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Effect of Emulsifiers on Some Chemicals and Sensory Properties of Low Fat Mozzarella Cheese

Kamal Awad Abdel Razig; Khansa Saeed Hadi; Mohamed Bakry Eltahe, Awad Elgead Taha Elkabashi, Mohammed Ahamed Kunna

Department of Food Science and Technology Faculty of Agriculture
Al- Zaeim Al- Azhari University P.O. Box1432, Khartoum North 13311, Sudan

Abstract: ***The effect of type and levels of emulsifiers on some quality of low fat Mozzarella cheese was investigated. Five Mozzarella cheeses samples were prepared from skim cow's milk (1% fat). Twoemulsifier's salts (disodium citrate and disodium phosphate) were used at two levels (0.2% and 0.4%). The resultant cheese was analyzed for physicochemical and organoleptic properties during storage of 0.15.30.45 and 60 days intervals at 5℃.The cheese yield was markedly increased by adding either of the two emulsifying salt, but the yield was higher with phosphate compared to the citrate treatments, since higher moistures were associated with the higher yields. The protein and fat contents increased during storage period due to the loss of moisture content, while the pH values decreased. In addition, both salt accelerated cheese ripening as indicated by the formal number. The quality of cheese, appearance, texture, flavour and over all acceptability, were improved by adding of the two emulsifying salts compared to the control.***

Keywords: Low fat mozzarella cheese, emulsifiers, chemicals, sensory properties

1. Introduction

Consumption of low fat and nonfat foods has become a way of life for many health conscious people. It is recommended (Singer and Moser, 1993), which not more than 30% of calories consumed in the human diet should come from fat, which has led to a surge in the development, production, and consumption of low fat and nonfat products. The 1994 revision of labeling standards by the food and drug administration has permitted the use of appealing names such as reduced fat, low fat and nonfat ice creams for products containing less than 10% milk fat that are targeted at health conscious consumers (Baer, 1999).

Mozzarella cheese, known for its meltability, stretchability, and shredability, is a semi-soft type of cheese popular in the USA especially in pizza. Processed Mozzarella cheese (PMC) is manufactured by utilizing Mozzarella cheese as raw material followed by heat processing in the presence of emulsifying salts (ESs) such as trisodium citrate and disodium phosphate to prolong the shelf life of Mozzarella cheese. To increase retail market penetration, longer shelf life Mozzarella cheese manufacture has been a growing trend among Indian cheese manufacturers. This has resulted in spurt increase in PMC production in India (Ganesh et al., 2015).

Emulsifying salts (ESs) are added in processed cheese to modify milk proteins which causes emulsifying action. In the presence of heat and shear, ESs causes sequestration of calcium present in colloidal calcium phosphate complex and hydration of para-casein (Guinee et al. 2004; Cunha and Viotto 2010). The resultant dispersion of protein results in casein micelle networks that dissolves and break down into particles roughly in the size range of casein sub-micelles. Sequestration of calcium enables the caseins to function as emulsifiers in the water–oil interface (Zehren and Nusbaum 1992). Surfaces of dispersed free fat droplets are coated with dispersed casein attributing to increased emulsification and to immobilization of a large amount of free water due to its hydrated nature (Guinee et al. 2004: Ganesh et al., 2015).

Emulsifying salts are essential in the formation of uniform structure of processed cheese. Sodium phosphates, polyphosphates and citrates represent the most commonly used ones. Their important role is to enhance the emulsifying capability of cheese proteins by removing calcium from caseins and by peptizing, hydrating and dispersing the protein. Additional effects the above salts show are pH increase (in the majority of cases) and buffering, stabilisation of oil-in-water emulsion and structure formation (Guinee et al., 2004; Mulsowet al., 2007).

The manufacture of mozzarella was first practiced in Khartoum dairy product company in 1992 and then practiced by Koko dairy and other few individual (small laboratories). There is a high demand for Mozzarella cheese by reputable senior class hotels and pizza center, because of public awareness of the positive correlationbetween serum cholesterol concentration and the risk of developing coronary heart disease. This point motivated search for processing Mozzarella cheese from different fat level to lower the effect of fat on cholesterol to meet the consumer demand and at the same time to choose the safest level of fat that minimize the risk of heart disease (Ali and Abdel Razig, 2010).Processed cheese is a dairy product manufactured from natural cheese and suitable emulsifying salts (e.g. sodium salts of phosphates, polyphosphates and citrates). Its matrix is formed under a partial vacuum, constant agitation and upon heating. Other optional dairy (butter, skim milk powder, whey powder, coprecipitates, caseinates, etc.) and non-dairy ingredients (water, vegetables, spices, flavourings, colourings, salt, hydrocolloids, etc.) can be added into the blen (Guinee et al., 2004; Lee et al., 2004).

