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Physicochemical Properties of Films Based on Bovine Gelatin Cross-linked with 1,4-Butanediol Diglycidyl Ether

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Abstract The effectiveness of 1,4-butanediol diglycidyl ether (BDDGE) as a cross-linking agent of bovine gelatin films plasticized with D-sorbitol intended for food packaging is reported. Free-standing films were produced by casting gelatin film-forming solutions containing D-soritol 30 % (g/100 g dry gelatin) and variable amounts of BDDGE (0–3 g/100 g dry gelatin). The addition of 1 % BDDGE (g/100 g dry gelatin) positively influenced the solubility, water vapor permeability, and mechanical properties of the produced films. Water solubility decreased about 77 %; the water vapor permeability enhanced 20 %, and the stretching ability increased 11 times while the tensile strength was reduced up to 7-fold with regard to the non-cross-linked counterpart. A simple extraction procedure was applied to remove the potential unreacted BDDGE and its hydrolysis products from BDDGE-treated gelatin films. Quantification was performed by gas chromatography combined with mass spectrometry (GC/MS), being lower than 0.25 μg/kg (below the limit of detection, 0.30 μg/L). Results suggested that water-soluble BDDGE may provide an effective cross-linking alternative to the usual aldehyde-based strategy to manufacture gelatin films with potential use as biodegradable food packaging materials.

Keywords Gelatin films · 1,4-Butanediol diglycidyl ether · Cross-linking · Functional properties · Quantification of residual cross-linking agent

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Introduction

Issues related to sustainability, petroleum shortage, and fluctuating oil prices together with the enormous increase in the use of polymer in great demand areas such as packaging, have intensified the development of new cost-effective greener alternatives (Di Franco et al. 2004). A variety of biopolymers such as proteins derived from animal and vegetal feedstock have emerged as renewable options to the depleting ones, particularly in disposable packaging items. Gelatin is a water-soluble protein obtained from the controlled hydrolysis of bone collagen or connective tissue skin of mammalian and fishes (Gennadios 2002), which has a wide range of functionalities and applications, including its biodegradable film-forming ability used in food coating/packaging applications (Sobral et al. 2001; Martucci and Ruseckaite 2009; Martucci et al. 2012; Núñez-Flores et al. 2013; Guo et al. 2014; Gupta et al. 2014). Unfortunately, gelatin-based films suffer from several issues when stored at high relative humidity due to the hydrophilic nature of gelatin (Gomez-Estaca et al. 2011).

The question is whether gelatin films can meet the fundamental technical requirements of the petroleum-based materials to be competitive. The traditional aldehyde-based cross-linking strategy has provided a powerful tool to tailor the physical properties of gelatin films (Robinson 1964; Bigi et al. 2001; de Carvalho and Grosso 2004; Martucci and Ruseckaite 2009; Martucci et al. 2012; Guo et al. 2014) although the suspected toxicity of such chemicals makes questionable their use in food contact materials (Bigi et al. 2001). Multifunctional epoxy compounds may afford a less explored approach to overcome this issue. Epoxy compounds are preferred as a stabilizing agent of collagen-based materials for biomedical applications owing to their lower toxicity compared with commonly used dialdehydes (Zeeman 1998; Sung et al. 1999; Di and Heath 2009; Enea et al. 2011; de

Boulle et al. 2013; Nicoletti et al. 2013; Zhang et al. 2014), but their use for food contact items is scarce. A few examples include wheat gliadin films modified by epiclhorydrine (Song et al. 2009), soy protein films stabilized by a polyamide–epichlorohydrin (Zhong et al. 2007), and gelatin films cross-linked with ethylene glycol diglycidyl ether (EGDE) (Vargas et al. 2008).

Amongst water-soluble polyepoxides, 1,4-butanediol diglycidyl ether (BDDGE) is commercially available as a cross-linking agent in dermal filler formulations (de Boulle et al. 2013). Although un-reacted BDDGE should be considered from slightly to moderately toxic (Zeeman 1998; Zhang et al. 2014; Materials Safety Data 2014), residual BDDGE might undergo hydrolysis yielding a diol-ether (3,3′(butane-1,4-diylbis(oxy)) bis propane-1,2-diol) which has been proved to be non-toxic (de Boulle et al. 2013) limiting safety risks. Accordingly, the present study analyses the ability of BDDGE as a cross-linking agent of bovine gelatin films plasticized by D-sorbitol. Moisture affinity (moisture content, solubility, water vapor permeability), tensile, and optical properties of the obtained films were investigated. In the context of the intended application, un-reacted BDDGE and BDDGE hydrolysis products in the films were quoted by gas chromatography coupled with mass spectrometry (GC/MS). This last information is critical because it may serve as a guide for the future application of these materials in food packaging.

Experimental

Materials

Bovine hide gelatin (Ge) (type B, 200 Bloom) was kindly supplied by Rousselot (Buenos Aires, Argentina). BDDGE (epoxy equivalent weight 0.017 eq/g, density 1.1 g/mL) was provided from Sigma-Aldrich (USA) and used as cross-linking agent without any further purification. D-sorbitol monohydrate and buffer solution pH 10 were purchased from Anedra (Buenos Aires, Argentina). Chromatography grade acetonitrile (≥99.8 %), methanol (≥99.8 %), acetic anhydride (≥99.0 %), and 4-(dimethylamino)pyridine (DMAP, ≥99 %) were provided by Sigma-Aldrich (St. Louis, MO, USA). All the other reagents were analytical grade.

Film Formation

All concentrations of the film-forming solutions (FFSs) and manufacturing conditions (i.e., dissolution conditions as well as the cure-drying cycle used in the casting procedure) were determined by preliminary studies (results not shown, Martucci 2008) in order to achieve free-standing films. Aqueous FFSs were prepared by dissolving 10 g of gelatin powder in distilled water with gentle heating at 50±2 °C and constant magnetic stirring for 30 min, to yield 10 % (g gelatin/100 g solution). An appropriate amount of D-sorbitol (30 g/100 g gelatin dry basis) was added under stirring for 15 min. The pH of the FFSs was monitored and adjusted to 10 with 1 M NaOH. Then, various amounts of BDDGE, i.e., 0, 0.5, 1, and 3 % (g/100 g dry gelatin) were added into the FFS and stirred again for 15 min at 50±2 °C. The produced FFSs were poured onto Teflon-coated petri dishes with an inner diameter of 10 cm and exposed to the following drying–curing cycle in a force air oven (Memmert, Germany): 4 h at 70 °C and 1 h at 120 °C (Martucci 2008). Afterward, films were peeled off from the molds and kept in an environmental chamber at 23 ±2 °C and 65±2 % relative humidity (RH). Control films without BDDGE were labeled as "Ge" while those containing BDDGE were labeled as "Ge-xBDDGE" where x defines the percentage in weight of BDDGE added.

