Cháfer Nácher, MT.; Sánchez González, L.; González Martínez, MC.; Chiralt A. (2012). Fungal decay and shelf life of oranges coated with chitosan and bergamot, thyme and tea tree essential oils. Journal of Food Science. 77(8):182-187. doi:10.1111/j.1750-3841.2012.02827.x

Fungal decay and postharvest quality of oranges coated with chitosan and essential oils

Cháfer et al. Decay and quality of oranges coated with essential oils

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Abstract

Chitosan coatings, containinig or not essential oils (bergamot (BO), thymol (TO) and tea tree oil (TTO)), were applied to oranges (cv. Navel Powell). Antifungal effect was evaluated by applying coatings before and after inoculating the fruit with *Penicillium italicum*, preventive and curative treatments respectively. The effect of coatings on the quality parameters (acidity, pH, soluble solids, juice percentage, weight loss, firmness, colour parameters and respiration rate) was controlled for the different oranges samples throughout the cold storage time. Preventive antimicrobial treatments with coatings containing TTO were the most effective, with a reduction of the microbial growth (expressed as the percentage of infected samples) of 50 %, as compared to the uncoated samples. The coatings did not lead to any relevant changes in the development of the sample quality parameters throughout the cold storage, except for a slightly reduced loss of both weight and firmness when the coatings contained BO.

Keywords: antifungal, bergamot, thymol, tea tree

Introduction

Citrus fruits are one of the most economically important crops in the Mediterranean area. The main postharvest losses are caused by the incidence of the citrus pathogens *Penicillium digitatum* (green mould) and *Penicillium italicum* (blue mould).

Synthetic chemicals have been widely used to control these fungal attacks. Nevertheless, current trends in both food industry and consumption are directed towards safer and healthier food production according to principles of sustainability and the need to be environmentally friendly. Numerous researchers have found an effective antifungal control by using food additives of low toxicity (Palou et al., 2008; Valencia–Chamorro et al., 2009) and natural compounds, such as essential oils (EO), which represent a possible alternative to disease control (Tripathi and Dubey, 2004; Palou et al., 2008). Different factors, like high volatility, odour, flavor, price and/or possible phytotoxicity, limit the commercial application of these compounds (Plaza et al., 2004). Their incorporation into biodegradable polysaccharide coatings represents an effective technique to control the high volatility of these compounds as the polysaccharide matrix entraps the bioactive agent, thus minimizing losses and slowing down its diffusion rate to the product surface (where the contamination is prevalent). Thus makes the process more effective at reducing the level of microorganisms than when applied directly on the surface of the product via a spray solution (Kristo et al., 2008: Quitavalla et al., 2002).

In addition to conferring antimicrobial properties to edible films, the incorporation of EO leads to modifications in terms of physical film properties, i.e, an improvement in the water vapor permeability due to the increment of the hydrophobic compound fraction in the films. This type of composite coatings also provides moderate barrier properties (to gases, water…) that contributes to reducing weight losses and slows down the fruit senescence (Vargas et al., 2008; González-Martínez et al., 2011).

Chitosan has been extensively used as a matrix to design polysaccharide films due to its excellent film forming properties, antimicrobial activity and biodegradable nature. The main drawback of chitosan films is its high water vapor permeability, which could be improved (as was commented on above) by adding some lipophilic compounds, such as essential oils (EO). At the same time, the EO could contribute to extend the antimicrobial capacity of pure chitosan films as some microorganisms have been shown to be insensitive to chitosan activity. In this sense, Roller

and Covill (1999) found, in chitosan film studies, that the growth of *Aspergillus flavus, Cladosporium cladosporioides and Penicillium aurantiogriseum* was not affected by the presence of chitosan. Sanchez-González at al., a and b also found that CH films were not effective against *Penicilium italicum* on PDA medium (*in vitro* studies).

