NUTRITIONAL COMPOSITION, TOXICITY STUDY OF SQUID AND CUTTLEFISH INK HYDROLYSATES AND FLAVOUR IMPROVEMENT USING MAILLARD REACTION

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by

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

%	Percentage
a*	Redness -greenness
AU	Enzyme unit
b*	Yellowness-blueness
cm	Centimetre
g	Gram
kg	Kilogram
L	Liter
L*	Lightness
mg	miligram
nm	nanometer
ΔE	Colour difference

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
ARASC	Animal Research and Service Centre
ASTM	American Society for Testing and Material
BW	Bodyweight
cGMP	Cyclic guanosine monophosphate
CI	Cuttlefish ink
CIE	Commission Internationale de l'Eclairage
CIEHP	Cuttlefish ink enzyme hydrolysates powder
CIEHP-mx	Cuttlefish ink enzyme hydrolysates powder with the addition of L-cysteine and D-xylose
CIEHP-x	Cuttlefish ink enzyme hydrolysates powder with the addition of D-xylose
DH	Degree of hydrolysis
EE	Encapsulation efficiency
FAA	Free amino acids
FAO	Food and Agriculture Organization
FCC	Food chemicals codex
FPH	Fish protein hydrolysates
GC-MS	Gas chromatography-mass spectrometry (GC-MS)
H&E	Hematoxylin and Eosin
HCl	Hydrochloric acid
IACUC	Institutional Animal Care and Use Committee
ISO	International Organization for Standardization
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JEPeM	Jawatankuasa Etika Penyelidikan Manusia

LD_{50}	Median lethal dose
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MRPs	Maillard reaction products
MSG	Monosodium glutamate
MTGase	Microbial transglutaminase
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NIST	National Institute of Standards and Technology
OECD	Organisation for Economic Co-operation and Development
OSHA	Occupational Safety and Health Administration
PCA	Principal component analysis
PUFA	Polyunsaturated fatty acids
QDA	Quantitative descriptive analysis
ROS	Reactive oxygen species
SCFA	Short-chain fatty acids
SI	Squid ink
SIEHP	Squid ink enzyme hydrolysates powder
SIEHP-mx	Squid ink enzyme hydrolysates powder with the addition of L-cysteine and D-xylose
SIEHP-x	Squid ink enzyme hydrolysates powder with the addition of D-xylose
UPD	Up and down
USM	Universiti Sains Malaysia
UV	Ultraviolet
WHO	World health organization

LIST OF APPENDICES

KOMPOSISI NUTRISI, KAJIAN KETOKSIKAN HIDROLISAT DAKWAT SOTONG DAN SOTONG KATAK DAN PENAMBAHBAIKAN PERISA MENGGUNAKAN REAKSI MAILLARD

ABSTRAK

Objektif kajian ini adalah untuk mengkaji komposisi pemakanan, ketoksikan oral akut dan peningkatan rasa serbuk enzim hidrolisat dakwat sotong (SIEHP) dan serbuk enzim hidrolisis dakwat sotong katak (CIEHP). SIEHP dihidrolisis oleh Alcalase® pada pH 8 dan 55°C, sementara CIEHP dihidrolisis oleh papain pada pH 6 dan 55°C selama 4 jam dan 3% (w/v) nisbah enzim-substrat, masing-masing. Analisis komposisi proksimat dan logam surih dilakukan. Kajian ketoksikan pada haiwan dilakukan mengikut Organisasi Kerjasama dan Pembangunan Ekonomi (OECD) No. 425. Satu kumpulan berfungsi sebagai kawalan dan dua kumpulan yang dirawat menerima 2000 mg/kg b.w (ujian had) SIEHP dan CIEHP pada satu dos secara oral. Semua kumpulan diperhatikan selama 14 hari dan organ-organ tikus yang telah dimatikan disimpan untuk pemeriksaan histopatologi. SIEHP dan CIEHP dipanaskan dengan D-xylose dan L-cysteine atau D-xylose pada suhu 120°C, pH 7.4 selama 2 jam dan dilabel sebagai SIEHP-mx atau SIEHP-x, CIEHP-mx atau CIEHP-x untuk menerokai ciri-ciri tindak balas Maillard Profil sensori dinilai berdasarkan analisis deskriptif kuantitatif (QDA) oleh 10 panelis terlatih, sementara kebolehterimaan sensori dilakukan kepada 30 pengguna terhadap penampilan, bau, rasa, sisa rasa dan penerimaan keseluruhan. Hasil kajian mendapati SIEHP dan CIEHP mempunyai kandungan protein dan abu yang lebih tinggi tetapi kandungan lemak dan kelembapan lebih rendah daripada dakwat tanpa hidrolisis. Kepekatan logam surih SIEHP dan CIEHP berada di bawah had yang dibenarkan dan urutan penurunannya adalah Zn>

Fe> Cu> Mn> Ni> Cd dan Zn> Fe> Mn> Cu> Ni> Cd, masing-masing. Dalam kajian *in vivo* melaporkan SIEHP dan CIEHP tidak mempunyai risiko toksik pada tikus dan tiada kematian yang dijumpai menunjukkan LD50>2000 mg/kg. Tidak ada perubahan ketara yang terdapat pada berat badan, pengambilan makanan dan air dan tidak ada kelainan kasar atau perubahan histopatologi pada semua organ viseral. Semasa tindak balas Maillard, perubahan ketara dijumpai pada SIEHP-mx, SIEHP-x, CIEHP-mx dan CIEHP-x yang meningkatkan pengumpulan produk keperangan, pengurangan pH, dan berubah menjadi warna yang lebih gelap, merah dan kuning. Sebatian mudah meruap yang berkaitan dengan aroma seperti hanyir berkurang, dan profil deria menggambarkan rasa umami yang lebih baik dan kurang sisa rasa pahit dalam SIEHP-x, CIEHP-mx, SIEHP-mx dan CIEHP-mx. Walau bagaimanapun, SIEHP-mx dan CIEHP-mx lebih disukai sebagai penerimaan keseluruhan. Secara keseluruhan, SIEHP dan CIEHP mempunyai kandungan protein yang lebih tinggi dan kepekatan logam surih berada di bawah had keselamatan. SIEHP dan CIEHP mempunyai ketoksikan yang sangat rendah tanpa tanda-tanda ketoksikan atau histopatologi yang luar biasa. Tindak balas Maillard dengan kehadiran D-xylose dan L-cysteine telah menambah baik perisa SIEHP-mx dan CIEHP-mx.

