Chemical and microbial quality indexes of Norwegian lobsters (*Nephrops norvegicus*) dusted with sulphites .

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Running title: Quality of Norwegian lobsters dusted with sulphites.

Abstract

The effect of dusting with a commercial sulphite-based anti-melanosis formulation on several chemical quality indexes of Norwegian lobsters (*Nephrops norvegicus*) was evaluated and compared with a control batch during iced storage. Dusting with 6% of the commercial product did not exceed the SO₂ residual levels permitted by the EU Directive and by the Food and Drug Administration. The additive retarded successfully the occurrence of black spots on the crustacean surface for at least 7 days and also bleached the samples. The prior addition of the sulphite-based compound did not affect either muscle pH or total volatile basic nitrogen content during storage, although it did produce increased lipid oxidation. The protease activity decreased, and differences in the pool composition of proteases were observed, serine and cysteine protease activities showing an increase. Finally, the sulphite-based compound delayed agmatine production and exerted an antimicrobial effect on H₂S-producers and Enterobacteria.

Key words: additives, polyphenoloxidase, antioxidants, chilling, fish.

Introduction

Seafood quality has been improving in recent years in response to a growing consumer demand and Norwegian lobster, which is widely distributed along the Atlantic coastline of Europe, is one of the most appreciated shellfish. Norwegian lobster, and seafood in general, are highly prized, but very perishable and this deterioration is primarily due to microbial spoilage, melanosis progression and endogenous enzymatic activity which leads to substantial financial loss. Freezing and refrigerating temperatures during distribution and retailing have been commonly used to prevent spoilage, and nowadays, different technologies, such as modified atmospheres, vacuum packaging or high-pressure treatment are also increasingly used as auxiliary technologies to prolong the shelf life of crustaceans (López-Caballero et al, 2000; Sivertsvik et al., 2002; Ruiz-Capillas et al., 2003). Furthermore, sulphite-derivatives, which are usually blended together with organic acids and chelants, are added immediately by dusting or dipping after capture to prevent the emergence of dark spots during chilled storage. Sulphites can also exert an antimicrobial effect, retarding microbial spoilage, as reported by Pyle and Koburger (1981) and Lambrecht (1995), despite not having been observed sometimes. (López-Caballero et al, 2000). Seafood spoilage also results from changes caused by the action of endogenous enzymes (Nip et al, 1985). Crustaceans possess high concentrations of digestive proteases, mainly serine proteases (Fernández Giménez et al., 2002), which are responsible for the decomposition of tissues and loss of freshness during chilled storage. Nip et al (1985) reported shelf life of iced fresh water prawns was no more than 3-4 days because of enzyme activity.. García-Carreño and Haard (1993) also reported rapid protease degradation of post-mortem krill and crayfish by endogenous proteases. The effect of sulphite-based compounds on these enzymes, as well as the effect of these preservatives on other physico-chemical freshness indexes, such as shear strength, oxidation of lipids and biogenic amines, though very important to the fishery industry, has hardly been studied. Only Ruiz-Capillas et al (2003) produced a paper about spoilage of Norwegian

lobsters during storage, despite their being stored in controlled and modified atmospheres.

The aim of this work was to evaluate the effect of a sulphite-based dusting on several physico-chemical and microbial freshness indexes of Norwegian lobster (*Nephrops norvegicus*) during chilled storage.

Materials and methods

Norwegian lobsters (*Nephrops norvegicus*) were caught off the south coast of Portugal and Spain by a commercial fishing vessel at the end of September. Average and sd sizes and weights were approximately 12.99 ± 1.1 cm (including the tail, carapace and clawed legs) and 30 ± 5 g, respectively. The experiment was carried out using Norwegian lobster treated conventionally with a 6% (w/w) commercial sulphite-based product called Melacide Fresh® (TEQUISA, Vigo, Spain, maximum SO₂ content 13.27%). Melacide Fresh® also includes sodium ascorbate, citric acid, and ethylenediaminetetraacetic acid (EDTA) in its composition. Untreated Norwegian lobsters were used for control purposes. Samples were placed in polystyrene boxes, covered with flaked ice and stored at low temperatures (2 °C). After the vessel reached port, the boxes were taken by refrigerated truck to the research centre of Instituto del Frío (Madrid, Spain), where they arrived within 40-48 hours after capture. The storage was carried out at 4 °C in polystyrene boxes, covering the crustaceans with ice, according to the traditional storage method.

Sulphite determination

The amount of sulphites in the muscles of various Norwegian lobsters was determined according to the Monier-Williams method (AOAC, 1984). Five determinations were done on peeled tails.

Muscle pH

Between 5-10 grams of muscle was homogenized with a double quantity (g/ml) of distilled water. After five minutes 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.

Digestive protease.

Protease activity was assayed according to the method employed by Hurtado et al (1999), using at least three replicates. Ten-gram aliquots of viscera were added to 20 ml of 0.02 M sodium phosphate buffer pH 6.5 and homogenized in an Omnimixer-Homogenizer (model 17106, OMNI International, Waterbury, USA) for 1 min. The homogenate was centrifuged for 40 min at 20000 x g (Beckman J2-MC Centrifuge, JA-20 Rotor, Beckman Instruments Inc., California, USA) at 4°C. The clear supernatant was used as an enzyme source.

