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G Oshadie De Silva
Department of Food Science and Technology, Faculty of Applied Sciences, University of Sri Jayewardenepura, Sri Lanka

RAUJ Marapana
Department of Food Science and Technology, Faculty of Applied Sciences, University of Sri Jayewardenepura, Sri Lanka

Ranmalie Manawaduge
Cargills Agrifoods Ltd, York Street, Colombo, Sri Lanka

Correspondence
G Oshadie De Silva
Department of Food Science and Technology, Faculty of Applied Sciences, University of Sri Jayewardenepura, Sri Lanka

Effect of naringinase enzymatic treatment on the bitter compound naringin in fresh juice of "Bibila sweet" oranges

G Oshadie De Silva, RAUJ Marapana and Ranmalie Manawaduge

Abstract
Enhanced bitterness in citrus juices is one of the common quality problem in beverage industry. Limonin and naringin are the two main responsible compounds for the bitterness. The aim of this study is to determine the effectiveness of adding naringinase enzyme on reducing naringin in "Bibila sweet" oranges to enhance its quality. Naringinase can decompose naringin in to less bitter precursors and it can be inactivated by heating up to 90 ^{0}C. Three enzyme concentrations as 1.0 g/L, 0.5 g/L and 0.25 g/L with two incubation time periods of 2 and 4 hours used at constant incubation temperature of 50^0 C were used as the variables. The concentration of Naringin was analyzed using Davis semi quantitative method and Ultra High Performance Liquid Chromatography. The optimum reduction can be obtained by adding 1.0g/L naringinase enzyme at 50 ^{0}C with 4 hours of incubation period with 86% reduction percentage.

Keywords: Naringinase, naringin, bitterness, Ultra high performance liquid chromatography

Introduction
Sweet orange is one of the prominent fruit of Citrus family which commonly grown in tropical and subtropical areas. This is grown over 3393 hectares in Sri Lanka while global production is approximately nine billion hectares [1, 2]. Considering Sri Lankan sweet orange production, there are six sweet orange varieties and they are Bibila sweet, Arogya, MKD, Sisila, BAN and MT.

The development of bitterness is one of the most common problem in Citrus industry with regards to quality of juice. The bitterness can be developed in two ways. This can be occurred in the presence of bitter compounds in higher concentrations or it can be developed after different physical treatments like heating. There are two main compounds which responsible for the bitterness in citrus fruits and they are limonin and naringin. This study depends on naringin present in "Bibila sweet" oranges.

Naringin (4',5,7-trihydroxyflavanone-7-rhamnoglycoside) is a flavonoid glucoside and it is one of the main bitter compound in citrus juices. Generally the amount of naringin depends on the maturity of the fruit. There can be higher amount of naringin in immature fruits than mature fruits. Further, naringin is a water soluble compound it can be easily extracted into juice. Therefore degree of damage done to the albedo, membranes and pith determines the amount of bitter component extracted [3]. Naringin is hydrolyzed by α-L-rhamnosidase to prunin and L-rhamnose when fruit starts to mature. Prunin which is 33% as bitter as naringin is further hydrolyzed to naringenin and D-glucose [4].

The reduction of naringin concentration can be done by enzymatic hydrolysis and it is a promising technique which can be used in industry level. Naringinase is an enzymatic complex with alpha-rhamnosidase activity that allows the hydrolysis of naringin to prunin (4,5,7-trihydroxy flavonone-7-glucoside) and rhamnose. β-glucosidase activity can convert prunin into naringenin (4,5,7-rihydroxyflavanone) and glucose due to its substrate specificity. These products have less bitterness than naringin and they are almost tasteless compounds [5, 6].

Materials and Methods
Plant Materials
Bibila sweet orange samples were collected from the Horticultural Research and Development institute, Bibila and from generous farmers in Thambuththegama area through Cargills Agrifoods Ltd. Samples with similar maturity stage were sorted before the analysis.

Reagents and Standards

HPLC grade Naringin standard (assay ≥ 95.0%), HPLC grade acetonitrile (assay ≥ 99.9%), HPLC grade methanol (assay ≥ 99.9%), Diethylene glycol (Assaay ≥ 99.5%, analytical standard) and 4M Sodium hydroxide (Analytical standard) were provided by Sigma-Aldrich. Furthermore Naringinase enzyme (100,000 μ/g) was provided by Sunson Industry Co. Ltd, China.

