Establishing processes in producing dicalcium phosphate, octacalcium phosphate and gelatin from monkfish (*Lophius vomerinus)* bones

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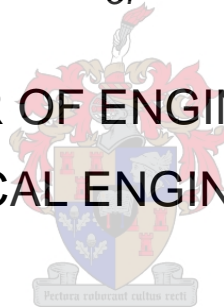
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Declaration

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Abstract

Extraction of valuable products from solid waste generated from fish processing can contribute towards improved utilisation of solid waste originating from fisheries. Fish heads are one of the solid wastes generated by monkfish processing in South African monkfish and hake fisheries. Recovery of protein nutrients from fish heads using a well-established technique, namely enzymatic hydrolysis, results in bones as by-product. Bones, naturally rich in calcium phosphate minerals and protein in small quantities, are a potential source of dicalcium phosphate (DCP) and gelatin protein. This study aimed to develop and optimise processes for the extraction of minerals from bones, precipitation of DCP from the extracted mineral liquor and extraction of gelatin from residual ossein, a by-product during the extraction of minerals from bones. The study entailed four main procedures: preliminary treatment, demineralisation, DCP precipitation and gelatin extraction. The bones utilised for the experimental work were recovered from monkfish heads by way of enzymatic hydrolysis using a simple treatment as the objective of the preliminary treatment.

A two-level, three-factor full factorial design was implemented for demineralisation optimisation. The design was applied to study the effect of H_3PO_4 concentration (% v/v), number of 24-hour extractions and ratio of solution (v) to raw material (w) at ambient temperature of 17 °C on ash and hydroxyproline contents of ossein. The results showed that the optimum conditions to obtain minimised ash content and maximised hydroxyproline contents in the ossein were 5% H_3PO_4 (v/v), four successive 24-hour extractions and 5:1 ratio of solution (v) to bones (w) at ambient temperature of 17 °C. Linear regression models were developed to predict ash content of 0 g kg^{-1} and hydroxyproline content of 75.9 g kg^{-1} in dry mass ossein at optimum conditions. The experimental results showed 2.8 g kg^{-1} dry mass and 69.7 g kg^{-1} dry mass ash and hydroxyproline contents in ossein, respectively, and within the ± 95% prediction interval of the models. The results showed that significant amounts of minerals contained in the monkfish bones were recovered in the mineral liquor while an amount of 69.7 g dry hydroxyproline per kg dry ossein was preserved in ossein.

A maximum precipitate of DCP from mineral liquor, having a maximum phosphorus (P) content and Ca:P molar ratio equal to one, was optimised using response surface methodology (RSM) with a three-factor, five-level central composite design (CCD). Quadratic regression models were proposed to study the combined effect of reaction temperature (°C), 1 M $Ca(OH)_2$:mineral liquor ratio (v:v) and reaction time (minutes) on the amount of DCP precipitate and the P content and Ca:P molar ratio thereof. The results showed that the optimum conditions were 75 °C reaction temperature, 0.95:1 v/v 1 M $Ca(OH)_2$:mineral liquor ratio and reaction time no longer than 17 minutes as reaction times longer than 17 minutes did not lead to increased DCP precipitate. At optimum conditions, the predicted values were 20.5 g dry mass of DCP precipitate per 150 ml starting mineral liquor, and the precipitate contained 221.6 g kg^{-1} dry mass P and 1.07 Ca:P molar ratio. The experimental results of 21.1 g dry mass of DCP precipitate per 150 ml starting mineral liquor, 215.2 g kg^{-1} dry mass P content and 1.16 Ca:P molar ratio were closely matched to the predicted values. The spent solution, as a by-product in the DCP

precipitation, contained very low levels of calcium (Ca) and P. Thus, there was an indication that there was a high recovery of total Ca and P inputs.

An X-ray powder diffraction analysis revealed that the DCP was precipitated in the form of dicalcium phosphate dihydrate (DCPD). The precipitated DCPD also contained essential micro minerals and very low levels of harmful heavy metals. The relative solubility of P in 2% citric acid solution for the precipitated DCPD was determined to be 95.1%, which was an estimate of the P bioavailability thereof and an indication of a potential good-quality inorganic P feed supplement. It was also found that octacalcium phosphate (OCP) was precipitated by using 25 °C reaction temperature, 1.2:1 v/v 1 M $Ca(OH)_2$:mineral liquor ratio and reaction time no longer than 17 minutes. It yielded 25.4 g dry mass of OCP precipitate per 150 ml starting mineral liquor, and the precipitate contained 18.3 g kg^{-1} dry mass P and 31.6 g kg^{-1} dry mass Ca, giving a Ca:P molar ratio of 1.34. Based on the literature, a composite of OCP has specific applications in the medical and dental fields; thus, OCP is regarded as an important biomaterial.

To establish the optimum gelatin extraction conditions from the ossein, RSM with a three-factor, five-level CCD was used. This design was applied to study the joint effect of extraction pH, temperature (°C) and time (minutes) on the hydroxyproline yield in the gelatin liquor at a fixed 1:10 ratio of ossein:water (w:v) and one extraction. The optimum conditions were extraction pH of 8, temperature of 80 °C and time of 125 minutes. The predicted hydroxyproline yield of the model at optimal conditions was 0.6% with an equivalent hydroxyproline recovery of 8.3%. The recovery of hydroxyproline was significantly lower than that from bones of cod species reported in literature on studies that utilised three or more successive extractions. Improving the hydroxyproline recovery (that will also improve hydroxyproline yield) in the present work can be achieved by employing two or more successive extractions of gelatin from ossein. This study demonstrated that gelatin could be extracted from monkfish ossein.

Based on the results of this study, there is a potential for extraction of valuable products DCPD, OCP and gelatin from monkfish bones using processes that were developed and optimised in this study. Consequently, this study can make future contributions towards improved utilisation of monkfish heads, which are currently viewed as a solid waste product from the South African monkfish and hake fisheries.

Opsomming

Die ekstraksie van waardevolle produkte uit die vaste afval wat van visverwerking afkomstig is, kan meewerk dat vaste afval uit visserye beter benut word. Viskoppe is een soort vaste afval wat uit monnikvisverwerking in die Suid-Afrikaanse monnikvis- en stokvisbedryf afkomstig is. 'n Goed gevestigde tegniek om proteïenvoedingstowwe uit viskoppe te herwin, te wete ensimatiese hidrolise, beteken dat visbeen as neweprodukt gebruik kan word. Been, wat 'n natuurlike bron van kalsiumfosfaatminerale en klein hoeveelhede proteïene is, is 'n potensiële bron van dikalsiumfosfaat (DKF) en gelatienproteïen. Die oogmerk van hierdie studie was om prosesse te ontwikkel en optimaal te benut vir die ekstraksie van minerale uit been, die presipitasie van DKF uit die geëkstraheerde mineraalvloeistof, asook die ekstraksie van gelatien uit die oorblywende osseïen (gedemineraliseerde been). Die studie het vier hoofprosedures behels: voorbereidende behandeling, demineralisering, DKF-presipitasie, en gelatienekstraksie. Die been wat vir die eksperimentele werk gebruik is, is deur ensimatiese hidrolise met 'n eenvoudige behandeling, wat met die voorbereidende behandeling in die vooruitsig gestel is, uit die monnikviskoppe herwin.

'n Tweevlak-, driefaktor-volfakulteitsontwerp is vir die demineralisasie toegepas. Die ontwerp is toegepas om die uitwerking van H_3PO_4-konsentrasie (% v/v), die getal 24-uur-ekstraksies en die verhouding oplossing (v) tot grondstof (w) teen 'n omringende temperatuur van 17 °C op die as- en hidroksiprolieninhoud van die osseïen te bestudeer. Die resultate het getoon die optimum toestande om geminimeerde asinhoud en gemaksimeerde hidroksiprolieninhoud in die osseïen te bereik, is 5% H_3PO_4 (v/v), vier opeenvolgende 24-uur-ekstraksies en 'n 5:1-verhouding oplossing (v) tot been (w) teen omringende temperatuur van 17 °C. Lineêre regressiemodelle is ontwikkel om asinhoud van 0 g kg^{-1} en hidroksiprolieninhoud van 75.9 g kg^{-1} in droëmaterieosseïen by optimale toestande te voorspel. Die resultate het onderskeidelik 2.8 g kg^{-1} droë materie (dm) en 69.7 g kg^{-1} dm as- en hidroksiprolieninhoud in die osseïen binne die ± 95%-voorspellingsinterval van die modelle aangedui. Die resultate het getoon dat beduidende hoeveelhede minerale in die monnikvisbeen uit die mineraalvloeistof herwin is, terwyl altesaam 69.7 g droë hidroksiprolien per kg droë osseïen in die osseïen behou is.

'n Maksimum presipitaat van DKF uit minerale vloeistof met 'n maksimum inhoud fosfor (P) en Ca:P-molêre verhouding gelyk aan een is deur middel van responsieoppervlakmetodologie (ROM) met 'n driefaktor-, vyfvlak- sentrale samestellingsontwerp (SSO) geoptimeer. Kwadratiese regressiemodelle is voorgestel om die gekombineerde uitwerking van reaksietemperatuur (°C), 1M $Ca(OH)_2$:minerale vloeistofverhouding (v:v) en reaksietyd (minute) op die hoeveelheid DKF-presipitaat, en die P-inhoud en Ca:P- molêre verhouding daarvan, te ondersoek. Die resultate het getoon dat die optimum toestande bereik is by 'n reaksietemperatuur van 75 °C, 0.95:1 v/v 1M $Ca(OH)_2$:minerale vloeistof-verhouding en 'n reaksietyd van nie meer nie as 17 minute, aangesien reaksietye van meer as 17 minute nie tot verhoogde DKF-presipitasie gelei het nie. Teen optimum toestande was die voorspelde waardes 20.5

g dm van DKF-presipitaat per 150 ml aanvangsoplossing, terwyl die presipitaat 221.6 g kg^{-1} P en 1.07 Ca:P- molêre verhouding bevat het. Die eksperimentele resultate van 21.1 g dm van DKF-presipitaat per 150 ml-aanvangsoplossing, 215.2 g kg^{-1} dm P-inhoud en 1.16 Ca:P- molêre verhouding het 'n sterk ooreenkoms met die voorspelde waardes getoon. Die gebruikte oplossing, as 'n neweproduk in die DKF-presipitasie, het baie lae vlakke van Ca en P bevat. Daar was dus 'n aanduiding van 'n hoë herwinning van totale Ca- en P-insette.

'n X-straalpoeierdiffraksieontleding het aangedui dat die DKF in die vorm van dikalsiumfosfaatdihidraat (DKFD) gepresipiteer word. Die gepresipiteerde DKFD het ook essensiële mikrominerale en beduidend lae vlakke van skadelike swaarmetale bevat. Die relatiewe oplosbaarheid van P in 'n 2%-sitroensuuroplossing vir die gepresipiteerde DKFD is vasgestel op 95.1%, wat 'n raming van die P-biobeskikbaarheid daarvan en 'n aanduiding van 'n potensieel goeie kwaliteit anorganiese fosforvoeraanvulling gee. Daar is ook bevind dat oktakalsiumfosfaat (OKF) by 'n reaksietemperatuur van 25 °C, 'n 1.2:1 v/v 1M Ca(OH)$_2$:mineraalvloeistofverhouding en 'n reaksietyd van nie meer nie as 17 minute presipiteer. Dit het 25.4 g dm van OKF-presipitaat per 150 ml- aanvangsoplossing opgelewer, terwyl die presipitaat 18.3 g kg^{-1} dm P en 31.6 g kg^{-1} dm kalsium (Ca) opgelewer het, met 'n gevolglike Ca:P- molêre verhouding van 1:34. Volgens die literatuur het 'n OKP-samestelling spesifieke toepassings in die mediese en tandheelkundige veld; en word OKP dus as 'n belangrike biomateriaal beskou.

Om die optimum gelatienekstraksietoestande vir die osseïen te bewerkstellig is ROM met 'n driefaktor-, vyfvlak-SSO gebruik. Hierdie ontwerp is toegepas om die gesamentlike uitwerking van ekstraksie-pH, temperatuur (°C) en tyd (minute) op die hidroksiprolienopbrengs in die gelatienvloeistof teen 'n vaste 1:10-verhouding van osseïen:water (w:v) en een ekstraksie te ondersoek. Die optimum toestande was 'n ekstraksie-pH van 8, temperatuur van 80 °C en tyd van 125 minute. Die voorspelde hidroksiprolienopbrengs van die model by optimale toestande was 0.6%, met 'n ekwivalente hidroksiprolienherwinning van 8.3%. Die herwinning van hidroksiprolien was beduidend laer as dié vir die been van kabeljouspesies wat in die literatuur genoem word in studies wat drie of meer opeenvolgende ekstraksies gebruik het. Verbeterde hidroksiprolienherwinning (wat hidroksiprolienopbrengs ook sal verbeter) kan in die huidige werk deur twee of meer opeenvolgende ekstraksies van gelatien uit die osseïen behaal word. Hierdie studie bewys dat gelatien uit monnikvisosseïen geëkstraheer kan word.

Gebaseer op die resultate van hierdie studie bestaan die potensiaal vir die ekstraksie van die waardevolle produkte DKFD, OKP en gelatien uit monnikvisbeen aan die hand van prosesse wat in hierdie studie ontwikkel en geoptimeer is. Gevolglik kan hierdie studie bydra tot die toekomstige nuttige gebruik van monnikviskoppe, wat op die oomblik as 'n afvalproduk van die Suid-Afrikaanse monnikvis- en stokvisbedryf beskou word.

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List of abbreviations

ANOVA	analysis of variance
AOAC	Association of Official Analytical Chemists
BSE	bovine spongiform encephalopathy
Ca	calcium
$CaSO_4$	calcium sulphate
$CaCl_2$	calcium chloride
CCD	central composite design
CEFIC	European Chemical Industry Council
CFR	Code of Federal Regulations
cm	centimetre
DCP	dicalcium phosphate
DCPA	dicalcium phosphate anhydrous
DCPD	dicalcium phosphate dihydrate
DF	degrees of freedom
DMAB	p-dimethylaminobenzaldehyde
dm	dry mass
EC	European Council
EU	European Union
FAO	Food and Agriculture Organization
FTIR	Fourier transform infrared spectroscopy
F-value	test statistics
g	gram
Gly	glycine
GMIA	Gelatin Manufacturers Institute of America
H_2SO_4	sulphuric acid
H_3PO_4	phosphoric acid
HCl	hydrochloric acid
Hyp	hydroxyproline
ICP	inductively coupled plasma
ICP-AES	inductively coupled plasma atomic emission spectroscopy
ICP-MS	inductively coupled plasma mass spectrometry
IFP	Inorganic Feed Phosphates
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
kg	kilogram
L	litre
M	molarity
MCP	monocalcium phosphate

MDCP	monodicalcium phosphate
MCPA	monocalcium phosphate anhydrous
MCPM	monocalcium phosphate monohydrate
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mol	mole
NaOH	sodium hydroxide
NCP	noncollagenous proteins
nm	nanometre
OCP	octacalcium phosphate
P	phosphorus
ppm	parts per million
Pro	proline
P-value	calculated probability
rpm	revolutions per minute
RSM	response surface methodology
µg	microgram
UK	United Kingdom
USA	United States of America
v	volume
w	weight
XRD	X-ray powder diffraction

Chapter 1
Introduction

This research was driven by the continuous global initiative to improve the utilisation of solid waste originating from global fisheries as this waste represents a significant amount of total capture fisheries output: it comprised 13% of the total world fish production in 2014 (Food and Agriculture Organization [FAO], 2016), which was equivalent to 21 million tonnes. Onshore fish processors are the major contributors to this solid waste; it can comprise up to 65% of the original material (FAO, 2014). This is an indication that also within the South African context, there are onshore raw materials available at fish processing facilities that present an opportunity for valorisation. Onboard fish processing also contributes to the solid waste as a result of processing of the catch on the fishing vessel. The amount of onboard fish processing waste is significant for South African hake, monkfish and sole fisheries and is estimated at 43 500 tonnes annually (Walmsley et al., 2007). Monkfish solid waste consists of heads and viscera; while the edible tail portion, which is only approximately one-third of the weight of the whole fish (Code of Federal Regulations [CFR], 2016a), is recovered. Thus, for the total monkfish catch of 7 800 tonnes in 2010 (Glazer and Butterworth, 2013), approximately two-thirds were discards. This implies that there are significant amounts of waste that need to be landed onshore, thus presenting a value-adding opportunity for fish processors.

Monkfish heads and viscera comprise nearly two-thirds of the whole monkfish weight; thus, the head portion of this fish represents a large proportion of the total catch. The head of the fish is made up of the head skeleton (Wheeler and Jones, 1989); hence, the number of bones in a monkfish head is likely substantial. Generally, on a dry mass (dm[1]) basis, bones consist of 60-70% inorganic substances, mainly calcium and phosphate (Kim, 2012; Talwar et al., 2016), and the balance comprises the organic component, mainly collagen (Boskey, 2013). This makes fish bones a potential source of calcium phosphate minerals and collagen proteins, and thus they may have future applications in agriculture (fertiliser and feed additives), food, nutraceutical and cosmetic industries (Graff et al., 2010; Lee et al., 2010; Malde et al., 2010; Noomhorm et al., 2014). The application of fish bones as fertiliser in agriculture is well established; however, as feed additive it is only employed as calcium supplement. An opportunity exists to utilise the mineral fraction in the fish bones as raw material in manufacturing feed additives such as inorganic monocalcium phosphate (MCP) and dicalcium phosphate (DCP).

Presently, the effective industrial and experimental methods of preparing MCP and DCP as feed additives are acidification of phosphate ore using sulphuric acid (H_2SO_4) or hydrochloric acid (HCl), followed by a purification step (defluorination) of the resultant solution and subsequent neutralisation of the purified solution with a calcium source to obtain feed-grade DCP. Besides DCP, by-product is also generated, such as insoluble calcium sulphate ($CaSO_4$) or soluble calcium chloride ($CaCl_2$) salts, aside from unreacted phosphate ore. Alternatively, several feed manufacturers employ neutralisation of feed-

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[1] Dry mass (dm) is the mass of a substance after the water content has been removed.

grade phosphoric acid (H_3PO_4) with lime to prepare MCP, DCP or a mixture thereof. Thus, the use of an alternative mineral acid such as H_3PO_4 in acidification of the fish bone material presents a practical means of DCP manufacture, with the potential advantage that a solid or liquid calcium salt by-product is possibly not generated.

Mammalian bone discards are alternatively used for phosphate ore using HCl in acidification process to prepare DCP. However, using a biological source such as mammalian bones is associated with strict quality control regulations as a result of the bovine spongiform encephalopathy (BSE) outbreak. Thus, in terms of industrial scale, mammalian bones are utilised to a smaller extent. Preparation of DCP by acidification of mammalian bones with HCl generates not only $CaCl_2$ by-product but also an ossein (demineralised bones). This implies that also in the context of biological origin, acidification of fish bones potentially generates fish bone ossein as by-product. Such a production strategy would enable complete utilisation of fish bones by preparing DCP as main product and collagen as co-product. In addition, the utilisation of fish bones as raw material could produce high-purity DCP without the need for purification steps. According to the literature, various temperatures ranging from 18 °C to 70 °C are used in acidification of phosphate ore. These levels of temperatures when employed during acidification of fish bones can possibly induce collagen denaturation of ossein by-product whereas an operating temperature of 4 °C is used in the collagen preparation to avoid denaturation. Thus, it is probable that the remaining ossein after enzymatic hydrolysis and mineral extraction will only be suitable for extracting gelatin (denatured collagen) and not native collagen.

The aim of this research was to develop and optimise processes in the extraction of minerals from monkfish bones, precipitation of DCP from the extracted minerals and extraction of gelatin from monkfish ossein, the residual following extraction of minerals from monkfish bones. In the literature, the enzymatic hydrolysis technique to recover proteins contained in fish processing solid waste (fish heads, frames, skin, tails, viscera, fins, guts, liver and roes) is well established. It specifically targets the protein component of the raw material such as fish heads, and any bones contained therein remain a by-product. Appropriately, in this study monkfish bones were recovered from monkfish heads using enzymatic hydrolysis as preliminary treatment of monkfish heads. The current work employed statistical experimental design procedures to optimise extraction of the different products, with subsequent analyses of all data obtained from experiments.

The subsequent chapters of this thesis consist of a literature review, the aim of the study, the materials and methods used, the results obtained and discussion thereof, and a conclusion. The chapters of this thesis are structured in a conventional thesis format. For ease of following the processes developed in this study, the procedures are set out sequentially in the materials and methods chapter. In sequence, these procedures are as follows: preliminary treatments of monkfish heads to recover the bones, extraction of minerals from bones resulting in two potentially valuable components (mineral liquor and ossein), precipitation of DCP from mineral liquor and, lastly, extraction of gelatin from ossein. This

sequence is also applied in reporting the results of the experiments in the results and discussion chapter.

This study showed that potentially valuable products such as DCP and gelatin could be derived from monkfish bones. The study thus contributes to the global initiative of improved utilisation of solid waste originating from global fisheries and more specifically, the study contributes towards improved utilisation of monkfish heads, which are currently viewed as a solid waste product from the South African monkfish and hake fisheries.

Chapter 2

Literature review

2.1   Fish processing solid waste

2.1.1   Global status quo

Solid fish processing waste is generated from a variety of discards originating both from onboard fishing vessel processing and from onshore fish processing and can amount to a large proportion of the total catch. The amount of waste originating from global fisheries is significant: of the total world fish production in 2014, it is estimated that 87% (146 million tonnes) was allocated to direct human consumption and the balance of 13% (21 million tonnes) was classified as waste (FAO, 2016). In some instances, land-based processing produces more waste compared to sea-based processing; for example, in the United Kingdom (UK), fishing discards, onboard fishing vessel processing waste and onshore processing waste account for 17%, 5% and 35% of the total amount of catch, respectively (Archer, 2001). Depending on the level of processing of fish to recover the edible portion, different types of fish solid waste is generated: heading and gutting produce heads and offal whereas filleting produces frames, trimmings and skin.

There are different driving forces that result in the production and discarding of fish processing waste. Fishing discards (or discarded catch) are defined as "the portion of the catch which is returned to sea for whatever reason" (Kelleher, 2005, p. xv). Discarding is a legal practice and normally driven by commercial and market consideration by the fishing operator and fisheries management policy, namely by-catch discards, quota discards and premarket selection, in other words high grading (Clucas, 1997). Fishing discards normally consist of whole, unprocessed fish (Kulka, 1996). Onboard fish processing waste results from processing of the catch on the fishing vessel and is normally dumped at sea due to its low value and lack of space onboard. The majority of these discards consist of heads (Shahidi, 2007), and the amount can be as high as 27-32% of the overall catch (Brooks et al., 2013). Onshore fish processing results in a larger variety of processing wastes, including heads, bones, viscera, gills, dark muscle, belly flaps and skin, and can comprise up to 65% of the original material, for example in the tuna canning industry (FAO, 2014).

According to Pearson and Dutson (1992), there are certain areas such as the North Sea and the Canadian Maritime Provinces where regulations that restrict landfill disposal of fish processing waste or limit the dumping of fish processing waste into the ocean are in place. These regulations oblige fishing vessels to reserve fish processing waste for recovery at onshore processing plants, protect the environment and enforce increased utilisation of fish processing waste. Onshore fish processing waste in the past was considered to be of low value and discarded. However, in the last two decades, the utilisation of this waste has gained attention because it represents a significant additional source of nutrition (FAO, 2016; Naylor et al., 2009).

2.1.2 Valorisation opportunities

Fish processing solid waste, such as fish heads and frames, contains bones that are a potential source of calcium phosphate minerals and collagen. In general, on a dry mass basis, bones consist of 60-70% inorganic substances, mainly calcium and phosphate (Kim, 2012; Talwar et al., 2016) known as hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, analogous to geologic hydroxyapatite (Kim, 2012). The balance comprises the organic component of which ~90% are type I collagen, ~5% are noncollagenous proteins (NCP) and ~2% are lipids (Boskey, 2013).

Proteins contained in fish processing solid waste (fish heads, frames, skin, tails, viscera, fins, guts, liver and roes) can be recovered in the form of peptides and amino acids that could exhibit biologically active properties using a well-established technique, namely enzymatic hydrolysis (Benhabiles et al., 2012; Benjakul and Morrissey, 1997; Chalamaiah et al., 2012; Hathwar et al., 2010; Kristinsson and Rasco, 2000; Nguyen et al., 2011; Ribeiro et al., 2014; Roslan et al., 2014). Protein hydrolysates have various industrial uses, including milk replacers, protein supplements, stabilisers in beverages and flavour enhancers (Brooks et al., 2013). Enzymatic hydrolysis specifically targets the protein component of the fish processing solid waste such as fish heads and frames, and any bones in the raw material remain a by-product.

Based on the report by the FAO (2016), the default avenue for valorisation of fish processing solid waste is fish meal and fish oil production. Fishmeal is mainly used for high-protein feed in aquaculture and livestock. Fish oil, the richest available source of long-chain highly unsaturated fatty acids, is used for human nutritional supplement as well as an ingredient in feeds in aquaculture. The global demand for both fish meal and fish oil is increasing, with a simultaneous increase in their prices. As a result, these products are no longer regarded as low-value products. However, only 35% of the global fishmeal production is obtained from fish processing waste (FAO, 2014) as the majority of the feedstock for fish meal and fish oil production is wild-caught whole oily fish species, for example anchoveta. This low percentage utilisation of fish processing waste for this purpose is due to the low-quality fishmeal that is produced from it: it has a high ash (mineral) content, a high proportion of small amino acids and a lower total protein content when compared to fish meal produced from whole fish (FAO, 2016; Naylor et al., 2009).

Other possible markets for fish processing solid waste are illustrated in Figure 2.1. Fish processing solid waste, for example fish heads, is also used for direct human consumption, and there is a growing demand for fish heads as food in Asian and African markets due to the nutritional value thereof (FAO, 2016). Fish heads can contain high-quality proteins, lipids with long-chain omega-3 fatty acids, micronutrients (such as vitamin A, riboflavin and niacin) and minerals (such as iron, zinc, selenium and iodine) (FAO, 2014). Nevertheless, the majority of fish consumers prefer to eat only clean, boneless fillets, a phenomenon that is strongly influenced by cultural factors; however, fish heads still contain essential nutrients that can be recovered for use in human or animal diets.

Marine sector	By-products (solid waste)	Marine products	Markets

Fish
-Catch
-Bycatch
	-Liver
	-Roe
	-Gut
	-Milt
	-Head
	-Skin
	-Frames
		-Proteins -Phospholipids
		-Peptides -Gelatin
		-Amino acids -Minerals
		-Enzymes -Nucleic acids
		-Oil -Extracts
		-Attractants
		-Docosahexaenoic acid
		-Eicosapentaenoic acid
			-Feed additives
			  -Fish
			  -Agriculture
			  -Pet food
			-Food industry
			-Food additives
			-Cosmetics
			-Biotechnology
			-Industrial applications

Figure 2.1: Overview of fish processing solid waste utilisation

Source: Kim (2014)

2.1.3 Monkfish processing solid waste in South Africa

The total annual onboard processing waste, specifically offal discarded at sea for South African hake, monkfish and sole fisheries, is 43 500 tonnes (Walmsley et al., 2007). Monkfish solid waste consists of heads and viscera as a result of the recovery of the edible tail portion of the monkfish. The monkfish tail weight is approximately a third of the weight of the whole monkfish based on the landing conversion factor of 2.91 for tail weight to whole weight (CFR, 2016a). Hence, for 7 800 tonnes of South African monkfish caught in 2010 (Glazer and Butterworth, 2013), approximately two-thirds of these catch were discards. This implies that there are significant amounts of waste that need to be landed onshore, thus presenting a value-adding opportunity for fish processors.

Typically, South African monkfish are caught as bycatch (nontarget catches) in the offshore demersal hake trawling industry. Monkfish catch accounts for 2-4% in hake and 33% in monkfish fisheries (Walmsley et al., 2007). The South African monkfish catch is almost equal to the monkfish sustainable catch limits (South African Sustainable Seafood Initiative, n.d.), an indication that monkfish discards such as heads and viscera are available from fish processors at a sustainable level. Additionally, the fact that monkfish heads and viscera comprise nearly two-thirds of the whole monkfish weight means that the head portion of this fish represents a large proportion of the total catch. The head of the fish consists of the head skeleton (Wheeler and Jones, 1989); hence, the number of bones in the monkfish head is likely substantial. These monkfish bones are a potential source of calcium phosphate minerals and collagen proteins and may have future relevance primarily in agriculture, food, and the cosmetics and medical industries.

2.2   Monkfish bones potential applications

Bones from various fish species have demonstrated diverse, direct applications as fertilisers (commercially available), calcium feed supplements (Graff et al., 2010; Lee et al., 2010) and calcium food supplements (Malde et al., 2010; Noomhorm et al., 2014). Fish bones are also a good potential source of collagen and gelatin (FAO, 2016) that can be used as supplements in food, nutraceuticals and cosmetics (Gildberg et al., 2002; Hall, 2011; Shahidi, 2007; Simpson et al., 2012; Silva et al., 2014; Venugopal, 2008). This suggests that there is a wide array of potential applications and valorisation opportunities for monkfish bones. The existing and potential use of fish bones are discussed below.

2.2.1   Fish bones as fertiliser

Fish bones are valued as a fertiliser due to the mineral content thereof. There are several existing companies that manufacture fish bone fertiliser: (1) Down to Earth in Oregon, United State of America (USA), uses steamed fish bone meal containing 14% Ca and 6.98% P; (2) Coast of Maine Organic Products Incorporated in Maine, USA, produces fish bone meal by dehydrating then grinding hake bones containing 18% Ca and 5.7% P; and (3) Alaska Mill and Feed in Alaska, USA, uses white cod bone to produce fish bone meal with 10-20% Ca and 6-8% P. Fish bones, with the majority of the constituents being calcium phosphates, could also be a potential source of MCP and DCP phosphate fertilisers. Naturally occurring phosphate ore in the form of hydroxyapatite or fluoroapatite are the primary resource utilised in producing MCP and DCP phosphate fertilisers (House, 2013). Agricultural fertilisers are the largest application of phosphates, accounting for 80% (IHS, 2016). Companies such as (1) Sap International Corporation (SICO) in Zoersel, Belgium, produces Sicalphos MCP with 13.5-16% Ca and 22% P; and (2) Aliphos in Vlaardingen, The Netherlands, produces Aliphos® DCP with 28% Ca and 18% P; these products are used not only as a fertiliser but also as a feed additive.

2.2.2   Fish bones as feed additive

Fish bones are not ideal as a direct feed additive, and their utilisation as such emphasises underutilisation in the context of their potential nutritional value. Several studies demonstrated that fish bones could be used as calcium feed supplement but not as phosphorus supplement for animals. Graff et al. (2010) ascertained that fish bones from salmon and cod could be a calcium source for growing pigs, and according to Lee et al. (2010), fish bone meal from Alaska seafood processing by-products could be used in rainbow trout fish feed formulations as a supplemental calcium source but not as a primary phosphorus source because of its low bioavailability to fish; 60-80% of the intake phosphorus is released undigested into the environment, posing an environmental challenge as well as causing waste of an expensive feed ingredient (Albrektsen, 2011). The bioavailable P content of feed ingredients of animal origin is generally lower than that of inorganic P sources counterparts, namely MCP and DCP (Viljoen, 2001a), even though they contain significant amounts of phosphorus; this is corroborated by Nordrum et al. (1997) who

found that the P bioavailability of inorganic calcium phosphates to Atlantic salmon was higher than that of hake fish bone meal.

However, fish bones with the majority of the constituents being calcium phosphates could be a potential source of inorganic calcium phosphates such as MCP and DCP. Currently, 5% of the phosphate natural resource (phosphate ore) in the form of hydroxyapatite and fluoroapatite is utilised for feed phosphate production (IHS, 2016). Feed phosphates include calcium phosphates (MCP and DCP), magnesium phosphates, sodium phosphates and ammonium phosphates. MCP and DCP are common inorganic calcium phosphate feed additives with MCP having a higher phosphorus bioavailability than DCP (Viljoen, 2001a; Viljoen, 2001b).

2.2.3 Fish bones as food, nutraceutical and cosmetic supplement

As calcium- and phosphorus-rich material, fish bones have been utilised as calcium-fortified food supplements. Alaska pollock and hoki backbones have been demonstrated as a source of soluble calcium and a potential calcium-fortified supplement as an alternative to calcium from dairy products (Noomhorm et al., 2014). Malde et al. (2010) also concluded that fish bones from salmon and cod were a well-absorbed calcium source for young, healthy men.

Additionally, fish bones, skin and scales can be an alternative source of collagen or gelatin to that obtained from land-based animals, for example bovine and porcine products. The underlying motive for the use of fish products aside from the rising interest in the valorisation of fish processing by-products (Gómez-Guillén et al., 2011) is the emergence of the BSE and foot-and-mouth disease crisis on top of the religious constraints on using bovine and porcine products (Alfaro et al., 2015; Herpandi et al., 2011; Karayannakidis and Zotos, 2016; Mariod and Fadul, 2013). The former results in health-related concerns among consumers while the latter is a sociocultural concern: some products are unacceptable to the Jewish and Muslim religions' kosher and halal diets, respectively.

