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Nile perch collagen and gelatin extraction and physico-chemical characterisation

By

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DEDICATION

To Alinda, Atiila, Faith and Birah Muyonga, the four ladies who give me reason to keep going.

DECLARATION

I declare that the thesis which I hereby submit for the degree PhD (Food Science) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at another University or institution of higher education.

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ABSTRACT

Nile perch collagen and gelatin extraction and physico-chemical characterisation

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Promoter: Dr Kwaku Gyebi Duodu
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Nile perch (*Lates niloticus*) is the most dominant fish species in Uganda, accounting for approximately 46% of all the fish landed. Industrial processing of Nile perch is estimated at 60,000 metric tonnes per annum, but processing waste, which represent approximately 50% of the raw material are under-utilised. This investigation was aimed at extracting and characterising collagen and gelatin from Nile perch waste in order to establish the potential of using these materials as sources for collagen and gelatin.

Acid soluble collagen (ASC) was extracted from young and adult Nile perch skins using 0.5 M acetic acid and precipitated using 0.9 M NaCl. The ASC yield, on dry basis, was 63.1 and 58.7%, respectively for young and adult fish skins while no collagen could be solubilised from bones of young and adult Nile perch by 0.5 M acetic acid. The skin collagens were found to consist of two alpha components (α1 and α2). Their imino acid content (19.3 and 20.0%, respectively for young and adult fish) and denaturation temperature (36°C) were higher than for most fish species. This confirmed that the imino acid content of collagen is a key determinant of the denaturation temperature of collagen. Fourier transform infrared (FTIR) spectroscopy showed a higher degree of molecular order in ASC from adult than from young Nile perch. This suggested a higher incidence of intermolecular crosslinks in adult Nile perch ASC.

Type A gelatins were extracted from skins and bones of young and adult Nile perch perch. Skins gave higher gelatin yield, higher 50°C extractability and skin gelatins generally exhibited superior functional properties to bone gelatins. Bone and skin gelatins had similar amino acid composition, with a total imino acid content of about 21.5%. Sodium dodecyl sulphate polyacrylamide gel electrophoresis revealed that skin gelatins had a higher content of polypeptides larger than β-chains (~200 kD) compared to bone gelatins. The functional properties of the gelatins were found to be correlated to the molecular weight distribution, with the >β fraction contributing positively to functional properties while the <α fraction contributed negatively. Passing gelatin extract through a column of activated carbon eliminated the fishy odour.

Forier transform infrared spectroscopy indicated that denaturation of collagen to gelatin leads to loss of molecular order and that the later gelatin extracts, derived from the more crosslinked collagen, possess higher molecular order than earlier gelatin extracts.

In general, the results showed that Nile perch skins and bones have potential for supplementing mammals as sources of gelatin. There is also potential of exploiting Nile perch skin as source of acid soluble collagen. It was also shown that there are no marked age-related changes in Nile perch collagen solubility as well as gelatin extractability and properties, probably because there is minimal development of mature crosslinks with animal age, in Nile perch collagen.

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1 INTRODUCTION & LITERATURE REVIEW

1.1 STATEMENT OF PROBLEM

Nile perch (*Lates niloticus*) belongs to the genus *Lates* and the family *Centropomidae*. It was introduced in Lake Victoria in the 1950s but has grown to become the most dominant fish species in the lake and other lakes in the region (Acere, 1993). In Uganda, Nile perch catches represent approximately 46% (~ 100,000 tonnes per year) of all the fish landed (UIA, 2001). The fish has been widely consumed within the country since its introduction but industrial processing has only become wide spread in the last decade. Currently about ten firms, within Uganda alone, are involved in industrial processing of fish. These firms are involved in production of Nile perch fillets, mainly for the export market. Industrial processing of Nile perch in Uganda is estimated at 60,000 metric tonnes per annum. The balance of about 40,000 metric tonnes is either sold unprocessed or processed (mainly by smoking) by informal set-ups. Approximately 50% of the raw material (fish) remains as processing waste after filleting (Shahidi, 1994). This is mainly in form of skeletons and skins. This creates approximately 30,000 metric tonnes of waste a year. Skins and bones are underutilised and pose a disposal problem for the processing firms.

Nile perch skins and bones could be used in the manufacture of gelatin. Gelatin is a denatured form of collagen that can be derived from any collagenous material. The traditional sources of gelatin are pigskins, bovine hides and bones. Recent outbreaks of Bovine spongiform encephalopathy (BSE) and increase in demand for kosher and halal foods have created a demand for alternative sources of gelatin for food applications. Gelatin from fish with scales such as Nile perch has potential for use in kosher and halal foods since it is generally acceptable for use as food by Muslims and Jews. It may also find market among the Hindu, who are not permitted to consume cattle products. Despite such potential, fish has generally not been widely used as a source of gelatin, probably because most species give gelatin of low quality compared to mammalian gelatin. Unlike mammalian gelatins, fish gelatins vary widely in composition, particularly with respect to their imino acids (proline and hydroxyproline) content and in their physico-chemical properties.

It is estimated that a total of about 700,000 people, out of Uganda's total population currently estimated at 24 million are engaged in fish-related enterprises, including fishing, manufacture of fishing gear, artisan processing, fish trade and industrial scale processing (MAAIF, 2000). Processing of fish waste to value-added products like gelatin would enhance the contribution of the fisheries sector to the national economy and is likely to lead to an improvement of the income of those involved in the sector.

The share of fisheries sector to Uganda's GDP is estimated at 2.2% and fish export revenue to Uganda has increased from less than US$ 1 million to more than US$ 50 million per annum in the last decade (MAAIF, 2000). The national vision is to triple the value of fish exports and to increase domestic consumption significantly over the next 25 years. The Uganda Investment Authority (UIA), which is responsible for promoting investment in the country and is supposed to guide potential investors on the promising areas for investment, has identified the processing of value added products from fish processing waste as one of the viable areas for investment (UIA, 2001). There is indeed adequate and inexpensive raw material for such an enterprise. Since Nile perch is also widely produced in neighbouring Kenya and Tanzania and the three countries have very favourable trade arrangements, an investment in processing of Nile perch waste could tap the raw materials and the markets of all the three countries.

1.2 LITERATURE REVIEW

1.2.1 Gelatin sources and uses

Gelatin is a high molecular weight polypeptide derived from collagen, the primary protein component of bones, skins and tendons (Poppe, 1992). It is obtained by denaturation and solubilisation of collagen derived from the skin, white connective tissue and bones of animals. Commercially, gelatin is mainly derived from pigskins and bovine hides and bones. It has been believed for a long time that fish skins generally yield low gel strength gelatin (Osborne, Voight & Hall, 1990; Holzer, 1996; Gudmundsson & Hafsteinsson, 1997) but recent studies (Grossman & Bergman, 1992; Montero, Fernández-Diaz & Gòmez-Guillen, 2002) have shown that it is possible to derive high gel strength gelatin from fish skins. The possibility of deriving

gelatin from fish bones has been less investigated. Osborne *et al.* (1990) reported use of headed and gutted, as well as, only gutted lung fish in gelatin extraction. The gelatin yield was rather low (4.9% in both cases) and the resulting gelatin had very high levels of ash (8.66 and 13.85%, respectively). Regenstein (2001) reported successful extraction of gelatin from shark bones but did not report the properties of the gelatin.

Fish gelatin is fast gaining popularity for use in kosher and halal foods. Kosher gelatin fetches 3 - 10 times the price of non-kosher gelatin and its availability is still rather low (Regenstein, 2001). Fish gelatins are available both in the gelling and non-gelling forms. The non-gelling fish gelatins find applications as stabilisers, film forming, binding and clarifying agents.

Estimated total world usage of gelatin is 200,000 metric tons per year with US usage being about 30,000 metric tons per year for food and 10,000 metric tons per year for pharmaceutical applications (Herz, 1995). Annual average use of gelatin for food applications is growing at about 3% (Poppe, 1992). Food applications of gelatin include gelling, thickening, stabilising, emulsification, binding, foaming, dispersing, aeration and glazing (Helcke, 2000). Gelatin is used because of its unique physical properties, i.e. its ability to form thermo-reversible gels, which melt below 37°C, normally between 24 and 32°C. Maturation time, gelatin concentration and presence of salts influence the exact melting point and Bloom gel strength. Gelatin swells when placed in cold water, absorbing 5 - 10 times its own volume of water. When heated to temperatures above its melting point, the swollen gelatin dissolves and forms a thermo-reversible gel when cooled. This characteristic is desired in many foods.

There are a number of reviews covering gelatin uses in food-related and other industries (Jones, 1977; Wood, 1977; Anon, 1980; Poppe, 1992). Gelatin is used as a stabilizer for frozen desserts, where it functions as an inhibitor of ice crystal growth and lactose crystallization during frozen storage of dairy products (Morley & Ashton, 1982; Fiscella, 1983; Morley, 1984). It is a texturizing agent in several dairy products, while in some, it adjusts viscosity, stabilizes emulsions, or improves foaming (Poppe, 1992). It has been shown to decrease syneresis (Fiszman, Llunch &

Salvador, 1999; Fiszman & Salvador, 1999) and to increase gel strength and solidity (Fiszman *et al.*, 1999) in yogurt and milk gels. It has been used to form milk gels at pH values outside that at which acid-induced coagulation occurs (Salvador & Fiszman, 1998). Gelatin has also been used in making low fat cheeses (Anon, 1996; Kurultay, Oksuz & Simsek, 2000). When added at a level of 0.1 – 0.3% to cheese milk, gelatin increases yield of cheese by 24 – 49% but this results in cheese with higher moisture content, water soluble nitrogen and pH (Kurultay *et al.*, 2000). Gelatin is also used in confectionery, meat and fish products, as well as in delicatessen products (Jones, 1977; Poppe, 1992) and as a clarifying and stabilizing agent for beverages (Jones, 1977; Poppe, 1992; Versari, Barbanti, Potentini, Mannuzzu, Salvucci & Galassi, 1998). When used in clarification treatment it reduces turbidity, total polyphenols, colour intensity and brown polymers (Versari *et al.*, 1998).

It is also used in low calorie spreads, imparting emulsion stability (Poppe, 1992). Gelatin has also been used in edible films and like other hydrocolloids imparts durability as well as good gas and lipid barrier properties (Greener & Fennema, 1994; Krochta & deMulder-Johnston, 1997). However, it has poor water barrier properties due to its hydrophilic nature. Film preparation temperature (Arvanitoyannis, Psomiadou, Nakayama, Aiba & Yamamoto, 1997; Arvanitoyannis, Nakayama &Aiba, 1998), plasticisers like sorbitol, glycerol, acetylated monogycerides, polyethylene glycol and sucrose (Greener & Fennema, 1994; Arvanitoyannis *et al.*, 1997, Arvanitoyannis *et al.*, 1998) and presence of other materials affect the strength and permeability of gelatin films. Glutaraldehyde crosslinking of gelatin leads to decrease in film extensibility, increase in stress at break and Young's modulus (Bigi, Cojazzi, Panzavolta, Rubini & Roveri, 2001).

A number of alternative approaches for manufacturing products without gelatin have been tried (Johnston-Banks, 1990). For water dessert gels, carrageenan and other gums have been used. However, the absence of a low temperature melting point and greater pH sensitivity has led to limitations in the usefulness of these alternative materials.

Non-food uses of gelatin include manufacture of both hard- and soft-type drug capsules (Wood, 1977), depending on the Bloom (gel strength) and as binders for light-sensitive "emulsions" in the manufacture of photographic films (Kragh, 1977). Gelatin is also used in cosmetics, as a sizing agent in paper manufacture, as an adhesive and as a floculant (Wood, 1977).

1.2.2 Manufacture of gelatin and its effect on gelatin properties

The procedure for the manufacture of gelatin varies and the raw material used determines the process adopted and the quality of gelatin formed (Poppe, 1992). The procedure generally involves acid and sometimes alkali pretreatment of skin or bones, aqueous extraction of gelatin followed by filtration, evaporation, cooling, drying and milling (Priebbenow, 1998). Gelatin extracted with only acid pretreatment is referred to as type A gelatin while those whose manufacture entails alkali pretreatment are referred to as type B. Type of acid used for extraction (Grossman & Bergman. 1992; Gudmundsson & Hafsteinsson, 1997; Priebbenow, 1998; Gòmez-Guillèn & Montero, 2001), thermal history (Cho & Song, 1996; Michon, Cuvelier, Relkin & Launay, 1997), extraction pH (Tawfeek, Khalid & Safwat, 1992; Priebbenow, 1998), and salts (Priebbenow, 1998) influence the yield as well as the properties of the resulting gelatin. Cho & Song (1996) showed that the yield of pigskin gelatin increases with extraction temperature, whereas gel strength, viscosity and molecular weight decrease. According to Chung, Kim, Ockerman and Min (1990), scalding of pigskins before gelatin extraction decreases viscosity and gel strength of resulting gelatin. Alkali-processed gelatin from chicken legs have been found to have higher melting point and viscosity than acid-processed gelatin from the same source (Tawfeek *et al.*, 1992). Acid-processed gelatins and gelatins extracted at high temperature normally have a higher proportion of low molecular weight peptides arising from hydrolysis and as a result may exhibit lower viscosity. The acid-processed gelatins also generally exhibit less thermo-hydrolysis when exposed to heat (Koepff, 1984). This is because they already contain a higher proportion of low molecular weight peptides and are less likely to undergo as much hydrolysis as the alkali-processed gelatins.

The type of acid used in extraction of gelatin has been shown to influence gelatin properties (Müller & Heidemann, 1993; Gòmez-Guillèn & Montero, 2001). Gòmez-

Guillèn and Montero (2001) reported lower gel strength, viscous modulus, elastic modulus and melting temperature for megrim skins gelatin extracted with citric acid compared to that extracted using acetic or propionic acid. Müller and Heidemann (1993) compared the degree of collagen hydrolysis by different acids during manufacture of gelatin from bovine hide and reported a higher degree of hydrolysis by organic acids than by inorganic acids. According to Gòmez-Guillèn and Montero (2001), the differences in the extent of collagen hydrolysis registered while using different acids in otherwise similar conditions can be attributed to differences in ionic strength. These authors proposed that more hydrolysis occurs at higher ionic strength.

The schematic below (Fig. 1.1), adapted from Poppe (1992) outlines the processes used in making gelatin from different sources.

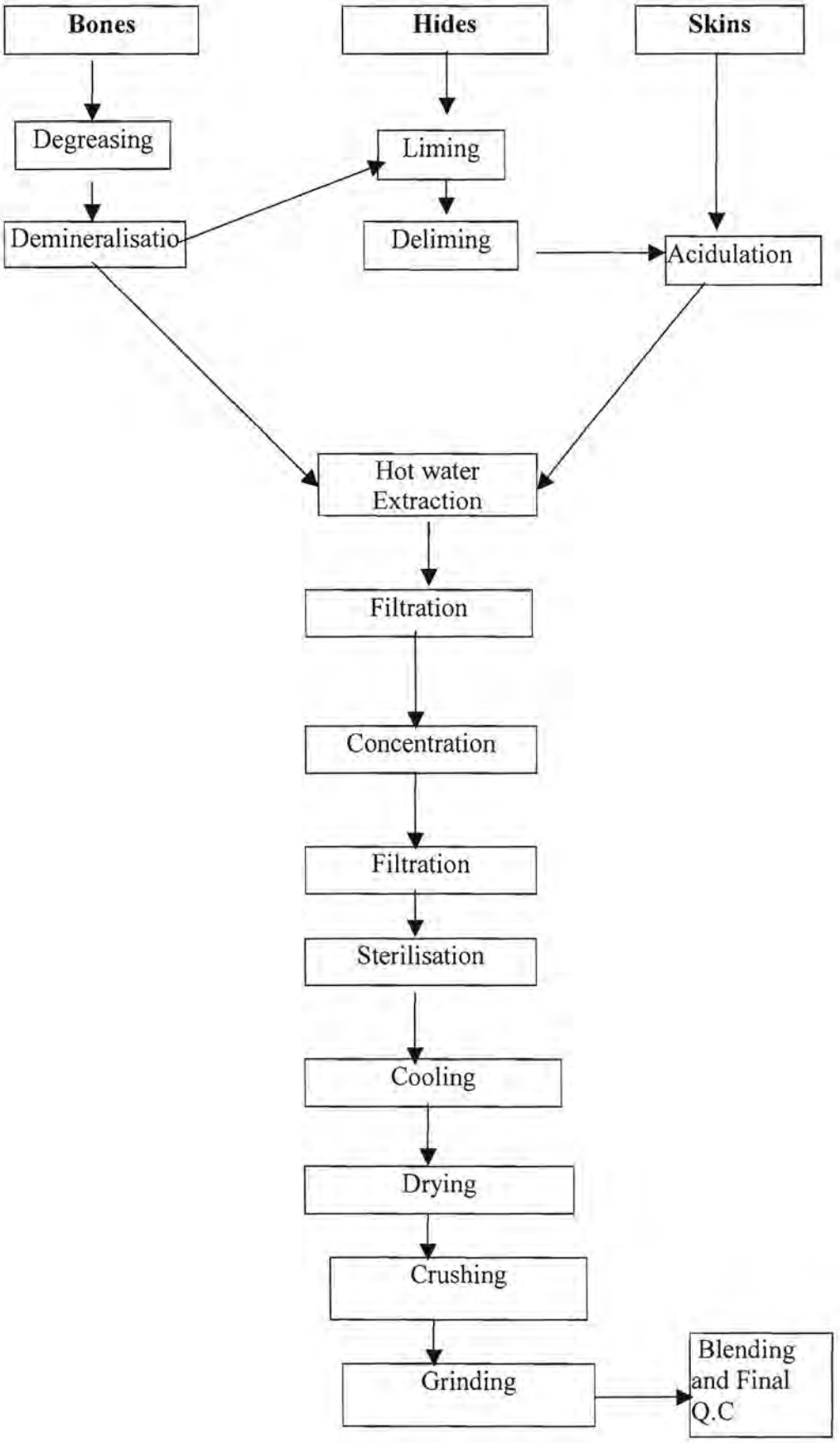


Figure 1.1: Procedures for gelatin extraction from different sources (Adapted from Poppe, 1992)

Fish skin gelatin has been produced using combined acid and alkali pretreatment (Grossman & Bergman, 1992; Gudmundsson & Hafsteinsson, 1997; Wanwimol & Worawattanamateekul, 1999) as well as by acid pretreatment only (Gòmez-Guillèn & Montero, 2001). The acids that have been used in fish skin gelatin production include sulphuric acid, hydrochloric acid and phosphoric acid (Gudmundsson & Hafsteinsson, 1997), propionic, acetic, citric, lactic and tartaric acid (Gòmez-Guillèn & Montero, 2001). Sodium hydroxide or calcium hydroxide are the main reagents used in alkali pretreatment. Gudmundsson and Hafsteinsson (1997) studied the characteristics of gelatin from fish skins and showed that the chemical treatments affected both the yield and properties of the gelatin.

1.2.3 Collagen

Collagen is the most abundant protein of animal origin, comprising approximately 30% of total animal protein. Collagen, extracted from different raw materials has been used for clarifying beverages (Taylor, 1997; Hickman, Sims, Miles, Bailey, de Mari & Koopmans, 2000), in cosmetics (Morimura, Nagata, Uemura, Fahmi, Shigematsu & Kida, 2002), in casings for meat products (Hood, 1987) and in a host of biomedical applications (Lee, Singla & Lee, 2001). Medical applications of collagen include use in drug delivery systems, sponges for burns and wounds and in tissue engineering. Collagen is also the raw material from which gelatin is derived. The properties of collagen from which gelatin is derived significantly influence the properties of gelatin.

1.2.3.1 Amino acid composition

Collagen has a repeating primary sequence of $(Gly\text{-}X\text{-}Y)_n$ with approximately one third of X and Y being imino acids (Franenkel-Conrat, 1963; Balian & Bowes, 1977; Darby & Creighton, 1993). Glycine makes up approximately one third of the collagen residues and alanine, approximately one ninth (Balian & Bowes, 1977). Generally, collagen contains very low levels of the sulphur-containing and aromatic amino acids and is among very few proteins that contain hydroxyproline (Veis, 1964).

Mammalian and avian collagens have very similar amino acid composition (Balian & Bowes, 1977).

Various authors have reported amino acid composition of fish collagens and generally reveal that the levels of the imino acids proline and hydroxyproline vary significantly among different fish species (Balian & Bowes, 1977; Poppe, 1992; Lu, Weilmeier, Chapman & Regenstein, 1994, Gudmundsson & Hafsteinsson, 1997; Lu, Chapman & Regenstein, 1997; Gilsenan & Ross-Murphy, 2000). The amount of imino acids, especially hydroxyproline depends on the environmental temperature at which the fish lives and it affects the thermal stability of the collagens (Rigby, 1968; Balian & Bowes, 1977; Kimura, Zhu, Matsui, Shijoh & Takamizawa, 1988). Collagens derived from fish species living in cold environments tend to have a lower content of hydroxyproline and they exhibit lower thermal stability than those from fish living in warm environments. Cold water fish species have also been reported to contain higher levels of the hydroxyamino acids, serine and threonine (Balian & Bowes, 1977). Studies on amino acid composition of collagens from warm water fish species have been quite limited. Grossman and Bergman (1992) showed that tilapia gelatin contains higher levels of imino acids than cold water fish collagens. The stabilising effect of hydroxyproline on the triple helical structure of collagen has been attributed to its involvement in inter-chain hydrogen bonding (Darby & Creighton, 1993). According to Holmgren, Taylor, Bretscher and Raines (1998), the stabilising effect of hydroxyproline results from the inductive effect of the hydroxyl group of hydroxyproline. They provided support for this hypothesis by substituting hydroxyproline with fluoroproline in peptides and found that the stability of the peptides, as shown by denaturation temperature, was in the order $(ProProGly)_{10} < (ProHypGly)_{10} < (ProFlpGly)_{10}$. The high stability of the ProFlpGly was attributed to the high electronegativity of fluorine.

Few studies have been conducted to compare amino acid composition of collagen from different tissues of the same organism. Eastoe and Leach (1958) and Balian and Bowes (1977), reported the amino acid composition for collagen from several sources, showing that collagens derived from bone and skin of the same animal generally had similar amino acid compositions.

1.2.3.2 Collagen super secondary structure

Collagen consists of three left handed helices (α-chains), normally twisted about each other (Figure 1.2) to form right handed tri-helical structures (Balian & Bowes, 1977; Darby & Creighton, 1993). The chains are held together by Van der Waals forces and hydrogen bonds between the NH group of glycine and a backbone carbonyl in one of the other chains and further stabilised by hydrogen bonding involving hydroxyproline (Darby & Creighton, 1993). Glycine allows the chains to come close enough for hydrogen bonding and water helps to stabilise the bonds (Balian & Bowes, 1977). The super helical aggregates of collagen are held together by covalent crosslinking of hydroxylysine residues, introduced after the helices are formed (Darby & Creighton, 1993).

Figure 1.2: Triple helical structure characteristic of collagen (Adapted from Bailey & Light, 1989)

X-ray diffraction shows that a collagen helix typically has a length of 100 nm and a diameter of 1.4 nm (Balian & Bowes, 1977; Darby & Creighton, 1993). Based on light and dark bands in collagen electron micrographs, Balian and Bowes (1977) proposed that individual molecules normally overlap with each other.

According to Bailey, Paul and Knott (1998) skin collagen fibres are randomly oriented and permit considerable extension. Tendon fibres are aligned in parallel while bone collagen fibres are organised in concentric layers to maximise strength.

1.2.3.3 Collagen variants

There are at least 19 variants of collagen, named type I – XIX (Bailey *et al.*, 1998) and more continue to be characterised. These collagen variants vary in their macromolecular structure (Bailey, 1987). Type I, II, III and V are the fibrous

collagens (Bailey & Light, 1989; Bailey *et al.*, 1998). These collagens are long (300 nm), rod-like molecules, which self assemble in a parallel, quarter staggered end overlap arrangement to form fibres possessing a characteristic band pattern with a periodicity of 67 nm (Bailey *et al.*, 1998).

Type I collagen is found in all connective tissue, including bones and skins (Bailey & Light, 1989; Bailey *et al.*, 1998). It is a heteropolymer of two α1 chains and one α2 chain. The α1 and α2 chains each have a molecular weight of about 95,000 Da but vary slightly in their amino acid composition. Type I collagen consists of one third glycine and contains no tryptophan and cysteine and is very low in tyrosine and histidine. Type II collagen is a homopolymer of three α1 chains and contains three times the amount of hydroxylysine as type I collagen (Bailey & Light, 1989). A high proportion of these residues are O-glycosylated to sugars. Type II collagen contain more methionine than type I collagen. The dominant collagen in cartilages is type II collagen.

Type III collagen is mainly found in embryonic tissue, scar tissue, arteries and intra-organ connections (Bailey & Light, 1989). It is composed of identical α1 chains and contains intra and possibly intermolecular disulphide bonds. Type V collagen contains α1 and α2 chains in a ratio of 1:2 as well as α3 chains. α3 chains contain more cysteine than α1 and α2 chains. Type IV collagen is high in hydroxyproline and hydroxylysine and in addition to the usual 4-hydroxyproline, it also contains 3-hydroxyproline.

Type IV, VII and X, belong to the category of network collagens. These form networks and are found in basement membranes. Type VI collagen occurs in loosely packed filamentous structure formed by end to end alignment of tetramers. Type IV, VI, VII, IX and X are filamentous and not important in tissues commonly used for gelatin extraction. The other collagen variants occur in minor amounts and are mainly found associated with the major variants. The functions of most of these minor collagen variants have not been established. The different collagen variants also vary in the nature of the constituent α-chains. Different α-chain types vary slightly in amino acid composition and as a result have slight differences in hydrophobicity.

Several studies have focused on the characterisation of different fish skin collagens (Piez, 1965; Rigby, 1968; Kimura & Ohno, 1987; Sato, Yoshinaka, Yoshiaki & Sato, 1989; Montero, Borderias, Turnay & Leyzarbe, 1990; Montero, Alvarez, Marti & Borderias 1995; Montero, Gòmez-Guillèn & Borderias, 1999; Nagai & Suzuki, 2000; Sivakumar, Arichandran, Suguna, Mariappan & Chandrakasan, 2000). Most fish skin collagens have been found to be type I, consisting of α1 and α2 chains (Nagai, Yamashita, Taniguchi, Kanamori & Suzuki, 2001; Gòmez-Guillèn, Turnay, Fernández-Diaz, Ulmo, Lizarbe & Montero, 2002; Nagai, Arakai & Suzuki, 2002). These α-chain variants though having approximately the same molecular weight (~95,000Da) can be separated by SDS PAGE due to their different affinity for SDS. Alpha 2 has a higher affinity for SDS and consequently exhibits a higher mobility than α1 (Kubo & Takagi, 1984). Piez (1965) isolated three variants of α-chains (α1, α2 and α3) from cod skin collagen and found that these variants differed in their amino acid composition. Alpha 3 has also been isolated from rainbow trout (Saito, Takenouchi, Kunisaki & Kimura, 2001), white sturgeon (Kimura, 1992), common horse mackerel (Kimura, *et al.*, 1988; Yoshida, Fujisawa, Mizuta & Yoshinaka, 2001) and eel (Kimura *et al.*, 1988).

1.2.3.4 Age-related changes in collagen

The most widely studied and probably most important change in collagen with age is crosslinking. According to Balian and Bowes (1977), esters, carbohydrates, aldehydes, γ-glutamyl and ε- amino groups are involved in collagen crosslinking. The mechanisms of collagen crosslinking have been extensively studied by Bailey and colleagues (Kent, Light, & Bailey, 1985; Bailey & Light, 1989; Sims & Bailey, 1992; Bailey, Sims, Avery, & Miles, 1993; Bailey *et al.*, 1998; Sims, Avery, & Bailey, 2000). Collagen crosslinking involves two different mechanisms. The initial crosslinking of collagen molecules during development involves a precisely controlled enzymic process (Figure 1.3). The products are divalent but mature spontaneously to form stable multivalent crosslinks.

is the dominant mature crosslink in dermal collagens. The keto-imines, which are the dominant immature crosslinks in bones, react with hydroxy-lysine aldehyde to form the pyridinoline hydroxylysyl-pyridinoline (Hyl-Pyr) or with lysine aldehyde to form pyrrole.

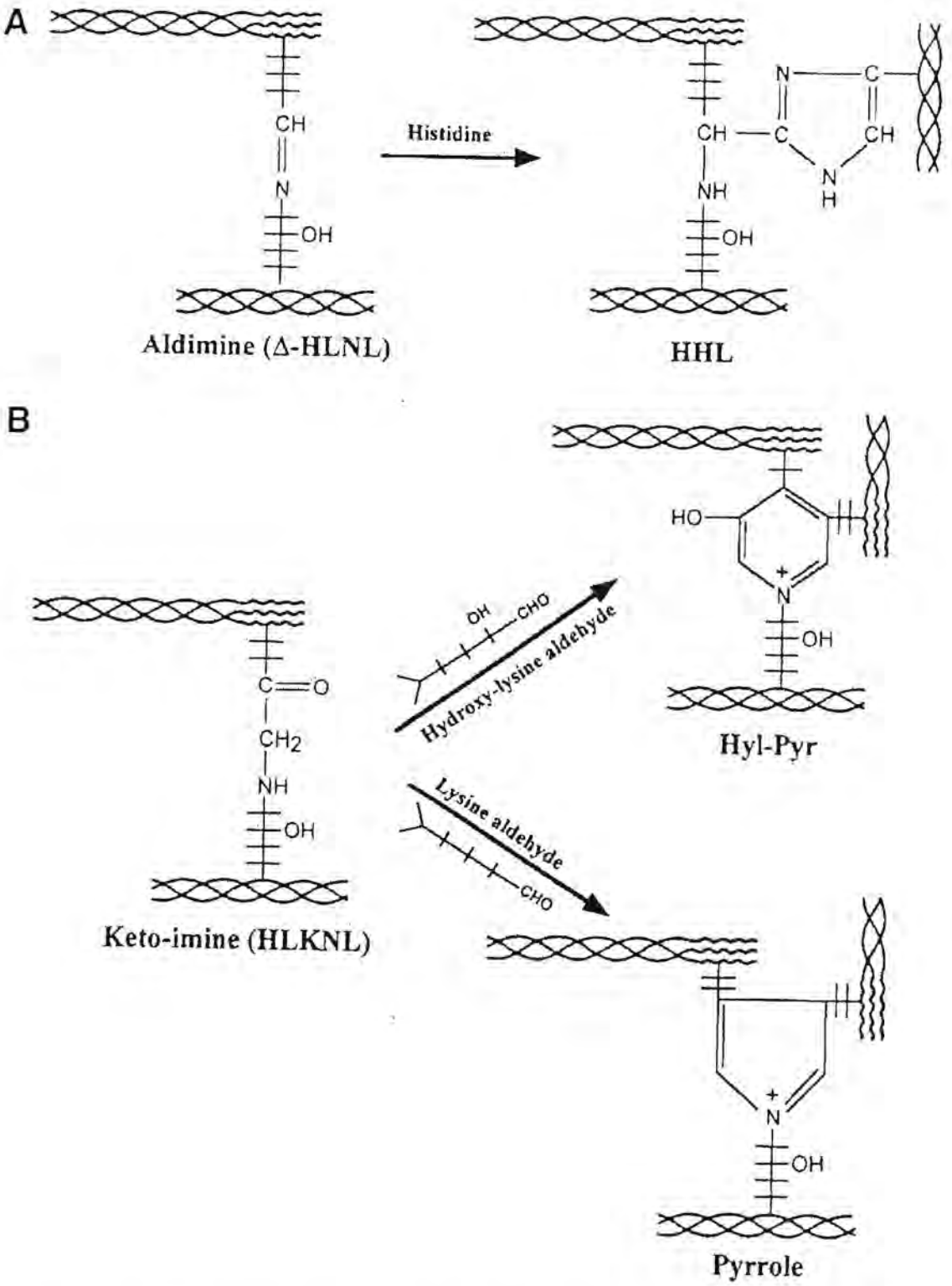


Figure 1.4: Maturation of immature collagen crosslinks A - aldimine & B - keto-imine (Source: Sims *et al.*, 2000).