Characterization

Thickness Average film thickness was determined using a micrometer (Venier, China), and measurements were taken at four random positions on the film slabs with an accuracy of ±0.01 mm. Average values were used to assess mechanical, optical, and barrier properties.

Determination of the Molecular Weight Between Cross-linking Points (M_c) Water uptake (WU) was determined according to the procedure described in ASTM D570-81. Gelatin samples (1 cm×3 cm×0.02 cm) were dried under vacuum and then weighed (w_d) before immersing in distilled water at 20 °C. After predetermined periods of time (t), the wet samples were recovered, blotted with tissue paper to remove the excess of water on the surface, and then weighed again (w_t). The WU was calculated as

$$\mathrm{WU}\ (\%) = 100\,(w_\mathrm{t} - w_\mathrm{d}) \,/\, w_\mathrm{d} \qquad (1)$$

Reported results are the average of three replicates. In order to estimate the network cross-linking density, the molecular weight of chain segments between two cross-linking points, M_c, was estimated by using the Flory–Renher equation (Flory and Rehner 1943).

$$M_c = \frac{\rho * V_1 * \left({\phi_g}^{1/3} - \frac{2\phi_g}{F} \right)}{\chi * {\phi_g}^2 + \ln\left(1 - \phi_g\right) + \phi_g} \qquad (2)$$

where ρ is the density of the dry gelatin determined by picnometry, V_1 is the solvent molar volume (18.06 cm^3 mol for water), χ is the Flory–Huggins polymer—solvent interaction parameter taken from the literature ($\chi = 0.49 \pm 0.05$) (Patil et al. 2000), and ϕ_g is the volume fraction of the swollen gelatin. This parameter was estimated from the experimental equilibrium water uptake (WU_{eq}) values, assuming the following relationship:

$$\phi_g = W_0 * \rho_w / \left[W\rho_g - W_0 * (\rho - \rho_w) \right] \tag{3}$$

where W_0 is the initial weight of the dry sample, W is the weight of the swollen sample which was measured after removing the excess liquid on the sample surface by a filter paper (WU_{eq}), ρ_w is the density of the water at room temperature, and ρ_g is the density of the dry and un-cross-linked gelatin.

Free Amino Groups and Cross-linking Extent The amount of amino groups involved in the cross-linking reaction was determined by UV-visible spectrometry using ninhydrin (2, 2-dihydroxy-1,3-indanedione (NHN)), in order to estimate the amount of free amino groups left after the chemical cross-linking (Martucci et al. 2012). Cross-linked gelatin films were dried under vacuum at room temperature until constant weight. A precise amount of sample (100±5 mg) was heated with ninhydrin solution (0.5 g/100 mL) for 20 min. Afterwards, the absorbance of the solution was recorded on a spectrophotometer Shimadzu 1601 PC at 570 nm (Tokyo, Japan). The free amino group content (FAG) was expressed by the following relationship:

$$FAG(\%) = \frac{(NHN\ \ reactive\ \ amine)_g - (NHN\ \ reactive\ \ amine)_{rg}}{(NHN\ \ reactive\ \ amine)_g} \tag{4}$$

where (NHN reactive amine)$_g$ is the total amount of amino groups in a gelatin film and (NHN reactive amine)$_{rg}$ is the amount of free amino groups presents in a gelatin film after the cross-linking reaction.

Tensile Tests Tensile parameters were determined at 25 °C by using an INSTRON 4467 Universal Testing Machine (High Wycombe, Buckinghamshire, England) equipped with a 0.5 kN cell at room temperature following the procedure described in ASTM D638-94b. Films were cut into dog bone-shaped specimens (30 mm×4.5 mm×0.2 mm) and conditioned at 65±2 % RH and 23±2 °C before testing. Samples were clamped into the metal grips for tensile testing and stretched at overhead crosshead speed of 3 mm/min. Elongation at break (ε%), Young modulus (E), and tensile strength (TS) were calculated as the average of nine replicates.

Moisture Content and Total Soluble Matter Total soluble matter was expressed as the percentage of film dry matter solubilized after 24-h immersion in distilled water. The wet method was used for total soluble matter (TSM) determinations (Rhim et al. 1998). Four samples (dimensions: 2 cm× 2 cm) from each formulation were carefully weighted (±0.0001 g) and subsequently dried in an air circulating oven (Memmert, Germany) at 105±2 °C for 24 h. After this time, specimens were recovered and re-weighed (±0.0001 g) to determine their initial dry matter (m_0). The initial moisture content (*MC*) was calculated as

$$\mathrm{MC}\ (\%)\ =\ 100 * (m_\mathrm{h} - m_0)\ /\ m_0 \tag{5}$$

Afterwards, four specimens of each film were weighed (m_h) and then directly immersed in 30 mL of distilled water with traces of sodium azide (0.02 g/100 mL) to inhibit microbial growth and stored in an environmental chamber at 25± 2 °C for 24 h. After this time, specimens were recovered, rinsed with distilled water, and dried in an air circulating oven at 105 °C until reaching constant weight (m_f). The TSM was expressed as following:

$$\mathrm{TSM}\ (\%)\ =\ 100 * (m_0 - m_\mathrm{f}) /\ m_0 \tag{6}$$

Initial dry matter values (m_0) used for TSM calculations were the ones obtained from MC measurements for the same film.

Water Vapor Permeability Water vapor permeability (WVP) was measured using a modified ASTM method as described by ASTM E96-95 desiccant method. The film was sealed on a glass permeation cup containing silica gel (0 % RH) using silicone vacuum grease and a rubber band to hold the film in place. The cups were placed at 25 °C in a humidity chamber at 65±2 % HR. The cups were weighed at 1-h intervals over a 10-h period. WVP of the film was calculated as follows:

$$WVP\left(kg \cdot m \cdot s^{-1} \cdot Pa^{-1} \cdot m^{-2}\right) = \frac{w}{A * t * \Delta P} * e \tag{7}$$

where w is the weight gain of the cup (kg) at time t (s); e is the film thickness (m); A is the exposed area of film (m^2); ΔP is the vapor pressure difference across the film (Pa). Three replicates of each film were used for WVP testing.

Fourier Transform Infrared Spectrometry Spectra were recorded on a Thermo Scientific Nicolet 6700 spectrometer (Wisconsin, EEUU). All runs were performed between 400 and 4000 cm^{-1} using an attenuated total reflection (ATR) accessory with a diamond ATR crystal using 32 scans with resolution of 4 cm^{-1}.

