

Structure conformational, physicochemical and rheological characterisation of red clover seed (*Trifolium pratense* L.) galactomannan

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ARTICLE INFO

Keywords:

Forage legumes
Polysaccharides
Viscoelasticity
Molecular properties
Chemical structure
Thermal properties

ABSTRACT

In the present work, a galactomannan isolated from the endosperm of red clover (*Trifolium pratense* L.) seeds was studied for its feasibility as a thickening and gelling agent in food applications. Red clover seed galactomannan (RCG) had a mannose-to-galactose ratio of 1.12, a molecular weight of 1500 kDa (SEC-MALS) and an intrinsic viscosity of 13.6 dL g⁻¹. The solvation affinity of RCG in water was equivalent to θ -solvent, according to the estimated Kraemer and Huggins coefficients. The critical coil overlap concentration of the RCG solutions in water was estimated to be $c^* = 0.35$ wt %, whereas the concentration dependence of specific viscosity in the dilute and semi-dilute regimes was $\propto c^{2.3}$ and $c^{4.2}$, respectively. The water vapour sorption dynamics of RCG were controlled by a solution-like sorption mechanism. RCG exhibited a semi-crystalline structure with the glass transition occurring at $T_{g, onset} \approx 44$ °C. The viscoelastic behaviour of the RCG solutions (1–7 wt %) obeyed the concentration superposition principle with a breakpoint in the relaxation dynamics to occur at $c \approx 2$ wt %. A time-temperature-concentration super master curve was successfully constructed for $c \geq 2$ wt % and temperatures between 5 and 65 °C with an Arrhenius kinetics calculated activation energy of 23.5 kJ/mol.

1. Introduction

Galactomannans constitute one of the most important groups of industrially relevant biopolymers. From a chemical structure viewpoint, galactomannans are heterogeneous polysaccharides comprising a β -(1 $\rightarrow$ 4) D-mannose backbone branched with α -(1 $\rightarrow$ 6) linked D-galactose monomeric units (Kontogiorgos, 2019). Although galactomannans are found in different bioresources, including fungi, yeasts and plants, the existing commercial galactomannans are isolated from the endosperm of leguminous plants, i.e., industrial crops or trees such as fenugreek (*Trigonella foenum graecum*), guar (*Cyamopsis tetragonoloba*), tara (*Tara spinosa*), locust bean (*Ceratonia siliqua*) and cassia (*Cassia obtusifolia*) (Mathur, 2011). Owing to their multifaceted techno-functionality and synergism with other commercial hydrocolloids (e.g., xanthan, carrageenans, starches, etc.), galactomannans are vastly exploited by the food, nutraceutical, cosmeceutical, and

pharmaceutical industry as binding, structuring, texturing, and stabilising agents (Sharma et al., 2021). In addition, galactomannans are widely used as copolymers in the production of biodegradable smart packaging films (including edible films and coatings) and 3D printable biocompatible hydrogel scaffolds for tissue engineering and living cell immobilisation (Mohammadinejad et al., 2020).

From a commercial perspective, galactomannans constitute the second-largest segment of the global plant-based industrial hydrocolloids market, following pectins. Guar and locust bean gum alone account for over 85 % of the total market share of galactomannans (Frost and Sullivan, 2023). Nonetheless, the adverse impacts of climate change, such as drought, excessive salinity, reduction in soil quality, etc., along with the increase in the galactomannan demand in sustainably reformulated end-products (e.g., plant-based food, biodegradable and biocompatible composite materials, etc.) pose significant supply-demand gap challenges (Frost and Sullivan, 2023). In view of

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<https://doi.org/10.1016/j.foodhyd.2025.111517>

Received 7 March 2025; Received in revised form 1 May 2025; Accepted 5 May 2025

Available online 6 May 2025

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this, the search for alternative, sustainable, and eco-resilient bioresources to produce industrially relevant hydrocolloids is highly desirable.

In a previous study (Hellebois, Soukoulis, et al., 2021), we documented that the seeds of the alfalfa (*Medicago sativa* L.) plant, a leguminous perennial forage crop, are rich in a fully substituted galactomannan. The alfalfa seed galactomannan has a mannose to galactose ratio of ~ 1.08 – 1.24 , molecular weight of ~ 2000 – 2200 kDa, intrinsic viscosity of ~ 9.2 – 9.4 dL/g, and a critical concentration $c^* \sim 0.31$ wt % (Hellebois, Soukoulis, et al., 2021). Apart from its thickening and gelling properties, alfalfa seed galactomannan is characterised by a soft cryogel-forming capacity at concentrations above its c^* , which was attributed to the ability of the polymer chain to form junctions via hydrogen bonding (Hellebois et al., 2022). In addition, alfalfa galactomannan was found to be a promising scaffolding material in cryogel templates for the delivery of bioactives such as living probiotic cells (Hellebois, Addiego, et al., 2024; Hellebois, Fortuin, et al., 2024).

Red clover (*Trifolium pratense* L.) is a short-lived perennial crop that is typically utilised for pasture and hay during the cool season in Europe and North America (Putnam & Orloff, 2014). Red clover is highly sustainable and ecologically resilient bioresource stemming from its high adaptability to poorly drained, low fertility and moderately acid soil types (Putnam & Orloff, 2014). Hitherto, the estrogenic isoflavones-rich aerial parts of red clover have been studied for their ability to alleviate menopause and metabolic syndrome-associated symptoms, inhibit melanogenesis (skin depigmentation) and offer anti-inflammatory properties (Cornara et al., 2016). Red clover seeds are primarily utilised as a food bioresource for sprouting purposes (Chiriac et al., 2020). From a nutritional viewpoint, red clover seeds are a good source of major mineral elements (Mg, K, Na and Ca) and trace elements (Zn, Fe, Cu, Ni and Se) (Chiriac et al., 2020). Besides, red clover seeds are good sources of free fatty acids (stearic, oleic, linoleic and linolenic acid), β -sitosterol, squalene, and free amino acids (Fahmy et al., 2023).

As regards the polysaccharidic content of red clover, the presence of a galactomannan exhibiting a mannose-to-galactose ratio of 58:42 has been reported (Grasdalen & Painter, 1980). From a chemical structure perspective, ^{13}C NMR spectra revealed the prevalence of highly D-galactose substituted D-mannose residue sequences (Grasdalen & Painter, 1980). Nonetheless, the molecular, physicochemical and rheological properties of red clover galactomannans remain unexplored. Therefore, in the context of circular economy as well as the evaluation of innovative and sustainable green materials, it is hypothesised that red clover seeds constitute a promising bioresource for the production of galactomannans of industrially relevant techno-functionality for food, nutraceutical, cosmeceutical, and pharmaceutical product applications. To support the hypothesis, the endospermic galactomannan matter of red clover seeds was assessed in terms of its conformational structure, thermophysical, water vapour sorption, steady-state flow and dynamic rheological properties.

2. Materials and methods

2.1. Materials

Red clover seeds (*Trifolium pratense* L.) were purchased from Naturapi (Clermont-Ferrand, France). All organic solvents and chemicals used for the extraction and the analyses of the gum were of analytical grade and obtained from Sigma-Aldrich (Leuven, Belgium).

2.2. Extraction and purification of the clover gum

The clover gum was extracted and purified according to the procedure detailed in (Hellebois, Soukoulis, et al., 2021) with minor modifications. In brief, red clover seeds were soaked in MilliQ water (seed to water ratio = 1:20) at 50 °C for 2 h. The swollen seeds were ground in a knife mill (Retsch GM200, Haan, Germany), mixed with acidified MilliQ

water (pH = 4.25 using HCl 1 M) and kept under mechanical stirring (IKA Eurostar® 60, Staufen, Germany) at 50 °C for 2 h to extract the water-soluble fibre fraction. Following centrifugation at 18,000g for 30 min, the supernatant was collected and precipitated with 3 vol of absolute ethanol. The ethanol-precipitated gum was solubilised in acidified MilliQ water (gum pellet to water ratio = 1:25, pH = 4.25) for 1 h under mechanical stirring, centrifuged at 18,000 g for 1 h, and the supernatant was precipitated with 3 vol of absolute ethanol. Eventually, the gum pellet was reconstituted in MilliQ water under mechanical stirring, neutralised with NaOH 1 M, dialysed (membranes cut-off 12.4 kDa) against MilliQ water for 72 h and freeze-dried at -80 °C for 5 days (Alpha 2–4 LSCplus, Christ, Germany). The obtained red clover seed gum lyophilisates were finely ground using a knife mill (A11 Analytical mill, IKA, Staufen, Germany) and stored in a Nalgene acrylic desiccating chamber at RH = 11 % (Thermo Fisher Scientific, Waltham, MA, United States).

2.3. Compositional and structure conformational characterisation

2.3.1. Proximate composition analysis

Moisture and ash content of the galactomannan gum extracts were gravimetrically determined according to the AOAC standard methods. The protein content was determined according to the Dumas method using a CHNS analyser (Elementar Vario Cube, Langensbold, Germany). Total carbohydrate content was determined using an enzymatic assay kit (Megazyme, K-ACHDF 08/16). Total lipid content was determined by difference.

2.3.2. Osidic composition analysis

In brief, 10 mg of the gum extract was transferred into an Eppendorf tube, mixed with 1 mL of sulphuric acid 2 M and incubated at 99 °C in a thermomixer (Thermomixer R, Eppendorf, Germany) for 10 h at 1300 rpm. On completion of incubation, the tubes were cooled down in an ice bath, centrifuged at 10,000 g for 5 min and the supernatants were carefully collected using a syringe. The sample preparation was carried out in four replicates.

The RCG hydrolysate was diluted 1:300 and 1:500 with MilliQ water and analysed using a Dionex™ ICS-5000+ Capillary HPIC™ System coupled with a pulsed electrochemical detector (Thermo Scientific™ Dionex™). The separation of sugar monomers was performed using a CarboPac SA10 analytic column (2×250 mm, Thermo Scientific™ Dionex™) and a CarboPac PA20 analytic column (3×150 mm, Thermo Scientific™ Dionex™). For the SA10 column, 100 % 1 mM NaOH was used as an eluent to separate the monosaccharide at a constant flow rate of 0.38 mL min^{-1} at 45 °C. For the PA20 column, the sugar monomers separation was performed using 4 % 1 mM NaOH at a flow rate of 0.5 mL min^{-1} at 30 °C. The retention time obtained with a single injection of each standard was used to identify each monosaccharide. The quantification of monosaccharides was performed using a calibration curve generated with varying standard concentrations (1, 2.5, 5, 7.5, 10, 25, 50, 75 and $100 \mu\text{mol L}^{-1}$). Data acquisition and analysis were carried out using Chromeleon™ Chromatography Data System Software (Thermo Scientific™).

2.3.3. Size exclusion chromatography (SEC-MALS)

The size exclusion chromatography (SEC) setup was composed of a SpectraSystem SCM 1000 degasser (Thermo Separation Products, San Jose, CA), a Shimadzu LC-20AR pump (Shimadzu Corporation, Tokyo, Japan), a $2 \mu\text{m}$ frit (Idex, Oak Harbor, WA), a GPC/SEC PL-Aquagel-OH 50×7.5 (8 μm) guard column (Varian Inc, Palo Alto, CA) and two tandem GPC/SEC PL-Aquagel-OH 300×7.5 mm analytical columns (Varian Inc, Palo Alto, CA); a Model 605 column oven (Scientific Systems Incorporated, State College, PA) set at 30 °C; a UV detector measuring at 280 nm (Rigas Labs, Thessaloniki, Greece) and a BI-MwA multiangle laser light scattering detector (MALS) (Brookhaven Instruments Corporation, Brookhaven, Holtsville, NY). The resulting data

were analysed with a dedicated chromatographic/scattering analysis software (Brookhaven Instruments Corporation, Brookhaven, Holtsville, NY). A 200 μL loop was used for injection at an eluent flow rate of 0.8 mL min^{-1} . Ultrapure water was used as an eluent. All measurements were repeated at least two times.

