UNIVERSITY OF NAIROBI

DETERMINATION OF THE AMINO ACID COMPOSITION OF GELATIN EXTRACTED FROM MARINE FISH SCALES AND POLYMER BLENDS CHARACTERIZATION.

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DECLARATION

I declare that this thesis is my original work and has not been submitted elsewhere for examination, award of a degree or publication. Where other people's work, or my own work has been used, this has properly been acknowledged and referenced in accordance with the University of Nairobi's requirement.

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DEDICATION

This work is dedicated to my family for the great support and encouragement.

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ABSTRACT

Whereas marine fish processing in Kenya is on one hand an important contributor to Kenya's food production and economy, it is on the other hand a source of bio waste disposed to the environment. Most of the by products, including fish skin, scales and bones, which are disposed as waste, can be utilized in the making of important products hence eliminating the environmental menace they pose. This study sought to utilize scales in the extraction of the protein gelatin. Gelatin in this study was extracted by an enzymatic process from the scales of three marine fish species; *Lutjanus sebea* (Red snapper), *Lethrinus harak* (Black spot emperor) and *Scalus ghobban* (Blue barred parrot fish). Concentration of the bacteria, *Bacillus cereus*, for mass production of enzyme was done in a fermentation medium using a bio reactor. Scales were hydrolysed at 45ºC and the pH maintained at 12. Complete hydrolysis took between 20 and 23 days for all species. The yield for dry gelatin was between 28.2% and 41.4% for the marine fish scales under study. Fourier transform infrared spectra showed the presence of amide bands and two other additional absorption bands indicating the presence of amide bonds for all the three fish species under study. The amino acid composition analysis for gelatin obtained from the fish species showed the presence of 16 amino acids. Glycine was the most abundant (35%), followed by Alanine, both adding up to around 50% of the total amino acid composition for all three marine fish species studied. Proline was higher for red snapper at over 14.2% compared to 11.1% and 11.6% for blue barred parrot fish and black spot emperor respectively. Polymer blends of three different compositions were prepared comprising of gelatin obtained from blue barred parrot fish scales and PLA. Thermal analysis of these polymer blends was conducted via TGA and DSC. Miscibility and compatibility were confirmed but were dependent on PLA/gelatin composition. The good yield of gelatin obtained in this study shows that alkaline protease was effective in the extraction of gelatin from the scales of the three marine fish species and that the scales are suitable sources of gelatin. This study also showed an improvement caused by polymer blending with the TGA analysis of the three polymer blends of amorphous PLA and gelatin showing slower rates of thermal decomposition than either of the polymers before blending.

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LIST OF ABBREVIATIONS

ASS	–	Amylase soluble starch
ApSS	–	Amyl pectin soluble starch
BBPF	–	Blue barred parrot fish
BSE	–	Black spot emperor
DSC	–	Differential Scanning Calorimetry
EAI	–	Emulsion Activity Index
ESI	–	Emulsion Stability Index
FPI	–	Fish protein isolate
FSG	–	Fish skin gelatin
FTIR	–	Fourier Transform Infra-Red Spectroscopy
GAH	–	Gelatin from Alkaline protease extraction
Gly	–	Glycine
GWE	–	Gelatin from water extraction
Hyp	–	Hydroxyproline
PLA	–	Poly lactic acid
Pro	–	Proline
RS	–	Red snapper
SD	–	Standard deviation
T_g	–	glass transition temperature
TGA	–	Thermogravimetric analysis
UV	–	Ultra violet radiation

CHAPTER ONE : INTRODUCTION

1.1 Background

1.1.1 Marine fish landing statistics in Kenya

Fishery, both marine and fresh water fishery, is a major economic activity in Kenya. Previously synonymous with certain communities living close to the large water bodies, fishing activities are now embraced by a large percentage of the population, including those in the highland regions in the form of fish farming. According to the annual fisheries statistical bulletin 2014 released by the state department of fisheries under the ministry of agriculture, livestock and fisheries of the government of Kenya, the overall marine fish production per annum in Kenya ranges from 7000-9000 metric tons, with the value of fish maintaining an upward trend as shown in Figure 1.1.

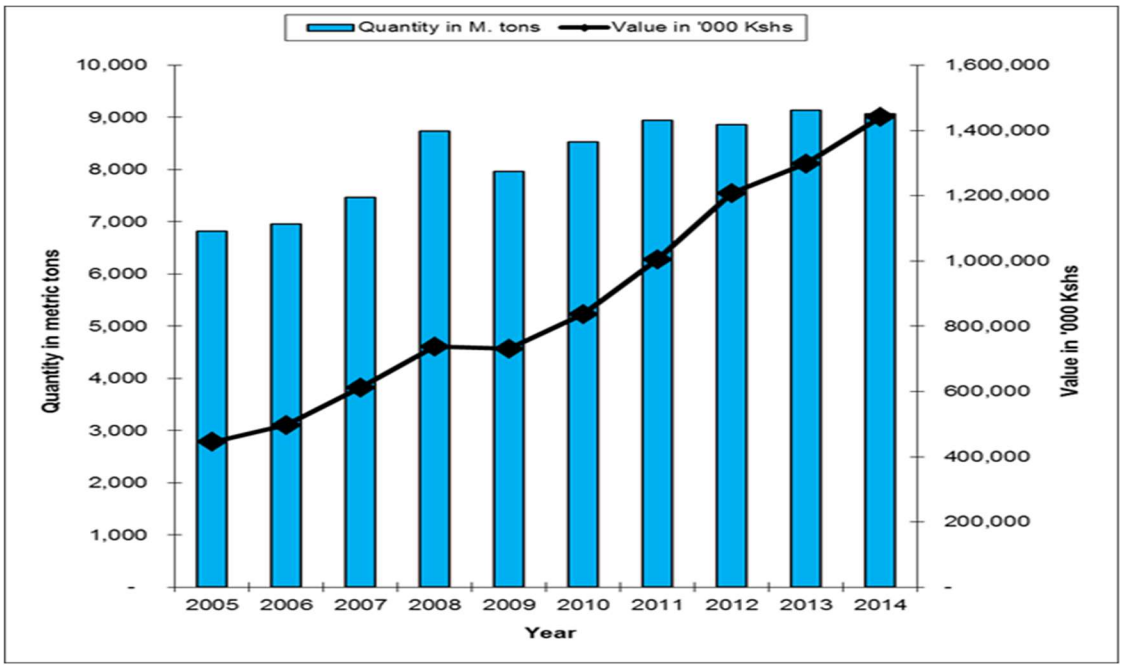

Figure 1.1: Trends of marine fish production by quantity and value 2004-2014

(Source: Fisheries Annual Statistical Bulletin, 2014)

Marine fishery on the Kenyan coast is carried out in Kale, Lama, Killifish, Mombasa and Tina River counties. Near shore landings, which is artisan and semi- industrial fishery, is carried out by the local communities while off shore landings, which is industrial fishery, is carried out by foreign industries. Table 1.1 lists the major marine fish species, both demurral and pelagic, landed in these counties as reported in the annual fisheries statistical bulleting of 2014.

Table 1.1: Marine fish landing by Species, Weight and Value and by Counties 2014

Species	Lamu		Tana River		Kilifi		Mombasa		Kwale	
DEMERSAL	M. Tons	000 Kshs	M. Tons	000 Kshs	M. Tons	000 Kshs	M. Tons	000 Kshs	M. Tons	000 Kshs
Rabbit fish	331	26,831	5	411	121	22,353	89	21,926	176	26,697
Scarvenger	290	28,560	9	1,094	148	31,050	68	17,970	171	28,359
Snapper	96	10,670	22	2,501	57	9,139	41	11,016	103	14,473
Parrot fish	257	22,299	0	26	62	11,702	51	9,255	137	20,704
Surgeon fish	16	1,558	3	251	67	7,803	3	876	41	5,704
Unicorn fish	16	1,605	-	-	100	13,822	15	3,593	44	5,166
Grunter	50	3,760	1	107	20	3,000	58	10,045	30	3,838
Pouter	79	7,960	-	-	17	3,665	52	7,643	46	6,709
Black skin	94	8,281	-	-	19	2,792	3	917	78	9,690
Goat fish	48	3,648	-	-	7	1,056	28	6,069	33	4,691
Steaker	3	321	-	-	10	1,591	-	-	19	2,173
Rock cod	48	4,405	9	924	38	5,483	10	2,425	54	7,391
Cat fish	62	4,501	36	3,229	30	5,229	14	3,267	42	5,486
Mixed dermasal	401	36,495	53	5,506	299	45,431	27	7,174	160	20,917
PELAGICS										
Crevallae jacks	64	5,112	21	1,683	39	6,744	39	8,406	79	9,411
Mullets	134	13,206	18	1,845	65	11,924	29	5,422	65	9,739
Little mackerels	-	-	3	303	204	28,522	57	8,539	184	20,913
Barracudas	63	5,031	4	414	70	10,510	64	13,928	101	12,533
Milk fish	33	2,690	-	-	30	4,858	0	65	19	2,949
King fish	10	962	14	2,084	60	10,591	21	4,606	22	3,736
Queen fish	28	2,252	42	6,727	30	5,246	29	4,643	23	2,410
Sail fish	8	760	3	367	127	21,455	28	6,224	10	1,520
Tunny	19	1,714	2	62	72	12,231	13	2,793	88	12,286
Dolphin fish	-	-	23	2,570	14	2,541	-	-	6	1,088
Mixed Pelagics	18	1,650	-	-	217	32,300	5	1,045	188	19,117

(Source: Fisheries Annual Statistical Bulletin, 2014)

The fish filleting processes that follow are accompanied by production of large amount of by products such as fish skin, bones, heads, scales and fins. It is estimated that of the total dry weight of fish, 75% accounts for these by products (Zakaria and Bakar, 2015). This is to say that, with the aforementioned high marine fish production, if no plans are in existence on how to utilize these by products, they then end up as bio waste and environmental pollutant. Currently a large percentage of these by products in Kenya are being disposed of as waste. Whereas a portion of this disposal is done in landfills, most of the fish processing stations dispose this waste in the environment both on land and in the drainage systems or nearby water bodies.

One way of solving this environmental problem caused by the disposal of the fish filleting waste is by improving the disposal of waste by the Government imposing stringent penalties on the involved industries. The other way is by value adding these bio wastes into valuable products that are in high demand in the market. Fish scales have been reported to contain an important protein called collagen (Jiang, 2013). This study therefore seeks to utilize marine fish scales in the production of gelatin derived from collagen.

Figure 1.2: A photograph of *Latrines hark* (Black spot emperor)

Lethrinus harak (black spot emperor) in Figure 1.2 is a marine fish species said to be benthic carnivore that is widely distributed in the shallow waters of Indian Ocean and western pacific (Nanaimo and Yamada, 2009).

Figure 1.3: A photograph of *Lutjanus setae* (Red snapper)

Lutjanus sebea (Red snapper) in Figure 1.3 is also a marine fish species found in the western pacific and the Indian Ocean and benthic carnivore, inhibits shallow near-shore waters (about 1m) to the depths of at least 150m (Newman and Dunk, 2002).

Figure 1.4: A photograph of *Scarus ghobban* (Blue barred parrot fish)

Scarus ghobban (Blue barred parrot fish) in Figure 1.4 from the parrot fish family are said to feed in groups, on the shallow benthic algae from dead coral. It attains on average a length size of 100 cm (Amin et al., 2002).

These three species are the marine fish species under this study selected due to the good scale cover and abundance in Kenyan shores of the Indian Ocean.

1.1.2 Gelatin

Gelatin has been an important name in households and industries globally. It is currently not produced locally but imported into the country. United Nations commodity trade database reported that as at 2010, Kenya spent close to 600,000 US dollars on gelatin importation only. Originally used to make glue, it has with time become a valuable raw material in many industries including food, material, pharmaceutical and photography industries, (Ahmad and Benjakul, 2011; Kim *et al.*, 2014). In the food industry for instance, which accounts for the biggest portion of gelatin's commercial application, it is utilized in the production of confectionary, jelled meat, bakery products and dairy products to provide water binding, gelling, texturization and stabilization (August, 1999; Ardekani *et al.*, 2013; Aisyah *et al.*, 2014). In pharmaceutical industries on the other hand, gelatin is used in making tablets, binding capsules, vitamin encapsulation and in making homeostatic sponges, while in photography, gelatin is used in film making (Aisyah *et al.*, 2014). Other fields where gelatin has found application are medicine and cosmetic products (Wangtueai and Noomhorm, 2009).

In appearance gelatin is a slightly yellow, odorless, brittle solid commercially available in granular powder form (Jaswir *et al.*, 2009). It dissolves in warm water and few highly polar organic solvents such as acetic acid, trifluoro ethanol and hexafluoro isopropanol (Finch and

Jobling, 1977). The commercial applicability in the earlier noted industries is mainly determined by its solubility, gel strength, viscosity, setting temperature and melting point (Mahmoodani *et al.*, 2014) with its ability to transform from sol to gel under low temperatures making it unique. The gel strength and the thermal stability are in turn dependent on the molecular weight distribution as well as the amino acid composition (Kim *et al.*, 2014).

Gelatin is a protein that does not occur naturally but is obtained by partial hydrolysis of collagen (Jaswir *et al.*, 2009). This collagen is a key structural protein in animals; in human beings for example, collagen accounts for one third of the total protein (Shoulders and Raines, 2009). Gelatin that is available in the market is mainly obtained from skin/hide, bones, tendons and ligaments of pig (porcine) and cattle (bovine) (Zakaria and Bakar, 2015). However, over the last couple of years, there has been health concerns and the religious restrictions facing this mammalian sourced gelatin, leading to the recent introduction of non-mammalian sources, that is, fish gelatin (fish skin and bones) and poultry gelatin (feathers, skin and feet).

1.1.3 Extraction process of gelatin

Gelatin extraction from collagen can either be done by chemical use, that is, using dilute acid or dilute base, or by an enzymatic process. A greater percentage of the gelatin that is available in the market is extracted via chemical means which is undertaken in two main steps. The first step is pre-treatment, carried out by either of two processes, that is, acid pretreatment (dilute Hydrochloric acid, Sulphuric acid, phosphoric acid, or acetic acid) or alkaline pretreatment (dilute Sodium hydroxide or Calcium hydroxide) (Jongjareonrak *et al.*, 2006). The choice between these two processes depends on the extent of cross linking in the mother collagen such that in fish skin and pig skin, where there is less cross linking, acid pretreatment

is used while alkaline pretreatment is used in cattle hide and bones where there is high cross linking (Herpandi *et al.*, 2011). Acid pretreatment yields type A gelatin with isoionic point of 6-9 while alkaline pretreatment yields type B gelatin with isoionic point of 5 (Jongjareonrak *et al.*, 2006). Isoionic point of a protein is the pH at which the amino acids present have a neutral charge on them; that is to say, the amino acids are neither basic nor acidic and the net charge is zero. It is important to know the isoionic point of gelatin as most of its applications involve production of consumable products an area in which charged species are often avoided due to the possibility of formation of free radicals in the body. Extraction of gelatin from the pretreated raw material is the second step done in hot water to convert insoluble collagen to soluble gelatin (Liu *et al.*, 2012).

