Investigating the effect of pectin methyl esterase (PME) and CaCl₂ infusion on the cell integrity of fresh-cut and frozen-thawed mangoes by 1D/2D NMR Relaxometry

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Abstract

A major quality defect of fresh-cut and frozen mangoes is texture loss owing to disruption of cell integrity. The objective of this study was to use Nuclear Magnetic Resonance (NMR) relaxometry (1D/2D) to understand the effect of pectin methyl esterase (PME) + CaCl₂ infusion under different conditions and the subsequent effect on cell integrity of fresh-cut and frozen mango slices. Mango slices were immersed in a solution composed of 0.001 U/mL of PME + 1% (w/w) CaCl₂. Infusion was performed at the following levels: atmospheric pressure/(PATM), vacuum of -15 in Hg (P50; 50 kPa pressure) and vacuum of -27 in Hg (P10; 10 kPa pressure). Samples were also frozen in a chest freezer at -80°C and subsequently stored at -20°C. Prior to analysis, the samples were thawed at 4°C for 2hrs. NMR relaxation experiments included: T₂ (spin-spin-relaxation), T¹ (spin-lattice-relaxation) and T₁-T₂ experiments with a 0.367T permanent magnet based NMR system using 5mm tubes for the three different treatments, in addition to untreated (Control) and frozen-thawed (FT) mangoes. As the severity of the vacuum treatment increased, the T₁ of the samples
decreased. The freeze-thaw process also resulted in an additional decrease in $T_1$. The 1D-$T_2$ distributions showed the presence of 4 proton compartments for all treatments in the relaxation spectra. A decrease in $T_2$ values was observed in the FT samples, and the number of compartments decreased to 2 for the Control and 3 for the PATM samples, whereas the number of compartments remained the same for the P50 and P10 samples. $T_1$-$T_2$ maps also showed that compartmentalization was retained after FT in the P50 and P10 samples with P10 showing the highest retention in cell structure. Results indicated that 1D relaxation experiments complemented with 2D-$T_1$-$T_2$ were a useful tool to understand and quantify the cell integrity of mango samples treated under different processing conditions.

**Keywords:** CalCl$_3$/pectin methyl esterase infusion, mangoes, NMR Relaxometry, freezing, thawing

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1. **Introduction**

*Mango* (*Mangifera indica* L.) is a popular tropical fruit that is native to South Asia$^1$. The fruit is mostly grown in tropical climates with India being the world’s largest producer$^2$. The high demand for mangoes from other parts of the globe requires overseas exportation of the fruit. However, a major
issue with mango and its products is texture loss, in particular in fresh cut and frozen mangoes. Fresh cut mangoes suffer from a significant reduction in firmness even upon cold storage. Charles et al. (2013) reported 1.5 N of firmness loss in the 'Kent' variety of mangoes after 2 days of storage at 6°C. Upon freeze thawing, Sriwimon & Boonsuphip (2011) reported approximately 50%-60% loss in texture. The deterioration in texture of frozen-thawed mangoes is related to the formation of ice crystals that damage cell walls and rupture cellular membranes, resulting in release of water and soluble components from intracellular compartments.

In previous studies, to prevent loss of firmness, a number of methods have been applied such as calcium infusion, ascorbic acid treatment, pectin methyl esterase treatment, ethylene ripening, sugar infusion, pulsed light treatment, infrared heat treatment and cryogenic or air-blast freezing. Among other treatments, infusion with calcium and the pectin methyl esterase enzyme has been shown to have positive effects on preservation of fruit texture. Pectin methyl esterase is an enzyme that cleaves the methyl groups from the galacturonic acid backbone of pectin. Calcium maintains fruit firmness by binding to the carboxylic acid groups of the pectin molecules that are de-esterified with PME; forming what is defined as the egg-box model.