2.3.4. Fourier transform infrared spectroscopic (FTIR) analysis

The freeze-dried RCG extract was ground with KBr at a 1:100 ratio and finally pressed at high pressure into a pellet. FTIR spectra were recorded on a Bruker Optics Vertex spectrometer and were carried out in the Attenuated Total Reflectance (ATR) mode. The ATR crystal is a diamond with an active sampling area of 0.8 mm in diameter and a reflection angle of 45°. Spectra were obtained from 50 scans. The baseline spectra were calculated without the sample and subtracted from the RCG obtained spectra.

2.3.5. Nuclear magnetic resonance (NMR) characterisation

Samples (~5 mg) were dissolved in 700 μL of D_2O . NMR analyses were carried out on a Bruker Avance 600 HDIII spectrometer, operating at 600.16 MHz, equipped with a 5 mm BBO probe and z-gradients. All spectra were recorded at 343 K and referenced versus the water peak (4.29 ppm). The ^1H spectrum was acquired using a recycling delay of 20 s and 16 scans. M/G ratios were determined from the integrals of the galactose and mannose h1 signals, at 5.03 ppm and 4.76 ppm, respectively. For $^{13}\text{C}\{^1\text{H}\}$ NMR, 10,240 transients were acquired with a recycling delay of 2 s.

2.3.6. X-ray diffraction analysis

X-ray diffraction was performed by a X'Pert Pro X-ray diffractometer (PANalytical, Almelo, Netherlands) using $\text{Cu K}\alpha$ as a radiation source and equipped with a PIXcel3D detector. The patterns were recorded in the range $2\theta = 5-75^\circ$ with a counting time of 247 s per 0.026° step in 1D mode. The crystallinity (%) of the gum extract was calculated using Eq. (2.1):

$$\text{Crystallinity (\%)} = \frac{\text{Total area of crystalline peaks}}{\text{Total area of amorphous and crystalline peaks}} \times 100 \quad (2.1)$$

2.4. Intrinsic viscosity measurements

The intrinsic viscosity $[\eta]$ of dilute red clover gum dispersions (0.01–0.08 wt %) in MilliQ water was measured using OC Ubbelohde capillary viscometer (Paragon Scientific, United Kingdom) at $25 \pm 0.1^\circ\text{C}$. The intrinsic viscosity was determined as the intercepts of the Huggins (Eq. (2.2)) and Kraemer (Eq. (2.3)) equations by extrapolating to an infinite dilute system as well as the slope of the Fedors equation (Eq. (2.4)):

$$\frac{\eta_{\text{sp}}}{C} = [\eta] + k_H [\eta]^2 C \quad (2.2)$$

$$\frac{\ln \eta_{\text{rel}}}{C} = [\eta] + k_K [\eta]^2 C \quad (2.3)$$

$$\frac{1}{2\left(\sqrt{\eta_{\text{rel}}}\right)} = \frac{1}{C[\eta]} - \frac{1}{C_{\text{max}}[\eta]} \quad (2.4)$$

where: η_{sp} and η_{rel} denote the specific and relative viscosity, respectively, C is the red clover gum concentration, k_H , k_K are the Huggins and Kraemer coefficients, respectively, and C_{max} is the upper concentration limit where the Fedors equation retains its linearity.

2.5. Steady state and dynamic rheological measurements

Aqueous solutions (0.1, 0.125, 0.25, 0.375, 0.5, 0.625, 0.75, 0.875, 1, 1.25, 1.5, 1.75, 2.0, 3.0, 4.0 and 5.0 wt %) of red clover gum in MilliQ

water (adjusted at pH = 7 using NaOH 0.1 M) were prepared for conducting the rheological measurements. All rheological measurements were performed in an Anton-Paar oscillatory rheometer (MCR 302, Graz, Austria) using either a concentric cylinder (steady-state flow behaviour measurements at $c_{\text{RCG}} \leq 1\%$ wt) or a sandblasted plate-plate geometry (dynamic and steady-state rheological measurements at $c_{\text{RCG}} > 1\%$ wt).

2.5.1. Steady state flow behaviour

Aliquots of the RCG solutions (ca. 15 mL) were transferred to the measuring geometry and equilibrated at ambient temperature ($25 \pm 0.05^\circ\text{C}$) for 20 min prior to the analysis. Upward shear rate sweeps in the range of $0.01-1000\text{ s}^{-1}$ were performed. The apparent viscosity ($\text{mPa}\cdot\text{s}$) – shear rate $\dot{\gamma}$ (s^{-1}) data were fitted to the Cross-Williamson model (Eq. (2.5)) as follows:

$$\eta = \eta_{\infty} + \frac{\eta_0 - \eta_{\infty}}{1 + (\tau \dot{\gamma})^m} \quad (2.5)$$

where η_0 and η_{∞} represent the zero and infinite shear viscosity (in $\text{mPa}\cdot\text{s}$), and τ is the relaxation time (s), and m is a dimensionless constant related to the power law exponent, i.e., $m = 1 - n$ (where n is the flow behaviour index), respectively.

2.5.2. Amplitude and frequency sweep measurements

For the dynamic rheological measurements, RCG gum solutions in the concentration range of 1–5 wt % were prepared. Amplitude sweeps (0.1 to 1000%) were conducted to determine the linear viscoelastic region (LVE) under controlled shear stress conditions, at 10 rad/s and 25°C . From the obtained amplitude sweep rheological spectra, the viscoelastic moduli and stiffness (G'_{LVE} and G''_{LVE} , $\tan\delta_{\text{LVE}}$) in the LVE regime, the yield stress at the limit of the LVE regime (τ_y) and the strain and stress flow-point ($G' = G''$) were calculated using the RheoCompass analysis software (Anton-Paar, Graz, Austria).

Frequency sweeps (0.1–100 rad/s) within the LVE regime (strain = 1 %) were performed at 25°C to evaluate the viscoelastic profile of the alfalfa gum dispersions. The slope of the double logarithmic storage modulus (G') – angular frequency (ω) curves was calculated. In addition, the frequency (f) at which the crossover of the viscoelastic moduli ($G' = G''$) takes place was calculated using RheoCompass analysis software. Time concentration superposition (TCS) master curves were constructed using the Reptate software (Boudara et al., 2020).

2.5.3. Time-Temperature Superposition (TTS) testing

To assess the impact of temperature on their viscoelastic response, RCG solutions (1–7 wt %) were subjected to frequency sweeps at 5, 15, 25, 35, 45, 55 and 65°C following the above-mentioned conditions. Time-temperature superposition (TTS) master curves were constructed using Reptate software.

2.5.4. Large amplitude oscillation shear (LAOS) measurements

LAOS analysis was conducted to investigate the nonlinear viscoelastic behaviour of the RCG solutions (Ewoldt et al., 2008). The meaningful viscoelastic moduli in the non-linear regime, i.e., the tangent modulus at $\gamma = 0$, G'_M (the stress-strain curve at zero strain, $G'_M = \left.\frac{d\tau}{d\gamma}\right|_{\gamma=0}$) and secant modulus G'_L (representing the ratio of the peak stress to peak strain, $G'_L = \frac{\tau_{\text{max}}}{\gamma_0}$), which reflect the local stiffness at the start of the deformation and the average stiffness across the entire strain amplitude, were measured. Moreover, dimensionless indices of nonlinearity, including strain stiffening ratio (S) and shear thickening ratio (T), were calculated, which are based on the viscoelastic moduli and dynamic viscosity. The software VAOS (Aarhus University, Denmark) was used for LAOS data treatments. From VAOS, different graphs and parameters such as elastic and viscous Lissajous-Bowditch curves, G'_M , G'_L , S , and T ratios were extracted to help understand the behaviour change

of galactomannan solutions under large deformation (Madsen et al., 2022, pp. 1–7).

2.6. Thermal characterisation

Thermal gravimetric analysis (TGA) of the red clover galactomannan was carried out in air using a TGA2 STAR System (Mettler Toledo, Switzerland) applying a heating rate of $5\text{ }^{\circ}\text{C min}^{-1}$ in the range of 25 to $800\text{ }^{\circ}\text{C}$. The onset weight loss temperature (T_{onset}) was determined as the point in the TGA curve at which a significant deviation from the horizontal was observed.

Thermal mechanical analysis (TMA) of red clover galactomannan was performed under inert atmosphere (He) using a DIL 402 select Expedis dilatometer (NETZSCH, Germany) with a constant load of 0.1 N at a heating rate of $5\text{ }^{\circ}\text{C min}^{-1}$ in the range of -80 to $220\text{ }^{\circ}\text{C}$. Heat distortion temperature (THDT) was determined as the temperature at which a noticeable deformation under applied load and scanning/heating rate was observed.

2.7. Scanning electron microscopy (SEM)

A scanning electron microscope from HITACHI (SU7000) was used for morphological observation. Lyophilised clover gum powder was deposited on carbon tape. A platinum-sputter coating of 5 nm was applied before the SEM analysis.

2.8. Water vapour sorption isotherms

The water vapour sorption isotherm of the RCG powder was determined as detailed in Hellebois et al. (2024). In brief, the RCG powder was desiccated in a glove box at $\text{RH} \approx 0$ for 5 days and aliquots of 200 mg were incubated for 3 weeks in diverse saturated salt solution-controlled water activity atmospheres in hermetically sealed Nalgene acrylic desiccator cabinets (Thermo Fisher Scientific, Waltham, MA, United States) until the water intake reached an equilibrium at $T = 20 \pm 1\text{ }^{\circ}\text{C}$. To this end, the following salts were considered: LiCl ($a_w = 0.11$), $\text{CH}_3\text{CO}_2\text{K}$ ($a_w = 0.23$), MgCl_2 ($a_w = 0.33$), K_2CO_3 ($a_w = 0.43$), $\text{Mg}(\text{NO}_3)_2$ ($a_w = 0.54$), NaCl ($a_w = 0.75$), NH_4Cl ($a_w = 0.79$), and KCl ($a_w = 0.85$). The relative water intake was then calculated gravimetrically.

2.9. Colourimetric analysis

One gram of RCG powder was placed in a 25 mm plastic Petri dish, and colour measurements were performed in triplicate using a CR-400 Minolta chromameter (Minolta Sarl, Roissy, France). The CIELab colour scale was used to measure the L^* (black to white), a^* (red to green), and b^* (yellow to blue) parameters (Soukoulis et al., 2014).

2.10. Statistical analyses

The normal distribution of the data was verified by means of the Shapiro-Wilk test and Q-Q plot representation. In addition, the equality of variance among the variables was verified using Levene's test. To determine the significance of the red clover gum concentration on the physicochemical and rheological properties, one-way ANOVA was performed using Origin software. Tukey's multiple range test was used to separate means of data when significant differences ($p < 0.05$) were detected.

