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OYSTER PRESERVATION BY HIGH PRESSURE TREATMENT

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ABSTRACT

The purpose of this study was to analyse the effect of 10 min continuous pressure and pulsed pressure in two 5-min steps (400 MPa / 7 ºC) on the microbial flora, total volatile bases, pH and texture of purified and unpurified oysters. High pressure treatment reduced the number of all the target microorganisms (total viable count, H_2S-producers microorganisms, lactic acid bacteria, *Brochothrix thermosphacta* and coliforms), in some cases by around 5 log units. The difference between the counts in the control and the pressurized oysters remained stable throughout 41 days of storage at 2 °C. No *Salmonella* spp. were detected either in the control batch or in the pressurized batches over this storage period. Deterioration of the oyster was accompanied by increased total volatile bases, mainly in the non-pressurized samples. The pH was practically constant in the pressurized oysters and fell slightly in unpressurized samples. As for mechanical properties, shear strength values were higher in pressurized than in unpressurized oysters. Step pulse pressurizing (400 MPa/7 °C in two 5-min pulses) produced no apparent advantages over continuous pressurizing based on any of the indices used.

Oysters are a high-value product and in Europe are generally eaten raw from the shell. Hence their shelf life is limited to the time they survive out of water, for which purpose they are kept at around 5 ºC in a saturated moisture ambient. It would therefore be highly advantageous economically to prolong their shelf life as much as possible without adversely affecting the sensory properties.

It is known that high pressure reduces the microorganism count *(10, 19, 29)* while retaining acceptable sensory properties of muscle foods *(12, 27*), although some authors describe modification of enzymatic activity *(4, 18)*. One proposal for reducing microorganism count while maintaining textural properties is to subject whole oysters to high pressure. Another useful avenue of exploration are means of applying high pressure. There is evidence in the literature that step-pulse pressurization in fish and other products is more effective than continuous pressurization, especially for the microbial stability (*1, 20).*

In general, molluscs differ in their chemical composition from fish and crustacean shellfish in containing significant levels of carbohydrate (glycogen) and a lower total quantity of nitrogen. For this reason, fermentative activities may be expected to occur as a part of microbial spoilage *(22)*. Pascual *(28)* reported that the predominant microorganisms during alteration of oyster are gram-negative proteolytic (*Pseudomonas* and *Vibrio*) and gram-positive saccharolytic (*Lactobacillus*) bacteria. There are references indicating that high-pressure treatment is highly lethal against the former and less lethal against the latter *(9, 11, 20)*. In addition, as a water-filtering organism, the oyster frequently accumulates pathogenic microorganisms, which cannot be removed by purification but may be killed by high pressure. This would allay the concern of a large proportion of the population who do not eat oysters for fear of contracting diseases.

As regards the appearance of pressurized oysters, preliminary trials have shown that

after pressurizing the meat is slightly more voluminous and has a very pleasant appearance, although there is occasional breakage of valves. Moreover, because pressurizing kills the animal, the oyster can easily be opened by hand. Another finding of preliminary trials was that at pressure as high as to 400 MPa the appearance of the meat was good. The appearance of the meat was better when pressurization was carried out under chilling (7 ºC) than at higher temperatures (20 and 37 ºC). The flavour was virtually unchanged, although Hoover *et al*. *(19)* described intensification of the flavour.

The object of this work was therefore to examine the possibility of prolonging the shelf life of chilled oysters by pressurization, continuous or pulsed, taking into account biochemical, physical and microbiological factors.

MATERIALS AND METHODS

Live cultured oysters (*Ostraea edulis*) were collected from a culture farm in Ría de Cambados (Pontevedra, Spain) in September. Half of the raw oysters were purified by placing them in filtered seawater during 48 h, while the other half were not purified. All were kept at about 5 °C for about 24 h before processing in the laboratory. Average size of shelled oysters was 7.2 ± 0.5 cm and average weight was 48.6 ± 5.5 g.