The following inhibitors were tested to classify proteases in the cephalothorax of Norwegian lobster: 1 mM iodoacetic acid (as a cysteine protease inhibitor), 1 mg/ml phenylmethyl sulfonyl fluoride (PMSF, inhibitor of serine and some cysteine proteases), 0.2 µg/ml pepstatin A (inhibitor of aspartic proteases), 10 mM EDTA (as a metalloprotease inhibitor), and 10 µM leupeptin (inhibitor of cysteine and serine proteases). All inhibitors were supplied by Sigma Chemical (St. Louis, USA). The concentrations were chosen on the basis of previously reported proteinase classification studies (An et al, 1994; Hurtado et al., 1999). In all cases, an aliquot of 100 µl of enzymatic extract was preincubated with 400 µl of protease inhibitors for 15 minutes at room temperature (22 °C). Phosphate buffer was used to measure activity without inhibitors. Later, 400 µl of 0.2 M sodium phosphate buffer pH 7 were added, and incubation was performed for 16 h at 10 °C. Following the incubation, the proteolysis was stopped by adding 100 µl of cold 50 % (w/v) trichloroacetic acid (TCA). The mixture was kept at 4 °C for 15 min to allow unhydrolyzed proteins to precipitate, followed by centrifugation at 5700 x g at 4 °C for 10 min (MicroSpin 24S, Sorvall Instruments, DuPont, Wilmington, Del., USA). The content of TCA-soluble oligopeptides in the supernatant was analyzed by the same method employed by Lowry et al (1951) using tyrosine as standard. Protease

activity was expressed as μ moles of tyrosine released per gram of muscle per hour of incubation (nmol Tyr/g/h). The results were the average of at least 3 determinations. In all cases the blank was the result obtained for an aliquot without inhibitor that was introduced in an iced bath at 0 °C for the incubation time.

Polyphenol oxidase (PPO) extraction.

PPO was extracted in accordance with Montero et al (2002). One part of the frozen powder (10 g) was added to three parts (w/v) of 0.05 M sodium phosphate buffer pH 7.2 containing 1 M NaCl, 2 % (w/v) Poly(vinylpyrrolidone) (Sigma-Aldrich, St Louis, USA) and 0.5 % (w/v) Brij 35 (Panreac química, Barcelona, Spain). The mixture was stirred for 3 h at 2 °C and then centrifuged (Beckman J2-MC Centrifuge, JA-20 Rotor, Beckman Instruments Inc., California, USA) at 20000 x g for 30 minutes at 4 °C. The clear supernatant was fractionated with solid ammonium sulphate at 0-40 and 40-70 % saturation at 2 °C. After subsequent centrifugation (20000 x g, 30 minutes, 4 °C), the pellet was dissolved in 2.5 ml of 0.1 M phosphate buffer pH 7.2. Finally, molecular exclusion filtration (PD-10 column, Sephadex G-25 M, Amersham Pharmacia Biotech) at 2 °C was used to remove salts. The sample were immediately frozen to -80 °C in order to prevent alterations prior to determination.

Measurement of PPO activity.

The enzyme activity was measured using the proline-catechol spectrophotometric assay (Rzepecki and Waite, 1989) under saturation conditions (calculated K_m and V_{max} were 3.3 mM and 2.33 Units/ml crude extract, respectively), according to Wang et al (1992). The absorbance at 530 nm was monitored at 24 °C for 5 minutes in a spectrophotometer (Shimadzu UV-1601, Kyoto, Japan) with a CPS-240 thermostatic controller. The enzyme activity was expressed as increment of optical density (OD) $\text{min}^{-1} \text{ml}^{-1}$ (Wang et al, 1992).

Melanosis score and sensorial analyses.

Over the 14-days storage period, melanosis development was evaluated by a group of 10 trained panellists every 3-4 days (10 individuals per treatment per evaluation).

Melanosis was scored separately in heads (cephalotorax), clawed legs, parapods, abdomen, telson, and pleopods. Melanosis was scored using a numeric scale from 1 to 4, as previously reported (Montero et al. 2004), where 1= complete absence of black spots; 2= a few small spots; 3= considerable spotting; 4= substantial spotting. The panellists also judged the marketability of both batches during chilled storage. The results were represented as %.

Colour measurements

Lightness (L^*), yellow-blue tendency ($\pm b^*$), and red-green tendency ($\pm a^*$) of well-pulverized cephalotorax carapace were measured with a Hunterlab colorimeter (Hunter Associates Laboratory, Inc., Reston, Virginia, USA), using a CIELab scale. With L^* , a^* and b^* values, whiteness (W^*) was calculated, according to Park et al (1995). Results are expressed as the mean of six measurements.

Shear strength.