Collagen and gelatin are potentially high-value products that can be obtained from fish bones. Collagen is the parent protein of gelatin, and both proteins have the same amino acid make up (Caballero et al., 2015; Kozlov and Burdygina, 1983). In collagen, the amino acids are arranged in ordered, long chains of rod-like, triple-helix structures whereas in gelatin, these chains are partially separated and broken (Domb et al., 1998) and transformed into coiled structures (Gómez-Guillén et al., 2011) upon denaturation by the application of heat (Podczeck and Jones, 2004). The functional properties of fish gelatin do not compare favourably with those of mammalian gelatin. Fish gelatin generally has lower gelling and melting temperatures and a lower gel modulus compared to mammalian gelatin (Shahidi, 2007); hence, its potential to replace mammalian gelatin is only applied to certain areas. These are as follows: (1) used as an additive in cold-stored products with a melting point as low as 10 °C in which gelling is not desirable, namely thermolabile compounds such as certain drugs that can be encapsulated at a lower

temperature; (2) used to enhance the shelf life of muscle foods such as fresh meat products due to gelatin coatings that act as a barrier to water loss and oxygen; (3) used in slimming diets when added to enzymatic hydrolysates of casein; (4) used along with other products to compensate for certain deficiencies during childhood and adolescence, pregnancy and lactation; and (5) used in the areas of light-sensitive coatings, low set-time gels and as an active ingredient in shampoo with protein (Venugopal, 2008). Fish gelatin is also used as an ingredient in cosmetics (Shahidi, 2007). Gelatin extracted from backbone fraction of Atlantic cod has a relatively low molecular weight and may be suitable for technical applications or as a nutraceutical (Gildberg et al., 2002).

Collagen, similar to gelatin, is also widely used as food ingredient and in pharmaceuticals, cosmetics and biomaterials (Hall, 2011; Silva et al., 2014; Simpson et al., 2012). Tropical fish collagen (Yamada et al., 2014), salmon skin collagen (Hoyer et al., 2012), salmon atelocollagen (Nagai et al., 2004) and tilapia atelocollagen (Yamamoto et al., 2014) could be used as scaffold to stimulate hard tissue formation in regenerative medicine aside from their nutritional benefits (Kim, 2014). Bioactive peptides obtained from marine collagen also could be applied in functional foods (Aleman and Martinez-Alvarez, 2013). Marine sponge collagen was investigated by Nicklas et al. (2009) as probable coating for gastroresistant tablets. Collagens from the skins of cod, haddock and salmon and the scales of silver carp are used in the cosmetic industry (Silva et al., 2014).

Therefore, with the intent of gearing up for complete utilisation of fish bones, feed additives in the form of inorganic calcium phosphate (MCP or DCP) and collagen or gelatin are potential applications of monkfish bones. To achieve this, the existing processes for the manufacturing of these products will be surveyed.

2.3 Industrial and experimental methods of preparing monocalcium phosphate and dicalcium phosphate as feed additive

There are several different approaches that can be followed to obtain MCP and DCP for use in animal feeds. Some of these techniques have been implemented at industrial scale. Methods that have already been commercialised and those that are currently limited to laboratory experimentation are both discussed below.

2.3.1 Acidification of phosphate ore and subsequent neutralisation with lime

Acidification of phosphate ore with mineral acids results in a solution containing calcium salt and H_3PO_4 solution, and subsequent neutralisation of the solution with lime produces DCP (De Waal, 2003; De Waal, 2002). The aim of the acidification procedure is to recover most of the phosphate contained in the phosphate ore. However, by-products are also generated, depending on the mineral acids that are used to solubilise the phosphate ore. Common mineral acids utilised for this purpose include H_2SO_4, HCl and H_3PO_4. Phosphate ore containing phosphate mineral in the form of calcium fluoroapatite, $3Ca(PO_4)_2CaF_2$, and hydroxyapatite, $3Ca_3(PO_4)_2Ca(OH)_2$

(Georgievskii et al., 1981), is the main global resource of phosphorus that is used in the production of animal feed supplements such as MCP and DCP (Ptáček, 2016). It contains high amounts of phosphate, but it is contaminated with 10-15 major impurities (Chaabouni et al., 2011) with high concentrations of fluorine (F), arsenic (As), cadmium (Cd) and uranium (U) (Syers et al., 1986). Removal of these impurities or reduction to an acceptable level is essential to produce feed-grade MCP and DCP. Hence, inclusion of purification steps is inevitable during the manufacturing process of feed-grade MCP and DCP from phosphate ore.

2.3.1.1 Sulphuric acid as solubilising agent for phosphate ore

The reaction of H_2SO_4 with phosphate ore results in a gypsum ($CaSO_4$) by-product, and often the gypsum is contaminated with naturally occurring U and thorium (Th) from phosphate ore. Thus, the gypsum exhibits weak radioactivity (Habashi et al., 1987) and creates a disposal problem (Habashi, 2014) although there is an attempt to recycle insoluble gypsum in the process, such as the work of De Waal (2002). Typically, the reaction of H_2SO_4 with phosphate ore proceeds in two steps (Sharma, 1991): (1) the diffusion of H_2SO_4 to the surface of phosphate ore particles until H_2SO_4 is consumed and $CaSO_4$ crystallises, thereby causing an exchange decomposition to proceed on the surface of solid phosphate particles with an excess of H_2SO_4, resulting in free H_3PO_4 formation; and (2) the diffusion of free H_3PO_4 into the pores of the undecomposed phosphate ore whereby MCP is formed in the solution and then begins to crystallise out when the solution becomes supersaturated. The formation and crystallisation of MCP is a slow process due to the low rate of diffusion of the H_3PO_4 through the crust of MCP formed on the surface of the phosphate ore and also due to the extremely low rate of crystallisation of the MCP. This process is also known as ripening of the superphosphate, and it is allowed to stand for about three months to make the reaction complete. Ripening is accelerated by lowering the temperature and removing the moisture, which results in more rapid crystallisation of the MCP and higher concentration of H_3PO_4 reacting with the unreacted apatite particles (Sharma, 1991). The MCP formed is known as single superphosphate, and it is contained with gypsum.

Rao (2004) utilised the single superphosphate as the starting material in the production of DCP. The superphosphate is agitated with water for about an hour, and the solution contains MCP with a pH between 2.2 and 2.6. The MCP solution is separated from the insoluble portion, and the pH of the filtrate is raised by adding hydrated lime solution to precipitate impurities such as iron (Fe), aluminium (Al) and fluorides (F^-). After separation of the impurities, the MCP solution under pH 3.2 is further added with hydrated lime to raise the pH to 6.5-6.7 to crystallise the DCP from the solution. Dried DCP contains 18.17% P, 24.45% Ca and 0.12% F.

De Waal (2003), Freitas and Giulietti (1997) and Giulietti (1994) utilised the impure H_3PO_4 solution that is generated in the first step reaction of H_2SO_4 and phosphate ore, as previously mentioned, in preparing DCP with insoluble gypsum and impurities as residual by-product. Once

the insoluble by-product is separated, the phosphoric acid solution is purified and defluorinated in order to obtain a H_3PO_4 solution with an acceptable level of impurities, such as shown in the work of De Waal (2003), Freitas and Giulietti (1997) and Giulietti (1994). Afterwards, it is neutralised with a calcium source such as calcium hydroxide, $Ca(OH)_2$, or calcium carbonate ($CaCO_3$) to produce DCP. It has been indicated that the impure H_3PO_4 solution is obtained by acidification of phosphate ore with concentrated H_2SO_4 at a temperature of 50 °C or 70 °C for four hours. The H_2SO_4 is added at such a rate that the total amount is added at approximately 50% or 60% of the reaction time. This procedure is then followed by a defluorination process to produce a defluorinated H_3PO_4 solution.

Furthermore, the neutralisation procedure of the defluorinated H_3PO_4 solution is controlled by the pH value of the solution while the temperature defines the form of the precipitated DCP crystals (Giulietti, 1994). The best operational conditions to produce an anhydrous form of DCP are a neutralisation time of one hour, a final pH of the solution of 3.5 and a neutralisation temperature of 95 °C (Giulietti, 1994) whereas at neutralisation temperature and time of 85 °C and 20 minutes, respectively, anhydrous DCP precipitates in the pH range between 2 and 5 (Freitas and Giulietti, 1997). For the process invented by De Waal (2003), DCP starts precipitating at a pH of about 5.5 up to 7. The same principle is adopted by industrial companies that manufacture feed-grade DCP, such as Ecophos (Belgium) and PotashCorp (USA), although there is no mention of specific industrial operating conditions thereof such as neutralisation temperature and time and final pH of the solution. The feed-grade DCP manufactured by Ecophos contains 18% P and 28% Ca while that of PotashCorp contains 18.5% P and 21% Ca.

2.3.1.2 Hydrochloric acid as solubilising agent for phosphate ore

Dissolution of phosphate ore in diluted HCl results in the formation of MCP and $CaCl_2$ solution. MCP is readily soluble in water; hence, it will not be precipitated. Therefore, additional calcium is added to saturate the solution and to change the molecular structure from MCP to DCP (Tessenderlo Group, 2006) with a soluble $CaCl_2$ solution as a by-product. This is reflected in the investigation carried out by Qadir et al. (2014) in producing DCP with a purity of 98.20% by solubilising phosphate ore with 10% w/w HCl for 1.5 hours at 30 °C to obtain an MCP and $CaCl_2$ solution. Thereafter, calcium hydroxide slurry is added to the solution until a final pH of 5 is reached to precipitate DCP. Alternatively, DCP is also produced by solubilising phosphate ore with 8% w/w HCl for 30 minutes at 18-26 °C to obtain an MCP and $CaCl_2$ solution. Thereafter, the solution containing 1.13% P is supplemented with calcium carbonate powder and milk of lime to precipitate DCP containing 17.44% P and 0.09% F (Loewy and Fink, 1976).

Further reuse of soluble $CaCl_2$ by-product within the process was also investigated. Zafar et al. (2006) suggested using H_2SO_4 to recover HCl from the $CaCl_2$ solution that could be used to recycle for the solubilisation of phosphate ore, leaving the $CaSO_4$ as insoluble salt by-product. Furthermore, De Waal (2002) investigated the decomposition of the $CaCl_2$ solution in a suitable

hydrocyclone. It entailed treating the $CaCl_2$ solution with water at an elevated temperature, preferably in the range of 1 000-1 200 °C to form $Ca(OH)_2$ and HCl. Prior to this, the $CaCl_2$ solution had been heated to its boiling point before it was introduced to the hydrocyclone. The HCl was recycled to the phosphate ore solubilising step to produce a $CaCl_2$ and MCP solution while the $Ca(OH)_2$ was caused to react with MCP to form DCP.

Principles analogous to the one described immediately above are also used by the feed manufacturer Ecophos (Belgium), although there is a lack of indicative operating conditions in manufacturing MCP and DCP whereby low-grade phosphate ore is used to react with HCl. Low-grade phosphate ore is normally a reject during beneficiation of phosphate ore and mostly contains low amounts of tricalcium phosphate and high amounts of carbonate (Zafar et al., 2006). Ecophos purifies the mixture of low-grade phosphate ore and HCl to remove significant amounts of the impurities. After the solid residue containing the impurities is separated, the resultant solution of MCP and $CaCl_2$ is caused to react with a calcium source to precipitate DCP from the $CaCl_2$ solution. The $CaCl_2$ solution is caused to react with H_2SO_4, resulting in a gypsum slurry and diluted HCl solution wherein the latter is recycled for phosphate ore digestion. Ecophos also manufactures MCP by causing DCP to react with purified feed-grade H_3PO_4 to form monodicalcium phosphate (MDCP) or MCP, which is a process similar to that invented by De Waal (2003). The MDCP is a mixture of MCP and DCP wherein the fraction of MCP in the MDCP is determined by the water-soluble P fraction as MCP is soluble in water while DCP is water-insoluble (Viljoen, 2001a). The Ecophos MCP contains 22.7% P and 17.5% Ca, the MDCP contains 21.8% P and 20.5% Ca and the DCP contains 18.0% P and 28.0% Ca.

2.3.1.3 Phosphoric acid as solubilising agent for phosphate ore

As in the case of manufacturing a single superphosphate using H_2SO_4 to solubilise the phosphate ore, the use of H_3PO_4 rather than H_2SO_4 generates triple superphosphate, composed of more or less pure MCP (Chavarria, 1978). As a common method for triple superphosphate manufacture, 50-60% H_3PO_4 is mixed with ground phosphate ore and the damp mixture is allowed to remain in the curing pile for about two weeks or longer. At the end of the curing period, maximum conversion of the phosphate transpires into an available form, principally MCP known as triple superphosphate. Marshall et al. (1933) investigated the factors affecting the H_3PO_4 and phosphate ore reaction. It was concluded that the mixtures prepared with 55-65% H_3PO_4 with four days or longer curing time yielded highly bioavailable phosphorus. However, due to a lack of procedures to remove the impurities contained in the phosphate ore, the triple superphosphate is not suitable as a feed additive; rather, its application is popular as agricultural fertiliser. Nonetheless, Rao (2004) utilised not only the single superphosphate as the starting material in the production of feed-grade DCP but also the triple superphosphate, using the same methodology employed in the manufacturing of the single superphosphate, as previously mentioned.

2.3.2 Neutralisation of phosphoric acid with lime

MCP and DCP are produced in both the hydrated and anhydrous forms such as monocalcium phosphate monohydrate (MCPM), monocalcium phosphate anhydrous (MCPA), dicalcium phosphate dihydrate (DCPD) and dicalcium phosphate anhydrous (DCPA). In order to obtain MCP or DCP, key parameters have to be controlled, including Ca:P ratio and reaction temperature (Gilmour, 2013).

MCP has been manufactured industrially since the mid-19th century, initially using a process based on manual mixing of H_3PO_4 with lime powder (Gilmour, 2013). At a later stage, this manual process developed into three different processes (Macketta, 1978). In the first process, equivalent amounts of lime and H_3PO_4 are mixed, followed by evaporation or crystallisation near the boiling point until MCP crystals have grown to the desired size. Thereafter, the crystals pass through a cooling zone wherein further crystallisation and growth take place. Once the crystals are separated, any remaining traces of free H_3PO_4 on the crystals are neutralised with a small amount of hydrated lime, converting the free acid to DCP. The addition of excess lime is intentional to remove residual free H_3PO_4 (Toy, 1973); thereby, DCP impurity is present in the industrial MCP product. The mixture of MCP and DCP is known as MDCP. In the second process, $Ca(OH)_2$ slurry and H_3PO_4 are mixed, which results in a reaction mixture with a desired Ca to P ratio. The mixture is spray dried at a temperature below 85 °C with final free moisture content of the MCP powder less than or equal to 0.5% to reduce the tendency of the MCP to cake on standing. The resultant MCP contains up to about 10% DCP. The third process entails addition of $Ca(OH)_2$ to 80% H_3PO_4 in a liquid-solid mixing apparatus at a rate to maintain the temperature of the reaction mixture between 150 °C and 80 °C. The heat of the reaction vaporises the water associated with the H_3PO_4, resulting in a dry, free-flowing MCPA. The dry product is sprayed with water and maintained at a temperature of approximately 50 °C to 85 °C for around 15-30 minutes until the MCPM is formed.

MCPM can be prepared by partial neutralisation of the H_3PO_4 with $Ca(OH)_2$, followed by evaporation of water at low temperature in acidic conditions. The anhydrous phase can be obtained with the same method but at higher H_3PO_4 concentration. It can also be formed by slow thermal dehydration of the hydrated phase above 100 °C (Ducheyne et al., 2011). In the presence of water, MCP decomposes partially to a more basic DCP and H_3PO_4. The extent of this reaction increases with the amount of water and temperature (Toy, 1973).

According to Ducheyne et al. (2011), DCPD crystals can be prepared simply by neutralisation of H_3PO_4 with $Ca(OH)_2$ at pH 3-4 at room temperature while DCPA is obtained by dehydration of DCPD at 180 °C, or it may also be directly precipitated in aqueous acidic solutions using pH 3-4 at mild temperature, namely 60 °C. Ferreira et al. (2003) produced DCP by mixing equal volumes of aqueous suspension of $Ca(OH)_2$ and aqueous solution of H_3PO_4, both of the same molar concentration, whereby the reaction temperature was maintained at 25 °C while Macketta (1978)

indicates that the reaction temperature should be maintained below 45 °C and the pH of the mixture should be roughly 6 in order to obtain DCP.

Moreover, there are feed manufacturers that also employ neutralisation of defluorinated H_3PO_4 with lime or $CaCO_3$ to produce MCP, MDCP and DCP. Some of these feed manufacturers include Elixir Prahovo (Serbia), Biominerale (South Africa), Yara (South Africa) and Mosaic Company (USA). The specifications of the products manufactured by these feed manufacturers in relation to P and Ca contents are shown in Table 2.1 with acceptable levels of undesirable elements such as F, As, Cd, Lead (Pb) and Mercury (Hg). The P content of the products varies from 18.0% to 22.7% while the Ca content ranges from 15.0% to 24.0%.

Table 2.1: MCPM, DCP, MDCP and DCPD specifications for several feed manufacturers in neutralising feed-grade phosphoric acid with lime or calcium carbonate

Feed manufacturers	Products	P (%)	Ca (%)	Reference
Elixir Prahovo (Serbia)	MCPM	22.7	15.0	Elixir Group Doo (2014)
Biominerale (South Africa)	MDCP	21.0	21.0	Biominerale (2007)
	DCP	18.0	24.0	
Yara (South Africa)	MDCP	21.0	16.0	Yara Animal Nutrition South Africa (2016)
	DCPD	18.5	23.0	
Mosaic Company (USA)	MCPM	21.0	15.0-18.0	The Mosaic Company (2016)
	MDCP	19.0	15.0-18.0	

Furthermore, Macha et al. (2013) utilised black mussel shells as the calcium carbonate source to neutralise phosphoric acid. Initially, as a pretreatment, black mussel shells were ground to 75-100 µm and organic matter was removed by using 5% sodium hypochlorite (NaClO). A sample suspended in distilled water was heated on a hot plate with temperature kept at 80 °C, then H_3PO_4 was added dropwise to the suspension for two hours. Alternatively, ultrasonic agitation was used during the addition of H_3PO_4. Thereafter, the solution was filtered and the DCP powder was dried in an oven at 100 °C for 24 hours and then calcined at 800 °C for three hours. The powder was identified as DCPA using X-ray powder diffraction (XRD), Fourier transform infrared spectroscopy (FTIR) and inductively coupled plasma mass spectroscopy (ICP-MS).

2.3.3 Acidification of mammalian source and subsequent neutralisation with lime

Mammalian bones also contain an inorganic component known as hydroxyapatite, analogous to geologic hydroxyapatite contained in phosphate ore (Boskey, 2013). Thus, acidification of mammalian source bones with mineral acids also results in a solution containing calcium salt and H_3PO_4, and subsequent neutralisation of the solution with lime produces DCP. This procedure is aimed at recovering most of the phosphate contained in the mammalian bones. Common mineral acids utilised for this purpose include HCl and H_3PO_4. Utilising HCl generates $CaCl_2$ by-product

while H_3PO_4 yields an MCP solution. Furthermore, if the mammalian bones are not initially incinerated, ossein by-product is produced during the acidification procedure.

2.3.3.1 Hydrochloric acid as solubilising agent for mammalian bones

According to Gilmour (2013), a typical process to manufacture DCP from animal bones commences by crushing the bones and then degreasing them in hot water at 80-85 °C. The bone chips are mixed with weak HCl over several days in a series of tanks of increasing HCl concentration; the range is 2-5% HCl. The resultant leachate is a mixture of $CaCl_2$ and MCP and is separated from the remaining solids. The leachate is further caused to react with lime to precipitate DCP, which is then separated and dried. The animal feed DCP from this process is particularly low in F^- and other undesirable constituents. In a similar manner, a feed manufacturer in the Netherlands (Sonac) utilises animal bones to produce feed-grade DCPD, which is suitable for application as feed additive in nonruminant animals and fish. The animal bones, after crushing and degreasing, are initially demineralised for five days in an HCl solution. Subsequently, the calcium phosphate solution is separated from ossein, and DCPD is precipitated through $Ca(OH)_2$ addition. However, as a result of the BSE outbreak (Scientific Steering Committee, 1998), for countries with a high or unknown BSE risk status, legislation that requires mammalian bones to be incinerated and not used as animal feed is in place to avoid the possibility of causing BSE transmission. In low-risk and BSE-free countries, the utilisation of mammalian bones is permitted but with stringent quality control systems.

2.3.3.2 Phosphoric acid as solubilising agent for mammalian bones

Calcium phosphate bone minerals that are contained in the ashes of the incinerated meat-bone wastes of oxen and pigs were treated with 37% H_3PO_4 at 90-95 °C to obtain a solution of MCP (Krupa-Żuczek et al., 2008). Acidification of bone ash with H_3PO_4 resulted in an MCP solution, and subsequent neutralisation of the solution with hydrated lime produced DCP (Rao, 2004). Krupa-Żuczek et al. (2008) treated the resultant MCP solution with H_2SO_4 to produce a filtrate of H_3PO_4 with $CaSO_4$ as by-product. It was indicated that no purification step was required to obtain feed-grade phosphoric acid solution. This solution could be a potential raw material for the neutralisation of H_3PO_4 with lime in manufacturing MCP, DCP or MDCP, as previously mentioned.

Primarily in the manufacturing of feed-grade MCP or DCP, the quality of the product serves as an indicator of its compliance with the feed additive specifications. Hence, it is of the utmost importance to examine the present specifications of inorganic calcium phosphate feed additives: MCP and DCP.

2.4 Monocalcium phosphate and dicalcium phosphate feed additive specifications

2.4.1 Importance of inorganic calcium phosphate

The inorganic component of the animal body is composed of minerals, which are classified into two categories based on the amount required by animals (Tisch, 2006): (1) major or

macrominerals such as P, Ca, sodium (Na), potassium (K), magnesium (Mg), chloride (Cl⁻) and sulphur (S); and (2) trace or microminerals such as Fe, iodine (I), manganese (Mn), copper (Cu), molybdenum (Mb), zinc (Zn), selenium (Se), chromium (Cr), and cobalt (Co). It is believed that these minerals are the structural components of the skeletal system (Webster and Lim, 2002). Some minerals participate in the catalytic activity of enzymes and other in the body's acid-base electrolyte balance (Tisch, 2006).

About 80% of the body's P is present in the skeleton (FAO, 1980; Vitti and Kebreab, 2010), and the balance is present in cells of the body that function in metabolism (Vitti and Kebreab, 2010). The metabolic role of P is as follows (Qadir et al., 2014; Webster and Lim, 2002): (1) development and maintenance of skeletal tissue; (2) maintenance of osmotic pressure and acid-base balance; (3) energy utilisation and transfer; (4) protein synthesis, transport of fatty acids and amino acid exchange; (5) growth and cell differentiation; and (6) appetite control, efficiency of feed utilisation and fertility.

P in the form of inorganic phosphates such as MCP or DCP products is routinely included in animal feeds to supply sufficient phosphorus to allow skeletal development and maintain high growth rates. Some phosphorus is obtained from plant sources such as barley, tapioca, maize, wheat, soybean meal and middlings in the form of phytate. However, phytate in such feedstuffs is only partially digested by monogastric animals (Viljoen, 2001a); hence, plant source feedstuffs do not contain enough bioavailable P to meet the requirements for animal production. As a result, supplemental P in the form of inorganic phosphates such as MCP or DCP is added to animal diets (Viljoen, 2001b).

However, according to Viljoen (2001b, p.1), "No element is ever completely absorbed or utilised; a fraction is inevitably lost in the normal digestive and metabolic processes." Therefore, the biological value, namely P availability and P digestibility of inorganic feed phosphates (MCP and DCP), has to be evaluated in order to calculate accurately the required phosphorus ration to closely match the animal's particular P nutritional requirements.

2.4.2 Quality evaluation of inorganic calcium phosphate

The *in vitro* chemical solubility tests of P from inorganic phosphates such as MCP and DCP in 2% citric acid and alkaline ammonium citrate solutions are routinely evaluated to estimate the P bioavailability thereof (Jamroz et al., 2011; Jamroz et al., 2012; Sullivan et al., 1992). As specified by the Inorganic Feed Phosphates (IFP), European Chemical Industry Council (CEFIC), the solubility of P in 2% citric acid indicates the level of P bioavailability while the solubility of P in alkaline ammonium citrate identifies the chemical nature of the feedstuff. As depicted in Table 2.2, MCP, DCPD and DCP are soluble in both solutions. It has also been established by the French National Agricultural Research Institute that phosphate's true digestibility increases linearly in relation to its solubility in both media, 2% citric acid and alkaline ammonium citrate,

whereby a solubility of greater than 95% in both solutions is a strong indication of good-quality inorganic P feedstuff (IFP CEFIC, 2016).

Table 2.2: Quality specification of feed phosphate in terms of *in vitro* chemical solubility tests

Feed phosphate solubility			
Inorganic phosphates	**Total P content (%)**	**Relative solubility of P (%)**	
		in citric acid	**in ammonium citrate**
Monocalcium phosphate	22.7	> 95	> 95
Monodicalcium phosphate	21.0	> 95	> 95
Dicalcium phosphate	17.0-21.0	> 95	> 95
Tricalcium phosphate	20.0	< 95	< 95
Natural tricalcium phosphate	14.0	< 95	< 95
> 95% = soluble; < 95% = poorly soluble			

Source: IFP CEFIC (2016)

In contrast, the *in vivo* P bioavailability test is quantified using the growth performance or bone parameters of animals such as breaking strength, tibia and toe ash contents (Fernandes et al., 1999; Petersen et al., 2011). Although complex and time consuming, these techniques seem to be the most appropriate in determining the P bioavailability (Viljoen, 2001b), considering that more than 80% of P is transferred to the skeleton (Waldroup, 1997). However, these techniques still do not measure the true or apparent P availability *per se* but rather provide an estimate (Li et al., 2016). Such techniques use a reference standard so that a relative biological value or ranking of P sources is determined. The standard source is normally given an arbitrary availability value of 100; hence, some studies may indicate P bioavailability > 100 (Waldroup, 1997).

Comparable *in vivo* assay such as P digestibility is also used to assess the true biological value of the inorganic calcium phosphate, and unlike P bioavailability, it is a quantitative determination of dietary P intake and P excretion in faeces (Li et al., 2016) as well as the endogenous P (Viljoen, 2001b; IFP CEFIC, 2016). Since this bioassay requires radioactive markers to measure levels of endogenous P, animal welfare becomes the flaw of the technique aside from its high cost and complexity; thus, this technique is not reproducible (IFP CEFIC, 2016).

Besides the biological value of the feedstuff, the physical and chemical properties and low amounts of undesirable substances (Bleukx, 2005) also reflect the quality thereof. Physical properties such as colour, particle size and free-flowing property provide advantages in mixing processes and ease of handling (Viljoen, 2001a). Finely ground inorganic mineral supplements tend to be dusty but more available than supplements that are more granular (Tisch, 2006). From the chemical perspective, the feedstuff's P and Ca contents and its Ca:P ratio are important evaluation criteria (IFP CEFIC, 2016) as these reflect the potential of the product though they do not necessarily indicate the biological value of the product (Viljoen, 2001a). It has been established that the efficacy of P absorption declines with increasing Ca intake; thus, adequate

P absorption has been correlated with Ca:P ratio. A target ratio of 1.5 to 2 parts Ca to 1 part P is recommended (Tisch, 2006).

In addition to ensuring that the feedstuff contains the desirable properties, elimination of unwanted substances (heavy metals) must be guaranteed. Nevertheless, the European Union Council and Parliament perceive the impossibility of fully eliminating the presence of undesirable substances; hence, provision is made for allowing the presence of these substances in low quantities to prevent undesirable and harmful effects. Therefore, undesirable substances may be present in feedstuff only in accordance with the conditions set out in Table 2.3.

Table 2.3: EU directive for undesirable substances in animal feed

Undesirable substances	Products intended for animal feed	Maximum content[2] (mg/kg)
Arsenic	Phosphates and feedstuffs obtained from the processing of fish or other marine animals	10
Lead	Phosphate feed materials	30
Fluorine	Feedstuffs of animal origin	500
Mercury	Feedstuffs produced by the processing of fish or other marine animals	0.5
Cadmium	Feed material of animal origin	2

Source: European Council (2002).

2.5 Experimental procedure of collagen and gelatin extraction

The extraction process for native collagen entails preservation of the triple-helix structure thereof whereas for gelatin, it necessitates denaturation of the collagen triple-helix structure, resulting in helix-to-coil transition. Collagen is one of the most abundant fibrous proteins and performs various mechanical functions. It is a sequence of amino acids composed of three chains with a repeating pattern of Gly-Pro-X or Gly-X-Hyp (Mocan et al., 2011; Yang et al., 2008), in which X can be any amino acid. Proline (Pro) or hydroxyproline (Hyp) comprises about one-sixth of the total sequence while glycine (Gly) accounts for one-third of the sequence. This pattern results in the formation of a right-handed triple-helical structure or super helix wherein the glycine residue is positioned at the interior axis of the helix while the rings of proline and hydroxyproline are pointing outward. It is believed that proline and hydroxyproline form the hydrogen bonds, which are responsible for the stabilisation of the super helix (Karayannakidis and Zotos, 2016). A single collagen molecule (tropocollagen) is a subunit of larger collagen aggregates such as fibrils. These collagen fibrils in bones are arranged in a parallel staggered array, allowing a gap region between tropocollagen subunits, which are presumed to be nucleation sites for the deposition of mineral components. The microfibrils within collagen fibrils are ordered as to be crystalline. Type I collagen provides bone with its tensile strength (Fratzl, 2008).

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[2] Relative to a feedstuff with a moisture content of 12%.

The crystalline structure of collagen molecules can be lost due to destruction of hydrogen bonds (Karim and Bhat, 2009) during heating. This process is referred to as denaturation and results in random coils of gelatin that form from the triple helix of collagen (Bandeira et al., 2014; Fratzl, 2008; Kim, 2015; Miles and Bailey, 1999). Gelatin is a cooked (Regenstein and Regenstein, 1984), disintegrated (Francis, 2000) or denatured (Nikoo et al., 2011) collagen, and it has been irreversibly hydrolysed. The mechanism of denaturation is a sharp contraction of collagen fibre to about a quarter of its original length on heating (Fratzl, 2008). Miles and Bailey (1999) correlate the shrinkage temperature of collagen with the temperature of the animal's habitat. For instance, cold-water fish have a low shrinkage temperature in a solution that is around 16 °C, warm-water fish have a higher shrinkage temperature in a solution of around 30 °C while for warm-blooded mammals it is 39-41 °C. It has been concluded that the denaturation temperature of the collagen molecules is therefore as little as 1-2 °C above the animal's habitat temperature. Cold-water fish such as cod have a preferential thermal habitat ranging from 3 °C to 15 °C (Freitas et al., 2016).

In the following paragraphs, the existing techniques utilised for the extraction of collagen or gelatin from fish bones will be surveyed. These techniques are based on the conventional processes for the extraction of collagen and gelatin from mammalian sources, namely pig skin, bovine hides and cattle bones as most used raw materials. Fish skins, fins and scales are also valuable marine sources of collagen and gelatin. However, there is hardly any existing industry that manufactures collagen or gelatin from fish bones although several studies have investigated the extraction of collagen or gelatin from fish bones. Hence, in the absence of proven industrial processes, only experimental procedures are surveyed.

2.5.1 Extraction of collagen from fish bones

Type I collagen as a main component in bones is the most abundant in nature (Omokanwaye et al., 2010; Visakh et al., 2016). Despite its abundance, the utilisation of collagen has been limited by its insolubility (Herold et al., 2010). Thus, to ensure its maximised recovery, techniques to extract collagen from fish bones have been composed into three important steps: sample preparation, extraction and purification (Simpson et al., 2012; Silva et al., 2014). Furthermore, these procedures are carried out at low temperature (4 °C) to avoid thermal denaturation of collagen. The procedures for collagen isolation from fish bones obtained from bigeye snapper, deep-sea redfish, tuna, Baltic cod, skipjack tuna, Japanese sea bass, ayu, yellow sea bream, chub mackerel, bullhead shark, horse mackerel, carp and rainbow trout (Kittiphattanabawon et al., 2005; Wang et al., 2008; Żelechowska et al., 2010; Takeshi and Suzuki, 2000; Duan et al., 2009; Tabarestani et al., 2012) are summarised below.

