# Influence of Crosslinkers and Crosslinking Method on the Properties of Gelatin Films Extracted from Leather Solid Waste

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**ABSTRACT:** Gelatin was extracted from chrome tanned leather waste with the aim to produce a durable coating or film. Crosslinking has shown to improve the physical performance of a film/coating. The effect of the method of crosslinking and the use of different crosslinking agents were studied. The extracted gelatin was crosslinked either by immersion of preformed films into a crosslinker solution (Method A) or by the addition of the crosslinking reagent to the gelatin solution prior to film formation (Method B). The different results obtained between both methods may be due to: the relative concentration of crosslinking reagent, the introduction of crosslinks within different regions of gelatin (triple helical regions and random coil regions), and the reaction rate. Method A of crosslinking is more likely to form crosslinks outside but close to the triple helical regions, disrupting the order and stability of the helical structure. Crosslinks may form preferentially within the random coil regions when Method B of crosslinking is used. Both methods led to the formation of chemical crosslinks in the extracted gelatin films, as demonstrated by the reduction of the degree of aqueous swelling and the proportion of low molecular weight fractions. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 119: 2105–2111, 2011

**Key words:** crosslinking; collagen; gelatine; biopolymer; film formation; swelling

## INTRODUCTION

Solid leather waste, such as chrome shavings, requires a feasible treatment to reduce its potential environmental hazard. Chrome tanned shavings may be treated to produce virtually chromium-free leather shavings, leaving the collagenic components of the leather unaffected.[1] Thermal denaturation or physical and chemical degradation of collagen involves the breaking of the triple-helix structure into random coils to produce gelatin. Previous studies have demonstrated that protein products can be isolated from chrome-tanned shavings using one- and two-step enzymatic processes.[2–4] The present one step process[1,5] is cost effective in terms of time, chemical, and waste production. Due to its collagen base, the molecular structure of gelatin may comprise of both triple helical and random coil regions imparting multifunctional properties.[6,7] Gelatin is therefore used for a wide range of applications, such as films or coatings in the food (casing) and pharmaceutical (encapsulation) industries.[5,6,8] The main goal of biological films is to replace existing synthetic, nonbiodegradable products at the lowest cost possible; focusing on improving quality and shelf life, protecting, and maintaining product integrity and enhancing product appearance.[6]

The practical use of gelatin extracted from leather waste, as a material, may be limited by its relatively poor physical properties whereupon the material may disintegrate upon handling.[6] To improve the product properties, it is often necessary to introduce exogenous crosslinking into the molecular structure of the gelatin.

The crosslinking mechanism has been classified into two types of crosslinks, intrahelical and interhelical.[9] The distance between gelatin molecules will determine if the crosslinks formed are intra or intermolecular.[10] The first type includes the bonds formed between two polypeptide chains in the same helices and influences the denaturation temperature. These crosslinks can stabilise the triple-helical regions of the gelatin, but do not increase mechanical strength.[11] The second type, interhelical crosslinks are formed between polypeptide chains of two adjacent helices, increasing the molecular weight of the gelatin, and affecting properties such as swelling and flexibility of the gelatin.

The application of gelatin as a coating involves formation of films directly on the surface of the object they are intended to protect or enhance.[6] For

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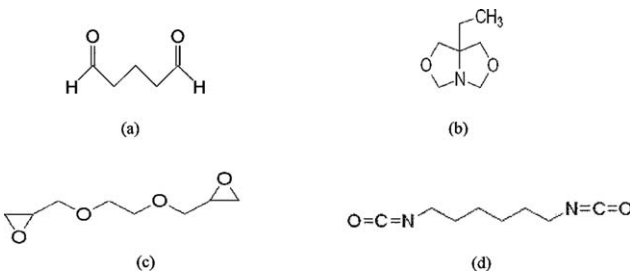


**Figure 1** Crosslinkers used in the study: (a) Glutaraldehyde (GTA), (b) 5-ethyl-1-aza-3,7-dioxabicyclo[3.3.0] octane (Oxazolidine II), (c) Ehylene Glycol Diglycidyl Ether (EGDE), and (d) Hexamethylene diisocyanate (HMDC).

a coating application, this crosslinking can be applied in different ways: the first Method A, is the formation of a protein/gelatin film on the surface of the object to coat, followed by the application of a crosslinking solution over the surface of the already formed film. The second Method B is based on the application of an already mixed solution of protein and crosslinker on the surface of the object.