NUTRITIONAL COMPOSITION, TOXICITY STUDY OF SQUID AND CUTTLEFISH INK HYDROLYSATES AND FLAVOUR IMPROVEMENT USING MAILLARD REACTION

ABSTRACT

The objectives of this study were to investigate the nutritional composition, acute oral toxicity and flavour improvement of squid ink enzyme hydrolysates powder (SIEHP) and cuttlefish ink enzyme hydrolysates powder (CIEHP). SIEHP was hydrolysed by Alcalase® at pH 8 and 55°C, while CIEHP was hydrolysed by papain at pH 6 and 55°C for 4 h and 3% (w/v) enzyme-substrate ratio, respectively. Proximate composition and trace metals analysis was conducted. Toxicity study on animals was performed according to Organization for Economic Co-operation and Development (OECD) No. 425. One group served as control and two treated groups received 2000 mg/kg b.w (limit test) of SIEHP and CIEHP at a single dose orally. All groups were observed for 14 days and the organs of euthanized rats were preserved for histopathological examination. The SIEHP and CIEHP were heated with D-xylose and L-cysteine or D-xylose at 120°C, pH 7.4 for 2 h and labelled as SIEHP-mx or SIEHP-x, CIEHP-mx or CIEHP-x to explored characteristics of Maillard reaction. The sensory profile was assessed based on quantitative descriptive analysis (QDA) by 10 trained panellists, while the sensory acceptability was performed on 30 consumers towards appearance, odour, flavour, aftertaste and overall acceptability. The findings revealed SIEHP and CIEHP had higher protein and ash content but lower fat and moisture content than ink without hydrolysis. The trace metals concentration of SIEHP and CIEHP were below recommended permissible limit and their decreasing order were Zn>Fe>Cu>Mn>Ni>Cd and

Zn>Fe>Mn>Cu>Ni>Cd, respectively. *In vivo* study reported SIEHP and CIEHP were devoid of any toxic risk in rats and no mortality found indicated LD_{50}>2000mg/kg. There was no significant alteration observed in body weight, food and water consumption and neither gross abnormalities nor histopathological changes were found in all visceral organs. During Maillard reaction, significant changes were found in SIEHP-mx, SIEHP-x, CIEHP-mx and CIEHP-x that increased accumulation of browning products, pH reduction, and turned to darker, redder and yellow colours. The volatile compounds associated with fishy-like aroma were reduced, and the sensory profile described better umami taste and a less bitter aftertaste in SIEHP-x, CIEHP-mx, SIEHP-mx and CIEHP-mx. However, SIEHP-mx and CIEHP-mx were more preferred as overall acceptance in rice porridge. Overall, SIEHP and CIEHP had higher protein content and the trace metals concentration were below safety limits. SIEHP and CIEHP possess very low toxicity with no sign of toxicity or any remarkable histopathological signs. The Maillard reaction with the presence of D-xylose and L-cysteine was improved the flavour of SIEHP-mx and CIEHP-mx.

CHAPTER 1

INTRODUCTION

1.1 Research background

Cephalopods play an essential role as keystone invertebrates in marine ecosystems and have been a valuable commercial fishery resource worldwide (Xavier et al., 2015). Cephalopods belong to phylum Mollusca and comprise soft-bodied animals and bilaterally symmetrical species (Derby, 2014). Cephalopods are the essential global fisheries resources representing squid, cuttlefish, nautilus, octopus, and Mediterranean countries are becoming the main exporter of Cephalopods to Japan (Kechaou et al., 2015). Hence, their economic value is increasing exponentially. The increased exploitation of squid contributes a considerable amount of byproducts, which account for up to 60% of the whole weight of squid (Ezquerra-Brauer & Aubourg, 2019). Visceral organs and ink sacs are the major squid byproducts discarded due to their low profitability (Vate & Benjakul, 2013). However, the underutilised marine byproducts are valuable raw material sources for recovering existing bioactive peptide constituents or producing bioactive peptides from protein components (Anal et al., 2013).

Numerous researchers have performed comparative melanogenesis experiments on squid ink (Derby, 2014; Wang et al., 2019). Melanogenesis is the formation of melanin pigment that gives the dark colour of squid ink and occurs in the presence of melanogenic enzymes, including tyrosinase, dopachrome rearranging enzyme and peroxidase (Ahamed et al., 2018). Melanin is a melanoprotein containing 10-15% protein composed of essential and non-essential amino acids in squid ink (Lestari et al., 2019). Squid ink possesses antioxidant, anti-tumour properties (Fahmy

& Soliman, 2013), anti-radiation, anti-retroviral and anti-bacterial effects (Nithya et al., 2011; Vennila et al., 2011). Besides, squid ink comprises several bioactive compounds widely used in homoeopathic medicine (Senan et al., 2013; Moustafa & Awaad, 2016).

Bioactive peptides remain inactive within the protein sequence but become active once released during digestion or enzymatic proteolysis. They show diverse biological behaviours based on amino acid composition and sequence (Ishak & Sarbon, 2018). Enzymatic hydrolysis is a promising process that converts underutilised marine wastes into a more marketable and acceptable form for direct human use (Salwanee et al., 2013). Even though chemical hydrolysis is commonly used in industry, enzymatic hydrolysis is possessed more desirable functional properties and high nutritional value under controlled proteolysis (Tadesse & Emire, 2020). In particular, the utilisation of fish protein hydrolysates (FPH) generates new functional food by allowing small peptides and amino acids production. These properties are enhanced water and oil absorption capacity, protein solubility, gelling activity, foaming capacity and emulsification capacity (Srikanya et al., 2017). The enzymatic degradation increases the number of amino acids, decreases the molecular weight of peptides and possesses unique taste properties, including sweet, salty, sour, bitter, and umami tastes and develops volatile compounds that enhance the flavour (Min, 2015). Simultaneously, this approach would improve the market value of low-valued fish byproducts and increase their utilisation (Shahidi et al., 2019).

Nevertheless, protein hydrolysates mainly derived from seafood are often accompanied by undesirable fishy and bitter off-flavour (Kouakou et al., 2014; Normah & Noorasma, 2018). The acceptance of fish protein hydrolysates has been

impeded due to the concerns of fishy off-flavours (Normah & Noorasma, 2018). The prevalent fish flavour is associated with rancid odour and/or flavour, affecting consumer acceptance and limit further application. Likewise, the presence of bitter peptides made of hydrophobic amino acids correlates with the bitterness of protein hydrolysates (Fu et al., 2019). The peptides hydrophobicity and length, amino acid sequence and spatial structure also develop bitter taste perception (Kim & Li-Chan, 2006). Food taste is an important factor determining consumer acceptance; therefore, enhancing the overall flavour of protein hydrolysates used as food ingredients (Clemente, 2000).

