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Determination of streptomycin in kiwifruit samples using LC-ESI-MS/MS

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LC-ESI-MS/MS를 이용한 키위 중 streptomycin 분석

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Abstract: In May 2012, a safety hazard issue arose because some kiwifruit growers in New Zealand had sprayed streptomycin to prevent kiwifruit canker. Therefore, for food safety management, analytical methods to determine streptomycin residues in kiwifruits are required. We developed an analytical method to determine streptomycin residues in kiwifruit samples using liquid chromatograph tandem mass spectrometer (LC-ESI-MS/MS). Streptomycin residues in samples were extracted using 1% formic acid in methanol, centrifugation for 10 min, and subsequent supernatant filtration. Purified samples were subjected to LC-ESI-MS/MS to confirm presence of and quantify streptomycin residues. Average streptomycin recoveries (6 replicates each sample) were in the range of 94.8%-110.6% with relative standard deviations of <10%. The linearity of the concentration range of 0.01-5.0 mg/kg using a matrix-matched calibration gave $R^2 = 0.9995$. The limit of quantification (LOQ) was 0.01 mg/kg. Results showed that our analytical method is rapid, simple, and sensitive, with easy sample preparation.

Key words: streptomycin, kiwifruit, LC-ESI-MS/MS

1. Introduction

In May 2012, a safety hazard issue arose because some kiwifruit growers in New Zealand had sprayed streptomycin to prevent kiwifruit canker. Most kiwifruits distributed in Korea are from New Zealand. Therefore, for food safety management, analytical methods to determine streptomycin residues in kiwifruits are required. This would require a specific, sensitive, and reliable analytical method for the determination of streptomycin.

Streptomycin is an antibiotic of the aminoglycoside class that was identified in 1944 during a search for water soluble and stable compounds that were active

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against gram-negative bacteria.[1] The antimicrobial activity of streptomycin is based on its ability to inhibit bacterial protein synthesis. The current primary use of this drug is treating infections caused by aerobic gram-negative bacteria.[2]

Streptomycin solubility in methanol is limited and it is practically insoluble in hydrophobic organic solvents. In aqueous solution, streptomycin is usually positively charged because of its amino groups and it is typically analyzed by high-performance liquid chromatography (HPLC) because of its polar ionic nature. Streptomycin usually does not partition well on reversed-phase columns. Pentane, hexane, and heptanes have been compared for their capability to separate streptomycin.[3] Streptomycin residues can be found in meat, liver, kidney, milk, and especially in honey. However, previous analytical methods used were very complex and time-consuming. Therefore, a more simple method is required.

Streptomycin is also used as a pesticide to control bacterial and fungal diseases of certain fruits, vegetables, seeds, and crops and for controlling algae in ornamental ponds and aquaria. Tolerances or maximum residue limits of 0.25 mg/kg were established in 40 Code of Fedral Regulations (CFR, USA) 180.245 for streptomycin residues in or on celery, peppers, tomatoes, potatoes, and pome fruits (apples, pears, crabapples, and quinces).[4] However, streptomycin is not registered in Korea.

Therefore, in this study, we developed and validated an analytical method to determine streptomycin residues in kiwifruit samples.

2. Materials and Methods

2.1. Standard, reagents, and chemicals

A certificated analytical streptomycin sodium sulfate standard (99.5%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Pesticide grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). LC grade water was obtained by purifying distilled water in a high performance reverse osmosis (RO) system (Sartorius Stedim Biotech, Göttingen, Germany). HPLC grade formic acid was purchased from Sigma-Aldrich. GHP syringe filters (0.22 μm) were purchased from Waters (Milford, MA, USA).

2.2. Sample sources and storage

Five types of kiwifruits with different origins were obtained from different local markets in Korea (Korea chamdarae, green and golden kiwifruits from Chile, green and golden kiwifruits from New Zealand). Prior to analysis, kiwifruits were stored in the dark in a refrigerator at 4 °C

2.3. Stock and working standard solutions

A standard stock solution of streptomycin was prepared in 50% methanol with water at 500 mg/kg. Streptomycin was dissolved in water to which methanol was added, because streptomycin solubility in methanol is limited. This stock was stored at 50 °C. Suitable concentrations of working standards were prepared from stock solutions by serial dilution using 50% methanol with water immediately prior to sample preparation.

