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High pressure treatment of green mussel *Perna viridis* Linnaeus, 1758: effect on shucking and quality changes in meat during chill storage

J. BINDU, J. GRINSON, C. K. KAMALAKANTH AND T. K. S. GOPAL

Fish Processing Division, Central Institute of Fisheries Technology, Matsyapuri, P. O., Kochi - 682 029, Kerala, India
e-mail: bindujaganath@gmail.com

ABSTRACT

Effect of high pressure on shucking of mussel meat from shell and quality changes in meat in terms of pH, total volatile base nitrogen (TVBN), thiobarbituric acid value (TBA), instrumental colour values (lightness, L*; redness, a* and yellowness, b*), instrumental hardness, total plate count (TPC) and sensory analysis during chill storage were evaluated. Shell-on mussels were pressure treated at 100, 200, 300 and 400 MPa with a holding time of 5 min at 30±3°C. The pressure treated meat was then aseptically removed from the shell and vacuum packed in laminated pouches made of polyester low density polythene and stored at 2±1°C for shelf life evaluation. Manually shucked mussel meat not subjected to pressure treatment served as control. pH and TBA values increased with increasing pressure treatment. Instrumental colour values (L*, a* and b*) and hardness were more for mussel meat subjected to 400 MPa pressure treatment. After high pressure treatment, TVBN and TPC decreased compared to control, however, these values increased during storage. Among the treated samples, 300 MPa shucked mussel meat was sensorially superior to others and had a shelf life of 28 days during chill storage (2±1°C).

Keywords: Chill storage, Green mussel, High pressure processing

Introduction

Mussels are commercially exploited bivalves that form an important item of food in many coastal countries. The nutritive value of mussels is attributed to its high mineral, vitamin as well as polyunsaturated fatty acid (PUFA) content of eicosapentaenoic acid and docosahexaenoic acid (Budge and Parrish, 2003) with less saturated fat when compared to the meat of animal origin (Emre *et al*., 2008). Mussels are transported shell-on in live conditions for shorter distance and also in modified atmospheric packaging (MAP) for keeping them alive (Pastoriza *et al*., 2004). The shelf life of green mussel meat stored in ice were reported as eight days with two days of prime quality (Chinnamma *et al*., 1970), six days (Payap *et al*., 2011) and four days (Erkan, 2005). Modified atmosphere packaging with different gases have been found to extend the shelf life as compared to ice storage. Shelf life of green mussels stored with gas concentration of 80% CO_2, 10% O_2 and 10% N_2 was twelve days (Payap *et al*., 2011) and for Mediterranean mussels packed with 80% CO_2 and 20% N_2, shelf life was more than 14 days at 4°C storage (Goulas *et al.,* 2007). During frozen storage, mussel meat had a shelf life of 40 weeks (Chinnamma, 1974).

Mussel meat is generally consumed raw, blanched or cooked. Removal of meat from the shell in large quantities is done by steaming or dipping in boiling water so that the shells open up easily. During this process there is loss of moisture from the meat, protein degradation and reduction in juiciness of the meat which render the texture rubbery. The original shape and structure of the mussels are also lost during cooking. High pressure (HP) processing is a useful technology for shucking the raw meat from the rigid shell of crustaceans and molluscs without cooking (Errol, 2007), thus making meat removal significantly more efficient without changing the size and shape of the meat retaining nutritional qualities. HP treated oysters were launched in U.S market by Motivatit Sea Foods Inc. as Gold Band Oysters and marketed by Nisbet Oyster Company. Release of off-odour and off-flavour compounds like H_2S, NH_3 and volatile metabolites by the action of microbes during spoilage leads to increase in the pH (Gennari *et al*., 1999) in the mussel meat. High pressure processing reduces the microbial content, does not affect the sensory or nutritional characteristics and hence can be used for raw or minimally processed foods (Indrawati *et al*., 2003). Majority of death due to seafood consumption in the United States of America is due to *Vibrio vulnificus* (Oliver and Kaper, 2001). HP treatment makes the seafood safe for eating in raw condition due to the inactivation of microorganisms (Lopez–Caballero *et al*., 2000a). A reduction in 6 log of *Vibrio* spp. in raw oysters was achieved at pressures ranging from

230-586 MPa (Koo *et al*., 2006). The effect of high pressure on bacterial flora of oysters, mussels, prawn and scallops has been studied in detail by Linton *et al*. (2003). Reduction of total plate count (TPC) was reported in HP treated clams by Narwankar *et al*. (2011) and HP treated oysters were rated superior to untreated ones based on sensory scores. Even though mussels are commercially exploited, very little work has been done on mussel meat preservation by applying high pressure. This paper studied the effect of HP treatment on shucking of mussel meat from the shells and quality changes in the meat during chill storage.

