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Effects of treatment with chitosan and antimelanogenesis agents on discoloration of chilled and frozen stored shrimp*)

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Summary

The effect of chitosan in combination with polyphenol oxidase (PPO) inhibitors and other additives (citric acid, rosemary extract) to inhibit or slow down melanosis during chilled and frozen storage was investigated. Fresh deepwater pink shrimps (Parapenaeus longirostris) were dipped in different solutions containing combinations of sodium metabisulphite (2500 mg/L), 4-hexylresorcinol (50 mg/L), chitosan (5 g/L), citric acid (200 mg/L) and rosemary extract (50 mg/L) and stored at 4°C for six days and at –18°C for twelve months. During storage, changes in colour were defined sensorially and instrumentally. Chitosan alone showed no effect on discoloration, but in combination with PPO inhibitors showed an additional inhibitory effect on melanosis formation. No noticeable darkening was observed during frozen storage in all treated shrimps, whereas signs of blackening were noticeable from the third month; 50 mg/L of 4-hexylresorcinol were more effective than 2500 mg/L of sodium metabisulphite in the prevention of melanosis development after shrimping.

Keywords: chitosan, polyphenol oxidase inhibitors, shrimp, melanosis

Shrimp is a highly perishable product and its post-mortem changes occur rapidly. The shrimp tissue is still biochemically active although the organism itself loses its viability soon after it is caught. The most significant change that occurs soon after capture is melanosis, which is characterized by discoloration (darkening in colour). This defect, that is also known as the Black Spot, or blackening, is caused by a system of enzymes that are naturally present in shrimps. These enzymes, in the presence of air, can chemically transform colourless compounds in the shrimp into complex brown pigments near the shrimp surfaces and shells (20). The reaction is a consequence of phenolic compounds' oxidation by polyphenol oxidase (PPO), which triggers the generation of dark pigments (16). As melanosis develops rapidly in shrimps and significantly reduces the marketability of the products within 24 hours (34), PPO inhibitors are frequently used in practice to prevent melanosis. From this point forth, sulphites and their derivatives, especially sodium metabisulphite, are the most effective anti-melanotic agents (28). However, residue of these compounds in shrimp may cause allergic reactions in certain individual consumers (15). Additionally, sulphites are unstable and decompose into sulphur dioxide gas when they have contact with water. Therefore, inhalation of this gas may cause serious health risks to consumers (27). 4-Hexylresorcinol ($C_{12}H_{18}O_2$) is proposed for use as a processing aid for the prevention of melanosis in shrimp and as an alternative to the currently approved sulphites. It is stable in saline water and does not lose its activity in the presence of organic materials (21, 27). 4-Hexylresorcinol has been evaluated by the Joint FAO/WHO Expert Committee on Food Additives in 1995 which was unable to establish a numerical ADI but concluded that the treatment of crustaceans at concentrations of up to 50 mg/L, resulting in residue levels of approximately 1 mg/kg in the edible portion, is not of toxicological concern. Subsequently, 4-hexylresorcinol is permitted as a food additive (E586) under

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part D of Annex III to Directive 95/2/EC, as amended by Directive 2006/52/EC, where it is permitted in fresh, frozen and deep-frozen crustaceans up to 2 mg/kg as residues in crustacean meat (10).

Chitosan is a biopolymer that is naturally obtained by the partial deacetylation of chitin, a natural polysaccharide present in the outer skeleton of shellfish including crabs and shrimps. It has been reported that chitosan extended the shelf-life of foods by means of versatile functions such as antimicrobial, antioxidative, moisture retention, film-forming, and enzyme immobilization (6-8, 11, 24, 33, 35, 37, 38). Chitosan was proposed to inhibit the browning in fruit and vegetable products as an alternative to sulphites (2, 18, 19, 41, 45). But, there are limited studies investigating the retarding effects of chitosan on the discoloration of shrimps during storage.

This study was conducted in order to investigate the effect of chitosan in combination with PPO inhibitors and other additives (citric acid, rosemary extract) to inhibit or retard melanosis during chilled and frozen storage of shrimp.