Sample preparation steps include (1) recovery of fish bones; (2) cleaning; (3) size reduction; and (4) chemical treatment to remove NCP and minerals. Bones were recovered from fish by removing the residual meat manually and then washing the bones with running tap water. Some protocols

utilised a mechanical separator to recover fish backbones from fish. Size reduction was achieved by cutting the bones into small pieces, breaking the bones with a hammer or further powdering the bones by mixing the samples in liquid nitrogen. A meat grinder was also used for bones containing residual meat. NCP represented a minor constituent (Olszta et al., 2007), approximately 5% on a dry mass basis, of the organic component of the fish bones (Boskey, 2013) and was commonly removed by using 0.1 M sodium hydroxide (NaOH). Different sample to alkali solution ratios (w/v) and extraction times were used: 1:2 and 24 hours (Zelechowska et al., 2010), 1:5 and 24 hours (Duan et al., 2009) or 1:10 and 6 hours (Kittiphattanabawon et al., 2005; Tabarestani et al., 2012), respectively.

However, some researchers perceived NCP removal from fish bone as unnecessary, possibly due to the minute quantity of NCP in fish bones. Fratzl (2008) indicates that there is no distinction between collagen and noncollagen components in bones; in general, organics in the bones are called 'collagen'. As for the removal of minerals (inorganic main components) in the bones, 0.5 M ethylenediaminetetraacetic acid was often used, with various ratios of bones to ethylenediaminetetraacetic acid solution (w/v) and extraction times ranging from one to five days, accompanied by a change of solution in between (Duan et al., 2009; Kittiphattanabawon et al., 2005; Takeshi and Suzuki, 2000; Tabarestani et al., 2012; Wang et al., 2008). Alternatively, 1 M HCl was also used with 1:3 bones to HCl solution ratio (w/v) and an extraction time of one hour (Zelechowska et al., 2010). A study by Skierka et al. (2007) demonstrated that the best operating conditions for demineralisation for a given 1:5 bones to HCl solution ratio (w/v) were 1 M HCl and three consecutive 24-hour extractions. For fatty bones, demineralisation was followed by defatting using hexane at a 1:10 w/v ratio for 24 hours (Wang et al., 2008) or using detergent overnight (Duan et al., 2009). Deep-sea redfish defatting resulted in a reduction of fat content from 26.8% to 3.0% on a dry mass basis (Wang et al., 2008).

Collagen extraction step employed 0.5 M acetic acid solution only (Duan et al., 2009; Kittiphattanabawon et al., 2005; Takeshi and Suzuki, 2000; Tabarestani et al., 2012; Wang et al., 2008) or with the addition of proteolytic enzymes (pepsin) for efficient solubilisation of collagen contained in the demineralised fish bones (Zelechowska et al., 2010). Lastly, for the purification step, the solubilised collagen in the solution was recovered by adding sodium chloride (NaCl) to a final concentration of 0.9 M, followed by precipitation of the collagen by further addition of NaCl at neutral pH. The precipitate was further purified by dissolving it in 0.5 M acetic acid and then dialysing it against 0.1 M acetic acid and distilled water to remove NaCl salt. The final collagen concentrated solution was then freeze-dried.

Based on the literature survey, the overall process to extract collagen from fish bones is outlined in Figure 2.2.

Fish

↓

Recovery of bones

↓

Size reduction

- - - alternatively - - - → NCP removal

↓

Demineralisation ← - - - - - - - -

↓

Defatting (only for fatty bones)

↓

Collagen extraction (proteolytic enzyme additive as optional)

↓

Precipitation of collagen from the solution

↓

Purification of collagen precipate by dialysis

↓

Freeze drying of collagen concentrated solution
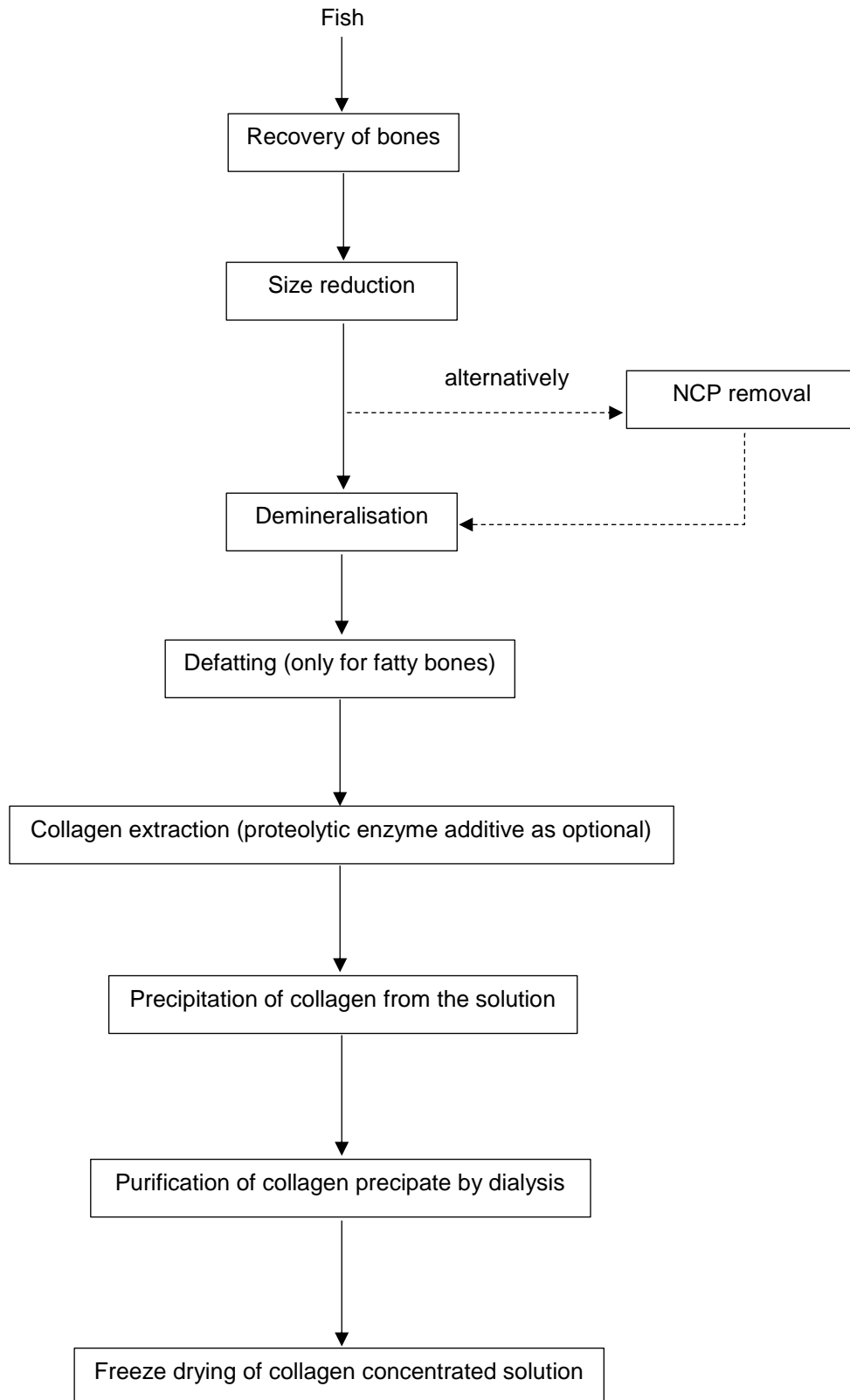
Figure 2.2: Overall process for collagen extraction from fish bones based on the existing process in literature

2.5.2 Extraction of gelatin from fish bones

The manufacturing process for gelatin consists of three main steps: pretreatment of the raw material, extraction of the gelatin and purification (Karim and Bhat, 2009). The general process of gelatin extraction from fish bones includes (1) bone crushing; (2) degreasing; (3) demineralisation to solubilise the mineral constituents in the bone; (4) gelatin extraction by heating the ossein in water; (5) separation of the gelatin solution by filtration; (6) purification of the gelatin solution using an ion exchange to remove any contaminants; (7) concentration; and (8) drying. This process has been adopted in several studies of gelatin extraction from fish bones obtained from either head or backbones of channel catfish, king weakfish, Atlantic cod, Nile perch, Atlantic mackerel and blue whiting; the experimental methodologies are discussed below.

Pretreatment involved recovery of bones, defatting, size reduction and demineralisation. Bones from fish heads were recovered using an alkali protease with the following conditions: hydrolysis in water at pH 9 and 50 °C for 140 minutes with mixing at 150 rpm (Liu et al., 2008). Alternatively, three sequential chemical treatments at room temperature were utilised using 3 M NaOH for the first two treatments and 3 M HCl for the last treatment (Arnesen and Gildberg, 2006a). Bones from fish backbones were recovered by manual removal of adhering meat or by using a mechanical meat bone separator to remove meat adhering to the backbones. Fatty bones were then defatted using water at 35 °C under constant shaking. Thereafter, defatted bones were crushed into less than 0.5 mm long, ground in a mill or manually segmented into small pieces. To recover the protein fraction in the fish bones, minerals contained in the bones were solubilised using HCl in different concentrations: 0.25 N (Khiari et al., 2013), 0.4 M (Liu et al., 2008), 0.6 M (Arnesen and Gildberg, 2006a; Gildberg et al., 2002), 3% (Da Trindade Alfaro et al., 2009; Muyonga et al., 2004) or 3.35% (Sanaei et al., 2013). Various ratios of bones to HCl solution (w/v) were used, with extraction times ranging from 7.5 to 48 hours, accompanied by a change of solution in between. The lengthy demineralisation of fish bone took 9-12 days with changing of the solution every 3 days (Muyonga et al., 2004). The operating temperature during demineralisation varied between 4 °C, 10 °C and 20 °C. Subsequently, demineralised bones were washed with tap water until the pH of the washwater was above 4 or neutral 7.

This was followed by a gelatin hot water extraction with different levels of bones to water ratio (1:2.5, 1:3 or 1:8 w/v), extraction temperatures (45, 60, 67 or 80 °C) and extraction times (2, 5.2 or 18 hours) (Da Trindade Alfaro et al., 2009; Khiari et al., 2013; Sanaei et al., 2013). Alternatively, three to five sequential gelatin extractions were employed. For five sequential extractions, the pH and temperature of the solution were 5.3 and 60 °C, 4.4 and 70 °C, 3.8 and 80 °C, 3.6 and 85 °C and 3.5 and 90 °C, respectively, employing 30 minutes for each extraction (Arnesen and Gildberg, 2006a). For three sequential extractions, the pH and temperature of the solution were 4.0 and 75 °C, 2.5 and 82 °C and 3.0 and 90 °C, respectively; the extraction times varied between two, three and four hours during the first, second and third extractions, respectively (Liu et al.,

2008). Lastly, the extracted gelatin solution was filtered, concentrated with a rotary evaporator and then freeze-dried.

In some cases, different treatments can be employed prior to gelatin extraction, including acid, alkaline or enzyme treatment. Alkali treatment of the demineralised fish bones is used before gelatin hot water extraction although acid treatment is also a viable treatment route for bones (Karayannakidis and Zotos, 2016). An alkali solution, namely $Ca(OH)_2$ (9 g/L) or NaOH (2 g/100 g and 4 g/100 g), was used for such purposes for a period of 48 or 144 hours at room temperature (Da Trindade Alfaro et al., 2009; Liu et al., 2008). Alternatively, H_2SO_4 or HCl are commonly used. The process of alkali or acid treatment of demineralised bones has initially been adopted from liming of ossein and hides from cattle sources using alkali and from acid pulping of pig skin using mineral acid. Liming of cattle bones and hides usually requires 8-12 weeks (Gelatin Manufacturers Institute of America [GMIA], n.d.) while acid pulping of pig skin requires only several hours or 24 hours at maximum (Karayannakidis and Zotos, 2016). Treatment time differs significantly due to the degree of collagen cross-linkage of the raw material (Karayannakidis and Zotos, 2016). However, it should be noted that the fish bone is light relative to mammalian bone and has a fibrous or 'woody' appearance (Wheeler and Jones, 1989). Figure 2.3 provides a summary of the overall process for the gelatin extraction from fish bones based on the surveyed literature.

Harris (1990) describes the design of a gelatin extraction process as the optimum balance between three factors: pH, temperature and extraction time for maximum yield. It is mentioned that the gelatin extraction rate is inversely proportional to the physical quality parameters of gelatin, bloom and viscosity values between pH 3 and 7. It is also indicated that the pH of the gelatin extraction can be selected either for favourable quantity (maximum extraction rate) at low pH or favourable quality (maximum in physical properties) at neutral pH, or a compromise between quantity and quality can be reached.

Furthermore, three or four separate extractions with increasing temperature for each extraction are normal practice. Common values are 55 °C, 60 °C, 70 °C, and 80-90 °C for first, second, third and fourth extractions, respectively (Harris, 1990). Similarly, first extraction takes place at 50-60 °C with successive increases in temperature of 5-10 °C for subsequent extractions (GMIA, n.d.). Each extraction resulted in gelatin of decreasing bloom strength and increasing colour (Harris, 1990) with the initial extraction providing a superior product: higher viscosity, higher gel strength and lighter colour (GMIA, n.d.). On a par with the gelatin quality is the gelatin yield. Like its parent protein (collagen), gelatin is also measured by its hydroxyproline content.

Fish

↓

Recovery of bones

↓

Defatting (only for fatty bones)

↓

Size reduction

↓

Demineralisation

↓

Washing with water (to remove excess acid)

----→ alternatively ----→ Alkali (liming), acid (pulping) or enzymatic treatment

↓

Gelatin extraction (using water)

↓

Filtration

alternatively

repeated sequential extraction (up to 5)

↓

Concentration

↓

Freeze drying of concentrated gelatin solution
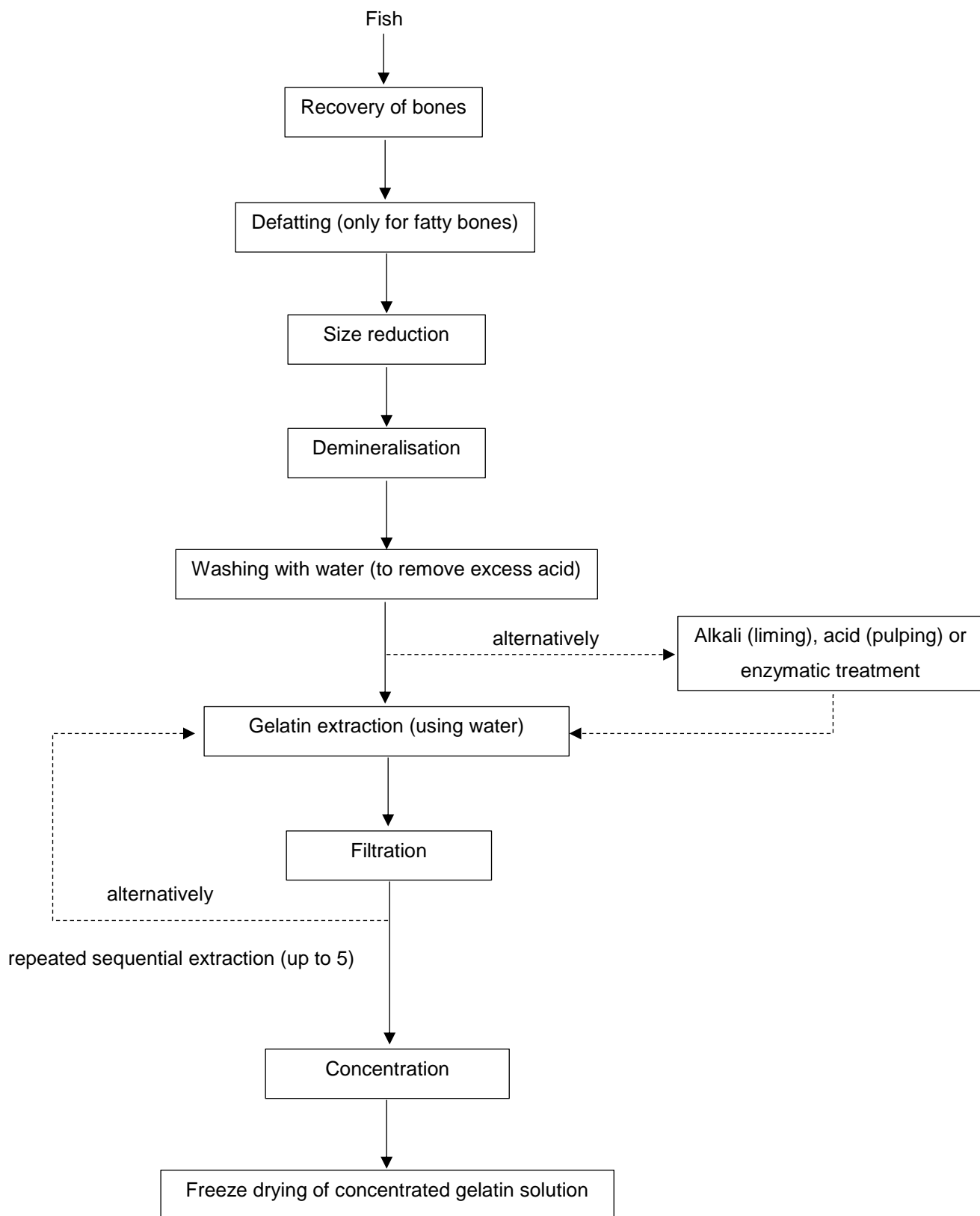
Figure 2.3: Overall process for gelatin extraction from fish bones based on the existing process in literature

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2.6   Collagen quantitation

Hydroxyproline is an amino acid that is found only in collagen protein and elastin proteins. However, the contribution of hydroxyproline from elastin is negligible compared to that from collagen (Caballero et al., 2015; Simpson et al., 2012) as the elastin content in muscle tissue is nearly 20-fold lower in concentration than collagen. For this reason, hydroxyproline quantification is a good measure of collagen content in a sample (Nollet and Toldrà, 2015), and hydroxyproline therefore is often used as a measure of collagen content in foods (Simpson et al., 2012). Since collagen is the parent protein of gelatin, hydroxyproline analysis is also used to analyse the gelatin content in foods and it is recognised as a definitive method providing a high degree of accuracy (GMIA, 2013; Harris, 1990). In addition to this, the hydroxyproline level in gelatin is correlated with its gel properties; high levels of hydroxyproline have a higher tendency to form gel (Silva et al., 2014). Hydroxyproline is regarded as the most important amino acid in the renaturation of gelatin subunits during gelling, and its only role is providing stability to the collagen triple helix, which is attributed to its hydrogen-bonding ability through its OH^- group. This is validated by the lower gel strength for fish gelatin extracted from cold water species, due to its low hydroxyproline content, as compared to the higher gel strength for fish gelatin from warm water species and mammalian gelatins that contain higher hydroxyproline levels (Gaonkar et al., 2014; Karim and Bhat, 2009; Shahidi, 2007; Szpak, 2011).

The determination of hydroxyproline content provides an indirect measure of the collagen content (Nollet and Toldrà, 2015). The specific quantity of collagen can be calculated when the hydroxyproline content is multiplied by a known conversion factor. This factor is normally species specific (Regenstein and Regenstein, 1984) as a result of a variable ratio of hydroxyproline to proline in collagen for each species although the sum of the hydroxyproline and proline content of collagen is roughly constant throughout the animal kingdom. Therefore, the hydroxyproline-to-collagen conversion factor of bovine collagen cannot be applied to fish. Typical conversion factors used by several studies are 14.7 for Baltic cod skin (Sadowska et al., 2003), 7.7 for bigeye snapper skin (Nalinanon et al., 2007), 8.6 for sardine scales (Bellali et al., 2013), 15.7 for Baltic cod backbone (Żelechowska et al., 2010), 10.6 for tiger pufferfish soft tissues (Tsukamoto et al., 2013), 8 for trash fish skin, bone and muscle (Muralidharan et al., 2013) and 8 for meat, meat products and muscle sample (Nollet and Toldrà, 2015). The conversion factor for hydroxyproline to collagen for a specific species is determined by extraction of collagen with the same methodology previously introduced in the extraction of collagen from fish bones. Thereafter, the dry mass and hydroxyproline content of purified collagen are determined. The conversion factor is defined as the ratio of the purified collagen dry mass to the hydroxyproline content thereof or as the ratio of the total amino acid residues to the hydroxyproline residues.

A colorimetric assay has been chiefly used for hydroxyproline quantitation (Kołodziejska et al., 2008; Mahmoodani et al., 2014; Sadowska et al., 2003) although full amino acid analysis using high-performance liquid chromatography instruments is also used. The colorimetric assay is

based on a reaction of oxidised hydroxyproline with p-dimethylamino benzaldehyde (DMAB), commonly known as Ehrlich's reagent. This reaction develops a chromophore that can be measured at a specific wavelength (typically 558 nm) using a spectrophotometer. There are different methods for the determination of hydroxyproline. Neuman and Logan's method (Neuman and Logan, 1950) oxidises hydroxyproline to pyrrole-2-carboxylic acid using hydrogen peroxide in the presence of copper sulphate and NaOH. The excess hydrogen peroxide is removed by heating and shaking. Oxidised hydroxyproline is coupled with DMAB in the presence of dilute H_2SO_4, giving a coloured product that can be measured at a specific wavelength using a spectrophotometer. However, along with hydroxyproline, tyrosine and tryptophan serve as interfering substances. An improved method using distillation apparatus (Serafini-Cessi and Cessi, 1964) and ion-exchange column (Stegemann and Stalder, 1967) is used to remove these interferences. Alternatively, Woessner's method (Woessner Jr., 1961) uses chloramine-T for hydroxyproline oxidation whereafter the solution is neutralised using perchloric acid before reaction with DMAB. Heating the mixture to 60 °C develops a colour that is stable for at least one hour and can be measured at a specific wavelength. These operating conditions are only applied to samples containing at least 2% hydroxyproline while for samples with less than 2%, benzene is added after the colour development. Finally, the International Organization for Standardization (ISO) method uses a chloramine-T that is mixed with buffer solution containing citric acid, NaOH, sodium acetate and 1-propanol for oxidising the hydroxyproline. Perchloric acid and DMAB are combined, with the addition of 2-propanol. Heating the mixture to 60 °C develops a colour that is stable for at least one hour and can be measured at a specific wavelength (ISO, 1994). This standard is also corroborated by the Official method 990.26: hydroxyproline in meat and meat products colorimetric method of the Association of Official Analytical Chemists (AOAC, 2005d).

Recent studies have alternatively employed alkaline hydrolysis with the intent to significantly reduce the 16-hour acid hydrolysis time of samples containing hydroxyproline in the conventional aforementioned ISO and AOAC methods. The use of 2 M and 2.2 M NaOH to hydrolyse solid and liquid samples, respectively, in an autoclave operating at 120 °C and 15 psi resulted in three-hour complete hydrolysis of samples (Hofman et al., 2011) while using 7 M NaOH in the autoclave operating at 120 °C resulted in 40-minute complete hydrolysis of samples (Da Silva et al., 2015). These methods have not gained widespread acceptance and have not been validated for a wide range of raw materials.

2.7   Literature review conclusion

Following the examination of the relative literature, the following conclusions can be drawn:

I.   The majority of the fish processing solid waste is generated from onshore fish processing. This implies that also within the South African context, onshore raw materials are available at fish processing facilities, which provides the opportunity for valorisation.

II. Significant amounts of monkfish onboard processing solid waste such as fish heads are generated from the South African monkfish and hake fisheries that need to be landed onshore, providing an opportunity to add value to this waste.

III. The enzymatic hydrolysis technique to recover proteins contained in fish processing solid waste (fish heads, frames, skin, tails, viscera, fins, guts, liver and roes) is well established. It specifically targets the protein component of the raw material such as fish heads, and any bones remain a by-product that can be further utilised as raw materials to produce value-added products.

IV. Bones mostly consist of inorganic matter, chiefly calcium and phosphate (~60-70% dry mass). The balance constitutes the organic matter, which consists mainly of collagen. This is an indication that fish bones are a potential source of calcium phosphate minerals and collagen proteins and thus have applications in agriculture (fertiliser and feed additives), food, and the nutraceutical and cosmetic industries.

V. The application of fish bones in agriculture as fertiliser is well established; however, as direct feed additives, they can only be used as a calcium source but not as a phosphorus source. An opportunity therefore exists to utilise the mineral fraction in the fish bones as raw material in manufacturing feed additives such as inorganic calcium phosphates (MCP and DCP).

VI. A new method that employs an alternative mineral acid such as H_3PO_4 in acidification of the fish bone material presents a practical means of DCP manufacture, with the potential advantage that a solid or liquid salt by-product is possibly not generated. Using HCl produces a $CaCl_2$ solution by-product while using H_2SO_4 produces $CaSO_4$ precipitate in acidification of fish bone. The new method could also produce high-purity DCP without the need for purification steps.

VII. Demineralisation of bones leaves ossein, which is a potential substrate for gelatin recovery.

VIII. The remaining ossein after demineralisation will only be suitable for gelatin extraction as previous stages will cause denaturation of collagen.

IX. Gelatin extraction from the bones of various species but not from monkfish has been investigated. This provides an opportunity to evaluate the suitability of ossein derived from monkfish bones as potential gelatin source.

Chapter 3
Aim of the study

Specific gaps in the literature were identified, which provided grounds for the aim of this study: to develop and optimise processes for the following:

(1) extraction of minerals from monkfish bones;
(2) precipitation of DCP from the extracted minerals; and
(3) extraction of gelatin from monkfish ossein.

Monkfish bones were obtained from enzymatic protein hydrolysis of monkfish heads. Consequently, this study could contribute towards improved utilisation of monkfish heads, which are currently viewed as a solid waste product from the South African monkfish and hake fisheries. To achieve the aim of the study, a simplified process flow diagram as depicted in Figure 3.1 was formulated.

Monkfish heads
↓
Pretreatment
↓
Monkfish bones
↓
Demineralisation

Mineral liquor   **Monkfish ossein**
↓   ↓
Precipitation   Gelatin extraction
↓   ↓
DCP   **Gelatin liquor**
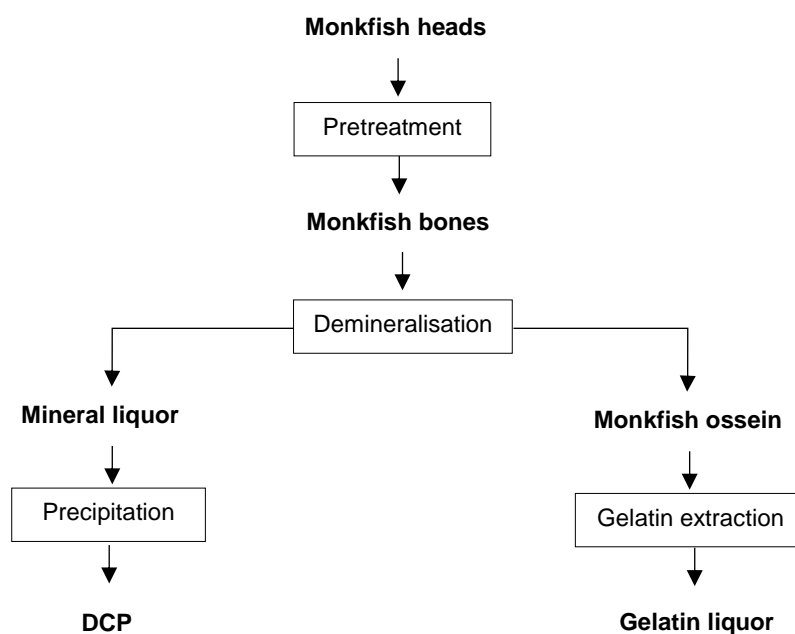
Figure 3.1: Schematic process flow diagram for producing DCP and gelatin from monkfish bones recovered from monkfish heads

Overall, the process diagram is made up of four main procedures: preliminary treatments, demineralisation, DCP precipitation and gelatin extraction. The objective of the preliminary treatments was to recover bones from monkfish heads by employing an enzymatic protein hydrolysis technique, which would produce protein hydrolysate and fish bone residue. Afterwards, the demineralisation procedure isolated the minerals from the recovered monkfish bones while preserving the organic phase of the bone. This process resulted in two potentially valuable components: mineral liquor and monkfish ossein. DCP was then precipitated from the mineral liquor, and gelatin was extracted from the ossein phase.

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Chapter 4
Materials and methods

4.1   Introduction

The intention of the study was to utilise the fish head bone residues, a by-product derived from extraction of protein nutrients from fish heads using enzymatic hydrolysis. To the best of my knowledge, there is no literature on the use of monkfish bone residues from enzymatic protein hydrolysis; however, a study of enzymatic hydrolysis of cod backbone (Gildberg et al., 2002) yielded bone sediments that were further utilised for gelatin recovery. Furthermore, a mild chemical treatment to extract proteins from cod heads (Arnesen and Gildberg, 2006) instead of using enzymes yielded bone residues for gelatin extraction. In the current study, monkfish bones were recovered from monkfish heads using a conventional method, namely enzymatic hydrolysis, as the objective of the preliminary treatments of monkfish heads. As the focus of this study was the recovery of bones from monkfish heads and not the production of protein hydrolysates, enzymatic protein hydrolysis conditions were not optimised or characterised; therefore, a simple treatment of monkfish heads was applied using an enzyme at a specific temperature, pH and time.

The proximate analysis of the recovered monkfish bones was determined to establish the moisture, ash, crude protein and crude fat composition thereof. The mineral content in the bones was recovered in a mineral liquor using a demineralisation process. The residual ossein, as a by-product of the demineralisation process, was presumed to contain purely collagen and a minimal amount of minerals. The collagen content in ossein was measured by the hydroxyproline amount while the mineral content in ossein was measured by the ash amount. This means that the objective of the demineralisation process was to ensure that the whole of the mineral fraction in the monkfish bones was contained in mineral liquor whereas the protein fraction was retained in the monkfish ossein. To address such an objective, a two-level full factorial design with three factors and one centre point was applied to determine the optimum conditions that maximised the hydroxyproline content and minimised the ash content in the monkfish ossein in g kg^{-1} dm.

The resulting mineral liquor was potentially a suitable raw material for the precipitation of DCP. To ensure that both DCP recovery and P content in the DCP were maximised, a CCD with three factors was used to find the optimum operating conditions. The by-product of demineralisation, the monkfish ossein, potentially contains high levels of collagen, which can be an ideal raw material for gelatin extraction. A further CCD with three factors was applied to establish the optimum operating conditions for the gelatin extraction from monkfish ossein that provided maximum hydroxyproline yield of gelatin liquor.

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4.2   Materials and methods

4.2.1   Preliminary treatments of monkfish heads

Monkfish heads were obtained from a local fish processor in Cape Town, South Africa. Whole heads were collected and transported on ice to Stellenbosch University where any remaining viscera attached to the heads were manually removed. Degutted monkfish heads were then washed with running tap water to remove mainly the thick mucus that blanketed the mouth and skin of the monkfish heads. Lastly, clean monkfish heads were stored in the freezer at -15 °C for subsequent processing.

Monkfish heads were minced before protein hydrolysis. This commenced by thawing frozen monkfish heads overnight in the laboratory at ambient temperature (approximately 17 °C). Thereafter, defrosted monkfish heads were minced with a bowl cutter (Universal Butchery Equipment Model Dampa CT55, South Africa) for 4 minutes using speed 1 and a further 10 minutes using speed 2 until a homogenous mixture was obtained.

Enzymatic protein hydrolysis was performed following the protocol as described by Wrolstad et al. (2005) for the preparation of a hydrolysis sample, which was specifically developed for using alcalase proteolytic enzyme. In summary, 2.5 kg of minced monkfish heads were mixed with 2.5 L of demineralised water in a 5 L glass beaker. The solids in the mixture were ensured to be fully suspended using an overhead mixer (DragonLab Model OS40-Pro, China) at 750 rpm. The mixture was heated in the water bath (Labotec Model 132, South Africa) until a temperature of 40 °C was attained. Thereafter, while the temperature was maintained, the pH of the mixture was adjusted to 8 using 1 M NaOH that was prepared by diluting 40 g sodium hydroxide pellets (Sodium Hydroxide Pellets Emparta, Merck) to 1 000 ml with demineralised water. As the desired temperature and pH of the mixture were obtained, 6.78 ml of protease enzyme Alcalase ® AF 2.4L (Novozymes) were added to the mixture to initiate the hydrolysis while mixing was continued. After 60 minutes of hydrolysis, the mixture was filtered using a strainer with an average aperture size of 1.3 mm. The filtrate was discarded whereas the monkfish bones residue on the strainer was washed with running tap water to remove any excess protein hydrolysate, and the excess water was allowed to drip off. Hydrolysis of 2.5 kg minced monkfish heads was repeated 25 times, and the monkfish bones yield was calculated as the amount of monkfish bones divided by the amount of wet minced monkfish heads. The moisture content of the monkfish bones was established by drying duplicate samples in an air drying oven at 100 °C until sample weight remained unchanged (120 minutes). The result was reported as mean ± standard deviation of the duplicate measurements.

4.2.2   Homogenising of monkfish bones

Monkfish bones were mixed with demineralised water in a 1:1 w/v ratio and blended using a food processor (Brabantia model BBEK1051). The bones were blended for 5 minutes at speed 3 and an additional 13 minutes at speed 5. The size reduction of monkfish bones aimed to ensure the

representative nature of the sample (Prichard et al., 1996; Proctor, 1994). Afterwards, the mixture was filtered using a filter cloth and then the residues were rinsed with running tap water to eliminate the froth that has formed during blending. The monkfish bone residues on the filter cloth were hand squeezed to expel the excess water. After thorough manual mixing of the monkfish bone residues that weighed approximately 2 800 g, 18 samples (35 g each) for subsequent demineralisation process, one sample (25 g) for proximate analysis and an additional one sample (10 g) for hydroxyproline analysis were obtained. Thereafter, the samples and the balance of monkfish bone residues were frozen until further use.

