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Preservation of spiced radish juice using hurdle technology

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Abstract

Consumer's acceptance towards white radish (*Raphanus sativus*) juice is very low due to its pungent flavour which can be overcome by blending suitable juice (sugarcane juice) with flavouring component (mint and coriander extract). But this blending would act as an obstacle in the way of preservation due to the nature of radish and sugarcane juice as they are highly prone to microbial attack. An attempt has therefore been made to preserve the blended radish juice with hurdle technology using different hurdles like pasteurisation temperatures (70°C and 80°C) at different durations (10 and 20 min) followed by the addition of potassium metabisulphite (KMS) (200, 300 and 400 ppm), respectively. All the samples were packed in glass bottles and stored in refrigerated condition for three months. With the increase in pasteurisation temperature, there was a significant decrease in total protein, total phenols, ascorbic acid, antioxidant activity, total plate count (TPC), yeast and mould count (YMC), and overall acceptability, whereas a significant increase in TSS (total soluble solids) and reducing sugars occurred. Similarly, an increase in pasteurisation duration resulted in a significant increase in TSS and reducing sugar, and a significant decrease in total protein, total phenol, ascorbic acid, antioxidant activity, TPC, YMC, and overall acceptability. The addition of potassium metabisulphite following pasteurisation significantly affected total protein, total phenol, antioxidant activity and ascorbic acid in a positive manner, whereas TSS, titratable acidity, reducing sugar, total sugar, TPC, YMC and overall acceptability in a negative manner. With enhancement of the storage period, almost all the parameters significantly decreased except TSS, titratable acidity, reducing sugar, total sugar, TPC and YMC. It was thus concluded that blended radish juice could be preserved for three months using hurdle technology (pasteurised at 70°C for 10 min followed by addition of 200 ppm KMS).

Introduction

Juices are excellent source of energy, vitamins, antioxidant, minerals and fibres (Afreen *et al.*, 2016). Recent years have witnessed an exorbitant increase in consumers' interests in vegetable juices, thereby providing vast possibilities for value added products to meet the consumers' demand. Sometimes, it is quite difficult to consume vegetables in an efficient manner, hence including 1 - 2 cups of vegetable juice daily is an effective and acceptable way for healthy adults to close the dietary vegetable gap (Shenoy *et al.*, 2010). Radish has an appreciable amount of vitamins B1, B2, B3, B6, B9, vitamin C, magnesium, copper, calcium, zinc, phosphorus and manganese (Nishadh and Mathai, 2014). It possesses a variety of health benefits, and is used in the treatment of diseases such as cancer, whooping cough, piles, liver problem, constipation, dyspepsia, gall problem, arthritis, gall stones, kidney stone and others (Agarwal and Varma, 2014). Beside all these health benefits, it also possesses antimicrobial activity against different pathogenic microorganisms (Gutiérrez and Perez, 2004). Despite its numerous health benefits, its juice's acceptability is very poor because of its pungent odour, which is due to the presence of bioactive compounds called glucosinolates. In an earlier attempt, the problem was overcome by blending it with sugarcane juice and spice extract along with salt (Kaur *et al.*, 2018). However, this blend was quite prone to microbial attack, which is an obstacle towards its preservation. Undoubtedly, it can be preserved using different

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preservation technologies like high temperature, low temperature and chemical preservatives (Sankhla *et al.*, 2012). Nevertheless, the retention of the phytochemical potential of the juice faces a great challenge and the option for the same is only hurdle technology.

With the introduction of hurdle technology concept by Leistner in 1978, there is a worldwide utilisation of this technique in food, which has been developed to address the consumers' demand for more natural and fresh foods, and is mainly used in food industry for the gentle and effective preservation of food (Sankhla *et al.*, 2012; Rawat and Pokhriya, 2014). The most important hurdles used in industries are high temperature, low temperature, modified atmosphere storage, preservatives and competitive microorganisms. Some of these hurdles influence the safety and quality of foods and at the same time improve the flavour of the products, thereby improving the total quality of food (Leistner and Gould, 2012). Different types of hurdles such as high temperature, low temperature, redox potential, water activity, and preservative have already been used by different researchers for the preservation of different juices i.e., sugarcane, strawberry, carrot, celery and beetroot juice (Sankhla *et al.*, 2012; Profir and Vizireanu, 2013; Wisal *et al.*, 2013).

