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LC-MS/MS Method for the Determination and Quantitation of Penicillin G and Its Metabolites in Citrus Fruits Affected by Huanglongbing

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ABSTRACT: In this study, we developed and validated a method for the extraction, identification, and quantitation of penicillin G and its metabolites (penilloic acid and penillic acid) in a variety of citrus fruits by employing sequential liquid/liquid and solid-phase extraction techniques in conjunction with UHPLC-MS/MS. Two product ion transitions per analyte were required for identification, which contributes to a high degree of selectivity. Corrected recoveries of penicillin G using an isotopically labeled internal standard were 90−100% at fortification levels of 0.1, 0.25, 1, and 10 ng/g. Absolute recoveries for penillic acid and penilloic acid were 50−75% depending on the matrix used. The limit of detection (LOD) of penicillin G and its metabolites was found to be 0.1 ng/g when 2 g of citrus was extracted. This method is useful in determining residue levels of penicillin G and its metabolites in citrus trees infected with huanglongbing bacteria after antibiotic treatment.

KEYWORDS: *antibiotic, penilloic acid, penillic acid, extraction, method development*

INTRODUCTION

In the past century, huanglongbing (HLB) has become one of the most devastating diseases threatening citrus trees worldwide. HLB, also known as citrus greening disease, is caused by bacteria in the genus *Candidatus Liberibacter*.[1−8] These bacteria are typically spread via small insects called the Asian citrus psyllid.[9,10] Once it becomes a vector for the bacteria, the psyllid carries and transfers the bacteria from one citrus tree to another, causing the disease. Infected trees experience stunted growth and produce irregularly shaped and bitter fruits; eventually, HLB leads to the death of the tree. The decrease in citrus production has cost the world economy several billion dollars in lost revenues and jobs.

Of the variety of antibiotics that have been tested to combat this disease, streptomycin, tetracycline, and penicillin are the most effective.[11,12] For example, Bove et al. reported the effectiveness of penicillin G, **1** (Figure 1), and tetracycline injections for the treatment of infected sweet orange trees.[13] In another paper, Chung and Zhisheng studied the effectiveness of various antibiotic application methods. Whereas tetracycline is more effective than penicillin under certain conditions, penicillin is considered less toxic and more cost-effective at high doses.[14] Recently, Duan et al. reported that a combination of penicillin G and streptomycin is effective in eliminating the bacterium with greater long-term effects than either antibiotic alone.[12]

Although these antibiotics have been used to treat the greening disease, there is little information regarding residue dissipation kinetics under field conditions. Research on the nature of these antibiotics and their accumulations in citrus fruits is crucial for the development of acceptable applications that comply with good agricultural practices (GAP) and may affect domestic and international trade.

Among the methods used to monitor the chemical residues of penicillin G in various food matrices, LC-MS is the most sensitive and widely used.[15−17] For example, Galey et al. described a sensitive method for the extraction, purification, and detection of penicillin G and other β-lactam antibiotics from milk using HPLC-MS.[18] In that paper, a liquid/liquid extraction with acetonitrile and water was used to extract penicillin followed by sample cleanup with solid phase extraction (SPE). The sample was then analyzed using ion trap LC-MS/MS, where the average recovery was 109% with a coefficient of variation (CV) of 3.2%. Subsequently, Wang used a similar analytical method for the quantitation of penicillin in honey.[19] In that work, penicillin G was extracted from the matrix using a phosphate buffer solution followed by cleanup with an SPE cartridge. LC-MS/MS was then used to quantitate penicillin G residues with the use of an internal standard. The limit of detection (LOD) for penicillin G was approximately 1 ng/g of matrix with a recovery of 93−109% (CV < 10%). More recently, penicillin G was extracted from ground pork using a 15:2 mixture of acetonitrile/water, followed by sample cleanup with an SPE cartridge. LC-MS/MS analysis showed an LOD of about 1 ng/g of matrix, but the recovery was relatively low at 43.6%.[20]

The efficient extraction of various compounds from food matrices is dependent on the molecule's structural features. Penicillin G features a labile β-lactam ring characterized by its susceptibility to ring-opening under various conditions including the presence of acids, bases, nucleophiles, or oxidizing

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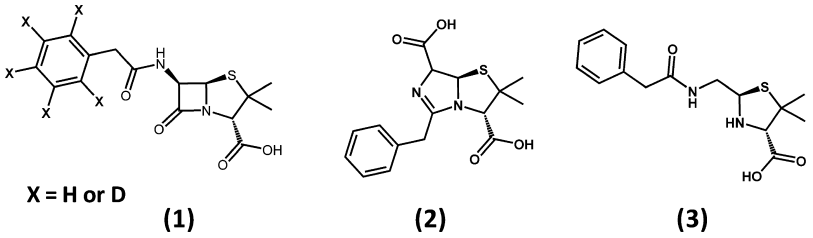

Figure 1. Schematic representation of the chemical structures of penicillin G or penicillin G-d_5 (**1**), penillic acid (**2**), and penilloic acid (**3**).

