Frozen storage of high-pressure and heat-induced gels of blue whiting (*Micromesistius poutassou*) muscle: rheological, chemical and ultrastructure studies

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ABSTRACT

This paper examines the influence of frozen storage over 34 weeks on rheological properties and chemical and microstructural characteristics of gels made from muscle of blue whiting (*Micromesistius poutassou*) subjected to different gelling treatments entailing three combinations of pressure, temperature and time: 200 MPa, <10 °C, 10 min (lot L), 375 MPa, 38 °C, 20 min (lot H) and atmospheric pressure 37 °C, 30 min and 90 °C, 50 min (lot T).

Freezing at -40°C causes certain changes in rheological parameters. In heat-induced gels, breaking force, elasticity and cohesiveness increase. Of the high-pressure-induced gels, breaking force increased and cohesiveness decreased in the lower-pressure gel, while the only change in the higher-pressure gel was some loss of elasticity. There was a general fall in water holding capacity (WHC) values. Lightness remained stable. Protein solubility: there was an increase in covalent bonds in lot T. As was the ultrastructure of the all gels matrixes were more disorganized as a result of freezing.

In the course of frozen storage, the greatest changes in rheological parameters generally took place during the first 8 weeks, and in all the gels there was a decrease in water holding capacity. In the heat-induced gel the changes were less marked over the storage period than in the high-pressure-induced gels, but the heat-induced gel was more brittle and did not maintain maximum folding test scores. Covalent bonds increased and hydrophobic interactions decreased in all lots. The general appearance of the structure in gel T remained more homogeneous, while the pressurized gels exhibited more and larger cavities

INTRODUCTION

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Gelling is the basis for manufacture of a large number of analogue products from fish mince, usually induced by heat treatment. According to some authors, high-pressure technology can be applied to develop gels with new textures which may serve as the basis for a whole new range of products (1, 2, 3).

A large proportion of gel-based seafood products are marketed in the frozen state, and it is therefore useful to examine the changes that occur during frozen storage of gels. There are quite a number of references to work on the changes undergone by proteins from fish mince or fillet, blue whiting being one of the species that undergoes most alteration (4). However, less work has been published on freezing and frozen storage of fish gels (5, 6, 7, 8). Since gelling is based on protein denaturation and subsequent aggregation and denaturation processes differ according to the pressure-time-temperature conditions applied, it may be that these conditions affect stability during gel storage.

Pérez-Mateos *et al.* (9) studied gelling of blue whiting mince under different pressure-time-temperature combinations and found that the best conditions for optimum gel strength were 200 MPa at 3 °C for 10 min or 375 MPa at 38 °C for 20 min. These conditions gave higher of work of penetration values than heat treatment.

The object of this experiment was to examine the rheological, chemical and ultrastructural changes caused by freezing at -40 °C and frozen storage at -18 °C in high-pressure-induced and heat-induced blue whiting mince gels.

MATERIALS AND METHODS

Blue whiting (*Micromesistius poutassou* Risso) used in this study was caught off the Cantabrian coast and kept at 4 °C with ice for about 24 hours. Average size was 23.4 ± 1.2 cm and average weight 77.8 ± 12.3 g. Fish were headed, gutted and washed. Skin and bones were removed with a deboning machine (Baader 694, Lübeck, Germany). Muscle was minced and washed in a solution of 0.2% NaCl at 0-3 °C, proportion 3:1 (solution: minced muscle), first with constant stirring for 10 min then without stirring for another 10 min. After draining, excess water was removed using a screw press (Baader 523). Sorbitol (4%) and tripolyphosphate (0.2%) were added as cryoprotectants. The mince was immediately vacuum-packed in bags (Cryovac BB-1, Grace, Barcelona, Spain) and frozen in a plate-freezer (Saabroe SMC, Denmark) to a temperature of -30 °C. The bags were stored at -80 °C in a freezer cabinet (Revco ULT, Giralt, Revco Scientific Inc., Asheville, N.C., USA) in order to minimize alteration during frozen storage up to gel preparation.