4.2.3 Demineralisation of monkfish bones

A two-level, three-factor (2^3) full factorial design was implemented for the demineralisation of monkfish bones. Three factors and their low and high level settings were investigated, namely (1) H_3PO_4 concentration (2% and 5% v/v); (2) number of extractions (2 and 4) with fixed 24-hour extraction time; and (3) ratio of solution (v) to raw material (w) (2:1 and 5:1). These levels were based on the optimum conditions, determined by a study of Skierka et al. (2007), for the demineralisation of the backbone of Baltic cod, a coldwater fish similar to monkfish, using HCl. It was established that 99% of the minerals contained in Baltic cod backbone were recovered using the following optimum conditions: 1 M HCl and three consecutive 24-hour extractions for a given 1:5 ratio of raw material to solution (w/v). The response variables measured were ash and hydroxyproline contents (g kg^{-1} dm) in the ossein.

The two-level full factorial design with three factors generated eight experimental runs, with each run having a unique treatment or combination of factor level settings. The design was also supplemented with a centre point run at the midpoint of the factor level settings, resulting in a total of nine independent treatments. This method provided protection against any potential curvature from second-order effects and also allowed an independent estimate of error to be obtained. As a result, there was an added parameter to the model, known as a pure quadratic curvature (Montgomery, 2000), curvature (STATISTICA) or centre-point term (Minitab). The full design was replicated once; thus, a total of 18 experimental runs were conducted. The complete experimental design is depicted in Table 4.1 in a randomised order.

Table 4.1: Two-level full factorial design of experiments with three factors, one centre point and one replicate in a random order for the demineralisation of monkfish bones

Experimental run	H_3PO_4 concentration, % v/v	Number of extractions	Ratio of solution (v) to raw material (w)
1	2.0	2	2:1
2	5.0	2	5:1
3	5.0	4	5:1
4	5.0	2	2:1
5	5.0	4	5:1
6	5.0	4	2:1
7	5.0	4	2:1
8	5.0	2	5:1
9	2.0	2	5:1
10	2.0	4	2:1
11	3.5	3	3.5:1
12	2.0	2	2:1
13	2.0	4	5:1
14	3.5	3	3.5:1
15	5.0	2	2:1
16	2.0	2	5:1
17	2.0	4	5:1
18	2.0	4	2:1

The different concentrations of H_3PO_4 solution such as 2.0% (pH 1.21), 3.5% (pH 1.00) and 5.0% (pH 0.96) v/v were prepared by diluting 24.13 ml, 41.73 ml and 60.33 ml of concentrated H_3PO_4 (85%, Merck), respectively, to 1 000 ml with demineralised water. For each experimental run, a 35 g frozen sample of monkfish bones was added with 70 ml, 122.5 ml or 175 ml of 2.0%, 3.5% or 5.0% v/v H_3PO_4 solution. The solid in the mixture was ensured to be completely suspended in the solution by using an overhead mixer (DragonLab Model OS40-Pro, China) at 250 rpm. Two identical mixers were randomly allocated to 18 experimental runs. After 24 hours extraction time at ambient temperature of 17 °C, the mixture was centrifuged using a 50 ml high-speed centrifuge (HunanHerexi Model H/T16MM, China) at 12 700 rcf for 20 minutes. Afterwards, the solid layer was separated and then re-extracted with new solution until two, three or four consecutive 24-hour extractions were completed. Excessive acid was subsequently removed by washing the monkfish ossein residue with demineralised water using a vacuum filter until the washwater pH was faintly acidic (5.5 to 6.8). Finally, samples of monkfish ossein from 18 experimental runs were analysed for the ash and hydroxyproline contents (% dry mass), which were the response variables in the demineralisation of monkfish bones experiment.

4.2.4   Preparation of feedstock for dicalcium phosphate precipitation and gelatin extraction

Four batches of 350 g monkfish bones were demineralised under optimal conditions: 5% v/v H_3PO_4 with a ratio of H_3PO_4 solution (v) to bones (w) of 5:1 and using four extractions. These

were the optimum operating conditions for the demineralisation of monkfish bones reported in the results and discussion chapter (see Section 5.3). The mixing speed of the overhead stirrer was 400 rpm. After 24 hours extraction time, the mixture was filtered using a filter cloth and the solids were re-extracted with fresh H_3PO_4 solution until four consecutive 24-hour extractions were completed. Mineral liquor from each extraction was retained after the filtration steps, and all four mineral liquor extracts were combined and stored in a plastic container at laboratory ambient temperature. The monkfish ossein was stored in the freezer after washing with demineralised water using a vacuum filter until the washwater pH was faintly acidic (5.5 to 6.8). Samples of monkfish ossein from two replicates of 350 g monkfish bones demineralisation were analysed for the ash and hydroxyproline contents to validate the fitted linear regression model for the demineralisation of monkfish bones. The results were reported as the average measurement of the duplicate samples.

The combined extracts of mineral liquor from one 350 g batch of monkfish bones demineralisation was utilised as feed materials for the DCP precipitation procedure. The combined extracts of the mineral liquor were initially mixed at 700 rpm speed for 10 minutes. Thereafter, while mixing was in progress, 35 samples (of 150 ml each) were extracted for performing DCP precipitation, and a sample (of 20 ml) was collected for Ca and P content analysis, respectively. The samples were stored in glass bottles at laboratory ambient temperature until further use.

The combined monkfish ossein from the four 350 g batches of monkfish bones demineralisation was utilised as feed materials for the gelatin extraction process. Frozen monkfish ossein was thawed at laboratory ambient temperature. The excess water on the monkfish ossein was expelled through hand squeezing using the filter cloth. The monkfish ossein that weighed approximately 415 g was mixed by hand, and 40 samples (of 10 g each) were obtained for subsequent gelatin extraction. The balance of the monkfish ossein was used for hydroxyproline and moisture content analyses. Thereafter, the samples were frozen until further use. The moisture content of the monkfish ossein was established by drying a sample in the air drying oven at 100 °C until the sample weight remained unchanged (120 minutes).

4.2.5   Precipitation of dicalcium phosphate from mineral liquor

For the precipitation of DCP from mineral liquor, a CCD was used to find the levels of three factors that maximised the total precipitate yield (g dm) and P content (g kg^{-1} dm) while targeting a Ca:P molar ratio of 1:1 as the experimental responses. The three factors were (1) reaction temperature (°C); (2) 1 M $Ca(OH)_2$: mineral liquor (v:v); and (3) reaction time (min.). The CCD consisted of two level factorial points, namely -1 and 1, with an additional centre point at 0 and two level star points positioned at an alpha value of -1.6818 and +1.6818 from the centre point. Briefly, these levels were -1.6818, -1, 0, 1 and 1.6818, which corresponded to 25, 35, 50, 65 and 75 °C for the reaction temperature; 0.2:1, 0.4:1, 0.7:1, 1:1 and 1.2:1 for 1 M $Ca(OH)_2$:mineral liquor (v:v); and 17, 35, 60, 85 and 102 min. for reaction time, respectively. These levels were determined based

on the various levels of operating conditions employed in the previous studies on the neutralisation of H_3PO_4 solution using $Ca(OH)_2$ to precipitate DCP (Ducheyne et al., 2011; Ferreira et al., 2003; Freitas and Giulietti, 1997; Giulietti, 1994; Macketta, 1978). It was found that the different levels of reaction time were 20 and 60 minutes, reaction temperatures were 25, 45, 60, 85 and 95 °C and the solution's final pH levels were 2, 3, 3.5, 4, 5, 6, and 7. In the present study, various levels of the solution's final pH were achieved by employing different levels of 1 M $Ca(OH)_2$:mineral liquor (v:v) ratio.

The CCD matrix for the three factors and five level settings is illustrated in Table 4.2 specifically for the DCP precipitation process. Additionally, the CCD with three factors contained 16 experimental runs; two runs were at centre point levels, and the balance had a unique treatment or combination of factor level settings. The design was replicated once; thus, a total of 32 experimental runs were conducted. This design is outlined in Table 4.3 in a random order.

Table 4.2: DCP precipitation process: CCD matrix for three factors and five level settings

Factors	Levels				
	-1.6818	-1	0	1	1.6818
	Actual values				
Reaction temperature, °C	25	35	50	65	75
1M $Ca(OH)_2$:mineral liquor (v:v)	0.2:1	0.4:1	0.7:1	1:1	1.2:1
Reaction time, min	17	35	60	85	102

Each experimental run was started by heating 150 ml of mineral liquor contained in a 400 ml glass beaker using a hot water bath (Labotec Model 132, South Africa) while mixing the mineral liquor with an overhead mixer (DragonLab Model OS40-Pro, China) at 300 rpm until the required temperature, namely 25, 35, 50, 65 or 75 °C was reached. Subsequently, while mixing was continued and the required temperature was maintained, an appropriate quantity, namely 30, 60, 105, 150 or 180 ml of 1 M $Ca(OH)_2$, was quickly added to the heated mineral liquor. The total volume of the mixture in the glass beaker was ensured to be fully submerged under the heated water in the water bath. The 1 M $Ca(OH)_2$ was prepared by diluting 74.09 g of calcium hydroxide (Calcium Hydroxide AR, ACE) to 1 000 ml with demineralised water. When the calcium hydroxide was completely added, the reaction time of 17, 35, 60, 85 or 102 min was started. At the end of the reaction time, the mixture was immediately vacuum filtered using a preweighed, ash-free filter paper (Macherey-Nagel, grade MN 640 d).

Table 4.3: CCD of experiments with three factors and one replicate in a random order for the precipitation of DCP from mineral liquor

Experimental run	Reaction temperature, °C	Reaction time, minutes	1 M Ca(OH)$_2$: mineral liquor (v:v)
1	50	60	0.7:1
2	50	60	0.7:1
3	75	60	0.7:1
4	35	35	0.4:1
5	25	60	0.7:1
6	50	102	0.7:1
7	65	35	1:1
8	35	85	0.4:1
9	50	17	0.7:1
10	50	102	0.7:1
11	35	35	1:1
12	65	85	0.4:1
13	50	60	1.2:1
14	50	60	0.2:1
15	65	35	0.4:1
16	50	60	0.2:1
17	50	60	0.7:1
18	65	85	1:1
19	65	35	1:1
20	50	17	0.7:1
21	65	35	0.4:1
22	35	85	0.4:1
23	35	85	1:1
24	65	85	0.4:1
25	35	85	1:1
26	25	60	0.7:1
27	75	60	0.7:1
28	35	35	1:1
29	65	85	1:1
30	50	60	1.2:1
31	35	35	0.4:1
32	50	60	0.7:1

The DCP residues on the filter paper were transferred to a preweighed watch glass and dried using an air drying oven at 100 °C for 5 hours, according to the AOAC Official method 950.01 (AOAC, 2005a). The weight of the DCP in dry mass was calculated as the difference of the constant weight and the combined weight of the filter paper and watch glass, and it was reported as the DCP yield (g dm). For each experimental run, the P and Ca contents (g kg^{-1} dm) of the DCP were analysed using inductively coupled plasma atomic emission spectroscopy (ICP-AES) wherein the P content was reported as total P. The fraction of total P soluble in water was extracted using the AOAC Official method 977.01 (AOAC, 2005c), and the P in the extract was

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determined using the ICP-AES technique. The Ca:P molar ratio of the dry precipitate was determined by dividing the Ca and P contents in mass (g) by the atomic mass (g/mol) of Ca and P, respectively.

A single new experimental run was carried out using 150 ml mineral liquor at optimised DCP precipitation conditions reported in the results and discussion chapter (see Section 5.4.1): 75 °C reaction temperature, 1 M Ca(OH)$_2$:mineral liquor (v:v) of 0.95:1 and reaction time no longer than 17 minutes as reaction times longer than 17 minutes did not lead to increased yield. The DCP yield (g dm), P and Ca contents (g kg^{-1} dm) and Ca:P molar ratio were determined using a similar method as mentioned previously in order to validate the fitted quadratic regression model for the precipitation of DCP. Additionally, to characterise the DCP, the relative solubility of P in water, 2% citric acid solution and alkaline ammonium citrate solution was determined as well as the micromineral content of the DCP. A method similar to the aforementioned one was employed in the extraction of P soluble in water while EC No. 2003/2003 methods 3.1.3 and 3.1.5.1 (EC, 2003) were used to extract P soluble in 2% citric acid and in alkaline ammonium citrate, respectively. The P of the extracts were determined using the ICP-AES method. The micromineral content of the DCP was determined using an ICP-MS. To conclusively identify the specific mineral phase of the precipitated DCP, XRD analysis was carried out as an additional characterisation of the nature of the DCP phase.

The Ca:P molar ratio of the precipitate obtained in experimental runs 13 and 30 (see Table B4) indicated the potential precipitation of octacalcium phosphate (OCP), a potentially high-value product. Thus, this was investigated further by altering the optimal operating conditions for the DCP precipitation such as using 25 °C instead of 75 °C for the reaction temperature and 1.2:1 instead of 0.95:1 for 1 M Ca(OH)$_2$:mineral liquor (v:v) ratio with a reaction time no longer than 17 minutes as reaction times longer than 17 minutes did not lead to increased yield (such as the case of DCP). The experiment was carried out in two 150 ml mineral liquor batches. Dry precipitate was obtained from the first batch after drying of crystals in an air drying oven to constant weight at 100 °C for 10 hours, and wet precipitate was obtained from the second batch after drying of crystals in an air drying oven at 100 °C for 5 hours. The precipitate yield (g dm), P and Ca contents (g kg^{-1} dm) and Ca:P molar ratio were determined. Furthermore, the dry and wet precipitate were characterised by FTIR to confirm whether that phase was indeed OCP.

4.2.6   Gelatin extraction from monkfish ossein

Optimisation of the gelatin extraction from monkfish ossein utilised a response surface design, namely CCD, to identify the operating conditions that maximised the hydroxyproline yield (%) in the gelatin extraction liquor. The CCD included three factors, namely extraction pH, extraction temperature (°C) and extraction time (min), and the design consisted of two level factorial points, namely -1 and 1, with an additional centre point at 0 and two level star points positioned at alpha value of -1.6818 and +1.6818 from the centre point. Briefly, these levels were -1.6818, -1, 0, 1,

and 1.6818, which corresponded to 3, 4, 5.5, 7 and 8 for the extraction pH; 50, 56, 65, 74 and 80 °C for the extraction temperature; and 25, 45, 75, 105 and 125 min for the extraction time, respectively. The factors and the levels utilised in the extraction of gelatin in the present study were chiefly adopted from Harris (1990). The CCD matrix for the three factors and five level settings is provided in Table 4.4 specifically for the gelatin extraction process at a fixed 1:10 ratio of ossein:water (w:v) and one extraction. The CCD with three factors contained 16 observations wherein two observations were at centre-point levels and the balance had a unique treatment or combination of factor level settings. The design was replicated once; thus, a total of 32 experimental runs were conducted. This design is outlined in Table 4.5 in a random order.

Table 4.4: Gelatin extraction process: CCD matrix for three factors and five level settings

Factors	Levels				
	-1.6818	-1	0	1	1.6818
	Actual values				
Reaction temperature, °C	50	56	65	74	80
Reaction time, min.	25	45	75	105	125
Reaction pH	3	4	5.5	7	8

For each experimental run, a 10 g frozen sample of monkfish ossein was added to 100 ml of demineralised water in a 400 ml glass beaker. The mixture was heated using a temperature-controlled water bath (Labotec Model 132, South Africa) until the required temperature was reached, namely 50, 56, 65, 74 or 80 °C, while mixing the mixture with an overhead mixer (DragonLab Model OS40-Pro, China) at 100 rpm to ensure complete suspension of ossein in the solution. Subsequently, while mixing was continued and the required temperature was maintained, 0.1 M H_3PO_4 or 0.1 M NaOH was added dropwise to the mixture until the required pH of the mixture, namely 3, 4, 5.5, 7 or 8, was reached and stabilised at pH level deviation of ± 0.05. The extraction time was initiated when the mixture was at the correct temperature and pH, and extraction took place for 25, 45, 75, 105 or 125 minutes while maintaining the required pH and temperature of the mixture. At the end of the extraction time, the mixture was vacuum filtered using an ash-free filter paper (Starlab, grade BIO-1-110) and then the volume of extracted gelatin liquor (as filtrate) was measured using a 100 ml graduated cylinder. Afterwards, the gelatin liquor was stored in the refrigerator at 4 °C and an aliquot of the gelatin liquor from 32 experimental runs was analysed for the hydroxyproline content. Thereafter, the hydroxyproline yield of each observation was calculated based on a previous study by Kołodziejska et al. (2008) with a slight modification (see Appendix D).

Table 4.5: CCD of experiments with three factors and one replicate in a random order for gelatin extraction from monkfish ossein

Experimental run	Extraction pH	Extraction temperature, °C	Extraction time, minutes
1	5.5	65	75
2	5.5	65	75
3	7.0	56	105
4	7.0	56	45
5	7.0	74	105
6	4.0	74	45
7	7.0	74	105
8	4.0	74	105
9	5.5	80	75
10	4.0	56	105
11	3.0	65	75
12	4.0	56	105
13	5.5	50	75
14	5.5	50	75
15	5.5	65	25
16	5.5	65	125
17	5.5	65	75
18	7.0	74	45
19	4.0	74	105
20	8.0	65	75
21	4.0	56	45
22	7.0	56	45
23	4.0	56	45
24	8.0	65	75
25	5.5	80	75
26	7.0	74	45
27	3.0	65	75
28	7.0	56	105
29	4.0	74	45
30	5.5	65	125
31	5.5	65	25
32	5.5	65	75

The protein content for each extracted gelatin liquor was determined by two methods: (1) nitrogen content determination by the Dumas method and (2) Bradford assay using lysozyme as standard. The nitrogen content was determined from a mixture of equal-volume aliquots of gelatin liquor from experimental runs with identical treatment conditions. The average of the protein content (using Bradford assay) and hydroxyproline content of gelatin liquor was also calculated for replicate experimental runs having identical treatment conditions. For the 32 experimental runs in the extraction of gelatin, there were 15 different treatments thereof (see Table A14). Hence, a

total of 15 mixtures of gelatin liquor were analysed for nitrogen content, and 15 average protein (using Bradford assay) and hydroxyproline contents were calculated.

The results for nitrogen content as measured by the Dumas method were given in the form of weight percentage; thus, the density of the gelatin liquor from each experimental run was determined in duplicate. Briefly, two aliquots of 1 ml gelatin liquor from each experimental run were separately weighed to the nearest 0.0001 g. Duplicate determination of the density was averaged and then multiplied by the total volume of the extracted gelatin liquor. Therefore, the equivalent weight of the extracted gelatin liquor for each experimental run was obtained. The average weight of the gelatin liquor was calculated for replicate experimental runs having identical treatment conditions. The protein content using nitrogen analysis from the Dumas method was calculated by multiplying the nitrogen content in the gelatin liquor by a factor of 5.55, according to Pomeranz and Meloan (1994). The protein content (as measured using Bradford assay) and the hydroxyproline content in the gelatin liquor were calculated by multiplying the protein concentration and hydroxyproline concentration of the extracted gelatin liquor, respectively, with the total volume of the extracted gelatin liquor.

A single validation run was carried out using 10 g frozen monkfish ossein and 100 ml demineralised water mixture, with pH 8, 80 °C and 90 min as the extraction pH, temperature and time, respectively. These were the optimum operating conditions for the extraction of gelatin from monkfish ossein reported in the results and discussion chapter (see Section 5.6); however, a shorter extraction time was utilised in a single validation run (from 125 min to 90 min). The hydroxyproline yield of the gelatin liquor was determined to validate the fitted quadratic regression model for the gelatin extraction from monkfish ossein. Furthermore, the hydroxyproline recovery in the gelatin liquor in mass was calculated relative to the hydroxyproline content of the monkfish ossein used on a dry mass basis, and it was expressed as mass percentage.

4.3 Analytical techniques

4.3.1 Proximate analysis

Proximate analysis such as moisture, ash, crude fat and crude protein contents was carried out based on AOAC Official method 934.01 (AOAC, 2002a), AOAC Official method 942.05 (AOAC, 2002b), a standard lipid extraction method (Lee et al., 1996), and AOAC Official Method 992.15 (AOAC, 1992), respectively. The crude protein was obtained from nitrogen analysis using the Dumas method in a nitrogen/protein determinator (LECO Model FP-528, USA). The nitrogen content was then multiplied by 6.25, which is traditionally used as a conversion factor in muscle foods (Nollet and Toldrà, 2009) or when the conversion factor of a specific protein is not known (FAO, 2003). The results were reported as the average of duplicate measurements.

4.3.2 Hydroxyproline analysis

Measurements of hydroxyproline (see Appendix C) in monkfish bones, monkfish ossein and gelatin liquor were determined based on AOAC Official method 990.26 (AOAC, 2005d). However, slight modifications were adopted for monkfish bones and ossein: (1) samples were dried for 270 minutes in an air drying oven at 100 °C before hydrolysis to eliminate any moisture variation in the samples and (2) triplicate measurements were determined to account for any hydroxyproline content variation in the samples. The hydroxyproline content variation in the samples can be ascribed to the unequal spatial distribution of collagen in tissue (Nollet and Toldrà, 2009). In addition to this, there were contributing factors to the hydroxyproline content variation in the samples that were beyond the control of the present study. Firstly, a bigger fish usually has a higher hydroxyproline content than a smaller one (Ministry of Fisheries, and Institute of Marine Research, 1995), and in South Africa, all size classes of monkfish are exposed to fishing (Walmsley et al., 2005). Secondly, a starving fish with poor diet produces more collagen than a well-fed fish (Silva et al., 2014), and in South Africa, an average of 43.15% of monkfish sampled had stomachs containing food while the stomachs of 56.85% were empty (Walmsley et al., 2005). A study of monkfish on the Agulhas Bank, South Africa, also showed that the stomachs of a large percentage of samples were empty (Griffiths and Hecht, 1986).

The hydroxyproline content of the hydrolysed test sample solution was determined by means of measuring the absorbance at 558 nm wavelength in a UV Vis Spectrophotometer (AandE Lab Model AE-S60-4U, UK). The equivalent hydroxyproline concentration was calculated using the fitted linear equation derived from the linear standard curve (see Figure A2) of duplicate absorbance measurements for nine different concentrations of trans-4-Hydroxy-L-proline standard (≥ 99% purity, Sigma-Aldrich). This equation is as follows:

$$Hydroxyproline\ concentration, \frac{\mu g}{ml} = \frac{Absorbance - 0.0017}{0.2002} \qquad \text{(Equation 4.1)}$$

The hydroxyproline contents of the monkfish bones and ossein and the gelatin liquor were reported as the average of the triplicate and duplicate measurements, respectively.

4.3.3 Major and trace elements analysis

Duplicate measurements of major elements, Ca and P, were determined using an ICP spectrometer (ThermoFisher Scientific Model iCAP 6200 Duo, England). Preparation of the DCP precipitate sample for the total P was based on AOAC Official method 957.02 (AOAC, 2005b). The extraction of P soluble in water was based on AOAC Official method 977.01 (AOAC, 2005c) and soluble in 2% citric acid and alkaline ammonium citrate solutions was based on EC no. 2003/2003 methods 3.1.3 and 3.1.5.1 (EC, 2003), respectively. The results were reported as the average of duplicate measurements of P and Ca.

Trace elements were analysed using an Agilent 7700 quadrupole ICP-MS. Samples were introduced via a 0.4 ml/min micromist nebuliser into a Peltier cooled spray chamber at a temperature of 2 °C with a carrier gas flow of 1.05 L/min. The elements vanadium (V), nickel (Ni), Cr, Mn, Fe, Co, Cu, Zn, As and Se were analysed under He-collision mode to remove polyatomic interferences while other elements used no-gas mode. The results were reported as the average of triplicate measurements of trace elements.

4.3.4 Particle characterisation analysis

The phase and crystallinity of the DCP sample were evaluated by an X-ray powder diffractometer (Bruker Energy Dispersive 1-D Detector and D2 Phaser) using copper tube as a source with 1.54184 Å wavelength over a 2Ө range of 4-40° at 0.18185° step increments. The infrared spectra of the OCP sample were measured with a NEXUS 630 FTIR instrument by ThermoNicolet instruments. The infrared spectra were recorded in reflectance mode using the Golden Gate diamond ATR from Thermo-Fischer equipped with ZnSe lenses. Finely powdered material of the solid samples was placed on the horizontal ATR. Duplicate spectra of each sample using 16 scans with a resolution of 4 cm^{-1}, representing data point spacing of just under 2 cm^{-1}, were accumulated. The final format of the spectra was in absorbance over the range 4 000-650 cm^{-1}. The spectrometer, equipped with a Ge-on-KBr beam splitter and DTGS/CsI detector, was not purged with HP nitrogen gas during recording. The operating and data manipulating software was the OMNIC (Version 7) package.

4.3.5 Gelatin liquor protein analysis

The protein content in the gelatin liquor was calculated using the Dumas method in a nitrogen/protein determinator (LECO Model TruSpec CN, USA). The nitrogen result was then multiplied by 5.55, which is traditionally used as a conversion factor for gelatin protein (Pomeranz and Meloan, 1994), to calculate the protein content. Using the linearised Bradford assay (Zor and Selinger, 1996), the protein content in the gelatin liquor was determined by means of measuring the absorbance at 590 and 450 nm wavelengths in a UV Vis Spectrophotometer (AandE Lab Model AE-S60-4U, UK). The equivalent protein concentration was calculated using the fitted linear equation (see Figure A3) derived from the linear standard curve of duplicate absorbance measurements for three different concentrations of lysozyme standard (~70 000 U/mg, Sigma-Aldrich). This equation is as follows:

$$Protein\ concentration, \frac{\mu g}{ml} = \frac{Absorbance,\ \frac{590}{450}nm - 0.853}{0.0488}$$

(Equation 4.2)

The results of both assays were reported as the average of duplicate measurements.

4.4   Statistical analysis

All experimental designs, namely the two-level full factorial design for the demineralisation of monkfish bones and CCD for the DCP precipitation and gelatin extraction, were generated using STATISTICA version 12 statistical software (StatSoft). All data obtained from experiments were also further analysed using this software package. All effects with a probability value of $p < 0.05$ were considered significant.

4.4.1   Demineralisation of monkfish bones

The analysis of variance (ANOVA) was used to identify main and interaction effects, and the magnitude of regression coefficients. These estimates provided regression coefficients to build a linear regression equation that related the response to the factor. Furthermore, the set of conditions or levels of the factors (optimal conditions) that provided minimised ash content and maximised hydroxyproline content in the monkfish ossein was determined using the response desirability analysis (StatSoft).

4.4.2   Precipitation of DCP from mineral liquor

The ANOVA using CCD provided an estimate of the quadratic effect and main and interaction effects of the three factors at a significance level of 0.05. A second-order regression equation was fitted to the observed response values with the corresponding treatments, which enabled prediction of the response from the given factor level settings. Thereafter, the desirability profiling (StatSoft) for the three responses was established based on a maximised yield and P content and a Ca:P molar ratio of approximately one. As a result, the set of levels (optimal conditions) for the DCP precipitation process that produced maximum yield of DCP crystals (at a Ca:P molar ratio of one) with highest possible P content was determined.

4.4.3   Gelatin extraction from monkfish ossein

The ANOVA using CCD provided an estimate of the quadratic effect and main and interaction effects of the three factors on the experimental response using a significance value of 0.05. A second-order regression equation was then fitted to the observed hydroxyproline response values with the corresponding treatments, which enabled prediction of the hydroxyproline response from the given factor level settings. Thereafter, the desirability profiling (StatSoft) for the three responses was established based on a maximised hydroxyproline yield. As a result, the set of levels (optimal conditions) for the extraction of gelatin from monkfish ossein that produced maximum hydroxyproline yield was determined.

Chapter 5
Results and discussion

5.1   Minced monkfish heads hydrolysis

Enzymatic hydrolysis of minced monkfish heads yielded 97.0 ± 10.5 g kg^{-1} wet weight of monkfish bones, and the moisture in the bones comprised 672.1 ± 1.4 g kg^{-1}. A significant amount of moisture was found in the bone residues as the excess water contained therein was only allowed to drip off. On a dry mass basis, the bone yield of minced monkfish heads was 31.8 ± 3.5 g dry bone material per kg wet starting material.

5.2   Monkfish bones proximate and hydroxyproline analyses

Proximate analysis of the homogenised monkfish bones revealed that it contained 464.8 g kg^{-1} moisture, 372.9 g kg^{-1} ash, 3.1 g kg^{-1} fat and 156.3 g kg^{-1} crude protein. The moisture content of the homogenised bones, from which the excess water had been removed by hand squeezing, was lower compared to the moisture content of the bone residues from enzymatic hydrolysis of heads. Based on the fat data, monkfish can be classified as a lean species, which is in agreement with prior findings according to which a fat content of 7.0 g kg^{-1} was seen in monkfish 'sawdust' waste collected from a filleting factory (Batista, 1999). In dry mass, ash or the mineral component of the monkfish bones constituted 696.8 g kg^{-1}, which is within the typical range of 600.0–700.0 g kg^{-1} dm inorganic fraction in the bone material (Kim, 2012; Gildberg et al., 2002) while protein represented 292.0 g kg^{-1} dm, which is mainly collagen (Boskey, 2013). However, fish species bones ash content reported in the literature (Toppe et al., 2007) varies from 222.2 to 573.7 g kg^{-1} dm, which is generally lower than the monkfish bones ash content, whereas the protein content ranges from 273.38 to 446.63 g kg^{-1} dm.

The hydroxyproline content of monkfish bones was found to be 20.2 g kg^{-1} dm. This value is higher compared to that for bigeye snapper, rainbow trout, blue whiting, salmon and brown trout bones: 15.1 g kg^{-1} (Kittiphattanabawon et al., 2005), 16.9 g kg^{-1} (Tabarestani et al., 2012), 13.0 g kg^{-1}, 17.2 g kg^{-1} and 18.9 g kg^{-1} dm (Toppe et al., 2007), respectively. This variation was attributed to the different fish species and the habitat temperature of the fish (Eastoe, 1957; Jongjareonrak et al., 2005; Kittiphattanabawon et al., 2005; Mahboob, 2015). The hydroxyproline content of monkfish bones in the current study is similar to that of cod and saithe bones: 20.2 g kg^{-1} and 21.3 g kg^{-1} dm (Toppe et al., 2007), respectively. This indicates that similar levels of hydroxyproline could be measured from the bones of lean and coldwater fish species.

5.3   Demineralisation of monkfish bones

The analysis of variance (see Table B1) indicated that H$_3$PO$_4$ concentration, number of extraction and ratio of solution (v) to raw material (w) had statistically significant effects ($P < 0.05$) on ash and

hydroxyproline contents in monkfish ossein. The pure quadratic curvature (c) is also significant. The fitted linear regression equations for ash and hydroxyproline contents responses are as follows:

$$Ash\ content, g\ kg^{-1}\ dm =\ 1696.8 – 235.2c – 180.0x – 185.5y – 147.1z\ + \ 6.5xyz \qquad \text{(Equation 5.1)}$$

$$Hydroxyproline\ content, g\ kg^{-1}\ dm =\ -54.5\ +\ 22.0c\ +\ 11.0x\ +\ 11.2y\ + 10.4z\ – 0.2xyz \qquad \text{(Equation 5.2)}$$

Where c = 1 if the point is a centre point and 0 if it is otherwise

x = H_3PO_4 concentration, % v/v

y = Number of extractions

z = Ratio of solution (v) to raw material (w)

The residual analysis of these models is satisfactory, wherein the points on the residual plot correspond to a straight line, reinforcing the model adequacy (see Figure B1). Furthermore, these two models explain about 80% and 97% of the variability in the ash and hydroxyproline content responses, respectively, based on the adjusted coefficient of determination, R^2. As an additional support to the model adequacy, the residual error in terms of lack of fit for these two models (see Table B2 and Table B3) when compared to the pure error is statistically insignificant ($P > 0.05$).

