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Effect of storage on the quality attributes of whey protein enriched milk chocolate

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Abstract
The storage stability of protein enriched products is a sensitive issue especially where the protein directly incorporated to meet the issue of protein energy malnutrition (PEM) in non-conventional/traditional food products. In this study, the whey protein enriched chocolate were packed in market replica and stored under refrigeration and ambient conditions. The stored samples were evaluated for a period of one month to check the immediate effect of addition of whey protein on the sensory, colour and physico-chemical properties. The developed product exhibited good shelf life upto one month of storage (which was considered as the symbolic shelf life) at refrigeration temperature.

Keywords: Whey protein, chocolate, storage study, HMF, tyrosine, sensory evaluation

1. Introduction
Milk chocolates are complex multiphase systems of particulate (sugar, cocoa, certain milk components) and continuous phases (cocoa butter, milk fat and emulsifiers) and prepared with series of refining, mixing, conching, tempering, moulding and packaging. It usually contains cocoa mass, whole milk powder, sugar and cocoa butter of about 12g; 19g; 48.5g; and 20 g per 100 g of chocolate, respectively (Beckett 2009, Schantz and Rohm 2005) [3, 12]. Usually, milk chocolate available in market contains about 20% milk powder (Afoakwa *et al.* 2008a, Lucisano *et al.* 2006) [1, 11] which significantly affects the organoleptic attributes of milk chocolate, chocolate processing and melting behaviour, as well as rheological characteristics of the final product (Taylor and Oliver 2009) [14]. The chocolate, up to now, enriched with various functional components like dried fruits (Komes *et al.* 2013) [8], probiotics, maltodextrin and lemon fibers (Ersan *et al.* 2014) [6], phytosterols (Botelho *et al.* 2014) [5], and natural sweeteners like stevia or others (Belscak *et al.* 2015) [4] have been extensively studied. The current study belongs to the storage of whey protein enriched milk chocolate. Further, to the best of author's knowledge, there is zilch regarding such type of studies in the literature concerning the impact of storage on the physic-chemical properties of whey protein enriched milk chocolate vis-à-vis control sample.

2. Materials and Methods
2.1 Materials
The skim milk powder (verka), cocoa powder ("Hershey's cocoa"), cocoa butter - *"Chocoville"* (Bakersville India Private Limited, Indore, India), whey protein concentrate and sugar were procured from local market of Ludhiana.

2.2 Milk Chocolate Preparation
The proportion of ingredients, i.e. sugar, skim milk powder, whey protein concentrate, cocoa powder and cocoa butter, for the preparation of milk chocolate was optimized using preliminary trials. The chocolate sample(s) were prepared as per the methods described (Wan *et al.* 2014) with slight modification.

2.3 Packing
Solidified chocolate was, then, packed in aluminum foil (as resemblance to market replica) and stored at refrigeration temperature (7°C±1°C) till further analysis.

2.4 Storage
Packed optimized product (market replica) was stored at refrigeration temperature (4±1ºC) and

ambient temperature (25±1ºC) to evaluate storage stability. During the storage period of 30 days, the product was evaluated at the interval of every seven days for physico-chemical (water activity, tyrosine value, free fatty acid content, TBA (Thiobarbituric acid), HMF (Hydroxymethyl furfural value); instrumental colour; sensory characteristics and microbiological analysis.

2.5 Analytical Methods

The extent of fat oxidation in protein enriched milk chocolate was measured in term of TBA value. The extraction method of Strange *et al.* (1977) [13] was followed. Hydroxymethylfurfural of milk chocolate was determined by using method described by Khatkar and Gupta (2011) [7] and the obtained HMF content was in the form of µM/Kg of chocolate sample. Tyrosine content of milk chocolate was determined using method described by Lin *et al.* (1982) and the obtained tyrosine content was in the form of mg/g for chocolate sample. Chocolate samples were analyzed for water activity using Aqua Lab water activity meter (Series 4TE). The instrument was calibrated with 6.0M NaCl at 25ºC and subsequently water activity of sample(s) was measured.

2.6 Instrumental Colour

Surface colour of chocolate samples was measured at 25°C as per the method described by Konar *et al.* (2014) [9] using portable colorimeter (Chroma Meter CR-400, Konica Minolta, Japan) and the results were expressed in terms of CIE-LAB system. Prior to analysing the samples, the instrument was calibrated with standard white tile as specified by the manufacturer of the equipment. Data was received through the software in terms of values for L* (lightness), 0 (black) to 100 (white); a* (redness), +60 (red) to -60 (green) and b* (yellowness), +60 (yellow) to -60 (blue). All data were expressed as the mean value of three replicates conducted on different samples of the same lot of each chocolate.