Several approaches have been made to eliminate or mask this unpleasant taste, including encapsulation, plastein reaction, exopeptidases treatment and Maillard reaction. Yet, the Maillard reaction modification seems to be an effective method due to the ability to modify the sensory properties and could be an effective way to enhance the acceptability of fish byproducts (Wei et al., 2018). Several studies described the new flavour of the fish protein hydrolysates (FPH) created via the Maillard reaction (Kouakou et al., 2014; Ogasawara et al., 2006). Maillard reaction products (MRPs) are widely used to produce flavour enhancers (Chen et al., 2019). Flavour is an essential criterion in evaluating the quality of products and consumer acceptance (Rehbein & Oehlenschläger, 2009). Hence, improving the flavour is a prerequisite to success as competitive functional ingredients (Chen et al., 2019).

1.2 Problem statement

The consumption of seafood has grown markedly because of its good nutritional characteristics and its rising demand worldwide. The increased exploration of seafood resources leads to a significant generation of byproducts. Commonly,

seafood processing byproducts are used to produce low market-value products such as animal feed, silage, or end up as discards despite being protein-rich byproducts. In face with the resulting disposal and environmental issues, attempts are undertaken to deal more efficiently with seafood wastes. In this realization, a practical approach is converting squid and cuttlefish ink byproducts to value-added protein hydrolysates via enzymatic hydrolysis. However, the major obstacle that could reduce the sensory acceptability of protein hydrolysates is the taste and aroma, particularly fishy and aroma defects. The various uses of squid and cuttlefish ink have gained much attention, yet the limited study of safety evaluation toward squid and cuttlefish ink hydrolysates might hinder consumer acceptance. Therefore, the knowledge of the nutritional content, safety assessment and organoleptic properties of ink hydrolysates may be significant for its potential inclusion.

1.3 Objectives

1.3.1 Main objective

To prepare the squid ink enzyme hydrolysates powder (SIEHP) and cuttlefish ink enzyme hydrolysates powder (CIEHP) and investigate their potential nutritional composition, safety evaluation and sensory characteristics after flavour improvement.

1.3.2 Specific objectives

1. To determine the nutritional composition and trace metal concentration of squid ink enzyme hydrolysates powder (SIEHP) and cuttlefish ink enzyme hydrolysates powder (CIEHP).
2. To examine the acute oral toxicity effect of squid ink enzyme hydrolysates powder (SIEHP) and cuttlefish ink enzyme hydrolysates powder (CIEHP).

3. To evaluate the organoleptic properties of flavour improvement effects of squid ink enzyme hydrolysates powder (SIEHP) and cuttlefish ink enzyme hydrolysates powder (CIEHP) with the addition of D-xylose and/or L-cysteine.

1.4 Significance of study

This research is carried out to address the gap and propose the potentiality of squid and cuttlefish ink utilization. Several reports accumulated in the present work have effectively emphasised the significance of seafood wastes protein hydrolysates as regards their nutritional and functional properties that are highly appealing for the development of functional ingredients. Besides, developing strategies to improve flavours and aromas of squid and cuttlefish ink hydrolysates are necessary to enhance consumer acceptability and thus widening its utilization. The efforts and advances in this area will surely discover new methods for detecting novel uses of protein hydrolysates derived from squid and cuttlefish ink byproducts in a broader food application.

CHAPTER 2

LITERATURE REVIEW

2.1 Cephalopods production and distribution

Cephalopods are a Mollusca class that has originated in the aquatic ecosystem around 500 million years ago (Leslie et al., 2018). The number of Cephalopods species is relatively low (about 700) and widely spread in many different oceanic regions (Derby, 2014). Cephalopods represent two main groups, namely Nautiloidea (nautilus) and Coleoidea (squid, cuttlefish, and octopus). The Coleoids are organised into *Octopodiformes* (*Vampyromorpha*, vampire squids; and Octopoda, octopuses) and *Decapodiformes* (squids and cuttlefishes) (Derby, 2014). However, the commercially captured Cephalopods species include squid, cuttlefish and octopus. Cephalopods are familiar with three groups of molluscs, namely gastropods, univalves with a single shell such as snails, slugs, and bivalves with two shells, scallops, oysters, and mussels (Derby, 2007). In nature, freshwater and marine molluscs possess their protective response against the predator, likely by their shells and shell-less molluscs such as squid and cuttlefish extensively used chemical defence (Fahmy & Soliman, 2013). Thailand is becoming the main exporter of Cephalopods and is preceded by other Southeast Asian countries (Vate & Benjakul, 2013). Approximately 70-80 thousand tons of Cephalopods are annually caught in Malaysia (Figure 2.1). Indian squid and cuttlefish are the significant sources available in Malaysia and the common fishery captured for domestic consumption and economic use (Rubaie et al., 2012).

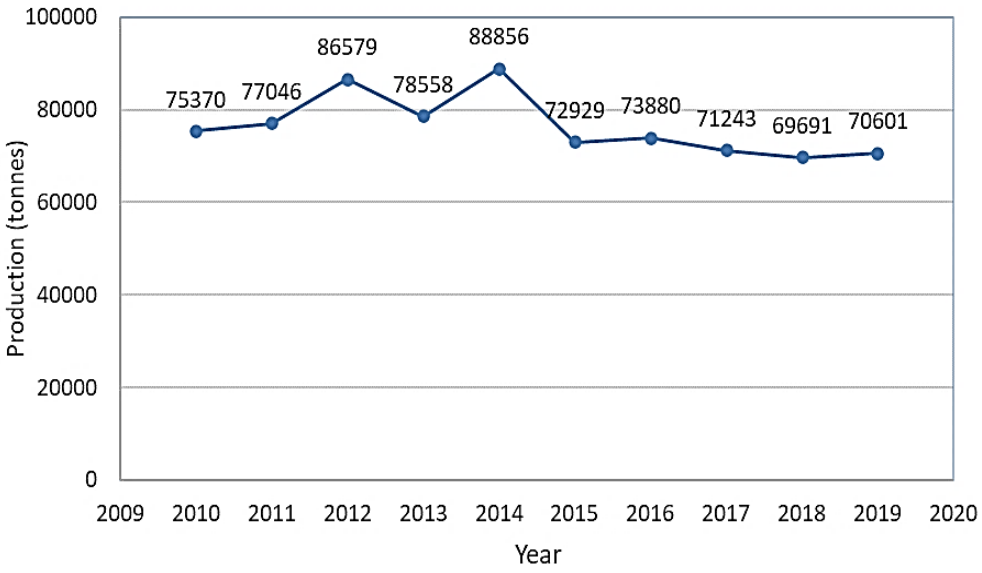

Figure 2.1. Cephalopods catch in Malaysia (tonnes) (FAO,2009-2021)

