# 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.

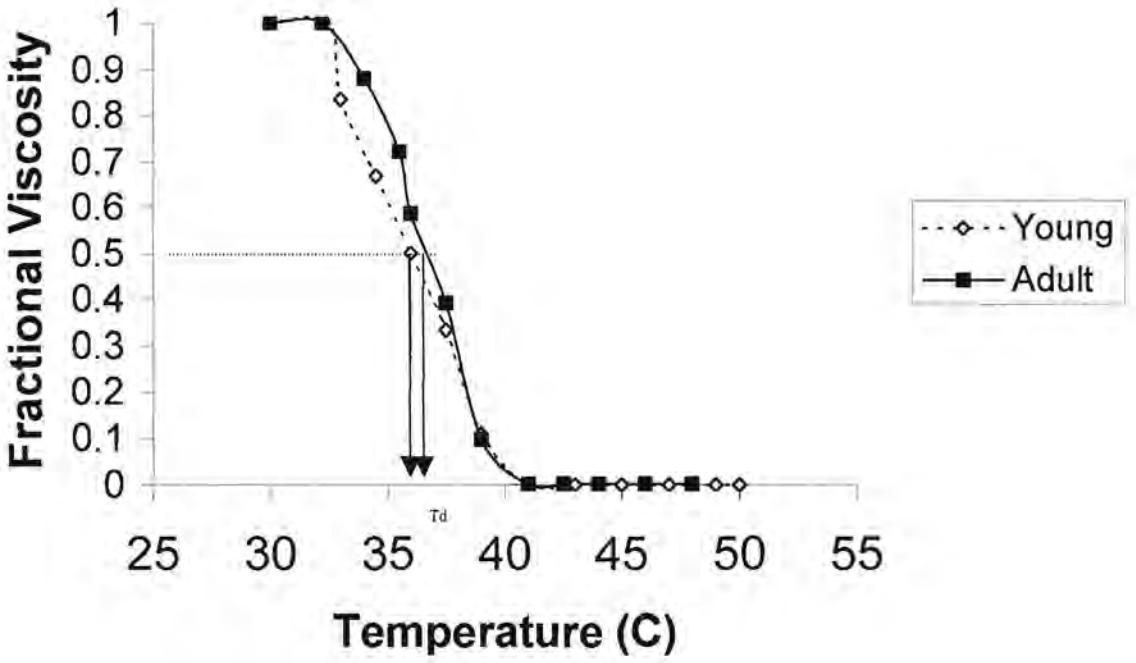


**Figure 2.1.1: Denaturation curve of collagen from skins of young and adult Nile perch as shown by change in fractional viscosity with temperature for 0.1% (m/v) solutions of collagen in acetic acid. Td is the denaturation temperature.**

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).

