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Original article

Potential of chitosan films immobilized with carboxylesterase on cucumber and grape preservation in post-harvest and degradation of carbamate and organophosphorus pesticides

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Summary

Introduction – **Much attention has been recently drawn to improve the shelf life of fresh fruits and vegetables by using biodegradable and edible film coatings. In addition, the minimization of hazardous pesticides in these products was recently established with the immobilization of pesticide-degradable enzymes into the coating film because of a public awareness of the health risks posed by pesticide residues in fruits and vegetables.** *Materials and methods* – **The present study reports the property of novel complexes of biopolymer chitosan/starch film-forming materials on the preservation quality of cucumber and grape fruits and degradation of carbamate and organophosphorus insecticides with immobilized pesticide-degradable enzyme (carboxylesterase, CbE) during cold storage. Principal component analysis (PCA) was performed on the data matrices consisting of two types of fruits and different sorts of parameters. The residues of tested insecticides were extracted from fruit samples using the Quick Easy Cheap Effective Rugged and Safe (QuEChERS) technique at different time intervals of cold storage.** *Results and discussion* – **The fruit quality parameters after 21 d of cold storage indicated that the chitosan coatings decreased the weight loss, total soluble solids (TSS), catalase, guaiacol peroxidase (G-POD), and polyphenol oxidase (PPO) compared to the control. However, the treatments increased the total soluble phenolics compared to the uncoated fruits. The PCA analysis reported that two (unrotated) significant principal components (PCs) explained more than 73% of the total variance in the data. The plots of component loadings showed significant groupings for tested parameters in fruits. The chitosan film-forming formulations degraded or chelated some residues of the insecticides however, the disappearance of the insecticides was increased with the formulations having CbE. The pesticides determination carried out by HPLC showed recoveries of these residues over 72%.** *Conclusion* – **These formulations could be used in coating of fresh fruits and vegetables containing low concentrations of organophosphorus and carbamate pesticides.**

Significance of this study

What is already known on this subject?

- Edible film coatings based on chitosan offer fruit and vegetable shelf life advantages including edibility, biocompatibility, barrier to gas properties, non-toxicity to mammals and having low cost comparing to synthetic polymers.

What are the new findings?

- The conditions for which these films incorporating immobilized pesticide-degradable enzymes have been established.

What is the expected impact on horticulture?

- This technology will combine extended shelf life of cold stored fresh fruits and vegetables and removal of harmful pesticide residues.

Keywords

chitosan edible coating, carboxylesterase, *Cucumis sativus*, *Vitis* spp., pesticide degradation

Résumé

Potentiel des films de chitosan avec carboxylestérase immobilisée sur la conservation du concombre et du raisin et sur la dégradation des pesticides du groupe des carbamates et organophosphorés.

Introduction – **Une grande attention a été portée récemment sur l'amélioration de la durée de conservation des fruits et légumes frais en utilisant un enrobage de film biodégradable et comestible. De plus, il a été récemment établi que l'immobilisation d'enzymes dans le film d'enrobage contribuait à atténuer la dangerosité des pesticides contenus dans ces produits. Notre étude a visé à établir les conditions pour lesquelles ces films pourraient être utilisés pour répondre aux risques pour la santé posés par les résidus de pesticides dans les fruits et légumes frais.** *Matériel et méthodes* – **La présente étude décrit les propriétés de nouveaux matériaux biopolymères filmogènes en chitosan/amidon sur la préservation de la qualité du concombre et du raisin au cours du stockage au froid, et sur la dégradation d'insecticides du groupe des**

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carbamates et organophosphorés grâce à une enzyme biodégradable, la carboxylestérase (CbE). Une analyse en composantes principales (ACP) a été effectuée sur les matrices de données constituées par les deux espèces et par différents types de paramètres. Les résidus d'insecticides testés ont été extraits à partir d'échantillons de fruits par la méthode Quick Easy Cheap Efficaces Rugged and Safe (QuEChERS) à différents intervalles de temps au cours du stockage au froid. *Résultats et discussion* **– Les attributs de qualité des fruits après 21 jours à 4 °C indiquent que les enrobages de chitosan diminuent la perte de poids, la matière soluble totale (TSS), les activités catalase, guaiacol péroxydase (G-POD) et polyphénol oxydase (PPO) comparativement au témoin. Cependant, les traitements ont augmenté les concentrations en composés phénoliques solubles par rapport aux fruits non enrobés. L'ACP a révélé que deux composantes principales (sans rotation) expliquaient de façon significative plus de 73% de la variance totale des données. La projection des composants en charge a montré des regroupements significatifs des paramètres testés sur fruit. Les formulations filmogènes de chitosan ont dégradé ou chélaté quelques résidus d'insecticide, mais la disparition de ces insecticides a réellement augmentée avec les formulations contenant la CbE. La mesure des pesticides effectuée par HPLC a permis de récupérer ces résidus à plus de 72%.** *Conclusion* **– Ces formulations peuvent être utilisées dans l'enrobage de fruits et légumes frais contenant de faibles concentrations de pesticides organophosphorés et de carbamates.**

Mots-clés
enrobage de film de chitosan, carboxylestérase, *Cucumis sativus*, *Vitis* spp., dégradation d'insecticides

Introduction

Synthetic pesticides are widely used in agriculture as they are considered economically important for improve crop quality and quantity production (Hoffman *et al*., 2000; Von Rumker, 1975; Hildebrandt *et al*., 2008). In addition, in today's world of extensive importing and exporting of food goods, the analysis and monitoring of pesticides is essential, although challenging (do Nascimento *et al*., 2016; Garcia and Teixeira, 2017). High residues of pesticides have been found in all fruits and vegetables (Fillion *et al*., 2000; Lehotay *et al*., 2005). Among these residues, chlorpyrifos as an organophosphorus insecticide and methomyl as a carbamate insecticide are marketed commercially in Egypt and used for foliar treatments of fruit and vegetable crops according to the Egyptian Agricultural Ministry (Khay *et al*., 2006; Zabik *et al*., 2000; Rashwan *et al*., 1991; Rabea *et al*., 2017). Public awareness about the health risks posed by pesticide residues in fruits and vegetables led to the development of many analytical techniques (Štajnbaher and Zupančič-Kralj, 2003; Storck *et al*., 2017) for detection and measuring these harmful residues (Chai and Tan, 2009; Multer *et al*., 2017; Badawy and El-Aswad, 2014). Now much attention was paid to improve the shelf life and degrade the pesticide residues in fruits and vegetables. Therefore, an immobilized-enzyme system was examined for its ability to hydrolyze residual pesticides in fruits and vegetables (Badawy and El-Aswad, 2014; Xie *et al*., 2010; Wheelock *et al*., 2005; Munnecke, 1976; Brooks, 1972; Gershater *et al*., 2006). Yun and others indicated that the immobilized enzyme might efficiently degrade fenvalerate as a pyrethroid insecticide (Yun, 1999). Degradation of organophosphorus pesticides using immobilized phosphotriesterase from *Pseudomonas diminuta* was also investigated (Caldwell and Raushel, 1991). However, the information regarding to the immobilization and characterization of enzymes that could be degraded chlorpyrifos and methomyl is still limited.

Recently, there has been a growing interest in the development of materials having a film forming ability such as chitosan that can be used to improve food safety and shelf life (Bosquez-Molina and Zavaleta-Avejar, 2016; Robertson, 2016). Chitosan is considered a conservative ideal coating material for fresh fruit because of its excellent film-forming and biochemical properties. It is a natural cationic co-linear polymer of β-(1-4)-D-glucosamine and *N*-acetyl-β-(1-4)-D-glucosamine units, and is obtained by deacetylation of chitin (Muzzarelli, 1983; Rabea *et al*., 2003). Chitosan coating is also safe as an edible film and has antimicrobial activity against a broad spectrum of plant pathogens (Badawy and Rabea, 2016). In addition, the structure of chitosan has many amino, hydroxyl, and acetyl groups that are very important functional sites for crosslinking and allowing for excellent complexing capacity with metal ions (Wan *et al*., 2010; Mohamed *et al*., 2013; Badawy *et al*., 2016).

