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Analytical Methods

Development and validation of a decigram-scale method for the separation of limonin from limonin glucoside by C-18 flash chromatography

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ABSTRACT

A preparative method for decigram-scale polishing of limonin glucoside (**1**) with regard to removing traces of limonin (**2**), the main bitter principle in Citrus, is reported. During the method development and up-scaling stages, sample purity, sample size, solvent amounts, and drying conditions were varied. The resulting polishing method consists of a simple step gradient that utilises food grade solvents, ethanol and water at 15% EtOH and 50% EtOH steps. Using a 75 × 300 mm C-18 column, this method is capable of processing 20 g of material per run in less than 3 h. Recovery of the purified limonin glucoside following evaporation of the solvent was 93.5% (±2.8, $n = 6$) and the limonin concentrations in the resulting materials were found to be reduced 10- to 15-fold.

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1. Introduction

Citrus limonoids are a family of highly oxygenated triterpenoid compounds that occur in a variety of citrus tissues in significant quantities as aglycones, glucosides or A-ring lactones (Fig. 1), the metabolic precursors to limonoid aglycones and glucosides (Manners, 2007). Results from in vitro and in vivo studies examining the bioactivity of these compounds suggest that citrus limonoids possess anti-tumor and anti-HIV activities and potentially cholesterol lowering properties (Manners, 2007). Limonin and limonin glucoside are the most abundant limonoids for the majority of citrus species. Limonin possess limited water solubility (<40 mg/l), is bitter and found at its highest concentrations in the non-edible portions of citrus fruits (e.g. peels, seeds). Juice concentrations of limonin are typically less than 20 mg/l (Abbasi Soleiman, Zandi Parvin, & Mirbagheri Esmaeil, 2005; Breksa & Ibarra, 2007), however, concentrations as low as 6 mg/l (Guadagni, Maier, & Turnbaugh, 1973; Guadagni, Maier, & Turnbaugh, 1974) will render citrus juices unacceptable to consumers due to excessive bitterness. In contrast, limonin glucoside is water soluble, tasteless, and found in orange juice at concentrations as high as 720 mg/l (Schoch, Manners, & Hasegawa, 2001).

The demonstrated bioavailability (Manners, Breksa, Schoch, Hasegawa, & Jacob, 2003) and dietary abundance of limonin glucoside, coupled with the potential to formulate functional foods or beverages utilising limonin glucoside led us to undertake the centigram isolation of this compound in order to further evaluate its physical properties and biological activities. The required quantities were successfully obtained from citrus processing waste streams utilising a modified version of the method of Schoch, Manners, and Hasegawa (2002). The isolated material was incorporated into beverages for stability studies; however, we soon learned that our isolation efforts were unfinished, when results from the taste tests of the beverages indicated that they were too bitter for consumption. Analysis of the test beverages allowed us to quickly ascertain that limonin was the culprit and that the limonin concentration in the beverage was four times the acceptable level. Compounding the challenge of removing the contaminating limonin, we faced the additional necessity of avoiding all but food grade materials and solvents because we wanted to retain the ability to use the material for human studies.

Review of the available literature revealed that, with few exceptions, the majority of limonoid isolation or analytical methods have focused exclusively on either aglycones or glucosides, but not separating aglycones from glucosides (Manners, 2007; Manners et al., 2003). In the present report, we present a straightforward, rapid, and efficient method for the polishing of relatively pure limonin glucoside with regard to removing traces of limonin.

2. Materials and methods

2.1. Materials

Solvents (acetonitrile, chloroform, methanol) were HPLC grade and along with formic acid (88%, ACS reagent grade), citric acid

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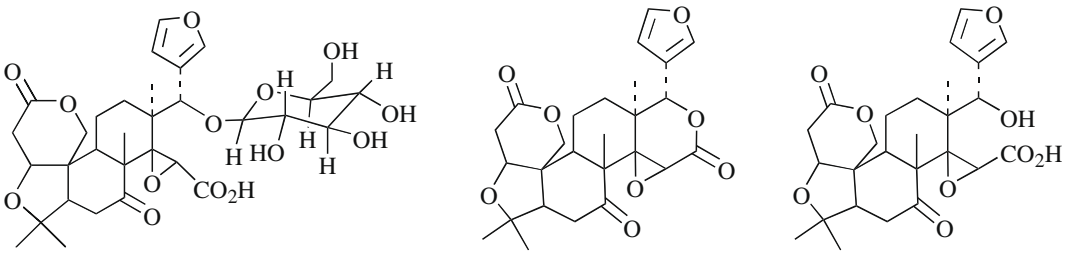

Fig. 1. Structures of limonin glucoside (**1**) and limonin (**2**) and limonoate A-ring lactone.

monohydrate (ACS), sodium citrate (enzyme grade) and sodium hydroxide (molecular biology grade) were purchased from Fisher Scientific Ltd. (Waltham, MA). Ethyl alcohol (190 proof, USP grade) was purchased from Equistar Chemicals, LP (Tuscola, IL) and filtered through a 0.45 μm type HA membrane filter (Millipore, Billerica, MA) prior to use. Water was deionized to $\geqslant$18.1 MΩ/cm resistance using a Barnstead NANOpure Deionisation System (Dubuque, IA). Water used to prepare HPLC mobile phases was filtered through a 0.45 μm type HA membrane filter prior to use. Limonin glucoside was isolated as previously described (Schoch et al., 2002). Modification of this method included the further purification of the isolated limonin glucoside by recrystallization from warm water.