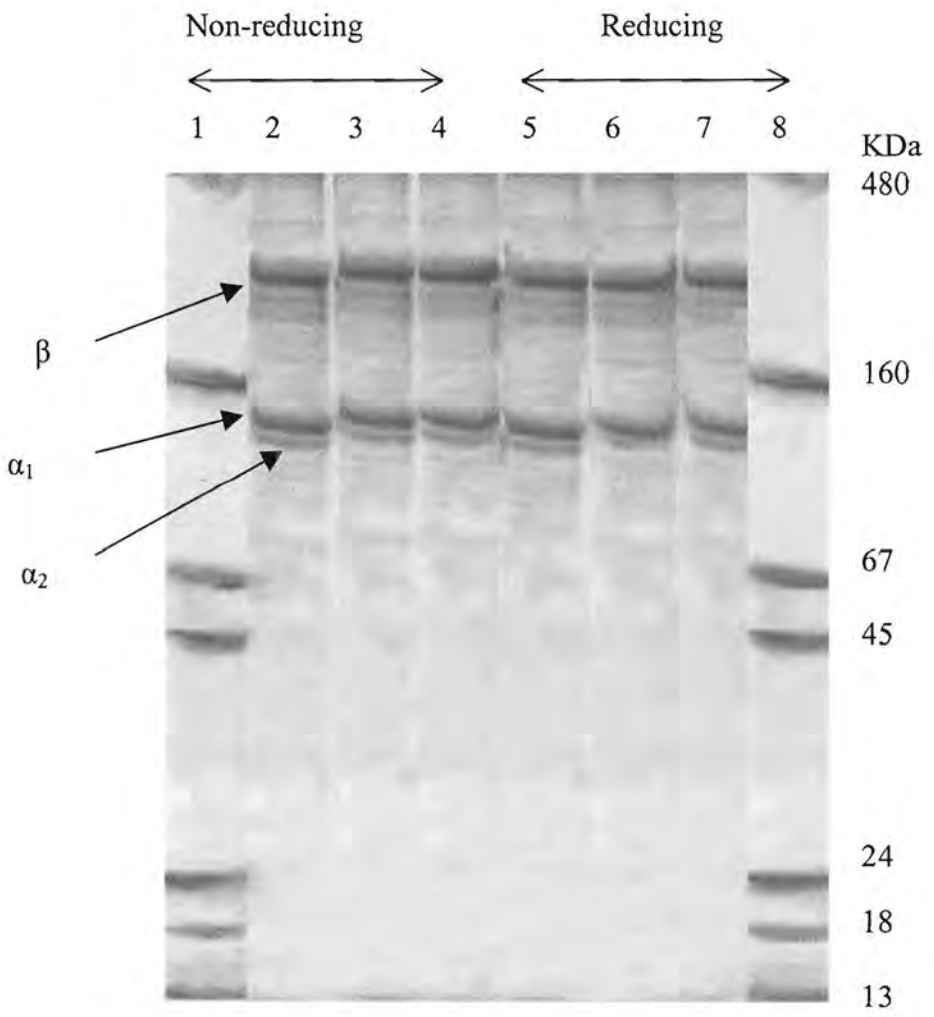


**Figure 2.1.2: SDS polyacrylamide gel (7.5%) electrophoretic pattern for acid soluble collagen under non-reducing and reducing conditions. 1 & 8 – Molecular weight markers, 2 & 5 – calfskin collagen, 3 & 6 – collagen from skin of adult Nile perch, 4 & 7 – collagen from skin of young Nile perch.**

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.

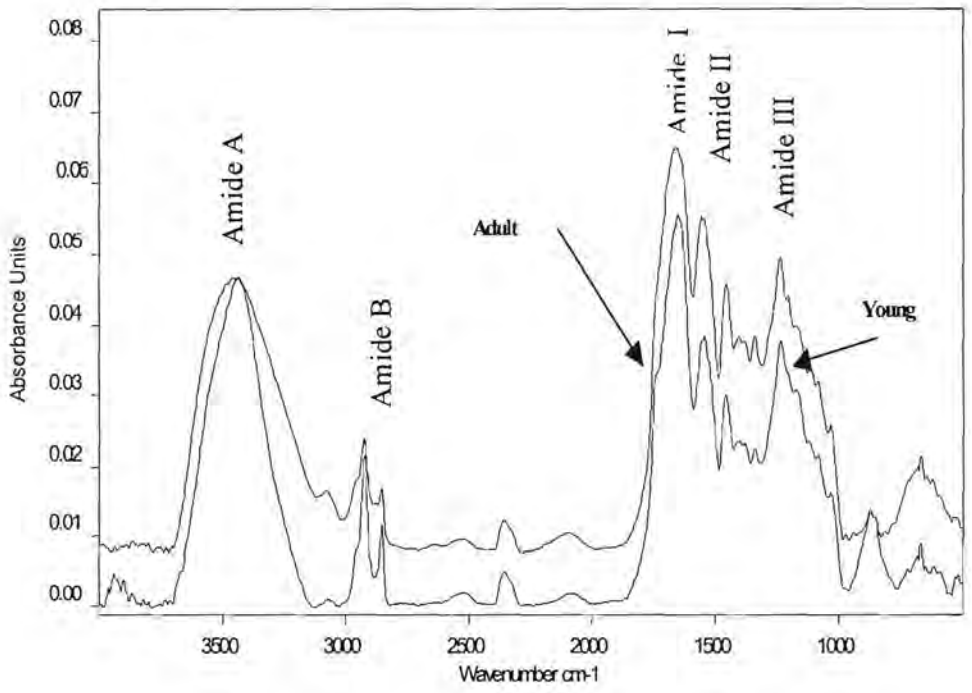


**Figure 2.1.3: Average FTIR spectra for triplicate samples of acid soluble collagen derived from skins of young and adult Nile perch**

