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ATR-FTIR Spectroscopy For Discrimination Of Gelatin From Different Sources: A Comparative Study.

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ABSTRACT

Gelatin is a fibrous protein that is widely used in several industrial food, cosmetic, pharmaceutical and medical applications. Gelatin is obtained from porcine and bovine sources. In this study, attenuated total reflection Fourier transform infrared spectroscopy (ATR-FRIR] was utilized to discriminate between gelatin from cold water fish skin (GF], type B from bovine skin (GB] and Type A from porcine skin (GA]. ATR-FTIR spectra of GF, GB, and GA were recorded and the absorption ratios, 1630 cm^{-1}/1527 cm^{-1}, 1239 cm^{-1}/1630 $cm^{-1}$and 1239 cm^{-1}/1448 cm^{-1} were calculated, difference spectra method, curve fitting analysis and second derivative of the amide I bands were performed. The results revealed that GA has the highest value of the absorption ratio of 1630 cm^{-1}/1527 cm^{-1} as compared to those of GF and GB. Moreover, all gelatin types have β sheet, helix, Random coil and β turn protein secondary structure but with different proportions. GB and GF were rich in β sheet content while GA possesses a large amount of random coil and triple helix. It can be concluded that conventional ATR-FTIR spectroscopy discriminated clearly between the three types of tested gelatin and it will be a simple and rapid method for differentiation between other types of gelatin. .

Keywords: ATR-FTIR spectroscopy, gelatin, fish, bovine, porcine.

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INTRODUCTION

Fourier transform Infrared spectroscopy (FTIR) is a powerful tool for structure determination of small molecules because of their sensitivity to the chemical composition and arrangement of molecules. FTIR spectroscopy has been used in the biological and medical fields. The biological materials proteins, carbohydrates, lipids, and nucleic acids have specific functional groups accordingly they obtain special spectral fingerprints in infrared region [1]. So FTIR spectroscopy is a valuable technique for the identification of protein structure and follows the protein reactions [2-4] and determination of protein conformation structure [5-6].

Gelatin is considered as a biopolymer and is widely used in several applications such as the food industry, cosmetic, pharmaceutical and medical applications. Gelatin is a fibrous protein that is obtained from the partial thermal hydrolysis or denaturation of collagen which is the main constituent of mammalian connective tissues. Skins, bones, and hides of mammalian are the main sources of collagen [7].

Different types of gelatin are obtained according to the treatment of the raw tissue. Alkaline treatment for bovine skin yield gelatin of type B while the acidic treatment for porcine skin gives type A gelatin [8, 9]. Fish gelatin, is another type of gelatin that is extracted from the skin of different fish species [10,11]. Fish gelatin had more attention to be alternative for the others mailman gelatin types that is for religious reasons and to avoid the infection with bovine spongiform encephalopathy virus [12,13].

The amino acid composition of gelatin was different according to its original sources of raw materials. Some studies for amino acids of gelatins extracted from bovine skin gelatin (type B) and porcine skin gelatin (type A) was found that both types contain different amount of glycine, arginine, proline, and hydroxyproline according to their original source [12, 13].

Different methods were reported on how to discriminate between various types of gelatin from different sources. For example, depending on the efficiency of these types on calcium phosphate precipitation, bovine bone gelatin was found to be more efficient to precipitate calcium phosphate than porcine skin [15]. Other researchers used electrophoresis and polymerase chain reaction for identification and verification of gelatin type in capsule shells [16]. FTIR-ATR combined with chemometrics analysis were used for distinguishing between different kinds of gelatin [17] and gelatin gummy candies according to their gelatin origin [18]. Others used FTIR spectroscopy for investigation of the relative relation between Amide I and Amide III components of collagen type I as a result of the thermal denaturation of the protein [19].

The objective of our work is to find out a rapid and simple spectroscopic marker/markers for discriminating between gelatin derived from porcine skin and gelatin of bovine and cold water fish skin origin.

MATERIALS AND METHODS

Type A gelatin from porcine skin, type B gelatin from bovine skin and gelatin from cold water fish skin was purchased from Sigma Aldrich.