2.2 Anatomy of Cephalopods

Cephalopods are among the most complex invertebrates. Squid belongs to phylum Mollusca, class of Cephalopoda, a subclass of *Coleoidea*, order of *Teuthoidea*, a family of *Loliginidae* and genus of *Loligo* (Anusha & Fleming, 2014). Squid consists of soft-bodied and bilaterally symmetrical, distinct head, eight-arm and two tentacles (Chavan et al., 2020). The body is covered with an outside skin-like mantle to protect the main body organs secret their internal shell (Norman & Reid, 2000). Squid is a fast swimmer and can "fly" off the water over a short distance. The water sucked into the mantle cavity and forcefully removed into a siphon rotates, allowing the squid to travel forward or backwards quickly (Wood et al., 2010). Cephalopods develop protean behaviours involving unpredictable, erratic escape behaviour like jetting and inking (Wood et al., 2010). Cephalopods have well-developed eyes and are aggressive predators (Okuzumi & Fuiji, 2000). The body is covered with skin containing pigment cells called chromatophores which can be observed in the Cephalopods behaviour through the colour changes (Okuzumi & Fuiji, 2000). Figure 2.2 showed a whole picture of Indian squid *(Loligo duvauceli)*.

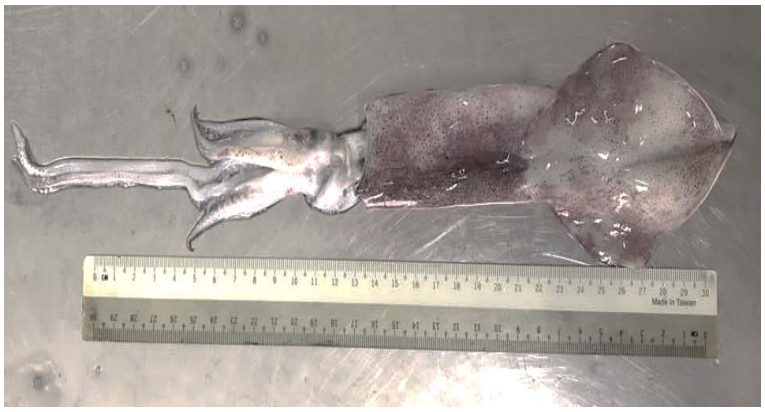

Figure 2.2. Indian Squid (*Loligo duvauceli*)

Similarly, cuttlefish is falling under the same phylum of Mollusca and the class of Cephalopoda. However, cuttlefish is gathered in order of *Sepioidea*, the family of *Sepiidae* and the genus of *Sepia* (Venkatesan & Mohamed, 2015). Unlike squid, cuttlefish consists of an internal shell identical to bones, a large W-shaped pupil, eight arms and two tentacles with suction serrulate used to catch prey (Mäthger et al., 2013). The primary function of an internal shell serves as a rigid buoyancy tank composed of a strong compressive force capable of protecting against hydrostatic pressure (Mäthger et al., 2013). Cuttlefish also has the same ink sac as squid and octopus comprising melanin compounds classified as a natural black pigment in a viscous colourless medium (Russo et al., 2003). Figure 2.3 showed a whole picture of cuttlefish (*Sepia Officinalis*).

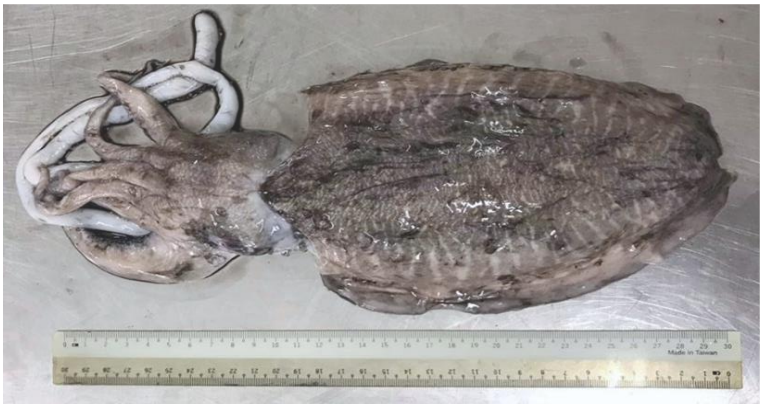

Figure 2.3. Cuttlefish (*Sepia Officinalis*)

2.3 Sources and utilisation of Cephalopods byproducts

Squids are distributed in several forms, including fresh, frozen, canned, dried, salted, and smoked (Suklim et al., 2003). The increased exploitation of squid contributes to a considerable amount of byproducts for up to 60% of the whole weight, defined as leftover after processing (Ezquerra-Brauer & Aubourg, 2019). Typically, these processing byproducts are utilised to produce low market-value products such as animal feed, silage, or direct disposal (Martínez-Alvarez et al., 2015). The byproducts are defined as leftover raw material after processing the main product, either edible or not. When recycled and processed, some of the byproducts turn out to be appropriate for human consumption, such as protein hydrolysates from fish processing co-products (He et al., 2013). According to USDA (2016), squid processing byproducts constitute about 52% of animal weight (Table 2.2). The main edible portion is the cone-shaped body trunk (mantle) which includes around 48% of the whole body weight. The byproducts are fins, head, tentacles, skin and viscera.

Instead of wasting these protein-rich raw materials, improving the utilisation has become hotspot research. Great attention has been accorded to utilising squid byproducts as sources of bioactive compounds that could be used for human nutrition or non-nutritional purposes (Ezquerra-Brauer & Aubourg, 2019). Marine byproducts are rich in biofunctional components such as vitamins, minerals, polysaccharides, polyunsaturated fatty acids (PUFA), enzymes, collagen, gelatine and bioactive peptides (Le Gouic et al., 2019). Previous research on fish protein hydrolysates (FPH) production presently focused on maximising the industrial value of fish wastes since their bioactive peptides hold tremendous interest (Halim et al., 2016). The draw has grown in investigating the possible means of using underutilised resources and industrial wastes to utilise high-quality protein sources better and increase commercial

food products economic value. Previous studies reported the potential added-value compounds from squid byproducts that are summarised in Table 2.2.

