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Characterization and development of different methods to extend shelf life of fresh cut fruit. Case study: novel controlled release system by layer-by-layer assembly

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The research project aimed at the investigation of different methods for the storage of fresh-cut fruit. This case study relates to the proposal of an innovative controlled release system to improve the shelf life of ready-to-eat fruit. The methods applied during three research years included the application of active molecules from natural substances, some widely used in commerce and other tested for the first time; first analyzed *in vitro* and then applied *in vivo*. The under consideration methods concerned dipping, coating and the layer-by-layer assembly. The analysis carried out on the fruit have monitored pomological traits performances (soluble solids content, titratable acidity, pH, color, flesh firmness), the chemical profile (polyphenoloxidase, carotenoids) and microbial growth.

Sviluppo e caratterizzazione di differenti metodi per l'estensione della shelf life della frutta di IV gamma. Caso studio: nuovo sistema a rilascio controllato tramite la tecnica del *layer-by-layer*

Il progetto di ricerca ha avuto come obiettivo l'investigazione di differenti metodiche per la conservazione della frutta di IV gamma. I metodi utilizzati nei tre anni di ricerca hanno riguardato l'applicazione di molecole attive di origine naturale, alcune ampiamente utilizzate in commercio e altre sperimentate per la prima volta; dapprima analizzate *in vitro* e poi applicate *in vivo*. Le metodiche prese in esame hanno riguardato il dipping, il coating e l'assemblaggio *layer-by-layer*. In questo lavoro verrà trattato il caso studio su un nuovo sistema a rilascio controllato, mediante la tecnica del *layer-by-layer*. Le analisi di *shelf life* sulla frutta hanno monitorato l'andamento dei parametri pomologici (solidi solubili, acidità titolabile, pH, colore, consistenza della polpa), l'evoluzione del profilo enzimatico e chimico (polifenolossidasi, carotenoidi) e l'andamento dello sviluppo microbico.

Keywords: ready-to-eat, layer-by-layer, antioxidant, antimicrobial, packaging.

1. Introduction

The growing demand for ready-to-eat food, associated to the need food loss reduction, have promoted many active packaging solutions for extending the shelf-life of foods. Active packaging materials are "materials and articles that are intended to extend the shelf-life or to maintain or improve the condition of packaged food. They are designed to deliberately incorporate components that would release or absorb substances into or from the packaged food or the environment surrounding the food" (European Commission, 2004). Several types of active packaging have been developed, containing: O_2 and ethylene scavenging (Cao *et al*., 2015), CO_2-scavengers and –emitters (Wang *et al*., 2015), moisture regulators, antimicrobial and antioxidant release (Mascheroni *et al*., 2010). Among antioxidant substances there are flavonoids from Green Tea extract, including catechins (Sutherland *et al*., 2006). Chitosan is one of the most used natural substance in active packaging; a linear polysaccharide, a deacetylated derivative of chitin. Chitosan has been extensively studied due to its low toxicity, biodegradability, and antimicrobial activity (Alvarez *et al*., 2013). Layer-by-layer (LbL) assembly is a basic technique for the fabrication of multicomponent films on solid supports by controlled adsorption from solutions or dispersions (Decher, 1997). With the LbL technique, polyelectrolyte multi-nanolayer films can be fabricated on various substrates through repeat deposition, mainly due to the electrostatic attraction between oppositely charged polyelectrolytes (Donath *et al*, 1998). One of the use of LbL assembly is the drug delivery in biomedical field, to release antibiotics or others substances (Wohl and Engbersen, 2012). One of the mechanism of drugs release is the immobilization of the active compound between two layers of LbL system, and release it by degradation of the layers (Flessner *et al*., 2011). The aim of this work is the development and characterization of a novel Controlled Release System (CRS) by the conjugation of Chitosan-Alginate LbL assembly, including catechin mix, and *in vivo* application on fresh-cut peach.

