The effect of inhibitors and high pressure treatment to prevent melanosis and microbial growth on chilled prawns (*Penaeus japonicus*)

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1 **ABSTRACT**

2
3 Ascorbic acid, citric acid, sodium benzoate, kojic acid and 4-hexylresorcinol were
4 evaluated alone and in combination as inhibitors of melanosis and microbial spoilage in
5 prawns (*Penaeus japonicus*) under non pressure and high pressure conditions. Sodium
6 benzoate and kojic acid were an effective combination for the inhibition of prawn
7 melanosis. 4-Hexylresorcinol alone and in combination with ascorbic or citric acid were the
8 most effective as inhibitors of melanosis and microbial spoilage in prawns. High pressure
9 treatment appeared to increase the melanosis process, but it did not alter the effect
10 performed by inhibitors. Lower total count of microorganisms seemed to be obtained with
11 the presence of ascorbic and citric acids under high pressure treatment.

12
13 **Key words:** melanosis, inhibitors, prawns, high pressure, microorganisms

1 INTRODUCTION

2
3 The appearance of melanosis or black spots on prawns, shrimps and other fresh crustaceans
4 is rapid even in chilled storage and involves some important economical losses for the fish
5 industry. Melanosis is triggered by a biochemical mechanism consisting of oxidation of
6 phenols to quinones by polyphenoloxidase (PPO). This is followed by non-enzymatic
7 polymerization of the quinones, giving rise to pigments of high molecular weight and very
8 dark or black coloring. These same reactions are responsible for enzymatic browning in
9 fruits and vegetables, for which there are numerous studies (Constantinides and Bedford
10 1967; Mayer and Harel 1979, Chen and others 1991b; Lee-Kim and others 1997;
11 Hernández and Cano 1998; Duangmal and Owusu 1999).

12
13 In crustaceans, PPO has various different locations. It is found on the exoskeleton, chiefly
14 on the shell of the cephalothorax, uropods and on the pleuron in the region of the
15 connection with the pleopods (Ogawa and others 1984). PPO is also found on the
16 haemolymph (Nakagawa and Nagayama 1981). Because of the intense irrigation of the
17 cephalothorax, this is where PPO is most commonly found. It remains active under
18 refrigeration (with or without ice) and in thawed products. This pigmentation spreads
19 rapidly and hence places a limit on the shelf life of crustaceans. In order to prevent this
20 phenomenon as far as possible, a variety of compounds have been used which act as
21 inhibitors. The most widely used of these are sulfites and derivatives. These chemical
22 substances interfere in the polymerization of the quinones, combining irreversibly with
23 them and forming colorless compounds (Embs and Markakis 1965). However, they are
24 invariably not effective, because they do not prevent melanosis entirely. It has been

1 necessary to search for alternatives that show effective inhibitory effects on melanosis but
2 are devoid of health hazards to consumers. However, the number of chemicals that can
3 actually be used in food systems to inhibit melanosis is limited by off-flavors, off-odors,
4 toxicity and economic feasibility (Chen and others 1991b).

5
6 There are other alternative compounds to sulfites and their derivatives. Most are of natural
7 origin, and their purpose is to inhibit or slow down the onset of melanosis. These include
8 ascorbic, benzoic, sorbic, kojic and phytic acids, proteases and resorcinol derivatives, all of
9 them considered to be safe compounds (Taoukis and others 1990; McEvily and others
10 1991; Chen and others 1991a, 1991b; Otwell and others 1992; Slattery and others 1995; Yu
11 and others 1996; Kubo and Kinst-Hori 1998). Several of them have already been included
12 in Food Legislation in different countries. This is the case with 4-hexylresorcinol,
13 considered as a safe product (GRAS) for human consumption (Otwell and others 1992) and
14 kojic acid in Japanese food products (Chen and others 1991a). The mode of action of these
15 compounds differs from one to another. Thus, the antioxidants seem to reduce the level of
16 quinones by reconvertng them to phenols; the phenolic derivatives of benzoic acid appear
17 to act as chelating agents of copper; and other antioxidants bind directly to the enzyme.
18 Moreover, it is well known that some of these compounds simultaneously inhibit microbial
19 growth.

