Original article

Quality of thawed deepwater pink shrimp (*Parapenaeus longirostris*) treated with melanosis-inhibiting formulations during chilled storage

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Summary This work investigates how the treatment of thawed deepwater pink shrimp (*Parapenaeus longirostris*) with several melanosis-inhibiting formulations, affects the quality of the shrimp during chilled storage. Formulations were as follows: a formulation containing 4-hexylresorcinol (0.1 and 0.05%), in combination with organic acids and chelating agents, a commercial formula based on sulphites, and a mixture of gluconic acid and commercial sulphites. No noticeable differences were observed for both trimethylamine and total volatile bases during chilled storage. pH evolution was irrespective of the treatment condition. Microbial load enlarged after the sixth day of chilled storage. Higher total bacteria counts were associated with the control and sulphite treatment conditions, while lactic acid bacteria growth seemed to be favoured under formulations based on 4-hexylresorcinol. The appearance of melanosis occurred more rapidly in control shrimp or in shrimp treated with commercial sulphites. 4-hexylresorcinol formulations preserved the quality of thawed shrimp and could replace traditional sulphites.

Keywords Chilled storage, freezing, 4-hexylresorcinol based on formulations, spoilage, sulphites, thawed shrimp.

Introduction

Sulphites are the most common and effective additives used to prevent melanosis in crustaceans. However, a search for alternative compounds was initiated, after the use of sulphites was found to be related to allergic and asthmatic reactions in some consumers (Taylor & Bush, 1987; McEvily *et al*., 1991).

In the last decade, several studies found evidence that 4-hexylresorcinol may be a good alternative to traditional sulphites. The effectiveness of 4-hexylresorcinol as a melanosis-inhibiting chemical has been demonstrated both in laboratory and on board experiments (McEvily *et al*., 1991; Otwell *et al*., 1992; Montero *et al*., 2001; Martínez-Alvarez *et al*., 2005b). Recently, Montero *et al*. (2004) reported that deepwater pink shrimp (*Parapenaeus longirostris*) were highly sensitive to melanosis, and that there was an increase in the inhibition of melanosis after the shrimp were treated with increasing concentrations of 4-hexylresorcinol. These investigators found that a concentration of 0.25% 4-hexylresorcinol was effective at extending the shelf life of this species for 1 week.

Frozen crustaceans frequently originate from locations that are a great distance from the countries where they are sold. According to the demands of consumers from Mediterranean countries, frozen crustaceans are usually thawed and commerced as fresh-like crustaceans, or alternatively, as frozen crustaceans, to be used as raw materials and further processed. The phenomena related to spoilage during frozen storage, although not interrupted, are slowed. Given this fact, and bearing in mind that melanosis and spoilage will continue in defrosted shrimp, it would be interesting to test additives with concentrations containing lower doses of 4-hexylresorcinol. To our knowledge, there is no information concerning the role of 4-hexylresorcinol in frozen crustaceans or the subsequent chilled storage.

The aim of this work was to evaluate the effect that the treatment of thawed shrimp with several melanosis-inhibiting formulations, used as alternatives to sulphites, has on the quality of the shrimp during chilled storage.

Materials and methods

Sample preparation

Deepwater pink shrimp (*P. longirostris*) were caught off the South coast of Spain (Cádiz) by trawl in November. On board, they were separated from the by-catch, and washed with seawater. Mean shrimp weights and average lengths at the time of capture were

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6.00 ± 1.5 g and 10.8 ± 0.5 cm. The shrimp were separated in five batches; one of these was considered as control with no additives (lot NA). A commercial sulphite-based product (Freskor®, Hasenosa S.A., Vigo, Spain, 60% maximum content of sulphites) at 4% (expressed as g compound per 100-g shrimps) was applied by immersion (shrimp:seawater:ice relation of 1:2:1), alone (lot CS), and accompanied by 10% gluconic acid (v/w) (lot CS-GLN). The melanosis-inhibiting blends were dissolved in seawater, and afterwards the shrimp were introduced and covered with ice for 1 hr. Once the treatment time ended, the shrimp were taken away, placed in perforated polystyrene boxes of 2-kg capacity, and covered with ice. For treatments with 4-hexylresorcinol, 0.05 and 0.1% (g of inhibitor per 100 g of shrimp) of 4-hexylresorcinol (H6250; Sigma, St. Louis, MO, USA) were used in combination with reagent grade citric acid (0.5%), ascorbic acid (0.5%), acetic acid (0.05 N), ethylene diamine tetra-acetic acid (EDTA) (450 ppm), and disodium dihydrogen pyrophosphate (PPi) (1.5%). All were diluted in approximately 1 L of seawater and sprayed on the surface of the crustaceans (R-0.1 lot and R-0.05 lot). Subsequently, shrimps were placed in perforated polystyrene boxes of 2-kg capacity, and covered with ice.

On arrival of the trawler in port, the boxes were deep frozen and stored at −18 °C during 48 h, and taken by refrigerated truck to the Instituto del Frío in Madrid (Spain), where they were stored at −18 °C for 3 months. Subsequently, the batches were defrosted at low temperature and conserved during 14 days at 2 °C. Further analyses were performed during the storage.

Proximate analysis

Proximate analyses of the raw pink shrimp was performed after being captured, according to the procedures of the Association of Official Analytical Chemists for moisture (method 24003), ash (method 1821) and crude protein (method 24024) (Association of Official Analytical Chemists, AOAC, 1995). Crude fat was determined according to the method of Bligh & Dyer (1959).

pH

Approximately 5–10 g of muscle was homogenised with a double quantity (g mL^{-1}) of distilled water. After 5 min at ambient temperature, pH was determined with a pHm93 pH-meter and a combined pH electrode (Radiometer, Copenhagen, Denmark). The experiments were repeated at least in triplicate.

Total volatile basic nitrogen

Total volatile basic nitrogen (TVB-N) determinations were carried out in triplicate over the storage period using the method of Antonacopoulos & Vyncke (1989). Ten grams ± 0.1 g of the ground sample were weighed in a suitable container and homogenised for 2 min with 100-mL 6% perchloric acid solution. After filtering, the extract was alkalinised with 20% sodium hydroxide solution, and submitted to steam distillation. The volatile base components were absorbed by an acid receiver and determined by titration of the absorbed bases. All analyses were performed at least in triplicate.