The response surface plots of the two factors at a time while holding the third factor at a fixed level (middle level) for ash and hydroxyproline content responses are depicted in Figure 5.1 and Figure 5.2, respectively. The examination of the response surface plots indicates that the minimised ash content and maximised hydroxyproline content in monkfish ossein are achieved at high levels of H_3PO_4 concentration, number of extraction and ratio of solution (v) to raw material (w): these values are 5% v/v, four extractions and a solution:raw material ratio of 5:1, respectively. In fact, a desirable monkfish ossein (see Figure B2) with minimised ash and maximised hydroxyproline contents is attained at high level settings as an optimal condition of the three investigated factors. Therefore, at optimal conditions, the model prediction intervals are -187.5 to 133.7 g kg^{-1} dm and 68.7 to 83.0 g kg^{-1} dm for ash and hydroxyproline contents, respectively of the final mass of the monkfish ossein. The negative values for the ash content were calculated using the model (see equation 5.1) for the ash content response. The equation contains high negative coefficient estimate values, which were calculated using the ANOVA, resulting in a potential negative value to the predicted ash response. However, an actual experimental run should yield a monkfish ossein containing a minimum of 0 g kg^{-1} dm of ash. Using similar optimal conditions, the actual experimental results repeated twice yielded a monkfish ossein of 2.8 g kg^{-1} dm ash and 69.7 g kg^{-1} dm hydroxyproline contents of the final mass of the monkfish ossein. The small content of ash and the highest possible content of hydroxyproline in the final mass of the monkfish ossein have shown a successful demineralisation of monkfish bones using the optimal conditions. Thus, a significant portion of the minerals contained in the monkfish bones was recovered in the mineral liquor, and the ossein, containing 69.7 g kg^{-1} dm hydroxyproline, is a suitable substrate for gelatin extraction.
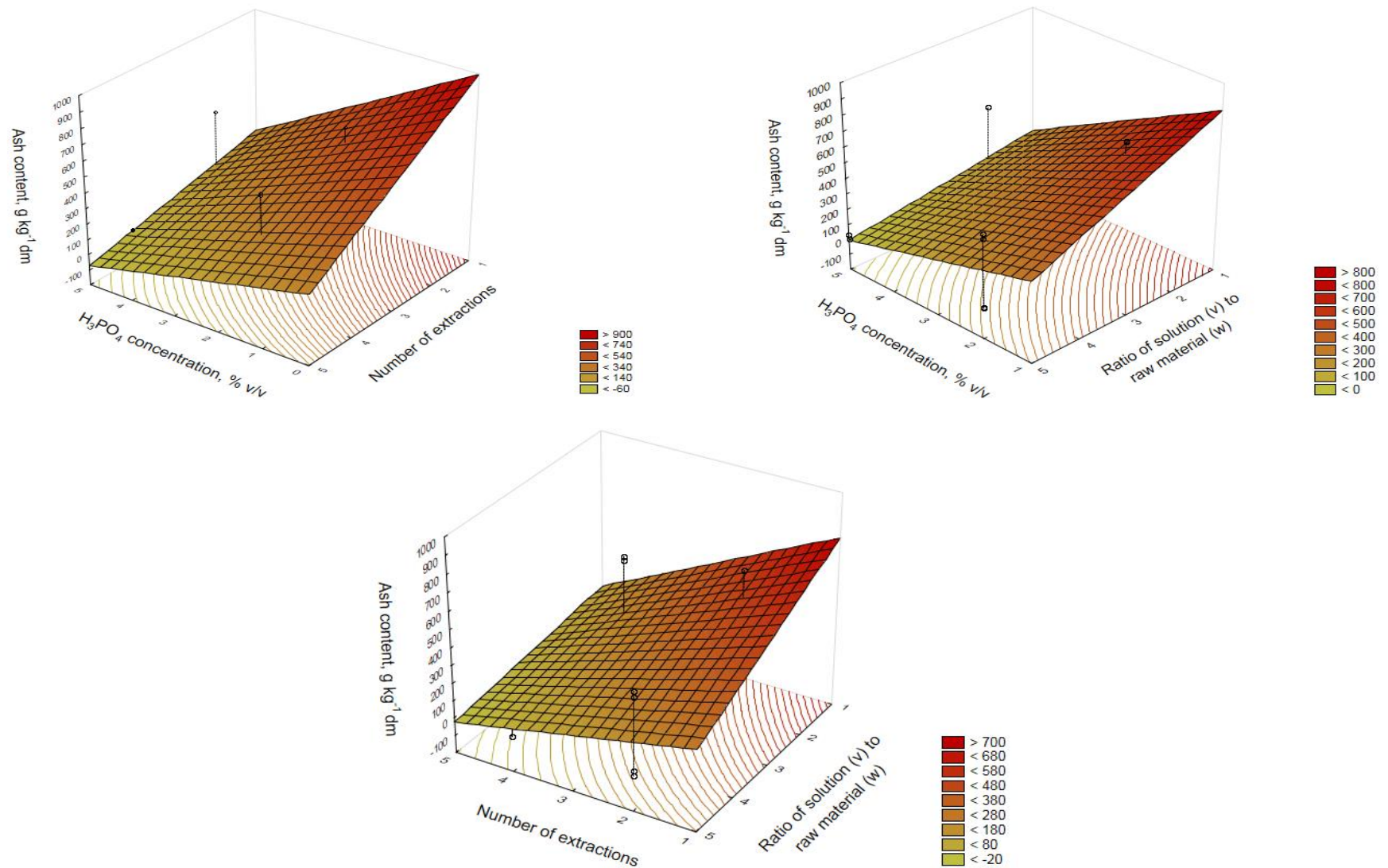
Figure 5.1: Surface plots for the ash content in the monkfish ossein response variable
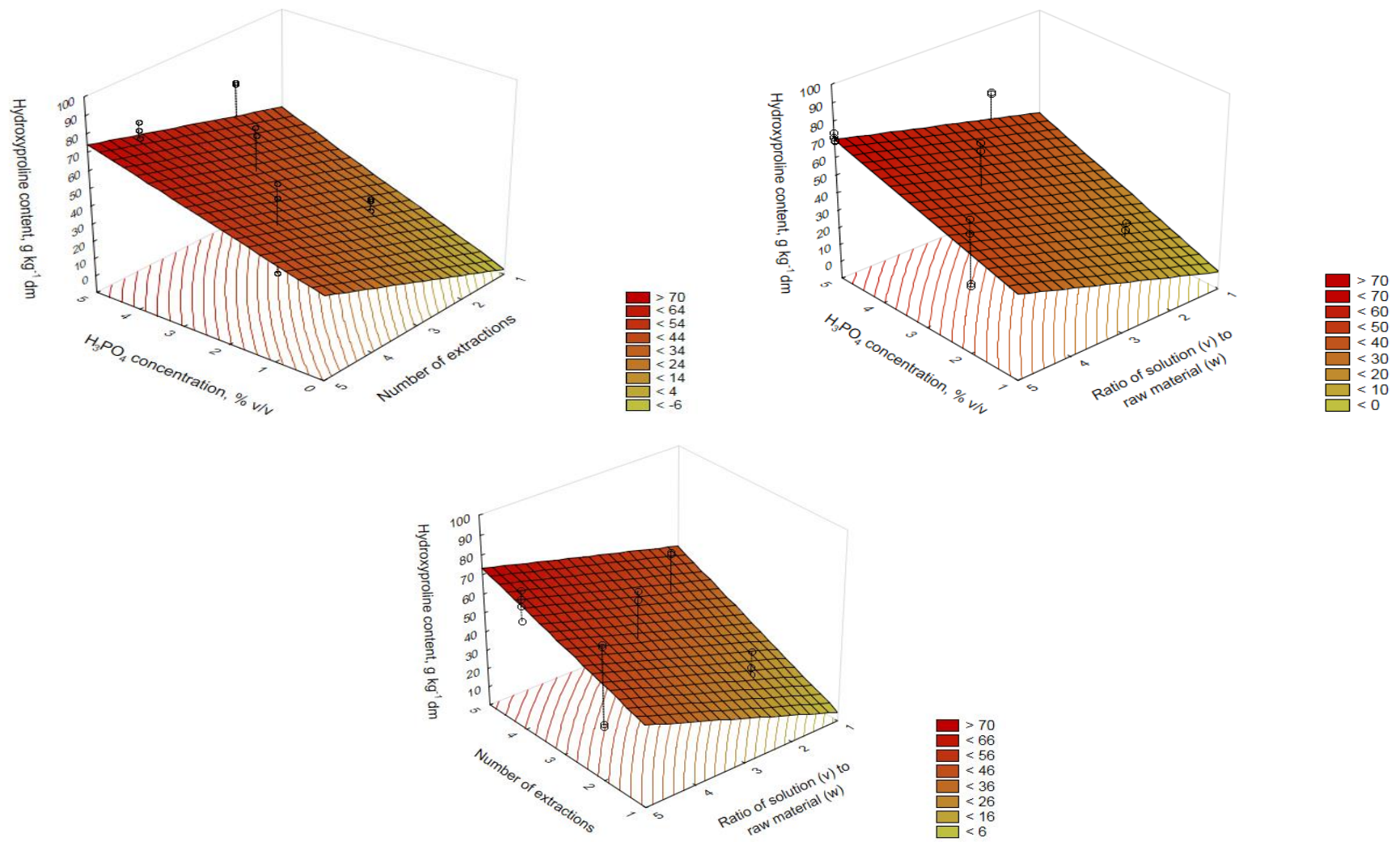
Figure 5.2: Surface plots for the hydroxyproline content in the monkfish ossein response variable

5.4 Precipitation of dicalcium phosphate from mineral liquor

5.4.1 Statistical optimisation

The complete ANOVA for this experiment is summarised in Table 5.1. It shows that the yield and Ca:P molar ratio of the precipitated DCP are both significantly affected ($P < 0.05$) by the linear and quadratic effects of reaction temperature and 1 M Ca(OH)$_2$:mineral liquor (v:v) ratio while reaction times longer than 17 minutes do not lead to increased yield. The linear interaction of these two factors is also significant. It is indicated in the ANOVA that the following has a significant influence ($P < 0.05$) on the P content of the precipitated DCP: (1) linear and quadratic effects of reaction temperature; (2) linear effect of 1 M Ca(OH)$_2$:mineral liquor ratio (v:v); (3) linear interaction of reaction temperature with 1 M Ca(OH)$_2$:mineral liquor ratio (v:v); and (4) linear interaction of reaction temperature with reaction time. However, the latter effect represents the higher order term and contains lower order terms thereof, namely reaction temperature and reaction time. Hence, in order to satisfy the hierarchy principle (Montgomery, 2000), the linear effect of reaction time (nonsignificant term) is incorporated into the prediction model of P content as response variable.

The fitted quadratic regression equations for the yield, P content and Ca:P molar ratio as the response variables of the precipitated DCP are as follows:

$$Yield, g \ = \ -21.7 \ - \ 0.003u^2 \ + \ 0.4u \ - \ 5.0 \ v^2 \ + \ 53.3v \ - \ 0.3uv \qquad \text{(Equation 5.3)}$$

$$P \ content, g \ kg^{-1} \ dm \ = \ 270.5 \ + \ 0.03u^2 \ - \ 2.1u \ - \ 27.4v \ - \ 0.3w \ - \ 0.9uv \ + \ 0.008uw \qquad \text{(Equation 5.4)}$$

$$Ca{:}P \ molar \ ratio \ = \ 1.2 \ - \ 0.00004u^2 \ + \ 0.002u \ + \ 0.8v^2 \ - \ 1.0v \ + \ 0.002uv \qquad \text{(Equation 5.5)}$$

Where u = Reaction temperature, °C

v = 1 M Ca(OH)$_2$:mineral liquor (v:v)

w = Reaction time, minutes

Table 5.1: ANOVA for the yield, P content and Ca:P molar ratio response variables in the precipitation of DCP from mineral liquor

Sources of variation	Sum of squares	DF[a]	Mean square	F-value	P-value
DCP yield					
(u) Reaction temperature, °C	14.739	1	14.739	36.315	0.000014
(u^2) Reaction temperature, °C	7.353	1	7.353	18.116	0.000533
(v) 1 M Ca(OH)$_2$:mineral liquor (v:v)[b]	2 618.035	1	2 618.035	6 450.418	0.000000
(v^2) 1 M Ca(OH)$_2$:mineral liquor (v:v)[b]	4.469	1	4.469	11.011	0.004066
uv Interaction	23.717	1	23.717	58.435	0.000001
Lack of fit	11.464	9	1.274	3.138	0.020296
Pure error	6.900	17	0.406		
Total sum of squares	2 684.121	31			
P content					
(u) Reaction temperature, °C	6 135.05	1	6 135.05	423.8118	0.000000
(u^2) Reaction temperature, °C	1 261.49	1	1 261.49	87.1441	0.000000
(v) 1 M Ca(OH)$_2$:mineral liquor (v:v)[b]	12 463.88	1	12 463.88	861.0102	0.000000
(w) Reaction time, minutes	13.66	1	13.66	0.9438	0.344912
uv Interaction	252.02	1	252.02	17.4093	0.000638
uw Interaction	128.26	1	128.26	8.8600	0.008467
Lack of fit	782.87	8	97.86	6.7602	0.000487
Pure error	246.09	17	14.48		
Total sum of squares	21 284.02	31			
Ca:P molar ratio					
(u) Reaction temperature, °C	0.002184	1	0.002184	13.0275	0.002164
(u^2) Reaction temperature, °C	0.001325	1	0.001325	7.9013	0.012026
(v) 1 M Ca(OH)$_2$:mineral liquor (v:v)[b]	0.133623	1	0.133623	797.0516	0.000000
(v^2) 1 M Ca(OH)$_2$:mineral liquor (v:v)[b]	0.117196	1	0.117196	699.0630	0.000000
uv Interaction	0.001056	1	0.001056	6.3004	0.022480
Lack of fit	0.002500	9	0.000278	1.6570	0.176819
Pure error	0.002850	17	0.000168		
Total sum of squares	0.279697	31			

Notes:

[a] = degrees of freedom

[b] = volume/volume

The adequacy of the three fitted models is supported by the residual analyses of the three response variables (see Figure B3). The points on the normal probability plot of the residuals for each response lie close to a straight line, suggesting that error terms are normally distributed. Further, 99% of the variation on the yield response and 94% of the variation on the P content response are explained by the fitted quadratic models for the yield and P content, respectively, although the residual error in terms of lack of fit for these two models when compared to the pure error is statistically significant ($P < 0.05$). This indicates that there is still some statistically significant variability that is not fully captured in the current model. For the Ca:P molar ratio response, 98% of the variation is explained by the fitted quadratic model for the Ca:P molar ratio and as an additional support to the model adequacy, the residual error in terms of lack of fit relative to the pure error is statistically insignificant ($P > 0.05$).
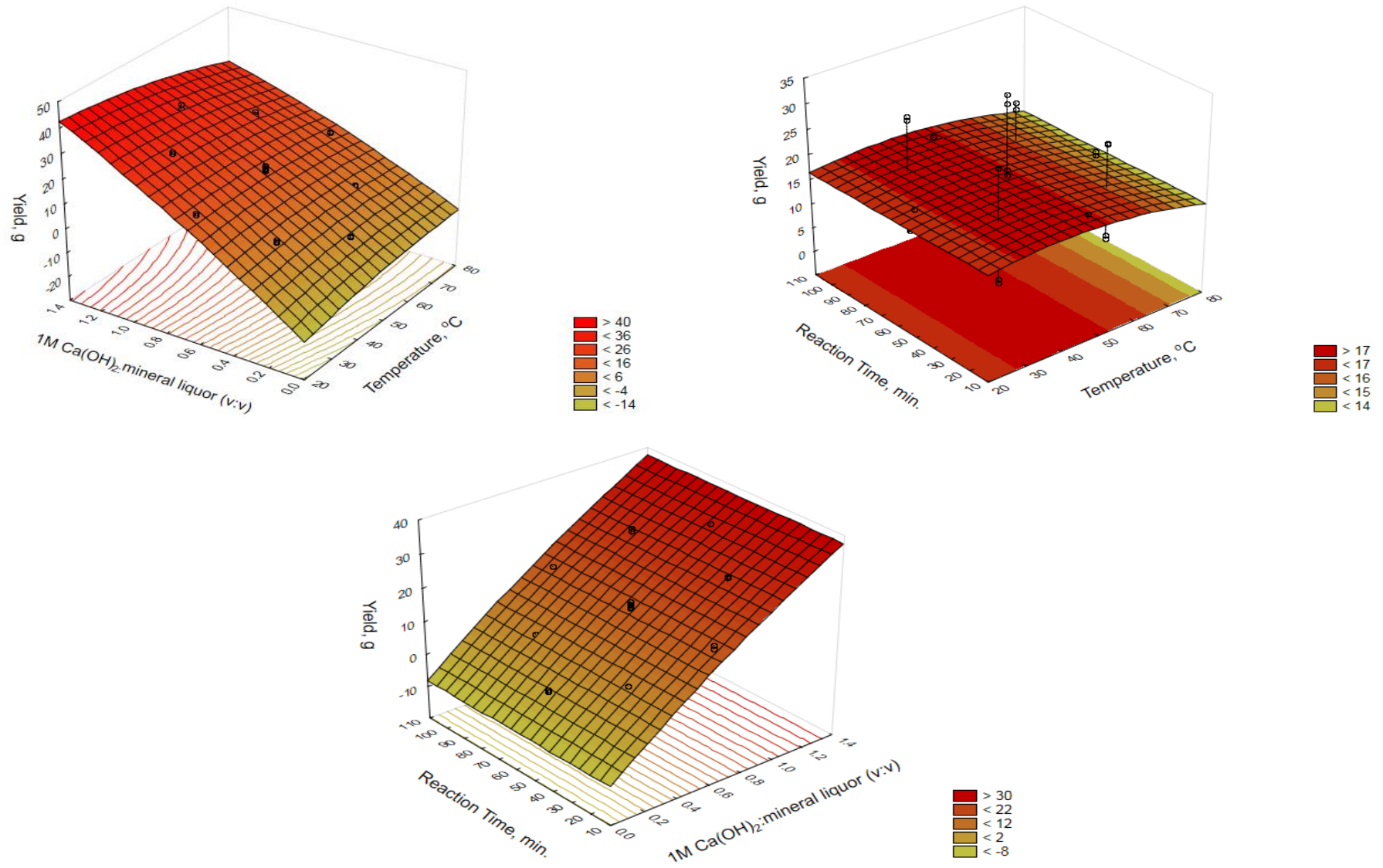
Figure 5.3: Surface plots for the yield response variable of the precipitated DCP from mineral liquor
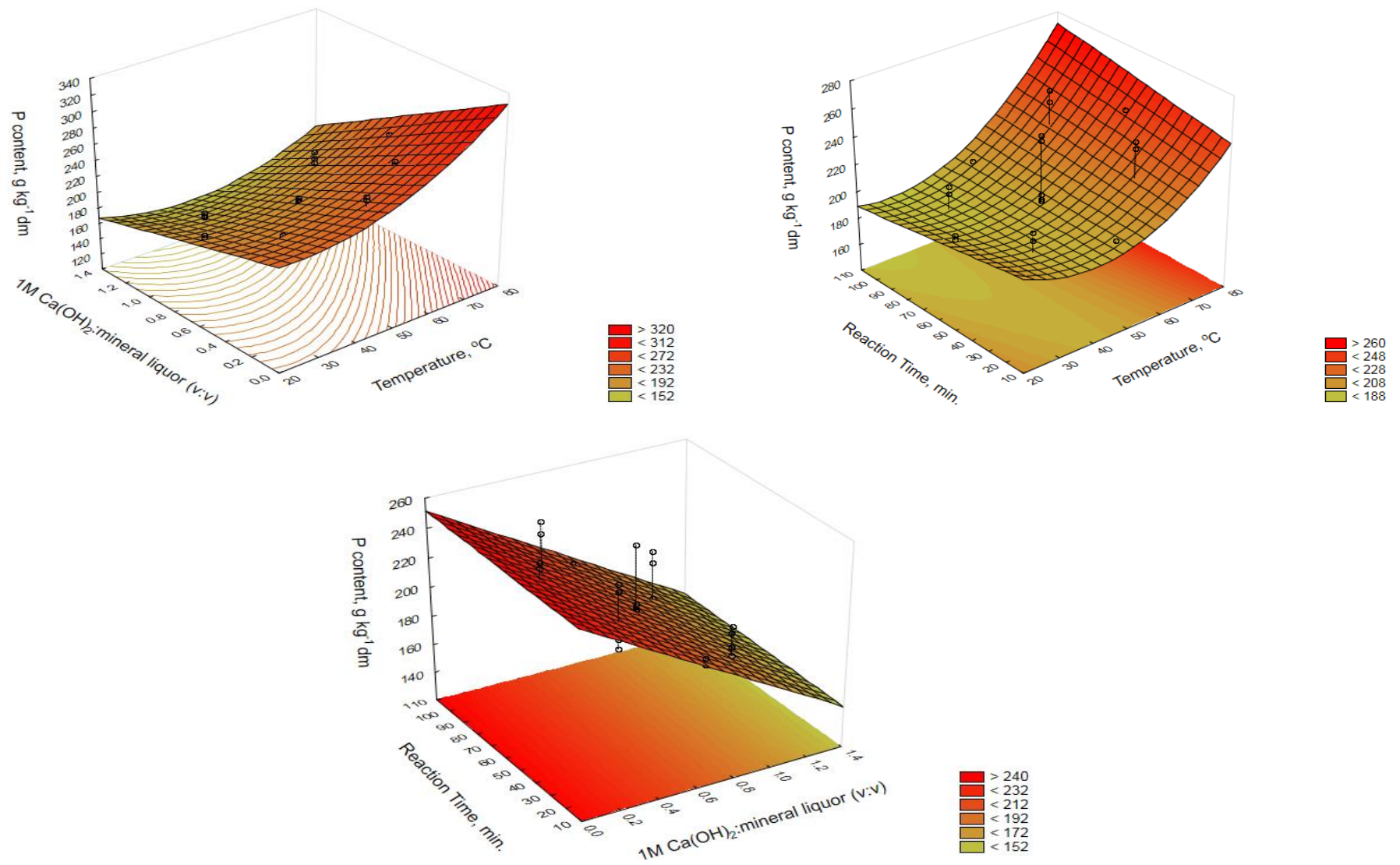
Figure 5.4: Surface plots for the P content response variable of the precipitated DCP from mineral liquor
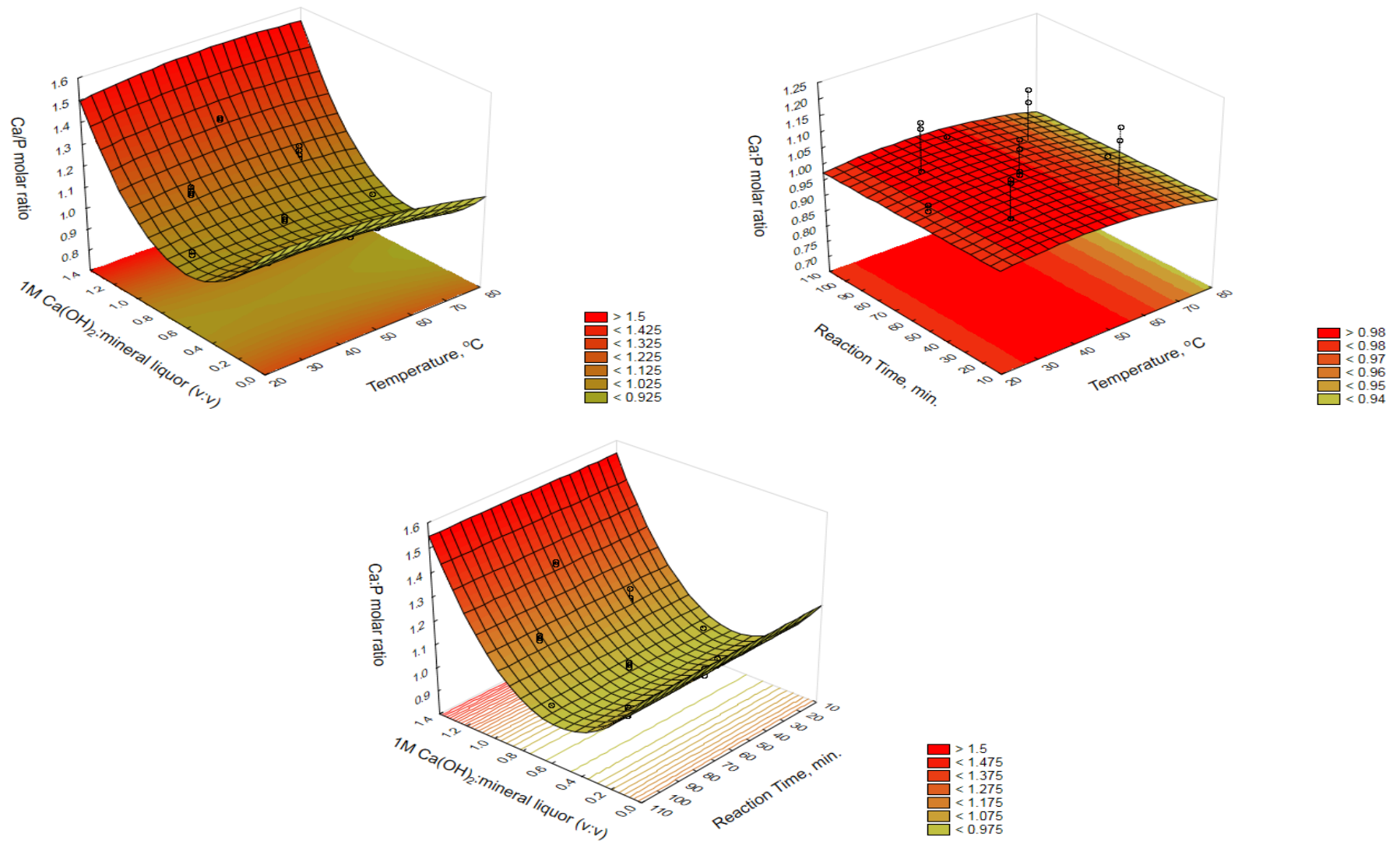
Figure 5.5: Surface plots for the Ca:P molar ratio response variable of the precipitated DCP from mineral liquor

Inspection of the yield response surface plots in Figure 5.3 indicates that the maximum yield of DCP is attained at a high level of 1 M $Ca(OH)_2$:mineral liquor ratio (v:v), regardless of the level of the reaction time, and preferably at a low to middle level of the reaction temperature. In like manner, based on the P content response surface plots in Figure 5.4, the maximum P content of DCP is attained at a low level of 1 M $Ca(OH)_2$:mineral liquor ratio (v:v) at any level of the reaction time and suitably at a high level of the reaction temperature. However, high levels of reaction temperature and time produce maximum P content of DCP. Furthermore, the Ca:P molar ratio response surface plots in Figure 5.5 show that DCP with a Ca:P molar ratio of 1 is attained at reaction temperature and a 1 M $Ca(OH)_2$:mineral liquor ratio (v:v), both ranging from middle to high levels, irrespective of the level of the reaction time. In summary, the optimal combination of level settings varies for each individual response, but the most desirable for the overall responses (see Figure B4) that maximises the yield and P content of DCP with a Ca:P molar ratio of 1:1 is achieved at the following optimal operating conditions: 75 °C reaction temperature and 1 M $Ca(OH)_2$:mineral liquor ratio (v:v) of 0.95:1 at any level of the reaction time. Since the reaction time factor for the yield and Ca:P molar ratio is not included in the model, it is practical to specify a reaction time no longer than 17 minutes as reaction times longer than 17 minutes do not lead to increased yield.

Therefore, at optimal conditions the model prediction intervals were 18.9 to 22.2 g dm of DCP precipitate per 150 ml starting mineral liquor, and the precipitate contained 211.9 to 231.2 g kg^{-1} dm P and 1.04 to 1.10 Ca:P molar ratio. Applying the same optimal conditions, the actual validation experiment resulted in 21.1 g dm of DCP precipitate per 150 ml starting mineral liquor, and the precipitate contained 215.2 g kg^{-1} dm P and 1.16 Ca:P molar ratio. Following separation of the precipitated DCP, 219.5 ml spent liquor was obtained containing 1.5 mg/ml P and 0.3 mg/ml Ca.

The process developed to precipitate DCP in the current study resulted in 93.4% and 99.0% recovery of total Ca and P inputs, respectively and a by-product (spent liquor) that potentially contains mainly water and very low levels of Ca and P. The total Ca and P inputs were obtained from 150 ml starting mineral liquor containing 32.4 mg/ml P and 8 mg/ml Ca and from 142.5 ml 1 M $Ca(OH)_2$, that was added to the starting mineral liquor based on 0.95:1 1 M $Ca(OH)_2$:mineral liquor ratio (v:v), containing 40 mg/ml Ca. According to McArthur and Spalding (2004), the chemical reaction of H_3PO_4 and $Ca(OH)_2$ results in precipitation of calcium phosphate salt such as DCP and water as a by-product. Thus, the addition of 142.5 ml 1 M $Ca(OH)_2$ to the 150 ml starting mineral liquor, mainly containing H_3PO_4, resulted in DCP precipitate and water as by-product.

5.4.2 Dicalcium phosphate product characterisation

Although the Ca:P molar ratio of the precipitated DCP in the current study using the optimal conditions exceeds the upper prediction interval by about 0.06, the DCP crystals were identified

specifically as DCPD (CaHPO$_4$•2H$_2$O; the International Union of Pure and Applied Chemistry [IUPAC] name is calcium hydrogen orthophosphate dihydrate; the mineral brushite) using XRD as depicted in Figure 5.6. The intense peak in the XRD pattern becomes apparent at the (020) experimental line for brushite (Jamroz et al., 2011), and the high intensity peaks at 2Ө angles of 11.8, 21.2, 29.5, 30.5 and 34.5° also indicate the formation of brushite as the main crystal structure (Li and Weng, 2007).
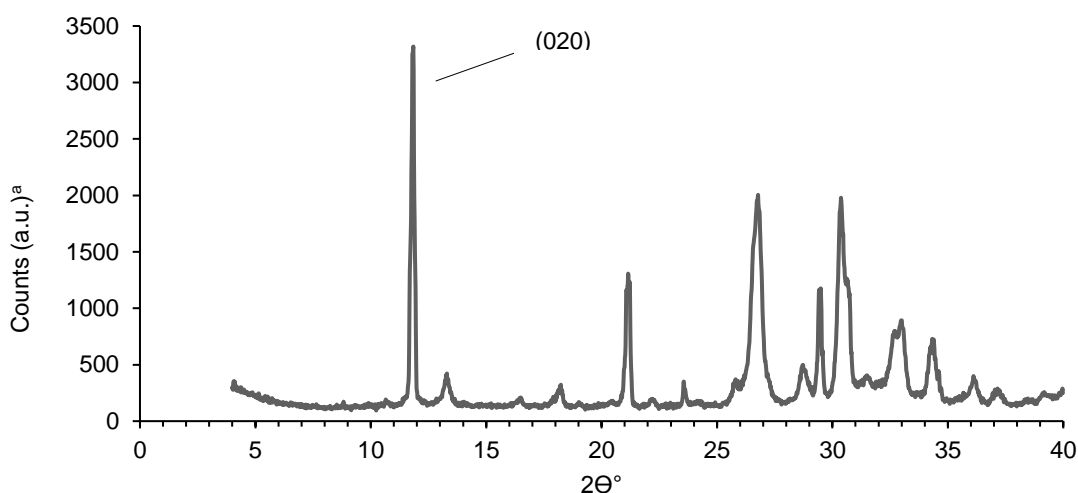
Figure 5.6: XRD patterns of the precipitated DCPD (CaHPO$_4$•2H$_2$O; the IUPAC name is calcium hydrogen orthophosphate dihydrate; the mineral brushite)
 Note: [a] = arbitrary unit

The precipitated DCPD in the current study contains 215.20 g kg^{-1} dm P, which is higher compared to that reported in the literature: 181 g kg^{-1} dm for DCPD (Rao, 2004), 182.93 g kg^{-1} dm for DCPD and an average of 195.45 g kg^{-1} dm for DCP samples originating from Brazil (Lima et al., 1999) and a range of 139.50 to 235.40 g kg^{-1} on a wet weight basis for DCP and DCPD samples originating from Polish and European factories (Jamroz et al., 2011). The fraction of total P in the precipitated DCPD that was soluble in water was only 3.29%. Furthermore, each of the 32 experimental runs for the precipitation of DCP resulted in a precipitate with relative solubility of P in water ranging from 1.54 to 6.02% of the total P. DCP is sparingly soluble in water, as corroborated by previous studies with water-soluble P that ranged from 0.9 to 11.2% of the total P (Jamroz et al., 2011; Lima et al., 1999; Mackay et al., 1980; Mullins et al., 1995).

Besides water-soluble P, the portion of total P in the precipitated DCPD that is soluble in 2% citric acid solution and alkaline ammonium citrate solution was also tested. The results are presented in Table 5.2 along with the results for the DCP or DCPD indicated in the literature and produced by commercial manufacturers. It should be noted that the neutral ammonium citrate solution is typically used in the literature to evaluate all phosphates but that alkaline ammonium citrate (Petermann's solution) is used specifically for evaluation of DCPD as per the International Fertilizer Development Center and the United Nations Industrial Development Organization

(1998). The solubility in 2% citric acid solution provides an estimate of the P bioavailability thereof (Jamroz et al., 2011; Jamroz et al., 2012; Sullivan et al., 1992; IFP CEFIC, 2016). Ideally, the P content in the feed supplement should be 100% bioavailable to animals. A solubility of greater than 95% in both solutions is a strong indication of good-quality inorganic P feedstuff (IFP CEFIC, 2016). The portion of P soluble in 2% citric acid solution for the DCPD in the current study is notably higher compared to the minimum level reported in the literature. It is also comparable to the maximum level reported in the literature and to the values for the commercial DCP or DCPD. Although scant information is provided in the literature for the relative solubility of P in alkaline or neutral ammonium citrate for the DCP product, the DCPD in the current study has a value on a par with the DCP animal feeds originating from Polish and European factories reported by Jamroz et al. (2011). The relative solubility of P in 2% citric acid solution for the precipitated DCPD was determined to be 95.1%, which is an estimate of the P bioavailability and an indication of a potential good-quality inorganic P feed supplement. However, *in vivo* evaluation of the DCPD in the current study is essential to determine the true P bioavailability thereof.

