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Validation of a Residue Analysis Method for Streptomycin and Tetracycline and Their Food Safety Evaluation in Pomegranate (*Punica granatum L.*)

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Supporting Information

ABSTRACT: A single-step methanol extraction based method was developed and validated for simultaneous estimation of the residues of streptomycin and tetracycline group compounds in pomegranate fruits by LC−MS/MS. The limits of quantification for all target compounds were ≤0.005 mg kg^{-1} with recoveries (%) at fortification levels of 0.005, 0.01, and 0.05 mg kg^{-1} being within 90−116% (RSD ≤ 9%) and interday precision RSD ≤ 12% at 0.01 mg kg^{-1}. A field experiment on the dissipation of streptomycin and tetracycline (including 4-epimers) residues in pomegranate fruits with regards to field applications of the commercial formulation Streptocycline SP (streptomycin sulfate 90% + tetracycline hydrochloride 10%) at 200 and 400 g a.i. ha^{-1} indicated preharvest intervals of 45 and 55 days for streptomycin and 12 and 15 days for tetracycline, respectively. The study will be useful in promoting effective residue monitoring and ensuring safe use of these antibiotics in managing bacterial diseases of pomegranate.

KEYWORDS: *streptomycin, tetracycline, residue dissipation, safety evaluation, pomegranate*

INTRODUCTION

Streptomycin (aminoglycoside group) and tetracycline (polyketide group) compounds are commonly used antibiotics, recommended in plant protection practices against bacterial diseases either singularly or in combinations, because of their broad spectrum bioefficacy, easy availability, and cost effectiveness. In many countries streptomycin and tetracycline are registered for use in agriculture, with the primary use in apple, pear, and related ornamental trees. In India, a combination product, Streptocycline (streptomycin sulfate 90% + tetracyclinehydrocloride 10%) manufactured by Hindustan Antibiotics Ltd. (Pune, India) is registered for use in apple (fire blight), beans (halo blight), citrus (citrus canker), potato (black leg and soft rot, bacterial brown wilt), tomato and chilies (bacterial leaf spots), etc.[1]

Bacterial blight caused by *Xanthomonas axonopodis pv Punicae* is a major challenge faced by the pomegranate growers in India over the past few years that poses a severe threat to the production and productivity of this commercially important crop.[2] Although the combination of streptomycin and tetracycline is used in commercial cultivation of pomegranate for the management of bacterial blight disease, neither of these antibiotics has any recommended preharvest interval (PHI) for safe use in pomegranate due to lack of information regarding their residue dissipation kinetics under field conditions. Agricultural applications without any supporting knowledge of the nature of residue dynamics might result in apprehension regarding residue accumulations of these antibiotics in pomegranate fruits at harvest, which in turn could affect domestic and international trade and also result in noncompliance to GAP (good agricultural practices) certification requirements. To ensure food safety to the consumers, the residues of these chemicals in plant products in the European Union (EU) are regulated at the default MRL of 0.01 mg kg^{-1}, and hence it is important to establish their individual PHIs to minimize accumulation of their residues to below EU-MRL[3] at the stage of harvest.

To the best of our knowledge, no method has been previously reported for simultaneous analysis of streptomycin and tetracycline compounds in plant matrix. In most of the earlier reported methods, the sample preparation for aminoglycosides and tetracycline compounds involved buffered aqueous extraction[4−8] that required further cleanup on an SPE (solid phase extraction) cartridge for removal of residual buffers and concentrating the residues. As such very few reports are available regarding individual analysis of these compounds in plant matrix.[4,7,9] The method prescribed by the EU Reference Laboratory on Single Residue Methods (Stuttgart, Germany) for polar compounds in the commodities of plant origin involves extraction of streptomycin by acidified methanol,[9] but this method does not include tetracycline compounds. In a review article on tetracycline analysis,[10] aqueous extraction has been presented as the primary method with McIlvaine/ethylenediaminetetraacetic acid (EDTA) (pH 4) buffer system being the most prevalent. The authors further summarized the comparative performance of ethyl acetate, acetonitrile, and methanolic trichloroacetic acid (TCA) as extraction solvents and found methanolic TCA offering the most effective performance (% recoveries) in serum samples.

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While tetracycline compounds have high solubility in alcohols, methanol has rarely been employed for its sample preparation possibly due to difficulty in further cleanup.

This publication reports development and validation of a simple, easy, and accurate method for the simultaneous analysis of streptomycin and tetracycline group compounds (TCs) comprising tetracycline, 4-epitetracycline, 4-epianhydrotetracycline, oxytetracycline, and chlortetracycline in pomegranate fruit matrix. A reverse phase HPLC method involving ion pair reagent was developed for optimum retention of streptomycin and good resolution of tetracyclines and their 4-epimers. The method is sensitive enough to offer LOQs at below 0.01 mg kg^{-1}. The method was applied successfully for residue monitoring in whole pomegranate fruits in a field dissipation study against single and double dose applications of Streptocycline considering its critical field use pattern by the farmers. The systemic nature of streptomycin and tetracycline was evaluated by analyzing edible (aril) and inedible (rind/peel) fruit parts. In the summary report of the "Committee for veterinary medicinal products",[11] the marker residues of tetracycline, oxytetracycline, and chlortetracycline are reported as the sum of the parent compounds plus corresponding 4-epimers. Thus in this study, formations of the known metabolites of tetracycline, i.e., 4-epitetracycline and 4-epianhydrotetracycline, in field conditions were also investigated. The dissipation data was evaluated in terms of PHI and half-life (DT_{50}) with simultaneous risk assessment in terms of acute toxicity evaluation on each sampling day to ensure their safe usage at farm level and also establish safety to the consumers.

