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Analysing Canonical Correlation of BBD-RSM Optimized Process Parameters for the Development of Spinach-Lemon-Tulsi Beverage

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HIGHLIGHTS

- The process parameters was standardized for blanching time, L-ascorbic acid and lemon juice using BBD RSM.
- Canonical Correlation Analysis (CCA) was done to see the effect of variables on the product quality.
- Physiochemical and antioxidant properties of all the runs were studied and presented in the paper.
- Numerical optimization of Beverage was carried out for recommendation to the end users.

Abstract: The effect of three independent variables *i.e*. blanching time (3- 7 minutes), L-ascorbic acid (25-75 mg) and lemon juice (2.5-7.5 mL) was studied on the quality of spinach- lemon- *tulsi* ready-to-serve (RTS) beverage. The process was optimized using BBD- RSM methodology and the linear effect of the independent variables on the quality parameter was studied using Canonical Correlation Analysis (CCA). A total of 15 runs including 3 centre points per block were obtained in BBD RSM and each run was analysed for different quality parameters *i.e.* total soluble solids (TSS), titratable acidity, reducing sugars, total sugars, ascorbic acid, antioxidant activity and overall acceptability. TSS, titratable acidity, reducing sugars, total sugars, ascorbic acid, DPPH free radical scavenging activity and overall acceptability ranged from 10.60 to 13.20°B, 0.37 to 0.50 % as citric acid, 1.70 to 3.10%, 6.30 to 8.20%, 42.30 to 64.90 mg/100 gm, 64.00 to 80.20%, and 6.6-8.3 (on a 9-point hedonic scale), respectively. The results analysed by BBD-RSM suggested a blanching time of 5.47 minutes, 75 mg L-ascorbic acid and 6.24 mL lemon juice are the optimum conditions for the preparation of spinach-lemon-*tulsi* RTS beverage. As per the CCA, the total variance of the independent variables on the dependent variables was 79%. L-ascorbic acid and lemon juice were found to have a positive

effect on dependent variables. The model also suggested that an increase in blanching time could harm the quality of the beverage.

Keywords: canonical correlation analysis; response surface methodology; physico-chemical properties; antioxidant activity; overall acceptability; beverage.

INTRODUCTION

Canonical correlation analysis (CCA) is one of the basic multivariate statistical methods other than principal component analysis (PCA) and partial least squares (PLS) which is majorly used in the analysis of complex data [1,2]. CCA calculate the effect of loadings of independent variables by analysing the equation to combine linear variables in a respective set and then evaluates their correlation from the derived equation [3]. It is the most appropriate method to see the effect of each independent variable on the quality parameters of the product [4]. In this study, the effect of process parameters viz. blanching time, L-ascorbic acid and lemon juice on the quality of spinach-*tulsi* beverage (pre-optimized in a separate process) was studied. Spinach (*Spinacia oleracea* L.) is the most widely grown green leafy vegetable in the world and is of high biological value [5]. It is a good source of carotenoids (a precursor of vitamin A), other vitamins especially C, E and K, minerals (Mn, Mg, Fe, K, Ca, Se) and dietary fibre [6]. Spinach leaves also possess a good antioxidant activity [7,8] and hypolipidemic activity [9]. *Tulsi* (*Ocimum tenuiflorum*) leaves are used in the Ayurvedic system of Indian medicine to cure several diseases like cough, cold and oxidative stress *etc*. Scientific studies also support this claim and this herb has been used in the preparation of health drinks and beverages [10]. The regular inclusion of vegetables especially leafy vegetables in the diet reduces the risk of cancer, cardiovascular disease and is also helpful in gut-related problems [11,12]. The preparation of novel products from vegetables like RTS beverages can further enhance their consumption. Therefore, a process was optimized to develop a spinach-*tulsi* based beverage. Generally, leafy beverages are rich in functional properties but poor in sensory attributes. Such beverages are not even stable in storage and the blackening of the product can be observed after a few days of storage. Therefore, in the present study, the quality parameters of the product were improvised by the intervention of the process parameters like blanching of the spinach leaves before juice extraction, the addition of the L-ascorbic acid to prevent blackening of juice and the addition of the lemon juice to prevent the blackening as well as improvement of the sensorial properties of the beverage. The effect of each process parameter on the quality attributes of the product was also studied using CCA. This can further help the researchers to understand the effect of these parameters on the quality of similar beverages.