**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).

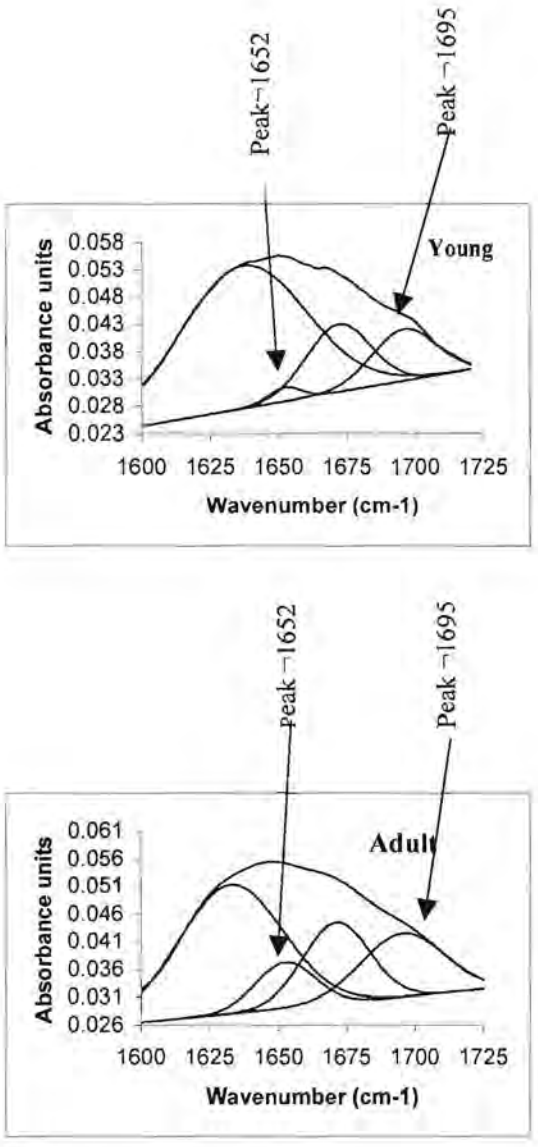


**Figure 2.1.4: Amide I band for collagen from the skins of young and adult Nile perch with fitted band components**

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 $\alpha$ and $\beta$ 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 $\gamma$) 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 $\text{Bloom}_m$ 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 $\alpha = 0.05$