Shear strength was determined on a tail muscle sample 5.5-8 g weight. This was divided in half lengthwise and spread on a Kramer cell perpendicular to the slots of the cell. A computer-controlled Instron Universal texturometer model 4501 was used (Instron Engineering Corp., Canton, MA, USA), with a cell load of 5 kN at a setting of 100 mm/min. Results were the average of four determinations and were expressed as Newtons per gram of muscle at the point of maximum load before sample breaking.

Lipid oxidation (TBA index)

TBA index (thiobarbituric acid) was determined following the method of Vyncke (1970), incubating at 90 °C for 40 min. Results were expressed as μg malonaldehyde per 100 g of muscle.

Total volatile basic nitrogen (TVB-N)

Total volatile basic nitrogen (TVB-N) determinations were carried out in triplicate over the storage period using the method of Antonacopoulos and Vyncke (1989). 10 g $\pm$ 0.1 g of the ground sample were weighed in a suitable container and homogenized for two

minutes with 100 ml 6 % perchloric acid solution. After filtering, the extract was alkalized 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.

Biogenic amines.

Agmatine (Agm), Cadaverine (Cad), Histamine (His), Putrescine (Put) and Tyramine (Tyr) were determined in 10% aqueous trichloroacetic acid extract by the post-column method described by Ritchie (1991). Detection was carried out by fluorescence measurement. Results of three replicates were expressed as μg of amine per millilitre ($\mu\text{g/ml}$) of wet sample.

Microbiological analyses.

A total amount of 10 g of muscle were collected under aseptic conditions and blended (1:9 dilution) with peptone water (Oxoid, Basingstoke, UK) containing 0.85 % added NaCl in a Stomacher model 400 type BA 7021 Lab Blender (Seward Medical, Colworth London, UK) for 1 minute. Total viable counts (TVC) were determined by spread plating on Long and Hammer's agar with 1 % NaCl (Spreekens, 1974), incubating at 20 °C for 5 days. H_2S -producing microorganisms, as black colonies, were determined on double-layered plates of Iron Agar, incubating at 20 °C for 3 days, and Enterobacteria on double-layered plates of Violet Red Bile Glucose Agar (30 °C, 2 days), according to López-Caballero et al. (2002).

Statistical analyses.

The significance of differences between mean values was evaluated using 2-way analyses of variance (ANOVA). Statistical processing was by the SPSS 12.0 computer programme (Chicago, Illinois, USA). The level of significance setting was $P \leq 0.05$.

RESULTS AND DISCUSSION.

Sulphite content in edible parts

Untreated Norwegian lobsters did not show residual SO₂ content in edible parts. In sulphite-treated Norwegian lobsters, a residual level of SO₂ in edible parts of 50±16.76 mg/ kg was determined. It was less than was reported by Gómez-Guillén et al (2005) in pink shrimp treated with the same concentration (~6%) of Melacide, probably because shrimp cuticle is softer. On the other hand, the residual levels of SO₂ in Norwegian lobster did not exceed the limits permitted by the EU Directive (1995), which has determined a maximum content of sulphites -SO₂- in edible parts of 150 mg/kg. The residual levels of SO₂ were also lower than the allowed limits set by both Food and Drug Administration (FDA) guidelines for residuals in treated shrimp and by the Codex Alimentarius established by a FAO/WHO Joint Commission (Anonymous, 2000, max content of 100 mg/kg).

Muscle pH

Three days after capture, the pH of both treated and untreated samples was about 7 (Table 1), increasing with time. During the first 7 days of storage the increment was significant ($P \leq 0.05$) in both samples, but from then on the muscle pH increased marginally, up to pH 8, due to the build-up of basic substances that was a result of enzymatic action, both endogenous and microbial (Huss, 1995). Dusting with the sulphite-based compound did not cause significant changes in the muscle pH. Similar pH values and tendencies were also observed by Ruiz-Capillas et al (2003) in Norwegian lobster from Grand Sole.

Digestive protease

In both studied groups, protease activity evolved similarly for the first six days after capture (Fig 1), decreasing steadily ($P \leq 0.05$). Live crustaceans possess very high proteolytic enzyme activity (García-Carreño et al., 1994), with the hepatopancreas responsible for synthesis, storage of zymogens and secretion (Fernández-Giménez et al., 2002). During post-mortem storage, the digestive enzymes, mainly serine proteases and some metalloproteases (García-Carreño and Haard, 1993), diffuse from

the digestive gland, exerting an intense activity and causing the spoilage of crustaceans together with microbial enzymes (Ferrer et al 1989).

After the first six days of chilled storage, the protease activity of proteases from control samples went up significantly; in contrast to the activity of proteases from sulphite-treated Norwegian lobsters, which did not change significantly (Fig 1). In both treatment groups, the increment may be due to the fact that several proteases, especially serine proteases, may have activated others by a proteolytic cleavage at a particular peptide bond (Jiménez-Vega et al., 2005). Post-death trypsin activity has also been reported to increase in Spiny lobster (*Panulirus homarus*) and penaeids (*P. aztecus* and *P. setiferus*) (Savagaon and Sreenivasan, 1978), but the possible contribution of microbial proteases, principally during the last days of storage, should not be dismissed either.