Trimethylamine

Trimethylamine (TMA-N) was determined using the Dyer method modified by Tozawa, as described in the Association of Official Analytical Chemists, AOAC (1995). Results were expressed in milligrams of TMA-N per 100 mg of sample.

Water holding capacity

Water holding capacity (WHC) was determined by the method described by Montero *et al.* (1996). Two grams of muscle was chopped, weighed and placed in a centrifuge tube, with three pipette filters (Gilson Pipetman, Paris, France) as absorbents. Samples were centrifuged for 10 min at 6000 ***g*** in a Sorvall RT60008 centrifuge (DuPont Co., Delaware, USA) at room temperature. Results were average of three determinations, and were expressed as grams of water per gram of protein in muscle.

Shear strength

Shear strength was determined on two tail muscle sample. These were spread on a Kramer cell perpendicular to the cell. A computer-controlled TA-XT2 Texture Analyser (Texture Technologies Corp., Scarsdale, NY, USA) was used, with a cell load of 50 N at a setting of 100 mm min^{-1}. Results were average of three determinations, and were expressed as Newtons per gram of muscle at the point of maximum load, before sample breaking.

Microbiological assays

At least twelve prawns per batch were used for microbiological analysis. A total amount of 10 g of muscle was collected and placed in a sterile plastic bag (Sterilin, Stone, Staffordshire, UK) with 90 mL of buffered 0.1% peptone water (Oxoid, Basingstoke, UK) in a vertical laminar-flow cabinet (mod. AV 30/70 Telstar, Madrid, Spain). After 1 min in a Stomacher blender (model Colworth 400; Seward, London, UK), appropriate dilutions were prepared for the following micro-organism determinations: (i) total bacterial counts (TBC) on spread plates of iron agar, 1% NaCl incubated at 15 °C

for 3 days; (ii) H_2S-producing organisms, as black colonies, on spread plates of iron agar, 1% NaCl incubated at 15 °C for 3 days; (iii) luminescent bacteria on spread plates of iron agar, 1% NaCl incubated at 15 °C for 5 days; (iv) *Pseudomonas* on spread plates of Pseudomonas agar base (Oxoid, JRH Biosciences, Lenexa, KS, USA) with added CFC (Cetrimide, Fucidine, Cephaloridine) supplement for *Pseudomonas* spp. (Oxoid), incubated at 25 °C for 48 h; (v) *Enterobacteriaceae* on double-layered plates of violet red bile glucose agar (VRBG; Oxoid), incubated at 30 °C for 48 h [after first adding 5 mL of tryptone soy agar (Merck, Darmstadt, Germany), and incubating at room temperature for 1 h]; (vi) lactic acid bacteria on double-layered plates of De Man, Rogosa & Sharpe (MRS) agar (Oxoid), incubated at 30 °C for 72 h. All microbiological counts are expressed as the log of the colony-forming units per gram (log CFU g^{-1}) of sample. All analyses were performed in triplicate.

Colour

Lightness (L^*), yellowness (b^*) and redness (a^*) of well-pulverised cephalothorax carapace were measured with a Hunterlab colorimeter (Hunter Associates Laboratory, Inc., Reston, VA, USA), using a CIELab scale (Young & Whittle, 1985). With L^*, a^* and b^* values, whiteness (W) was calculated, according to Park (1994). Results were expressed as the mean of six measurements.

Polyphenoloxidase activity determination

The crude enzyme was obtained from head carapace. They were separated from the viscera on sampling days during chilled storage – frozen and stored at −80 °C until analysis. Crude extracts were obtained according to Wang *et al.* (1992), with slight modifications (Martínez-Álvarez *et al.*, 2005b).

The enzyme activity was measured using the proline-catechol spectrophotometric assay (Rzepecki & Waite, 1989) at saturating conditions, according to Wang *et al.* (1992). The results were expressed as units per millilitre of crude enzyme, considering the unity as an increment of 0.01 absorbance min^{-1}. Analyses were performed in duplicate.

Sensory evaluation

Over the 15-day storage period, a group of trained panellists routinely evaluated shrimp appearance every 2 days (ten individuals per treatment per evaluation), scoring melanosis according to a scale from 1 to 4, where 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. Odour was evaluated according to a scale from 0 to 3 where 0 = typical, 1 = neutral, 2 = slightly ammonia, 3 = off-odour. Appearance of yellow-green colouring in viscera (presence or absence) were also evaluated by the panellists.

Statistical analyses

The significance of differences between mean values was evaluated using two-way ANOVA. Statistical processing was by the SPSS 12.0 computer program (Chicago, IL, USA). The level of significance setting was $P < 0.05$.

Results and discussion

Proximate analysis results of the fresh shrimp were: crude protein 20.80 ± 0.62%; moisture 78.87 ± 0.15%, total fat 0.41 ± 0.06% and ash 1.49 ± 0.04%. These values are similar to others found from pink shrimp (Huidobro *et al.*, 2002). Furthermore, the pH value of the fresh shrimp was 7.3. This value is in accordance with pH value reported for deepwater pink shrimp from the Portuguese coast (Mendes *et al.*, 2002; Gonçalves *et al.*, 2003). After 3 months of frozen storage and subsequent thawing (day 0 of chilled storage), the pH value was 7.2, regardless of the treatment condition (Fig. 1). All treatment groups evolved similarly until day 7 (Table 1). At the end of the period, the pH value recorded for all groups was approximately 8.5, and no significant differences were detected ($P \leq 0.05$). The increment of 1.5 units in the pH value was the result of the accumulation of basic compounds that were a result of the enzymatic action, both endogenous and microbial.