Table 1. Recovery of Penicillin G, Penillic Acid, and Penilloic Acid from Three Citrus Matrices Fortified in Triplicate[a]

	LC-MS absolute recovery (RSD, %)			LC-MS corrected recovery (RSD, %) using internal standards
analyte concn (ng/g)	penicillin G 335 → 114	penillic acid 335 → 128	penilloic acid 309 → 174	penicillin G 335 → 114
orange matrix				
0.1	63 (23)	70 (20)	73 (11)	100 (17)
0.25	89 (8)	63 (5)	73 (8)	118 (8)
1	69 (8)	66 (3)	69 (5)	99 (6)
10	60 (14)	67 (6)	62 (9)	101 (3)
grapefruit matrix				
0.1	43 (15)	50 (10)	60 (2)	80 (10)
0.25	51 (12)	61 (10)	57 (8)	88 (7)
1	59 (2)	63 (5)	57 (2)	93 (7)
10	50 (9)	64 (2)	51 (4)	97 (5)
lemon matrix				
0.1	33 (6)	56 (6)	57 (6)	77 (5)
0.25	40 (16)	73 (17)	61 (12)	87 (2)
1	41 (5)	65 (1)	57 (4)	97 (10)
10	38 (4)	67 (5)	55 (7)	99 (4)

[a]Only the most selective ion products are shown.

agents.[21,22] For example, in the presence of acids or bases, the highly strained β-lactam ring opens to give an array of products (metabolites) such as penicilloic acid, penicillinic acid, penicillamine, penilloaldehyde, aminopenicillanic acid, penaldic acid, penamaldic acid, isopenicillic acid, penillic acid, and penilloic acid. Of these, the most abundant are penillic acid, **2**, and penilloic acid, **3**.[22−29] The instability of penicillin G emphasizes the importance of designing an analytical method that identifies not only penicillin G but also potential metabolites.

To the best of our knowledge, no method has been reported for the analysis of penicillin G and its metabolites in citrus matrices. In this paper, we present a sensitive method to quantitate penicillin G and its metabolites (penillic and penilloic acids) with high selectivity in a variety of citrus matrices using ultra-high performance liquid chromatography coupled with triple-quadrupole tandem mass spectrometry (UHPLC-MS/MS). Throughout method development, we systematically investigated the extraction process and matrix effects to optimize quantitative accuracy and precision. Because of the potential use of penicillin G to treat citrus greening disease, it is necessary to develop a reliable method for the extraction and identification of penicillin G residues. This method is useful to quantitatively monitor the presence of penicillin G and its metabolites in citrus fruits post-treatment.

MATERIALS AND METHODS

Reagents. Penicillin G potassium salt (99.4%) and internal standard (penicillin G-d_5 potassium salt) (98.4%) were provided by o2si Smart Solutions (Charleston, SC, USA). Penilloic acid (96.3%) and penillic acid (99.3%) were purchased from LGC Standards (Wesel, Germany). HPLC grade hexane, acetonitrile (with and without 1% formic acid), methanol, and water (with and without 1% formic acid) were provided by Fisher Scientific (Pittsburgh, PA, USA). Anhydrous monosodium phosphate, disodium phopshate, and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Buffer Solution Preparation. The phosphate buffer solution (PBS) (0.1 M, pH 7) was prepared by dissolving anhydrous NaH_2PO_4 (5.8 g, 0.0483 mol) and Na_2HPO_4 (8.15 g, 0.0574 mol) in 1 L of deionized (DI) water. Ammonium acetate ($NH_4C_2H_3O_2$) buffer (0.05 M, pH 6.7) was prepared by dissolving ammonium acetate (4.0 g, 0.0519 mol) in 1 L of DI water.

Preparation of Standard Stock Solutions. Individual stock solutions of penicillin G potassium salt, penilloic acid, penillic acid, and internal standard penicillin G-d_5 at 1000 μg/mL were made by dissolving the standard (after adjusting for salt content and purity) in a mixture of water and acetonitrile (1:1, v/v). These stock standard solutions were stored at −20 °C for an extended period of time (>5 months) with no issues.

Preparation of Mixed Intermediate Standard Solutions (Int-A) (1 μg/mL = 1 ng/μL). The above stock solutions, each at 1000 μg/mL, were used to prepare a mixed standard solution (Int-A) containing penicillin G, penillic acid, and penilloic acid, each at a concentration of 1 μg/mL. These were prepared by adding 100 μL of each standard stock solution into the same 100 mL volumetric flask. The flask was filled to the mark with phosphate buffer (0.1 M, pH 7). Preparation of intermediate solutions at concentrations of 2, 5, 20, 100, 200, and 300 ng/mL was carried out on each day of batch extraction/analysis by further diluting Int-A. The intermediate internal standard solution containing 1 μg/mL penicillin G-d_5 was prepared by adding 100 μL of the penicillin G-d_5 stock solution (1000 μg/mL) into a 100 mL volumetric flask, which was subsequently filled to the mark with phosphate buffer (0.1 M, pH 7). This was further diluted with

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phosphate buffer to make an internal standard intermediate solution at a concentration of 40 ng/mL.