Sample preparations. All shelled oysters (900 individuals) were washed in cold water (5 °C) with 3.5 % (w/v) marine salt for 30 min. After draining off excess drip solution, oysters were wrapped individually in plastic bags and then samples of 6 oysters were vacuum-packed in bags with oxygen permeability of 60 cm^3/24 h/m^2 /atm at 23 ºC (Cryovac BB-1, Grace, Barcelona, Spain). Shelled oyster was treated in a high-pressure pilot unit (ACB 665, Gec Alsthom, Nantes, France): 400 MPa at 7 °C for 10 min (non-purified batch: 400-NP) or 400 MPa at 7 °C for 5 min in two consecutive steps (non-

purified batch: 400 pulses-NP; purified batch: 400pulses-P). The pressure was increased by 2.5 MPa/s and depressurization lasted a total of 15 s. In the case of pulsed application, a delay of 10 s was set between pulses. Pressurized oysters were stored at about 2 °C, while the raw shelled control (non-purified batch: C-NP; purified batch: C-P) was stored at about 5 °C. Unpressurized vacuum packed oysters (non-purified batch: V-NP; purified batch: V-P) were prepared for comparison and stored at 2 °C. The oysters (24 individuals per treatment) were periodically shucked manually in order to analyse the meat, taking care to avoid cross-contamination of the product in handling. All analyses were performed in duplicate.

Proximate analyses of raw shucked oyster meat were performed according to AOAC procedures *(3):* moisture *(*method 24003), ash (method 1821), protein (method 24024), and crude fat was determined using the method of Bligh and Dyer *(8).*

The pH of oyster meat was measured using a pHmeter (MeterLab pHM 93, Radiometer Analytical, Denmark) after blending 10 g meat with 100 ml distilled water. Determination of total volatile bases (TVB) was based on the method of Antonacopoulos and Vyncke *(2)*. A homogenate of shucked oyster meat was made with perchloric acid (6 %) to precipitate the muscle proteins. This was followed by centrifugation at 4000*g* 5 min 5 °C and distillation of the supernatant (Tecator AB, Kjeltec System, mod.1002, Höganäs, Sweden). The distillate was collected in boric acid (3 %) and titrated with hydrochloric acid (0.05 N). Results were expressed in mg of nitrogen/100 g of sample.

At least 12 oysters per lot were used for microbiological analysis as follows: 10 g (and 25 g to detect *Salmonella* spp.) from different parts of individuals for each different treatment were taken aseptically in a vertical laminar-flow cabinet (Telstar mod. AV 30 / 70, Spain) and placed in a sterile plastic bag (Sterilin, U.K.) with 90 ml of buffered peptone water (Oxoid, U.K.). After 2 min in a stomacher blender (mod. Colwoth 400, Seward, UK),

dilutions were made in the same diluent to determine the following microorganisms: double-layered plates on Iron Agar (IA, Adsa Micro, Spain) for the total viable count (TVC) and H_2S-producing microorganisms after 72 h of incubation at 20 °C; spread plates on Iron Agar + 0.5 % NaCl for total viable count and *Photobacterium phosphoreum* count after 5 days of incubation at 15 ºC; double-layered plates on M.R.S. agar (Oxoid) for the lactic acid bacteria (LAB) after 72 h of incubation at 30 °C; spread plates on Streptomycin Thallous Acetate Agar (STAA, Adsa-Micro, Spain, containing streptomycin, cycloheximide and thallous acetate as selective agents) for the *Brochothrix thermosphacta* count after 48 h of incubation at 20 °C; double-layered plates on COLI ID medium (bioMérieux, France) to enumerate coliforms and presumptive *Escherichia coli* after 48 h of incubation at 37 °C; and finally, spread plates on SM ID medium (bioMérieux, France) to detect the presence/absence of *Salmonella* spp. after 24 h of incubation at 37 °C. Microbiological counts were expressed as log CFU/g of sample, except for *Salmonella*, which was expressed as absence/presence in 25 g of sample.