Crosslinking by glycation occurs during aging and is most important in determining the properties of collagen during senescence (Bailey *et al.*, 1998). It has been

demonstrated using *in vitro* studies that glycation causes increase in the tensile strength (Kent *et al.*, 1985; Bailey *et al.*, 1993) and denaturation temperature (Bailey *et al.*, 1993) of collagen. It seems therefore that even for adult animals (after achieving sexual maturity), the ease with which collagen can be transformed to gelatin decreases with age.

Fish skin collagens have been reported to develop minimal amounts of mature crosslinks (Hickman *et al.*, 2000). Cohen-Solal, Le lous, Allain and Meunier (1981) also demonstrated by measuring hydrothermal isometric tensions that fish skin collagen crosslinks do not mature to thermally stable bonds. As a result of its low content of mature crosslinks, even adult fish skin collagen is soluble in dilute acids (Sikorski & Borderias, 1988).

1.2.3.5 Stability of collagen to chemicals, enzymes and heat

Balian and Bowes (1977) reviewed the effects of heat, chemicals and enzymes on collagen. Generally, native collagen is very stable and resistant to enzyme and chemical treatments at ambient temperatures. Prolonged alkali treatment however, leads to loss of amide nitrogen and conversion of a small fraction of arginine to ornithine. This leads to change of the isoelectric point (pI) from about 9.3 to 4.8 - 5.0. Treatment of acid soluble collagen with dilute alkali solutions (pH 11) at 40°C for short periods leads to limited breakdown to form $\alpha 1$ and $\alpha 2$ fractions. Treatment of insoluble hide and bone collagen with 5 - 8% NaOH, saturated with sodium phosphate leads to greatly increased solubility in acid and the resulting eucollagen has properties comparable to those of soluble collagen extract. Hydrazine and hydroxylamine break ester-like bonds and increase collagen solubility. Proteolytic enzymes may cleave a few intra-molecular linkages and may significantly increase solubility, reduce viscosity and tensile strength. Most enzyme activity occurs on the non-helical part of collagen and significant breakdown of the collagen helix can only be achieved by collagenases.

Heat treatment of collagen leads to denaturation and collapse of the helical structure and disentanglement of the chains. The collagen also shrinks (at the shrinkage temperature) causing textural changes in the tissue (meat) in which it is contained.

The stability of collagen depends on the extent of crosslinking in the collagen and as discussed earlier, this increases with age of the animal and varies with tissue. Thermal stability of collagen also depends on the imino acid content (Rigby, 1968; Purslow, 1987). The denaturation temperature for mammalian collagens is about 36 - 41°C (Hickman *et al.*, 2000; Leikina, Mertt, Kuznetzova & Leikin, 2002). Denaturation temperature of fish collagens vary widely and are related to the environmental temperature in which the fish lives. Collagen denaturation temperature has been reported for cod (15°C) (Rigby, 1968), Alaska pollack (16.8°C) (Kimura & Ohno, 1987), muscle of carp (32.5°C), eel (29.3°C), common mackerel (26.1°C), chum salmon (19.4°C) (Kimura *et al.*, 1988), Japanese seabass (30°C), skip jack tuna (29.7°C), ayu (29.7°C) (Nagai & Suzuki, 2000) halibut (17°C) and bigeye-tuna (31°C) (Rose, Kumar & Mandal, 1988).

1.2.4 Changes associated with conversion of collagen to gelatin

According to Johns and Courts (1977) transition of mature mammalian collagen to gelatin entails the hydrolysis of lateral, mainly non-peptide linkages, to effect a depolymerisation of multi-chain units; hydrolysis of peptide bonds; and disruption of hydrogen bonding. Some of the lateral bonds are acid labile. They are only slowly affected by alkali up to pH 13, although the effect is faster above this pH. Collagens with minimal crosslinking e.g. fish skin collagen require only the disruption of hydrogen bonding.

Hydrolysis of peptide bonds mainly appear to involve linkages at glycine (Johns & Courts, 1977) and at arginine (Müller & Heidemann, 1993). Müller and Heidemann (1993) found arginine to the left of the COOH end in 50% of peptides derived from calfskin collagen by the acid gelatin manufacture process. In 40% of the peptides, it was found to occupy the second position to the right of the split position. Proline and hydroxyproline were never found at the terminal ends of the peptide sequences. Koepff (1984) compared 250 g Bloom acid-processed pigskin gelatin to similar gel strength bovine hide and bone gelatins made by the acid and alkali processes and found the proportion of peptides smaller than the α chain, to vary in the order limed hide > acid pigskin > limed ossein. The proportion of the fractions with molecular

weight less than α were 35%, 32% and 25% respectively for the limed hide, acid pigskin and limed ossein gelatins. It seems both the raw material and the process used in gelatin manufacture determine the extent of peptide bond hydrolysis in the manufacture of gelatin.

The composition of gelatin is similar to that of the source collagen, except for changes arising from hydrolysis of amide groups of asparagine and glutamine, split off of urea from arginine to give ornithine and concentration of dominant residues (glycine, alanine, proline and hydroxyproline) due to purification (Eastoe & Leach, 1977). The split off of urea occurs only to a limited extent while the extent of the amide hydrolysis depends on the severity of the gelatin manufacturing process (Eastoe & Leach, 1977; Poppe, 1992). Severe acid pretreatment such as that used in demineralisation of bones causes hydrolysis of a significant proportion of amide groups, while mild acid pretreatment causes minimal change. The alkali process normally entails longer pretreatment and results in conversion of most amide groups to carboxyls and yields purer gelatin (Eastoe & Leach, 1977). According to Johns and Courts (1977), the long soak in alkali during lime pretreatment removes a considerable amount of non-collagenous material.

1.2.5 Properties of gelatin

The properties of gelatin vary with source (Gilsenan & Ross-Murphy, 2000), extraction procedure (Poppe, 1992; Gudmundsson & Hafsteinsson, 1997) and additives present in the gelatin or the system in which the gelatin is analysed (Choi & Regenstein, 2000; Sarabia, Gómez-Guillén & Montero, 2000; Fernández-Diaz, Montero & Gómez-Guillén, 2001).

1.2.5.1 Composition

Commercial gelatins contain approximately 87.5% protein, with the 12.5% constituted almost exclusively moisture and ash. The amino acid composition of gelatin is determined by the amino acid composition of the collagen from which it is derived and is therefore dependent on the source species. Gelatin contains all the essential amino acids found in proteins except tryptophan (Leach & Eastoe, 1977).

Cysteine is either absent or present in trace quantities (Poppe, 1992). In contrast to mammalian gelatins, fish gelatins vary widely in composition, particularly with respect to the imino acids proline and hydroxyproline (Norland, 1990; Poppe, 1992; Lu *et al.*, 1994, Gudmundsson & Hafsteinsson, 1997; Lu *et al.*, 1997; Gilsenan & Ross-Murphy, 2000; Yoshimura, Terashima, Hozan, Ebato, Nomura, Ishii & Shirai, 2000). Table 1.1 below gives the amino acid composition for Ox hide, tilapia and cod skin gelatins. The imino acid content is ~30% for mammalian gelatins (Eastoe, 1967; Poppe, 1992) and 16 - 18% for most fish species (Osborne *et al.*, 1990; Norland, 1990; Gudmundsson & Hafsteinsson, 1997; Gilsenan & Ross-Murphy, 2000). The amino acid content of gelatins vary from one source to another but always consists of large amounts of proline, hydroxyproline and glycine (Gilsenan & Ross-Murphy, 2000). The ratio of glycine to glutamic acid has been found to be higher in cod skin gelatin than in tilapia skin gelatin, whereas the levels of proline and hydroxyproline are lower in cod skin gelatin (Gudmunsson & Hafsteinsson, 1997). The level of imino acids is important in gelatin since their pyrrolidine groups act as nucleation sites for the formation of potential junction zones during gelation (Ledward, 1986). Gelatin with high imino acid content, therefore tend to exhibit better gelling properties than those with low levels of imino acids.

Table 1.1: Amino acid composition (g/1000 g protein) of some gelatins

Amino Acid	Ox hide Gelatin[a]	Tilapia Gelatin[b]	Cod Skin Gelatin[c]
Aspartic Acid	66	52.9	61.7
Threonine	22	25.5	24.2
Serine	40.8	31.2	62.5
Glutamic Acid	116	88.5	100
Alanine	110	92.2	88.2
Cysteine	Nd	Nd	<1
Valine	25.7	13.2	18.7
Methionine	8.6	12.3	19.2
Isoleucine	17.2	10.7	13.6
Leucine	34.1	25.6	23.3
Tyrosine	3.8	7.2	6.3
Phenylalanine	22.6	19.7	17.7
Histidine	8.2	10.1	13.9
Hydroxylysine	9.1	12.7	Nd
Lysine	42.6	35.4	34.4
Arginine	87	87.8	82.4
Hydroxyproline	134	103.1	67.7
Proline	165	150.5	105.1
Glycine	276	204.0	248.8
Ornithine	Trace	0.4	6.0
Ammonia	Nd	16.1	4.8

[a] – Eastoe (1967)

[b] - Grossman & Bergman (1992)

[c] - Gudmundsson & Hafsteinsson (1997)

Nd – Not detected

Generally, cold-water fish gelatins have low hydroxyproline content (Gilsenan & Ross-Murphy, 2000; Gudmundsson, 2002). As a result, cold-water fish gelatins have a higher critical concentration (minimum gelatin concentration required to form a gel)

and lower melting point than bovine and warm water fish gelatins. Sarabia *et al.* (2000) reported a higher level of imino acids in tilapia than in megrim. The two were mainly found to differ in the amount of hydroxyproline. Megrim was found to contain more serine and methionine. Since fish gelatins vary widely in their functional properties, different fish species may be targeted depending on the desired properties.

1.2.5.2 Molecular weight distribution

Since gelatin is a poly-dispersed (heterogenous) protein it is not helpful to think of average molecular weight. Instead the molecular weight distribution is of greater value. Generally, most gelatins consist of molecules with molecular size ranging from more than 1,000,000 Da to about 10,000 Da (Tavernier, 1989). The α-chain (molecular weight approximately 95,000 Da) is the basic unit in gelatin and the large molecules are polycondensates of α-chains as dimers (β-chains), trimers (γ-chains) and oligomers (microgels). Gelatin also consists of variable amounts of peptides with molecular weight less than 95,000 Da. These result from hydrolysis of α-chains. The molecular weight distribution of gelatin is determined by the extent of crosslinking in the source collagen and on the severity of the extraction process (Reich, Walther & Stather, 1962; Cole & Roberts, 1996). Acid-processed gelatin normally contains more fragments than alkali-processed ones (Koepff, 1984; Cole & Roberts, 1996). This is a result of cleavage of more peptide bonds in the acid process. According to Stainsby (1990), the content of α-chains in gelatin falls and low molecular weight (< α) peptides increase as extraction advances.

As high as 30 - 50% of high Bloom gelatins are normally made of molecules of size in the range between α and β-chains (Poppe, 1992). Some gelatins also contain aggregates with molecular weight up to 10 million Da and polypeptides with molecular weights less than 80,000 Da. In-depth discussions of the relationship between molecular weight distribution and individual functional properties are presented later.

Different techniques have been used to study the molecular weight distribution of gelatin and collagen. SDS PAGE has been widely applied to gelatin and collagen

(Koepff, 1984; Ming-Zhi, Jin-Kang & Chi, 1984; Chalepakis, Tanay & Heidemann, 1985; Hayashi, Kawamura, Ohtsuka, & Itoh, 1990; Norland, 1990; Reddy, Hudson, Bailey & Noelken, 1993; Cole & Roberts, 1996; Gómez-Guillén, Sarabia, Solas & Montero, 2001). The conditions used by different authors vary, but in all cases, SDS PAGE shows gelatin to be a polydispersed protein. According to Koepff (1984) SDS PAGE with densitometric analysis provides accurate and highly repeatable results for molecular weight distribution of gelatin. This, combined with the relatively low cost of electrophoresis equipment and ease of the technique has made SDS PAGE a widely used tool for the study of molecular weight distribution of gelatin.

Size exclusion chromatography has also been applied to study the molecular weight distribution of collagen and gelatin, either using the low pressure techniques (Gel filtration chromatography) (Piez, 1965; Miller, Martin, Piez & Powers, 1967; Cole & McGill, 1988) or by applying high pressure size exclusion chromatography (Dupont, 2002). In several studies, gel filtration chromatography has been followed by electrophoresis (Kimura & Ohno, 1987; Kimura *et al.*, 1988; Nagai & Suzuki, 2000; Nagai *et al.*, 2001). This allows separation of more molecular species.

One problem faced in the determination of molecular weight distribution of collagen and gelatin is finding suitable molecular weight standards. Globular proteins are not suitable molecular weight standard markers for gelatin and collagen. Kubo, Isemura and Takagi (1982) found that the collagen α-chain which consists of 1052 amino acid residues has the same effective hydrodynamic volume as a standard globular polypeptide consisting of about 800 residues. This is because of the unique amino acid profile of collagen. The difference between the mobility of collageneous proteins and globular protein has been attributed to the former's high content of the relatively small amino acid residues, glycine, proline and alanine (Noelken, Wisdom, & Hudson, 1981). The molecular weight distribution of collagenous materials can nonetheless be compared to each other by SDS PAGE since the relative migration (R_f) of these proteins also exhibit a high correlation to their molecular weight. Noelken *et al.* (1981) showed that a plot of R_f against molecular weight for collagen was parallel to that of globular protein, with collagenous materials having lower R_f values for a given molecular weight. In studies where globular proteins have been used as molecular weight markers during SDS PAGE of collagen (Mizuta, Hwang &

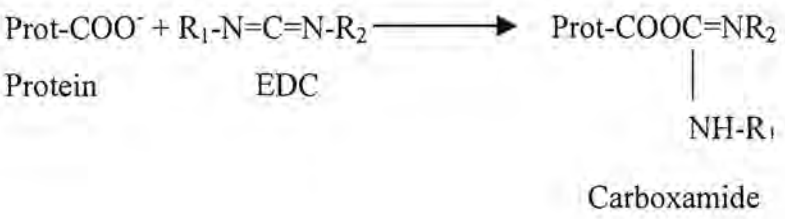
Yoshinaka, 2002; Mizuta, Isobe & Yoshinuka, 2002; Mizuta, Hwang & Yoshinaka, 2003) the mobility of α-chains (molecular weight ~95,000 Da) has been found to be lower than that for β-galactosidase (molecular weight 116,000 Da). Based on such results, α-chain could be interpreted to have molecular weight of about 120,000 Da. This is much higher than the true molecular weight of approximately 95,000 Da determined by sedimentation (Piez, 1967). This proves that the mobility of collagen follows a different behaviour from that followed by globular proteins and when these proteins are used as molecular weight markers for collagen, results should be interpreted with caution. Some workers have opted for other molecular weight markers when conducting SDS PAGE for gelatin or collagen. Cole and Roberts (1996) used acid soluble collagen from calfskin as the molecular weight marker for gelatins during SDS PAGE.

1.2.5.3 Isoelectric point

Differences in the isoelectric point (pI) of gelatins are related to the extent of modification during preparation (Poppe, 1992). The extent of modification determines the proportion of amide groups, which in turn determine the isoelectric point (Eastoe & Leach, 1977). The pI of gelatin may vary from 9.4, which is the isoelectric point of unmodified collagen, to 4.8, which represents maximum modification, i.e. 90 - 95% of potential carboxylic acid groups exist in the free state. Gelatins produced by mild acid pretreatment for example, pigskin gelatin, have high pI values because the process is less severe and less modification of collagen occurs. On the other hand, alkali or lime pretreatments lead to more modification due to the prolonged treatment. As a result only a few amide groups survive and their pI are in the range 4.8 - 5.2. Bone gelatin produced without lime treatment usually exhibits intermediate pI values.

Hayashi *et al.* (1990) caused amidation of lime-processed gelatin by reacting with ammonia and water-soluble 1-ethyl-3-dimethylaminopropyl carbodiimide (EDC). They reported an increase of pI from 5.0 to 10.4, decrease in gel strength while viscosity reduced and then increased as amidation was increased. Amidation was found not to cause inter- or intra-molecular crosslinking and the changes in functional properties were attributed to inhibition of gelatin renaturation into collagen-like

helical structures. It seems the negative charge of the carboxyl group is important in gelatin renaturation and amidation removes the negative charge on the carboxyl group of proteins, converting the carboxyl groups to carboxamide groups (Equation 1.1).



Equation 1.1

1.2.5.4 Gel strength

When concentration is sufficiently high and temperature sufficiently low, gelatin solutions change into gels (Wainewright, 1977; Stainsby, 1977). The standard measure of the gel strength of gelatin gels is Bloom gel strength. This is defined as the mass in grams which is necessary to apply to the surface of a gel, by means of a piston 12.7 mm in diameter, in order to produce a depression 4 mm deep (BSI, 1975; Poppe, 1992). The gel must be contained in a standard flask at a concentration of 6.67% (m/v) and must have been matured at 10 °C for 16 - 18 hours. The concentration, maturation time and temperature are standardised because gel strength increases with concentration of gelatin and maturation time but varies inversely with temperature (BSI, 1975; Ledward, 1986; Shehata, Shalaby & Hassan, 1994). Gel strength also varies with pH and reduces with amidation (Hayashi *et al.*, 1990). Additionally, gel strength depends on the molecular weight distribution of gelatin. Hydrolysis has been reported to cause a drop in the gelling properties of gelatin (Poppe, 1992) while crosslinking of gelatin with transglutaminase leads to increase in gel strength (Fernández-Diaz *et al.*, 2001). The higher the proportion of α and β fractions, together with larger peptides the higher the Bloom gel strength is likely to be (Johnston-Banks, 1990). One is likely however, to get gelatin of the same Bloom gel strength with quite different molecular weight distributions, indicating that the relationship between gel strength and molecular weight distribution is not one of direct proportionality. Koepff (1984) observed that increasing of peptides in acid processed pigskin gelatin from 33% to 63% by thermohydrolysis did not lead to

change in gel strength and concluded that the gel strength above a certain average molecular weight is independent of the average molecular weight.

Fernández-Diaz *et al.* (2001) showed that crosslinking of gelatin using transglutaminase and also addition of magnesium sulphate and glycerol leads to increase in gel strength for cod and hake gelatin. The increase was found to be higher for hake than for cod and this was attributed to the higher imino acid content and higher content of low molecular weight fraction of the hake gelatin.

In many applications, high Bloom gelatin can be used to replace a low Bloom version, with a reduced level of use (Pye, 1996). Cold water fish gelatins generally have lower gel strength and this has been attributed to their low content of hydroxyproline and proline (Gilsenan & Ross-Murphy, 2000). Warm water fish species contain more of these imino acids (Grossman & Bergman, 1992) and some studies have shown that they give gels of higher gel strength (Leuenberger, 1991; Grossman & Bergman, 1992).

1.2.5.5 Melting point

Two definitions of gelatin melting point as given by the British Standards Institution and the Japanese Standards Association are presented here. Both of them require determination of a critical temperature at which gelatin softening begins to occur. According to The British Standards Institution (BSI, 1975) melting point of gelatin refers to the temperature at which a gelatin gel containing 6.67% gelatin in water and matured at 10°C for 16 – 18 hours softens sufficiently to allow drops of carbon tetrachloride to sink through the gel. According to the Japanese Standards Association (JSA, 1996), the melting point of gelatin is the temperature at which a gel containing 10% gelatin in water and matured at 10°C for 16 – 18 hours starts to flow, when gradually heated from 10°C. Molecular weight distribution is an important determinant of melting point. According to Ledward (1986), lower molecular weight gelatin fractions have a lower propensity to form gels and gelatins high in low molecular weight fractions tend to exhibit low melting temperatures. Melting point increases with concentration but is depressed by sodium chloride and is affected by maturation conditions (Choi & Regenstein, 2000). The values for melting

temperature recorded using the BSI and JSA standard methods for the same gelatin, would differ since the two methods use different gelatin concentrations. Amino acid composition is an important determinant of the temperature of gel formation and gel melting (Johnston-Banks, 1990). The low melting point and gel formation temperature for fish gelatins has been attributed to their low content of the imino acids proline and hydroxyproline (Gilsenan & Ross-Murphy, 2000; Fernández-Díaz *et al.*, 2001). Low imino acid content is associated with reduced propensity for intermolecular helix regeneration (Gilsenan & Ross-Murphy, 2000).

Salts such as magnesium sulphate, sodium phosphate and ammonium sulphate have been reported to increase the melting temperature of gelatin (Sarabia *et al.*, 2000). The change was attributed to interaction between the salts and the surrounding water, leading to greater screening of electrostatic interactions. Because of their large size, sulphates and phosphates, unlike small ions like Cl^-, are believed to remain further from the positively-charged centres of proteins. Instead, they interact with water and increase likelihood for formation of protein-protein junctions. Sarabia *et al.* (2000) proposed that in the case of hake lower ionic strength (0.1 - 0.5 M magnesium sulphate) may be suitable for promoting useful junctions by promoting protein unfolding without distorting the assembly of chains into collagen-like helical rods. This however, was not the case with tilapia gelatin and the difference was attributed to differences in conformation of gelatin molecules and to intrinsic factors such as amino acid composition and isoelectric point.

Gilsenan and Ross-Murphy (2000) studied the effect of blending different gelatins and reported that addition of 2% bovine gelatin to cod gelatin increased the melting point of cod gelatin by 5°C and proposed that presence of bovine gelatin stabilises cod gelatin helices.

1.2.5.6 Setting point and setting rate

When gelatin in solution is adequately cooled, it sets to form a gel. The temperature at which a gelatin solution in water gels is called the setting point (BSI, 1975; JSA, 1996). The concentration of gelatin must be specified. The setting temperature is lowered by mechanical action such as shear and increased by slow cooling.

According to Poppe (1992) setting point for a 10% solution varies from 24 to 29°C, depending mainly on Bloom gel strength and type of pretreatment used.

Setting time of gelatins is one of the most important physical properties with respect to the fast coating of thin layers such as those encountered in photographic applications. The log of setting time varies linearly (negative slope) with the log of gelatin concentration and setting time varies inversely with the square of gelatin molecular weight (Stainsby, 1977). This observation is rather misleading since gelatins exhibit a wide molecular weight distribution and therefore average molecular weight is of little importance. Tavernier (1989) by controlling manufacture conditions produced bone gelatins varying in the different molecular weight fractions. Gelatins with a high proportion of microgels were found to gel faster. The increase in setting rate was steepest between 3 and 10% microgels. According to Stainsby (1990), racemization occurring during prolonged exposure of collagen to alkali pH leads to gelatins with poorer setting properties.

Cold water fish gelatin, due to its low content of imino acids, shows rather low gelation temperature compared to mammalian gelatins (Leuenberger, 1991; Gilsenan & Ross-Murphy, 2000). The critical concentration (minimum concentration required to form a gel upon cooling) has also been shown to vary with source of gelatin. Gilsenan and Ross-Murphy (2000) showed that critical concentration for bovine gelatin is slightly lower than that of tilapia, tuna and megrim but much lower than that for cod. This too was attributed to differences in imino acid content, mainly, to differences in hydroxyproline. Addition of salts to fish skin gelatins, has been found to prolong setting time (Sarabia *et al.*, 2000) and this can be attributed to destabilisation of the gelatin structure. Mechanical disturbances also delay setting and setting temperature is higher when cooling is done slowly (Stainsby, 1977).

1.2.5.7 Viscosity

According to Poppe (1992) the viscosity of concentrated gelatin solutions depends mainly on interactions between gelatin molecules. At lower concentrations, the solvent and individual gelatin molecules play a more significant role. Above 40°C,

viscosity decreases exponentially with temperature. Viscosity of gelatin is lowest at the isoelectric point and increases exponentially with concentration.

At a concentration of 2% and the temperature range of 20 – 80°C, gelatins have been shown to exhibit Newtonian behaviour (Wulansari, Mitchell, Blanshard & Paterson, 1998; Marcotte, Taherian-Hoshahili & Ramaswamy, 2001). Based on this observation, Wulansari *et al.* (1998) proposed that gelatin coils are sufficiently compact to reduce the importance of entanglements but not so compact as to resist deformation when coils approach one another. Increase in shear rate therefore does not lead to change in apparent viscosity.

Fernández-Diaz *et al.* (2001) reported an increase in viscosity due to the addition of magnesium sulphate and glycerol to cod and hake skin gelatins. This was attributed to unfolding of the gelatins due to interactions with the additives as explained earlier. Molecular weight is of key importance in determining viscosity of gelatin solutions. Gelatins high in high molecular weight peptides exhibit the highest viscosities (Johnston-Banks, 1990). This has been attributed to high molecular weight gelatin molecules developing more helices during cooling. Viscosity degradation due to gelatin hydrolysis is generally more pronounced than Bloom degradation and depends on the rate of generation of low molecular weight peptides (Koepff, 1984). For gelatins with an initial high proportion of low molecular weight peptides, hydrolysis results in a less rapid loss in viscosity compared to gelatins with a low proportion of the low molecular weight fraction. Tavernier (1989) observed a high linear correlation (r^2 = 0.979) between increase in the content of microgel (450,000 - 900,000 Da) fraction and increase in gelatin viscosity. Variations in peptides smaller than α, the α and β fractions were found to influence viscosity to a lower extent.

1.2.5.8 Turbidity

The clarity of gelatin solutions depends on the conditions during and after extraction as well as on the pH (Poppe, 1992; Fernández-Diaz *et al.*, 2001). Early extractions of gelatin (lower temperature extracts) tend to be of higher clarity than later extracts. At low temperatures (< 30°C), gelatin extracts have the highest turbidity at their isoelectric point (Poppe, 1992). Ineffective filtration is a cause for high gelatin

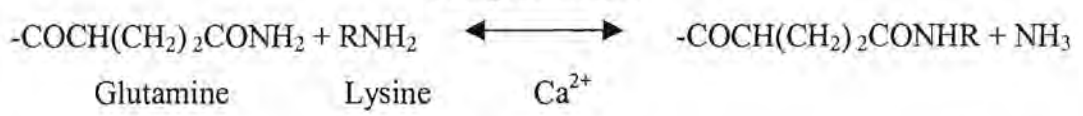
turbidity. High gelatin turbidity is undesirable since it may limit the use of gelatin in applications where clarity is essential.

1.2.5.9 Colour

Gelatin colour depends on the raw materials, the processing method and the order of extraction (Poppe, 1992). When gelatin has high colour intensity, it introduces the colour into the food or other materials in which it is applied. This may not be desirable, especially in applications where colour is critical to quality such as in water desserts. It is therefore advantageous that gelatin colour intensity is low and colour measurements are part of quality control protocols. Gelatin colour may be measured by use of tristimulus colorimeters or by visual observation (Cole & Roberts, 1997). Cole (1995) observed higher colour intensity for gelatins derived from older animal hides and attributed the colour to Maillard reactions involving gelatin and glucose. As noted earlier, glycation of collagen, which involves introduction of glucose moieties, occurs at advanced age (Bailey *et al.*, 1998). Such collagens have a higher degree of Maillard reaction and therefore higher colour intensity

1.2.6 Effect of crosslinking on the functional properties of gelatin

As noted earlier, the molecular weight distribution of gelatin is a key determinant of functional properties. Low molecular weight fractions generally do not enhance the functional properties of gelatin. Several studies have been conducted on the crosslinking of gelatin and its effect on the functional properties. Crosslinking of gelatin with transglutaminase has been the main means of enzymatic crosslinking applied to gelatin and there has been much interest in this in recent years (Fernández-Diaz *et al.*, 2001; Gòmez-Guillèn, Sarabia, Solas & Montero, 2001). Transglutaminase causes crosslinking by catalysing an acyl-transfer reaction in presence of Ca^{2+}, introducing a covalent crosslink between glutamine and lysine residues (Spector, 1982; Dickinson, 1997; Fernández-Diaz *et al.*, 2001) (Equation 1.2).



Equation 1.2

Fernández-Diaz *et al.* (2001) crosslinked gelatin with transglutaminase and reported increase in gel strength. Gòmez-Guillèn *et al.* (2001) reported increase in gelatin gel strength, hardness, elasticity and cohesiveness after crosslinking of megrim skin gelatin with transglutaminase. High levels of the enzyme (approaching 0.9 g/kg), however, reduced gel strength and hardness. This may have been due to creation of very high molecular weight aggregates. According to Stainsby (1977) abundance of very high molecular weight molecules leads to low gel strength. This is because very long molecules may fold back on themselves, forming intra-molecular junctions, which are not useful in promoting gel strength (Ledward, 1986). The effect of transglutaminase treatment on the physicochemical properties of different gelatins may vary due to differences in reactive residues, secondary and tertiary structures of the gelatins.

Crosslinking has also been shown to cause increase in melting temperature (Fernández-Diaz *et al.*, 2001; Gòmez-Guillèn *et al.*, 2001). Fernández-Diaz *et al.* (2001) reported increase in melting temperature of cod and hake skin gelatin following crosslinking with transglutaminase.

1.2.7 Effect of age of source animals on the properties of gelatin

The age of the source animals has been shown to affect the extractability and properties of gelatins. Reich *et al.* (1962) reported a much lower gelatin yield from acid-conditioned pigskins from animals aged 2.5 years compared to animals aged 1.5 years. This decrease in yield with age was accompanied by a decrease in viscosity and melting point of gelatin. For cattle hides, the yield decreased more slowly between the age of 11 weeks and 5 years and the changes in gelatin melting point and viscosity were less pronounced. Cole and McGill (1988) reported decrease in Bloom gel strength and viscosity of bovine hide gelatin with increase in animal age. Gelatin from the older animals was also found to contain higher levels of the low molecular

weight fraction. This is in agreement with the observation by Johnston-Banks (1990) that gelatin viscosity is proportional to the amount of the high molecular weight fraction. The older animals contain more stable collagen crosslinks and conversion of their collagen to gelatin results in a higher extent of peptide bond cleavage, since a more severe process is required. This results in a high concentration of low molecular weight fragments in the gelatin.

Reich *et al.* (1962), however, observed that before the general age-related reduction in viscosity and melting point, newborns and animals a few weeks old gave gelatin of lower viscosity. These authors unfortunately did not report any measurements for gel strength. They also reported high content of water and salt soluble protein in skins from newborn and very young (a few weeks old) animals. By comparing the influence of age on the yield and functional properties of gelatin, Reich *et al.* (1962) concluded that the age-related differences are not dependent on the absolute chronological age, but age in relation to the average life-span of the animal species concerned.

Cole and McGill (1988) reported decrease in extractability at 45°C for alkali treated hides between ages 6, 18 and 60 months, although hides from older animals gave a higher total yield. Bloom strength and viscosity were higher for the more easily extracted gelatins derived from the younger animal hides. Turbidity (Cole, 1995) and colour intensity (Cole & Roberts, 1996) have also been shown to increase with animal age for bovine hide gelatin. The increase in colour intensity was attributed to *in vivo* Maillard reaction.

1.2.8 Use of Fourier transform infrared (FTIR) spectroscopy in the study of collagen and gelatin

When infrared radiation interacts with matter, it may be absorbed, causing bonds to vibrate (Smith, 1996). Chemical structural fragments within molecules (functional groups) tend to absorb infrared radiation of the same wave number range regardless of the structure of the rest of the molecule. This means that there is a correlation between the wave numbers at which a molecule absorbs infrared radiation and its structure. This correlation allows the study of the structure of molecules.

Infrared spectroscopy has been recognised for a long time as a useful technique for estimating the secondary structure of proteins (Ambrose & Elliot, 1951). Computerised FTIR helped to improve sensitivity and the subtraction of spectra arising from solvents (Surewicz & Mantsch, 1988). There has been a lot of development in the interpretation of protein secondary structure from FTIR spectra in recent years (Surewicz & Mantsch, 1988; Prystupa & Donald, 1996; Pelton & McLean, 2000; Dzwolak, Kato & Taniguchi, 2002).

The amide groups of polypeptides and proteins possess several characteristic vibrational modes or group frequencies (Miyazawa, Shimanouchi & Mizushima, 1956; Durrani & Donald, 1996). Proteins and peptides normally show absorption bands at around 3300 cm^{-1} (amide A), 1660 cm^{-1} (amide I), 1550 cm^{-1} (amide II), 1350 – 1220 cm^{-1} (amide III) and 700 cm^{-1} (Kretschmer, 1957). The amide I band between 1600 and 1700 cm^{-1} is the most useful for infrared spectroscopic analysis of protein secondary structure. Amide II and III bands have also been applied to the study of protein secondary structure. However, amide II sensitivity is not well established while, though sensitive to the secondary protein structure, the amide III band of proteins is very weak (Surewicz & Mantsch, 1988).