Several compounds with protease inhibitory capacity were tested in order to determine changes in the pool of digestive proteases during chilled storage. Diverse researchers have described the composition of this pool in different crustaceans, although it depends on several endogenous and exogenous factors, such as ontogeny, the moulting stage, circadian rhythm and the feeding regime (Albuquerque-Cavalcanti et al., 2001), which affect the enzyme activity in the digestive gland of live crustaceans. The protease activity is lower during intermoulting and premoulting stages, because the feeding activity declines (Fernández-Giménez et al, 2002). Furthermore, García-Carreño et al (1999) described the presence of non-competitive inhibitors in live crustaceans which inactivate proteases, thus varying enzymatic activity.

The current study suggests some differences in the composition of the pool of proteases of both samples throughout chilled storage (Fig 1). In control samples, the increasing inhibition by PMSF ($P \leq 0.05$) together with the constant inhibitory effect of iodoacetic acid and leupeptine under storage conditions, suggests a growing activation of serine-proteases during chilled storage (Fig 2a,b,c). García-Carreño and Haard (1993) reported the presence of serine proteases in the pool of proteases of langostilla (*Pleuroncodes planipes*) and crayfish (*Pacifastacus astacus*) which play a very

important role in the spoilage of crustaceans, not only by their effect on endogenous protein, but also by their involvement in the PPO activation process (Wang et al., 1992). Serine proteases are a diverse group, but trypsin and chymotrypsin have been considered as the major proteolytic enzymes in many crustaceans. Ezquerro et al. (1997) reported trypsin and chymotrypsin as the main proteases in white shrimp (*Penaeus vannamei*), with trypsin activity being responsible for more than half of the total protein hydrolysis. Moreover, chymotrypsin has also been detected in four shrimp species of genus *Penaeus* (Albuquerque-Cavalcanti et al, 2001), despite the fact that García-Carreño et al (1994) have indicated that chymotrypsin is absent from some decapod digestive systems. In the present study, the inhibitory effect exerted by iodoacetic acid and leupeptin also indicates the existence of cysteine proteases in the pool of proteases. The existence of cysteine-proteases such as cathepsin C has been demonstrated in different crustacean species such as langostilla (*Pleuroncodes planipes*) and crayfish (*Pacifastacus astacus*) by García-Carreño et al. (1994).

Regarding the effect of EDTA, figure 2d shows the slight contribution of metallo-proteases only in the last days of storage and they may well be serine proteases, as the effect of PMSF suggests (Fig 2a). Doke and Ninjoor (1987) have also reported the presence of an alkaline metal dependent serine protease, with activity at pH 7 in shrimp (*Penaeus indicus*).

Finally, pepstatin A induced activation of protease activity (data not shown), probably because pepstatin A produced an inhibitory effect of aspartic proteases that gave rise to an increment in activity of other proteases. The reason could be that pepstatin A induced changes in the protein conformation. In line with this, Jian et al (1991) reported an inhibitory effect of reducing agents, such as L-ascorbic acid and glutathione, on trypsin-like protease, although both agents induced activation of chymotrypsin-like enzymes.

Regarding sulphite-treated Norwegian lobsters, figures 2a,b,c reflect fluctuations in the activity of cysteine and serine proteases during chilled storage. Cysteine and serine-

proteases diminished their activity gradually for the first 7 days of storage (Fig 2 a,c), but from then on the activity of these enzymes increased, as especially the effect of leupeptin (Fig 2b) indicates, diminishing again at the end. The increment could be produced by the increase of pH in the cephalotorax, because trypsin-like serine proteases typically show pH-optima under neutral or slightly alkaline conditions. Moreover, it is possible that the addition of a sulphite-based compound affected the activity of these enzymes. Additionally, the pool of proteases in sulphite-treated samples was relatively insensitive to EDTA inhibition (Fig 2d), possibly because metalloproteases were previously inhibited by the EDTA included in the commercial formulation. However, the absence of inhibition in control samples during the first days of storage indicates metalloproteases were not active after mortem. Nevertheless, metalloproteases becomes active again at the end of storage. Concerning the determination of higher peptide content after treatment with pepstatin A, a powerful inhibitor of aspartic proteases, it could reflect an increase in the activity of other proteases due to the fact that aspartic proteases are not active.

PPO activity during chilled storage

The greatest activity of PPO from untreated Norwegian lobsters was found in the 40-70 % ammonium sulphate fraction. The activity, expressed as Δ OD/min/ml, remained constant for the first week after capture (Table 2a), but from then on it decreased gradually ($P \leq 0.05$). When PPO activity was expressed as Δ OD/min/mg protein (Table 2b), enzymatic activity fluctuated over the first ten days, but diminishing during the last days of storage, probably because PPO was irreversibly inactivated during the oxidation of substrate to product (Ramírez et al, 2003). The inactivation could be due to a free radical-catalyze fragmentation of one or more of the histidine residues that bind copper at the active site, and results in a subsequent release of copper (Ramírez et al., 2003).