MATERIAL AND METHODS

Raw materials

The raw materials *i.e.* spinach, tulsi leaves and lemon were collected from Vegetable Experimental Farm, Medicinal and Aromatic garden, and Fruit Demonstration Farm of the Rani Lakshmi Bai Central Agricultural University, Jhansi. Table sugar was procured from the local market.

Pretreatments

Freshly harvested leaves were sorted and washed under running tap water to remove the dust and other adhering particles. The surface moisture of the leaves was further air-dried by spreading under the fan and further used for the preparation of the beverage.

Optimization of the process parameters

To get the best combination and wider acceptable beverage BBD-RSM (Box and Behnken Design of Response surface methodology) method was applied. The independent variables selected for optimization were lemon juice (A) concentration *viz*. 2.5, 5 & 7.5 ml, L-ascorbic acid (B) *viz*. 25, 50 & 75 mg/100g, (C) blanching time *viz*. 3, 5 & 7 minutes in boiling water. The software (Design Expert, 13) generated a total of 15 runs including 3 centre points per block. The responses studied were Total soluble solids (TSS), titratable acidity (TA), reducing sugars, total sugars, ascorbic acid, antioxidant activity and OAA (overall acceptability). Fisher test was applied to see the best fit model for the individual parameter. The equation in terms of coded factors was used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for

identifying the relative impact of the factors by comparing the factor coefficients. After analysis, the best combination suggested by the software (Design Expert, 13) was prepared and comparison was made between predicted and experimental values to see the desirability.

Preparation of the beverage

The spinach leaves were blanched for the time optimized from BBD-RSM Design, *tulsi* leaves were added (1:50), crushed in a mixer by adding 10% water, and the juice was collected by filtering through the muslin cloth. The ready-to-serve (RTS) beverage was prepared as per guidelines of the FSSAI, 2006 *i.e.* to 100 ml of juice, 100 g of sugar was added and the final volume was made 1 litre by the addition of the potable grade water.

Physico-chemical analysis

Total soluble solids (TSS) were measured using a hand-held refractometer having a scale of 0-32°B (Erma), titratable acidity (TA) was estimated by titrating the sample against 0.1N NaOH as per the standard procedures [13], reducing sugars and total sugars were estimated by titrating against a known quantity of Fehling's A and Fehling's B solution using methylene blue as an indicator [14]. The ascorbic acid content was measured as per the standard AOAC method (AOAC, 2000). Antioxidant activity (DPPH free radical scavenging activity) was measured as per the method of Brand-Williams and coauthors [15].

Sensory analysis

The sensory evaluation of the product was conducted by a panel of 6 semi-trained judges (three males and three females) on a 9-point hedonic scale (whereby, 9 = Like extremely; 8 = Like very much; 7 = Like; 6 = Like slightly; 5 = Neither like nor dislike; 4 = Dislike slightly; 3 = Dislike moderately; 2 = Dislike; 1 = Dislike extremely) (Joshi 2011). The judges were provided with coded samples in separate chambers and were allowed to rinse their mouths with water in between each sampling.

Statistical analysis

Table 1. Effect of treatment combinations (blanching time, ascorbic acid and lemon juice) on quality parameters of optimization of spinach-lemon-tulsi RTS beverage

