IMPACT OF CALCIUM ON VISCOELASTIC PROPERTIES OF FORTIFIED APPLE TISSUE

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ABSTRACT

The objective of this study was to analyze the linear viscoelastic behavior of calcium impregnated apples by creep and sinusoidal tests and to correlate the changes in rheological parameters with some structure features. The effect of different Ca concentrations of impregnation solutions (0, 0.1 and 0.53%, w/w), system pressure (vacuum or atmospheric) and process time was studied. All samples showed a viscoelastic solid behavior with the storage modulus (G′) dominating the viscoelastic response. Treated apple samples with or without calcium showed a pronounced decrease in G′ as compared with fresh tissue. Instantaneous compliance (J_0), decay compliances (J_1 and J_2) and fluidity significantly increased after treatments, while retardation times were approximately constant. Impregnation at high calcium concentrations provoked severe folding of walls and/or a general inner disruption of cells with plasmolysis and membrane breakage in a different way according to the pressure applied and the treatment time, explaining the viscoelastic behavior. Changes in compliances and storage modulus values were significantly lower for apples treated under vacuum than when the treatment was performed at atmospheric pressure, suggesting that vacuum impregnation is an effective methodology to fortify apple tissue avoiding serious damage in viscoelastic properties and presumably in texture.

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PRACTICAL APPLICATION

Incorporation of physiologically active food components (PACs) into vegetable matrices to obtain functional foods opens new product opportunities for the food industry. Impregnation processes performed at atmospheric pressure or under vacuum conditions may be employed to incorporate PACs, among them calcium. Because of alterations in structure, these processes can strongly modify rheological properties and hence texture of fruits. The results of this research can help to select operative impregnation conditions that allow the incorporation of great calcium quantities in apple tissue minimizing changes in viscoelastic properties. This objective is of primordial interest when designing minimally processed "fortified apples" preserved by refrigeration and to be consumed as is.

INTRODUCTION

Impregnation techniques of vegetables consist of immersing the cellular material in a suitable solution. According to the use, the solution may contain water activity (a_w) or pH depressors like sugars or salts, antimicrobials, etc. Recently, new formulations including minerals, vitamins or other physiologically active components have been proposed to develop functional foods (Ortiz *et al.* 2003; Alzamora *et al.* 2005; Anino *et al.* 2006).

When impregnation processes are carried out at atmospheric pressure (AI), plant cellular structure acts as a semipermeable membrane, and the solute is transferred from the concentrated solution to the cell by a process usually considered as diffusion driven. Vacuum impregnation (VI) consists of exchanging the internal gas or liquid occluded in open pores (intercellular spaces) of the material for the external liquid. This impregnation solution penetrates the pores by capillary action and by the pressure gradient imposed to the system through hydrodynamic mechanisms (Fito 1994). VI operation is restricted to porous products; short process times are used and it can involve volume change of solid, associated with the expansion, release and further compression of air inside the pores. The final concentration achieved in the matrix is often affected by the coupling of penetration–deformation phenomena, depending on the mechanical response of the material. During AI, a longer processing is required to reach the same solute incorporation, exposing the tissue structure to an eventual stress (plasmolysis, folding of cell walls, etc) due to an extensive contact to gradient solute concentration for long periods of time.

Mechanical properties play an important role in quality attributes of processed fruits and vegetables. Several physical, ultrastructural, microstruc-

tural and macrostructural modifications often occur during treatments due to the complex morphology of plant tissues, which affect mechanical properties. At the cellular and tissue levels, the three major structural elements that contribute to mechanical behavior of plant-based foods are the turgor pressure exerted on the cell membrane by intracellular fluids, the cell wall rigidity and the cell–cell adhesion, determined by the integrity of the middle lamella and the plasmodesmata (Jackman and Stanley 1995; Waldron *et al.* 1997; Alzamora *et al.* 2000; Oey *et al.* 2007). Calcium has been extensively used in low concentration as firming agent to improve postprocessing quality characteristics and extend shelf life of fruit products (Poovaiah 1986; Luna-Guzmán and Barret 2000; Martín-Diana *et al.* 2007). Resistance to softening resulting from calcium addition has been attributed to the stabilization of membrane systems and the formation of Ca-pectates by cross-linking free carboxyl groups on adjacent polygalacturonate chains present in the middle lamella, contributing to cell–cell adhesion and cohesion (Jackman and Stanley 1995). Recently, the interest in calcium-enriched fruits has intensified as a result of evidences linking osteoporosis, hypertension and cancer to calcium deficiency (Alzamora *et al.* 2005; Anino *et al.* 2006; Martín-Diana *et al.* 2007). These enriched products would help in incorporating more calcium in the usual diet, avoiding the consumption of dietary supplements (Salvatori *et al.* 2007).

Texture changes of products are often evaluated through mechanical measurements carried out with tests under large deformation conditions. Uniaxial compression is probably the most commonly employed because of their similarities with the mastication process. Although fruits are viscoelastic, they are often treated as elastic, so they are generally measured until rupture. The modulus of deformability, calculated in most of cases, is a measure of the product stiffness. It is the stress/strain ratio determined from the slope of the force–deformation curve before the elastic limit. Beyond this point, the force required to attain rupture can also be determined. The results of these tests can be correlated to changes in microstructure caused by compression. On the contrary, small deformation tests (linear viscoelasticity) are commonly performed in shear and allow the evaluation of viscoelastic properties with minimal physical damage to the sample. Therefore, dynamic rheology can be correlated to microstructure of the sample at rest (i.e., static microstructure) (Khan *et al.* 1997). Dynamic mechanical analyses (small-amplitude oscillatory tests) can be used to study the rheological properties of materials at different frequencies. In creep tests, the elastic, viscoelastic and viscous flow characteristics can be predicted separately. This way, more information for discrimination between samples is provided which allows researchers to do a better relationship between rheological parameters and sample's molecular structure (Alvarez *et al.* 1998; Gunasekaran and Mehmet 2000; Varela *et al.* 2007; Alzamora *et al.* 2008).

Literature reports about calcium incorporation to vegetable matrices and its effect on tissue texture and structure are extensive and quite diverse, depending on the calcium source and concentration, the application method and the type of produce (Abbot *et al.* 2000; Betoret *et al.* 2001; Gras *et al.* 2003; Alzamora *et al.* 2005; Aguayo *et al.* 2008). Therefore, no general conclusions can be derived. On the other hand, mechanical properties of calcium-containing vegetable tissues had been usually evaluated in terms of tests at large deformations (Martín-Diana *et al.* 2007). The objective of this study was to analyze the linear viscoelastic behavior of apple as determined by creep and sinusoidal tests during calcium impregnation at AI and under VI and to correlate the changes in rheological parameters with some structure features. The purpose is to present an integral approach for a better understanding of the mechanical behavior of apple material after incorporation of calcium by impregnation processes and its relationship to material structure and its organization.