Therefore, the objective of the current study was to prepare novel complexes of chitosan/starch film-forming materials with Ca(II) and Mg(II) ions with immobilized pesticide-degradable enzyme (carboxylesterase, CbE). The effect of these coatings on fruit of cucumber and grape was studied for the preservation quality and degradation of carbamate and organophosphorus insecticides during the storage in cold conditions. Cucumber and grapes were selected as common important agricultural commodities in Egypt and around the world and could be rapidly destroyed by microbial attack in postharvest stage. They contain a high abundance of health-benefiting substances that have antioxidant activity, cleansing action of toxins and waste, and prevention of constipation. In this study, the residues of carbamate and organophosphorus insecticides were extracted and purified using "quick, easy, cheap, effective, rugged, and safe" (QuEChERS) method and determination by HPLC. These formulations could be extended to the biodegradation and chelating of other pesticides having ester group. It can also be used as a platform for the rational design of film coatings for various applications as well as for the manufacture of other types of enzymatic hydrolysis of organic pollutants.

Materials and methods

Chemicals and insecticides

Chitosan derived from shrimp shell with a degree of acetylation ≤15%, D-sorbitol ≥98%, ethanol, α-naphthyl acetate (α-NA), α-naphthol, Fast Blue B salt, bovine serum albumin (BSA), *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD), gallic acid, polyvinylpyrrolidone (PVP), pyrocatechol, sodium-potassium tartarate, guaiacol, disodium EDTA, thiobarbituric acid (TBA), and Folin-Ciocalteu phenol reagent were purchased from Sigma-Aldrich Co. (Spruce Street, St. Louis, MO, USA). Carboxylesterase (CbE) in lyophilized form was isolated and purified from rabbit liver (MW estimated to be 30 kDa) (Rabea *et al*., 2017). The enzyme showed specific activity of 1.36 µmol α-naphthol hydrolyzed mg protein^{-1} min^{-1} using α-naphthyl acetate.

Corn starch, calcium chloride, magnesium chloride, tween 80, and all other commercially available solvents and reagents were from of analytical grade and purchased from Algomhoria Chemical Co., Alexandria, Egypt. Acetonitrile and water of HPLC quality were from Merck (Darmstadt, Germany). PTFE syringe filter (0.2 µm) and the bulk amino sorbent (primary secondary amine, PSA, bonded silica) was obtained from Supelco, Sigma-Aldrich Co. (Spruce Street, St. Louis, MO, USA). The organophosphorous insecticide chlorpyrifos (≥ 98% technical grade) was obtained from Dow AgroSciences LLC Co. and the carbamate insecticide methomyl (≥ 99.4% technical grade) was obtained from DuPont Crop Protection Co. Formulated chlorpyrifos (clorzan 48% EC) and methomyl (Neomyl 90% SP) were obtained from Kafr El Zayat Pesticides and Chemicals Co. (Kafr El Zayat, Gharbia, Egypt). Technical pesticides stock solutions were prepared in acetonitrile however their formulations were freshly prepared in distilled water and were not used for more than 3 h after preparation to minimize the degradation.

Fruit samples

Cucumber and grape samples grown under organic conditions were selected as common important agricultural commodities in Egypt. Fresh untreated samples were collected from the Agricultural Dana Farm at the Nobaria city, El Beheira Governorate for the laboratory experiments. The fruits were transported to the laboratory and then they were graded for uniform size, shape and color and for being free of visible defect and decay.

Preparation of edible chitosan film-forming formulations

Chitosan stock solution (2%, w/v) was prepared in 1% (v/v) aqueous acetic acid solution. To achieve complete dispersion of chitosan, the solution was stirred overnight at room temperature, filtrated through three layers of cheesecloth to remove impurities. Starch solution having a concentration of 2% (w/v) was prepared by dispersing corn starch in distilled water and heating the mixture on hotplate with stirring until it gelatinized and then cooled to 25 °C. Coating solutions were prepared by mixing solutions of chitosan and starch to give final ratios of 1:1 (%) at pH 4.0 and at 60 °C. The resulting mixtures were gently stirred at 40 °C for 30 min. Sorbitol as a plasticizer was added to the coating film forming solutions at 0.5% (w/v) based on the final solution of the polysaccharide. Chitosan-Ca and chitosan-Mg films were also prepared by the same previous composite polymers with molar ratio of 1:1 (chitosan: $CaCl_2$ and chitosan: $MgCl_2$) calculated to glucosamine unit of chitosan molecule. The mixture was stirred for homogenization under aseptic conditions for 5 min. Tween 80 as a surfactant (0.05%) was added based on the final formulation. The solutions were incubated at 40 °C for 30 min with gentle stirring. The formulations were mixed with 1,000 mg L^{-1} of the lyophilized CbE with 10 mmol L^{-1} of glutaraldehyde as a crosslinking agent and stored at 4 °C until use. The protein content in the solutions was determined by Lowry method (Lowry *et al*., 1951) and a protein standard curve was generated using BSA. The treatments were as follows: C^-: negative control (treated with sterile distilled water); C^+: positive control (treated with pesticide and inoculated with fungus); T1: chitosan (1%) + starch (1%) + sorbitol (0.5%) + tween (0.05%); T2: chitosan (1%) + starch (1%) + sorbitol (0.5%) + tween 80 (0.05%) + enzyme; T3: chitosan (1%) + starch (1%) + sorbitol (0.5%) + tween 80 (0.05%) + $CaCl_2$; T4: chitosan (1%) + starch (1%) + sorbitol (0.5%) + tween 80 (0.05%) + $CaCl_2$ + enzyme; T5: chitosan (1%) + starch (1%) + sorbitol (0.5%) + tween 80 (0.05%) + $MgCl_2$; and T6: chitosan (1%) + starch (1%) + sorbitol (0.5%) + tween 80 (0.05%) + $MgCl_2$ + enzyme.

Application of insecticides on fruit samples

Under laboratory conditions, the fruits were treated with methomyl and chlorpyrifos formulations by dipping in the field application rate of both insecticides (1.5 g L^{-1} for methomyl and 3.75 g L^{-1} for chlorpyrifos) for one minute.

Coating application with chitosan film forming formulations

Fruit samples were randomly distributed into eight groups. Two groups provided the untreated controls (negative and positive) and six groups were assigned to the coating formulation treatments (T1–T6 as indicated before). The fruits were dipped into each treatment for one min in and then allowed to dry for 1 h at 25 °C. Negative control was dipped and treated with sterile distilled water. Fruits were then packed in covered plastic boxes (200 × 130 × 70 mm) and stored for 21 d at 4 °C and 95% RH. Three replicates (250 g each) were used for each treatment.

Fruit quality parameters

Weight loss

Fruit samples were weighed at the beginning of the experiment (*i.e.*, day 0) and at the end of experiment (21 d) during storage at 4 °C and 95% relative humidity (RH). The difference between the initial and final weight of the fruit was considered as a total weight loss and the results were expressed as the percentage loss of the initial weight as the standard method of AOAC (1995).

Total soluble solids (TSS)

The TSS content was determined at 20–22 °C using digital refractometer (Atago Co., Tokyo, Japan) in fruit juice and a direct reading was taken as described in AOAC method (Cunniff and Jee, 1995). The values are mean of six replicates and given as mean ± standard error.

Total soluble phenolics (TSP) assay

A weight (0.5 g) of fruit was frozen for 48–72 h with 10 mL of 95% ethanol and then homogenized with a Tissue Tearor (BioSpec 985370-04 Homogenizer). The homogenate was centrifuged at 10,000 rpm for 10 min. A milliliter of the resulting supernatant was combined with 1 mL of 95% ethanol, 5 mL of distilled water and 0.5 mL of 50% Folin-Ciocalteu phenol reagent. After five min incubation period at room temperature, 1 mL of 5% (w/v) sodium carbonate was added followed by brief vortexing to mix. The mixture was incubated for 1 h in a dark-cupboard and the absorbance was measured at 725 nm by spectrophotometer (Alpha-1502 UV-Visible Spectrophotometer, LAXCO, Inc., Bothell, Washington, USA). A standard curve was established using a gallic acid (GA) in 95% ethanol (0.05–0.50 mg L^{-1}). Total phenolics content was standardized against gallic acid and absorbance values were converted to µg GA equivalents (GAE) g^{-1} on a fresh weight basis using a constant K of 1.0883 obtained from GA standard curve with $R^2 = 0.9961$ (y = 1.0883x, where y is the absorbance value of the unknown sample and x is the concentration of total phenolics based on GA in mg L^{-1}) (Burguieres *et al*., 2007).