Calibration Curve Preparation. To prepare 800 μL of the individual calibration standards, 100 μL of the penicillin G-d_5 internal standard intermediate standard (40 ng/mL) was added to each of six different autosampler vials. Then to each vial was added 100 μL of each intermediate standard (2, 5, 20, 100, 200, 300 ng/mL). This was followed by the addition of 600 μL of ammonium acetate buffer. It should be noted that the calibration curve was prepared out of matrix for the quantitation of penicillin G because an internal standard was used to compensate for ion suppression and recovery losses during the extraction procedure. Because no isotopically labeled standards are available for penillic or penilloic acids, a calibration curve in matrix was used. The in-matrix curve was prepared by carrying six separate 2 g samples of matrix through the extraction process. The extracted matrix samples were evaporated in 50 mL centrifuge tubes and labeled 1−6. To each sample of dried matrix was added 100 μL of the correspondingly labeled mixed intermediate standard (2, 5, 20, 100, 200, 300 ng/mL) along with 700 μL of ammonium acetate buffer.

Sample Fortification. The fortified samples were made by spiking 2 g of the different citrus matrices (lemon, orange, and grapefruit) with 100 μL of four intermediate mixed standard solutions (2, 5, 20, and 200 ng/mL) and 100 μL of the internal standard intermediate solution (40 ng/mL). Overall, four spikes were made for each matrix to reach final concentrations of 0.1, 0.25, 1, and 10 ng/g of penicillin G, penillic acid, and penilloic acid in each spike. The concentration of the internal standard spiked into the matrix was constant in all samples (2 ng/g). Reagent (2 g of water) and matrix blanks were not fortified with internal standard and were used as negative controls.

Penicillin G Extraction from Citrus Matrices. Citrus fruit samples were purchased (from retail stores) and immediately stored at −80 °C. The entire frozen fruits were then homogenized to a powder-like consistency using a robot coupe with a 7 mm thick stainless steel blade (Robot Coupe, Ridgeland, MS, USA), and a 2 g portion was immediately weighed into a 50 mL polypropylene centrifuge tube. Then, 4 mL of PBS buffer (0.1 M, pH 7) was added, and the samples were fortified with 100 μL of internal standard. Each of four separate samples was then spiked with the intermediate standards, in triplicate, as shown in Table 1. The samples were shaken using a vortex mixer for 10 min at 1100 rpm. Four milliliters of hexane was added to the mixture followed by additional shaking in the vortex mixer (10 min at 1100 rpm). Then, the samples were centrifuged at 4000 rpm for 15 min to separate the aqueous and organic layers. The hexane layer was then discarded using a Pasteur pipette, and the aqueous layer was then transferred to an Amicon Ultra 50000 M_w membrane filtration device (Millipore, Darmstadt, Germany). Centrifugation at 4000 rpm for 15 min filtered solids and residual hexane from the aqueous layer. The filtrate was washed with 2 mL of phosphate buffer followed by further centrifugation (15 min, 4000 rpm) resulting in a filtered aqueous solution containing extracted penicillin G and its metabolites.

SPE Cleanup of Penicillin G. Oasis HLB, 6 cc, 200 mg SPE extraction cartridges (Waters, Milford, MA, USA) were prewashed sequentially with 3 mL of methanol, 3 mL of H_2O, and 3 mL of PBS buffer. The sample extract was loaded onto the SPE column, and the column was washed with 2 mL of phosphate buffer. Then, a slight vacuum was applied to dry the SPE columns. Penicillin G and metabolites were eluted from the SPE columns with 3 mL of acetonitrile into clean 50 mL polypropylene centrifuge tubes. The eluate was evaporated to dryness using a stream of N_2 at 40 °C for 1 h. Ammonium acetate buffer (800 μL) was then immediately added. The extract was mixed by vortexing for 20 s and followed by filtration through a 0.2 μm PVDF filter for LC-MS/MS analysis.