The objective of this investigation is to study the effect of emulsifiers on some quality of low fat Mozzarella cheese during storage.

2. Materials and Methods

Fresh cow's milk was obtained from a private farm in Shambat, Khartoum North. Salt (sodium chloride) was obtained from local market Khartoum North and rennet powder was obtained from Chr-Hansen's laboratory,French.

Emulsifier's salts
Two emulsifiers salts, disodium citrate (RhodiaUkLtd, Watford, UK.) and disodium phosphate (BK GiuliniGmb H, Ludwigshafen, Germany). Were selected and used at two levels (0.2% and 0.4 %)**.**

Preparation and manufacture of Mozzarella cheese
Mozzarella cheese was made as described by Ali and Abdel Razig (2011), five Mozzarella cheese samples were prepared from cow milk, which were standardized at 1% fat level and divided equally into 5 vats. The fist was used as a control. To the second and third 0.2% and 0.4% disodium citrate were added, and to the fourth and fifth disodium phosphate at the same rate were added, heated to 45C° and cool to 37C°. The rennet powder was introduced at rate 0.05% (for each 20Kg of milk) and left for 30-40 min.for coagulation. After coagulation, the curd was cut into about 3 centimeter cubes with stainless steel knives and healed for 10 min. before stirring and the whey is drained. The curds were formed into blocks and left in open areas to drain off the remaining whey. The drain curd exposed to warm temperature until required acidity reached at critical pH (5.2-5.3). The curd was then put in hot water at 75-80℃ and mixed properly for 5 min until a smooth elastic mass was obtained, stretched into proper form using stainless steel container for 3h. Slightly salted in 5% cold brine solution (sodium chloride) for 2 h. The cheese removed from the brine, dried lightly, weighted and package in polyethylene. The resultant cheese was analyzed for physicochemical and organoleptic properties, when fresh and during storage periods of 0.15, 30, 45 and 60 days intervals at 5℃.

Physicochemical analysis of cheese
Cheese samples were analyzed for moisture content, fat content and protein content according to AOAC (2002) method. The pH-value was determined according to Newlander and Atherton (1964), while formol ripening index was determined according to Abdel-Tawab and Hofi (1966).

Organoleptic quality of cheese
Ten panelists were chosen to judge the quality of Mozzarella cheese in term of appearance, flavour, texture and overall acceptability. The sensory evaluation of Mozzarella cheese was evaluated by scoring procedure (headonic scale) described by Ihekoronye and Ngoddy (1985), where 5: excellent, 4: very good, 3: good, 2: acceptable and 1: poor.

Statistical analysis
Data were analyzed as complete randomized design with three replicates using statistical analysis system program SAS (1997). Means were separated using Duncan's Multiple range test.

3. Result and Discussion

Cheese yield: The yield of Mozzarella cheese was significantly (P≤ 0.05) affected by the type and levels of emulsifiers (Table1).Sample 0.4% disodium phosphate recorded the highest yield (13.51%), compared to values 0.2% disodium phosphate (12.85%), 0.4% disodium citrate (12.00%), 0.2% disodium citrate (11.75%) and control (10.60%). The yield of Mozzarella was always higher when emulsifiers were added. This could be attributed to higher moisture retention. These results, are in agreement with Abo-Dawood and Abdou, (1973) and Amer*et al*., (1977). Kosikowski (1982) found the yield of Mozzarella cheese was 13.2% while ElOwni and Osman (2009) stated 11.65%.Also Wadhwani, (2011) who investigated the strategies to improve the quality of low-fat mozzarella and cheddar cheeses, and stated the mean yield of cream and emulsion (W) 1/O/W2 or O/W2incorporated low-fat cheeses were ranged from 7.7 to 7.2%.