Run Order	Process variables			Responses						
	Lemon juice (mL)	Ascorbic acid (mg)	Blanching (min.)	TSS (°B)	Titratable Acidity (%)	Reducing Sugars (%)	Total Sugars (%)	Ascorbic acid (mg/ 100g)	Antioxidant activity (%)	OAA (Maximum 9)
1	5	25	7	10.60	0.42	2.80	7.40	42.30	64.00	7.60
2	2.5	75	5	11.80	0.38	1.70	6.50	64.30	79.80	7.20
3	5	75	3	11.80	0.43	2.90	7.30	64.50	80.20	8.30
4	7.5	50	7	12.80	0.49	3.10	8.00	52.50	75.00	7.00
5	7.5	50	3	13.00	0.50	3.10	8.20	54.30	75.80	6.80
6	5	75	7	11.60	0.41	3.00	6.40	63.80	79.10	8.20
7	2.5	50	7	11.00	0.39	1.90	6.30	52.10	74.50	6.80
8	2.5	50	3	11.40	0.42	2.10	6.50	53.10	74.50	6.80
9	7.5	25	5	12.80	0.47	2.60	7.30	46.50	68.50	6.60
10	2.5	25	5	11.20	0.37	1.70	6.60	43.90	65.50	7.00
11	7.5	75	5	13.20	0.49	2.90	8.20	64.90	79.50	6.70
12	5	50	5	11.40	0.41	2.10	6.80	53.60	74.90	8.10
13	5	25	3	11.60	0.40	2.00	7.10	48.40	71.60	8.10
14	5	50	5	11.40	0.40	2.00	7.10	53.80	75.20	8.20
15	5	50	5	11.20	0.40	2.20	7.10	53.80	75.60	8.20

Table 2a. Analysis of variance and regression analysis of fractional factorial design for estimated coefficient to the preparation of spinach-lemon-tulsi RTS beverage

Variables	df	Estimated coefficient						
		TSS (°B)	Titratable Acidity (%)	Reducing Sugars (%)	Total Sugars (%)	Ascorbic acid (mg/ 100g)	Antioxidant activity (%)	OAA (Maximum 9)
Model	9	11.33	0.403	2.100	7.120	53.73	74.250	8.170
A-Lemon	1	0.80	0.049	0.538	0.725	0.600	0.563	-0.088
B-Ascorbic acid	1	0.28	0.006	0.175	0.000	9.550	6.130	0.138
C-Blanching	1	-0.23	-0.005	0.088	-0.125	-1.200	-1.190	-0.050
AB	1	-0.05	0.003	0.075		-0.500		-0.025
AC	1	0.05	0.005	0.050		-0.200		0.050
BC	1	0.20	-0.010	-0.175		1.350		0.100
A^2	1	0.78	0.030	0.000		-0.292		-1.250
B^2	1	0.13	-0.005	0.125		1.460		-0.046
C^2	1	-0.07	0.017	0.450		-0.442		-0.071
Lack of fit	3							
R^2		0.99	0.984	0.934	0.775	0.997	0.909	0.957
Adj R^2		0.97	0.956	0.814	0.714	0.991	0.885	0.880
C.V. %		1.18	2.08	9.34	4.74	1.31	2.27	3.12

*Significant at $p<0.05$

Table 2b. Analysis of variance of fractional factorial design for F-values to the preparation of spinach-lemon-tulsi RTS beverage

Variables	df	F-values						
		TSS (°B)	Titratable Acidity (%)	Reducing Sugars (%)	Total Sugars (%)	Ascorbic acid (mg/ 100g)	Antioxidant activity (%)	OAA (Maximum 9)
Model	9	49.77	34.58	7.83	12.66	167.54	36.78	12.44
A-Lemon	1	264.8*	242.7*	45.77*	36.89*	5.70	0.89	1.14*
B-Ascorbic acid	1	31.29*	3.99	4.85	0.00	1443.84*	105.48*	2.81
C-Blanching	1	20.95*	2.55	1.21	1.10	22.80*	3.96	0.37
AB	1	0.52	0.32	0.45		1.98		0.05
AC	1	0.52	1.28	0.20		0.32		0.19
BC	1	8.28*	5.11	2.43		14.43*		0.74
A^2	1	117.2*	41.25*	0.00		0.62		106.46
B^2	1	3.40	1.38	1.14		15.54*		0.14
C^2	1	0.85	13.76*	14.81*		1.43		0.34
Lack of fit	3	1.75	3.25	7.75	4.42	62.50	27.98	26.25

*Significant at $p<0.05$

Table 3. Numerically optimized conditions for preparation of spinach lemon-*tulsi* RTS beverage