LC-MS/MS Method. The LC-MS/MS included an ACQUITY UPLC I-Class System (Waters) connected to an API 6500 Qtrap (AB Sciex, Toronto, ON, Canada) triple-quadrupole mass spectrometer. Analyses were carried out using electrospray ionization (ESI) in positive-ion mode using multiple reactions monitoring (MRM). Optimized mass spectrometer parameters for the detection of penicillin G and its metabolites were obtained through infusion studies. The parameters optimized were declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP). The MS/MS product ion spectra of the standards and experimental samples were obtained using a target-oriented screening approach in enhanced product ion (EPI) scan mode. The source parameters (ion source voltage (4500 V), curtain gas (30 psi), heater gas (45 psi), and ion source temperature (400 °C)) were identical in MRM and EPI scan modes. Separation of the test compounds was achieved using a 150 mm × 2.1 mm i.d., 1.7 μm, Acquity C18 column. The mobile phase composition was (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The column temperature was maintained at 40 (±1) °C. The gradient began at 95:5 of A:B with a flow of 0.2 mL/min. The gradient was changed to 5:95 of A:B over a course of 10 min followed by a 5 min hold at 5:95 of A:B. Over 0.1 min, the gradient was changed to 95:5 of A:B with a concomitant change in flow rate to 0.4 mL/min. After 4 min, the flow was reduced to 0.2 mL/min over the course of 0.1 min. This was held for 0.8 min to complete the LC gradient (20 min total run time).

Method Validation. This analytical method was validated by independently analyzing three different matrices (lemon, orange, and grapefruit); each matrix was studied in triplicate. A multipoint, in-matrix calibration curve was used to calculate the concentration of penillic acid and penilloic acid in the spikes. The penicillin G is calculated from a multipoint, in-solvent calibration curve with an isotopically labeled internal standard (penicillin G-d_5). Calibrants were used at levels ranging from 0.25 ng/g (equivalent to 0.1 ng/g in sample) through 37.5 ng/g (equivalent to 15 ng/g in sample). A minimum of five data points for linear and quadratic regression lines were required, but any combination of standards may be used for quantitation. Curves were fit with $1/x$ weighting with an R^2 value >0.99. It is the policy of the laboratory to set limit of detection (LOD) and limit of quantitation (LOQ) values at levels greater than the minimum 3× and 10× general requirements, respectively, such that daily instrument variance, matrix effects, procedural errors, etc., do not result in failures to meet validated LODs and LOQs for studies taking place over extended periods of time. It is also the policy of the laboratory to verify LOD and LOQ reporting levels using spiked sample matrix. In short, the laboratory may set LOD and LOQ values to levels greater than the minimum detected signal at 3× or 10× on the instrument on the basis of the needs of the method and the desired ruggedness of the method. The LOD for this method was set to the lowest concentration validated in this study at 0.1 ng/g in matrix, whereas the LOQ was set at 0.25 ng/g. Both of these levels exceed the 3× and 10× requirements for the primary transition. Values below the LOD are reported as not detected (ND). Values between the LOD and LOQ are reported as below the quantitation limit (BQL). Values at and above the LOQ are reported as actual values.

RESULTS AND DISCUSSION

Citrus matrices contain a variety of compounds including sugars, citric acid, vitamin C, folic acid, potassium, phytochemicals (e.g., limonoids, flavanoids, etc.), and essential oils (e.g., limonene, octanal, copaene, dodecanal, etc.).[30−33] These various products are distinct in their structures and properties; this must be taken into consideration when attempts are made to extract specific molecules of interest such as penicillin G and its metabolites (penillic acid and penilloic acid) (Figure 1). These products are amphiphilic, containing a benzyl group at one end and one or two carboxylic acids at the other. The presence of carboxylic acid groups increases the polarity of these molecules, which makes them highly soluble in water and easily extracted using an aqueous medium. It is worth noting that penicillin G contains a fused β-lactam ring system, which is susceptible to ring-opening under acidic and alkaline conditions, as well as under enzymatic activity. It can easily be transformed or isomerized into many other compounds such as penillic, penilloic, and penaldic acids, penicillamine, and