In an enzymatic extraction process, enzymes are used rather than hot water. Since proteases are enzymes that facilitate the hydrolysis of proteins, they are therefore the enzymes used in the extraction of gelatin. The different types of proteases include; trypsin, neutral protease, papain and alkaline protease (Jiang, 2013). Protease enzymes have numerous industrial applications including leather tannery, detergent industries and pharmaceutical industry, therefore accounting for 60% of all industrial enzyme applications (Sevinc and Demirkan, 2011). However, despite their popularity in other sectors these enzymes have not been keenly considered in the extraction of gelatin for commercial purposes. Protease enzymes are largely produced by bacteria, namely *Bacillus spp*, which are mostly extracellular, with the concentration being done in a fermentation media (Sevinc and Demirkan, 2011). *Bacillus cereus* has been seen to exhibit good protease activity (Wanyonyi *et al.*, 2014) while alkaline protease is said to be the best for gelatin extraction from fish scales, when compared to the other proteases (Jiang, 2013). The extent of the bacterial multiplication in the concentration

process and the eventual enzyme production is dependent on the nutrients present especially the carbon and nitrogen sources, as well as physical factors such as inoculums concentration, temperature, pH and incubation time (Lakshmi *et al.*, 2014).

1.1.4 Polymer

Polymers are categorized into two categories; natural polymers such as proteins and polysaccharides and synthetic polymers for example poly (lactic acid) and poly (glycolic acid) (Gentile *et al.*, 2014). Polymers in either of the categories have advantageous and disadvantageous characteristics that affect their functionalities. Natural polymers have the advantage of being bio-degradable and bio-compatible but also have the disadvantage of being structurally unstable (Vijayakumar and Subramanian, 2014). Synthetic polymers on the other hand, are costly to produce with some being non-biodegradable and thermally unstable. One way of merging the positive attributes of different polymers and minimizing the disadvantages associated with their individual use is by polymer blending. Polymer blending is the mixing of two polymers with the aim of improving their properties depending on whether miscible or immiscible blends are obtained. This way, resultant polymer blends may have advantageous physical and mechanical properties that either of the constituent polymers did not have by itself (Ferreira *et al.*, 2001).

1.2 Statement of the problem

Fish scales despite being valuable fish by products are disposed of as waste. At the marine fish processing plants and markets in Kenya, scales are disposed by being washed off through the drainage system to the environment owing to their light mass. However, these scales are hard and rigid and therefore tend to persist in the environment for a long time before their degradation (Kim *et al.*, 2014). This makes them a nuisance to the populace around the fish filleting sites and an environmental pollutant. A suitable way of utilizing them ought to be sought which will help solve the environmental problem they cause. Further, there are challenges associated with porcine (pig) gelatin and bovine (cattle) gelatin which are the sources dominating gelatins market. Diseases like Bovine Spongiform Encephalopathy (mad cow disease) and foot and mouth disease associated with cattle are some of the health concerns being raised against bovine sourced gelatin (Zakaria and Bakar, 2015). Consumption of porcine products is unacceptable for populations with Muslim and Judaism faith while the consumption of bovine products is not allowed for the Hindus (Ardekani *et al.*, 2013). Key sources of gelatin are reducing due to competition from other industries. For instance, cattle hides, aforementioned as one of the key sources of commercial gelatin, are increasingly being used in leather tannery (August, 1999). There are limitations in using gelatin as a natural polymer by itself in applications like controlled drug delivery due to its high solubility and low thermal stability in the physiological environment (Vijayakumar and Subramanian, 2014).

1.3 Objectives

1.3.1 General objective

To characterize gelatin extracted from marine fish scales and use it in formulation of different polymer blends.

1.3.2 Specific objectives

1. To study and determine the optimal conditions for the extraction process of gelatin from marine fish scales of the different species using enzymes, at specified conditions.
2. To determine the amino acid composition of the gelatin extracted, and its spectrometric analysis.
3. To characterize polymer blends to be prepared, composed of the gelatin extracted and PLA, using TGA and DSC.

1.4 Justification and Significance

Value addition in marine fish scales has not been carefully considered in Kenya. Additionally, marine fish scales have not been studied thoroughly as sources of gelatin and therefore an additional study is required. This study therefore aims at closing these gaps by extracting gelatin from the scales of different marine fish species. Successful development of new technologies for the extraction of gelatin can be economically beneficial to the marine fish filleting stations, in turn reducing waste emanating from them.

Owing to the fact that consumption of gelatin from bovine and porcine sources is prohibited for certain religions, the fact that there are health related reservations held against bovine source and since there is reduction in the availability of some key raw materials for the production of gelatin, a suitable alternative source of gelatin ought to be considered. Marine fish scales are readily available and there are no religious and health concerns that have been raised against them.

The applicability of gelatin in different fields could be improved by blending it with a polymer that is more mechanically and thermally stable that it such as Poly (lactic acid), that though synthetic, it is biodegradable and biocompatible (Onyari *et al.*, 2008).

CHAPTER TWO : LITERATURE REVIEW

2.1 LITERATURE REVIEW

2.1.1 Collagen

Collagen from which gelatin is obtained is the most abundant protein in animals (Muyonga *et al.*, 2004). It is composed of three polypeptide chains joining to form a unique triple helix structure (Gelse *et al.*, 2003). There have been discovered 28 types of collagen (namely collagen I to collagen XXVIII) and 46 types of poly peptide chains, the difference being in the sequencing of amino acids (Shoulders and Raines, 2009). Fish scales contain type I collagen (Zhang *et al.*, 2011). Being a key structural element, collagen's main function is to give the different tissues and organs structural stability (Gelse *et al.*, 2003).

The constituent polypeptide chains are made of a repetitive amino acid sequence in the form Gly-X-Y (Gómez-Guillén *et al.*, 2002). Glycine must appear in this position after every repetition in the sequencing. This is especially possible due to Glycine's small size required for the close packing of the triple helix structure (Shoulders and Raines, 2009). Often, amino acids Proline (Pro) and Hydroxyproline (Hyp) occupy the X and Y positions respectively (Díaz-Calderón *et al.*, 2014), making Gly-Pro-Hyp the most repetitive triplet (Shoulders and Raines, 2009).

As shown in Figure 2.1, the triple helix structure is maintained by the hydrogen bonding between N-H of Glycine in one chain and C=O of the amino acid occupying the X position in another chain (Shoulders and Raines, 2009). The amount of Hydroxyproline also increases the stability of this structure as well as thermal stability via its involvement in inter-chain hydrogen bonding by its OH group (Muyonga *et al.*, 2004). However, for this hydrogen

bonding involving Hydroxyproline to be effective, Hydroxyproline must occupy the Y position in the repetitive Gly-X-Y amino acid sequence (Shoulders and Raines, 2009).

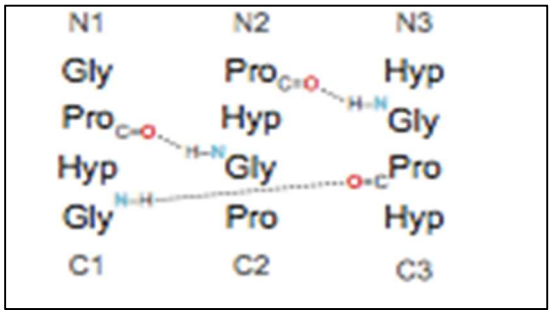

Figure 2.1: Interchain hydrogen bonding in collagen **(Shoulders and Raines, 2009)**

The triple helix structure extends throughout the entire collagen molecule except for short C and N terminal regions, called telopeptide depicted in Figure 2.2, where most of the intra and inter cross linking takes place (Gómez-Guillén *et al.*, 2002). This cross linking is dependent on factors including age, tissue and animal species (Kim *et al.*, 2014). The amino acids involved in cross linking are Lysine and Hydroxylysine as well as their aldehyde derivatives (Gómez-Guillén *et al.*, 2002).

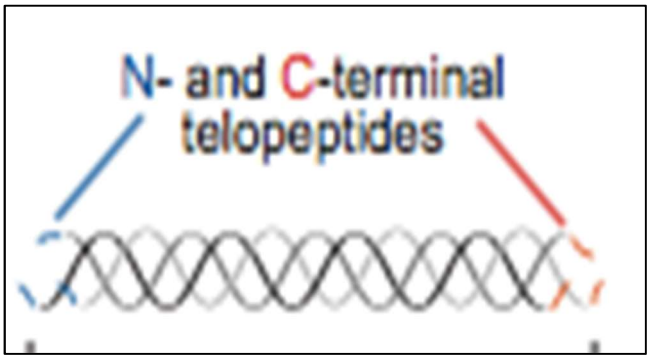

Figure 2.2: Telopeptide regions of collagen **(Shoulders and Raines, 2009)**

2.1.2 Conversion of collagen to gelatin

The key difference between collagen and gelatin is in their structures. In gelatin, the peptide chains are not held in triple helix structure but appear as single coils. This therefore indicates that the conversion process involves the destabilization of the triple helix structure. In other words, the process of converting collagen to gelatin involves denaturing and partially hydrolysing collagen (Mahmoodani *et al.*, 2014). Pre-treatment process aids in the removal of superfluous matter and breaking covalent bonds mainly seen in cross-linked regions (Kim *et al.*, 2014). Extraction process, done either by hot water or enzyme, involves breaking hydrogen bonds that stabilize the triple helix structure (Liu, *et al.*, 2008; August, 1999). The severity of the extraction process in terms of the operating conditions (Temperature, duration and pH) is dependent on the extent of cross linking in the parent collagen (Mahmoodani *et al.*, 2014).

Collagen molecule has a large structure but as the inter chain hydrogen bonds are being broken in the conversion process, amide bonds in the peptide chains are broken as well in a manner that is not homogenous, resulting in gelatin with peptides chains that are smaller but of different sizes (August, 1999). The extraction process would be best if the extent of this peptide chain degradation is low. This would ensure that gelatin obtained has high enough molecular weight and reasonably low molecular weight distribution resulting in good gel strength (Liu, *et al.*, 2008).

Several studies have been done on different ways of extracting gelatin other than the conventional acid/base pre-treatment followed by hot water extraction. Gomez-Guillen *et al* (2005) did a study on the extraction of gelatin from the skin of the fish Dover sole (*Solea vulgaris)* by application of high pressure (250-400 MPa). The aim of the experiment was to

compare the properties of gelatin when the high-pressure application was done at the pre-treatment step done mild acid at 10ºC or when pressure application was done at the extraction step done in water at 45ºC. The results showed that the yield of gelatin was higher when pressurization was done during pre-treatment than in extraction. However, pressurization at the extraction stage gave the preferred high molecular weight gelatin than pressurization at the pre-treatment step. According to them, pressurization at the extraction step gave rise to pressure-induced aggregation increasing the molecular weight. From this study, it is seen that gelatin extracted under high pressure had higher melting point and better renaturation ability upon subsequent cooling than gelatin extracted under acid pre-treatment and hot water extraction without any pressurization (Gómez-Guillén *et al.*, 2005).

Jiang (2013) prepared gelatin from carp fish *(Cyprinus carpio*) scales (a fresh water fish) by mild hydrolysis using enzyme protease. Upon testing different types of protease enzymes; trypsin, neutral protease, papain and alkaline protease, he concluded that alkaline protease gave a good yield of 48.1% (w/w) under hydrolysis conditions of 50ºC, pH of 9 for 6 hours (Jiang, 2013). He compared gelatin from water extraction (GWE) with gelatin from alkaline protease extraction (GAH) and noted a difference in that GWE lead to less hydrolysis of peptide bonds and thus gave high molecular weight gelatin while GAH gave low molecular weight gelatin. He then characterized the two using emulsion activity index (EAI) and emulsion stability index (ESI) using an oil in water interface. He concluded that GAH had higher EAI than GWE but lower ESI. The activity was higher for GAH due to the larger surface area of the gelatin, therefore forming a film around the oil droplet at a higher rate and the stability was lower due to the lower molecular weight of the gelatin (Jiang, 2013).

2.1.3 Gelatin

The properties of gelatin extracted are similar to those of collagen used. Gelatin extracted from different sources such as different tissues or animals of different species is however expected to have different properties. Amino acid composition of gelatin said to be species specific is a good example (Kim *et al.*, 2014). Nearly all of the essential amino acids, whose structures are shown in Figure 2.3, are said to constitute gelatin.

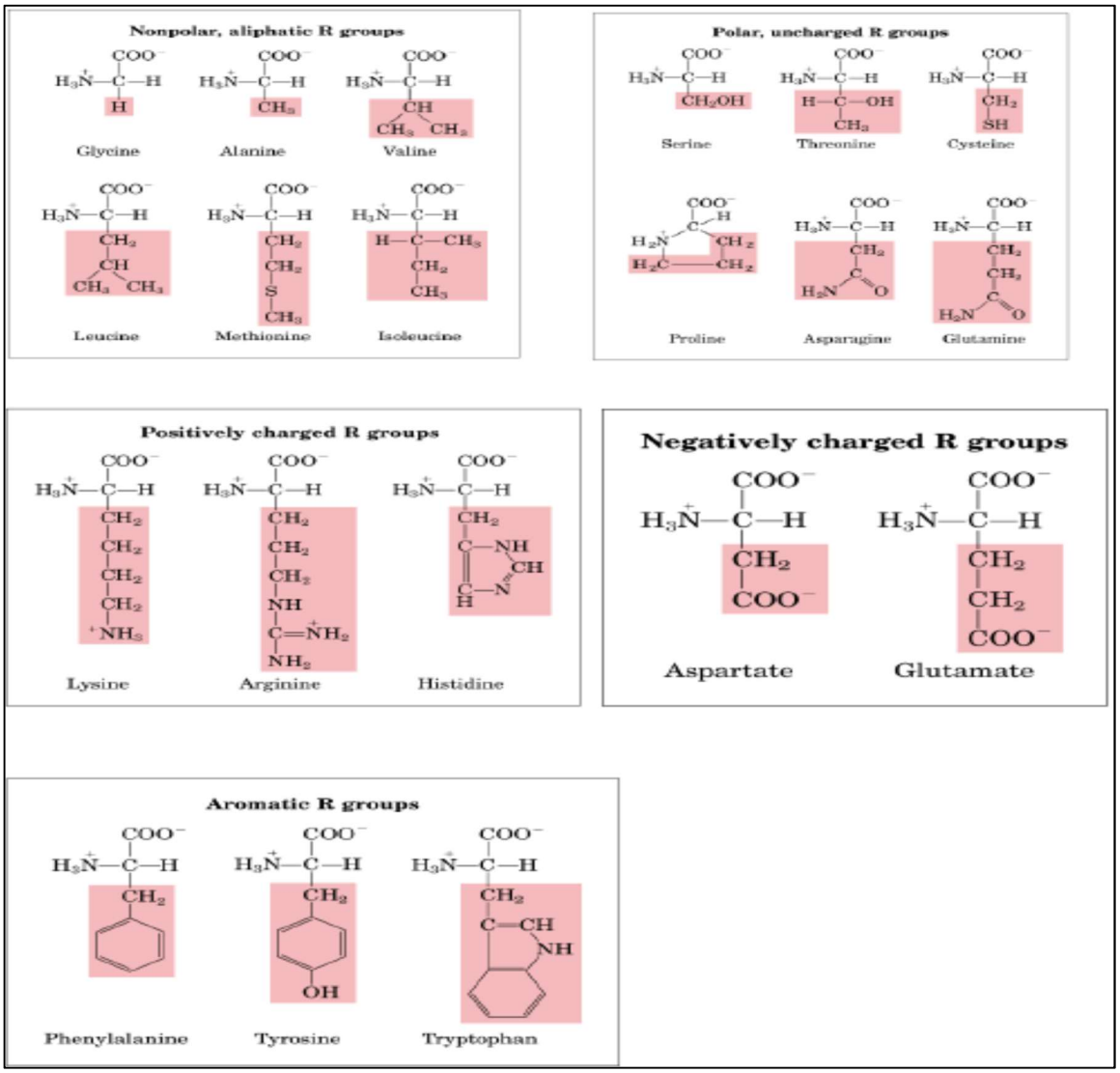

Figure 2.3: Structures of the different amino acids **(Heyrovsky *et. al.*, 2008)**