Materials and methods

Raw material

Green mussels (*Perna viridis*) harvested from the unpolluted seawater off the coast of Tikkoti near Calicut, India and brought to the laboratory in live condition. The shells of the mussels having a size of 10±2 cm were washed thoroughly to remove any foreign particle on the shell and the mussels were vacuum packed in 12 µm polyester 300 gauge polythene laminated pouches of size 6x10 inches. The packed mussels were again repacked in another pouch to prevent any breakage or leaking during HP treatment. The sealing was done in a vacuum sealing machine (Sevana Quick Seal Machine, India). The samples were immediately subjected to high pressure treatment.

High pressure treatment

High pressure treatment was carried out in a high pressure processing machine (Stansted Fluid Power, Stansted, Essex, UK). Thirty percent monopropylene glycol in distilled water was used as pressure transmitting liquid in a pressure vessel having 2 l capacity. The packed mussels were subjected to pressure treatment of 100, 200, 300 and 400 MPa with a holding time of 5 min. The temperature involved in the process was 30±3°C. Two K-type thermocouples were used to record the rise in temperature. Mussel meat was manually removed from the shell after processing and packed (100 g each) aseptically in laminated pouches made of polyester polythene. The samples from each pressure treatment were stored immediately in ice at 1:1 ratio in insulated boxes and samples were drawn periodically for microbiological and biochemical analysis. For control, the meat was manually removed by plying open the two shells and teasing out the meat with a stainless steel knife. The melted water was drained and replaced with flake ice every day.

Biochemical and microbial analyses

Biochemical parameters like pH (APHA 1998) and total volatile base nitrogen (TVB-N) were determined by micro diffusion method as per Conway (1950) and thiobarbituric acid value (TBA) as per Tarladgis *et al*. (1960). Texture profile analysis (TPA) was done using universal testing machine (Lloyd instruments LRX plus, UK) equipped with a load cell of 50 N. Instrument color values (Lightness L* redness, a* and yellowness b*) were measured using Hunter lab Colorimeter Model No D/8-S (Miniscan XE Plus) with geometry of diffuse /8° (sphere 8 mm view) and an illuminant of D65/10°. Total plate count was enumerated as per methodology given in Bacteriological Analytical Manual (Maturin and Peeler, 2001). Sensory analysis of the samples was carried out by the method described by Peryam and Pilgrims (1957). Samples were analysed in triplicate and mean values were taken.

Statistical analysis

One-way analysis of variance (ANOVA) was performed to find the effect of pressure on biochemical and microbiological parameters during different storage days ($p<0.05$). Tukey's test was performed to compare the means of different levels of pressure on storage days. All the statistical analyses were performed using SAS 9.2.

Results and discussion

Shucking of mussel meat

The ease in removal of meat from the shell of mussel varied with the level of high pressure treatment. Mussel meat treated at 100 MPa, was found difficult to be shucked and remained attached to the shells by the adductor muscle and also at the edges of the mantle. In mussels treated at 200 MPa, the meat was easily removed, but the adductor muscles remained on the shells. The meat got easily detached from the shell, when subjected to 300 and 400 MPa pressure treatment. He *et al*. (2002) observed that there was 100% meat extraction from the oysters treated at 310 MPa and Hsu *et al*. (2010) found that the optimum pressure treatment for HP treated oyster was from 240 and 300 MPa.

pH during chill storage

The changes in pH content during the storage period in treated and untreated mussel meat is given in Table 1. Statistical analysis revealed that there was significant effect of pressure on pH ($p<0.05$). However no significant difference was observed between control and 100 MPa treated sample and between 300 MPa and 400 MPa treated samples during the initial days of storage. However a slight increase in pH was found in HP treated mussel meat during chill storage. This may be due to protein denaturation and the exposure of the basic ions during the pressure treatment (Anguspanisch and Ledward, 1998; Anguspanish *et al*., 1999). During chill storage, the pH gradually increased in control and in all pressure treated

