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A Rapid and Selective method for the Quantification of Naringenin in order to monitor Naringinase activity

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Abstract : Sodium acetate (NaOAc) can selectively ionize 7-OH of naringenin generating a bathochromic shift of 41nm in its UV absorbance. This principle is used in the described spectrophotometric method that can detect naringenin amidst naringin and prunin present in the incubation mixture of naringinase. The method could be adopted for real sample analysis as it remains unaffected by the presence of phenolics such as ascorbic acid, gallic acid, citric acid and cinnamic acid.
Keywords: Naringinase; Shift reagents; Naringenin; Naringin; Prunin.

Introduction

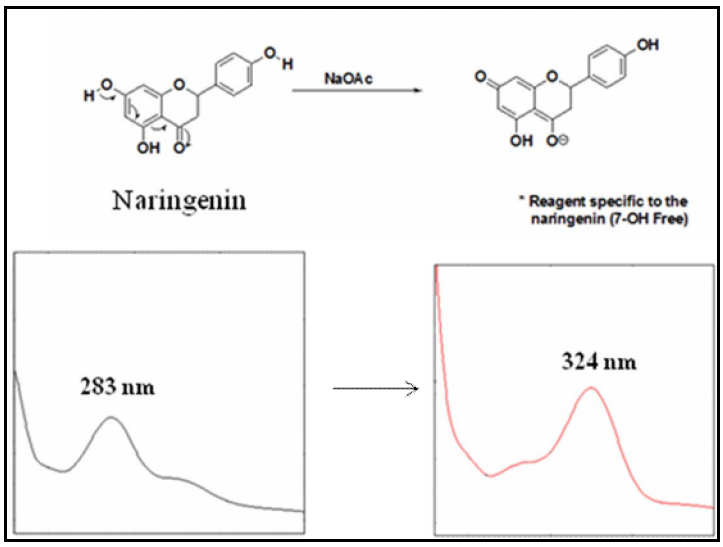
The bitterness in the citrus juice is mainly due to the presence of naringin (naringenin-7-O-glucorhamnoside). Hence, the conversion of naringin to its aglycone moiety results in the removal of bitterness. Enzymatic conversion using naringinase is preferred as it does not interfere with the organo-leptic properties or the nutritional qualities of the fruit juices unlike the other debittering strategies[1]. Naringinase (EC 3.2.1.40) is a two subunit complex with rhamnosidase and glucosidase activities. Hydrolysis is sequential with rhamnosidase initially converting naringin to prunin (4'-5,7-trihydroxyflavanone-7-glucoside) with the release of rhamnose, and finally, glucosidase hydrolyse prunin to naringenin (4′,5,7-trihydroxyflavanone) with the release of glucose[2].

In order to track the naringinase activity various assay methods are described in the literature[3]. Amongst which, Davis method[4] is the most extensively used. The method developed by Habelt and Pittner[5] is versatile in being able to distinguish naringin, prunin, and naringenin present in the incubation mixture. But, both the methods suffer from the disadvantage of being affected by other phenolics due to non-specific action of sodium hydroxide used in these assays.

The present method on the other hand uses NaOAc. NaOAc is routinely used as shift reagent in conjunction with UV spectrophotometry to identify the OH functionality in flavonoids due to their selective nature of the mode of action[6,7]. They can selectively ionize the free 7-OH group present in flavanoids. This capability is exploited in developing the described method for quantifying the naringenin present in the incubation mixture of naringinase. Spectrophotometric analysis of the mixture will give an indiscrete broad peak at 283nm, representing naringin, prunin and naringenin. On the addition of NaOAc the peak resolves into two, namely 1) 283 nm peak representing naringin and prunin 2) 324 nm peak representing naringenin. This enables effective estimation of naringenin from the mixture.

The method basically involves 1) Ethyl acetate extraction of the flavanoids from the incubation mixture 2) Addition of the shift reagent, NaOAc 3) Measurement of absorbance at 324 nm 4) Deduction of the amount of naringenin from its absorbance by extrapolation in the standard graph. The non-interference of ascorbic acid, gallic acid, citric acid and cinnamic acid on the method was demonstrated. An experiment on the application of the assay method on the aqueous orange peel extract was done to demonstrate the suitability of the assay in real sample analysis.

Theoretical background: Naringin possesses two free hydroxyl groups at 5 & 4' positions and a hydroxyl group at the 7 position that is bound to a rutinoside (disaccharide-rhamnose and glucose) via a glycosidic bond. But in naringenin the 7-OH is free as it is an aglycone (absence of a rutinoside). NaOAc selectively ionizes the free 7-OH since it is the most acidic when compared to the others. This ionization brings about a bathochromic shift in the UV absorption peak of naringenin but not in the combined peaks representing naringin and prunin (**Scheme 1**).