2.4. Sample preparation

A laboratory sample of blank material of a whole kiwifruit was divided into sub-samples to provide sufficient number of samples. These sub-samples were stored at approximately 50 °C to prevent modifications to the kiwifruit. After thawing, these samples were homogenized and a 10 g sample was transferred to a centrifuge. A completely homogenized sample (10 g) was weighed in a 50 mL plastic centrifuge tube, 10 mL of acidified methanol (1% formic acid in methanol) was added, and this solution was vigorously shaken for 1 min. After centrifuging for 10 min at >2500 G, the solution was filtered using a 0.22 μm syringe filter. One or more aliquots (1 mL each) of the filtered extract were transferred to auto-sampler vials.

2.5. Accurate mass analysis

A LC system, Waters Acquity (Waters, Milford, MA, USA), was interfaced to a Quattro premier XE LC mass spectrometer (Waters, Milford, MA, USA). This was equipped with an HPLC packed C_{18} column (2 mm I.D. × 150 mm, 3 μm) that was thermostatically maintained at 40 °C. The flow rate was 0.2 mL/min and the injection volume was 5 μL. The mobile phase was 0.1% formic acid in water, i.e., 0.1% formic acid in acetonitrile gradient in which the percentage of the organic modifier was changed linearly as follows: 0 min, 100%; 3 min, 100%; 5 min, 0%; 11 min, 0%; and 15 min, 100%.

Detection was performed using Quattro LC (quadrupole-hexapole-quadrupole) mass spectrometer with an orthogonal Z-spray-electrospray interface (Waters). Positive ions were detected using the multiple reaction monitoring (MRM) modes with a dwell time of 0.2 s. The nebulizer gas flow was set to approximately 20 L/h and the desolation gas flow was set to 600 L/h. Infusion experiments were performed using a single syringe pump, directly connected to the interfaces.

MS optimization was achieved by infusing standard solutions of each individual compound (1 mg/kg) in acetonitrile at a flow rate of 200 μL/min. For operation in the MS/MS mode, the collision gas was argon (99.999%) with a pressure of 1.2×10^{-3} mbar in the collision cell. Capillary and extractor voltages of 1 kV and 5 voltage (V), respectively, were used in the positive ionization mode. The RF lens was set to 0.2 and the interface and source temperatures were set to 300°C and 120°C, respectively. Dwell times were 0.2 s per scan. Resolutions of 15/15 (low mass resolution/high mass resolution) were set for both quadrupoles and ion energies of 0.5 and 1.0 were set for the first and the third quadrupoles, respectively. Finally, the electron multiplier was set to 655 V and the entrance and exit voltages were set to 2 V to perform MS/MS.

Table 1. LC-MS/MS conditions for determination of streptomycin residue

Instrument	LC-ESI-MS/MS (Quattro Premier XE, Waters, USA)		
Column	HPLC packed C_{18} column (2 mm I.D. × 150 mm, 3 ìm)		
Mobile phase	Time (min)	0.1% formic acid in water (%)	0.1% formic acid in acetonitrile (%)
	0	0	100
	3	0	100
	5	100	0
	11	100	0
	15	0	100
Flow	0.2 mL/min		
Column temperature	40°C		
Ionization mode	ESI positive-ion mode		
Cone voltage	80 V		
Collision energy	35 eV		
Injection volume	5 μL		

Masslynx NT v 4.1 software was used to process the quantitative data obtained for calibration standards and samples. The LC-ESI-MS/MS conditions used are summarized in *Table* 1.