3. Results and discussion

3.1. Proximate and osidic composition

To enhance the purity of the final gum, the aqueous extraction was conducted at the isoelectric point of clover seed proteins (i.e., $\text{pI} = 4\text{--}4.25$) followed by the removal of the soluble protein residual ($<5\%$)

via high-speed centrifugation of the 1:10 diluted galactomannan extract (Hellebois, Soukoulis, et al., 2021). This extraction method allows satisfactory deproteinisation of the gum without the need for processing aids such as proteases or organic solvents (Soukoulis et al., 2018). Under these conditions, the CGC isolation yield was estimated at $6.24 \pm 0.52\text{ g g}^{-1}$ of red clover seeds (on a dry basis), which is comparable to that of alfalfa (Hellebois, Soukoulis, et al., 2021) but lower than that of fenugreek gum (Brummer et al., 2003). As reported in Table 1, RCG consisted of 97.5 wt % carbohydrate matter, whilst the residual protein and ash solids were below 1 and 1.6 wt % (on a dry basis), respectively, which is very close to the proximate composition of alfalfa seed galactomannan (Hellebois, Soukoulis, et al., 2021) and protease-treated (Pronase) fenugreek gum (Brummer et al., 2003).

The RCG extract comprised almost exclusively mannose and galactose, whilst traces of glucose and arabinose were identified. Parameters such as the botanical origin and phenotype of the leguminous seeds as well as the implemented extraction conditions (i.e., temperature, pH and ionic strength) are known to impact the sugar monomers profile of galactomannans. According to our findings, the RCG had a mannose to galactose (M/G) ratio of 1.13, suggesting that RCG consists of a fully galactose-substituted mannan backbone similar to that of alfalfa, $\text{M/G} = 1.04\text{--}1.29$, fenugreek, $\text{M/G} = 1.05\text{--}1.26$ and *Mimosa scabrella* L., $\text{M/G} = 1.10$, seed galactomannans (Brummer et al., 2003; Ganter et al., 1995; Hellebois, Soukoulis, et al., 2021).

3.2. Structural characterisation of the red clover galactomannan by NMR measurements

1D and 2D NMR measurements were conducted to investigate the structure of the red clover seed galactomannan. The ^1H NMR spectrum (Fig. 1A) of the RCG is quite similar to that reported in the case of fenugreek (Muschin & Yoshida, 2012), *Cassia grandis* (Albuquerque et al., 2014), *Dimorphandra gardneriana* (Cunha et al., 2009), and *Prosopis juliflora* (Vieira et al., 2007) galactomannans. The well-separated anomeric protons signal peaks at $\delta = 5.03$ and 4.76 ppm were assigned to H-1 of α -D-galactose and β -D-mannose, respectively (Albuquerque et al., 2014; Cunha et al., 2009). The ratio of the areas of the anomeric signals was estimated to $^1\text{H}(\text{Man})/^1\text{H}(\text{Gal}) = 1.12$, which is in keeping with our osidic composition analysis findings as well as the findings of Grasdalen and Painter (1980) on alfalfa and red clover galactomannan extracts.

The ^{13}C NMR spectrum (Fig. 1B) of RCG was in very close proximity to other fully galactose-substituted galactomannans (i.e., $\text{M/G} \sim 1$) such as fenugreek (Muschin & Yoshida, 2012), mesquite gum (Vieira et al., 2007) and alfalfa (Grasdalen & Painter, 1980). In agreement with the ^1H NMR anomeric proton signals, the ratio of the anomeric carbon signal areas (at $\delta = 100.7$ and 99.4 ppm) was estimated at $^{13}\text{C}(\text{Man})/^13\text{C}(\text{Gal}) = 1$ (Suppl. Fig. 2A). The slight differences in the Man – Gal ratios of the anomeric proton and carbon signals can be primarily attributed to differences in the relaxation behaviour and signal intensity among the ^1H

Table 1

Proximate and osidic composition of the red clover galactomannan extract (values represent the average of three technical replicates, $n = 3$).

Proximate composition ($\text{g } 100\text{g}^{-1}$ of dry matter)	
Total carbohydrates	97.5 ± 0.6
Protein	0.91 ± 0.41
Ash	1.59 ± 0.28
Lipids	traces
Sugar composition ($\text{g } 100^{-1}$ of total carbohydrate matter)	
Arabinose	0.1 ± 0.0
Galactose	47.0 ± 1.1
Glucose	0.1 ± 0.0
Mannose	52.8 ± 1.2
Fucose	nd
Rhamnose	nd
M/G	1.13 ± 0.01

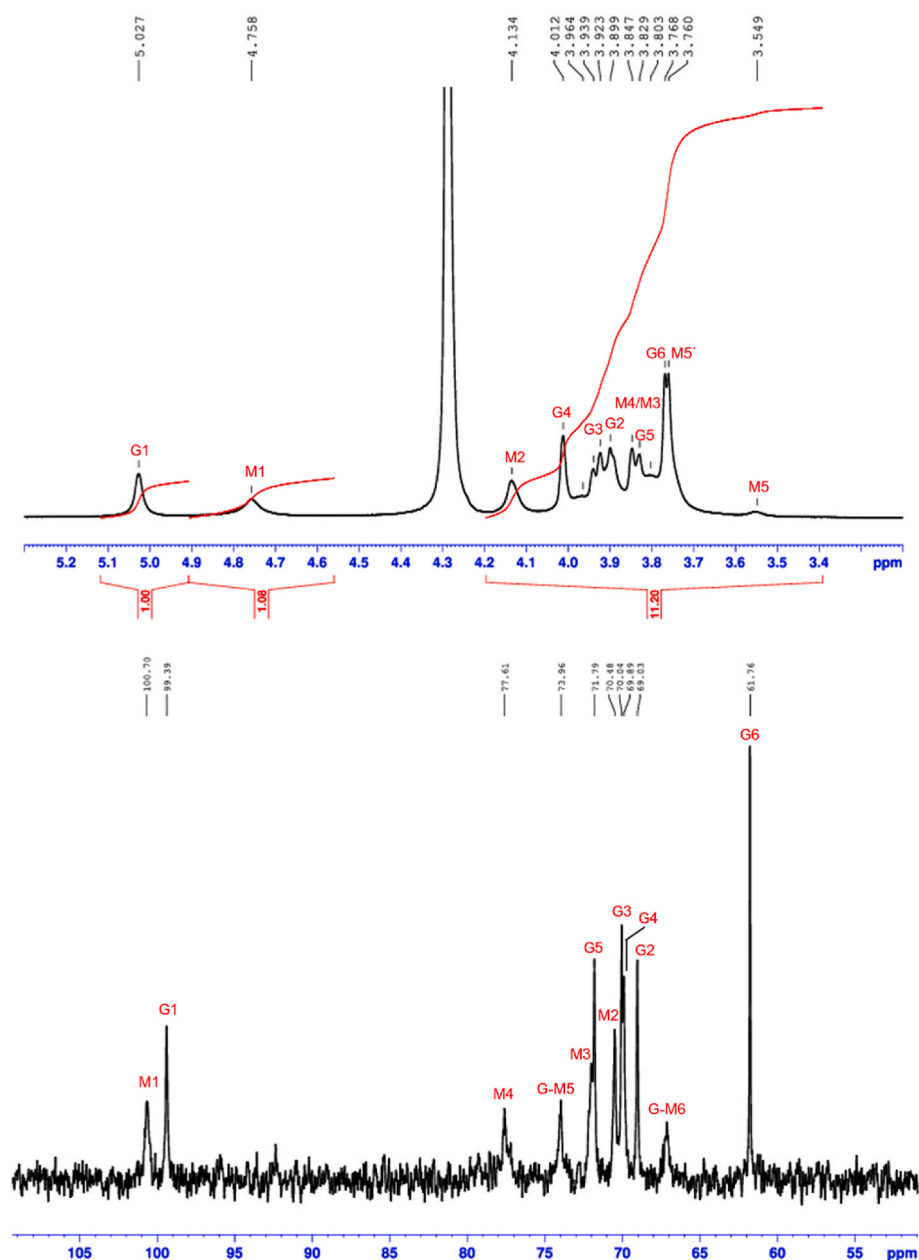


Fig. 1. ^1H (top) and ^{13}C (bottom) NMR spectra of a red clover seed galactomannan solution (10 mg/mL) in D_2O . G and M represent signals from α -D-galactose and β -D-mannose, respectively.

NMR and ^{13}C NMR spectra as well as in the local chemical environment around Man and Gal molecules. The signals at $\delta = 61.3$ and 61.8 ppm were assigned to the C-6 resonance of the unsubstituted β -D-mannose and α -D-galactose residues, respectively (Cunha et al., 2009). Previous studies have shown that the ^{13}C NMR region of galactomannans at $\delta = 77$ – 78 ppm displays three distinct peaks as follows: Peak I ($\delta = 77.8$ ppm) corresponds to two continuous galactose substituted mannose units (i.e., $-\text{Gal}(\alpha1\rightarrow6)\text{-Man}(\beta1\rightarrow4)\text{-Man}(\beta1\rightarrow4)\text{-Gal}(\alpha1\rightarrow6)-$); Peak II ($\delta = 77.3$ ppm) refers to a mannose dyad with a single galactose substitution (i.e., $-\text{Gal}(\alpha1\rightarrow6)\text{-Man}(\beta1\rightarrow4)\text{-Man}(\beta1\rightarrow4)-$ or $-\text{Man}(\beta1\rightarrow4)\text{-Man}(\beta1\rightarrow4)\text{-Gal}(\alpha1\rightarrow6)-$); Peak III ($\delta = 77$ ppm) is associated with the presence of galactose depleted mannose dyads (i.e., $-\text{Man}(\beta1\rightarrow4)\text{-Man}(\beta1\rightarrow4)-$) (Cunha et al., 2009; Ganter & Reicher, 1999). The intensity of the peaks was in the decreasing order of Peak I > Peak II > Peak III confirming the high degree of galactose substitution of the mannan backbone (Suppl. Fig. 2B).

3.3. Physicochemical characterisation of the red clover galactomannan

In Fig. 2A and B the visual aspects of the red clover seeds and the obtained freeze-dried gum extract are displayed. No discolouration defects were identified in the extract implying the effectiveness of the gum isolation and purification process implemented. According to Table 2, the luminosity L^* of the RCG was 91.5, which is comparable to that of spray-dried tara seed galactomannan (Moscoso-Moscoso et al., 2024). SEM assessment of the freeze-dried RCG powder confirmed the presence of microparticulated gum agglomerates (Fig. 2C). The assessment of the surface morphology of the gum agglomerates confirmed the presence of an evenly filamentous macroporous polymeric network (Fig. 2D), similar to that of biopolymer cryostructures, which is presumably formed via the interchain hydrogen bond polymeric bridging upon cryogenic processing (Gun'ko et al., 2013).