The amide I and II vibrational modes are mixtures of bond bending and bond stretching vibrations resulting from complex motion of the atoms (Fraser & Suzuki, 1970). The amide II band (~1550 cm^{-1}) involves a mixture of CN stretching (40%) and in-plane NH bending (60%) while amide I involves 80% C=O stretching, 10% CN stretching and 10% in-plane NH bending contributions. The amide I band represents primarily the C=O stretching vibrations of the amide groups coupled to the in-plane NH bending and CN stretching modes (Surewicz & Mantsch, 1988). The exact frequency of this vibration depends on the nature of hydrogen bonding involving the C=O and NH moieties, which in turn is determined by the particular secondary structure adopted by the polypeptide chains. Because proteins are heteropolypeptides, they contain polypeptide fragments in different conformations. These manifest as complex composites consisting of overlapping component bands in the amide I band. These component bands can be separated by resolution enhancement achieved by band narrowing. For some proteins, the components of the

amide I band have been assigned to specific polypeptide conformations (Byler & Susi, 1986; Jaworsky, Brauner & Mendelsohn, 1986; Surewicz & Mantsch, 1988). A band centered at 1650 – 1658 cm^{-1} in ^2H_2O (deuterated water) is believed to represent α-helical structure, while polypeptides in a non-ordered conformation have a peak at 1640 – 1648 cm^{-1}. In H_2O however, the amide I peak corresponding to unordered peptides appears at 1650 – 1660 cm^{-1} and is therefore not easily resolved from the amide I band corresponding to orderly (α-helix) conformation. Peaks between 1620 and 1640 cm^{-1} or even below 1620 cm^{-1} are assigned to β-strands protein secondary structure. Amide I bands between 1670 and 1675 cm^{-1} have been attributed to β-components.

Fibrous proteins have extended regions of α and β-secondary structure (Gans, 1980). Consequently, their vibrational spectra tend to show features similar to those found in model compounds (homopolypetides). Gans (1980) noted that collagen molecules have a structure distinctly different from the α-helix and β-sheet structures of other peptides. Thus the spectral band assignments applied to these conformations do not necessarily apply to collagen. The amido nitrogen of the pyrrolidine rings of proline and hydroxyproline residues in collagen carry no hydrogen for intra-chain hydrogen bonding while the flatness of the amide group prevents extension of the peptide chain as in beta arrangement and interferes with the compact coiling of the alpha helix (Morrison & Boyd, 1992).

Fourier transform infrared (FTIR) spectroscopy has been used to study changes in the secondary structure of collagen and gelatin. It has been used to study collagen crosslinking (Paschalis, Verdelis, Doty, Boskey, Mendelesohn & Yamauchi, 2001), denaturation (Friess & Lee, 1996) and thermal self-assembly (Jakobsen, Brown, Hutson, Finc & Veis, 1983; George & Veis, 1991) as well as gelatin melting (Prystupa & Donald, 1996). The spectral changes which are indicative of changes in collagen secondary structure have been identified to include changes in the amide A (Milch, 1964), amide I (1636 –1661 cm^{-1}), amide II (1549 – 1558 cm^{-1}) (Renugopalakrishnan, Chandarakan, Moore, Hutson, Berney & Ravejendra, 1989) and in the amide III (1200 - 1300 cm^{-1}) regions (Friess & Lee, 1996).

Fibrillogenesis (self assembly) of collagen has been found to be associated with broadening and a slight shift to lower wave number of the amide A peak (Milch, 1964), increase in intensity and slight shift to lower wave number of amide III peak (Jakobsen *et al.*, 1983), band broadening and shift of amide I peak to lower wave number (Jakobsen *et al.*, 1983; George & Veis, 1991; Prystupa & Donald, 1996) and shift of amide II peak to lower wave number (Jakobsen *et al.*, 1983; George & Veis, 1991). Shift of amide I, II and III peaks to lower wave numbers, increase in intensity of amide III and broadening of amide I are therefore associated with increased intermolecular interactions (by hydrogen bonding) in collagen.

Denaturation of collagen on the other hand has been found to lead to reduction in the intensity of amide A, I, II and III peaks (Friess & Lee, 1996), narrowing of amide I band (Prystupa & Donald, 1996), increase in amide I component found around 1630 cm^{-1} and reduction in the intensity of amide I component found around 1660 cm^{-1} (George & Veis, 1991; Payne & Veis, 1988; Renugopalakrishnan *et al.*, 1989).

Prystupa and Donald (1996) studied gelatin melting and found it to be associated with reduction in the 1678 cm^{-1} peak and 1660/1690 cm^{-1} peak intensity ratio and increase in amide I components occurring around 1613, 1629 and 1645 cm^{-1}. These authors assigned the bands occurring at 1645 – 1657 cm^{-1} to random coils and the 1660 cm^{-1} band to triple helix, with contribution from α-helix and β-turns. The amide I component at 1690 cm^{-1} has been attributed to helices of aggregated collagen-like peptides (Doyle, Bendit & Blout, 1975; Prystupa & Donald, 1996). According to Doyle *et al.* (1975) this peak vanishes with hydration of collagen or gelatin.

FTIR spectroscopy has therefore developed into a very useful technique for studying changes or differences in collagen and gelatin secondary structure. This technique requires very small quantities of sample and a short time to complete. It allows studies of aqueous systems as well as dry samples. These advantages make FTIR a robust technique.

1.3 GAPS IN KNOWLEDGE

Only a few warm water fish species have been studied with respect to their potential as sources of collagen and gelatin and these have been shown to contain more imino acids and to exhibit superior functional properties in comparison to cold water fish species (Grossman & Bergman, 1992; Gilsenan & Ross-Murphy, 2001). Nile perch is one of the largest warm water fish species, growing to over 250 kg and over 13 years of age. The utilisation of its waste material is still low and could be enhanced by a better understanding of the functional properties of the materials derived there-from. To date, literature on the chemical and functional properties of Nile perch collagen and gelatin is lacking.

Studies on fish collagen and gelatin have been limited, almost exclusively, to fish skin and yet, bones represent a sizeable fraction of the fish waste and contains substantial amounts of collagen. Bone and skin collagen vary in the type and quantity of crosslinks they contain (Sims *et al.*, 2000). It was not clear, however, how this influences the properties of gelatin derived from these materials.

Previous studies on fish gelatin have not addressed differences between gelatins extracted from fish of varying age. In mammals, it has been demonstrated that gelatin and collagen extractability reduces and gelatin functional properties become poorer as animal age increases (Reich *et al.*, 1962; Cole & Roberts, 1996). These age-related changes in case of mammals have been attributed to increase in stable collagen crosslinks. Some authors (Cohen-Solal *et al.*, 1981; Hickman *et al.*, 2000) have, however, shown that there is minimal development of stable collagen crosslinks in fish. It was not apparent therefore, whether age-related reduction in collagen and gelatin extractability and in gelatin quality also occurs in fish.

Factors affecting the functional properties of fish gelatin are also not completely understood. For gelatins containing microgels (fraction with molecular weight > 450 kDa), the proportion of this fraction has been shown to be highly positively correlated to viscosity and setting temperature and negatively correlated to setting time (Tavernier, 1989). Gelatins derived from collagen low in heat stable crosslinks, such as fish skin gelatins, however, do not contain appreciable amounts of microgels. It

was not clear for such gelatins, how their functional properties are related to their molecular weight distribution. It was also not clear what differences exist between early (low temperature) and later (high temperature) gelatin extracts obtained from the same raw material in a sequential extraction process.

1.4 HYPOTHESES

- Because Nile perch is a warm water fish species, its collagen and gelatin should contain high levels of imino acids and exhibit functional properties different from those of cold water fish species.
- The extractability and functional properties of gelatin from Nile perch skins should differ from those from Nile perch bones because collagen from the two tissues differ in extent of stable crosslinks and as a result differ in their stability to heat denaturation.
- The functional properties of Nile perch skin and bone gelatins should vary with age of the source fish and these differences arise from differences in the extent of collagen crosslinking.

1.5 OBJECTIVES

The goal of this study was to provide information that can be used to promote commercial utilisation of fish skins and bones as a raw material for gelatin and collagen. The specific objectives were to;

- Determine the physico-chemical properties of Nile perch collagen.
- Determine and compare the extractability of Nile perch skin and bone gelatins.
- Determine and compare the chemical and functional properties of Nile perch skin and bone gelatins.
- Compare chemical and functional properties of Nile perch skin and bone gelatins to those of bovine bone and commercial fish skin gelatins.
- Determine the relationship between the chemical and functional properties of Nile perch gelatins.

2 RESEARCH

2.1 CHARACTERISATION OF ACID SOLUBLE COLLAGEN FROM SKINS OF YOUNG AND ADULT NILE PERCH (*Lates niloticus*)*

Running title; Characterisation of Nile perch collagen

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2.1.1 Abstract

Acid soluble collagen (ASC) was extracted from the skins of young and adult Nile perch (*Lates niloticus*) using 0.5 M acetic acid and precipitation using 0.9 M NaCl. The ASC yields, on a dry weight basis, were 63.1 and 58.7%, respectively for young and adult fish skins. SDS PAGE showed the collagens to contain two alpha components (α1 and α2). ASC from Nile perch was found to contain more imino acids (19.3 and 20.0%, respectively for young and adult fish) than most fish species. The denaturation temperature for the collagens from the skins of young and adult Nile perch was determined to be 36°C, which is also higher than that for most other fish species. Fourier transform infrared spectroscopy showed a higher degree of molecular order in ASC from adult than from young Nile perch. The results indicate that age-related changes in Nile perch skin collagen are not very pronounced, probably because there is minimal development of mature cross-links.

Key Words: Nile perch, fish collagen, imino acids, fish waste, denaturation temperature

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2.1.2 Introduction

Collagen is the most abundant protein of animal origin, comprising approximately 30% of total animal protein. There are at least 19 variants of collagen, named type I – XIX (Bailey, Paul & Knott, 1998). Type I, II, III and V are the fibrous collagens. Type I collagen is found in all connective tissue, including bones and skins. It is a heteropolymer of two α1 chains and one α2 chain. It consists of one-third glycine, contains no tryptophan and cysteine and is very low in tyrosine and histidine.

Several studies have focused on the characterisation of different fish collagens (Piez, 1965; Rigby, 1968, Kimura & Ohno, 1987; Sato, Yoshinaka, Yoshiaki & Sato, 1989; Montero, Alvarez, Marti & Borderias 1995; Montero, Gòmez-Guillèn, & Borderias, 1999; Nagai & Suzuki, 2000; Sivakumar, Arichandran, Suguna, Mariappan & Chandrakasan, 2000). Most fish collagens have been found to consist of two α-chain variants, which are normally denoted as α1 and α2 (Nagai, Yamashita, Taniguchi, Kanamori & Suzuki, 2001; Gòmez-Guillèn, Turnay, Fernández-Diaz, Ulmo, Lizarbe & Montero, 2002). These α-chain variants though having approximately the same molecular weight (~95,000Da) can be separated by SDS PAGE due to their different affinity for SDS. Alpha 2 has a higher affinity for SDS and consequently exhibits a higher mobility than α1 (Kubo & Takagi, 1984). Piez (1965) isolated three variants of α-chains (α1, α2 and α3) from cod skin collagen and found that these variants differed in their amino acid composition. Alpha 3 has also been isolated from rainbow trout (Saito, Takenouchi, Kunisaki & Kimura, 2001), common horse mackerel (Kimura, Zhu, Matsui, Shijoh and Takamizawa, 1988; Yoshida, Fujisawa, Mizuta & Yoshinaka, 2001) and eel (Kimura *et al.*, 1988).

In addition to differences in molecular species, fish collagens have been shown to vary widely in their amino acid composition. In particular, the levels of imino acids (proline and hydroxyproline) vary significantly among fish species (Balian & Bowes, 1977; Poppe, 1992; Gudmundsson & Hafsteinsson, 1997). The amount of imino acids, especially hydroxyproline, depends on the environmental temperature in which the fish lives and it affects the thermal stability of the collagens (Rigby, 1968; Balian

& Bowes, 1977; Kimura *et al.*, 1988). Collagens derived from fish species living in cold environments have lower content of hydroxyproline and they exhibit lower thermal stability than those from fish living in warm environments. This is because hydroxyproline is involved in inter-chain hydrogen bonding which stabilises the triple helical structure of collagen (Darby & Creighton, 1993). Cold water fish species are also reported to contain higher levels of hydroxyamino acids, serine and threonine (Balian & Bowes, 1977). Grossman & Bergman (1992) showed that gelatin from tilapia, a warm water fish species, contains higher levels of imino acids than cold water fish collagens.

Nile perch (*Lates niloticus*), like tilapia, is a warm water fish species. It is the most important commercial fish species in East Africa. Approximately 100,000 tonnes of Nile perch are processed, annually, in Uganda alone. The fish landed vary greatly in size. Young (< 80 days old) fish measure as little as 6.4 cm and the largest adult (13+ years old) fish measure up to 160 cm long for males and 190 cm for females (Acere, 1993). The smallest length for sexually mature fish is 53.5 cm and 67.5 cm for males and females, respectively (Acere, 1993; Ogutu-Ohwayo, 2000). This occurs between the age of 1 and 2 years.

About 50% of the total fish weight remains as waste, mainly in the form of skins and bones, during preparation of fish fillets (Shahidi, 1994). This waste if utilised in the manufacture of value added products such as collagen, could contribute significantly to the economic value of the fish. The characteristics of collagen, the main component of these waste materials influences their potential for utilisation. The aim of this study was to characterise collagen from skins of Nile perch and to investigate the effect of fish age on collagen properties. It has been demonstrated for mammals that the solubility of their collagen reduces as they age due to changes in the amount and type of cross-links (Bailey *et al.*, 1998).

2.1.3 Materials and Methods

2.1.3.1 Raw materials

Skins of Nile perch (*Lates niloticus*) were procured from Nge-ge Fish Ltd, Kampala, Uganda. These were by-products of fillet processing. The very small skins from young fish (skin thickness < 0.4 mm) and the large ones from adult fish (skin thickness > 1.5 mm) were selected and used in this study. Portions were taken for the determination of chemical composition. These were immediately refrigerated (~ 7°C) and analysed within 48 hours. The rest of the selected skins were frozen until required for extraction of collagen.

2.1.3.2 Proximate analysis

Proximate analysis was conducted at the Department of Food Science and Technology, Makerere University. Portions were taken from different parts of the skins, blended together and used for proximate analysis. Moisture, lipid, ash and protein contents of skins from young and adult Nile perch were determined by AOAC (1995) methods 950.46, 960.39, 900.2A and 928.08, respectively. Protein digestion was done for 2 hours, using a catalyst made by mixing 0.75 g of selenium powder and 100 g of mercuric sulphate (Eastoe & Eastoe, 1952). A conversion factor of 5.4 was used in calculating the protein content from the Kjeldahl nitrogen content since collagen; the main protein in skin contains approximately 18.7% nitrogen (Eastoe & Eastoe, 1952).

2.1.3.3 Extraction of collagen

The method described by Gòmez-Guillèn and Montero (2001) was used to obtain collagen from skins of young and adult Nile perch. The method involves washing the skins with chilled (~ 5°C) water for a period of 10 minutes. During this time, the skins were pressed intermittently by hand. The skins were then washed with 0.8 M NaCl for 3 periods of 10 minutes each followed by rinsing in running water. The

volume and solids content of the wash liquors (water and NaCl solution) were determined and used to calculate the amount of solids lost in the wash liquors. Collagen was then extracted using 0.5 M acetic acid solution (1 g of skin per 20 ml of 0.5 M acetic acid). The extraction was conducted for 16 hours, with intermittent stirring. The viscous collagenous material was separated from the insoluble components by sieving through cheesecloth. The volume and solids content of the filtrate were determined and used to determine the total acid soluble solids from the skins. The collagen solution was then centrifuged and salt (to make 0.9 M NaCl solution) was added to the supernatant to precipitate the collagen. The precipitated collagen (acid soluble collagen) was separated by centrifugation at 2500 x *g* for 30 minutes. To further purify the collagen, it was re-dissolved in acetic acid and re-precipitated as described above. The collagen extraction, precipitation and separation were conducted at room temperature (approximately 15°C). The acid soluble collagens from young and adult Nile perch were separately freeze-dried and used for analysis.

2.1.3.4 Determination of collagen denaturation temperature

Determination of denaturation temperature was based on the method described by Kimura *et al.* (1988). An Ostwald's viscometer was filled with 0.1% (m/v) collagen solution in acetic acid. The viscometer was then immersed in a water bath held at 30°C and left to stand for 30 minutes, to allow the collagen solution to equilibrate to the water bath temperature. The temperature was raised stepwise up to 50°C and maintained at each temperature for 10 minutes. Collagen solution viscosities were measured at temperature intervals of about 2°C from 30°C up to 50°C. Fractional viscosities were computed for each temperature as follows:

$$\text{Fractional viscosity} = \frac{\text{maximum viscosity - measured viscosity}}{\text{maximum viscosity - minimum viscosity}}$$

Thermal denaturation curves were then obtained by plotting the fractional viscosities against temperature for young skin and adult skin collagen. The denaturation temperature was taken to be the temperature at which fractional viscosity was 0.5.

2.1.3.5 Amino acid analysis

Amino acid analysis was conducted by the Pico.Tag method (Bidlingmeyer, Cohen & Tarvin, 1984) at the Deartment of Biochemistry, University of Pretoria. This method involves derivatisation of amino acids using phenylisothiocyanate (PITC) and determination of the phenylthiocarbamyl derivative of amino acids (PTC amino acids) using reversed phase HPLC. Dry collagen (10 - 20 mg) from skins of young and adult Nile perch was mixed with 6 M HCl (1 ml) containing 1% phenol (v/v). The mixture was evacuated, blown with N_2 and vacuum-sealed before hydrolysis at 110°C for 24 hours. After hydrolysis the samples were cooled and diluted to 5 ml with de-ionised water. A portion (25 µl) was then dried and derivatised. Derivatisation involved addition of 10 µl of a mixture of methanol, water and trimethylamine (2:2:1), mixing and then drying for 5 minutes. This was followed by addition of 20 µl of a mixture of methanol, water, trimethylamine and phenylisothiocyanate (7:1:1:1). The sample was left to stand for 20 minutes at room temperature (20 - 25°C), dried under vacuum and then dissolved in 200 µl of pH 7.4 phosphate buffer and filtered with a 0.45 µm filter. Portions (20 µl) of the filtered samples were injected using an automatic loader (WISP™) (Millipore Corp, Milford, MA, USA) into the Pico.Tag column (part no 88131, 3.9 mm X 13 cm) (Millipore Corp, Milford, MA, USA) for amino acid analysis.

2.1.3.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis (SDS-PAGE) was conducted using the discontinuous Tris-HCl/glycine buffer system (Laemmli, 1970), with 7.5% resolving gel and 4% stacking gel. Samples containing approximately 5 µg of solids per µl were prepared by dissolving 10 mg of collagen in 2 ml sample buffer prepared with and without 2-mercaptoethanol. A portion (20 µl) of sample was loaded per well. Calfskin acid soluble collagen (Sigma Chemical Co, St Louis, MO, USA) and molecular weight markers were loaded alongside the Nile perch collagen samples. The molecular weight markers (ICN Biomedicals Inc., Aurora, OH, USA) contained cytochrome C (horse heart), myoglobin (horse heart), chymotrypsinogen A, ovalbumin, bovine serum albumin, gamma globulin (human) and apoferritin. These were mixed in

sample buffer to a make a solution containing 2 μg of each protein per μl and 10 μl were loaded per well.

Electrophoresis was conducted using the Protean II xi vertical cell and the 1000 powerpac (Bio-Rad laboratories, Hercules, CA, USA) at a constant current of 30 mA and a temperature of 10°C. Gels were stained using 0.1% Coomassie Brilliant Blue R250 dissolved in water, methanol and trichloroacetic acid (5:4:1) and de-stained using a solution containing methanol, distilled water and acetic acid in a ratio of 5:4:1.

2.1.3.7 Fourier transform infrared spectroscopy

FTIR was conducted at the Department of Chemistry, University of Pretoria. FTIR spectra were obtained from discs containing 2 mg collagen in approximately 100 mg potassium bromide (KBr). All spectra were obtained using a Bruker infrared spectrophotometer (Bruker Instruments, Billerica, MA) from 4000 to 500 cm^{-1} at data acquisition rate of 2 cm^{-1} per point. Background was subtracted using the Opus software (Bruker Instruments, Billerica, MA). Triplicate samples of collagen from young and adult Nile perch skins were analysed and spectra for the triplicate runs averaged. Fourier self deconvolution (achieved by band narrowing) was conducted on the average spectra for the amide I band, using a resolution enhancement factor of 1.8 and full height band width of 13 cm^{-1}. The self deconvolution provided information on the number and location of sub-bands. Curve fitting was then performed using peakfit software (SPSS Inc., Chicago, IL, USA).

2.1.3.8 Statistical analysis

Means for the properties for the adult and young fish skin collagens were compared using t-test and p-values are presented wherever applicable.

2.1.4 Results and Discussion

2.1.4.1 Proximate composition of Nile perch skins

The skins from young and adult Nile perch were found to contain similar amounts of protein (20 – 22%) (Table 2.1.1). The lipid content was however, higher for the skins of adult fish than for the skins of young fish ($p = 0.02$). It seems that the fish accumulate subcutaneous fat as they age. The ash content was also considerably higher for skins of adult fish probably because of increased scale mineralisation with age.

Table 2.1.1: Proximate composition of skins from young and adult Nile perch

	Young fish	Adult fish	p-value
Moisture	72.7 (1.3)	68.4 (0.6)	0.54
Protein	20.3 (2.0)	21.60 (1.3)	0.16
Lipid	5.0 (0.7)	6.8 (0.3)	0.02
Ash	3.7 (0.5)	6.0 (0.2)	0.16

Values in brackets are standard deviations for triplicate samples

2.1.4.2. Solubility of Nile perch skin solids

The solubilities of solids in water, salt solution and in acetic acid were not significantly ($p > 0.05$) different for the skins of young and adult fish (Table 2.1.2). Working with pigskin, Reich, Walther and Stather (1962) found that the component soluble in water consisted only of non-collagenous matter but that the salt soluble component contained both non-collagenous matter and collagen. The amount of stable crosslinks in collagen have been reported to increase with age in mammals (Sims, Avery & Bailey, 2000). As a result, the solubility of mammalian collagen in salt solution and cold acid solutions reduces with age (Reich *et al.*, 1962). The consistently slightly lower solubilities for adult compared to young fish skin collagen may be indicative of some slight increase in the amount or extent of stable crosslinks.

Table 2.1.2: Solubility of solids from skins of young and adult Nile perch in solutions used in collagen preparation

	% of total solids solubilised		
Component	Young Fish	Adult Fish	p-value
Water-soluble (%)	3.5 (0.2)	2.5 (0.1)	0.52
Salt-soluble (%)	3.4 (0.3)	2.4 (0.3)	0.29
Acid-soluble (%)	63.1 (3.3)	58.7 (3.4)	0.13
Insoluble (%)[1]	30.0 (1.7)	36.4 (3.3)	0.18

Values in brackets are standard deviations for triplicate experiments

[1] Obtained by difference

Fish skin collagens have been reported to develop minimal amounts of mature crosslinks (Hickman, Sims, Miles, Bailey, de Mari, & Koopmans, 2000). Cohen-Salal, Le Lous, Allain, and Meunier (1981) also demonstrated by measuring hydrothermal isometric tensions that fish skin collagen crosslinks do not mature to thermally stable bonds. As a result of its low content of stable crosslinks, fish skin collagen can easily be solubilised even from adult fish.

2.1.4.3 Denaturation temperature of Nile perch skin collagens

Figure 2.1.1 shows the changes in fractional viscosity with increasing temperature for young and adult Nile perch skin collagens. Both the young and adult skin collagens exhibited a rapid loss of viscosity with heating. This can be attributed to denaturation of collagen. The thermal denaturation temperature (Td) was determined to be about 36.0°C for collagen from the skin of young fish and about 36.5°C for collagen from the skin of adult fish. The minimal difference in denaturation temperatures of collagens from young and adult Nile perch is also indicative of minimal differences in the extent of stable crosslinks.

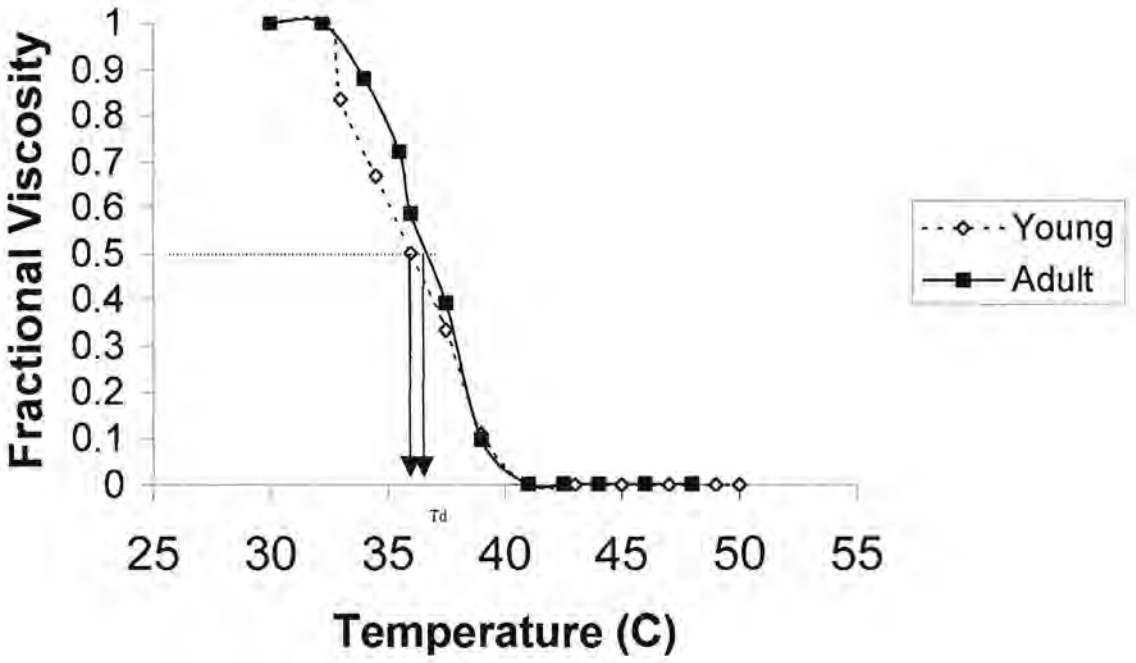

Figure 2.1.1: Denaturation curve of collagen from skins of young and adult Nile perch as shown by change in fractional viscosity with temperature for 0.1% (m/v) solutions of collagen in acetic acid. Td is the denaturation temperature.

The denaturation temperature recorded in this study for collagen from the skin of Nile perch is higher than the values reported for those from temperate fish species. Collagen denaturation temperatures have been reported for cod (15°C) (Rigby, 1968), Alaska pollack (16.8°C) (Kimura & Ohno, 1987), muscle of carp (32.5°C), eel (29.3°C), common mackerel (26.1°C), chum salmon (19.4°C) (Kimura *et al.*, 1988), Japanese seabass (30°C), skip jack tuna (29.7°C) and ayu (29.7°C) (Nagai & Suzuki, 2000). The higher denaturation temperature for collagen of Nile perch may be attributed to the higher imino acid content compared with cold-water fish collagens.

2.1.4.4 Amino acid composition of Nile perch skin collagens

Table 2.1.3 shows the amino acid composition of the acid soluble collagen extracted from young and adult fish skins of Nile perch. The amino acid contents of collagens from the skins of young and adult fish were not significantly different from each other, suggesting that amino acid composition of collagen is independent of age. The collagens were found to contain no tryptophan and cysteine. They were also very low

in methionine, tyrosine and histidine, like other collagens (Balian & Bowes, 1977; Grossman & Bergman, 1992; Gudmundsson & Hafsteinsson, 1997; Yoshida, Fujisawa, Mizuta & Yoshinaka, 2002). A significant observation was the high total imino acid content (20.03 and 19.26 %, respectively, for young and adult fish skin collagen) of acid soluble Nile perch skin collagen in comparison to other fish collagens. The total imino acid content of Nile perch skin collagens, though lower than the 25.36% for tilapia (Grossman & Bergman, 1992) is among the highest reported for fish collagen. Collagen from cold-water fish species contains 16 - 18% imino acids (Gilsenan & Ross-Murphy, 2000; Gudmundsson & Hafsteinsson, 1997; Norland, 1990). The higher imino acid content and higher denaturation temperature of collagen of Nile perch, in comparison with cold-water fish species are in agreement with observations by Rigby (1968) that thermal stability of collagen increases with imino acid content.

The high imino acid content, especially the hydroxyproline content is also significant because it affects the functional properties of gelatin that can be derived from collagen (Gilsenan & Ross-Murphy, 2000; Gòmez-Guillèn *et al.*, 2002). Fish gelatin has potential for use in several applications (Norland, 1990; Osborne, Voight & Hall, 1990), however, low gel strength is a major problem hindering increased production and use of fish gelatins. The low gel strength of fish gelatins has been attributed to the low imino acid content of fish collagens (Gilsenan & Ross-Murphy, 2000; Gòmez-Guillèn *et al.*, 2002). Nile perch skin collagen contains more imino acids and therefore has potential for use in the manufacture of gelatins with good gelling properties.

Table 2.1.3: Amino acid composition of acid soluble collagen from skins of young and adult Nile perch

	Amino acid content g/100 g protein		
	Young fish	Adult fish	p-value
Asp	6.14 (0.04)	5.91 (0.02)	0.24
Gln	10.04 (0.01)	9.85 (0.01)	0.05
Hyp	7.88 (0.01)	8.05 (0.03)	0.83
Ser	3.47 (0.01)	3.34 (0.03)	0.58
Gly	21.11 (0.11)	22.10 (0.11)	0.81
His	1.16 (0.05)	1.10 (0.02)	0.74
Arg	8.10 (0.01)	8.15 (0.02)	0.24
Thr	3.24 (0.01)	3.04 (0.01)	0.05
Ala	9.77 (0.02)	10.09 (0.02)	0.64
Pro	11.38 (0.11)	11.98 (0.14)	0.11
Tyr	0.96 (0.03)	0.86 (0.02)	0.26
Val	2.47 (0.02)	2.35 (0.02)	0.56
Met	1.72 (0.01)	1.58 (0.04)	0.56
Ile	1.38 (0.01)	1.26 (0.02)	0.72
Leu	3.19 (0.01)	2.83 (0.03)	0.85
Phe	2.48 (0.02)	2.31 (0.05)	0.74
Lys	4.07 (0.01)	3.77 (0.15)	0.55
Hyl	1.44 (0.01)	1.43 (0.05)	0.39

Values in brackets are standard deviations for duplicate samples

Table 2.1.4: Proportion of total imino acids and percent hydroxylation of lysine and proline in collagen from skins of young and adult Nile perch

	Young fish	Adult fish
Total imino acids (%)	19.26	20.03
% Hydroxylation		
Lysine	26.1	27.5
Proline	40.9	40.2
Total	37.6	37.6

The degree of hydroxylation of proline and lysine, influences the thermal stability of collagen (Kimura *et al.*, 1988). A higher degree of hydroxylation is associated with higher denaturation temperature, for collagens with similar amino acid profiles. The total degree of hydroxylation of proline and lysine for Nile perch collagen (Table 2.1.4) was found to be similar to that reported for pike (34%) and cod (32%) skin collagens (Piez & Gross, 1960) but higher than that reported by Gòmez-Guillèn *et al* (2002) for sole (25.3%), megrim (25%), and hake (24.6%). The denaturation temperature for cod has been reported to be 15°C (Rigby, 1968). It appears that it is the higher imino acid content, rather than the extent of hydroxylation that seems to be the reason for the higher denaturation temperature observed for Nile perch skin collagen.