Proximate analysis was performed according to AOAC procedures (10) and crude fat by Bligh and Dyer (11). For washed mince the composition was: crude protein $12.3\% \pm 0.3$, moisture $82.9\% \pm 1.2$, crude fat $0.50\% \pm 0.04$ and ash $0.62\% \pm 0.01$. The analyses do not show the cryoprotectants added.

Gel preparation. Washed blue whiting mince was semithawed at about -4 °C and placed in a refrigerated vacuum homogenizer (Stephan UM5, Stephan u. Söhne GmbH & Co., Hameln, Germany). The muscle was ground for 1 minute at 3,000 rpm. 1% sodium chloride (Panreac, Montplet & Esteban S.A., Barcelona, Spain) was added and the mixture homogenized for 3 min; a proportion of 1% NaCl has been reported by Pérez-Mateos *et al.* (3) to give the highest values of

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work of penetration in blue whiting gel. Then 5% starch (Clearam CH 20, Laisa, Barcelona, Spain) was added with crushed ice to give the required final gel moisture (78%) and homogenizing continued for 6 min at 1,500 rpm in vacuum conditions. The resulting batters were stuffed into cases (Krehalon Soplaril, Barcelona, Spain) of 40 μ m thickness and 3.5 cm diameter. The filled casings were subjected to either of two pressure-time-temperature combinations: 200 MPa for 10 min at <10 °C (lot L) and 375 MPa for 20 min at 38 °C (lot H) in a high-pressure pilot unit (ACB 665, Gec Alsthom, Nantes, France). For comparison with the traditional gelling procedure, heat-induced gels were made at atmospheric pressure by waterbath immersion: 37 °C for 30 min then 90 °C for 50 min (lot T). All the casings were immediately cooled with running water and kept at 4 °C overnight (chilled control), after freezing at -40 °C (quick-frozen control) in a tunnel freezer (AGA Frigoscandia, Helsingborg, Sweden) and kept in frozen storage at -18 °C for 2, 4, 8, 12, 17, 21 and 34 weeks. All the samples were completely thawed before analysis and were kept at 4 °C one night before the control day.

Folding test (12). A slice 3.5 cm in diameter and 3 mm high is folded over twice. If it does not break, a score of 5 is awarded. If it breaks on the second fold, it scores 4. If a crack shows only on part of the folded edge, it scores 3. If the crack runs the length of the folded edge, it scores 2. If the slice breaks apart completely, it scores 1.

Rheological analysis (3, 9). For texture analyses, samples were removed from their casings, cut (3.5 cm diameter, 3 cm height) and tempered at 20 °C. **Puncture test**: Gels were penetrated to breaking-point using a texturometer (Instron 4501, Instron Engineering Corp., Canton M.A., USA) with a round-ended stainless steel plunger ($\varnothing$ = 5 mm). Cross-head speed was 10 mm/min and a 100 N load-cell was used. Breaking deformation [mm] and breaking force [N] were carried out at least in quadruplicate. **Texture Profile Analysis (TPA)**. Samples were placed on the flat plate of

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the texturometer. Compression was applied by a cylindrical plunger ($\varnothing = 58$ mm) adapted to a 5 kN load cell at a deformation rate of 50 mm/min. On the basis of previous trials to establish a compression limit that would ensure no cracking and recoverability of most samples, it was decided to compress samples to 50% of height. In the test, each sample was compressed twice running. The parameters determined were hardness [N], elasticity [%], cohesiveness [%] and adhesiveness [g.cm]. All determinations were performed at least in quadruplicate.

Water holding capacity. The method of Montero *et al.* (13) was used. About 1.5 g of sample was placed in a centrifuge tube, along with two dried pipet filters (Gilson Pipetman, Villiers le Bel, France). It was expressed as water retained per 100 g of water present in the sample prior to centrifuging (Sorvall RT6000B, DuPont Co., Wilmington, DE, USA) at 4000g for 15 min at ambient temperature. All determinations were carried out in triplicate.