Sample Preparation

"Bibila sweet" orange samples were washed properly to remove any dirty material and then they were peeled and squeezed manually using a juice squeezer. Furthermore, it was strained to remove seeds and filtered through four layers of nylon cloth to remove juice sacs. The filtered juice was kept in a hot water bath and it was heated at 90 ^{0}C for 5 minutes. The PET bottles were washed from hot water and filtered with the heated juice and capped. Then they were kept in cold water bath. Afterwards, once they were reached to room temperature, the bottles were kept inside the freezer at -18 ^{0}C.

The frozen juice were allowed to thaw and they were centrifuged using centrifuge (Spectrafuge 6c, Labnet international.inc) at a speed of 1207.44 ×g for 15 min prior to commence analysis. The supernatant was decanted the sediment was removed. This was done for every juice sample. The centrifuged juice samples were filtered through 0.45 μm nylon membrane into vials prior to commence HPLC and supernatant was directly used for the Davis method.

Enzymatic Treatment

The effect of naringinase enzyme on reducing naringin content was analyzed. Moreover, the most suitable concentration and the incubation time combination were tested for "Bibila sweet" orange samples using the method explained by Patil and Dhake in 2014 with slight modifications [7].

Initially sorted sweet oranges were peeled manually and the juice was squeezed using juice squeezer. The seeds were removed and it was filtered through four layer nylon cloth to remove juice sacs. The filtered juice was separated into equal amounts. Naringinase enzyme was purchased from Sunson industry group Co Ltd, China. In this experiment the concentration of the Naringinase enzyme and the incubation time were taken as two variables. At the particular incubation temperature a sample was kept as the control. Naringinase enzyme was added according to three concentrations (1.0, 0.5 and 0.25g/L). Each concentration was kept at 50 ^{0}C for 1 hour and 2 hours. After the particular incubation time, each sample were heated in hot water bath at 90 ^{0}C for 5 minutes. PET bottles washed with hot water and they were filled with the heated juice and capped. Then they were kept in cold water bath. Afterwards, once they reached room temperature, the bottles were kept inside the freezer at -18 ^{0}C.

The frozen juice was allowed to thaw and then centrifuged at 3000 rpm for 15 minutes prior to commence analysis. The supernatant layer was decanted and the sediment was removed. This was done for every juice sample in the lot. The centrifuged juice samples were filtered through 0.45 μm nylon membrane into vials.

Determination of naringin content using HPLC

The concentration of naringin was measured as the given method by Yusof *et al*., 1990 and Kimball in 1991 with some slight modifications.

Naringin was dissolved and the 1000 ppm stock solution was prepared. Then the stock solution was filtered through 0.45 μm nylon membrane. The standard series was prepared using filtered de-ionized water as the solvent.

An isocratic mobile phase was made using acetonitrile and de-ionized water (20:80, v/v) and filtered through 0.45 μm ×47 mm membrane (Supelco 58067-nylon 66). Then it was de-gassed by using ultra sound de-gassing unit.

The analysis was performed using UltiMate 3000, Thermo Scientific UHPLC unit with C_{18} reverse phase column (150 mm × 3 mm, 5 μm particle size) and diode array detector. The analysis temperature was set to 25 ^{0}C and the flow rate was adjusted to 1.0 ml/min. Naringin was analyzed at 280 nm using diode array detector [3, 8].

Determination of naringin using Davis method

The concentration of naringin was analyzed using Davis colorimetric method as explained by Cavia-Saiz, Muniz, Ortega and Busto in 2011. Naringin was in warm de-ionized water and 100 ppm, 200 ppm, 300 ppm, 400 ppm and 500 ppm were prepared from the 1000 ppm stock solution by dissolving 1.00 ml, 2.00 ml, 3.00 ml, 4.00 ml and 5.00 ml in 10 ml volumetric flasks respectively.

Then 0.1 ml of the standard solution was added to 5 ml 90% diethylene glycol and mixed well. After that 0.1 ml of 4M sodium hydroxide was added to the solution and mixed well. The absorbance was measured at 420 nm after 15 minutes of time using HACH DR 6000 spectrophotometer. A volume of 0.5 ml of de-ionized water was added instead of standard to prepare the blank [6]. Then 0.1 ml of juice samples were added instead of standard solution to measure the concentration of naringin and each was triplicated.

Statistical analysis

All the gathered data was statistically analyzed using Minitab 17th version software using one way ANOVA, Turkey pair wise comparison and two way ANOVA where required.