Catalase activity

Fruit tissue (2 g) was homogenized in 10 mL of 0.1 mol L^{-1} phosphate buffer (pH 7.0) and the homogenate was filtered through three layers of cotton cloth to remove the cell fragments. The clear supernatant after centrifugation at 10,000 rpm for 15 min at 4 °C was collected as enzyme extract. Catalase was determined according to Change and Maehly method (Change and Maehly, 1955) by monitoring the disappearance of H_2O_2 by recording the decrease in absorbance at 240 nm of a reaction mixture containing 50 mmol L^{-1} sodium phosphate buffer (pH 7.0), 12.5 mmol L^{-1} H_2O_2, and 20 µL of enzyme extract. Enzyme activity was calculated as U mg^{-1} min^{-1}. One unit of catalase activity is defined as the amount of enzyme that decomposes 1 mmol of H_2O_2 mg^{-1} protein min^{-1} under the conditions of the assay.

Guaiacol peroxidase (G-POD) assay

G-POD was determined according to the method described by McCue and co-authors (2000). The enzyme reaction mixture consisted of 0.1 mol L^{-1} potassium phosphate buffer (pH 6.8), 50 mmol L^{-1} guaiacol, and 0.2 mmol L^{-1} hydrogen peroxide. To 1 mL of the above reaction mixture was added 50 µL of the enzyme extract in phosphate buffer (pH 7.0). The increase in absorbance (*i.e.*, production) of tetraguaiacol was assayed over a period of 5 min at 470 nm. The rate of change in absorbance obtained was then used to quantify the enzyme activity in the mixture using the extinction coefficient of tetraguaiacol (the oxidized product). The millimolar extinction coefficient of tetraguaiacol at 470 nm is 26.6 mmol L^{-1} cm^{-1}. Guaiacol peroxidase activity was reported as nmol tetraguaiacol mg^{-1} min^{-1}.

Polyphenol oxidase (PPO) activity

The activity of PPOase was determined by mixing 1.5 mL of pyrocatechol (0.2 mol L^{-1}), 1.4 mL phosphate buffer (pH 6.8) and 0.1 mL enzyme extract (Han *et al*., 2008). The mixture was read immediately at 420 nm by Spectrophotometer, changes in A_{420} recorded for 5 min, and expressed as U mg^{-1} min^{-1} (one unit equal the change in A_{420} of 0.001 per minute at pH 6.8 and reaction mixture at 25 °C).

Extraction of insecticides by QuEChERS method

QuEChERS procedure involves initial extraction with acetonitrile followed by a step of extraction/separation after adding a mixture of salts. An aliquot of the crude extract is then cleaned by solid phase extraction with dispersive PSA solid-phase extraction (Payá *et al*., 2007; Anastassiades *et al*., 2007). Samples were taken at 0, 1, 3, 7, 10, 14, and 21 d of the application. Accurately weight of 50 g was taken of sample in a clean beaker and homogenized. Two homogenized grams of cucumber and grape samples were weighed into centrifuge tubes of 25 mL. The main extraction involved the addition of 5 mL of 1% acetic acid in acetonitrile. The tube was closed and stirred vigorously by hand for 1 min. To induce phase separation and pesticides partitioning, buffer and salt mixture (composed of 0.5 g of anhydrous magnesium sulfate, 0.1 g sodium chloride, 0.15 g of sodium acetate) was added to the slurry from the first extraction. The tube was closed, shaken vigorously by hand for 1 min and was centrifuged for 1 min at 2,000 rpm.

Clean-up by D-SPE technique

The cleanup step was carried out following the QuEChERS method as described before with dispersive SPE (D-SPE) technique (Payá *et al*., 2007; Anastassiades *et al*., 2007). The organic layer (acetonitrile) was transferred to a 10-mL centrifugation tube containing 0.05 g of PSA, 0.15 g of $MgSO_4$ and 0.05 g activated charcoal per mL extract to remove water and undesired co-extractives. The tube was closed, shaken vigorously by hand for 30 s, and centrifuged for 5 min at 3,000 rpm. Clear supernatant was collected into HPLC vials.

Pesticides standard preparation for HPLC analysis

For preparation of stock solution, standards of chlorpyrifos and methomyl were dissolved in acetonitrile (10 mg L^{-1}), considering standard purity, by accurately weighing individual analytical standards into volumetric flasks, dissolving and diluting them to volume with acetonitrile and stored at 4 °C in the dark. Working standard solutions were prepared daily by appropriately diluting multiple stock solutions with acetonitrile.

HPLC analysis

Chlorpyrifos and methomyl residues in cucumber and grape fruits were quantitatively analyzed using an Agilent 1260 HPLC Infinity system (Germany) equipped with an Agilent variable wavelength ultraviolet detector (VWD). Ten microlitre of each sample was injected onto the HPLC column using the autosampler apparatus. The separation was performed with a reversed-phase column (RP-C_{18}, ZORBAX Eclips Plus C18, 4.6 × 250 mm, 5 µm) with the column oven at 25 °C. The system consisted of quaternary gradient solvent pump to control the flow rate of the mobile phase and an autosampler for automatic injection. The flow rate was 1 mL min^{-1} (acetonitrile and acetonitrile/water, 60:40 for chlorpyrifos and methomyl, respectively), and the variable wavelength UV detector was set at 233 nm for methomyl and 289 nm for chlorpyrifos. Residue peaks were tentatively identified on the basis of retention times (chlorpyrifos 3.73 ± 0.01 min and methomyl 2.74 ± 0.01 min). Residue amounts were calculated by comparing peak height to peak height obtained from known amount of each insecticide. Standard curves for chlorpyrifos and methomyl were also obtained by HPLC. Different quantities (0.0125–0.075 µg) of chlorpyrifos and methomyl were injected into HPLC with constant injected volumes of 5 µL. The calibration curve was linear up to 0.30 µg of each compound and the correlation coefficient was > 0.99. Detection limits were approximately 0.001 µg per injection taken as a signal/noise ratio.

Statistical analysis

Experimental data are presented as mean ± standard error and the statistical analysis was performed by the SPSS program (ver. 21.0, USA). Analysis of variance (ANOVA) of the data was conducted and means property values were separated ($p \leq 0.05$) with Duncan's multiple range test. In addition, data collected from various parameters were subjected to Principal Component Analysis (PCA) using SPSS software. Fruit quality parameters included weight loss, TSS, TSP, catalase, G-POD, and PPO were taken as variables. The method of PCA makes use of intercorrelations starting from the correlation matrix of the variables. It eliminates the redundancy from the data; that is, it reduces the dimensionality of the data by revealing several underlying components. The underlying components are represented by new variables called principal components. Their values are the component scores. The principal components are, in fact, linear combinations of the original variables and vice versa. The linear coefficients of the inverse relation of linear combinations are called the component loadings, that is, the correlation coefficients between the original variables and the principal components.

Results and discussion

Fruit quality

The quality parameters include weight loss, TSS, TSP, catalase, G-POD, and PPO in the cucumber and grape fruits coated and uncoated after 21 d of storage at 4 °C were determined. The weight loss rate is one of the most direct indices used to assess the conservation impact of fresh fruits and vegetables. It is mainly caused by respiration and evaporation of the moisture content through the fruit skin. Figure 1A showed that the weight loss of the coated cucumber and grape fruits significantly decreased during storage (1.31–3.07%) compared with the uncoated fruits (4.13 and 10.72% for cucumber and grape, respectively). In comparison to the controls (C^+ and C^-), fruits coated with chitosan/starch-Ca film-forming formulation immobilized with CbE exhibited the lowest weigh loss values (1.31 and 1.34% for cucumber and grape, respectively).

The TSS contents of the uncoated and coated fruits with different chitosan composite formulations after storage for 21 d at 4 °C are shown in Figure 1B. The highest TSS of cucumber fruits was found at the initial time (3.58%) and then declined after 21 d of storage. All the TSS of cucumber fruits coated by chitosan/starch formulations were slightly lower (2.83–3.57%) than in positive control (3.77%). However, the value in C^- was significantly lower (2.10%) than initially obtained and C^+. This may be referring to that some deterioration occurred with this uncoated and untreated with the insecticide. These results confirmed that the chitosan/starch film-forming formulations were better for maintaining the TSS content compared to the controls.