Table 2.1: Amino acid composition of gelatin from the scales of sea bream fish, big head carp fish and grass carp fish in g/1000g

Amino acid	**Sea bream scales gelatin**	**Big head carp scales gelatin**	**Grass carp fish scales gelatin**	**Porcine skin gelatin**
Hydroxyproline	72	56	70	82
Asparagine/Aspartic acid	45	48	47	46
Threonine	24	27	25	18
Serine	40	33	39	36
Glutamine/Glutamic acid	70	80	77	83
Proline	113	100	87	90
Glycine	345	350	367	355
Alanine	123	125	129	116
Cysteine	1	0	1	1
Valine	17	21	18	24
Methionine	15	16	13	5
Isoleucine	5	12	10	12
Leucine	18	22	21	25
Tyrosine	4	3	4	3
Phenylalanine	14	14	13	12
Histidine	5	6	5	5
Lysine	27	28	25	29
Arginine	50	54	50	53
Hydroxylysine	6	6	0	0

(Zhang *et al.*, 2011; Liu *et al.*, 2012; Akagündüz *et al.*, 2014)

Taking an example of the amino acid composition of gelatin from scales of sea bream fish, big head carp fish and grass carp fish, a variation in the composition from one species to the other is seen as shown in Table 2.1. Although there is the similarity in the general abundance of Glycine, Alanine and Proline as well as in the low contents of Histidine and Tyrosine for gelatin from the scales of the three species, there are variations in the amount of the different amino acids. Additionally, the amount of Hydroxyproline is seen to be higher than the amount of Glutamine/Glutamic acid in gelatin obtained from the scales of sea bream, which is not the case for the other two species. Aside from the species specificity, the environment that the animal from which collagen was sourced is exposed to, also affect the amount of certain amino acids. For instance, in fish gelatin, the amount of Hydroxyproline is dependent on the environmental temperature of the fish habitat, in that, gelatin sourced from fish living in cold environment have lower amounts of the amino acid than those living in warm environments (Muyonga *et al.*, 2004).

Fish gelatin and mammalian gelatin have been seen to have different amino acid composition. Comparing gelatin from porcine and the fish scale gelatin from the species in Table 2.1, it is evident that the contents of some amino acids vary significantly.

One variation is in the amount of Methionine in both gelatins, with the amino acid being in higher contents in fish gelatin than in mammalian gelatin (Zhang et al., 2011). The amount of imino acids Proline and Hydroxyproline is lower in fish gelatin than in mammalian gelatin, which has been seen to result in a reduction in its gel strength, in turn limiting its applicability in different areas (Gómez-Guillén et al., 2002). There are however similarities between the two gelatins including the abundance of Glycine and the richness of Alanine, as well as the negligible amounts of Cysteine (Liu et al., 2012). A general conclusion however is that

gelatins with different amino acid compositions have different setting temperature, melting temperature and gel strength (Liu *et al.*, 2012).

Several comparative studies have also been done on gelatin extracted from different tissues of one fish species. Akagunduz *et al* (2014) characterized gelatin extracted from sea bream bones and scales. Gelatin yield in this study was higher for the scales than bones. The gel strength of gelatin from the scales was as well higher than that from the bones. According to them, even though the difference in the amount of amino acids Proline and Hydroxyproline being the main reason for the difference in gel strength, it could not have been the reason as the amino acid composition was similar in the two tissues. The difference was attributed to the higher protein content in the scales than bones, given that gel strength is dependent on protein concentration (Akagündüz *et al.*, 2014).

Liu *et al,* (2012) sought to characterize pepsin-solubilised collagen from fins, scales, skins, bones and swim bladders of big head carp *(Hypophthalmichthys nobilis)*. From their findings, the skins and swim bladders gave a higher yield than the fins, scales and bones. The amino acid composition for gelatin from the five tissues was similar while the thermal stability of gelatin from the swim bladders and bones was better than that from fins, scales and skins (Liu *et al.*, 2012).

2.1.4 Poly Lactic acid

Poly lactic acid (PLA) is an aliphatic polyester in which the monomer is hydroxyl carboxylic acid (Lactic acid) (Babu *et al.*, 2013). Lactic acid is mainly in three diasterioisomeric forms; pure D-Lactic acid and pure L-Lactic acid which are crystalline with melting points of about 180ºC and DL-Lactic acid which is amorphous with glass transition temperature at 50-57ºC made of equimolar mixtures of D and L lactic acids (Ahmed *et al.*, 2008). Lactic acid is

produced by bacterial fermentation of sugars obtained from renewable sources and the polymerization carried out by direct poly-condensation reaction of the lactic acid monomer to form PLA (Babu *et al.*, 2013). Its properties such as biodegradability, biocompatibility, high mechanical strength and high transparency are some of the reason why it is preferred in applications such as controlled release of drug delivery and food packaging (Ahmad and Benjakul, 2011; Noori and Ali, 2014).

2.1.5 Polymer blends

Polymer blends are classified as either homogeneous or heterogeneous, with heterogeneous polymer blends either partially miscible or immiscible. Miscibility occurs due to the presence of hydrogen bonding or dipole-dipole forces in the polymer mixture (Soliman and Furuta, 2014). Miscible polymer blends have a single phase, are often optically transparent and have one glass transition temperature (T_g), partially miscible polymer blends have partial phase separation and two T_g while immiscible polymer blends have complete phase separation and two T_g (Prud'homme, 1982; Parameswaranpillai *et al.*, 2014). Partially miscible blends, though comprising of two phases and thus heterogeneous, indicate the presence of interactions at molecular level between the two polymers and hence can be said to be compatible (Kolbuk *et al.*, 2013).

Gelatin being a natural polymer has been blended with other natural polymers to make either polysaccharide-protein blends or protein-protein blends. Arfat *et al.* (2014) blended fish protein isolate (FPI) from yellow stripe trevally muscle with fish skin gelatin (FSG) in a bid to reduce the rigidity of FPI caused by strong covalent bonding. According to their findings, it is possible to improve the properties of FPI by blending it with gelatin in the ratio of 1:1

where the gelatin contained 30% glycerol. The blends at this ratio were compatible with the main interaction being via hydrogen bonding (Arfat *et al.*, 2014).

Soliman and Furuta (2014) did a comparative study on the miscibility and mechanical properties between the blends of Amylase soluble starch (ASS) and gelatin and blends of Amyl pectin soluble starch (ApSS) and gelatin. From the DSC in this study, ASS and gelatin blends at 50/50 ratio gave thermal grams that exhibited one endothermic peak missing indicating homogeneity of the films and high miscibility. ApSS/gelatin blends gave thermal grams exhibited melting peaks indicating partial miscibility. It is due to the good miscibility between ASS and gelatin at 50/50 ratio that gave blend better tensile strength and water permeability resistance (Soliman and Furuta, 2014).

CHAPTER THREE : METHODOLOGY

3.1 Sample collection and preparation

The marine fish scales from the three marine fish species were collected separately from sea food markets in Nairobi, Kenya. The fish were obtained from Malindi, along the Kenyan coast. Collection and parking for the scales of all the three species was done separately in plastic bags and transported to the laboratory where they were stored in a freezer at -80ºC.

Species identification was done at the Nairobi National Museum, Nairobi, Kenya with the three marine fish species being identified as; *Lutjanus sebea* (Red snapper)*, Lethrinus harak* (Black spot emperor) and *Scalus ghobban* (Blue barred parrot fish).

Scales were washed several times separately using tap water to remove dirt and meat present and later sun dried. The dry scales were then packed in clean dry plastic bags and stored under room temperature awaiting the hydrolysis process.

3.2 Enzyme production

The bacteria (*Bacillus cereus stain wwcp1*), initially isolated from the mud waters from Lake Bogoria, Kenya and re-plated, was used for enzyme production. The media for mass production of enzyme was prepared as outlined by Wanyonyi *et al*. (2014) containing 5.00g K_2HPO_4, 3.75g KH_2PO_4, 0.75g $MgSO_4.7H_2O$, 0.75g Urea, 0.75g $CaCl_2$, 1.25g Yeast extract and 12.50 Casein.

This enzyme production was done using a bioreactor (R'ALF plus duet fermenter, 3.7L).

The weights above were prepared in two sets, dissolved in 2.5 L distilled water separately in the two bio reactor vessels and their pH adjusted to 9 (Lakshmi *et al.*, 2014) using NaOH solution by a pH meter. The media in the two vessels was then sterilized using a steam auto clave (Tuttnauer steam sterilizer, 121ºC) for 30 minutes. After this, the sterile media was

allowed to cool to room temperature and the bioreactor was set in place. Inoculation was done in the two compartments using 5% overnight grown seed bacterial culture. The temperature was set at 36ºC, stirrer was set at 120 rpm and the fermentation process allowed to proceed as shown in Figure 3.1;

Figure 3.1: Fermentation taking place in the bioreactor

After four days of continuous fermentation the enzyme was harvested, centrifuged in Sorvall ST16R centrifuge (1500rpm, 4ºC for 4 minutes) and placed in the cold room (4ºC) ready for use in the hydrolysis process.

3.3 Gelatin extraction

200 grams of scales from the three marine fish species were separately packed in 1L conical flasks. In each, 500mLs of enzyme was added; the pH adjusted to 12, plugged using cotton wool and covered with aluminium foil. A side experiment was carried out simultaneously; whereby 5g of each type of scales was weighed and placed in 100mL conical flasks, 50mLs distilled water added to each, pH adjusted to 12, plugged using cotton wool and sealed using aluminium foil. These were the blanks. The six conical flasks were moved to the oven at a temperature of 50ºC (Jiang, 2013). After every 24 hours, the pH was re-adjusted to 12 and

the swirling and stirring done to ensure uniform and complete hydrolysis in the conical flasks. Upon complete hydrolysis, filtration was done separately for the three species under study to separate the solution containing gelatin and the insoluble fine particles on the bottom of the conical flask. These gelatin solutions were then freeze dried in the freeze drier to obtain a solid as shown in Figure 3.2;

Figure 3.2: Freeze drier drying the gelatin solution

The gelatin for the three species was stored in different air tight containers and placed in a desiccator containing calcium chloride to keep off moisture. These were ready for analysis. The hydrolysis process for the blanks proceeded under the same set of conditions with the final observations being recorded.

3.4 Determination of the yield

The yield for dry gelatin from the three species was calculated using the formulae;

%Yield= (Mass of the dried gelatin / Mass of the dry scales hydrolysed) × 100 (Zakaria and Bakar, 2015).

3.5 Fourier transform infrared spectrometry

This analysis was done for the pure gelatin from the three marine fish species using a Fourier transform infrared spectrometer IR Affinity-1S Shimadzu model. The gelatin samples were separately ground to very fine particles and placed on the sample compartment. The sample compartment containing the sample was then clamped onto the mount of the FTIR for analysis. The number of scans ran were 20. Upon completion, spectra in the range of 500 to 4500 cm^{-1} were produced and printed.

3.6 Amino acid composition analysis

Amino acid composition analysis of gelatin from the three marine fish species under study was performed by an Agilent Liquid Chromatograph using a narrow bore, (2.1 x 200 mm Hypersil AA-ODS 5 µm reverse phase column) purchased from Thermo Electron (part # 30105-202130). Samples were weighed and placed in a 13 x 100 mm Pyrex tube along with 1mL N HCl and 11 µmoles of Internal Standards (Norvaline and Sarcosine). After adding Internal Standards, the samples along with controls and blanks were exposed to liquid-phase 6N HCl for 22 hours at 100°C. Amino acids were separated on an Agilent 1260 with column heater, automatic injection programming UV and Fluorescence detection. 5µL of the hydrolysate was dried down and re-suspended in 250 µL of 0.4 M Borate buffer. 1µL was injected. The G1367E auto sampler was used to perform pre-column derivatization and multiple sample handling. The derivatized amino acids were then eluted from the reverse phase column. Primary amino acids (tagged with OPA, Agilent #5061-3335) were detected at 338/390 nm by the Variable Wavelength (UV) detector (G1365D) and secondary amino acids (tagged with FMOC, Agilent 5061-3337) at 266/324 nm. The fluorometric detector (G1321B) was used to monitor the primary ones at excitation/emission 340/450nm and the

secondary ones at 266/305nm. The assay was calibrated by a standard (Agilent 5061-3331) which was subjected to the same treatment as the samples and control, including hydrolysis. The assay was controlled by a known protein, Human Serum Albumin. An aliquot from the same batch of HSA was run with every assay.

3.7 Polymer blends preparation

The polymer blends were prepared between the gelatin extracted from *Scalus ghobban* (Blue barred parrot fish) and Polylactic acid (PLA) of molecular weight of 100.00 purchased from Fisher Scientific. The polymer blends, gelatin/PLA, were in the ratios of 20/80, 50/50, 80/20. PLA solution was prepared by dissolving each of the following weights; 0.2g, 0.5g and 0.8g in 20mL Dichloromethane in 100mL conical flasks at room temperature. Gelatin solution on the other hand was prepared by dissolving each of the following weights; 0.2g, 0.5g and 0.8g in 10mL distilled water in 100mL beakers at room temperature.

The different sets of PLA solutions were added to the corresponding sets of gelatin solution to obtain the above-mentioned ratios. The mixing for each set was done using a magnetic stirrer at room temperature to obtain a uniform mixture and immediately poured on glass Petri dishes.

The Petri dishes were left on a flat bench to dry at room temperature for 4 days. These polymer blends were ready for analysis.

3.8 Thermal Analysis

3.8.1 Thermogravimetric Analysis

This analysis was done for PLA and the different PLA/gelatin blends using TGA Q 500 V20.13 Build 39 (Universal V4.54 TA instruments) instrument under nitrogen. Sample sizes

ranging from 10-28mg were heated in a platinum pan from room temperatures to 750°C at a rate of 10°C/min and the graph was recorded.

3.8.2 Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) was also done on PLA and the different PLA/gelatin blends using DSC Q100 V9.9 Build 303 (Universal V4.54 TA instruments) instrument under nitrogen. Samples taken in sample sizes ranging from 4.46-9.83mg were heated separately from room temperature to 200°C at a scanning rate of 10°C/min.

CHAPTER FOUR : RESULTS AND DISCUSSION

4.1 Sample preparation

Figure 4.1 shows photographs of the of the clean sun-dried fish scales for each of the three marine fish species studied

a) Red snapper

b) Black spot emperor

c) Blue barred parrot fish

Figure 4.1: The scales from a) Red snapper (*Lutjanus sebea*), b) Black spot emperor (*Latrines hark*) and c) Blue barred parrot fish (*Scalus ghobban*) after washing and drying

The scales of the different species had different colours and sizes. Red snapper scales were white in colour and had the smallest size, black spot emperor scales were grey and slightly

bigger than those of red snapper, while blue barred parrot fish scales were grey in colour with blue and green patches and had the largest size.