At the beginning of the chilled conservation period, there were significant differences ($P \leq 0.05$, Table 1) between the TVB-N content of control shrimp (30 mg N per 100 g) and that of shrimp treated with 4-hexylresorcinol (34.3 mg per 100 g). These values are usually

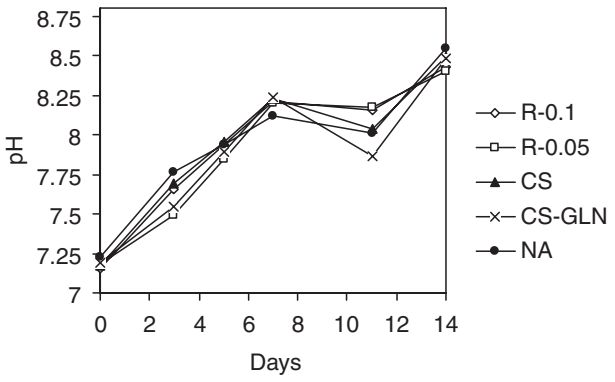

Figure 1 pH during the chilled storage (2 ± 1) °C of thawed shrimp. R-0.1: 0.1% 4-hexylresorcinol; R-0.05: 0.05% 4-hexylresorcinol; CS: commercial sulphites; CS-GLN: commercial sulphites + gluconic acid; NA: control shrimp. Formulations are detailed in materials and methods.

Table 1 Analyses of variance of pH, total volatiles bases (TVB-N), trimethylamine (TMA-N), water-holding capacity (WHC) and shear strength

		Days of storage					
Indexes	**Lots**	**0**	**3**	**5**	**7**	**11**	**14**
pH	R-0.1	a/x	a/y	a/z	a/w	a/w	ab/v
	R-0.05	a/x	b/y	b/z	ab/w	b/w	a/v
	CS	a/x	ac/y	a/z	a/w	c/z	bc/v
	CS-GLN	a/x	b/y	abc/z	a/w	d/z	bc/v
	NA	a/x	c/y	ac/z	b/w	c/z	c/v
TVB-N	R-0.1	a/x	a/x	ab/y	a/x	a/x	a/x
	R-0.05	a/x	ab/x	a/y	b/y	a/x	ab/x
	CS	ab/x	bc/y	b/yz	b/y	a/xz	a/xz
	CS-GLN	ab/x	c/y	ab/y	b/y	a/x	b/x
	NA	b/x	ab/y	ab/z	b/z	a/y	c/z
TMA-N	R-0.1	a/x	a/x	a/x	a/x	a/x	a/x
	R-0.05	a/xy	a/x	a/xy	ab/x	a/y	a/xy
	CS	a/x	a/x	a/x	ab/x	a/x	a/x
	CS-GLN	a/xz	a/y	a/xyz	ab/xyz	a/xz	a/xz
	NA	a/x	a/x	a/x	a/x	a/x	b/y
WHC	R-0.1	a/x	a/x	a/x	a/x	a/x	–
	R-0.05	a/x	a/x	ab/x	a/x	a/x	–
	CS	a/xy	a/xy	b/x	a/y	a/y	–
	CS-GLN	a/xy	a/xyz	b/x	a/xyz	a/yz	–
	NA	a/x	a/x	ab/x	a/x	a/x	–
Shear strength	R-0.1	a/xy	a/x	a/y	a/y	a/z	–
	R-0.05	a/x	a/x	b/x	b/x	b/y	–
	CS	a/x	a/y	b/y	c/z	c/v	–
	CS-GLN	a/x	a/x	ab/x	d/y	d/z	–
	NA	a/xz	a/xy	b/y	a/z	c/v	–

R, 4-hexylresorcinol (0.1 and 0.05%) with organic acids and chelating agents; CS, commercial sulphites; CS-GLN: commercial sulphites and gluconic acid; NA: non-additives (control).
Different letters (a, b, c ...) in the same column indicate significant differences ($P \leq 0.05$) as a function of treatment; different letters (x, y, z...) in the same row indicate significant differences ($P \leq 0.05$) as a function of storage time. (–), not determined.

found in high-quality fresh crustaceans (Matches, 1982; Mendes *et al.*, 2002). During the subsequent storage period, the total base content of all batches increased; however, this growth was only slightly moderate in the shrimp treated with resorcinol (Fig. 2). The early increase in TVB-N content, when bacterial counts were rather low, indicated that autolytic processes were involved in the production of volatile bases (Finne, 1982). Freezing could cause tissue membrane disruption, making endogenous enzymes more active after thawing. It is likely that adenosine and adenosine monophosphate (AMP) deaminases play a major role in this process, the former retaining close-to-optimum activity during the early part of the storage period. During days 5–7, the maximum values of TVB-N content were raised in all batches, with the exception of the R-0.1 group. This group registered a TVB-N content of 35 mg N per 100 g ($P \leq 0.05$), and this value remained constant until

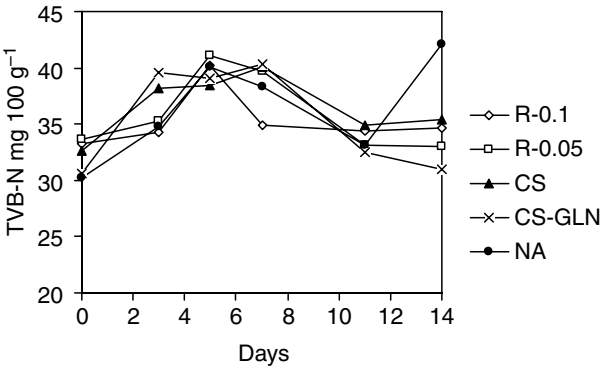

Figure 2 Total volatile bases (TVB-N) during the chilled storage (2 ± 1) °C of thawed shrimp. Formulations are detailed in M&M.

the end of the study period. The remaining batches also presented a decrease in TVB-N content from this point on. This subsequent decrease was most likely a result of the shrimp being washed by the melting ice, a phenomenon that has previously been reported by other researchers (Cintra *et al.*, 1999; Martínez-Alvarez *et al.*, 2005a). However, TVB-N content of the control shrimp (NA) significantly increased and registered 43 mg N per 100 g on day 14 ($P \leq 0.05$).

