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Application of Humidity-Controlled Dynamic Mechanical Analysis (DMA-RH) to Moisture-Sensitive Edible Casein Films for Use in Food Packaging

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Abstract: Protein-based and other hydrophilic thin films are promising materials for the manufacture of edible food packaging and other food and non-food applications. Calcium caseinate (CaCas) films are highly hygroscopic and physical characterization under broad environmental conditions is critical to application development and film optimization. A new technology, humidity-controlled dynamic mechanical analysis (DMA-RH) was explored to characterize CaCas/glycerol films (3:1 ratio) during isohume temperature (T) ramps and steps, and isothermal RH ramps and steps, to determine their mechanical and moisture-sorption properties during extensive T and RH variations. When RH and/or T increased, CaCas/Gly films became strongly plasticized and underwent several primary and secondary humidity-dependent transition temperatures (or transition humidities); the CaCas/Gly network hypothetically rearranged itself to adapt to the increased water-content and heat-induced molecular mobility. Between 5–40 °C and 20%–61% RH, moisture-sorption was rapid and proportional to humidity between transition points and accelerated greatly during transitions. CaCas/Gly films seemed unsuitable for storage or utilization in warm/humid conditions as they lost their mechanical integrity around $T_m \sim 40$ °C at 50% RH and T_m decreased greatly with increased RH. However, below T_m , both moisture- and heat-induced structural changes in the films were fully reversible and casein films may withstand a variety of moderate abuse conditions.

Keywords: Hydrophilic thin films; dynamic mechanical analysis; environmental conditions; temperature; humidity; edible packaging; casein; tensile properties

1. Introduction

Edible packaging films made from milk proteins are a “green” potential solution to replace some petroleum-based food-packaging films and improve the quality and preservation of food, while at the same time reducing waste, since the films biodegrade and can be eaten with the food they are protecting. Casein (Cas), the main protein of cow’s milk, is precipitated from milk by acidification and then neutralized with $\text{Ca}(\text{OH})_2$ or NaOH base to re-solubilize and form calcium caseinate (CaCas) or sodium caseinate (NaCas) before spray-drying. The caseinates have a random-coil structure due to the presence of many ring-shaped proline residues that stiffen the backbone and prevent the formation of tertiary structures, through steric hindrance [1]. The proline residues are also hydrophobic and their distribution pattern along the proteins contributes to the caseinates’ amphiphilic character that is exploited in the film-making process. Films manufactured from CaCas or NaCas blended with glycerol (Gly) are strong, transparent, tasteless and excellent oxygen barriers [2], but their mechanical properties vary strongly with the ambient humidity present during their manufacture, storage and utilization [3]. To date, edible films intended for food packaging applications have been prepared from a variety of proteins, carbohydrates, and protein/carbohydrate combinations, and are generally highly hydrophilic, soluble in water and extremely sensitive to moisture during storage and utilization; however, the tensile properties of hydrophilic edible films are typically measured at normal temperature (T) and relative humidity (RH) conditions (23 ± 2 °C and $50\% \pm 5\%$ RH) only. For example, Cas/Gly film strips are stored in normal conditions for at least 40 h according to the standard ASTM method for testing tensile properties of thin films [4], then mounted on an Instron-type texture analyzer or an opened dynamic mechanical analyzer (DMA), in a room or chamber also controlled at $\sim 50\% \pm 5\%$ RH and 23 ± 2 °C, to measure the stress-strain curves of Cas/Gly films in normal conditions [5–7]. In order to measure the stress-strain curves under different RH conditions and obtain the Young’s modulus, Y , tensile strength, TS , and elongation at break, D (%), of casein films as a function of RH, films have been left to equilibrate in the open in the laboratory and tested at different times of the year, with RH varying from as low as $\sim 10\%$ to as high as $\sim 70\%$ RH [3,8]. At constant film thickness, the elongation at break of CaCas/Gly films increased considerably with RH, while the tensile strength and elastic modulus decreased somewhat linearly with RH and could in theory be extrapolated to ~ 0 MPa at $\sim 80\%$ RH [3]. Using casein films grafted with acrylonitrile, Somanathan *et al.* found that the transient tensile properties (*i.e.*, stress-strain curves) of casein-based films were strongly affected by the amount of moisture absorbed by the films [9]. During the DMA measurement of stress-strain curves of beta-casein films preconditioned in an environmental chamber at 50% RH, Mauer *et al.* postulated that the 10 min needed to test each sample at the variable ambient RH present in the laboratory at the time of measurement had a negligible effect on the moisture-content of the films, and that the tensile properties measured were representative of the properties at 50% RH [7].

Environmental conditions (T and RH) are known to affect the mechanical properties of biopolymer films in several ways. For instance, hydrophilic soy-protein/Gly films were found to possess high moisture-sorption rates and to absorb increasing amounts of water when RH rises [10]. Because the humidity (H) in the air increases near-exponentially with temperature at constant RH [11], variations in either T or RH can considerably affect the films' water-content, which acts as a plasticizer and generally reduces the tensile strength and increases the elongation at break of the films [10]. Protein-based thermoplastics resemble semi-crystalline polymers and can have several structural relaxations and transition temperatures, including a glass transition (T_g) and a melting point (T_m), as well as various secondary transition temperatures that correspond to different changes in protein-protein interactions, or partial denaturation of the tertiary or secondary structure of the proteins. Unlike polymers, T_g and T_m do not necessarily imply complete unfolding of the proteins into a fully amorphous structure, as α -helices and β -sheets can remain [12]. The thermal behaviors of casein or whey protein films—plasticized with Gly or not—have been determined at 0% RH at a wide range of temperatures using differential scanning calorimetry (DSC) or dynamic mechanical analysis (DMA; also termed dynamic thermo-mechanical analysis, DMTA) [13–15]. Recently, the dynamic thermo-mechanical behaviors of soy-protein/Gly films were characterized with DMA from -100 to 150 °C with a heating rate of 5 °C/min at 0% RH [16].

However, since water strongly plasticizes protein networks, relative humidity values above 0% are expected to heavily influence the different transition temperatures of protein-based films. Different levels of moisture absorbed into casein films during thermo-mechanical analyses are expected to lower T_g and T_m to different extents, as well as trigger new unfolding and reconfiguration patterns for the proteins, that may be indicated by new, or shifted, secondary transition temperatures. To date, the thermo-mechanical properties of milk-based films have not been measured other than in dry (0% RH) conditions, and few other edible films and bioplastics have been characterized with DMA after equilibrating under varied RH conditions. In order to measure the RH-dependent thermo-mechanical properties of hydrophilic films and bioplastics, materials have been left to equilibrate for at least a week at ~ 23 °C in air-tight chambers containing different saturated salt solutions, such as: LiCl, CH₃COOK, MgCl₂, K₂CO₃, Mg(NO₃)₂, NaNO₂, NaCl or KCl, which generate constant RH conditions of 11%, 24%, 33%, 44%, 54%, 66%, 76% or 85%, respectively [17], then loaded onto a DMA for thermo-mechanical analysis with a T -ramp at 0% RH. For example, Hochstetter *et al.* evaluated uncoated wheat gluten/Gly sheets from -150 to 50 °C at 0% RH with a T -ramp of 2 °C/min at different frequencies of 1, 2.5, 5, 10 and 20 Hz [17]. Wongsasulak *et al.* found that the moisture content played a key role in the thermo-mechanical properties and microstructure of egg albumen/cassava starch composite films and added a wet cotton ball in the DMA chamber to reduce moisture-loss during T -ramps. After ramping T from 25 to 130 °C at a rate of 2.5 °C/min, water loss in the samples was reduced to $\sim 5\%$ in the presence of wet cotton, compared to 20% without it [18]. The effective RH created within the DMA chamber was not tracked and most certainly fluctuated with T during the experiment, from a low or normal RH at 25 °C, to $\sim 100\%$ RH once water in the cotton vaporized above 100 °C, possibly affecting the thermo-mechanical results in different ways. A different technique employed to perform DMA T -ramps at specific moisture contents consists of coating samples with silicone oil or silicone-based hydrophobic grease after equilibration at the desired RH, to limit water evaporation during heating and maintain a constant moisture content within the film or thin sheet. Using this technique, Zárate-Ramírez *et al.* characterized the thermal properties of wheat gluten

bioplastics, preconditioned at 53% RH (14% moisture content), from -30 to 160 °C while keeping the moisture content hypothetically constant [19,20]. Ditudompo *et al.* employed three different RH levels to precondition extruded starch ribbons at 33%, 75% and 98% RH to yield moisture-contents of $\sim 13\%$, 19% and 31%, then coated the samples with silicon grease to perform DMA T -ramps; the grease was found to effectively keep water-loss below 5% [21].

In this paper, we assess a novel technology called DMA-RH that precisely controls the relative humidity inside the DMA chamber during a test by balancing the flow rates of dry and water-saturated nitrogen flowing through the test chamber to produce any desired value of RH between 0% and 80% at $5-90$ °C. RH is a strong function of temperature: the mass of water per unit volume of air is termed “humidity”, $H(T)$; at constant pressure, the mass of water at saturation (the dew point, $H_s(T)$) increases nearly exponentially with T [11]; the relative humidity is defined as $RH = H(T)/H_s(T)$, and therefore, at constant RH, $H(T)$ increases exponentially with T . The DMA-RH technology is able to perform isohume (iso-RH) T -ramps, as well as isothermal (iso- T) changes in RH, both in transient and dynamic mode. Isohume T -ramps at different RH values and isothermal RH-steps or RH-ramps are expected to provide new information on the thermo-mechanical properties of hydrophilic thin films, since the broad variations of $H(T)$ in the atmosphere around the sample may profoundly affect the moisture-content, protein configuration, network structure and molecular mobility of the films. Although DMA-RH has recently been employed to characterize some of the humidity-dependent properties of different green food-packaging materials, including Kraft and Clupak papers, biodegradable polyesters, and carboxymethylcellulose-based films [22–24], it has not yet been applied to edible and/or protein-based films. CaCas/Gly films with a constant composition and physical attributes were chosen as a template for method-development using the DMA-RH technology to evaluate its performance and usefulness to characterize the hydro-thermo-mechanical properties of hydrophilic, protein-based thin films. Exploratory transient and dynamic tensile tests were performed under a wide variety of (T , RH) conditions by designing various experimental programs to simulate potential real-life conditions during the fabrication, storage and/or utilization of the films, such as refrigeration, “normal” handling conditions, heating, cold/dry or hot/humid climates, *etc.*, which may be encountered during the life of food packaging films.