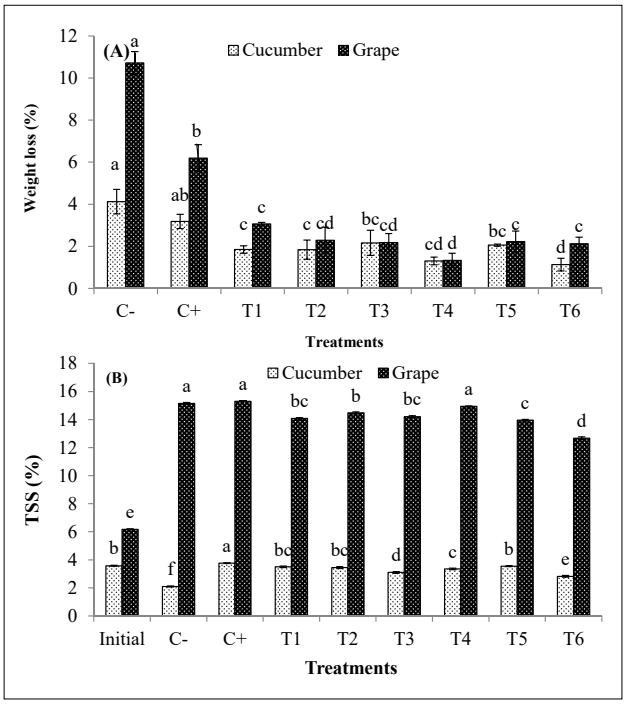

Figure 1. Effect of chitosan film-forming formulations on weight loss (A) and TSS (B) in the uncoated and coated cucumber and grape fruits after 21 days of storage at 4 °C. C^-: Negative control (treated with sterile distilled water); C^+: Control positive (treated with pesticide); T1: Chitosan/starch formulation; T2: Chitosan/starch-CbE formulation; T3: Chitosan/starch-Ca formulation; T4: Chitosan/starch-Ca-CbE formulation; T5: Chitosan/starch-Mg formulation; and T6: Chitosan/starch-Mg-CbE formulation. Values are mean of six replicates and given as mean ± standard error. Different letters (a-f) on the bars indicate the range from higher to lower rank as significant differences according to the Duncan's multiple range test ($P \leq 0.05$).

In grape fruits (Figure 1B), the TSS values were higher than that obtained in cucumber and grapes coated by chitosan/starch formulations reached their maximum values (12.68–15.30%) at day 21 compared to 6.18% at zero time. This result is referring to that the grapes presented a high soluble sugar content and maturation development during the storage period. Among all film-forming formulations, chitosan/starch-Mg (T5), and chitosan/starch-Mg-CbE (T6) showed high effect in maintaining lower TSS accumulation (13.95 and 12.68%, respectively) at day 21 compared to the other formulations. These results are consistent with other studies that reported the fruits coated with chitosan showed low level of TSS resulting delaying the ripening process (Mali and Grossmann, 2003; Gol *et al*., 2011, 2013; Ali *et al*., 2011; Chien *et al*., 2007). Ghasemnezhad and others reported that there was no significant difference for TSS content in coated and uncoated of apricot fruits storage with chitosan (Ghasemnezhad and Shiri, 2010). However, Moalemiyan and Ramaswamy reported an increase in the TSS of cucumber fruits (coated with a Pectin-based film and control) up to 10 days of treatment, then a decrease up to day 15 (Moalemiyan and Ramaswamy, 2012). Arjun *et al*. showed negligible or very little changes of TSS during storage for fresh-cut apple, papaya, carrot and cucumber coated by chitosan-soy based edible coating formulation (Arjun *et al*., 2015).

The changes in the TSP contents in cucumber and grape fruits after 21 d with storage at 4 °C are presented in Figure 2A. Usually the accumulation of phenolic compounds occurred in many fruits and vegetables in postharvest phase, where cells rapidly synthesize a larger quantity of phenolic acids as a defense for wound healing and to provide disease resistance (Ali *et al*., 2011; Zhang and Quantick, 1997). In the present study, it was observed that the TSP content significantly increased during storage of fruits in chitosan/starch film-forming formulations (T1–T6) as well as in the control samples compared to the initial (9.53 and 10.97 mg kg^{-1} for cucumber and grape, respectively). Similar results were confirmed by other studies reporting that the chitosan coatings maintained the high level of the TSP content or induced the production of phenolic compounds in grapes, tomatoes, litchi, apricot, pepper, cucumber, and strawberry fruits (Gol *et al*., 2013; Zhang and Quantick, 1997; Badawy and Rabea, 2009; Sánchez-González *et al*., 2011; Xing *et al*., 2011; Liu *et al*., 2007; Poverenov *et al*., 2014; Zhang *et al*., 2015; Wang and Gao, 2013).

Application of chitosan-based edible coating on cucumber reduced significantly the catalase activity after 21 days of storage at 4 °C (16.95–47.45 U mg^{-1} min^{-1}) compared to 136.10, 212.66, and 62.39 for initial, negative control, and positive control, respectively (Figure 2B). In addition, it was significantly decreased in grapes to the range from 3.39 to 14.56 U mg^{-1} min^{-1} (T1–T6) compared to 30.67, 22.66, and 28.00 for initial, negative control, and positive control, respectively.

The data shown in Figure 2C indicate that the untreated control samples reached the highest levels of G-POD after 21 days of storage. Values of 98.81 and 135.17 nmol tetraguaiacol min^{-1} mg^{-1} found in C^- and C^+ of cucumber, respectively. However, the values of 39.40 and 34.11 for C^- and C^+ of grapes respectively compared to 30.21 and 21.80 at initial

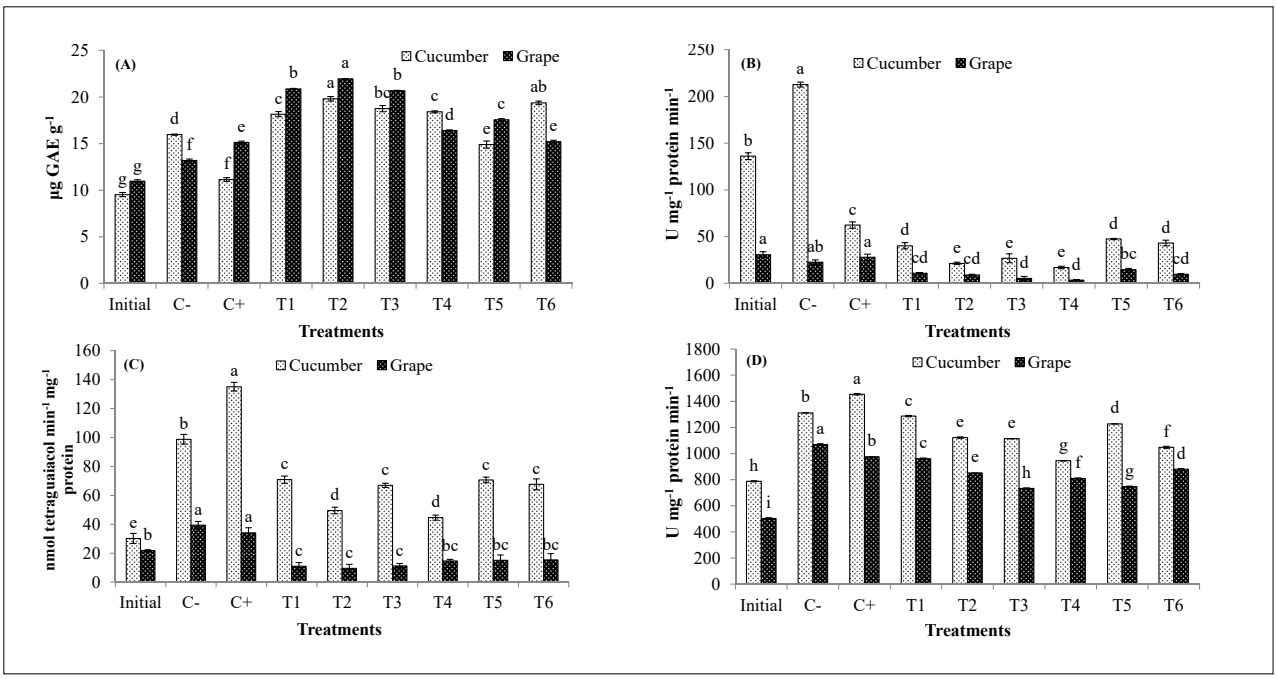

Figure 2. Effect of chitosan film forming formulations on TSP (A), catalase (B), G-POD (C), and PPO (D) in the uncoated and coated cucumber and grape fruits after 21 days of storage at 4 °C. C⁻: Negative control (treated with sterile distilled water); C⁺: Control positive (treated with pesticide); T1: Chitosan/starch formulation; T2: Chitosan/starch-CbE formulation; T3: Chitosan/starch-Ca formulation; T4: Chitosan/starch-Ca-CbE formulation; T5: Chitosan/starch-Mg formulation; and T6: Chitosan/starch-Mg-CbE formulation. Values are mean of three replicates and given as mean ± standard error. Different letters (a-i) on the bars indicate the range from higher to lower rank as significant differences according to the Duncan's multiple range test ($P \leq 0.05$).

for cucumber and grape respectively. However, the G-POD activities in the cucumber and grapes treated with chitosan/starch film-forming formulations dramatically inhibited the increase of G-POD during the cold storage. The maximal values were 70.85 and 15.34 in cucumber and grape, respectively, which were 47.58 and 55.03% lower than the positive controls of cucumber and grape, respectively.

