Effect of different chemical compounds as coadjutants of 4-hexylresorcinol on appearance of deepwater pink shrimp (*Parapenaeus longirostris*) during chilled storage

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ABBREVIATED RUNNING TITLE: Appearance of pink shrimp immersed in 4-hexylresorcinol-based formulas

ABSTRACT

Different chemical compounds (kojic acid, cumic acid phytic acid, sodium metabisulphite, magnesium carbonate, sorbic acid, and different protease inhibitors) were used as coadjutants in 4-hexylresorcinol-based melanosis-inhibiting formulas tested by immersion on pink shrimp (*Parapenaeus longirostris*). Increasing concentrations of 4-hexylresorcinol delayed the occurrence of melanosis during storage. However, they could not prevent the appearance of yellow-greenish colouration in the cephalothorax, which diminished the consumer acceptability of shrimps. The incorporation of protease inhibitors (EDTA, disodium dihydrogen pyrophosphate, iodoacetic acid, egg white and PMSF) into the 4-hexylresorcinol-based blends improved acceptability through storage, suggesting protease activity post mortem contributes to the final acceptability of crustaceans.

Keywords: melanosis, pink shrimp, *Parapenaeus longirostris*, polyphenol oxidase, 4-hexylresorcinol.

INTRODUCTION

Melanosis is a surface colouration that initiates as soon as crustaceans are removed from water and come into contact with the oxygen in the atmosphere. The reaction involves the oxidation of phenols to quinones by polyphenol oxidase (PPO), providing the precursors of insoluble polymeric pigments (Otwell & Marshall, 1986). Currently, prevention of black spots involves application of sulphite derivatives, although the authorised residual content in flesh is not very effective to delay the appearance of melanosis in some species such as pink shrimp (*Parapenaeus longirostris*). The use of high doses of sulphites is not a good alternative given that it is related to allergic reactions, as well as other health risks associated with its use (Taylor & Bush, 1987). It emphasizes the need for a safe effective sulphite alternative. 4-hexylresorcinol (4-HR), is for example a competitive inhibitor of PPO "generally recognized as safe" (GRAS) for use in the prevention of crustacean melanosis (Frankos et al., 1991). The use of 4-HR is permitted in the US, Canada, Australia, and some Latin American countries (Montero, Martínez-Alvarez & Gómez-Guillén, 2004). In 2003, the Scientific Committee on Food of the European Commission considered 4-HR to be toxicologically acceptable for the prevention of melanosis in shrimps under specific conditions of use (provided residues in crustacean meat do not exceed 2 mg/kg). In 2004, a proposal of amending the EU Directive 95/2/EC on food additives other than colorants and sweeteners was adopted by the European Commission and presented to the European Parliament in order to authorize 4-HR as an alternative to sulphites for preventing the browning of crustaceans. Its effectiveness as melanosis-inhibiting compound has been demonstrated both in laboratory tests and on board (McEvily, Iyengar & Otwell, 1991; Otwell, Iyengar & McEvily, 1992; Guandalini, Ioppolo, Mantovani, Stacchini & Giovannini, 1998; Montero, López-Caballero & Pérez-Mateos,

49 2001a; Montero et al., 2004). However, the effective doses are different depending on
50 several factors such as species, physiological states, and application method. In addition,
51 the experimental conditions cannot be held in the same account, as those used in
52 commercial boats, where fishermen manipulate a considerable amount of shrimp and
53 accurate weight measurement is difficult. That results in amount of melanosis-inhibiting
54 formulations used on board not corresponding to those used in the laboratory.
55 A commercial formulation composed of 4-HR and NaCl (Everfresh®, Opta Food
56 Ingredients) is used in different countries with positive results in delaying melanosis.
57 However, for deepwater pink shrimp (*Parapenaeus longirostris*) this product has proved to
58 be quite ineffective using the concentrations and application times recommended by the
59 manufacturer (unpublished data). That encourages the search for more potentially effective
60 4-HR-based formulations, mainly for species very susceptible to melanosis such as pink
61 shrimp. Montero et al. (2004) reported the application of citric acid, ascorbic acid and
62 acetic acid in association with 4-HR to enhance the appearance of shrimp, accentuating
63 their natural pink colouration. Despite the fact that this formulation inhibited melanosis
64 itself, the viscera turned yellowish during storage, which is attributed to primary post
65 mortem spoilage. The incorporation of coadjutants in 4-HR based formulations could result
66 in the prevention of the prevalence of yellow shades in the head, thereby improving
67 appearance. These chemicals could be different food-grade compounds for which anti-
68 browning action has been demonstrated in vegetables, fruits and crustaceans. Phytic acid is
69 a natural antioxidant (Graf, Empson & Eaton, 1987) and chelating agent which
70 demonstrates melanosis-inhibiting activity, as been described in vegetables (Hicks et al.,
71 2004), but rarely in crustaceans (Guang-Li, Yuan-hong, Shu-qing & Xue-lin, 1996). Kojic
72 acid is an antibiotic substance produced by several species of *Aspergillus* and *Penicillium*,

