


# Sustainable collagen-based films cross-linked with *Vitex agnus-castus* for enhanced water retention and thermal stability

Cemile Ceren Kahraman<sup>1,\*</sup> , Nima Pourrasoul Sardroudi<sup>2</sup>, Hande Eski<sup>2</sup>, and Arife Candaş Adıgüzel Zengin<sup>2</sup>

<sup>1</sup> Usak University, Leather Textile and Ceramics Design Application and Research Center, Usak, Turkey

<sup>2</sup> Engineering Faculty, Leather Engineering Department, Ege University, Izmir, Turkey

Received: 29 July 2025 / Received in final form: 21 November 2025 / Accepted: 21 November 2025

**Abstract.** This study presented a sustainable methodology for designing collagen-based biomaterials by using natural reinforcing agents and environmentally friendly cross-linking to improve water absorption, water retention and thermal stability – key features for biomedical and environmental applications. Collagen films were enhanced with keratin and natural polysaccharides (carboxymethyl cellulose, microcrystalline cellulose, acacia gum, soy protein, and carrageenan), then cross-linked by *Vitex agnus-castus* (VAC) extract as an eco-friendly alternative for synthetic cross-linkers. Glutaraldehyde was used for comparative analysis. A series of comprehensive analyses were conducted, including Fourier-transform infrared spectroscopy (FTIR), thermogravimetric analysis (TGA), differential scanning calorimetry (DSC), and water absorption testing. Among all compositions, films containing 30% acacia gum and 3% VAC demonstrated the highest water absorption and retention capacity (5.90 g/g after 48 h) and enhanced thermal stability, with the minimal weight loss observed at 600 °C. FTIR analysis confirmed enhanced molecular connections through cross-linking, while DSC results validated increased structural resilience. The results indicated that VAC serves as both a structural and functional cross-linker, facilitating the creation of biodegradable, thermally resilient, and moisture-retentive films. This natural system presents significant potential for wound dressing and other biomedical applications within a sustainable material framework.

**Keywords:** Collagen films / *Vitex agnus-castus* / water absorption / thermal stability / polysaccharides / keratin

## 1 Introduction

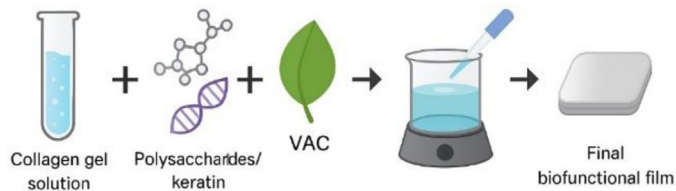
The skin is the largest and one of the most complex organs in the human body, acting as a dynamic barrier for protection, hydration, and temperature regulation [1]. Wounds and burns can significantly undermine its integrity, resulting in moisture depletion, microbial invasion, and protracted recovery. Modern wound care seeks better dressing materials that maintain moisture, enhance cellular regeneration, and endure physiological stress [2]. Collagen-based biomaterials are notably advantageous owing to their superior biocompatibility, biodegradability, and structural resemblance to the extracellular matrix [3–5].

However, native collagen suffers from limitations such as low thermal stability and mechanical strength, particularly after extraction process [6]. To overcome these issues, it is frequently combined with other natural polymers.

Polysaccharides, including carboxymethyl cellulose (CMC), microcrystalline cellulose (MCC), carrageenan, soy protein, and acacia gum, offer hydrophilicity, structural reinforcement, and a sustainable resource [7,8]. Moreover, keratin, a fibrous protein derived from animals such as wool, has attracted attention for its mechanical properties, hydrophilicity, and biocompatibility in film-based biomedical applications [9,10].

A critical aspect in designing stable collagen-based films lies in the choice of cross-linking strategy. Conventional agents such as glutaraldehyde (GA) provide efficient structural stability; despite this, their cytotoxicity and non-biodegradability restrict their biomedical use [11]. Recent initiatives have concentrated on creating natural and environmentally sustainable cross-linkers. *Vitex agnus-castus* (VAC), a Mediterranean medicinal plant abundant in iridoit glycosides and phenolic compounds, offers a promising eco-friendly alternative [12]. Iridoit-derived compounds have been documented to create Schiff bases with amine groups in collagen, potentially improving cross-linking efficacy while reducing toxicity [13,14].

\* e-mail: [cemile.kahraman@usak.edu.tr](mailto:cemile.kahraman@usak.edu.tr)



**Fig. 1.** Schematic illustration of VAC-crosslinked collagen film preparation.

Although there is an increasing literature on polysaccharide- and keratin-reinforced collagen films, research including VAC as a natural cross-linker remain very limited. Moreover, there is a lack of comparative assessments specifically focusing on water retention capacity and thermal stability which are two critical characteristics for wound dressings subjected to dynamic physiological conditions.

This study aims to fulfill this gap by developing hybrid collagen-based films strengthened with keratin and polysaccharides, cross-linked with VAC extract (See Fig. 1). The effects of VAC concentration and keratin as well as the polysaccharide type on film performance are assessed by FTIR, TGA, DSC, and water absorption tests. The findings intend to illustrate that VAC can work as an efficient, sustainable alternative for synthetic agents, improving both the functional and environmental efficacy of collagen-based films in biomedical applications.

## 2 Material and methods

### 2.1 Materials

Type I bovine collagen gel was sourced from the Leather and Footwear Research Institute, Collagen Department, located in Bucharest, Romania. Polysaccharides used in the formulations were carboxymethyl cellulose (CMC, Carl Roth, Germany), microcrystalline cellulose (MCC, Alfa Aesar, USA), soy protein (SP, Carl Roth, Germany), carrageenan (Carr, Duchefa, Netherlands), and acacia gum (AG, Galenik, Turkey). Hydrolyzed keratin, containing at least 85% protein, a maximum of 6% moisture, and a pH range of 4 to 6.5, was supplied from Carbosynth (UK).

The extract of *Vitex agnus-castus* (VAC), used as a natural cross-linker, was provided by the Faculty of Pharmacy at Ege University in Turkey. Glutaraldehyde (GA) solution was acquired from Sigma-Aldrich (USA).

### 2.2 Preparation of film biomaterials

A total of 77 collagen-based film biomaterials were synthesized using diverse ratios of polysaccharides, keratin, and cross-linking agents.

The collagen gel's solid content was reduced from 2.43% to 1%, and its pH was adjusted from 2.0 to a range of 7.0–7.4 using 1M NaOH. The final weight of each batch was determined using the equation:  $n \times 2.43 = M$ , where  $n$  represents the gel weight to be measured, and  $M$  denotes the final gel mass. Following pH adjustment, the gel was diluted with deionized water and equilibrated for 24 h at 4 °C.

Collagen gel was subsequently combined with keratin and polysaccharides (carboxymethyl cellulose (CMC), microcrystalline cellulose (MCC), carrageenan (Carr), soy protein (SP), and acacia gum (AG)) at weight/weight ratios of 15%, 20%, or 30%. VAC was incorporated at concentrations of 1%, 2%, or 3% w/w, whereas glutaraldehyde (GA) was maintained at a constant level of 0.05% for comparative analysis. Formulation example for a 20 g batch is; 15 g collagen gel, 4 g polysaccharide/keratin, and 1 g cross-linker (VAC or GA).

Mixtures were homogenized and subsequently cast into glass petri dishes. Film biomaterials were dried at room temperature in an oven for a duration of 24 h.

Table 1 presents a selection of representative collagen-based film samples prepared with various polysaccharides/keratin and cross-linking agents. The representative DSC peak data for selected samples is summarized in Table 2. The complete list of 77 samples with their compositions and codes is provided in the Supplementary Material (Tab. S1).

### 2.3 Assessment of water absorption capacity

The water absorption capacity was determined using an analytical balance with a precision of 0.0001 g (Ege University, Chemical Laboratory). Samples were weighed ( $W_0$ ) and subsequently immersed in 2 mL of deionized water at room temperature for a duration of up to 48 h. Weights were measured at 30 min, 1 h, 2 h, 4 h, 24 h, and 48 h intervals ( $W_s$ ). The swelling ratio was determined using the following formula: Water Absorption (g/g) =  $(W_s - W_0) / W_0$ . Measurements were conducted in six replicates for each sample.

### 2.4 Structural analysis via FTIR

Fourier Transform Infrared Spectroscopy (FTIR) analyses were performed using a PerkinElmer Spectrum Two at the Instrumental Analysis Laboratory of Ege University. Spectra were obtained within the range of 4000–600  $\text{cm}^{-1}$ , utilizing a resolution of 0.5–4  $\text{cm}^{-1}$ , with each sample undergoing 8–12 scans.

### 2.5 Thermal analysis (TGA and DSC)

Thermogravimetric analysis was conducted using a PerkinElmer TGA8000 instrument. Each sample, weighing approximately 1–3 mg, was analyzed at a heating rate of 10 °C/min, spanning temperatures from 30 °C to 600 °C.

Differential scanning calorimetry was performed utilizing a Shimadzu DSC 60-Plus instrument. Samples (approximately 4–5 mg) were pre-soaked in deionized water for 1 h and subsequently sealed in aluminum pans. Heating occurred from 30 °C to 120 °C at a rate of 10 °C/min under standard conditions.

## 3 Results

### 3.1 Water absorption capacity

The capacity for water absorption is a critical characteristic of biomaterials designed for biomedical applications, including wound dressings, tissue-engineering scaffolds,

**Table 1.** Selection of representative collagen-based film samples prepared with various polysaccharides/keratin and cross-linking agents.

Sample code	Polymer type	Polymer ratio (%)	Cross-linker	Cross-linker ratio (%)
601BC	–	–	–	–
1906_12	Keratin (glycerolized)	50	–	–
9-10/12/23-1	–	–	Glutaraldehyde	0.05
1002Coll_2H	–	–	VAC	2
1002Coll_3H	–	–	VAC	3
1003_5 Koll	Acacia Gum	30	VAC	3
601CM30H	Carboxymethyl Cellulose	30	VAC	2
2704_12	Keratin	20	VAC	3
1906_3	Carrageenan	20	Glutaraldehyde	0.05
601SB15H	Soy Protein	15	VAC	2
601MC15H	Microcrystalline Cellulose	15	VAC	2

**Table 2.** Peak points and enthalpy values obtained from DSC analysis.

Samples	Td peaks	$\Delta H$ total J/g	Mean Td
601BC	67.57, 77.41, 163.31	–3,37	102,76
1002_Coll_3H	91.32, 118.71	–0,36	105,02
1003_5K	66.9, 72.84, 148.41	–8,58	96,05
2704_15	57.28, 67.92, 145.5	–18,23	90,23
1002_Karr15_3H	56.94, 77.98	–4,3	67,46

and drug delivery systems. The ability of a material to absorb and retain moisture impacts its interaction with biological tissues and influences processes including cellular growth and nutrition transport [15]. As emphasized in recent studies, optimizing water uptake properties is essential for customizing biomaterials for applications, thereby enhancing their performance and practical usage [16]. This study assessed the water absorption capacity of collagen-based films reinforced with different biopolymers and cross-linked with either *Vitex agnus-castus* (VAC) extract or glutaraldehyde (GA), with the objective of evaluating their performance applications.

