Heilbrigðisvísindasvið

Novel protein sources from fish processing side streams and underutilised species for human consumption

Hang Thi Nguyen

Thesis for the degree of Philosophiae Doctor

April 2023

HÁSKÓLI ÍSLANDS

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Ný prótein til manneldis úr hliðarstraumum fiskvinnslu og vannýttum fisktegundum

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Ágrip

Talsvert magn af uppsjávarfiski og fjölbreyttir hliðarstraumar sem falla til við fiskvinnslu fara í framleiðslu á fiskmjöli og lýsi sem nýtist í fóðurframleiðslu. Aukin eftirspurn síðustu ára eftir sjávarafurðum og fiskmjöli til aukins fiskeldis hefur hins vegar leitt til ofnýtingar fiskistofna. Því hafa rannsóknir og nýsköpun undanfarna áratugi beinst að því að finna önnur prótein til fóðurframleiðslu, sem hefur dregið úr eftirspurn á fiskmjöli. Samhliða þessari þróun hefur áhugi á að nýta uppsjávartegundir og hliðarstrauma frá fiskvinnslu til framleiðslu afurða sem eru ætlaðar til manneldis eða sérsniðis fóðurs aukist til muna, sem mun auka verðmætasköpun og draga úr umhverfisáhrifum innan sjávariðnaðarins.

Heildarmarkmið rannsóknarinnar var að meta möguleika á bættri nýtingu á hliðarstraumum frá vinnslu Tra catfish (Pangasius) í Víetnam annars vegar og hins vegar að rannsaka ferlastrauma í íslenskri fiskmjölsvinnslu meðal annars frá vinnslu á mögrum fiski (kolmunna) og feitum fiski (makríl og síld). Með endurbættri kælitækni um borð og í öllu framleiðsluferlinu fæst nú hágæða hráefni og um leið eru prótein- og fituríkir straumar frá vinnslunni af hærri gæðum og hentar því sem hráefni í vinnslu afurða til manneldis. Þó nokkrar rannsóknir hafa verið gerðar á nýtingu próteina úr hliðarstraumum við vinnslu uppsjávartegunda, en lítil þekking er fyrir hendi um breytingar á efnafræðilegum eiginleikum próteina við vinnsluna. Almennt eru mismunandi hliðarstraumar sameinaðir við vinnslu í stað þess að halda þeim aðskildum, sem leiðir til aukinnar hættu á krossmengun milli dýrmæts vöðvavefs og óæskilegum efnum svo sem örverum og blóði. Ef vinnslustraumunum er haldið aðskildum er hægt að nýta sérkenni hvers hliðarstraums um sig, og samtímis myndast tækifæri á vinnslu sérsniðinna og verðmætari prótein- og lýsisafurða.

Við vinnslu á Pangasius í Víetnam fellur árlega til um ein milljón tonna af hliðarstraumum og ef þetta hráefni væri unnið sérstaklega væri hægt að auka verðmæti framleiðslunnar umtalsvert.

Til að nýta próteinríka hliðarstrauma frá vinnslu Pangasius var ráðist í að rannsaka tvær nýstárlegar nýtingaraðferðir, þ.e. annars vegar framleiðslu á fiskpróteinísólati (FPI) með pH-shift aðferðinni og hins vegar framleiðslu á fiskpróteini með ensímhvöttu vatnsrofi (FPH) beint frá FPI. Allar FPI-afurðirnar voru með ákjósanlega amínósýrusamsetningu, sem gefur til kynna góðan möguleika á nýtingu þeirra sem innihaldsefni í gæðamatvæli og/eða bætiefni. FPI-efnin höfðu hærra próteininnihald og lægra fitu- og öskuinnihald en surimi framleitt úr sama hráefni. FPH-efnin reyndust hafa andoxunareiginleika sem opnar mikla möguleika á notkun þeirra sem þráarvarnarefni í matvælum.

Eiginleikar afurðanna úr hinum ýmsu hliðarstraumum, svo sem litur, andoxunarvirkni, eðliseiginleikar o.fl., voru breytilegir þó straumarnir hefðu svipaða efnasamsetningu. Þessi niðurstaða ítrekar enn nauðsyn þess að nýta hvern hliðarstraum frá flakavinnslu Pangasius sér í aðskildar vinnslur, þar sem sérhanna megi afurðir með tilliti til mismunandi eiginleika þeirra og verðmæti.

Í verkefninu var einnig ráðist í að meta gæði próteinríkra strauma í fiskmjölsvinnslu á Íslandi. Niðurstöður sýndu að saltleysanleg prótein, lífræn amín (BA) og basísk rokgjörn köfnunarefnissambönd (TVB-N), eins og trímetýlamín (TMA) og dímetýlamín (DMA), lækkuðu verulega í gegnum vinnsluna. Flest köfnunarefnissambönd sem ekki voru prótein, þar á meðal BA, TVB-N, TMA og DMA, fylgdu almennt vatnsmeiri straumunum við vinnsluna, sem leiddi til lægri gildi þessara efna í föstu straumunum (pressukökunni og soði). Annars vegar var fylgst með gæðabreytingum próteina við kolmunnavinnslu (fitulitlu hráefni), og hins vegar við vinnslu á blöndu af makríl og síld (fitumiklu hráefni). Samsetning hráefnis hafði veruleg áhrif á eiginleika vinnslustraumanna og ekki síst á lokaafurðirnar, fiskmjölin. Í báðum fiskmjölsferlunum innihélt pressukakan hátt próteininnihald og lítið af óæskilegum próteinefnasamböndum, sem gefur til kynna að nota megi þessi hráefni til að þróa verðmætari próteinafurðir en nú er gert. Að auki bentu efnasamsetning föstu straumanna (pressuköku, soð, þykkni) til þess að framleiða megi verðmætari vöru, þar á meðal til manneldis ef hver straumur er unninn sérstaklega.

Heilt á litið bendir rannsóknin til þess að þau hráefni sem í dag eru helst nýtt til fiskmjölsframleiðslu bæði í Víetnam og á Ísandi megi nýta á skynsamlegri hátt með beitingu nýrra vinnsluaðferða og bestun vinnsluferla. Þannig má framleiða verðmætari próteinafurðir til manneldis og annarra sérsniðinna afaurða úr þessu vannýttu hráefni.

Lykilorð:

Prótein, nýting, uppsjávarfiskur, fiskpróteinísolat, fiskpróteinhýdrólýsat, fiskmjöl, Pangasius, hliðarstraumar

Abstract

A significant amount of small pelagic species and side streams obtained from industrial fish processing are currently converted to fishmeal and fish oil, and primarily used in feed production. The demand for fish and seafood products and fishmeal for expansion of aquaculture has resulted in the overexploitation of fish stocks, which challenges global food security. In recent decades, research and innovation have focussed on finding alternative proteins for feed production, leading to decreased fishmeal prices. Hence, there is now an incentive to try alternative ways to process small pelagic species and industrial side streams to produce products intended for human consumption which would bring added value and reduce environmental impacts.

The overall aim of this study was to assess the potential of improving protein utilization from the side streams from Tra catfish industrial processing in Vietnam, and protein-rich streams obtained during Icelandic fishmeal processing, including from lean species (blue whiting) and fatty fish (Atlantic mackerel and Atlantic herring blend). Although several studies have been conducted on utilising proteins from industrial processing side streams and pelagic species in recent years, there is still little detailed knowledge available on the chemical properties and quantities of different side streams. Generally, the industrial side streams are combined during processing, leading to contamination of valuable muscle tissue with various enzymes and microorganisms, lipids, and blood, which promotes spoilage and makes their further uses for human consumption challenging. Therefore, looking at the possibility of procuring proteins, including isolates and hydrolysates, from each protein-rich side stream separately is necessary.

Protein recovery from pelagic fish species has been carried out using innovative methods, but because of the small size of these species, and as they are often caught when actively feeding, they are highly perishable which is reflected in the quality of the products. However, improved cooling technologies on-board and throughout the value chain can result in high-quality raw materials. Protein-rich streams from fishmeal production can then be used as human food ingredients if the processing processes are optimized and re-designed.

Vietnam's Tra catfish filleting processing industry produces about one million tons of side streams annually, which if processed separately could be utilised to maximise their practical value.

To utilise the protein-rich Tra catfish side streams for protein products for human consumption, two innovative methods were studied, including fish protein isolate (FPI) production using the pH-shift method, and fish protein

hydrolysate (FPH) production directly from the FPIs using enzymatic hydrolysis. All the FPIs had an excellent amino acid composition, indicating a good possibility of using them as food ingredients and/or supplements. The FPIs had higher protein content, and lower lipid and ash contents than commercial surimi made from the same raw material. The resulting FPHs constitute a natural source of antioxidants with great potential for food application as antioxidative additives. Although the different protein-rich side streams resulted in protein products with similar proximate chemical properties, other attributes, such as colour (between FPIs) and different antioxidative activities (among FPHs), were dependent on the side stream being processed each time. Therefore, the different side streams from Tra catfish filleting should be utilised separately, adjusting each stream towards producing a specific value-added product.

The quality of the protein-rich streams from pelagic fishmeal and fish oil processing in Iceland was determined. Overall, salt soluble proteins (SSP), biogenic amines (BA), and total basic volatile nitrogen compounds (TVB-N), such as trimethylamine (TMA) and dimethylamine (DMA), decreased significantly, likely due to heat treatment during processing. Most of the non-protein nitrogen compounds, including BA, TVB-N, TMA, and DMA, were separated into the liquid streams during processing, resulting in lower values in the solid streams (the press cake and sludge). The fish species processed, including blue whiting (BW), and Atlantic mackerel and herring blend (MHB), and the composition of the initial raw materials also affected the characteristics of each processing stream, as well as the final fishmeal. In both fishmeal processes studied, the press cake had high protein content, and low content of unwanted non-protein nitrogen compounds, indicating a potential of using this material for the development of higher-value protein products. In addition, different chemical characteristics of the different solid streams (press cake, sludge, concentrate) indicated a promising potential for producing a wide range of products, including high-value products for human consumption if these streams were to be processed separately.

Overall, the study indicated that the current raw materials for fishmeal processing in Vietnam (Tra catfish side streams) and Iceland (small pelagic species and side streams) could be utilized by applying new methods, and/or optimising the current processing processes to obtain higher-value protein ingredients, which could be used for human consumption.

Keywords:

Protein, utilisation, pelagic species, fish protein isolate, fish protein hydrolysate, fishmeal, Tra catfish, side streams

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Contents

List of abbreviations

ACO	Abdominal cut-offs
ACO-FPH	Fish protein hydrolysate produced from the ACO-FPI
ACO-FPI	Fish protein isolate produced from the abdominal cut-off
BW	Blue whiting
BW-FM	Blue whiting fishmeal
DH	Degree of hydrolysis
DM	Dark muscle
DMA	Dimethylamine
DM-FPH	Fish protein hydrolysate produced from the DM-FPI
DM-FPI	Fish protein isolate produced from the dark muscle
DPPH	2,2-diphenyl-1-picrylhydrazyl
DPPH-RSA	2,2-diphenyl-1-picrylhydrazyl-radical scavenging activity
Equiv.	Equivalent
FFDM	Fat-free dry matter
FPH	Fish protein hydrolysate
FPI	Fish protein isolate
FPI-DMR	Dry matter recovery of the fish protein isolate compared to the initial raw material
FPI-PR	Protein recovery of the fish protein isolate compared to the initial raw material
HBB	Head and backbone blend
HBB-FPH	Fish protein hydrolysate produced from the HBB-FPI
HBB-FPI	Fish protein isolate produced from the head and backbone blend
HM-PP	Protein powder for human consumption
LC-MS	Liquid chromatography-mass spectrometry
LC PUFA	Long-chain polyunsaturated fatty acids
MHB	Mackerel and herring blend
PER	Protein extractable recovery
PES	Protein extraction solution
PET-PP	Protein powder for pet food
pI	Isoelectric protein point

PP	Protein powder
PR	Protein recovery of the fish protein hydrolysate compared to the initial substrate
PUFA	Polyunsaturated fatty acids
SDS-PAGE	Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis
SSP	Salt soluble protein
TC1-FM	Tra catfish 1 fishmeal
TC2-FM	Tra catfish 2 fishmeal
TRPC	Total reducing power capacity
TMA	Trimethylamine
TVB-N	Total volatile base nitrogen
Tuna-FM	Tuna fishmeal
ww	Wet weight

List of figures

List of tables

List of original papers

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-IV):

I. Nguyen, H. T., Bao, H. N. D., Dang, H. T. T., Tómasson, T., Arason, S., & Gudjónsdóttir, M. (2023). Value adding potential of side streams from industrial filleting of Tra catfish (*Pangasius hypophthalmus*). Submitted to the Journal of Food Processing and Preservation.

II. Nguyen, H. T., Bao, H. N. D., Dang, H. T. T., Tómasson, T., Arason, S., & Gudjónsdóttir, M. (2022). Protein recovery of Tra catfish (*Pangasius hypophthalmus*) protein-rich side streams by the pH-shift method. *Foods*, 11(11), 1531. https://doi.org/10.3390/foods11111531.

III. Nguyen, H. T., Bao, H. N. D., Dang, H. T. T., Tómasson, T., Arason, S., & Gudjónsdóttir, M. (2022). Protein characteristics and bioactivity of fish protein hydrolysates from Tra catfish (*Pangasius hypophthalmus*) side stream isolates. *Foods,* 11(24), 4102. https://doi.org/10.3390/foods11244102.

IV. Nguyen, H. T., Hilmarsdóttir, G. S., Tómasson, T., Arason, S., & Gudjónsdóttir, M. (2022). Changes in protein and non-protein nitrogen compounds during fishmeal processing—Identification of unoptimized processing steps. *Processes*, 10(4), 621. https://doi.org/10.3390/pr10040621.

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Declaration of contribution

Paper I. The PhD candidate and the co-authors designed the experiment. The PhD candidate carried out the sampling and analysis, wrote the original draft, and prepared the manuscript for publication in collaboration with co-authors.

Paper II. The PhD candidate and the co-authors designed the experiment. The PhD candidate carried out the sampling and analysis, wrote the original draft, and prepared the manuscript for publication in collaboration with co-authors.

Paper III. The PhD candidate and the co-authors designed the experiment. The PhD candidate carried out the sampling and analysis, wrote the original draft, and prepared the manuscript for publication in collaboration with co-authors.

Paper IV. The PhD candidate and the co-authors designed the experiment and wrote the manuscript in collaboration. The PhD candidate carried out the sampling, analysed the data, wrote the original draft, and prepared the manuscript for publication in collaboration with co-authors.

1 Introduction

Fish is important for food security and nutrition as it is rich in protein with essential amino acids, long-chain polyunsaturated fatty acids (LC PUFA), vitamins, and minerals (FAO, 2022; Henchion et al., 2017; Tacon et al., 2020). In recent decades, increasing income, urbanisation, and a better understanding of the potential health benefits associated with fish consumption have led to an increased demand for fish and seafood products (Henchion et al., 2017; Shahidi and Ambigaipalan, 2015). In 2019, fish provided about 17% of the total animal protein consumption, and 7% of the total protein consumption worldwide. Globally, the average per capita fish consumption was 20.5 kg in 2020, and had more than doubled since the 1960s (FAO, 2022). Most fish is processed and used directly and/or indirectly (i.e. as fishmeal and fish oil as the ingredients of aquafeed) for human consumption (Béné et al., 2015; Koehn et al., 2022) (**Figure 1**).

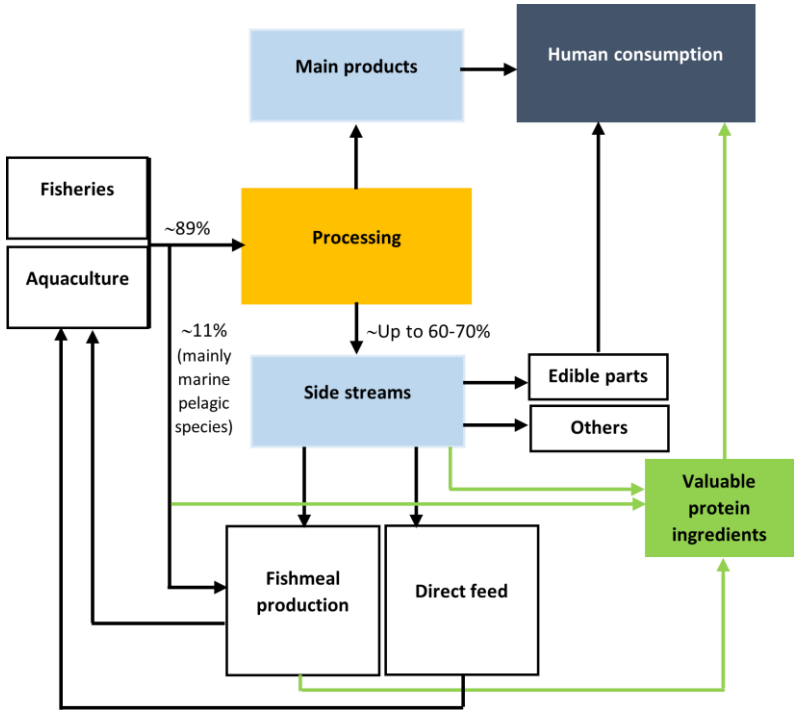

Figure 1. Overview of the value chain of fish production and uses. Black lines: current common practice. Green lines: the potential of increased protein sources for human consumption by utilising raw materials in more sustainable ways.

Most fish is consumed fresh and chilled (44%), followed by frozen (35%), prepared and preserved (11%), or cured (10%) (FAO, 2022). Most fish processing follows these basic steps: stunning (for farmed fish), bleeding, scaling, heading, gutting, removing fins, washing, filleting or portioning, packaging, labelling, and distribution (Borderías and Sánchez-Alonso, 2011; Ghaly et al., 2013). Side streams during fish processing are considered to be all processing streams, edible or inedible, that remain once the main products, such as fillets, or whole and gutted fish have been produced (Adegoke and Tahergorabi, 2021). Side streams usually include heads, backbones, viscera, dark muscle, and trimmings, and can account for up to 60–70% of the raw material, depending on species, size, and processing methods (Arason et al., 2010; FAO, 2020; Ghaly et al., 2013) (**Figure 1**). Traditionally, side streams were often discarded as waste, used directly for feed or for the production of feed, silage or fertilizers (Rustad et al., 2011). Currently, significant amounts of side streams are used for fishmeal and fish oil production of relatively low economic value (FAO, 2020). Some parts of the side streams, especially of large species, are edible and used for direct human consumption, such as eggs (roe), mince, tongues, stomachs, cheeks, collars, and swim bladders (Rustad et al., 2011; Thilsted et al., 2014; Vasconi et al., 2020) (**Figure 1**). However, with increased understanding of the nutritional properties of the side streams, other ways to use them have been gaining attention over the past couple of decades. The main products currently converted from fish side streams are protein concentrates, isolates, hydrolysates, collagen, gelatine, fish oils, and enzymes (Ucak et al., 2021; Välimaa et al., 2019; Zamora-Sillero et al., 2018). The muscle proteins, such as myofibrillar and sarcoplasmic proteins, isolates, and hydrolysates are recovered from cut-offs, heads, backbones and the dark muscle. These products are valuable protein ingredients, often with higher nutritional value than the intact proteins and possess various functional properties, and are thus widely used in food industries (Aspevik et al., 2021; Atef and Mahdi Ojagh, 2017; Egerton et al., 2018; Thawornchinsombut and Park, 2007; Välimaa et al., 2019; Zamora-Sillero et al., 2018). Bony side streams, skin, and scales have been be used to produce collagen and gelatine, which are commonly used as film products, to increase water-holding, emulsion stabilisation, to increase adhesiveness in meat and fish products (meat binding), as well as in the cosmetic industry (Gómez-Guillén et al., 2009; Karayannakidis and Zotos, 2016; Khiari, 2011; Milovanovic and Hayes, 2018; Nurilmala et al., 2021; Wasswa et al., 2007). Enzymes used in food and pharmaceutical products may also be extracted from the viscera (Shahidi and Kamil, 2001). Fish oils may be produced from the backbone, heads, viscera, cut-offs, and skin in fatty fish, and from the liver in lean fish (Alfio et al., 2021; Pateiro et al., 2021).

About 11% of the world's fisheries production, mainly small pelagic species accounting for about 20% of the total capture fisheries, are used for fishmeal

and oil production (FAO, 2020) (**Figure 1**). Fishmeal and fish oil are the most nutritious and digestible ingredients in aquafeeds, including high-quality proteins and LC PUFA, especially suitable for fish feed at specific stages of production such as seed, brood stock, and finishing diet (FAO, 2022; Izquierdo et al., 2001). In 2020, about 86% of the fishmeal produced, and 73% of the fish oil were used for aquafeed production. Most of the remaining proportion was used as ingredients in pet food and animal feed. About 11% of fish oil was used for biofuel and pet food, while only 16% was intended for human consumption (FAO, 2022).

Vietnam and Iceland are among the biggest fishmeal producers in the world (Deutsch et al., 2007; Granada et al., 2016). In Vietnam, side streams from Tra catfish (*Pangasius hypophthalmus*) processing constitute an important source of raw materials for fishmeal and fish oil production (Edwards et al., 2004). Generally, these raw materials result in low-quality fishmeal with high lipid and ash content and relatively low protein content (Edwards et al., 2004; Thuy et al., 2007). Fishmeal production for aquaculture is of major environmental and socioeconomic concern and should be decreased to the extent possible (Henriksson et al., 2014; Thuy et al., 2007). In particular, the Tra catfish industry is producing more fishmeal than needed for feed production. The large use of fishmeal in aquafeeds has been reported as a significant environmental impactor, increasing global warming and eutrophication (Nhu et al., 2016). Fishmeal and fish oil can to a large extent be replaced by various alternative feed ingredients of plant origin, insects, microalgae, microbial proteins, and seaweed at lower cost. Therefore, the Tra catfish side streams should be utilized to produce value-added products, which can bring profit to processing companies far beyond the margins of selling fish fillets, rather than aiming for low value fishmeal production, as is the current practice. In Iceland, fishmeal is mainly produced from small marine pelagic species such as blue whiting (*Micromesistius poutassou*) and capelin (*Mallotus villosus*), along with cut-offs from Atlantic mackerel (*Scomber scombrus*) and Atlantic herring *(Clupea harengus*) (Hilmarsdóttir et al., 2022). Fishmeal and oil have for many decades been produced using old and outdated processes and technologies, resulting in relatively low-quality products (Einarsson et al., 2019; Hilmarsdóttir et al., 2022).

A future increase in demand for protein rich ingredients is predicted due to the growing global population. Shifting protein sources up the available supply chain and using novel sources should therefore be encouraged (Boland et al., 2013; Henchion et al., 2017). Although increasing aquaculture is to some extent meeting this demand, underutilized species and side streams in fish processing should be utilized more efficiently (FAO, 2022; Henchion et al., 2017; Lam et al., 2020; Lam et al., 2016; Love et al., 2017) to provide a more sustainable production from existing sources of proteins for direct human consumption (as demonstrated in **Figure 1**). Small pelagic species commonly used for fishmeal

and oil production contain high-quality proteins, as well as valuable LC PUFA and micronutrients. In combination with recent improvements in fishing and on-board handling, production processes can be redesigned and optimised to produce higher value products even for human consumption (Beveridge et al., 2013; Cashion et al., 2017; Henchion et al., 2017).

While fillets are the most valuable part of the fish, the side streams are also excellent sources of highly nutritional compounds such as proteins, omega-3 LC PUFA, vitamins and minerals (Chen et al., 2022a; Love et al., 2017). Hence, converting these side streams into ingredients for new products should be a viable alternative to the current low value products. By doing so producers are not only adding value, but also decreasing environmental impacts, as well as simultaneously supplying consumers with nutritious, low-cost, and convenient food (FAO, 2022; Sasidharan and Venugopal, 2020). However, the side streams often have high microbial, enzyme, and blood content, and different physicochemical properties compared to the main products. This often makes the side streams more susceptible to rapid quality degradation, limiting their utilisation into higher-value products, especially if they are collected and processed together. The quality degradation can though be limited by collecting, storing, and processing the side streams properly and quickly, as appropriate for their individual physicochemical composition (van Berlo et al., 2023; Wu et al., 2022a, 2022b).

To maximize the utilization and quality of protein-rich materials obtained from fish side streams, further information regarding the chemical characteristics of the protein-rich materials during the process is needed. It is also necessary to look for innovative methods to recover proteins efficiently from these side streams.

2 Literature review

2.1 Underutilised fish species

Small pelagic fish contribute by far the largest biomass removed by current industrial fishing (Alder et al., 2008; Péron et al., 2010; Watson et al., 2015). High demand for fishmeal and oil has contributed to increased fishing pressure and overfishing of several stocks (Watson et al., 2015). In Vietnam, side streams from the processing of Tra catfish constitute a major source of raw material for fish meal and oil production. In Iceland, side streams from pelagic fish species, such as blue whiting, Atlantic mackerel, and Atlantic herring play a similar role. The fish species included in the current study are described in the following sections.

2.1.1 Tra catfish

Tra catfish (**Figure 2**) is a freshwater fish commonly farmed in Southeast Asian countries, such as Vietnam, Thailand, and Indonesia, as well as in India and Bangladesh. It is currently the most important farmed species in Vietnam (FAO, 2020; Nam et al., 2020). Tra catfish is the key species in the aquaculture industry in Vietnam, accounting for most of the revenues from fish and fish products. Vietnam is also the biggest producer of Tra catfish in the world, with nearly 1.6 million tons in 2019 (FishStatJ, 2022) which is mostly processed into frozen white fillets (**Figure 3**). Tra catfish has a relatively low market price, but the fillets are of good quality, with a high protein (18.9–20.2%) and low lipid content (2.1–2.6%), and the taste is comparable with other whitefish species, such as cod or haddock (Chakma et al., 2022; Dang et al., 2018b; Thong et al., 2020).

Figure 2. Tra catfish (*Pangasius hypophthalmus*). Photo taken during the present study by Hang (2020).

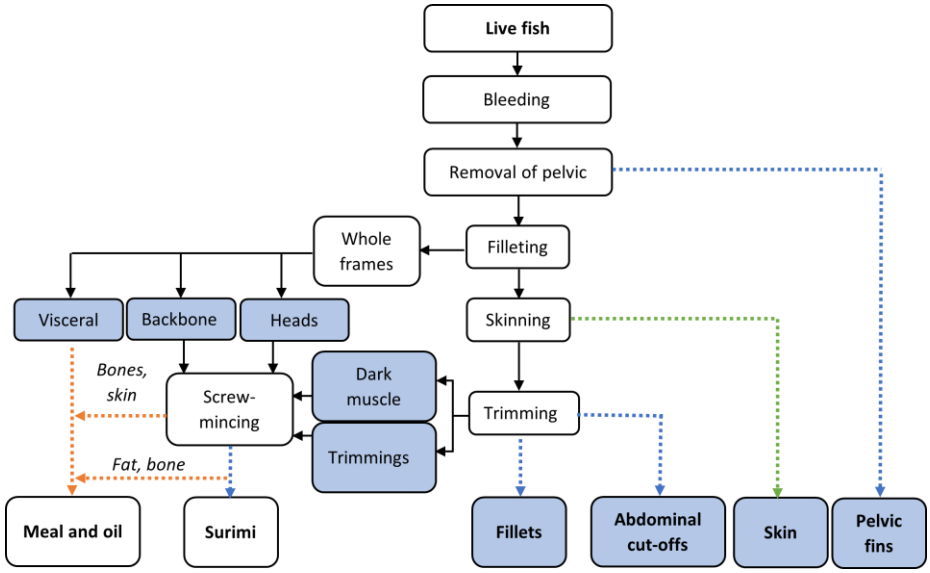

Figure 3. Industrial Tra catfish processing process. The blue lines indicate the streams producing products for human consumption, the orange represent the streams used to produce animal feed, and the green colour shows material for producing functional products. Blue-filled boxes indicate the main streams from the fillet processing.

In Vietnam, there are competing uses for small marine fish and bycatch for the production of livestock and aquaculture feed, fish sauce and fishmeal, and direct human consumption. Tra catfish filleting results in large amounts of side streams, which account for 62-67% of the production (Thuy et al., 2007). The side streams from the production of fillets were estimated to be about 1 million tons in 2019 (FishStatJ, 2022). However, side streams from Tra catfish processing often have high lipid and ash content, ranging from 15.3–29.8% and 2.4–5.7%, respectively (Nam et al., 2020). This makes the utilization of proteins from the side streams challenging due to their oxidative sensitivity. Traditionally, the side streams, including heads, backbones, viscera, trimmings, and dark muscle are combined for reduction into fishmeal and fish oil (Nhu et al., 2015) (**Figure 3** & **Figure 4**). Some of these side streams have, in recent years, been utilized for making surimi (**Figure 3**).

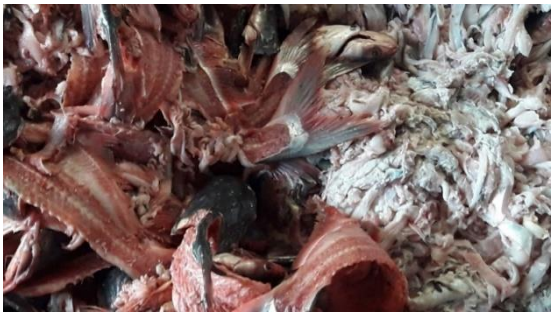

Figure 4. Tra catfish side streams are combined before processing into fishmeal and fish oil. Photo was taken during the present study by Hang (2020).

2.1.2 Blue whiting

Blue whiting (**Figure 5**) is a small abundant pelagic planktivorous gadoid species. Blue whiting is distributed in the Northeast Atlantic, including southern part of Greenland, Norway, around Iceland, the western Mediterranean, south along the African coast, southeast of Canada, and in the northeast of the USA. Blue whiting spawn and spend the larval stage, and part of the juvenile stage, in the waters west of the British Isles, Ireland, and the Norwegian Sea, before making annual migrations in early summer to the main feeding areas (FishBase, 2022; Gastauer et al., 2016; Mahe et al., 2016; Payne et al., 2012). The fish is lean with a fat content below 1% in the fillets. The water content in the fillets is about 80–83%, and the protein content 19–20% (Aubourg et al., 1998; Egerton et al., 2018). The average length of the fish at capture is usually around 26–32 cm (Gastauer et al., 2016). Blue whiting made up about 50% of the pelagic catch around Iceland in 2020 (Statistics Iceland, 2021). This species is used primarily for fish meal production, and is generally not considered tasty enough for direct human consumption (FAO, 2022).

Figure 5. Blue whiting (*Micromesistius poutassou*) ©, drawing by Jón Baldur Hlíðberg. (Source: https://www.fauna.is).

2.1.3 Atlantic mackerel

Most of the mackerel and herring catches in Iceland are processed for direct human consumption either as frozen, whole, headed-gutted (HG) fish or as fillets. The side streams, including cut-offs, heads, guts, viscera, and backbones

are collected and used for fishmeal and oil production, along with any bycatch (Hilmarsdottir et al., 2020; Sveinsdóttir et al., 2020). The mackerel and herring seasons overlap, and these species are thus often caught and processed together into fishmeal and oil (Gänsbauer et al., 2016; Langøy et al., 2012).

Atlantic mackerel (*Scomber scombrus*) belongs to the Scombridae family, commonly found on both sides of the North Atlantic Ocean, including the Baltic Sea (Langøy et al., 2012; Spijkers and Boonstra, 2017). Atlantic mackerel is a highly migratory species (Gänsbauer et al., 2016; Kvaavik et al., 2019). It is a fatty fish, and the proximate composition may vary according to season and geography, with a fat content ranging between 4.5–26.5%, a water content from 55.0 to 72.3%, and protein content ranging between 18.7–27.6% (Guizani and Moujahed, 2015; Romotowska et al., 2016). It is considered a healthy fish for consumption because it is not only high in LC PUFA? omega-3 fatty acids but is also an excellent source of selenium, fat-soluble vitamins, such as vitamin A, D, and E, as well as water-soluble vitamins, including niacin and vitamins B6 and B12 (Aminullah Bhuiyan et al., 1993; Romotowska et al., 2016). An adult mackerel can be 32–38 cm weighing 255–665 g (Korneliussen, 2010) (**Figure 6**).

Figure 6. Drawing of Atlantic mackerel (*Scomber scombrus*) © by Jon Baldur Hlidberg (source: www.fauna.is).

2.1.4 Atlantic herring

Atlantic herring (*Clupea harengus*) (**Figure 7**) is a migratory species commonly found in the North Atlantic (Libungan et al., 2015). The species is characterised by very flexible migration patterns due to changes in feeding, spawning, and wintering areas (Gänsbauer et al., 2016; Libungan et al., 2015). Herring is a highly nutritious fatty fish which is rich in PUFA, especially omega-3 fatty acids and minerals such as calcium, phosphorus, iron, magnesium, and potassium (Dang et al., 2018a; Egerton et al., 2020; Jensen et al., 2007). In Iceland, the Atlantic herring is commonly processed into frozen and salted fillets for human consumption, but the rest material is used for fishmeal and fish oil production (Dang et al., 2018a; Saevaldsson and Gunnlaugsson, 2015).

Figure 7. Atlantic herring (*Clupea harengus*) © by Jon Baldur Hlidberg (www.fauna.is).

2.2 Fish proteins

Proteins are required for health, reproduction, growth and the optimal performance of humans and animals. Not only the protein content, but also the amino acid profile within the proteins are important properties of food products (Boland et al., 2013). Fish proteins are generally easily digestible and of high quality, with a well-balanced amino acid composition, containing sufficient levels of the essential amino acids required from the human diet (Henchion et al., 2017; Tahergorabi et al., 2011). Fish proteins are particularly rich in the essential methionine and lysine (Henchion et al., 2017). Fish proteins are also a good source of bioactive peptides, which can have different health benefits, such as helping control blood pressure and inflammation, maintenance of bone health, and improved mental health (Abachi et al., 2022; Daneault et al., 2017; Hei, 2020; Hokmabadinazhad et al., 2022; Ko et al., 2016).

Proteins generally account for 15–25% of the total weight of the fish, depending on the species, diet, stage of maturity, season, and specific properties of the different parts of the body. Along with non-protein nitrogen compounds, proteins play an important role in the nutritional value and sensory quality of seafood products (Hall, 1997; Sikorski et al., 1995; Tahergorabi et al., 2011). However, improper handling, processing, or storing may cause protein changes responsible for the quality degradation of products, such as unwanted flavour and texture, and loss of digestibility and nutritional value (Ghaly et al., 2010).

2.2.1 Muscle proteins in fish

Muscle tissue is the major edible part of fish. Fish muscles include two types, striated and smooth muscles. The striated muscles are further divided into white and dark muscles. Both the white and dark muscle content, composition, and distribution depends on the species (Ochiai and Ozawa, 2020; Tahergorabi et al., 2011). Generally, white muscle exists in all parts of the fish, but dark muscle is generally found underneath the skin (**Figure 8**). In some fish species, the dark muscle is also located near the backbone, such as in tuna species (Elena et al., 2011) (**Figure 8**). Fish muscle consists of numerous muscle fibres bound together by connective tissue. These muscle fibres are comprised of myofibrils, which consist of multiple myofibrillar proteins (**Figure 9**).

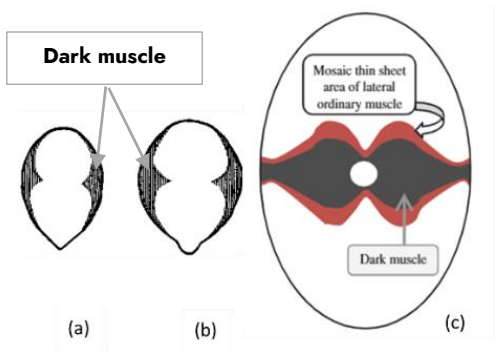

Figure 8. A diagram and cross-section of fish indicating the white and dark muscle distribution in herring (a), mackerel (b) (Love, 2001), and in Pacific bluefin tuna (*Thunnus orientalis)* (b) (adapted from Roy et al. (2012).

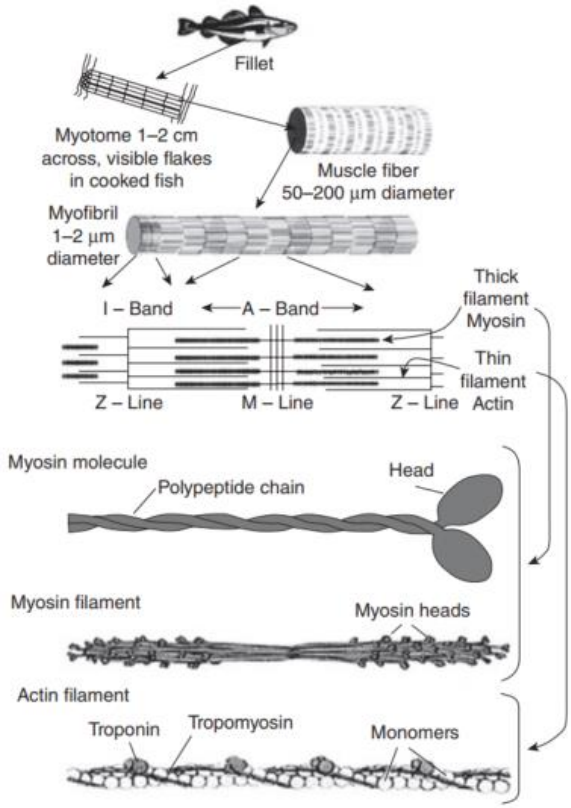

Figure 9. Fish muscle tissue structure (adapted from Goodband (2002) and Tahergorabi et al. (2011)).

Muscles contain several classes of proteins that serve different functions in the organism. Muscle proteins are classically divided into three groups based on their solubility in aqueous solutions, including myofibrillar, sarcoplasmic, and stroma proteins (**Table 1**).

Table 1. Classification of fish muscle proteins

Muscle protein	Solubility[1]	Main components[2]	Percentage[3]	Function/ application[4]
Myofibrillar proteins	Extractable with neutral salt solution of high ionic strength (0.3–1 M)	Myosin Actin Tropomyosin Troponin	66–77	Meat binding, water holding, and texture of products
Sarcoplasmic proteins	Extractable with water or neutral salt solution of low ionic strength (0.1–0.15 M)	Enzymes pigments (heme proteins)	20–30	Colour, water binding, and fat emulsification
Stroma proteins	Not extractable with neutral salt solution, nor in diluted acids and alkalis	Collagen Elastin	3–5	Texture of products

[1 2, 3] (Sikorski et al., 1995; Tahergorabi et al., 2011), [4] (Ustunol, 2014)

Myofibrillar proteins are the most abundant proteins in fish muscle that form myofibrils, accounting for 66–77% of the total muscle proteins (Adegoke and Tahergorabi, 2021; Hall, 1997; Kristinsson and Rasco, 2000). Myofibrillar proteins are further divided into contractile proteins, such as myosin and actin (65% of total muscle protein), regulatory proteins, such as tropomyosin and troponin, and other minor proteins (Adegoke and Tahergorabi, 2021; Ustunol, 2014) (**Table 1**).

Myosin is the major muscle protein in fish, accounting for 55–60% of the myofibrillar proteins. Myosin is the most important component of the muscle proteins with regards to functional properties such as water-holding capacity and gelation (Jafarpour and Gorczyca, 2012; Ustunol, 2014). However, both the amino acid profile and the conformation of the protein are responsible for the amount of water bound by a protein (Thorarinsdottir et al., 2011). In addition, myosin is the main protein responsible for heat-induced aggregation and is the only myofibrillar protein that forms gels (Kristinsson and Hultin, 2003; Ramiarez et al., 2000).

Actin accounts for approximately 20% of the total amount of myofibrillar proteins in fish muscle (Tahergorabi et al., 2011), and can be easily extracted. Myofibrillar proteins in fish muscle are less thermal-stable than those in the mammalian muscle (Adegoke and Tahergorabi, 2021; Sikorski et al., 1995). Their stability depends on fish species, the habitat temperature, and other conditions influencing the state of the proteins in vitro, such as pH, ionic strength, temperature, and salt concentrations (Sikorski et al., 1995). Solubility of myofibrillar proteins varies depending on the temperature, pH, and ionic strength, which can be adjusted to extract and recover them from seafood side streams (Tahergorabi et al., 2011). Myofibrillar proteins are the most important functional proteins recovered from fish muscle because they provide excellent water-holding capacity, gelling properties, flavour and binding properties, indicating their wide application potentials within the food industry (Adegoke and Tahergorabi, 2021; Gehring et al., 2009).

Sarcoplasmic proteins account for 20–30 % of total muscle proteins (**Table 1**). They are water-soluble and generally found in the cell plasma or the fluid surrounding myofibrils. The content of sarcoplasmic protein is generally higher in pelagic than in demersal species (Gokoglu and Yerlikaya, 2015; Lam et al., 2020; Sikorski et al., 1995). Most sarcoplasmic proteins are of a globular structure with a high density of exposed polar and charged side chains. This explains why they are readily soluble in water, and at low ionic strength (Xiong, 2018). Sarcoplasmic proteins have poor water-holding capacity, and inhibit gel formation of myofibrillar proteins (Tahergorabi et al., 2011). However, muscle colour is highly correlated with the content of sarcoplasmic proteins (Joo et al., 1999). On the other hand, several studies indicate that sarcoplasmic proteins could support gel strength, perhaps as a proteinase inhibitor, by catalysing the cross-linking of myosin heavy chains and improving the thermal gelation of myofibrillar proteins (Jafarpour and Gorczyca, 2012; Joo et al., 1999; Kim et al., 2005; Tahergorabi et al., 2011). Sarcoplasmic proteins in fish consist mainly of enzymes, oxygen carrier proteins (pigments), and other albumins (Belitz et al., 2004; Gokoglu and Yerlikaya, 2015; Sikorski et al., 1995).

Heme proteins exist in the flesh of fish and play important roles related to colour change, especially in tuna species (Lee et al., 2003). They also act as pro-oxidants, and accelerate lipid and protein oxidation during handling, processing, and storage of fish products (Carlsen et al., 2005; Gokoglu and Yerlikaya, 2015; Lund et al., 2011). However, since these proteins are soluble in water or in low ionic salt solutions, they are easily removed from the myofibrillar proteins by pressing or washing the fish muscle in low concentration saline solutions (Tahergorabi et al., 2011).

Stroma or connective proteins in fish muscle comprise about 3–5 % of the total protein (**Table 1**), which is a much lower proportion than observed in meat proteins (10–15%) (Fellows, 2017; Tahergorabi et al., 2011). Connective proteins include collagen and elastin, which comprise the connective tissues surrounding

the muscle fibres. Stroma proteins are mostly located in the interstitial spaces between muscle cells. In fish muscle, the connective tissue is mainly composed of collagen and provides extracellular support for the fibres. The distribution of collagen is species-dependent and reflects their swimming behaviour. Stroma proteins also play an important role in the textural characteristics of fish muscle and products (Kristinsson and Rasco, 2000; Sikorski et al., 1995; Tahergorabi et al., 2011; Ustunol, 2014). The connective tissues are responsible for the integrity of fish fillets and the rheological characteristics of seafood products. There is a positive relationship between the firmness of the fish muscle and its collagen content (Listrat et al., 2016; Sikorski et al., 1995; Tahergorabi et al., 2011). Generally, the dark muscle contains more stroma proteins and fewer sarcoplasmic proteins than the white muscle (Tahergorabi et al., 2011). The stroma proteins are not soluble in water, salt solutions, nor acid or alkaline solutions. Therefore, they are readily retained in the sediment during protein recovery from fish muscles (Kim and Park, 2007; Kristinsson and Rasco, 2000; Tahergorabi et al., 2011).

2.2.2 Protein quality changes

Fish are highly perishable post mortem due to their high water and lipids content, as well as their weak muscle tissue structure, neutral pH, and abundancy of resident bacteria, which promote biochemical and bacterial spoilage (Ghaly et al., 2010; Nie et al., 2022). Improper handling and temperatures also play important roles in the spoilage of fish and seafood products. The spoilage process begins immediately after death and includes several states, including autolytic, enzymatic, oxidative, and microbial spoilage mechanisms (Ghaly et al., 2010) (**Figure 10**). Beside lipid changes (including hydrolysis and oxidation), protein disintegration, caused by endogenous enzymes and bacterial activities, is the main reason for deterioration post mortem, and has a strong negative impact on the quality of fish and processed fish products (Abd El-Hay, 2022; Ghaly et al., 2010; Nie et al., 2022) (**Figure 10**).

Fish proteins are highly susceptible to enzymatic deteriorative changes due to the high presence of proteolytic enzymes in the fish muscle and viscera (Ghaly et al., 2010). Protein spoilage processes in fish generally begins with autolytic breakdown of the proteins, forming peptides and free amino acids. These peptides and free amino acids are then decarboxylated by endogenous enzymes and bacterial activities, forming biogenic amines, such as putrescine, histamine, and cadaverine (Nikoo et al., 2022; Tavares et al., 2021). Microbial growth also decomposes the peptides and free amino acids into highly volatile compounds, such as ammoniac, organic acids, sulphides, alcohols, aldehydes, and ketones, leading to the formation of common spoilage-indicating off-flavours and off-odours (Ghaly et al., 2010; Wu and Bechtel, 2008) (**Figure 10**).