Table 5.2: Comparison of relative solubility of P in 2% citric acid and alkaline or ammonium citrate solutions for DCP or DCPD in the current study, literature and industries

Product	Relative solubility of P (%)		Reference
	in 2% citric acid solution	in alkaline or neutral ammonium citrate	
DCPD	95.1	85.2	Current study
DCP	86.0–98.0	NG[a]	Lima et al., 1995
DCP	51.0–98.0	70.0–85.0	Jamroz et al., 2011
DCP	99.0	99.0	Aliphos, 2014
DCPD	96.0	NG[a]	Yara Animal Nutrition South Africa, 2016
DCPD	90.0	NG[a]	Yichang Municipal Pacific Chemicals Co., Ltd., 2014

Note:

[a] = not given

The precipitated DCPD in the current study is also a source of other essential microminerals besides P and Ca and contains very low levels of harmful heavy metals and nonessential metals. All these constituents and their corresponding levels are summarised in Table 5.3, and the values were found to be lower than the maximum tolerable levels in animal feeds as set by the National Research Council (2005). The levels of the potentially harmful elements are significantly below the maximum acceptable limits set by the EU (EC, 2002): 34.09 mg kg^{-1}, 2.27 mg kg^{-1}, 11.36 mg kg^{-1} and 0.57 mg kg^{-1} in dry mass for Pb, Cd, As and Hg, respectively. Nonessential metals such as antimony (Sb) and beryllium (Be) in the precipitated DCPD are present in minute quantities. Sb in the form of antimony trioxide and pentoxide was tested on dogs and cats at doses above 100 ppm for months without toxic manifestations although in humans the estimated average daily intake of Sb from food and water is 4.6 µg per day (Nordberg et al., 2015), and as food colour additive, Sb should not be more than 2 ppm (CFR, 2016b). The oral reference dose for Be is set

at 0.002 mg kg^{-1} per day (Agency for Toxic Substances and Disease Registry, 2002). Furthermore, the Ca:P ratio on a dry mass basis of the precipitated DCPD is 1.5, which is within the recommended target ratio of 1.5 to 2 for animal feeds (Tisch, 2006). This is significant for adequate phosphorus absorption by animals in conjunction with Ca intake.

Table 5.3: Essential macro- and microminerals, potentially harmful elements and nonessential metals composition of the precipitated DCPD

Elements	Quantity
Essential macro minerals (g kg^{-1} dry mass)	
P	215.2
Ca	323.9
Essential micro minerals (mg kg^{-1} dry mass)	
Mg	1900.0
S	1660.0
Si	515.4
Sr	350.7
Na	195.6
Fe	190.3
Al	163.3
Mn	92.1
K	42.7
Zn	37.5
Ba	23.7
B	5.2
V	4.1
Cr	2.7
Ni	0.8
Cu	0.7
Co, Mo, Se, Li	< 0.02
Potentially harmful elements (mg kg^{-1} dry mass)	
Pb	1.9
Cd	0.3
As	0.2
Hg	0.02
Nonessential metals (mg kg^{-1} dry mass)	
Sb	1.4
Be	< 0.02

5.5   Precipitation of octacalcium phosphate from mineral liquor

Two experimental runs with the same treatment in the precipitation of DCP from mineral liquor (see Table A4, experimental runs 13 and 30) had Ca:P molar ratio of 1.30 and 1.31. These values indicate a potential precipitation of octacalcium phosphate (OCP), a potentially high-value product. In order to

investigate results, two further experimental runs were performed at the following reaction conditions: 25 °C for the reaction temperature, 1.2:1 for 1 M Ca(OH)$_2$:mineral liquor (v:v) ratio and a reaction time no longer than 17 minutes. The experiment was carried out in two 150 ml mineral liquor batches.

The two experimental runs resulted in the formation and precipitation of OCP crystals. It yielded 25.4 g dm of OCP precipitate per 150 ml starting mineral liquor, containing 18.3 g kg^{-1} dm P and 31.6 g kg^{-1} dm Ca, giving a Ca:P molar ratio of 1.34. An inherent property of OCP is its ability to contain nonstoichiometric ratios of Ca:P, and it can either be Ca deficient or contain excess Ca, resulting in Ca:P molar ratios ranging from 1.26 up to 1.48 (Dorozhkin, 2016); however, OCP typically has a Ca:P molar ratio of 1.33 (Elliott, 1994). The operating conditions to precipitate OCP are as follows: 25 °C for the reaction temperature and 1.2:1 for 1 M Ca(OH)$_2$:mineral liquor (v:v) ratio with reaction time no longer than 17 minutes (such as the case of DCP). Figure 5.7 illustrates the characteristic FTIR spectra of both precipitated dry OCP (no adsorbed water) and wet OCP. The spectra of dry and wet OCP are similar, with the water molecule band stretching from 3 700–2 700 cm^{-1} and at 1 645 or 1 644 cm^{-1}. The PO$_4^{3-}$ stretching vibrations occur at   1 025, 1 113 or 1 021 cm^{-1}. A thin band is also detected at 872 or 870 cm^{-1} for HPO$_4^{2-}$ compound bending vibrations. Thus, the OCP crystals are identified specifically as OCP (Ca$_8$(HPO4)$_2$(PO$_4$)$_4$•5H$_2$O; the IUPAC name is tetracalcium hydrogen orthophosphate diorthophosphate pentahydrate).

The spectra of the precipitated OCP from this research resembles that of apatite: (1) the water molecule band is relatively wide from 3 600–2 600 cm^{-1} (Berzina-Cimdina and Borodajenko, 2012) and 3 000–3 700 cm^{-1} (Drouet and Drouet, 2013); (2) the PO$_4^{3-}$ compound stretches from 1 100–1 000 cm^{-1} (Berzina-Cimdina and Borodajenko, 2012; Coates, 2000) and is intense at 1 048 and 1 090 cm^{-1} (Drouet and Drouet, 2013); and (3) the HPO$_4^{2-}$ chemical group has an absorption band at 875 or 880 cm^{-1} (Berzina-Cimdina and Borodajenko, 2012). Furthermore, Sun et al. (2010) indicate that the FTIR of the materials with a Ca:P molar ratio of 1.33–1.67, such as OCP and apatite, displays absorbed water bands at 1 650 cm^{-1} and 2 700–3 700 cm^{-1}, phosphate bands at 573, 968 and 1 030–1 090 cm^{-1} and acid phosphate bands, HPO$_4^{2-}$ at 887 and 989 cm^{-1}. However, the precipitated OCP from this research is not an apatite due to the absence of OH$^-$ stretching vibration at 3 572 ± 2 cm^{-1}, which is a narrow peak (Berzina-Cimdina and Borodajenko, 2012). Furthermore, the absence of CO$_3^{2-}$ ions noticeable in the region 1 570–1 350 cm^{-1} indicates a nonapatitic structure (Drouet and Drouet, 2013). Nevertheless, a similarity in the spectral features of nonstoichiometric nanocrystalline apatites and OCP exists that can be attributed to the structural relationship (Drouet and Drouet, 2013; Dorozhkin, 2016). The OCP structure can indeed be described as the alternative stacking of 'apatitic' layers (with crystallographic positioning of ions very close to those in Hyp) and 'hydrated' layers that enclose in particular all the HPO$_4^{2-}$ ions contained in OCP (Drouet and Drouet, 2013; Dorozhkin and Epple, 2002) and Ca^{2+} ions (Ducheyne et al., 2011).
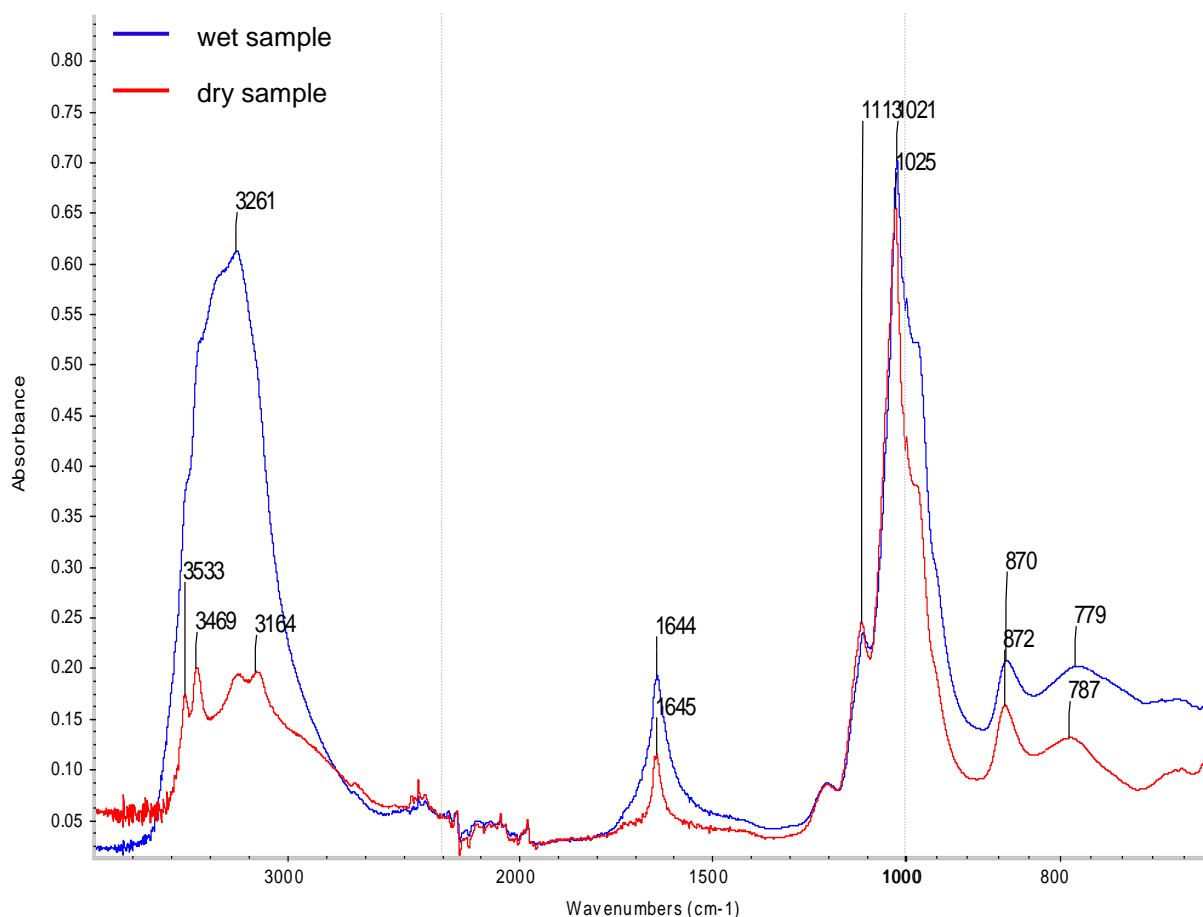
Figure 5.7: FTIR spectra of the precipitated OCP (Ca$_8$(HPO4)$_2$(PO$_4$)$_4$•5H$_2$O; the IUPAC name is tetracalcium hydrogen orthophosphate diorthophosphate pentahydrate)

OCP is a potentially high-value product and it is regarded as an important biomaterial. It has specific applications in the medical and dental fields due to its natural osteoinductive quality, which is the ability to induce bone formation (Komlev et al., 2014), and it is a precursor of apatite in the bone and teeth mineralisation process (Ducheyne et al., 2011; Dorozhkin and Epple, 2002). An OCP-collagen composite (OCP making up 77% of the weight in the OCP and collagen mixture) has been used to enhance bone regeneration in human bone defects (Kawai et al., 2014) and implants in tooth extraction sockets and cyst holes (Kawai et al., 2016). Moreover, when tested on rats with calvaria defects, the OCP-based materials consisting of OCP and natural polymers, such as gelatin, induced bone regeneration at a rate over 70%; thus, OCP-based materials are probable good candidates for an advanced material compatible to autologous bone implantation (Suzuki, 2013). Furthermore, the stromal cells derived from mouse bone marrow when cultured *in vitro* on the OCP hydrolysate and gelatin composite have shown osteoblast proliferation and differentiation, thus indicating that the composite could be a candidate as a scaffold in bone tissue engineering (Ezoe et al., 2015). A mixture of OCP and porcine atelocollagen sponge (OCP/Col) enhanced bone healing in a dog tooth extraction socket model (Iibuchi et al., 2009).

5.6   Extraction of gelatin from monkfish ossein

It is specified in the ANOVA illustrated in Table 5.4 that the linear and quadratic effects of the extraction pH and temperature have a significant influence (P < 0.05) on the amount of hydroxyproline contained in the gelatin liquor. Similarly, the linear effects of the extraction time as well as the linear interaction of the extraction temperature and time are also significant. The fitted quadratic regression equation for the hydroxyproline yield as the response variable in the extraction of gelatin from monkfish ossein is as follows:

$$Hydroxyproline\ Yield, \% \ = \ 1.5 - 0.2x^2 \ + \ 0.02x - 0.03y^2 \ + 0.0003y - 0.002z \ + \ 0.00006yz \qquad \text{(Equation 5.6)}$$

Where x = Extraction pH

y = Extraction temperature, °C

z = Extraction time, minutes

Table 5.4: ANOVA for the hydroxyproline yield response variable in the extraction of gelatin from monkfish ossein

Sources of variation	Sum of squares	DF	Mean square	F-value	P-value
(x) Extraction pH	0.02136	1	0.02136	38.08	0.000010
(x^2) Extraction pH	0.03463	1	0.03463	61.74	0.000000
(y) Extraction temperature, °C	0.3639	1	0.3639	648.77	0.000000
(y^2) Extraction temperature, °C	0.01512	1	0.01512	26.96	0.000073
(z) Extraction time, minutes	0.04273	1	0.04273	76.18	0.000000
yz Interaction	0.003844	1	0.003844	6.853	0.018002
Lack of fit	0.006565	8	0.000821	1.463	0.241808
Pure error	0.009536	17	0.000561		
Total sum of squares	0.4877	31			

The residual analysis of the model is satisfactory, wherein the points on the normal probability plot of the residuals for the response (see Figure B5) lie close to a straight line. This suggests that the error terms are normally distributed. Furthermore, the model explains 96% of the variability in the hydroxyproline yield response based on the adjusted coefficient of determination, R^2. As an additional support to the model adequacy, the residual error in terms of lack of fit for the model relative to the pure error is statistically insignificant (P > 0.05).

The response surface plots for hydroxyproline yield response are depicted in Figure 5.8. Inspection of the response surface plots indicates that the maximised hydroxyproline yield is obtained at a high level of extraction temperature and time and at both low and high levels of extraction pH. A desirability of one (see Figure B6) that maximises the hydroxyproline yield is achieved at the following optimal operating conditions: pH 8, 80 °C extraction temperature and 125 minutes extraction time at fixed 1:10 ratio of ossein:water (w:v) and one extraction. The predicted hydroxyproline yield of the model at optimal

conditions is 0.6% with an equivalent hydroxyproline recovery[3] of 8.3%. However, a verification run using the optimal conditions resulted in a gelatin liquor in unsubstantial amount, possibly as an effect of the combination of long extraction time and high temperature. This could possibly be resolved by using an increased amount of starting material, namely 30 g of monkfish ossein. With an insufficient amount of stored material at hand, a validation run using optimum conditions was not feasible. Furthermore, within the limitations of the study, preparation of new material for gelatin extraction was not feasible.

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[3] Hydroxyproline recovery is the percentage amount of hydroxyproline recovered in the gelatin liquor in weight relative to the amount of hydroxyproline in the dry monkfish ossein used in weight.
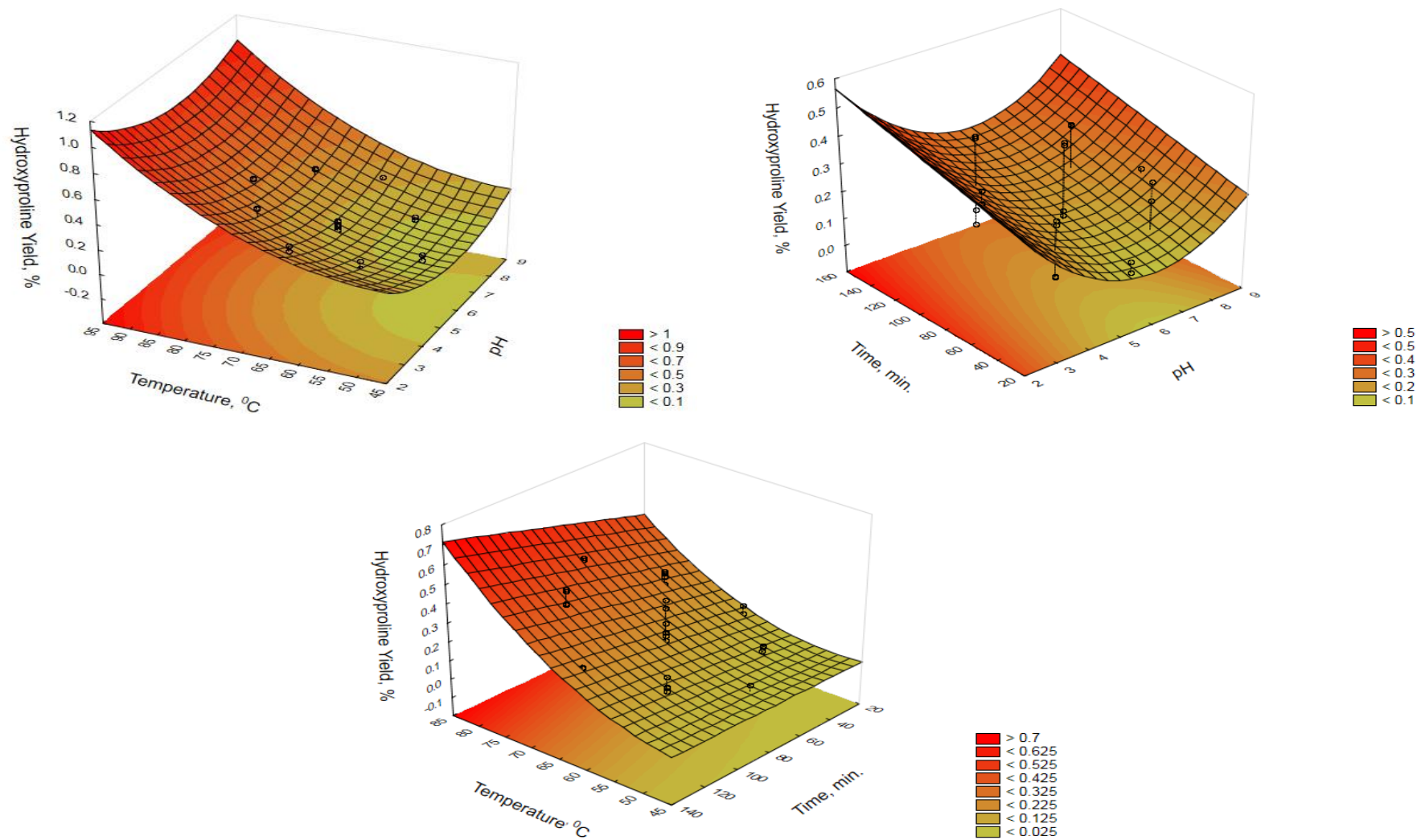
Figure 5.8: Surface plots for the hydroxyproline yield response variable of the extracted gelatin liquor from monkfish ossein

For the purpose of model validation, a shorter extraction time, namely 90 minutes, that also provided a high desirability but gave a lower prediction value of hydroxyproline yield by about ~0.08% was used. Therefore, the validation run employed extraction pH of 8, temperature of 80 °C and time of 90 minutes, whereby the model prediction intervals for these operating conditions were 0.47–0.60% hydroxyproline yield. A single validation run using the latter operating conditions resulted in a gelatin liquor with 0.57% hydroxyproline yield with an equivalent hydroxyproline recovery of 7.7%. The hydroxyproline percentage recovery is low compared to 64% and 52% recovery of hydroxyproline in gelatin supernatant from cod backbones and heads, respectively, using 90 minutes extraction time and 70 °C extraction temperature (Kołodziejska et al., 2008). The high disparity of hydroxyproline recovery is mainly attributed to repeated extractions of gelatin up to three times from cod backbones and heads whereas there is only a single extraction of gelatin from monkfish ossein in the present work. The hydroxyproline recovery in the gelatin extraction from Atlantic cod fish head bones by Arnesen and Gildberg (2006) at first and second extraction is also low, 3.7 and 4.4% respectively, but the recovery increases to 12.0, 20.1 and 14.3% during the third, fourth and fifth extractions, respectively, although increasing temperature and acidity were used during each extraction. Hence, employing two or more successive extractions or studying the effect thereof on the extracted gelatin from monkfish ossein could potentially improve the hydroxyproline recovery in the gelatin liquor.

The protein content in the gelatin liquor using nitrogen analysis by the Dumas method provides higher values compared to the colorimetric estimation of protein content using the linearised Bradford assay (see Figure A4). The Dumas method gives sixfold to thirtyfold higher estimates of protein than the Bradford assay. Nollet and Toldrà (2009) indicate that the major problem of colorimetric protein assays is the high variability of protein concentration estimates from different individual proteins versus the true concentration. Thus, the choice of standard for colorimetric assay has a significant effect on the protein concentration estimates for different proteins, specifically with strongly divergent amino acid compositions in proteins such as gelatin. Colorimetric assays are adequate if an absolute protein concentration of gelatin liquor is not required; otherwise, a more reliable approach is required, for example the Kjeldahl or Dumas methods. However, inspection of the protein content (by the Dumas method) and the hydroxyproline content in the gelatin liquor from 15 different treatments (see Figure A5) suggests a variable ratio of protein to hydroxyproline; it ranges from 12 to 174. This ratio is expected to be almost similar for 15 different treatments if the gelatin liquor contains pure gelatin. The variation is only ascribed to the presence of a variable amount of nitrogen-containing proteins other than gelatin or possibly to the presence of pure gelatin in the extracted gelatin liquor. Protein estimates using the Dumas method to quantify gelatin in the gelatin liquor are not completely dependable. Hence, measurement of hydroxyproline appears to be the best approach for quantification of gelatin. By using a precise hydroxyproline-collagen conversion factor, the gelatin content in the gelatin liquor can be calculated.

5.7   Overall material balance

The overall material balance in developing and optimising processes for the extraction of minerals from monkfish bones, precipitation of DCP from the extracted minerals and extraction of gelatin from monkfish ossein are presented below. A total of 914.32 g dm DCPD or alternatively 1 100.74 g dm OCP was produced from 5 889 g of wet minced monkfish heads as the starting material. The bones recovered from the hydrolysis of the starting material amounted to 187.32 g dm, as described in Figure 5.9. The recovery of mineral fraction from these bones required a total of 7 000 ml 5% H_3PO_4 (v/v), as illustrated in Figure 5.10, resulting in mineral liquor and ossein as a by-product. Furthermore, saturation of mineral liquor with 6 178 ml 1 M $Ca(OH)_2$ precipitated DCPD, or alternatively OCP was precipitated from the mineral liquor saturated with 7 847 ml 1 M $Ca(OH)_2$. It is also outlined in Figure 5.11 that a mixture of 3.1 g dm ossein and 100 ml H_2O produced 28 ml gelatin liquor containing 627.62 µg/ml hydroxyproline.
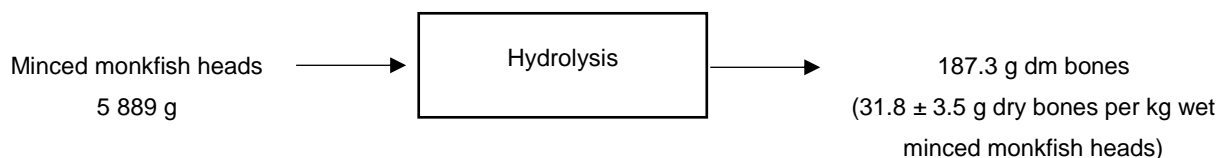
Figure 5.9: Material balance for the recovery of monkfish bones by enzymatic hydrolysis of minced monkfish heads
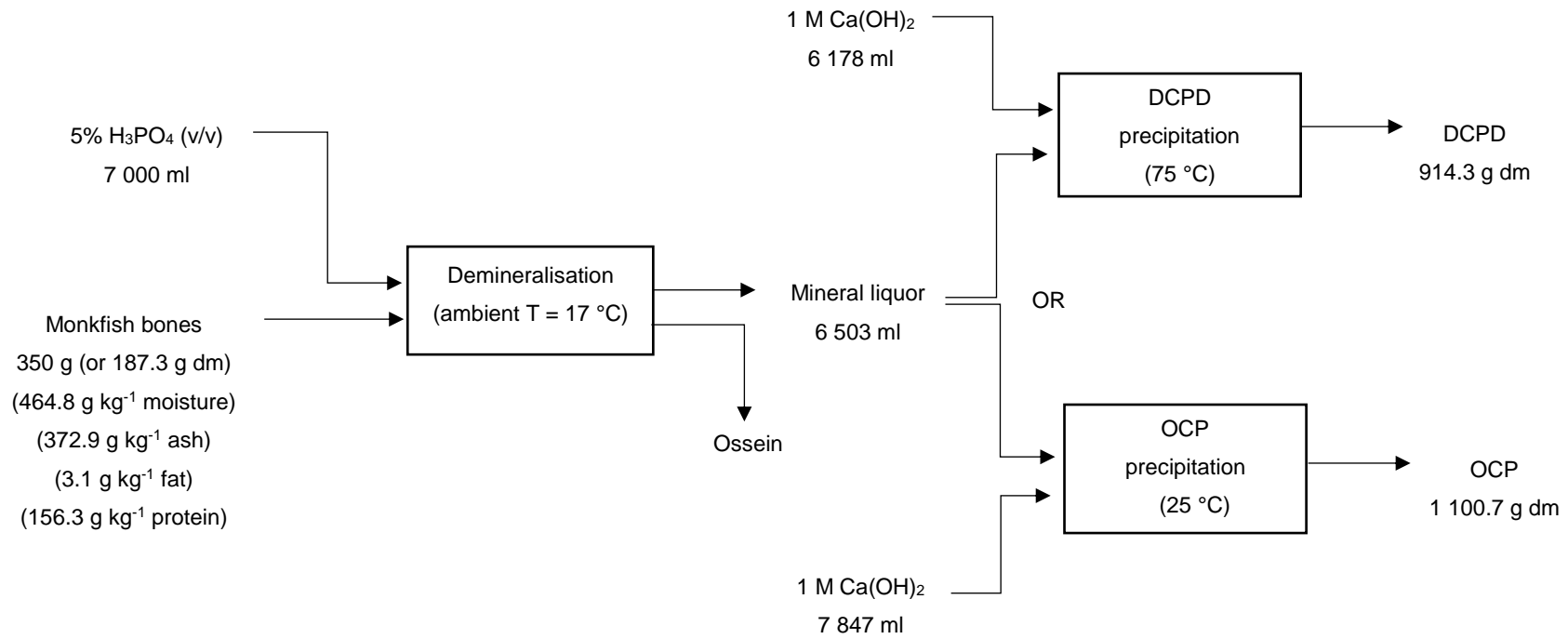
Figure 5.10: Material balance for the recovery of mineral liquor from monkfish bones and precipitation of DCPD or OCP from the recovered mineral liquor

Demineralised water
100 ml

Monkfish ossein

10 g (or 3.1 g dm)

(690.0 g kg^{-1} moisture)

(73.5 g kg^{-1} dm hydroxyproline)

Gelatin extraction

(80 °C)

Gelatin liquor

28 ml

(627.6 µg/ml hydroxyproline)
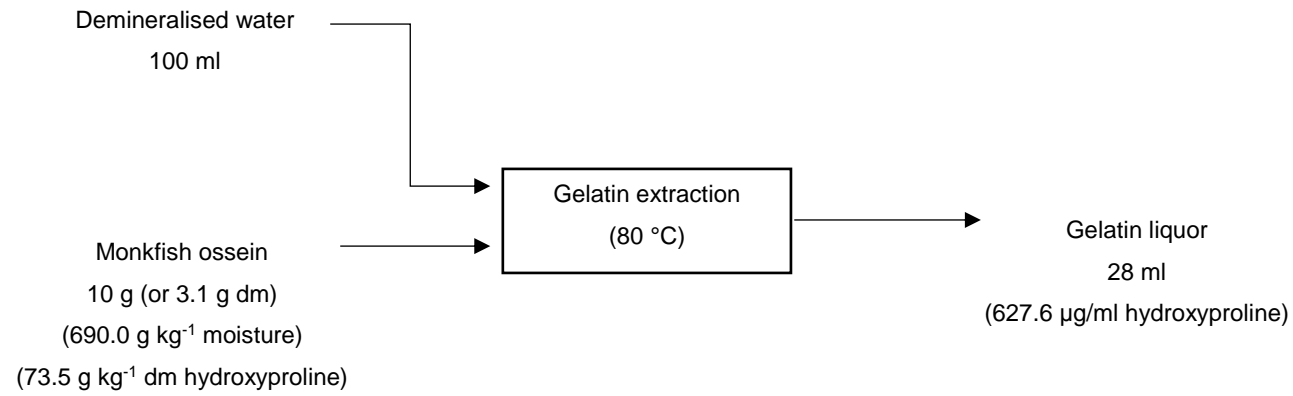
Figure 5.11: Material balance for the extraction of gelatin from monkfish ossein (by-product in the demineralisation of monkfish bones)

Chapter 6
Conclusion

This research has proved that employing the processes that were developed and optimised in this study, the potentially valuable products DCPD, OCP and gelatin can be derived from monkfish bones. The process developed to precipitate DCP resulted in 93.4% and 99.0% recovery of total Ca and P inputs, respectively: an implication that the new method employed in the present work resulted in a by-product (spent liquor) that potentially contains mainly water and very low levels of Ca and P. The DCPD from this study has promising applications in animal feeds and is superior to existing commercial products as it contains essential microminerals and low levels of harmful heavy metals. The OCP has potential applications in the medical and dental fields. The gelatin extracted in this study requires further examination to determine its potential use in the pharmaceutical, food, nutraceutical, technical and/or cosmetics industries. Consequently, this study contributes towards improved utilisation of monkfish heads, which are currently viewed as a solid waste product from the South African monkfish and hake fisheries.

Novel processes were developed and optimised in the extraction of minerals from monkfish bones and the precipitation of DCP from the extracted minerals. It was found that the optimum conditions for the extraction of minerals from monkfish bones were 5% H_3PO_4 (v/v), 5:1 ratio of solution (v) to bones (w) and four successive 24-hour extractions at ambient temperature of 17 °C. The resultant monkfish ossein was determined to contain 2.8 g kg^{-1} dm ash, signifying that significant amounts of minerals contained in the monkfish bones were recovered in the mineral liquor, while 69.7 g dry hydroxyproline per kg dry ossein was preserved in the residual monkfish ossein. Thus, the resultant mineral liquor is a potential raw material for DCP precipitation while the monkfish ossein is a potential raw material for gelatin extraction.

The DCP precipitation from the mineral liquor that produces 21.1 g dm of DCPD precipitate per 150 ml starting mineral liquor, with precipitate containing 215.2 g kg^{-1} dm P and 1.16 Ca:P molar ratio, can be prepared provided that the optimum conditions are employed. It was established that these optimum conditions were 75 °C reaction temperature, 0.95:1 v/v 1 M Ca(OH)$_2$:mineral liquor ratio and reaction time no longer than 17 minutes as reaction times longer than 17 minutes do not lead to increased DCPD precipitate. An XRD analysis revealed the DCPD nature of the precipitate. The DCPD in the current study has an estimate of 95.1% P bioavailability, and it is potentially a good-quality animal feed supplement. Furthermore, the DCPD in this study is a source of other essential microminerals (Mg, S, Si, Sr, Na, Fe, Al, Mn, K, Zn, Ba, B, V, Cr, Ni, Cu, Co, Mo, Se and Li) besides P and Ca and contains very low levels of harmful heavy metals (Pb, Cd, As and Hg) and nonessential metals (Sb and Be).

This study has also demonstrated that OCP crystals can be produced at conditions different from the optimal operating conditions to precipitate DCP. The precipitated OCP was identified using a Ca:P

65

molar ratio and FTIR spectra. It was found that the operating conditions to precipitate OCP were 25 °C reaction temperature, 1.2:1 v/v 1 M $Ca(OH)_2$:mineral liquor ratio and reaction time no longer than 17 minutes. These operating conditions yielded 25.4 g dm OCP precipitate per 150 ml starting mineral liquor, and the precipitate contained 18.3 g kg^{-1} dm P and 31.6 g kg^{-1} dm Ca, giving a Ca:P molar ratio of 1.34. Based on the literature, a composite of OCP has specific applications in the medical and dental fields; thus, OCP is regarded as an important biomaterial.