MATERIALS AND METHODS

Chemicals and Reagents. Certified reference standards (CRS) of streptomycin sulfate (98%), dihydrostreptomycin sesquisulfate hydrate (99%), tetracycline hydrochloride (96.5%), and oxytetracycline hydrochloride (97%) were procured from Dr Ehrenstorfer GmbH (Augsburg, Germany). CRS of 4-epitetracycline hydrochloride (98.6%), 4-epianhydrotetracycline hydrochloride (90%), chlortetracycline hydrochloride (93.1%), and doxycycline hyclate (≥98%) were purchased from Sigma-Aldrich (Steinheim, Germany). Formic acid (98% pure) and gradient grade methanol were obtained from Merck India Ltd. (Mumbai). HPLC grade water was obtained through Sartorius water purification system (Göttingen, Germany). Ammonium formate was purchased from Thomas Baker (Mumbai, India). Perfluorobutyric acid (PFBA) (98%) was purchased from Sigma-Aldrich (Steinheim, Germany).

Individual stock solutions of streptomycin and dihydrostreptomycin standards were prepared by dissolving about 10 mg (accurate weight recorded) of the respective compounds in 10 mL of water and storing the solution in plastic bottles. Similarly, working stock solutions of TCs were prepared in methanol and stored in amber colored bottles. The working standard solution mixture of TCs (10 $\mu g\ mL^{-1}$) and internal standard (IS) solution of doxycycline (10 $\mu g\ mL^{-1}$) were prepared freshly every time in methanol from the stock solution. The individual working standard solution of streptomycin and its IS dihydrostreptomycin (10 $\mu g\ mL^{-1}$) were freshly prepared in water at each time of use. From this, the intermediate working standard solutions were prepared by appropriate dilution with respective solvents. The calibration standard solutions were prepared in 0.1% formic acid and 5 mM PFBA in methanol + water (50:50; v/v). Dihydrostreptomycin and doxycycline as IS were added at 0.05 and 0.025 $\mu g\ mL^{-1}$ levels, respectively. Similarly, matrix matched calibration standards were prepared using the blank extract obtained out of the untreated pomegranate fruits, which was diluted in 1:1 proportion with HPLC mobile phase A as mentioned below.

LC−MS/MS Analysis. The LC−MS/MS system included an HPLC (Shimadzu UFLC XR) connected to an API 5500 Qtrap (AB Sciex, Toronto, Canada) triple quadrupole mass spectrometer. Measurements were done with electrospray ionization (ESI) in positive polarity and multiple reaction monitoring (MRM). For this purpose, the compound specific mass parameters of all the analytes, viz., declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP), were optimized by means of direct infusion into the source to generate specific mass transitions. Using a target oriented screening approach in enhanced product ion (EPI) scan mode, MS/MS product ion spectra were generated for the standards and experimental samples during method optimization. By using the information dependent acquisition (IDA) method editor, an MRM≫EPI experiment was designed (optimized MRMs were used for this experiment). For the dependent EPI scan, two different energies (20 and 35 V) were used with a scan rate of 10000 amu/s and default dynamic fill time. The source parameters, viz., ion source voltage (5500 V), nebulizer gas (30 psi), heater gas (60 psi), and ion source temperature (500 °C), were similar while running the mass spectrometer in MRM as well as MRM≫EPI mode.

Separation of the test compounds was achieved using an Atlantis T3 column (3 μm, 100 mm length, 2.1 mm i.d.; Waters Corporation, Milford, USA). The mobile phase composition was (A) water/methanol (90:10) with 5 mM PFBA as ion pair reagent, 0.1% formic acid, and 2.5 mM ammonium formate and (B) methanol/water (80:20) with 0.1% formic acid; with gradient 0−1 min 2% B, 1−8 min 2−100% B, 8−11 min 100% B, 11−11.5 min 2% B, 11.5−15 min 2% B. The column oven temperature was maintained at 35(±1) °C, and the mobile phase flow rate was 0.45 mL min^{-1}.

The column eluate was directed through a programmable divert valve (six port, two positions A and B), which enabled time-dependent switching of the valve guiding the flow either to the direction of waste (position B) or into the source (position A). The advantage of using this feature was that the column eluate was diverted from the mass spectrometer at the times when no analytes were present, thus avoiding transfer of unnecessary contaminants to the MS source. The column eluate was directed into the ionization source during 5−9 min, whereas during 0−5 and 9−15 min, the column flow was directed to pass to the waste.

Final Protocol for Sample Preparation. Treated whole fruit samples of pomegranate without any washing step were subsampled as per the procedure validated in our laboratory,[12] which in brief involved cutting the individual fruits into eight pieces and randomly selecting two diagonal pieces from each fruit to account for 1 kg of representative sample from a total of 5 kg field sample. This representative sample was chopped into small pieces (approximately 1 cm^2 surface area) followed by thorough crushing in a blender after addition of 0.5 L of water. Approximately 300 g of the crushed sample was further homogenized, and from this, a portion of 15 g sample was drawn into a 50-mL polypropylene centrifuge tube to which IS solutions (0.025 mg kg^{-1} doxycycline and 0.05 mg kg^{-1} dihydrostreptomycin, respectively) were added. The sample was vortexed for 30 s, followed by addition of 15 mL of methanol and 0.1 mL of formic acid, respectively. The mixture was then vortexed for 2 min and centrifuged at 7000 rpm for 10 min at 20 °C. An aliquot of 1 mL was withdrawn into an Eppendorf tube and diluted with 1 mL of mobile phase A containing 5 mM PFBA. The diluted sample extract was again centrifuged at 12000 rpm for 5 min and filtered through 0.2 μm nylon membrane filter and analyzed by LC−MS/MS. The injection volume was 20 μL.