Table 2.1
Composition of squid processing and utilisation (USDA, 2016)

	Body parts	Composition (%)
Byproducts	Mantle	48
	Head and tentacles	25
	Fin	15
	Visceral	8
	Skin	3
	Pen	1

The primary component, including viscera, pens, and skins, are estimated for over 40% of the overall body weight of squid, which may be hydrolysed to produce peptides and free amino acids (Wang et al., 2019). The viscera, head and eyes may be processed as food additives and fertilisers (Kim, 2013). Furthermore, squid skin is an excellent source of collagen (Veeruraj et al., 2015). Because of their binding abilities, collagen-based biomaterials are commonly employed. Squid collagen in the production of biofilms with chitosan is a potential plasticizer (Uriarte-Montoya et al., 2010). The squid pen is a rich source of β-chitin with low amounts of inorganic compounds (Wang & Liang, 2017). Also, squid pens have been identified as a source of chitin, a precursor of chitosan that has been used in medicine and as a thickener in food preparation (Wang & Liang, 2017). The melanin component has earned attention among squid byproducts and has been used in comparative melanogenesis studies. Melanogenesis is the reaction of melanin pigment formed by the dark colour of ink produced by tyrosinase, dopachrome rearranging enzyme and peroxidase (Ahamed et

al., 2018). Due to the popularity of squid ink as a flavouring agent, the awareness of this material slowly increased (Derby, 2014).

Table 2.2
Potential added-value compounds from squid byproducts

Byproducts	Added-value compounds	References
Arms, fins, head, tentacles, skin	Collagen and gelatine	Fu et al. (2013), Deng et al. (2015), Suárez-Jiménez et al. (2015), Chan-Higuera et al. (2016)
Head, fins, tentacles, skin	Bio-plasticiser	Giménez et al. (2009), Arias-Moscoso et al. (2011)
Cartilage, pen	Chitin	Jung and Zhao (2014), Li et al. (2016)
Digestive gland, testis, arms, integument	Eicosapentaenoic and docosahexaenoic acids	Saito et al. (2014)
Arms, head, mouth, pens, viscera, pens, tentacles	Food additive	Ramírez-Suárez et al. (2012)
Skin	Pigments as preservative agents	Aubourg et al. (2016), Ezquerra-Brauer et al. (2018)
Proteases	Hepatopancreas	Osuna-Ruiz et al. (2010), Márquez-Ríos et al. (2016)
Fins	Protein concentrated	Márquez-Álvarez et al. (2015)
Arms, head, tentacles, viscera	Replacement fish meal	Toyes-Vargas et al. (2017)
Arms, fins, head, ink, skin, tentacles	Bioactive compounds	Chan-Higuera et al. (2016), Shangyun et al. (2016),
Ink	Functional foods	Zhou et al. (2015), Dong et al. (2017)
Ink	Wound-healing sponge	Huang et al. (2018)
Ink	Antioxidant, anti-inflammation	Fahmy and Soliman (2013), Guo et al. (2014)
Ink	Anti-neoplastic	Soliman et al. (2015)
Ink	Antihypertensive	Kim et al. (2003)

2.3.1 Added-value compounds of Cephalopods ink

A recent medical study suggested that Cephalopod ink is a multifunctional bioactive marine drug with anticancer, antioxidant and anticoagulant properties. The ink is a significant source of tyrosinase, an enzyme that acts as a barrier to microbial activity and is employed as an antimicrobial agent against *Staphylococcus aureus* and biofilm bacteria (Singh et al., 2020). Senan (2015) investigated the antibacterial activity of a methanolic extract of cuttlefish (*Sepia pharaonic)* ink against pathogenic bacterial strains. According to Karim et al. (2016), bacteria growth is extremely slow in squids treated with 0.25% squid ink when stored at 4°C. The inks of squid (*Loligo duvauceli*) and cuttlefish (*Sepioteuthis lessoniana*) have been discovered to have a potent antibacterial effect against biofilm-causing pathogens (Nicomrat & Tharajak, 2015). Besides, sepia ink is employed as a coagulant for internal haemorrhages in ancient Chinese medicine, particularly gynaecology (Zhang et al., 2014). The squid ink polysaccharide-chitosan sponge (SIP-CS) study on the haemostatic properties suggests that natural squid ink polysaccharides (SIPs) may exhibit procoagulating activity (Huang et al., 2018). Sepia ink can reduce kidney dysfunction caused by bile duct ligation and the invasive of pulmonary aspergillosis (Soliman et al., 2014).

The antioxidant characteristics of squid and cuttlefish ink exist in the melanin and melanin-free fractions of the ink (Derby, 2014). Liu et al. (2011) reported the excellent growth performance, antioxidant activities and immunity of broiler chickens affected by squid ink. Vate and Benjakul (2013) studied the influence of splendid squid *Loligo formosana* melanin-free ink on the ability of antioxidants, in which different tests are performed *in vitro* tests including DPPH radical activity for scavenging, ABTS radical activity to scavenge, ferric reducing antioxidant power (FRAP) and chelating activity towards Fe^{2+} that found to be positive. Melanin-free ink

of squids (*Loligo formosana*) may prevent the oxidation of lipids in surimi gels during cooled storage (Vate et al., 2014). Wang et al. (2014) discovered the antioxidant properties of melanin-Fe squid ink while utilising it as a therapy for iron deficiency anaemia (IDA) in rats.

2.4 Cephalopods ink

The ink sac is present when it comes to hatching, and even a tiny and young Cephalopod may generate and release inks (Derby, 2014). Squid and cuttlefish defend themselves by fast jetting escape movement, colouration changes that can be cryptic, disruptive or shocking, autotomy of weapon, venom, and inking (Derby, 2007). The ink appears like a smokescreen when Cephalopods escape and draw the attention of predators (Bush & Robison, 2007) or may be used as a warning signal to others (Wood et al., 2008). Cephalopods release dark cloudy ink from their sacs as an adaptive response when confronted with danger and physical threats (Liu et al., 2011). The cloud of inks forms is believed as an effective screen behind when animals escape, and the alkaloids found in ink may paralyse the olfactory senses of a predator (Jereb & Roper, 2010). Each Cephalopod species produces slightly different coloured ink, as the octopus creating black ink, the squid producing blue-black ink, and the cuttlefish producing brown ink (Derby, 2014).