Triplicate ATR-FTIR spectra of each tested gelatin powder (GA, GB & GF) were recorded by using Bruker FTIR (VERTEX 70/80) instrument in the range between 4000 cm^{-1} to 500 cm^{-1} with a spectral resolution of 4 cm^{-1} in the form of potassium bromide (KBr) discs. Typically, 40 scans were signal-averaged for a single spectrum to decrease the signal to noise ratio. Background spectra, which were collected under identical conditions, were subtracted from the sample spectra automatically. Each spectrum was normalized and baseline corrected by using OMNIC 8.3 software program (Thermo Fisher Scientific Inc., Massachusetts, USA). To increase the resolution of the overlapping bands second derivative and Gaussian decomposition were used to localize the position of the bands in the spectra over the amide I and amide II bands in the range (1700 cm^{-1}-1500 cm^{-1}) by using the same software. The second derivative obtained under the conditions -Savitsky-Golay derivative, third grade polynomial and 7 point.

The difference method between the examined average spectra were carried by using IR solution software.

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RESULTS AND DISCUSSION

Some previous works for discrimination different types of gelatin were based on chemical methods. One of them is built on calcium phosphate precipitation according to the different affinity of gelatin types to precipitate it [15]. Whereas other authors distinguish between bovine and porcine skin gelatin depending on their chemical properties such as their amino acids content and polypeptide profile [12]. Other workers applied polyacrylamide gel electrophoresis coupled with principal component analysis, and polymerase-chain-reaction-restriction fragment length polymorphism for detecting and distinguish the type of gelatin content in capsule shells [13]. Some researchers determined the authentication of gelatin by species-specific polymerase chain reaction method [20, 21].

It is known that the chemical methods are more expensive and required long time for preparations and measurements whereas FTIR spectroscopy region (4000 cm^{-1}- 400 cm^{-1}) in particular supplies information on very large number of functional groups, and the absorption band are sensitive to the physical and chemical content of individual constituents. FTIR spectroscopy is a chipper and fast technique, it has no requirement for sample preparation, and its spectrum is recorded in a few minutes.

The averaged ATR-FTIR spectra of the tested gelatin types (GA, GB and GF) were showed in Figure (1a). Visual examination of this Figure showed that all gelatin spectra, regardless of their type, have the same spectral signatures apart from slight changes in some bands intensities and bands position shift. Over the region 3300 up to 3000 for all spectra a broad high intense absorption band was observed at about 3280 cm^{-1} which was assigned mainly to the overlapping of OH and NH stretching vibration modes of the peptide groups in amide A [22]. The absorption band at 3072 cm^{-1} which was assigned to amide B that arises from NH stretching modes in proteins [23]. Whereas the spectral region between 3000- 2800 cm^{-1} has the absorption band at 2931 cm^{-1}, 2934 cm^{-1}, and 2928 cm^{-1} for GA, GB and GF respectively, this absorption band was interpreted for the C-H asymmetric of CH_2 group [24]. Also another band was observed in the GA and GB spectra at 2876 cm^{-1} which was originated from C-H symmetric vibration of CH_3 [25]. The absorption band localized at 1630 cm^{-1} for GA and GB spectral samples and at 1632 cm^{-1} for GF was assigned to amide I which arises mainly from the C=O stretching vibration of peptide backbone with minor participations from the out-of-phase CN stretching vibration, the CCN deformation and the NH in-plane bend. [26]. The appearance of the amide I at 1630 cm^{-1} -1632 cm^{-1} for gelatin in our study was in coincidence-with the result of other researcher. They referred to the absorption peak at about 1633 cm^{-1} to the characteristic of the coiled structure of gelatin [27]. The amide II mode observed at 1529 cm^{-1} for GA and at 1527 cm^{-1} for GB and GF arises from the N-H bending vibration strongly coupled to the C-N stretching vibration of protein amide groups [28]. Asymmetric CH3 bending and deformation of the methyl groups of proteins were represented by the presence of the absorption bands at about 1447 cm-1 and 1406 cm-1, respectively [29, 30]. The absorption band at 1334 cm^{-1} was assigned to CH_2 wagging, and that at1239 cm-1 was referred to amide III as well as asymmetric phosphate [PO2] stretching vibrations. The amide III is the combination peaks between C-N stretching vibrations and N-H deformation from amide linkages in addition to wagging vibrations from CH2 groups of the glycine backbone and proline side-chains [2]. The band at 1081 cm-1 was interpreted as collagen and phosphodiester groups of nucleic acids [31]. The absorbance ratios of the bands 1630 cm-1/1527 cm-1, 1239 cm-1/1630 cm-1, 1239 cm-1/1527 cm-1 and 1239 cm-1/1448 cm-1 are represented as histograms in Figure (1b). It is observed from this Figure that GA has the highest value for amide I to amid II ratio among the other types while GF has the lower value than that of GB and this trend is reversed to the ratio of 1239 cm-1 /1448 cm-1 which indicated that GA has the highest amount of unfolded protein as compared to GB and GF. This result is confirmed with other researchers. They found that the decrease in the ratio of 1239 cm-1 /1448 cm-1 is a result of partial denaturation for collagen and gelatin [13, 32].