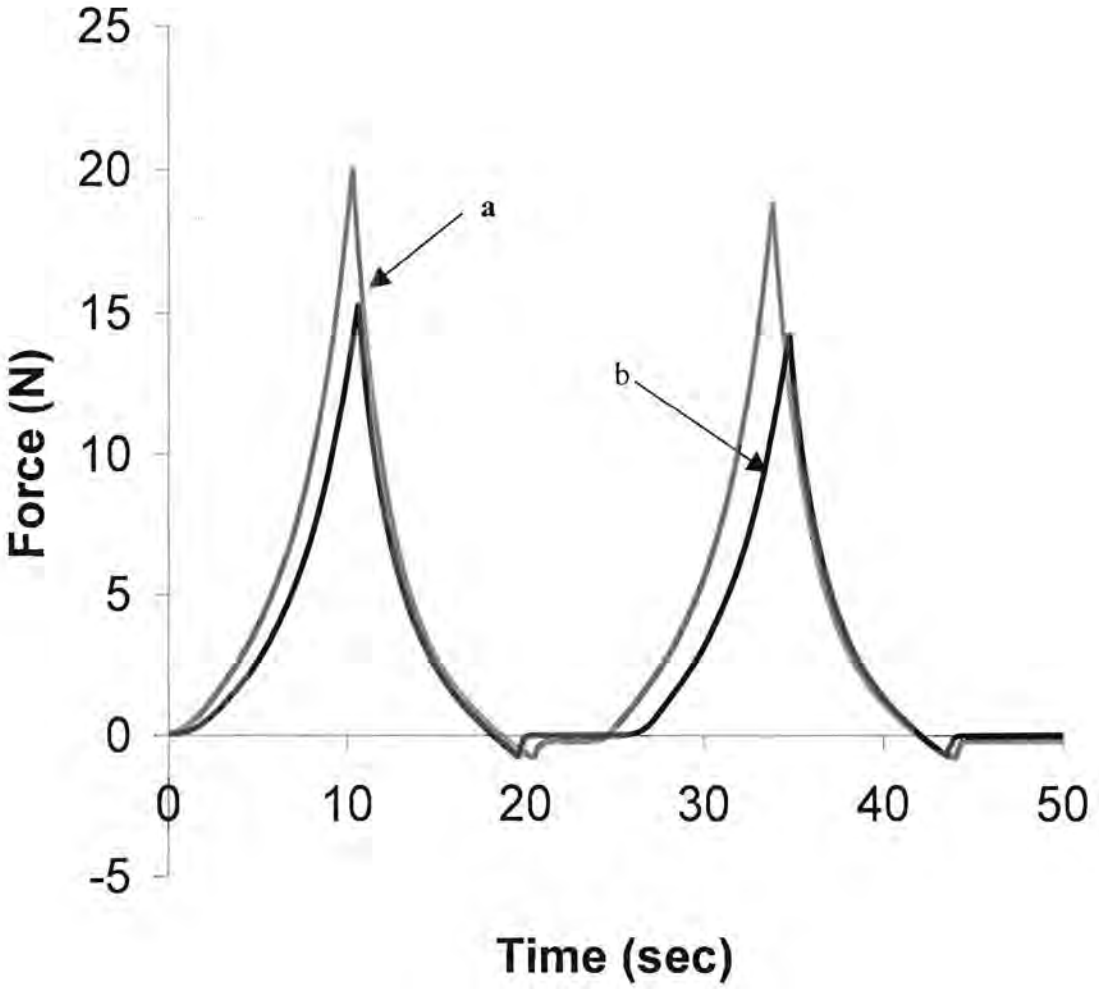


**Figure 2.2.1: Typical texture profile for gels containing 6.67% of Nile perch skin (a) and bone (b) gelatin**

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 $\alpha = 0.05$

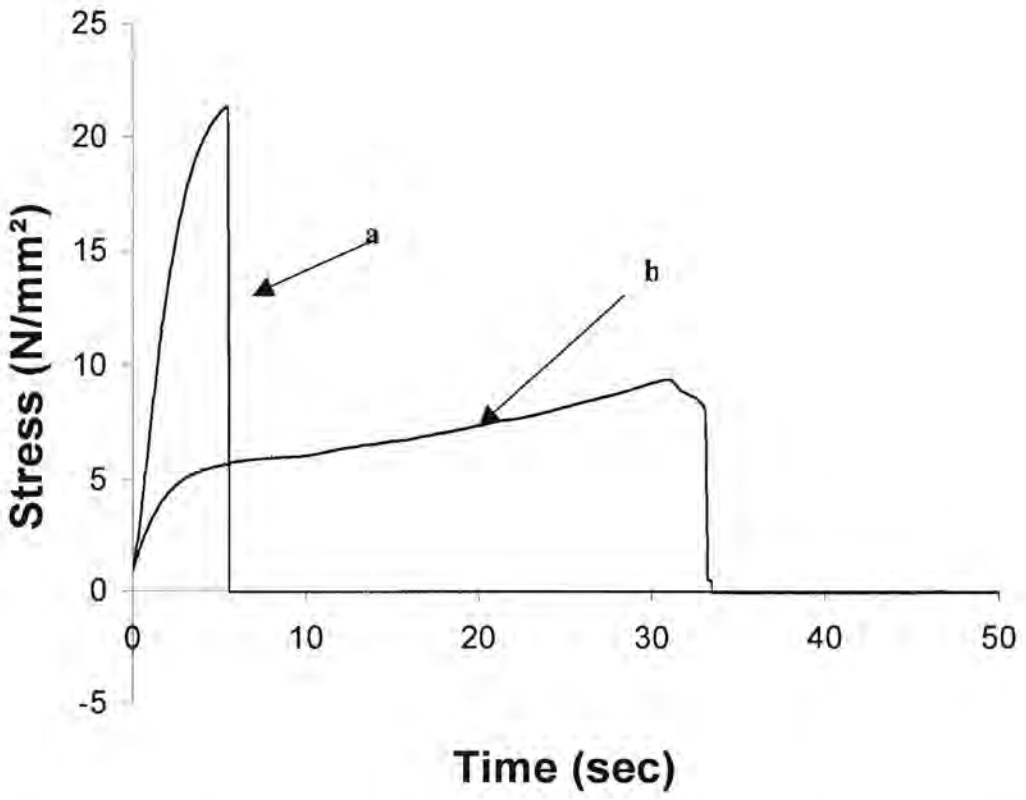


**Figure 2.2.2: Typical stress - strain curves for films ~ 0.6 mm thick made by casting 5% gelatin solutions of Nile perch skin (a) and bone (b) gelatin in 0.5 M acetic acid, with 0.2 g glycerol per g of gelatin**

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.