Nuclear Magnetic Resonance (NMR) proton relaxometry has been widely used in the analysis of physiological and biochemical changes in fruits and vegetables. The variations in longitudinal (T₁) and transverse (T₂) relaxation times can yield information on microstructural changes in plant tissues.
The NMR relaxation curve obtained from systems that consist of several distinct water compartments such as plant cells is expressed by a multiexponential decay curve. By mathematical transformation of the decay curve with the non-negative least squares (NNLS) method, a 1D relaxation spectrum can be obtained. The spectrum features several distinct peaks with each peak referring to a different proton compartment inside the fruit tissue. The changes in areas of the peaks and T₂ relaxation times for each compartment can then be used to interpret physiological changes. The use of 2D relaxation spectra (e.g. T₁-T₂) assure a more sensitive analysis for samples compromised of different water compartments. In a 2D experiment, T₁ and T₂ are measured at the same time. This way, compartments having the same T₂ values could be differentiated based on differences in T₁. NMR spectra are especially valuable in investigating complex systems such as plant tissue that display heterogeneity in terms of water distribution both in a macroscopic and microscopic scale. This method has proven effective in analysis of apple mealiness, avocado quality, internal-browning of pears and starch granules distribution in potato tissues.

The purpose of this study was to determine the effects of various PME-CaCl₂ infusion treatments on the microstructure of fresh cut and frozen-thawed mangoes using changes in signal intensities, T₁ and T₂ relaxation times and evaluate the utilization of 1D and 2D NMR relaxometry as a tool for analysis of changes in plant tissue.

2. Materials and Methods
‘Kent’ mango variety of ripe mangoes (M. indica L.) were purchased from a local supermarket in Davis, CA, USA. The selected fruits were chosen regarding the uniformity in size and maturity. Maturity was evaluated based on fresh colour, texture and soluble solid content. The chosen mangoes’ soluble solid content was in the range of 16-19 ° Brix and firmness was in the range of 26-30 N. During preparation of samples, to minimize variation between samples, the highly diverse parts that correspond to approximately 20% of the total length from both the stem and the blossom end of the fruit were discarded. The mangoes were washed, peeled and cut into cubes of 1.5 cm. For infusion treatments, a stock solution of pectin methyl esterase (commercial PME from Aspergillus aculeatus, Novoshape, Novozymes, Bagsvaerd, Denmark) with an activity of 11.53 U/ml was used.

Infusion experiments: As control, untreated mango cubes were used. The infusion solution consisted of 10-g/l calcium chloride and 0.001 U/mL PME. The PME concentration was selected based on the preliminary studies. To ensure complete immersion of sample into the infusion solution, the sample/infusion solution ratio was chosen to be 1:3 (w/w). Infusion was performed under 3 different conditions:

- **Infusion at atmospheric pressure (PATM)**
- **Infusion at -15 inches Hg (P50; 50 kPa pressure)**
- **Infusion at -27 inches Hg (P10; 10 kPa pressure)**
In all treatments, mango cubes were immersed in the infusion solution at room temperature (25°C) for 10 min. To submerge the samples into the infusion solution, an aluminium mesh was used. For infusion under vacuum conditions, the infusion solution containing the cubes was placed in plastic containers. The containers were then placed in a vacuum oven (Isotemp Vacuum Oven Model 280A, Thermo Fisher Scientific, Pittsburgh, PA, USA) and the vacuum level was adjusted accordingly. The samples were infused for 10 min at both 50 and 10 kPa. After infusion, samples were laid on paper towels for 5 min, packed into plastic bags and kept at 25°C for 2h before analysis or freezing. This ensured enough time for the reaction between calcium, pectin and the enzyme to take place. Some of the mango cubes were also frozen after infusion in a chest freezer at -80°C and subsequently stored at -20°C. Prior to NMR experiments the samples were thawed at 4°C for 2h and kept at 25°C for 30 min. These samples were denoted as “FT”, or frozen-thawed samples in the rest of the study. For both before and after freezing experiments an untreated control sample was also used.