2.1.4.5 Electrophoretic pattern of Nile perch skin collagens

SDS PAGE showed that both young and adult fish skin acid soluble collagen consisted of α chains and their dimers (β chains) (Figure 2.1.2). The α components showed two distinct species varying in their mobility, for both reducing and non-reducing conditions. It may be concluded therefore, that Nile perch acid soluble collagen is made up of at least two α species (α1 and α2). This is similar to the pattern observed for several other fish species (Nagai *et al.*, 2001; Gòmez-Guillèn *et al.*, 2002) and is typical of type I collagen (Bailey & Light, 1989). The electrophoretic pattern of Nile perch skin collagen was generally similar to that of calfskin collagen (Figure 2.1.2). The calfskin collagen species (α and β chains)

however exhibited slightly higher mobility than their fish collagen counterparts. This may be due to differences in amino acid composition or pI.

The α2 was the minor component of the two species and it seems Nile perch collagen exists as trimers consisting of two α1 and one α2 chains. This is typical of type I collagen (Bailey & Light, 1989), which is the major collagen in dermal tissue (Bailey & Light, 1989; Bailey *et al.*, 1998).

There was no clear difference in the electrophoretic pattern under reducing and non-reducing conditions, suggesting absence of disulphide bonds. This is consistent with the observation that the collagen was almost devoid of sulphur-containing amino acids. No consistent difference was observed in the electrophoretic pattern of collagen from young and adult fish skins.

As observed by Hayashi and Nagai (1979), the mobility of alpha chains was lower than would be expected for globular proteins of similar molecular weight (ca 95 kDa) and when globular proteins are used as molecular weight markers, the molecular weight of collagen could be overestimated. This is because of the unique amino acid profile of collagen. The difference observed in mobility between collagenous proteins and globular protein has been attributed to the high content of the relatively small amino acid residues, glycine, proline and alanine of the former (Noelken, Wisdom, & Hudson, 1981). The estimated molecular weight for α-chain, using globular protein standards was approximately 120 kDa (Figure 2.1.2).

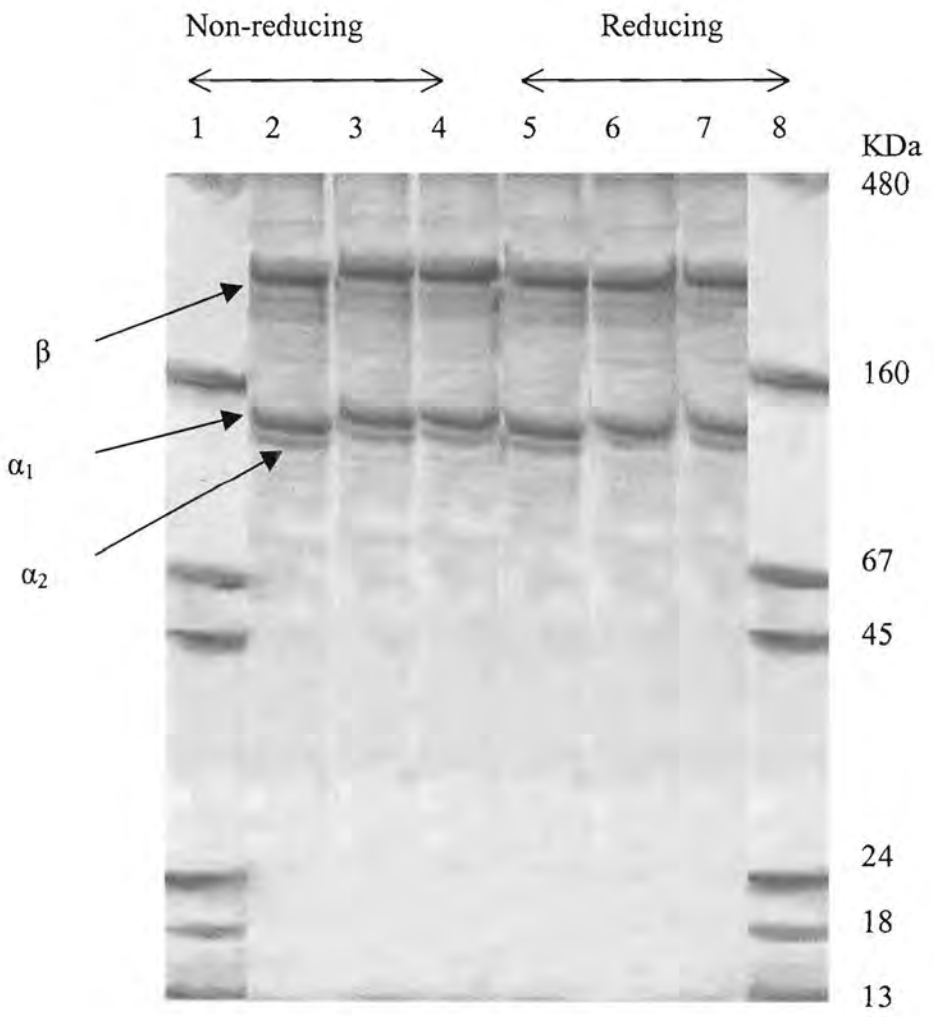

Figure 2.1.2: SDS polyacrylamide gel (7.5%) electrophoretic pattern for acid soluble collagen under non-reducing and reducing conditions. 1 & 8 – Molecular weight markers, 2 & 5 – calfskin collagen, 3 & 6 – collagen from skin of adult Nile perch, 4 & 7 – collagen from skin of young Nile perch.

2.1.4.6 Fourier transform infrared spectra for acid soluble collagens

The Nile perch acid soluble collagens exhibited FTIR spectra (Figure 2.1.3) similar to that exhibited by other collagens (Jackson, Choo, Watson, Halliday, & Mantsch, 1995; Liu, Dixon, & Mantsch, 1998; Sai & Babu, 2001).

The spectra for acid soluble collagen from young and adult fish skins differed slightly, indicating some differences in the secondary structure of the two proteins. Table 2.1.5 is a summary of the major peaks identified in the FTIR spectra of ASC from young and adult Nile perch skins, and their assignments. Generally, most of the peaks for the young fish collagen appeared at a lower frequency compared to the corresponding peaks for the adult fish collagen.

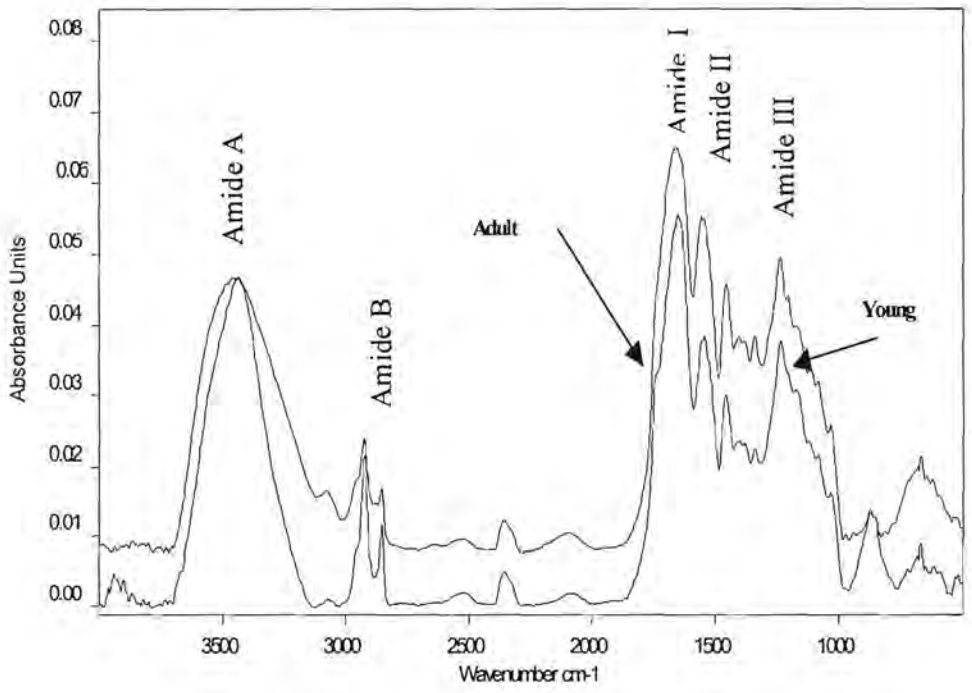

Figure 2.1.3: Average FTIR spectra for triplicate samples of acid soluble collagen derived from skins of young and adult Nile perch

Table 2.1.5: FTIR spectra peak positions and assignments for acid soluble collagen from skins of young and adult Nile perch

Region	Peak wavenumber (cm^{-1})		Assignment	Reference
	Young	Adult		
Amide A	3434	3458	NH stretch, coupled with hydrogen bonding	Sai & Babu (2001)
Amide B	2924	2926	CH_2 asymmetrical Stretch	Abe & Krimm (1972)
-	2853		CH_2 symmetrical Stretch	Abe & Krimm (1972)
Amide I	1650	1654	C=O stretch/hydrogen bonding coupled with COO-	Jackson *et al.* (1995)
Amide II	1542	1555	NH bend coupled with CN stretch	Jackson *et al.* (1995)
-	1457	1455	CH_2 bend	Jackson *et al.* (1995)
-		1340	C H_2 wagging of proline	Jackson *et al.* (1995)
Amide III	1235	1238	NH bend	Jackson *et al.* (1995)
-	871	875	Skeletal stretch	Abe & Krimm (1972)
-	670	670	Skeletal stretch	Abe & Krimm (1972)

- No common name for the spectral region

The amide I and amide II peaks were at a lower frequency for the young fish skin (1650 and 1542 cm^{-1}, respectively) compared to adult fish skin (1654 and 1555 cm^{-1}, respectively) collagen. Based on the location of the amide I and amide II peaks, it would seem that the acid soluble collagen from the young skins had a lower degree of molecular order, since a shift of these peaks to lower wave numbers is associated with a decrease in the molecular order (Payne & Veis, 1988). It would appear therefore that there were more intermolecular crosslinks in the adult fish collagen. Amide I components (Figure 2.1.4) showed adult Nile perch ASC amide I band to consist of a higher proportion of the component at 1695 cm^{-1} than the young fish ASC (Table 2.1.6). This band is linked to the extent of intermolecular interactions in collagen and collagen-like peptides (Doyle, Bendit, & Blout, 1975; Prystupa & Donald, 1996; Paschalis, Verdelis, Doty, Boskey, Mendelesohn, & Yamauchi, 2001).

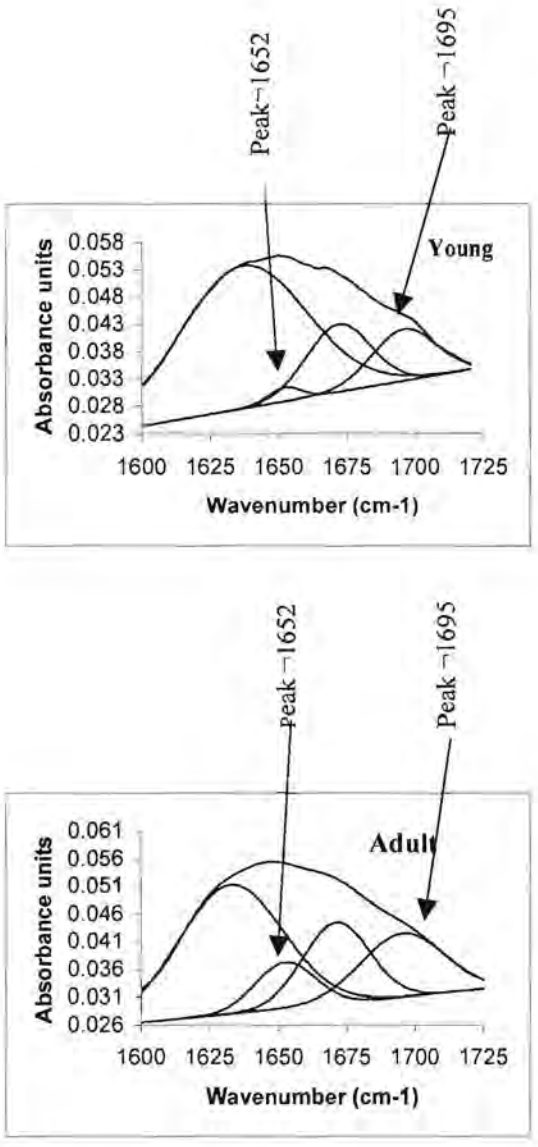

Figure 2.1.4: Amide I band for collagen from the skins of young and adult Nile perch with fitted band components

The other considerable difference was the lower intensity of the component with peak at 1652 cm^{-1} in young fish ASC (Table 2.1.6). This component has been attributed to random coils (Prystupa & Donald, 1996), suggesting a lower extent of unwinding of the triple helix in the young fish ASC. It seemed therefore that adult fish ASC retained more intermolecular crosslinks during solubilisation with acetic acid but the triple helical structure, normally held together by intramolecular hydrogen bonds (Darby & Creighton, 1993) was extensively destroyed. The young fish ASC on the other hand, because of its lower content of stable intermolecular bonds could be solubilised more easily and perhaps retained triple helices to a greater extent. The

minimal differences in the extent of collagen crosslinking with age were therefore reflected in differences in the FTIR spectra of the collagens.

Table 2.1.6: Peak location (cm^{-1}) and percent area (in brackets) of fitted components of amide I band for collagen from skins of young and adult Nile perch

	Young	Adult
1	1637 (69)	1634 (49.1)
2	1652 (1.8)	1653 (10.4)
3	1672 (16.7)	1671 (20.5)
4	1696 (12.6)	1695 (20)

2.1.5 Conclusions

Based on solubility and amino acid composition, it may be concluded that collagen from the skin of Nile perch differs considerably from mammalian and coldwater fish collagens. The collagen was easily solubilised from skins of both young and adult Nile perch using 0.5 M acetic acid, indicating that it had a low content of stable crosslinks. The solubility and denaturation temperature of collagen from skins of young and adult Nile perch were similar, indicating that age-related changes in collagen were less pronounced in Nile perch skin than in mammalian collagen.

Based on the electrophoretic profile and amino acid composition, it may be concluded that collagen from Nile perch skins, like collagens from skins of most other fish species, is Type 1 collagen. Collagen from Nile perch skins however differs from collagens from skins of other fish species in some respects. The denaturation temperature and the imino acid content of collagen from the skin of Nile perch were found to be higher than those reported for most fish species and closer to those for mammalian collagens. Due to its high imino acid content, Nile perch collagen may be a source of gelatin with good gelling properties, since the gelling properties of gelatin are related to its imino acid content. The high acid solubility of Nile perch collagen has implications for gelatin manufacture from skins of Nile perch since prolonged

acid pre-treatment, before extraction of gelatin from the skins, would lead to high losses of collagen and low gelatin yield.

2.1.6 Acknowledgements

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2.2 EXTRACTION AND PHYSICO-CHEMICAL CHARACTERISATION OF NILE PERCH (*Lates niloticus*) SKIN AND BONE GELATIN*

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2.2.1 Abstract

Type A gelatins were extracted from skins and bones of young and adult Nile perch and analysed to determine their functional and chemical properties. Total gelatin yield (for sequential extraction at 50, 60, 70 and 95°C) was in the order adult fish skins > young fish skins > adult fish bones > young fish bones, while percentage gelatin recovery at 50°C was in the order young fish skins > adult fish skins > young fish bones > adult fish bones. The gelatins obtained were free of fishy odour. Nile perch skin gelatin colour, turbidity and composition was within the range reported for bovine gelatins. Nile perch bone gelatin however exhibited high ash content and turbidity. The 50°C extracted gelatin from both young and adult fish skins exhibited gel strength greater than 220 g. This was significantly higher than the gel strength for the corresponding bone gelatins (179 g and 134 g, respectively for young and adult fish). Gelatin from adult Nile perch skins also exhibited higher viscosity and lower setting time than bone and the young fish skin gelatins. Skin gelatins were found to exhibit higher film tensile strength but lower film percent elongation than bone gelatins. Bone and skin gelatins had approximately the same amino acid composition, with a total imino acid content of about 21.5%. SDS PAGE revealed that skin gelatins had a higher content of polypeptides with molecular weight greater than β compared to bone gelatins. The differences in functional properties between the skin and bone gelatins appeared to be related to differences in molecular weight distribution of the gelatins.

Key Words: Nile perch, fish gelatin, bone gelatin, gel strength, imino acids, molecular weight distribution

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* Limited version of this chapter accepted for publication by *Food Hydrocolloids*.

2.2.2 Introduction

Processing of fish leads to enormous amounts of waste. It is estimated that fish processing waste after filleting accounts for over 50% of the total fish weight (Shahidi, 1994) and 30% of the waste is in the form of bones and skins (Gòmez-Guillèn, Turnay, Fernández-Diaz, Ulmo, Lizarbe & Montero, 2002). The fish skins and bones can be processed into gelatin, thus contributing to solving the problem of waste disposal and in addition creating a value-added product. Recent outbreaks of Bovine spongiform encephalopathy (BSE) and increase in demand for kosher and halal foods have created a demand for fish gelatin for food applications. Use of fish gelatin however, remains limited, mainly because most species give low Bloom gelatins.

A number of studies have addressed properties of fish skin gelatins (Grossman & Bergman, 1992; Holzer, 1996; Gudmundsson & Hafsteinsson, 1997; Choi & Regenstein, 2000; Fernández-Diaz, Montero & Gòmez-Guillèn., 2001; Gómez-Guillén & Montero, 2001; Gudmundsson, 2002) showing that their properties differ from those of mammalian gelatins and vary between species. Literature on fish bone gelatin is, however, limited.

The functional properties of gelatin are related to their chemical characteristics. The gel strength, viscosity, setting behaviour and melting point of gelatin depend on their molecular weight distribution and the amino acid composition (Johnston-Banks, 1990). It is generally recognised that the imino acids proline and hydroxyproline are important in the renaturation of gelatin subunits during gelling (Johnston-Banks, 1990). As a result, gelatins with high levels of imino acids tend to have higher gel strength and melting point. The molecular weight distribution is also important in determining the gelling behaviour of gelatin. According to Johnston-Banks (1990), the sum of intact α and β fractions together with their peptides is proportional to the gel strength while the viscosity, setting rate and melting point increase with increase in the amount of the high molecular weight (greater than γ) fraction.

While the amino acid composition is mainly dependent on the source species (Eastoe & Leach, 1977), the molecular weight distribution of gelatin depends to a large extent on the extraction process (Müller & Heidemann, 1993). During conversion of

collagen to gelatin, the inter- and intra-molecular bonds linking collagen chains as well as some peptide bonds are broken. The more severe the extraction process, the greater the extent of hydrolysis of peptide bonds and therefore the higher the proportion of peptides with molecular weight less than α. The age of the source animal may influence the ease with which gelatin can be extracted and the extent of peptide hydrolysis during the extraction (Reich, Walther & Stather, 1962; Cole & McGill, 1988). Older animal collagen is more crosslinked and a more severe process is required to denature it to form gelatin (Reich *et al.*, 1962). There are differences in the extent and type of crosslinking found in bones and skins (Sims & Bailey, 1992). This may also affect the ease with which collagen may be solubilised and transformed to gelatin and may result in differences between the properties of gelatins extracted from the two tissues.

Only a few studies have been conducted on warm water fish gelatin and these show that these fish species give gelatin of better functional properties than cold water fish species (Leuenberger, 1991; Grossman & Bergman, 1992; Gilsenan & Ross-Murphy, 2000; Jamilah & Harvinder, 2002). This has been attributed to their higher content of imino acids.

Nile perch (*Lates niloticus*) is a warm water fish species. The catch of Nile perch in Uganda alone is estimated at 100,000 tonnes per year (UIA, 2001). According to Acere (1993) the length of Nile perch increases with age, with young (less than 80 days old) fish measuring as little as 6.4 cm and the largest adult (13+ years old) measuring up to160 cm long for males and 190 cm for females. The smallest length for sexually mature fish is 53.5 cm and 67.5 cm for males and females, respectively and this occurs between the age of 1 and 2 years (Acere, 1993). Studies with pig skins (Reich *et al.*, 1962) and cattle hides (Cole & Roberts, 1996) have shown that the quality and extractability of gelatin reduce with age of the animal.

The objective of this study was to determine and compare the properties of Nile perch bone and skin gelatins and to determine the effect of the age of the source fish.

2.2.3 Materials and Methods

2.2.3.1 Raw materials

Fish skins and skeletons were obtained from Nge-ge Fish Limited, Kampala, Uganda. These were by-products from fillet processing. The very small skins (skin thickness < 0.4 mm) and bones (skeleton length < 40 cm) from young fish and the very large skins (skin thickness > 1.5 mm) and bones (skeleton length > 95 cm) from adult fish were selected and used for the study.

Portions were taken from different parts of the skins and skeletons, scrapped to remove attached flesh, blended together and used for proximate analysis. Moisture, lipid, ash and protein were determined by AOAC (1995) methods 950.46, 960.39, 900.2A and 928.08, respectively. Protein digestion was done for 2 hours, using a catalyst made by mixing 0.75 g of selenium powder and 100 g of mercuric sulphate (Eastoe & Eastoe, 1952). A conversion factor of 5.4 was used for calculating the protein content from the Kjeldahl nitrogen content since collagen, the main protein in skin, contains approximately 18.7% nitrogen (Eastoe & Eastoe, 1952).

The skins and skeletons for gelatin manufacture were frozen immediately upon delivery at the laboratory and thawed just before the gelatin extraction process. All extractions were conducted at the Uganda Industrial Research Institute, Kampala, Uganda.

2.2.3.2 Pre-treatment

Skins were pre-treated by acidulation with 0.01 M sulphuric acid liquor (pH of 2.5 - 3.0) and this pH range was maintained throughout the pre-treatment period (16 hours) by adding more acid solution until the skins were adequately conditioned. The skin-to-liquor ratio was about 1:2 (w/v). The conditioned skins were washed twice, each time with a volume of water equal to the volume of the conditioning liquor, until a final pH of 3.5 – 4.

Bones used for gelatin extraction were cleaned by scraping with a knife to eliminate some of the flesh and then degreased by tumbling in warm (35°C) water. The degreased bones were then demineralised using 3% HCl, at ambient temperature (20 - 25°C) until the bones did not have any hard cores. The acidulation liquor was changed at 3 day intervals. The time required for complete demineralisation was 9 - 12 days. The spent liquor from the demineralisation process was analysed for ash and organic matter to determine the acid-use efficiency and the extent of organic matter loss during the demineralisation process. All the spent liquor from the demineralisation process for a given batch of bones was collected and duplicate portions (5 ml each) were drawn and used to determine the acid-use efficiency. The liquor portions were dried and ashed to determine the ash content. Acid consumed was determined by titrating duplicate portions of the spent liquor against 0.1 M NaOH. The leached bones (ossein) were washed with water until the wash water pH was greater than 4. This required 6 - 7 washes (ossein to water ratio of 1:2).

An attempt was also made to leach the bones at refrigeration temperatures (3 - 5°C). This was aimed at reducing the possibility of loss of collagen in the demineralisation liquor. Under refrigeration, the leaching process required 21 days to complete, with the liquor being changed every 3 days.

2.2.3.3 Gelatin extraction

The pre-treated materials were transferred to beakers, covered with warm (~ 60°C) water and gelatin extracted in water baths by 3 sequential 5 hour extractions at 50, 60 and 70°C, followed by boiling for 5 hours. For the extraction of gelatin from skins of young Nile perch, the higher (70°C) temperature extraction was omitted because the shrinkage of skins resulting from earlier extractions was very high and the remaining mass of skins very small. In all cases, extraction pH was between 3.5 and 4.

The volume of the extracts obtained at the different temperatures used and the mass of the residue ("scutch") after boiling for 5 hours were recorded. Portions of the gelatin extracts (light liquor) were filtered through Whatman 1 filter paper and used for determining the solids concentration. The light liquor concentrations were determined

by evaporating duplicate 10 ml portions to a stable weight (48 hours at 105°C) and the concentration was used to calculate % gelatin extractability as follows:

Amount of gelatin (g) extracted at a given temperature = Light liquor concentation (g/l) X liquor volume (l)

$$\frac{\text{Amount of gelatin extracted at a given temp}}{\text{sum of gelatin extracted at all temp}} \times 100\% = \%\ \text{gelatin extractability at a given temp}$$

For each of the extractions, yield was calculated, both based on total weight and on dry ash-free basis.

The remainder of gelatin extracts (light liquors) were filtered through compressed cotton wool. The light liquors were then passed through a column of activated carbon (GRC 22, BHT water treatment, Chloorkop, South Africa) at a rate of approximately 5 bed volumes per hour. This was aimed at removing the fishy odour. The pH of the light liquors was adjusted to about 5 using 5% ammonia solution and the extracts were dried in a cross-flow air drier at 42°C, until brittle sheets were formed. The brittle sheets were broken into small pieces and milled using a domestic coffee grinder to pass through a 1mm mesh sieve.

Bovine bone gelatin was obtained from Davis Gelatin, Brazil and commercial fish gelatin from AquaGel Inc., London, UK.

2.2.3.4 Analysis of gelatins

Colour, turbidity, gel strength and viscosity were determined at Davis Gelatine, SA (now Gelita, SA) at Krugersdorp.

2.2.3.4.1 Proximate composition of gelatins

Proximate analysis was conducted at Makerere University, Kampala, Uganda. The moisture, ash and fat content of the extracted gelatins were determined by the BSI 757 methods (BSI, 1975). Protein content was determined by Kjeldahl method (AOAC, 1995) and a nitrogen conversion factor of 5.4 was used (Eastoe & Eastoe, 1952). Protein digestion was done as described by Eastoe and Eastoe (1952) to ensure complete hydrolysis of collagen.

2.2.3.4.2 Determination of isoionic point

The isoionic point (pI) was determined by passing a 1% solution of gelatin through a column of mixed bed resin (Rohm and Hass MB3) at a flow rate of approximately 10 bed volumes per hour and measuring the pH of the deionised solution.

2.2.3.4.3 Determination of gelatin colour and turbidity

Colour (in Davis Gelatin Units) and turbidity (in Nepholemeter turbidity units) were determined using Nessler tubes and a turbidimeter (ICM, Hillsboro, OR, USA), respectively, as described by Cole and Roberts (1996). The colour was determined on a 4% gelatin solution while the turbidity was determined on 6.67% (w/v) gelatin solution.

2.2.3.4.4 Determination of gel strength

The Bloom gel strength was determined by the British Standard 757: 1975 method (BSI, 1975), using a texture analyser (Stevens Weighing & Measuring Specialists, Loughton, UK). A solution containing 6.67% (w/v) gelatin was prepared by mixing 7.5 g of gelatin and 105 ml of distilled water in a Bloom bottle. The mixture was swirled and let to stand for 30 minutes at room temperature to allow the gelatin to absorb water and swell. The Bloom bottles were then transferred to a water bath maintained at 42°C and held for 30 min during which they were swirled intermittently. The samples were then transferred to a cold water bath maintained at 10 ± 0.1°C and held at this temperature for 16 - 18 hours before determination of gel strength. The Bloom gel strength (in g) was determined with the texture analyser set to make a 4 mm depression at a rate of 0.5 mm/sec. Corrected gel strength (assuming 87.5% protein) was calculated from the equation;

$$\text{Corrected Bloom} = \text{Bloom}_m \text{ X } (87.5/ (100 - \text{Moisture\%} - \text{Ash\%}))^2$$

Where Bloom_m was measured Bloom.

2.2.3.4.5 Determination of viscosity

Viscosity (in mSt) was determined by British Standard 757: 1975 method (BSI, 1975) using a U-tube Ostwald's viscometer. Samples used for Bloom gel strength determination were melted in a water bath maintained at 45°C and then poured into the viscometer. The viscometer was held in a water bath maintained at 60°C for 15 min before the viscosity was determined.

2.2.3.4.6 Determination of setting point and setting time

Setting point and time were determined on 10% (w/v) gelatin solutions dissolved in thin wall (12 mm x 75 mm) test tubes in the same way as described for the Bloom samples. The dissolved samples from the warm water bath were transferred to another water bath held at 40°C. The bath was then cooled slowly by adding chilled water (~ 2°C) at intervals of 15 sec. A thermometer was inserted into the sample and lifted out at 15 sec intervals. The temperature of the mixture at which the gelatin solution no longer dripped from the tip of the thermometer was recorded as the setting temperature.

Setting time was determined on samples prepared in the same way as those for the determination of the setting temperature. Samples were transferred to a water bath maintained at 10°C. A rod was inserted in the gelatin solution and raised at intervals of 15 sec. The time at which the rod could not detach from the gelatin sample was recorded as the setting time.

2.2.3.4.7 Determination of melting point

Determination of melting point was based on the JIS K6503 (JSA, 1996) method. Solutions containing 6.67% (w/v) gelatin were prepared in thin wall (12 mm x 75 mm) screw cap test tubes. The test tubes were filled to leave some headspace and closed. The dissolved samples were held in a refrigerator (7°C) for 16 - 18 hours, after which they were transferred into a water bath (10°C) and inverted so that the headspace was at the bottom. The water bath was warmed gradually (about 1°C per

min) by adding warm (~ 45°C) water at intervals of about 60 sec. The temperature at which the gel melted, to allow the gas in the headspace to start moving up was recorded as the melting point.

2.2.3.4.8 Texture profile analysis

Texture profile was determined using a TA-XT2 Texture profile analyser (Stable Microsystems, Surrey, UK). Only samples with Bloom greater than 200 g for Nile perch skin gelatins and greater than 150 g for bone gelatins were analysed. Gels containing 6.67% gelatin (corrected to protein content of 87.5%) were prepared from Nile perch skin, bovine bone and the commercial fish gelatins. For the Nile perch bone gelatins, two levels of gelatin concentration were used, i.e. gels containing 6.67% solids and those with concentration adjusted to give a gel strength of 225 g. The concentration corresponding to Bloom of 225 g for the Nile perch gelatins was calculated based on the formula;

$$Concentration(\%) = 6.67\sqrt{\frac{225}{SampleBloom}}$$

The samples were dissolved in the same way as the samples used for Bloom determination and then poured into cylindrical plastic containers with a diameter of 30 mm and a height of 40 mm. The samples were stored in a cold room (9 – 10°C) for 16 – 18 hours. Before testing, the samples were equilibrated to room temperature (~ 15°C) for 30 min. The samples were removed from the plastic moulds and sections (20 mm length) cut off and tested by imparting a 50% strain, double compression, using 50 mm diameter aluminium probe. Pre-test, test and post-test speed were set at 1 mm/sec and trigger force at 0.49 N. The hardness, springiness, cohesiveness, chewiness and gumminess were determined as described by Pye (1996).

2.2.3.4.9 Sensory evaluation

Sensory evaluation was conducted using a 20 member panel consisting of students and staff of the Department of Food Science and Technology at Makerere University. Only individuals who were able to detect off odour in gelatin samples having a slight putrid odour and one contaminated with an extract from dry fish skins were selected.

Samples for sensory evaluation were prepared by dissolving 0.5 g of gelatin in 7 ml of distilled water, to obtain a solution containing approximately 6.67% gelatin. The samples were prepared in test tubes with screw caps and dissolved as described for the Bloom samples. The samples were held in a water bath at 50°C, with the screw caps lightly closed. Panellists were instructed to remove the screw caps, sniff the contents and identify the odour they perceived as well as indicate the odour intensity, using a six point scale (0 = No odour, 1 = Very mild and only perceivable on careful assessment, 2 = Mild but easily perceivable, 3 = Strong but not offensive, 4 = Strong and offensive, 5 = Very strong and very offensive).

2.2.3.4.10 Determination of film properties

Gelatin films were prepared by dissolving 1 g of sample in 15.7 ml of 0.5 M acetic acid and 0.2 g of glycerol. A portion (10 ml) of the mixture was cast per 9 cm petri dish and the films dried for 48 hours in a fume hood at room temperature (~ 25°C). The films were then cut into 60 X 6 mm strips. For each film, thickness was determined at 6 different points using a micrometer. The average thickness was entered into the texture analyser program and used to calculate film area needed for expressing the stress in N/mm^2. The film strips were conditioned in a desiccator at room temperature and 50% relative humidity for 40 – 48 hours after which tensile stress and % strain were determined using a TA-XT2 texture analyser (Stable Microsystems, Surrey, UK) with tensile rig grip A/TG attachment. The determinations were done at pre-test speed of 1.0 mm/s, test speed of 0.4 mm/s, post-test speed of 8 mm/s and trigger force of 0.49 N.

