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# Fourier transform infrared (FTIR) spectroscopic study of acid soluble collagen and gelatin from skins and bones of young and adult Nile perch (*Lates niloticus*)

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**Abstract**

Fourier transform infrared (FTIR) spectroscopy was conducted on type A gelatins derived from skins and bones of young and adult Nile perch (*Lates niloticus*) 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 the 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.


*Keywords:* Nile perch; Gelatin; Collagen; FTIR; Protein structure

## 1. 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 et al., 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 shown to include changes in the amide A (Milch, 1964), amide I (1636–1661 $cm^{-1}$), amide II (1549–1558 $cm^{-1}$) (Renugopalakrishnan et al., 1989) and 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

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reduction in the intensity of amide I component, found around 1660 $cm^{-1}$ (George & Veis, 1991; Payne & Veis, 1988; Renugopalakrishnan et al., 1989).

Prystupa & 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 et al., 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 are manifested 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, affects the stability of collagen to denaturation processes, e.g. heat. Collagens 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 structures of gelatins derived from young and old animals differ. Nile perch (*Lates niloticus*) is a warm water fish species, with potential for giving gelatin with gelling properties more similar to mammalian gelatins than cold water fish species.

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.

## 2. Materials and methods

### 2.1. 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 & 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 h, 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.

### 2.2. 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 h) by adding more acid solution until the skins were adequately swollen. 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 h 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 all 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 decreased 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 1.

### 2.3. Fourier transform infrared spectroscopy

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) 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

Table 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 |

Table 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 and Babu (2001) |
| – | 2924 | 2923 | 2924 | DM | 2923 | 2924 | DM | Sh | Sh | $CH_2$ asymmetrical Stretch | Abe and Krimm (1972) |
| – | 2853 | 2853 | 2853 | | | 2853 | DM | | | $CH_2$ symmetrical Stretch | Abe and 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, Choo, Watson, Halliday, and Mantsch (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 | | $CH_2$ 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 and Krimm (1972) |
| – | 670 | 670 | 660 | 669 | 670 | 870 | 866 | | 874 | Skeletal stretch | Abe and 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.

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).

## 3. Results and discussion

### 3.1. Frequencies

The frequencies at which major peaks occurred for acid soluble collagen and the different gelatins and collagens are summarised in Table 2.

### 3.2. Spectra for skin gelatins

Nile perch gelatins, extracted at 50 °C, exhibited high Bloom (>200 g) and therefore have potential for substituting mammalian gelatins in gelling applications. 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 (Fig. 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 was observed for the low temperature-extracted gelatins and for acid-soluble collagen. The amide A band in the high temperature-extracted gelatins was in fact 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 gelling of high molecular weight gelatins. During drying, therefore, it seems that 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 they are 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

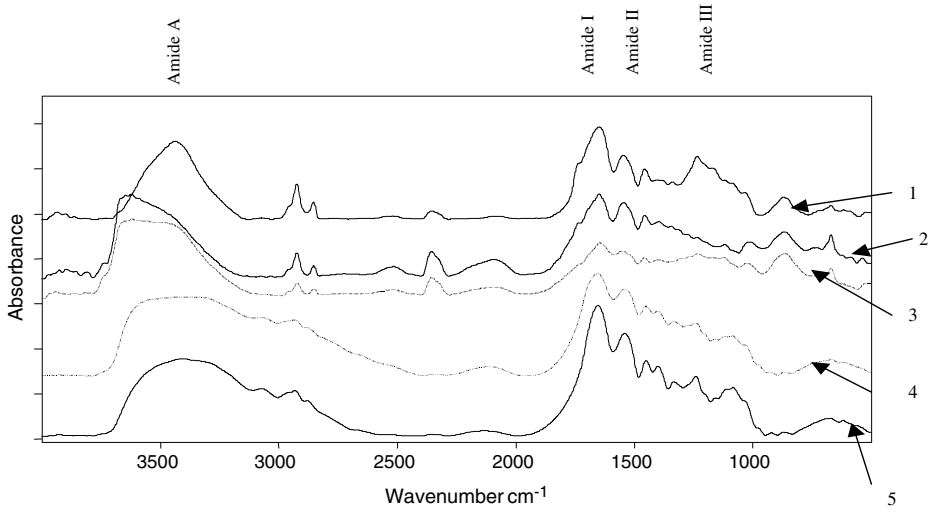


Fig. 1. FTIR spectra for young Nile perch skin acid-soluble collagen (1), adult Nile perch skin gelatin extracted at 50 °C (2), young Nile perch skin gelatin extracted at 50 °C (3), young Nile perch skin gelatin extracted at 60 °C (4) and adult Nile perch skin gelatin extracted at 70 °C (5).