1D/2D NMR Relaxometry Experiments: For all treatments and for an untreated mango (before and after freezing), cubic mangoes were cut into cylindrical pieces with a 4 mm cork to fit into a 10 mm NMR tube. $T_1$, $T_2$, $T_1-T_2$ experiments were performed in a 0.367T (15.635 MHz) system (Spin Core Inc, Gainesville, FL, U.S.A) with a 10 mm rf coil. Spin-lattice relaxation ($T_1$) measurements were performed using an inversion recovery pulse sequence with a delay time changing in the range of 0.5 ms to 4.61s with 512 acquisition points, and 3 scans with 5 replicates. $T_1$ values were analyzed
using MATLAB. For $T_2$ measurements, a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence was used with an echo time (TE) of 4 ms, 4000 echoes, and 16 scans. 1D Non-Negative Least Square (NNLS) applied to the $T_2$ decay curves to obtain relaxation spectra. PROSPA (Magritek Inc., Wellington, New Zealand) was used for 1D-NNLS analysis. 2D $T_1$-$T_2$ experiments were performed with an Inversion Recovery-CPMG experiment. The number of steps in the $T_2$ domain was 512 whereas in the $T_1$ domain 64 inversion times were used with 4 scans. Echo times and inversion times were the same as in the 1D experiments. 2D data sets were analyzed with the NNLS GUI interface developed by the Callaghan Group (Wellington, New Zealand). 6 mango cubes for each treatment conditions were used for the NMR experiments as replicates.

**Statistical Analysis:** To compare the means of the relaxation times, analysis of variance (ANOVA) with Tukey’s multiple range test, was used. Differences were considered significant for $p<0.05$.

### 3. Results & Discussion

#### 3.1. $T_1$ Relaxation Time Measurements

Fig. 1 shows the longitudinal relaxation times for each treatment. With respect to the control, $T_1$ relaxation times did not exhibit a significant change after Ca-PME infusion under atmospheric conditions (PATM). With increasing severity of vacuum treatment, a general decreasing trend in $T_1$ times was observed (though not always significant). The freeze-thaw process also resulted in an additional decrease in $T_1$ times. This could be attributed to a decrease in moisture content as $T_1$ is
related to water content more directly \(^2\). However, in a previous study carried out by the authors \(^6\) in
which the same treatments were used to monitor textural changes in ‘Kent’ mango, after vacuum
treatment with either water or Ca-PME solution, an increase in moisture content of the samples was
observed. The moisture content was highest in control water samples with a 10 kPa vacuum treatment.
The increase in water penetration with increasing vacuum severity was explained by the replacement of
air-filled pores inside the fruit with water, which is referred to as waterlogging. This increase in
moisture should result in an increase in \(T_1\) values. The decrease in \(T_1\) despite the increase in moisture
content can be explained by the interaction of water with surrounding macromolecules. Ca-PME
infusion causes the water to be entrapped within an egg-box shaped cross-link structure produced by
the complex between carboxylic acid groups of pectin and calcium ions, which results in gel formation.
Formation of a gel structure is known to decrease \(T_1\) dramatically \(^2^0\). The decrease in \(T_1\) times due to
gel formation might be high enough to compensate for the effect of increasing moisture content. The
fact that, among the samples, P10 samples exhibited the highest PME activity but also has the
shortest \(T_1\) is in agreement with this observation \(^6\).

