Annals of Biology **38** (1) : 88-95, 2022

Impact Score : 0.32 (Scopus)

Minimization of Limonin and Naringin Content in Kinnow Juice Using Response Surface Methodology

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(Received : August 30, 2021; Accepted : October 09, 2021)

ABSTRACT

The present study was carried out to evaluate the effect of three variables viz., naringinase, florisil and thermosonication on limonin and naringin content of kinnow juice. Box-Behenken design was used for design of experiment. Florisil and naringinase content varied from 20 to 40 g/l and 0.6 to 1.0 ml/100 ml, while thermosonication of juice was performed from 20 to 40 KHz at 30 ^{0}C for 20 min in a portable thermosonicator machine. Limonin and naringin content of treated samples, fresh juice and different parts of kinnow fruits were estimated, their values were as follows : fresh juice (7.8 and 212.0 ppm), peel (46.0 and 13121.0 ppm), pomace (190.0 and 150.0 ppm) and albedo (negligible and 3928.0 ppm). Reduction in limonin and naringin content was found varying from 45.6 to 51.2% and 33.3 to 40.9%, respectively. The least deviation in actual values (limonin–43.8% reduction and naringin–40.0% reduction) of response against predicted values (limonin–45.64% reduction and naringin–40.3% reduction) was found in condition at naringinase (1.0 ml/100 ml), florisil (20 g/l) and thermosonication treatment (37.21 KHz).

Key words : Limonin, naringin, kinnow juice

INTRODUCTION

Citrus fruits are India's third most popular fruits with an estimated production 13976000 MT and area under cultivation 1054000 ha after bananas (31504000 MT) and mangoes (20444000 MT), as per a recent report released by National Horticulture for 2nd advance estimate of year 2019-2020. For the year 2018-2019, citrus fruits production in Haryana was estimated 549332 MT, while the total area under cultivation was 20789 ha. District Sirsa was ranked first with production of 306724 MT followed by Fatehabad (70515 MT), Bhiwani (55226 MT), Narnaul (39687 MT) and Hisar (28417 MT) (Horticulture Department of Haryana, 2018-19) for production of citrus fruit. Mosambi, kinnow, orange, lemon, lime, galgal, tangerine and grapefruit are common citrus fruits grown in India (Purewal and Sandhu, 2021).

Kinnow (*Citrus reticulata* Blanco) is a citrus fruit from family Rutaceae. It is a versatile fruit and grows well at altitudes of 500-1500 MSL, 150-250 cm rainfall, in sub-tropical climate with moderate winters and warm summers. Under adequate management, the tree bears the fruit from third year and continues so for 15-20 years. Kinnow at the maturity time of mid-January to mid-February has a TSS/acid ratio of 12 : 1-14 : 1 and a golden orange colour, while in the initial periods of growth it is green in colour. Kinnow peel is categorized into two sections : exterior brightly coloured layer known as flavedo which contains bright pigments and oil cells, which is used as a raw material to extract essential limonene oil, while albedo is the inner white colour fiber layer, which is high in pectin and is bitter due to presence of naringin. Seeds are found in the middle of the fruit axis in numbers ranging from 5 to 23 which vary according to the size, variety and maturity of the fruit. The seeds are bitter and contain limonin. Expressing, hydraulic pressing and squeezing can all be used to extract juice from citrus fruits (peeled or intact). The juice obtained by peeling, squeezing fruit and soft press extraction in such a manner that seeds are not crushed was shown to be superior to other techniques, with the sole drawback being a lower juice recovery. The average vitamin C content of kinnow is 31.0 mg/100 g, iron 0.4 mg/100 g, calcium 40 mg/100 g, average TSS% 11.5, phosphorus 18 mg/100 g and average acidity is 0.9% (Rattanpal *et al.*, 2017). Currently 95% of kinnow output is destined for the fresh fruit market and during seasonal peaks, there is a

surplus in the market, prices decrease and 25-30% kinnow is wasted. The total post-harvest waste from farms to kinnow customers ranged from 14.87% in the Delhi market to 21.91% in Bengaluru market. Processing of kinnow juice into value added product can be possible solution to these post-harvest losses. Apart from highly perishable and lesser shelf-life citrus juices also possesses another problem of bitterness and this problem is difficult to overcome for any citrus industry. Excessive bitterness in citrus juice is a big issue in the citrus business across the world since it lowers the product's quality and economic value.