PPO activity from sulphite-treated Norwegian lobsters was detected only slightly at the end of storage (Table 2a,b). Initially, PPO activity was not observed, probably because

sulphites inhibited PPO by disrupting disulfide bonds present in the enzyme (Ferrer et al 1989; Lambrecht, 1995;). Complexing agents included in the antimelanosic formulation, such as EDTA and citric acid, could have also chelated the copper present in the enzyme-active site, affecting enzyme activity. On the other hand, both sulphites and L-ascorbic acid (that is included in the formulation) could have reduced the coloured orthoquinones back to the colourless and less reactive diphenols (Lambrecht, 1995).

Melanosis score and sensory analyses.

As expected, melanosis increased gradually in untreated samples during chilled storage (Fig 3a,b) due to the high activity of PPO and to the presence of natural substrates such as free tyrosine and tyramine (Rolle et al, 1991). Significantly, black spots appeared in all anatomical parts after two days of storage (Fig 3a, Table 3), especially in the heads (in the rostrum and where both clawed legs and parapods are connected to the cuticle). Dusting with sulphites was effective in delaying blackening at least for 7 days (Fig 3a), but then black spots were observed (Table 3) on the sides of the head, pleopods, telson, and tail carapace (outlining the sections of the tail, specifically in the connective tissue). According to Markakis and Embs (1966), the L-ascorbic acid could react first with the quinones, facilitating the inhibition of the PPO by sulphites. Then, when all the L-ascorbic acid had been oxidized, the quinones could have interacted with the sulphites. After both L-ascorbic acid and sulphites had been gradually consumed in the reaction (Lambrecht, 1995), the formed quinones could have polymerized and/or reacted with amino acids and proteins, forming melanins (Lambrecht, 1995; Riquebourg et al., 1996). This implies that sulphites present in the flesh could not have irreversibly inhibited PPO as a whole, (Ferrer et al. 1989; Lambrecht, 1995), and therefore the initial sulphite content in edible parts could be enough to stop PPO activity throughout the shelf life period.

Regarding clawed legs, melanosis was absent for ten days. Moreover, the presence of black spots was notorious in broken parts of the cuticle and clawed legs of some

individuals from the first day of storage. On considering acceptability during chilled storage, melanosis was the main cause of rejection by the panellists in both groups of treatments. All sulphite-treated individuals were considered marketable during the first seven days after capture, but not the control samples.

Colour measurements (CIE L*a*b*)

Norwegian lobsters treated conventionally with a commercial sulphite-based product presented a significantly higher lightness (L*) and whiteness (W*) than those of the control samples (with no inhibitors) throughout chilled storage (Table 4), coinciding with a minor prevalence of black spots on the shells. Different authors (Ferrer et al, 1989, Lambrecht, 1995) have reported that sulphites are potent reducing agents, so it is possible they could have bleached the cuticle. In both groups, lightness and whiteness showed a steady and significant decline during storage, due to the gradual appearance of black spots on the heads.

Regarding redness (a*), in all cases it tended to decrease during storage which meant that samples turned greenish. It is important to emphasise that, significantly, the sulphite-treated samples showed ($P \leq 0.05$) the highest values from the third day on of storage. This is very important because consumers consider the red colour to be a parameter of quality in Norwegian lobsters. On the other hand yellowness (b*), did not show any definite tendency in Norwegian lobsters treated with sulphite-based compounds during storage, unlike control samples, where yellow tended to decrease gradually.

Shear strength

Hardness of muscle tended to increase during storage in both groups studied (Table 5), though in no case was the increment found to be significant. The use of sulphite-based compounds did not affect hardness. These results could also explain the absence of a serious breakdown of muscle tissue produced by proteases, which may be because the activity of these enzymes on tail muscle diminished during ice-storage.

Lipid oxidation (TBA index)

TBA analysis is an important quality index, indicating lipid oxidation. In Norwegian lobster, the TBA index was less than 40 µg malonaldehyde/ 100 g sample (Table 6), probably because lipid content was very low (Rosa and Nunes, 2003). In both treatment groups, the TBA index was constant until the ninth day of storage, but from then on it began to come down, especially in the sulphite-treated samples ($p \leq 0.05$). The descent may be due to the fact that malonaldehyde could have interacted with substances generated by a protein degradation process. In any case, TBA content in sulphite-treated samples was always higher than those determined in control, although the differences were significant ($p \leq 0.05$) only at the beginning of storage. Lavoie et al (1994) suggested that sulphites might reduce or prevent oxidation, but in the case of crescent concentrations the reduction of hydroperoxides by metabisulphites could transform this compound into an oxidant, such as a sulphite radical.

Total volatile basic nitrogen (TVB-N)

The initial TVB-N level was about 20 mg/100 g in both sample groups (Table 7). Similar values have also been reported at the beginning of storage in other crustacean species, such as pink shrimp, brown shrimp, and tiger prawns (López-Caballero et al., 2002). Crustaceans may have high TVB-N values peculiar to themselves (Cadun et al 2005), and TVB-N concentrations higher than 25 mg/100 g can be found in good quality crustaceans (Matches, 1982; Mendes et al., 2002). During the first week after capture, TVB-N content remained constant and evolved similarly in both treatment groups (Table 7), Then, a sharp and significant ($P \leq 0.05$) increase was observed, exceeding the upper limit considered unfit for human consumption in fishery products (30-35 mg/100 g of muscle, Huss, 1988). Nevertheless, the differences between samples were not significant ($P \leq 0.05$) during storage. From day 10 onwards, TVB-N increased marginally in both treatment groups, due to the combined results of tissue enzymes and microbial activity.