Results and Discussion

Naringinase enzyme has α-rhamnosidase and β-glucosidase activities sequentially. α-rhamnosidase hydrolyzes naringin to prunin and it is converted to naringenin (4,5,7-trihydroxyflavonone) by β-glucosidase. These resultant compounds are less bitter than naringin. Generally prunin is only one third bitter of naringin and naringenin is a tasteless compound [4].

The Figure 1 represents calibration plot of naringin, Figure 2 represents the chromatogram of control sample and the Figure 3 represents an enzyme treated sample.

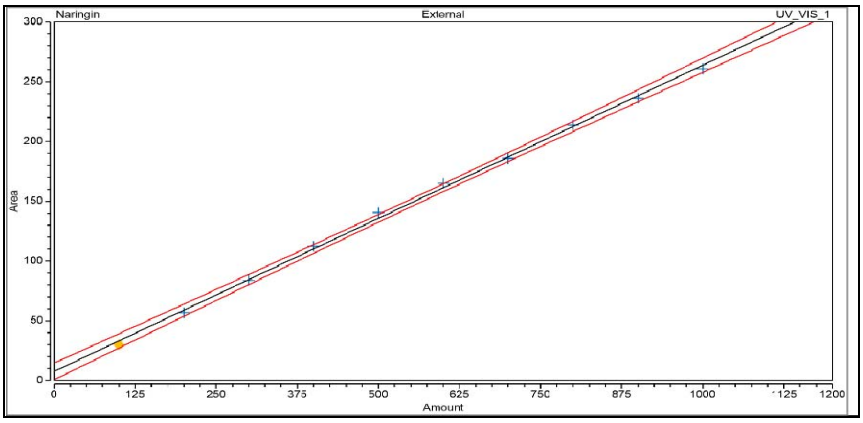

Fig 1: Calibration plot of naringin standard which is obtained from UHPLC technique

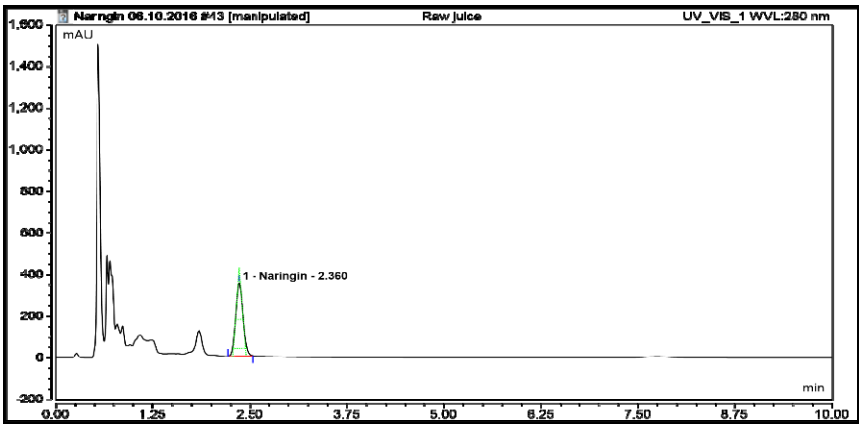

Fig 2: Chromatogram before treating with enzyme

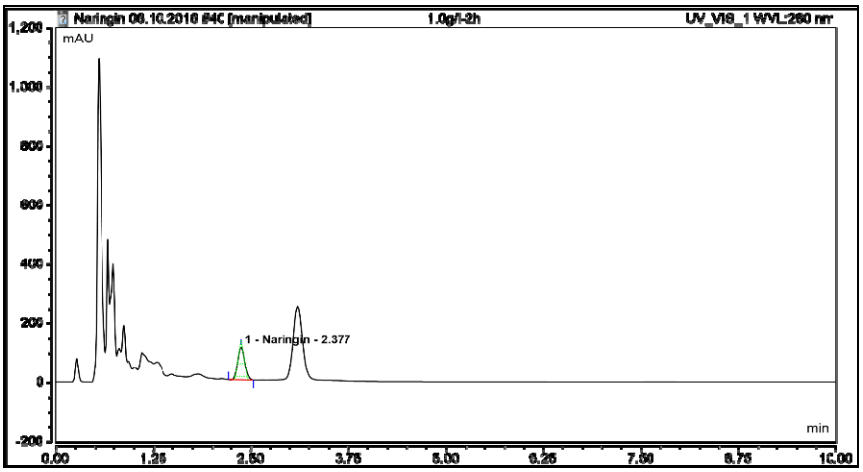

Fig 3: Chromatogram after the treating with enzyme (1.0g/L-2 hours)

Further, Table 1 represents average concentration of naringin obtained after naringinase enzymatic treatment which the samples were analyzed using HPLC technique.