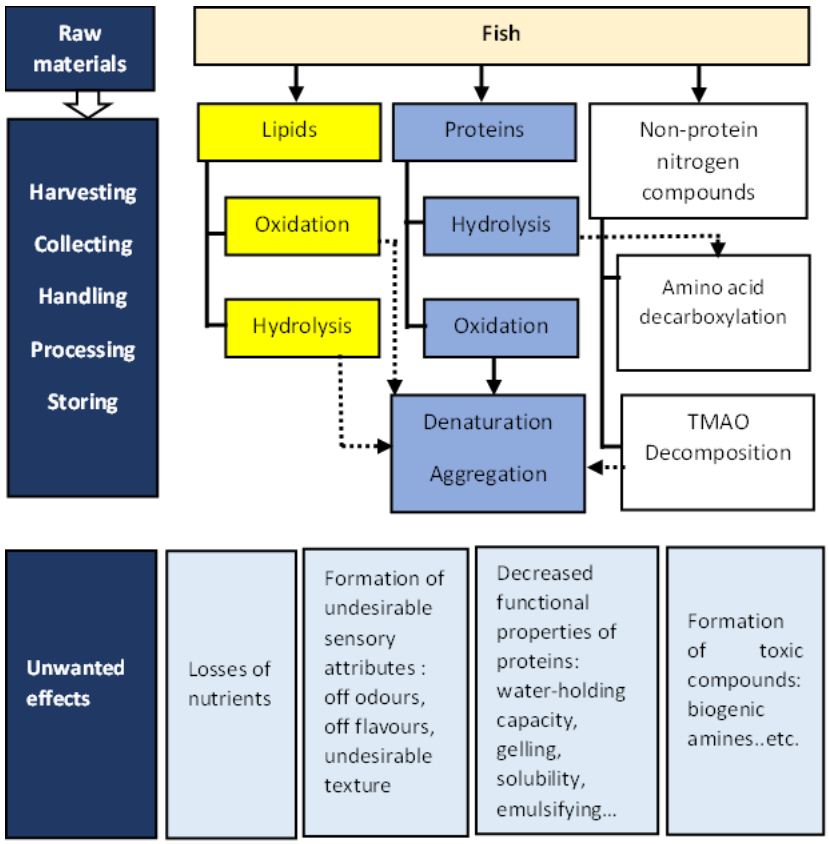

Figure 10. Overview of fish protein quality changes during main degradation processes, and their subsequent effect on quality.

Another common spoilage process is the breakdown of trimethylamine oxide (TMAO), either by microbial activities forming trimethylamine (TMA), or by endogenous enzymes in the fish forming dimethylamine (DMA) and formaldehyde (Wu and Bechtel, 2008). TMA has a characteristic "fishy" smell and contributes significantly to off-flavours (Huss, 1988). Meanwhile, DMA and formaldehyde are considered to relate to protein oxidation and denaturation processes since they cause the formation of cross-links between muscle proteins (Sotelo et al., 1995). For those reasons, TMA and DMA contents have been widely used as freshness indicators of marine fish and their products (Badii and Howell, 2002; Benjakul and Visessanguan, 2011; Sotelo and Rehbein, 2000; Wu and Bechtel, 2008). This spoilage commonly occurs in marine rather than freshwater species, which contain no TMAO or only small amounts (Sotelo and Rehbein, 2000).

Fish proteins are also susceptible to denaturation and aggregation (Eymard et al., 2009; Lund et al., 2011). The state of the protein structures is driven by different non-covalent forces such as hydrogen bonds, hydrophobic interactions, ionic interactions and van der Waal forces (Rehman et al., 2021). The functional or activated conformation of a protein is called native conformation, which is stable under specific environmental conditions such as temperature, pH, ionic strength, and pressure. When these specific environmental conditions of proteins change, the protein structure may become unstable and unfold. These changes may result in alternative protein conformations, or structure degradation into smaller proteins or peptides (Foegeding and Davis, 2011; Walter and Buchner, 2002; Xiong, 1997). Freezing and frozen storage (Shenouda, 1980; Thawornchinsombut and Park, 2006; Zhang et al., 2018), thermal treatments (Ohshima et al., 1993; Skipnes et al., 2008; Wu et al., 1985), and acid or alkaline treatments (Zhang et al., 2021) can cause denaturation and aggregation of fish proteins (Davis and Williams, 1998). The degree of denaturation and aggregation is associated with the conformational changes in the protein and its decreased solubility (Foegeding and Davis, 2011).

2.3 Current utilization of side streams and small pelagic species

2.3.1 Fishmeal

Fishmeal can be produced from almost any type of seafood but is generally made from wild-caught small and medium-sized pelagic marine species (65–75%), such as the Peruvian anchoveta, menhaden, blue whiting, capelin, sardine, mackerel and herring, which have a relatively high fat and bone content and are, therefore, usually not considered suitable for human consumption (FAO, 2022; Shepherd and Jackson, 2013; Tacon and Metian, 2009). Other raw materials for fish meal and oil production include side streams from seafood processing and bycatch (FAO, 2022; Hall, 2010; Miles and Chapman, 2006; Shepherd and Jackson, 2013). In 2020, over 27% of the global fishmeal production, and 48% of the total fish oil production were obtained from side streams (FAO, 2022).

Fishmeal is a powder which is traditionally obtained in a complex process including several processing steps, such as cooking, pressing, drying, and milling raw fish and side streams derived from fish processing (Shepherd and Jackson, 2013). During the cooking step, tissue proteins are thermally coagulated, and fat depots are ruptured and liberated, which helps the subsequent separation steps. Pressing is then performed to separate the solid and liquid phases from the cooking mixture. Other separation techniques, such as centrifugation and ultrafiltration, can potentially be used to further purify the

liquid phase. After that, the press cake, decanter sludge, and stick water concentrate are mixed and dried to form the final fishmeal product (FAO, 1986; Nygaard, 2010). The traditional industrial processing of fishmeal and fish oil is illustrated in **Figure 1** & **Figure 11**.

Figure 11. Flow diagram of traditional fishmeal and fish oil processing.

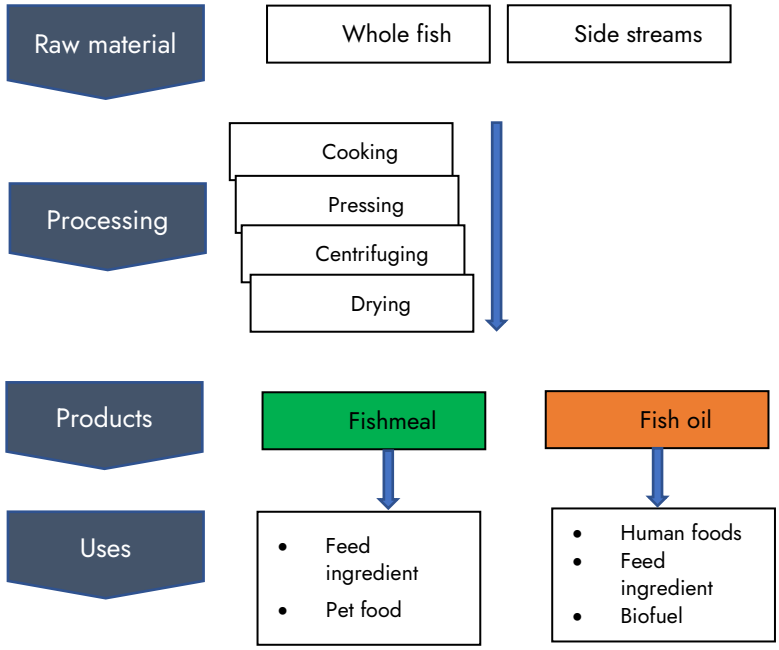

Figure 11. Flow diagram of traditional fishmeal and fish oil processing.

Fishmeal is commonly used in the production of pet food, and animal and aquaculture feed due to its high energy and protein content, highly digestible essential amino acids, fatty acids, vitamins, and minerals (Cho and Kim, 2011; Miles and Chapman, 2006). However, little is known about the detailed protein quality changes that occur during processes applied in fishmeal production. Few studies exist on the quality changes during fishmeal processing even though they have remained mostly unchanged for decades (Einarsson et al., 2019; Hilmarsdottir et al., 2020; Hilmarsdottir et al., 2021; Köse et al., 2003). Recent studies have reviewed the effects of traditional fishmeal and oil processing on oil and water separation and lipid and water quality (Hilmarsdottir et al., 2020; Hilmarsdottir et al., 2021). However, other nutrient composition (i.e. proximate composition) and quality indicators, including biogenic amines and volatile nitrogen compounds, which are of high concern in fishmeal and fish protein powder products have not been studied in detail before (FAO, 1986). As

mentioned above, fishmeal is an important ingredient in aquafeeds. Feed composition strongly affects the growth rate and fillet quality of farmed fish (Rasmussen, 2001). Opstvedt et al. (2000) showed that Atlantic salmon fed on fishmeal produced from rancid herring, which may have higher content of biogenic amines and volatile nitrogen compounds, had reduced feed consumption and growth than when fed on fishmeal made from fresh herring.

Fishmeal typically contains 5–10% moisture, 60–70% protein, 5–12% lipid and 10–20% ash. Traditional production results in low-quality products intended for animal and aquaculture at a relatively low value (Shepherd and Jackson, 2013). Recent technological advances and a deeper understanding of the pelagic fishes used as raw materials, however, provide opportunities for improvement of these processes for higher quality and value. Furthermore, fishmeal production for use in aquaculture is a major environmental and socioeconomic concern and should be decreased to the extent possible (Henriksson et al., 2014; Thuy et al., 2007). Several studies indicate that raw materials used for fishmeal can be used to produce protein ingredients for human consumption (Hilmarsdottir et al., 2020; Hilmarsdottir et al., 2021; Hilmarsdóttir et al., 2022). However, such changes call for improved knowledge and optimized processing methods in the fishing industry (Hilmarsdóttir et al., 2022).

2.3.2 Potential future changes in fish side stream processing and underutilised species

Knowledge about the quality and composition of materials during fishmeal processing may be necessary to assess the status of current processes as well as the potential for value adding processing. Optimization of the traditional fishmeal processes or redesigning them towards the production of more valuable products would support both higher production and economic yield for the producer, and lead to an increase of the availability of healthier products, even for human consumption, on the market (Rustad et al., 2011).

Aquaculture is currently the fastest growing food production system in the world (Watson et al., 2015). Therefore, the demand for aquaculture feed is expected to increase. Crustacean and finfish culture is still highly dependent on marine capture fisheries for sourcing key dietary nutrients, including fish oil and fishmeal (Tacon and Metian, 2008). Most of the fishmeal and fish oil production is used in aquaculture (FAO, 2022). However, increasing the catching of pelagic fish is unsustainable and alternative protein sources are needed (Hua et al., 2019). In fact, aquaculture has been the fastest growing food production sector over the last three decades. Feed inputs account for 40–75% of aquaculture production costs (FAO, 2018), and using a large portion of fishmeal in feed formulations generally increases production costs. This has led to

increased use of plant-based protein sources (Gatlin et al., 2007; Gupta et al., 2020; Voorhees et al., 2019), animal by-product meals (Fontinha et al., 2021; Galkanda-Arachchige et al., 2020; Lunger et al., 2007), dried yeast biomass (Rosales et al., 2017; Shurson, 2018), and microalgae meal (de Cruz et al., 2018) in the fish feed. However, eliminating fishmeal completely in the feed is not recommended since that would remove necessary omega-3 fatty acids and other nutrients from the feed (Bourre, 2005; Miles and Chapman, 2006; Naylor et al., 2009).

It has been predicted that the use of fishmeal and fish oil for general aquafeeds will decline in the long term and will instead increasingly be used for the production of high-value speciality feed, such as a higher-value feed starter, finisher, and broodstock feeds, or even be improved for human consumption (Tacon and Metian, 2008). With improvements in fishing and on-board fish handling and processing techniques, an increased proportion of small pelagic fish can be used directly for human food rather than for non-food fishmeal and fish oil (FAO, 2022; Hua et al., 2019; Saevaldsson and Gunnlaugsson, 2015; Tacon and Metian, 2009). In addition, an important source to future protein nutrition for humans will have to come from novel proteins, such as isolates, hydrolysates, and protein powders, which are currently used as animal feed. The protein sources need to be improved and their utilization converted for human consumption to meet increased protein demand (Boland et al., 2013), as demonstrated in **Figure 1**.

2.4 Recovery of muscle proteins from fish processing side streams and underutilised species

The direct uses of side streams for human consumption or as food ingredients have been limited due to their bony (heads, backbone, fins) or oily properties (i.e., fatty fish side streams) (Tahergorabi et al., 2011). Utilisation of side streams which have high contents of pro-oxidants (heme proteins), endogenous enzymes (viscera), and microorganisms (skin, gills, viscera), are especially challenging (Sajib, 2021). However, side streams may contain valuable protein, lipids, vitamins, and minerals, which can be used in various products and markets. This indicates a good practical potential for utilising seafood processing side streams if the protein fraction can be efficiently recovered and separated from the lipids (Tahergorabi et al., 2011). Several fish-derived protein products are even considered to be of higher value than intact fish because they are highly extractable and digestible (Chen et al., 2022a; Khan et al., 2022).

One of the most common techniques used to extract proteins in fish muscles from processing side streams and low-value species is applying a conventional water washing and refining to obtain a washed fish mince or surimi (Khan et al., 2022; Kim and Park, 2007; Kim et al., 2005). Surimi is an inexpensive

intermediate ingredient which can be used for producing a variety of seafood products (Kim and Park, 2007). In the conventional process of surimi production, the fish muscle is washed and dewatered to obtain concentrated myofibrillar proteins. Water-soluble sarcoplasmic proteins are then removed, resulting in considerable protein loss and excessive water usage (Kim and Park, 2007; Kim et al., 2005). The surimi processing water can contain significant quantity of nutrients such as proteins, lipids, nitrogen and phosphorus compounds, and minerals (Venugopal and Sasidharan, 2021). Inappropriate disposal of surimi waste water may also have a negative impact on the environment (Martín-Sánchez et al., 2009) as it may favour microbial growth. Anaerobic decomposition of proteins and other nitrogenous compounds form unwanted gas, such as carbon dioxide, methane, amines, diamines, ammonia and hydrogen sulphide, which may change the colour and odour of water and contribute to climate change (Venugopal and Sasidharan, 2021). Therefore, finding proper applications for the recovered proteins so that the processing can be economically and environmentally sustainable is necessary.

The pH-shift method and enzyme or acid hydrolysis have been developed to obtain high-value proteins, such as isolates, hydrolysates, peptides and amino acids while reducing protein losses and environmental impacts (Khan et al., 2022; Kim and Park, 2007; Kim et al., 2005; Tadpitchayangkoon et al., 2010b).

2.4.1 Isolate preparation with the pH shift method

In recent decades, much attention has been paid to the recovery of fish muscle proteins using novel isoelectric solubilisation/precipitation process. This method is commonly referred to as the ***pH-shift method*** (Gehring et al., 2011; Tahergorabi et al., 2011; Tahergorabi et al., 2015). When a food protein is at its isoelectric point (pI), the protein maintains a zero net electrostatic charge, in which protein-protein hydrophobic interactions overcome protein-water electrostatic interactions, and the minimum solubility of the proteins is obtained (**Figure 12**). The proteins then precipitate and can be collected easily (Gehring et al., 2009; Tadpitchayangkoon et al., 2010b). The principle of this method is based on the differences in protein solubilisation at low and high pH ($\leq$ 3.5 or $\geq$ 10.5). Shifting the pH generally makes the muscle proteins soluble. Separation techniques, such as high-speed centrifugation, are then applied to separate and remove fat (upper layer), from the protein solution (middle layer), as well as removing insoluble impurities (sediments), such as connective tissues, skin, bones, and scales. Subsequently, the protein solution is precipitated at its isoelectric point (pI, ~pH 5.5), and collected as a ***fish protein isolate (FPI)*** (Hultin et al., 2005; Hultin and Kelleher, 2000; Tadpitchayangkoon et al., 2010b).

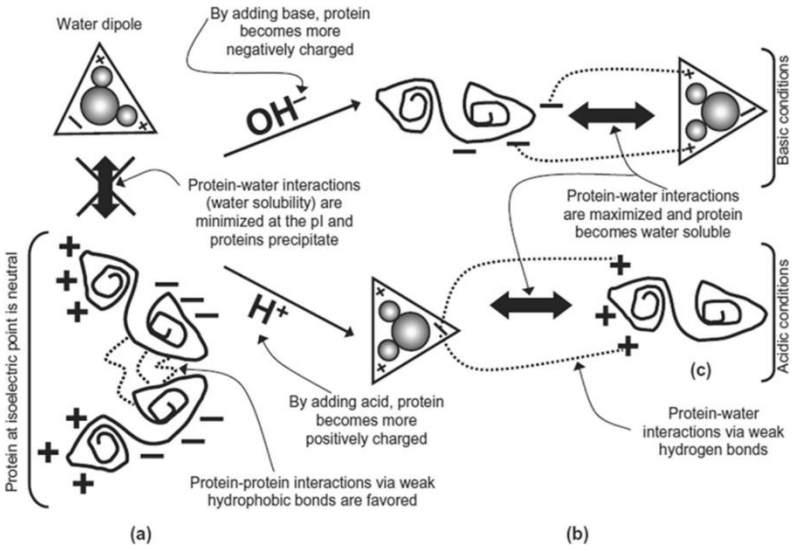

Figure 12. The biochemical principle for FPI production (a) At its pI, protein–water interactions are at their minimum, while protein-protein interactions via weak hydrophobic bonds are at their maximum, causing protein precipitation. (b) Protein-water interactions prevail under acidic or basic conditions far from the pI, resulting in protein solubility (Adapted from Torres et al. (2007)).

The pH shift method has been shown to allow efficient recovery of muscle proteins that retain several functional properties such as gel-forming ability and water holding capacity (Gehring et al., 2009; Gehring et al., 2011; Tahergorabi et al., 2011). The pH shift method has been widely used to recover proteins from low-value species and fish processing side streams (Khan et al., 2022), such as Atlantic salmon (*Salmo salar*), Atlantic cod (*Gadus morhua*) and herring (*Clupea harengus*) (Abdollahi and Undeland, 2018, 2019)

Side streams are usually combined during industrial processing (Pounds et al., 2022). However, different side streams have specific water, protein, lipid, ash, and pigment composition, and fish proteins from side streams may also be contaminated by other tissues, such as skin, backbone and blood (Hultin et al., 2005). This can limit protein recovery from the side streams and may challenge the stability of the FPI produced. High lipid and pigment contents, such as haemoglobin and myoglobin from the blood, may cause rancid, fishy odours in the final product (Hultin et al., 2005). Blood contamination often occurs, where the side streams are processed together. Producing FPIs from each side stream separately could increase the quality and value of the final products.

2.4.2 Fish protein hydrolysates

Fish protein hydrolysates (FPH) are a breakdown product of fish muscle proteins consisting of a mixture of peptides and amino acids of various molecular weight depending on the degree of hydrolysis (Siddik et al., 2021). Different methods, including autolysis, bacterial fermentation, or chemical and enzymatic hydrolysis, have been used to recover FPHs from side streams and underutilised species. However, enzymatic hydrolysis is considered the most efficient method (Gao et al., 2021; Kristinsson and Rasco, 2000; Siddik et al., 2021; Zamora-Sillero et al., 2018). FPHs are rich sources of amino acids and peptides that benefit human health as they include naturally bioactive compounds that can prevent, manage, or treat various human diseases (Ahn et al., 2012; Dale et al., 2019; Galanakis, 2012). FPHs have been reported as suitable nutraceutical foods due to their multiple biological activities, having numerous beneficial properties for neurological, cardiovascular, metabolic, intestinal, and immune health (Gao et al., 2021; Ozogul et al., 2021; Peighambardoust et al., 2021; Samaranayaka et al., 2010; Theodore and Kristinsson, 2007). FPHs have been considered to have a potential role in formulating a high-quality protein diet, especially for older adults, as they support skeletal muscle health and anabolism during ageing (Lees and Carson, 2020; Lees et al., 2021).

Proteases from various sources, such as animals, plants, bacteria, and fungi, have been used during FPH production. Alcalase® is an alkaline bacterial protease produced from *Bacillus licheniformis*. This enzyme has been recommended by several researchers as one of the best to produce FPHs with high yield and high antioxidant activity (Dong et al., 2008; Kristinsson and Rasco, 2000; Liceaga-Gesualdo and Li-Chan, 1999; Nam et al., 2020). Several studies have been conducted to find the optimal operating conditions for Alcalase® during preparation from side streams of Tra catfish. They all show a similar optimal temperature range, from 50 °C to 60 °C. However, the optimal hydrolysis time varies between studies, from 2 to 9 hours, and the ratio of enzyme/substrate may be dependent on the different raw material sources (Amiza et al., 2013; Minh, 2014; Nam et al., 2020). These studies focused on protein hydrolysate directly from Tra catfish side streams, but there is limited information available about the protein hydrolysate process from FPIs recovered from Tra catfish side streams. A detailed analysis of the protein changes occurring during FPI and FPH processing from separate side streams is necessary for the development of higher value products from side streams produced during the processing of Tra catfish.

3 Aims and objectives

The main aim of the study was to assess the potential of recovering high quality proteins from side streams from industrial processing and small pelagic species. One objective was to perform a detailed analysis of the protein changes occurring during the tested processes, including side streams from Tra catfish filleting processing in Vietnam and fish meal processing from small pelagic species in Iceland, in order to make them more efficient and valuable. A second objective was to propose changes in processing which would allow production of higher quality products for human consumption, instead of using these raw materials in fish meal and fish oil production, as traditionally done.

This included the following sub-objectives:

- Exploring the quantity and chemical properties of different side streams from industrial Tra catfish processing (**Paper I**).
- Investigating the possible utilisation of protein-rich side streams from industrial Tra catfish processing to produce value-added protein products for human consumption (**Papers II-III**)
- Evaluating the protein quality changes in the protein-rich production streams during traditional fishmeal and fish oil products from blue whiting, Atlantic mackerel and herring, seeking possible applications for human consumption by optimisation and re-designing current processes in fishmeal production (**Paper IV**).
- Comparing the protein characteristics of current common fishmeal products in Iceland and Vietnam studied in **Papers I-IV** to several commercial protein powders (**Section 5.7 in Thesis**).

4 Materials and methods

4.1 Experimental design

The overall experimental approach of the research is shown in **Figure 13** and describes how the publications are connected. The protein-rich side streams from Tra catfish fillet processing (indicated in **Paper I**), which is normally used for fishmeal and fish oil production, were used for protein recovery using the pH-shift method (**Paper II**). The fish protein isolates prepared according to the optimal conditions obtained in **Paper II** were the initial raw material for hydrolysate production (**Paper III**). The chemical properties of protein-rich streams were also analysed during Iceland's fishmeal processing to assess their utilization potential for human consumption (**Paper IV**). In addition, the quality of common fishmeal products in Iceland and Vietnam were evaluated and compared to several commercial protein powders to assess the potential of utilising these protein streams for human consumption. The experimental design of each individual study is described in detail in **Papers I-IV**.

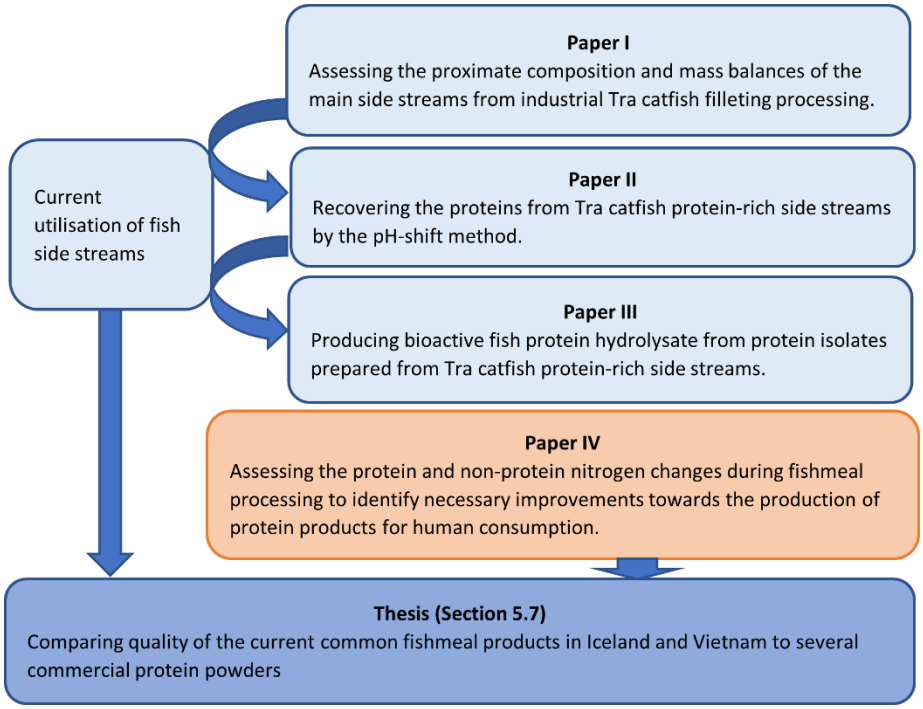

Figure 13. Flowchart of the overall experimental design of the study.

4.1.1 Experimental design for papers I - III

The objective of **Paper I** was to investigate the proximate composition of different side streams obtained from industrial Tra catfish filleting (**Figure 14**). The proximate composition and mass flow of each side stream was assessed to determine their utilisation potential for the development of protein products for human consumption. Samples (ten fish/group, n = 3 groups) from each side stream were collected separately, and each sample was weighed and recorded for mass balance calculations.

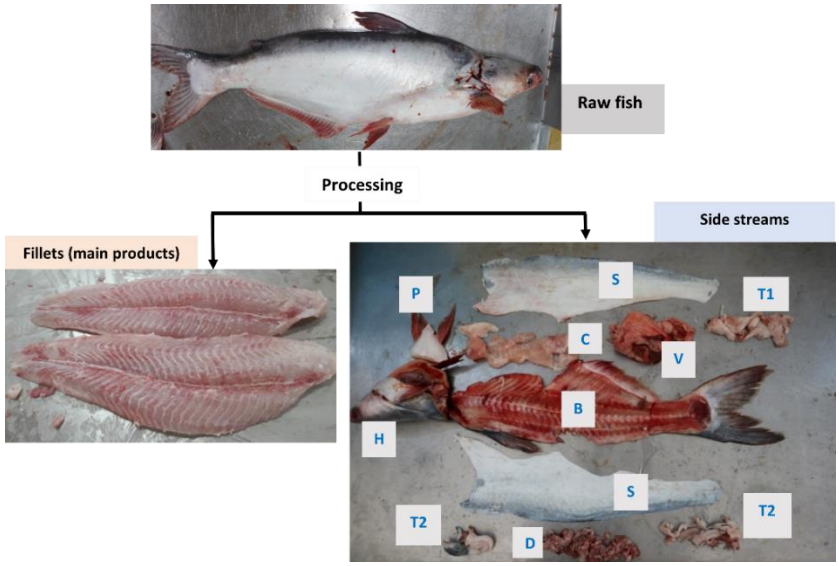

Figure 14. The main product (fillets) and side streams from Tra catfish industrial processing. Abbreviations: H: head; B: backbone; V: viscera; S: skin; C: abdominal cut-offs; T: trimmings (T1: fat collected from both sides of the belly, T2: fat collected from the skin-side dorsal portions); D: dark muscle; and P: pelvic fin. Photographs were taken during the present study by Hang (2020).

The objective of **Paper II** was to optimise the protein recovery from the Tra catfish protein-rich side streams by applying the pH-shift method. Production of FPI using the pH-shift method was performed at different pH values, extraction ratios, and extraction times of the protein solubilisation step to find the optimal FPI production settings. A flow chart for the optimisation process applied during the protein recovery trials from the dark muscle is shown in **Paper II**. The protein extractable recovery (PER), protein recovery (FPI-PR), and dry matter recovery (FPI-DMR) were measured to indicate the effect of those factors on protein recovery efficiency by the pH-shift method. These parameters have been used to evaluate the efficiency of protein recovery by pH-shift method in several

previous studies (Abdollahi et al., 2016; Hultin et al., 2005; Surasani et al., 2018). FPIs were then produced from the dark muscle (DM-FPI), head and backbone blend (HBB-FPI), and abdominal-cut offs (ACO-FPI) at the optimised conditions. FPI-PR, FPI-DMR, lipid and ash removal efficiencies were compared for all FPIs. In addition, quality properties including the proximate composition, amino acid profiles, protein patterns (SDS-PAGE), and colour of the FPIs, were evaluated and compared with commercial surimi produced at the company from the same batch of side streams. The results from **Paper I** indicated that different protein rich Tra catfish side streams differ in proximate composition, especially the lipid and ash contents, which may limit protein recovery. Therefore, the efficacy of each side stream's protein recovery and quality properties may differ. The comparison is thus necessary to show which streams are suitable for recovering protein and whether these side streams should be processed separately or together.

The objective of **Paper III** was to optimise the FPH production from proteins obtained from the Tra catfish protein-rich side streams by the pH-shift method (as a follow-up of **Paper II**). FPH was prepared by hydrolysis of the fish protein isolate produced from the dark muscle (DM-FPI) using the Alcalase® enzyme. Trials were carried out at different enzyme ratios (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 5.0% (volume/weight % compared to the protein substrate), different hydrolysis temperatures (at 45 °C , 50 °C, 55 °C, 60 °C, 65 °C and 70 °C), and for different hydrolysis times (1.5, 3, 4.5, 6, 7.5, and 9 hours) to ascertain the optimal conditions for FPH production. A flow chart for the optimisation process of the FPH production from the DM-FPI is shown in **Paper III**. The degree of hydrolysis (DH), protein recovery (PR), and antioxidant activities, including 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH-RSA), and the total reducing power capacity (TRPC) of the FPHs, were determined to evaluate the effects of hydrolysis conditions on the yield and bioactive properties of the FPHs. The FPHs were produced from the HBB-FPI and ACO-FPI at the optimal procedure as determined in this study. DH, PR, DPPH-RSA, TRPC, and amino acid profiles of each FPH were analysed and compared for all FPHs. The comparison is necessary to show which streams are promising for the production of bioactive hydrolysates and whether these side streams should be processed separately or together.

4.1.2 Experimental design for paper IV

The objective of **Paper IV** was to evaluate the quality changes of protein and non-protein nitrogen compounds during industrial fishmeal processing from blue whiting, as well as a blend of Atlantic mackerel side streams and Atlantic herring side streams. Samples were analysed for their proximate composition, biogenic amines (BA), total volatile base nitrogen (TVB-N), trimethylamine (TMA), and dimethylamine (DMA) in critical processing steps (including the raw material, press cake, sludge, and concentrate) as those processing streams are later combined

into the final fishmeal. Mass flows of these parameters were also studied to identify processing steps that should be optimised to improve the utilisation potential of different streams for human consumption. Details of the raw materials used in the study can be seen in **Table 2**

Table 2. Details of the raw material used in the fishmeal and fish oil production studied in Paper IV *.

Name in text	Blue whiting	Mackerel/herring blend
Species and by-catch	100% ***Blue whiting*** (*Micromesistius poutassou*) (whole)	58% ***Atlantic mackerel*** (*Scomber scombrus*) (cut-offs) 37% ***Atlantic herring*** (*Clupea harengus*) (cut-offs) 4.5% ***Blue whiting*** (*Micromesistius poutassou*) (whole) <0.5% By-catch
Fishing date	30.04.19	03.09.17–07.09.17
Dates fishmeal processed	02.05.19–03.05.19	07.09.17–08.09.17
Fishing grounds	South of Faroe Island	East and southeast off Iceland
Fishing gear	Midwater trawling	Midwater trawling
Temperature at landing	2.1°C (one trawler)	3 ± 1.5°C
Salt content at landing	0.5 g/100 g sample	0.9 ± 0.4 g/100 g sample

* The table is adapted from (Hilmarsdottir et al., 2021).

4.2 Analytical methods

Various analytical methods were used throughout the study to determine the proximate composition of each side stream during the industrial Tra catfish filleting and evaluate the quality of the industrial fishmeal. The focus was set on the quality properties of the available side streams, and the quality changes occurring during various processing steps to gain insight into the potential value-adding involved in utilising the studied side streams. Then, analytical methods were used to indicate the value-added protein-producing efficacy.

A summary of the main parameters investigated in the study is shown in **Table 3**. Each of the analytical methods is described in detail in Papers I-IV and will, therefore, not be described further in this section. However, the section provides insight into the importance of applying these quality assessment methods.

Table 3. Summary of the main parameters focused on in the research.

Parameter	Papers				Section 5.7 in the thesis
	I	II	III	IV	
Proximate composition	x	x	x	x	x
Yield (mass balance)	x			x	
Protein properties					
· Soluble protein content		x	x	x	x
· SDS-PAGE[1]		x			
· Amino acid composition		x	x		
Non-protein nitrogen compounds					
· Biogenic amines				x	x
· TVB-N, TMA, DMA[2]				x	x
Efficiency of the production of fish proteins					
· Protein extractable recovery		x			
· Dry matter recovery		x			
· Protein recovery		x	x		
· Lipid removal		x			
· Ash removal		x			
· Degree of hydrolysis			x		
Antioxidant activities					
· DPPH-Radical scavenging activity			x		
· Total reducing power capacity			x		
Colour		x			

[1] Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis.

[2] TVB-N: Total volatile basic nitrogen; TMA: Trimethylamine; DMA: Dimethylamine.

4.2.1 Yield and proximate composition

The proximate composition and yield at different processing steps were assessed. These parameters are important for raw material characterization and mass balance calculations. Detailed raw material characterization helps with identification of promising processing methods for each raw material. The proximate composition was also measured to evaluate the nutritional value of the fish protein isolates and the protein-rich streams in fishmeal processing.

Water content in samples was determined according to ISO 6496 (1999). The crude protein content (total nitrogen content) of the samples was measured

using the Kjeldahl method according to ISO 5983-2 (2009). Lipid content was determined using the Bligh and Dyer (1959) method, while the ash content was measured according to the Association of Official Analytical Chemists (AOAC,2000). Water, protein, lipid, and ash contents were expressed as a percentage of wet weight.

4.2.2 Protein properties

The proteins recovered from protein-rich side streams using the pH-shift method and/or hydrolysis method were evaluated based on their physicochemical properties and efficiency (as expressed by the protein recovery as described in Sections 4.2.4 & 4.2.5). Both parameters were important to evaluate the potential utilisation of the processes. Protein recovery was determined by measuring the protein content in key processing streams (**Paper II** and **Paper III**). The proteins of the FPIs and the initial raw materials waere extracted according to (Mæhre et al., 2018). The soluble proteins of samples were then measured based on the method by Bradford (1976).

Sodium dodecyl sulphate slab polyacrylamide gel electrophoresis (SDS-PAGE) with dimensions of 140 x 140 x 1 mm was performed according to the modified method of Laemmli (1970), using a precast gel Mini-protean TGX 10% (Bio-Rad Lab., Inc., California, United States). Insoluble protein was retained in the sediments, while some other proteins were not precipitated and thus lost in the liquid phase after centrifugation (**Paper II**) (Hultin et al., 2005). Production of isolates involves several steps that can change the characteristics of the proteins, including their structure and functions (Tadpitchayangkoon et al., 2010a). The SDS-PAGE was, thus, used to indicate changes in the composition and degradation state of the proteins after processing.

The amino acid composition is one of the most important quality attributes of protein products because it influences both their nutritional value and functional properties (Lee et al., 2023). The amino acid composition of the FPI and FPH samples (g/100 g protein) was therefore analysed using the liquid chromatography-mass spectrometry (LC-MS) method according to ISO 13903:2005.

Several steps in fishmeal processing include thermal treatment (**Paper IV**), causing protein denaturation and aggregation, leading to decreased content of salt soluble proteins (SSP) (Chen et al., 2022b; Li et al., 2022; Odoli et al., 2019). SSP content is related to protein solubility, which is an important functional characteristic. Protein solubility is the primary property of proteins used in liquid foods (Yousefi and Abbasi, 2022; Zayas, 2012). Salt soluble proteins (SSP) (g/100 g ww) were extracted from the samples with a NaCl buffer (1 M NaCl and 0.02 Na_2CO_3, pH 7.0) according to the method described by

(Kelleher and Hultin, 1991). The soluble proteins were then measured based on the method from Bradford (1976).

4.2.3 Non-protein nitrogen compounds

Fish is highly perishable due to the high content of water, protein, and lipids. It is more challenging to control the quality of fish than that of other animal products as the fish muscle provides an ideal environment for autolytic and hydrolytic enzymes (Venugopal, 2002). If fish is not cooled or handled properly at harvest, spoilage may be rapid. Therefore, total volatile base nitrogen compounds (TVB-N), mainly trimethylamine (TMA) and ammonia (NH_3) are commonly used as a quality criteria to evaluate the freshness of raw materials, as well as the quality of fishmeal (Haaland and Njaa, 1989; Wu et al., 2009). TVB-N was determined using the steam distillation method described by Malle and Poumeyrol (1989). The TMA and DMA were measured according to the liquid chromatography-mass spectrometry method described by Baliño-Zuazo and Barranco (2016).

Fishmeal is an important part of most fish feeds. High contents of biogenic amines in fishmeal have been associated with decreased growth and quality of farmed fish (Aksnes and Mundheim, 1997; Jasour et al., 2018). Therefore, biogenic amines have also been used as a quality criterion for fishmeal. Samples were thus tested for biogenic amines, including tyramine, putrescine, cadaverine and histamine, using a method developed by Olajos (2015).

4.2.4 Efficiency of the production of fish protein isolates

Protein extractable recovery (PER) was measured as a percentage of the protein extracted during the solubilisation step compared to the raw material. The protein recovery of the fish protein isolate production (FPI-PR) was determined as the amount of protein recovered as compared to the protein content of the initial raw material. Dry matter recovery of the FPI production (FPI-DMR) was assessed as the dry matter content recovered compared to the dry matter from the initial raw material during the FPI production. These parameters were determined to optimise the working conditions of the protein recovery when applying the pH-shift method and are described in detail in **Paper II**.

The FPI production aims to remove lipids and other impurities such as skin, scale and bones, to obtain the final protein concentrate (Hultin et al., 2005). Lipid and ash removal (%) during the FPI production were assessed by the proportional weight loss of the lipid and ash content, respectively, during the production compared to the lipid and ash contents in the raw material. The methods used are described in **Paper II.**

4.2.5 Efficiency of the fish protein hydrolysate production

The degree of hydrolysis (DH) has been used as an indicator for the peptide bond cleavage, while protein recovery (PR) shows the yield obtained from the hydrolysis (Benjakul and Morrissey, 1997). The methods used are described in **Paper III.**

4.2.6 Antioxidant activities of fish protein hydrolysates (FPHs)

Antioxidative activities are important properties of fish protein hydrolysates as they indicate the biological and functional values of the FPH (Chalamaiah et al., 2012). Antioxidant activities of the FPHs were evaluated through measuring the 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH-RSA) based on the method described by Fu et al. (2002). DPPH is a comparatively stable radical used as a substrate to determine antioxidant efficacy (Najafian and Babji, 2014). The total reducing power capacity (TRPC) of the FPH was measured using the method from Oyaizu (1986). The reducing capacity of a compound may serve as an indicator of its antioxidant activity. The presence of reducers (i.e., antioxidants) leads to the decrease of the Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}). In this assay, the yellow colour of the test mixture changed to various shades of blue and green, depending on the reducing power of each sample. The methods used are described in **Paper III**.

4.2.7 Colour

The colour intensity of the samples was determined with a Minolta Chroma Meter CR-400 (Minolta, Osaka, Japan) using the CIE Lab system as described by Abdollahi et al. (2016). The instrument recorded the L^* value (brightness), a^* value (redness) and b^* value (yellowness) of the samples. The whiteness was also determined for FPI and surimi samples.

The whiteness was calculated using the following equation, as described by Surasani et al. (2018):

$$\text{Whiteness} = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}}$$

4.3 Statistical analysis

All data summaries and statistical analyses were carried out in Microsoft Office Excel 365 (Microsoft Inc., Redmond, Wash., U.S.A) and IBM SPSS Statistics software (Version 22, IBM, 1 New Orchard Road, Armonk, New York, NC 10504-1722, United States). One-way analysis of variance (ANOVA) and Tukey's HSD tests were performed on means of the variables to assess statistical differences between treatments and samples. Pearson's correlation analysis was performed to find correlations between variables. All statistical analyses were performed assuming a significant difference set to the 5% level ($p < 0.05$).

5 Results and discussions

The main results of the study are presented in the following chapter and in more detail in **Papers I-IV**.

5.1 Yield and proximate composition of industrial Tra catfish side streams (Paper I).

Fillet yield from the bled fish was 31.8 ± 0.8%, while side streams accounted for 65.3 ± 0.4%, and material loss during processing was 2.9 ± 0.4% (**Figure 15**). Significant proportions of protein (63% of that available in the raw material), lipids (97%), and ash (92%) were left in the side streams (**Paper I**). A total annual production of 1.6 million tons in 2019 (FishStatJ, 2022) would thus have generated approximately 1.1 million tons of side streams, containing 153 thousand tons of protein, 314 thousand tons of lipids, and 43 thousand tons of ash. As mentioned before are the side streams from Tra catfish processing currently mainly combined and used to produce fishmeal and fish oil. Innovative ways need to be found to utilise these side streams more sustainably, and with greater respect for the raw materials to achieve products for human consumption.

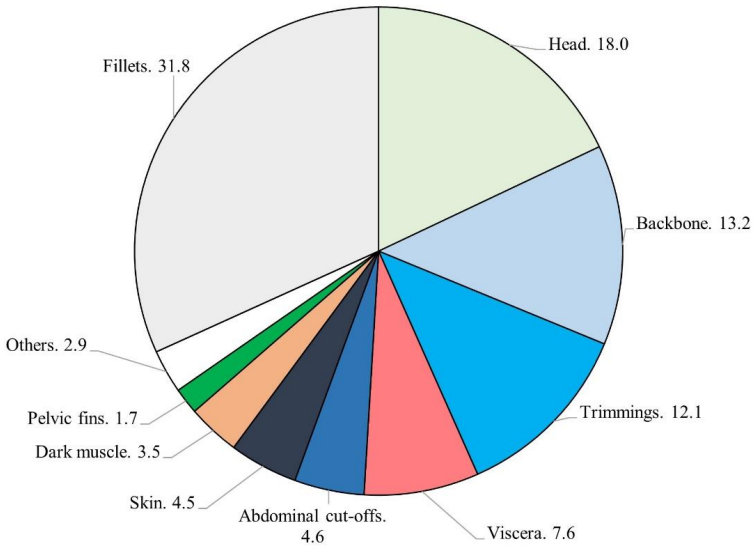

Figure 15. Mass ratios between the Tra catfish streams (% of the total weight of bled fish).

Heads and backbones contributed about 48% to the total amount of the side streams (**Figure 15**). This stream was rich in lipid (17–29%), protein (15–15.5%), and ash content (6.6–8.7%) (**Figure 16**). The ***trimmings***, accounting for 18% of the total side streams, had the highest lipid content (59.2 ± 1.1. ***Abdominal cut-offs*** and ***dark muscle*** contained 14.7–15.3% protein and 0.8–1.0% ash (**Figure 16**). However, due to high lipid contents and potentially high blood content, it is challenging to recover proteins from Tra catfish side streams for human consumption. This especially applies when the side streams are combined before processing. Valuable tissues may be contaminated with undesirable enzymes, lipids, pigments and blood, which accelerate lipid oxidation and quality degradation (Hultin et al., 2005). Side streams should be utilised separately to maximise the value of each stream, and to obtain the best possible quality and stability of the end products.

The proximate composition differed among Tra catfish side streams. The fillets had the highest water (80.4 ± 1%) and the lowest lipid content (2.1 ± 0.2) (**Figure 16**). However, the lowest water content (33.6 ± 1.4%) and the highest lipid content (59.2 ± 1.1%) were observed in the trimmings, indicating that most of the lipid in the Tra catfish is located in the skin-side dorsal portions and the outer part of the belly. This is in agreement with the study on farmed Atlantic salmon (*Salmo salar*) conducted by Aursand et al. (1994), which showed that lipids are primarily located in the dorsal fat depot and belly flaps. A study on farmed silver carp (*Hypophthalmichthys molitrix*) also showed the highest lipid content to be in the belly flaps (Kunyaboon et al., 2021). The trimming step and depth of skinning (regular or deep skinning) can therefore, affect the lipid content of the fillets (Dang et al., 2018b). The differences in chemical composition between the side streams further support that they should be processed separately for optimal utilisation and for broadening the possibilities in product development.