Lemon juice (mL)	Ascorbic acid (mg)	Blanching (min.)		Responses							
				TSS (°B)	Titratable Acidity (%)	Reducing Sugars (%)	Total Sugars (%)	Ascorbic acid (mg/ 100g)	Antioxidant activity (%)	OAA (Maximum 9)	Desirability
6.24	75	5.47	Predicted (through RSM)	12.47	0.45	2.89	7.42	64.50	80.06	7.91	0.87
			Experimental	12.20	0.45	2.95	7.30	64.35	79.30	7.45	
			Differences	0.27	0.00	-0.07	0.12	0.15	0.76	0.46	

Table 4. Eigenvalue, variance and variance explained of canonical correlations analysis of spinach lemon-*tulsi* RTS beverage

Set no.	Correlation	Eigen-value	Variance (%)	Wilks Statistic	Set 1 by Self	Set 1 by Set 2	Set 2 by Self	Set 2 by Set 1	F	Num D.F	Sig.
1	0.997	149.51	79.0	0.000	0.333	0.331	0.402	0.399	15.50	21.00	<0.001
2	0.987	38.34	20.8	0.019	0.333	0.325	0.287	0.279	6.27	12.00	0.002
3	0.506	0.35	0.2	0.744	0.333	0.085	0.068	0.017	0.48	5.00	0.780

To see the individual effect of independent variables *i.e.* blanching time, L-ascorbic acid and lemon juice concentration on the quality parameters *i.e.* total soluble solids, titratable acidity, reducing sugars, total sugars, ascorbic acid, antioxidant activity and overall acceptability the results of 15 runs suggested by BBD RSM was further statistically evaluated by using CCA, IBM-SPSS statistical software version 20. The software generates an eigenvalue that is used to calculate the level of variance and determine the effect of independent variables on the quality parameters of the product. The variance in *per cent* is calculated manually by dividing each set of eigenvalues by the total number of eigenvalues. Since there were three sets of canonical correlation the only one which showed the maximum variance was selected for the study and the rest two were rejected as CCA is used to estimate canonical coefficients (x_{i1}, x_{i2},…, x_{ip} and y_{i1}, y_{i2},…, y_{ip}) [16].

RESULTS AND DISCUSSION

Effect on TSS

Due to the interaction between different independent variables the TSS values varied from 10.6 to 13.2°B (Table 1). The quadratic model of F-value (49.77) implies the model is significant for TSS. All three process parameters *i.e*. lemon juice (mL), L-ascorbic acid (mg) and blanching time (min) had a significant effect on TSS at the linear level, further, ascorbic acid and blanching time also had a significant effect at the interactive level. The lime juice also had a significant effect on TSS at the quadratic level (p<0.05) (Table 1). The regression equation (R^2) accounts for 99% of the total variation in TSS (Table 2 a&b). Further, from Figure 1(a), it is very clear that there was an increment in TSS with the addition of lemon juice and L-ascorbic acid, while, the increase in blanching time from 3 to 7 min. had a significant negative effect on TSS. The increment in TSS content might be due to the contribution of soluble solids by the addition of ascorbic acid and lemon juice. A similar increment in TSS was also reported in a previous study on cashew nut ameliorated in mango juice [17] and microgreen and fruit-based RTS beverages [18].

Effect on titratable acidity

The titratable acidity ranged from 0.37-0.50% as CA. It could be seen from the data in table 1 that lemon juice had a significant effect on titratable acidity at the linear level and lemon juice and blanching time had a significant effect ($p \leq 0.05$) at a quadratic level. The lack of fit was non-significant which indicated that the model was significant and the regression equation (R^2) accounts for 98.4% of the total variation in titratable acid content in the beverages (Table 1). Figure 1(b) shows that the addition of L-ascorbic acid from 25 to 75 mg and lemon juice from 2.5 to 7.5 mL had significantly increased the titratable acidity of the beverage. Both L-ascorbic and lime juice imparts acidity to the food products [19]. Rodbotten and coauthors [20] also observed similar changes in titratable acidity in the preparation of different acceptable combinations in apple juice.