Material and methods

Shrimp samples. Deepwater pink shrimps (*Parapenaeus longirostris*) were caught using a drag net (beam trawl) in the Marmara Sea (Tekirdağ offshore, Turkey). The shrimps were immediately mixed with ice at a 2: 1 ratio in polystyrene boxes after being caught and kept within them till packaging in order to avoid any negative atmospheric effects, and were immediately brought to the processing laboratory within 90 minutes. The shrimping was carried out on three different dates in May-June 2011. Approximately 25 kg of shelled shrimps were used in each experimental trial.

Treatment of shrimps. Shrimps were subjected to washing with tap water in a plastic container. The washed shrimps were randomly divided into eight equal batches of 3 kg each and then treated for 10 minutes by dipping in 6 L of solutions containing combinations of sodium metabisulphite (Merck 106528), 4-hexylresorcinol (Merck 820647), deacetylated (75-85%) chitosan (Sigma-Aldrich 448,877), acetic acid (Merck 100056), citric acid (Merck 100244), and rosemary extract (Sigma-Aldrich, W299200) as indicated in the ratios shown in Tab. 1. One of the batches was treated with tap water (without additive) for an equal time and used as an untreated control sample. The chemicals for groups A and B were directly dissolved in tap water, while for other groups (C, D, E, F, G), chitosan was first dissolved into 1% acetic acid solution and then other chemicals were added.

Treated and untreated shrimps were removed and allowed to drain for 10 min on a pre-disinfected colander. After the draining of the excess treatment solution, each group of shrimps were distributed to Styrofoam plates and sealed with polyethylene film. All packages were kept according their storage condition in a refrigerator (4°C) for 6 days and in a deep-freezer (–18°C) for 12 month. Duplicate samples were taken at 2, 4, and 6 days of chilled storage and 3, 6, 7, 8, 9 and 12 month of frozen storage for colour (instrumental and sensorial) analysis. Results are recorded as the arithmetic means of three experimental trials.

Instrumental colour analysis. The surface colour of the shrimp samples was measured using a Colorflex Hunter-Lab Spectrophotometer (Hunter Associates Laboratory Inc., Reston, VA, USA). Colour was evaluated using diffuse illumination (D65 2° observer) with 8 mm viewing aperture and a 25 mm port size with the specular component excluded (3). Measurements were made directly on the surface of shrimp immediately after opening the pack. Samples were placed in a special cup of the instrument, which fitted within the sample port of the colorimeter. The colour of three by three of 15 pieces of raw shelled shrimp was evaluated after removal of head, tail and legs and was averaged at each sampling day, immediately after opening each package in terms of CIE L* (lightness), a* (redness) and b* (yellowness) values.

Sensorial colour evaluation. Eight trained panellists, staff of Istanbul University from the Food Hygiene and Technology Department (3 females and 5 males, ranging in age between 26 and 45 years) evaluated the colour of the shrimp samples by using a 100 mm of graphic rating scale from unacceptable (extremely dark, pale) to perfect (clear and bright). Prior to the analysis, vocabularies of the sensory attribute were developed by the panellists in a round-table session, using a standardized procedure (17), and training was conducted in two separate sessions approximately 2 h for the evaluation of selected attributes and was followed by an open-discussion session to familiarize panellists with the attribute and the scale to be used. All assessments took place in individual temperature-controlled booths in day light conditions. Each sample was labelled at random, with a three-digit code number and the panellists were received a set of 6 samples (with 3 samples of in each plate) served in a complete randomized order. It was requested to mark

Tab. 1. Experimental design and chemicals used

Group	Acetic acid	Chitosan	Rosemary extract	Citric acid	Sodium metabisulphite	4-Hexyl-resorcinol
A	–	–	–	–	2500 mg/L	–
B	–	–	–	–	–	50 mg/L
C	10 ml/L	5 g/L	–	–	2500 mg/L	–
D	10 ml/L	5 g/L	–	–	–	50 mg/L
E	10 ml/L	5 g/L	50 mg/L	200 mg/L	2500 mg/L	–
F	10 ml/L	5 g/L	50 mg/L	200 mg/L	–	50 mg/L
G	10 ml/L	5 g/L	–	–	–	–
Control	–	–	–	–	–	–

a position on the unstructured line scale for the colour of the examined samples. Ratings were measured by a ruler and converted to numerical scores, and means of the scores given by the panellists were defined as a result of sensory panel (5).