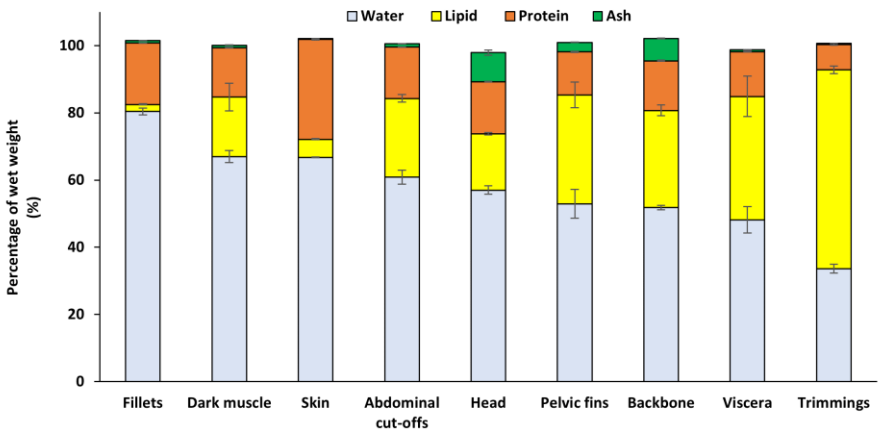

Figure 16. Proximate composition of different streams from Tra catfish filleting.

5.2 Optimized protein extraction conditions for FPI production from Tra catfish side streams (Paper II).

The pH-shift method is commonly used to recover or isolate proteins from fish side streams, producing fish protein isolates (FPIs). In this study, protein extractable recovery (PER), and protein and dry matter recoveries of the FPI (FPI-PR and FPI-DMR) were evaluated at different pH, extraction ratios, and extraction times in the protein solubilisation step, in order to find the optimal FPI production settings. The dark muscle side stream was used for determining the optimal extraction conditions.

pH had a strong effect on protein extraction from the dark muscle (**Figure 17A**). The least PER was observed at pH 5 (17.9 ± 3.7%), but the PER gradually increased as the pH moved either up or down (**Figure 17A**). These results are in agreement with the findings obtained for carp muscle (*Cyprinus carpio L.*) (Tian et al., 2017) and tilapia (Chomnawang and Yongsawatdigul, 2013). This effect of pH on the protein extraction may be because pH influences protein solubility. Protein solubility depends on electrostatic and hydrophobic interactions between protein molecules. When hydrophobic interactions are lower than the electrostatic repulsion, protein solubility increases, and vice versa (Zayas, 2012). At the isoelectric point pH (PI) (~ 5.5 for fish protein (Hultin et al., 2005; Tian et al., 2017)), a balance is reached between positive and negative charges, which minimizes the electrostatic repulsion, leading to decreased protein solubility. At pH lower or higher than their PI, net positive charges or net negative charges are formed on the proteins, creating electrostatic repulsive forces between molecules and the protein–water interactions increase, resulting in increased solubility (**Figure 12**) (Gehring et al., 2009). The highest PER was obtained at pH 12 (95.3 ± 3.3%) ($p < 0.05$) (**Figure 17A**). This is in agreement with the observations by Zayas (2012) and Abdollahi and Undeland (2019), who found protein extraction yields to be higher under alkaline than acid conditions. This may be because the proteins are denatured during harsh acid treatments, and retained in the sediment after the centrifugation of the extraction mixture (Kristinsson et al., 2005). Brenner et al. (2009) also observed that the rate of irreversible aggregation of cod protein increased as the pH declined and was especially rapid at pH below 8. Higher FPI protein recovery (FPI-PR) and FPI dry matter recovery (FPI-DMR) were obtained at higher PER, as expected. The extraction pH had a similar influence on the PER, FPI-PR, and FPI-DMR in this study. The highest FPI-PR and FPI-DMR were obtained at pH 12, with values of 70.9 ± 4.8% and 30.3 ± 0.4%, respectively ($p < 0.05$).

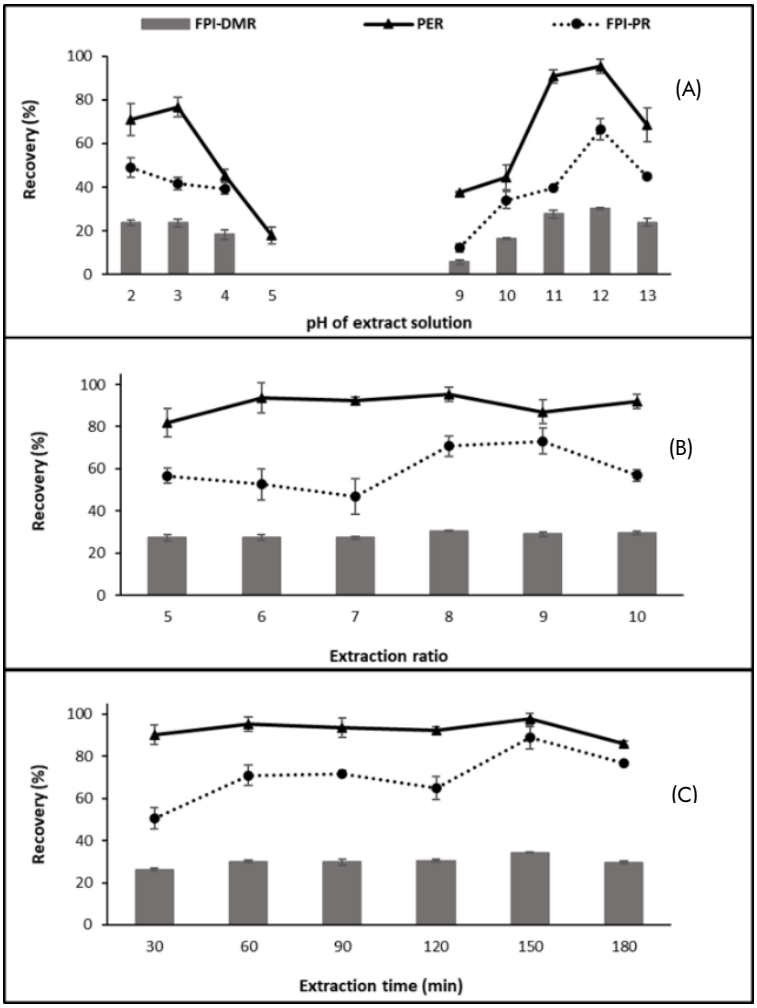

Figure 17. Effects of extracting conditions, including pH (A), extraction ratio (B) and extraction time (C), on protein extractable recovery (PER, %), protein recovery (FPI-PR, %) and dry matter recovery (FPI-DMR, %) of fish protein isolates produced from the Tra catfish dark muscle.

The ratio of the extraction solution to the raw material (***extraction ratio***) had little effect on protein and dry matter recoveries. PER increased slightly when the extraction ratio increased from 5 to 6 and decreased again at a ratio of 9 (**Figure 17B**). However, the effect of different extraction ratios on PER was not significant ($p > 0.05$). This could be because of the low protein content of the raw material, so that even the lowest volume of the extract solution was enough to extract most of the proteins. However, the FPI-PR was significantly higher at an

extraction ratio of 8 ($p < 0.05$) than at lower ratios. The highest FPI-PR was obtained at a ratio of 8 to 9 (70.9–73.2%) (**Figure 17B**). The extraction ratio ranging from 8–10 resulted in higher FPT-DMR compared to extraction ratios between 5–7 ($p < 0.05$), although the difference was not statistically significant.

As with the extraction ratio, the ***extraction time*** had little effect on protein and dry matter recoveries. When the extraction time was increased from 30 min to 60 min, the PER, FPI-PR, and FPI-DMR increased, followed by stable values from 60 min to 120 min, reaching a maximum at 150 min, and decreasing thereafter (**Figure 17C**). This decrease may be associated with protein denaturation and aggregation under the alkaline solubilisation when the extracting time is extended, as observed in the protein of Indian mackerel (*Rastrelliger kanagurta*) (Chanarat and Benjakul, 2013). The denatured proteins could have been retained in the sediment during the precipitation as discussed above.

Optimal protein extraction conditions in the dark muscle were thus obtained at pH 12, a solvent to raw material extraction ratio of 8, and an extraction duration of 150 min. These settings were then used to compare the extraction efficiency of fish protein isolates from the other side streams.

5.3 Comparison of the fish protein isolates (FPIs) produced from different Tra catfish side streams and surimi (Paper II).

The highest FPI-PR and FPI-DMR were obtained in the dark muscle (88.9 ± 5.3% and 34.3 ± 0.2%, respectively), followed by the ACO (83.0 ± 2.9% and 32.7 ± 2.7%) of the Tra catfish (**Paper II**). Although these different side streams had a similar crude protein content (**Table 4**), the protein profile may differ. Al Khawli et al (2020) showed that the HBB may have high residual blood content, which contains water-soluble haemoglobin and myoglobin, as well as stromal proteins, which can thus not be precipitated into isolates (Gehring et al., 2011). Abdollahi et al. (2016) and (Kristinsson et al., 2005) reported that heme proteins are mostly removed from the FPI production if the alkaline pH-shift method is applied, although this method also results in a lower protein recovery. However, this is beneficial in FPI production because the residual heme proteins in FPI act as the main pro-oxidants causing lipid and protein oxidation (Abdollahi et al., 2016). Removing them from the FPI does thus generally lead to more stable products. Meanwhile, the HBB may contain high content of stroma proteins, which are not soluble regardless of the pH or ionic strength of the solution (Kristinsson et al., 2005; Matak et al., 2015), and thus remain in the sediment. Also, some proteins may not be recovered because they were still stuck to the bone fractions, while others may be lost in the sediment in the HBB-FPI

processing (Gehring et al., 2011), or in the top lipid layer after centrifugation (Kristinsson et al., 2005), resulting in the lowest FPI-PR and FPI-DMR in the HBB-FPI production (68.2 ± 4.8% and 19.1 ± 1.4%) (**Paper II**).

Table 4. Proximate composition (%) of the raw materials and fish protein isolates (FPIs) produced from Tra catfish dark muscle, head, and backbone blend (HBB), abdominal cut-offs (ACO), and surimi made from the same batch of raw materials. Results are expressed as means ± SD based on triplicate measurements (n = 3)*.

		Water Content	Crude Protein	Lipid Content	Ash Content
Raw material	Dark muscle	66.5 ± 1.0^{An}	14.7 ± 0.2^{Bn}	17.6 ± 1.5^{Bm}	0.8 ± 0.1^{Bm}
	HBB	54.8 ± 1.0^{Cn}	15.2 ± 0.1^{An}	21.9 ± 0.6^{Am}	7.8 ± 0.4^{Am}
	ACO	60.8 ± 2.0^{Bn}	15.3 ± 0.1^{An}	23.5 ± 1.1^{Am}	1.0 ± 0.1^{Bm}
FPIs and surimi	DM-FPI	73.9 ± 0.7^{bcm}	23.5 ± 0.9^{am}	3.2 ± 0.0^{bn}	0.1 ± 0.0^{bn}
	HBB-FPI	77.3 ± 0.8^{am}	20.4 ± 0.5^{bm}	2.8 ± 0.4^{bn}	0.1 ± 0.0^{bn}
	ACO-FPI	73.1 ± 2.1^{cm}	24.4 ± 1.4^{am}	3.1 ± 0.1^{bn}	0.0 ± 0.0^{bn}
	Surimi	77.0 ± 0.1^{ab}	17.5 ± 0.3^{c}	5.4 ± 0.1^{a}	0.2 ± 0.0^{a}

* Different uppercase superscript letters indicate significant differences within the column for the raw material; different lowercase superscript letters a–c shows significant differences within the column for the FPIs and surimi; different lowercase superscript letters m–n indicate significant differences between raw material and the corresponding FPI for same parameter at $p < 0.05$.

Most lipids and ash were removed during the FPI production (90.4–95.2% and 86.3–91.5%, respectively), although the effect depended on initial lipid and ash contents of the raw materials (**Paper II**). This indicates a high capacity of the pH-shift processing in removing lipids and ash from the protein isolates. The three side streams examined had all different lipid content. However, all the corresponding FPIs had similar low lipid content (2.8–3.2%). This result may reflect that the remaining lipids in the FPIs were protein-linked lipids, while the storage lipids may be removed with the upper layer in the first centrifugation, and the membrane phospholipids may be separated into the first sediment (Kristinsson et al., 2013). The surimi had higher lipid content than the FPIs (**Table 4**). This may be due to the higher lipid content of the raw material (including trimmings with high lipid content (**Figure 16**), and lower lipid removal in the water washing process in surimi production compared to the pH-shift method, in agreement with observations on broiler meat (Chaijan and Panpipat, 2017), channel catfish (Kristinsson et al., 2005), and Atlantic croaker (*Micropogonias undulatus*) (Kristinsson and Liang, 2006). As with the removal of pro-oxidants, lipid removal during FPI production is advantageous for further use

of the FPIs since muscle lipids are highly susceptible to oxidation, increasing the risk of the formation of rancidity. This especially applies to fatty fish species like the Tra catfish, which has high lipid content in the side streams (**Figure 16**) and high presence of pro-oxidants, such as haemoglobin and myoglobin from the blood. This may form rancid, fishy odours in the final products, which limits the further use of the FPHs (Abdollahi et al., 2016; Undeland et al., 2004). Pro-oxidants in the substrate may react with antioxidative peptides generated during and after hydrolysis. Lowering these pro-oxidant compounds can thus preserve the antioxidant properties of the FPHs (Halldorsdottir et al., 2014; Khantaphant et al., 2011). Therefore, pre-treatment of protein substrates, such as defatting, washing, and/or centrifuging, are necessary to remove the excess lipid and pro-oxidants before using them for FPH production (Intarasirisawat et al., 2012; Klompong et al., 2007; Kristinsson and Rasco, 2000). Another advantage of using FPIs as raw materials for the FPHs production compared to the traditional method is that the loss of protein during the production is expected to be close to zero (**Paper III**).

The FPI products produced from the dark muscle (DM) and abdominal cut-offs (ACO) had a similar proximate composition, and all the studied FPIs had a similar protein pattern (**Paper II**), and amino acid profiles (**Paper II**). The chemical composition of the FPIs fulfils the FAO/WHO/UNU recommendations for adults (FAO, 2007), indicating that these FPIs may be useful as additives or ingredients in developing protein rich food products for adults, including the essential amino acids (Chen et al., 2007).

FPI produced from ACO had the highest colour lightness (L-value) and whiteness (**Paper II**), which may be due to a low content of heme proteins in this raw material (Richards and Hultin, 2002). Meanwhile, HBB-FPI had the lowest lightness and whiteness values, potentially due to a higher risk of residual blood and heme proteins contamination of this side stream (Abdollahi et al., 2016; Undeland et al., 2004). The high redness value (a^*-value) in the DM-FPI may be explained by the fact that dark muscle material has a high heme protein content, as shown in mackerel and trout (Richards and Hultin, 2002). Based on the colour assessment, the ACO-FPI is most promising of the FPIs as a food ingredient because of its whiteness. The FPIs produced from dark muscle (DM-FPI) and HBB (HBB-FPI) had similar chemical properties but showed less beneficial colour attributes (**Table 4**). These raw materials should thus be processed into FPIs separately, adjusting each FPI towards the production of a specific value-added food product. All FPIs had significantly higher protein contents and lower lipid contents than the surimi (**Table 4**), indicating a high efficiency of the pH-shift method to recover proteins from industrial Tra catfish side streams. The FPI made from ACO (ACO-FPI) had especially high whiteness, increasing its potential for the development of a high-value product.

5.4 Producing bioactive fish protein hydrolysates (FPHs) from Tra catfish side streams isolates (Paper III)

The effectiveness of different enzymatic hydrolysis conditions (enzyme concentrate, temperature, and time) during FPH production from the isolated protein from the Tra catfish dark muscle (FPH-DM) were investigated by evaluating the degree of hydrolysis (DH), protein recovery (PR), and antioxidative activities, including DPPH-RSA and TRPC of the FPHs.

The ***enzyme-protein substrate ratio*** had significant effects on ***DH***. Hydrolytic enzymes split the peptide bonds of the protein substrates through their active sites, which may initiate catalysis through covalent interaction with the protein substrates (Nothling et al., 2018). Therefore, increasing the enzyme concentration speeds up the reaction, as long as there is enough substrate available to bind to, leading to the DH increase when the enzyme-protein substrate ratio increased from 0 to 3.0%, and reached the highest DH value of 33.3 ± 4.0% at an enzyme ratio of 3.5% (**Figure 18A**). This result was in agreement with the FPH production from Tra catfish by-products using Alcalase® as carried out by Nam et al. (2020), where the highest DH (30.7%) was obtained at the enzyme-protein substrate ratio of 3.4%. A higher DH value has been obtained at higher enzyme ratios in earlier studies (Guerard et al., 2002; Intarasirisawat et al., 2012; Klompong et al., 2007; Nam et al., 2020). However, in the current study the DH decreased slightly when the enzyme concentration exceeded 3.5%. This may due to some of the peptides generated in the FPH may have been further hydrolysed, forming free amino acids and smaller peptides when the enzyme ratio increased (Noman et al., 2018). In addition, since enzymes are proteins, the ***hydrolysis temperature*** influences the stability and activity of enzymes (Eisenthal et al., 2006). Enzyme activity increases with temperature as long as the enzyme is stable. However, higher temperatures may result in increased internal energy of the enzyme (Daniel and Danson, 2010) and more covalent interaction between specific enzyme groups (active groups) and the protein substrate (Eisenthal et al., 2006). This would have led to increases in the DH, especially when the temperature increased from 50 °C to 65 °C. When the temperature exceeded 65 °C, the enzyme may become denatured and inactivated, resulting in a decreased DH (**Figure 18B**). DH increased, from 18.9 ± 1.4 % to 31.5 ± 1.1%, when the ***hydrolysis time*** was increased from 1.5 to 9.0 hours ($p < 0.05$) (**Figure 18C**). A similar observation was reported by Nam et al. (2020), which showed that DH increased when the time was increased up to 15 hours.

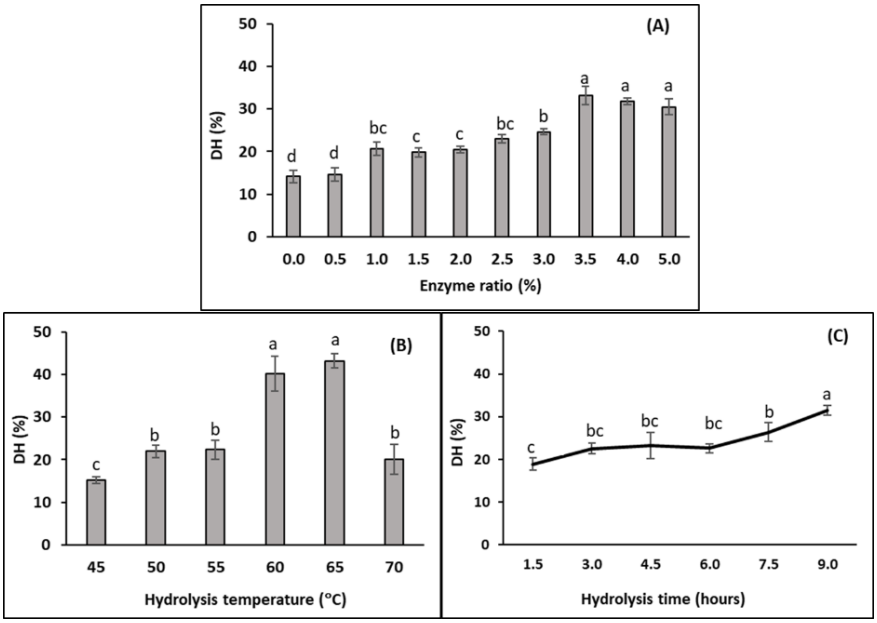

Figure 18. Effects of the hydrolysis conditions: enzyme/substrate protein ratios (A), temperature (B) and time (C) on the degree of hydrolysis (DH, %). Different lowercase letters show significant differences within hydrolysis condition at a significance level of $p < 0.05$.

The ***PR*** increased significantly from 8.0 ± 0.6% to 58.1 ± 0.8% when the enzyme ratio increased from 0% to 3.0% (**Figure 19A**). However, PR remained stable when the enzyme ratio was increased up to 5% ($p > 0.05$). This stability may be due to the saturation of the peptide bonds, especially of the soluble peptides in the hydrolysis solution, and the thermal denaturation of the enzyme (Benjakul and Morrissey, 1997; Nam et al., 2020). The DH can affect the PR and other functional properties, such as antioxidative activities (Benjakul and Morrissey, 1997; Wei et al., 2018). During hydrolysis (i.e. with Alcalase®), the enzyme divides the peptide bonds in the initial proteins into smaller protein molecules and peptides with higher solubility, which is positively correlated to the PR (Tacias-Pascacio et al., 2020). However, although the DH generally increased when the reaction temperature increased from 45 °C to 65 °C (**Figure 18B**), the PR increase was not statistically significant over this temperature range (**Figure 19B**). The initial substrate may be partly hydrolysed during the FPI production before entering the FPH production. Similar to the DH, the PR significantly decreased when the temperature reached 70 °C (41.3 ± 2.6 %). By then the enzyme may already have been partially thermally denatured, leading to a lower enzymatic activity (Benjakul and Morrissey, 1997). The PR remained constant during the first 4.5 hours, reaching a maximum value at a hydrolysis

time of 6 hours (67.5 ± 2.0%) (**Figure 19C**), although the DH changed significantly with time (**Figure 18C).** This may be because the FPH was made from FPI, where any impurities containing insoluble proteins had already been removed, leading to higher concentrations of extractable proteins. The decrease in PR when the hydrolysis is extended to 9 hours may be due to further degradation of peptides to free amino acids, and/or the formation of other volatile compounds (Wu et al., 2003).

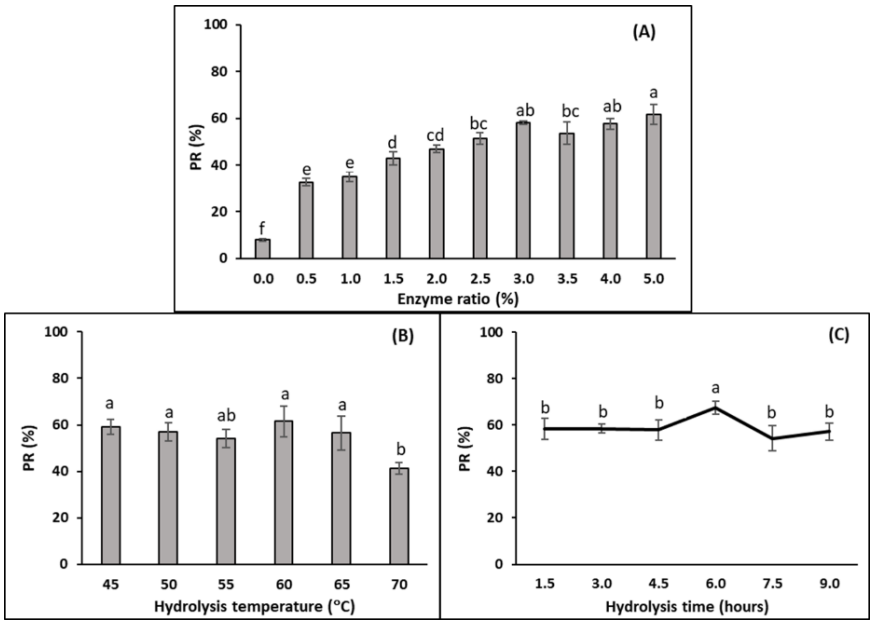

Figure 19. Effects of conditions during hydrolysis: enzyme/substrate protein ratios (A), temperature (B) and time (C) on protein recovery (PR, %). Different lowercase letters indicate significant differences within hydrolysis condition at a significance level of $p < 0.05$.

DPPH is a comparatively stable radical used as a substrate to determine antioxidant efficacy (Najafian and Babji, 2014; Tanuja et al., 2014). The reducing capacity of a compound may also be used as an indicator of its antioxidant activity. The effect of the enzyme ratio on the ***parameters indicating bioactive activites,*** including DPPH-RSA and TRPC, were similar (**Figure 20A, B**). The DPPH-RSA and TRPC increased slightly, from 22.0 ± 1.2% to 38.5 ± 2.0%, and from 0.74 ± 0.9 equiv. mg vitamin C/g FPH protein to 2.0 ± 0.2 equiv. mg vitamin C/g FPH protein, respectively, when the enzyme ratio increased from 0.5% to 2.5%. The DPPH-RSA then increased significantly when the enzyme ratio exceeded 2.5%, reaching its highest value (79.8 ± 6.9%) when the enzyme was used at a concentration of 3.5%. These results reflect that more

antioxidative peptides are released at higher enzyme concentrations. The antioxidative groups of the peptides generated are normally inactive within the sequence of the precursor protein molecules. However, when the enzyme ratio exceeded 3.5% the already formed antioxidative peptides during the early stages of hydrolysis may brake down, resulting in the reduction of DPPH-RSA.

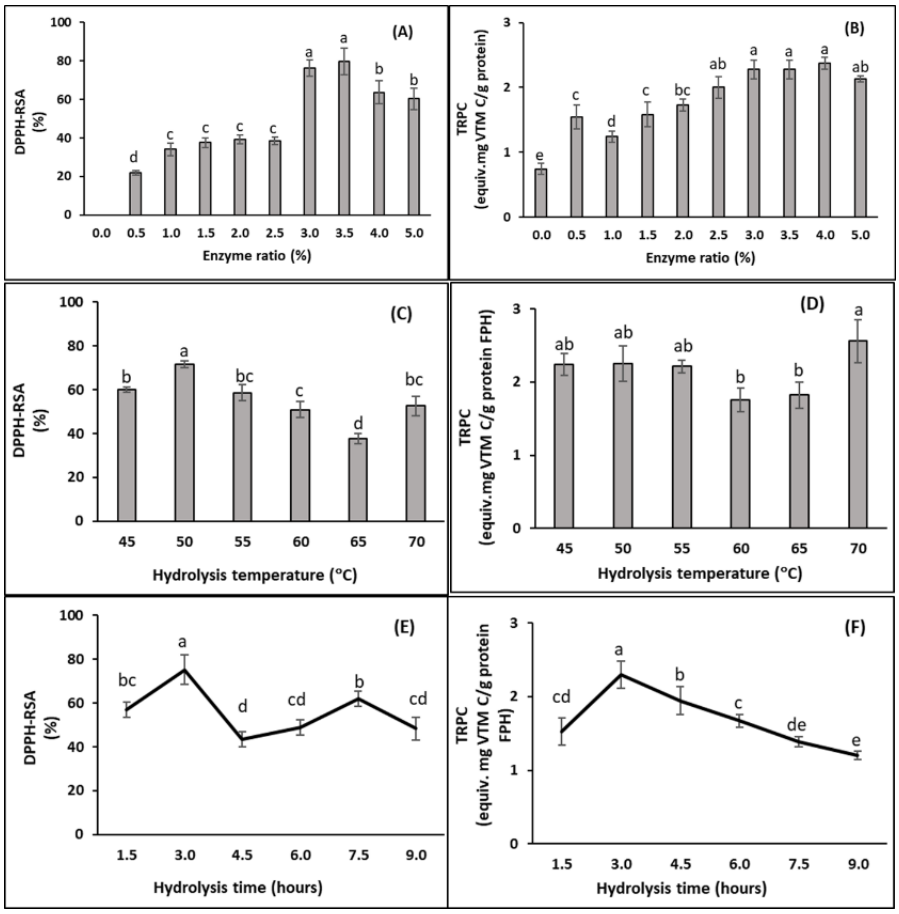

Figure 20. Effects of conditions during hydrolysis: enzyme/substrate protein ratios (A,B), temperature (C,D) and time (E,F) on DPPH-radical scavenging activity (DPPH-RSA, %) and total reducing power capacity (TRPC, equiv. mg vitamin C/g FPH protein), respectively. Different lowercase letters show significant differences within hydrolysis condition at a significance level of $p < 0.05$.

Increased enzyme activity when the temperature was raised from 45 °C to 50 °C may have released antioxidative peptides, which function as free radical scavengers, which in turn resulted in significant increase of DPPH-RSA, from 57.0 ± 3.4% to 75.1 ± 6.8%, ($p < 0.05$) (**Figure 20C**). However, at

temperatures above 50 °C, the DPPH-RSA decreased significantly again. TRPC changes were not completely consistent with changes in DPPH-RSA at the same temperatures (**Figure 20C,D**). The peptide contributors for these two antioxidant activities may have differed, as reported by Zou et al. (2016). The TRPC was stable in at hydrolysis temperatures ranging from 45 °C to 55 °C ($p > 0.05$), followed by a slight reduction when the hydrolysis temperature was increased to 60 °C to 65 °C. The DPPH-RSA and TRPC changes were in the opposite direction to the DH changes (**Figure 18B and Figure 20C,D**) and were different compared to the correlation between these parameters as effect by the enzyme ratio, (i.e, the DPPH-RSA and TRPC increased along with the increased DH, and vice versa) (**Figure 18A and Figure 20A,B**). The enzyme divides the peptide bonds of the precursor proteins into smaller protein molecules and peptides, as evidenced by increased DH, resulting in higher antioxidant activities. In this trial, however, the antioxidant decreased when the DH increased, possibly because that peptides exhibiting antioxidant activities were generated and reaching the maximum at 55 °C. Higher DH obtaining at 60–65 °C indicated stronger hydrolysis in this period, where the exceeded hydrolysis may break down the generated antioxidant peptides, resulting in DPPH-RSA and TRPC reduction (García-Moreno et al., 2014; Klompong et al., 2007). Therefore, the highest TRPC was obtained (2.6 ± 0.3 equiv. mg VTM C/g FPH protein), when the hydrolysis was limited due to denatured and inactivated enzymes at 70 °C,

DPPH-RSA fluctuated when the hydrolysis was extended from 1.5 to 9.0 hours (**Figure 20E**). These fluctuations reflect that there are two concurrent processes in progress, one which includes the generation of antioxidative peptides, and a second process which breaks down the peptides generated in the first one. TRPC increased significantly when the hydrolysis was extended from 1.5 to 3 hours, followed by a significant decrease when the hydrolysis was extended further to 9 hours (**Figure 20F**). The reduction in TRPC is likely due to further oxidative processes of the antioxidant peptides, which were generated in the FPH during hydrolysis (Halldorsdottir et al., 2014), and/or due to the breakdown of the antioxidative peptides when hydrolysis (with increased DH) was extended beyond 3 hours (Noman et al., 2018). The highest antioxidative activities were observed when the hydrolysis was extended to 3.0 hours, resulting in a DPPH-RSA value of (75.1 ± 6.8%), and a TRPC value of 2.3 ± 0.2 equiv. mg vitamin C/g FPH protein.

Optimal hydrolysis of the FPI-DM by the Alcalase® enzyme in this study was thus obtained at an enzyme/substrate protein ratio of 3% (v/w), and a hydrolysis temperature of 50 °C for 3 hours.

5.5 Comparison of the fish protein hydrolysates (FPHs) prepared from different fish protein isolates (FPIs) (Paper III)

All the FPHs had a similar amino acid profile (**Paper III**) although they originated from different side streams. The amino acid compositions of the FPHs were similar to those in the corresponding initial substrates (FPIs). Essential amino acids in all FPHs accounted for 46.3 ± 0.1 g/100 g protein. Glutamic acid, aspartic acid, lysine, and leucine were the main components of all FPHs, similar to FPHs produced from capelin (*Mallotus villosus*), Pacific whiting (*Merluccius productus*), and herring (*Clupea harengus*) (Chalamaiah et al., 2012). The amino acid composition of food products plays important roles in various physiological and biological processes to maintain human health. Glutamic acid, arginine and cysteine can enhance the immune response (Grimm and Kraus, 2001). Arginine and glycine can improve healing of wounds (Chyun and Griminger, 1984; Wu et al., 2014). In addition, all the FPHs have amino acid compositions that comply well with the human amino acid requirements for adults, as recommended by FAO/WHO/UNU (FAO, 2007). It could be concluded that these FPHs may be useful as additives or ingredients when developing products for adults providing a balanced protein diet (Chalamaiah et al., 2012).

The degree of hydrolysis (DH) was not significantly different between the three FPH from the different FPIs ($p > 0.05$). However, the protein recovery (PR) was highest in the ACO-FPH (80 ± 6.3 %), compared to 58.4 ± 2.0% in the DM-FPH, and 60.4 ± 5.7% in the HBB-FPH (**Table 5**). This may be because the ACO-FPH's initial substrate (ACO-FPI) had higher soluble protein content than the DM-FPI and HBB-FPI. The antioxidant activities of a FPH is determined by the molecular weight of the proteins, amino acid profile and sequences of peptides (Sabeena Farvin et al., 2016; Zou et al., 2016). In this study, the antioxidative activities were different between the FPHs, although they had a similar amino acid composition, indicating that molecular weight and peptide sequences of the FPHs may play a primary role in the antioxidant activities. Other compounds, such as pro-oxidants and other antioxidant in the products may also affect their antioxidative properties.

Table 5. Effectivity of the fish protein hydrolysate (FPH) processing from fish protein isolates obtained from Tra catfish dark muscle (DM-FPH), head and backbone blend (HBB-FPH), and abdominal cut-offs (ACO-FPH). Results are expressed as means ± SD from triplicate measurements (n = 3)*.

Production	DH (%)	PR (%)	DPPH-RSA (%)	TRPC (equiv. mg vitamin C/ g FPH protein)
DM-FPH	22.5 ± 1.3[a]	58.4 ± 2.0[b]	75.1 ± 6.8[a]	2.3 ± 0.2[b]
HBB-FPH	22.9 ± 1.6[a]	60.4 ± 5.7[b]	57.9 ± 3.6[b]	2.0 ± 0.2[b]
ACO-FPH	24.0 ± 3.5[a]	81.5 ± 4.3[a]	86.1 ± 1.6[a]	6.4 ± 0.4[a]

* Different superscript letters indicate significant differences within each column at $p < 0.05$. Abbreviation: DH: degree of hydrolysis; PR: protein recovery, DPPH-RSA: DPPH-radical scavenging activity, TRPC: Total reducing power capacity.

The ACO-FPI contain lower amounts of pro-oxidants, and thus results in the highest DPPH-RSA (86.1 ± 1.6%) and TRPC (6.4 ± 0.4 equiv. mg vitamin C/g FPH protein) in the ACO-FPH (**Table 5**). In contrast, The HBB-FPI may contain a higher content of pro-oxidants, such as myoglobin and iron (**Paper III**), which can react with the antioxidant peptides during hydrolysis. This agrees with the observations that the HBB-FPH exhibited the lowest bioactivity as reflected by the DPPH (57.9 ± 3.6%) and TRPC (2.0 ± 0.2 equiv. vitamin C/g FPH protein), respectively.

5.6 Protein and non-protein nitrogen compounds in protein-rich streams during fishmeal and fish oil processing (Paper IV)

The protein and non-protein quality changes, including protein content, salt soluble protein (SSP), biogenic amines (BAs) and volatile nitrogen compounds were investigated during the fishmeal processing of the blue whiting (BW) and the mackerel herring blend (MHB) (**Figure 21**). The importance of these parameters regarding product quality are discussed in the literature review (**Section 2.2.2**).

5.6.1 Water and lipid removal during fishmeal processing

Most of the water and the lipids followed the liquid processing streams after the separation steps, resulting in increased crude protein and reduced lipid and water contents in the press cake and sludge in both BW and BMH. The crude

protein content significantly increased while the lipid content decreased in the final MHB fishmeal (65.2 ± 0.3% protein and 14.3 ± 0.2% lipid) compared to the raw material (**Paper IV**). This indicates that a significant amount of lipid was separated into the oil stream, as expected (**Figure 21**). The protein and lipid contents were significantly higher in the final BW fishmeal (69.1 ± 0.5% and 9.4 ± 0.1%, respectively) than in the raw material (13.0 ± 0.9% and 1.8 ± 0.1%, respectively), mainly due to water removal. The water and lipid removal resulted in a relative increase of ash content in both the BW and MHB press cake (13.7 ± 1.9% and 5.7 ± 1.5%, respectively), and final fishmeal (15.6 ± 0.4% and 15.9 ± 0.7%, respectively). The BW and MBH fishmeal could both be classified as type C fish protein concentrates (FPC), according to their lipid content. To classify as a type A FPC, the lipid content should be below 0.75% (Einarsson et al., 2019; FAO, 1986). Substantial changes are thus required during processing of both species to obtain a type A FPC classification of the products.

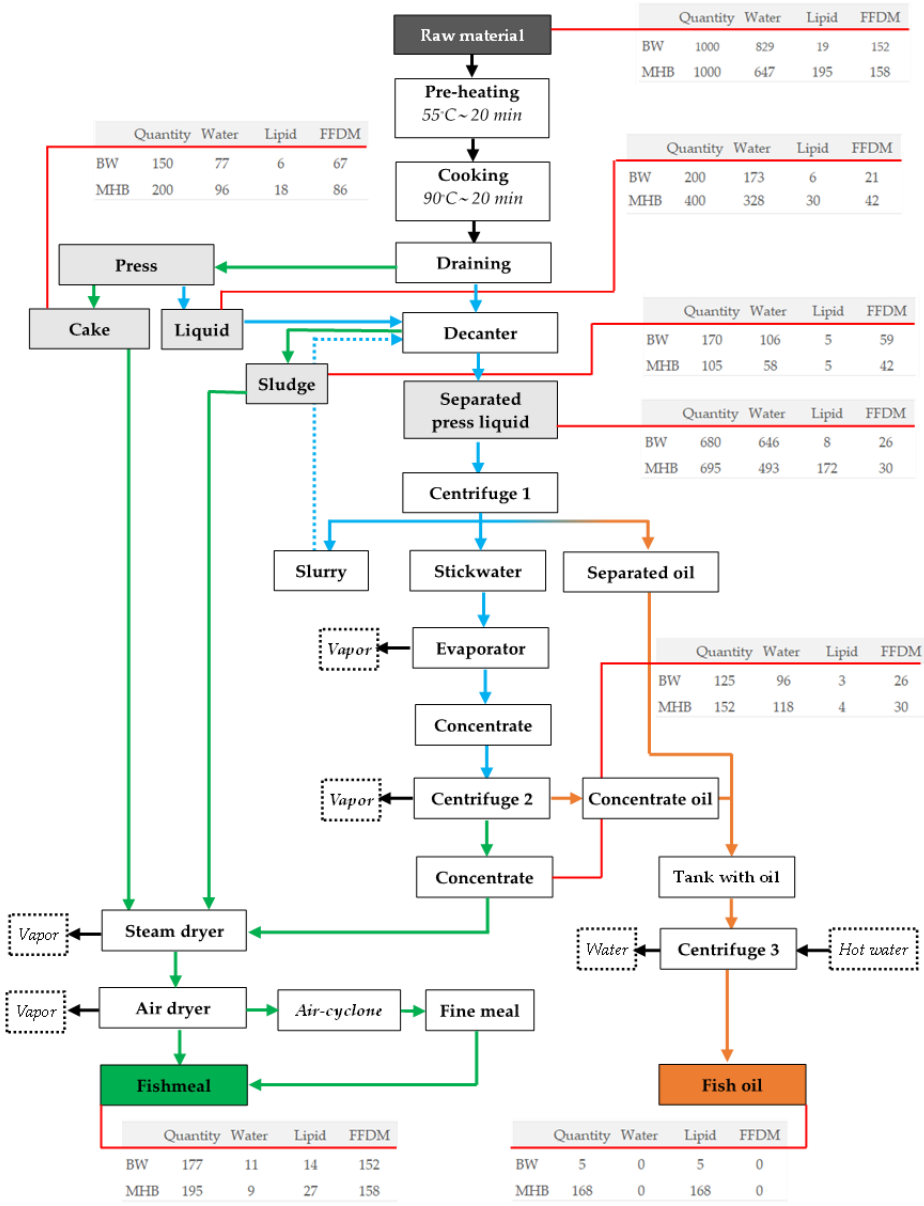

Figure 21. Industrial fishmeal and fish oil production. The green colour indicates the ***solid streams*** throughout the process, the blue represents the ***liquid streams***, and the yellow colour expresses the ***oil streams***. Mass balance from 1000 kg of raw material was calculated for blue whiting (BW) and mackerel/herring blend (MHB), shown by red lines. The quantity of each processing stream and the amounts of water, lipid, and fat-free dry matters (FFDM) in each stream are shown in kg. The flow chart was adapted from Hilmarsdottir et al. (2020).

After evaporation of the press liquid, the concentrate was mixed with the press cake and the sludge before entering the drying steps. Although most of the lipids followed the separated press liquid, a significant amount of lipids was left in the press cake (approximately 4% and 9% in the BW and MHB, respectively) and sludge (3% and 5% in the BW and MHB, respectively) (**Paper IV**). The press cake was the most significant contributor of lipids (contributed to 39% of the lipids in the BW, and to 67% in the MHB) in the fishmeal, indicating that the initial processing steps, especially the pressing step, require optimization for the removal of lipids during the processing of both species. Most of the lipids present in the separated press liquid were effectively extracted with the two centrifuges, forming the final fish oils and lowering the lipid content of the fishmeal. Nevertheless, only 26% of the lipid content of the BW raw material was extracted to form the BW fish oil. Improving the lipid separation from the solid streams and directing them toward the liquid streams would therefore not only increase the oil yield but also improve the fishmeal quality. In BW, the highest contributor to water was the sludge (38%) compared to the MHB sludge (21%), showing that the decanter did not operate properly, potentially due to overloading (**Figure 21**). Overload of the decanter should thus be avoided.

5.6.2 Protein changes during fishmeal processing

The **salt soluble protein (SSP)** content, which is a measure of protein solubility, is considered the first functional characteristic during the development and testing of a new protein ingredient. SSP declined significantly during processing, from 5.7 ± 0.7% to 0.9 ± 0.0% in the BW and from 6.1 ± 0.5% to 0.9 ± 0.0% in the MHB (**Paper IV**). This reflects that proteins were denatured and heavily aggregated, with an associated loss of protein solubility during the processing of both species. Protein denaturation during fishmeal processing may be due to heating, causing irreversible changes to the protein structure, such as protein unfolding, exposing previously hidden hydrophobic groups, or heat-induced aggregation (Yada, 2004; Zayas, 2012). These changes occurred already during the cooking steps, resulting in the very low SSP in the press cake in both the BW and MHB (0.5 ± 0.0 g/100 g ww and 0.3 ± 0.0 g/ 100 g ww, respectively). The low SSP in the fishmeal may limit their practical uses. The heat treatment applied in the current fishmeal processing is too rough. For the products to be fulfil for human consumption, the heating step thus requires optimising.

Biogenic amines (BAs) are created from the decarboxylation of free amino acids by endogenous enzymes of raw material or by bacterial activities under unhygienic conditions during storage and processing (Hazards, 2011; Shalaby, 1996). Histamine, tyramine, putrescine, and cadaverine are the most common biogenic amines occurring in fish and seafood products, which are products of the decarboxylation of histidine, tyrosine, ornithine and lysine, respectively

(Hazards, 2011; Santos, 1996). The presence of BAs poses a considerable toxicological risk in some food and feed products if their levels reach a critical threshold (Hazards, 2011; Ruiz-Capillas and Herrero, 2019; Shalaby, 1996; Visciano et al., 2020). Histamine poisoning is one of the most common foodborne illness caused by consuming certain species of marine fish and fishery products containing unusually high levels of histamine, especially those derived from histidine-rich species, such as mackerel, herring, tuna, and sardine (Visciano et al., 2014). Its toxic impacts are characterized by various symptoms similar to allergic reactions, including hypotension, flushing, headache, urticaria, abdominal cramps, diarrhoea, nausea, and vomiting (Visciano et al., 2014). Tyramine may also cause migraines and hypertensive crises in sensitive individuals (Latorre-Moratalla et al., 2008). The dietary biogenic amines tyramine and histamine inducing cytotoxicity has been reported *in vitro* previously (Del Rio et al., 2017; Linares et al., 2016). BAs may also be considered as carcinogens since they can react with nitrites to form potentially carcinogenic nitrosamines (Bulushi et al., 2009). In addition, BAs are heat stable during normal cooking, and can therefore be used as an indicator of quality and/or acceptability in some food and feed products (Hazards, 2011). The BW and MHB raw materials had high contents of biogenic amines (BAs). This may have resulted from bacterial activity during the delay between catching and processing, or indicating improper cooling and handling of the raw materials before entering into the fishmeal processing (FAO, 1986; Hilmarsdottir et al., 2020). The histamine in the raw materials was higher than the acceptable limit for human consumption (<0.2 g/kg ww), as established by FAO/WHO (2013) but within acceptable limits for fishmeal production in the blue whiting (<1 g/kg ww) (Pike and Hardy, 1997). Cadaverine was the most abundant BA in all streams of both BW and MHB fishmeal processing. Histamine and tyramine are the most toxic BAs (Hazards, 2011). However, cadaverine accumulation can accelerate the histamine toxicity (Bjeldanes et al., 1978; Shalaby, 1996). Generally, at the same processing steps, the MHB had higher BA levels, especially histamine levels, than the BW (**Paper IV**). This may be because the MHB fishmeal was produced from side streams of mackerel and herring, which are histidine-rich species and histidine is the precursor to histamine. The side streams may have higher ratios of gills and viscera, containing high amounts of endogenous enzymes and microorganism, which can convert the amino acid precursors into the corresponding biogenic amines (Moro et al., 2020).