and is widely used in Japan as a food additive for preventing enzymatic browning. Kojic acid acts by interfering with the uptake of O_2 required for the enzymatic reaction and/or reduction of quinone compounds to diphenols to prevent melanin formation (Chen, Wei, & Marshall, 1991a). Cumic acid, abundant in seeds of *Cuminum cyminum* (cumin), acts by binding to the coupled binuclear copper active site with the carboxylic group and can be classified as a non-competitive inhibitor (Kubo & Kinst-Hori, 1998). Another chemical, magnesium carbonate, is decomposed into carbon dioxide, which interferes with the utilization of oxygen by PPO (Gutierrez Alsina, 1976). Furthermore, several food preservatives, such as potassium sorbate and sulphite derivatives, could also be used to delay the appearance of melanosis mediated by some spoilage bacteria, according to Chinivasagam, Bremner & Reeves, (1998). Other substances show a double role, such as ethylenediaminetetraacetic acid (EDTA), and disodium dihydrogen pyrophosphate (PPi) (Iyengar & McEvily, 1992; Montero et al., 2004). They can chelate the copper prosthetic group at the PPO active site, slowing down the enzymatic reaction, and can also inhibit digestive metalloproteases responsible for PPO activation. Moreover, the inhibition of digestive proteases should diminish free tyrosine concentration in flesh, which is a natural substrate for PPO. Egg white can also be used as protease inhibitor, as it contains serine and cysteine proteases. Other specific non-food grade inhibitors such as phenylmethylsulphonyl fluoride (PMSF) and Iodoacetic acid (IAA) could also be used to study the role of different proteases in the decomposition of viscera during storage. PMSF is an inhibitor of serine proteases, while Iodoacetic acid (IAA) is an inhibitor of cysteine proteases.

The main objective of this work was to assess the effect of several chemicals used as coadjutants in 4-HR-based formulations on the occurrence of black spots and yellow-green

97 shades in the head of the deepwater pink shrimp (*Parapenaeus longirostris*) during chilled
98 storage. All formulations were used by dipping on board, simulating conditions habitually
99 used by fishermen. Preservatives, natural melanosis-inhibiting chemicals and protease
100 inhibitors infrequently studied in crustaceans were studied. The effect of these formulations
101 on acceptability was also evaluated through storage.

102 **MATERIALS AND METHODS.**

103 Deepwater pink shrimp (*Parapenaeus longirostris)* were caught off the South coast of
104 Spain (Cádiz) by trawl in March and May. Mean shrimp weights were 8.03 ± 1.54 g. On
105 board they were separated from the by-catch, washed with seawater and separated into
106 groups. Different 4-HR based formulations (Table 1) were then applied by immersion for 1
107 hour (shrimp:seawater relation of 1:2), one group immersed in seawater being considered
108 as control. All melanosis-inhibiting blends (Table 1) included 0.1% or 0.25 % of 4-
109 hexylresorcinol (Sigma-Chemical, St Louis, MO, USA), and in almost all blends it was
110 used in combination with 0.5 % L-ascorbic acid (Sigma Chemical, St Louis, USA), 0.5 %
111 citric acid (Panreac Chemical, Barcelona, Spain), and 0.3% acetic acid (Panreac Chemical,
112 Barcelona, Spain). For simplicity, these formulations were designated as ACRA 0. 1%
113 (with 0.1% 4-HR) and ACRA 0.25% (with 0.25% 4-HR). Resorcinol alone or these basic
114 mixtures were accompanied by different chemical compounds to determine their capacity
115 to improve the appearance of shrimps: magnesium carbonate, di sodium di hydrogen
116 pyrophosphate (PPi), and potassium sorbate (Panreac Chemical, Barcelona, Spain);
117 iodoacetic acid (IAA, Sigma Chemical, St Louis, MO, USA), phenylmethylsulphonyl
118 fluoride (PMSF, Sigma Chemical), kojic acid (5-hydroxy-2 hydroxy-methyl-γ-pyrone,
119 Sigma Chemical), phytic acid, and ethylenediaminetetraacetic acid (EDTA) (Sigma
120 Chemical); cumic acid (4-isopropilbenzoic acid, Aldrich Chemical, Milwaukee, WI, USA),

sodium metabisulphite (Sigma Aldrich Chemie, Germany); and dried egg white powder (Degussa, Barcelona, Spain). Each formulation was assayed in two lots (1.5 kg of shrimps per lot). Once the treatment time was finished, the shrimps were removed, placed in perforated polystyrene boxes with a capacity of 2 kg, and covered with flaked ice. On arrival of the trawler at the port, the boxes were taken by refrigerated truck to the Instituto del Frío in Madrid, where they were stored in ice at 2 ºC.

