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Composite Films of Thermoplastic Starch and CaCl₂ Extracted from Eggshells for Extending Food Shelf-Life

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Abstract: Calcium chloride (CaCl₂) has been widely used to maintain the quality of fresh-cut fruits and vegetables because it stabilizes and strengthens the membrane system against fungal attacks. It is mainly applied via spray coating and dip coating techniques. This study explored a method of incorporating calcium chloride extracted from eggshells in a packaging material, thermoplastic starch (TPS), via a hot-melt extrusion process. The composites were characterized by FTIR, DSC, SEM-EDX and tensile testing. FTIR confirmed the chemical reactions between CaCl₂ and TPS. DSC results showed a significant decrease in the heat of fusion by adding 20 wt% of CaCl₂ content in TPS, indicating a drop in the degree of crystallinity. The Young's modulus of TPS was not significantly affected by the incorporation of 10 wt% CaCl₂ ($P = 0.968$), but reduced notably with the addition of 20 wt% CaCl₂ ($P = 0.05$), indicating the plasticizer effect of the CaCl₂. Physicochemical analysis of fresh-cut apple slices was assessed. Samples placed on the surface of the TPS/CaCl₂ composites displayed less pH reduction, reduced antioxidant activity, more weight loss and increased reducing sugar compared to the samples placed on the surface of virgin TPS films. CaCl₂ released from the TPS/CaCl₂ films was measured and their antimicrobial activity was confirmed by bacterial inhibitory growth assessment. Fungal growth was observed on apple slices placed on virgin TPS film by day 21 while apple slices placed on TPS/CaCl₂ 20 wt% composites did not support any fungal growth for 28 days. In summary, TPS and eggshell-extracted CaCl₂ showed the ability to maintain the quality of fresh-cut apples, and TPS/CaCl₂ 10 wt% composite could be a good option as a packaging material for fresh-cut fruits due to active antimicrobial activity and maintained Young's modulus.

Keywords: thermoplastic starch; eggshell waste; calcium chloride; composite films; hot melt extrusion; antimicrobial activity; food shelf-life



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1. Introduction

The demand for fresh fruits has increased considerably in recent years. However, fresh-cut fruits have increased respiratory rates, biochemical reactions, microbiological spoilage and quality deterioration [1]. Many methods have been studied in an attempt to extend the postharvest life of fresh-cut fruits. To this end, researchers have been employing treatments with ozone [2], peroxyacetic acid [3], chlorine dioxide [4], hydrogen peroxide [5], organic acid [6], oxalic acid [7], calcium-based solutions [8], antioxidant and antimicrobial coating [9,10], irradiation [11], cold plasma treatment [12], pulsed light [13], ultrasound [14], modified atmosphere packaging [15], active and intelligent packaging [16], and nanocomposite packaging [17].

Some natural materials and recovered wastes have been reported to maintain the quality of fresh fruits. Lotus leaves have a strong metabolism of free radicals and antioxidant activity. The extract of lotus leaves has been coated on fruits to extend the life after harvest [18]. Aloe vera suppresses phenolic oxidation and inhibits enzyme activity, and is coated on harvested fruits [19]. Mushroom cell wall contains chitin, which can be converted

into chitosan, and chitosan has excellent antibacterial and antifungal activities. Chitosan is produced from mushroom waste and coated with fresh-cut melons [20].

Chicken eggshells are by-products from food industry that have been considered as hazardous waste by the European Commission regulations, but are generally disposed of in landfills contributing to pollution problems. Given the amount of agricultural waste generated and the ever-increasing cost of waste disposal, some egg processing facilities pay a high cost to dispose their waste in landfills [21]. However, eggshells are a valuable source of biomaterials rich in calcium salt, which can be recovered from waste and used to stabilize the membrane and strengthen the vegetal cell wall against fungal attacks. The extraction of calcium chloride from eggshells has been reported to be used by dip coating on fruits [8]. The spraying of calcium solutions has also proven to be one of the most effective way to apply calcium to fresh fruits to prevent losses [22].

In this current study, we propose a novel approach to manufacture biodegradable packaging material that releases calcium chloride as an alternative method for preserving quality and extending shelf life of fresh-cut fruits. Calcium chloride was chemically extracted from eggshells and embedded in a typical packaging material, thermoplastic starch (TPS), by a hot-melt extrusion process. Starch is the main component of a naturally occurring, biodegradable polymer structure belonging to the polysaccharides group. Starch is considered the second most important biopolymer after cellulose and can be obtained from a range of renewable plant resources, such as maize, wheat and potatoes. Starch can be converted into TPS by adding a plasticizing agent under appropriate shear and temperature conditions [23]. The characterization of the TPS/CaCl₂ composites was carried out by Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), scanning electron microscopy (SEM) coupled with energy dispersive X-ray (EDX) and tensile testing in this study. Fresh-cut apple slices were placed on virgin TPS and TPS/CaCl₂ films to investigate the changes in physicochemical properties and potential antimicrobial activity of the TPS-based composites. The physicochemical properties of the fresh-cut apple slices were investigated by monitoring pH, mass, reducing sugar, antioxidant activity and colour changes. The release of calcium chloride from the composites and antimicrobial activities were also investigated.

2. Materials and Methods

2.1. Materials

Thermoplastic potato starch (TPS) commercially available as Solanyl[®] C2002 was obtained from Rodenburg Biopolymers (Oosterhout, The Netherlands) and used as received. Chicken eggshells were collected for the experiment. Pink Lady[®] Cripps Pink cpv apples originating from France were purchased from a local supermarket.

Hydrochloric acid, sodium potassium tartrate, ethylenediaminetetraacetic acid (EDTA), sodium chloride and sodium hydroxide were obtained from TCI Europe (Zwijndrecht, Belgium). Calconcarboxylic acid (Patton and Reeder's indicator), 3,5-Dinitro-2-hydroxybenzoic acid (DNS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ethanol and glucose were purchased from Merck (Darmstadt, Germany).