Moisture content: Table 2. Showschanges in moisture contents of Mozzarella cheese during storage period as affected by type and levels of emulsifiers. The moisture content of cheese decreased significantly (P≤ 0.05) with progress in storage period. Sample 0.4% disodium phosphate recorded the highest moisture content (56.90%), compared to values 0.2% disodium phosphate (56.40%), 0.4% disodium citrate (53.10%), 0.2% disodium citrate (51.65%) and control (49.30%). The average moisture percentage of the cheeses explained and confirmed the yield results, since higher moistures were associated with the higher yields. However, moisture changes as a result of ripening were normal and expected. Ali and Abdel Razig (2011) stated that the moisture content of Mozzarella cheese decreased during storage period. Approximately withWadhwani, (2011) who investigated the strategies to improve the quality of low-fat mozzarella and cheddar cheeses, and stated the mean moisture content of low-fat cheeses were ranged from 57.7to 55.8%.

Protein and fat contents: The type and levels of emulsifiers significantly (P≤ 0.05) affected the protein and fat contents of Mozzarella cheese (Table 3). Samples 0.2% disodium citrate recorded the lowest (27.13%) protein while the control recorded the highest in protein (32.38%). Storage period significantly (P≤ 0.05) affected the protein contents of Mozzarella cheese, where they increased gradually till the end of storage (Mehanna, 1975). Ali and Abdel Raig (2011) found the protein content ranged between 19.20%-21.51%. Srbinovska*et al*., (2001) stated the protein content was 14.04%. Approximately with Wadhwani, (2011) who investigated the strategies to improve the quality of low-fat mozzarella and cheddar cheeses, and stated the mean protein content of low-fat cheeses were ranged from 33.6to 30.4%.

Fat contents: The type and levels of emulsifiers significantly (P≤ 0.05) affected the fat contents of Mozzarella cheese (Table 3). Samples 0.2% disodium citrate recorded the lowest (6.69%) while the control recorded the highest (7.00%). Storage period significantly (P≤ 0.05) affected the fat contents of Mozzarella cheese, where they increased gradually till the end of storage (Mehanna, 1975). Similar with Wadhwani, (2011) who investigated the

strategies to improve the quality of low-fat mozzarella and cheddar cheeses, and stated the mean fat content of low-fat cheeses were ranged from 6.0 - 6.5%.

pH values: It is clear from Table 4.that the type and levels of emulsifiers had significant (P≤ 0.05) effect on pH values. The highest pH values were(5.85) and (5.90)in samples with 0.2% and 0.4% disodium phosphate respectively, followed (5.55) and (5.70) by samples containing disodium citrate, while the lowest record (5.30) in control sample. A gradual decrease in pH values of all samples during storage period. As the storage period advanced, a slight increase in pH in emulsifier's treatment started at the end of storage period. Tessier and Rose (1958) and Amer*et al.,* (1977) reported that, sodium citrate raised the pH of cheese at the end of the storage period. Casein dispersion ability of Emulsifying salt (ES) is pH dependent which is low near pH 5.0 (Dimitreli et al. 2005). PMC made during the study had pH of 5.2 near which ES should be efficient in causing casein dispersion. Similar with Wadhwani, (2011) who investigated the strategies to improve the quality of low-fat mozzarella and cheddar cheeses, and stated the mean pH value content of low-fat cheeses were ranged from 5.1.According to scientific literature, the pH optimum for obtaining suitable structural and sensory properties of processed cheese should oscillate between 5.5 and 6.0 (Guinee et al., 2004; Lee &Klostermeyer, 2001; Lu et al., 2007; Marchesseau et al.,1997).

Formol ripening index: Table 4. Also showed that the type and levels of emulsifiers had significant (P≤ 0.05) effect on formol ripening. The highest formol ripening were (24) and (21) in samples with 0.2% and 0.4% disodium citrate respectively, followed (19) and (16) by samples containing disodium phosphate, while the lowest record (14) in control sample.

The rate of cheese ripening is more explained and confirmed in Table 4. Both emulsifying salts accelerated ripening compared with the control treatment. It is obvious that adding sodium citrate was more effective in accelerating ripening than disodium phosphate.

Organoleptic properties of Mozzarella cheese

The sensory evaluation of the Mozzarella cheese (Table 5. and 6.) indicated that, there were differences (P≤ 0.05) among the sample in their organoleptic attributes. Sample 0.2% disodium citrate was superior in appearance, texture, flavour and over all acceptability. Storage period significantly (P≤ 0.05) affected the acceptability. The best score was obtained at the day 30, and the worst at the beginning of the storage.