Differential Scanning Calorimetry Differential scanning calorimetry (DSC) analysis was carried out on a Perkin Elmer calorimeter model Pyris 1 (USA) equipped with a liquid nitrogen cooling system and calibrated with an indium standard. Samples of 5–7 mg were placed in the measurement heating cell in aluminum pans. An empty pan was used as reference. All runs were performed from room temperature

(23±2 °C) up to 225 °C, under nitrogen atmosphere and at a heating rate of 5 °C/min. The glass transition temperature (T_g) was determined from a second run after quenching, and it was taken as the mid-point of the heat capacity jump.

Scanning Electron Microscopy Microstructure was examined by scanning electronic microscopy on a Phillips 505 microscope (Eindhoven, The Netherlands) with an operating voltage of 15 kV. Samples were cryo-fractured, and then, the cross sections were sputter-coated with gold.

Opacity Opacity was determined using a LoviBond Colorimeter RT500 (Amesbury, UK) with a measuring area of 8 cm of diameter, following the procedure described in ASTM D 523 using a D65 daylight source. The opacity (%) of the samples was calculated from the reflectance measurements of each sample with a black backing and each sample with a white backing according to Eq. (8):

$$Opacity(\%) = 100 * \frac{Y^*_{black}}{Y^*_{white}} \tag{8}$$

where Y is a three-stimulus value. All reported values are the average of three replicates.

Gas Chromatography Coupled with Mass Spectrometric Detection Analysis

Extraction Method The extraction of residual BDDGE from Ge-1BDDGE film was performed by gridding the sample into very small pieces in order to increase the surface area and improve the extraction efficiency. Basically, 0.6 g of gridded sample was extracted with 5 mL of acetonitrile at 20±2 °C in a glass vial for 10 days. Afterwards, the extract was recovered by centrifugation, and the obtained extract was filtered off through a glass-fiber filter. Five milliliters of the extract was concentrated to 500 μL for quoting the analyte (c.a. BDDGE) with no further derivatization. In order to check the presence of hydrolysis products of BDDGE, extracted samples were acetylated in situ in order to improve the chromatographic response. Basically, 900 μL of the extract was chemically reacted with acetic anhydride (100 μL) in the presence of DMAP as catalyst (1 μL of 1 % DMAP solution in acetonitrile) followed by heating in a convection oven for at 70 °C for 2 h.

Instrumentation Samples were analyzed by GC employing an Agilent GC model 7890 A (Agilent, Palo Alto, USA) equipped with an auto-sampler ALS and coupled to an Agilent single quadrupole MS model 5975C (Agilent, Palo Alto, USA). The GC was equipped with an Agilent 5MS column (30 mm×0.25 mm internal diameter and 0.25 μm thickness), and helium was used as carrier (1 mL/min) in constant flow mode, with a total GC run time of 30 min. The injector temperature was kept at 250 °C in a splitless mode and using an injection volume of 4 μL. The oven temperature was programmed to increase from 50 °C, hold 2 min, increased to 280 at 10 °C/min, and then hold for 5 min. The mass spectrometer was operated in electron impact (EI) mode at 70 eV with anion source temperature at 230 °C and quadrupole temperature 150 °C. Spectra were recorded in selective ion monitoring (SIM) mode for the characteristic ions m/z 201 and 203 for the internal standard (2-bromo-1-nitrobencene), and then, selective monitoring of m/z 100 and 129 for BDDGE was tested in order to ensure good resolution. Hydrolysis products were quoted in scanning mode. The peak areas were reported as a function of the injected concentration, and the calibration curves were obtained by linear regression. Standard solutions of BDDGE were prepared in acetonitrile (0.5–4 μg/L) with the addition of the internal standard (5 μg/L). Each value of the calibration curves was the average of three replicates. The limit of detection (LOD) was calculated from the apparent measured value of blank injections (mean+3×standard deviation); the limit of quantification (LOQ) was calculated using the apparent measured value of blank injections (mean+10×standard deviation).

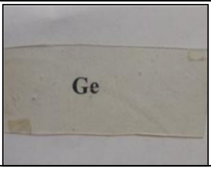
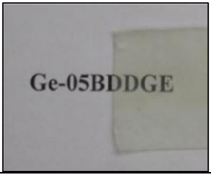
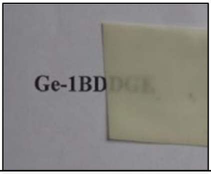
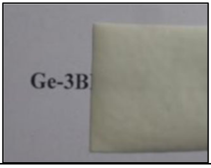
Statistics Data values obtained in the experiments were statistically analyzed by one-way analysis of variance (ANOVA) employing Origin Lab 8 software. Differences between pairs of means were assessed on the basis of confidence intervals using the Tukey test, with the significance of the difference set at $P<0.05$.

Results and Discussion

Physical Appearance, Opacity, and Morphological Analysis

Free-standing Ge-BDDGE films were obtained after cure-drying the film-forming solutions for 4 h at 70 °C and post curing 1 h at 120 °C. It seems that the inclusion of BDDGE brings dimensional stability to gelatin films as it was directly evidenced by the feeling of comparative hardness on handling. Visually, Ge-BDDGE films showed homogeneous appearance regarding the absence of particles and bubbles, but they turned translucent with increasing BDDGE (Table 1). Confirming the visual inspection, Ge-BDDGE films showed higher opacity values than control film ($P<0.05$) (Table 1), c.a. from 10.8±0.2 to 68.7±3.6 % for Ge and Ge-3BDDGE films, respectively. This result suggests that BDDGE, especially at high levels, contributes to limit the light transmission of the films in the visible range. Since thickness values slightly varied with BDDGE (Table 1), the increased translucency could be caused by an increment of the surface roughness of

Table 1 Physical appearance, thickness, and opacity of Ge-BDDGE films plasticized by D-sorbitol (30 g/100 g gelatin)

Film	Physicalappearance	Thickness (µm)	Opacity (%)
Ge		220±10.0 a	10.8±0.2 a
Ge-0.5BDDGE		200±10.0 ab	14.5±1.2 b
Ge-1BDDGE		170±20.0 b	51.2±2.1 c
Ge-3BDDGE		0.19±0.02 ab	68.7±3.6 d

Mean values within the same column followed by the same letter are not significantly different ($P>0.05$, Tukey test)

BDDGE-containing films (Park et al. 2008). In order to verify this assumption, scanning electron micrographs of the top surfaces of control and Ge-BDDGE films were obtained, and results are presented in Fig. 1. The images illustrate that the untreated films had smooth and dense surface typical for protein films, with no visible holes or cracks (Fig. 1a). With the addition of BDDGE, the top surface of the resultant films became progressively rough and uneven (Fig. 1b–c). The cross-sectional micrographs of the films (Fig. 1d–f) revealed obvious differences in the inner morphology of the films. Ge control film was compact and even whereas many porous and channels growing from the bottom to the top appeared in the Ge-3BDDGe film. This phenomenon was because the viscosity of the film-forming solutions was so high that it hampers to remove solvent bubbles during the cure-drying process, causing the appearance of channels and holes in the films (Martucci 2008).