2. Experimental Section

2.1. Materials

Calcium caseinate powder (CaCas) was purchased from American Casein Co. (Burlington, NJ, USA). According to the manufacturer, CaCas contained 93% protein on a dry basis and 88% as is, 1.7% fat, 4.0% ash, $<1\%$ carbohydrate and 5% moisture. pH was 6.9. Glycerol (Gly) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Solutions of CaCas and Gly were prepared with de-ionized (DI) water produced by a Milli-Q Synthesis water purification system (Millipore, Billerica, MA, USA).

2.2. Films Preparation

Identical CaCas/Gly films were prepared periodically using a standardized method: the film-making solution was prepared with DI water to a total solid concentration of 15%, with a constant CaCas/Gly

ratio of 3:1 (75% CaCas, 25% gly). CaCas was first mixed in water with a magnetic stirrer at ~1000 rpm for 1 h, then Gly was added and the solution again stirred for 1 h. Red food coloring (McCormick & Co. Inc., Hunt Valley, MD, USA) was added at the end (1 drop/20 mL solution) to facilitate observation of the appearance and macro-structure of the dried films. The pH was measured with a pH/ion meter (Mettler Toledo, Schwerzenbach, Switzerland). The film-making solution was then transferred to a vacuum filtration flask and connected to the laboratory vacuum system until degassed, to prevent air bubbles in the dried films.

Films were cast on silicone baking mats (Weston Products LLC, Strongsville, OH, USA) [3]. In between film-casting, the mats were cleaned with diluted Contrex detergent, wiped dry, then a 70/30 percent ethanol/DI water solution was applied to remove any detergent residue. To cast a film, a silicone mat was placed on a K-101 Control Coater apparatus (RK Print-Coat Instruments Ltd., Royston, UK) set to speed setting #3. Rectangular films of approximately 15 cm × 25 cm with two different dry thicknesses (~0.02–0.03 mm and ~0.04–0.05 mm) were spread using two different spirally-wound meter bars: #200 or #300 (RK Print-Coat Instruments Ltd.). After spreading the solution, silicone mats were transferred to level tables at ambient temperature and relative humidity (T and RH) for an hour to set. Since variations in ambient RH during drying and storage reportedly have drastic effects on the properties of casein films [3], the films were then placed to dry in an environmental chamber at 50% RH and 20 °C (Model 6020-1, Caron, Marietta, OH, USA). After 1 day, the films were peeled from the mats, then placed between sheets of paper and stored in the chamber for a minimum of 4 days before testing. Films were cast in multiple independent repeats. Defect-free areas of the films were cut into 5 mm × 40 mm strips with a razor blade; strip thickness was measured at five points using a Mitutoyo absolute thickness gage (Mitutoyo Corp., Kanagawa, Japan), and averaged; then one extremity of each film strip was taped to a paper label and strips were returned to the environmental chamber until testing.

2.3. Humidity-Controlled Dynamic Mechanical Analyses (DMA-RH)

2.3.1. Equipment

The viscoelastic properties of select films under precise T and RH conditions were measured via dynamic mechanical analysis (DMA), where an oscillatory displacement (strain) is applied to film strips and the resulting force (stress) is measured. The DMA instrument was a Q800 from TA Instruments (New Castle, PA, USA) equipped with a DMA-RH attachment (also TA Instruments) that can control RH from 0% to 80% between 5 and 90 °C by modulating the flow rates of dry nitrogen and wet (saturated) nitrogen coming into the test chamber. RH initializes at 0% then increases to the desired value at a rate of 2%/min. A sensor measures the effective RH near the sample with ±2% accuracy. T is controlled with a Peltier heating/cooling system. The DMA-RH system is fully programmable and results can be viewed in real-time and programs edited as needed during a run using the software listed below.

2.3.2. Sample Mounting

Films strips were carefully mounted into the Film Tension Clamp with the gauge-length set at 15 mm. Strips were checked for accurate vertical positioning and the absence of slack. The DMA-RH chamber was bolted close before each test, except for “open” stress-strain tests.

2.3.3. Principles of DMA

During a test in sinusoidal mode, the input strain, the resulting stress, and the lag between the two (phase angle δ) are measured, and complex moduli are automatically calculated:

- Storage Modulus, E' (MPa): $E' = \text{stress/strain} \times \cos\delta$, is the ability of the material to store energy, and represents the elastic properties of the film (E' is similar to the stiffness);
- Loss Modulus, E'' (MPa): $E'' = \text{stress/strain} \times \sin\delta$, is the heat dissipated by the material as a result of molecular motions (damping), and represents the viscous properties of the film (similar to viscosity);
- Complex modulus: $E^* = E' + iE''$, is the material's resistance to deformation;
- $\text{Tan}\delta = E''/E'$, is the ratio of the dissipated energy to the energy stored per cycle of sample deformation and characterizes the material's damping properties. For purely elastic materials, $\text{Tan}\delta = 0$; for pure liquid, $\text{Tan}\delta = \infty$ (*i.e.*, $\delta = 90^\circ$); visco-elastic materials have intermediate values.

2.3.4. Stress-Strain Measurements in Transient Mode

In transient mode, strips were mounted as above, equilibrated at the desired T and RH for 2–4 h or the current ambient conditions for at least 1 h, then a static force increase of 1 N/min was applied until breaking. For each film, 5 or 6 strips were tested and the Young Modulus, Y (MPa), was taken as the initial slope of the stress-strain curve; the tensile strength, TS (MPa), was taken as the point of maximum stress on the curve; and the elongation at break, D (%), was taken as the strain when the film ruptures. Statistical analysis of the results was performed using ANOVA in Microsoft Excel 2010.

2.3.5. Iso-Strain Study of the Effects of T and RH in Dynamic Mode

The appropriate oscillatory parameters (amplitude and frequency) for thin casein films were first defined according to traditional DMA methodology, then kept constant during the entire study:

- The linear response region for frequency was determined using iso-strain frequency-sweeps at 20 °C and 50% RH at different strain values. A frequency of 4 Hz, which was consistently in the middle of the linear region for E' and E'' independently of strain, was selected;
- Strain-sweeps were performed at 20 °C and 50% RH at different frequencies to determine the lower and upper bounds of the films' linear stress-strain regime. A strain of 0.1%, near the lower-bound of the linear region, was selected;
- A pre-load force of 0.1 N was applied and the “force track” option selected to prevent any slack in the films strips during oscillations.

All the T - and RH-dependent DMA-RH programs were performed at 4 Hz and 0.1% strain, with a pre-load force of 0.1 N and $15 \times 5 \times 0.03$ – 0.05 mm^3 film strips. The different programs written comprised:

- Temperature ramps (T -ramp) at constant RH: isohume T sweep consisted of 1–6 h equilibration at 0%, 10%, 20%, 30%, 40%, 50%, 60% or 70% RH and 5 °C, followed by slow heating of the film at a rate of 0.33 °C /min up to 90 °C. T -ramps were designed to fit within 8 h when possible;
- Relative humidity ramps (RH-ramp) at constant T : isothermal RH sweeps consisted of 6 h equilibration at 20 °C and 20% RH, followed by a series of miniature RH steps of 0.5% every 4 min (or, 0.125%/min), up to 80% RH. RH Sweeps were designed to fit within a 16 h overnight shift;
- Temperature steps (T -steps) at 50% RH: after 6 h equilibration at 20 °C and 50% RH, T was changed to different values and held for 3 or 4 h at 50% RH with each step;
- Humidity steps (RH-steps) at constant T : isothermal RH steps consisted of 2–12 h equilibration at 20 °C or 40 °C and 0%–50% RH, followed by quick RH-steps (2%/min) of different amplitude and length. T -steps and RH-steps were designed to observe moisture-sorption kinetics during each step and to obtain equilibrium values of the dynamic mechanical properties at various sets of (T , RH);
- Instant RH-steps: humidity steps using a sample equilibrated at ambient RH (RH_a) were performed by leaving the DMA-RH opened and deflecting the N₂ inlet gas from the sample while the inlet RH increased to the target value (RH_1); when the inlet RH reached RH_1 , the chamber was closed and RH around the sample rapidly jumped from RH_a to RH_1 .

During each program listed above, the following values were recorded every 1 s (ramps) or 10 s (steps) and monitored in real-time: E' , E'' and $\tan\delta$; T and sample RH; and clamp displacement d (μm). The displacement was later converted to % elongation (or swelling/shrinkage) by dividing d with the initial sample length, as measured at the end of the equilibration period.

All the DMA-RH programs were written, performed, and recorded with the Advantage for Q Series Version software (Thermal Advantage Release 5.2.6). Stress-strain data and dynamic mechanical data were plotted and analyzed using the Universal Analysis 2000 (Version 4.7A) software (both from TA Instruments-Waters LLC, New Castle, PA, USA). Figures were designed using Universal Analysis 2000, as well as Microsoft Excel and Powerpoint 2010.

3. Results and Discussion

3.1. Stress-Strain Tests

3.1.1. Open Tests

Transient stress-strain tests at ambient RH and T were performed by letting samples equilibrate in the laboratory for at least 30 min, then leaving the test chamber opened and deflecting the continuous flow of dry N₂ away from the sample by inserting a small cardboard shield in front of the N₂ inlet. RH values with a stability of $\pm 2\%$ were obtained around the tensile clamp, and the daily RH ranged from 10% to 60% over the course of a year, with $T = 20$ – 23 °C. The averaged tensile strength (TS) and Young's Modulus (Y) as a function of ambient RH are reported in Figure 1.