Limonin and naringin are two key components, responsible for bitterness in kinnow juice. Limonin is a type of limonoid, which is mainly present in seeds of fruit, while naringin is a type of flavonoid which is present in skin and juice of the kinnow fruit. The concentration of limonin and naringin in citrus fruits may vary with the type of fruit, cultivar, agroclimatic condition where it is grown. It also varies in different parts of fruit (peel, seeds, albedo, etc). Limonin is generated in leaves as a non-bitter form (limonoate A ring lactone) and is transferred to fruit and seeds. As a result, the amount of limonin in each section of the fruit varies.The entire fruit contains almost no limonin but its non-bitter precursor which is limonate-A-ring lactone (LARL) is shown to be intracellularly present in the cytoplasm of cells in membranous sacs, most likely at a neutral to slightly alkaline pH. When these sacs burst during juice extraction, the LARL comes in contact with the juice's total acidic pH which catalyses ring closure to form limonin. The presence of an endogenous enzyme limonin D-ring lactone hydrolase accelerates this conversion by catalysing D-ring lactoneization under acidic circumstances or opening the o-ring of limonin under alkaline circumstances. Kumar *et al.* (2020) reported that limonin content kinnow fruits varied in different parts, seeds (224.37 ppm), pulp (114.91 ppm), flavedo (56.95 ppm) and juice (20.33 ppm) whereas negligible amount was found to be in the albedo portion. Kaur *et al.* (2018) reported limonin content of fresh kinnow juice to be 7.50 ppm. Naringin is a water-soluble bitterness causing compound present in the fruit membrane and albedo and is extracted into fruit juice. Peel has maximum naringin content followed by seeds of citrus fruits, that's why citrus fruits are scraped before extraction of juice. It is most bitter flavonoids amongst naringin, poncirin, neohesperidin and neoeriocitrin. Kumar *et al.* (2020) reported the naringin content in different parts of kinnow fruit and found that the highest concentration was in flavedo (13589.82 ppm), albedo (4037.83 ppm) seed (710.82 ppm), pulp (131.84 ppm) and the least was found to be in juice (105.67 ppm).

Removal of bitterness causing compounds from juice, removal of physical barriers, use of flavour enhancers and bitter compounds scavengers (salt, sugar, florisil), enzymatic (naringinase and α-L-rhamnosidase) treatment, genetic engineering techniques are some basic approaches used to minimize bitterness. Researchers have already used lye treatment, addition of sugars, β-cyclodextrin, hot water treatment, cellulose acetate layers and enzymatic methods using microbial consortia as a solution of this problem. Attempts have already been made to reduce these two principal components in the citrus juices but it has not been eliminated totally also the treatments given changed the sensory and nutritional quality of the juice. The present study focused on the combination of techniques to reduce the bitterness. Response surface methodology was used for design of experiments. Three variables viz., crude naringinase, florisil and thermosonication were used in Box-Behenken Design. The present study was carried out to evaluate the effect of naringinase enzyme (crude form), thermosonication and florisil content in juice to minimize the concentration of limonin and naringin. Florisil is an odorless, white colour adsorbent which is used as a debittering agent, chemically it is activated magnesium silicate (Purewal and Sandhu, 2020). Treatment of kinnow and other citrus juices with florisil resulted in reduction in the limonin and naringin content (Kumar *et al.*, 2020). Thermosonication is a treatment that combines heat and sonication in the product is treated with moderate heat (Aadil *et al.*, 2015), it has minimum effects on juice quality. Gao *et al.* (2021) found that due to sonication the degrees of enzymatic hydrolysis of limonin and naringin were increased to 36.16 and 89.90%, respectively, while the enzymatic hydrolysis duration was reduced by 33%. It was