Yeast extract glucose chloramphenicol agar was purchased from Merck and MacConkey Agar, mannitol salt agar and plate count agar were purchased from Lab M (Bury, UK). *Staphylococcus aureus* ATCC 25,923 and *Escherichia coli* ATCC 9001 were obtained from LGC Standards (Middlesex, UK). Luria-Bertani broth (LB) was prepared by adding 10 g·L⁻¹ of sodium chloride, 10 g·L⁻¹ of tryptone and 5 g·L⁻¹ of yeast extract (Neogen Europe, Ayr, UK) and adjusting the pH to 7.2.

2.2. CaCl₂ Extraction from Eggshell Waste

Eggshells were collected, washed and dried at room temperature for 2 days, followed by a grinding process with an ultra centrifugal mill ZM 200 (Retsch GmbH, Haan, Germany). 30 g of the resulting eggshell powder was treated with 450 mL of 4% HCl solution (1:15 *w/v* ratio). The mixture was magnetically stirred 5 times for 15 min, with a 30 min

interval between each run, after which gas bubbles were no longer dispersed. This was followed by centrifugation at $4025 \times g$ for 15 min using a MIKRO 220R centrifuge (Andreas Hettich GmbH, Tuttlingen, Germany). The supernatant was collected, dried at $110\text{ }^{\circ}\text{C}$ for 48 h and stored until further use.

2.3. Composite Film Preparation and Characterization

TPS, TPS/CaCl₂ 10 wt% and TPS/CaCl₂ 20 wt% films were prepared by hot-melt extrusion using a bench-top Prism™ twin-screw extruder (Thermo Electron GmbH, Karlsruhe, Germany) with 16 mm diameter screws and a 25/1 length to diameter ratio, at a temperature profile of 70, 130, 150, 160 and $170\text{ }^{\circ}\text{C}$, from feeding to die zone, with a screw speed of 60 rpm, followed by a 3-roll calendar system to form films. Processing temperature is usually determined by the melting temperature of the polymer. The zones near to the die maintain the molten phase of the polymer with a processing temperature slightly higher than the melting temperature of the polymer. While the feeding zone has a lower temperature to prevent the clogging and bridging of the polymer, the temperature is increased gradually through the barrel [24]. The thickness of all the films was below 1 mm. The resulting films were characterized by FTIR, DSC, SEM-EDX and tensile testing.

2.3.1. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR was carried out on a Perkin Elmer Spectrum One fitted with a universal attenuated total reflectance (ATR) sampling accessory (PerkinElmer, Waltham, MA, USA). All data was recorded at $21\text{ }^{\circ}\text{C}$ in the spectral range of $4000\text{--}650\text{ cm}^{-1}$ against the air as background, using a 4 scan per sample cycle at a resolution of 0.5 cm^{-1} and a fixed universal compression force of 70–80 N. Subsequent analysis was carried out using spectrum software.

2.3.2. Differential Scanning Calorimetry (DSC)

DSC was carried out using a DSC 2920 Modulated DSC (TA Instruments, New Castle, DE, USA) with a nitrogen flow rate of $30\text{ mL}\cdot\text{min}^{-1}$ to prevent oxidation. Calibration of the instrument was performed using indium as standard. Test specimens weighed between 6 and 8 mg were measured on a Sartorius Entris analytical balance (Sartorius AG, Göttingen, Germany), capable of reading five decimal places. Samples were crimped in non-perforated aluminium pans, with an empty crimped aluminium pan used as reference. The thermal history was removed by heating samples from $30\text{ }^{\circ}\text{C}$ to $200\text{ }^{\circ}\text{C}$ at the rate of $10\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$, and then held isothermally at $220\text{ }^{\circ}\text{C}$ for 10 min. The samples were then cooled down from $220\text{ }^{\circ}\text{C}$ to $20\text{ }^{\circ}\text{C}$ at $30\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$. Finally, the thermal properties of the samples were recorded by heating samples from 30 to $200\text{ }^{\circ}\text{C}$ at a rate of $10\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$.

2.3.3. Scanning Electron Microscopy Coupled with Energy Dispersive X-ray (SEM-EDX)

The cross section of the TPS films was examined using a Mira XMU SEM instrument (Tescan Mira, Brno, Czech Republic) in back scattered electron mode. Energy dispersive X-ray (EDX) was performed using an Oxford Instruments (Abingdon, UK) detector to determine the elemental composition of TPS and TPS/CaCl₂ composites. The accelerating voltage used was 10 kV for all materials. Prior to analysis, samples were freeze-fractured by liquid nitrogen, placed on an aluminium stub, and coated with gold-sputtering using a Baltec SCD 005 system at 0.1 mbar vacuum for 110 s.

2.3.4. Tensile Testing

Tensile testing was carried out on a Lloyd Lr10k tensometer (Ametek Ltd., West Sussex, UK) using a 2.5 kN load cell on ASTM standard test specimens. Tests were recorded at a strain rate of $10\text{ mm}\cdot\text{min}^{-1}$, room temperature, initial gauge length of 20 mm, and a pull to limit setup (stop at strain of 30%). Data was recorded using Nexygen™ software. The tensile tests were carried out in accordance with ASTM D882. A minimum of five replicates were analyzed per group, and prior to testing, the thickness of each sample

was measured. The stress–strain curves were recorded and the Young’s modulus of each sample was calculated.