The RCG extract was studied regarding its component populations using size exclusion chromatography coupled with multi-angle laser

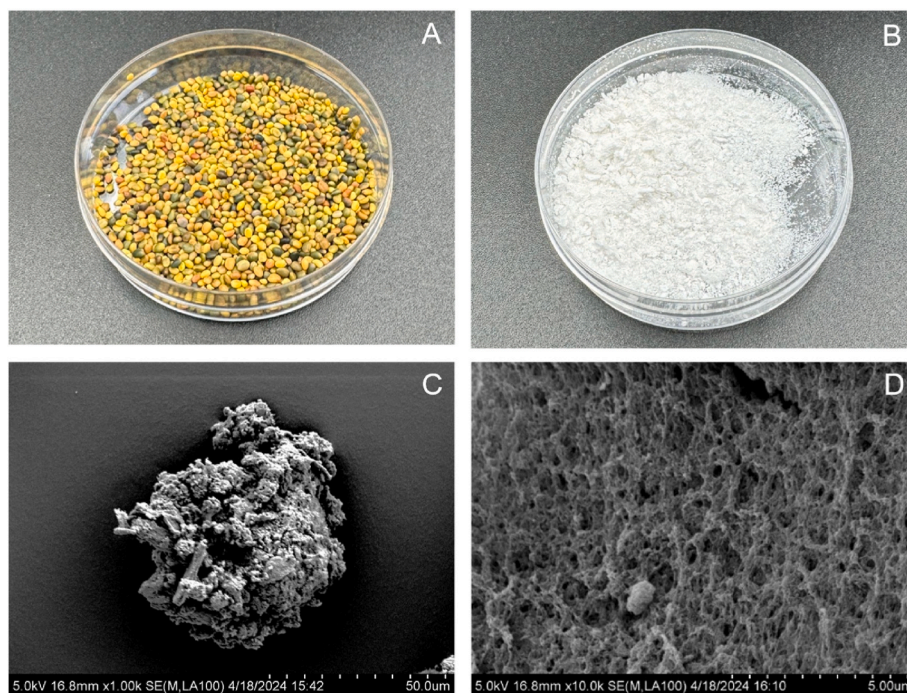


Fig. 2. Visual aspects of the red clover (*Trifolium pratensis* L.) seeds (A) and the obtained galactomannan (B). Scanning Electron Microscopy images of the red clover galactomannan acquired at 1000 × (C) and 10,000 × (D).

Table 2

Structure conformational and physicochemical properties of the red clover seed galactomannan extract (values represent the average of three technical replicates, $n = 3$).

Red clover seed gum property	
$\overline{M}_w$ ($\times 10^6$ Da) – SEC/MALS	1.50 ± 0.02
$\overline{M}_w$ ($\times 10^6$ Da) – GPC/SEC	1.23 ± 0.03
PDI – GPC/SEC	1.04 ± 0.01
DP_n – GPC/SEC	3389 ± 152
z -diameter (nm)	120.0 ± 4.3
Polydispersity (DLS)	0.33 ± 0.12
ζ -potential (mV)	-0.63 ± 0.07
L^*	91.54 ± 0.5
a^*	0.5 ± 0.03
b^*	1.13 ± 0.01

light scattering (SEC-MALS) (Fig. 3A). According to the acquired SEC-MALS chromatographs, two molecular weight populations were identified. The first eluted at 7 mL, was composed of very large macromolecular assemblies of a molecular weight size approximately 1500 kDa (Table 2). Additionally, the mild absorption observed at 280 nm, suggests the presence of galactomannan conjugates with either peptides or polyphenols, which are naturally present in clover seeds (Drużyńska et al., 2024). Proteins as originators of the UV absorbance should be ruled out, as no amide bands were seen in the FTIR plots. The second elution peak at 14 mL showed no response in the SLS detector, while it absorbed at 280 nm. The molecular weight of the latter corresponded to a standard dextran of approximately 12 kDa and is possibly associated with peptide and phenolic structures. In line with the SEC-MALS findings, GPC-SEC chromatograms (Suppl. Fig. 3) revealed that the RCG extract had a $M_w = 1227$ kDa, PDI = 1.04 and $DP_n = 3289$ (Table 2). In this context, RCG has a similar M_w to locust bean gum but significantly lower than other fully galactose-substituted galactomannans such as alfalfa (Hellebois, Soukoulis, et al., 2021) and fenugreek gum (Brummer et al., 2003; Youssef et al., 2009).

As expected, RCG did not convey any significant surface charge density (Table 2), which is in line with the non-ionic character of

galactomannans (Kontogiorgos, 2019). The hydrodynamic radius of RCG was estimated to be approximately 120 nm, which is comparable to that of locust bean gum but relatively higher than alfalfa, fenugreek and guar gum (Hellebois, Soukoulis, et al., 2021). These differences postulate that RCG is more prone to associative aggregation compared to other fully galactose-substituted galactomannans such as fenugreek and alfalfa.

A representative FTIR spectrum of the RCG is illustrated in Fig. 3B. As seen, the spectrum exhibits a typical carbohydrate peak distribution with the major absorbance peaks found in the $1000\text{--}1200\text{ cm}^{-1}$ and $3300\text{--}3400\text{ cm}^{-1}$ region in agreement with previous studies on galactomannans (Albuquerque et al., 2014; Cerqueira et al., 2011). The signal obtained between $2800\text{--}3000$ and $3100\text{--}3600\text{ cm}^{-1}$ is primarily ascribed to the C–H and O–H stretching, respectively (Yuen et al., 2009). In keeping with the findings of Silva Neto et al. (2023), ethanol-assisted purification of galactomannans favours the formation of new hydrogen bonds in the galactomannan structure, which is reflected in the broad width of the convoluted amide A and B peaks. Absorption common to galactomannans was detected at 1147 cm^{-1} , which is associated with the C–O bending of the pyranose ring (Tamaki et al., 2010). The peaks identified at 870 and 814 cm^{-1} are known to be associated with the presence of β -linked D-mannopyranose and α -linked D-galactopyranose units, which is in agreement with the molecular structure conformation of galactomannans (Figueiró et al., 2004). According to the observations of Silva Neto et al. (2023) the signal at 1641 cm^{-1} is associated with the stretching of the mannopyranose and galactopyranose rings. Nonetheless, the $1600\text{--}700\text{ cm}^{-1}$ (Amide I) peak is also well-documented for characterising the stretching vibrations of the C=O of the peptide bonds due to the presence of the residual protein in the RCG extract (Hellebois et al., 2023; Rashid et al., 2018).

To gain insight into its physical structure, the RCG extract was analysed by means of XRD, as seen in Fig. 3C. The obtained X-ray diffractogram suggested that RCG exhibits a semi-crystalline structure presenting four well-defined peaks associated with crystalline structures at $2\theta = 15.7, 20.3, 31.8$ and 33.1° . According to equation (2), the total crystallinity of the RCG was estimated at 16.4 %. Previous studies have shown that parameters such as the gum purification and drying process

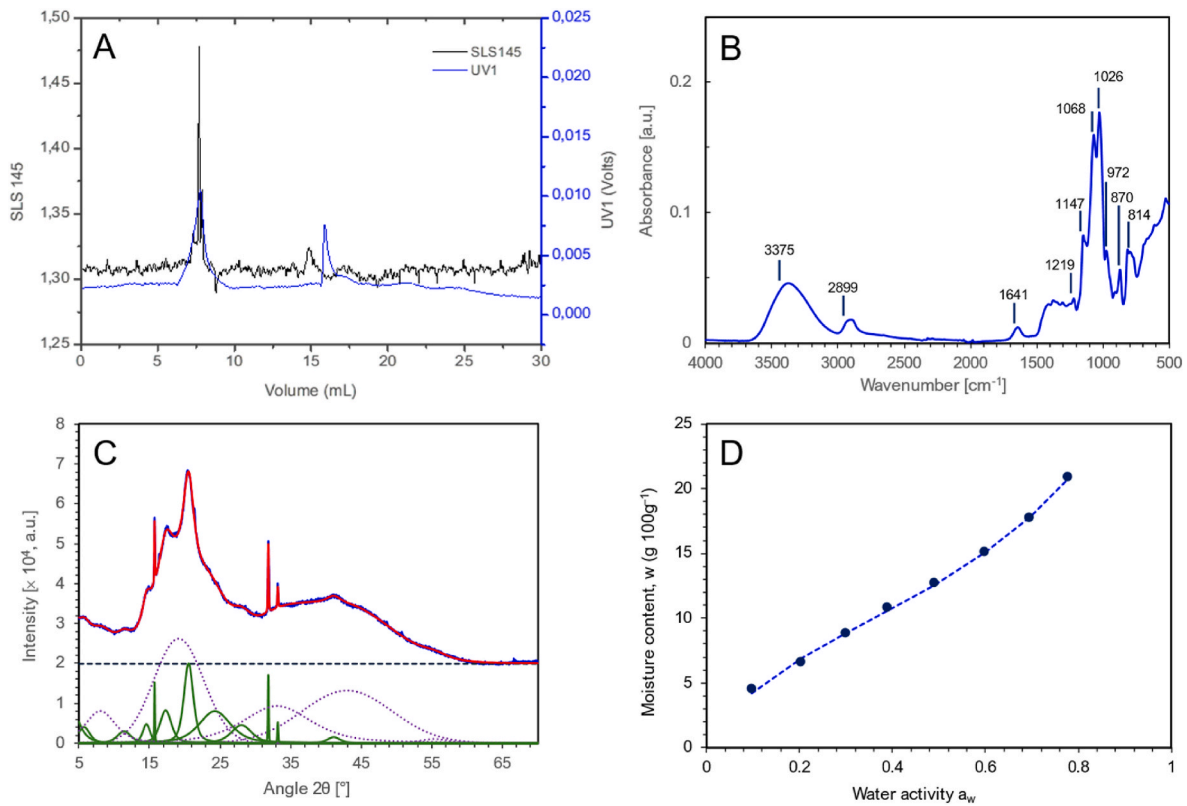


Fig. 3. Physicochemical characteristics of the red clover seed galactomannan. A: SEC-MALS chromatographs for molecular weight determination, B: FTIR spectrum, C: X-ray diffractogram, and D: Water vapour sorption isotherm (at 25 °C).

can significantly alter their physical structure. In line with our findings, [Silva Neto et al. \(2023\)](#) reported that the purification of crude extracts of *Adenanthera pavonina* L. seed galactomannan via ethanol-assisted precipitation induced a significant reduction in the total amount of the amorphous states leading to a semi-crystalline physical state. In another study, [Vendruscolo et al. \(2009\)](#) demonstrated that oven drying induces an increase in the crystallinity of *Mimosa scabrella* L. seed galactomannan compared to spray drying.

To determine its water vapour sorption and thermophysical properties, RCG was pre-conditioned at absolute dryness conditions (RH = 0, P₂O₅) in a glove box for 5 days prior to the analyses. At such conditions, the residual moisture of the gum was estimated at 0.05 g g⁻¹. As illustrated in [Fig. 3D](#), the water vapour sorption isotherm of RCG exhibited a sigmoidal shape, i.e., type II isotherm according to the Brunauer classification. In general, type II isotherms are commonly described by the GAB model, and they assume the presence of multilayers in the internal surface of a material. In order to achieve a quantitative classification of the sorption curve of RCG, the original water vapour sorption curve was transformed into an $a_w/w - a_w$ plot ([Suppl. Fig. 1](#)) and fitted to the following equation as proposed by [Blahovec and Yanniotis \(2009\)](#):

$$\frac{a_w}{w} = X_1 + (X_2 - X_1 X_4) a_w - (X_3 + X_2 X_4 - X_1 X_4^2) \frac{a_w^2}{1 + X_4 a_w} \quad (3.1)$$

Derivation of equation (3.1), allows the calculation of the parameter D_{10} at $a_w = 0$:

$$\left[\frac{d\left(\frac{a_w}{w}\right)}{da_w} \right]_0 = D_{10} = X_2 - X_1 X_4 \quad (3.2)$$

the $a_{w,m}$ represents the water activity at which $\frac{d\left(\frac{a_w}{w}\right)}{da_w} = 0$ and the ratio of the first derivative of the $a_w/w - a_w$ plot, R_{fi} :

$$R_{fi} = \frac{1 - X_3 \frac{2+X_4}{D_{10}}}{(1 + X_4)^2} \quad (3.3)$$

The above-mentioned parameters for RCG were estimated at $D_{10} = 0.099$, $a_{w,m} = 0.589$, $R_{fi} = -0.40$ and $X_4 = 0.456$, which according to the sorption isotherm quantitative classification criteria ($D_{10} > 0$, $R_{fi} < 0$ and $X_4 > 0$) introduced by [Blahovec and Yanniotis \(2009\)](#), suggest a Type-IIb sorption isotherm (nearly common GAB isotherm). Similar results were reported for other hydrocolloids like karaya gum ([Yanniotis & Blahovec, 2009](#)). It was previously demonstrated that the overall water vapour sorption in food materials stems from the superposition of the localised monolayer adsorption and the dilution of some parts of solids in solution. Based on the obtained X_4 values ($0 < X_4 < 0.5$) RCG water vapour sorption dynamics are mainly controlled by a solution-like sorption mechanism and to a lesser extent by the forces acting between the product surface and the water condensed from the vapour as a monomolecular layer. This can be attributed primarily to the hydrophilicity of RCG and the low number of strong water molecules binding sites (COO^- , NH_3^+ , $-\text{OH}$ phenolic) on the microporous polymer internal surface. The monolayer water content of RCG was estimated at 0.0958 g g⁻¹ (GAB model), which is significantly higher than that of higher M/G galactomannans such as guar and locust bean gum ([Vishwakarma et al., 2011](#)), and thus, implying its higher hygroscopicity.