Numerous *in vitro* studies have demonstrated the antifungal effectiveness of some EOs against major citrus pathogens such as thymol, oregane, cinnamon, tea tree, birch, cumin and clove oils (Arras et al., 1993, Arras 1999, Plaza et al., 2004, Sanchez-Gonzalez et al., 2011, Yigit et al 2000, Daferera et al., 2000, Giamperi et al., 2002).

Nevertheless, there is very little data about this *in vitro* effectiveness (against major citrus pathogens) when EOs are incorporated into film matrices. Sánchez et al. (2011a and b) *in vitro* studies showed that antifungal activity of composite films against *Penicilium. italicum* was dependent on EO concentration used. Chitosan composite films incorporating Tea Tree Oil (TTO) and Bergamot Oil (BO) delayed the fungal growth of *P. italicum*, films containing 3:1 BO–CH and 2BO:CH ratios exhibited the strongest power of inhibition on PDA medium. However, the antifungal efficiency observed *in vitro* has not always been found *in vivo*, thus probably due to the volatile nature of the EO constituents (Plaza el al., 2004) and the interactions established between film components and the vegetal tissue.

Limited information has been found about the application of coatings based on EOs to citrus fruit (in vivo applications), to the best of our knowledge. Du Plooy et al. (2009) incorporated *Mentha spicata* and *Lippia scaberrima* essential oils into a variety of commercial coatings to control of disease and water loss successfully in 'Tomango' oranges. Plaza et al. (2004) found that thyme and oregano oils incorporated in a commercial wax coating failed to reduce the incidence of *P. digitatum* in 'Salustiana' and 'Valencia' oranges.

One approach followed to simulate fruit commercial situations is to submit the commodities to curative or preventive treatments. In the first one, the fruit (or commodity) is inoculated with the pathogen after the application of the postharvest treatment (to simulate pre-existing infections) and in the second, the inoculation is carried out before (to simulate possible re-infections of fruit during handling or storage).

In this work, chitosan based coatings incorporating Bergamot oil, Tree Tea Oil and Thymol Oil were applied to oranges to evaluate: (1) the antifungal efficiency of these coatings against conidia of the citrus pathogen *P. italicum* in both preventive and curative treatments and (2)

postharvest quality of cold-stored oranges coated with these CH-based films in terms of pH, acidity, weight losses, respiration rates, colour and mechanical properties.

Materials and methods

Raw materials

Oranges (*Citrus sinensis* cv. Navel Powell), cultivated in organic production systems, were harvested in Carricola (Valencia, Spain) and immediately transported to the laboratory, washed under tap water and allowed to dry at room temperature. The oranges that were free of any signs of mechanical damage or fungal decay were selected and standardized as to homogeneity of size, shape and colour.

High molecular weight chitosan (CH), with a deacetylation degree of 82.7% (CAS Number 9012-76-4, Sigma–Aldrich, EEUU), glacial acetic acid and essential oil (EO) of bergamot (BO), thymol (TO) and tea tree oil (TTO) (Herbes del Moli, Alicante, España) were used to obtain film-forming solutions.

Preparation of film-forming dispersions

Chitosan (1%, w/v) was dispersed in an aqueous solution of glacial acetic acid (0.5%, v/v), at 25ºC. After an overnight agitation, each essential oil (EO) was added to the chitosan (CH) solution to reach a final concentration of 2% (w/w) (Sánchez et al., 2010b). CH-EO mixtures were emulsified at room temperature using a rotor-stator homogenizer (Ultraturrax DI 25 basic-Yellowline, Janke & Kunkel, Staufen, Germany) at 13,500 rpm for 4 minutes.

Antimicrobial effectiveness

Stock culture of *Penicillium italicum* (CECT 2294), supplied by Colección Española de Cultivos Tipos (CECT, Burjassot, Spain), was kept frozen (-25ºC) in Potato Dextrose Agar (Scharlab, Barcelona, Spain) supplemented with 30% glycerol (Panreac, Barcelona, Spain). The fungus was inoculated on Potato Dextrose Agar (PDA) and incubated at 25ºC until sporulation. The inoculum's concentration was adjusted by means of a haemocytometer at 10^5 spores per mL.