The gravimetric swelling test was performed in distilled water at ambient temperature for a duration of 48 h. The test was conducted in six repetitions to guarantee repeatability. This method, known for its simplicity and cost-efficiency, enables extended monitoring, hence enhancing the comprehension of both absorption and retention throughout time. The findings validated that films exhibiting elevated cross-linking densities and denser internal architectures absorbed reduced amounts of water, aligning with existing research [17–20]. The incorporation of hydrophilic functional groups, such as –OH and –COOH, the pore structure, and external conditions including temperature were identified as significant factors impacting variations in swelling behavior.

The dense configurations of collagen film biomaterials demonstrate reduced permeability to water and increased resistance to degradation, making them suitable for applications that necessitate prolonged structural stability

[20,21]. Nonetheless, this structure limits water absorption and controls swelling behavior [22]. Consequently, materials with either fast or limited swelling characteristics may be advantageous for many biological applications. The swelling results obtained in this study are presented graphically in three distinct groups based on formulation characteristics, enabling a comparative interpretation across biomaterial categories.

The biofilm produced by combining polysaccharide and keratin exhibited generally lower swelling ratios in VAC-crosslinked samples compared to those GA-crosslinked samples. Water absorption capacity measurements could not be obtained from samples containing carboxymethyl cellulose (CMC), since the samples disintegrated after 30 min.

The graphs demonstrate that the acquired values support the expected results from the water absorption capacity study. Following that, the selection of biomaterials must be based on their efficacy in the intended application. The water absorption properties of the VAC-crosslinked films support their use in environments necessitating controlled hydration and extended stability, such as wound dressings or slow-release systems. Conversely, GA-crosslinked or keratin-rich films exhibit enhanced swelling capacity, making them potentially more appropriate for short-term, high-fluid environments. The ability to tailor swelling behavior through polymer composition and cross-linker type offers considerable flexibility in the production of biological materials.

### 3.2 FTIR spectroscopy results

Fourier Transform Infrared (FTIR) spectroscopy was used to assess the structural integrity and conformational alterations of collagen-based films after reinforcing and cross-linking. It is a prevalent analytical method for evaluating the molecular fingerprint of materials, especially effective in determining the existence and stability of protein secondary structures [23]. The investigation concentrated on the amide I, II, and A bands, which signify protein backbone integrity, hydrogen bonding, and potential denaturation events.

The amide I band, mainly associated with C=O stretching vibrations, indicates the secondary structure of proteins, including  $\alpha$ -helix and  $\beta$ -sheet conformations. In contrast, the amide II band is responsive to N-H bending and C-N stretching vibrations, both of which are influenced by environmental or chemical changes [24,25]. Shifts or fluctuations in intensity within these bands indicate alterations in hydrogen bonding networks and protein stability. Furthermore, the disparity in wavenumber between the amide I and II bands ( $\Delta V$ ) is regarded as a crucial measure for evaluating structural order or disruption in collagen matrices. An increased  $\Delta V$  often indicates conformational irregularity, frequently due to insufficient cross-linking or environmental denaturation [26].

The comparative study of the spectra showed the impact of various cross-linkers (VAC vs. GA) and biopolymer reinforcements on molecular interactions and stability. The results provide further understanding of the chemical interactions and structural integrity of the biofilms, acting as supplementary evidence to swelling and thermal behavior assessments.

The FTIR graphs of the biofilm samples containing keratin showed no amide III bands. Collagen structure's  $1250\text{ cm}^{-1}$  peak correlates to organic molecules' C-O, C-N stretching, or C-H bending vibrations. The dense matrix structure of the sample makes peak identification difficult. Hydrogen connections between keratin and collagen and cross-linkers may reduce vibrations in this area, making them hard to detect. Samples containing higher amounts of crosslinker or polysaccharide/keratin exhibited increased density ratios, indicating enhanced crosslinking strength. In films cross-linked with 3% VAC, the AI/AA density ratio was nearly 1.0, compared to 0.777 in 601BC (control sample). The value decreases slightly (0.88 to 0.99) when mixed with polysaccharides, although it stays over 0.8 in most samples.  $\Delta V$  values, indicating protein structure, exceeded 100 in certain samples. The film structure obtained by free drying at room temperature is dense and exhibits a lack of porosity in both surface area and structure, which is expected to induce structural conformational distortions during biomaterial production, an outcome that is undesirable.

For a clear and comparative analysis of the structural integrity of the films, the FTIR data were categorized into four distinct categories according to compositional characteristics: (i) films without keratin or polysaccharide reinforcement, (ii) GA-crosslinked films, (iii) keratin-reinforced VAC-crosslinked films, and

(iv) polysaccharide-reinforced VAC-crosslinked films (see Figs. 2, 3, 4). This categorization facilitated the systematic assessment of cross-linking effects and reinforcement strategies on the molecular structure of collagen. A dual-axis line chart was used for each group to concurrently display two essential FTIR parameters: the AI/AA ratio, which indicates the preservation of protein secondary structure, and the  $\Delta V$  (I-II), which signifies conformational regularity or disorder. The simultaneous plotting of these metrics facilitates the assessment of molecular integrity and structural deviation, providing a thorough understanding of the impact of each formulation strategy on the collagen matrix at the molecular level. Figures 5–8 illustrate dual-parameter comparisons, facilitating a group-wise structural analysis.

To provide a clearer interpretation of the FTIR findings, representative spectra of selected formulations (601BC, 1002\_coll\_3H, 1002\_Karr15\_3H, 1003\_5K, and 2704\_15) are presented in the Supplementary Material. These spectra help visualize the characteristic amide bands and the spectral modifications associated with cross-linking and biopolymer incorporation. These spectra allow a clearer visualization of the characteristic amide bands and the modifications induced by keratin, polysaccharides and VAC/GA cross-linkers.

The control collagen film (601BC) exhibited the typical amide bands of type-I collagen:

- Amide A ( $\sim 3300\text{--}3325\text{ cm}^{-1}$ ): N-H stretching overlapped with bound water; broad and intense due to the highly hydrated structure of unmodified collagen.
- Amide I ( $\sim 1630\text{--}1655\text{ cm}^{-1}$ ): C&O stretching; the peak position suggests partial preservation of  $\alpha$ -helix/ $\beta$ -sheet conformations.
- Amide II ( $\sim 1530\text{--}1550\text{ cm}^{-1}$ ): N-H bending and C-N stretching.
- Amide III ( $\sim 1230\text{--}1250\text{ cm}^{-1}$ ): Weak in the control film due to the dense, low-porosity structure formed during free drying.

Upon VAC cross-linking (1002\_coll\_3H), Amide A shifted to lower wavenumbers, indicating stronger hydrogen bonding. The Amide I band also became sharper, supporting improved structural order. This is consistent with previous findings on VAC-protein interactions.

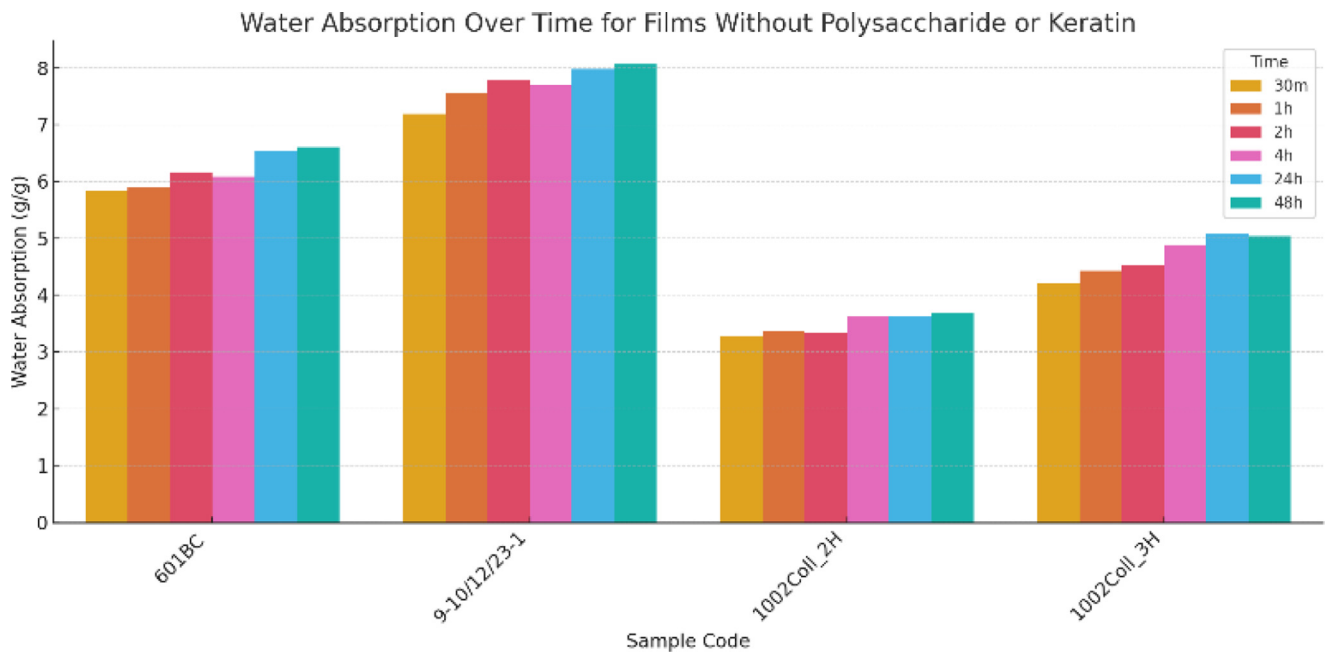
Keratin-reinforced films (1003\_5K and 2704\_15) showed noticeable reduction or disappearance of the Amide III band ( $\sim 1240\text{ cm}^{-1}$ ). This may be attributed to:

- (i) overlapping of C-O/C-N stretching in keratin,
- (ii) dense intermolecular packing caused by keratin-collagen H-bonding,
- (iii) restricted molecular mobility induced by VAC cross-linking.