In general, chitosan maintained better fruit quality with retarded the increase of the antioxidant enzymes activity such as catalase and G-POD (Ali *et al*., 2011; Wang and Gao, 2013). Wang and Gao reported that catalase activity in strawberries treated with chitosan decreased during storage at 5 °C and 10 °C and this response was dose-dependent. The activity of G-POD in strawberries increased during storage at 5 °C or 10 °C. However, chitosan treatment reduced or delayed the increase in G-POD activity. This indicated that chitosan treatment could inhibit oxidative enzyme activity in fruits during storage (Wang and Gao, 2013).

PPO catalyzes the *o*-hydroxylation of mono phenols to *o*-diphenols and catalyzes the oxidation of *o*-diphenols to *o*-quinones. Rapid polymerization of *o*-quinones produces polyphenols that cause browning of the fruit that accompanies senescence, wounding, and responses of fruits to pathogens (Mayer and Harel, 1991; Friedman, 1997). Changes in the activity of PPO in the fruit coated with chitosan formulations during storage for 21 days at 4 °C are shown in Figure 2D.

Table 1. Variance explained and cumulative proportion of total variance accounted by each factor derived from the PCA.

Factor	Initial eigenvalues			Extraction sums of squared loadings		
	Total	% of variance	Cumulative %	Total	% of variance	Cumulative %
1	5.21	43.40	43.40	5.21	43.40	43.40
2	3.57	29.78	73.18	3.57	29.78	73.18
3	0.91	7.52	80.70			
4	0.78	6.48	87.17			
5	0.61	5.03	92.21			
6	0.29	2.44	94.64			
7	0.25	2.06	96.70			
8	0.18	1.52	98.22			
9	0.09	0.72	98.94			
10	0.07	0.57	99.51			
11	0.05	0.40	99.90			
12	0.01	0.10	100.00			

Extraction method: Principal Component Analysis.

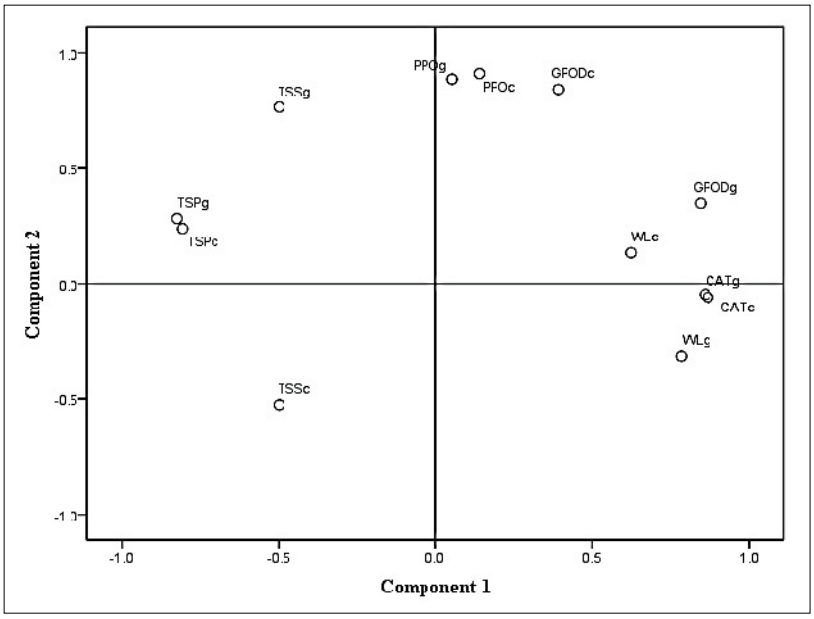

Figure 3. Unrotated principal component matrix scores for the tested parameters with two components extracted. Component 2 vs. component 1 obtained from the PCA of the various quality parameters of coated fruits. WLc: weight loss of cucumber; WLg: weight loss of grapes; TSSc: total soluble solids of cucumber; TSSg: total soluble solids of grapes; TSPc: total soluble phenolics of cucumber; TSPg: total soluble phenolics of grapes; CATc: catalase of cucumber; CATg: catalase of grapes; GPODc: guaiacol peroxidase of cucumber; GPODg: guaiacol peroxidase of grapes; PPOc: polyphenol oxidase of cucumber; and PPOg: polyphenol oxidase of grapes.

The specific activity of PPO (U mg^{-1} min^{-1}) in cucumber increased significantly in the uncoated fruit (from 787.52 at zero time of the experiment to 1310.84 and 1453.68, in the negative and positive controls, respectively). Chitosan coating treatments significantly reduced or delayed the increases in PPO activities with a range from 944.83 to 1286.47 in cucumber and from 732.69 to 960.32 in grape compared to the controls. This indicated that chitosan treatment could inhibit oxidative enzyme activity in fruits during storage. Similar results reported that chitosan retarded the increase of PPO activity in fruits during cold storage (Ali *et al*., 2011; Wang and Gao, 2013). Wang and Gao reported that PPO activity in strawberries increased during storage at 5 °C and 10 °C however, chitosan treatment reduced or delayed this increase (Wang and Gao, 2013).

Chemometric analyses of the resulting data have been performed using PCA to transform a set of original variables into new uncorrelated variables (axes), which are called principal components. PCA provides an objective way of finding indices of this type, so that the variation in the data can be accounted as concisely as possible. Data of fruit quality for the two types of fruits examined in the present study (Figures 1 and 2) were subjected to PCA in attempts to identify parameters affected significantly by chitosan film-forming formulations. When six original variables were entered in the PCA program, two factors were returned that met the criteria of their eigenvalues exceeding 1.0 (Table 1). The total Eigenvalues ranged from 5.21–0.07 for the factor analyzed (Table 1) with two components extracted having 43.40 and 29.78% of variance and cumulative % of 43.40 and 73.18%. Sum of the squares of the factor loadings computed for the original variables (Table 1) is equal to the eigenvalue of each factor 1 and 2 (Table 1), thus representing the extent of contribution of each factor to the total variance. It is customary to consider only those factors that have eigenvalues of 1.0 or greater as having any practical significance (Jeffers, 1967; Yada and Nakai, 1986). The two factors or principal components obtained accounted more than 73% of the total variance (Table 1), where the total variance is the sum of the individual variances for each of the original variables.

Figure 3 shows the first two PCs against each other. As PCA is invariant to the mirroring through the origin, all fruit quality parameters including weight loss, TSS, TSP, catalase, G-POD, and PPO in the cucumber and grape fruits belong to four groups. The data measured here indicate a significant positive correlation between weight loss, PPO, and G-POD of cucumber and grapes and components 1 and 2. However, the negative correlation observed between TSS of cucumber with both components 1 and 2 with -0.499 and -0.526, respectively, whereas TSS of grapes and TSP of cucumber and grapes showed significant negative correlation with component 1 (-0.499, -0.807, and -0.825, respectively). The point for TSP and catalase of cucumber is close to that of grapes because they contain the approximately the same content. PPO can also be similar variable. A PCA plot could have confirmed the positive role of the chitosan formulation in the preservation of the cucumber and grape fruits.

Identification and quantification of the pesticides

The experimental conditions with retention time (Rt) of chlorpyrifos and methomyl by Agilent 1260 HPLC-VWD with Agilent ZORBAX Eclipse Plus C18 are indicated in Table 2. The pesticides were identified by comparing their retention times with respect to the technical grade reference standard

Table 2. Retention time and experimental conditions of chlorpyrifos and methomyl by Agilent 1260 HPLC-VWD with Agilent ZORBAX Eclipse Plus C18.

Insecticide	Solvent system	Optimum wavelength (nm)	Retention time (min)	Calibration range (µg injected)	Correlation coefficient (r^2)	LoD	LoQ
Chlorpyrifos	Acetonitrile	289	3.68±0.01	0.0125–0.0750	0.9974	0.0009	0.0030
Methomyl	Acetonitrile:Water (60:40)	233	2.48±0.01	0.0125–0.0750	0.9991	0.0005	0.0016

LoD is a limit of detection and LoQ is a limit of quantification.