2.7 Sensory Evaluation

For the evaluation of sensory attributes of whey protein enriched milk chocolate, the chocolate samples were served to a panel of semi-trained panellists (seven members) selected from the faculty of College of Dairy Sciences and Technology, GADVASU, Ludhiana at 16°C±2°C. Each panellist evaluated chocolate sample for sensory attributes such as appearance, flavour, mouth feel, melting behaviour, texture (biting behaviour), sweetness and overall acceptability on 9 point Hedonic scale.

2.8 Microbiological Analysis

Standard plate count, yeast and mold count, and coliform count were recorded as per procedure given by Aidoo *et al.* (2010) [2] using the media Nutrient agar for standard plate count, Potato dextrose agar for yeast and mold and Macconics agar for coliform count.

2.9 Statistical Analysis

The data obtained from the various experiments during experimental work were analyzed for overall mean, standard deviation, one-way and two way analysis of variance (ANOVA) and critical difference (with CD_{LSD}), wherever required, using Microsoft Excel (Microsoft office 2010).

3. Results and Discussion

3.1 Storage study of optimized product

The optimized protein enriched chocolate was packed in aluminum foil and stored at refrigeration temperature (4±1˚C) and ambient temperature (25±1˚C) for one month. During storage, product was evaluated at 7 days interval for changes in their Hydroxymethyl furfural value, thiobarbituric acid value, free fatty acid, tyrosine content, instrumental colour values (L*, a*, b*); textural properties (hardness). During the storage the changes in the microbiological quality of chocolate were also studied in terms of SPC, coliform and yeast and molds at first and last day of storage. The sensory qualities (appearance, flavour, mouthfeel, melting behaviour, texture, sweetness and overall acceptability) of chocolate stored at both the temperatures were also studied.

3.2 Effect of storage period on sensory attributes of optimized milk chocolate

Data presented in Table 1 shows the sensory scores of the protein enriched milk chocolate during storage at refrigeration and ambient temperature, respectively. The data for the changes in the sensory scores for the milk chocolate stored at refrigeration temperature were tabulated in Table 1. The sensory scores for the appearance, flavour, texture and sweetness were not affected at 0.01% level of significance however the sensory scores were significantly affected at 0.05% level of significance, except for the sensory scores of the flavour and sweetness which were non significantly (P<0.01,0.05) decreased during storage.

The appearance scores of the milk protein enriched chocolate were decreased significantly (P<0.05) from 8.5 to 7.75 during the one month storage period. No significant decrease was observed in the sensory scores of flavor during storage period at refrigeration temperature, whereas mouth feel scores were significantly (P<0.01, 0.05) decreased from 8.06 to 7.08.Sensory scores for the melting behavior of chocolate also significantly (P<0.01, 0.05) decreased from 7.95 to 7.33 during the 28 days of storage at refrigeration temperature. Sensory scores for the texture of the chocolate during the storage period were also significantly (P<0.05) decreased from 7.88 to 7.25. Sweetness scores of milk chocolate were not significantly affected during storage period however they decreased from 7.85 to 7.67. The overall acceptability scores of the protein enriched milk chocolate were found to decrease significantly (P<0.01, 0.05) with progress in the storage period from 8.08 to 7.4 after 28 days of storage. The decrease in the sensory scores was significant in different aspects but, minimal although the product was still acceptable after one month of storage period at refrigeration temperature.

On other side, a significant (P<0.01, 0.05) decrease in all the sensory scores was observed as the storage period increased. During storage, changes in the sensory scores followed a linear trend and highest scores for all the sensory attributes were observed on the zero day and lowest scores were observed on the last day (14^{th} day) of storage.