Sensorial analyses

A taste panel composed of eleven trained panellists visually inspected 14 shrimp per treatment group every two days during the time of chilled storage. This conventional sensory assessment was used to identify those significant sensory appearance attributes which best define the quality loss of pink shrimp during storage. Those included general outward appearance (acceptability), the condition of the cephalothorax-tail junction, the presence of yellow-greenish colouration beneath the head cuticle and also that of black spots on the shell. The outward appearance was assessed as total percentage of shrimps considered as acceptable for being sold on the market. The presence of yellow-greenish colourations was evaluated as percentage of individuals with visual presence of this shade in the head. The condition of the cephalothorax-tail junction was evaluated as percentage of individuals with cephalothorax and tail separated. Finally, the presence of black spots in the shell was assessed according to a visual scale from 1 to 4 (Montero et al., 2004), in which 1 = complete absence of black spots; 2 = a few small spots on the carapace; 3 = considerable spotting on the carapace; 4 = substantial spotting over the entire shrimp. The cephalothorax-tail junction was not affected by the different formulas tested, and for that reason this parameter was not further discussed.

Statistical analyses

145 The melanosis scores were regressed on time in storage using the SPSS computer software
146 program (SPSS Inc., Chicago, Ill., USA). Linear or polynomial regressions were plotted
147 through data dispersion, showing in all cases regression coefficients $R^2 \geq 0.90$. The data
148 from the different variables analysed were used as the data matrix in different principal
149 component analyses (PCA).

150 **RESULTS AND DISCUSSION.**

151 **Non treated samples (Control samples).** A noticeable increase in all parameters was
152 observed during iced storage, but especially in the case of melanosis, which showed the
153 earliest and more pronounced changes. Firstly, melanosis was observed mainly in the
154 cephalothorax and pleopods. Later, melanosis was also detected in abdomen cuticle and
155 telson, covering the whole surface after seven days of chilled storage (Fig 1). Regarding
156 acceptability, black spots decreased the perceived quality of the shrimp by the test panel,
157 and only approximately 25 % of shrimps were considered as acceptable two days after
158 capture.

159 **Inhibitory effect of 4-hexylresorcinol combined with acids.** The use of the melanosis-
160 inhibiting formula composed of 0.1% 4-HR, 0.5% L-ascorbic acid, 0.5 % citric acid, and
161 0.3% acetic acid led to an absence of black spots in shrimps for the first four days of chilled
162 storage (Fig. 1a). This formula with 0.25 % 4-HR proved to be more effective at inhibiting
163 melanosis (Fig 1b). The effectiveness of 4-HR as inhibitor of PPO activity from crustaceans
164 has been extensively reported (McEvily et al., 1991; Otwell et al., 1992; Guandalini et al.,
165 1998; Montero et al., 2004), although the effect depends on the application method, the
166 quantity applied, and mainly on the crustacean specie. The presence of organic acids in the
167 4-HR-based formula produces an acidic pH, that should negatively affect the PPO activity
168 (Gökoglu, 2004). Furthermore, ascorbic acid causes the chemical reduction of the pigment

169 precursors, and citric acid may have a dual inhibitory effect on PPO: lowering the pH and
170 chelating the copper at the active site of the enzyme (Iyengar & McEvily, 1992). Both
171 ascorbic and citric acid could synergistically interact in the inhibition process, according to
172 Lee-Kim, Hwang & Kim (1997).
173 Despite the finding that ACRA formula inhibited the PPO activity, the heads (beneath
174 cuticle) turned greenish-yellow during storage, affecting acceptability (Table 2). The
175 factor(s) which promote(s) the appearance of this colouration is/are unknown. However, it
176 coincides with the appearance of black spots on carapace, so it is possible that yellow-
177 greenish colouration is in some way related to melanosis process. Autolysis of shrimp
178 viscera, or spoilage bacteria capable of producing melanin (Chinivasagam et al., 1998)
179 could also be involved in the appearance of yellow-greenish shades in heads.
180 Kojic acid was incorporated in 4-HR based formulas as it would complement the inhibitory
181 effect of 4-HR (Chen et al., 1991a). The activity of kojic acid in the prevention of
182 melanosis involves two mechanisms: direct inhibition of PPO, and the chemical reduction
183 of the pigment or pigment precursors to colourless compounds (Iyidogan & Bayindirli,
184 2004). Direct inhibition of PPO is produced as kojic acid is a competitive, slow-binding
185 inhibitor, like 4-HR (Jiménez & García-Carmona, 1997). The melanosis-inhibiting effect of
186 kojic acid has been observed in several crustacean species, such as *Penaeus monodon*
187 (Chen, Wei, Rolle, Otwell, Balaban & Marshall, 1991b) *Penaeus japonicus* (Montero,
188 Avalos & Pérez-Mateos, 2001b), but the effect of this chemical together with 4-HR has not
189 been studied in crustaceans. In this context positive results of melanosis-inhibiting
190 formulations including 4-HR and kojic acid have been described on apples (Iyidogan &
191 Bayindirli, 2004). The effectiveness of kojic acid against melanosis could be higher when
192 mixed with ascorbic acid and citric acid, and those chemicals constitute a Japanese product