Several different methods of crosslinking to improve durability and strength of films have been reported in literature. Physical methods include dehydrothermal treatment, photo-oxidation, segmental orientation, and ultraviolet and gamma radiation.[12–15] Chemical crosslinking uses agents, such as aldehydes, carbodiimides, epoxy compounds, and acyl azides.[13,14,16] These agents chemically form covalent inter and/or intramolecular links between protein chains.[6]

Glutaraldehyde (GTA) [Fig. 1(a)] is currently the most widely used aldehydic crosslinker[16–20] and for this reason it was used as a reference crosslinker. The reaction is rapid, complex, and essentially irreversible; GTA introduces crosslinks through Schiff's bases.[10] Olde-Damink[21] (1995) explains the crosslinking of collagen with GTA through the reaction of the amine groups of lysine and hydroxylysine residues of collagen with the aldehyde groups of GTA, forming intra and intermolecular crosslinks.[15,16] Due to GTA polymeric nature, crosslinks of various lengths may be formed.[18] A concentration of 0.05% (v/v) GTA is able to crosslink about 60% of the ε-amino groups.[16]

Oxazolidines [Fig. 1(b)] are heterocyclic compounds used as retanning agents in leather processing. The reaction with the leather is achieved through the opening of the cyclic rings of its molecule, and the subsequent reaction with the amine groups of the collagen.[22–26] Research studies have been conducted with oxazolidine and collagen in retanning applications, however, further research is required for its use as a protein crosslinker.

Ethylene glycol diglycidyl ether (EGDE) [Fig. 1(c)] has been widely applied in the chemical industry; however, its use as a protein crosslinker is relatively recent. Reaction with epoxides can involve either the acidic or amino groups, the reaction rate is determined by the pH of the solution.[7,9] The epoxy functionality predominantly reacts with the amino groups on lysine,[27] similar to GTA, although the crosslinking reaction rate is slower.[28] The physical properties may improve and rendering the material more flexible.[29]

Hexamethylene diisocyanate (HMDC) [Fig. 1(d)] forms crosslinks with two amino groups via urea-type fractions.[30,31] The resultant material is a strong and resistant collagen material.

The aim of the present research is to find and efficient method to improve the stability and physical properties of the extracted gelatin for films and coatings. The effect of the method of crosslinking and the use of alternative crosslinking agents to GTA, such as epoxy compounds, oxazolidine, and diisocyanates will be investigated.

## EXPERIMENTAL

### Materials

Dechromed shavings were obtained after applying a dechroming process[32–34] on chrome containing leather shavings supplied by the tannery of the British School of Leather Technology of The University of Northampton, UK. GTA solution (50% (v/v)), EGDE, HMDC, 5-ethyl-1-aza-3,7-dioxabicyclo[3,3,0] octane (oxazolidine II), phosphate buffered saline (PBS), and 2-(N-morpholino)ethanesulfonic acid solution were of analytical grade and supplied by Sigma-Aldrich, UK. Disodium tetraborate and 2-propanol were supplied by BDH Chemicals, UK and Fisher Scientific, UK, respectively.

### Methods

#### Gelatin extraction and film preparation

Based on previous work,[1,5] the gelatin used in the following studies was obtained by thermal hydrolysis of 200 g/L of dechromed shavings in deionised water for 5 h at 80°C. To prepare gelatin films, a solution of the extracted gelatin (10 mL) was placed in a small Petri dish (55 mm diameter) and allowed to air dry at a constant temperature (20°C) and 60% relative humidity.[16,35] For all analyses, uncrosslinked gelatin was used as a control.

