

The DH of beef bone extract hydrolysed by Protamex®, bromelain and Flavourzyme® at E/S ratio of 0-4% w/w are shown in **Figure 4-1**. Bone extract has a DH of 0.33±0.01%, without the addition of proteases. This could be due to the manufacturing process of bone extract, where the bones are pressure-cooked in water for at least two hours at 121°C. Similar hydrolysis due to heat treatment had been reported for WG proteins (Elmalimadi et al., 2017). Bone extract hydrolysed by Flavourzyme® showed the highest DH compared to Protamex® and bromelain at E/S ratio of 0.5-4% w/w. The high DH in bone extract hydrolysed by Flavourzyme® could be due to its preferential specificity, as Flavourzyme® contains a mixture of endo- and exoproteases. Protein hydrolysed by the mixture of endo- and exoproteases could generate higher DH, as endoproteases digest both the secondary and tertiary structure of protein substrates, followed by removing a single amino acid, a dipeptide or a tripeptide from one of the free N- or C-terminals by exoproteases. Both Protamex® and bromelain contain only endoproteases. Therefore, bone extract hydrolysed by these two enzymes exhibited a lower DH than Flavourzyme®.

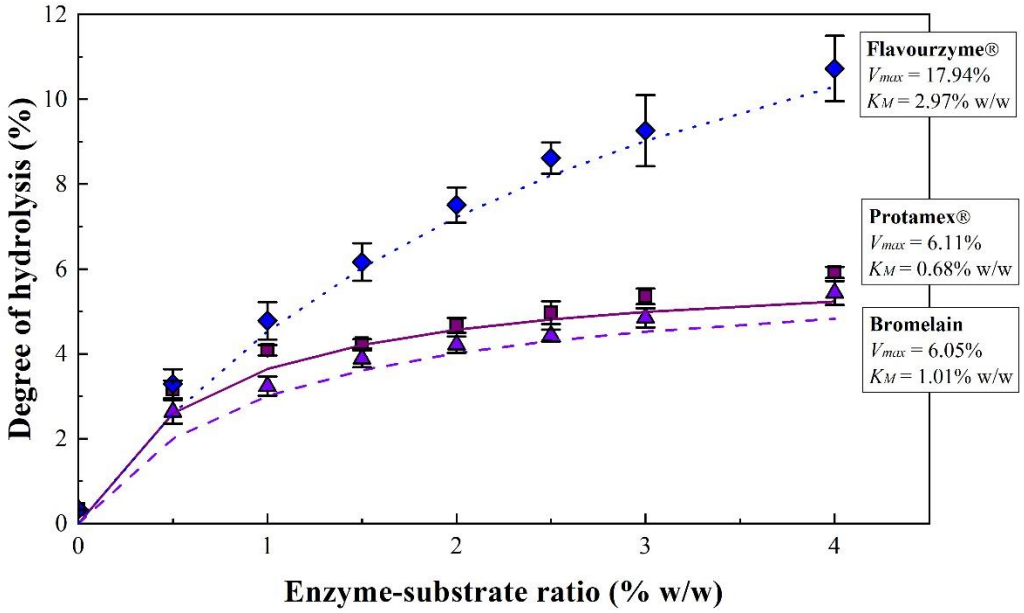

Figure 4-1 Degree of hydrolysis (DH) of beef bone extract hydrolysed by Protamex® (■), bromelain (▲) and Flavourzyme® (◆) at different enzyme-substrate (E/S) ratio ranging 0-4% w/w at 120 min of hydrolysis. The symbols and lines represent experimental and calculated DH, respectively (fitted using Michaelis-Menten equation). The modelled maximum DH ($\boldsymbol{V_{max}}$) and E/S ratio ($\boldsymbol{K_M}$) at 50% $\boldsymbol{V_{max}}$ for Protamex (—), bromelain (—) and Flavourzyme (—) were presented. Data represent the mean and error bars represent the standard deviation. The values of DH among different enzymes were significantly different ($p \leq 0.05$) according to Tukey's post-hoc test.

There was no stationary phase for the three enzymes with increasing enzyme concentration, thus making it difficult to determine the optimum E/S ratio for each enzyme. Michaelis-Menten model is generally used to calculate the substrate concentration rather than enzyme concentration in an enzymatic reaction. However, in this study, the Michaelis-Menten equation was used to determine the optimum E/S ratio in order to prevent enzyme saturation in case excess enzymes are being added:

$$v = \frac{V_{max}[S]}{K_M + [S]} \quad \textbf{(6)}$$

Where V is the degree of hydrolysis (DH; %) after 120 min. V_{max} is the maximum DH (%) at the maximum (saturating) E/S ratio after 120 min. The Michaelis constant K_M is the E/S ratio (% w/w) at which the reaction rate was 50% of V_{max} after 120 min. $[S]$ is the E/S ratio (% w/w). Based on the Michaelis-Menten equation using Microsoft Excel's Solver analysis tool, the modelled V_{max}

and K_M values for the three enzymes fitted using the Michaelis-Menten model are shown in **Figure 4-1**. In order to obtain a reasonable rate of hydrolysis, the ideal E/S ratio (K values) were calculated based on 80% V_{max}, and were found to be 1.10, 1.60 and 4.70% w/w for Protamex®, bromelain and Flavourzyme®, respectively.

4.4.3 Effects of hydrolysis treatments on the degree of hydrolysis (DH) of beef bone hydrolysates

The DH of beef bone extract hydrolysed by Protamex®, bromelain and Flavourzyme® at different hydrolysis treatments are shown in **Figure 4-2**. Bone extract hydrolysed by Flavourzyme® exhibited highest DH compared to Protamex® and bromelain in single hydrolysis treatment. This was due to the high E/S ratio used to hydrolyse bone extract by Flavourzyme®, as well as larger activity of Flavourzyme® according to **Figure 4-1** at a range of E/S ratios.

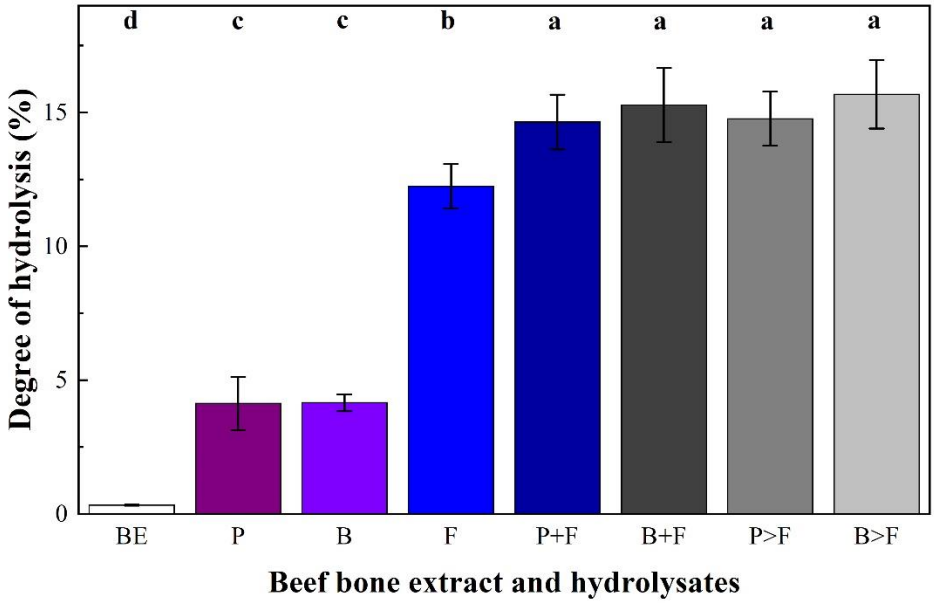

Figure 4-2 Degree of hydrolysis of beef bone extract hydrolysed by Protamex®, bromelain and Flavourzyme® at different hydrolysis treatments (i.e. single, simultaneous and sequential). Data represent the mean and error bars represent the standard deviation. Values bearing different lowercase letters were significantly different ($p \leq 0.05$) according to Tukey's post-hoc test.

When combining Flavourzyme® with Protamex® or bromelain in simultaneous and sequential hydrolysis treatments, there was a significant increase in DH compared to single

hydrolysis treatment. This correlated well with the previous study by Nchienzia et al. (2010), where simultaneous and sequential hydrolysis treatment of poultry meal using Acalase® and Flavourzyme® had higher DH than single hydrolysis treatment using Acalase® or Flavourzyme®. Bone extract hydrolysed using sequential hydrolysis treatment showed higher DH than simultaneous hydrolysis treatment, which was in accordance with Nchienzia et al. (2010). This could be due to longer reaction time, a total of four hours for sequential hydrolysis treatment and two hours for simultaneous hydrolysis treatment, allowing the enzymes to have more time to hydrolyse bone extract. Besides that, the conditions used for simultaneous hydrolysis treatment followed by Flavourzyme® at its recommended temperature, hence, Protamex® and bromelain might not be able to perform at its optimum when hydrolysing bone extract. However, there was no significant difference in simultaneous and sequential hydrolysis treatment when using the same enzyme combinations. The small gains of DH in sequential hydrolysis treatment may not be worthwhile, as it required twice as long to hydrolyse bone extract as compared to simultaneous hydrolysis treatment.

4.4.4 Effects of hydrolysis treatments on the molecular weight distribution of beef bone hydrolysates

The *Mw* distribution of beef bone extract hydrolysed by Protamex®, bromelain and Flavourzyme® at different hydrolysis treatments are shown in **Table 4-4**. Different molecular standards were injected in separate runs and a regression equation that relates *Mw* and elution time was established (**Equation (5)**). The samples were separated into five fractions (i.e. <1000, 1000-5000, 5000-10000, 10000-30000, >30000 Da) by SEC column at UV wavelength of 214 nm.

Table 4-4 Molecular weight distribution (range between <1000 and >30000 Da) of beef bone extract hydrolysed by Protamex®, bromelain and Flavourzyme® at different hydrolysis treatments (i.e. single, simultaneous and sequential)

Extract and hydrolysates [3]	Molecular weight (Da) [1,2]				
	>30000	10000-30000	5000-10000	1000-5000	<1000
Bone extract	24.33±3.65^{a}	52.17±3.39^{c}	3.59±2.18^{a}	10.41±2.44^{c}	9.50±2.26^{c}
Protamex®	2.84±0.38^{b}	71.68±4.68^{a}	0.91±0.34^{b}	13.71±3.11^{b}	10.86±1.53^{bc}
Bromelain	2.92±0.53^{b}	71.44±3.99^{a}	0.87±0.77^{b}	13.87±3.33^{b}	10.90±0.90^{bc}
Flavourzyme®	2.83±0.35^{b}	64.90±1.88^{b}	0.49±0.26^{b}	18.34±1.11^{a}	13.44±2.31^{ab}
P+F	2.95±0.37^{b}	64.08±1.22^{b}	0.75±0.10^{b}	18.82±0.83^{a}	13.40±1.61^{ab}
B+F	2.19±0.99^{b}	66.44±1.43^{ab}	0.67±0.24^{b}	18.44±0.52^{a}	12.26±0.27^{abc}
P>F	2.07±1.08^{b}	64.24±0.83^{b}	0.94±0.23^{b}	18.77±0.56^{a}	13.98±2.26^{a}
B>F	2.54±0.55^{b}	67.49±3.26^{ab}	0.83±0.07^{b}	17.40±1.39^{a}	11.74±1.32^{abc}

[1] Values are expressed as the mean and standard deviation of three replicates.
[2] Values bearing different lowercase letters in the same column were significantly different ($p \leq 0.05$) according to Tukey's post-hoc test.
[3] Peptides in bone extract or hydrolysates expressed as a percent of total area from SEC-HPLC intensity curves (%).

The *Mw* distribution was related to DH, where hydrolysates with higher DH showed a higher proportion of low molecular peptides (<5000 Da), which was in accordance with Dong et al. (2014) and Zhang et al. (2017). Bone extract contained the highest proportion of peptides with *Mw* >30000 Da and the lowest proportion of peptides with *Mw* <5000 Da compared to the hydrolysates. Bone extract hydrolysed by Flavourzyme® displayed the highest proportion of peptides with *Mw* <5000 Da for single hydrolysis treatments. This correlated with the DH and could be due to Flavourzyme® containing a mixture of endo- and exoproteases. When combining Flavourzyme® with Protamex® or bromelain in simultaneous and sequential hydrolysis treatments, there was no significant difference in *Mw* distribution compared to single hydrolysis treatment. This showed that Flavourzyme® is effective in breaking down proteins into smaller peptides, without the addition of other enzymes. Besides that, there was also no significant difference in *Mw* distribution between simultaneous and sequential hydrolysis treatments.

4.4.5 Effects of hydrolysis treatments on exposed sulfhydryl and disulphide bond contents of beef bone hydrolysates

The SH and SS contents of beef bone extract hydrolysed by Protamex®, bromelain and Flavourzyme® at different hydrolysis treatments are shown in **Figure 4-3**. It was observed that

with increasing DH, the hydrolysates tended to have a decrease in exposed SH content and an increase in SS content. All samples contained a similar proportion of total SH contents (results not shown) as there was complete extraction of SH groups in bone extract and hydrolysates using tris-glycine buffer containing urea and SDS, due to the comparable protein contents in all samples. Bone extract hydrolysed by bromelain showed highest exposed SH contents compared to Protamex® and Flavourzyme® in single hydrolysis treatment. This could be due to bromelain, a cysteine protease containing one reactive SH group per molecule, that reacts stoichiometrically with DTNB (Murachi, 1976). As there was no centrifugation step in the enzymatic hydrolysis process, the SH group in the bromelain residue could contribute to the exposed SH contents. When combining Flavourzyme® with Protamex® or bromelain in simultaneous and sequential hydrolysis treatment, there was no significant difference in exposed SH contents when compared with Flavourzyme® only, except for B+F. Besides that, there was no significant difference in SH contents for the hydrolysates in simultaneous and sequential hydrolysis treatments. Enzymatic hydrolysis led to the decrease of exposed SH contents in bone hydrolysates relative to bone extract, which was also reported by Zhao, Liu, Zhao, Ren, and Yang (2011). Proteases caused the partial unfolding of proteins or peptides, by uncovering the buried hydrophobic or SH group, leading to exposure of the thiol groups to the molecular surface and could then form aggregates (e.g. sulfhydryl-disulphide bond interchange) (Zhang et al., 2017).

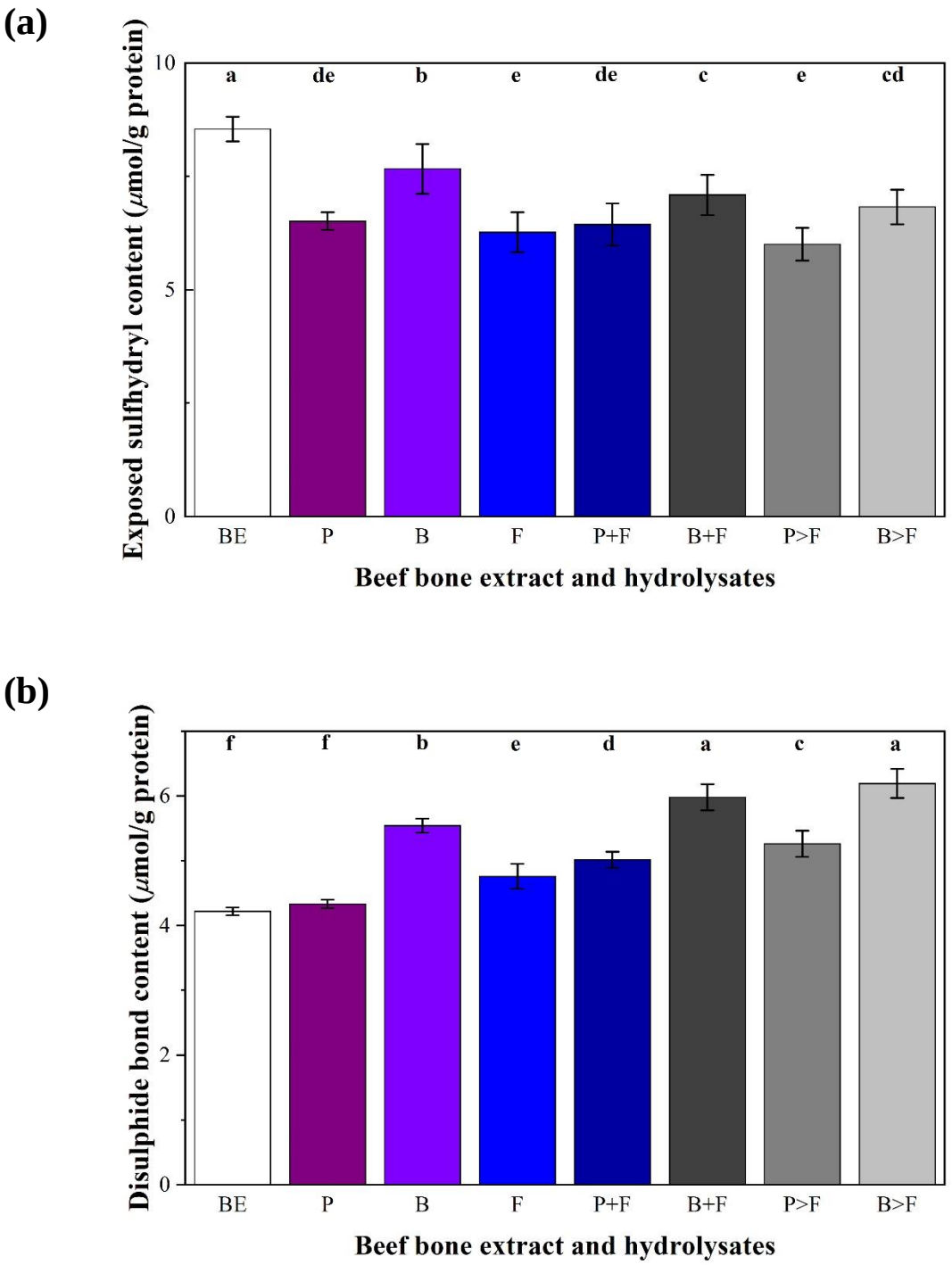

Figure 4-3 (a) Exposed sulfhydryl and (b) disulphide bond contents of beef bone extract hydrolysed by Protamex®, bromelain and Flavourzyme® at different hydrolysis treatments (i.e. single, simultaneous and sequential). Data represent the mean and error bars represent the standard deviation. Values bearing different lowercase letters in the same column were significantly different ($p \leq 0.05$) according to Tukey's post-hoc test.

Similar to exposed SH contents, bone extract hydrolysed by bromelain showed highest SS contents compared to Protamex® and Flavourzyme® in single hydrolysis treatment. When combining Flavourzyme® with Protamex® or bromelain in simultaneous and sequential hydrolysis treatment, there was only significant difference in SS contents for B+F and B>F when compared with single hydrolysis treatment. There was also no significant difference in SS contents between simultaneous and sequential hydrolysis treatments. It was observed that increased DH led to significant changes in SS contents, except for hydrolysates produced using bromelain. The SH group in the bromelain residue could undergo SH/SS interchange that led to the increase in SS contents for hydrolysates produced using bromelain. The increasing DH could promote exposure and formation of SS during the enzymatic hydrolysis which was also reported by Zhang et al. (2017).

4.4.6 Effects of hydrolysis treatments on viscosity of beef bone hydrolysates

The apparent viscosity of beef bone extract decreased from 7.48±0.51 to 5.24±0.03 Pa.s as the shear rate increased from 1 to 100 s^{-1}, showing slight shear thinning behaviour. Shear-thinning behaviour was attributed to the stretching of fibrous meat protein (mainly collagen) and parallel alignment with flow stream during shearing (Tornberg, 2005). The apparent viscosity of bone extract was approximately 10× higher than the hydrolysates that exhibited Newtonian behaviour. The apparent viscosities of seven hydrolysates were found to be lower than bone extract. This could be due to the higher DH, where a larger proportion of high *Mw* peptides being cut into smaller fragments by the enzymes, resulted in reducing the viscosity of the hydrolysates.

Bone extract hydrolysed by Flavourzyme® (0.181±0.009 Pa.s at a shear rate of 100 s^{-1}) in single hydrolysis treatment exhibited the lowest apparent viscosity. Protamex® and bromelain were 0.379±0.007 and 0.349±0.017 Pa.s at a shear rate of 100 s^{-1}, respectively. When combining Flavourzyme® with Protamex® or bromelain in simultaneous and sequential hydrolysis treatment, there was no significant difference in apparent viscosities at a shear rate of 100 s^{-1} when compared with Flavourzyme® only. This shows that Flavourzyme® is efficient in reducing the viscosity of bone extract, without the addition of other enzymes. Overall, the results indicated that enzymatic hydrolysis affected beef bone extract by reducing the viscosity of hydrolysates.

4.5 Conclusion

The objective of this study was met where Protamex®, bromelain, Flavourzyme® and its combinations, had successfully hydrolysed beef bone extract into bone hydrolysates. Bone extract contained high protein content, low-fat level and was rich in flavour amino acids (aspartic and glutamic acids), making it a potential ingredient in food applications. The use of the Michaelis-Menten model for optimum E/S ratio determination had effectively shown the hydrolysis kinetics for the three enzymes. Of the three enzymes in single hydrolysis treatment, Flavourzyme® provided the greatest increase in DH, and consequentially the largest proportion of small *Mw* peptides (<5000 Da) and the greatest reduction in viscosity. Combining Flavourzyme® with Protamex® or bromelain significantly increased DH. However, simultaneous or sequential hydrolysis treatments made little difference in any of the measured parameters. Hence, simultaneous hydrolysis treatment was preferred as it required a shorter hydrolysis time. The enzymatic hydrolysis of bone extract demonstrated potential in converting these low-value meat products into high-value functional ingredients with low viscosity and non-gelling characteristics. The impact of these changes on meat flavour development will be further studied.

Chapter 5: [2] Changes in the physicochemical properties and flavour compounds of beef bone hydrolysates after Maillard reaction

5.1 Abstract

This study investigated the changes in physicochemical properties and volatile compounds of beef bone hydrolysates during heat treatment as a result of the MR. Five beef bone hydrolysates obtained from single (P-Protamex®, B-bromelain, and F-Flavourzyme®) and simultaneous (P+F and B+F) enzymatic hydrolysis treatments were combined with ribose in aqueous solutions and heated at 113°C to produce MRPs. Total FAA decreased after heat treatment indicating the occurrence of the MR. MRPs showed a decrease in pH and an increase in browning intensity as the DH of hydrolysates increased. The volatile compounds generated during heat treatment were evaluated using gas chromatography-mass spectrometry (GC-MS) with headspace solid-phase microextraction (SPME) sampling. A total of 40 volatile compounds were identified in all MRPs and their concentration was found to increase with increasing DH. Pyrazines were the most abundant class of compounds produced as a result of the MR. F-MRP showed the highest peak area intensity for 17 volatile compounds in single hydrolysis treatment followed by heat treatment. There was also no significant difference in those major volatile compounds between F-MRP and P+F-MRP or B+F-MRP from simultaneous hydrolysis treatment after heating. F-MRP obtained the highest score for meaty taste and overall acceptability. Hence, the use of Flavourzyme® alone to increase the flavour intensity of beef bone extract is recommended. Overall results indicated that enzymatic hydrolysis and MR could be used to modify the flavour characters of beef bone extract.

Keywords: Maillard reaction, beef bone hydrolysates, peptide contents, free amino acids, volatile compounds

[2] This chapter is published as Chiang, J.H., Eyres, G.T., Silcock, P.J., Hardacre, A.K., & Parker, M.E. (2019). Changes in the physicochemical properties and flavour compounds of beef bone hydrolysates after Maillard reaction. *Food Research International, 123, 642-649*. doi: https://doi.org/10.1016/j.foodres.2019.05.024

5.2 Introduction

The MR, also known as Maillard browning or non-enzymatic browning, plays an important role in the development of volatile flavour compounds and the appearance of cooked food (Van Ba et al., 2012). MR was described by French chemist Louis Maillard (1912) when he investigated the browning reaction between lysine and glucose. MR takes place with the involvement of primary precursors, such as compounds with an available amino group (e.g. amines, FAA, peptides or proteins) and reducing sugars (e.g. ribose, xylose, glucose or fructose) at specific heating conditions to produce MRPs (Reineccius, 2005; Van Boekel, 2006; Van Ba et al., 2012). These precursors react during heating in primary reactions to form intermediate products. The intermediate products further react with other degradation products to form a complex combination of volatiles responsible for flavour, aroma, and dark-coloured pigments.

The MR plays an important role in meat flavour development, together with lipid oxidation, caramelisation or ascorbic acid browning (Reineccius, 2005). The most abundant flavour compounds formed via MR are aliphatic aldehydes, ketones, diketones, and short-chain fatty acids. However, heterocyclic compounds containing oxygen, nitrogen, sulphur, or combinations of these atoms, are much more numerous and play a significant role in the flavour development of thermally processed foods. The development of meat flavour is often influenced by reacting sulphur-containing compounds, such as amino acids, with reducing sugars (Kerler et al., 2010). Flavour development via MR largely depends on the type of amino acids and reducing sugars present, and the reaction conditions such as temperature, time and pH (Jousse et al., 2002). The choice of sugar type is of minimal importance in determining flavour character while the choice of the amino acid is very important (Reineccius, 2005). The type of sugar tends to play a larger role in determining the rate of reaction, rather than influencing the flavour character (Izydorczyk, 2005). In this study, the pentose sugar ribose was used to study MRPs made with beef bone hydrolysates. This monosaccharide is known to be associated with the ribonucleotides in meat muscle, highly involved in MR during thermal processing of meat flavour, and has a greater order of reactivity and rate of reaction than hexoses, disaccharides, trisaccharides, etc. (Jayasena et al., 2013). The type of amino acids determines the flavour compounds formed, for instance, flavour-active sulphur-containing compounds are usually generated from MR between cysteine and ribose (Elmore et al., 2002; Cerny & Davidek, 2003).

Meat hydrolysates or meat extracts have been used as condiments to impart meat-like flavours to food products (Varavinit et al., 2000). However, research has shown that meat extracts impart inferior flavour and odour characteristics compared to meat hydrolysates. Studies by Sun et al. (2014) and Song et al. (2016) showed that animal bone extracts could be processed into flavour ingredients through enzymatic hydrolysis and MR. Dong et al. (2014) demonstrated the use of Flavourzyme® to improve the flavour quality of chicken bone extracts by significantly increasing the proportion of pyrazine and sulphur compounds in the hydrolysates. In a further study, Sun et al. (2014) used Protamex® and Flavourzyme® in a sequential hydrolysis treatment and reported that MR significantly reduced the bitter taste and improved the overall flavour of hydrolysed chicken bone extracts. Song et al. (2016) reported high scores for mouthfeel, umami and meat sensory attributes of beef bone protein hydrolysed with a combination of lipase and papain, followed by MR with xylose.

The use of enzymes including lipase, papain, Protamex® and Flavourzyme® in single or sequential hydrolysis treatments followed by MR to produce MRPs from animal bone extracts has been previously reported (Sun et al., 2014; Song et al., 2016). However, there are no studies regarding MRPs made from hydrolysates using combinations of plant and microbial proteases using a simultaneous hydrolysis treatment. Therefore, the objective of this study was to compare the effects of single and simultaneous enzymatic hydrolysis treatments using Protamex®, bromelain and Flavourzyme® on the physicochemical properties and volatile compounds of beef bone hydrolysates with ribose after heat treatment due to the progression of the MR. The changes in pH, browning intensity, FAA and peptide contents of MRPs were compared. The changes in volatile profiles of MRPs were evaluated using headspace solid-phase microextraction (SPME) with gas chromatography-mass spectrometry (GC-MS).

5.3 Materials and methods

5.3.1 Materials

Beef bone extract (≥44% protein, ≤55% moisture, ≤3% ash, ≤1% fat and ≥53°Brix total soluble solids) supplied by Taranaki Bio Extracts Ltd (Hawera, New Zealand) was used as the substrate for hydrolysis. Protamex® (1.5 Anson Units/g, Batch: PW2A1117) and Flavourzyme® 1000L (1000 Leucine Amino Peptidase Units/g, Batch: HPN00539) were obtained from

Novozymes (Bagsværd, Denmark); and Enzidase® bromelain (1200 Gelatine Digesting Units/g, Batch: 190117) was sourced from Zymus International Ltd (Auckland, New Zealand). D-ribose was obtained from Amtrade NZ Ltd (Auckland, New Zealand). 1,2-Dichlorobenzene and γ-aminobutyric acid were obtained from Sigma-Aldrich, New Zealand. Disodium phosphate and sodium tetraborate decahydrate were obtained from ThermoFisher Scientific, New Zealand. Methanol and sodium azide was obtained from Merck Life Science, New Zealand. Acetonitrile was obtained from BDH VWR Analytical, Australia. The chemicals were of ≥98-99% purity. Ultrapure water purified by treatment with a Milli-Q apparatus; Millipore Corporation (Bedford, Massachusetts, USA) was used in all experiments. All other chemicals and reagents used were of analytical grade.

5.3.2 Enzymatic hydrolysis of beef bone extract

The proteolysis of beef bone extract (BE) was conducted as described by Chiang, Loveday, Hardacre, and Parker (2019b). The different enzyme systems used in the hydrolysis of BE are shown in **Table 5-1**. The DH of beef bone hydrolysates was conducted using the OPA method as described by Chiang et al. (2019b), to determine the concentration of α-amino groups in the hydrolysates.

Table 5-1 Enzyme systems used and degree of hydrolysis (%) for single and simultaneous hydrolysis treatment of beef bone extract

Sample	Enzyme system [1]	Conditions for enzymatic hydrolysis [2]	Degree of hydrolysis [3] (%)
P	Protamex®	Temperature: 40°C E/S: 1.10% w/w	4.13 ± 0.99^{c}
B	Bromelain	Temperature: 55°C E/S: 1.60% w/w	4.16 ± 0.32^{c}
F	Flavourzyme®	Temperature: 50°C E/S: 4.70% w/w	12.24 ± 0.83^{b}
P+F	Protamex® + Flavourzyme®	Temperature: 50°C E/S: 1.10% w/w (P), 4.70% w/w (F)	14.65 ± 1.03^{a}
B+F	Bromelain + Flavourzyme®	Temperature: 50°C E/S: 1.60% w/w (B), 4.70% w/w (F)	15.28 ± 1.38^{a}

[1] "P+F", "B+F" represent simultaneous hydrolysis using Protamex® or Bromelain with Flavourzyme®.
[2] Enzyme-substrate (E/S) ratio is based on enzyme weight to protein weight.
[3] Data are presented as the mean and standard deviation of three replicates.
Values bearing different lowercase letters in the same column were significantly different ($p \leq 0.05$) according to Tukey's post-hoc test.

5.3.3 Preparation of Maillard reaction products (MRPs)

Beef bone hydrolysates and D-ribose were mixed at a ratio of 1:0.068 (protein weight to reducing sugar weight) in screw-capped laboratory glass bottles and capped loosely before heat treatment to facilitate the MR. The mixtures were adjusted to pH 6.5 with 1 M NaOH as the mixture decreased after mixing with ribose. The mixtures were then heated at 170 kPa (113°C) for 10 min in a pressure cooker (Model No. 921, All American, Wisconsin, USA) for the production of MRPs. After heating, five MRP solutions (marked as P-MRP, B-MRP, F-MRP, P+F-MRP and B+F-MRP) were allowed to cool in the pressure cooker for 15 min, to ensure the internal pressure decreased slowly to ambient before sample removal. The MRPs were then placed in a cold-water bath for another 15 min and then stored at 4°C before further analysis. A control sample (BE-MRP) was prepared from BE (without any hydrolysis treatment) and ribose using the same preparation conditions described above.

5.3.4 pH analysis

The pH of BE, hydrolysates and MRPs were measured using a benchtop pH meter (SG23, SevenGo Duo™, Mettler-Toledo AG, Switzerland). The pH meter was calibrated using buffer solutions (pH 4 and 7) before analysis. The pH values were recorded once the readings stabilised.

5.3.5 Browning intensity analysis

The browning intensities of BE, hydrolysates and MRPs were analysed using absorbance readings at 420 nm according to Sun et al. (2011) and Tan, Abbas, and Azhar (2012), using a spectrophotometer (Ultrospec II, Pharmacia, England). Samples were diluted to a protein concentration of 5 mg/mL with ultrapure water. The absorbance at 550 nm was also measured to correct for any turbidity in the samples:

$$A^{*}_{420} = A_{420} - A_{550} \quad \textbf{(1)}$$

where, A^{*}_{420}; browning index, A_{420}; absorption at 420 nm and A_{550}; absorption at 550 nm for turbidity correction.

5.3.6 Molecular weight distribution analysis

The *Mw* distribution of BE, hydrolysates and MRPs was determined by SEC-HPLC as described by Chiang et al. (2019b). The mobile phase containing 0.1 M PB (pH 7.0) and 0.02% w/v sodium azide in ultrapure water, was filtered through a 0.22 μm filter followed by a 0.025 μm filter and degassed prior to analysis. Each sample was diluted to a concentration of 20 μL/mL with mobile phase and filtered through a 0.22 μm syringe filter before sample loading. Sample separation was conducted using an SEC column (Shodex SB-806M HQ, Japan) connected to a guard column (Shodex OHpak SB-G 8B, Japan). The mobile phase was sparged with helium and pumped through the HPLC system (Shimadzu, Japan) to the SEC column at a flow rate of 0.5 mL/min at 1.5 MPa, then through the UV detector at 214 nm. The samples (50 μL) were loaded into the column through an injection port and were separated at 35°C, over an elution period of 45 min. Calibration curves were obtained using cytochrome C (12400 Da), aprotinin (6511 Da), insulin chain B (3495 Da) and leucine enkephalin (555 Da) as *Mw* standards from Sigma-Aldrich, USA. The data was analysed using LabSolutions software (version 5.73, Shimadzu, Japan) to determine the *Mw* distribution.