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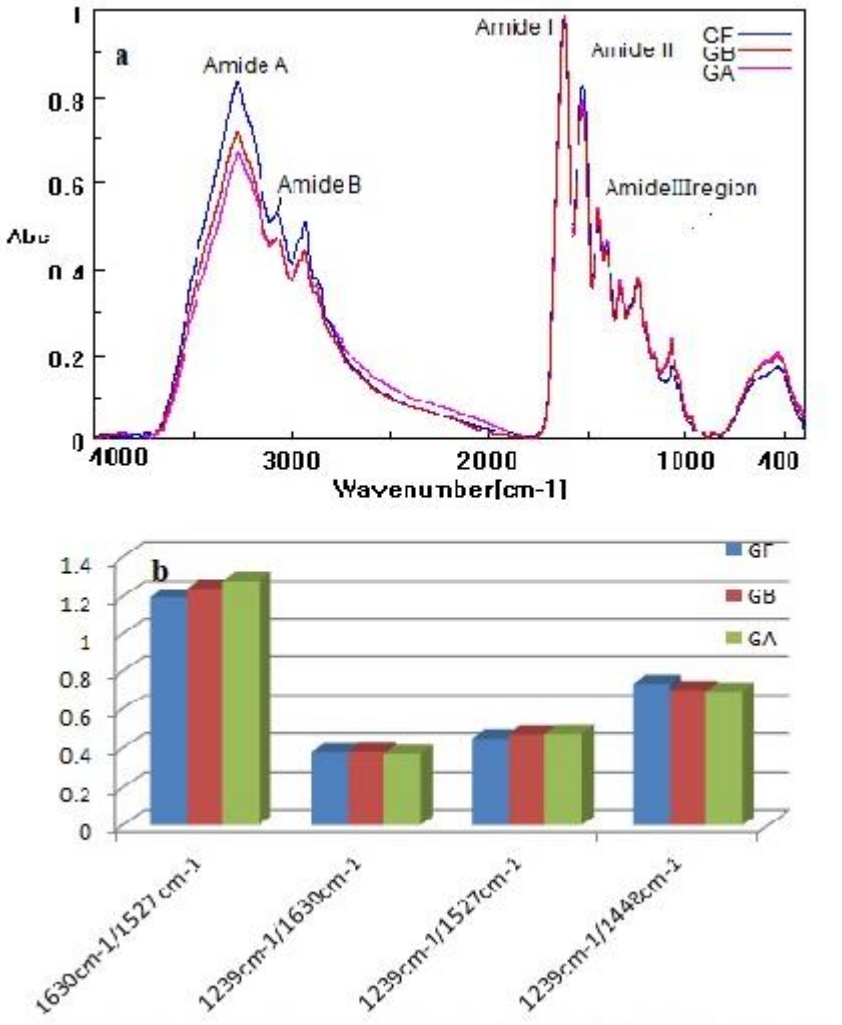

Figure1: ATR-FTIR spectra of gelatin types used (a) and their absorbance ratios of the bands at 1630cm^{-1}/1527cm^{-1}, 1239cm^{-1}/1630cm^{-1}, 1239cm^{-1}/1527cm^{-1} and 1239cm^{-1}/1448cm^{-1} (b).

Whereas the two other ratios 1239 cm^{-1} /1630 cm^{-1} and 1239 cm^{-1} /1527 cm^{-1} are nearly equal for all types of gelatin used.