Furthermore, a process has been developed and optimised for the extraction of gelatin from monkfish ossein although reoptimisation embodying two or more successive extractions to improve hydroxyproline recovery in the extracted gelatin is recommended. This study has demonstrated that gelatin can be extracted from monkfish ossein. In one extraction, the optimum conditions to extract gelatin with a 0.6% hydroxyproline yield from the mixture of ossein and water at 1:10 ratio (w/v) were extraction pH of 8 and extraction temperature and time of 80 °C and 125 minutes, respectively. At these optimum conditions, the hydroxyproline recovery was determined to be 8.3%. The recovery of hydroxyproline is significantly lower than that from the bones of cod species reported in the literature on studies that utilised three or more successive extractions. The potential of improving the hydroxyproline recovery (that will also improve hydroxyproline yield) in the present work can be achieved by employing two or more successive extractions of gelatin from ossein. It was also determined in this study that the colorimetric method such as Bradford assay and nitrogen determination by the Dumas method were not definitive methods to quantify the amount of gelatin. The potential of quantifying the gelatin amount in the present work can be achieved when the hydroxyproline-to-collagen conversion factor in the monkfish bones has been determined. Thus, there is a need to determine the hydroxyproline-to-collagen conversion factor in the monkfish bones to quantify the amount of gelatin protein extracted from monkfish ossein.

This study has highlighted areas that require future study:

I.   Evaluate the true biological value of the DCPD product in the present work by means of *in vivo* animal feed trials.

II.   Investigate the utilisation of two or more successive extractions of gelatin from monkfish ossein. This can be done by evaluating the effect thereof on the hydroxyproline content in the gelatin in conjunction with the variable factors in the present study, such as extraction pH, time and temperature. This approach could potentially improve the hydroxyproline recovery in the gelatin liquor.

III.   Determine the hydroxyproline-collagen conversion factor in the monkfish bones to quantify the amount of gelatin protein extracted from monkfish ossein. This can be achieved by extracting the collagen in the monkfish ossein followed by a series of purification steps until a purified, concentrated collagen solution is obtained. A hydroxyproline-collagen conversion factor can then be determined from the full amino acid profile of the extracted collagen using amino acid analysis.

IV.   Characterise the gelatin obtained from monkfish ossein to assess the grade and quality of the gelatin. This can be achieved by measuring different functional, nutritional and chemical characteristics, namely the bloom value (gel strength), viscosity, pH, isoelectric point, ash content and amino acid profile of the gelatin. By doing this, the relevant application of the extracted gelatin from monkfish ossein can be determined.

V.   Evaluate the economic viability of the optimised process developed in DCP precipitation or alternatively the process developed for OCP precipitation in the current study. For gelatin, however, economic viability can only be evaluated once the hydroxyproline recovery in the extracted gelatin is improved in significant quantity and the gelatin thereof is characterised.

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Appendix A: Experimental data

Figures

(a)

(b)

(c)

(d)

(e)

(f)

Figure A1: An illustration of (a) whole monkfish, (b) monkfish head obtained from local fish processor in Cape Town, South Africa, (c) degutted and clean monkfish head, (d) minced monkfish heads using a bowl cutter, (e) monkfish bones recovered from enzymatic hydrolysis of minced monkfish heads and (f) homogenised monkfish bones using a food processor

Absorbance = (0.2002 x Hydroxyproline Concentration) + 0.0017

R^2 = 0.9963
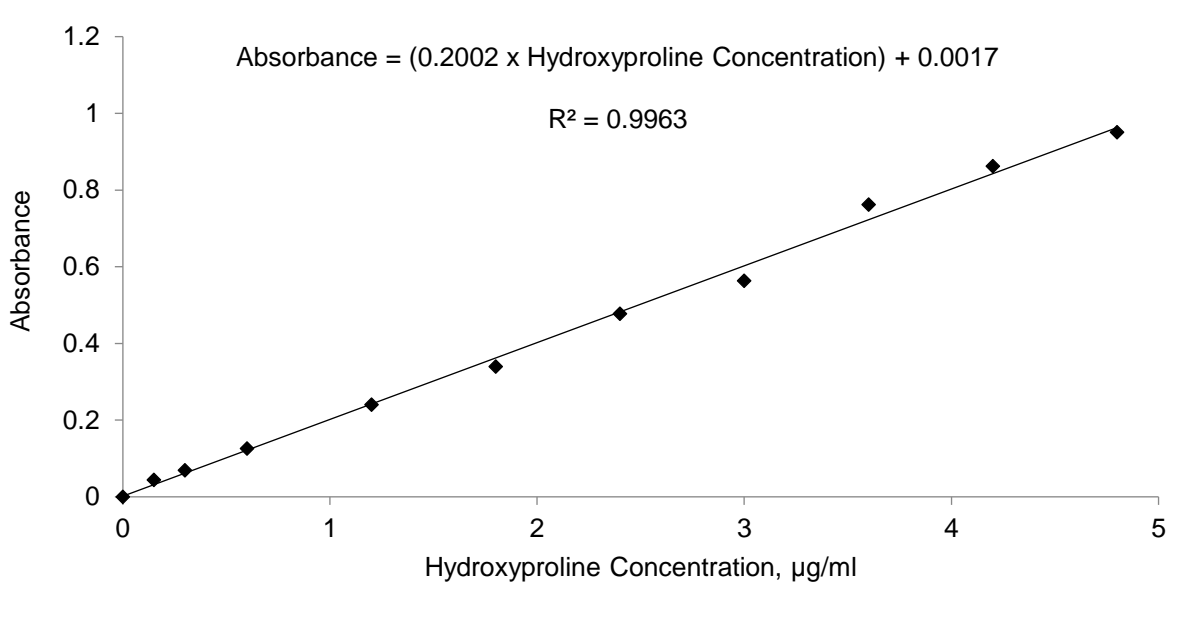
Figure A2: Hydroxyproline standard curve

Abosrbance (590/450 nm) = (0.0488 x Protein Concentration) + 0.853

R^2 = 0.9945
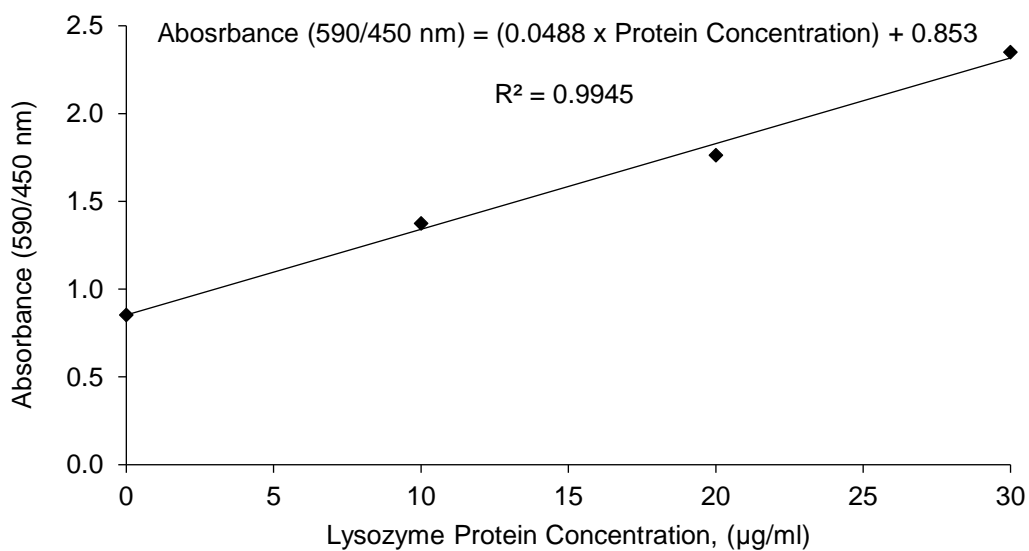
Figure A3: Standard curve for the linearised Bradford assay, using lysozyme as standard
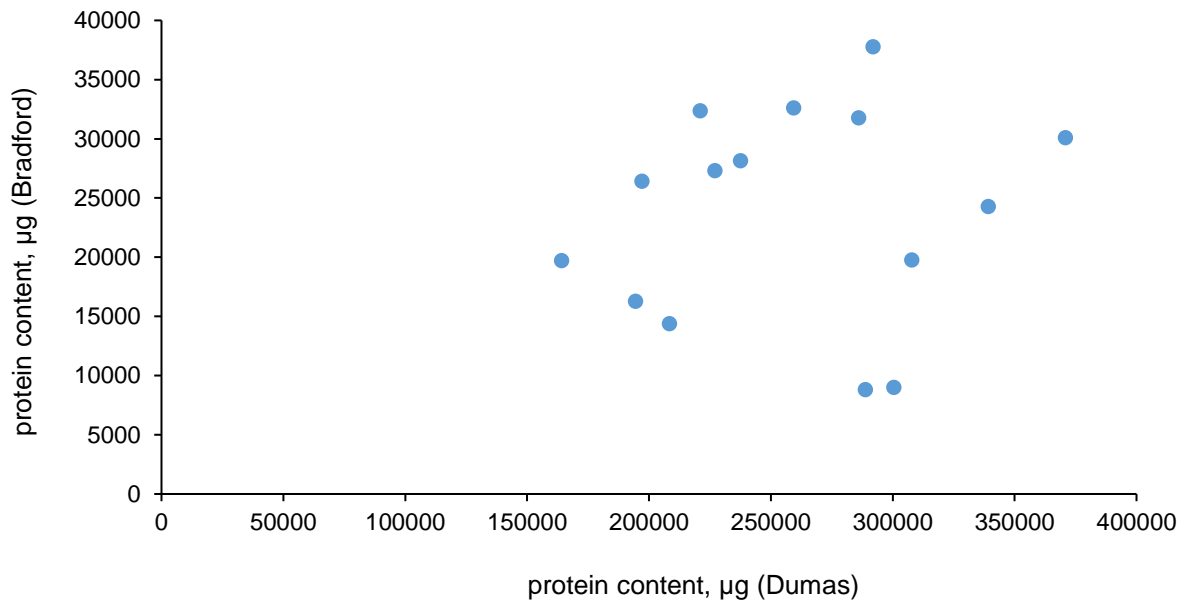
Figure A4: Correlation between protein content using the Dumas method and Bradford assay of the extracted gelatin liquor from 15 different treatments utilised in the 32 experimental runs for the extraction of gelatin from monkfish ossein
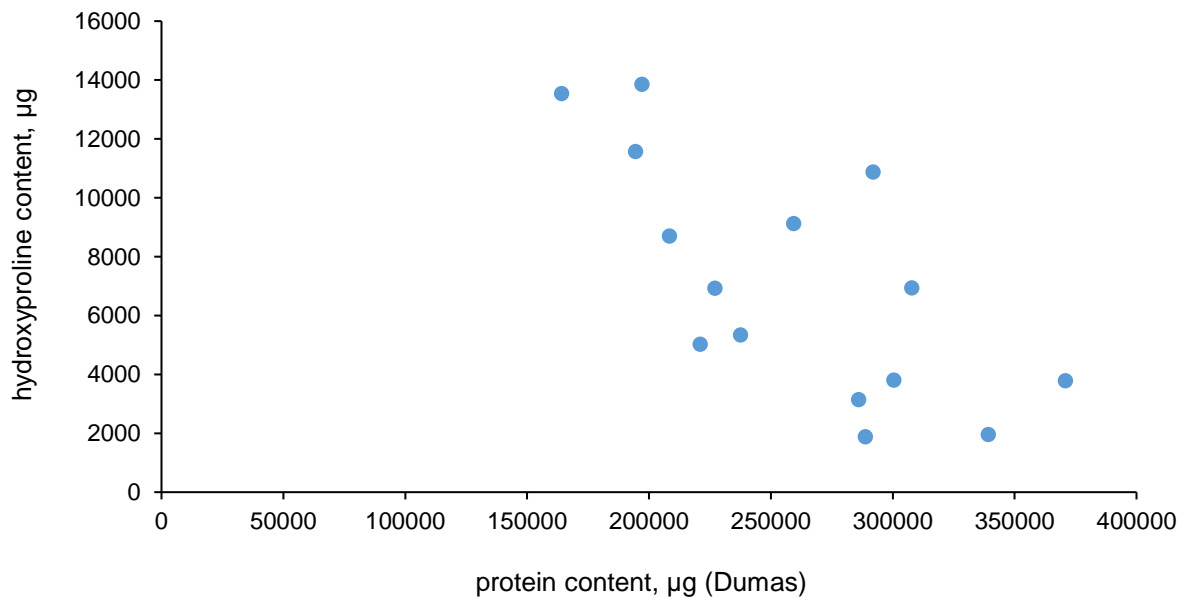
Figure A5: Correlation between protein content using the Dumas method and the hydroxyproline content of the extracted gelatin liquor from 15 different treatments utilised in the 32 experimental runs for the extraction of gelatin from monkfish ossein

Tables

Table A1: Sizes of 13 randomly selected degutted and clean monkfish heads

Monkfish heads	Weight, kg
1	1.48
2	0.56
3	1.11
4	2.77
5	2.62
6	1.51
7	1.00
8	1.89
9	1.13
10	1.57
11	1.44
12	1.28
13	4.59

Table A2: Monkfish bones yield from enzymatic hydrolysis of minced monkfish heads

Hydrolysis	Minced fish heads, kg wet weight	Fish bones yield, g kg^{-1} wet weight	Fish bones moisture content, g kg^{-1}	Fish bones yield, g kg^{-1} dry mass
1	2.5	87.2	674.3	28.6
2	2.5	87.2	674.3	28.6
3	2.5	87.5	671.2	28.7
4	2.5	91.0	671.1	29.8
5	2.5	85.0	674.5	27.9
6	2.5	92.5	671.0	30.3
7	2.5	98.9	672.1	32.4
8	2.5	90.3	672.6	29.6
9	2.5	84.4	673.0	27.7
10	2.5	80.6	673.3	26.4
11	2.5	89.2	672.7	29.2
12	2.5	89.3	672.7	29.3
13	2.5	101.3	671.9	33.2
14	2.5	104.4	670.5	34.2
15	2.5	93.3	673.8	30.6
16	2.5	99.1	669.4	32.5
17	2.5	110.2	670.3	36.1
18	2.5	99.0	672.1	32.4
19	2.5	101.4	671.9	33.2
20	2.5	120.4	671.1	39.5
21	2.5	95.8	670.8	31.4
22	2.5	101.2	671.9	33.2
23	2.5	107.3	671.6	35.2
24	2.5	114.0	670.2	37.3
25	2.5	115.4	673.6	37.8

Table A3: Data from the demineralisation of monkfish bones experiment using two-level full factorial design

Experimental run	H_3PO_4 concentration, % v/v	Number of extractions	Ratio of solution (v) to raw material (w)	Ash content, g kg^{-1} dm	Hydroxyproline content, g kg^{-1} dm
1	2.0	2	2	637.2	23.2
2	5.0	2	5	30.5	71.0
3	5.0	4	5	1.1	69.3
4	5.0	2	2	404.8	35.6
5	5.0	4	5	2.8	73.7
6	5.0	4	2	0.0	63.4
7	5.0	4	2	0.0	64.4
8	5.0	2	5	0.0	69.7
9	2.0	2	5	469.7	30.0
10	2.0	4	2	507.6	27.1
11	3.5	3	3.5	0.0	65.9
12	2.0	2	2	639.5	14.8
13	2.0	4	5	0.0	65.8
14	3.5	3	3.5	1.2	70.3
15	5.0	2	2	474.9	27.0
16	2.0	2	5	437.4	28.6
17	2.0	4	5	3.4	58.0
18	2.0	4	2	527.8	15.7

Table A4: Data from the precipitation of DCP from mineral liquor experiment using CCD

Experimental run	Reaction temperature, °C	Reaction time, minutes	1 M Ca(OH)$_2$:mineral liquor (v:v)	Yield, g	P content, g kg^{-1} dm	Ca:P molar ratio
1	50	60	0.7	16.8	203.7	0.99
2	50	60	0.7	16.2	203.5	0.98
3	75	60	0.7	14.9	242.8	0.95
4	35	35	0.4	5.0	208.3	0.99
5	25	60	0.7	17.5	198.2	1.00
6	50	102	0.7	17.2	197.1	0.99
7	65	35	1.0	24.6	197.8	1.11
8	35	85	0.4	5.3	206.3	0.99
9	50	17	0.7	17.1	195.9	0.98
10	50	102	0.7	17.3	199.9	0.99
11	35	35	1.0	27.7	177.6	1.11
12	65	85	0.4	6.6	249.9	0.95
13	50	60	1.2	31.6	158.0	1.30
14	50	60	0.2	0.7	246.6	1.07
15	65	35	0.4	5.3	240.1	0.96
16	50	60	0.2	0.4	243.2	1.10
17	50	60	0.7	17.5	198.8	0.99
18	65	85	1.0	22.2	212.0	1.09
19	65	35	1.0	24.5	193.2	1.15
20	50	17	0.7	18.2	200.4	0.98
21	65	35	0.4	6.0	245.0	0.96
22	35	85	0.4	5.7	201.3	0.98
23	35	85	1.0	27.64	179.1	1.14
24	65	85	0.4	6.49	241.9	0.96
25	35	85	1.0	28.40	171.7	1.12
26	25	60	0.7	16.53	195.1	0.98
27	75	60	0.7	15.55	243.1	0.95
28	35	35	1.0	27.79	183.6	1.10
29	65	85	1.0	23.40	204.0	1.13
30	50	60	1.2	33.28	148.8	1.31
31	35	35	0.4	5.76	202.5	0.99
32	50	60	0.7	18.57	200.2	1.00

Table A5: Data from the extracted gelatin liquor from monkfish ossein experiment using CCD

Experimental run	Extraction pH	Extraction temperature, °C	Extraction time, minutes	Hydroxyproline yield, %
1	5.5	65	75	0.199
2	5.5	65	75	0.182
3	7.0	56	105	0.113
4	7.0	56	45	0.072
5	7.0	74	105	0.372
6	4.0	74	45	0.302
7	7.0	74	105	0.375
8	4.0	74	105	0.450
9	5.5	80	75	0.432
10	4.0	56	105	0.188
11	3.0	65	75	0.373
12	4.0	56	105	0.136
13	5.5	50	75	0.043
14	5.5	50	75	0.083
15	5.5	65	25	0.142
16	5.5	65	125	0.226
17	5.5	65	75	0.150
18	7.0	74	45	0.247
19	4.0	74	105	0.444
20	8.0	65	75	0.248
21	4.0	56	45	0.103
22	7.0	56	45	0.049
23	4.0	56	45	0.100
24	8.0	65	75	0.199
25	5.5	80	75	0.441
26	7.0	74	45	0.313
27	3.0	65	75	0.329
28	7.0	56	105	0.132
29	4.0	74	45	0.286
30	5.5	65	125	0.220
31	5.5	65	25	0.101
32	5.5	65	75	0.157

Table A6: Amount of gelatin liquor extracted from monkfish ossein and the equivalent density and weight

Experimental run	Extracted amount, ml	Density$_1$, g/ml[a]	Density$_2$, g/ml[a]	Average density, g/ml[b]	Weight, g[c]
1	90	0.9682	0.9668	0.9675	87.08
2	83	0.9500	0.9589	0.9545	79.22
3	69	0.9484	0.9618	0.9551	65.90
4	47	0.9465	0.9485	0.9475	44.53
5	90	0.9477	0.9612	0.9545	85.90
6	81	0.9430	0.9578	0.9504	76.98
7	64	0.9443	0.9602	0.9523	60.94
8	37	0.9384	0.9504	0.9444	34.94
9	78	0.9380	0.9538	0.9459	73.78
10	72	0.9513	0.9658	0.9586	69.02
11	92	0.9543	0.9522	0.9533	87.70
12	43	0.9497	0.9433	0.9465	40.70
13	87	0.9646	0.9385	0.9516	82.78
14	62	0.9571	0.9316	0.9444	58.55
15	76	0.9664	0.9390	0.9527	72.41
16	71	0.9586	0.9368	0.9477	67.29
17	89	0.9598	0.9443	0.9521	84.73
18	84	0.9573	0.9492	0.9533	80.07
19	70	0.9677	0.9642	0.9660	67.62
20	46	0.9630	0.9616	0.9623	44.27
21	91	0.9597	0.9637	0.9617	87.51
22	81	0.9579	0.9612	0.9596	77.72
23	67	0.9576	0.9590	0.9583	64.21
24	45	0.9520	0.9559	0.9540	42.93
25	80	0.9575	0.9547	0.9561	76.49
26	73	0.9545	0.9534	0.9540	69.64
27	91	0.9527	0.9582	0.9555	86.95
28	35	0.9468	0.9525	0.9497	33.24
29	88	0.9520	0.9626	0.9573	84.24
30	61	0.9508	0.9620	0.9564	58.34
31	75	0.9531	0.9616	0.9574	71.80
32	77	0.9553	0.9612	0.9583	73.79

Notes:

[a] - Density$_1$ and density$_2$ were determined from two aliquots of 1 ml gelatin liquor from each experimental run that were separately weighed to the nearest 0.0001 g.

[b] - Average density was calculated as the average of density$_1$ and density$_2$.

[c] - Weight was calculated by multiplying average density by extracted amount.

Table A7: Hydroxyproline and protein contents of the extracted gelatin liquor from monkfish ossein

Experimental run	Extracted amount, ml	Hydroxyproline content, µg	Protein content, µg[a]
1	90	3 102	30 403
2	83	4 208	30 971
3	69	9 357	31 348
4	47	13 945	25 274
5	90	1 528	8 706
6	81	4 107	9 446
7	64	9 718	13 446
8	37	11 532	17 026
9	78	10 188	33 423
10	72	7 696	20 663
11	92	1 333	22 252
12	43	13 403	19 336
13	87	4 411	31 495
14	62	7 014	27 723
15	76	5 656	25 538
16	71	6 171	29 952
17	89	3 179	33 142
18	84	5 832	33 749
19	70	8 881	33 867
20	46	13 763	27 565
21	91	2 227	8 932
22	81	3 500	8 548
23	67	7 664	15 310
24	45	11 610	15 545
25	80	11 548	42 129
26	73	6 162	18 877
27	91	2 568	26 337
28	35	13 663	20 107
29	88	3 143	28 720
30	61	6 825	26 889
31	75	4 645	27 959
32	77	4 865	29 114

Note:

[a] - Determined using Bradford assay

Table A8: List of experimental runs in gelatin extraction with identical treatment conditions. A total of 15 different treatments were utilised in the 32 experimental runs for the extraction of gelatin

Treatment	Experimental run replicates
1	15, 16, 31 and 32
2	4 and 20
3	6 and 22
4	5 and 21
5	8 and 24
6	3 and 19
7	12 and 28
8	2 and 18
9	9 and 25
10	11 and 27
11	13 and 29
12	14 and 30
13	7 and 23
14	10 and 26
15	1 and 17

Appendix B: Statistical output

Figures
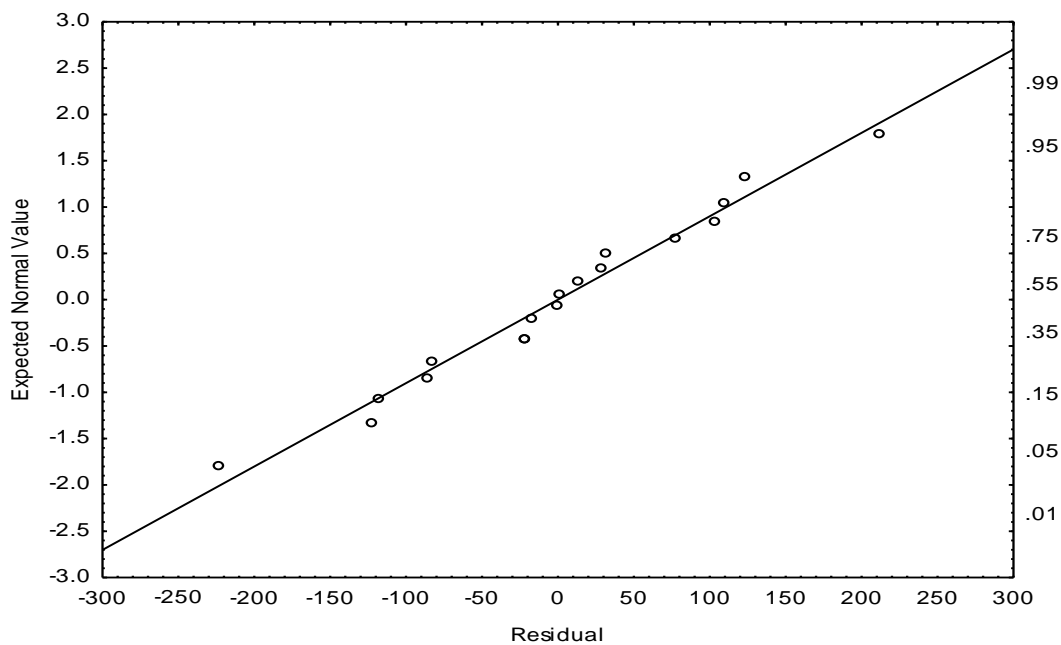
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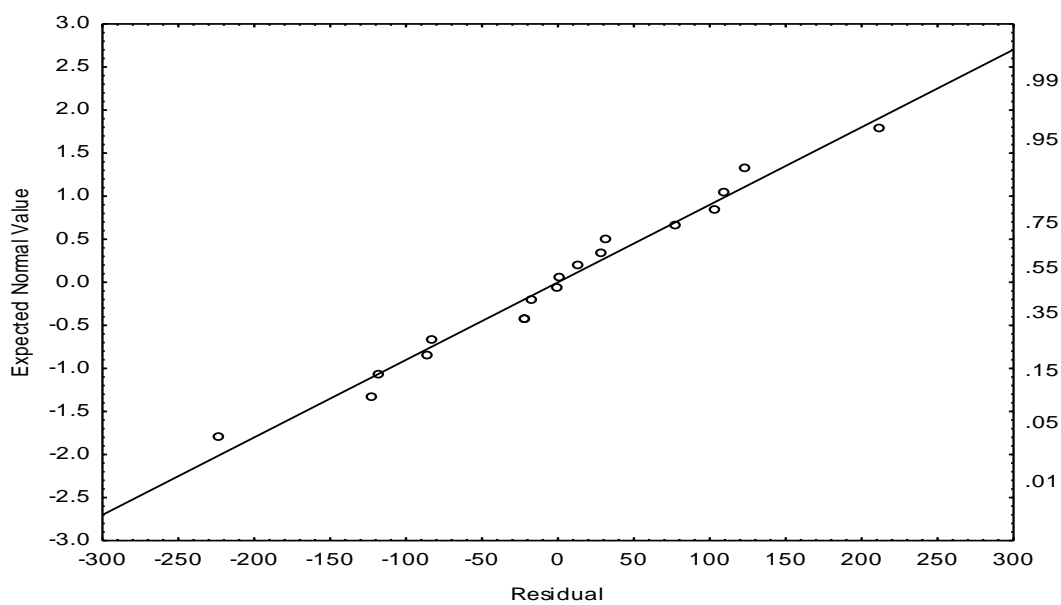
(b)

Figure B1: Normal probability plot of the residuals for the (a) ash and (b) hydroxyproline contents response variables in demineralisation of monkfish bones

95
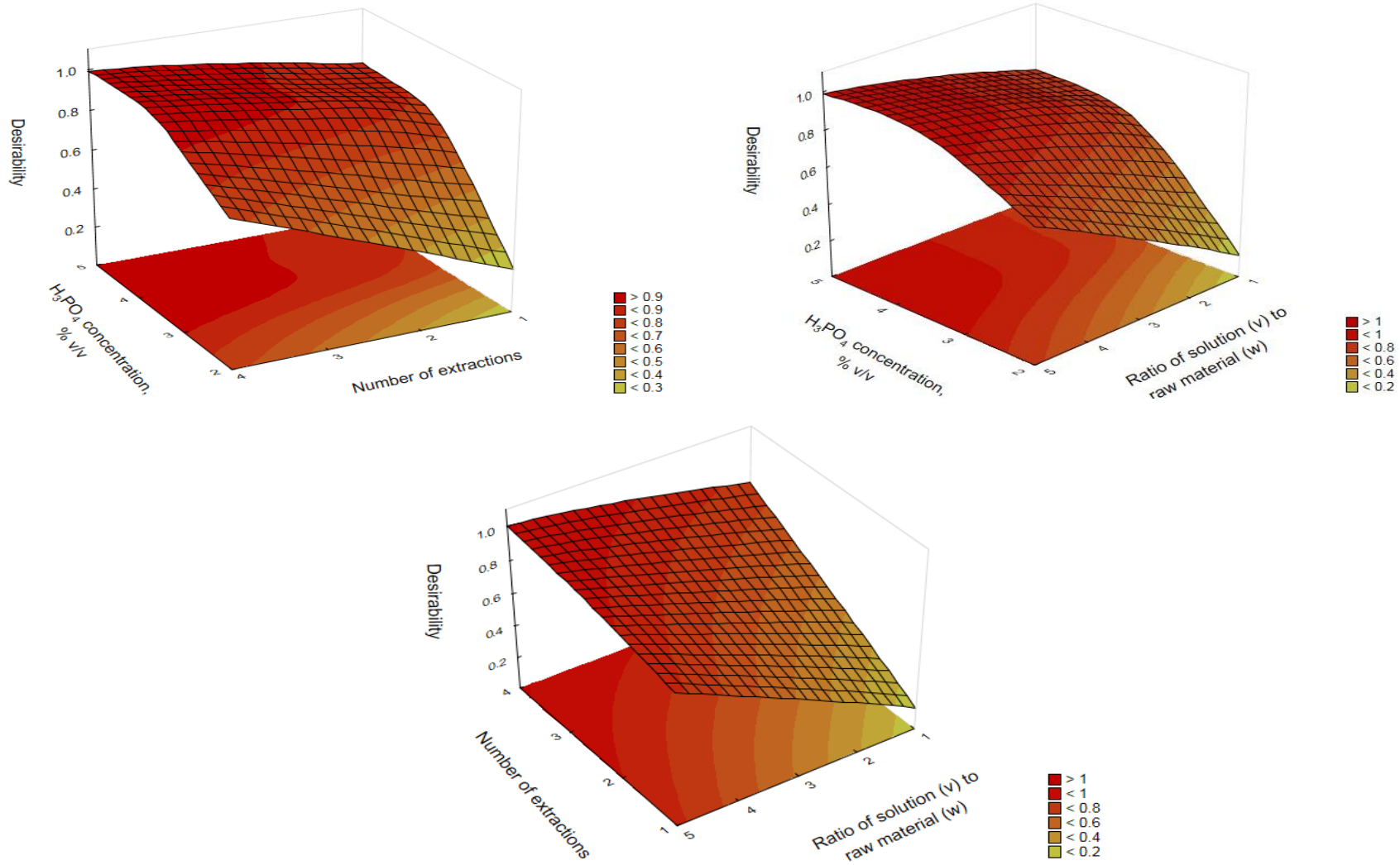
Figure B2: Desirability surface plots for the demineralisation of monkfish bones with minimised ash and maximised hydroxyproline contents in the monkfish ossein
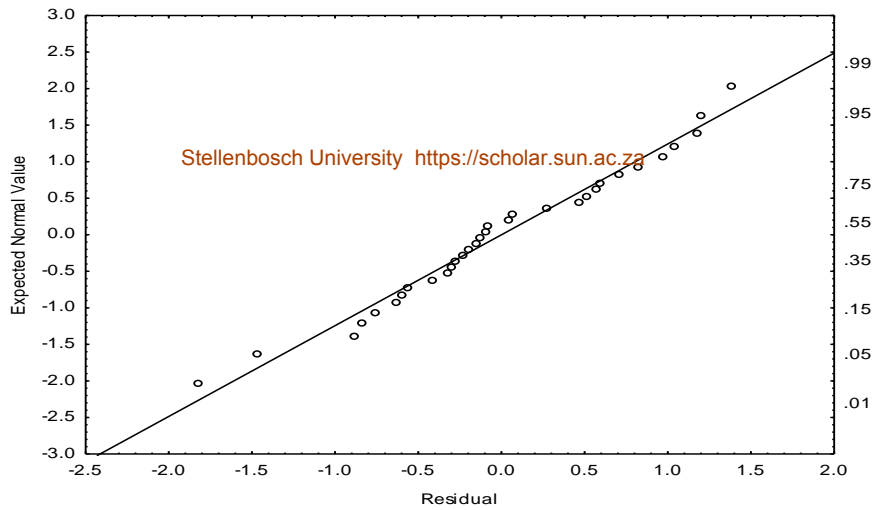
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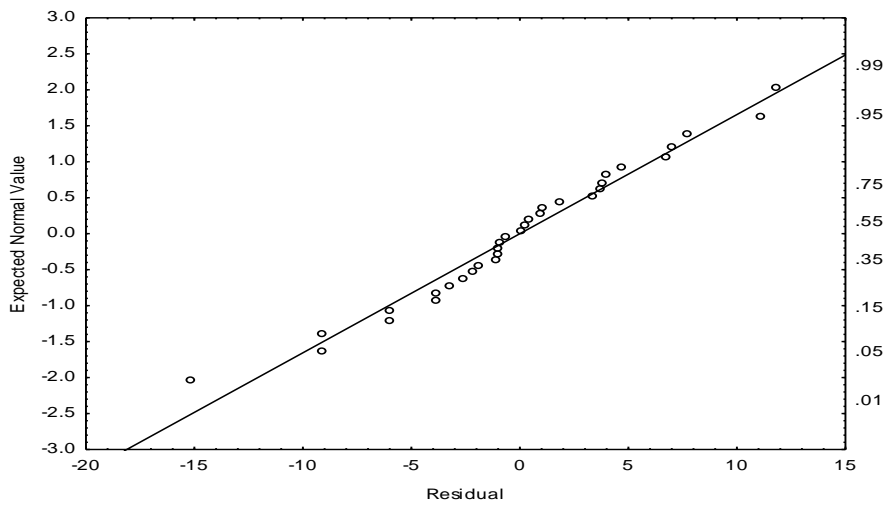
(b)
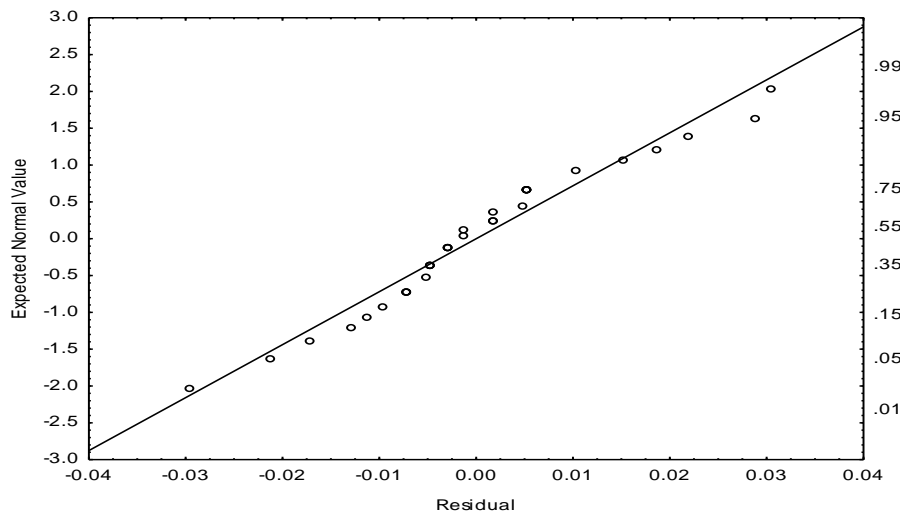
(c)