20
21 The use of technologies like high pressure which can slow down or inhibit not only the
22 melanosis process but also the growth of microorganisms could lead to the extension of the
23 shelf life of crustaceans. In this connection, previous studies with chilled prawn (López-
24 Caballero and others 2000) have shown that the combination of vacuum packaging and

high pressure prevents the appearance of black spots and the growth of microorganisms in prawns longer than 20 d. However, the results so far have been insufficient to determine the precise effect of high pressure in relation to this discoloration. The purpose of this work was to study the action of compounds like ascorbic acid, citric acid, benzoate, kojic acid and 4-hexylresorcinol – alone or in combination – as possible inhibitors of melanosis and microbial growth in pressurized and non-pressurized prawns.

MATERIALS & METHODS

Sampling procedure

The species utilized was farmed live imperial tiger prawn (*Penaeus japonicus*) (Acuinova Andaluçia, S.A., Ayamonte, Spain), caught in December. Average lengths and weights of individuals were 13.0 ± 0.9 cm and 21.5 ± 4.5 g, respectively. Samples were stored at -50°C until the start of the experiment. The prawns were thawed at 4°C overnight prior to analysis. All were submerged in cold water (5°C) with 3.5 % (w/v) sea salt and a concentration of 0.5 % (w/v) of the appropriate inhibitor: L (+) – ascorbic acid (Panreac Químic, Montcada i Rixac, Barcelona, Spain); citric acid (Panreac); sodium benzoate (Panreac); kojic acid (Sigma, Chemical Co., St. Louis, MO, USA) and 4-hexylresorcinol (Sigma). This concentration of inhibitors helped to maintain an acid pH for the ascorbic and citric acid solutions and permitted cold solubilization of 4-hexylresorcinol. The prawns were kept for 120 min in a water bath at $3 \pm 1^{\circ}\text{C}$ with these solutions at a ratio of 40 individuals per litre. The pH values of the solutions were: control (8.1), ascorbic acid (3.0),

1 citric acid (2.8), sodium benzoate (7.7), kojic acid (5.6), 4-hexylresorcinol (6.6), 4-
2 hexylresorcinol + ascorbic acid (3.0) and 4-hexylresorcinol + citric acid (2.7).

3
4 After draining, some of the prawns were vacuum packed in bags (Cryovac BB-1, Grace,
5 Barcelona, Spain) and pressurized in a high-pressure pilot unit (ACB 665, Gec Alsthom,
6 Nantes, France) at 400 MPa at 7 °C for 10 min. Finally, the packs were pierced and kept
7 stored at 4° C.

8 9 **Melanosis score**

10 At least 12 prawns per batch, placed in a plastic tray and kept at chilled temperature, were
11 evaluated during the storage period by a panel of eight previously trained judges. Panels of
12 eight members were all trained together as follows: slaughtered prawns were examined
13 immediately, establishing and evaluating the characteristic colors in each anatomical region.
14 These prawns were kept in chilled storage and examined everyday until they reached a high
15 degree of sensory deterioration. In this way, types of spots were established, ones due to
16 intrinsic color of species and others due to melanosis. In addition, from the beginning of
17 training, parts of the prawns were treated with inhibitors of melanosis, allowing the
18 evaluation of the different colors caused by inhibitors. Melanosis as manifested by the
19 black spots on the prawns was determined following a visual scale (modification of the one
20 developed by Otwell and Marshall, 1986). The scale was: 0 = absent; 2 = slight (up 20% of
21 prawns surface affected); 4 = moderate (20-40% of prawns surface affected); 6 = notable
22 (40-60% of prawns surface affected); 8 = severe (60-80% of prawns surface affected); 10 =
23 extremely heavy (80-100% of prawns surface affected).

Microbial analyses

At least 12 prawns per lot were used for microbiological analysis as follows: 10 g (and 25 g to detect *Salmonella* spp. and *Shigella* spp.) from different parts of individuals were taken in a vertical laminar-flow cabinet (mod. AV 30/70, Telstar, Madrid, Spain) in sterile conditions and placed in a sterile plastic bag (Sterilin, Stone, Staffordshire, U.K.) with 90 mL buffered peptone water (Oxoid, Basingstoke, UK). After 2 min in a stomacher blender (mod. Colworth 400, Seward, London, UK), dilutions were made with buffered peptone water (Oxoid) to determine the following microorganisms: double - layered plates on Iron Agar, IA (Adsa Micro, Barcelona, Spain) for the total viable count (TVC) and H₂S - producers after 72 h incubation at 20 °C; double-layered plates on MRS (Oxoid) to enumerate lactic acid bacteria after 72 h at 30 °C; spread plates on Pseudomonas Agar Base (Oxoid) supplemented with CFC supplement (Oxoid) for *Pseudomonas* spp. after 48 h at 20°C, spread plates on SM ID medium (bioMérieux, Marcy l'Etoile, France) and SS medium (bioMérieux) to detect the presence/absence of *Salmonella* spp., and *Shigella* spp., respectively, after 24 h incubation at 37 °C (sample was first enriched in buffered peptone water, Adsa Micro, 37 °C/20 h and then seeded in Rappaport-Vassiliadis broth, Merck, Darmstadt, Germany, at 43 °C/24 h). Microbiological counts were expressed as CFU/g of sample, except for *Salmonella* and *Shigella* which were expressed as absent/present in 25 g sample.