Scheme 1. Mechanism of NaOAc based UV shift

Experimental

Chemical and enzyme

Naringin and naringenin were purchased from Sigma and Spectrochem respectively. Naringinase enzyme was prepared from a locally isolated and characterized *Aspergillus niger*. The analytical grade methanol and anhydrous NaOAc used in the study were purchased from Spectrochem.

Instrumentation

Spectroscopic measurement was made with UV-Spectrophotometer (Shimadzu UV-1800).

Standardized procedure for naringenin estimation

100 µL of naringin (from a stock of 1mg/mL prepared in methanol) was incubated with 500 µL of naringinase and 400 µL of distilled water at 40 ^{o}C for 2 hrs. With regard to aqueous orange peel extract 1mL was taken directly for the reaction. After incubation, flavanoids were extracted twice with 200 µL of ethyl acetate and transferred into a fresh tube. Ethyl acetate was removed under reduced pressure and the residue was re-dissolved in 100 µL methanol. To it, 3.3 mL of analytical grade methanol and 200 mg of anhydrous NaOAc were added. The mixture was vortexed for 30 seconds. The content was then transferred to a quartz cuvette and spectrophotometrically read at a wavelength of 200 to 800 nm against methanol as the blank. Peak at 324nm represents that of naringenin shift and the concentration was deduced by extrapolating its absorbance in the standard graph.

Calibration curve

For generating the standard graph different concentrations (36.76, 73.53, 110.29, 147.06, 183.82 µM) of standard solutions of naringenin were made and their absorbances were measured at 324 nm in the presence of anhydrous NaOAc reagent (200 mg). Absorbance and the respective concentrations were used for plotting the calibration curve.

Interference of phenolic compounds on the assay

Interference of other phenolics on the method was analyzed using compounds that were readily available in the lab and those that predominantly present in the citrus fruit juices. 30 µg each of ascorbic acid, gallic acid, citric acid and cinnamic acid were used for the experiment. The effect of NaOAc on individual compounds were tested initially using the above mentioned procedure. They were also analyzed by spiking with 110.29 µM naringenin.

Results and discussion

Analysis of the Naringinase incubation mixture

Reaction mixture of naringinase was analysed with the described method in order to estimate the amount of naringenin produced during hydrolysis. In the absence of NaOAc a mixture containing naringin, prunin and naringenin displayed an indiscrete peak at 283nm. On the other hand, in the presence of NaOAc a bathochromic shift of 41nm generated a new peak of naringenin at 324nm (**Figure 1**). The amount of naringenin present in the mixture was deduced by extrapolation from the standard graph (**Figure 2** and **Table 1**).

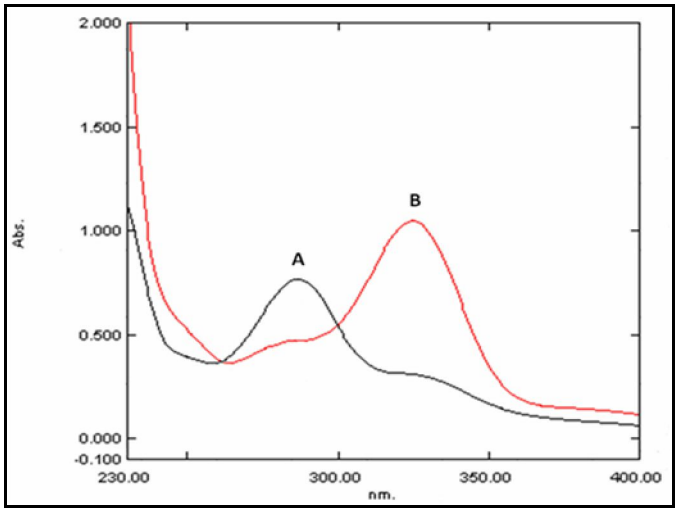


Figure1. Shift of naringenin present in the incubation mixture.

A. Mixture of naringin, prunin and naringenin in the absence of NaOAc. B. Shift in naringenin peak with the addition of NaOAc.

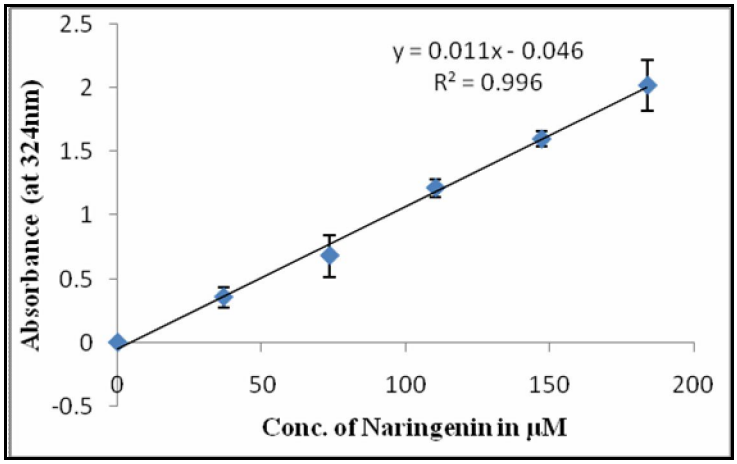


Figure 2. Naringenin standard curve.