Statistical analysis. All data were analyzed by one way analysis of variance (ANOVA) for investigating the effect of treatments during storage time. The trial was performed in triplicate and significant differences were defined with SPSS 13.0 for Windows (40) using Duncan's multiple range tests ($P < 0.05$).

Results and discussion

Coating with chitosan may inhibit browning of the fruits and vegetables through the protection phenolic compounds from enzymatic oxidation by creating a barrier against atmospheric oxygen (2, 11, 18, 42). Similar attitudes could be seen in fishery products. Mohan et al. (29), who investigated the effect of chitosan coating (1% and 2%) on colour in sardine, reported a rapid decline in colour scores of the untreated group in comparison to those treated with chitosan. However, in the present study, treatment of shrimp with chitosan alone (5 g/L) did not have a significant effect on delaying the blackening. Chitosan treated and untreated (control) shrimps showed high level melanosis and completely darkened at the second days of chilled storage. Therefore, these groups are not included in the sensory and instrumental colour evaluation. For other groups, the variations in the sensorial colour scores of the shelled shrimp during refrigerated storage were shown in Tab. 2. Despite the use of anti-melanosis agents, the discoloration appeared rapidly. All groups scored less than half of maximum score (100) on the 4th day. However, the mean colour scores of the samples treated with sodium metabisulphite (groups A, C and E) were lower than those treated with 4-hexylresorcinol (groups B, D and F). The differences in means were statistically significant on days 2 and 4 of storage ($P < 0.05$). Similar results were reported by other previously conducted studies. Guandalini et al. (14), McEvily et al. (27) and Martinez-Alvarez et al. (25) highlighted that 4-hexylresorcinol showed a marked ability to inhibit or slow down melanosis in shrimp and in lobsters.

In the present study, although a fast discoloration was observed in all groups, the samples treated with combination of chitosan with sodium metabisulphite or with 4-hexylresorcinol graded by panellists with lower scores compared to the samples treated with only PPO inhibitors (Tab. 2). This finding indicated that chitosan enhances the effect of anti-melanosis agents and contribute to the protection of original colour. The addition of citric acid and rosemary extract along with chitosan into the treatment solutions did not make a further improvement. Montero et al. (30) also reported that combining the 4-hexylresorcinol with citric acid did not increase the extent of melanosis inhibition, but did noticeably improve shrimp appearance. Some studies showed a positive effect of citric acid (4, 23, 32). Pardio et al. (32) explained that the inhibitory effect combination with citric acid on melanosis is correlated with a more effective inhibition of PPO at a low pH level. According to Marshal et al. (23), citric acid exerts its inhibitory effect on PPO by lowering the pH as well as by chelating the copper at the active site of the enzyme.

A rapid colour change was observed in shrimps in the present study despite the use of melanosis inhibitors. The enzyme concentration, enzyme activity and phenolic substrate levels were known to vary among shrimp species (39). The deepwater pink shrimp (Parapenaeus longirostris), which was the material of the present study, was considerably sensitive to melanosis due to high PPO activity (30, 44). Guandalini et al. (14) and Montero et al. (31) emphasized that melanosis could only be delayed solely for 2-4 days on deepwater pink shrimps while treated with various concentrations of 4-hexylresorcinol.

The rate of enzyme-catalysed reactions is controlled to a great extent by temperature. For every 10°C temperature increase, there is a two-fold increase in the rate of an enzyme-catalysed reaction. On the other hand, for every 10°C reduction in temperature, a similar decrease in the rate of biological activity occurs (23). As the experiments were carried out during a warm season and time consuming while shrimping on the boat, the melanosis process could be initiated before chilled storage stage depending on the increased PPO activity.

The sensory scores of colour of shrimp were correlated well with instrumental measured changes. Lightness (L*) value is directly associated with melanosis. In all

Tab. 2. Mean sensory colour scores of shrimps during chilled storage (over a hundred points)

Group	Chilled storage (day)			
	0	2	4	6
A	86.2 ± 1.81	50.6 ± 2.50^{c}	18.0 ± 1.13^{d}	7.0 ± 0.69^{e}
B	85.8 ± 1.62	68.2 ± 1.80^{a}	35.2 ± 2.38^{b}	19.4 ± 1.37^{ab}
C	86.9 ± 1.35	54.3 ± 1.82^{bc}	25.7 ± 1.58^{c}	11.0 ± 1.62^{de}
D	86.9 ± 1.82	69.7 ± 1.58^{a}	40.7 ± 1.62^{a}	16.5 ± 1.35^{bc}
E	86.5 ± 1.81	60.1 ± 1.37^{b}	28.6 ± 2.33^{c}	12.5 ± 1.37^{cd}
F	87.6 ± 1.56	71.9 ± 2.63^{a}	41.8 ± 1.62^{a}	21.3 ± 2.03^{a}