Table 1. pH of green mussel meat in control and pressure treated samples during chill storage

Days of storage	Control	100 MPa	200 MPa	300 MPa	400 MPa
0	6.24[a]	6.2[a]	6.45[b]	6.65[c]	6.65[c]
4	6.66[a]	6.37[b]	6.88[c]	7.12[d]	7.02[e]
8	7.12[a]	6.98[b]	7.23[c]	7.32[c]	6.77[d]
12	7.68[a]	7.22[b]	6.87[c]	7.22[b]	7.11[d]
15	N.D	7.42[b]	7.27[c]	7.42[b]	7.39[d]
18	N.D	N.D	7.47[a]	7.47[a]	7.58[b]
22	N.D	N.D	7.60[a]	7.60[a]	7.37[b]
26	N.D	N.D	7.70[a]	7.76[a]	7.56[b]
28	N.D	N.D	N.D	7.50[a]	7.40[b]

* Values bearing different superscripts are significantly different ($p< 0.05$)N. D : not determined

samples mainly due to the production and accumulation of volatile compounds which could be attributed to the metabolic activity of bacteria present in the mussel meat. The control as well as 100 MPa treated mussel meat had a pH of 7.68 and 7.42 respectively, on the 12th and 15th day of storage. On 28th day of storage, 300 and 400 MPa treated samples had pH value of 7.5 and 7.4 respectively.

TVB-N

One-way ANOVA revealed that pressure treatment had significant effect on TVB-N during the storage period ($p<0.05$) (Table 2). A reduction in TVB-N was observed in all samples after HP treatment (control : 13.06; 100 MPa : 10.26; 200 MPa : 11.15; 300 MPa : 9.23 and 400 MPa : 10.65). Karim et al. (2011) reported significant reduction in initial TVB-N value in haddock muscle after HP treatment. The TVB-N values increased slowly during the initial period and in the later days of storage there was a rapid increase which is attributed to the increase in the microflora of the samples. This increase in microflora resulted in production of off odours due to the accumulation of undesirable primary metabolites such as trimethylamine, other amines and ammonia (Colby *et al*., 1995). Varlık *et al*. (2000) also found that TVB-N had a tendency to increase with days of storage due to enzymatic degradation as well as microbial activity. An increase in TVB-N values during storage of HP treated gilthead seabream (Sparuss aurata) was reported by Erkan and Uretener (2010). Limit of acceptability of TVB-N value is 30-35 mg N_2 100 g^{-1} (Connell, 1995). In control, the value exceeded the limit and reached 38.24 on 12th day of storage. In 100 MPa treated sample, it was 34.22 on 15th day of storage, whereas in 200 MPa, the limit of acceptability of TVB-N value exceeded on 22nd day of storage and for 300 and 400 MPa, it was on 28th day of storage.

Table 2. Changes in TVB-N of green mussel meat in control and pressure treated samples during chill storage

Days of storage	Control	100 MPa	200 MPa	300 MPa	400 MPa
0	13.06[a]	10.26[b]	11.15[c]	9.23[d]	10.65[e]
4	18.26[a]	19.56[b]	15.37[c]	12.38[d]	11.67[e]
8	20.15[a]	22.69[b]	19.28[c]	18.43[d]	14.32[e]
12	38.24[a]	28.65[b]	24.62[c]	22.37[d]	19.26[e]
15	N.D	34.22[a]	25.68[b]	24.08[c]	22.32[d]
18	N.D	N.D	32.17[a]	28.36[b]	27.84[c]
22	N.D	N.D	35.66[a]	32.33[b]	33.16[c]
26	N.D	N.D	40.4[a]	36.46[b]	36.52[c]
28	N.D	N.D	N.D	37.15[a]	37.08[b]

* Values bearing different superscripts are significantly different ($p< 0.05$)N. D : not determined

TBA value

Changes in TBA content during the storage period in treated and untreated mussel meat is given in Table 3. Pressure treatment was found to have significant effect on TBA values during storage days ($p<0.05$). TBA values were found to increase after HP treatment. Increase in the lipid oxidation may be due to the effect of pressure in releasing the metal ions like Cu and Fe present in the meat of mussels (Cruz-Romero *et al*., 2007). High lipid oxidation was reported with increasing pressure for cod muscle (Angsupanich and Ledward, 1998) and they also opined that pressure damages the cell structure and expose intercellular lipid which gets readily oxidised on storage. Increased lipid oxidation with increase in level of pressure treatment was reported in oysters (*Crassostrea gigas*) by Cruz-Romero *et al*. (2008). Chevalier *et al*. (2001) found that 200 MPa pressure treatment for 30 min increased oxidation in turbot muscle. In control and 100 MPa, TBA values reached almost the value of rejection limit on 12th and 15th days of storage, whereas in 200 MPa treated samples TBA values exceeded the limit on 26th day of storage and for 300 and 400 MPa, it reached the limit on 28th day of storage.

