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Fourier transform infrared (FTIR) spectroscopic study of extracted gelatin from shaari (*Lithrinus microdon*) skin: effects of extraction conditions

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Abstract: Fourier Transform Infrared (FTIR) Analysis was used to characterize secondary structure of gelatins extracted from shaari skin and compared with bovine and porcine gelatin obtained from commercial source. The concentration and temperature of extracted solutions were varied from 0.01 to 1.0 N and 4 to 80°C, respectively. The intensity ratio of amide III and I as a measure of denaturation process showed that all samples had almost the same protein structure at 0.1 and 1.0 N concentration for all extraction temperatures. At low acid concentration (0.01 N) and low temperature (4°C) significant amount of triple helix remained intact (i.e. less denaturation). As the temperature increased from 20°C, the random coil structure increased as the protein became denatured and protein losses its triple structure. The cluster analysis showed that secondary structure of gelatin extracted using mild treatment (concentration: 0.01 N and temperature: 4°C) behaved completely different from other extracted gelatin.

Keywords: Fish skin, gelatin, Fourier Transform Analysis (FTIR), glass transition, state diagram

Introduction

Food and pharmaceutical industries all over the world are witnessing an increasing demand for collagen and gelatin (Karim and Bhat, 2009). Skin and bone from bovine and porcine sources have usually been utilized commercially in gelatin production. In recent years however, fish gelatin has gained importance as the demand for non-bovine and non-porcine gelatin has decreased due to socio-cultural and health problems. In addition fish gelatin (especially from warm-water fish) reportedly possesses similar characteristics to procine gelatin and may thus be considered as an alternative to mammalian gelatin for use in food products (Karim and Bhat, 2009).

Gelatin is a protein compound derived from denatured collagen composed of long chains of amino acids connected by peptide bonds (Ockerman and Hansen, 1988). The amino acid composition of collagen, and consequently gelatin, is almost completely lacking in tryptophan and is low in methionine, cystine and tyrosine (Jamilah and Harvinder, 2002). The quality of gelatin depends on its physical, chemical and structural characteristics. The most important physical properties of gelatin are its gel strength and viscosity. Factors affecting gelatin properties and production processes are the species, breed, age, manner of feeding the source animal, and also the storage conditions of the raw materials (Hinterwaldner, 1977; Jamilah and Harvinder, 2002). The number and distribution of polypeptides, which are influenced by the manufacturing method and pH, also affect gelling properties (Yoshimura *et al.*, 2000). The properties of the gelatin also depend on the molecular weight distribution of the collagen components and the ratio of α-chain to β-component (Gomez-Guillen *et al.,* 2002).

Infrared (IR) spectroscopy is among the most powerful spectroscopic techniques for food analysis since it covers the details on the functional group as well as chemical composition that are contained in the infrared spectrum of specific substances (Kumosinski and Farrell, 1993). The Fourier Transform infrared (FTIR) spectroscopy together with attenuated total reflectance (ATR) or transmission accessories are being used to determine chemical, physico-chemical, structural, morphological, and intermolecular cross-linking of foods and biomaterials (Hashim *et al.,* 2010).

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FTIR spectroscopy has been used to monitor the changes in the functional groups and secondary structure of gelatin. Kong and Yu (2007) reviewed the applications of FTIR spectroscopy in determining protein secondary structure, conformational changes, structural dynamics and their stability. Hashim et al. (2010) used FTIR for differentiation of bovine and porcine gelatins. The FTIR spectra were analyzed using a chemometric method and principal component analysis successfully classify and characterize gelatin compounds of the FTIR spectra in the range of 3290-3280 cm^{-1} and 1660-1200 cm^{-1}.

