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Naringin in Turkish Orange Juices and Its Reduction by Naringinase

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ABSTRACT

Turkey is a major producer of fresh oranges and related products. The juice making process involves the debittering of naringin. Few studies have been reported on this compound in the fruits and products in Turkey. Furthermore, the process to remove this bitterness was also of interest in this study. Samples of 97 freshly hand-squeezed orange juice and orange peel juice, 30 commercial orange juice and peel concentrates collected from the Mediterranean Region of Turkey were analyzed for their naringin concentrations by HPLC following the Fisher and Wheaton method. Naringin concentration of 53 juice samples from freshly squeezed orange and 87 peel juice samples ranged from 0.12 to 2.63 mg L^{-1} and 0.50 to 15.7 mg L^{-1}, respectively. On the other hand, the naringin concentration of the commercial orange juice and peel concentrates were higher and ranged from 0.61 to 19.4 mg L^{-1} and 95 to 995 mg L^{-1} for 30 samples, respectively. Due to higher naringin levels in orange peel concentrates, these samples were subjected to a debittering process using free naringinase with a K_m value of 0.098 mM naringin, at pH 4.0 and 60˚C. After 85 min of debittering process, 35.3% of the naringin was found to be hydrolyzed.

Key words: naringin, naringinase, debittering, orange juice, orange peel juice, orange juice concentrate, orange peel concentrate

INTRODUCTION

Turkey, an important orange producer in the world, exports oranges as fresh fruits, orange juice and concentrates. Turkish orange juice industry recovers the juice from orange pulp by extraction[1], and the peels from extractors are used in cloudy peel concentrate production, providing an excellent cloudy agent for juice drinks with a high degree of stability[2,3]. Many fruit juice and beverages are preferred to be turbid rather than transparent due to consumer's choice. Recently, due to the prohibited use of brominated oils and artificial cloudifiers in citrus beverages in some countries, the demand has increased for suitable and natural cloudifiers as commercial orange peel concentrate by-products from citrus raw material[4].

Some varieties of orange juice and peel concentrates have an undesirable bitterness originating from flavonoid glycosides and limonoids. Naringin (4',5,7-trihydroxyflavanone-7-rhamnoglycoside) as a flavonoid glycoside is the main bitter component of several citrus fruits[3]. Debittering of citrus fruit juices is an important process used to control quality and improve the commercial values[5,6]. Naringin hydrolysis, with subsequent decrease in the bitterness, is of industrial concern. Due to this commercial interest, the flavor should be acceptable by consumers[7]. Naringin may be found in different varieties of oranges or blends with other citrus fruits. So far, there is no data available for naringin in Turkish orange fruits and its products.

Most commercial methods for debittering juices are based on the use of ion exchange technology, free or immobilized naringinase, supercritical carbon dioxide extraction and adsorption[5,8,9]. Naringinase, a crude enzyme obtained from the fungus *Penicillium sp*. or *Aspergillius sp*. with α-L-rhamnosidase and β-D-glucosidase activities, have been widely used to hydrolyze naringin to naringenin, which is not bitter[6,9,10]. Recently, the gene of α-L-rhamnosidase has been cloned and expressed with marked activity in *Esherichia coli*. The recombinant α-L-rhamnosidase provides an economical source of debittering enzymes and reveals a practical revolution in industrial debittering of citrus juices[6]. Tsen and Tsai (1988) reported that the optimum pH of naringinase from *Penicillium sp*. is 3.7, and this enzyme possesses about 75-85% of its maximum activity in the natural fruit juice (pH = 3-3.4). Naringinase from *Penicillium sp*. has also been reported to have a better operational stability[9].

In this study, naringin levels of most important varieties for industrially processed oranges in the Mediterranean Region of Turkey were investigated. The optimum conditions (such as pH, temperature) were investigated for naringin hydrolysis by free naringinase and this procedure was employed for high naringin levels in orange juices. The results gave important information on naringin levels of oranges of the Mediterranean Region in Turkey and very usefull hydrolyzing procedure for debittering process in commercial citrus juice production.