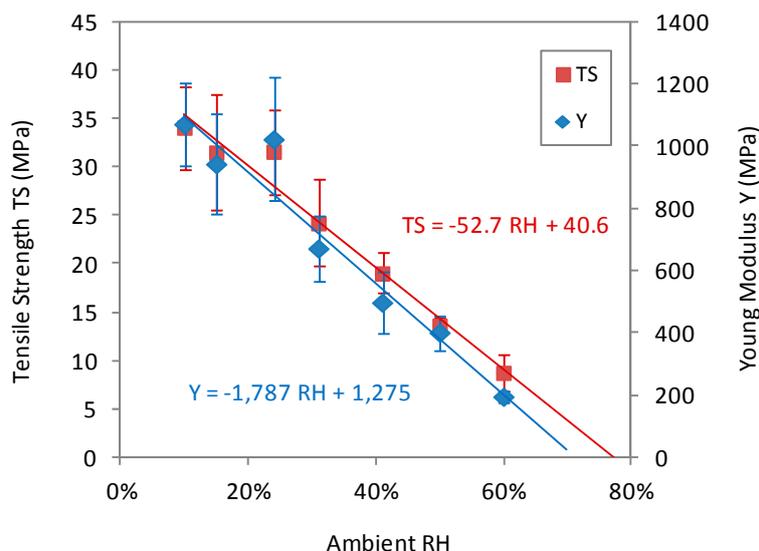


Figure 1. Tensile properties of ~ 0.04 mm CaCas/Gly films measured in transient stress-strain mode with the DMA chamber opened to ambient conditions (~ 20 °C and varying RH depending on the day). Samples were stored at 60% RH for 5–12 days before testing.

At the extension rate of 1 N/min, CaCas/Gly films appeared strong and stiff and highly sensitive to ambient moisture. TS and Y decreased linearly when RH increased and ambient moisture plasticized the films, comparable to the results of Bonnaillie *et al.* [3]. The elongation at break (D) was low and varied from $D \sim 8\%$ at 10% RH to $D \sim 27\%$ at 60% RH. Linear extrapolation of TS and Y to zero suggests the existence of a point of null strength for the films between 70% and 80% RH, where the protein network becomes “fluid”, similarly to a heat-induced melting point.

In normal conditions (50% RH), the films showed a high modulus of $Y = 400$ MPa, and a high strength of $TS = 13.6$ MPa, with a low elongation at break of $D \sim 20\%$. These results cannot readily be compared with literature data for other Cas/Gly films, as the sample geometry and extension rate greatly affect the tensile properties of viscoelastic materials [25]: TS and Y typically increase, while D decreases, with shorter and/or thinner film strips and higher extension rates, as in this study, compared to Bonnaillie *et al.* [3] and others [2].

For example, in prior works, Tomasula *et al.* reported values of $TS = 1.6, 1.9,$ or 7.0 MPa and $D = 67\%, 76\%,$ or 66% for CaCas/Gly films (2.33:1 ratio) of thicknesses 0.11, 0.15, or 0.10 mm, respectively, at 23 °C and 50% RH [26,27]; in two other studies, CaCas/Gly films (2:1 ratio), or beta-Cas/Gly films (also 2:1 ratio), with similar ~ 0.11 mm thickness, reportedly possessed $TS \sim 4.25$ MPa and $D \sim 1.45\%$ [28], or $TS \sim 6.0$ MPa and $D \sim 274\%$ [7], respectively, also in normal environmental conditions. With a ratio of 4:1, slightly thinner (0.085 mm) NaCas/Gly films reportedly possessed $TS \sim 17\text{--}27$ MPa and $D \sim 10.5\%$ [29], also in normal conditions, which is more similar to the values obtained in this work.

Repeatability of the stress-strain tests was good at high RH (Figure 1). Data scattering increased at low RH when the films became brittle and tended to break early in the presence of any defect.

Opened stress–strain tests at ambient RH are quick and enable testing and comparing multiple film strips in a same day at approximately the same environmental conditions; this can be useful to screen different film compositions and formulations. Since CaCas films are highly moisture-sensitive,

small changes in RH would render difficult the observation of more subtle formulation- or composition-induced property-changes unless T and RH are carefully kept constant.

3.1.2. Closed Tests

Stress-strain tests were performed with the DMA-RH chamber closed to maintain both a constant RH and T . The selected set of (T , RH) may correspond to “normal” conditions (*i.e.*, ~ 23 °C and 50% RH), or may match any other desired value, such as the conditions during storage, to evaluate the mechanical properties of films in various storage conditions.

In a prefatory shelf-life study, CaCas/Gly films were stored at 20 °C and 60% RH for 21 days to evaluate the effect of high moisture on the integrity of the films with time. Beginning on day 7, films were removed from storage every few days to cut strips; each strip was then mounted onto the tensile clamp inside the DMA-RH chamber and allowed to re-equilibrate to 60% RH before performing stress-strain measurements (Figure 2). Because DMA-RH programs always begin at 0% RH, 30 min were necessary to reach 60% RH, during which the film strip stiffened and shrank due to drying. Lengthwise shrinkage was clearly observed through negative clamp “displacement” (d) readings on the DMA-RH control panel, which tracked the position of the mobile half of the tension clamp, in μm . A re-equilibration time of 1–2 h after reaching 60% RH was allotted for each strip tested. Film strips were considered equilibrated when d plateaued for at least 30 min. Because of the 1–2 h equilibration time needed, only 3–5 strips were tested on a given day.

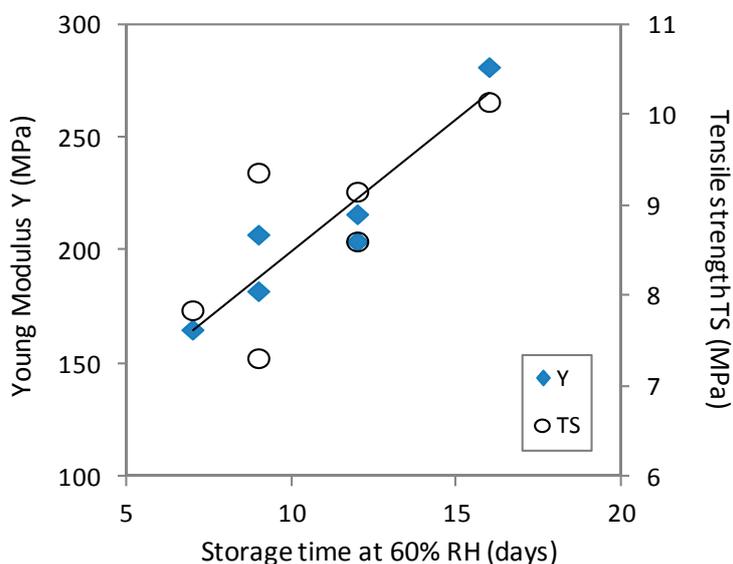


Figure 2. Isohume stress-strain tests of ~ 0.04 mm CaCas/Gly films in the closed DMA-RH chamber: TS and Y as a function of length-of-storage at 60% RH.

Figure 2 shows that the tensile strength and modulus of CaCas/Gly films stored and measured at 60% RH tended to increase with time from 7 to 16 days in storage, signifying that the films became stiffer and more brittle with time, hypothetically due to bacterial-degradation at the high water activity, since the films and environmental chamber were not sterile. At 21 days, films became too brittle to cut into strips and could not be tested, suggesting a shelf-life of less than three weeks for CaCas/Gly films under humid conditions.

According to these results, CaCas/Gly films were subsequently stored at 50% RH and 20 °C and tested within two weeks of manufacture. Fresh films were made periodically so the age of each film tested remained between 4 and 14 days at 50% RH.

3.2. Rate of Moisture-Sorption

Figure 3 presents the variations of strain for a CaCas/Gly film strip held statically during an instant RH-step from 30% RH (ambient) to 62% RH (N₂ inlet). The film strip was well equilibrated at 30.5% RH and the strain was plateauing near −0.8% for ~30 min. Once the DMA-RH chamber was closed at $t = 33.4$ min, moist N₂ flushed the atmosphere in approximately 2 min. The film strip began absorbing water immediately, as indicated by a sharp increase in strain at $t = 33.6$ min indicative of water-swelling. CaCas/Gly films are highly hygroscopic; swelling is not only rapid, but substantial: after 10 min, the sample gained 3.5% in length; after 1 h, the sample's length increased by 5.2%. Thermogravimetric moisture-sorption analyses will be necessary to determine the water-sorption kinetics of casein films as a function of changing environmental conditions, as well as the equilibrium water content and stoichiometric water-to-casein ratio as a function of T and RH.

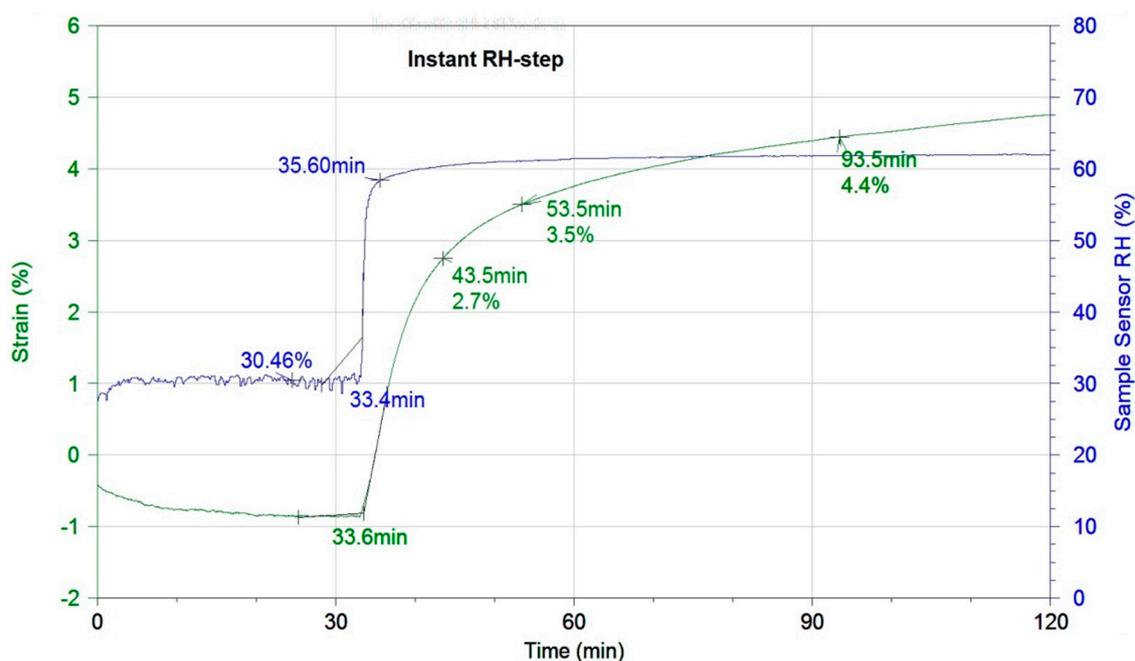


Figure 3. Instant RH-step at 20 °C, for a ~0.04 mm CaCas/Gly film stored at RH_s = 50%: step from RH_a = 30% to RH₁ = 62%. Legend: Blue = RH; Green = Strain% (sample elongation).