Table 3. TBA of green mussel meat in control and pressure treated samples during chill storage

Days of storage	Control	100 MPa	200 MPa	300 MPa	400 MPa
0	0.19[a]	0.32[b]	0.25[c]	0.36[d]	0.25[c]
4	0.88[a]	0.68[b]	0.35[c]	0.46[d]	0.73[b]
8	1.24[a]	1.05[b]	0.93[c]	0.86[d]	0.82[d]
12	1.9[a]	1.44[b]	1.25[c]	1.12[d]	1.36[e]
15	N.D	1.92[a]	1.54[b]	1.77[c]	1.44[d]
18	N.D	N.D	1.62[a]	1.84[b]	1.67[c]
22	N.D	N.D	1.69[a]	1.88[b]	1.74[c]
26	N.D	N.D	2.12[a]	1.97[b]	1.86[c]
28	N.D	N.D	N.D	2.3[a]	2.05[b]

* Values bearing different superscripts are significantly different ($p< 0.05$)N. D : not determined

Instrumental colour values (L, a* and b*)*

Pressure treatment had significant effect on colour values (during storage ($p<0.05$) (Table 4). L* value of the samples increased proportionally with pressure level and during storage it showed an increasing trend. Changes in L* value could be attributed to the denaturation of

Table 4. Changes in L* value of green mussel meat in control and pressure treated samples during chill storage

Days of storage	Control	100 MPa	200 MPa	300 MPa	400 MPa
0	64.1[a]	67.19[b]	68.02[c]	69.64[d]	70.78[e]
4	64.55[a]	68.34[b]	70.22[c]	71.65[d]	72.45[e]
8	66.37[a]	69.44[b]	70.81[c]	71.96[d]	73.56[e]
12	69.63[a]	68.63[b]	72.43[c]	73.24[d]	74.66[e]
15	N.D	70.11[a]	73.98[b]	74.12[c]	76.44[d]
18	N.D	N.D	73.59[a]	74.18[b]	76.87[c]
22	N.D	N.D	75.36[a]	75.69[b]	77.64[c]
26	N.D	N.D	76.88[a]	76.98[b]	78.46[c]
28	N.D	N.D	N.D	76.86[a]	78.64[b]

* Values bearing different superscripts are significantly different ($p< 0.05$)N. D : not determined

myofibrillar and sarcoplasmic proteins as reported by Angsupanich and Ledward (1998) and Chevalier *et al*. (2001). Johnson *et al*. (2003) reported a cooked appearance in pressure treated oyster and Hoover *et al*. (1989) found cooked appearance at higher pressures of 300 MPa. Similar trend was reported in HP treated oyster by Cruz-Romero *et al*. (2004). There was significant effect of pressure on a* value ($p<0.05$). The samples had an initial redness of 1.49 for control; 2.18 for 100 MPa; 3.17 for 200 MPa; 3.01 for 300 MPa and 2.89 for 400 MPa. During storage, redness increased in all samples (Table 5). A final value of 3.39 and 2.94 was obtained for 300 and 400 MPa on the final day of rejection *i.e.,* 26 and 28 days of storage. b* value was 12.33, 13.45, 13.48, 14.98 and 16.8 for control and the high pressure treated samples which indicated that yellowness increased with pressure and during chill storage. At the end of the storage period, mussel meat subjected to 300 and 400 MPa had values of 21.35 and 22.01 respectively (Table 6). The L*, a* and b* values obtained for mussel meat during storage are in agreement with the findings in high pressure processed squid meat (Nagashima *et al*., 1993) and oyster meat (Cruz-Romero *et al*., 2004).