MATERIALS AND METHODS

Sample Preparation and Treatments

Fresh apples (*Malus pumila*, Granny Smith var.) at similar ripening stage ($a_w = 0.98 \pm 0.01$; soluble solids content $\cong 12 \pm 1$°Brix; pH = 3.5 ± 0.1) were obtained from a local market and stored at 4C until use. Each fruit was hand peeled and cut parallel to the main axes using a lathe to obtain parenchyma slabs ($\cong 60$ mm × 60 mm × 6 mm).

Aqueous solutions containing calcium salts (food grade, Saporiti S.A., Argentina) and/or glucose (food grade, Refinerías de Maíz S.A., Argentina) were used as immersion media. Glucose concentration (12–12.5% w/w) was calculated for obtaining isotonic media regarding the content of apple native soluble solids to avoid water transfer mechanisms. A mixture of Ca lactate and Ca gluconate was chosen as calcium source because of its relatively high solubility at room temperature and the neutral taste imparted to the fruit (Anino *et al.* 2006). For calcium impregnation, isotonic glucose solutions were prepared with two calcium levels: 0.53% w/w Ca (5,266 μg/g calcium) in one case and 0.1% w/w Ca (994 μg/g calcium) in the other one. A third isotonic glucose solution without calcium salts was also prepared to assess the effect of only immersion treatment. Potassium sorbate (1,500 μg/g) was also added to the solutions and the pH was adjusted to 3.5 (natural pH of the fruit) by addition of citric acid to inhibit and/or retard microbial growth. The three sets of experiments (0, 0.1 and 0.53% w/w Ca) were carried out with apples from different lots.

Two impregnation techniques were carried out by immersing the cut apples in the impregnation medium with forced convection at room temperature under specific conditions of pressure: VI or atmospheric impregnation (AI). Fruit-to-solution ratio was 1:16 w/w. For VI, a vacuum pressure of 50 mmHg was applied to the system containing the apple slabs for 10 min and then AI was restored and maintained for 10 min. AI was conducted under conditions of internal control to mass transfer and fruit samples were taken out of the solutions at different immersion times (0, 2, 6, 10 and 22 h) (Anino *et al.* 2006). As immersion control, apple samples were processed in the same conditions as described above, at AI or under vacuum (V), but in the isotonic glucose solution without calcium salts.

After being treated, apple slabs were taken out of the solutions, examined for calcium concentration and viscoelastic characteristics and observed by light microscopy (LM) and transmission electron microscopy (TEM).

Determination of Calcium Content

After being desiccated in a vacuum oven at 60C, samples (two replicates for each treatment) were divided into two parts and calcium content was analyzed. Each half sample was digested with nitric acid (Merck, Darmstadt, Germany) in Parr bombs (Parr Instrument Company, Moline, IL) and a microwave oven. Then, 2–3 aliquots of each digested sample were measured by atomic absorption spectrometry, with $LaCl_3$ (6,500 mg/g) (Merck) as interference suppressor, using a Varian Spectrophotometer SpectrAA-20, model (Mulgrave, Victoria, Australia), air-acetylene flame, 0.5 nm slit and 422.7 nm wavelength (AOAC 2000). The reference material RM 8435 (whole milk powder) was also subjected to identical treatment to verify the accuracy of the analytical procedures. Triplicates were run with each set of apple samples and reference material. Values within 5% of the certified value for reference material and 55% relative standard deviation for apple samples were required for acceptance of the data. Mean values of calcium content (expressed as total content of wet apple) were reported.

Determination of pH, A_w and Soluble Solids

The a_w was measured at 25C with a psychrometer (Aqua-Lab CX-2, Decagon Devices Inc., Pullman, WA), calibrated with nonsaturated NaCl aqueous solutions. Soluble solids content percent in the liquid phase was analyzed by measuring the refraction index in a refractometer (Atago, PR 101, Tokyo, Japan) at 25C. The pH was measured with a pH meter Mettler Toledo (model MP 220, Schwerzenbach, Switzerland). All measurements were made in triplicate and the average values were informed.

Measurement of Viscoelastic Properties

Fresh and treated apple slices (6 mm thick, 30 mm in diameter) were loaded between parallel plates (30 mm in diameter) of a Paar Physica CR300 rheometer (Anton Paar GmbH, Graz, Austria), using only as much compression as was necessary to provide maximum contact area and minimum slip.

Dynamic oscillatory tests were performed at 20C in the controlled strain mode. The linear viscoelastic range (LVR) was determined at an angular frequency (ω) of 10 s^{-1} by the strain dependence data of the storage modulus (G'). Storage (G') and loss (G'') modulus were obtained through the frequency sweep test (0.1–100 s^{-1}), using an amplitude of 0.05% (within the LVR previously established).

Creep-recovery tests were conducted by applying a constant shear (35 Pa) during 60 s. After removal of the stress, sample recovery was registered for an additional period of 120 s. A previous stress sweep by varying the applied stress from 10 to 50 Pa indicated that in the selected condition, the deformation was proportional to the stress applied. Before the creep assay, the sample was subjected to repeat loading and unloading cycles in order the material loss the long time memory and to remove any surface irregularity in the specimen (Mittal and Mohsenin 1987).

Temperature was controlled by an external liquid bath thermostat model Viscotherm VT2 (Anton Paar GmbH). For both tests, data were obtained using a minimum of 12 replicates for each condition assayed.

Analysis of Viscoelastic Data

The LVR for amplitude sweep was determined with the Paar Physica U.S. 200 software package (Anton Paar GmbH). Variations with frequency of G' values were analyzed by using a bilogarithmic regression:

$$\log G' = k + n \log \omega \tag{1}$$

where n is the slope of regression and k is the origin ordinate.

Creep compliance data were fitted to a 6-element Burgers mechanical model consisting of a spring connected in series with two Kelvin–Voigt elements (each Kelvin–Voigt element has a spring and a dashpot in parallel) and a dashpot element, described by the equation:

$$J(t,\tau) = (J_0) + \sum_{i=1}^{2} (J_i)\left(1 - e^{-t/\lambda_i}\right) + \frac{t}{\eta_N} \tag{2}$$

where $J(t, \tau)$ is the creep compliance (= $\gamma(t)/\tau$ with $\gamma(t)$ being the strain at the time t and τ the constant stress applied), J_0 is the instantaneous compliance at

$t = 0$, J_i are the retarded compliances, λ_i ($= \eta_i \times J_i$) are the retardation times and η_i are the coefficients of viscosity associated with the Voigt elements, and η_N is the coefficient of viscosity associated with Newtonian flow and its inverse is the steady-state fluidity of the material.

