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Naringin and naringenin determination and control in grapefruit juice by a validated HPLC method

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Abstract

The main goal of this work was to develop and validate a fast, effective HPLC method for the simultaneous determination and control of naringin and naringenin, in several samples of citrus juices, namely, natural and commercial grapefruit and orange juices. The reason for developing this HPLC procedure is to control the debittering process using naringinase.

A successful resolution and retention times were obtained with a C_{18} reversed phase column, at a 1 mL min^{-1} flow rate, with a gradient of acetonitrile:water and at the temperature of 25 °C. The method was linear in the working range of 55–95 μg mL^{-1} for naringin and 10–60 μg mL^{-1} for naringenin. Repeatability was determinate at three concentration levels obtaining a RSD (%) always lower than 2.5%, a limit of detection (LOD) and a limit of quantification (LOQ) of 2.83, 8.57 and 1.11, 3.37 μg mL^{-1} for naringin and naringenin, respectively, were obtained.

Keywords: Naringin; Naringenin; Validation; Linearity; HPLC-DAD

1. Introduction

The bitterness of some fruit juices is an undesirable quality for the juices industry, mainly of orange and grapefruit. The principal groups of compounds present in these fruits responsible for bitterness are flavonoids (*e.g.* naringin) and limonoids (*e.g.* limonin). Naringin (4′,5,7-trihydroxyflavanone-7-β-L-rhamnoglucoside-(1,2)-α-D-glucopyranoside) is the main bitter flavonoid in grapefruit juices. It has been reported that when naringin is present in water solutions in concentrations higher than 20 μg mL^{-1} the bitter taste can be detected, however in grapefruit juices, bitterness is only, detectable in concentrations higher than 300–400 μg mL^{-1} (Soares & Hotchkiss, 1998).

In order to debitter citrus juices, adsorption techniques (Ribeiro, Silveira, & Ferreira-Dias, 2002) and enzymatic hydrolysis have been reported to achieve this goal (Pedro et al., 2007; Prakash, Singhal, & Kulkarni, 2002; Puri & Banerjee, 2000; Sekeroglu, Fadiloglu, & Gogus, 2006; Vila-Real, Alfaia, Calado, & Ribeiro, 2007). Adsorption debittering has some disadvantages such as the loss of juice acidity, of flavor, sweetness, and turbidity as well as less efficiency.

To control the quality and improve the commercial value of the citrus juices, namely of grapefruit, maintaining the health properties and increasing the acceptance by the consumer, the reduction of naringin concentration by enzymatic hydrolysis appears as one promising technique with industrial application (Chien, Sheu, & Shyu, 2001). Naringinase is an enzymatic complex with α-rhamnosidase activity responsible for naringin hydrolysis to prunin (4,5,7-trihydroxy flavovone-7-glucoside) and rhamnose, and β-glucosidase activity that hydrolyses prunin into naringenin (4′,5,7-trihydroxyflavanone) and glucose. Prunin, has one-third of naringin bitterness, and naringenin is almost tasteless (Puri, Marwaha, Kothari, & Kennedy, 1996). A great number of antioxidants are naturally presents in citrus juice, which are responsible for the potential

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protective action of these juices against certain degenerative diseases. Naringenin, the product of the enzymatic hydrolysis of naringin may be potentially useful as pharmacological agent, as anticancer, in the treatment or prevention of atherosclerosis, with a number of antiatherogenic activities, such as antioxidant, anti-inflamatory, antithrombotic and vasodilator (Chen, Shen, & Lin, 2003).

In order to identify and quantify flavonoids in several matrixes (*e.g.* blood, urine, fruit juices) a number of analytical methods have been developed, especially High Performance Liquid Chromatography (HPLC) methods (Belajová & Suhaj, 2004; Ishii, Furita, & Kasuya, 1997; Kanaze, Gabrieli, Kokkaçou, Georgarakis, & Niopas, 2003; Kanaze, Kokkaçou, Georgarakis, & Niopas, 2004; Widmer, 2000). Some of these methods (Chien et al., 2001; Norouzian, Hosseinzadeh, Inanlou, & Moazami, 1999; Pellati, Benvenuti, & Melegari, 2004) were tested for the analysis of naringin and naringenin in grapefruit juice however some were time-consuming with high retention times for naringenin, with a bad resolution and not directly applicable for the simultaneously quantification of naringin and naringenin. Two validated official international methods are available to determine naringin (AOAC, 1999; EN, 1997). Therefore, the objective of the present work was the development and validation of a fast and efficient gradient HPLC method for the simultaneous determination of naringin and naringenin in citrus juices, namely in grapefruit juice. The developed method will, also allow the follow up of the enzymatic reaction, with naringinase as the biocatalyst, in the debittering of grapefruit juice.