Figure B3: Normal probability plot of the residuals for the (a) yield, (b) P content and (c) Ca:P molar ratio response variables
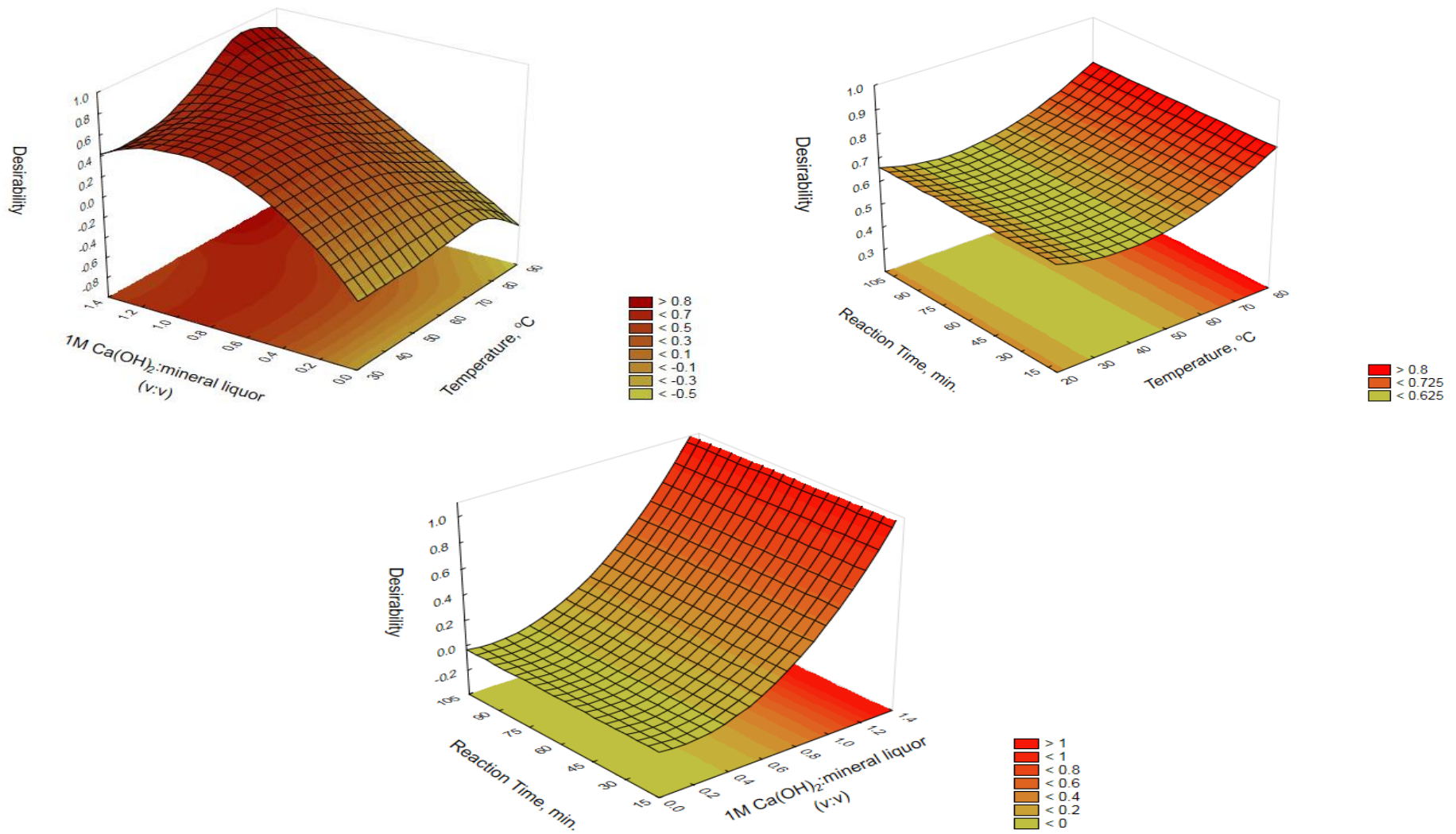
Figure B4: Desirability surface plots for the precipitation of DCP from mineral liquor with maximised yield and P content, and a Ca:P molar ratio of approximately one
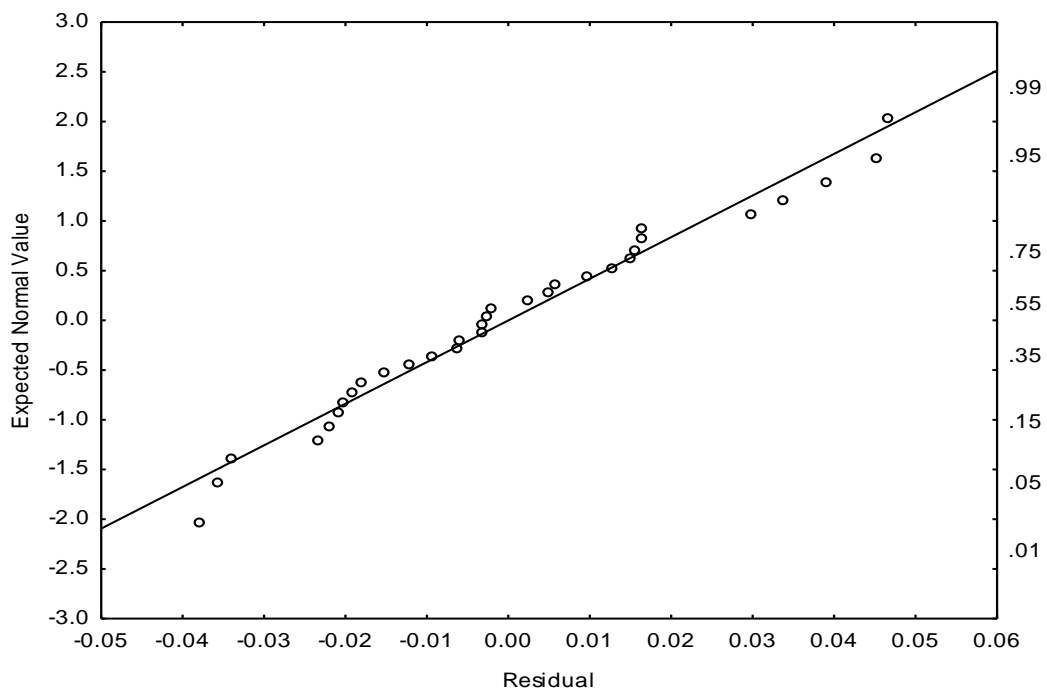
Figure B5: Normal probability plot of the residuals for the hydroxyproline yield response variable

Figure B6: Desirability surface plots for the extraction of gelatin from monkfish ossein with maximised hydroxyproline yield

Tables

Table B1: Factor effect estimates for the demineralisation of monkfish bones with ash and hydroxyproline contents as the response variables
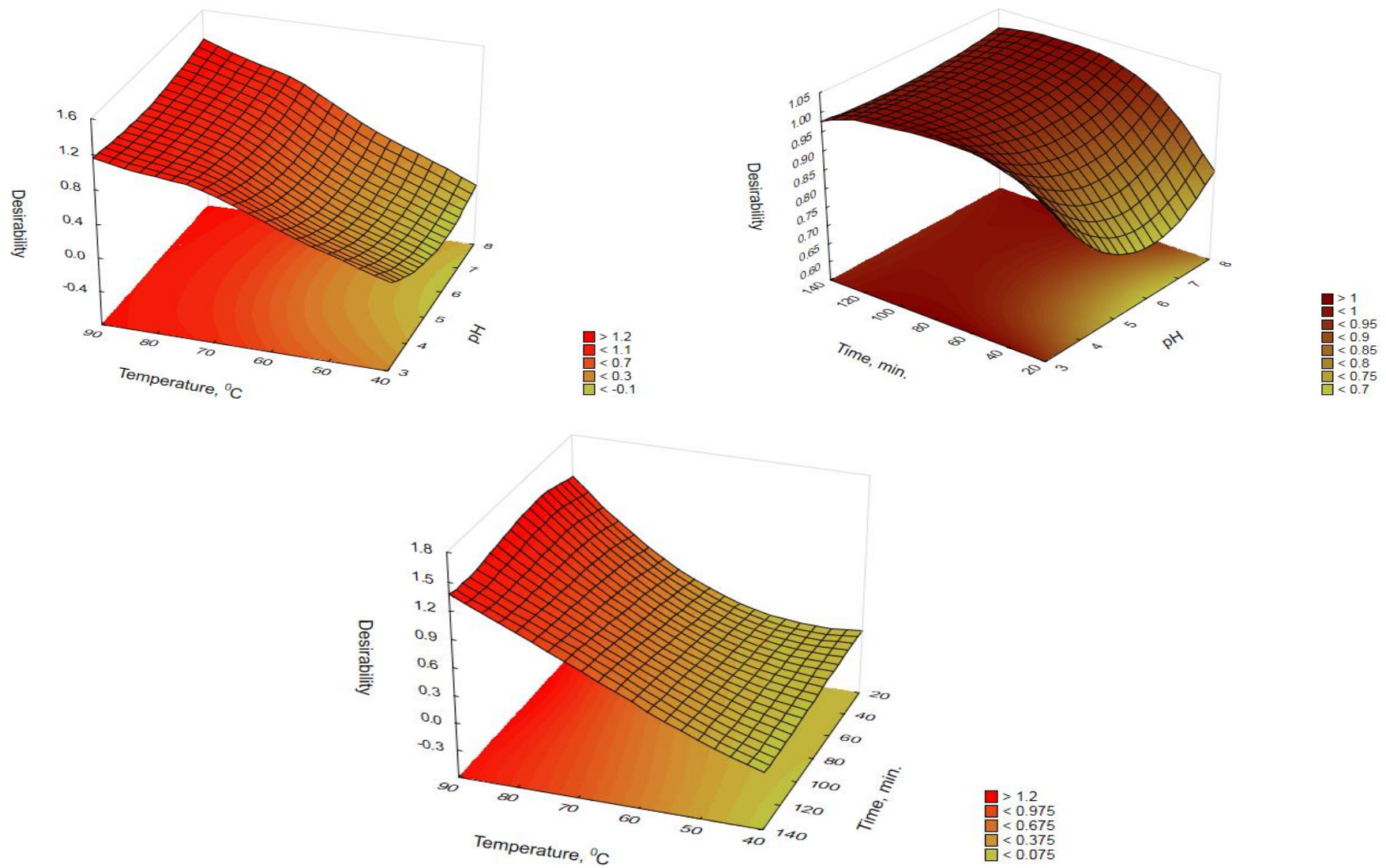
Factor	Effect estimate	Standard error	P-value
Ash content			
Intercept	235.774	25.761	0.000007
(c) Pure quadratic curvature	-470.348	154.565	0.013949
(x) H_3PO_4 concentration, % v/v	-334.103	51.522	0.000113
(y) Number of extractions	-210.873	51.522	0.002705
(z) Ratio of solution (v) to raw material (w)	-235.323	51.522	0.001352
xyz Interaction	143.898	51.522	0.020953
Hydroxyproline content			
Intercept	46.0813	1.147	0.000000
(c) Pure quadratic curvature	44.0375	6.880	0.000125
(x) H_3PO_4 concentration, % v/v	26.3625	2.293	0.000001
(y) Number of extractions	17.1875	2.293	0.000037
(z) Ratio of solution (v) to raw material (w)	24.3625	2.293	0.000002
xyz Interaction	-15.4125	2.293	0.000087

Table B2: Demineralisation of monkfish bones ANOVA for the ash content response

Sources of variation	Sum of squares	DF	Mean square	F-value	P-value
(c) Pure quadratic curvature	98 323	1	98 323.0	9.26006	0.013949
(x) H_3PO_4 concentration, % v/v	446 498	1	446 497.9	42.05115	0.000113
(y) Number of extractions	177 869	1	177 868.8	16.75168	0.002705
(z) Ratio of solution (v) to raw material (w)	221 507	1	221 506.7	20.86149	0.001352
xyz Interaction	82 826	1	82 826.0	7.80054	0.020953
Lack of fit	77 582	3	25 860.8	2.43557	0.131705
Pure error	95 562	9	10 618.0		
Total sum of squares	1 200 167	17			

Factor	Coefficient estimate	DF	Standard error	- 95% Confidence limit	+ 95% Confidence limit
Intercept	1 696.798		218.5489	1 202.406	2 191.190
(c) Pure quadratic curvature	-235.174	1	77.2827	-409.999	-60.348
(x) H_3PO_4 concentration, % v/v	-179.994	1	31.4135	-251.056	-108.932
(y) Number of extractions	-185.501	1	40.0665	-276.137	-94.864
(z) Ratio of solution (v) to raw material (w)	-147.067	1	31.4135	-218.130	-76.005
xyz Interaction	6.536	1	2.5051	0.869	12.203

Table B3: Demineralisation of monkfish bones ANOVA for the hydroxyproline content response

Sources of variation	Sum of squares	DF	Mean square	F-value	P-value
(c) Pure quadratic curvature	861.912	1	861.912	40.9686	0.000125
(x) H_3PO_4 concentration, % v/v	2 779.926	1	2 779.926	132.1362	0.000001
(y) Number of extractions	1 181.641	1	1 181.641	56.1661	0.000037
(z) Ratio of solution (v) to raw material (w)	2 374.126	1	2 374.126	112.8476	0.000002
xyz Interaction	950.181	1	950.181	45.1643	0.000087
Lack of fit	5.087	3	1.696	0.0806	0.968921
Pure error	189.345	9	21.038		
Total sum of squares	8 342.216	17			

Factor	Coefficient estimate	DF	Standard error	- 95% Confidence limit	+ 95% Confidence limit
Intercept	-54.5044		9.7282	-76.5112	-32.4977
(c) Pure quadratic curvature	22.0188	1	3.4401	14.2368	29.8007
(x) H_3PO_4 concentration, % v/v	11.0197	1	1.3983	7.8565	14.1829
(y) Number of extractions	11.1980	1	1.7835	7.1635	15.2325
(z) Ratio of solution (v) to raw material (w)	10.3530	1	1.3983	7.1898	13.5162
xyz Interaction	-0.2126	1	0.1115	-0.4648	0.0397

Table B4: Factor effect estimates for the precipitation of DCP from mineral liquor with yield, P content and Ca:P molar ratio as the response variables

Factor	Effect estimate	Standard error	P-value
Yield			
Intercept	17.189	0.2170	0.000000
(u) Reaction temperature, °C	-1.475	0.2447	0.000014
(u^2) Reaction temperature, °C	-1.160	0.2725	0.000533
(v) 1 M $Ca(OH)_2$:mineral liquor (v:v)	19.654	0.2447	0.000000
(v^2) 1 M $Ca(OH)_2$:mineral liquor (v:v)	-0.904	0.2725	0.004066
uv Interaction	-2.435	0.3185	0.000001
P content			
Intercept	199.771	0.9419	0.000000
(u) Reaction temperature, °C	30.086	1.461	0.000000
(u^2) Reaction temperature, °C	14.532	1.557	0.000000
(v) 1 M $Ca(OH)_2$:mineral liquor (v:v)	-42.883	1.461	0.000000
(w) Reaction time, minutes	1.408	1.449	0.344912
uv Interaction	-7.938	1.902	0.000638
uw Interaction	5.663	1.902	0.008467
Ca:P molar ratio			
Intercept	0.9849	0.004411	0.000000
(u) Reaction temperature, °C	-0.01795	0.004973	0.002164
(u^2) Reaction temperature, °C	-0.01557	0.005538	0.012026
(v) 1 M $Ca(OH)_2$:mineral liquor (v:v)	0.1404	0.004973	0.000000
(v^2) 1 M $Ca(OH)_2$:mineral liquor (v:v)	0.1464	0.005538	0.000000
uv Interaction	0.01625	0.006474	0.022480

Table B5: Precipitation of DCP from mineral liquor ANOVA for the yield response

Sources of variation	Sum of squares	DF	Mean square	F-value	P-value
(u) Reaction temperature, °C	14.739	1	14.739	36.315	0.000014
(u^2) Reaction temperature, °C	7.353	1	7.353	18.116	0.000533
(v) 1 M $Ca(OH)_2$:mineral liquor (v:v)	2 618.035	1	2 618.035	6 450.418	0.000000
(v^2) 1 M $Ca(OH)_2$:mineral liquor (v:v)	4.469	1	4.469	11.011	0.004066
uv Interaction	23.717	1	23.717	58.435	0.000001
Lack of fit	11.464	9	1.274	3.138	0.020296
Pure error	6.900	17	0.406		
Total sum of squares	2 684.121	31			

Factor	Coefficient estimate	DF	Standard error	- 95% Confidence limit	+ 95% Confidence limit
Intercept	-21.6574		2.1573	-26.2090	-17.1058
(u) Reaction temperature, °C	0.3980	1	0.06594	0.2589	0.5371
(u^2) Reaction temperature, °C	-0.0026	1	0.000606	-0.0039	-0.0013
(v) 1 M $Ca(OH)_2$:mineral liquor (v:v)	53.3169	1	2.7911	47.4282	59.2057
(v^2) 1 M $Ca(OH)_2$:mineral liquor (v:v)	-5.0236	1	1.5139	-8.2177	-1.8295
uv Interaction	-0.2706	1	0.03539	-0.3452	-0.1959

Table B6: Precipitation of DCP from mineral liquor ANOVA for the P content response

Sources of variation	Sum of squares	DF	Mean square	F-value	P-value
(u) Reaction temperature, °C	6 135.05	1	6 135.05	423.8118	0.000000
(u^2) Reaction temperature, °C	1 261.49	1	1 261.49	87.1441	0.000000
(v) 1 M $Ca(OH)_2$:mineral liquor (v:v)	12 463.88	1	12 463.88	861.0102	0.000000
(w) Reaction time, minutes	13.66	1	13.66	0.9438	0.344912
uv Interaction	252.02	1	252.02	17.4093	0.000638
uw Interaction	128.26	1	128.26	8.8600	0.008467
Lack of fit	782.87	8	97.86	6.7602	0.000487
Pure error	246.09	17	14.48		
Total sum of squares	21 284.02	31			

Factor	Coefficient estimate	DF	Standard error	- 95% Confidence limit	+ 95% Confidence limit
Intercept	270.4817		13.7419	241.4888	299.4746
(u) Reaction temperature, °C	-2.0621	1	0.40877	-2.9245	-1.1996
(u^2) Reaction temperature, °C	0.0323	1	0.00346	0.0250	0.0396
(v) 1 M $Ca(OH)_2$:mineral liquor (v:v)	-27.3741	1	10.8457	-50.2565	-4.4916
(w) Reaction time , minutes	-0.3493	1	0.13009	-0.6238	-0.0749
uv Interaction	-0.8819	1	0.21137	-1.3279	-0.4360
uw Interaction	0.0076	1	0.00254	0.0022	0.0129

Table B7: Precipitation of DCP from mineral liquor ANOVA for the Ca:P molar ratio response

Sources of variation	Sum of squares	DF	Mean square	F-value	P-value
(u) Reaction temperature, °C	0.002184	1	0.002184	13.0275	0.002164
(u^2) Reaction temperature, °C	0.001325	1	0.001325	7.9013	0.012026
(v) 1 M Ca(OH)$_2$:mineral liquor (v:v)	0.133623	1	0.133623	797.0516	0.000000
(v^2) 1 M Ca(OH)$_2$:mineral liquor (v:v)	0.117196	1	0.117196	699.0630	0.000000
uv Interaction	0.001056	1	0.001056	6.3004	0.022480
Lack of fit	0.002500	9	0.000278	1.6570	0.176819
Pure error	0.002850	17	0.000168		
Total sum of squares	0.279697	31			

Factor	Coefficient estimate	DF	Standard error	- 95% Confidence limit	+ 95% Confidence limit
Intercept	1.2263		0.04385	1.1338	1.3188
(u) Reaction temperature, °C	0.001597	1	0.001340	-0.00123	0.004425
(u^2) Reaction temperature, °C	-0.000035	1	0.000012	-0.00006	-0.000009
(v) 1 M Ca(OH)$_2$:mineral liquor (v:v)	-0.9952	1	0.05673	-1.1149	-0.87550
(v^2) 1 M Ca(OH)$_2$:mineral liquor (v:v)	0.8135	1	0.03077	0.74860	0.87843
uv Interaction	0.001806	1	0.000719	0.00029	0.003323

Table B8: Factor effect estimates for the extraction of gelatin from monkfish ossein with hydroxyproline yield as the response variable

Factor	Effect estimate	Standard error	P-value
Intercept	0.1692	0.008069	0.000000
(x) Extraction pH	-0.05614	0.009097	0.000010
(x^2) Extraction pH	0.07960	0.01013	0.000000
(y) Extraction temperature, °C	0.2317	0.009097	0.000000
(y^2) Extraction temperature, °C	0.05260	0.01013	0.000073
(z) Extraction time, minutes	0.07940	0.009097	0.000000
yz Interaction	0.03100	0.01184	0.018002

Table B9: Extraction of gelatin from monkfish ossein ANOVA for the hydroxyproline yield response

Sources of variation	Sum of squares	DF	Mean square	F-value	P-value
(x) Extraction pH	0.02136	1	0.02136	38.08	0.000010
(x^2) Extraction pH	0.03463	1	0.03463	61.74	0.000000
(y) Extraction temperature, °C	0.3639	1	0.3639	648.77	0.000000
(y^2) Extraction temperature, °C	0.01512	1	0.01512	26.96	0.000073
(z) Extraction time, minutes	0.04273	1	0.04273	76.18	0.000000
yz Interaction	0.003844	1	0.003844	6.853	0.018002
Lack of fit	0.006565	8	0.000821	1.463	0.241808
Pure error	0.009536	17	0.000561		
Total sum of squares	0.4877	31			

Factor	Coefficient estimate	DF	Standard error	- 95% Confidence limit	+ 95% Confidence limit
Intercept	1.5229		0.3070	0.8753	2.171
(x) Extraction pH	-0.2133	1	0.02495	-0.2659	-0.1607
(x^2) Extraction pH	0.01769	1	0.002251	0.01294	0.02244
(y) Extraction temperature, °C	-0.03364	1	0.008309	-0.05117	-0.01611
(y^2) Extraction temperature, °C	0.000325	1	0.000063	0.000193	0.000457
(z) Extraction time, minutes	-0.002408	1	0.001433	-0.005432	0.000616
yz Interaction	0.000057	1	0.000022	0.000011	0.000104

Appendix C: Hydroxyproline assay procedure

AOAC Official method 990.26 (Hydroxyproline in meat and meat products: colorimetric method)

Preparation of buffer solution

This solution was prepared by dissolving 30 g citric acid monohydrate (≥ 98%, Sigma-Aldrich), 15 g NaOH (Emparta, Merck), and 90 g sodium acetate trihydrate (≥ 99%, Sigma-Aldrich) in approximately 500 ml water. Afterwards, 290 ml 1-propanol (≥ 99.9%, Sigma-Aldrich) was added and then the pH of the solution was adjusted to pH 6 by adding acid or base. The solution was transferred to a 1 L volumetric flask and diluted to volume with water. The solution was then transferred to a dark bottle, stored in the refrigerator at 4 °C and used within two months.

Preparation of oxidant solution

This solution was prepared by dissolving 1.41 g chloramine-T hydrate (95%, Aldrich) in 100 ml buffer solution. The solution was transferred to a dark bottle, stored in the refrigerator at 4 °C and used within a week.

Preparation of colour reagent

This solution was prepared by dissolving 10 g 4-dimethylaminobenzaldehyde (≥ 99%, Fluka Analytical) in 35 ml 60% w/w perchloric acid (70% assay, Merck). Thereafter, 65 ml 2-propanol (LC-MS Chromasolv, Fluka Analytical) was slowly added with mild agitation. The reagent was used within a day of preparation.

Hydrolysis of sample

Duplicate sample portions of 4 g were weighed to the nearest 0.0001 g into an Erlenmeyer flask. Afterwards, 30 ml 3.5 M H_2SO_4 (3.5 M, Kimix) was added to the sample and the flask was covered with watch glass and placed in a 105 ± 1 °C drying oven for 16 hours. Thereafter, the hot hydrolysate was quantitatively transferred to a 500 ml volumetric flask using water and diluted to volume with water. The solution was filtered using an ash-free filter paper (Macherey-Nagel, grade MN 640 d). The sample filtrate was stored in the refrigerator at 4 °C and was analysed within two weeks.

Colour development and measurement of the sample

The filtrate was diluted with water in a volumetric flask so that the hydroxyproline concentration of the final dilution was in the range of the hydroxyproline standard concentration given in the linear plot calibration (see Figure A2). From the final dilution, 2 ml was extracted and transferred to a test tube and into another test tube, 2 ml of water was pipetted as blank. To each test tube, 1 ml of oxidant solution was added. The test tubes were manually shaken for 1 minute and left to stand for 20 ± 2 minutes at room temperature. Thereafter, 1 ml of colour reagent was added to each test tube and the test tubes were manually shaken for 2 minutes. The test tubes were capped with aluminium foil and immediately placed in a 60 °C ± 0.5 °C water bath (Labotec Model 132, South Africa) for exactly 15 minutes. The

tubes were then cooled under running tap water for three minutes. The absorbance of the sample solution was measured against the blank in 10 mm glass cells at 558 ± 2 nm with aUV Vis Spectrophotometer (AandE Lab Model AE-S60-4U, UK).

Appendix D: Hydroxyproline yield formula

The following formula was used to calculate the hydroxyproline yield for the gelatin extraction from monkfish ossein experimental runs:

$$Hydroxyproline\ yield, \% = \frac{hydroxyproline\ content\ in\ gelatin\ liquor\ \left(\frac{g}{ml}\right) x\ total\ volume\ of\ gelatin\ liquor\ (ml)}{weight\ of\ monkfish\ ossein\ used\ (g\ dm)}\ x\ 100$$

Appendix E: Preparation of sample for total phosphorus determination

AOAC Official method 957.02 (Total phosphorus in fertilizers: preparation of test solution)

Procedure

A sample portion of 1 g was boiled gently for 30 – 45 minutes with 20 – 30 ml HNO_3 (65%, Merck) in a suitable flask to oxidise all oxidisable matter and then cooled. The mixture was added with 10 – 20 ml $HClO_4$ (70%, Merck) and boiled very gently until white fumes appeared in the flask. The solution was cooled, added with 50 ml demineralised water and boiled for a few minutes. Thereafter, the solution was cooled and transferred to a 250 ml volumetric flask, added to the mark with demineralised water and mixed. The solution was filtered using an ash-free filter paper (Macherey-Nagel, grade MN 640 d), and a sample portion of the filtrate was taken out for P content analysis.

Appendix F: Procedure to extract phosphorus soluble in water

AOAC Official method 977.01 (Water-soluble phosphorus in fertilizers: preparation of test solution)

Procedure

A sample portion of 1 g was placed on ash-free filter paper (Macherey-Nagel, grade MN 640 d) and washed with demineralised water until the filtrate measured approximately 250 ml. The demineralised water was added in fine stream directed around the periphery of the filter paper in a circular path, ensuring that the water and solid sample were thoroughly mixed with each addition. Each portion was ensured to pass through the filter paper before more water was added. The filtrate was transferred to a 250 ml volumetric flask, added to the mark with demineralised water and mixed. A sample portion of the solution was taken out for P content analysis.

Appendix G: Procedure to extract phosphorus soluble in 2% citric acid

EC no. 2003/2003 method 3.1.3 (Extraction of phosphorus soluble in 2% citric acid)

Preparation of 2% citric acid solution

This solution was prepared by diluting 20.0001 g citric acid monohydrate (≥ 98%, Sigma-Aldrich) to 1 000 ml with demineralised water. The concentration of the citric acid solution was verified by titrating 10 ml of the solution with 28.9 ml of standard solution of 0.1 M sodium hydroxide, using three drops of phenolphthalein as an indicator.

Extraction of phosphorus procedure

A sample portion of 5 g was weighed to the nearest 0.0001 g into an Erlenmeyer flask. Afterwards, 500 ml 2% citric acid solution was added to the sample. When the first millilitres of the solution were added, the mixture in the flask was shaken vigorously by hand to stop the formation of lumps and to prevent the sample from sticking to the sides. The flask was sealed with a rubber stopper and shaken in a rotary shaker (SMC Model 36L unit, South Africa) at 38 turns per minute for exactly 30 minutes at a temperature of 20 ± 2 °C. The solution was filtered using an ash-free filter paper (Macherey-Nagel, grade MN 640 d), discarding the first 20 ml of the filtrate, and a sample portion of the collected filtrate was analysed for P content.

Appendix H: Procedure to extract phosphorus soluble in alkaline ammonium citrate

EC no. 2003/2003 method 3.1.5.1 (Extraction of soluble phosphorus according to Petermann at 65 °C)

Preparation of alkaline solution of ammonium citrate (Petermann)

This solution was prepared by dissolving 86.5005 g citric acid monohydrate (≥ 98%, Sigma-Aldrich) in 250 ml of demineralised water in a 500 ml Erlenmeyer flask. The flask was then placed in an ice bath, and 132.15 ml of NH_4OH (25% assay, Kimix) was slowly added to the citric acid solution using a glass funnel, the stem of which was immersed in the solution while shaking the flask constantly. The temperature of the resultant solution was then adjusted to 20 °C using a hot water bath. The solution was transferred to a 500 ml volumetric flask and added to the mark with demineralised water. The solution contained 41.9 g/L of ammoniacal nitrogen and 173 g/L citric acid, with a pH of 9.46 at 21.4 °C: the characteristics of a Petermann's solution.

Extraction of phosphorus procedure

A sample portion of 1 g was weighed to the nearest 0.0001 g into an Erlenmeyer flask. Afterwards, 200 ml Petermann's solution was added to the sample. The flask was sealed with a rubber stopper and shaken vigorously by hand to avoid the formation of lumps and to prevent any adherence of the substance to the sides. The flask was placed in the water bath (Labotec Model 132, South Africa) at 65 °C and shaken every 5 minutes during the first 30 minutes. Thereafter, the flask was shaken every 10 minutes for a further 60 minutes. After each shaking, the rubber stopper on the flask was raised gently to equilibrate the pressure therein. The level of water in the water bath was ensured to be above the level of solution in the flask. The flask was removed and cooled to a temperature of about 20 °C. The solution was transferred to a 500 ml volumetric flask, added to the mark with demineralised water and mixed. The solution was filtered using an ash-free filter paper (Macherey-Nagel, grade MN 640 d), discarding the first 20 ml of the filtrate, and a sample portion of the collected filtrate was analysed for P content.