Method Validation. The performance of the analytical method was assessed as per the single laboratory validation approach of Thompson et al.[13] The linear calibration in solvent and matrix matched standards (whole pomegranate fruit) were established using seven concentration levels ranging between 0.0005 and 0.05 $\mu g\ mL^{-1}$ employing the IS calibration method. Calibration equation was obtained by plotting peak area ratio of the standard peak to the IS peak on the *Y*-axis and the concentration ratio of the standard to that of the IS on *X*-axis through the LC−MS/MS software Analyst 1.5.1. For estimation of the matrix effect (ME, %) the average peak area

responses of the matrix-matched standards in six replicates at 0.05 μg mL^{-1} (peak area of postextraction spike) were compared with the corresponding average peak areas of the solvent standards. The ME was evaluated in whole pomegranate fruit and fruit parts, viz., peel (crushed in 1:1 ratio with water) and arils (crushed without adding water). For all of these matrixes, the same extraction method as described previously was followed to obtain control matrix extract for preparation of matrix matched standards. The ME was quantified as % suppression or % enhancement in peak area using the following equation:

$$\text{ME (\%)} = [(\text{peak area of matrix matched standard} - \text{peak area of solvent standard}) \times 100] / (\text{peak area of solvent standard})$$

Negative values of ME (%) indicate matrix induced signal suppressions, whereas positive values indicate enhancement in the signal. The sensitivity of the method was determined in terms of limit of detection (LOD) and limit of quantification (LOQ) of the test compounds in matrix matched standards. The LOD and LOQ were determined by considering a signal-to-noise ratio (S/N) of 3 and 10, respectively, for the quantifier MRM ensuring that the qualifier MRM has S/N of ≥3:1 at the LOQ level. The method was characterized with respect to accuracy and associated relative standard deviation (RSD) by performing recovery experiments on fresh untreated whole pomegranate fruit matrix. The fortified samples at three concentration levels, viz., 0.005, 0.01, and 0.05 mg kg^{-1}, were extracted as per the protocol. The recovery (%) obtained against matrix-matched standard calibration + IS were used to evaluate the performance of the method, where quantification of residues was done using calibration equation obtained as described above.

Precision was estimated in terms of repeatability expressed as RSD (%) associated with the accuracy experiment. To evaluate intraday precision, single analyst performed the accuracy experiment at the spiking level of 0.01 mg kg^{-1} in a single day in six replicates (intraday, $n = 6$). For interday precision three analyst performed the accuracy experiment with six replicates each on three consecutive days (interday, $n = 18$).

Field Experiment. Field experiments were conducted on a cultivated variety, "Aarakta", at a farm located in Rajapur (Latitude 19°35′30.67′ N, Longitude 74°11′39.77′ E), Ahmednagar, Maharashtra State as per the EU guidelines for crop field trials.[14] The plant to plant and row to row distances were 8 and 10 ft, respectively. Streptocycline was applied using a knapsack sprayer at the rate of 200 g a.i./ha (single dose) and 400 g a.i./ha (double dose) in separate plots at 15 days interval after 60 and 75 days of flowering stage (usual duration between fruit setting to fruit ripening is 135−150 days). An untreated control was simultaneously maintained during the study. The crop was grown under drip irrigation. Around 20 fruits (approximately 5 kg) were collected at random from each replicate of the treated and control plots (1 ha) separately at a regular time interval on 0 (1 h after spraying), 1, 3, 5, 7, 10, 15, 30, 45, and 60 days after the final foliar spray. The fruits hidden inside the canopy or those showing signs of infestation of insect pests, diseases or any physiological disorders were not considered during sampling. On the 60th day of sampling the fruits were ready for harvest. All the samples were transported to the laboratory at a controlled temperature (4 °C) and immediately stored at 0 °C until analysis to prevent any degradation losses of the residues. The atmospheric temperature in the field during the study period ranged between 20 and 42 °C, and no rainfall was recorded during the period of foliar spray and harvesting (final sampling).

Analysis of Field Samples. Whole pomegranate fruits collected from the field study were extracted using the final protocol described above, and the residues of streptomycin, tetracycline, and its 4-epimers were estimated. In pomegranate fruits, only the arils are consumed by humans, while other fruit parts could find their use in cattle feed or as components of medicinal formulations. Thus, the residues in the peel and arils were also evaluated in all of the collected samples separately. Homogenization of the peel samples involved prior addition of water in the ratio of 1:1 due to inherent low moisture content of this fraction, whereas for aril samples water addition was not required. The final residue contents were calculated on fresh weight basis.

Data Analysis. A number of publications have shown that simple first-order kinetics cannot adequately explain the degradation behavior of pesticides in natural systems, where the degradation pattern may follow a nonlinear path.[15,16] In view of this, the time-wise residue data were analyzed by linear as well as nonlinear regression using following equations.