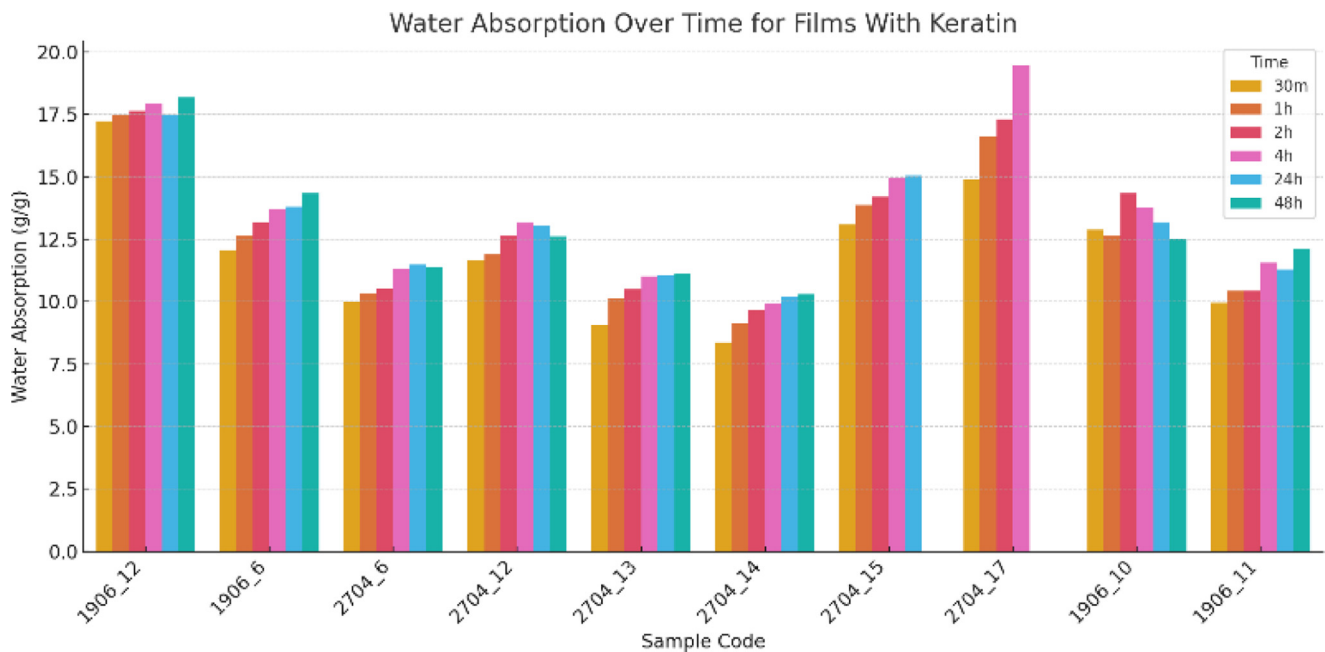
These trends are also reflected in their AI/AA ratios approaching 1.0, indicating high structural preservation.

In polysaccharide-containing films (1002\_Karr15\_3H), additional features appeared:

- Sulphate peaks from carrageenan ( $\sim 1210\text{--}1260\text{ cm}^{-1}$ ;  $\sim 1020\text{--}1070\text{ cm}^{-1}$ ).
- C-O-C glycosidic vibrations from acacia gum ( $\sim 1030\text{--}1100\text{ cm}^{-1}$ ).



**Fig. 2.** Water absorption overtime for films without polysaccharide or keratin.



**Fig. 3.** Water absorption overtime for films with keratin.

These overlaps contribute to peak broadening in the fingerprint region, complicating the assignment of Amide III while also confirming successful incorporation of biopolymers.

Across all reinforced or cross-linked films, the increase in AI/AA ratios and reduced  $\Delta V$  values compared to the control sample demonstrate improved conformational stability. GA-crosslinked films showed the most pronounced enhancement, while VAC-keratin combinations

produced the most balanced structure (high AI/AA with controlled  $\Delta V$ ), reflecting synergistic hydrogen-bonding effects.

The control group displayed low AI/AA ratios and moderate  $\Delta V$  values, suggesting a partial disruption of protein secondary structures resulting from insufficient cross-linking. The structural irregularity supports the recognized limitations of unmodified collagen films. GA-crosslinked films exhibited enhanced AI/AA ratios

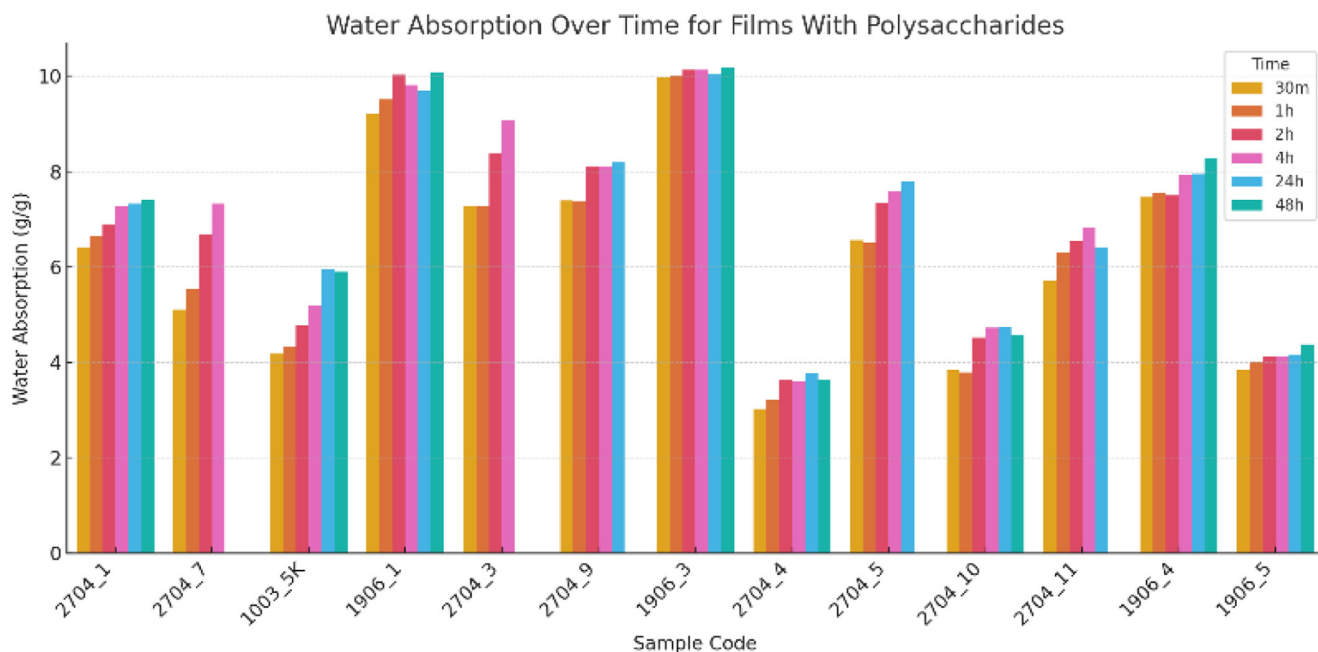


Fig. 4. Water absorption overtime for films with polysaccharide.

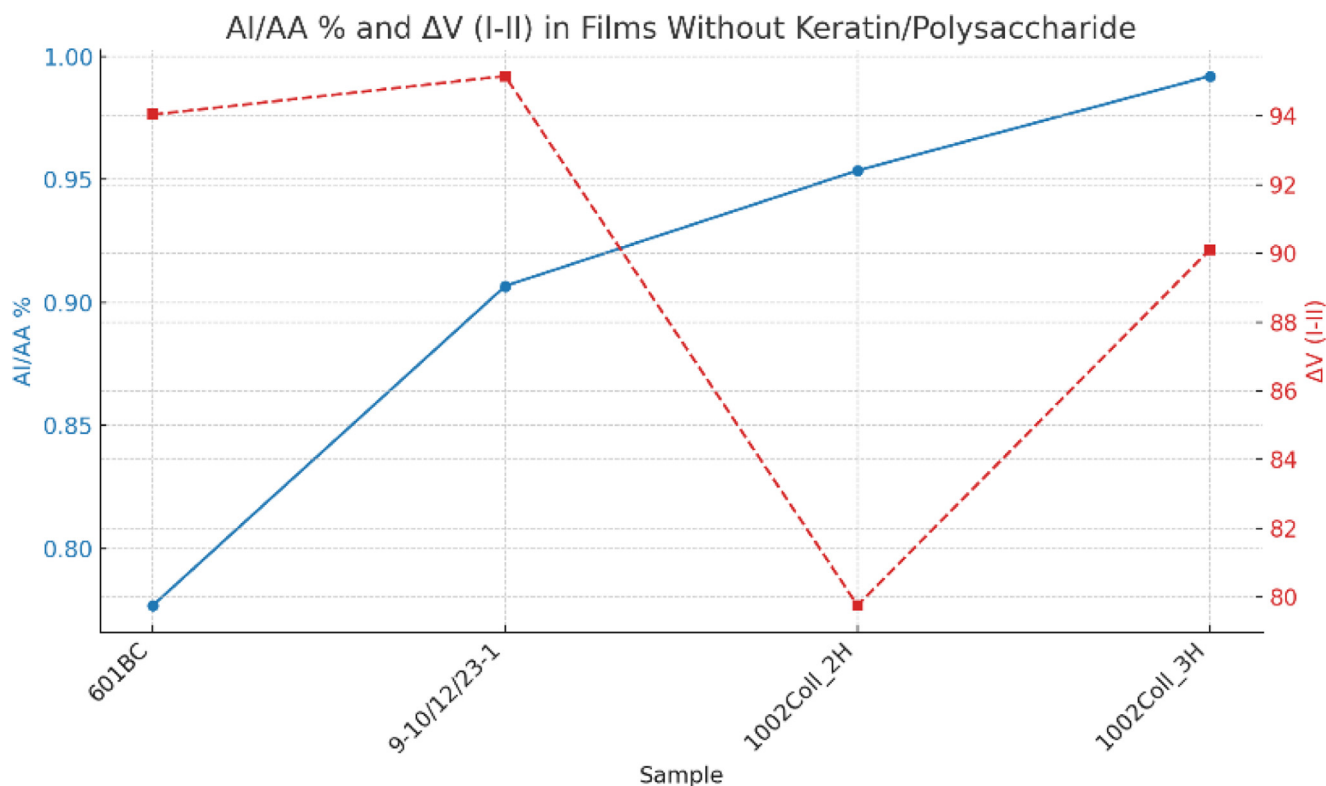
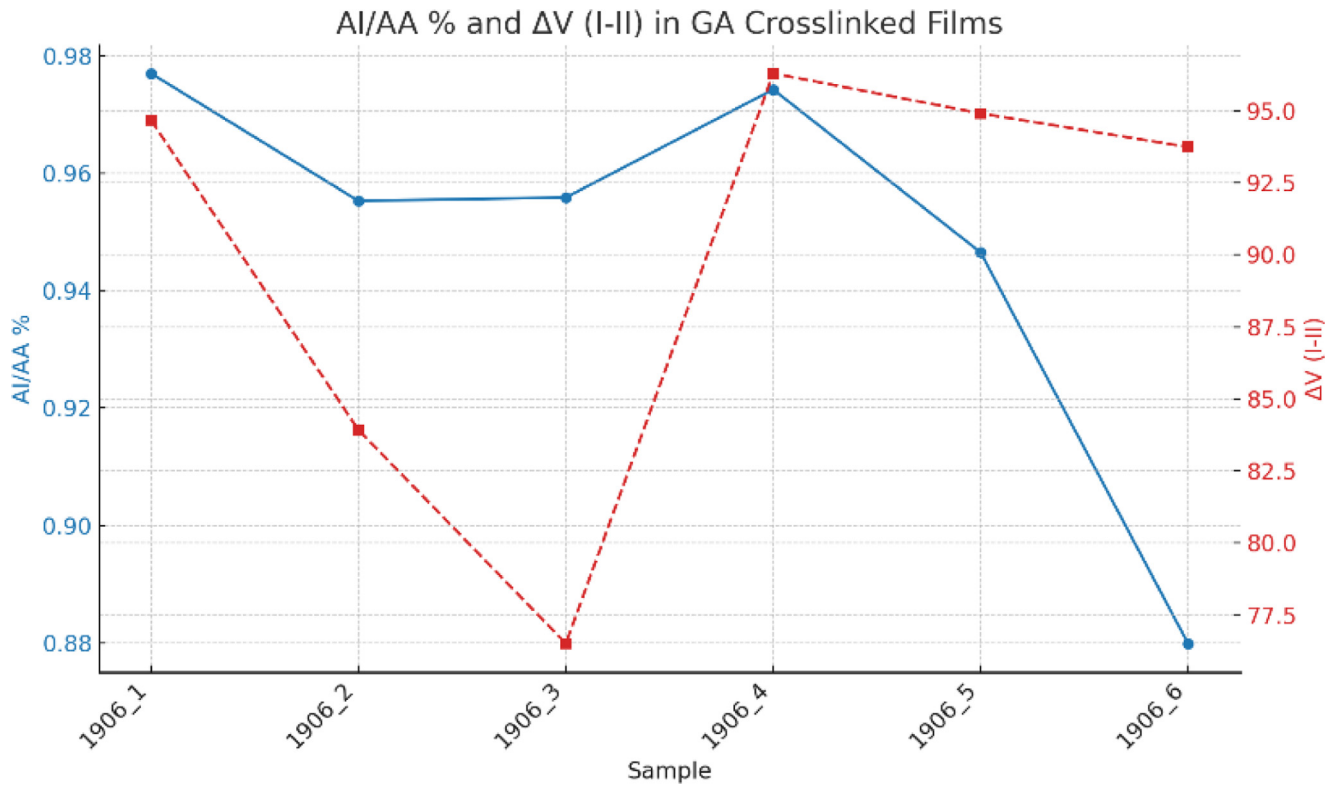