Thermal gravimetric analysis (TGA) was conducted to determine the thermal stability and decomposition pattern of the RCG extract ([Fig. 4A](#)). As expected, two major weight loss events were detected in the TGA curves: a mass loss of about 5 % in the range of 25–100 °C (T_e , midpoint = 75 °C) representing the amount of the evaporated unbound water, and a second steep mass loss in the range of 250–450 °C ($T_{d,onset} = 255$ °C, $T_{d, midpoint} = 311$ °C), which represents the thermal decomposition of the gum extract. In the latter case, the thermal decomposition temperature of RCG is very close to that of galactomannans from *Gleditsia triacanthos* L. seeds ($T_{d,mid} = 310$ °C), comparable to that of

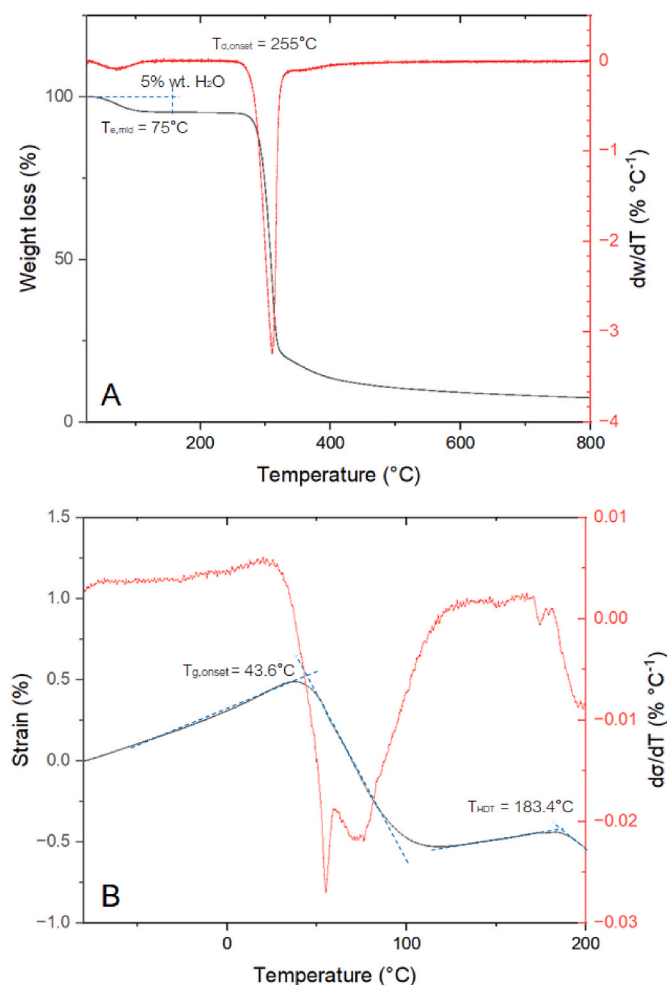


Fig. 4. Thermophysical characterisation of the red clover galactomannan. A: Thermogravimetric analysis (TGA) curve and B: Thermomechanical analysis (TMA) curve for glass transition temperature determination.

fenugreek, *Caesalpinia pulcherrima* and *Adenanthera pavonina* L. seeds ($T_{d,mid} = 312\text{--}322\text{ }^{\circ}\text{C}$) but higher than that reported for guar gum ($T_{d,onset} = 235\text{ }^{\circ}\text{C}$) (Cerqueira et al., 2011; Dhull et al., 2020; Varma et al., 1997). Parameters such as the presence of impurities (e.g. ash and protein) as well as the crystallinity of the galactomannans are known to influence their thermal stability.

The determination of the glass transition temperature (T_g) employing DSC was not possible mainly due to the very low changes in the specific heat capacity ΔC_p during the 1st and 2nd heating ramp. Such behaviour has been also reported in the case of galactans (Mitsuiki et al., 1998). In view of this, thermomechanical analysis (Fig. 4B) was carried out allowing the reproducible determination of the onset of two thermal transitions: a low temperature ($T_{g,onset} = 44\text{ }^{\circ}\text{C}$) which was attributed to the glass transition temperature point and a high-temperature thermal transition accompanied by a substantial deformation under the applied load and heating rate, which is defined as the heat distortion temperature ($T_{HDT,onset} = 184\text{ }^{\circ}\text{C}$). Several studies have demonstrated that the T_g of galactomannans (residual water content in the range of 4–6 wt %) is detected at intermediate temperature zones, i.e., $\sim 40\text{ }^{\circ}\text{C}$ for guar gum, $\sim 57\text{ }^{\circ}\text{C}$ for *Gleditsia triacanthos*, $\sim 53\text{ }^{\circ}\text{C}$ for *Adenanthera pavonina*, and $\sim 59\text{ }^{\circ}\text{C}$ for locust bean gum (Cerqueira et al., 2011; Chouaibi et al., 2019; Chaires-Martínez et al., 2008). On the other hand, Busch et al. (2019) adopting a multi-analytical approach employing DSC, ^1H NMR and DMA techniques, reported the existence of low (between -80 and $-10\text{ }^{\circ}\text{C}$), intermediate (in the vicinity of $50\text{ }^{\circ}\text{C}$) and high temperature (between 50 and $100\text{ }^{\circ}\text{C}$) thermal transitions. The intermediate

temperature occurring thermal transition was accompanied by a change in the solid characteristics of the galactomannans as manifested by the decrease in T_2 values (^1H NMR) and by a maximum loss modulus E'' (DMA). This appears to be in line with the thermal transitions observed in the RCG TMA curve at ~ 44 , 55 and $73\text{ }^{\circ}\text{C}$. Yet, in line with the observations of Busch et al. (2019), the identification of an accurate T_g was not achievable, which can be attributed to the parameters affecting the polymer chain immobilisation such as crystallinity, conformational order and inter-residue hydrogen bonding.

3.4. Determination of the intrinsic viscosity

The intrinsic viscosity $[\eta]$ of RCG was determined at the dilute regime i.e., $1.15 < \eta_{rel} < 2.31$, which was corresponding to $c \leq 0.08$ wt %. The experimental data were fitted with the Kraemer (Fig. 5A), Huggins (Fig. 5A), and Fedors (Fig. 5B) equations, whilst the modified Mark-Houwink equation (3.4), as proposed by Pollard et al. (2010), was also used to obtain the theoretical $[\eta]$ based on the experimentally (SEC) calculated M_w and the molecular weight of the repeating unit M_0 , as follows:

$$[\eta] = 1800 \pm 200 \cdot M_0^{-1.61} \cdot M_w^{0.61 \pm 0.01} \quad (3.4)$$

In this context, the $[\eta]$ of RCG was estimated to: 12.9 (Huggins), 13.8 (Kraemer), 13.8 (Fedors) and 13.7 (modified Mark-Houwink), resulting in a $[\eta] = 13.55\text{ dL g}^{-1}$. A very good approximation of the $[\eta]$ using the Fedors and Kraemer equations has been also reported in the case of other galactomannans (Nwokocha et al., 2018). The C_{max} (i.e., limiting concentration of the Fedors plot) was estimated to be 0.33 dL g^{-1} , which is generally lower than that of other galactomannans including *Peltophorum pterocarpum* (0.84 dL g^{-1}) (Nwokocha & Williams, 2014) and *Delonix regia* (0.41 dL g^{-1}) (Nwokocha et al., 2018) seeds as well as *Adansonia digitata* leaves (1.24 dL g^{-1}) (Nwokocha & Williams, 2016). The Kraemer and Huggins constants were estimated at $k_K = -0.012$ and $k_H = 0.852$. In general, $k_K < 0$ and $k_H < 0.5$ indicate good polymer solvation and dilute polymer solutions in good solvents, respectively (Yousefi & Razavi, 2019). Hereby, the calculated k_H indicates a theta solvent quality i.e., the polymer-polymer and polymer–water molecular interactions are equally favoured. Thus, RCG is molecularly dissolved in water adapting a random coil conformation with the size of the polymer coil being directly proportional to its molecular weight. In the case of RCG, $k_K + k_H = 0.84 > 0.5$, which suggests the self-association of the polymer chains in the solvent (MilliQ water). The differences in the Huggins and Kraemer constants (i.e. $k_H \gg k_K$) most probably stem from differences in the RCG polymer chain length and the sequence pattern of the galactose depleted (smooth) and substituted (hairy) pattern (Gillet et al., 2017). A similar to RCG behaviour has been also observed in the case of alfalfa (*Medicago sativa* L.) seed galactomannan ($M/G \sim 1.1$) where $k_K + k_H = 0.83$ (Hellebois, Soukoulis, et al., 2021).

3.5. Steady-state flow behaviour

The steady-state flow behaviour curves of RCG solutions ($0.125\text{--}2$ wt %) in MilliQ water are displayed in Fig. 6A. For the entire range of concentrations tested, the RCG solutions exhibited a shear thinning behaviour ($n < 1$), which is in line with our previous observations for alfalfa seed galactomannans (Hellebois, Soukoulis, et al., 2021). Fitting the apparent viscosity – shear rate data with the Cross-Williamson model, the zero viscosity, relaxation time (τ) and the critical shear rate (i.e., the shear rate at which the shear thinning behaviour is established) were determined. The dependence of the relaxation time on concentration was $\tau \propto c^{3.2}$ for $0.75 < c < 2$ and $\tau \propto c^{0.56}$ for $0.25 < c < 0.75$, which are characteristic of semi-dilute entangled and unentangled regimes, respectively. According to the master curve (Fig. 6B), the critical coil overlap of RCG was estimated at $c^* = 0.35$ wt %, which is in good approximation with the $c^* \sim 4/[\eta]$ as previously reported by

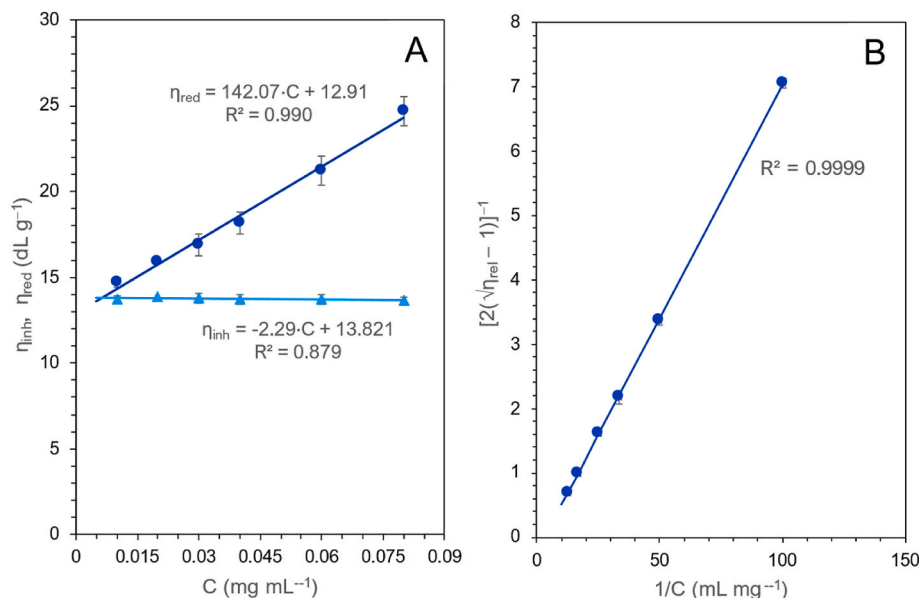


Fig. 5. Estimation of intrinsic viscosity of red clover galactomannan solution (pH = 7, MilliQ water, 25 °C) according to the Kraemer (η_{inh}), Huggins (η_{rel}) and Fedors ($\sqrt{\eta_{rel}}$) equations.