2.2.3.4.11 Amino acid analysis

Amino acid analysis was conducted using the Pico.Tag method (Bidlingmeyer, Cohen & Tarvin, 1984) at the Department of Biochemistry, University of Pretoria. This method involves derivatisation of amino acids using phenylisothiocyanate (PITC) and determination of the phenylthiocarbamyl (PTC) amino acids using reversed phase HPLC. Dry gelatin (10 - 20 mg) was mixed with 6 M HCl (1 ml) containing 1% phenol (v/v). The mixture was evacuated, blown with N_2 and vacuum sealed before

hydrolysis at 110°C for 24 hours. After hydrolysis the samples were cooled and made up to 5 ml with deionised water. A portion (25 µl) was then dried and derivatised. Derivatisation involved addition of 10 µl of a mixture of methanol, water and trimethylamine (2:2:1), mixing and then drying for 5 minutes. This was followed by addition of 20 µl of a mixture of methanol, water, trimethylamine and phenylisothiocyanate (7:1:1:1). The sample was held for 20 minutes at room temperature (20 - 25°C), dried under vacuum and then dissolved in 200 µl of pH 7.4 phosphate buffer and filtered with a 0.45 µm filter. Portions (20 µl) of the filtered samples were injected using an automatic loader (WISP™) (Millipore Corp, Milford, MA, USA) into the Pico.Tag column, part no 88131 (3.9 mm X 13 cm) (Millipore Corp, Milford, MA, USA), for amino acid analysis.

2.2.3.4.12 Sodium dodecyl sulphate gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted using the discontinous Tris-HCl/glycine buffer system (Laemmli, 1970), with 7.5% resolving gel and 4% stacking gel. Samples containing approximately 5 µg of solids per µl were prepared by dissolving 0.01 g gelatin in 2 ml sample buffer containing 2-mercaptoethanol. A portion (20 µl) of sample was loaded per well. Calfskin acid soluble collagen (Sigma Chemical Co., St Louis, MO, USA) and molecular weight markers were loaded alongside the Nile perch gelatin samples. The molecular weight markers (ICN Biomedicals Inc., Aurora, OH, USA) contained cytochrome C (horse heart), myoglobin (horse heart), chymotrypsinogen A, ovalbumin, bovine serum albumin, gamma globulin (human) and apoferritin. These were mixed in sample buffer to make a solution containing 2 µg of each protein per µl and 10 µl were loaded per well. Electrophoresis was conducted using the Protean II xi vertical cell and the 1000 powerpac (Bio-Rad laboratories, Hercules, CA) at a constant current of 30 mA and a temperature of 10°C. Gels were stained using 0.1% Coomassie Brilliant Blue R250 dissolved in water, methanol and trichloroacetic acid (5:4:1) and de-stained using a solution containing methanol, distilled water and acetic acid in a ratio of 5:4:1. The gels were scanned at the Department of Microbiology, University of Pretoria, using a GS-300 transmittance/reflectance scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA, USA) using the transmittance mode.

2.2.3.5 Statistical analysis

Data for the different parameters were compared using analysis of variance (ANOVA) and means were separated using LSD. In all cases data from triplicate experiments were used.

2.2.4 Results and Discussions

2.2.4.1 Proximate composition of raw materials

The protein content of the fish skins was found to be approximately 20 - 22% and that for bones was approximately 13% (Table 2.2.1). The protein content of the collagenous material represents the maximum possible yield of gelatin expected from them. This was higher for skins than for bones, but did not significantly vary with age for either skins or bones. The bones also generally contained higher ash and lower moisture than the skins. Skins from adult fish were found to contain more lipid than skins from young fish skin, probably because the fish accumulate subcutaneous fat as they age. Ash content was also considerably higher for adult than for young fish skins, probably because of increased calcification of scales with age.

Table 2.2.1: Proximate composition of Nile perch skins and bones obtained from young and adult fish

	Skin		Bone	
	Young	**Adult**	**Young**	**Adult**
Moisture	72.7 (1.3) a	68.4 (0.57) a	36.8 (2.6) b	36.3 (1.6) b
Protein	20.3 (2.0) a	21.6 (1.3) a	13.2 (1.2) b	13.1 (1.3) b
Lipid	4.96 (0.67) b	6.8 (0.3) a	7.1 (1.3) a	7. 8 (1.3) a
Ash	3.7 (0.5) b	6.0 (0.2) b	38.4 (1. 8) a	39.1 (2.6) a

- Values in brackets are standard deviations of triplicate samples
- Values in the same row followed by same letter are not significantly different at $\alpha = 0.05$

2.2.4.2 Gelatin yield and extractability

The proportion of total protein recovered was much higher from skins than from bones. For the skins, protein recovery, as shown by the percentage yield on dry ash-free basis (Table 2.2.2) was about 64%, indicating a loss of about 36%. The unrecovered protein includes both the protein lost in acidulation liquor and the protein in the residue after boil out.

Table 2.2.2: Extractability, isoionic point and yield of gelatin from Nile perch bones and skins

	Skins		Bones			
			Room temp leaching		Cold leaching	
	Young	Adult	Young	Adult	Young	Adult
Extractability (%)						
50°C	87.3 (6.1) a	66.3 (5.3) b	36.6 (4.7) c	29.1 (5.5) c	35.5 (7.2) c	34.4 (3.4) c
60°C	8.2 (5.2) c	24.2 (2.3) b	39.9 (6.4) a	33.8 (7.9) ab	38.1 (4.8) a	32.8 (0.6) ab
70°C	Ne	5.7 (2.2) c	11.3 (4.6) b	15.5 (8.6) ab	19.2 (2.1) a	20.6 (0.3) a
95°C	4.2 (2.1) c	3.9 (3.1) c	12.2 (3.1) b	21.5 (4.9) a	12.3 (6.8) b	12.2 (4.2) b
pI						
50°C	8.8 (0.2) a	9.4 (0.3) a	7 (0.6) b	7.2 (0.7) b	6.81 (0.4) b	7.44 (0.4) b
60°C	Nd	8.8 (0.2) a	7.3 (0.4) b	7.5 (0.6) b	Nd	Nd
70°C	Ne	8.5 (1.1)	Nd	Nd	Nd	Nd
Yield (%) on wet basis	12.3 (2.1) b	16.0 (0.3) a	1.3 (1.0) c	2.4 (0.7) c	6.6 (5.2) c	9.8 (6.7) bc
Yield (%) on dry ash free basis	64.3 (4.9) a	64.3 (1.2) a	6.1 (0.9) d	11.5 (2.9) c	26.2 (19.4) b	39.3 (25.5) b

- Values in brackets are standard deviations of triplicate experiments
- Values in the same row followed by same letter are not significantly different at $\alpha = 0.05$

Ne – No extraction at these conditions

The young fish skins gave a lower total gelatin yield, on wet basis (12.3 %) than the adult fish skins (16%). Grossman and Bergman (1992) and Gudmundsson and Hafsteinsson (1997) reported gelatin yield of about 15% for tilapia and cod, respectively. Jamilah and Harvinder (2002) reported yields of 7.81 and 5.39% for red and black tilapia. Assuming moisture content of 12.5% for gelatin, the yield reported by Grossman and Bergman (1992) and Gudmundsson and Hafsteinsson (1997) would be approximately 13% on dry ash free basis. This is between the yield recorded for young and adult Nile perch skins in this study. The difference in gelatin yield from young and adult Nile perch skins may be due to a higher loss of soluble components from the young fish skins, higher degree of skin disintegration and the slightly higher moisture content of skins from young fish. Reich *et al.* (1962) compared the

proportion of protein solubilised by water, NaCl and citrate buffer from pigskin and cattle hides of varying age. They found that for skins from newborn pigs 25% of the total proteins were solubilised by sequential extractions with these solutions while for skins from 2.5 year old pigs only 5.6% of the proteins were solubilised. The corresponding values for hides from newborn and 5 year old cattle were found to be 21.7 and 4.8%. Solubility of proteins in epidermal tissue of animals therefore decreases with age. This may be attributed to increase in extent and stability of collagen crosslinks.

Nile perch bones generally gave a lower yield of gelatin than skins, both on the basis of total mass and on dry ash free basis (Table 2.2.2). The difference may be attributed to two factors; the high proportion of flesh attached to the bones compared to the skins and to higher loss of collagen due to the long leaching process. The flesh mainly consisted of non-collagenous material and this was solubilised during the acidulation process. Up to 76% of the original bone organic matter content was lost in the leaching liquor. It must be noted that this largely consisted of the non-collagenous flesh on the skeletons. Approximately 10 mmol of HCl was required to leach 1g of ash from the bones. Leaching under refrigeration (3 - 5°) resulted in significantly higher yield in comparison to room temperature leaching. The higher yield from bones leached at refrigeration temperature was probably due to reduction in loss of organic matter from the bones. The low temperature extractability, was however, not significantly affected by the leaching temperature. There was wide variation in the yield of gelatin from bones and this seems to have resulted from the differences in the amount of flesh that was attached on the bones.

Extractability data (Table 2.2.2) also showed differences between bone and skins. First (50°C) extractability was higher for young and adult fish skin (> 65%) than for bone (< 40%) gelatin. This is consistent with reports that the two types of tissue differ in the type and quantities of crosslinks (Sims, Avery, & Bailey, 2000). Low temperature extractability is expected to be higher if the collagen is less crosslinked. In mammals, the extent of crosslinking of collagen increases and the type of crosslinks change as animals age (Sims & Bailey, 1992). According to Sims *et al.* (2000) collagen from skins of immature animals mainly contain the intermediate crosslinks dehydroxylysinonorleucine (deHLNL) whereas collagen from bones of

immature animals contain hydroxylysinoketonorleucine (HLKNL). These intermediate divalent crosslinks are respectively converted to the more stable trivalent histidinohydroxylysinonorleucine (HHL) and pyridolines (PYR) during maturation. The PYR crosslinks are more stable to heat than the HHL crosslinks (Bailey, Paul & Knott, 1998). The data in this study suggests that Nile perch skin collagen contain markedly lower amounts of stable crosslinks than bone collagen.

Gelatin extractability at 50 °C was significantly higher for young than for adult Nile perch skins. Studies on mammals (Reich *et al.*, 1962; Cole & McGill, 1988) have shown markedly higher extractability at low temperature for younger cattle hides and pigskins. The acid pretreatment process was found unsuitable for extraction of gelatin from hides of adult animals. In the case of Nile perch, however, extractability of gelatin was high (66.3% at 50°C) even from adult fish skins. It seems therefore, that the age-related changes in collagen are less pronounced in Nile perch than in mammals. According to Hickman, Sims, Miles, Bailey, de Mari, & Koopmans (2000) there is minimal maturation of crosslinks in fish collagen. This is probably the reason for high fish skin gelatin extractability at low temperature, even from adult fish.

2.2.4.3 Isoionic point

The isoionic point was generally lower for bone than for skin gelatins (Table 2.2.2). The lower pI for bone gelatins may be attributed to the prolonged exposure of bones to acid treatment during demineralisation. According to Eastoe and Leach (1977), deamidation of asparagine and glutamine occur during prolonged exposure of collagenous material to acid or alkali, leading to decrease in pI values.

2.2.4.4 Proximate composition of gelatins

The proximate composition of gelatin was found to vary with the type of tissue used as raw material but was unaffected by age of the fish (Table 2.2.3). Generally, the gelatin samples extracted were almost free (< 0.5%) of fat. This showed that the processes used had eliminated fat as desired. The skin gelatins were generally low in ash, with most having ash content lower than the recommended maximum of 2.6% (Jones, 1977). The bone gelatins, however, had much higher ash content (most in the

range 3 - 10%), indicating that the leaching process was inadequate. The leaching temperature did not affect the proximate composition of the bone gelatins. Manufacture of fish bone gelatin may therefore, require an ion exchange step to remove the salts or improvement of the leaching process, for example, by application of a counter-current process.

Table 2.2.3: Proximate composition of gelatins derived from skins and bones of young and adult Nile perch

	Skins		Bones			
			Room temp leaching		Cold leaching	
	Young	Adult	Young	Adult	Young	Adult
Protein (%)						
50°C	88.8 (3.1) a	88.0 (4.7) a	83.3 (3.9) ab	78.4 (2.5) b	81.2 (3.1) ab	79 (2.1) b
60°C	87.4 (5.6) a	88.7 (2.4) a	82.0 (2.1) b	82.9 (4.3) b	81.9 (0.9) b	81.4 (41.6) b
70°C	Ne	87.9 (4.8) a	85.5 (1.6) a	86.1 (4.2) a	84.3 (1.3) a	85.8 (2.2) a
Moisture (%)						
50°C	10.4 (0.9) a	10.5 (0.6) a	10.8 (0.4) a	10.3 (1.1) a	10.8 (0.7) a	10.5 (1.1) a
60°C	11.5 (1.0) a	10.7 (1.1) a	9.8 (1.1) a	9.0 (1.1) a	10.8 (1.7) a	10.9 (1.6) a
70°C	Ne	11.0 (1.1) a	10.0 (1.3) a	10.0 (1.0) a	10.1 (0.9) a	9.5 (1.3) a
Ash (%)						
50°C	1.7 (0.4) c	1.4 (0.4) c	8.4 (0.4) b	11.2 (0.5) a	7.5 (0.7) b	9.4 (1.0) ab
60°C	0.8 (0.2) c	0.5 (0.1) c	4.4 (0.2) b	8.1 (0.4) a	6.7 (0.2) b	7.1 (0.7) ab
70°C	Ne	1.0 (0.1) b	5.9 (0.3) a	4.9 (0.6) a	5.5 (0.4) a	6.0 (0.7) a
Lipid (%)						
50°C	0.0 (0.0) a	0.1 (0.0) a	0.2 (0.1) a	0.0 (0.0) a	0.2 (0.1) a	0.0 (0.0) a
60°C	0.0 (0.0) a	0.2 (0.0) a	0.0 (0.0) a	0.2 (0.1) a	0.0 (0.0) a	0.21 (0.1)a
70°C	Ne	0.0 (0.0) a	0.0 (0.0) a	0.0 (0.0) a	0.0 (0.0) a	0.0 (0.0) a

- Values in brackets are standard deviations of triplicate samples
- Values in the same row followed by same letter are not significantly different at $\alpha = 0.05$

Ne – No extraction at these conditions

2.2.4.5 Gelatin odour

Sensory evaluation did not reveal any differences in odour between Nile perch gelatins and bovine bone or commercial fish gelatins studied. The gelatins were found to be free of fishy odour and to have a mild putrid odour (mean hedonic score of 2 - 2.5). It seems therefore that the activated carbon treatment eliminated the fishy odour from fish gelatins.

2.2.4.6 Gelatin colour and turbidity

The colour of the gelatins derived from Nile perch skins and bones (Table 2.2.4) were within the range reported for bovine hide gelatins (Cole & Roberts, 1996). Turbidity values are largely dependent on efficiency of the clarification (filtration) process. In the process used in this study, unlike in commercial gelatin manufacture, filtration was only done on the light liquor. In the commercial process, filtration is done on both the light and the heavy (concentrated) liquors. The heavy liquor filtration eliminates particles that precipitate as a result of concentration. This leads to further improvement in gelatin clarity. The range of turbidity values recorded in this study (20 – 945 NTU) was very wide. Higher values may have resulted from inadequate filtration. High turbidity values interfere with colour measurements (Cole & Roberts, 1996). The results however, show that it is possible to obtain Nile perch skin gelatin with turbidity in the range reported for bovine hide gelatins (Cole & Roberts, 1996) even with a single filtration. The turbidity of the bone gelatins were however higher than values reported for bovine skin gelatins, and may require a more efficient filtration process than was used in this study.

2.2.4.7 Gelatin viscosity and gelling properties

The gelatins extracted from Nile perch skins at 50°C generally exhibited higher gel strength (222 and 229 g, respectively for young and adult fish) than corresponding bone gelatins (179 and 134 g, respectively for young and adult fish bones leached at room temperature and 73 and 70 respectively for the bones which were leached at 3 – 5° C) (Table 2.2.4). Low temperature leaching was found to give low Bloom gelatins. Since Bloom is a very important property in commercial gelatins, the low temperature

leaching process followed by type A extraction was not found suitable for Nile perch bone gelatin extraction and as a result this gelatine was not included in subsequent analyses.

Gelatin extracted from skins at higher temperature exhibited lower gel strength but this was not necessarily the case for bone gelatins. Gelatin extracted from adult fish skins at 50°C also exhibited higher viscosity (42.3 mSt) than bone gelatins. There was no significant difference ($p > 0.05$), however, between the viscosity of young Nile perch skin and the Nile perch bone gelatins. The setting and melting temperatures were found to be similar for adult fish skin and the bone gelatins but lower for young fish skin gelatin, while setting time was lowest for adult fish skin gelatin, lower for the bone gelatins and highest for the young fish gelatins.

Table 2.2.4: Functional properties of gelatin extracted from different raw materials at various temperatures

	Nile Perch Gelatins						Other Gelatins[1]	
	Skins		Bones				Bovine bone	Commercial Fish
			Room temp leaching		Cold leaching			
	Young	Adult	Young	Adult	Young	Adult		
Corrected Bloom (g)							221	216
50°C[2]	222 (5) a	229 (10) a	179 (6) b	134 (12) c	73 (7) d	70 (10) d		
60°C	81 (23) c	175 (13) a	147 (4) b	151 (21) ab	24 (4) e	68 (3) d		
70°C	Ne	134 (17) b	Nd	160 (11) a	Ns	39 (5) c		
Viscosity (mSt)							46	40
50°C	21.6 (2.2) b	42.3 (2.1) a	28.2 (2.8) b	30.0 (2.9) b	24.9 (4.1) b	23.5 (6.6) b		
60°C	13.2 (4.2) c	28.6 (1.3) a	26.3 (1.6) a	26.1 (1.7) a	20.4 (1.4) b	17.8 (0.0) b		
70°C	Ne	21.4 (3.6) a	Nd	24.8 (2.1) a	10.8 (0.8) c	19.5 (1.5) b		
Setting Temp (°C)							25.3	22.5
50°C	13.8 (0.1) d	19.5 (1.1) a	18.5 (0.7) a	19.0 (0.4) a	14.6 (0.5) c	16.5 (0.0) b		
60°C	Ns	18.5 (0.4) a	19.1 (1.1) a	18.5 (0.7) a	16.9 (0.9) b	14.5 (0.5) b		
70°C	Ne	15.5 (1.1) b	Nd	18.0 (0.9) a	Ns	12.3 (0.3) c		
Setting Time (s)							60	60
50°C	135 (0) a	60 (0) c	90 (0) b	90 (15) b	150 (0) d	150 (0) d		
60°C	Ns	75 (0) a	75 (0) a	75 (0) a	120 (0) b	150 (0) c		
70°C	Ne	105 (0) a	Nd	90 (0) b	Ns	195 (0) c		
Melting Temp (°C)							31.6	26.3
50°C	21.4 (0.3) b	26.3 (1.2) a	26.5 (0.7) a	25.5 (1.3) a	24.3 (1.3) a	24.9 (0.9) a		
60°C	Ns	25.5 (2.1) a	25.6 (1.1) a	25.4 (0.4) a	24.7 (1.1) a	23.2 (0.6) a		
70°C	Ne	24.7 (1.4) a	Nd	25.0 (1.8) a	Ns	21.5 (1.5) b		
Colour (DGU)							3.1	3.1
50°C	3.7 (0.0) b	5.2 (0.0) a	5.1 (2.7) a	4.0 (1.2) b	4.3 (0.0)	Nm		
60°C	Nm	7.4 (1.4) a	3.1 (0.0) c	4.8 (1.4) b	Nm	Nm		
70°C	Ne	Nm	Nd	6.1 (1.2) ab	7 (0.7) a	5.7 (1.1) b		
Turbidity (NTU)							44	21
50°C	42(0) c	20 (1) d	517 (47) a	190 (10) c	352 (22) b	254.5 (40.1) bc		
60°C	158 (97) c	27(3) d	165 (16) c	109 (7) c	944.5 (116.4) a	624 (7.1) b		
70°C	Ne	116 (34) a	Nd	113 (14) a	50 (11) b	136 (16) a		

[1] - Extraction temperature, unknown. [2]-Extraction temperature

Ne – No extraction at these conditions, Nd- Not determined due to inadequate sample
Ns- did not set at 10°C after 30 minutes, Nm- Not measurable due to high turbidity.

- Values in brackets represent standard deviation for triplicate samples
- Values in the same row followed by the same letter are not significantly different at $\alpha = 0.05$

Gel hardness was found to be higher for Nile perch skin than bone gelatins (Table 2.2.5), although the compression graphs for the two categories of gelatin were of similar shape (Figure 2.2.1). There was a high correlation ($r^2 = 0.98$) between gelatin Bloom gel strength and gel hardness. Hardness can therefore be used to compare the gel strengths of gelatins. The gelatins had a cohesiveness of 0.88 – 0.97 and a springiness of 0.94 – 1.0 (Table 2.2.5). There were no significant differences in cohesiveness and springiness between the Nile perch gelatins and bovine bone and commercial fish skin gelatins. The Nile perch bone gelatins however, gave significantly lower hardness, gumminess and chewiness when used in the same concentrations (6.67%) as the Nile perch skin and bovine bone and commercial fish gelatin. At concentrations corresponding to 225 g Bloom gel strength, there was no significant difference between the texture profile of Nile perch bone gelatins and that of the other gelatins tested. It seems therefore that Nile perch gelatins could be used to replace the other gelatins in some food applications without significantly altering the texture. Since melting point also determines the sensory quality of some food products in which gelatin is used (Choi & Regenstein, 2000), substitution of mammalian gelatins with fish gelatins cannot be based only on their texture profile.

Table 2.2.5: Texture profile of Nile perch, bovine bone and commercial fish gelatin

Source	Bloom (g)	Hardness (N)	Cohesiveness	Springiness	Gumminess (N)	Chewiness (N)
Young Nile perch skins	217	20.16 (1.96) a	0.93 (0.05) a	0.94 (0.03) a	18.72 (2.76) a	17.51 (1.15) a
Adult Nile perch skins	240	22.40 (2.40) a	0.94 (0.04) a	0.98 (0.04) a	21.09 (3.11) a	20.57 (3.09) a
Young Nile perch bones	163	17.51 (0.54) b	0.93 (0.01) a	0.96 (0.02) a	16.25 (0.74) b	15.62 (0.97) b
Young Nile perch bones	225*	24.68 (2.93) a	0.90 (0.05) a	0.95 (0.05) a	22.22 (2.88) a	21.09 (3.14) a
Adult Nile perch bones	150	16.51 (2.17) b	0.87 (0.04) a	0.98 (0.06) a	14.41 (0.21) b	14.13 (0.44) b
Adult Nile perch bones	225*	22.33 (2.20) a	0.90 (0.04) a	0.96 (0.03) a	20.09 (2.43) a	19.23 (2.74) a
Aquagel fish	216	20.07 (0.07) a	0.97 (0.05) a	0.99 (0.05) a	19.42 (1.05) a	19.51 (1.38) a
Bovine ossein	221	20.15 (2.47) a	0.94 (0.07) a	0.95 (0.03) a	18.97 (2.45) a	18.17 (2.87) a

*Calculated Bloom values based on dry ash free matter.

- Values in brackets represent standard deviation for triplicate samples
- Values in the same column followed by the same letter are not significantly different at $\alpha = 0.05$

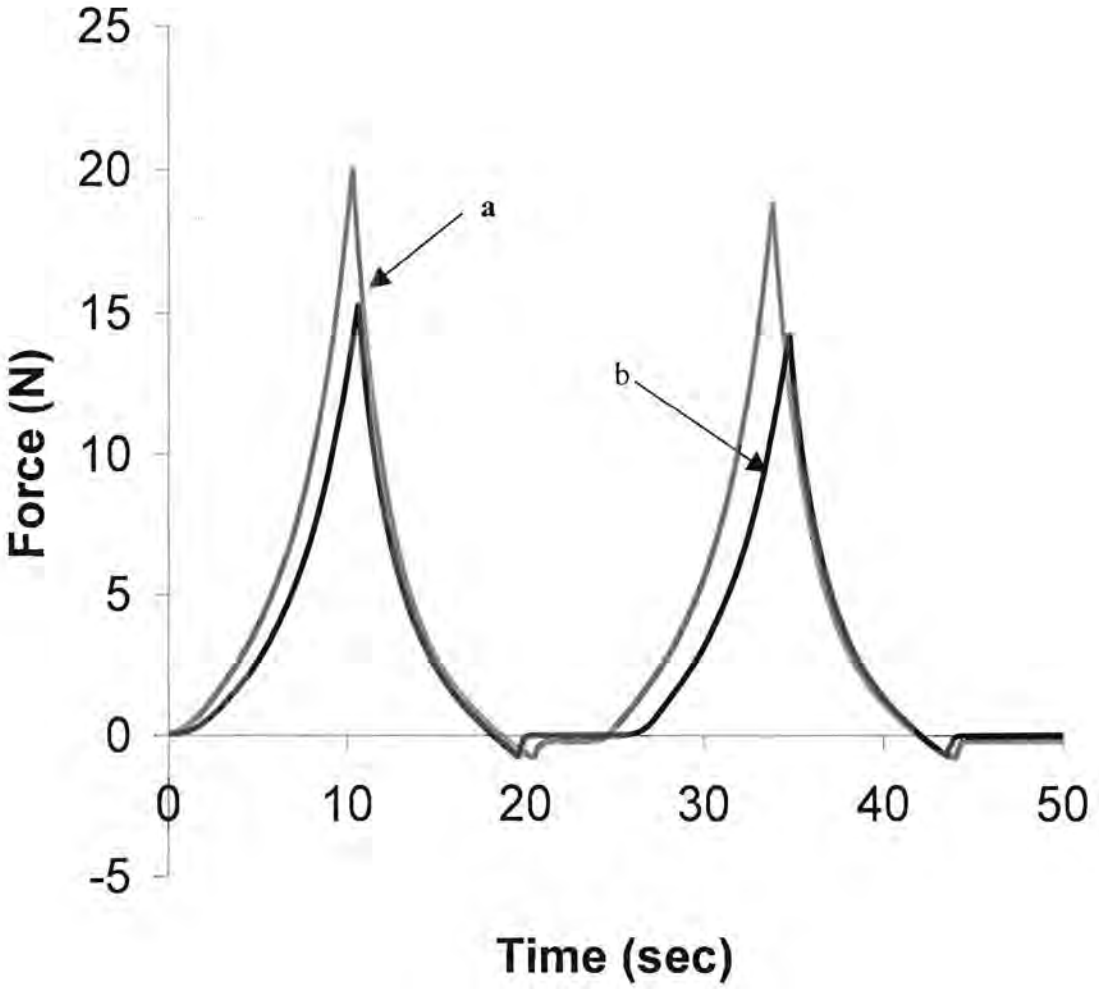

Figure 2.2.1: Typical texture profile for gels containing 6.67% of Nile perch skin (a) and bone (b) gelatin

2.2.4.8 Film properties

Nile perch skin gelatin films were found to exhibit film strength (stress at break) and % strain (% elongation at break) similar to that of bovine bone gelatin (Table 2.2.6). The Nile perch bone gelatin however, exhibited lower film strength and higher % elongation (Table 2.2.6 and Figure 2.2.2). The higher gel strength of Nile perch skin gelatins suggests a greater extent of protein entanglement in the fish skin than in the bone gelatin (De Graaf, 2000). It is not clear how differences in ash content of the gelatins may have impacted their film forming properties.

Table 2.2.6: Film properties of Nile perch skin and bone gelatin, bovine bone and commercial fish gelatin

Sample	Stress at Break (N/mm^2)	% Elongation at Break
Young Nile perch skin gelatin	21.4 (2.7) a	7.8 (2.2) b
Adult Nile perch skin gelatin	17.6 (4.4) a	11.9 (6.9) b
Young Nile perch bone gelatin	6.4 (1.6) b	39.2 (7.6) a
Adult Nile perch bone gelatin	6.9 (4.1) b	40.7 (11.8) a
Commercial fish gelatin	14.9 (4.1) a	24.5 (6.8) ab
Bovine bone gelatin	16.2 (1.6) a	12.2 (1.7) b

- Values in brackets represent standard deviation for triplicate samples
- Values in the same column followed by the same letter are not significantly different at $\alpha = 0.05$

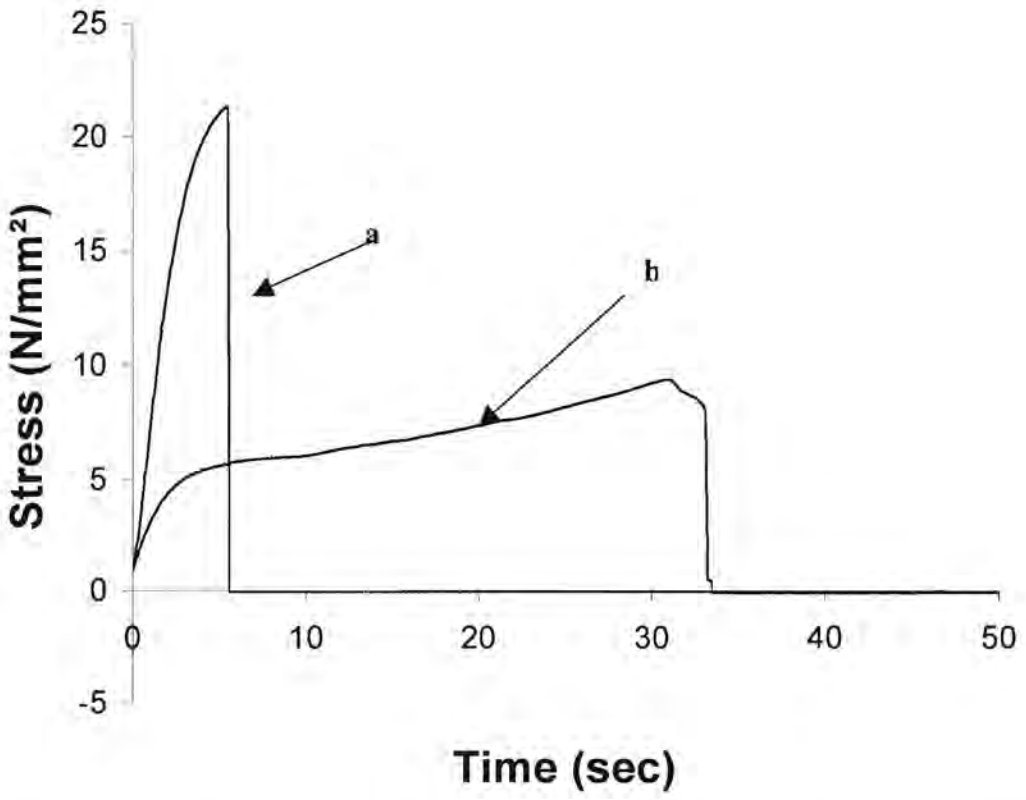

Figure 2.2.2: Typical stress - strain curves for films ~ 0.6 mm thick made by casting 5% gelatin solutions of Nile perch skin (a) and bone (b) gelatin in 0.5 M acetic acid, with 0.2 g glycerol per g of gelatin

2.2.4.9 Molecular weight distribution

SDS PAGE showed that the molecular weight distribution of Nile perch gelatins varied with the collagenous tissue used as raw material (Fig 2.2.3). Nile perch skin gelatins were generally found to contain higher proportions of the > β fraction than the Nile perch bone gelatins (Table 2.2.7). This is consistent with higher incidence and/or stability of crosslinks in the bone than in the skin collagen, resulting in more cleavage of peptide bonds during the manufacture of bone gelatins. It seems the thermal stable crosslinks are more resistant to cleavage than the collagen peptide bonds.