4.2 Rate of hydrolysis

Complete hydrolysis occurred after 20 days, 22days and 23 days for black spot emperor (BSE), red snapper (RS) and blue barred parrot fish (BBPF) respectively. After complete hydrolysis of the scales an insoluble fine powder called hydroxyapatite remained at the bottom of the conical flasks while the gelatin was present in solution as shown in Figure 4.2 for the three fish species;

a)

b)

c)

Figure 4.2: Fully hydrolyzed scales for the a) red snapper, b) black spot emperor and c) blue barred parrot fish, with gelatin in solution and hydroxyapatite settled at the bottom of the flask

As hydrolysis of the scales occurred the pH lowered prompting re-adjustments back to 12 after every 24 hours of hydrolysis. The Table 4.1 shows the pH before re-adjustment for the species under study until complete hydrolysis was observed

Table 4.1: pH before adjustment after 24 hours before complete hydrolysis under enzyme

Days	**BSE**	**RS**	**BBPS**
1	8.37	8.68	8.05
2	9.07	8.91	9.19
3	8.99	9.13	9.66
4	9.57	10.24	9.83
5	10.08	10.41	10
6	10.16	10.43	10.14
7	10.52	10.65	10.36
8	10.8	10.73	10.57
9	10.92	10.88	10.44
10	10.98	10.76	10.73
11	11.04	10.9	10.93
12	10.99	11.01	10.98
13	11.13	11.22	11
14	11.19	11.07	10.87
15	11.27	11.18	11.19
16	11.43	11.38	11.25
17	11.74	11.44	11.4
18	11.69	11.53	11.35
19	11.76	11.49	11.43
20	11.89	11.67	11.71
21		11.71	11.66
22		11.87	11.83
23			11.87

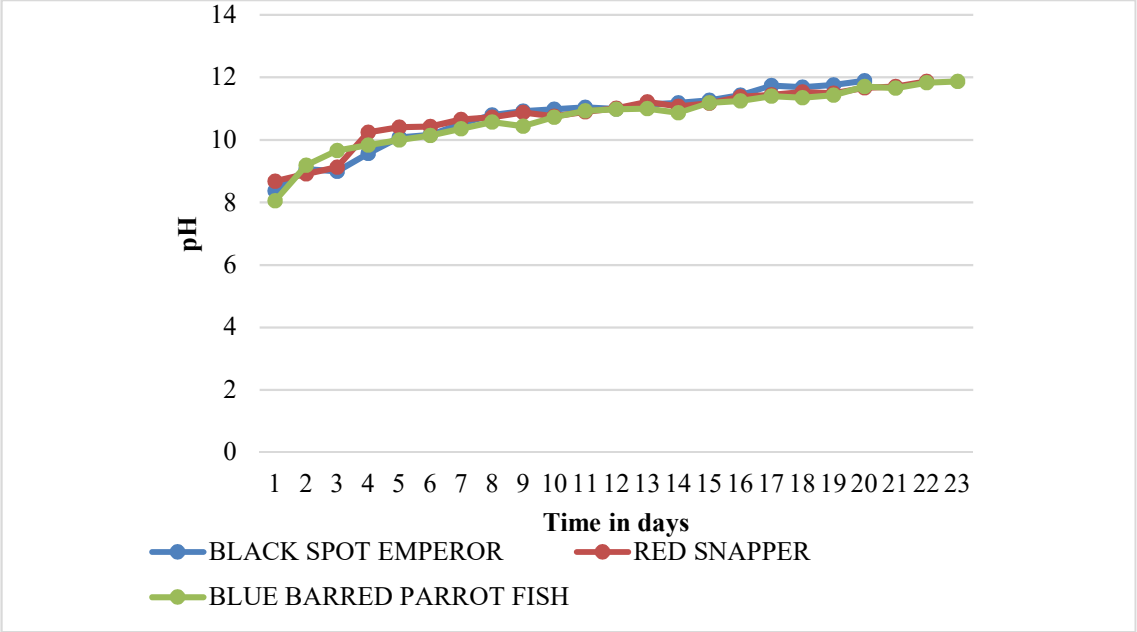

Figure 4.3: Graph showing pH prior adjustment as the hydrolysis process progressed.

As shown in Figure 4.3, the pH drops from 12 were similar for all the three fish species. Taking the pH drop as the indicator of the rate of hydrolysis of the scales, it can therefore be seen that the rate of hydrolysis was similar for the scales of three species.

One important parameter in the extraction of gelatin from any type of collagen is the duration used. The time used here for the species under study was long. A reason for the long duration required to completely hydrolyse the scales is that there was no preliminary pre-treatment done on the scale, an important example being grinding of the scale to increase the surface area for contact with the enzyme.

Hydrolysis of the blue barred parrot fish took the longest time. This is attributed to their larger size when compared to the scales of the other two species. Despite the similar sizes of the scales from red snapper and black spot emperor, red snapper's scales took a longer time to fully hydrolyse, this being attributed to the compactness of these scales from physical examination. It is however expected that scales would take a longer time to completely

hydrolyse depending on the degree of cross linking. Cross linking affects the severity of the process required to extract gelatin and is dependent on factors such as the age of the animal and the tissue from which collagen was sourced. It has been reported that collagen is more cross linked in older animals as compared to younger animals and the cross linking in bones is different than that in skins (Muyonga *et al.*, 2004; Kim *et al.*, 2014).

The extraction of gelatin for blanks under distilled water was done for the same period as the fish scales as a control.

4.3 Gelatin yield for the three marine fish species

After the freeze-drying process, a slightly yellow brittle hygroscopic solid was obtained from the three fish species.

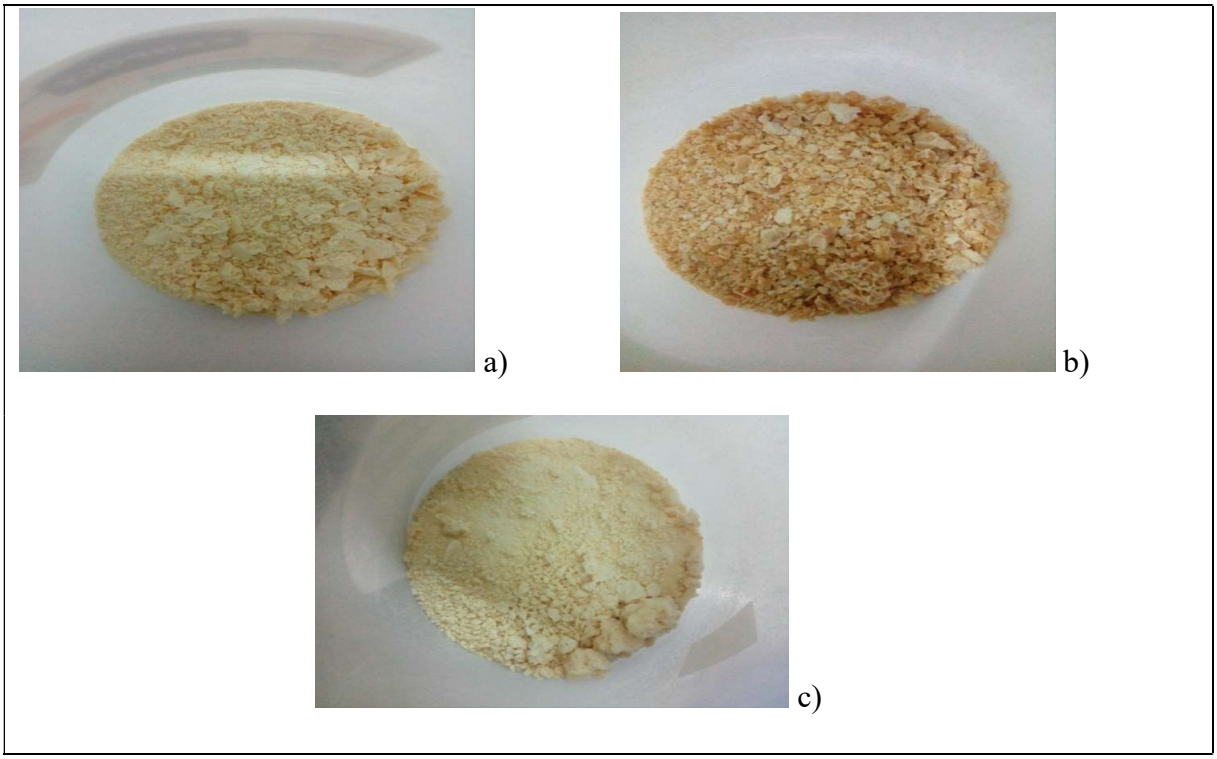

Figure 4.4: Gelatin from a) black spot emperor, b) blue barred parrot fish and c) red snapper after freeze drying

Upon crushing the brittle solid with walls of the containers they were held in, the gelatin obtained from each of three fish species appeared as shown in Figure 4.4.

The percentage yield of gelatin obtained from the scales of each species using the formula indicated in the methodology section was; 38.7%, 28.2% and 41.4% for black spot emperor, red snapper and blue barred parrot fish respectively.

The large size of the scales of blue barred parrot fish contributed to the higher yield of gelatin when compared to the other two fish species under study. The low yield in red snapper scales could mean there is more hydroxyapatite in these scales when compared to collagen.

4.4 FTIR analysis

The Fourier transform infrared spectrometry for the gelatin obtained from the scales of the three marine fish species gave similar spectra as shown in figures 4.5 to 4.7 with five Amide bands namely Amide A, B, I, II, III and two other bands.

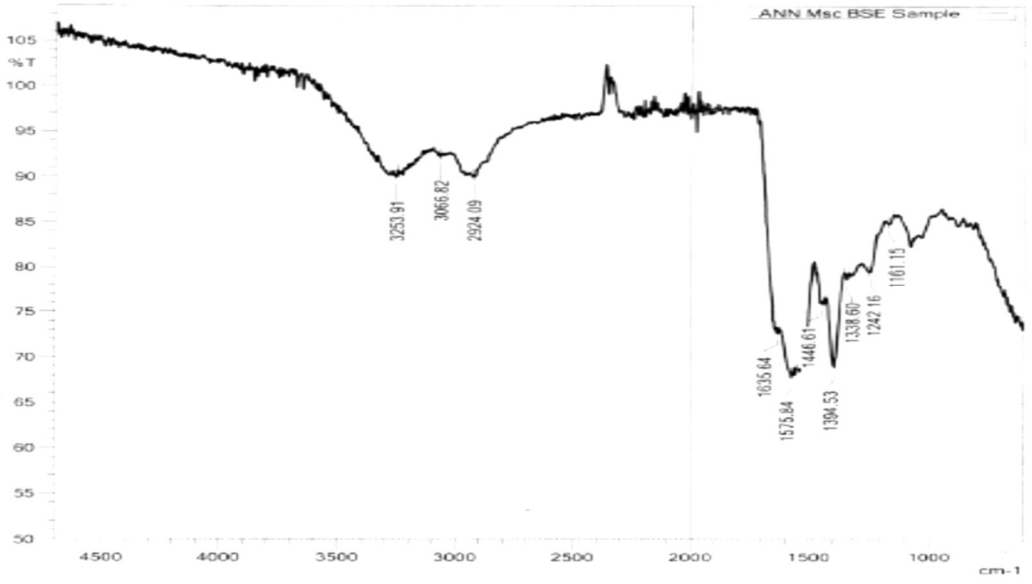

Figure 4.5: Fourier Transform Infrared spectra for the gelatin obtained from the scales of Black spot emperor

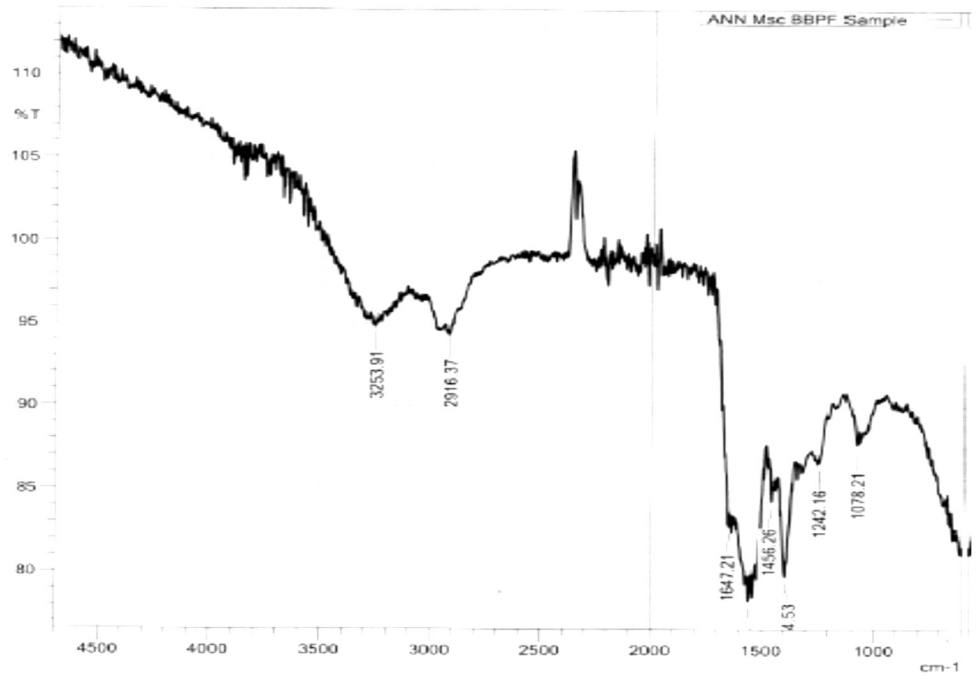

Figure 4.6: Fourier Transform Infrared spectra for the gelatin obtained from the scales of Blue barred parrot fish

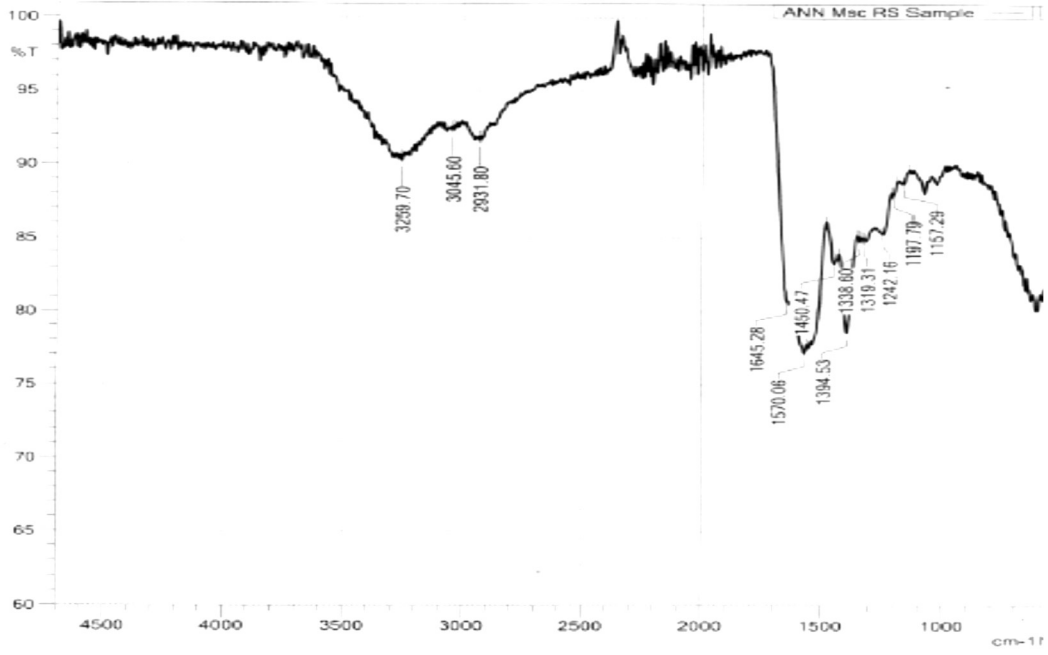

Figure 4.7: Fourier Transform Infrared spectra for the gelatin obtained from the scales of Red snapper