(Robinson et al., 1982) in the case of galactomannans of varying M/G. A second breakpoint at $c^* \sim 1.82$ wt %, has been assigned to the transition from the Rouse (semi-dilute) to the entangled solution regime. In comparison to previous studies, the critical coil overlap concentration of RCG is higher than that of locust bean gum ($c^* = 0.14\text{--}0.20$ wt %) and guar gum ($c^* = 0.1\text{--}0.20$ wt %) but comparable to that of fully substituted galactomannans such as alfalfa ($c^* = 0.31$ wt %) (Doublier & Launay, 1981; Gillet et al., 2017; Hellebois, Soukoulis, et al., 2021; Pollard & Fischer, 2014). It is well documented that the critical coil overlap concentration is a measure of the molecular weight and structure conformation of a polymer: the higher the molecular weight and the more rigid the conformation, the lower the c^* (Rubinstein & Colby, 2003). An estimation of the chain flexibility of RCG was made by computing the persistence length (L_p) according to the worm-like chain (WLC) model for semi-flexible (random-coil) polymers under dilute solution conditions:

$$R_g^2 = 2L_p L \left[1 - \frac{L_p}{L} \left(1 - e^{-\frac{L}{L_p}} \right) \right] \quad (3.5)$$

where L denotes the contour length i.e., $L = \ell_0 N$ ($\ell_0 = 0.54$ nm and $N = 306$ g mol^{-1} for galactomannans, (Sayko et al., 2021)) and R_g denotes the radius of gyration as calculated using the Flory – Fox equation assuming a random coil polymer:

$$[\eta] = \frac{1}{\sqrt{6}} \frac{\Phi R_g^3}{M_w} \quad (3.6)$$

where Φ is the Flory-Fox constant ($\Phi = 2.86 \times 10^{23} \times (1 - 2.63\varepsilon + 2.86\varepsilon^2)$, where $\varepsilon = \frac{2\alpha - 1}{3}$, and $\alpha = 0.5$ for θ -solvents), M_w is the average molecular weight of the galactomannan. Using the experimentally determined intrinsic viscosity and average molecular weight of the RCG, and assuming that $L_p \ll L$, the persistence length was calculated to be approximately 6.59 ± 0.62 nm, which falls within the range of the experimentally determined L_p values reported in the literature (Picout et al., 2001, 2002). According to previous studies by (Morris et al., 2008; Pollard et al., 2010), the persistence length of galactomannans ranges from 3 to 12 nm and appears to be independent of the M/G, botanical origin and subfamily of the galactomannan extracts. In the context of the WLC theory, the dimensional stiffness parameter of RCG (C_∞) was

estimated as follows:

$$C_\infty = \frac{\langle R^2 \rangle}{n l^2} \approx \frac{2L_p}{l_0} \quad (3.7)$$

where $\langle R^2 \rangle$ is the mean-square end-to-end distance of the real chain, l is the bond length, and n is the number of backbone bonds. According to the obtained $C_\infty = 24.4 \pm 2.3$, RCG exhibits moderate chain flexibility stemming from its highly branched mannan backbone. The estimated C_∞ is comparable to that reported (Pollard et al., 2010) for native galactomannans of the Fabaceae ($C_\infty = 19.9 \pm 1.3$) and Caesalpinioideae ($C_\infty = 19.9 \pm 1.3$) subfamilies, but significantly higher (and therefore stiffer) than galactomannan hydrolysates ($C_\infty = 12.2$ and 12.6 for guar and locust bean gum hydrolysates, respectively) (Picout et al., 2001, 2002).

3.6. Viscoelastic properties

3.6.1. Large amplitude oscillatory shear (LAOS) measurements

Amplitude sweeps were carried out at an angular frequency of 10 rad/s to determine the linear viscoelastic regime of the RCG solutions at the concentration range of 1–5 wt %. As given in Table 3, the shear strain offset of the LVE regime of the solutions was inversely proportional to the RCG concentration. Increasing the stress beyond the LVE regime, an overshoot of the G'' was observed at $c \geq 3$ wt %, which is indicative of LAOS type III (i.e., weak strain overshoot) behaviour. LAOS type III behaviour is observed in polymer solutions where the formation of new interchain molecular junctions occurs more slowly than the breakdown of the existing ones as shear strain increases (Hyun et al., 2011). As expected, the progressive increase in the shear strain resulted in the collapse of the entangled polymer networks, as shown by the transition from the elastic to the viscous-dominant viscoelastic behaviour at shear flow stress (flow point). The proportionality to the RCG concentration increase at the flow points is primarily associated with the increase in the inertia of the entangled polymer solutions. The findings are generally comparable to those reported in the case of alfalfa seed galactomannan (Hellebois, Soukoulis, et al., 2021).

In Fig. 7, the Lissajous-Bowditch curves for the galactomannan solutions illustrate the relationship between shear stress and strain or strain rate amplitude, as influenced by the RCG concentration. As illustrated in Fig. 7, beyond the boundary of the LVE regime (i.e.

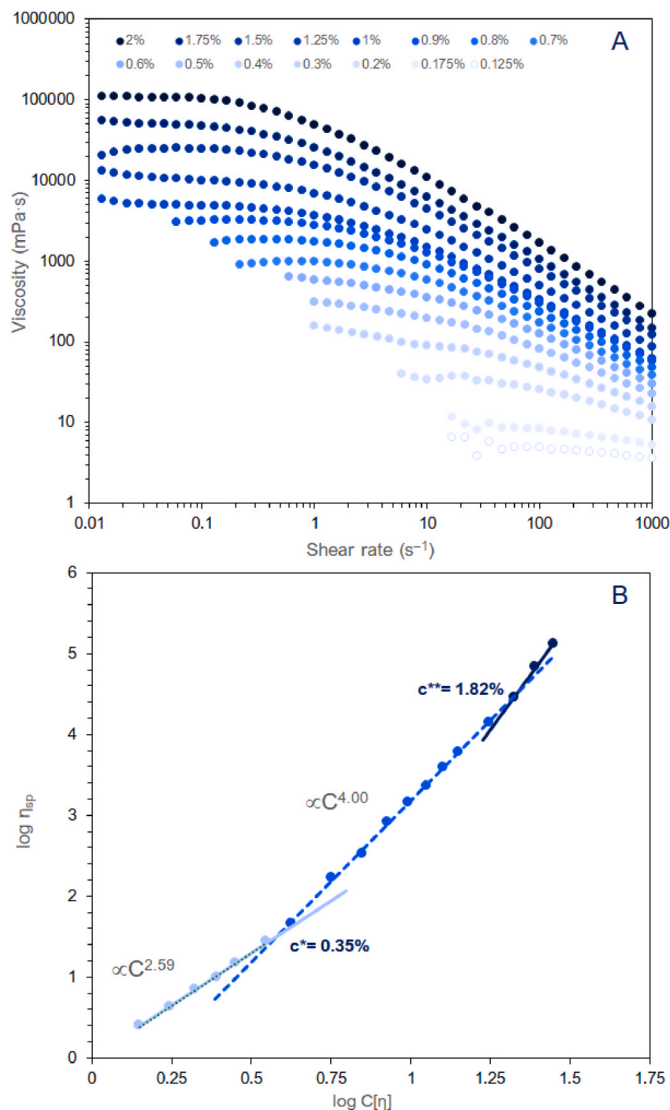


Fig. 6. Steady state flow behaviour curves of red clover galactomannan solutions (pH = 7, 25 °C) as influenced by the gum concentration (A) and double logarithmic plot of specific viscosity at zero shear rate ($\eta_{sp,0}$) as a function of coil overlap parameter c [η] (B). In Fig. 6B, the dilute ($c < c^*$), semi-dilute ($c^* < c < c^{**}$) and concentrated ($c > c^{**}$) solution regimes are illustrated by different colours.

Table 3

Viscoelastic properties i.e. shear strain amplitude sweeps ($\omega = 10$ rad/s) of the red clover seed galactomannan solutions ($c = 1\text{--}5$ % wt., pH = 7, 25 °C).

C_{gum} (% wt.)	G'_{LVE} (Pa)	γ'_{LVE} (%)	Flow point, τ_f (Pa)	G'_f (Pa)
1	6.01	25.3	nd	nd
1.5	36.5	14.7	20.7	32.8
2	176	14.4	101.6	107.5
3	264	13.7	180.1	146.7
4	878	11.3	665.1	374.3
5	1251	10.8	919	458.4

approximately 10 % for $c \geq 1.75$ wt %) and for shear strains below 100 %, all systems exhibited a balanced viscoelastic behaviour as indicated by the elliptical shape of the elastic (Fig. 7A) and viscous (Fig. 7B) Lissajous curves. For strain amplitudes below 100 %, the hysteresis loop area remained relatively unaltered; therefore, the RCG solutions retained their viscoelastic character. Conversely, the hysteresis loop of

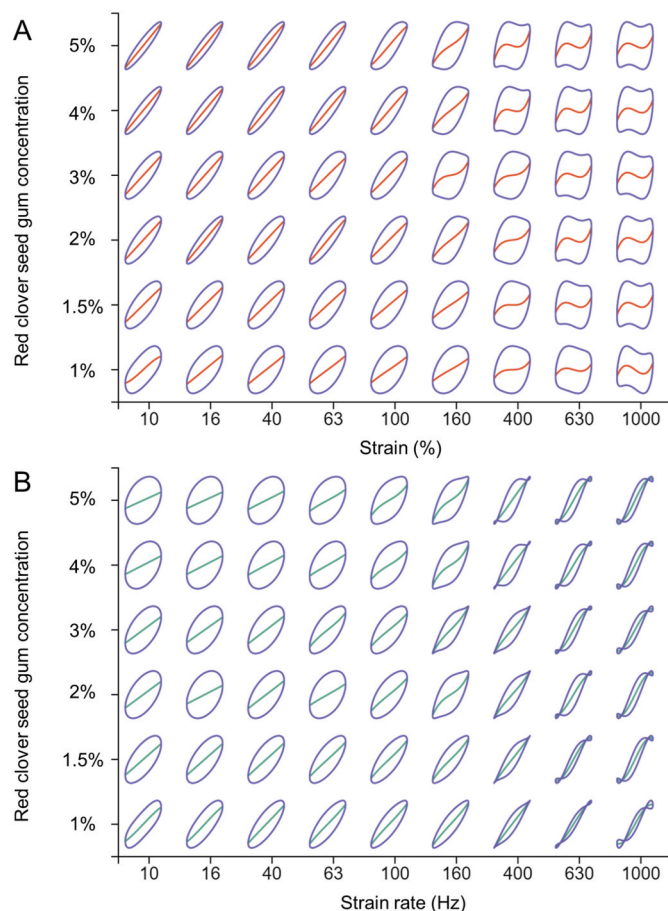


Fig. 7. Lissajous-Bowditch plots of the red clover seed galactomannan solutions in MilliQ water as influenced by the gum concentration (1–5 % wt.) at 25 °C.