5.3.7 Analysis of free amino acids

The FAA content for BE, hydrolysates and MRPs were determined as described by Chungchunlam, Henare, Ganesh, and Moughan (2015) with modifications. Samples were diluted (5× for hydrolysates and 10× for BE and MRPs) with ultrapure water and deproteinised by ultrafiltration using a polyethersulfone (PES) membrane centrifugal concentrators (Vivaspin 500, 5 kDa *Mw*CO, Sigma-Aldrich, New Zealand) at 13000 rpm for 60 min with a mini centrifuge (Heraeus Fresco 17, Thermo Scientific, USA). After centrifugation, 50 μL or 100 μL of the supernatant of diluted hydrolysates or MRPs, respectively, were added with 0.02 N hydrochloric acid (HCl) containing 50 nmol/mL of γ-aminobutyric acid (internal standard) into an HPLC vial and to make up to the final volume of 1 mL with ultrapure water.

FAAs were determined by injecting 0.5 μL of each sample into HPLC (Agilent 1200SL, Agilent Technologies, USA) after pre-column derivatisation with OPA. A 150 mm × 2.1 mm id, 3.5 µm particle size, C18 reverse-phase column (ZORBAX Eclipse Plus, Chrom Tech, USA) was used for separation at a flow rate of 0.42 mL/min. The column was operated at 40°C. Two mobile phases were used: Mobile A was composed of 0.01 M disodium phosphate, 0.01 M sodium tetraborate decahydrate and 0.5 mM sodium azide at pH 8.2. Mobile B was composed of methanol: acetonitrile: ultrapure water, (45: 45: 10 v/v/v). A fluorescence detector operated at 230 nm (excitation) and 450 nm (emission) was used to estimate amino acid concentrations in the sample. Amino acids standards (Sigma-Aldrich, New Zealand) were used for the identification of compounds based on retention time. Quantification was determined using external calibration curves. The system used for amino acids analysis gave poor resolution for proline, therefore, no data for this amino acid are shown.

5.3.8 Volatile components analysis using Gas Chromatography-Mass Spectrometry (GC-MS)

Volatile extraction by headspace solid-phase microextraction (HS-SPME) was conducted according to Richter, Eyres, Silcock, and Bremer (2017) with modifications. Beef bone extract and MRPs (1.0 ± 0.1 g) along with 1.0 g of 30% (w/w) sodium chloride solution were placed in 20 mL flat-bottom headspace vials and sealed with PTFE-coated silicone septa screw caps. 1,2-Dichlorobenzene in methanol (50 μL, 5 μg/mL) was used as an internal standard. A 2 cm SPME

fibre coated with 50/30 μm DVB/CAR/PDMS (Supelco, Bellefonte, USA) was preconditioned prior to analysis at 270°C for 30 min. The extraction was carried out with a multipurpose autosampler (PAL RS185, Agilent, USA) at 60°C. Each sample had a 2 min equilibrium time with agitation before the fibre was exposed for 45 min in static headspace mode. After the extraction, the compounds were thermally desorbed at 240°C for 5 min in spitless mode (split/ splitless inlet, Agilent, USA).

GC analyses were performed using an Agilent 6890N GC equipped with an Agilent 5975B VL mass spectrometer (Agilent Technologies, USA). The chromatographic separation was performed using a 60 m × 0.32 mm id × 0.5 μm Zebron ZB-WAX capillary column (Phenomenex, California, USA) with helium as the carrier gas at a flow rate of 1.0 mL/min. The oven temperature was held at 40°C for 5 min, increased at a rate of 4°C/min until 210°C and subsequently increased at a rate of 10°C/min, and held at 240°C for 5 min. The transfer line to the MS was set to 230°C and the quadrupole was set to 150°C. The mass spectrometer was operated at a scan speed of 1562 u/s and mass spectra were recorded in the range of 30-300 m/z. Carryover between GC runs was assessed using blank sample analyses and was not detected. Volatile compounds were identified by comparing the mass spectra to the National Institute of Standards and Technology (NIST14) database and supported by retention indices (RI). The RI values were calculated relative to an *n*-alkanes series (C9-C24) separated under the same analytical conditions as the samples. Approximate quantities of the volatile compounds were estimated by comparison of their peak areas with that of the internal standard, obtained from the total ion chromatograms, assuming that the relative response factor was 1 and the recovery ratio was 100%.

5.3.9 Sensory evaluation

BE and MRPs were used in the formulation of beef gravy (**Table 5-2**) for sensory evaluation. Beef broth was prepared by diluting BE or MRPs with water in the ratio of 1:4 to a total soluble solids' concentration equivalent to ~11°Brix. The beef broth was then heated in a saucepan over medium heat, the dry ingredients were added with continuous stirring for 5 min until the gravy boiled and thickened as the starch gelatinised.

Table 5-2 Formulation of beef gravy made from beef bone extract or MRPs

Ingredients	Amount (%)
Water	74.4
Beef bone extract/ Maillard reaction products (MRPs)	18.6
Modified starch (Pure-flo®)	4.0
Sugar	2.0
Salt	0.9
White pepper powder	0.1

The sensory acceptability of gravies made from MRPs and the control prepared with BE were evaluated. A total of 30 panellists (24 women and 6 men, who were 18 to 55 years old of age) participated in this study. Consumer testing was conducted at Massey University's Sensory Laboratory. The samples were coded with randomly selected 3-digit numbers. The sample presentation order for the panellists was balanced in order to control any order effects. Each panellist was presented with a tray containing five samples (BE, BE-MRP, P-MRP, F-MRP and P+F-MRP) in 20 mL plastic sampling cups. The evaluation session was conducted in individual air-conditioned booths (20°C) under normal lighting. To eliminate carryover factors, panellists were provided with unsalted crackers and room temperature water for palate cleansing between samples. The panellists were asked to rate their scores for appearance, meaty aroma, meaty taste and overall acceptance using a 9-point hedonic scale (1 = 'dislike extremely', 5 = 'nether like nor dislike' and 9 = 'like extremely'). Approval to use human subjects for the sensory evaluation was granted by the Massey University Human Ethics Committee, Southern A.

5.3.10 Data analysis

All experimental work was carried out in three replicates, on freshly prepared samples and the results were reported as means ± standard deviations of the measurements. Data were analysed using Minitab® 18 statistical software (Minitab Inc, USA). Statistical analyses of observed differences among means consisted of one-way analysis of variance (ANOVA), followed by Tukey's pairwise comparison of means ($p \leq 0.05$).

5.4 Results and discussions

5.4.1 Changes in pH and browning intensity after Maillard reaction

To assess the extent of MR, differences in pH and browning intensity were determined (**Table 5-3**). BE had a native pH of 6.68 ± 0.01. The pH of hydrolysates was similar to BE (pH 6.52-6.67), with only P+F and B+F being significantly different, although the magnitude of the difference was small. Free protons (H^+) caused a decrease in pH of the hydrolysates due to proton exchange that occurred between the deionised carboxyl group and the amino group of the protein during enzymatic hydrolysis (Kristinsson & Rasco, 2000; Rutherfurd, 2010). The mixtures were then adjusted to pH 6.50 before heat treatment. The pH of BE and hydrolysates were significantly different after MR (p<0.05; Tukey's test results not shown). The pH of MRPs decreased after 10 min of pressure cooking at 170 kPa (113°C) to between 5.40 and 5.50, with no significant differences between sample treatments. The decrease in pH could be due to the formation of organic acids (e.g. acetic acid) during MR, through mechanisms such as the degradation of sugar, peptides and FAA (Lan et al., 2010; Eric et al., 2013; Wang et al., 2016).

Table 5-3 Changes in pH and browning intensity of beef bone extract, hydrolysates and Maillard reaction products (MRPs)

Sample	pH [1]	Browning intensity (A^*_{420}) [1]
Before Maillard reaction (Beef bone extract and hydrolysates)		
BE	6.68 ± 0.01^{a}	0.17 ± 0.02^{a}
P	6.67 ± 0.01^{a}	0.17 ± 0.02^{a}
B	6.66 ± 0.01^{a}	0.15 ± 0.01^{ab}
F	6.66 ± 0.01^{a}	0.13 ± 0.01^{b}
P+F	6.59 ± 0.01^{b}	0.13 ± 0.02^{b}
B+F	6.52 ± 0.01^{c}	0.16 ± 0.01^{a}
After Maillard reaction (MRPs)		
BE-MRP	5.50 ± 0.01^{a}	0.27 ± 0.03^{e}
P-MRP	5.47 ± 0.01^{a}	0.57 ± 0.03^{c}
B-MRP	5.48 ± 0.01^{a}	0.51 ± 0.01^{d}
F-MRP	5.43 ± 0.01^{a}	0.76 ± 0.01^{b}
P+F-MRP	5.40 ± 0.06^{a}	0.79 ± 0.01^{ab}
B+F-MRP	5.45 ± 0.04^{a}	0.81 ± 0.01^{a}

[1] Data are presented as the mean and standard deviation of three replicates.
Values bearing different lowercase letters (a, b and c) in the same column within 'Before Maillard reaction' or 'After Maillard reaction' for 'pH' or 'browning intensity' were significantly different (p≤0.05) according to Tukey's post-hoc test.

Browning during heat treatment resulted in brown coloured products (de Oliveira, Coimbra, de Oliveira, Zuñiga, & Rojas, 2016). Increasing absorbance values are used as an indication for the degree of browning caused by MR at more advanced stages. The dark brown colour for the treated samples was a clear indicator of the progress of the MR. BE and hydrolysates exhibited a browning index that ranged between 0.13 and 0.17 (**Table 5-3**). The browning intensities of BE and hydrolysates were significantly different after MR ($p<0.05$; Tukey's test results not shown). A substantial increase in the browning intensity was observed when bone hydrolysates and ribose underwent MR in the pressure-cooker with intensity values ranging between 0.27 for BE-MRP to 0.81 for B+F-MRP. Results also showed that the browning intensity of MRPs increased significantly with increasing DH. This could be because a higher DH provides a greater proportion of low *Mw* peptides to react with ribose to generate a higher amount of brown pigments such as melanoidins (heterogeneous nitrogen-containing brown pigment) during the MR (Wang, Qian, & Yao, 2011).

5.4.2 Changes in peptide contents after Maillard reaction

The *Mw* distribution of peptides for BE changed during enzymatic hydrolysis and the MR (**Table 5-4**). Compared with BE, the percentage of each *Mw* fractions (>30000 Da, 10000-30000 Da, 5000-10000 Da, 1000-5000 Da and <1000 Da) for hydrolysates showed significant differences with increasing DH. This showed that the enzymes had reduced the average *Mw* of the protein of BE significantly.

Table 5-4 Changes in molecular weight distribution (range between <1000 and >30000 Da) of beef bone extract, hydrolysates and Maillard reaction products (MRPs)

Sample	Molecular weight (Da) [1,2]				
	>30000	**10000-30000**	**5000-10000**	**1000-5000**	**<1000**
Before Maillard reaction (Beef bone extract and hydrolysates)					
BE	24.33 ± 3.65^{a}	52.17 ± 3.39^{c}	3.59 ± 2.18^{a}	10.41 ± 2.44^{c}	9.50 ± 2.26^{b}
P	2.84 ± 0.38^{b}	71.68 ± 4.68^{a}	0.91 ± 0.34^{b}	13.71 ± 3.11^{b}	10.86 ± 1.53^{ab}
B	2.92 ± 0.53^{b}	71.44 ± 3.99^{a}	0.87 ± 0.77^{b}	13.87 ± 3.33^{b}	10.90 ± 0.90^{ab}
F	2.83 ± 0.35^{b}	64.90 ± 1.88^{b}	0.49 ± 0.26^{b}	18.34 ± 1.11^{a}	13.44 ± 2.31^{a}
P+F	2.95 ± 0.37^{b}	64.08 ± 1.22^{b}	0.75 ± 0.10^{b}	18.82 ± 0.83^{a}	13.40 ± 1.61^{a}
B+F	2.19 ± 0.99^{b}	66.44 ± 1.43^{ab}	0.67 ± 0.24^{b}	18.44 ± 0.52^{a}	12.26 ± 0.27^{a}
After Maillard reaction (MRPs)					
BE-MRP	32.65 ± 1.95^{a}	40.38 ± 1.75^{c}	9.05 ± 0.51^{a}	7.81 ± 0.54^{ab}	10.11 ± 0.28^{a}
P-MRP	2.29 ± 0.28^{b}	82.06 ± 1.60^{a}	0.79 ± 0.65^{b}	6.93 ± 0.57^{b}	7.93 ± 0.74^{b}
B-MRP	2.62 ± 0.47^{b}	80.67 ± 1.75^{a}	1.16 ± 0.85^{b}	7.30 ± 1.04^{ab}	8.25 ± 0.43^{b}
F-MRP	4.19 ± 1.57^{b}	76.27 ± 1.98^{b}	1.02 ± 0.68^{b}	8.20 ± 0.55^{ab}	10.32 ± 0.75^{a}
P+F-MRP	2.63 ± 0.17^{b}	78.94 ± 1.31^{ab}	0.96 ± 0.64^{b}	8.71 ± 0.70^{a}	8.76 ± 0.20^{b}
B+F-MRP	3.43 ± 0.63^{b}	75.45 ± 1.34^{b}	1.94 ± 0.39^{b}	8.09 ± 0.43^{ab}	11.09 ± 0.72^{a}

[1] Data are presented as the mean and standard deviation of three replicates.
[2] Peptides in beef bone extract, bone hydrolysates and MRPs are expressed as a percent of total area from SEC-HPLC intensity curves (%).
Values bearing different lowercase letters (a, b and c) in the same column within 'Before Maillard reaction' or 'After Maillard reaction' for each *Mw* fraction were significantly different ($p \leq 0.05$) according to Tukey's post-hoc test.

The *Mw* distribution of BE and hydrolysates at each *Mw* fraction were significantly different after MR ($p<0.05$; Tukey's test results not shown). The *Mw* distribution for medium *Mw* fractions (5000-10000 Da and 10000-30000 Da) increased after MR. This could be due to polypeptide aggregation and/ or cross-linking (Van Boekel, 1998; Jousse et al., 2002). The increase in *Mw* is largely at 10-30 kDa rather than larger. In contrast, the *Mw* distribution for low *Mw* fractions (1000-5000 Da and <1000 Da) decreased, especially for the 1000-5000 Da fraction, illustrating a decrease in peptide contents after heat treatment due to the progression of the MR. The increase in high *Mw* peptides and the decrease in low *Mw* peptides were consistent with findings from Lan et al. (2010) and Eric et al. (2013), who reported that small peptides could act as important reactants in the MR to produce peptides with flavour enhancing capacities through peptide cross-linking. Furthermore, MRPs derived from 1000-5000 Da peptides could influence the mouthfeel, umami and kokumi taste of food products (Ogasawara, Katsumata, & Egi, 2006). The decrease in the proportion of 1000-5000 Da peptides after MR could also lead to an increase

in the generation of nitrogen-containing heterocyclic compounds such as pyrazines and pyrroles (**Table 5-6**). The *Mw* distribution results indicated that low *Mw* peptides at 1000-5000 Da could be the main participant in cross-linking reaction during MR.

5.4.3 Changes in free amino acids after Maillard reaction

The proportion of FAA in BE, hydrolysates and MRPs is shown in **Table 5-5**. The proportion of total FAA in MRPs decreased after heat treatment, which supports the occurrence of MR during heating. This could be due to the interactions (i.e. cross-linking) between amino acids and sugar or their degradation products (Lan et al., 2010). Alternatively, it could be associated with the formation of volatile compounds from the amino acids (Sun et al., 2014). Although the DH achieved using Protamex® and bromelain were somewhat similar (**Table 5-1**) in single hydrolysis treatment, B-MRP (1.57 mg/g protein) had a larger change in total FAA than P-MRP (0.18 mg/g protein), in terms of the magnitude change. MRPs derived from simultaneous hydrolysis treatment (P+F-MRP; 7.30 mg/g protein) had a lower change in total FAA compared to F-MRP (9.40 mg/g protein) from single hydrolysis treatment.

Other FAAs such as hydrophobic, umami and sulphur-containing for each MRP decreased after heat treatment. Amino acids with hydrophobic side chains usually have an unpleasant bitter taste (Chen & Zhang, 2007). It was shown that with the usage of Flavourzyme® for the hydrolysis treatments, the amount of hydrophobic amino acids in F-MRP, P+F-MRP and B+F-MRP decreased greatly after MR, but the final contents in MRPs were still higher than BE-MRP, P-MRP and B-MRP. Yu et al. (2018) reported that umami amino acids could be increased by utilising MR, due to the preferential degradation of peptides containing glutamic acid or aspartic acid. However, this effect was not encountered in this study. It was shown that umami amino acids decreased after heat treatment, which could be due to these amino acids participating in the formation of volatile compounds via the MR. F-MRP and B+F-MRP had the greatest loss in sulphur-containing amino acids in single and simultaneous hydrolysis treatment, respectively.

Table 5-5 Free amino acid composition of beef bone extract, hydrolysates and Maillard reaction products (MRPs)

Amino acids	Free amino acids content (mg/g protein) [1]											
	Before Maillard reaction (Beef bone extract and hydrolysates)						After Maillard reaction (MRPs)					
	BE	P	B	F	P+F	B+F	BE-MRP	P-MRP	B-MRP	F-MRP	P+F-MRP	B+F-MRP
Essential amino acids												
Histidine	0.02±0.01^{b}	0.03±0.01^{b}	0.08±0.02^{b}	0.28±0.09^{a}	0.30±0.02^{a}	0.41±0.00^{a}	0.02±0.01^{y}	0.01±0.00^{y}	0.02±0.00^{y}	0.14±0.00^{z}	0.16±0.00^{z}	0.18±0.05^{z}
Isoleucine	0.02±0.01^{b}	0.04±0.00^{b}	0.04±0.03^{b}	0.91±0.05^{a}	1.12±0.24^{a}	1.01±0.04^{a}	0.02±0.00^{w}	0.02±0.00^{w}	0.01±0.00^{w}	0.59±0.01^{x}	1.08±0.02^{z}	0.70±0.03^{y}
Leucine	0.06±0.02^{b}	0.08±0.01^{b}	0.11±0.02^{b}	2.83±0.23^{a}	2.93±0.38^{a}	2.69±0.02^{a}	0.04±0.01^{x}	0.05±0.00^{x}	0.06±0.00^{x}	2.23±0.02^{y}	2.55±0.06^{z}	2.16±0.18^{y}
Lysine	0.05±0.01^{b}	0.04±0.02^{b}	0.09±0.03^{b}	0.95±0.10^{a}	0.68±0.02^{a}	0.80±0.15^{a}	0.02±0.00^{y}	0.02±0.00^{y}	0.03±0.00^{y}	0.53±0.05^{z}	0.54±0.03^{z}	0.65±0.14^{z}
Methionine	0.01±0.01^{b}	0.02±0.01^{b}	0.05±0.02^{b}	0.61±0.06^{a}	0.59±0.08^{a}	0.59±0.02^{a}	0.00±0.00^{y}	0.02±0.00^{y}	0.01±0.00^{y}	0.20±0.02^{z}	0.22±0.00^{z}	0.18±0.03^{z}
Phenylalanine	0.06±0.02^{b}	0.05±0.01^{b}	0.08±0.03^{b}	1.68±0.22^{a}	1.63±0.21^{a}	1.62±0.14^{a}	0.03±0.01^{y}	0.03±0.00^{y}	0.03±0.00^{y}	1.35±0.01^{z}	1.44±0.02^{z}	1.35±0.09^{z}
Threonine	0.04±0.02^{b}	0.04±0.00^{b}	0.08±0.03^{b}	1.23±0.21^{a}	1.06±0.07^{a}	1.36±0.16^{a}	0.03±0.01^{x}	0.05±0.00^{x}	0.04±0.00^{x}	0.62±0.02^{y}	0.74±0.01^{zy}	0.81±0.09^{z}
Valine	0.04±0.01^{b}	0.05±0.01^{b}	0.12±0.02^{b}	1.14±0.08^{a}	1.46±0.30^{a}	1.37±0.02^{a}	0.03±0.00^{x}	0.05±0.00^{x}	0.04±0.00^{x}	0.82±0.03^{y}	1.29±0.00^{z}	0.95±0.11^{y}
Non-essential amino acids												
Alanine	0.16±0.03^{c}	0.19±0.02^{c}	0.33±0.18^{c}	2.08±0.17^{b}	2.56±0.18^{ab}	3.03±0.25^{a}	0.16±0.01^{x}	0.33±0.00^{x}	0.20±0.00^{x}	1.40±0.07^{y}	1.88±0.07^{z}	2.09±0.16^{z}
Arginine	0.08±0.05^{c}	0.07±0.02^{c}	0.17±0.05^{c}	1.84±0.36^{b}	2.13±0.21^{ab}	2.71±0.08^{a}	0.02±0.03^{y}	0.02±0.00^{y}	0.06±0.00^{y}	1.06±0.00^{z}	1.27±0.03^{z}	1.51±0.45^{z}
Aspartic acid	0.04±0.00^{c}	0.05±0.01^{c}	0.08±0.03^{bc}	0.16±0.02^{b}	0.16±0.03^{b}	0.26±0.03^{a}	0.04±0.01^{x}	0.04±0.00^{x}	0.05±0.00^{x}	0.13±0.01^{y}	0.13±0.00^{y}	0.18±0.02^{z}
Asparagine	0.14±0.02^{d}	0.20±0.01^{d}	0.49±0.03^{cd}	1.65±0.16^{ab}	1.33±0.01^{bc}	2.38±0.49^{a}	0.14±0.03^{x}	0.17±0.01^{x}	0.34±0.04^{x}	0.93±0.03^{y}	0.92±0.01^{y}	1.47±0.16^{z}
Cysteine	0.11±0.09^{c}	0.19±0.02^{c}	0.62±0.03^{bc}	0.90±0.12^{b}	0.90±0.06^{b}	1.72±0.32^{a}	0.06±0.07^{x}	0.16±0.00^{x}	0.22±0.00^{x}	0.61±0.03^{y}	0.74±0.07^{y}	1.05±0.07^{z}
Glutamic acid	0.07±0.01^{c}	0.12±0.02^{c}	0.15±0.02^{c}	0.45±0.11^{b}	0.41±0.08^{b}	1.04±0.02^{a}	0.08±0.03^{x}	0.05±0.00^{x}	0.09±0.01^{x}	0.33±0.02^{y}	0.33±0.00^{y}	0.70±0.12^{z}
Glutamine	0.03±0.04^{b}	0.03±0.02^{b}	0.10±0.01^{b}	3.43±0.47^{a}	3.65±0.04^{a}	3.65±0.15^{a}	ND	ND	0.06±0.00^{y}	0.46±0.02^{z}	0.44±0.01^{z}	0.53±0.12^{z}
Glycine	0.24±0.04^{c}	0.28±0.03^{c}	0.37±0.06^{c}	1.30±0.08^{b}	1.14±0.03^{b}	2.10±0.12^{a}	0.19±0.01^{x}	0.34±0.01^{x}	0.29±0.00^{x}	0.81±0.04^{y}	0.92±0.03^{y}	1.37±0.09^{z}
Serine	0.06±0.01^{b}	0.08±0.01^{b}	0.12±0.02^{b}	0.53±0.13^{ab}	0.62±0.02^{ab}	1.12±0.36^{a}	0.06±0.01^{x}	0.10±0.00^{x}	0.07±0.00^{x}	0.49±0.02^{y}	0.49±0.02^{y}	0.65±0.06^{z}
Taurine	0.20±0.05^{a}	0.19±0.01^{a}	0.17±0.09^{a}	0.15±0.01^{a}	0.10±0.02^{a}	0.20±0.10^{a}	0.13±0.05^{y}	0.10±0.01^{y}	0.12±0.01^{y}	0.21±0.02^{zy}	0.31±0.01^{z}	0.31±0.07^{z}
Tyrosine	0.03±0.01^{b}	0.01±0.01^{b}	0.08±0.06^{b}	0.49±0.04^{a}	0.37±0.05^{a}	0.44±0.04^{a}	0.02±0.00^{x}	0.01±0.00^{x}	0.02±0.00^{x}	0.34±0.01^{z}	0.35±0.01^{z}	0.30±0.01^{y}
Total FAA	**1.46±0.10**	**1.74±0.06**	**3.34±0.25**	**22.6±0.8**	**23.1±0.7**	**28.5±0.8**	**1.10±0.10**	**1.56±0.02**	**1.77±0.04**	**13.2±0.1**	**15.8±0.1**	**17.1±0.6**
Essential FAA	**0.30±0.04**	**0.34±0.03**	**0.65±0.07**	**9.63±0.42**	**9.76±0.59**	**9.83±0.27**	**0.19±0.02**	**0.24±0.00**	**0.25±0.00**	**6.49±0.07**	**8.01±0.07**	**6.98±0.29**
Non-essential FAA	**1.16±0.10**	**1.40±0.06**	**2.69±0.23**	**13.0±0.7**	**13.4±0.3**	**18.7±0.8**	**0.90±0.10**	**1.32±0.02**	**1.52±0.04**	**6.75±0.10**	**7.78±0.11**	**10.2±0.5**
Hydrophobic FAA[2]	**0.55±0.05**	**0.69±0.04**	**1.59±0.20**	**12.1±0.5**	**12.9±0.6**	**14.2±0.5**	**0.41±0.07**	**0.72±0.00**	**0.66±0.00**	**8.30±0.08**	**10.4±0.1**	**9.77±0.31**
Umami FAA[3]	**0.12±0.01**	**0.17±0.02**	**0.23±0.03**	**0.62±0.11**	**0.57±0.09**	**1.30±0.04**	**0.12±0.03**	**0.09±0.00**	**0.14±0.01**	**0.46±0.02**	**0.46±0.00**	**0.88±0.12**
Sulphur-containing FAA[4]	**0.12±0.01**	**0.21±0.02**	**0.67±0.03**	**1.51±0.14**	**1.49±0.10**	**2.31±0.32**	**0.07±0.07**	**0.18±0.00**	**0.23±0.00**	**0.81±0.03**	**0.97±0.07**	**1.23±0.08**

[1] Data are presented as the mean and standard deviation. ND = Not Detected

Values bearing different lowercase letters (a, b, c and d) in the same row for 'Before Maillard reaction' were significantly different ($p \leq 0.05$) according to Tukey's post-hoc test.

Values bearing different lowercase letters (w, x, y and z) in the same row for 'After Maillard reaction' were significantly different ($p \leq 0.05$) according to Tukey's post-hoc test.

[2] Hydrophobic amino acids: Alanine, Cysteine, Histidine, Isoleucine, Leucine, Methionine, Phenylalanine, Proline, Threonine, Tyrosine and Valine (Damodaran, 2008).

[3] Umami amino acids: Aspartic acid and Glutamic acid (Sun et al., 2014).

[4] Sulphur-containing amino acids: Cysteine and Methionine (Liu et al., 2015).

5.4.4 Changes in volatile compounds after Maillard reaction

The volatile compounds formed after heat treatment can be classified into three groups such as (1) sugar dehydration or fragmentation products (e.g. furans, pyrones, cyclopentenes, carbonyls and acids), (2) amino acid degradation products (e.g. aldehydes) and (3) volatiles produced by further interactions (e.g. pyrroles, pyridines, imidazoles, pyrazines, oxazoles, and thiazoles) (Nursten, 2005). A total of 40 compounds, consisting of pyrazines, thioethers, furans, pyrroles, aldehydes, ketones, esters, alcohols, acids, and other miscellaneous compounds that might have no contribution to the aroma characteristics, were identified in BE and MRPs (**Table 5-6**). It was observed that most pyrazines, 2-furanmethanol and benzaldehyde gradually increased when the DH of the hydrolysates increased. Pyrazines were the main class of compounds produced as a result of the MR based on total peak area. Normalised peak responses for these compounds were calculated by comparison of peak area with that of the internal standard. Some of them were the major volatile components, and others were intermediates in the formation of other volatiles.

The BE had the lowest number of compounds detected and the lowest total normalised peak response of all treatments, with pyrazines present in the highest relative peak area intensity relative to the other compounds present in BE. BE contained the lowest proportion of pyrazines when compared with the MRPs. This indicated the weak flavour intensity of the original extract, supported by informal odour assessment. BE also contained the lowest proportion of acetic acid among all samples. This corresponded with the higher pH of BE compared to the MRPs. After heat treatment, BE-MRP showed the generation of previously undetected volatile compounds such as 2-methylpyrazine, dimethyl disulphide and dimethyl trisulphide, which are considered to contribute to a roasted, sulphurous and meaty flavour (Mahajan, Goddik, & Qian, 2004). The relative content of pyrazines doubled from a peak area ($\times 10^6$) of 410.7 ± 18.1 to 878.4 ± 15.5 as a result of the heat treatment. The relative content of acetic acid increased greatly from a peak area ($\times 10^6$) of 6.3 ± 1.2 to 189.6 ± 30.4. This correlated with pH analysis, where BE-MRP achieved a pH of 5.50 ± 0.01 after heat treatment. There was also an increase in 2-furanmethanol, which may contribute a burnt and bitter character in BE-MRP, as reported by Mahajan et al. (2004) and Naudé and Rohwer (2013), who investigated the aroma compounds of sweet whey powder and coffee flavour in Pinotage wine, respectively.

When BE was hydrolysed by either Protamex®, bromelain or Flavourzyme® in single hydrolysis treatment followed by heat treatment, F-MRP showed the highest peak area intensity for most volatile compounds (**Table 5-6**). This could be due to its high DH, where a higher proportion of FAA or peptides were available to react with ribose during heat treatment (**Table 5-5**). F-MRP showed a significantly higher proportion of some pyrazines, thioethers and other volatile compounds when compared with P-MRP and B-MRP. Volatile compounds such as 2,5-dimethyl-3-isopentylpyrazine and 2-acetyl-3,5-dimethylpyrazine were generated in F-MRP but were not present in P-MRP and B-MRP in single hydrolysis treatment. This could be due to Flavourzyme® being a mixture of endo- and exo-proteases, which can hydrolyse peptide bonds within polypeptides and from either the N or C-terminal ends (O'Sullivan et al., 2017), and thus generate volatile compounds that endoproteases (i.e. Protamex® and bromelain) are unable to generate during MR. There was no significant difference in the proportion of acetic acid for P-MRP, B-MRP and F-MRP, which correlated with similar pH results. There were no significant differences for any of the volatile compounds between P-MRP and B-MRP.

When combining Flavourzyme® with Protamex® or bromelain in simultaneous hydrolysis treatment followed by MR, there was no significant difference in the proportion of most volatile compounds between P+F-MRP and B+F-MRP (**Table 5-6**). The magnitude of the differences was small for some volatile compounds even when they were significantly different. When comparing F-MRP in single hydrolysis treatment with both P+F-MRP and B+F-MRP in simultaneous hydrolysis treatment, there were no significant differences between the major volatile compounds for the three MRPs. This showed that Flavourzyme® is effective in generating major volatile compounds during MR, without the need for the addition of other enzymes.

It has been reported that meaty flavour is associated mainly with sulphur compounds and their derivatives (Van Boekel, 2006). However, in this study, only two sulphur-containing volatiles, dimethyl disulphide and dimethyl trisulphide were detected in the MRPs. BE-MRP contained the least amount of these thioethers, while F-MRP had the highest amount. Both B-MRP and B+F-MRP obtained from hydrolysates made using bromelain was found to have a lower proportion of thioethers in their respective hydrolysis treatments. It was also shown that B-MRP and B+F-MRP contained a higher proportion of furans, which typically have a sweet, caramel-like flavour (Song

et al., 2016). This could potentially decrease the meaty aroma in the two MRPs. Interestingly, compounds such as butylated hydroxytoluene (BHT), and sorbic acid having antioxidant and antimicrobial activity, respectively, were detected in the MRPs. BHT was found in BE-MRP, P+F-MRP and B+F-MRP, while sorbic acid was found in F-MRP, P+F-MRP and B+F-MRP.

The occurrence for the hazardous compounds such as carcinogenic (e.g. acrylamide) and mutagenic (e.g. heterocyclic aromatic amines) products formed during MR was assumed low. There are no exact regulation limits (e.g. tolerable daily intake) on these hazardous compounds specified by FAO/WHO (Food and Agriculture Organisation/ World Health Organisation), FDA (Food and Drug Administration), EFSA (European Food Safety Authority) or FSANZ (Food Standard Australia New Zealand). Acrylamide is a substance that forms through the chemical reaction between asparagine (containing an amide group) and reducing sugars. Therefore, to minimise the amount of acrylamide formed during MR, the amount of free asparagine to react with reducing sugar should be as low as possible (Nursten, 2005). Heterocyclic aromatic amines (HAA) are formed when amino acids, reducing sugars, and creatine or creatinine (substances found in muscle) react at high temperatures. However, creatine or creatinine was not analysed in this study. It was expected to be low as the extract was obtained from beef bone with a low amount of muscle meat. Both acrylamide and HAA form during high-temperature cooking, such as frying, roasting, grilling and baking (above 120°C) and in low moisture contents (Food Drug Administration, 2016).

In this study, the formation of acrylamide was not measured. However, the highest amount of free asparagine was 2.38 mg/g protein (or 1.13 mg/g product) for B+F treatment and over half of this reacted during MR (**Table 5-5**). In comparison, Song et al. (2016) found that the amount of free asparagine contained in beef bone hydrolysates was much higher 8.6-19.7 mg/g product.

The heat treatment for MR was conducted at 115°C, and in high moisture content (>50%) and these conditions are not optimal for the formation of acrylamide. Furthermore, these MRPs were intended as a high-intensity flavour ingredient to be used at low usage levels, thus limiting the concentration of hazardous compounds in final food products. Therefore, it is assumed that acrylamide and HAA values are low, but these could be tested in further work.