Effect on reducing sugars

The reducing sugars ranged in-between 1.7 to 3.1% for different runs (Table 1). Lemon juice had a significant positive effect on the reducing sugars at the linear level and blanching time had a significant negative effect on the reducing sugars at the quadratic level (Table 1). A similar trend can be seen in Figure 1(c). The reducing sugars content was decreased with the increase in blanching time (3 to 5 minutes), while, an increase in blanching time from 5 to 7 minutes increased the reducing sugars level significantly. The regression equation (R^2) accounted for 93.4% of the total variation in reducing sugars content. The increment in reducing sugars was contributed by the addition of lemon juice *i.e.* ranged from 4.89 -6.31%. Similar results were observed by Salehi [12] in the preparation of vegetable juices. The decrease in reducing sugars on blanching might be due to the formation of hydroxymethyl.

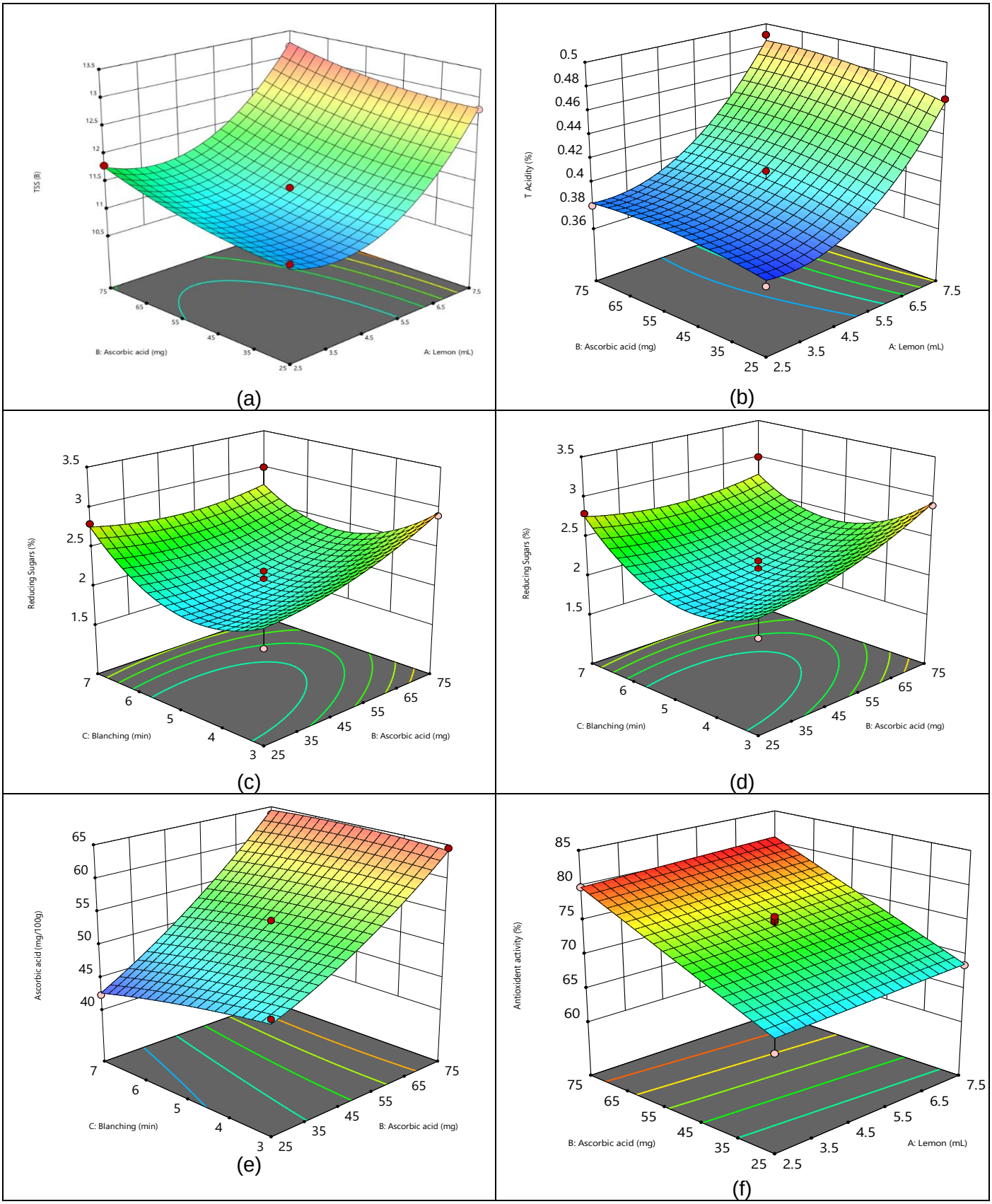

Figure 1. Effect of lemon juice, L-ascorbic acid and blanching time on (a) TSS (°B), (b) titratable acidity (%), (c) reducing sugars (%), (d) total sugars (%), (e) ascorbic acid (mg/ 100g) and (f) antioxidant activity (%) of spinach lemon-*tulsi* RTS beverage furfural (HMF) from reactions involving amino acids and reducing sugars [21,22]. Such formation might have resulted in the degradation of reducing sugars in the beverage with an increase in blanching up to 5 min. While the increase in the reducing sugars with an increase in blanching time might be due to the degradation of higher carbohydrates.

Effect on total sugars