Figure 3 reveals that atmospheric humidity changes cause rapid and pronounced swelling of casein films, and thus have a strong effect on the moisture content of the films, and environmental conditions before and during mechanical analyses, therefore having critical effects on the results. For example, the length (and moisture content) of film strips changes if they are taken out of storage for only a few minutes to be tested under an ambient RH value (RH_a) different from the storage RH (RH_s). If RH_s and RH_a are different, casein-based films should be equilibrated for at least 1–2 h at RH_a (see next section) or the effective water content of the films will be unknown and somewhere between the equilibrium values at RH_s and RH_a.

3.3. Sample Equilibration at (T , RH) before Mechanical Testing

Because film strips typically shrank before the beginning of a test, a pre-conditioning step was added to every test-program to allow samples to equilibrate after closing the DMA-RH chamber. Figure 4 presents the typical pre-conditioning pattern for CaCas/Gly film strips. Shrinkage and swelling of the sample were monitored via strain changes as a function of time and RH around the sample. The sample was stored at $RH_s = 50\%$; ambient relative humidity was $RH_a = 23\%$; and target testing conditions were $RH_1 = 62\%$, all at $\sim 20^\circ\text{C}$.

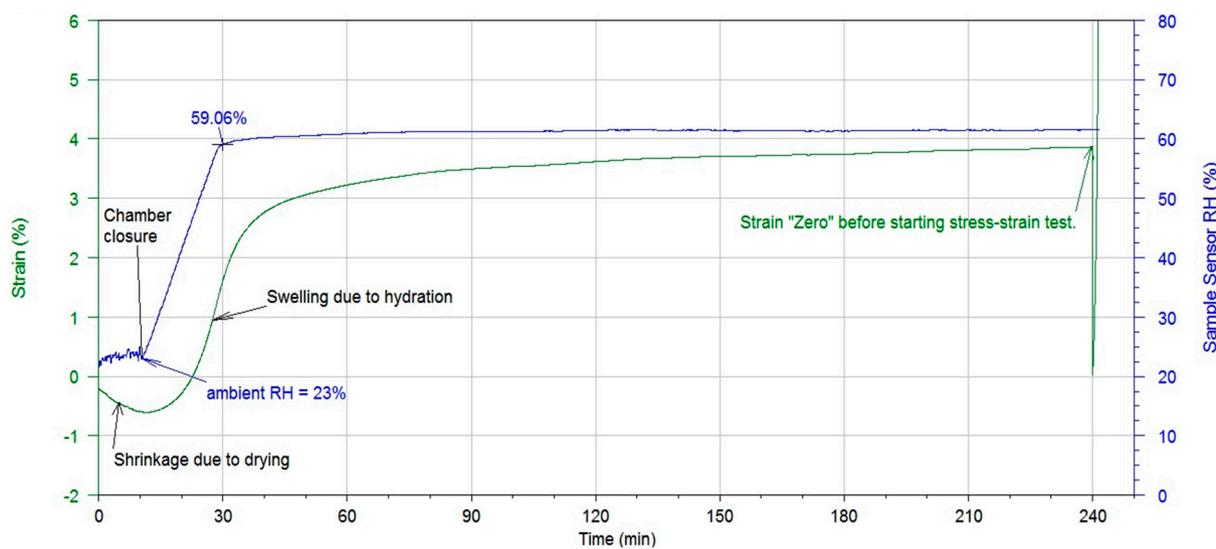


Figure 4. Pre-conditioning of a ~ 0.05 mm CaCas/Gly film sample inside the DMA-RH chamber: storage at $RH_s = 50\%$; ambient $RH_a = 23\%$; target $RH_1 = 60\%$. Legend: Blue = RH, Green = Strain% (sample elongation).

When $RH_a < RH_s$, film strips initially dried and shrank (negative Δ -strain) during and after installation on the tensile clamp (Figure 4, $t < 12$ min). The chamber was kept opened until $RH_1 = RH_a$ (23%) to minimize shrinkage while RH_1 automatically rose from 0% to 23%. After closing the chamber ($t = 12$ min), RH_1 increased to 59% at a rate of 2%/min and the sample quickly reverted to swelling (positive Δ -strain). Swelling was fast and near-linear while RH_1 increased linearly, and slowed when RH_1 stabilized. After equilibrium (Δ -strain ~ 0) is considered reached at $t = 240$ min, the sample was re-measured to initialize the strain and the test-program began.

All the film strips were pre-conditioned similarly. The time required to reach a strain (or displacement, d) plateau strongly depended on RH_1 and T and required from 30 min to >12 h. The time needed for equilibration increased when $|RH_1 - RH_s|$ increased, T decreased, or RH_1 decreased; in general, hydration of film strips was faster than drying.

The adequate equilibration time needed for each program type was determined and then kept constant for all samples, to minimize variations between samples.

3.4. Dynamic Isohume Temperature Ramps

Film strips were pre-conditioned for 2 h inside the DMA-RH chamber, in dynamic (oscillating) mode, to equilibrate at 5°C and 50% RH. At $t = 120$ min, T was ramped slowly and linearly to 90°C while

RH was automatically maintained at 50%. Because the humidity, $H(T)$, increases exponentially with T at constant RH, the flow rates of wet and dry N_2 were constantly adjusted by the software, causing small undulations of RH with time with an amplitude of $\pm 0.5\%$ that translated into small undulations of other signals monitored: the clamp displacement (d), storage modulus (E') and loss modulus (E''), as seen on Figure 5.

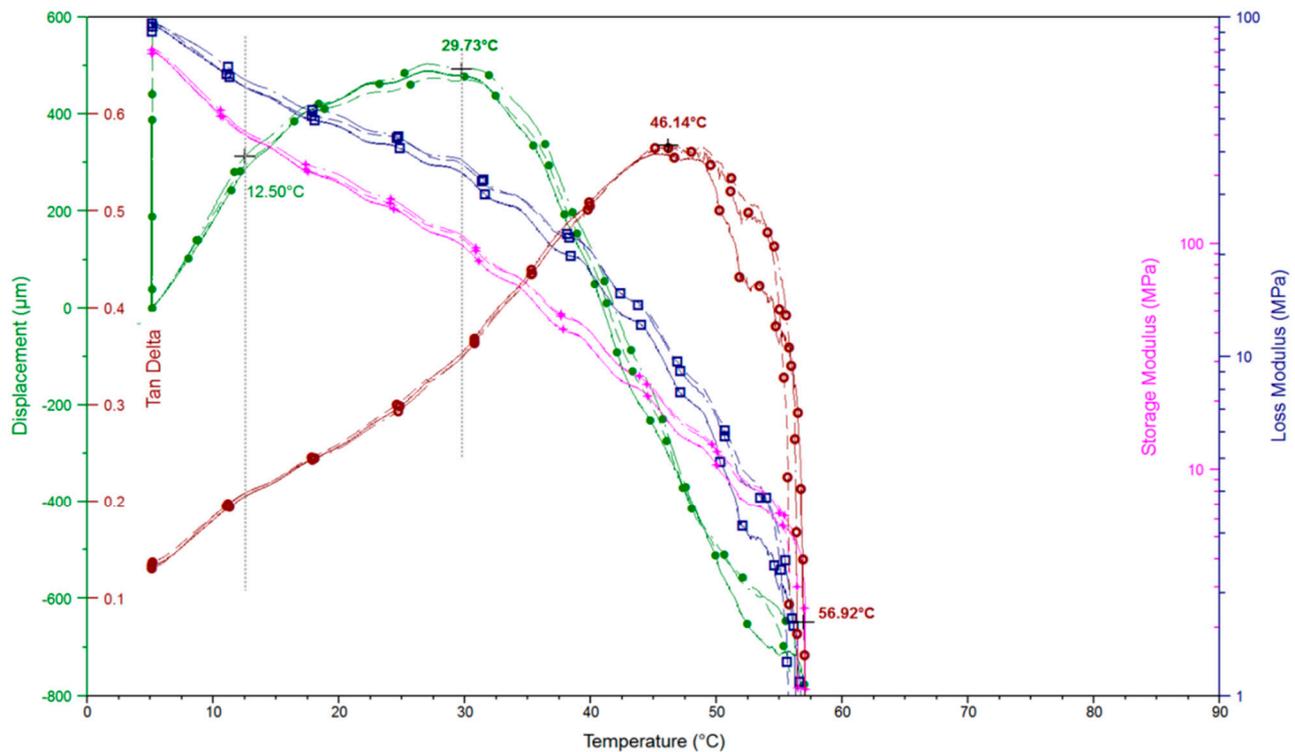


Figure 5. Dynamic temperature ramp, from 5 to 90 °C, at 50% RH: independent triplicate using three strips from the same CaCas/Gly film (~ 0.04 mm), tested on three consecutive days. Legend: green (\bullet) = clamp displacement (d); pink (+) = storage modulus (E'); dark blue (\square) = loss modulus (E''); brown (\circ) = Tan Delta (δ).