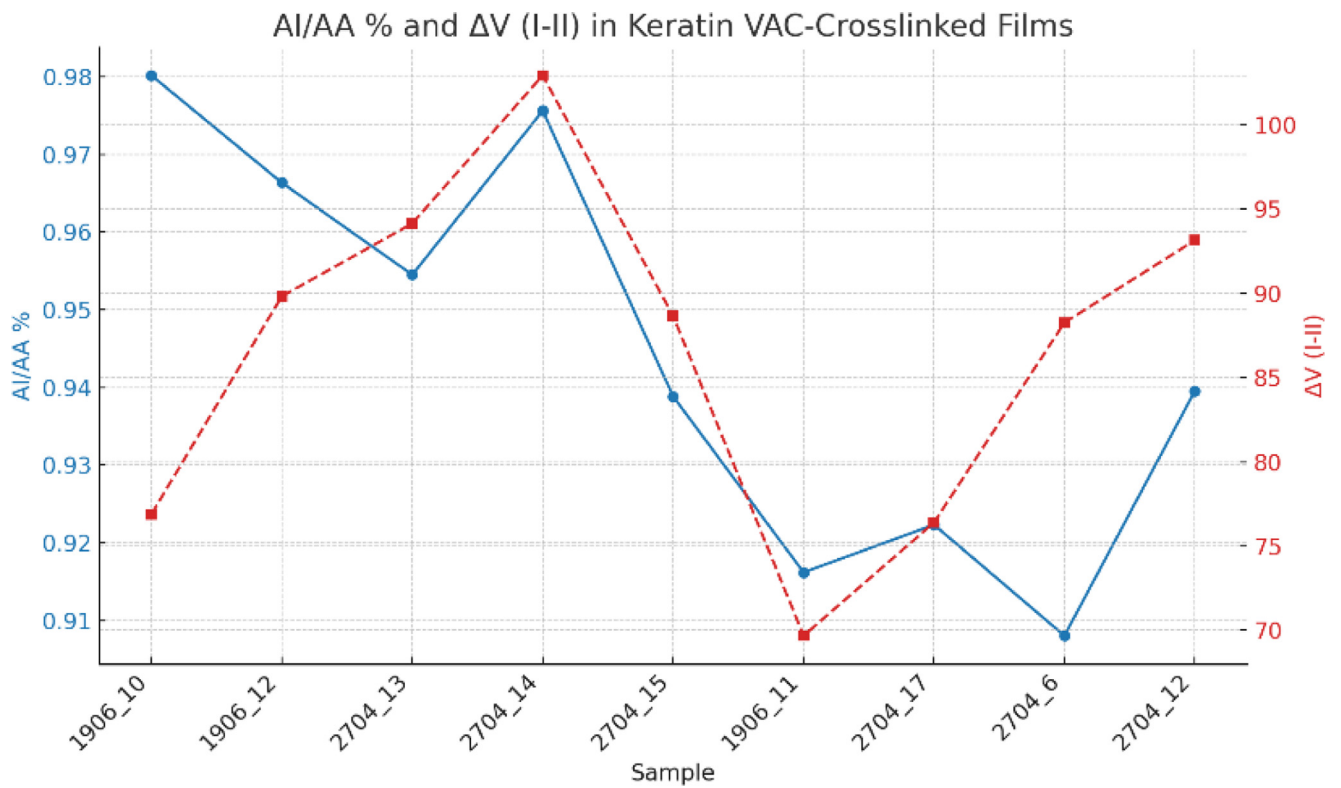
Fig. 5. AI/AA % and  $\Delta V$  (I-II) in films without keratin/polysaccharide.

and more uniform  $\Delta V$  values, indicating improved stabilization of the collagen network. The results confirm the established effectiveness of glutaraldehyde in improving molecular cohesion, despite its associated toxicity issues. Keratin-reinforced VAC-crosslinked films exhibited

high AI/AA ratios near 1.0, accompanied by relatively stable  $\Delta V$  values. This indicates that keratin and VAC work together to maintain collagen integrity by strengthening hydrogen bonding [27]. Samples reinforced with polysaccharides demonstrated varying AI/AA values and



**Fig. 6.** AI/AA % and  $\Delta V$  (I-II) in films GA-crosslinked.



**Fig. 7.** AI/AA % and  $\Delta V$  (I-II) in films keratin reinforced VAC-crosslinked.

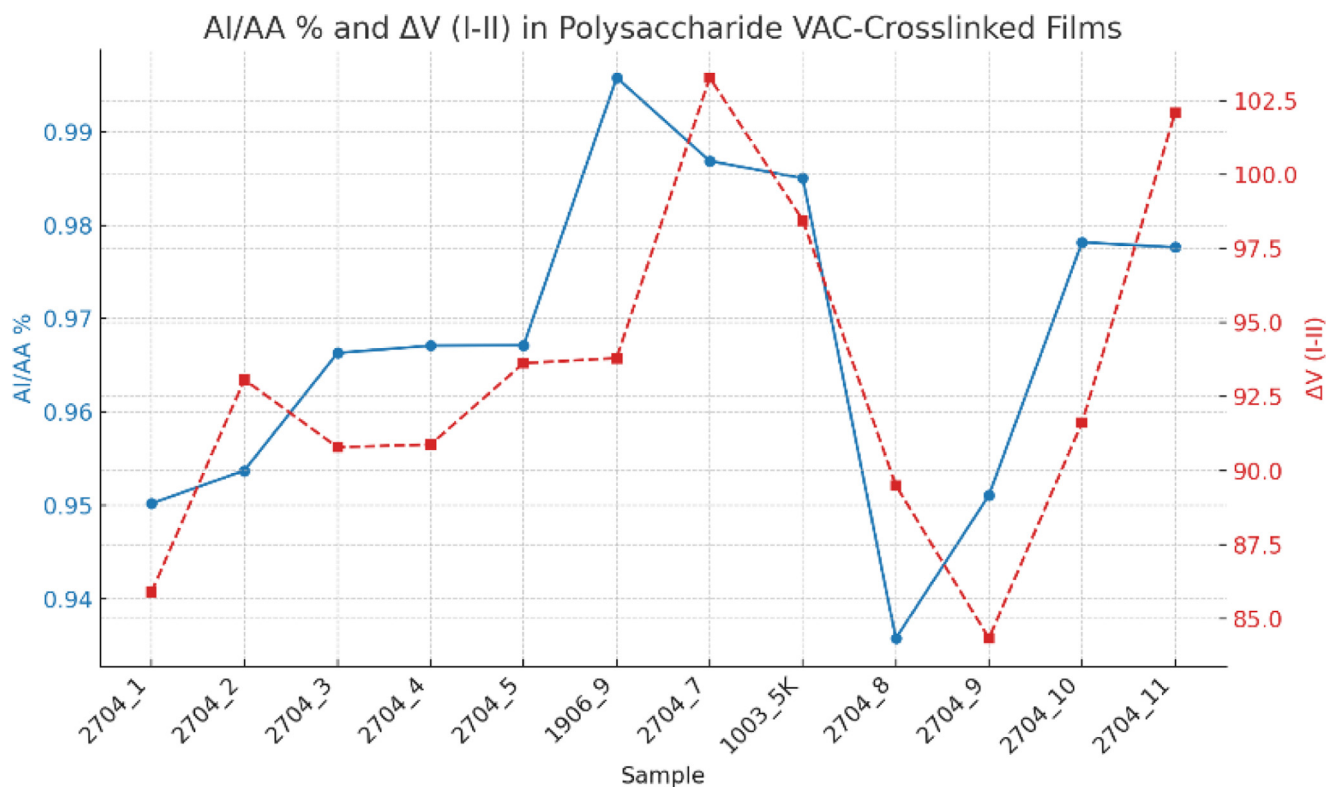


Fig. 8. AI/AA % and  $\Delta V$  (I-II) in films polysaccharide reinforced VAC-crosslinked.

an increased  $\Delta V$  range, reflecting different interaction levels based on the polymer type. Certain formulations demonstrated significant structural integrity, suggesting effective biopolymer integration through VAC.