Biogenic amines

Biogenic amines are generated by decarboxylase activity of both endogenous and microbial enzymes on free aminoacids, and their presence could serve as an indicator of fish spoilage (López-Caballero, 2002).

Tyramine has been reported to be the primary post-mortem substrate for PPO-catalyzed melanosis (Rolle et al., 1991). However, the tyramine content of both batches was below the threshold limit (< 7.51 mg/kg) throughout the entire storage period (Table 8). Similar low tyramine levels have been detected by López-Caballero et al. (2002) in pink shrimp. Regarding histamine content, it too was under threshold limits (< 5.80 mg/kg) during chilled storage, as López-Caballero et al (2002) also reported in pink shrimp. Putrescine levels were higher in control samples at the end of the storage period, while cadaverine content was superior in sulphite-treated samples throughout the storage period. Mietz and Karmas (1978) obtained high levels of putrescine and cadaverine during chilled storage of shrimp and lobster. With respect to the agmatine content of control samples, it was found in higher concentrations during the first days of storage, but the level decreased dramatically thereafter. Agmatine results from the decarboxilation of free arginine, which is very abundant in Norwegian lobster (Rosa and Nunes, 2003). Unlike agmatine levels in control samples, agmatine production was delayed in sulphite-treated samples at the beginning of storage. This could suggest differences in the pool of decarboxylating enzymes, as is shown in Fig 3d, where metalloproteases activity, which includes carboxypeptidases activity, is slightly superior in control samples. However, differences in the prevalence of microorganisms with decarboxylic activity should be taken into consideration, too.

Microbiological analyses.

Seafood deterioration is primarily due to bacterial action, so hygienic handling on board is very important to retard microbial growth and prolong shelf life. Regarding TVC, the counts evolved similarly in both batches. Initially, the counts were quite low (Table 9), indicating that Norwegian lobsters were of high quality. Then, TVC increased significantly ($P \leq 0.05$) in both groups throughout storage. The counts were less than 10^6

cfu/g (the microbiologic limit in accordance with UE legislation) during the first 7 days. At the end of the storage period, the TVC content reached about 7 or 8 log cfu/g, which coincides with a significant increment of TVB-N values (Table 9). The absence of any antimicrobial effect of sulphites throughout the spoilage period may be due to the commercial compound being added at the recommended use level, but not in excess (Pyle and Koburger, 1981). With regards to H₂S-producing micro-organisms, of which *Shewanella putrefaciens* makes up the greater part (López-Caballero et al., 2000), prior dusting with a sulphite-based compound retarded growth for the first seven days. However, both groups had similar counts from then onwards. The inhibitory effect on *Shewanella* is of importance, because together with *Pseudomonas* they usually dominate the microflora in fish and fishery products after 1-2 weeks of chilled storage (Sivertsvik et al., 2002).

The highest differences were observed for presumptive *Enterobacteriaceae*, which indicates the sanitary quality of the product (ICMSF, 1998). A lag phase in bacterial growth was observed in both groups during the first 7 days, with counts of less than 3 log cfu/g, because of the low temperature during chilled storage. That indicated that samples were properly handled before the experiments but from this point on, the enterobacteria content increased substantially ($P \leq 0.05$) up to the 10th day, and remained constant from then on. Sulphite-treated samples almost always registered ($P \leq 0.05$) significantly lower counts than those of control samples. This indicates that dusting with a sulphite-based compound may be effective at extending the shelf life of Norwegian lobsters, taking into account that IFST (1999) fixed levels of 10⁴ cfu/g for processed products (including fishery products), and this in spite of the manipulation associated with the dusting process.

To summarize, the sulphite-based treatment is effective in preventing melanosis development for at least 7 days, inhibiting PPO activity and having an antimicrobial effect, mainly against H₂S-producers and Enterobacteria. Moreover it has an inhibiting

effect on protease activity although an increases in serine and cysteine protease activity was observed.

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LEGENDS TO FIGURES

Figure 1.- Protease activity (mean $\pm$ sd) of Norwegian lobsters during chilled storage. Different letters (a, b, c...) indicate significant differences ($P \leq 0.05$) as a function of storage time. Different letters (x, y, z...) indicate significant differences ($P \leq 0.05$) as a function of treatment.

Figure 2.- Effectiveness of several inhibitors (mean $\pm$ sd) on protease activity in both groups of treatments. a) PMSF (1 mg/ml); b) Leupeptin (10 μ M); c) Iodoacetic acid (1 mM); d) EDTA (10 mM). Different letters (a, b, c...) indicate significant differences ($P \leq 0.05$) as a function of storage time. Different letters (x, y, z...) indicate significant differences ($P \leq 0.05$) as a function of treatment.

Figure 3.- Melanosis score (mean $\pm$ sd) of a) heads of Norwegian lobster and b) tails, telson, clawed legs and pleopods of Norway lobsters during chilled storage.