The TMA-N level in the shrimp was 0.5 mg N per 100 g at the beginning of the chilled storage period (Fig. 3). Similar values have been found in iced shrimp (*Pandalus borealis*) (Bullard & Collins, 1978) and pink shrimp under ice stored (López-Caballero *et al.*, 2002). This species could present a low concentration of TMA-N, owing to the fact that the major part of the volatile basic fraction in crustaceans is ammonia (Yeh *et al.*, 1978). Nearly all batches evolved similarly over 14 days, with some fluctuations in TMA-N levels ranging from 0.4 to 1.2 mg N per 100 g. However, the level of TMA-N in control shrimp suddenly increased on day 11,

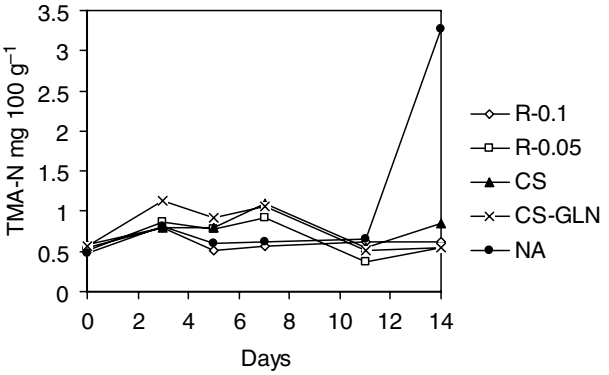

Figure 3 Trimethylamine (TMA-N) during the chilled storage (2 ± 1) °C of thawed shrimp.

reaching a significant value of 3.4 mg N per 100 g at the end of the period ($P \leq 0.05$, Table 1). This occurrence could be related to an increase in microbial load, mainly of *Shewanella putrefaciens* (Table 2) as species capable of reducing TMA-N oxide to TMA-N (Gram & Dalgaard, 2002).

At the beginning of the study, the WHC of the shrimp was approximately 80%. The WHC slightly decreased in all the batches over the storage period (Fig. 4). This decrease in WHC coincided with an increase in the muscle moisture of the defrosted shrimp during chilled storage (data not shown), probably as a result of absorption of water from ice melting, indicating that the extraction of this water could be easy. Significant differences were detected among treatments about WHC, with most of these differences occurring on day 5 (Table 1).

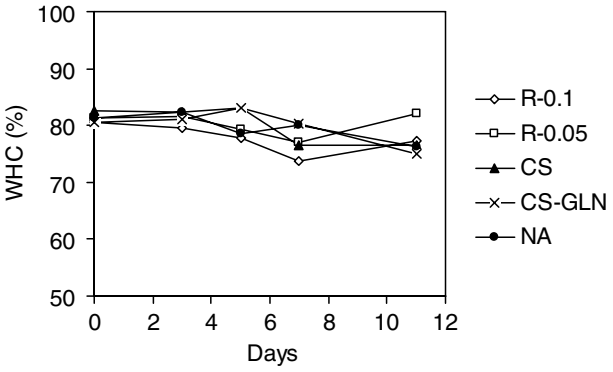

Figure 4 Water holding capacity (WHC) evolution during the chilled storage (2 ± 1) °C of thawed shrimp.

Table 2 The total bacteria count, H_2S-producing micro-organisms, *Pseudomonas* spp., *Enterobacteriaceae* and lactic acid bacteria

		Days of storage					
Micro-organisms	**Lots**	**0**	**3**	**5**	**7**	**11**	**14**
Total bacteria count	R-0.1	<2	<2	<2	$3.6^{ab/y}$	$4.7^{a/z}$	$6.5^{ac/v}$
	R-0.05	<2	<2	<2	$3.1^{a/y}$	$5.2^{ac/z}$	$6.0^{a/v}$
	CS	$2.0^{b/x}$	$3.0^{b/y}$	$3.1^{b/y}$	$4.2^{ab/y}$	$6.4^{b/z}$	$7.5^{bd/v}$
	CS-GLN	$2.3^{b/x}$	$2.3^{c/x}$	$3.0^{b/y}$	$3.9^{b/z}$	$5.4^{c/v}$	$6.9^{bc/w}$
	NA	<2	$2.6^{bc/y}$	$3.6^{b/z}$	$4.1^{b/z}$	$6.4^{b/v}$	$7.9^{d/w}$
H_2S-producing micro-organisms	R-0.1	<2	<2	<2	<2	<2	<2
	R-0.05	<2	<2	<2	<2	<2	<2
	CS	<2	<2	<2	<2	<2	<2
	CS-GLN	<2	<2	<2	<2	<2	<2
	NA	$2^{a/x}$	<2	<2	<2	<2	$5.0^{b/y}$
Pseudomonas spp.	R-0.1	<2	<2	<2	$2.4^{a/y}$	$3.7^{a/z}$	$5.4^{a/w}$
	R-0.05	<2	<2	<2	<2	$4.1^{a/y}$	$4.6^{b/z}$
	CS	<2	<2	$2^{b/y}$	3.3c/z	$4.2^{a/w}$	5.5^{a}/v
	CS-GLN	<2	<2	$2.3^{b/y}$	$2.3^{a/y}$	$3.9^{a/w}$	$5.2^{a/v}$
	NA	<2	<2	$2.0^{b/y}$	$3.3^{c/z}$	$4.1^{a/w}$	$5.1^{ab/v}$
Enterobacteriaceae	R-0.1	<1	<1	<1	$2.2^{a/y}$	$2.0^{a/y}$	$2.3^{a/y}$
	R-0.05	<1	<1	$1.1^{b/y}$	$1.0^{b/y}$	<1	<1
	CS	<1	<1	<1	$1^{b/y}$	<1	$2.1^{c/z}$
	CS-GLN	$1^{b/x}$	$1.3^{b/xz}$	$2.2^{c/y}$	$2.1^{a/y}$	$1.3^{c/z}$	$1.2^{b/z}$
	NA	<1	<1	<1	<1	<1	<1
Lactic acid bacteria	R-0.1	$1.0^{a/x}$	$1.1^{a/x}$	$1.0^{a/x}$	$2.3^{a/y}$	$1.9^{ab/y}$	$4.1^{a/z}$
	R-0.05	$1.2^{a/x}$	$1.3^{ac/x}$	$1.6^{b/x}$	$1.0^{b/x}$	$1.6^{a/x}$	$3.1^{b/y}$
	CS	$2.3^{b/x}$	$2.3^{b/x}$	$1.5^{ab/y}$	$1.3^{c/y}$	$2.3^{bc/x}$	$2.0^{c/y}$
	CS-GLN	$2.3^{b/xy}$	$2.0^{b/x}$	$1.9^{b/x}$	$2.1^{a/xy}$	$2.6^{c/y}$	$2.3^{c/xy}$
	NA	$1.0^{a/x}$	$1.3^{c/x}$	$1.4^{ab/xy}$	$1.0^{b/x}$	$2.0^{ac/y}$	$2.0^{c/y}$