193 for inhibiting PPO in foods (Chen et al., 1991b). A mixture of ascorbic acid and kojic acid
194 has also been patented for use as an anti-browning agent in foods (Fukusawa, Wakabayashi
195 & Natori, 1982). Nevertheless, the use by immersion of formulations with 4-HR, organic
196 acids and 0.1% kojic acid had no significant effect on appearance of shrimps from two days
197 of chilled storage (Fig 1 and Table 1). It is worth noting that the bleaching mechanism of
198 quinones by kojic acid seems to be related to a redox reaction leading to an oxidised yellow
199 derivative of the inhibitor (Kahn, 1995), and it could enhance the intensity of yellow colour
200 in head during storage. The author suggested that kojic acid may not be desirable in certain
201 products, but that would depend on the concentration of the inhibitor and the type of food
202 in which it is used.
203 The incorporation of 0.1% cumic and/or phytic acid in the 4-HR-based formulations was
204 also tested (Fig. 1a and 1b). Cumic acid is a non-competitive inhibitor of PPO (Kubo &
205 Kinst-Hori, 1998). Furthermore, phytic acid is an antioxidant and chelating agent, so it
206 would inhibit not only PPO, but also digestive metalloproteases. Intensity of melanosis was
207 attenuated only when those chemicals were included in 0.1% 4-HR-based formulations, and
208 for 9 days of chilled storage. When cumic and/or phytic acid were incorporated in the
209 0.25% 4-HR based formula, the intensity of yellow shade in the head was lower after 7
210 days of chilled storage. However, this was not enough to improve the general appearance of
211 shrimps, according to the test panel.
212 **Inhibitory effect of 4-hexylrerorcinol combined with sulphites.** Sodium metabisulphite
213 was tested in association with 4-HR. This chemical is usually incorporated in commercial
214 melanosis-inhibiting formulations. The use of sulphite derivatives is limited by the
215 regulatory authorities of many countries, which have indicated a maximum concentration of
216 sulphites and derivatives in different foods. However, the appearance of melanosis occurs

more rapidly in several shrimp species such as pink shrimp, and for that reason dosage of sulphites exceeds the usually permitted concentrations (Gómez-Guillén, Martínez-Alvarez, Llamas Marcos & Montero, 2005). The use of 4-HR together with sulphite-derivatives in commercial formulations could therefore be of high interest to reduce sulphite concentrations in the flesh of shrimp. Figure 2 depicts the effect of 0.1% 4-HR combined with variable concentrations of sodium metabisulphite routinely used on board (0.62% and 1.25%). In both cases, their effect on melanosis and also on the evidence of yellow-greenish colouration beneath cuticle was similar to that achieved by dipping shrimps in a solution of 4-HR alone (Table 3). Nonetheless, lower concentrations of 4-HR were more effective to prevent melanosis than that used for sodium metabisulphite.

Inhibitory effect of 4-HR combined with acids and magnesium carbonate. Magnesium carbonate exerts a positive effect on melanosis prevention when it is used together with citric, ascorbic, and sodium metabisulphite (Gutierrez Alsina, 1976). Magnesium carbonate is decomposed into carbon dioxide, which interferes in the utilization of oxygen by PPO, preventing decomposition of the viscera. Therefore, the effect of both magnesium carbonate and 4-HR on melanosis could be complementary. The incorporation of 0.5% magnesium carbonate in ACRA-0.1% formulations slightly prevented melanosis, but only after 9 and 11 days of storage (Fig 3). This effect was not noticeable when magnesium carbonate was used together with 0.25 % 4-HR. Moreover, the intensity of yellow-greenish colouration was not reduced (Table 3), registering figures of 100% after 4 days of storage. After 7 days, this colouration was not observed by the panellists due to the intensity of melanosis in the head. Higher concentrations of magnesium carbonate (7 %) decreased final quality after 7 days of storage and did not have any effect on the other parameters judged.

241 **Inhibitory effect of 4-hexylrerorcinol combined with acids and protease inhibitors.**
242 The use of protease-inhibitors (EDTA and PPi) together with 4-HR and organic acids
243 increased acceptability, as shrimps were preserved well during nine days of chilled storage.
244 It should also be noted that, although exhibiting a decrease in sensorial appraisal during
245 storage, melanosis did not reach the threshold rejection value (≤2) after 9 days of iced
246 storage, except when egg white was used (Fig. 4). Despite the presence of yellow shades
247 beneath cuticle registering figures higher than 56% after 4 days of storage, almost 100% of
248 shrimps were surprisingly considered acceptable by the panellists during the whole period.
249 That is because the use of protease inhibitors decreased the intensity of the yellow shade in
250 head (Table 4), maintaining the fresh pink appearance. This result is ascribed to the natures
251 of EDTA and PPi, which are metalloprotease inhibitors and consequently may, at least
252 partially, inhibit the formation of free tyrosine and phenylalanine, substrates for the action
253 of PPO. In addition, they can inhibit the PPO activity by chelating the copper prosthetic
254 group at the PPO-active site or reducing the level of copper available for incorporation into
255 the enzyme (McEvily, Iyengar & Otwell, 1992).
256 On the other hand, egg white was added as it contains several inhibitor compounds,
257 including ovomucoid and ovoinhibitor, which are serine-protease inhibitors, and cystatin,
258 which is a cysteine-protease inhibitor (Stevens, 1991). Nonetheless, egg white exerts a
259 detrimental effect on appearance (Table 5). It could be due to the fact that egg white could
260 not be completely dissolved into the dipping solution. Egg white also contains proteins
261 which can be hydrolyzed by endogenous and microbial proteases, producing free
262 aminoacids which could be good substrates for PPO.
263 PMSF and IAA were also incorporated in the 4-HR-based formulas. Both were used only to
264 gain scientific knowledge about their possible mechanism of action in the context of