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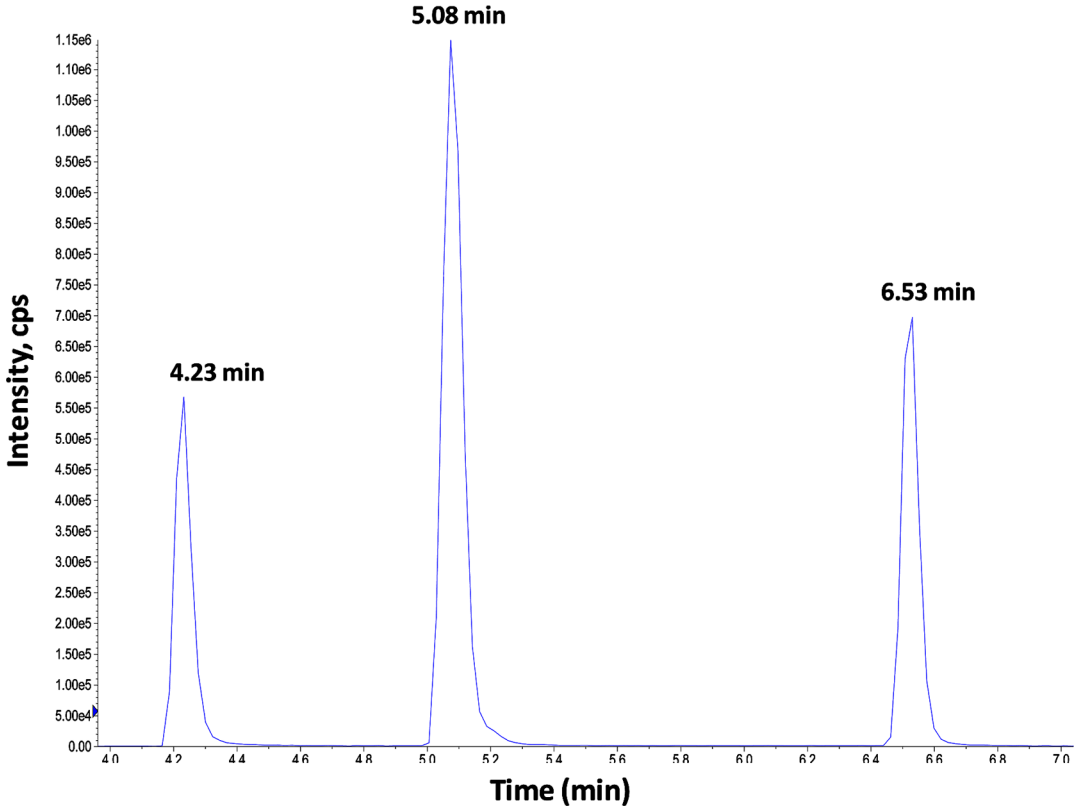

Figure 2. LC chromatogram of the three analytes showing their retention times with 6.53 min for penicillin G, 5.08 min for penilloic acid, and 4.23 min for penillic acid.

penilloaldehyde. Once infected citrus trees are treated with penicillin G, we believe various environmental conditions including heat, rain, sunlight, and contact with biological nucleophiles, acids, or bases might lead to the decomposition of penicillin G and formation of penicillin metabolites.[21] Therefore, it is important to identify these metabolites when fully assessing the treatment of citrus trees with penicillin G. This motivated us to incorporate penilloic acid and penillic acid metabolites (commercially available) into our extraction and analytical method because they may be present even after penicillin G has fully decomposed. This information guided us in the development of our method for the extraction of penicillin G and its metabolites from citrus matrices as observed by LC-MS/MS analysis.

Extraction Method Optimization. Samples were fortified with an internal standard solution containing only penicillin G-d_5 as well as an intermediate mixed standard solution containing penicillin G, penilloic acid, and penillic acid. After fortification, liquid/liquid extraction using a phosphate buffer (pH 7) and hexane solvent was carried out. The buffer helps to prevent degradation of penicillin G because it is more stable at pH 7, whereas the hexane removes nonpolar components from the sample. This increases the longevity of the C18 columns used on the liquid chromatograph instruments and decreases the probability of ion enhancement or suppression. Next, the aqueous phase was cleaned using a membrane filtration device to remove completely insoluble components. Finally, we used an amphiphilic OASIS HLB column to trap and purify the analytes from hydrophilic contaminants. The analytes were then eluted with acetonitrile, dried, reconstituted with an organic buffer (ammonium acetate), and injected in the LC-MS instrument. This method of extraction has been successfully used to extract penicillin G, penillic acid, and penilloic acid with absolute recoveries ranging from 35 to 70% depending on the matrix used, the compound of interest, and the fortification level. Recoveries as calculated for penicillin G using the internal standard had a range of 77−118% (Table 1).

LC-MS/MS Analysis. Solutions (1 ng/μL) of penicillin G, penicillin G-d_5, penillic acid, and penilloic acid were independently infused into the AB Sciex 6500 mass spectrometer to verify precursor/product ion masses. A Q1MS scan mode was used to identify the precursor ion of each compound. Once identified, the DP was optimized for each precursor ion. Then, product ion scan mode was used to find the product ions. A scan range from 100 to 340 Da was used to allow for observation of low mass product ions as well as the precursor ion. A collision energy ramp (5−80 eV) allowed for determination of the best confirming product ions for each precursor in terms of sensitivity and unique mass values. Using a MRM scan mode, in conjunction with automated compound optimization mode, the DP, CE, EP, and CXP were optimized for each product ion. The precursor to product ion transitions were found to be 335 → 176, 335 → 160, and 335 → 114 (amu) for penicillin G, 309 → 263, 309 → 174, and 309 → 128 (amu) for penilloic acid, 335 → 289, 335 → 243, 335 → 176, and 335 → 128 (amu) for penillic acid, and 335 → 181, 335 → 160, and 335 → 114 (amu) for penicillin G-d_5. Using the optimized parameters, subjection of the standards to LC-MS analysis allowed for observation of retention times and ion ratios between precursor and product ions. Penicillin G and its metabolites were separated on a reversed-phase LC column under the given gradient conditions within 20 min. Figure 2 shows the LC chromatogram of these three compounds. Typical retention times of 6.53, 5.08, and 4.23