Microscopic Observations

For LM of fresh and impregnated samples, cubes ($\cong$3 mm^3) taken from the internal zone of the sample were fixed in glutaraldehyde solution (3 g/100 g) and then in 0.1 M potassium phosphate buffer (pH = 7.4) overnight at room temperature. Cubes were then rinsed three times with distilled water, postfixed in OsO_4 solution (1.5 g/100 g) at room temperature and dehydrated in a graded acetone series prior to embedding in low-viscosity Spurr resin. Sections (1–2 μm thick) of the Spurr-embedded tissue were cut on a Sorvall MT2-B Ultracut microtome and stained with toluidine blue (1 g/100 g) and basic fuchsin (1 g/100 g) solutions and examined in a Axioscop 2 Plus light microscope (CARL ZEISS, Jena, Germany). For TEM, ultrathin sections were stained with uranyl acetate/lead citrate and examined with a Siemens Elmiskop I TEM at 80 kV (Siemens AG, Berlin, Germany).

All reagents were from Merck Química Argentina S.A. (Argentina).

Statistical Analysis

Statistical analyses were carried out using the STATGRAPHICS Plus package (StatPoint Technologies, Inc., Warrenton, VA). Differences between treatments were tested by analysis of variance, and intragroup comparisons were established by applying Tukey's multiple range test.

The STATGRAPHICS Plus software (StatPoint Technologies, Inc.) was also used for nonlinear and linear regression analyses and for creating an average data series (average best-fit parameters, with an asymptotic 95% confidence intervals for each of the unknown parameters) resulting from several experimental data series. The estimation method used to minimize residual sum of squares in the nonlinear regression was Marquardt.

RESULTS AND DISCUSSION

Calcium Concentration of Apples

Average calcium content of raw apple flesh was 22 μg/g. Figure 1 shows the incorporation of calcium into apple samples after VI and atmospheric impregnation. At AI, when the solution with the lowest calcium concentration was used, calcium content increased till 500 μg/g and 1,000 μg/g after 10 and

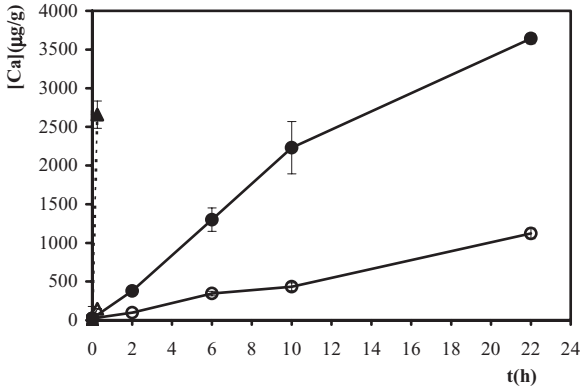

FIG. 1. CALCIUM CONCENTRATION OF APPLES AFTER IMPREGNATION TREATMENTS (AI AND VI) IN ISOTONIC GLUCOSE SOLUTIONS CONTAINING CALCIUM
● AI with 0.53% W/W CA; ○ AI with 0.1% W/W CA; ▲ VI with 0.53% W/W CA; Δ VI with 0.1% W/W CA

22 h of immersion, respectively. The employment of the solution with the highest calcium concentration considerably increased calcium uptake in the early stages of the process and then it continued increasing with time up to 3,500 μg/g at 22 h. A vacuum process in the 0.53% w/w calcium solution was approximately equivalent to a 12-h process at AI in the same solution regarding calcium incorporation.

As expected, no changes in physicochemical properties (pH, a_w and soluble solid content) were observed in samples after treatments. The impregnation processes did not provoke tissue dehydration or soluble solids content changes, as glucose solutions were approximately isotonic with respect to soluble solids of apples and calcium quantities incorporated into apple matrices, although significant from the point of view of fortification, were too low.

Viscoelastic Behavior

LVR was determined on fresh apple and on apple samples immersed in the different solutions from the strain sweeps in the region at which G' was independent of the strain amplitude. The LVR ranged between 0 and 0.102% in fresh apples and between 0 and 0.154% in treated ones. Therefore, a 0.05% strain value was selected to assure linearity during subsequent frequency sweep test. Immersion treatments caused significant changes in the mechanical spectra of apple tissue. Both storage (G') and loss (G'') moduli were reduced due to processing, which indicated that tissue became less viscous and less elastic after treatments. Average G' values for apple samples subjected to

TABLE 1.
DYNAMIC STORAGE MODULUS G' (kPA)* AT DIFFERENT ANGULAR FREQUENCIES (ω) AND PARAMETERS OF EQ. (1) FOR FRESH APPLE TISSUE AND APPLE TISSUE SUBJECTED TO IMMERSION PROCESSES IN ISOTONIC AQUEOUS GLUCOSE SOLUTIONS WITH AND WITHOUT CALCIUM SALTS AT ATMOSPHERIC PRESSURE OR UNDER VACUUM

Experimental conditions	$\omega = 100\ s^{-1}$	$\omega = 10\ s^{-1}$	$\omega = 1\ s^{-1}$	$\omega = 0.1\ s^{-1}$	n (Eq. 1)	k (Eq. 1)	R^2 (for Eq. 1)
Without Ca							
Raw tissue	268 ± 71^{a}	240 ± 68^{a}	213 ± 59^{a}	213 ± 58^{a}	0.033	5.302	0.947
AI, 2 h	191 ± 11^{b}	165 ± 11^{b}	150 ± 10^{b}	143 ± 9^{b}	0.041	5.183	0.965
AI, 6 h	102 ± 16^{c}	88 ± 14^{c}	79 ± 13^{c}	72 ± 12^{c}	0.050	4.900	0.990
AI, 10 h	91 ± 18^{c}	78 ± 15^{c}	69 ± 14^{c}	62 ± 12^{c}	0.055	4.843	0.996
AI, 22 h	93 ± 11^{c}	80 ± 10^{c}	74 ± 9^{c}	64 ± 8^{c}	0.053	4.856	0.997
VI	186 ± 42^{b}	159 ± 36^{b}	144 ± 33^{b}	139 ± 32^{b}	0.042	5.166	0.955
With 0.1% w/w Ca							
Raw tissue	259 ± 60^{a}	229 ± 50^{a}	210 ± 46^{a}	205 ± 47^{a}	0.035	5.329	0.958
AI, 2 h	163 ± 30^{c}	142 ± 27^{c}	130 ± 26^{b,c}	119 ± 22^{c}	0.038	5.127	0.951
AI, 6 h	143 ± 57^{c}	124 ± 49^{c}	112 ± 44^{c}	104 ± 40^{cd}	0.049	5.048	0.972
AI, 10 h	73 ± 18^{d}	63 ± 15^{d}	57 ± 13^{d}	54 ± 12^{e}	0.048	4.758	0.977
AI, 22 h	91 ± 22^{d}	79 ± 18^{d}	70 ± 17^{d}	73 ± 8de	0.048	4.852	0.921
VI	207 ± 50^{b}	178 ± 42^{b}	161 ± 38^{b}	73 ± 8^{b}	0.042	5.214	0.949
With 0.53% w/w Ca							
Raw tissue	332 ± 71^{a}	294 ± 59^{a}	270 ± 52^{a}	265 ± 48^{a}	0.034	5.442	0.924
AI, 2 h	134 ± 44^{b}	115 ± 38^{b}	104 ± 34^{b}	96 ± 31^{b}	0.048	5.020	0.987
AI, 6 h	80 ± 7^{cd}	69 ± 6^{c}	60 ± 5^{c}	55 ± 5^{c}	0.055	4.785	0.994
AI, 10 h	80 ± 12^{cd}	68 ± 10^{c}	59 ± 9^{c}	52 ± 8^{c}	0.063	4.773	0.998
AI, 22 h	74 ± 13^{d}	63 ± 10^{c}	55 ± 9^{c}	50 ± 8^{c}	0.058	4.744	0.996
VI	120 ± 9^{bc}	103 ± 7^{b}	92 ± 7^{b}	84 ± 7^{b}	0.050	4.968	0.991