Table 5-6 Volatile compounds in the beef bone extract and Maillard reaction products (MRPs) with different enzymatic hydrolysis treatment

Compounds	RI [2]	Peak area (×10^6) [1]						
		BE	BE-MRP	P-MRP	B-MRP	F-MRP	P+F-MRP	B+F-MRP
Pyrazines								
2-Methylpyrazine	1311	ND	57.8 ± 2.1^{d}	74.0 ± 2.0^{c}	89.5 ± 1.8^{b}	117.6 ± 5.6^{a}	112.3 ± 5.5^{a}	119.2 ± 6.7^{a}
2,5- and 2,6-Dimethylpyrazine (coeluting)	1378	147.7 ± 14.6^{d}	249.4 ± 0.9^{c}	547.0 ± 23.6^{b}	568.9 ± 9.3^{b}	741.2 ± 31.9^{a}	794.4 ± 50.6^{a}	802.6 ± 27.5^{a}
2-Ethyl-6-methylpyrazine	1441	6.6 ± 1.1^{d}	19.0 ± 1.9^{c}	23.1 ± 1.2^{bc}	26.1 ± 1.3^{b}	40.7 ± 1.5^{a}	42.4 ± 1.3^{a}	43.7 ± 1.8^{a}
2-Ethyl-5-methylpyrazine	1447	75.2 ± 3.3^{d}	60.4 ± 0.5^{e}	106.6 ± 1.9^{c}	100.4 ± 4.4^{c}	145.3 ± 2.0^{b}	168.2 ± 3.2^{a}	163.5 ± 5.3^{a}
Trimethylpyrazine	1470	35.9 ± 2.0^{e}	60.2 ± 0.6^{d}	95.5 ± 2.9^{c}	101.0 ± 2.8^{c}	158.3 ± 3.6^{b}	171.0 ± 3.8^{a}	171.6 ± 6.8^{a}
3-Ethyl-2,5-dimethylpyrazine	1506	134.5 ± 10.0^{d}	363.3 ± 15.1^{c}	451.9 ± 18.7^{b}	432.8 ± 14.6^{b}	512.8 ± 18.5^{a}	533.8 ± 14.8^{a}	538.1 ± 25.6^{a}
3,5-Diethyl-2-methylpyrazine	1579	10.7 ± 0.1^{c}	68.4 ± 1.0^{b}	81.8 ± 4.4^{a}	79.2 ± 2.1^{a}	81.0 ± 3.1^{a}	84.9 ± 1.5^{a}	84.3 ± 4.2^{a}
2,5-Dimethyl-3-isopentylpyrazine	1723	ND	ND	ND	ND	64.7 ± 2.2^{b}	76.5 ± 0.7^{a}	68.1 ± 5.5^{b}
2-Acetyl-3,5-dimethylpyrazine	1766	ND	ND	ND	ND	7.5 ± 0.2^{ab}	8.3 ± 0.6^{a}	7.4 ± 0.5^{b}
Total pyrazines		**410.7 ± 18.1**	**878.4 ± 15.5**	**1379.8 ± 30.7**	**1397.9 ± 18.3**	**1869.3 ± 37.7**	**1991.8 ± 53.3**	**1998.5 ± 39.8**
Thioethers								
Dimethyl disulphide	1043	ND	15.5 ± 6.4^{c}	31.6 ± 5.5^{bc}	27.6 ± 1.6^{c}	52.5 ± 7.5^{a}	47.2 ± 7.5^{ab}	48.7 ± 9.4^{ab}
Dimethyl trisulphide	1428	ND	18.3 ± 9.7^{b}	28.8 ± 2.1^{b}	21.8 ± 2.8^{b}	87.6 ± 5.9^{a}	87.9 ± 8.9^{a}	75.4 ± 11.7^{a}
Total thioethers		**-**	**33.8 ± 11.7**	**60.4 ± 5.9**	**49.4 ± 3.2**	**140.1 ± 9.5**	**135.1 ± 11.6**	**124.1 ± 15.0**
Furans								
2-Methylfuran	840	ND	41.6 ± 9.9^{ab}	69.8 ± 10.3^{a}	68.2 ± 10.8^{a}	47.7 ± 3.7^{ab}	41.7 ± 1.9^{ab}	34.5 ± 18.3^{b}
Furfural	1531	ND	79.6 ± 3.6^{a}	ND	ND	ND	ND	76.7 ± 3.4^{a}
2-Propanoylfuran	1647	ND	32.0 ± 2.6^{a}	ND	34.6 ± 2.1^{a}	ND	ND	ND
3-Phenylfuran	1914	ND	ND	MD	ND	36.5 ± 2.5^{b}	38.7 ± 0.4^{b}	42.7 ± 1.0^{a}
Total furans		**-**	**153.1 ± 10.8**	**69.8 ± 10.3**	**102.8 ± 11.0**	**84.2 ± 4.4**	**80.4 ± 1.9**	**153.9 ± 18.6**
Pyrroles								
1-Furfurylpyrrole	1879	ND	26.7 ± 2.5^{c}	42.1 ± 1.5^{a}	33.8 ± 1.4^{b}	43.1 ± 1.8^{a}	40.2 ± 2.9^{a}	37.6 ± 3.2^{ab}
2-Acetylpyrrole	2010	ND	ND	ND	ND	6.6 ± 0.2^{c}	8.0 ± 0.6^{b}	12.7 ± 0.3^{a}
2-Formylpyrrole	2057	ND	ND	9.4 ± 0.5^{a}	9.7 ± 0.7^{a}	ND	ND	ND
Total pyrroles		**-**	**26.7 ± 2.5**	**51.5 ± 1.6**	**43.5 ± 1.6**	**49.7 ± 1.9**	**48.2 ± 2.9**	**50.4 ± 3.2**
Ketones								
5-Methyl-2-hexanone	1130	ND	ND	ND	ND	25.0 ± 2.0^{a}	26.7 ± 2.4^{a}	22.9 ± 7.3^{a}
6-Methyl-2-heptanone	1253	ND	ND	ND	ND	15.7 ± 0.3^{a}	15.7 ± 0.6^{a}	14.0 ± 2.0^{a}
Total ketones		**-**	**-**	**-**	**-**	**40.7 ± 2.0**	**42.3 ± 2.4**	**36.9 ± 7.6**
Esters								
Furfuryl acetate	1591	ND	37.6 ± 1.9^{a}	38.1 ± 1.5^{a}	39.6 ± 1.8^{a}	ND	ND	ND
3-Hydroxy-2,2,4-trimethylpentyl isobutyrate	1911	23.6 ± 14.7^{a}	26.2 ± 13.0^{a}	37.2 ± 15.0^{a}	22.8 ± 6.9^{a}	24.8 ± 9.3^{a}	36.9 ± 3.2^{a}	25.1 ± 12.9^{a}
Total esters		**23.6 ± 14.7**	**63.9 ± 13.2**	**75.3 ± 15.0**	**62.4 ± 7.1**	**24.8 ± 9.3**	**36.9 ± 3.2**	**25.1 ± 12.9**
Alcohols								

2-Furanmethanol	1714	60.3 ± 6.4^{d}	182.9 ± 2.8^{c}	255.9 ± 11.2^{b}	281.5 ± 9.6^{b}	399.7 ± 12.6^{a}	399.6 ± 13.2^{a}	407.7 ± 4.3^{a}
Alpha-terpineol	1756	20.8 ± 5.2^{c}	25.4 ± 6.8^{bc}	48.7 ± 6.8^{a}	36.3 ± 4.7^{abc}	39.9 ± 7.4^{ab}	50.9 ± 3.9^{a}	43.8 ± 9.2^{a}
5-Methyl-2-Furanmethanol	1775	ND	ND	ND	21.0 ± 0.6^{b}	20.2 ± 1.1^{b}	22.1 ± 0.7^{b}	35.8 ± 1.0^{a}
exo-2-Hydroxycineole	1903	ND	ND	4.0 ± 0.5^{a}	ND	ND	ND	ND
Total alcohols		**81.1 ± 8.3**	**208.3 ± 7.4**	**308.6 ± 13.1**	**338.7 ± 10.7**	**459.8 ± 14.7**	**472.6 ± 13.8**	**487.3 ± 10.2**
Acids								
Acetic acid	1498	6.3 ± 1.2^{b}	189.6 ± 30.4^{a}	142.3 ± 1.4^{a}	162.6 ± 23.6^{a}	166.3 ± 10.9^{a}	160.0 ± 25.3^{a}	172.4 ± 28.0^{a}
Butanoic acid	1680	ND	27.5 ± 0.7^{b}	30.1 ± 1.4^{a}	30.7 ± 1.0^{a}	ND	ND	ND
3-Methyl-pentanoic acid	1834	ND	ND	ND	ND	50.0 ± 1.5^{c}	81.8 ± 0.8^{a}	66.2 ± 3.9^{b}
4-Methyl-pentanoic acid	1844	ND	ND	ND	ND	274.1 ± 6.5^{a}	285.5 ± 2.2^{a}	280.8 ± 17.9^{a}
Hexanoic acid	1883	ND	32.2 ± 1.2^{b}	36.6 ± 1.9^{b}	34.8 ± 1.6^{b}	45.3 ± 0.7^{a}	47.9 ± 1.8^{a}	47.2 ± 3.7^{a}
Heptanoic acid	1976	ND	ND	13.5 ± 3.7^{a}	9.6 ± 1.1^{a}	11.9 ± 0.1^{a}	13.8 ± 0.7^{a}	13.1 ± 1.9^{a}
Octanoic acid	2065	ND	18.6 ± 4.1^{c}	26.5 ± 5.4^{abc}	23.7 ± 2.4^{bc}	28.0 ± 0.9^{abc}	34.4 ± 1.1^{a}	32.1 ± 5.5^{ab}
Sorbic acid	2144	ND	ND	ND	ND	31.0 ± 0.6^{b}	35.0 ± 1.4^{ab}	40.1 ± 4.7^{a}
Nonanoic acid	2150	ND	20.1 ± 5.3^{b}	23.9 ± 7.8^{ab}	23.6 ± 2.4^{ab}	29.7 ± 4.7^{ab}	35.7 ± 2.4^{a}	30.8 ± 6.4^{ab}
Total acids		**6.3 ± 1.2**	**288.0 ± 31.2**	**272.9 ± 10.6**	**285.0 ± 24.0**	**636.3 ± 13.7**	**693.9 ± 25.7**	**682.8 ± 35.1**
Others								
Styrene	1289	49.3 ± 3.6^{c}	58.3 ± 4.4^{bc}	73.1 ± 6.1^{b}	57.7 ± 3.5^{c}	50.8 ± 8.9^{c}	60.4 ± 6.1^{bc}	95.4 ± 3.4^{a}
Benzaldehyde	1606	33.6 ± 3.9^{e}	53.1 ± 4.8^{d}	92.8 ± 3.7^{c}	93.4 ± 6.6^{c}	122.7 ± 3.9^{b}	131.6 ± 6.5^{b}	152.0 ± 7.1^{a}
Cyclodecane	1811	ND	ND	ND	12.7 ± 3.3^{c}	ND	54.7 ± 1.5^{a}	29.4 ± 2.3^{b}
3,4-Dimethyl-2,5-furandione	1818	ND	7.9 ± 0.4^{a}	6.8 ± 0.4^{a}	6.6 ± 1.2^{a}	ND	ND	ND
Butylated hydroxytoluene	1946	ND	4.5±0.4^{c}	ND	ND	ND	15.9±0.7^{a}	12.3±0.7^{b}
Total		**82.9 ± 5.3**	**123.8 ± 6.6**	**172.6 ± 7.2**	**170.4 ± 8.3**	**173.6 ± 9.7**	**262.7 ± 9.1**	**289.1 ± 8.2**

[1] Data are presented as the mean and standard deviation of three replicates.
Values bearing different lowercase letters in the same row were significantly different ($p \leq 0.05$) according to Tukey's post-hoc test.
[2] RI = Retention Indices on polar ZB-Wax column.
ND = Not Detected

5.4.5 Sensory evaluation of beef gravy

B-MRP and B+F-MRP were not included in the sensory evaluation of beef gravy. This was due to no significant difference observed between P-MRP and B-MRP in single hydrolysis treatment, as well as P+F-MRP and B+F-MRP in simultaneous hydrolysis treatment for GC-MS results. The hedonic acceptance scores of beef gravy are shown in **Table 5-7**. BE had the lowest scores for appearance, meaty aroma and overall acceptability. BE exhibited a light brown colour that may have been unappealing to the panellists. BE had only 13 identified volatile compounds, where its relative contents of pyrazines were the lowest among all samples (see **Table 5-6**), which could have led to the lowest score in the meaty aroma. After heat treatment, BE-MRP obtained higher scores for all attributes than BE, however, there was no significant difference except for appearance. This indicated that MR has a slight contribution to the flavour development of BE.

Table 5-7 Mean hedonic scores on the three attributes and overall acceptance of beef gravy in consumer sensory evaluation (n=30)

Sample	Mean score [1,2]			
	Appearance	**Meaty aroma**	**Meaty taste**	**Overall acceptance**
BE	4.03 ± 1.45^{b}	4.80 ± 1.24^{b}	5.10 ± 1.84	4.70 ± 1.60^{b}
BE-MRP	6.10 ± 1.24^{a}	5.27 ± 1.36^{ab}	5.50 ± 1.85	5.30 ± 1.56^{ab}
P-MRP	6.87 ± 1.33^{a}	6.00 ± 1.44^{a}	5.40 ± 1.54	5.67 ± 1.24^{ab}
F-MRP	6.73 ± 1.20^{a}	5.63 ± 1.54^{ab}	5.60 ± 1.69	5.83 ± 1.51^{a}
P+F-MRP	6.57 ± 1.25^{a}	5.80 ± 1.16^{a}	4.80 ± 1.92	5.07 ± 1.53^{ab}
F-value	**24.34**	**3.68**	**1.02**	**2.80**
***p*-value**	**0.000**	**0.007**	**0.400**	**0.028**

[1] Data are presented as the mean and standard deviation.
[2] Sensory evaluation scores are normally distributed.
Values bearing different lowercase letters in the same column were significantly different ($p \leq 0.05$) according to Tukey's post-hoc test.

After enzymatic hydrolysis and heat treatment as a result of MR, P-MRP, F-MRP and P+F-MRP obtained higher scores for appearance than BE and BE-MRP. This could be due to a higher amount of melanoidins produced due to higher DH, which may have been more appealing in appearance to the panellists. P-MRP, F-MRP and P+F-MRP also obtained higher scores for meaty aroma than BE and BE-MRP. Based on the GC-MS results (**Table 5-6**), it showed that P-MRP, F-MRP and P+F-MRP had significantly higher relative contents of pyrazines such as 2-methylpyrzaine, 2,5- and 2,6-dimethylpyrazine (co-eluting), 3-ethyl-3,5-dimethylpyrazine, and thioethers such as dimethyl disulphide and dimethyl trisulphide. This resulted in panelists able to

perceive the aroma more distinctly. There were no significant differences in meaty taste among all samples. F-MRP scored the highest in overall acceptability but was only significantly different to BE. This indicated that enzymatic hydrolysis and MR could be used as an effective approach to increase the flavour quality of BE as a natural meat flavour enhancer. As F-MRP obtained the highest score for meaty taste and overall acceptability, further works can be conducted using F-MRP to optimise the beef gravy or other food product development.

5.5 Conclusion

In this study, MRPs produced using beef bone extract or hydrolysates from single and simultaneous enzymatic hydrolysis treatments were compared. The decrease in pH, low *Mw* peptides and total FAA, and the increase in browning intensity and high *Mw* peptides after heat treatment indicated the occurrence of MR during pressure-cooking. Low *Mw* peptides at 1000-5000 Da could be the main participant in the cross-linking reaction during MR. A total of 40 volatile compounds were identified with GC-MS and their concentration was found to increase with increasing DH. F-MRP showed the highest peak area intensity for the major volatile compounds in single treatment followed by heat treatment. However, combining other enzymes with Flavourzyme® in simultaneous treatment followed by heating did not result in significant differences in the detected levels of volatile compounds. F-MRP obtained the highest score for meaty taste and overall acceptability. The changes in volatile profiles and sensory scores of beef bone hydrolysates after MR indicated that enzymatic hydrolysis and heat treatment as a result of MR could be used to modify the flavour characters of beef bone extract as a natural meat flavour product.

Chapter 6: [3]Effects of soy protein to wheat gluten ratio on the physicochemical properties of extruded meat analogues

6.1 Abstract

This study reported on the effects of SPC to WG ratio at a constant mass of SPC and WG on the physicochemical properties of extruded meat analogues. Meat analogues (~57%MC) were extruded at a maximum barrel temperature of 170°C, at a dry and water feed rate of 2.8 kg/h and 3.6 kg/h, respectively. The physical, chemical and textural properties of meat analogues were studied, where meat analogues containing 30%WG showed the highest degree of texturisation, fibrous structure, hardness and chewiness using instrumental and sensory analysis. Layered or fibrous microstructure of meat analogues was observed using SEM and LM. Meat analogues containing 20%WG and 30%WG were found to exhibit fibrous microstructure with large fibrous structures interconnected with much smaller fibres. The types of chemical bonding in meat analogues were tested with urea, DTT and SDS; findings suggested that a large portion of aggregated proteins were linked with hydrogen bonds. Disulphide bonds became increasingly important as the amount of WG was increased, and synergies between hydrogen bonds and disulphide bonds were evident from increased solubility effects with mixed solvents, especially U and DTT. It was concluded that processing conditions during extrusion denatured the proteins, allowing increased crosslinking, which facilitated the formation of fibrous structures.

Keywords: meat analogue; wheat gluten; soy protein; high-moisture extrusion cooking; degree of texturisation; fibrous structure

6.2 Introduction

Meat analogues are food products that are designed to have sensory properties that are similar to meat but are made from plant proteins (Wild et al., 2014; Malav et al., 2015). Extruded chunked products with porous meat-like structure were first made during the early 1960s by

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Wenger laboratory (Sadler, 2004; Strahm, 2005), and the product was typically sold dry as texturised vegetable protein (TVP). The development of high-moisture meat analogues using extrusion cooking technology began in the early 1990s (Wild et al., 2014), and was probably dependent on the development of the TS food extruder. Meat analogues resemble meat in terms of its aesthetic properties such as structure, texture, flavour, colour, and appearance (Strahm, 2005; Asgar et al., 2010). They provide a high amount of protein and are designed as low-calorie food products for the human diet (Riaz, 2004).

The conventional development of meat analogues consists of two main steps, which are mixture preparation and chunk formation (Orcutt et al., 2006; Malav et al., 2015). The mixture is prepared either prior to extrusion or within the extruder by blending, chopping and mixing the proteins, fat, salts, and other ingredients to form a matrix of proteins that encapsulate the fat and non-soluble components. After blending of ingredients, the mixture is then extruded as water is being added during processing to obtain a final MC of about 60% (Lin et al., 2002; Chen et al., 2010). After water is injected into the extruder, the ingredients are heated to above 150°C before being forced through a cooling die (Cheftel et al., 1992). During extrusion processing heat and shear denature the proteins, allowing new chemical bonds to form between adjacent protein molecules and creating fibrous meat-like structure (Cheftel et al., 1992).

In this study, high moisture meat analogues were mainly made from SPC and WG. SPC is extracted from soybeans (*Glycine max*) and comprises 65-70% w/w soy protein (Golbitz & Jordan, 2006; Day, 2013). SPC-based meat analogues were reported to be easier to extrude and texturise compared to formulations based on SPI under similar conditions (Cheftel et al., 1992). SPI-based analogues exhibited homogenous structure while those that contained only SPC demonstrated an anisotropic structure with layers or coarse fibres in the direction of flow through the die. The primary storage protein group in wheat (*Triticum* spp.) grains is termed WG (Asgar et al., 2010; Day, 2013). It forms a cohesive viscoelastic network that is important in the production of many food products. WG is comprised of two protein classes: gliadin and glutenin. Gliadins are low or medium *Mw* monomeric proteins with intramolecular S-S bonds, while glutenins are much larger molecules containing different polypeptides connected by intermolecular S-S bonds (Wieser,

2007). WG holds the fibre together in the matrix for meat analogues (Rizvi et al., 1980), by serving as the main binding agent in the system to stick the product together and remain stable.

The use of soy protein only (i.e. SPI) in extruded meat analogues using different process parameters such as MC, cooking temperatures and SME on the textural and chemical characteristics has been previously reported (Chen et al., 2010; Chen et al., 2011; Fang et al., 2014). SME (kJ/kg) is the mechanical energy input required to obtain a unit weight of material through the extruder (Muthukumarappan & Karunanithy, 2012). The textural, microstructural, chemical and sensory characteristics of SPI:WS (9:1)-based extruded meat analogues have been investigated (Lin et al., 2000, 2002). In this study, different textural (i.e. cutting force) and microstructural (i.e. rapid freezing/ cryosectioning for LM) techniques were used to gather the latest information on the meat analogues. Lastly, Liu and Hsieh (2007) and Liu and Hsieh (2008) studied the hardness attribute and protein solubility of SPI:WG:WS (60:40:5)-based extruded meat analogues at different MC. There are also reports of meat analogues made from soy proteins and WG using Couette Cell (a concentric cylinder device comprising an inner rotating cylinder and an outer stationary cylinder) technology (Grabowska, Tekidou, Boom, & van der Goot, 2014; Krintiras et al., 2014; Krintiras et al., 2015; Krintiras, Diaz, Van der Goot, Stankiewicz, & Stefanidis, 2016). However, there are no studies reported on the effects of WG on the physicochemical properties of extruded SPC-based meat analogues. Therefore, in this study, the effects of SPC to WG ratio (89:0, 79:10, 69:20 and 59:30% w/w dry ingredient) on the physicochemical properties of extruded meat analogues are reported. In addition, microstructures and chemical linkages in meat analogues were measured, which provide understanding of the interactions among proteins when formed into fibrous meat-like structures.

6.3 Materials and methods

6.3.1 Materials

SPC (ALPHA® 11 IP, Solae™, 70.2% protein, 4.8% moisture, 4.4% ash, 18.8% carbohydrate and 1.8% fat) was purchased from Tari International NZ Ltd (Auckland, New Zealand). WG (FLOURG25, 75% protein, 10% moisture, 1.5% ash, 12.5% carbohydrate and 1% fat) and WS (FLOURCW25, 0.4% protein, 12.1% moisture, 0.5% ash and 87% carbohydrate) were purchased from Davis Trading (Palmerston North, Zealand). Chicken breasts were bought from

the local supermarket, New World (Palmerston North, New Zealand). Bovine serum albumin (BSA, Fraction V, Low Endotoxin, 98.9% protein and 1.1% moisture) powder was obtained from Invitrogen Corporation, New Zealand. Di-potassium hydrogen orthophosphate and potassium dihydrogen orthophosphate were obtained from Ajax Finechem, New Zealand. SDS and Bradford reagent were obtained from Sigma-Aldrich, New Zealand. DTT was obtained from Merck Life Science, New Zealand. Urea was obtained from ThermoFisher Scientific, New Zealand. Ultrapure water purified by treatment with a Milli-Q apparatus; Millipore Corporation (Bedford, Massachusetts, USA) was used in analytical experiments. All other chemicals and reagents used were of analytical grade.

Table 6-1 Proximate composition of ingredients used to produce extruded meat analogues specified by the manufacturers

Ingredient	Proximate composition (%)				
	Protein	Moisture	Ash	Carbohydrate	Fat
Soy protein concentrate	70.2	4.8	4.4	18.8	1.8
Wheat gluten	75.0	10.0	1.5	12.5	1.0
Wheat starch	0.4	12.1	0.5	87.0	0

6.3.2 High-moisture extrusion cooking

All extrusion experiments were performed using a pilot-scale, co-rotating, and intermeshing TS extruder (Clextral BC-21, Firminy Cedex, France). The extrusion formulation (% w/w of non-water ingredients) of four meat analogues with different SPC to WG ratio is based on 89:0 (0%WG), 79:10 (10%WG), 69:20 (20%WG) and 59:30 (30%WG), with 5% vegetable oil, 3% pumpkin powder, 2.7% WS and 0.3% salt (**Table 6-2**). The extrusion formulation was obtained based on previous work conducted at Massey University and from the guidelines given in **Table 2-4**. The operating parameters were set as followed: screw diameter (D_s), 25 mm; total screw length (L_s), 700 mm; length/ diameter ratio of screw (L_s/D_s), 28:1; barrel diameter (D_b), 26 mm; and a long cylindrical cooling die with a diameter of 10/355 mm was attached at the end of the extruder. The screw profile comprised (from feed to exit) of: two 50 mm length, 20 mm pitch, forward screw (100 mm); three 50 mm length, 15 mm pitch, forward screw (150 mm); two 50 mm, 10 mm pitch, forward screw (100 mm); one 50 mm, 15 mm pitch, forward screw (50 mm), one 25 mm, 7 mm pitch, reverse screw (25 mm); one 50 mm, 15 mm pitch, forward screw (50 mm), one 25 mm, 7 mm pitch, forward screw (25 mm); and four 50 mm, 7 mm pitch, forward screw (200

mm). The barrel was segmented into the feeding zone (T1) and six temperature-controlled zones (T2 to T7), which was heated by steam and cooled by running water pipes (~25°C). A gravimetric feeder (K-ML-D5-KT20 and LWF D5, Coperion K-Tron, Switzerland) was used to feed the dry ingredients into the extruder at a rate of 2.8 kg/h. Water was injected into the extruder through an inlet port at a constant flow of 3.6 kg/h to obtain the MC of approximately 60% w/w (wet basis) in the final product. The screw speed was 400 rpm and the barrel temperatures were set at 20, 50, 80, 110, 150, 170 and 150°C in the seven zones from feed to die.

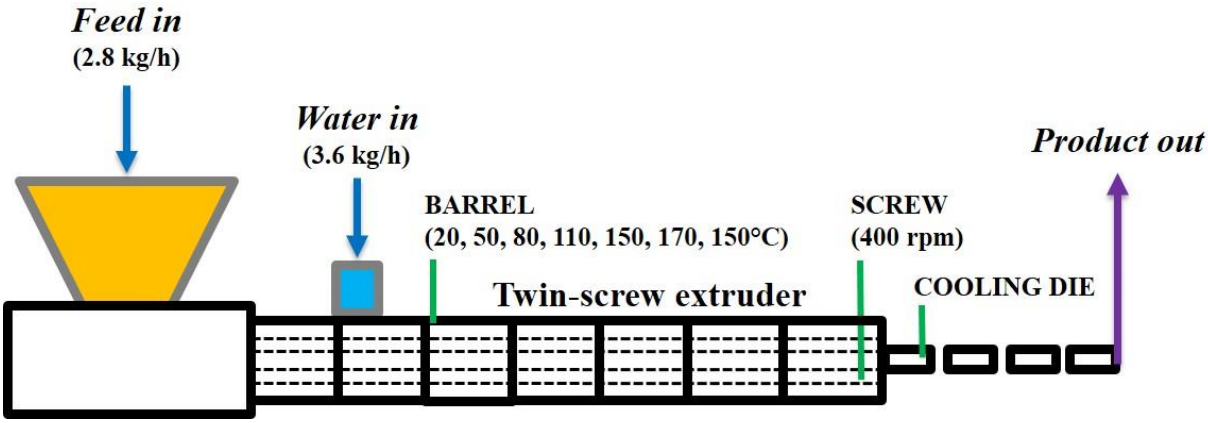

Figure 6-1 Schematic illustration of twin-screw extruder used to produce meat analogues.

Table 6-2 Formulation of extruded meat analogues at different soy protein concentrate (SPC) to wheat gluten (WG) ratio

Sample	Formulation (% non-water ingredients)					
	Soy protein concentrate	Wheat gluten	Wheat starch	Oil	Salt	Pumpkin powder
0%WG	89	0	2.7	5	0.3	3
10%WG	79	10	2.7	5	0.3	3
20%WG	69	20	2.7	5	0.3	3
30%WG	59	30	2.7	5	0.3	3

6.3.3 Preparation of cooked chicken breast

Cooked chicken breasts were prepared according to Lyon and Lyon (1991) and Morey and Owens (2017) with modifications. Chicken breasts were individually packaged in plastic bags and cooked in a heated water bath. The breasts were cooked to an internal temperature of 75-80°C, removed from the water bath and cooled at room temperature for 30 min, drained and sectioned for cutting force and TPA to ensure uniform sampling temperature.

6.3.4 Protein, moisture, pH and colour analysis

The protein content of the meat analogues and cooked chicken breasts were determined by the Kjeldahl method using Kjeltec system with 2006 Digestor and 2100 Distilling Unit (Foss Tecator Inc, Höganäs, Sweden). The resulting nitrogen content was multiplied by 6.25 for meat and SPC, and 5.7 for WG to determine protein content.

The MC of the meat analogues and cooked chicken breasts were determined using the air-oven method as described by Nielsen (2010) with modifications. Pans and lids were dried in the oven (Contherm Scientific, New Zealand) at 108°C for an hour and cooled in the desiccator for another hour before analysis. The weights of empty pans and lids were then recorded, and 2 g of shredded samples were placed into numbered pans and lids and put into the oven for 24 hours. The weight of the pans, lids and samples after drying were recorded after cooling in a desiccator for an hour.

The pH of the meat analogues and cooked chicken breasts were measured using a benchtop pH meter (SG23, SevenGo Duo™, Mettler-Toledo AG, Switzerland) as described by Liu and Hsieh (2007). The pH meter was calibrated using buffer solutions (pH 4 and 7) before analysis. The pH values were measured after blending samples using a high-shear mixer (DIAX600, Heidolph, Germany) at 24,000 rpm, with ultrapure water at 20% w/w concentration for one minute. The pH values were recorded once the readings were stabilised.

The colour of the meat analogues and cooked chicken breasts was determined using a hand-held chroma meter (CR-400, Minolta Co, Japan) as described by Fang et al. (2014) with modifications. The instrument was calibrated with a white tile, and colours were expressed in CIE-Lab parameters as L^*, a^*, and b^*. Three measurements were taken at random surface locations of the samples. Coordinate L^* represents the lightness of colour (0 = black and 100 = white), -*a*/+*a* represents the greenness or redness, and –*b*/+*b* represents the blueness or yellowness. The standard values for the white tile calibration were $L_s^* = 94.56$, $a_s^* = -0.40$, $b_s^* = 3.66$.

6.3.5 Textural properties analysis

6.3.5.1 Cutting force

The cutting force of the meat analogues and cooked chicken breasts were analysed using a texture analyser (TA.XT Plus, Stable Micro Systems, UK) as described by Chen et al. (2010) and Osen et al. (2014) with modifications. The sample was cut into square shape and dimension (15×15×8 mm) and a craft knife blade probe was used to cut the sample to 75% of its original thickness at a speed of 1 mm/s along the direction vertical (F_L) and parallel (F_V) to the direction of meat analogues outflow from the extruder, respectively. The degree of texturisation (DT) was used to indicate fibrous structure formation and was expressed as the ratio of F_L and F_V.

6.3.5.2 Hardness and chewiness

The hardness and chewiness of the meat analogues and cooked chicken breasts were analysed using the 2-bite test with a texture analyser (TA.XT Plus, Stable Micro Systems, UK) as described by Fang et al. (2014) with modifications. The sample was cut into square shape and dimension (15×15×8 mm) and compressed using a P/51 probe to 50% of its original thickness at a speed of 1 mm/s for the first bite, returned to original position over 5 sec, and followed by the second bite at 1 mm/s to 50% of the first compressed thickness.

6.3.6 Sensory evaluation

The sensory attributes difference test for meat analogues and the cooked chicken breast was evaluated as described by Choi (2013) with modifications. A total of 46 panellists (25 women and 21 men, who were 18 to 55 years old of age) participated in the study. Sensory testing was conducted at Massey University's Sensory Laboratory. Meat analogues and chicken breast were fan-grilled in the oven at 180°C for 10 min and 15 min, respectively and cut into pieces of 25×15×8 mm before serving to panellists.

The samples were coded with randomly selected 3-digit numbers and placed in balanced order for tasting. Each panellist was presented with a tray containing five samples in 50 mL plastic sampling containers. The evaluation session was conducted in individual booths at an air-conditioned temperature under normal lighting. To eliminate carryover factors, consumers were provided with room temperature water for mouth cleaning between samples. The consumers were

asked to rate their intensity scores for fibrous structure (visual observation by tearing the sample into half), hardness (bite completely through the sample between the molar teeth) and chewiness (chew the sample for at least 24 chews) using a 9-point scale (fibrous structure: 1 = 'not fibrous' and 9 = 'very fibrous'; hardness: 1 = 'soft', 5 = 'firm' and 9 = 'hard'; chewiness: 1 = 'tender', 5 = 'chewy' and 9 = 'tough'). Approval to use human subjects for the sensory evaluation was granted by the Massey University Human Ethics Committee, Southern A.

6.3.7 Microscopy analysis

6.3.7.1 Scanning electron microscopy

For visualisation under the scanning electron microscope, samples of meat analogues and cooked chicken breasts were cut into approximately 10×10×10 mm pieces and were fixed in primary Modified Karnovsky's fixative (3% glutaraldehyde, 2% formaldehyde in 0.1 M phosphate buffer, pH 7.2) and allowed to fix for at least 8 hours at room temperature. After rinsing three times (10-15 min each) in 0.1M phosphate buffer solution at pH 7.2, the samples were dehydrated in a graded series of ethanol solutions (25%, 50%, 75%, 95% and 100%) for 15 min each and a final 100% ethanol wash for 1 hour. The samples were then critical point dried using liquid CO_2 as the CP fluid and 100% ethanol as the intermediary (Polaron E3000 series II critical point drying apparatus). Once dried, the samples were mounted onto an aluminium stub using double-sided tape and silver conductive paint (RS Components, UK) and sputter coated with approximately 100 nm of gold (Baltec SCD 050 sputter coater) and viewed in the scanning electron microscope (Quanta 200 Environmental, FEI Company, USA) at 250× magnification, at an accelerating voltage of 20 kV.