Shear strength was determined using a texturometer (Instron 4501, Instron Engineering Corp., Canton M.A., USA). The shucked oyster meat was placed in a Kramer cell for shearing at 100 mm / min, using a 5 kN load cell. The maximum force was expressed in proportion to the sample weight (Newton/gram) on the basis of at least six replicates.

Changes in general appearance were monitoring during storage by a visual method, taking into account shape, size and colour.

Statistical analysis. The difference of means between pairs was resolved by means of confidence intervals using non-parametric statistics by the Kruskal-Wallis test (BMP3S) in the BMDP computer programme (BMDP Statistical Software, Inc., Cork Technology Park, Cork, Ireland). The level of significance was set for a z-value ≤ 0.10.

RESULTS AND DISCUSSION

The proximate composition of the oyster meat was: moisture: 76.04 ± 1.29; ash: 2.31 ± 0.11; crude protein 11.68 ± 0.15; crude fat 1.81 ± 0.35. The residual percentage is attributed to glycogen, which according to some authors *(22, 24)* is about 5-8% Other authors have reported similar composition, bearing in mind that this composition varies according to species and season when caught *(6, 7, 21)*.

The pH of live oyster meat (Fig. 1) was 6.3 for non-purified and 6.2 for purified samples. This is consistent with the reports of other authors *(6, 23)*. There was a slight decrease of pH in the control and the vacuum-packed oysters; towards the end of storage values fell to nearly 6.0, which is the lower limit of acceptability for oysters *(7)*. This may be due to the relatively high level of glycogen and the fact that spoilage of mollusc shellfish is basically fermentative *(22)*. This behaviour was absent in the oysters pressurized continuously for 10 min or in two 5-min pulses, possibly due to microbial reduction.

This being a fresh product, the initial total volatile base content of the raw oysters was low (13.3 mg nitrogen/100 g in non-purified oysters and 11.2 mg/100 g in purified oysters). After about 10 days in storage, the controls reached total volatile base content of 25-30 mg/100 g (normally considered spoilage levels) and off-odours were detected. This did not happen in the vacuum-packed and the pressurized samples until two and six weeks' storage respectively (Fig. 2). Murata *et al*. *(26)* reported that the TVB content in shucked oysters stored in ice was initially relatively low (10.5 mg nitrogen/100 g) and thereafter increased significantly in two weeks up to 25 mg nitrogen/100 g. The reason why TVB values were relatively low at the point of spoilage may be that, as noted earlier, the oyster undergoes general acidification as the high glycogen content is converted to lactic acid.

With the exception of total viable count at 20 °C, microorganism counts were higher in unpurified than in purified oysters, probably due to recontamination in the purifying tank *(5)* (Tables 1 and 2). The counts of TVC at 20 °C and coliforms were below the recommended microbiological limits for fresh bivalve molluscs *(28)*.

A variety of factors have been proposed to account for the inactivation of microorganisms in pressurized foods, including the type of microorganism, the level, time and temperature of pressurization and the specific foodstuff concerned *(10, 17, 27, 29)*. In the present case pressurizing (400 MPa, 10 min, 7 ºC) of oysters reduced the counts of the target microorganisms, in some cases to below the detection threshold (Tables 1 and 2). This was the case for coliforms, presumptive *E*. *coli*, H_2S-producing microorganisms and total viable microorganisms at 15 °C; in this last case the reduction was as low as 5 log cycles. In minced meat pressurized at 400 MPa/20 min Carlez *et al*. *(10)* reported total inactivation of *Pseudomonas*, *Lactobacillus* and coliforms with the total flora reduced by 3-5 log cycles, a higher figure than in the present case. In a model system of inoculated pork slurries pressurized at 400 MPa/10 min/25 ºC, Shigehisa *et al*. *(29)* achieved a 6 log cycle reduction of *Escherichia coli*, *Campylobacter yeyuni, Pseudomonas aeruginosa, Salmonella typhimirium,* and *Yersinia enterocolitica.* These authors reported that higher pressures were required to inactivate some gram-positive species, which they suggested was because these microorganisms are more resistant to pressure than gram-negative species *(11, 19, 30)* This could account for the inactivation of coliforms, presumptive *E. coli* and H_2S-producers in pressurized purified oysters stored for 41 days.