Table 1: Effect of storage period on the sensory attributes of the milk protein enriched milk chocolate

	Temperature	Storage period (days)					CD	
		0	7	14	21	28	0.01[a]	0.05[1]
Appearance	Ambient	8.33±0.45^{b,3}	7.67±0.41^{b,2}	6.53±0.45^{a,1}	-	-	0.85216	0.599091
	Refrigeration	8.5±0.46^{3}	8.37±0.28^{23}	8.05±0.50^{12}	8.32±0.4^{23}	7.75±0.76^{1}	NS	0.43507
Flavour	Ambient	8.08±0.3^{c,3}	7.55±0.23^{b,2}	6.9±0.49^{a,1}	-	-	0.481422	0.338452
	Refrigeration	8.23±0.33	8.02±0.28	7.8±0.24	7.83±0.68	7.58±0.8	NS	NS
Mouth feel	Ambient	7.98±0.29^{c,3}	7.47±0.19^{b,2}	6.52±0.29^{a,1}		-	0.462504	0.325152
	Refrigeration	8.06±0.23^{b,2}	8.07±0.27^{b,2}	7.78±0.25^{b,2}	7.75±0.69^{b,2}	7.08±0.2^{a,1}	0.558932	0.409775
Melting behaviour	Ambient	7.91±0.12^{c,3}	7.47±0.05^{b,2}	6.62±0.2^{a,1}	-	-	0.213911	0.150385
	Refrigeration	7.95±0.12^{b,2}	7.97±0.21^{b,2}	7.78±0.24^{ab,2}	7.72±0.6^{ab,12}	7.33±0.41^{a,1}	0.55384	0.406041
Texture	Ambient	7.85±0.16^{b,3}	7.4±0.2^{b,2}	6.7±0.41^{a,1}	-	-	0.479098	0.336818
	Refrigeration	7.88±0.32^{2}	7.83±0.21^{2}	7.7±0.4^{2}	7.8±0.21^{2}	7.25±0.42^{1}	NS	0.417026
Sweetness	Ambient	7.8±0.2^{b,3}	7.32±0.25^{ab,2}	6.91±0.38^{a,1}	-	-	0.527864	0.371102
	Refrigeration	7.85±0.16	7.78±0.21	7.62±0.38	7.78±0.21	7.67±0.82	NS	NS
Overall acceptability	Ambient	7.99±0.16^{c,3}	7.48±0.1^{b,2}	6.69±0.06^{a,1}	-	-	0.207262	0.145711
	Refrigeration	8.08±0.23^{b,2}	8.01±0.17^{b,2}	7.78±0.27^{ab,2}	7.87±0.41^{ab,2}	7.44±0.43^{a,1}	0.431488	0.316341

Data are presented as mean ± SD (n = 3). Means with the same superscript in a row are not significantly different, *P<0.01 (for a, b, c…) and P<* 0.05 (*for 1, 2, 3,…*) from each other. ** Significant a < 0.01, * Significant at P< 0.05.

Sensory scores for the appearance of milk chocolate decreased significantly (P<0.01; 0.05) from 8.33 to 6.53 after 14 days of storage. Similar trend was also observed in the flavour, mouthfeel, melting behaviour, texture, sweetness and overall acceptability scores which also decreased significantly (P<0.01, 0.05) with progress in the storage period. Reduction in the sensory scores showed that the chocolate samples stored at room temperature were not acceptable to the panellists after the two weeks of storage, which might be due to the melted form of chocolate.

3.3 Effect of storage period on instrumental colour values of optimized milk chocolate

The results from experimental and statistical analysis for the changes in the instrumental colour values of the milk chocolate during storage at refrigeration and ambient temperature were tabulated in Table 2. The L* value of milk chocolate stored at refrigeration temperature increased significantly (P<0.01) during one month of storage period. The L* value which represents the whiteness/glossiness of the chocolate increased from 30.52 to 34.64 and a* value from 10.81 to 11.71 after 28 days of refrigeration storage. All the values for lightness and redness were significantly different from each other at 0.01 and 0.05% level of significance. The b* value of milk chocolate increased significantly (P<0.01/0.05) from 11.53 to 13.03. Yellowness or b* value was observed to decrease in first week of storage and then it tended to increase during the remaining storage period.

The L* value which represents the lightness and glossiness of the chocolate was significantly (P<0.01/0.05) increased from 30.52 to 32.43 after 7 days of storage and then to 35.18 after 14 days of storage period at ambient temperature. The increase in L* value with increase in the storage period might be due to the formation of fat bloom on the surface of milk chocolate. The a* value of the chocolate was also significantly (P<0.01/0.05) affected by the progress in the storage period. At 0 day of storage, the a* value of the chocolate was 10.81 which significantly increased to 11.63 after 14 days of storage at ambient temperature. Significant difference (P<0.01/0.05) in the b* value of optimized milk chocolate during storage period was also observed (Table 2). Yellowness or b* value of the product increased linearly during storage. Initially, the b* value of the chocolate was 12.76 and after 14 days of storage it was observed to be 13.08.