Textural aspects perceived in the mouth play a primitive role in determining overall quality of products. In the present study, ES content affected the body and texture of PMC significantly which resulted in appropriate hard texture PMC. Thus, high scores for body and texture were obtained on increasing the content of ES in formulation (Ganesh et al., 2015). Increasing KCl content had the inverse role and the cheese was softer with pasty body. Replacement of NaCl with KCl must be carefully studied as at high concentrations (>1%), KCl tends to decrease cheese firmness (Cruz et al. 2011). Bhaskaracharya and Shah (1999) found that, the hardness of Mozzarella cheese decreased with increase in moisture content.

Also results were reported by the study of Chen and Liu (2012) in which effect of different types of ES was investigated on hardness of processed cheese made from Mozzarella. Similarly, Shirashoji et al. (2010) investigated the effect of varying concentration (0.25–2.75%) of sodium hexametaphosphate on hardness of pasteurized process cheese, wherein cheese with higher ES administered to have greater hardness. Amer*et al.,* (1977) stated that, cheese texture became smoother and softer when emulsifiers were added to low-fat milk. The worst body, solid and hard, was that of the control.

In the present study, with increasing levels of KCl, flavour score of PMC was shown to decrease with enhanced bitterness perception. Similar findings for imitation cheese (El-Bakry et al. 2011) and process cheese (Zehren and Nusbaum 2000) were proven to cause bitter taste perception on more than 50% substitution of NaCl with KCl. Utilization of potassium-based emulsifying salts in preparation of processed cheese was also encountered to affect the flavour such as a decrease in the overall saltiness and development of bitter and chemical-metallic offflavors(Gupta et al. 1984; Karahadian and Lindsay 1984; Henson 1997).

Zisu and Shah, (2004); Abd El-Rafee *et al.,* (2004) and Sameen *et al.,* (2008) concluded that, the sensory attribute of Mozzarella cheese were improved during storage period. It may be concluded that emulsifying salts improve the quality of low fat Mozzarella cheese.

4. Conclusion

Emulsifying salts play an important role in quality of low fat Mozzarella cheese. Moisture content and pH values decreased during storage, while the fat, protein and formol ripening index increased. Over all acceptability of cheese containing 0.2% disodium citrate was the best than the other samples, 30 days storage is quite satisfactory for low-fat Mozzarella cheese to obtain good quality.

Table 1: Effect of emulsifiers on yield of Mozzarella cheese

Item	Control	Type and level of emulsifiers			
		Disodium citrate (%)		Disodium phosphate (%)	
		0.2	0.4	0.2	0.4
Kg cheese/ 100kg milk	10.60±0.16 [e]	11.75±0.18 [d]	12.00±0.15 [c]	12.85± 0.17 [b]	13.51±0.19 [a]

* Mean ± SD. Having different superscript letters on row are significantly different (P≤0.05).

Table 2: Effect of emulsifierson moisture content (%) of Mozzarella cheese

Storage period (Days)	Control	Type and level of emulsifiers			
		Disodium citrate (%)		Disodium phosphate (%)	
		0.2	0.4	0.2	0.4
0.0	49.30±0.09 ^{hi}	51.65±0.08^{ef}	53.10±0.06 ^{c}	56.40± 0.18 ^{a}	56.90±0.17 ^{a}
15	47.60±0.11 ^{j}	49.10±0.13^{i}	51.45±0.16^{ef}	52.00±0.09 ^{e}	54.40± ^{d}
30	46.50±0.14 ^{k}	47.00±0.15^{J}	50.30±0.08^{g}	51.10±0.11 ^{f}	52.80±0.13 ^{d}
45	45.60±0.19 ^{m}	46.30±0.12^{kl}	49.80±0.09^{h}	50.45±0.16 ^{g}	52.15±0.18 ^{de}
60	45.10±0.08 ^{m}	46.00±0.07 ^{L}	48.75±0.17 ^{hi}	49.70±0.12 ^{d}	51.85±0.14^{ef}

*Mean±SD. Having different superscript letters on columns and rows are significantly different (P≤0.05).