3.2. 1D \(T_2\) Relaxation Spectra

The spin-spin relaxation (\(T_2\)) and spin-lattice relaxation (\(T_1\)) times are known to yield
information on water content, physical properties of water and interaction of water with the
surrounding macromolecules \(^1^3\). The relaxation spectrum is acquired by multi-exponential inversion of
the $T_2$ relaxation curves. This way, it is possible to differentiate dissimilar microstructural water
compartments with different $T_2$ times $^{22}$. The variations in relaxation spectrum can be used as an
indicator for numerous proton related changes within food systems such as increase in water content,
movement of proton containing molecules and incidences causing proton exchange within various
proton pools such as those in hydroxyl or amino groups $^{20,23}$. For living cells, these proton pools refer
to water compartments within the cell. Hence, by monitoring changes in relaxation times and signal
intensities, it is possible to gain information regarding the changes in cell structure $^{24,25}$. In all of the
fresh cut mango samples, a total of 4 peaks were observed (Fig.2). In most of the previous studies that
involve use of NMR relaxometry on fruit and vegetable tissues, either 3 or 4 peaks were observed $^{13,16,18,26,27}$. The peak with the lowest $T_2$ (and a low peak area) is usually attributed to cell wall, the second
to the cytoplasm protons, and the third and largest compartment generally assigned to vacuole since in
plant cells most of the water is stored inside the vacuoles. The fourth peak (having the highest $T_2$) was
previously thought to be associated with extracellular water or starch or non-exchangeable solute
protons, such as the ones coming from sugar or proteins $^{16,18,26}$. The fourth peak, that is observed in
fresh cut mango relaxation spectra having a high signal intensity, was assigned to extracellular water
that might be formed due to deformation of the fruit taking place during sample preparation.

In Table 1, the spin-spin relaxation times and the percent relative areas (RA) that correspond
to each peak in the relaxation spectrum (Figure 2) are shown. The RAs are correlated with the
magnitude of signal intensity coming from each proton pool within the samples. Relative areas denote the contribution of that component to the overall signal.

It is known that a decrease in water concentration or an increase in solute concentration will enhance relaxation rate thus decreasing relaxation times. It is challenging to interpret relaxation spectra with 100% accuracy since there are numerous mechanisms involved such as diffusive and chemical exchange between water compartments as well as cross relaxation. Nevertheless, in proton transverse relaxation, the effect of cross relaxation is negligibly small, hence the changes in relaxation times will be explained by diffusive and chemical exchange in this study.

3.2.1. Effect of freeze-thawing on untreated fresh-cut mango samples

For untreated mango samples, the number of peaks appearing in the relaxation spectrum decreased from 4 to 2 after freeze thawing (Table 1). This decrease in peak number refers to a decompartmentalization within fruit cells. After freeze thawing, four compartments were replaced by two compartments (with 93% of signal coming from one, 7% from the other). The $T_2$ of the first peak that remained after thawing was not different from the first peak of the control ($p<0.05$), but there was a significant decrease in relative signal intensity (15% to 7% of total peak area). The second peak of the frozen-thawed samples was observed at a $T_2$ value that is significantly different from all four peaks of the control and was marginally larger in relative area. The results clearly indicated a disruption in cell structure. Crystal formation during freezing is known to cause irreversible damage to cellular
compartments. The difference between the NMR relaxation spectra of control and frozen-thawed samples resulted from the rupture of internal cellular membranes along with the cell wall. The water that is now highly mobile merges and presumably forms the larger proton pool, surrounded by the remains of cell wall and membrane (which constitutes the smaller proton pool). In a previous study carried out by the authors, using the exact same treatments to monitor textural changes in ‘Kent’ mango, the control sample exhibited a marginal decrease in firmness after freeze thawing. This was attributed to total disruption of cell integrity, which was confirmed with confocal microscopy images.

This result is consistent with the relaxation spectrum obtained in this study for the control samples.