Table 2: Effect of storage period on instrumental colour values of optimized milk chocolate

	Temperature	Storage period (days)					CD	
		0	7	14	21	28	0.01[a]	0.05[1]
L*	Ambient	30.52±0.07^{a,1}	32.43±0.16^{b,2}	35.18±0.55^{c,3}	-	-	1.010136	0.666694
	Refrigeration	30.52±0.07^{a,1}	31.81±0.06^{b,2}	32.68±0.21^{c,3}	33.42±0.29^{d,4}	34.64±0.2^{e,5}	0.48	0.34
a*	Ambient	10.81±0.06^{a,1}	11.27±0.08^{b,2}	11.63±0.1^{c,3}	-	-	0.246541	0.162718
	Refrigeration	10.81±0.06^{a,1}	11.02±0.03^{b,2}	11.26±0.02^{c,3}	11.54±0.1^{d,4}	11.71±0.06^{e,5}	0.16	0.11
b*	Ambient	12.76±0.4^{a,2}	12.15±0.05^{a,1}	13.08±0.15^{ab,2}		-	0.752723	0.4968
	Refrigeration	12.75±0.4^{c,3}	11.53±0.07^{a,1}	12.14±0.03^{b,2}	12.58±0.23^{bc,3}	13.03±0.16^{c,3}	0.58	0.41

Data are presented as mean ± SD (n = 3). Means with the same superscript in a row are not significantly different, *P<0.01 (for a, b, c…) and P<* 0.05 (*for 1, 2, 3,…*) from each other.
** Significant at P< 0.01, * Significant at P< 0.05.

3.4 Effect of storage period on water activity a_w of milk chocolate

The data for effect of storage temperature (ambient and refrigeration) and period on the changes in the water activity (a_w) of the milk chocolate enriched with protein is presented in Table 3. There was significant (P<0.01/0.05) difference between the water activity values observed during the storage period at ambient and refrigeration storage temperature. The water activity of the milk chocolate increased from 0.394 to 0.456 after 14 days of storage at ambient temperature, while when storage was done at refrigeration temperature the water activity value increased from 0.394 to 0.454 after 14 days of storage and then to 0.515 after 28 days of storage. The water activity results were in the range from 0.394 to 0.465. All the observed values for water activity were significantly different from each other.

3.5 Effect of storage period on hydroxyl methyl furfural content of milk chocolate

The data for the hydroxyl methyl furfural content of the milk chocolate stored at ambient temperature and refrigeration temperature is presented in Table 3. The initial HMF value of the protein enriched milk chocolate was 5.12% which was significantly (P<0.01/0.05) affected by storage temperature and period. When chocolate was stored at ambient temperature, the HMF content of the chocolate increased significantly to 8.51 % after 14 days of storage period and when chocolate was stored at refrigeration temperature the HMF content was increased to 7.34% after 14 days of storage period, however the increase in the HMF content was higher at ambient temperature as compared to refrigeration temperature which may be due to the slow rate of physicochemical changes in the chocolate at lower temperature. The HMF content of the chocolate after 28 days of storage at refrigeration temperature was significantly increased to 8.7%.

3.6 Effect of storage period on thiobarbituric acid content of milk chocolate

The data for the effect of the storage period on the thiobarbituric acid content of the milk chocolate fortified with protein was presented in Table 3. The thiobarbituric acid content of the freshly prepared milk chocolate was 0.004. When chocolate was stored at ambient temperature, the thiobarbituric acid content was increased to 0.024 after 14 days of ambient temperature and to 0.020 when chocolate was stored at refrigeration temperature. The thiobarbituric acid content was increased to 0.026 after 28 days of storage at refrigeration temperature. The increase in the TB acid content was found to be statistically significant at 0.01 and 0.05% level of significance. It was also observed from the results that the increase in the TBH was higher at ambient storage conditions as compared to refrigeration storage as rate of biochemical changes was higher at room temperature than at low temperature. TBA content was increased due to the further degradation of free fatty acid and formation of malondialdehyde (MDA).