In order to determine the antifungal activity, two types of in vivo assays were carried out, simulating a preventive or curative effect of coatings, respectively. The methodology used was adapted from Valencia-Chamorro et al. (2009).

In preventive assays, oranges were dipped for 1 min in the different chitosan formulations (20 fruits per treatment), completely dried (about 6-8 hours at room temperature) and then inoculated with the pathogen *(Penicillium italicum).* Fruit inoculation was carried out by wounding each fruit once on the fruit equator and inoculating at two points located on opposite sides of the fruit equator. Inoculated but uncoated fruits were used as controls (20 fruits).

In curative assays, the fruit was first inoculated as described above and, 24 h later, was coated by dipping it in the film forming dispersions for 1 min. Surface drying was also carried out at room temperature.

Every fruit was placed on perforated metallic trays and then incubated for a storage period of up to 26 days at 25 °C and 90% RH to simulate the optimal conditions for microbial life. Disease incidence was assessed as the percentage of decayed fruit after 5, 10, 17, 19 and 26 days and disease severity was determined as the diameter of the lesion (mm).

Postharvest quality of coated oranges

Selected oranges were dipped in the film-forming dispersions for 1 min. Samples were dried by natural convection for 2-3 h at room temperature and were stored on perforated metallic trays in an incubator (EC-1400-HR, Radiber S.A., Spain) at 5°C and 90% RH. Before the fruit characterization at different cold storage times (15, 30 and 60 days), fruits were kept for a week at 20°C and 60% RH to simulate commercialization period.

20 oranges per treatment and type of coating were used to control the cumulative weight losses at different storage times, expressed as a percentage loss of the initial weight.

The juice of each orange was extracted and the percentage of juice was expressed with respect to the weight of the same orange.

Acidity, pH, soluble solids and ascorbic acid were analysed at 20ºC in previously extracted juice. Four juice samples were considered for each treatment/time. Each juice sample corresponded to 5 oranges. Acidity was analysed by following the AOAC 942.15 method (AOAC, 1995), and was expressed as g of citric acid per 100 g of fruit. Total soluble solids in citrus juice were measured by a refractometer (3T, Atago Co., Ltd., Japan).

The mechanical properties were measured by using a texture analyser (TA-XTplus, Stable Micro Systems, UK) with a 50 kg load cell, using a 75 mm diameter cylindrical probe. The machine compressed the samples (20 for each treatment and storage time) in the equatorial zone until there was a deformation of 5% at a 1 mm/s speed. Results were expressed as the force required to reach this deformation level. Measurements were taken in 20 oranges for each treatment and storage time.

A closed system was used to measure the respiration rate (Castelló et al. 2006). At each sample time during storage, two samples (each one about 150-200 g) were placed in 2 L hermetic glass jars with a septum in the lid for sampling gas in the headspace at different times. The O_2 y CO_2 contents were measured using an O_2 and CO_2 meter (Checkmate 9900, PBI Dansensor, Denmark). The respiration rate (RRi, mg kg^{-1} h^{-1}) of the samples, in terms of CO_2 generation and O_2 consumption, was determined from the slope of the fitted linear equation, as described by Fonseca et al. (2002). Analyses were carried out in triplicate and samples were only used for these analyses.

Colour was measured using a spectrocolorimeter (CM-3600d, Minolta Co., Japan) with a 10 mm diameter window. Measurements were taken in 20 samples fruits/treatment throughout the entire storage period. To avoid the effects of heterogeneity in the fruit, measurements were always taken in the same previously marked sample zone in the orange. CIE-L*a*b* coordinates, hue (h^*_{ab}) and chrome (C^*_{ab}) (CIE, 1986) were obtained from the reflection spectra of the samples using D65 illuminant/10º observer.

Statistical analysis

Results were analysed by a multifactor analysis of variance and discriminant analysis with 95% significance level using Statgraphics® Plus 5.1. Multiple comparisons were performed through 95% LSD intervals.