the elastic Lissajous curves increased inversely to the concentration of RCG, particularly when $c < 1.75$ wt %. In general, a larger area implies higher damping or energy loss, typical of materials with significant viscous behaviour. At shear strains above 100 %, the asymmetry of the elastic Lissajous curves became more evident, in the form of distorted rectangular shapes, indicating a yielding (plastic) behaviour. At shear strain rate amplitudes above 100 Hz, a transition in the shape of the viscous Lissajous curves was observed, shifting a pinched single loop ($100 < \text{strain rate} < 400$ Hz) to a figure-eight-shaped double loop (strain rate > 400 Hz). It has been well documented that increased asymmetry in Lissajous curves is associated with changes in the polymer entanglement dynamics and closely related to molecular conformation (e.g. length of the polymer chain, degree of branching, type of side functional groups, etc) (Kamkar et al., 2022). Similar to other fully galactose-substituted galactomannans, e.g. alfalfa or fenugreek gum, RCG can form interchain junctions via hydrogen bonding (Hellebois, Gaiani, et al., 2021). Beyond the LVE regime and within the MAOS regime, the RCG solutions predominantly maintained their elastic or viscoelastic character, depending on concentration. At higher strain rate amplitudes, the energy dissipated was insufficient to break down the polymer-polymer junction zones, leading to nonlinear viscoelastic behaviour similar to that of branched polymers, as indicated by the pinched viscous Lissajous loops. As the shear strain rate further increased, the formation time scale of new interchain polymer junction zones became shorter than the oscillatory deformation time scale, leading to the rupture of existing junction zones, which was reflected in the self-intersected shape of the viscous Lissajous loops (Schreuders et al., 2021).

To provide insight into the structural changes occurring at large

deformations, the strain stiffening ($S = \frac{G'_s - G'_0}{G'_0}$) and shear thickening ($T = \frac{\eta'_s - \eta'_0}{\eta'_0}$) ratios as influenced by the RCG concentration were plotted in Fig. 8A and B, respectively. As illustrated in Fig. 8A, all RCG solutions exhibited pronounced shear stiffening behaviour when shear strain exceeded 100 %. Yet it was not possible to identify a clear impact of the RCG concentration on the S shear strain dependency. Looking at the intracycling thickening behaviour plot (Fig. 8B), a maximum in the T values of all RCG solutions was observed, followed by a steep decrease at the highest range of shear strain rates. A similar behaviour for the S and T plots has been reported in the case of tomato paste (Duvarci et al., 2017b), mayonnaise (Duvarci et al., 2017a) and wheat gluteins (Yazar et al., 2017). The changes in the intensity of the T peak values increased proportionally to the RCG concentration.

3.6.2. Small amplitude oscillatory (SAOS) measurements

3.6.2.1. Time concentration superposition (TCS). The RCG solutions ($c = 1$ to 7 wt %) were subject to frequency sweeps (angular frequency of 0.1–100 rad/s) within the LVE regime at a strain of 0.1 % (Fig. 9A). As shown in Fig. 9A at $c \geq 1.5$ wt % the RCG aqueous solutions exerted a typical entangled concentrated galactomannan solution behaviour i.e., viscous and elastic dominant viscoelasticity at low and high-frequency ranges, respectively (Barrera et al., 2022). The frequency dependence of the G' - G'' crossover points, f_c , (Fig. 9B–Table 4) was inversely proportional to the RCG concentration. Galactomannan molecular structure conformation parameters such as the M/G ratio, degree of polymerisation, hairy to smooth region sequencing pattern etc. as well as their purity are known to impact their solution relaxation dynamics (Gillet et al., 2017; Pollard & Fischer, 2014; Sittikijyothin et al., 2005). In general, the RCG solutions exhibited shorter relaxation times (τ) compared to partially galactose-depleted galactomannans such as locust bean gum, *Gleditsia triacanthos*, and guar gum, yet comparable to the relaxation time of fully substituted galactomannans such as alfalfa and fenugreek solutions at the same total biopolymer concentration (Barrera et al., 2022; Brummer et al., 2003; Gillet et al., 2017; Hellebois, Gaiani, et al., 2021; Sittikijyothin et al., 2005). At $1 < c < 2$ wt %, the relaxation time was $\tau \propto c^{3.73}$, indicating that RCG solutions comply with reptation dynamics and therefore, they are classified as entangled biopolymers. Yet due to measurement limitations, it was not possible to confirm the c_e where the transition from the semi-dilute ($\tau \propto c^{2\nu-1}$, $\nu = 0.588$ presuming θ -solvent conditions) to the terminal entangled regime ($\tau \propto c^3$) occurs. Similar constraints have been reported by Pollard and Fischer (2014) in the case of guar gum. When $c > 2$ %, the relaxation time of the

RCG solutions became notably less dependent on concentration ($\tau \propto c^{0.47}$) most probably due to polymer chain crowding effects; at such conditions the increase in the biopolymer concentration results in a reciprocal diminishment of the diameter of the reptation tube model minimising the impact on relaxation dynamics. As a next step, the dynamic rheological spectra (Fig. 9A) were time-concentration superposed (TCS) as illustrated in Fig. 9C. A very satisfactory superposition of the G' and G'' spectra was achieved for the concentration range (1–7 wt %) tested hereby. As clearly illustrated in Fig. 9C, the G'' plateaued at high RCG concentrations indicating a saturation in viscous energy dissipation most probably due to sterically hindered rearrangement of the galactomannan chains. Plotting the horizontal shift factor ($\log a_c$), associated with changes in the relaxation dynamics as a function of the biopolymer concentration-against $\log(c/c_{ref})$ (Fig. 9D), a breakpoint in the slopes of the curves was observed, i.e., $\log a_c \sim c^{3.58}$ ($1 < c \leq 2$ wt %) and $\log a_c \sim c^{0.67}$ ($c \geq 2$ wt %). Therefore, our findings postulate a transition from the reptation dynamics ($\log a_c \propto c^{3.4-4}$) to the concentrated entangled solution regime. In the latter case, the reinforcement of the non-specific physical entanglements results in the formation of a less flexible polymer network and gives rise to specific physical interactions between the polymer chains, hindering the molecular motion of the solvent (Barrera et al., 2022). In addition, it is postulated that hyperentanglement, i.e. weaker, fleeting associations in the crystallographic α direction (Doyle et al., 2009), may also contribute to the observed changes in the relaxation dynamics of the RCG solutions.

3.6.2.2. Time temperature superposition (TTS). To gain insight into the relaxation dynamics of the RCG solutions (1–7 wt %) as a function of temperature, frequency sweeps ($\omega = 0.1$ –100 rad/s, strain = 0.1 %) were conducted at various temperatures (i.e., 5–65 °C, in 10 °C increments). Master curves applying the Time-Temperature Superposition (TTS) principle were constructed as illustrated in Fig. 10A, using ambient temperature (25 °C) as the reference temperature, T_r . The calculated frequency-temperature shift factors (a_T) at various RCG concentrations (1–7 wt %) were plotted against temperature, and the activation energies (E_a) as influenced by the RCG concentration were calculated according to the Arrhenius equation (Eq. (3.5)):

$$\ln a_T = \frac{E_a}{R} \left(\frac{1}{T} - \frac{1}{T_r} \right) \quad (3.5)$$

Except for galactomannan concentration, other factors, including the galactomannan molecular structure conformation, and the solvent quality, are known to impact the activation energy of galactomannan solutions. As illustrated in Fig. 10B, the E_a values of the galactomannan

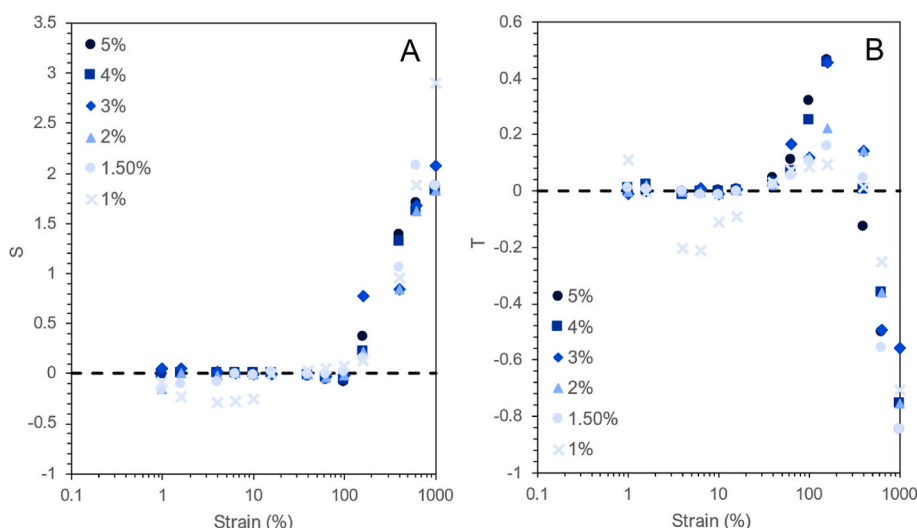


Fig. 8. Shear strain dependent stiffening (S) and thickening (T) behaviour of the red clover seed galactomannan solutions (1–5 % wt.) at 25 °C.

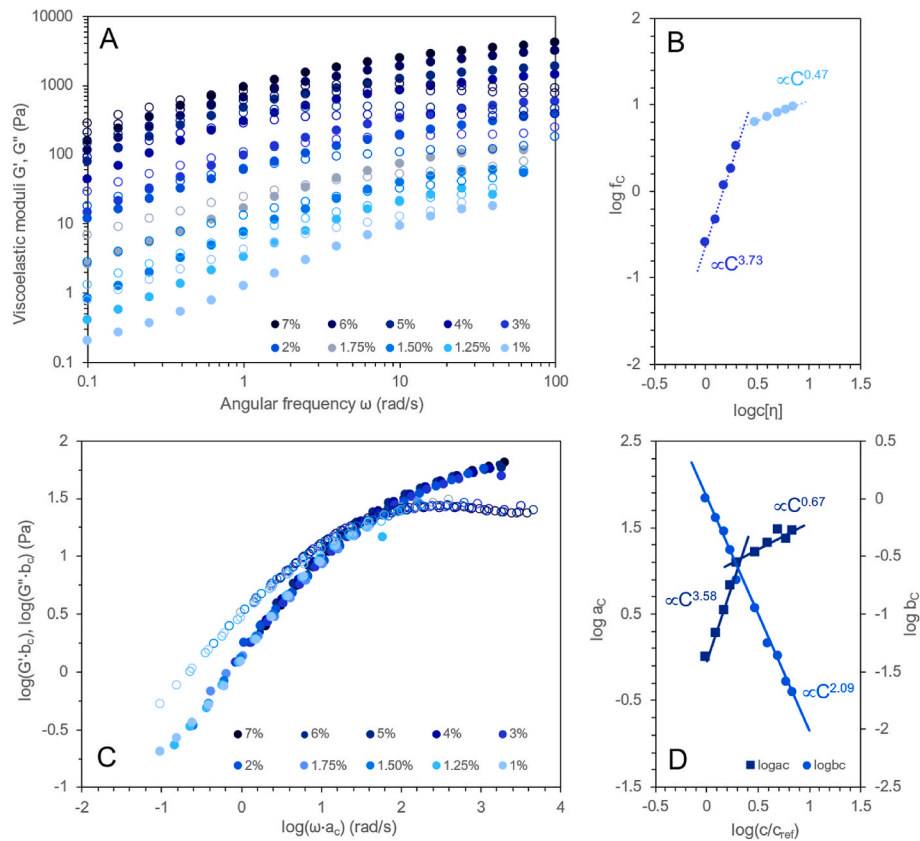


Fig. 9. Application of the time concentration superposition (TCS) principle. A: Viscoelastic spectra (strain = 0.1 %) of the red clover seed galactomannans (1–7 % wt.) in MilliQ water at 25 °C. B: Time concentration master curve, C: Time concentration superposed viscoelastic spectra and D: Shift factor a_c (squares) and b_c (circles) concentration master curves.