3.7 Effect of storage period on tyrosine content of milk chocolate

The data for the effect of the storage temperature and period on the tyrosine content of the chocolate was presented in Table 3. Storage of chocolate at ambient and refrigeration temperature was found to have a no effect on the tyrosine content and it was not affected by the progress in the storage period at both the conditions. The tyrosine content of the milk chocolate enriched with protein before and storage at each condition as remained constant at 28.33%.

3.8 Effect of storage period on FFA content of optimized milk chocolate

The data for the effect of the storage period on the free fatty acids content of the milk chocolate enriched with whey protein was presented in Table 3. The FFA content of the freshly prepared milk chocolate was 0.97%. When, chocolate was stored at ambient temperature, the FFA content was increased to 1.09% after 14 days of ambient temperature and to 1.25% after 28 days of storage at refrigeration temperature. The increase in the FFA content was probably due to the degradation of fat present in the milk chocolate. It was also observed from the results that the increase in the FFA was higher at ambient storage conditions as compared to refrigeration storage, as rate of biochemical changes was higher at room temperature compared to low temperature.

Table 3: Effect of storage period on the water activity, thiobarbituric acid and tyrosine content of the milk protein enriched chocolate

	Temperature	Storage period (days)					CD	
		0	7	14	21	28	0.01[a]	0.05[1]
a_w	Ambient	0.394±0.001^{a,1}	0.453±0.001^{b,2}	0.465±0.002^{c,3}	-	-	0.005	0.003
	Refrigeration	0.394±0.001^{b,2}	0.33±0.003^{a,1}	0.454±0.008^{d,4}	0.441±0.001^{c,3}	0.515±0.002^{e,5}	0.011	0.008
HMF	Ambient	5.12±0.02^{a,1}	7.48±0.33^{b,2}	8.51±0.15^{c,3}	-	-	0.64	0.42
	Refrigeration	5.12±0.02^{a,1}	6.27±0.4^{b,2}	7.34±0.02^{c,3}	8.02±0.04^{d,4}	8.7±0.02^{e,5}	0.070	0.049
TBA	Ambient	0.004274	0.015085	0.023897	-	-	NS	NS
	Refrigeration	0.00427	0.00816	0.0201	0.0251	0.0259	NS	NS
Tyrosine	Ambient	28.33	28.33	28.33	-	-	NS	NS
	Refrigeration	28.33	28.33	28.33	28.33	28.33	NS	NS
FFA	Ambient	0.9744	1.045	1.09	-	-	-	-
	Refrigeration	0.9744	1.0208	1.0672	1.16	1.2528	-	-

Data are presented as mean ± SD (n = 3). Means with the same superscript in a row are not significantly different, *P<0.01 (for a, b, c…) and P<* 0.05 (*for 1, 2, 3,…*) from each other. ** Significant at P< 0.01, * Significant at P< 0.05.

3.9 Microbial analysis of optimized milk chocolate during storage

Microbial count of the developed product was done at zero days and the last day of the storage study and is tabulated in table 4. At zero day of storage, the standard plate counts was observed 2.097 $\log_{10}$CFU/, whereas the coliform count was observed nil. The counts for yeast and mold were 1.903 $\log_{10}$ CFU/g at zero day of the storage. At the last day of storage, the microbial counts of the stored product were slightly increased, but not significantly (P<0.05). The standard plate counts and yeast & mold counts of the developed product was observed to increase to 2.301 $\log_{10}$CFU/g and 1.978 $\log_{10}$CFU/g, respectively, at last day of storage.

Table 4: Effect of storage period on microbiological count of optimized milk chocolate

	0 day	7 day	14 day	21 day	28 day
SPC ($\log_{10}$CFU/g)	2.097	-	-	-	2.301
Coliform ($\log_{10}$CFU/g)	Nil	-	-	-	Nil
Yeast & mold ($\log_{10}$CFU/g)	1.903	-	-	-	1.978

– not observed

4. Conclusion

From the results obtained during storage study, it was concluded that there was no major perceivable defect observed in optimized product upto 14th days of storage at ambient temperature and upto 28th day of storage at refrigeration temperature. The overall sensory scores of the chocolate decreased from 7.99 to 6.69 after 14 days of storage at ambient temperature and when chocolate was stored at refrigeration temperature the overall acceptability scores decreased from 8.08 to 7.44. The lightness value of the chocolate also increased as the storage period progressed at each storage conditions.

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