For each experiment set, means in the same column with the same superscripts were not significantly different ($P < 0.05$).

* Values represent means and standard deviations of parameters (≥12 determinations).

immersion processes at different times of treatment and glucose aqueous solutions with different calcium content (0, 0.1 and 0.53% w/w) are summarized in Table 1. Treated apples showed a pronounced decrease ($P < 0.05$) in G' as compared with fresh tissue. At AI, G' diminished with time until a plateau was reached after 6 h treatment, when samples were immersed in the glucose solution without Ca (62–65% decrease) as well as in the 0.53% w/w Ca solution (76–78% decrease). Then, as immersion processes proceeded, G' values maintained constant. In the 0.1% w/w Ca solution, the decrease in G' value was significant until 10 h treatment (72%). Under vacuum, the effect of immersion treatments (solution with or without calcium) on G' was not so significant, being similar to the effect of 2 h atmospheric treatment in the 0 and

0.53% w/w calcium solutions. As G' is related to the rigidity of the tissues, it would be concluded that softening due to loss of tissue integrity took place due to immersion processes.

As also shown in Table 1, the G' modulus slightly increased with increasing angular frequency, with a greater slope of log G' – log ω lines (Eq. 1) for treated apples (n ranging between 0.038 and 0.063) than for raw samples. This fact would indicate more elasticity in the fresh fruits than in the treated ones. However, the very weak frequency dependence for both raw and treated tissues corresponded to a network-type microstructure, with strongly attractive interparticle forces and high particle concentrations (Khan *et al.* 1997). It is worth mentioning that, in spite of different apple lots used in experiments with different calcium concentrations, the frequency dependence of G' for fresh tissues was similar in all cases ($n = 0.033$–0.035).

The magnitudes of viscous modulus G'' decreased with a similar trend to G' values for apple tissues with the different treatments (data not shown). G'' values of fresh and processed tissues also varied with the frequency of oscillations. At low frequencies, the curves log G'' versus log ω exhibited a negative (nearly a plateau) slope, while at high frequencies, they showed a positive slope. The same frequency dependence pattern for G' and G'' has been reported for other authors in minimally processed apple and melon (Martínez *et al.* 2005; Martínez *et al.* 2007). G'' and G' magnitudes were used to calculate the loss tangent values (tan $\delta = G''/G'$) supplied in Table 2. It can be observed that all fruits assayed behaved as viscoelastic materials, with G' dominating the viscoelastic response over the whole frequency range ($G''/G' = 0.12$–0.20). Therefore, samples had clearly dominant solid characteristics. The small differences observed in tan δ values for the different treatments did not show any clear trend. This parameter was not sensitive for distinguishing physical differences between raw or processed apple tissues, or between immersion treatments.

Creep-recovery behavior was significantly affected by immersion treatments. The compliance versus time curves of fresh and treated fruit during the creep phase were well characterized (correlation coefficients ≥ 0.999) by the mathematical model represented by Eq. (2) and the corresponding rheological parameters are supplied in Table 3. The mechanical model applied provided excellent approximation of the creep data in the time range of the experiment. The rheological behavior of the apple tissue was defined in terms of four separate compliances. J_0 would be related to those bonds of structural units that are stretched elastically when the stress is applied, and show instantaneous and complete recovery when the stress is removed. J_i parameters would be related to bonds that break and reform at different rates, the weaker bonds breaking at smaller values of time than the stronger ones. They show retarded elastic recovery. The linear region of Newtonian compliance t/η_N would be

TABLE 2.
AVERAGE LOSS TANGENT (G''/G')* AT DIFFERENT ANGULAR FREQUENCIES (ω) FOR FRESH APPLE TISSUE AND APPLE TISSUE SUBJECTED TO IMMERSION PROCESSES IN ISOTONIC GLUCOSE SOLUTIONS WITH AND WITHOUT CALCIUM SALTS AT ATMOSPHERIC PRESSURE OR UNDER VACUUM

Experimental conditions	$\omega = 100\ s^{-1}$	$\omega = 10\ s^{-1}$	$\omega = 1\ s^{-1}$	$\omega = 0.1\ s^{-1}$
Without Ca				
Raw tissue	0.149 ± 0.02^{b}	0.14 ± 0.02^{ab}	0.138 ± 0.018^{b}	0.153 ± 0.016^{a}
AI, 2 h	0.146 ± 0.007^{bc}	0.128 ± 0.006^{bc}	0.135 ± 0.003^{b}	0.163 ± 0.007^{ab}
AI, 6 h	0.129 ± 0.001^{e}	0.113 ± 0.003^{c}	0.134 ± 0.003^{b}	0.176 ± 0.002^{bc}
AI, 10 h	0.139 ± 0.004^{bcd}	0.123 ± 0.003^{c}	0.134 ± 0.003^{b}	0.177 ± 0.011^{c}
AI, 22 h	0.133 ± 0.004^{de}	0.114 ± 0.002^{c}	0.132 ± 0.007^{b}	0.175 ± 0.008^{bc}
VI	0.165 ± 0.016^{a}	0.148 ± 0.017^{a}	0.154 ± 0.014^{a}	0.173 ± 0.007^{bc}
With 0.1% w/w Ca				
Raw tissue	0.141 ± 0.022^{a}	0.127 ± 0.020^{ab}	0.138 ± 0.019^{ab}	0.155 ± 0.024^{a}
AI, 2 h	0.148 ± 0.008^{ab}	0.130 ± 0.073^{bc}	0.138 ± 0.009^{ab}	0.176 ± 0.012^{ab}
AI, 6 h	0.139 ± 0.002^{a}	0.121 ± 0.003^{ab}	0.124 ± 0.001^{a}	0.159 ± 0.007^{ab}
AI, 10 h	0.138 ± 0.006^{a}	0.120 ± 0.008^{ab}	0.130 ± 0.007^{a}	0.173 ± 0.014^{b}
AI, 22 h	0.134 ± 0.03^{a}	0.116 ± 0.004^{a}	0.127 ± 0.008^{a}	0.163 ± 0.009^{b}
VI	0.158 ± 0.017^{b}	0.140 ± 0.015^{c}	0.147 ± 0.014^{b}	0.17 ± 0.02^{ab}
With 0.53% w/w Ca				
Raw tissue	0.156 ± 0.004^{a}	0.141 ± 0.012^{a}	0.147 ± 0.010^{a}	0.163 ± 0.014^{a}
AI, 2 h	0.142 ± 0.007^{b}	0.121 ± 0.007^{b}	0.129 ± 0.011^{b}	0.179 ± 0.016^{bc}
AI, 6 h	0.138 ± 0.004^{b}	0.119 ± 0.004^{b}	0.135 ± 0.008^{b}	0.189 ± 0.014^{c}
AI, 10 h	0.143 ± 0.002^{b}	0.125 ± 0.004^{b}	0.149 ± 0.009^{a}	0.204 ± 0.009^{d}
AI, 22 h	0.138 ± 0.003^{b}	0.119 ± 0.005^{b}	0.135 ± 0.007^{b}	0.183 ± 0.074^{bc}
VI	0.141 ± 0.005^{b}	0.122 ± 0.003^{b}	0.137 ± 0.001^{b}	0.175 ± 0.009^{b}