2.4. Physicochemical Analysis of the Fresh-Cut Apple Slices

Physicochemical analysis of the fresh-cut apple slices (pH, mass, reducing sugars, antioxidant activity) was determined after 14 days of contact with TPS film and TPS/CaCl₂ 20 wt% composite films. Fresh-cut apple slices from day 0 were used as reference. Each apple slice was immersed in 10 mL of distilled water and vortexed for 30 s. The solution was used for pH, reducing sugars and antioxidant activity measurements.

2.4.1. Reducing Sugars

The reducing sugars of the fresh-cut apple slices were measured by the DNS method [25]. The DNS reagent was prepared by dissolving 45 g sodium potassium tartrate in 75 mL of distilled water, dissolving 1.5 g of DNS with 10 mL of 2 mol·L^{−1} NaOH solution, and adding distilled water until 150 mL. 5 mL of sample solution was added with 1 mL of DNS reagent, heated at 100 °C for 10 min, then cooled to room temperature. The absorbance was read at 540 nm by a UV-VIS 1280 spectrophotometer (Shimadzu, Kyoto, Japan). A standard curve was obtained with commercial glucose. The concentration of glucose from each sample solution was calculated against the standard curve. Three replicates were used for this study.

2.4.2. Antioxidant Activity

The antioxidant activity (AOA) was measured by DPPH assay [26]. 0.4 mmol DPPH solution was prepared by adding 16 mg of DPPH in 100 mL of ethanol. 1 mL of sample solution was added with 4 mL of DPPH solution, vortexed for 5 s and set for 30 min. The absorbance was read at 515 nm by UV-VIS spectrophotometry. The control used was the DPPH solution. The antioxidant activity was calculated by (1):

$$AOA\% = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100\%, \quad (1)$$

2.4.3. Colorimetry

Colour measurements (L^* , a^* , b^* and c^* values) of the specimens were determined by using a Lovibond RT Series Reflectance Tintometer (Lovibond Tintometer, Amesbury, UK) with OnColor software. Prior to measuring the colour of the specimens, the instrument was standardized by placing black and white standard plates and L^* , a^* and b^* colour values were recorded. The L^* values correspond to lightness/darkness (0 for black and 100 for white), the a^* values correspond to the specimen’s colour dimension from red to green (the greater the a^* value, the redder), the b^* values correspond to the specimen’s colour dimension from yellow to blue (the greater the b^* value, the yellower), and c^* values correspond to chroma (0 for achromatic colour and the greater the c^* value, the brighter).

2.5. Estimation of CaCl₂ Release from Composite Films

To evaluate the release of CaCl₂ from virgin TPS (control), TPS/CaCl₂ 10 wt% and TPS/CaCl₂ 20 wt% composite films, samples of known weight were incubated in distilled water at 4, 20 and 30 °C to simulate refrigerated and ambient storage conditions. Water samples of 4 mL each were withdrawn at 0, 3, 6, 24, 48, 72 and 192 h of incubation to mimic packaging conditions of food with over one week of shelf life, and suitably diluted to estimate the concentration of calcium released from composite films by complexometric titration method [27]. EDTA complexometric titration was employed with Patton and Reeder’s (PR) as an indicator. This blue dye indicator forms a dye-metal ion complex with calcium ions, but in the presence of EDTA molecules it produces a more stable EDTA-metal ion complex, and this process causes the colour to change from blue to pink/red. The concentration of Ca²⁺ in tested samples was calculated based on the volume of EDTA

used to complex the PR indicator ($\text{Ca}^{2+}:\text{EDTA} = 1:1$). A calibration curve was previously prepared using starting solutions of $500 \text{ mg}\cdot\text{L}^{-1}$ eggshell-based CaCl_2 , $0.005 \text{ mol}\cdot\text{L}^{-1}$ EDTA and $8 \text{ mol}\cdot\text{L}^{-1}$ NaOH. All tests were carried out over two independent experiments.

2.6. Microbial Studies

2.6.1. Microbial Growth on Fresh-Cut Apple Slices

TPS and TPS/ CaCl_2 20 wt% composite films were cut into $\sim 1 \text{ cm}^2$ segments, and each sample was individually placed in an empty well of a six-well plate. Apple slices with a dimension of $1 \text{ cm} \times 1 \text{ cm} \times 3 \text{ mm}$ were placed on the surface of TPS and TPS/ CaCl_2 20 wt% composite films and incubated at $20 \text{ }^\circ\text{C}$ for 28 days. Triplicate samples were removed at days 2, 7, 9, 14, 16, 21, 23 and 28 to quantify microbial growth on the samples and apple segments. The microbial load of each sample and apple segment was quantified via a method previously described elsewhere [2]. Each sample and corresponding apple slice was placed in 10 mL of phosphate buffered saline and homogenized by agitation in a stomacher for 5 min. The spread plate method was used to quantify total coliform bacteria (MacConkey Agar), total yeasts/moulds (Yeast Extract Glucose Chloramphenicol Agar), and *S. aureus* (Mannitol Salt Agar) incubated at $37 \text{ }^\circ\text{C}$ for 48 h, $25 \text{ }^\circ\text{C}$ for 72 h, and $37 \text{ }^\circ\text{C}$ for 48 h, respectively. The pour plate method was used to count total mesophilic bacteria (Plate Count Agar), incubated at $37 \text{ }^\circ\text{C}$ for 24 h.

2.6.2. Antibacterial Activity of the TPS/ CaCl_2 Composite Films

Antimicrobial activity was evaluated against *Staphylococcus aureus* (ATCC 25,923) to represent Gram-positive bacteria and *Escherichia coli* (ATCC 9001) to represent Gram-negative bacteria. Tests were conducted in LB broth using an adapted standard broth microdilution assay for bacteria that grow aerobically, as recommended by the CLSI standard [28]. Maximal tested concentration of CaCl_2 extracted from eggshell was $200 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$. Composite films ($0.5 \times 0.5 \text{ mm}$) were tested using the same method. The test was carried out in triplicate with LB medium and TPS films acting as a negative control and tested microorganisms as a positive control. Growth of the respective tested microorganisms after 24 h of incubation at $37 \text{ }^\circ\text{C}$ was measured as optical density (OD) at 600 nm using a Biotek Synergy HT Microplate Reader (Biotek Instruments GmbH, Bad Friedrichshall, Germany) with Gen5 microplate reader software from Biotek Instruments.