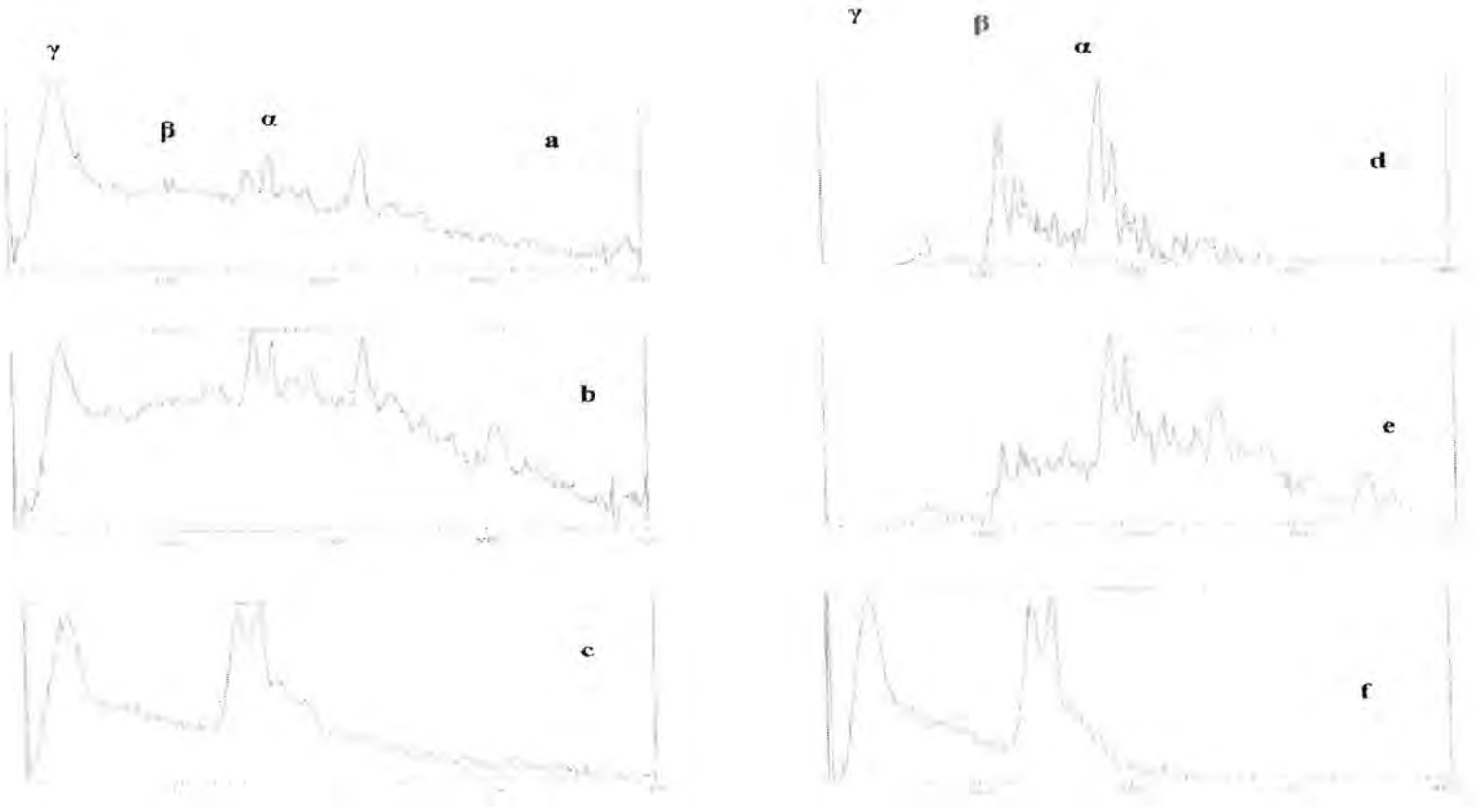

Figure 2.2.3: SDS PAGE densitograms for gelatins from skin of adult Nile perch (a), skins of young Nile perch (b), commercial fish gelatin (c), gelatin from bones of adult Nile perch (d), bones of young Nile perch (e) and bovine bones (f). Nile perch gelatins were extracted at 50°C. Extraction temperature for the other gelatins not known.

Table 2.2.7: Molecular weight distribution of gelatins obtained from different raw materials at varying extraction temperatures

Sample source (extraction temperature)	Proportion (%) of different fractions			
	> β	β - α	α	< α
Young Nile perch skin (50°C)	17.8 (2.9) b	25.9 (2.9) b	7.9 (1.0) c	48.4 (3.1) b
Adult Nile perch skin (50°C)	19.7 (3.9) b	25.4 (3.1) b	22.3 (2.8) b	32.6 (4.1) c
Adult Nile perch skin (60°C)	17.8 (4.2) b	34.7 (3.4) a	9.9 (1.1) c	37.6 (3.9) bc
Young Nile perch bone (50°C)	3.3 (0.7) cd	10.0 (1.4) d	28.4 (3.3) a	58.3 (6.7) ab
Young Nile perch bone (60°C)	2.9 (0.4) d	15.7 (2.1) c	18.8 (2.1) b	62.6 (7.1) a
Young Nile perch bone (70°C)	0.7 (0.3) e	4.1 (0.8) f	26.3 (2.1) a	70.0 (6.1) a
Adult Nile perch bone (50°C)	5.0 (1.0) c	37.5 (2.9) a	30.0 (4.1) a	27.5 (4.1) c
Adult Nile perch bone (60°C)	7.9 (2.0) c	11.3 (1.7) d	17.9 (2.1) b	63.0 (7.1) a
Adult Nile perch bone (70°C)	0.1 (0.0) e	8.3 (1.0) e	18.3 (2.1) b	73.5 (6.2) a
Bovine bone (U*)	28.8 (3.8) a	27.1 (3.1) b	29.7 (3.6) a	14.4 (1.9) d
Commercial fish skin (U*)	18.4 (2.0) b	29.5 (3.6) b	27.3 (3.1) a	24.0 (2.7) c

*U = Temperature of extraction unknown

- Values in brackets represent standard deviation for duplicate samples
- Values in the same column followed by the same letter are not significantly different at $\alpha = 0.05$

When gelatins from same raw materials were compared, later (higher temperature) extractions were found to contain more peptides (molecular weight less than α chain) and lower proportion of high molecular weight (greater than β) fractions than earlier (low temperature) extractions. The gelatins extracted from Nile perch skins at higher temperature (60 and 70°C) also exhibited lower gel strength, melting point, setting temperature and longer setting time (Table 2.2.4). The functional properties of the gelatins studied were correlated to the proportion of the different molecular weight fractions (Table 2.2.8). The lower content of high molecular weight fractions (> β) for bone gelatins was associated with lower viscosity, melting and setting point and longer setting time exhibited by these gelatins in comparison to the adult fish skin gelatins. The proportion of low molecular weight (< α) fraction (peptides) was higher for young than for the adult fish skin gelatin. This may be responsible for the lower viscosity, setting and melting temperature and longer setting time for the young fish skin gelatin. According to Tavernier (1989), high incidence of low molecular weight peptides is associated with low viscosity, melting point, setting point and high setting time. In this study, viscosity, Bloom, hardness and film strength had a high positive correlation ($r^2 > 0.7$) to the > β fraction, while the α fraction was highly positively correlated ($r^2 > 0.7$) to melting and setting temperature but negatively correlated ($r^2 <$ -0.7) to film strength. Viscosity was also highly negatively correlated ($r^2 = -0.79$) to < α fraction.

Despite the difference in the level of peptides, the gel strength was similar for gelatins from young and adult fish skins. Earlier studies (Koepff, 1984; Graesser, 1985) have also shown no simple correlation between gelatin gel strength and molecular weight distribution for high gel strength gelatins.

The low setting temperature, melting point, viscosity and very high setting time of young Nile perch skin gelatin may partly be due to the very wide molecular weight distribution exhibited by this gelatin. According to Yau, Kirkland and Bly (1979), wide molecular weight distribution negatively affects some functional properties of macromolecules.

The bovine bone and commercial fish skin gelatins were found to exhibit functional

properties quite similar to those of adult Nile perch skin gelatin, except for melting and setting temperature, which were higher for bovine bone gelatin. The difference between the properties of fish gelatin and bovine bone gelatin may partly be attributed to differences in molecular weight distribution. The bovine bone gelatin and the commercial fish skin gelatin were found to be lower in peptides smaller than α-chain (Table 2.2.7) and had densitograms (Figure 2.2.3) with fewer distinct fragments. Such densitograms are characteristic of alkali-processed gelatins (Koepff, 1984; Cole & Roberts, 1996). The bovine bone gelatin also had a much higher content of γ components.

Table 2.2.8: Correlation coefficients between % of different molecular weight fractions and some functional properties of gelatin

Functional property	Fraction			
	>β	β-α	α	<α
Bloom	0.81*	0.24	-0.02	-0.52
Viscosity	0.70*	0.38	0.61	-0.79*
Setting Temperature	0.45	0.20	0.71*	-0.61
Setting Time	-0.28	-0.09	-0.51	0.39
Melting Temp	0.41	0.07	0.71*	-0.51
Hardness	0.78*	-0.02	-0.41	-0.24
Film strength	0.75*	0.17	-0.74*	-0.15
Film elongation	-0.68	-0.13	0.67	0.13

- Values with asterisks were significant at $\alpha = 0.05$

2.2.4.10 Amino acid composition

The amino acid composition of Nile perch gelatins was found to be similar for all the Nile perch gelatins (Table 2.2.9). It would seem therefore that the bone and skin collagens have similar amino acid composition and that differences observed in the functional properties of gelatins were not due to differences in amino acid content.

The amino acid composition of Nile perch gelatins was, however, different from those reported for other species. Imino acid content of Nile perch gelatins (~ 21.5%) was

higher than ~ 17% reported for cod gelatin (Grossman & Bergman, 1992; Gudmunsson & Hafsteinsson, 1997) but lower than ~ 25% (Grossman & Bergman, 1992) reported for tilapia and ~ 30% for mammalian (Poppe, 1992) gelatins. The imino acid content of gelatins has a strong influence on their functional properties (Gilsenan & Ross-Murphy, 2000). Nile perch gelatins were found to contain imino acids at levels between those of mammalian gelatin and those of cold water fish species. The melting and setting temperatures of the gelatins were also found to lie between those reported for mammalian gelatin and those for gelatin from cold water fish species. It seems therefore, that imino acid content is a key determinant of these properties.

Table 2.2.9: Amino acid composition of gelatin from skins and bones of young and adult Nile perch

	Amino Acids g/100g Protein			
	Young fish skin gelatin	**Adult fish skin gelatin**	**Young fish bone gelatin**	**Adult fish bone gelatin**
Asp	5.26 (0.23)	5.29 (0.02)	4.67 (0.08)	5.17 (0.22)
Glu	9.41 (0.00)	9.41 (0.06)	9.41 (0.01)	9.42 (0.07)
Hyp	9.08 (0.02)	8.82 (0.04)	9.52 (0.02)	9.76 (0.05)
Ser	3.00 (0.01)	3.08 (0.01)	3.02 (0.02)	3.13 (0.02)
Gly	23.65 (0.01)	23.76 (0.04)	23.51 (0.15)	23.55 (0.15)
His	1.02 (0.01)	1.01 (0.01)	1.04 (0.03)	1.04 (0.04)
Arg	8.14 (0.04)	8.31 (0.03)	7.94 (0.10)	8.17 (0.07)
Thr	2.71 (0.04)	2.80 (0.01)	2.81 (0.04)	2.86 (0.03)
Ala	10.53 (0.10)	10.56 (0.02)	10.46 (0.03)	10.32 (0.15)
Pro	12.47 (0.15)	12.81 (0.10)	12.27 (0.03)	12.00 (0.26)
Tyr	0.55 (0.01)	0.55 (0.02)	0.60 (0.01)	0.62 (0.01)
Val	2.08 (0.02)	2.02 (0.01)	2.12 (0.01)	2.05 (0.02)
Met	1.74 (0.02)	1.32 (0.01)	1.75 (0.02)	1.45 (0.04)
Ile	0.98 (0.01)	0.95 (0.02)	1.11 (0.03)	1.00 (0.02)
Leu	2.28 (0.02)	2.21 (0.02)	2.40 (0.03)	2.30 (0.05)
Phe	2.09 (0.01)	2.09 (0.02)	2.24 (0.07)	2.15 (0.07)
Lys	3.60 (0.06)	3.56 (0.03)	3.43 (0.07)	3.58 (0.12)
Hyl	1.42 (0.04)	1.45 (0.10)	1.72 (0.01)	1.42 (0.11)
Hyp + Pro	21.55	21.63	21.79	21.76
%Hydroxylation				
Lys	24.96	28.94	33.40	28.4
Pro	42.14	40.78	43.69	44.85

- Values in brackets represent standard deviation for triplicate samples
- Values for the different gelatins were not significantly different at $\alpha = 0.05$

2.2.5 Conclusions

There are considerable differences between extractability and yield of gelatin from Nile perch skins and bones. Nile perch skin and bone gelatins also differ in their functional properties and molecular weight distribution. These properties also vary with age of source fish, although the influence of age is less pronounced. This supports earlier reports that fish collagen crosslinks do not appreciably mature into stable forms.

The differences in functional and chemical properties of Nile perch gelatin from different raw materials seem to arise from differences in the ease with which collagens in these materials can be transformed into gelatin. Nile perch skin collagen easily denatures to give gelatin at low temperature, even from adult fish. Nile perch bone collagen on the other hand requires a more severe heat treatment. As a result, Nile perch bone gelatin consists of a high proportion of low molecular weight fractions, which are associated with poor gelling properties.

Nile perch gelatins exhibit functional properties, which are more similar to mammalian gelatins than cold water fish skin gelatins. The superior functional properties of Nile perch gelatin compared to cold water fish gelatins may be explained by their higher content of imino acids. There is, therefore, a potential for exploitation of Nile perch processing waste for gelatin extraction. The potential is higher for Nile perch skins than bones because Nile perch skins give higher gelatin yield and the skin gelatin exhibits better functional properties than Nile perch bone gelatin.

2.2.6 Acknowledgements

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2.3 FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPIC STUDY OF ACID SOLUBLE COLLAGEN AND GELATIN FROM SKINS AND BONES OF YOUNG AND ADULT NILE PERCH*

Running title: FTIR spectroscopy of Nile perch gelatin

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2.3.1 Abstract

Fourier transform infrared (FTIR) spectroscopy was conducted on type A gelatins derived from skins and bones of young and adult Nile perch by a sequential extraction process. Spectra for gelatins were compared to each other and to that of acid soluble collagen from young Nile perch skins, in order to elucidate changes in protein secondary structure during collagen to gelatin transformation. The first gelatin extracts showed diminished amide III bands while the last gelatin extracts showed distinct amide III bands and their amide I bands consisted of a higher percent area of a component around 1690 cm^{-1}. The differences suggested that collagen to gelatin transition leads to loss of molecular order. The later gelatin extracts, exhibited higher molecular order than earlier gelatin extracts probably because the former contained surviving crosslinks or/and because renaturation of the low molecular weight gelatin fractions (later gelatin extracts) led to formation of more protein-protein linkages.

Key words: Nile perch, Gelatin, collagen, FTIR, protein structure

* Accepted by *Food Chemistry*.

2.3.2 Introduction

Fourier transform infrared (FTIR) spectroscopy has been used to study changes in the secondary structure of collagen and gelatin. It has been used to study collagen crosslinking (Paschalis, Verdelis, Doty, Boskey, Mendelesohn & Yamauchi, 2001), denaturation (Friess & Lee, 1996), thermal self assembly (Jakobsen, Brown, Hutson, Fink & Veis, 1983; George & Veis, 1991) as well as gelatin melting (Prystupa & Donald, 1996). The spectral changes which are indicative of changes in collagen secondary structure have been identified to include changes in the amide A (3300 – 3500) (Milch, 1964), amide I (1636 – 1661 cm^{-1}), amide II (1549 – 1558 cm^{-1}) (Renugopalakrishnan, Chandarakasan, Moore, Hutson, Berney & Ravejendra, 1989) and in the amide III (1200 - 1300 cm^{-1}) regions (Friess & Lee, 1996).

Fibrillogenesis (self assembly) of collagen has been found to be associated with broadening and a slight shift to lower wave number of the amide A peak (Milch, 1964), increase in intensity and slight shift to lower wave number of amide III peak (Jakobsen *et al.*, 1983), band broadening and shift of amide I peak to lower wave number (Jakobsen *et al.*, 1983; George & Veis, 1991; Prystupa & Donald, 1996) and shift of amide II peak to lower wave number (Jakobsen *et al.*, 1983; George & Veis, 1991). Shift of amide I, II and III peaks to lower wave numbers, increase in intensity of amide III and broadening of amide I are therefore associated with increased intermolecular interactions (by hydrogen bonding) in collagen.

Denaturation of collagen on the other hand has been found to lead to reduction in the intensity of amide A, I, II and III peaks (Friess & Lee (1996), narrowing of amide I band (Prystupa & Donald, 1996), increase in amide I component found around 1630 cm^{-1} and reduction in the intensity of amide I component found around 1660 cm^{-1} (George & Veis, 1991; Payne & Veis, 1988; Renugopalakrishnan *et al.*, 1989).

Prystupa and Donald (1996) studied gelatin melting and found it to be associated with reduction in the 1678 cm^{-1} peak and 1660/1690 cm^{-1} peak intensity ratio and increase in amide I components occurring around 1613, 1629 and 1645 cm^{-1}. These authors assigned the bands occurring at 1645 – 1657 cm^{-1} to random coils and the 1660 cm^{-1}

band to triple helix, with contribution from α-helix and β-turns. The amide I component at 1690 cm^{-1} has been attributed to helices of aggregated collagen-like peptides (Doyle, Bendit & Blout, 1975; Prystupa & Donald, 1996). According to Doyle *et al.* (1975) this peak vanishes with hydration of collagen or gelatin.

As animals age the extent of crosslinking of their collagen increases and the type of crosslinks change (Sims & Bailey, 1992; Bailey, Paul & Knott, 1998; Sims, Avery & Bailey, 2000; Hickman, Sims, Miles, Bailey, de Mari & Koopmans, 2000). According to Bailey *et al.* (1998) collagen from skins of immature animals mainly contain the intermediate crosslinks dehydroxylysinonorleucine (deHLNL) whereas collagen from bones of immature animals contain hydroxylysinoketonorleucine (HLKNL). These intermediate divalent crosslinks are respectively converted to the more stable trivalent histidinohydroxylysinonorleucine (HHL) and pyridolines (PYR) during maturation. It has been shown that differences in the quantities of the two types of crosslinks manifest in the amide I region of the FTIR spectra of collagenous tissue (Paschalis *et al.*, 2001). There is a positive correlation between the ratio of the components (1660/1690 cm^{-1}) and the relative abundance of PYR and HHL crosslinks.

Age-related increase in stability of collagen through increase in the amount and stability of crosslinks affect the stability of collagen to denaturation processes e.g. heat. Collagen with more extensive crosslinks e.g. those from mature bovine hide require a more severe process to break the crosslinks and allow collagen denaturation and solubilisation into gelatin (Reich, Walther & Stather, 1962). During such severe processes, more peptide bonds are broken but some intermolecular crosslinks survive. The triple helices of collagen from young animals are mainly held together by hydrogen bonds and Van der Waals forces. In such collagens, heat treatment mainly leads to breaking of hydrogen bonds and the triple helical structure is more likely to decompose mainly to intact alpha chains. It is not clear, however, whether the secondary structure of gelatins derived from young and old animals differ. In this study the FTIR spectra of gelatins derived from young and adult Nile perch skins and bones were determined and compared to those of acid soluble collagen from the same species, in an effort to elucidate changes in secondary structure that occur during the

conversion of collagen to gelatin. The first and last gelatin extracts from skins and bones of young and adult Nile perch were studied. Nile perch is a warm water fish species, with potential for giving gelatin with gelling properties more similar to mammalian gelatins than cold water fish species.

2.3.3 Materials and Methods

2.3.3.1 Preparation of acid soluble collagen

Acid soluble collagen was prepared from skins of young Nile perch (skin thickness < 0.4 mm) as described by Gòmez-Guillèn and Montero (2001). Briefly the method involved washing of the skins with chilled (~ 5°C) water for a period of 10 min. During this time, the skins were pressed intermittently by hand. The skins were then washed with 0.8 M NaCl for 3 periods of 10 min each followed by rinsing in running water after each wash with NaCl. Collagen was then extracted using 0.5 M acetic acid solution (1:20 w/v). The extraction was conducted for 16 hours, during which the skins were stirred intermittently. The viscous collagenous material was separated from the insoluble components by sieving through cheesecloth and collagen was precipitated using 0.9 M NaCl, washed with distilled water and freeze-dried.

An attempt was made to extract collagen from bones using 0.5 M acetic acid, but no collagen could be precipitated from the acetic acid liquor, after 5 days holding at room temperature.

2.3.3.2 Preparation of gelatins

The gelatins used in this study were derived from Nile perch skins and bones by the acid process. Gelatin was extracted from young (skin thickness < 0.4 mm and skeleton length < 40 cm) and adult (skin thickness > 1.5 mm and skeleton length > 95 cm) fish. Briefly, extraction of skin gelatin involved acidulation with concentrated sulphuric acid to a pH of 2.5 - 3.0 and maintaining this pH range throughout the swelling period (16 hours) by adding more acid solution. The skins were then transferred to beakers, covered with warm (~ 60°C) water and gelatin extracted in

water baths at 50°, 60° and 70°C, in a sequential process. In the case of young fish skins, extraction was conducted at only 50 and 60°C, because after the 60°C extraction, the residue left was very small and would give very small amounts of gelatin at 70°C. The gelatin extracts (light liquors) were filtered through compressed cotton wool. The light liquor concentrations were determined by evaporating duplicate 10 ml portions to a stable weight (48 hours at 105°C) and the concentration was used in calculation of % gelatin extractability as follows:

$$\frac{\text{Amount of gelatin extracted at a given temp}}{\text{sum of gelatin extracted at all temp}} \times 100\% = \%\ \text{gelatin extractability at a given temp}$$

The light liquors were then passed through a column of activated carbon (GRC 22, BHT water treatment, Chloorkop, South Africa) at a rate of ~ 5 bed volumes per hour. The pH of the light liquors was adjusted to ~ 5.0 using 5% ammonia solution and the gelatin extract was dried in a cross-flow air drier at 42°C, until brittle. The brittle sheets were broken into small pieces and milled using a domestic coffee grinder to pass through a 1 mm mesh sieve.

Bones used for gelatin extraction were cleaned, by scraping with a knife, to reduce the flesh contamination. They were then degreased by tumbling in warm (35°C) water and demineralised using 3% HCl at room temperature (20 - 25°C) for a period of 9 - 12 days, with the liquor changed after every three days, until the bones did not have any hard cores. The demineralised bones were then treated in the same way as the acidulated skins. The extractability and Bloom of the gelatins are presented in Table 2.3.1.

Table 2.3.1: Source, extractability and Bloom of gelatins used

Source	Extraction temperature (°C)	Extractability (%)	Bloom (g)
Fish skin gelatins			
Adult fish	50	70.0	240
Young fish	50	86.5	217
Adult fish	70	10.6	134
Young fish	60	12.9	0
Fish bone gelatins			
Adult fish	50	33.0	84
Young fish	50	33.3	156
Adult fish	70	9.6	155
Young fish	70	22.6	0

2.3.3.3 Fourier transform infrared spectroscopy

Fourier transform spectroscopy was conducted at the Department of Chemistry, University of Pretoria. FTIR spectra were obtained from discs containing 2 mg sample in approximately 100 mg potassium bromide (KBr). All spectra were obtained using a Bruker infrared spectrophotometer (Bruker Instruments, Billerica, MA, USA) from 4000 to 500 cm^{-1} at data acquisition rate of 2 cm^{-1} per point. Background was subtracted using the Opus software (Bruker Instruments, Billerica, MA). Triplicate samples of collagen and gelatins were analysed and spectra for the triplicate runs averaged. Fourier self deconvolution was conducted on the average spectra for the amide I band, using a resolution enhancement factor of 1.8 and full height band width of 13 cm^{-1}. The self deconvolution provided information on the number and location of components. Curve fitting was then performed using peakfit software (SPSS Inc., Chicago, IL, USA).

2.3.4 Results and Discussions

The frequencies at which major peaks occurred for acid soluble collagen and the different gelatins and collagens are summarised in Table 2.3.2.

Table 2.3.2: FTIR spectra peak position and assignments for Nile perch skin and bone gelatins

Region	Peak Wave Number cm^{-1}									Assignment	Reference
	ASC	YS(50)	AS(50)	YS(60)	AS(70)	YB(50)	AB(50)	YB(70)	AB(70)		
Amide A	3434	3623	3648	3411	3404	3421	3456	3310	3478	NH stretch, coupled with HB	Sai & Babu (2001)
-	2924	2923	2924	DM	2923	2924	DM	Sh	Sh	CH_2 asymmetrical Stretch	Abe & Krimm (1972)
-	2853	2853	2853			2853	DM			CH_2 symmetrical Stretch	Abe & Krimm (1972)
-	2355	2355	2356			2355	DM				
Amide I	1650	1648	1650	1654	1653	1647	1644	1656	1652	C=O stretch/HB coupled with COO-	Jackson *et al.* (1995)
Amide II	1542	DM	1541	1542	1541	1558	DM	1544	1540	NH bend coupled with CN stretch	Jackson *et al.* (1995)
-	1457	1458	1457	1452	1451	DM	1457	1451	1450	CH_2 bend	Jackson *et al.* (1995)
-		DM		DM	1335		1402	1335		C H_2 wagging of proline	Jackson *et al.* (1995)
Amide III	1235	1234	DM	DM	1240			1243	1236	NH bend	Jackson *et al.* (1995)
-		1026	1011		1082	1122	1107		1127	C-O stretch	Jackson *et al.* (1995)
-	871	863	867				1006	1082	1076	Skeletal stretch	Abe & Krimm (1972)
-	670	670	660	669	670	870	866		874	Skeletal stretch	Abe & Krimm (1972)
-						670		701	671		

ASC – Young Nile perch skins acid soluble collagen, YS – Gelatin extracted from young fish skins, AS - Gelatin extracted from adult fish skins,
YB – Gelatin extracted from young fish bones, AB - Gelatin extracted from adult fish bones.
Numbers in brackets represent extraction temperature (°C) for the gelatin,
Sh - Peak appearing as shoulder
- No common name for the spectral region
DM - Diminished peak
HB – Hydrogen bonding

2.3.4.1 Spectra for skin gelatins

Gelatins derived from young fish skins at 50°C exhibited spectra very similar to those for gelatins derived from adult fish skins at the same temperature (Figure 2.3.1), but quite different from those extracted at higher temperature (70°C for the adult and 60°C for the young fish skins) and from those of acid soluble collagen. Compared to the spectra for acid soluble collagen, the low temperature extracted gelatins showed lower intensity amide I and II bands and the amide III band was almost non-existent. These changes are indicative of greater disorder (Friess & Lee, 1996) in gelatin and are associated with loss of triple helix state. This is consistent with changes expected as a result of denaturation of collagen to gelatin. The gelatin extracted at the higher temperatures, however, exhibited distinct amide III peaks. It seems therefore, that the extent of order in the high temperature extracted gelatins may be higher than that in low temperature extracted gelatins.

The gelatins extracted at higher temperature exhibited a much broader amide A than observed for the low temperature extracted gelatins and for acid soluble collagen. The amide A band in the high temperature extracted gelatins was infact merged with the CH_2 stretching band expected to occur at around 2930 cm^{-1}. According to Kemp (1987) amide A tends to merge with the CH_2 stretch peak when carboxylic acid groups exist in stable dimeric (intermolecular) associations. It seems therefore, that there are more associated components in the high temperature extracted gelatins. The high temperature extracted gelatins consist mainly of low molecular weight peptides and according to Ledward (1986) gelling of low molecular weight gelatin fractions entails more protein-protein linkages than for high molecular weight gelatins. During drying therefore, it seems the low molecular weight, high temperature extracted gelatin fractions renatured slowly, forming a network with more protein-protein linkages than the high molecular weight low temperature extracts.

It is also possible that the high temperature extracted gelatins contain some covalent intermolecular bonds (surviving crosslinks) since it is derived from the most crosslinked collagen, after the less crosslinked collagen is extracted during earlier (low temperature) extractions. The stable intermolecular crosslinks may not break during extraction of gelatin. Instead, solubilisation may be achieved by cleavage of

peptide bonds. As a result, the high temperature extracted gelatin may contain a significant amount of intermolecular crosslinks. This may produce FTIR spectra showing a higher degree of molecular order. Paschalis *et al.* (2001) isolated stable (PYR and HHL) crosslinks from bovine bone gelatin, supporting the assertion that intermolecular crosslinks may survive the process of gelatin extraction.

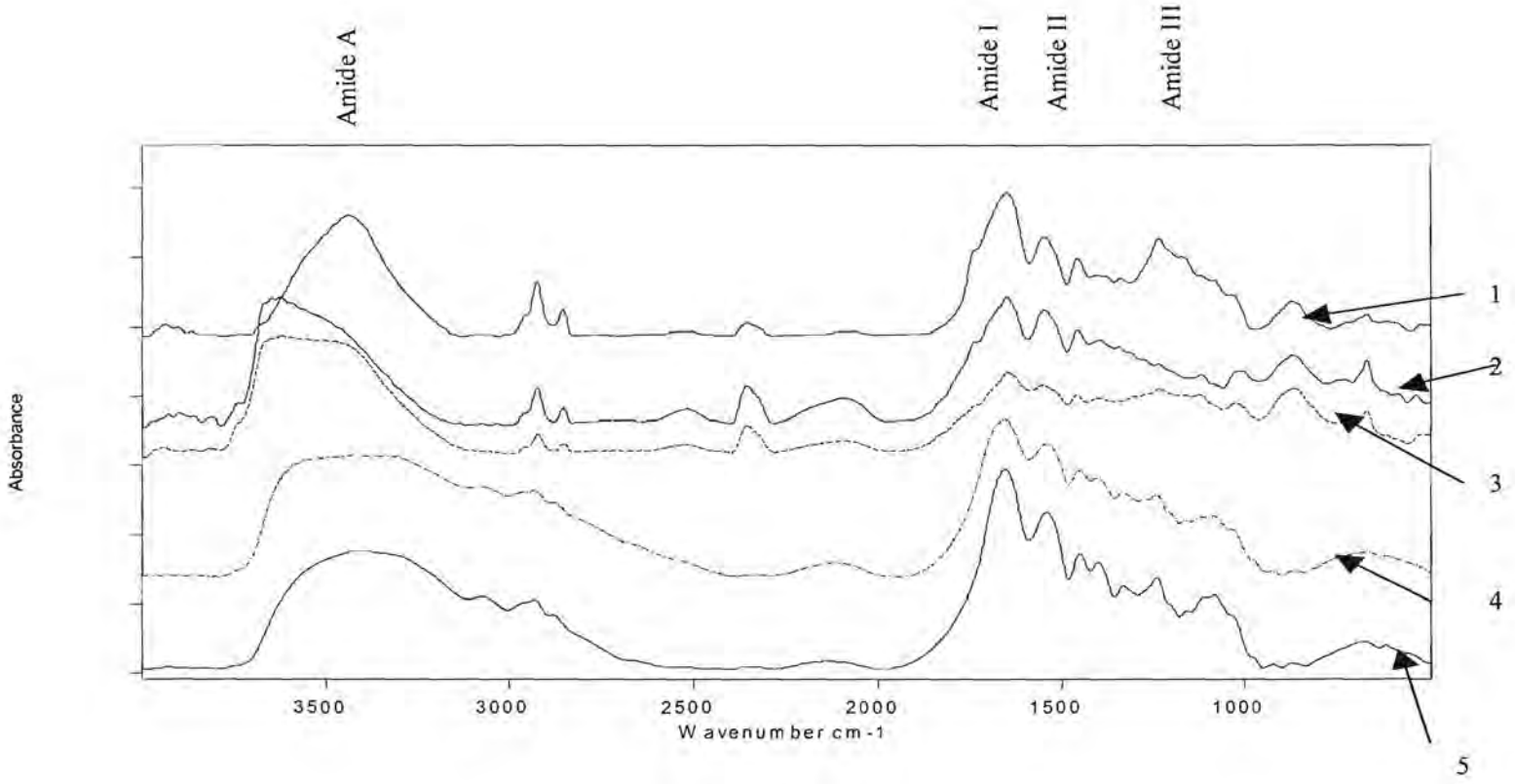


Fig 2.3.1: FTIR spectra for young Nile perch skin acid soluble collagen (1), adult Nile perch skin gelatin extracted at 50°C (2), young Nile perch skin gelatin extracted at 50°C (3), young Nile perch skin gelatin extracted at 60°C (4) and adult Nile perch skin gelatin extracted at 70°C (5).