265 enzymatic browning of crustacean, since they are non food grade chemicals. PMSF is a
266 characteristic inhibitor of serine proteases, while IAA is a thiol-blocking reagent used to
267 inhibit cysteine proteases in many studies (Hameed & Haard, 1985; Yamashita &
268 Konagaya, 1990; Gómez-Guillén, Hurtado & Montero, 2002). Shrimps treated with this
269 formulation showed the lowest melanosis index during storage, probably because the use of
270 protease inhibitors could at least partially inhibit the formation of free tyrosine and
271 phenylalanine, both substrates for the action of PPO. On the other hand, overall quality was
272 improved, but only after 9 days of chilled storage (Table 5), probably because the formula
273 with PMSF and IAA attenuated the yellow shade of head. The slight improvement in the
274 visual aspect could indicate that the appearance of greenish colouration and decomposition
275 of viscera are connected, since serine and cysteine proteases are the main enzymes implied
276 in this process. The use of cystein and serine protease inhibitors should also be important as
277 these proteases are implied in the mechanism of activation of pro-PPO post mortem
278 (Söderhall & Cerenius, 1992; Zotos & Taylor 1997).
279 **Effect of preservatives in 4-HR-based formulations.** Spoilage bacteria could cause
280 melanosis in stored prawns, according to Chinivasagam et al. (1998). To detect the effect of
281 microorganisms on appearance of melanosis or greenish colouration, the preservative
282 potassium sorbate was incorporated in formulations containing 4-HR plus acids. Melanosis
283 in samples treated with and without preservatives evolved similarly during storage (Fig 5),
284 which evidenced the negligible effect of microorganisms on appearance of melanosis.
285 Similar results were observed regarding greenish colouration (Table 5). Furthermore,
286 almost 100 % of shrimps were considered acceptable by the panellists after 7 days of
287 storage.

Principal Component Analyses. Different principal component analyses (PCA) were performed in addition to sensorial analyses. The aim here was to identify significant differences that might have been overlooked previously in simple sensorial analyses. These further analyses were performed separately for samples treated with formulations including the same chemical compound. The corresponding principal component analyses (PCA) data matrix are shown in Table 7. In all cases, the global data matrix was reduced to 2 principal components (PC), which together account for 84-92 % of the explained variance.

The analyses of PC1 showed in all cases that yellow-greenish colouration correlated positively with the time of storage and negatively with the acceptability. That means none of the chemicals used prevented the appearance of yellow shades beneath the head cuticle, which led, together with the appearance of melanosis, to the progressive loss of quality. Even though 4-HR could not stop the appearance of the yellow-greenish colouration, the acceptability and melanosis inhibition were increased with higher concentrations of this chemical, as it is shown by the second component (PC 2, 36 % variance, Table 7a). The incorporation of magnesium carbonate, phytic acid, kojic acid, cumic acid or sulphites in the 4-HR-based formulas did not improve the general acceptability of shrimps (Table 7b-f). On the other hand, the presence of protease inhibitors in the melanosis-inhibiting formulas was related to better general acceptability (Table 7g, PC 2, 26% variance). However, PC 1 confirm that parameter acceptability is greatly influenced by both dark and yellow spots. As yellow-greenish colouration was judged only as presence or absence, it is possible that the protease inhibitors improved general appearance by reducing the intensity of this tone beneath the head cuticle.

CONCLUSIONS

311 The use of increasing concentrations of 4-hexylresorcinol is related to better consumer
312 acceptability of pink shrimp (*Parapenaeus longirostris*) during chilled storage, also
313 inhibiting the evidence of melanosis. Although 4-hexylresorcinol can lessen the appearance
314 of black spots in the carapace, it can not prevent the previous manifestation of yellow-
315 greenish colourations underneath head cuticle. The incorporation of protease inhibitors as
316 coadjutants in 4-hexylresorcinol-based formulations improved general appearance, but this
317 effect was not statistically significant. This suggests that the appearance of greenish
318 colourations could be related to decomposition of the viscera mediated by proteases, which
319 could facilitate liberation of substrates for PPO such as tyrosine. On the other hand, the
320 incorporation of another natural chemicals as kojic, cumic and phytic acid in 4-
321 hexylresorcinol based formulations did not improve the overall acceptability of shrimps
322 during storage. Finally, the incorporation of preservatives did not have any effect on
323 appearance, suggesting micro-organisms do not participate in the appearance of anomalous
324 colourations in the cephalothorax.
325
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FIGURE CAPTIONS

- Figure 1: Melanosis during the chilled storage of shrimp treated with 4-HR based formulas including kojic acid (0.1%), cumic acid (0.1%) or phytic acid (0.1%). a) Formulas with 0.1% 4-hexylresorcinol plus organic acids; b) Formulas with 0.25% 4-HR plus organic acids. ACRA: 4-HR+ascorbic acid (0.5%)+acetic acid (0.05 N)+citric acid (0.5%); Koj: kojic acid; Cum; cumic acid; Phy: phytic acid.