Interestingly, all the BAs decreased during the processing in both species. About 82% and 89% of the total BA content in the raw materials was removed during processing of BW and MHB respectively (**Figure 21**). No histamine was found in the BW fishmeal, while a significantly proportion of histamine was removed during the MHB fishmeal processing, decreasing from 3.5 ± 0.2 g histamine/kg ww in the raw material to 0.8 ± 0.1 g/kg ww in the final fishmeal

(**Paper IV**). This reduction may be because BAs were decomposed into smaller volatile compounds, which may be easily removed through water removal during the high-temperature cooking and drying steps (Köse et al., 2003).

Total volatile base nitrogen (TVB-N) in fish and fish products primarily includes ammonia, trimethylamine (TMA), and dimethylamine (DMA) (FAO, 1986). High TVB-N and TMA levels were observed in the raw materials (with TVB-N contents of 83.9 ± 0.6 and 68.1 ± 3.4 mg N/100 g ww in the BW and MHB, respectively, and TMA content of 60.3 ± 5.3 and 35.8 ± 4.6 mg N/100 g ww in the BW and the MHB, respectively. It is necessary to maintain proper collection, handling, and storing of the raw materials before processing to improve the safety and quality of the final products. The TVB-N values indicate spoilage in the raw material during the delay between catching and processing, , and in agreement with BA formation in the raw material before processing, as discussed above. The higher TVB-N, TMA, and DMA values obtained in the BW was not surprising due to the BW being a gadoid fish, which has high levels of TMAO and TMA-ase enzymes (Mizuguchi et al., 2011; Rey-Mansilla et al., 1999). The TVB-N levels decreased during processing of both the BW and MHB before the drying steps (**Paper IV**). Water removal during drying may have resulted in a relative increase in the TVB-N content in both final products. Interestingly, approximately 81% and 62% of the TVB-N in the raw material evaporated during processing of BW and MHB, respectively (**Figure 21** IV). Since TMA is a volatile amine (Wu and Bechtel, 2008), a part of the TMA content that existed in the raw material may have evaporated during the cooking and pressing steps in a similar way as the TVB-N. This resulted in lower TMA levels in the press cake and press liquid (in BW), after the drying steps, as well as in the final fishmeal compared to other processing streams in both species. More than 90% of the TMA in the raw materials was removed during both BW and MHB processing (**Paper IV**).

The DMA changes did on the other hand not follow the same trend as TMA and TVB-N. The DMA content was stable before entering the centrifugation step. Drying may have resulted in a relative increase in the DMA content in both final products, similar to the TVB-N. Although the TVB-N contents in the raw material and intermediate processing streams were generally higher in the BW than in the corresponding MHB samples, the MHB fishmeal had a significantly higher TVB-N level than the BW fishmeal. Meanwhile, the TMA and DMA were higher in the BW than in the MHB fishmeal. This could be due to potentially higher ammonia formation during the pre-processing delay of the MHB side stream blend than in the BW due to a higher proportion of viscera in the MHB raw materials. Viscera, rich in enzymes and bacteria, can promote protein changes and spoilage, forming amino acids and ammonia (Wu and Bechtel, 2008). Ammonia formation during the thermal degradation of protein and amino acids has also been observed in earlier studies (Biswal et al., 2020; Sohn and Ho, 1995). The formation of free amino acids, especially those that play a role as substrates for

biogenic amine formation, can have negative effects on the quality of fishmeal (Hazards, 2011). It has been reported that conditions of potentiated or accelerated proteolysis also increases BA formation (Leuschner et al., 1998; Linares et al., 2012; Özogul and Özogul, 2019). Ammonia accumulation in fish and seafood product leads to a pungent odour (Wu and Bechtel, 2008).

The **non-protein nitrogen compounds** measured are water soluble and were thus released into the liquid streams (separated press liquid, concentrate), resulting in lower values in the solid streams (the press cake and sludge). This results in a higher BA amount in the press liquid than press cake (in the BW) and higher BA in the separated press liquid than the sludge (BW and MHB). The concentrates contributed less than 20% to the quantity of fishmeal produced (**Figure 21**). Mixing the liquid streams back into the fishmeal processing, as is the current practice, could thus cause problems when processing raw materials with high BA content, and should be avoided, at least for histamine-rich species such as mackerel and herring. Processing the solid streams individually could lead to lower undesirable non-protein nitrogen compounds in the final products, giving them more promising potential for human consumption applications. Most undesirable non-protein nitrogen compounds were removed during the processing of both species, especially during the drying step. The lipid quality was also highly influenced by heating in this step, as described earlier by Hilmarsdottir et al. (2020). This indicates that the drying step requires optimization. Spray drying of the processing streams could potentially provide milder drying and higher quality.

5.7 Quality of common traditional fishmeal products in Iceland and Vietnam in comparison with several commercial protein powders

To assess the potentials of using side streams evaluated in this study for human consumption a comparison of the fishmeal in this study with commercial fish protein products (PP), which are used in protein sources in foods for humans (HM-PP) and pets (PET-PP) was conducted (**Figure 22**). Since the quality and stability of fishmeal and protein powders are dependent on the nutrient composition (i.e. proximate composition), and safety related indicators, such as biogenic amines and volatile nitrogen compounds, the comparison between products focused on these parameters.

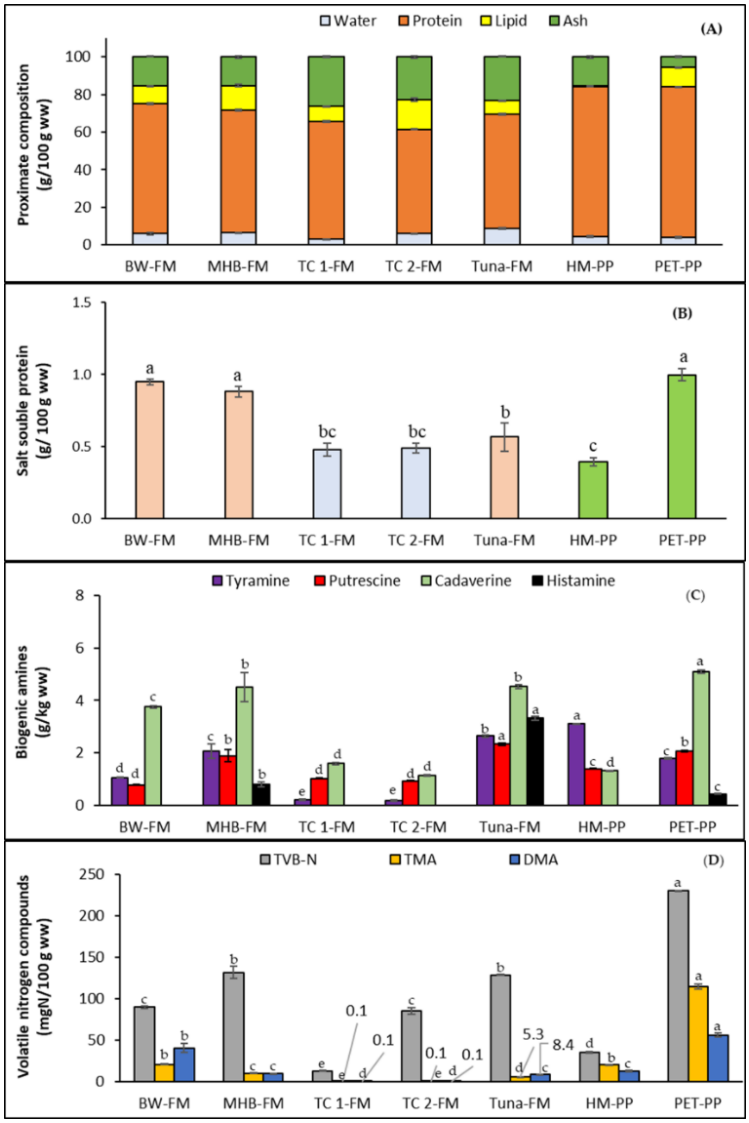

Figure 22. Chemical properties of two Icelandic fishmeal (BW-FM and MHB-FM) and three Vietnamese fishmeal (TC 1-FM, TC2-FM and Tuna-FM) in comparison with two commercial protein powders (HM-PP and PET-PP), indicating proximate composition (g/100), salt soluble protein (SSP, g/100 g), and four biogenic amines (BA, g/kg ww), TVB-N (g/100 g), TMA (g/100g) and DMA (g/100g).

The HM-PP is a commercial protein powder produced from Atlantic cod (*Gadus morhua*) cut-offs without skin and bones from cod fillet processing, and is available in capsules (Protis; Kópavogur, Iceland, www.protis.is). Commercial PET-PP was obtained from the hydrolysis of fresh rest materials from several

gadoid species as formulated by a company in France (Copalis; Le Portel, France www.copalis.fr). Three fishmeal products from Vietnam were collected from different fishmeal factories right after processing, including tuna fishmeal (Tuna-FM) and tra catfish fishmeal. The tuna-FM was produced from tuna side streams, mostly including heads (without eyes), backbones and visceral (without stomach) from yellowfin tuna (*Thunnus albacares*) from tuna processing companies in Khanh Hoa, Vietnam. Two tra catfish (*Pangasius hypophthalmus*) fishmeal (TC-FM) products obtained from two different big companies in Mekong Delta. Both were made from the side streams from the local tra catfish processing factories. After processing, in the side streams, stomachs, pelvic fins and low-fat cut-offs were collected using directly for human consumption, skin was used for gelatin production. The all remaining side streams entered the fishmeal (TC 1-FM) and oil production or apart of head and backbone were used to produce surimi and the remaining materials are combined to produce fishmeal (TC 2-FM) and oil (**Figure 3**).

Large variations were observed in the chemical properties of the traditional fishmeal products (**Figure 22**). Water content is an important quality parameter for fish meal and fish protein products. Low water content can inhibit protein browning and bacterial-induced deterioration. However, too low water activity in overdried fish powders increases lipid oxidation and loss of protein solubility (Shaviklo, 2015; Sun et al., 2002). Therefore, water content ranging from 5% to 12% is generally recommended for fishmeal products (Einarsson et al., 2019; FAO, 1986). The BW-FM, MHB-FM and TC 2-FM had similar water contents of 6.0–6.5% and lower than the Tuna-FM (8.8 ± 0.3%) (**Figure 22A**). The water contents of these fishmeal products are comparable to earlier published results (Ariyawansa, 2000; Ween et al., 2017). The HM-PP, PET-PP and TC 1-FM had water contents below 5% making them sensitive to oxidation, especially the TC 1-FM (3.0 ± 0.1%), which had high lipid content. The quality of the fishmeal products and protein powders were highly affected by the fish species and forms of the initial raw materials. The fishmeal produced from whole small pelagic fish (blue whiting) (BW-FM) had the highest protein content (69.1 ± 0.5%) among the fishmeal products, but was lower than the two commercial PPs, which had protein contents of about 80%. This may be because the HM-PP was produced from the cut-offs from fillet processing, while the PET-PP was made by enzymatic hydrolysis of side streams from several gadoid species, which removed most bones during processing. Therefore, by separating the ash from the protein contents of fishmeal products can be increased to the same level as the PET-PP and MH-PP. The three Vietnamese fishmeal products, which were produced from side streams, had relatively low protein contents (below 63%), while having high ash contents (22.9–26.2%). The ash content in Vietnamese fishmeal products ranged from 22.9–26.2% and was higher than in any of the other products, probably due to the use of heads and backbones. The ash content in fishmeal and protein powder

products is associated with mineral and inorganic compounds (Janbakhsh et al., 2018). Fishmeal is a good source of minerals, especially calcium, phosphorus, and magnesium, essential for the growth of farmed fish and other farmed animals (Boyd, 2015; Miles and Chapman, 2006). However, Shearer et al. (1992) indicated that when ash content in dietary increased from 10% to 17.5%, the growth of juvenile Atlantic salmon decreased. High ash content may limit the use of these products as protein ingredients for human consumption. The BW-FM, TC 1-FM and Tuna-FM have lower lipid contents (9.4 ± 0.1%, 8.1 ± 0.2% and 7.2 ± 0.3%, respectively) than the pet food (10.4 ± 0.0%) (**Figure 22A)**. Hence, these fishmeal products could be used as pet food based on the lipid quality assessment. Nevertheless, the protein powder for human consumption only contained 0.3 ± 0.0% lipids. It is therefore, necessary to focus on lipid removal during the phase that contributes to lipids to the final products.

Generally, salt soluble fish proteins are mostly made up of sarcoplasmic and myofibrillar proteins, which can account for 85–90% total protein content (11–24% wet weight) (Sikorski et al., 1995; Ustunol, 2014). Low SSP (below 1.0 g/ 100 g ww) (**Figure 22B**) was observed in all the products studied, which indicates significant protein denaturation and aggregation under heating. Thermal processing is a major treatment in the processing of fishmeal and protein powders, with temperatures up to 100 °C (Ween et al., 2017). Heating thus causes irreversible denaturation and aggregation of the proteins, resulting in decreased salt solubility (Cai et al., 2018; Chen et al., 2022b). Myosin, actin, and sarcoplasmic proteins are the main components of fish proteins, constituting for 85 - 95% of the total fish proteins. Fish myosin begins to denature at around 35°C; actin is denatured in the temperature range of 58–68 °C, while sarcoplasmic proteins are denatured at around 44 °C (Skipnes et al., 2008; Vieira et al., 2018; Yu et al., 2014). Most fish proteins have thus denatured when temperatures have reached around 75 °C (FAO, 1986). The denaturation temperature of Pacific oyster (*Crassostrea gigas*) salt soluble proteins is about 55 °C (Zhang et al., 2020). The difference in SSP in the different products may be because the available fish proteins denature dependent on the species and/or the different heating applications during the production (Poulter et al., 1985). Excessive heating treatment during drying in traditional fishmeal processing furthermore results in poor functional properties, such as water holding and emulsifying capacities (Ween et al., 2017), and loss of digestibility (Gulati et al., 2017).

The mackerel/herring blend (MHB-FM) and tuna fishmeal (Tuna-FM) showed high **BA** levels, with 9.3 g/kg and 12.8 g/kg, compared to the others, especially with histamine levels (0.8 ± 0.1 g/kg and 3.3 ± 0.1 g/kg, respectively) much higher than the threshold level for human consumption (<0.2 g/kg ww (FAO/WHO, 2013)) (**Figure 22C**). The BW-FM, TC-FMs were free of histamine and met the criteria to be considered as products for human consumption. The animal feed-

intended products originating from marine species (i.e., BW-FM, MHB-FM, Tuna-FM, and PET-PP) had high TVB-N levels compared to the fishmeal from freshwater species (TC-FMs) (**Figure 22D**). Meanwhile, the TC-FMs had lower TMA and DMA contents than the other products. The contents of biogenic amines in TC-FMs were even lower than the HM-PP. The HM-PP, which is produced as a supplement, showed a high nutrient value and low content of unwanted non-protein nitrogen compounds (BA, TVB-N, TMA, and DMA). The fishmeal made from marine species contained high contents of biogenic amines and volatile nitrogen compounds may be due to the improper collecting and storing the initial raw materials. Therefore, improving the quality of raw materials and further process optimization from the studied raw materials are necessary for product development for human consumption.

The results of this study can be used as references in the case of re-designing the current fishmeal processes towards producing protein ingredients for human consumption. The current fishmeal processes need to be redesigned if protein ingredients for human consumption are to be produced.

6 Conclusions

A significant proportion of small pelagic species caught and side streams from industrial fish processing are used to produce fishmeal and fish oil. Meanwhile, demand for high quality nutritious fish protein for human consumption is increasing which provides an opportunity to add value by utilising proteins from these raw materials. The present study was carried out to provide a deeper understanding on the utilisation potential of these materials to produce high-value proteins. The results provide systematic information on the proximate compositions and yield of each side stream from the Tra catfish filleting, which can help the catfish industry in Vietnam to develop appropriate utilisation for each side stream. The study also established optimal conditions to produce fish protein isolates and fish protein hydrolysates from protein rich side streams, and their suitability as ingredients in novel food products.

Each side stream of the Tra catfish production has a unique proximate composition, indicating that combining the side streams, as currently done, limits their utilisation for different purposes. Heads and backbones (HBB) are rich in lipids, proteins, and ash. The trimmings have the highest lipid content, presenting utilisation opportunities to produce lipid rich products. Abdominal cut-offs (ACO) and dark muscle have high protein and low ash content. They may thus be good raw material sources for producing high value protein products. Heads and backbones (HBB), abdominal cut-off (ACO), and dark muscles (DM) are the three main protein-rich streams with a protein content ranging from 14.7–15.5%. However, they differ in lipid and ash contents, and should thus be processed separately into protein products.

Tra catfish protein-rich streams, especially dark muscle, and abdominal cut-offs, can be processed into high-value protein ingredients, which can be used for human consumption, such as isolates and hydrolysates. The optimal extraction conditions to produce protein isolates were obtained at pH 12, a solvent to raw material ratio of 8, and an extraction duration of 150 min, conditions providing optimal protein solubilisation. Most lipid and ash fractions were removed in the production (>90% and >85%, respectively), resulting in a high protein content of the FPIs. The FPIs had a higher protein content and lower lipid and ash contents than industrial surimi, indicating high efficiency of producing protein isolates from industrial Tra catfish side streams using the pH-shift method. All FPIs had good amino acid profiles which agree with the dietary recommendations for adults. The FPIs can be used as protein ingredients in gel-based snack foods,

like surimi and mince, or as ingredients in developing other-value added products.

FPHs were of good nutritional value with high content of essential amino acids, indicating that the FPH can be used as food supplements. Tra catfish side streams should be processed into these high-value proteins separately, adjusting each type of raw material towards producing a specific value-added food product.

The study also investigated the protein quality of the processing streams during fishmeal and fish oil production from small pelagic species in Iceland (**Paper IV**). Cooking, lipid separation, and drying steps need to be improved and optimized in order to produce high-quality products in the future, especially for human consumption. In addition, removal of the viscera and proper collecting, handling, stable cooling, and storing of the raw materials before processing should be applied to improve the safety and quality of the final protein products. Most of the unwanted non-protein nitrogen compounds, including biogenic amines and volatile nitrogen compounds, released into the liquid processing streams, contain mostly water and lipids, while high-molecular-weight proteins were retained in the solid streams. The main streams entered to final fishmeal (press cake, sludge, and latter concentrate) had different chemical properties, supporting that these streams should be processed further separately into final products. This would allow production of higher-quality protein products from the press cake, while the sludge and concentrate could contribute to lower value products.

7 Future perspectives

Although many questions were answered, the study also indicated several new questions for the scientific community and industry to tackle together in the near future. The study assessed the potential of utilising Tra catfish side streams separately, which could add substantial value. However, the study focused on protein content and characteristics, while most earlier studies have focused on the lipid quality. Future studies should investigate the potential utilization of these raw materials in a holistic manner, considering the nutritional, economic, and environmental factors. FPIs and FPHs have good amino acid and proximate composition and are promising as protein ingredients in food applications. Other properties of these FPIs and FPH should be addressed, such as their sensory attributes, functional characteristics and stability during storage and further use. The applications of these ingredients in different food products should be studied in detail.

Protein-rich streams in fishmeal processing of both the BW and MHB such as the press cake, sludge, and concentrate, could potentially be used to produce human food. First the products from the optimized processes should be studied further regarding amino acid composition, digestibility, sensory attributes, functional properties, and stability. It would also be of interest to study applications of the optimized products as ingredients for the development of value-added products intended for human consumption, such as supplements, sport nutrition, and food additives.

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Original publications

Paper I

Value adding potential of side streams from industrial filleting of Tra catfish *(Pangasius hypophthalmus)*

Short title: Side stream composition of Tra catfish

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Abstract

Systematic information gathering on the characteristics of Tra catfish side streams is necessary to evaluate their utilisation potential. Different side streams were collected individually, and their proximate composition and mass flows were analysed. Significant proportions of protein (63%), lipids (97%), and ash (92%) were left underutilised in the side streams. Heads and backbones, contributing to 48% of the total side streams, was rich in lipid (17–29%), protein (15–15.5%), and ash content (6.6–8.7%), showing high potential for protein, lipid and ash-based product development. The trimmings, accounting for 18% of the total side streams, had the highest lipid content (59.2%), allowing effective use in lipid products. Abdominal cut-offs and dark muscle had a high protein content (14.7–15.3%), and low ash content (0.8–1.0%) and may thus be good raw materials for producing high–value protein products. Each side stream should be utilised separately to maximise the value and assure the best possible end products.

Practical applications

The study indicates the quantity and proximate composition of side streams created from the industrial Tra catfish filleting processing. Annually, about 1.1 million tons of side streams, which contain significant proportions of high-quality protein, lipids, and ash, are mostly used underutilized in fishmeal and fish oil production. Each side stream has different proximate composition, thus, the side streams should be collected for producing higher quality products separately to maximise the value and assure the best quality of end products.

Keywords: Backbone; trimmings; dark muscle; cut-offs; proximate composition; mass balance

1. INTRODUCTION

Freezing is the primary method of processing fish for human consumption, contributing to 27% of the total fish production, and 62% of the global consumption of processed fish in 2018 (FAO, 2020). The production of frozen fillets leads to considerable quantities of side streams, or up to 70% of the weight of the fish, including heads, backbones, viscera, cut-offs, trimmings, skin, and blood (Arason et al., 2010; FAO, 2020; Olsen, Toppe, & Karunasagar, 2014; Ucak et al., 2021; Välimaa et al., 2019). Historically, fish side streams have often been thrown away as waste, been used directly as feed, or processed into fish silage, fishmeal and fish oil used in animal feed. However, side streams represent a significant source of nutrition and functional compounds, and their further use has gained increasing attention during the last two decades (FAO, 2020; Zamora-Sillero, Gharsallaoui, & Prentice, 2018).

The proximal composition of fish tissues is highly heterogeneous, which is reflected in the physicochemical properties and composition of different side streams (Alfio, Manzo, & Micillo, 2021), affecting their suitability as raw materials for producing specific functional ingredients for food and health care products (Ucak et al., 2021; Välimaa et al., 2019). Food supplements derived from fish side streams have been shown to positively affect human health and add value (Pateiro, Domínguez, Varzakas, Munekata, Movilla Fierro, & Lorenzo, 2021; Ucak et al., 2021). The main products currently derived from fish side streams are protein concentrates, isolates, hydrolysates, collagen, gelatine, fish oils, and enzymes (Nguyen, Bao, Dang, Tómasson, Arason, & Gudjónsdóttir, 2022; Ucak et al., 2021; Välimaa et al., 2019; Zamora-Sillero et al., 2018). The muscle proteins are produced from the fish muscle tissue, such as myofibrillar and sarcoplasmic proteins, isolates, and hydrolysates. These proteins have been shown to have practical applications in oil and water-holding, fat emulsification, and textural and structural retaining of various restructured food products, such as sausages and fish cakes (Kim & Mendis, 2006; Välimaa et al., 2019; Zamora-Sillero et al., 2018). Collagen and gelatine, which are commonly used as film products, to increase water-holding, emulsion stabilisation, and to increase adhesiveness in meat and fish products (meat binding), are, on the other hand, produced from connective tissue, skin, scales, and bones (Karayannakidis & Zotos, 2016; Kim et al., 2006). Enzymes used in food and pharmaceutical products may also be recovered from the viscera (Fereidoon Shahidi & Kamil, 2001). Fish oils may be produced from the backbone, heads, viscera, cut-offs and skin in fatty fish, and from the liver in lean fish (Alfio et al., 2021; Pateiro et al., 2021). However, little is known about the potential utilisation of the Tra catfish side streams.

Tra catfish is endemic to the waters of the Mekong basin in Southeast Asia. It is the most popular farm-raised fish species in Vietnam, Thailand, Indonesia,

Cambodia, India, and Bangladesh (FAO, 2020; Nam, Van Hoa, Anh, & Trung, 2020; Nguyen et al., 2022) (Figure 1). Tra catfish farming started at the beginning of the 1960s in the Mekong Delta in Vietnam on a small scale to supply daily food for local households. Since the end of the 1990s, the production has grown fast, and in the last two decades, Tra catfish has become the key species in the aquaculture industry in Vietnam, with production increasing from 40 thousand tons in 1997 to 1.6 million tons in 2019 (Figure 1). Vietnam accounts for over half of the global Tra catfish production (Figure 1), and 90% of the international market (Trang Thuy Nhu, Schaubroeck, De Meester, Duyvejonck, Sorgeloos, & Dewulf, 2015; Thong, Ankamah-Yeboah, Bronnmann, Nielsen, Roth, & Schulze-Ehlers, 2020).

Tra catfish has a relatively low market price but is of good quality, and the taste is comparable with other whitefish species such as cod or haddock (Trang Thuy Nhu et al., 2015; Thong et al., 2020). It is predicted that together with tilapia, Tra catfish will account for 62% of the global aquaculture production by 2030 (FAO, 2020). Tra catfish in Vietnam is commonly processed into four products: frozen fillets, fishmeal, fish oil, and extra parts (Trang Thuy Nhu et al., 2015). The frozen white fillets are the most common product, mainly intended for export, accounting for 98% of the current total export value of this species (Dang, Gudjónsdóttir, Tómasson, Nguyen, Karlsdóttir, & Arason, 2018; Nguyen et al., 2022; Thong et al., 2020). However, filleting generates large amounts of varying side streams, consisting of heads, backbones, cut-offs, trimmings, viscera, skin, fins, and blood, accounting for 62–67% of the production, amounting to almost 1 million tons in 2019. Most of these side streams are processed together to produce fishmeal and fish oil. A small percentage of the side streams, such as stomachs and swim bladders, are sold in the local market as edible foods and/or animal feeds (Trang Thuy Nhu et al., 2015).

The inadequate management of fish side streams is one of the main issues the fish industry faces nowadays. The mismanagement of this raw material causes both economic loss and environmental problems. Several studies have shown significant potential to increase the sustainability of the fisheries industry by maximising the edible yield of each species for human consumption via strategic management of side stream utilisation (Campanati, Willer, Schubert, & Aldridge, 2022; Stevens, Newton, Tlusty, & Little, 2018). Effective utilisation of side streams would thus simultaneously improve both the economic and environmental sustainability of a production process. Several studies have been performed on the utilisation of Tra catfish side streams (Nam et al., 2020; Nguyen et al., 2022). However, there are limited data on the quantity (yield) and chemical properties of each side stream of Tra catfish as processed in Vietnam. This limited data and lack of knowledge on the proximate composition of the Tra catfish side streams is a barrier to the potential utilisation of those side streams.

In this study, the proximate composition and mass balances of the main side streams from industrial Tra catfish filleting processing were investigated to identify the side streams that show the most potential for value adding and product development for human consumption.

2. MATERIALS AND METHODS

2.1. Raw materials and sampling

2.1.1 Preparation of fish samples for measurements of yield and proximate composition

Live Tra catfish (*Pangasius hypophthalmus*) were transported in a well boat from a farm to the processing factory in the Mekong Delta in Vietnam in March 2020. The fish were allowed to rest for about 6–10 hours before entering the processing area. The fish were washed and bled for 20–30 min in a water tank at 14 ± 2 °C before entering the filleting process. Three samples of ten fish were randomly collected after bleeding for further studies (n = 3).

A flowchart of the filleting process can be seen in Figure 2. The ***pelvic fins*** were manually cut off before filleting. The rest raw materials from the filleting were manually separated into ***backbones*** (including the fins, tail, some muscle and skin attached to the backbone), ***heads*** (also consisting of some muscle)***,*** and ***viscera***. The ***skin*** was mechanically removed (Nam Dung, Ho Chi Minh, Vietnam), and the skinned fillets entered the trimming area, which had a room temperature of 18 ± 2 °C. ***Trimmings,*** primarily consisting of visible fat tissue on both sides of the belly and skin-side dorsal portions, were manually removed from the skinned fillets. Then the **dark muscle** was manually separated, and the belly flap was cut off the trimmed and skinned fillets to obtain the ***abdominal cut-offs*** and final ***fillets,*** respectively. An overview of the main products (fillets) and side streams from the Tra catfish fillet processing can be seen in Figure 3.

Samples from each side stream were collected separately, and each sample was weighed (IPC WP, Ishida, Kyoto, Japan), and recorded for mass balance calculations. Each sample was minced in a meat grinder (TA57D, Didacta, Torino, Italia) at 16 ± 2 °C, and the minced samples were then block frozen (about 5 kg each block) for 3 hours at −35 to −40 °C in a contact freezer (Mycom, Tokyo 135-8482, Japan), reaching a core temperature of −18 °C to −20 °C. The frozen blocks were cut into 0.5–1 kg pieces, appropriate for sampling for each chemical analysis. The pieces were individually packaged in polyethylene bags, and then placed in styrofoam boxes for transport by a cold truck at −18 ± 1 °C for about 8 hours to the laboratory. Upon arrival, the samples were stored at −25 ± 1 °C until they were analysed within two months. Prior to analysis, samples were thawed at 2–4 °C for 24 hours in a refrigerator. Each sample was measured for proximate composition in duplicate, including water, lipid, protein, and ash contents, as described in Section 2.2.

2.1.2. Chemicals

All chemicals used in the study were of analytical grade and purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich Company (Missouri, United States).

2.2. Proximate composition analyses

Water content of the samples was measured according to ISO 6496 (1999). Samples of 5.0 g were weighed out and placed in small porcelain bowls. The bowls were dried at 103 ± 1 °C for 4 hours (Memmert INE- 600, Germany). The samples were then left in a desiccator to cool to ambient temperature. The water contents of the samples were gravimetrically determined.

Crude protein content was determined using the Kjeldahl method according to ISO 5983–2 (2009). Two grams of minced sample, two Kjeldahl catalyst tablets (each tablet contains 0.4 g $CuSO_4$ and 3.5 g K_2SO_4), and 17.5 mL concentrated H_2SO_4 were put into a Kjeldahl tube for digesting at approximately 420 °C for 2.5 h. The digested product was made alkaline with NaOH before distillation into a 1% boric acid solution. The amount of ammonia nitrogen in the solution was quantified by titration with a standardised 0.2 N HCl solution. The chosen multiplication factor used to convert the nitrogen content into crude protein content was 6.25.

Lipids were determined using the Bligh and Dyer (1959) method. Approximately 25 g sample was homogenised with 50 mL of chloroform, 50 mL of methanol, and 25 mL of 0.88% KCl on ice for 4 min. The homogenised product was centrifuged for 20 minutes at 4 °C at 2500 rpm (MF 600, Biobiz, Incheon, Korea). The chloroform phase (the liquid bottom part), which contained the lipids, was then collected, and filtrated through a glass microfiber filter under vacuum suction. Exactly 2 mL of the chloroform phase were transferred to a glass tube and placed in a vacuum dryer at 55 °C to remove all chloroform solvent. The remaining mixture was weighed to calculate the total lipid content.

The ash content was measured according to the AOAC standard method (AOAC, 2000). About 5 g of sample was weighed into a crucible. The sample was heated at 550 ± 3 °C overnight, and then left in a desiccator for cooling to ambient temperature. The ash content was determined gravimetrically. Water, lipid, protein and ash contents were expressed as a percentage of the total sample wet weight.

2.3. Mass balance during processing

The average quantities and proximate composition of the raw materials (input) and the fillets and side streams (output streams) were used for mass balance calculations. The factory can process 300 tons of raw material per day at full capacity. However, a basis of 1000 kg of bled fish was set as a functional unit

for the mass balance calculations. Materials lost during processing, which included small pieces of meat, blood and liquid (mainly water and lipids), were calculated as the difference between the input weight of bled fish and the combined weight of all output products and side streams.

2.4. Statistical analysis

All data summaries and statistical analyses were carried out using the IBM SPSS Statistics software (Version 22, IBM, 1 New Orchard Road, Armonk, New York, NC 10504-1722, United States) and Microsoft Office Excel 2013 (Microsoft Inc., Redmond, Wash., U.S.A). One–way ANOVA and Tukey's HSD tests were performed on the means of each variable. Pearson's correlation analysis was performed to find the correlations between variables. Significance of difference was defined at the 5% level ($p < 0.05$) for all analyses.

3. RESULTS AND DISCUSSION

3.1. Proximate composition

The proximate composition varied among the filets and different side streams (Figure 4). A significant inverse relationship was observed between the water and lipid contents in the streams in this study ($r = -0.95$), which has been indicated earlier in different parts of Asian catfish (Thammapat, Raviyan, & Siriamornpun, 2010), as well as in different fish species (Egerton, Mannion, Culloty, Whooley, Stanton, & Ross, 2020; Yeannes & Almandos, 2003). The highest water (80.4 ± 1%) and protein content (18.3%), along with the lowest lipid content (2.1 ± 0.2%), were observed in the fillets. However, the lowest water content (33.6 ± 1.4%) and the highest lipid content (59.2 ± 1.1%) were observed in the trimmings, indicating most of the lipid in the Tra catfish flesh is positioned in the subcutaneous adipose tissue and the outer part of the belly. Therefore, the effectiveness of the trimming step affects the lipid content of the final fillet product (Manthey-Karl, Lehmann, Ostermeyer, & Schröder, 2016). The subcutaneous tissue contains a high proportion of dark muscle, which may be rich in polyunsaturated fatty acids (PUFA), which have been linked to certain health benefits (Aursand, Bleivik, Rainuzzo, Leif, & Mohr, 1994; Karlsdottir, Sveinsdottir, Kristinsson, Villot, Craft, & Arason, 2014). However, high PUFA contents also challenge the storage stability of the muscle, as the risk of lipid oxidation is increased with higher PUFA content (Dulavik, Sørensen, Barstad, Horvli, & Olsen, 1998; Karlsdottir et al., 2014). Removing the subcutaneous tissue during skinning will consequently lead to a lower lipid content in the fillets, and can thus be recommended with regards to fillet storage stability (H.T.T Dang et al., 2018). Since the trimmings also make up a high proportion of the side streams (Figure 6), and due to their high lipid content, the trimmings side stream is a promising source for producing fish oil products. However, proper collecting and storing of the trimmings are required to inhibit lipid oxidation. Moreover, attention should be paid to the lipid profile and bioactive compounds

in the trimmings. Further studies on the fatty acid composition and screening of potential target consumer groups will allow production of oil products for human consumption rather than using this material for feed or biofuel, as currently done. Omega-3 polyunsaturated fatty acids are essential to the health of humans. However, several fatty acids cannot be synthesised by the human body. These fatty acids must be supplied through diet, especially by consuming fish, fishery products and fish oil supplements, mainly from the marine capture fisheries (Steffens & Wirth, 2005; Tocher, Betancor, Sprague, Olsen, & Napier, 2019). However, due to the limitation of these sources when facing global population growth, it is necessary to obtain more supplies from aquaculture and fish side streams (Tocher et al., 2019). The fatty acid composition of fish is strongly affected by the fatty acid profile of their feed (Rasmussen, 2001). Fish meal is increasingly being substituted by plant-based or insect-based feed ingredients in fish feed for the Tra catfish (Da, Lundh, Lindberg, & Berg, 2016; Hung, Thanh, Pham, & Browdy, 2015). Although such substitution may lower the production cost, and even lead to some environmental sustainability gains, the quality of the Tra catfish products may suffer since little or no omega-3 unsaturated fatty acids are then provided in the fish feed (Huong Thi Thu Dang et al., 2018; Trang T Nhu, Schaubroeck, Henriksson, Bosma, Sorgeloos, & Dewulf, 2016; Tocher et al., 2019). This indicates that finding a balance between fishmeal versus plant– and insect–based feed ingredients is necessary. Using large amounts of fishmeal and fish oil derived from marine fisheries is an important way to ensure high levels of omega-3 polyunsaturated fatty acids for farmed fish like Tra catfish. More studies on the effect of feed composition on the fatty acid composition of the Tra catfish fillets and side streams should be carried out. By doing that, the market prices of the fillets with substantial levels of omega-3 polyunsaturated fatty acids can potentially be increased, and more valuable oil products could be processed from the Tra catfish side streams.

The crude protein content was highest in the skin (29.7 ± 0.2%), followed by the fillets (18.3 ± 0.1%) (Figure 3). The protein content of the Tra catfish skin in this study was higher than in Alaskan pollock (*Gadus chalcogrammus*) and Pacific cod (*Gadus macrocephalus*) (25% and 24.5%, respectively) (Bechtel, 2003), but lower than in giant catfish (*Pangasianodon gigas*) (43.0%) (Sai-Ut, Jongjareonrak, & Rawdkuen, 2012) and rainbow trout (*Oncorhynchus mykiss*) (41.1%) (Tabarestani, Maghsoudlou, Motamedzadegan, Mahoonak, & Rostamzad, 2012). This shows that the side stream composition is highly dependent on the fish species (Al Khawli et al., 2020). With this high protein content, mainly consisting of collagen and elastin (Le, Nguyen, Tran, Takahashi, & Osako, 2020), the Tra catfish skin could effectively be used as raw materials for the production of collagen and gelatine. On the other hand, the lowest protein content was observed in the trimmings (7.6 ± 0.2%). This is likely because this stream had a high lipid content, as mentioned above. A

significantly negative correlation between protein and lipid content was observed in this study ($r = -0.78$). The proportion of nitrogen in dry weight in different fish parts as affected by the lipid content has been reported by Sikorski, Pan, and Shahidi (1994). A higher lipid but lower protein content in the bones of fatty fish compared to lean fish was also observed by Toppe, Albrektsen, Hope, and Aksnes (2007). Feed composition significantly affects the proximate composition of farm fish, as mentioned earlier. High dietary lipid levels resulted in higher fat deposition in whole farm fish, especially leading to significant subcutaneous fat accumulation (Chaiyapechara, Casten, Hardy, & Dong, 2003). Even though a high lipid content of feed has positive influences on the growth of fish, an increase in body lipid content leads to a decrease in fillet yield (Rasmussen, 2001; Regost, Arzel, Cardinal, Robin, Laroche, & Kaushik, 2001). Also, low protein but high lipid content may lower the utilization potential of the side streams due to increased lipid oxidation and contributed off-flavours (Chaiyapechara et al., 2003).

The water, lipid, and protein content of the fillets were similar as those observed in Tra catfish by Huong Thi Thu Dang et al. (2018), which were 78.5%, 2.6% and 18.8%, respectively, but the water content was higher than observed in the study by Deepitha, Xavier, Layana, Nayak, and Balange (2021) (75.8%). Fluctuations in the proximate composition are common in several fish species, and can be influenced by diet, age, season, and growing conditions (Rasmussen, 2001; Taşbozan, Gökçe, & Erbaş, 2016). Which parts of the fish are sampled also influence the proximate composition, as reflected in the compositional analysis of the different side streams in the current study.

The head had the highest ash content (8.7%), followed by the backbone (6.6%). These bone-rich streams of the Tra catfish (heads and backbone) contained 51.8–57.0% water, 14.8–15.5% protein, and 16.8–28.9% lipid. These side streams can thus be considered valuable sources of protein, collagen, and lipids (Al Khawli et al., 2020; Nam et al., 2020). The boneless side streams, including the dark muscle, abdominal cut-offs, viscera, and trimmings, had similar ash contents (from 0.3 to 1%). A high standard deviation in the lipid content of the dark muscle and pelvic fins indicate high variation in the lipid content between sample replicates. This may be due to the heterogeneous distribution of lipids within the subcutaneous tissue. The handling and precision of the skinning and trimming processes can affect the amount of these tissues attached to the dark muscle and pelvic fins, leading to a large variation in the lipid content both of the pelvic fins, and of the removed subcutaneous dark muscle. The lipid location and distribution in the Tra catfish obtained in the current study are different compared to the lipid distribution observed in white, lean fish species, which have the most lipid content located in the liver (2.3–6%), while trimmings, head, and backbone of the lean species have a very low lipid content (<1%) (Falch, Rustad, & Aursand, 2006). However, the lipid

distribution of the Tra catfish is in agreement with other fatty fish species. Particularly, Aursand et al. (1994) found that in Atlantic salmon, lipid-rich parts were obtained in the dorsal fat region, belly flap, dark muscle, backbone, head, viscera and skin (18.1–38.4%), while the white muscle had a significantly lower value of 9.6%. High lipid contents were obtained in the backbone, head, skin and viscera (17–34%), while the muscle had a lower lipid value (7.9%) in gilthead sea bream (*Sparus aurata*) (Pateiro et al., 2020). The lipid-rich properties of the subcutaneous tissue (making up the majority of the lipid content of the trimmings and dark muscle) have been observed in Atlantic mackerel (*Scomber scombrus*) (24.3–26.2%) (Sveinsdóttir, Sverrisdóttir, Karlsdóttir, Rustad, Arason, & Gudjónsdóttir, 2021). High lipid contents were also obtained in herring (*Clupea harengus*) heads and backbones (32.3%), and in salmon heads (56.4%) (Zhang, Abdollahi, Alminger, & Undeland, 2022). The lipid-rich properties of the fatty fish side streams indicate a high potential for lipid utilization and oil production. However, this is a challenge when using them for protein recovery because high lipid levels are linked to increased risk of oxidation, which may limit further use of the protein products derived from the side streams if not handled properly. Also, fish side streams may be rich in blood, which contains haemoglobin and accelerates lipid oxidation. Using antioxidants may control haemoglobin–mediated lipid oxidation of the side streams, thus stabilising them for further uses, as suggested by several authors (Sajib, Wu, Fristedt, & Undeland, 2021; Wu, Abdollahi, & Undeland, 2021). However, the fish side streams must be processed immediately after filleting unless antioxidants are added (Wu et al., 2021).

The boneless streams, including the abdominal cut-offs and dark muscle, which have high protein content, can be utilised as food fortification ingredients for food products, or to increase the protein content in products, such as mince, protein isolate, hydrolysate products, peptides, and amino acids (Nguyen et al., 2022).

3.2. Mass balances during processing

The weight proportions and mass flows of the different Tra catfish processing streams are displayed in the mass balance shown in Figure 5. The fillet yield from the bled fish was 31.8 ± 0.8%, side streams accounted for 65.3 ± 0.4%, and material loss during processing was 2.9 ± 0.4%. Fillet yield generally ranges between 30–50%, while the side streams constitute up to 70% of fish, depending on species and processing specifications (Olsen et al., 2014). Cultured salmon (*Salmo salar*) and striped bass (*Morone saxatilis*) have much higher fillet yield (> 50% and > 40%, respectively) (Borderías & Sánchez-Alonso, 2011; Rustad, Storrø, & Slizyte, 2011), which may be due to the fact that these fish species are normally processed as skin-on fillets. The fillet yield of the Tra catfish was also lower than commonly obtained in Atlantic cod (*Gadus morhua*) (33.6-43%) (Arason et al., 2010; Falch et al., 2006), saithe (*Pollachius*

virens) (36.9%), and tusk (*Brosme brosme*) (32.3%) (Falch et al., 2006), but comparable with haddock (*Melanogrammus aeglefinus*) (31.9%) (Falch et al., 2006). The main difference in the fillet yield may be because a large proportion of fat edges and dark muscle were removed from the Tra catfish final fillets, while the leaner species, such as cod, saithe and tusk, do not contain a large proportion of fat or dark muscle, and do thus not require these removal steps. However, the fillet yield of the Tra catfish was higher than for several fatty fish, such as horse mackerel (*Trachuru mediterraneus*), which have fillet yields ranging between 27.5–29.9%) (Tzikas, Ambrosiadis, Soultos, & Georgakis, 2007). The fillet yield in this study was comparable with a previous Tra catfish studied by Phan, Kals, Masagounder, Mas-Muñoz, La, and Schrama (2021), with a fillet yield of 31.8%, but lower than the observed value by Nortvedt (2007) (33.9%). The handling can affect the amount of tissues attached to the backbone and trimmings, influencing the fillet yield. Furthermore, catching time, location, the size of the fish, natural variability, different standards of trimming, structural anatomy of the fish, and their farming conditions can also influence the fillet yield of the fish (Borderías et al., 2011; Crouse et al., 2018). Farmed fish may present significant variability in fillet yield. Fish fillet, as the main edible stream of fish, plays the primary economic and nutritional value of fish production for species sold as fillets. Low fillet yield is considered as an unexpected carcass quality property because it leads to economic loss and a higher quantity of side streams with a lower value and utilised limitation. However, flesh quality is the primary consumer concern, dependent on protein and lipid contents, colour, texture and flavour. Improving fillet yield without any negative impact on fillet quality is a significant challenge for farm fish production. This is not a simple task because quality attributes are particularly important for consumers. Genetic selection has been applied to improve the fillet yield but not result in a lower-quality product, as observed in rainbow trout (*Oncorhynchus mykiss*) (Bugeon, Lefevre, Cardinal, Uyanik, Davenel, & Haffray, 2010) and common carp (*Cyprinus carpio*) (Prchal et al., 2018). Also, fillet yield is expected to increase with body weight (Gjerde, Mengistu, Ødegård, Johansen, & Altamirano, 2012), and also lower fat content in body fish can result in a higher fillet yield, as discussed above. However, these higher body weight and lower fat content may link to the climatic conditions (including feeding and stocking biomass) and lead to a higher production cost (Bauer & Schlott, 2009).