Explanations: $^{a\text{-}e}$ means within a column with different letters are significantly different ($P < 0.05$); (A) sodium metabisulphite (2500 mg/L), (B) 4-hexylresorcinol (50 mg/L), (C) chitosan (5 g/L) + sodium metabisulphite (2500 mg/L), (D) chitosan (5 g/L) + 4-hexylresorcinol (50 mg/L), (E) chitosan (5 g/L) + citric acid (200 mg/L) + rosemary extract (50 mg/L) + sodium metabisulphite (2500 mg/L), (F) chitosan (5 g/L) + citric acid (200 mg/L) + rosemary extract (50 mg/L) + 4-hexylresorcinol (50 mg/L)

samples L* value decreased continuously as the storage time progressed (Fig. 1). But, the L* value of the shrimps treated with sodium metabisulphite alone was significantly lower than the samples treated with 4-hexylresorcinol alone on the 2nd and 4th days ($P < 0.05$). The results indicated that 4-hexylresorcinol significantly delayed melanosis when compared with sodium metabisulphite. A similar decrease was observed for the redness (a*) values. However, the difference between the two groups was not statistically significant. Yellowness (b*) value is associated with deterioration. In the present study, the value was increased in relation to the microbial growth in all groups during storage period.

Addition of chitosan into the treatment solutions slowed down the rate of changes in L*, a* and b* values (Fig. 1). The differences between the groups that were treated with and without chitosan were statistically significant in most cases ($P < 0.05$). Mohan et al. (29) reported that the changes in the instrumental colour parameters in chitosan treated sardines were slower than in untreated samples. In the present study, the slower increase in b* values in shrimp samples that were treated with solutions containing chitosan may be explained by the antimicrobial effect of chitosan delaying the decay process. Addition of rosemary extract and citric acid into the treatment solutions with chitosan did not cause a significant difference in the instrumental colour values. Some studies on different meat products reported that rosemary contributed to the colour (redness) stability during storage (12, 13, 36), whereas others did not (1, 26).