also reported that sonication increased the activity of enzymes helped in breaking of CO bonds in naringin. Naringinase is a multifunctional enzyme that utilizes naringin as a substrate and converts it to rhamnose and prunin using its alpha-L-rhamnosidase activity subsequently beta-D-glucosidase breaks down prunin into glucose and naringenin. The quantity of bitter naringin is reduced by producing naringenin which has a sweet flavour therefore the enzyme is commonly employed in the debittering of citrus juice (Silva *et al.,* 2017; Kaur *et al.*, 2018; Narnoliya and Jadaun, 2019). The enzyme alpha-L-rhamnosidase hydrolysis naringin to prunin (33% as bitter as naringin) and L-rhamnose as the fruit grows and the least amount of naringin is found in ripened fruit. Prunin is further acted upon by beta-D-glucosidase which converts it to naringenin and D-glucose. Thus, naringin hydrolysis mediated by alpha-L rhamnosidase and beta-D-glucosidase generates substantially debittered, consumer acceptable citrus juice.

MATERIALS AND METHODS

The present study was carried out in the Department of Food Technology, Guru Jambheshwar University of Science & Technology, Hisar. Fresh kinnow fruits were procured from the local market of Hisar, Haryana. *Aspergillus niger* was grown in 250 ml conical flask having 150 ml sterilized nutrient broth mixed with naringin (1 g). Flask was kept in shaking incubator at 25-27°C followed by centrifugation (5000 rpm for 10 min). Supernatant thus collected was used in the present study as crude form of the naringinase enzyme. Florisil used in study was supplied by Central Drug House (CDH) Private Limited. Other chemicals used in this study were also laboratory grade and supplied by reputed agencies.

Fresh and healthy fruits free from blemish and any kind of spots were taken for juice extraction. Fruits were washed and dried before slicing them into two halves with the help of a knife. Two halves thus obtained were placed on a cone (perforated) of a potable juice extracting machine. The fruit sacs were compressed between fixed cone and an upper moving arm, manually. Compression led to extraction of juice which was collected in glass beaker (500 ml), later it was transferred to bottles after removal of fibrous part by straining through a sieve. This technique did not crush the seeds and also did not allow the pulp to mix in the juice, which was required for present research work. Incorporation of pulp and crushing of seed added bitterness to juice. The albedo portion of the fruit was also removed manually. Pomace used in study was collected from juicer machine after extraction of juice (albedo free fruit). Peel, pomace and albedo were subjected to cutting into smaller pieces with help of kitchen knife. Freeze drying was carried out by placing the smaller pieces of each type of sample in petri plates to access the actual content of naringin and limonin. Samples were freezed for 3 h at – 2°C and then were freeze-dried for 10 h. Samples collected after drying were grounded using kitchen grinder (Sujata).

To minimize the concentration of bitterness causing compounds, A Box-Behenken design was used. Three variables, namely, naringinase enzyme, thermosonication and florisil were selected to decrease the limonin and naringin content of freshly extracted kinnow juice. The level of this variable was selected on the basis of preliminary studies and literature reviewed. Florisil and naringinase content varied from 20 to 40 g/l and 0.6 to 1.0 ml/100 ml, respectively (Table 1). Thermosonication of juice samples was performed from low (20 KHz) to high frequency (40 KHz) at 30°C for 20 min in a portable thermosonicator machine. Each variable was varied at (-1), (0) and (+1) levels. Total 17 experiments were suggested by Design expert trial version (6.0) software. Out of these 17 experiments, there were five center point experiments. For each experiment, 200 ml kinnow juice was taken in 500 ml glass beaker. Initially florisil treatment was given by transferring the weighed amount of florisil calculated according to design of experiment, followed by stirring at 2 min using high speed

Table 1. Values of independent variables at three levels of the Box-Behnken design

Independent variables	Code	Levels in coded form		
		-1	0	+1
Naringinase (ml/100 ml)	x_1	0.6	0.8	1.0
Florisil (g/l)	x_2	20	30	40
Thermosonication (KHz)	x_3	20	30	40

laboratory stirrer. After stirring samples were allowed to rest for 2 min for sedimentation of adsorbent and then straining of juice was carried out by using double muslin cloth. In the next step thermosonication (Power sonic 410) of juice sample treated with florisil, was carried out for 20 min at 30°C at different frequencies by transferring the juice in falcon tube (45 ml) and for each sample 03 tubes were taken. In the end naringinase treatment was given by transferring the calculated amount of enzyme in juice sample (50 ml) and incubating it for 12 h at room temperature. After treating the samples with all the three techniques, the samples were analyzed for limonin and naringin content.