#### Crosslinking reagent solutions

All the crosslinking reactions were carried out at an equimolar concentration of 0.07*M* (equivalent to the GTA concentration 0.625% (v/v)[12,36,37]). The different crosslinking solutions (Fig. 1) were prepared as follows: the GTA solution was prepared in PBS

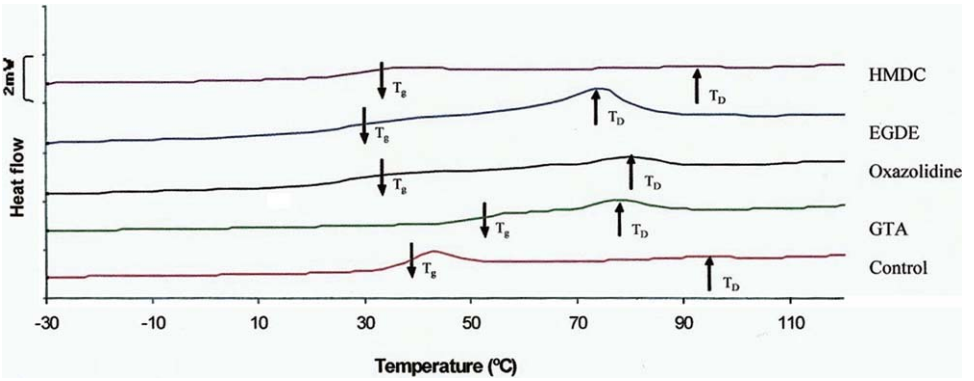


**Figure 2** Thermal plots of the control and films crosslinked with GTA, Oxazolidine, EGDE, and HMDC by Method A of crosslinking. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

buffer (pH 7.4). Oxazolidine II was prepared in 0.01*M* PBS (pH 7.4).[22,23] The EGDE solution was prepared by diluting the crosslinker in 0.025*M* disodium tetraborate solution (pH 9.0).[38] Gelatin films were equilibrated in a 2-propanol solution for 30 minutes and then immersed in a HMDC solution in 2-propanol.[39]

### Crosslinking films (Method A)

Samples were crosslinked by immersing the already prepared uncrosslinked extracted gelatin films in the different crosslinking reagent solutions. All the samples were crosslinked for 24 h at room temperature. After the prescribed period, the samples crosslinked with GTA were immersed in a 2% (w/v) glycine solution for 2 h to react with unreacted aldehyde groups in GTA and so prevent unwanted side reactions.[40] All the films were repeatedly washed with deionised water once the crosslinking reaction had been completed. The films were air dried at room temperature and stored at 20°C and 65% RH until required.

### Crosslinking gelatin solutions (Method B)

The crosslinking reagent was added to the extracted gelatin solution and mechanically stirred for 5 minutes. Agitation was required due to the speed of the crosslinking reaction with the gelatin. The mixture was poured into a Petri dish and allowed to air dry at room temperature (20°C and 65% RH).

## Characterisation of modified gelatin films

### Thermal analysis

Samples were stored in a humidity chamber at the prescribed relative humidity (60%) for a minimum of 48 h and 20°C, prior to analysis. The dry gelatin film (10 mg) was hermetically sealed in an aluminium pan and subjected to a double scan in a differential scanning calorimeter. The scans were carried out at a heating rate of 5°C/minute in the temperature range −50 to 120°C. All samples were run in triplicate.

### Swelling[35,41,42]

The films were weighed and immersed in a PBS solution for different periods of time. Wet samples were blotted with filter paper, to remove the surface water not taken into the gel, and reweighed. The amount of absorbed water was calculated as follows:

$$\text{Swelling } (\%) = 100(W_{\text{wet}} - W_{\text{dried}})/W_{\text{dried}}$$

where $W_{\text{wet}}$ is the weight of the film after being immersed in PBS solution for a determined period of time and $W_{\text{dried}}$ is the initial weight of the gelatin film.