Instrumental hardness

Pressure had significant effect on hardness which increased proportionately with pressure levels and also

Table 5. Changes in a* value of green mussel meat in control and pressure treated samples during chill storage

Days of storage	Control	100 MPa	200 MPa	300 MPa	400 MPa
0	1.49[a]	2.18[b]	3.17[c]	3.01[d]	2.89[e]
4	1.59[a]	2.37[b]	3.14[c]	3.24[d]	2.69[e]
8	2.16[a]	2.41[b]	2.97[c]	3.16[d]	2.98[c]
12	2.88[a]	2.63[b]	3.13[c]	3.15[c]	3.18[d]
15	N.D	2.31[a]	3.44[b]	3.26[c]	3.18[d]
18	N.D	N.D	3.65[a]	3.44[b]	3.27[c]
22	N.D	N.D	3.81[a]	3.31[b]	3.36[c]
26	N.D	N.D	3.88[a]	3.41[b]	3.43[b]
28	N.D	N.D	N.D	3.39[a]	2.94[b]

* Values bearing different superscripts are significantly different ($p< 0.05$)N. D : not determined

Table 6. Changes in b* value of green mussel meat in control and pressure treated samples during chill storage

Days of storage	Control	100 MPa	200 MPa	300 MPa	400 MPa
0	12.33[a]	13.45[b]	13.48[c]	14.98[d]	16.8[e]
4	12.65[a]	14.58[b]	15.36[c]	15.46[d]	17.25[e]
8	13.56[a]	15.64[b]	16.45[c]	16.58[d]	18.26[e]
12	14.15[a]	15.68[b]	16.84[c]	18.47[d]	20.27[e]
15	N.D	16.02[a]	18.47[b]	19.54[c]	21.54[d]
18	N.D	N.D	18.36[a]	20.44[b]	21.35[c]
22	N.D	N.D	19.75[a]	21.66[b]	21.55[c]
26	N.D	N.D	20.17[a]	20.99[d]	21.94[c]
28	N.D	N.D	N.D	21.35[a]	22.01[b]

* Values bearing different superscripts are significantly different ($p< 0.05$)N. D : not determined

during storage period ($p<0.05$). For control it was 17.49 and for pressure levels of 100, 200, 300 and 400 MPa it was 25.28, 26.68, 28.09 and 38.47 respectively. An increasing trend in hardness was observed with increase in the storage period (Table 7). Several authors have conducted experiments to understand the textural changes by high pressure processing in seafood such as fish muscle (Angsupanich and Ledward, 1998). Increase in the shear strength has been observed for oysters processed at 400 MPa for 5 and 10 min (Lopez- Caballero *et al*., 2000b). An increase in the hardness was observed in prawns processed at 200 and 500 MPa for 10 min (Lopez- Caballero *et al*., 2000b). A final value of 19.08 was observed for control samples on 12th day of storage. On the 15th day of storage mussel meat subjected to 100 MPa had a value of 25.54 N and 200, 300 and 400 MPa had final values of 30.54, 31.65 and 43.65 N respectively. This increase in the texture may be due to the protein-protein interactions which results in tissue elasticity and hardness (Oshsima *et al*., 2003). In pressure treatment above 200 MPa, there was a compaction of fibres and the formation of a protein gel network wherein the muscles are unlikely to be affected by the proteases and the muscles remain more compact than the control samples (Cheftel and Culoli, 1997) whereas in control, the proteases act on the myofibrillar proteins and collagen. Similar results have been observed in cod muscles (Anguspanisch *et al*., 1999).

Table 7. Changes in hardness of green mussel meat in control and pressure treated samples during chill storage

Days of storage	Control	100 MPa	200 MPa	300 MPa	400 MPa
0	17.49[a]	25.28[b]	26.68[c]	28.09[d]	38.47[e]
4	18.45[a]	26.35[b]	26.45[c]	29.5[d]	38.69[e]
8	19.18[a]	25.64[b]	27.65[c]	28.65[d]	40.51[e]
12	19.08[a]	26.48[b]	28.43[c]	30.56[d]	41.37[e]
15	N.D	25.54[a]	27.35[b]	32.65[c]	39.54[d]
18	N.D	N.D	29.25[a]	31.65[b]	42.65[c]
22	N.D	N.D	29.86[a]	32.84[b]	42.65[c]
26	N.D	N.D	30.54[a]	31.65[b]	43.5[c]
28	N.D	N.D	N.D	31.65[a]	43.65[b]

* Values bearing different superscripts are significantly different ($p< 0.05$)N. D : not determined