2.2. Preparation of in-house test beverages

In order to evaluate the limonin content in the purified limonin glucoside prior to and following polishing, small volumes of test beverages were prepared in house. These test beverages were prepared in a citrate solution (pH 4.0). To prepare the citrate solution citric acid (0.1 M, 33 ml) was combined with sodium citrate (0.1 M, 17 ml), brought up close to a total volume of 100 ml with water and the pH then adjusted to 4.0 with sodium hydroxide (1.0 N). Samples of the polished or unpolished limonin glucoside (20 mg) were weighed out, added to 15 ml of citrate buffer, sonicated for 30 min and finally filtered through a 0.2 μm Nalgene syringe filter (SFCA, Rochester, NY). The resulting test beverages were extracted and analysed as described below.

2.3. Determination of limonin in samples and test beverages

A 1.0 ml aliquot of sample was combined with 2 ml of chloroform and vortexed for 1 min at speed #4 on a VWR multi-tube vortexer (West Chester, PA). The resulting emulsion was clarified by centrifugation for 2 min at 2800 rpm using a Fisher Scientific Marathon 8 K Centrifuge (Waltham, MA). Half of the chloroform layer (1.0 ml) was transferred to a 1.2 ml polypropylene tube and evaporated to dryness under a stream of warm nitrogen gas (Jones Chromatography SPE Dry Dual, Charlottesville, VA). Prior to HPLC analysis, samples were reconstituted in a solution (500 μl) comprising of 30% ACN and 70% H_2O, vortexed for 1 min at speed #4 and the sample pooled to the bottom of its tube by centrifugation for 2 min at 4000 rpm (ICE Centra CL4, Needham Heights, MA).

Limonin concentrations present in the chloroform extracts of the samples were determined by HPLC using a Waters 2695 System (Milford, MA) coupled to a Waters 996 PDA detector set to scan from 190 to 250 nm or Waters Dual Absorbance Detector 2487 set at 210 nm. Standards and samples (20 μl) were injected on a 50 × 2.0 mm Phenomenex Phenosphere-Next-5 μ Phenyl column (Torrance, CA) equipped with a guard column of the same stationary phase, maintained at 30 °C and flowing isocratically at a rate of 0.5 ml/min. The solvent composition was 30:70 acetonitrile: 10 mM formic acid and the total run time was 5.5 min. Instrument control and data acquisition was accomplished using Masslynx (version 4.0). Quantitative results were obtained by comparing the area under curve at 210 nm for the sample to a standard curve generated from limonin (1, 2.5, 5.0, 10.0 and 25.0 ppm) standards prepared in 20% acetonitrile. Values were reported as the mean of three replicate analyses.

2.4. HPLC conditions for the analysis of limonin glucoside and limonin in a single run

The HPLC analysis of limonin glucoside and limonin in samples in a single run was accomplished using a binary gradient composed of (A) acetonitrile and (B) formic acid (25 mM). Analysis was conducted on a Waters 2695 system coupled to a Waters model 996 diode array detector (190–250 nm) and Sedex 55 ELS detector (50 °C, 2.5 bar N_2, S.E.D.E.R.E., Alfortville, France). A 50 × 2.0 mm Phenomenex Phenosphere-Next-5 μ Phenyl column (Torrance, CA) equipped with a guard column of the same stationary phase and maintained at 30 °C was used. The flow rate was 0.5 ml/min and the column was eluted starting with a linear gradient from 15/85 (A:B) to 30/70 (A:B) in 5 min, continuing with an isocratic stretch at 30/70 (A:B) for 5 min, followed by a linear gradient in 2 min back to 15/85 (A:B) and re-equilibrating at this composition for 3 min. The total run time was 15 min. Sample injection volumes were 20 μl. Samples with ethanol contents in excess of 20% were diluted with water to give a final ethanol content of 20% or less.

2.5. HPLC-ESI/MS analysis

The HPLC-ESI/MS analysis of samples was conducted on a system controlled by Xcalibur (version 1.4) that consisted of a Waters 2695 coupled to a TSP UV 2000 detector (210 nm) and Thermo Finnigan LCQ Advantage ion-trap mass spectrometer (San Jose, CA). The same binary gradient described above was utilised with 10 μl injections. MS analysis was conducted in negative mode for the first 6.8 min, whereupon analysis was conducted in positive mode for the remaining 8.2 min. The mass spectrometer was tuned through optimisation on the signal generated by introduction of a limonin glucoside (5 ppm, negative mode, *m/z* 649.3) or limonin (5 ppm, positive mode, *m/z* 471.1) solution into the mass spectrometer in the LC mobile phase at the flow rate used for analysis. Following tuning, the mass spectrometer was operated in negative ion mode with a capillary temperature of 380 °C, spray voltage of 4.50 kV, capillary voltage of 9.0 V and scan range 375–975 *m/z*, and in positive ion mode with a capillary temperature of 380 °C, spray voltage of 4.50 kV, and capillary voltage of 3.0 V and scan range 375–975 *m/z*.