In addition to measuring the physico-chemical characteristics, different techniques are being used to characterize gelatins from different sources. These techniques include the measure of the gelling characteristics of gelatin by mechanical or rheological methods (Zhou and Regenstein, 2005; Rahman and Al-Mahrouqi, 2008; Avena-Bustillos *et al*., 2006; Haug *et al.*, 2004; Yang *et al*., 2008), nano-structure by atomic force microscopy (AFM) (Zhou and Regenstein, 2005), molecular weight measurement (Nalinanon *et al.,* 2008), and thermal characteristics by Differential Scanning Calorimetry (DSC) (Rahman *et al*., 2008; Sobral and Habitante, 2001; Vanin *et al.*, 2005). However, negligible work was conducted to characterize the gelatin using FTIR techniques from same source while different treatment conditions were used. In this study, FTIR spectra of skin gelatins derived from shaari (a warm water fish) using different conditions were determined and compared to bovine and porcine gelatins, in an effort to elucidate changes in secondary structure that occur during the conversion of collagen to gelatin.

Materials and Methods

Source of raw materials

In the month of August 2008, one batch of Shaari (Lithrinus microdon) skin (age ≈ 6 months; 60-80 cm in length; mixed gender) was collected from local super market in Muscat. The skin was stored at -40°C until used for the extraction and analysis.

Sample preparation

Commercial gelatins were bought from Sigma, Saint Louis, MO, USA (porcine powder: catalogue number G 2500 and bovine powder: catalogue number: G 9382). Frozen shaari skin was thawed at room temperature for about 1 hour and then the attached flesh was removed by scratching with a knife and de-scaled. Skin was washed with running tap water and was then divided into three batches. The first batch was used for gelatin extraction. Second batch of the skin was dried in a convection oven at 80°C for 18 hr. The third batch was dried in a desiccator containing silica gel at 20°C. Both dried skin w\samples were ground into powder by a hammer mill with sieve size 1.0 mm (Model MF 10 Basic, IKA Works, USA).

Extraction procedure followed that described by Gomez-Guillen and Montero (2001). The concentration and temperature of acetic acid solution used in this study were 0.01, 0.1 and 1 N, and 4, 20, 50 and 80°C, respectively. The solution-skin mass ratio for extraction was 6:1. Gelatin extracted in solution was separated using two layer filter cloth. The solution was then dried in an oven initially at 60°C for 24 hours and then dried by storing it in a desiccator containing silica gel at 20°C for two days. The dried sample was ground in a hammer mill using sieve of 1.0 mm screen (MF 10 basic from IKA Works, Inc, USA).

The powder samples were equilibrated in desiccator maintained at 11.3% relative humidity environment by placing saturated lithium chloride solution (20°C). The equilibration time was around 4 weeks. The equilibrated samples were stored in an air tight glass bottle at -20°C. The moisture contents of all equilibrated samples were determined by drying 2 g of samples in a mechanical convection oven for 24 hours at 105°C (AOAC, 1990). All equilibrated samples' moisture content was raised to 16.0 g/100 g sample by placing it in a desiccator with water at the bottom until it reached a predetermined weight. All samples were stored in air tight glass bottles at -20°C until used for FTIR analysis.

Fourier Transform Infrared (FTIR) spectroscopy

FTIR spectra were obtained from discs containing 10 mg dried sample (moisture content: 16.0 g/100 g sample) in approximately 90 mg potassium bromide (KBr). All spectra were obtained using a Bruker infrared spectrophotometer (Bruker Instruments, Billerica, MA) equipped with a DTGS detector. Background (pure potassium bromide) was subtracted using the Opus software (Bruker Instruments, Billerica, MA). All spectra were collected by co-adding 128 scans at a resolution of 2 cm^{-2} and a gain of 1.0. Twelve replicates of each sample were analyzed and spectra for the replicated runs were 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. The peaks were identified by software and assigned according to the literature values.