2.3.4.2 Spectra for bone gelatins

The spectra exhibited by bone gelatins differed from those exhibited by acid soluble collagen and skin gelatins (Figures 2.3.2). The amide I peaks in the bone gelatins were at lower frequencies than for acid soluble collagen. There were also differences in the amide III region. The 50°C extracted Nile perch gelatins basically did not show absorption peaks in this region while the 70°C extracted gelatins showed peaks. Nile perch bone gelatins also exhibited sizeable peaks between 1000 and 1100 cm^{-1}. Absorption in this region is attributed to C-O vibration due to carbohydrates (Jackson, Choo, Watson, Halliday & Mantsch, 1995). Carbohydrates in collagen are associated with glycation of collagen (Bailey *et al.*, 1998) and carbohydrates are required in the formation of pentosidine crosslinks (Kent, Light, & Bailey, 1985). It seems Nile perch bone gelatins are more likely to contain pentosidine crosslinks than Nile perch skin gelatins and acid soluble collagen. Cole (1995) reported presence of pentosidine crosslinks in bovine hide collagen but studies on fish skin collagen with hydrothermal isometric tension show that they do not contain substantial amounts of stable crosslinks, such as pentosidine crosslinks, even at advanced age (Cohen-Solal, Le Lous, Allain, & Meunier, 1981). Hickman *et al.* (2000) reported different types of crosslinks in fish swim bladder collagen. The stable crosslinks reported included HHL and PYR but these were in concentrations of less than 10% those reported for bovine collagen.

Differences in the amide III region of the bone gelatins compared to acid soluble collagen and skin gelatins are worthy of note, since the intensity of the amide III band has been associated with the triple helical structure. The high temperature (70°C) extracted bone gelatins were found to exhibit low intensity peaks at around 1240 cm^{-1}. These peaks were not observed in the low temperature (50°C) extracted gelatins. It seems, similar to the case of skin gelatins, the 70°C extracted bone gelatins had more intermolecular associations than the 50°C extracted gelatins.

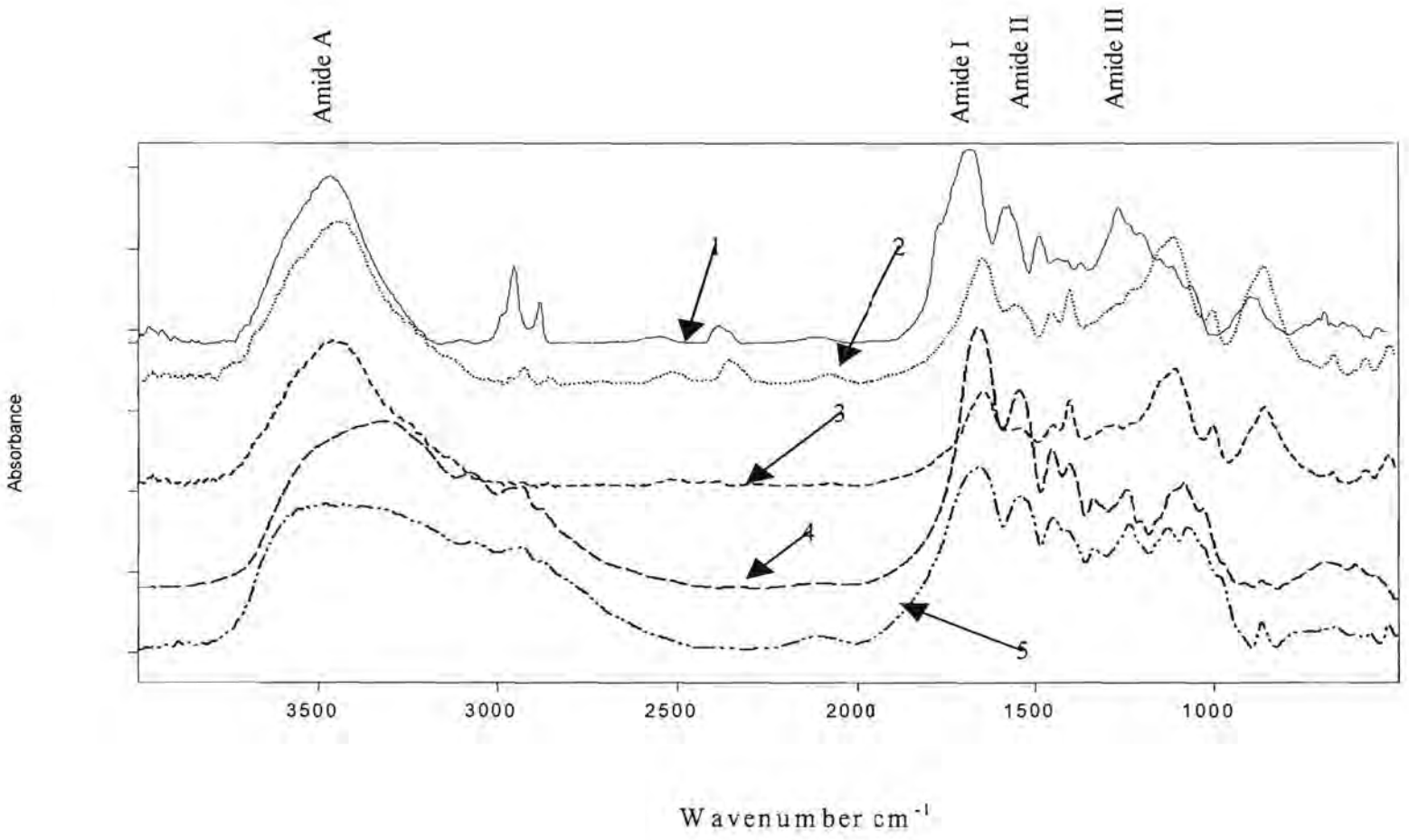


Fig 2.3.2: FTIR spectra for young Nile perch skin acid soluble collagen (1), gelatin from young (2) and adult (3) Nile perch bones extracted at 50°C and from young (4) and adult (5) Nile perch bones extracted at 70°C.

2.3.4.3 Amide I band components for Nile perch skin and bone gelatin

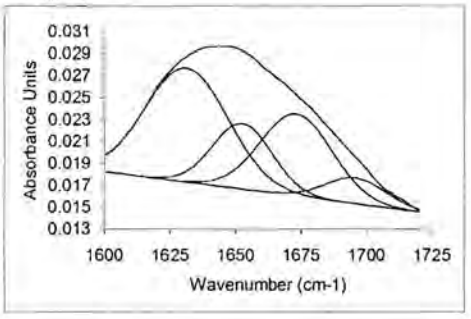
The amide I band between 1600 and 1700 cm^{-1} is the most useful for infrared spectroscopic analysis of the secondary structure of proteins (Surewicz & Mantsch, 1988). Deconvolution of the amide I band showed the band to consist of four components. The component peaks, their location and % areas are shown in Figure 2.3.3 and Table 2.3.3.

Table 2.3.3: Location and percent area contribution of amide I components for Nile perch skin and bone gelatin and skin acid soluble collagen

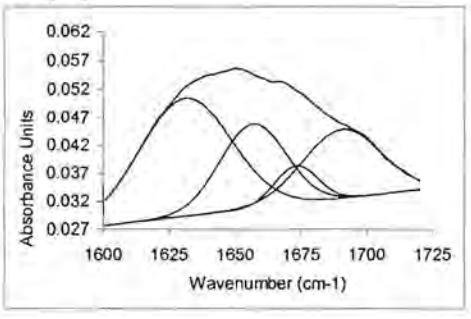
Material	Component peak location (cm^{-1}) and percent area (in brackets) contribution of total band			
	1	2	3	4
Young fish skin acid soluble collagen	1637 (69.0)	1652 (1.8)	1672 (16.7)	1696 (12.6)
Adult fish skin gelatin 50°C	1634 (49.6)	1652 (10.9)	1674 (30.4)	1699 (9.2)
Adult fish skin gelatin 70°C	1631 (18.8)	1658 (50.8)	1674 (2.1)	1690 (28.3)
Young fish skin gelatin 50°C	1633 (45.1)	1652 (18.7)	1674 (25.2)	1697 (10.9)
Young fish skin gelatin 60°C	1633 (32.9)	1657 (23.8)	1675 (8.4)	1694 (35.0)
Adult fish bone gelatin 50°C	1632 (44.8)	1652 (19.0)	1673 (28.8)	1695 (7.4)
Adult fish Bone gelatin 70°C	1631 (45.4)	1657 (24.4)	1673 (7.1)	1690 (23.1)
Young fish bone gelatin 50°C	1633 (49.2)	1651 (15.0)	1674 (26.0)	1699 (9.8)
Young fish bone gelatin 70°C	1631 (31.5)	1658(31.8)	1672 (1.8)	1688 (34.9)

- Figures derived from average spectra for triplicate determinations
- Fit quality (r^2) between original and fitted spectra ≥ 0.9998

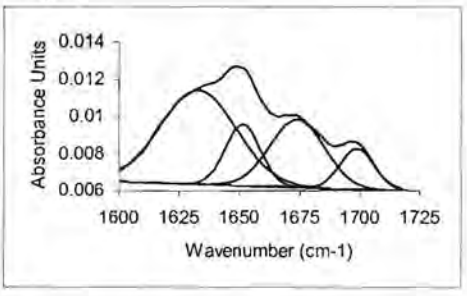
AB (50)



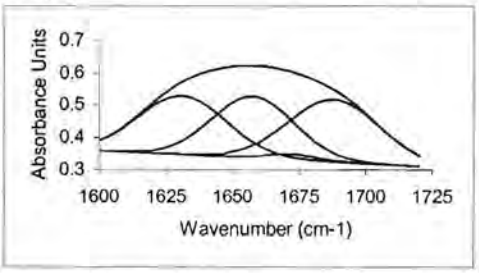
AB (70)



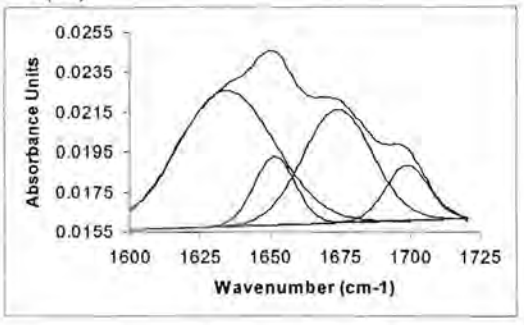
YB (50)



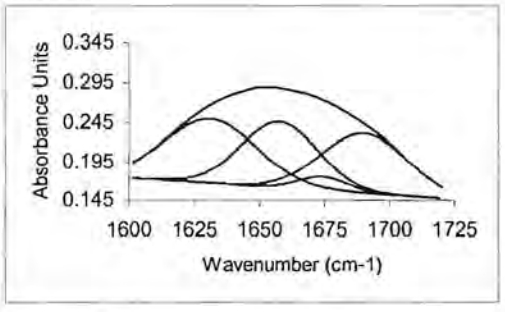
YB (70)



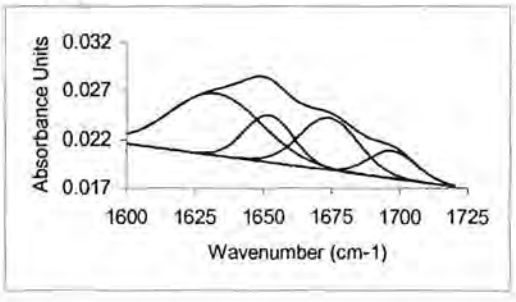
AS (50)



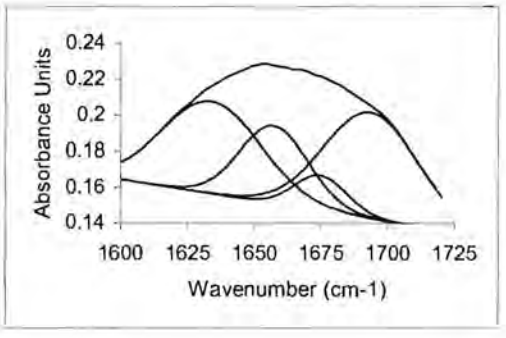
AS (70)



YS (50)



YS (60)



ASC

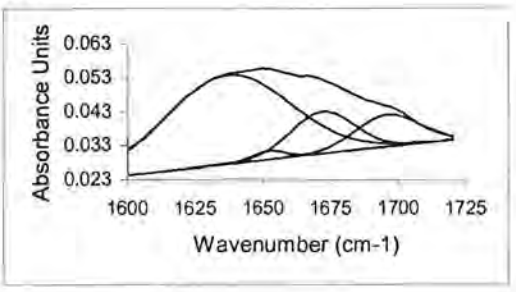


Fig 2.3.3: Amide I band for Nile perch gelatins and collagens with fitted band components

ASC – Young Nile perch skin acid soluble collagen, AB - Gelatin extracted from adult fish bones, YB – Gelatin extracted from young fish bones, AS - Gelatin extracted from adult fish skins, YS – Gelatin extracted from young fish skins. Numbers in brackets represent extraction temperature for the gelatin.

In agreement with Byler and Susi (1986), it is clear from Table 2.3.3 that protein segments with similar structures do not necessarily show band components with the same frequencies. Overall, the variation in frequencies for particular band components in this investigation was not very different from that reported by Byler and Susi (1986). They observed a variation of approximately 15 cm^{-1} for frequencies attributable to β-structures of various proteins.

Quantitative band-fitting analysis of amide I band areas, as applied in this investigation, has proved useful in studying the nature and the extent of protein conformational changes (Surewicz & Mantsch, 1988). Using this method, good correlations have been found for secondary structure estimates obtained by X-ray data and from infrared analysis (Byler & Susi, 1986; Surewicz & Mantsch, 1988).

One major observation in the amide I band components in this study was the consistently higher % area contributed by the 1690 cm^{-1} component for the higher temperature extracted gelatins. In addition, the 1690 cm^{-1} component occurred at lower wave numbers in the higher temperature extracted gelatins than in their low temperature extracted counterparts, while the 1650 cm^{-1} component occurred at lower wave numbers for the low temperature extracted gelatins than their high temperature extracted counterparts. An amide I component at around 1690 cm^{-1} has been reported for gelatin (Payne & Veis, 1988; Prystupa & Donald, 1996; Paschalis *et al.*, 2001) and collagen-like peptides (Doyle *et al.*, 1975) and has been attributed to intermolecular associations. The bands around 1630, 1650 and 1675 cm^{-1} have been assigned to imide residues (and partly to β-sheet), random coils and β-turns respectively (Prystupa & Donald, 1996) while the helical state is reported to show at 1660 cm^{-1} (Payne & Veis, 1988; George & Veis, 1991). The 70°C extracted gelatins, however, had their component peaks showing at 1657 - 1658 cm^{-1}. The corresponding peaks were found at 1651 – 1652 cm^{-1} for 50°C extracted gelatins. These differences may be suggestive of differences in the secondary structure of these gelatins. As earlier proposed, it seems, the 70°C extracted gelatins contain a higher degree of molecular order than the 50°C extracted gelatins, probably due to protein-protein linkages formed during drying of these low molecular weight gelatins. Based on their high content of the 1650 and 1675 cm^{-1} components, the 50°C extracted dry gelatins seem to be made up, predominantly of random coils and β-turns.

The differences between bone and skin gelatins extracted at the same temperature may be due to structural differences between bone and skin collagens from the same species. Sims *et al.* (2000) reported that the two types of tissue have different types of crosslinks.

2.3.5 Conclusions

FTIR spectroscopy showed that conversion of collagen to gelatin leads to loss in the triple helical structure and decrease in molecular order. The extent of these changes, in the case of Nile perch seem to be affected by the order (in a sequential extraction process) of gelatin extraction and the collagenous tissue from which gelatin is

extracted. The secondary structure of gelatin obtained from the same raw material by sequential extractions may vary, with later extraction (higher temperature) containing more intermolecular associations in the dry state. The early extractions are obtained from the least crosslinked collagen. Due to the relatively milder extraction temperature, peptide hydrolysis is not expected to be extensive and higher molecular weight gelatin fractions are produced. During drying, these form some protein-protein linkages but these are not likely to be many. On the other hand later extracts are obtained from the more crosslinked collagen and contain more low molecular weight fractions. These are likely to form more protein-protein linkages which manifest as higher molecular order.

2.3.6 Acknowledgements

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3 DISCUSSION

3.1 DISCUSSION OF METHODS USED

3.1.1 Gelatin manufacture process and quality of resulting Nile perch gelatin

Two general methods are used in the manufacture of gelatin (Hinterwaldner, 1977a; Johnston-Banks, 1990; Poppe, 1992). The first method entails alkali pre-treatment of the raw material for up to 12 weeks followed by extraction in acidic media at temperatures ranging from 45 to 90°C. This technique results in type B gelatin and is suitable for raw materials that contain substantial quantities of heat stable intermolecular collagen crosslinks. It is used in the manufacture of gelatin from adult bovine hides and from bones. Alkali pre-treatment preferentially breaks covalent intermolecular crosslinks (Grand & Stainsby, 1975), making it easier to solubilise gelatin at relatively low temperature. This technique generally takes long (up to 12 weeks) and is avoided for raw materials that contain small quantities of stable intermolecular crosslinks such as pigskins and fish skins.

The second technique involves a short acid conditioning, followed by washing out some of the acid and extraction in acidic aqueous media. This technique gives type A gelatin. It is applicable to raw materials that are low in stable intermolecular crosslinks and has been widely applied to pigskins and fish skins. It has also been used for extraction of gelatin from ossein. Extraction of gelatin from bones is generally preceded by acid leaching of ash (Hinterwaldner, 1977b). This is because bones contain substantial amounts of ash, which would otherwise contaminate the gelatin. Ash also makes it difficult to extract gelatin from bones. Leaching temperature and acid concentration affect the extent of collagen loss during the leaching process. The difference in yield between bones leached at refrigeration temperature and those leached at room temperature (Table 2.2.2) in this study may be due to excessive loss of organic matter in the latter process. The acid concentrations used normally range from 3 to 6%, with the lower concentrations normally used when leaching temperature is high.

In this investigation, type A process was chosen for extraction of gelatin because fish collagen has been reported to contain minimal quantities of stable intermolecular crosslinks (Cohen-Solal *et al.*, 1981; Hickman *et al.*, 2000). Judging from the extractability of gelatin obtained in this investigation, this process was generally adequate for extraction of gelatin from Nile perch skins, regardless of the age of the source animal. The extractability of Nile perch bone gelatin was, however, low. It may be desirable to test type B process for the extraction of gelatin from Nile perch bones.

Elimination of the fishy odour has been a major concern in fish gelatin manufacture processes. The process commonly used to eliminate fishy odour from fish gelatin involves washing fish skins several times with acid and alkali (Grossman & Bergman, 1992; Holzer, 1996). This process is not only expensive but also time consuming, tedious and leads to plenty of effluent. In this study, it was shown that activated carbon eliminates fishy odour, eliminating the need for the numerous washes. One advantage with activated carbon is that a single lot can be used on several thousand times its volume of gelatin solution and the carbon can be regenerated, if necessary, by volatising the adsorbed odorous compounds.

It is important that gelatin does not exhibit high colour intensity or turbidity as these may negatively affect the usefulness of gelatin in some food products. Gelatins derived from bovine hides of adult cattle tend to have a darker colour than gelatin from calfskins, bones and pigskins. This has been attributed to presence of pentosidine crosslinks in collagen of adult bovine hide (Cole, 1995). Formation of pentosidine crosslinks entails Maillard reaction and therefore results in dark products. Industrial processing of gelatin sometimes entails bleaching of the gelatin using sulphur dioxide or hydrogen peroxide. In this study, the colour of the gelatins derived from Nile perch without bleaching, were found to be within the range reported for bovine hide gelatins (Cole & Roberts, 1996) and no differences in colour were observed between gelatins derived from skins or bones from young and adult fish. It was therefore not necessary to include a bleaching step during the manufacture of gelatin from Nile perch skins and bones and colour is not likely to be an obstacle to utilisation of Nile perch bones and skins in the manufacture of gelatin.

Turbidity is largely dependent on efficiency of the clarification (filtration) process as well as the isoionic point of the gelatin. The commercial process of gelatin manufacture often entails filtration of both the light and the heavy (concentrated) liquors. The heavy liquor filtration eliminates particles that precipitate as a result of concentration. This leads to further improvement of gelatin turbidity. The process used in the manufacture of gelatin in this study involved only a single filtration process, conducted on the light liquor. It was not possible to conduct a second filtration because the light liquor was dried directly, without an initial concentration step. The range of turbidity values recorded in this study (20 – 944 NTU) was very wide. Higher values may have resulted from inadequate filtration. To ensure low turbidity it may be advisable to always use the double filtration process.

Gelatin ash and fat content are also important quality parameters. Fat is generally eliminated during the numerous washes and during the bone degreasing process. Generally, the gelatin samples in this study were almost free (< 0.5%) of fat. This showed that the processes used eliminated fat as desired. The skin gelatins were generally low in ash, with most having ash content lower than the recommended maximum of 2.6% (Jones, 1977). The cleaning process before extraction of gelatin from fish skins significantly reduces the amount of scales which would otherwise contribute to high ash levels in fish skin gelatin. The bone gelatins, however, had much higher ash content (most in the range 3 - 10%). Manufacture of fish bone gelatin may therefore, require an ion exchange step or alternative steps such as electro-dialysis to remove the salts.

3.1.2 Methods for determination of gelatin functional properties

To facilitate comparison of gelatins from different sources, standard industrial methods have been developed for the analysis of the functional properties of gelatin. These were used in this study. These methods strictly define how samples should be treated before determination of the functional properties. In the determination of gel strength for example, BSI (1975) defines the temperature and duration of maturation as 10 ± 0.1°C and 16 – 18 hours, respectively and the concentration of gelatin as

6.67%. Choi and Regenstein (2000) demonstrated that differences in maturation times have significant effects on the Bloom and melting point values recorded. Gelatin concentration (Choi & Regenstein, 2000) and maturation temperature (Michon *et al.*, 1997; Choi & Regenstein, 2000; Arnesen & Gildberg, 2002) have also been shown to significantly affect the values recorded for functional properties of gelatin. Efforts were made, therefore, to ensure that these factors are strictly controlled when measuring functional properties. Since the actual concentration of protein in a solution containing the same amount of gelatin sample varies with the purity (protein content) of the gelatin, efforts have been made to develop formulae which allow comparison of gelatins varying in purity. For gel strength, gelatins may be compared based on corrected Bloom. This takes into account the non-protein content of the gelatin. Corrected Bloom can be derived from the formula;

$$\text{Corrected Bloom} = \text{Bloom}_m \times (87.5/(100 - \text{Moisture\%} - \text{Ash\%}))^2$$

Where Bloom_m is the measured Bloom

(Cole & McGill, 1988; Pye, 1996)

The correction of other factors for non-protein material is however, less straight forward, partly because the non-protein solid (ash) may also contribute to properties such as viscosity, film properties, melting point and even setting temperature and time.

3.1.3 Methods for chemical analysis of gelatin

3.1.3.1 SDS PAGE

SDS PAGE with densitometric analysis has been recognized as a reliable, simple and inexpensive technique for the determination of molecular weight distribution of collagen and gelatin (Koepff, 1984; Ming-Zhi, *et al.*, 1989; Chalepakis *et al.*, 1985; Hayashi *et al.*, 1990; Norland, 1990; Reddy *et al.*, 1993; Cole & Roberts, 1996; Gómez-Guillén *et al.*, 2001). Globular proteins, commonly used as molecular weight markers, however, give erroneous results when used to estimate molecular weight of collagenous proteins. In this study, globular proteins and commercial Type I calfskin

acid soluble collagen were used as molecular weight markers. The mobility of α-chains was found to correspond to a molecular weight of about 120,000 Da, when globular proteins were used as molecular weight markers. This is much higher than the true molecular weight (approximately 95,000 Da) determined by sedimentation (Piez, 1967). Other authors (Kubo *et al.*, 1982; Mizuta *et al.*, 2002a; Mizuta *et al.*, 2002b; Mizuta *et al.*, 2003) have also reported this anomaly and this has been attributed to collagen's high content of the relatively small and hydrophobic amino acid residues, glycine, proline and alanine (Noelken *et al.*, 1981). These amino acids tend to cling together due to their hydrophobic side chain and this may hinder neutralisation of their positive charge by SDS. In this study, use of acid soluble collagen, alongside globular protein markers allowed interpretation of the molecular weight relative to γ, β and α chains and facilitated comparison of molecular weight distribution of different samples.

3.1.3.2 Amino acid analysis

Amino acid composition of collagens and gelatins was determined by reversed phase high performance liquid chromatography following hydrolysis and phenylthiocarbomyl (PTC) derivitisation as described by Bidlingmeyer *et al.* (1984). This technique has been reported to be rapid, efficient, sensitive and is specific for both primary and secondary amino acids in protein hydrolysates. The method uses ultraviolet detection and analysis time is only 12 minutes. Its sensitivity is up to concentrations in the order of pmols.

Several other approaches can be used for amino acid analysis of proteins. Separation and quantitation of amino acids in most of the techniques for amino acid analysis is achieved by chromatographic methods (Blau, 1981; Sarwar *et al.*, 1983; Bildlingmeyer *et al.*, 1984; Miller, Juritz, Barlow & Wessels, 1989). Amino acid analysis based on ion exchange chromatography was developed by the Nobel laureates Moore and Stein in the 1950s. This has facilitated automation and rapid amino acid analysis (Jakubke & Jeschkeit, 1977; Tristram & Rattenburg, 1981; Bildlingmeyer *et al.*, 1984). The earlier methods used in amino acid analysis by ion exchange chromatography involved separation on a sulfonated cation-exchange resin using a series of buffers as eluents (Bildlingmeyer *et al.*, 1984). Detection of the

separated amino acids was done by colorimetry via a post-column reaction with ninhydrin. Later, conventional liquid chromatographs were configured specifically for amino acid analysis. In these approaches, the post-column derivitisation required special reaction chambers as part of the chromatographic system. This limited the apparatus to amino acid analysis and the ion exchange separation required approximately one hour. Pre-column derivitisation was developed to overcome these limitations (Bidlingmeyer *et al.*, 1984). It involves derivatisation of amino acids before separation. Pre-column derivatisation, especially with reversed-phase columns, offers greater efficiency, ease of use and higher speed of analysis than conventional ion-exchange techniques. Dansyl (Dns), phenylthiohydantoin (PTH), o-phthalaldehyde (OPA) and phenylisothiocynate (PITC) are among the reagents that have been used for pre-column derivatisation. Dns and PTH derivatisation while rapid, efficient and sensitive suffers from poor derivative stability. PTH also gives less than quantitative yields while Dns shows reagent interference peaks. OPA on the other hand does not react with secondary (modified) amino acids.

3.1.3.3 Fourier transform infrared (FTIR) spectroscopy

Infrared spectroscopy is one of the widely used techniques for estimating the secondary structure of proteins (Ambrose & Elliot, 1951). Compared to X-ray crystallography, FTIR spectroscopy is quite simple and yet it has now been developed to give quantitative information of the amounts of the different secondary structure conformations. While nuclear magnetic resonance (NMR) spectroscopy is widely used for the study of protein secondary structure, interpretation of NMR spectroscopy results for large proteins is complex and the technique is currently limited to study of small (< 30,000 Da) proteins (Haris & Severcan, 1999). Circular dichroism (CD) spectroscopy, on the other hand, only provides qualitative information on the different protein conformations (Darby & Creighton, 1993).

FTIR spectroscopy requires small quantities of sample (~2 mg per test) and can be conducted both on solid (in form of pellets prepared by mixing sample with KBr) and aqueous samples. The FTIR spectra obtained from pellets of Nile perch collagen and gelatin with KBr, in this study, were quite similar to spectra obtained in other studies of collagen in solution (Payne & Veis, 1988; Friess & Lee, 1996). One difference,

however, was the observation of an amide I component at around 1690 cm^{-1}. This component was also observed by Doyle *et al.* (1975) who reported its disappearance with hydration and related its intensity to intermolecular associations. Use of KBr discs may, therefore, provide additional information, over FTIR in aqueous environments, with regard to intermolecular associations.

3.2 DISCUSSION OF STUDY RESULTS

Low imino acid content is associated with poor gelling properties of gelatin and low thermal stability of collagen. It has been reported that the imino acid content of fish collagens increases with the environmental temperature at which the fish grows (Rigby, 1968; Mathews, 1975; Kimura *et al.,* 1988). There are limited studies on warm water fish collagens and these indicate that collagen from skins of warm water fish species contain more imino acids than collagen from cold water fish species. Warm water fish species have also been found to give gelatin of superior gelling properties compared to gelatin from cold water fish species (Grossman & Bergman, 1992; Gilsenan & Ross-Murphy, 2000; Sarabia *et al.,* 2000; Gómez-Guillén *et al.*, 2002; Jamilah & Harvinder, 2002). Many warm water fish species have, however, not been studied with respect to their gelatin making properties. In this study, Nile perch, a warm water fish species was used as a source of gelatin and collagen and the chemical and functional properties were determined to test conformity to the reported trend in imino acid content and gelatin functional properties. Additionally, it was deemed necessary to determine the potential of fish bones as a source of gelatin and to determine if the age of the fish influenced the extractability and properties of gelatin. It was hypothesised that Lake Victorian Nile perch (*Lates niloticus*) collagen, like collagen from other warm water fish species contains high levels of imino acids and that gelatin derived from the collagenous tissues of Nile perch would exhibit gelling properties superior to those of cold water fish species. Since imino acid content of collagen also affects its thermal stability, it was anticipated that Nile perch collagen would exhibit higher thermal stability than collagen from cold water fish species.

Amino acid analysis showed Nile perch collagen to contain higher levels of imino acids (20%) than collagen from cold water fish species, which contain on average 17% imino acids (Rigby, 1968; Grossman & Bergman, 1992; Gudmundsson &

Hafsteinsson, 1997). The total imino acid content of Nile perch collagen was among the highest reported for fish. Tilapia gelatin was reported to contain 25% imino acids (Gross & Bergman, 1992)

The thermal denaturation temperature (Td) for Nile perch collagen was determined to be about 36°C. This is higher than the values reported for temperate fish species. Collagen denaturation temperature has been reported for Alaska pollack (16.8°C) (Kimura & Ohno, 1987), muscle of carp (32.5°C), eel (29.3°C), common mackerel (26.1°C), chum salmon (19.4°C) (Kimura *et al.*, 1988), Japanese seabass (30°C), skip jack tuna (29.7°C) and ayu (29.7°C) (Nagai & Suzuki, 2000).

The high imino acid content and denaturation temperature of Nile perch collagen, in comparison to cold water fish species is in agreement with observations by Rigby (1968) that thermal stability of collagen increases with imino acid content. The collagen and gelatin were found to contain no tryptophan and cysteine, and were low in methionine, tyrosine and histidine, like other type I collagens (Balian & Bowes, 1977; Grossman & Bergman, 1992; Gudmundsson & Hafsteinsson, 1997; Yoshida *et al.*, 2001).

Electrophoresis (SDS PAGE) showed that both young and adult Nile perch skin acid soluble collagen consisted of α chains plus their dimers. The α components showed two distinct species varying in their mobility, for both reducing and non-reducing conditions. It could be concluded therefore, that Nile perch acid soluble collagen is made up of at least 2 α species (α1 and α2). The electrophoretic pattern of Nile perch skin collagen was similar to that of type I calfskin collagen.

The α2 species was the minor component of the two species. Based on the electrophoretic profile, it seems Nile perch collagen exists as $(\alpha 1)_2\alpha 2$ trimers (i.e. two chains of α1 and one chain of α2) . Skins of some fish species, such as cod (Piez, 1965) and trout (Saito *et al.*, 2001) have been reported to contain α1, α2 and α3 species. The α3 species contains cysteine and migrates slightly slower than the α1 species during SDS PAGE under reducing conditions. The electrophoretic pattern of Nile perch collagen, however, showed only two α species, even under reducing

conditions. In addition, no cysteine was detected in the collagen, suggesting that Nile perch skin collagen is made up exclusively of α1 and α2 species. This is typical of type I collagen, which is the dominant collagen in dermal tissue (Bailey & Light, 1989; Bailey *et al.*, 1998).