2.6. Matrix-matched calibration standards and recovery experiments

Suitable aliquots of a blank extract were transferred to auto-sampler vials and processed as shown in *Table* 2. Pesticide concentration was calculated using matrix-matched calibration standards. A weighed portion of an appropriate blank homogenate was placed in a 50 mL centrifuge tube and spiked with a suitable

Table 2. Scheme for preparing matrix-matched calibration standards

Calibration levels in μg pesticide/mL		Calibration standards				
		0.01	0.05	0.1	0.25	0.5
Blank extract		900 μL	900 μL	900 μL	900 μL	900 μL
Mixture of water and acidified methanol		90	50	-	50	-
Pesticide working solution	1 μg/mL	10	50	100	-	-
	5 μg/mL	-	-	-	50	100
Total volume		1,000 μL	1,000 μL	1,000 μL	1,000 μL	1,000 μL

pesticide working solution. Spiking was performed directly to the matrix prior to any solvent addition. Suitable aliquots of a blank extract were then transferred to auto-sampler vials. Samples for matrix calibration were spiked with different volumes of the working solution. A streptomycin standard was added to a test sample.

2.7. Method validation

For this study, we considered linearity, specificity, accuracy, precision, and the limits of detection (LOD) and quantification (LOQ). Linearity was determined using different known concentrations of streptomycin (0.01, 0.05, 0.1, 0.25, and 0.5 mg/kg). These were prepared by diluting the stock solution. The specificity of the method was evaluated by injecting a procedural blank, kiwifruit blank samples, and blank samples spiked with the lowest fortification level assayed. The accuracy of the method was determined by a recovery test using samples spiked with 3 concentration levels of streptomycin: 0.01, 0.05, and 0.5 mg/kg.

Recovery experiments used 6 replicates for each of these concentrations. Precision was evaluated within the same day at each recovery level and was determined as the relative standard deviation (RSD) for 6 replicates. LOD (mg/kg) was determined as the lowest concentration that gave a response of 3 times the baseline noise level defined from the analysis of a control (untreated) sample. LOQ was determined as the lowest validated concentration that provided acceptable recoveries (94.8%-110.6%) and precision (<10%) or a response of 10 times the baseline noise level.

2.8. Identification and quantification

Confirmation of streptomycin was on the basis of the ratio of the intensities of the 2 most abundant product ions obtained from the precursor. The ratio of the areas of the most intensive product ions of streptomycin (m/z 263) was used for quantification. Calculations of the concentrations of the spiked samples for the determination of recovery were performed using matrix calibration curves that included 5 concentration levels. These matrix calibration samples were spiked with the working solution.

3. Results and Discussion

3.1. Extraction and purification optimization

In this study, we used acidified methanol as the extraction solvent because streptomycin is a hydrophilic compound. Acidified methanol (1% formic acid in methanol) provides for a better extraction condition for both polar and non-polar pesticides from vegetable samples. This extraction method is simple and fast, although extracts still require further purification steps.

Previous studies generally used a C_{18} and an Oasis HLB SPE cartridge because streptomycin compounds are highly polar, and hence 2 solid phase sorbents were chosen, C_{18} and Oasis HLB. Both of these have been widely used in research in environmental pollutant chemistry and proved to be suitable for pesticide extraction from samples. In preliminary extraction experiments, we used both sorbents to evaluate the efficiency of each cartridge for streptomycin analysis.

Recovery rates showed that SPE cartridge C_{18} and Oasis HLB had recoveries of <60% for most of the streptomycin. These results suggested that our SPE without cartridge method worked appropriately and our experimental procedures were satisfactory. In contrast, to increase the recovery percentage and simplify the pretreatment step, we attempted to remove the SPE cartridge step. Treatment without a cartridge gave high recoveries of >90%. Thus, we established a simple extraction cartridge without pretreatment by filtration.

3.2. Instrument optimization

LC-ESI-MS/MS conditions were chosen to provide a good peak shape within a short run time. It was not necessary to chromatographically separate each peak because unique MRM transitions corresponded to each analyte. However, peaks that could be baseline resolved provided an opportunity to divide our method into more MRM functions with fewer MRM transitions for each function. This allowed for each MRM transition to have a longer dwell time and consequently better sensitivity.