Limonin content of kinnow juice was determined using spectrophotometric method. Reagents used were : Acetonitrile, chloroform, glacial acetic acid, perchloric acid (70%), 4 (dimethylamino) benzaldehyde and D limonin. Burnham's reagent was prepared by -0.1 g DMAB, 3 ml acetic acid and 2.4 ml perchloric acid. Standard stock solution of limonin (10 ppm) : 0.5 ml limonin was taken in 50 ml volumetric flask and volume was made up to 50 ml by using acetonitrile. For preparation of standard curve 0.2, 0.4, 0.6, 0.8 and 1 ml of stock solution was taken and the volume was made up to 1 ml by distilled water and further following procedure was followed. The juice samples were diluted two times and then centrifuged for 10 min at 3,000 rpm and the supernatant was taken for further estimation and in the case of freeze-dried samples. One g of sample was weighed and diluted 100 times with distilled water; it was then centrifuged at 4000 rpm for 5 min and was filtered using Whatman Paper No. 4.

In order to eliminate polar substances, 1 ml of the supernatant was placed into a test tube, succeeded by 2 ml chloroform. A shaker was used to thoroughly mix the mixture for 2 min which led to phase separation, 1.5 ml of Burnham reagent (0.1 g 4- (dimethylamino) benzaldehyde, glacial acetic acid (3 ml), and perchloric acid (2.4 ml) was added to 1 ml of the chloroform phase. To get the most reddish colour, this combination was left at room temperature for 30 min. At 503 nm, the top phase's absorbance was observed. After noting down the absorbance, the concentration of the samples was calculated from the standard curve graph (absorbance vs. concentration) in ppm.

Ten mg of standard naringin was weighed and the volume was made up to 50 ml with distilled water. 0.2 , 0.4 , 0.6, 0.8 and 1 ml of the stock solution was added in separate test tubes and the volume was made up to 1 ml with distilled water followed by 10 ml of 90% diethylene glycol and 0.2 ml of 4 M sodium hydroxide. The test tube was incubated for 5 min at room temperature and the readings were noted using UV spectrophotometer at 420 nm. The juice samples were diluted 10 times and in the case of freeze-dried samples 1 g of sample was weighed and diluted 100 times with distilled water, it was then centrifuged at 4000 rpm for 5 min and was filtered using Whatman Paper No. 4. The readings were noted down for the absorbance of each sample. The concentrations were calculated from the standard curve graph (absorbance vs. concentration).

Per cent minimization of limonin and naringin content = Initial content (Fresh juice) - Final content (processed juice)/Initial content (Fresh juice) × 100

A Box-Behnken experimental design was used to study the effect of independent variables on dependent variables. The independent variables level was selected through preliminary trials and feasibility of operating condition. The experimental design involved 17 experiments with five combinations of the central point and presented in Table 2. Design Expert trial version 13 (State-Ease, Minneapolis, MN) was used for analysis of variables. The response (limonin and naringin content) for different experimental combinations was related to the coded variables (xi, i=1, 2 and 3) by a second-degree polynomial equation.

$$Y=\beta0+\beta1x1+\beta2x2+\beta3x3+\beta12x1.x2+\beta13\,x1.x3+\beta23x2.x3+\beta11x12+\beta22x22+\beta33x32$$

Where, Y is the estimated response, the coefficients of the polynomial were

Table 2. Limonin and naringin content different parts of kinnow fruit and juice

S. No.	Part of fruit	Naringin (ppm)	Limonin (ppm)
1.	Fresh juice	212.0	7.8
2.	Peel (flavedo)	13121.0	46.0
3.	Pomace	150.0	190.0
4.	Albedo	3928.0	Negligible

represented by β0 (constant), β1, β2, β3 (linear effects); β12, β13, β23 (interaction effects); β11, β22, β33 (quadratic effects). The adequacy of the model was determined by evaluating the lack of fit, coefficient of correlation (R^2) and the Fisher test value (F-value) obtained from the analysis of variance (ANOVA). The regression coefficient was used to generate response surface three-dimensional plots by keeping one response at the centre level.