Results and discussion

Curative and preventive antifungal activity of coatings

In this work, preventive treatment was evaluated using coated oranges inoculated with Penicillium italicum after the coating dried, whereas curative treatment implied the previous infection of the fruit before coating.

Figures 1a and 2a show the development of the incidence of disease (expressed as the percentage of affected fruits) in both the curative and preventive applications of coatings. In every case, the percentage of affected fruits increased throughout the storage time to reach an asymptotic value from 15 storage days onwards. This percentage was lower for samples coated with films containing TO and TTO, for curative and preventive applications, respectively. Figures 1 and 2 also show the diameter of mould growth (1b and 2b), where a linear increase of the diameter of the affected fruit zone was observed in every case. This linear increase did not differ significantly when referring to the treatments for preventive applications, although in curative treatments, a slightly lower growth rate was observed for the samples coated with the film containing TO, coinciding with the lowest incidence of disease observed for this treatment. In general, whereas no antifungal effect was observed in CH coated oranges, the incorporation of TTO, in the case of preventive applications, and TO, in the curative applications, provided the coatings with the highest fungal control. As compared with the uncoated samples, there was a reduction of the infected fruits of 50 and 35%, respectively.

In the case of curative treatments, on the fifth storage day the pure CH and the CH-BO coatings also showed a good fungal control, but this effect was no longer maintained. In the case of preventive treatments, after 10 storage days the CH-TO and CH-BO coatings showed a similar antifungal effect (with an incidence of disease of about 80%), both being less effective than those containing TTO (around 50%). Whereas CH-TO was more effective in curative treatments, CH-TTO had a greater antifungal effect in preventive treatments, where the final inhibition of mould growth was promoted. The results show that the antifungal effect of the essential oil incorporated in the coating formulation depended on whether the applied treatment was curative or preventive, in agreement with the conclusions reported by Valencia-Chamorro *et al.* (2009). These authors also shown the fruit cultivar and the adaptation level of the pathogen to the fruit played an important role.

Taking into account our results, it can be concluded that when the pathogen is already adapted to the fruit (such as in curative treatments), the TO provide the CH based coatings with the greatest disease control.

No antifungal effect was observed for pure CH coatings in either preventive or curative treatments as it has been observed by others authors in similar studies carried out in *cv.* ‘Valencia’ oranges (Valencia-Chamorro et al, 2009). Nevertheless, some authors (Chien et al 2006, 2007) found

chitosan coatings to be effective against blue and green postharvest citrus decay in Tankan and Murcott tangor citrus fruit, respectively. Differences related to the different molecular weight, degree of polymerization and deacetylation of chitosan (Jung and Kim, 1999) and citrus cultivar used could explain the observed differences.

Postharvest quality

Weight loss and compositional changes

Table 1 shows average values, standard deviation and ANOVA results for compositional variables (pH, acidity, ºBrix), percentage of juice and weight loss of samples, both uncoated and coated at different cold storage times.

The acidity values of the samples showed a significant decrease throughout storage time, regardless of the coating. This decrease is more marked at the beginning of storage (acidity reduction of about 39% during the first 2 weeks) than in the following period, where a decrease of 33% was registered over 6 weeks of storage. Few changes were detected for pH values that only showed a slight increase at the end of storage, regardless of the coating. The soluble solids fluctuated throughout the storage time with no clear treatment dependent tendency. This could be explained by a greater contribution of the natural variability of the sample than that of the treatment or storage time.

In general, the compositional changes described above are coherent with the low metabolic activity of this non-climateric fruit throughout its postharvest. The scarce differences among the compositional variation pattern for the different samples throughout the storage, point to the fact that no detrimental effect was provoked by the coatings.

Both storage time and coating type were found to have a significant effect ($p<0.05$) on sample weight loss. This weight loss increased over the storage time and tended to be lower for all the coated oranges than the uncoated (4-4.7%). Coatings containing BO gave rise to the lowest weight losses; the reduction of weight loss ranging between 34-40% with respect to the control samples. At the end of storage, CH-BO coated oranges maintained the lowest weight loss and very few differences were detected among the other coatings. Other studies have also reported the

effectiveness of polysaccharide coatings as a water barrier in citrus fruit and its enhancement by the incorporation of lipids (Du Plooy et al., 2009; Rojas-Argudo et al., 2009; Valencia-Chamorro et al., 2009). Thus, the addition of essential oils to CH coatings showed a slight improvement of the water barrier properties, as can be expected from the polar nature of some of their components and their liquid state.