### 3.3 Thermal analysis results

Thermogravimetric analysis (TGA) is a widely used method for evaluating the thermal stability, compositional changes, and degradation behavior of polymeric biomaterials. This method is important in assessing the impact of biopolymer reinforcements and cross-linkers on the thermal resistance and structural integrity of collagen-based films [28]. TGA quantifies the mass reduction of a sample upon heating, offering insights into moisture evaporation, thermal degradation of organic materials, and the existence of thermally stable residues.

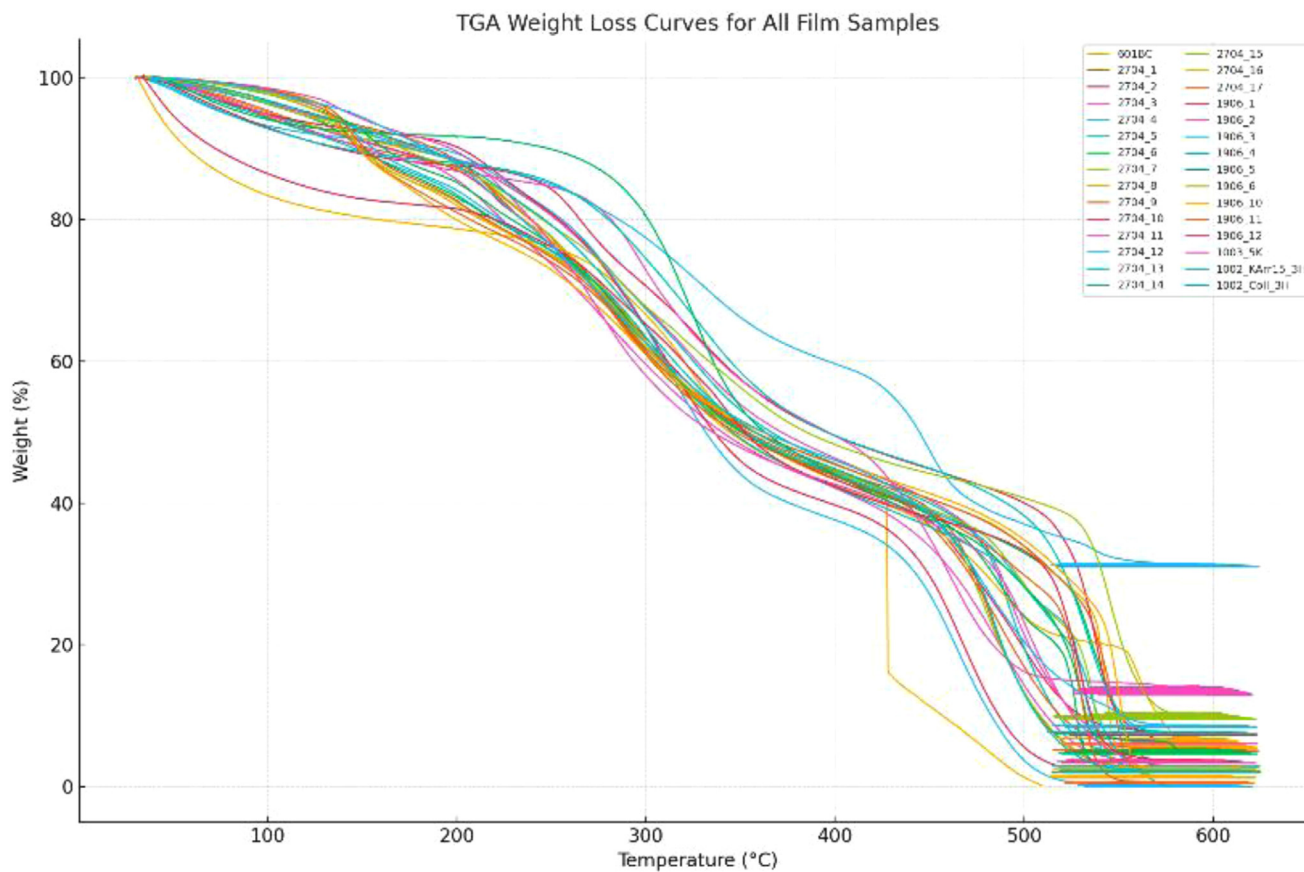
The analysis identifies three discrete degradation phases: the initial phase (approximately 30–100 °C), linked to the evaporation of physically absorbed and loosely bound water; a subsequent phase (200–400 °C), related to the decomposition of the collagen backbone; and a final phase exceeding 500 °C, indicative of the disintegration of highly stable carbonaceous residues [29]. The principal decomposition step, typically observed around 300 °C in DTG curves, is the most significant aspect, as it reflects the structural compactness and cross-linking density of the film. The delayed onset of degradation, higher peak decomposition temperature, and increased residual mass

indicate enhanced thermal stability, typically linked to stronger intermolecular interactions and a more densely cross-linked polymer network [30].

In this study TGA is used to analyze the thermal profiles of film samples and to evaluate the efficacy of *Vitex agnus-castus* (VAC) as a natural cross-linker in improving thermal resistance. The observed variations in DTG peak temperatures and total mass loss support the influence of biopolymer type and cross-linking technique on the thermal properties of collagen matrices.

All film formulations had a distinct three-phase thermal degradation profile, starting with moisture evaporation (30–100 °C), followed by the major breakdown of organic components (200–400 °C), and concluding with the destruction of residual substances. The difference in thermal resistance across formulations is seen in both the initiation of significant breakdown and the amount of residue preserved at elevated temperatures, with some VAC-crosslinked samples – especially those reinforced with polysaccharides – demonstrating improved thermal stability.

Collagen-based films, owing to their biodegradable and organic characteristics, often produce little residue during thermal decomposition. Nonetheless, significant variation exists due to disparities in graphical formulation. The maximum residue was noted in the gluteraldehyde-crosslinked carrageenan reinforced film (1906\_3, 30.99%), indicating the stabilizing influence of synthetic crosslinkers on sulfate-rich polysaccharide matrices. On the other hand,



**Fig. 9.** TGA weight loss curves for all samples.

VAC-crosslinked samples like 1002\_KArr15\_3H (15% carrageenan + 3% VAC) demonstrated efficient natural crosslinking while sustaining low residue levels (8.39%). The control film of collagen (601BC) demonstrated one of the lowest residual weights, affirming its natural biodegradability and highlighting the roles of biopolymer reinforcement and crosslinker selection in heat resistance. Most VAC-crosslinked samples exhibit a weight loss of at least 5% upon the heating process.

The crosslinking effect is recognized for its ability to reduce early weight loss; thus, it is expected that all samples will demonstrate a decrease compared to the unmodified film sample. The absence of crosslinkers in the structure of the 1906\_12 sample led to diminished thermal stability. It is noteworthy that some GA-crosslinked samples exhibit lower strength compared to the VAC-crosslinked samples.

The DTG profiles demonstrate the thermal degradation characteristics of films cross-linked with 2–3% VAC extract and reinforced with various biopolymers (except 1002\_Coll\_3H). All samples demonstrate a primary degradation peak between 250 and 350°C that indicate the breakdown of collagen-polysaccharide/keratin networks. Among the evaluated formulations, the film with 30% acacia gum and 3% VAC (1003\_5K) exhibited the narrowest and sharpest DTG peak, indicating a more cohesive and densely cross-linked structure. The keratin

reinforced film containing 2% VAC (2704\_15) and the carrageenan reinforced film with 3% VAC (1002\_Karr15\_3H) exhibited wider degradation bands, suggesting a more gradual thermal decomposition. The film with 3% VAC (1002\_Coll\_3H) demonstrated the lowest degradation intensity, thereby highlighting the role of biopolymer support in enhancing thermal resilience. The findings indicate that both VAC concentration and polysaccharide reinforcement have a significant impact on the thermal decomposition kinetics and structural integrity of the films.

Differential Scanning Calorimetry (DSC) is a commonly used method in biomaterials research for examining thermal transitions and structural stability under regulated heating conditions. In collagen-based materials, DSC mostly assesses the denaturation temperature ( $T_d$ ) as an indicative of the thermal stability of the triple-helical collagen configuration. The location and morphology of the endothermic peak in the DSC thermogram are strongly correlated with the molecular interactions inside the matrix, particularly hydrogen bonding and the degree of cross-linking [31,32].

$T_d$  is important for evaluating the durability of scaffolds and wound dressings under physiological settings. An elevated  $T_d$  indicates increased resistance to heat degradation, typically resulting from enhanced cross-link density due to the incorporation of stabilizing agents such

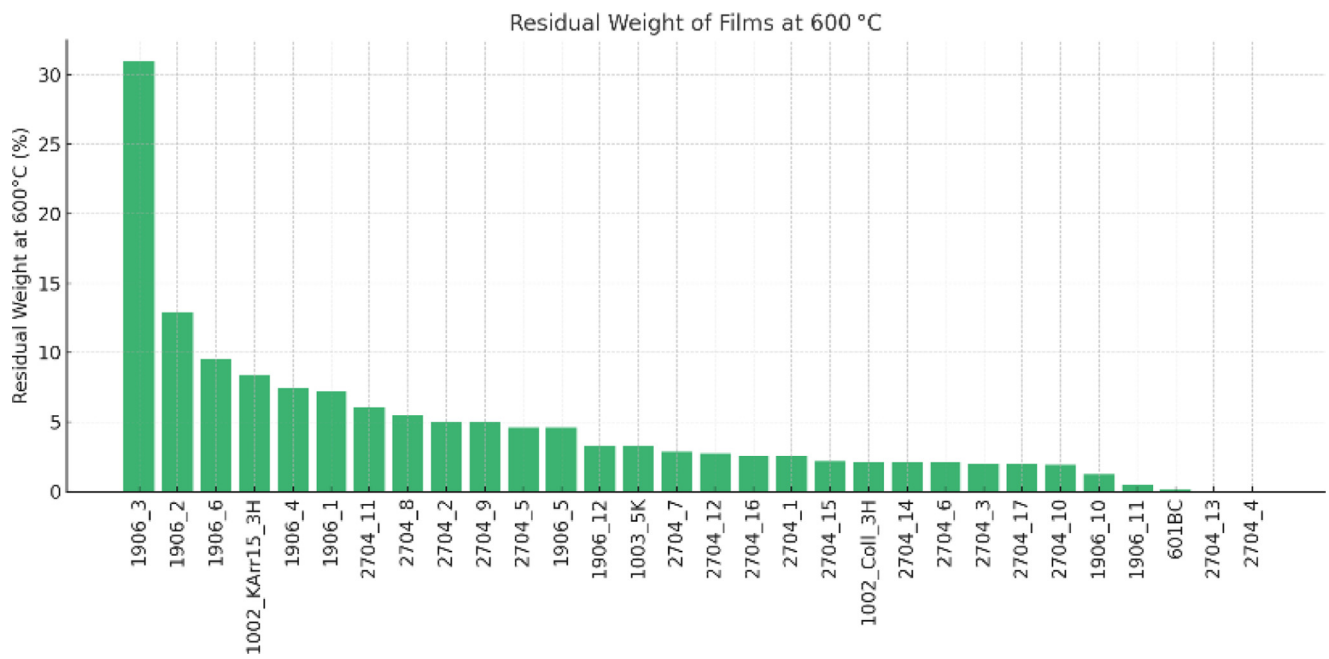


Fig. 10. Residual weight of films at 600 °C.