RESULTS AND DISCUSSION

Limonin and naringin contents in different parts of kinnow fruits were fresh juice (7.8 and 212.0 ppm), peel (46.0 and 13121.0 ppm), pomace (190.0 and 150.0 ppm) and albedo (negligible and 3928.0 ppm) as shown in Table 2. Limonin content was highest in the pomace, while naringin was highest in the albedo. Similar results were also reported by Kumar *et al.* (2020). Results indicated that if the peel, pomace and albedo parts were removed carefully from the fruit then the amount of limonin and naringin in the juice could be reduced to a significant amount.

Reduction in limonin and naringin content was found varying from 45.6 to 51.2% and 33.3 to 40.9%, respectively (Table 3). F-value 129.64 implied that model term was significant for naringin content of juice (Table 4). Only linear effect of variables was found significant, whereas interactive effect and quadratic effect was not observed. R square value 0.9676 and adj-R square value was also closer to 1. The cube plot (Fig. 1) was used to illustrate the combined effect of three variables on naringin percentage reduction of kinnow juice. The values indicated in the plots were predicted by software after analysis of variance. It was realized that the maximum reduction in naringin per cent could be obtained at highest level of all selected variables. While the minimum reduction in naringin per cent would be at minimum level of variables. From the cube plot for naringin, it was predicated that the maximum reduction in naringin (41.50%) was at (+1) level of all selected

Table 3. The Box-Behnken design matrix used for treatment and the responses of kinnow juice

S. No.	Naringinase (ml/100 ml)	Florisil (g/l)	Thermosonication (KHz)	Naringin (% reduction)	Limonin (% reduction)
1.	0.6	20	30	33.3	45.9
2.	1.0	20	30	40.9	45.6
3.	0.6	40	30	34.4	49.7
4.	1.0	40	30	41.5	49.0
5.	0.6	30	20	34.0	48.6
6.	1.0	30	20	39.6	46.0
7.	0.6	30	40	34.7	49.0
8.	1.0	30	40	40.0	48.2
9.	0.8	20	20	36.0	45.8
10.	0.8	40	20	37.0	49.5
11.	0.8	20	40	37.1	46.2
12.	0.8	40	40	38.6	51.2
13.	0.8	30	30	37.4	48.5
14.	0.8	30	30	37.4	48.5
15.	0.8	30	30	37.4	48.5
16.	0.8	30	30	37.4	48.5
17.	0.8	30	30	37.4	48.5

Table 4. Analysis of variance for naringin content of kinnow juice

Source	Sum of squares	d. f.	Mean square	F value	Prob > F	
Model	85.93	3	28.64333	129.7433	< 0.0001	Significant
x_1	81.92	1	81.92	371.0662	< 0.0001	
x_2	2.205	1	2.205	9.987805	0.0075	
x_3	1.805	1	1.805	8.175958	0.0134	
R-squared	0.96768					
Adj R-squared	0.960222					
Pred R-squared	0.932889					

Level of significance : *P<0.1, **P<0.05, ***P<0.01 and d. f. : degree of freedom.