Table 1: Average concentration of naringin and the reduction percentage before and after the treatment using UHPLC

Sample	Concentration of naringinase (g/L)	Incubation time (hours)	Average concentration of naringin	Reduction of naringin as a percentage (%)
Control	-	-	135.482^{a}±1.146	-
Enzyme added	1.0	2	20.207^{cd}±0.290	85.08^{ab}±0.21
	0.5	2	22.451^{bc}±1.142	83.43^{c}±0.84
	0.25	2	24.586^{b}±0.619	81.85^{d}±0.46
	1.0	4	18.285^{d}±0.732	86.50^{a}±0.54
	0.5	4	20.789^{c}±0.842	84.66^{bc}±0.62
	0.25	4	22.241^{c}±0.778	83.58^{bc}±0.57

Data represented as mean ± SE (n=3). Mean values in a column superscripted by different letters are significantly different at $P < 0.05$.

The activity of naringinase enzyme highly depends on the incubation temperature and time. Previous studies have been emphasized that the optimum activity of naringinase enzyme depicts between 40^0 C and 50^0 C with 1.0 g/L – 0.25 g/L [7, 9]. Therefore the incubation temperature was selected as 50 ^{0}C and naringinase was added as 1.0 g/L, 0.5 g/L and 0.25 g/L. In order to identify the optimum activity two incubation periods were selected. Hence each sample was kept at 50 ^{0}C for 2 hours and 4 hours separately.

The linear regression equation of the calibration plot is y=7.6084 + 0.2563x where y is area under the curve and x is the naringin concentration which obtained in HPLC. Furthermore, the calibration plot is fit to the model since R^2 = 0.9986.

There's a significant difference in naringin concentration between some of samples because P < 0.05. When considering the two hours of incubation period, 1.0g/L and 0.25g/L combination has P < 0.05. Hence there's a significant difference in naringin content between 1.0g/L and 0.25g/L added samples. Considering the reduction percentage, the average reduction percentage of 0.25g/L added samples is 81.853±0.457 % while 85.085 ± 0.214% in 1.0g/L added samples. But there's no significant difference between 1.0g/L and 0.5g/L combination and 0.5g/L and 0.25g/L combination since P is greater than α.

Furthermore, considering four hours of incubation period, the P value between 0.5g/L and 0.25g/L combination is greater than 0.05. Hence there's no significant difference of concentration of naringin between those samples. In addition there's a significant difference in naringin content between 1.0g/L, 0.5g/L added samples and 1.0g/L and 0.25g/L samples because P < 0.05.

In contrast the P > 0.05 between 1.0g/L-2 hours and 1.0g/L-4 hours samples. This indicates there's no significant difference in naringin content between two samples. Theoretically the concentration of naringin should be decreased as the incubation period increases [7]. On the other hand there's no significant difference in 0.5g/L-2hours, 0.25g/L-4hours and 1.0g/L-2hours, 0.5g/L-4hours samples.

However the highest reduction in naringin was obtained by adding 1.0g/L for 4 hours. When considering reduction percentage it is almost 86.503±0.541%. But there's not much difference in reduction of naringin content, whether it is incubated for 2 hours or 4 hours.

The least reduction of naringin was determined in 0.25g/L of naringinase concentration incubated for 2 hours and it was approximately 81.853±0.457 %. Therefore these results implies that the hydrolysis of naringin increases as the concentration of naringinase enzyme and incubation time increasing.

pH of the juice is important for the hydrolysis of naringin apart from concentration of naringinase enzyme, incubation temperature and the incubation period. The optimum activity of naringinase enzyme is when the pH 4.5 [10]. But the pH of "Bibila sweet" orange juice varies between 3.5 and 3.9. Hence the optimum activity of naringinase enzyme did not be obtained at above conditions.

In addition the activity of naringinase enzme is inhibited at 90 ^{0}C and previous studies have been depicted that inactivation of naringinase enzyme can be obtained by heating 90 ^{0}C for 5 minutes [11]. Therefore effect from naringinase enzyme would not be present after the pasteurizing treatment.

When determining the interaction between the naringinase concentration and incubation time period P > 0.05 and there's no interaction effect between naringinase enzyme concentration and incubation time on the naringin content.

However there is a variation in naringin content with respect to the enzyme concentration and the incubation time when considering the analysis of one way ANOVA. But this is not emphasized in two way ANOVA as the interaction effect is not significant. As interpret above as the concentration increased with respect to particular incubation period the concentration of naringin was decreased.