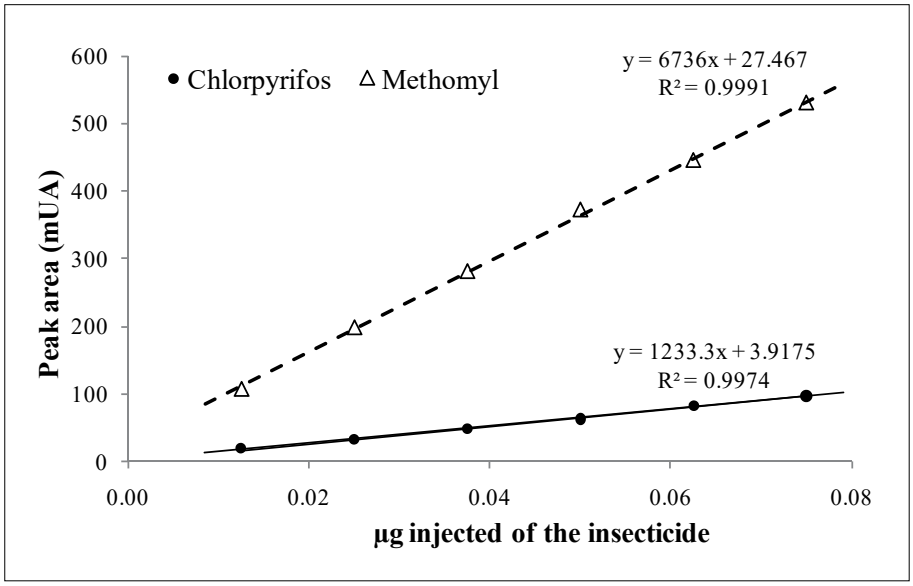

Figure 4. Calibration curves of chlorpyrifos and methomyl using Agilent 1260 HPLC-VWD.

using the suitable solvent system. The optimum wave length (nm) was 233 for methomyl and 289 for chlorpyrifos. Chlorpyrifos was eluted at Rt of 3.68 ± 0.01 min using acetonitrile at flow rate of 1 mL min^{-1}. Methomyl was eluted at Rt of 2.48 ± 0.01 min using acetonitrile/water (60:40) at flow rate of 1.0 mL min^{-1}. The quantitative determination of each insecticide was performed using the calibration curve obtained from chromatography experiments with standard solutions. For quantification, the external calibration curves with different concentrations of each insecticide as shown in Figure 4 for chlorpyrifos and methomyl. The linear regression ($y = a + bx$) parameters for method calibration were taken and the correlation coefficient (R^2) of the analytical curves was higher than 0.99 (Figure 4), with linearity for each compound, which allows the quantization of these compounds by the method external standardization.

Limit of detection (LoD) and limit of quantification (LoQ)

LOD is the lowest concentration of the analyte in a sample that can still be detected by the method of analysis, but not having to quantify as an appropriate value. However, LoQ is the lowest concentration of the sample that can still be detected quantitatively with accuracy and acceptable precision (Health U.D.o., 2000). The LoD was obtained from the peak intensity at 0.05 µg mL^{-1} and blank. LoD was defined as 3σ/S and LoQ was defined as 10σ/S, where σ is the standard deviation of the 10 times of the peak intensity at 0.05 µg mL^{-1} of each insecticide and the zero calibrator (blank) and S is the slope of the calibration curve. The LoD of chlorpyrifos and methomyl were 0.0009 and 0.0005 mg kg^{-1}. LoQ values for chlorpyrifos and methomyl were 0.0030 and 0.0016 mg kg^{-1} (Table 2).

Recovery

Recovery experiments were carried out to investigate the efficiency of extraction and cleaning steps. The untreated cucumber and grape fruits were spiked with three levels 10, 25, and 50 mg kg^{-1} of the standard solution of each insecticide and the extraction and clean-up were performed as described before. The concentration of each pesticide in the final extracts was calculated and the data showed in Table 3. The recovery of chlorpyrifos in cucumber and grape

Table 3. Recovery of chlorpyrifos and methomyl in the spiked cucumber and grape samples.

Insecticide	Fruit sample	Spiked concentration (mg kg^{-1})	Found (mg kg^{-1}) ± SE (n = 3)	Recovery (%) ± SE
Chlorpyrifos	Cucumber	10	7.28±0.22	72.76^{d}±2.21
		25	22.59±0.58	90.37^{c}±2.34
		50	44.80±1.17	89.61^{c}±2.34
	Grape	10	7.37±0.20	73.69^{d}±1.97
		25	23.36±0.58	93.44^{c}±2.34
		50	45.95±1.11	91.90^{c}±2.21
Methomyl	Cucumber	10	9.41±0.13	94.11^{c}±1.28
		25	26.09±1.00	104.38^{ab}±3.98
		50	52.73±0.22	105.47^{ab}±0.44
	Grape	10	9.78±0.10	97.79^{bc}±1.00
		25	26.00±0.70	104.01^{ab}±2.80
		50	53.71±0.14	107.42^{a}±0.29

Different letters (a-d) on the values in the same column indicate the range from higher to lower rank as significant differences according the Duncan's multiple range test ($P \leq 0.05$). Values are mean of three replicates and given as mean ± standard error.

ranged from 72.76 to 93.44% and the recovery of methomyl in cucumber and grape ranged from 94.11 to 107.42%. These results meet the requirement of pesticide residue analysis.

Residues of chlorpyrifos and methomyl in cucumber and grape samples

The residues of chlorpyrifos and methomyl (mg kg^{-1} based on the fresh weight) in/on cucumber and grape fruits coated with immobilized and non-immobilized chitosan/starch films with carboxylesterase at different time intervals during the storage at 4 °C as shown in Tables 4–7. The insecticides residues were detected in all samples up to 21 d of cold storage with decline in their concentrations with the time of storage. The residues in cucumber control samples slightly decreased during the storage period (4.98 mg kg^{-1} at zero time to 3.45 mg kg^{-1} at day-21) compared with those coated with the chitosan/starch film non-immobilized or immobilized with the enzyme (Table 4). It can be noted that the chitosan film coatings absorbed some of pesticide residues directly after treatment as shown in the residues found after 1 h (zero time). This result may be referring to the chelation characteristics of chitosan molecule as the presence of amino groups (Yoshizuka *et al.*, 2000; Wu *et al.*, 2010).

Results in Table 5 showed that the initial deposit of chlorpyrifos in the untreated grape fruit was 4.75 mg kg^{-1} whereas it decreased at 3.05 mg kg^{-1} after 21 days of cold storage. A rapid disappearance was noticed with the chitosan/starch coating treatments, after one day of application with values of 2.19, 2.00, 2.04, 1.75, 2.00, and 1.94 mg kg^{-1} with chitosan/starch film (T1), chitosan/starch-CbE film (T2), chitosan/starch-Ca (T3), chitosan/starch-Ca-CbE (T4), chitosan/starch-Mg (T5), and chitosan/starch-Mg-CbE (T6), respectively. The progression of time after application resulted in more dissipation of the pesticide residues. The residues after 21 d were 0.73, 0.43, 0.56, 0.38, 0.66, and 0.47 mg kg^{-1} for T1, T2, T3, T4, T5, and T6, respectively.

The extracted residues of methomyl in/on cucumber control fruit were 4.90 mg kg^{-1} (one hour after application) and 3.01 mg kg^{-1} after 21 d at cold storage when applied at the field recommended dose (Table 6). However, the residues in/on grape control fruit ranged from 4.41 mg kg^{-1} (1 h after application) to 2.47 mg kg^{-1} in 21 d at cold storage (Table 7). Generally, a reduction of methomyl residues was observed with chitosan/starch coating treatments. In cucumber, the methomyl residues were 1.37, 1.24, 1.80, 1.57, 1.79, and 1.63 mg kg^{-1} for T1, T2, T3, T4, T5, and T6, respectively after 21 d of the treatments compared to 3.01 mg kg^{-1} in the control. On the other hand, the residues in grape fruits were 1.21, 1.05, 1.58, 1.49, 1.19, and 0.84 mg kg^{-1} for T1, T2, T3, T4, T5, and T6, respectively after 21 d of the treatment compared to 2.47 mg kg^{-1} in the control.