First-order model (linear):

$$[A]_t = [A]_1 \exp^{-k_1 t} \quad (1)$$

First + first-order model (nonlinear):

$$[A]_t = [A]_1 \exp^{-k_1 t} + [A]_2 \exp^{-k_2 t} \quad (2)$$

$[A]_t$ is the residue concentration (mg kg^{-1} of pomegranate) of A at time t (days). $[A]_1$ and $[A]_2$ are the initial concentrations of A at time 0 degraded through first-order processes 1 and 2, and k_1 and k_2 are the degradation rate constants for 1 and 2, respectively. Half-life (DT_{50}), which is the time at which the concentration of the initial deposits reaches at 50% level signifies the speed of degradation and was determined by the following equations:

$$DT_{50}\ (\text{first-order model}) = DT_{50}\ (\text{first-order model}) = \ln(2)\ k^{-1} \quad (3)$$

Preharvest interval (PHI), i.e. the time period (in days) required for dissipation of the initial residue deposits to the MRL was determined by the following equation:

$$\text{PHI} = [\log(\text{intercept}) - \log(\text{MRL})] / \text{slope of the first-order equation} \quad (4)$$

Since the first + first-order model cannot be described in a differential form, the DT_{50} and PHI could be calculated only by an iterative procedure.

The acceptable daily intake (ADI) of streptomycin is 0.03 mg kg^{-1} body weight day^{-1} (JMPR 1995) and the EU-MRL is 0.01 mg kg^{-1}.[3] There is no prescribed ADI or EU-MRL for tetracycline, although the same are available for oxytetracycline. The ADI of 0.003 (EMEA 1995) and EU-MRL of 0.01 mg $kg^{-1}$for oxytetracyclin[3] was therefore used for calculating the DT_{50}, PHI, and safety evaluation of day-wise combined residues of tetracycline + 4-epitetracycline. The data processing for method optimization, validation, and dissipation study were performed using Microsoft Office Excel 2007.

Safety Evaluation. The food safety of each compound was evaluated by comparing their dietary exposure (theoretical maximum daily intake) with that of maximum permissible intake (MPI). Multiplying the ADI by body weight of an average child (16 kg), the MPIs were estimated at 0.48 mg $person^{-1}$ day^{-1} for streptomycin and 0.048 mg $person^{-1}$ day^{-1} for tetracycline. The dietary exposure values for acute toxicity assessment were calculated by multiplying the residue levels in each sample (mg kg^{-1}) with combined average daily consumption of fruits and vegetables of 0.24 kg.[17]

RESULTS AND DISCUSSION

Method Optimization. To determine the optimum ratio of sample weight to extraction solvent volume, three different ratios, viz., 1:1, 1:1.5, and 1:2 were evaluated and compared for achieving the best possible results with respect to accuracy, precision, and LOQ. Control matrix obtained by crushing untreated pomegranate fruits with water in the ratio of 1:0.5 was spiked with streptomycin and tetracycline (as a representative of TCs) working standards to achieve 0.05 mg kg^{-1} of concentration. From this, 15 g of subsamples corresponding to 10 g of actual weight of pomegranate were drawn in separate sets of six replicates. Extraction was carried

Table 1. Optimized LC−MS/MS (MRM) Parameters for Analysis of Streptomycin and TCs

analyte name	t_R[a] (min)	parent (m/z)	DP[b] (V)	quantifier (m/z)	CE[c] (V)	CXP[d] (V)	qualifier (m/z)	CE[c] (V)	CXP[d] (V)
oxytetracycline	5.87	461	55	426.5	26	11.8	201	51	8.7
tetracycline	5.82	445	71	410	26	20	154	38.7	5
4-epitetracycline	5.62	445	71	410	27	20	428	21	16
4-epianhydrotetracycline	7.06, 7.2	427.5	70	410	35	9.5	154	35	6.6
doxycycline	7.04	445	70	428[e]	25	8	321	45	9
chlortetracycline	6.52	479	70	444	28	8	154	38	5
streptomycin	5.56	614	181	582	30	15	263	47	20
dihydrostreptomycin	5.49	584.6	40	263[e]	41	12	246	59	10

[a]Retention time. [b]Declustering potential. [c]Collision energy. [d]Collision cell exit potential. [e]Used as IS.

out after addition of 10, 15, and 20 mL of methanol and acidification with 1% formic acid. The samples were quantitatively analyzed against the solvent calibration standards, and the results were compared.

Recovery (%) of streptomycin for the sample/solvent ratio of 1:1, 1:1.5 and 1:2 were 92(±2.0)%, 91(±2.3)%, and 96(±2.0)%, respectively, whereas for tetracycline it was 94(±3.2)%, 99(±4)%, and 98(±4.6)%, respectively, showing no significant difference. For calculation of recoveries, appropriate dilution factors were applied that were decided on the basis of inherent water content of pomegranate fruits, volume of water added during homogenization, and volume of methanol used for extraction. Though good recoveries and precision were obtained in the cases of all of the above-mentioned sample/solvent combinations, in the case of a 1:1 ratio, the supernatant appeared hazy because of finely suspended particles. The supernatants for 1:1.5 and 1:2 ratios appeared clear and transparent and could be easily filtered. The resulting LOQs of streptomycin and tetracycline for the sample to solvent ratio of 1:1.5 and 1:2 were almost similar and ≤50% of the EU default MRL (0.01 mg kg^{-1}). Thus, for the final method, the sample to solvent ratio of 1:1.5 was selected.

In the initial studies, addition of formic acid was observed to significantly enhance the extraction recovery of streptomycin, but at the same time, the acidic pH (pH ≈ 4) of pomegranate fruit matrix and addition of formic acid triggered the formation of 4-epimers to a small extent. To serve the purpose of simultaneous extraction of streptomycin and TCs with acceptable recoveries, the concentration of formic acid was optimized. A homogenized pomegranate sample was spiked at 0.05 mg kg^{-1} with streptomycin and TCs, viz., tetracycline, oxytetracycline, and chlortetracycline. From this, 15 g subsamples were drawn in three sets of three replicates each. Extraction was carried out after addition of 15 mL of methanol and formic acid at 0, 0.1%, 0.5%, 1%, 2%, and 3% (v/w) to the sample, respectively, and pH of the extract in each set of addition was recorded. The corresponding recoveries (%) of streptomycin and TCs were compared.