Statistical analyses

The prawns were divided in two groups - pressurized or not pressurized - in order to facilitate the interpretation of statistical analyses. The statistical differences were evaluated by means of linear regression and the melanosis score was studied during the storage time

using the BMDP computer program (BMDP Statistical Software, Inc., Cork Technonogy Park, Cork, Ireland). The level of significance was set at $p \leq 0.05$.

RESULTS & DISCUSSION

Melanosis of untreated prawns

The natural evolution of the melanosis in chilled prawns without additives (Fig. 1: C) was very rapid, attaining a score of 4 in 4 d. Otwell and Marshall (1986) considered a score of 4 or more as indicative of product degradation, although Otwell and others (1992) did not consider the product to be unacceptable until it scored 8. A score of 4 for extent of melanosis in 3 to 4 d has also been reported for prawns of the *Penaeus* genus (Otwell and Marshall 1986; Otwell and others 1992).

High pressure treatment on melanosis of prawns

High pressure treatment (400 MPa, 10 min, 7 °C) did not favor the inhibition of melanosis, so at the outset the score was higher in the pressurized prawns with a significant level ($p \leq 0.05$) for linear regression. The inhibition of PPO by high pressure has been studied in a variety of vegetable products – chiefly purées, juices and extracts (Quaglia and others 1996; Hernández and Cano 1998) . Most of these authors reported that the higher the pressure, the more PPO is inhibited. However, López-Caballero and others (2000) have found that the melanosis score was higher in pressurized prawns than in control batches, while the score was not related to high pressure level (200 or 400 MPa, 7°C, 10 min).

Effect of inhibitors and high pressure treatment on melanosis of prawns

The two acids assayed – ascorbic (A) and citric (CI), see Fig.1 – did not slow down the progress of melanosis compared to the control batch ($p \leq 0.05$). The antioxidant effect of these compounds, whereby the quinones are reduced to original phenols, was not effective under the experimental conditions. The acidity of the solution did not slow down melanosis either, despite the fact that in a model system it has been found that the enzyme is inhibited in acid medium (Simpson and others 1987; Ferrer and others 1989; Ali and others 1994). However, Duangmal and Owusu Apenten (1999) in potato indicated that the inhibiting effect of ascorbic acid at 5 mM is greatest at pH 6.8.

The prawns with ascorbic and citric acids scored higher in pressurized than non-pressurized batches ($p \leq 0.05$), and the evolution of the score was similar to that of the control (Fig 1: C). Asaka and Hayashi (1991) found that in pear fruit extract pressurized at 400 MPa, 25 °C, 10 min, PPO activation was higher in slightly acid conditions (p H 5 - 6). Therefore, it could be interesting to study the effect of acid conditions at higher level and different exposure time in non-pressurized prawns.

Sodium benzoate (Fig. 1: B) was somewhat more effective and scored lower than ascorbic and citric acids ($p \leq 0.05$). Its presence in the pressurized prawns tended to keep values lower than in the control. The action mechanism of sodium benzoate is similar for the entire group of benzoates and phenolic derivatives like cumic acid (Kubo and Hinst-Hori 1998). These compounds act as chelating agents of Cu^{2+} – a metal necessary for the activity of PPO – either by bonding through the hydroxyl group to the active center of the enzyme or

1 else by forming a *Shiff* base through its aldehyde group, although the first of these
2 mechanisms is the predominant one (Kubo and Hinst-Hori 1998).

3
4 At the experimental concentrations, kojic acid clearly inhibited melanosis (Fig. 2: K). The
5 prawns treated with this acid displayed practically no black spots until 5 d of storage and
6 continued to score 4 for a further 4 d ($p \leq 0.05$). This effect was also observed after high
7 pressure treatment (Fig. 2: K-H). The references to vegetable products in the literature
8 indicate that kojic acid has an irreversible inhibiting effect on quinones. Moreover, this acid
9 can be mixed with ascorbic and citric acids to make up a Japanese product for inhibiting
10 tyrosinase in foods (Chen and others 1991a), although the information available on the
11 effect of kojic acid is very limited at present.