Triplicate measurements of the same film on three consecutive days showed an excellent repeatability (Figure 5). After equilibration at 5 °C and 50% RH, the samples' dynamic properties were: $\text{Tan}\delta = 0.1335 \pm 0.0029$, $E' = 711 \pm 11$ MPa and $E'' = 95.8 \pm 1$ MPa. When T increased, the combined effects of heat and increased humidity $H(T)$ plasticized the CaCas/Gly network, which swelled, as indicated by the positive clamp displacement (d). E' and E'' dropped when T rose, signifying a loss of elasticity as well as a loss of resistance to plastic deformation, also demonstrated by the rise of $\text{Tan}\delta$ that is significant of a less crystalline and/or a more rubbery network.

While a traditional glass-transition (T_g) is not observed under these test conditions, different slope changes indicate intermediate transition temperatures (T_i) that may possibly correspond to changes in the conformation of casein proteins; or, reconfiguration of the casein-glycerol-water network as more water molecules are absorbed and heat further enhances molecular mobility; or, even the loss of certain bonds between the casein proteins as water expands the network and heat activates a wide variety of chain-motions. The first change of slope was consistently observed around $T_1 = 12.5$ °C at 50% RH in the E' , $\text{Tan}\delta$ and d curves, corresponding to a minor transition of the CaCas/Gly network.

Around $T_2 = 30$ °C, the rise of $\text{Tan}\delta$ and drop of E' and E'' accelerated, while the sample length abruptly shifted from swelling to shrinking. This surprising phenomenon was observed consistently; as more water and heat were added into the film strip and the casein network continued to soften, we expected that the film's expansion would also accelerate due to swelling and the gentle tugging of the dynamic oscillations. Two possible explanations for the opposite, shrinking behavior observed, may be that: (a) the network has become sufficiently liquid-like for surface-tension forces to cause the softened film to contract with each dynamic oscillation, in order to minimize the surface-area of the sample; or (b) the casein proteins may be adopting more tightly-coiled configurations.

The peak of $\text{Tan}\delta$ around 46 °C was adopted as the traditional “melting temperature”, T_m .

While the T -ramp was programed to continue until 90 °C, CaCas/Gly films typically broke between 40 and 60 °C at 50% RH. The knowledge of the mechanical behavioral changes of CaCas/Gly films as a function of T in humid conditions is useful to investigate potential food packaging applications; for example, at 50% RH, the films are most soft and pliable around 30–40 °C and could possibly be shrink-wrapped, but lose their structural integrity and may possibly become sticky above 40 °C; thus, CaCas/Gly films may not be suited for manufacture, handling, storage and utilization in humid climates where T routinely exceed 40 °C.

Using a new batch of film, a series of isohume T -ramps was performed on the entire range of RH available (0%, 10%, 20%, 40%, 50%, 60% and 70%) to map the thermo-mechanical properties of CaCas/Gly films as a function of both T and RH. Figure 6 presents the storage modulus, E' , and the sample-length variation (swelling/stretching and shrinkage), D , during seven different isohume T -ramps.

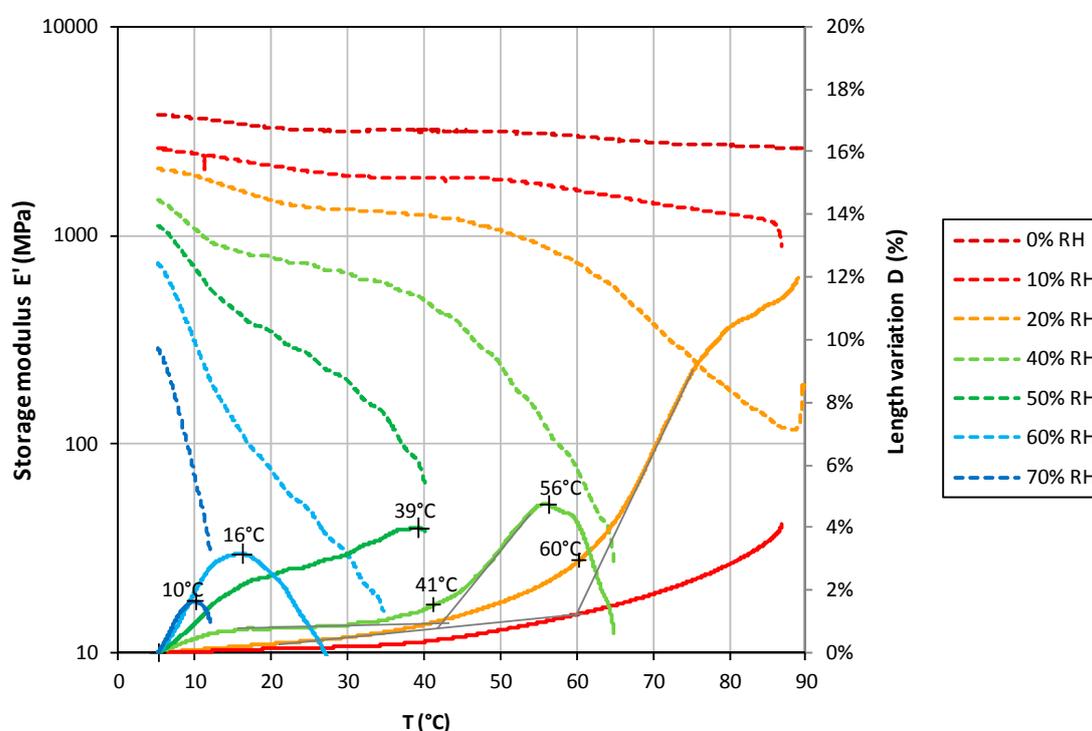


Figure 6. Storage modulus (E') and variation in sample length (D) vs. T , at 0%–70% RH, for 0.04–0.05 mm CaCas/Gly films. The dashed line represent E' ; solid lines represent D .

Similarly to Figure 5, E' dropped and samples initially swelled when T (and $H(T)$) increased. After pre-conditioning at 5 °C for 1–6 h (depending on RH), the initial value of E' became considerably

lower as RH increased. At a given constant temperature greater than T_g , which is the case here, E' of a polymer is proportional to its degree of crystallinity [30], thus an increasing moisture content in the film was assumed to render the CaCas/Gly/water network increasingly amorphous. In this series of T -ramps, several samples broke immediately above the melting point indicated by the peak of $\text{Tan}\delta$; thus, T_m was considered the temperature at break. Table 1 lists T_m as well as several secondary transition temperatures (T_1 , T_2 , T_3) as a function of RH, and the values for E' (RH) at 20 °C.

Table 1. Transition temperatures of CaCas/Gly films during isohume T -ramps at 0%–70% RH. Data extracted from the E' curves (E') or D curves (D) of Figure 6.

RH	1 st Inflexion Point (Reduced Slope) T_1	2 nd Inflexion Point (Increased Slope) T_2	Sample Shrinkage, T_3	Melting Point, T_m	E' at 20 °C (MPa)
0%	~25 °C (E')	~50 °C (E')	–	>90 °C	3,277
10%	~35 °C (E')	~50 °C (E')	–	>90 °C	2,150
20%	~22 °C (E')	~42 °C (E'), 60 °C (D)	–	>90 °C	1,450
40%	12.5 °C (E' and D)	41 °C (E' and D)	56 °C (D)	65 °C	780
50%	12.5 °C (E' and D)	–	39 °C (D)	40 °C	344
60%	12.5 °C (E')	–	16 °C (D)	34 °C	75
70%	–	–	10 °C (D)	12 °C	–

The strength of CaCas/Gly films dropped extremely rapidly at 70% RH, with a low T_m of 12 °C. The films were less and less affected by T as RH decreased, with slower drops in E' and higher T_m values. At 60% RH and lower, three types of secondary transitions temperatures (T_i) were observed during T -ramps:

1. Around $T_1 = 12.5$ °C, the rapid, linear drop of E' (and linear rise of D) slowed, indicated by a reduced slope (or even a plateau) for both E' and D , similar to a rubbery plateau. T_1 increased to 22–35 °C below 20% RH;
2. At low RH ($\leq 40\%$), T_2 marks an inflexion point where the slope of the E' and/or D curves increased, signifying a greater effect of T (and $H(T)$) on the structure and integrity of the casein network;
3. At high RH ($\geq 40\%$), samples reverted from swelling to shrinking around T_3 .

The four transition temperatures generally decreased with higher RH: the plasticization and microstructure of the CaCas/Gly network being a function of water content and heat-induced molecular mobility, greater water contents in the films may diminish the amount of heat needed to trigger the different transitions.

According to Table 1 and Figure 6, CaCas/Gly films have rubbery-plateau-type behavior and may be useful for the manufacture and utilization of food packaging between conditions of ~12.5–40 °C and 10%–50% RH, which provide a combination of strength, pliability, and low sensitivity to T changes (up to 40 °C). At lower T and RH, the films may be too crystalline and brittle for handling and folding, but may be stored for prolonged shelf-life; on the other hand, excessive T and RH may compromise the strength and integrity of the casein network and impair use as well as shorten the shelf-life (Section 3.1.2).

3.5. Dynamic Isothermal Humidity Ramps

Because T -ramps raise both the amount of heat provided to the sample and the amount of moisture, $H(T)$, in the atmosphere around the sample, isothermal RH-ramps were performed to isolate the effect of moisture on the dynamic mechanical properties of CaCas/Gly films at constant T .

After closing the DMA-RH, samples were equilibrated for 6 h at 20% RH and 20 °C; longer equilibration times were necessary than with T -ramps because the hygroscopic casein films tended to dry slowly at 20% RH.

Figure 7 presents the clamp displacement (d), storage modulus (E'), loss modulus (E'') and $\text{Tan}\delta$ for three RH-ramps of the same film, performed on three consecutive days.