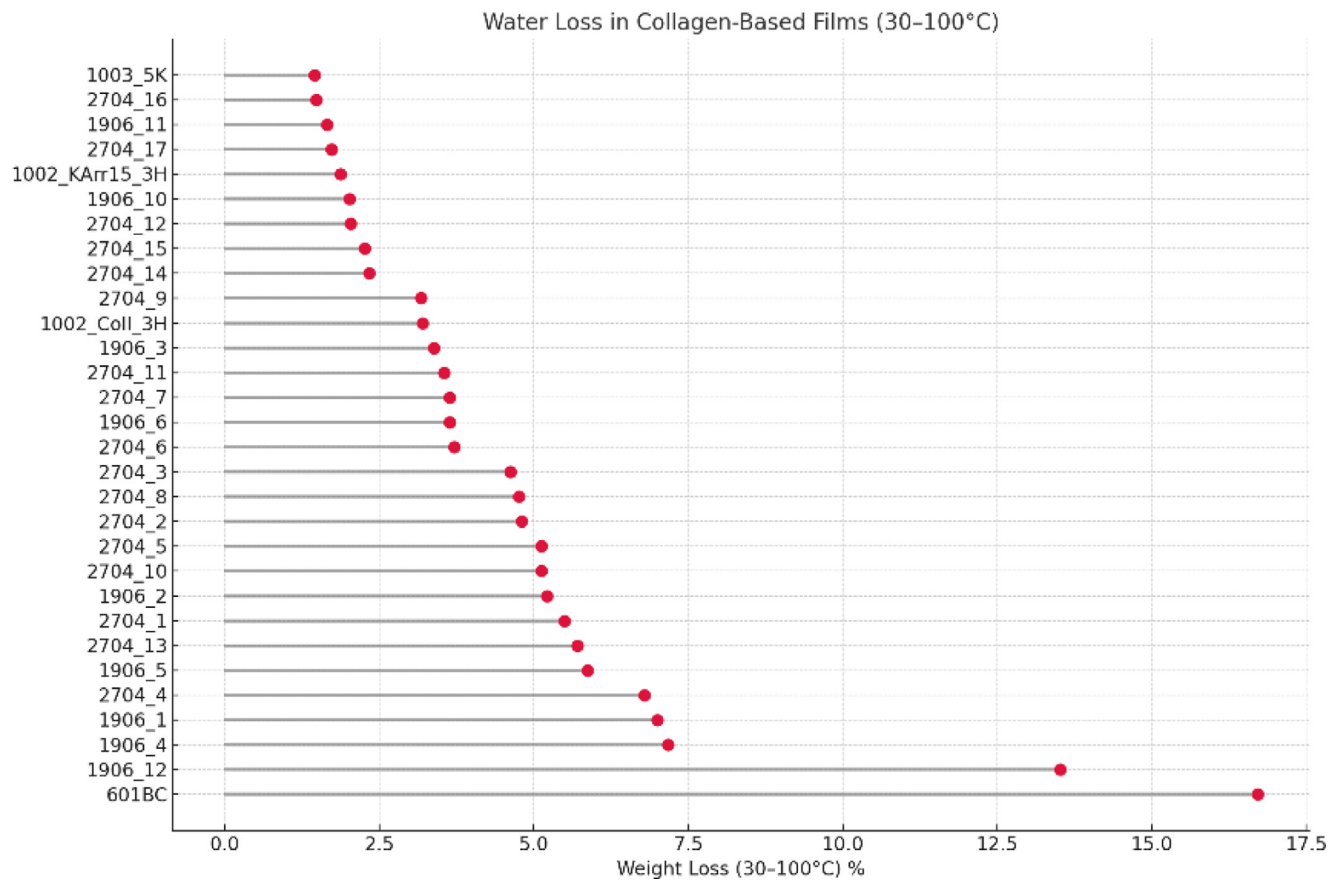


Fig. 11. Water loss in collagen-based films (30-100°C).

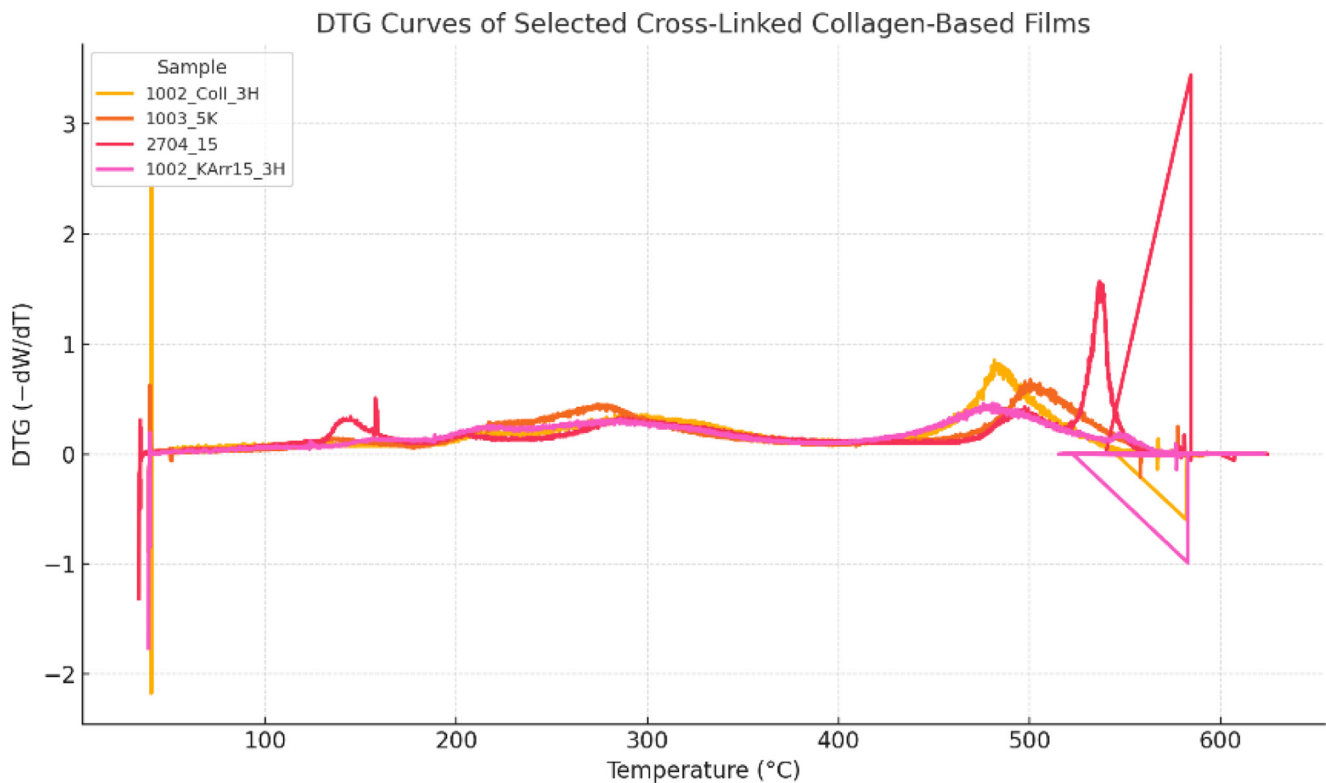


Fig. 12. DTG curves of selected cross-linked films.

as polysaccharides or keratin [33]. This work used DSC to analyze the thermal properties of certain collagen-based films that were crosslinked with VAC extract and reinforced with acacia gum, keratin, or carrageenan.

This approach provided insights into the cross-linking efficiency and structural integrity of each formulation by examining the beginning of denaturation and the enthalpy of the transition. These thermal measurements are essential for forecasting the long-term efficacy of biomaterials under physiological stress and validating their appropriateness for biomedical applications, including wound healing Figures 9–14.

The denaturation temperatures ( $T_d$ ) and total enthalpy values ( $\Delta H$ ) were assessed to ascertain the structural integrity and thermal stability of each formulation.

The control film of collagen (601BC) had the lowest thermal stability, with denaturation transitions occurring at about 67.57 °C, 77.41 °C, and 163.31 °C, with a total enthalpy of  $-3.37$  J/g. The existence of several peaks and modest energy release indicates a diverse collagen structure with decreased resistance to thermal breakdown.

The film cross-linked with 3% *Vitex agnus-castus* (VAC) extract (1002\_coll\_3H) exhibited the maximum thermal stability, characterized by increased peak temperatures (91.32 °C, 118.71 °C) and a significantly diminished enthalpy value ( $-0.36$  J/g). This outcome validates the crosslinking efficacy of VAC, which improved the heat stability of the collagen matrix by incorporating supplementary molecular connections and structural rigidity.

The addition of 15% carrageenan with 3% VAC (1002\_Karr15\_3H) resulted in enhanced thermal stability compared to the control. However, its  $T_d$  values (56.94 °C, 77.98 °C) were lower than those of the VAC-only film, despite a significantly elevated enthalpy value ( $-4.30$  J/g). This suggests that while carrageenan improved matrix cohesiveness, it might have hindered crosslinking efficiency, leading to increased energy release during the transition.

The film reinforced with 30% acacia gum and 3% VAC (1003\_5K) demonstrated a comparable improved thermal profile, with  $T_d$  peaks at around 66.90 °C, 72.84 °C, and 148.41 °C. Although the temperature values were similar to the control, the increased enthalpy ( $-8.58$  J/g) indicates enhanced molecular mobility and potentially more complex thermal dissociation pattern attributed to the branched polysaccharide structure of acacia gum.

On the other hand, the film comprising 30% keratin and 2% VAC (2704\_15) had the highest total enthalpy ( $-18.23$  J/g) despite exhibiting mild thermal transition temperatures (57.28 °C, 67.92 °C, 145.50 °C). This can be related to the heterogeneity contributed by keratin, which creates an uneven network that absorbs and release greater energy during heating, while lacking the uniform thermal resistance afforded by VAC alone.