The settings for the mass spectrometer were optimized while infusing a 0.2 mg/kg aqueous solution of the analytes acidified with 0.1% formic acid in methanol into the mass spectrometer. The mass spectrum recorded the product ions of streptomycin with the collision energy set at 35 eV. Streptomycin displayed positive ionization and MS parameters were optimized to provide at least 2 transitions with an acceptable sensitivity. The most sensitive was selected as the quantitative transition and the other was used as a confirmative transition. The full scan spectrum (optimized at a cone voltage of 80 V) showed abundant ions at *m/z* 582 corresponding to $[M+H]^+$ ions. The MS/MS spectrum showed major peaks at *m/z* 263 and *m/z* 246 optimized at a collision energy of 35 eV. Therefore, 582→263 and 582→246 were selected as quantitative and confirmative transitions, respectively (*Fig.* 1).

The mobile phase initially used for streptomycin determination consisted of methanol and water mixtures. However, this mixture displayed broad, asymmetric peaks. Addition of 0.1% formic acid to the mobile phase provided the most appropriate condition for analysis. The use of a gradient of this solution allowed for a rapid elution and the correct peak shape for streptomycin. It appeared that adding formic acid to the mobile phase improved the peak shape and the signal-to-noise-ratio. Adding 0.1% formic acid resulted in a more symmetrical peak shape and the separation efficiency could be further improved with a higher acid concentration. The signal-to-noise-ratio appeared to be optimal when adding 0.1% formic acid to the mobile phase.[5]

The effect of the mobile phase buffer pH on streptomycin separation was previously investigated at pH 3-6 with octane or hexane sulfonate as the counter ion. When hexane sulfonate was used as the ion-pairing reagent, streptomycin could not be separated at pH 6. Only when the pH was reduced to 3 or less, a satisfactory separation was achieved. The pH and the ionic strength of the injected sample have an effect on ion pairing results.[3, 4, 6-9]

A gradient elution method was established with an

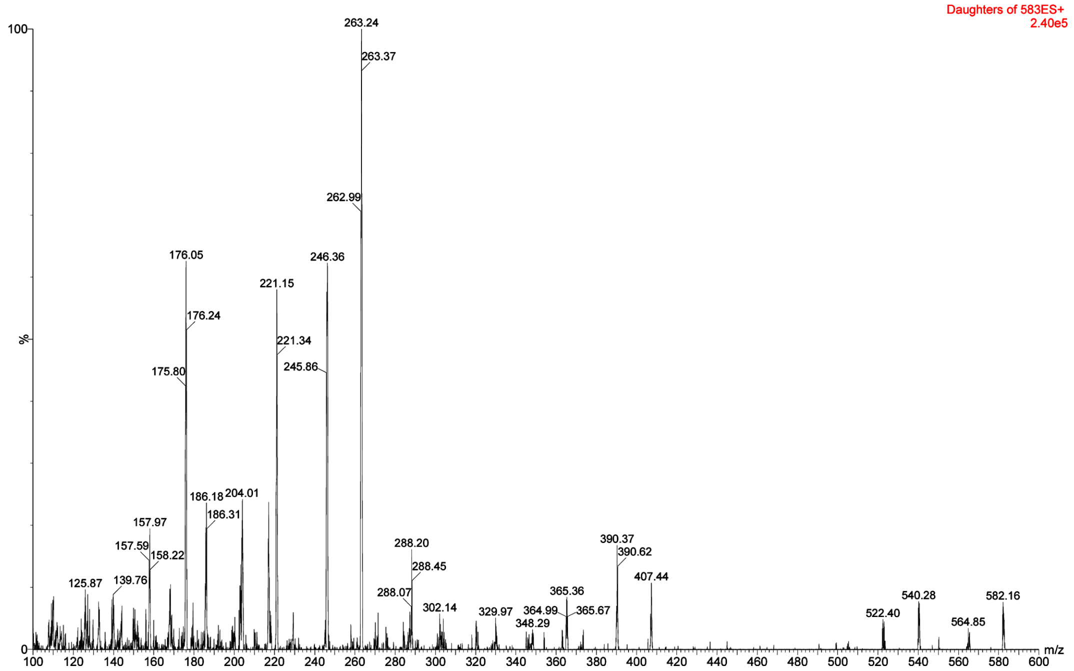

Fig. 1. Positive ion electro-spray MS/MS full scan spectrum at a collision energy of 35 eV.

acidic binary mobile phase, with phase A (0.1% formic acid in acetonitrile) and phase B (0.1% formic acid in D.W.), each containing 0.1% formic acid following the method of Quilliam *et al.*[10] Chromatographic separation was achieved using a HPLC packed C_{18} column (2 mm I.D. × 150 mm, 3 μm). The flow rate was set at 0.2 mL/min and the injection volume was 5 μL. The spiked samples and matrix-matched standards were run in 6 replicates against methanol standards using our in-house validated and accredited methods for analysis.