To evaluate the difference between GA, GB and GF samples over the amide I region, difference spectra method was carried (Figure 2). The results Figure (2a) showed several positive peaks of protein secondary structure of GA, which are dominated by β turn and random coil structure. Meanwhile, the negative peaks of the GB protein secondary structure are mainly of the β sheet. On the other hand Figure (2b) it was observed that GA has a broad positive peak centered at 1617 cm^{-1} that assigned as side chain [33], while several negative peaks for the proteins secondary structure of GF are mainly representing to α helix and β turn and random coil structure. The subtraction of GF from GB spectrum (Figure 2c) resulted in three positive peaks for protein secondary structure for the GB that is assigned to β sheet structure, on the other side, seven negative peaks are observed for GF those indicated to α helix and β turn structure composition of GF.

For further investigations of the previous IR results, the second derivative of the FTIR spectra of the examined samples were carried over the amide I region (Figure 3). The amide I vibration is more resistive to the nature of the side chain effect. It mainly depends on the secondary structure of the backbone of the peptide chain therefore it is most common used for the determination of protein secondary structure [25].

Bands at about 1658 cm^{-1} and 1650 cm^{-1} were assigned to the α-helix structure as a result of the study of the IR spectra of α-helical proteins [34]. Also they coincide with the theoretical calculation [35]. Bands

near 1663 cm^{-1} were assigned to 3_{10} helices [34-36], also other authors referred these bands to a triple helix structure [39- 40]. Several authors assigned the bands in the regions of 1640 cm^{-1} - 1620 cm^{-1} and 1695 cm^{-1} - 1690 cm^{-1} to β-sheet protein secondary structure [34, 35]. The assignment of bands at about 1670 cm^{-1}, 1683 cm^{-1} and 1688 cm^{-1} were referred to β-turns. Turns are also associated with a characteristic band around 1665 cm^{-1}. The random coil conformation was assigned to the band between 1640 cm^{-1} and 1648 cm^{-1} [34].

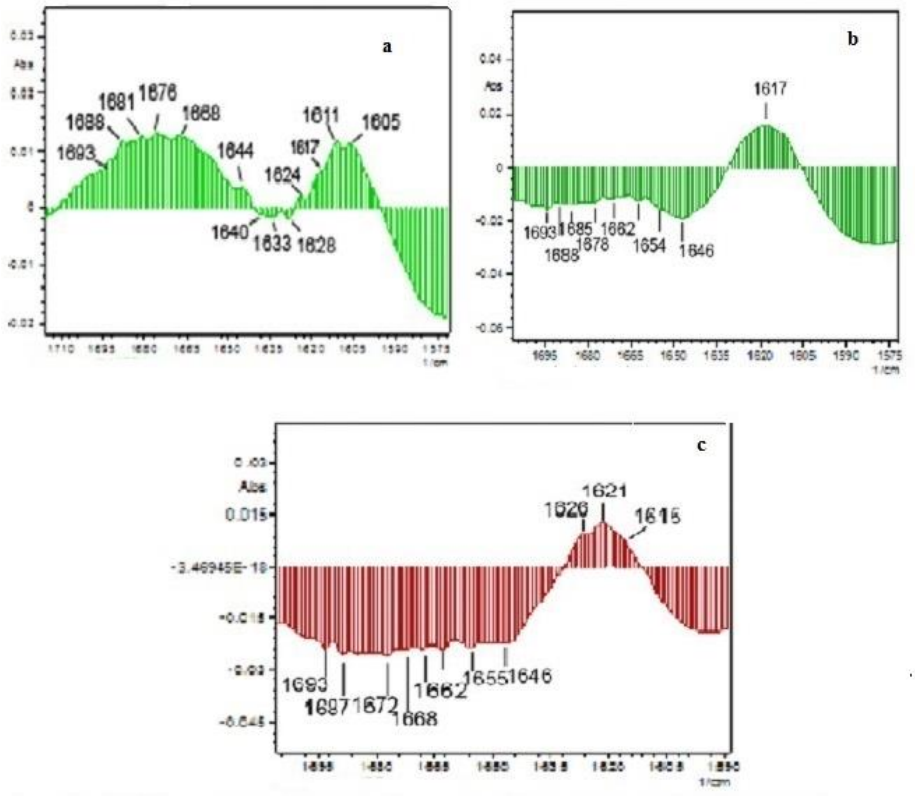

Figure 2: The difference spectra of amide I region of ATR-FTIR spectra of gelatin types under investigation, subtraction of GB from GA (a), GF from GA (b) and GF from GB (c).