2.7. Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) with a Tukey post hoc test to determine differences. Differences were considered significant when $p \leq 0.05$. The software used to perform statistical analysis was SPSS (IBM Version 22) for Windows. All data collected in this study were expressed as mean \pm standard deviation. A sample size of 5 was used for colorimetry and tensile testing, and a sample size of 3 was used for DSC testing, physiochemical analysis of fresh-cut apple slices and microbial studies.

3. Results and Discussion

3.1. Starch-Eggshell Extract Composite Films Characterization

3.1.1. Chemical Interaction Mechanism between CaCl_2 and TPS

FTIR analysis was conducted to study chemical interactions of the composite components, evidenced by changes of characteristics spectral bands. ATR-FTIR spectra of the eggshell-based CaCl_2 , virgin TPS and the resulting composite films are presented in Figure 1. The spectrum of CaCl_2 extracted from eggshells showed peaks at 3487 and 3396 cm^{-1} assigned to asymmetric O–H stretch, a peak at 2164 cm^{-1} for symmetric O–H stretch, and H–O–H bending vibration bands at 1629 and 1618 cm^{-1} due to the presence of water in CaCl_2 . The appearance of multiple bands instead of a usual single band for asymmetric O–H stretching and H–O–H bending is indicative of different hydrogen bonding environments resulting from interactions with chloride ions as well as water molecules [29].

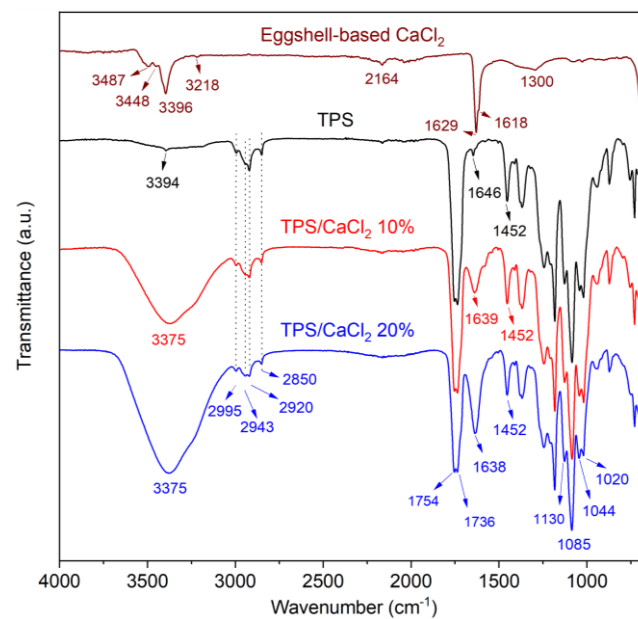


Figure 1. FTIR spectra of CaCl_2 extracted from eggshells, virgin TPS, TPS/ CaCl_2 10 wt% and TPS/ CaCl_2 20 wt% composite films.

The spectrum of virgin TPS exhibited typical C–H stretching vibration bands at 2995, 2920, 2850 cm^{-1} and C–O stretching vibration bands at 1130, 1085, 1044 and 1020 cm^{-1} . Frequencies at 1646 cm^{-1} and 1452 cm^{-1} were believed to be an attribution of tightly bonding water and glycerol molecules present in TPS, respectively. Similar results for thermoplastic starch-based films were reported elsewhere [30,31]. The presence of highly polar hydroxyl groups in starch could easily form intermolecular and intramolecular hydrogen bonds, which can be identified at 3394 cm^{-1} in the FTIR spectrum. The absorption vibration is affected by the number and intensity of hydrogen bonds present in starch molecules [32]. A broad band at 3375 cm^{-1} in both TPS/ CaCl_2 10 wt% and TPS/ CaCl_2 20 wt% composites indicate interactions with hydroxyl groups between CaCl_2 and TPS [32,33].

3.1.2. The Effect of CaCl_2 on the Thermal Behaviour of TPS

The changes in the thermal properties of the TPS films after the incorporation of CaCl_2 extracted from eggshell were evaluated by DSC analysis. Thermograms of virgin TPS, TPS/ CaCl_2 10 wt% and TPS/ CaCl_2 20 wt% composite films are shown in Figure 2. The glass transition temperature (T_g) slightly changed from 56.17 ± 0.89 °C for virgin TPS to 56.78 ± 0.51 and 55.81 ± 0.97 °C for TPS/ CaCl_2 10 wt% and TPS/ CaCl_2 20 wt% composites, respectively. This small decrease in the T_g values may indicate a lower barrier of activation energy for molecular motions to overcome in order to reach the rubber-to-glass transformation. It seemed that the hydrogen bonding formed between calcium ions and polysaccharide chains favoured chain mobility and reduced the T_g value. This glass-transition behaviour has been referred to as plasticization effect in the literature [32,34].