A matrix-matched standard curve was prepared to compare response factors over a range of concentrations that were representative of naturally contaminated kiwifruit extracts. Injecting a small volume of extract (5 μL) into the LC-MS/MS had more impact in reducing matrix effects than increasing the reconstitution volume of the extract and injecting larger volumes. A small injection volume and a mobile phase gradient for streptomycin analysis were analyzed to establish the best conditions.

3.3. Matrix effect

Quantification using matrix-matched standards requires generating a calibration curve using solutions with the exact compositions as the samples by extracting blank material or by artificially reconstructing the matrix and spiking with analytes at different concentrations. Although this approach is perfectly acceptable when the sample matrix is identical in all samples being analyzed, its application for monitoring kiwifruit has not been established. As a result, a matrix-matched standard curve for the analysis of samples contaminated by interfering substances should compensate for this to some extent.

3.4. Linearity

In recent years, external matrix-matched standard calibration is being widely used in pesticide residue analysis of plant matrices by LC-ESI-MS/MS. However, in this study we used external solvent standard calibration because we found that the response of an analyte in a pure solvent standard (y =

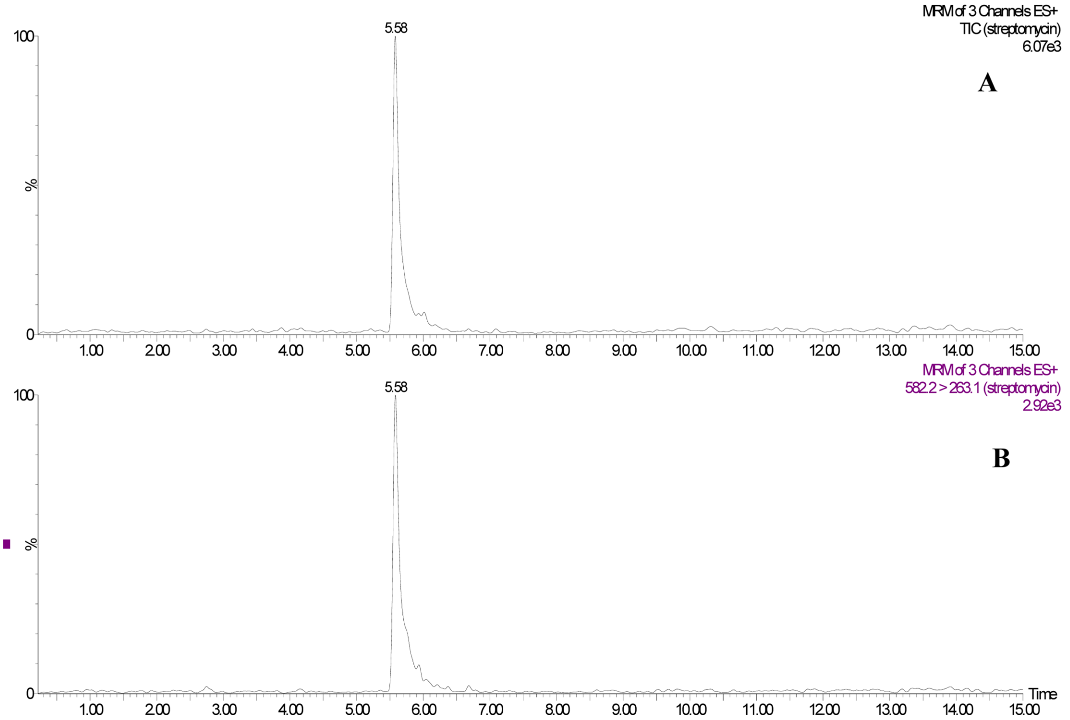

Fig. 2. LC-ESI-MS/MS chromatograms of a standard streptomycin solution at 1 mg/kg. A. Total ion chromatograph (TIC); B. *m/z* 582→263.