Table 1. Enzymatic hydrolysis of standard and real samples.

S.No.	Substrate	Naringenin concentration (µM)
1	Naringin	115
3	Orange peel-Aqueous extract	173

Interference from other phenolics

Interference of the chosen phenolics (as they are present in citrus juice) such as ascorbic acid, gallic acid, citric acid and cinnamic acid on the method was studied. None of them exhibited a shift in their peaks in the presence of NaOAc. In a mixture containing the phenolic acids and naringenin, the later alone shifted to form a new peak at 324nm (**Figure 3**)

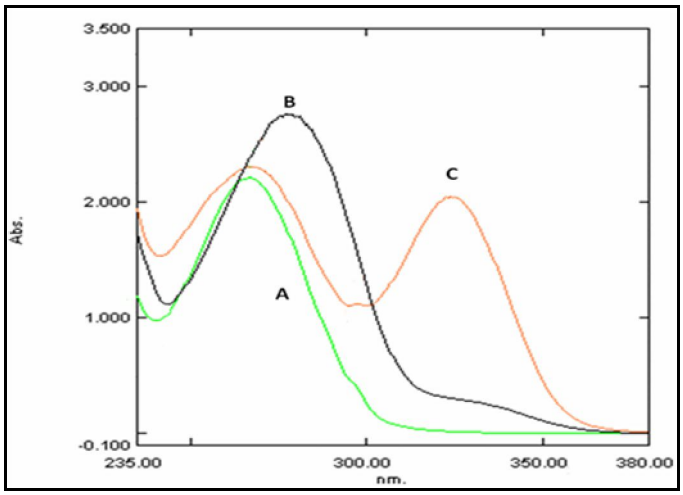


Figure 3. Analysis of the interference of phenolics acids on the assay.

A. Mixture of phenolics ascorbic acid, gallic acid, citric acid and cinnamic acid (phenolic acids). B. Phenolic acids along with naringenin in the absence of NaOAc . C. Phenolic acids along with naringenin in the presence of NaOAc.

Applications of the present method to real sample analysis

Aqueous orange peel extract was incubated with the enzyme and the reaction mixture was analyzed spectrophotometricaly. Selective shift of naringenin peak was also noticed here. This can help in estimating the naringenin formed during naringinase hydrolysis (**Figure 4**).

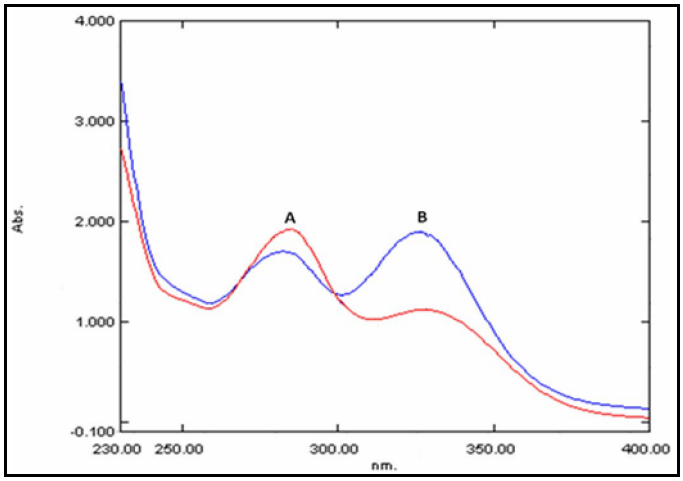


Figure 4. Analysis of orange peel extract.

A. Mixture of naringin, naringenin and prunin. B. Naringenin

Conclusions

A spectrophotometric method to quantify Naringenin based on the use of NaOAc as shift reagent was developed. This method is rapid and selective. It can be used to monitor the hydrolytic activity of the naringinase enzyme. Notable advantage of this method is that it remains unaffected by the presence of other phenolic acids such as ascorbic acid, gallic acid, citric acid and cinnamic acid. This characteristic is ideal for the real sample analysis. The disadvantage of the method is its inability to differentiate the glycosides- naringin and prunin that are also present in the reaction mixture. In order to overcome this lacuna the method in combination with the existing methods to assay aldohexoses (rhamnose and glucose) [8].

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