The change in the heat of fusion (ΔH_f) was calculated from the peak area in the second heating scan of DSC curves. ΔH_f value increased from 4.59 ± 0.24 and 7.97 ± 0.27 $\text{J}\cdot\text{g}^{-1}$ for virgin TPS and TPS/ CaCl_2 10 wt%, respectively, and decreased considerably to 0.20 ± 0.06 $\text{J}\cdot\text{g}^{-1}$ for TPS/ CaCl_2 20 wt% composite. The endothermic peak observed is attributed to the melting of the starch material, which occurs by the disruption of hydrogen bonding at the molecular level [35,36]. The enthalpy of melting of a 100% crystalline TPS depends on the starch source, such as maize, wheat or potato [37], and the moisture content [35]. Therefore, the exact degree of crystallinity of TPS was not calculated in this study. However, the significant reduction in the area under the melting peak observed when 20 wt% of CaCl_2 was added indicated that it could effectively reduce the degree of

crystallinity of TPS/CaCl₂ composites. It has been previously explained that CaCl₂ could effectively destroy the crystals of starch [32].

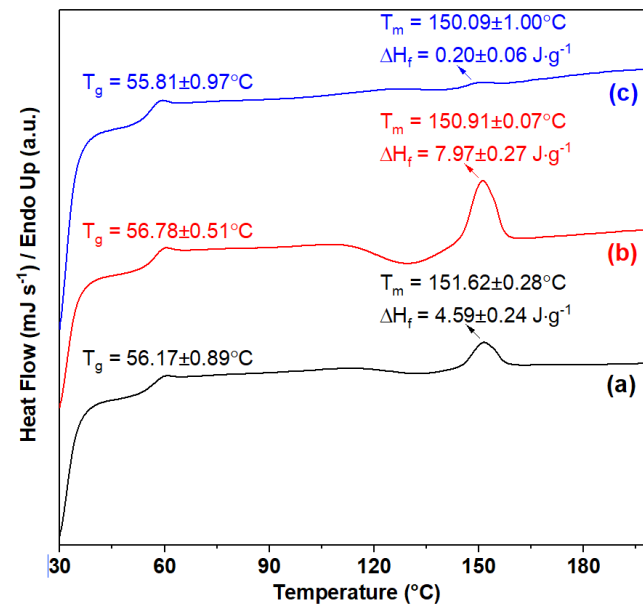


Figure 2. Thermograms of (a) virgin TPS, (b) TPS/CaCl₂ 10 wt% and (c) TPS/CaCl₂ 20 wt% composite films. T_g is the glass transition temperature, T_m is the melting temperature and ΔH_f is the fusion enthalpy change obtained from the peak area in the second heating scan of DSC curves. Average and mean values are referent to analysis in triplicates.

3.1.3. Mechanical Properties

The effect of the addition of CaCl₂ on the mechanical behaviour of the thermoplastic starch films was evaluated by tensile testing (Figure 3). It was found that the Young's modulus of TPS was not significantly affected by the incorporation of 10 wt% of CaCl₂, with 2100.3 ± 322.9 MPa ($P = 0.968$), but significantly reduced to 650.9 ± 32.7 MPa with the incorporation of 20 wt% of CaCl₂ ($P < 0.05$). This can be explained by the plasticizing mechanism and effect of CaCl₂ on the crystallization mechanism. Plasticizers increase the space between polymer chains, reduce and intermolecular bonding, thus decrease mechanical properties [38]. However, TPS/CaCl₂ 10 wt% composite with the maintained Young's modulus appeared to be a promising packaging material. The possible reason for a significant reduction in Young's modulus caused by high content of CaCl₂ (20 wt%) might be because of the plasticizing effect of CaCl₂ on TPS [39].

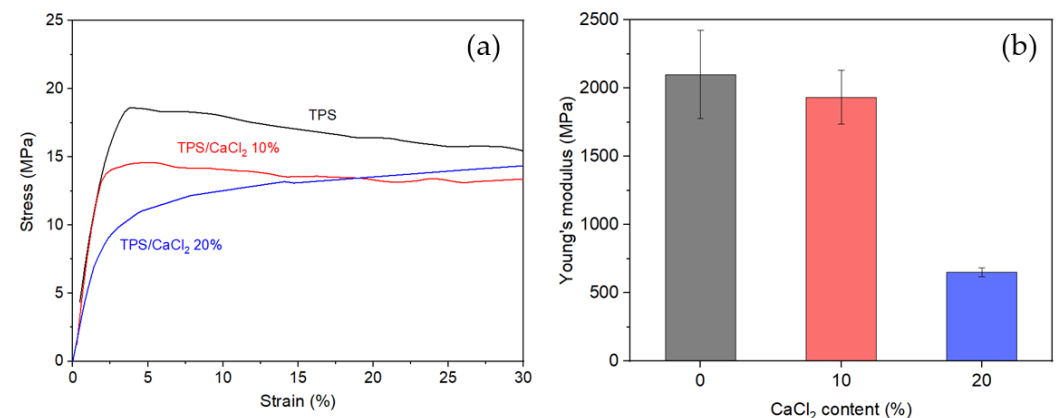


Figure 3. Effect of CaCl₂ addition on the mechanical properties of thermoplastic starch. (a) stress-strain curves and (b) Young's modulus of virgin TPS, TPS/CaCl₂ 10 wt% and TPS/CaCl₂ 20 wt% composite films.

3.1.4. The Effect of CaCl₂ on the Morphology of TPS

Scanning electron microscopy was used to investigate the morphology of TPS/CaCl₂ composite films. SEM micrographs of the virgin TPS (a) TPS/CaCl₂ 10 wt% (b) and TPS/CaCl₂ 20 wt% (c) material composites are shown in Figure 4. The elemental analysis by EDX confirmed that TPS is basically composed of carbon ($56.85 \pm 4.0\%$) and oxygen ($43.15 \pm 4.0\%$) while TPS/CaCl₂ 10 wt% contained $6.47 \pm 5.41\%$ of Ca and $9.30 \pm 6.08\%$ of Cl, and TPS/CaCl₂ 20 wt% incorporated $10.38 \pm 1.89\%$ of Ca and $17.31 \pm 0.77\%$ of Cl. SEM images showed that a uniform texture has been achieved for all films. Additionally, the TPS matrix is observed as a continuous grey background and the patches of the calcium salt can be observed and identified by EDX elemental analysis.