The statistical significance of treatments was conducted with Duncan's multiple range tests using generalized linear model (GLM) procedure at 5% significance level (SAS, 2001). Two multivariate exploratory methods were used to identify structures in the overall profiles of the consumers in this survey: clustering analysis and Non-metric Multidimensional Scaling (MDS). Both analyses were performed with PAST software (Hammer *et al*., 2001). A hierarchical clustering using Ward's method (Ward, 1963) of variance minimization within groups was applied to the untransformed FTIR spectra. The number and the composition of the clusters retained were estimated by visual observation of the clustering tree. The principal component analysis was carried out on the FTIR spectra considering all samples. The different gelatin and skin samples were identified in the biplot space by their group in the clustering analysis.

Results and Discussion

Figure 1 shows a typical FTIR spectrum for extracted fish gelatin at 4°C using 0.01 N acetic acid solution. It shows three major peak regions marked as 1 (3600-2700 cm^{-1}), 2 (1900-900 cm^{-1}), and 3 (400-900 cm-1). The regions are assigned to the bonds: Amide A and B; Amide I, II and III; and Amide IV, V and VI (Kong and Yu, 2007; Pelton and McLean, 2000; Jackobsen *et al.*, 1983). Similar results were also observed by Hashim *et al.* (2010), Muyonga *et al.* (2004), and Ahmad and Benjakul (2011).

Figure 2 shows similar FTIR spectra for fish skin, bovine and porcine gelatin. The ruggedness of the curves between regions 2 and 3 (Figure 3) was more pronounced in the cases of fish skin compared to the bovine and porcine gelatin. Similarly ruggedness in the regions 2 and 3 for fish skin collagen and gelatin was observed (Woo *et al.,* 2008; Muyonga *et al*., 2004; Bhat and Karim, 2009; Yakimets *et al*., 2005). In addition the peak corresponding to Amide III was clearer in the case of fish skin gelatin, whereas this peak was significantly reduced in the cases of bovine and porcine gelatin.

Figure 3 shows the effect of extraction temperature on the FTIR Spectra at low acid (0.01 N). At low temperature the region 1 was more close to the bell shaped, whereas at higher temperature the base of the second half of the peak was shifted to the right to a lower wave number. In addition the intensity of the peaks in the regions 2 and 3 decreased with the increase of temperature. Similar effect was observed for medium (0.1 N) and high (1.0 N) concentration, Figures 4 and 5 also shows different shape of the FTIR spectra as a function of extraction temperature.

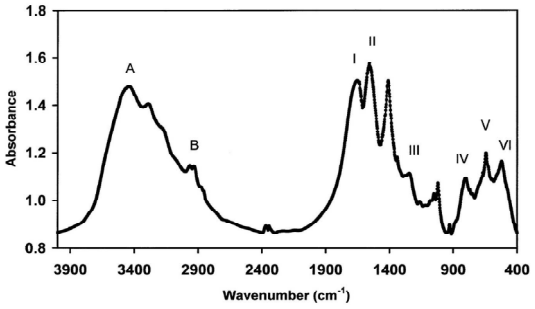

Figure 1. A typical FTIR spectrum for extracted fish skin gelatin at 4°C using 0.01 N acetic acid solution

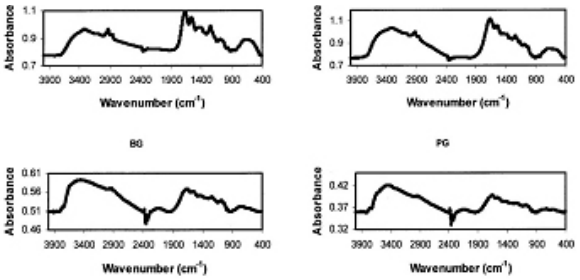

Figure 2. FTIR spectra for whole fish skin and commercial gelatin. SDC: Skin dried in silica gel at room temperature, SDO: Skin dried in oven at 80°C, BG: Bovine gelatin, PG: Porcine gelatin