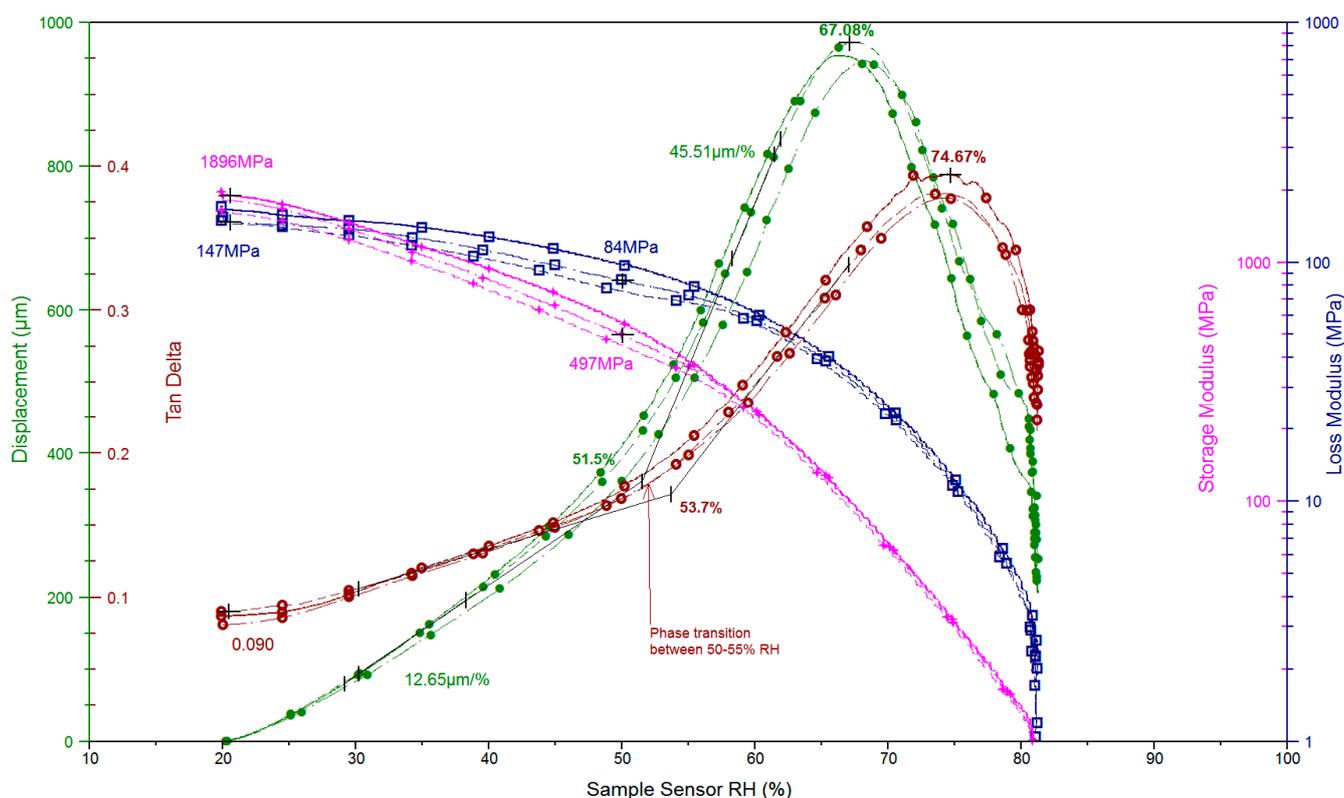


Figure 7. Isothermal RH-ramp on ~ 0.04 mm CaCas/Gly films after 6 h equilibration at 20 °C and 20% RH; triplicate performed after 4, 5 and 6 days in storage at 20 °C and 50% RH. Legend: green (\bullet) = d ; pink (+) = E' ; dark blue (\square) = E'' ; brown (\circ) = $\text{Tan}\delta$.

As RH slowly and linearly increased from 20% to 80%, d and $\text{Tan}\delta$ increased while E' and E'' dropped, all being synonymous to water absorption and plasticization of the casein network. Between 20% and 50% RH, $\text{Tan}\delta$ and the rate of swelling of the film, indicated by d , were proportional to RH, suggesting that the amount of water absorbed in the film is proportional to the amount of water present in the atmosphere around the film. Around 50%–55% RH, a first transition, indicated by an inflexion of all the curves, was observed: above 55% RH, the rise of $\text{Tan}\delta$ and the rate of swelling accelerated greatly, while E' and E'' began to plummet. The rate of linear swelling almost quadrupled, from 12.65 $\mu\text{m}/\%$ between 25% and 50% RH, to 45.51 $\mu\text{m}/\%$ between 55% and 65% RH. This first transition humidity, RH_1 , appears to correspond to a critical water-content inside the CaCas/Gly films

that triggered a reconfiguration within either the casein proteins themselves (e.g., loosening of the random-coil structure), or within the CaCas/Gly/water network, to form a looser structure that absorbs more water molecules per unit of water present in the atmosphere. Around RH₁, the casein proteins hypothetically uncoiled, or some of the hydrogen or ionic bonds within the network broke, resulting in the plummeting of E' and a loss of crystallinity of the network and accelerated rise of Tan δ . The inflexion point on the Tan δ curve (53.7%) is slightly higher than that on the displacement curve (51.5%), suggestive of a delay (similar to super-saturation) between the physical absorption of water molecules inside the film and the reconfiguration of the casein network once the critical water content has been reached.

A second transition humidity, RH₂, was observed around 67.0% when sample swelling sharply reverted to shrinkage as the moisture content of the films reached a critical fraction that enabled contraction of the network, possibly due to surface tension, or coiling of the caseins (similar to polymer annealing), as with T -ramps. A melting-point, RH_m, indicated by the peak of Tan δ , lastly occurred near 74.7% RH when the films began to behave more like liquids than solids. In a prior study, Mauer *et al.* found that glycerol migrated out of beta-Cas/Gly films around 76% RH [7].

Although the strength (E' and E'') of the film was very low at the end of each test, the three samples in Figure 7 continued to shrink and soften after reaching 81% RH but did not rupture.

For further study, the different critical moisture contents related to RH₁, RH₂ and RH_m, and the corresponding water-to-casein stoichiometric ratios, may be determined with thermo-gravimetric moisture-sorption analysis.

The repeatability of RH-ramps experiments was excellent for samples cut from the same film and tested on consecutive days using an identical pre-conditioning (equilibration) program (as in Figure 7). Repeatability was also good between samples cut from separate films of identical composition that were manufactured and then tested on different days. Table 2 lists the equilibration values for E' , E'' and Tan δ of eight independent samples, after pre-conditioning at 20% RH and 20 °C for 6 h and measuring the equilibrated length.

Table 2. E' , E'' and Tan δ of eight independent samples equilibrated at 20 °C and 20% RH for 6 h.

Sample #	E' (MPa)	E'' (MPa)	Tan δ
1	2,196	158.8	0.072
2	1,995	161.2	0.081
3	1,985	152.8	0.077
4	1,899	165.1	0.087
5	1,608	145.0	0.091
6	1,810	146.9	0.081
7	1,588	138.4	0.089
8	1,772	159.2	0.090
Average	1,857	153.4	0.083
Standard deviation	206	9.2	0.007
Error	±11.1%	±6.0%	±8.0%

In Table 2 and in general, possible sources of data scattering were identified as variations in the age of the films (from 3 days to 3 weeks), differing film thickness and crosslink density due to gradients

formed during drying, user-error (e.g., slack; poor vertical alignment) when mounting film strips, and structural defects in the strip (e.g., air bubble, rough edge, etc.).

3.6. Effects of Equilibration Time before T-Ramps and RH-Ramps

Equilibration of film strips to RH values lower than the storage RH (50%) required several hours, depending on the equilibration RH and T . Insufficient pre-conditioning times retained excessive moisture within the film samples, which corrupted the mechanical measurements. Figure 8 presents T -ramps at 30% RH performed with four different samples stored at 50% RH, then equilibrated at 5 °C and 30% RH for either 4 h or 6 h. While all four samples had similar initial E' and E'' values after pre-conditioning, the two samples equilibrated for only 4 h retained more moisture and possessed a less crystalline structure, as indicated by an artificially higher (~30% higher) initial value of $\text{Tan}\delta$. Additionally, when T increased, E' and E'' began decreasing, and $\text{Tan}\delta$ rising, at lower T and with a higher rate than for samples equilibrated for 6 h. Samples equilibrated for 6 h underwent a first transition around $T_1 \sim 59$ °C and did not break at 90 °C; the two samples equilibrated for 4 h had inflexion points of ~47 °C and ~51 °C, and broke at ~61 °C and ~70 °C, respectively, due to the excess moisture present within the film. Thus, it is critical to allow adequate equilibration time before beginning each test program.