R, 4-hexylresorcinol (0.1 and 0.05%) with organic acids and chelating agents; CS, commercial sulphites; CS-GLN, commercial sulphites and gluconic acid; NA, non-additives (control). Detection limit is <2 log CFU g^{-1} for total bacteria count, H_2S-producing micro-organisms and *Pseudomonas* spp.; <1 log CFU g^{-1} for *Enterobacteriaceae* and lactic acid bacteria.

Different letters (a, b, c ...) in the same column indicate significant differences ($P \leq 0.05$) as a function of treatment; different letters (x, y, z ...) in the same row indicate significant differences ($P \leq 0.05$) as a function of storage time.

The shear strength of peeled tails changed slightly during chilled storage until day 5, with values of firmness of approximately 5 N g^{-1} (Fig. 5). Similar values were reported in freshly caught deepwater pink shrimp (Huidobro *et al*., 2002). At the end of the storage period, the shear strength in CS-GLN and CS batches significantly increased, with values of 14 and 17 N g^{-1}, respectively (Table 1). The higher shear strength of the CS-GLN group could be associated with the fact that this treatment displayed the highest concentration of acid compounds. On day 11, the control shrimp (NA) also obtained values of shear strength of 14 N g^{-1}, while the shrimp treated with 4-hexylresorcinol registered the lowest values.

After the shrimp were thawed, the TBC were below the detection limit (R-0.1, R-0.05 and NA groups) or very close to it (CS and CS-GLN groups). These results are similar to those reported by some authors in newly caught pink shrimp (Huidobro *et al*., 2002; López-Caballero *et al*., 2002). Our results suggest that the

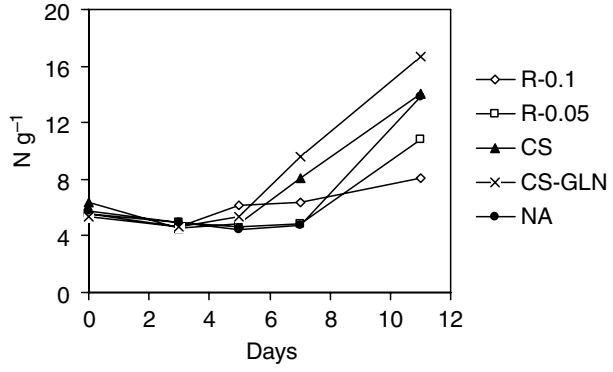

Figure 5 Shear strength (N g^{-1}) during the chilled storage (2 ± 1) °C of thawed shrimp.

freezing process did not eliminate the microflora, given that the TBC of the shrimp in this study, before freezing, was <2 log CFU g^{-1} for the R-0.1 and R-0.05 groups, 3.2 log CFU g^{-1} for the CS group, 3 log CFU g^{-1} for the CS-GLN group and 3.1 log CFU g^{-1} for the NA group. However, our results indicate that the freezing process could produce certain damages to the micro-organisms, resulting in a reduction of 1–3 log cycles. Microbial growth was detected in the control group on day 3, while microbial growth in shrimp treated with resorcinol was observed on day 7 (Table 2). After this point, significant differences of between 0.5–2 log cycles in TBC ($P \leq 0.05$) were observed. On day 14, control and CS batches obtained the highest counts (7.9 and 8 log CFU g^{-1}, respectively). In a previous study, deep-water pink shrimp (*Penaeus longirostris*), treated with sulphites, registered 7 log CFU g^{-1}, when they were stored on ice for 9 days (López-Caballero *et al*., 2002). In the present work, the tested formulations with 4-hexylresorcinol or gluconic acid inhibited microflora compared with shrimp without additives (i.e. the control lot). In addition, the inhibition of microbial growth was enhanced in these batches after day 7. This is an important finding, given that microbial spoilage is usually irrelevant at the beginning of storage. The formulations that were based on resorcinol were the most efficient in the inhibition of microbial growth, even at lower concentrations (0.05%). Microbial inhibition by 4-hexylresorcinol has previously been observed in tiger prawns (Martínez-Álvarez *et al*., 2006). Alternatively, it has been reported that a solution of 0.05% 4-hexylresorcinol did not effectively inhibit microbial growth (European Commission, 2003).

H_2S-producing micro-organisms were only detected in control shrimp after thawing and on day 14 (Table 2). In this study, the freezing process seemed to affect the proliferation of this group of organisms, most of them being *Shewanella putrefaciens* (Table 2). Along the same lines, the presence of luminous colonies was not detected during the storage period. López-Caballero *et al*. (2006) found luminous colonies (up 5 log CFU g^{-1}) during the iced storage, at 2 ± 1 °C, of fresh lobster treated with 4-hexylresorcinol and commercial sulphites.

Pseudomonas spp. were found on day 5, except in the treatment groups treated with resorcinol (Table 2). On day 7, the counts of *Pseudomonas* colonies slightly increased in all groups except the R-0.05 group. In the R-0.05 group, counts still remained below the detection limit. After day 7, all batches evolved similarly, with registered counts between 4.6 and 5.5 log CFU g^{-1} at the end of storage. The counts were not significantly different from the counts obtained in the R-0.05 group.