Pasteurisation is used to extend the shelf life of juice by inactivating pathogenic microorganisms and enzymes (Kathiravan *et al.*, 2014). However, high temperature processing for long time can result in undesirable changes that account for nutrient loss, colour alteration as well as the overall acceptability of the juice (Gao and Rupasinghe, 2012). Different chemical preservatives (sodium benzoate, potassium sorbate, citric acid, potassium metabisulphite) are also commonly used in the beverage industries to extend beverages' shelf-life. Potassium metabisulphite (KMS) is one of the preservatives which has an effective control over browning thereby retaining the original colour of juice. The addition of 0.2% KMS helps in controlling microbial growth by reducing the pH and maintaining the sensory attributes of juice (Sakhale *et al.*, 2012). On the other hand, however, high concentration of KMS in the juice can lead to decreased acceptability of the juice as it may impart pungent smell to the juice (Talasila *et al.*, 2012). Studies have reported that cold temperature storage is also effective in prolonging the shelf life of juices with less decrease in total sugar at low temperature storage in comparison to storage at room temperature (Chauhan *et al.*, 2002; Sankhla *et al.*, 2012).

It is clear from the literature that a high temperature processing for long time as well as high concentration of chemical preservatives can deteriorate the phytochemical and quality potential of juice. Therefore, the present work was aimed to employ different hurdles such as low temperature pasteurisation (70 - 80°C) for shorter time (10 and 20 min) with low concentration of KMS (200, 300 and 400 ppm) along with low temperature storage (4°C) to maintain the quality attributes of the blended radish juice during storage.

Materials and method

Preparation and preservation of spiced radish juice

Radish (var. Pusa Chetki) roots, mint, coriander leaves and fresh sugarcane juice (var. Co 0238) were procured from the local market in Jalandhar, Punjab, India. Spiced radish juice was prepared by using radish juice (67.5%), herbal extract (1%), sugarcane juice (30%) and a mixture of salt (1.5%) as per the standard procedure (Kaur *et al.*, 2018). The beverage prepared was filled into steam-sterilised bottles, and pasteurised at 70°C and 80°C for 10 and 20 min respectively. Different concentrations of KMS (200, 300 and 400 ppm) were separately added into these bottles followed by crown corking. A total of 12 treatments were given to the blended juice (Table 1) which were refrigerated (4°C) for three months, and analysed at one-month interval for various physico-chemical, sensory and microbiological attributes. In order to study the effect of multiple replicate on the quality characteristics of radish beverages, each combination as prepared earlier was made and preserved three times at the same condition.

Table 1. Treatment details for the preservation of blended juice.

Treatments	Pasteurisation temperature (°C)	Pasteurisation time (min)	KMS concentrations (ppm)
T_1	70	10	200
T_2	70	10	300
T_3	70	10	400
T_4	70	20	200
T_5	70	20	300
T_6	70	20	400
T_7	80	10	200
T_8	80	10	300
T_9	80	10	400
T_{10}	80	20	200
T_{11}	80	20	300
T_{12}	80	20	400

Analytical methods

The total soluble solids (TSS), titratable acidity and pH of blended beverages were determined as per the standard methods (AOAC, 1984). The reducing and total sugars were determined according to Lane and Eynon method as described by Ranganna (1986). The total proteins were estimated using the Lowry's method, whereas the total phenolic content was determined by modified Folin-Ciocalteau method as described by Sadasivam and Manickam (1992). The ascorbic acid content was estimated by titration method with 2,6-dichlorophenol-indophenol dye solution (AOAC, 1984). The antioxidant activity (DPPH free radical scavenging activity) was measured as per the standard method of Brand-Williams *et al.* (1995).

Microbiological analysis

Pour plate method was performed for the estimation of viable bacterial and fungal counts. Plate Count Agar and Potato Dextrose Agar were used for the two microbial counts, respectively (Krishnakumar and Devadas, 2006).