3.2.2. Effect of Calcium–PME infusion under atmospheric pressure on fresh cut and frozen-thawed mangoes

Following Ca-PME infusion of mango slices at atmospheric pressure (PATM), in the relaxation spectrum, all four peaks seem to be preserved with only minor changes in $T_2$ times and signal intensities. While the signal intensities coming from the first two compartments did not change significantly ($p<0.05$), the areas of the remaining two peaks showed some variations. The area of the third peak decreased almost 40%, whereas the fourth peak displayed a dramatic increase. Nevertheless, looking at these values, it is possible to say that Ca-PME infusion at atmospheric pressure did not alter cell structure significantly. However, after FT treatment of PATM samples, 3 peaks were observed in the relaxation spectrum. The decrease in the total compartment number from 4 to 3
indicated a possible disruption in cell integrity. There were no significant changes in T₂ and area of the
first compartment after FT, which denotes that cell wall structure was mostly conserved despite crystal
formation. One very radical change that is worth noting is that after FT, there was formation of a
second peak that confers significantly different T₂ times and a very high signal intensity (79% of total
peak area). This new compartment might be related to the change in compartmentalization resulting
from to Ca-PME infusion. PME can de-esterify pectin by cleaving the methyl groups. Carboxylic acid
groups of the resulting low methyl esterified pectin can then form complexes with Ca²⁺ ions resulting in
an egg-box structure that can entrap water⁶.⁹. The entrapment of water within a continuous solid
matrix is crucial to fruit texture, and is highly related with the firmness of fruits and vegetables³²,³³. In
a previous study by the researchers, samples that had been infused with Ca-PME at atmospheric
pressure had higher firmness (though not significant for p<0.05) than the control. Samples infused
with water at atmospheric pressure instead of the Ca-PME solution (water control samples) displayed
considerably lowered firmness. Nevertheless, Ca-PME infusion was merely enough to compensate for
the loss in texture due to water penetration into the fruit. This finding and the NMR results of the FT
samples, to a certain extent indicated that Ca-PME infusion was mildly effective at preserving the
firmness of mango slices after freezing⁶.

3.2.3. Effect of Calcium-PME infusion under 50 kPa vacuum on fresh cut and frozen-thawed mangoes

Ca-PME infusion under 50 kPa vacuum (P50) resulted in some minor changes in areas and
T₂ times of the four peaks. Peak 1 that is attributed to the protons inside the cell wall showed no
change in area and spin-spin relaxation times. The $T_2$ times coming from the remaining compartments all exhibited a minor decrease. Additionally the peak area of the fourth compartment (which refers to signal intensity) increased significantly ($p<0.05$). The fourth compartment was assigned to extracellular water, and the increase in area of peak 4 can be related with increasing moisture content due to increased rate of water penetration into open pores under vacuum. The similarity of NMR relaxation spectrum of the 50 kPa vacuum treated samples with the control clearly demonstrates the minimal effect of vacuum treatment on the cellular structure of mangoes.

After freeze thawing, all four peaks were conserved, with significant decreases in $T_2$ values. The signal intensity coming from the first compartment remained the same whereas the signal intensities of other compartments were significantly altered, with peak 2 changing the most (416% increase in relative area). The fact that the relaxation times and signal intensity coming from peak 1 (cell wall) showed no change after freeze thawing likely indicates that cell wall integrity was mostly preserved even after freeze thawing. The decrease in $T_2$ times for the other compartments can be explained by proton exchange between cellular compartments and the egg-box structure of crosslinking entrapping the water. The radical increase in the area of peak 2 (assigned to water inside the cytoplasm) might refer to a disruption of intracellular membranes surrounding subcellular organelles, which is further supported with the decreases in signal intensities coming from other cellular compartments. Still the fact that vacuum infusion at 50 kPa preserved all four peaks is a
positive indication for texture conservation. This outcome is in agreement with the previous study conducted by the authors, that reported significantly higher firmness for Ca-PME infusion under 50 kPa vacuum compared to control samples.

3.2.4. Effect of Calcium-PME infusion under 10 kPa vacuum on fresh cut and frozen mangoes

The relaxation spectrum results for Ca-PME infusion under the highest vacuum treatment (10 kPa pressure) followed similar trends with 50 kPa vacuum treatment. The $T_2$ times of all four compartments were not significantly different from the $T_2$ times of 10 kPa vacuum treated mangoes ($p<0.05$). Again similar to the P50 results, after P10 treatment, the first compartment was highly conserved. As in the P50 treatment, after freeze thawing the signal intensity of the first compartment remained the same, but there was a smaller decrease in $T_2$ relaxation time. This outcome can be interpreted as the positive effect of the higher vacuum treatment on preservation of cell wall integrity. Additionally, after FT, as with the P50 treatment, $T_2$ times for all four compartments displayed a decrease.