During storage of both vacuum-packed and pressurized oysters there was an increase in the lag phase (LAB, TVC at 20 °C) compared to the controls (Tables 1 and 2). In pressurized oysters there was also some delay in growth (coliforms, LAB, H_2S-producers, TVC at 15 °C and TVC, if only slight, at 20 °C), which was attributed to sublethal damage

or cellular stress induced by high pressure *(12, 19)*, resulting in a lower growth rate at the end of the experimental storage period (Tables 1 and 2). Cellular stress induced by high pressure processing, causing apparent inactivation but with resumption of growth after a repair phase, occurred in the genus *Pseudomonas (10).*

To determine whether microorganisms are affected in the same way by continuous (10 min) pressure and two 5-min pressure pulses, both procedures were tried on unpurified oysters. Step pulse pressurizing at 400 MPa did not increase inactivation of the flora. Microbial counts on non-purified vacuum packed oyster pressurized in two 5-min pulses at 400 MPa, 7 ºC (400pulses-NP) were higher in most cases than for non-purified vacuum-packed oyster pressurized at 400 MPa, 10 min, 7 ºC (400-NP) (Tables 1 and 2). These results do not agree with the findings of Alemán *et al*. *(1)* in pineapple and Hurtado *et al*. *(20)* in octopus, who found that pulsed pressure treatments – at 240 and 400 MPa, respectively – reduced microbial populations more effectively than continuous pressure treatment.

The incidence of H_2S-producers (of which *Shewanella putrefaciens*is the predominant species), which are considered to be specific to spoilage of fish and fish products *(14)*, was low, and they become predominant only in the non-purified control (C-NP). Some presumptive non-luminescent *P. phosphoreum* were detected on spread plates of Iron Agar in most of the non-purified lots in the early stages of storage. *P. phosphoreum* could be responsible for the spoilage of vacuum-packed fish from marine waters along with *S. putrefaciens (15)*, given their ability to reduce TMAO to TMA *(13),* but in the present study they did not constitute the majority flora. The pressure sensitivity of H_2S-producers has been described by Myllymäki *et al*. *(25)*, who reported a reduction of about 10^4 CFU/g (to the limit of detectability) in fish products from Finland pressurized (500 and 700 MPa) for 5 min at 20 ºC. This reduction in numbers was greater than the reduction in total aerobic flora. To our knowledge, there is no information about the behaviour of *P*. *phosphoreum*

under high pressure treatment.

Pascual *(28)* reported that the bacteria predominating during oyster spoilage included gram-negative proteolytic species (*Pseudomonas* and *Vibrio*), with production of amines, and ammonia and saccharolytic species (*Lactobacillus*), which reduced the pH. This would account for the high TVB counts in the vacuum-packed lots; in the pressurized lots, TVB levels were lower because of the lethal effect of high pressure on these species. In this connection it should be noted that oysters have been found to content TMAO, although usually in small amounts compared to other molluscs *(16).* Total viable counts on spread plates on Iron Agar at 15 °C were higher than counts on pour plates on Iron Agar at 20 °C which once again brings into question the use of only one method for enumeration of microorganisms in a food *(13)*.

No *Salmonella* spp. were detected during the experimental storage period, while counts of coliforms were reduced dramatically by high pressure treatment (Table 2). Presumptive *E*. *coli* was detected in the control oyster and in non-pressurized vacuum-packed oyster. Presumptive *E. coli* was otherwise consistently below the detection threshold (<10 CFU/g) in pressurized oysters. These results would indicate that the pressurized samples were more hygienic and hence presented less potential risk of the presence of pathogenic microorganisms.