6.3.7.2 Light microscopy (rapid freezing and cryosectioning)

Each piece of meat analogue and cooked chicken breast was placed onto a specimen holder and embedded in O.C.T. compound (Tissue-Tek, ProSciTech, New Zealand). The specimen holder was gently placed in a shock freezer at –50°C and was rapidly frozen for at least 10 min. Subsequently, the frozen sample was affixed to a cryostat (Jung CM1800, Leica Microsystems, Germany) at –20°C. The block was positioned on the stage of the cryostat for thin sectioning. After the sample surfaces were removed, the antiroll plate was set under the blade, and the samples were sliced to 18 μm. Because the thinly sectioned samples remained on the blade, a glass slide (IHC,

FLEX, Dako) was pressed on the top of the blade for adhesion. All sliced samples were air-dried on microscope glasses. A progressive H&E method according to Gill, Frost, and Miller (1974) was performed on all slides using an autostainer XL staining platform (ST5010, Leica Biosystems, Germany). Sections were washed in water, dehydrated using 100% alcohol, cleared in xylene and permanently mounted with DPX (a mixture of distyrene, plasticiser and xylene) and a coverslip (CV5030, Leica Biosystems, Germany). Sections were then viewed under a bright-field light microscope (Zeiss Axiophot, Germany) at 100× magnification, fitted with a colour top mount digital camera (DFC320, Leica Microsystems, Germany) using Leica Application Suite software (Version 3.8.0, Leica Microsystems, Switzerland).

6.3.8 Protein solubility analysis

The solubility of protein from the meat analogues and cooked chicken breasts was analysed as described by Chen et al. (2011) and Osen et al. (2015) with modifications. Different extracting solutions (1–8) were used to dissolve specific chemical bonds within the protein networks as shown in **Table 6-3**.

Table 6-3 Eight extracting solutions with selecting reagents and their combinations used for assessing protein solubility

S/N	Extracting solution	Chemical bond and their interactions
1	PB (P); 0.1 M phosphate buffer consisting of KH_2PO_4 and K_2HPO_4 with a pH of 7.5	Native state protein
2	PB+Urea (PU); 8M urea	Hydrogen bonds
3	PB+DTT (PD); 0.05 M dithiothreitol	Disulphide bonds
4	PB+SDS (PS); 1.5 g/100 ml sodium dodecyl sulphate	Hydrophobic interactions
5	PB+U+DTT (PUD)	Interactions between hydrogen bonds and disulphide bonds
6	PB+U+SDS (PUS)	Interactions between hydrogen bonds and hydrophobic interactions
7	PB+DTT+SDS (PDS)	Interactions between disulphide bonds and hydrophobic interactions
8	PB+U+DTT+SDS (PUDS)	Interactions among hydrogen bonds, disulphide bonds and hydrophobic interactions

A 0.5 g sample was extracted in a polystyrene bottle with 10 mL of each extracting solution for 30 min on a shaker, followed by blending the mixture using a high-shear mixer (DIAX600,

Heidolph, Germany) at 24,000 rpm for 1 min. The mixture was then agitated for another 30 min, followed by centrifugation at 4500 rpm for 10 min. The supernatant was then filtered through a 0.22 μm PES (polyethersulfone) syringe filter and collected. The soluble proteins in the supernatants were determined using the Bradford protein assay at 595 nm with a multi-plate reader (SPECTROstar Nano, BMG LABTECH, Germany) using bovine serum albumin (BSA) powder as a standard. The total nitrogen in the original samples was measured using the Kjeldahl method described earlier, and the protein content was calculated with a conversion factor of 6.25 for SPC and 5.7 for WG. The protein solubility was calculated as the ratio of soluble protein in the supernatant to total protein in the samples.

6.3.9 Data analysis

All experimental work was carried out in three replicates, on freshly prepared samples and the results were reported as means ± standard deviations of the measurements. Data were analysed using Minitab® 16.2.1 statistical software (Minitab Inc, USA). Statistical analyses of observed differences among means consisted of one-way analysis of variance (ANOVA), followed by Tukey's pairwise comparison of means ($p \leq 0.05$). Figures were exported from Origin Software 8.5 (OriginLab Corp., MA, USA).

6.4 Results and discussions

6.4.1 Effects of SPC to WG ratio on the protein content, moisture content, pH and colour properties of soy-based meat analogues

The meat analogues made with different SPC to WG ratio in comparison with cooked chicken breast meat are shown in **Figure 6-2**. The protein content, MC, pH and colourimetric properties of meat analogues at different SPC to WG ratio are shown in **Table 6-4**. The protein content of all four meat analogues ranged between 25.38 and 26.76%. Although these differences were significant, they were rather small and only slightly below that of the cooked chicken breast.

Table 6-4 Protein content (% w/w of wet material), moisture content, pH level and *L** value of extruded meat analogues at different soy protein concentrate to wheat gluten ratio

Sample	Protein content (%)	Moisture content (%)	pH	Colour (*L** value)
0%WG	25.38 ± 0.33^{c}	56.95 ± 0.18^{bc}	7.13 ± 0.01^{a}	58.01 ± 2.55^{c}
10%WG	25.86 ± 0.13^{bc}	57.36 ± 0.14^{bc}	7.10 ± 0.02^{a}	60.72 ± 1.20^{b}
20%WG	26.27 ± 0.41^{bc}	56.48 ± 0.10^{c}	7.06 ± 0.03^{ab}	60.47 ± 0.75^{bc}
30%WG	26.67 ± 0.31^{b}	57.47 ± 0.30^{b}	6.98 ± 0.03^{b}	61.44 ± 0.68^{b}
Boiled chicken	28.97 ± 0.57^{a}	69.11 ± 1.23^{a}	6.26 ± 0.02^{c}	76.68 ± 1.95^{a}

Data are presented as the mean and standard deviation of three replicates.
Values bearing different lowercase letters were significantly different ($p \leq 0.05$).

Meat analogues contained MC that ranged between 56.48 and 57.47%, which was slightly below the targeted MC of 60% in the literature. Although the raw chicken breast (~75% w/w moisture) lost moisture during cooking, cooked chicken breast had the highest MC among the samples. The loss of water was due to denaturation of muscle proteins at high temperatures, inducing transverse and longitudinal shrinkage (Warner, 2017).

The pH of the meat analogues ranged between 6.98 and 7.13. Although there were significant differences between meat analogues, the differences were rather small. Cooked chicken breast showed a slightly higher pH than literature values (6.09 and 6.21) (Fletcher, Qiao, & Smith, 2000).

The *L** value of the meat analogues increased slightly with increasing WG levels (**Figure 2**). This showed that meat analogues containing 30%WG had most lightness compared to the other three samples. Cooked chicken breast exhibited higher *L** values than meat analogues due to its different composition.

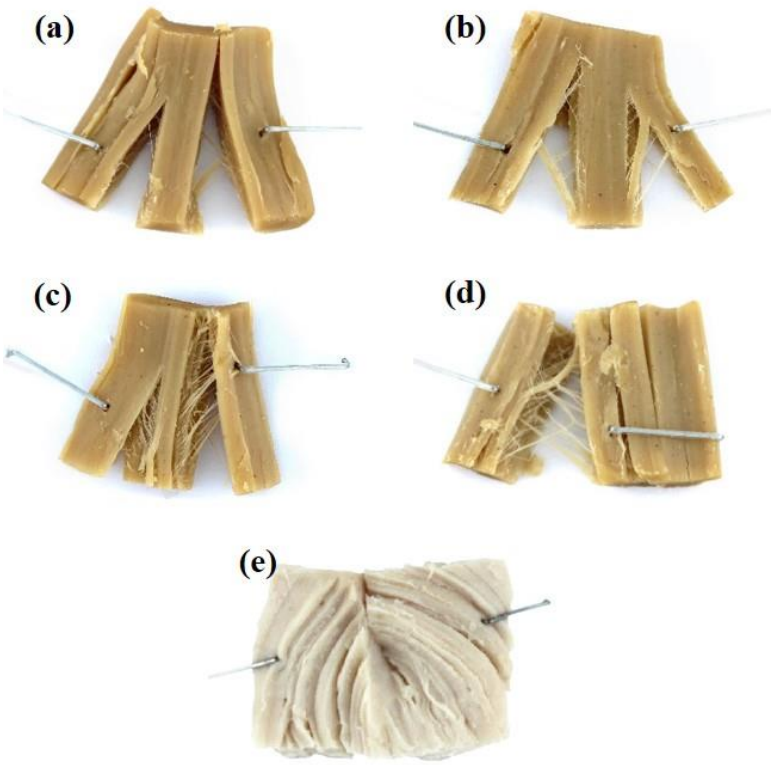

Figure 6-2 Visual images of extruded meat analogues with different soy protein concentrate to wheat gluten ratio (a) 0%WG, (b) 10%WG, (c) 20%WG, (d) 30%WG, and (e) visual image of boiled chicken breast.

6.4.2 Effects of SPC to WG ratio on the textural properties of soy-based meat analogues

6.4.2.1 Instrumental analysis

The degree of texturisation (DT = F_L/F_V) is an indicator of fibrous structure formation (Chen et al., 2010), where F_L/F_V should exhibit dimensionless value >1, because more force should be required to cut across the fibres than is needed to cut between the fibres (i.e. when the cut is made parallel to the fibres). If the fibrous structure is not present, then F_L and F_V will be similar and DT will be closer to 1. The DT of meat analogues with different SPC to WG ratio is shown in **Table 6-5**.

Table 6-5 Textural (instrumental and sensory) properties of meat analogues with different soy protein concentrate to wheat gluten ratio

Sample	Instrumental textural properties		
	Degree of texturisation	**Hardness (N)**	**Chewiness (N)**
0%WG	1.30 ± 0.14^{c}	45.40 ± 4.69^{c}	36.28 ± 4.14^{ab}
10%WG	1.35 ± 0.16^{bc}	44.88 ± 7.54^{c}	34.02 ± 7.14^{b}
20%WG	1.53 ± 0.24^{abc}	60.88 ± 9.57^{bc}	41.79 ± 7.79^{ab}
30%WG	1.74 ± 0.18^{a}	78.61 ± 4.18^{a}	45.32 ± 5.18^{a}
Boiled chicken	1.63 ± 0.24^{ab}	55.01 ± 7.37^{b}	18.84 ± 3.01^{c}
Sample	**Sensory properties**		
	Fibrous structure	**Hardness**	**Chewiness**
0%WG	5.72 ± 2.23^{b}	5.63 ± 1.83^{b}	5.39 ± 1.57^{a}
10%WG	6.04 ± 1.86^{ab}	5.74 ± 1.86^{ab}	5.33 ± 1.58^{a}
20%WG	6.28 ± 2.15^{ab}	6.15 ± 1.74^{ab}	5.52 ± 1.72^{a}
30%WG	6.85 ± 1.65^{a}	6.67 ± 1.61^{a}	5.87 ± 1.67^{a}
Grilled chicken	5.59 ± 1.92^{b}	2.72 ± 1.22^{c}	3.07 ± 1.55^{b}

Data are presented as the mean and standard deviation.
Values bearing different lowercase letters were significantly different ($p \leq 0.05$).

The DT increased with a decreasing ratio of SPC to WG. The DT was higher than that reported by Fang et al. (2014) and Chen et al. (2010) on meat analogues made from SPI without WG; in those cases, the DT ranged between 1.06 and 1.20. Meat analogues containing 30%WG had the highest DT among the meat analogues. This indicated that WG was an important ingredient for the formation of the fibrous structures in meat analogues. Extrusion SME for meat analogues containing 0%, 10%, 20% and 30%WG were 700, 646, 592 and 475 kJ/kg, respectively. An increase in DT was observed when the calculated SME decreased, which was in accordance with Fang et al. (2014). Boiled chicken breast showed DT between 20%WG and 30%WG, with no significant difference.

Textural Profile Analysis (TPA) uses a double compression test to imitate chewing and calculates sensory-relevant parameters from the resulting force-time curves (Bourne, 2002). The textural properties of meat analogues with different SPC to WG ratio are shown in **Table 6-5**. Hardness is the maximum force of the first compression, while chewiness applies only to solid products and is calculated as $Hardness \times Cohesiveness \times Springiness$ (Bourne, 2002; TTC, 2016). The instrumental hardness and chewiness of meat analogues increased with decreasing SPC to WG ratio. Decreased SME correlated with an increase in instrumental hardness and chewiness. However, Fang et al. (2014) reported that the instrumental hardness and chewiness of texturised

soy proteins increased 22.47% and 17.01%, respectively, as SME rose from 820 to 1259 kJ/kg, indicating that lower SME correlated with extrudates having lower hardness and chewiness. Boiled chicken breast was observed to have hardness in between meat analogues containing 10%WG and 20%WG, and a significantly lower chewiness than all the meat analogue samples. The high chewiness in meat analogues corresponds with low SME values, which are indicative of low melt viscosity (Day & Swanson, 2013). This is consistent with the extrusion melt having higher plasticity as more WG is substituted for SPC, which perhaps resulted in better dissolution of proteins within the melt (Day & Swanson, 2013) and a more dense, cohesive structure on cooling (Cheftel et al., 1992).

6.4.2.2 Sensory analysis

An attribute difference test was conducted to quantitatively evaluate differences in texture between the meat analogues. The sensory properties of the meat analogues in terms of fibrous structure, hardness and chewiness of meat analogues with different SPC to WG ratio are shown in **Table 6-5**. Panellists were asked to tear the meat analogues apart to observe the fibrous network, by looking for the presence of long visible strands that were pulled apart longitudinally.

The fibrous structure scores increased with decreasing SPC to WG ratio. This correlated with DT results from cutting force analysis. Meat analogues containing 30%WG showed significant difference to meat analogues containing 0%WG. This indicated that WG improved the formation of a fibrous network and 30%WG was required to demonstrate the difference in fibrous network in meat analogues using sensory analysis. Grilled chicken breast received the lowest fibrous structure score when compared with meat analogues. Panellists may have mentally conflated the term ‘fibrous’ with concepts such as ‘hard’ and ‘tough’, as used in common parlance, and given grilled chicken lower fibrous scores based on lower hardness and chewiness (**Table 6-5**).

Hardness is defined as the force required to compress a substance between the molar teeth for solid foods to a given deformation or penetration (Szczesniak, 2002). Chewiness is defined as the energy required to masticate solid food to a state ready for swallowing. Sensory hardness and chewiness scores increased with decreasing SPC to WG ratio. This correlated with instrumental

results from TPA. Boiled chicken breast had hardness in between meat analogues containing 10%WG and 20%WG in TPA. However, grilled chicken scored the lowest hardness in sensory analysis. This could be due to the loss of moisture from meat analogues during grilling, thus increasing the hardness. Results from both instrumental and sensory analysis indicated that the two methods were useful in differentiating the textural properties of meat analogues at different SPC to WG ratio.

6.4.3 Effects of SPC to WG ratio on the microstructural properties of soy-based meat analogues

Scanning electron and light micrographs for meat analogues with different SPC to WG ratio are shown in **Figure 6-3**. From the SEM micrographs, a layered structure was observed for meat analogues containing 0%WG and 10%WG (**Figure 6-3a(i) and b(i)**) ranged between 90-160 μm and 40-100 μm, respectively, with multiple segmented layers observed for meat analogues containing 10%WG. Fibrous microstructure was observed for meat analogues containing 20%WG and 30%WG (**Figure 6-3c(i) and d(i)**) ranged between 10 and 40 μm. Similar trends were also observed for light micrographs, where meat analogues containing 0% WG and 10% WG (**Figure 6-3a(ii) and b(ii)**), showing layered structure, while a fibrous network was observed for meat analogues containing 20% WG and 30% WG (**Figure 6-3c(ii) and d(ii)**).

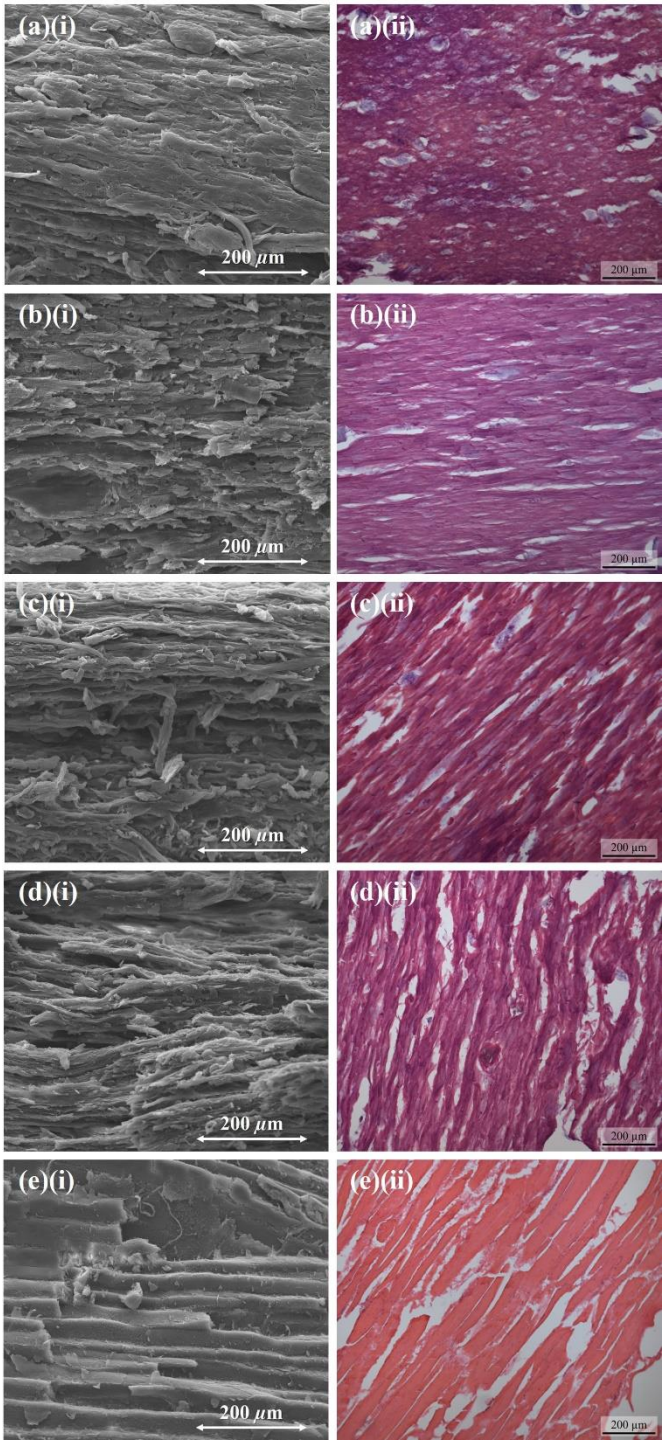

Figure 6-3 (i) Scanning electron and (ii) light micrographs of extruded meat analogues with different soy protein concentrate to wheat gluten ratio (a) 0%WG, (b) 10%WG, (c) 20%WG, (d) 30%WG, and (e) micrographs of boiled chicken breast, at 250× magnification and 100× magnification, respectively.

It was also noted that the large fibrous structures observed in meat analogues containing 20%WG and 30%WG were interconnected by much smaller fibres, which were also reported in the study by Krintiras et al. (2015) on meat analogues made from SPI containing 22.6%WG. Krintiras et al. (2015) suggested that those smaller fibres were probably WG. This indicated evidence for the existence of anisotropic structures in the four meat analogues and higher WG levels contributed to the fibrous microstructures. The SEM micrograph for cooked chicken breast (**Figure 6-3e**) demonstrated the muscle fibres with connective tissues, which was in accordance with Takei et al. (2016). The muscle fibres in cooked chicken breast ranged between 25 and 55 μm, which were larger than those in meat analogues containing 20%WG and 30%WG.

6.4.4 Effects of SPC to WG ratio on the protein solubility of soy-based meat analogues

The effects of SPC to WG ratio on protein solubility were conducted by determining the amount of protein from meat analogues that were solubilised by eight different extracting solutions consisting of four selective reagents (**Figure 6-4**). These solutions solubilised the protein by breaking different classes of intermolecular chemical bonds (Lin et al., 2000). Phosphate buffer (PB) extracted the least amount of protein in all samples. This reflects the strength and diversity of intermolecular bonding in meat analogues. Increasing WG led to decreasing PB extractability, which illustrates that WG is a more effective binder than SPC (Liu & Hsieh, 2007).

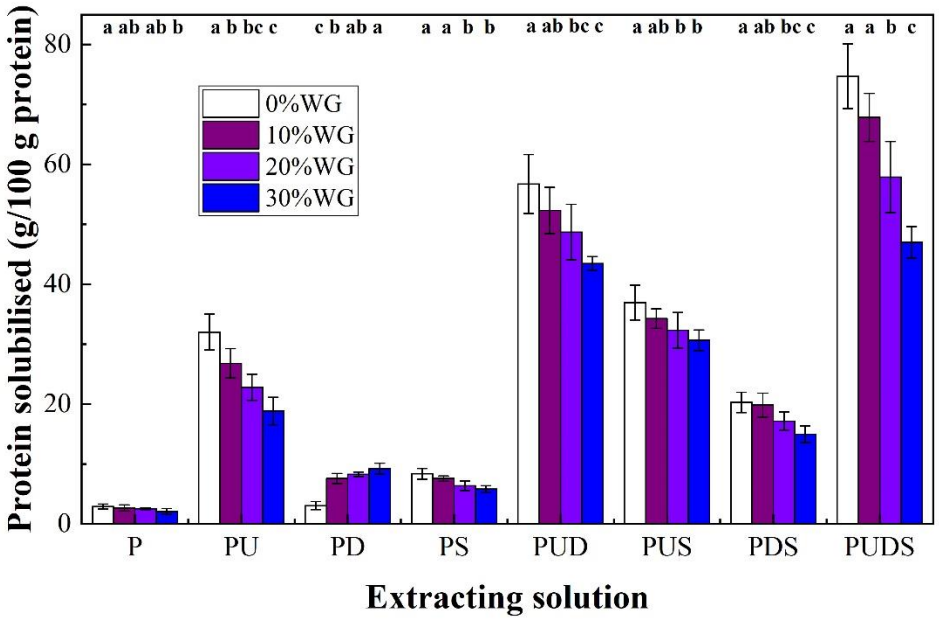

Figure 6-4 Effects of soy protein concentrate to wheat gluten ratio on the protein solubility of extruded meat analogues induced by different extracting solutions PB (P), PB+U (PU), PB+DTT (PD), PB+SDS (PS), PB+U+DTT (PUD), PB+U+SDS (PUS), PB+DTT+SDS (PDS), and PB+U+DTT+SDS (PUDS). Data represent the mean and error bars represent the standard deviation. Values bearing different lowercase letters were significantly different ($p \leq 0.05$).

When Urea, DTT or SDS was combined with PB, the amount of protein solubilised for meat analogues increased. This suggested that the protein in the meat analogues was aggregated with more than one type of chemical bond as mentioned by Lin et al. (2000). The amount of protein solubilised by PB+Urea was the highest for the two-component solvents. This indicated a large portion of the protein was linked with H-bonds. The lower amount of protein solubilised by PB+DTT and PB+SDS showed that S-S bonds and hydrophobic interactions were less important. Increasing DTT-solubilised protein with increasing WG level correlates with DT (**Table 6-5** and **Figure 6-3d(i) and (ii))**, which suggests that S-S bonds play a role in the formation of fibrous structure. The majority of these S-S bonds could be attributed to WG itself.

When two or more reagents were combined with PB, the amount of protein solubilised for meat analogues further increased. This suggested that there were interactions between the chemical bonds. Firstly, protein solubility was greater for all samples in PB+U+DTT, compared to the sum

of PB+Urea and PB+DTT. This could be due to the synergistic effect of the two reagents. Liu and Hsieh (2007) reported a similar synergy between urea and DTT.

The protein solubilised by a combination of PB+DTT+SDS (S-S bonds and hydrophobic interactions) was approximately equal to the sum of PB+DTT and PB+SDS (**Figure 6-4**), and the same could be said for PB+U+SDS *vs.* PB+U and PB+SDS. Lin et al. (2000), Rareunrom et al. (2008) and Chen et al. (2011) reported that the disruption of non-covalent forces (PB+U+SDS) solubilised the lowest amount of protein. However, in this study, the lowest amount of protein was solubilised by PB+DTT+SDS. On the other hand, for the combination of PB+U+DTT+SDS, the amount of protein solubilised was greater than 45%. This indicated that the protein structure of meat analogues was supported by H-bonds, S-S bonds, hydrophobic interactions and their combinations, which was in accordance with Liu and Hsieh (2008) and Chen et al. (2011).

6.5 Conclusion

The objective of this study was met where four extruded meat analogues at different SPC to WG ratio had successfully been produced using a TS extruder. The change in SPC to WG ratio affected the formation of fibrous structure in the meat analogues, where decreasing SPC to WG ratio showed increased fibrous structure using textural, sensory and microscopy analysis. Meat analogues containing 30%WG exhibited the highest DT, fibrous structure, hardness and chewiness when compared with others. The use of boiled chicken breast as a reference food helped for comparison purposes. Meat analogues containing 20%WG and 30%WG were found to be the closest in terms of structural properties rather than textural and sensory properties. H-bonds were the major force in the formation and stabilisation of the structure of meat analogues. However, S-S bonds were the key force in forming the fibrous structure of the meat analogues made from high moisture extrusion. Overall, WG played an important role by contributing to the increase in S-S bonds in meat analogues to form fibrous structure.

Chapter 7: [4]Effects of Maillard-reacted beef bone hydrolysate on the physicochemical properties of extruded meat alternatives

7.1 Abstract

Meat analogues are made from plant proteins using high-moisture extrusion processing, to have the same sensory properties as meat. However, meat analogues exhibit very weak aroma and are almost tasteless which has resulted in limited market success. Maillard-reacted beef bone hydrolysate (MRP) provides important sensory characteristics of heat-treated food products, by contributing to their appearance, texture, flavour and aroma. Therefore, MRP added at different concentrations to the plant proteins before extrusion may produce meat alternatives with high aroma and taste quality whilst maintaining a fibrous structure. This study investigated the effects of MRP at different concentrations (0, 10, 20, 30 and 40% wet weight) with plant proteins on the textural, microstructural, chemical and sensory properties of meat alternatives. Meat alternatives consisting of 40%MRP showed the lowest degree of texturisation and were observed to have multiple-segmented layers accompanied with some fibrous microstructure. Results from protein solubility analysis suggested that a large proportion of the aggregated proteins were held together by hydrogen bonds. While the key force in the formation of fibrous structure in meat alternatives was disulphide bonds. Meat alternatives containing 20%MRP obtained the highest sensory scores for appearance, meaty aroma, meaty taste and overall acceptability. Overall, results showed that the addition of MRP to produce meat alternatives changed the textural, structural and sensory properties significantly.

Keywords: Maillard reaction products; extrusion cooking; meat alternatives; degree of texturisation; fibrous microstructure; sensory quality

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7.2 Introduction

The meat industry in New Zealand produced about 1.10 million tons of red meat (beef, lamb and mutton) for export in the Year 2017-18 (Beef + Lamb New Zealand, 2018). The meat sector is a large and growing industry and plays an important role in economic development across New Zealand. However, the meat industry also produces large amounts of low-value by-products (also referred as co-products) including bone, reclaimed meat, meat trimmings, skin, fatty tissues, offal, blood, etc., during the production and processing of meat (Mullen et al., 2017; Shen, Zhang, Bhandari, & Gao, 2018). If these by-products are not effectively utilised, valuable sources of potential revenue will be lost, while adding and increasing the cost of disposal of these products. New techniques or new products that use these by-products along with some scientific developments have enormous potential to value-add and ensure the sustainability of the meat industry (Toldrá, Aristoy, Mora, & Reig, 2012).

Scientific research and development work can help to convert low-value meat by-products into a product which is capable of offsetting all processing and disposal costs and also reducing environmental damage. Henchion, McCarthy, and O'Callaghan (2016) discussed some of the proposed pathways toward the commercialisation or increased use of by-products. One option is to develop food products that can be used as edible ingredients rather than as finished products. However, their compatibility with other ingredients must be established so that they can be incorporated into an existing product using a processing technology that is linked with benefits. For instance, edible meat by-products from beef cattle such as meaty beef bones (6-12% of carcass weight) are one possibility. These are pressure-cooked in water, to extract gelatine and the soluble components of meat. The resulting solution is defatted and then concentrated, yielding beef bone extract which has low fat, is viscous, and is a good source of minerals and amino acids (Song et al., 2016; Chiang et al., 2019b). Value can be added to the bone extract by using enzymatic hydrolysis to increase the proportion of FAA, followed by MR with added reducing sugars and using a high-temperature thermal treatment to produce MRP (Shen et al., 2018). MRP provides important sensory aspects of heat-treated food products, by contributing to their appearance, texture, flavour and aroma (Bastos, Monaro, Siguemoto, & Séfora, 2012).

In previous studies, the meat flavour of beef bone extract was successfully enhanced through different enzymatic hydrolysis treatments using plant and/ or microbial proteases (Chiang et al., 2019b), followed by MR with ribose in a pressure cooker (Chiang, Eyres, Silcock, Hardacre, & Parker, 2019a). This study aims to incorporate the Maillard-reacted beef bone hydrolysates into meat analogues (Chiang, Loveday, Hardacre, & Parker, 2019c). Meat analogues are made from plant proteins using high-moisture extrusion processing, to have the same sensory properties as meat (Malav et al., 2015). However, the meat analogues exhibit a very weak aroma and are almost tasteless, which has resulted in limited market success (Wild et al., 2014). Therefore, there could be a possibility to add Maillard-reacted beef bone hydrolysate at different concentrations with the plant proteins before extrusion to assess whether it was possible to produce meat alternatives with expected high aroma and taste quality, whilst still preserving a fibrous texture.

To date, there are no studies that have investigated the interactions between meat and plant proteins to form meat alternatives using a TS extruder. Therefore, the objective of this study was to understand the effects of Maillard-reacted beef bone hydrolysate at different concentrations (0, 10, 20, 30 and 40% wet weight basis) with plant proteins (SPC and WG) on the physicochemical properties of extruded meat alternatives. The textural properties (cutting force, hardness and chewiness), microstructures, chemical linkages in meat alternatives and sensory evaluation were studied.

7.3 Materials and methods

7.3.1 Materials

Beef bone extract used as the substrate for hydrolysis and subsequent MR was obtained from Taranaki Bio Extracts Ltd, New Zealand. Flavourzyme® 1000L (Batch: HPN00539) was given by Nutura, New Zealand. D-ribose was obtained from Amtrade NZ Ltd. SPC (ALPHA® 11 IP, Solae™) was purchased from Tari International NZ Ltd. WG (FLOURG25) and WS (FLOURCW25) were bought from Davis Trading, New Zealand. The proximate composition of these raw materials is shown in **Table 7-1**. Bovine serum albumin (BSA) powder (Fraction V, 98.9% protein) was obtained from Invitrogen, New Zealand. Mono-potassium phosphate and di-potassium phosphate were supplied by Ajax Finechem, New Zealand. Urea was purchased from ThermoFisher Scientific, New Zealand. DTT was supplied by Merck Life Science, NZ. Bradford

reagent and SDS were purchased from Sigma-Aldrich, New Zealand. Ultrapure water obtained through purification treatment using a Milli-Q apparatus (Millipore Corporation, USA) was used for all analytical experiments. All other chemicals and reagents used were of analytical grade.

Table 7-1 Proximate composition of ingredients used to produce extruded meat alternatives specified by the manufacturers

Ingredient	Proximate composition (%)					
	Protein	Carbohydrate	Fat	Moisture	Ash	Total soluble solids
Beef bone extract	≥44	-	≤1	≤55	≤3	≥53°Brix
Soy protein concentrate	70.2	18.8	1.8	4.8	4.4	-
Wheat gluten	75.0	12.5	1.0	10.0	1.5	-
Wheat starch	0.4	87.0	0	12.1	0.5	-

7.3.2 Preparation of Maillard-reacted beef bone hydrolysate

The enzymatic hydrolysis of beef bone extract was conducted as described by Chiang et al. (2019b), using Flavourzyme® 1000L (Batch: HPN00539, Novozymes, Denmark) at an E/S ratio of 4.70% w/w. The preparation of Maillard-reacted beef bone hydrolysate (MRP) was carried out as described by Chiang et al. (2019a), where hydrolysate and D-ribose (Amtrade NZ Ltd, New Zealand) were mixed at a ratio of 1:0.068 (protein weight to reducing sugar weight) at pH 6.5, and pressure-cooked at 170 kPa (113°C) for 10 min in a pressure cooker (Model No. 921, All American, Wisconsin, USA). The MRP was stored at 4°C before extrusion cooking.

7.3.3 Extrusion cooking

All extrusion cooking was carried out using a pilot plant-scale, co-rotating and intermeshing TS extruder (Clextral BC-21, Firminy Cedex, France), and the operating parameters were set as described by Chiang et al. (2019c), where water and MRP (liquid feed) were pumped into the side of the extruder through an inlet port at a steady rate of 3.6 kg/h. The extrusion formulation (% w/w) of meat alternatives at different concentrations of MRP is summarised in **Table 7-2.** MRP (approximately 55% water and 45% solids) was added in the formulation where the proportion of water and dry ingredients were set constant.

Table 7-2 Extrusion formulation of meat alternatives at different concentrations of Maillard reaction products (MRP)

Sample	Formulation (% w/w)							
	Soy protein concentrate	Wheat gluten	Wheat starch	Oil	Salt	Pumpkin powder	MRP	Water
0%MRP	23.60	12.00	1.08	2.00	0.12	1.20	0	60.0
10%MRP	20.95	10.65	0.96	1.78	0.11	1.07	10	54.5
20%MRP	18.29	9.30	0.84	1.55	0.09	0.93	20	49.0
30%MRP	15.64	7.95	0.72	1.33	0.08	0.80	30	43.5
40%MRP	12.98	6.60	0.59	1.10	0.07	0.66	40	38.0

7.3.4 Moisture, protein, pH and colour analysis

The methodologies for MC, protein content, pH level and L^* value of the meat alternatives were reported in **Section 6.3.4**.