TPC values

There was significant effect of pressure on TPC during storage period ($p<0.05$) (Table 8). Reduction of microbial count in HP treated oyster was reported by Lopez-Caballero *et al*. (2000). Control had an initial TPC of 2.01 cfu g^{-1}, whereas 400 MPa had least value of 0.11. Reduction of microbial load could be due to breakdown of the cell membrane and altering its permeability which in turn leads to lack or loss of nutrients and ultimately death of microorganisms by high pressure. Control sample reached the limit of acceptability of 5.11 on 12th day and sample subjected to 100 MPa on 16th day of storage whereas 200 and 300 MPa were rejected on 24th and 28th days of storage. At 400 MPa, the count was within the limit on 28th day of storage.

Table 8. Changes in TPC of green mussel meat in control and pressure treated samples during chill storage

Days of storage	Control	100 MPa	200 MPa	300 MPa	400 MPa
0	2.01^{a}	1.34^{b}	0.64^{c}	0.22^{d}	0.11^{e}
4	3.62^{a}	2.77^{b}	1.21^{c}	0.95^{d}	0.53^{e}
8	4.45^{a}	3.34^{b}	2.18^{c}	1.76^{d}	1.31^{e}
12	5.11^{a}	4.45^{b}	2.88^{c}	2.66^{d}	2.12^{e}
15	6.71^{a}	4.95^{b}	3.78^{c}	3.42^{d}	2.89^{e}
18	N.D	5.72^{a}	3.99^{b}	3.87^{c}	3.10^{d}
22	N.D	N.D	4.42^{a}	4.12^{b}	3.21^{c}
26	N.D	N.D	5.70^{a}	4.66^{b}	3.86^{c}
28	N.D	N.D	N.D	5.12^{a}	4.3^{b}

* Values bearing different superscripts are significantly different ($p< 0.05$)N. D : not determined

Changes in sensory score during storage

Sensory characteristics were evaluated using a 9-point hedonic scale. The overall acceptability scores were obtained by pooling the scores for each attribute *viz*., colour, appearance, texture, taste and odour. There were significant changes in sensory scores in all samples during storage ($p<0.05$). Sensory score was observed to decrease with increase in the storage period in all samples. Sensory evaluation is used in estimating seafood quality and these results are correlated with microbiological and chemical parameters (Karungi *et al*., 2004). At higher pressures, the samples were found to be harder and had a cooked appearance. Control and 100 MPa samples were sensorally rejected on 12th and 16th day of storage respectively whereas samples subjected to 200, 300 and 400 MPa were rejected on 28th day of storage. Among the treated samples, 300 MPa sample was found to be superior while considering the overall quality parameters.

High pressure treatment had a significant effect on the shucking of meat from the shell and on extension of shelf life of green mussel meat in chill storage. In this study it was seen that at higher pressures of 300 and 400 MPa, denaturation of the adductor muscels were greater and the detachment of meat from the shell was total. Significant effect ($p<0.05$) of high pressure treatment on quality parameters were observed in all the samples. Among the treated samples, it was observed that 300 MPa pressure was optimum among the different pressure levels studied for wholesome removal of meat from mussel shell. Considering the overall acceptability, storage of mussel meat at this pressure had a shelf life of 28 days during chill storage (2±1°C).

Table 9. Sensory score of green mussel meat in control and pressure treated samples during chill storage

Days of storage	Control	100 MPa	200 MPa	300 MPa	400 MPa
0	8.5^{a}	8^{b}	8.1^{c}	8.4^{d}	8.2^{e}
4	6.1^{a}	7.6^{b}	7.2^{c}	7.6^{b}	7.2^{c}
8	5.1^{a}	6.4^{b}	6.9^{c}	7.1^{d}	6.8^{e}
12	4.0^{a}	5.9^{b}	6.2^{c}	6.8^{d}	6.2^{c}
15	N.D	5.4^{b}	5.5^{b}	6.5^{c}	5.7^{d}
18	N.D	3.99^{a}	5.0^{a}	6.3^{c}	4.9^{d}
22	N.D	N.D	4.8^{a}	5.7^{b}	4.7^{c}
26	N.D	N.D	4.1^{a}	5.1^{b}	4.1^{a}
28	N.D	N.D	3.91^{a}	4.0^{b}	3.95^{a}

* Values bearing different superscripts are significantly different ($p< 0.05$)N. D : not determined

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