The lemon juice had a significant effect on total sugars content at the linear level. The same can also be seen in figure 1(d). The addition of lemon juice had a direct relationship with total sugars content while L-ascorbic acid and blanching time had no major impact on the total sugars content of the beverage. Similar observations were recorded by Sharma and coauthors [18] in the preparation of microgreen and fruit-based ready-to-serve beverages. The addition of lemon juice (having 7.04 to 8.69 % of total sugars) might also result in an increment in total sugars content in the beverage.

Effect on ascorbic acid and antioxidant activity

The ascorbic acid ranged from 42.3 to 64.9 mg/100g in different beverages prepared as per the suggested runs of prepared beverages. The effect of L-ascorbic acid was significant (p<0.05) on the total ascorbic acid content of beverage at linear, interactive and quadratic levels (Table 2 a&b). The same is evident from Figure 1(d) that the addition of L-ascorbic acid from 25 mg to 70 mg had a positive effect on the ascorbic acid content. The increase in blanching time from 3 to 7 min had a significant negative (p<0.05). effect on the ascorbic acid content at the linear and interactive level. Ascorbic acid is water-soluble and highly labile to heat and this might be responsible for a decrease in ascorbic acid content on blanching [23-25]. The DPPH radical scavenging activity ranged from 64 to 80.2% in the prepared beverage. Similarly, the addition of lemon juice also imparted ascorbic acid to the prepared RTS beverage and has a slightly positive influence on antioxidant activity, while, an increase in blanching time decreased the antioxidant activity (Figure 1(f)). The presence of ascorbic acid and other phenolic compounds are responsible for increased antioxidant activities of the beverage. Fu and coauthors [26] evaluated 62 different fruit types and concluded that the antioxidant capacity of fruits varied and the different combinations enhanced the antioxidant activity of the mixed beverage samples and similar supporting results were obtained by Pereira and coauthors [27], Moura and coauthors [28].