For each experiment set, means in the same column with the same superscripts were not significantly different ($P < 0.05$).
* Values represent means and standard deviations of parameters.

related to those bonds that are ruptured during the shear creep step and the time required for them to reform is longer than the creep recovery period; the released units will flow and part of the structure is not recovered. It is to be noted that one difficulty encountered in measuring and characterizing the physical properties of fresh or minimally processed plant tissues is that they are usually alive and respiring, and also can be dehydrated during the measurement, changing very rapidly and requiring tissue extension to be observed in short periods of time. So the interpretation of the creep behavior in this research corresponded to the time scale over which creep occurs (Alzamora *et al.* 2008). Another drawback of particular concern in creep experiments is the enormous variability within and between fruits. Fruit tissues are anisotropic and exhibit a distribution of rheologic properties, which also depend on the stage of development, agronomic practices and time of harvest in the field

(Petrell *et al.* 1979; Carpita and Gibeaut 1993; Alzamora *et al.* 2008) Therefore, relatively large standard deviations associated with creep data are not uncommon and a sufficient number of replicates is necessary to obtain an acceptable level of confidence in creep parameters determined by instrumental tests.

Figure 2 illustrated the adjustment of average compliance curves using the mechanical model represented by Eq. (2). Fresh tissues exhibited lower values of instantaneous compliance (J_0), retarded compliances (J_1, J_2) and fluidity ($1/\eta_N$) than those of treated samples. An increase of compliances with time (from 0 to 6 h) was observed in tissues immersed in the solution without Ca. No significant differences ($P < 0.05$) were obtained between compliance values of samples immersed for long periods of time (6–22 h). In general, a 0.1% w/w Ca concentration in the solution affected similarly the creep response, excepting for 10-h immersed samples, where significant greater compliances were observed. The treatment with the highest Ca concentration provoked great compliance values, with the same behavior along process time. Tissues subjected to vacuum treatment (with and without Ca) showed compliance values (J_0, J_1, J_2, $1/\eta_N$) similar to those exhibited by samples immersed for 2 h at AI.

Overall retardation times λ_1 and λ_2 (i.e., the time required for the strain on structural elements associated with viscoelastic behavior to reach 63% of their maximum strain) did not differ significantly between fresh and treated samples. These results indicate that the structural elements associated reached equilibrium at the same velocity in both fresh and treated tissues. There were no significant differences ($P < 0.05$) between steady-state viscous compliance ($1/\eta_N$) values of apple samples treated for different times, but these values were significantly greater than the one of raw tissue, that is, a higher tissue fluidity associated with a greater permanent strain was denoted after processing.

The overall compliance at the end of the creep phase showed a significant increase as immersion process proceeded from 0 to 6 h, mainly in 0.53% w/w Ca solution (see values in Table 3, calculated as percentage of the compliance for raw apple, ΔJ%). Overall compliances were related to calcium concentration: the greatest deformations were found for 0.53% w/w Ca treated apples, while compliances of apples immersed in the 0.1% w/w Ca solution were, in general, lower than those of samples immersed in the solution without calcium. For VI treatments, this increase was less pronounced (73, 227 and 82% for solutions without Ca, 0.53% w/w Ca and 0.1% w/w Ca, respectively). For fresh and processed apples, the relative contribution of each type of compliance to the overall compliance was in the range 48–67% for instantaneous elastic, 14–20% for the slow-rate viscoelastic, 13–23% for the fast-rate viscoelastic and 1–17% for the steady-state viscous compliance or plasticity

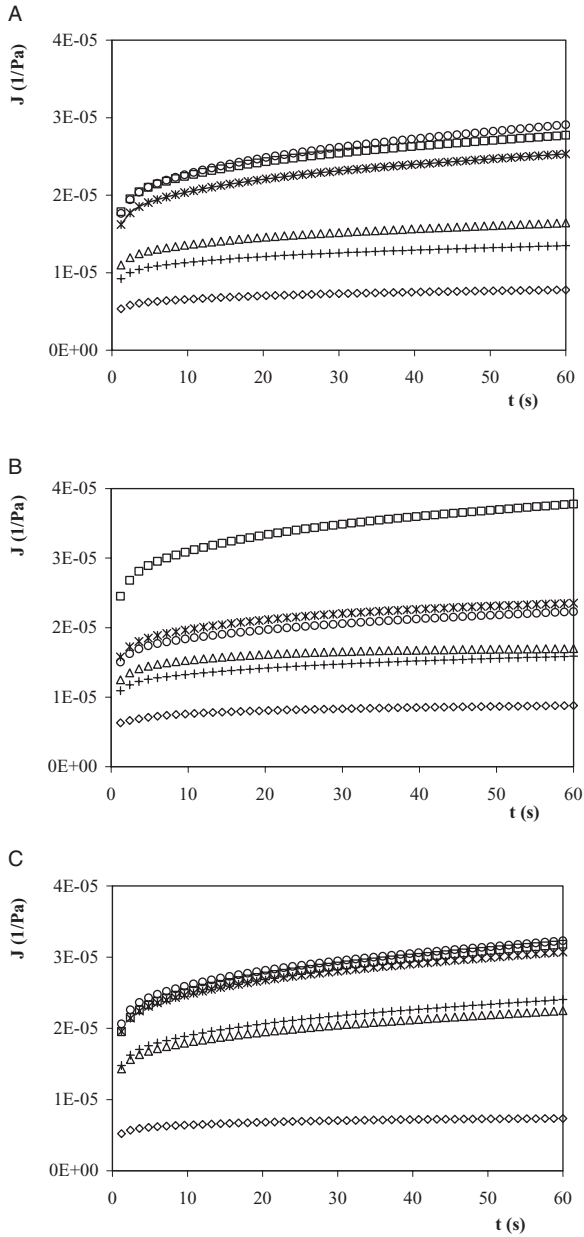

FIG. 2. CREEP CURVES PREDICTED BY USING EQ. (2) WITH VISCOELASTIC PARAMETERS FROM TABLE 3 FOR RAW AND TREATED APPLE TISSUES SUBJECTED TO IMMERSION PROCESSES AT ATMOSPHERIC PRESSURE OR UNDER VACUUM IN ISOTONIC GLUCOSE SOLUTIONS
(A) Without CA; (B) with 0.1% W/W CA; (C) with 0.53% W/W CA.