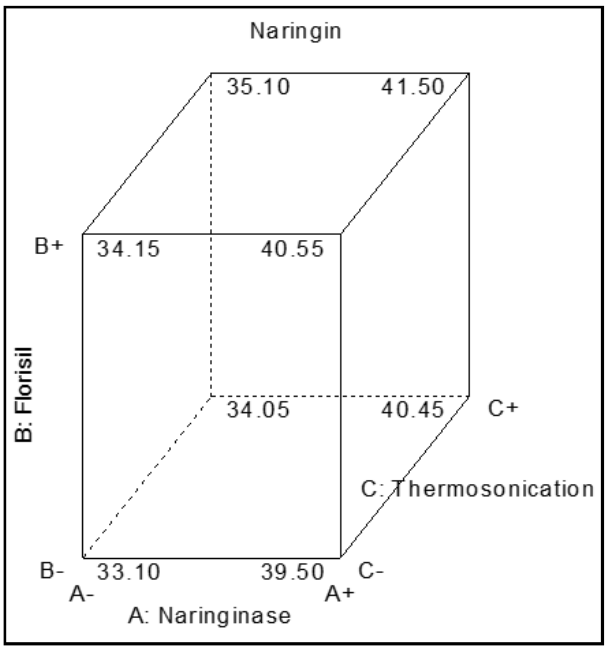

Fig. 1. Cube plot for effect of naringinase, florisil and thermosonication treatment on minimization naringin content of kinnow juice.

variables i. e. 1 ml/100 ml Naringinase, 40 g/l florisil and 40 KHz Thermosonication frequency, while minimum reduction in naringin content of kinnow juice was expected at (-1) level of all three variables. Highest reduction in naringin per cent of kinnow juice was followed at combination of (+1) level of A and B (-1) and (-1) level of C. With increasing concentration of enzyme naringinase, the percentage reduction in naringin was found increasing. It was found that with increasing concentrations of enzyme naringinase (from 0.6 to 1 ml/100 ml) there was a linear increase in the amount of per cent naringin reduction, because naringinase was a multifunctional enzyme that performed two functions : alpha-L-rhamnosidase and beta-D-glucosidase, for naringin as a substrate and converted it to rhamnose and prunin using its alpha-L-rhamnosidase activity; while beta-D-glucosidase activity broke down the prunin into glucose and naringenin. As it lowered the quantity of bitter naringin and produced naringenin (sweet flavour), it decreased the amount of bitter principle naringin in the juice. Silva *et al.* (2017) observed that best reduction was achieved by adding 1.0 g/l naringinase enzyme at 50°C for 4 h with an 86% decrease in bibla sweet oranges. Similar results were also reported by Patil and Dhake (2014), Kaur *et al.* (2018) and Housseiny and Aboelmagd (2019). It was also seen that with increasing concentrations of florisil the per cent reduction of naringin of the juice increased linearly. Kumar *et al.* (2020) used florisil on kinnow juice and observed that it reduced the limonin content to 50%. Florisil decreased the amounts of both naringin and limonin in the kinnow juice. It was also observed that with the increasing frequencies of sonication treatment, the per cent reduction in naringin was found to increase in the juice and a possible reason for this decrease was reported by Gao *et al.* (2021) that sonication increased the activity of -l -rhamnosidases, -limoninases and glucosidases and also helped in breaking of CO bonds in naringin. Relationship established between dependent and independent variables for naringin content of juice was predicted as follows :

$$\text{Naringin (\%)} = +37.30 + 3.20 \times x_1 + 0.53 \times x_2 + 0.48 \times x_3$$

F value for given model was 30.25 (Table 5) the value of "Prob. > F" for model was less than 0.05 indicated that the model was significant as desired. Linear, quadractic and interactive effects were found for following model terms, x_1, x_2, x_3, ..., x_{12} and x_{13}. The value or R-squared in this case was 0.9749 and close to 1, which was desired. Fig. 2 reveals that maximum reduction in limonin (50.68%) was found at highest level (40 g/l and 40 KHz) of B and C variables, respectively, by keeping naringinase content at 0.6 ml/100 ml level. With increasing concentration adsorbent florisil, limonin per cent of the juice was found to be increasing. It was observed that with the increasing concentration of enzyme naringinase the amount of per cent reduction in limonin decreased a little and it was possibly be due to the long incubation time. The application of adsorbent florisil resulted in a sharp increase in the per cent reduction of limonin of the juice, attributed to the adsorbing power of florisil. Results are in accordance with findings of Kumar *et al.* (2020). It was only when additional pulp was added to the juice that limonin content was slightly increased. Similarly, in the present study, there was no pulp of fibrous part in the juice and, therefore, a slight decrease was seen in the limonin content.