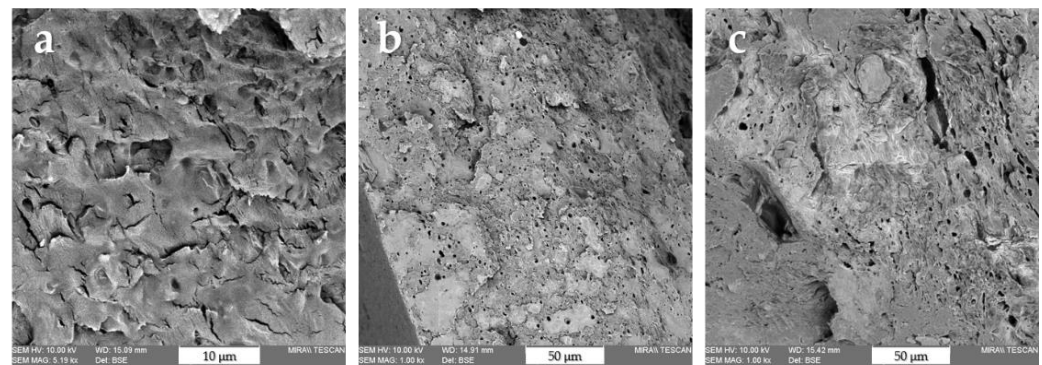


Figure 4. SEM micrographs of (a) virgin TPS, (b) TPS/CaCl₂ 10 wt% and (c) TPS/CaCl₂ 20 wt% composite films.

3.2. Physicochemical Analysis and Colorimetry of the Fresh-Cut Apple Slices

The pH value, weight loss ratio, reducing sugar, antioxidant activity and colour change are summarized in Table 1. The pH value is an important indicator for food freshness or shelf stability. After 14 days, all apple slices had a significant reduction in pH, from 4.41 ± 0.32 for fresh-cut apple slices to 3.91 ± 0.1 for apple slices placed on TPS/CaCl₂ 20 wt% composite films ($P = 0.049$), and 3.42 ± 0.08 for apple slices placed on TPS films ($P = 0.002$). The incorporation of CaCl₂ resulted in less of a decrease in the pH value. These results are aligned with the findings from Thakur's group, which investigated the effects of CaCl₂ solution on fresh-cut fruits, and reported that the fruits immersed in CaCl₂ solution exhibited higher pH value compared to the non-treated fruits [8]. The explanation could be that the reduction of pH value is related to the organic acid generated by microorganisms, and the embedded CaCl₂ inhibited the growth of microorganisms, thus resulting in a higher pH value.

Table 1. Colourimetry results and physicochemical analysis (pH, weight loss, reducing sugars, antioxidant activity) of fresh-cut apple slices determined after 14 days of contact with TPS and TPS/CaCl₂ 20 wt% films.

Apple Slices	Colourimetry				pH Value	Weight Loss %	Reducing Sugar %	Antioxidant Activity %
	<i>L</i> ¹	<i>a</i> ²	<i>b</i> ³	<i>c</i> ⁴				
Fresh-cut (day 0)	82.66 ± 0.86	1.09 ± 0.08	18.76 ± 0.47	18.79 ± 0.48	4.41 ± 0.32	N/A	0.21 ± 0.02	28.1 ± 3.4
14 days on TPS	72.97 ± 2.34	5.43 ± 0.98	24.8 ± 2.3	25.41 ± 2.15	3.42 ± 0.08	28 ± 2.2	0.58 ± 0.03	20.9 ± 1.6
14 days on TPS/CaCl ₂ 20 wt%	76.07 ± 1.34	4.13 ± 0.78	25.44 ± 2.53	25.77 ± 2.6	3.91 ± 0.1	32.7 ± 1.01	1.05 ± 0.26	24.8 ± 3.5

¹ *L* value corresponds to lightness/darkness (0 for black and 100 for white). ² *a* value corresponds to the specimen's colour dimension from red to green (the greater *a** value, the redder). ³ *b* value corresponds to the specimen's colour dimension from yellow to blue (the greater *b** value, the yellower). ⁴ *c* value corresponds to chroma (0 for achromatic colour and the greater the *c** value, the brighter).

The size of all the apple slices reduced noticeably, which indicated dehydration of the apple samples. The mass of the apple slices was measured at the beginning and by the end of the 14th day of the test, the weight loss was calculated. The apple slices placed on TPS/CaCl₂ 20 wt% composite films showed more weight loss with $32.7 \pm 1.01\%$ than the apple slices placed on TPS films with a weight loss of $28 \pm 2.2\%$ ($P = 0.029$). However, a study reported that papaya, muskmelon and guava samples immersed in CaCl₂ solution exhibited less weight loss than the samples not treated with CaCl₂ solution. One reason for this could be that the calcium strengthened the cell wall and preserved the structure and integrity of the cell membranes, thus resulting in less weight loss [8]. In this study, instead of being immersed in liquid, all the samples were placed on the top of the polymer films, dehydration occurred and the incorporated CaCl₂ increased the water loss, which was supported by a study on the effect of calcium on pineapple dehydration kinetics [40].

The reducing sugar is any sugar that contains free aldehyde or the ketone groups and is capable of acting as a reducing agent. The reducing sugar increases during the fruit ripening [25]. After 14 days, all the apple slice samples displayed a significant increase in reducing sugar ($P = 0.001$ for all comparisons), and the samples placed on TPS/CaCl₂ 20 wt% composite films showed a more significant increase than the samples placed on virgin TPS films ($P = 0.003$). However, several studies of fresh-cut fruits immersed in calcium solution exhibited a lower increase in reducing sugar than non-treated fruit samples, and this is because calcium solution delays the fruit ripening by inhibiting the activity of cell wall degrading enzymes [8,41]. In this study, the increased reducing sugar of the apple samples placed on TPS/CaCl₂ 20 wt% composite films was related to the dehydration of the samples.