Figure 3 shows the appearance of oysters with and without high-pressure treatment. It is noted that after high pressure treatment the oyster was slightly more voluminous and juicy. However there were only slight apparent differences initially in the shear strength of lots pressurized in one step and in two consecutive steps (Fig.4). After a week in storage, the initial value had doubled in the pressurized oysters, remaining stable thereafter until the end of storage. This increased shear strength may have been due to aggregation and water loss induced by denaturation in the myofibrillar fraction.

In some cases, packages were punctured by the sharp oyster shell. This caused heavy blackening of pressurized oysters, possibly due to enzymatic activity, which will be studied in subsequent papers.

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Table 1. Microorganisms (log CFU/g) in purified and non-purified oyster

Microorganisms	Treatments	Storage (days)				
TVC (20°C)		0	6	13	28	41
	C-NP	3.13	4.60_{x}	$5.79_{x,y}$	-	-
	V-NP	3.13^{a}	$3.33^{a,b}{}_{x,y}$	$3.91^{a,b}{}_{x,y}$	3.77^{b}	$5.12^{a,b}{}_{x,y}$
	400-NP	$2.72^{a,b}$	$2.69^{a,b}{}_{x,y}$	$2.73^{a,b}{}_{x,y}$	4.72^{a}	$2.07^{b}{}_{x}$
	400pulses-NP	3.00	$3.00_{x,y}$	4.58_{x}	2.87	4.19_{y}
	C-P	3.38^{a}	$2.92^{a,b}{}_{x,y}$	$4.01^{b}{}_{x,y}$	-	-
	V-P	$3.38^{a,b}$	$3.46^{a,b}{}_{x,y}$	$3.75^{a}{}_{x,y}$	3.00^{b}	$6.79^{a,b}{}_{x,y}$
	400pulses-P	1.39	1.30_{y}	2.32_{y}	4.38	$3.85_{x,y}$
H_2S-Producers						
	C-NP	<1	<1	6.00	-	-
	V-NP	<1	1.00	1.65	4.09	2.67
	400-NP	<1	<1	<1	<1	<1
	400pulses-NP	<1	<1	2.29	<1	<1
	C-P	1.00	1.00	2.93	-	-
	V-P	1.00	1.17	1.39	1.54	4.26
	400pulses-P	<1	<1	<1	<1	<1
TVC (15 °C)						
	C-NP	5.39	5.69	7.50	-	-
	V-NP	$5.39^{a,b}$	$4.85^{a,b}$	4.55^{a}	$6.13b_{x,y}$	$6.50^{a,b}{}_{x,y}$
	400-NP	<2	<2	2.74	6.71_{x}	3.68_{x}
	400pulses-NP	<2	<2	4.43	$5.48_{x,y}$	6.56_{y}
	C-P	4.56^{a}	$5.00^{a,b}$	5.88^{b}	-	-
	V-P	4.56^{a}	$4.24^{a,b}$	4.97^{b}	$4.57a,b_{y}$	$7.77^{a,b}{}_{x,y}$
	400pulses-P	<2	<2	<2	$5.42_{x,y}$	$4.60_{x,y}$

C-NP, control non-purified; V-NP, non purified vacuum-packed; 400-NP, non-purified vacuum-packed and pressurized at 400 MPa, 10 min, 7 ºC; 400pulses-NP, non-purified vacuum packed and pressurized at 400 MPa, 7 ºC in two 5-min pulses; C-P, control purified; V-P, purified vacuum-packed and 400pulses-P, purified vacuum-packed and pressurized at 400 MPa, 7 ºC in two 5-min pulses. Different letters (a, b) mean significant differences ($z \leq 0.10$) during the storage time and different letters (x, y) mean significant differences among treatments.