In general, VAC-crosslinked films, especially the 3% VAC-only system, demonstrated enhanced heat resistance. The incorporation of carrageenan, acacia gum, or keratin resulted varying effects: carrageenan and keratin

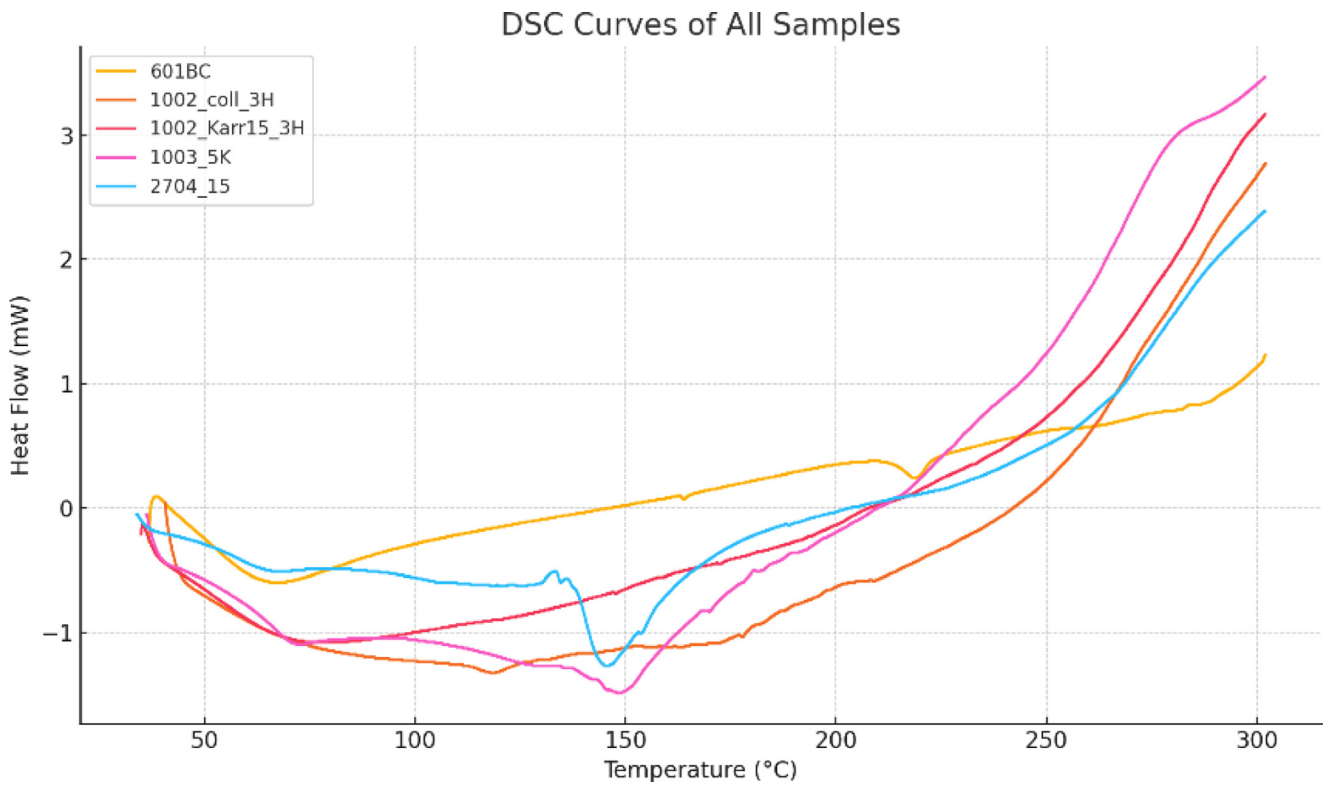


Fig. 13. DSC curves of all samples.

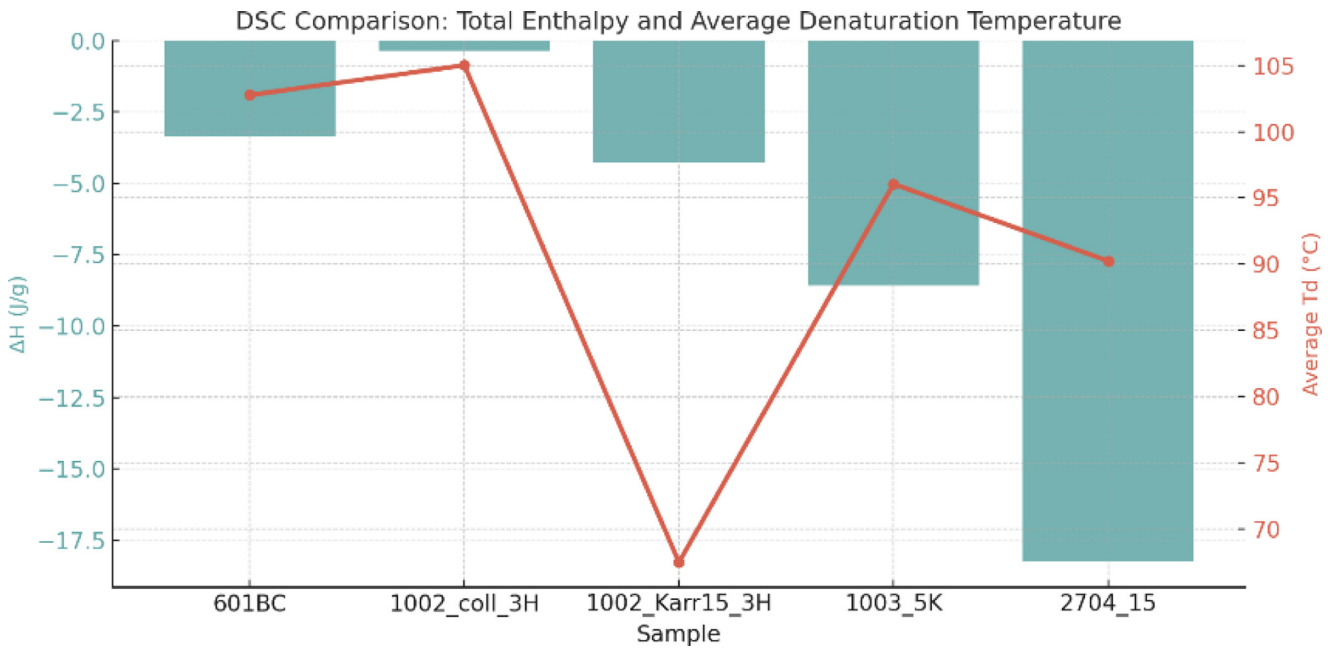


Fig. 14. DSC comparison for total enthalpy and average denaturation temperature.

reinforced ethalpic response without significantly increasing Td, whereas acacia gum led to intermediate thermal performance. These discoveries underscore the adjustability of collagen film characteristics via changes based on polysaccharides and proteins.

### 4 Discussion

The production of collagen-based films for biomedical applications necessitates a meticulous balance of structural integrity, moisture regulation, and thermal resistance.

This study incorporated natural biopolymers – acacia gum, carrageenan, carboxymethyl cellulose, microcrystalline cellulose, and keratin – into collagen matrices and cross-linked those using *Vitex agnus-castus* (VAC) extract to create biodegradable and sustainable films with tailored physicochemical properties. Our findings enhance the potential of VAC as a functional cross-linker that can modulate its fundamental material properties, while also providing fresh insights into the synergistic roles of polysaccharide and protein-based additives.

DSC and TGA tests demonstrated that VAC substantially enhanced thermal stability. The film, including only 3% VAC (1002\_coll\_3H), showed a high average denaturation temperature and a low enthalpy change, signifying effective crosslinking and enhanced molecular stiffness. The addition of 3% VAC and 30% acacia gum (1003\_5K) resulted in a film with improved water retention and heat degradation properties, supporting the notion that the effectiveness of VAC is preserved or possibly enhanced in environments rich in polysaccharides. This aligns with prior literature detailing the beneficial impact of acacia gum on hydrogel stability and water retention, as the branched polysaccharide structure facilitates uniform hydration and structural reinforcement [34,35].

On the contrary, films with keratin (2704\_15) or carrageenan (1002\_Karr15\_3H) had wider thermal degradation patterns and elevated enthalpy reactions, suggesting a more diverse internal structure. Although keratin provides mechanical support, its interaction with VAC at a 2% concentration is insufficient for establishing uniform crosslinks within the matrix, resulting in increased energy release and diminished denaturation temperatures. These data emphasize that whereas VAC contributes in crosslinking, the form and concentration of included biopolymers significantly affect the ultimate material properties. The results suggest that 2% VAC might be inadequate in systems necessitating robust inter-polymer cohesion, such as keratin-rich matrices.

The water absorption study reinforces also these observations. VAC cross-linked films demonstrated moderate swelling ratios, indicating controlled moisture absorption and structural stability, which are critical attribute for prolonged wound treatments. Among the samples analyzed, the film containing 30% acacia gum and 3% VAC demonstrated superior water absorption and retention performance while maintaining its structural integrity during the immersion duration. This illustrates a balanced network that combines robust internal cohesion with surface hydrophilicity, creating an optimal environment for moisture-sensitive biomedical applications. In contrast, CMC-based films dissolved swiftly, underscoring the importance of formulation compatibility and crosslinker interactions.

The FTIR data further supports the crosslinking action of VAC. Samples exhibiting elevated VAC content, particularly in conjunction with acacia gum, demonstrated AI/AA ratios approaching 1.0 and  $\Delta V$  values, signifying the stabilization of protein structure. Samples with keratin demonstrated good AI/AA ratios; nevertheless, the elevated enthalpy values in DSC suggest a less homogenous crosslinking network. These structural insights emphasize

the VAC's capacity to maintain or enhance the molecular architecture of collagen, particularly when integrated with appropriate polysaccharides.

This study also fills a notable gap in the literature; the application of *Vitex agnus-castus* as a collagen cross-linker has not been previously documented. The composition of iridoid glycosides seems to promote Schiff base formation with collagen's amino groups, serving both a structural enhancer and a possibly bioactive compound. The dual function of VAC, enhancing both crosslinking and heat and hydration properties, indicates a novel category of multifunctional additives for sustainable biomaterial advancement.

As a conclusion, ideal film composition was identified as 30% acacia gum and 3% VAC, which provides the most favorable balance of water absorption, structural integrity, and thermal performance. The results not only illustrate the effectiveness of VAC in biomedical matrix design but also promote future investigation of plant-derived iridoids as eco-friendly substitutes for synthetic crosslinking agents.

In addition to the physicochemical and thermal outcomes discussed above, the material system developed in this study also holds broader relevance within sustainable material design frameworks. The use of a plant-derived cross-linker (VAC) and the reinforcement of collagen with naturally sourced polysaccharides and keratin demonstrate a bio-based approach that can support circular material flows by valorizing agricultural and animal by-products. The improved thermal stability, moisture regulation, and structural integrity obtained particularly in VAC–acacia gum formulations suggest that these films may hold potential not only for biomedical use but also for future low-impact applications that require tunable performance and biodegradability. While industrial scalability, end-of-life behavior, and comparative eco-efficiency require further investigation, the present findings illustrate how material engineering strategies centered on natural chemistry can contribute to emerging bioeconomic and design-for-circularity paradigms.