MATERIALS AND METHODS

Fresh orange fruits (97 samples) were obtained from local farmers. The fruits were at the mature stage, had a uni-

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form color and no signs of spoilage. Orange products including 30 concentrated commercial orange juices and peel samples were obtained from a food company (Mersin, Turkey).

High Pressure Liquid Chromatograph (HPLC) (Shimadzu, LC-10ATVP model, Japan) was used with a C-18 HD (250 × 4 mm i.d.) column. Injections, each 20 μL, were applied using a solvent of acetonitrile/water/acetic acid (20/80/2.5) to detect the naringin with a UV detector at 280 nm. Flow rate was 1.0 mL min^{-1}.

Naringin and naringinase from *Penicillium sp*. were supplied from Sigma (USA). Commercial pectic enzyme (Rohapect PTE) was supplied from AB Enzymes Abitec Group (Germany). All solvents and other chemicals were obtained from Merck (Germany). Stock solution of naringin was prepared at a concentration of 120 μg mL^{-1} in dimethylformamide (DMF)/0.01 M acetic acid (1/4). The working solutions were prepared at desired concentrations by diluting the stock solution with DMF/0.01 M acetic acid. For the naringinase solution, 1000 mg was dissolved in 1 L of 0.1 M sodium acetate buffer adjusted to pH 4.0.

I. *Sample Preparation*

Orange fruits were hand-squeezed and the juice was filtered through a stainless steel sieve (0.41 mm) to remove the seeds and pomace. The fruit peel (raw albedo and flavedo) was mashed in a blender. One liter of deionized water was added to 500 g of mashed peel and mixed. The mixture was heated to 45˚C, 3 mg of commercial pectic enzyme was added, and it was held at this temperature for 30 min. Both the juice and the mashed peel were pasteurized at 95˚C for 2 min and cooled to room temperature. This product was orange peel juice.

Concentrated orange juice (50˚ Brix) and concentrated orange peel juice (50˚ Brix) were diluted to 11.8˚ Brix with deionized water to compare with fresh orange juices.

II. *Determination of Naringin in Samples*

The analyses were conducted according to the Fisher and Wheaton method[3]. Commercial orange peel, juice concentrates and fruit juice samples (10 mL) were mixed with 10 mL of DMF and 10 mL of 0.025 M ammonium oxalate, and the volume was made up to 50 mL with ultra deionized water. Samples were placed in a water bath at 90˚C for 10 min, cooled to room temperature and filtered through a 0.45 μm membrane filter. Twenty- μL aliquots of filtrate were injected to HPLC, and naringin was identified in the samples by comparing the retention time with that of standards and quantified by comparing the peak areas.

III. *Enzymatic Hydrolysis*

Naringinase activity was determined from the naringin hydrolysis rate. For enzymatic hydrolysis, naringinase solution was added to naringin solutions, and the reaction was performed at 60˚C and pH 4.0 for up to 150 min. Right after the reaction time was fulfilled, the hydrolyzed samples were prepared and the naringin amounts were determined again by the Fisher and Wheaton Method[3].

RESULTS AND DISCUSSION

I. *Freshly Squeezed Orange and Peel Juices, Commercial Orange Juice and Peel Concentrates*

The 97 freshly hand-squeezed orange juice and peel juice samples were analyzed to determine the naringin levels. Among these, 53 orange juice and 87 orange peel juice samples had detectable naringin concentrations (Table 1). Samples from freshly hand-squeezed orange juices of Washington Navel, Shamouti and Yerli gave naringin concentrations of 0.12-1.52 mg L^{-1}, 0.12-1.55 mg L^{-1} and 0.16-2.63 mg L^{-1}, respectively. On the other hand, samples from freshly hand-squeezed orange peel juices of Washington Navel, Shamouti and Yerli gave naringin concentrations of 0.50-15.70 mg L^{-1}, 0.70-12.23 mg L^{-1} and 1.61-13.00 mg L^{-1}, respectively. In general, naringin concentrations of hand-squeezed orange peel juices were higher than those of hand-squeezed orange juices, showing higher concentrations in the rind.