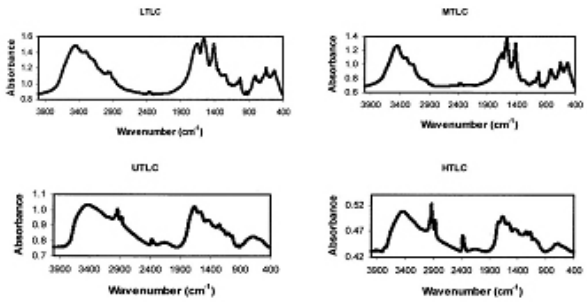

Figure 3. FTIR spectra of fish skin gelatin extract using 0.01 N acetic acid solution at different temperature. LTLC: 4°C and 0.01 N, MTLC: 20°C and 0.01 N, UTLC: 50°C and 0.01 N, HTLC: 80°C and 0.01 N

In general 14 peaks were identified by the software program. Eight peaks for amide bonds were identified based on their wave number presented in the literature earlier (Figure 1). These are amide A, B, I, II, III, IV, V, and VI (Badii and Howell, 2006; Kong and Yu, 2007; Muyonga *et al.*, 2004; Pelton and McLean, 2000; Jackobsen *et al.*, 1983). In the literature peak shape, wave number at the peak, intensity and whole range of intensity-wave number data are being used to characterize the differences in the structure of a material. Tables 1 and 2 show the wave number and absorbance values of the eight amide peaks.

Comparison between gelatins and the conformational changes of their structure has been based on the comparison of absolute peak intensities. Amide I band, between 1600 and 1700 cm^{-1}, was the most useful for infrared red spectroscopic analysis of the structure of proteins (Surewicz and Mantsch,

Table 1. FTIR spectra peak wavenumber^{-1} for (Amides) in skin and gelatin from shaari and commercial mammalian gelatins

Sample	Amide A	Amide B	Amide I	Amide II	Amide III	Amide IV	Amide V	Amide VI
LTLC	3449 a (1.5)	2964 a (0.6)	1654 b (0.8)*	1560 a (0.1)	1244 a (0.4)	801 c (0.7)	644 c (0.4)	520 c (0.8)
MTLC	3443 ab (5.1)	2929 c (0)	1638 c (0.2)	1561 a (1.9)	-	808 b (1.0)	643 c (0.6)	520 c (2.0)
UTLC	3423 ed (0)	2926 egf (0.5)	1655 b (0.6)	1555 c (0)	1241 ba (1.9)	-	685 a (14.4)	-
HTLC	3424 ed (6.2)	2925 gf (0.5)	1654 b (0.5)	1549 cd (0)	1239 bc (0)	718 e (0)	-	-
LTMC	3415 e (7.1)	2926 edf (0.7)	1654 b (0.3)	1552 cb (0)	1245 ba (0)	-	667 ba (0)	-
MTMC	3421 ed (11.0)	2925 gf (1.1)	1654 b (0.7)	1541 f (0)	1239 bc (0)	-	689 a (0)	-
UTMC	3414 e (16.5)	2925 hg (0.7)	1653 b (0)	1546 f (0)	1239 bc (0)	721 d (0)	-	-
HTMC	3430 dc (4.5)	2927 d (0.1)	1629 b (0.3)	1541 f (0)	1236 c (0)	-	671 ba (0)	-
LTHC	3430 dc (9.8)	2927 ed (0.5)	1654 b (2.0)	-	-	-	-	-
MTHC	3425 ed (4.0)	2926 egf (0.3)	1654 b (0)	-	-	-	-	-
UTHC	3432 dc (6.7)	2926 egf (0.2)	1653 b (0)	-	1241 ba (0)	-	-	-
HTHC	3423 ed (11.9)	2925 gf (0.5)	1654 b (1.1)	1544 ef (0)	-	-	670 ba (0)	618 a (5.0)
BG	3437 bc (12.2)	2929 c (0)	1655 b (0.5)	1549 cd (0)	-	712 f (0)	-	609 b (0)
PG	3441 bac (8.7)	2935 b (0)	1654 b (0.2)	1555 b (0)	-	718 e (0)	-	-
SDC	3326 f (13.2)	2924 h (1.7)	1662 a (4.8)	1540 a (0.1)	-	875 a (0)	650 bc (16.5)	-
SDO	3318 f (7.5)	2926 egf (1.0)	1663 a (0.6)	1546 ed (0.9)	-	875 a (0)	684 a (12.0)	-