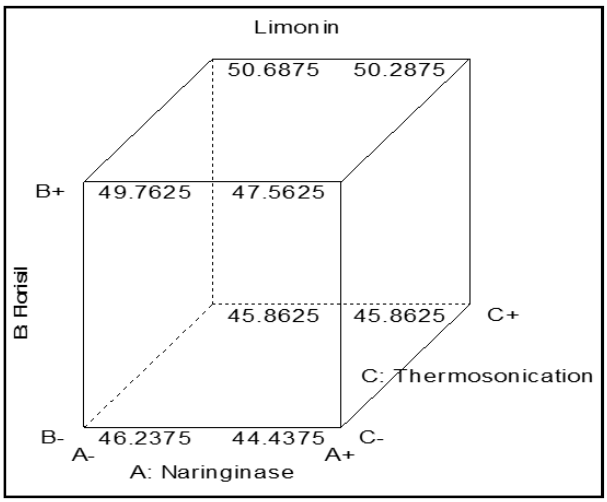

Fig. 2. Cube plot for effect of naringinase, florisil and thermosonication treatment on minimization limonin content of kinnow juice.

Model predicted for limonin content of juice, is as follows :

$$\text{Limonin} = +48.50 - 0.55 \times x_1 + 1.99 \times x_2 + 0.59 \times x_3 - 0.59 \times x_1^2 - 0.36 \times x_2^2 + 0.038 \times x_3^2 - 0.10 \times x_{12} + 0.45 \times x_{13} + 0.32 \times x_{23}$$

The numerical multi-response optimization technique with desirability function was used to estimate the optimum level of naringinase, florisil and thermosonication. The least deviation in actual values (limonin–43.8% reduction and naringin–40.0% reduction) of response against predicted values (limonin–45.64% reduction and naringin–40.3% reduction) was found in condition at naringinase (1.0 ml/100 ml), florisil (20 g/l) and thermosonication treatment (37.12 KHz) (Table 6). Therefore, it was found best among respective three optimum conditions having less than 5% deviation. Hence, the optimized conditions were considered further to study.

CONCLUSION

Bitterness causing compound was minimized by the selected combination of variables, although they were present comparatively in lower amount because fruits were procured in month of March. Reduction in limonin and naringin content was found varying from 45.6 to 51.2% and 33.3 to 40.9%, respectively. Naringin content kinnow juice was found decreasing with increasing level of all three variables; naringinase, florisil and thermosonication. Impact of naringinase enzyme was more profound for minimization naringin content. For limonin degradation, florisil showed a significant effect. Every year production of kinnow fruit is increasing in Sirsa, Fatehabad and Bhiwani districts, outcome of this research can benefit both farmers and processers in future.

Table 5. Analysis of variance for limonin content of kinnow juice

Source	Sum of squares	d. f.	Mean square	F value	Prob > F	
Model	40.16279	9	4.462533	30.25446	< 0.0001	Significant
x_1	2.42	1	2.42	16.40678	0.0049	
x_2	31.60125	1	31.60125	214.2458	< 0.0001	
x_3	2.76125	1	2.76125	18.72034	0.0035	
x_1^2	1.453289	1	1.453289	9.85281	0.0164	
x_2^2	0.553289	1	0.553289	3.751115	0.0940	
x_3^2	0.005921	1	0.005921	0.040143	0.8469	
x_{12}	0.04	1	0.04	0.271186	0.6186	
x_{13}	0.81	1	0.81	5.491525	0.0516	
x_{23}	0.4225	1	0.4225	2.864407	0.1344	
R-squared	0.9749					
Adj R-squared	0.9427					
Pred R-squared	0.5990					

Level of significance : *P<0.1, **P<0.05, ***P<0.01 and d. f.: Degree of freedom.

Table 6. Optimized level of variables and responses

	Naringinase (ml/100 ml)	Florisil (g/l)	Thermosonication (KHz)	Naringin (% reduction)	Limonin (% reduction)
Predicted	1.00	20.00	37.12	40.3111	45.64
Actual	1.00	20.00	37.12	40.0	43.8

ACKNOWLEDGEMENT

The authors are highly thankful to UGC for providing financial support via a research project.

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