### SDS-PAGE

Aliquots of 10 mg of gelatin were dissolved in 1 mL of sample buffer. The samples were denatured at 90°C for 5 minutes, and loaded in appropriate volumes (15 μL) onto a vertical acrylamide gel (4% (v/v) stacking gel, 7.5% (v/v) resolving gel). Standard markers, from 6.5 to 205 kDa were loaded with the samples. The gels were run at 0.01 mA/gel. Gels were stained overnight with Coomassie Brilliant Blue solution and then destained with methanol (10% (v/v)) and acid acetic (7% (v/v)) solution prior to analysis.

## RESULTS AND DISCUSSION

### Thermal analysis

The results of thermodynamic analysis are shown in Figures 2 and 3. The glass transition ($T_g$) and denaturation ($T_D$) temperatures for the gelatin films are shown in Table I. Whilst the glass transition was defined for all samples, the denaturation peak was

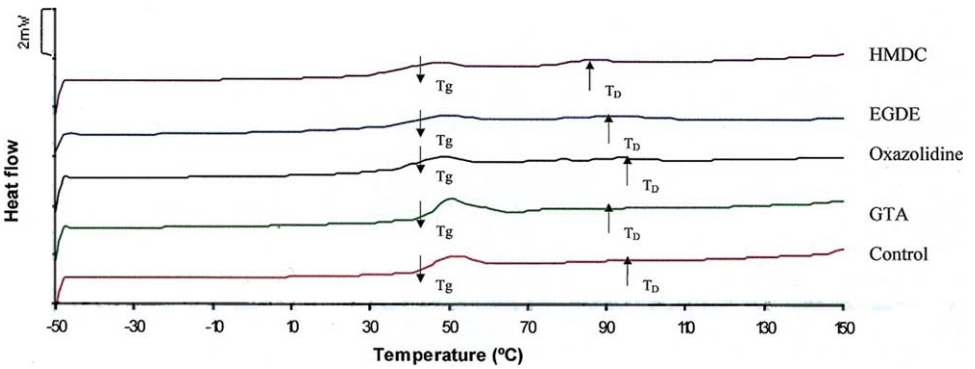


**Figure 3** Thermal plots of the control and films crosslinked with GTA, oxazolidine, EGDE, and HMDC by Method B of crosslinking. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

in some cases small and difficult to discern, particularly for material crosslinked by Method B. The results from the thermal analysis showed a clear difference between the two methods of crosslinking. A hypothesis to explain these results may be: the relative degrees of formation of crosslinks outside or inside the helical regions, the differences in the diffusion rates of the crosslinker into the extracted gelatin structure, and the relative probabilities of crosslinks forming.

The denaturation temperature ($T_D$) of collagen indicates the degree of its resistance to thermal denaturation. Research studies of crosslinked collagen[11,16,18,39] found that the denaturation temperature increased after crosslinking, this thought to be due to the stabilisation of the collagen structure. However, in this study, the denaturation temperature was found to decrease when comparing the crosslinked films with the control (Table I). Research[43,44] has shown a correlation between the glass transition temperature and crosslinker type. The studies showed that the glass transition temperature may decrease with crosslinking due to the crosslinks pushing polymer chains apart and so increasing free volume. The longer the crosslinker molecule, the more flexible the resulting crosslinked material and consequently the lower the glass transition temperature will be.[43]

The results obtained by Method A crosslinking (Fig. 2) showed that films crosslinked with longer chain crosslinkers, such as EGDE and HMDC presented the lowest glass transition temperatures, close to room temperature (25°C). Both crosslinkers have a long chain, which permits the gelatin molecules to have partial freedom of movement at a lower temperature, resulting in semiflexible films at room temperature. The films crosslinked with GTA showed a higher $T_g$ (51.7°C), which was evidenced by the hardness of the films at room temperature. GTA is a short chain molecule and an effective crosslinker with a high crosslinking density.[18,19,36] The short GTA crosslink restricts freedom of movement of the gelatin molecule, thus producing a high glass transition temperature.