Amide I bands for black spot emperor (BSE), red snapper (RS) and blue barred parrot fish (BBPF) were 1635.64, 1645.28 and 1647.21 cm^{-1} respectively corresponding to C=O stretching vibrations. The peaks had a low amplitude which is due to the interactions between C=O and adjacent chains via hydrogen bonding (Ahmad and Benjakul, 2011). This hydrogen bonding is also responsible for reduction in the frequency from the expected 1715 cm^{-1}. Amide II bands for BSE, RS and BBPF were at 1575.84, 1570.06 and 1572.95 cm^{-1} respectively corresponding to C-N stretching and N-H in-plane bending. Amide III bands were similar for the three species occurring at 1242.16 cm^{-1} representing C-N and N-H in-plane bending as well. Amide A bands for BSE, RS and BBPF were 3253.91, 3253.91 and 3259.70 cm^{-1}respectively, representing N-H stretching vibration. BSE and RS exhibited Amide B bands at 3066.82 and 3045.60 respectively, corresponding to N-H stretching vibrations, with BBPF spectra missing this band. These N-H stretching vibrations bands were of low amplitudes and lower frequency than the expected 3400-3550 cm^{-1} due to hydrogen bonding. The other bands exhibited by BSE, RS and BBPF were 2924.09, 2931.80 and 2916.37 cm^{-1} which were C-H stretching vibrations, for a methylene group. The same methylene group exhibited bending vibrations at 1394.53 cm^{-1} for the spectra of all three species. These peaks corresponding to methylene groups gave an indication that there was abundance of an amino acid with methylene group. These FTIR spectra were similar to the spectra for gelatin extracted from chicken feet by acid which exhibited the Amide I, II, III, A and B bands at 1658.74cm^{-1}, 155.44cm^{-1}, 1236.36cm^{-1}, 3322.12cm^{-1} and 2924.36cm^{-1} respectively (Widyasari and Rawdkuen, 2014)

4.5 Amino acid composition

The HPLC chromatograms showing the retention time for the different amino acids are as shown in figures 4.8 to 4.10 with the different peak heights showing the relative abundance of a given amino acid.

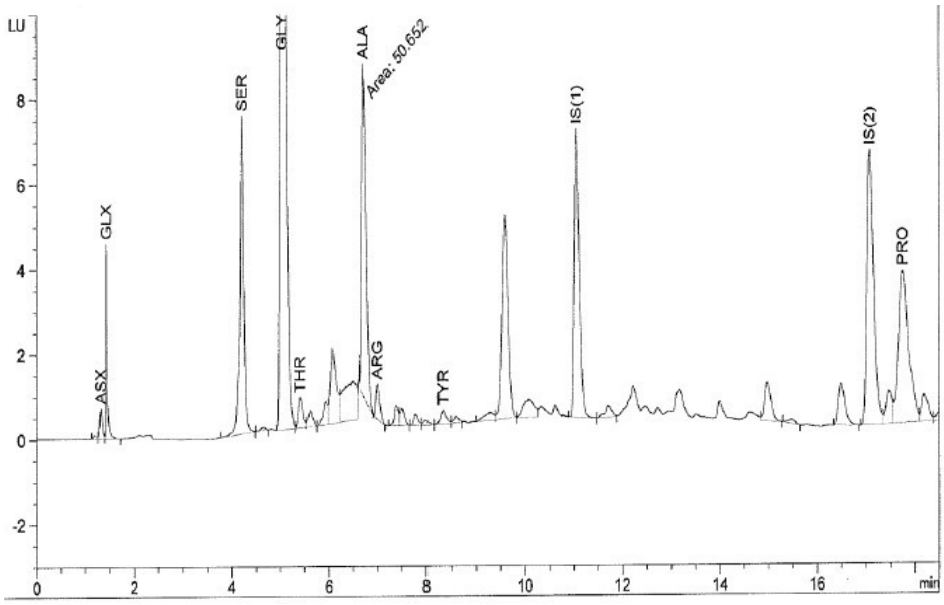

Figure 4.8: HPLC chromatogram of gelatin obtained from scales of Red snapper

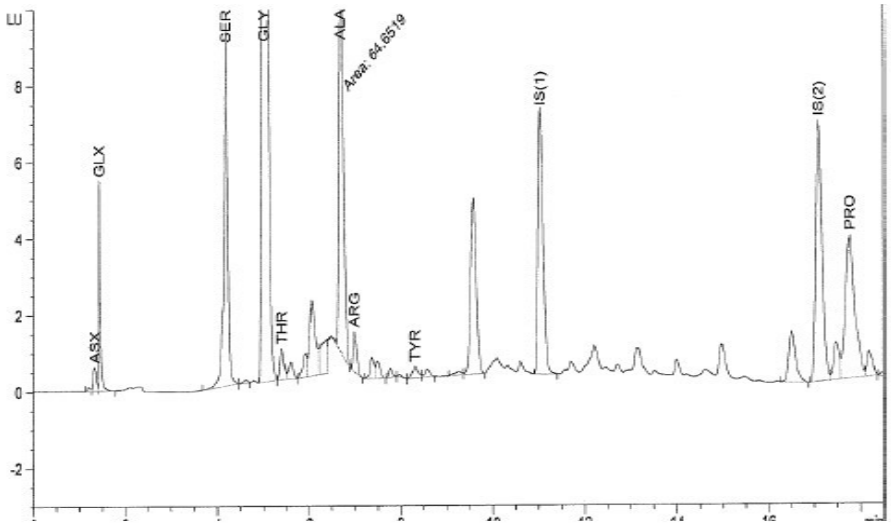

Figure 4.9: HPLC Chromatogram for gelatin obtained from the scales of Blue barred parrot fish

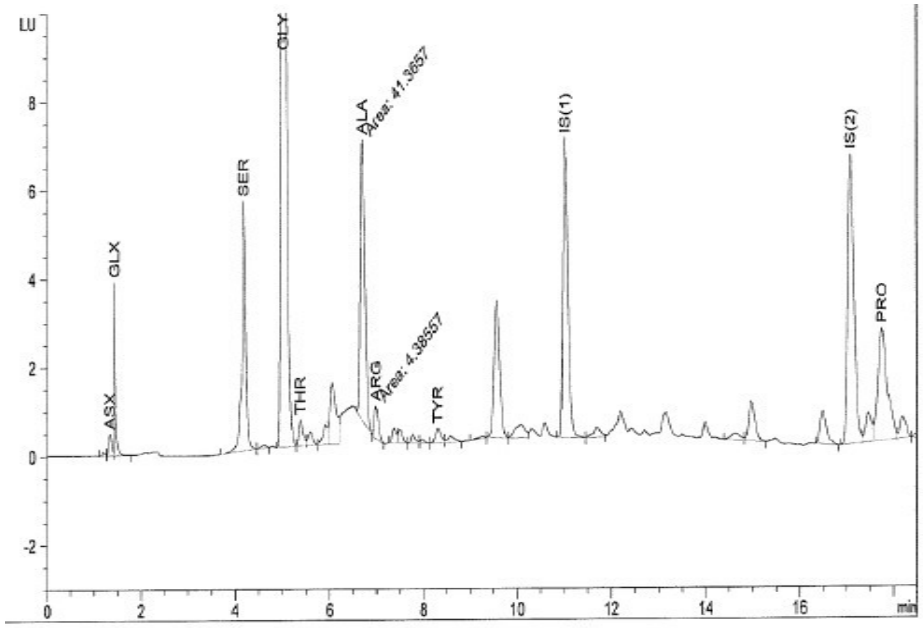

Figure 4.10: HPLC Chromatogram for gelatin obtained from scales of Black spot emperor

Sixteen (16) of the amino acids shown in Figure 2.3 were obtained from this analysis. The amino acid composition (weight %) for the three species is shown in Table 4.2. The samples were analyzed in duplicates and the standard deviation (SD) is also indicated. The results show that Glycine is the most abundant amino acid. This high amount of Glycine is typical of gelatin due to the repeating sequence of (Glycine-X-Y) amino acid triplets in its constituent chains resulting in gelatin having at least one third of its amino acid composition as Glycine (Muyonga *et al.*, 2004). Alanine content is also high for the three species.

Alanine, found in the non-polar region of collagen occupying the Y position of the earlier mention repetitive amino acid triplet, together with Proline and Hydroxyproline are said to be responsible for the high viscoelasticity of gelatin (Gómez-Guillén *et al.*, 2002).

The key difference between gelatin extracted in this study and gelatin extracted from other sources like bovine skin and Pangasius catfish skin (Mahmoodani *et al.*, 2014), porcine skin and Grass carp fish scales (Zhang *et al.*, 2011), is the absence Hydroxyproline.

Table 4.2: Amino acid composition of gelatin from the scales of Red snapper, Black spot emperor and Blue barred parrot fish in percentage

Amino acid	% Composition ± SD in RS scales gelatin	% Composition ± SD in BBPF scales gelatin	% Composition ± SD in BSE scales gelatin
Glycine	34.84± 0.0288	35.79± 0.045	35.85± 0.0968
Alanine	14.81± 0.0005	15.42± 0.0072	15.15± 0.0085
Proline	14.16± 0.0002	11.05± 0.2048	11.63± 0.0481
Glutamic acid	8.67± 0.0013	9.05± 0.0025	8.93± 0.0001
Aspartic acid	5.15± 0.0013	5.62± 0.0013	5.58± 0.0072
Arginine	3.51± 0.0001	3.15± 0.0005	3.58± 0.0018
Leucine	2.93± 0.0002	3.26± 0.0032	2.89± 0.0005
Lysine	2.73± 0	2.84± 0.0025	2.87± 0.0001
Valine	2.55± 0	2.81± 0.0001	2.64± 0.0041
Serine	2.45± 0.0001	2.76± 0.0002	2.50± 0.0041
Methionine	2.09± 0.0098	1.62± 0.0001	2.09± 0.0265
Phenylalanine	1.92± 0.0002	2.06± 0.0032	2.04± 0.0001
Isoleucine	1.38± 0	1.64± 0	1.29± 0.0001
Threonine	1.21± 0.0018	1.09± 0.0001	1.27± 0.0013
Tyrosine	0.87± 0.0005	0.98± 0.0001	0.94± 0.0013
Histidine	0.76± 0.0008	0.94± 0.0001	0.86± 0.0008
Cysteine	TRACE	TRACE	TRACE
Total	100	100	100

This absence in Hydroxyproline could have resulted from a low rate of hydroxylation of Proline or due to the conditions applied during the denaturation process such as pH or extraction duration used (Muyonga *et al.*, 2004). Jamilah and Harvinder (2002) reported a similar absence of Hydroxyproline as well as Hydroxylysine in gelatin obtained from black and red Tilapia skin. Of particular importance to note is that Proline was higher in gelatin

from the scales of red snapper at 14.16% compared to gelatin from scales of black spot emperor and blue barred parrot fish at 11.63% and 11.05% respectively. Cysteine does not take part in the structure of type I collagen; its presence in the gelatin from the scales of the three species could indicate the presence of another type of protein such as elastin or keratin (Giménez *et al.*, 2009). Methionine content was greater than 1.6%, higher than that found in porcine gelatin said to be 0.5%, supporting the conclusion that fish gelatin has higher amounts of Methionine compared to mammalian gelatin (Zhang *et al.*, 2011). Gelatin from the three species under study was low in amino acids Tyrosine and Histidine which is an inherent property of all gelatins (Mahmoodani *et al.*, 2014).Amino acid Tryptophan was also absent in gelatin extracted in this study for the three fish species. Gelatin is said not to contain Tryptophan (Muyonga *et al.*, 2004).

4.6 Polymer blends

Figure 4.11: Photographs of dry polymer blends

Upon drying very brittle films were formed as shown in Figure 4.11. This brittle nature could be reduced by adding plasticizers such as glycerol or sorbitol as they improve the mechanical strength of the film network (Jongjareonrak *et al.*, 2006). In this study, no plasticizers were used and therefore the polymer blends were broken to very fine particles and placed in air tight plastic zip lock bags.

4.7 Differential Scanning Calorimetry (DSC) Analysis results

4.7.1 DSC Analysis for PLA (D, L-Lactic acid) and gelatin

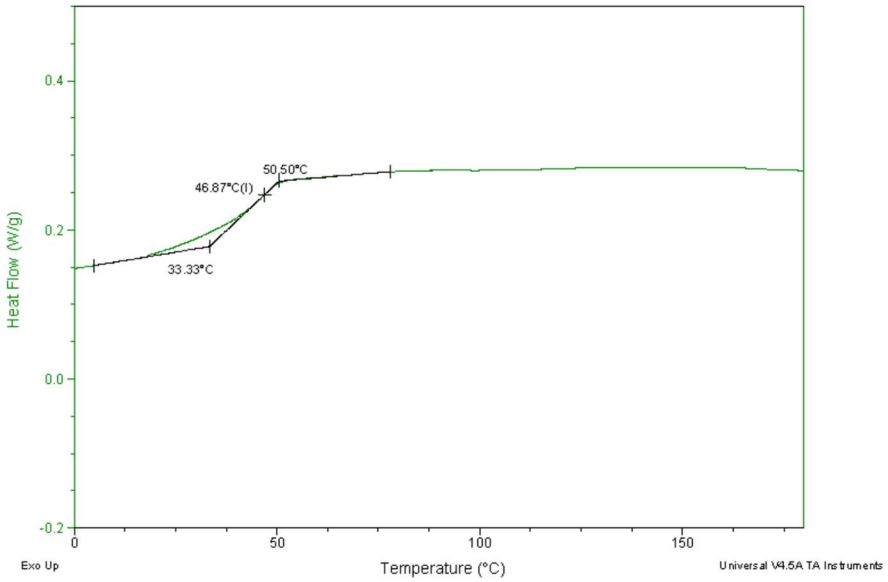

Figure 4.12: DSC thermogram for D, L-PLA

From the DSC thermogram of PLA in Figure 4.12, only one transition that corresponds to the glass transition temperature is seen. There is no peak relating to melting temperature. This indicates that the PLA used in this study was amorphous, derived from the diastereomeric form of D, L-Lactic acid.