8742 × 5.2442) did not differ from external matrix-matched standard calibration (y = 8666 × 3.3492). Small amounts of a sample (5 g) and the aliquot step (5 times dilution) contributed to eliminating or compensating for the matrix effect.

Different known concentrations of streptomycin (0.01, 0.05, 0.1, 0.25, and 0.5 mg/kg) were prepared in methanol by diluting the stock solution. Each solution was prepared in 6 replicates. Standard solutions were injected into an LC column and peak areas were measured to calculate streptomycin residue concentrations. A calibration curve was generated by plotting the concentrations of the injected standards versus the peak areas observed and the linearity of this method was evaluated by analyzing 5 working solutions. The peak areas obtained with different streptomycin concentrations were used to calculate the residue concentrations ($R^2 = 0.999$).

3.5. Specificity

The specificity (matrix effect) of an analytical

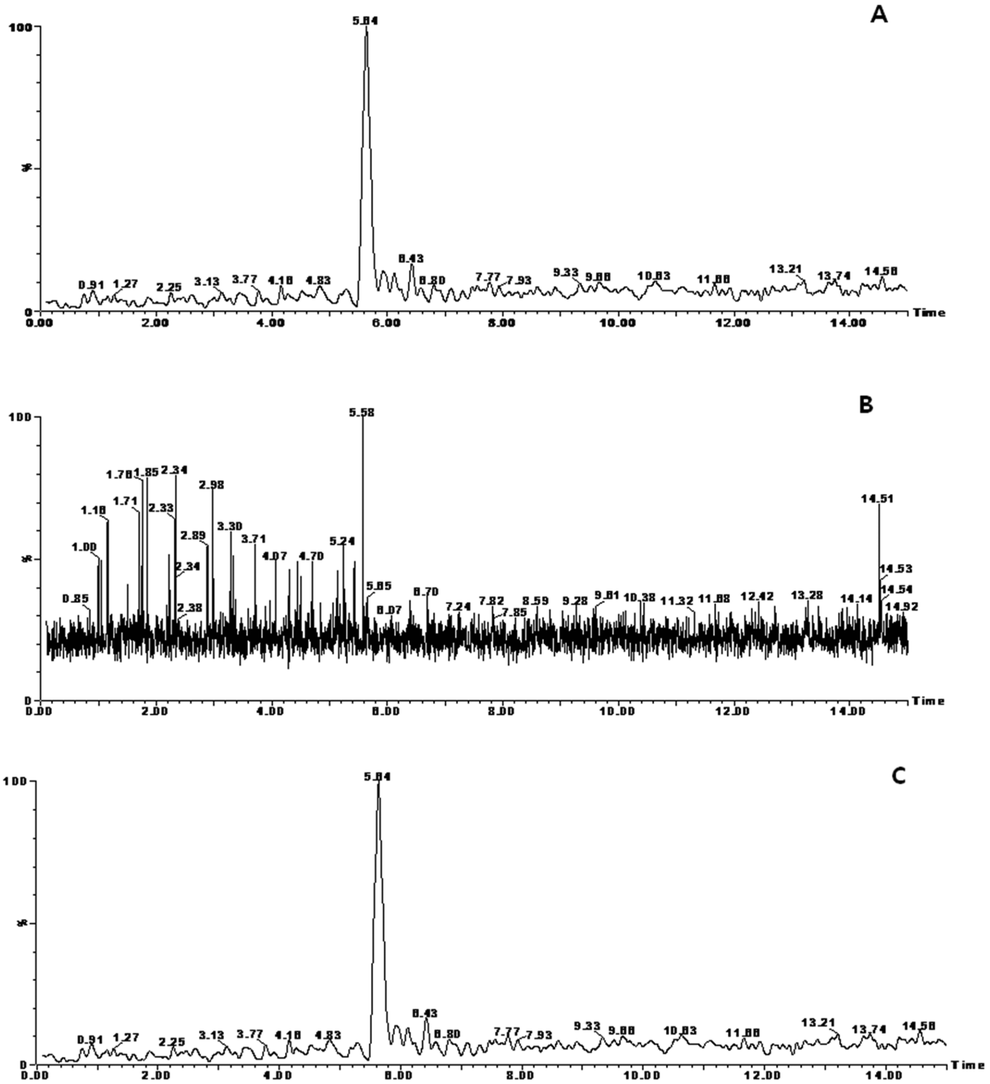

Fig. 3. LC-ESI-MS/MS chromatograms and spectrum; A. Streptomycin standard solution at 0.5 mg/kg; B. blank kiwifruit sample; C. kiwifruit sample spiked with 0.05 mg/kg of streptomycin.

Table 3. Quantification limit (mg/kg), mean recovery rates (%), and relative standard deviations (%) (n = 6) for our analytical procedure

Sample	Spiking Level (mg/kg)	*Recovery±RSD (%)	LOQ (mg/kg)
Kiwifruit	0.01	101.2 ± 0.17	0.01
	0.05	110.6 ± 0.47	
	0.5	94.8 ± 4.71	

*Mean values of 6 replicates ± standard deviation.

method is assessed on the basis of the absence of interfering peaks at the retention times for the analytes. The retention time for streptomycin was constant at 5.54±0.1 min, and there were no interfering peaks at this retention time for streptomycin (*Fig.* 2).

3.6. Accuracy and precision

Recovery studies were done using 0.01, 0.05, and 0.5 mg/kg fortification levels for streptomycin in blank kiwifruit (*Fig.* 3). The recovery data and the relative standard deviations obtained by this method are summarized in *Table* 3. These data were calculated from 6 replicates of the given samples made by a single analyst in 1 day. The repeatability of this method was satisfactory (RSD <10%).

3.7. Detection and quantification limits