TABLE 3.
VISCOELASTIC PARAMETERS*, PLASTICITY (P)† AND TOTAL COMPLIANCE INCREASE (ΔJ)‡ FOR FRESH APPLE TISSUE AND APPLE TISSUE SUBJECTED TO IMMERSION PROCESSES IN ISOTONIC GLUCOSE SOLUTIONS WITH AND WITHOUT CALCIUM SALTS AT ATMOSPHERIC PRESSURE OR UNDER VACUUM

Experimental conditions	J_0 (1/Pa) ($\times 10^6$)	J_1 (1/Pa) ($\times 10^6$)	J_2 (1/Pa) ($\times 10^6$)	λ_1 (s)	λ_2 (s)	η_N (Pa s) ($\times 10^{-7}$)	P (%)*	ΔJ (%)
Without Ca								
Raw tissue	4.3 (0.3)^{a}	1.4 (0.17)^{a}	1.55 (0.15)^{a}	16 (3)^{a}	1.25 (0.10)^{a}	10 (3)^{a}	7.66	–
AI, 2 h	8.9 (1.2)^{b}	2.5 (0.4)^{b}	2.9 (0.4)^{b}	12.4 (0.4)^{a}	1.29 (0.06)^{a}	2.9 (0.3)^{b}	12.83	111
AI, 6 h	14.0 (0.7)^{c}	4.9 (0.3)^{c}	5.2 (0.3)^{c}	12.9 (0.3)^{a}	1.36 (0.05)^{a}	1.22 (0.11)^{b}	16.94	274
AI, 10 h	14.3 (1.4)^{c}	4.6 (0.4)^{cd}	5.1 (0.5)^{c}	12.9 (0.4)^{a}	1.31 (0.03)^{a}	1.59 (0.16)^{b}	13.66	256
AI, 22 h	13.0 (0.5)^{c}	4.1 (0.17)^{d}	4.56 (0.17)^{c}	13.0 (0.4)^{a}	1.30 (0.04)^{a}	1.64 (0.07)^{b}	14.43	226
VI	7.5 (0.8)^{b}	2.02 (0.16)^{a,b}	2.5 (0.2)^{b}	13.9 (1.2)^{a}	1.34 (0.07)^{a}	4.1 (0.6)^{b}	10.75	73
With 0.1% w/w Ca								
Raw tissue	5.9 (0.8)^{a}	1.3 (0.3)^{a}	1.1 (0.3)^{a}	16 (2)^{a}	3.2 (0.4)^{a}	11 (2)^{a}	6.35	–
AI, 2 h	10.5 (1.0)^{b,c}	3.1 (0.3)^{b,c}	3.1 (0.4)^{b,c}	14 (3)^{a}	1.5 (0.5)^{b}	2 (3)^{b}	13.91	122
AI, 6 h	12.8 (1.0)^{c}	3.6 (0.3)^{c,d}	3.8 (0.4)^{c,d}	19 (3)^{a}	1.6 (0.5)^{b}	3 (3)^{b}	9.99	155
AI, 10 h	20.0 (1.0)^{d}	6.5 (0.3)^{e}	7.0 (0.4)^{e}	14 (3)^{a}	1.5 (0.5)^{b}	1 (3)^{b}	11.64	332
AI, 22 h	12.8 (1.0)^{c}	4.5 (0.3)^{d}	4.6 (0.4)^{d}	16 (3)^{a}	1.4 (0.5)^{b}	3 (3)^{b}	7.54	169
VI	9.1 (1.0)^{b}	2.5 (0.3)^{b}	2.8 (0.4)^{b}	17 (3)^{a}	1.4 (0.5)^{b}	4 (3)^{a,b}	9.75	82
With 0.53% w/w Ca								
Raw tissue	4.1 (0.3)^{a}	1.52 (0.12)^{a}	1.70 (0.2)^{a}	19 (3)^{a}	1.31 (0.15)^{a}	60 (34)^{a}	1.35	–
AI, 2 h	11.8 (1.1)^{b}	3.5 (0.3)^{b}	3.8 (0.3)^{b}	13.1 (0.5)^{a}	1.4 (0.05)^{a}	1.73 (0.13)^{b}	15.42	206
AI, 6 h	16.3 (0.6)^{c}	5.2 (0.3)^{d}	6.1 (0.3)^{c}	12.8 (0.6)^{a}	1.27 (0.05)^{a}	1.27 (0.09)^{b}	14.69	340
AI, 10 h	15.2 (0.8)^{c}	5.3 (0.2)^{d}	6.2 (0.3)^{c}	13.1 (0.5)^{a}	1.30 (0.06)^{a}	1.17 (0.06)^{b}	16.11	333
AI, 22 h	15.6 (1.0)^{c}	4.9 (0.3)^{c,d}	5.8 (0.3)^{c}	12.9 (0.4)^{a}	1.33 (0.04)^{a}	1.32 (0.06)^{b}	14.80	318
VI	11.8 (1.0)^{b}	4.2 (0.5)^{b,c}	4.3 (0.3)^{b}	13.2 (0.5)^{a}	1.34 (0.05)^{a}	1.58 (0.14)^{b}	15.82	227

* Values represent means and standard errors (in brackets) of parameters derived by fitting Eq. 2 to average compliance curve from creep phase. Means in the same column with the same superscripts were not significantly different ($P < 0.05$).

† Values represent the contribution of the steady-state viscous compliance ($1/\eta_N$) to the overall compliance.

‡ Capacitance increase percentage relative to fresh tissue.

(% P). As can be concluded from the plasticity values (the ratio of unrecoverable or permanent deformation, t/η_N, to the total deformation, $J(t,\tau)$) shown in Table 3, all samples exhibited plastic strain which remained unrecovered in the creep recovery test. In general, AI processes in 0.1% w/w Ca concentration solution provoked a permanent deformation more reduced than that obtained when immersion was performed in the other solutions.