Color of mince and gels was determined on a colorimeter (HunterLab MiniScan MS/S- 4000S, Hunter Associates Laboratory Inc., Reston, VA, USA) using the CIELab scale (D65/10 °) where L^* is the parameter that measures lightness. The result was the average of six measurements taken at ambient temperature at different points on the sample.

Protein solubility of gel. In order to measure the types of bonds present in the three gels over the period of frozen storage, the gels were solubilized in four solutions at pH = 7.0 (3, 14): 0.6 M sodium chloride (S1), 1.5 M urea + 0.6 M sodium chloride (S2), 8 M urea + 0.6 M sodium chloride (S3) and 0.5 M 2- β -mercaptoethanol + 8 M urea + 0.6 M sodium chloride (S4). Two grams of chopped gel was homogenized with 10 mL of S1 (Ultraturrax TP18/10, Janke & Kunkel, Ika-Werk, Staufen, Germany) for 2 min at maximum speed. The resulting homogenate was stirred at 4 °C for one hour, then centrifuged for 20 min at 20,000g in a centrifuge (Beckman J2-MC, Beckman Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung A-Food Research and Technology 1997;205(5):335-342

Instruments Inc., Palo Alto, CA, USA). The pellet obtained was homogenized in solution S2 by the same process, then again in S3 (in this case, twice) and finally in S4. Protein concentration was determined in triplicate: in the supernatants, based on the method of Lowry (15) using a commercial preparation (DC Protein Assay Reagent S n° 500-0116, Bio-Rad Laboratories, Hercules, CA, USA), and in the insoluble protein by the method of Kjeldhal. Where necessary the solutions were dialysed to prevent interference in protein determination. Colorimetry was performed in a spectrophotometer at 750 nm (UV-1203, Shimadzu, Hyoto, Japan). Results are the average of two determinations and are expressed as percentage of solubilized protein with respect to total protein.

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Scanning electron microscopy: Cubes of 2 to 3 mm were cut from inside the gels for microscopic examination. Samples were fixed in 2% glutaraldehyde in phosphate buffer (pH = 7.3) and dehydrated in increasing series of acetone (from 40 to 100%). They were then critical-point dried with CO₂ as transition fluid in a dryer (Balzer CPD030, Liechtenstein) and mounted on copper sample holders, followed by sputter-coating with gold in a metallizer (Balzer SCD004). Samples were kept in a dryer until examination by scanning microscope (Jeol, JSM 6400, Japan) at 20 kV. Micrographs were taken of each gel at x100, x500 and x3,000 magnifications.

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Statistical analysis. Two-way analysis of variance was carried out using a statistical computer programme (Statgraphics, STSC Inc, Rockville, MD, USA). The difference of means between pairs was resolved by means of confidence intervals using a LSD range test. Level of significance was set for $p \leq 0.05$.

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RESULTS AND DISCUSSION

Effect of freezing on the gel

5 Folding test scores did not alter after freezing in any of the lots ($p \leq 0.05$), all of which scored the maximum.

After freezing there was little change in the rheological parameters (Fig. 1). Breaking deformation increased slightly in the heat-induced gels ($p \leq 0.05$). There was no change in the deformation of
10 the high-pressure-induced gels; these produced high values, especially gel L, which also exhibited high breaking force. The heat-induced gels lost hardness and adhesiveness, but these values were still much higher than in the high-pressure-induced gels ($p \leq 0.05$). There were some changes ($p \leq 0.05$) in elasticity values, the most pronounced being the fall in values in gel H; however, values were very high in all cases. Although there were changes ($p \leq 0.05$) in
15 cohesiveness of gels T and L, these were not very great. Gel L was the most cohesive of all the lots (3).

Other authors, such as Martí de Castro *et al.* (7) in heat-induced gels made from sardine, have found no significant changes in rheological parameters after freezing at -40°C (except a decrease
20 in breaking force and elasticity values). However, other studies have reported an increase in work of penetration (the product of breaking deformation and breaking force) as a result of freezing in crab analogues (6).