12
13 4-Hexylresorcinol (Fig.2: R) was the most effective inhibitor at the concentrations assayed
14 ($p \leq 0.05$), with which the prawns were kept for at least a wk in very acceptable condition.
15 The inhibiting effect works by means of an irreversible interaction that allows it to remain
16 effective (McEvily and others 1991). Some studies have been carried out on ice stored pink
17 shrimp (*Penaeus duorarum*) in which it was found that 50 ppm of 4-hexylresorcinol for 1
18 min was more effective than the habitual 12500 ppm of sulfite and maintained acceptable
19 levels of melanosis at a lower level and for longer (up to 12 d) (Otwell and others 1992).
20 There are also more recent studies on the inhibiting effect of hexylresorcinol in prawns
21 (*Penaeus duorarum*, *Penaeus aztecus*) (Benner and others 1994) and shrimps (*Penaeus*
22 *esculentus*, *Penaeus plebejus*, *Metapenaeus enchavouri*, *Metapenaeus bennettiae*) (Slattery
23 and others 1995). The differences found by the various authors in the effectiveness of 4-

1 hexylresorcinol could be due to the intrinsic characteristic of the species. The pressurized
2 prawns containing 4-hexylresorcinol maintained lower values than the pressurized control.

3
4 Consideration was also given to the combined action of 4-hexylresorcinol with ascorbic
5 acid and with citric acid (see Fig.2: R+A, R+CI, respectively). In both cases there was a
6 similar mechanism that enhanced the effect exerted by each one separately, and the batches
7 treated in this way scored lowest throughout the storage period ($p \leq 0.05$). This suggested
8 that each of these substances could suppress the pigment formation mechanism at different
9 stages or else favor the action of 4-hexylresorcinol. There has been a study of simultaneous
10 use of melanosis-inhibiting substances. In PPO isolated from burdock roots, mixtures of
11 these same compounds were found to have a strong inhibiting effect even when used in
12 smaller proportions than each one (0.1 %). Their effect was greater than with bisulfite (Lee-
13 Kim and others 1997). These authors also reported different effectiveness depending on the
14 model system used. The differences in inhibiting effect in the present study could have
15 been due both to the species and to the intrinsic differences between muscle and extracts
16 (real or model system, respectively). Inhibition was likewise found in pressurized batches,
17 which suggested that the effect was not altered by the treatment. In crustaceans, the
18 combined action of kojic acid and ascorbic acid appeared to be mutually enhancing, the
19 ascorbic acid reducing the formation of quinones and the kojic acid interacting irreversibly
20 with them, leading finally to less polymerization of these pigmented compounds (Chen and
21 others 1991a, 1991b). The combination of 4-hexylresorcinol and lactic acid has proved
22 satisfactory in some crustaceans, as lactic acid inhibits microbial growth while 4-

hexylresorcinol inhibits melanosis, thus prolonging their shelf life (Benner and others 1994).

The regression analysis showed significant differences among all the inhibitors assayed, as it has been reported. The differences between batches, attributed to the use of inhibiting substances, were magnified in the unpressurized prawns (statistic $f=23.45$ against $f=8.49$). These results seem to indicate that high pressure treatment could partially mask the effect of the inhibitors assayed.

Microorganisms in untreated and treated prawns

Related to the studied microorganisms (total viable count, lactic acid bacteria and *Pseudomonas* spp.), the initial loads were very low in all cases (Table 1). During the first week of storage the number of microorganism increased very slightly in most of the unpressurized batches. This did not occur in the pressurized prawns, which could indicate damage caused by the treatment (Carlez and others 1993). There are references to the use of additives and pressurization as they relate to microbial growth. Thus, Pepper and Knorr (1990) reported that the addition of sorbic or benzoic acids could enhance the effect of pressure on microorganisms. The counts in the present study were so low that this effect could have been masked. At the end of the assay the prawns treated with 4-hexylresorcinol (R) attained higher counts, while those treated with this compound in combination with ascorbic acid (R+A) and citric acid (R+CI) still had very low values. This could be due to some anti-microbial effect of ascorbic and citric acids. In this connection, Benner and others (1994) found in *Penaeus aztecus* that the addition of L-lactic acid to the 4-hexylresorcinol dip solution prevented bacterial growth in the dip solution and did not

diminish melanosis inhibition, whereas the 4-hexylresorcinol showed no antimicrobial activity when it was used on its own. There was no sign of *Salmonella* spp., *Shigella* spp. or H₂S-producers microorganisms during the assay.