7.3.5 Textural properties analysis

The methodologies for cutting force, hardness and chewiness (2-bite test) of meat alternatives were reported in **Section 6.3.5**.

7.3.6 Microscopy analysis

The methodologies for SEM and LM of meat alternatives were reported in **Section 6.3.7**.

7.3.7 Protein solubility and chemical cross-linking analysis

The methodology to determine the amount of protein solubilised for meat alternatives was reported in **Section 6.3.8**.

7.3.8 Sensory evaluation

Extruded meat alternatives were used in the formulation of minced meat alternatives (see **Table 7-3**) for sensory evaluation. The samples were ground with a food processor (Compact 3100, Magimix, Australia) equipped with a 9 cm mini blade for 10 min at the highest speed. The minced meat alternatives were then stir-fried with other ingredients using a cooking pan at medium heat for 5 min.

Table 7-3 Formulation of minced meat alternatives

Ingredients	Amount (%)
Extruded meat alternatives	87.38
Soy sauce	7.77
Sugar	1.75
White pepper powder	0.19
Vegetable oil	2.91

The sensory acceptability of cooked minced meat alternatives was evaluated. A total of 55 panellists (18 men and 37 women, who were between 18 and 55 years old) participated in this study. Consumer testing was conducted at the Sensory Laboratory at Massey University, Palmerston North. The samples were coded with randomly selected 3-digit numbers. The sample presentation order for the panellists was balanced in order to control any order effects. Each person was given a tray containing five samples (0%MRP, 10%MRP, 20%MRP, 30%MRP and 40%MRP) in 20 mL plastic sample cups. The study was conducted in individual air-conditioned booths (20°C) under normal lighting. The panellists were provided with unsalted crackers and room temperature water for palate cleansing between samples so as to eliminate carryover factor. The panellists were then asked to rate their scores for appearance, meaty aroma, meaty flavour and overall acceptance using a 9-point hedonic scale (1 = 'dislike extremely', 5 = 'nether like nor dislike' and 9 = 'like extremely'). The Massey University Human Ethics Committee (Southern A) had granted the approval to used human subjects for the sensory analysis.

7.3.9 Data analysis

All experimental work was carried out in three replicates, and the results were reported as means ± standard deviations of the measurements. One-way analysis of variance (ANOVA) and post-hoc Tukey's pairwise comparison of means ($p \leq 0.05$) was carried out to determine significant differences on analysed data using Minitab® 18 statistical software (Minitab Inc, USA). Figures were plotted and exported using Origin Software 2018 (OriginLab Corp., USA).

7.4 Results and discussions

7.4.1 Moisture, protein, pH and colour properties of meat alternatives at different concentrations of MRP

The photographs of the meat alternatives at different concentrations of MRP are shown in **Figure 7-1**. The moisture, protein, pH and colourimetric properties of the meat alternatives at different concentrations of MRP are shown in **Table 7-4**. The moisture and protein content of the meat alternatives decreased and increased, respectively, when MRP concentration increased. The measured moisture and protein content of MRP was 53.27 ± 0.11% and 47.36 ± 0.38%, respectively. The change in moisture and protein content was due to the replacement of water with MRP in the formulation, as there was no change in the extrusion processing conditions. This led to the reduction of MC and increase of protein content as MRP concentration increased. Meat alternatives with the addition of MRP had significantly lower MC and higher protein contents than boiled chicken breast.

Table 7-4 Moisture, protein (% w/w of wet material), pH and *L** value of extruded meat alternatives at different concentrations of Maillard reaction products (MRP)

Sample	Moisture content [1] (%)	Protein content [1] (%)	pH [1]	Colour [1] (*L** value)
0%MRP	56.03 ± 0.30^{b}	26.41 ± 0.04^{e}	6.83 ± 0.01^{a}	61.24 ± 0.49^{b}
10%MRP	50.58 ± 0.03^{c}	31.82 ± 0.14^{c}	6.61 ± 0.01^{b}	49.81 ± 0.39^{c}
20%MRP	46.20 ± 0.32^{d}	34.93 ± 0.18^{b}	6.46 ± 0.01^{c}	42.63 ± 0.71^{d}
30%MRP	43.41 ± 0.07^{e}	37.70 ± 0.17^{a}	6.31 ± 0.02^{d}	40.57 ± 1.13^{e}
40%MRP	43.61 ± 0.20^{e}	38.21 ± 0.11^{a}	6.29 ± 0.01^{de}	39.29 ± 0.64^{e}
Boiled chicken[#]	69.11 ± 1.23^{a}	28.97 ± 0.11^{d}	6.26 ± 0.02^{e}	76.68 ± 1.95^{a}

[1] Data are presented as the mean and standard deviation of three replicates.
Values bearing different lowercase letters were significantly different ($p \leq 0.05$) according to Tukey's post-hoc test.
[#] Retrieved from Chiang et al. (2019c).

The pH of the meat alternatives decreased with increasing MRP concentrations. The pH of MRP was 5.43 ± 0.01, and this ingredient, therefore, increased the acidity of meat alternatives as the MRP concentration was increased. Meat alternatives had a higher pH than boiled chicken breast.

The L^* value of the meat alternatives decreased with increasing MRP concentrations (**Figure 7-1**). There was an increase in darkness as MRP increased in the meat alternatives. Sample containing 40%MRP was the darkest. MRP was dark in colour and hence had an L^* value of 23.47 ± 0.28. The dark colour of MRP was due to the generation of melanoidins (heterogeneous nitrogen-containing brown pigment) during MR (Wang et al., 2011), which led to the darkening of meat alternatives with increasing MRP concentrations.

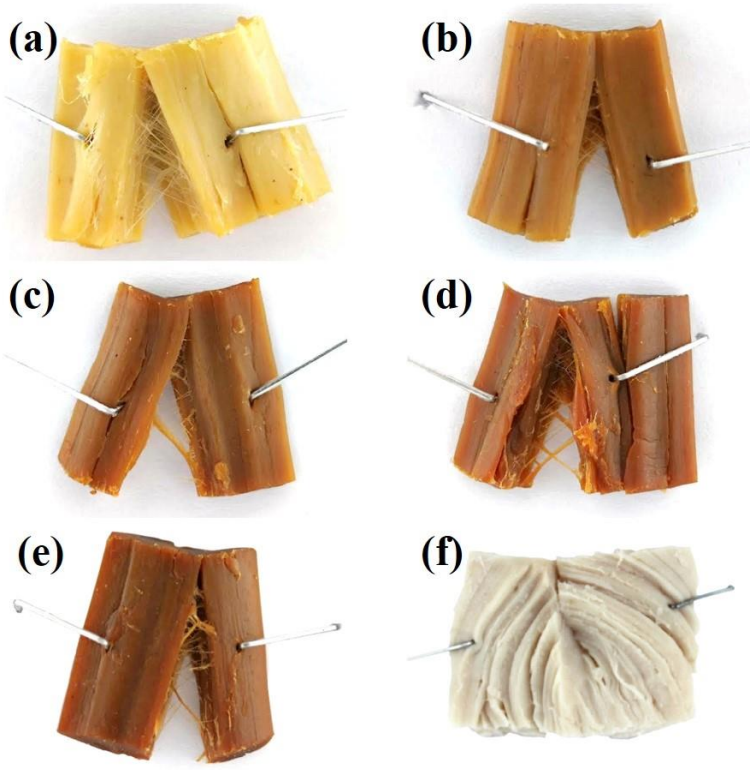


Figure 7-1 Pictures of extruded meat alternatives at different concentrations of Maillard reaction products (MRP) (a) 0%MRP, (b) 10%MRP, (c) 20%MRP, (d) 30%MRP, (e) 40%MRP, and (f) picture of boiled chicken breast.

7.4.2 Textural properties of meat alternatives at different concentrations of MRP

Fibrous structure is known to have form when the degree of texturisation (DT = F_L/F_V, dimensionless value) is >1, where F_L and F_V represent the parallel and vertical fibre network across the meat alternatives, respectively (Chen et al., 2010). The value of F_L should be higher than F_V, as greater force is required to cut across the fibres that run parallel to the direction of extrusion than the force required to cut along the fibres in the meat alternatives. The DT of meat

alternatives with different MRP concentrations is shown in **Table 7-5**. The DT decreased with increasing MRP concentration. This indicates that MRP decreased the formation of fibrous structure in the meat alternatives. Meat alternatives with 10% or greater MRP had significantly lower DT than boiled chicken breast.

Table 7-5 Textural properties of extruded meat alternatives at different concentrations of Maillard reaction products (MRP)

Sample	Textural properties [1]		
	Degree of texturisation	Hardness (N)	Chewiness (N)
0%MRP	1.75 ± 0.23^{a}	55.19 ± 3.72^{a}	40.34 ± 3.34^{a}
10%MRP	1.35 ± 0.13^{b}	23.84 ± 1.85^{c}	20.02 ± 2.37^{c}
20%MRP	1.32 ± 0.07^{b}	29.62 ± 4.95^{c}	20.89 ± 3.85^{c}
30%MRP	1.27 ± 0.12^{b}	37.55 ± 1.51^{b}	26.46 ± 2.76^{b}
40%MRP	1.24 ± 0.11^{b}	42.66 ± 2.60^{b}	30.66 ± 2.44^{b}
Boiled chicken[#]	1.63 ± 0.24^{a}	55.01 ± 7.37^{a}	18.84 ± 3.01^{c}

[1] Data are presented as the mean and standard deviation of three replicates.
Values bearing different lowercase letters were significantly different ($p \leq 0.05$) according to Tukey's post-hoc test.
[#] Retrieved from Chiang et al. (2019c).

Hardness, as measured by the texture analyser, is the peak force that occurs during the first compression, while chewiness is the product of $Hardness \times Cohesiveness \times Springiness$ (Bourne, 2002). The hardness and chewiness of the meat alternatives increased as MRP was raised from 10% to 40%, but were all significantly lower than 0%MRP for hardness and chewiness. The results were in accordance with Sun, Zhao, Cui, Zhao, and Yang (2010), who reported a decrease in hardness and chewiness for Cantonese sausages when incorporated with 2%MRP of mechanically deboned chicken residue and xylose heated for 60 min at 90°C and 100°C. Possible explanations for the decrease in hardness with the addition of MRP are; disruption to the formation of fibrous structure of the plant protein powders by the presence of MRP, or the dilution of the protein powders due to the addition of MRP (Sun et al., 2010; Cavalheiro et al., 2014; Ketnawa, Benjakul, Martínez-Alvarez, & Rawdkuen, 2016; Chiang et al., 2019c). Both effects could lead to poorer formation of protein cross-linking that resulted in softer texture compared to meat alternatives containing 0%MRP. The increase in hardness for meat alternatives observed as the MRP increased from 10% to 40% could be due to the decrease in MC that occurred due to the increase in MRP. Meat alternatives with added MRP had significantly lower hardness than boiled

chicken breast. Boiled chicken breast had the lowest chewiness compared to meat alternatives, but this was not significantly different to meat alternatives containing 10%MRP and 20%MRP.

7.4.3 Microstructural properties of meat alternatives at different concentrations of MRP

SEM and light micrographs for meat alternatives at different concentrations of MRP are shown in **Figure 7-2**. From the SEM micrographs, the extent of fibrous microstructure for meat alternatives appeared to decrease with increasing MRP concentrations. Fibrous microstructure was observed for meat alternatives consisting of 0%MRP to 20%MRP (**Figure 7-2a(i), b(i) and c(i)**) and fibre diameters ranged from 10 and 50 μm. Multiple segmented layers accompanied with some fibrous microstructure was noticed for meat alternatives consisting of 30%MRP and 40%MRP (**Figure 7-2d(i) and e(i)**) and fibre diameters ranged from 15 and 90 μm. The loss of fibrous microstructure for meat alternatives with increasing MRP concentration could be due to dilution of plant protein, in particular WG that has been shown to influence the formation of fibrous structure (Chiang et al., 2019c). The presence of MRP may also disrupt the formation of fibrous networks by the plant proteins. The muscle fibres in boiled chicken breast (**Figure 7-2e(i)**) were thicker than meat alternatives consisting of 0%MRP to 20%MRP.

Similar trends were detected for the light micrographs, where meat alternatives consisting of 0%MRP to 20%MRP (**Figure 7-2a(ii), b(ii) and c(ii)**) exhibited fibrous microstructure, while a layered structure was observed for meat alternatives consisting of 30%MRP and 40%MRP (**Figure 7-2d(ii) and e(ii)**). Further to that, it was observed that the proportion of non-uniform voids (white areas) decreased with increasing MRP concentrations in the light micrographs. These voids could be formed either due to escaping steam as the pressure in the hot mixture decreased as it passed through the die or shear stresses as the cooling meat alternatives dragged against the wall of the die. These voids could also be the air that was entrapped during the structuring of the meat alternatives (Dekkers, Boom, & van der Goot, 2018). Air pockets could be formed in the meat alternatives during extrusion, as air bubbles were entrapped and elongated in the shear flow direction of the extruder, and expansion of the final product due to water evaporation.

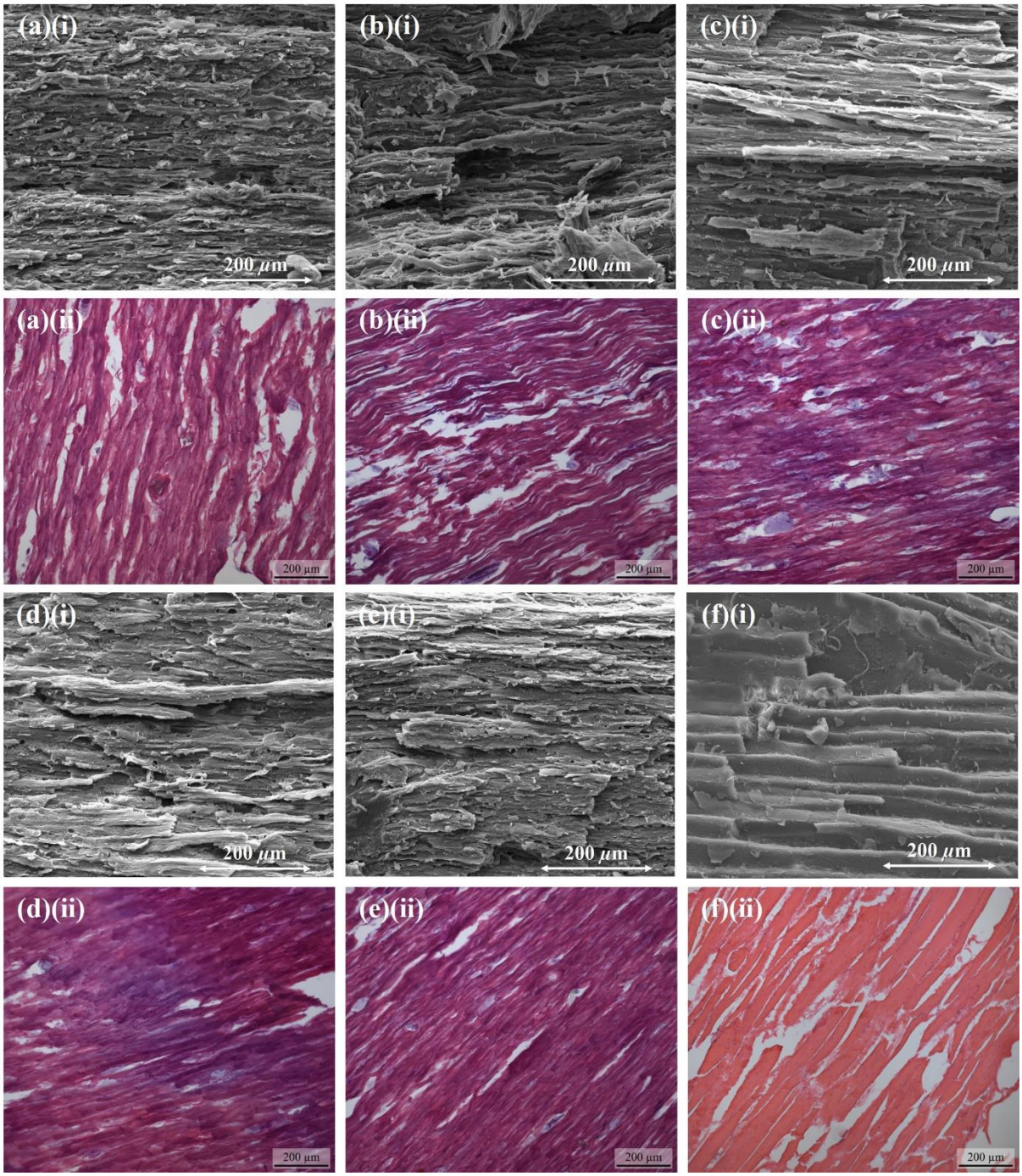


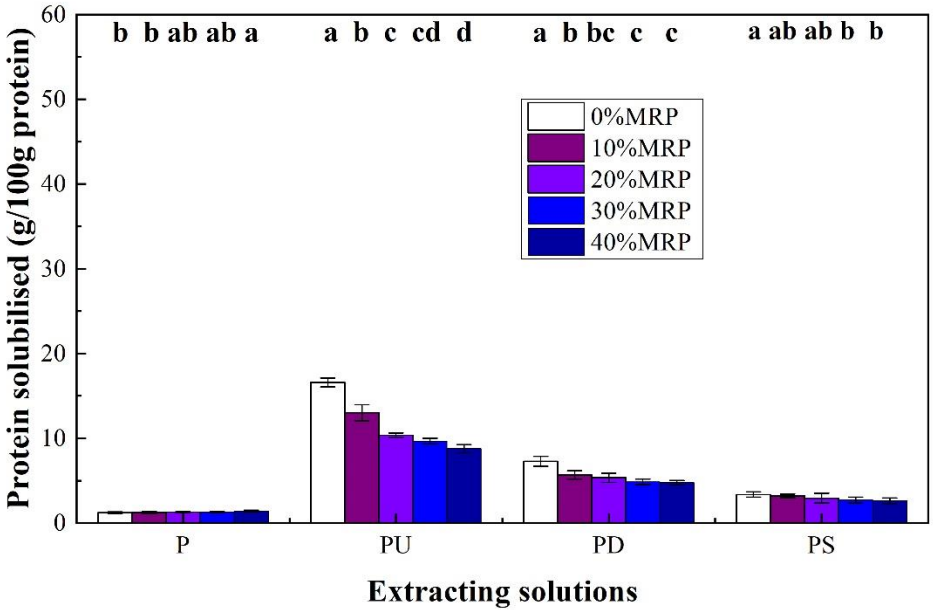
Figure 7-2 (i) SEM and (ii) light micrographs of extruded meat alternatives at different concentrations of Maillard-reaction products (MRP) (a) 0%MRP, (b) 10%MRP, (c) 20%MRP, (d) 30%MRP, (e) 40%MRP, and (f) boiled chicken breast[#], at 250× and 100× magnification, respectively.

[#] Retrieved from Chiang et al. (2019c).

7.4.4 Protein solubility of meat alternatives at different concentrations of MRP

The amount of protein solubilised by the eight extracting solutions comprising four selective reagents for meat alternatives at different concentrations of MRP was determined (**Figure 7-3a and b**). These extracting solutions solubilised the proteins in meat alternatives by breaking down different intermolecular chemical bonds (Lin et al., 2000). Phosphate buffer which solubilises proteins in their native state (Hager, 1984; Horvath & Czukor, 1993), extracted the least amount of protein in all samples, which found to be in accordance with Liu and Hsieh (2007), Chen et al. (2011), Osen et al. (2015) and Chiang et al. (2019c). The low solubility of protein in phosphate buffer indicates that the proteins in meat alternatives were denatured and polymerised by the heat and pressure during extrusion cooking (Osen et al., 2015). There was a trend of increasing solubilised protein in phosphate buffer when MRP concentrations increased. The trend was expected as the MRP is a water-soluble extract and hence increased solubility would be expected in the meat alternatives at higher concentrations of MRP (Oliver, Melton, & Stanley, 2006).

(a)



(b)

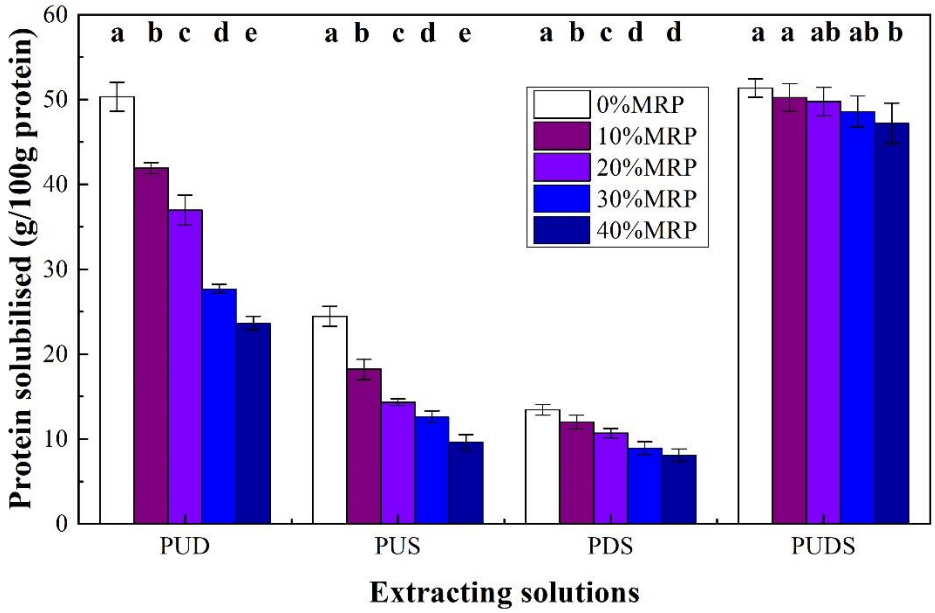


Figure 7-3 Protein solubilised by different extracting solutions (a) PB (P), PB+U (PU), PB+DTT (PD), PB+SDS (PS), and (b) PB+U+DTT (PUD), PB+U+SDS (PUS), PB+DTT+SDS (PDS), PB+U+DTT+SDS (PUDS) for extruded meat alternatives at different concentrations of Maillard reaction products (MRP). Data represent the mean and error bars represent the standard deviation of three replicates. Values bearing different lowercase letters were significantly different ($p \leq 0.05$) according to Tukey's post-hoc test.

The amount of solubilised protein in the meat alternatives increased as one of the other reagents (Urea, DTT or SDS) was mixed with phosphate buffer, indicating that the protein aggregated as a consequence of more than one type of chemical bond (Lin et al., 2000). For the two-component solvents system, the amount of protein solubilised was less than 20% with PU achieving the highest solubility followed by PD with PS having the lowest. As the MRP concentration increased from 0%MRP to 40%MRP, there was a decrease in solubilised protein with PU, PD and PS. The results from the two-component solvents system indicate that the order of decreasing importance of bond type was H-bonds, S-S bonds and finally hydrophobic interactions. The fact that the amount of protein solubilised with two-component solvents system decreased with MRP indicates that the types of bond being broken down are associated with the plant proteins rather than the protein from MRP. But given the low solubilities achieved with the two-component solvents system suggests that there is significantly more complex bonding than simple H-bonds, S-S bonds and hydrophobic interactions. Decreasing DTT-solubilised protein

with increasing MRP concentrations correlates with DT (**Table 7-5** and **Figure 7-2a(i) to e(i)**), which indicates that S-S bonds played an important role in the development of fibrous structure (Chiang et al., 2019c).

The amount of protein solubilised for meat alternatives further increased as two or more reagents were mixed with phosphate buffer, confirming that there were interactions between the chemical bonds. Protein solubility was found to be higher for all samples in PUD when compared with the sum of PU and PD. This was because of the synergistic effect of Urea and DTT which was reported by Liu and Hsieh (2007) and Chiang et al. (2019c). The protein solubilised by the combination of PDS was approximately equal to the sum of PD and PS (**Figure 7-3b**), and also the same for PUS *vs.* PU and PS. The combination of PDS solubilised the lowest amount of protein when compared with PUD and PUS, which was in accordance with Chiang et al. (2019c).

Lastly, the amount of protein solubilised by the combination of PUDS was greater than 47%. This suggests that almost half of the protein structure of the meat alternatives was supported by H-bonds, S-S bonds, hydrophobic interactions and their combinations, which was again in agreement with Liu and Hsieh (2008), Chen et al. (2011) and Chiang et al. (2019c). Other types of intermolecular chemical bonds could be involved such as electrostatic interactions. However, the amount of protein solubilised using acids, alkali or salt solutions (Liu & Hsieh, 2008) showing electrostatic interactions were negligible in the study by Azzollini et al. (2019) on the protein-protein interactions of insect-based (lesser mealworm) meat analogue.

7.4.5 Sensory evaluation

The sensory scores of minced meat alternatives at different concentrations of MRP are shown in **Table 7-6**. There was an observation that scores for all attributes (appearance, meaty aroma and meaty taste) and overall acceptance for meat alternatives containing 0%MRP to 30%MRP were on the ‘like’ side of the 9-point hedonic scale except for meat alternatives containing 40%MRP. Meat alternatives containing 20%MRP obtained the highest scores for all three attributes and overall acceptability. However, due to the variation between panellists, statistically significant differences were not seen between meat alternatives containing 0%MRP

and 20%MRP except for appearance. Meat alternatives containing 40%MRP obtained a lower score than meat alternatives containing 0%MRP for all three attributes and overall acceptability.

Table 7-6 Mean hedonic scores on the three attributes and overall acceptance of minced meat alternatives in consumer sensory evaluation (*n*=55)

Sample	Mean score [1,2]			
	Appearance	**Meaty aroma**	**Meaty taste**	**Overall acceptance**
0%MRP	5.38 ± 1.68[b]	5.71 ± 1.47	5.95 ± 1.64[a]	5.76 ± 1.45[a]
10%MRP	6.51 ± 1.20[a]	5.73 ± 1.35	6.20 ± 1.46[a]	6.20 ± 1.35[a]
20%MRP	6.60 ± 1.53[a]	5.93 ± 1.40	6.40 ± 1.62[a]	6.36 ± 1.53[a]
30%MRP	5.80 ± 1.47[ab]	5.91 ± 1.38	6.16 ± 1.72[a]	6.00 ± 1.47[a]
40%MRP	5.24 ± 1.86[b]	5.55 ± 1.56	5.04 ± 1.76[b]	4.93 ± 1.55[b]
F-value	**8.88**	**0.67**	**5.84**	**8.04**
***p*-value**	**0.000**	**0.616**	**0.000**	**0.000**

[1] Data are presented as the mean and standard deviation.
[2] Sensory evaluation scores are normally distributed.
Values bearing different lowercase letters in the same column were significantly different ($p \leq 0.05$) according to Tukey's post-hoc test.

As the MRP concentrations increased in the meat alternatives, the amount of melanoidins also increased. The meat alternatives containing 40%MRP had the most intense brown colour (**Table 7-4 and Figure 7-4**), where it appeared to be burnt-like, which might explain why it had the lowest score for appearance. Meat alternatives containing 0%MRP obtained the second-lowest score for appearance. This could be due to its pale brown colour that did not appeal to the panellists.

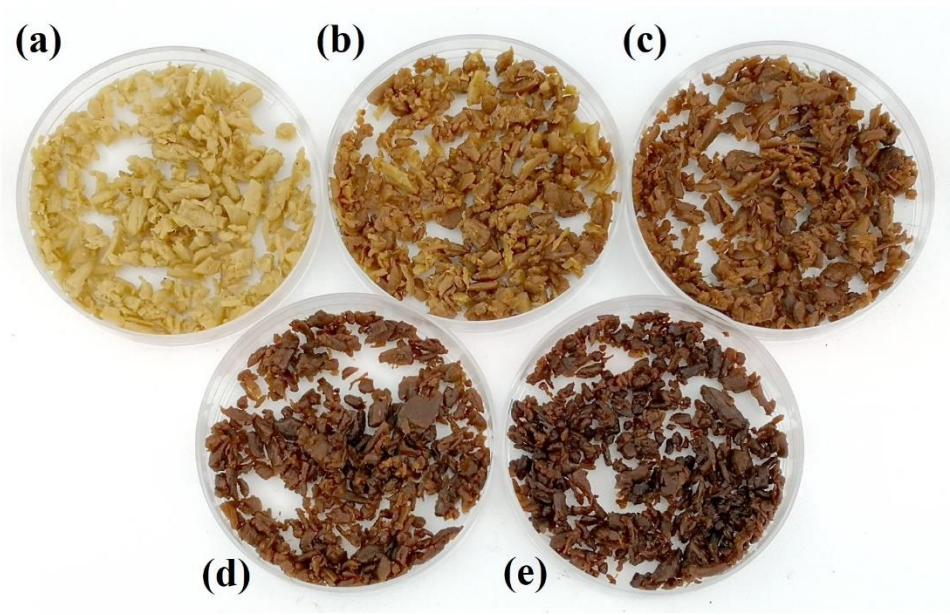


Figure 7-4 Pictures of minced meat alternatives at different concentrations of Maillard reaction products (MRP) for sensory evaluation (a) 0%MRP, (b) 10%MRP, (c) 20%MRP, (d) 30%MRP, and (e) 40%MRP.

The addition of flavours is known to have an optimum level, too low and the flavour is not detectable or considered too weak, while if the level is too high, it can be overwhelming and unpleasant. MRP contained a large number of compounds which contribute to flavour in cooked foods including meat (Mottram, 1998). The optimum level of MRP was 20%MRP. Meat alternatives containing 40%MRP obtained the lowest score for meaty taste. This could be due to its very high concentration of 2-furanmethanol (Chiang et al., 2019a). High levels of 2-furanmethanol cause the sample to be most undesirable due to its intense burnt and bitter taste (Lee, Moon, & Lee, 2010).

There were no significant differences among all samples for the meaty aroma attribute. Overall acceptability results indicate that meat alternatives containing 20%MRP attaining the highest score were well-received by the panellists. This suggests that further work can be conducted using meat alternatives containing 20%MRP to develop new food products.

7.5 Conclusion

In this study, the interactions between Maillard-reacted beef bone hydrolysates at different concentrations with plant proteins on the physicochemical properties of extruded meat alternatives were investigated. The change in MRP concentrations affected the formation of fibrous structure in meat alternatives, where increasing MRP concentrations showed decreased fibrous structure using textural and microscopy analysis. Meat alternatives containing 40%MRP showed the lowest DT, but highest hardness and chewiness when compared with meat alternatives incorporated with at least 10%MRP. Boiled chicken breast used as reference food suggested that meat alternatives containing 10%MRP and 20%MRP were the closest in terms of structural properties rather than textural properties. The major forces responsible for developing and stabilising the structure of the meat alternatives were H-bonds as well as the interactions between H-bonds and S-S bonds. The main force involved in fibrous structure in meat alternatives is S-S bonds. Meat alternatives containing 20%MRP obtained the highest sensory score for appearance, meaty aroma, meaty taste and overall acceptability among all meat alternatives. Overall, the addition of MRP to meat analogues changed the textural, structural and sensory properties of the meat alternatives significantly.

Chapter 8: [5] Extruded meat alternatives made from Maillard-reacted beef bone hydrolysate and plant proteins. Part I - Effect of moisture content.

8.1 Abstract

This study investigated the effects of MC on the physicochemical properties of extruded meat alternatives made from Maillard-reacted beef bone hydrolysate and plant proteins. Samples were extruded at 170°C (maximum barrel temperature), at 3.6 kg/h (liquid feed rate), and at 1.8, 2.2, 2.6 and 3.0 kg/h (dry feed rates) to obtain MC of 60%MC, 56%MC, 52%MC and 49%MC, respectively. Meat alternatives at 52%MC showed the greatest degree of texturisation. However, meat alternatives at 49%MC were the closest in terms of both textural and microstructural properties to the reference sample; boiled chicken breast. Results from protein solubility analysis suggest that the aggregated proteins were held together by hydrogen bonding and the fibres were held together by S-S bonds. Results showed that change in MC as a process parameter plays an important role in the formation of fibrous structure in extruded meat alternatives.

Keywords: meat alternatives; extrusion cooking; moisture content; degree of texturisation; fibre formation; protein solubility

8.2 Introduction

Food extrusion is a process where a molten or paste-like material is cooked, under a range of conditions of mixing, heating and shear, after which it is shaped by forcing the product through a die (Rossen & Miller, 1973). This technology was introduced to the food industry in the late 1950s and enabled the manufacture of a large number of products with novel shapes, textures, and tastes (Riaz, 2013; Alam et al., 2016). Extrusion processing is typically a short-time process (30 to 100 seconds) and may be carried out at low temperatures (≤ 100°C; confectionary and pasta), medium temperatures (100-150°C; snack foods and pet foods), or high temperatures (≥ 150°C;

[5] This chapter is published as Chiang, J.H., Hardacre, A.K., & Parker, M.E. (2020). Extruded meat alternatives made from Maillard-reacted beef bone hydrolysate and plant proteins. Part I - Effect of moisture content. *International Journal of Food Science and Technology, 55(2), 649-659*. doi: https://doi.org/10.1111/ijfs.14319

texturised proteins) (Riaz, 2000; Steel et al., 2012). Extrusion processing is preferred over other conventional processing methods (high-temperature/ long-time, e.g. baking, roasting. boiling, deep-frying, sterilization, etc.), as the operation is automated, continuous, versatile, energy-efficient, has a low operating cost, and has high capacity and productivity. In addition, extrusion processing has the capability to produce a broad range of high-quality finished products with minimum processing time using inexpensive ingredients.