Sensory analysis

A nine-point hedonic sensory score card as described by Amerine *et al.* (1965) and Joshi (2006) was followed to analyse the sensory characteristics of the blended beverage samples at different storage intervals. Blended beverage samples were evaluated by a panel of 10 semi-trained members from the Department of Food Technology and Nutrition, Lovely Professional University, Phagwara, India, for colour, aroma, flavour and overall acceptability. Prior to sensory analysis, the judges were familiarised with the blended beverage samples and the hedonic scale in the training session. The judges were asked to score the blended beverage samples for the aforementioned sensorial quality attributes from 1 = dislike extremely to 9 = like extremely depending upon their respective intensity. Sensory evaluations were carried out in the isolated booths at room temperature (27 ± 2°C). During the session, the panellist used plain water to rinse their mouth in between each blended beverage sample (Joshi and Kumar, 2017). The mean score of all these attributes was used to draw the overall acceptability of the product.

Statistical analysis

Data obtained from the physico-chemical, microbiological and sensory attributes of blended beverage samples were analysed by using GraphPad Prism (La Jolla, CA, USA) (version 5.01) software. Results were expressed as means ± SD. Differences between the means were tested for statistical significance using a 2-way ANOVA, followed by Bonferroni post-hoc test. The significance level was set at 5% ($p < 0.05$). To obtain the effect of different measures (pasteurisation duration, pasteurisation temperature, KMS concentration, storage duration) independently, the means of the respective measure are given in the tables, irrespective of the others.

Results and discussion

Effect of different pasteurisation temperatures

With increasing pasteurisation temperature from 70°C to 80°C, there was a significant increase in TSS and reducing sugar and a significant decrease in total protein, total phenol, ascorbic acid, antioxidant activity, TPC, YMC, and overall acceptability (Table 2). An increase in TSS might be due to the evaporation of water which causes concentration of juice to some extent by heat processing (Pareek *et al.*, 2011). Similar opinions were put forth by Dar *et al.* (1992) in apple juice. Further studies reported that an increase in TSS might also be attributed to the breakdown of the complex carbohydrates into simple soluble carbohydrates (Raj *et al.*, 2011). The increase in reducing sugars might be due to the heat treatment which leads to an increase in hydrolysis of complex compound (Nisar *et al.*, 2015). The decrease in total protein was observed with increasing pasteurisation temperature from 70 to 80°C, as higher pasteurisation temperature could lead to the loss of some essential amino acid resulting in the decrease in total protein content (Matua, 2013). There was a significant decrease in phenolic content due to the increase in pasteurisation temperature which may be attributed to the breakdown of polymeric phenols and thus responsible for its degradation at high temperature (Guo *et al.*, 2013). The ascorbic acid content in blended beverage samples decreased with increasing pasteurisation temperature as ascorbic acid is sensitive to heat, light and oxygen (El-Ishaq and Obirinakem, 2015). A similar trend of decrease was also observed in antioxidant activity along with the increase in pasteurisation temperature as was observed in phenolics and ascorbic acid. Various researchers have documented that antioxidant activity totally depends upon pigments (carotenoids, anthocyanin), ascorbic acid and total phenolic content in the sample (Rice-Evans *et al.*, 1997; Kapasakalidis *et al.*, 2006). The decrease in TPC and YMC was due to the high temperature as bacteria, yeasts and moulds are heat-sensitive and are easily destroyed by heat treatment. Similar findings were put forth by Sankhla *et al.* (2012) in sugarcane juice. The overall acceptability

of the blended beverage samples pasteurised at 70 and 80°C showed a decline in sensory scores. This could be due to the thermal effect which may decrease the taste and flavour of the juice and thus affected the overall acceptability (Kathiravan *et al.*, 2014).

Table 2. Effect of pasteurisation temperatures on the physico-chemical, microbial and sensory properties of the blended juice during storage.