Table 1. The concentration of naringin (mg L^{-1}) in freshly hand-squeezed orange juices and peel juice from oranges

Area	Variety of orange	Orange juice				Orange peel juice			
		Sample number[a]	Range	Average	Standard deviation	Sample number[a]	Range	Average	Standard deviation
Adana	Washington Navel	5	0.18-0.48	0.31	0.14	6	2.60-9.73	6.22	3.18
	Shamouti					3	3.27-4.93	4.10	0.83
	Yerli	7	0.51-1.20	0.74	0.23	13	1.88-10.50	4.78	2.94
Antalya	Washington Navel	4	0.16-0.96	0.46	0.37	6	1.38-9.08	5.24	2.69
	Yerli	3	0.16-2.63	1.28	1.25	4	3.50-9.53	2.82	6.27
Hatay	Washington Navel	4	0.12-0.71	0.37	0.25	6	3.56-15.70	7.15	4.87
	Shamouti	2	0.12-1.03	0.58	0.64	3	1.63-6.39	4.71	2.67
	Yerli					4	1.63-13.00	5.46	5.20
Mersin	Washington Navel	19	0.16-1.52	0.71	0.33	27	0.50-14.24	5.50	3.51
	Shamouti	5	0.23-1.55	0.64	0.52	9	0.70-12.23	6.55	3.34
	Yerli	4	0.58-1.19	0.85	0.25	6	1.61-9.04	5.50	3.01

[a]Given only naringin detectable samples.

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Table 2. Naringin concentration (mg L^{-1}) in commercial orange juice and peel concentrates

Samples	Number of samples	Range	Average	Standard deviation
Orange Juice Concentrates	30	0.61-19.40	7.34	4.98
Orange Peel Concentrates	30	95-955	369.50	161.50

When the naringin concentrations of each fresh orange juice and peel juice were compared within each other in terms of variety and region, the *t*-test indicated that there were no significant differences between them at the 95% confidence level.

No data on naringin in Turkish citrus fruits and orange juices have been reported. Trotta *et al*. (2002) reported that many orange cultivars in southern Italy had a relevant amount of naringin and limonin resulting in an undesired bitterness within a few hours after the extraction[11]. Other published data[12,13] referred to the varieties of citrus fruit flavanoids owing to significant quantities of hesperidin in oranges and naringin in grapefruits. The naringin concentrations in commercial orange juices and peel concentrates were found to be 0.61-19.4 mg L^{-1} and 95-955 mg L^{-1}, respectively (Table 2). Relatively higher naringin levels were found in these commercial products compared to those of hand-squeezed orange and peel juices. These high naringin values may be caused by the higher level of naringin in different varieties of oranges or blends with other citrus fruits during extraction and the concentration processes.

The taste threshold of naringin to impart bitterness is about 50 mg L^{-1}[3]. As observed, naringin levels of fresh orange juices, orange peel juices and orange juice concentrates were much lower than the threshold value. However, the commercial orange peel concentrates have very high naringin contents resulting in a need for the debittering process.

II. *Hydrolysis of Naringin in Buffer Solution or Commercial Orange Peel Concentrates*

The effect of pH on the reaction rate was investigated spanning a pH range of 3.5 to 4.0 in 0.1 M acetate buffer solutions at temperatures of 40 to 60˚C. The optimum pH value was found to be 4.0 at 60˚C, agreeing with the literature values[7,9,10]. Additionally, effects of substrate and enzyme concentrations on the reaction rate were also investigated, and the optimum concentrations were found to be 200 and 300 mg L^{-1} for naringin and naringinase, respectively[14].

The Km value for naringinase for naringin was determined using a Lineweaver-Burk plot. K_m value of the free naringinase was 0.098 mM[14], much lower than the values 0.87 and 7.8 mM reported by Tsen (1984)[15] and Tsen and Tsai (1988)[10], respectively. The differences could be due to the different buffer solutions, ionic strengths and temperatures, as well as the source and purity of naringinase and the difference between free and immobilized enzymes.