Figure (3) shows that the second derivative of GA spectra has a broad and low number of troughs. Whereas those of the two other types are nearly similar and have many troughs with different depths. The observed troughs in all types second derivatives spectra indicated that all gelatin types under investigation have β-sheet, helical, random and β-turn protein secondary structure with different amounts.

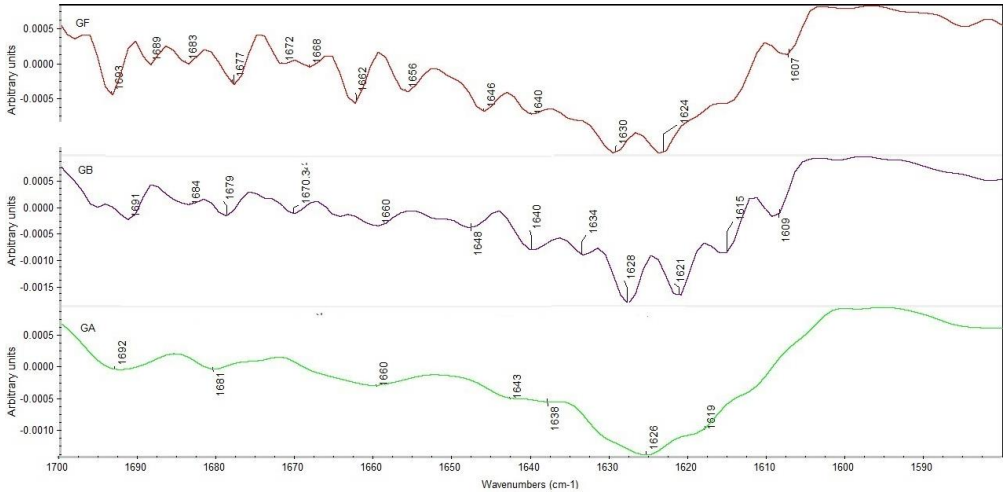

Figure 3: The second derivative of amide I region of ATR-FTIR spectra of gelatin types under study.

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The curve fitting of the amide I and amide II for ATR-FTIR spectra of gelatin types under examination are represented in Figure (4) and the amide I components positions, half band width [HBW] and areas are collected in table 1. The assignment of these component bands and their percentage area are recorded in table 2.

Figure (4) and table 1 show that the curve fitting of the amide I region for each gelatin is different in the numbers, sub-bands positions, HBW and areas of its components. The amide I component bands for GF are observed at 1621.84 cm^{-1}, 1630.19 cm^{-1}, 1638.37 cm^{-1} and 1695.40 cm^{-1} for β sheet, 1668.93 cm^{-1}, 1676.03 cm^{-1}, 1682.52 cm^{-1} and 1688.68 cm^{-1} for β turns and 1646.53 cm^{-1}, 1654.43 cm^{-1}, 1661.84 cm^{-1} for random coil, α-helical and triple helix structure respectively. Meanwhile GB amide I sub-bands located at 1622.66 cm^{-1}, 1636.03 $cm^{-1}$and 1693.54 cm^{-1} represent β sheet structure while the component bands at 1669.33 cm^{-1}, 1680.84 cm^{-1} are assigned to β turns conformation. The random coil and α-helix of GB amide I component bands found at 1647.57 cm^{-1} and 1658.55 cm^{-1} respectively. The curve fitting for amide I of GA result in the component bands at 1629.29 cm^{-1} and 1692.68 cm^{-1}, 1643.56 cm^{-1}, 1660.64 cm^{-1} and 1677.45 cm^{-1} which are indicted to β-sheet, Random coil, β-turns, and triple helix respectively.