Table 2. Microorganisms (log CFU/g) in purified and non-purified oyster

Microorganisms	Treatments	Storage (days)				
LAB		0	6	13	28	41
	C-NP	2.90	3.72	$4.81_{x,y}$	-	-
	V-NP	2.90	2.65	$3.00_{x,y}$	$2.65_{x,y}$	$5.09_{x,y}$
	400-NP	<1	$2.70^{a,b}$	$2.65^{a,b}{}_{x,y}$	$5.03^{b}{}_{x}$	$5.00^{a,b}{}_{x}$
	400pulses-NP	$1.60^{a,b}$	$2.75^{a,b}$	$3.69^{a}{}_{x}$	$3.00^{b}{}_{y}$	$1.17^{a,b}{}_{y}$
	C-P	1.00^{a}	$1.47^{a,b}$	$2.39^{b}{}_{x,y}$	-	-
	V-P	1.00^{a}	$2.46^{a,b}$	$2.77^{b}{}_{x,y}$	$2.30^{a,b}{}_{x,y}$	$4.54^{a,b}{}_{x,y}$
	400pulses-P	$1.25^{a,b}$	$1.60^{a,b}$	$1.95^{a,b}{}_{y}$	$1.00^{a}{}_{x,y}$	$4.51^{b}{}_{x,y}$
B.thermospacta						
	C-NP	<2	2.60	3.97	-	-
	V-NP	<2	2.30	2.00	<2	<2
	400-NP	<2	<2	<2	<2	<2
	400pulses-NP	<2	<2	2.30	<2	<2
	C-P	<2	<2	<2	-	-
	V-P	<2	2.00	<2	<2	<2
	400pulses-P	<2	<2	4.42	<2	<2
Coliforms						
	C-NP	2.63	2.87	$4.66_{x,y}$	-	-
	V-NP	$2.63^{a,b}$	$2.39^{a,b}$	$4.03^{a}{}_{x}$	<1	$1.17^{a,b}$
	400-NP	<1	<1	$1.69_{x,y}$	1.17	<1
	400pulses-NP	<1	<1	$3.34_{x,y}$	1.00	<1
	C-P	1.00	<1	$2.44_{x,y}$	-	-
	V-P	1.00	<1	$2.51_{x,y}$	<1	<1
	400pulses-P	<1	<1	<1	<1	<1
E. coli						
	C-NP	+	-	-		
	V-NP	+	+	-	-	-
	400-NP	-	-	-	-	-
	400pulses-NP	-	-	-	-	-
	C-P	+	+	+		
	V-P	+	+	+	-	+
	400pulses-P	-	-	-	-	-