Stabilizing Interactions and Thermal Properties

The potential interactions between gelatin and BDDGE in the films resulting from the addition of different BDDGE contents at constant gelatin and D-sorbitol concentrations were assessed by ATR-Fourier transform infrared spectrometry (FTIR) analysis, and spectra of control gelatin (without BDDGE) and completely cured Ge-BDDGE films containing variable BDDGE concentrations 0, 0.5, 1, and 3 % (g/100 g dry gelatin) are collected in Fig. 2. Gelatin control film showed peaks of relevance at wavenumber values corresponding to amide A (~3278 cm^{-1}), amide I associated with C=O stretching (~1634 cm^{-1}), amide II (~1533 cm^{-1}) assigned to N–H bending and C–N stretching, and amide III (~1239 cm^{-1}) associated with N–H in-plane bending and C–N stretching coupled with C–H and N–H deformation (Núñez-Flores et al. 2013). Moreover, it was easy to observe several changes of some specific gelatin bands after BDDGE treatment. Indeed, significant signal increment was detected in the wavenumber region ranging from 3200 to 3500 cm^{-1} (attributed to the intermolecular bonded hydroxyl group and the stretching of N–H bond) associated with the appearance of new OH groups and the transformation from primary amino groups into secondary ones. The shift experienced by amide A band toward higher wavenumbers was coincident with the progressive increase in the intensity of the absorption band in the range of 1150–1100 cm^{-1} related to C–O stretching being most likely caused by the appearance of secondary alcohol groups upon epoxy–amine reaction (Nicoletti et al. 2013). The peak assigned to C–O stretching of D-sorbitol is located at the same wave number region (1000–1100 cm^{-1}) (Colthup et al. 1964). Since D-sorbitol concentration was kept invariable in all films, i.e., 30 % (g/100 g dry gelatin), the observed shift of such peaks toward lower wave number with BDDGE suggests the occurrence of additional hydrogen interactions (Bergo and Sobral 2007) arising from the increasing amount

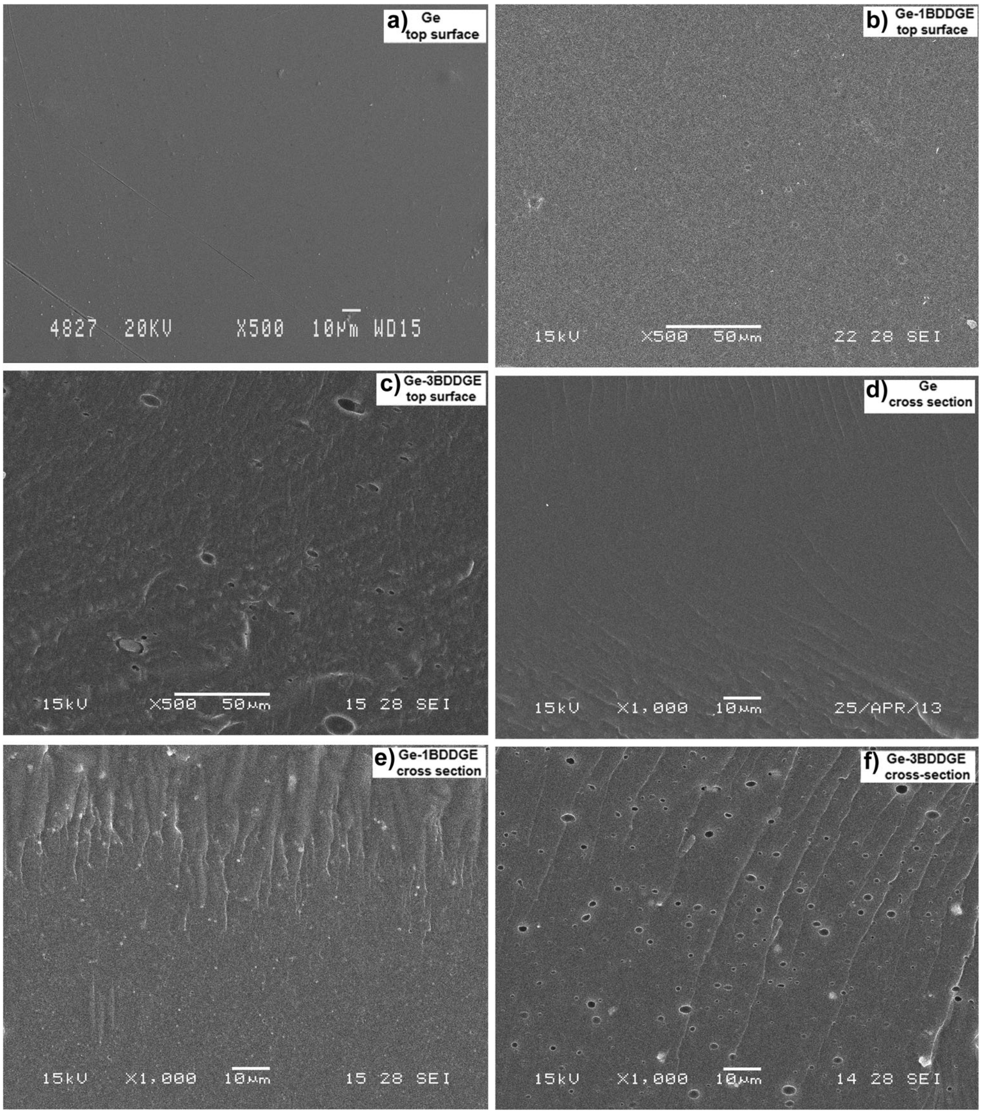


Fig. 1 Scanning electron micrograph surfaces (**a**–**c**) and cross sections (**d**–**f**) of control and Ge-BDDGE films