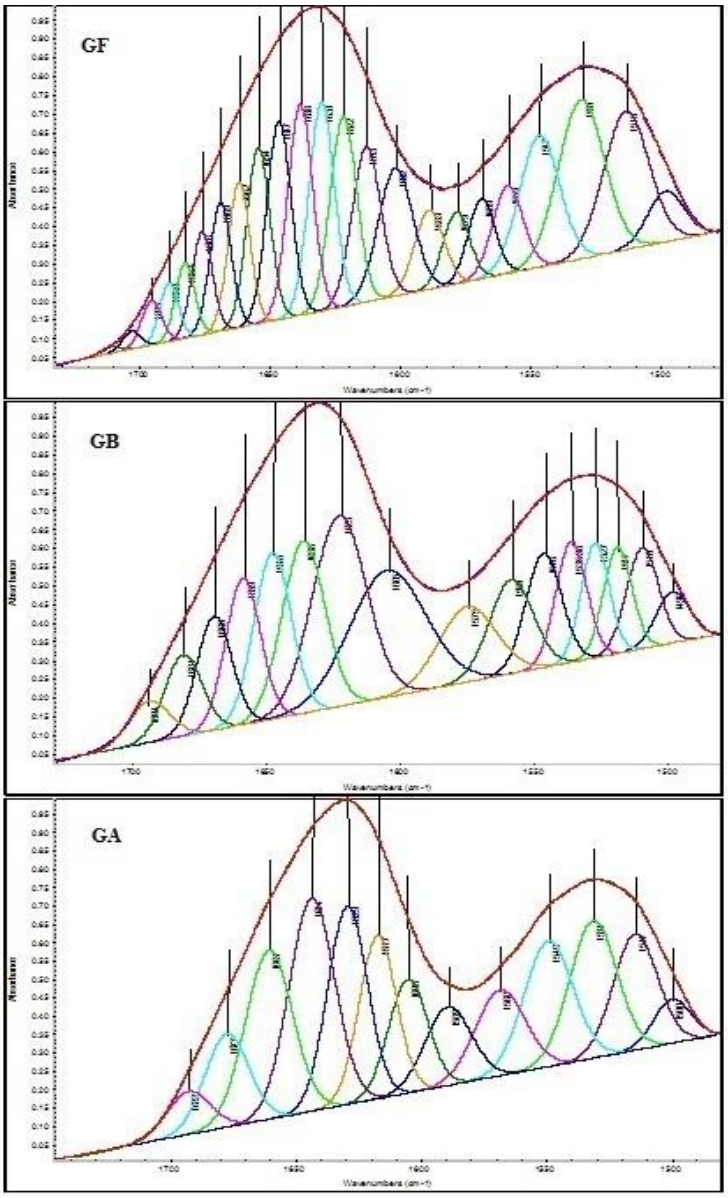

Figure 4: The component bands of amide I and amide II curve fitting of the gelatin types under investigation [based on their derivative]. The red line represent the original spectrum and the blue line represent the fitted one.

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Table 1: The band position, HBW and the area of the component bands of amide I curve fitting for ATR-FTIR spectra of GF, GB and GA.

GF			GB			GA		
Band position cm^{-1}	HBW	Area	Band position cm^{-1}	HBW	Area	Band position cm^{-1}	HBW	Area
1601.829	15.516	5.6498	1604.764	33.17	12.0129	1605.077	18.298	6.0419
1612.957	12.862	5.6921				1617.252	17.393	8.2493
1621.837	11.78	6.4079	1622.662	23.611	12.853			
1630.191	11.309	6.7432				1629.294	18.535	10.6716
1638.372	11.082	6.6649	1636.028	18.688	9.1715			
1646.53	10.779	6.1049	1647.566	16.318	7.741	1643.555	21.319	13.1712
1654.432	10.343	5.1934	1658.552	15.233	6.3672			
1661.837	9.651	4.0143				1660.644	22.097	10.8124
1668.929	9.554	3.5097	1669.325	15.206	4.956			
1676.026	9.1	2.6786				1677.447	20.521	5.5523
1682.516	8.405	1.8275	1680.838	16.268	3.7824			
1688.682	8.297	1.3909						
1695.396	9.002	1.1408	1693.5375	17.801	2.0911	1692.677	20.19	2.5173

The above results show that all gelatin types have β sheet, random coil and β turn protein secondary structure with different percentages. GA has the highest percentage of random coil (23.1%) content than GB (13.1%) and GF (10.7%) which is consistent with the result obtained by the difference method. GF and GB have 9.11% and 10.8% α-helix structure from amide I respectively while GA has no component represented α-helix but it has one at 1660.64 cm^{-1} and also GF has one at 1661.84 cm^{-1} which acts to triple helix. These difference in gelatin types composition may be referred to their difference in the origin and the pre-treatment of the parent collagen. This result is in coincidences with those reported that collagen contains a triple helix structure fiber. During the acid or alkaline or enzymatic hydrolysis of collagen hydrogen bonds of its triple helix were breakdown resulting in one, two or three random chain gelatin molecules. On the cooling process for the resulting gelatin some chains partially restored to triple helix structures like the parent collagen [40-42] Generally, the chemical content of gelatin is similar to that of the parent collagen [43].