In contrast to Method A, crosslinking with Method B, showed no large differences in $T_D$ between the different crosslinkers and the control (Fig. 3). All the films showed a glass transition and a denaturation temperature of $\sim$ 40 and 90°C, respectively, similar to the values found by Yakimets et al.,[45] who identified the glass-rubbery transition at 40°C and a denaturation temperature of 95°C for commercial type B gelatin films. The most likely explanation of the similarity in the thermal values for all the crosslinking agents used could be that, in Method B, crosslinking takes place in solution,

**TABLE I**
**Effect of Different Crosslinking Methods and Agents on the Thermodynamic Properties of Gelatin Films, at a Relative Humidity of 60%**

| | Method A | | Method B | |
|---|---|---|---|---|
| Crosslinker | $T_g$ (°C) | $T_D$ (°C) | $T_g$ (°C) | $T_D$ (°C) |
| Control | 37.5 ± 2.4 | 96.4 ± 2.5 | 37.5 ± 2.4 | 96.4 ± 2.5 |
| GTA | 51.7 ± 4.2 | 83.5 ± 3.6 | 42.7 ± 0.5 | 89.1 ± 0.6 |
| Oxazolidine | 34.6 ± 4.6 | 76.0 ± 5.1 | 39.7 ± 0.0 | 94.2 ± 0.7 |
| EGDE | 28.2 ± 5.6 | 73.9 ± 1.5 | 37.1 ± 0.5 | 91.8 ± 0.5 |
| HMDC | 26.8 ± 0.4 | 92.3 ± 0.3 | 36.9 ± 0.2 | 86.8 ± 1.2 |

Average and standard deviation are given.

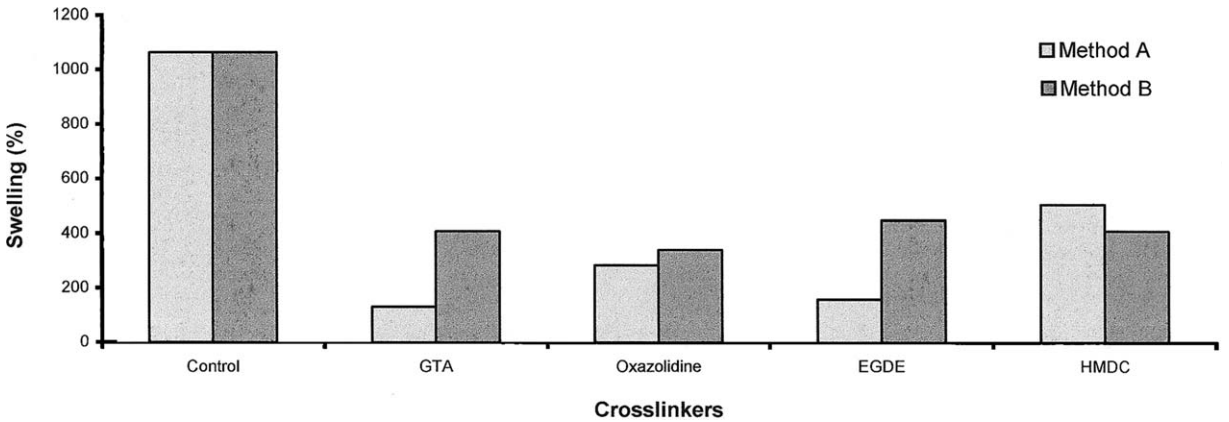


**Figure 4** Effect of the crosslinking method on the swelling of crosslinked gelatin films.

where gelatin is found completely in a state of random coils and the rapid diffusion of crosslinker molecules in the aqueous solvent allows chemical crosslinks to be formed rapidly throughout the random coils of the gelatin. When the crosslinked gelatin cooled and the helices formed, intrahelical crosslinking may not occur, and so reducing the influence on helical stability. As mentioned earlier, for gelatin crosslinked by Method B the denaturation peak was small, which may implies that the amount of triple helical material present in the sample was at a low concentration. If crosslink occurs before the formation of triple helices (as in Method B), it is likely that the triple-helical formation will be hindered as the altered molecular geometry, due to crosslinking, may not accommodate the formation of the triple-helix. Such helices will form in a less restrictive environment where no crosslinks have formed.