The head was the largest side stream, followed by the backbone and trimmings, with weight proportions of 18.0 ± 0.4%, 13.2 ± 0.5% and 12.1 ± 0.7% compared to the bled fish, respectively (Figure 6), equivalent to 27.5 ± 0.6%, 20.3 ± 0.4% and 18.6 ± 0.9% of the total weight of the combined side streams, respectively. The Tra catfish head yield was comparable with values obtained in gadoid species (Falch et al., 2006). However, the yields of trimmings and backbone of the Tra catfish were higher, while the yield of viscera

was lower than the gadoid, which had yield values of 8.2–9.3%, 8.4–10.6%, and 12–15%, for the head, backbone, and trimmings, respectively (Falch et al., 2006). The pelvic fins was the smallest side stream, making up 1.7% of the bled fish, following the dark muscle (3.5%) in the Tra catfish in this study. The skin and abdominal cut-offs had similar yields (4.5% and 4.6% of the total side streams, respectively).

Total unexplained yield losses during the processing process after bleeding (Figure 6) were 2.9%, as mentioned before. The highest contributor to this material loss was in the splitting step (1.7%), followed by the trimming (1.1%), and filleting steps (0.1%). The losses during the filleting and splitting steps may be due to the residual blood and other organic liquids (mainly water and lipids) released from the fish during processing and handling (Uttamangkabovorn, Prasertsan, & Kittikun, 2005). Furthermore, during trimming, some muscle and fat got stuck in the processing machinery, resulting in an overall material weight loss of 1.1%.

After the filleting process, about 37.1% of the total protein remained in the fillets (main products), while only 3.2% of the total lipids and 7.6% of the total ash followed the fillets. Most of the total lipids (96.8%), ash (92.4%) content, and a large part of the protein content (62.9%) were left in the side streams. These results show that there is still high potential in recovering and utilising the side streams into high–value products. The possibility of producing food–grade fish protein, such as isolates and hydrolysates, and oils can bring higher benefits than the current fishmeal or traditional silage processes, as indicated by Venslauskas et al. (2021). Among side streams, the lipid contribution was highest in the trimmings (37% of the total side stream lipids), followed by the backbone (19%), head (16%), and viscera (15%). Meanwhile, the head, backbone, and skin were the side streams containing significant amounts of protein (30%, 20% and 14% of the total protein in side streams). However, the composition and quality of the proteins may be different in different streams (Nanton, Vegusdal, Rørå, Ruyter, Baeverfjord, & Torstensen, 2007; Sikorski et al., 1994). The muscles contain mainly myofibrils (40–60% of the total crude protein), sarcoplasmic proteins (30% of the total crude protein), and a small proportion of connective proteins (collagens) (F Shahidi, 1994). Meanwhile, the proteins of bones, skin, fins, and heads are principally collagens, indicating that the latter Tra catfish side streams are promising for collagen and gelatin production (Karayannakidis et al., 2016).The composition and quality of the lipids may also differ in different streams (Nanton et al., 2007; Sikorski et al., 1994). Different fatty acid profiles from different fish streams have been shown in sardine (*Sardinella lemuru*) (Khoddami, Ariffin, Bakar, & Ghazali, 2009) and Indian mackerel (*Rastrelliger kanagurta*) (Sahena, Zaidul, Jinap, Yazid, Khatib, & Norulaini, 2010) in support of this notion.

Most ash content was found in the bony streams, with 55% of the composition obtained in the head, and 30% in the backbone side streams. Studies have demonstrated that the bony streams are rich in calcium, phosphorus, sodium, and potassium (Kandyliari et al., 2020; Välimaa et al., 2019). Therefore, these streams can be utilised for the production of minerals or mineral-containing compounds, which are used as additives or ingredients in human food/food supplements and animal feed or medical materials, as investigated in earlier Tra catfish study (Nam et al., 2020).

4. CONCLUSIONS

The production of Tra catfish has been growing steadily during the current decade in Vietnam. Most is used to produce frozen fillets for export (97% total production). Side streams constituted 65.3% of the total fish production, including about 63% of the total proteins, 97% of the lipids, and 92% of ash. It means that, with an annually production of 1.6 million tons (2019), it forms about 1.1 million tons side streams, containing 153 thousand tons of protein, 314 thousand tons of lipid and 43 thousand tons of ash. Traditionally, the Tra catfish side streams are mainly used in unsorted form to produce fishmeal and fish oils. To produce various value-added products, it will be necessary to recover and utilise the side streams more effectively.

There is promising potential to utilise the side streams and produce various valuable products based on their specific chemical composition. Different proximate compositions of the side streams may result in different nutritional and functional properties. Therefore, using the side streams all together in their unsorted forms, as currently done, limits the utilisation of the different types of side streams for different purposes. Furthermore, mixing the side streams together may contaminate valuable tissues with undesirable enzymes, lipids, pigments and blood, which accelerate lipid oxidation and quality degradation. Therefore, side streams should be sorted and utilised separately to maximise the value of each stream and assure the best possible end products.

The proximate compositions suggested the potential exploitation of processing these side streams into various end products. In the Tra catfish flesh, most of the lipids are found in the subcutaneous tissue and belly regions, resulting in high lipid content in the trimmings (59.2 ± 1.1%). With a high proportion (19% of total rest material), the trimming is thus a high potential source for producing fish oil products. Proper collecting and storing of these parts are necessary to inhibit lipid quality changes, and further studies on the fatty acid composition are necessary to find the optimal utilization for the fish oils produced from these parts.

The head and backbone side stream may be used to recover protein/collagen/gelatine, lipids, and minerals. Further studies on the protein,

fatty acid, amino acid, and ash profiles are necessary to show the potential for utilisation of these side streams into high–value products. The Tra catfish industry produces a considerable quantity of fillets. A significant lipid amount remains in the side streams. It is necessary to have more studies on the effect of the fish feed composition to improve the nutritional and bioactive characteristics of Tra catfish. Improved feed composition may result in fillet products and side streams with high levels of omega-3 polyunsaturated fatty acids, leading to higher fillet market value and producing high value fish oils from the side streams. The boneless streams, including the abdominal cut-offs and dark muscle, contain a high protein content, and can be utilised as ingredients for food products, or for the production of protein isolates, peptides or hydrolysate products.

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Figures legends

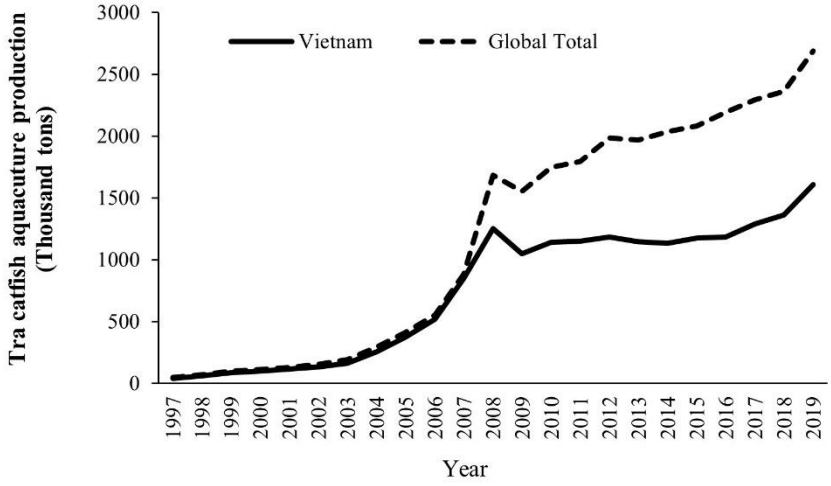

Figure 1. Tra catfish aquaculture production (thousand tons) from 1997 to 2019 (FishStatJ, 2022).

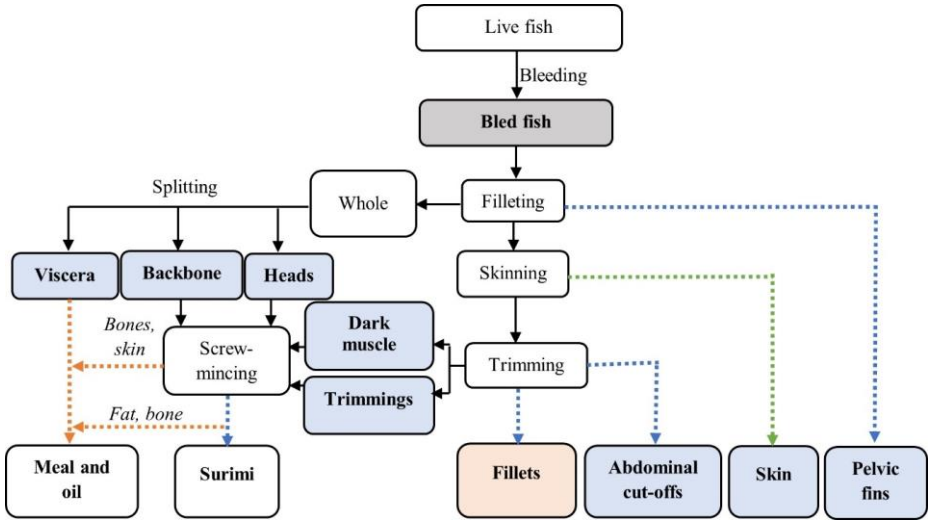

Figure 2. The industrial Tra catfish processing streams. The blue lines indicate the processing streams destined for human consumption, the orange lines represent processing streams intended for animal feed products, and the green indicates side stream material for producing collagen/gelatine. Colour-filled boxes: samples were collected, weighed, and measured chemical composition. The flow chart was adapted from (Nguyen et al., 2022).

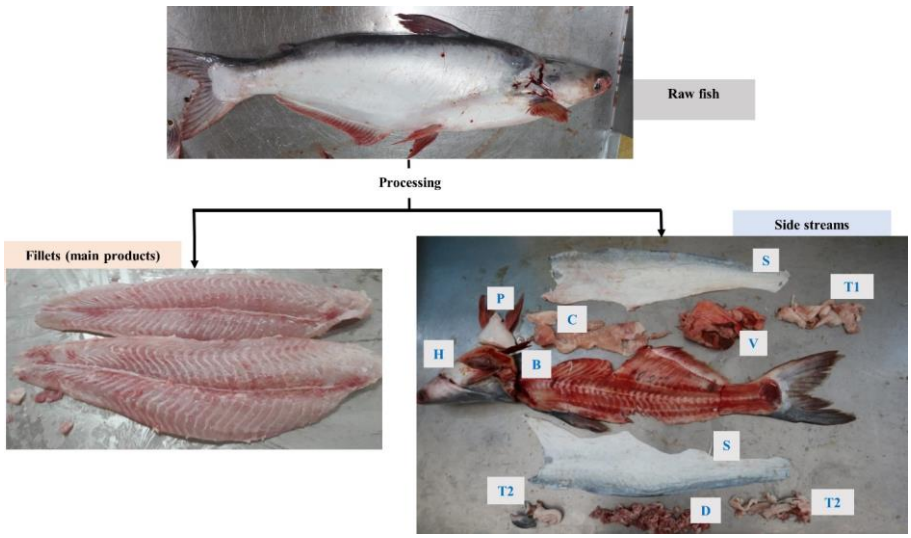

Figure 3. Overall main products (fillets) and side streams from the Tra catfish industrial fillet process.

Abbreviations: H: head; B: backbone; V: viscera; S: skin; C: abdominal cut-offs; T: trimmings (T1: fat collected from both sides of the belly, T2: fat collected from the skin-side dorsal portions); D: dark muscle and P: pelvic fins.

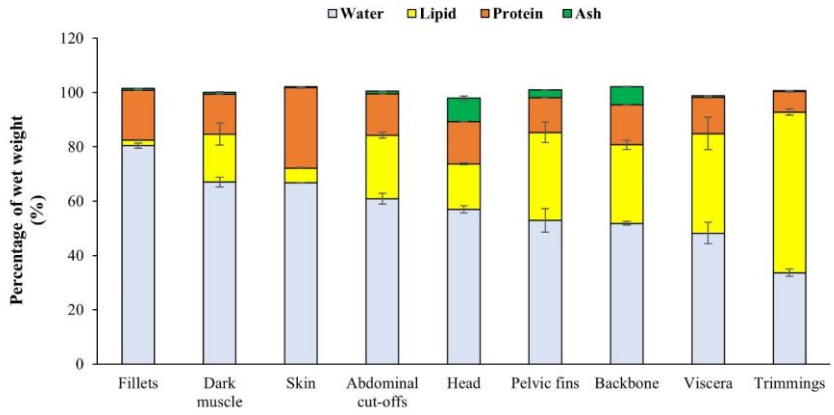

Figure 4. Proximate composition of different streams from Tra catfish filleting processing (n = 3, mean ± SD).

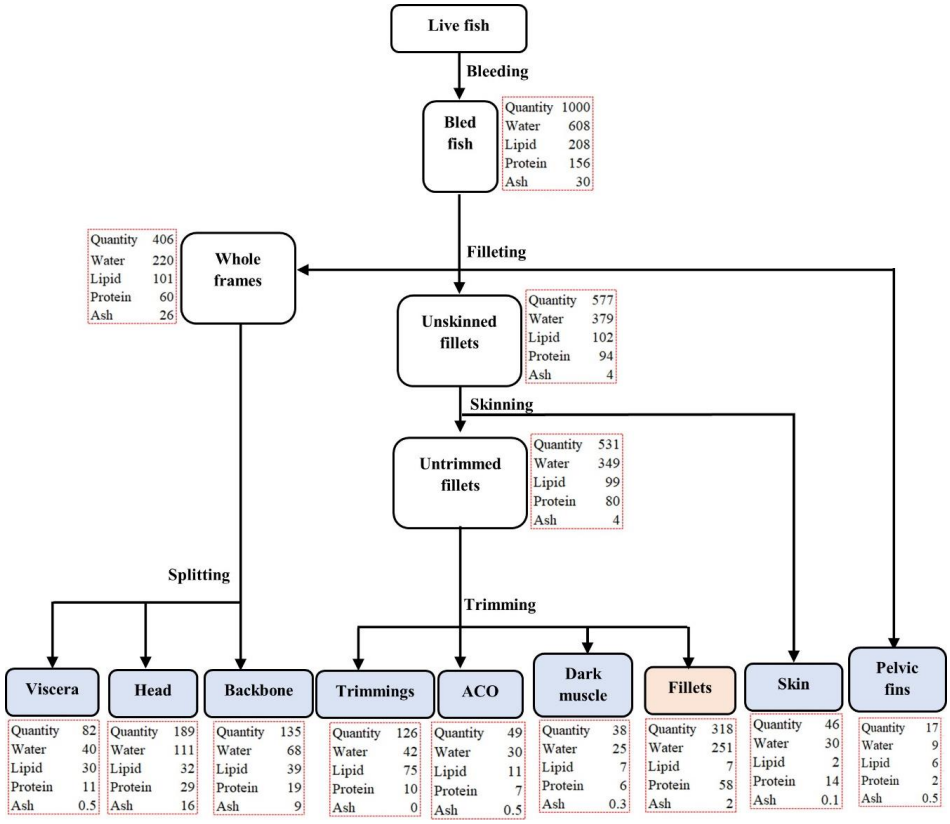

Figure 5. Mass balance of the Tra catfish fillet production process. Blue-filled boxes indicate the separated streams matching Figures 1&2. Mass balance from 1000 kg bled fish was calculated and shown in red boxes. The quantity of each processing stream and the amounts of water, lipid, protein, and ash in each stream were expressed in kg. ACO: abdominal cut-offs.

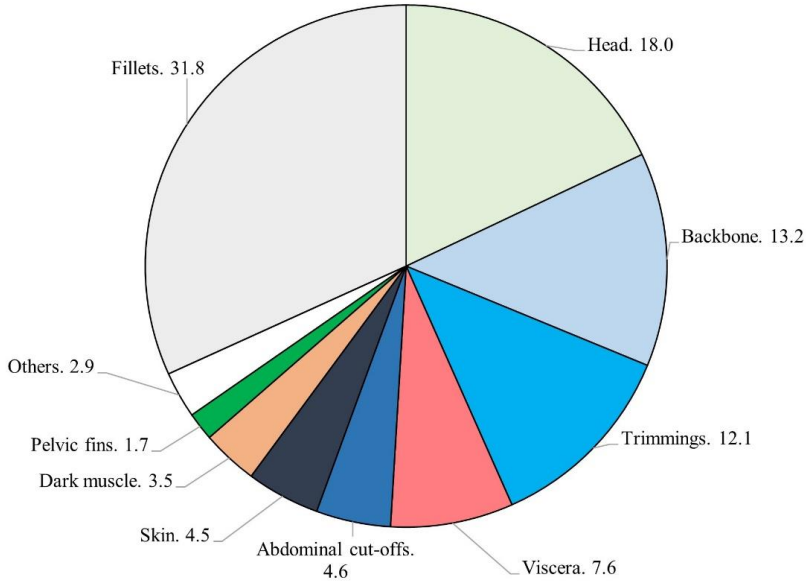

Figure 6. Mass ratios between the Tra catfish streams (% of the total weight of bled fish

Paper II

foods

MDPI

Article

Protein Recovery of Tra Catfish (*Pangasius hypophthalmus*) Protein-Rich Side Streams by the pH-Shift Method

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Abstract: Increasing protein demand has led to growing attention being given to the full utilization of proteins from side streams in industrial fish processing. In this study, proteins were recovered from three protein-rich side streams during Tra catfish (*Pangasius hypophthalamus*) processing (dark muscle; head-backbone; and abdominal cut-offs) by an optimized pH-shift process. Physicochemical characteristics of the resulting fish protein isolates (FPIs) were compared to industrial surimi from the same raw material batch. The pH had a significant influence on protein extraction, while extraction time and the ratio of the extraction solution to raw material had little effect on the protein and dry matter recoveries. Optimal protein extraction conditions were obtained at pH 12, a solvent to raw material ratio of 8, and an extraction duration of 150 min. The resulting FPI contained <10% of the fat and <15% of the ash of the raw material, while the FPI protein recovery was 83.0–88.9%, including a good amino acid profile. All FPIs had significantly higher protein content and lower lipid content than the surimi, indicating the high efficiency of using the pH-shift method to recover proteins from industrial Tra catfish side streams. The FPI made from abdominal cut-offs had high whiteness, increasing its potential for the development of a high-value product.

Keywords: Tra catfish; dark muscle; cut-offs; protein isolate; protein; lipid removal; amino acids

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1. Introduction

Fish is a limited resource, and the global demand for fish protein is increasing faster than can be met with traditional resources [1–3]. Therefore, recovering protein for human food from side streams in industrial fish processing is of great interest. Side streams can be used directly as food or processed into fish-based protein foods such as sausages, cakes, snacks, sauces, or other products such as gelatin, dietetic products, pharmaceuticals, natural pigments, cosmetics, or constituents in other products [4–6]. The improved utilization of fish side streams may thus increase products for human consumption and add value.

Proteins are found in all parts of fish, but different side streams may have different protein content and composition [7,8]. There are three main categories of proteins in fish: structural proteins, sarcoplasmic proteins, and connective tissue proteins, which have different physiochemical properties [9]. About 30–50% of the muscle is usually left on the frame during filleting [9]. These muscle proteins are highly nutritious and digestible, and flesh from frames and abdominal-cut offs can be used to produce mince, surimi, or surimi-based products [10]. There are two types of muscle in finfish: white and dark muscle. The dark muscle is used for continuous swimming action, while the white muscle supplies rapid energy bursts. Due to high levels of pro-oxidants, such as heme protein and iron, and its often-unwanted colour, the dark muscle is commonly removed from the fillets in

the trimming step of fatty fish processing [11–14]. However, the dark muscle is used to produce fishmeal, pet food, fish silage, and fertilizer at a low economic value. Nevertheless, as it contains high-quality proteins and bioactive protein-derived compounds, it also has potential for use in food and healthcare products [15–17]. Several studies have been carried out on processing the dark muscle from several species into functional proteins such as hydrolysates, isolates, and peptides [16–18].

Tra catfish are commonly farmed in Southeast Asia, including Vietnam, Thailand, Indonesia, and Cambodia. They are currently the most important farmed freshwater species in Vietnam [19,20]. Globally, Vietnam is the third-largest exporter of fish and fish products, with most of its revenues coming from farmed Tra catfish. Vietnam is also the biggest producer of Tra catfish, with nearly 1.42 million tonnes in 2018 [21]. Over 97% is processed into frozen, white fillets for export [22]. However, only about 40% of the fish processed is used for human consumption [23]. The growth in Tra catfish production means an increase in its side streams (head, backbone, skin, cut-offs, trimmings, and viscera), which account for 62–67% of production [24,25]. The side streams from the production of fillets thus amounted to over 800,000 tonnes in 2018 [19]. Traditionally, these side streams have mostly been used to produce fishmeal and silage which are of low economic value and generate limited profits [24–28]. Some of the fishmeal produced is used to produce feed for Tra catfish which does not require a high proportion of animal protein in its diet [29]. Recently, the industry is producing more fishmeal than needed for feed production. The large use of fishmeal in aqua-feeds has been reported as a significant environmental impactor, causing global warming and eutrophication [30]. Fishmeal and fish oil in their diets can be significantly replaced by various alternative feed ingredients from plant origin, insects, microalgae, microbial proteins, and seaweed, which have lower prices. Another common use of Tra catfish fishmeal is for tilapia, shrimp, poultry and pig feeds [29]. Some of these side streams have, in recent years, been utilized for producing surimi, although the surimi industry started in Vietnam in the 1990s [31]. However, the processing industry has the potential to make more profit from the production of protein isolates and hydrolysates used in food ingredients in value-added products, and/or for feed intended for juvenile fish and other farmed animals that require protein-rich and highly digestible diets. The utilization of these side streams could thus bring profit to processing companies far beyond the margins of selling fish fillets and only recycling the side streams within the industry, as is the current situation.

Side streams have high lipid and ash content, ranging from 15.3 to 29.8% and 2.4 to 5.7%, respectively [19], which can limit their direct use as human foods. Several authors have studied the use of Tra catfish side streams to recover proteins [19,32–36]. Still, there has been limited work completed on the use of dark muscle and on comparing the properties of FPIs recovered from different side streams. In order to maximize the utilization of individual side streams, the physicochemical characteristics of different side streams should be studied.

The pH-shift method is commonly used to recover or isolate proteins from fish side streams [17,37]. The preparation of fish protein isolates includes three main steps: (i) the solubilization of proteins at low or high pH ($\leq$3.5 or $\geq$10.5); (ii) the removal of fat and other impurities by using a high-speed centrifuge; and (iii) the precipitation of the protein at their isoelectric point (pH = 5.5) [37]. FPI can be frozen for later use, such as for surimi or mince production [38]. However, as mentioned earlier, different side streams may have specific water, protein, lipid, ash, and pigment composition. Fish proteins from side streams may also be contaminated by other tissues, such as the skin, backbone, or blood [37], which can be barriers to successful protein recovery from the side streams and may challenge the stability of the protein product during storage. High lipid and pigment content, such as haemoglobin and myoglobin from the blood, may cause rancid, fishy odours in the final product [37]. Blood contamination often occurs in current production systems where side streams are commonly processed together. However, processing FPIs from each side stream separately could potentially increase the quality and value of the final products.

In this study, FPI was produced from the dark muscle, head and backbone blends, and abdominal cut-offs using the pH-shift method. The proximate composition, colour and protein composition, and properties of the different FPIs and surimi produced from the same processing side stream batches were analysed and compared.

2. Materials and Methods

2.1. Raw Materials and Sampling

2.1.1. Collection of Side Streams

A flowchart of the industrial Tra catfish filleting process is presented in Figure 1. The fish were bled and the pelvic fins manually removed before filleting. The rest raw materials from the filleting were separated into ***heads*** (also consisting of some flesh), ***backbones*** (including the tail, fins, and some skin and muscle), ***viscera,*** and ***pelvic fins***. The untrimmed fillets were then passed through a **skin** removal system (Nam Dung, Ho Chi Minh, Vietnam). The skinned fillets were then transferred to the trimming area, which had a room temperature of 18 ± 2 °C. Fat edges (mainly subcutaneous fat and some flesh) removed from the skinned fillets were considered ***trimmings***. Thereafter, the ***dark muscle*** of the fillet was removed manually, and the belly flap was cut off the trimmed and skinned fillets to obtain the ***final fillets*** and ***abdominal cut-offs.***

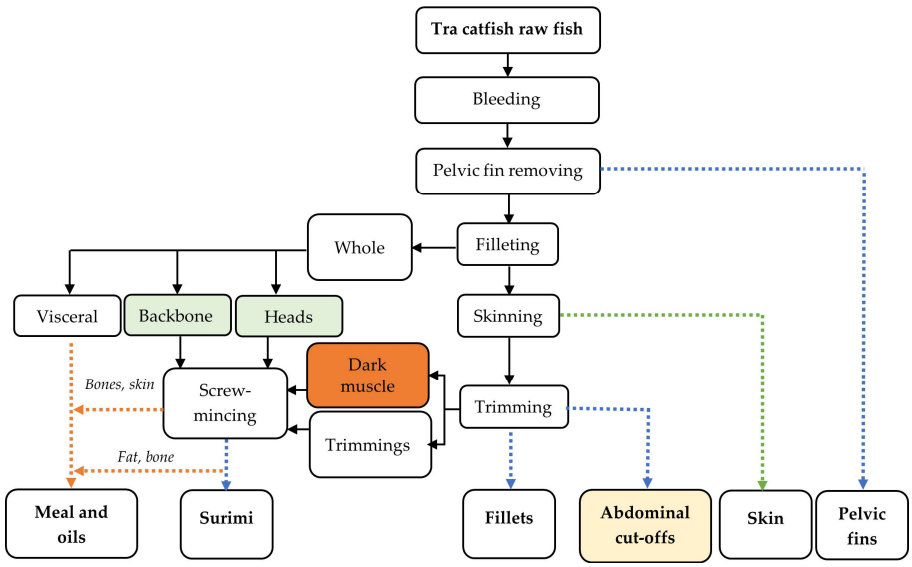

Figure 1. Industrial Tra catfish processing streams. The blue line indicates the stream producing products for human consumption, the orange represents the stream used to produce animal feed, and the green colour shows material for producing functional products. Colour-filled boxes indicate the material collected for optimization protein recovery by the pH-shift method.

Dark muscle, head and backbone blend (HBB), and abdominal cut-offs (ACO) were collected and measured separately for this study (see the further list of abbreviations in Table S1). Each sample was minced evenly using a meat grinder (TA57D, Didacta, Torino, Italia) in a cool room (16 ± 2 °C). The minced samples were then block frozen (5 kg per block) in a contact freezer (Mycom, Tokyo 135-8482, Japan) for 3 h at −35 to −40 °C, reaching a core temperature of −18 °C to −20 °C. The frozen blocks were then cut into 0.5–1 kg pieces appropriate for chemical analyses and trials. The pieces were individually packaged in polyethylene bags, and then put in styrofoam boxes for transport to the laboratory by a cold truck for 8 h. Upon arrival, the samples were stored at −25 ± 1 °C

until they were analysed and were used within three months. Prior to use, samples were left in a refrigerator at 2–4 °C for 24 h to thaw.

2.1.2. Chemicals

All chemicals used in the study were of analytical grade and purchased from Sigma-Aldrich (Missouri, TX, USA) and Merck (Darmstadt, Germany).

2.1.3. Preparation of Protein Isolate from the Dark Muscle

The production of FPI using the pH-shift method [39] was performed at different ***pH*** values, ***extraction ratios,*** and ***extraction times*** of the protein solubilization step in order to find the optimal FPI production settings. A flow chart for the optimization of protein recovery from dark muscle is shown in Figure 2.

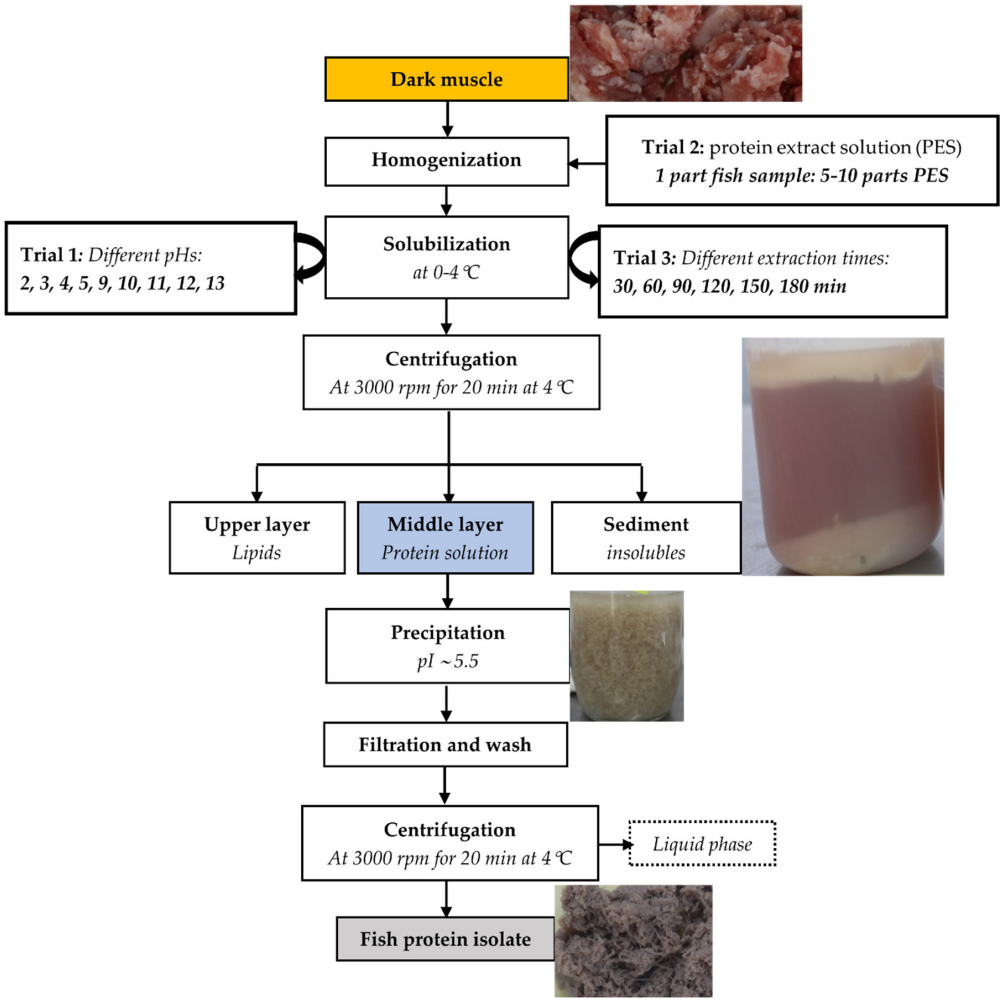

Figure 2. Experimental design for the optimization of protein recovery from Tra catfish dark muscle, carried out through 3 trials. Orange-filled box: protein and water content measured; blue-filled box: weight of the protein solution recorded and its protein content determined; grey-filled box: weight recorded, water and protein content determined.

First, the pH of the protein solubilization was optimized during Trial 1 (Figure 2). Different amounts of either 2N HCl or 2N NaOH solutions were added to distilled water to obtain ***protein extract solutions (PES)*** with acidic pH 2, 3, 4 and alkaline pH 9, 10, 11, 12, and 13, adjusting the pH using a pH meter (Portavo 904 X, Knick, Berlin, Germany). About 200 g of minced dark muscle was homogenized (ULTRA- TURAX® T18 basic, IKA, Germany) with 1600 mL of cold PES for 1 min, and the pH of the mixture was adjusted with 2N HCl and 2N NaOH solutions to obtain the targeted pH. The blends were kept in a fridge for 60 min at 0–4 °C for protein solubilization. The cold suspension was then centrifuged at 3000 rpm for 20 min at 4 °C (MF 600, Biobiz, Incheon, Korea). The supernatant (***protein solution***) was weighed and determined for protein content using the Bradford method [40] to evaluate the ***protein extractable recovery (PER)***. PER was also measured at pH 5. The protein solution was then adjusted to pH 5.5 (isoelectric protein point (PI)) using 2N NaOH and 2N HCl to precipitate the protein [41,42]. The aggregated precipitates were filtered through a nylon monofilament bag (mesh size: 25 micron; Dong Son Ltd., Vietnam), and the mixture was washed with distilled deionized water to a neutral pH. The mixture was then centrifuged at 3000 rpm for 20 min to dewater and remove the NaCl to form the final ***fish protein isolate (FPI)***. The FPI was weighed and measured for water and protein contents to evaluate the ***protein recovery (FPI-PR)*** and ***dry matter recovery*** (FPI-DMR), as described in the analysis section.

Trial 1 indicated that pH 12 was optimal for the solubilization step of the FPI production and was, therefore, selected for the subsequent trials. The effects of using different ratios between PES and dark muscle (***extraction ratio***, *v*:*w*) were evaluated, with ratios ranging between 5 and 10 times (*v*:*w*). The PER, FPI-PR, and FPI-DMR were determined as above.

During Trial 2, a ratio between PES and the raw material of 8 (*v*:*w*) was found to be optimal for protein extraction and was thus used in further experiments. The FPI was then produced at ***different extraction times***, including 30, 60, 90, 120, 150, and 180 min in Trial 3. The PER, FPI-PR and FPI-DMR were investigated in the same procedures as above. Each experiment was performed in triplicate.

2.1.4. FPI Preparation from Different Side Streams at the Optimal Conditions

The protein isolates were produced from the HBB and ACO at the above-optimized conditions. FPI-PR, FPI-DMR, lipid and ash removal effectivity were compared at the previously determined optimal conditions for all protein isolates made from dark muscle, HBB, and ACO. In addition, quality properties, including proximate composition, amino acid profiles, protein patterns, and colour of the protein isolates, were evaluated and compared with commercial surimi produced at the company from the same batch of rest materials.

2.2. Analyses

2.2.1. Proximate Composition

Water content of the samples was determined according to ISO 6496:1999. Approximately 5.0 g of sample was placed in a small porcelain bowl. The bowl was dried in an oven at 103 ± 1 °C for 4 h, then allowed to cool to ambient temperature for about 30 min in a desiccator before the weight was recorded again.

The crude protein content of the samples was measured using the Kjeldahl method according to ISO 5983-2:2008. About 2 g of minced sample was digested in 17.5 mL concentrated sulphuric acid with copper sulphate added as a catalyst at approximately 420 °C. The digested mixture was made alkaline with NaOH, and the nitrogen was distilled off as NH_3. The NH_3 was "trapped" in a 1% boric acid solution. The amount of ammonia nitrogen in the solution was measured by titration with a standardized H_2SO_4 solution. A nitrogen conversion factor of 6.25 was used to calculate crude protein content.

Lipids were measured according to the Bligh and Dyer [43] method. About 25 g of the sample was homogenized with 50 mL of chloroform, 50 mL of methanol and 25 mL

of 0.88% KCl for 4 min. The mixture was centrifuged at 4 °C for 20 min at 2500 rpm. The chloroform fraction (the liquid bottom part) which contained the lipids was then collected and filtrated through a glass microfiber filter under vacuum suction. Exactly 2 mL of the chloroform fraction was pipetted into a glass tube and placed in a vacuum dryer at 55 °C to remove the chloroform solvent. The remaining mixture was weighed to measure the total lipid content.

The ash content was determined according to the method of the Association of Official Analytical Chemists (AOAC, 2000). About 5 g of the sample was weighed into a crucible. The sample was heated overnight at 550 ± 3 °C and then cooled down in a desiccator before being weighed. Ashes were quantified gravimetrically. The water, crude protein, lipid and ash contents were expressed as a percentage of wet weight.

2.2.2. Protein Extractable Recovery (PER), FPI Protein, and FPI Dry Matter Recoveries

Protein extractability was defined as a percentage of the protein extracted into the solution during the solubilization step compared to the initial raw material protein, calculated using Equation (1).

$$\text{PER } (\%) = \frac{\text{Protein content of the protein solution}}{\text{Protein content of the raw material}} \times 100 \quad (1)$$

FPI protein recovery was defined as the recovered protein amount compared to the protein content of the initial raw material, as calculated by Equation (2).

$$\text{FPI-PR } (\%) = \frac{\text{Protein content of the FPI}}{\text{Protein content of the raw material}} \times 100 \quad (2)$$

The protein content of the solution was measured using the Bradford method [40]. Exactly 50 µL of soluble protein sample was mixed with 2.5 mL of Bradford reactive solution, then incubated for 25 min at ambient temperature. The absorbance was read at 595 nm in a DR6000 UV-VIS spectrophotometer (HACH, Düsseldorf, Germany). The protein content was calculated using a standard curve made with bovine serum albumin with concentrations ranging between 0.1 and 1.4 mg/mL.

The protein content of the FPIs and the initial raw material was extracted according to Mæhre et al. [44]. Approximately 1 g sample was homogenized with 60 mL of 0.1N NaOH in 3.5% NaCl solution. The mixture was then incubated for 90 min at 60 °C following centrifugation at 5000 rpm at 4 °C for 30 min (TJ-25 Centrifuge, Beckman Coulter, CA, USA). The supernatants were measured for protein content using the Bradford method, as described above.

FPI-DMR was defined as the dry matter content recovered compared to the dry matter from the initial raw material during the FPI production, as calculated according to Equation (3).

$$\text{FPI-DMR } (\%) = \frac{\text{Dry matter content of the FPI}}{\text{Dry matter content of the raw material}} \times 100 \quad (3)$$

2.2.3. Lipid and Ash Removal during FPI Production

Lipid and ash removal (%) during FPI production was assessed by the proportional weight loss of lipid/ash during the production compared to the lipid/ash amount in the raw material. The weight loss of lipids and ash during FPI production was calculated by the initial amount in the raw material minus the amount in the FPI produced.

2.2.4. Amino Acid Analysis

The amino acid composition of the FPI samples was measured using the liquid chromatography–mass spectrometry (LC-MS) method according to ISO 13903:2005. Amino acids were liberated from the protein using an EZ: faast LC-MS kit system (Phenomenex, Torrance, CA, USA). An ion-exchange chromatography separated the individual amino

acids through the EZ: faast AAA-MS column (250 mm × 2.0 mm, 4 µm) (Phenomenex, Torrance, CA, USA). They were then detected by ninhydrin reaction at λ 570 nm (λ 440 nm for proline) in the Shimadzu LC-MS 8030 system (Kyoto, Japan). Amino acid content was expressed as g/100 g crude protein.

2.2.5. SDS-PAGE Pattern

Sodium dodecyl sulfate (SDS) slab gel electrophoresis with dimensions of 140 × 140 × 1 mm was performed according to the modified method of Laemmli [45] using a precast gel Mini-protean TGX 10% (Bio-Rad Lab., Inc., Hercules, CA, USA). The running buffer contained 3 g/L Tris base, 14.4 g/L glycine, and 1g/L SDS in deionized water (pH 8.3). About 5 g of the sample was extracted with 25 mL of 1M NaCl buffer (pH 7.0) and diluted to a 2 mg/mL concentration. Then, 10 µL of the protein extracts were mixed well with 10 µL 4x Laemmli SDS loading buffer (1610747, Bio-Rad Lab., Inc., CA, USA) containing β-mercaptoethanol and placed into a 96-well plate. The sample wells were then entirely covered by an appropriate tape piece and heated at 100 °C for 5 min in a PCR heating block (Bio-Rad Lab., Inc., CA, USA). Then, 10 µL of the mixture was loaded into the electrophoresis gel well. Electrophoresis was conducted in a Mini-protean®Tetra Cell (Bio-Rad Lab., Inc., CA, USA) coupled with an electrophoresis power supply (Pharmacia Biotech, Uppsala, Sweden) at a constant current of 30 mA per gel until the tracking blue dye front reached the end of the gel. The gels were collected and stained in a dye solution (containing 0.05% Coomassie blue, 25% isopropanol and 10% acetic acid) overnight. The gels were then de-stained with a 10% acetic acid solution until the gel background was clear for photography by a Canon photo scanner (Tokyo, Japan). The molecular weight of protein bands was estimated using the Spectra^{TM} multicolour broad range protein ladder (10-260 kDa) (Thermo Fisher Scientific, Waltham, MA, USA).

2.2.6. Colour

The intensity of the colour was determined with a Minolta Chroma Mette CR-400 (Minolta, Osaka, Japan) using the CIE Lab system as described by Abdollahi et al. [46]. The instrument recorded the L * value (brightness) on a scale of 0 to 100 from black to white; the a * value (redness) from −60 to 60, where a > 0 represents the red component and a < 0 represents the green component; and the b * value (yellowness) from −60 to 60, where (+) stands for the yellow component and (−) stands for the blue component. The whiteness was calculated using Equation (4), as described by Surasani et al. [47]:

$$\text{Whiteness} = 100 - \sqrt{(100 - \text{L}*)^2 + \text{a}*^2 + \text{b}*^2} \quad (4)$$

2.2.7. Statistical Analysis

All data summaries and statistical analyses were carried out in Microsoft Excel 365 (Microsoft Inc., Redmond, Washington, DC, USA) and IBM SPSS Statistics software (Version 22, IBM, 1 New Orchard Road, Armonk, New York, NY, USA). One-way analysis of variance (ANOVA), Tukey's HSD tests, and Student's t-tests were performed on means of the variables. All statistical analyses were performed assuming a significant difference set to the 5% level ($p < 0.05$).

3. Results and Discussion

3.1. Optimization of Protein Recovery from Dark Muscle

3.1.1. Effects of pH on Extraction Levels (Trial 1)

The minimum PER was observed at pH 5 (17.9 ± 3.7%) and gradually increased as the pH moved up or down (Figure 3). Protein extraction recovery (PER) is affected by the protein solubility at a given pH, and the protein solubility depends on the electrostatic and hydrophobic interactions between the protein molecules. When electrostatic repulsion is higher than the hydrophobic interactions, protein solubility increases and vice versa [48]. Proteins become positively or negatively charged at a pH lower or higher than their PI. This

leads to electrostatic repulsion between molecules and the hydration of charged residues (protein–water interactions increase). The PI of fish protein is commonly around 5.5 [37,49].

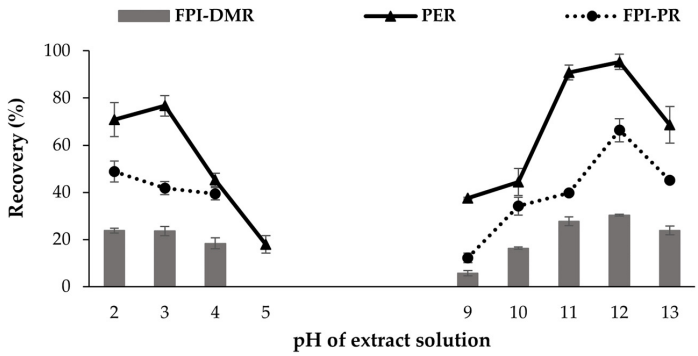

Figure 3. Effect of pH on protein extraction recovery (PER, %), FPI protein recovery (FPI-PR, %), and dry matter recovery (FPI-DMR, %) of the fish protein isolate produced from Tra catfish dark muscle. The protein extraction was carried out for 60 min with a volume/weight extraction ratio of 8.

Protein extraction yield is greater under alkaline than acid conditions, as reported by Zayas (2012) and Abdollahi and Undeland [50]. This may be due to the denaturation of proteins during harsh acid treatment. Denatured proteins will be lost in the sediment after the centrifugation of the extraction mixture [51,52]. The highest PER was obtained at pH 12 (95.3 ± 3.3%) ($p < 0.05$). Higher PER also resulted in higher FPI protein recovery (FPI-PR) and FPI dry matter recovery (FPI-DMR), as expected. A similar effect of the extraction pHs on the PER, FPI-PR, and FPI-DMR was indicated in this study. The highest FPI-PR and FPI-DMR were obtained at pH 12, with values of 70.9 ± 4.8% and 30.3 ± 0.4%, respectively ($p < 0.05$). Although the protein recovery values at pH 11 were lower than the corresponding values at pH 12 ($p < 0.05$), the FPI-DMR was not significantly different between these two pH values ($p > 0.05$). These results reflect that the lipid that remained in the FPI at pH 11 was higher than at pH 12. This is in agreement with the study of Chen and Jaczynski (2007), which stated that the pH of extraction buffers can influence the lipid content of the final FPI. Alkaline washing has, furthermore, been found to be more effective in removing lipids than using water with a lower pH for washing [42].