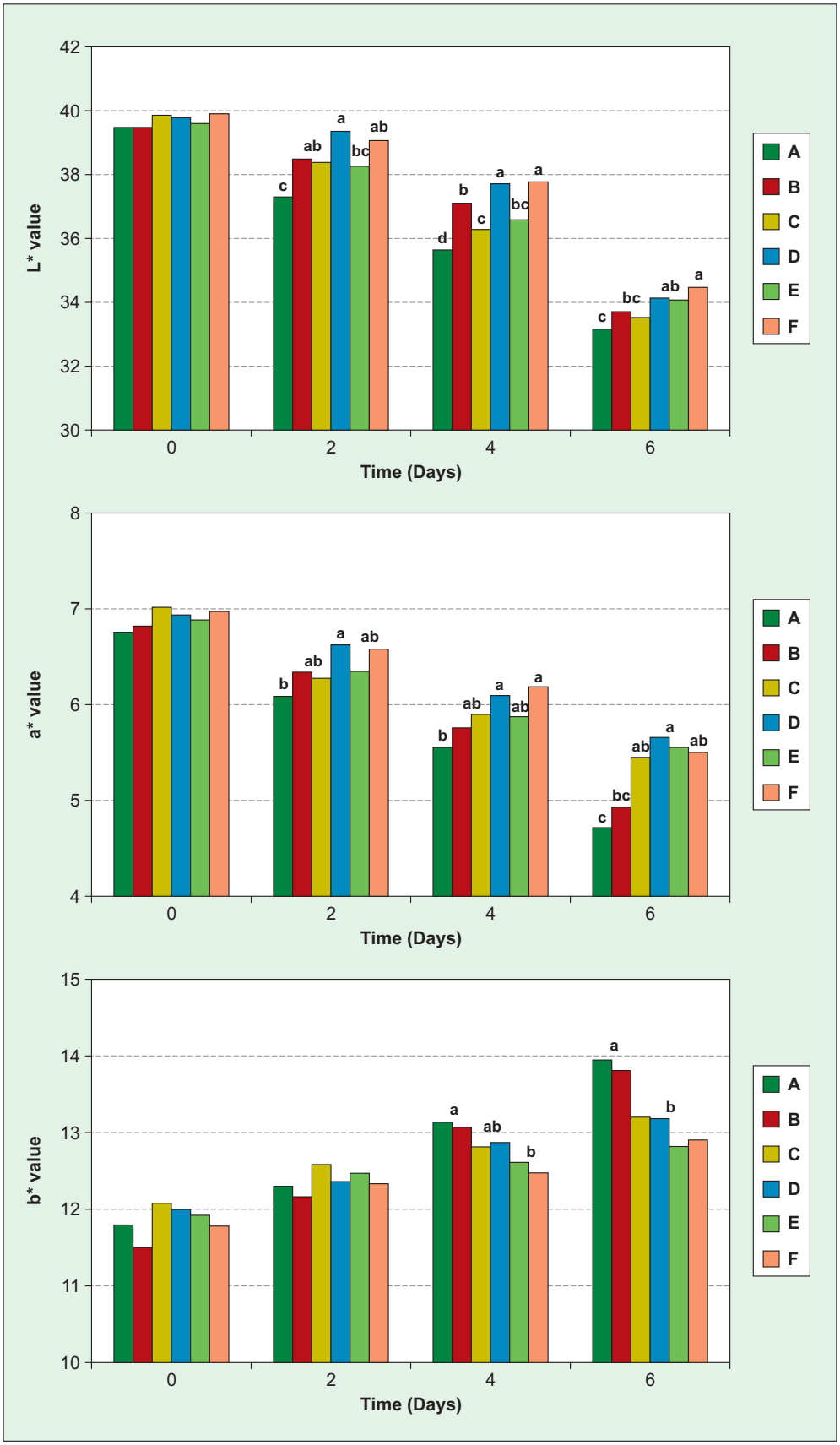

Fig. 1. Changes of colour (L*, a*, b*) values of shrimp during chilled storage
Explanations: [a-c] means within a column with different letters are significantly different ($P < 0.05$); (A) sodium metabisulphite (2500 mg/L), (B) 4-hexylresorcinol (50 mg/L), (C) chitosan (5 g/L) + sodium metabisulphite (2500 mg/L), (D) chitosan (5 g/L) + 4-hexylresorcinol (50 mg/L), (E) chitosan (5 g/L) + citric acid (200 mg/L) + rosemary extract (50 mg/L) + sodium metabisulphite (2500 mg/L), (F) chitosan (5 g/L) + citric acid (200 mg/L) + rosemary extract (50 mg/L) + 4-hexylresorcinol (50 mg/L)

Tab. 3. Mean sensory colour scores of shrimps during frozen storage (over a hundred points)

Group	Frozen storage (month)					
	3	6	7	8	9	12
A	86.22 ± 0.46[a]	82.74 ± 0.50[a]	82.42 ± 0.54[a]	82.02 ± 0.50[a]	81.36 ± 0.44[a]	76.86 ± 0.54[a]
B	85.95 ± 0.44[a]	83.15 ± 0.45[a]	82.68 ± 0.48[a]	82.33 ± 0.51[a]	81.95 ± 0.51[a]	77.15 ± 0.43[a]
C	86.36 ± 0.54[a]	83.26 ± 0.51[a]	82.74 ± 0.49[a]	82.22 ± 0.47[a]	80.97 ± 0.41[a]	77.35 ± 0.41[a]
D	86.39 ± 0.53[a]	83.99 ± 0.41[a]	83.23 ± 0.55[a]	82.62 ± 0.58[a]	82.34 ± 0.55[a]	78.24 ± 0.37[a]
E	85.10 ± 1.01[a]	83.05 ± 0.55[a]	82.72 ± 0.50[a]	82.27 ± 0.53[a]	82.06 ± 0.51[a]	77.43 ± 0.43[a]
F	86.85 ± 0.51[a]	83.79 ± 0.51[a]	83.16 ± 0.57[a]	82.40 ± 0.56[a]	82.22 ± 0.56[a]	79.04 ± 0.66[a]
Control	73.77 ± 0.77[b]	65.11 ± 0.57[b]	64.81 ± 0.90[b]	63.10 ± 0.85[b]	60.32 ± 0.53[b]	51.66 ± 0.62[b]