The free radical scavenging activity of the apple slice samples was evaluated with DPPH. After 14 days, all the samples displayed a decrease in antioxidant activity ($P = 0.003$). The reason could be that free radicals were scavenged by the antioxidant molecule with time, thus reducing the amount of free radical and free radical scavenging activity [8]. A less reduction in antioxidant activity was observed in apple slices placed on TPS/CaCl₂ 20 wt% composite films, which could be explained by the less reduced antioxidant enzymes caused by CaCl₂, in comparison to virgin TPS [42].

The colour change in food is linked with pigment degradation and oxidation of ascorbic acid. After 14 days, all apple slices turned yellow. The *L* value, which indicates the brightness, decreased significantly after 14 days placed on both TPS films and TPS/CaCl₂ 20 wt% composite films ($P = 0.03$ for all comparisons), while the *a*, *b*, and *c* values increased significantly ($P = 0.05$ for *a* value; $P = 0.002$ for *b* value; $P = 0.001$ for *c* value). There was no significant difference in *L*, *a*, *b* and *c* values between apple slices placed on TPS films and apple slices placed on TPS/CaCl₂ 20 wt% composite films ($P = 0.512$ for *L* value; $P = 0.997$ for *a* value; $P = 1$ for *b* value; $P = 1$ for *c* value). However, Thakur's group reported that all the *L*, *a* and *b* values decreased significantly for both CaCl₂ treated and non-treated fruit samples, and CaCl₂ treated fruit samples displayed higher *L*, *a* and *b* values than non-treated samples [8]. The reason could be due to the fruit samples being immersed in CaCl₂ solution and thus became whiter. In contrast, the samples were dried and turned yellower in this study. The result of increasing *a*, *b*, and *c* value agreed with a study of the fruit drying process [43].

3.3. CaCl₂ Release from Starch-Eggshell Extract Composite Films

TPS/CaCl₂ 10 wt% and TPS/CaCl₂ 20 wt% composite film samples of approximately 100 mg each were employed in the releasing tests. A calibration curve was constructed using eggshell-based CaCl₂ solutions ranging from 18 to 325 mg·L⁻¹ following a linear relationship with the volume of EDTA consumed in the titrations to form the EDTA-Ca²⁺ ion complex. This curve was used to determine the amount of CaCl₂ released from the composite films and the results are summarized in Table 2. For comparison, virgin TPS control samples were also studied and, as expected, all tests were negative for the presence of calcium ions in the aqueous solution. The release of CaCl₂ from both composite films

did not seem to depend on the temperature over the first 48 h of assessment. However, after a period of 192 h, the amount of CaCl₂ released at 30 °C was slightly higher for both TPS/CaCl₂ 10 wt% (ca. 0.5 mg) and TPS/CaCl₂ 20 wt% (ca. 1.0 mg) when compared to the same tests at 4 °C. Results indicated that the release of CaCl₂ from the composite films was more sustained at 4 °C when compared with ambient temperature conditions (20 and 30 °C) after a week of storage.

Table 2. Amount of CaCl₂ released (mg) from TPS/CaCl₂ 10 wt% and TPS/CaCl₂ 20 wt% composite films after different incubation periods at 4, 20 and 30 °C.

Time (h)	TPS/CaCl ₂ 10 wt%			TPS/CaCl ₂ 20 wt%		
	4 °C	20 °C	30 °C	4 °C	20 °C	30 °C
3	1.08 ± 0.03	0.68 ± 0.01	0.97 ± 0.03	2.86 ± 0.03	3.32 ± 0.01	3.27 ± 0.03
6	1.30 ± 0.03	0.92 ± 0.03	1.14 ± 0.03	3.12 ± 0.07	3.68 ± 0.07	3.94 ± 0.11
24	2.05 ± 0.06	1.87 ± 0.01	2.14 ± 0.03	5.73 ± 0.01	6.76 ± 0.07	6.16 ± 0.06
48	2.47 ± 0.03	2.29 ± 0.01	2.50 ± 0.06	6.87 ± 0.01	7.57 ± 0.03	7.74 ± 0.08
72	2.54 ± 0.04	2.71 ± 0.01	2.91 ± 0.08	7.49 ± 0.17	8.38 ± 0.17	8.57 ± 0.08
192	2.60 ± 0.01	2.93 ± 0.04	3.16 ± 0.06	8.45 ± 0.01	8.84 ± 0.01	9.49 ± 0.17

Figure 5 displays the accumulative release in percentage over the releasing time studied. Based on the maximum filler content, calculated from the weight of the salt initially added to the films, it was clear that the composite material containing 20 wt% of CaCl₂ released around 15% more CaCl₂ than the TPS/CaCl₂ 10 wt% film after 192 h of testing.

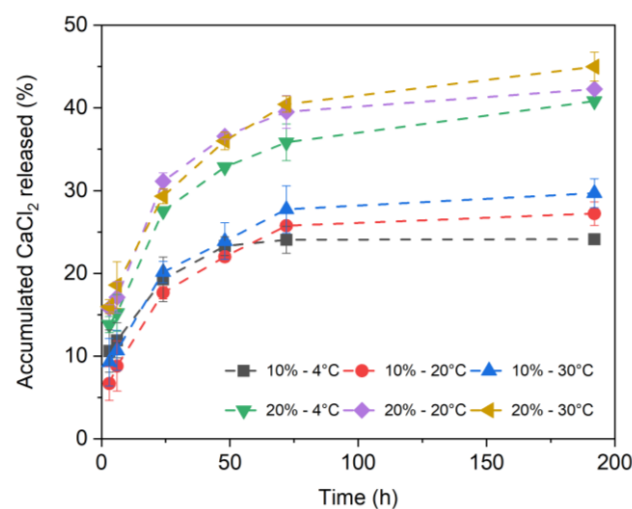


Figure 5. Accumulated CaCl₂ released (%) from composite films after different incubation periods at 4, 20 and 30 °C.