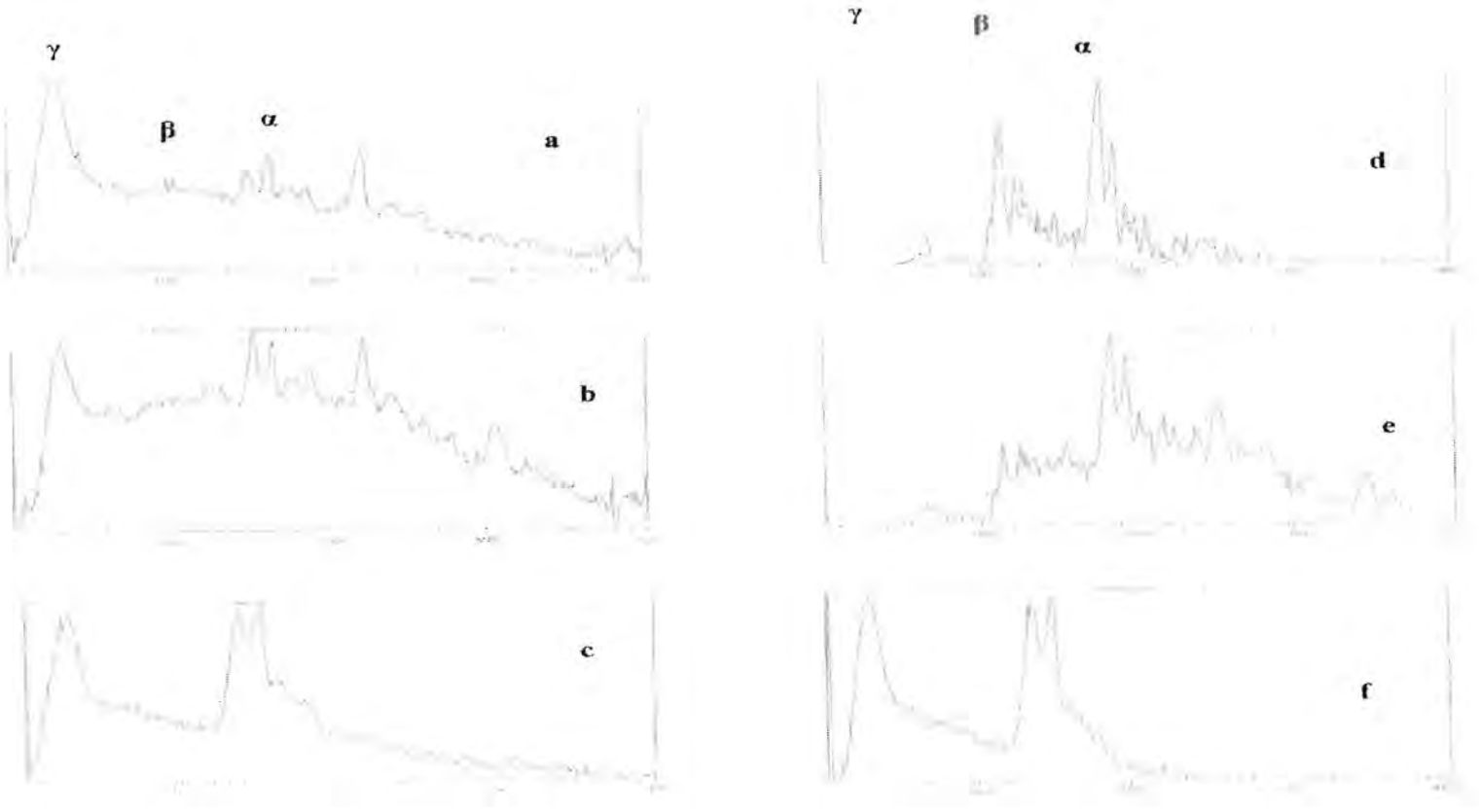


**Figure 2.2.3: SDS PAGE densitograms for gelatins from skin of adult Nile perch (a), skins of young Nile perch (b), commercial fish gelatin (c), gelatin from bones of adult Nile perch (d), bones of young Nile perch (e) and bovine bones (f). Nile perch gelatins were extracted at 50°C. Extraction temperature 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.