Enterobacteria were only detected in the CS-GLN group, with levels barely reaching the detection limit (Table 2) at the beginning of the chilled storage period. During storage, low concentration counts, even below <1 log CFU g^{-1}, were found for this group. Some authors (Huidobro *et al*., 2002) have reported higher enterobacteria counts (2.7 log CFU g^{-1}) in newly caught pink shrimp. In this same species, counts in shrimp treated with sulphites were 1.9 log CFU g^{-1} when the shrimp were freshly caught. These values rose to 5 log CFU g^{-1} on day 9 of chilled storage (López-Caballero *et al*., 2002).

Lactic acid bacteria were detected in all treatment groups at the beginning of storage (Table 2). These counts varied depending on the treatment condition, with groups CS and CS-GLN registering significantly higher counts than the other groups. During the storage period, the level of lactic acid bacteria measured in the control group increased 1 log cycle ($P \leq 0.05$), while batches containing sulphites practically remained constant. However, the level of lactic flora in shrimp treated with 4-HR increased, especially at the end of storage. The finding that 4-HR favoured the growth of this type of bacteria has previously been observed (López-Caballero *et al*., 2006). In addition, a high concentration of gluconic acid was used in the formulation applied to the CS-GLN group, indicating that this substance could also favour the growth of lactic flora and the selection of acid-resistant species. However, the drop in pH in the muscle produced by gluconic acid was not clearly observed in the present work. This finding could be attributed to the buffering capacity of the muscle.

Changes in the colour parameters of the head carapace in thawed pink shrimp during chilled storage are indicated in Fig. 6. The colour parameters of the head include lightness (L^*), redness (a^*) and yellowness (b^*). Originally, lightness (L^*) differed depending on the treatments (Fig. 6a). The higher value corresponded to the shrimp treated with sulphites and gluconic acid (CS-GLN) ($P \leq 0.05$, Table 3). This may be attributed to the fact that sulphites are potent reducing agents and are able to bleach the samples (McEvily *et al*., 1991; Rotllant *et al*., 2002). However, shrimp treated only with sulphites did not show higher values of L^*. This result may have occurred because these shrimp showed slight melanosis prior to freezing, or the gluconic acid may have enhanced the blanching produced by the sulphites. The significantly lower value of L^* in control shrimp was determined by the appearance of black spots (Table 3). As the storage period advanced, levels of L^* in all groups, excluding the groups treated with 4-hexylresorcinol (R-0.1 and R-0.05), decreased. After the shrimp were thawed, significant differences were found between the red-green tendencies (a^*), exhibited by shrimp treated with 4-hexylresorcinol (with $a^* +$ values, Fig. 6b) and the remaining treatment groups (Table 3). However, two days later $a^* +$ values diminished in the groups treated with 4-hexylresorcinol, resulting in similar a^* values across all groups. These results suggest the appearance of green colourations on the shrimp heads.

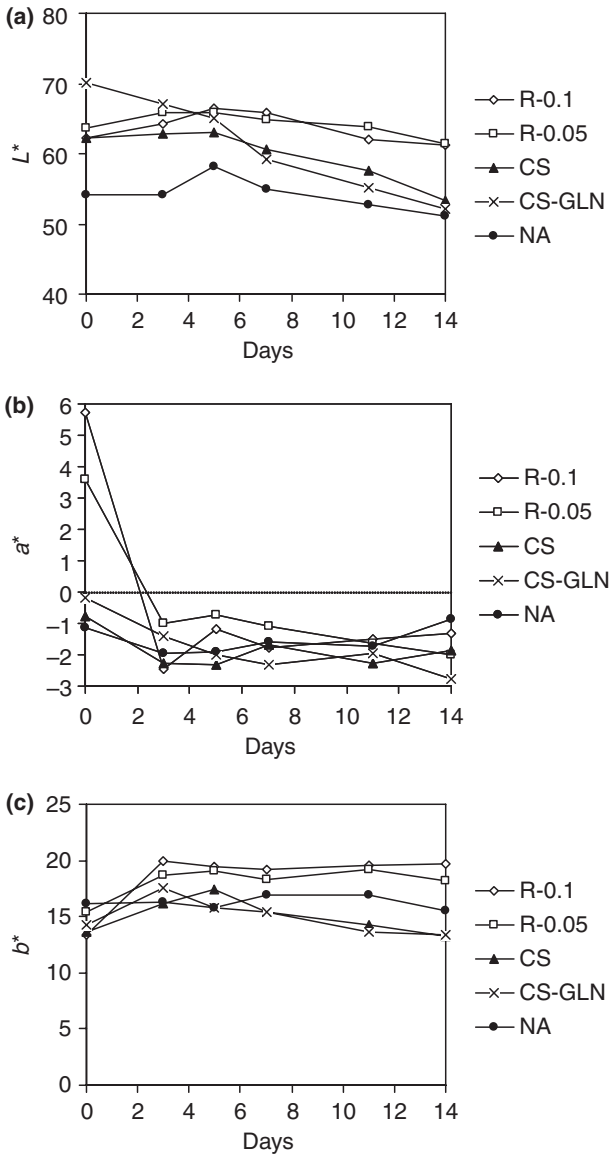

Figure 6 (a) Lightness (L^*) during the chilled storage (2 ± 1) °C of thawed shrimp. (b) Redness (a^*) during the chilled storage (2 ± 1) °C of thawed shrimp. (c) Yellowness (b^*) during the chilled storage (2 ± 1) °C of thawed shrimp.

Panellists determined the appearance of these colourations and the results are described as follows. After this time period, green tendency remained constant in all groups up to the end of storage. Figure 6c showed changes in the yellowness colour parameter (b^*). During storage, significantly higher values of b^*+ were found in shrimp treated with crescent concentrations of 4-hexylresorcinol (Table 3). The evolution of this paramenter was similar in all groups after day 2 of storage.