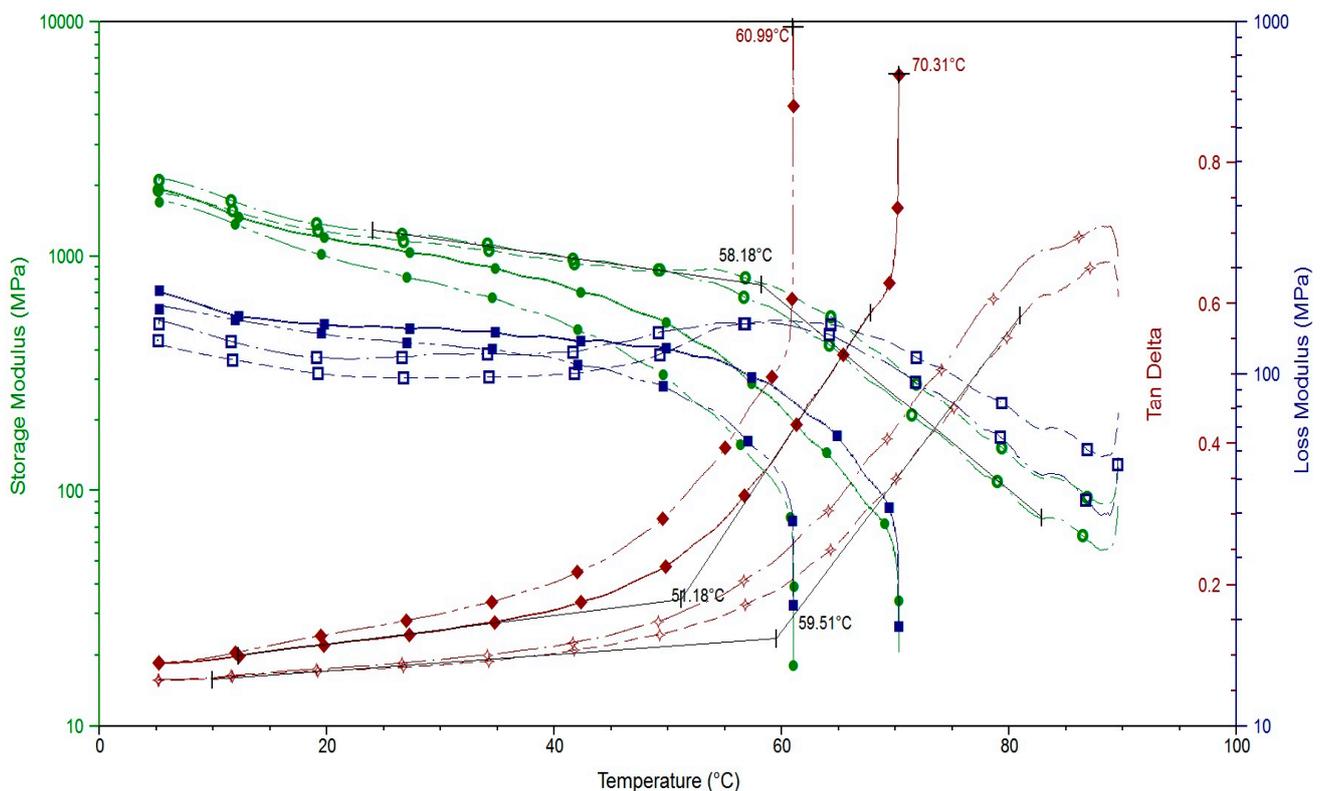


Figure 8. T -ramp duplicates at 30% RH for 0.04–0.05 mm CaCas/Gly films after pre-conditioning for four hours (green (●) = E' ; dark blue (■) = E'' ; brown (◆) = $\text{Tan}\delta$) or six hours (green (○) = E' ; dark blue (□) = E'' ; brown (◇) = $\text{Tan}\delta$).

3.7. Dynamic Humidity-Steps

At constant $T = 20\text{ }^{\circ}\text{C}$, samples were cycled from normal conditions (50%) to high ($\geq 60\%$) then low (20%–40%) RH for 3–6 h each, to simulate normal, humid and dry storage and utilization conditions that may be encountered by casein films. The rate of change and equilibrium values of the dynamic mechanical properties and sample length were studied for each RH-step. Figure 9 presents the storage modulus (E'), $\text{Tan}\delta$, and the sample length (L) as a function of time during a 23 h-long RH-steps program.

During each step, E' greatly dropped and $\text{Tan}\delta$ and L rose when RH increased, due to the absorption of plasticizing water molecules into the film. When RH decreased, water-desorption caused the opposite changes. Changes in E' , $\text{Tan}\delta$ and L occurred simultaneously to each RH steps and tapered within 1–10 min of RH stabilization, signifying that both water sorption and desorption into/from casein films were rapid between 20% and 61% RH. The inflexion point for each signal (E' , $\text{Tan}\delta$ and L) during each RH step was measured using the “onset point” function of the Universal Analysis 2000 software; an example is shown at 680 min for E' (Figure 9). The stabilization delays, calculated from: $\text{delay} = t(\text{inflexion point}) - t(\text{RH stabilization})$, are listed in Table 3.

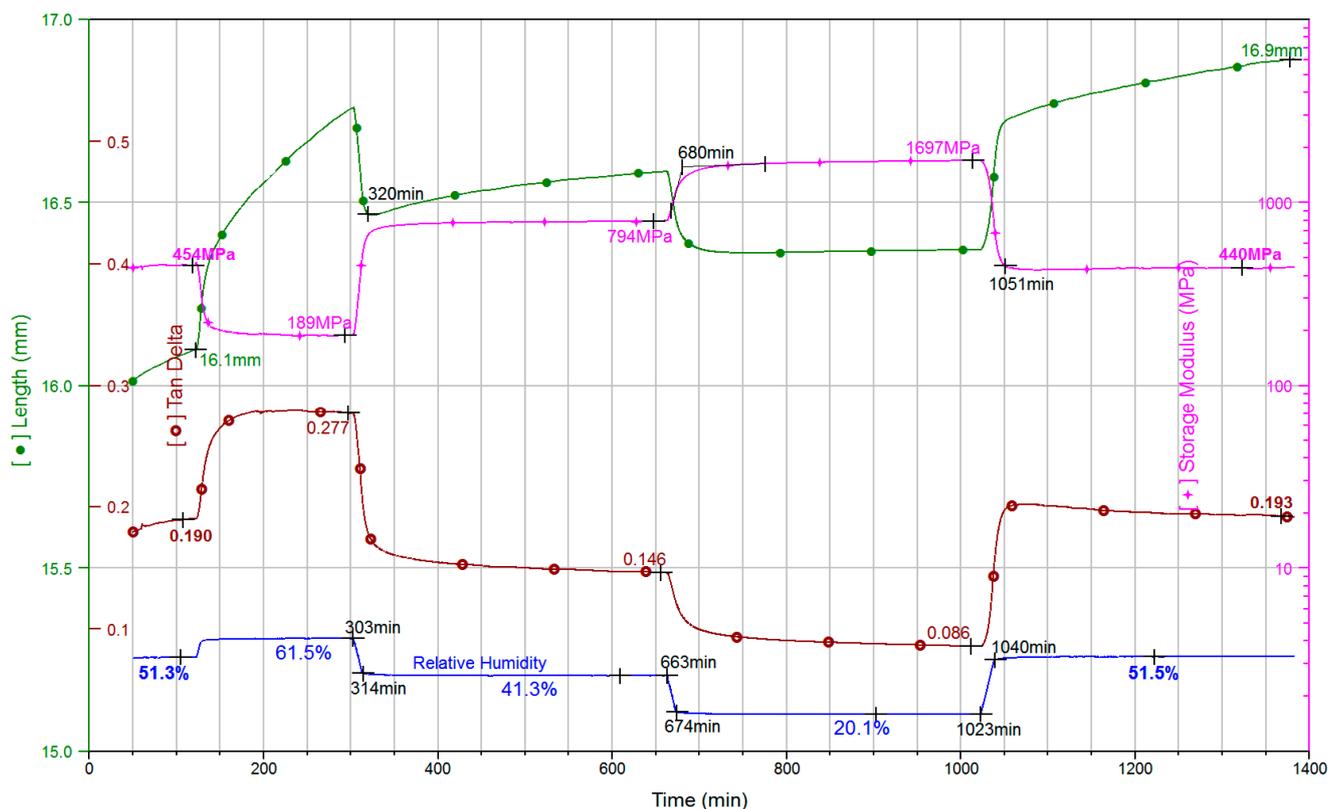


Figure 9. RH steps at 20 °C for 0.042 mm CaCas/Gly films: dynamic mechanical variations due to fast humidity changes between 20% and 61.5% RH. Legend: green (●) = sample length, L ; pink (+) = E' ; blue (-) = RH; brown (○) = $\text{Tan}\delta$.

Table 3. Delay (min) between RH-stabilization and inflexion of the E' , $\text{Tan}\delta$ and L curves during RH-steps of Figure 9.

RH Step	E' Delay	$\text{Tan}\delta$ Delay	L Delay
51.3% to 61.5%	4.9 min	10.3 min	9.0 min
61.5% to 41.3%	2.6 min	5.2 min	1.4 min
41.3% to 20.1%	6.0 min	8.7 min	5.5 min
20.1% to 51.5%	3.5 min	4.0 min	3.5 min

Equilibrium for E' was considered reached ~ 50 min after each RH step. However, $\text{Tan}\delta$ slowly and continually decreased after E' leveled off and did not reach a plateau after 6 h at 20%, 41% and 51% RH. This suggests a slow tightening phenomenon during which the casein network progressively adopted a more crystalline configuration with each new water-content.

In-between RH steps, the length of the film increased continuously, not due to swelling since H is constant, but because the sample was stretched by a fraction of 1 μm during each dynamic oscillation. The rate of stretching was minimal at 20% RH (0.03 $\mu\text{m}/\text{min}$) and increased greatly with RH as additional moisture increased the mobility of the network, with stretching rates of 0.22, 0.35, and 2.0 $\mu\text{m}/\text{min}$ at 41%, 51%, and 61% RH, respectively. As a result, L increased by $\sim 5\%$ during the whole test.

The values of E' and $\text{Tan}\delta$ after equilibration at 51.3% and 51.5% RH at the beginning and end of the test were almost identical: $E' = 454$ MPa and $\text{Tan}\delta = 0.190$ in the beginning; $E' = 440$ MPa and $\text{Tan}\delta = 0.193$ at the end; which means that although application of high and low humidity altered the water-content of CaCas/Gly films radically and changed their configuration, the change was fully reversible. Thus, storage or utilization of casein films under temporary abuse conditions (such as, high RH) or temporary uncontrolled atmosphere should not affect the mechanical properties of the films after normal storage conditions are restored.

The equilibrium values of E' and $\text{Tan}\delta$ obtained with the RH-steps program matched the transient values obtained with the RH-ramp program well (Table 4), suggesting a good repeatability and accuracy of both testing methods, with less than 11% difference between the two; thus, either method may be used, depending on whether discrete sorption kinetics and equilibrium data are desired, or near-equilibrium values and transition points on the entire RH-range are of interest.

Table 4. Comparison of values of E' and $\text{Tan}\delta$ obtained with the RH-ramp and RH-step methods of Figures 7 and 9.