Parameter	Pasteurisation temperature	
	70°C	80°C
TSS (°B)	10.25 ± 0.1[a]*	10.42 ± 0.08[b]
Titratable acidity (%)	0.40 ± 0.03[a]	0.38 ± 0.03[a]
pH	4.20 ± 0.03[a]	4.22 ± 0.03[a]
Reducing sugars (%)	0.59 ± 0.06[a]	0.68 ± 0.07[b]
Total sugars (%)	9.77 ± 0.57[a]	9.89 ± 0.61[a]
Total proteins (mg/100 ml)	163.6 ± 15.76[a]	132.1 ± 16.48[b]
Total phenols (mg/100 ml)	25.26 ± 0.83[a]	20.17 ± 0.79[b]
Ascorbic acid (mg/100 ml)	15.68 ± 1.01[a]	11.11 ± 0.64[b]
Antioxidant activity (%)	71.32 ± 2.41[a]	64.18 ± 1.92[b]
Total plate count (Log)	2.04 ± 0.3[a]	1.77 ± 0.2[b]
Yeast and mould count (Log)	1.80 ±0.4[a]	1.54 ± 0.2[b]
Overall acceptability	7.33 ± 0.64[a]	6.80 ± 0.77[a]

Mean ± SD (n = 3). Different lowercase superscripts in the same row indicate significant difference ($p < 0.05$) between temperatures.

Effect of different pasteurisation durations

With the extension of pasteurisation duration from 10 to 20 min, there was a significant increase in TSS and reducing sugar, and a significant decrease in total protein, total phenol, ascorbic acid, antioxidant activity, overall acceptability, TPC and YMC (Table 3). The increase in TSS could be due to the increase in pasteurisation duration as this caused more evaporation of water which would further concentrate the blended beverage samples thereby leading to the increase in TSS. It could also be due to the breakdown of complex carbohydrates into simple soluble carbohydrates as earlier discussed. There was an increase in reducing sugars with the extension in pasteurisation duration from 10 to 20 min, which could be due to the increase in the hydrolysis of complex molecule (Nisar *et al.*, 2015). Table 3 clearly shows the decline in total protein content of the samples with increasing pasteurisation duration as longer pasteurisation time could lead to the denaturation of protein thereby resulting in decreased protein content. The decrease in the total phenols could be due to the breakdown of polymeric phenols thereby resulting in the thermal degradation of these compounds (Guo *et al.*, 2013). The decrease in ascorbic acid could be due to the sensitivity of ascorbic acid to high temperature at longer period (Pareek *et al.*, 2011). The antioxidant activity of the blended beverage samples decreased when pasteurisation duration was prolonged from 10 to 20 min, which could be due to the decrease in phenolic content and the ascorbic acid as earlier discussed (Rice-Evans *et al.*, 1997; Kapasakalidis *et al.*, 2006). The decrease in TPC and YMC could be due to longer pasteurisation time as more heating time could kill more microorganisms. The overall acceptability of the samples decreased with increasing pasteurisation duration as prolonged heat processing could affect the taste and flavour of the juice.

Table 3. Effect of pasteurisation time on the physico-chemical, microbial and sensory properties of the blended juice during storage.

Parameter	Pasteurisation time	
	10 min	20 min
TSS (°B)	10.23 ± 0.08[a]*	10.33 ± 0.08[b]
Titratable acidity (%)	0.40 ± 0.03[a]	0.38 ± 0.04[a]
pH	4.22 ± 0.04[a]	4.22 ± 0.04[a]
Reducing sugars (%)	0.61 ± 0.10[a]	0.67 ± 0.07[a]
Total sugars (%)	9.72 ± 0.65[a]	9.94 ± 0.50[a]
Total proteins (mg/100 ml)	161.87 ± 14.76[a]	133.95 ± 13.67[b]
Total phenols (mg/100 ml)	24.47 ± 1.21[a]	19.97 ± 1.03[b]
Ascorbic acid (mg/100 ml)	16.13 ± 0.72[a]	11.66 ± 0.57[b]
Antioxidant activity (%)	74.89 ± 3.40[a]	63.60 ± 5.34[b]
Total plate count (Log)	1.91 ± 0.3[a]	1.69 ± 0.2[b]
Yeast and mould count (Log)	1.78 ± 0.3[a]	1.53 ± 0.2[b]
Overall acceptability	7.22 ± 0.80[a]	6.92 ± 0.69[a]

Mean ± SD (n = 3). Different lowercase superscripts in the same row indicate significant difference ($p < 0.05$) between times.