The limit of quantification was determined to be 0.01 mg/kg. The quantification limit was defined as the lowest fortification level evaluated at which acceptable average recoveries were achieved (94.8% -110.6%; RSDs of <10%). This quantification limit also reflects the fortification level at which an analytic peak is consistently generated at approximately 10 times the baseline noise level in the chromatogram. The limit of detection was determined to be 0.002 mg/kg at approximately 6 times the background level of a control injection around the retention time for the peak of interest.

These results revealed that our developed, validated analytical method could be used for the determination of streptomycin in agricultural product samples and will be used as an official analytical method.

3.8. Application of our method to actual samples

The amounts of streptomycin in kiwifruit samples were not detected in these kiwifruit samples.

4. Conclusions

A method was developed for streptomycin analysis in kiwifruit. A rapid and easy sample preparation procedure was developed and optimized. In the first step, spiked kiwifruit was added to 10 g of homogenized sample along with 10 mL of 1% formic acid in methanol for 5 min. After vigorous shaking of the extraction mixture and centrifuging for 10 min at 2,500 × G, the upper extraction 1% formic acid in methanol layer was filtered using a 0.22 μm syringe filter. The final step of analysis used liquid chromatography-tandem mass spectrometry. The average recoveries of streptomycin (6 replicates for each concentration) were 94.8%-110.6% with relative standard deviations of <10%. LOD and LOQ were 0.002 and 0.01 mg/kg, respectively. These results showed that our developed analytical method was simple, rapid, sensitive, and suitable for streptomycin determination in kiwifruit samples. The linearity in the concentration range of 0.01-5.0 mg/kg gave R^2 = 0.9995. The average recoveries of streptomycin were 94.8%-110.6%.

The results obtained for calibration, recovery, and linearity showed that our method was practical, efficient, and reliable for determination of streptomycin in agricultural products. For streptomycin, the sensitivity of this method was sufficient to ensure a reliable determination at pesticide levels that were much lower than the respective MRLs established by the Korea MFDS. Therefore, our proposed analytical method could be satisfactorily used for regular monitoring of streptomycin residues in agricultural products as well as other matrices with slight modifications.

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