2. Materials and Methods

2.1 Preparation of layer-by-layer system

Chitosan and alginate water dispersion, 0.2 % (w/v), was separately prepared by dissolving the powder in 2 % (v/v) of acetic acid, only water for alginate, at 25 °C for 3 h under stirring. After that, the pH was adjusted to 3.8 and 6.5 for chitosan and alginate solution, respectively. Into the alginate solution was added 0.35% of green tea tannins extract. The solutions were used in LbL assembly. This (Fig. 1) was carried out using the method of Li *et al*. (2013). A-PET sheets were cleaned with distilled water and methanol for removing lipids and contaminants. After drying, they were submitted to corona treatment (BD-20 high frequency generator, Electro-Technic Products, Inc., Chicago, IL, USA) to increase surface energy and generate a negative-charge surface. The A-PET sheet was cut in 7 x 3 cm strips, each strip was dipped into the chitosan solution for 10 min; after, the strips were rinsed in distilled water for 5 min to remove the excess of chitosan; strips coated by chitosan were dried by filtered compressed air. Following, strips were dipped into the alginate-tannin solution for 10 min; the rinsing and drying steps were the same as the one for chitosan. Finally, 20 bilayers were achieved by repeating the previous steps. All the samples were stored into desiccators at 0% RH and put into a cold chamber at 4 °C for the following measurements.
For the characterization (antioxidant, antimicrobial and absorbance tests) of A-PET coated by LbL assembly and to understand kinetic of release, the strips were stirred for 48 h. A portion of strip, 3 x 3 cm, was cut and put into the glass flask and immersed in 5 mL of acid aqueous solution (acidified by citric acid at pH 3.8) as food simulant. The flasks with samples were shaken for 6, 24 and 48 h using Flask Dancer 270292 (Boekel Scientific, Feasterville, PA, USA). The obtained solution were stored into cold chamber for the next analysis.

Figure 1. *Layer-by-Layer assembly procedure.*

2.2 Optical Contact Angle (OCA)

On uncoated A-PET surface and on for each successive alginate and chitosan layers, contact angle (θ) measurement was performed using the sessile drop method (Newman and Kwok, 1999) with optical contact angle apparatus (OCA 15 Plus – Data Physics Instruments GmbH, Filderstadt, Germany) equipped with a video measuring system with a high-resolution CCD camera and a high performance digitizing adapter. SCA20 software (Data Physics Instruments GmbH, Filderstadt, Germany) was used for data acquisition. Onto the A-PET surface 4 ± 0.5 µL droplet of Milli-Qwater was placed by syringe (Hamilton, Switzerland), the measures were made at 0 s and 20.5 ± 0.3 °C, 15 replicates of contact angle measurements were carried out.

2.3 UV-Visible spectrophotometry, antioxidant and microbiological assays

The UV–Vis analyses were carried out using a spectrophotometer Lambda 25 (Perkin Elmer, MA). The absorbance at 280nm was measured on the extracted solution of A-PET-LBL at different time, after 6, 24 and 48 h. Analysis of the antioxidant capacity of the A-PET strips coated by LbL, was carried out employing the DPPH assay, following the method of Brand-Williams *et al*. (1995) with some modifications. The DPPH solution was diluted in ethanol to obtain 1.00 ± 0.03 absorbance units at 515 nm. The extracts samples were dissolved in ethanol (20 g/L) and, after centrifugation, they were serially diluted. The DPPH solution (2.94 mL) was placed in a cuvette where 60 µL sample were added. The absorbance readings were carried out after incubation for 50 min at 20 ± 1 °C. A calibration curve was prepared by adding increasing concentration of Trolox ranged from 50 to 1000 µM; each concentration was assayed in triplicate, as well as each sample. Results were expressed as mol Trolox per 100 g of powder. Antimicrobial activity carried out against strains belonging to official collection, i.e. Escherichia coli CECT 434 (Spanish Type Culture Collection), Staphylococcus aureus ATCC 29213, Aspergillus niger NRRL 565 (Agricultural Research Service Culture Collection) and Penicillium chrysogenum CECT 2802. Bacterial strains were weekly maintained on TSB (Tryptic Soy Broth), incubated at 30 °C for 24 h and then stored at 4 °C, while yeasts on MEB (Malt Extract Broth, g/L: malt extract 20, glucose 20, soy peptone 1, pH 5.8), incubated at 28 °C for 24-48 h and then stored at 4 °C until use. Moulds were maintained on MEA solid culture (MEB added with 15 g/L agar), incubated at 25 °C for 5-7 d and then stored at 4 °C until use. Qualitative determination of antimicrobial activity was performed as follows: thirty mL of soft TSA or MEA (TSB or MEB added with 8 g/L agar) were poured in a Petri Dish and inoculated with 300 µL of a microbial suspension prepared in sterile distilled water (OD 600nm: 0.300 ± 0.050); moulds were inoculated as spores

suspension in sterile distilled water (OD 600 nm: 0.300 ± 0.050). Once solidified, holes were made by using a sterile tip and 150 µL of extracts were poured inside. Cultures were all incubated at each appropriate temperature for 24 h (up to 7 d for moulds). The presence of a growth inhibition halo around holes indicates an antimicrobial activity.