Effect of KMS concentrations

With increasing concentration of KMS from 200 to 400 ppm, a significant increase in total protein, total phenol, ascorbic acid and antioxidant activity, and a significant decrease in TSS, titratable acidity, reducing sugar, total sugar, TPC, YMC and overall acceptability were observed (Table 4). The decrease in TSS and sugars could be due to the lowered rate of hydrolysis of polysaccharides into monosaccharides which ultimately reduced the enhancement in TSS and sugars by the addition of KMS (Pareek *et al.*, 2015).

The decrease in titratable acidity could be due to the non-involvement of sugar in hydrolysis and thus not promoting the formation of acid by the breakdown of polysaccharides (Wisal *et al.*, 2013). The increase in total proteins could be due to the low interaction of organic acids with sugar as suppressed by KMS (Pareek *et al.*, 2015). The increase in total phenols and ascorbic acid was observed with increasing concentrations of KMS. The possible reason for this could be due to the inactivation of enzymes responsible for the oxidation of total phenols and ascorbic acid by KMS (Pareek *et al.*, 2015). There was an increase in the antioxidant activity of blended beverage samples as the concentrations of KMS were increase which could be due to the increase in the phenolic content and ascorbic acid of the blended beverage samples (Pareek *et al.*, 2015). The addition of KMS decreased the TPC and YMC because KMS could restrict the microbial activity in blended beverage samples by reducing their pH (Chauhan *et al.*, 2002; Sakhale *et al.*, 2012). The overall acceptability of the blended beverage samples showed a decline in sensory scores from 7.68 to 6.23, which could be due to the addition of preservatives responsible for the loss of volatile aromatic substances which in turn affected the taste (Rawat and Pokhriyal, 2014).

Table 4. Effect of KMS concentrations on the physico-chemical, microbial and sensory properties of the blended juice during storage.

Parameter	KMS concentration		
	200 ppm	300 ppm	400 ppm
TSS (°B)	10.35 ± 0.06^{a}*	10.30 ± 0.08^{b}	10.20 ± 0.08^{c}
Titratable acidity (%)	0.42 ± 0.01^{a}	0.39 ± 0.02^{a}	0.37 ± 0.03^{b}
pH	4.22 ± 0.04^{a}	4.22 ± 0.05^{a}	4.22 ± 0.03^{a}
Reducing sugars (%)	0.69 ± 0.10^{a}	0.66 ± 0.06^{a}	0.56 ± 0.10^{b}
Total sugars (%)	10.52 ± 0.23^{a}	9.67 ± 0.16^{b}	9.30 ± 0.21^{c}
Total proteins (mg/100 ml)	129.52 ± 11.18^{a}	141.52 ± 18.56^{b}	167.69 ± 19.47^{c}
Total phenols (mg/100 ml)	18.60 ± 0.92^{a}	21.58 ± 0.96^{b}	24.97 ± 1.70^{c}
Ascorbic acid (mg/100 ml)	9.75 ± 0.34^{a}	10.53 ± 0.59^{b}	12.41 ± 1.94^{c}
Antioxidant activity (%)	66.16 ± 3.38^{a}	71.88 ± 6.33^{a}	79.20 ± 3.16^{b}
Total plate count (Log)	1.97 ± 0.1^{a}	1.78± 0.1^{b}	1.65 ± 0.2^{c}
Yeast and mould count (Log)	1.61 ± 0.2^{a}	1.46 ± 0.1^{b}	1.40 ± 0.1^{c}
Overall acceptability	7.68 ± 0.30^{a}	7.30 ± 0.36^{b}	6.23 ± 0.46^{c}

Mean ± SD (n = 3). Different lowercase superscripts in the same row indicate significant difference ($p < 0.05$) between concentrations.