The optimal pH for FPI production from the dark muscle occurred at pH 12, which is within the range obtained in a previous study, which stated an optimal pH of 11.5 and 12.5 for salmon and herring rest materials, respectively [50]. Several previous studies have reported the maximum solubility of fish muscle protein at pH 12, such as in tilapia frame rest materials [53] and salmon rest materials [50]. Nevertheless, this pH value was slightly different from the pH of 10~11 recommended by Kristinsson, Lanier, Halldorsdottir, Geirsdottir and Park [17], and the pH 13 as recommended by Rohu for rest materials [47].

3.1.2. Effects of Using Different Proportions of Extract Solution (Trial 2)

Solubilization is performed using extraction solution in a 5–10 times ratio compared to the raw material [17]. PER increased slightly as the extraction solution and the raw material ratio increased from 5 to 6, and decreased again at a ratio of 9 (Figure 4). However, these differences in PER with different extraction ratios were not significant ($p > 0.05$). This could be due to low protein content in the raw material, so that even the lowest volume of the extract solution was enough to extract most of the proteins. However, the FPI-PR increased significantly at an extraction ratio of 8 ($p < 0.05$) compared to the lower ratios. However, no significant differences were seen in FPI-PR for extraction ratios between 5 and 7. This result is in agreement with Zhang and Chang [54], who showed that changing the ratio from 4 to 7 did not affect the FPI-PR produced from catfish by-products. The highest recovery rates were obtained at a ratio of 8 to 9 (70.9–73.2%).

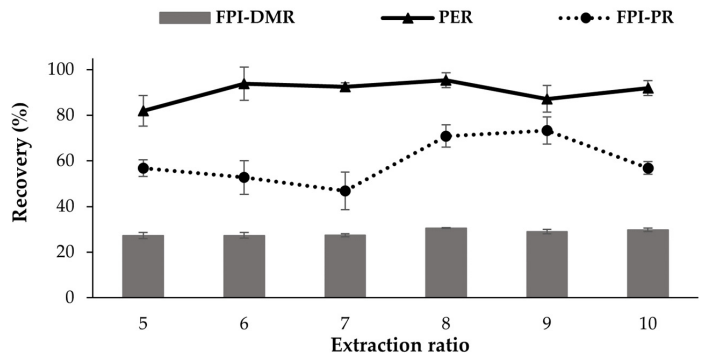

Figure 4. Effect of extraction ratio (extract solution volume/raw material weight) on PER (%), FPI-PR (%), and FPI-DMR (%) of the fish protein isolate (FPI) produced from the Tra catfish dark muscle. The protein extraction was carried out at pH 12 for 60 min.

The FPT-DMR was higher when the ratio of the extract solution was 8–10 than when it was 5–7 ($p < 0.05$), but was not significantly different within these ranges. Batista [55] suggested that a low ratio of extract solution may result in increased viscosity, limiting the effectiveness of the centrifugation and separation of solid particles, and resulting in more proteins being lost during the separation steps. In contrast, a high ratio can give a very diluted protein extract, reducing the recovery of protein in the precipitation and resulting in a decrease in FPI-PR at an extraction ratio of 10. The volume of water used for the fish protein isolate production plays an important role in industrial processing due to increased water use and discharge. Therefore, the ratio of 8 was chosen as the optimal condition for the FPI produced by the pH-shift method from the Tra catfish dark muscle.

3.1.3. The Effects of Extraction Time (Trial 3)

PER, FPI-PR, and FPI-DMR increased when the extraction time was increased from 30 min to 60 min, followed by stable values from 60 min to 120 min, reaching a maximum at 150 min and decreasing after that (Figure 5). An increase in PER when the extraction time was extended from 20 min to 120 min was reported by Batista [55]. Increased FPI-PR and FPI-DMR occurred when the extraction time was extended from 30 to 75 min in alkali-aided protein extraction from catfish by-products [54].

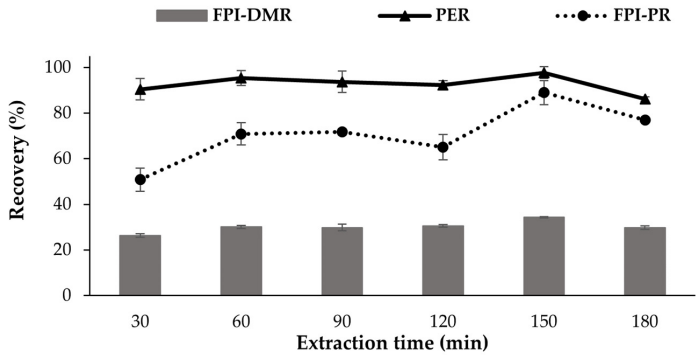

Figure 5. Effect of extraction time on the PER (%), FPI-PR (%), and FPI-DMR (%) of the fish protein isolate (FPI) produced from Tra catfish dark muscle. The proteins were extracted at pH 12 with an extraction ratio of 8.

The denaturation of proteins resulted in decreased PER, FPI-PR and FPI-DMR when the extraction time was prolonged to 180 min in this study, but extending the extraction time may promote the denaturation, as seen by the aggregation and polymerization of myofibrillar/cytoskeletal proteins in alkaline treatments [56]. Some proteins may thus unfold under alkaline conditions, forming hydrophobic interactions and disulfide bonds. These formations link protein chains together, resulting in protein aggregations and polymerization [57–59]. These protein fractions can be retained together in the sediment during acid precipitation [58].

3.2. Comparison of the FPI Produced from Different Rest Raw Materials and Surimi

3.2.1. FPI Processing Effectivity

FPI-PR is a good indicator of the economic feasibility of the pH-shift method. In previous studies, FPI-PR has been observed to range between 42% and 90%, depending on methods used to measure protein concentration, fish species, types of raw material, centrifugation force, and the content of water-soluble sarcoplasmic proteins [60]. The FPI-PR and FPI-DMR were highest in the dark muscle (88.9 ± 5.3% and 34.3 ± 0.2%, respectively), followed by the ACO (83.0 ± 2.9% and 32.7 ± 2.7%), and lowest in the HBB (68.2 ± 4.8% and 19.1 ± 1.4%). Although these different side streams had similar crude protein contents (Table 2), the protein composition differed. The HBB may contain high residual blood containing water-soluble haemoglobin and myoglobin and stromal proteins [61] which are lost during water processes [60]. The heme proteins were removed mostly from the FPI production using the alkaline pH-shift method—as Abdollahi et al. (2016), Kristinsson, Theodore, Demir and Ingadottir [52] observed—resulting in a lower protein recovery. However, this is a desirable feature in FPI production because the residual heme proteins in FPI act as the main pro-oxidants, causing lipid and protein oxidation [46,62,63]. At the same time, stroma proteins are not soluble, regardless of the pH or ionic strength of the solution [52,64]. Therefore, these proteins remained in the bone and skin fractions (sediment). Additionally, some proteins may not have been recovered because they were still stuck to the bone fractions and lost in the sediment in the HBB-FPI processing [60], or could have been lost in the top lipid layer after centrifugation [52]. Zhang and Chang [54] also showed a low FPI-DMR (below 17%) during protein recovery from the catfish head and frame blend.

Most lipids were removed during FPI production (90.4%–95.2%), depending on the lipid content of the raw materials (Table 1), indicating the high capacity of the pH-shift processing in separating and removing lipids from raw materials. The lipids are separated during the pH-shift process based on their density and polarity [52]. At pH 12, proteins were solubilized and separated from the storage lipids and membrane phospholipids [65]. Most of the storage lipids may come to the surface in the first centrifugation due to their lower density, while most unsaturated membrane phospholipids may be separated into the first sediment [66].

Table 1. Process effectivity of the fish protein isolate (FPI) processing from Tra catfish dark muscle (DM-FPI), head and backbone blend (HBB-FPI), abdominal cut-offs (ACO-FPI), and surimi made from the same batch of raw material. Results are expressed as means ± SD of triplicate measurements (n = 3) *.

Production	FPI-PR (%)	FPI-DMR (%)	Lipid Removal (%)	Ash Removal (%)
DM-FPI	88.9 ± 5.3 ^{a}	34.3 ± 0.2 ^{a}	90.4 ± 1.0 ^{b}	86.3 ± 0.8 ^{b}
HBB-FPI	68.2 ± 4.8 ^{b}	19.1 ± 1.4 ^{b}	95.2 ± 0.7 ^{a}	91.5 ± 0.6 ^{a}
ACO-FPI	83.0 ± 2.9 ^{a}	32.7 ± 2.7 ^{a}	93.6 ± 0.2 ^{a}	87.2 ± 0.5 ^{b}

* Different superscript letters show significant differences within column at $p < 0.05$.

Ash content was reduced by 86.3 to 91.5% during FPI production (Table 1). In the HBB, the ash was removed mainly from the insoluble bone fraction separated into the first sediment due to the bony property of the raw material. The highest mineral removal

was indicated in the HBB-FPI production, reflecting that most of the bone fraction was removed from the FPI. Ash reduction in the dark muscle and ACO-FPI may be due to the release of blood containing iron and heme proteins in the water phase during FPI production [46]. Impurities with high mineral content could possibly be used in animal feeds as a mineral additive [67] or produced for collagen/gelatin, orthopedics, or dental materials (for example, the HBB fraction with a high level of Ca and p), as suggested by Nam, Van Hoa, Anh and Trung [19].

3.2.2. Proximate Composition

The crude protein content of the FPIs was much higher than in the corresponding raw materials, although the water content was also higher (Table 2). The alkaline pH-shift process thus appeared to effectively separate insoluble impurities (bone, skin and connective tissues) and lipids. The trends of the changes in the proximate composition of the FPI products compared to their raw material agreed with studies performed on salmon and herring by-products [50,66] and cod by-products [50]. The lipid and ash contents of the FPIs were much lower compared to their corresponding raw materials. This is because most of the lipids and ash were removed during FPI processing, as discussed above. Lipid removal from the FPI is important because muscle lipids are susceptible to oxidation, leading to rancidity [46,56,60].

Table 2. Proximate composition (%) of the raw materials and fish protein isolates (FPIs) produced from Tra catfish dark muscle, head and backbone blend (HBB), abdominal cut-offs (ACO), and surimi made from the same batch of raw materials. Results are expressed as means ± SD of triplicate measurements ($n = 3$) *.

		Water Content	Crude Protein	Lipid Content	Ash Content
Raw material	Dark muscle	66.5 ± 1.0^{An}	14.7 ± 0.2^{Bn}	17.6 ± 1.5^{Bm}	0.8 ± 0.1^{Bm}
	HBB	54.8 ± 1.0^{Cn}	15.2 ± 0.1^{An}	21.9 ± 0.6^{Am}	7.8 ± 0.4^{Am}
	ACO	60.8 ± 2.0^{Bn}	15.3 ± 0.1^{An}	23.5 ± 1.1^{Am}	1.0 ± 0.1^{Bm}
FPIs and surimi	DM-FPI	73.9 ± 0.7^{bcm}	23.5 ± 0.9^{am}	3.2 ± 0.0^{bn}	0.1 ± 0.0^{bn}
	HBB-FPI	77.3 ± 0.8^{am}	20.4 ± 0.5^{bm}	2.8 ± 0.4^{bn}	0.1 ± 0.0^{bn}
	ACO-FPI	73.1 ± 2.1^{cm}	24.4 ± 1.4^{am}	3.1 ± 0.1^{bn}	0.0 ± 0.0^{bn}
	Surimi	77.0 ± 0.1^{ab}	17.5 ± 0.3^{c}	5.4 ± 0.1^{a}	0.2 ± 0.0^{a}

* Different uppercase superscript letters indicate significant differences within the column for the raw material; different lowercase superscript letters $^{a–c}$ show significant differences within the column for the FPIs and surimi; different lowercase superscript letters $^{m–n}$ indicate significant differences between raw material and the corresponding FPI at same parameter at $p < 0.05$.

Significantly higher lipid content in the surimi than the FPIs may be due to the higher lipid content of the raw material (including trimmings with high lipid content) and lower lipid removal in the water washing process in surimi production. Membrane lipids are retained during the water washing process and the storage lipids co-aggregate with proteins [65]. A lower efficiency of lipid-lowering (82%) by water washing than the alkaline pH-shift method of FPI production (94%) has also been observed in broiler meat [65], Atlantic croakers [56], and catfish [52]. Lower protein content in the surimi may reflect lower protein recovery during surimi processing as compared to FPI processing [52]. Only myofibrillar proteins are recovered in surimi processing, while water-soluble proteins (sarcoplasmic proteins) are removed into the water streams [68]. Additionally, some muscle proteins are attached to bone and skin and are lost in the separation step [60].

Although the three raw side stream materials examined had different lipid contents, the corresponding FPIs all had a similar lipid content. This result may reflect that the remaining lipids in the FPIs were protein-linked lipids. Both membrane lipids and storage lipids are removed effectively after centrifugation during FPI processing, as Kristinsson et al. (2013) observed. The FPI products produced from the dark muscle and ACO had a similar

proximate composition. These two fractions could be combined to produce FPI, unless their functional properties differ.

3.2.3. Amino Acid Profiles

The amino acid profiles of the FPIs influence both nutritional value and functional properties. All three FPIs had a similar amino acid composition (Table 3). Glutamic acid, aspartic acid, lysine and leucine were the primary components of all FPIs, similar to FPIs obtained from gutted herring [69] and rainbow trout [67]. Essential amino acids accounted for almost 50% of the total amino acids. All FPIs basically complied well with the FAO/WHO/UNU [70] recommendations for adults, indicating that these FPIs may be useful as additives or ingredients in developing proteinaceous food products for adults needing essential amino acids [67].

Table 3. Amino acid composition (g/100 g crude protein) of the fish protein isolates produced from Tra catfish dark muscle (DM-FPI), head and backbone blend (HBB-FPI), and abdominal cut-offs (ACO-FPI).

Amino Acids	DM-FPI	HBB-FPI	ACO-FPI	FAO/WHO/UNU [70] *
Hydroxyproline	ND	ND	0.6	
Alanine [b]	6.3	6.5	6.4	
Arginine [a]	6.6	6.9	6.3	
Aspartic acid	**11.2**	**12.1**	**10.5**	
Cysteine/Cystine	1.1	1.1	1.0	
γ-Aminobutyric acid	ND	ND	ND	
Glutamic acid	**18.4**	**19.4**	**17.2**	
Glycine [b]	3.7	3.9	5.1	
Histidine [a]	2.6	2.6	2.8	1.5
Isoleucine [ab]	4.7	5.0	4.6	3.0
Leucine [ab]	**8.8**	**9.1**	**7.9**	5.9
Lysine [a]	**9.3**	**10.1**	**8.8**	4.5
Methionine [ab]	3.3	3.5	2.9	2.2
Phenylalanine [ab]	3.8	4.2	3.8	3.8
Proline [b]	4.6	4.3	5.0	
Serine	4.2	4.4	4.1	
Threonine [a]	4.6	4.9	4.2	2.3
Tyrosine	2.2	3.5	3.1	
Valine [ab]	5.0	5.3	4.8	3.9
Total amino acids	100.5	106.8	99.1	
Total essential amino acids	48.8	51.7	46.2	
Total Hydrophobic amino acid	40.3	41.8	40.6	

[a] Essential amino acid for infants. [b] Hydrophobic amino acids. * FAO/WHO/UNU recommendations for adults (2007).

Hydroxyproline was not detected in FPI obtained from the dark muscle and HBB, and only 0.6 g/100 g protein was present in ACO. This amino acid is primarily found in collagen. The collagen fraction was thus effectively removed during the pH-shift processing, in agreement with the study by Marmon and Undeland [69]. The cysteine/cystine content was also comparable with the herring FPI studied by [69], with content levels from 1.0 to 1.1 g/100 g protein.

The residual cysteine/cystine and hydrophobic amino acids play an important role in the FPI application. Under food processing treatments such as heating and mechanical shear, proteins' tertiary structure may be unfolded, exposing sulfhydryl, cystine and hydrophobic groups and forming disulfide bonds and hydrophobic interactions. These chemical activities contribute to the gel-forming ability of FPIs. The FPIs might be used in gelling-based food products, similar to surimi or mince [57,71]. However, the natural

disufide bond and hydrophobic interaction formations in FPIs during storage should be avoided, as this could lead to aggregation and decrease their gel-forming ability [72].

3.2.4. SDS-PAGE

Tropomyosin, a protein with a molecular weight (MW) ranging from 35 to 40 kDa, was dominant in the FPIs produced. Other protein bands were obtained in ranges of 70–100 kDa, 140–260 kDa, 40–50 kDa, and approximately 15 kDa (Figure 6). A different protein pattern was observed in FPIs made from Atlantic croaker, which showed myosin to be most abundant [56]. G-actin (46–49 kDa) and tropomyosin were the two main proteins recovered in the surimi, similar to the surimi produced from Atlantic croaker [56]. A higher intensity of myosin heavy chain and heavy meromyosin (140–260 kDa) were observed in the commercial surimi than in the FPIs, while protein bands from 70 to 100 kDa were only identified in the FPIs. Several studies have shown the protein pattern of surimi to be similar to that of its raw material [59,73]. These results reflect that myosin may be partly hydrolysed during FPI processing to form lower molecular weight bands [46,47,56]. In addition, myosin heavy chain denaturation and aggregation might already occur in FPIs, leading to the loss of protein extractability and missing myosin heavy chain bands in FPIs [59,72]. Myosin is well known as being primarily responsible for the functionality of muscle foods, especially gelation [71]. The degradation of myosin heavy chain and heavy meromyosin in the FPIs may lower their gel-forming ability. Small cysteine-containing proteins or microbial transglutaminase additions should be considered in cases using these FPIs in gelling-based products [71,74]. The intensity of the protein bands varied slightly across the samples. A protein band ranging from 15 to 25 kDa (myosin light chain) was clearly identified for ACO-FPI, but was weak in DM-FPI and HBB-FPI.

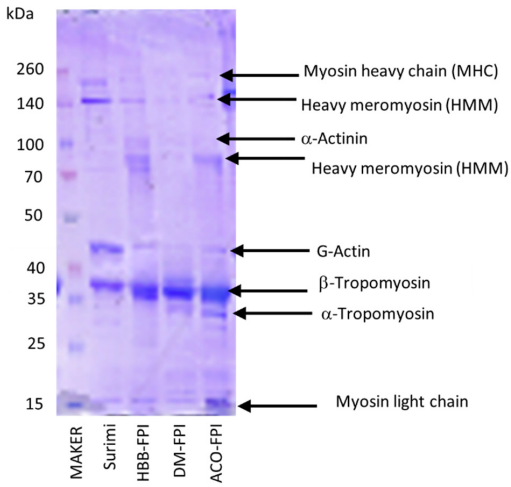

Figure 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of surimi and FPI made from head and backbone blend (HBB-FPI), dark muscle (DM-FPI), and abdominal cut-offs (ACO-FPI).

3.2.5. Colour

When compared to the surimi, all FPIs produced had lower lightness and whiteness values and higher yellowness and redness components, except for the redness of the ACO-FPI (Table 4). A similar result was observed in surimi and protein isolates made from mackerel (*Rastrelliger brachysoma*) [75]. Colour can be influenced by the amount of residual blood and dark muscle, as well as the presence of pigments such as heme proteins and melanin [76,77]. Conventional washing during surimi processing may reduce the pro-oxidants such as heme proteins and metal ions which cause colour changes [12,46,68,78].

Table 4. Colour of the fish protein isolates produced from Pangasius dark muscle, head and backbone blend (HBB), abdominal cut-offs (ACO), and surimi. Results are expressed as means ± SD of triplicate measurements (n = 3) *.

		Lightness (L*)	Redness (a*)	Yellowness (b*)	Whiteness
FPIs	DM-FPI	47.8 ± 1.0^{cA}	1.8 ± 0.1^{bA}	11.4 ± 0.5^{aA}	46.6 ± 1.1^{cB}
	HBB-FPI	34.0 ± 4.1^{dC}	$\mathbf{3.9 \pm 1.6^{aC}}$	5.4 ± 2.0^{cB}	33.6 ± 4.0^{dC}
	ACO-FPI	$\mathbf{64.9 \pm 0.3^{bB}}$	-1.2 ± 0.1^{cB}	$\mathbf{11.7 \pm 0.2^{aA}}$	$\mathbf{63.0 \pm 0.4^{bA}}$
Industrial surimi		$\mathbf{73.5 \pm 0.6^{a}}$	-0.2 ± 0.1^{c}	4.2 ± 0.5^{c}	$\mathbf{73.2 \pm 0.6^{a}}$

* Different uppercase superscript letters indicate significant differences within the column; different lowercase superscript letters show significant differences within the column between FPI products at $p < 0.05$.

Lower whiteness and higher yellowness and redness were likely due to blood residue and the oxidation of heme proteins (especially myoglobin) in the recovered proteins catalysed by the alkaline pH [46,75]. The HBB raw material may have contained high residual blood content due to the presence of the main blood vasculature along the backbone. Previous studies have shown that the alkaline and acid conditions during FPI processing promote the auto-oxidation of heme proteins and the discolouration of FPIs [12,75]. The presence of blood and/or residual heme proteins in fish side streams and FPIs produced from them can thus affect several quality properties, such as oxidative stability and whiteness [46,62]. The FPI produced from ACO was the lightest and of the highest whiteness, while HBB-FPI had the lowest. The ACO-FPI was produced from ACO, which may have had a low content of heme proteins, thereby resulting in the lowest redness value [79]. The high redness value in the DM-FPI may have been due to high levels of residual heme proteins because the dark muscle material had a high heme protein content, as has also been shown in mackerel and trout [79]. Among the FPIs the ACO-FPI is most promising as a food ingredient because of its high whiteness. However, prewashing the raw material before solubilization and a high water ratio used during homogenizing with the raw material may improve the whiteness of the FPIs even further, as suggested by Abdollahi, Marmon, Chaijan and Undeland [46].

4. Conclusions

The objective of the study was to investigate the potential for using different Tra catfish side streams for fish protein isolate production by assessing the chemical composition and characteristics of the side streams and the optimization of the protein isolate extraction conditions.

The pH of the extraction solution had a substantial effect on FPI production. In contrast, the ratio of extraction solution to raw material had a relatively minor effect on the FPI process. The protein extracted at pH 12 with a ratio of 8 (extraction solution/raw material, volume/weight) for 150 min gave the best results.

Most of the lipid and ash content was removed in the production of the FPIs, resulting in high protein content. The FPIs had a higher protein content and a lower lipid and ash content than the industrial surimi. All the FPIs had good amino acid compositions that could be used in food products for adults. In addition, with high protein and dry matter recoveries, Tra catfish dark muscle and ACO are potential sources for producing protein isolates using the pH-shift method. However, the prewashing and homogenisation steps should be studied to improve the whiteness of the FPIs.

The FPIs produced from dark muscle and ACO had similar chemical properties but different colour attributes. These raw materials should thus be processed into FPIs separately, adjusting each FPI towards the production of a specific value-added food product. However, other physicochemical properties, such as texture attributes, gel-forming ability, and lipid stability, should be studied further.

Overall, the study showed that Tra catfish FPIs have great potential as food ingredients in gel-based snack foods, similar to surimi and mince, and can be used as ingredients in the development of other higher-value products.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods11111531/s1, Table S1: List of abbreviations.

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Paper III

foods

MDPI

Article

Protein Characteristics and Bioactivity of Fish Protein Hydrolysates from Tra Catfish (*Pangasius hypophthalmus*) Side Stream Isolates

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Abstract: Enzymatic hydrolysis is a novel method to recover highly potent bioactive fish protein hydrolysates (FPHs) from fish processing side-streams. The common way of producing FPHs directly from fish side-streams may be inappropriate due to the excess of lipids and pro-oxidants, especially in lipid-rich streams, as obtained from Tra catfish. This study aimed to optimise the hydrolysis conditions for a commercial enzyme (Alcalase®2.4 L) (enzyme concentrate, temperature, and time) in FPH production from the fish protein isolate obtained from Tra catfish dark muscle (DM-FPI) using the pH-shift method. The degree of hydrolysis (DH), protein recovery (PR), and antioxidant properties, including DPPH radical scavenging activity (DPPH-RSA) and total reducing power capacity (TRPC), were measured to evaluate the effects of the hydrolysis conditions on the FPHs. Optimal hydrolysis was obtained at an enzyme/substrate protein ratio of 3% (*v*/*w*) and a hydrolysis temperature of 50 °C for 3 h. The FPHs obtained from different substrates, including DM-FPI, abdominal cut-off (ACO) FPI, and head and backbone blend (HBB) FPI, had similar DHs under these optimum conditions, ranging from 22.5% to 24.0%. However, the FPH obtained from abdominal cut-off isolate (ACO-FPH) showed the highest PR of 81.5 ± 4.3% and the highest antioxidant properties, with a DPPH-RSA of 86.1 ± 1.6% and a TRPC of 6.4 ± 0.4 equivalent mg vitamin C/g protein. The resulting FPHs present a natural source of antioxidants with great potential for food applications, especially the ACO-FPH. In addition, all FPHs had excellent amino acid profiles, indicating strong potential for their use as supplements. Tra catfish protein-rich side-streams can thus be processed into high-value bioactive FPHs using Alcalase for human consumption.

Keywords: enzymatic hydrolysis; dark muscle; side streams; antioxidant; fish protein isolates; amino acids

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1. Introduction

Fish has long been recognised as a valuable and nutritious food. Fish is low in saturated fatty acids and cholesterol compared to other animal-origin foods, such as meat, poultry, and eggs. Fish and fish products have been recommended as an important part of a healthy diet, especially if they replace other protein-rich foods with high contents of saturated lipids and cholesterol. Fish and seafood provide substantial essential nutrients, especially proteins, polyunsaturated fatty acids, and minerals [1–3]. Several studies have shown that the consumption of fish-derived proteins, such as peptides, fish protein hydrolysates (FPHs), and fish protein isolates (FPIs), also brings more health benefits than consuming intact fish proteins due to their high absorption and digestion properties [2,4].

In recent decades, increasing urbanization among the world population, as well as increased consumer awareness of the health benefits of fish consumption, have led to an

increased demand for fish and seafood products [5,6]. In 2019, fish provided about 17% of the total animal proteins and 7% of the total proteins consumed worldwide [7]. Food security issues, especially the supply of sufficient high-quality, nutrient-rich proteins, are a challenge due to the increased growth in the global population and overexploited marine resources [8,9]. Moreover, processing of raw materials from the fishing and fish-farming industries generates substantial underutilized side-streams, many of which have high biological and nutritional value [10,11]. Side streams from fish processing are considered to be all the processing streams that do not contribute to the main production, such as fillets or whole and gutted fish. In developing countries, such as Vietnam, these materials either go to waste or are converted into animal feed, fishmeal, and fertilizer, leading to the underutilization of available resources [12,13]. The Tra catfish is a freshwater species commonly farmed in Vietnam, Thailand, Indonesia, India, and Bangladesh. It is currently the most important farmed freshwater species in Vietnam [7,14]. Tra catfish have a relatively low market price, but their fillets are of good quality, with high protein (18.9%) and low lipid content (2.6%), and the taste is comparable to other whitefish species, such as cod or haddock [15,16]. Frozen white fillets are the most common product of Tra catfish production in Vietnam, resulting in the creation of significant amounts of side streams from the filleting processing [12]. In the case of Tra catfish production, common side-streams originate from the removal of the head and backbones, viscera, dark muscle, and abdominal cut-offs. Fish side-streams often contain high-quality proteins that may be utilized to develop protein-containing foods beneficial for human consumption whilst enhancing economic and environmental sustainability. It is necessary to investigate the potential of converting these fish side-streams into value-added products rather than only producing low-value products, such as fishmeal, fish oil, silage, and fertilizers, as is traditionally the case.

Enzymatic modification of proteins has been applied widely in the food industry for a long time [17,18]. Enzymes help improve the nutritional value of various foods by splitting complex proteins, fats, and carbohydrates into smaller and simpler compounds, increasing their digestibility, absorption, and metabolism [17]. Fish protein hydrolysates (FPHs) are fish-derived proteins containing mixtures of peptides and amino acids of various molecular weights depending on the extent of the enzymes' hydrolysis. FPHs were among the most important novelty fishery products in the last decade [19]. FPHs enhance the functional aspects of proteins and can be used as additives and/or as ingredients in food applications. Their surface properties, such as hydrophobic and hydrophilic surface groups, can stabilise oil-in-water emulsion and increase foaming activity and water-holding capacity [3,11,20]. FPHs with antioxidant activities are used widely in various industries, such as in health food, aquaculture, pharmaceuticals, cosmetics, and food processing/preservation industries [21–26]. In intensive farming systems, fish are kept at high densities, which may increase stress and cause increased susceptibility to diseases, resulting in economic production losses [27]. FPHs and bioactive peptides have, on the other hand, been shown to have positive effects on fish health, immunity, and growth during farming [28–30].

FPHs can be produced using different methods, including autolysis, bacterial fermentation, or chemical and enzymatic hydrolysis [3,31,32]. Enzymatic hydrolysis is considered the most efficient method to recover protein hydrolysates from fish side-streams [18,33]. By using different proteolytic enzymes, it may be possible to produce a broad spectrum of food ingredients for a wide range of applications. Enzymatic hydrolysis generates precise hydrolysates, retaining the nutritive value of the source protein with high protein recovery in a short time, and it is beneficial for targeting specific derived products [18]. Several authors have studied the enzymatic proteolysis and solubilization of proteins from different types of fish side-streams in recent decades [18,23,33,34]. In particular, several FPHs with antioxidant activities have been prepared from side streams of fish species, such as Pacific hake [32], round scad [34], cod [35], hoki, pollock, sandfish, tilapia [23], and rainbow trout [36]. Proteases from various sources, such as from animals, plants, bacteria, and fungi, have been used during FPH production. Alcalase® is a commercially available alkaline bacterial protease produced from *Bacillus licheniformis*. This enzyme has been

highly recommended as one of the most effective enzymes to produce FPHs with high yield and high antioxidant activities [14,37–39].

Traditionally, FPHs are produced directly from fish processing side-streams, such as the heads, frames, dark muscles, cut-offs, and viscera, either as combined streams or separated streams. The raw materials are minced and homogenized with water and then enzymes are added, after which the hydrolysis process is initiated under the given working conditions [18]. However, high lipid content and the presence of pro-oxidants, such as haemoglobin and myoglobin from the blood, in the side streams may cause rancid, fishy odours in the final product, which limits the further use of the FPHs [40–42]. This can be especially challenging during utilization of Tra catfish side-streams, which often have high lipid and ash content ranging from 15.3 to 29.8% and 2.4 to 5.7%, respectively [14]. Therefore, pre-treatment of protein substrates, such as defatting, washing, and/or centrifuging, are necessary to remove the excess lipid and pro-oxidants before using them for FPH production [18,43,44]. Alternatively the FPH can be stabilized by adding antioxidants [45]. Producing FPH from fish protein isolates (FPIs) may thus both reduce unwanted components and improve quality.

The pH-shift method is commonly used to recover proteins from fish side-streams. The proteins are solubilised at either low or high pH ($\leq$3.5 or $\geq$10.5), then fat and other impurities are removed using high-speed centrifugation, after which the proteins are collected following precipitation at their isoelectric point (pH = 5.5) [41,46]. During FPI production using the pH-shift method, most lipids, ash, and contaminates are removed, resulting in high-quality raw material for further FPH production [12,42]. Kakko et al. [47] found that proteins isolated with the pH-shift method had a higher nutrient value than the enzymatically extracted hydrolysates from the same raw material. Nisov et al. [48] also found that the pH-shift method resulted in higher protein recovery yield than the enzymatic method when treating Baltic herring (*Clupea harengus membras*) and roach (*Rutilus rutilus*).

The current study aimed to investigate the feasibility of obtaining functional fish protein hydrolysates (FPHs) by enzymatically hydrolysing FPIs recovered from protein-rich side-streams from Tra catfish (*Pangasius hypophthalmus*) fillet processing, including the dark muscle (DM-FPI), abdominal cut-offs (ACO-FPI), and a head and backbone blend (HBB-FPI). The hydrolysis conditions for the FPH production from FPI-DM was optimised using a one-factor-in-a-time method [14]. The effects of varying enzyme-substrate ratios (trial 1), hydrolysis temperatures (trial 2), and hydrolysis times (trial 3) on the obtained FPHs were evaluated based on their degrees of hydrolysis, protein recoveries, and antioxidant activities. Furthermore, the amino acid composition and the properties of the FPHs were analysed and compared.

2. Materials and Methods

2.1. Raw Materials and Sampling

2.1.1. Preparation of Fish Protein Isolates (FPIs)

Dark muscle (DM), a head and backbone blend (HBB), and abdominal cut-offs (ACO) were collected from industrial Tra catfish fillet processing (Nam Viet, Can Tho, Vietnam), as described in detail by Nguyen et al. [12]. The corresponding FPIs (i.e., DM-FPI, HBB-FPI, and ACO-FPI) obtained from these side streams were prepared according to optimised conditions as described by Nguyen, et al. [12]. The raw material was minced and then homogenised in distilled water at pH 12 and a water-to-raw-material ratio of 8 (volume/weight) for 1 min. The mixture was left in a fridge at 0−4 °C for 150 min for protein solubilization. The homogenate was then centrifuged at 3000 rpm for 20 min at 4 °C (MF 600, Biobiz, Incheon, Korea), and the middle fraction, containing soluble proteins, was recovered. This fraction was adjusted to a pH of 5.5 to precipitate the proteins (Portavo 904×, Knick, Berlin, Germany). The aggregated precipitates were then filtered using a nylon monofilament bag (mesh size: 25 micron; Dong Son Ltd., Ho Chi Minh City, Vietnam), and the obtained proteins were washed with distilled deionized water to a neutral pH and centrifuged at 3000 rpm for 20 min to dewater them, remove the NaCl, and form the

fish protein isolates (FPIs). Each obtained FPI was mixed evenly, divided, and weighed into 50 g samples, which were then packed in sealed polyethylene bags and stored at -25 ± 1 °C until use.

2.1.2. Preparation of Fish Protein Hydrolysate (FPH) from DM-FPI

The preparation of FPH through the hydrolysis of the DM-FPI using the Alcalase® enzyme was carried out with different enzyme ratios, temperatures, and durations to obtain the optimal conditions for FPH production. A flow chart of the optimization trials for FPH production from the DM-FPI is shown in Figure 1.

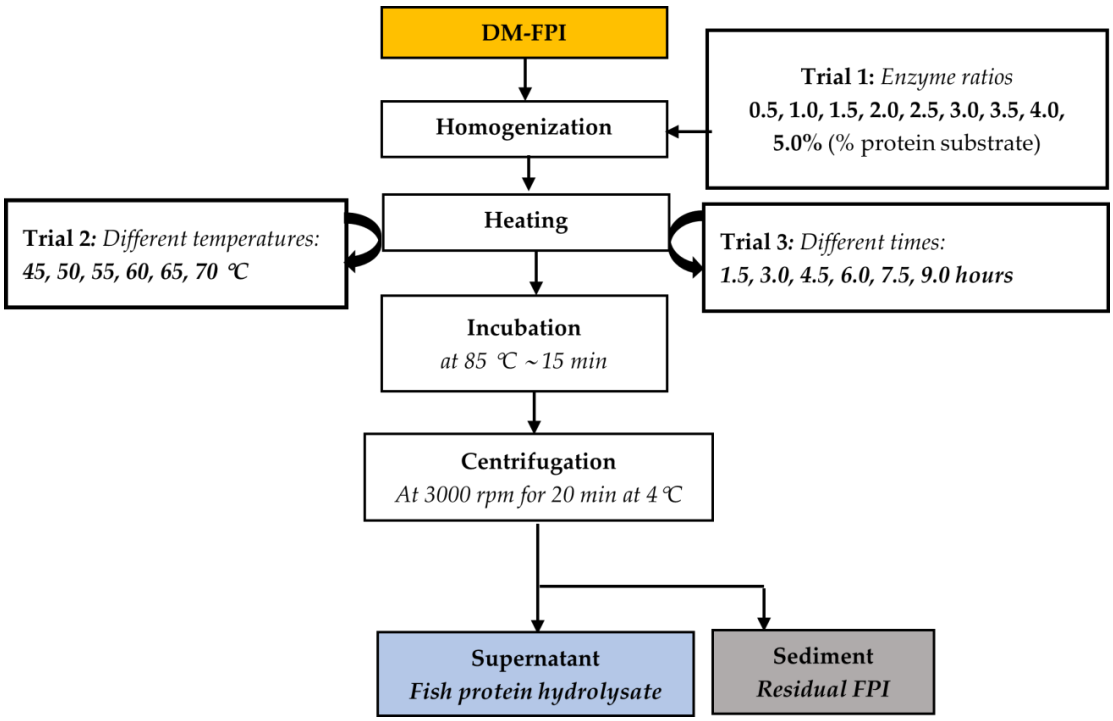

Figure 1. Experimental design for the optimization of FPH production from Tra catfish DM-FPI (dark muscle fish protein isolate) performed with three trials. Orange-filled box: protein, water-lipid, and ash content measured; blue-filled box: weight of the protein solution recorded and its protein content determined; grey-filled box: weight recorded and water content determined.

First, the enzyme–substrate protein ratio (volume/weight, %) was optimized during trial 1. About 50 g of DM-FPI sample was added to distilled water to obtain a protein substrate concentration of 7.5% [37]. The pH of the mixture was adjusted to 7.5 using 0.1 N NaOH and 0.1 N HCl ((Portavo 904×, Knick, Berlin, Germany). Different ratios of enzyme, including 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 5.0%, were then mixed with the 7.5% protein DM-FPI mixture. The samples were then incubated at 50 °C for 3 h in a shaking water bath for the hydrolytic reaction (VS-1205SW1, Vision Bionex, Bucheon-si, Gyeonggi-do, South Korea). After hydrolysis, the mixture was heated and incubated at 85 °C for 15 min to terminate the enzyme activity. The heated mixture was then centrifuged and separated into two fractions: a protein solution (fish protein hydrolysate (FPH)) and a sediment (residual FPI). The degree of hydrolysis (DH), protein recovery (PR), antioxidant activities (including 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH-RSA)), and total reducing

power capacity (TRPC) of the FPHs were determined to evaluate the effects of enzyme ratios on the yield and bioactive properties of the FPH, as described in Section 2.2.

An enzyme ratio of 3% was found to be optimal for protein hydrolysis and, thus, was used in further trials. The FPHs were then prepared at different hydrolysis temperatures, including 45 °C, 50 °C, 55 °C, 60 °C, 65 °C, and 70 °C, in trial 2. The DH, PR, DPPH-RSA, and TRPC were evaluated as above.

Trial 2 indicated that the hydrolysis temperature of 50 °C was optimal for FPH production and it was selected for the subsequent trials. The FPH was then produced with different hydrolysis times, including 1.5, 3, 4.5, 6, 7.5, and 9 h, in trial 3. The DH, PR, DPPH-RSA, and TRPC were determined with the same procedures as mentioned above. Each experiment was carried out in triplicate.

2.1.3. FPH Preparation from Different FPIs at the Optimal Conditions

The HBB-FPHs and ACO-FPHs were produced from the corresponding HBB-FPIs and ACO-FPIs with the optimal procedure obtained in this study. The DH, PR, DPPH-RSA, TRPC, and amino acid profile of each FPH were measured and compared.

2.1.4. Chemicals

All chemicals used in the study were of analytical grade and purchased from Sigma-Aldrich Company (Missouri, TX, USA) and Merck (Darmstadt, Germany).

Alcalase® 2.4 L was purchased from Sigma-Aldrich (Missouri, TX, USA). This protease product was obtained from *Bacillus licheniformis*, Subtilisin A. The Alcalase® was stored at 4 ± 1 °C until use.

2.2. Analyses

2.2.1. Proximate Composition of the Tra Catfish Side-Streams and their Corresponding FPIs

Water content was determined according to ISO 6496:1999 [49]. About 5.0 g of sample was weighed in a small porcelain bowl. The bowl was dried in an oven for 4 h at 103 ± 1 °C and then allowed to cool to ambient temperature for about 30 min in a desiccator before being weighed.

The crude protein content (total nitrogen content) was measured using the Kjeldahl method according to ISO 5983-2:2009 [50]. Approximately 2 g of minced sample was digested in 17.5 mL concentrated H_2SO_4 with two Kjeldahl tablets (each tablet included 3.5 g K_2SO_4 and 0.4 g $CuSO_4$) as a catalyst at 420 °C for 2.5 h. The digested mixture was made alkaline with NaOH and the nitrogen distilled off as NH_3. The NH_3 was "trapped" in a 1% boric acid solution. The amount of ammonia nitrogen in the solution was quantified using a standardized H_2SO_4 solution by titration. A nitrogen conversion factor of 6.25 was used to calculate crude protein content.

Lipids were analysed according to the Bligh and Dyer [51] method. Approximately 25 g of the sample was homogenized for 4 min with 50 mL of chloroform, 50 mL of methanol, and 25 mL of 0.88% KCl. The homogenized sample was centrifuged at 2500 rpm for 20 min at 4 °C. The chloroform phase containing lipids (the liquid bottom part) was collected and filtrated through a glass microfiber filter under vacuum suction. Exactly 2 mL of the chloroform fraction was transferred into a glass tube and placed in a vacuum dryer at 55 °C to remove the chloroform solvents. The remaining sample was weighed to calculate the total lipid content.

The ash content was determined according to the method described by the Association of Official Analytical Chemists (AOAC, 2000) [52]. About 5 g of sample was placed into a crucible. Each sample was heated overnight at 550 ± 3 °C and then cooled down in a desiccator before being weighed. Ashes were quantified gravimetrically. The water, crude protein, lipid, and ash contents were expressed as percentages of wet weight (ww).

2.2.2. Amino Acid Analysis

The total amino acid profiles of the FPH samples were determined using the liquid chromatography–mass spectrometry (LC-MS) method according to ISO 13903:2005 [53]. Approximately 1 g of sample was hydrolysed for 24 h in 25 mL of 6 N HCl at 110 °C in a sealed vessel. Amino acids were extracted from the sample using an EZ:faast LC-MS kit system (Phenomenex, Torrance, CA, USA). Ion-exchange chromatography was used to separate the individual amino acids through the EZ:faast AAA-MS column (250 mm × 2.0 mm, 4 µm) (Phenomenex, Torrance, CA, USA). The amino acids were then detected using a ninhydrin reaction at λ 570 nm (λ 440 nm for proline) in a Shimadzu LC-MS 8030 system (Kyoto, Japan). Amino acid content was expressed as g/100 g crude protein.

2.2.3. Degree of Hydrolysis (DH)

The degree of hydrolysis (DH) is defined as the ratio between the number of broken peptide bonds (*h*) and the total number of peptide bonds per mass unit (h_{tot}) [54], as described by Equation (1):

$$\mathrm{DH}\ (\%) = \frac{\mathrm{h}}{\mathrm{h}_{tot}} \times 100 \quad (1)$$

h was determined by measuring the amount of free α-amino group formed in the hydrolysed protein products. The method is based on the formation of a yellow complex between the amino groups in the amino acids with a dinitrofluorobenzene (DNFB) reagent. The absorbance of the solution was read at 410 nm in a DR6000 UV–Vis spectrophotometer (HACH, Düsseldorf, Germany). h_{tot} is the number of peptide bonds, with 8.6 mol peptide equivalent/kg for fish protein [55]. The equation can then be rewritten as:

$$\mathrm{DH}\ (\%) = \frac{\mathrm{A} \times 0.001 \times \text{dilution factor}}{\mathrm{P} \times 8.6 \times 0.001} \times 100 \quad (2)$$

A × 0.001 indicates the amount of amino groups (mol/mL) formed based on a standard curve made with glycine with concentrations ranging from 0.0002 to 0.001 mM/mL, and P is the total protein content (g) in 1 g of the hydrolysate solution sample.

2.2.4. Protein Recovery

The protein recovery (PR) from the FPHs was calculated using the following Equation (3):

$$\mathrm{PR}\ (\%) = \frac{\text{Total protein in the FPH}}{\text{Total protein in the initial substrate (FPI)}} \times 100 \quad (3)$$

The protein content of the solution fraction of the FPHs was determined using the Bradford method [56]. For this procedure, 50 µL of the sample was mixed with 2.5 mL of the Bradford reactive solution and then incubated for 25 min at ambient temperature. The absorbance was read at 595 nm using a DR6000 UV–Vis spectrophotometer (HACH, Düsseldorf, Germany). The protein content was calculated based on a standard curve made with bovine serum albumin with concentrations ranging between 0.1 and 1.4 mg/mL.

The protein content of the FPIs was extracted following Mæhre et al. [57]. About 1 g sample was homogenized with 60 mL of 0.1 N NaOH in 3.5% NaCl solution. The homogenates were then incubated in a water bath at 60 °C for 90 min, which was followed by centrifugation at 4 °C for 30 min at 5000 rpm (TJ-25 Centrifuge, Beckman Coulter, Brea, CA, USA). The supernatants were measured for protein content using the Bradford method as described above.