Table 1: Muscle pH (mean $\pm$ sd) of samples during chilled storage.

	DAY 3	DAY 7	DAY 10	DAY 14
Control	7.30 $\pm$ 0.05 ^{a/x}	7.80 $\pm$ 0.06 ^{b/x}	7.89 $\pm$ 0.03 ^{b/x}	7.74 $\pm$ 0.05 ^{b/y}
Sulphites	7.40 $\pm$ 0.08 ^{a/x}	7.76 $\pm$ 0.14 ^{b/x}	7.83 $\pm$ 0.05 ^{bc/x}	7.97 $\pm$ 0.08 ^{c/x}

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

Table 2: Enzymatic activity (mean $\pm$ sd) of partially purified PPO from cuticle of cephalotorax of Norwegian lobster during chilled storage. a) Enzymatic activity expressed as Δ OD/min/ml crude extract; b) enzymatic activity expressed as Δ OD/min/mg of protein.

a)	DAY 3	DAY 7	DAY 10	DAY 14
Control	0.067 $\pm$ 0.003 ^{c/y}	0.067 $\pm$ 0.001 ^{c/y}	0.050 $\pm$ 0.005 ^{b/y}	0.024 $\pm$ 0.003 ^{a/x}
Sulphites	0.000 $\pm$ 0.000 ^{a/x}	0.000 $\pm$ 0.000 ^{a/x}	0.000 $\pm$ 0.000 ^{a/x}	0.026 $\pm$ 0.002 ^{b/x}
b)				
Control	0.013 $\pm$ 0.000 ^{d/y}	0.010 $\pm$ 0.000 ^{b/y}	0.012 $\pm$ 0.001 ^{c/y}	0.006 $\pm$ 0.001 ^{a/y}
Sulphites	0.000 $\pm$ 0.000 ^{a/x}	0.000 $\pm$ 0.000 ^{a/x}	0.000 $\pm$ 0.000 ^{a/x}	0.001 $\pm$ 0.000 ^{b/x}

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

Table 3: Statistical analyses of melanosis score determined in different anatomical parts of Norwegian lobster.

		DAY 3	DAY 7	DAY 10	DAY 14
Head	Control	a/y	b/y	b/y	c/y
	Sulphites	a/x	a/x	b/x	c/x
Tail	Control	a/x	a/y	b/y	c/y
	Sulphites	a/x	a/x	b/x	c/x
Telson	Control	a/y	b/y	bc/y	c/y
	Sulphites	a/x	a/x	b/x	c/x
Claws	Control	a/x	b/y	b/y	c/y
	Sulphites	a/x	a/x	a/x	b/x
Pleopods	Control	a/y	b/y	c/y	c/y
	Sulphites	a/x	a/x	b/x	c/x

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

Table 4: Lightness, yellowness and redness (mean $\pm$ sd) of samples during chilled storage.

		DAY 3	DAY 7	DAY 10	DAY 14
L*	Control	46.76 $\pm$ 0.82 ^{a/y}	37.44 $\pm$ 0.95 ^{b/y}	37.08 $\pm$ 1.78 ^{b/y}	32.92 $\pm$ 2.38 ^{c/y}
	Sulphite	50.09 $\pm$ 1.05 ^{a/x}	48.76 $\pm$ 1.22 ^{a/x}	46.51 $\pm$ 1.17 ^{b/x}	42.96 $\pm$ 1.22 ^{c/x}
a*	Control	4.75 $\pm$ 0.57 ^{a/x}	-0.66 $\pm$ 0.63 ^{b/y}	0.49 $\pm$ 1.00 ^{b/y}	-0.90 $\pm$ 0.40 ^{b/y}
	Sulphite	3.37 $\pm$ 1.64 ^{a/x}	4.75 $\pm$ 0.57 ^{ab/x}	5.55 $\pm$ 1.19 ^{b/x}	0.94 $\pm$ 0.98 ^{c/x}
b*	Control	11.20 $\pm$ 1.51 ^{a/x}	10.23 $\pm$ 0.48 ^{ab/x}	9.07 $\pm$ 1.30 ^{bc/x}	7.43 $\pm$ 0.72 ^{c/x}
	Sulphite	10.06 $\pm$ 1.87 ^{ab/x}	11.79 $\pm$ 1.88 ^{b/x}	10.57 $\pm$ 0.64 ^{ab/x}	9.13 $\pm$ 1.08 ^{a/x}
W*	Control	45.36 $\pm$ 0.71 ^{a/y}	36.60 $\pm$ 0.94 ^{b/y}	36.40 $\pm$ 1.66 ^{b/y}	32.50 $\pm$ 2.29 ^{c/y}
	Sulphite	48.93 $\pm$ 1.38 ^{a/x}	47.17 $\pm$ 1.41 ^{ab/x}	45.18 $\pm$ 1.31 ^{b/x}	42.21 $\pm$ 1.25 ^{ac/x}

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

Table 5: Shear strength (mean $\pm$ sd) of samples during chilled storage.