For a better characterization of the texture of fruit and vegetable tissues, it is essential to understand their mechanical behavior in terms of different rheological parameters, not only those obtained with small deformation measurements but also the ones obtained under large deformation conditions, which better correlate to changes in microstructure caused by deformation (Khan *et al.* 1997). In previous studies, a uniaxial compression test was performed in an Instron Machine on apple samples subjected to the same experimental conditions with 0.53% w/w Ca solutions (Anino *et al.* 2006). In these high deformations tests, a force was applied on the vertical axis to deform the sample at a constant speed and force–distance curves were recorded. From these curves, some parameters related to product firmness were obtained: values of force when tissue rupture was detected (F_{rup}) and modulus of deformability (E_d) up to 10% compression. Calcium impregnated tissues exhibited force–distance curves with some differences as compared with the one of the raw fruit, showing a decrease in the values of F_{rup} and E_d. A pronounced decrease in F_{rup} values was evident in calcium impregnated apple tissues (ΔF_{rup} 7% after 2 h, 47% after 6 h, 41% after 10 h and 34% after 22 h AI treatment; 41% after VI treatment) or in samples immersed in the solution without incorporation of Ca (18% after 2 h, 47% after 6 h, 63% after 10 h and 69% after 22 h A treatment; 14% after V treatment), which indicates a softening of the tissues after both AI and vacuum processes, with and without calcium.

Compliances and storage modulus values for apples impregnated with calcium under vacuum were significantly higher or lower, respectively, than those obtained for raw apple, although in a lesser extent than when the treatment was performed under AI for long periods of time. After VI in 0.53% w/w Ca solution, rheological behavior of the tissue (oscillatory and creep tests) was similar to that obtained after 2 h AI, even when calcium penetration was significant and equivalent to that achieved after 10 h AI process (~2,600 μg/g). Therefore, VI could be a good industrial choice to fortify apple, considering the texture maintenance and the short time periods of immersion.

Structural Features

The viscoelastic response was intended to correlate with tissue ultrastructure and microstructure. Microscopic observations of apples treated in 0.53% w/w Ca solution along atmospheric treatments and after vacuum process were

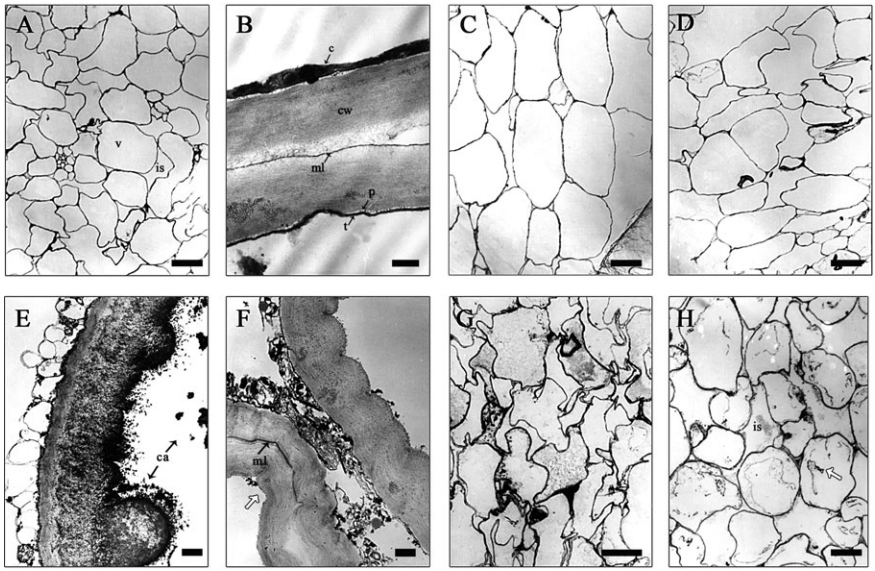

FIG. 3. LIGHT MICROSCOPY (LM) AND TRANSMISSION ELECTRON MICROSCOPY (TEM) MICROPHOTOGRAPHS ILLUSTRATING THE EFFECT OF EXPOSURE TO CALCIUM SALT SOLUTIONS (0.53% W/W CA) AFTER 6 H AT ATMOSPHERIC PRESSURE AND AFTER VACUUM PROCESS

A, C, D, G, H: LM; B, E, F: TEM. (A,B) Fresh apple; (C) in solution without Ca salts at atmospheric pressure; (E–G) AI in solutions with Ca salts; (D) in solutions without Ca salts under vacuum; (H) VI in solutions with CA salts. c, cytoplasm; cw, cell wall; v, vacuole; ml, middle lamella; p, plasmalemma; t, tonoplast; ca, calcium crystals. Scale: A, C, D, G, H: 50 μM; B, F: 500 nm; E: 1 μM.

performed by LM and TEM. Some micrographs are illustrated in Fig. 3. Immersion processes per se and calcium incorporation resulted in many structural changes. In the fresh tissue, cells showed central vacuoles, parietal cytoplasmic layers and darkly stained walls with a tight longitudinal fiber pattern (Fig. 3A). Arrangement of cells and intercellular spaces was inhomogeneous and anisotropic. Most cells exhibited a very neat middle lamella cementing adjacent cells (Fig. 3B). The 6-h immersion process per se affected the integrity of cell walls as seen in Fig. 3C. After 6 h treatment in the glucose solution without calcium, in spite of the fact that the cells appeared turgid, the lower electronic density of the walls would indicate solubilization and/or hydrolysis of pectin substances in the cell wall and the middle lamella. The extension of the reactions was greater as immersion time increased (micrographs not shown), during the early stages of immersion until 6 h, contributing to wall loosening and disintegration. Tissue behavior was different after calcium incorporation. After 6 h immersion in the glucose solution with 0.53%

w/w Ca, cells exhibited very darkly stained cell walls (Fig. 3G), with a middle lamella in some cells clearly reinforced, probably due to calcium–pectin interaction (Fig. 3F). In other cells, instead of a well-defined middle lamella, a high-density staining of central region of cell walls was noted (Fig. 3E). Therefore, the presence of calcium in the wall matrix would allow maintaining middle lamellae and/or pectin network integrity, as it promoted cross-linking of pectin polymers, as demonstrated by TEM and LM photographs. In addition, calcium would make pectin macromolecules of cell wall less soluble through the formation of bridges between them. The incorporation of calcium would also help in counteracting the solubilization and depolymerization of pectin of the wall occurring during immersion in the aqueous solution. Cell walls, in general, appeared with a very high electronic density, although with loose intermixed network pattern, very different from the longitudinal one of the fresh fruit. From these considerations, it would be expected that calcium impregnation increased the mechanical strength of the tissue compared with the immersion treatment in the solution without calcium. However, calcium penetration provoked other two notorious structural changes. One of them was a severe internal disruption in the cells. As seen in Fig. 3E, crystals of calcium salts appeared between the cell wall and the plasmalemma, detaching the cytoplasm and pushing it further into the cell. Not only did the cytoplasm appear separated from the wall, but in most cells, the membranes looked broken with vesicle formation (Fig. 3G). Deposition of calcium could be seen in the intercellular spaces, along the walls and in the lumen of the cells of impregnated apples (Fig. 3E). During the first hours, as calcium incorporation proceeded, the number of cells that lost turgidity due to breakage of membranes by calcium crystals increased (micrographs not shown). In second place, cells appeared very deformed with folded and angular walls (Fig. 3F,G). In spite of the clearly more stained cell walls in tissues fortified with calcium, this extensive folding (Fig. 3F) would indicate that wall networks of calcium-containing tissues had a mechanical resistance very much reduced as compared with that of raw fruit.