Table 3 Analyses of variance of Lightness (L), redness (a^*), yellowness (b), polyphenoloxidase (PPO), melanosis and odour

		Days of storage					
Indexes	**Lots**	**0**	**3**	**5**	**7**	**11**	**14**
L^*	R-0.1	a/x	ab/y	a/z	a/yz	a/x	a/x
	R-0.05	a/x	ac/y	a/y	a/xy	a/xy	a/z
	CS	a/xy	b/x	b/x	a/y	b/z	b/v
	CS-GLN	b/x	c/y	a/z	b/v	c/w	b/u
	NA	c/xz	d/xz	c/y	c/x	c/z	b/v
a^*	R-0.1	a/x	a/y	a/y	a/z	a/y	a/v
	R-0.05	b/x	b/y	b/z	b/v	b/z	b/w
	CS	c/x	c/y	b/y	a/v	c/v	c/w
	CS-GLN	d/x	d/x	d/x	a/x	d/x	d/x
	NA	d/x	d/x	d/x	d/x	d/x	d/x
b^*	R-0.1	a/x	a/y	a/z	a/v	a/w	a/u
	R-0.05	b/x	b/x	b/x	b/x	b/x	b/x
	CS	b/x	b/x	b/x	b/x	b/x	b/x
	CS-GLN	b/x	b/x	b/x	b/x	b/x	b/x
	NA	b/x	b/x	b/x	b/x	b/x	b/x
PPO	R-0.1	a/x	a/y	a/z	a/x	a/v	a/w
	R-0.05	a/z	b/y	b/x	b/z	b/y	a/y
	CS	b/x	c/y	c/z	c/v	ab/vw	a/v
	CS-GLN	a/y	b/zx	c/y	d/z	a/x	a/z
	NA	c/x	c/y	a/y	c/z	a/z	a/v
Melanosis	R-0.1	a/x	a/x	a/y	a/z	a/v	a/v
	R-0.05	a/x	b/x	b/y	a/y	a/z	a/v
	CS	b/x	c/y	c/y	b/z	b/v	b/v
	CS-GLN	b/x	d/y	d/z	c/v	b/w	b/w
	NA	c/x	e/y	e/z	d/z	c/z	c/z
Odour	R-0.1	a/x	a/xy	a/y	a/xy	a/y	a/y
	R-0.05	a/x	a/xy	a/xy	a/xy	a/y	a/y
	CS	a/w	a/wx	a/xy	a/xy	a/yz	b/z
	CS-GLN	a/x	a/xy	a/y	a/y	a/y	b/z
	NA	a/x	a/x	a/xy	a/x	a/yz	ab/z

R, 4-hexylresorcinol (0.1 and 0.05%) with organic acids and chelating agents; CS, commercial sulphites; CS-GLN, commercial sulphites and gluconic acid; NA, non-additives (control).
Different letters (a, b, c,...) in the same column indicate significant differences ($P \leq 0.05$) as a function of treatment; different letters (x, y, z,...) in the same row indicate significant differences ($P \leq 0.05$) as a function of storage time.

The PPO activity of thawed shrimp that were periodically extracted from the cephalothorax carapace during refrigeration is shown in Fig. 7. The tested formulations did not completely inhibit the PPO activity. Initially, the significant highest activity was observed in the control samples ($P \leq 0.05$, Table 3). This activity tended to diminish during the storage period, especially after the fifth day. These different behaviours may be explained by the presence of the diverse melanosis-inhibiting agents. Sulphites and D-gluconic acid may have a synergic effect against melanosis. Sulphites have been found to irreversibly inhibit PPO (Ferrer *et al.*, 1989; Lambrecht, 1995; Ricquebourgh *et al.*, 1996). Sulphites have also been found to interact with formed quinones and change coloured orthoquinones back to colourless

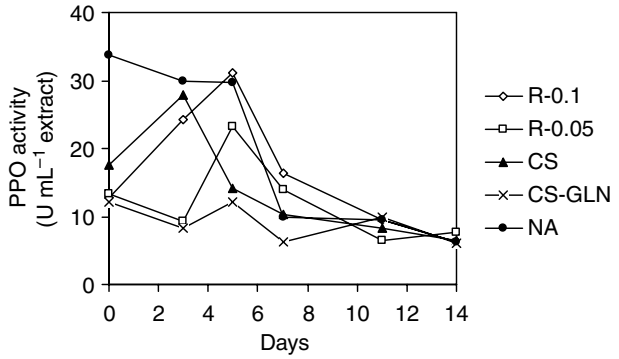

Figure 7 Polyphenoloxidase (PPO) during the chilled storage (2 ± 1) °C of thawed shrimp.

and less reactive diphenols (Ricquebourgh *et al*., 1996). The organic acid, D-gluconic acid, may act as an inhibitor of PPO in two different ways: (i) by diminishing the pH, or (ii) chelating the copper in the active site of the enzyme (Gyurcsik & Nagy, 2000). Given that the pH of the solutions was buffered by the pH of the muscle, the effect of D-gluconic acid on PPO in this study could mainly be based on its chelating capacity. It has been shown that 4-hexylresorcinol is a competitive inhibitor (Jiménez & García-Carmona, 1997) and it has widely been described as a good alternative to the use of sulphites by dipping (Frankos *et al*., 1991; McEvily *et al*., 1991; Iyengar & McEvily, 1992; Otwell *et al*., 1992; Guandalini *et al*., 1998). The melanosis-inhibiting effect of 4-hexylresorcinol, accompanied by several organic acids and chelating agents, has also been reported in shrimp (Montero *et al*., 2004) and prawns (Montero *et al*., 2001). The L-ascorbic acid present in the melanosis-inhibiting mixture may act, reducing quinones into original phenols, whereas citric acid will help to chelate the copper present in the active site of the enzyme (Iyengar & McEvily, 1992) and significantly to decrease the pH of the medium, as acetic acid does, which makes PPO unstable (Ferrer *et al*., 1989; Ali *et al*., 1994). Furthermore, EDTA and sodium pyrophosphate are chelating agents, which may act directly on the active site of PPO (Iyengar & McEvily, 1992; Lambrecht, 1995; Ramírez *et al*., 2003).