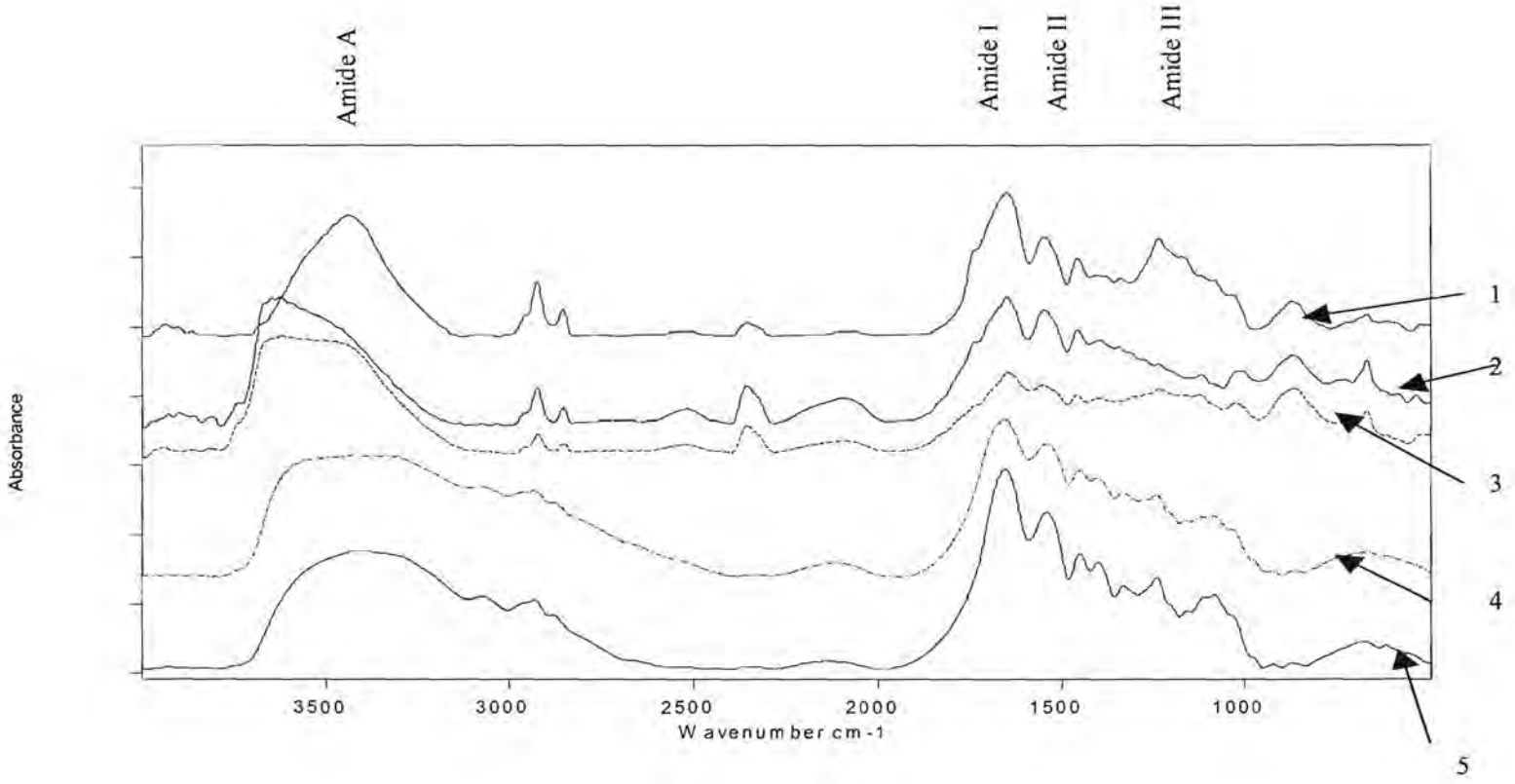



**Fig 2.3.1: FTIR spectra for young Nile perch skin acid soluble collagen (1), adult Nile perch skin gelatin extracted at 50°C (2), young Nile perch skin gelatin extracted at 50°C (3), young Nile perch skin gelatin extracted at 60°C (4) and adult Nile perch skin gelatin extracted at 70°C (5).**

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.