Observations by LM of samples immersed in isotonic solutions and subjected to vacuum showed a slight decrease in cell wall staining when compared with fresh apple (Fig. 3D). When the process was carried out in the solution containing calcium, walls looked more stained and cells seemed to be more rounded than in fresh apple (Fig. 3H). However, rupture of membranes or plasmolysis was also detected in most of cells.

Creep model parameters and G' values may thus be associated with structural components of the fruit tissue, reflecting changes that occur at cellular level. Various structural modifications may contribute to disassembly and loosening of primary cell walls and cell turgidity of fruits during processing affecting creep response and storage modulus: degree of tissue turgidity,

cellulose microfibrils slipping through the amorphous matrix of the wall, matrix flow, molecular regrouping of constituents (especially cellulose) or a combination of these (Alvarez *et al.* 1998; Alzamora *et al.* 2008).

The complexity of these changes has made it difficult to determine the significance of the individual structural elements on viscoelastic behavior. Jackman and Stanley (1995) proposed an interpretation of a similar 6-element creep model to analyze the multiple softening mechanisms in tomato pericarp tissue during ripening. This interpretation has been successfully used for explaining cooked potato and osmotically dehydrated melon and apple creep behavior (Alvarez *et al.* 1998; Martínez *et al.* 2005; Martínez *et al.* 2007). Instantaneous elastic compliance J_0 would be related to the combination of turgor and primary cell wall strength as dictated by cellulose. Viscoelastic compliances J_1 and J_2 could be attributed to time-dependent changes in pectins and hemicelluloses, respectively. Steady-state viscosity could be related to cell wall fluidity arising from exosmosis and/or solubilization and degradation of polymers and less water-binding capacity due to treatments.

On other hand, cellulose, the turgor pressure and the air occluded in the matrix have been suggested as responsible for the elastic behavior of plant tissues and could influence G' values. Cellulose, the main component of the cell wall, constitutes a stretch-resistant cellulose/hemicellulose network, which provides individual cells with rigidity and resistance to rupture (Carpita and Gibeaut 1993). The turgor pressure (i.e., the outwardly directed pressure exerted by the content of the cell on the wall) leads to the rigidity of plant cells and tissues and, together with the cell wall, provides the mechanical support for maintaining cell and tissue shape.

All calcium immersion treatments provoked loss of turgor, decreasing, in a similar way, G' values. In AI treatments, as calcium incorporation proceeded, the number of cells that lost turgidity due to breakage of membranes by calcium crystals increased. Accordingly, G' values diminished with treatment time. When a generalized rupture of tonoplast and plasmalemma occurred (~after 6–10 h immersion), a further exposure to calcium solutions did not modify the value of the storage modulus. Assuming that G' is also related to the state of cellulose microfibrils, the progressive disorganization of wall networks during the first hours of immersion in the solution without calcium would be responsible for the decrease in the elastic modulus in the early periods of time. Again, at longer immersion times into the solution, when this disorganization was generalized, G' value reached a plateau. The reduction of the intercellular air spaces in V and VI samples and the loss of turgor in VI ones could explain the small reduction in G' values occasioned by vacuum processes.

The low η determined in treated tissues indicated an increase in fluidity of the cell wall matrix and could be attributed to: (1) the greater amount of

apoplastic water due to plasmolysis or membrane breakage by calcium and the regrouping of constituents in the wall networks of pectins, cellulose and hemicelluloses in AI and VI apples; and (2) the decrease in viscosity due to solubilization and degradation of pectins and other wall biopolymers during immersion in A-treated apples or the greater hydration of walls due to air–liquid exchange in V-treated apples. Although this creep parameter is affected by great standard errors due to biological variability (Alzamora *et al.* 2008), it can be observed that the decrease in η values was very much severe in samples treated in the 0.53% w/w Ca solution (Table 3) accordingly to the exosmosis produced by the disruption of inner membranes.

The increase in the instantaneous compliance J_0 in treated samples could be ascribed to the structure changes in a similar way to the decrease in G' values, as these parameters are both influenced by the same structure elements. The change in wall characteristics (i.e., degradation of cell walls due to solubilization and degradation of wall polymers in A-immersed apples and microfibrillar reorganization and changes in wall network patterns in AI samples) would be traduced in greater compliances J_1 and J_2.

Although immersion processes per se significantly decreased the storage modulus and increased the compliance values, these changes appeared to be more pronounced when calcium salts at the highest concentration were added in the medium. On the contrary, the impregnation with the lowest calcium level solution would appear to have a slight beneficial influence.

The effect of calcium on the rheological behavior of apples in this study is not in agreement with some findings reported by other authors who used dipping treatments (typical dipping times and concentrations of calcium salts ranging from 1 to 5 min and from 0.5 to 1%, respectively) for preventing softening, or impregnation treatments for fortifying apples (using lower calcium concentrations) or different histological tissues (eggplant, carrot) (Betoret *et al.* 2001; Gras *et al.* 2003; Martín-Diana *et al.* 2007). Although all these authors evaluated mechanical properties at large deformations, the calcium amounts incorporated into the different tissue matrices (including apple) were much lower than those obtained in the present work. This fact would indicate that calcium concentration of the impregnation medium would have an effect on the way calcium modified the structure in the tissue and its impact on rheological characteristics. Also, it is important to mention that the response to impregnation is highly produce-specific (Alzamora *et al.* 2005).

CONCLUSIONS

The effect of calcium incorporation on the viscoelastic properties of apple tissue depended on the impregnation conditions (pressure, calcium

concentration in the solution, process time). An increase in tissue fluidity and elastic and viscoelastic compliances (creep test) and a decrease in storage modulus (oscillatory test) occurred in apples due to the immersion process per se (treatments in the isotonic glucose solution without calcium). The presence of 0.53% w/w calcium in the solution slightly accentuated this behavior. Although calcium penetration would counteract soluble component losses and hydrolysis of polymers from the cell wall, rending much stained walls as observed in the micrographs, high calcium concentrations also provoked a general inner disruption of cells with plasmolysis and membrane breakage and severe folding of walls. For great quantities of calcium incorporated, changes in compliances and storage modulus values were significantly lower for apples treated under vacuum than when the treatment was performed under AI, suggesting that VI is an effective methodology to fortify apple tissue avoiding serious damage in viscoelastic properties and presumably in texture.

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