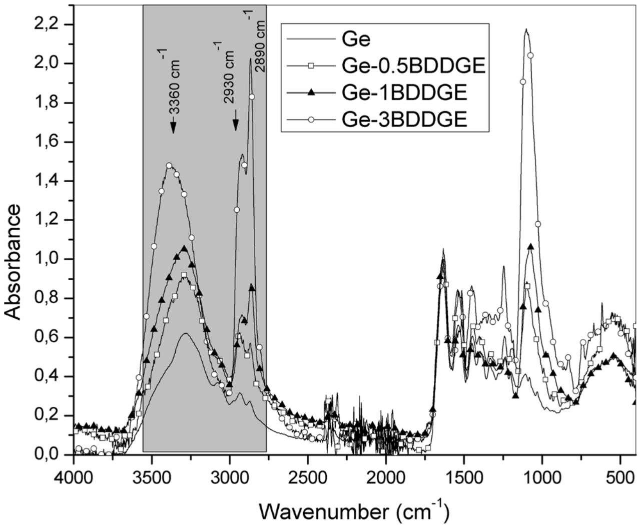


Fig. 2 FTIR spectra of Ge (*line*), Ge-0.5BDDGE (*square*), Ge-1BDDGE (*triangle*), and Ge-3BDDGE (*circle*) films, containing D-sorbitol 30 % (g/100 g dry gelatin)

of hydroxyl groups attached to the film network originated during epoxy–amine reaction and/or from the hydrolysis under casting of un-reacted pendant epoxide groups. Other significant intensity changes were observed at 2890 and 2930 cm^{-1}, associated with the contribution of aliphatic moieties from BDDGE, confirming the incorporation of BDDGE to gelatin matrix. Changes in amplitude and intensity of amide I and II bands produced by BDDGE were hardly noticeable suggesting a possible interaction through hydrogen bonds at this level to be negligible (Nicoletti et al. 2013). All these experimental evidences agreed well with a reaction mechanism taking place at alkaline pH level involving the opening of oxirane rings in BDDGE by the nucleophilic attack of amino side chain groups in gelatin with the concomitant appearance of secondary hydroxyl groups (Zeeman 1998; Nicoletti et al. 2013). It should be keep in mind that not only cross-linking but masking (one-point fixation) reaction as well will account for this observations (Zeeman 1998); therefore, no conclusions about mono- or bis-epoxy linking can be drawn from FTIR analysis.

Thermal properties of gelatin matrices can be analyzed by DSC with some precision. The thermograms of the first scan from room temperature up to 170 °C (results not shown) were dominated by a broad endothermic event peaking at 100 °C arising from the removal of residual or bonded water in gelatin films (Martucci et al. 2012), and no other thermal change was detected at least up to 170 °C. On the other hand, the traces of the second scan after quenching from 170 °C to room temperature (Fig. 3) were characterized by an endothermic peak representing the overall T_g. The variation of T_g values with BDDGE content in a non-monotonic fashion (see arrows in Fig. 3) gave experimental evidence of the relative contribution of covalent bridges and one-point fixation structures to the gel formation with increasing BDDGE, in accordance with studies performed on collagen—BDDGE (Zeeman 1998).

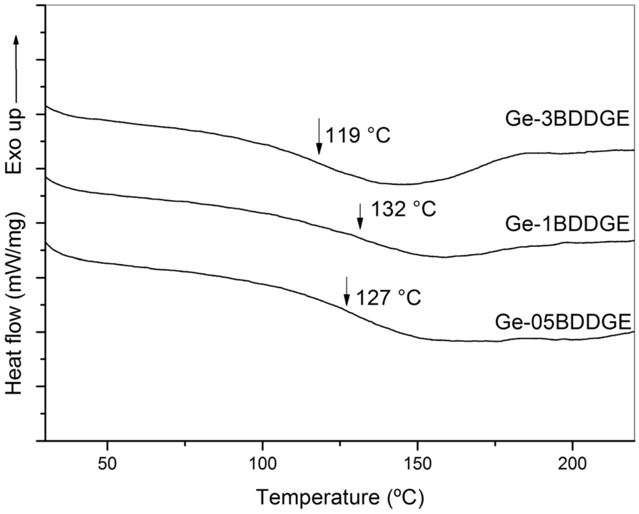


Fig. 3 DSC heating curves (second scan) for D-sorbitol plasticized—gelatin films treated with BDDGE (5 °C min^{-1}, nitrogen atmosphere). *Arrows* indicate the glass transition temperature (T_g)

Tensile Properties

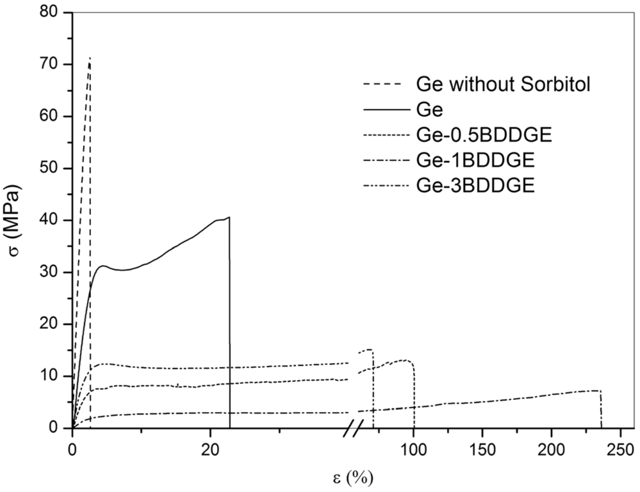


Fig. 4 Representative stress–strain curves of gelatin films modified with varying BDDGE content