Gelatin is amorphous in nature as well. The glass transition temperature for gelatin has been reported to be as high as 200ºC (Matveev *et al.*, 1997). High T_g are reported in cases where

there is presence of crosslink of chemical groups which are able to form hydrogen bonds with water molecules (Patil *et al.*, 2000). Rivero et al. (2010) reported a glass transition temperature of 183.5ºC for bovine sourced gelatin while Kiplagat (2016) reported a glass transition temperature of 157ºC for gelatin extracted from Nile perch scales. The DSC thermogram for gelatin obtained from Nile perch scales is as shown in Figure 4.13;

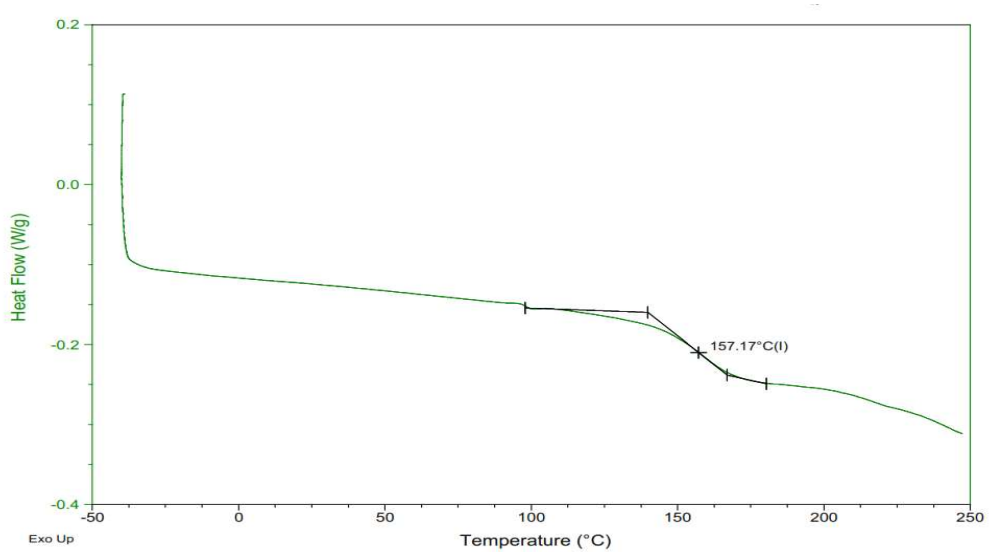

Figure 4.13: DSC thermogram for gelatin extracted from the scales Nile perch,

4.7.2 DSC Analysis for polymer blends

Differential scanning calorimetry gives the glass transition temperature(s) of the polymer blends thus assisting in the deduction of miscibility or immiscibility (Kolbuk *et al.*, 2013). The DSC thermogram obtained for 20/80 D, L-PLA/gelatin blend is as shown in Figure 4.14. As observed, the DSC thermogram exhibited two distinct glass transition temperatures (T_g) indicating phase separation and immiscibility in the polymer blend. The lower T_g corresponds to D, L-PLA while the higher T_g corresponds to gelatin. The DSC thermogram in Figure 4.14 shows a slight shift in position of T_g for the blend compared to D, L-PLA homopolymer. This

indicates that there is slight interaction between the polymers with the molecules in D, L-PLA and gelatin. This interaction slightly diffuses the interface between the polymers leading to that small shift observed.

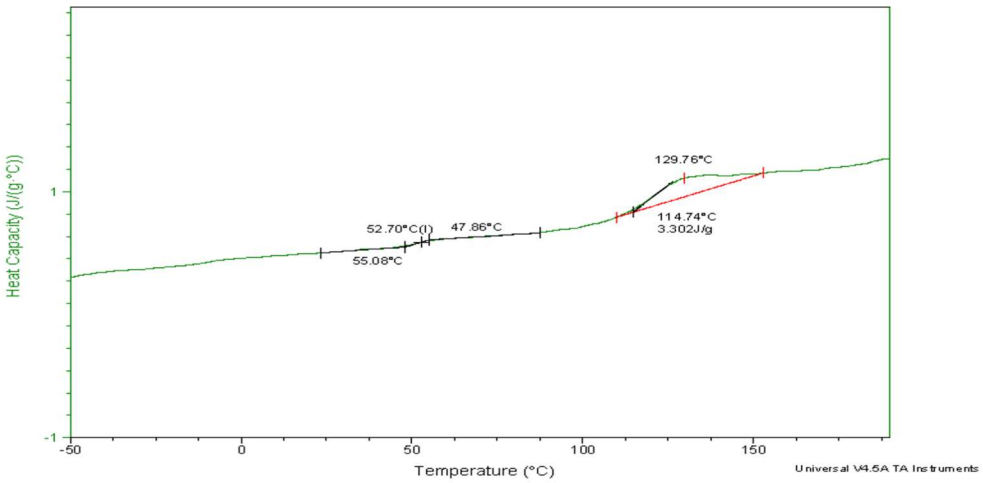

Figure 4.14: DSC thermogram for D, L-PLA/gelatin polymer blends in the ratio 20/80.

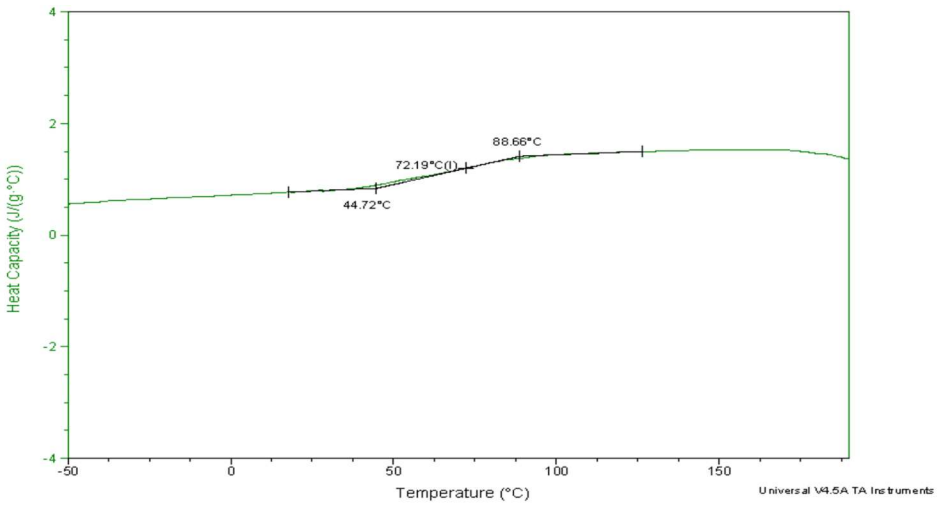

Figure 4.15: DSC thermogram for D, L-PLA/gelatin polymer blends in the ratio 50/50.

The DSC thermogram obtained for 50/50 D, L-PLA/gelatin blends was as shown in Figure 4.15. Thermogram shows one T_g indicating the presence of one phase. This shows that there is complete miscibility between amorphous PLA and gelatin at the ratio 50/50. This miscibility could have been as a result of hydrogen bonding between the OH in D, L-PLA and C=O in gelatin.

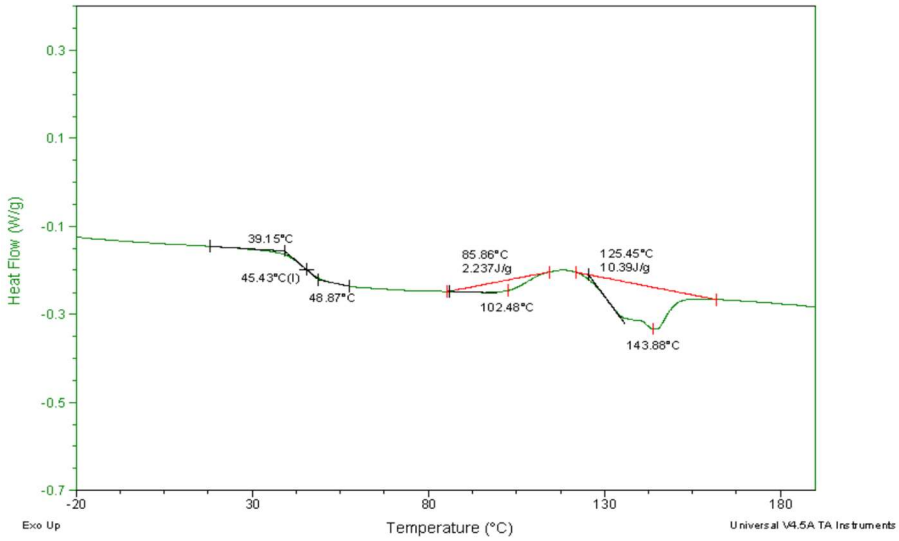

Figure 4.16: DSC thermogram for D, L-PLA/gelatin polymer blends in the ratio 80/20.

The thermogram in Figure 4.16 for the 80/20 D, L-PLA/gelatin ratio gave two T_g indicating the presence two separate phases. This shows that at when D, L-PLA and gelatin are blended at this ratio there is immiscibility. Looking at the T_g of D, L-PLA in this thermogram and comparing them with the T_g of the pure D, L-PLA, there is no difference in their positions. This shows that at this ratio there a likelihood that no interactions between the polymers at molecular level took place. The spectra obtained indicates slight crystallization of D, L-PLA crystalline segments.

Comparing the DSC thermograms of D, L-PLA/gelatin at 20/80 and 80/20 ratios, interactions between amorphous D, L-PLA/gelatin is better seen when the amount of gelatin is higher

than when the amount of D, L-PLA is higher. Complete miscibility is however achieved at D, L-PLA/gelatin ratio of 50/50 as illustrated in Figure 4.15. As shown, only one T_g was obtained at 72.2ºC.

4.8 Thermogravimetric analysis

Thermogravimetric analysis (TGA) aids in defining the limits of thermal stability of the pure polymers as well as the polymer blends (Fernandes *et al.*, 2002). From the TGA graphs, different regions on the curve can be identified as the weight of the sample reduces with increase in temperature, that is, region corresponding to loss of solvent or water, thermal decomposition of the sample, loss of atmospheric components such as nitrogen and oxygen, combustion of carbon and finally the region exhibiting the residue in the form of inorganic and inert residue (ash).

4.8.1 TGA analysis for PLA (D, L-Lactic acid) and gelatin

The TGA graph corresponding to D, L-PLA in Figure 4.17 shows two weight loss regions as the temperature increased.

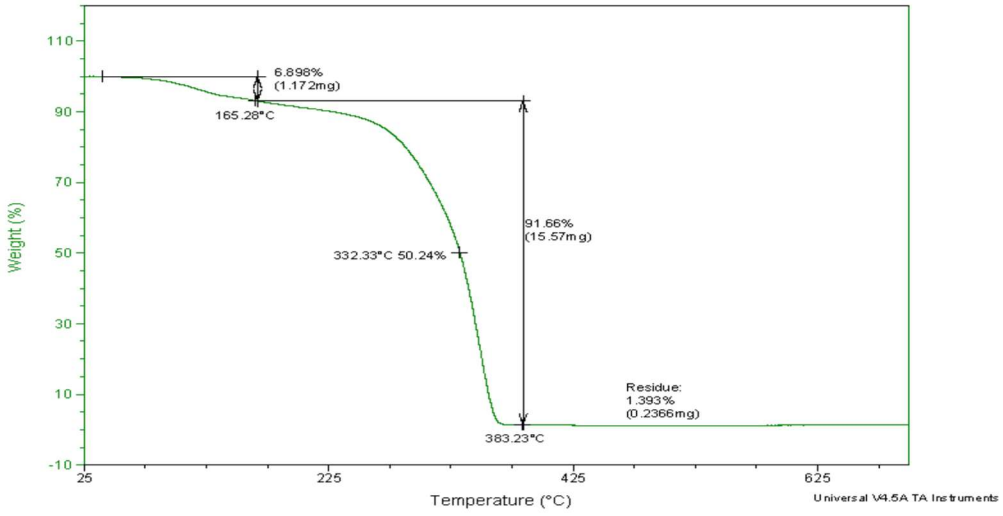

Figure 4.17: TGA graph for D, L-PLA.

The first region gave a drop-in weight of about 6.9%, relating loss of a volatile portion that was the solvent used, of which in this study dichloromethane was used. The second weight loss region corresponded to the thermal decomposition of the polymer. The onset of this decomposition was at 165.28ºC to end at 383.23ºC. This thermal decomposition accounted for 91.66% of weight loss to leave an ash residue of 1.39%. The peak relating to this weight loss region is steep, indicating a fast rate of thermal decomposition.

Gelatin is said to have a lower onset temperature for thermal decomposition than D, L-PLA. Kiplagat *et al*. (2017) reported on onset temperature of 100ºC for gelatin obtained from Nile perch scales.

4.8.2 TGA Analysis of the D, L-PLA/gelatin polymer blends

TGA thermogram of the polymer blends at the ratio 20/80 in the Figure 4.18 shows two regions of weight loss with increasing temperature. The first region represented the thermal decomposition of the polymer blend involving the highest amount weight loss of 81.96%.

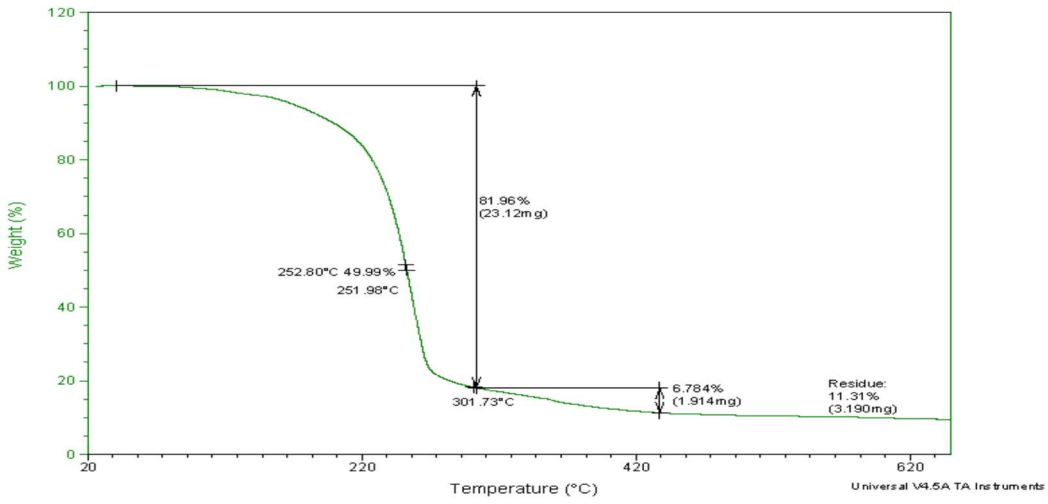

Figure 4.18: TGA graph for D, L-PLA/gelatin polymer blend in the ratio 20/80.

The onset temperature was around 100ºC, which was about 65ºC lower than the onset temperature of D, L-PLA. This showed that PLA/gelatin polymer blends at this ratio had a lower thermal stability than D, L-PLA. The peak for this decomposition (100ºC to 301.73ºC) was however less steep compared to that of D, L-PLA, indicating a slower rate of decomposition. The second region gave a reduction in weight of 6.78% which corresponded to the combustion of carbon in the polymer blend. A residue of 11.3% was left.

In the TGA thermogram in the Figure 4.19 relating to the polymer blends in the 50/50 ratio, there are three weight reduction regions with increase in temperature.