Worth nothing here is that, compared to the 50 kPa vacuum treatment, in the 10 kPa samples, the decrease in $T_2$ times were significantly lower ($p<0.05$). Area distributions also followed similar trends with the area of the second peak rising dramatically (around 350%) with the FT treatment. Compared to P50, the P10 treatment exhibited smaller changes in $T_2$ times and signal intensities, both before and after FT. This indicates a positive correlation between texture preservation
and vacuum treatment severity. However, in the previous study, the firmness of the P10 samples was found to be the same as the control, indicating no positive effect of P10 treatment on texture. However, this was explained with the increased crystal formation due to the higher rates of penetration of water into the fruit pores.

3.3. 2D Relaxation Profiles

2D relaxation spectrums of each system yields a unique $T_1$-$T_2$ profile that acts as a finger-print for water distribution inside the tissue. The profiles in Fig.3 were obtained by digitally overlaying the 2D relaxation profiles of all replicates for each treatment. The ‘Y’ shape trend shown in the control mangoes represents the cell structure of fresh mangoes. The peaks on the left part of ‘Y’ above the diagonal are exchange peaks and do not represent real compartments since it is not possible for spin-spin relaxation time, $T_2$, to be larger than spin-lattice relaxation time, $T_1$. These peaks arise due to the mathematical transformation made by Non Negative Least Squares (NNLS) Method.

It is clear from the 2D maps that the ‘Y’ shape structure is disrupted in untreated FT samples and PATM FT samples. However, vacuum treated samples kept the ‘Y’ shape to an extent despite freezing. The P10 treatment, which applies the highest vacuum, was particularly successful in conserving the ‘Y’ shape. This agrees with NMR relaxation spectrum results and the previous study by the authors, where vacuum infusion with PME and calcium at 10kPa was reported as the most suitable method for preserving the texture of fresh cut mangoes.
4. Conclusion

NMR relaxometry was successful in providing insight on the extent and type of disintegration taking place within mango tissues by freezing followed by various pretreatments. The combined information gathered from 1D and 2D relaxation profiles was enough to demonstrate the effects of various pretreatments on texture of mangoes. According to the NMR results, infusion of PME and calcium was confirmed to maintain the cellular structure of both fresh cut and frozen mangoes. Additionally the highest vacuum level used for Ca-PME infusion (at 10 kPA) was shown to be the most suitable pretreatment for preservation of cell structure upon freeze thawing. All the information gathered from 1D and 2D NMR experiments was in agreement with a previous study carried out in our laboratory. Hence, with this study it was shown that 1D/2D NMR experiments were excellent tools in understanding cellular damage in plant tissues.
Figures

Figure 1. Average $T_1$ relaxation times of mango samples after different treatments. Untreated (Control), control after freeze thawing (Control FT); samples infused with 0.001 U/mL of PME + 1% (w/w) CaCl$_2$ solution at atmospheric pressure (PATM), at atmospheric sample after freeze thawing (PATM FT); at 50 kPa (P50), at 50 kPa after freeze thawing (P50 FT); at 10 kPa (P10), at 10 kPa after freeze thawing (P10 FT).

Figure 2. A representative 1D NMR spectrum of a fresh-cut mango sample (control).

Figure 3. 2D profiles of relaxation times (x axis: log$_{10}$ (T1), y axis: log$_{10}$ (T2)) for (a) Untreated (control) (b) Control after freeze thawing; samples infused with 0.001 U/mL of PME + 1% (w/w) CaCl$_2$ solution at (c) PATM (d) PATM after freeze thawing; at (e) 50 kPa (f) 50 kPa after freeze thawing; at (g) 10 kPa (h) 10 kPa after freeze thawing,

Figure 4. 3D Representation of a $T_1$-$T_2$ map
Figure 1
Figure 2
Figure 4
Table 1. Average $T_2$ relaxation times and percent relative areas (RA) of mango samples after different treatments. Untreated (Control); samples infused with 0.001 U/mL of PME + 1% (w/w) CaCl$_2$ solution at atmospheric pressure (PATM), at 50 kPa (P50), at 10 kPa (P10).

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5. References