Cephalopod ink comprises mucus, so it does not quickly disperse in water but retains a concentrated ink layer made of two gland secretions (Derby, 2014). The ink gland in the ink sac is blackened by melanin (Derby, 2014). The funnel organ is a mucus-producing gland located on the internal surface of the funnel and comprises two lateral pads and a median pad of the same histological structure, which the lateral pad is composed of the epithelial and sub-epithelial layers (Ali et al., 2017). The

epithelial layer shows a single layer of columnar cells, while the subepithelial layer consists of many secretory cells (Ali et al., 2017). The ink sac lumen stores the ink gland, which immediately secretes ink to the hindgut via a duct near the anus (Derby, 2014). Their muscular walls and sphincters are also highly specialised in controlling the ejection of ink from the sac (Derby, 2014). However, it seems the ink sac and the funnel organ are innervated by distinct natural routes and may be controlled separately (Derby, 2014). Some of these organs have to be defined by the innervation and significance, including the shape of the generated ink, such as the relative secretion amount of these two organs, which is influenced by the cephalopods' perception of danger (Derby, 2014). Cephalopod ink has existed for millennia in several commercial and industrial uses in food components (Derby, 2014). Other applications that used calamari ink is model edible particulate ingredients for the food industry (Baalousha et al., 2011).

2.4.1 Constituents of Cephalopods ink

The melanin of Cephalopods has gained the most interest so far, likely because it offers a distinctive black colour. Moreover, Cephalopods have been regarded for a long time as a convenient model system for investigating melanogenesis (Derby, 2014). While the glutamate, nitric oxide and cGMP help regulate ink production by activating the pathway to increase the tyrosinase and production of melanin pigments (Derby, 2014). These ink glands contain various melanogenic enzymes, including tyrosinases, peroxidases, and dopachrome-rearranging enzymes (Derby, 2014). Peroxidase is involved in the later stages of melanin biosynthesis and facilitates eumelanin polymers from monomers (Derby, 2014). Melanin is a complex amino acid biopolymer that usually occurs in two forms, eumelanin and pheomelanin (Derby, 2014). Eumelanin is commonly present in Cephalopods ink which likely the structure

and synthesis of melanin are better described for cuttlefish, particularly *Sepia Officinalis* (Derby, 2014).

Each ink sac in *Sepia* produces 1 g of eumelanin and makes up around 15% of the total wet ink weight and proteins, another 5-8% of the *Sepia* wet ink (Derby, 2014). Eumelanin is an oligomeric structure arranged into particles formed from 75% of DHICA (5,6-dihydroxyindole 2-carboxylic acid)-derived units and only 20% DHI (5,6-dihydroxyindole)-derived units (Figure 2.4). All components are involved in melanin production during melanogenesis, such as hydrogen peroxide, enzymes, initial products, and intermediates (Neifar et al., 2009).

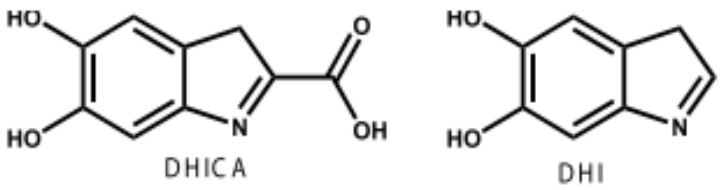

Figure 2.4. *Sepia* melanin is a copolymer of eumelanin (Magarelli et al., 2010)

2.4.2 Chemical composition of Cephalopods ink

Sepia ink is mainly composed of black melanin, which confers its distinctive black colour, viscosity and density (Neifar et al., 2009). It is also noted the melanin contains peptide fragments called melanoproteins which generally indicated the approximate composition of the ink varies from species to species (Neifar et al., 2009). Over the past decade, the squid (*Ommastrephes bartrami*) melanin is classified as melanoprotein, consisting of a significant melanin pigment, protein, carbohydrate, and traced amounts of amino acids, such as glycine, aspartic acid and glutamic acid (Mimura et al., 1982). Squid ink contains the pigment melanin, proteins, lipids, glycosaminoglycans, and mucopolysaccharides (USDA, 2016). The two layers structural melanin particle comprises high-density melanin and an organic compound

lysine externally low-density (Nair et al., 2011). The predominant component of Cephalopod ink is melanin and polysaccharides complexes containing free amino acids (FAAs) in the highest concentration, such as taurine, glutamic acid, aspartic acid, alanine and lysine (Nair et al., 2011).

Indeed, the composition of proteins, minerals, fats and carbohydrates are present in dried squid ink samples (Lopez-González et al., 2003). Melanin is a melanoprotein containing 10-15% protein composed of essential and non-essential amino acids in squid ink (Lestari et al., 2019). Shakthi et al. (2016) reported the crude ink and melanin free ink cuttlefish (*Sepia prabahari*) are made from protein (1.16–1.49 mg/ml), lipid (0.25–1.42 mg/ml), carbohydrates (0.01–1.14 mg/ml), ash (0.4 & 0.1%) and moisture is (93.33%), respectively. The higher protein and carbohydrates in squid ink are found via estimated through the Folin-Ciocalteu reagent and Anthrone method (Nisha & Suja, 2018). A similar report suggested that crude ink extracts of squid (*Loligo duvauceli*) possess a high amount of protein, followed by the mineral, moisture and fat content (Fatimah Zaharah & Rabeta, 2017). Ganesan et al. (2017) reported the cuttlefish inks, namely *Sepia pharaonis, Sepia prabahari* and *Sepia ramanias* consists of protein content in the range of 12.59% and 16.47%. The ash and carbohydrates content is less than 3%, while the fat content estimated for cuttlefish ink varies from 0.19-0.21%. In particular, the squid ink is considered high in protein due to the major constituent is 80% of myofibrillar protein, 12-20% of myoplastic protein and 2-3% of myostroma protein. Besides, the mineral content presented differs from species, maturity, biological stage, habitat, species, and mineral concentration in seawater (Okuzumi & Fuiji, 2000). The common mineral element reported in Cephalopods ink is calcium (Takaya et al., 1994; Chen, 2000; Zheng et al., 2002; Liu et al., 2011).