T and RH conditions	RH-steps	RH-ramp	Difference
20 °C and 20% RH (6 h equilibration)	$E' = 1,697$ MPa, $\text{Tan}\delta = 0.086$	$E' = 1,896$ MPa, $\text{Tan}\delta = 0.090$	11%, 4.6%
20 °C and 51.5% RH	$E' = 440$ MPa, $\text{Tan}\delta = 0.193$ (6 h equilibration)	$E' = 454$ MPa, $\text{Tan}\delta = 0.177$ (no equilibration)	3.2%, 9%
20 °C and 61.5% RH	$E' = 189$ MPa, $\text{Tan}\delta = 0.277$ (3h equilibration)	$E' = 203$ MPa, $\text{Tan}\delta = 0.266$ (no equilibration)	7.4%, 4.1%

3.8. Dynamic Temperature-Steps

At constant 50% RH, the sample was cycled from normal conditions (20 °C) to refrigeration (5 °C) and warm climate (40 °C) to observe the effects of the combined variation of T and $H(T)$ on moisture sorption and mechanical properties (Figure 10).

Similarly to RH-steps, the mechanical changes caused by moisture desorption at 5 °C and moisture absorption at 40 °C were reversible, with near identical equilibrated values of E' and $\text{Tan}\delta$ at the beginning and end of the test program. The CaCas/Gly network seemed to adopt the same equilibrium configuration at 20 °C and 50% RH independently of its temperature history up to 40 °C.

When T was reduced (at $t = 180$ min, and $t = 910$ min in Figure 10) and $H(T)$ lowered exponentially, CaCas/Gly films instantly stiffened (E' increase and drop of $\text{Tan}\delta$) and shrank (L reduction). An overshoot of E' and undershoot of L and $\text{Tan}\delta$ occurred as the film cooled and water molecules desorbed from the casein network, hypothetically placing it into a temporary, stoichiometrically-unstable configuration. This was followed by a decrease of E' and increase of $\text{Tan}\delta$ and L after T and $H(T)$ stabilized and the network adopted a new equilibrium configuration, suggesting that the rates of cooling and water-desorption are faster than reconfiguration of the casein network. Inversely, when T and $H(T)$ were raised (at $t = 383$ min, and $t = 643$ min), the immediate water-sorption and warming of the network caused an undershoot of E' and overshoot of $\text{Tan}\delta$ and L , as the network became supersaturated with water and over-plasticized. Once T and $H(T)$ stabilized, the casein network rearranged into a more stable configuration involving the new water molecules, and E' increased while $\text{Tan}\delta$ and L decreased, before equilibration. Each T -step, from T_i to T_{i+1} , occurred within 19 min (15 °C-amplitude) or 23 min (20 °C-amplitude), whereas equilibration of the film's network at T_{i+1} required ~70 min, corresponding to a ~50 min delay caused by slow rearranging of the casein network to adapt to the new water content and the different chain-mobility imparted by T_{i+1} . This equilibration time is much shorter than that recommended in prior literature: the "Standard Test Method for Tensile Properties of Thin Plastic Sheeting" recommends an equilibration time of 40 h at 23 ± 2 °C and $50\% \pm 5\%$ RH before testing films (ASTM, [4]); while Mauer *et al.* found that β -casein films reached equilibrium relative humidity within 48 h inside an environmental chamber [7].

During each temperature plateau, the film length (L) increased continuously due to dynamic oscillations, as described in Section 3.8. The rate of stretching was minimal at 5 °C then increased greatly with T , as added heat and moisture increased the mobility of the network.

Around $t = 650$ min, the expected overshoot of L during the T -step from 20 °C to 40 °C, was interrupted and became a sharp and pronounced undershoot instead, signifying shrinkage of the film. It is hypothesized that, when T reached ~25 °C, "over-plasticizing" of the casein network reached the critical point that triggers film contraction, as described in Sections 3.4 and 3.5; the film's liquid-like behavior also appears in the jagged shape of the $\text{Tan}\delta$ peak between $t = 660$ and 670 min, and the very low minimum value of 7.2 MPa for E' .

As with RH-steps, the equilibrium values of E' and $\text{Tan}\delta$ at 50% RH and 20 °C obtained with the T -step program approximately match the results of the T -ramp program at 20 °C. Since equilibrium was reached in ~70 min with a T -step amplitude of 20 °C, more accurate T -ramps may be obtained by reducing the ramping rate to $20/70 = 0.28$ °C/min or less, instead of the 0.33 °C/min used in this work, to produce constant equilibrium condition.

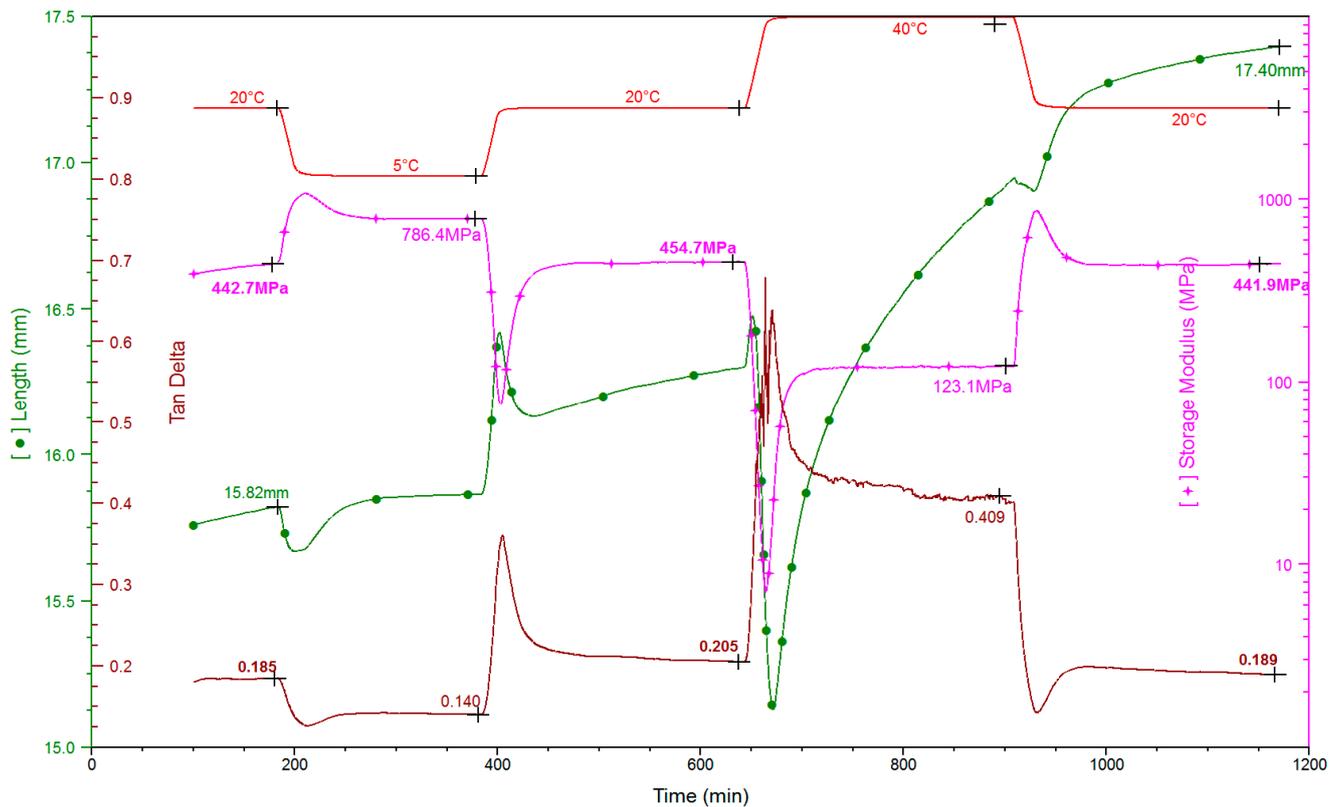


Figure 10. *T*-steps at 50% RH for a 0.042 mm CaCas/Gly film: dynamic mechanical variations due to fast *T* and *H*(*T*) changes between 5 and 40 °C. Legend: green (●) = *L*; pink (+) = *E'*; red (–) = *T*; brown (–) = *Tan*δ.

4. Conclusions

The DMA-RH technology permitted the analysis of the dynamic mechanical properties and certain physico-chemical behaviors of hydrophilic films as a function of temperature in isohume conditions, or between 0% and 80% relative humidity in isothermal conditions. A method developed to characterize CaCas/Gly films in this work showed excellent precision and repeatability and yielded different types of results useful to the design of edible food packaging. CaCas/Gly films appeared highly hygroscopic and extremely sensitive to both RH and *T* variations. Moisture-sorption and desorption are immediate and proportional to humidity around the film, therefore, pre-conditioning the films at the intended testing-RH prior to mechanical testing is critical, whether with DMA or texture analysis.

As the moisture content inside films increased with RH and/or *T* changes, the CaCas/Gly network underwent several secondary transitions, assumed to be rearrangements of the casein network and/or conformational changes of the casein proteins to accommodate extra water molecules, as well as a melting point. While moisture sorption is rapid, network reconfiguration required over an hour depending on RH, therefore sufficient pre-conditioning and equilibration times are important for accuracy. The different transition temperatures varied strongly with RH, as *T* and moisture appeared to act in synergy to plasticize the casein network. At 20 °C, CaCas/Gly films underwent a secondary transition near 50% RH that accelerated both moisture sorption and plasticization. Precise RH control is therefore critical during mechanical testing, particularly near 50% RH, to prevent large data scattering due to the film's different behaviors below and above this transition point. On the other

hand, changes to the CaCas/Gly network caused by moisture sorption between 5–40 °C and 20%–60% RH proved reversible.

One of the main limitations of the DMA-RH technology applied to hydrophilic films is the long time needed to pre-condition each sample and the long programs necessary to keep samples near a constant state of equilibrium, which generally limited data-collection to one sample a day.

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Author Contributions

Peggy Tomasula and Laetitia Bonnaille conceived and directed the research; Laetitia Bonnaille developed the DMA-RH theories and programs, analyzed and interpreted the data, and prepared the figures and the manuscript; Peggy Tomasula reviewed and approved the manuscript.

Conflicts of Interest

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

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