	DAY 3	DAY 7	DAY 10	DAY 14
Control	8.99 $\pm$ 0.44 ^{a/x}	10.71 $\pm$ 1.13 ^{a/x}	10.20 $\pm$ 1.35 ^{a/x}	11.11 $\pm$ 1.13 ^{a/x}
Sulphites	9.26 $\pm$ 0.73 ^{a/x}	10.12 $\pm$ 0.97 ^{a/x}	9.95 $\pm$ 1.00 ^{a/x}	10.23 $\pm$ 0.73 ^{a/x}

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

Table 6: TBA index (mean $\pm$ sd) in samples during chilled storage.

	DAY 3	DAY 6	DAY 9	DAY 13
Control	21.44 $\pm$ 1.25 ^{a/x}	19.04 $\pm$ 3.65 ^{a/x}	23.61 $\pm$ 1.60 ^{a/x}	12.75 $\pm$ 1.94 ^{a/x}
Sulphites	36.41 $\pm$ 1.80 ^{b/y}	32.98 $\pm$ 6.62 ^{b/x}	33.89 $\pm$ 1.06 ^{b/x}	18.01 $\pm$ 1.02 ^{a/x}

Different letters (a, b, c...) in the same row indicate significant differences ($P \leq 0.05$) as a function of storage time; different letters (x, y, z...) in the same column indicate significant differences ($P \leq 0.05$) as a function of treatment. Results are expressed as μg malonaldehyde per 100 g sample

Table 7: TVB-N (mean $\pm$ sd) of samples during chilled storage.

	DAY 3	DAY 6	DAY 9	DAY 13
Control	18.35 $\pm$ 0.42 ^{a/x}	19.89 $\pm$ 1.96 ^{a/x}	39.78 $\pm$ 0.10 ^{b/x}	41.47 $\pm$ 0.56 ^{b/x}
Sulphites	17.65 $\pm$ 0.28 ^{a/x}	18.21 $\pm$ 1.40 ^{a/x}	46.37 $\pm$ 1.26 ^{b/x}	48.82 $\pm$ 10.16 ^{b/x}

Different letters (a, b, c...) in the same row indicate significant differences ($P \leq 0.05$) as a function of storage time; different letters (x, y, z...) in the same column indicate significant differences ($P \leq 0.05$) as a function of treatment. Results are expressed as mg TVB-N per 100 g sample.

Table 8: Biogenic amine content in muscle (means $\pm$ sd) during chilled storage. Results are expressed as mg of biogenic amines per kg of sample

		DAY 3	DAY 6	DAY 9	DAY 13
Tyramine	Sulphites	< 7.51	< 7.51	< 7.51	< 7.51
	Control	< 7.51	< 7.51	< 7.51	< 7.51
Putrescine	Sulphites	7.43 $\pm$ 0.41	10.18 $\pm$ 0.69	15.26 $\pm$ 1.93	6.53 $\pm$ 0.62
	Control	9.42 $\pm$ 1.44	< 6.53	< 6.53	28.95 $\pm$ 4.81
Cadaverine	Sulphites	8.45 $\pm$ 1.17	< 4.45	11.61 $\pm$ 2.96	8.52 $\pm$ 2.04
	Control	7.56 $\pm$ 1.56	< 4.45	< 4.45	< 4.45
Histamine	Sulphites	< 5.80	< 5.80	< 5.80	< 5.80
	Control	< 5.80	< 5.80	< 5.80	< 5.80
Agmatine	Sulphites	< 8.52	19.25 $\pm$ 5.37	57.41 $\pm$ 11.21	40.01 $\pm$ 12.17
	Control	110.27 $\pm$ 20.21	164.73 $\pm$ 37.33	< 8.52	< 8.52

Table 9: Total viable count (TVC), H₂S producers and Enterobacteria content (mean ± sd) of samples during chilled storage.

		DAY 3	DAY 7	DAY 10	DAY 14
TVC	Control	< 3 ^{a/x}	5.95 ± 0.13 ^{b/x}	6.70 ± 0.02 ^{c/x}	7.58 ± 0.03 ^{d/x}
	Sulphites	< 3 ^{a/x}	5.27 ± 0.01 ^{b/x}	6.47 ± 0.22 ^{c/x}	7.19 ± 0.04 ^{c/x}
H₂S producers	Control	< 3 ^{a/x}	5.35 ± 0.17 ^{b/y}	6.50 ± 0.03 ^{c/x}	7.49 ± 0.00 ^{d/x}
	Sulphites	< 3 ^{a/x}	3.87 ± 0.17 ^{b/x}	6.14 ± 0.27 ^{c/x}	7.11 ± 0.03 ^{d/x}
Enterobacteria	Control	2.01 ± 0.11 ^{a/y}	2.85 ± 0.03 ^{b/y}	4.22 ± 0.22 ^{c/y}	4.04 ± 0.04 ^{c/x}
	Sulphites	1.15 ± 0.18 ^{a/x}	1.59 ± 0.11 ^{a/x}	3.63 ± 0.04 ^{b/x}	3.82 ± 0.09 ^{b/x}

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

FIG 1

FIG 2

FIG 3