Although there were generally significant changes ($p \leq 0.05$) in the percentages of soluble protein
25 in the different solutions and the insoluble fraction (Table 1), these were minor except for an
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increase in covalent bonding in gel L following freezing. Montero *et al.* (8) have reported that non-covalent bonds were not affected by freezing at -40 °C.

Freezing caused a small decrease ($p \leq 0.05$) in water holding capacity values (Fig. 2) in T and H gels. WHC was higher in the heat-induced gel than in the pressure-induced gels (3). This may have been due to gelatinization of the starch in heat-induced gels, something which did not occur in high-pressure-induced gels in the experimental conditions. WHC was even lower in gel H, probably because this was a weaker gel to judge by the rheological and chemical data. Other authors (7) did not however find that freezing induced changes in WHC values of gels made from sardine mince.

The freezing of the gels produced no significant changes in the values of lightness (Fig. 2). The highest values were found in the heat-induced gel (T) and in the moderate-temperature high-pressure-induced gel (H), probably due to the heat as has been observed by some other authors (3, 9).

Effects of frozen storage of the gels

The change in folding test scores over time was in the lot of heat-induced gels, which fell to 4 by the 8th week of storage probably the brittleness of the gel may be due to large cavities. The high-pressure-induced lots, on the other hand, maintained the maximum score throughout. This could mean that gel-forming capacity is retained better in high-pressure-induced gels but declines in heat-induced gels.

There were only very slight changes in breaking deformation and breaking force values (Table 2)

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of gels T and H gels; the change was somewhat greater in gel L. The values were lower in the heat-induced gel than in the other two lots, particularly lot L.

Some authors have reported increased values of the puncture test in the first 30 days of frozen storage (5, 7) as a result of reorganisation in the gel matrix.

In the TPA test (Table 3) the characteristics analyzed were generally quite stable with only some small changes ($p \leq 0.05$). Both hardness and adhesiveness were much greater in the heat-induced gel than in the high-pressure gels. Cohesiveness was much greater in gel L (3).

Other authors like Martí de Castro *et al.* (7) have found the TPA rheological characteristics to be quite constant over the storage period, with slight fluctuations at the outset and close of storage.

A small, gradual decrease was detected in the evolution of water holding capacity (Table 4) in high-pressure gels. WHC in the heat-induced gel was very stable for the first 5 months, declining thereafter.

Loss of water holding capacity in gels has also been reported by other authors (7). According to Sikorsky and Kotakowska (16) the decline of WHC is due to dehydration of the protein networks which lose their water binding ability. This dehydration of the network may be prevented in part by the binding of water to gelatinized starch inside the network cavities. This would account for the difference in WHC values of heat-induced and high-pressure-induced gels; in the former, heating fully gelatinizes the starch, whereas this does not occur, or at least does so only partially, in high-pressure-induced gels (17).

Lightness values (Table 4) remained stable in all cases, with only slight variation ($p \leq 0.05$) by the Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung A-Food Research and Technology 1997;205(5):335-342

end of storage.

Table 5 depicts protein solubility in different solutions as a measurement of the types of bond present in the three gels over the frozen storage period. Slight but significant changes ($p \leq 0.05$) were found in protein fractions S1 and S2. The most pronounced change in all lots was an increase in insoluble fraction in relation with the fraction S3 which means an increase in covalent bonding to the detriment of hydrophobic interactions. In lot L, the reduction in fraction S3 (comprised hydrophobic interactions) over the first few weeks was accompanied by a reduction in fraction S2 (comprised hydrogen bonds); there was no appreciable increase in fraction S4 (comprised disulfide bonds), probably because this lot was made in refrigerated conditions. The reduction in fraction S3 (comprised hydrophobic interactions) in lots H and T was smaller over the first month. There, disulfide and other covalent bonds increased, probably because of moderate heating. This increase in disulfide bonds may be due to the heating temperatures applied in the gelling process in lots H and T.