The pHs of the methanolic extract with the addition of 0, 0.1%, 0.5%, 1%, 2%, and 3% (v/w) formic acid were 4.59, 4.40, 4.10, 3.44, 3.16, and 3.10, and the corresponding recoveries of streptomycin were 42(±6.9)%, 61(±2.6)%, 91(±2.8)%, 101(±2.0)%, 104(±1.5)%, and 105(±4.0)%, respectively, showing significant enhancement in extraction with increased acidity. It was observed that even before addition of formic acid, small quantities of tetracycline and chlortetracycline were converted into the respective 4-epimers, and such conversion was further accelerated by 2−10% in response to further addition of formic acid in the range of 0−3%, although no conversion into 4-epianhydrotetracycline was recorded. In the case of chlortetracycline (retention time t_R = 6.52 min in Supplementary Figure 3), a small peak was observed at t_R = 6.31 min with similar MRM transitions. The retention behavior and fragmentation pattern indicated it to be the 4-epimer of chlortetracycline and was in complete agreement with the reported data.[18] Formic acid concentration of 1% was set for the final extraction method, which facilitated optimum recovery of streptomycin (Supplementary Figure 4) with minimum epimerization.

LC−MS/MS Analysis. Streptomycin is a highly polar compound that is difficult to retain on a regular C_{18} column, which is why the use of hydrophilic interaction chromatography (HILIC)[19] or volatile ion pair reagents is a common practice for its analysis; however, it is reported that such ion pair reagents adhere to the ESI capillaries and affect the sensitivity of the mass spectrometer.[19] The complex nature of TCs resulting in difficulties in chromatographic analysis has been discussed in a review article.[20] According to this article, TCs might form chelate complexes with metal ions and get adsorbed on the silanol groups in a reversed phase column, resulting in peak tailing. Use of an end-capped modified silica column, and use of organic acids (e.g., oxalic acid) or ion pair reagent in the mobile phase are the key solutions to ensure the separation of TCs without peak tailing.

In the current study, the main objective was to optimize a comprehensive chromatographic method for both streptomycin and the TCs. Atlantis T3, which is an end-capped column, was selected to avoid peak tailing of TCs. A gradient program was optimized for sufficient retention of streptomycin and good resolution and peak shapes of TCs. For streptomycin analysis, use of PFBA as ion pair reagent was found to be a good option. For the optimization of PFBA concentration, different volumes leading to 1, 2.5, 5, and 10 mM of PFBA, respectively, were used to prepare the mobile phase A. Using each concentration of PFBA, the column (Atlantis T3) was conditioned, and subsequently standard streptomycin solution (0.05 μg mL^{-1}) was injected. Retention time, proper elution, and peak shape of the analyte were compared at each PFBA concentration. The concentration of PFBA was optimized to avoid an unutilized surplus residual amount while maintaining good retention and peak shape of streptomycin. It was observed that performance of 5 and 10 mM PFBA were on par, and thus 5 mM concentration was considered as optimum (Supplementary Figure 1). Streptomycin formed the $[M + H + CH_3OH]^+$ adduct (m/z = 614)[21] in the ESI source, and use of this ion as precursor made it possible to achieve around nine times higher S/N over the protonated pseudomolecular ion of streptomycin $[M + H]^+$, m/z = 582). Addition of ammonium formate (2.5 mM) in the mobile phase promoted the formation of the $[M + H + CH_3OH]^+$ adduct.