However, a strong dark coloring was detected in the flasks containing buffered peptone water with portions of prawn muscle pressurized at 400 MPa, 10 min, 7 °C (used for pre-enriching of *Salmonella* spp. and *Shigella* spp.) after 2 h incubation at 37 °C. The explanation for this may be that the PPO enzyme became activated during high pressure treatment and entered the culture medium after homogenization in the peptone water. Chinivasagam and others (1998) also reported this effect, although they attributed it to growth of some strains of *Pseudomonas fragi* incubated at 5° and 30 °C in a medium containing L-tyrosine with and without added peptone water. These authors proposed the theory that in some circumstances some spoilage bacteria may be capable of producing melanin. Vanderzants and others (1987) reported a black pigmentation induced by indole-producing microorganisms and *Acinetobacter* sp. when determining the total viable bacteria of beef carcasses.

CONCLUSION

Combinations of antioxidants and PPO inhibitors are particularly useful to prevent melanosis in prawns. Thus, it is necessary to standarize the most suitable conditions for their application because the effect of these compounds could be different depending of the nature of the food. High pressures (400 MPa, 10 min, 7 °C) activated the melanosis process on prawns but without altering the effect of the assayed inhibitors. Although the effect of

high pressure on microorganisms was not very clear owing to the low counts, its effectiveness in reducing the number of microorganisms is dealt with widely in the literature, and this, combined with the use of melanosis inhibitors, could lead to delay the prawns spoilage. The combined action of 4-hexylresorcinol and citric or ascorbic acids is especially interesting, because this reduced the appearance of melanosis and there was no apparent microbial proliferation. However, the high pressure treatment tested here did not inhibit the appearance of melanosis in whole prawn, but actually tended to activate it.

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1 Table 1- Microorganisms in prawns (CFU/g)

Microorganisms	Batches	Storage days at 2 °C		
		4	6	10
Total viable count	A	10	30	-
	B	<10	10	-
	C	10	<10	-
	CI	<10	80	-
	K	<10	99	-
	R	60	70	570
	R+A	<10	25	20
	R+CI	<10	<10	10
	A-H	<10	<10	-
	B-H	<10	<10	-
	C-H	<10	<10	-
	CI-H	<10	<10	-
	K-H	<10	<10	-
	R-H	10	<10	-
	R+A-H	<10	10	-
	R+CI-H	<10	<10	-
Lactic acid bacteria	A	10	<10	-
	B	10	10	-
	C	<10	<10	-
	CI	<10	10	-
	K	<10	10	-
	R	10	15	20
	R+A	<10	<10	40
	R+CI	<10	10	15
	A-H	<10	20	-
	B-H	10	20	-
	C-H	<10	<10	-
	CI-H	<10	10	-
	K-H	10	10	-
	R-H	<10	10	-
	R+A-H	15	10	-
	R+CI-H	10	10	-
<i>Pseudomonas</i> sp.	A	50	<100	-
	B	<100	<100	-
	C	<100	<100	-
	CI	<100	<100	-
	K	<100	100	-
	R	<100	100	3700
	R+A	<100	<100	<100
	R+CI	<100	<100	<100
	A-H	<100	100	-
	B-H	<100	<100	-
	C-H	<100	<100	-
	CI-H	<100	<100	-
	K-H	<100	<100	-
	R-H	<100	<100	-
	R+A-H	<100	<100	-
	R+CI-H	<100	<100	-

2 Batches: Control (C), with ascorbic acid (A), citric acid (CI), sodium benzoate (B), kojic acid (K), 4-
3 hexylresorcinol (R), 4-hexylresorcinol and ascorbic acid (R+A), 4-hexylresorcinol and citric acid (R+CI).
4 High pressure treatment in prawns are reflected with -H.
5

- 1 Figure 1- Score of melanosis of prawns during chilled storage treated with: Control (C) and
2 pressurized (C-H), with ascorbic acid (A) and pressurized (A-H), citric acid (CI) and
3 pressurized (CI-H), sodium benzoate (B) and pressurized (B-H)
4
- 5 Figure 2- Score of melanosis of prawns during chilled storage treated with: kojic acid (K)
6 and pressurized (K-H), 4-hexylresorcinol (R) and pressurized (R-H), 4-hexylresorcinol and
7 ascorbic acid (R+A) and pressurized (R+A-H), 4-hexylresorcinol and citric acid (R+CI)
8 and pressurized (R+CI-H)