- Figure 2: Intensity of melanosis during the chilled storage of shrimp treated with 4-hexylresorcinol (0.25 % or 0.1%) combined with citric acid (0.5%), ascorbic acid (0.5%), acetic acid (0.05 N), and sodium metabisulphite (1% or 0.625%). ACRA: 4-HR+ascorbic+acetic+citric acid; Sul: sodium metabisulphite.

- Figure 3: Appearance of melanosis during the chilled storage of shrimp treated with 4-hexylresorcinol (0.25 % or 0.1%) combined with citric acid (0.5%), ascorbic acid (0.5%),

426 acetic acid (0.05 N), and magnesium carbonate (7% or 0.5%). ACRA: 4-
427 HR+ascorbic+acetic+citric acid; Mg Car: magnesium carbonate.
428 - Figure 4: Intensity of melanosis during the chilled storage of shrimps treated with 4-
429 hexylresorcinol (0.25 % or 0.1%) combined with organic acids, and the protease inhibitors
430 EDTA (225 ppm), PPi (1%), egg white (1% and 2%), Iodoacetic acid (210 ppm),and PMSF
431 (1 %)). ACRA 0.25%: 4-HR (0.25%)+ascorbic acid (0.5%)+acetic acid (0.05 N)+citric acid
432 (0.5%); EDTA: Ethylenediaminetetraacetic acid; PPi: Sodium pirophosphate; Ew: Egg
433 white; IAA: Iodoacetic acid; PMSF: Phenylmethylsulphonyl fluoride.
434 - Figure 5: Melanosis during the chilled storage of shrimp treated with 4-hexylresorcinol
435 (0.25 % or 0.1%) combined with organic acids, the protease inhibitors EDTA (225 ppm)
436 and PPi (1%) and potassium sorbate. ACRA 0.25%: 4-HR (0.25%)+ascorbic acid
437 (0.5%)+acetic acid (0.05 N)+citric acid (0.5%). Sor: Potassium sorbate (2%).
438

439 **TABLES**

440 Table 1. Melanosis-inhibiting blends used by immersion after capture. ACRA: 0.5%
441 ascorbic acid+0.5% citric acid+0.3% acetic acid+0.1% 4-HR (ACRA 0.1%) or 0.25 % 4-
442 HR (ACRA 0.25%). R: 4-HR; Cit: Citric acid; Koj: Kojic acid; Cum: Cumic acid; Phy:
443 Phytic acid; Mg Car: Magnesium carbonate; Sul: sodium metabisulphite; EDTA:
444 Etilenediaminetetraacetic acid; Sor: Potassium sorbate; PPi: Sodium pirophosphate; Ew:
445 Egg white; IAA: Iodoacetic acid; PMSF: Phenylmethylsulphonyl fluoride.

446

MELANOSIS-INHIBITING BLEND	Koj (%)	Cum (%)	Phy (%)	Mg Car (%)	Sul (%)	Sor (%)	EDTA (ppm)	PPi (%)	EW (%)	IAA (%)	PMSF (%)
CONTROL											
ACRA 0.1%											
ACRA 0.1%+Koj	0.1										
ACRA 0.1%+Cum		0.1									
ACRA 0.1%+Phy			0.1								
ACRA 0.1%+Cum+Phy		0.1	0.1								
ACRA 0.1%+Mg Car				0.5							
ACRA 0.25%											
ACRA 0.25%+Koj	0.1										
ACRA 0.25%+Cum		0.1									
ACRA 0.25%+Phy			0.1								
ACRA 0.25%+Cum+Phy		0.1	0.1								
ACRA 0.25%+EDTA+PPi							225	1			
ACRA 0.25%+EDTA+PPi+Ew 1%							225	1	1		
ACRA 0.25%+EDTA+PPi+Ew 2%							225	1	2		
ACRA 0.25%+EDTA+PPi+IAA+PMSF							225	1		1	1
ACRA 0.25%+Sor						2					
ACRA 0.25%+EDTA+PPi+Sor						2	225	1			
ACRA 0.25%+Mg Car				0.5							
ACRA 0.25%+Mg Car 7%				7							
R 0.1%											
R 0.1%+Sul 1.25%					1.25						
R 0.1%+Sul 0.625%					0.625						
Sul 1.25					1.25						