Effect of different storage durations

Storage durations affected TSS, titratable acidity, reducing sugar, TPC and YMC in a positive manner and total protein, total phenols, ascorbic acid, antioxidant activity and overall acceptability in a negative manner (Table 5, Figure 1). The TSS content of blended juice slightly increased during storage which could be due to the hydrolysis of polysaccharides into monosaccharides and soluble disaccharides (Gould, 1983). The increase in titratable acidity could be due to the degradation of sugars into carboxyl acids or degradation of pectin into pectinic acid during storage (Kinh *et al.*, 2001). These results agree with the findings of Sharma *et al.* (2014) who reported an increase in the titratable acidity at different storage temperatures i.e., 6°C and 37°C. They further reported that the intensity of the increase in titratable acidity was more at 37°C as compared to 6°C of storage in lemon drink. The reducing sugars of blended beverage samples in the present work increased with increasing storage period, which could probably be due to the conversion of sucrose into reducing sugar primarily due to acids (Wisal *et al.*, 2013). The effect of the storage on the total sugars was non-significant. Similar results were put forth by Lanjhiyana *et al.* (2010) in lime-ginger blended squash. The decrease in total protein content could be due to the involvement in non-enzymatic browning by interaction with organic acids and sugar during storage (Pareek *et al.*, 2015). The decrease in the total phenols could be due to the decrease in individual polyphenols since certain polyphenols which are water soluble, sensitive to oxidation which results in their degradation during storage (Bhattacherjee *et al.*, 2011). The ascorbic acid content decreased with the progression of storage time, which could be due to oxidation by residual oxygen, followed by the decomposition which might have been accelerated due to prolonged storage duration. These findings agree with the studies of Ibrahim (2016). The decrease in antioxidant activity could be attributed to the loss of ascorbic acid and total phenolics due to oxidation and other reactions (Kapasakalidis *et al.*, 2006; Oszmiański and Wojdyło, 2009). The TPC and YMC increased during storage. Similar trend has been reported by Chauhan *et al.* (2002) in sugarcane juice. The overall acceptability of the samples decreased with the advancement in storage duration. This could be due to the increase in acidity upon storage, decreased flavour and loss of colour of the blended beverage samples thereby resulting in browning (Wisal *et al.*, 2013). Although the sensory scores for quality characteristics decreased during storage, they remained well within the acceptable limit.

Table 5. Effect of different storage durations on the physico-chemical, microbial and sensory properties of the blended juice during storage.

Parameter	Storage duration			
	0 day	30 days	60 days	90 days
TSS (°B)	10.22 ± 0.09[a]*	10.36 ± 0.07[b]	10.38 ± 0.07[c]	10.46 ± 0.06[d]
Titratable acidity (%)	0.39 ± 0.03[a]	0.42 ± 0.01[a]	0.43 ± 0.01[b]	0.44 ± 0.03[c]
pH	4.22 ± 0.03[a]	4.19 ± 0.03[a]	4.17 ± 0.03[b]	4.16 ± 0.03[c]
Reducing sugars (%)	0.64 ± 0.03[a]	0.68 ± 0.02[b]	0.70 ± 0.03[c]	0.71 ± 0.03[d]
Total sugars (%)	9.83 ± 0.57[a]	9.85 ± 0.69[a]	9.86 ± 0.61[a]	9.86 ± 0.66[a]
Total proteins (mg/100 ml)	142.91 ± 19.41[a]	136.12 ± 16.13[a]	133.87±10.55[a]	131.86± 11.60[a]
Total phenols (mg/100 ml)	25.72 ± 1.51[a]	23.44 ± 2.12[b]	20.93 ± 1.18[c]	19.58 ± 1.58[d]
Ascorbic acid (mg/100 ml)	14.90 ± 1.59[a]	12.61 ± 1.08[b]	11.04 ± 1.82[c]	8.62 ± 1.89[d]
Antioxidant activity (%)	68.75 ± 3.27[a]	64.31 ± 2.56[b]	62.63 ± 4.52[c]	62.19 ± 4.13[d]
Total plate count (Log)	1.81±0.1[a]	1.83 ± 0.1[a]	1.84 ± 0.1[b]	1.89 ± 0.2[c]
Yeast and mould count (Log)	1.78 ± 0.1[a]	1.79 ± 0.1[a]	1.82 ± 0.1[b]	1.85 ± 0.2[c]
Overall acceptability	7.07 ± 0.53[a]	7.02 ± 0.56[a]	6.42 ± 0.51[b]	6.10 ± 0.57[c]

Mean ± SD (n = 3). Different lowercase superscripts in the same row indicate significant difference ($p < 0.05$) between durations.