Representative stress–strain curves of the films are shown in Fig. 4. The mechanical behavior of un-plasticized gelatin films shows the typical characteristics of brittle and rigid materials, since they exhibited high values of stress and low values of strain at break in accordance with the development of a rigid protein network (Ge without D-sorbitol in Fig. 4). The incorporation of D-sorbitol increase chain mobility and the plasticized gelatin film (Ge in Fig. 4) displayed yield-like deformation followed by a large deformation region, indicating enhanced ductility (Sobral et al. 2001; Martucci et al. 2012). Table 2 summarizes the mechanical parameters of Ge-BDDGE films, in terms of tensile strength (TS), elongation at break (ε%), and Young modulus (E). The TS and E values of Ge film were 45.9±2.5 and 1113 ±123 MPa, respectively. The addition of BDDGE up to 1 % (g/100 g dry gelatin) reduced TS and E ($P<0.05$) to 6.8±0.5 and 79.7±12.1 MPa, respectively (Table 2). This trend was accompanied by the concomitant increment in the elongation at break from 20.7±3.2 to 235.6±8.7 % for control and Ge-1BDDGE films, respectively (Table 2). The significantly lower ($P<0.05$) TS and E values and higher ε% values of Ge-BDDGE films (irrespective of BDDGE content) compared with control one (Table 2) suggest that sites present on gelatin chains that were originally joined in physical cross-links (i.e., control film) are prevented to rejoin due to the presence of chemical cross-links and pendant groups which may alter the spatial organization of the gelatin chains (Kuijpers et al. 1999). Our results reveal that at low BDDGE level, i.e., BDDGE ≤1 % (g/100 g dry gelatin), cross-linking is counterbalanced by the plasticizing effect exerted by the secondary hydroxyl groups (from masking or cross-linking) and hydroxyl-terminated pendant groups from hydrolyzed un-reacted epoxides. All these hydroxyl-containing compounds which are attached to gelatin can take part in weakening the interactions between protein chains enhancing the mobility of the macromolecules (plasticizing effect), reducing TS and E values, and enhancing the extensibility of Ge-BBDGE films (Table 2). Such effect was previously demonstrated for gelatin films cross-linked with dialdehyde starch (Martucci and Ruseckaite 2009) and epoxide-cross-linked collagen materials (Di and Heath 2009; Enea et al. 2011). Conversely, the addition of larger amounts of BDDGE resulted in an increase in TS with the corresponding decrease in the elongating capacity compared with films with lower BDDGE content (Table 2). This was in accordance with a more reticulated material achieved at high BDDGE level. This was verified by the fact that the molecular weight between two cross-linking points (M_c) estimated by Flory–Rehner equation (Flory and Rehner 1943) dropped from 5757±108 to 232±33 Da for control and Ge-3BDDGE films (Table 2). Although such BDDGE level can make the

Table 2 Molecular weight between cross-linking points (M_c) and tensile properties of Ge-BDDGE films plasticized by D-sorbitol (30 g/100 g gelatin)

Film	M_c (Da)	E (MPa)	TS (MPa)	ε (%)
Ge	5757±108 a	1113±123 a	45.9±2.5 a	20.7±3.2 a
Ge-0.5BDDGE	3450±530 b	304.6±22.3 b	13.0±1.2 b	95.6±10.2 b
Ge-1BDDGE	1197±43 c	79.7±12.1 c	6.8±0.5 c	235.6±8.7 c
Ge-3BDDGE	232±33 d	568.3±70.7 d	13.0±2.2 b	65.63±7.9 d

Mean values within the same column followed by the same letter are not significantly different ($P>0.05$, Tukey test)

network more rigid, the contribution of secondary hydroxyl groups as well as hydroxyl-terminated pendant groups may account for to the extensibility of such films which is about three times that of control film.

Moisture Content, Total Soluble Matter, and Water Uptake

The MC is an important factor that affects the properties of films because water was the most common plasticizer. MC significantly reduced ($P<0.05$) from 17.7±1.0 to 11.4±1.5 %, for control and Ge-1BDDGE films, respectively (Table 3). The small but significant drop in MC at BDDGE ≤1 % (g/100 g dry gelatin) agreed well with the fact that reaction between gelatin and BDDGE at alkaline pH proceeds through amino groups in gelatin blocking such as hydrophilic positions capable of association with water (Zeeman 1998). At higher BDDGE level, the limited amount of free amino groups left after reaction (7.2±0.1 %, Table 3) is counterbalanced by the concomitant appearance of secondary and terminal hydroxyl pendant groups, and MC became almost invariable with BDDGE. Comparable behavior was observed in dialdehyde-cross-linked gelatin films (Martucci and Ruseckaite 2009; Martucci et al. 2012) and oxidized xantan gum-gelatin (Guo et al. 2014).

Similarly, the addition of BDDGE provoked statistically lower ($P<0.05$) soluble material (Table 3). Control film underwent complete dissolution (TSM=100 %) in comparison with the cross-linked films, in accordance with the hydrophilic nature of gelatin and D-sorbitol (Gennadios 2002; De Carvalho and Grosso 2004). The incorporation of 3 % BDDGE (g/100 g dry gelatin) markedly reduced ($P<0.05$) TSM by 77 % (Table 3). It seems that a partially cross-linked network contributes introducing severe restrictions into gelatin matrix, leading to a blockage of gelatin ability to interact with water molecules. Improvements in water-resistant ability by cross-linking protein films was previously reported (Rhim et al. 1998; De Carvalho and Grosso 2004; Martucci and Ruseckaite 2009; Martucci et al. 2012).

The level of water uptake at the equilibrium (WU_{eq}) was considerably reduced with BDDGE ($P<0.05$) with 161±12 % for Ge-3BDDGE versus 1854±85 % for the control film (Table 3). The reduction of WU in gelatin films due to cross-linking with epoxide compounds was already reported by Vargas and co-workers (2008) for gelatin-cross-linked with EDGE. This behavior can be explained by the equilibrium swelling theories of cross-linked polymer networks, such as the model propose by Flory and Rehner (1943), which postulates that the equilibrium extent of swelling is determined by two opposite driving forces: the thermodynamic compatibility of the polymer chains and the swelling liquid, and the elastic retractive forces of the polymer chains. When these two opposite forces counterbalance each other, the swelling reaches equilibrium. As the average length of the polymer chains between network junctions decreases, the retractive forces intensify and cross-linked films exhibit much lower water uptake capacity.

Water Vapor Permeability

Because the main function of film is often to impede moisture transfer between the packed food and the surrounding atmosphere, it is essential that the WVP is as low as possible. Table 3 shows that the addition of BDDGE up to 1 % (g/100 g dry gelatin) has the potential to reduce WVP. However, upon an addition level of BDDGE >1 % (g/100 g dry gelatin), the WVP

Table 3 Percentage of free amino groups (FAGs), moisture content (MC), total soluble matter (TSM), water uptake at the equilibrium (WU_{eq}), and water vapor permeability (WVP) of Ge-BDDGE films

Sample name	FAG (%)	MC (%)	TSM (%)	WVP×10^{14} (kg m/Pa s m^2)	WU_{eq} (%)
Ge	100.0±0.1 a	17.7±1.0 a	100.0±0.1 a	2.42±0.27 ab	1854±85 a
Ge-0.5BDDGE	54.2±0.3 b	15.7±0.1 b	36.8±0.2 b	2.07±0.10 ac	501±31 b
Ge-1BDDGE	46.5±0.2 c	11.4±1.5 c	23.4±0.7 c	1.97±0.13 c	325±5 c
Ge-3BDDGE	7.2±0.1 d	12.2±0.5 c	22.8±3.2 c	2.53±0.11 b	161±12 d

All samples are plasticized by D-sorbitol (30 g/100 g gelatin)