Explanations: [a-b] means within a column with different letters are significantly different ($P < 0.05$); (A) sodium metabisulphite (2500 mg/L), (B) 4-hexylresorcinol (50 mg/L), (C) chitosan (5 g/L) + sodium metabisulphite (2500 mg/L), (D) chitosan (5 g/L) + 4-hexylresorcinol (50 mg/L), (E) chitosan (5 g/L) + citric acid (200 mg/L) + rosemary extract (50 mg/L) + sodium metabisulphite (2500 mg/L), (F) chitosan (5 g/L) + citric acid (200 mg/L) + rosemary extract (50 mg/L) + 4-hexylresorcinol (50 mg/L), (Control) non-treated

Sensorial colour changes during frozen storage of shrimp are shown in Tab. 3. Discoloration (blackening) in untreated samples (control group) was noticeable at the third month of storage, and continued to increase steadily throughout the storage period. Sensory colour score, which are 86.93 at beginning, decreased to drop to 51.66 at the end of frozen storage period. On the other hand, all treated shrimps showed no significant colour changes during storage and decrease in colour scores was very low level even at the 12th month of storage. Similarly, Tsironi et al. (43) reported that sulphite treated shrimps stored at –12°C and –15°C had acceptable appearance, as judged by the sensory panellists, for approximately 8 and 11 months of storage, respectively. The mean colour scores of the control group at all stages of storage significantly lower than of treatment groups ($P < 0.05$). In general, the mean colour scores of the shrimps treated with 4-hexylresorcinol were higher than of those treated with sodium metabisulphite. Similarly, the addition of chitosan into the dipping solution resulted in higher scores. However, the differences among treatment groups were not statistically significant ($P < 0.05$). In fact, the extent of colour change was not sufficient to compare the treatment groups. Instrumental colour analysis gave similar results and the L* values associated with melanosis in all groups showed a decreasing trend during frozen storage (Fig. 2). In control group shrimps, the L* values decreased more quickly compared to the treated groups ($P < 0.05$). The L * values of the treated groups was found very close to each other. Redness (a*) values of shrimps showed similar changes during storage. Frozen storage did not affect the yellowness (b*) values and there were no significant differences among all groups during storage.

These results indicated that the activity of PPO continues slowly at –18°C, and colour problem may occur in the frozen shrimps without any inhibitor. But, Rotllant et al. (34) reported that quick freezing appeared to be a good method to prevent melanosis simultaneously and frozen storage for three months did not affect the appearance of melanosis. This difference may be due to freezing methods or the type of shrimps. On the other hand, the time between capture and freezing of shrimps in the present study was about six hours. The increased enzyme activity in this time period may be caused to early blackening in the frozen shrimps. Therefore, freezing is required to be made promptly after shrimping (22). Frozen storage can effectively retard physicochemical changes in the shrimps. But, it is likely that during thawing, the inactive form of PPO stored in tissues are easily released and activated, and in the presence of suitable substrates and oxygen, melanosis develops more rapidly (9).

Melanosis is an important factor which reduces the marketability of the products. This phenomenon develops rapidly in shrimps; therefore, anti-melanosis agents are frequently used in crustaceans after being caught to prevent their darkening in colour. From this point of view, the results of the present study indicated that even when it was used at lower concentration, 4-hexylresorcinol was more effective in delaying melanosis in shrimps during the storage period in comparison to the widely used anti-melanosis agent – sodium metabisulphite. Moreover, the combination of anti-melanosis agents with chitosan would succeed in a greater retarding of discoloration, whereas the addition of rosemary extract and citric acid into the treatment solution would result only in a slight improvement on the appearance of shrimps.