Comparing both crosslinking methods, some interesting differences on $T_D$ and $T_g$ (Figs. 2 and 3) have been noted. The differences are thought to be due to the introduction of a degree of disorder into the triple helical regions with the Method A of crosslinking. Gelatin resembles a semicrystalline polymer with a low degree of crystallinity and in this respect is markedly different from a highly crystalline fibre such as collagen. Two different phases can be found within the gelatin structure, triple helical regions and random coil regions, and chemical crosslinks may be formed in either phase. In the film form, biopolymer chains in the random coil phase are close to each other and so can be easily crosslinked together. Thus it is reasonable to assume that crosslinking by Method A promotes intramolecular crosslinks in the random coil region outside the helical regions, inducing differences to the glass transition temperature depending on the type of crosslinker and chain length. The formation of chemical crosslinks in the random coil phase, but close to triple helical regions, may act to distort and disrupt the ordered molecular packing in these regions.

For future research, a study about the crosslinking fixation index would be plausible, the ninhydrin method[29] can be used to determine free amino groups, thus obtaining a percentage of the amino groups reacted with respective crosslinking agent.

## Swelling

The degree of swelling of the film provides an indication of the matrix network characteristics and the stability of the gelatin films.[16,35] Measuring the degree of swelling of polymers in solvents is a very common procedure to assess the degree of crosslinking: the higher the degree of crosslinking, the lower the swelling,[46] since less water can be held by the crosslinked network. As seen in Figure 4, Method A crosslinking shows that GTA produced the highest degree of crosslinking followed by EGDE, oxazolidine, and HMDC. GTA and EGDE is thought to form a more "closed" matrix structure, the shorter the chain length between chains, the greater the density of crosslinks, the tighter the network and the less water can be held by the structure.

For GTA and EGDE, swelling was found to be higher using Method B (408 and 450%, respectively) than Method A (130.6 and 158%, respectively), suggesting that Method B imparted a lower crosslinking density. It may be argued that in an aqueous solution, whilst diffusion of the crosslinking agent through the system is faster, the gelatin chains are more often further apart than in the film so that the probability of crosslinking occurring at any time is lower in solution, which ultimately leads to a lower crosslink density.

The percentage of swelling obtained with films crosslinked using Method A was found to be lower than with Method B, as shown by Figure 4; this is probably due to a more extensive formation of inter and intramolecular chemical crosslinks,[47] except for the film crosslinked with HMDC. However, as expected, both methods produced a large decrease

in the percentage of swelling with respect to the control (1100%). The differences between crosslinkers are more pronounced for Method A (average of 270 ± 71%) than for Method B crosslinking (402 ± 45%).

The difference between Method A and B may be due to the smaller molecules that are able to diffuse more easily, into the film and crosslink the closely packed polymer chains (Method A). In Method A (films) chain segments are packed closer together and crosslinking is more efficient, especially for lower molecular size crosslinkers, which are able to diffuse more rapidly into the film. With the larger molecule (HMDC) diffusion into the film may be hindered, with less effective crosslinking. In Method B, chains in solution are more mobile, further apart than in the film, and so hinder the formation of crosslinks. The probability of two chain segments coming close enough to form a crosslink is reduced.

## SDS-PAGE

The results of the molecular weight distribution analysis by SDS-PAGE for the samples crosslinked by Methods A and B are shown in Figures 5 and 6, respectively. The uncrosslinked control film showed a molecular weight range of 6.5 to 205 kDa. Gelatins do not give the distinct bands that are usually seen with other proteins; this is possibly due to the heterogeneous nature of the protein,[48] which is formed by different size polypeptides giving an uneven distribution in the high-, mid- and low-molecular weight ranges of 50 to 200 kDa.