2.4 *In vivo* shelf life trials on peach slices

Peaches (*Prunus persica* L. Batch cv 'Alexandra") were purchased at the wholesale market (10 kg) at commercial maturity and stored for 1 d at 4 °C until use. Peaches were homogenous in size and ripening (6 ± 0.7 kg/cm^2, 9.70 ± 0.1 °Brix, 12 ± 0.6 g/L titratable acidity). Fruits were pre-washed with distilled water, sanitized for 2 min in chlorinated water (1.5 g/L sodium hypochlorite), rinsed with distilled water and gently dried by hand. Peaches with skin were cut into slices of about 1.5 cm thickness (15 g each slice), using a sterile stainless-steel knife. Four slices (60 g) were placed on PET tray, among each slice and on the bottom of the tray were insert A-PET-LbL strips, 7 x 3 cm and 7 x 7 cm respectively (LBL); in uncoated samples (UNC) slices were placed with untreated strips and in control samples (CTR) without strips; the trays with slices were put into low-density polyethylene (LDPE) bags which were stored at 4 ± 1 °C up to 7 d. Each treatment was carried out in triplicate. Samples were then collected after 3, 5 and 7 d, for a total of 3 sampling.

2.5 Physical and chemical evaluations

Flesh colour was evaluated using the CIE L*a*b* System by a Minolta CR-300 chromameter (Konica Minolta Sensing, Inc., Japan). Three measurements were performed on each side of slices. The instrument was calibrated using a standard white plate. The Chroma (C) was calculated as following reported:

$$C^* = \sqrt{a^{*2} + b^{*2}} \qquad (1)$$

Fruit firmness was measured with digital penetrometer (53205, TR Turoni, Forlì, Italia) and expressed as kg/cm^2. Weight loss (WL) was calculated by the difference between the initial and final weight (after 7 d) of samples. The value was expressed as a relative percentage and calculated as follows:

$$WL = \frac{(\mathrm{Wi} - \mathrm{Wf})}{\mathrm{Wi} * 100} \qquad (2)$$

Where Wi is the initial weight and Wf is the weight measured during storage.
Total soluble solids (TSS, %) and titratable acidity (TA, g/L) were measured on the juice obtained from slices (50 g for each sample) by an electronic juicer (Moulinex, France). TSS was determined by a digital refractometer (Atago Co., Ltd, Tokyo, Japan model PR-32). Titratable acidity was measured on by titrating 1:10 diluted juice (obtained from 50 g of samples) using sodium hydroxide 0.1 M by automatic titrator (Compact 44-00, Crison Instruments, SA, Barcelona, Spain). To determine the carotenoids content the De Ritter and Purcell (2010) method was used. Briefly, 5 g of sample were mixed for 20 min with 50 mL of extracting solvent (hexane/acetone/ethanol, 50:25:25, v/v). The organic phase was recovered and then used for analyses after suitable dilution with hexane. Total carotenoid determination was carried out on an aliquot of the hexane extract by measuring absorbance at 450 nm in a spectrophotometer Lambda 25 (Perkin Elmer, MA). Total carotenoids were calculated as follows:

$$\beta carotene\ content(\mu g\ 100\ g) = \frac{Abs * V * D * 100 * 100}{W * Y} \qquad (3)$$

Where V is total volume extract, D the dilution factor, W the sample weight and Y the percentage of dry matter content of the sample. The analyses were performed in triplicate for each sample.For the determination of polyphenol oxidase (PPO) activity, the buffer solution (1:1) at pH 6.5 was prepared using 1M NaCl and 5% polyvinylpolypyrrolidone, peach sample (5 g) was mixed with a buffer solution and homogenized using an Ultra-Turrax DI25 (IKA Works, Germany). The homogenate mix was centrifuged at 12,000 rpm for 30 min at 4 ◦C (Centrifuge Rotofix 32A, Hettich, Germany.). The supernatant was collected and filtered, to obtain the enzymatic extract, required for enzyme activity determination. According to the method of Soliva-Fortuny *et al*. (2001) and Kołodziejczyk *et al*. (2010) PPO activity was determined spectrophotometrically, adding 3 mL of 0.05 M catechol and 75 µL of extract into a quartz cuvette. The changes in absorbance at 400 nm were recorded every 1 min up to 3 min. One unit of PPO activity was defined as a change in absorbance of 0.001 min^{-1} mL^{-1} of enzymatic extract immediately after extract addition. All determinations were performed in triplicate.