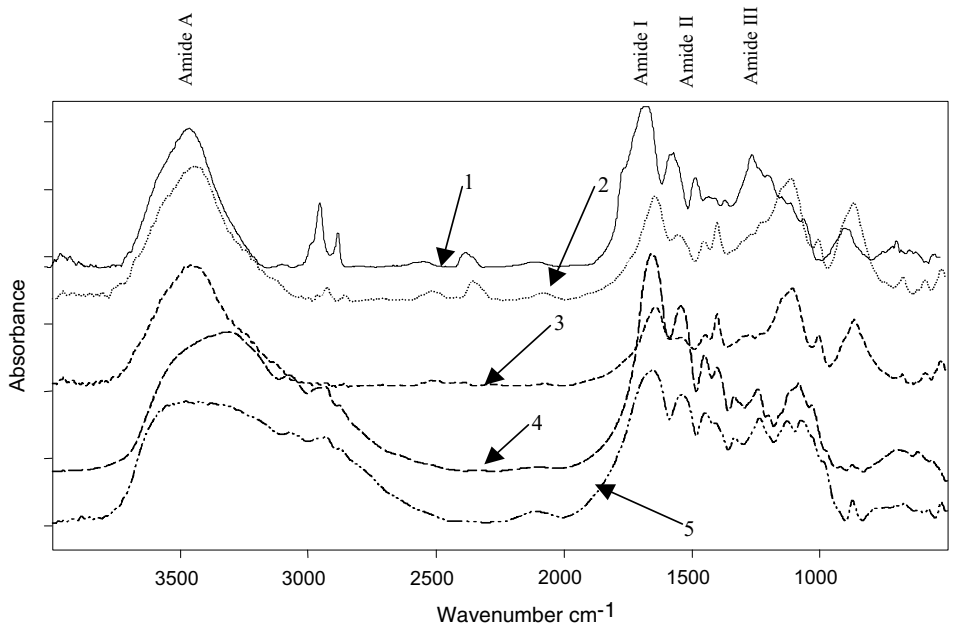


Fig. 2. FTIR spectra for young Nile perch skin acid-soluble collagen (1), gelatin from young (2) and adult (3) Nile perch bones extracted at 50 °C and from young (4) and adult (5) Nile perch bones extracted at 70 °C.

bone gelatin, supporting the assertion that intermolecular crosslinks may survive the process of gelatin extraction.

### 3.3. Spectra for bone gelatins

The spectra exhibited by bone gelatins differed from those exhibited by acid-soluble collagen and skin gelatins (Fig. 2). The amide I peaks in the bone gelatins were at lower frequencies than those of acid-soluble collagen. There were also differences in the amide III region. The 50 °C-extracted Nile perch bone 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 et al., 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 that 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 less than 10% of 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, that the 70 °C-extracted bone gelatins had more intermolecular associations than the 50 °C-extracted gelatins.

### 3.4. 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 Fig. 3 and Table 3.

In agreement with Byler & Susi (1986), it is clear from Table 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 & Susi (1986). They reported a variation of approximately 15