Table 8-1 Process parameters such as moisture content, temperature, screw speed that were widely studied to influence the product properties of extruded meat analogues/ alternatives

References	Process parameters		
	Moisture content (%)	Temperature (°C)	Screw speed (rpm)
Lin et al. (2000) and Lin et al. (2002)	60, 65, 70 [a]	138, 149, 160	150
Rehrah et al. (2009)	40, 50, 60 [a]	150, 160, 170	60, 80, 100
Chen et al. (2010)	28, 36, 44, 52, 60 [a]	140, 150, 160	160
Osen et al. (2014)	55 [b]	100, 120, 140, 160	150
Pietsch, Emin, & Schuchmann (2017)	40 [a]	90, 100, 120, 155, 170	300
Palanisamy et al. (2018a)	40, 47, 55, 62, 68 [a]	135, 145, 155, 165, 180	400, 800, 1200, 1600
Palanisamy, Töpfl, Berger & Hertel (2019)	50, 55 60 [a]	145, 160, 175	500, 800, 1200
Pietsch, Bühler, Karbstein, & Emin (2019)	60 [a]	100, 140, 160	180, 500, 800
Samard, Gu, & Ryu (2019)	30, 70 [a]	160	150, 200

[a] Feed moisture content
[b] Moisture content of extruded product

The development of high-moisture meat analogues using extrusion processing began in the early 1990s (Wild et al., 2014). One of the main functions of an extruder in the development of meat analogues is texture alteration, where the physical textures of the ingredients are altered using different extrusion parameters (Riaz, 2000). Meuser and Van Lengerich (1984) recommended a basic system analysis model to categorise extrusion parameters that mostly influenced the product properties. They are namely (a) *process parameters* (**Table 8-1**) which include MC, barrel/ cooking/ extrusion temperature, screw speed, screw configuration, die dimension, raw material characteristics (i.e. ingredient variations or protein contents of the ingredient), (b) *system parameters* which comprise energy input, RTD, SME, and (c) *product properties* such as colour,

texture, microstructure, sensory, nutrition. Among these three groups of parameters, process parameters have the most effects on the final product properties (fibrous structure in meat analogues) by affecting extrusion system parameters (Chen et al., 2010).

Water plays an important role due to its effects on heat transfer during extrusion processing (Chiang, 2007). Higher MC lowers the viscosity, shear and friction during extrusion processing, and improves heat transfer from extruder barrel to the material being processed (Lin et al., 2000). Water also helps in the separation of protein and facilitates the formation of protein fibrous structure. During extrusion processing, the protein component unfolds, denatures and aggregates which leads to a change in physical state (Qi & Onwulata, 2011). Large amounts of water, when combined with starch, could lead to a phase separation which enhances protein-protein interaction (Chiang, 2007; Zhang, Li, Zhang, Drago, & Zhang, 2016). This phase separation could then cause the proteins to form fibrous structure during the texturisation stage that occurs in the cooling die.

The effects of MC on meat analogues have been extensively studied. Lin et al. (2000) observed that as MC decreased from 70% to 60%, the structure of SPI meat analogues became more directionally aligned. The authors also reported that MC was more important input variable on the product texture and sensory characteristics (Lin et al., 2002) than cooking temperature. Liu and Hsieh (2008) reported that among the three SPI-based meat analogues extruded at MC of 60.11, 66.78 and 72.12%, only extrudates at 60.11%MC exhibited well-defined fibre orientation. Rehrah et al. (2009) stated that the fibrous structure of peanut-based meat analogues was more apparent at about 55%MC. Chen et al. (2010) found that MC (at a broad range of 28, 36, 44, 52 and 60%MC) had a significant effect on the degree of texturisation on extruded SPI, where the extrudates at 60%MC had the best fibrous structure. Palanisamy et al. (2018a) reported that the cutting force of extruded lupin protein was mostly affected by water feed, where meat analogues with 55% water feed exhibited long aligned layers. These previous studies show that meat analogues at MC of 55-60% exhibit the most prominent fibrous structure.

Based on our previous study on the interactions between MRP of beef bone hydrolysate at different levels (0, 10, 20, 30 and 40% wet weight) and plant proteins on extruded meat alternatives (Chapter 6), we found that meat alternatives incorporating MRP had a fibrous structure at MC

between 46.20 to 50.58%. These deviated from literature value of 55-60%MC. Furthermore, there are no studies on the effects of MC on extruded meat alternatives incorporated with MRP. Therefore, in this study, the aim was to determine the effects of MC on the physicochemical properties of extruded meat alternatives made from Maillard-reacted beef bone hydrolysate and plant proteins. The textural and microstructural properties, with chemical linkages and protein aggregation in meat alternatives were measured, to provide an understanding of fibre formation by changing the MC.

8.3 Materials and methods

8.3.1 Materials

Commercial beef bone extract obtained from Taranaki Bio Extracts Ltd, New Zealand, was used as the substrate for enzymatic hydrolysis followed by MR. SPC (ALPHA® 11 IP, Solae™) was purchased from Tari International NZ Ltd, New Zealand. WG (FLOURG25) and WS (FLOURCW25) were purchased from Davis Trading, New Zealand. The proximate composition of the raw ingredients is shown in **Table 7-1**. All chemicals and reagents used were of analytical grade.

8.3.2 Preparation of Maillard-reacted beef bone hydrolysate and extruded meat alternatives

The enzymatic hydrolysis of beef bone extract was conducted as described by Chiang et al. (2019b), using Flavourzyme® 1000L (Batch: HPN00539, Novozymes, Denmark) at an E/S ratio of 4.70% w/w. The preparation of Maillard-reacted beef bone hydrolysate (MRP) was carried out as described by Chiang et al. (2019a), where hydrolysate and D-ribose (Amtrade NZ Ltd, New Zealand) were mixed at a ratio of 1:0.068 (protein weight to reducing sugar weight) at pH 6.5, and pressure-cooked at 170 kPa (113°C) for 10 min in a pressure cooker (Model No. 921, All American, Wisconsin, USA). The MRP was stored at 4°C before extrusion cooking.

Extrusion cooking was conducted using a TS Clextral BC-21 food extruder (Firminy Cedex, France). The formulation (% w/w) of extruded meat alternatives was based on liquid feed (i.e. 71% water and 29% MRP), and dry ingredients (i.e. 59% SPC, 30% WG, 5% vegetable oil, 3% pumpkin powder, 2.7% WS and 0.3% salt). The extruder and operating parameters were set according to

Chiang et al. (2019c), where the dry ingredients were fed into the extruder at a different dry feed rate of 1.8, 2.2, 2.6 and 3.0 kg/h, in order to obtain meat alternatives with different MC marked as 60%MC, 56%MC, 52%MC and 49%MC, respectively. The MC of the meat alternatives were determined using the air-oven method as described by Chiang et al. (2019c). Shredded samples (2 g) in numbered pans and lids were placed in the oven at 108°C for 24 hours.

8.3.3 Protein content, pH level and colour (*L** value) analysis

The methodologies for MC, protein content, pH level and *L** value of meat alternatives were reported in **Section 6.3.4**.

8.3.4 Textural properties analysis

The methodologies for cutting force, hardness and chewiness (2-bite test) of meat alternatives were reported in **Section 6.3.5**.

8.3.5 Microstructural properties analysis

The methodologies for SEM and LM of meat alternatives were reported in **Section 6.3.7**.

8.3.6 Protein solubility analysis

The methodology to determine the amount of protein solubilised for meat alternatives was reported in **Section 6.3.8**.

8.3.7 SDS-PAGE electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted with Mini-Protean® 3 Cell (Bio-Rad Laboratories, USA) using Mini-Protean® Precast TGX 4-20% gradient gels with 10 × 30 μL wells (Bio-Rad Laboratories, USA). Running buffer contained 25 mM Tris, 192 mM glycine and 0.1% SDS (pH 8.3 at 20°C) and the sample buffer contained 62.5 mM Tris-HCl at pH 6.8, 2% SDS, 10% glycerol and 0.003% bromophenol blue (pH 6.8 at 20°C). The supernatant of PUS and PUDS from protein solubility analysis were used as non-reduced and reduced samples, respectively. Each supernatant was diluted to a protein content of 50 mg/mL with PB and then diluted again with the sample buffer at a ratio of 1:1 to obtain a final concentration of 25 mg/mL to obtain sharp and visible bands.

Precision Plus Protein™ (Bio-Rad Laboratories, USA) unstained standards (ranged from 10 kDa to 250 kDa) were used as molecular size markers. BSA (Sigma Aldrich, USA) at the protein content of 2 mg/mL were prepared as standard protein. All samples and standards were heated in a 90°C water bath for 5 min before being loaded into the gel. Gels were loaded with 10 μL of molecular size markers and diluted reduced samples, 15 μL of diluted non-reduced samples, and 5 μL of BSA using a 25 μL Hamilton syringe (Hamilton Company, Switzerland) into each lane. Electrophoresis conditions were 200V at 20°C. After electrophoresis, gels were fixed using the fixing solution (40% absolute ethanol and 10% acetic acid) for 15 min. Staining was done using QC Colloidal Coomassie stain (Bio-Rad Laboratories, USA) for 20 hours and destained using ultrapure water (purified using Milli-Q apparatus, Millipore Corporation, USA) for 3 hours. Destaining water was changed every hour. The quantification of intact proteins and polypeptides was conducted using gel scanning densitometry (Gel Doc XR+ and Image Lab™ software version 6.0.0, Bio-Rad Laboratories, USA).

8.3.8 Data analysis

All experimental work was carried out in three replicate samples from the extruder, and two measurements were recorded from each sample. Figures were plotted and exported using Origin Software 2018 (OriginLab Corp., USA). Data were analysed using Minitab® 18 statistical software (Minitab Inc, USA), reported as means ± standard deviations of the measurements. Data were also analysed for statistical significance using one-way analysis of variance (ANOVA), while post-hoc Tukey's pairwise comparison of means ($p \leq 0.05$) was used to identify significant differences.

8.4 Results and discussions

8.4.1 Moisture content, protein content, pH level and *L** value of meat alternatives at different moisture contents

The images of meat alternatives at different MC and boiled chicken breast reference are shown in **Figure 8-1**. Meat alternatives at 49%MC and 52%MC **(Figure 8-1a and b)** exhibited visible fibre strands at the tear opening of the extruded samples. The MC, protein content, pH level and *L** value of meat alternatives at different MC are shown in **Table 8-2**. As the liquid feed rate

was fixed at 3.6 kg/h for all extruded samples, reducing the dry feed rate, increased the MC of the meat alternatives.

Table 8-2 Extrusion dry feed rate, moisture content, protein content (% w/w of wet material), pH level and *L** value of extruded meat alternatives at different moisture contents

Sample	Dry feed rate (kg/h)	Moisture content [1] (%)	Protein content [1] (%)	pH [1]	Colour [1] (*L** value)
49%MC	3.0	49.02 ± 0.51^{e}	36.16 ± 0.35^{a}	6.48 ± 0.01^{a}	42.68 ± 0.95^{b}
52%MC	2.6	52.26 ± 0.57^{d}	33.48 ± 0.31^{b}	6.45 ± 0.01^{b}	41.06 ± 1.12^{bc}
56%MC	2.2	55.93 ± 0.25^{c}	30.96 ± 0.46^{c}	6.41 ± 0.01^{c}	38.87 ± 2.23^{cd}
60%MC	1.8	60.12 ± 0.17^{b}	28.38 ± 0.19^{d}	6.37 ± 0.01^{d}	37.10 ± 0.93^{d}
Boiled chicken[#]	-	69.11 ± 1.23^{a}	28.97 ± 0.57^{d}	6.26 ± 0.02^{e}	76.68 ± 1.95^{a}

[1] Data are presented as the mean and standard deviation of three replicates.
Values bearing different lowercase letters were significantly different ($p \leq 0.05$) according to Tukey's post-hoc test.
[#] Retrieved from Chiang et al. (2019c).

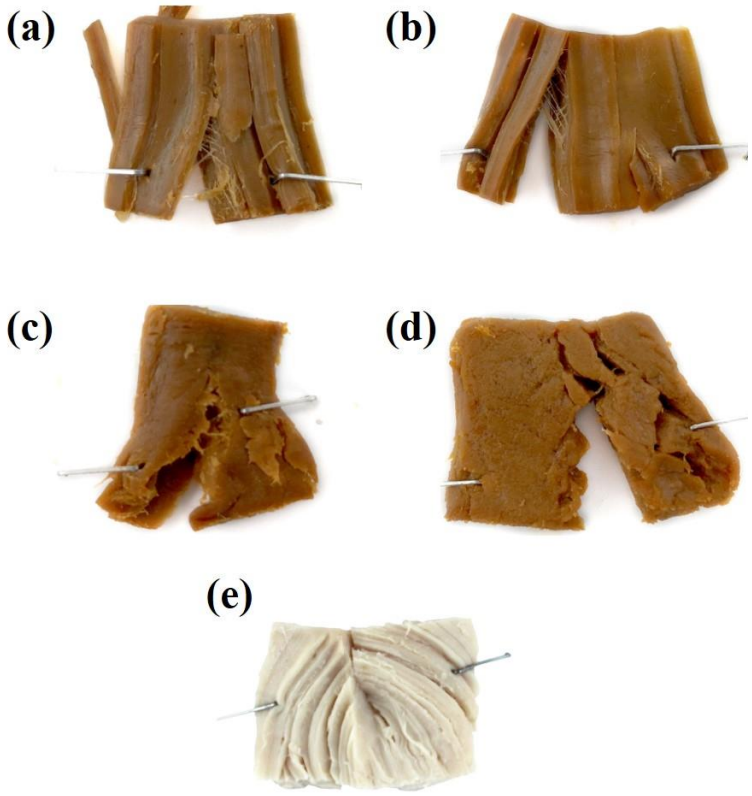


Figure 8-1 Photographed images of extruded meat alternatives at different moisture contents (a) 49%MC, (b) 52%MC, (c) 56%MC, (d) 60%MC, and (e) image of boiled chicken breast.

The protein content, pH and L^* values of the meat alternatives decreased with increasing MC. Therefore, meat alternatives at 60%MC had the lowest protein content, pH and were the darkest (lowest L^* value). The darkness of the samples was due to the MRP which has an L^* value of 23.47 ± 0.28, much lower than all meat alternatives. The dark colour of MRP could be due to the generation of melanoidins (heterogeneous nitrogen-containing brown pigment) during the MR (Wang et al., 2011). Samples with higher MC contained a greater proportion of MRP resulting in the increased darkness. The liquid feed consisting of MRP (pH 5.43 ± 0.01) and water, pumped at a fixed rate of 3.6 kg/h, decreased the protein content of meat alternatives with increasing MC, due to lesser amount of dry ingredients (lesser protein) used during extrusion. It also increased the acidity of meat alternatives with increasing MC, due to lesser amount of dry ingredients (pH 6.98 ± 0.03) used during extrusion.

In comparison with the reference sample, all meat alternatives had significantly lower MC than boiled chicken breast. There was no significant difference in protein content with meat alternatives containing 60%MC and boiled chicken breast. The differences in pH were rather small and only slightly higher than that of the boiled chicken breast, although there were significant differences observed. Meat alternatives had significantly lower L^* value (darker colour) than boiled chicken breast.

8.4.2 Textural properties of meat alternatives at different moisture contents

The degree of texturisation (DT = F_L/F_V, dimensionless value of >1), where F_L and F_V represent the vertical and parallel fibre network across the extrudates, respectively, determine the formation of fibrous structure in meat alternatives (Chen et al., 2010). The DT of meat alternatives at different MC is shown in **Table 8-3**. Meat alternatives at 52%MC had the highest DT, while meat alternatives at 60%MC had the lowest DT. The DT for all meat alternatives at different MC were greater than those reported by Chen et al. (2010) and Fang et al. (2014) on meat analogues made only from SPI (ranged between 1.06 and 1.20) at MC of 50-60%. Results indicated that higher MC (60%MC) decreased the formation of fibrous structures in meat alternatives. Meat alternatives at 52%MC had significantly higher DT than boiled chicken breast.

Table 8-3 Textural properties of extruded meat alternatives at different moisture contents

Sample	Textural properties [1]				
	F_L	F_V	DT	Hardness (N)	Chewiness (N)
49%MC	8.05 ± 0.82^{b}	4.07 ± 0.70^{b}	1.98 ± 0.20^{ab}	40.25 ± 5.73^{b}	28.20 ± 3.81^{a}
52%MC	7.56 ± 1.10^{b}	2.92 ± 0.70^{bc}	2.59 ± 0.28^{a}	28.36 ± 3.64^{c}	19.21 ± 2.35^{b}
56%MC	6.21 ± 1.89^{b}	3.26 ± 0.18^{bc}	1.91 ± 0.31^{ab}	30.80 ± 2.99^{c}	13.79 ± 2.21^{c}
60%MC	2.91 ± 0.30^{c}	2.33 ± 0.05^{c}	1.25 ± 0.11^{b}	18.18 ± 3.91^{d}	6.24 ± 1.99^{d}
Boiled chicken[#]	16.84 ± 1.13^{a}	10.35 ± 1.38^{a}	1.63 ± 0.24^{b}	55.01 ± 7.37^{a}	18.84 ± 3.01^{b}

[1] Data are presented as the mean and standard deviation.
Values bearing different lowercase letters were significantly different ($p \leq 0.05$) according to Tukey's post-hoc test.
[#] Retrieved from Chiang et al. (2019c).

Instrumental hardness is the peak force that occurs during the first compression, while chewiness for solid materials is the product of $Hardness \times Cohesiveness \times Springiness$ (Bourne, 2002). It was reported that with increasing MC in meat analogues to 60-70%MC, the hardness and chewiness of meat analogues decreased (Lin et al., 2000). The authors explained that this was due to the higher amount of water contained within the samples, or at higher MC, the lower viscosity of the mixture resulted in an incomplete texturisation process and led to a softer texture. Results showed that meat alternatives at 49%MC and 60%MC had the highest and lowest hardness and chewiness, respectively. Horita, Messias, Morgano, Hayakawa, and Pollonio (2014) explained that chewiness is evidently influenced by hardness, therefore the interpretation of the results could be similar. Meat alternatives at all MC had significantly lower hardness than boiled chicken breast. Boiled chicken breast showed no significant difference in chewiness with meat alternatives at 52%MC.

8.4.3 Microstructural properties of meat alternatives at different moisture contents

SEM and LM micrographs for meat alternatives at different MC are shown in **Figure 8-2**. From the SEM micrographs, meat alternatives at 49% and 52%MC (**Figure 8-2a(i) and b(i)**) had a fibrous microstructure ranging in size between 10 and 50 μm in length. Meat alternatives at 56%MC (**Figure 8-2c(i)**) had a layered structure with thickness ranging between 35 and 150 μm, while meat alternatives at 60%MC (**Figure 8-2d(i)**) had a disoriented-pattern structure. The loss of fibrous microstructure in meat alternatives was observed with increasing MC. This could be due to the dilution effect with water has on the dry ingredients due to the increase of MC during

extrusion that led to poor formation of protein cross-linking and WG being an important ingredient in the formation of fibrous structure by increasing the amount of S-S bonds (Ketnawa et al., 2016; Chiang et al., 2019c). Another possible reason could be due to higher MC, the meat alternatives were unable to build up enough die pressure before the cooling die because of its lower viscosity (Lin et al., 2002), which resulted in meat alternatives with a more sponge-like texture with little fibrous structure. The fibres in boiled chicken breast reference (**Figure 8-2e(i)**) ranged between 25 and 55 μm, which were thicker than the meat alternatives at 49% and 52%MC.

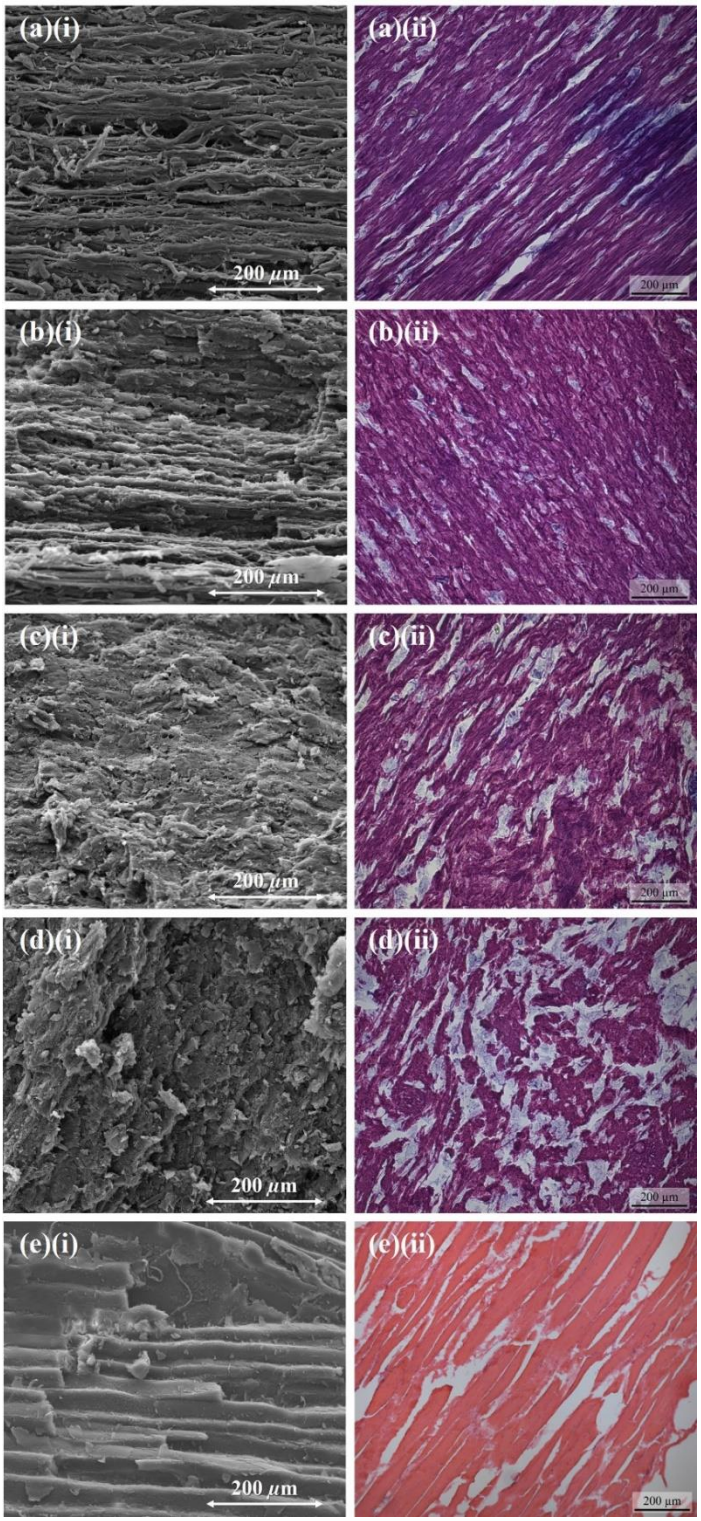


Figure 8-2 (i) SEM and (ii) LM micrographs of extruded meat alternatives at different moisture contents (a) 49%MC, (b) 52%MC, (c) 56%MC, (d) 60%MC, and (e) micrographs of boiled chicken breast[#], at 250× magnification and 100× magnification, respectively.

[#] Retrieved from Chiang et al. (2019c).

Similar observations were made for LM micrographs, where meat alternatives at 49% and 52%MC (**Figure 8-2a(ii) and b(ii)**), exhibited fibrous microstructure. A combination of fibrous and layered structure was observed for meat alternatives at 56%MC (**Figure 8-2c(ii)**), while meat alternatives at 60%MC (**Figure 8-2d(ii)**) exhibited disoriented-pattern structure. Further to that, there was an observation of non-uniform voids in the meat alternatives using rapid freezing and cryosectioning technique. These voids could be the air pockets formed due to the expansion of material due to water evaporation. These air pockets may also contribute to the fibrous properties of meat alternatives as described by Dekkers et al. (2018). These voids could correspond with the results from DT, as the voids allowed the craft knife blade probe to cut the meat alternatives more easily, which led to lower DT. We speculated that the higher number of voids led to lower DT values of the meat alternatives (**Table 8-3**). From both types of micrographs, meat alternatives at 49%MC and 52%MC showed prominent fibrous microstructures, showing deviation from literature values where meat analogues with fibrous structure contained MC of 55 to 60%. The results suggest that not all extruded products exhibited prominent fibrous network at 55-60%MC, and by adjusting the MC of extruded products led to the change in the physical structure which could be due to the cross-linking of proteins (Palanisamy et al., 2018a).

The DT is defined as F_L/F_V. The microstructures have been compared with both their corresponding F_L and F_V values. F_L refers to the force applied to cut the vertical fibre network across the meat alternatives, and was observed to decrease with increasing MC. Although meat alternatives at 49%MC (**Figure 8-2(a)(i)**) were observed to have more voids (white spaces) when compared with meat alternatives at 52%MC (**Figure 8-2(b)(i)**). The higher F_L could be due to the hardness of the meat alternatives at 49%MC. This caused the craft knife blade probe to have more resistance when cutting along the vertical fibre network. While F_V is the force require to cut the parallel fibre network. However, F_V value for meat alternatives at 52%MC was slightly lower than meat alternatives at 56%MC though the difference was not significant different. This could be due to the arrangement of the fibres for meat alternatives at 52%MC (**Figure 8-2(b)(i)**) which were more parallelly aligned, resulting in less resistance to cut, and hence lower F_V value.

8.4.4 Protein solubility of meat alternatives at different moisture contents

The protein solubility of the meat alternatives at different MC was conducted by determining the amount of protein solubilised by different extracting solutions that consisted of selective reagents such as phosphate buffer, urea, DTT and SDS (**Figure 8-3**). These eight extracting solutions are designed to establish what types of intermolecular bonding are involved in structure formation (Lin et al., 2000). Phosphate buffer (P), which disrupt proteins in their native states (Liu & Hsieh, 2007; Osen et al., 2015), had the lowest amount of protein solubilised in all samples (Chiang et al., 2019c). This showed that the proteins in the meat alternatives at different MC were denatured and polymerised by the heat and pressure during extrusion (Osen et al., 2015). There was an increasing trend of solubilised protein in P with increasing MC, which was in accordance with Chiang (2007). At lower MC, this could be due to localised chemical modification of proteins leading to lower solubility, while at higher MC, the proteins were dispersed and solubilised in the extrudates (Qi & Onwulata, 2011). It could be also due to the amount of protein denaturation and viscosity inside the extruder which decreased at higher MC, thus reducing the protein interactions and cross-linking (Palanisamy et al., 2018a). The same explanation could be used for the increasing trend of solubilised proteins in PU, PS, PUD, PUS, PDS and PUDS with increasing MC.

The amount of protein solubilised in the meat alternatives at different MC increased as P was combined with one of the other reagents (Urea, DTT or SDS). This indicated that more than one type of chemical bond was involved in the aggregated protein in the meat alternatives (Lin et al., 2000). The amount of protein solubilised by PU was found to be the greatest for two-component solvents. This showed a large portion of protein was linked with H-bonds. The amount of protein solubilised by PD followed by PS showed that S-S bonds and hydrophobic interactions were lower than PU. The amount of DTT-solubilised protein correlates with DT and microstructural properties (**Table 8-3** and **Figure 8-2a(i) to d(i)**), where the highest amount of protein solubilised by PD showed the highest DT and fibrous microstructure in the meat alternatives. This indicated that S-S bonds played an essential role in fibrous structure formation (Chiang et al., 2019c).

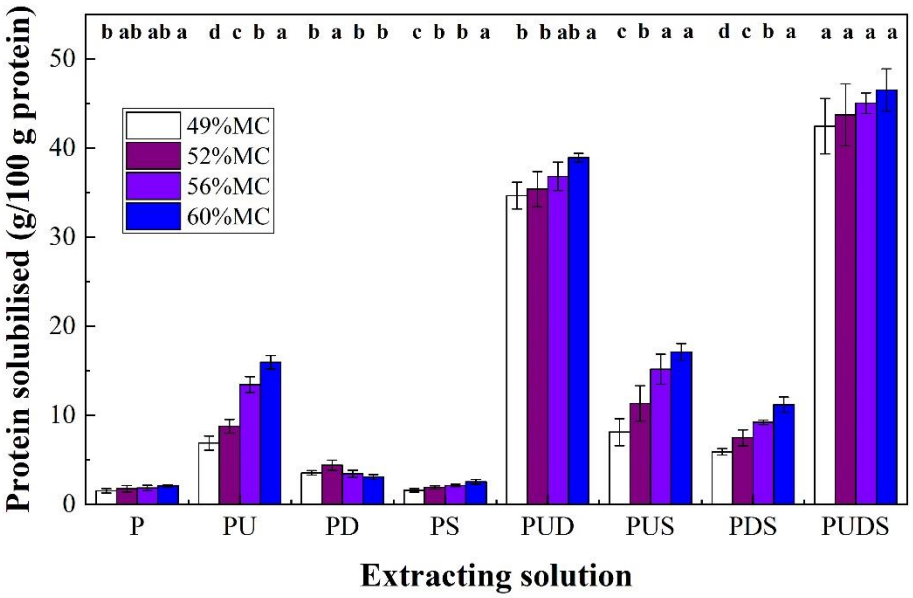


Figure 8-3 The amount of protein solubilised from extruded meat alternatives at different moisture contents induced by eight extracting solutions P, PU, PD, PS, PUD, PUS, PDS, and PUDS. Data represent the mean and error bars represent the standard deviation. Values bearing different lowercase letters were significantly different ($p \leq 0.05$) according to Tukey's post-hoc test.

Furthermore, the amount of protein solubilised from the meat alternatives at different MC further increased when P was combined with two or more reagents. This indicated that there were interactions between the H-bonds, S-S bonds or hydrophobic interactions. Protein solubility was found to be higher for all samples in PUD for three-component solvents, compared to the sum of PU and PD. This was due to the synergistic effect of the two reagents (urea and DTT) which was in accordance with Liu and Hsieh (2007) and Chiang et al. (2019c). The protein solubilised by the combination of PDS (S-S bonds and hydrophobic interactions) was approximately equal to the sum of PD and PS, and the same for PUS (H-bonds and hydrophobic interactions) *vs.* PU and PS. The combination of PDS solubilised the lowest amount of protein compared with PUD and PUS, which was in accordance with Chiang et al. (2019c).

Lastly, the amount of protein solubilised was greater than 42% for the combination of PUDS. This suggested that H-bonds, S-S bonds, hydrophobic interactions and their combinations supported almost half of the protein structure of the meat alternatives, which was in accordance with Liu and Hsieh (2008), Chen et al. (2011) and Chiang et al. (2019c).

8.4.5 Aggregation of meat alternatives at different moisture contents

The relevance of disulphide-mediated polymerisation of meat alternatives was further investigated by SDS-PAGE. The samples (PUS and PUDS in non-reduced and reduced conditions, respectively) from the protein solubility analysis were used to observe the difference in *Mw* distribution between the two treatments. The electrophoresis of meat alternatives at different MC under non-reduced (Lane 2-5) and reduced (Lane 7-10) conditions is shown in **Figure 8-4**. Comparing both non-reduced and reduced lanes, it was observed that the 7S (*Mw* of 72 and 49 kDa) and 11S (*Mw* of 35 and 19 kDa) bands become darker as the MC increased. This could be due to the fact that large *Mw* peptides in meat alternatives were unable to penetrate the pores of the gel (Chen et al., 2010; Chen et al., 2011). This indicated that the amount of peptides at the bands became lower to higher, as increasing MC caused the raw protein to undergo polymerisation and cross-linking during extrusion, which led to the formation of protein-protein polymers with large *Mw*.

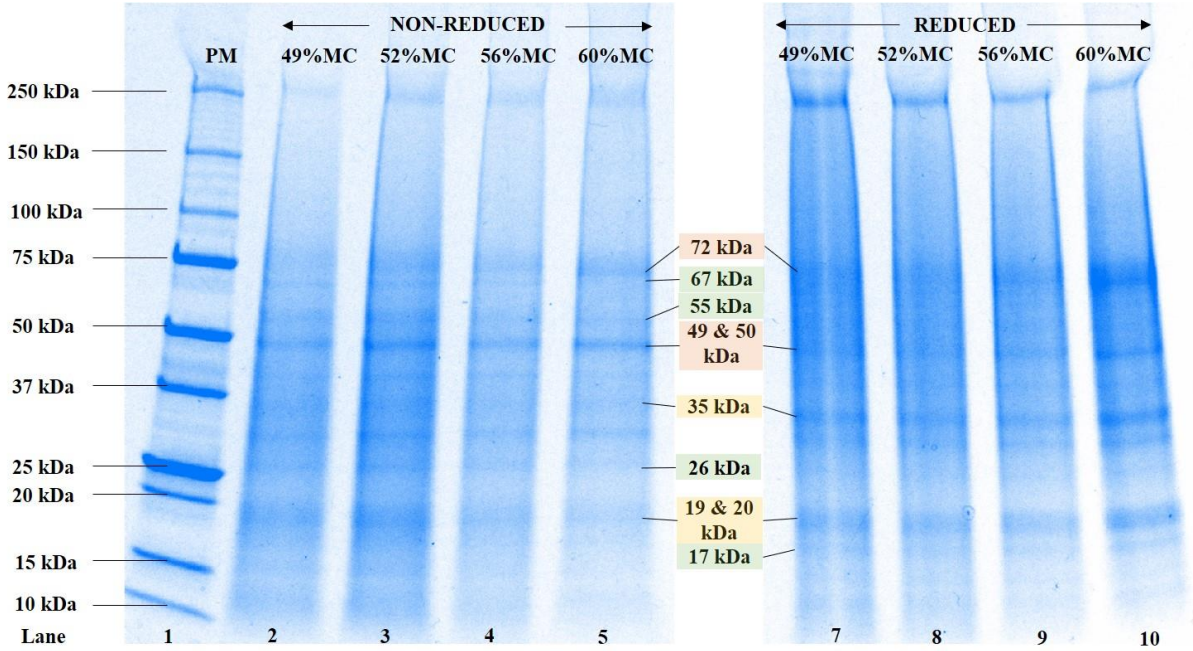


Figure 8-4 SDS-PAGE of extruded meat alternatives at different moisture contents under non-reduced (Lane 2-5) and reduced (Lane 7-10) conditions. PM referred to the molecular size markers.