In all experiments, when comparing the degraded amounts of chlorpyrifos and methomyl using the three types of chitosan formulations (chitosan/starch, chitosan/

Table 4. Residues of chlorpyrifos (mg kg^{-1}) in cucumber samples coated with immobilized and non-immobilized chitosan/starch films with carboxylesterase at different time intervals during the storage at 4 °C.

Treatment	Residues of chlorpyrifos (mg kg^{-1}) ± SE at time (d)						
	0	1	3	7	10	14	21
C+	4.98^{a}±0.56	4.94^{a}±0.44	4.75^{a}±0.15	4.20^{a}±0.45	3.85^{a}±0.96	3.70^{a}±0.36	3.45^{a}±0.64
T1	4.02^{a}±0.96	3.50^{a}±0.44	3.48^{a}±1.31	2.71^{a}±0.94	2.32^{ab}±0.04	1.61^{b}±0.41	1.15^{b}±0.14
T2	3.82^{a}±0.69	3.64^{a}±0.48	3.21^{a}±1.20	1.88^{a}±0.61	1.45^{b}±0.02	0.94^{b}±0.55	0.89^{b}±0.10
T3	3.86^{a}±0.85	3.34^{a}±0.70	2.97^{a}±0.24	2.71^{a}±1.00	2.29^{ab}±0.02	1.11^{b}±0.02	0.88^{b}±0.20
T4	3.40^{a}±1.16	3.13^{a}±0.79	2.20^{a}±0.24	1.99^{a}±0.60	1.34^{b}±0.30	1.06^{b}±0.55	0.76^{b}±0.14
T5	3.51^{a}±0.22	3.16^{a}±0.45	2.65^{a}±0.66	2.19^{a}±0.48	1.55^{b}±0.10	1.40^{b}±0.22	0.82^{b}±0.02
T6	3.21^{a}±0.23	2.45^{a}±0.77	1.99^{a}±0.73	1.48^{a}±0.27	1.28^{b}±0.55	0.94^{b}±0.58	0.71^{b}±0.32

C+: Control positive (treated with pesticide); T1: Chitosan/starch formulation; T2: Chitosan/starch-CbE formulation; T3: Chitosan/starch-Ca formulation; T4: Chitosan/starch-Ca-CbE formulation; T5: Chitosan/starch-Mg formulation; and T6: Chitosan/starch-Mg-CbE formulation. Different letters (a-d) on the values in the same column indicate the range from higher to lower rank as significant differences according to the Duncan's multiple range test ($P \leq 0.05$). Values are mean of three replicates and given as mean ± standard error.

Table 5. Residues of chlorpyrifos (mg kg^{-1}) in grape samples coated with immobilized and non-immobilized chitosan/starch films with carboxylesterase at different time intervals during the storage at 4 °C.

Treatment	Residues of chlorpyrifos (mg kg^{-1}) ± SE at time (d)						
	0	1	3	7	10	14	21
C+	4.75^{a}±0.12	4.57^{a}±0.76	4.52^{a}±0.55	4.36^{a}±0.44	4.26^{a}±0.46	3.49^{a}±0.25	3.05^{a}±0.42
T1	4.08^{ab}±0.46	2.19^{b}±0.66	1.68^{b}±0.29	1.44^{b}±0.80	1.05^{b}±0.49	0.87^{b}±0.61	0.73^{b}±0.11
T2	4.05^{bc}±0.69	2.00^{b}±0.76	1.27^{b}±0.11	1.18^{b}±0.12	0.97^{b}±0.57	0.65^{b}±0.34	0.43^{b}±0.13
T3	3.56^{c}±0.65	2.04^{b}±0.14	1.42^{b}±0.33	1.19^{b}±0.03	0.93^{b}±0.29	0.87^{b}±0.24	0.56^{b}±0.16
T4	3.40^{c}±0.18	1.75^{b}±0.55	1.10^{b}±0.05	0.91^{b}±0.32	0.80^{b}±0.61	0.60^{b}±0.34	0.38^{b}±0.24
T5	3.50^{c}±0.49	2.00^{b}±0.49	1.60^{b}±0.16	1.27^{b}±0.07	1.03^{b}±0.24	0.72^{b}±0.48	0.66^{b}±0.07
T6	3.35^{c}±0.48	1.94^{b}±0.07	1.20^{b}±0.13	0.93^{b}±0.08	0.78^{b}±0.31	0.64^{b}±0.68	0.47^{b}±0.10

C+: Control positive (treated with pesticide); T1: Chitosan/starch formulation; T2: Chitosan/starch-CbE formulation; T3: Chitosan/starch-Ca formulation; T4: Chitosan/starch-Ca-CbE formulation; T5: Chitosan/starch-Mg formulation; and T6: Chitosan/starch-Mg-CbE formulation. Different letters (a-d) on the values in the same column indicate the range from higher to lower rank as significant differences according to the Duncan's multiple range test ($P \leq 0.05$). Values are mean of three replicates and given as mean ± standard error.

Table 6. Residues of methomyl (mg kg^{-1}) in cucumber samples coated with immobilized and non-immobilized chitosan/starch films with carboxylesterase at different time intervals during the storage at 4 °C.

Treatment	Residues of methomyl (mg kg^{-1}) ± SE at time (d)						
	0	1	3	7	10	14	21
C+	4.90^{a}±0.25	4.87^{a}±0.91	4.50^{a} ±0.81	4.22^{a}±0.44	3.80^{a}±0.19	3.41^{a}±0.05	3.01^{a}±0.50
T1	3.96^{a}±0.46	3.68^{a}±0.47	3.58^{ab}±0.32	2.40^{b}±0.01	1.73^{a}±0.06	1.47^{a}±0.61	1.37^{b}±0.52
T2	3.87^{a}±0.65	3.35^{a}±0.84	3.23^{ab}±0.31	2.05^{b}±0.57	1.67^{a}±0.83	1.34^{a}±0.19	1.24^{b}±0.51
T3	3.93^{a}±0.43	3.38^{a}±0.48	2.87^{ab}±0.01	2.80^{b}±0.14	2.10^{a}±0.56	2.03^{a}±0.97	1.80^{b}±0.58
T4	3.40^{a}±0.56	2.63^{a}±0.55	2.58^{b}±0.01	2.20^{b}±0.50	1.81^{a}±0.76	1.81^{a}±0.41	1.57^{b}±0.02
T5	3.85^{a}±0.84	3.32^{a}±0.01	3.04^{ab}±0.17	2.85^{b}±0.12	2.18^{a}±0.87	2.14^{a}±0.67	1.79^{b}±0.50
T6	3.76^{a}±0.73	2.99^{a}±0.36	2.95^{ab}±0.01	2.77^{b}±0.28	1.87^{a}±0.82	1.71^{a}±0.45	1.63^{b}±0.49

C+: Control positive (treated with pesticide); T1: Chitosan/starch formulation; T2: Chitosan/starch-CbE formulation; T3: Chitosan/starch-Ca formulation; T4: Chitosan/starch-Ca-CbE formulation; T5: Chitosan/starch-Mg formulation; and T6: Chitosan/starch-Mg-CbE formulation. Different letters (a-d) on the values in the same column indicate the range from higher to lower rank as significant differences according to the Duncan's multiple range test ($P \leq 0.05$). Values are mean of three replicates and given as mean ± standard error.

Table 7. Residues of methomyl (mg kg^{-1}) in grape samples coated with immobilized and non-immobilized chitosan/starch films with carboxylesterase at different time intervals during the storage at 4 °C.