Mean values within the same column followed by the same letter are not significantly different ($P>0.05$, Tukey test) increased again reaching a value similar to that of control film (Table 3). The initial drop in WVP from 2.42±0.27×10^{-14} kg m/m^2 s Pa for control films up to 1.97±0.13×10^{-14} kg m/m^2 s Pa for Ge-1BDDGE was attributed to the more tortuous path to the water molecules, offered by the tight protein network induced by cross-linking, which occupies hydrophilic amino groups, and to the small contribution of hydroxyl groups from epoxy–amine reaction and hydrolyzed pendant groups. The increment in WVP of Ge-3BDDGE film could be explained by the presence of multiple small porous in Ge-3BDDGE film (surface and cross section, Fig. 1), thus potentially allowing the water vapor to pass through easily. Comparison of SEM observations of the cross section of the films revealed that Ge-1BDDGE had more compact structure than Ge-3BDDGE film (Fig. 1e, f). The well-recognized thickness effect of hydrophilic films (McHugh et al. 1993) seems to play a minor role in determining WVP since increasing BDDGE level induces not statistically significant (P>0.05) variations in such parameter as discussed earlier in the text (Table 1). The Ge-1BDDGE films had lower WVP values than those reported for glycerol plasticized-soy protein isolate, whey protein isolate, and gelatin films, all cross-linked with DAS (15.0–16.5×10^{-13}, 5.43×10^{-13}, and 1.7 ±0.5×10^{-13} kg m/m^2 s Pa, respectively) (Rhim et al. 1998; Martucci and Ruseckaite 2009). However, WVP was still higher than those of synthetic polymers, i.e., high-density polyethylene (HPDE) (2.4×10^{-16} kg m/m^2 s Pa), polyvinyl chloride (PVC) (0.7–2.4×10^{-16} kg m/m^2 s Pa), and low-density polyethylene (LDPE) (3.6–9.7×10^{-16} kg m/m^2 s Pa) (Morillon et al. 2002), more likely due to their hydrophilic nature.

Residual BDDGE

1,4-Butanediol diglycidyl ether is a type of low-toxicity compound that can have harmful effects on health, so it is mandatory to ensure that BDDGE content is relatively low in the obtained films. GC/MS was used to identify (full scan) and quantify (SIM mode) residual BDDGE as well as its hydrolysis products from extracted film samples in acetonitrile. The chromatogram of the commercial BDDGE (Fig. 5a) exhibited

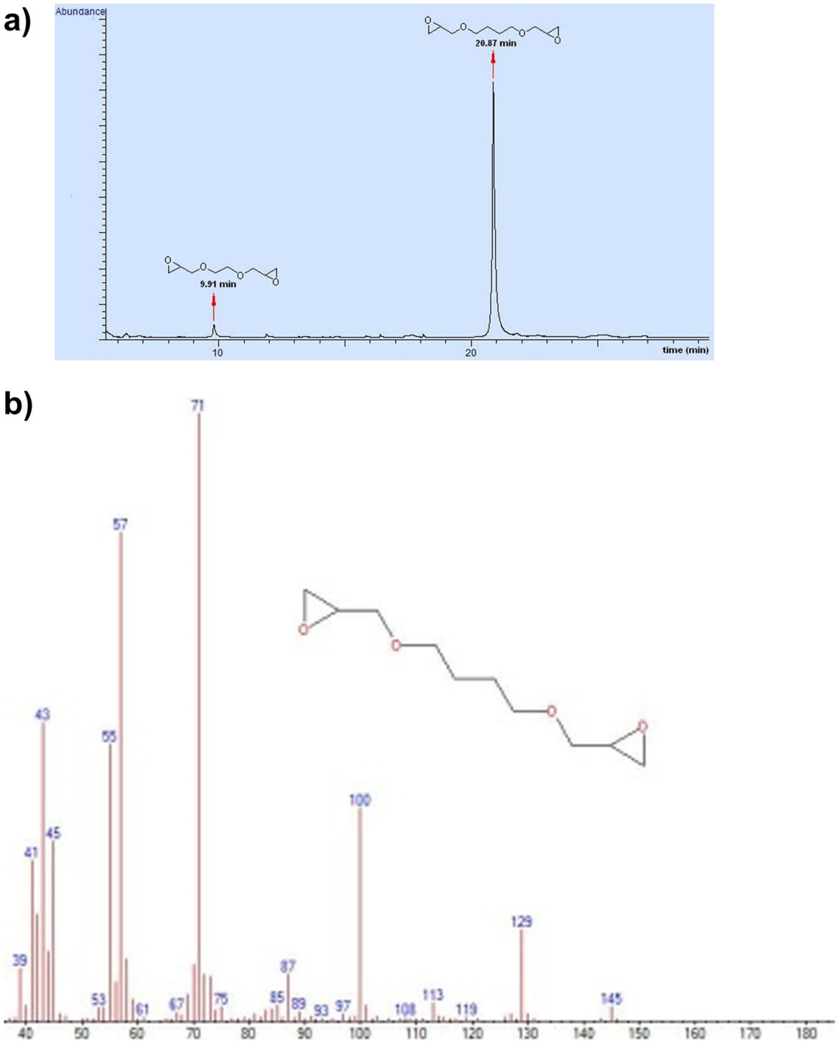


Fig. 5 **a** GC elution profile and **b** mass spectrum of commercial BDDGE

an intense peak at retention time (RT) 20.87 min. The dominant fragments in the mass spectrum (*m*/*z*=40–190 Da) of such peak were observed, in order of abundance, at *m*/*z* 71, 57, 45, 100, 129, and 87 Da (Fig. 5b). Relevant peaks (Fig. 5b) agreed well with data reported elsewhere for BDDGE (NIST/EPA/NIH 2008). The minor peak in GC at RT 9.91 min was positively identified by MS to ethylene glycol diglycidyl ether, suggesting the presence of low level of this last analyte in commercial BDDGE.

The quantification of the un-reacted BDDGE in Ge-1BDDGE film was performed on extracted samples by using a calibration curve with correlation coefficient *R*=0.998. Results revealed that residual level of BDDGE in Ge-1BDDGE films was below the level of detection (c.a. LOD=0.30 μg/L), corresponding to a free BDDGE <0.25 μg/kg. Residual BDDGE level in Ge-1BDDGE film was far below the toxicity data, reported as the lethal dose (LD_{50}) in rats, c.a. LD_{50}=1134 μg/kg (Materials Safety Data 2014), the limit of tolerance required by the cosmetic industry of injected BDDGE—sodium hyaluranate gels, c.a. 2 μg/g (Zhang et al. 2014), and the maximum value of epoxy compounds in food contact materials imposed by the Commission Regulation 1895 (2005) which require a maximum value of 9 mg/kg for free diglycidyl ether of bisphenol A (DGEBA) and its hydrolysis products. Therefore, Ge-1BDDGE films can be classified as safe materials.