Firmness, respiration activity and optical properties

Table 2 shows the results of compression test in oranges, respiration rates, in terms of oxygen consumption and CO_2 generation, and optical properties. There was a significant decrease throughout the storage time in the firmness values of every sample of Navel Powell oranges, evaluated by means of compression force, which is coherent with the previously described weight loss development and the subsequent loss of fruit turgor. The loss of fruit firmness after the storage period was about 46-57% with respect to the initial force values. Although the firmness of oranges was not notably affected by the coating treatment, coatings with BO and TTO did significantly ($p<0.05$) delay the loss of firmness. This has also been previously reported by other authors working on coated citric fruits (Hagenmaier, 2000; Pérez-Gago et al., 2002 and Valencia-Chamorro et al., 2009), where a notable influence of the citric cultivar was observed.

The respiration rate was evaluated through the oxygen consumption and CO_2 production (Table 2) and provides essential information about fruit metabolic activity during postharvest. ANOVA showed that only storage time had a significant effect.

Measurements of the color coordinates for the same sample before and after coating revealed total color differences of ΔE 2.5±2.0 units, regardless of the type of coating, which indicates that the coatings have a very low impact on the product color. Table 2 shows luminosity, chrome and hue colour coordinates of samples at different storage times, where a mean value was considered at time 0 for every sample, since no significant differences were found. The luminosity values of every sample decreased significantly during storage (around 6%). This can be explained by the surface drying during storage which implies a greater concentration of pigments per surface area, thus promoting light absorption. L* values were slightly higher for CH-BO coated samples, which also showed the lowest water loss during storage. Every sample experienced a significant reduction in its hue values (around 6%), showing a more orange-less yellow hue at the end of storage, regardless of the

treatment. Chrome values fluctuate slightly throughout the storage time, depending on the sample. The observed changes in color coordinates could also be affected by the increase in the carotenoid content which occurs during citric postharvest as a function of the maturity index of the harvested fruit (Spiegel-Roy and Goldschmidt, 1996).

Every sample experienced a decrease in both oxygen consumption and CO2 generation while stored. In the case of CO2 generation, this effect was more marked at the beginning of storage, whereas after 2 storage weeks no significant decrease was observed. Nevertheless, oxygen consumption decreased both during the first two storage weeks and in the last storage weeks. This can be explained by the metabolic activity of this kind of fruit which slows down immediately after harvesting and is accelerated when senescence of the fruit occurs, after several storage weeks. The differences between oxygen consumption and CO2 generation suggest the development of anaerobic pathways to obtain the cell energy mainly during the final storage period when fruits become senescent. The coatings were observed to have no effect ($p>0.05$) on the respiration pattern of the fruit throughout the storage time, except for the fact that the lowest level of CO2 is generated at the initial time of uncoated samples, which can be attributed to the stress that the coating process exerts on the fruit.

Conclusion

Pure CH films did not show any antifungal activity against *Penicillium italicum*. A microbiological analysis showed that the antifungal properties of chitosan composite films were dependent on whether the treatment was preventive or curative. So, the greatest antifungal effectiveness against *Penicillium italicum* was displayed by the CH-TO coatings for the curative treatments of oranges and the coatings containing TTO were more effective when applied in a preventive way. The quality parameters of oranges obtained throughout postharvest suggest that the coatings did not have a notable effect on the fruit development and color appearance, but they provide the fruit surface with enhanced water barrier properties. CH coatings containing TTO applied as a preventive treatment are recommended for the studied citrus fruit cultivar.