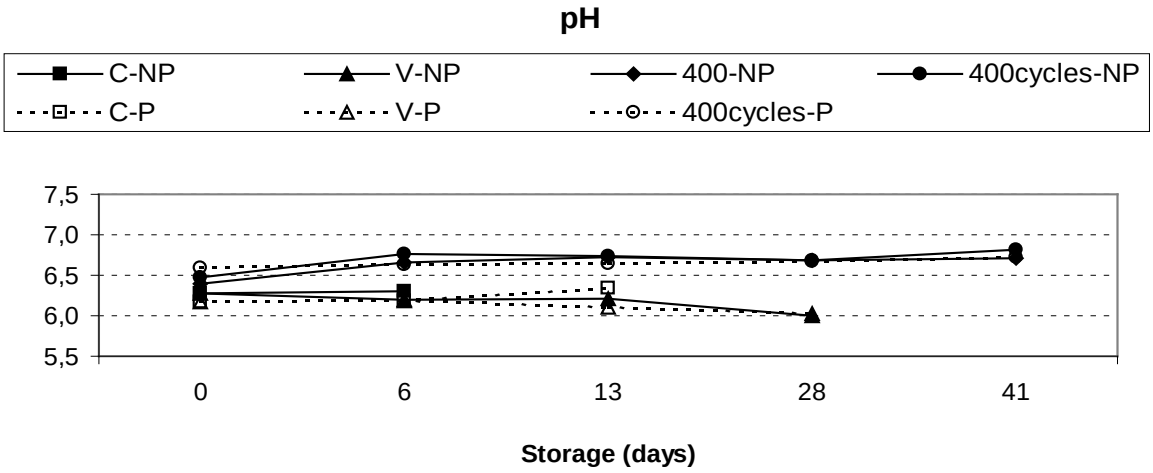
C-NP, control non-purified; V-NP, non purified vacuum-packed; 400-NP, non-purified vacuum-packed and pressurized at 400 MPa, 10 min, 7 ºC; 400pulses-NP, non-purified vacuum packed and pressurized at 400 MPa, 7 ºC in two 5-min pulses; C-P, control purified; V-P, purified vacuum-packed and 400pulses-P, purified vacuum-packed and pressurized at 400 MPa, 7 ºC in two 5-min pulses.+ /- indicates presence or absence for *E. coli*. Different letters (a, b) mean significant differences ($z \leq 0.10$) during the storage time and different letters (x, y) mean significant differences among treatments

Figure 1.- pH of lots of oysters (C: control; V: vacuum-packed; 400: vacuum-packed and pressurized at 400 MPa, 10 min, 7 ºC; 400pulses: vacuum packed and pressurized at 400 MPa, 7 ºC in two 5-min pulses; P: purified lot and NP: non purified lot). Different letters (a, b) mean significant differences ($z \leq 0.10$) during the storage time and different letters (x, y) mean significant differences among treaments

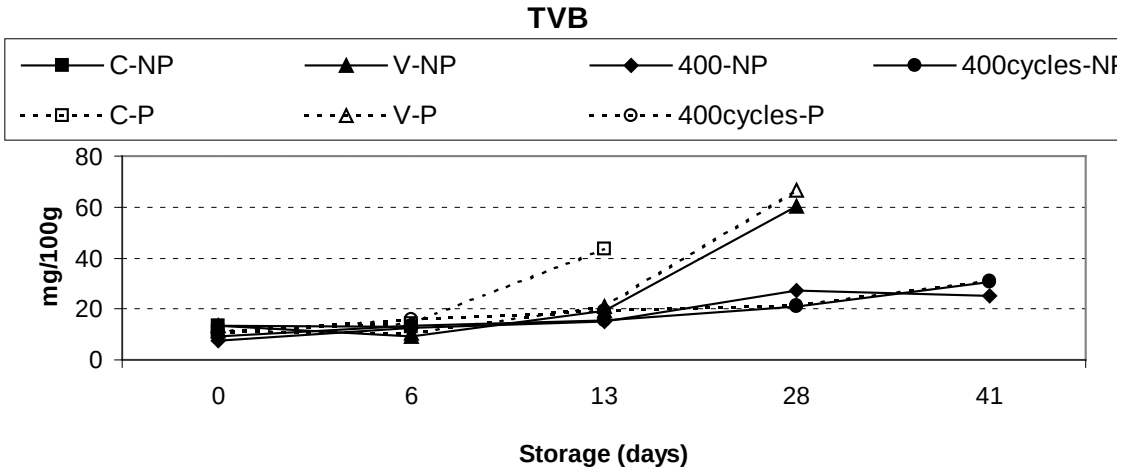
Figure 2.- Total volatile bases (TVB) of lots of oysters (C: control; V: vacuum-packed; 400: vacuum-packed and pressurized at 400 MPa, 10 min, 7 ºC; 400pulses: vacuum packed and pressurized at 400 MPa, 7 ºC in two 5-min pulses; P: purified lot and NP: non purified lot). Different letters (a, b) mean significant differences ($z \leq 0.10$) during the storage time and different letters (x, y) mean significant differences among treaments