Table 3: Effect of emulsifierson protein and fatcontents of Mozzarella cheese

Storage period (Days)	Protein content (%)					Fat content (%)				
	Control	Type and level of emulsifiers				Control	Type and level of emulsifiers			
		Disodium citrate (%)		Disodium phosphate (%)			Disodium citrate (%)		Disodium phosphate (%)	
		0.2	0.4	0.2	0.4		0.2	0.4	0.2	0.4
0.0	32.38± 0.15 ^{d}	27.13± 0.16 ^{m}	28.46± 0.19^{l}	29.50± 0.21^{Jk}	30.60± 0.12^{gb}	7.00± 0.02 ^{i}	6.69± 0.03 ^{J}	6.51± 0.05^{Jk}	6.35± 0.04^{Jk}	6.15± 0.06 ^{k}
15	33.25± 0.18 ^{c}	28.80± 0.09 ^{L}	29.70± 0.07 ^{J}	30.45± 0.11g^{h}	31.15± 0.13 ^{f}	9.35± 0.05^{ef}	9.04± 0.09 ^{f}	8.66± 0.07 ^{g}	8.50± 0.08^{gh}	8.30± 0.03 ^{h}
30	34.05± 0.14 ^{b}	29.10± 0.15 ^{k}	30.10± 0.08 ^{h}	30.80± 0.11 ^{g}	31.70± 0.17^{ef}	10.40± 0.06^{bc}	10.10± 0.08 ^{c}	9.71± 0.02 ^{de}	9.55± 0.04 ^{e}	9.10± 0.05 ^{f}
45	34.65± 0.11^{ab}	29.70± 0.09 ^{J}	30.45± 0.14^{gh}	31.20± 0.16^{ef}	31.90± 0.13 ^{e}	11.20± 0.03^{ab}	10.90c± 0.07^{b}	10.50± 0.06^{bc}	10.33± 0.08 ^{c}	9.80± 0.04 ^{d}
60	34.90± 0.13 ^{a}	29.85± 0.19 ^{i}	30.75± 0.16^{gh}	31.40± 0.18^{ef}	32.00± 0.17 ^{de}	11.82± 0.04 ^{a}	11.50± 0.06^{ab}	11.10± 0.05 ^{b}	10.85± 0.07^{bc}	10.40± 0.03 ^{c}

* Mean ± SD. Having different superscript letters on columns and rows are significantly different (P≤0.05).

Table 4: Effect of emulsifiers on pH value and formol ripening index of Mozzarella cheese

Storage Period (Days)	pH value					Formol repining index (%)				
	Control	Type and level of emulsifiers				Control	Type and level of emulsifiers			
		Disodium citrate (%)		Disodium phosphate (%)			Disodium citrate (%)		Disodium phosphate (%)	
		0.2	0.4	0.2	0.4		0.2	0.4	0.2	0.4
0.0	5.30±0.06 ^{d}	5.55±0.07 ^{c}	5.70±0.11 ^{b}	5.85± 0.08 ^{a}	5.90±0.05 ^{a}	14±0.18 ^{m}	24±0.19^{iJ}	21±0.13 ^{J}	19± 0.14 ^{k}	16±0.16 ^{L}
15	4.70±0.03 ^{g}	5.00±0.09 ^{e}	5.10±0.02 ^{e}	5.35±0.06 ^{d}	5.50±0.08 ^{c}	19±0.15 ^{k}	36±0.12 ^{f}	31±0.09^{gh}	25±0.11^{iJ}	21±0.17 ^{J}
30	4.35±0.08 ^{i}	4.70±0.06 ^{g}	4.80±0.05 ^{f}	5.10±0.09 ^{e}	5.25±0.11 ^{de}	22±0.16^{iJ}	45±0.14 ^{de}	40±0.19 ^{e}	32±0.13 ^{g}	28±0.18 ^{i}
45	4.20±0.07^{IJ}	4.45±0.08 ^{hi}	4.50±0.05 ^{hi}	4.80±0.0 ^{I}2	4.90±0.03^{ef}	30±0.11 ^{h}	60±0.15 ^{b}	50±0.17 ^{c}	45±0.16 ^{g}	41±0.11 ^{e}
60	4.10±0.04 ^{J}	4.60±0.07 ^{h}	4.70±0.06 ^{g}	4.85±0.09^{ef}	5.00±0.05 ^{e}	35±0.13 ^{f}	75±0.16 ^{a}	60±0.14 ^{b}	52±0.12 ^{c}	48 ±0.16 ^{d}

* Mean ± SD. having different superscript letters on columns and rows are significantly different (P≤0.05).