When S-S bonds were broken under the reduced condition with the addition of DTT, the bands at 67 (7S alpha), 55 and 26 kDa dissociated, revealing new bands at 17 kDa. Furthermore, the bands at 50 (7S beta or low *Mw* glutenins) kDa became less intense, and more pronounced

bands observed at 35 (11S acid) and 20 (11S basic) kDa, which was in accordance with Chiang (2007). Overall results showed from the difference in bands intensities and new bands formed indicated the importance of S-S bonds contribution to protein aggregation and the formation of fibrous structure in meat alternatives.

8.5 Conclusion

The change in MC affected both the textural and the microstructural properties of the meat alternatives, where meat alternatives at 52%MC and 60%MC had the highest and lowest DT, which did not correspond with published literature value for 55-60%MC that was reported as forming the most prominent fibrous structure in meat analogues. Meat alternatives at 52%MC exhibited fibrous microstructure, while meat alternatives at 60%MC had a disoriented structural pattern under SEM and LM. The use of boiled chicken breast as a reference food showed that meat alternatives at 49%MC could be the closest in terms of both textural and microstructural properties. H-bonds were the major force responsible for forming and stabilising the structure of meat alternatives, while S-S bonds were the main force in the formation of fibrous structure. In conclusion, not all extruded products at 55-60%MC exhibited fibrous structures and by adjusting MC as a process parameter played an important role in the formation of fibrous structure in meat alternatives.

Chapter 9: [6] Extruded meat alternatives made from Maillard-reacted beef bone hydrolysate and plant proteins. Part II - Application in sausages.

9.1 Abstract

This study investigated the physicochemical properties of sausages made from meat alternatives that included from Maillard-reacted beef bone hydrolysate and plant proteins (soy protein and WG) at different MC (S49%MC, S52%MC, S56%MC and S60%MC). S49%MC had the highest hardness and chewiness, observed with chunks of long fibres under SEM. The hardness and chewiness of sausages decreased as MC increased. S60%MC exhibited a soft and mushy texture, and no fibre structure was observed. Sausages made from meat alternatives had higher protein oxidation as compared with reference sausage made from the chicken breast (SCB), which could be due to longer storage period, as meat alternatives were extruded, frozen and stored before making into sausages. Sensory results showed that SCB obtained the highest scores for all attributes except for appearance, among all sausages. Overall results showed that further improvements can be made when using extruded meat alternatives to make sausages.

Keywords: meat alternatives; sausage; texture properties; microscopy; protein oxidation; sensory quality

9.2 Introduction

Meat analogues can be formulated to have protein, fat and MC that resemble whole muscle meats such as chicken breast (Lin et al., 2002; Chiang et al., 2019c). They exhibit striated and anisotropic structures which are similar to chicken breast in terms of visual appearance and texture. Meat analogues can be used to imitate coarse ground meat and other products that are available in various shapes and sizes. In recent years, there have been many studies on the development of meat analogues using different types of plant proteins such as soy, pea, lupin and peanut (Lin et

[6] This chapter is published as Chiang, J.H., Hardacre, A.K., & Parker, M.E. (2020). Extruded meat alternatives made from Maillard-reacted beef bone hydrolysate and plant proteins. Part II - Application in sausages. *International Journal of Food Science and Technology, 55(3), 1207-1217.* doi: https://doi.org/10.1111/ijfs.14362

al., 2002; Rehrah et al., 2009; Chen et al., 2010; Osen et al., 2014; Krintiras et al., 2015; Palanisamy et al., 2018a; Chiang et al., 2019c). However, there are limited studies on the application of these meat analogues in food systems.

Previous studies have reported on the use of meat analogues or textured proteins to make products such as nuggets, sausage patties and ground meat in puff pastry snacks. Kumar, Sharma, Kumar, and Kumar (2012b) prepared three analogue meat nuggets made from ground textured soy protein to WG paste ratio (35.5:10, 31.5:14 and 27.5:18% w/w), ground mushroom, etc., and studied their physicochemical and sensory properties. The cooking yield, protein and fat content of the three analogue meat nuggets were significantly different. Sensory results showed that incorporation of the highest level of WG (18%), obtained significantly higher scores for appearance, flavour, texture, binding and overall acceptability. However, when compared to economy-grade chicken nuggets, the analogue meat nuggets containing 18% WG had significantly lower MC, protein content, fat content, cooking yield and texture properties (hardness, chewiness, springiness and cohesiveness) (Kumar, Sharma, & Kumar, 2011). The mean sensory scores for most attributes such as flavour, juiciness, texture and overall acceptability were significantly lower for analogue meat nuggets as compared to chicken nuggets. However, the authors concluded that analogue meat nuggets can be a suitable substitute for chicken nuggets based on their product profiles (e.g. low fat, sodium and energy content).

Lin (2014) developed imitation sausage patties from textured soy protein or textured pea proteins (fixed at 15% w/w), with varying levels of soy or pea protein isolates (3, 6 and 9% w/w) and king oyster mushrooms (0, 3.5 and 7% w/w). Soy-based patties were found to have significantly higher cooking yield than pea-based patties. The highest values of hardness and chewiness of soy-based patties resulted when SPI (9% w/w) and king oyster mushrooms (7% w/w) were both at their highest levels. While the highest values of hardness and chewiness of pea-based patties were achieved with the lowest level of pea protein isolate (3% w/w) and no addition of king oyster mushrooms. However, no sensory analysis was conducted to understand the consumers' acceptance of the imitation sausage patties.

Rehrah et al. (2009) prepared puff pastry snacks using ground extruded peanut-based meat analogue and compared them with a control commercial soy-based meat analogue to evaluate its sensory acceptability. Meat analogues were flavoured with beef flavour extract and flavour enhancers, cooked and ground into ground beef analogues. The ground beef analogues were then used in the preparation of puff pastry snacks, which were used as carriers to test consumer acceptability of ground beef analogues. Peanut-based meat analogue comprised of beef flavour, sugar, crushed red peppers and soy sauce, had the best sensory attributes compared to the other peanut-based meat analogue formulations and were liked equally to the control, a soy-based meat analogue. The authors concluded that meat analogues produced from low-cost defatted peanut flour had the potential to compete with commercial meat analogues and appeal to health-conscious consumers and vegetarians.

Sausages are defined as ground meat products (contain no less than 50% of fat-free meat) made from red meat and/ or poultry with water, binders and seasoning (Essien, 2003; Food Standards Australia New Zealand, 2019). They are usually stuffed into a casing and may be cured, smoked or cooked. Sales of processed meat such as sausages, bacon, ham, salami, canned meat and meat-based sauces grew 5% in current retail value and 4% in retail volume to reach NZD492 million and 29,900 tonnes in 2018 (Euromonitor, 2017), where convenience was the key driver behind consumer decision making. It was reported that the actual sales of meat analogues in New Zealand grew from NZD23.7 million in 2013 to NZD33.5 million in 2018, and it is expected to grow further from 2018 to 2023 to NZD56.1 million, where the key driver is expected to be due to increasing concern about the environmental impact of animal farming (Euromonitor, 2017).

No studies were found in the literature that used extruded meat alternatives as the main ingredient in sausages. Furthermore, no studies are comparing the application of products made from extruded meat analogues/ alternatives and real meat. Therefore, in this study, extruded meat alternatives at different MC were made into the form of a sausage. Chicken breast was also made into sausages as a reference sample. The physicochemical properties of sausages made from extruded meat alternatives at different MC were reported. The oxidative stability and sensory evaluation of sausages were studied, to provide an understanding of how the textural and

microstructural properties of sausages at different MC affect the protein stability and sensory results.

9.3 Materials and methods

9.3.1 Materials

Commercial beef bone extract was obtained from Taranaki Bio Extracts Ltd, New Zealand. SPC (ALPHA® 11 IP, Solae™) was purchased from Tari International NZ Ltd, New Zealand. WG (FLOURG25) and WS (FLOURCW25) were purchased from Davis Trading, New Zealand. The proximate composition of the ingredients is shown in **Table 7-1**. Chicken breasts were bought from the local supermarket, New World (Palmerston North, New Zealand). All chemicals and reagents used were of analytical grade. Ultrapure water purified by treatment with a Milli-Q apparatus; Millipore Corporation (Bedford, Massachusetts, USA) was used in analytical experiments.

9.3.2 Preparation of Maillard-reacted beef bone hydrolysate and extruded meat alternatives

The enzymatic hydrolysis of beef bone extract was conducted as described by Chiang et al. (2019b), using Flavourzyme® 1000L (Batch: HPN00539, Novozymes, Denmark) at an E/S ratio of 4.70% w/w. The preparation of Maillard-reacted beef bone hydrolysate (MRP) was carried out as described by Chiang et al. (2019a), where hydrolysate and D-ribose (Amtrade NZ Ltd, New Zealand) were mixed at a ratio of 1:0.068 (protein weight to reducing sugar weight) at pH 6.5, and pressure-cooked at 170 kPa (113°C) for 10 min in a pressure cooker (Model No. 921, All American, Wisconsin, USA). The MRP was stored at 4°C before extrusion cooking.

Table 9-1 Composition of extruded meat alternatives at different moisture contents

Sample	Dry ingredients (% w/w)						Liquid feed (% w/w)	
	Soy protein concentrate	Wheat gluten	Vegetable oil	Pumpkin powder	Wheat starch	Salt	Water	MRP
49%MC	26.38	13.41	2.24	1.34	1.21	0.13	41.46	13.83
52%MC	24.79	12.60	2.10	1.26	1.13	0.13	44.04	13.95
56%MC	22.88	11.64	1.94	1.16	1.05	0.12	47.13	14.08
60%MC	20.71	10.53	1.75	1.05	0.95	0.11	50.66	14.24

Extrusion cooking was conducted using a TS Clextral BC-21 food extruder (Firminy Cedex, France). The formulation (% w/w) of extruded meat alternatives was based on liquid feed (i.e. 71% water and 29% MRP), and dry ingredients (i.e. 59% SPC, 30% WG, 5% vegetable oil, 3% pumpkin powder, 2.7% WS and 0.3% salt). The extruder and operating parameters were set according to Chiang et al. (2019c), where the dry ingredients were feed into the extruder at a different feed rate of 1.8, 2.2, 2.6 and 3.0 kg/h, to obtain meat alternative with different MC at 60.12 ± 0.17, 55.93 ± 0.25, 52.26 ± 0.57 and 49.20 ± 0.32%, respectively. The composition of extruded meat alternatives at different MC is shown in **Table 9-1**.

9.3.3 Formulation and manufacture of sausages

Meat alternatives or chicken breast was size reduced with a food processor (Compact 3100, Magimix, Australia) equipped with a 9-cm mini blade for 10 min at the highest speed. Dry ingredients were slowly added to the ground samples as powders while processing. Afterwards, cold water was added into the mixture. The addition of ingredients took less than 5 min and the final temperature was approximately 15°C. The mixture was then extruded into collagen reconstituted casing (30 mm diameter, Viscofan, Germany) and hand-linked to form approximately 12 cm links in length. The sausages were then placed in "cook-in" clear vacuum bags (90 mm × 250 mm, 95 μm thickness, Cas-Pak Products Ltd, New Zealand), putting two sausages per bag and vacuum packaged (C200 tabletop vacuum chamber machine, MULTIVAC, Germany). The bags were then heat-processed in a temperature-controlled water bath maintained at 80°C until a final internal temperature of 75°C was reached using a thermometer with needle-tip probe. Then, samples were cooled immediately in an ice-water bath. The packages were stored at 4°C for 0, 7, 14 and 21 days before protein oxidation analysis. The formulation of the various sausages made from meat alternatives or chicken breast was obtained based on earlier work carried out at Massey University by the candidate as an undergraduate student (**Table 9-2**), and the sausages were marked as S49%MC, S52%MC, S56%MC and S60%MC based on the MC of meat alternatives, and SCB for chicken breast.

Table 9-2 Formulation of sausages made from extruded meat alternatives or chicken breast

Ingredients	Amount (%)	Justification for use
Extruded meat alternatives/ chicken breast	75	Main ingredients: protein/ meat source
Cold water	17.5	Contributes to meat binding, keep the temperature low to reduce bacteria growth
Modified starches	4.0	Acts as a thickener and provides good freeze-thaw stability
Salt	1.1	Aids in water-binding
Sugar	1.0	Acts as a flavour enhancer
Sodium tripolyphosphate (STPP)	0.5	Acts as a preservative, increases water-holding capacity
Kappa-carrageenan	0.5	Improves the texture of low-fat products, i.e. juiciness, tenderness and cooking yield
Ground white pepper	0.2	Contributes to flavour, aroma and taste
Ground black pepper	0.2	

9.3.4 Cooking yield

The cooking yield was determined by calculating weight differences for samples before and after cooking using the following formula (Serdaroglu, 2006):

$$Cooking\ yield\ (\%) = \frac{cooked\ sausage\ weight\ (g)}{uncooked\ sausage\ weight\ (g)} \times 100\% \qquad \textbf{(1)}$$

9.3.5 Protein, moisture, pH and colour analysis

The methodologies for MC, protein content, pH level and L^* value of meat alternatives were reported in **Section 6.3.4**.

9.3.6 Textural properties analysis

The textural properties of the sausages were analysed using the 2-bite test with a texture analyser (TA.XT Plus, Stable Micro Systems, UK) as described by Chiang et al. (2019c). The sausages were cut perpendicular to their axis into a thickness of approximately 10 mm per piece and compressed in the direction of the axis using a P/61 mm probe to 50% of its original thickness at a speed of 1 mm/s for the first bite, returned to the original position over 5 sec, followed by the second bite at 1 mm/s, to 50% of the first compressed thickness. The following parameters were determined using Exponent software (Version 6.1.15.0, Stable Micro Systems, UK): hardness (N)

= peak force requires for first compression ($Force\ at\ Peak\ 1$), cohesiveness (dimensionless) = ratio of positive force area during the second compression to that in the first compression ($Area\ 2/Area\ 1$), adhesiveness (N.mm) = the negative force area for the first bite representing the work necessary to pull the compressing probe away from the sample ($Area\ 3$), springiness (dimensionless) = distance sample recovers after the first compression ($Distance\ 2$) and chewiness (N) = applies only to solid products and is the product of $Hardness \times Cohesiveness \times Springiness$ (Pereira et al., 2011).

9.3.7 Scanning electron microscopy (SEM) analysis

The methodology for SEM of meat alternatives was reported in **Section 6.3.7.1**.

9.3.8 Protein oxidation analysis

Protein oxidation was measured through protein carbonyl content of the sausages using the DNPH (Sigma-Aldrich, New Zealand) derivatisation method according to Feng, Sebranek, Lee, and Ahn (2016) and Turgut, Soyer, and Işıkçı (2016) with modifications. A sausage sample without the casing (1 g) was added to 10 mL of pyrophosphate buffer (2.0 mM $Na_4P_2O_7$ (BDH Chemicals, England), 10 mM Tris (BDH VWR Analytical, Australia), 100 mM KCl (ThermoFisher Scientific, New Zealand), 2.0 mM $MgCl_2$ (ThermoFisher Scientific, New Zealand), and 2.0 mM EDTA (BDH VWR Analytical, Australia), pH 7.4) and blended using a high-shear mixer (Ultra Turrax® T25 Basic, Ika Works Asia, Malaysia) at 12,000 rpm for 1 min to form a smooth slurry. After blending, two equal aliquots of slurry (45 μL) were transferred into microcentrifuge tubes containing 1 mL of 10% trichloroacetic acid (TCA, Fisher Scientific, New Zealand). Samples were centrifuged at 12000 × g for 5 min and the supernatant was discarded. After which, one pellet was treated with 1 mL of 0.01 M DNPH dissolved in 2 M HCl (for carbonyl content), and the other pellet was incubated with 2 M HCl (for protein quantification). Samples were then incubated for 1 h at room temperature, with vortexing every 20 min. After incubation, the proteins were further precipitated with 0.6 mL of 10% TCA and centrifuged at 12,000 × g for 5 min. The supernatant was carefully discarded by not damaging the pellets. DNPH in the pellets was removed by washing the sample three times with 1 mL of 0.01 M HCl in 1:1 (v/v) ethanol/ ethyl acetate (ThermoFisher Scientific, New Zealand) followed by centrifugation at 12,000 × g for 5 min after each wash. The pellet was finally solubilized in 1.5 mL of 6.0 M guanidine

hydrochloride (Sigma-Aldrich, New Zealand) dissolved in 0.02 M potassium dihydrogen phosphate (pH 2.3 adjusted with HCl). The samples were vortexed and centrifuged at 12,000 × *g* for 5 min to remove insoluble materials. The absorbance of the final solution was measured using a spectrophotometer at 370 nm against 6.0 M guanidine hydrochloride in 0.02 M potassium dihydrogen phosphate (Ajax Finechem, New Zealand) buffer. The absorbance values of the blank samples were subtracted from their corresponding sample values. The carbonyl content was calculated using an absorption coefficient of 22,000 $M^{-1}cm^{-1}$, the protein concentration of samples (determined at 280 nm) and was expressed as nmol/mg of protein.

9.3.9 Sensory evaluation

The sensory acceptability of the sausages was evaluated by 50 panellists (36 women and 14 men, who were 18-25 (23 panellists), 26-35 (17 panellists), 36-45 (6 panellists) and 46-55 (4 panellists) years old of age) participated in the study. Consumer testing was conducted at Massey University's Sensory Laboratory. The sausages were fan-grilled in the oven at 180°C for 15 min and cut into a thickness of approximately 10 mm per piece before serving to panellists. The samples were coded with randomly selected 3-digit numbers. The sample presentation order for the panellists was balanced in order to control any order effects. Each panellist was presented with a tray containing five samples (S49%MC, S52%MC, S56%MC, S60%MC and SCB) in 20 mL plastic sampling cups. The evaluation session was conducted in individual air-conditioned booths (20°C) under normal lighting. To eliminate carryover factors, panellists were provided with unsalted crackers and room temperature water for palate cleansing between samples. The panellists were asked to rate the resulting sausages for appearance, hardness (bite completely through the sample between the molar teeth), chewiness (chew the sample for at least 12 chews), meaty aroma, meaty flavour and overall acceptance using a 9-point hedonic scale (1 = 'dislike extremely', 5 = 'nether like nor dislike' and 9 = 'like extremely'). Purchase intention was also evaluated using a 5-point scale (1 = 'would certainly not buy' to 5 = 'would certainly buy'). Approval to use human subjects for the sensory evaluation was granted by the Massey University Human Ethics Committee, Southern A.

9.3.10 Data analysis

All experimental work was carried out on sausages made from three replicate sausage samples from the extruder (one sausage sample made from each replicate for analytical experiment), where two measurements were recorded from each sample (n=6). Figures were plotted and exported using Origin Software 2018 (OriginLab Corp., USA). Data were analysed using Minitab® 18 statistical software (Minitab Inc, USA), reported as means ± standard deviations of the measurements. Data were also analysed for statistical significance using one-way analysis of variance (ANOVA), while post-hoc Tukey's pairwise comparison of means ($p \leq 0.05$) was used to identify significant differences.

9.4 Results and discussions

9.4.1 Cooking yield, protein, moisture, pH and colour properties of sausages

The cross-sectional views of sausages made from the meat alternatives at different MC and SCB are shown in **Figure 9-1**. Small bits of meat alternatives were observed for S49%MC and S52%MC, while coarse surfaces were detected in S56%MC and S60%MC. S60%MC was unable to maintain its circular cross-sectional view (slight dent on the left-hand side) due to its soft and mushy texture. SCB exhibited a fine and smooth surface with some air pocket voids.

Table 9-3 Cooking yield, moisture content, protein content, pH level and L^* value of sausages made from extruded meat alternatives at different moisture contents and chicken breast sausage

Samples	Cooking yield [1] (%)	Moisture content [1] (%)	Protein content [1] (%)	pH [1]	Colour [1] (L^* value)
S49%MC	99.72 ± 0.14^{a}	54.86 ± 0.80^{d}	25.41 ± 0.40^{a}	6.82 ± 0.01^{a}	43.44 ± 0.72^{b}
S52%MC	99.38 ± 0.85^{a}	56.21 ± 0.98^{d}	24.52 ± 0.22^{a}	6.76 ± 0.03^{b}	41.89 ± 1.94^{b}
S56%MC	99.08 ± 0.88^{a}	58.52 ± 0.92^{c}	22.83 ± 0.32^{b}	6.75 ± 0.02^{b}	38.92 ± 1.05^{c}
S60%MC	99.56 ± 0.36^{a}	61.59 ± 0.84^{b}	21.51 ± 0.67^{c}	6.76 ± 0.03^{b}	38.20 ± 0.46^{c}
SCB	99.45 ± 0.45^{a}	73.52 ± 0.32^{a}	17.60 ± 0.06^{d}	6.58 ± 0.01^{c}	72.55 ± 0.64^{a}

[1] Data are presented as the mean and standard deviation of three replicates.
Values bearing different lowercase letters were significantly different ($p \leq 0.05$) according to Tukey's post-hoc test.

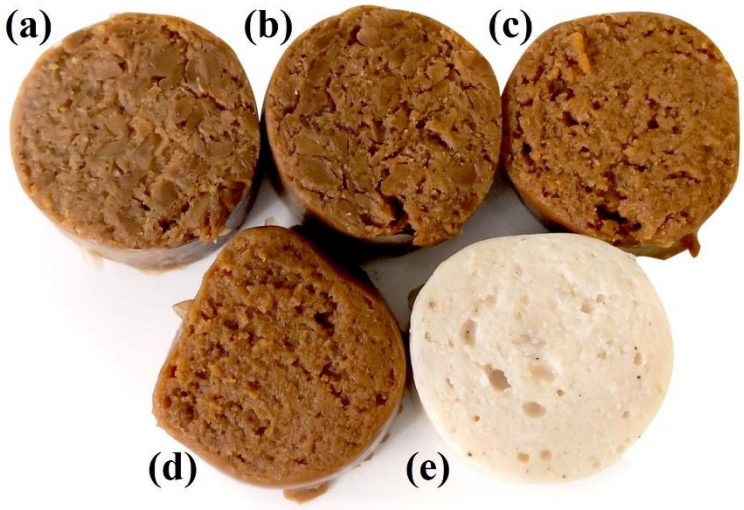


Figure 9-1 Visual images of cross-sectional view of sausages made from extruded meat alternatives at different moisture contents (a) S49%MC, (b) S52%MC, (c) S56%MC, (d) S60%MC, and chicken breast (e) SCB.

The cooking yield, MC, protein content, pH and colourimetric properties of the sausages are shown in **Table 9-3**. The average cooking yield of all sausages was found to be above 99%. The collagen casing of each sausage did not rupture after vacuum packing and water-bath cooking, hence there were no leakage or moisture loss, and this may explain the high cooking yield. The use of modified starches, STPP and kappa-carrageenan may also contribute to a high cooking yield. Modified starch has been shown by Mohammadi and Oghabi (2012) to improve water-holding capacity and cooking yield of sausage due to the swelling of the starch granules embedded in the protein gel matrix. STPP has been widely used at a level of up to 0.5% of the final product to reduce moisture losses during cooking (Young, Lyon, Searcy, & Wilson, 1987). STPP can also be used in combination with sodium chloride as the two act synergistically to improve moisture-binding much more than when alone. Trius and Sebranek (1996) highlighted that the addition of kappa-carrageenan contributed to gel formation and water retention in meat products, by absorbing and trapping water during the heating process, which resulted in a higher cooking yield and lesser purge during storage.

The use of meat alternatives with higher MC resulted in sausages with higher MC. Overall due to the addition of water in the sausage formulation, the MC of sausages were higher than MC of meat alternatives they were made from. The protein content and L^* value of sausages decreased

when the MC of sausages increased, which follow a similar trend of results from meat alternatives reported in a previous study by Chiang, Hardacre, and Parker (2020). The protein content of the sausages decreases with increasing MC (**Table 9-2**). Sausages with higher MC contained a greater proportion of MRP resulting in the increased darkness (lower *L** value). In comparison with the reference sample, sausages made from meat alternatives had significantly higher protein content and pH, and significantly lower MC and *L** value (darker colour) than SCB.

9.4.2 Textural properties of sausages

The textural properties of sausages made from meat alternatives at different MC and chicken breast are shown in **Table 9-4**. Sausages made from meat alternatives had a decrease in hardness when MC increased indicating that a higher MC (lower solid content) resulted in sausages with softer textural properties. In this study, the sausages exhibited lower hardness and chewiness than the main ingredients (i.e. meat alternatives or chicken breast) from a previous study (Chiang et al., 2020). This could be due to the addition of water in the formulation or grinding of the main ingredients which disrupted its intact structure that led to lower hardness and chewiness. Cohesiveness is a measurement of the degree of difficulty in breaking down the internal structure of the sausages (Yang, Choi, Jeon, Park, & Joo, 2007). Sausages made from meat alternatives had an increase in cohesiveness when MC increased. This indicates that sausages with higher MC were able to withstand a second deformation relative to its resistance under the first deformation.

Table 9-4 Textural properties of sausages made from extruded meat alternatives at different moisture contents and chicken breast sausage

Samples	Textural properties [1]				
	Hardness (N)	Cohesiveness	Adhesiveness (N.mm)	Springiness	Chewiness (N)
S49%MC	30.50 ± 1.68^{b}	0.282 ± 0.014^{c}	-3.91 ± 1.04^{b}	0.470 ± 0.048^{b}	4.04 ± 0.88^{b}
S52%MC	24.08 ± 1.07^{c}	0.313 ± 0.043^{bc}	-5.43 ± 1.88^{bc}	0.475 ± 0.048^{b}	3.59 ± 0.62^{b}
S56%MC	13.79 ± 1.17^{d}	0.376 ± 0.043^{ab}	-6.54 ± 1.81^{c}	0.574 ± 0.136^{b}	3.04 ± 1.08^{bc}
S60%MC	5.72 ± 0.64^{e}	0.398 ± 0.075^{a}	-3.96 ± 0.23^{b}	0.462 ± 0.025^{b}	1.05 ± 0.20^{c}
SCB	41.73 ± 3.95^{a}	0.439 ± 0.017^{a}	-0.05 ± 0.03^{a}	0.867 ± 0.097^{a}	15.92 ± 2.90^{a}

[1] Data are presented as the mean and standard deviation of three replicates.
Values bearing different lowercase letters were significantly different ($p \leq 0.05$) according to Tukey's post-hoc test.

Adhesiveness is the work required to overcome the sticky forces between the samples and the probe. Sausages made from meat alternatives had significantly higher adhesiveness than SCB. The increase in adhesiveness in sausages made from meat alternatives could be due to the adhesive properties of WG in the meat alternatives formulations and this could lead to a sticky consistency and higher adhesion (Day, 2011).

Springiness is the rate at which a deformed sample returns to its original size and shape and is sometimes referred to as 'elasticity' (Yang et al., 2007). There were no significant differences in springiness between sausages made from the meat alternatives. Sausages made from meat alternatives had a decrease in chewiness when MC increased. As chewiness is evidently influenced by hardness, the interpretation of the results could be similar (Horita et al., 2014).

In comparison with the reference sample, SCB had the highest hardness, springiness and chewiness among all samples, as only SCB contained real muscle meat, while the rest of the sausages were made mainly from plant proteins and liquid meat protein. There was no significant difference between the cohesiveness for S60%MC and SCB. SCB had the lowest adhesiveness among all samples. A typical meat sausage is characterised as having a smooth, firm surface without adherence to touch (Ayadi, Kechaou, Makni, & Attia, 2009). Overall results showed that none of the sausages made from meat alternatives was close to SCB in terms of textural properties.

9.4.3 Microstructural properties of sausages

As previously mentioned by Chiang et al. (2020), there was a loss of fibrous microstructure in meat alternatives when MC increased. Fibrous microstructure was observed for meat alternatives at 49%MC and 52%MC, whereas a layered structure was observed for meat alternatives at 56%MC, and a disoriented-pattern structure was observed for meat alternatives at 60%MC. After grinding the meat alternatives and chicken breast with a food processor for 10 min at its highest speed for the preparation of sausages, the structure of all sausages was disrupted and changed significantly. Scanning electron micrographs for sausages made from extruded meat alternatives at different MC and SCB are shown in **Figure 9-2**. The proportion of fibrous structure decreased in sausages made from meat alternatives as MC of the meat alternatives increased. S49%MC exhibited chunks of long fibres, while S52%MC showed some long fibres under SEM.

This could be due to higher hardness in 49%MC (40.25 ± 5.73N) than 52%MC (28.36 ± 3.64N), which resulted in less disruption of the structure of S49%MC as the grinding settings for all sausages were set the same. Small and short fibres were observed in S56%MC, while no fibre was spotted in S60%MC. This could be its softer texture due to higher MC, which led to larger disruption on its structures after grinding. SCB had somewhat similar disoriented-pattern structure as S60%MC, but their textural properties were significantly different.

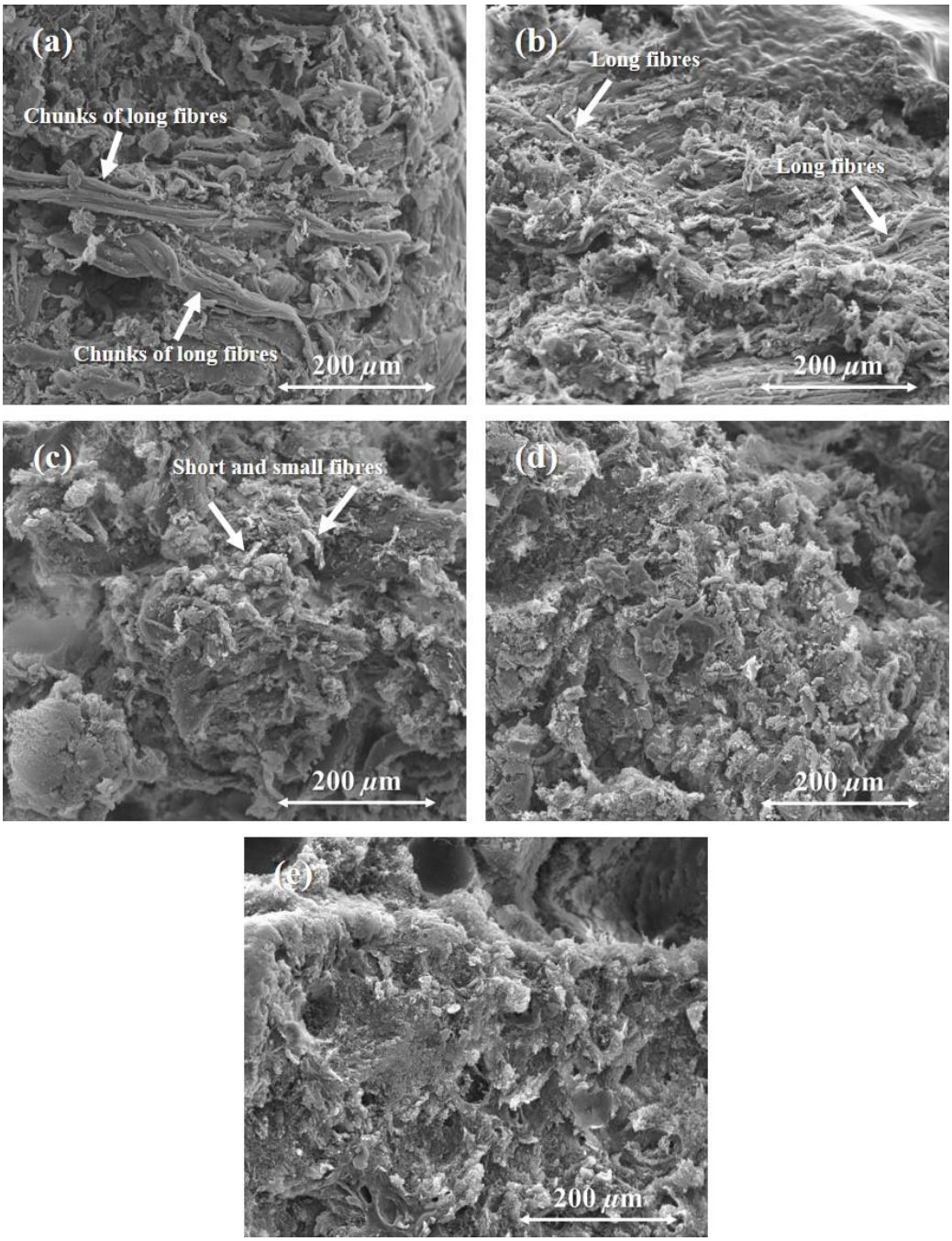


Figure 9-2 Scanning electron micrographs of sausages made from extruded meat alternatives at different moisture contents (a) S49%MC, (b) S52%MC, (c) S56%MC, (d) S60%MC, and chicken breast sausage (e) SCB, at 250× magnification.

9.4.4 Oxidative stability of sausages

Protein oxidation is one of the main causes of quality deterioration during the processing and storage of food products (Zhang et al., 2013). Some of the major protein oxidative modifications take place at the side chains of amino acids, which includes thiol oxidation, aromatic

hydroxylation, and generation of carbonyl compounds. The quantification of carbonyl compounds using the DNPH method has been widely used as a general measurement of protein oxidation in foods (Lund et al., 2011). Carbonyl content was determined as a measure of protein oxidation to assess the chemical stability of sausages made from meat alternatives at different MC and SCB at 4°C for 21 days (**Figure 9-3**).