Liu et al. (2011) demonstrated the cuttlefish (*Sepialla maindroni*) ink consists of 16 amino acids, high acid content, and 9 fatty acids, which approximately (1.34%) constituted by unsaturated (43.4%) and saturated (56.6%) ones. Neifar et al. (2009) worked on cuttlefish (*Sepia officinalis*) specimens, expressed that melanin consists of carbohydrates, polymer and proteins, which indicated the crude ink is higher in protein. Minerals in melanin substances generally maintain normal metabolic and physiological activities and are essential elements of enzymes and structural proteins. The squid Boreo Pacific gonate (*Gonatopsis borealis*) ink is discovered to contain high amounts of protein compared to other squid species, such as neon flying (*Ommastreohes bartrami*) and boreal club-hook (*Onychoteuthis borealijaponica*) (Shirai et al., 1997). All these inks comprise high amounts of carbohydrate, homarine, glycine, betaine and are rich in taurine and hydroxyproline, consisting of trimethylamine oxide (Shirai et al., 1997).

Matsue et al. (1995) proposed the squid ink is high in molecular weight sugars. Derby (2014) reported that Cephalopods ink is rich in dissolved free amino acids. Six species, including squid (*Doryteuthis Loligo pealeii* and *Lolliguncula brevis*), octopus (*Vulgaris* and *Bimaculoides*) and cuttlefish (*Sepia officinalis*) consist of total dissolved free amino acids ranging from 0.5-132mM (Derby, 2014). The ink constitutes 50% of taurine and glutamate in 3-7% from five out of six species. The total dissolved free amino acids are approximately 0-2%, containing 0-2.9 mM of tyrosinase (Derby, 2014).

2.5 Enzymatic hydrolysis of Cephalopods ink

Enzymatic hydrolysis increased the number of cleaved peptides and formed smaller peptides that may change the functional properties of the protein and enhance

the sensory properties (Yang et al., 2020). Enzymatic hydrolysis requires a small amount of easily deactivated enzymes, mild temperature conditions, and pH during hydrolysis (Herpandi et al., 2011). The enzymes should be food-grade and non-pathogenic if derived from the microbial origin (Herpandi et al., 2011). The proteases responsible for protein hydrolysis are endopeptidases or endopeptidases that cleave peptides within the protein chain, while exopeptidases are cleave peptides from the terminal ends (Benjakul et al., 2014). Commonly, the enzymes used for hydrolysis in the fish protein found in animals (pepsin, trypsin, pancreatic, and chymosin), plants (papain, bromelain, and ficin) and mainly of microbial origin (glucoamylase, α-amylase, and pectinase) (Herpandi et al., 2011). Their "specificity" of an enzyme enables them to identify a particular substrate they intended to target. They are helpful in industrial operations and are able to catalyse the reaction between certain substances (Patel et al., 2017).

Enzyme selection is crucial for protein hydrolysis because different enzymes have different specificity, which is particularly associated with functional properties (Korhonen & Pihlanto, 2006). Compared to animal or plant origin enzymes, microbial enzymes provide several benefits, such as more versatile catalytic activity and stability at greater pH and high temperature (Herpandi et al., 2011). Plant and animal proteases such as papain, bromelain, ficin, and pepsin proteases have also been used for hydrolysis (Himonides et al., 2011). From a technical and economic point of view, microbial enzymes such as Alcalase® are one of the most effective enzymes to produce fish protein hydrolysate (FPH) attributed to their ability to achieve a high degree of hydrolysis in a relatively short period and under mild conditions, possess an increased preference for hydrophobic amino acids that eventually develop low

bitterness yield products with high nutrient content and good functional properties (Amiza et al., 2011; Binsi & Zynudheen, 2019).

2.6 Toxicity studies

According to The General Food Law (Commission of the European Communities, 2002), the safety of any newly developed products should be assessed before market introduction and focus on the contrasts with similar conventional foodstuffs shall be evaluated with a documented safe use report. The reasonable evaluation of the protein hydrolysates safety and their fractions could be achieved by comparing their predicted intake of amino acids with those amounts which should reasonably be ingested under normal consumption conditions for a balanced and varied diet or would otherwise pose a potential risk to consumers (Schaafsma, 2009). Given the possible adverse consequences of peptides-enriched formulations, every peptide-based substance must be tested for further clinical studies (Barkia et al., 2020). The comprehensive food safety tests, including the histamine content, microbiological and allergy tests, are essential to ensure the protein hydrolysates comply with food safety standards (Bhandari et al., 2020). Ideia et al. (2019) suggested additional *in vivo* studies to validate the results of fish hydrolysates health-sustaining compounds. The toxicity study provides critical fundamental information on safe acute doses, the possible acute effects and target toxicity organs prior to the repeated dose toxicity study.

2.6.1 Acute oral toxicity of Cephalopods ink

Acute oral toxicity refers to the adverse consequences of a single or several doses administered orally within 24 hours (OECD, 2008). According to the literature, the oral route is the most effective and extensively used for assessing acute toxicity

(Syahmi et al., 2010). Although the absorption is slow, this technique is less costly and less painful to the animal (Syahmi et al., 2010). The animals would have fasted prior dosed with food and other substances in the digestive system that may affect the responses (Syahmi et al., 2010). The LD_{50} (median lethal dose) test to estimate the dose of a test substance that causes 50% mortality in a particular animal species (OECD, 2008). Although its primary endpoint is death, the acute non-lethal effect may be a toxicity sign depending on the tested chemical (Maheshwari & Shaikh, 2016).

The up-and-down (UPD) method for acute oral toxicity involves assessing young adult animals in a staircase mode one at a time (Rispin et al., 2002). The result of the preceding animal determines the next dose level and whether the requirements for passing the test have been met (Rispin et al., 2002). If the previous animal survived, the subsequent dose increases; if the last animal died, the following dose decreases (Rispin et al., 2002). The method enables the trials to converge on the LD_{50} area, which corresponds to the median response of the normal dose-response curve in the log (Rispin et al., 2002). The methodology is tested in 1987 in laboratory experiments (ICCVAM, 2001). Lipnick et al. (1995) stated the findings showed the UPD predicted both the LD_{50} of these materials and the conventional test. The concept of the up and down acute toxicity study as a standard test method has been acknowledged as an alternative to the OECD guideline 401 (1987) by the American Society for Testing and Material (ASTM) in 1987 and (revised in 1998) and the OECD guideline 425 in 1998.