Table 5: Effect of emulsifiersonappearance and texture of Mozzarella cheese

Storage Period (Days)	Appearance					Texture				
	Control	Type and level of emulsifiers				Control	Type and level of emulsifiers			
		Disodium citrate (%)		Disodium phosphate (%)			Disodium citrate (%)		Disodium phosphate (%)	
		0.2	0.4	0.2	0.4		0.2	0.4	0.2	0.4
0.0	2.0±0.11^{n}	3.0±0.12^{J}	2.8±0.09^{k}	2.5± 0.07^{L}	2.2±0.08^{m}	1.8±0.11^{L}	3.4±0.09^{h}	3.4±0.07^{h}	3.2± 0.08^{J}	3.3±0.06^{i}
15	3.2±0.09^{i}	4.4±0.03^{c}	4.0±0.06^{e}	3.8±0.05^{fg}	3.5±0.07^{h}	2.4±0.08^{k}	4.2±0.06^{de}	4.3±0.04^{d}	4.0±0.08^{f}	4.1±0.07^{e}
30	4.2±0.05^{d}	4.8±0.01^{a}	4.7±0.02^{ab}	4.5±0.03^{bc}	4.4±0.04^{c}	4.0±0.06^{f}	4.8±0.02^{ab}	4.9±0.01^{a}	4.6±0.03^{bc}	4.7±0.02^{b}
45	3.9±0.08^{f}	4.6±0.03^{b}	4.4±0.04^{c}	4.2±0.05^{d}	4.0±0.06^{e}	3.8±0.09^{g}	4.6±0.03^{bc}	4.7±0.02^{d}	4.4±0.04^{cd}	4.5±0.03 c
60	3.5±0.09^{h}	4.2±0.04^{d}	4.1±0.07^{de}	3.9±0.07^{f}	3.7 ±0.08^{g}	3.4±0.11^{h}	4.4±0.05^{cd}	4.6±0.03^{bc}	4.0±0.06^{f}	4.2 ±0.07^{de}

* Mean ± SD. Having different superscript letters on columns and rows are significantly different (P≤0.05).

Table 6: Effect of emulsifiersonflavour and over all acceptability of Mozzarella cheese

Storage period (Days)	Flavour					Over all acceptability				
	Control	Type and level of emulsifiers				Control	Type and level of emulsifiers			
		Disodium citrate (%)		Disodium phosphate (%)			Disodium citrate (%)		Disodium phosphate (%)	
		0.2	0.4	0.2	0.4		0.2	0.4	0.2	0.4
0.0	1.9±0.12 ^{p}	2.8±0.09^{kL}	2.6±0.08 ^{m}	2.4± 0.11^{N}	2.1±0.07 ^{O}	2.0±0.15 ^{p}	3.0±0.09 ^{L}	3.8±0.07 ^{m}	2.6± 0.07 ^{N}	2.4±0.11 ^{O}
15	2.7±0.05 ^{L}	3.7±0.08 ^{g}	3.4±0.11 ^{i}	3.1±0.07 ^{J}	2.9±0.09 ^{k}	3.1±0.09 ^{k}	4.0±0.06 ^{g}	3.8±0.07 ^{h}	3.6±0.04 ^{i}	3.4±0.08 ^{J}
30	4.1±0.07 ^{e}	4.8±0.01 ^{a}	4.7±0.02 ^{b}	4.5±0.03 ^{c}	4.3±0.05 ^{d}	4.1±0.07 ^{f}	4.9±0.01 ^{a}	4.7±0.02 ^{b}	4.5±0.03 ^{d}	4.4±0.05 ^{de}
45	3.7±0.09 ^{g}	4.5±0.02 ^{c}	4.2±0.04 ^{de}	4.0±0.05^{ef}	3.9±0.06 ^{f}	3.9±0.08^{gh}	4.8±0.02^{ab}	4.6±0.03 ^{c}	4.3±0.04 ^{e}	4.1±0.07 ^{f}
60	3.3±0.12^{iJ}	4.1±0.07 ^{e}	3.9±0.11 ^{f}	3.7±0.08 ^{g}	3.6 ±0.12 ^{h}	3.6±0.11 ^{i}	4.6±0.05 ^{de}	4.4±0.05 ^{de}	4.2±0.06^{ef}	4.0 ±0.08 ^{g}

* Mean ± SD. having different superscript letters on columns and rows are significantly different (P≤0.05).

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