Consequently, it appeared that the CaCl₂ release mechanism depended upon the initial concentration of the salt used in the composition of the composite films. In a previous study, Longano et al. evidenced that the release of copper ions from a thermoplastic polymer (Polylactic acid) composite for antibacterial food packaging applications is also dependent on the concentration of the metal ions [44].

3.4. Microbial Growth on Fresh-Cut Apple Slices

The effect of calcium chloride extracted from eggshell in preventing the microbial colonization on fresh-cut apple slices incubated at 20 °C can be seen in Table 3. Throughout the 28-day incubation, no microbial colonization was observed on any apple slices placed on TPS/CaCl₂ 20 wt% composites. Regarding the virgin TPS, fungal colonization was observed from day 21. On day 21 one out of three replicates presented with visible fungal

growth on the surface of the apple flesh; on day 23, two out of three replicates were contaminated with fungi; by the final day of the study all samples placed in TPS film were supporting fungal growth. These results would propose that TPS/CaCl₂ 20 wt% composites could prevent cut fruit spoilage for ≥ 28 days.

Table 3. Effect of calcium chloride extracted from eggshell on preventing the microbial colonization on fresh-cut apple slices incubated at 20 °C.

Day	Colony Forming Units·mL ⁻¹							
	Total Mesophilic Bacteria		Coliforms		<i>Staphylococcus aureus</i>		Yeasts/Moulds	
	TPS	TPS/CaCl ₂ 20 wt%	TPS	TPS/CaCl ₂ 20 wt%	TPS	TPS/CaCl ₂ 20 wt%	TPS	TPS/CaCl ₂ 20 wt%
2	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
7	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
9	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
14	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
16	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
21	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	445 ± 770	0 ± 0
23	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	879 ± 826	0 ± 0
28	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	843 ± 67	0 ± 0

3.5. Antibacterial Activity of the TPS/CaCl₂ Composite Films

Antibacterial activity was assessed by OD assay to evaluate the inhibitory capacity of the composite films against the growth of two common Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria strains (Figure 6). Minimal inhibition concentration of eggshell extract itself was estimated to the value of 100 µg·mL⁻¹ against *S. aureus*.

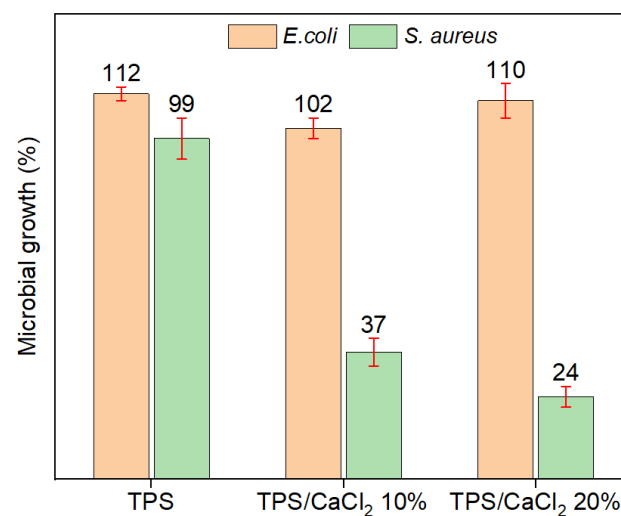


Figure 6. Microbial growth (%) dependent on the initial percentage of CaCl₂ in composite films. 100% is relative to the value for positive control sample (maximal bacterial growth).

It was shown that eggshell extract composites expectedly had no inhibitory effect on tested Gram-negative *E. coli*, while in the case of Gram-positive *S. aureus* strain, growth was reduced by ca. 63% and 76% for the TPS/CaCl₂ 10 wt% and TPS/CaCl₂ 20 wt% composite films, respectively. The growth inhibitory behaviour observed for *S. aureus* is in accordance with the CaCl₂ release results, which suggest that the inhibition activity is enhanced by increasing the concentration of salt in the formulation and, consequently, the release of Ca²⁺ ions from the TPS/CaCl₂ composites. The initial salt loading effect is supported by a previous work that reported the relationship between copper ions release and antimicrobial behaviour of polymer/metal composites in which the bactericidal effect against *S. aureus* was found to be proportional to the metal ion concentration [45]. Although the mechanism by which Ca²⁺ ions affect the bacterial growth has not been completely unveiled, the use of divalent cations has been reported as one of the most effective chemical treatments to

inhibit bacterial growth by neutralizing the surface charges of the bacteria and weakening bacterial membranes with the formation of pores induce bacterial transformation through a dual-role process of neutralizing the charges and weakening of cell surface to promote the formation of pores on bacterial membranes [46].

4. Conclusions

Calcium salt is used as a firming agent to maintain the quality of fresh fruits and vegetables. It is usually applied to fruits and vegetables via dip coating and spraying. In this study, a method of incorporating calcium chloride into a packaging material, thermoplastic starch, by means of a hot-melt extrusion process was described. Calcium chloride leached out from the packaging material, thus preserved the fruits. In addition, the calcium chloride used in this study was extracted from eggshell waste, since eggshells are rich in calcium salt. It was found that thermoplastic starch with 10 wt% calcium chloride might be a good option as a packing material for fresh-cut fruits, due to the active antimicrobial activities and maintained Young's modulus.

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