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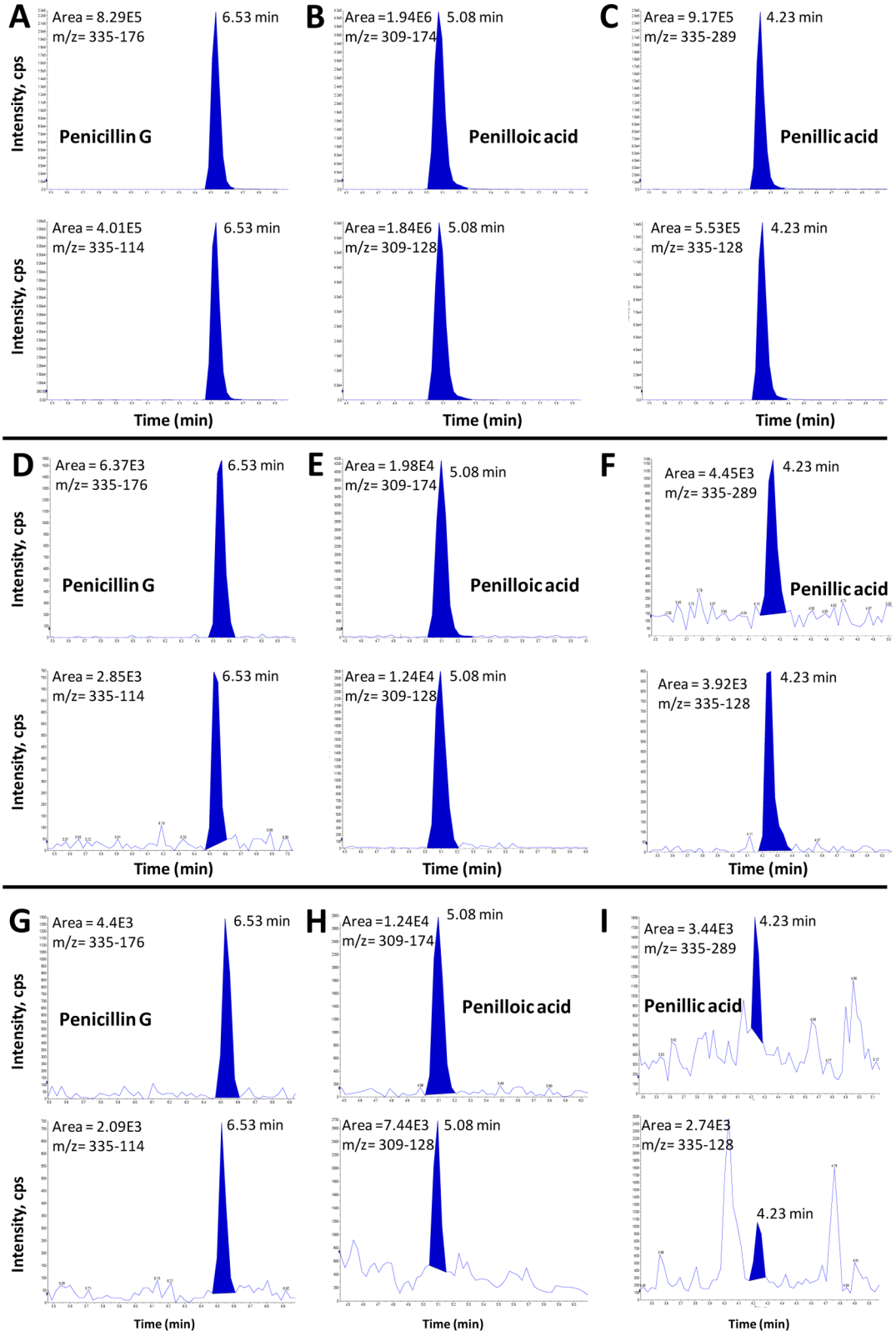

Figure 3. Extracted ion chromatograms collected at standard concentrations of 15 ng/mL in solvent (A–C), 0.1 ng/mL in solvent (D–F), and 0.1 ng/mL in grapefruit matrix (G–I) for penicillin G (*m/z* 176 and 114), penilloic acid (*m/z* 174 and 128), and penillic acid (*m/z* 289 and 128), respectively. These ion products were used for quantitation and precursor confirmation.

min were observed for penicillin G, penilloic acid, and penillic acid, respectively. These retention times reflect expectations based on the chemical structures (i.e., polarity) of these compounds. On one hand, the shortest retention (for penillic acid) can be explained by the high polarity of the two carboxylic acid groups along with the imidazoline heterocycle. Penicillin G, on the other hand, features only one carboxylic acid group along with a nonpolar benzyl group contributing to its longer