Note:
*Another peak was observed at 1638 ± 0.3
Means with same letter in the same column are not significantly different ($p<0.05$)

Table 2. FTIR spectra peak intensity Absorbance for (Amides) in skin and gelatin from shaari and commercial mammalian gelatins

Sample	Amide A	Amide B	Amide I	Amide II	Amide III	Amide IV	Amide V	Amide VI
LTLC	1.8 a (0.3)	1.3 a (1.0)	1.9a (0.4)	2.0 a (0.4)	1.3 a (0.2)	1.3 a (0.2)	1.4 a (0.2)	1.4 a (0.2)
MTLC	1.2 b (0.3)	0.8 dc (0.2)	1.1b (0.3)	1.2 b (0.3)	-	0.9 b (0.2)	1.0 b (0.2)	1.0 b (0)
UTLC	0.8 dc (0.1)	0.8 dc (0.1)	0.8dc (0.1)	0.8 dc (0.1)	0.7 cb (0.1)	-	0.7 d (0.1)	-
HTLC	0.5 gfe (0.1)	0.6 gef (0.2)	0.5gf (0.2)	0.5 fe (0.2)	0.5 d (0.2)	0.4 e (0.1)	-	-
LTMC	0.6 dfe (0.2)	0.7 def (0.2)	0.6dgfe (0.2)	0.6 dfe (0.1)	0.6 cd (0.1)	-	0.6 d (0.1)	-
MTMC	0.8 dc (0.2)	0.9 c (0.2)	0.9c (0.2)	0.9 c (0.2)	0.8 b (0.2)	-	0.7 cd (0.2)	-
UTMC	0.7 dce (0.1)	0.8 dc (0.1)	0.8dce (0.1)	0.7 dce (0.1)	0.7 cb (0.1)	0.7 cd (0.1)	-	-
HTMC	0.7 dce (0.1)	0.7 def (0.1)	0.7dfe (0.1)	0.6 dfce (0.1)	0.6 cd (0.1)	-	0.6 d (0.1)	-
LTHC	0.6 gfe (0.2)	0.6 gef (0.2)	0.6gfe (0.2)	-	-	-	-	-
MTHC	0.5 gfe (0.1)	0.5 gf (0.1)	0.6gf (0.1)	-	-	-	-	-
UTHC	0.5 gf (0.1)	0.6 gef (0.1)	0.5gf (0.1)	-	0.5 d (0.1)	-	-	-
HTHC	0.7 dfe (0.2)	0.7 de (0.2)	0.7dfe (0.1)	0.7 dce (0.2)	-	-	0.6 d (0.1)	0.6 c (0.1)
BG	0.6 gfe (0.1)	0.6 gef (0.1)	0.6gfe (0.1)	0.6 dfe (0.1)	-	0.5 ed (0.1)	-	0.4 d (0.1)
PG	0.5 g (0.1)	0.4 g (0.1)	0.4g (0.1)	0.4 f (0.1)	-	0.4 e (0.1)	-	-
SDC	0.8 c (0.4)	0.8 dc (0.4)	0.9c (0.5)	0.9 c (0.5)	-	0.7 cd (0.4)	0.7 cd (0.4)	-
SDO	1.1 b (0.1)	1.1 b (0.1)	1.2b (0.1)	1.1 b (0.1)	-	0.8 cb (0.1)	0.9 cb (0.1)	-

Note: Means with same letter in the same column are not significantly different ($p<0.05$)