2.2.5. Antioxidant Activities

Antioxidant activities of the FPHs were evaluated through 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH-RSA) based on the method described by Fu et al. [58]. The total reducing power capacity (TRPC) of the FPHs was measured using the method described by Oyaizu [59].

Determination of DPPH-RSA

Approximately 2.0 mL of FPH sample was mixed with 1 mL of 96% ethanol and 1 mL of DPPH solution (0.1 mM in 96% ethanol). The reaction tubes, in triplicate, were wrapped in aluminium foil, shaken well, and then left to incubate for 30 min at room temperature. The absorbance of the solutions was read at 517 nm (DR6000 UV–Vis, Hach, Düsseldorf, Germany). For the control sample, 2 mL of 96% ethanol was used instead of the FPH sample. The DPPH-RSA was calculated using Equation (4):

$$\text{DPPH} - \text{RSA}\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (4)$$

where $A_{control}$ and A_{sample} are the absorbances of the control and sample solution read at 517 nm, respectively.

Determination of TRPC

Exactly 2 mL of FPH sample was mixed with 2 mL of 0.2 M phosphate buffer (pH 6.6) and 2 mL of 1% potassium ferricyanide. The mixture was then incubated for 20 min at 50 °C. After that, 2 mL of 10% TCA was added to the mixture. Approximately 2 mL of the incubated mixture was mixed with 0.4 mL of 1% ferric chloride and 2 mL of distilled water in a test tube. The absorbance of the solution was read at 700 nm after a 10 min reaction (DR6000 UV-VIS, Hach, Düsseldorf, Germany). Each sample was measured in triplicate. A standard curve was made using a standard vitamin C solution spanning a concentration range of 0 to 20 μg/mL, and the TRPC was expressed as equivalent (equiv.) mg vitamin C/g FPH protein.

High DPPH-RSA and TRPC values indicated that the FPHs had high antioxidant activities.

2.2.6. Statistical Analysis

All data summaries and statistical analyses were carried out in Microsoft Excel 365 (Microsoft Inc., Redmond, WA, USA) and IBM SPSS Statistics software (Version 22, IBM, 1 New Orchard Road, Armonk, New York, NC 10504-1722, United States). One-way analysis of variance (ANOVA) and Tukey's HSD tests were performed on the means of each variable. Significant levels were defined as $p < 0.05$ for all statistical analyses.

3. Results and Discussion

3.1. Proximate Composition of the Raw Materials and FPIs

The crude protein content was significantly higher, and the lipid and ash contents significantly lower, in the FPIs than in the corresponding raw materials (Table 1). Thus, the alkaline pH-shift process effectively removed insoluble impurities (bone, skin, and connective tissues) and lipids from the FPIs. Nguyen et al. [12] found that over 90% of the total lipids and 85% of the total ash in the raw materials were removed during FPI processing. Lipid removal during FPI production is advantageous for further processes, since muscle lipids are highly susceptible to oxidation, increasing the risk of the formation of rancidity [42,60,61]. Other common pro-oxidants in the side streams, such as myoglobin (Mb), haemoglobin (Hb), and iron, can also be removed successfully during FPI processing [12,42], thus reducing the risk of oxidation even further. However, the pro-oxidants present in the substrate may react with antioxidative peptides generated during and after hydrolysis. Lowering these pro-oxidant compounds may thus preserve the antioxidant properties of the FPHs obtained [62,63].

Table 1. Proximate composition (%) of the fish protein isolates (FPIs) produced from Tra catfish dark muscle, head and backbone blend (HBB), and abdominal cut-offs (ACOs) *. Results are expressed as means ± SD from triplicate measurements (n = 3) **.

		Water Content	Crude Protein	Lipid Content	Ash Content
Raw material	Dark muscle	66.5 ± 1.0 ^{A}	14.7 ± 0.2 ^{B}	17.6 ± 1.5 ^{B}	0.8 ± 0.1 ^{B}
	HBB	54.8 ± 1.0 ^{C}	15.2 ± 0.1 ^{A}	21.9 ± 0.6 ^{A}	7.8 ± 0.4 ^{A}
	ACO	60.8 ± 2.0 ^{B}	15.3 ± 0.1 ^{A}	23.5 ± 1.1 ^{A}	1.0 ± 0.1 ^{B}
FPIs	DM-FPI	73.9 ± 0.7 ^{ab}	23.5 ± 0.9 ^{a}	3.2 ± 0.0 ^{a}	0.1 ± 0.0 ^{a}
	HBB-FPI	77.3 ± 0.8 ^{a}	20.4 ± 0.5 ^{b}	2.8 ± 0.4 ^{a}	0.1 ± 0.0 ^{a}
	ACO-FPI	73.1 ± 2.1 ^{b}	24.4 ± 1.4 ^{a}	3.1 ± 0.1 ^{a}	0.0 ± 0.0 ^{a}

* The data were adapted from Nguyen et al. [12]. ** Different uppercase letters indicate significant differences within the column for the raw material (A, B, C); different lowercase letters show significant differences within the column for the FPI products (a, b) at a significance level of $p < 0.05$.

Lowering the lipid content from the lipid-rich side-streams through FPI processing is beneficial, and it is in agreement with Kristinsson and Rasco [18], who suggested that excess lipids must be removed from lipid-rich raw materials before they are used for FPH production. Halldórsdóttir et al. [64] also reported applying the pH-shift method to recover proteins and remove undesirable components from saithe (*Pollachius virens*) mince. The hydrolysis process in the Halldórsdóttir et al. [64] study resulted in higher quality FPHs than when processed the traditional way without dewatering. In addition, Khantaphant, et al. [63] observed that lowering the lipid content and impurities by applying membrane separation followed by washing to brownstripe red snapper (*Lutjanus vitta*) mince before hydrolysis can form FPHs with higher antioxidative activities than FPHs prepared directly from the original mince.

3.2. Optimization of FPH Processing from DM-FPIs

3.2.1. Effects of Enzyme Ratios (Trial 1)

The degree of hydrolysis (DH) is used as an indicator for peptide bond cleavage, while protein recovery (PR) indicates the yield obtained from the hydrolysis process. The DH can affect the PR and other functional properties, such as antioxidative activities [65,66]. Overall, the DH and PR increased when the enzyme–protein substrate ratio increased from 0 to 3.0% (Figure 2A,B). During the hydrolysis process (i.e., with Alcalase), the enzyme divides the peptide bonds in the initial proteins into smaller protein molecules and peptides with higher solubility [67]. The hydrolytic enzyme breaks down the peptide bonds of the protein substrates through their active sites, which may initiate catalysis through covalent interaction with the protein substrates [68]. Therefore, increasing the enzyme concentration speeds up the reaction, as long as there is enough substrate available to bind to, resulting in an increase in both the DH and PR. The PR and antioxidant activities significantly increased when the 0.5% enzyme was added compared to the autolysis of the substrate (no enzyme added). However, the DH was not significantly different with these two enzyme ratios ($p > 0.05$). When no exogenous enzyme was added, the DH was 14.1%. This value was higher than the DH from the autolysis of Tra catfish side-streams under the same hydrolysis conditions (i.e., temperature of 50 °C for 3 h) studied by Nam et al. [14], who obtained a value of 6.5%. In the autolysis of yellowfin tuna (*Thunnus albacares*) performed for 3 h at 45 °C, the resulting DH was about 5% [69]. These results may reflect the fact that the hydrolysis had already occurred in the substrate (i.e., DM-FPI) in this study, as discussed by Nguyen et al. [12]. There was no significant difference in the DH when the enzyme ratios ranged from 1.0% to 2.5%. However, the DH significantly increased when the enzyme ratio exceeded 2.5%, and the highest DH value of 33.3 ± 4.0% was obtained at an enzyme ratio of 3.5%. This result was in agreement with the production of FPHs from Tra catfish by-products using Alcalase studied by Nam et al. [14], where the highest DH (30.7%) was obtained at the enzyme–protein substrate ratio of 3.4%. The increase in the DH with higher enzyme ratios has been shown in early studies [14,43,44,69]. However, in the

current study, the DH only decreased slightly when the enzyme concentration exceeded 3.5%. Moreover, some of the peptides generated in the FPH may be further hydrolysed, forming free amino acids and smaller peptides when the enzyme ratio increases, leading to a decline in the DH [70].

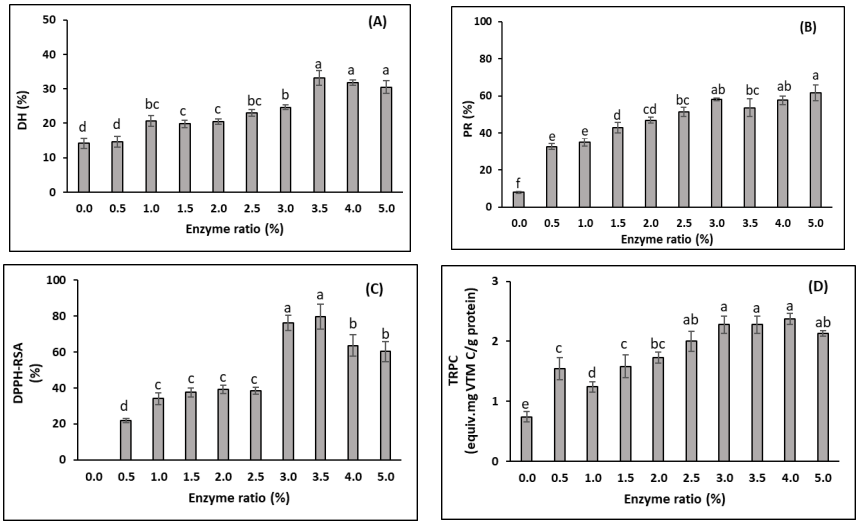

Figure 2. Effects of the enzyme/substrate ratios on properties of FPHs obtained from DM-FPI (trial 1), including: (**A**) degree of hydrolysis (DH, %); (**B**) protein recovery (PR, %); (**C**) DPPH radical scavenging activity (DPPH-RSA, %); and (**D**) total reducing power capacity (TRPC, equiv. mg vitamin C/g FPH protein). The hydrolysis reaction was carried out at 50 °C for 3 h. Different lowercase letters show significant differences at a significance level of $p < 0.05$.

The PR increased significantly, from 8.0% to 58.1%, when the enzyme ratio increased from 0% to 3.0%. Afterwards, the PR remained stable when the enzyme ratio was increased to 5% ($p > 0.05$). Nam et al. [14], who prepared FPHs from a mixture of Tra catfish side-streams using the enzyme Alcalase, also found that the highest nitrogen recovery (82.2%) was obtained at an enzyme–protein substrate ratio of 3.4%. This may have been due to saturation of the existing peptide bonds, especially soluble peptides in the hydrolysis solution, and thermal denaturation of the enzyme [14,65].

DPPH is a comparatively stable radical used as a substrate to determine antioxidant efficacy [71]. The DPPH-RSA increased slightly, from 22.0% to 38.5%, when the enzyme ratio increased from 0.5% to 2.5% (Figure 2C). Furthermore, there was a significant increase in DPPH-RSA when the enzyme ratio exceeded 2.5%, and the DPPH-RSA reached the highest value (79.8 ± 6.9%) when the enzyme was used at a concentration of 3.5%. These results reveal that the FPHs studied possibly contained amino acids or peptides, which can act as electron donors, reacting with free radicals to improve the stability of products and terminate radical chain reactions. The increase in DPPH-RSA when the enzyme ratio increased from 0.5% to 3.5% may have been due to the increase in the extent of hydrolysis at higher enzyme concentrations, releasing more antioxidative peptides, which are normally inactive within the sequence of the precursor protein molecules [72]. However, the DPPH-RSA significantly decreased when the enzyme ratio exceeded 3.5%. This may have been due to the breakdown of the already formed antioxidative peptides during the early stages of the hydrolysis process. Similar results were observed by Tanuja et al. [73], who showed a reduction in the DPPH-RSA of the FPH produced from Tra catfish frame meat when the Alcalase concentration (% v/w of substrate protein) was increased from 0.5% to 2.5%.

The reducing capacity of a compound may serve as an indicator of its antioxidant activity. The presence of reducers (i.e., antioxidants) leads to a reduction of the Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}). In this assay, the yellow colour of the test mixture changed to various shades of blue and green, depending on the reducing power of each sample. Therefore, measurement of the formation of Perl's Prussian blue at 700 nm can be used to monitor the Fe^{2+} concentration. In this study, the TRPC had a similar change trend as the DPPH-RSA, with increases ranging from 0.74 equiv. mg vitamin C/g FPH protein to 2.0 equiv. mg vitamin C/g FPH protein when the enzyme ratio increased from 0 to 2.5% (Figure 2D). The hydrolysis of DM-FPI (as expressed by the increased DH) into peptides may give rise to both the release of sequences with antioxidant properties and the exposure of previously hidden amino acid residues and side chains with antioxidant activity, as discussed above, resulting in an increase in TRPC. No significant change was observed in the TRPC as the enzyme concentration was increased from 3.0−5.0%. In this trial, the changes in the antioxidant activities and the DH showed similar trends. This result was consistent with the study by Klompong et al. (2007), who showed that the TRPC increased with an increased DH.

Based on the results of trial 1, the enzyme–substrate protein ratio of 3.0 was chosen as the optimum enzyme ratio for the FPHs produced from the DM-FPI.

3.2.2. Effects of Hydrolysis Temperature (Trial 2)

The hydrolysis temperature had significant effects on the DH (Figure 3A). The DH slightly increased when the temperature increased from 45 °C to 50 °C. The DH then increased sharply, from 17.0% to 43.2%, when the hydrolytic temperature increased from 50 °C to 65 °C. However, the DH significantly dropped to 20.1% when the temperature reached 70 °C. According to Eisenthal et al. [74], temperature influences the stability and activity of enzymes. As mentioned above, the hydrolysis reaction is based on the covalent interaction between specific enzyme groups (active groups) and the protein substrate. Hence, this reaction rate depends on the enzyme's specific three-dimensional structure [68]. In addition, the increased hydrolysis temperature may increase the internal energy of the enzyme [75]. Enzyme activity increases with temperature as long as the enzyme is stable, which resulted in increases in the DH, especially when the temperature increased from 50 °C to 65 °C. However, the enzyme is a protein and may be denatured and inactivated at high temperature [76] (in this study, when the temperature was above 65 °C), resulting in a decreased DH. These results were in agreement with an observation by Amiza et al. [77], who found that the effect of temperature on the DH of Tra catfish frame hydrolysis showed a bell-sharped pattern. Below the optimal temperature, the DH increased. However, above the optimal temperature value, the DH was reduced due to enzyme denaturation and inactivation. In addition, the enzyme catalyses rapidly with the insoluble protein molecules and then polypeptide chains that are poorly bonded to the surface are hydrolysed. The proteins that are cleaved the slowest are the more compacted core proteins [44]. At the temperature of 70 °C, the substrate proteins may be partly aggregated and less susceptible to enzymatic hydrolysis, leading to a lower DH than at lower temperatures.

Several authors have noted a positive correlation between the DH and the PR during FPH production. However, in this study, although the DH strongly increased when the reaction temperature was increased from 45 °C to 65 °C, the increase in the PR was not statistically significant, with values ranging from 54.1% and 61.4% (Figure 2B). This may have been due to the initial substrate being partly hydrolysed before entering the FPH production process, as discussed above. The initial substrate used in this study was obtained with the pH-shift method and mainly composed of sarcoplasmic and myofibrillar proteins, some of which were even partly hydrolysed, resulting in high solubility [12]. Therefore, the initial substrate had high solubility that could be extracted to the FPHs even at a low DH. The PR only significantly decreased when the temperature reached 70 °C (41.3%), which was consistent with the DH reduction at this temperature. This may have been because the enzyme was thermally denatured, leading to lower activity [65].

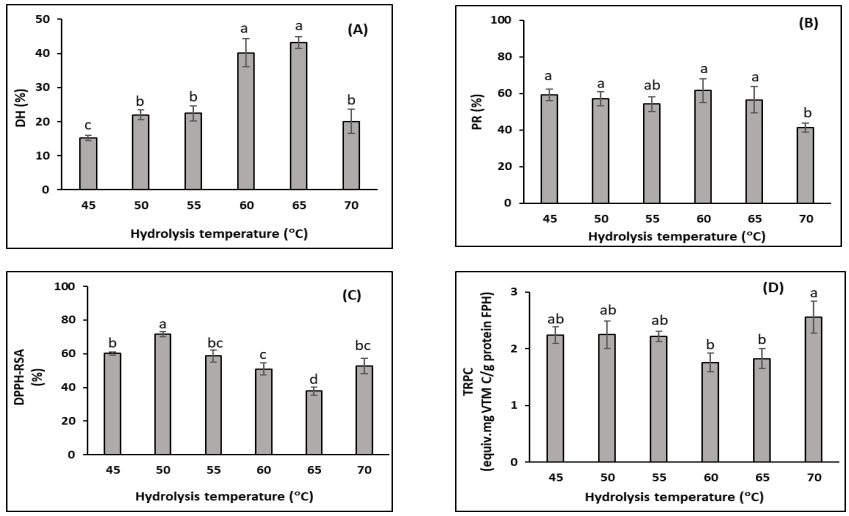

Figure 3. Effect of hydrolysis temperature (°C) on FPH properties from the DM-FPI (trial 2), including: (**A**) degree of hydrolysis (DH, %); (**B**) protein recovery (PR, %); (**C**) DPPH radical scavenging activity (DPPH-RSC, %); and (**D**) total reducing power capacity (TRPC, equiv. mg vitamin C/g FPH protein). The hydrolysis reaction was carried out for 3 h with an enzyme ratio of 3%. Different lowercase letters show significant differences at a significance level of $p < 0.05$.

The DPPH-RSA significantly increased, from 57.0% to 75.1%, when the hydrolysis temperature increased from 45 °C to 50 °C ($p < 0.05$) (Figure 3C). The enzymatic hydrolysis led to the release of antioxidative peptides, which function as free radical scavengers. This may have been due to the higher enzyme activity obtained at the higher temperatures, resulting in more hydrolysis. The increase in hydrolysis may have resulted in a higher content of low-weight molecular peptides, which are the main components related to radical scavenging activity [78,79]. However, there was a significant reduction in the DPPH-RSA when the temperature continued to increase to 65 °C. These findings are comparable with those from a study on mackerel (*Pneumatophorus japonicus*) FPH production with the use of Alcalase that showed that the highest DPPH-RSA was obtained when the hydrolysis was performed at a temperature of 46 °C, which was followed by a significant reduction when the temperature increased to 60 °C [80]. The DPPH-RSA changes were the opposite of the DH changes and this relationship differed from the correlation between these two parameters in trial 1. This reflects the fact that, after obtaining the highest DPPH-RSA value, the excess hydrolysis (expressed by a higher DH) could break the released antioxidative compounds in the FPH, resulting in a decrease in the DPPH-RSA. This finding is in agreement with those of Klompong et al. [44], who showed that, as the DH increased, from 5% to 25%, the antioxidant activities of the FPH produced from yellow stripe trevally (*Selaroides leptolepis*) with Alcalase decreased. Furthermore, these antioxidative compounds may be sensitive to temperature, since oxidation occurs more rapidly at higher temperatures.

The TRPC changes were not completely consistent with the DPPH-RSA changes in this trial (Figure 3D). This may have been because the peptide contributors for these two antioxidant activities differed. The TRPC was not significantly different in the hydrolysis temperatures ranging between 45 °C and 55 °C ($p > 0.05$). There was a slight decrease when the hydrolysis temperature was increased to the temperature range of 60 °C to 65 °C. This reduction in the TRPC when the DH obtained the maximum value may have been due to the breakdown of ferric reducing agents at the stage of excessive hydrolysis, similarly to the DPPH, as discussed above. Therefore, the highest TRPC was obtained (2.6 ± 0.3 equiv.

mg VTM C/g FPH protein) when the degree of hydrolysis was limited at the temperature of 70 °C.

The optimum temperature for the hydrolysis of DM-FPI with Alcalase in this study was 50 °C. This is consistent with the FPH production from Tra catfish frames with Alcalase studied by Amiza et al. [81]. Wasswa et al. [82] also reported an optimum temperature of 50 °C for grass carp skin (*Ctenopharyngodon Idella).* However, other studies show different optimal temperatures for Alcalase activity in FPH production, indicating that the optimal temperature is species-dependent, as well as being dependent on different substrate and reaction conditions [65,83,84].

3.2.3. Effects of Hydrolysis Time (Trial 3)

DH increased, from 18.9% to 31.5%, as the hydrolysis time was increased from 1.5 to 9.0 h ($p < 0.05$) (Figure 4A). This was consistent with the findings from a study on FPH production from Tra catfish by-products [14] that showed an increase in the DH when the time was increased up to 15 h. Other studies on other fish species have also shown the same positive relationship between hydrolysis time and the DH, including species such as Pacific whiting (*Merluccius productus*) [65], silver carp (*Hypophthalmichthys molitrix*) [39], and skipjack tuna (*Katsuwonus pelamis*) [85].

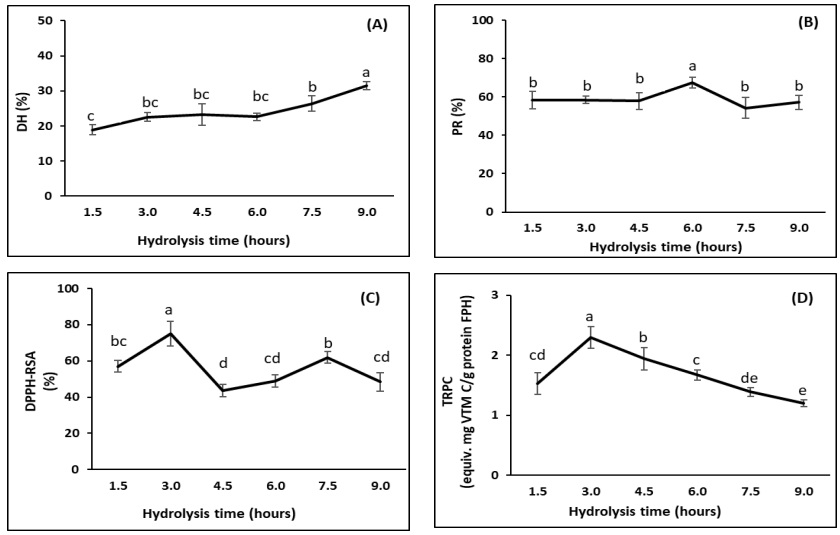

Figure 4. Effect of hydrolysis time (hours) on characteristics of FPHs obtained from the DM-FPI (trial 3), including: (**A**) degree of hydrolysis (DH, %); (**B**) protein recovery (PR, %); (**C**) DPPH radical scavenging activity (DPPH-RSC, %); and (**D**) total reducing power capacity (TRPC, equiv.mg vitamin C/g FPH protein). The hydrolysis reaction was carried out at 50 °C with an enzyme ratio of 3%. Different lowercase letters show significant differences at a significance level of $p < 0.05$.

PR did not change much, although the DH changed significantly with time (Figure 4B). This may have been due to the fact that the FPHs were produced from FPI and any impurities containing insoluble proteins had already been removed, leading to higher concentrations of extractable proteins in the initial substrate. The PR remained constant during the first 4.5 h, reaching a maximum value at a hydrolysis time of 6 h (67.5 ± 2.0%), and decreased when the hydrolysis was extended to 9 h. This decrease may have been due to the further degradation of peptides to free amino acids and/or other volatile compounds [79]. The highest PR in this study was lower than that obtained by Nam et al. [14] (81.9%) for FPHs produced from Tra catfish by-products using Alcalase. This difference is likely due to the differences in evaluation methods, raw materials, and hydrolysis conditions.

DPPH-RSA significantly increased, from 57.0% to a maximum value of 75.1%, when the hydrolysis was prolonged from 1.5 to 3.0 h (Figure 4C). This may have been due to the increase in hydrolysis over this period, resulting in greater amounts of compounds with DPPH radical scavenging potential. Oxidative processes may occur during hydrolysis [62]. Therefore, extending the hydrolysis beyond 3.0 h decreased the DPPH to 48.9% for 6 h hydrolysis. However, the DPPH-RSA increased when the hydrolysis was further extended to 7.5 h, followed by a reduction after that. These fluctuations reflected a possibility that two concurrent processes may have affected the DPPH-RSA during enzymatic hydrolysis. The first process was the release of the antioxidative peptides and the second was the breakdown of the generated antioxidative peptides. Dong et al. [39] found that the DPPH-RSA of FPHs was affected by the hydrolysis time, with the highest value obtained after 2 h of hydrolysis and a slight reduction after 6 h of hydrolysis. Wang et al. [80] studied FPH production from mackerel (*Pneumatophorus japonicus*) and showed that the DPPH-RSA increased with time, obtaining a maximum value after 5 h followed by a decline when the hydrolysis was prolonged to 8 h.

Similarly to the DPPH-RSA, the TRPC significantly increased, from 1.5 equiv. mg vitamin C/g FPH protein to 2.3 equiv. mg vitamin C/g FPH protein, when the hydrolysis was prolonged from 1.5 to 3 h (Figure 4D). However, there was a significant decline in the TRPC when the hydrolysis was extended to 9 h down to 1.2 equiv. mg vitamin C/g. This may have been due to further oxidative processes related to the antioxidant peptides released in the FPHs during hydrolysis, as observed by Halldorsdottir, et al. [62]. Furthermore, the excessive hydrolysis (with an increased DH) after 3 h may have degraded the obtained antioxidative peptides and led to a reduction in the TRPC [70].

A hydrolysis time of 3 h was thus regarded as optimal for the production of FPHs with high PR and antioxidant activities. This was similar to the value obtained by Amiza et al. [81], who indicated that 163 min was the optimal reaction time for the hydrolysis of Tra catfish frames with Alcalase. The optimal hydrolysis time for the preparation of FPHs from visceral waste proteins of Catla (*Catla catla*) with Alcalase was 135 min, as indicated by Bhaskar et al. [86].

3.3. Comparison of the FPHs Prepared from Different FPIs

3.3.1. Amino Acid Composition

The amino acid compositions of food proteins play important roles in various physiological activities of the human body. They also relate to foods' functional roles and potential within the food industry [72]. All the FPHs studied had similar amino acid compositions (Table 2). The amino acid compositions of the FPHs were similar to those in the corresponding initial substrates (FPIs), which were reported by Nguyen et al. [12]. However, the hydrophobic proteins were lower in the FPHs compared to the corresponding FPIs. This may have been because the hydrophobic proteins remained in the sediment after the production of the FPHs.

Furthermore, gamma-aminobutyric acid and cysteine were observed in all FPHs, although these amino acids were not detected in the corresponding substrates [12]. The presence of the cysteine in the FPHs may have been because cystine was reduced to form cysteine during hydrolysis, as indicated by the lower cystine content in the FPHs compared to the FPIs. Glutamic acid, aspartic acid, lysine, and leucine were the main components of all FPHs, similarly to FPHs produced from capelin (*Mallotus villosus*), Pacific whiting (*Merluccius productus*), and herring (*Clupea harengus*) [72]. The amino acid compositions of all the FPHs met the human amino acid requirements for adults recommended by the FAO/WHO/UNU [87], indicating that these FPHs may be useful as additives or ingredients when developing products for adults, providing valuable input to a balanced protein diet [72].

Table 2. Amino acid compositions (g/100 g protein) of the fish protein hydrolysates produced from fish protein isolates prepared from dark muscle (DM-FPH), head and backbone blend (HBB-FPH), and abdominal cut-offs (ACO-FPH).

Amino Acids	DM-FPI **	HBB-FPI **	ACO-FPI **	DM-FPH	HBB-FPH	ACO-FPH	FAO/WHO/UNU *
4-Hydroxyproline	ND	ND	0.6	ND	ND	ND	
Alanine [b]	6.3	6.5	6.4	6.6	6.5	6.6	
Arginie [a]	6.6	6.9	6.3	6.4	5.9	6.6	
Aspartic acid	11.2	12.1	10.5	10.7	10.7	10.9	
Cystein	ND	ND	ND	0.6	0.7	0.6	
Cystine	1.1	1.1	1.0	0.7	0.6	0.7	
Gamma-aminobutyric acid	ND	ND	ND	0.5	0.6	0.6	
Glutamic acid	18.4	19.4	17.2	20.4	19.2	20.4	
Glycine [b]	3.7	3.9	5.1	3.3	3.4	3.4	
Histidine [a]	2.6	2.6	2.8	2.4	2.5	2.2	1.5
Isoleucine [ab]	4.7	5.0	4.6	4.0	4.2	3.9	3.0
Leucine [ab]	8.8	9.1	7.9	8.5	8.8	8.5	5.9
Lysine [a]	9.3	10.1	8.8	10.2	9.6	10.4	4.5
Methionine [ab]	3.3	3.5	2.9	2.8	2.8	3.2	2.2
Phenylalanine [ab]	3.8	4.2	3.8	3.1	3.1	2.9	3.8
Proline [b]	4.6	4.3	5.0	3.6	4.0	3.4	
Serine	4.2	4.4	4.1	4.5	4.8	4.6	
Threonine [a]	4.6	4.9	4.2	4.5	4.5	4.4	2.3
Tyrosine	2.2	3.5	3.1	2.6	2.8	2.7	
Valine [ab]	5.0	5.3	4.8	4.3	4.8	4.4	3.9
Total amino acids	100.5	106.8	99.1	99.6	99.5	100.2	
Total essential amino acids	48.8	51.7	46.2	46.2	46.3	46.4	
Total hydrophobic amino acid	40.3	41.8	40.6	36.3	37.6	36.2	

[a] Essential amino acid for infants. [b] Hydrophobic amino acids. * FAO/WHO/UNU recommendations for adults [87]. ** The data were adapted from Nguyen et al. [12].

3.3.2. DH, PR, and Antioxidant Activities

The DH was not significantly different between the three FPHs produced from different side streams ($p > 0.05$). However, the PR was highest in the ACO-FPH (80 ± 6.3%) compared to 58.4 ± 2.0% in the DM-FPI and 60.4 ± 5.7% in the HBB-FPH (Table 3). This may have been due to the ACO-FPH's initial substrate (ACO-FPI) having higher soluble protein content than the DM-FPI and HBB-FPI [12].

Table 3. Efficiency of the fish protein hydrolysate (FPH) production from fish protein isolates obtained from Tra catfish dark muscle (DM-FPH), head and backbone blend (HBB-FPH), and abdominal cut-offs (ACO-FPH). Results are expressed as means ± SD from triplicate measurements ($n = 3$) *.

Production	DH (%)	PR (%)	DPPH (%)	TRPC (Equiv. mg Vitamin C/g FPH Protein)
DM-FPH	22.5 ± 1.3 [a]	58.4 ± 2.0 [b]	75.1 ± 6.8 [a]	2.3 ± 0.2 [b]
HBB-FPH	22.9 ± 1.6 [a]	60.4 ± 5.7 [b]	57.9 ± 3.6 [b]	2.0 ± 0.2 [b]
ACO-FPH	24.0 ± 3.5 [a]	81.5 ± 4.3 [a]	86.1 ± 1.6 [a]	6.4 ± 0.4 [a]

* Different superscript letters indicate significant differences within each column at $p < 0.05$.

The antioxidant activity of peptides is closely related to their amino acid constituents and their sequences [78]. In this study, all the FPHs had similar amino acid profiles (Table 2). However, their antioxidant activities were significantly different. Therefore, the peptide sequences of the FPHs may play a primary role in the antioxidant activities. Furthermore,

the pro-oxidant content in the initial substrates (myoglobin, heme, iron, etc.) may have also affected the antioxidant activities of the obtained FPHs. The HBB-FPI may have contained higher amounts of pro-oxidants, such as myoglobin and iron [12], which could have reacted with the antioxidant peptides during hydrolysis, resulting in the lowest bioactivity values, as reflected by the DPPH (57.9 ± 3.6%) and TRPC (2.0 ± 0.2 equiv. vitamin C/g FPH protein), respectively in the HBB-FPH. In contrast, the ACO-FPI contained lower amounts of pro-oxidants and, correspondingly, resulted in the ACO-FPH having the highest DPPH (86.1 ± 1.6%) and TRPC (6.4 ± 0.4 equiv. mg vitamin C/g FPH protein). The radical scavenging activity was found to correlate with the Met, Arg, Val, His, Pro, and Asp content in the peptide sequences of the FPHs [71,88]; when combined, these amino acids reached a significant amount (about 25 g/100 g protein) in all FPHs (Table 2). Overall, the results suggest that the FPHs from Tra catfish side-streams have good potential as natural antioxidant ingredient in foods.

4. Conclusions

The present study clearly indicated that the conditions of hydrolysis with Alcalase, including the enzyme concentration, temperature, and hydrolysis time, had significant effects on the properties of the FPHs produced from the Tra catfish dark muscle FPIs using the pH-shift method. An enzyme ratio of 3.0%, hydrolysis temperature of 50 °C, and hydrolysis time of 3 h were established as the optimal hydrolysis conditions, as the highest antioxidant activities (DPPH-RSA and TRPC) and relatively high protein recovery were obtained at these conditions.

The FPHs produced from DM-FPI, HBB-FPI, and ACO-FPI had different chemical properties. These materials should thus be processed into FPHs separately, adjusting each FPI towards the production of a specific value-added food product. However, all FPHs showed high antioxidant activities, especially the ACO-FPH and DM-FPH, which had high DPPH-RSA and TRPC, indicating the great potential of these FPHs as food antioxidants. Furthermore, the obtained FPHs showed high nutritional value with significant essential amino acid contents, indicating that the FPHs could also be used as food supplements.

However, other physicochemical properties, such as solubility, foaming ability, water- and oil-holding capacity, emulsifying properties, and lipid stability, should be studied further to shed more light on the potential application range of FPHs. In addition, FPHs may contain various types of peptides, which have specific bioactive properties and different bioavailability. Therefore, peptide groups from these FPHs should be purified further, and their properties should be identified to obtain more information about the bioactive and bioavailability characteristics of each peptide.

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Paper IV

processes

MDPI

Article

Changes in Protein and Non-Protein Nitrogen Compounds during Fishmeal Processing—Identification of Unoptimized Processing Steps

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Abstract: Quality changes of protein and non-protein nitrogen compounds during industrial fishmeal processing of fatty pelagic species (mackerel/herring rest material blend, MHB) and lean fish (whole blue whiting, BW) were studied to identify processing steps that require optimization to allow production of products for human consumption. Samples from protein-rich processing streams throughout the fishmeal production were analyzed for proximate composition, salt soluble protein content (SSP), biogenic amines (BA), total volatile basic nitrogen (TVB-N), trimethylamine (TMA), and dimethylamine (DMA). Mass flows throughout processing were balanced based on the total mass and proximate composition data. The quality of the final fishmeal products was highly dependent on the fish species being processed, indicating that the processes require optimization towards each raw material. The chemical composition changed in each processing step, resulting in different properties in each stream. Most of the non-protein nitrogen compounds (including BA, TVB-N, TMA, and DMA) followed the liquid streams. However, the concentrate contributed less than 20% to the produced fishmeal quantity. Mixing of this stream into the fishmeal processing again, as currently carried out, should thus be avoided. Furthermore, the cooking, separating, and drying steps should be optimized to improve the water and lipid separation and avoid the formation of undesired nitrogen compounds to produce higher-value products intended for human consumption.

Keywords: fishmeal; protein; biogenic amines; trimethylamine; dimethylamine; TVB-N

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1. Introduction

Fish is a nutrient-dense food containing high-quality proteins with a well-balanced amino acid composition, long-chain polyunsaturated fatty acids (LC PUFA), and micronutrients [1,2]. In 2015, fish contributed to about 17% of the human intake of animal proteins and 7% of the world's total protein consumption [3]. Global fish production reached about 179 million tons in 2018, of which approximately 88% went to human consumption [3]. Nevertheless, fish is a limited resource, and the depletion of marine fisheries resources and growing environmental challenges are global issues that require action [3]. At the same time, the growing global human population will increase the need for fish and fishery products [1,4]. It is predicted that more than 10% of the world's human population could face micronutrient and fatty acids deficiencies due to the reduced availability of fish over the coming decades [1]. Many underutilized fish species and protein-rich rest materials are used for low-value fishmeal intended for aquaculture or other animal feed. Most small fish species caught on an industrial scale are used for fishmeal and fish oil production rather than for direct human consumption [3,5,6]. The Food and Agriculture Organization of the

United Nations (FAO) suggests that more attention should be paid to utilizing low-value nutrient-rich fish species, such as small pelagic fish, for human food. Decreasing the use of wild fish for fishmeal and fish oil production and redirecting fish reduction facilities towards the processing of fish protein for human consumption can be a valuable step in meeting the future human protein demand [4–6]. Small-sized fish species, which have a valuable source of essential micronutrients and high-quality protein [7], can be used for human consumption or as a start feed in aquaculture or agriculture if the processes are updated and optimized. Fish protein products from underutilized species can also be used in functional food or ready-to-eat products, which has encouraged food manufacturers to develop new methods to process fish protein powders [8,9].

Fish muscle is, however, highly perishable and susceptible to degradation during handling, processing, and storage. Protein stability is one of the most important characteristics of processed fish products, affecting the nutritional, digestive, and sensory quality. Protein changes can lead to the loss of essential amino acids, an overall decrease in nutritional value, and protein functionality and digestibility [10–13]. Several studies have shown a loss of salt soluble proteins (SSP) in refrigerated, frozen, and salted fish during processing and storage [10–12,14]. However, there is little information available on protein changes driven by heat and drying treatment during fishmeal processing.

Non-protein nitrogen compounds formed during the degradation of proteins play a significant role in determining the taste and smell of fishery products. Free amino acids, peptides, purine bases, urea, and trimethylamine oxide (TMAO) are the main components of this chemical group [15]. Post-catch handling and processing may cause changes in proteins due to the formation of undesirable non-protein nitrogen compounds such as biogenic amines, total volatile basic nitrogen (TVB-N), trimethylamine (TMA), dimethylamine (DMA), and ammonia [13]. Therefore, it is important to control and minimize the formation of these compounds if the products are intended for human consumption.

Globally, 65–75% of the fishmeal and fish oil is produced from small pelagic fish [3,6,16]. Pelagic fish species constitute a large part of the captured fish in Iceland and made up 51% of the total catch in 2020. Blue whiting (*Micromesistius poutassou*), Atlantic mackerel (*Scomber scombrus*), and Atlantic herring (*Clupea harengus*) are the three dominant pelagic species, accounting for 42% of the total catch. However, these species only accounted for about 13% of the total value [17]. Therefore, much can be gained from developing higher-value products from these species. In Iceland, most of the herring and mackerel catches are processed for human consumption as frozen, headed, and gutted or filleted fish. The side streams (cut-offs, heads, guts, viscera, backbone, etc.) are collected and used for fishmeal and fish oil production, along with any bycatch [18,19]. The mackerel and herring seasons overlap, and these species are thus often processed into fishmeal and oil simultaneously. Mackerel and herring are histidine-rich species [20,21], and fish guts are rich in a wide variety of enzymes and bacteria. Biogenic amines are generated from the decarboxylation of free amino acids by endogenous enzymes of raw material or by bacterial activities. Biogenic amines, such as histamine, tyramine, putrescine, and cadaverine, are a potential health risk because of their toxic characteristics [22,23]. It is thus of high importance to limit the formation of biogenic amines during production. This is one of the main challenges when producers want to optimize the utilization of side streams from mackerel and herring fishmeal processing for human consumption into the development of other high-value-added products.

Blue whiting made up about 50% of the pelagic catch around Iceland in 2020 [17]. This species is used primarily for fish meal production and is generally not considered tasty enough for direct human consumption. Blue whiting is a lean fish of the gadoid family, with high trimethylamine oxide (TMAO) and TMAOase levels [24]. During post-catch handling and processing, TMAO may be broken down by spoilage bacteria into trimethylamine (TMA), generating pungent and undesirable fishy flavours and odours. Moreover, TMAO may be split into dimethylamine (DMA) and formaldehyde (FA) under TMAOase catalysis.

It has been shown that TMAO decomposition plays an important role in the total volatile base nitrogen (TVB-N) production in this species [24].

Traditional fishmeal/oil products have been processed with the same technology for decades, forming low-quality products of relatively low economic value. These production processes are primarily purposed toward water removal. Meanwhile, the protein quality, lipid removal, and separation of unwanted non-protein nitrogen compounds have not been considered in detail [16,25]. Furthermore, the processes are not optimized towards variations in the raw materials or the processing of different species.

This study therefore aimed to indicate how proteins and unwanted non-protein nitrogen compounds change and/or separate during processing from the initial raw materials to the final products during processing of different pelagic species. Evaluating changes in protein characteristics during each step of the current fishmeal processes is crucial in order to systematically change these processes towards producing high-quality, protein-rich products for human consumption. The changes in protein and non-protein nitrogen compounds during traditional processing of fatty pelagic fish species and leaner fish were investigated to identify necessary improvements towards the production of protein products for human consumption, as affected by species and raw material characteristics.

2. Material and Methods

2.1. Raw Material and Sampling

2.1.1. Raw Materials

Raw materials were collected on two occasions to compare the efficiency of a fishmeal factory during the processing of a fatty (a mackerel/herring blend, MHB) raw material and lean raw material (blue whiting, BW).

The Atlantic mackerel/herring was caught from 3 September to 7 September 2017, off the southeast coast of Iceland, by midwater trawling. The mackerel and herring were mechanically headed and gutted (Baader 221: Automatic Pelagic Processing Line) upon arrival to the processing facility. The cut-offs were collected along with the bycatch, and the material was pumped into the fishmeal processing facilities, where it was stored in a receiver tank and kept at 3 ± 1.5 °C until processed 1–3 days upon arrival to shore. The raw materials for the MHB fishmeal production contained 58% of Atlantic mackerel (*Scomber scombrus*) cut-offs, 37% of Atlantic herring (*Clupea harengus*) cut-offs, 4.5% of blue whiting (*Micromesistius poutassou*), and about 0.5% of bycatch species.

The blue whiting was caught on 30 April 2019, south of the Faroe Islands, by midwater trawls. The BW was refrigerated at 2 ± 2 °C on board for 24 h before being transferred to the fishmeal processing facility, where it was processed in the same way as the MHB as described in the following section. More detailed information about the raw materials and their handling were described by Hilmarsdottir et al. [16].

2.1.2. Sampling during Industrial Fishmeal and Oil Processing

A detailed flow chart of the industrial fishmeal and oil production processes is presented in Figure 1. Upon arrival at the factory, the raw material was preheated for 20 min at 55 °C. Next, the mixture entered a cooking step at 85–95 °C for 20 min before being drained and pressed to remove excess water. Then, the press liquid and drained liquid were transferred to a decanter, forming a liquid mixture called ***separated press liquid***. The separated press liquid entered centrifuges and evaporators to separate the fish oil from the solid processing streams. The liquid streams were led through two evaporators to produce a ***concentrate***, which was combined with the ***press cake*** and ***sludge*** before drying.

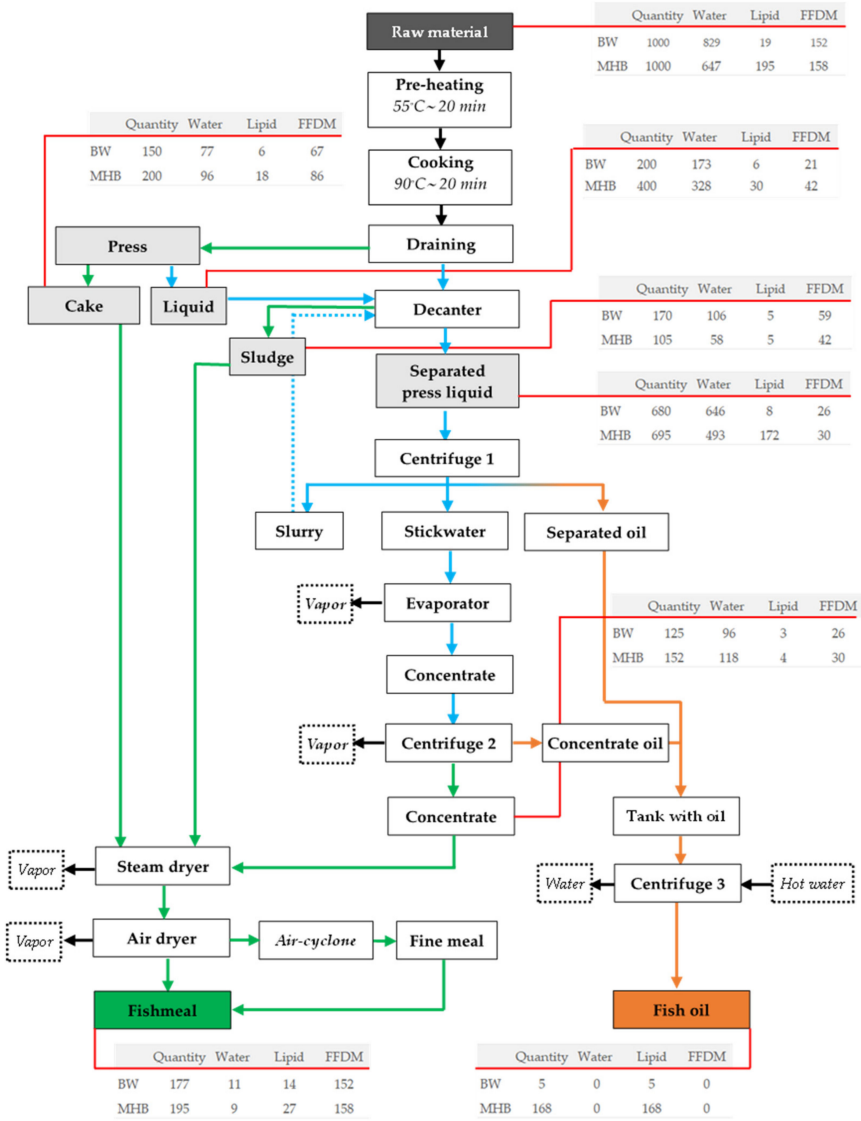

Figure 1. Industrial fishmeal and fish oil production process. The green colour indicates the solid streams throughout the process, the blue represents the liquid streams, and the yellow colour expresses the oil streams. Mass balance from 1000 kg of raw material was calculated for blue whiting (BW) and mackerel/herring blend (MHB), shown by red lines. The quantity of each processing stream and the amounts of water, lipid, and fat-free dry matters (FFDM) in each stream are shown in kg. The flow chart was adapted from Hilmarsdottir et al. [18].