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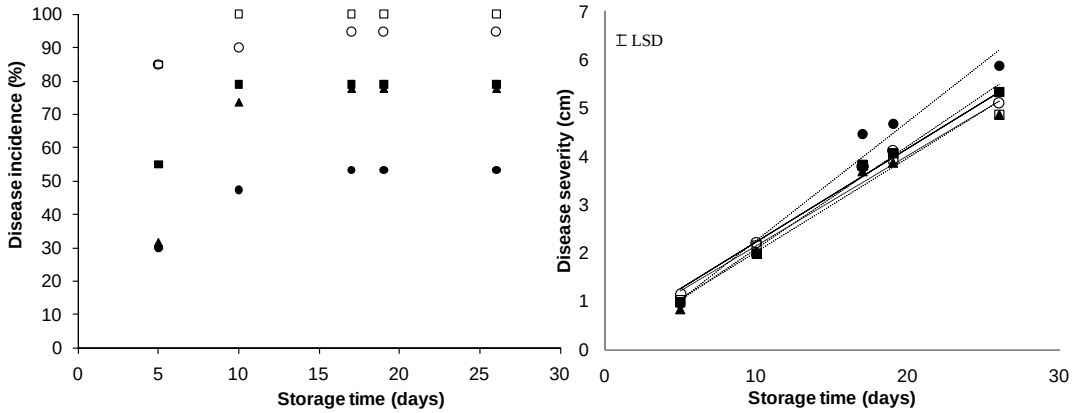
FIGURE CAPTIONS

Figure 1. Development of the disease incidence (a), expressed as the percentage of affected fruits, and disease severity (b), expressed as diameter of mould growth, in preventive application of coatings.

Figure 2. Development of the disease incidence (a), expressed as the percentage of affected fruits, and disease severity (b), expressed as diameter of mould growth, in curative application of coatings.

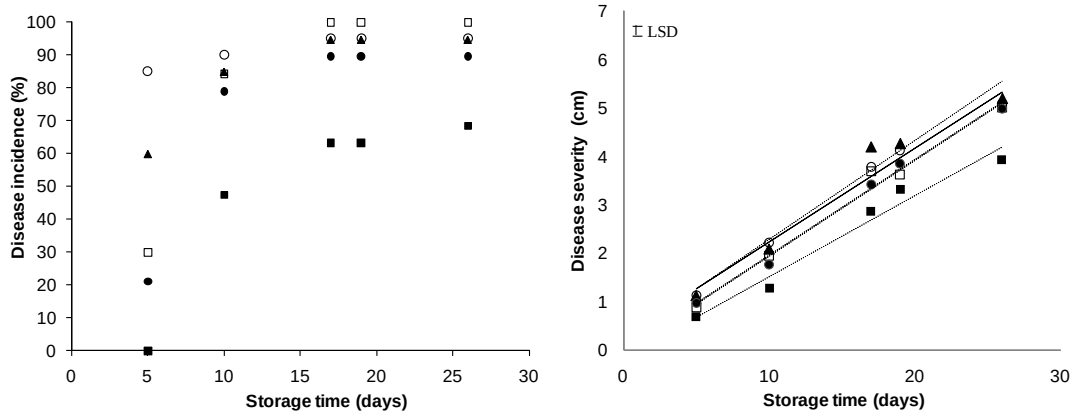
LSD= 0.237



○ Control, □ CH, ■ T, ▲ BO, ● TTO

Figure 1.

LSD=0.242



○ Control, □ CH, ■ T, ▲ BO, ● TTO

Figure 2.

Table 1. Compositional variables (pH, acidity, ºBrix), percentage of juice and weight loss of samples uncoated and coated at different cold storage times. Average values, standard deviation and ANOVA.