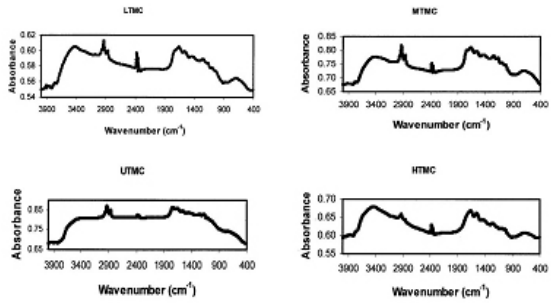

Figure 4. FTIR spectra of fish skin gelatin extract using 0.1 N acetic acid solution at different temperature. LTMC: 4°C and 0.1 N, MTMC: 20°C and 0.1 N, UTMC: 50°C and 0.1 N, HTMC: 80°C and 0.1 N

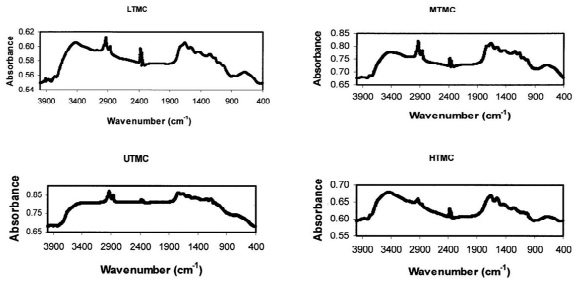

Figure 5. FTIR spectra of fish skin gelatin extract using 1 N acetic acid solution at different temperature. LTHC: 4°C and 1 N, MTHC: 20°C and 1 N, UTHC: 50°C and 1 N, HTHC: 80°C and 1 N

1988). The intensity of amide III band has been also associated with the triple helical structure (Muyonga *et al*., 2004). In this study the ratio between the amide III band (1245-1240 cm^{-1}) and amide I band (1660-1650 cm^{-1}) was used to observe the loss of secondary structure of collagen and formation of random coil structure. This technique was used due to the difficulty faced in using absolute peak intensity to compare between different samples. An increase in amide I band intensity is related to an increase in random coil at the expense of the ordered secondary structure. Figure 6 shows the intensity ratio of amide III and I as a function of extraction concentration and temperature. The graph shows that all samples had almost similar protein structure at 0.1 and 1.0 N concentration for all extraction temperatures. At 0.01 N concentration and low temperatures (4, 20, and 50°C), significant amount of triple helix remained intact. As the temperature increased from 20°C, the random coil structure increased as the protein become denatured and protein lost its triple structure. Thus, the low temperature/low acidity condition showed little effect on both yield (results not shown) and denaturation power as it is shown in the graph in Figure 6. Earlier reports showed similar results suggesting use of high temperature for extraction

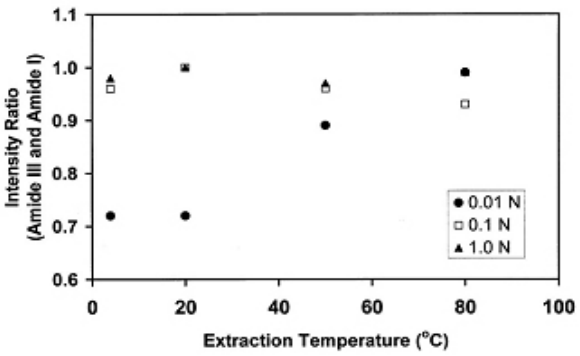

Figure 6. Ratio of amide III and amide I as a function of extraction concentration for different concentration

and impracticality of low temperature for gelatin extraction (Bailey *et al*., 1998). This study shows that low temperature could be compensated with medium (0.1 N) to high acid (1.0 N) concentration of acid condition. As the temperature increased (50°C) the loss of triple helical structure was seen to be more pronounced at low acid concentration and the protein is more likely to have random structure. Earlier observation indicated that high temperature and high acidity caused degradation on protein chains and produced protein with low molecular weight. These low molecular weight proteins at the time of drying (by freeze drying) came together and formed helical structure. This rearrangement is not possible with higher molecular weight proteins which were produced by low acid condition (Muyonga *et al*., 2004). However in these studies this degradation was not observed as the ratio of amide III/amine I at high concentration and high temperature was reduced.