Figure 3.- Control oyster (A) and pressurized osyter (B)

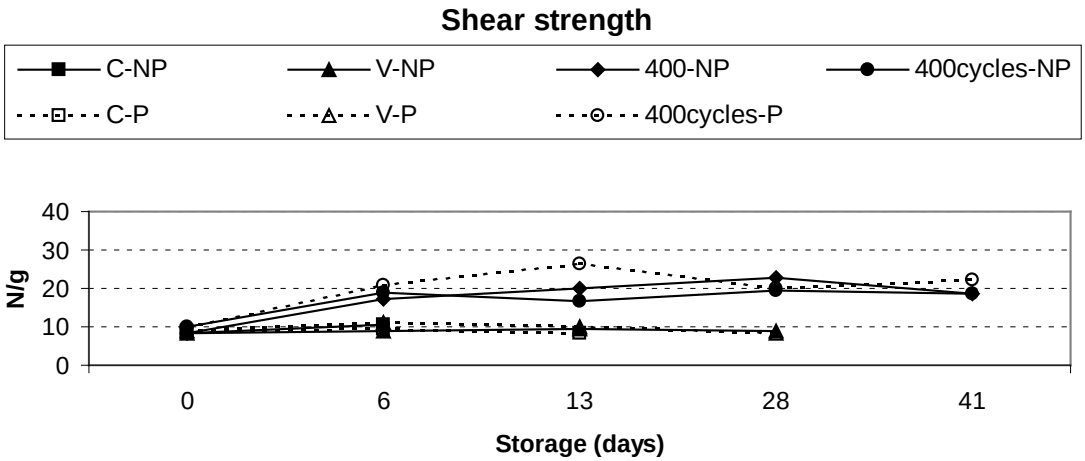
Figure 4.- Shear strength of lots of oysters (C: control; V: vacuum-packed; 400: vacuum-packed and pressurized at 400 MPa, 10 min, 7 ºC; 400pulses: vacuum packed and pressurized at 400 MPa, 7 ºC in two 5-min pulses; P: purified lot and NP: non purified lot). Different letters (a, b) mean significant differences ($z \leq 0.10$) during the storage time and different letters (x, y) mean significant differences among treatments



treatment/time	0 days	6 days	13 days	28 days	41 days
C-NP	a/xy	a/xyz	a/xyz	-	-
V-NP	a/xy	ab/xz	ab/xy	b/x	-
400-NP	a/xy	ab/xy	b/xz	ab/x	ab/x
400pulses-NP	a/xy	ab/y	ab/z	ab/x	b/x
C-P	a/x	ab/z	b/xyz	-	-
V-P	a/x	a/xz	ab/y	b/x	-
400pulses-P	a/y	a/xyz	a/xyz	a/x	b/x



treatment/time	0 days	6 days	13 days	28 days	41 days
C-NP	-/x	-/xyz	-	-	-
V-NP	ab/xy	a/x	ab/xyz	b/xy	-
400-NP	a/z	ab/xyz	ab/y	b/xy	ab/x
400pulses-NP	a/y	ab/xyz	ab/yz	ab/x	b/x
C-P	a/xyz	ab/yz	b/x	-	-
V-P	ab/xyz	a/xy	ab/x	b/y	-
400pulses-P	a/xyz	a/z	a/xy	a/x	b/x



treatment/time	0 days	6 days	13 days	28 days	41 days
C-NP	-/xy	-/xyz	-	-	-
V-NP	ab/xy	a/z	b/x	ab/x	-
400-NP	a/y	ab/xyz	ab/xy	b/y	ab/x
400pulses-NP	a/x	b/x	ab/xy	ab/xy	ab/x
C-P	a/xy	a/yz	a/x	-	-
V-P	a/xy	a/xyz	a/x	a/x	-
400pulses-P	a/x	ab/xy	b/y	ab/xy	ab/x