	Time (weeks)	pH	Acidity (g citric acid/100 g sample)	ºBrix	% juice	Weight loss (%)
Control	0	3.606 (0.005)^{aw}	0.982 (0.013)^{aw}	9.36 (0.05)^{aw}	37 (9)^{aw}	-
	2	3.596 (0.015)^{aw}	0.57 (0.02)^{ax}	10.3 (0.2)^{ax}	39 (6)^{bw}	4.0 (0.7)^{aw}
	4	3.673 (0.015)^{ax}	0.553 (0.013)^{ax}	9.63 (0.15)^{ay}	39 (4)^{aw}	3.5 (0.4)^{bw}
	8	3.706 (0.005)^{ay}	0.422 (0.009)^{ay}	9.36 (0.05)^{aw}	41 (6)^{aw}	4.7 (0.8)^{bx}
CH	0	3.606 (0.005)^{aw}	0.982 (0.013)^{aw}	9.36 (0.05)^{aw}	37 (9)^{aw}	-
	2	3.54 (0.02)^{bx}	0.566 (0.013)^{ax}	9.36 (0.05)^{bw}	44 (3)^{aw}	3.3 (0.3)^{cw}
	4	3.77 (0.02)^{by}	0.54 (0.02)^{ax}	9.3 (0.2)^{abw}	37 (6)^{aw}	4.2 (0.7)^{cx}
	8	3.823 (0.005)^{bz}	0.425 (0.004)^{ay}	9.86 (0.05)^{bx}	37 (4)^{aw}	4.1 (0.5) ^{bcx}
CH-BO	0	3.606 (0.005)^{aw}	0.982 (0.013)^{aw}	9.36 (0.05)^{aw}	37 (9)^{aw}	-
	2	3.52 (0.02)^{bx}	0.630 (0.013)^{ax}	9.80 (0.05)^{cx}	48 (7)^{acx}	2.4 (0.4)^{bw}
	4	3.656 (0.005)^{ay}	0.560 (0.013)^{ay}	9.96 (0.05)^{cy}	40 (6)^{aw}	3.2 (0.4)^{abx}
	8	3.776 (0.005)^{cz}	0.441 (0.009)^{abz}	9.43 (0.05)^{aw}	39 (9)^{awx}	3.1 (0.5)^{ax}
CH-T	0	3.606 (0.005)^{aw}	0.982 (0.013)^{aw}	9.36 (0.05)^{aw}	37 (9)^{aw}	-
	2	3.543 (0.005)^{bx}	0.592 (0.013)^{ax}	9.03 (0.05)^{dx}	49 (5)^{cx}	3.4 (0.4)^{cwx}
	4	3.593 (0.015)^{cw}	0.636 (0.013)^{bx}	9.63 (0.15)^{ay}	42 (4)^{aw}	3.0 (0.5)^{aw}
	8	3.72 (0.02)^{dy}	0.46 (0.02)^{by}	8.46 (0.05)^{cz}	42 (5)^{aw}	3.8 (0.6)^{cx}
CH-TTO	0	3.606 (0.005)^{aw}	0.982 (0.013)^{aw}	9.36 (0.05)^{aw}	37 (9)^{aw}	-
	2	3.87 (0.02)^{cx}	0.620 (0.013)^{awx}	8.46 (0.05)^{ex}	37 (5)^{bw}	3.0 (0.6)^{cw}
	4	3.72 (0.03)^{dy}	0.505 (0.009)^{cxy}	9.2 (0.2)^{by}	35 (8)^{aw}	3.4 (0.6)^{abw}
	8	3.83 (0.02)^{bz}	0.384 (0.009)^{cy}	8.43 (0.05)^{cx}	40 (7)^{aw}	4.2 (0.7)^{bcx}

^{a,b,c,d,e} Different superscripts within a column indicate significant differences among samples for the same time($p<0.05$).
^{w,x,y,z} Different superscripts within a column indicate significant differences between times for the same sample ($p<0.05$).

Table 2. Colour coordinates, respiration rates and firmness of samples uncoated and coated at different cold storage times. Average values, standard deviation and ANOVA.