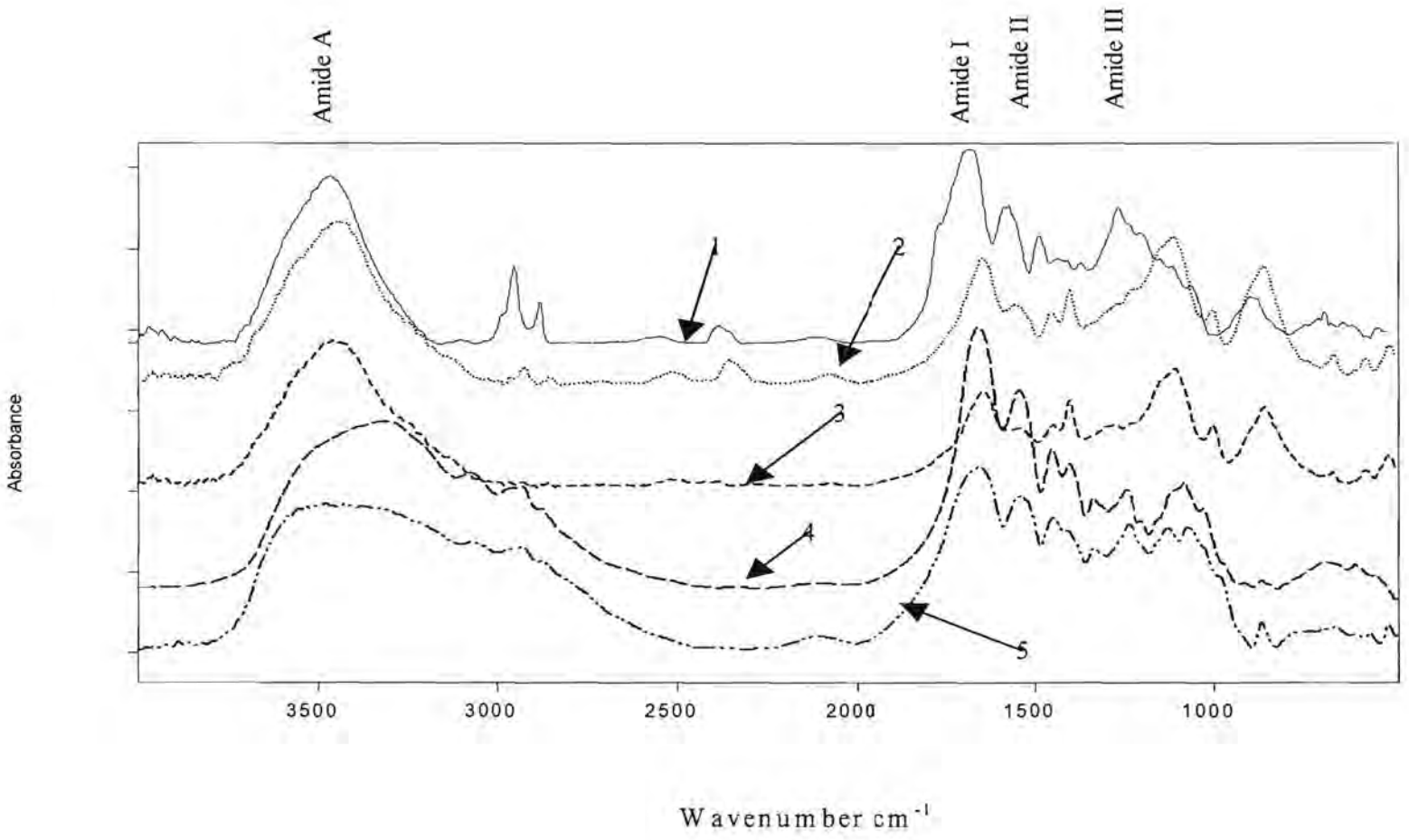



**Fig 2.3.2: FTIR spectra for young Nile perch skin acid soluble collagen (1), gelatin from young (2) and adult (3) Nile perch bones extracted at 50°C and from young (4) and adult (5) Nile perch bones extracted at 70°C.**

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

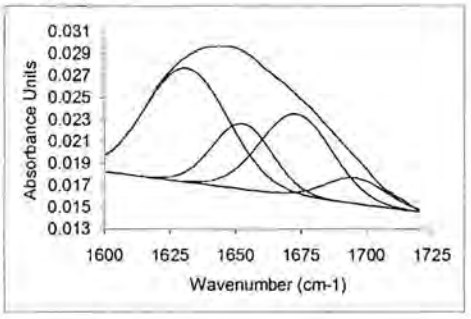
The amide I band between 1600 and 1700 $cm^{-1}$ is the most useful for infrared spectroscopic analysis of the secondary structure of proteins (Surewicz & Mantsch, 1988). Deconvolution of the amide I band showed the band to consist of four components. The component peaks, their location and % areas are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