PPO activity in groups treated with melanosis-inhibiting compounds significantly increased in all cases during the first 5 days of chilled storage. According to Adachi *et al*. (2001), haemocyanin can be converted into a phenoloxidase-like enzyme (HdPO). In addition, a cooperative reaction between haemocyanin and a purified 160-kDa protein (Adachi *et al*., 2003) contributes to the black spot development in thawed prawns. This 160-kDa protein, presumably located in the cuticle, is very stable under freezing conditions. Owing to damage in the tissue, this protein could have reacted with HdPO after thawing. Furthermore, the increment in PPO activity may also be attributed to the stimulation of inactive proPPO, especially by the action of serin proteases (Söderhäll & Cerenius, 1992; Ali *et al*., 1994; Adachi *et al*., 2001), and also by diminishing the residual content of melanosis-inhibiting agents. In all cases in the current study, the trend changed and PPO activity decreased considerably, from 5 to 7 days, after thawing to the end of storage period. This could be related to PPO degradation by proteases, especially thiol proteases, as described by Wang *et al*. (1994) in Norway lobster. This phenomenon could also be associated with an irreversible inactivation of the enzyme (Ramírez *et al*., 2003). Considering these results, it might be deduced that the quantity of the formulations inhibiting melanosis that were added after the capture of the shrimp in November was not enough to prevent the progression of enzymatic browning after thawing.

Initially, the shrimp treated with mixtures that included 4-hexylresorcinol did not show melanosis after thawing (Fig. 8), while control samples presented a slight and significant presence of black spots on their heads (Table 3). The early presence of black spots on the heads of the samples may be attributed to the season during which the shrimp were caught, which coincides with the molting cycle of the shrimp (Ogawa *et al*., 1984). It should be noted that the shrimp were captured in autumn, when moulting usually takes place and melanosis develops faster. During chilled storage, melanosis increased gradually in all cases, chiefly in control samples. Adachi *et al*. (2001) have also reported a significant and rapid development of black spots in Kuruma prawn after thawing. Shrimp treated with 4-hexylresorcinol showed the best appearance during conservation, with only a slight melanosis over a minimum of 8 days. Regardless of the concentration of 4-hexylresorcinol, the evolution was relatively similar and generally without differences across groups ($P \leq 0.05$, Table 3).

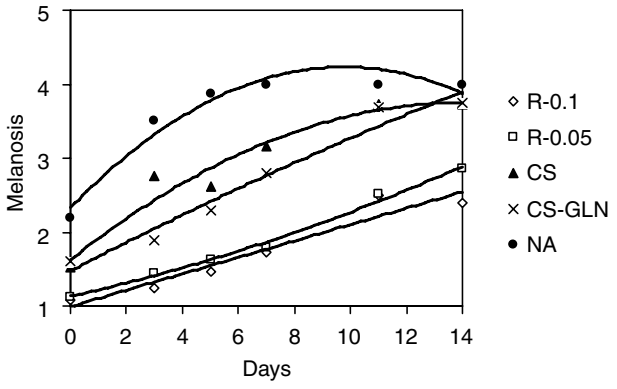

Figure 8 Melanosis during the chilled storage (2 ± 1) °C of thawed shrimp.

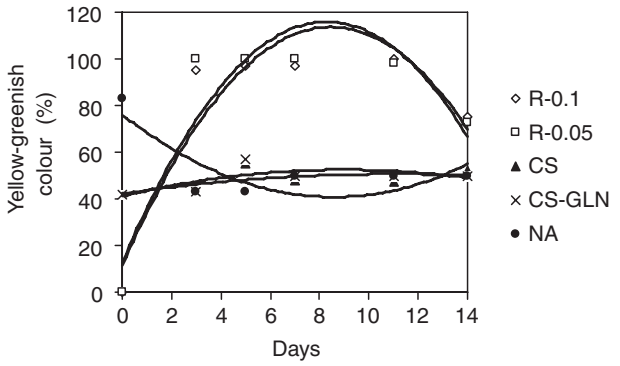

Figure 9 Yellow-green colourations during the chilled storage (2 ± 1) °C of thawed shrimp.

The appearance of yellow-greenish colouring in the cephalothorax was a part of the sensory assessment by the panellists (Fig. 9). Initially, control shrimp registered figures higher than 80%, while 4-HR-treated shrimps registered at 0%. However, the evolution of this parameter during chilled storage showed that resorcinol conferred green colouration on the cephalothorax of the shrimp. This fact was noted around the middle of the storage period and could have effects on the market value. The remaining batches attained intermediate values of approximately 40% throughout most of the study period.

At first, all groups scored between 0.5 and 1 ('typical'-'neutral') on measures of odour (Fig. 10, Table 3). All groups quickly evolved from 'neutral' to 'slightly ammonia' or even 'off-odour' during chilled storage. On day 14, shrimp treated with 4-HR did not exceed a score of 2 on measures of odour (limit of the acceptability of shrimp).

To summarise, formulations based on 4-hexylresorcinol may be used as substitutes for the traditional sulphite formulations. Our finding that 4-hexylresorcinol formulations prevent the appearance of melanosis in thawed pink shrimp, even more efficiently than sulphites, supports this substitution. Moreover, the formulations in this study that contained 4-hexylresorcinol delayed the phenomena associated with spoilage (i.e. the production of basic compounds, off-odours and microbial growth). The combined use of sulphites and gluconic acid also constitutes a promising alternative, as it contributed to a decrease in the deterioration of the shrimp after defrosting.

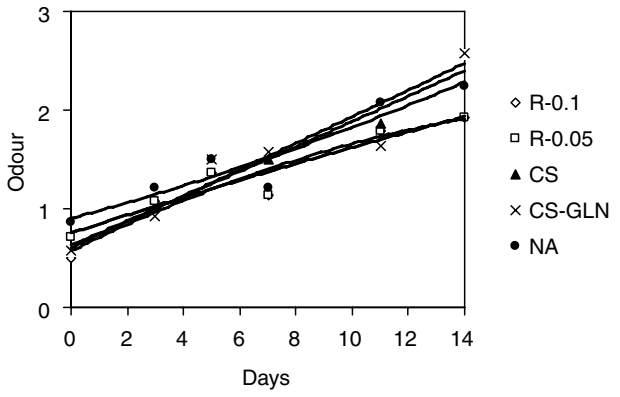

Figure 10 Odour evaluation during chilled storage (2 ± 1) °C of thawed shrimp.

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