Some changes could be observed before and after crosslinking: as expected and found in previous literature[48–50] the crosslinking of gelatin displaces the molecular weight distribution from low to high molecular weight ranges. This indicates that the formation of larger chains is as a result of the crosslinking between the polypeptides.

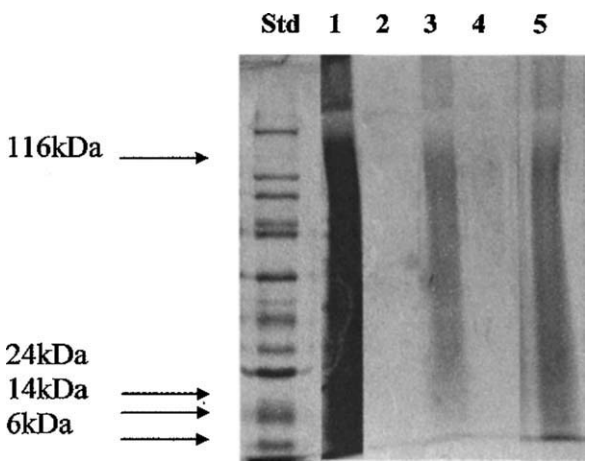


**Figure 5** Molecular weight analysis of gelatins crosslinked by Method A, using SDS-PAGE: Std: standard molecular weight marker, Lane 1: control, Lane 2: GTA crosslinked film, Lane 3: Oxazolidine crosslinked film, Lane 4: EGDE crosslinked film, Lane 5: HMDC crosslinked film.

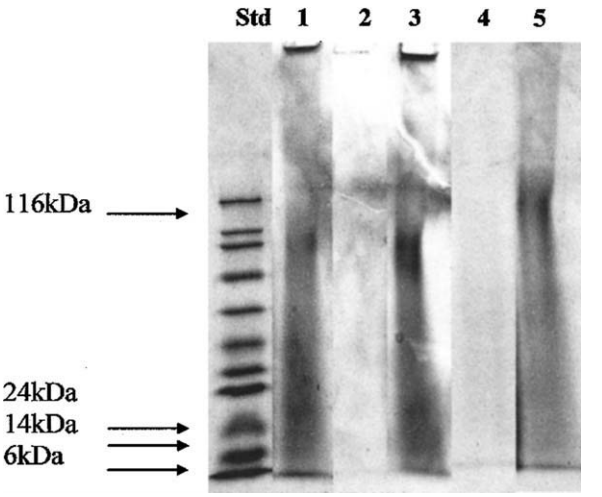


**Figure 6** Molecular weight analysis of gelatins crosslinked by Method B, using SDS-PAGE. Std: standard molecular weight marker, Lane 1: control, Lane 2: GTA crosslinked film, Lane 3: Oxazolidine crosslinked film, Lane 4: EGDE crosslinked film, Lane 5: HMDC crosslinked film.

Due to incomplete solubilisation in the electrophoresis sample buffer, the results of SDS-PAGE for GTA and EGDE were not evidenced.

Comparing Method A and B of crosslinking for oxazolidine, the swelling results were confirmed, Method A was more efficient than Method B. Oxazolidine crosslinking produced gelatin solutions and films over the whole range of molecular weights, with a higher concentration in the high molecular weight range, 84–116 kDa and lack of distribution in the low molecular range (6–14 kDa) for Method A of crosslinking.

Similar results for the swelling experiments were found for HMDC for both crosslinking methods. Molecular weight analysis, however, showed a higher proportion of stronger bands of protein in the high molecular weight range around 116 kDa for Method B of crosslinking.

## CONCLUSIONS

It has been demonstrated that the thermal properties, swelling, and molecular weight distribution of films produced from gelatin, extracted from tannery waste, can be modified and controlled by chemical crosslinking. The results of thermal studies have shown that crosslinkers with longer molecular chain lengths, such as EGDE and HMDC reduce the glass transition temperature of gelatin films, imparting flexibility to the film. The control of the glass

transition may be useful for potential future applications in coatings or films.

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