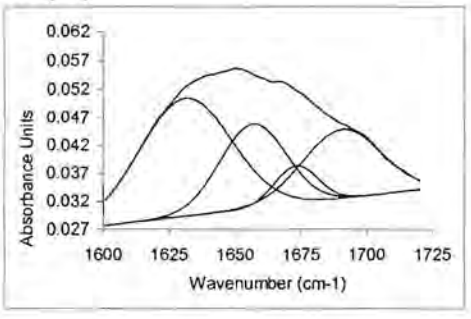
| Material | Component peak location ($cm^{-1}$) and percent area (in brackets) contribution of total band | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| Young fish skin acid soluble collagen | 1637 (69.0) | 1652 (1.8) | 1672 (16.7) | 1696 (12.6) |
| Adult fish skin gelatin 50°C | 1634 (49.6) | 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 $\geq 0.9998$

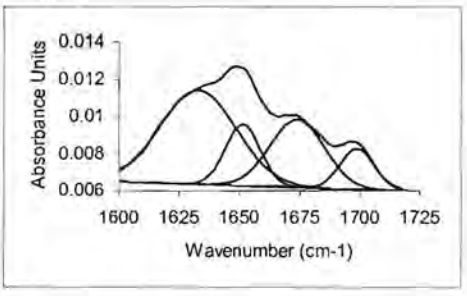
AB (50)



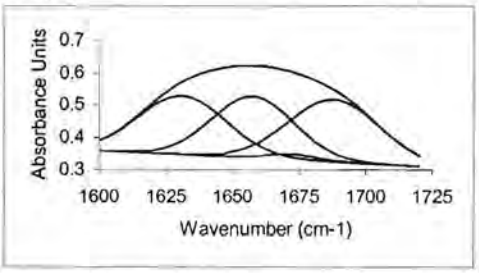

AB (70)



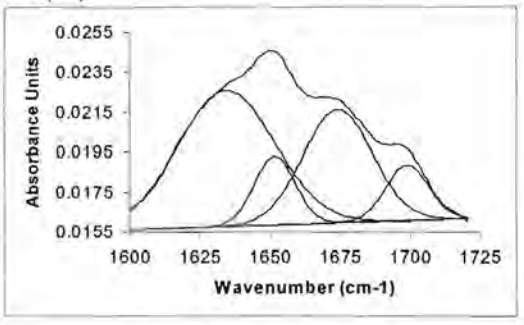

YB (50)



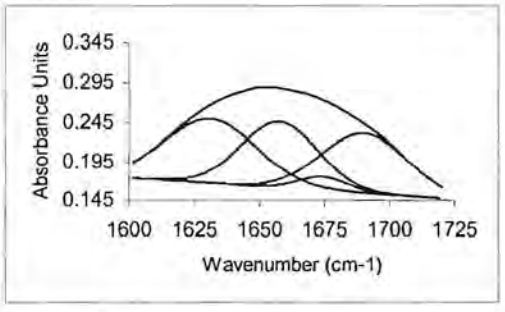

YB (70)



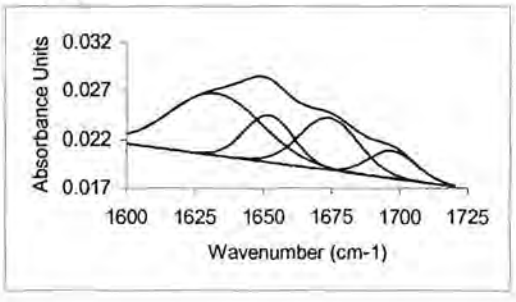
AS (50)



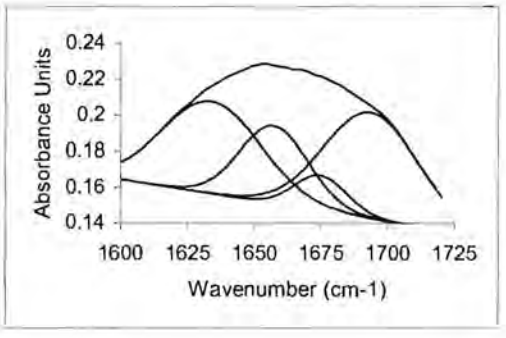

AS (70)




YS (50)




YS (60)



ASC

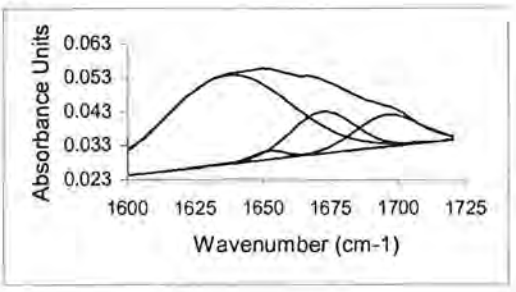



**Fig 2.3.3: Amide I band for Nile perch gelatins and collagens with fitted band components**

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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