2. Materials and methods

2.1. Chemicals

Naringin (4′,5,7-trihydroxyflavanone-7-β-L-rhamnoglucoside-(1,2)-α-D-glucopyranoside) 96.6%, naringenin (4′,5,7-trihydroxyflavanone) 99%, limonin (limonoic acid 3,19.16,17-dilactone) >75% and naringinase (CAS Number 9068-31-9) were from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile HPLC grade, sodium acetate trihydrate, glacial acetic acid, absolute ethanol were from Merck (Darmstadt, Germany). K-carrageenan was obtained from Fluka (St. Louis, MO, USA). All other chemicals were analytical grade and obtained from various sources. Grapefruits were bought in local supermarkets. Oranges were from an orchard in the South of Portugal. A sample of commercial orange juice and orange concentrate juice was a gift from a Portuguese Fruit Juice Industry.

2.2. Equipment

The analyses were performed using a high-performance liquid chromatographic system – HPLC Waters 2690 Separation Module (quaternary solvent delivery pumps, in-line degasser, automatic injector with a 100 μL loop and a column oven), with a Photo Diodes Array (PDA), detector (Model Waters 996), and the results processed by Millennium®32, Waters software (Waters Corporation, Milford, Ireland). Separations were performed on a Merck analytical column, Lichrospher® 100, RP-18 (5 μm particle size, 250 × 4 mm *i.d.*).

Mettler Toledo AB204-5 analytical balance, Branson 3200 ultrasonic bath, Sigma 3K20 centrifuge and Metrohm 744 pH meter were also used.

2.3. Chromatographic conditions

The mobile phase consisted on acetonitrile (A)/water (B) and each solvent was filtered through a 0.2 μm pore size hydrophilic polypropylene filter and degassed in an ultrasonic bath before use. Separation was performed using a gradient programme: 0–8 min 23% A; 8–15 min 23–65% A linear; 15–20 min 65–70% A linear; 20–21 min 70–23% A linear; 21–22 min 23% A. The photodiode array detector (PAD) was set at 200–400 nm wavelength and the chromatogram detected at 280 nm. The analysis were performed at 25 °C (column oven temperature), with a 1 mL min^{-1} flow rate and the injection volume was 20 μL.

2.4. Standard solutions

Stock solutions of naringin and naringenin of 1000 μg mL^{-1} were prepared in a mixture of absolute ethanol:sodium acetate buffer 0.02 M, pH 4.0 (1:1) (v:v). Appropriate dilutions (v:v) of the stock solutions were made with sodium acetate buffer pH 4.0, to obtain working solutions from 50 to 100 μg mL^{-1} of naringin and from 10 to 60 μg mL^{-1} of naringenin. These solutions were used for the study of linearity, repeatability, limit of detection (LOD) and limit of quantification (LOQ).

The linearity of the method was established by using 9 standard solutions levels for naringin (concentration range of 55–95 μg mL^{-1}) and 11 standard solutions levels for naringenin (concentration range of 10–60 μg mL^{-1}), assayed in triplicate on three separate experiments. In linearity studies the determination factor, must be greater than 0.99, the deviation obtained from the distribution of normalized values for each concentration level should be less than 15% for organic compounds.

The limit of detection (LOD) (lowest concentration of analyte in a sample matrix that can be detected) and the limit of quantification (LOQ) (lowest that can be quantified with acceptable accuracy and precision) were determined in two different ways, through: (i) the residual standard deviation obtained by linear regression analysis; (ii) standard deviation of the response of 10 (working range lower limit) standard solutions.

Diode-array detection (DAD) in HPLC was used to make a spectral scan of the peaks, which was used to check for selectivity/specificity of the method.