Table 2. Prevalence of yellow-greenish colourations and acceptability (%) of shrimp treated with 4-HR based formulas including kojic acid (0.1%), cumic acid (0.1%) or phytic acid (0.1%). a) Formulas with 0.1% 4-hexylresorcinol plus organic acids; b) Formulas with 0.25% 4-HR plus organic acids. ACRA: 4-HR+ascorbic acid (0.5%)+acetic acid (0.05 N)+citric acid (0.5%); Koj: kojic acid; Cum; cumic acid; Phy: phytic acid.

a)

	DAYS OF STORAGE					
Yellow-greenish shades (%)	**0**	**2**	**4**	**7**	**9**	**11**
Control	0	12.50	12.50	33.33		
ACRA 0.1%	0	16.67	42.86	70	75	100
ACRA 0.1%+Koj	0	16.67	100	100	100	100
ACRA 0.1%+Cum	0	41.67	50	80	75	100
ACRA 0.1%+Phy	0	33.33	57.14	80	100	100
ACRA 0.1%+Cum+Phy	0	33.33	57.14	80	100	100
Acceptability (%)	**0**	**2**	**4**	**7**	**9**	**11**
Control	100	27.58	0	0	0	0
ACRA 0.1%	100	100	100	80	0	0
ACRA 0.1%+Koj	100	100	100	0	0	0
ACRA 0.1%+Cum	100	83.3	100	50	8.33	0
ACRA 0.1%+Phy	100	83.3	100	50	0	0
ACRA 0.1%+Cum+Phy	100	100	100	50	0	0

b)

Yellow-greenish shades (%)	**0**	**2**	**4**	**7**	**9**	**11**
Control	0	12.5	12.5	33.33		
ACRA 0.25%	0	8.33	28.57	80	100	100
ACRA 0.25%+Koj	0	33.33	14.29	80	91.67	100
ACRA 0.25%+Cum	0	33.33	21.43	50	90	81.25
ACRA 0.25%+Phy	0	16.67	14.29	30	80	87.5
ACRA 0.25%+Cum+Phy	0	33.33	21.43	50	80	87.5
Acceptability (%)	**0**	**2**	**4**	**7**	**9**	**11**
Control	100	27.58	0	0	0	0
ACRA 0.25%	100	100	100	90	8.33	0
ACRA 0.25%+Koj	100	100	100	100	8.33	6.25
ACRA 0.25%+Cum	100	100	100	100	16.67	0
ACRA 0.25%+Phy	100	100	100	100	8.33	6.25
ACRA 0.25%+Cum+Phy	100	100	100	100	0	0

455 Table 3. Presence of yellow-greenish colouration beneath cuticle and acceptability (%) of
456 shrimps dipped in melanosis-inhibiting solutions including 4-HR and/or sodium
457 metabisulphite. R: 4-HR; Sul: sodium metabisulphite.
458

	DAYS OF STORAGE				
Yellow-greenish shades (%)	**0**	**2**	**4**	**7**	**9**
Control	0	12.50	12.50	33.33	
R 0.1%	0	14.29	59.38	98.61	88.89
Sul 1.25%	0	24.40	25	33.33	
R 0.1%-Sul 1.25%	0	9.52	75	77.78	100
R 0.1%-Sul 0.625%	0	15.48	62.50	84.26	100
Acceptability (%)	**0**	**2**	**4**	**7**	**9**
Control	100	27.58	0	0	0
R 0.1%	100	80.95	69.64	0	0
Sul 1.25%	100	42.86	2.08	0	0
R 0.1%-Sul 1.25%	100	79.76	29.17	27.78	0
R 0.1%-Sul 0.625%	100	75.60	45.83	16.67	0

459

460 Table 4. Presence of yellow-greenish colouration beneath head cuticle (%) and
461 acceptability (%) of shrimps treated with different melanosis-inhibiting blends including
462 magnesium carbonate. ACRA: 4-HR (0.1 or 0.25%)+ascorbic acid (0.5%)+acetic acid (0.05
463 N)+citric acid (0.5%); Mg Car: Magnesium carbonate.

464

	DAYS					
Yellow-greenish shades (%)	**0**	**2**	**4**	**7**	**9**	**11**
Control	100	27.58	0	0	0	0
ACRA 0.1%	100	100	100	80	0	0
ACRA 0.25%	100	100	100	90	8.33	0
ACRA 0.1%+Mg Car 0.5%	100	100	100	20	0	0
ACRA 0.25%+Mg Car 0.5%	100	100	100	60	0	6.25
ACRA 0.25%+Mg Car 7%	100	100	100	0	0	0
Acceptability (%)	**0**	**2**	**4**	**7**	**9**	**11**
Control	100	27.58	0	0	0	0
ACRA 0.1%	100	100	100	80	0	0
ACRA 0.25%	100	100	100	90	8.33	0
ACRA 0.1%+Mg Car 0.5%	100	100	100	20	0	0
ACRA 0.25%+Mg Car 0.5%	100	100	100	60	0	6.25
ACRA 0.25%+Mg Car 7%	100	100	100	0	0	0

465

466 Table 5: Presence of yellow-greenish shades beneath cuticle (%) and acceptability (%) of
467 shrimps treated with different melanosis-inhibiting blends. ACRA 0.25%: 4-HR
468 (0.25%)+ascorbic acid (0.5%)+acetic acid (0.05 N)+citric acid (0.5%); EDTA:
469 Etilenediaminetetraacetic acid; PPi: Sodium pirophosphate; Ew: Egg white; IAA:
470 Iodoacetic acid; PMF: Phenylmethilsulphonil fluoride.