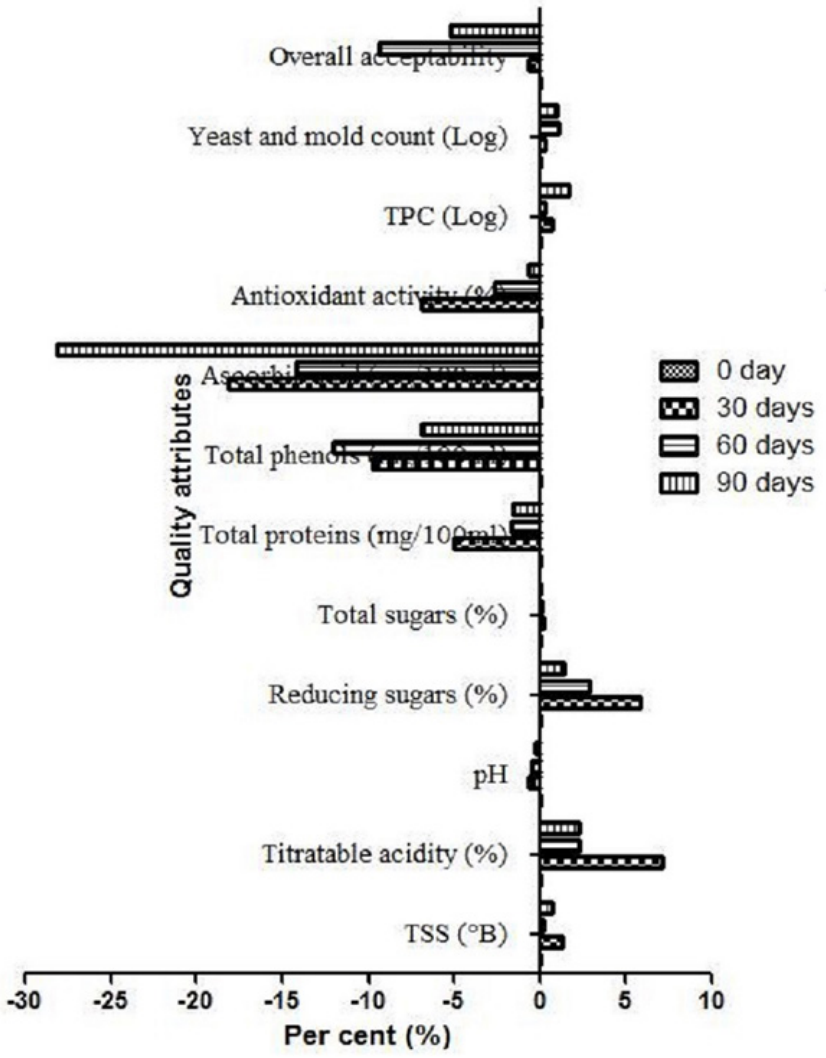

Figure 1. Effect of different storage durations on the physico-chemical, microbial and sensory properties of the juice during storage (in term of percentage of increase or decrease as compared to zero day of storage).

Conclusions

The present work demonstrated that different temperatures, durations, preservatives and storage durations had an independent and significant effect on the quality attributes of the blended beverage samples. With increase in the temperature, duration and KMS concentration; a small but significant decrease in the sensory and phytochemicals was observed. But in fact, it was low which indicated that even at lower intensity, these hurdles were effective. For storage, similar trend was observed but in a negative manner. However, blended beverage samples were acceptable up to three months of storage based on the different quality attributes. Therefore, it can be concluded that the blended radish juice could be preserved using the hurdle technology; pasteurised at 70°C for 10 min followed by addition of 200 ppm KMS for three months at refrigerated condition.

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