The first drying step was performed in a rotary disc steam dryer for 30 ± 5 min (steam temperature of 160 °C, drying temperature of 95 °C), reducing the moisture content of the material to approximately 40–50%. The material underwent a second drying step in a Hetland air dryer for 16 ± 2 min (maximum input air temperature 450 °C, drying temperature 150 °C at the middle of the dryer, wet bulb temperature of about 65 °C). Some fine particle meal (***fine meal***) was blown out through the air duct during the air drying.

This meal was recovered and combined with the rest of the dried meal, forming the final ***fishmeal***, which had a moisture content of 5–10%.

Samples were collected at key locations throughout the processing, as indicated in Figure 1. After collecting, the samples were cooled overnight to 0 ± 2 °C and transported the following morning to the laboratory. The samples were then stored at −25 °C until analysis, which took up to six months for the MHB and three months for the BW. Prior to analysis, samples were left to thaw at 0–4 °C for 12–36 h. Three samples (n = 3) were collected at each location, and chemical analyses were performed in duplicate for each individual sample. In order to assess the effectiveness and quality changes occurring during processing, a combination of well-known and/or accredited analytical methods, which are commonly applied during food production, were applied. The same analytical methods were furthermore applied to both raw materials, as that allows quantitative and qualitative comparisons of processing of the two raw materials. The applied analytical methods are described in detail in Sections 2.2–2.4.

2.1.3. Chemicals

All chemicals used in the study were of analytical grade and purchased from the Sigma-Aldrich Company (Missouri, TX, USA).

2.2. Proximate Composition Changes during Processing

Water content was measured according to ISO 6496:1999. About 5.0 g of sample was weighed and placed in a small porcelain bowl. The bowls were left to dry for 4 h at 104 ± 2 °C and allowed to cool to ambient temperature in a desiccator for 30 min before being weighed again.

Crude protein content of the samples was measured according to ISO 5983-2 (2009). About 2 g of homogenized sample was digested in 17.5 mL concentrated H_2SO_4 in the presence of two Kjeldahl tablets (each tablet contains 0.4 g $CuSO_4$ and 3.5 g K_2SO_4) as an oxidative catalyst at approximately 420 °C for 2.5 h. The digested sample was made alkaline by adding NaOH, and the nitrogen distilled off as NH_3. The NH_3 was then "trapped" in a 1% boric acid solution. The amount of ammonia nitrogen in the solution was quantified by titration with a standardized H_2SO_4 solution. The nitrogen content was multiplied by 6.25 to obtain the ratio of crude protein.

Lipids were extracted from 25 g samples with 50 mL of chloroform, 50 mL of methanol, and 25 mL or 0.88% KCl according to the Bligh and Dyer method [26]. After homogenizing for 4 min, the mixture was centrifuged at 2500 rpm for 20 min at 4 °C. The lower chloroform phase, containing the lipid fraction, was collected and filtrated on a glass microfiber filter paper under vacuum suction. The extracts were then removed from the upper phase and filled with chloroform to reach a volume of 50 mL. Exactly 2 mL of the chloroform phase was pipetted in a glass tube and blown by a nitrogen jet at 55 °C to remove the solvent. The remaining solution was weighed to determine the total lipid content.

Ash was defined as the remaining components of the dry matter. The ash content was calculated as the total wet weight (100%) after removing the water, lipid, and crude protein contents. Fat-fee dry matter (FFDM) was calculated as the total wet weight (100%) minus the water and the lipid contents. The water, crude protein, lipid, and ash content were expressed as a percentage of wet weight.

2.3. Mass Balances during Processing

Material balances are essential for effective process development in the food industry [27] and aid in assessment of the quantity of products and side streams.

As the fishmeal and fish oil production was assessed under steady-state conditions, the mass of the raw materials entering the process facilities equalled the mass of the products and other exiting processing streams [28]. The raw materials are composed of three major components: solids (FFDM), lipids, and water. The primary purpose of the fishmeal process lies in the separation of these major components [13]. The mass balances were established

throughout the production, and the quantity of side streams was estimated based on changes in these components after each processing step. The overall mass balances were calculated based on an input of *1000 kg of raw materials*. When fitting the mass balance between operation steps, average values were used on an FFDM base.

2.4. Protein Changes during Processing

2.4.1. Salt Soluble Protein Content (SSP)

Salt soluble proteins (SSP) were extracted from the samples with a NaCl buffer (1 M NaCl and 0.02 Na_2CO_3, pH 7.0) according to the method described by Kelleher and Hultin [29]. Exactly 190 mL of buffer solution was added to 10 g sample, and the mixture was homogenized in an Ultra-Turrax homogenizer (Ika Laborttechnik, T25 basic, Staufen, Germany) for 1 min. The mixture was incubated on ice for an hour before being centrifuged at 4 °C for 15 min at 10,000 rpm (Avanti Centrifuge J.10, Beckmann Coulter, Fullerton, CA, USA). The SSP were measured by quantifying the amount of solubilized protein in the supernatant based on the Bradford method [30]. The diluted supernatant and the Bradford reactive solution were placed in a 96-well microplate, and the absorbance read at 595 nm (Sunrise Microplate Reader, Tecan GmbH, A-5082 Grodig, Austria). The SSP were calculated based on a calibration curve made with bovine serum albumin with concentrations ranging between 0.1–1.4 mg/mL. Results were expressed as a percentage of the wet weight.

2.4.2. Biogenic Amines (BA)

Samples were tested for biogenic amines, including tyramine, putrescine, cadaverine, and histamine, using a method developed by Olajos [31]. About 5 g of the sample was homogenized with 45 mL of 0.6 M perchloric acid using an Ultra-Turrax homogenizer for 1 min. The homogenate was then filtered through a Whatman pleated filter paper 113 V. The filtrate was pressed using a disposable syringe assembled into a membrane filter (pore size 0.45 µm). This extract was then used for the measurement by using liquid chromatography (LC-30/20 AD with two low-pressure pumps high-performance liquid chromatography (HPLC) system) (Shimadzu, Kyoto, Japan). The BA were separated on a reversed-phase column (Zorbax Eclipse Plus C 18 4.6 × 250 mm, 5 µm), and after online derivatization (post-column derivatization) using ortho-phthalaldehyde, they were measured by fluorescence detection. A standard curve was made using a mixture of standard solutions, including tyramine hydrochloride, putrescine dihydrochloride, cadaverine dihydrochloride, and histamine dihydrochloride solutions, spanning a range of 2.5–100 mg/L. The BA contents were calculated and expressed as g/kg wet weight (ww).

2.4.3. Total Volatile Basic Nitrogen (TVB-N), Trimethylamine (TMA) and Dimethylamine (DMA)

TVB-N was determined using the steam distillation method described by Malle and Poumeyrol [32]. Approximately 50 g of sample was homogenized with 100 mL of 7.5% aqueous trichloroacetic acid solution. The blend was filtrated through a Whatman pleated filter paper 113 V. Then, 25 mL of the extract was transferred into a distillation flask with 6 mL of 10% NaOH solution. Steam distillation was then performed using a Kjeldahl-type distillatory, and the TVB-N was collected under a condenser into a beaker containing 10 mL solution of 4% boric acid and indicators (0.04 mL of methyl red and bromocresol green), which turned green when alkalinized by the TVB-N. The alkalized mixture was titrated with a standardized H_2SO_4 (0.037 N) solution using a 0.05 mL graduated burette. Complete neutralization was achieved when the colour turned pink on addition of a further drop of sulphuric acid solution.

The TMA and DMA were measured according to the liquid chromatography–mass spectrometry method described by Baliño-Zuazo and Barranco [33]. About 2.5 g of sample was homogenized with 50 mL of 10 mM acetic acid solution and centrifuged at 13,400 rpm at 4 °C. Twenty µL of the supernatant of the extract was mixed with 480 µL of tetraethy-

lammonium chloride hydrate 3.2 µg/mL in acetonitrile/water (6:4), 20 µL of 0.5 M bicarbonate buffer, and 1 mL of tert-butyl bromoacetate (5 mg/mL in acetonitrile). The mixture was incubated in a water bath for 1 h at 60 °C for a derivatization reaction. The derivatized samples were analyzed using a Luna HILIC column (150 × 2 mm I.D., 3 µm) (Phenomenex Torrance, CA, USA) in a Dionex Ultimate 3000 HPLC (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a TSQ Quantiva mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The TVB-N, TMA, and DMA were calculated and indicated as mg N/100 g ww.

2.5. Statistical Analysis

All data summaries and statistical analyses were performed using the IBM SPSS Statistics software (Version 22, IBM, 1 New Orchard Road, Armonk, New York, NC 10504-1722, USA) and Microsoft Office Excel 2013 (Microsoft Inc., Redmond, WA, USA). One-way analysis of variance (ANOVA), Tukey's HSD tests, and Student t-tests were performed on means of the variables. Significant difference was set at the 5% level ($p < 0.05$) for all statistical analyses.

3. Results and Discussion

3.1. Changes in Proximate Composition during Processing

The water content of the blue whiting (82.9 ± 0.6%) was significantly higher than in the mackerel/herring blend (64.6 ± 3.3%) ($p < 0.05$). By contrast, the MHB had a significantly higher lipid content (19.5 ± 2.0%) than the BW (1.8 ± 0.1%). The difference in water and lipid contents between the different raw materials agrees with the species-dependent lipid content, as water and lipid content have an inverse linear relationship in fish muscle [34,35]. The BW had slightly higher water and lower crude protein content than the blue whiting reported by Egerton et al. [34], which had lipid, crude protein, and water contents ranging between 3–5%, 16–18%, and 75–80%, respectively. These differences can mainly be explained by different location and time of fishing [36].

Water content decreased significantly during pressing to 51.5 ± 2.3% and 47.9 ± 1.6% in the BW and MHB press cakes, respectively (Figure 2). The water content of the sludge was 62.4 ± 0.6% and 56.3 ± 0.9% in the BW and MHB, respectively, which was significantly lower than the water content in the separated press liquid (91.1 ± 0.0% and 71.2 ± 2.1%). These results confirm that the press and decanter play an important role in water removal. Water content is an important quality parameter of fishmeal. Low water content can inhibit protein browning and bacterial-caused deterioration. However, too low water activity increases the risk of lipid oxidation and loss of protein solubility [37]. Therefore, a water content of 5–12% is generally recommended for fishmeal [13,25]. The final BW and MHB fishmeal had water contents close to the suggested range (6.4 ± 0.1% and 4.6 ± 0.2%, respectively) and are comparable to earlier published results for these species [38,39].

Most of the lipids followed the liquid processing streams after the separation steps, resulting in increased crude protein and decreased lipid content in the press cake and sludge (Figures 1 and 2), more so in the MHB processing due to the higher lipid content in the raw material. The MHB press cake had a crude protein content of 37.5 ± 2.4% and a lipid content of 8.8 ± 0.6%, compared to 12.0 ± 0.3% crude protein and 19.5 ± 2.0% lipid content in the raw material. Similarly, after passing the decanter, the sludge contained 34.6 ± 0.7% protein and 4.7 ± 0.2% lipid, compared to 10.0 ± 0.2% protein and 18.2 ± 3.3% lipid in the separated press liquid. The crude protein content significantly increased while the lipid content decreased in the final MHB fishmeal (65.2 ± 0.3% protein and 14.3 ± 0.2% lipid) compared to the raw material (Figure 2b). Both the proportional protein and lipid contents were significantly higher in the final BW fishmeal than in the raw material, mainly due to water removal. The BW fishmeal had a similar crude protein content to the BW fishmeal in earlier studies (68–70.5%) [38,40]. The protein contents of the fishmeal were comparable to fishmeal made from other pelagic species reported in the literature [38,41,42] and higher than the protein content in fishmeal made from both cod and saithe (61.9%) [39] and

tuna cut-offs (56.2–59.1%) [43]. The lipid content of fishmeal from pelagic fish is typically between 6–10% [39,41]. The MHB fishmeal had a considerably higher lipid content and lower protein content than the BW fishmeal, reflecting the different composition of the raw materials used [13]. Lipid separation is thus of special importance during processing of fatty fish species. However, both the BW and MHB fishmeal had a high lipid content and were thus classified as type C fishmeal and should not be used for human food under current processing conditions [13,18,25]. The high lipid content indicates inefficiency in lipid separation and removal during processing of both species. The processes thus require optimization with regards to the lipid separation if the fishmeal is intended for human consumption. However, the optimal processing changes might be different while processing the two different species. Hilmarsdottir et al. [18] suggested that optimization of early processing steps, including the heating steps, would improve lipid separation during fishmeal processing of a mackerel–herring blend. Furthermore, their study suggested that drying the press cake, the sludge, and the latter concentrate individually could result in more flexibility in processing and process control, ultimately leading to the production of higher-quality products.

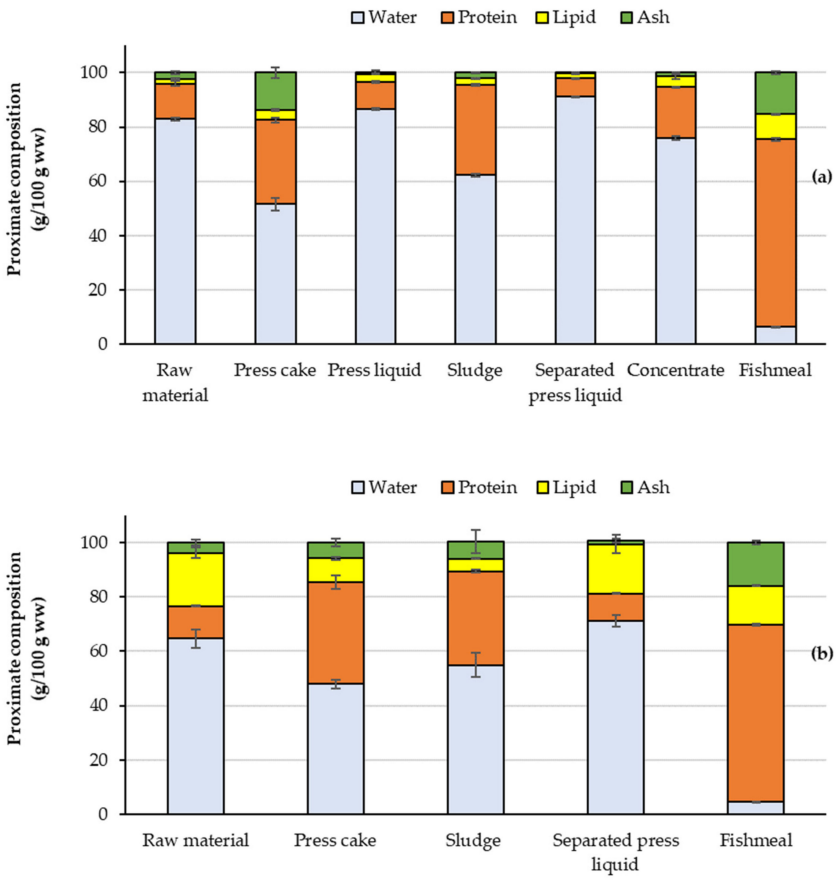

Figure 2. Proximate composition (%, g/100 g ww) in BW (**a**) and the MHB (**b**) fishmeal products during industrial processing.

The press cake of both raw materials contained higher amounts of ash than other intermediate stages of processing or 13.7 ± 1.9% and 5.7 ± 1.5% in the BW and MHB, respectively. The high ash content of the press cake reflects that the bones of the fish were

mainly left in the press cake. This was especially evident in the BW processing. After entering the decanter, the remaining ash content remained in the sludge, resulting in a lower ash content in the separated press liquid (0.3 ± 0.0% in BW and 1.3 ± 0.8% in MHB). The water and lipid removal led to a relative increase of ash content in both the BW and MHB fishmeal (15.6 ± 0.4% and 15.9 ± 0.7%, respectively), of which results are comparable with values observed in anchovy fishmeal (15.0%) [41] but lower than those seen in fishmeal made from cod and saithe off-cuts (22.4%) [39].

3.2. Mass Balances during Processing

The factory produces fishmeal and fish oil from around 1200 tons of raw material per hour when operating at full capacity [18]. However, to ease the assessment of yield and size of processing streams, a basis of 1000 kg of raw materials was set for the mass balance calculations. The different raw materials resulted in very different proportions of mass flow through the press cake, sludge, and concentrate, as well as differences in the yield of fishmeal and oil (Figure 1), but processing yields are highly dependent on the chemical composition of the raw materials [13]. The ratios between the press cake and separated press liquid were approximately 3:4 and 1:2 for the BW and MHB, respectively, indicating that the chemical composition had a high impact on the balance between the liquid and solid streams during processing, and thus the effectiveness of the process. The obtained ratios between the press cake and press liquid furthermore indicate that the pressing was more efficient in the MHB than in the BW. In agreement with this, the MHB, which had higher FFDM and lipid content in the raw material, resulted in a higher production yield of both fishmeal and oil than the BW (Figure 1).

Although most of the lipids followed the separated press liquid, a significant amount of lipids remained in the press cake (approximately 4% and 9% in the BW and MHB, respectively) and sludge (3% and 5% in the BW and MHB, respectively). After evaporation of the press liquid, the concentrate was mixed with the sludge and press cake prior to entering the drying steps. The press cake contributed the biggest proportion (44% and 54%) of the total mass in the combined solid stream, followed by the sludge (39% and 27%), and the smallest proportion originated from the concentrate (17% and 19%) during the BW and MHB fishmeal productions, respectively. The press cake was the highest contributor of lipids (39% in BW and 67% in MHB) in the fishmeal. In BW, the highest contributor to water was the sludge (38%), whereas the concentrate was the highest contributor of water to the MHB fishmeal (44%). The different contributions of the solid streams towards the composition of the final fishmeal during processing of the two different raw materials are of high concern and highlight the importance of adjusting the processing towards the optimal efficiency and quality of each raw material. The high water content in the BW production indicates that the decanter did not operate properly, potentially due to overload, leaving a higher water proportion in the BW sludge (38%) compared to the MHB sludge (21%) (Figure 1). Overload of the decanter should thus be avoided. Assessment of the fishmeal composition, furthermore, identified the high importance of efficient lipid separation, both from the BW sludge and the MHB press cake, indicating that the initial processing steps require optimization for the removal of lipids during processing of both species.

Most of the lipids present in the separated press liquid were effectively extracted with the two centrifuges, forming the final fish oils and decreasing the lipid content of the fishmeal. However, only 26% of the lipid content of the BW raw material was extracted to form the BW fish oil, whereas 86% of the MHB lipid content in the MHB raw material was extracted to form the MHB fish oil. This could partially be dependent on differences in the total lipid content as well as the lipid composition and availability of lipid classes between the species [16]. Improving the lipid separation from the solid streams and directing them toward the liquid streams would therefore not only increase the fishmeal quality but also increase the oil yield. The different efficiency of the processing steps due to the variation in chemical compositions of the different raw materials indicate that further optimizations of the processing of each species are necessary.

The BW and MBH fishmeal could both be classified as type C fish protein concentrate (FPC), according to their lipid content. For a type A FPC, the lipid content should be lower than 0.75% [13,25]. Substantial changes are thus required during processing of both species to obtain a type A FPC classification of the products. However, as the solid streams differ in proximate composition (Figure 1), a suitable end product needs to be aligned with the properties of each raw material and each processing stream, including the quality of the proteins, which are discussed in the following section.

3.3. Protein Quality Changes during Processing

3.3.1. Salt Soluble Protein Content (SSP)

Salt soluble protein content (SSP) decreased significantly during processing, from 5.7 ± 0.7% to 0.9 ± 0.0% in the BW and from 6.1 ± 0.5% to 0.9 ± 0.0% in the MHB (Figure 3a,b), indicating substantial protein denaturation and associated loss of protein solubility during the processing of both species. Generally, the total protein content in fish muscle ranges from 11–24% wet weight, in which SSP account for 85–90% [2,44]. Low SSP content was expected in this study due to the high content of connective tissues (stroma protein) in the raw material [45]. Moreover, the low SSP in both BW and MHB raw materials may be due to protein denaturation during cold storage before processing and the frozen storage of the samples until they were analyzed. The SSP content in the BW was slightly higher than in the blue whiting studied by Derkach et al. [46], which had an SSP content of 5.2%.

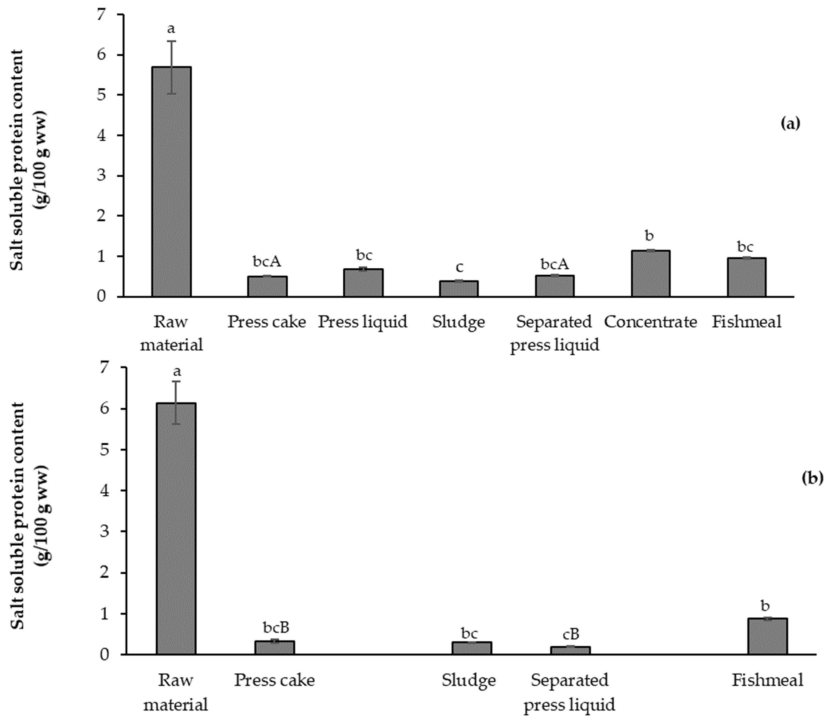

Figure 3. Salt soluble proteins (%, g SSP/100 g ww) in chosen BW (**a**) and MHB processing samples during industrial fishmeal production (**b**). Lowercase letters indicate significant differences in SSP between sampling locations, while uppercase letters indicate significant differences between two types of raw materials at the same processing step. Statistical significance levels were set to $p < 0.05$ for all analyses.

The SSP significantly decreased right after the cooking steps and remained stable during the following steps, reflecting that the proteins were already mostly denatured during cooking. This is in agreement with earlier studies, which have shown that protein denaturation occurs mainly during the cooking step as cell membranes break down, and the fat depots rupture, separating the oil and water from the fish muscle [13]. Heating thus causes irreversible changes to the protein structure, such as protein unfolding, exposing previously hidden hydrophobic groups, or heat-induced aggregation, resulting in decreased content of salt soluble proteins [45,47]. The very low SSP content after the cooking step indicates that most myofibrillar proteins denatured, and sarcoplasmic proteins precipitated during the heat treatment. These protein changes can furthermore decrease protein digestibility in vitro [48]. Actin, myosin, and sarcoplasmic proteins account for 85–95% of total fish proteins [44]. Fish myosin begins to denature at around 35 °C; sarcoplasmic proteins are denatured at around 44 °C, while actin is denatured in the temperature range of 58–68 °C [49–51]. Most fish proteins have thus denatured at temperatures around 75 °C [13]. Significant decreases in protein solubility due to heat processing have also been reported earlier [52,53]. A proportional increase was observed in SSP in the final fishmeal compared to the solid streams entering the dryers. Although some further protein denaturation is expected to occur during the drying step, this increase in SSP can mainly be explained by the removal of water and lipids during drying.

SSP content is related to protein solubility, which is considered the first functional characteristic during the development and testing of a new protein ingredient. Protein solubility is the primary property of proteins used in liquid foods [45]. The low SSP in the fishmeal hence limit their practical uses. The heat treatment applied in the current fishmeal processing appears to be too rough. For the products to be fit for human consumption, the heating step thus requires changing. Adding a suitable amount of polyphosphate or sucrose could potentially protect the proteins from denaturation during drying, as suggested by Shaviklo [8]. In addition, potential alternative processing solutions could involve the reduction of the temperature but extending the heating step duration both during cooking and drying or use tailored enzymes (proteases) to facilitate more effective protein breakdown without losing the SSP. The use of enzymes may decrease the processing time, lower the energy input, and increase the economic effectiveness as shown in various industrial food processing, such as fish protein concentrate or hydrolysate production [54]. Protein hydrolysates are good nutritional supplements since they have high bioavailability and can be utilized for various metabolic activities [55]. The enzymatic process could be performed at a temperature range from 45–60 °C [56,57] for an extended time. However, these temperature conditions can also promote microbial, biochemical, and chemical spoilage during processing [58–60] and should thus be applied with care. The products derived from an enzymatic protein hydrolysis can have a bitter taste, which is one of the key issues that limits its application in food products [57]. However, this showcases the wide potential that lies in pelagic fish processing and high-quality product development and innovation.

3.3.2. Biogenic Amines (BA)

The four BA, tyramine, putrescine, cadaverine, and histamine, decreased during processing and were more strongly indicated in the MHB than in the BW processing (Figure 4a,b). Cadaverine was the most abundant biogenic amine in all sampling locations during processing of both BW and MHB. In the BW process, histamine was only detected in the raw material (0.3 ± 0.1 g/kg ww) and the liquid streams (press liquid and separated press liquid, each with the content of 0.01 g/ kg ww). No histamine was detected in the BW fishmeal, indicating that the histamine was successfully removed during the processing. The histamine level in the initial MHB raw material was 3.5 ± 0.2 g/kg ww and decreased to 0.8 ± 0.1 g/kg ww in the final fishmeal. However, higher histamine levels were detected in all processing streams in the MHB processing than what is acceptable for human food (<0.2 g/kg ww), as established by the European Commission Regulation No 2073/2005. The histamine in the raw materials was higher than the acceptable level for

human consumption but was at acceptable levels for fishmeal in BW (<1 g/kg ww) [61]. High BA levels in the BW and MHB raw materials may have resulted from bacterial activity during the delay between catching and processing [13,18]. Since both mackerel and herring are histidine-rich species, and the raw material used for fishmeal processing contained a large ratio of viscera and dark muscle, the risk of bacterial growth is high [62]. Furthermore, the generation of BA in mackerel and herring has been shown to occur even when stored at low temperatures, such as 2 °C [21].

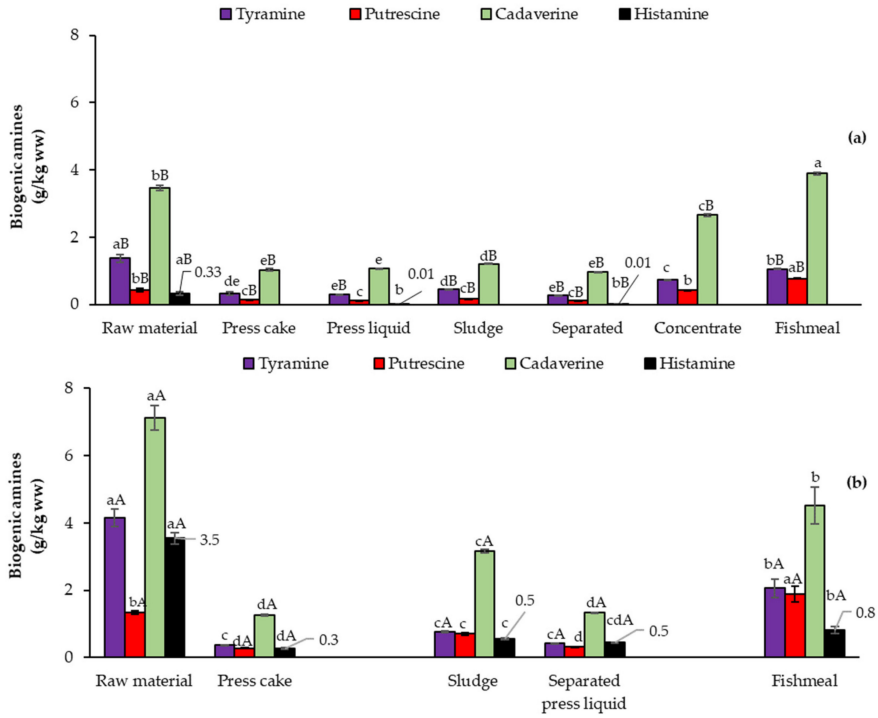

Figure 4. Biogenic amines (BA) (g/kg ww) obtained during fishmeal and oil processing from BW (**a**) and MHB (**b**). Within each BA type, lowercase letters indicate a significant difference between sampling locations, while uppercase letters show significant differences between raw materials at the same processing step. Statistical significance levels were set to $p < 0.05$ for all analyses.

The BA content was significantly higher in the MHB than the BW raw material, and the same trend was seen in the corresponding fishmeal. This indicates that the BA formation is species specific [63,64]. Histidine, the precursor to histamine, exists in abundance in the dark muscle of fish. Thus, dark-muscle-rich fish species generally contain more histidine than leaner species [59,63]. Furthermore, there is a positive correlation between the amino acid histidine and the amount of histamine formed [65]. Tuna and mackerel, which belong to the scombroid family, thus often have high histidine levels [60,64], resulting in high histamine contents in products from these species. This is in good agreement with the different BA levels in the species in the current study, but no histamine was found in the BW fishmeal, while a histamine content of 0.8 g/kg ww was obtained in the MHB.

Several previous studies have indicated that BA are thermally stable even during boiling [66,67] and are thus not likely to be primarily affected by the thermal steps. However, in this study, significant decreases were observed in the BA after the cooking and pressing steps, during which the total content of the four studied BA went from 5.6 g/kg ww in the raw material to 1.5 g/kg ww in both the press cake and press liquid of the BW and from 16.2 g/kg ww in the raw material to 2.2 g/kg ww in the press cake for the MHB.

Overall, about 82% and 89% of the total BA content in the raw materials was removed during the BW and MHB processing, respectively (Figures 1 and 4). These overall decreases in BA content after processing could possibly be explained by their complex decomposition into other volatile compounds under heating [67]. BA are of low molecular weight and mostly water-soluble [60,68] and are thus released into the liquid streams rather than the oil and solid streams. This results in a higher BA amount in the press liquid than press cake (in the BW) and higher BA in the separated press liquid than the sludge (BW and MHB). Mixing the liquid streams back into the fishmeal processing, as is currently carried out, could thus cause problems when processing raw materials with high BA content and should be avoided, at least for histamine-rich species such as mackerel. However, the effect of the BA levels of other species, such as BW, should not be neglected since high levels of individual BAs (such as cadaverine) can cause problems when adapting the processes towards human consumption. The relative increase in total BA observed in the concentrate (BW) and both fishmeal products (BW and MHB) may then be due to the water and lipid removal during processing, as discussed above.

3.3.3. Total Volatile Basic Nitrogen (TVB-N), Trimethylamine, and Dimethylamine

TVB-N in fish and fishery products primarily includes ammonia, TMA, and DMA [13]. TVB-N levels are often used as a quality criterion for the freshness of raw materials destined for fishmeal processing. Threshold values are set to not exceed 60 mg N/100 g in the raw material if the products are intended for human consumption (European Commission Implementing Regulation No 2019/627) [13] and should be less than 80 mg N/100 g in the raw material for fishmeal production [13]. High TVB-N and TMA levels were observed in the raw materials (with TVB-N contents of 83.9 $\pm$ 0.6 and 68.1 $\pm$ 3.4 mg N/100 g ww in the BW and MHB, respectively, and TMA content of 60.3 $\pm$ 5.3 and 35.8 $\pm$ 4.6 mg N/100 g ww in the BW and the MHB, respectively. These values indicate spoilage in the raw material during the delay between catch and processing, in agreement with the observation of the BA formation in the raw material prior to processing. Furthermore, the raw materials contain viscera, which have a high content of bacteria and enzymes that can promote spoilage during the transport and storage of the fish on board the fishing vessel before entering the fishmeal processing [13,24]. The TVB-N level of the BW was higher than the recommended level for the raw material and substantially higher than reported values in the light muscle of blue whiting, even after six days of storage on ice at 0 °C (22.7 $\pm$ 1.6 mg N/100 g ww) [69]. The DMA level of the BW (11.9 $\pm$ 1.1 mg N/100 g ww) was higher than in the light muscle of blue whiting as studied by Rey-Mansilla et al. [24], who detected DMA levels of only 4 mg N/100 g ww after seven days of iced storage. The TVB-N, TMA, and DMA were significantly higher in the BW raw material than in the MHB. This may be due to the BW being a gadoid fish, which has high level TMAO and TMAase enzyme [24,70]. According to the study by Mizuguchi et al. [70] DMA is formed faster in dark muscle than light gadoid muscle, and that DMA formation was triggered by two main factors, i.e., nonheme iron and taurine levels, which are both abundant in gadoid dark muscle. DMA formation is therefore of special concern during processing of BW cut-offs, which contain a high proportion of dark muscle. Furthermore, the TMAO may already have partially decomposed into DMA in the raw material during the delay between catch and processing, explaining high DMA levels in the BW raw material, as discussed earlier.

The TVB-N levels decreased during the fishmeal processing of both the BW and MHB before the drying steps (Figure 5). The water removal during drying may have resulted in a relative increase in the TVB-N content in both final products, in a similar manner as seen in the BA and SSP results. About 81% and 62% of the TVB-N in the raw material evaporated during BW and MHB processing, respectively (Figures 1 and 5). TMA levels showed similar trends as the TVB-N levels during processing (Figure 5), indicating that TMA is a dominant component of the TVB-N, as shown by Howgate [71]. Since TMA is a volatile amine [72], a part of the TMA content that existed in the raw material may have evaporated during the cooking and pressing steps, resulting in lower TMA content

in both the press cake and press liquid (in BW). More than 90% of the TMA in the raw materials was removed during both BW and MHB processing (Figures 1 and 5). However, the same trend was not as clearly indicated in the DMA changes. The DMA content was stable before entering the centrifugation step. After evaporation and centrifugation, the removal of water and oil led to significantly higher levels of DMA, TMA, and TVB-N in the concentrate than the separated press liquid in BW (Figure 5a). TVB-N, TMA, and DMA are water-soluble compounds; thus, they are mainly dispersed into the liquid phases during processing, resulting in a significantly higher amount in the liquid streams (BW press liquid and BW- and MHB-separated press liquid) than in the solid ones (BW press cake and BW and MHB sludge, respectively). TMAO is decomposed into TMA, DMA, and formaldehyde (FA) during thermal processing [73]. Therefore, the remaining TMA and DMA in the products may result from two concurrent processes, the generation from TMAO decomposition and loss due to volatilization. Rapid DMA non-enzymatic formation was observed in fish muscle dried at 90 °C by Spinelli and Koury [74]. This may explain why the DMA was not lost during the fishmeal processing in this study in the same manner as the TVB-N and TMA (Figure 5). DMA levels increased significantly during evaporation in the BW fishmeal processing, from 11.0 ± 0.5 mg N/kg ww in the separated press liquid to 30.2 ± 0.6 mg N/kg ww in the concentrate. During the drying step, a large part of the water was removed, which could lead to a relative increase in the TMA such as other dry matter components. However, the TMA in the final fishmeal products was lower than during processing (press cake, sludge, and concentrate). This indicates that the TMA was removed in the drying steps, probably mainly due to the removal of water.

Although the TVB-N contents in the raw material and intermediate processing streams were generally higher in the BW than the corresponding MHB samples, the MHB fishmeal had a significantly higher TVB-N level than the BW fishmeal. Meanwhile, the TMA and DMA were higher in the BW than in the MHB fishmeal. This could be due to potentially higher ammonia formation during the pre-processing delay of the MHB by-product blend than in the BW due to a higher proportion of viscera in the MHB raw materials. Viscera, which are rich in enzymes and bacteria, can promote protein changes and spoilage, forming amino acids and ammonia [72], resulting in the formation of undesirable odours and flavours. Ammonia generation during thermal degradation of protein and amino acids has also been observed in earlier studies [75,76].

The fact that the non-protein nitrogen compounds followed the liquid streams, resulting in lower values in the solid streams (the press cake and sludge), indicates that processing these streams individually could lead to lower volatile nitrous compounds in the final products, especially if the BA-rich liquid streams are not redirected into the process. This is in agreement with the observations of Hilmarsdottir et al. [18], who identified inefficient water removal during the draining and concentration steps and that the lipid separation from the fishmeal was insufficient for the production of high-quality products, such as for human consumption or even fish feed. Hilmarsdottir et al. [18] thus recommended that the main streams entered to final fishmeal (press cake, sludge, and latter concentrate) should be processed separately. This would allow production of higher-quality protein products from the press cake, while the sludge and concentrate could contribute to lower value products. The current observations on BA, TVB-N, and TMA content support this notion as well. However, the sludge had a high protein ratio (88 g/100 g dry matter (DM) in BW and 78 g/100 g DM in MHB) and a low lipid and ash proportion, comparable with the proximate composition of fish protein hydrolysates [9]. This stream, therefore, could potentially be used to produce high-value products, such as special feeds, animal feed enrichments, or nutritional supplements and healthy foods for human consumption, such as fish protein hydrolysates or fish protein concentrates [8,9]. These potential uses may bring more economic value than traditional fishmeal production. However, to develop high-quality products for humans, other quality properties of this part, such as TVB-N and biogenic amines of the sludge, need to comply with safety requirements. Processing should thus primarily be optimized to reduce these unwanted non-protein nitrogen compounds.

Adding membrane filtration at appropriate settings to the processing could potentially provide a solution to this problem.

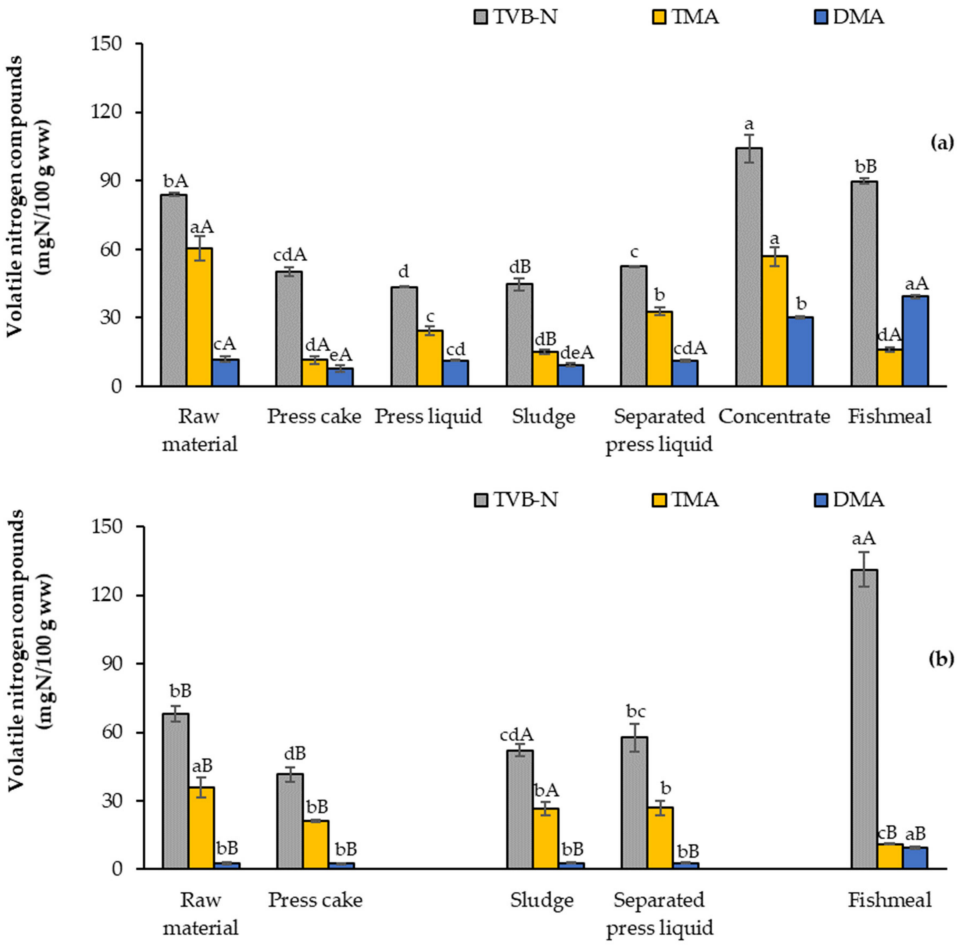

Figure 5. Volatile nitrogen content (mg N/100 g ww) in streams from the industrial BW fishmeal production (**a**) and MHB fishmeal production (**b**). Within each parameter, lowercase letters investigate significant differences between sampling locations, uppercase letters show significant differences between raw materials at the sample processing step. Statistical significance levels were set to $p < 0.05$ for all analyses.

4. Conclusions

Chemical characteristics of the protein-rich processing streams in two industrial fishmeal processes of BW and MHB were evaluated in this study. The raw materials and processing samples were collected at the fishmeal factory. With an input of 1200 tons of raw materials per day, the quality of raw materials entering the factory can be highly variable. The fishmeal production processes were conducted three days post-catch to ensure enough raw materials to fulfil the capacity criteria of the factory. However, this pre-processing delay resulted in considerable heterogeneity and quality degradation in the raw material. In addition, the analysis showed that processing conditions at each step could fluctuate significantly, and the processing efficiency was highly dependent on the species being processed. These variations furthermore influenced the chemical properties of the samples and the quality of the resulting fishmeal products.

Large amounts of non-protein nitrogen compounds were observed in the raw materials, probably due to the three-day pending time from catch to entering the fishmeal processing. Removing the viscera and proper collecting, handling, stable cooling, and storing of the raw materials before processing would improve the safety and quality of the final protein products. This can widen the utilization of the final protein products, potentially even for human consumption, and simultaneously bring more economic benefits of the production.

The BW fishmeal had a higher protein content (69.1 $\pm$ 0.5%) than typical fishmeal (64–67%), and BA and TVB-N levels were within acceptable thresholds for fishmeal. The MHB fishmeal had a protein content of 65.2 $\pm$ 0.3% and a histamine content below 1 g/kg, currently making it acceptable for animal feed. However, the histamine (0.8 $\pm$ 0.1 g/kg) and TVB-N (131.4 $\pm$ 7.3 mg N/100 g) in the fishmeal were higher than acceptable for human consumption. These two products can thus be graded as type C fishmeal with lipid contents above 3%.

Soluble protein content and non-protein nitrogen compounds were readily released into the liquid processing streams together with most of the water and lipids, while high-molecular-weight proteins were retained in the solid streams. Most undesirable non-protein nitrogen compounds were removed during the processing of both species, especially during the drying step. The lipid quality was also highly affected by heating in this step, as described earlier by Hilmarsdottir et al. [18]. This indicates that the drying step requires optimization. Spray drying of the processing streams could potentially provide milder drying and higher quality. Other unoptimized processing steps, such as pressing and concentration, were also identified, which need to be improved in order to produce high-quality products in the future. The testing of alternative processing is left for future studies. Comparison between the two species, moreover, showed that the processes need to be adapted to each raw material for higher-value product production.

In both industrial fishmeal processes (BW and MHB), the press cake had high protein contents and low contents of non-protein nitrogen compounds, making the press cake a promising material for the development of higher-value products. Furthermore, separate processing of the solid streams (press cake, sludge, concentrate) thus shows promising potential for production of a wider range of products, including high-value products for human consumption. However, to be used as human food, the products from the optimized processes should be studied further regarding amino acid profiles, digestibility, and sensory attributes. It would also be of interest to study applications of the optimized products as ingredients for the development of other value-added products included for human consumption.

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