471

	DAYS					
Yellow-greenish shades (%)	**0**	**2**	**4**	**7**	**9**	**11**
ACRA 0.25%	0	8.33	28.6	80	100	100
ACRA 0.25%+EDTA+PPi+Ew 1%	0	58.3	57.1	70		
ACRA 0.25%+EDTA+PPi+Ew 2%	0	41.7	71.4	40	40	
ACRA 0.25%+EDTA+PPi+IAA+PMSF	0	0	56.3	56.3	60	80
Acceptability (%)	**0**	**2**	**4**	**7**	**9**	**11**
ACRA 0.25%	100	100	100	90	8.33	0
ACRA 0.25%+EDTA+PPi+Ew 1%	100	100	100	0	0	0
ACRA 0.25%+EDTA+PPi+Ew 2%	100	100	83.3	0	0	0
ACRA 0.25%+EDTA+PPi+IAA+PMSF	100	100	100	83.3	83.33	67

472

473 Table 6: Development of yellow-greenish colouration in the cephalothorax and
474 acceptability of shrimps dipped in different melanosis-inhibiting solutions. EDTA:
475 Etilenediaminetetraacetic acid; PPi: Sodium pirophosphate; Sor: Potassium sorbate.

476

	DAYS OF STORAGE					
Yellow-greenish shades (%)	**0**	**2**	**4**	**7**	**9**	**11**
ACRA 0.25%+Sor	0	41.7	42.9	75	100	100
ACRA 0.25%+EDTA+PPi+Sor	0	41.7	57.1	80	80	100
ACRA 0.25%	0	8.33	28.6	80	100	100
Acceptability (%)	**0**	**2**	**4**	**7**	**9**	**11**
ACRA 0.25%+Sor	100	100	85.7	100	0	0
ACRA 0.25%+EDTA+PPi+Sor	100	100	85.7	100	16.67	0
ACRA 0.25%	100	100	100	90	8.33	0

477

Table 7: Principal Component Analyses (data matrix) on the basis of the increasing concentrations of 4-HR in the melanosis-inhibiting formulas (a), or else of the presence of kojic acid (b), cumic acid (c), phytic acid (d), sulphites (e), magnesium carbonate (f), and protease inhibitors (EDTA, PPi, Ew, IAA, PMSF) (g) in the 4-HR-inhibiting formulas.

a)	**PC 1 (49% variance)**	**PC 2 (36 % variance)**
4-hexylresorcinol content	**0.316**	**0.864**
Storage period	**0.971**	**-0.199**
Yellow-greenish colouration	**0.966**	**0.056**
Acceptability	**-0.593**	**0.689**
Melanosis score	**0.349**	**-0.745**

b)	**PC 1 (62 % variance)**	**PC 2 (27 % variance)**
Kojic acid	**0.008**	**0.985**
Storage period	**0.960**	**-0.047**
Yellow-greenish colouration	**0.908**	**0.109**
Acceptability	**-0.833**	**0.339**
Melanosis score	**0.960**	**0.095**

c)	**PC 1 (68 % variance)**	**PC 2 (22 % variance)**
Cumic acid	**0.035**	**0.990**
Storage period	**0.972**	**0.043**
Yellow-greenish colouration	**0.938**	**0.104**
Acceptability	**-0.886**	**0.251**
Melanosis score	**0.893**	**0.159**

d)	**PC 1 (68 % variance)**	**PC 2 (24 % variance)**
Phytic acid	**0.017**	**0.987**
Storage period	**0.967**	**-0.003**
Yellow-greenish colouration	**0.952**	**-0.069**
Acceptability	**-0.832**	**0.458**
Melanosis score	**0.927**	**0.181**

e)	PC 1 (67 % variance)	PC 2 (22 % variance)
Sulphites	**-0.002**	**0.968**
Storage period	**0.978**	**-0.070**
Yellow-greenish colouration	**0.886**	**-0.211**
Acceptability	**-0.972**	**-0.089**
Melanosis score	**0.824**	**0.322**

490

f)	PC 1 (67 % variance)	PC 2 (23 % variance)
Magnesium carbonate	**0.004**	**0.989**
Storage period	**0.965**	**-0.012**
Yellow-greenish colouration	**0.923**	**0.002**
Acceptability	**-0.888**	**0.341**
Melanosis score	**0.873**	**0.276**

491

g)	PC 1 (58 % variance)	PC 2 (26 % variance)
Protease inhibitors	**0.033**	**0.979**
Storage period	**0.944**	**-0.054**
Yellow-greenish colouration	**0.856**	**-0.038**
Acceptability	**-0.725**	**0.577**
Melanosis score	**0.867**	**-0.004**

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