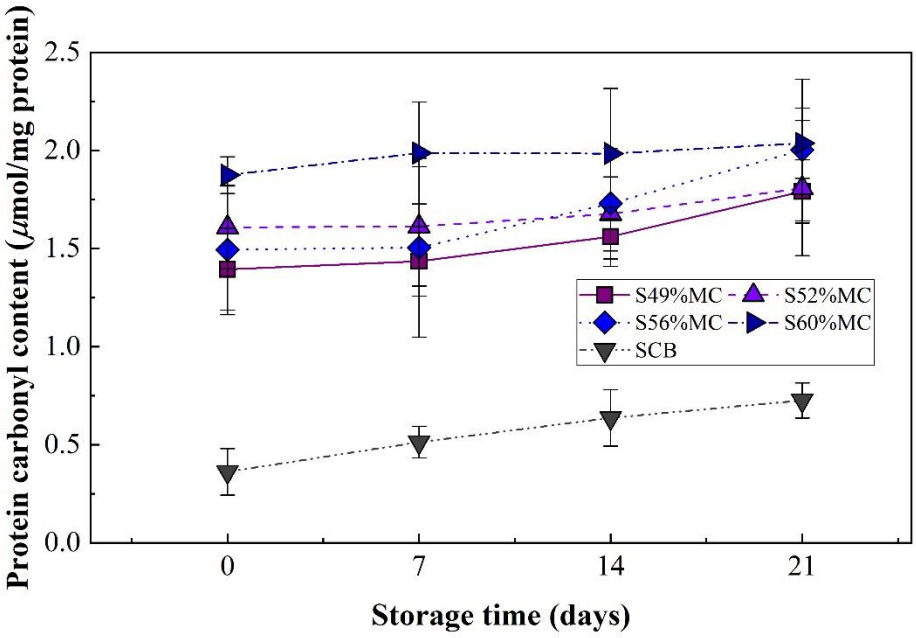


Figure 9-3 Protein carbonyl content of sausages made from extruded meat alternatives at different moisture contents (S49%MC, S52%MC, S56%MC, S60%MC) and chicken breast sausage (SCB). Data represent the mean and error bars represent the standard deviation of three replicates.

There was an increase in carbonyl content for all sausages as the storage time increased. This could be due to the high level of available oxygen in the packaging with decreased ability to maintain its antioxidant system and resulted in an increased level of protein oxidation (Astruc, Marinova, Labas, Gatellier, & Santé-Lhoutellier, 2007) even though the sausages were vacuum-packaged, which was in accordance with Zakrys-Waliwander, O'Sullivan, O'Neill, and Kerry (2012). Other possible reasons could be due to different pathways such as (i) binding of non-protein carbonyl compounds from lipid peroxidation by Michael addition (4-hydroxy-2-nonenal (HNE) and malondialdehyde) to protein amino acid side chains including cysteine sulfhydryl and lysine amino groups (Refsgaard, Tsai, & Stadtman, 2000), or (ii) direct oxidation of amino acid side chains including arginine, lysine, proline, and threonine (Amici, Levine, Tsai, & Stadtman, 1989) or (iii) addition of reactive carbonyl derivatives such as ketoamines, ketoaldehydes, and

deoxyosones generated by reducing sugar and their oxidation products after reacting with lysine (Xiong, 2000).

Sausages made from meat alternatives at different MC had higher carbonyl content as compared with SCB. This could be due to longer storage period for meat alternatives, as it was produced by extrusion, then frozen and stored before being made into sausages (e.g. early onset of protein oxidation). However, chicken breast was freshly purchased from the supermarket, chilled overnight and then made into sausages the following day. On Day 0, the carbonyl content of sausages made from meat alternatives increased when MC increased. There were no significant differences ($p \leq 0.05$) on the carbonyl contents for Day 0, 7, 14 and 21 between S49%MC, S52%MC and S56%MC. A similar trend was still evident on Day 21 on the carbonyl content of sausages made from meat alternatives. There were significant differences ($p > 0.05$) between S49%MC and S60%MC on the carbonyl contents at Day 0, 7 and 14. There was no significant difference ($p \leq 0.05$) between all sausages made from meat alternatives on Day 21. Structural modification can induce protein oxidation as mechanical energy (e.g. grinding of meat) during the processing of meat products can destroy the integral cell structure and break up antioxidant defence systems, resulting in high susceptibility to protein oxidation (Zhang et al., 2013). This could explain why S49%MC with chunks of long fibres had a lower proportion of carbonyl contents than S60%MC which had a disoriented-pattern structure under SEM (**Figure 9-2**).

There was a significant difference ($p > 0.05$) for SCB on carbonyl contents at every 7-day interval. There was no significant difference ($p \leq 0.05$) between all sausages made from meat alternatives for carbonyl contents from Day 0 to 21. This could be due to Maillard-reacted beef bone hydrolysate (MRP) incorporated into the meat alternatives being capable of retarding the development of rancidity in foods. Anti-oxidative compounds are supposedly formed during MRP production (Lingnert & Lundgren, 1980).

9.4.5 Sensory evaluation of sausages

The hedonic acceptance scores and purchase intention for all sausages made from extruded meat alternatives are shown in **Table 9-5**. SCB had the highest scores for all attributes except for appearance indicating that sausage made from the chicken breast was well-liked by the panellists.

This was expected as chicken meat is one of the common raw material used in the production of sausages other than beef, veal, lamb or pork and is likely familiar to the panellists. The SCB had a white appearance and looked like a German/ Bavarian Weisswurst. It scored slightly lower for appearance than S49%MC but the difference was not significant. S60%MC obtained the lowest score for appearance among all samples. This could be due to its soft and mushy texture, which resulted in a distorted shape when sliced (**Figure 9-1d**).

Panellists were asked to bite completely through the sample between their molar teeth and to chew the sample for at least 12 chews, in order to rate their acceptance for hardness and chewiness, respectively. The scores for hardness and chewiness of sausages made from meat alternatives decreased when MC increased indicating that sausages made from meat alternatives with higher MC were softer and less chewy. This correlated with instrumental results from TPA. Both sensory scores and instrumental results for hardness and chewiness of sausages decreased when MC increased. This suggests that the sausage with a soft texture and low chewiness had the lowest hedonic score. S60%MC had the lowest score for hardness and chewiness among all samples. This could be due to its soft and mushy texture (like pâté or mashed potato) and was therefore not well-liked as a sausage. There was no significant difference in the meaty aroma for sausages made from meat alternatives. This could be due to the same amount of Maillard-reacted bone hydrolysate (MRP) being used in the formulation of the meat alternatives to make sausages. Interestingly, different texture of sausages made from meat alternatives resulted in different score in meaty taste, although the amount of MRP used in the production of meat alternatives at different MC was similar. S60%MC with soft and mushy texture had the lowest score in meaty taste.

Table 9-5 Mean scores on the five attributes, overall acceptance and purchase intention of sausages made from meat alternatives at different moisture contents and chicken breast sausage in consumer sensory evaluation (*n*=50)

Sample	Mean score [1, 2]						
	Appearance	**Hardness**	**Chewiness**	**Meaty aroma**	**Meaty taste**	**Overall acceptance**	**Purchase intention**
S49%MC	6.08 ± 1.47^{a}	5.64 ± 1.82^{b}	5.76 ± 1.78^{b}	5.14 ± 1.70^{b}	4.70 ± 1.85^{b}	5.10 ± 1.73^{b}	2.66 ± 1.14^{b}
S52%MC	5.84 ± 1.38^{a}	4.94 ± 1.92^{b}	5.26 ± 1.80^{b}	5.12 ± 1.42^{b}	4.80 ± 1.58^{b}	5.02 ± 1.44^{b}	2.46 ± 0.95^{b}
S56%MC	5.62 ± 1.60^{a}	3.48 ± 1.64^{c}	3.56 ± 1.53^{c}	5.16 ± 1.57^{b}	3.98 ± 1.53^{bc}	3.72 ± 1.40^{c}	1.88 ± 0.82^{c}
S60%MC	4.50 ± 1.92^{b}	2.16 ± 1.22^{d}	2.40 ± 1.40^{d}	4.80 ± 1.67^{b}	3.66 ± 1.87^{c}	2.88 ± 1.49^{d}	1.46 ± 0.68^{c}
SCB	6.04 ± 1.76^{a}	6.86 ± 1.07^{a}	6.92 ± 1.40^{a}	6.76 ± 1.70^{a}	7.66 ± 0.92^{a}	7.18 ± 1.16^{a}	3.78 ± 1.06^{a}
F-value	**7.89**	**68.79**	**63.76**	**11.56**	**49.73**	**62.96**	**43.82**
***p*-value**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**

[1] Data are presented as the mean and standard deviation.

[2] Sensory evaluation scores are normally distributed.

Values bearing different lowercase letters in the same column were significantly different ($p \leq 0.05$) according to Tukey's post-hoc test.

SCB obtained the highest score in overall acceptance and purchase intention among all sausages, followed by S49%MC and S52%MC with no significant difference between these two samples. Overall sensory results show that the use of extruded meat alternatives in the manufacture of sausages produced far inferior sausages compared to sausages made from conventional meat ingredients. Although S49%MC obtained the highest scores (i.e. appearance, hardness, chewiness, overall acceptance and purchase intention) among all sausages made from meat alternatives, it still scored lower than SCB for all sensory characteristics. Furthermore, S49%MC had softer textural properties than SCB. From our previous results, it was noted that even though meat alternatives at 49%MC might have very close textural and microstructural properties to boiled chicken breast (Chiang et al., 2020). However, when made into sausages, the grinding process disrupted the intact structure of meat alternatives, causing S49%MC to lose its textural and microstructural properties compared to ground SCB. Therefore, it is recommended that meat alternatives at even lower MC (<49%MC) may be required to retain higher hardness when the meat alternatives are made into sausages, in order to match SCB in terms of textural and sensory properties.

9.5 Conclusion

In this study, the physicochemical properties of sausages made from extruded meat alternatives at different MC were investigated. S60%MC had the lowest hardness and chewiness due to its high MC that resulted in a soft and mushy texture. S49%MC exhibited chunks of long fibres, while S52%MC showed some long fibres under SEM. Small and short fibres were observed in S56%MC, and no fibre was spotted in S60%MC. Sausages made from meat alternatives had higher protein oxidation as compared with SCB. However, there was no significant difference in sausages made from meat alternatives at different MC for protein oxidation from Day 0 to 21. Sensory results showed that SCB obtained the highest scores for all attributes except for appearance among all sausages with significant differences. Although S49%MC obtained the highest sensory scores among all sausages made from meat alternatives, it is recommended that meat alternatives at even lower MC (<49%MC) be considered to match SCB in terms of textural and sensory properties. Overall results showed that the use of extruded meat alternatives in the making of sausages was far inferior from sausages made from conventional meat ingredients.

Chapter 10: Overall conclusion and recommendations

10.1 Overall conclusion

The research outline in this thesis used enzymatic hydrolysis and the Maillard reaction to improve the flavour character of beef bone extract, which is a low-value high protein meat by-product. The resulting flavoursome protein extract (MRP) was then used as an ingredient together with plant proteins to develop extruded meat alternatives. Addition of MRP at 20% produced meat alternatives with a meaty aroma and taste, while achieving a desirable fibrous structure. Using the meat alternatives to make sausages showed that further work needs to be done to increase its consumers' acceptability to match the control (chicken breast meat). The following research questions were answered:

10.1.1 How to modify the flavour character of beef bone extract to become flavoursome protein ingredients?

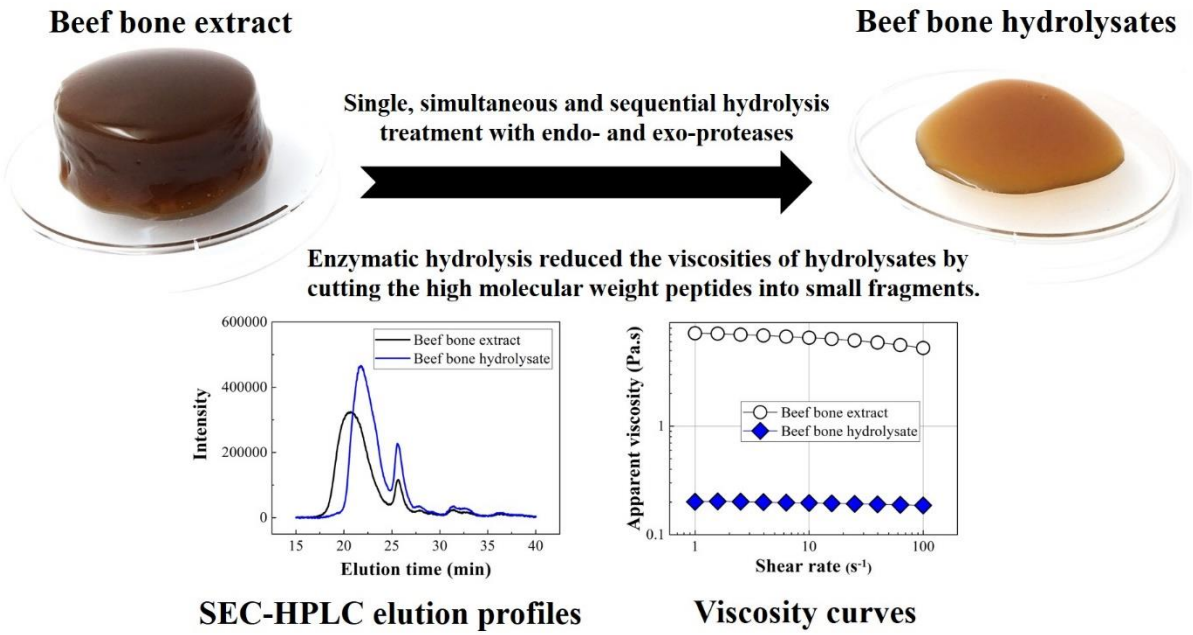


Figure 10-1 Enzymatic hydrolysis of beef bone extract using single, simultaneous and sequential treatment with endo- and exo-proteases.

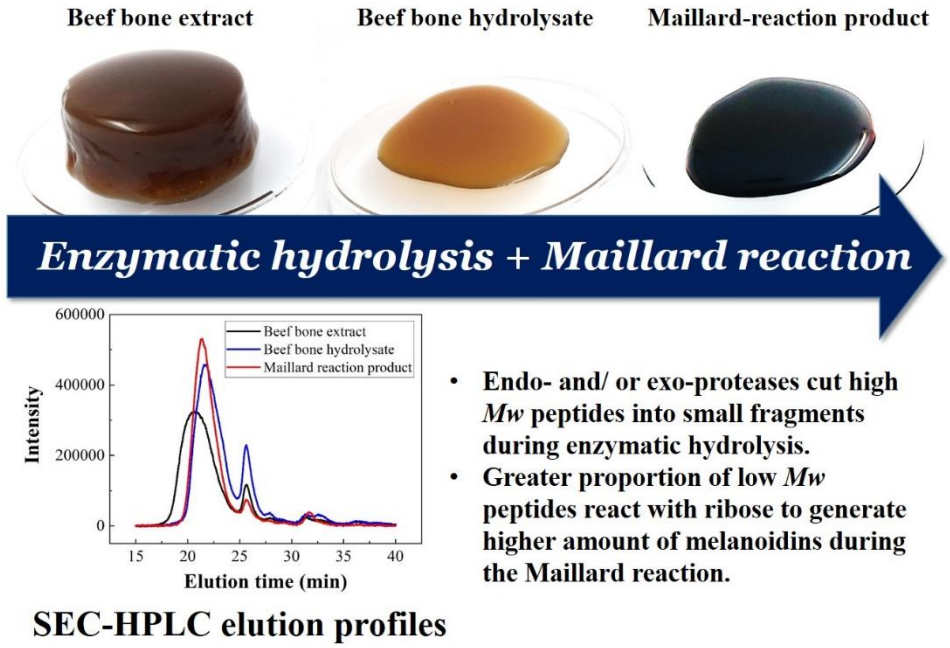


Figure 10-2 Changes in the SEC-HPLC elution profiles of beef bone extract, hydrolysate and Maillard reaction product after heating due to the progression of Maillard reaction.

The use of enzymatic hydrolysis followed by the MR modified the flavour character of beef bone extract and turned it into a flavoursome protein ingredient. We studied three types of enzymatic hydrolysis treatments of beef bone extract, namely single, simultaneous and sequential. Simultaneous hydrolysis treatment (2 hours) was found to be the most favourable as there were no significant differences in terms of DH, *Mw* distribution and sulfhydryl content when compared with sequential treatment (4 hours). We were also able to determine the hydrolysis kinetics of Protamex®, bromelain and Flavourzyme® in this research. The Michaelis-Menten model was used to obtain the ideal E/S ratio, which was assessed by the DH. This novel approach was considered as a practical method as it was easy to understand, reliable and not time-consuming. It allowed us to estimate the maximum DH for the three enzymes on beef bone extract. This approach can be used for the hydrolysis of other meat by-products for future work.

Lastly, the pressure cooker was used as heat treatment for MR to produce MRPs. The whole process took a total of 45 min (including pre-heating, holding, and cooling), which include heat treatment due to the progression of MR at 113°C for 10 minutes. The process to produce MRPs has a total processing time of less than 3 hours and uses relatively simple equipment heat the

reaction mixture and therefore the manufacture of the flavoursome protein ingredients could be a viable industrial process.

The use of enzymatic hydrolysis followed by MR significantly increased the volatile compounds of interest. Further to that, the use of aminopeptidase (e.g. Flavourzyme®) in single hydrolysis treatment followed by the MR heat treatment showed that the enzyme is capable of increasing the proportion of flavour compounds and obtaining the highest sensory scores, without the addition of other enzymes.

10.1.2 How to improve the structural and textural properties of current extruded meat analogues?

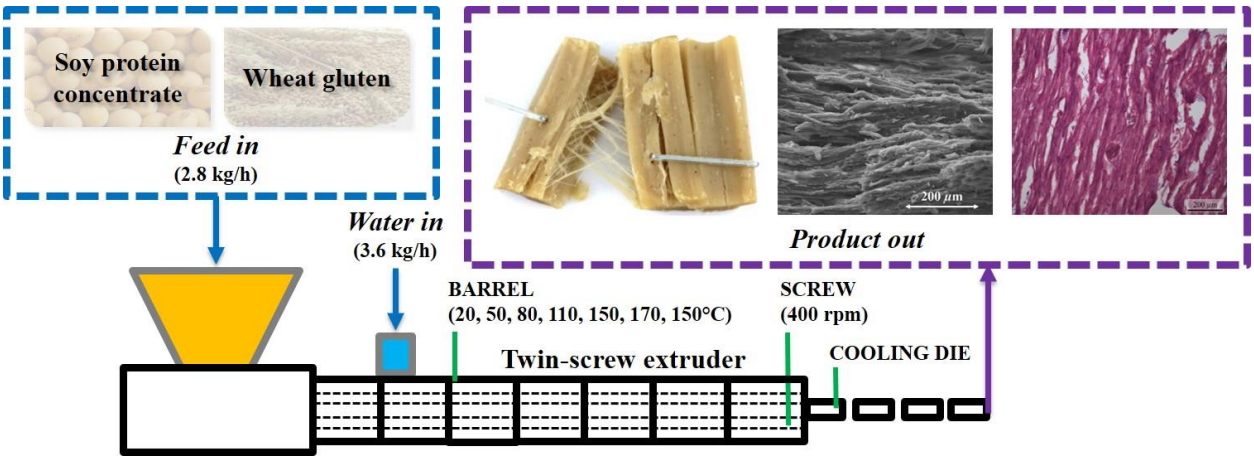


Figure 10-3 Effects of soy protein concentrate to wheat gluten ratio on the physicochemical properties of extruded meat analogues, where meat analogues containing 30% wheat gluten exhibited fibrous microstructure.

Results showed that WG played an important role by contributing to the increase in S-S bonds in meat analogues to form fibrous structure. Meat analogues containing 30%WG exhibited the highest degree of texturisation, fibrous microstructure, hardness and chewiness when compared with others (0, 10 and 20%WG). When meat analogues were compared with boiled chicken breast, it was found that meat analogues containing 20%WG and 30%WG are the closest in terms of structural properties to chicken breast. But comparing the textural properties, chicken breast was found to be softer and less chewy than meat analogues containing 20%WG and 30%WG when measured by both TPA and sensory methods.

10.1.3 How does the flavoursome protein ingredient interact with plant proteins to generate meat alternatives with high acceptability?

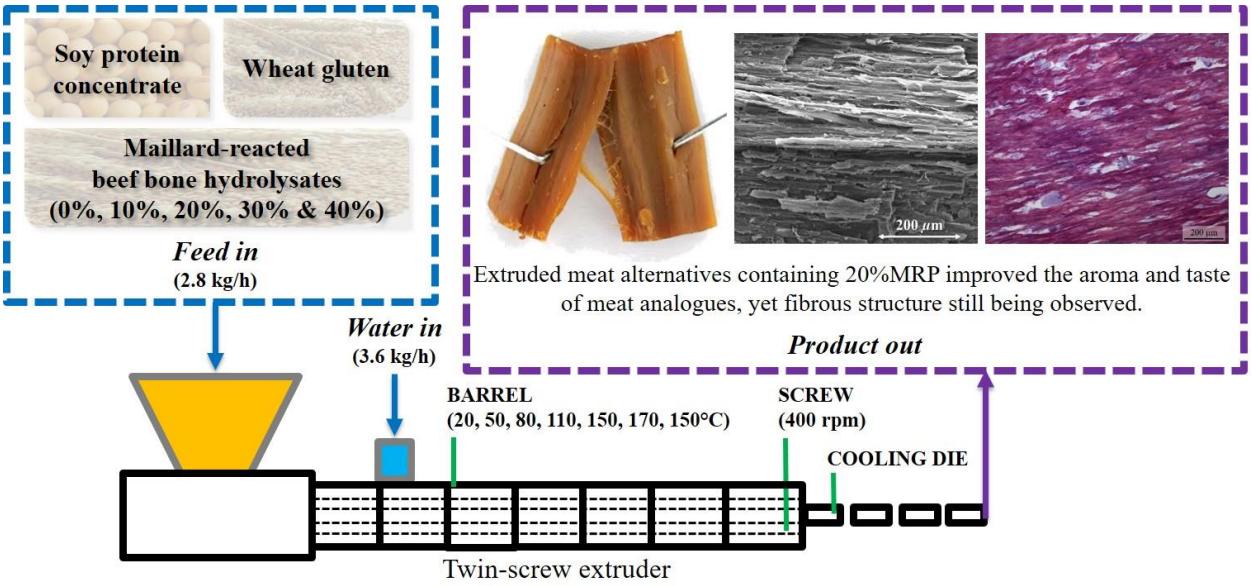


Figure 10-4 Interactions between Maillard-reacted beef bone hydrolysate and plant proteins on the physicochemical properties of extruded meat alternatives, where meat alternatives containing 20%MRP improved the aroma and taste, with fibrous structure still being observed.

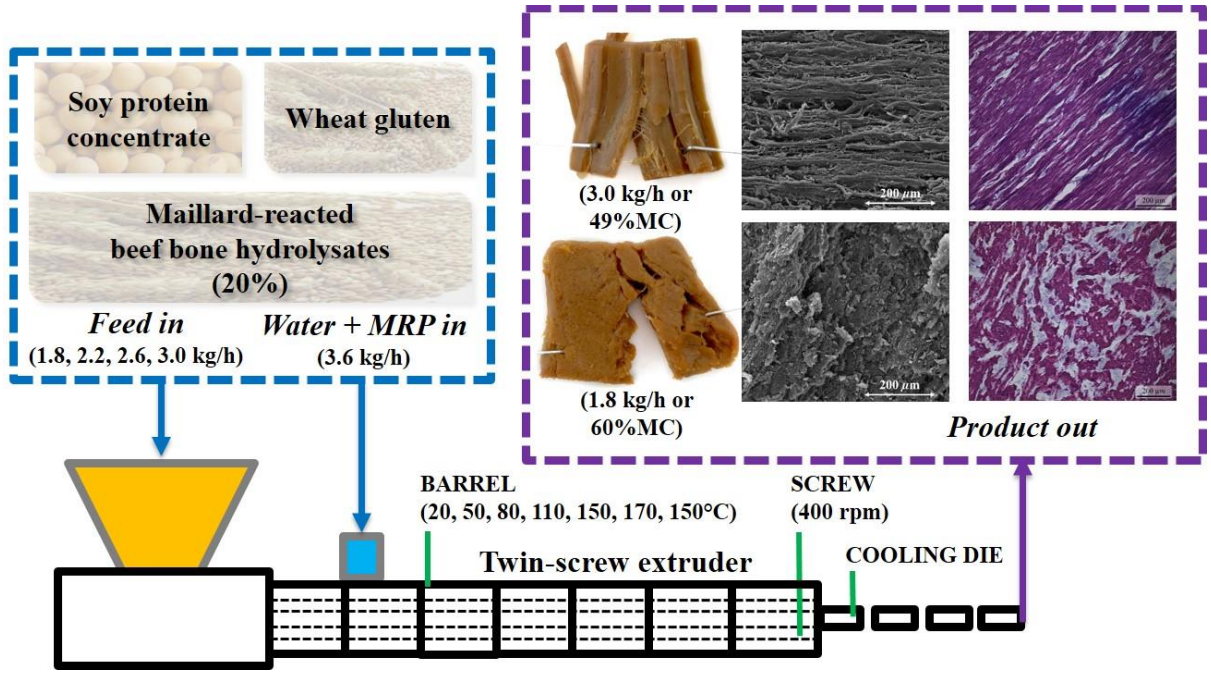


Figure 10-5 Effects of moisture contents on the physicochemical properties of extruded meat alternatives, where meat alternatives at 49%MC exhibited most fibrous microstructure.

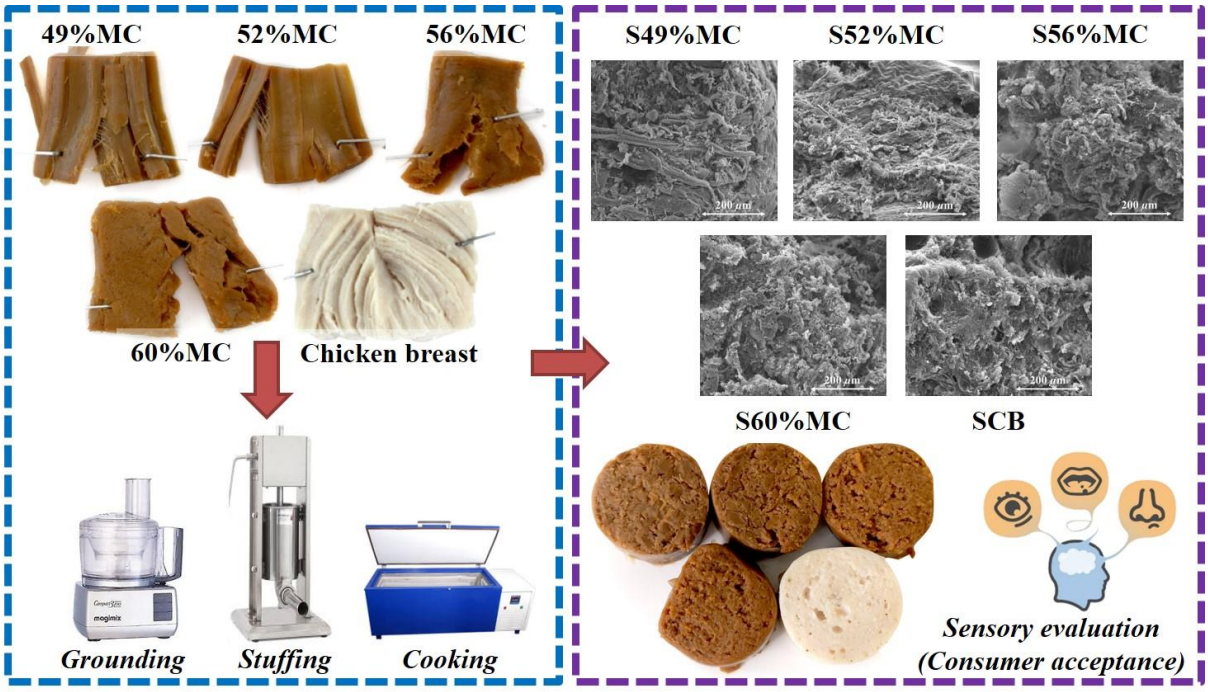


Figure 10-6 Sausages made from extruded meat alternatives at different moisture contents, where sausages made from meat alternatives at 49%MC scored the highest in appearance, hardness, chewiness, overall acceptance and purchase intention during consumers' acceptability test.

Results showed that the addition of MRP to meat analogue formulations to produce meat alternatives changed the textural, structural and sensory properties significantly. Meat alternatives containing 20%MRP improved the aroma and taste of meat analogues as measured by sensory evaluation, with fibrous microstructure still observed.

The MC was an important parameter in the formation of fibrous microstructure for meat alternatives. Meat alternatives at 49%MC were the closest in terms of both textural and structural properties to chicken breast.

Sausages made from meat alternatives at 49%MC scored the highest in appearance, hardness, chewiness, overall acceptance and purchase intention during consumers' acceptability test. However, overall results showed that sausages made from extruded meat alternatives were far inferior to sausages made from conventional meat ingredient such as chicken breast.

10.2 Recommendations

Future research should be focused on overcoming barriers to the transfer of positive laboratory outcomes to industrial production. Some of the key future research directions are recommended below.

10.2.1 Choice of other meat by-products as substrate for enzymatic hydrolysis

Commercial beef bone extract was used in this research thesis. Other meat by-products for flavour development could be considered such as desinewed minced beef and mechanically separated chicken using enzymatic hydrolysis with aminopeptidase (e.g. Flavourzyme®) and MR with ribose.

10.2.2 Choice of other reducing sugars for Maillard reaction

The work was conducted using ribose as reducing sugar. Other sugars could be considered such as xylose or glucose. However, ribose and xylose are known to be expensive (~$30 per kg). Hence, it would be of economic value to investigate whether a cheaper source, glucose (~$1 per kg) is able to produce similar MRPs as ribose. It will reduce raw material' expenses significantly.

10.2.3 Additional characterisation techniques for beef bone hydrolysates and Maillard reaction products

Additional characterisation techniques could be conducted to obtain more information on beef bone hydrolysates and MRPs. For the hydrolysates, it will be interesting to determine some of the functional and bioactive properties of beef bone hydrolysates such as solubility, emulsifying properties, antioxidant activity, ACE inhibiting activity and antimicrobial activity as they may offer useful additional commercial opportunities for the meat industry. For MRPs, the use of gas chromatography-olfactometry-mass spectrometry (GC-O-MS) can identify the odour quality and chemical structure of volatile compounds positively and quickly at one injection (Song & Liu, 2018). Other information such as flavour dilution (FD) factor and odour activity value (OAV) are the main parameters used to measure the role of each compound in the overall aroma of food even though they are calculated parameters. Lastly, the food safety aspect of MRPs should be taken into consideration, in terms of microbiological aspect or hazardous compounds (e.g. carcinogenic or mutagenic products) formed during MR.

10.2.4 Choice of other plant proteins in the development of meat analogues

Soy and wheat proteins are widely used as the basic ingredients for meat analogues at the moment. However, soy and wheat cause food allergies to humans. Furthermore, people with celiac diseases have chronic, multiple-organ autoimmune disorder to gluten which affect the small intestine. Alternative protein-rich raw materials with high consumer's acceptance must be identified, such as pea protein, lupin protein or even insect protein which have been studied, or proteins containing high S-S bonds can be explored. It was because meat analogues with prominent fibrous microstructure such as containing 30%WG were found to have high S-S bonds.

10.2.5 Additional characterisation techniques for extruded meat analogues/ alternatives

Additional characterisation techniques can be conducted to gather more information on extruded meat analogues/ alternatives. For instance, the use of Fourier-transform infrared spectroscopy (FT-IR) should be investigated in order to understand the secondary structural changes of proteins during extrusion (Zhang et al., 2019). The use of x-ray tomography should be used to determine the air pockets formed due to the expansion of material due to water evaporation, as Dekkers et al. (2018) hypothesized that the air pockets may contribute to the fibrous properties of meat analogues. A *in vitro* digestion of meat analogues/ alternatives is recommended, as it stimulates the physiological conditions of sequential oral, gastric and small intestinal phases to obtain the physiological response to meat analogues/ alternatives. Lastly, the food safety (i.e. microbiological and shelf-life) aspect of meat analogues/ alternatives should be evaluated as well.

10.2.6 Scaling up or industrialisation of the production of Maillard reaction products or meat alternatives

This study used a laboratory-scale method by generating MRP from beef bone hydrolysates. Scale-up should be studied at the pilot-plant scale level to see if the range of flavour compounds generated at the laboratory-scale are produced at the pilot-plant scale. For instance, a batch process using a temperature and pressure-controlled double jacket machine such as STEPHAN Universal Machine can be used to produce hydrolysates by maintaining the system at 50°C at 200 rpm for 120 min. After the hydrolysis, ribose could be added directly into the system and heated at 170 kPa (113°C) for 10 min to produce Maillard-reacted beef bone hydrolysate. Similar

characterisation techniques will be used to see if there are differences between laboratory-scale and pilot-plant scale production.

Meat analogues and meat alternatives were produced on a pilot-plant scale (kg/h) in this research thesis. Hence, an industrial size TS extruder (kg×10^3/h) with higher throughput is recommended for the scale-up production. During the industrialisation, some of the process variables such as dry feed flow rate, liquid feed flow rate and screw speed may need to be adjusted, to obtain samples with prominent fibrous meat-like structure.

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We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

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Name/title of Primary Supervisor:	Dr Michael Parker / Lecturer
Name of Research Output and full reference:	
Chiang, J. H., Henderie, A. K., & Parker, M. S. (2019). Extruded meat alternatives made from Maillard-reacted beef bone hydrolysate and plant proteins. Part I - Effect of moisture content. International Journal of Food Science and Technology.	
In which Chapter is the Manuscript /Published work:	Chapter 8
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Chiang, J.H., Hardacre, A.K., & Parker, M.E. (2020). Extruded meat alternatives made from Maillard-reacted beef bone hydrolysate and plant proteins. Part II - Application in sausages. International Journal of Food Science and Technology, 55(3), 1267-	
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