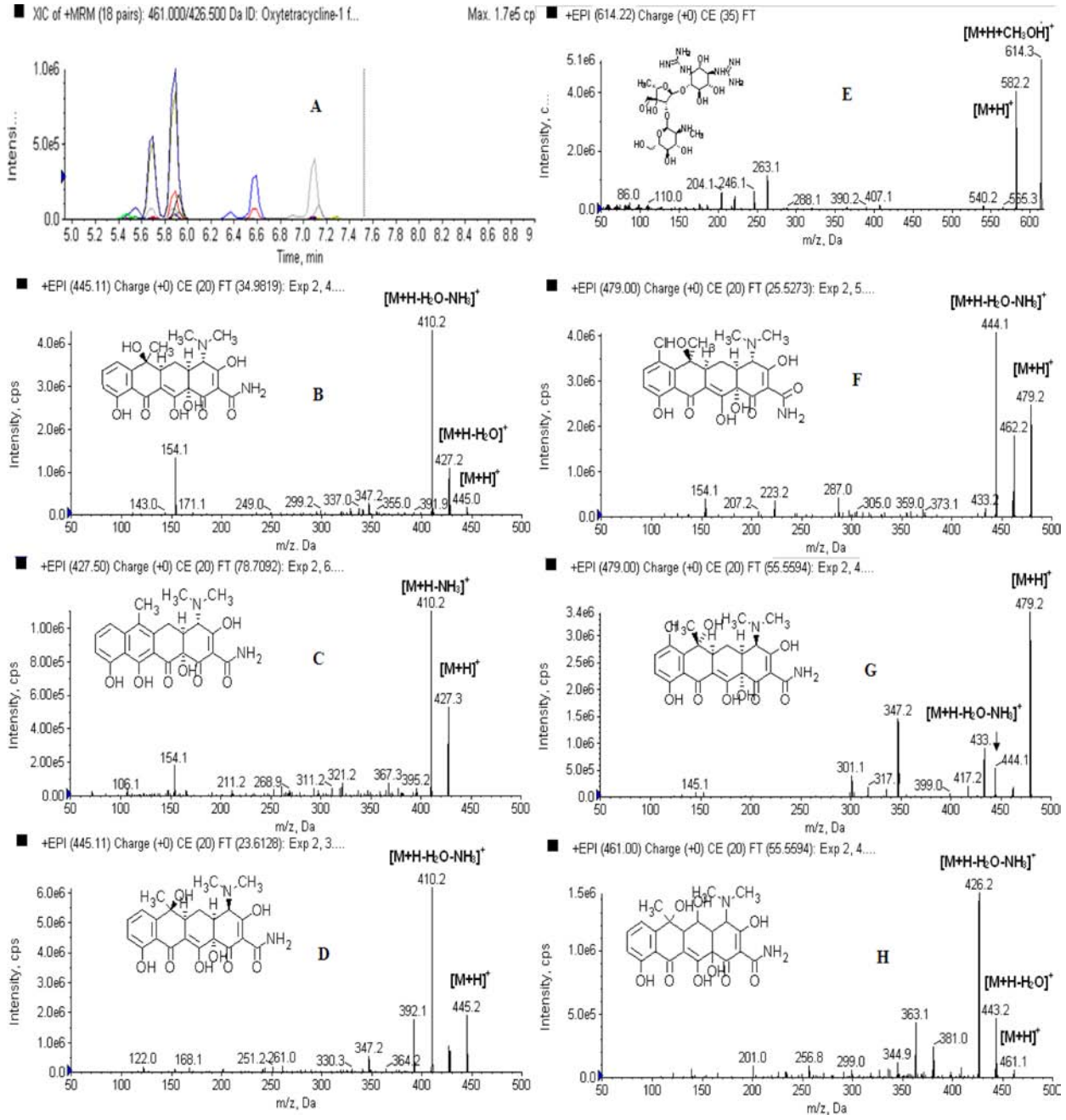

Figure 1. LC−MS/MS chromatogram and MS/MS spectra of streptocycline and TCs at 0.05 μg mL^{-1}. (A) Total ion chromatogram of streptomycin and TCs and MS/MS spectra of (B) tetracycline [$C_{22}H_{24}N_2O_8$], (C) 4-epianhydrotetracycline [$C_{22}H_{22}N_2O_7$], (D) 4-epitetracycline [$C_{22}H_{24}N_2O_8$], (E) streptomycin [$C_{21}H_{39}N_7O_{12}$], (F) chlortetracycline [$C_{22}H_{23}ClN_2O_8$], (G) 4-epichlortetracycline [$C_{22}H_{23}ClN_2O_8$], and (H) oxytetracycline [$C_{22}H_{24}N_2O_9$].

Initially, while optimizing the chromatographic program, vertical peak splitting was observed in the case of streptomycin and dihydrostreptomycin when injected in the ion pair chromatographic system. This was creating difficulties in automated peak integration during quantification. The extent of peak splitting was more in matrix as compared to the solvent standard. In literature, such phenomenon has been discussed and explained for aminoglycosides,[22] and according to this explanation the pH and ionic strength of the injected samples influence the ion pairing mechanism. Because of the inconsistency caused by sample addition into the mobile phase, which moves at a slower pace than the solvent front, there are nonuniform retentions of the sample components, which in turn cause peak splitting. Therefore, to make the nature of the sample in harmony with the ion pair mobile phase, dilution of calibration standard and sample extract was done with 5 mM PFBA (mobile phase A), which effectively resolved the problem. The use of a programmable divert valve was also beneficial in minimizing blockage of the opening of the ESI capillary due to the sticky and sugary depositions.

The compound-specific mass parameters with the corresponding retention times are presented in Table 1. The MRM transition with greater signal-to-noise ratio was used for quantification, while the next most abundant MRM transition was used for confirmation of the identity. For confirmation, the ion ratios were calculated as percent ratio of peak areas of the

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Table 2. Method Validation Data for Streptomycin and TCs in Pomegranate

		recovery ± RSD (%) (n = 6) (whole pomegranate fruit)			interday precision RSD (%) (n = 18)	ME (%)		
name of the compound	LOQ (mg kg^{-1})	0.005 (mg kg^{-1})	0.01 (mg kg^{-1})	0.05 (mg kg^{-1})	0.01 (mg kg^{-1})	whole fruit	peel	arils
streptomycin	0.005	102(±7)	116(±5)	103(±4)	6	−19	−26	−2
tetracycline	0.0025	109(±5)	108(±8)	105(±5)	7	5	2	1
4-epitetracycline	0.0025	104(±6)	109(±7)	102(±6)	6	8	20	5
4-epianhydrotetracycline	0.005	104(±9)	96.1(±5)	90(±7)	12	6	3	2
oxytetracycline	0.005	108(±4)	109(±4)	107(±7)	7	11	13	3
chlortetracycline	0.0025	101(±9)	106(±7)	93(±6)	7	6	16	7