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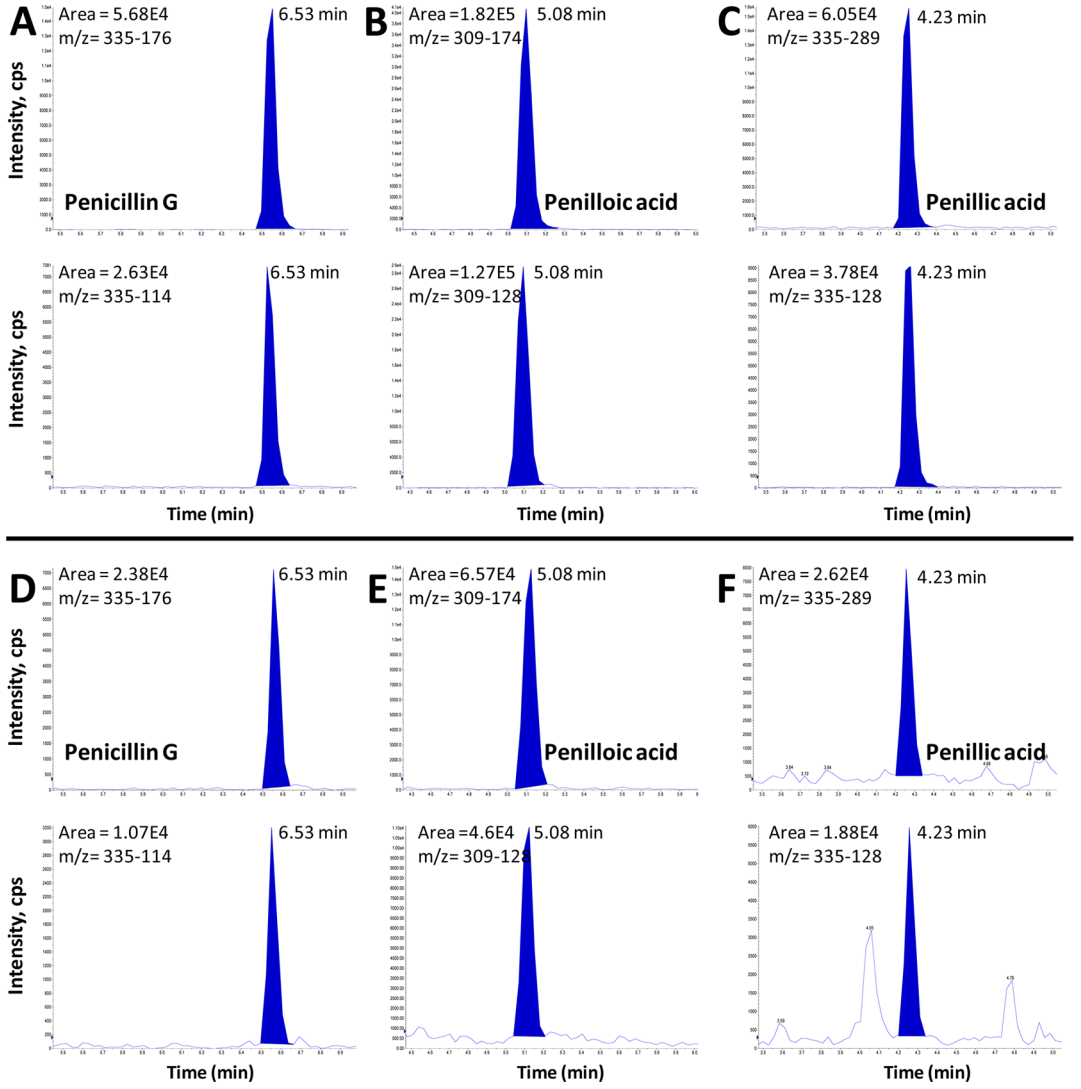

Figure 4. Chromatograms of two product ions of standards in solvent (A–C) and of fortified (spiked) samples in grapefruit matrix (D–F), each at a concentration of 1 ng/mL. The absolute difference in ion ratios between standards in solvent and fortified grapefruit matrix were found to be 1.3, 0.2, and 9% for penicillin G, penilloic acid, and penillic acid, respectively.

retention time of 6.53 min (Figure 1). Panels A–C of Figure 3 show representative LC-MS chromatograms of the most sensitive two product ion signals at a concentration of 15 ng/mL sample in solvent. To demonstrate the ability of this method to function at the LOD, the same chromatograms are shown in Figure 3D–F and in Figure 3G–I at a concentration of 0.1 ng/mL in solvent and grapefruit matrix, respectively. At the LOD level (100 ppt), matrix interference is clearly observed; however, detection of the product ions is still possible (Figure 3I). The product ion transitions used for quantitation purposes were selected on the basis of both the sensitivity and the presence of a clean background. The transitions used for quantitation are 335 → 114, 309 → 174, and 335 → 128 for penicillin G, penillic acid, and penilloic acid, respectively.