2.6 Antimicrobial activity of CRS on peach slices

After 0, 3, 5 and 7 d, a portion of sample (10 g) were transferred aseptically into a Stomacher bag (400 mL PE, Barloworld, France) containing 135 mL of sterile peptoned water (10 g/L bacteriological peptone, Costantino, Italy) and blended in a Stomacher (Star Blender LB 400, Biosystem, Belgium) at high speed for 3 min. Ten-fold

dilution series of the obtained suspension were made in the same solution for plating. The following culture media were used: TSA (Merck, Germany) for mesophiles, *Pseudomonas* Agar base (Himedia, India) for *Pseudomonas spp*, VRBLA (Violet Red Bile Agar, Merck, Germany) for *Enterobacteriaceae* and MEA for yeasts and fungi. Colonies were counted after incubation at 30 °C for 24 h for mesophiles, 30 °C for 5 d for yeasts and fungi and 25 °C for 24 h for *Pseudomonas*. Counts were performed in triplicate and reported as logarithms of the number of colony forming units (log cfu/g peach), and means and standard deviations (SD) were calculated.

3. Results

3.1 *In vitro* analysis

Contact angle measurement (Fig. 2A) showed the different values for each assembled layer. Uncoated A-PET showed an angle of 43.32° ± 0.95, after 20 layers (10 of chitosan and 10 of alginate), the final angle value reach 67.70° ± 0.49. The angle values depend on different layer compound, chitosan layers causes the increase of contact angle value, in opposite way, alginate layers induces contact angle decrease.
The extracted solution of CRS was measured by the absorbance increase at 280 nm, the wavelength of maximum absorbance of catechin. In this measurement, the increase in absorbance value depend by the time of extraction. After 48 h of extraction from the CRS, the extracted solution An higher absorbance than the other two samples (Fig. 2B). Antioxidant assay on the extracted solution of CRS was carried out. DPPH decay was increase by time of extraction. After 6 h the antioxidant power of extracted solution was around 2-3 % (Fig. 2C), similar values were obtained for different final layer of the CRS. A net difference was evident between extracted CRS and untreated A-PET solutions. After 48 h of extraction the antioxidant capacity was increase up to 7 %. In terms of final layer, alginate layer showed higher values than chitosan, this could be attributed to the higher presence of tea tannins by the addition of one layer more in to the LbL assembly.

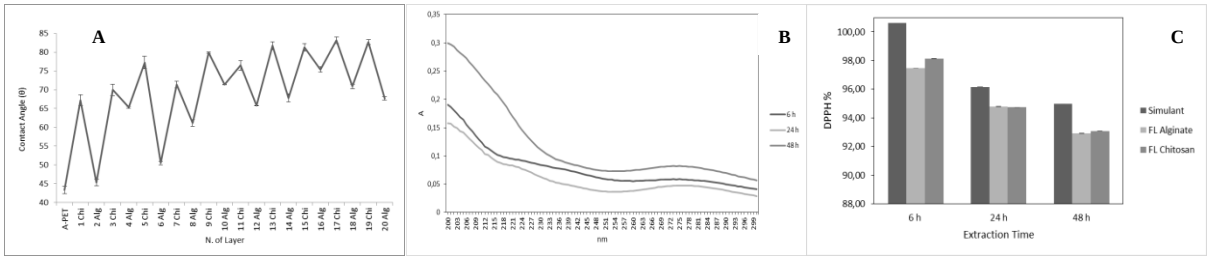

Figure 2. Contact angle values (A) of uncoated A-PET and A-PET coated by 20 LbL (measured 0 s after water drop deposition). Data points are the means ± SD; *UV-spectra (B) of different LbL extracts at three different time; antioxidant assay (C) of CRS extract expressed as percentage of DPPH decay during the three extraction times (6, 24, 48 h), for different final layer (FL), chitosan or alginate.*

In microbiological qualitative trials no activity against bacteria was shown. Missed antibacterial capacity depends on the low concentration of chitosan into the LbL assembly. At the same time, any antifungal activity was present against *A. niger* (data not shown) but it was observed against *P. chrysogenum* (Fig. 3). The antifungal activity of the chitosan was evident in the sample after 48 h of extraction. In this case, P. chrysogenum has not grown into the hole. After the fungal growth, the culture was sporulated, around the hole of 48 h sample was present the inhibition halo.