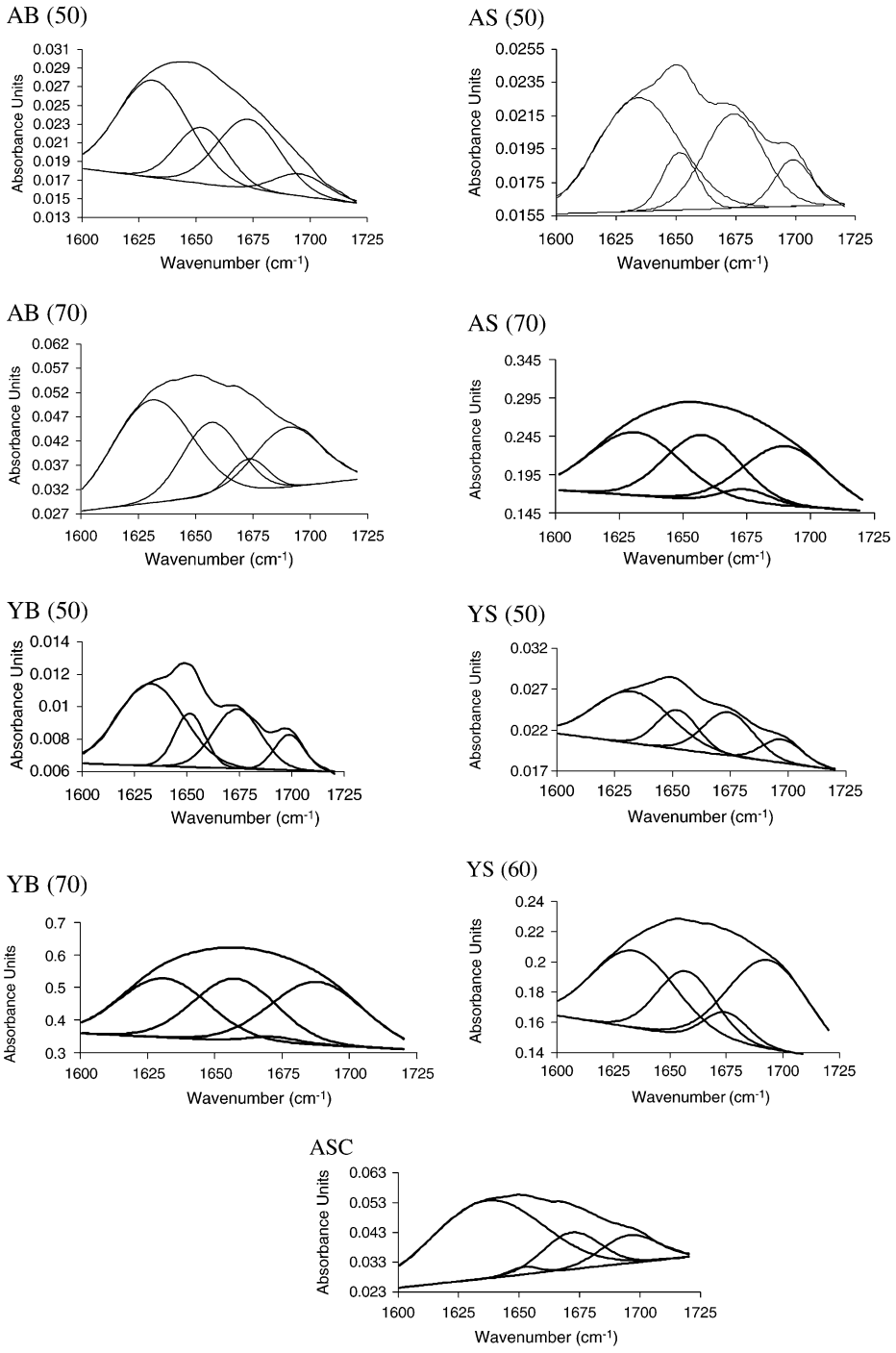


Fig. 3. Amide I band for Nile perch gelatins and collagens with fitted band components ASC – Young Nile perch skin acid soluble collagen, AB – Gelatin extracted from adult fish bones, YB – Gelatin extracted from young fish bones, AS – Gelatin extracted from adult fish skins, YS – Gelatin extracted from young fish skins. Numbers in brackets represent extraction temperature for the gelatin.

$cm^{-1}$ for frequencies, attributable to β-structures of various components.

Quantitative band-fitting analysis of amide I 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 was the consistently higher % area contributed by the 1690 $cm^{-1}$ component for the higher temperature-extracted gelatins. In addition, the component around 1690 $cm^{-1}$ occurred at higher wave

Table 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 $\geqslant 0.9998$.

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 that 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 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. (1992) reported that the two types of tissue have different types of crosslinks.

## 4. 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, seems 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 are manifested as higher molecular order.

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