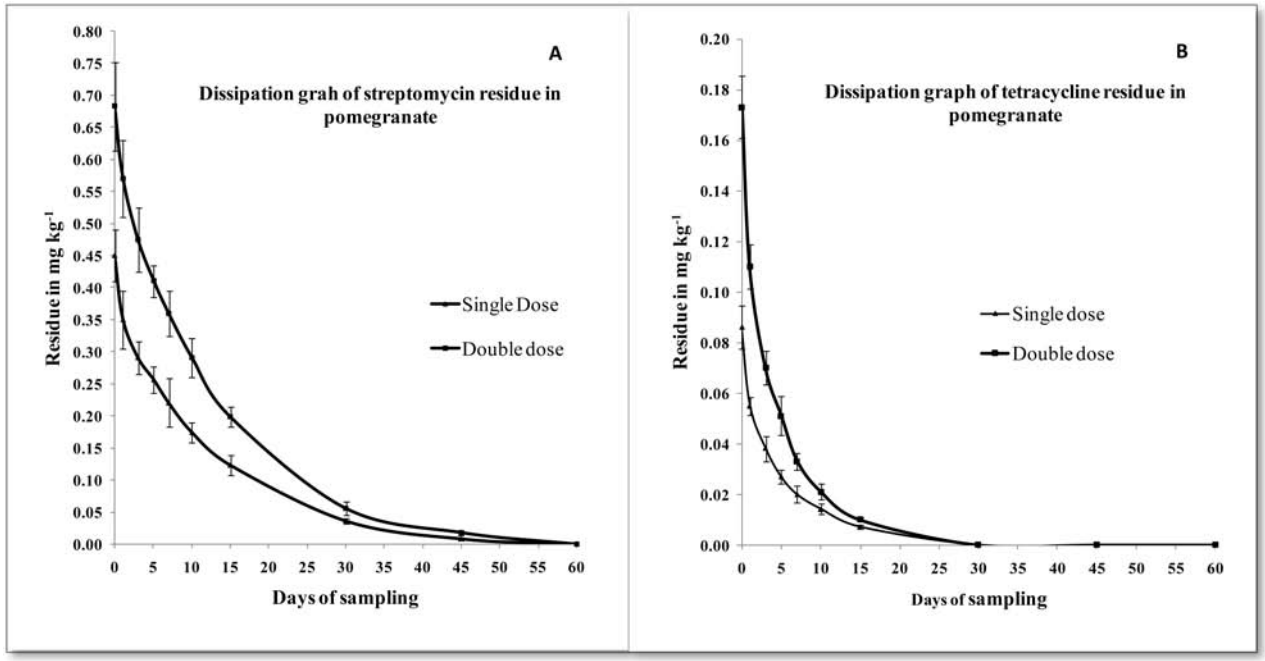

Figure 2. Dissipation of (A) streptomycin and (B) tetracycline in whole pomegranate fruits.

qualifier and quantifier MRMs in accordance with the DG-SANCO Guidelines.[23] Extracted ion chromatograms of streptomycin and TCs are presented in Supplementary Figure 3. The MS/MS spectra generated using MRM≫EPI mode of all analytes (Figure 1) was an additional information and used for the tentative identification of the 4-epimer of chlortetracycline, which was formed during the extraction process as depicted in the previous section.

Method Validation. The calibration curves obtained by plotting seven concentration levels ranging between 0.0005 to 0.05 μg mL^{-1} in solvent and matrix matched standards were linear (linear regression with weighing $1/x$), with $R^2 \geq 0.995$ (Supplementary Figure 2). From the results for ME (%) (Table 2), it is clear that significant matrix induced signal enhancement (10−20%) was observed in the case of TCs mainly for oxytetracycline (whole fruit and peel), 4-epitetracycline (peel), and chlortetracycline (peel). In case of streptomycin, significant signal suppression was observed for streptomycin mainly in whole fruit (−19%) and peel (−26%). The significant difference in ME (%) in different fruit parts can be attributed to the peculiar matrix composition of peel and arils.[12]

The method LOQs of all the test compounds were below the EU-MRL of 0.01 mg kg^{-1}. The recovery of all the compounds was within 90−116% at all the spiking levels. The precision (RSD) in the condition of repeatability (intraday, n = 6) at all the spiking levels was ≤9%, whereas the reproducibility (interday, n = 18) at 0.01 mg kg^{-1} was ≤12% for all the compounds. The recovery and precision data thus fulfill the requirement of the DG-SANCO guideline. [23] The calculated peak area ratios of the analyte to IS in spiked samples were in close agreement with those of matrix matched standards (<10%). The recovery and precision data presented in Table 2 justify the selection and use of IS with matrix matched standards for accurate estimation of residues.

Quantification of Residues. Addition of IS before extraction of the residues enabled accurate quantification through correction of error contributed by dilution of the extract during sample preparation and variations in detector response over time. As it was difficult to get the isotopically labeled IS for aminoglycosides and TCs, dihydrostreptomycin and doxycycline, which are not commonly used in plant protection practices, were selected as IS, and in addition, matrix matched calibrations were employed to compensate the matrix effect.

The signal for 4-epianhydrotetracycline consisted of two peaks representing two epimers, and summing of the areas of these two peaks enabled correct quantification of 4-epianhydrotetracycline. In the field study, residues of 4-epitetracycline were detected in all of the samples to an extent of about 38−41% of the tetracycline residues from 0 day until 12 days at