	Time (weeks)	L	h	c	RRO_2 (mL/kg.h)	$RRCO_2$ (mL/kg.h)	Firmness Force (N)
Control	0	67 (2)^{aw}	61 (2)^{aw}	75 (2)^{aw}	11 (3)^{aw}	12 (2)^{aw}	35 (5)^{aw}
	2	65.8 (1.8)^{awx}	60 (2)^{awx}	78.0 (0.8)^{ax}	7.6 (1.3)^{abx}	10.4 (1.9)^{abw}	19 (7)^{ax}
	4	64.4 (1.5)^{axy}	58.5 (1.2)^{axy}	76.8 (1.4)^{ax}	9.6 (0.6)^{awx}	9.0 (0.5)^{aw}	16 (3)^{ax}
	8	62.5 (1.3)^{ay}	56.9 (0.8)^{ay}	77.5 (0.8)^{ax}	0.05 (0.04)^{ay}	9 (3)^{aw}	15 (2)^{ax}
CH	0	67 (2)^{aw}	61 (2)^{aw}	75 (2)^{aw}	12 (2)^{aw}	26 (7)^{bw}	35 (5)^{aw}
	2	67 (2)^{aw}	60 (2)^{awx}	76.1 (0.8)^{bcw}	8.7 (0.7)^{awx}	13.42 (1.16)^{bx}	20 (3)^{ax}
	4	64.7 (1.7)^{abwx}	57 (2)^{ay}	72.0 (1.4)^{cx}	6.5 (1.9)^{awx}	6 (2)^{ax}	19 (6)^{ax}
	8	63.0 (1.8)^{ax}	57.0 (1.5)^{ay}	76.0 (1.7)^{aw}	4 (6)^{bx}	11.6 (1.7)^{ax}	16 (3)^{ax}
CH-BO	0	67 (2)^{awx}	61 (2)^{awx}	75 (2)^{aw}	12 (2)^{aw}	26 (7)^{bw}	35 (5)^{aw}
	2	69 (2)^{bwx}	63 (4)^{aw}	75.5 (1.8)^{cw}	5.1 (1.3)^{bx}	7.3 (1.4)^{ax}	24 (5)^{bx}
	4	67 (3)^{bw}	60 (5)^{awx}	73 (2)^{bcw}	6 (3)^{ax}	8 (4)^{ax}	16 (3)^{ay}
	8	64 (2)^{ax}	57 (3)^{ax}	75 (2)^{aw}	0.02 (0.06)^{ay}	6 (2)^{ax}	18 (5)^{ay}
CH-T	0	67 (2)^{aw}	61 (2)^{aw}	75 (2)^{awx}	12 (2)^{aw}	26 (7)^{bw}	35 (5)^{aw}
	2	66.4 (1.4)^{aw}	60 (2)^{awx}	77.1 (0.5)^{abw}	7 (2)^{abw}	12 (2)^{abx}	19 (2)^{ax}
	4	65.7 (1.9)^{abw}	58 (3)^{ax}	74.8 (0.9)^{abx}	8 (7)^{aw}	10 (8)^{ax}	18 (2)^{ax}
	8	64.2 (0.3)^{aw}	58.5 (0.9)^{awx}	75.7 (0.2)^{awx}	0.05 (0.03)^{ax}	10 (4)^{ax}	17 (2)^{ax}
CH-TTO	0	67 (2)^{aw}	61 (2)^{aw}	75 (2)^{aw}	12 (2)^{aw}	26 (7)^{bw}	35 (5)^{aw}
	2	66.15 (1.03)^{awx}	61.0 (0.7)^{aw}	77.6 (1.5)^{abx}	5.2 (1.3)^{bx}	12 (5)^{abx}	26 (4)^{bx}
	4	65.3 (0.6)^{abx}	58.4 (1.2)^{ax}	74.7 (1.8)^{bw}	5.6 (0.5)^{ax}	6.6 (0.8)^{ax}	21 (8)^{ay}
	8	62.9 (0.6)^{ay}	57.1 (1.4)^{ax}	77.2 (1.4)^{ax}	0.05 (0.02)^{ay}	9 (2)^{ax}	19 (5)^{ay}

^{a,b} Different superscripts within a column indicate significant differences among samples for the same time($p<0.05$).
^{w,x,y} Different superscripts within a column indicate significant differences between times for the same sample ($p<0.05$