Over time, the un-reacted BDDGE undergoes hydrolysis producing non-reactive chemicals which can experience

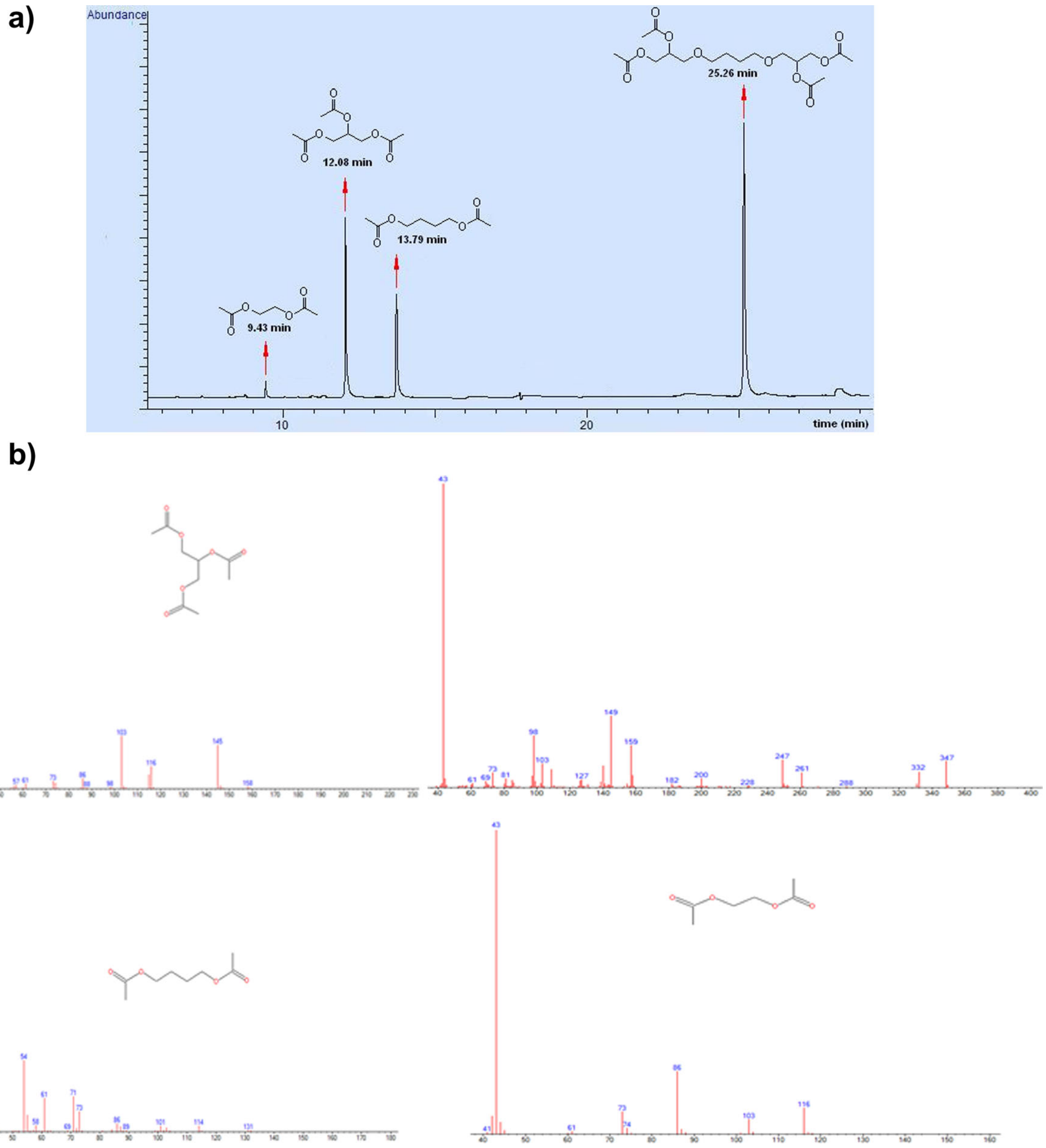


Fig. 6 **a** GC elution profiles and **b** mass spectra of BDDGE hydrolysis products (as acetyl derivatives)

further metabolic degradation to yield two main products: glycerol and butanediol (de Boulle et al. 2013). Accordingly, Ge-1BDDGE films were extracted, and then, the acetylated extract was analyzed by GC/MS. Four analytes were detected in GC which closely matched the reference in the internal MS data library for a diol-ether resulting from the hydrolysis of the epoxide groups in BDDGE, glycerol, 1,4-butanediol, and ethanediol, respectively (Fig. 6b). The presence of the diol-ether in the extracted films infers that un-reacted BDDGE hydrolyses under the film processing conditions to this non-toxic and non-genotoxic compound (De Boulle et al. 2013) which is usually excreted by urine (INSER M. 1999). The other three analytes detected emerge from the subsequent hydrolysis of the diol-ether and are known to be eliminated through metabolic cycles: 1,4-Butanediol is oxidized and then metabolized to CO_2 through the Krebs cycle (Irwin 1996); glycerol is converted into CO_2 by glycolysis metabolized (Robergs and Griffin 1998). These findings sustain the assumption that most of the un-reacted BDDGE potentially left in the films was hydrolyzed to safe product.

Conclusions

Gelatin films plasticized with D-sorbitol and cross-linked with water-soluble BDDGE were successful prepared by casting using reaction conditions and a cure-drying cycle determined from exploratory studies. The contribution of cross-linking points and one-point fixation structures resulted in a broad distribution of molecular motions in the produced materials. This was reflected in the non-monotonic variation of T_g, the increased elongation at break and reduced TS experienced as BDDGE increased up to BDDGE 1 % (g/100 g dry gelatin), associated with the plasticization effect of the pendant segments originated by masking which hydrolyze under casting to hydroxyl-terminated pendant groups. In terms of the performance, the formulation with BDDGE 1 % (g/100 g dry gelatin) showed the best set of final properties for the intended application: Significant and desirable reductions in solubility (77 %), moisture content (35 %) and water uptake (6-folds), and WVP (about 20 %) were obtained. The stretchability of Ge-1BDDGE films increased 11-folds without inducing significant brittleness (TS reduced 7-fold). The low (undetectable) level of free BDDGE in Ge-1BDDGE probed that such films are safe and promising food contact materials.

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