Fortifications and Recoveries Results. The method was tested in triplicate to determine its accuracy and precision, and the results are shown in Table 1. Penicillin G, penilloic acid, and penillic acid were each examined in three different citrus commodities (orange, grapefruit, and lemon) at four different concentrations (0.1, 0.25, 1, and 10 ng/g of matrix). The commodities were extracted within the same day to limit the extent of penicillin G degradation in the citrus matrices. The three analytes were then extracted and analyzed by UHPLC/ESI-MS/MS along with a set of samples prepared in solvent at identical concentrations. Preliminary experiments indicated that the instrumental response varies with the absence or presence of citrus matrix. Therefore, to compensate for the effects of the matrix suppression, matrix-matched calibration standard curves (in-matrix curves) were used to quantitate the analytes and obtain what we call absolute recovery. Because of the availability of the standard, penicillin G can be quantified using isotopically labeled penicillin G-d_5 internal standard, which accounts for recovery losses, ion suppression, and degradation of the analytes during the extraction process. Absolute recoveries of the three analytes used in this study from fortified citrus were calculated using a matrix-matched calibration standard curve, and the correlation of coefficient values (R^2) were consistently >0.99. To generate these curves, standards were dissolved in a solution (containing the matrix components) at concentrations varying from 0.1 to 15 ng/g. To quantitate the absolute recoveries of the analytes, chromato-

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grams were used to plot the in-matrix calibration points, which were fitted using a $1/x$ weighted quadratic curve. On the other hand, the calibration curve obtained using the isotopically labeled internal standard, penicillin G-d_5, was fitted using $1/x$ linear regression.

The effect of matrix on the area counts for all of the analytes in the three matrices was also investigated. We determined the effect of ion suppression on the signal in all of the citrus matrices. The matrix ion suppression was found to be about 18, 39, and 19% in lemon, 25, 42, and 32% in orange, and 32, 42, and 28% in grapefruit for penicillin G, penilloic acid, and penillic acid, respectively. These data indicate that there is a slight difference in the ion suppression when the matrix is varied. Table 1 shows the absolute recoveries of penicillin G, penillic acid, and penilloic acid along with their relative standard deviations (RSD). Samples were analyzed in triplicate for each matrix as measured by the in-matrix curve. It also shows the recovery of penicillin G as calculated by the internal standard. The recoveries of penilloic acid and penillic acid at each of the four concentrations ranged from 50 to 73% with an average RSD value of <10%. However, the matrix seems to affect the recovery of penicillin G with an average of 70, 50, and 38% recovery in orange, grapefruit, and lemon, respectively. This is probably due to the instability of penicillin G at low pH during the extraction process. We anticipated having lower pH in the lemon matrix than in the grapefruit and orange matrices. To confirm this, we measured the pH of each matrix after stabilization with 4 mL of phosphate buffer and found the pH to be 4.0 for lemon, 5.5 for grapefruit, and 6.0 for orange. Furthermore, we studied the stability of penicillin G and penicillin G-d_5 in the final extract from lemon over the course of 1 week. The final extract, with a pH 7.0 after reconstitution with ammonium acetate buffer, was stored at 10 °C in the LC autosampler. Under these conditions, no significant changes were observed in the concentrations of penicillin G and penicillin G-d_5.

The recovery of penicillin G obtained when corrected with internal standard was nearly 100%. Low recoveries were observed in grapefruit and lemon (about 70−80%) at low levels of fortification (0.1 and 0.25 ppb). Differing matrices did not affect ion ratios, which closely corresponded to the ion ratios observed for in-solvent standards (Figure 4). Figure 4 shows an example of the chromatograms of two ion products of penicillin G, penillic acid, and penilloic acid dissolved in solvent and in fortified/extracted samples (spikes) at 1 ng/mL. These chromatograms demonstrate the consistency of ion ratios (RSD < 10%). The recoveries and RSDs indicate that our method provides good accuracy and precision for the determination of penicillin G and its metabolites in citrus.

In conclusion, an efficient method for the extraction, identification, and quantitation of penicillin G and its metabolites in a variety of citrus fruits (lemon, orange, and grapefruit) has been developed and optimized using UHPLC-MS/MS. Combining liquid/liquid extraction with solid phase extraction cartridges served as a simple and rapid technique for the extraction of penicillin G and the metabolites of interest (penilloic acid and penillic acid). Two product ion transitions per analyte, along with the ion ratios, were used for data acquisition and analyte confirmation, resulting in a highly selective method. Penicillin G and its metabolites can be detected with a limit of detection (LOD) of 0.1 ng/g when 2 g of citrus is extracted. This method is currently used by the Florida Department of Agriculture and Consumer Services to test actual citrus for the presence of penicillin G and its metabolites from trees that have been affected by the greening disease and treated with penicillin G.

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The authors declare no competing financial interest.

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