Montero *et al.* (8) reported that the decline in hydrophobic interactions accompanied by increased in weak bonds occurring during frozen storage in heat-induced gels made from sardine with added egg white could be due to aggregation and destabilization of the gel network.

The changes in the rheological parameters were too small to account for the observed changes in solubility. This is probably because of reorganization of the common bonds, which were relatively strong (hydrophobic interactions and covalent bonds).

SEM (Fig. 3) revealed a major difference between heat-induced (Fig.3 A) and high-pressure-induced (Fig. 3 B,C) gels, the former being vacuolar whereas the latter exhibited a

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relatively compact matrix. After freezing, there was visibly more disgregation or disorganization than in the corresponding chilled samples. In gel T (Fig.3 D) there was augmentation of the globular zones, could account an increase in breaking deformation, and the cavities became less homogeneous in shape and size (lower WHC values). Gel L (Fig. 3 E) exhibited loss of compactness as the globular and fibrous structures combined (could increase values of work of penetration). Gel H (Fig. 3 F) had a more porous appearance (lower WHC values) but no great uniformity.

At the end of the frozen storage period (Fig. 4), the general appearance of the structure in the heat-induced gel (Fig. 4 A) was more homogeneous. The matrix in the high-pressure-induced gels was less uniform, the compact zones being interrupted by large, irregular cavities (Fig. 4 B,C). At greater magnification (x3.000) there were considerable differences between lots. The heat-induced gel (Fig. 4 D) presented a highly globular appearance while the high-pressure-induced gels (Fig. 4 E,F) were more organized without actually forming a true three-dimensional network. The matrix of gel L was very fibrous, which could account for its higher cohesiveness and breaking force values. The gel H matrix, on the other hand, was more amorphous, as reflected in lower rheological parameter values.

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Figure 1.- Puncture test and compression parameters of lots T, L and H (T: 37 °C, 30 min/ 90 °C, 50 min; L: 200 MPa, <10 °C, 10 min; H: 375 MPa, 38 °C, 20 min). Different letters (a,b,c,d) indicate significant differences ($p \leq 0.05$) amongst gels

Figure 2.- Water holding capacity and lightness of lots T, L and H (T: 37 °C, 30 min/ 90 °C, 50 min; L: 200 MPa, <10 °C, 10 min; H: 375 MPa, 38 °C, 20 min). Different letters (a,b,c,d) indicate significant differences ($p \leq 0.05$) amongst gels

Figure 3.- Scanning electron microscope image of gels T, L and H (T: 37 °C, 30 min/ 90 °C, 50 min; L: 200 MPa <10 °C 10 min; H: 375 MPa, 38 °C, 20 min) at x500 magnifications before freezing (A, B, C respectively) and after freezing (D, E, F respectively)

Figure 4.- Scanning electron microscope image of gels T, L and H (T: 37 °C, 30 min/ 90 °C, 50 min; L: 200 MPa, <10 °C, 10 min; H: 375 MPa, 38 °C, 20 min) at x100 (A, B, C respectively) and at x3,000 magnifications (D, E, F respectively) at 34 weeks of frozen storage

Table 1.- Protein fractions of lots T, L and H (T: 37 °C, 30 min/ 90 °C, 50 min; L: 200 MPa, <10 °C, 10 min; H: 375 MPa, 38 °C, 20 min) in different solutions: 0.6 M sodium chloride (S1), 1.5 M urea + 0.6 M sodium chloride (S2), 8 M urea + 0.6 M sodium chloride (S3) and 0.5 M 2-β-mercaptoethanol + 8 M urea + 0.6 M sodium chloride (S4) at pH = 7.0 and insoluble fraction (In). Different letters (a, b, c) indicate significant differences ($p \leq 0.05$) between controls for each lot, and different letters (x, y, z) indicate significant differences ($p \leq 0.05$) among lots for each control