Muyonga *et al*. (2004) determined the FTIR spectra of Nile perch skin collagen and gelatin at 50 and 70°C. They showed that in some regions the spectra was so complex and rugged as result differentiation between different extracted gelatins was not possible. In this case multivariate analysis (RCA) was performed on the spectra for discrimination and classification. Hashim *et al.* (2010) used the deformation of N-H bonds in the range 3290-3280 cm^{-1} and 1660-1200 cm^{-1} for discriminate analysis. These regions were found to give information on the origin of the gelatin. Principal component analysis of the spectra in these regions clearly showed different locations of porcine and bovine gelatin in the biplot. The spectra in this study showed minimal variation within 2900-1900 cm^{-1}, thus this region was not included in our analysis. Figure 7 shows dendogram based on the cluster analysis. Three major clusters could be identified, however gelatin extracted at low temperature and concentration was completely different from other samples. The 3 cluster groups could be visualized by

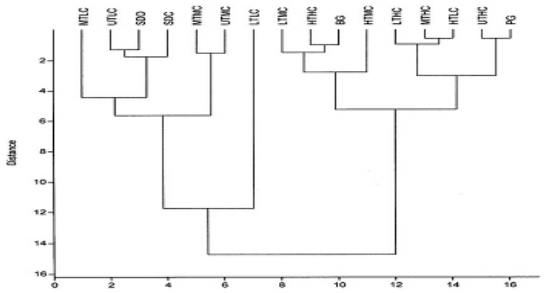

Figure 7. Dendogram showing 3 major classes of gelatin extracted using different extraction conditions

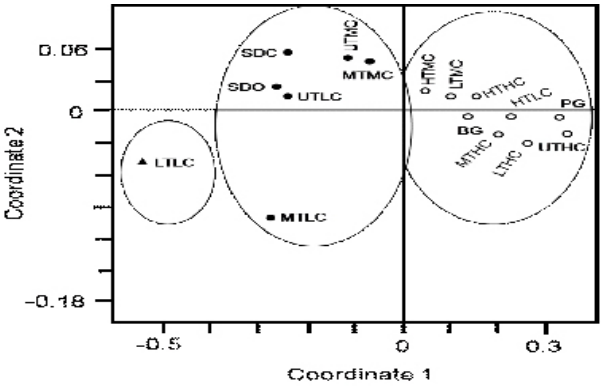

Figure 8. Principal component analysis showing 3 cluster groups in the biplot

principal component analysis as shown in the biplot of Figure 8. The gelatin extracted at 4°C and 0.1 N, 50°C and 1.0 N, 80°C and 0.01 N, and 20°C and 1.0 N are the closest neighbors of the bovine gelatin. This indicated that a combination of temperature and concentration extraction could be used to develop the desired structural characteristics of the extracted gelatin.

Conclusions

Commercial bovine and porcine gelatins; and shaari fish skin gelatin extracted at different temperature (4, 20, 50 and 80°C) and acetic acid concentrations (1.0 N, 0.1N and 0.01) were studied by FTIR technique. FTIR analysis showed similar spectra for commercial bovine and porcine gelatins and for shaari fish skin gelatin extracted at a temperature of 4°C and at 0.01 N acetic acid concentrations. It also showed that acid concentration and temperatures of extraction had an effect on the conversion of collagen to gelatin and on the loss of molecular order of triple helix. Low concentrations and low temperatures caused low disturbance to the native structure of collagen, i.e. a significant amount of triple helix remained. Samples extracted at all temperatures for medium and high acid concentration showed almost same type of random structures with denatured protein. The cluster analysis on the spectra identified 3 classes of gelatin produced by different treatments. Mild treatment (4°C and 0.01 N) showed completely

different spectra as compared to other treatments. Different desired structural characteristics of fish skin were possible to develop using a specific temperature and concentration of extraction solution.

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