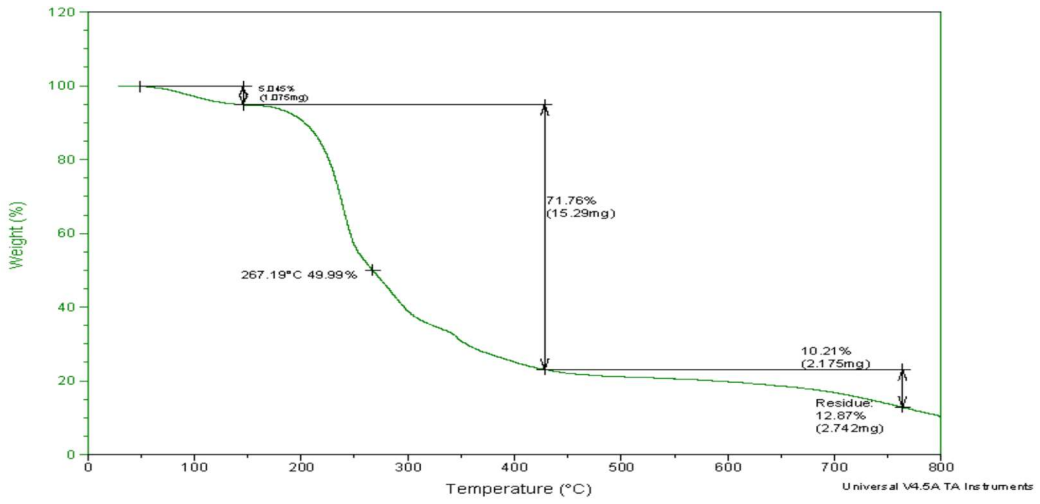

Figure 4.19: TGA graph for D, L-PLA/gelatin polymer blends in the ratio 50/50.

The first weight loss region represented the loss of water or solvent in the polymer blend which was a decrease of 5.05%. The second region corresponded to the thermal decomposition of the polymer blend representing the highest weight reduction of 71.8%. The onset temperature for this decomposition was 150^0C which was an intermediate onset temperature between the onset temperature in the thermal decomposition of D, L-PLA and gelatin. The peak for this thermal decomposition is less steep compared to those of D, L-PLA and pure gelatin. This shows that the rate of decomposition of the polymer blend between D,

L-PLA/gelatin at this ratio was slower than that of D, L-PLA and of gelatin. The third weight reduction region of 10.21% represented the combustion of carbon leaving a residue of 12.9%.

TGA graph for the polymer blend in the ratio 80/20, given in the Figure 4.20, shows two weight loss zones. The first zone representing about 7.13% was due to the loss of solvent.

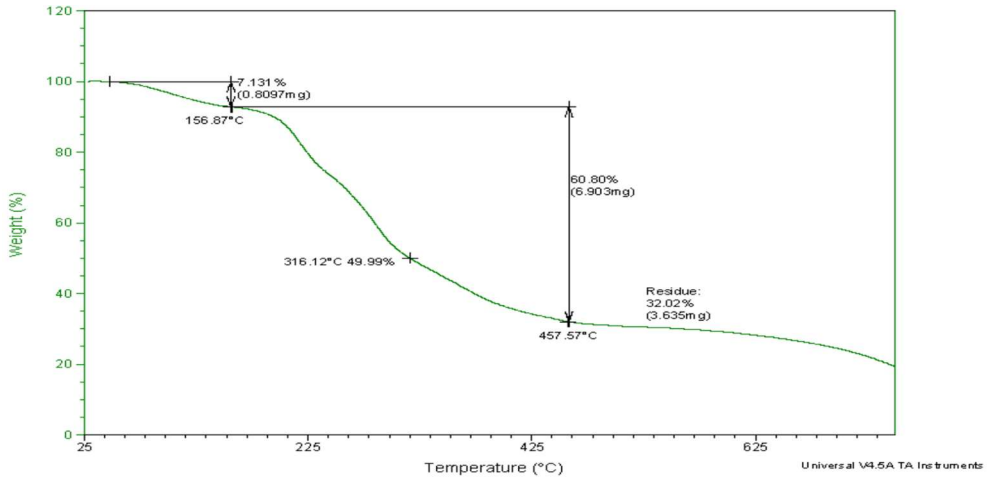

Figure 4.20: TGA graph for D, L-PLA/gelatin polymer blend in the ratio 80/20.

The second region corresponding to a weight loss of 60.80% was as a result of thermal decomposition. The onset temperature of this decomposition of was 156.9ºC. The peak for this thermal decomposition between 156.9ºC to 457.6ºC was less steep than the peaks relating to thermal decomposition of both D, L-PLA and gelatin, indicating that the rate of thermal decomposition was slower. The resultant residue was 32.0%. The onset temperatures for the thermal decomposition of the polymer blends depended on which polymer was in higher amounts in the polymer blends. In the 80/20 D, L-PLA/gelatin blend this temperature was closer to that of D, L-PLA while in the 20/80 ratio the temperature was close to that of gelatin. At 50/50 ratio the onset temperature was intermediate. The rate of thermal decomposition was however slower for the polymer blends than for both polymer in their pure form.

CHAPTER FIVE : CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

This aim of this study was to establish the suitability of marine fish scales, currently being discarded as waste, in the production of gelatin. Three marine fish species were used, and characterization done. The effectiveness of extraction of gelatin from marine fish scales using alkaline protease enzyme has not been studied in Kenya.

High yield of gelatin obtained (28.2% to 41.4%) is a good indicator that scales from these marine fish species are potential sources of gelatin. This high yield also demonstrates the viability of extraction of gelatin by use of alkaline protease enzyme at a pH of 12 and temperature of 50ºC. The more environmentally friendly method developed in this study for gelatin extraction using enzymes can be used to replace the commonly used acid/base extraction method.

Fourier transform infrared spectra are similar for gelatin obtained from the scales of the three fish species with all showing five Amide bands namely Amide A, B, I, II, III. The amino acid composition analysis also indicated great similarities among the gelatin of the three fish species in terms of composition and the abundance of the constituent amino acids. The key difference was in the contents of Proline which was higher for red snapper at over 14.2% compared to 11.1% and 11.6% for blue barred parrot fish and black spot emperor respectively.

Methionine was high for the gelatin obtained from the three fish species in this study at over 1.6%, higher than that found in porcine gelatin said to be 0.5%. However, even with this difference, there were similarities between gelatin extracted in this study and mammalian

gelatin with the abundance of Glycine, Alanine and Proline, the low contents of Tyrosine and Histidine seen in mammalian gelatin as well as the absence of Cysteine and Tryptophan being reported here. This shows that gelatin extracted from the scales of the three fish species under study can be used in the different applications of gelatin as an alternative to gelatin extracted from mammalian sources.

Polymer blends made in this study were between two amorphous polymers. The DSC analysis of the polymer blends between amorphous PLA and gelatin revealed that there was miscibility and compatibility between the polymers which was dependent on their ratio. Miscibility was reported at the D, L-PLA/gelatin ratio of 50/50 with immiscibility being seen at the ratios 20/80 and 80/20. TGA analysis showed that the onset temperatures for the thermal decomposition of the polymer blends depended on which polymer was in higher amounts in the polymer blends while the rate of thermal decomposition being lower for the polymer blends than for both polymer in their pure form and decreasing as the amount of D, L-PLA increased.

With successful extraction of good quality gelatin from the scales of these marine fish species and with the attainment of compatible polymer blend of gelatin and D, L-PLA the objectives of this study were fulfilled.

5.2 Recommendations

- It is recommended that a study be done for the scales of the three species by enzyme under the same conditions with the scales been ground before hydrolysis. A comparison should then be made on the yield of the gelatin obtained and the time required for complete hydrolysis to occur when the scales are ground before

hydrolysis and (as is done in this study) when the scales are not ground before hydrolysis.

- The time required for hydrolysis of scales reported in this study was quite long. The time can be reduced by replacing the enzyme used after a specified time of hydrolysis. The enzyme could be filtered, and fresh enzyme used to proceed with the hydrolysis process, with all the filtrate being freeze dried. This could hasten the hydrolysis process as the enzyme loses its activity with time. In this study the same enzyme was used for the whole hydrolysis period.
- The gel strength and viscoelasticity of gelatin obtained from the three species should be carried out. Given that the amount of Proline varied, there could be a difference in these properties in the gelatin.

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APPENDICES

Appendix 1: % Amino acid composition of gelatin obtained from the scales of Black spot emperor

Amino acid	Amount in nano moles		% comp. in rep.1	% comp. in rep.2	Average % comp. ±SD
	Replicate 1	Replicate 2			
ASX	36.80	35.00	5.52	5.64	5.58 ± 0.0072
GLX	59.52	55.38	8.92	8.93	8.93 ± 0.0001
SER	16.37	15.73	2.45	2.54	2.50 ± 0.0041
HIS	5.84	5.13	0.88	0.83	0.86 ± 0.0013
GLY	240.62	220.97	37.07	35.63	35.85 ± 0.0968
THR	8.27	8.00	1.24	1.29	1.27 ± 0.0013
ALA	100.56	94.29	15.08	15.21	15.15 ± 0.0085
ARG	23.71	22.40	3.55	3.61	3.58 ± 0.0018
TYR	6.16	5.98	0.92	0.96	0.94 ± 0.0008
VAL	17.85	16.09	2.68	2.59	2.64 ± 0.0041
MET	14.65	12.19	2.20	1.97	2.09 ± 0.0001
PHE	13.51	12.63	2.03	2.04	2.04 ± 0.0265
ILE	8.59	7.94	1.29	1.28	1.29 ± 0.0001
LEU	18.89	17.58	2.87	2.84	2.86 ± 0.0005
LYS	19.13	17.74	2.87	2.86	2.87 ± 0.0001
PRO	76.53	73.04	11.47	11.78	11.63 ± 0.0481
TOTAL	667.01	620.10	100	100	

Appendix 2: % Amino acid composition of gelatin obtained from the scales of Blue barred parrot fish

Amino acid	Amount in nano moles		% comp. in rep.1	% comp. in rep.2	Average % comp. ±SD
	Replicate 1	Replicate 2			
ASX	42.61	38.28	5.64	5.59	5.62 ± 0.0013
GLX	68.56	61.67	9.08	9.01	9.05 ± 0.0025
SER	20.51	18.84	2.77	2.75	2.76 ± 0.0002
HIS	7.09	6.34	0.94	0.93	0.94 ± 0.0001
GLY	271.34	243.95	35.94	35.64	35.79 ± 0.0968
THR	8.22	7.37	1.09	1.08	1.09 ± 0.0001
ALA	116.87	105.09	15.48	15.36	15.42 ± 0.0072
ARG	23.85	21.43	3.16	3.13	3.15 ± 0.0005
TYR	7.40	6.62	0.98	0.97	0.98 ± 0.0001
VAL	21.21	19.14	2.81	2.80	2.81 ± 0.0001
MET	12.17	11.06	1.61	1.62	1.62 ± 0.0001
PHE	15.23	14.37	2.02	2.10	2.06 ± 0.0032
ILE	12.40	11.22	1.64	1.64	1.64 ± 0
LEU	24.88	22.07	3.30	3.22	3.26 ± 0.0032
LYS	21.65	19.13	2.87	2.80	2.84 ± 0.0025
PRO	80.97	77.85	10.73	11.37	11.05 ± 0.2048
TOTAL	754.94	684.42	100	100	

Appendix 3: % Amino acid composition of gelatin obtained from the scales of Red snapper

Amino acid	Amount in nano moles		% comp. in rep.1	% comp. in rep.2	Average % comp. ±SD
	Replicate 1	Replicate 2			
ASX	33.09	30.03	5.17	5.12	5.15 ± 0.0013
GLX	55.65	50.71	8.69	8.64	8.67 ± 0.0013
SER	15.66	14.30	2.45	2.44	2.45 ± 0.0001
HIS	4.74	4.59	0.74	0.78	0.76 ± 0.0008
GLY	222.31	205.14	34.72	34.96	34.84 ± 0.0288
THR	7.95	6.95	1.24	1.18	1.21 ± 0.0018
ALA	94.70	86.97	14.79	14.82	14.81 ± 0.0005
ARG	22.43	20.62	3.50	3.51	3.51 ± 0.0001
TYR	5.44	5.14	0.85	0.88	0.87 ± 0.0005
VAL	16.34	14.97	2.55	2.55	2.55 ± 0
MET	13.86	11.85	2.16	2.02	2.09 ± 0.0098
PHE	12.25	11.30	1.91	1.93	1.92 ± 0.0002
ILE	8.83	8.07	1.38	1.38	1.38 ± 0
LEU	18.85	17.15	2.94	2.92	2.93 ± 0.0002
LYS	17.48	16.03	2.73	2.73	2.73 ± 0
PRO	90.75	83.01	14.17	14.15	14.16 ± 0,0002
TOTAL	640.33	586.84	100	100	

Appendix 4: Publication done on this study

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Amino acid Composition of Gelatin Extracted from the Scales of Different Marine Fish Species in Kenya

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ABSTRACT

Gelatin in this study was extracted by an enzymatic process from the scales of three marine fish species; *Lutjanus sebea* (Red snapper), *Lethrinus harak* (Black spot emperor) and *Scalus ghobban* (Blue barred parrot fish). Concentration of bacteria for mass production of enzyme was done in a fermentation medium using a bio reactor. Scales were hydrolyzed at 50°C and the pH maintained at 12. Complete hydrolysis took between 20 and 23 days for all species. The yield for the dried gelatin was between 28.2% and 41.4% for the marine fish scales under study. Fourier transform infrared spectra showed the presence of amide bands and two other additional absorption bands, indicating the presence of amide bonds for all the three species. The amino acid composition analysis for the gelatin of three species was then done showing the presence of 16 amino acids. Glycine was the most abundant for all the three species with about 35% followed by Alanine both adding up to around 50% of the total amino acid compositions. The amount of Proline was high for red snapper at over 14.2% compared to 11.1% and 11.6% for blue barred parrot fish and black spot emperor respectively.

Keywords: Gelatin, Enzymatic Process, Marine Fish Scales, Amino Acid Composition.

I. INTRODUCTION

Gelatin is a protein that does not occur naturally but is obtained by thermal denaturation of collagen (Gomez-Guillen *et al.,* 2002). Collagen is a key structural protein in animals including humans (Shoulders and Raines, 2009). This thermal denaturation is a hydrolysis process that involves breaking of covalent and hydrogen bonds in the chains of collagen's triple helix structure, in turn converting the insoluble collagen to soluble gelatin (Kim *et al.*, 2014).

The extraction of gelatin can be done chemically using acid or alkaline solutions or by use of enzymes. In an enzymatic process, alkaline protease is the best enzyme for gelatin extraction (Jiang, 2012). The protease enzymes are largely produced by bacteria, namely *Bacillus spp*, and are mostly extracellular, with the concentration being done in a fermentation media (Sevinc and Demirkan, 2011). *Bacillus cereus* stain wwcp is specifically said to exhibit good protease activity (Wanyonyi *et al.,* 2014). The extent of the bacterial multiplication and the eventual enzyme production is dependent on the nutrients present especially the carbon and nitrogen sources and the physical factors such as inoculums concentration, temperature, pH and incubation time (Lakshmi *et al.,* 2014).

The gelatin that is available in the market is mainly obtained from pig (porcine) skin and cattle (bovine) hide and bones (Ahmad and Benjakul, 2011). However, there has been health concerns (Zakaria and Bakar, 2015) and the religious restrictions ((Ardekani *et al.,* 2013) facing this mammalian sourced gelatin, leading to the recent entry of non mammalian sources, that is, fish gelatin (fish skin and bones) and poultry gelatin (feathers and feet) (Kim *et al.*, 2014). Fish skin and bone have been the main focus as sources of gelatin, with most of the extraction being done by chemical means.

This study involved the extraction of gelatin from the scales of three marine fish species; *Lutjanus sebea* (Red

snapper), *Lethrinus harak* (Black spot emperor) and *Scalus ghobban* (Blue barred parrot fish) selected in relation to abundance on the Kenyan coast. The extraction was done using enzyme.