Treatment	Residues of methomyl (mg kg^{-1}) ± SE at time (d)						
	0	1	3	7	10	14	21
C+	4.41^{a}±0.66	4.23^{a}±0.03	4.20^{a}±0.01	3.80^{a}±0.53	3.54^{a}±0.01	3.01^{a}±0.09	2.47^{a}±0.01
T1	2.39^{a}±0.66	2.24^{bc}±0.08	1.88^{b}±0.01	1.73^{b}±0.40	1.62^{b}±0.42	1.22^{b}±0.02	1.21^{b}±0.67
T2	2.10^{a}±0.43	1.98^{bc}±0.15	1.70^{b}±0.50	1.55^{b}±0.53	1.11^{b}±0.00	1.10^{b}±0.02	1.05^{b}±0.72
T3	3.38^{a}±0.49	2.67^{b}±0.52	2.08^{b}±0.66	2.02^{b}±0.34	1.84^{b}±0.31	1.76^{b}±0.64	1.58^{b}±0.02
T4	3.26^{a}±0.98	2.46^{b}±0.01	1.95^{b}±0.25	1.77^{b}±0.21	1.61^{b}±0.21	1.55^{b}±0.74	1.49^{b}±0.08
T5	2.30^{a}±0.60	1.85^{bc}±0.01	1.75^{b}±0.10	1.72^{b}±0.19	1.28^{b}±0.09	1.25^{b}±0.30	1.19^{b}±0.13
T6	1.81^{a}±0.42	1.43^{c}±0.20	1.38^{b}±0.06	1.26^{b}±0.09	1.13^{b}±0.01	1.09^{b}±0.28	0.84^{b}±0.01

C+: Control positive (treated with pesticide); T1: Chitosan/starch formulation; T2: Chitosan/starch-CbE formulation; T3: Chitosan/starch-Ca formulation; T4: Chitosan/starch-Ca-CbE formulation; T5: Chitosan/starch-Mg formulation; and T6: Chitosan/starch-Mg-CbE formulation. Different letters (a-b) on the values in the same column indicate the range from higher to lower rank as significant differences according to the Duncan's multiple range test ($P \leq 0.05$). Values are mean of three replicates and given as mean ± standard error.

starch-Ca, and chitosan/starch-Mg) which immobilized or non-immobilized with CbE, we found that the films immobilized with the enzyme resulted in the greatest degradation compared to the non-immobilized films. The comparison of the results from the different films is indicated that the films of chitosan/starch-metal ions (Ca^{++} and Mg^{++}) with enzyme showed high efficiency in the degradation of the both insecticides compared to the chitosan/starch film alone. This finding was supported by Zhang and co-authors (2015), who reported that the chitosan-magnesium, -calcium, -iron(III), and -zinc coordination complexes showed high efficiency for degradation of dichlorvos, omethoate, dimethoate, and chlorpyrifos pesticides at in a heterogeneous system. Moreover, it be noted that chitosan based edible coating application was more effective in grape than cucumber on the reduction of both pesticides.

Dynamics of chlorpyrifos and methomyl

Kinetic equations for the pesticides were calculated from the simple first-order kinetic equation $C_t = C_0 e^{-kt}$ where C_t is the concentration (mg kg^{-1}) at time t (d) after application, C_0 is the initial concentration (mg kg^{-1}) first-order rate constant (day) and k is the first-order state constant (day^{-1}) (Ma *et al.*, 2004). The half-life (DT_{50}, d) of chlorpyrifos and methomyl residue dissipation in cucumber and grape fruits were calculated from the experimental data and summarized in Table 8. The data showed that the DT_{50} of chlorpyrifos in cucumber and grape without chitosan/starch based edible coating application (C+) were 36.97 and 35.16, respectively. However, the DT_{50} values with the treatments (T1–T6) dramatically decreased compared with the control and ranged from 5.26 to 9.83 d in cucumber and from 2.52 to 3.25 d in grape. The DT_{50} of methomyl in cucumber and grape fruits coated with chitosan/starch formulations were ranged from 7.49 to 10.88 d in cucumber and from 6.01 to 13.41 d in grape compared to 31.92 and 26.67 d in controls of cucumber and grape, respectively. The DT_{50} of chlorpyrifos was almost the same with that observed by Zhang and co-authors (2007) who studied the multi-residual dynamics of some pesticides including chlorpyrifos in the spring cabbage. Liang and co-authors showed a steady decline in the residues of chlorpyrifos and dichlorvos in cucumber under greenhouse conditions and the declines of exceeded 70% (Liang *et al.*, 2012). They reported that the degradations of both insecticides applied at 1.5 times recommended dose and 1 spraying times in cucumber coincided with $C_t = 0.0753e^{-0.433t}$, $C_t = 0.235e^{-0.199t}$, respectively, and the DT_{50} values obtained were 1.60 and 3.48 d for chlorpyrifos and dichlorvos, respectively.

In fruit and vegetable samples collected from different farmers' fields, higher pesticide residues, exceeding the maximum residue level (MRL) values were observed (Dejonckheere *et al.*, 1995; Yen *et al.*, 1999; Yu *et al.*, 2016). The current study indicated that the application of chitosan/starch base edible coating was enough effective to reduce

Table 8. First-order kinetic equations and half-life values of chlorpyrifos and methomyl in cucumber and grape samples coated with immobilized and non-immobilized chitosan/starch films with carboxylesterase after 21 d of storage at 4 °C.

Treatment	Half-life (DT_{50}, d)			
	Chlorpyrifos		Methomyl	
	Cucumber	Grape	Cucumber	Grape
C+	36.97	35.16	31.92	26.67
T1	9.83	2.98	10.88	13.41
T2	8.78	2.52	8.17	13.19
T3	8.42	3.11	9.23	6.77
T4	7.54	2.60	7.49	6.01
T5	8.62	3.25	10.28	8.60
T6	5.26	2.92	8.01	8.36

DT_{50} (day) values were calculated according to the first order reaction as follows: DT_{50} (d) = 0.693/k. k is a kinetic constant from the first-order kinetic equation ($C_t = C_0 e^{-kt}$). C+: Control positive (treated with pesticide); T1: Chitosan/starch formulation; T2: Chitosan/starch-CbE formulation; T3: Chitosan/starch-Ca formulation; T4: Chitosan/starch-Ca-CbE formulation; T5: Chitosan/starch-Mg formulation; and T6: Chitosan/starch-Mg-CbE formulation.

the residues of chlorpyrifos on cucumber and grapes after 14 or 21 days of storage below the acceptable levels (MRL ranged from 0.7 to 1.0 mg kg^{-1} according to the Joint FAO/WHO Meeting on Pesticide Residues) (Pesticides A., 2013). Therefore, the fruits coated with chitosan/starch formulations could be consumed safely, 14–21 d after the treatment.

Navarro and others studied the disappearance of chlorpyrifos, penconazole, fenarimol, vinclozolin, metalaxyl, and mancozeb in grapes (Navarro *et al*., 2001). They found that the residue levels immediately after application were 6.91, 0.14, 0.53, 5.82, 0.89, and 0.97 mg kg^{-1}, but fell to 0.14, 0.03, 0.06, 0.44, 0.10, and 0.22 mg kg^{-1} 28 d after the application for chlorpyrifos, penconazole, fenarimol, vinclozolin, metalaxyl, and mancozeb, respectively. The half-life times calculated were 4.4, 6.6, 7.8, 8.3, 12, and 13.5 d, respectively. Xie and co-authors studied some treatments including water, different concentrations of crude intracellular enzyme obtained from fungus named WZ-I, 0.5% detergent, and 0.5% saleratus in degradation of the chlorpyrifos residues on the surface of cabbage and cucumber (Xie *et al*., 2006). The results showed that the degrading enzyme was effective reaching a 60.2% of degradation within 10 min and being greatly affected by the enzyme concentration. The rates of chlorpyrifos removal on the surface of the cabbage using the enzyme at a concentration of 0.5% and 5.0% were 49.8% and 55.2%, respectively, and the rate of removal of pesticides from cucumber, were 30.8% and 54.2%, respectively.

Ahmed and Ismail reported that the methomyl residues by HPLC reached levels of 0.55, 0.2 and 0.6 mg kg^{-1} fruit seven days after application on strawberries, tomatoes and cucumbers, respectively (Ahmed and Ismail, 1995). The samples of these fruits collected from local markets at Ismailia Governorate, Egypt showed that the methomyl residues in 12.5% of tomato and 25% of strawberry samples were above 0.2 mg kg^{-1}. In addition, rates of degradation or dissipation of methomyl residues in grape fruit followed first-order rate kinetics with similar patterns at standard and double-dose applications (Banerjee *et al*., 2006). Residues of methomyl were lost with Pre-Harvest Interval (PHI) of 55.0 and 61.0 d, following applications at 1 and 2 kg a.i. ha^{-1}, respectively.

Conclusion

In this study, new biopolymer composites immobilized with pesticide-degradable CbE enzyme, were properly prepared and applied to extend the shelf life of cucumber and grapes in the postharvest phase with degradation of pesticide residues during cold storage. The data indicate that film-forming formulations have relatively valuable effects on the quality of fruits with significant degradation of chlorpyrifos and methomyl insecticides over the control. Therefore, these biodegradable formulations could be used as interesting alternatives to improve the preservation qualities and degrade pesticide residues of perishable fresh cut fruit and vegetables resulting maintain the health of the consumer. This technology could be transferred to both organic and conventional producers when they begin exporting tropical products.

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Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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