Table 2: The band position and the percentage area of the component bands of amide I curve fitting for ATR-IR spectra of GF, GB and GA.

GF		GB		GA		
Band position cm-1	% area	Band position cm^{-1}	% area	Band Position cm^{-1}	% area.	assignment
1601.83	9.91	1604.76	20.37	1605.08	10.6	Side chain
1612.96	9.98			1617.25	14.47	Side chain
1621.84	11.24	1622.66	21.79			β sheet
1630.19	11.83			1629.29	18.72	β sheet
1638.37	11.69	1636.03	15.55			β sheet
1646.53	10.71	1647.57	13.13	1643.56	23.1	Random coil
1654.43	9.11	1658.55	10.80			α helix
1661.84	7.04			1660.64	18.96	triple helix
1668.93	6.16	1669.33	8.40			β turn
1676.03	4.7			1677.45	9.74	β turn
1682 .52	3.21	1680.84	6.41			β turn
1688.68	2.44					β turn
1695.40	2	1693.54	3.55	1692.68	4.42	β sheet

The total percentage area of amide I sub-bands for each conformation of protein secondary structure are illustrated by histograms Figure (5). It is clear from this Figure that β sheets are the most protein secondary structure content of GB (41%) and GF (37%) comparing with their content of random coil, α-helix and β turn while GA has nearly equal percentage for β sheets (23.2%) and random coil (23.1%) structure. The high

amounts of the total percentage area for β sheets, β turn, and random coil content of GB, GF, and GA respectively are confirmed with our result obtained by the difference method. Also, GA has a high quantity of triple helix (19 %) as compared to that of GF (7%) content. Whereas GB has no triple helix but it has an α-helix structure that is more than that of GF content. These results are confirmed with that obtained by other workers who stated that the extracted gelatin contains a

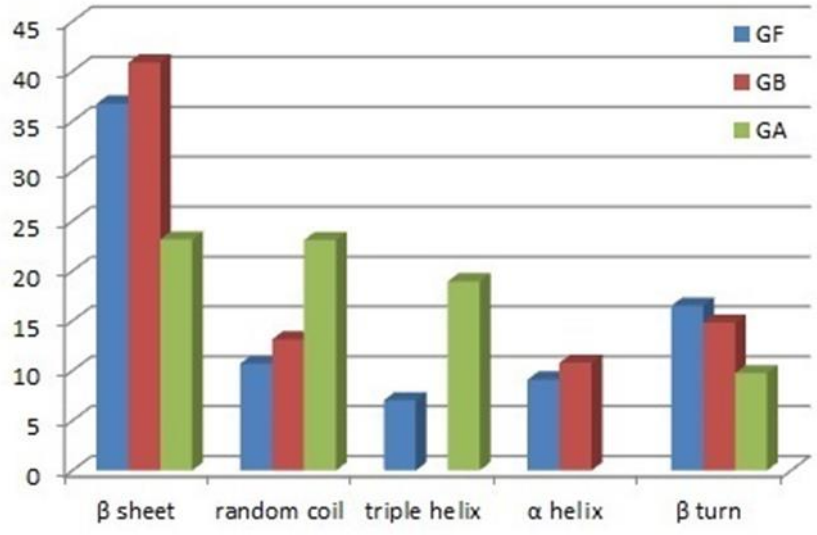

Figure 5: The total percentage area of amide I sub-bands of different conformation protein second structure

mixture of polypeptide/polymer chains with different molecular weights containing α-chains, β -chains, γ-chains and their fragments. α-Chains are intact polypeptide chains of collagen, while β are covalently cross-linked double and γ - chains are triple compositions of α –chains [39].

CONCLUSION

The study of ATR-FTIR spectra for Amide I of GF, GB and GA indicated that all these types of gelatin used have β-sheet, helical, random and β-turn protein secondary structure but with different amounts. GB is characterized by having a high amount of β sheets structure content as compared to GF and GA. It can be differentiated between GF and GB by the higher quantity of β turn and the appearance of triple helix sub-band in GF spectra. Whereas GA is characterized by having a large amount of random coil and triple helix protein secondary structure. FTIR spectroscopy can discriminate between gelatin types used in this study so it acts as a signal light that may be used as a simple and rapid method for differentiation between other types of gelatin but needs further study.

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