II. METHODS AND MATERIAL

2.1 Scales collection and preparation.

The marine fish scales from the three species were purchased separately from sea food markets in Nairobi, Kenya. The fish were obtained from Malindi, along the Kenyan coast. Species identification was done at the Nairobi National Museum, Nairobi, Kenya with the marine fish being identified as Lutjanus sebea (Red snapper), Lethrinus harak (Black spot emperor) and Scalus ghobban (Blue barred parrot fish). The scales were washed separately using tap water to remove dirt and meat present and later sun dried. The dry scales were then packed in clean dry plastic bags and stored under room temperature awaiting the hydrolysis process.

2.2 Enzyme production

The bacteria (Bacillus cereus stain wwcp1) initially isolated from the mud waters from Lake Bogoria, Kenya (Wanyonyi et al., 2014) and re-plated was used for enzyme production. The enzyme production was done using a bioreactor (R'ALF plus duet fermenter, 3.7L). Media for enzyme production was prepared containing 0.25% casein, 5% glucose, K_2HPO_4, KH_2PO_4, $MgSO_4.7H_2O$, $CaCl_2$, urea and yeast extract in distilled water. The pH of the media was adjusted to 9 using NaOH solution by a pH meter and sterilized for 30 minutes in an auto clave (Tuttnauer steam 121^{0}C sterilizer).The media was then allowed to cool under room temperature after which inoculation was done with a 5% overnight grown seed bacterial culture. Temperature was set 36^{0}C, and stirrer was set at 120 rpm. After four days of continuous fermentation the enzyme was harvested, centrifuged in Sorvall ST16R centrifuge (1500rpm, 4^{0}C for 4 minutes) and placed in the cold room (4^{0}C) ready for use in the hydrolysis process.

2.3 Gelatin extraction

200g of scales from the three marine fish species were weighed and packed separately in 1L conical flasks and 500mLs of enzyme solution was added to each. The pH was adjusted to 12 after which the conical flasks were plugged using cotton wool and covered with aluminum foil. The hydrolysis process was done at a temperature of 50^{0}C (Jiang, 2012). After every 24 hours, the pH for was re-adjusted to 12 and stirring done, to ensure uniform and complete hydrolysis in the conical flasks. The hydrolysis process took 20 days, 22days and 23 days for black spot emperor, red snapper and blue barred parrot fish respectively. Gelatin solution was filtered and freeze dried.

2.4 The yield

The yield of gelatin for the scales of each species was calculated using the formulae;
%Yield= (Mass of the dried gelatin / Mass of the dry scales hydrolyzed) × 100 (Zakaria and Bakar, 2015).

2.5 Fourier Transform Infrared Spectrometry

This analysis was done for the pure gelatin from the three marine fish species using a Fourier transform infrared spectrometer IR Affinity-1S Shimadzu model. The process was carried as described by Ahmad and Benjakul (2011), but the spectra obtained in the 500 to 4500 cm^{-1}range.

2.6 Amino acid composition analysis

Amino acid composition analysis of gelatin from the three marine species understudy was performed using a narrow bore, (2.1 x 200 mm, Hypersil AA-ODS, 5 µm reverse phase column) purchased from Thermo Electron (part # 30105-202130). Samples were weighed and placed in a 13 x 100 mm Pyrex tube along with 1mL N HCl and 11 µmoles of Internal Standards (Norvaline and Sarcosine). After adding Internal Standards, the samples along with controls and blanks were exposed to liquid-phase 6N HCl for 22 hrs hours at 100^{0}C. Amino acids were separated on an Agilent 1260 with column heater, automatic injection programming UV and Fluorescence detection. 5µL of the hydrolysate was dried down and resuspended in 250 µL of 0.4 M Borate buffer. 1µL was injected. The G1367E auto sampler was used to perform pre-column derivatization and multiple sample handling. The derivatized amino acids were then eluted from the reverse phase column. Primary amino acids (tagged with OPA, Agilent #5061-3335) were detected at 338/390 nm by the Variable Wavelength (UV) detector (G1365D) and secondary amino acids (tagged with FMOC, Agilent 5061-3337) at 266/324 nm. The fluorometric detector (G1321B) was used to monitor the primary ones at excitation/emission

340/450nm and the secondary ones at 266/305nm. The assay was calibrated by a standard (Agilent 5061-3331) which was subjected to the same treatment as the samples and control, including hydrolysis. The assay was controlled by a known protein, Human Serum Albumin. An aliquot from the same batch of HSA was run with every assay.

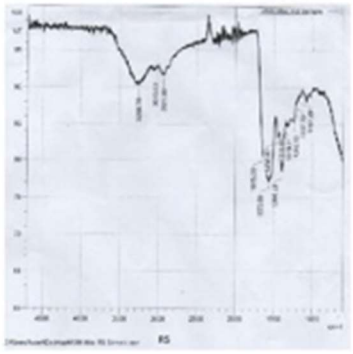
III. RESULTS AND DISCUSSION

3.1 Yield

Gelatin obtained was slightly yellow and very hygroscopic. The yield was 28.2%, 38.7% and 41.4% for the scales of red snapper (RS), black spot emperor (BSE) and blue barred parrot (BBPF) fish respectively. In general, higher yields were obtained, for long extraction periods used for the enzyme hydrolysis reaction (up to 23 days). However, even though extended extraction duration leads to increased yield, the expected downside is that the gelatin obtained could have short chains (Ahmad and Benjakul, 2011). Peptide bonds are broken alongside hydrogen bonds connecting the chains in the triple helix structure. Consequently, high reduction in the size of the peptide chains leads to the lowering of the gelling strength of gelatin (Liu *et al.*, 2008). The large size of the scales of blue barred parrot fish also contributed to the higher yield when compared to the other two species. The low yield in red snapper scales is attributable to the compactness of the scales compared to the other fish species (black spot emperor and blue barred parrot fish) investigated.

3.2 Fourier Transform Infrared Spectrometry

The Fourier transform infrared spectrometry spectra for the three species showed five Amide bands namely Amide A, B, I, II, III and two other bands as shown in Figure 1;



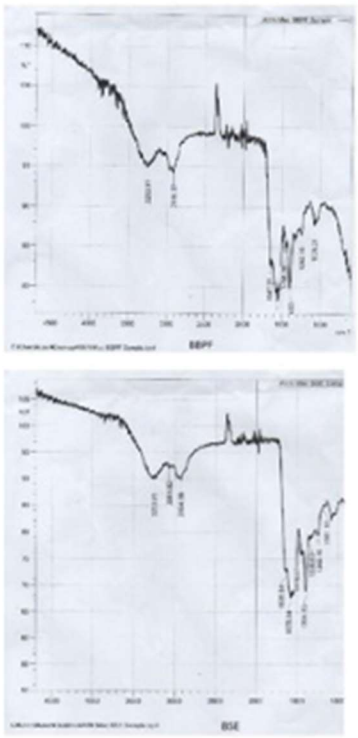


Figure 1: Fourier Transform Infrared spectra for the Red snapper, Blue barred parrot fish and Black spot emperor respectively.

Amide I bands for BSE, RS and BBPF were 1635.64, 1645.28 and 1647.21 cm-1 respectively corresponding to C=O stretching vibrations. The low amplitude were due to the interactions between C=O and adjacent chains via hydrogen bonding (Ahmad and Benjakul, 2011). This hydrogen bonding is also responsible for reduction in the frequency from the expected 1715 cm-1. Amide II bands for BSE, RS and BBPF were at 1575.84, 1570.06 and 1572.95 cm-1 respectively corresponding to C-N stretching and N-H in-plane bending. Amide III bands were similar for the three species occurring at 1242.16 cm-1 representing C-N and N-H in-plane bending as well. Amide A bands for BSE, RS and BBPF were 3253.91, 3253.91 and 3259.70 cm-1 respectively, representing N-H stretching vibration. BSE and RS exhibited Amide B bands at 3066.82 and 3045.60 respectively, corresponding to N-H stretching vibrations, with BBPF spectra missing this band. These N-H stretching vibrations bands were of low amplitudes and lower frequency than the expected 3400-3550 cm-1 due to hydrogen bonding. The other bands exhibited by BSE, RS and BBPF were 2924.09, 2931.80 and 2916.37 cm-1 which are attributable to the C-H stretching vibrations,

for the methylene groups of Glycine and Alanine indicating their relative abundance relative to other present amino acids present. The same methylene group exhibited bending vibrations at 1394.53 cm-1 for the spectra of all three species. These FTIR spectra were similar to the spectra for gelatin extracted from chicken feet by acid method which exhibited the Amide I, II, III, A and B bands at 1658.74cm-1, 155.44cm-1, 1236.36cm-1, 3322.12cm-1 and 2924.36cm-1 respectively (Widyasari and Rawdkuen, 2014).

3.3 Amino acid composition

The HPLC chromatograms showing the retention time for the different amino acids are as shown in figures 2 to 4 with the different peak heights showing the relative abundance of a given amino acid.

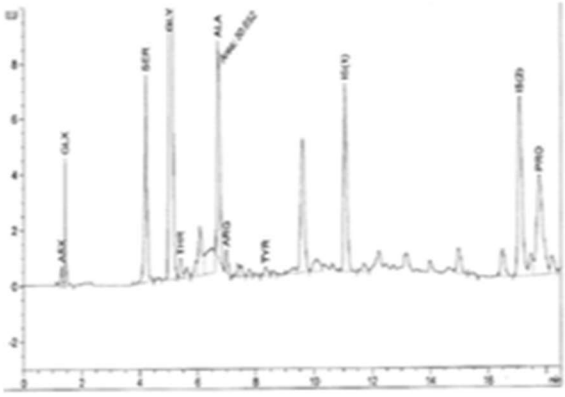


Figure 2: HPLC chromatogram of gelatin obtained from scales of Red snapper.

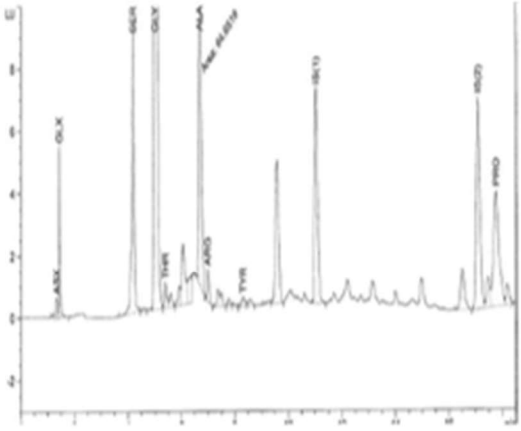


Figure 3: HPLC Chromatogram for gelatin obtained from the scales of Blue barred parrot fish

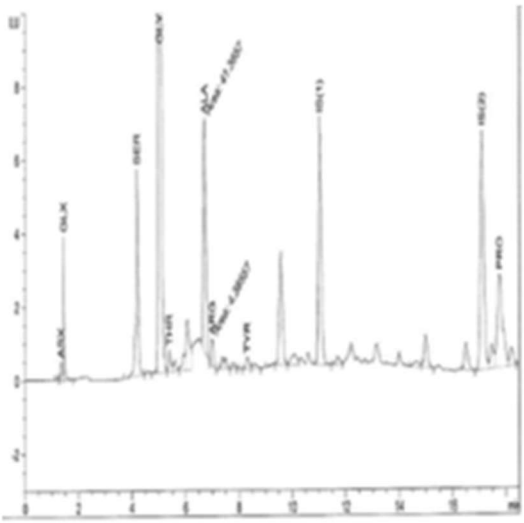


Figure 4: HPLC Chromatogram for gelatin obtained from the scales of Black spot emperor

The amino acid composition (weight %) for the three fish species is shown in table 1. The samples were analyzed in duplicates and the standard deviation (SD) is also indicated.

Table 1: Amino acid composition (%) of gelatin from the scales of Red snapper, Black spot emperor and Blue barred parrot fish.

Amino acid	% Composition ± SD in RS scales	% Composition ± SD in BBPF scales	% Composition ± SD in BSE scales
Glycine	34.84± 0.0288	35.79± 0.045	35.85± 0.0968
Alanine	14.81± 0.0005	15.42± 0.0072	15.15± 0.0085
Proline	14.16± 0.0002	11.05± 0.2048	11.63± 0.0481
Glutamic acid	8.67± 0.0013	9.05± 0.0025	8.93± 0.0001
Aspartic acid	5.15± 0.0013	5.62± 0.0013	5.58± 0.0072
Arginine	3.51± 0.0001	3.15± 0.0005	3.58± 0.0018
Leucine	2.93± 0.0002	3.26± 0.0032	2.89± 0.0005
Lysine	2.73± 0	2.84± 0.0025	2.87± 0.0001
Valine	2.55± 0	2.81± 0.0001	2.64± 0.0041
Serine	2.45± 0.0001	2.76± 0.0002	2.50± 0.0041

Methionine	2.09± 0.0098	1.62± 0.0001	2.09± 0.0265
Phenylalanine	1.92± 0.0002	2.06± 0.0032	2.04± 0.0001
Isoleucine	1.38± 0	1.64± 0	1.29± 0.0001
Threonine	1.21± 0.0018	1.09± 0.0001	1.27± 0.0013
Tyrosine	0.87± 0.0005	0.98± 0.0001	0.94± 0.0013
Histidine	0.76± 0.0008	0.94± 0.0001	0.86± 0.0008
Cysteine	Trace	Trace	Trace
Total	100	100	100

The results show that Glycine is the most abundant amino acid. This high amount of Glycine is attributed to the repeating sequence of (Glycine-X-Y) amino acid triplets in the constituent chains of gelatin, which is specific to gelatin, where X and Y are mainly Proline and Hydroxyproline respectively. The key difference of the gelatin extracted from the scales of the marine fish species under study using the operating conditions used here, when compared to the gelatin extracted from other sources like porcine skin and Grass carp fish scales (Zhang et al., 2011) and Pangasius catfish skin (Mahmoodani et al., 2014), is the absence of Hydroxyproline. The absence of Hydroxyproline as well as Hydroxylysine has also been reported by Jamilah and Harvinder (2002) in gelatin obtained from black and red Tilapia skin. The presence of Proline and Hydroxyproline in gelatin is important as this enhances gelatin's thermal stability. Zang et al., (2011) reported that the amount of Hydroxyproline is low in fish gelatin when compared to mammalian gelatin due to the low hydroxylation of Proline in turn leading to the low thermal stability. Of particular importance to note, is that Proline was higher in gelatin from the scales of red snapper at 14.16% compared to gelatin from scales of black spot emperor and blue barred parrot fish at 11.63% and 11.05% respectively. Cysteine does not take part in the structure of type I collagen; its presence in the gelatin from the scales of the three species could indicate the presence of another type of protein such as elastin or keratin (Gimenez et al., 2009). Methionine content was greater than 1.6%, higher than that found in porcine gelatin said to be 0.5%, supporting the conclusion that fish gelatin has higher amounts of Methionine compared to mammalian gelatin (Zhang et al., 2011). Gelatin from the three species under study was also low in amino acids Tyrosine and Histidine which is an inherent property of all gelatins (Mahmoodani et al., 2014).

IV. CONCLUSION

The yield of gelatin obtained (28% to 41%) shows that fish scales are a good source of gelatin. The use of alkaline protease enzyme at pH 12 and at 500C was effective in extraction of gelatin from fish scales. The amino acid composition of the different species varied slightly especially in the amount of Proline. The key difference between the gelatin extracted in this study and the mammalian sourced gelatin is the absence of Hydroxyproline. However, the two gelatins share key attributes meaning that the scales of these three marine fish species abundant in Kenya can be used as sources of gelatin on a large scale.

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