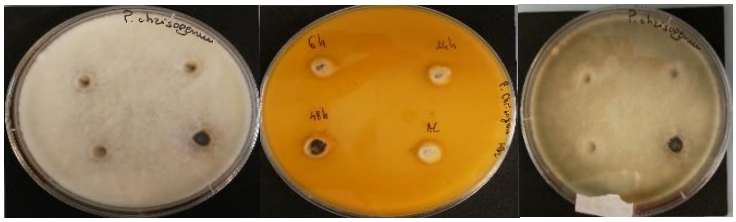

Figure 3. *Culture of P. chrysogenum, front (left) and back (center) of Petri-dish, after 6 d of incubation with different CRS extracts; after 10 d (right).*

3.2 *In vivo* analysis

After 7 d of storage, Lightness (L*) was higher in LBL sample, the same result occurred for the Chroma (C*). In terms of flesh firmness, is possible to see an increment of values, may due to a leatheriness phenomenon affected by storage temperature (Lurie and Crisosto, 2005). Comparing samples, LBL showed the higher value in flesh firmness. During storage, weight loss (WL) was measured, the values showed high weight loss for control in

comparison with other treatment. The presence of PET strips affected the loss of weight of the peach slices, avoiding loss around 2.5% of initial weight. About TSS and TA, the values follow the normal postharvest behavior, no significant differences were observed. Chemical analysis showed the decrease in total carotenoids content for all the samples. LBL treatment showed the lowest reduction of total carotenoids content, in accord to Srinivasa *et al*. (2002). Also the presence of PET strips could reduce the carotenoids decay.
As regards microbiological trials, the developed films were found to limit psycrophiles population from near 2 (CTR sample) to less than 1 log cfu/g. Yeasts and moulds were always in the range 1-2 log cfu/g. No significant changes were evidenced for *Enterobacteriaceae*, *Pseudomonas* and mesophiles population, confirming that shelf life of fresh cut fruit is limitedly affected by microbial growth.

Table 1. *Evolution of chemical and physical indexes of peach slices after 7 d of storage (T7), A-PET coated by LbL sample (LBL), A-PET uncooated sample (UNC) and control (CTR). Data are means ± SD. Minor letters show significant differences ($p \leq 0.05$) among treatments.*

	L*		C*		Firmness kg cm^{-1}		WL %	TSS %		TA % of malic acid	
	T0	T7	T0	T7	T0	T7	T7	T0	T7	T0	T7
CTR	72.04 ±0.37	74,47^{c} ±2.60	17.77 ±1.15	20.87^{c} ±3.03	3.97 ±0.46	4.08^{b} ±0.73	5.22 ±0.09	8.25 ±0.55	9.55ns ±0.02	0.808 ±0.09	0.710ns ±0.02
UNC	72.04 ±0.37	76.16^{ab} ±2.45	17.77 ±1.15	21.39^{ab} ±2.29	3.97 ±0.46	4.86^{b} ±1.33	2.48 ±0.68	8.25 ±0.55	8.99ns ±0.59	0.808 ±0.09	0.720ns ±0.06
LBL	72.04 ±0.37	76.58^{a} ±2.29	17.77 ±1.15	22.89^{a} ±1.59	3.97 ±0.46	5.89^{a} ±1.54	2.50 ±0.70	8.25 ±0.55	9.53ns ±0.01	0.808 ±0.09	0.670ns ±0.08

Table 2. *Evolution of chemical indexes of peach slices after 7 d of storage (T7), for A-PET coated by LbL sample (LBL), A-PET uncooated sample (UNC) and control (CTR). Data are means ± SD. Minor letters show significant differences ($p \leq 0.05$) among treatments.*

	Total carotenoids content (µg 100 g^{-1})		PPO activity (U mL^{-1} min^{-1})	
	T0	T7	T0	T7
CTR	75.64 ±16.6	35,04^{c} ± 4.56	0.014 ± 0.005	0.021ns ± 0.008
UNC	75.64 ±16.6	58,02^{b} ± 2.27	0.014 ± 0.005	0.023ns ± 0.005
LBL	75.64 ±16.6	64,86^{a} ± 2.68	0.014 ± 0.005	0.011ns ± 0.003

4. Conclusions and Future Perspectives

The novel CRS developed showed the migration of chitosan and catechin modulated by time. Chitosan was effective against *P. chrysogenum*, inhibiting the fungal growth. Tea catechin, immobilized into the alginate layers, carried out its antioxidant capacity that increase according to the extraction time. *In vivo* trials showed the delay of slices senescence, due to presence of chitosan and catechin from green tea extract, slowing the decay of several quality factors.

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