Table 3 shows the hydrolysis of naringin in commercial orange peel concentrates (having 167.74 mg L^{-1} of naringin), commercial grapefruit juice concentrates (515.65 mg L^{-1}), orange and grapefruit juice mixtures I (231.01 mg L^{-1}) and II (68.77 mg L^{-1}) and naringin solutions (200 mg L^{-1}) with naringinase at 60˚C as a function of time. Naringin concentrations decreased from 200 mg L^{-1} to 3.20 and 5.50 mg L^{-1} at 86 and 97 min in naringin solutions at pH 4.0 and 3.5, respectively. These results showed that the naringinase activity was maximized at pH 4.0 and 60˚C. In general, it was observed that the naringin concentrations in all samples decreased with the enzymatic hydrolysis, and at the end of the hydrolysis process, 78.45, 64.75, 69.98 and 74.92% of naringin remained in the commercial grapefruit peel (obtained from a food product company) and orange peel concentrates, orange and grapefruit juice mixtures I and II, respectively, while less than 5% of naringin remained in the naringin solutions.

Table 3. Hydrolysis of naringin in different orange - grapefruit juice mixtures, commercial orange and grapefruit peel concentrates and naringin solutions with naringinase at 60˚C

Sample	pH	Time (min)	Naringin (mg L^{-1})	% Remained
Commercial grapefruit peel concentrates (diluted to 11.8˚ Brix)	2.88	0	515.65	100.00
		8	513.37	99.56
		15	476.75	92.46
		21	465.06	90.19
		30	446.67	86.62
		40	437.11	84.77
		49	430.61	83.51
		64	414.83	80.45
		74	410.93	79.69
		96	404.51	78.45
Commercial orange peel concentrates (diluted to 11.8˚ Brix)	4.03	0	167.74	100.00
		7	164.87	98.29
		35	130.32	77.69
		45	125.45	74.79
		85	108.61	64.75
Orange - grapefruit juices mixture I (1:3) (10.1˚ Brix)	3.42	0	231.01	100.00
		16	204.36	88.46
		39	179.03	77.50
		55	161.67	69.98
Orange - grapefruit Juices mixture II (3:1) (12.3˚ Brix)	3.71	0	68.77	100.00
		27	59.34	86.29
		37	56.79	82.58
		64	51.52	74.92
200 ppm naringin solution	3.5	0	200.40	100.00
		30	68.71	34.28
		62	26.30	13.12
		97	5.50	02.74
200 ppm naringin solution	4.0	0	200.40	100.00
		28	49.10	24.50
		49	26.80	13.37
		86	3.20	01.59

Natural grapefruit juices contain 1-1.5% (w/w) citric acid, 3.4-5.0% reducing sugars, approximately 3% sucrose, and 0.017-0.025% naringin with a pH value of 3.3-3.4[15].

Natural orange juice, on the other hand, contains 1% (w/w) organic acids and 10% soluble sugars. Sugars and citric acid levels in the commercial orange peel concentrates are higher than those in orange, grapefruit and lemon juices. Soluble sugars are primary constituents of peel, pulp, and rag dry solids. In these fractions, glucose and fructose are about equal to the sucrose contents. Besides these major sugars, the peel also contains smaller amounts of xylose and rhamnose[2]. Soares and Hothkiss (1998) reported that rhamnose and glucose, products of naringin hydrolysis[16], are competitive inhibitors of naringinase. Table 3 shows that the remaining naringin levels in all juices and concentrations are higher than those in naringin solutions. The differences could be due to the effects of grapefruit and orange compositions on naringinase activity, especially the presence of glucose and rhamnose as inhibitors. Therefore, reduction of naringin in commercial orange peel concentrates is much lower than that in naringin solution.

In conclusion, naringin in Turkish orange products generally originating from orange fruits, does not exceed acceptable naringin level in fruit juices (except commercial orange peel concentrates). Recently, the use of commercial orange peel concentrates, having an average of 369.5 mg L^{-1} naringin, has rapidly increased in the flavored orange beverages. We suggest to the industry that naringin originating from orange peel may be reduced by free naringinase. At the end of enzymatic reaction, the naringin was hydrolyzed to naringenin which is harmless and not bitter.

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