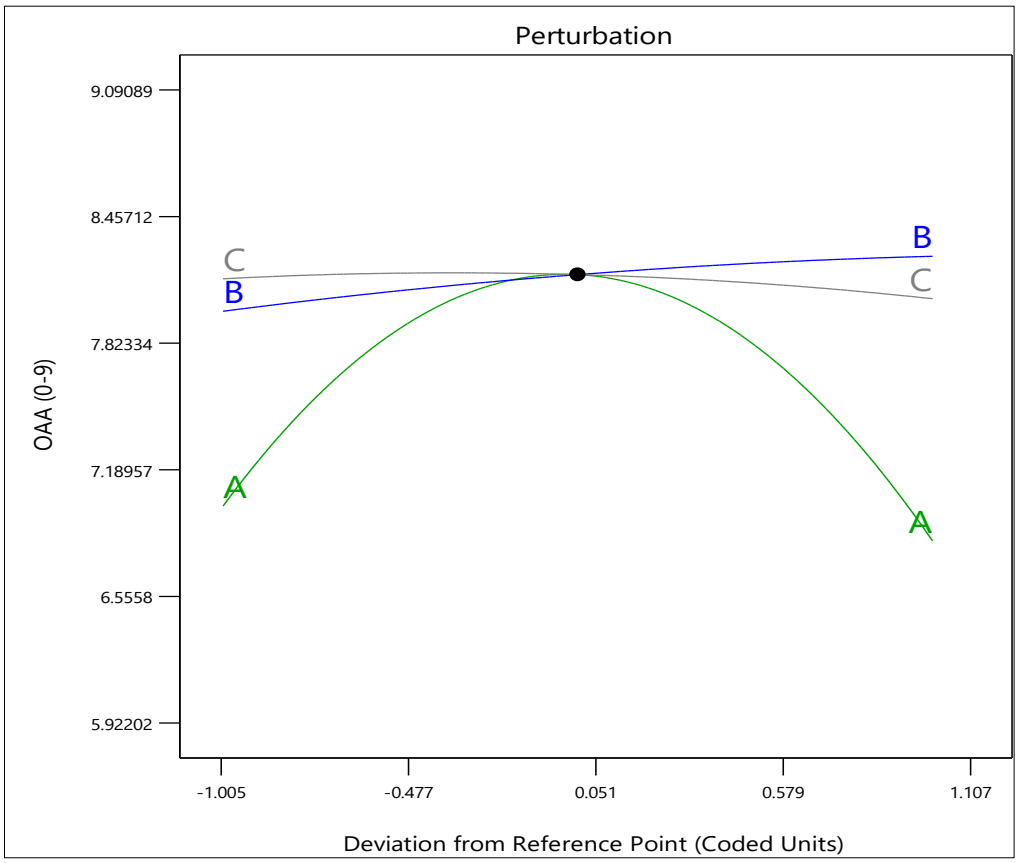

Figure 2. Effect of lemon juice, L-ascorbic acid and blanching time on sensory attribute OAA.

Effect on overall acceptability

The OAA increased significantly (p<0.05) on the addition of lemon juice up to a concentration of 5.0 ml, however, a decrease in OAA was observed with a further increase in the concentration of lemon juice (Figure 2). A higher concentration (5.0 ml) of lemon juice in the beverage might be contributing to acidity which may result in its decreased acceptability. The OAA of juices is greatly influenced by TSS: acid and their suitable ratio are very important for the acceptability of juices [29,30].

Numerical optimization

Based on the studied treatments the software suggested the optimum process parameters *i.e*. lemon juice, L-ascorbic acid, and blanching time for the development of spinach-lemon-*tulsi* RTS beverage as 6.24 mL, 75 mg, and 5.47 min, respectively. This combination had a desirability of 0.87. A detail of the optimized process parameters and their effect on quality attributes is also provided in Table 3.