## Acknowledgments

This research forms part of Cemile Ceren Kahraman's doctoral thesis, "Synthesis of Polysaccharide and Keratin Reinforced Collagen-Based Composite Biomaterials and Determination of Their Potential Biomedical Applications", completed at Ege University in 2024. The authors gratefully acknowledge the financial support provided by the Ege University Scientific Research Projects Coordination Unit (BAP) under Project No. 25234. The authors also thank the BAP Coordination Unit for their technical and administrative assistance throughout the project.

## Funding

This study was supported by Ege University Scientific Research Projects Coordination Unit (BAP), Project No: 25234.

## Conflicts of interest

The authors have nothing to disclose.

## Data availability statement

The datasets generated and analyzed during the current study are available from the corresponding author upon request.

## Author contribution statement

C.C. Kahraman — Conceptualization, methodology, writing—original draft, data interpretation. N. Pourrasoul Sardroudi — Experimental work, data acquisition, water absorption & thermal measurements. H. Eski — FTIR–DSC/TGA support, result evaluation, visualization. A.C.A. Zengin — Supervision, review & editing.

## Supplementary material

**Supplementary Table S1.** Full list of collagen-based film samples with their compositions and codes.

**Supplementary Figure S1.** FTIR spectra of selected collagen-based films: (a) 601BC (control), (b) 1002\_coll\_3H (VAC-crosslinked), (c) 1002\_Karr15\_3H (VAC–carrageenan formulation), (d) 1003\_5K (VAC–keratin formulation), and (e) 2704\_15 (keratin-rich VAC-crosslinked film). The spectra illustrate the characteristic amide bands (Amide A, I, II, and III) and the spectral modifications associated with cross-linking, keratin incorporation, and polysaccharide reinforcement. These representative curves support the interpretation of hydrogen-bonding interactions, shifts in protein secondary structure, and changes in the fingerprint region discussed in the main text.

The Supplementary Material is available at <https://www.rees-journal.org/10.1051/rees/2025003/olm>.

## References

1. A. Sorushanova, L.M. Delgado, Z. Wu, N. Shologu, A. Kshirsagar, R. Raghunath, A.M. Mullen, Y. Bayon, A. Pandit, M. Raghunath, D.I. Zeugolis, The collagen suprafamily: from biosynthesis to advanced biomaterial development, *Adv. Mater.* **31**, 1801651 (2019)
2. P. Yadav, H. Yadav, V.G. Shah, G. Shah, G. Dhaka, Biomedical biopolymers, their origin and evolution in biomedical sciences: a systematic review, *J. Clin. Diagn. Res.: JCDR.* **9**, ZE21 (2015). S.H. Mok, G. Bi, J. Folkes, I. Pashby, Deposition of Ti – 6Al – 4V using a high power diode laser and wire, Part I: investigation on the process characteristics, 2015. <https://doi.org/10.1016/j.surfcoat.2008.02.008>
3. M. Meyer, Processing of collagen based biomaterials and the resulting materials properties, *Biomed. Eng. online.* **18**, 24 (2019)
4. S. Pradhan, A.K. Brooks, V.K. Yadavalli, Nature-derived materials for the fabrication of functional biodevices, *Mater. Today Bio.* **7**, 100065 (2020)
5. T. Biswal, Biopolymers for tissue engineering applications: a review, *Mater. Today: Proc.* **41**, 397–402 (2021)
6. C. Dong, Y. LV, Application of collagen scaffold in tissue engineering: recent advances and new perspectives, *Polymers.* **8**, 42 (2016)
7. Z. Morsada, M.M. Hossain, M.T. Islam, M.A. Mobin, S. Saha, Recent progress in biodegradable and bioresorbable materials: from passive implants to active electronics, *Appl. Mater. Today.* **25**, 101257 (2021)
8. S.S. Shaji, K. Kamalasanan, S. Harika, A. Tiwari, B. Raj, Natural biopolymer for 3D printing, *J. Polymer Sci. Eng.* **6**, 3016 (2023)
9. C. Ferroni, G. Varchi, Keratin-based nanoparticles as drug delivery carriers, *Appl. Sci.* **11**, 9417 (2021)
10. J. McLellan, S.G. Thornhill, S. Shelton, M. Kumar, *Keratin-based biofilms, hydrogels, and biofibers. In Keratin as a protein biopolymer: extraction from waste biomass and applications* (Springer International Publishing, Cham, 2018), pp. 187–200
11. A. Oryan, A. Kamali, A. Moshiri, H. Baharvand, H. Daemi, Chemical crosslinking of biopolymeric scaffolds: current knowledge and future directions of crosslinked engineered bone scaffolds, *Int. J. Biol. Macromol.* **107**, 678–688 (2018)
12. H. Kirmızıbekmez, D. Demir, Iridoid glycosides and phenolic compounds from the flowers of vitex agnus-castus, *Helvetica Chimica Acta.* **99**, 518–522 (2016)
13. M. Schröpfer, M. Meyer, Research article investigations towards the binding mechanisms of vegetable tanning agents to collagen, *Res. J. Phytochem.* **10**, 58–66 (2016)
14. A.P. Antunes, G. Attenburrow, A.D. Covington, J. Ding, Utilisation of oleuropein as a crosslinking agent in collagenic films, *J. Leather Sci.* **2**, 1 (2008)
15. S.N. Park, J.C. Park, H.O. Kim, M.J. Song, H. Suh, Characterization of porous collagen/hyaluronic acid scaffold modified by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide cross-linking, *Biomaterials.* **23**, 1205–1212 (2002)
16. P. Hartrianti, L.T. Nguyen, J. Johannes, S.M. Chou, P. Zhu, N.S. Tan, M.B. Tang, K.W. Ng, Fabrication and characterization of a novel crosslinked human keratin-alginate sponge, *J. Tissue Eng. Regen. Med.* **11**, 2590–2602 (2017)
17. N. Davidenko, C.F. Schuster, D.V. Bax, N. Raynal, R.W. Farndale, S.M. Best, R.E. Cameron, Control of crosslinking for tailoring collagen-based scaffolds stability and mechanics, *Acta Biomater.* **25**, 131–142 (2015)
18. B.B. Mandal, J.K. Mann, S.C. Kundu, Silk fibroin/gelatin multilayered films as a model system for controlled drug release, *Eur. J. Pharm. Sci.* **37**, 160–171 (2009)
19. S. Sadeghi, J. Nourmohammadi, A. Ghaee, N. Soleimani, Carboxymethyl cellulose-human hair keratin hydrogel with controlled clindamycin release as antibacterial wound dressing, *Int. J. Biol. Macromol.* **147**, 1239–1247 (2020)
20. A. Joorabloo, M.T. Khorasani, H. Adeli, Z. Mansoori-Moghadam, A. Moghaddam, Fabrication of heparinized nano ZnO/poly (vinylalcohol)/carboxymethyl cellulose bionanocomposite hydrogels using artificial neural network for wound dressing application, *J. Ind. Eng. Chem.* **70**, 253–263 (2019)
21. W. Shao, J. Wu, S. Wang, M. Huang, X. Liu, R. Zhang, Construction of silver sulfadiazine loaded chitosan composite sponges as potential wound dressings, *Carbohydr. Polym.* **157**, 1963–1970 (2017)
22. Q.L. Loh, C. Choong, Three-dimensional scaffolds for tissue engineering applications: role of porosity and pore size, *Tissue Eng. Part B: Rev.* **19**, 3 (2013)
23. K. Belbachir, R. Noreen, G. Gousspillou, C. Petibois, Collagen types analysis and differentiation by FTIR spectroscopy, *Anal. Bioanal. Chem.* **395**, 829–837 (2009)
24. A. Martínez Cortizas, O. López-Costas, Linking structural and compositional changes in archaeological human bone collagen: an FTIR-ATR approach, *Sci. Rep.* **10**, 17888 (2020)

25. T. Riaz, R. Zeeshan, F. Zarif, K. Ilyas, N. Muhammad, S.Z. Safi, A. Rahim, S.A. Rizvi, I.U. Rehman, FTIR analysis of natural and synthetic collagen, *Appl. Spectrosc. Rev.* **53**, 703–746 (2018)
26. B. de Campos Vidal, M.L. Mello, Collagen type I amide I band infrared spectroscopy, *Micron.* **42**, 283–289 (2011)
27. C. Stani, L. Vaccari, E. Mitri, G. Birarda, FTIR investigation of the secondary structure of type I collagen: new insight into the amide III band, *Spectrochim. Acta Part A: Mol. Biomol. Spectrosc.* **229**, 118006 (2020)
28. T.W. Sun, W.L. Yu, C. Qi, F. Chen, Y.J. Zhu, Y.H. He, Multifunctional simvastatin-loaded porous hydroxyapatite microspheres/collagen composite scaffold for sustained drug release, angiogenesis and osteogenesis, *J. Control. Release.* **259**, e130 (2017)
29. H.S. Canbay, M. Dogantürk, Compatibility studies of sildenafil with different excipients by using TGA, DSC, XRD and FTIR, *Celal Bayar Univ. J. Sci.* **15**, 401–407 (2019)
30. P. Knauth, H. Hou, E. Bloch, E. Sgreccia, M.L. Di Vona, Thermogravimetric analysis of SPEEK membranes: thermal stability, degree of sulfonation and cross-linking reaction, *J. Anal. Appl. Pyrol.* **92**, 361–365 (2011)
31. P. Budrugaec, L. Miu, The suitability of DSC method for damage assessment and certification of historical leathers and parchments, *J. Cult. Herit.* **9**, 146–153 (2008)
32. Y. Zhang, Z. Chen, X. Liu, J. Shi, H. Chen, Y. Gong, SEM, FTIR and DSC investigation of collagen hydrolysate treated degraded leather, *J. Cult. Herit.* **48**, 205–210 (2021)
33. H. Capella-Monsonís, J.Q. Coentro, V. Graceffa, Z. Wu, D.I. Zeugolis, An experimental toolbox for characterization of mammalian collagen type I in biological specimens, *Nat. Protoc.* **13**, 507–529 (2018)
34. N. Prasad, N. Thombare, S.C. Sharma, S. Kumar, Gum Arabic—a versatile natural gum: a review on production, processing, properties and applications, *Ind. Crops Prod.* **187**, 115304 (2022)
35. D.A. Safta, C. Bogdan, S. Iurian, M.L. Moldovan, Optimization of film-dressings containing herbal extracts for wound care—a quality by design approach, *Gels.* **11**, 322 (2025)

**Cite this article as:** Cemile Ceren Kahraman, Nima Pourrasoul Sardroudi, Hande Eski, Arife Candaş Adıgüzel Zengin, Sustainable collagen-based films cross-linked with *Vitex agnus-castus* for enhanced water retention and thermal stability, *Renew. Energy Environ. Sustain.* **10**, 3 (2025), <https://doi.org/10.1051/rees/2025003>