2.7 Organoleptic properties

2.7.1 Flavour improvement approaches

A practical approach to reducing the unfavourable fishy odour and bitterness of protein hydrolysates without affecting the quality is essential. Although different methods have been implemented, each of them has limitations.

2.7.1(a) Plastein reaction

Plastein reaction is a protease-catalysed response correlated with plastein production. A plastic gel-like, insoluble and protein-like material are produced from peptide mixtures when a high concentration of protein hydrolysates/peptides is incubated with particular proteases under optimal circumstances named 'plastein' (Idowu & Benjakul, 2019). Plastein reactions are an efficient method for minimising bitterness without losing the structure and functionality of protein hydrolysates (Fu et al., 2019). The glutamic acid diethyl ester response resulted in a lower proportion of hydrophobic amino acids, which enhanced the product flavour (Synowiecki et al., 1996). Pepsin-treated soy protein hydrolysates are displayed reduced in bitterness, which indicated the bitter dipeptides played the leading role in developing plastein chains by condensation (Fujimaki et al., 1970). The rising studies demonstrated the plastein reaction had improved the bioactivity of peptides. However, plastein formation during transpeptidation and condensation requires excessive enzymes during the process, leading to an altered peptide series and affecting bioactivity (Udenigwe & Rajendran, 2016).

2.7.1(b) Encapsulation

Several methods for encapsulating protein hydrolysates have been discovered such as spray cooling, spray drying, coacervation and liposomes (Fu et al., 2019).

Spray drying is widely used to trap peptides in protein and polysaccharide-based carriers due to comparatively low production costs and good product stability (Fu et al., 2019). Nevertheless, high temperatures used during spray drying may cause protein carrier denaturation and peptide structure modification due to the Maillard reaction, where reducing sugar is present in the solution (Mohan et al., 2015). Meanwhile, liposomes are amphipathic colloidal vesicles consisting of hydrophilic, lipophilic, and amphiphilic areas suitable for peptide entrapment. Liposome encapsulation carriers may achieve greater encapsulation capacity (Allen & Cullis, 2013). The chitosan-coated liposome method utilising the milk fat globule membrane has been established to encapsulate Atlantic salmon protein hydrolysates with a high encapsulation efficiency (EE) (Li et al., 2015). However, the consequence of encapsulating on the bioactivity and bioavailability of peptides *in vivo* remains to be further investigated (Fu et al., 2019).

2.7.1(c) Exopeptidases

Exopeptidases may selectively cleave terminal peptides and release free amino acids from bitter peptides (Fu et al., 2019). It is classified as either aminopeptidase or carboxypeptidase depending on the amino acid at the N- or C-terminal (Raksakulthai & Haard, 2003). Exopeptidases have been reported to improve the protein hydrolysate flavour derived from animal byproducts (Fu et al., 2018). The simultaneous use of endopeptidase and exopeptidase can ensure effective hydrolysis and cleavage of hydrophobic amino acids. Consequently, protein hydrolysates have less bitterness and sustain the amino acid composition (Cheung & Li-Chan, 2014). The additional exopeptidases may further cleave amino acids pertinent to bioactive peptides, resulting in bioactivity and functional properties changes (Cheung et al., 2015). The controlled enzymatic debittering involving various forms of exopeptidases are a commonly used

and economical process (Fu et al., 2019). However, this process must be optimised to eradicate bitterness for each application.

2.7.1(d) Maillard reaction

The Maillard reaction is well-known as a non-enzymatic browning pathway that occurs between carbonyl groups and amine groups (Normah & Noorasma, 2018). Maillard reaction products (MRPs) formed naturally in food during heat treatment by reducing sugars. Available amino acids may interact and modify the significant aspects of food, such as colour, flavour, and stability (Luo et al., 2014). Furthermore, the Maillard reaction pathway and mechanisms are among the most complex systems and produce many intermediate products besides melanoidins, such as aldehydes, ketones, and heterocyclic compounds (Jiang et al., 2018). Nevertheless, the Maillard reaction is notoriously difficult to control. The complexity of the Maillard reaction lies in the flavour formation influencing factors including temperature, pH, time, water activity, reaction media, amino acid, or protein hydrolysates and sugar (Taylor & Linforth, 2010). These factors have led to a change in reaction rate, pathways and end-product (Taylor & Linforth, 2010). The varieties of reactants in Maillard reaction generally affect the flavour profile, whereas the other conditions determine the kinetics (Yu et al., 2017). The Maillard reaction rate is inversely related to the size of the sugar molecule. Pentoses like xylose and ribose respond quicker than hexoses (Ye et al., 2017).

The food industry widely implements Maillard reaction technology to improve appearance, similar aroma, and taste characteristics of heat-treated products such as meat, chocolate, coffee, caramel, popcorn, and bread (Taylor & Linforth, 2010). Also, the Maillard reaction plays an essential role in the food chemical process to develop

tasty flavour by carbonyl of sugar-reducing and amino acid or peptide to produce a unique flavour in roasted meat and baked bread (Damodaran et al., 2007). The past few years, the increased study showed the Maillard reaction products (MRPs) derived from food protein peptides contribute to the flavour, colour, and bioactive properties (Karnjanapratum et al., 2016; Song et al., 2017; Yu et al., 2018; Fu et al., 2019). The flavour of MRPs mainly relies on different types of reducing sugar and amino acid Maillard, such as producing meat aroma synthesis or barbecue shrimp essence (Luo et al., 2014). Even though the Maillard response is valuable to enhance the flavour of peptides, it is vital to note that the Maillard reaction may alter the structure of peptides, which might result in their changed bioactivity (Fu et al., 2019). To some extent, the Maillard reaction is provided with an upgradable process that could assist in debittering and fishy flavour removal than other treatments.

2.7.2 Preparation of Maillard Reaction Products (MRPs)

The MRPs can be prepared through wet or dry heating treatments (Fu et al., 2019). The dry heating process starts with the combination of sugar and peptides, followed by heat treatment at a specific temperature and relative humidity (Kato et al., 1993). The Maillard reaction in the dry condition is limited due to the lengthy process and irregular exposure of raw materials (Qi et al., 2010). On the other hand, the wet heating process comprises a peptide-sugar mixture in aqueous-based solutions heated at a certain temperature and typically employed in altering peptides via the Maillard reaction (O'Mahony et al., 2017). The Maillard reaction changes to food protein peptides have a vital impact on their flavour and bioactivity. The sequence, length and composition of peptides are also included (Van Lancker et al., 2011). The glycation of proteins induced by sugar conjugation via the Maillard reaction is widely