Nile perch skin collagen from both young and adult fish showed high solubility in 0.5 M acetic acid. This was indicative of low content of stable collagen crosslinks, since stable crosslinks are resistant to cleavage by dilute acid and when they are present in collagen, they make it insoluble. Earlier work (Cohen-Solal *et al.*, 1981; Hickman *et al*, 2000) provided evidence that fish collagen does not develop appreciable amounts of stable crosslinks with age. This was further supported by the observation that acid soluble collagen from the young and old Nile perch skins exhibited basically similar denaturation curves, with a denaturation temperature of about 36°C. However, hardly any Nile perch bone collagen could be solubilised by 0.5 M acetic acid. It would seem therefore that Nile perch bone collagen contain more stable crosslinks than skin collagen.

According to Sims *et al.* (2000), collagen from bones and skins vary significantly in the type and quantities of crosslinks and as animals age, the quantities and types of collagen crosslinks change. Collagen from skins of immature animals mainly contain the intermediate crosslinks dehydroxylysinonorleucine (deHLNL) whereas collagen from bones of immature animals contain hydroxylysinoketonorleucine (HLKNL). HLKNL is stable to dilute acids, which explains the insolubility of bone collagen even in infancy (Bailey *et al.*, 1998). The intermediate divalent crosslinks, deHLNL and HLKNL are respectively converted to the more stable trivalent histidinohydroxylysinonorleucine (HHL) and pyridolines (PYR) during maturation. This explains the increase in stability of collagens to heat and dissolving agents with age. The PYR crosslinks are more stable to heat than the HHL crosslinks (Bailey *et al.*, 1998) and therefore bone collagens are more resistant to denaturation by heat than skin collagens.

FTIR spectra for acid soluble collagen from young and adult fish skins were, however, found to differ slightly, indicating some differences in the secondary

structure of the two. The amide I and amide II peaks were at a lower frequency for the young fish skin (1650 and 1542 cm^{-1}, respectively) compared to adult fish skin (1654 and 1555 cm^{-1}, respectively) collagen. Based on the location of the amide I and amide II peaks, it seemed that the ASC from the young skins had a lower degree of molecular order, since decrease in molecular order is associated with shift of these peaks to lower wave numbers (Payne & Veis, 1988). It seemed therefore, that there were more intermolecular bonds in the adult fish collagen. Amide I components showed adult Nile perch acid soluble collagen amide I band to consist of a higher proportion of the component around 1690 cm^{-1} than the young fish ASC. This band is linked to the extent of intermolecular interactions in collagen and collagen-like peptides (Doyle *et al.*, 1975; Prystupa & Donald, 1996; Paschalis *et al.*, 2001). The other noticeable difference was the lower intensity of the component with peak around 1650 cm^{-1} in young fish ASC. This component has been attributed to random coils (Prystupa & Ronald, 1996), suggesting a lower extent of unwinding of the triple helix and less random coils in the young fish ASC. It seemed therefore, that adult fish ASC retained more intermolecular crosslinks during solubilisation with acetic acid but the triple helical structure, normally held together by intramolecular hydrogen bonds (Darby & Creighton, 1993) was extensively destroyed. The young fish ASC on the other hand, because of its lower content of stable intermolecular bonds could be solubilised more easily and retained triple helices to a greater extent. The apparently minimal differences in the extent of collagen crosslinking with age were therefore reflected in differences in the FTIR spectra of the collagens.

Earlier studies have shown that the extent of collagen crosslinking differs between bones and skins and increases with age (Sims *et al.*, 2000). It was hypothesised that the differences between bone and skin collagens would lead to differences in gelatin extractability and functional properties. Additionally, it was hypothesised that gelatin from skins and bones of fish of different ages would also vary in their extractability and functional properties.

Low temperature (50°) extractability of gelatin was expected to be higher for Nile perch skins if the skin collagen was less crosslinked than the bone collagen. The results of this study showed skins to give markedly higher low temperature gelatin

extractability than bones. This confirmed assertions (Bailey *et al.*, 1998; Sims *et al.*, 2000) that the bone collagen contains more stable crosslinks than skin collagens. However, there were no pronounced age-related differences in extractability of gelatin from either Nile perch bones or skins. This confirmed earlier reports (Cohen-Solal *et al.*, 1981; Hickman *et al.*, 2000) that fish collagen crosslinks do not substantially mature to the stable forms, since gelatin extractability reduces with increase in amounts of stable crosslinks. Low temperature (3 - 5°) leaching led to increase in yield from Nile perch bones. This was probably due to reduction in organic matter loss. However, the resulting gelatins were of poorer functional properties (gel strength and setting properties) than gelatins derived from bones leached at ambient temperature. The reason for this was not established but it was probably due to higher rentention of non-collagenous proteins during the cold leaching process. It may therefore be necessary to consider deboning of the fish bones before gelatine extraction.

It has been suggested that the pyrrolidine rings of imino acids give collagen a rigid structure, allowing only restricted rotation about bonds adjacent to the pyrrolidine rings (Piez & Gross, 1960). This ensures close proximity of adjacent chains and facilitates inter-chain hydrogen bonds and Van der Waals attractions. Hydroxyproline is also believed to cause stabilisation of collagen, by involvement in intra-molecular hydrogen bonding (Darby & Creighton, 1993) or some form of inductive effect (Holmgrem *et al.*, 1998). During gelling, gelatin essentially undergoes partial renaturation into collagen-like structures. Imino acid content of gelatin determines the strength of the renatured structures and is therefore a key determinant of the functional properties of gelatin.

The potential of most fish species as sources of gelatin to substitute mammalian gelatins is limited, mainly by the poor gelling properties of their gelatins. The poor gelling properties of fish gelatins have been attributed to their low content of the imino acids proline and hydroxyproline (Eastoe & Leach, 1977). This study showed that the imino acid content of Nile perch gelatin was higher than that reported for cold water fish species. This confirms earlier reports (Rigby, 1968; Grossman & Bergman, 1992) that collagen and gelatin derived from warm water fish species contain higher

levels of imino acids than those from cold water fish species. The general amino acid composition was found not to vary with age or between bone and skin gelatins. Apparently, amino acid composition seems independent of age. Eastoe and Leach (1958) gave the amino acid composition for collagen from bones and skins of mammals, showing that collagens derived from bones and skins of the same animal generally have similar amino acid compositions.

Alongside the high imino acid content, Nile perch gelatins were found to exhibit functional properties quite similar to those of mammalian gelatins and superior to those reported for gelatin from cold water fish species. This supported the assertion (Gilsenan & Ross-Murphy, 2000; Gòmez-Guillèn *et al.*, 2002) that imino acid content has a strong influence on the functional properties of gelatin. The problem of low gel strength and generally poor gelling properties is, therefore, not universal to fish gelatins, but limited to cold water fish species. This is in agreement with the report by Grossman and Bergman (1992) of high gel strength (263 g Bloom) for tilapia gelatin.

It should be noted, however, that while the amino acid composition of the different Nile perch gelatins studied was basically similar, the functional properties varied. The gelatins extracted from Nile perch skins at 50°C generally exhibited higher gel strength (222 and 229 g, respectively for young and adult fish) than corresponding bone gelatins (179 and 134 g, respectively for young and adult fish bones leached at room temperature). Gelatin extracted from skins of young and adult Nile perch at higher temperature exhibited lower gel strength but this was not always the case for bone gelatins. Gelatin extracted from adult fish skins also exhibited higher viscosity (42.3 mSt) than bone gelatins (28.2 and 30.0 mSt, respectively for young and adult fish). There was no significant difference, however, between the viscosity of young fish skin gelatin and the bone gelatins. The setting and melting temperatures were found to be similar for adult fish skin and the bone gelatins but lower for young fish skin gelatin, while setting time was lowest for adult fish skin gelatin, lower for the bone gelatins and highest for the young fish gelatins. Generally, the adult fish skin gelatins exhibited superior gelling properties to the other gelatins.

Gel hardness was found to be higher for Nile perch skin than bone gelatins, although

the compression graphs for the two categories of gelatin were similar. There were no significant differences in cohesiveness and springiness between the Nile perch gelatins, bovine bone gelatin and commercial fish skin gelatin. The Nile perch bone gelatins however, gave significantly lower hardness, gumminess and chewiness when used in the same concentrations (6.67%) as the Nile perch skin and commercial gelatins. At concentrations corresponding to 225 g Bloom gel strength, there was no significant difference between the texture profile of Nile perch bone gelatins and that of the other gelatins tested. It seems therefore that Nile perch gelatins could be used to replace the other gelatins in some food applications without significantly altering the texture. Nile perch gelatins, however, exhibited lower melting points than bovine bone gelatin. This may affect the sensory profile of food products made with fish gelatin for example low melting point of fish gelatins has been associated with better flavour release when the gelatins are used in gelled food products (Choi & Regenstein, 2000).

Nile perch skin gelatin films were found to exhibit film strength and % strain at break similar to that of bovine ossein gelatin. The Nile perch bone gelatin however, exhibited lower film strength and higher % elongation. It seems the film properties follow the same pattern as gel strength. According to De Graaf (2000), film strength of proteins increases with increase in protein entanglement (formation of network). Based on the higher film strength of Nile perch skin gelatins, it seems these exhibited more entanglements than the lower molecular weight bone gelatins. Bigi and colleagues (Bigi *et al.*, 2001; Bigi, Cojazzi, Panzavolta, Roveri & Rubini, 2002) demonstrated that crosslinking of gelatin leads to increase in film strength and reduction in percent elongation, showing that the higher the proportion of high molecular weight fractions of the gelatin, the higher the film strength and the lower the elongation. This is in agreement with the trend recorded in this study.

Evidently, the functional properties of Nile perch gelatin depend on other factors in addition to amino acid composition. The molecular weight distribution of gelatin seemed to be a key determinant for most functional properties. Table 3.1 below shows the proposed relationships between the functional and chemical properties of Nile perch gelatin.

Table 3.1 Summary of the proposed relationship between functional and chemical properties of Nile perch gelatin

Functional property	Related chemical properties	Relationship	Determinant of chemical property
Gel strength	Imino acid content	Gel strength increases with imino acid content	Species from which gelatin is extracted
	Molecular weight distribution	Gel strength increases with fraction of molecular weight between α and γ	• Content of crosslinks in source collagen • Severity of extraction process
	Crosslinks content	Gel strength reduces with increase in intramolecular crosslinks	• Content of crosslinks in source collagen • Order of extraction
Viscosity	Imino acid content	Viscosity increases with imino acid content	Species from which gelatin is extracted
	Molecular weight distribution	Viscosity increases with high molecular weight (>β) fraction and decreases with the content of low molecular weight (<α) peptides	• Content of crosslinks in source collagen • Severity of extraction process
Melting temperature	Imino acid content	Melting temperature increases with imino acid content	Species from which gelatin is extracted
	Molecular weight distribution	Melting temperature decreases with gelatin content of <α fraction and increases with α fraction	• Content of crosslinks in source collagen • Severity of extraction process
Setting temperature	Imino acid content	Setting temperature increases with imino acid content	Species from which gelatin is extracted
	Molecular weight distribution	Setting temperature decreases with gelatin content of <α fraction and increases with α	• Content of crosslinks in source collagen • Severity of extraction process
Setting time	Imino acid content	Setting time reduces with increase in imino acid content	Species from which gelatin is extracted
	Molecular weight distribution	Setting time increases with gelatin content of <α fraction	• Content of crosslinks in source collagen • Severity of extraction process
Film strength	Molecular weight distribution	Film strength increases with fraction of molecular weight between α and γ	• Content of crosslinks in source collagen • Severity of extraction process
Film elongation	Molecular weight distribution	Film elongation increases with fraction <α	• Content of crosslinks in source collagen • Severity of extraction process

Electrophoresis showed that the molecular weight distribution of Nile perch gelatins varied with the collagenous tissue used as raw material. Skin gelatins were generally found to contain higher proportions of the high molecular weight (> β) fraction than Nile perch bone gelatins. When collagen contains few intermolecular crosslinks, its conversion to gelatin is associated with minimal cleavage of peptide bonds, since solubilisation is easily achieved by cleavage of the weak intermolecular forces (hydrogen bonds, Vander Waals forces and intermediate crosslinks). As a result, the gelatin contains a high proportion of intact α-chains as well as dimers and oligomers of α-chain. When collagen contains a high concentration of stable intermolecular crosslinks, more peptide bonds are broken to facilitate solubilisation, resulting in gelatin with a lower concentration of high molecular weight fractions. Figure 3.1 demonstrates the conversion of collagen to gelatin in both crosslinked and monomeric collagens. Nile perch bone collagen, because of its high content of stable crosslinks, even at a young age, is likely to have undergone more peptide bond hydrolysis during its conversion to gelatin than the skin collagen, resulting in gelatin with a higher content of low molecular weight peptides.

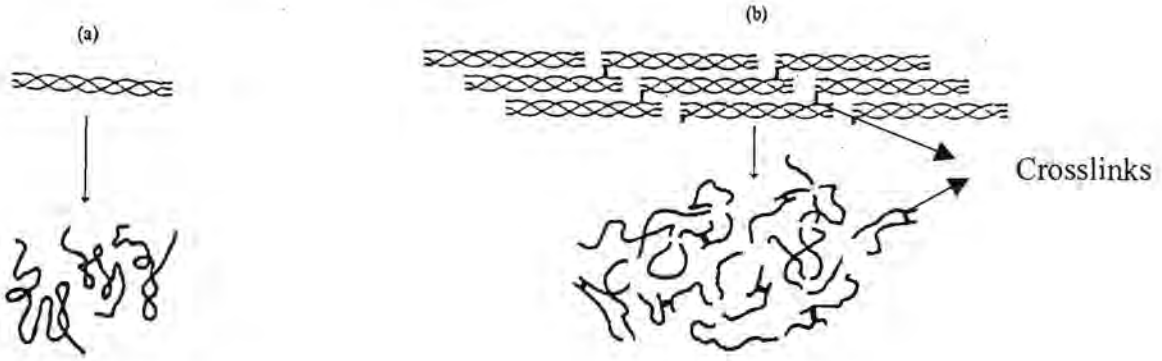

Figure 3.1: Schematic representing proposed changes occurring during the conversion of collagen to gelatin. a) monomeric collagen; b) crosslinked collagen. Adapted from Bailey (1991).

According to Ledward (1986), gelation is associated with formation of intermolecular junctions (linkages) and the number of junctions required to form a gel depends on the molecular weight distribution of the gelatin used. The gelatin fractions of low molecular weight require more junctions to form gels and result in weaker gels than gelatin fractions of higher molecular weight. Koepff (1984) observed that the gel phase of gelatin is made up mainly of the high molecular weight molecules while the low molecular weight (smaller than α) molecules tend to stay in the sol phase.

The functional properties of gelatin are also affected by the types of junctions (linkages) formed during renaturation of gelatin. Ledward (1986) observed that the junctions that contribute most to gelatin physical properties such as gel strength are those that form between adjacent molecules (inter-chain junctions), while those formed between different parts of the same molecule (intra-chain junctions) do not contribute significantly to these properties. When gelatin contains a high proportion of intermolecular crosslinks, it is more likely to form intra-chain junctions (Figure 3.2) and therefore to exhibit poorer functional properties.

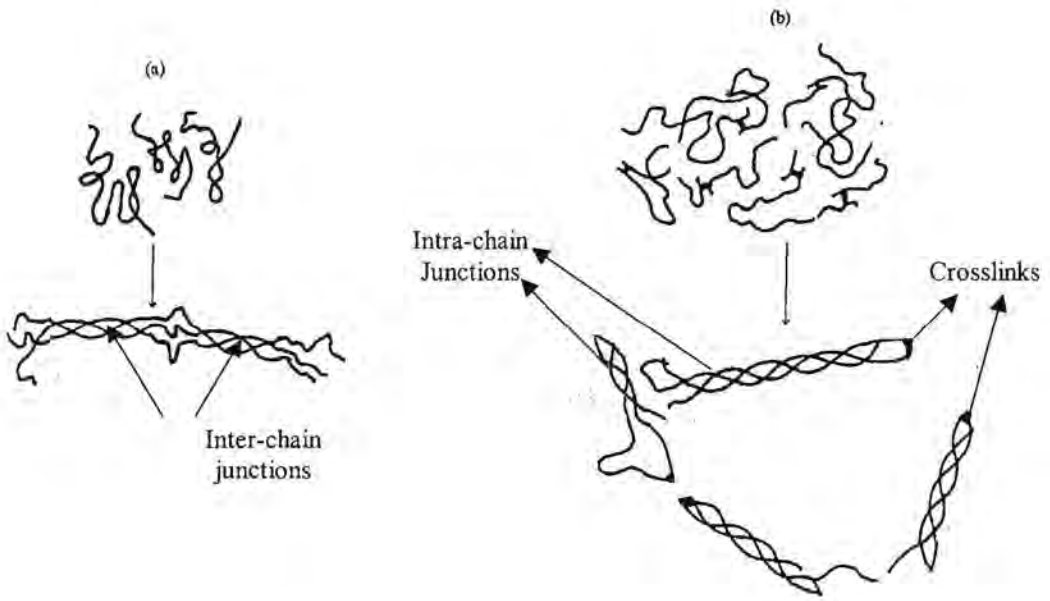

Figure 3.2: Schematic representing proposed changes during gelation of gelatin derived from a) gelatin without intermolecular crosslinks; b) gelatin containing a high concentration of intermolecular crosslinks (Adapted from Bailey, 1991).

Bone gelatins had a higher content of low molecular weight protein fractions and formed weaker gels as shown by their lower gel strength and gel hardness. Films made from bone gelatins also had lower tensile strength and exhibited higher % elongation at break. It seems that during film formation, there is also formation of protein-protein linkages between gelatin molecules and the lower molecular weight gelatin fractions require more of such junctions, leading to a weaker network, which stretches and breaks easily. This was supported by results from FTIR spectroscopy. FTIR spectra of gelatins seemed to vary with the collagenous tissue from which the gelatin was extracted and with the order of extraction. The FTIR spectroscopy study led to the proposition that the secondary structure of gelatin obtained from the same raw material by sequential extractions may vary, with later extractions containing more intermolecular associations in the dry state. The first gelatin extractions were obtained from the least crosslinked collagen and consisted largely of high molecular weight protein fractions. Later extractions are obtained from the more crosslinked

collagen that could not be solubilised by the mild first extraction. During drying of the gelatin extracts, the first gelatin extracts (made up predominantly of high molecular weight fractions) seem to have formed fewer protein-protein linkages than later extracts obtained from the more crosslinked collagen and containing more low molecular weight fractions. Because of the higher content of stable crosslinks in bone collagen, the bone gelatins are likely to have contained more intermolecular crosslinks and therefore were more likely to form intra-chain linkage than the skin gelatins.

When the molecular weight distribution of gelatins from the same raw materials were compared, later (higher temperature) extractions were found to contain more low molecular weight peptides and lower proportion of α chains than earlier (low temperature) extractions. The gelatins extracted from adult Nile perch skins at higher temperatures also exhibited lower gel strength, melting point, setting temperature and longer setting time. The proportion of low molecular weight (< α) fraction was higher for young than for the adult fish skin gelatin. Reich *et al* (1962) observed an increase in viscosity and yield with animal age for gelatin derived from bovine hides and pigskins, until the age of 36 and 28 weeks, respectively. Thereafter, gelatin viscosity dropped with age. Gelatin yield was related to animal age in the same way as viscosity. Gelatin viscosity has been reported to increase with the proportion of high molecular weight fraction and to reduce with the amount of low molecular weight fraction (Tavernier, 1989). The high incidence of low (< α) molecular weight peptides may therefore be responsible for the lower viscosity, setting and melting temperature and longer setting time observed for the young Nile perch skin gelatin. In the case of Nile perch, the gelatin from the adult fish skins exhibited higher viscosity, setting and melting temperature and shorter setting time than gelatin from young Nile perch fish skins but similar gel strength. Adult fish skin also gave higher yield. It seems from this observation, that the age-related changes in collagen, which are responsible for lower yield and viscosity for bovine hide and pigskin gelatin also occur in Nile perch but are less pronounced in the latter. This is consistent with reports by Hickman *et al* (2000) that the crosslinks found in young fish collagens are retained to a large extent and only a minor proportion of these crosslinks mature to stable forms.

The functional properties of the gelatins studied were found to be correlated to the proportion of the different molecular weight fractions. Viscosity, Bloom, hardness and film strength had a high positive correlation ($r^2 > 0.7$) to the > β fraction. This is in accord with observations by Graesser, Koepff and Tomka (1983) that high molecular weight gelatin protein fractions have a higher propensity to form gels than the lower molecular weight fractions. The Viscosity was also highly negatively correlated ($r^2 = -0.79$) to < α fraction. High incidence of peptides has been associated with low viscosity, melting point, setting point and high setting time (Koepff, 1984; Tavernier, 1989). Generally, low molecular weight peptides did not contribute positively to the functional properties of gelatins. Low molecular weight peptides arise from extensive collagen hydrolysis, either due to the severity of the extraction or because of a high degree of collagen crosslinking. It may be desirable, in the case of highly crosslinked collagen to break some of the collagen crosslinks, for example by alkali pre-treatment before gelatin extraction.

Despite the difference in the levels of high and low molecular weight protein fractions between gelatin from skins of young and adult Nile perch, there was no difference in gel strength, suggesting no direct relation between molecular weight distribution and gel strength. Earlier studies (Koepff, 1984; Graesser, 1985) have also shown no simple correlation between gelatin gel strength and molecular weight distribution for high gel strength gelatins.

The differences in functional properties between Nile perch skin and bone gelatins seem to have arisen from differences in the ease with which collagens in the two collagenous tissues can be transformed to gelatin. Nile perch skin collagen easily solubilised to give gelatin at low temperature, while only a small fraction (< 40%) of the bone collagen could be solubilised at 50°C. The bone collagen generally seemed to require a more severe heat treatment to facilitate denaturation to gelatin. Bone gelatins also contained more of the low molecular weight peptides compared to skin gelatins extracted at the same temperature. Nile perch bone collagen, therefore, seemed to undergo more peptide bond hydrolysis, resulting in gelatins with a higher content of low molecular weight fractions compared to the gelatin extracted from Nile perch skins. The inferior functional properties of bone gelatin seem therefore to be a

result of the more stable crosslinks in the bone collagen.

The bovine bone and commercial fish skin gelatins studied were found to exhibit functional properties quite similar to those of adult fish skin gelatin, except for melting and setting temperature, which were higher for bovine bone gelatin. The difference between the properties of fish gelatin and bovine bone gelatin may be attributed to differences in amino acid composition and molecular weight distribution. Bovine gelatin is known to contain ~ 30% (w/w) imino acids (Eastoe, 1967; Poppe, 1992) which is significantly higher than the 21% recorded for Nile perch. The higher imino acid content contributes to the higher setting and melting temperature. Similarity in gel strength despite differences in molecular weight distribution reaffirms the lack of direct correlation between these properties. The bovine bone gelatin and the commercial fish skin gelatin were found to be lower in low molecular weight ($< \alpha$) peptides and had densitograms with fewer distinct fragments, characteristic of alkali-processed gelatins (Koepff, 1984). The bovine bone gelatin also had a much higher content of $> \beta$ components.

Melting and setting temperatures of the Nile perch gelatins were found to lie between those reported for mammalian gelatin and those for gelatin from cold water fish species. It seems amino acid composition is a key determinant of these properties since the imino acid content of Nile perch gelatin was also between that of mammalian and cold water fish gelatins.

Differences between collagen and gelatin can be used to elucidate changes that occur in the transformation of collagen to gelatin. In this study, several differences were observed between Nile perch collagen and gelatins. One noteworthy difference was the slightly lower imino acids (proline and hydroxyproline), glycine and alanine content of Nile perch skin collagen in comparison to gelatins. This is attributable to the fact that gelatin is derived mainly from the helical part of collagen because much of the telopeptide regions are lost during pretreatment. The helical part is higher in the amino acids dominant in collagens (proline, hydroxyproline, glycine and alanine). As a result, gelatin normally contains higher levels of these amino acids in comparison to the collagen from which it is derived (Eastoe & Leach, 1977).

In addition, unlike Nile perch collagen, which consisted, almost exclusively of α and β chains, gelatins were found to consist of numerous fragments varying widely in molecular weight. This showed that conversion of collagen to gelatin was accompanied by cleavage of inter and intramolecular bonds. Stainsby (1987) suggested on the basis of the continuous molecular weight distribution of gelatin that during gelatin manufacture, backbone peptide bonds and collagen crosslinks are cleaved with equal ease. Paschalis *et al.* (2001) reported presence of collagen crosslinks in bovine bone gelatin, also confirming that collagen to gelatin transformation does not eliminate all the crosslinks. Collagen to gelatin transformation therefore involves cleavage of some peptide bonds and some intermolecular crosslinks.

FTIR spectra for gelatins were also quite different from those of collagen. The major differences were the lower intensities of amide I, II and III bands exhibited by gelatins. These differences are indicative of a higher molecular order in collagen than gelatin. This is consistent with changes expected as a result of denaturation of collagen to gelatin. FTIR spectroscopy, therefore showed that conversion of collagen to gelatin leads to loss in the triple helical structure and decrease in molecular order. The extent of these changes, in the case of Nile perch seemed to be affected by the order of gelatin extraction and the collagenous tissue from which gelatin is extracted.

4. CONCLUSIONS AND RECOMMENDATIONS

- Collagen from the skins of young and adult Nile perch is highly soluble in dilute acid. It seems there is minimal development of stable crosslinks with age in Nile perch skin collagen. Nile perch bone collagen is, however, insoluble in dilute acid, even from bones of young fish. This indicates that Nile perch bone collagen like mammalian bone collagens contains more stable crosslinks than the skin collagen.
- As a result of the low content of stable intermolecular crosslinks in Nile perch skin collagen, the acid extraction process (with extraction done around pH 4) is adequate for the extraction of Nile perch skin gelatin, even from skins of adult Nile perch. Extractability of gelatin from Nile perch skins at 50°C was quite high (> 65%).
- While the acid extraction process also gave gelatin from Nile perch bones, the extractability was rather low (< 40 %) and functional properties of the resulting gelatin were poorer than those for Nile perch skin gelatin. This supports the hypothesis that Nile perch skins and bones differ in their content of stable crosslinks, with bones having a higher content. The crosslinks that normally occur in skeletal matter, even at early age tend to be stable and resistant to cleavage by dilute acids. This seems to be the case in Nile perch as well.
- No marked age-related differences were found in collagen solubility and gelatine extractability for Nile perch. This confirms the proposition that aging in fish is not accompanied with significant development of stable collagen crosslinks.
- The acid soluble collagen (ASC) derived from Nile perch skins was found to contain approximately 20% imino acids. This is intermediate between values reported for cold water fish and mammalian collagen. The denaturation temperature for Nile perch ASC (36°C) was also found to be intermediate between values reported for cold water fish species and mammalian collagen. This confirms earlier reports that denaturation temperature of collagen is positively correlated to imino acid content and that these parameters are dependent on the environmental temperature at

which the fish grow.

- The electrophoretic profile and amino acid composition showed that Nile perch skin collagen is of the Type 1 variant.
- Conversion of Nile perch collagen to gelatin was found to lead to loss of molecular order and increase in the concentration of proline, hydroxyproline, alanine and glycine. The difference in amino acid composition between collagen and gelatin arises from the loss of telopeptide non-helical parts during pre-treatment. These regions contain lower levels of the four amino acids than the helical part.
- Nile perch skin and bone gelatin was found to have similar amino acid composition, with imino acid content of approximately 21.5%. This is intermediate between the imino acid content reported for cold water fish species and mammals. The functional properties of Nile perch skin gelatins were also intermediate between those for the two groups. This confirms assertions that the functional properties of gelatin are a function of their imino acid content.
- Nile perch skin gelatin, however, exhibited superior functional properties to Nile perch bone gelatin, despite the similar imino acid content. The functional properties of Nile perch skin and bone gelatins were found to be related to the molecular weight distribution of the gelatins. Nile perch bone gelatins were found to contain more low molecular weight peptides and less fraction with molecular weight > β. The high incidence of low molecular weight peptides seems to arise from their content of more stable intermolecular crosslinks. Transformation of Nile perch bone collagen to gelatin by the acid extraction process seem to entail significant hydrolysis of peptide bonds while in the case of the less crosslinked skin collagen, solubilisation was easily achieved by breaking the weak intermolecular bonds.
- FTIR spectroscopy led to the proposition that the gelatin obtained at higher temperature during the later extractions in a sequential process in which temperature is increased gradually, contain more intermolecular linkages, in the dry state, than the earlier extracts obtained at lower temperature. It was not clear, however, whether these were intermolecular covalent crosslinks or hydrogen bonding occurring during renaturation of gelatin to

collagen-like networks. The study also showed the potential of FTIR spectroscopy in the study of collagen and gelatin in the anhydrous state.

- Generally, it is clear from the study that Nile perch skins have potential for supplementing mammals as a source of gelatin and collagen. The gelatin yield of 12.3 and 16% recorded, respectively, for young and adult Nile perch are in the commercially acceptable range. The functional properties of the gelatins recorded in this study, especially for the Nile perch skin gelatin are superior to those reported for most commercial fish gelatins. The high solubility of Nile perch skin collagen is an attribute that makes it attractive as a commercial source. In addition, Nile perch collagen was found to exhibit reasonably high denaturation temperature (36°C). This implies that Nile perch collagen, unlike cold water fish collagens would not denature (and lose functionality) during handling at ambient temperature prevailing in most parts of the world. There is, therefore, real potential for exploitation of Nile perch processing waste, especially skins, for commercial production of collagen and gelatin. In the case of Nile perch bones, gelatin yield was low (< 10% for low temperature leaching and < 2.5% for ambient temperature leaching) and the functional properties rather poor. In addition, Nile perch bone collagen was found to be insoluble in dilute acid. This study therefore, did not demonstrate high potential for the exploitation of Nile perch bones as sources of gelatin or collagen.
- Further work is required to determine the types and quantities of different crosslinks in bones and skins collagen derived from Nile perch of varying age. Determination of the types and quantities of different crosslinks in gelatin is also recommended. This would shed more light on the collagen-gelatin transformation and reveal the cause of the differences in molecular order shown by FTIR spectroscopy in this study.
- Further work on optimisation of the extraction of gelatin from fish bones is also recommended. It is recommended, that the alkali extraction process be attempted, since the low temperature extractability of the bone gelatin was considerably lower by the acid process and the functional properties poorer than the skin gelatin. There is also need for optimisation of the

leaching process.

- Studies on management of the waste generated in the manufacture of gelatin as well as analysis of the cost and environmental impact of using activated carbon instead of the alternative multiple acid and alkali washes for elimination of the fishy odour may be worthwhile.
- Research involving use of Nile perch collagen and gelatin in different applications is also recommended. Nile perch gelatin was found to have a considerably lower melting temperature than mammalian gelatin of equal Bloom. It would be interesting to study how this may affect the sensory properties of food products made from these gelatins, especially flavour release.

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LIST OF PUBLICATIONS

Journal Papers

- Muyonga, J. H., Cole, C.G.B. & Duodu, K.G. (2004) CHARACTERISATION OF ACID SOLUBLE COLLAGEN FROM SKINS OF YOUNG AND ADULT NILE PERCH (*Lates niloticus*). *Food Chemistry.* 85, 81 - 89.

- Muyonga, J. H., Cole, C.G.B. & Duodu, K.G. EXTRACTION AND PHYSICO-CHEMICAL CHARACTERISATION OF NILE PERCH (*Lates niloticus*) SKIN AND BONE GELATIN. *Food Hydrocolloids.* Accepted.

- Muyonga, J. H., Cole, C.G.B. & Duodu, K.G. FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPIC STUDY OF ACID SOLUBLE COLLAGEN AND GELATIN FROM SKINS AND BONES OF YOUNG AND ADULT NILE PERCH. *Food Chemistry.* Accepted.

Oral Presentation

- Muyonga, J.H., Cole, C.G.B. & Duodu, K.G. 2003. EXTRACTION AND PHYSICO-CHEMICAL CHARACTERIZATION OF NILE PERCH (*Lates niloticus*) SKIN AND BONE GELATIN. SOUTH AFRICAN ASSOCIATION OF FOOD SCIENCE AND TECHNOLOGY BIANNUAL CONFERENCE, PRETORIA SOUTH AFRICA. SEPTEMBER $1^{ST} - 5^{TH}$ 2003.