Table 4

Small amplitude oscillatory shear properties (strain = 0.1 %) of the red clover seed galactomannan solutions ($c = 1$ –5 % wt., pH = 7, 25 °C).

C_{gum} (% wt.)	Crossover frequency, ω_c (rad/s)	$\log G' - \log \omega$ slope	$\log G'' - \log \omega$ slope
1	24.0	0.893	0.609
1.25	13.3	0.864	0.577
1.5	5.41	0.826	0.506
1.75	3.41	0.689	0.422
2	1.41	0.541	0.289
3	0.99	0.507	0.276
4	0.86	0.486	0.175
5	0.78	0.441	0.161
6	0.72	0.469	0.152
7	0.66	0.456	0.143

solutions were not remarkably influenced by the RCG concentration ranging from 23 to 26 kJ/mol. In general, the hereby calculated E_a values are in range with the experimentally calculated for guar gum (i.e., 20–30 kJ/mol), fenugreek seed paste (~26 kJ/mol) and *Bauhinia monandra* (~21 kJ/mol) (İşikli & Karababa, 2005; Nwokocha et al., 2017; S. Wang et al., 2015). To evaluate the interplay between RCG concentration and temperature (TTCS) a super master curve was constructed as follows:

$$a_{cT} = a_c \cdot a_T = \exp \left[\frac{E_a}{R} \left(\frac{1}{T} - \frac{1}{T_r} \right) + [\beta(c - c_{ref}) + \beta_0] \right] \quad (3.6)$$

Where: (a_{cT}) is the concentration–temperature shift factor, E_a denotes the activation energy, R is the universal gas constant, T_{ref} and c_{ref} are the reference temperature and concentration, respectively. Due to changes in their physical state as a function of concentration, the super master

curve was applied only over the concentration range where the RCG solutions comply with the concentrated entangled solution state dynamics (i.e., $c \geq 2$ wt %). A similar approach has been adopted in polymeric systems where changes in the mobility of the entangled network are encountered at a molecular level (Suarez-Martinez et al., 2019). As illustrated in Fig. 10C, a very satisfactory superposition was obtained for the entire concentration range. The crossover of the TTC superposed viscoelastic moduli G' & G'' was identified at $\log(a_c a_T f) = 0.09$ Hz with the relaxation time to be estimated at $\tau \approx 11$ s. The short relaxation time of the RCG solutions over the assessed temperature range implies that the entangled galactomannan networks involve transient, dynamic physical interactions (mainly hydrogen bond bridging) rather than permanent, long-lasting ones, e.g. chemical crosslink junctions. To confirm this, RCG (1 % wt) aqueous systems in the presence of urea (0.25–2 M), employed as a hydrogen bond disrupting agent, were subject to frequency sweeps. As illustrated in Fig. 11, a steep increase in the complex viscosity of the RCG in the urea–water solvent environment was observed at 0.25 M. Our findings are in good agreement with the study of (Winkworth-Smith et al., 2016), who reported a significant increase in the intrinsic and zero shear viscosity of fenugreek gum in a chaotropic solvent environment (urea–LiCl–water) due to the enhancement of the chain flexibility by weakening the intra-chain hydrogen bonds facilitating the molecular expansion. Indeed, DLS characterisation of the RCG in urea–water solvent environment showed an increase by 21–26 % in the hydrodynamic radii compared to the RCG in water exemplar exemplifying the urea-mediated conformational changes of RCG. At higher urea concentrations a power-dependent decrease in the complex viscosity of the RCG solutions was observed, which assumes the progressive disruption of the inter-chain hydrogen bond junctions (Wang et al., 2019).

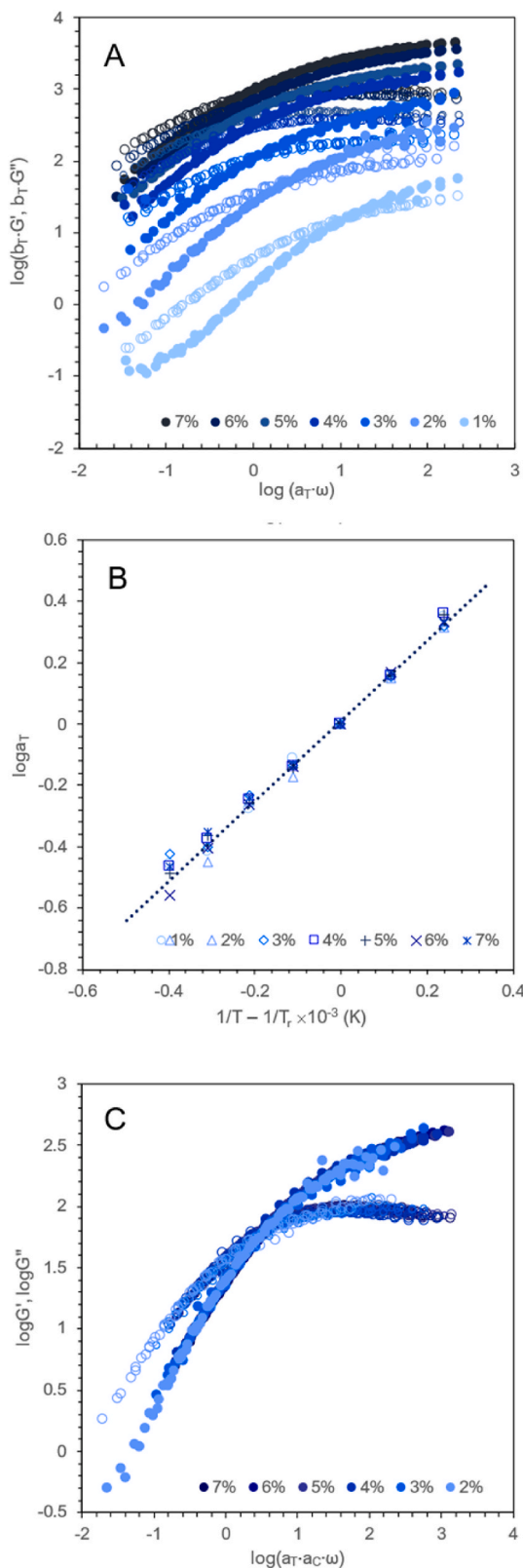


Fig. 10. A: Temperature time superposition (TTS) of complex viscosity (η^*) as influenced by the red clover seed galactomannan concentration ($c = 1\text{--}7$ % wt.), B: Arrhenius plot of horizontal shift factor $\log a_T$ versus inverse temperature $1/T - 1/T_{ref}$ ($T_{ref} = 25$ °C), and C: Time temperature concentration super master curve (TTCS) of the RCG solutions at $c \geq 2$ % wt.

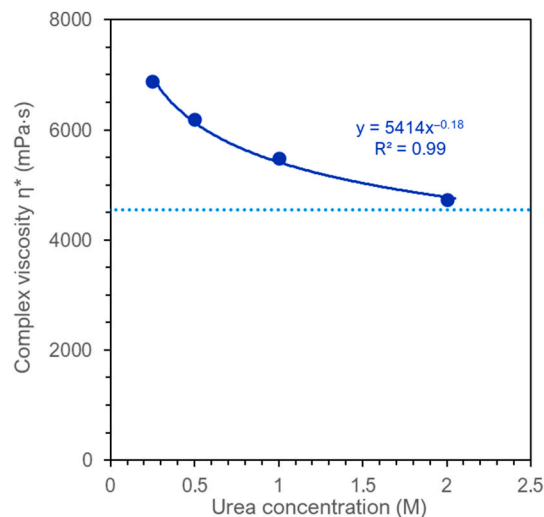


Fig. 11. Impact of urea (0.25 – 2 M) on the complex viscosity η^* of red clover seed galactomannan solutions (1 % wt.) at 25 °C. The dashed line denotes the complex viscosity of the RCG aqueous solution in the absence of urea.

4. Conclusions

RCG is a non-ionic heteropolymer having an M/G ratio of 1.12, a molecular weight of approximately 1500 kDa, an intrinsic viscosity of 13.6 dL g^{-1} and a hydrodynamic radius of 120 nm. RCG exhibited theta-solution behaviour in deionised water, which was attributed to inter-chain polymer associations. The critical coil overlap concentration of RCG in deionised water was estimated to be $c^* = 0.35$ wt %, whereas the concentration marking the transition from the Rouse to the entangled solution state was approximately 1.82 wt %. A steep slope in the $\eta_{sp} - C$ [η] master curve (i.e., $\propto C^{4.0}$), characteristic of galactomannans, was observed, indicating the occurrence of non-specific interchain polymer interactions. Large amplitude oscillatory shear tests revealed that RCG solutions, beyond the LVE and within the MAOS regime, retained their viscoelastic character, whilst at higher strain rate amplitudes, a predominant non-linear viscoelastic behaviour was observed due to the rupture of the interchain polymer junction zones. According to SAOS testing, at $1 < c < 2$ wt %, the relaxation time was $\tau \propto c^{3.73}$, suggesting that RCG solutions complied with reptation dynamics, whilst at higher concentrations (up to 7 wt %) the relaxation time became less responsive to concentration, i.e., $\tau \propto c^{0.38}$. A time-concentration-temperature super master curve was successfully constructed for $c \geq 2$ wt %, allowing the determination of a universal activation energy (E_a) of 23.5 kJ/mol in the temperature range of 5 to 65 °C. In conclusion, it was demonstrated that RCG is a promising, sustainable cold-water-soluble galactomannan possessing industrially relevant thickening and gelling properties useful for food, nutraceutical, cosmeceutical, and pharmaceutical product applications. Further studies to assess its synergism with other industrial hydrocolloids and functionality in model food systems are needed.

CRediT authorship contribution statement

Lingxin You: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Reiner Dieden:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Yves Fleming:** Writing – review & editing, Investigation, Formal analysis. **Jennyfer Fortuin:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Alexander Shaplov:** Writing – review & editing, Writing – original draft, Validation, Investigation, Formal analysis. **Davide Odelli:** Writing – review & editing, Investigation, Formal analysis. **Denis Pittois:** Writing – review & editing, Investigation, Formal analysis. **Christos Ritzoulis:** Writing –

review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Christos Soukoulis**: Writing – review & editing, Writing – original draft, Validation, Resources, Project administration, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Dr. T. Hellebois is gratefully thanked for assisting with the procurement of the materials and the gum extraction.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodhyd.2025.111517>.

Data availability

Data will be made available on request.

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