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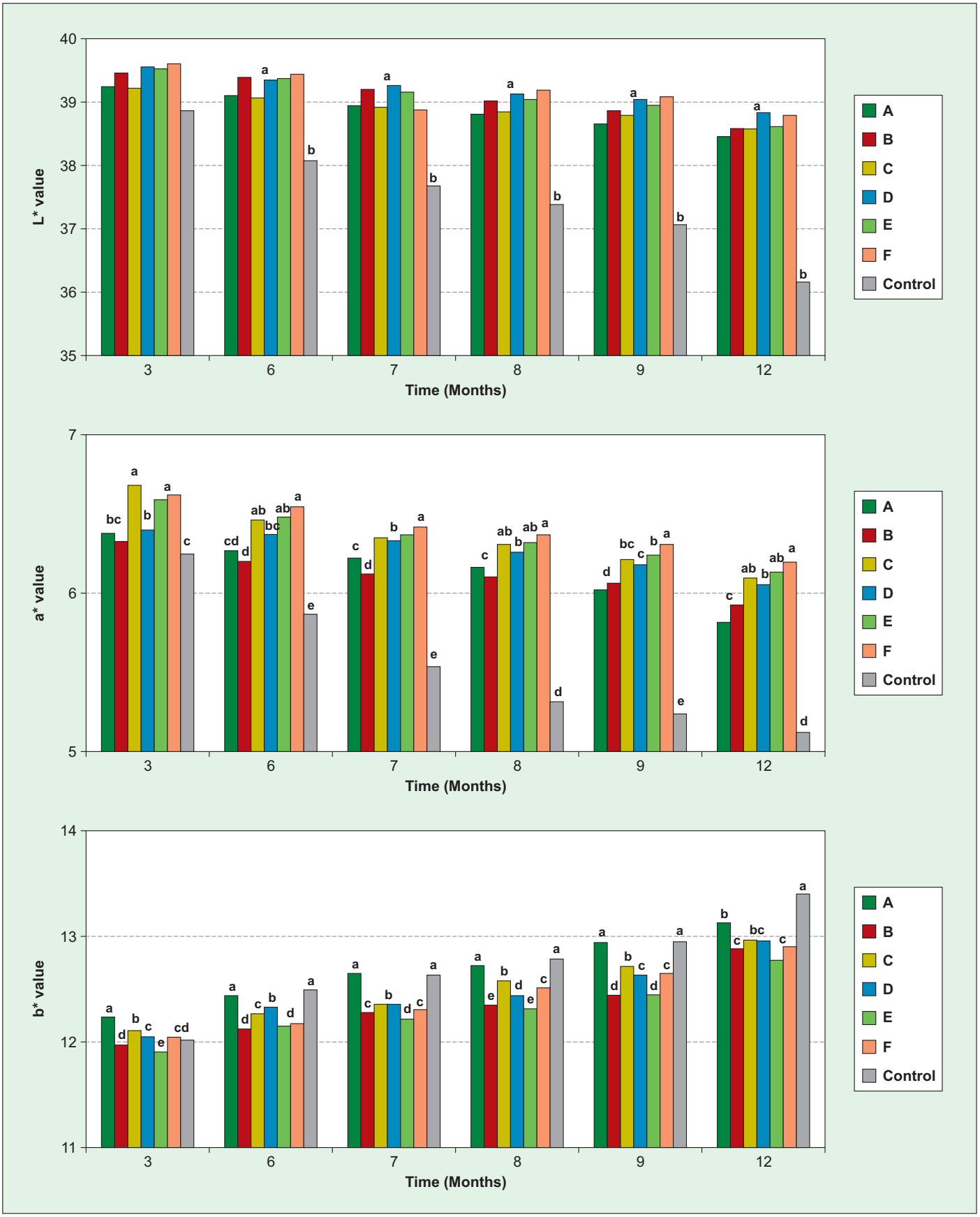

Fig. 2. Changes of colour (L*, a*, b*) values of shrimp during frozen storage

Explanations: [a-e] means within a column with different letters are significantly different ($P < 0.05$); (A) sodium metabisulphite (2500 mg/L), (B) 4-hexylresorcinol (50 mg/L), (C) chitosan (5 g/L) + sodium metabisulphite (2500 mg/L), (D) chitosan (5 g/L) + 4-hexylresorcinol (50 mg/L), (E) chitosan (5 g/L) + citric acid (200 mg/L) + rosemary extract (50 mg/L) + sodium metabisulphite (2500 mg/L), (F) chitosan (5 g/L) + citric acid (200 mg/L) + rosemary extract (50 mg/L) + 4-hexylresorcinol (50 mg/L), (Control) non-treated

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