Table 2.- Puncture test parameters of lots T, L and H (T: 37 °C, 30 min/ 90 °C, 50 min; L: 200 MPa, <10 °C, 10 min; H: 375 MPa, 38 °C, 20 min). Different letters (a, b, c) indicate significant differences ($p \leq 0.05$) between controls for each lot, and different letters (x, y, z) indicate significant differences ($p \leq 0.05$) among lots for each control

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Table 4.- Water holding capacity and lightness of lots T, L and H (T: 37 °C, 30 min/ 90 °C, 50 min; L: 200 MPa, <10 °C, 10 min; H: 375 MPa, 38 °C, 20 min). Different letters (a, b, c) indicate significant differences ($p \leq 0.05$) between controls for each lot, and different letters (x, y, z) indicate significant differences ($p \leq 0.05$) among lots for each control

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mercaptoethanol + 8 M urea + 0.6 M sodium chloride (S4) at pH = 7.0 and insoluble fraction (In).

Different letters (a, b, c) indicate significant differences ($p \leq 0.05$) between controls for each lot,

and different letters (x, y, z) indicate significant differences ($p \leq 0.05$) among lots for each control

		ionic bonds	hydrogen bonds	hydrophobic interactions	disulfide bonds	covalents bonds
4°C	T	11.6a	8.2a	45.5a	11.1a	23.7a
	L	17.0b	13.6b	43.3ab	7.3b	18.8b
	H	14.1c	7.8a	58.4c	5.5b	14.2c
-40°C	T	12.1a	6.4c	47.6a	13.2a	20.8ab
	L	16.8b	13.8b	39.1b	5.1b	25.2d
	H	16.0b	9.1d	56.4c	6.5b	12.0c

	Storage time (weeks)							
	0	2	4	8	12	17	21	34
breaking deformation								
T	6.3ab/x	6.3ab/x	6.8a/x	5.7b/x	6.4ab/x	6.2ab/x	6.1ab/x	5.3b/x
L	22.1a/y	20.4b/y	21.5a/y	19.2c/y	19.9bc/y	18.5d/x	16.9e/x	16.9e/x
H	11.9ab/z	12.4b/z	14.4c/z	11.1b/z	9.3cd/z	10.9c/z	9.8d/z	10a/z
breaking force								
T	1.9a/x	1.8a/x	2.1a/x	2.0a/x	2.0a/x	2.2a/x	2.6a/x	2.0a/x
L	8.3a/y	8.9a/y	8.9a/y	7.7b/y	9.1a/y	8.7a/y	7.0c/y	7.8b/y
H	2.4a/x	1.9a/x	2.9b/z	2a/x	1.7a/x	2.1a/x	2.5ab/x	3.2b/z

	Storage time (weeks)							
	0	2	4	8	12	17	21	34
hardness								
T	80.4a/x	83.0a/x	77.2a/x	81.8a/x	88.9a/x	86.8a/x	100.6b/x	83.8a/x
L	39.8a/y	50.5b/y	44.4ab/y	45.5ab/y	57.4b/y	57.4b/y	68c/y	62.8c/y
H	39.7a/y	33.0a/z	33.0a/z	38.5a/y	45.4a/z	41.1a/z	58.3b/z	70.2c/z
elasticity								
T	95.5a/x	83.3b/x	93.5a/x	90.4a/x	92.9a/x	92.0ac/x	87.3c/x	89.0c/x
L	97.1a/x	95.3a/y	92.4a/x	94.3a/x	95.8a/x	94.1a/x	93.7a/x	89.1b/x
H	76.1a/y	97.8b/y	91.9b/x	88.8c/z	93.1b/x	87.7bc/x	92.8bc/x	92.6b/y
adhesiveness (.1000)								
T	2.8a/x	3.0ab/x	2.7a/x	3.0ab/x	3.2b/x	3.0ab/x	3.6c/x	2.9ab/x
L	0.9a/y	1.1ab/y	0.9a/y	0.9a/y	1.1ab/y	1.1ab/y	1.3b/y	1.3b/y
H	1.2a/z	1.1a/y	1.0a/y	1.2a/y	1.4ab/y	1.2a/y	1.7b/z	2.0b/z
cohesiveness								
T	61ab/x	60ab/x	61b/x	60ab/x	59a/x	61ab/x	59a/x	60ab/x
L	76a/y	78bc/y	81c/y	80bc/y	80bc/y	79b/y	77ab/y	77ab/y
H	63a/z	59b/x	62a/x	61ab/x	59b/x	62a/x	62ab/x	64a/z