Previous studies had been emphasized that there's no effect on the concentration of limonin by the activity of naringinase enzyme. It is proved that there's no reduction of limonin unless there's a polymer support which can be absorbed [12, 13].

Furthermore, samples were analyzed using Davis method and Figure 4 represents the calibration plot of naringin standard and Table 2 represents the average concentration of naringin in each sample obtained from Davis test method.

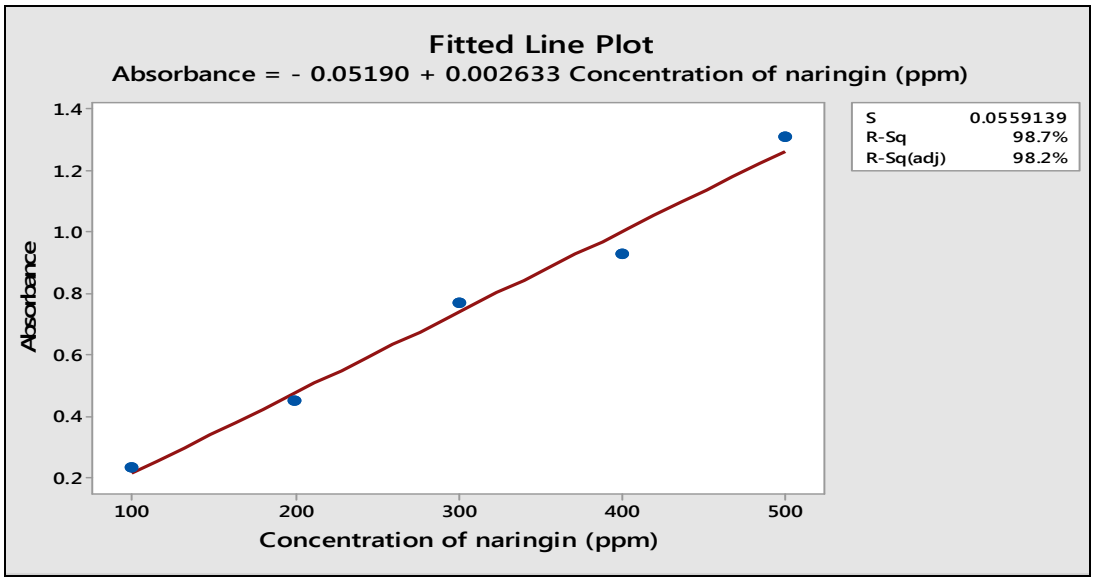

Fig 4: Calibration plot of naringin standard in Davis test method

Table 2: Average concentration of naringin using Davis test method

Sample	Concentration of naringinase (g/L)	Incubation time (hours)	Average concentration of naringin
Control	-	-	335.1^{ab}±23.7
Enzyme added	1.0	2	363.2^{ab}±24.5
	0.5	2	412.7^{a}±52.6
	0.25	2	329.9^{b}±24.2
	1.0	4	381.3^{ab}±26.7
	0.5	4	362.2^{ab}±23.3
	0.25	4	360.4^{ab}±19.5

Data represented as mean ± SE (n=3). Mean values in a column superscripted by different letters are significantly different at $P < 0.05$.

In the Davis method naringin reacts with diethylene glycol in alkali medium and produce corresponding chalcone which depicts in yellow in color. The naringin concentrations of each sample are hundred folds when comparing with results of UHPLC analysis. This can be due to reaction of 4'-hydroxy-7-alkoxy or 4'-hydroxy-7-glycosidoxy flavanones present in orange juice apart from naringin which reacts with diethylene glycol in alkali medium to give yellow color chalcones. And this is not a direct indication of bitterness as some non-bitter precursors respond with alkaline diethylene glycol.

The P value of whole analysis is greater than 0.05. Hence there's no significant difference between treatments. Considering the results of UHPLC, there is a significant difference between treatments with respect to the concentration of naringin. This can be due to the response of other compounds with diethylene glycol.

Considering the interaction effect between enzyme concentration and incubation time, $P > 0.05$ and this emphasizes that there's no interaction effect between two variables.

Conclusion

Naringinase enzyme can be used to reduce the concentration of naringin in "Bibila sweet" orange. The most effective conditions on reducing naringin is 1.0 g/L for 4 hours at 50 ^{0}C. The reduction percentage of naringin is approximately 86%.

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