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Table 3. Dissipation Rate Kinetics Data of Streptomycin and Tetracycline in Whole Pomegranate Fruits

	streptomycin		tetracycline	
	single dose (200 g a.i./ha)	double dose (400 g a.i./ha)	single dose (200 g a.i./ha)	double dose (400 g a.i./ha)
MRL (mg kg^{-1})	0.01		0.01	
	1st + 1st order reaction			
$[A]_1$	0.082	0.084	0.028	0.065
k_1	2.302	1.830	1.676	0.845
$[A]_2$	0.368	0.599	0.058	0.108
k_2	0.075	0.075	0.146	0.163
R^2	0.999	0.999	0.999	0.999
DT_{50} (days)	6.5	8.0	2.5	2.5
PHI as per MRL (days)	45.0	55.0	12.0	15.0
	1st order reaction			
[A]	0.410	0.643	0.079	0.164
k_1	0.088	0.084	0.209	0.236
R^2	0.983	0.991	0.974	0.989
DT_{50} (days)	8.0	8.0	3.3	2.9
PHI (days)	20.3	21.8	4.2	5.0

single dose and 15 days at double dose. The formation of 4-epimers of chlortetracycline depending on pH and temperature in the solution form has been reported by Schwartzmanet et al.[24] In a study on the metabolism of TCs in eggs, Zurhelle et al.[25] reported formation of 4-epimers under the influence of temperature and time. Therefore, it was concluded that 4-epitetracycline residues detected in the pomegranate field samples were formed due to exposure to the field atmospheric conditions (heat, sunlight, etc.) and acidic pH of the fruit matrix. When calculating the tetracycline concentration in field samples, the residues of tetracycline and 4-epitetracycline were added up to get the final residues.

Dissipation of Residues in the Field Samples, Estimation of PHI. The dissipation pattern of the residues of tetracycline and streptomycin (Figure 2) had a nonlinear nature implying that the first-order rate kinetics was inadequate to explain the behavior of their residue dissipation pattern, which is governed by physical factors (such as sunlight, heat, moisture, etc.), plant metabolic processes, interaction with chemical factors, etc. The comparative results of the first and first + first-order rate kinetics are presented in Table 3. The correlation coefficient (R^2) values for the first + first-order model were closer to 1, indicating better applicability of this model against the first-order model reflecting partitioning of the residues into two phases, where one fraction of the applied compound (which was immediately available in free form) degraded rapidly, leaving the other part possibly remaining in dynamic equilibrium as an adsorbed fraction on cellular components that becomes available for degradation slowly over time. The PHIs calculated by the first + first-order model provided realistic values of 45 and 55 days for streptomycin and 12 and 15 days for tetracycline at single and double dose applications, respectively. Streptocycline is a combination product with a higher proportion of streptomycin (90%), and therefore, a longer persistence of streptomycin was recorded as compared to tetracycline, which was indicated by respective 0 day residues (*Y*-intercept) in Figure 2 and PHI values. The PHIs estimated through first-order kinetics were inadequate to achieve dissipation of the residue load of these chemicals to the MRL, indicating unsuitability of this model to explain their residue dynamics.

The RSDs for the concentrations in the samples collected in replicates were within 20% at both the standard and double dose applications. The average initial (1 h after application) residue deposits of tetracycline and streptomycin were 0.086(±10%) and 0.45(±9%) mg kg^{-1}, respectively, at single dose with the corresponding levels of 0.173(±7%) and 0.683(±10%) mg kg^{-1} at double dose. More than 50% of the initial deposits of tetracycline dissipated within 3.5 days of field applications at both single and double doses against 8 days required for streptomycin.

From the day-wise analysis of the residues in different parts of the fruits, viz., peel and arils, the systemic nature of these compounds was well recognized. It was observed that the translocation of residues of streptomycin and tetracycline into arils was rapid. The estimated quantities of streptomycin in arils increased from 0.03 mg kg^{-1} on 0 day to 0.21 mg kg^{-1} on the third day in the case of single dose of application. Similarly, the measured residues of tetracycline in arils increased from the initial concentration of 0.012 mg kg^{-1} on 0 day to the peak value of 0.081 mg kg^{-1} on the very next (first) day. In case of streptomycin, the highest concentration of residues in arils (third day) was about 20% of the highest residues measured in the peel fractions (0 day). On the other hand, in the case of tetracycline, the highest estimated concentration in arils was about 50% (first day) as compared to that of peel (0 day). However, the residues of streptomycin and tetracycline in arils dissipated to below MRL within the estimated PHIs.

Safety Evaluation. The dietary exposure of streptomycin and tetracycline on all the sampling days (including 0 day) were less than the respective MPI both at the single and double doses of applications, which ensures safety of the fruits with incurred residues to the consumers in terms of possible incidence of acute toxicity. From the field study results and estimated PHIs, it is suggested that application of streptomycin and tetracycline should be avoided at the later growth stage of pomegranate especially when the fruit maturity is close to harvest. Farmers should strictly follow the recommended dose of application to minimize initial residue deposits and ensure dissipation of these systemic compounds within the estimated PHI period.

In conclusion, in this endeavor a simple and reliable residue analysis method has been established that offers simultaneous analysis of streptomycin and TCs in pomegranate. The method was applied for the study of dissipation of streptomycin and tetracycline residues in pomegranate under field condition. The

PHIs of 45 and 55 days for streptomycin and 12 and 15 days for tetracycline were established at single and double dose applications, respectively. The dietary exposure was found to be less than the calculated MPI of 0.48 mg person^{-1} day^{-1} for streptomycin and 0.048 mg person^{-1} day^{-1} on 0 day itself. The rising concern about the use of these antibiotic compounds in plant protection practices worldwide and stringent MRL regulation highlights the importance of this study. The method of analysis reported here should potentially be applicable in other food matrixes belonging to the commodity group of high water content fruits and vegetables for which application of these antibiotics is common. Moreover, for any research or commercial testing laboratories it will offer an efficient solution for screening of samples for simultaneous detection and quantification of the residues of streptomycin and TCs.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary figures as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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