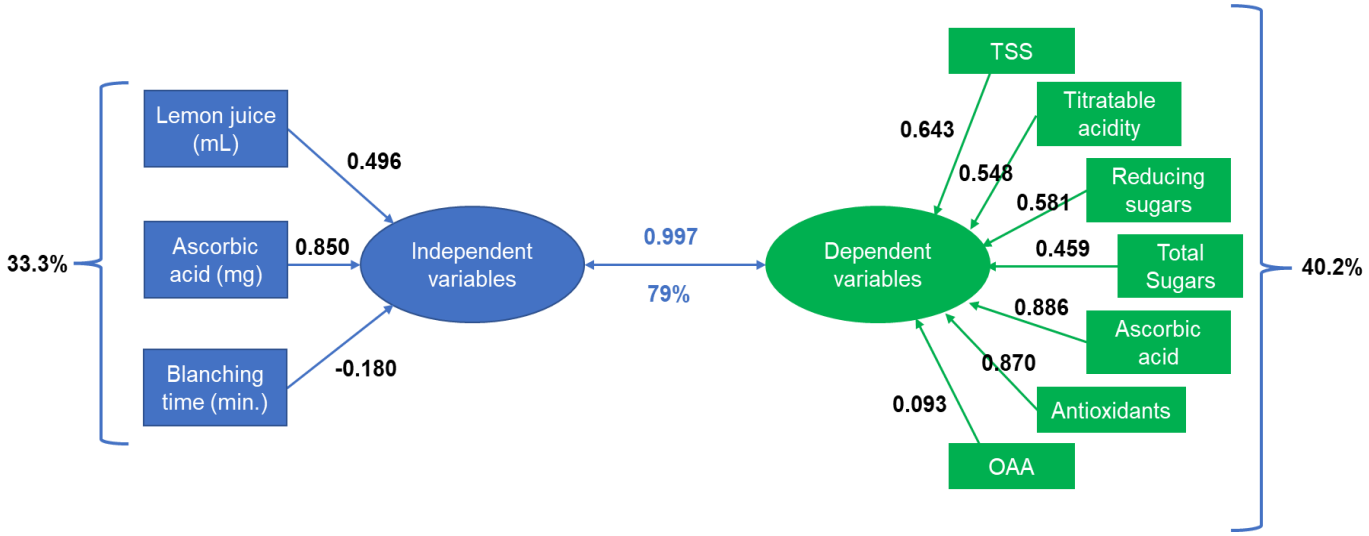

Figure 3. Canonical correlation of independent variables on quality parameter of spinach lemon-*tulsi* RTS beverage.

Canonical correlation analysis (CCA)

As per the canonical correlation analysis, the overall variation of the independent variables on the responses was 79% (Table 4). The values of independent canonical loadings for the variables *i.e*. lemon juice, L-ascorbic acid and blanching time were 0.496, 0.850, and -0.180, respectively (Figure 3). The total variance of these three on the responses is 33.3%, which was obtained from the proportion of variance explained for 'Set 1 by Self' after converting that into *per cent* value. The canonical loading values obtained for the responses *i.e.* TSS, Titratable acidity, reducing sugars total sugars, ascorbic acid antioxidant activity and OAA were 0.643, 0.548, 0.581, 0.459, 0.886, 0.870 and 0.093, respectively. The variance explained by responses on the dependent variables is 40.2% which was obtained from 'Set 2 by Self' of the proportion variable explained (Table 3). Further, from the figure, it is clear that the loading value (+0.496) of lemon juice that even a small increment of lemon juice has a positive influence on responses and the increase in lemon juice content will increase or retain the ascorbic acid concentration in the prepared spinach-lemon-*tulsi* beverage. This will also increase the antioxidant activity and a similar trend is also obtained for other responses except for OAA which did not have much effect. The canonical loading of the independent variable L-ascorbic acid is on the higher side (+0.850) and it can be seen from Figure 3, that it had a positive influence on all studied responses except OAA. The most significant effect of L-ascorbic acid was on ascorbic acid content followed by an antioxidant activity, TSS and reducing sugars. The loading value obtained for the blanching time was -0.180. This suggests that if the blanching time is reduced, an increase in ascorbic acid content and other responses will be observed.

CONCLUSIONS

The optimization process of spinach-lemon-*tulsi* RTS showed that treatments lemon juice, L-ascorbic acid addition and blanching time had a significant role. The addition of lemon juice significantly positively influenced the TSS, titratable acidity, reducing sugars, total sugars and overall acceptability. The addition of L-ascorbic acid in the beverages influenced the TSS, ascorbic acid and antioxidant activity, while the

blanching time had its effect on TSS, titratable acidity reducing sugars and ascorbic acid. The CCA showed the loadings lemon juice and ascorbic acid are positive on independent variables while blanching time was found to harm independent variables. Further, from the present study, it is evident that the numerically optimized juice based on different physico-chemical and sensory properties that the 5 mL spinach juice, blanching for 5.47 minutes followed by addition of 6.24 mL lemon juice, 2mL *tulsi* juice and 75 mg L-ascorbic acid can be recommended for the preparation of spinach lemon *tulsi* RTS beverage.

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