	Storage time (weeks)							
	0	2	4	8	12	17	21	34
water holding capacity								
T	91.6a/x	95.1b/x	92.9ab/x	91.5a/x	93.9ab/x	92.6ab/x	92.4ab/x	81.8c/x
L	87.9a/y	91.3b/y	85.5a/y	86.4a/y	89.5ab/y	82.2c/y	80.2c/y	71.5d/y
H	85.0a/z	89.9b/y	86.0a/y	79.8c/z	84.7a/z	71.0d/z	74.6e/z	64.0f/z
lightness								
T	68.1a/x	67.7a/x	65.5b/x	66.7b/x	67.7a/x	64.9b/x	69.1c/x	60.0d/x
L	63.1a/y	64.7b/y	62.3c/y	64.0d/y	64.3b/y	62.6ac/y	64.5db/y	77.0e/y
H	69.5ab/z	71.6c/z	69.1a/z	69.2a/z	69.7ab/z	68.4d/z	70.0b/z	64.0e/z

		Storage time (weeks)							
		0	2	4	8	12	17	21	34
T	S1	12.1a/x	11.8ab/x	12.4a/x	13.6c/x	10.9b/x	11.1ab/x	10.8b/x	12.2a/x
	S2	6.4a/x	6.4a/x	6.8a/x	5.3b/x	4.5bc/x	4.3c/x	3.2d/x	4.8bc/x
	S3	47.6a/x	43.2b/x	43.4b/x	20.2c/x	12.5d/x	12.4d/x	11.7d/xy	30.4e/x
	S4	13.2a/x	18.0b/x	17.2b/x	27.4c/x	17.0b/x	16.1 ab/x	16.5ab/x	15.2a/x
	In	20.8a/x	20.6a/x	20.3a/x	33.6b/x	55.2c/x	56.1c/x	57.8c/x	37.5b/x
L	S1	16.8a/y	16.1ab/y	14.9b/y	15.6b/y	14.1c/y	14.6bc/y	14.6bc/y	16.1ab/y
	S2	13.8a/y	11.3b/y	11.7b/y	6.6c/y	7.7d/y	7.9d/x	8.3d/y	13.7a/y
	S3	39.1a/y	22.5b/y	17.8c/y	16.2c/y	11.9d/x	10.4d/x	10.3d/x	12.2d/x
	S4	5.1a/y	6.8ab/y	5.3a/y	8.1ab/y	5.5a/y	4.4a/y	9.9b/y	9.8b/y
	In	25.2a/y	43.2b/y	50.4c/y	53.5c/y	60.8d/x	62.6e/y	56.9de/x	47.7bc/y
H	S1	16.0a/y	17.2ab/y	17.0ab/z	18.4b/z	14.2c/y	15.6c/y	13.5a/z	14.0c/y
	S2	9.1a/z	10.8b/y	9.7a/z	7.7c/z	7.1c/y	4.8d/y	5.2d/z	7.3c/y
	S3	56.4a/z	48.7b/z	51.3b/z	21.1c/x	16.4d/y	17.7d/y	14.6d/y	19.3c/y
	S4	6.5a/y	8.8ab/y	11.0b/z	15.9c/z	16.7c/z	17.4c/z	19.8c/x	12.7b/z
	In	12.0a/z	14.5a/z	11.0a/z	37.0b/z	45.6c/y	44.4c/z	46.9c/y	46.7c/y